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DETERMINATION OF CREATININE IN URINE SAMPLES USING

GOLD NANOPARTICLES AND PAPER-BASED JAFFE REACTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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งานวิจัยนี้นำเสนอวิธีการตรวจวัดครีแอทินินในตัวอย่างปัสสาวะของมนุษย์ด้วยวิธีเทียบสี
2 วิธี คือ การใช้อนุภาคระดับนาโนเมตรของทองคำ และปฏิกิริยาจาฟเฟ่ฐานบนกระดาษ สำหรับ
วิธีการตรวจวัดครีแอทินินด้วยอนุภาคระดับนาโนเมตรของทองคำ มีการใช้การเตรียมตัวอย่างโดยการ
สกัดสารด้วยเฟสของแข็งซิลิกาเจลที่ดัดแปรหมู่ฟังก์ชันบนผิวด้วยหมู่ซัลโฟนิกเพื่อแยกครีแอทินินออก
จากเมทริกซ์ของตัวอย่างปัสสาวะก่อนทำการตรวจวัดด้วยอนุภาคระดับนาโนเมตรของทองคำ โดย
ประจุบวกของหมู่อะมิโนบนครีแอทินินเหนี่ยวนำให้เกิดการรวมตัวกันของอนุภาคระดับนาโนเมตรของ
ทองคำ ส่งผลให้เกิดการเปลี่ยนแปลงสีของสารละลายจากสีแดงเป็นสีม่วงหรือสีน้ำเงิน ซึ่งสามารถ
สังเกตเห็นได้ด้วยตาเปล่า เซอร์เฟซพลาสมอนสเปกตรัมของอนุภาคระดับนาโนเมตรของทองคำเลื่อน
ไปในตำแหน่งที่ความยาวคลื่นสูงขึ้นเมื่อความเข้มข้นของครีแอทินินเพิ่มขึ้น ซึ่งสามารถตรวจวัดได้ด้วย
เครื่องยูวี-วิสิเบิลสเปกโทรโฟโตมิเตอร์ ผลการทดลองแสดงความสัมพันธ์เชิงเส้นตรงในช่วงความ
เข้มข้นครีแอทินิน 15-40 มิลลิกรัมต่อลิตร ให้ค่าความเข้มข้นต่ำสุดของการตรวจวัดครีแอทินินอยู่ที่
13.7 มิลลิกรัมต่อลิตร จากผลการทดลองพบว่าค่าร้อยละการได้กลับคืนของครีแอทินินจากตัวอย่าง
(%recovery) อยู่ในช่วง 94.0-104.0% และค่าความเที่ยงในการวิเคราะห์ (%RSD) เท่ากับ 9.4%
สำหรับการตรวจวัดครีแอทินินด้วยปฏิกิริยาจาฟเฟ่บนฐานกระดาษ จะเคลือบกระดาษกรองด้วย
3-โพรพิลซัลโฟนิกแอซิด ไตรเมทอกซีไซเลน ก่อนนำไปใช้สำหรับสกัดครีแอทินิน โดยครีแอทินินที่ถูก
ดูดซับอยู่บนกระดาษกรองจะถูกตรวจวัดด้วยวิธีมาตรฐานของจาฟเฟ่ การเปลี่ยนแปลงสีบนกระดาษ
กรองสามารถสังเกตได้ด้วยตาเปล่า และความเข้มของสีบนกระดาษกรองสามารถวิเคราะห์ได้ด้วย
โปรแกรม Image J ช่วงความเป็นเส้นตรงของวิธีการตรวจวัดอยู่ในช่วง 0-60 มิลลิกรัมต่อลิตร
ค่าร้อยละการได้กลับคืนของครีแอทินินจากตัวอย่าง (%recovery) อยู่ในช่วง 91.9-113.8% และค่า
ความเที่ยงในการวิเคราะห์ (%RSD) เท่ากับ 8.6% และได้ประยุกต์ใช้วิธีการวิเคราะห์ที่นำเสนอนี้ใน
การตรวจวัดครีแอทินินในตัวอย่างปัสสาวะของมนุษย์ด้วยความแม่นและความเที่ยงที่ยอมรับได้

ภาควิชา เคมี	ลายมือชื่อนิสิต	_____
สาขาวิชา เคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก	_____
ปีการศึกษา 2557		

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KEYWORDS: GOLD NANOPARTICLES / CREATININE / SURFACE PLASMON RESONANCE (SPR) / HUMAN URINE / SOLID PHASE EXTRACTION / JAFFE REACTION / PAPER-BASED

JARINYA SITTIWONG: DETERMINATION OF CREATININE IN URINE SAMPLES USING GOLD NANOPARTICLES AND PAPER-BASED JAFFE REACTION. ADVISOR: ASST. PROF. FUANGFA UNOB, Ph.D., 74 pp.

This work proposed two new colorimetric methods; gold nanoparticles (AuNPs) method and paper-based Jaffe reaction, for the determination of creatinine in human urine samples. For the AuNPs method, sample extraction by sulfonic acid-functionalized silica gel was introduced to separate creatinine from urine matrix before the detection with citrate-capped AuNPs. The assembly of AuNPs was induced by attachment of positively charged amino groups on creatinine, resulting in a change of the color of AuNPs solution from red to purple or blue, which could be observed by naked eyes. The surface plasmon absorption band was shifted towards longer wavelengths when the concentration of creatinine increased which could be measured with a UV-Visible spectrophotometer. This method had a linear range of 15-40 mg/L and limit of detection (S/N = 3) of 13.7 mg/L. The recovery of creatinine in sample observed by the proposed method was 94.0-104.0% and the relative standard deviation was less than 9.4%. For the paper-based colorimetric method, 3-propylsulfonic acid trimethoxysilane was coated on the filter paper before application for creatinine extraction. The absorbed creatinine on the filter paper was detected with the standard Jaffé method. The color change on filter paper could be observed visually and the color intensity was determined by Image J software. The linearity range was observed in the range of 0-60 mg/L. The recovery of creatinine in sample observed by the proposed method was 91.9-113.8% and the relative standard deviation was 8.6 %. These methods were applied to determine creatinine in urine samples with acceptable accuracy and precision.

Department:	Chemistry	Student's Signature	....
Field of Study:	Chemistry	Advisor's Signature	....
Academic Year:	2014		

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LIST OF ABBREVIATIONS

M	Molar
mmol/L	Millimole per liter
nm	Nanometer
mg/L	Milligram per liter
µL	Microliter
mL	Milliliter
m^2/g	Square meter per gram
cm^3/g	Cubic centimeter per gram
cm	Centimeter
A	Absorbance
Å	Angstrom
^0C	Degree Celsius
K	Kelvin
LOD	Limit of detection
SPE	Solid phase extraction
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
TGA	Thermal gravimetric analysis
TEM	Transmission electron microscopy

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

Creatinine is a final waste product from creatine and phosphocreatine metabolism in skeleton muscles [1]. Creatinine is excreted by glomerular filtration and released into the blood and finally excreted out into urine. Creatinine detection is necessary in clinical diagnosis as it is a biomarker to monitor renal and muscle function [2]. If high level of creatinine in plasma and low level of creatinine in urine are observed, it indicates that the filtration by the kidney is not functioning well. The normal levels of creatinine in human urine are between 250-2590 mg/L depending on age, gender, weight and muscle mass [3, 4].

There are many publication reporting the methods for creatinine detection such as a spectrometric method (base on Jaffé's reaction) [5-9], chromatographic methods (e.g. HPLC, LC-MS, GC-MS, CE) [7, 10-17], enzymatic method coupled with electrochemical techniques (e.g. potentiometry, amperometry, voltammetry [5, 8, 18, 19]. The spectrometric method which is a colorimetric method based on Jaffé's reaction is normally used for creatinine determination in blood and urine. This reaction is based on the reaction between creatinine and picric acid under a strong basic condition. The obtained product, an orange-red complex, can be measured by a UV-Visible spectrophotometer in 490-520 nm wavelength ranges [7, 9, 14]. This method is simple and low operating cost; nevertheless, low selectivity and accuracy. Other interfering species in biological fluid can react with alkaline picrate to give a product that can absorb the visible light at the same wavelength range as does the creatinine-picrate complex. Hence, such species can affect method sensitivity and results accuracy [14]. The similar matrix effect may also reduce the method efficiency. Therefore, a simple, sensitive and accurate method is preferred for creatinine detection. To improve the method efficiency, the matrix effects have to be minimized by using a sample preparation technique. Furthermore, colorimetric

detection is an interesting choice for analysis due to its simple detection by either naked eyes or simple instruments.

At present, metal nanoparticles, especially gold nanoparticles (AuNPs) have been widely used in many applications as colorimetric sensors or biosensors for determination of biological molecules, organic molecules and metal ions. They show interesting physical, chemical and optical properties that are different from their bulk materials; in particular their optical properties e.g. surface plasmon resonance (SPR). The change in their SPR leads to change in the color of the solution detectable by naked eyes or spectrophotometry. Moreover, with their simple and fast synthesis, having large surface area to volume ratio and ease to bind with organic molecules, biological molecules or metal ions [20], the nanoparticles have become important materials in biomedical applications. Therefore, the use of AuNPs is a good choice for colorimetric sensing of creatinine. However, the interactions of AuNPs and analyte molecules through electrostatic interaction or covalent bonding are nonspecific interaction; therefore, the matrix interfering species may have an effect on creatinine assays.

To overcome the effect of urinary matrix and improve the sensitivity and accuracy of the proposed method, sample preparation by solid phase extraction, based on a cation exchange principle has become a technique of interest prior to the measurement by the proposed methods. Silica gel is generally used in many applications due to its hydrophilic surface, high surface area and ease of surface modification. In this research, sulfonic acid-functionalized silica gel was prepared and used as a strong cation exchanger for extraction of cationic creatinine from urine samples. This strong cation exchanger was synthesized by grafting 3-mercaptopropyltrimethoxylsilane on the silica surface to obtain the thiol groups (-SH) on the surface that were further oxidized with H_2O_2 solution to the sulfonic acid group (-SO_3H) on the silica surface. Before extraction, creatinine in sample solutions was converted to its cationic form by adjusting the pH of solutions to an acidic condition. The positive charges of creatinine replace ions on the cation exchanger. Neutral and negative charges compounds in urine sample would not be extracted by

the adsorbent; therefore, these interfering species are eliminated. Afterwards, the extracted cationic creatinine was detected with negatively charged citrate-capped AuNPs.

Another method of interest for creatinine detection is to use a paper-based colorimetric sensor which is a cost-effective and simple analysis tool. The color on the paper was developed after the target molecules react with a specific reagent and are then detected by naked eyes. To improve the specificity and selectivity for creatinine detection, the filter paper surface was modified with a specific reagent for selective creatinine extraction. The concept of ion exchange was applied. Under acidic condition, creatinine has positive charges that can be attached on the negatively charged surface. Therefore, sulfonic acid groups were selected for surface modification. After the extraction of creatinine in urine samples onto the paper, a solution of picrate reagent was dropped onto the paper to generate a color product with creatinine, which could be detected visually or recorded as photo for color intensity analysing with Image J program. The color intensity on the filter paper was related to the concentration of creatinine.

This research is focused on the method development for creatinine determination. Two colorimetric methods i.e. AuNPs method and the paper-based Jaffé reaction are proposed.

1.2 Research objectives

1.2.1 To prepare sulfonic acid-functionalized silica gel and the filter paper for creatinine extraction and detection by an AuNPs method and the Jaffé reaction.

1.2.2 To investigate the effect of extraction parameters and detection parameters to get the optimal condition for creatinine detection.

1.2.3 To apply both proposed methods to detect creatinine in human urine samples.

1.3 Scope of the research

For AuNPs method, the matrix effect was eliminated using sample preparation by solid phase extraction. A cation exchanger adsorbent was prepared by modifying the surface of silica gel with sulfonic acid group ($-SO_3H$). The modified adsorbent was characterized by thermogravimetric analyzer (TGA), surface area analyzer and acid-base titration, compared to native silica gel. The effect of extraction parameters on creatinine extraction including extraction time, elution time, pH values of creatinine solution and interfering species were examined. For creatinine detection with the AuNPs method, the extracted creatinine standard solution or the extracted urine sample was mixed with AuNPs solution and analyzed by a UV-Visible spectrophotometer to record the absorption data and compared with the standard Jaffé method. The influence of the concentration of AuNPs and incubation time between AuNPs and creatinine solution were investigated to obtain an optimal condition.

For the paper-based creatinine detection, the sulfonic acid group was coated on surface of the filter paper before applying the paper for creatinine extraction. Then, the extracted creatinine on the paper reacted with alkaline picrate solution to generate yellow complex resulting in a change of the paper color that could be observed by naked eyes and the color intensity of the taken photos was determined by using Image J program. The obtained results were compared to the standard Jaffé method.

1.4 The benefit of this research

To obtain a simple and accurate method for creatinine detection in human urine samples with short analysis time and easy detection by either naked eyes or a simple instrument.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Creatinine

Creatinine (2-Amino-1-methyl-2-imidazoline-4-one) is a final waste product of human creatine and creatine phosphate as a result of muscle metabolic [1]. Creatinine is excreted by glomerular filtration, released into blood and finally eliminated into urine by the kidneys. It is formed at a relatively constant rate in the body [1]. Creatinine plays an important role as an indicator of the kidney function. High creatinine level in plasma indicates a problem with the kidney [2]. The normal levels of creatinine in human urine are between 280-2590 mg/L and 6-12 mg/L in blood [3, 4].
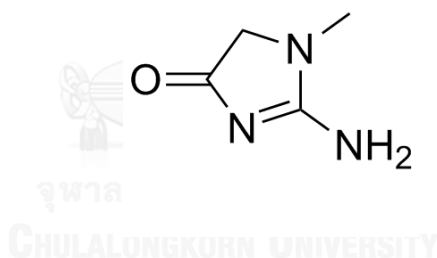
Figure 2.1 Structure of creatinine.

2.2 Method for creatinine determination

Several methods have been proposed for creatinine determination, including enzymatic method, chromatographic method and spectrometric method (based on the Jaffé reaction) which are described hereafter in more details.

2.2.1 Spectrometric method

A spectrometric method based on the Jaffé reaction is the most generally used colorimetric method for creatinine determination. The forming orange-red color product [5, 15] when creatinine reacted with picric acid in an alkaline medium can be measured by a UV-Visible spectrophotometer at wavelengths in the range from 490 to 520 nm [7, 9, 14], the mechanism of this method is shown in Figure 2.2. The signal intensity depends on creatinine concentration in samples. This method is simple with low operating cost, nevertheless poor specificity and time-consuming. Several publications reported the weak points of the Jaffé method concerning poor specificity due to the effect of common interfering species in biological fluids, such as ascorbic acid, uric acid, glucose, ketones and proteins if present at high concentrations [14, 21, 22]. These interferences can react with alkaline picrate and the resulted products may absorb the UV-Vis light at the same region as do creatinine-picrate complex, hence affecting the sensitivity and accuracy of this method [14, 23]. Therefore, it is necessary to overcome this problem.

Creatinine   Picric acid   Yellow creatinine-picrate complex
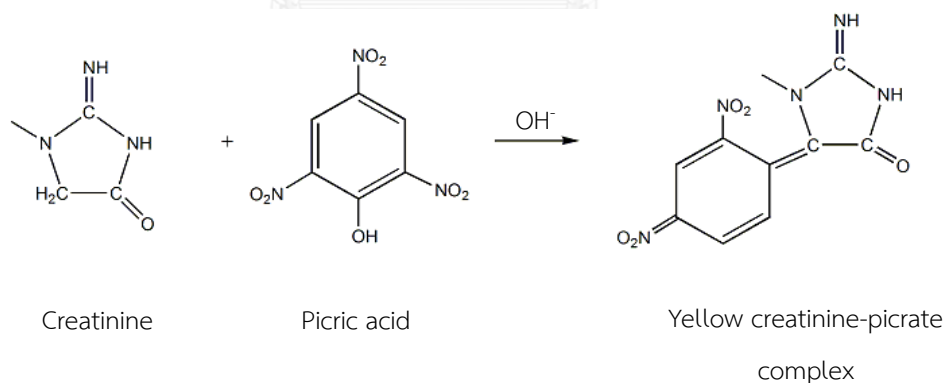
Figure 2.2 The mechanism of the Jaffé reaction.

2.2.2 Enzymatic method

An enzymatic method is based on the determination of H_2O_2 or NH_3 products [4, 24] resulted from the reaction of creatinine in urine with enzymes including creatinine amidohydrolase, creatine amidinohydrolase or creatinine deiminase, and sarcosine oxidase. These products can be detected by electrochemical techniques (i.e. amperometry, potentiometry and voltammetry methods). The reaction sequences are shown in the equations below. The enzymatic method reveals several advantages compared to the Jaffé method, including use of small sample volume, high specificity and accuracy. However, this method has disadvantages as it is time-consuming and the enzymes are unstable and cannot be stored for long time [5, 18, 21, 25].

$$\text{Creatinine} + H_2O \xrightarrow{\text{Creatine amidohydrolase}} \text{Creatine} \tag{2.1}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatine amidinohydrolase}} \text{Sarcosine} + \text{urea} \tag{2.2}$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{\text{Sarcosine oxidase}} \text{Glycine} + \text{Formaldehyde} + H_2O_2 \tag{2.3}$$

or

$$\text{Creatine} + H_2O \xrightarrow{\text{Creatinine deiminase}} \text{methyl-hydantoine} + NH_3 \tag{2.4}$$

2.2.3 Chromatographic method

A chromatographic method is one of the alternative methods for creatinine separation from urinary matrix. Currently, there are many separation methods for creatinine determination such as high performance liquid chromatography (HPLC–UV) [11, 14, 17], gas chromatography-tandem mass spectrometry (GC–MS) [12], liquid chromatography-mass spectrometry (LC–MS) [15, 16], and capillary electrophoresis (CE) [7, 10, 13]. These methods are based on the separation of the composition of mixtures from each other by different affinities of the substances with a selective

stationary phase. Nevertheless, these methods are time consuming and complicated and they require high skilled operators and expensive equipment [26].

From previous methods, matrix interferences remain a significant problem in biological sample measurements. Therefore, sample preparation technique is required to remove matrix interference and improve the selectivity and accuracy of the methods.

2.3 Nanoparticles

Nowadays, metallic nanoparticles [27-32] (having diameters in a range between 1 and 100 nm) have drawn strong interests and also play an important role in many applications due to their unique optical properties that are different from their bulk substances, especially surface plasmon resonances (SPR). It is easy to control their size, shape, structure and composition when compared with other nanostructures in order to make a change or tune their optical properties. Therefore, they are useful in biomedical applications as a colorimetric sensor or biosensor for determination of biological molecules, organic molecules and metal ions. There are numerous types of metal nanoparticles that comprise silver, gold, titanium oxide and iron nanoparticles. Gold nanoparticles (AuNPs) show interesting and unique optical properties which make them very interesting and popular as sensors. Compared with other metallic nanoparticles, AuNPs are good choice for sensors application due to their ease of synthesis, small size to volume ratio, extensive thermal stability and ability in binding with organic molecules or metal ions, relatively low cytotoxic and strong SPR.

The interaction of a nanoparticle with incident light, an electromagnetic field, at a certain wavelength induces a collective oscillation of the free conduction electrons across the particle. These resonances are known as surface plasmon resonance (SPR) as shown in Figure 2.3. Due to SPR, the particle absorbs and scatters (extinction cross section) the electromagnetic radiation strongly. The frequency of surface plasmon absorption band and color of a gold nanoparticle depend strongly

on the size, shape, inter-particle distance, surface composition of the particle and the dielectric properties of the surrounding medium. Citrate-capped AuNPs have a negative charge on the surface, which can interact with the positively charged molecules through electrostatic interaction and/or covalent bonding leading to AuNPs disaggregation or aggregation. The change in inter-particle distance caused by AuNPs disaggregation or aggregation results in a change of solution color and their SPR absorption band which can be detected visually or by spectrophotometry. AuNPs with a size of 13 nm exhibiting the maximum absorption wavelength (λ_{max}) around 520 nm [32] are observed as red color solution. When AuNPs aggregate, the color of AuNPs solution changes from red to purple and then blue depending on the degree of aggregation, the position of the λ_{max} of AuNPs solution shifts to longer wavelength (red shift) and also the bandwidth increases [29].
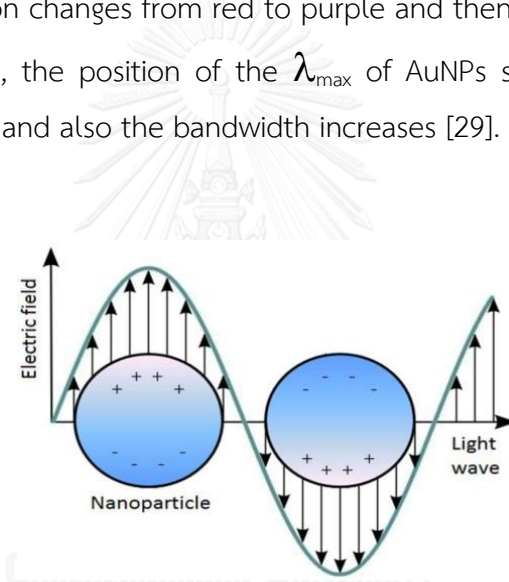
Figure 2.3 Schematic demonstration of plasmon oscillation of electrons on a gold nanoparticle under light irradiation [33].

There are many methods of AuNPs preparation based on the reduction of chloroauric acid (Au^{3+}) to elemental gold (Au^{0}) which are stabilized to prevent the aggregation by using stabilizing agent (e.g. polymer, surfactants, DNA, protein [31, 32, 34-36]. The most commonly used method for AuNPs preparation is citrate reduction method. AuNPs can be prepared simply by using tri-sodium citrate as reducing agent

and also stabilizing agent. They can be synthesized in various sizes by tuning tri-sodium citrate concentration.

From above notice, it makes AuNPs an interesting nanomaterial for many applications, in particular sensor or biosensor for biomolecule assay.

2.4 Sample preparation by solid phase extraction (SPE)

Solid phase extraction (SPE) is an excellent method for sample extraction and clean-up or preconcentration by adsorption of analytes onto solid adsorbent and elution with a suitable eluent. SPE is often used for resolving the problem of matrix interferences in urine samples before the analysis with other analytical methods. Creatinine in urine can adsorb on the adsorbent to separate it from other species prior to the detection process. There are several adsorbents that are used for sample treatment such as an ion-exchange resin and clay minerals, depending on the goal of work and target analytes. The application of ion-exchange adsorbents will be described below.

2.5 Extraction by cation exchange resin

Ion exchange chromatography is one type of chromatographic methods for determination and purification of organic molecules, proteins, amino acids or other ions based on charge separation. The positively or negatively charged ions on an ion exchanger are replaced by other ions of the same charge in the solution which contacts the adsorbent. Ion exchange chromatography can be classified into 2 types, cation exchange chromatography and anion exchange chromatography and only cation exchange chromatography will be referred hereafter.

Cation exchanger can be divided into two types which are strong acid and weak acid cation exchange resins. Strong cation exchanger and weak cation exchanger contain sulfonic acid group and carboxylic acid group as cation exchange site, respectively. However, strong cation exchangers are normally used in many

applications because it can be used in wide pH range. In case of using a strong cation exchange resin, the positively charged molecules in sample can replace these counter ions of sulfonate acid group on the resin (e.g. H^+, Na^+) and adsorb on resin via electrostatic interaction, whereas negatively charged and neutral molecules are not retained (Fig. 2.4). Afterwards, cationic ions adsorbed on the resin are eluted with a suitable solution. From the above mentioned, this method is chosen for the extraction of creatinine from matrix interferences in urine samples to overcome interference problem before analysis with the proposed method. However, the commercial cation exchangers resin have low capacity, therefore a silica-based strong cation exchanger was synthesized in this study to increase the capacity of material.

Positively charged protein binds to negatively charged bead

Negatively charged protein flows through
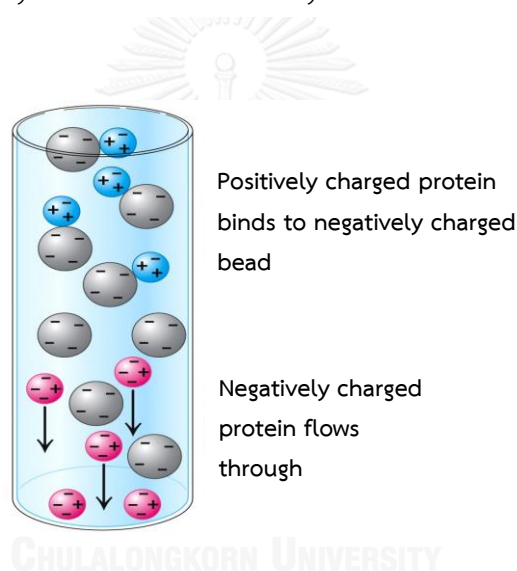
Figure 2.4 Cation exchange procedures for positively charged protein separation [37].

Silica gel is commonly used as adsorbent because it has high surface area and a number of silanol groups on its surface which can be functionalized easily. It is less expensive than some organic polymers [38]. Silica gel is normally used as adsorbent in solid phase extraction. To increase the selectivity and expand the application of silica material, its surface is usually modified with other functional groups. In this research, a sulfonic acid-functionalized silica gel was prepared and used as strong

cation exchanger for extraction of creatinine in human urine samples before detection with the proposed method.

2.6 Paper-based for creatinine detection

Currently, paper is widely used as a supporting platform for colorimetric sensor and devices for environmental analysis, clinical diagnosis and food analysis such as test strips for urine analysis, microfluidic device coupled with electrochemical techniques and lateral-flow immunoassay due to its low price, thin and small size. The main component of paper is cellulose fiber that can be functionalized with other functional groups to change their properties depending on the main goal of analysis. The image of the paper-based sensors can be captured by scanner or digital camera and the intensity of color can be measured by Adobe or Image J software.

2.7 Literature review

As a result of the unique optical properties of AuNPs, in particular surface plasmon resonance (SPR), a number of researchers have reported the successful use of AuNPs as colorimetric sensor for detection of biological molecules, organic molecules or metal ions. Wang *et al*. [34] developed a new method for lysozyme detection in saliva and human urine samples based on gold nanoparticle plasmon resonance light scattering (PRLS) measurement and visual detection by using lysozyme DNA aptamer as the recognition element. The mechanism was based on the change of ionic strength of the solution that induced AuNPs aggregation. The sensitivity of this method could be improved by changing the concentration of salt (NaCl). The aggregation of AuNPs occurred after adding lysozyme to the DNA aptamer stabilized AuNPs in the solution containing specific salt concentration, resulting in a change of solution color from red to blue and enhancing PRLS. The linear range depended on AuNPs concentrations and it was in the range of 0.2-4 nM for 0.7 nM

AuNPs, 0.3-6 nM for 1.4 nM AuNPs and 0.6-8 nM for 2.1 nM AuNPs with the recovery in the range of 95.8-104.5%.

Li *et al.* [39] studied the interaction between cysteine and gold nanoparticles for cysteine determination. The proposed method was based on self-assembly of gold nanoparticles. AuNPs was prepared by using tri-sodium citrate as reducing agent and stabilizer therefore the surface of AuNPs were negatively charged. At the same time, cysteine bearing −SH group and −NH$_3^+$ groups could be attached onto the negatively charged surface of AuNPs via the covalent binding and the electrostatic interaction. As a result, a self-assembly of AuNPs occurred which enhanced resonance light scattering. The linear range for the detection was from 0.01 to 0.25 μg/mL with the detection limit of 2.0 ng/mL.

Cao and Li [31] proposed a simple colorimetric method for heparin detection using cysteamine-stabilized AuNPs solution. The surface of AuNPs would have positive charge (NH$_3^+$). After adding heparin, the negatively charged heparin could interact with the positively charged AuNPs through electrostatic attraction leading to AuNPs aggregation and a solution color changed from red to blue which could be detected by naked eyes and a UV-Visible spectrophotometer. The linear range of heparin determination was found to be in the range from 0.09 to 3.12 mg/mL with the limit of detection of heparin in water of 0.03 mg/mL and the relative standard deviation (RSD) was approximately 2%.

Xiao *et al.* [40] modified the surface of AuNPs with fluorosurfactant and used it as a resonance light scattering (RLS) probe for cysteine and homocysteine determination in human urine samples based on cysteine induced AuNPs aggregation. The linear range of the method was in the range of 0.08–6.0 μM for cysteine and homocysteine with a detection limit of 0.04 μM and the recovery of analytes from spiked urine samples observed by this method was found in the range of 96.0–113.0%.

Pu *et al.* [41] developed a simple and rapid method for arginine detection based on the aggregation of citrate-capped AuNPs. When solution pH was lower than pI of arginine, the positively charged of guanidino group on arginine would interact with the negatively charged citrate-capped AuNPs through electrostatic interactions and hydrogen-bonding that induced AuNPs aggregation and a change of the solution color from red to blue. The absorption spectra were monitored using a UV–Visible spectrophotometer. The linear range and the detection limit (3σ/slope) were found to be 0.08–13.2 µM and 16 nM, respectively. Furthermore, this method could be used to detect arginine easily at the concentration as low as 0.4 µM by naked eyes without using complicated or expensive instruments.

Mirza *et al.* [42] prepared AuNPs and modified their surface with doxorubicin. Under weak acidic condition, the amino groups of doxorubicin were protonated and adsorbed on the surface of AuNPs through electrostatic interaction. This phenomenon led to the change of the absorption intensity and the wavelength of the maximum absorption of UV-Vis light of AuNPs solution. On the other hand, under basic condition the protonation of amino group did not occurred, therefore, doxorubicin could not be adsorbed on AuNPs surface. This idea would be applied for nanosized drug-delivery system.

Chen *et al*. [43] developed a novel method for thrombin detection in plasma using fibrinogen conjugated gold nanoparticle (Fib-AuNPs) as a colorimetric biosensor. AuNPs modified with fibrinogen had slightly extinction of light which was higher than that of unmodified AuNPs. The solution of Fib-AuNPs appeared in pink color. After adding thrombin, the aggregation of Fib-AuNPs took place. The SPR absorbance of the mixture solution decreased when the concentration of thrombin increased. The range of linearity of this work was from 0.1 to 10 pM with the detection limit of 0.04 pM.

From previous research, AuNPs were used in many biomedical assays as colorimetric sensor. The negatively charged AuNPs were easily modified with organic molecules or biomolecules bearing positive charge. Therefore, in this research,

we focused on using citrate-capped AuNPs as colorimetric sensor for creatinine detection because creatinine have positive charges under acidic condition and can be adsorbed on AuNPs surface through electrostatic interaction. The change in their SPR bands and color of solution was observed by naked eyes or a UV-Visible spectrophotometer.

However, the interaction between label-free AuNPs and biological molecules are non-specific. The matrix in biological samples may have effect on creatinine determination. Therefore, sample preparation technique was required to separate creatinine from urine sample prior to the determination with the proposed method. Rockerble *et al*. [44] used cation-exchange resin to remove interferences from human serum and urine samples before detection with the Jaffé reaction. Samples were diluted in 0.10 N HCl solution before an extraction process. Creatinine adsorbed on a cation-exchange resin was eluted by phosphate buffer solution at a pH of 12.4. After that, the extracted solution was mixed with alkaline picrate for 30 minutes and the absorbance of solution was measured by a UV-Visible spectrophotometer at a wavelength of 510 nm.

Mitchell [6] developed a method for determination creatinine in serum and urine samples by separating creatinine from matrix interferences using cation-exchange resin. Samples were diluted with buffer at a pH of 3.0 and extracted onto the adsorbent. Then, creatinine on the adsorbent was eluted by using phosphate buffer solution (pH 12.2). Creatinine in the supernatant was detected by the Jaffé reaction. After 30 minutes of reaction time, the absorbance of the mixture solution was recorded by a UV-Visible spectrophotometer at a wavelength of 505 nm.

Haeckel *et al.* [45] proposed a serum creatinine assay using clay mineral (Fuller's earth) to remove interferences. Human serum was deproteinized with tungstic acid and then extracted with clay mineral. Under acidic condition, *ca.* 92% of creatinine can be adsorbed on the adsorbent. After the supernatant was discarded, an alkaline picrate solution was added to adsorbent to elute the adsorbed

creatinine. The absorbance of the eluted orange color product was measured by a UV-Visible spectrophotometer at a wavelength of 490 nm.

Ambrose *et al.* [46] proposed a chromatographic method (HPLC) for determination of human serum creatinine. To eliminate serum matrix, a strong cation-exchange resin was introduced to separate creatinine in serum. At a pH below pKa value of creatinine, the creatinine molecule was positively charged and adsorbed by the resin. The adsorbed creatinine on resin surface was eluted by lithium acetate buffer of pH of 7.1 and then the amount of creatinine was detected with HPLC technique equipped with UV detector at 234 nm.

In this work, sample preparation by solid phase extraction was introduced to overcome the effect of matrix interferences in urine samples prior to the analysis with AuNPs. Sulfonic acid-functionalized silica gel was prepared and used as a strong cation exchanger for extraction of creatinine in urine via an ion-exchange mechanism.

Furthermore, a paper-based method of detection had gained a lot of interest due to its simplicity and possibility of on-site analysis. Ferreira *et al.* [47] proposed a new silver nanoparticles-paper based sensor for ascorbic acid detection using paper as substrate. Paper platform consisted of hydrophilic (test zone) and hydrophobic parts produced by wax printing. AgNPs and silver ions (AgNO$_3$ solution) were dropped onto the paper on the hydrophilic zone (7 mm in diameter), followed by samples. The color of AgNPs paper-based sensor changed from light yellow to gray color due to nanoparticle growth and clusters creation. The color intensities which depended on ascorbic acid concentration were measured by using a scanner or a homemade portable transmittance colorimeter. For real samples analysis, the sensitivity of this sensor was comparable to the standard titration method.

Gomes *et al.* [48] developed a low cost and disposable paper test strip for screening of drugs (oxytetracycline; OXY) in aquaculture environment by visual comparison of color changes. The paper was modified with 3-triethoxysilylpropylamine (APTES) to form amine layer that allowed metal ions (e.g. Fe (II) or Cu (II)) binding by coordination. The metal ion would react with the

drug to produce a color compound. The images of the paper were captured with a camera and the color intensity was determined by the Paint program. After that, the paper strips were applied for the analysis of drug spiked environmental water and the concentrations of OXY as low as 30 ng/mL could be detected.

Zhou *et al*. [49] developed method for detection of H_2O_2, glucose and protein biomarker by using a paper-based colorimetric biosensor platform as probe. The filter paper was modified with 3-aminopropyltriethoxysilane (3-APTMS) which was further cross-linked with glutaraldehyde (GA) to form brick-red complex (APTMS-GA). This complex could be oxidized by H_2O_2 leading to a visual color change on the paper and Image J software was used to measure the color intensity of the filter paper photo. For glucose detection, glucose oxidase (GOx) was immobilized on the modified paper. Glucose could be detected through the reaction of between APTMS-GA and enzymatic product H_2O_2. For the analysis of protein biomarker, prostate specific antigen (PSA), the APTMS-GA modified paper was coated with chitosan and subsequently AuNPs. The anti PSA antibody (Ab_1) was further dropped onto the paper and attached on AuNPs as the site for PSA capturing. After adding PSA, gold nanorods functionalized with GOx and anti-PSA antibody (Ab_2) (GNR-GOx-Ab_2) were further dropped to bind to PSA on the paper. Then a glucose solution was added onto the paper and H_2O_2 was generated by reaction between GOx and glucose, resulting in the change of the paper color. The linear range of H_2O_2, glucose and PSA determination were 2.5-500 mM, 0.5-30 mM and 0.1-10 ng/mL, respectively.

In this study, we were interested in using the filter paper for extraction of creatinine in human urine samples and detection by using the Jaffé reaction and observing the color change. The filter paper surface was modified with sulfonic acid groups before use for creatinine extraction. The creatinine adsorbed on the paper was detected with the Jaffé reaction. The color intensity was proportional to the concentration of creatinine on the paper which can be determined by Image J software.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals

All chemicals were of analytical reagent grade (AR). The list of chemicals is shown in Table 3.1.

Table 3.1 List of chemicals.

Chemicals	Supplier/ Grade
Gold(III) chloride tri-hydrate ($HAuCl_4.3H_2O$ ≥99.9%)	Sigma-Aldrich
3-mercaptopropyltrimethoxysilane 95%	Aldrich/reagent grade
Creatinine	Aldrich/reagent grade
Sodium chloride	Merck/for analysis
Sodium hydroxide	Merck/ for analysis
Ethanol	Merck/for analysis
Picric acid	Sigma-Aldrich/reagent grade
Hydrogen peroxide 30%	Merck/AR grade
Tri-sodium citrate dihydrate	Fisher Scientific
Urea	Merck/for analysis
Bovine serum albumin (BSA)	Sigma-Aldrich
Potassium chloride	Merck/for analysis
Hydrochloric acid (37% W/W)	Merck/for analysis
Nitric acid (65% W/W)	Merck/for analysis

3.2 Instruments

Table 3.2 List of instruments

Instruments	Model
UV-visible spectrophotometer	HP/8453
Thermogravimetric analyzer	Perkin-Elmer/Pyris1
Surface area analyzer	BEL Japan/BELSORP-mini
Transmission electron microscope	JEOL/JEM-2100
pH meter	Hanna instruments/pH 211
Magnetic stirrer	Gem/MS 101
Centrifuge	MES/TAUR 2

3.3 Solutions preparation

Gold (III) ions solution

A 1.0 mM gold (III) ions solution was prepared by dissolving 0.0394 g of gold(III) chloride tri-hydrate ($HAuCl_4.3H_2O$) in 100 mL of deionized water. The stock solution was further used for gold nanoparticles (AuNPs) preparation.

Tri-sodium citrate solution

A 0.5% (w/v) tri-sodium citrate solution was prepared by dissolving 0.5 g of tri-sodium citrate dihydrate in deionized water and the volume was adjusted to 100 mL. The obtained solution was used as a reducing agent and stabilizer in the preparation of gold nanoparticles (AuNPs).

Creatinine solutions

A 10,000 mg/L creatinine standard stock solution was prepared by dissolining creatinine powder with deionized water. The working standard solutions in the concentration range of 15-40 mg/L for the use in the gold nanoparticles (AuNPs) method and 0-60 mg/L for the use in a paper-based method were prepared by diluting creatinine standard stock solution with 1.0 mmol/L of HCl solution.

Sodium chloride solution

A 0.5 mol/L sodium chloride solution was prepared by dissolving 2.14 g of sodium chloride powder in deionized water and the volume was adjusted to 250 mL. This solution was used as an eluent for creatinine elution from solid phase.

Sodium hydroxide solution

A 0.75 mol/L sodium hydroxide solution (NaOH) was prepared by dissolving 3.0 g of sodium hydroxide in 100 mL of deionized water. The obtained solution was used in creatinine analysis by the standard Jaffé method.

Picric acid solution

A stock solution of picric acid was prepared by dissolving 1.5 g of picric acid with deionized water. A 0.04 mol/L picric acid solution was generated by diluting the saturated stock solution of picric acid with deionized water. This solution was used in the Jaffé method for creatinine determination.

3.4 Preparation of gold nanoparticles (AuNPs) solutions

Gold nanoparticles solutions (AuNPs) were prepared by using tri-sodium citrate as reducing agent and stabilizer. In brief, 20 mL of 0.50 mmol/L gold(III) chloride tri-hydrate (HAuCl$_4$.3H$_2$O) solution was heated to a temperature of approximately 90 °C. Afterwards, 2.0 mL of 0.5% (w/v) tri-sodium citrate solution was added under continuous stirring for 15 minutes. The solution color changed from yellow to deep red indicating the formation of AuNPs. Then AuNPs solution was cooled to room temperature and kept in a glass bottle at 4 °C for further use.

3.5 Preparation of sulfonic acid-functionalized silica gel

Sulfonic acid-functionalized silica gel was prepared by mixing and heating 4.0 g of silica gel with the mixture solution of 15.9 mL of 3-mercaptoproplytrimethoxysilane (3-MPTMS), 160 mL of ethanol, and 160 mL of deionized water at 120 $^\circ$C under reflux and nitrogen atmosphere for 5 hours. Consequently, the solid was filtered off and washed with deionized water and dried in an oven at 120 °C overnight. To prepare sulfonic acid-functionalized silica gel, the thiol groups on the silica surface were oxidized by mixing the obtained solid with 160 ml of 30% H$_2$O$_2$, 160 mL of ethanol and 160 mL of deionized water. The mixture was continuously stirred at room temperature for 24 hours. Then the solid was filtered, washed with deionized water and dried in an oven at 120 °C overnight. The sulfonic acid-functionalized silica gel was characterized by thermogravimetric analyzer (TGA), surface area analyzer and the titration technique. The reaction scheme is presented in Figure 3.1.
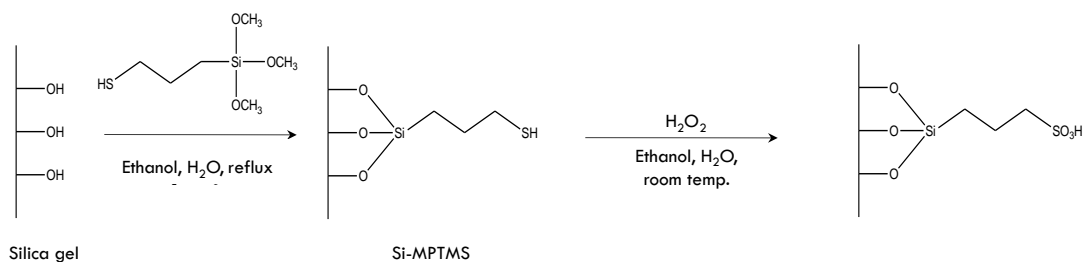
Figure 3.1 Surface modification of silica gel.

3.6 Characterization method

The absorption spectra of AuNPs solutions were recorded by a UV-Visible spectrophotometer. The thermal stability of sulfonic acid-functionalized silica gel was investigated under nitrogen atmosphere by using a thermogravimetric analyzer. A surface area analyzer was used to measure surface area, pore size distribution and total pore volume of the sulfonic acid-functionalized silica gel by means of N_2 adsorption and data were calculated by using the BJH model. The morphology of AuNPs and AuNPs aggregates was observed by a transmission electron microscope (TEM).

The acid capacity of native silica gel and silica gel bearing sulfonic acid groups was determined by acid-base titration [50]. Briefly, 100 mg of solid was weighted into a test tube. After that, 25.0 mL of 1.0 mmol/L HCl solution was added to protonate the acidic functional groups on the solid under vigorous stirring at room temperature. The solution was removed after centrifugation and 25.0 mL of 0.5 mol/L NaCl solutions was subsequently added to the solid to replace H^+ on the exchangeable functional groups. Afterwards, the mixture was centrifuged to separate the solid and then a 20.0 mL of supernatant was collected for titration with 0.05 mol/L NaOH solution using phenolphthalein as an indicator. The observed amount of NaOH was used to calculate the acid capacity (mol proton/g of adsorbent) of the solid.

3.7 Sample extraction by sulfonic acid-functionalized silica gel

3.7.1 Creatinine extraction procedure

Sulfonic acid-functionalized silica gel was used as a cation exchanger to separate creatinine from urine sample matrix or standard solutions. Before extraction process, urine samples and working creatinine standard solutions were diluted in 1.0 mmol/L HCl solution to convert creatinine into its cationic form before extraction by the modified adsorbent. In the extraction process, the amount of 50 mg adsorbents was weighted into a test tube and then 5.0 mL of creatinine standard solution or a diluted urine sample was added. The mixture was stirred continuously at room temperature for a specific period of time. After separating the solid by centrifugation and washing the solid with deionized water, the creatinine adsorbed on solid was eluted with 5.0 mL of 0.5 mol/L NaCl solution. The supernatant was kept for creatinine analysis with the AuNPs method, compared to the standard Jaffé method. The influence of creatinine extraction parameters including extraction time, elution time, sample pH and interfering species were investigated to find a suitable condition for creatinine determination. The procedure of sample extraction by solid phase is presented in Figure 3.2.
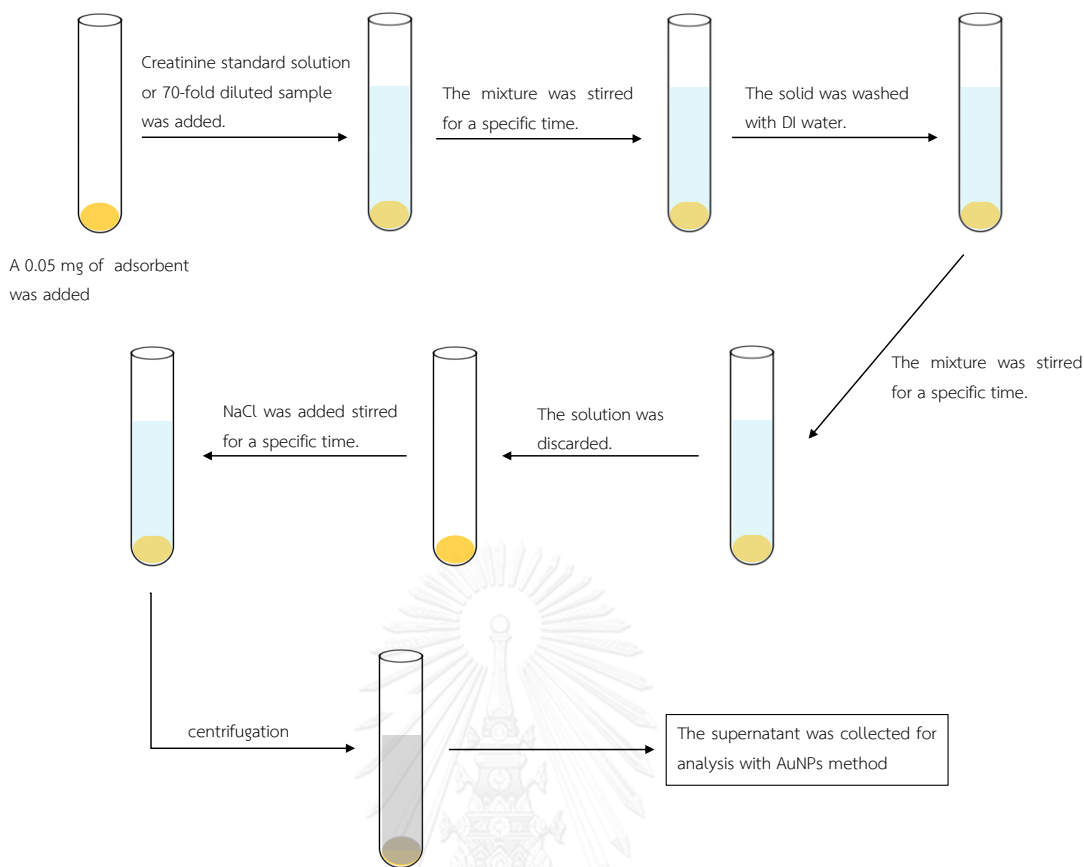
Figure 3.2 Sample extraction by solid phase.

3.7.2 Influence of extraction parameters

To find a suitable condition for creatinine extraction and elution, the effects of extraction parameters including extraction time, elution time, pH of the creatinine solution and interferences were investigated.

3.7.2.1 Effect of extraction time and elution time

Creatinine standard solutions at a concentration of 16 mg/L were selected for this study. The extraction and elution time were varied in the range of 10 to 30 minutes. When different extraction times were applied, the elution time was fixed at 30 minutes. In the same manner the extraction time was fixed at 30 minutes

when the elution time was varied. The creatinine adsorbed on the adsorbent was eluted by 0.5 mol/L NaCl solution and then analyzed with the AuNPs method.

3.7.2.2 Effect of initial pH of the creatinine solution

The effect of initial pH of the solution on creatinine extraction was studied using the pH range of 4-7 which are the normal pH range of human urine [51]. Hence, creatinine standard solutions at the concentration of 1050 and 2800 mg/L were prepared with pH of 4.0, 5.0, 6.0, and 7.0, respectively by using NaOH or HNO_3 solutions to adjust the pH value. Then, the prepared solutions were diluted 70-fold with 1.0 mmol/L HCl solution prior to the extraction process according to the protocal discribed previously.

3.7.2.3 Effect of interfering species

To investigate the effects of interferences on creatinine extraction, the substances commonly present in urine such as urea, bovine serum albumin (BSA) and KCl were added to creatinine solution at their maximum concentration found in human urine. Creatinine standard solutions with concentration of 1050 and 2800 mg/L were mixed with different concentrations of each interfering species such as 17.5 and 35 g/L urea, 35 and 350 mg/L BSA or 17.5 g/L KCl, respectively. Then, the binary mixtures were diluted 70-fold prior to the sample extraction process described previously in section 3.7.1. Afterwards, the extracted creatinine solutions were analyzed by the AuNPs method according to the analytical method proposed herein using a UV-visible spectrophotometer for recording the data.

3.8 Determination of creatinine by gold nanoparticles (AuNPs)

3.8.1 Optimization of creatinine determination

A volume of 70 µL of extracted solution was added into 1.0 mL of 0.25 mmol/L AuNPs solution under vigorous stirring for a specific period of time. Immediately afterwards, the absorption spectrum of the mixture solution was measured with a UV-Visible spectrophotometer in the wavelength range of 400 to 800 nm (as shown in Figure 3.3). Calibration curves were constructed by plotting the ratio of absorbance observed at 600 nm over that measured at 520 nm ($A_{600/520}$) versus concentration of creatinine standard solutions. The influence of AuNPs concentration and reaction time between AuNPs and creatinine solution were investigated. Subsequently, the performance of the proposed method was compared with the standard Jaffé method. Lastly, the AuNPs method was applied to analyze creatinine in human urine samples. The morphology of AuNPs in solution before and after loading creatinine was observed by TEM.

To analyze creatinine by the standard Jaffé method, a 3.0 mL of extracted creatinine standard solution or extracted urine sample was added into a test tube. After that, 1.0 mL of 40 mmol/L picric acid solution and 1.0 mL of 0.75 mol/L NaOH solution was added subsequently under stirring at room temperature. After 30 minutes, the absorbance of solution was measured using a UV-Visible spectrophotometer at a wavelength of 505 nm.
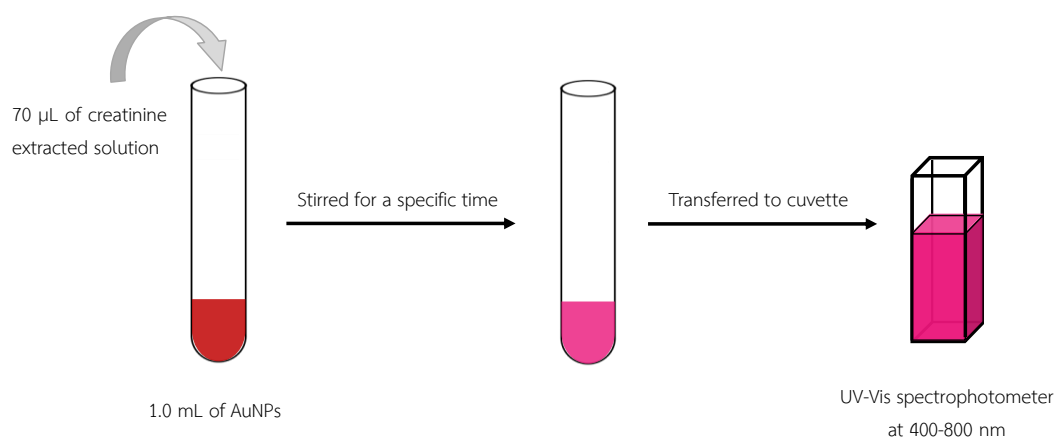
Figure 3.3 The analytical procedure for creatinine detection by AuNPs.

3.8.2 Effect of AuNPs solution concentration

To obtain the suitable AuNPs concentration for creatinine analysis, AuNPs solutions at different concentrations were prepared by diluting a 0.5 mmol/L AuNPs solution with deionized water to get AuNPs solutions with concentration in the range from 0.15-0.5 mmol/L. Subsequently, 70 µL of creatinine standard solutions having different concentrations (10-60 mg/L) was added to AuNPs solutions. The mixture solution was analyzed by a UV-Visible spectrophotometer.

3.8.3 Effect of reaction time between creatinine and AuNPs solution

To study the effect of reaction time between creatinine and AuNPs solutions, a 70 µL of a 25 mg/L creatinine standard solution was mixed with 1.0 mL of 0.25 mmol/L AuNPs solution under vigorous stirring. The absorption spectra of the mixture solution were recorded in the time range of 1 to 10 minutes using a UV-Visible spectrophotometer. The ratios of the absorbance at 600 nm to that at 520 nm ($A_{600/520}$) was observed at different reaction times.

3.9 Method validation

Validation of the proposed AuNPs method for creatinine determination in human urine samples is performed. The accuracy, precision, limit of detection (LOD), and linear range of the proposed method were evaluated under a selected condition.

3.9.1 Calibration curve and linearity

The linearity of the method calibration curve was observed in the concentration range of 15-40 mg/L. Standard solutions of creatinine were extracted following in the extraction procedure and then the extracted solutions were determined by AuNPs solution. The measurement was repeated at least 4 times. The calibration curve for creatinine detection was constructed by plotting absorbance ratio at 600 nm and 520 nm ($A_{600/520}$) versus concentrations of creatinine standard solution.

3.9.2 Limit of detection (LOD)

To obtain the limit of detection (LOD) of this method, the extracted reagent blank were analyzed by the AuNPs method in 10 replicates. The obtained results were used to calculate LOD values gained from the signal that is three times the standard deviation (3σ) of the blank solution signal.

3.9.3 Accuracy and precision

To validate the proposed method, human urine samples were extracted according to the extraction processes as described in section 3.7.1. Analytical recoveries were observed by using spike method. A creatinine standard solution at the concentration of 10 or 15 mg/L was added into urine samples. After sample

extraction by the solid phase, the extracted solutions were analyzed with the AuNPs method under suitable condition. The obtained results from the analysis of spiked and non-spiked urine sample were employed for calculation of percent recovery (% recovery), following equation 3.1 and percent relative standard deviation (% RSD) which represented the accuracy and precision of the proposed method, respectively. Moreover, the extracted solutions were also measured with the Jaffé method and the results were compared to those observed by the proposed method.

$$\text{\% recovery} \quad = \quad \frac{(Xs-Xb)}{S} \times 100 \qquad (3.1)$$

Xs is   the mass of creatinine found in the spiked sample

Xb is   the mass of creatinine found in non-spike sample.

S   is   the mass of creatinine spiked in the sample

3.9.4 Determination of creatinine in human urine samples

For real sample application, two urine samples were collected from Environmental analysis research unit members. The urine samples were diluted 70-fold with 1.0 mmol/L HCl solution before the sample extraction process and analysis by AuNPs solution. The obtained results were compared to results from the standard Jaffé method.

3.10 Paper-based creatinine detection

3.10.1 Preparation of paper platform

In order to prepare the filter paper coated with sulfonic acid group, the mixture solution containing 1.0 mL of deionized water, 1.0 mL of H_2O_2, 1.0 mL of ethanol and 0.5 mL of 3-MPTMS was stirred at room temperature for 24 hours. Afterwards, 40 µL of the obtained solution was dropped onto 1.5×1.5 cm filter paper (Whatman No.1) and dried in a desiccator.

3.10.2 Optimization of paper-based creatinine determination method

The coated filter papers were immersed into 2.0 mL of creatinine standard solution of different concentrations for a specific period of time. Afterwards, the filter paper was washed with deionized water and the Jaffé reaction was used for creatinine determination by dropping 40 µL of alkaline picrate solution onto the filter paper. The alkaline picrate reacted with creatinine adsorbed on the filter paper resulting in color change from yellow to orange-red or red-brown which could be observed by naked eyes. A photo of the filter paper was taken and subjected to Image J software to observe the color intensity. To obtain a suitable condition for creatinine extraction and detection, the influence of extraction time, reaction time, pH of the creatinine solution and interfering species were investigated. Lastly, this method was applied for creatinine determination in human urine samples.

3.10.2.1 Effect of extraction time

To obtain the suitable condition for creatinine extraction by using the filter paper, the extraction time was varied in the range of 2-10 minutes and creatinine concentration ranging from 0-60 mg/L was selected for this study. After creatinine extraction, the filter paper was washed with deionized water before

reacting with alkaline picrate solution. The color intensity of creatinine complex on the filter paper was measured using Image J software.

3.10.2.2 Effect reaction time

After creatinine extraction using the filter paper coated with sulfonic acid group, creatinine adsorbed on the filter paper was determined with the Jaffé reaction. The reaction time between adsorbed creatinine on the paper and alkaline picrate solution on color development was monitored in the time range of 2-10 minutes. The suitable time for this reaction was selected for further study.

3.10.2.3 Effect of sample pH

The effect of sample pH on creatinine extraction was examined in the pH range of 4-7. Creatinine standard solutions at the concentration of 1000 and 3000 mg/L were prepared at solution pH of 4.0, 5.0, 6.0, and 7.0, respectively using NaOH or HNO_3 to adjust pH of the solution. After that, all prepared solutions were diluted 50-fold with 1.0 mmol/L HCl solution prior to the extraction process with the filter paper coated with sulfonic acid group and the color intensity was then measured by Image J software.

3.10.2.4 Effect of interfering species

The effects of interfering species were studied by adding potential interfering substances into creatinine standard solutions having concentration of 1000 and 3000 mg/L to obtain the concentration of 15 g/L urea, 36 and 360 mg/L bovine serum albumin (BSA), and 15 g/L KCl or 1.5 g/L glucose in the solution. The concentration of each interfering species in creatinine standard solution was the maximum concentration possibly found in human urine. Later, the binary mixtures were diluted 50-fold with 1.0 mmol/L HCl solution before creatinine extraction by

the filter paper coated with a sulfonic acid group following the procedure described in section 3.10.2.

3.10.3 Application to human urine samples

Four fresh urine samples were collected from Environmental analysis research unit members and analyzed with the proposed paper-based method. Before the extraction creatinine on the filter paper, urine samples were diluted 50-fold with 1.0 mmol/L HCl solution and then the filter paper coated with sulfonic acid group was immersed into a diluted sample solution. After the filter paper was washed with deionized water, creatinine adsorbed on the filter paper was detected with the Jaffé reaction by dropping alkaline picrate onto the filter paper. After a specific period of reaction time, the color of the paper was observed and analyzed by Image J software. The color intensity (mean gray values) obtained from non-spiked urine samples and spiked urine samples were used to calculate the concentration of creatinine by comparison with standard calibration curve and %recovery and %RSD were evaluated.

3.10.4 The method performance

3.10.4.1 Accuracy and precision

To evaluate the method performance, the spiked sample method was performed under suitable condition by adding creatinine standard solution into urine samples to obtain the spiked concentration of 5 and 10 mg/L in samples. Then creatinine was extracted according to the protocal discribed previously. After taking a photo of the papers and the analysis by Image J software, the obtained results from non-spiked urine samples and spiked urine samples were compared to standard calibration curve and the data were used to calculate %recovery and %RSD.

3.10.4.2 Calibration curve and linearity

In this section, the linearity of the method was investigated in the concentration range of 0-60 mg/L. All creatinine standard solutions were extracted by the filter paper under the suitable condition and detected by the Jaffé reaction. The obtained color intensity (mean gray values) from Image J software was used in the plot against creatinine concentration to construct the calibration curve.

CHAPTER IV

RESULTS AND DISCUSSION

In this research, two colorimetric methods are proposed for determination of creatinine in human urine samples by visual detection or using a UV-Visible spectrophotometer. The first method is based on AuNPs aggregation induced by creatinine leading to a change in the color of AuNPs solution. The second method is a paper-based colorimetric detection using the filter paper coated with sulfonic acid group ($-SO_3H$) for creatinine extraction and detection with the Jaffé method. Therefore, this chapter is divided into two main parts.

4.1 Determination of creatinine by gold nanoparticles solution

For AuNPs method, the sample preparation by solid phase extraction, based on cation exchange was used to separate creatinine from human urine samples. A cation exchanger, sulfonic acid-functionalized silica gel was prepared in our laboratory and used for extraction of creatinine before detection with AuNPs method.
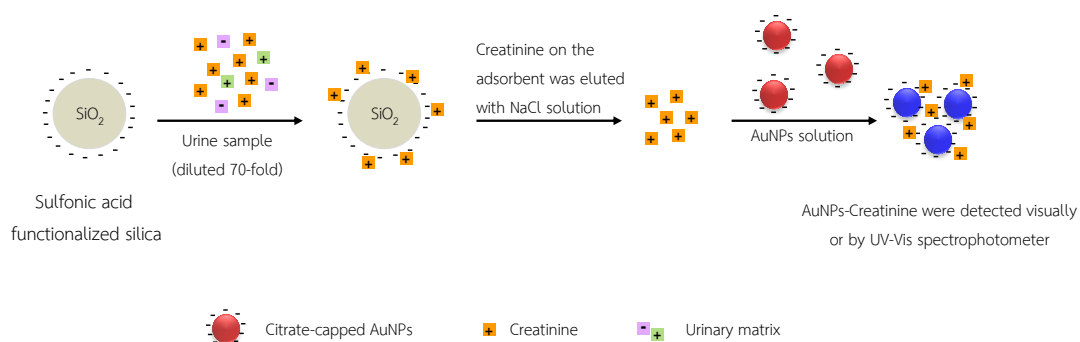
Scheme 4.1 The proposed method of creatinine determination by AuNPs solution.

4.1.1 Synthesis and characterization of sulfonic acid-functionalized silica gel

The sulfonic acid-functionalized silica gel was synthesized by grafting method (Fig. 4.2). Silica gel was mixed with the solution of 3-mercaptopropyltrimethoxysilane (3-MPTMS), ethanol and deionized water. After 5 hours reflux, the functionalized thiol groups on the silica gel were further oxidized by H_2O_2 to convert thiol groups to sulfonic acid groups. Lastly the solid was washed with deionized water and dried in an oven overnight.
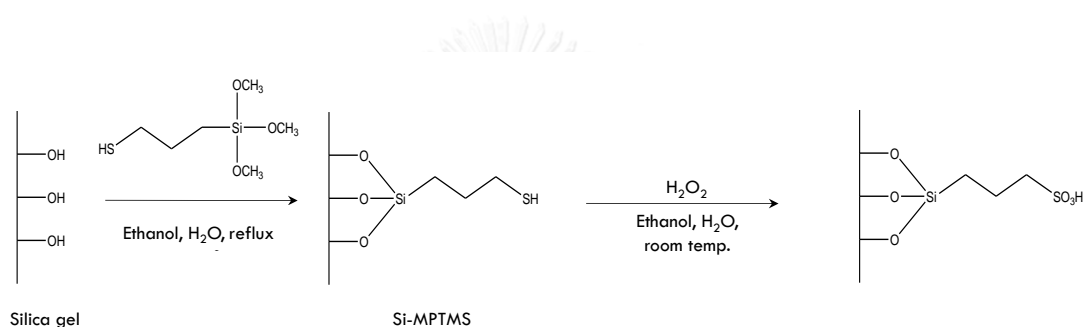
Figure 4.2 Surface modification of silica gel.

The obtained adsorbent was characterized by the thermal gravimetric analyzer (TGA) to verify the presence of organic functional groups on the sulfonic acid-functionalized silica gel. The surface area analyzer was used to determine the surface area, pore size distribution and total pore volume of the functionalized silica gel. Finally, the acid capacity of the adsorbents was determined by the titration technique.

The thermal stability of native silica gel and modified silica gel investigated by TGA was compared. The weight-loss was observed in the temperature range of 30-800 °C at a heating rate of 20 °C/min under nitrogen atmosphere. The thermograms are shown in Fig. 4.3 to 4.4. The weight loss observed at temperature below 100 °C due to the loss of moisture in the adsorbent materials was found in both

thermograms, whereas in the temperature range of 200-580 °C, the profile of the thermograms of silica gel and functionalized silica gel was different, demonstrating different composition of these materials. The weight loss observed at high temperature range could be attributed to the decomposition of organic moiety on the adsorbent including propyl sulfonic acid groups and MPTMS residues [50]. The results indicated that the functionalized silica gel was successfully prepared.
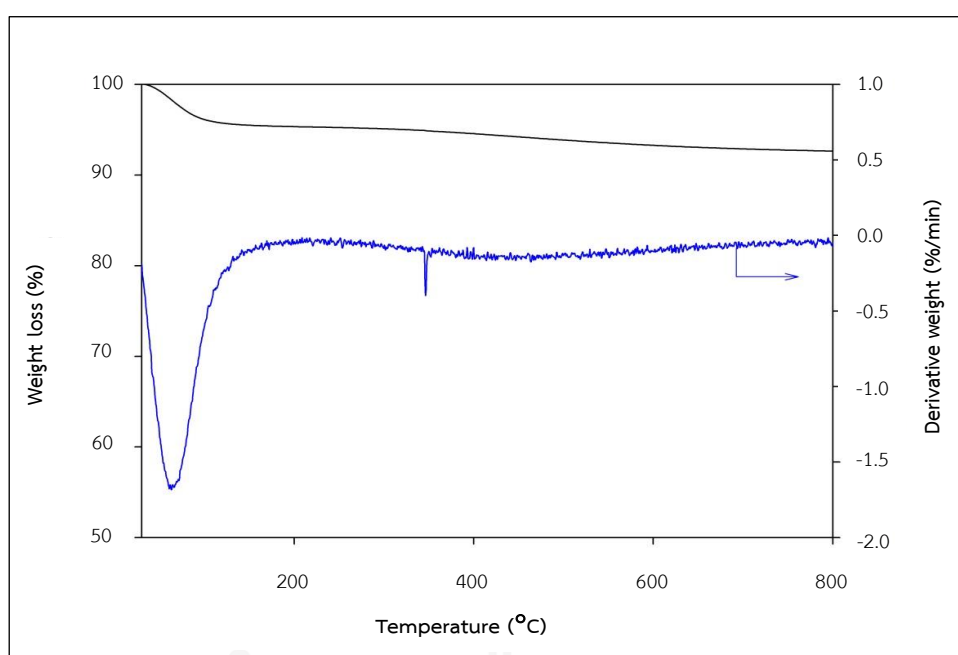
Figure 4.3 Thermogram of the native silica gel.
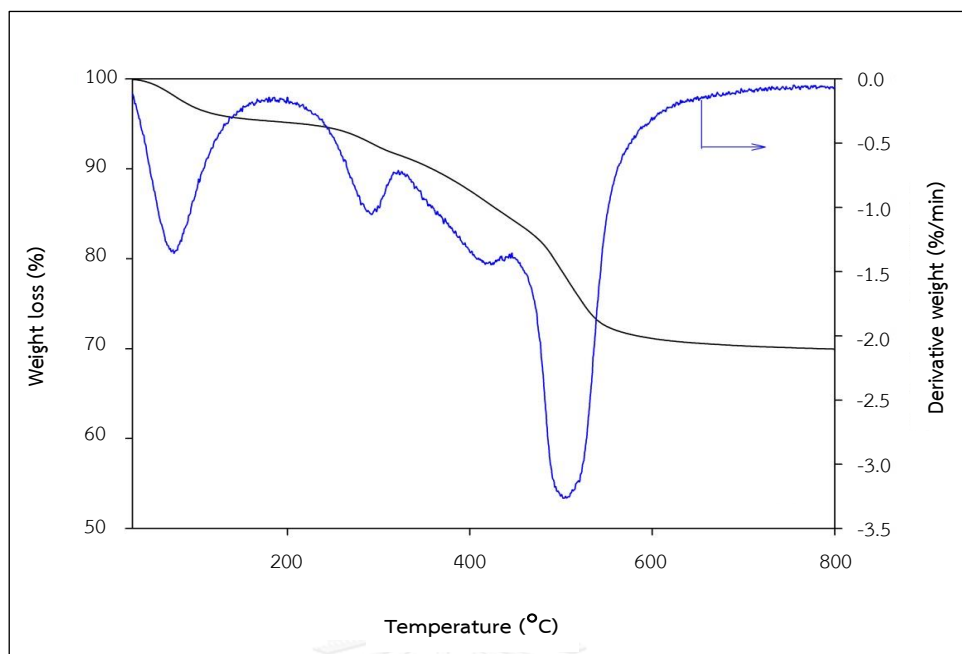
Figure 4.4 Thermogram of the prepared sulfonic acid-functionalized silica gel.

Afterwards, the BET surface area, pore diameter and total pore volume of the adsorbents were determined. The results were calculated from the experimental data of nitrogen adsorption-desorption isotherms at 77 K. The obtained results are shown in Table 4.1. It is clearly seen that the surface area of silica gel decreased from 366.75 to 136.23 m^2/g after the functionalization.

Finally, to confirm the presence of the sulfonic acid functional groups on silica gel surface, the acid capacity of the modified silica gel was determined using acid-based titration technique and then the results were compared with the native silica gel. The obtained results indicate that the acid capacity of native silica gel and synthesized material were 0.003470±0.0003 mmol H^+/g of adsorbent and 0.3580±0.0008 mmol H^+/g of adsorbent, respectively. The acid capacity of the modified silica gel was much higher than that of native silica gel which confirmed the successful functionalization of the sulfonic acid group on the surface of silica gel.

Table 4.1 BET surface area, pore diameter and total pore volume of native silica gel and modified silica gel

Samples	BET surface area (m^2/g)	pore diameter (Å)	Total pore volume (cm^3/g)
Native silica gel	366.7	72.1	0.661
Sulfonic acid functionalized silica gel	136.2	57.7	0.197

4.1.2 Determination of creatinine by AuNPs solutions

To obtain a suitable condition for creatinine detection by AuNPs solution, the influence of detection parameters including the concentration of AuNPs solution and the reaction time between AuNPs and creatinine solution were investigated.

4.1.2.1 Reaction between AuNPs and creatinine solution

In this work, AuNPs were prepared by citrate reduction method and therefore AuNPs would bear negative charge on its surface. The citrate-cap AuNPs solution was in red color. After the solution was analyzed by a UV-Visible spectrophotometer, an SPR absorption band (500-800 nm) of AuNPs solutions displayed a maximum absorption at wavelength of 520 nm, corresponding to the presence of particles in the size of *ca.* 13 nm [32]. After creatinine standard solution was added, the absorption at 520 nm decreased while a new absorption band at longer wavelength (550-800 nm) appeared, demonstrating the aggregation of AuNPs. The positively charged amino group on creatinine would interact with the negatively charged citrate-cap AuNPs through electrostatic interactions, causing a decrease in the inter-particle distances between particles and resulting in AuNPs aggregates. These phenomena led to a change in color of AuNPs solution from red to purple or

to blue depending on the concentration of creatinine (Fig. 4.5). This mechanism was confirmed by TEM images (Fig. 4.6).
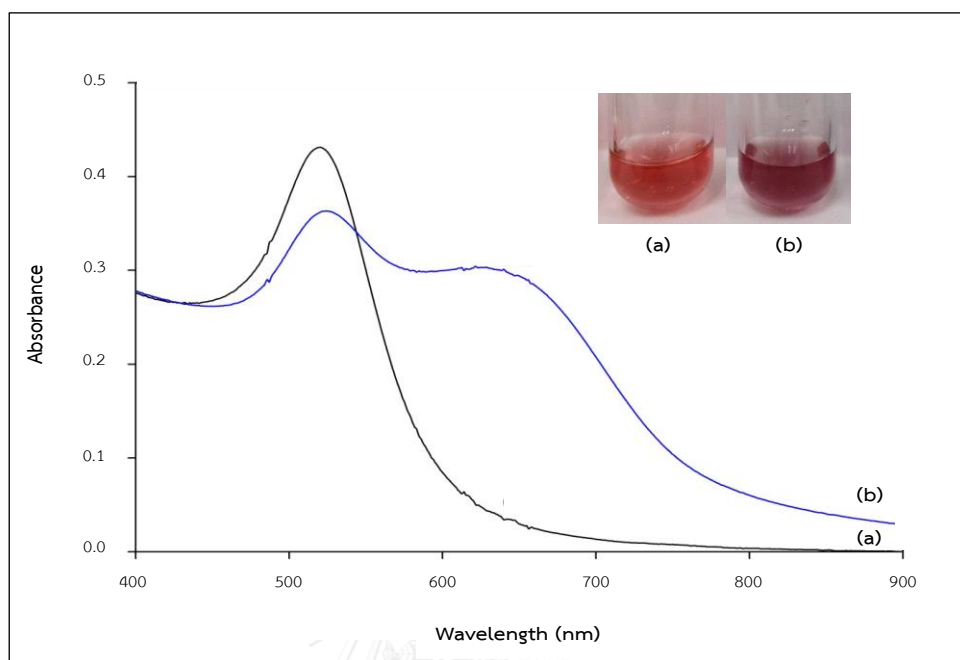
Figure 4.5 UV-Vis absorption spectra of AuNPs solution (a) with and (b) without 60 mg/L creatinine standard solution.
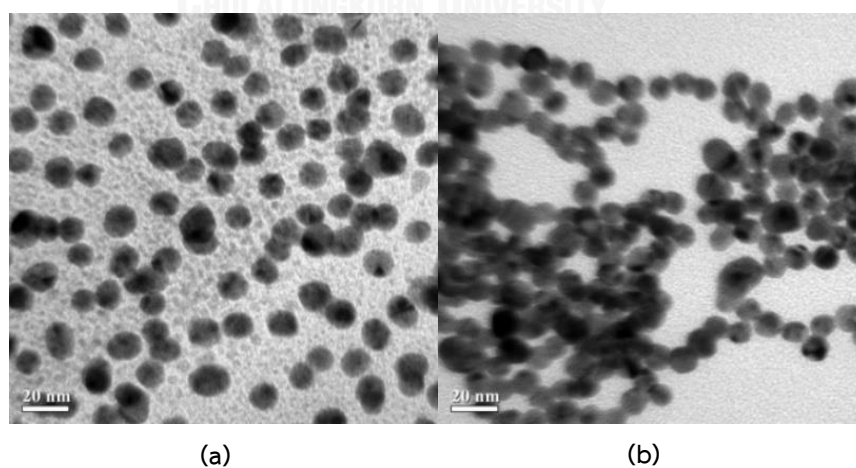
Figure 4.6 TEM images of (a) AuNPs solution and (b) AuNPs solution after adding 60 mg/L creatinine standard solution.

4.1.2.2 Preliminary study of creatinine detection in urine sample

From preliminary study, when a urine sample or a urine sample spiked with creatinine standard solution (700 mg/L) was diluted 70-fold and added into AuNPs solution, there was no change in the absorption band of AuNPs and also the color of the solutions, as seen in Fig. 4.7 (a-c). It indicated that the urinary matrix inhibited the aggregation of AuNPs. Therefore, sample extraction by sulfonic acid-functionalized silica was performed to extract creatinine from urine samples. After adding the extracted solution into AuNPs solution, the absorption at longer wavelength was observed indicating the aggregation of AuNPs and solution color also changed as shown in Fig. 4.7 (d-e). From these results, the sample preparation was necessary in the determination of creatinine by AuNPs.
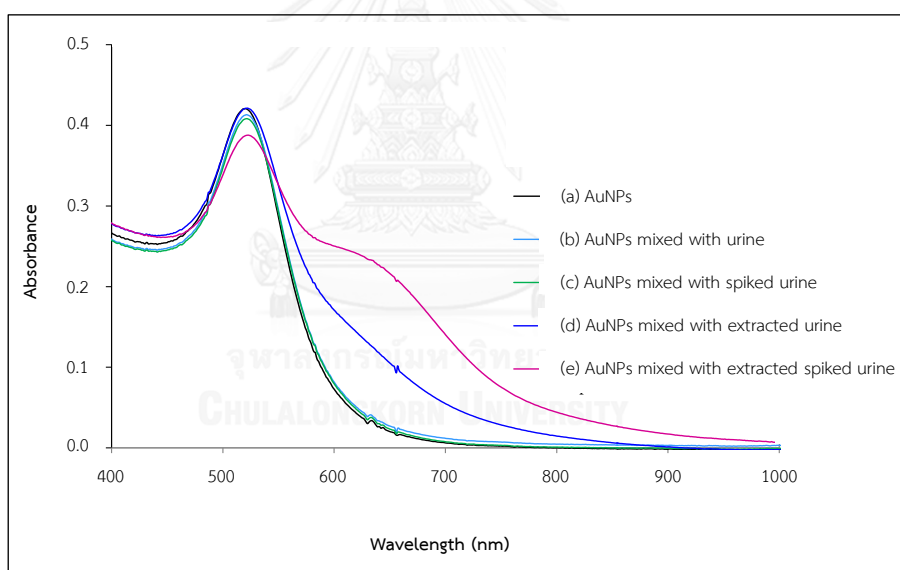
Figure 4.7 UV-Vis absorption spectra of AuNPs solution in the presence of urine sample and urine spiked with creatinine (700 mg/L) before and after solid phase extraction and the solutions color (sample dilution of 70-fold).

4.1.2.3 Effect of AuNPs solution concentration

The effect of AuNPs concentration was investigated to find a suitable condition for creatinine detection. Creatinine standard solutions of various concentrations in the range of 10-60 mg/L were prepared in aqueous solution. The creatinine solution was then added to AuNPs solution having different concentrations (0.15-0.5 mmol/L). It was found that at low concentration of AuNPs, the aggregation of AuNPs occurred slowly and the analysis time was long. When the concentration of AuNPs increased up to higher than 0.3 mmol/L, the aggregation of AuNPs occurred very quickly and AuNPs in solution was then precipitated at high concentration of creatinine. It can be concluded that the concentration of the AuNPs in solution could affect the degree of aggregation of AuNPs which would also have influence on the analytical linear range for creatinine analysis. Therefore, the concentration of AuNPs solution of 0.25 mmol/L was chosen for creatinine detection in further study. It should be noted that by using this AuNPs solution in the analysis of creatinine in NaCl solution, which was used as eluent, the same linear range of the calibration curve was also observed.

4.1.2.4 Effect of reaction time between creatinine and AuNPs solution

From preliminary study, the aggregation of AuNPs occurred immediately after adding creatinine and continuously and the degree of aggregation depended strongly on the reaction time. The effect of reaction time between AuNPs and creatinine solution on the signal development was investigated in the time period ranging from 1 to 10 minutes. When the reaction time increased, the signal intensity of AuNPs solution at 520 nm and at 550-800 nm continually decreased and increased, respectively (Fig. 4.8 inset), and the color of AuNPs solution changed from red to purple and then to blue. The aggregation of AuNPs in solution was monitored using absorbance ratio at 600 and 520 nm ($A_{600/520}$). The obtained $A_{600/520 \ nm}$ data were plotted against the time as presented in Fig. 4.8. The results of the study

showed that the value of the $A_{600/520}$ dramatically increased at the beginning during 1 to 4 minutes, gradually increased from 5 to 7 minutes and remained relatively constant after 7 minutes of reaction time. When constructed calibration curves of creatinine standard solution (15-40 mg/L) by plotting between the concentration of creatinine standard solution and the $A_{600/520}$, it was found that a good linearity of the calibration curve was obtained at the reaction time of 3 minutes. Using long reaction times, AuNPs in solution precipitated at high concentration of creatinine, therefore the linear range of the calibration curve was narrow (Fig. 4.9). The reaction time of 3 minutes was selected for creatinine analysis.
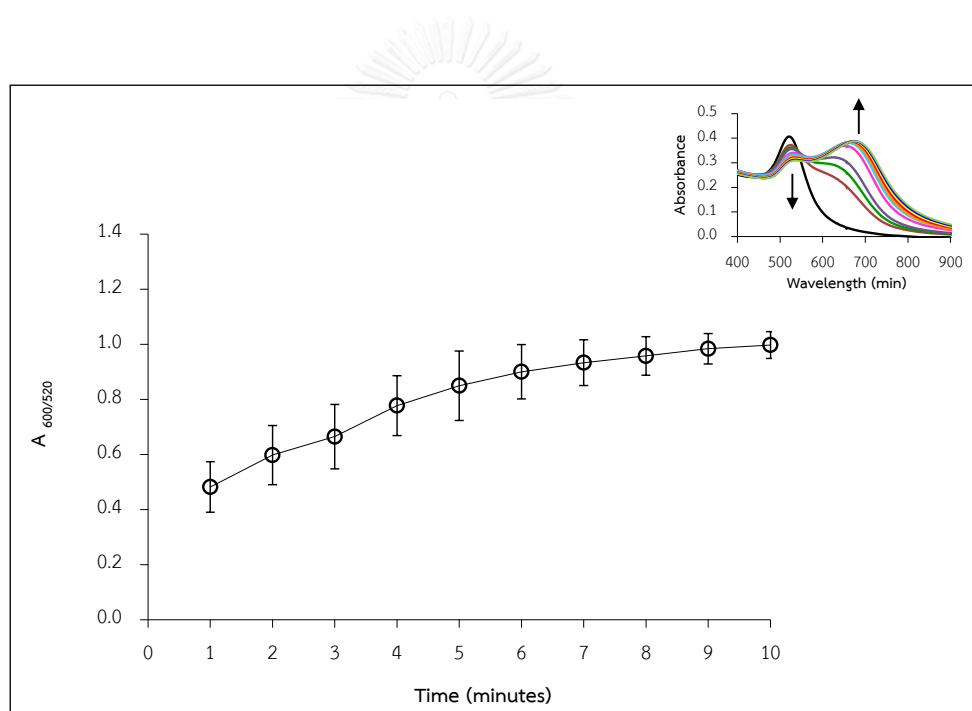
Figure 4.8 The $A_{600/520}$ of AuNPs solution after adding 25 mg/L creatinine solution recorded at 1 to 10 minutes (inset – absorption spectra of the AuNPs solution).
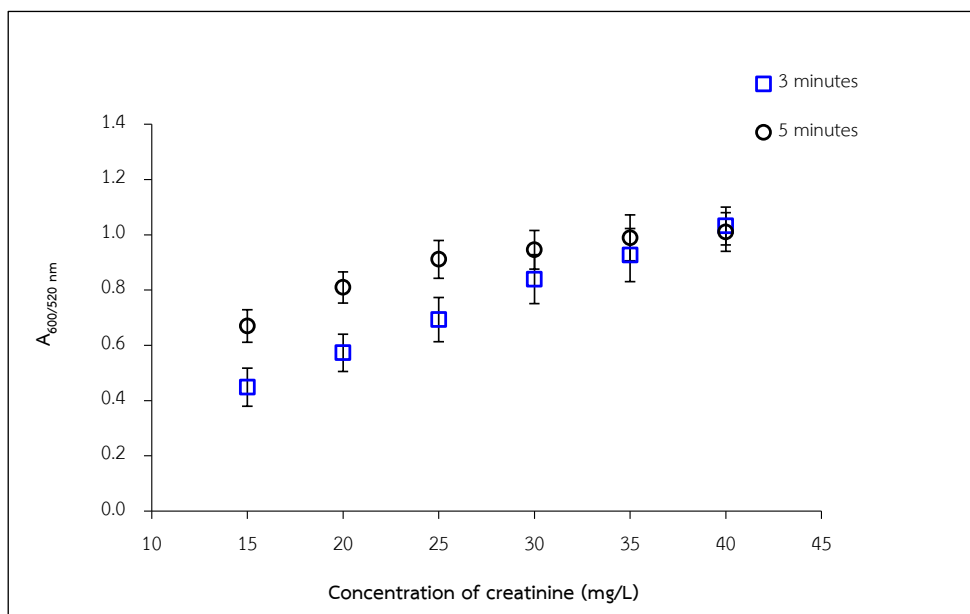
Figure 4.9 The calibration curves of creatinine standard solution (15-40 mg/L) at the reaction time of 3 and 5 minutes.

4.1.3 Creatinine extraction

The influence of various parameters including extraction time, elution time, sample pH and interfering species on the efficiency in creatinine extraction by the solid phase was investigated to obtain a suitable condition for sample extraction.

4.1.3.1 Effect of extraction time and elution time

In this section, the effect of extraction and elution time in the range of 10 to 30 minutes was studied using the sulfonic acid-functionalized silica gel as strong cation exchanger. A creatinine standard solution at the concentration of 16 mg/L was selected to study the effect of this parameter. In the extraction process, creatinine in the standard solution or a urine sample was converted to its cationic form by decreasing pH of solution to *ca.* 3 with HCl solution before extraction via an ion exchange mechanism; therefore the neutral and anionic interfering species in

urine sample solution was eliminated. Afterwards, the adsorbed creatinine on the adsorbent was eluted by sodium chloride solution and detected with AuNPs method using a UV-Visible spectrophotometer to record absorbance signal. The obtained results in Fig. 4.10-4.11 show that the absorbance signals of the AuNPs solutions after adding creatinine extracted at the selected extraction and elution time range were not significantly different at 95% confidence level. Therefore, an extraction time and elution time of 10 minutes was selected for creatinine extraction and elution to reduce the sample preparation time. Under selected condition, approximately 80% of creatinine in solution could be extracted by the adsorbent. Therefore, to construct calibration curve for creatinine quantification, all creatinine standard solutions was undergone the same sample preparation procedure as urine samples before detection with the AuNPs method. For urine samples extraction, human urine samples were diluted 70-fold with 1.0 mmol/L HCl solution before sample extraction process.
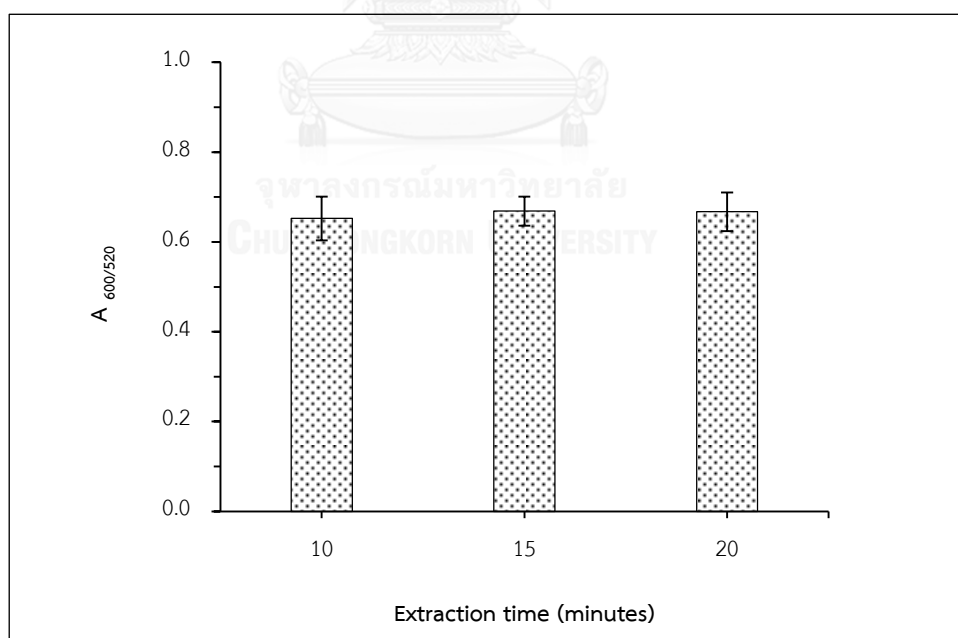
Figure 4.10 The $A_{600/520}$ ratio of AuNPs solution after adding creatinine solution extracted from 16 mg/L creatinine standard solution with different extraction time (elution time of 30 minutes).
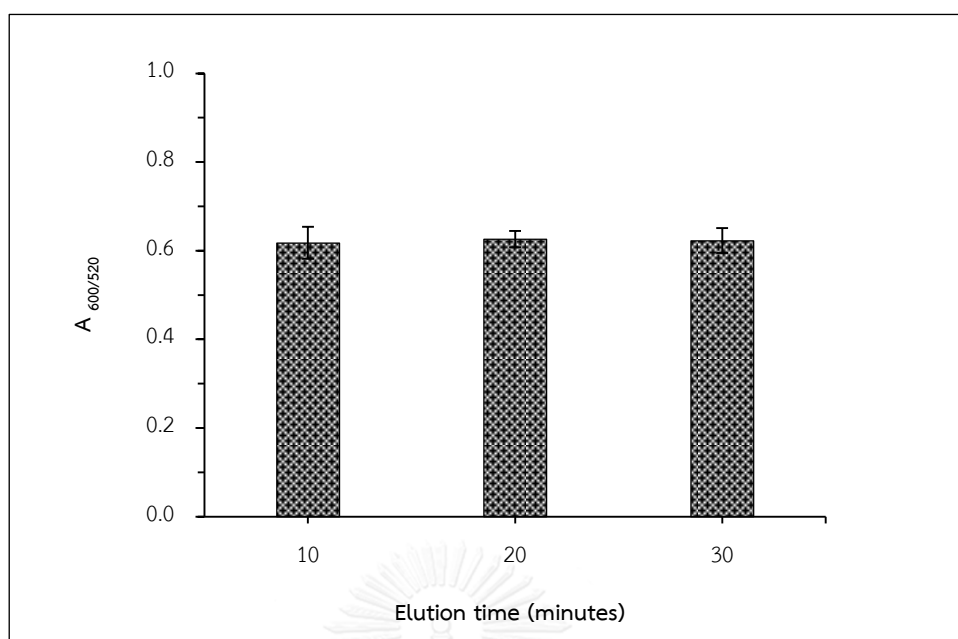
Figure 4.11 The $A_{600/520}$ ratio of AuNPs solution after adding creatinine solution extracted from 16 mg/L creatinine standard solution with different elution time (extraction time of 30 minutes).

4.1.3.2 Effect of initial pH of the creatinine solution

Normally the pH values of human urine are in the range from 4.5 to 8 depending on the time of urine collection and amount of water the person drinks on the day [51]. Therefore, the effect of initial sample pH on the extraction efficiency was monitored in the pH range of 4-7 by preparing creatinine standard solutions with the concentration of 1050 and 2800 mg/L having different pH values. Then, these solutions were diluted 70-fold with 1.0 mmol/L HCl solution before to the extraction process. The obtained results (Fig. 4.12) show that the absorbance singnal of AuNPs solutions was not significantly different at 95% confidence level. The different initial sample pH did not affect creatinine extraction efficiency because the pH of the all solution was controlled by 1.0 mmol/L HCl solution used to dilute the samples and to transform creatinine to its cationic form prior to the extraction procedure.
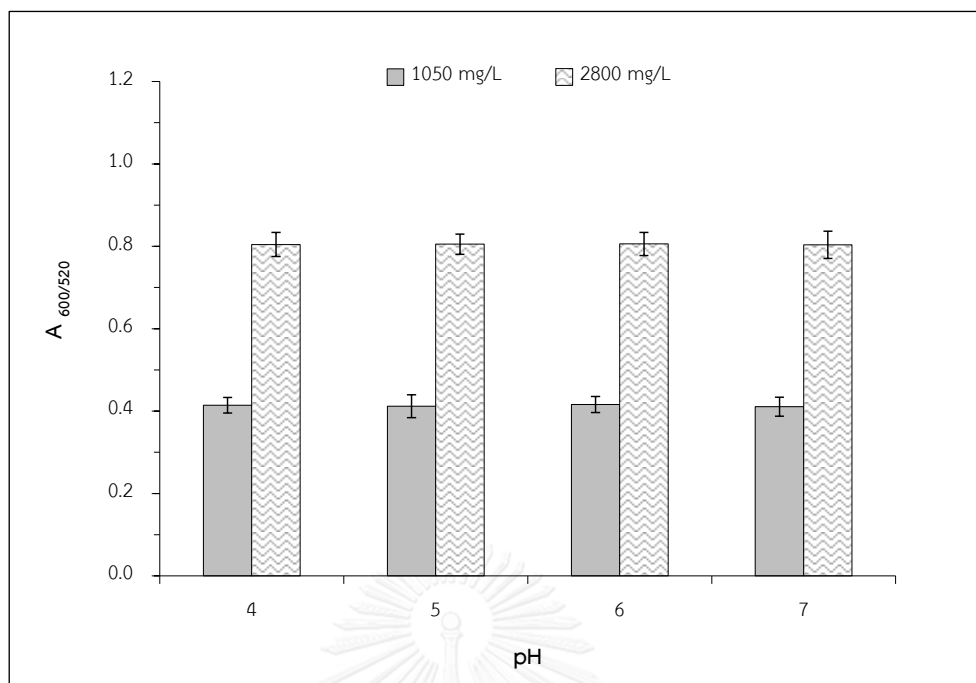
Figure 4.12 The $A_{600/520}$ ratio of AuNPs solution after adding creatinine solution extracted from 15 and 40 mg/L creatinine solution with different pH values.

4.1.3.3 Effect of interfering species

Finally, the effect of potential interferences species including KCl, urea and bovine serum albumin (BSA) on the efficiency of creatinine extraction was investigated. The creatinine standard solution with the concentration of 1050 and 2800 mg/L were chosen in this study. Each of interferences having maximum concentration possibly found in human urine was added into a creatinine standard solution to produce a binary mixture and then the mixture was diluted 70-fold with 1.0 mmol/L HCl solution before sample preparation and detection with the proposed method. The obtained results shown in Fig.4.13 indicate that the signal of AuNPs solutions in the analysis of the extract from creatinine solutions with and without these interfering species were not significantly different at 95% confidence level. In the case of urea (pK^+_{BH} 0.18), in the solution having pH 3.0, urea was present in neutral form and then it would not retain on the adsorbent. Therefore, it did not

have impact on creatinine extraction and detection. Moreover, in a high level of KCl salt condition, the extraction efficiency was also not affected. For the BSA case (pI 4.7), the BSA concentration used in this study was 35 and 350 mg/L which can be found in normal human urine [52]. The absorbance signals of AuNPs solution mixed with extracted solutions from creatinine solution with and without BSA were not significantly different at 95% confidence level. The sample preparation technique by solid phase extraction can eliminate these interfering species.
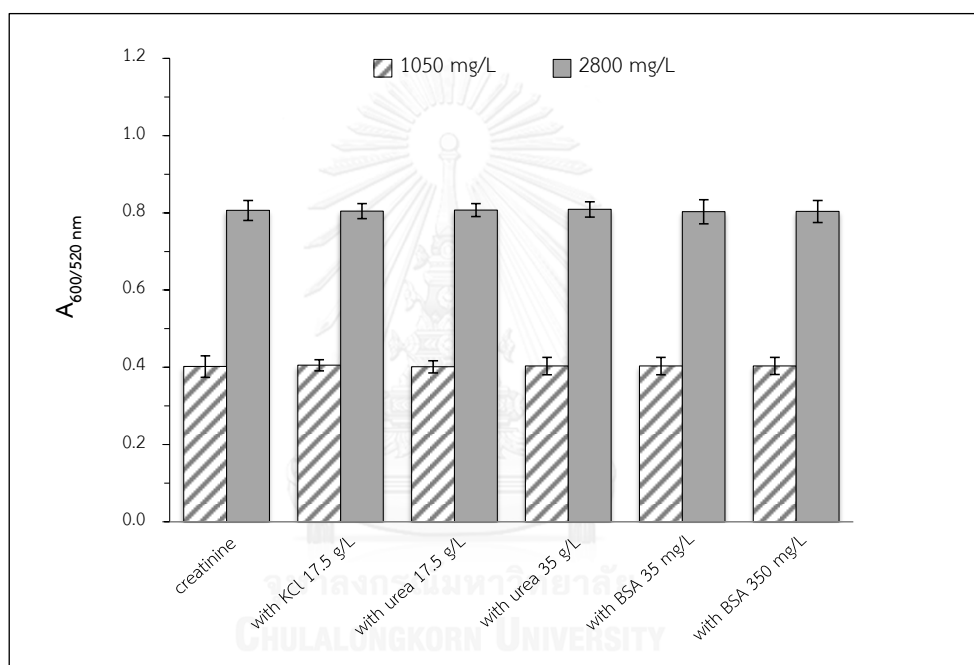
Figure 4.13 The $A_{600/520}$ of AuNPs solution after adding the extracted of creatinine standard solutions at the concentration of 15 and 40 mg/L in the presence of various interfering substances.

To confirm the necessity of solid phase extraction, a BSA solution with a concentration of 5.0 mg/L as presented in diluted urine solution was undergone the extraction process. BSA solution before and after extraction were added to AuNPs solutions. The observed UV-Vis spectra are compared as shown in Fig. 4.14.

From the results, it is clearly demonstrated that BSA could also induce AuNPs aggregation, which would lead to positive false error in creatinine determination. After the sample extraction of BSA solutions, the aggregation of AuNPs was not observed. Therefore, BSA could not affect creatinine detection by AuNPs using the proposed sample preparation method. The effect of other amino acids possibly found in human urine sample was not investigated because of the low concentration of these species in human urine when compared with that of creatinine. Furthermore, their concentration would be reduced after sample dilution.
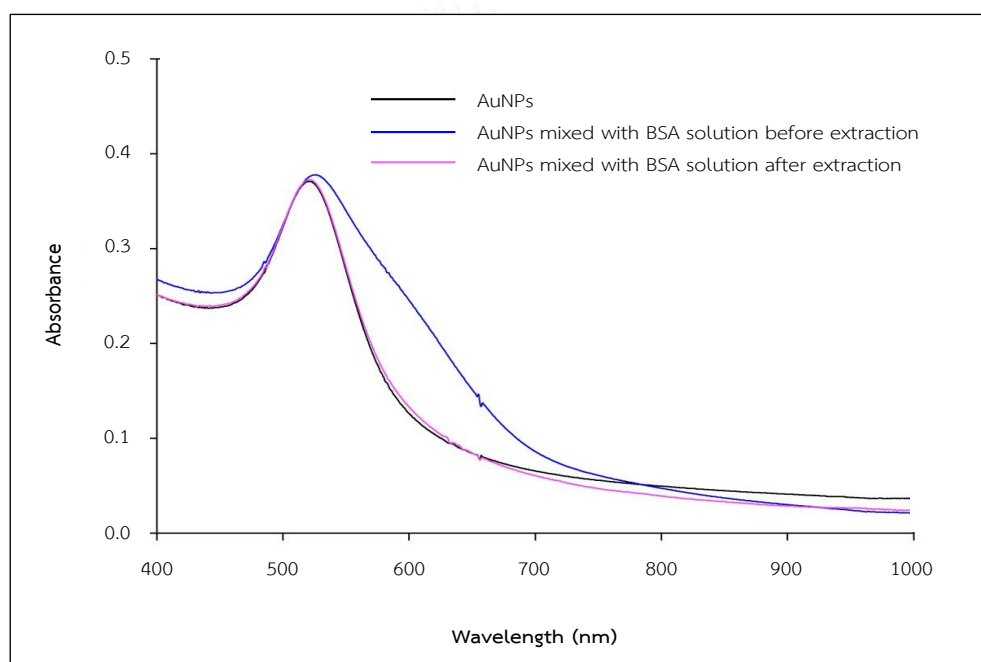
Figure 4.14 UV-Vis absorption spectra of AuNPs solution after adding of 5.0 mg/L BSA solution before and after extraction by the solid phase.

4.1.3.4 Method validation

In this section, the proposed method was validated under the suitable condition; 5.0 mL of sample or standard was extracted with sulfonic acid-functionalized silica gel for 10 minutes and eluted with 5.0 mL of 0.5 mol/L NaCl for 10 minutes. A 70 μL of the extracted solution was mixed with 1.0 mL of 0.25 mmol/L AuNPs solution for 3 minutes before the detection with a UV-Visible spectrophotometer. The validation parameters including the linear range, the limit of detection (LOD), accuracy and precision of the proposed method were observed and the results of urine samples analysis were compared with the standard Jaffé method.

4.1.3.4.1 Calibration curve and linearity

To quantify the concentration of creatinine in urine samples, creatinine standard solution at the concentration in the range of 15-40 mg/L were undergone the sample preparation procedure and analyzed by the AuNPs method. A change in AuNPs solution color could also observed by naked eyes, as shown below. The change in solution color depended on the concentration of creatinine and was related to the change in absorbance signal (Fig. 4.15). The calibration curve was plotted between the absorbance ratio of 600 nm to 520 nm ($A_{600/520}$) of AuNPs solution and the concentrations of creatinine as illustrated in Fig. 4.16. The calibration plot exhibited a good linear relationship over the working concentration range of 15-40 mg/L with the linear regression equation of y = 0.021x + 0.083 and correlation coefficients (R^2 value) of 0.9980. Unfortunately, this working range did not cover the low level of creatinine in human urine but the high level of creatinine.
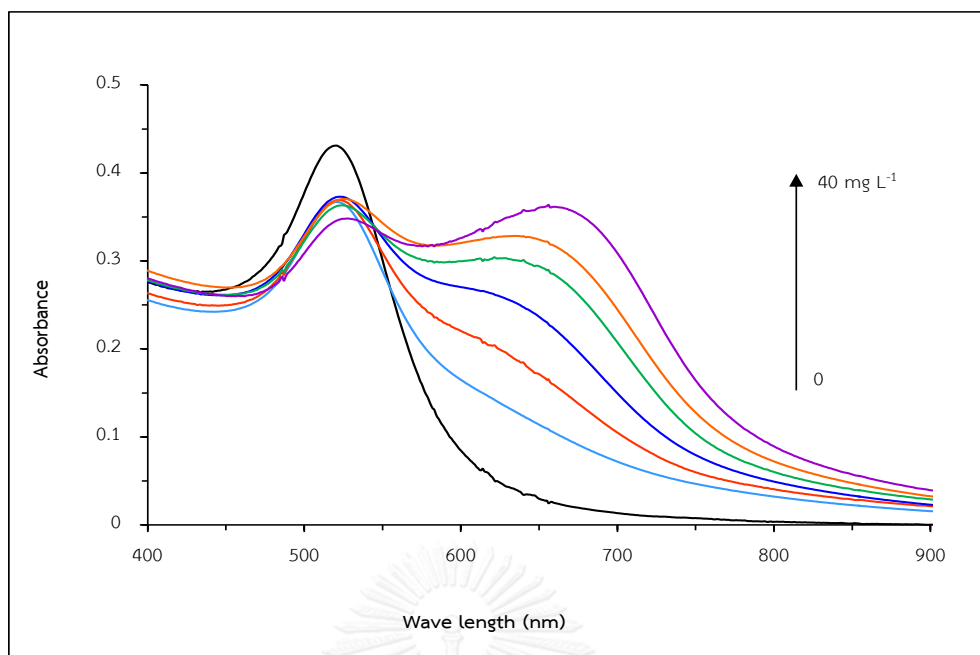
Figure 4.15 UV-Vis absorption spectra of AuNPs solutions in the presence of creatinine in the concentration range of 0-40 mg/L and color chart of AuNPs solution for naked eyes observation.
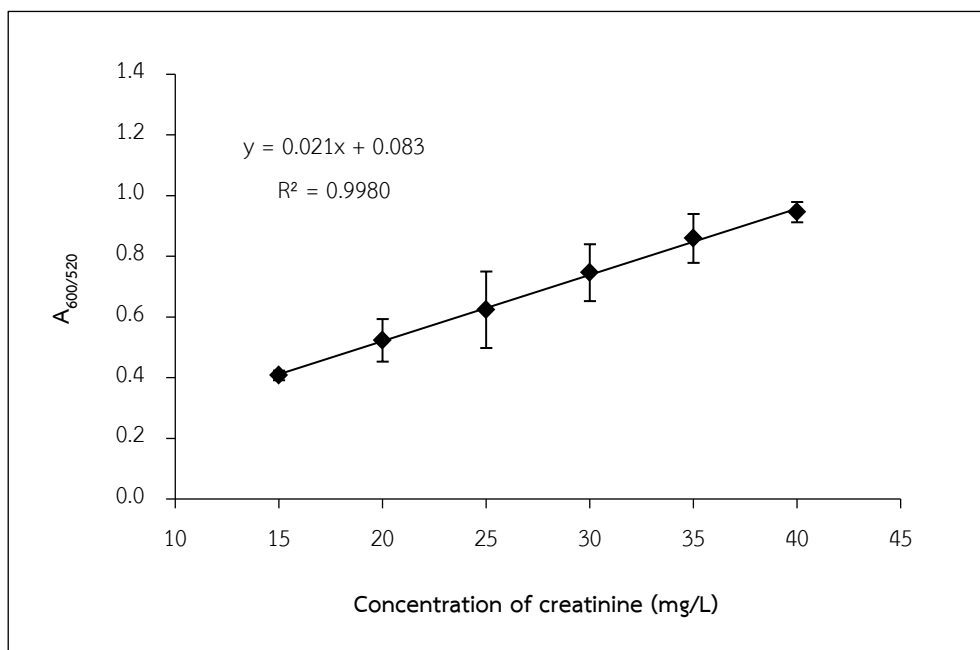
Figure 4.16 The calibration curve for creatinine determination.

4.1.3.4.2 Limit of detection

The limit of detection (LOD) of the proposed method was achieved from the measurement of the blank solution. The blank solution was obtained from the extraction of 1.0 mmol/L HCl solution following the extraction procedure as described in section of 3.7 under the suitable condition. The LOD value of the proposed method obtained by comparing the signal (which has the signal to noise ratio three times of the standard deviation (3σ) of the blank solution signal) to the standard curve and it was found to be 13.7 mg/L.

4.1.3.4.3 Accuracy and precision of urine sample analysis

The accuracy of the proposed method was evaluated by using the spike method. Under suitable condition, creatinine standard solution with a concentration of 10 or 15 mg/L was spiked into diluted urine sample prior to the sample extraction process. The accuracy and precision of these methods are presented in term of %recovery and %RSD, respectively.

Human urine samples were collected from Environmental analysis research unit (EARU) members. Fresh urine samples were diluted 70-fold with 1.0 mmol/L HCl solution before the extraction by the prepared adsorbent and then the creatinine extracted were detected with the proposed method and compared with the standard Jaffé method. The results of urine analysis by the AuNPs method and the standard Jaffé method were not significantly different at 95% confidence level (Table 4.2). The recoveries of creatinine from spiked samples were found in the range of 96.1–104.5% and 98.6–100.2% when used the proposed method and the standard Jaffé method, respectively. The relative standard deviation (%RSD) of results obtained by using the proposed method was found in the range of 5.6–9.7% and 2.1–3.5% by the standard Jaffé method. These results demonstrate that accuracy and precision of the proposed method are acceptable for determination of creatinine in human urine samples [53].

Table 4.2 The recovery of creatinine in urine samples, as determined by the AuNPs and the standard Jaffé methods after sample preparation by solid phase extraction

Samples*	Amount added (mg/L)	AuNPs method			Jaffé method		
		Found (mg/L)	Recovery (%)	RSD (%)	Found (mg/L)	Recovery (%)	RSD (%)
Urine 1	0	16.1±1.2	—	—	17.6±0.4	—	—
	10	26.1±1.8	99.3±9.4	9.5	27.5±0.4	98.7±2.1	2.1
	15	30.6±2.0	96.1±8.2	8.5	32.4±0.2	98.6±3.4	3.4
Urine 2	0	18.8±2.4	—	—	17.9±0.5	—	—
	10	29.5±2.3	104.5±5.9	5.6	27.9±0.4	100.2±2.1	2.1
	15	33.2±1.8	97.3±9.4	9.7	32.9±0.3	100.1±3.5	3.5

* Samples were 70-fold diluted.

4.2 Paper-based creatinine detection

4.2.1 Determination creatinine by paper-based

In this section, the filter paper (1.5×1.5 cm) was coated with 3-propylsulfonic acid trimethoxysilane and used to extract creatinine in solution. Before sample extraction by the paper, creatinine solution or sample was diluted with 1.0 mmol/L HCl solution to convert creatinine to its protonated form. Therefore, creatinine can be extracted onto the paper via an ion exchange mechanism between cations on the filter paper and creatinine cations. After the extraction process, the filter paper was washed with deionized water and then creatinine on the paper was detected with the Jaffé reaction by dropping an alkaline picrate solution onto the filter paper. The color of the filter paper changed from yellow to orange-red or red-brown due to the presence of complex of creatinine and picrate that could be observed by naked eyes. In order to quantify the amount of creatinine adsorbed on the filter paper, a photo of the filter paper was taken and subjected to Image J software to obtain the color intensity (mean gray values). The color intensities were used to construct a standard calibration curve for creatinine determination. Finally, this method was applied for creatinine determination in human urine samples. The process of the proposed method was schematically presented in Fig. 4.17.
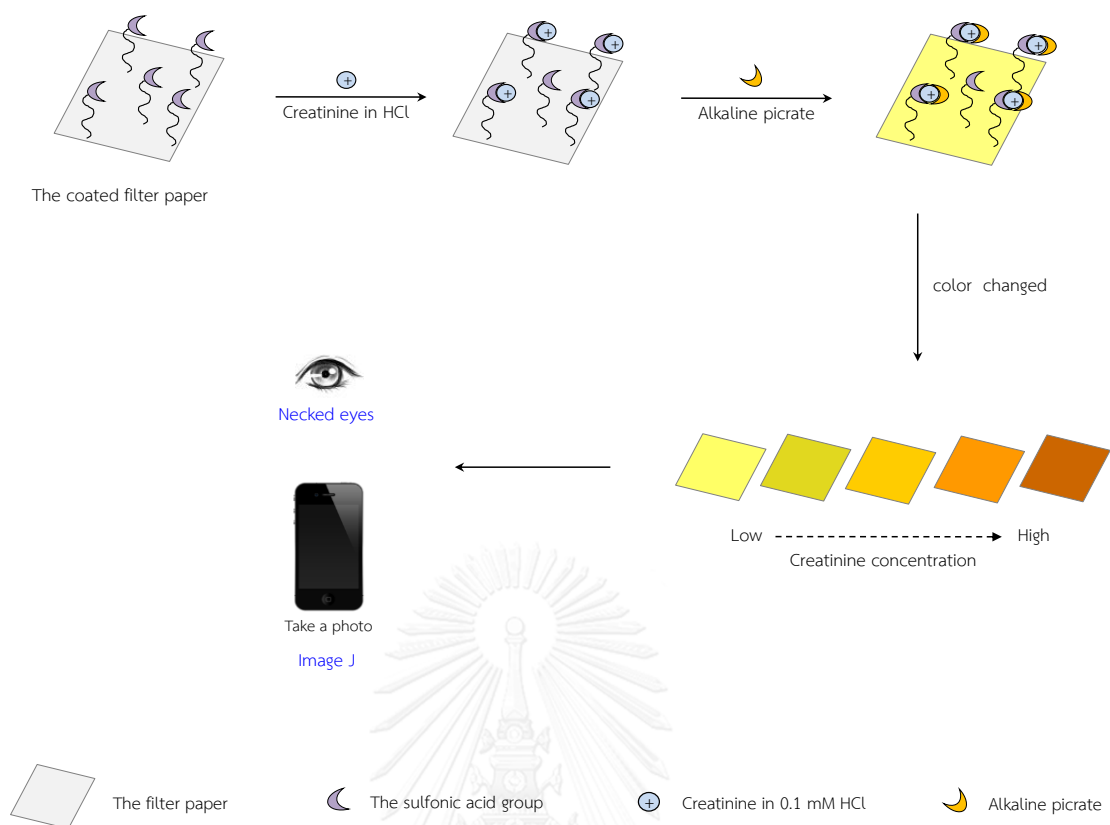
Figure 4.17 The proposed paper-based method for creatinine determination.

4.2.2 Effect of extraction time

To obtain a suitable condition for creatinine extraction by using the coated filter paper, the extraction time was varied from 2 to 10 minutes. The creatinine concentrations in the range of 0-60 mg/L were chosen for this study. After creatinine extraction and detection with the Jaffé reaction, the intensity of complex color on paper was determined by Image J software. In general observations, when the yellow color of paper became dark yellow and brown as the concentration of creatinine solution increased, the color intensity determined by Image J as mean gray values would decrease as shown in Fig. 4.18. When the extraction time was varied, the color intensity was slightly decreased when the extraction time increased. However, being immersed in solution with long extraction time, the filter paper became too soft to handle. Therefore, long extraction time was not suitable for creatinine

extraction by the coated filter paper. Extraction time of 2 minutes was selected for the method.
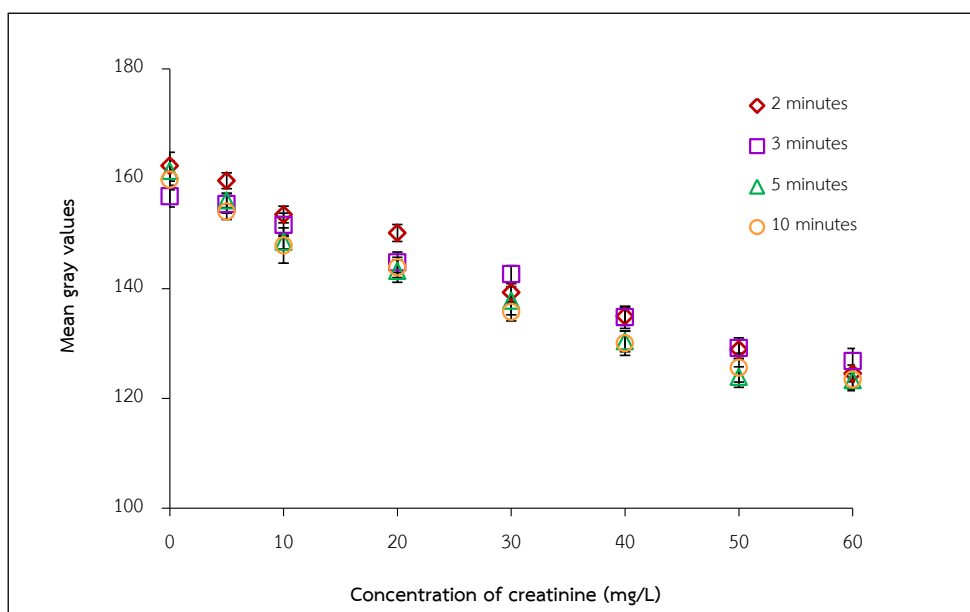
Figure 4.18 The color intensity of creatinine complex on paper after extraction of creatinine in solutions with various times (2–10 minutes); the concentration of creatinine standard solution in the range of 0-60 mg/L.

4.2.3 Effect of reaction time between creatinine adsorbed on paper and alkaline picrate

After a series of solutions containing 0 to 60 mg/L creatinine was extracted by the coated filter paper for 2 minutes and the creatinine on the paper were detected with the standard Jaffé method. The reaction time between creatinine and alkaline picrate solution on color development was monitored in the range from 2 to 10 minutes. After dropping the alkaline picrate solution onto the paper, the yellow brown color was developed and the color became darker when reaction time increased. On the other hand, the intensity of complex color as mean gray values

obtained from Image J software decreased. The results are shown in Fig. 4.19. At high concentration of creatinine, when reaction time of longer than 2 minutes was applied, the color intensity was significantly lower, indicating that the complex could form in higher extent. Reaction time of longer than 3 minutes did not yield significant change in the color intensity. To reduce the analysis time, the reaction time of 3 minutes was selected for further study.
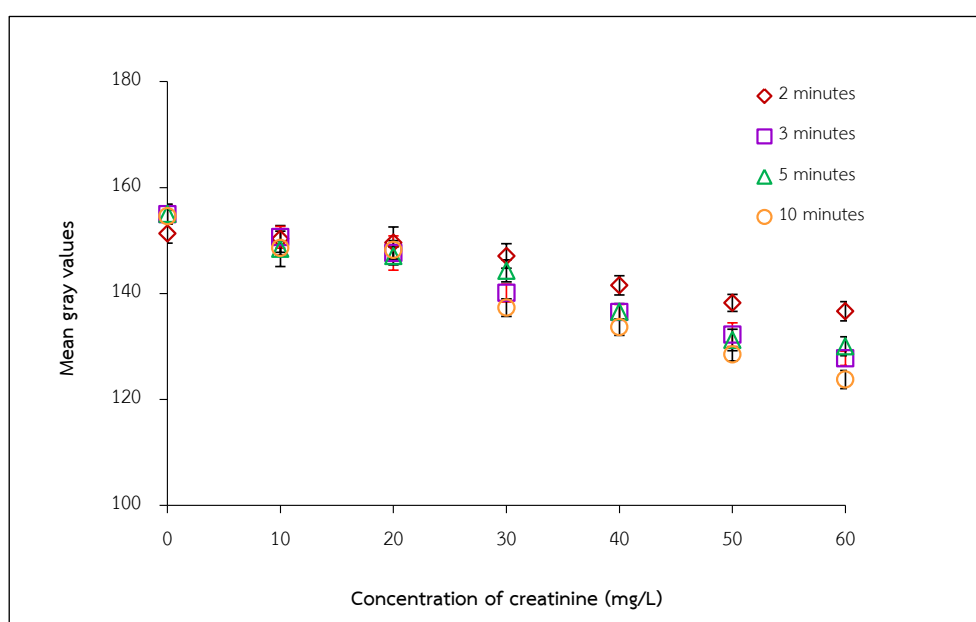
Figure 4.19 The color intensity of creatinine complex on papers developed by using various reaction times between alkaline picrate solution and creatinine extracted from standard solutions (0-60 mg/L).

4.2.4 Effect of sample pH

In addition, the effect of initial pH of the solution on creatinine extraction was investigated by adjusting the pH of creatinine standard solutions (1000 and 3000 mg/L) to the pH values in the range of 4-8. Afterwards, all prepared solutions were diluted 50-fold with 1.0 mmol/L HCl solution before to the extraction and detection

process following previously mentioned procedure. The obtained results are shown in column chart in Fig. 4.20. Despite of different solution pH, there was not significant difference in the color intensity observed which was also confirmed by the photo of the papers color. It is probably because the pH of solution after sample dilution with 1.0 mmol/L HCl solution was relatively constant and hence creatinine could be converted to its cationic form.
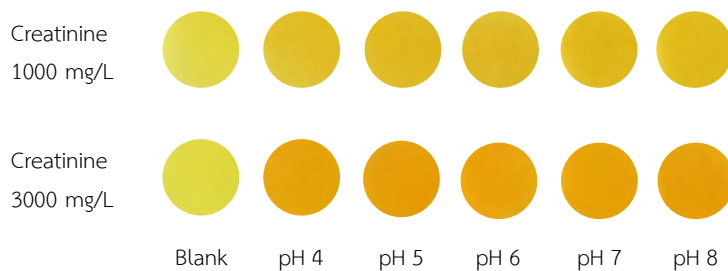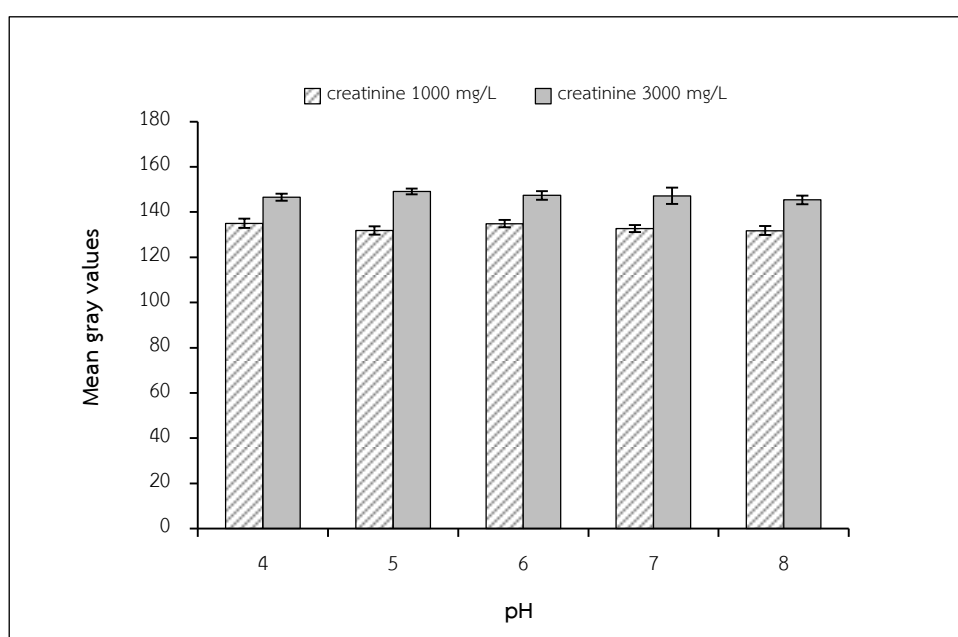
Figure 4.20 The color intensity and chart of paper color observed in the detection of creatinine in standard solutions (1000 and 3000 mg/L) having different initial solution pH.

4.2.5 Effect of interfering species

Finally the effect of interfering species possibly found in urinary matrix on the efficiency in creatinine extraction and detection was studied in binary system. In this section, the experiment was performed into two systems; standard solution system and urinary system. Each of interfering substances including 15 g/L urea, 36 and 360 mg/L bovine serum albumin (BSA), and 15 g/L KCl and 1.5 g/L glucose was added into urine samples and creatinine standard solutions having the concentration of 1000 mg/L. Later, all binary mixtures were diluted 50-fold before creatinine extraction following the procedure described in section 3.10.2. Figure 4.21 shows the results achieved from Image J software, the intensity of color on the paper with and without interfering species was not different which was confirmed by the photo of the paper. The same results were also observed in urinary samples spiked with interfering species as presented in Fig. 4.22. In both systems, all interfering species did not affect creatinine extraction and determination.

In the case of glucose (pK_a 12.28) and urea (pK^+_{BH} 0.18), they did not affect creatinine extraction because in solution of pH 3 (pH of solution diluted with 1.0 mmol/L HCl before extraction by the filter paper), these species were present in neutral form and hence, they were not extracted on the coated filter paper. Moreover, the presence of high concentration of KCl salt did not have effect on creatinine extraction. For the effect of BSA on creatinine extraction, at the selected concentration possibly found in human urine, BSA seems not to have influence on creatinine extraction.
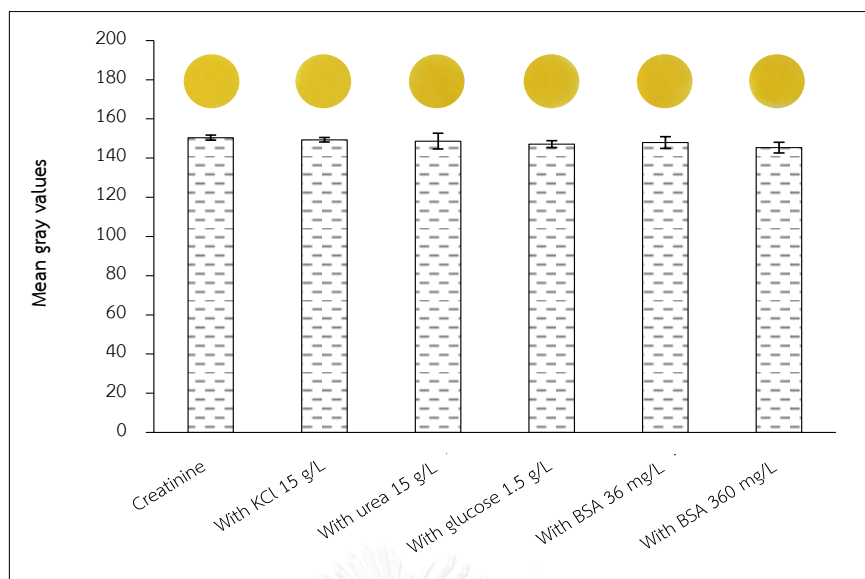
Figure 4.21 The color intensity and chart of paper color observed in the detection of creatinine in standard solution (1000 mg/L) in the absence (creatinine blank) and in the presence of various interfering substances.
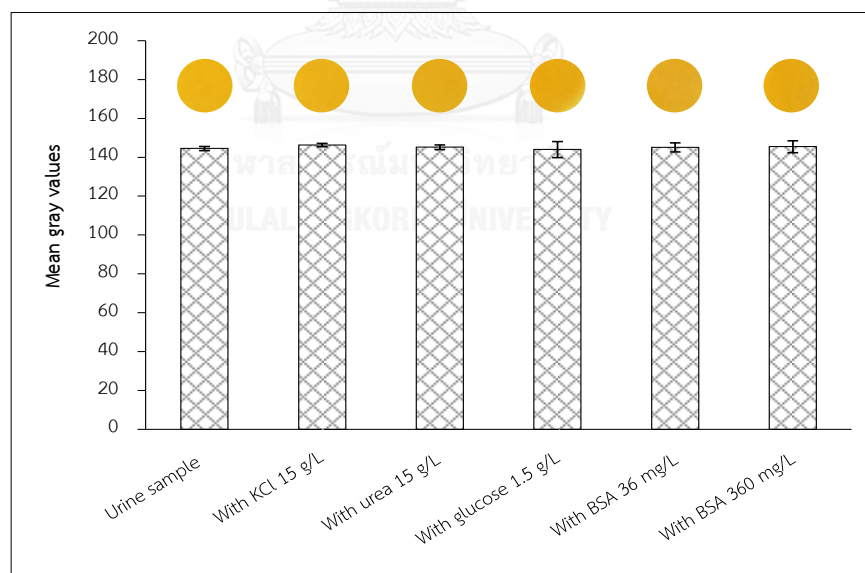
Figure 4.22 The color intensity and chart of paper color observed in the detection of urine sample in the absence (urine sample) and in the presence of various interfering substances.

4.2.6 Method performance

In this section, the proposed method was validated under the chosen condition; 2.0 mL of sample or standard solution was extracted with the coated filter paper for 2 minutes and detected with the standard Jaffé reaction by dropping 40 µL of alkaline picrate solution onto the paper. After reaction time of 3 minutes, the color of the filter paper was observed by naked eyes. A photo of the paper was taken and subjected to Image J software to determine the color intensity. The method performance including the linear range, accuracy and precision were evaluated and the results of urine sample analysis by the proposed method were compared with the standard Jaffé method.

4.2.6.1 Calibration curve and linearity

The linear relationship between paper color intensity (mean gray values) and creatinine concentrations was observed in the creatinine concentration range of 0-60 mg/L. The calibration curve is shown in Fig. 4.23. The chart of color for naked eyes detection is also presented.
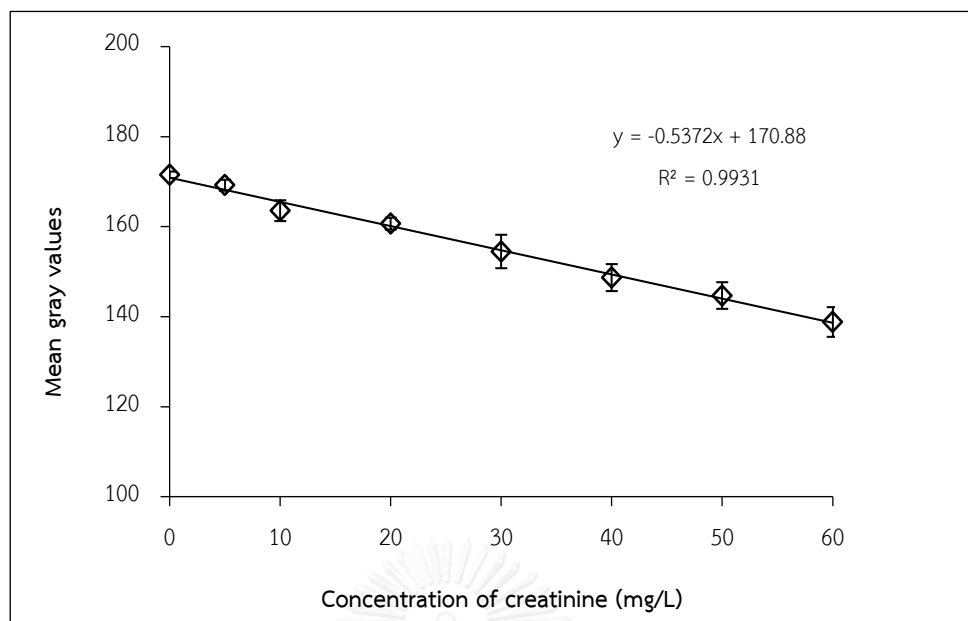
Figure 4.23 The calibration curve for creatinine determination.

4.2.6.2 Accuracy and precision in urine sample analysis

To evaluate the accuracy of the method, the spiked method was used under suitable condition. Creatinine at the concentration of 5 and 10 mg/L was spiked into 50-fold diluted urine samples prior to the sample extraction and detection by the paper-based method. The results were compared to those observed by using the standard Jaffé method. The accuracy and precision of these methods are presented in term of %recovery and %RSD.

For human urine detection, fresh urine samples were obtained from Environmental analysis research unit (EARU) members. The results obtained from urine analysis by the paper-based method and the standard Jaffé method was not significantly different at 95% confidence level. The results in Table 4.3 show that the recovery of creatinine from samples analyzed by the paper-based method using

Image J software was found in the range of 91.9 to 113.8% and 101.2 to 109.5% for the standard Jaffé method. The relative standard deviation of the results obtained from the proposed method and the standard Jaffé method was 2.7-8.6% and 0.2-5.4%, respectively. The colorimetric detection of paper-based results obtained by naked eyes, by comparing the paper color after sample analysis with the standard color is summarized in Table 4.4.

Table 4.3 The recovery of creatinine analysis in urine samples, as determined by paper-based and standard Jaffé methods after sample preparation by solid phase extraction

Samples*	Amount added (mg/L)	Paper-based method			Jaffé method		
		Found (mg/L)	Recovery (%)	RSD (%)	Found (mg/L)	Recovery (%)	RSD (%)
Urine 1	0	12.0±0.3	—	—	10.2±0.1	—	—
	5	17.3±0. 5	106.0±4.7	4.4	15.4±0.1	103.4±1.5	1.4
	10	23.3±1.0	112.4±6.8	6.0	20.4±0.2	101.2±2.2	2.2
Urine 2	0	29.1±1.0	—	—	27.5±0.1	—	—
	5	33.7±1.1	91.9±3.6	3.9	32.6±0.1	102.0±1.2	1.2
	10	38.5±1.2	94.6±4.4	4.6	37.9±0.1	104.8±0.2	0.2
Urine 3	0	35.8±1.6	—	—	39.3±0.6	—	—
	5	41.2±2.1	106.3±9.2	8.6	44.3±0.9	109.5±5.9	5.3
	10	45.3±2.0	94.5±3.2	3.4	49.9±0.0	106.8±5.8	5.4
Urine 4	0	12.4±1.7	—	—	11.6±0.1	—	—
	5	17.9±1.3	108.6±7.4	6.8	16.8±0.3	104.2±1.5	1.4
	10	23.8±2.0	113.8±3.1	2.7	21.9±0.2	102.8±1.6	1.6

*Samples were 50-fold diluted

Table 4.4 The colorimetric detection of paper-based by naked eyes by comparing with the chart of color of creatinine

Samples*	Non spiked sample			Spiked level					
				5 mg/L			10 mg/L		
	Paper color	Concentration (mg/L)		Paper color	Concentration (mg/L)		Paper color	Concentration (mg/L)	
		Naked eyes detection	Standard method		Naked eyes detection	Standard method		Naked eyes detection	Standard method
Urine 1		5-10	10.2		10-20	15.4		20-30	20.4
Urine 2		10-20	27.5		30-40	32.6		40-50	37.9
Urine 3		30-40	37.9		40-50	39.3		50-60	44.3
Urine 4		5-10	11.6		10-20	16.8		20-30	21.9

*Samples were 50-fold diluted

For the naked eyes detection of spiked samples, the intensity of color on the paper related to the concentration of creatinine. The values observed by naked eyes were in agreement with the values observed by the standard Jaffé method.

CHAPTER V

CONCLUSION

5.1 Conclusion

In this work, two colorimetric methods namely AuNPs method and paper-based method are proposed for determination of creatinine in human urine samples. The detection method can be either naked eyes detection or a UV-Visible spectrophotometer. Sample extraction by solid phase based on cation exchange principle was introduced to overcome the effect of urinary matrix before the determination with both methods.

For the AuNPs method, functionalized silica gel was used for sample preparation. Silica gel was grafted with 3-MPTMS followed by the oxidation of the thiol group (-SH) by H_2O_2 solution to the sulfonic acid group (-SO$_3$H). The obtained adsorbent was characterized by TGA, surface area analysis and the titration technique. The results confirmed the successful of surface functionalization. Before creatinine extraction, creatinine was transformed to its cationic species by decreasing sample pH to acidic pH and subsequently extracted on sulfonic acid-functionalized silica through cation exchange mechanism. The creatinine adsorbed on the adsorbent was eluted with NaCl solution followed by reaction with citrate-capped gold nanoparticles (AuNPs). The negative charge on AuNPs surface could interact with the positively charged creatinine through electrostatic interaction, resulting in the assembly of AuNPs and a change of the color of AuNPs solution from red to purple and then blue depending on the creatinine concentration, which could be observed by naked eyes. The surface plasmon absorption band shifted towards longer wavelengths when creatinine concentration increased which could be measured by a UV-Visible spectrophotometer. The morphology of AuNPs and AuNPs aggregates was characterized by TEM method. In the proposed method, the sample extraction time and elution time was 10 minutes. An extract was mixed with 0.25 mmol/L AuNPs for 3 minutes prior detection with a UV-Visible spectrophotometer. The study

of the effect of sample pH and interfering ions show that the solutions pH did not have effect on creatinine extraction by the strong cation exchanger. Finally, the obtained condition was applied to extract creatinine in urine samples and determine by the AuNPs method compared with the standard Jaffé method. The results from the proposed method showed a good linear correlation with the linear range of 15-40 mg/L. The recoveries were in the range of 96.1-104.5% and the relative standard deviation was 9.7%. The results obtained from the proposed were not significantly different from the standard method. This method could be applied to determine the creatinine in human urine samples with short analysis time and acceptable accuracy and precision. The detection limit (LOD) of the proposed method was found to be 13.7 mg/L.

For the paper-based method, the modified filter paper was used as a platform for creatinine extraction and detection. The surface of the filter paper was coated with sulfonic acid groups before being applied to extract creatinine via an ion-exchange mechanism. After the extraction process, the creatinine adsorbed on the filter paper was detected with the Jaffé reaction by dropping an alkaline picrate solution onto the filter paper to generate the color product which could be observed visually. The photo of filter paper was taken and the color intensity determined by Image J software was used to construct calibration curve for quantification. The obtained results were compared to the standard Jaffé method. To achieve a suitable condition for creatinine extraction and detection, the influence of extraction time, reaction time, pH of the creatinine solution and interfering species were investigated. The extraction time of 2 minutes and reaction time of 3 minutes were selected for the method. For the effect of initial pH of the solution, the results showed that sample pH did not affect creatinine extraction by the coated filter paper. Lastly, the potential interfering species did not have effect on the detection of creatinine. Under the suitable condition, the linear range of the proposed method was 0-60 mg/L. The paper-based method was further applied for determination of creatinine in human urine samples compared with the standard Jaffé method. The recoveries of the proposed method and the standard Jaffé method were in the range

of 91.9 to 113.8% and 101.2 to 109.5%, respectively. The relative standard deviation (%RSD) of the proposed method was less than 8.6%. The proposed method could be applied for creatinine determination in human urine samples with easy detection by naked eyes.

5.2 Suggestion for future work

The AuNPs solution detection system should be developed for determination of creatinine in blood.

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