PAPER-BASED DEVICE FOR URINARY OXALATE DETECTION

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ในงานวิจัยนี้นำเสนอการพัฒนาวิธีวิเคราะห์ปริมาณออกซาเลตในปัสสาวะด้วยกระดาษตรวจวัดที่ดัดแปรด้วยสารประกอบเชิงซ้อนของ Fe(III) และเคอร์คูมินซึ่งมีสีน้ำตาลแดง โดยออกซาเลตในตัวอย่างจะเกิดปฏิกิริยากับ Fe(III) เกิดเป็นสารประกอบเชิงซ้อนของ Fe(III) และออกซาเลต ส่งผลให้ปริมาณของสารประกอบเชิงซ้อนของ Fe(III) และเคอร์คูมินบนกระดาษตรวจวัดน้อยลง ปริมาณสารประกอบเชิงซ้อนที่เหลือบนกระดาษตรวจวัดแปรผกผันกับปริมาณออกซาเลตในตัวอย่าง โดยสังเกตการเปลี่ยนสีของกระดาษตรวจวัดได้จากการเปลี่ยนจากสีน้ำตาลแดงเป็นสีเหลือง และทำการหาความเข้มสีด้วยโปรแกรม ImageJ โดยจากการศึกษาผลของตัวแปรต่างๆ พบว่าภาวะที่เหมาะสมในการเตรียมกระดาษตรวจดวัด คือ ใช้สารละลายเคอร์คูมินเข้มข้น 7.5 มิลลิโมลาร์ และสารละลาย Fe(III) เข้มข้น 0.25 มิลลิโมลาร์ ในกรดไฮโดรคลอริคเข้มข้น 0.01 โมลาร์ ซึ่งการตรวจวัดออกซาเลตสามารถทำได้ในตัวอย่างที่มีค่าความเป็นกรด-เบสเท่ากับ 2.0 และใช้ปริมาตรของสารละลายตัวอย่าง 3 มิลลิลิตร และระยะเวลาการตรวจวัด 10 นาที โดยทำการตรวจวัดตัวอย่างด้วยกระดาษตรวจวัดจำนวน 3 ซ้ำ ซึ่งวิธีการตรวจวัดนี้มีช่วงความสัมพันธ์เชิงเส้นตรงในช่วงความเข้มข้นของออกซาเลต 0-100.0 ไมโครโมลาร์ และขีดจำกัดของการตรวจวัดที่ 0.8 ไมโครโมลาร์ นอกจากนี้จากการศึกษาพบว่าเมทริกซ์ในปัสสาวะส่งผลต่อการวิเคราะห์ จึงทำการตรวจวิเคราะห์ปริมาณออกซาเลตในปัสสาวะด้วยวิธี standard addition ผลการทดลองพบว่าค่าร้อยละการได้กลับคืนของออกซาเลตในปัสสาวะตัวอย่าง (%recovery) อยู่ในช่วง 95-109% และค่าความเที่ยงในการวิเคาะห์ (%RSD) อยู่ใช่วง 3.2-7.6% โดยวิธีการตรวจวิเคราะห์ที่พัฒนาขึ้นนี้ สามารถประยุกต์ใช้ในการตรวจวิเคราะห์ออกซาเลตในปัสสาวะตัวอย่างด้วยความแม่นและความเที่ยงที่ยอมรับได้

สาขาวิชา เคมี ลายมือชื่อนิสิต .....

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In this work, a simple method for urinary oxalate determination was developed using testing paper modified with reddish-brown Fe(III)-curcumin complex. Oxalate in sample interacted with Fe(III) ions on the surface of the testing paper to form a Fe(III)-oxalate complex in solution, resulting in a decrease of the amount of Fe(III)-curcumin complex on the paper surface. The amount of the remaining Fe(III)-curcumin complex on the testing paper was inversely proportional to the oxalate level in the sample solution. A change of the testing paper color from reddish-brown to yellow could be observed and the color intensity was determined by ImageJ program. The effect of various parameters was investigated. The suitable condition for the testing paper preparation was by using 7.5 mM of curcumin and 0.25 mM of Fe(III) in 0.01 M of hydrochloric acid. The detection of oxalate was achieved at pH 2 with the sample volume of 3 mL and 10 minutes detection time. The sample detection by the testing paper should be repeated 3 times. The proposed method provided a linear range from 0 to 100.0 μM with a limit of detection of 0.8 μM. The effect of the urine sample matrix was also examined and the standard addition method was applied to detect oxalate in urine samples. The percentage of recovery and the standard deviation of the results observed were 95-109% and 3.2-7.6%, respectively. This method could be applied to screen the oxalate level in human urine sample with acceptable accuracy and precision.

Field of Study:	Chemistry	Student's Signature .....
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LIST OF ABBREVIATIONS

mg	Milligram
ng/mL	Nanogram per liter
mg/mL	Milligram per liter
µM	Micromolar
mM	Millimolar
mL	Milliliter
nm	Nanometer
LOD	Limit of detection
LOQ	Limit of quantitation
°C	Degree Celsius
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2SO_4	Sodium sulfate
$MgSO_4$	Magnesium sulfate
NH_4Cl	Ammonium chloride

CHAPTER I
INTRODUCTION

1.1 Statement of background the problem

Urinary oxalate analysis is the primary investigation to indicate hyperoxaluria [1]. Hyperoxaluria can cause the inhibition of calcium absorption in the blood resulting in the formation of insoluble complex with calcium and calcium oxalate stones in the kidney and urinary tract. This might lead to other complications in the kidney function or genitourinary system, which might cause death [2]. Essentially, oxalate in human body comes from food consumption [3], particularly vegetables and fruits. It is also the product from the metabolisms of amino acids, glycine, glyoxylate [4-6], and ascorbate in the body [6, 7]. As humans does not have oxalate-degrading enzymes, oxalate is excreted from the body through the genitourinary system. The normal oxalate level in blood and urine is in the range of 0.800-2.500 μM and 100-300 μM [8], respectively. If the urinary oxalate is higher than 300 μM, there might be a risk of developing kidney and urinary tract stones. Therefore, the screening of urine oxalate level is necessary.

Several oxalate analysis methods have been developed including ion chromatography [9-11], high performance liquid chromatography [12-14], enzymatic analysis [8], and spectrophotometry [15-17]. Although these methods are accurate, sensitive, and selective toward oxalate determination, they are high cost and require the analytical instruments. Hence, a simple and convenient detection is highly desired. A paper-based colorimetric detection method that is simple, low cost, and environmentally friendly is a promising alternative for oxalate detection [8, 15, 16].

In this research, a testing paper for colorimetric detection of oxalate was developed. A filter paper was modified with reddish-brown Fe(III)-curcumin complex before use. Curcumin is a natural yellow pigment obtained from the extracts of dried

turmeric roots. It is classified as insoluble polyphenolic compounds that has chelating properties to form complexes with many metals such as iron, boron, and mercury [18, 19]. This natural extract has been applied in many researches as a chromogenic probe for the determination of several analytes [17-21]. Herein, the filter paper was modified with curcumin and Fe(III) solutions consecutively. The Fe(III) ions formed complexes with curcumin on the paper surface and the paper color changed from yellow to reddish-brown. The obtained testing paper was further used to detect oxalate in sample solutions. The detection mechanism was based on the competitive reaction of oxalate in the sample solution with Fe(III) to form Fe(III)-oxalate complex resulting in a decrease of Fe(III)-curcumin complex content on the testing paper. The amount of the remaining Fe(III)-curcumin complex on the paper was inversely related to the concentration of oxalate in solution. The paper color changed from reddish-brown to yellow in the presence of oxalate and it could be observed by naked-eye. The color intensity of the testing paper in green mode was also determined by subjecting the photo of the testing paper to the ImageJ program. When the paper color turned from the reddish-brown to orange or yellow upon increasing the oxalate concentration in solution, the mean color intensity increased. With this relationship, the colorimetric detection of oxalate was achieved. This method could be used to indicate the urinary oxalate level for a further diagnosis of hyperoxaluria. Under the optimized condition, the testing paper was applied to detect oxalate in urine samples.

1.2 Research objectives

1. To prepare the testing paper modified with Fe(III)-curcumin complex and investigate the effect of preparation parameters

2. To optimize the parameters affecting the oxalate detection and apply the method to determine the oxalate level in human urine samples.

1.3 Scope of this research

A testing paper method for urinary oxalate analysis based on the formation of Fe(III)-oxalate complex was developed. The colorimetric detection was employed to determine the remaining Fe(III)-curcumin complex on the testing paper. The relationship between color intensity of the testing paper and the oxalate concentration was investigated. The effect of parameters related to the preparation of the testing paper (*e.g.* pH, curcumin and Fe(III) concentration) and the parameters affecting the detection of the oxalate (*e.g.* pH of oxalate solution, sample volume, detection time, and number of detection) were studied. For this method, the concentration of oxalate in the range of 0-100.0 μM was applied. The color intensity of the testing paper was determined by submitting the photo to the ImageJ program to analyze the intensity in green mode. The efficiency of the testing paper was evaluated by applying the testing paper to detect the oxalate concentration in the urine samples. The results obtained from the proposed method were also compared with the results from ion chromatography method.

1.4 The benefit of this research

To obtain a testing paper method for a simple colorimetric detection of oxalate in human urine samples.

CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 Urinary oxalate

The measurement of urinary oxalate is one of the initial tests for evaluating kidney stone disease. Oxalate is the crystallization stimulating substance called lithogenic substance [3] leading to calcium oxalate stone in the kidney and urinary tract [1, 3]. Kidney stone is a public health problem found across the world with higher incidence [22]. From the report of Thailand's public health ministry, it was found that the number of kidney and urinary tract stone patients is consistently increasing, especially in the Northeastern area. The growing rate of kidney stone patients is around 16% with a trend of the recurrent illness of 39% within 2 years after the treatment [3, 23] and 50% within 10 years after the treatment [24, 25]. The patients have to pay for a high treatment cost and prevent the recurrence of illness. Moreover, the occurrence of stones in the urinary tract may result in complications causing death. As kidney stone disease highly affects human quality of life [3, 26], the screening of oxalate level in human urine is important for a rapid treatment and reducing the risk of the disease.

Normally, human body does not have the oxalate digesting enzymes and therefore, the body gets rid of oxalate through the urinary tract. High level of oxalate in the body could be a result from food consumption or body metabolism and it may cause oxalate toxicity. The acute toxicity causes stomachache, vomit, diarrhea, and might cause death. The chronic toxicity might be from a long-term exposure to oxalate leading to calcium-oxalate complex in the kidney and urinary tract [2]. The average level of oxalate in blood and urine for a healthy individual should be in the range from 0.8-2.50 μM and 100-300 μM, respectively [8]. If the urinary oxalate level is higher than 300 μM, it indicates that the person could be at risk of an early kidney and urinary tract stones. When the body contains high oxalate level, it results in the formation of

calcium-oxalate complex in blood that is further eliminated through the urinary system. This causes accumulation of the complex and then kidney and urinary tract stones [3, 23]. The main factors affecting the amount of oxalate in the body is from the diet [1] and metabolic processes of glycine, glyoxylate, and ascorbate in the body [4-7] (Figure 2.1). The abnormality of stone inhibitor level such as low citrate, potassium and magnesium condition can also lead to accumulation of calcium-oxalate complex [26]. To prevent the kidney stone disease, the urinary oxalate level should be monitored.

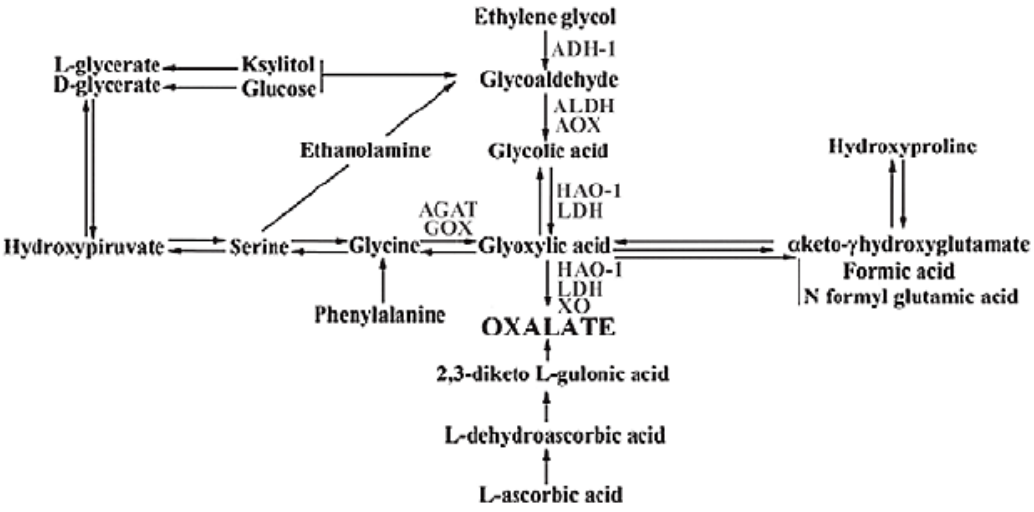


Figure 2.1 Metabolism and oxalate production in human body [4-7].

2.2 Method for oxalate detection

A large number of methods have been reported for measuring oxalate in water, food, and urine samples including ion chromatography (IC) [9-11], high performance liquid chromatography (HPLC) [12-14], enzymatic analysis [8], indicator-displacement assay (IDA) using spectrophotometry [15-17], and paper-based colorimetric devices [8, 15, 16]. Some of the methods are summarized in Table 2.1.

Table 2.1 Detail and performance of some previously reported methods for oxalate determination

Method	Linearity range LOD (μM)	Sample	Reaction/Condition	Ref.
IC	0.625-10.0 LOD < 0.3	Plasma	Plasma sample was injected to an ion chromatograph using NaOH as the mobile phase with a linear concentration gradient from 5 mM to 52.5 mM over 21 minutes.	[9]
IC	55.56-666.44 LOD = 17.18	Honey	The solid-phase extraction using anionic cartridges and 0.01 M chromate as eluent was chosen as sample preparation step. The resulting anions were analyzed by an IC with an anion column, borate gluconate buffer as mobile phase, and conductimetric detection.	[10]
IC	0.26-75.00 LOD = 0.19	Urine Beer	The separation was performed with a surfactant coated octadecyl-silica monolithic columns. Oxalate was separated from the sample matrix in the column and detected after post-column reaction with tris(2,2′-bipyridyl)ruthenium(III) with chemiluminescence detection.	[11]
HPLC	- LOD lower than 0.1	Urine Plasma	Oxalate was separated by ion-pair reversed-phase chromatography with a mobile phase of 10 % methanol in 100 mM phosphate buffer at pH 7.0. Tris (2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] was electrogenerated and added to react with oxalate post-column for further detection.	[12]
HPLC	555-4,440	Forage grasses	Oxalate in forage grasses was extracted with water or 1M HCl. Sample was analyzed in HPLC with an anion column (Shodex 1C SI-90 4E) and $NaHCO_3$ as eluent, and detected at 210 nm by a diode array detector.	[13]

Table 2.1 Detail and performance of some previously reported methods for oxalate determinations (contd.)

Method	Linearity range LOD, (μM)	Sample	Reaction/Condition	Ref.
LC/MS	5.56-5,000 LOD = 16.22	Urine	Liquid-liquid extraction with ethyl acetate was adopted for sample preparation. Chromatographic separation was performed in a C18 column by gradient elution with methanol and 1 M formate buffer at 35 °C. Oxalate was monitored with a quadrupole MS system.	[14]
IDA and UV-Vis spectrophotometer	1.76-49.40 LOD = 0.62	Urine Vegetable	The light blue complex of Cu(II) and 1-Amino-4-[3-(4,6-dichlorotriazin-2-ylamino)-4-sulfophenylamino]anthraquinone-2-sulfonic acid (RB4) was used as an indicator. Oxalate competed with RB4 to form complex with Cu(II). In the presence of oxalate, solution color changed from light blue of Cu(II)-RB4 complex to dark blue of free RB4.	[15]
IDA and UV-Vis spectrophotometer	0.83-113 LOD = 0.54	Urine	The Eriochrome Cyanine R (ECR) and VO(II) complex was used as an indicator. Oxalate competed with ECR to form complex with VO(II) resulting in a color change from purple of ECR-VO(II) complex to yellow of free ECR.	[16]
IDA and UV-Vis spectrophotometer	1.12-12.70 LOD = 0.57	Urine Water Food	The curcumin nanoparticles (CURNs) and Fe(III) complex was used for oxalate detection at pH 2.0–2.5. In the presence of oxalate, oxalate would form the Fe(III)-oxalate complex resulting in the reduction of the CURNs-Fe(III) complex content and a color change from reddish brown of CURNs-Fe(III) complex to yellow of free CURNs.	[17]

For chromatographic methods, ion chromatographic method based on the separation of components in the sample by the ion affinity toward the ion exchanger and electrical conductivity detection is one of the most popular methods for oxalate determination. Both ion chromatographic method and high-performance liquid chromatography methods allow the separation and detection of oxalate with high sensitivity and selectivity [9-14]. However, these methods are time-consuming and require a skilled operator and a high-cost equipment.

The colorimetric detection of oxalate has gained its popularity and become an alternative strategy for point-of-care monitoring due to its simple operations, visual observation, and low-cost instrument. In general, the method is based on the change of optical property of complexes in the sample solution or on the surface of substrate. The colorimetric methods can be used for qualitative, semi-quantitative, and quantitative analysis of oxalate by a variety of methods including enzymatic analysis [8], UV-Vis spectrophotometry and paper-based colorimetric device [8, 15-17].

2.3. Paper-based colorimetric device

Paper-based colorimetric device has become popular for the point-of-care detection of various analytes. It is potable, easy to fabricate and use. It consumes much less reagent and requires a small sample volume. Moreover, the devices can also be discarded immediately after measurements. Therefore, the device has been used for sample analysis in many fields, such as health diagnostics, environmental monitoring, as well as food quality analysis [27].

Paper is an abundant material and it can be generally found. Various type of papers has been used for fabricating paper-based device including filter papers. Paper is flexible and has porous matrix composed of hydrophilic cellulose fiber with an intrinsic capillary. Liquid can flow on the paper with capillary force without using external force. Its hydrophilic surface makes the paper a suitable candidate for loading and absorbing a variety of reagents [28, 29] according to its application. Furthermore,

by creating the hydrophobic barrier, the liquid can be transported in the pre-designed pathways on paper [30-32].

A large number of researches on the fabrication and application of paper-based devices have been reported. It has been applied for colorimetric detection of various analytes including compounds in serum and urine samples (*e.g.* glucose, lactate, uric acid [33-36]). The color on the paper could be observed by naked eyes and recorded by a digital camera, scanner, or a smart phone. The photo is inputted into a computer in order to determine the color intensity with ImageJ program. This is convenient for processing experimental results. The example of paper-based devices developed for the colorimetric detection of oxalate is described hereafter.

Worramongkona and coworkers [8] developed a paper-based colorimetric device for detection of urinary oxalate based on enzymatic reactions (Figure 2.2). Oxalate was turned to formate by oxalate decarboxylase. The obtained formate reacted with ion of NADH turned it into NAD^+ using formate dehydrogenase as catalyst. The NAD^+ was transformed back into NADH by the reaction with 1-methoxy-5-methyl-phenazinium methyl sulfate (PMS), resulting in PMS in reduced form. The latter further react with 3-(4,5-dimethylthiazol-2-yl) 2,5diphenyltetrazoliumbromide (MTT) to transform back to its oxidized PMS form. The MTT color changed from yellow of its oxidized form to purple of its reduced form or 3-(4,5–dimethylthiazolyl-2)-2,5-diphenyl-2H-tetrazoliumbromide (MTT Formazan). The color change could be observed by naked eyes. The detection range was from 10 to 1000 μM oxalate with a limit of detection of 10 μM.

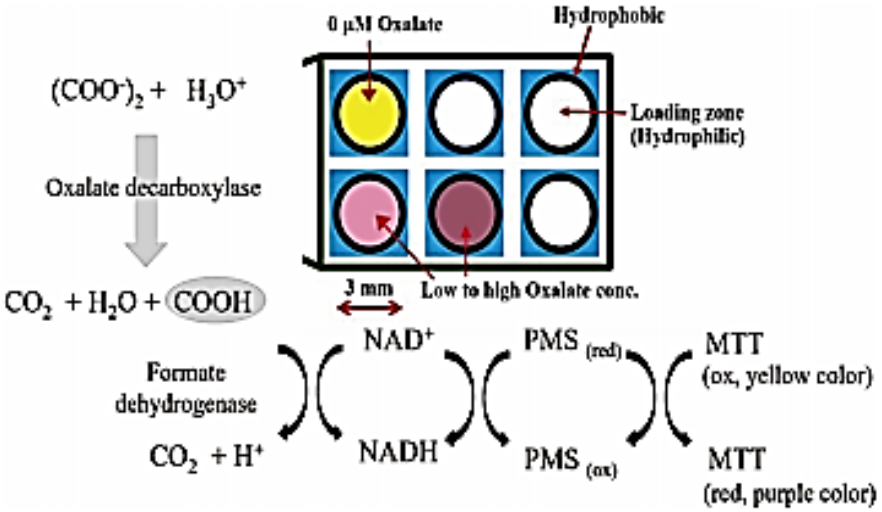


Figure 2.2 Pattern of device and reaction for detection of oxalate [8].

Tavallal and coworkers [15] developed a method based on indicator-displacement assay (IDA) for oxalate detection in sample solutions by UV-Vis spectrophotometer and by a paper-based device. The detection mechanism was summarized in Table 2.1 and Figure 2.3. The reaction was also performed on a paper-based device with a detection range from 1.76 to 49.40 µM. The method could be applied to detect oxalate in human urine and vegetable samples.

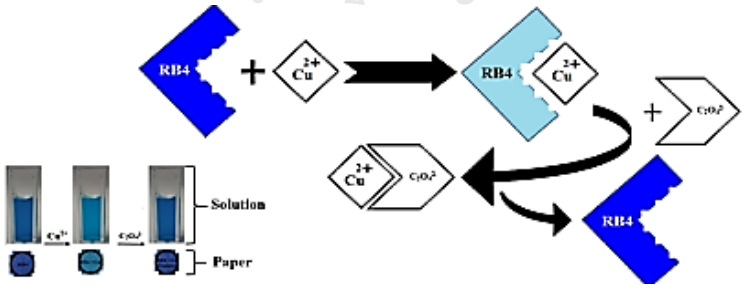


Figure 2.3 Illustration of the proposed reaction mechanism of RB4 with Cu(II) and oxalate [15].

There have been a few researches demonstrating the detection of oxalate by paper-based method. In this research, a paper based method was developed using

the concept of indicator displacement assay (IDA) and Fe(III)-curcumin complex as colorimetric probe.

2.4 Curcumin

Curcumin ((E,E)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione) is a polyphenol compound that has been recognized as a natural yellow pigment and a component of the spice, turmeric. It is extracted from the dried root of the rhizome Curcuma longa [17]. Several chemical and optical properties of curcumin are shown in Table 2.2 [37]. Curcumin appears in different tautomerism forms depending on the pH of the media (Figure 2.4). Under basic condition (pH > 8), the enolate form is present and its color is red. While under acidic conditions (pH 3-7), curcumin color is yellow and it demonstrates the proton donor characteristic attributed to its keto form [20].

Table 2.2 The chemical and optical properties of curcumin

Property	
State of matter	Light yellow powder
Molecular weight	368.38 g/mol
Melting point	183 °C
Water solubility	Insoluble
Solubility	In Ethanol
Absorption wavelength	408-430 nm
Emission wavelength	460-560 nm

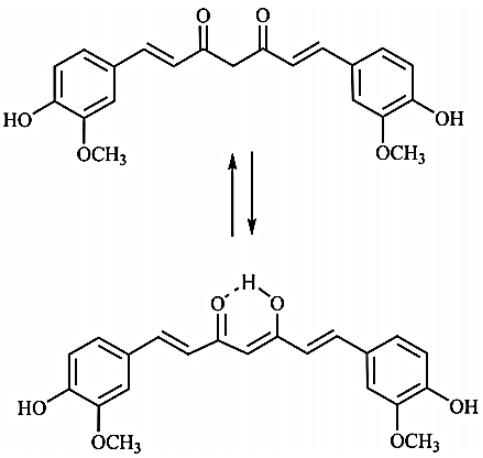


Figure 2.4 Keto (top) and enolate (bottom) tautomeric forms of curcumin [20].

Considering its chemical properties, curcumin has been applied in many researches as a chromogenic probe for colorimetric detection of various analytes (e.g. sulfide, oxalate, boron, Hg(II) [17-20]) and for pH sensing [21]. Examples of curcumin application are described hereafter.

Pourreza and Golmohammadi [20] presented the method for sulfide detection in aqueous solution by using curcumin nanoparticle (CURNs) in micelle mediated system. In the absence of both Cu(II) and sulfide, CURNs of orange-red color could be extracted in non-ionic surfactant phase and exhibited high surface plasmon absorption intensity. In the presence of Cu(II) in basic solution, the CURNs-Cu(II) complex was obtained with positively charged surface. As a result, it was not extracted into surfactant phase. The absorption intensity in surfactant phase decreased and the phase color changed from orange-red to yellow. Upon the addition of sulfide, the absorption intensity increased again due to the formation of CuS complex, releasing orange-red CURNs to the surfactant phase. With this concept, CURNs and Cu(II) were used for sulfide detection. The detection range was from 0.5–200.0 ng/mL with a detection limit of 0.4 ng/mL. This method was successfully applied to detect sulfide in different water samples. (Figure 2.5)

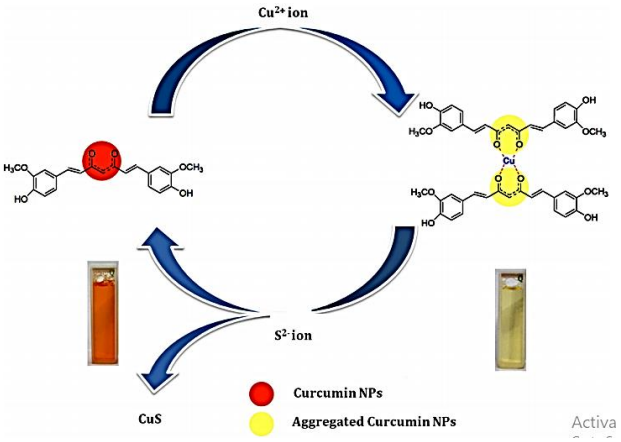


Figure 2.5 Schematic representation for the colorimetric sensing of sulfide using CURNs in the presence of Cu(II) [20].

Pourreza and coworkers [17] proposed a colorimetric method for the determination of oxalate using CURNs-Fe(III) complex. With the concept of indicator-displacement assay (IDA) described in Table 2.1 and Figure 2.6, the sample solution was analyzed by a UV–Vis spectrophotometer. This developed oxalate detection method could be applied to water, food, and urine samples with satisfactory results.

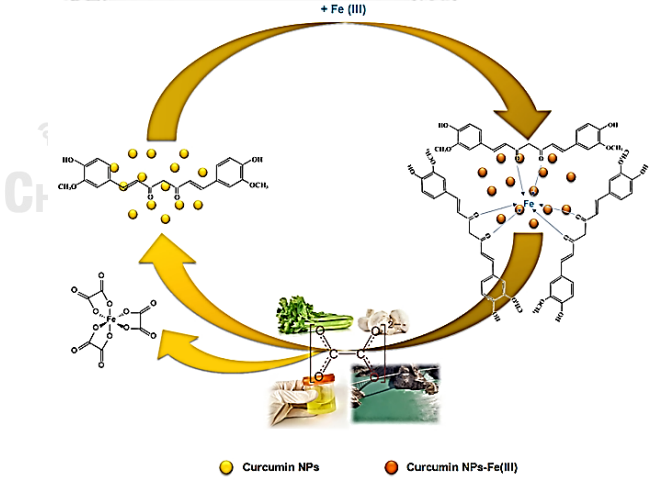


Figure 2.6 Proposed mechanism for the formation of CURNs-Fe(III) complex and effect of oxalate [17].

Furthermore, curcumin was also used as chromogenic probe to modify the paper platform for colorimetric detection of several analytes.

Francisco and coworkers [18] presented the combination of paper-based devices and information technology equipment for non-instrumental detection of boron in water samples. The device was prepared with curcumin and ethanolic extracts of Curcuma longa L. powder. The colorimetric detection is based on a two step-strategy involving initially the formation of rosocyanin in the device under acidic conditions. In the presence of boron, the color changed from red to blue-green related to boron concentration under alkaline condition. The color change in the device was then exploited for determination of boron by digitization and image processing with IT devices (scanner and tablet camera) and a photo analysis program, respectively. The linear range for boron detection of 0.2–0.8 mg/L was obtained (Figure 2.7)

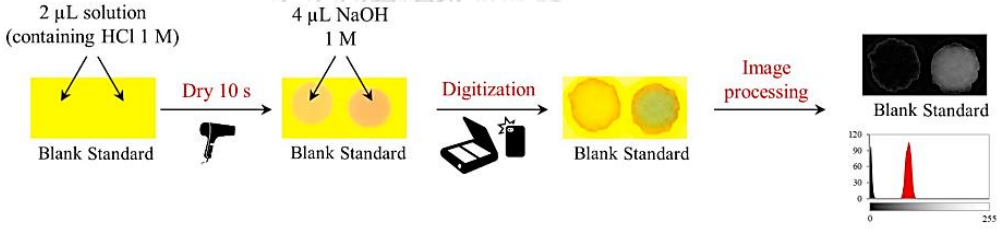


Figure 2.7 Schematic representation of the experiment procedure for boron detection [18].

Pourreza and coworkers [19] developed a novel paper based platform for monitoring Hg(II) using CURNs as a selective and green probe. The mechanism was based on the complex formation between Hg(II) and CURNs and subsequent change of optical properties of CURNs upon the addition of Hg(II). The yellow color of CURNs is gradually faded to light yellow which could be distinguished by naked eyes (Figure 2.8). The absorption intensity of CURNs decreased depending on the Hg(II) levels. The linear range for Hg(II) determination was 0.5–20 mg /mL (LOD = 0.17 mg/mL) without-preconcentration and 0.01–0.4 mg/mL (LOD = 0.003 mg/mL) with 50 times-

preconcentration by repeated additions of the sample solution onto the same test zone.

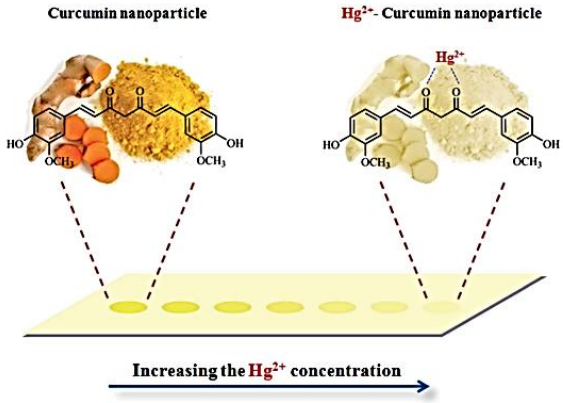


Figure 2.8 Schematic representation of Hg(II) sensing using CURNs on paper-based device [19].

Pourreza and Golmohammadi [21] developed a novel lab-on-paper device for pH sensing using CURNs (Figure 2.9). In order to fabricate the lab-on-paper, the wax dipping method was used. The developed sensor was successfully applied to the determination of pH in different water samples with satisfactory results.

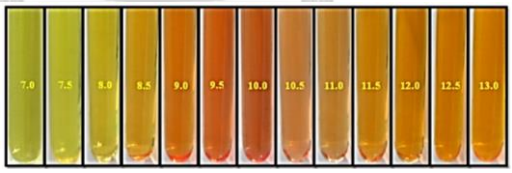


Figure 2.9 Photographical images of the CURNs solutions [21].

From the literature review up to date, there is no report on the development of paper-based method for oxalate detection using curcumin and indicator displacement assay concept.

2.5 Color intensity determination by ImageJ program

ImageJ program is a free Java image processing program and analysis software for determining the color intensity. The color intensity from image or photo can be determined in red, green, blue channels (RGB), and gray mode. For the color intensity determination with ImageJ program, it analyzes the color intensity of the photo color using the spectral responsivity of the red, green, and blue channels which are roughly Gaussian functions with typical ranges of 580-700, 500-580, and 400-500 nM, respectively. The value for a channel indicates the total photons in that formula in Equations. (2.1–2.3) [38]. In this work, the green channel was chosen to analyze the color intensity.

$$R = \int_{\lambda} P(\lambda) S_R(\lambda)\, d\lambda \quad (2.1)$$

$$G = \int_{\lambda} P(\lambda) S_G(\lambda)\, d\lambda \quad (2.2)$$

$$B = \int_{\lambda} P(\lambda) S_B(\lambda)\, d\lambda \quad (2.3)$$

P = incident intensity

S = spectral responsivity for a particular channel

CHAPTER III

EXPERIMENTAL

3.1 Instruments

Table 3.1 Instrument list

Instruments	Model (Brand)
Analytical balance	SI-234 (Metter-Toledo Ltd.)
Pipette	(Brand Transferpette)
pH meter	8603 Schwerzenbach AG (Metter-Toledo Ltd.)
Magnetic stirrer	Gem/MS 101 (LMS)
Centrifuge	Zentrifugen D-78532 Tuttlingen (Hettich)
UV-Vis spectrophotometer	HP 8453 (Hewlett Packard)
Ion chromatograph	Dionex Integrion RFIC System (Thermo Fisher Scientific)
Digital camera	Power Shot SX50 HS (Canon)
Cube light box	(Brand Udiobiz)

3.2 Chemicals and reagents

3.2.1 Chemicals

Milli-Q-purified water was used for all solution preparation except for curcumin solution. All of the chemicals used in this research were of analytical grade. The list of chemicals is shown in Table 3.2. Whatman No.42 filter paper was used as paper substrate to prepare testing papers.

Table 3.2 Chemical list

Chemicals	Supplier
Oxalic acid	Sigma-Aldrich
Curcumin	Sigma-Aldrich
Iron(III) chloride	Sigma-Aldrich
Ethanol 95% v/v	Merck
Hydrochloric acid	Merck
Nitric acid	Merck
Sodium Hydroxide	Merck

3.2.2 Preparation of reagents

A solution of curcumin (10 mM) was prepared by dissolving 36.84 mg of curcumin powder in ethanol in a 10 mL volumetric flask. A solution of Fe(III) (10 mM) was prepared by dissolving 18.50 mg of $FeCl_3$ in 0.01 M HCl in a 10 mL volumetric flask. A stock solution of oxalate (100 mM) was prepared by dissolving 126.03 mg of $C_2H_2O_4.2H_2O$ in 0.01 M HNO_3 and the pH of oxalate solution was adjusted to 2.0 using 1.0 M NaOH solution before making up the volume to 10 mL by 0.01 M HNO_3 solution. These solutions were used as stock solutions for preparing diluted solutions. All solutions were freshly prepared before use.

3.3. Mechanism study

To confirm that Fe(III)-curcumin complex could be used for oxalate detection, the reaction mechanism was investigated using a UV-Vis spectrophotometer. The experimental procedure was performed as follows. One milliliter of 0.3 mM curcumin solution was mixed with 1.00 mL of 0.1 mM Fe(III) in 0.01 M HCl solution and 1.00 mL of oxalate solution (pH 2.0). The final concentration of oxalate in these mixtures was 20.0, 60.0, or 200.0 μM. A blank solution was prepared in the same manner without adding the oxalate solution. The spectrum of the prepared solutions was recorded in a wavelength range from 200 to 700 nm.

3.4 Preparation of Fe(III)-curcumin testing paper

A piece of Whatman No. 42 filter paper (5x5 cm) was submerged in 10 mL of a curcumin solution for 15 min. The paper was allowed to dry at room temperature and further submerged in 10 mL of a Fe(III) in 0.01 M HCl solution for 15 min (Figure 3.1). The resulting Fe(III)-curcumin paper was dried by hot air from a hair dryer and cut into 5x5 mm pieces for further use in oxalate determination.

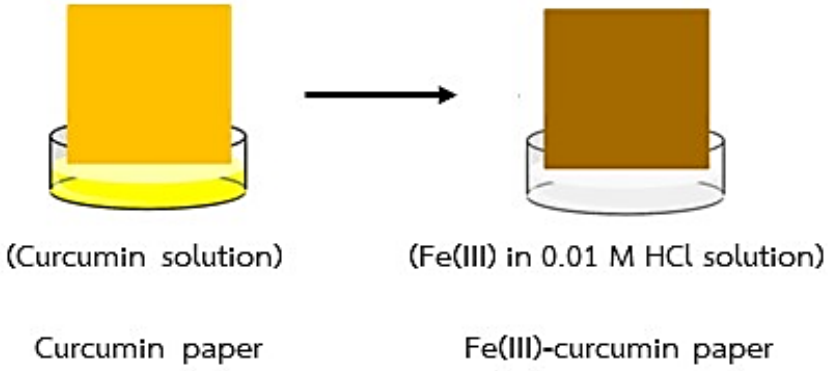


Figure 3.1 The procedure for preparation of Fe(III)-curcumin testing papers.

To obtain the optimum condition for the preparation of Fe(III)-curcumin testing paper, the effect of Fe(III) solution pH, curcumin concentration, and Fe(III) concentration were studied. The range of values of the studied parameters is shown in Table 3.3. The performance of the obtained testing papers was evaluated by using the testing papers in the detection of oxalate.

In the detection procedure, the testing paper was submerged in a 3.00 mL oxalate standard solution (1.0-100.0 μM) for 10 minutes. The detection by the same testing paper was repeated 3 times and a new standard solution of the same concentration was used each time. The color of the testing paper was observed by naked eyes and the color intensity (I) in green mode was determined by analyzing the photo of the papers in ImageJ program. In the absence of oxalate (blank), the color of the testing paper was reddish-brown and the green intensity value was low. When the color of the testing paper changed to paler tone or yellow in the presence of oxalate,

the green intensity value was higher. The delta color intensity (ΔI) was calculated by comparing the color intensity of testing paper used in an oxalate solution ($I_{oxalate}$) to that observed in the blank solution (I_{blank}) of that experiment as shown in Equation (3.1).

$$\Delta I = I_{oxalate} - I_{blank} \quad (3.1)$$

Table 3.3 The range values of the studied parameters for preparation of Fe(III)-curcumin testing paper

Reagents	Range of concentration
HCl acid in Fe(III) solution	0.1 M (pH 1), 0.01 M (pH 2)
Curcumin solution	1.875 – 7.500 mM
Fe(III) solution	0.125 – 0.250 mM

3.5 Oxalate determination by the testing paper

In the determination of oxalate by the testing paper, a piece of Fe(III)-curcumin testing paper was fully submerged in a 3.00 mL oxalate solution for a specific time. The solution was discarded and the photo of the testing paper was recorded by a digital camera in a light box with controlled brightness. The color of the testing paper was observed by naked eyes and the color intensity in green mode was determined by analyzing the photo of the papers in ImageJ program. The delta color intensities were calculated following Equation 3.1 using I_{blank} of each experimental condition. The method is schematically shown in Figure 3.2.

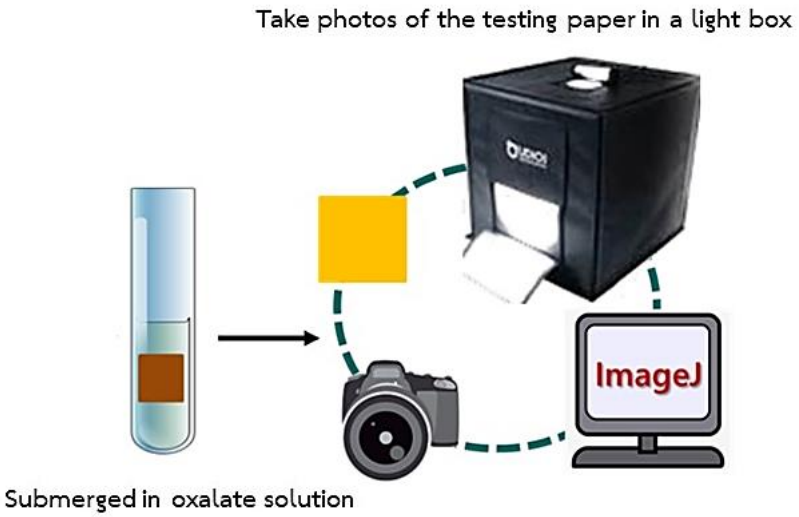


Figure 3.2 The analytical procedure for oxalate colorimetric detection.

To obtain the optimum condition for oxalate determination, the effect of various parameters including pH of oxalate solution, sample volume, detection time, and number of detections were investigated. The range of values of the studied parameters is shown in Table 3.4.

Table 3.4 The range values of studied parameters for oxalate determination

Parameters	Range of values
pH of oxalate solution	2.0 - 4.0
Sample volume	3.00, 5.00 mL
Detection time	10, 15 minutes/time
Number of detections	1 - 3 times

3.6 Stability of the testing paper

The Fe(III)-curcumin testing papers were prepared using 7.5 mM curcumin and 0.25 mM Fe(III) in 0.01 M HCl. These testing papers were stored in refrigerator (≈ 0°C) and protected from the light with aluminum foil. To investigate the stability of these testing papers, the testing papers were stored for different period and the performance

of the papers was evaluated weekly by using the paper to detect oxalate in standard solutions following the method described in section 3.5 under the optimum conditions.

3.7 Method performance

The performance characteristic of the method including the linear working range, the limit of detection, and limit of quantitation were evaluated under the optimum condition.

Linear working range

In this method, the color of the testing paper was reddish-brown in the absence of oxalate (blank) and the green intensity value was low. When the color on the testing paper changed to paler tone or yellow in the presence of oxalate, the green intensity value was higher. By observing the change in color intensity, the external calibration curve for oxalate determination was plotted between the concentrations of oxalate in the range of 0-100.0 μM against the delta color intensity (ΔI). In addition, the linear equation and the correlation coefficient (R^2) was determined.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of the method were obtained by the measurement of blank solution containing 0.01 M HNO_3 (pH 2.0) using the testing papers. The experimental procedure was performed following the section 3.5 under the optimum condition in nine replicates. The value of LOD and LOQ were calculated from the external calibration curve plotted between concentrations of oxalate in the range of 0-8.75 μM and the delta color intensity, according to Equations (3.2) and (3.3), respectively, where;

$$LOD = 3SD_{blk}/Slope \quad (3.2)$$

$$LOQ = 10SD_{blk}/Slope \quad (3.3)$$

SD_{blk} = the standard deviation of ΔI values of testing papers used in blank solution

Slope = the slope of the linear calibration curve

3.8 Effect of possible interfering species

The effect of compounds possibly found in human urine samples (*e.g.* NaCl, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, Na_2SO_4, $MgSO_4$, KCl, $CaCl_2$, NH_4Cl, citrate, creatinine, urea, and uric acid) on the oxalate determination was studied. The testing paper was applied in a single solution containing each compound at the level normally found in urine [39]. Moreover, the effect of glucose and ascorbic acid were also investigated. The concentration of compounds studied is shown in Table 3.5.

Additionally, the effect of potential interfering species including NaH_2PO_4, Na_2SO_4, $MgSO_4$, citrate and ascorbic acid was studied in a binary mixture containing each species and 5.0 μM oxalate. These mixtures were analyzed by the testing paper and the proposed method. The results were compared to that observed in the single oxalate standard solutions.

Table 3.5 Concentration of compounds studied

Compounds	Concentration (mM)
NaCl	54
$NaHCO_3$	2
Na_2HPO_4	0.35
NaH_2PO_4	0.32
Na_2SO_4	9
$MgSO_4$	2
KCl	30
$CaCl_2$	3
NH_4Cl,	15
Citrate	5
Creatinine	4
Urea	200
Uric acid	1
Glucose	0.8
Ascorbic acid	1

3.9 Determination of oxalate in human urine samples

3.9.1 Effect of urine sample matrix

In order to determine the oxalate level in real samples, urine samples were collected from healthy adult volunteers from our research group. The fresh urine sample was diluted at least 25 times by 0.01 M HNO_3 (pH 2.0) without any further sample preparation step. These diluted urine solutions were spiked with the oxalate standard solutions to obtain the spiked concentration range from 2.5-8.75 μM oxalate. These solutions were used for the analysis by the testing paper and the obtained results were used to plot a standard addition calibration curve (ΔI against spiked oxalate concentration). The effect of real urine samples matrix was investigated by

comparing the slope of the external calibration curve to that of the standard addition calibration curve.

3.9.2 Analysis of real urine samples

The standard addition method was applied to overcome the effect of urine sample matrix. For the analysis of a urine sample, oxalate standard solutions were added into a set of 50.00 mL of the urine sample with a fixed volume to produce a set of spiked samples for standard addition method. In order to evaluate the accuracy of the method, urine samples spiked with 100.0 µM oxalate were analyzed under optimum conditions and the recovery was determined according to Equation 3.4 where;

$$\%\text{recovery} = ((C_s - C_b)/S) \times 100 \quad (3.4)$$

C_s = the concentration of oxalate found in the spiked sample

C_b = the concentration of oxalate found in non-spiked sample

S = the concentration of oxalate spiked in the sample

For the concentration level studied in this research, the recovery of the spiked analyte should be in the range of 80-110% for the acceptable accuracy of the method (Table 3.6) [40]. The precision of this method was evaluated and presented in terms of relative standard deviation (%RSD) which should not be higher than 11%.

The results from this proposed method were compared with the results from ion chromatography in the analysis of the same urine samples. The analytical condition for the sample analysis by ion chromatography is shown in Table 3.7 [41]. The external calibration method was applied to determine oxalate by ion chromatography. Moreover, the detail of the digital camera and the condition of recording photos in all experiments are listed in Table 3.8.

Table 3.6 Acceptable values of analyte recovery and relative standard deviation for the determination at different concentration according to AOAC international

Analyte (%)	Mass fraction	Unit	Mean recovery, (%)	RSD, (%)
0.01	10^{-4}	100 ppm	90-107	5.3
0.001	10^{-5}	10 ppm	80-110	7.3
0.0001	10^{-6}	1 ppm	80-110	11
0.00001	10^{-7}	100 ppb	80-110	15
0.000001	10^{-8}	10 ppb	60-115	21
0.0000001	10^{-9}	1 ppb	40-120	30

Table 3.7 Conditions of ion chromatography method

Parameters	Values
Column	Dionex IonPac AG 19 (4 x 50 mm) guard column and Dionex IonPac AS 19 (4 x 250 mm) separation column
Eluent	Potassium hydroxide concentration gradient, 1 mM for 1.5 min, 1 mM to 20 mM from 1.5 to 5 min. 20 mM to 40 mM from 5 min to 7 min, respectively
Flow rate	1.0 mL/min
Injection volume	25 µL
Detector	Conductivity detector

Table 3.8 Details of digital camera conditions

Conditions	Details
Brand	Canon
Camera model	Canon PowerShot SX50 HS
Camera mode	M Mode
F-stop	f/7.1
Exposure time	1/500 sec.
ISO speed	ISO-200

CHAPTER IV
RESULTS AND DISCUSSIONS

In this research, a testing paper for colorimetric detection of oxalate in urine samples was developed by modifying a filter paper with Fe(III)-curcumin complex. The detection was based on the reaction between Fe(III) and oxalate in sample solution which decreased the quantity of Fe(III)-curcumin complex on the paper surface. The amount of Fe(III)-curcumin complex remained on the paper was inversely proportional to the oxalate level in the sample solution. The possible mechanisms are presented in Equations (4.1-4.2). The color of the testing paper changed from reddish-brown of Fe(III)-curcumin complex to orange and yellow upon an increase of the oxalate level in sample solution.

$$\text{Curcumin} + \text{Fe(III)} \xrightarrow{\text{Acidic Media}} \underset{\text{(reddish-brown)}}{\text{Fe(III)-curcumin complex}} \qquad (4.1)$$

$$\underset{\text{(reddish-brown)}}{\text{Fe(III)-curcumin complex}} \xrightarrow{\text{Oxalate ion}} \text{Fe(III)-oxalate complex} + \underset{\text{(yellow)}}{\text{Curcumin}} \qquad (4.2)$$

4.1 Mechanism study

To investigate the detection mechanism, the interaction between Fe(III) ions and curcumin in solution in the presence and absence of oxalate was observed by a UV-Vis spectrophotometer as shown in Figure 4.1. It was found that the absorption spectrum of free curcumin is different from that of the mixture of curcumin and Fe(III) solution. The free curcumin spectrum shows an absorption spectrum with the absorption maxima (λ_{max}) at 427 nm, while the absorption spectrum of the curcumin-

Fe(III) solution has a new absorption maxima at 413 nm. The color of the solution also changed from yellow of free curcumin to pale orange in the presence of Fe(III). These results indicated the formation of Fe(III)-curcumin complex [17].

When there was oxalate ions present in the Fe(III)-curcumin complex solution, the absorption spectra of the mixture was similar to that of free curcumin, revealing the absence of Fe(III)-curcumin complex. The absorption maxima shifted from 413 nm of Fe(III)-curcumin complex to around 427 nm of free curcumin. The solution color also changed from pale orange to yellow of free curcumin upon the addition of oxalate. The results indicated that oxalate ions in the solution interacted with Fe(III) ions through complex formation owing to a greater affinity compared to curcumin. As a result, free curcumin was increasingly released in solution with a rise of oxalate concentration.

Although the absorption spectrum of Fe(III)-oxalate complex was not observed under this experimental condition due to its low molar absorptivity, the reappearance of curcumin absorption band in the mixture also confirmed this mechanism. According to this mechanism and the observation of color change, the Fe(III)-curcumin complex was further used to modify the testing paper for oxalate detection.

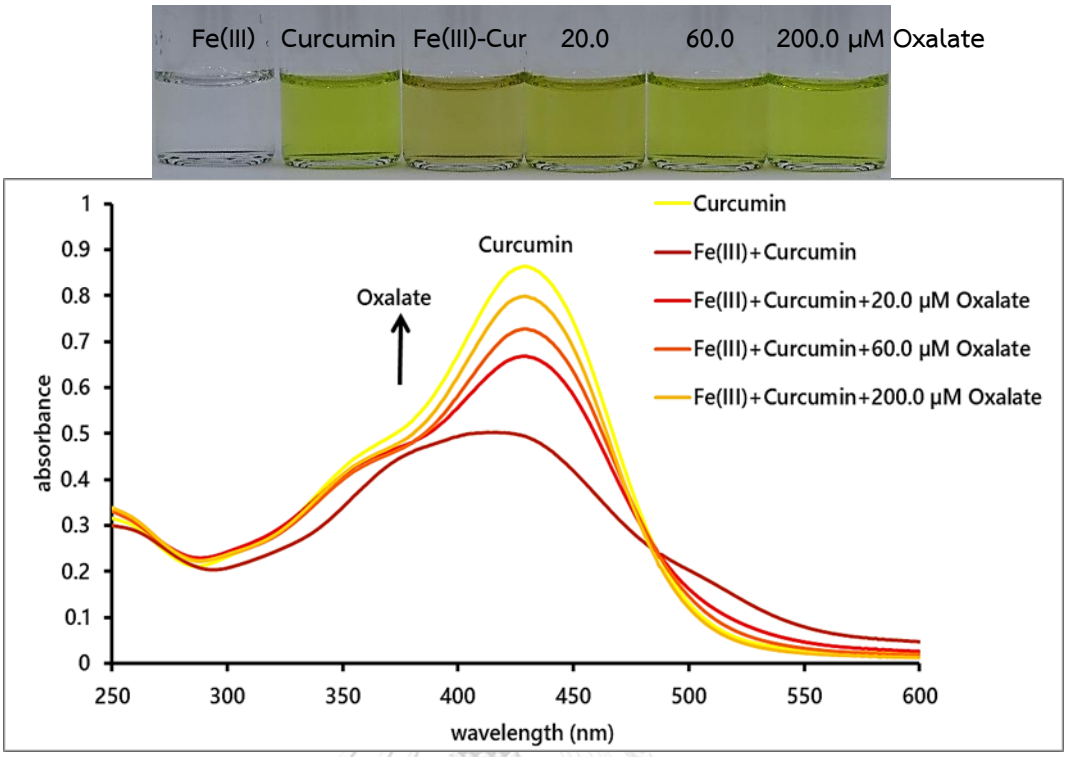


Figure 4.1 UV-Vis Spectra of curcumin solution and the mixture of curcumin and Fe(III) in the presence of oxalate at pH 2.0. (curcumin 20.0 μM, Fe(III) 6.7 μM)

4.2 Colorimetric determination of oxalate by the testing paper

In this research, the oxalate concentration in the range of 1.0-100.0 μM was applied to evaluate the performance of the obtained testing papers. These studied levels represented the level of urinary oxalate for a healthy individual (100-300 μM). The method efficiency should be adequate for the determination of normal urinary oxalate concentration and lower. If a urinary oxalate concentration was high or higher than 300 μM, the crystal of calcium-oxalate would be observed in urine sample by a microscope. Hence, it was not necessary to develop a method to detect very high-level oxalate.

4.2.1 Preparation of Fe(III)-curcumin testing paper

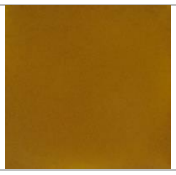
In the detection of oxalate by this method, the testing paper containing Fe(III)-curcumin complex was prepared by modifying the filter paper with curcumin solution,

followed by Fe(III) solution. The effect of various parameters affecting the testing paper performance was investigated. The performance of the obtained testing papers prepared under different conditions was evaluated by using the obtained testing papers to detect oxalate in standard solutions. The color change of the testing paper was observed by naked eyes and the color intensity of the testing paper photos was determined using ImageJ program in green mode.

Effect of Fe(III) solution pH for testing paper preparation

The solution pH plays an important role in the metal complex formation. In this study, it may affect the complex formation between Fe(III) ions with curcumin on the paper. From the preliminary study, the Fe(III)-curcumin complex formation reaction should be performed under acidic condition to avoid the precipitation of $Fe(OH)_3$ at pH higher than 2. In this experiment, a HCl solution of 0.1 M (pH 1.0) and 0.01 M (pH 2.0) were used for preparing 0.25 mM Fe(III) solution. The filter paper modified with 7.5 mM curcumin was submerged in these Fe(III) solutions to prepare testing papers. The photos of the obtained testing papers are shown in Table 4.1. When the testing paper was prepared with Fe(III) in 0.01 M HCl, the paper color was reddish-brown with a darker tone than the paper prepared with Fe(III) in 0.1 M HCl, revealing a higher content of complex on the paper. The results indicated that the suitable pH for Fe(III)-curcumin complex formation was pH 2 and thus, 0.01 M HCl was used in Fe(III) solution for the testing paper preparation.

Table 4.1 The photo of testing papers prepared by using different Fe(III) solution pH

Solutions for testing paper preparation		
7.5 mM Curcumin	**7.5 mM Curcumin with 0.25 mM Fe(III) in 0.1 M HCl**	**7.5 mM Curcumin with 0.25 mM Fe(III) in 0.01 M HCl**
		

Effect of curcumin and Fe(III) concentration for testing paper preparation

The concentrations of curcumin and Fe(III) used for paper modification would have influence on the performance of the obtained paper as they would directly affect the amount of the Fe(III)-curcumin complex on the paper and hence the starting color of the testing paper. In this study, their effect was concurrently investigated. The testing papers obtained under different conditions were used to detect oxalate in the range of 1.0-100.0 μM and compared. The paper photos and the color intensities are shown in Table 4.2. It should be noticed that the more intense red-brown color, the lower the color intensity in green mode. The paper color of yellow gave the highest color intensity in green mode regarding the whole color change of testing papers in this method (red-brown to yellow).

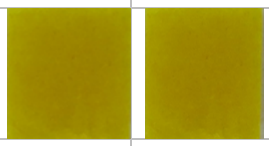
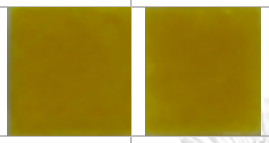
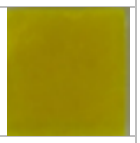
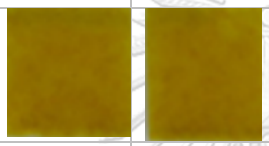
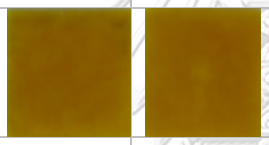
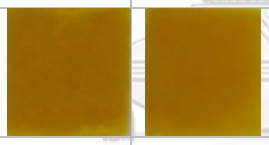
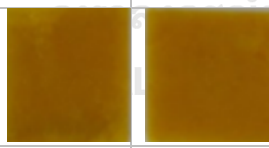
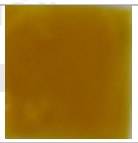
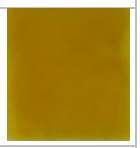
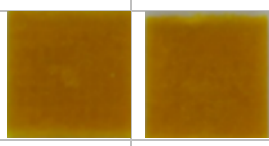
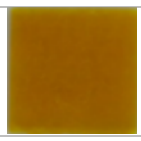
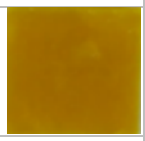
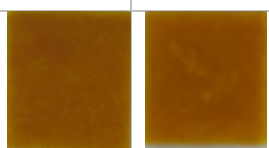
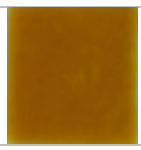
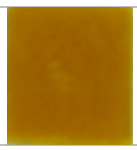
When 1.875-5.000 mM of curcumin and 0.125-0.500 mM of Fe(III) concentration range were applied, the starting color of the obtained testing papers was pale reddish-brown due to low amount of Fe(III)-curcumin complex on the paper surface. The color change on the testing paper was difficult to observe by naked eyes. However, when compared the color intensities of the papers used in blank solution and in 1.0 μM or 5.0 μM oxalate solution using t-Test, the results showed that the color intensities of testing papers were significantly different at the 95% confidence level (α= 0.05). A distinguished color change could only be observed in 10.0 μM oxalate solution compared to blank solution.

In contrast, using 7.5 mM curcumin solution and 0.125 or 0.250 mM Fe(III) for the paper preparation, the initial paper color appeared in darker tone in comparison with the other conditions. By increasing the curcumin concentration, it increased the number of ligand on the paper surface to form complex with Fe(III). The paper color was darker red-brown indicating a higher content of Fe(III)-curcumin complex on the paper surface and the color intensities in green mode also decreased. When these papers were used to detect 5.0 μM of oxalate, the color change on the testing paper

to pale reddish-brown could be observed by naked eyes. The color intensities of the papers used to detect oxalate solutions (1.0–100.0 μM) were significantly different from that of blank solution at the 95% confidence level (α= 0.05) (t-Test).

By comparing the color intensities change in increasing oxalate concentration, a sharper change in color intensities was obtained by using the testing papers prepared with 7.5 mM of curcumin and 0.25 mM of Fe(III). These results also indicated that a good sensitivity was obtained under this condition. Therefore, this condition was selected to prepare the testing paper in further experiments.

Table 4.2 Effect of curcumin and Fe(III) concentration for testing paper preparation on the detection of oxalate (color intensity as green values are given under each photo)

[Curcumin] (mM)	[Fe(III)] (mM)	Testing papers color and color intensity/ Oxalate concentration (μM)					
		blank	1.0	5.0	10.0	50.0	100.0
1.875	0.250						
		147.41	149.50		159.53		
2.500	0.250						
		122.21	125.31		128.34		140.25
	0.500						
		116.53	118.11		123.32		133.56
3.750	0.250						
		96.38	97.90	106.42	113.70	118.10	124.12
5.000	0.125						
		98.40	103.72	109.20	114.63	126.90	135.70
	0.250						
		90.27	92.14	94.23	98.63	111.13	116.70
7.500	0.125						
		91.81	94.00	95.39	100.0	112.44	121.32
	0.250						
		79.64	85.40	92.98	102.73	107.56	114.14

(Condition: pH 2.0, sample volume = 3 mL, number of detections = 3 with 10 min each)

4.2.2 Oxalate determination

Effect of pH on oxalate detection

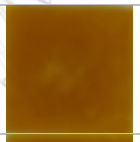
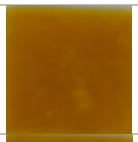
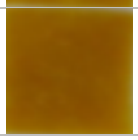
Given that the pH value is an important factor on the formation of metal complexes, it may have an influence on the complexation between oxalate and Fe(III) on the testing paper. In this study, the effect of sample solution pH on oxalate detection was investigated. The pH of oxalate solutions was varied from 2.0 to 4.0. The solutions containing 5.0 and 10.0 μM oxalate and the testing paper prepared by using 7.5 mM curcumin and 0.25 mM Fe(III) in 0.01 M HCl were used in this study. The effect of pH was evaluated based on the change of the testing paper color and the color intensity, compared to those observed in blank solutions. The color of the testing papers before and after the detection at different solution pH is shown in Table 4.3. The color intensity of the testing paper was determined using ImageJ program in green mode and the delta color intensities was calculated by comparing the intensity of the testing paper used in blank solution and in oxalate solution ($\Delta I = I_{oxalate} - I_{blank}$) as shown in Figure 4.2.

It was observed that the testing paper color changed from intense reddish-brown to paler reddish- brown in blank solution at pH 2.0, 3.0 and 4.0, compared to the milli-q water solution (pH 5.0-6.0). This observation revealed that a small amount of Fe(III) ions was released from the Fe(III)-curcumin testing paper under acidic condition. Consequently, it affected the starting content of Fe(III)-curcumin complex on the testing paper, and hence the initial color of the paper. Upon an increase of solution pH from 2 to 4, the change of paper color from reddish brown to paler color could be slightly better observed by naked eyes in the detection of 5.0 and 10.0 μM oxalate. The highest delta color intensities were also obtained in the analysis of oxalate solutions at pH 4.0. These results indicated that oxalate could form complex with Fe(III) at pH 4 better than lower pH conditions, resulting in a lower amount of Fe(III)-curcumin complex left on the testing paper. Considering the pK_a of the oxalic acid (pKa_1 = 1.25-1.46 and pKa_2 = 3.81-4.40) [42], it was obvious that at pH 4.0, oxalic acid was

deprotonated to oxalate anion in a higher extent than at pH 3 and pH 2. These anions were more readily to form complex with Fe(III) ion on testing paper, compared to oxalic acid.

However, it was observed in our preliminary study that the effect of several urine matrix species (*e.g.* citrate) on the analytical response was also prominent at pH 3.0-4.0. Therefore, to get accurate results from the method, the sample pH value of 2.0 was selected for further study, despite lower detection sensitivity.

Table 4.3 Effect of pH on the testing paper color used for oxalate detection

Solution pH	Oxalate concentration (μM)		
	Blank	5.0	10.0
milli-Q water (pH 5.0-6.0)			
2.0			
3.0			
4.0			

(Condition: pH 2.0, 3.0, 4.0, sample volume = 3 mL, number of detections = 3 with 10 min each)

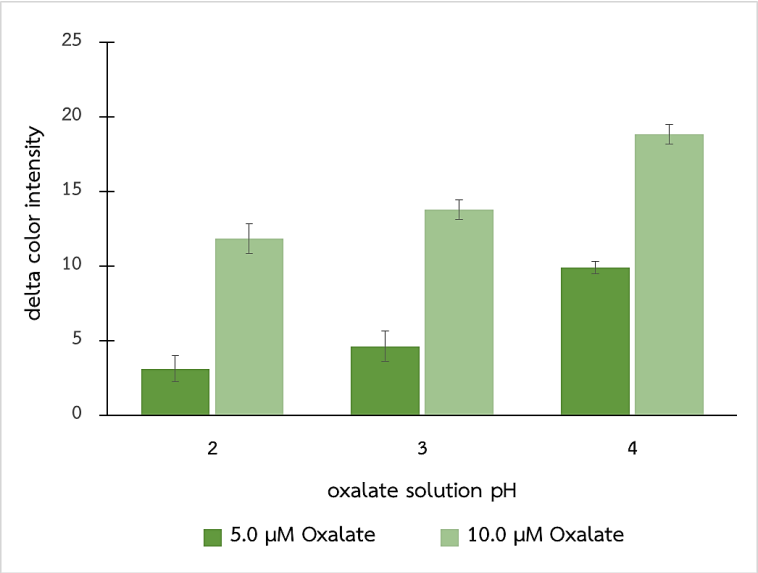
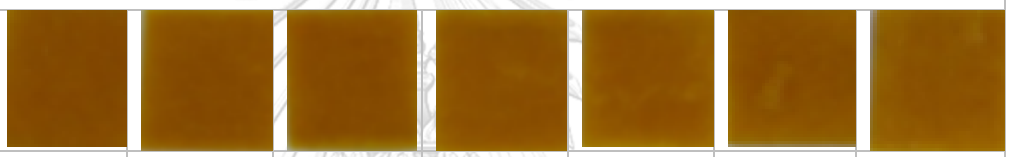
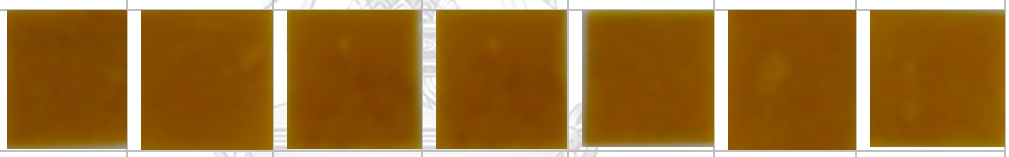
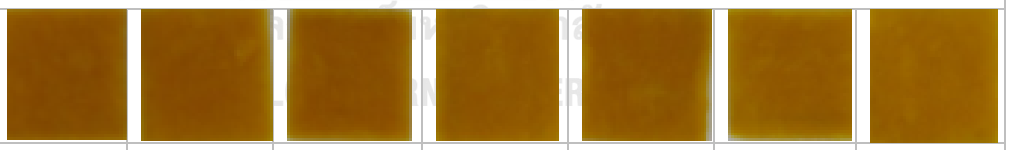


Figure 4.2 Effect of pH on the color intensity change observed in 5.0 and 10.0 µM oxalate solution.

Effect of sample volume, detection time, and number of detections on oxalate detection

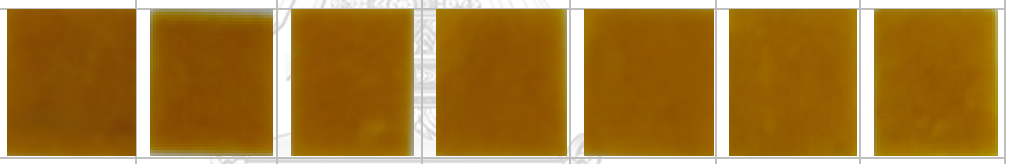
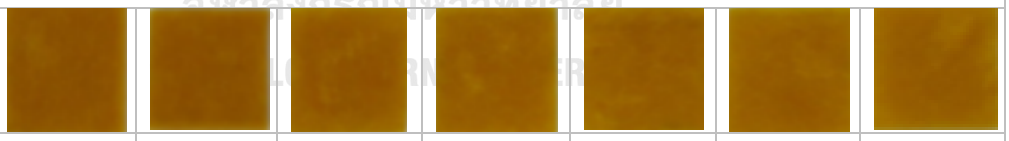
In an attempt to obtain a good detection sensitivity, the effect of sample volume, detection time, and number of detections was investigated concurrently. The Fe(III)-curcumin testing papers were used in the detection of 2.5-8.75 µM oxalate solutions. The concentration of 1.0 µM oxalate was excluded due to low precision of the analytical results was observed. A sample volume of 3 mL and 5 mL was applied in this work. A solution of 0.01 M HNO_3 was added in all sample solutions with a volume of 10% of sample volume to keep the pH of sample solution at 2.0. The detection time was 10 or 15 minutes for each detection and the number of detections was varied from 1 to 3 times. For each detection, a new oxalate solution of the same concentration studied in that specific experiment was used. The color of the testing papers is shown in Table 4.4-4.5. The color intensities of all the testing papers in green mode were determined. The delta intensity values ($\Delta I = I_{oxalate} - I_{blank}$) were calculated and used to plot the curve against oxalate concentrations observed under each condition (Table 4.6).

Table 4.4 Effect of sample volume, detection time, and number of detections on testing papers color and delta color intensities observed in the detection of 2.5-8.75 μM oxalate

Sample volume (mL)	Oxalate concentration (μM)						
	Blank	2.5	3.75	5.0	6.25	7.5	8.75
	n=1 / 10 min						
3.00							
	74.40±0.10	75.60±0.30	77.98±0.04	79.70±0.23	82.88±0.15	84.35±0.28	86.59±0.32
5.00							
	74.86±0.19	76.12±0.34	78.20±0.40	80.76±0.33	82.49±0.06	84.93±0.08	87.53±0.05
	n = 2 / 20 min						
3.00							
	75.01±0.14	78.52±0.66	81.79±1.19	83.62±0.75	85.93±1.10	87.66±0.46	89.82±1.47
5.00							
	75.5±0.28	78.41±0.22	80.50±0.46	82.60±0.55	84.51±0.58	87.16±1.21	89.76±0.46
	n = 3 / 30 min						
3.00							
	81.80±0.55	84.87±0.08	88.59±0.50	92.02±0.08	94.37±0.03	96.72±1.08	99.12±0.21
5.00							
	82.47±0.70	86.33±0.04	88.95±0.10	92.48±0.61	95.36±0.46	98.12±0.41	100.20±0.56

Note: Number of detections (n) / Total detection time, (condition: pH 2.0 of oxalate)

Table 4.5 Effect of sample volume, detection time, and number of detections on testing papers color and delta color intensities observed in the detection of 2.5-8.75 μM oxalate

Sample volume (mL)	Oxalate concentration (μM)						
	Blank	2.5	3.75	5.0	6.25	7.5	8.75
	n=1 / 15 min						
3.00							
	77.59±0.27	79.65±0.53	82.55±0.44	85.00±0.92	86.22±0.11	88.48±0.58	90.75±0.43
5.00							
	76.96±0.07	79.32±0.33	81.94±1.36	84.00±0.15	86.16±0.67	88.22±0.47	90.45±0.47
	n = 2 / 30 min						
3.00							
	81.34±0.13	84.52±0.54	88.35±0.67	91.34±0.25	93.53±0.16	95.70±0.24	99.18±1.25
5.00							
	83.22±0.12	86.95±0.68	89.59±0.51	92.88±0.99	95.84±0.44	98.13±0.32	101.02±0.03
	n = 3 / 45 min						
3.00							
	84.22±0.34	88.15±0.66	91.96±0.57	95.55±0.08	98.16±0.40	101.05±0.38	103.45±0.18
5.00							
	85.96±0.67	89.19±0.42	92.24±0.50	96.06±0.31	99.19±0.52	101.94±0.50	104.61±0.90

Note: Number of detections (n) / Total detection time, (condition: pH 2.0 of oxalate)

Table 4.6 The linear equation of the calibration curve for oxalate determination constructed from results obtained using different sample volume, detection time, and number of detections

Sample volume (mL)	Number of detections (n) / total detection time		
	n=1 / 10 min	**n=2 / 20 min**	**n=3 / 30 min**
3.00	y = 1760.3x - 3.1 R^2 = 0.9936	y = 1747.1x - 1.2 R^2 = 0.991	y = 2228.7x - 1.7 R^2 = 0.9893
5.00	y = 1804.3x - 3.3 R^2 = 0.9975	y = 1798.6x - 1.8 R^2 = 0.996	y = 2279.3x - 1.7 R^2 = 0.9951
	Number of detections (n) / total detection time		
	n=1 / 15 min	**n=2 / 30 min**	**n=3 / 45 min**
3.00	y = 1703.9x - 1.7 R^2 = 0.9901	y = 2252.2x - 1.8 R^2 = 0.9929	y = 2431.6x - 1.5 R^2 = 0.9923
5.00	y = 1752.3x - 1.8 R^2 = 0.9987	y = 2261.6x - 2.9 R^2 = 0.998	y = 2430.8x - 2.9 R^2 = 0.9986

The results show that increasing the sample volume in the studied range did not affect the change of color on the testing papers under every conditions. By using 3-mL and 5-mL sample volume, the color of the testing papers obtained from the analysis of oxalate at different concentrations were similar (Table 4.4-4.5). This is likely because the oxalate diffusion from bulk solution to the paper surface for the reaction with Fe(III) did not alter, nor specifically increase by using a larger sample volume under the same period of time. Hence, at the same oxalate concentration and same detection time, the content of remaining Fe(III)-curcumin complex on the paper surface in 3-mL and 5-mL sample was not significantly different at 95% confidence level (T-test). There are therefore no advantages from using larger sample volumes.

Furthermore, the effect of both detection time and the number of detections was investigated. Regarding the paper color observed in the detection of oxalate solutions (2.5-8.75 μM), a fading of testing paper color was clearly observed after being repeatedly submerged in a fresh oxalate solution for the 2nd and 3rd time. By repeating the detection by the same paper, the time period that the paper contacted with the

oxalate solution was also prolonged and hence, increased the number of oxalate to react with Fe(III)-curcumin complex on the paper. The results of prolonging the detection time from 10 to 15 min for each detection also show a clearer fading of paper color. In Table 4.4-4.5, when the detection time was 10 and 15 minutes, the dark reddish-brown color was still observed on the testing paper but paler at 15 minutes. The delta color intensities were also higher at 15 minutes, indicating a clearer fading of color. The same results were observed when repeated the detection.

For better evaluating the effect of detection time, the delta color intensity of the testing papers obtained under each condition was used to construct calibration curves against oxalate concentrations (2.5-8.75 μM) and compared as shown in Table 4.6. The linear relationship was observed under every experimental condition studied with $R^2 > 0.99$. Hence, the sensitivity of the detection was evaluated using the linear slope. It is clearly seen that a higher slope or a higher detection sensitivity was obtained in increasing detection time or number of detections. At 30 minutes of total detection time (n = 3, 10 minutes each and n = 2, 15 minutes each), the testing papers exhibited the same color change when observed by naked eyes. These results were confirmed by statistical calculation using t-Test and it showed that color intensities of the testing papers used to detect same oxalate concentration were not significantly different at the 95% confidence level (α= 0.05). Similar calibration slopes were also observed.

The results from using 45 minutes of detection show the highest sensitivity and good linear relationship but the analysis time was long. The total detection time of 30 min (n = 3, 10 minutes each) was selected in this work to shorten the analysis time. According to linear regression of results obtained by using a 3-mL and 5-mL sample volume, similar sensitivity was obtained under every experimental condition. Hence, the sample volume of 3 mL was selected in this method.

4.3 Stability of the testing paper

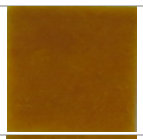
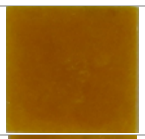
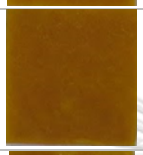
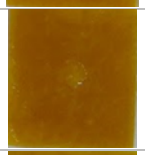
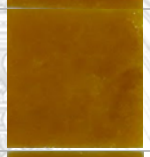
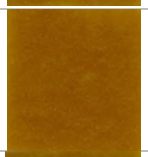
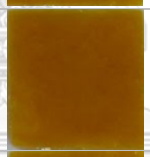
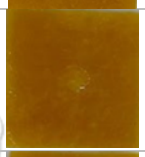
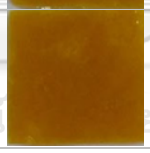
The stability of the testing papers was evaluated by observing the performance of the Fe(III)-curcumin papers in the oxalate detection. The condition of testing paper preparation was described in section 3.4. The papers were kept at ≈ 0°C in the dark for six weeks. For each week, several of the stored papers were used to detect oxalate solutions (2.5, 5.0, and 8.75 μM) by the method described in section 3.5 under the optimum conditions. These levels of oxalate represented low, medium, and high level of the working range, respectively.

The stability of the testing papers was evaluated based on the color change on the testing papers preserved in different time periods, observed in the oxalate detection (Table 4.7). The color intensities were also determined and the delta intensities are compared as shown in Figure 4.3. The results show that increasing of shelf life from 1 to 6 weeks did not affect the performance of the testing papers. Similar change of Fe(III)-curcumin complex color on testing paper were observed. The color intensity results of using freshly prepared papers were statistically compared to those stored for 2-6 weeks (one-way Anova). It showed that the color intensities of testing paper stored for 1 to 6 weeks and used to detect each level of oxalate were not significantly different (F=0.0070 (week-2), F=0.0084 (week-3), F=0.0070 (week-4), F=0.0002 (week-5) and F=0.0005 (week-6)) at 95% confidence level (α= 0.05). The results indicated that the amount of Fe(III)-curcumin complex on the paper surface was not likely to degrade when stored at ≈0°C and protected from the light before use.

On the other hand, when the store time was longer than 6 weeks, it resulted in a deteriorated performance of the testing paper. Paler reddish-brown color on the testing paper was observed in the detection of every oxalate level. The color intensities were significantly different from those observed by using papers stored for 1 to 6 weeks. These results revealed that the shelf life of the testing papers is up to 6 weeks

when stored in a refrigerator and protected from the light with aluminum foil before use.

Table 4.7 Color of the testing papers kept at different time observed in the detection of oxalate

Shelve time (week)	Oxalate concentration (μM)			
	Blank	2.5	5.0	8.75
1				
2				
3				
4				
5				
6				

(Condition: pH 2.0, sample volume = 3 mL, number of detections = 3 with 10 min each)

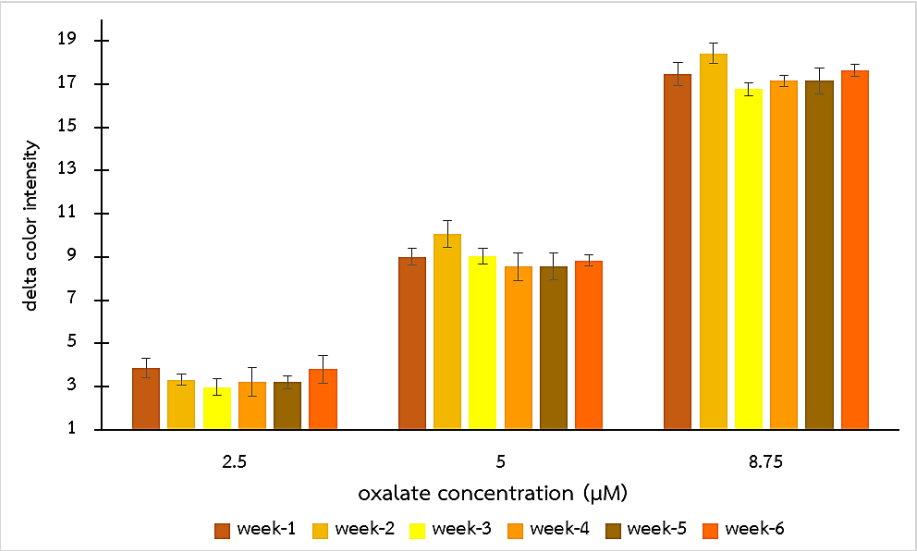


Figure 4.3 The delta intensities of testing paper for oxalate deterimination in each week.

4.4 Method performance

To evaluate the method performance, the method was performed to detect oxalate standard solutions under the chosen condition as followed. The Fe(III)-curcumin testing papers prepared by using 7.5 mM curcumin and 0.25 mM Fe(III) in 0.01 M HCl (pH 2.0) solutions were used to detect oxalate in 3 mL sample at pH 2.0. The number of detections was 3 times with 10 minutes each. The color changes of testing papers were observed by naked eyes and the color intensity of the testing paper was determined using ImageJ program in green mode. The delta color intensity (ΔI) of the testing papers used to detect oxalate solution compared to that in blank solution was calculated and used for calibration curve construction. Under these chosen conditions, the linear working range, the limit of detection, and the limit of quantitation were determined.

Linear working range

The calibration curve exhibited 2 ranges of linear relationship with different sensitivities. The linear relationship with higher sensitivity was obtained in the oxalate concentration in the range from 0-8.75 μM with a linear equation of y=2050x-0.7 (R^2 = 0.9929). While lower sensitivity was observed in the detection of 8.75-100.0 μM of oxalate with a linear equation of y=151.37x+16.8 (R^2 = 0.9932) as shown in Figure 4.4. With the limited surface of the paper, a limited amount of Fe(III)-curcumin complex could be modified on the paper surface. Therefore, the content of Fe(III)-curcumin complex on the paper surface was not sufficient for the detection of high level oxalate. Consequently, the color intensity of the paper used to detect high oxalate level did not sharply change when oxalate concentration changed.

With appropriate dilution of sample, this working range could cover the average range of oxalate found in human urine samples (100-300 μM) for healthy person. Considering the color change observed by naked eyes and the sensitivity in oxalate detection under this experimental condition, the working range of 0-8.75 μM was focused for further study. The repeatability of this method was evaluated. The relative standard deviations (%RSD) of calibration curve slope and intercept for inter-day measurements (n = 7) of oxalate in the range 0-8.75 μM were 0.03 % and 0.22 %, respectively with linear equation y = (2115.2±55.8)x - (0.7±0.1). It reveals that this method has good repeatability.

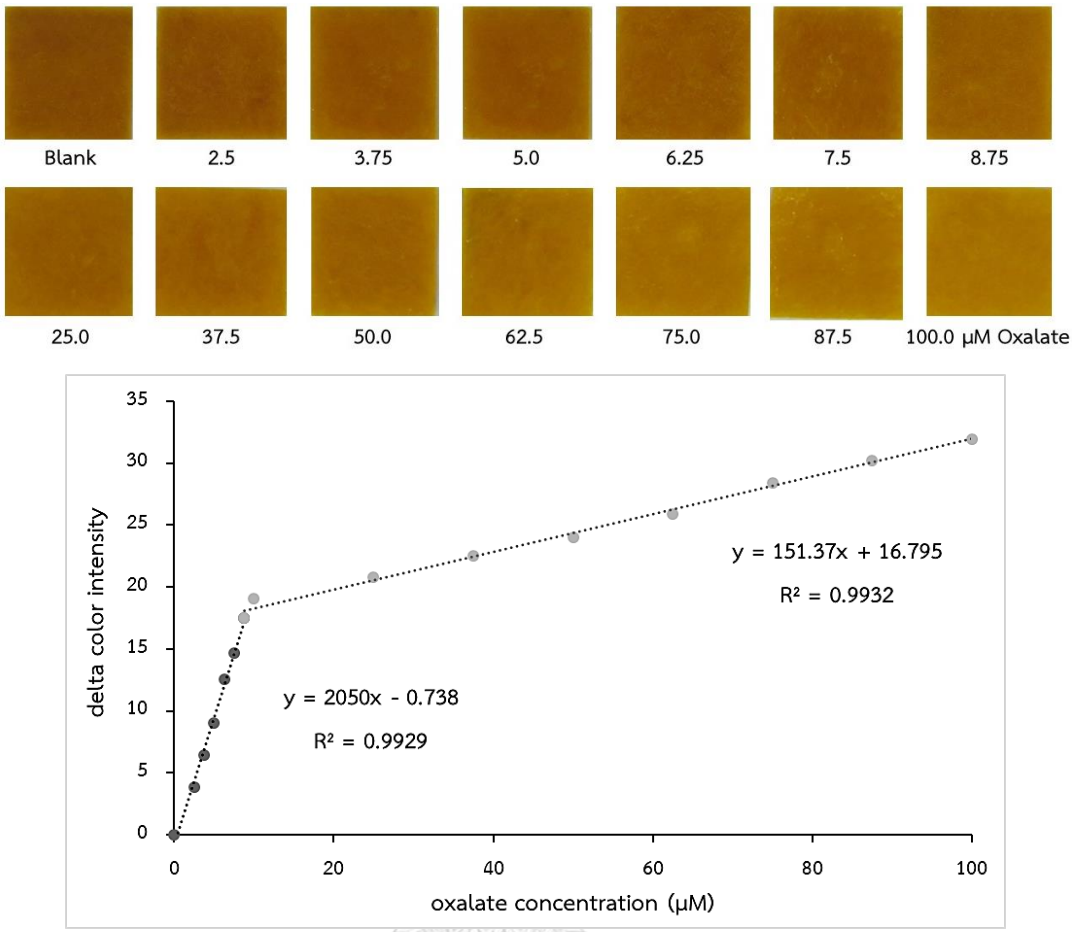


Figure 4.4 The calibration curve for 0-100.0 µM oxalate determination by using Fe(III) curcumin testing papers and testing paper color chart.

Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) of the proposed method were obtained by using the testing paper with the blank solution containing 0.01 M HNO_3 (pH 2.0). The experimental procedure was performed following the section 3.5 under optimum condition. In nine replicates, The mean color intensity of blank solution obtained at LOD and LOQ were calculated according to Equation 3.2, 3.3, respectively. The LOD value of 0.8 µM and the LOQ value of 2.5 µM were obtained while the LOD by naked-eye was 5.0 µM.

4.5 Effect of possible interfering species

To evaluate the applicability of the method to detect oxalate in human urine sample, the effect of different compounds commonly found in urine sample was investigated. The testing paper was used with a solution containing NaCl, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, Na_2SO_4, $MgSO_4$, KCl, $CaCl_2$, NH_4Cl, citrate, creatinine, urea, and uric acid with an average level related to the synthetic urine solution (Table 3.5). Moreover, the effect of common compounds such as glucose and ascorbate were also studied.

The color and the color intensity of the testing papers used for the analysis of the studied compounds in single solution were compared to those observed in blank solution and 0.1 mM oxalate solution as shown in Table 4.8. The delta color intensities were also calculated and shown in Figure 4.5. Compared to the paper color observed in blank solution, the testing paper color did not change when the papers were used with the solution of NaCl, $NaHCO_3$, Na_2HPO_4, KCl, $CaCl_2$, NH_4Cl, creatinine, urea, uric acid, or glucose. On the other hand, the paper color was faded when used in the solution of NaH_2PO_4, Na_2SO_4, $MgSO_4$, or citrate. These species showed interfering effect due to the ability of complex formation with Fe(III) in case of NaH_2PO_4 [43]. Na_2SO_4, $MgSO_4$ could also react Fe(III) [44, 45], while ascorbic acid may reduce Fe(III) in Fe(III)-curcumin complex to Fe(II) lowering the amount of Fe(III)-curcumin complex on the testing paper [46]. The presence of these compounds would result in a positive error.

Furthermore, the tolerant limit of these compounds was determined by using binary mixture containing oxalate and studied compound. The color of the testing papers used to detect 0.005 mM oxalate in the absence and in the presence of these compounds was compared. The tolerant limit of NaH_2PO_4, Na_2SO_4, $MgSO_4$, citrate, and ascorbic acid was found to be 0.03 mM, 0.9 mM, 0.2 mM, 0.2 mM, and 0.01 mM, respectively. The results are shown in Table 4.9 and Figure 4.6. The delta color intensities of testing papers observed in oxalate solution with and without these compounds were not significantly different at 95% confidence level (α= 0.05) confirmed by t-test ($MgSO_4$ (P=0.417), Na_2SO_4 (P=0.123), NaH_2PO_4 (P=0.984), citrate

(P=0.220), ascorbic acid (P=0.074)). These concentration levels of these compounds could be found in urine sample diluted at least 10 times and 25 times (for citrate) in respect to the average level in synthetic urine, except for ascorbic acid.

Citrate exhibited a strong interfering effect on the analytical response of the testing paper because it could competitively react with Fe(III) to form Fe(III)-citrate complex, especially at pH 2 and greater [58]. Considering the pKa values of citric acid (pKa_1 = 3.13, pka_2= 4.76, pka_3 = 5.40) [58], citric acid was deprotonated in solution having pH value higher than 3. Citrate anions were likely to form complexes with Fe(III) better than its acid form. Hence, the presence of citrate in sample solution resulted in false positive results. To overcome this problem, the pH of sample solutions should be lower than pH 3.0 to keep it in its protonated form. In addition, the level of citrate in sample should not exceed its tolerant limit.

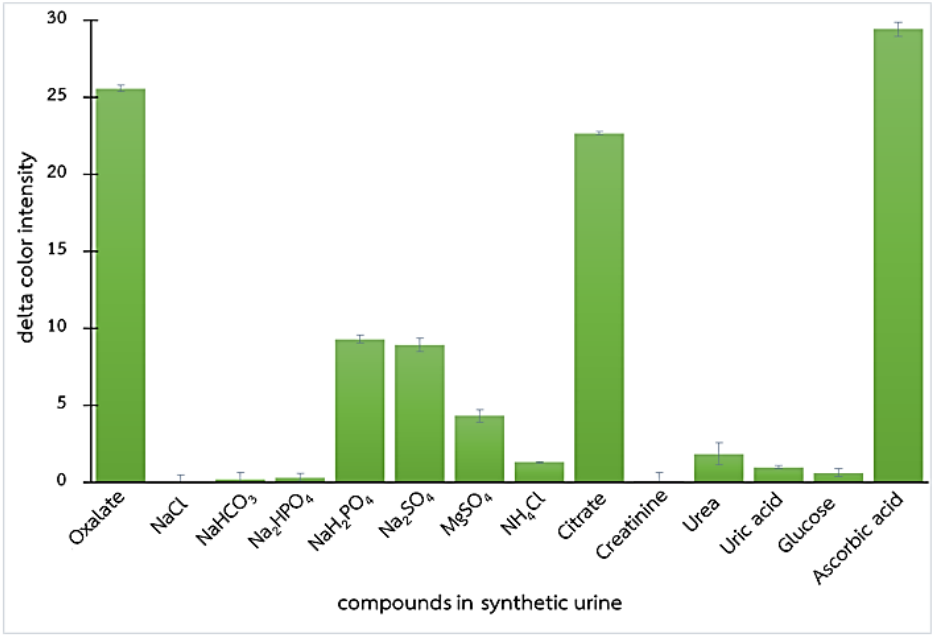
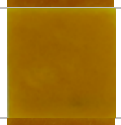
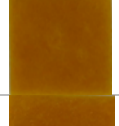
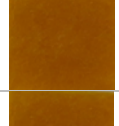
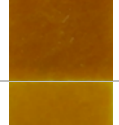


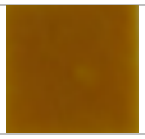
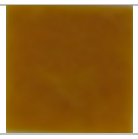
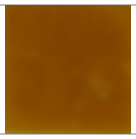
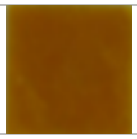
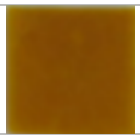
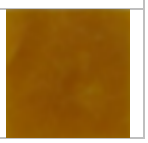
Figure 4.5 The delta color intensities observed in the detection of single solutions using the testing paper

Table 4.8 Effect of synthetic urine matrix in the single solution process

Compounds	Concentrations (mM)	Testing papers color	Color intensity
Blank	-		80.14±0.22
Oxalate	0.1		106.72±0.21
NaCl	54		80.15±0.26
$NaHCO_3$	2		80.33±0.25
Na_2HPO_4	0.35		80.45±0.70
NaH_2PO_4	0.32		89.43±0.10
Na_2SO_4	9		89.05±0.44
$MgSO_4$	2		84.46±0.43
NH_4Cl	15		81.40±0.41
Citrate	5		102.82±0.52
Creatinine	4		80.24±0.48
Urea	200		81.99±0.07
Uric acid	1		81.12±0.08
Glucose	0.8		80.77±0.25
Ascorbic acid	1		109.55±0.44

(Condition: pH 2.0, sample volume = 3 mL, number of detections = 3 with 10 min each)

Table 4.9 The color of paper used to detect oxalate in binary mixture solutions

[Oxalate] (mM)	Compounds					
	Oxalate	Oxalate + 0.03 mM $NaHPO_4$	Oxalate + 0.9 mM $NaSO_4$	Oxalate + 0.2 mM $MgSO_4$	Oxalate + 0.2 mM Citrate	Oxalate + 0.01 mM Ascorbic acid
0.005						

(Condition: pH 2.0, sample volume = 3 mL, number of detections = 3 with 10 min each)

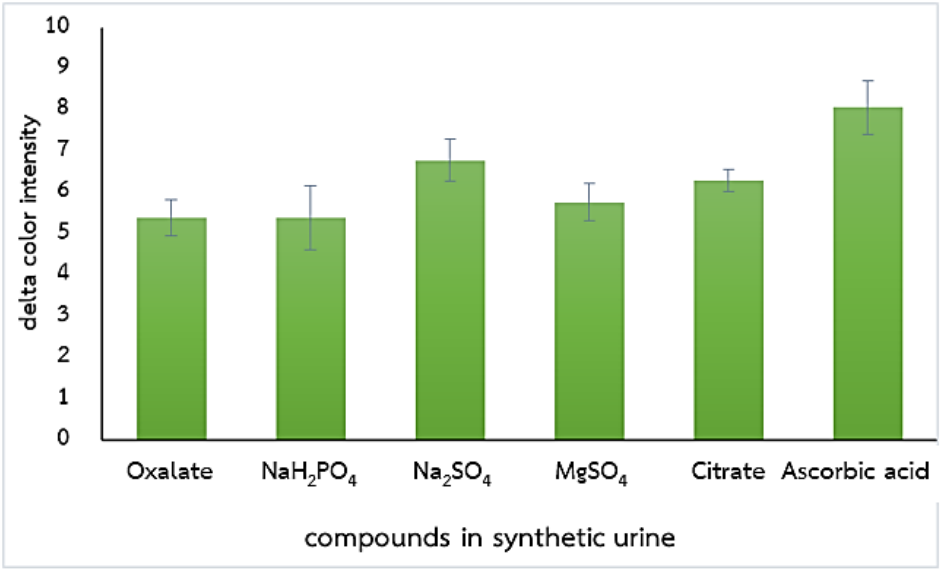


Figure 4.6 The delta color intensities observed in the detection of binary mixture solutions using the testing paper

In summary, to prevent the effect of urine matrix, the urine sample should be diluted at least 25 times and the sample solution pH should be adjusted to pH 2.0 prior to the detection. The presence of ascorbic acid coming from food uptake should not be higher than 0.01 mM in diluted sample solution.

4.6 Determination of oxalate in urine samples

Urine samples were collected from healthy volunteers in our research group and informed consent was obtained from all individual participants. The fresh urine sample was diluted at least 25 times by 0.01 M HNO_3 solution (pH 2.0) without other sample pretreatment step and analyzed by the method described in section 3.5 under the optimum condition.

4.6.1 Effect of urine matrix

From the previous study, it was found that certain species possibly found in urine sample could interfere the analytical response to some extent. To evaluate the applicability of the testing paper with real sample analysis, the effect of urine matrix was investigated by comparing analytical response of oxalate (0-8.75 μM) in standard solutions, synthetic urine solutions, and spiked real urine samples. External calibration curves were plotted between the concentrations of oxalate against the delta color intensity ($\Delta I = I_{oxalate} - I_{blank}$) in green mode of the testing papers and compared to examine the effect of matrix. The synthetic urine was prepared following the method of Chutipongtanate and Thongboonkerd [39] and diluted 25 times to reduce the interference effect as described in section 4.8. The results are shown in Figure 4.7.

It was observed that the external calibration curve obtained in the analysis of oxalate standard had higher slope than that of oxalate in synthetic urine solutions or spiked urine sample solutions. On the other hand, the slope of the calibration curves obtained from the analysis of oxalate in synthetic urine solutions and spiked urine sample solutions were similar. These results indicated that the urine matrix still had effect on the detection resulting in a slightly lower sensitivity in the real sample analysis. Therefore, the standard addition method was applied to the determination of oxalate level in urine overcome the matrix effect. In this case, the delta color intensity (ΔI) was calculated by comparing the color intensity of testing paper used in a non-spiked sample solution ($I_{non\text{-}spiked}$) or spiked sample solution (I_{spiked}) to that observed in blank solution of 0.01 M HNO_3 (I_{blank}) as shown in Equations (4.3-4.4). These

ΔI values were used to plot standard addition calibration curve to determine the oxalate level in that sample.

$$\Delta I = I_{non\text{-}spiked} - I_{blank} \quad (4.3)$$

$$\Delta I = I_{spiked} - I_{blank} \quad (4.4)$$

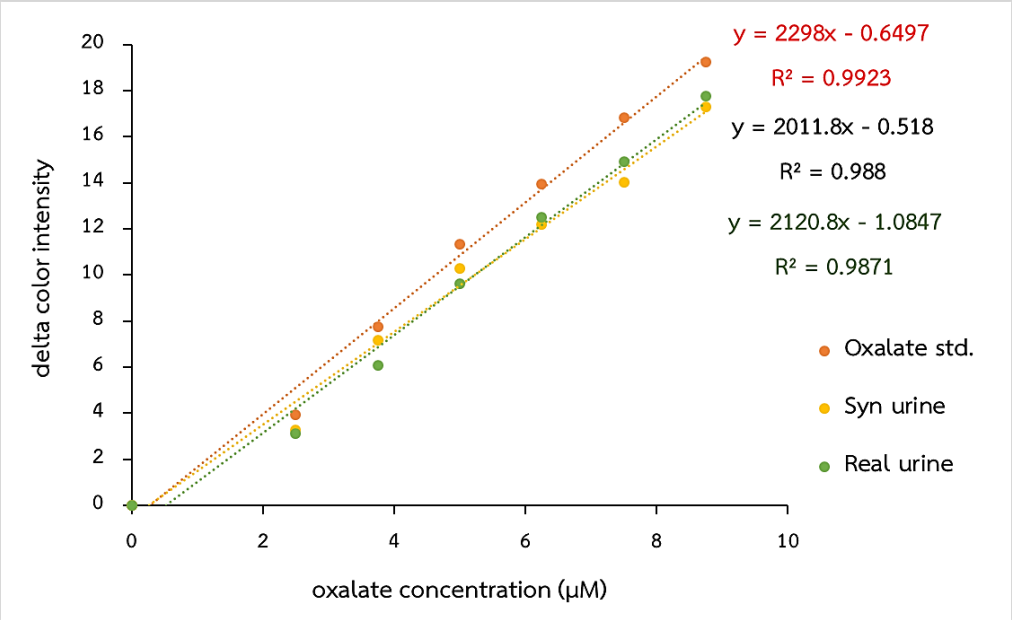


Figure 4.7 External calibration curve obtained from the analysis of oxalate standard solutions, oxalate in synthetic urine solutions, and spiked urine sample solutions.

4.6.2 Real sample analysis

To evaluate the accuracy of the method in urine sample analysis, the results from the proposed method was compared to the results obtained by using ion chromatography technique in the analysis of the same urine samples. The urine samples were diluted at least 25 times with 0.01 M HNO_3 prior to the analysis by the testing paper under the optimum condition and the standard addition method. The oxalate concentration range for standard addition method was 2.5-8.75 μM. On the other hand, the external standard method was applied to the sample analysis by ion chromatography with the linear working range of 6.0-100.0 μM oxalate and the

detection limit of 5.6 μM. The samples spiked with known concentration of oxalate were analyzed by both methods to determine the recovery of analyte. The accuracy and precision of these methods were evaluated and presented in terms of %recovery and %RSD, respectively. The results obtained from both methods are compared in Table 4.10.

The %recovery obtained by the proposed method was in the range of 95–108%, compared to 100–105% by ion chromatography technique. The relative standard deviation (%RSD) of the proposed method was found in the range of 3.2–7.6%, compared to 0.5–4.5% of the ion chromatography technique. These results indicate that both the proposed method and ion chromatography method can be used for oxalate detection in urine samples with acceptable accuracy and precision according to the criteria of AOAC international.

By comparing the results obtained from both methods, it was found that the results from the proposed method could be both close or significantly different from those by ion chromatography method depending on the starting concentration of oxalate in urine samples. For sample-1, the urine sample contained a high concentration of oxalate, the results of both methods were not significantly different by pair T-test at 95% confidence level. However, when the starting concentration of oxalate in sample was too low for the proposed method to detect such as in sample 2-4, the results from spiked samples observed by the method was slightly lower than those from the instrumental analysis. This observation could be explained by the sample dilution required in this method to overcome the matrix effect, while the ion chromatography technique would not have this matrix problem due to the species separation in the column. In addition, the method sensitivity was not high enough to distinguish a slight change in micromolar level in diluted samples. Hence, the results from the proposed method were different from the instrumental method in this case.

Table 4.10 Determination of urinary oxalate in real urine samples by the proposed method and ion chromatography method

Samples	Spiked level (μM)	Proposed method			Ion chromatography method		
		Found level (μM)	%RSD	%Recovery	Found level (μM)	%RSD	%Recovery
Sample-1	-	177.0±8.5	4.8	-	168.8±5.7	3.4	-
	100.0	261.5±8.3	3.2	95%	271.0±4.4	1.6	102%
Sample-2	-	N.D.	-	-	14.2±0.0	0.49	-
	100.0	109.1±5.5	5.1	109%	116.2±0.0	0.53	102%
Sample-3	-	N.D.	-	-	34.9±2.0	4.5	-
	100.0	108.5±8.3	7.6	108%	140.2±5.0	3.9	105%
Sample-4	-	N.D.	-	-	40.3±1.5	3.8	-
	100.0	108.9±5.1	4.7	108%	141.0±2.4	1.7	100%

Note; N.D. = Non detectable, mean ± SD (n=3)

The analytical performance of this method was compared to other paper-based methods for oxalate detection as presented in Table 4.11. It can be seen that the performance of the Fe(III)-curcumin testing paper method was comparable to the other works. Furthermore, the testing paper preparation is simple and low cost. It can be a good alternative for oxalate determination with a consideration regarding matrix effect.

Table 4.11 Comparison of oxalate determination with different colorimetric method

Method	Linear range (μM)	LOD (μM)	Reaction	Sample	Ref.
Paper-based device	10-1000	10	Enzymatic reactions with colorimetric detection	Urine	[8]
Paper-based device	1.76-49.40	0.62	Indicator displacement assay (IDA) with RB4-Cu(II) complex as indicator	Urine Vegetable	[15]
Paper-based device	0.83-113.00	0.54	Indicator displacement assay (IDA) with the complex of Eriochrome Cyanine R (ECR) and VO(II) as indicator.	Urine	[16]
Fe(III)-curcumin testing paper	0-100.0	0.8	Indicator displacement assay (IDA) with Fe(III)-curcumin complex as indicator	Urine	**[This work]**

CHAPTER V
CONCLUSION

5.1 Conclusion

In this research, a simple colorimetric method was developed to determine oxalate level in urine samples using the testing paper modified with Fe(III)-curcumin complex. The detection mechanism was based on the reaction between Fe(III)-oxalate complex in the sample solution which decreased the amount of Fe(III)-curcumin complex on the testing paper. The mechanism was proved by a UV-Vis spectrophotometer. The change of testing paper color from reddish-brown of Fe(III)-curcumin complex to yellow of free curcumin in the presence of oxalate could be clearly observed by naked eyes. The effect of parameters affecting the paper preparation was studied. The optimum conditions for the preparation of Fe(III)-curcumin testing paper was to use 7.5 mM curcumin and 0.25 mM Fe(III) in 0.01 M HCl. The testing paper was used in the oxalate detection in standard solutions and the influence of different parameters were investigated. The optimum pH for oxalate detection was pH 2.0. The detection was performed by immerging the testing paper in 3 mL of sample for 10 minutes. The detection should be repeated 3 times.

For the quantitative analysis, the color intensity of the testing paper was determined by using ImageJ program with green mode. Under the optimum conditions, the linear working range of this method was 0 to 100.0 μM with a correlation coefficient (R^2) > 0.99, limit of detection of 0.8 μM, and limit of quantitation of 2.5 μM.

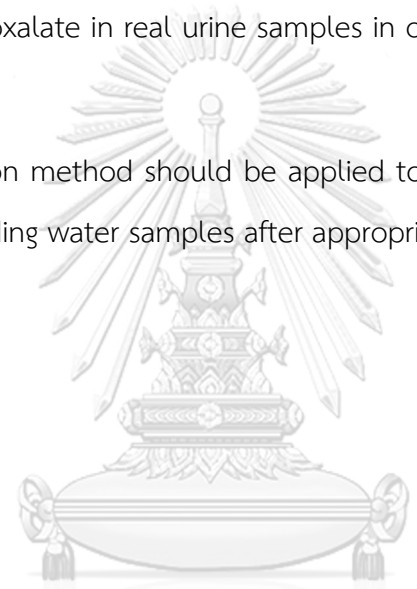
To apply the testing paper for urine analysis, the effect of urine matrix and compounds generally found in human urine samples was investigated. Most compounds studied show strong effect on the analysis of oxalate. To overcome the matrix effect, the standard addition method was applied for the quantification of oxalate in real urine samples. The percent recovery (%recovery) obtained by this method was in the range of 95-108%, compared to 100-105% by ion chromatography

method. The relative standard deviation (%RSD) of the results obtained was found in the range of 3.2-7.6%, compared to 0.5-4.5% by ion chromatography method. The testing paper showed a potential as an alternative method for screening of oxalate levels in human urine samples.

5.2 Suggest for future work

1. The sample preparation should be applied to overcome the matrix effect in the determination of oxalate in real urine samples in order to improve the detection sensitivity.

2. The detection method should be applied to detect oxalate level in other type of samples including water samples after appropriate sample preparation.



REFERENCES

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