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DETERMINATION OF URINARY GLUCOSE BY FLUORESCENCE SPECTROSCOPY OF CURCUMIN USING FENTON REACTION

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งานวิจัยนี้นำเสนอทางเลือกของการตรวจวัดกลูโคสในปัสสาวะโดยอาศัยปฏิกิริยากลูโคสออกซิเดส และปฏิกิริยาเฟนตัน โดยให้ไฮโดรเจนเปอร์ออกไซด์ซึ่งเป็นผลิตภัณฑ์จากปฏิกิริยาระหว่างกลูโคสและเอนไซม์กลูโคสออกซิเดส ทำปฏิกิริยากับเฟอร์รัสไอออนในปฏิกิริยาเฟนตัน โดยผลิตภัณฑ์จากปฏิกิริยาเฟนตันประกอบด้วยอนุมูลอิสระไฮดรอกซิล และเฟอร์ริกไอออน ซึ่งสามารถทำปฏิกิริยากับเคอร์คูมินด้วยการเกิดปฏิกิริยาออกซิเดชัน และการเกิดสารประกอบเชิงซ้อนตามลำดับ ค่าสัญญาณฟลูออเรสเซนซ์ของเคอร์คูมินลดลงตามความเข้มข้นที่สูงขึ้นของไฮโดรเจนเปอร์ออกไซด์ หรือกลูโคส มีการศึกษาผลของตัวแปร ได้แก่ ความเข้มข้นของไฮโดรคลอริก เวลาที่ใช้ในการทำปฏิกิริยาระหว่างรีเอเจนต์และสารตัวอย่าง ปริมาณรีเอเจนซ์ และเวลาสำหรับการทำปฏิกิริยากลูโคสออกซิเดส นอกจากนี้ได้ศึกษาผลกระทบของเมทริกซ์ในปัสสาวะและตัวรบกวนอื่น โดยพบว่าเมื่อสารตัวอย่างถูกเจือจางอย่างน้อยสองเท่า เมทริกซ์ในปัสสาวะไม่ส่งผลกระทบต่อการวิเคราะห์กลูโคสในปัสสาวะด้วยวิธีการของงานวิจัยนี้ ช่วงเวลาที่ใช้ในการเกิดปฏิกิริยากลูโคสออกซิเดส และปฏิกิริยาระหว่างรีเอเจนซ์กับสารตัวอย่างล้วนใช้เวลาอย่างละ 30 นาที ภายใต้สภาวะที่เหมาะสมสามารถตรวจวัดกลูโคสได้โดยมีช่วงความสัมพันธ์เชิงเส้นตรงในช่วงความเข้มข้นกลูโคส 0.30-1.40 มิลลิโมลาร์ ให้ค่าความเข้มข้นต่ำสุดที่สามารถตรวจวัดได้ คือ 0.10 มิลลิโมลาร์ และค่าความเข้มข้นต่ำสุดของกลูโคสสำหรับการวิเคราะห์ปริมาณ คือ 0.30 มิลลิโมลาร์ องค์ประกอบอื่นที่สามารถพบในปัสสาวะไม่มีผลกระทบต่อการตรวจวัดกลูโคสด้วยวิธีของงานวิจัยนี้ ยกเว้นวิตามินซี ค่าความเข้มข้นสูงสุดของวิตามินซีที่ไม่มีผลกระทบต่อการวิเคราะห์ คือ 12.5 มิลลิกรัมต่อลิตร นอกจากนี้เมื่อเปรียบเทียบค่าค่าความเข้มข้นของกลูโคสที่ตรวจวัดได้ด้วยวิธีการของงานวิจัยนี้กับผลจากการใช้วิธีมาตราฐานเฮกไซไคเนส พบว่าค่าความเข้มข้นของกลูโคสที่ตรวจวัดในตัวอย่างปัสสาวะจากทั้งสองวิธีไม่มีความแตกต่างกันอย่างมีนัยสำคัญ ค่าความเที่ยงในการวิเคราะห์ (%RSD) อยู่ในช่วง 0.9-3.9% นั่นคือประสบความสำเร็จในการประยุกต์ใช้วิธีการที่นำเสนอนี้ในการตรวจวัดกลูโคสในปัสสาวะของมนุษย์ด้วยความแม่นและความเที่ยงที่ยอมรับได้

ภาควิชา เคมี	ลายมือชื่อนิสิต .....
สาขาวิชา เคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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SUTTHIDA RUAMARAM: DETERMINATION OF URINARY GLUCOSE BY FLUORESCENCE SPECTROSCOPY OFCURCUMIN USING FENTON REACTION. ADVISOR: ASST. PROF. FUANGFA UNOB, Ph.D., CO-ADVISOR: ASST. PROF. NARONG PRAPHAIRAKSIT, Ph.D., pp.

This research presents an alternative method to determine glucose level in human urine based on the glucose oxidase reaction and Fenton reaction. Hydrogen peroxide produced in the reaction between glucose oxidase and glucose would react with ferrous ion via Fenton reaction. The products of Fenton reaction including hydroxyl radicals and ferric ions further reacted with curcumin through oxidation reactions and complex formation, respectively. The fluorescence intensity of curcumin decreased with an increase of hydrogen peroxide or glucose concentration. The effects of various parameters were investigated including HCl concentration, reaction time periods, quantity of reagents and time periods for glucose oxidase reaction. Moreover, the urine matrix effect and potential interfering species were also studied. As a result, the urine matrix did not have impact on glucose detection after a 2-fold dilution of urine sample. The time period required in glucose oxidase reaction and Fenton and curcumin reaction were both 30 minutes. By using the proposed condition, the linear range for glucose determination was 0.30-1.40 mM with the limit of detection and quantification of 0.10 and 0.30 mM, respectively. The co-existing species in urine sample did not affect the determination of glucose except ascorbic acid. The tolerant value of ascorbic acid in urine for glucose analysis by the proposed method was 12.5 mg/L. Moreover, the analytical results obtained by using the proposed method in the analysis of urine samples were not significantly different from the results observed by the standard hexokinase glucose-6-phosphate dehydrogenase method. The percent of relative standard deviation of glucose analysis by using the proposed method were found in the range of 0.9-3.9%. The proposed method was successfully applied to detect glucose in human urine samples with acceptable accuracy and precision.

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

M	Molar
mM	Millimolar
ppm	Part per million
mg/L	Milligram per liter
mg/mL	Milligram per milliliter
mg	Milligram
g	Gram
L	Liter
µL	Microliter
mL	Milliliter
nm	Nanometer
min	Minute
LOD	Limit of detection
LOQ	Limit of quantitative
°C	Degree Celsius
DMSO	Dimethyl-sulfoxide
BSA	Bovine serum albumin
DI	Deionized water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HSAB	Hard Soft Acid Base

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

Glucose, a small unit of carbohydrate, is a primary source of energy for the living body. The determination of glucose can be used to indicate certain diseases in clinical analysis such as diabetes [1-3]. The data from the International Diabetes Federation (IDF) shows that the increase in the number of patients with diabetes is a result of heredity and lifestyle. Poor eating behavior and lack of exercise are the main causes of diabetes [4]. Moreover, according to the World Health Organization, in 2004 over 150 million people in the world were affected with diabetes and the number is expected to increase to 366 million in 2030 [5, 6]. Nonetheless, approximately 50% of the people with diabetes are not even aware of this. People diagnosed with diabetes in the early state can be treated before the severe illness develops. Diabetes can cause a number of short and long-term health complications, including hypoglycemia, heart disease, nerve damage, and vision problems. Therefore, the screening of diabetes in people who have high risk for diabetes is very important [4, 6].

The current approach to diagnose diabetes is the determination of blood glucose levels by fasting plasma glucose test (FPG) and oral glucose tolerance test (OGTT) [1, 3]. However, the FPG and OGTT methods do not allow patients to eat for at least eight hours before the test, and OGTT requires two blood tests. These practices are prone to infection; as a result, skilled people are required to collect samples, and the practices are not suitable for people who are needle phobic (trypanophobia). While blood glucose testing is the primary way to test high glucose, urine testing can also be useful because excess glucose in body passes through urine. The urine test is more practical to screen diabetes with glucose levels because urine can be collected anytime without restrictions. The advantage of testing urine for glucose is that it is inexpensive, noninvasive, painless, convenient and especially suitable for children (painless). Moreover, the content of urinary glucose was an

indicator for renal and hepatic diseases [7] or tubular disease such as Fancomi syndrome as well [8]. The glucose in urine is called glucosuria, which resulted from the amount of glucose in the glomerular filtrate that exceeds the capacity of the renal tubule to reabsorb it [7-9].

The determination of urinary glucose can be performed in many different ways; for example, o-toluidine method [10, 11], and enzymatic method which comprises of glucostat [12] (using glucose oxidase and horseradish peroxidase) and hexokinase glucose-6-phospate dehydrogenase method [9, 13, 14]. O-toluidine method is based on the reaction between o-toluidine and glucose in glacial acetic acid, which results in a change of o-toluidine color. On the other hand, in the glucostat method glucose oxidase, horseradish peroxidase and chromogen react with glucose and its content can be measured by the changes of color in chromogen. O-dianisdine [14-17] and quinomeimine [18] are common chemicals which are used as chromogen. The hexokinase glucose-6-phospate dehydrogenase method utilizes reagents which include adenosine triphosphate (ATP), glucose-6-phosphate dehydrogenase (G6P-DH) and nicotine-amide-adenine-dinucleotide-phosphate (NADP). NADPH is the final product obtained in this reaction and it can be measured by the color which occurs after the reaction is finished. The enzymatic method usually was measured with colorimetry using either naked-eye technique (test kit, strip test) or spectroscopy. The naked-eye technique is cheap and fast; however, only semi-quantitative results can be obtained as the color of urine affects its accuracy and precision. [19]. Spectroscopic method can provide better quantitative results and more reliability in terms of accuracy and precision [20, 21]. Therefore, an alternative determination of glucose in urine to indicate the disease is presented in this proposed method.

Fluorescence spectroscopy is a highly sensitive and specific method to identify certain analytes. With the use of curcumin being a reducing agent and having fluorescence properties, it can be used to detect glucose by reacting with the products from Fenton reaction between ferrous ion and hydrogen peroxide produced from the reaction of glucose with glucose oxidase. This reaction produces hydroxyl radical and ferric ion which react with curcumin. The reduction of glucose is

related to the reaction of curcumin; therefore, the decrease in curcumin fluorescence can be related to the amount of glucose in the tested solution.

From the information above, the screening of the abnormal glucose concentration is important to indicate the problem health. Hence, this research focuses on the method to detect glucose in human urine by fluorescence spectroscopy which is highly sensitive and selective detection technique. The optimal condition was evaluated through with various parameters and the method was tested the performance via LOD, LOQ, linear range, accuracy and precision. The accuracy and precision were assessed by comparing the results of glucose analysis with the standard method, hexokinase glucose-6-phospate dehydrogenase method.

1.2 Objective and scope of the research

The objective of this research was to develop a method for the urinary glucose determination with fluorescence spectroscopy of curcumin using Fenton reaction.

The proposed urinary glucose detection method is based on the use of curcumin, glucose oxidase reaction and Fenton reaction. Various parameters were investigated such as the reagent solvents, mole ratio between analyte and reagents, matrix effect in synthetic urine. Moreover, LOD, LOQ, linearity, precision and accuracy of urinary glucose were studied in validation of the developed method. The precision and accuracy were compared with the standard hexokinase glucose-6-phospate dehydrogenase method.

1.3 The benefit of the research

A new method to detect glucose in human urine sample was obtained.

CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 Glucose

Glucose, a biomarker of human health, can indicate certain diseases such as diabetes. Glucose is a small unit of carbohydrate normally obtained from diet and is the basic source of energy for human [1-3]. Glucose is transported into cells through multiple metabolic pathways and stored as glycogen [22]. Glucose has a molecular formula $C_6H_{12}O_6$ and a molecular weight of 180. Glucose is divided into D-glucose isomer which exists in nature and L-glucose isomer which is formed by synthesis. However, D-glucose isomer is considered an indicator of the disease because this isomer exists in human [23].Moreover, D-glucose has two forms of stereoisomer, alpha-D-glucose and beta-D-glucose. The structure of alpha form has an OH group on the far right bottom, and the beta form has the OH group on the far right top as shown in Figure 2.1. D-glucose is an equilibrium mixture of alpha and beta forms [24].

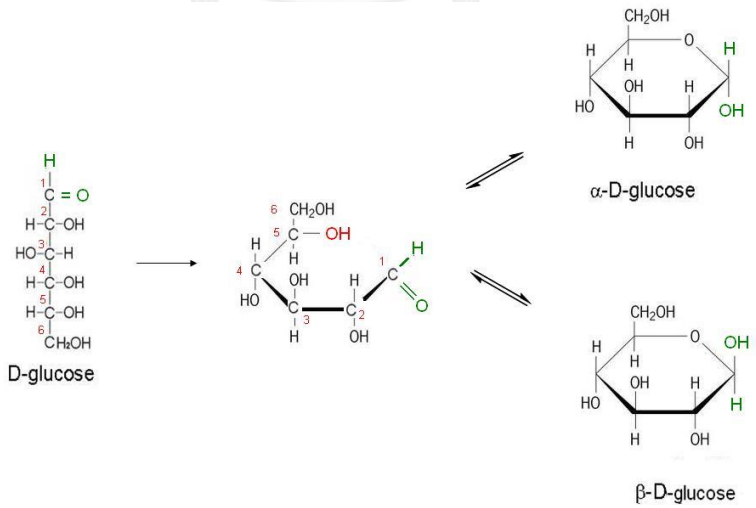


Figure 2.1 The structure of glucose

Various studies have reported different ranges of diagnostic glucose levels. The measurement varies according to the methods employed. For example, the concentration of glucose above 0.83, 1.11 mM are declared abnormal ranges of glucose in urine as reported by Fine et al. [8] and Bitzen et al. [25] respectively. Fine et al. and Bitzen et al studied glucose content based on glucostat method, but Fine et al. used o-dianisidine as chromogen with UV-Vis spectroscopy while Bitzen et al. used orthotoluidine as chromogen with colorimetric method (naked eye method). In addition, Scherst et al. [9] studied normal and abnormal ranges of glucose in urine by using hexokinase glucose-6-phospate dehydrogenase method, and later detected glucose with UV-Vis spectroscopy. They reported that an abnormal glucose range is higher than 1.11 mM. Any abnormal glucose level in urine called glucosuria, which indicates a health problem. However, the hexokinase glucose-6-phosphate dehydrogenase method is the standard and reference method used in this work, hence the normal glucose range of this method was then adapted to be less than 1.11 mM.

2.2 Determination of glucose in urine

The most common methods to determine glucose in urine have been proposed, including o-toluidine method and enzymatic method, which are divided into glucostat method (glucose oxidase couple horseradish peroxidase) and hexokinase glucose-6-phospate dehydrogenase method. Enzymatic method was mostly used for glucose analysis and was determined with naked eye (test kit, strip test) or spectroscopic techniques. Naked eye technique provides a cheap and fast approach, but it gives only semi-quantitative information as a result of interference from the color of urine. Spectroscopic techniques, on the other hand, can present quantitative data with accuracy and precision. The principles of these methods are described in more detail below.

2.2.1 O-toluidine method

O-toluidine method is a direct method to detect glucose in urine. O-toluidine is a primary aromatic amine that reacts with glucose in a hot glacial acetic acid which produces a blue-green color and its absorbance at 630 nm with UV Vis spectroscopy is related to the concentration of glucose (Figure 2.2) [10, 11]. However, the Word Health Organization has declared o-toluidine as a potential carcinogenic substance; thus, this method is scarcely used nowadays [26].

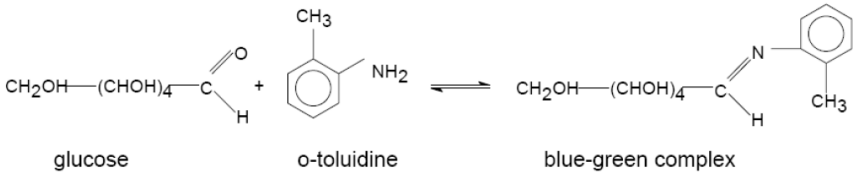


Figure 2.2 The reaction of o-toluidine method to detect glucose

2.2.2 Enzymatic method

2.2.2.1 Glucostat method

Glucostat method employs reagents which consist of chromogen and two types of enzymes which is glucose oxidase and horseradish peroxidase. There are two reactions in this method as shown in Figure 2.3. The detail about glucostat method is explained below.

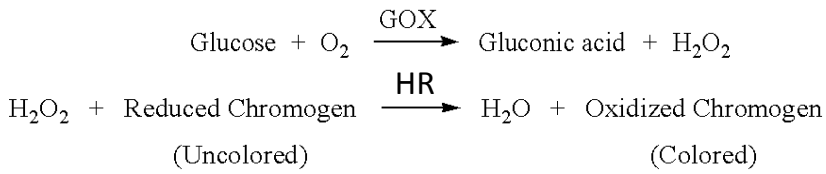


Figure 2.3 The reaction of glucostat to detect glucose

Glucose oxidase was purified from the genus Aspergillus. Glucose oxidase can be found in a lot of researches which report that glucose oxidase is a specific catalyst of the reaction of beta-D-glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor with the simultaneous production of hydrogen peroxide [18]. Glucose oxidase is a highly specific beta-anomer of D-glucose, while alpha-anomer does not appear to be a suitable substrate. The alpha and beta forms of D-glucose are reciprocal in equilibrium and thus there is no delay reactivity. The beta-D-glucose is available for immediate reaction with enzyme [5, 6, 18, 24]. The reaction between glucose and glucose oxidase produces hydrogen peroxide which reacts with chromogenic substrate using horseradish peroxidase (HRP) as a catalyst to produce oxidized-chromogen. The color which is changed by chromogen was measured with naked eye or spectroscopic technique [17, 27, 28]

In addition, there are many chromogens, which are commonly used indicators in this method such as o-dianisidine [8, 15, 17, 18], quinoneimine [18], tetramethybenzidine (TMB) [19, 29, 30]. They can indicate absorbance at 430, 500, 652 nm after the completion of the reaction respectively. However, there are several disadvantages in glucostat method which are the high cost of the two enzymes, chromogenic substrate triggering cancer and giving inaccuracy if urine contains high level of uric acid or albumin.

2.2.2.2 Hexokinase glucose-6-phospate dehydrogenase method

The hexokinase glucose-6-phospate dehydrogenase method features two reactions like glucostat method, but uses different enzymes. The detail of the reaction is described below (Figure 2.4).

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

Figure 2.4 The reaction of hexokinase glucose-6-phosphate dehydrogenase method to detect glucose.

Hexokinase catalyzes a specific reaction, based upon the inversion of glucose to glucose 6-phosphate (G6P) by adenosine triphosphate (ATP) with hexokinase. Glucose-6-phosphate (G6P) is oxidized to 6-phosphogluconate with the reduction of nicotine-amide-adenine-dinucleotide-phosphate (NADP) to NADH by G6PDH. The amount of NADH formed is related to the amount of glucose in the sample and can be measured with UV-vis spectroscopy, with its absorbance at 340 nm.

In terms of the urinary glucose test, the enzymatic method is more popular than o-toluidine method because the o-toluidine reagent is a potential carcinogenic substance, and the process of preparing reagents is complicated. However, the enzymatic method has a restriction due to the high cost of the enzymes. Moreover, horseradish peroxidase in glucostat method is an unstable enzyme which is decomposed easily under the poor condition [9, 13, 14]. Thus, a method for the determination of urinary glucose is developed in this work using reaction between glucose and a reagent mixture (curcumin, ferrous ion, glucose oxidase) to overcome those weaknesses in the determination of urinary glucose. The concept of this proposed method is using one enzyme (glucose oxidase) which helps decrease the

cost of reagents when compared with glucostat method. Furthermore, the technique which is used in this work to detect glucose in urine is fluorescence spectroscopy. Fluorescence spectroscopy is a good technique which is highly sensitive and specific for certain analytes. The results from fluorescence spectroscopy are superior in both accuracy and precision, along with the wide linear range, when compared with UV-Vis spectroscopy.

2.3 Fluorescence spectroscopy

2.3.1 The principle of fluorescence spectroscopy

Fluorescence is a category of luminescence which is the emission of light from any substance and occurs from electronically excited states. Fluorescence is the emission of photons which are released from the excited state to the ground state. The emission rate of fluorescence is typically $10^{8}s^{-1}$. Fluorescence occurs in conjugated molecules of aliphatic and aromatic rings called fluorophore [31]. The fluorescence process is illustrated with Jablonski's diagram.

Jablonski'sdiagram is an energy diagram which illustrated the absorption and emission. This diagram is named after Professor Alexander Jablonski, who is regarded as the father of fluorescence spectroscopy. Jablonski's diagram involves electronic transition. The singlet ground, first and second electronic states, is depicted by S_0, S_1, and S_2, respectively. The first transition in Jablonski's diagram is the absorption of a photon of a particular energy by the molecule of our interest. This is indicated by a straight arrow pointing up as shown in Figure 2.5.

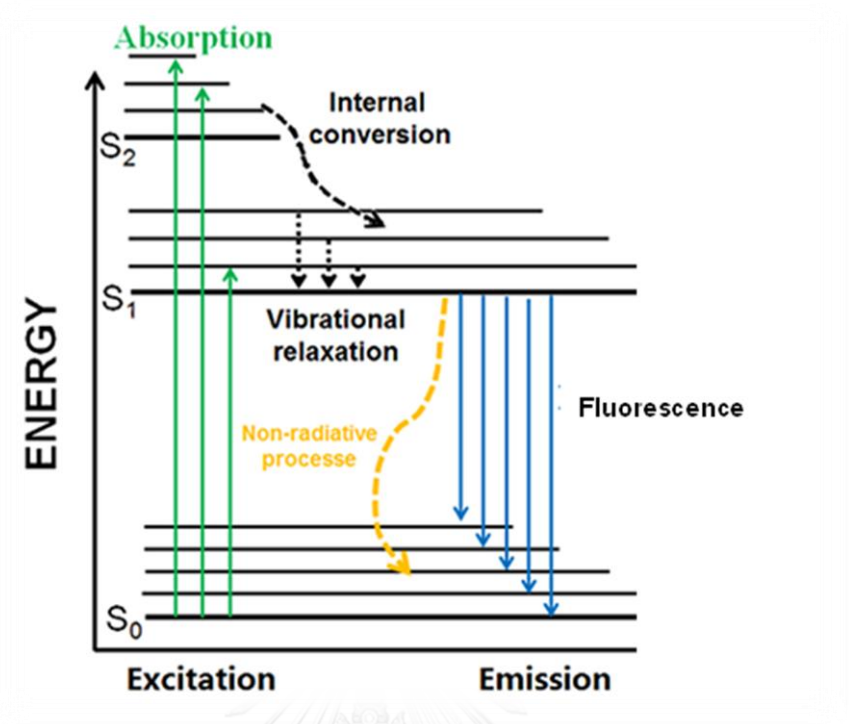


Figure 2.5 Jablonski diagram

Absorption process of an electron takes place very rapidly, with the speed of 10^{-15} seconds, from a lower to a higher energy level. On the other hand, fluorescence is the emission of photons from the excited to the ground state as shown in Figure 2.5 [31, 32], with a lower speed of 10^{-9} to 10^{-7} seconds. The electrons stay in the same multiplicity manifold. Fluorescent photons lose energy on the way to the ground state due to vibrational relaxation and internal conversion.[31].

Fluorescence spectral

A fluorophore is a fluorescent compound which can emit light upon light excitation. Each of fluorophore has a unique of spectra of fluorescence. The fluorescence spectra of fluorophore are relative fluorescence intensity versus wavelength (nanometers). The structure of the fluorophore affects the emission spectra. The emission intensity (Em_1, Em_2) depends on the energy at

the excitation wavelength (Ex_1, Ex_2). The highest emission intensity occurs when fluorophore is excited with the maximum absorption wavelength. Thus, the intensity of emission varies according to the input amount of the energy, as shown in Figure 2.6 [31].

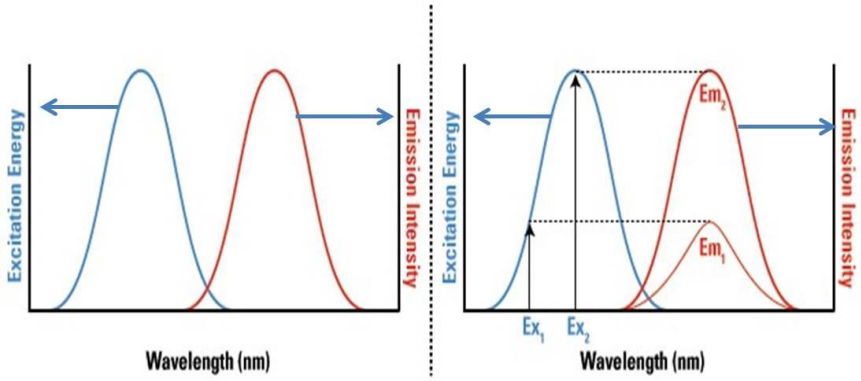


Figure 2.6 The excitation and the emission spectra of fluorophore

Stokes shift

Stokes Shift is the difference between the excitation and emission wavelengths as the energy of emission dissipate during the excited state lifetime. These phenomena result in the lower energy and longer wavelength of the emission photons than those of the exciting photons as shown in Figure 2.7.

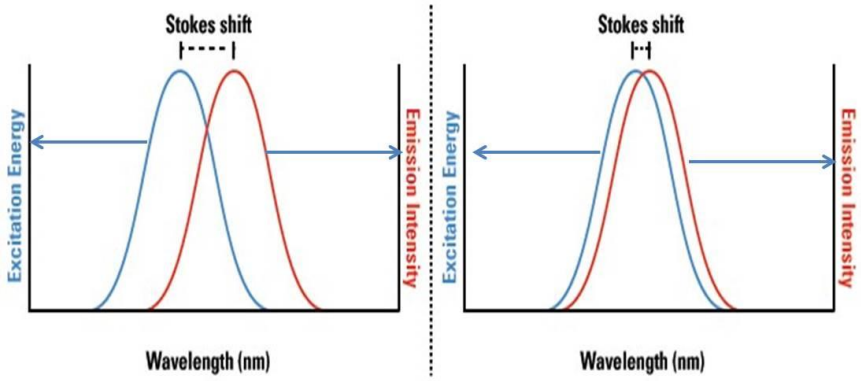


Figure 2.7 Fluorophores with large (left) and small Stokes shift (right). [31]

The Stokes shift is also a distinct characteristic of each fluorophore. Larger Stokes shifts of fluorophore (left) show a clear distinction between the excitation and the emission light in the sample, while fluorophores with smaller Stokes shifts (right) exhibit greater background signal because of the smaller gap between the excitation and the emission wavelengths from their great overlapping.

2.3.2 Quenching of fluorescence

The phenomenon of decreasing intensity of fluorescence is called quenching. The quenching can occurs by a variety of molecular interactions which depend on the chemical properties of the individual molecules. The phenomena which affect the quenching can be divided to static (complex formation) and dynamic (collisional) quenching. Both static and dynamic quenching require molecular contact between the fluorophore (sample) and the quencher (inhibitor of fluorescence).

Static (complex formation) quenching

The phenomenon of static quenching takes place when the fluorophore containing molecules and the quencher form a complex in the ground state before the excitation occur. This complex is non-fluorescent and the only observed fluorescence is from uncomplexed fluorophores. Thus, static quenching removes a part of the fluorophores from observation.

Dynamic (collisional) quenching

Dynamic quenching involves the diffusion of fluorophore during its lifetime in the excited state which results in the fluorophore returning to ground state, without the emission of photon. It has been reported that the dynamic quenching occur by a mixed mechanism which includes intersystem crossing, electron exchange quenching, photo-induced electron transfer (PET), depending on the structure of the fluorophore. Intersystem crossing involves the electron transfer from excited singlet state to become an excited triplet

and returns to the ground state by the same quencher, or non-radiative decay. While electron exchange quenching is a process in which the donor (fluorophore) and acceptor (quencher) exchange their electron. The electron of excited donor (D* in LUMO) is transferred to the excited acceptor (A* in LUMO) while the electron from the ground acceptor (A in HOMO) is transferred back to the ground donor (D in HOMO). The electron exchange transfer occurs when wavelength of emission spectra of donor overlap with wavelength of absorption spectra of acceptor. The diagram electron exchange transfer is schematically shown in Figure 2.8.

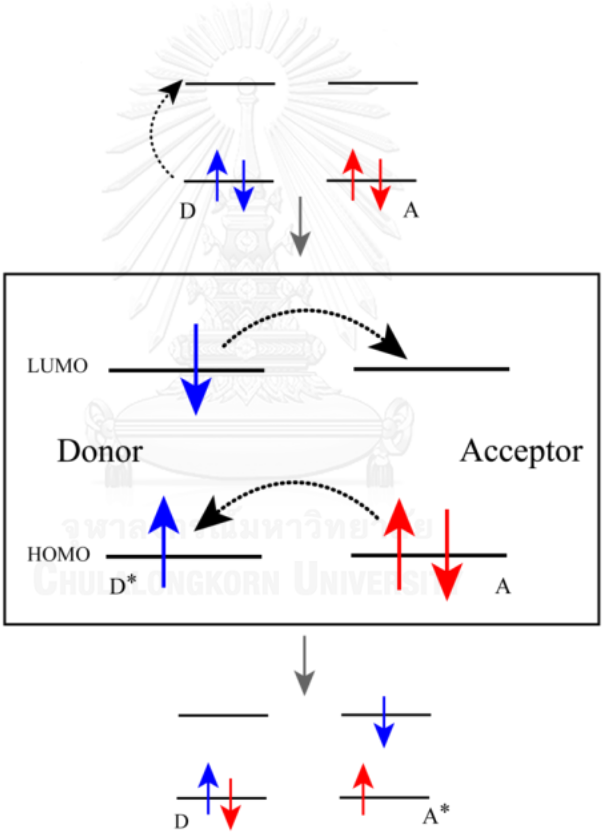


Figure 2.8 The diagram of electron exchange transfer

Photo-induced Electron Transfer (PET) is the last mechanism of dynamic quenching. PET quenching involves the redox potentials of the fluorophore and quencher. The energy change of PET occurs by electron

transfer between donor and acceptor. The diagram of PET is shown in Figure 2.9, D_P and A_P are the electron donor and electron acceptor respectively while D_P+A_P is a charge transfer complex. The fluorophore (D_P*) is excited from the ground state (HOMO) to the excited state (LUMO). The electron donor in HOMO transfers an electron to the acceptor, forming the charge-transfer complex (D_P+A_P*).

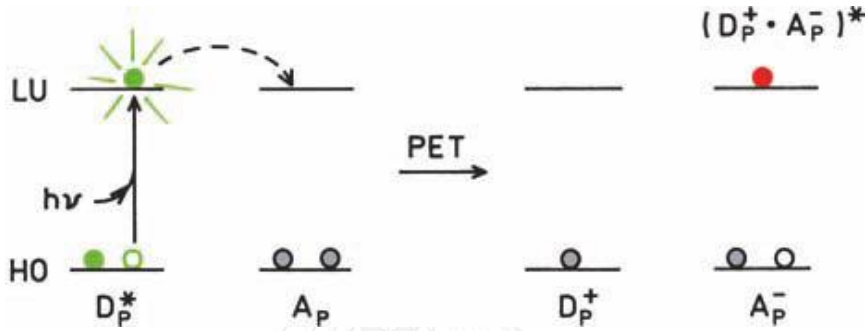


Figure 2.9 Molecular orbital schematic for the photo-induced electron transfer.

The charge transfer complex can return to the ground state without emission of a photon, but in some cases excited charge transfer complex emission is observed as seen in Figure 2.10. However, D_P and A_P do not form a complex when both are in the ground state because this is energetically unfavorable. Finally, the extra electron on the acceptor is returned to the electron donor. When discussing PET the term donor refers to the electron-rich species that donates an electron to an acceptor [31].

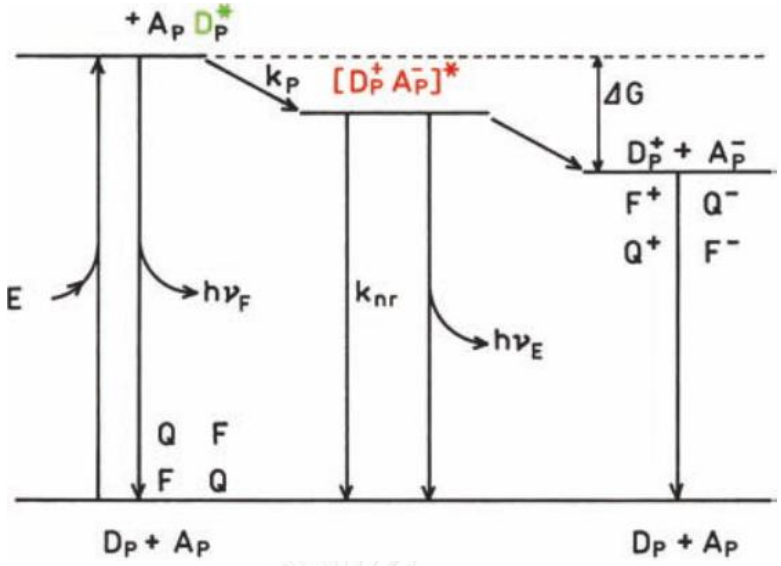


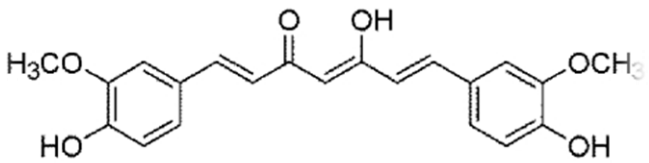
Figure 2.10 The energy diagram for photo-induced electron transfer

2.5 Literature review

A number of researchers have proposed methods to detect glucose by fluorescence spectroscopy based on synthetic fluorophore. For example, Liu, Y. et al. [33] and Karnati, V.V., et al. [34] synthesized Tetraphenylethene-cored diboronic acid and Diphenylboronic acid fluorescent sensor, respectively. These synthetic fluorophores can detect glucose based on covalent interaction (H-bond) between boronate anion and hydroxyl groups of glucose. The synthetic fluorophore has many steps which are complicated and time consuming thus this work presented easier way to detect glucose base on the common fluorophore (not synthetic) cooperated general reactions.

Curcumin is a common fluorophore which produce the fluorescence intensity to monitor glucose level in this work. Curcumin (1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione) is a natural orange-yellow crystalline powder. Curcumin is one of the three curcuminoids present in turmeric. It belongs to the family of Zingiberaceae. Curcumin has a molecular formula $C_{21}H_{20}O_6$ and a molecular weight of 368.91. This substance is nontoxic to animals and humans except for some bacteria. Curcumin is insoluble in water, while soluble in organic solvents such as

methanol, ethanol, dimethyl-sulfoxide (DMSO). The structure of curcumin depends on the solution pH [35], [36]. At pH 1 to 7, the color of curcumin solution is yellow, the enol form in the pH range is the dominant species. The structure of curcumin in this pH range contains a conjugated double bond and polyphenol (Figure 2.11) which can be detected by fluorescence spectroscopy. While, at pH>7 condition the red color is presented, the structure of curcumin is degraded to ferulic acid, feruloylmethane, vaniline (Figure 2.12).



Curcumin
(keto-enol form)

Figure 2.11 The structure of curcumin in the enol form [35].

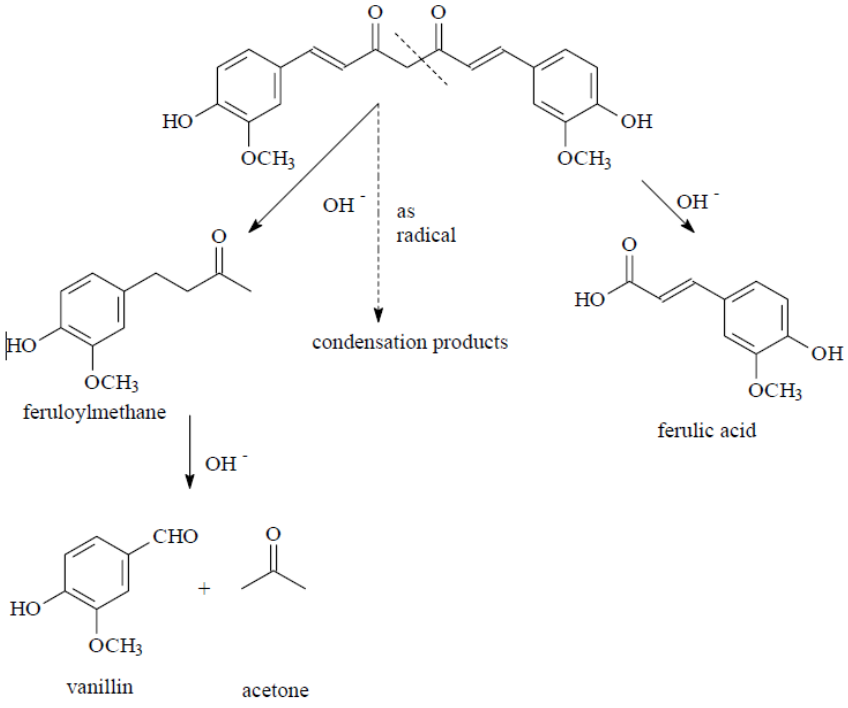


Figure 2.12 The degradation of curcumin

Moreover curcumin displays a hard Lewis base character of enol form and a powerful antioxidant activity (highly reducing agent) [37, 38] which was used to detect many analytes in previous works. The hard Lewis base of curcumin was used to detect ferric ion as presented in Saithongdee et al's research. [39] They created a sensor to determine ferric ion in solution with zein membrane containing curcumin, fabricated by electrospining and crosslinking with citric acid. The concentration of analyte could be determined by naked-eye detection after diping this sensor into sample. Zein membrane containing curcumin was evaluated as a function of pH from 1 to 5 in terms of its affinity toward various types of metal ions (Ag^{+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, Na^{+}, K^{+} and As^{5+}). As a result, only ferric ion was reacted with zein membrane containing curcumin and produced the color change from yellow to brown at pH 2. Because the enol form of curcumin can bind with metal as a ligand in the curcumin-metal complex, the color of zein membrane containing curcumin changes. This selectivity was described by the Hard Soft Acid Base (HSAB) principle. Ferrous ion is a hard Lewis acid and prefers to combine with enol form of curcumin which is a hard Lewis base.

Meanwhile, the property of curcumin as a reducing agent was used to detect hydrogen peroxide which is an oxidizing agent as presented in Iwunze's research [40]. Iwunze used the fluorescence technique to monitor the signal of curcumin when it reacts with hydrogen peroxide. In this experiment, curcumin concentration was kept constant (8.57×10^{-4} mM) while the concentration of hydrogen peroxide were varied from 9.0 to 93.2 mM. The fluorescence of curcumin decreases in accordance with the increasing concentration of hydrogen peroxide (quenching). These phenomena of quenching take place when curcumin (fluorophore), as a reducing agent is oxidized by hydrogen peroxide (quencher). Furthermore, hydrogen peroxide can be produced from glucose oxidase reaction according to many previous researches presented [5, 6, 18, 24]. The glucose oxidase has been widely employed in glucose analysis. This enzyme is highly specific to catalyze the conversion of glucose and oxygen to gluconic acid and hydrogen peroxide.

Reddy et al. [41] studied the role of curcumin as an inhibitor of lipid peroxidation. Lipid peroxidation is initiated by reactive oxygen species. The

generation of reactive oxygen species was suppressed by curcumin as a result of its antioxidant activity. In addition, this research reported the little amount of time that generates ferric ion and hydroxyl radical by ferrous ion and hydrogen peroxide. This reaction is called Fenton reaction [42, 43]. The hydroxyl radical which is the product of Fenton reaction has a relatively higher oxidation than hydrogen peroxide. The products from Fenton reaction are ferric ion and hydroxyl radical which react with curcumin and these can be used to detect glucose by fluorescence spectroscopy.

Fenton reaction was first described by H.J.H. Fenton who first observed the oxidation of tartaric acid by hydrogen peroxide in the presence of ferrous ion [44]. The application of Fenton reaction concerns an oxidizing process that eliminates hazardous organics of industrial wastewater such as aromatic amines, [45] pesticides [46, 47] and surfactants [48]. Fenton reaction is composed of oxidation processes which generate hydroxyl radical through the interaction between hydrogen peroxide and ferrous ion as shown in Eq. 2.1. Then, the hydroxyl radical can attack and initiate the oxidation of an organic pollutant molecule (R) by several degradation mechanisms as shown in the Eq. 2.2-2.4 [49], [50].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (2.1)$$

$$OH^{\bullet} + RH \longrightarrow H_2O + R^{\bullet} \quad (2.2)$$

$$R^{\bullet} + Fe^{3+} \longrightarrow Fe^{2+} + R^{+} \quad (2.3)$$

$$R^{\bullet} + H_2O_2 \longrightarrow ROH + R^{+} \quad (2.4)$$

Fenton reaction is an important source of hydroxyl radical. Ferrous ion is oxidized to ferric ion within a few seconds to minutes in the presence of excessive hydrogen peroxide while hydrogen peroxide is decomposed to hydroxyl radicals [42, 51, 52]. The efficiency of the reaction depends mainly on the concentration of hydrogen peroxide, ferrous ion/hydrogen peroxide ratio, pH, reaction time and temperature. A wide variety of work report the effect of temperature and pH on the

Fenton reaction [50, 53, 54]. The optimum condition for Fenton reaction is normally carried out at 30-40℃ and pH 2 to 3. This reaction only takes place in acidic condition because ferrous ion precipitates in basic condition as $Fe(OH)_3$ and hydrogen peroxide decomposes into oxygen, water, and heat, which can create hazardous condition.

As the literature reviews lead to concept of glucose analysis using the properties of curcumin, glucose oxidase reaction and Fenton reaction. Glucose which is the analyte was turned to gluconic acid and hydrogen peroxide from glucose oxidase reaction. This hydrogen peroxide was mixed with ferrous ion in Fenton reaction. The products of Fenton reaction is ferric ion and hydroxyl radical which relate to glucose level. Ferric ion can combine with curcumin to obtain a curcumin-Fe(III) complex at pH 2 and hydroxyl radical as a very strong oxidizing agent reacts with curcumin. Curcumin is a fluorescent compound. When curcumin reacts with the products from Fenton reaction, the fluorescence quenching is observed. The fluorescence intensity of curcumin decreases with the increasing concentration of glucose after all chemical activities (glucose oxidase and Fenton reaction). Therefore, the decrease of fluorescence intensity of curcumin is related to the level of glucose in urine sample.

CHAPTER III

EXPERIMENTAL

3.1 Analytical instrument

Fluorescence spectrofluorometer

All fluorescence measurements were performed on a Cary Eclipse spectrofluorometer (Agilent Technologies). The operating parameters are listed in **Table 3.1**.

Table 3.1 The operating parameters used for the measurement of fluorescence by curcumin

Operating conditions	Curcumin
Mode	Emission
Excitation (nm)	419
Emission wavelength start (nm)	439
Emission wavelength stop (nm)	800
Excitation slit (nm)	10
Emission slit (nm)	5

pH meter

The pH readings were obtained throughout the experimentation using a pH/mv meter of the model UltraBASIC-10(Denver).

3.2 Chemicals

The chemicals used in this research are listed in Table 3.2. All the chemicals were of analytical grade.

Table 3.2 The list of chemicals

Chemicals	Supplier
Acetic acid	Sigma-Aldrich
Albumin	Sigma-Aldrich
Ammonium chloride	Riedel-de Haen
Ascorbic acid	Sigma-Aldrich
Calcium chloride	Baker analyzed
Creatinine	Sigma-Aldrich
Curcumin	Sigma-Aldrich
Dimethyl sulfoxide, (DMSO)	Lab scan
Enzyme glucose oxidase from Aspergillus	Sigma-Aldrich
Ferrous ammonium sulfate	RAMKWM
Glucose standard	Sigma-Aldrich
Hydrochloric acid (37%), (HCl)	Merck
Hydrogen peroxide (30%)	Merck
Magnesium chloride	Merck
Potassium chloride	Univar
Potassium dihydrogen phosphate	Merck
Sodium acetate	Fisher Scientific
Sodium chloride	Carlo ERBA
Sodium sulphate	Fisher Scientific
Sodium oxalate powder	Baker analyzed
Tri-Sodium citrate	Fisher Scientific
Urea	Sigma-Aldrich

3.3 Solution preparation

All solutions were prepared with de-ionized water, except for curcumin which was dissolved in DMSO.

Curcumin solution

Curcumin solution was freshly prepared by weighing of curcumin and dissolved in DMSO.

Hydrochloric acid (HCl)

Hydrochloric acid solution was prepared by diluting the appropriate amount of the concentrated hydrochloric acid solution (37 %w/w) with de-ionized water.

Ferrous ammonium sulfate

Ferrous ammonium sulfate solution was prepared daily by dissolving of ferrous ammonium sulfate with 0.10 M hydrochloric acid solution.

Glucose, Hydrogen peroxide, Bovine serum albumin (BSA), Ascorbic acid

Glucose, hydrogen peroxide, bovine serum albumin (BSA), ascorbic acid solution were prepared by dissolving glucose standard, bovine serum albumin and ascorbic acid with de-ionized water.

Glucose oxidase

Glucose oxidase (15 mg/mL) was prepared by weighing exactly 15 mg from the stock glucose oxidase into 1.00 mL of de-ionized water

Acetate buffer solution

Sodium acetate-acetic acid buffer solution pH *ca.* 5 was prepared by using 1.00 M of sodium acetate and acetic acid solution. The pH 5 of acetate buffer solution was adjusted by the addition of 1.00 M sodium acetate/acetic acid.

Synthetic urine

The compositions of synthetic urine are listed in Table 3.3 [55]. All of the compounds were weighed accordingly and dissolved in de-ionized water.

Table 3.3 The list of chemicals in synthetic urine

Chemicals	Weight (mg) in 100.00 mL
Ammonium chloride	100
Calcium chloride	56
Creatinine	110
Magnesium chloride	31
Potassium chloride	160
Potassium dihydrogen phosphate	280
Sodium chloride	460
Sodium sulphate	230
Sodium oxalate powder	2
tri-Sodium citrate	65
Urea	2500

The generation of glucose by glucose oxidase

Glucose oxidase reaction is a specific reaction to change glucose to gluconic acid and hydrogen peroxide under oxygen. The condition to generate glucose is to take 1.00 mL of glucose standard or urine sample, then a solution of acetate buffer (0.20 ml) is added to adjust the pH to ca. 5 and 0.02 mL of glucose oxidase (15 mg/mL) to react with glucose at 37-40 degree Celsius [8, 15].

3.4 Fluorescence intensity of curcumin under different conditions

To confirm the feasibility of the proposed method, the mixture solutions of curcumin with products from Fenton reaction were considered and were compared with curcumin solution (blank) to observe the phenomenon of fluorescence quenching which should occur. The solution of curcumin (blank) and the mixture of curcumin with hydrogen peroxide, ferric ion and mixture of ferrous ion and hydrogen peroxide were prepared according to Table 3.4 and analyzed by fluorescence spectroscopy after 30 minutes of reaction time.

Table 3.4 The concentration and volume of each reagent to investigate the concept in this work

Solutions	Preparation
solution 1 (blank)	1.00 mL of 0.05 mM curcumin (adjust the volume to 3.00 mL with DI water)
solution 2 (H_2O_2 reaction)	1.00 mL of 0.05 mM curcumin + 0.10 mL of 0.40 mM H_2O_2 (adjust the volume to 3.00 mL with DI water)
solution 3 (ferric ion reaction)	1.00 mL of 0.05 mM curcumin + 2.00 mL of 0.0066 mM ferric ions
solution 4 (Fenton reaction)	1.00 mL of 0.05 mM curcumin + 0.10 mL of 0.40 mM H_2O_2 + 1.90 mL of 0.225 mM ferrous ions

3.5 Optimization of glucose detection condition

3.5.1 Effect of HCl concentration

An error in the determination of glucose in this work would occur when the added ferrous ions were oxidized to ferric ions by oxygen in the air. To suppress the oxidation, hydrochloric acid (HCl) was chosen as a solvent for ferrous ammonium sulfate preparation. Each of the two HCl concentrations

(0.07, 0.10 M) was used to dissolve ferrous ammonium sulfate and mixed with curcumin solution before these solutions were detected with fluorescence spectroscopy. To prepare the mixture, 1.00 mL of 0.05 mM curcumin was mixed with 1.90 mL of 0.255 mM, ferrous ion prepared in HCl of different concentration. The final volume of solution was 3.00 mL. The fluorescence intensity from each condition was compared to blank solutions (only curcumin).

3.5.2 Effect of reaction time periods

The reaction time between the reagents (ferrous ammonium sulfate and curcumin) and hydrogen peroxide required to complete the reaction was investigated. Hydrogen peroxide represents the concentration possibly generated in glucose standard solution after mixing with glucose oxidase and acetate buffer. In the experiment, the fluorescence intensity was monitored at 5 minute intervals from 5 to 45 minutes in the presence of 0.40, 1.30, 2.30 mM hydrogen peroxide.

3.5.3 Effect of quantity of reagents on hydrogen peroxide detection

Hydrogen peroxide is a product from glucose oxidase reaction. The concentration of hydrogen peroxide is related to the level of glucose in the sample after the enzyme generation. The goal upper limit of hydrogen peroxide in this experiment was 2.30 mM. Curcumin is dissolved in DMSO and the volume of curcumin is limited to 20% of the total volume of the mixture to reduce the solvent vaporization which may affect the precision of the result. Under the mole ratios between reagents and hydrogen peroxide in Table 3.5, the concentrations of glucose which were measured by fluorescence spectroscopy were 0.40, 0.80, 1.30, 1.80 and 2.30 mM.

Table 3.5 The concentration and the volume of reagents used to detect hydrogen peroxide

Condition	A	B	C	D
Volume of H_2O_2 (mL)	0.10			
Concentration of curcumin (mM)	0.345	0.287	0.230	0.307
Volume of curcumin (mL)	2.00			1.50
Concentration of Fe(II) ion (mM)	0.05			0.115
Volume of Fe(II) solution (mL)	8.00			6.00
Total volume (mL)	10.10			7.60
mole ratio of curcumin: Fe(II): H_2O_2(2.3mM)	3:2:1	2.5:2:1	2:2:1	2:3:1

3.5.4 Effect of quantity of reagents on glucose detection

The best condition of mole ratio between reagents and hydrogen peroxide from the previous experiment was used to establish a calibration curve for the determination of glucose in urine. In order to reduce the amount of waste, the total volume of the mixture was reduced in condition D while keeping the percentage of an organic solvent in the mixture at 20% v/v for curcumin dissolution. In addition, the goal upper limit was extended to 2.80 mM (glucose) because the concentration of glucose was diluted in the step of glucose oxidase reaction. At this level of glucose, 2.30 mM hydrogen peroxide was expected. The concentrations of glucose studied were 0.50,

1.10, 1.50, 2.30, 2.80 mM. The optimum condition to generate glucose was to take 1.00 mL of glucose standard or urine sample, into 0.20 mL of acetate buffer to control the pH to ca. 5, and to add 0.02 mL of glucose oxidase (15 mg/mL) to react with glucose at 37-40 °C. The generation time of glucose oxidase reaction was 60 minutes [8, 15]. The mixture between analyte (0.10 mL) and reagent solutions as mentioned in condition D were measured with fluorescence spectroscopy and were presented in terms of the calibration curve between delta fluorescence intensity and the concentration of analyte.

3.6 Determination of glucose in urine.

3.6.1 Effect of urinary matrix

The selectivity of this proposed method was assessed by way of the effects of matrix toward glucose detection in synthetic urine. The standard curves are constructed by using the results from the analysis of glucose standards (0.50, 1.00, 1.40, 2.00, 2.50, 2.80 mM) prepared in de-ionized water and in the synthetic urine (matrix matched standards). The results from the glucose analysis with both calibration curves are compared by the statistics from t-test.

3.6.2 Effect of potential interfering species

The effect of interference species in the determination of urinary glucose with the proposed method was studied. The interferences of interest were added as the binary mixtures in synthetic urine. Glucose solutions of 0.70, 1.60, 2.50 mM containing various interference species and several concentrations (Table 3.6) were represented as a normal and an abnormal glucose value in urine. Bovine Serum Albumin (BSA), creatinine and ascorbic acid were chosen as potential interfering species. Then, glucose in synthetic urine with the interference species was compared to the one without the species with fluorescence spectroscopy.

Table 3.6 The list of interfering species and studied level

Interfering species	Studied value
Bovine serum albumin	300 mg/L[56]
Creatinine	2590 mg/L[57]
Ascorbic acid	630, 310, 160, 50, 25 mg/L[57]

3.6.3 Effect of time period for glucose oxidase reaction

Three incubation periods, 15, 30 and 60 minutes, of hydrogen peroxide with glucose oxidase were studied to find the shortest time for glucose oxidase reaction under the glucose concentration levels of 1.00 and 2.80 mM, which represent a normal and an abnormal value in urine.

3.7. Method performance.

The performance of proposed method was considered based on the optimal condition. The limit of detection (LOD), limit of quantification (LOQ) and linear range of the proposed method were determined.

3.7.1 Limit of detection (LOD) and the limit of quantification (LOQ).

The fluorescence intensity of a solution of curcumin without glucose (blank solution) was measured under the optimal condition in ten replicates. Based on fluorescence quenching which was used to monitor glucose level in this work, the signal of fluorescence was shown in terms of delta fluorescence intensity. Thus, in this work, the limit of detection (LOD) and

limit of quantification (LOQ) were calculated according to Eq. 3.1-3.2, respectively.

$$\Delta I_{LOD} = \Delta I_{Blk} + 3SD_{\Delta BLK} \quad (3.1)$$

$$\Delta I_{LOD} = \Delta I_{Blk} + 10SD_{\Delta BLK} \quad (3.2)$$

Where,

ΔI_{LOD} = the delta fluorescence intensity of curcumin at LOD concentration.

ΔI_{LOQ} = the delta fluorescence intensity of curcumin at LOQ concentration.

ΔI_{Blk} = the mean of delta fluorescence intensity of blank

SD_{BLK} = the standard deviation of delta fluorescence intensity of blank

The LOD and LOQ were obtained by comparing ΔI_{LOD} and ΔI_{LOQ} values to the linear equation from standard calibration curve. The standard calibration curve was plotted between delta fluorescence intensity of curcumin and concentration of glucose in range of 0.00-0.40 mM.

3.7.2 Linearity and working range

The calibration curve for glucose detection was established based on the optimal condition in this work. The calibration curve was plotted between delta fluorescence intensity and the concentration of standard glucose solution. The linearity of the proposed method was reported in terms of correlation coefficient (R^2 value).

3.8 Application in real human urine sample

3.8.1 Accuracy and precision

For the analysis of human urine, the samples were collected from members of Environmental Analysis Research Unit. The samples which

originally contained no glucose were spiked with glucose standard at 1.00 and 2.30 mM, which is a normal and abnormal glucose range in human urine (before diluting the sample). Glucose which is an analyte in human urine sample was detected according to the procedure in this work, and the glucose results were presented in terms of fluorescence intensity of curcumin. The obtained results were compared to the ones from the hexokinase glucose-6-phospate dehydrogenase method, a standard method from Pro-laboratory international company to investigate the accuracy and precision. The precision was presented in terms of percentage of relative standard deviation (%RSD).

CHAPTER IV
RESULTS AND DISCUSSIONS

The concept of this method was based on the detection of hydrogen peroxide generated in the reaction between glucose oxidase (GOX) and glucose in a sample (Eq. 4.1). Ferrous ions were added to react with hydrogen peroxide in order to increase its sensitivity via a reaction known as Fenton reaction (Eq. 4.2). The products from Fenton reaction further reacted with curcumin and quenched its fluorescence intensity. The amount of curcumin used in the reactions was related to the level of glucose in sample.

$$\text{Glucose} + \text{GOX} + O_2 \longrightarrow \text{Gluconic acid} + H_2O_2 \quad (4.1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (4.2)$$

4.1 Fluorescence intensity of curcumin under different conditions

In this method, the fluorescence quenching was expected when curcumin reacted with the products from Fenton reaction. To verify this concept, the fluorescence intensities of solutions containing curcumin with hydrogen peroxide, ferric ions and a mixture of ferrous ion and hydrogen peroxide were observed. Hydrogen peroxide is the product from glucose oxidase reaction, while ferric ions and hydroxyl radicals are the products from Fenton reaction. The fluorescence intensities of curcumin in each mixture solution are shown in Figure 4.1.

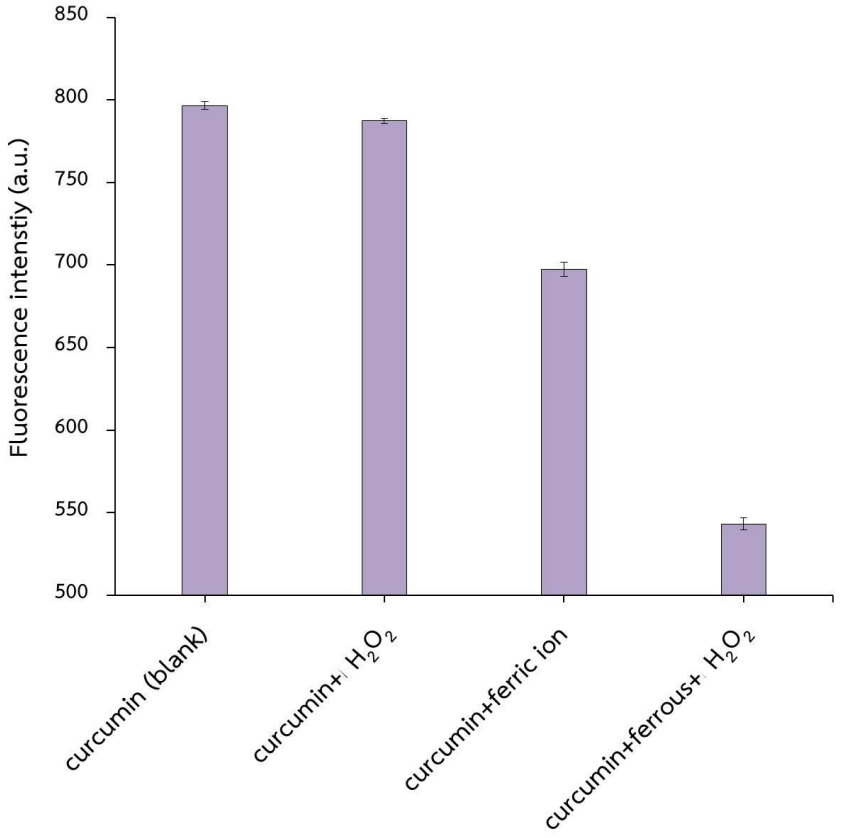


Figure 4.1 The fluorescence intensities of curcumin in the studied mixture solutions.

When comparing the fluorescence intensities of the mixtures with curcumin solution, the highest extent of fluorescence quenching was obtained in curcumin solution mixed with hydrogen peroxide and ferrous ion followed by curcumin with ferric ions. Only a slight fluorescence quenching was observed in the mixture solution of curcumin and hydrogen peroxide. The reaction of curcumin with hydrogen peroxide may take place through oxidation reaction as hydrogen peroxide acted as a moderate oxidizing agent [42, 43] while curcumin with one enol group may show a reducing property. However, this reaction did not favorably occur and only a small amount of curcumin was consumed by this reaction. On the other hand, in the mixture of curcumin and ferric ion, curcumin could form complex with ferric ions with the general formula of ML_2 (M as ferric ion, L as curcumin). This structure model

was one of several possible complex structures of metal-curcumin (ML, ML_2) [58]. As a result, lower number of free curcumin was left in the mixture resulting in lower fluorescence intensity in this case. Furthermore, in the mixture of curcumin, ferrous ion and hydrogen peroxide, the highest extent of fluorescence quenching was obtained. In this case, curcumin would react with the products from Fenton reaction not only through complex formation with ferric ion but also oxidation reaction with hydroxyl radical known as a strong oxidizing agent. It was thus clearly demonstrated that with Fenton reaction, the sensitivity of glucose determination was improved and the method was further developed.

4.2 Optimization of glucose detection condition

4.2.1 Effect of HCl concentration

In this work, the products from Fenton reaction react with curcumin, and affect the fluorescence intensity. However, ferrous ion can also be converted to ferric ion with oxygen in the air. This phenomenon can potentially lead to a positive error because the source of ferric ion is not only from Fenton reaction, but also from the oxidation of ferrous ion with oxygen in the air.

Furthermore, the optimum pH for Fenton reaction and complex formation between curcumin and ferric ion is the acidic condition (pH 2-3). Thus, HCl was used to dissolve ferrous ammonium sulfate to suppress the oxidation of ferrous ions by oxygen. The optimum concentration of HCl for dissolving ferrous ammonium sulfate was found to control positive error and to optimize an acidic condition in this work. The concentrations of HCl which were studied were 0.07 and 0.10 M. The ferrous ammonium sulfate solutions in these concentrations of HCl were mixed with curcumin solutions prior to the analysis by fluorescence spectroscopy. The fluorescence intensities of curcumin in these mixtures are compared with the fluorescence intensity of curcumin of the same concentration without ferrous ions (Figure 4.2).

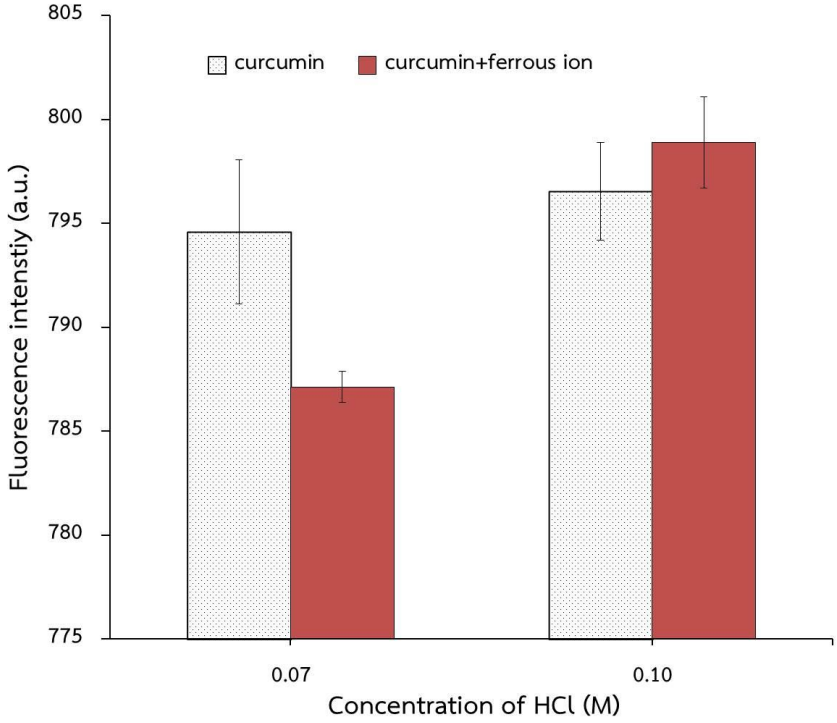


Figure 4.2 The effect of different HCl concentration levels on fluorescence intensity of curcumin solutions in the presence of ferrous ions.

The fluorescence intensities of the mixture solution of curcumin and ferrous ammonium sulfate in 0.07 M HCl was lower than that observed in curcumin solution of the same concentration (blank). The fluorescence quenching was the result of the presence of ferric ions in the mixture that could form complexes with curcumin. In contrast, the fluorescence intensities of curcumin solution and the mixture solution between curcumin and ferrous ammonium sulfate in 0.10 M HCl was not significantly different indicating that the oxidation of ferrous ions did not occur or it was negligible. Therefore, a 0.10 M HCl solution was chosen to be a solvent of ferrous ammoniums sulfate.

4.2.2 Effect of reaction time period

The time periods required for the reaction between reagents and hydrogen peroxide was determined by following the fluorescence intensity of curcumin in the reaction mixtures containing 0.40, 1.30 or 2.30 mM hydrogen peroxide. These concentration levels represented the concentration of hydrogen peroxide that could be generated by glucose oxidase reaction in urine sample with normal and abnormal glucose ranges. Figure 4.3 presents the delta fluorescence intensity or the extent of fluorescence quenching calculated by subtracting the fluorescence intensity of curcumin solution by that of the mixture of reagents and hydrogen peroxide and observed every 5 minutes (45 minutes in total).

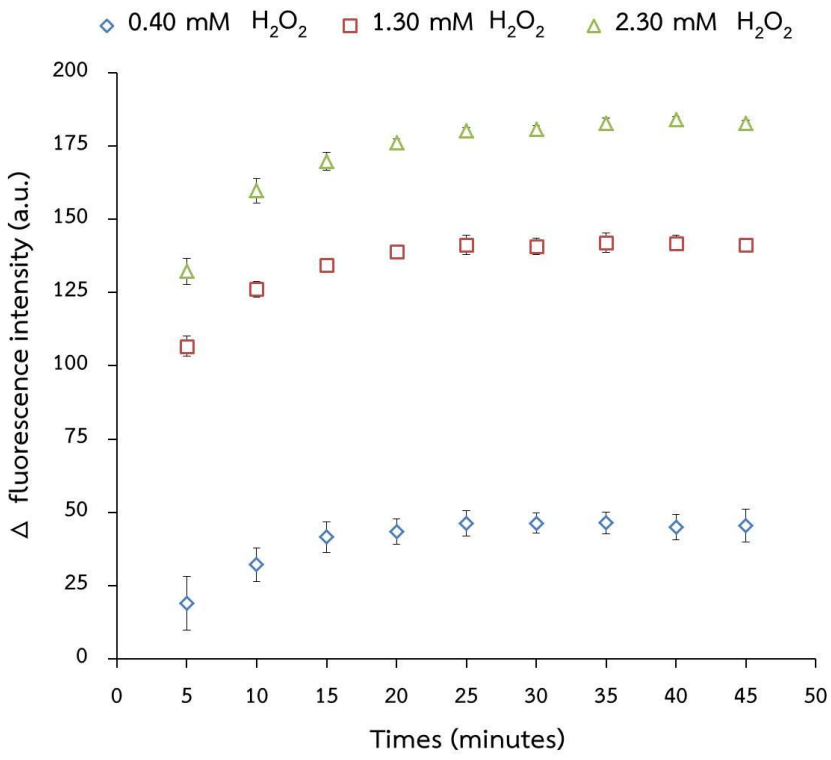


Figure 4.3 The effect of different reaction times between reagents and hydrogen peroxides on the quenching of curcumin fluorescence.

As shown in Figure 4.3, the extent of fluorescence quenching was proportional to the concentration of hydrogen peroxide. The extent of fluorescence quenching in each mixture solution increased with a prolongation of reaction time and subsequently reached a constant level indicating the completeness of the reactions. The results show that the reaction time period of 20, 25 and 30 minutes were required for the reaction of 0.40, 1.30 and 2.30 mM hydrogen peroxide to complete, respectively. Hence, the reaction period of 30 minutes was chosen for further analyses.

4.2.3 Effect of quantity of reagents on hydrogen peroxide detection

Based on glucose oxidase reaction, hydrogen peroxide quantitatively represents the glucose level in sample. Thus, the amount of reagents required in the reaction with hydrogen peroxide was determined before applying the most suitable condition in the detection of glucose. Under a suitable condition, a linear relationship between the extent of fluorescence quenching and hydrogen peroxide concentration should be obtained.

For a quantitative analysis, the amount of reagents should be sufficient for the reaction with analytes at the concentration range of interest. In this work, the amount of curcumin and ferrous ions added into the mixture were adjusted with respect to hydrogen peroxide at high concentration (2.30 mM) possibly generated in a urine sample. Firstly, the number of moles of ferrous ions was fixed at twice the number of moles of hydrogen peroxide at high concentration, while the mole number of curcumin was varied. The mole ratios of curcumin : ferrous ions : hydrogen peroxide (2.30 mM) used in this study was 3:2:1 (ratio A), 2.5:2:1 (ratio B) and 2:2:1 (ratio C), respectively. The fluorescence quenching observed as a function of hydrogen peroxide concentration with the variation of reagent quantity are shown in Figure 4.4.

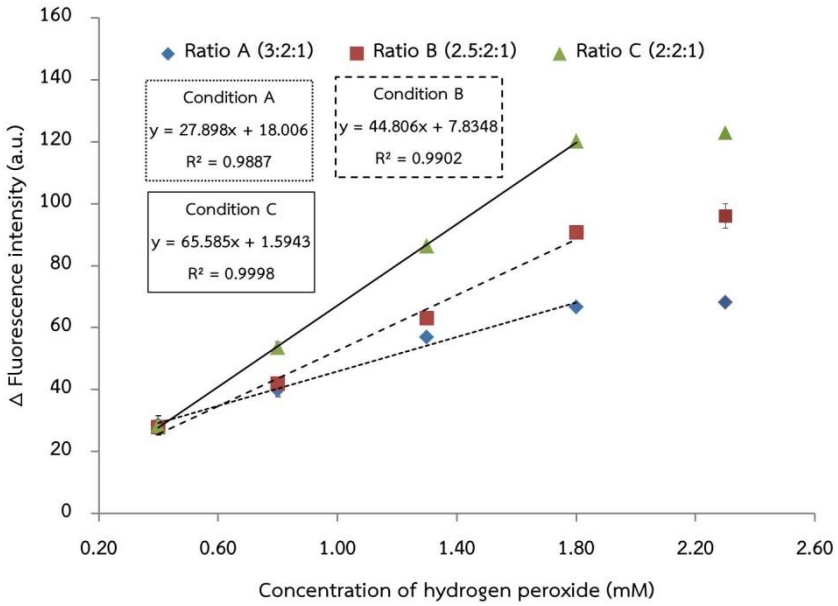


Figure 4.4 The effect of reagents mole ratios on the fluorescence quenching and standard calibration curves for hydrogen peroxide determination.

Under these conditions, a linear relationship between the extent of fluorescence quenching and hydrogen peroxide in the concentration range of 0.40-1.80 mM was observed. Regarding the sensitivity of the detection, it was found that the calibration curve obtained by using the condition C had the highest slope compared to the results from other conditions. However, the extent of fluorescence quenching by the presence of 1.80 and 2.30 mM hydrogen peroxide were not significantly different (data not shown). It should be noticed that the latter concentration was the highest concentration of hydrogen peroxide used to set the mole ratio of the other reagents. In an attempt to extend the upper limit of quantitation, the condition C was adopted with a modification. The amount of ferrous ammonium sulfate was further increased while the amount of curcumin was fixed so as to obtain the mole ratio of curcumin : ferrous ions : hydrogen peroxide of 2:3:1 (condition D). The results of fluorescence quenching vs concentration of glucose under mole ratio of condition D are shown in Figure 4.5.

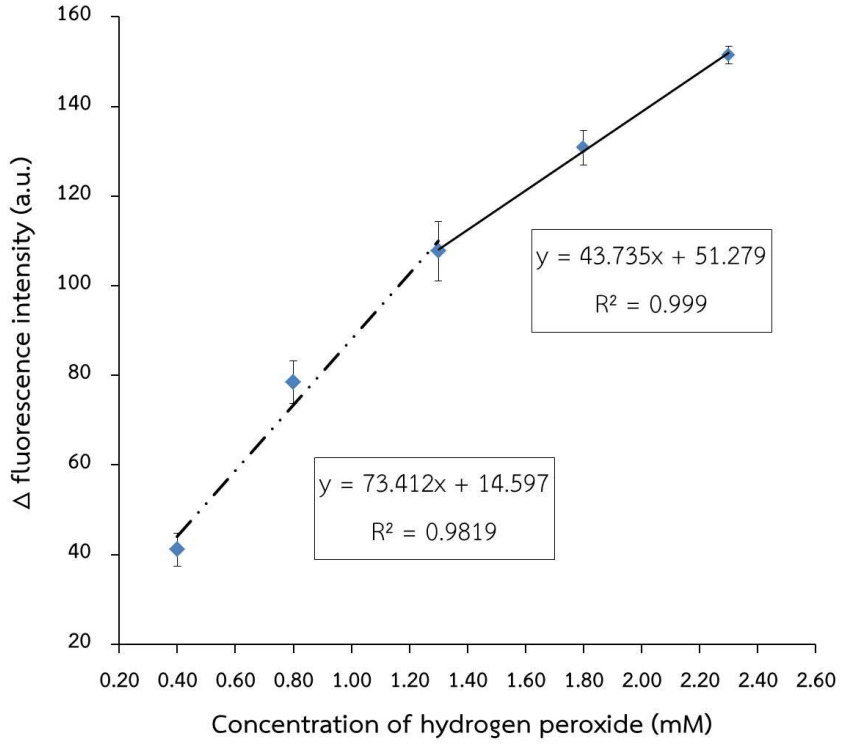


Figure 4.5 The standard calibration curve of hydrogen peroxide obtained by using mole ratio of condition D.

Under the condition D, the linear range could be extended to 2.30 mM hydrogen peroxide with two different slopes. A slope of the calibration curve in the range of 0.40-1.30 mM was higher than that of the concentration range of 1.30-2.30 mM hydrogen peroxide. The lower detection sensitivity observed in high concentration range could be attributed to the loss of some content of hydroxyl radicals through free radicals recombination. As a high quantity of hydroxyl radicals would be generated in the system at high concentration levels of hydrogen peroxide via Fenton reaction, these reactive free radicals can combine with each other to form a stable non radical species as occurred in a termination step of radical chain reaction.

4.2.4 Effect of quantity of reagents on glucose detection

The reagent quantity required for the detection of hydrogen peroxide (condition D) was used to establish a calibration curve for glucose determination. A step of hydrogen peroxide generation through glucose oxidase reaction was added. To perform this reaction, glucose oxidase and acetate buffer solution were mixed with glucose standard solution and the concentration of glucose in standard or sample was diluted by a dilution factor of 1.22. Thus 2.30 mM hydrogen peroxide which was the upper limit would be obtained from the reaction of 2.80 mM glucose. With the application of the chosen mole ratio (curcumin : ferrous ion : analyte of 2:3:1), the fluorescence spectra of curcumin observed in the detection of glucose of different concentration levels are shown in Figure 4.6. The calibration curve plotted between delta fluorescence intensity and glucose concentration is shown in Figure 4.7.

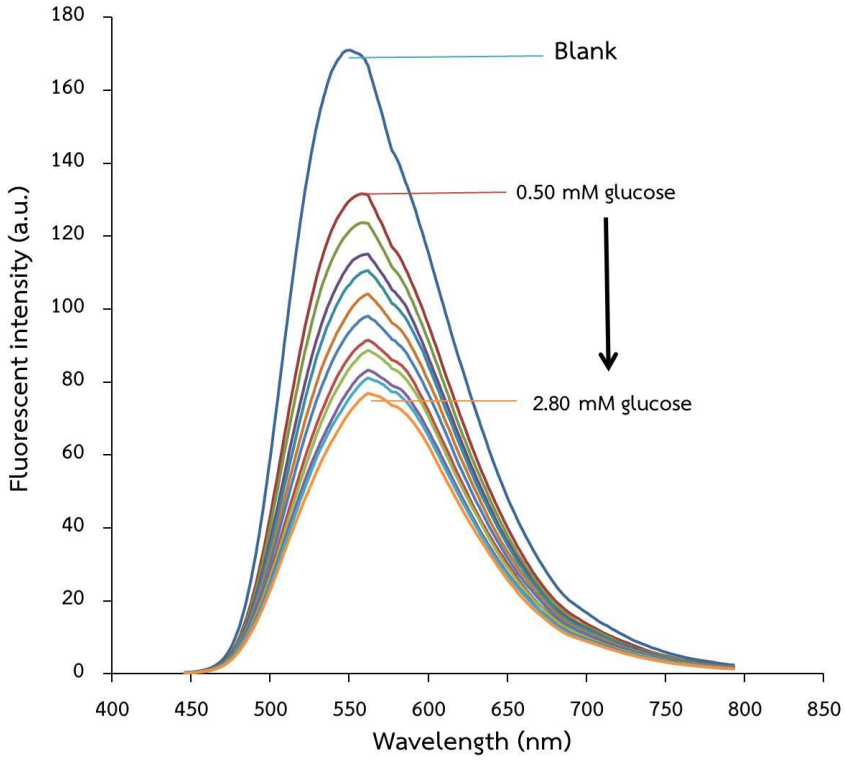


Figure 4.6 The fluorescence spectra of curcumin in the detection of glucose of different concentrations.

The fluorescence intensity of curcumin was reciprocal to the glucose concentration levels due to the presence of hydrogen peroxide generated at different contents in the system (Figure 4.6). As expected, the standard calibration curve of glucose also had two different slopes as previously observed in the detection of standard hydrogen peroxide (Figure 4.5). Nevertheless, the slope of the calibration curve constructed by using the results from the glucose analysis (Figure 4.7) was lower than the slope of the calibration curve of hydrogen peroxide. The lower sensitivity was possibly a result of the presence of several compounds including gluconic acid generated from the oxidation of glucose in glucose oxidase reaction. These compounds may react with highly reactive hydroxyl radicals. Consequently, a certain quantity of hydroxyl radicals was consumed by these side reactions and only the residual hydroxyl radicals would react with curcumin, resulting

in a lower fluorescence quenching, compared to a pure hydrogen peroxide system. However the obtained calibration curve for glucose analysis could cover the goal upper limit of glucose detection in this work (2.80 mM glucose).

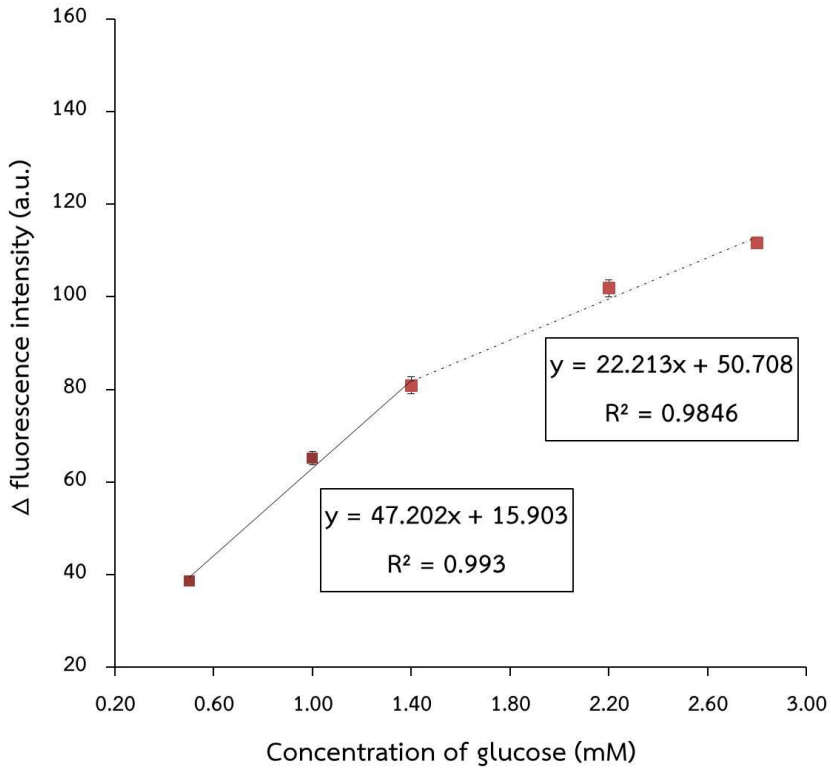


Figure 4.7 The standard calibration curve for glucose determination.

4.3 Determination of glucose in urine

4.3.1 Effect of urinary matrix

The effect of human urine matrix was investigated by comparing the external standard calibration curves constructed in the concentration range from 0.50 to 2.80 mM glucose using glucose standard solutions and matrix

matched standard solutions as shown in Figure 4.8. The matrix matched standard solutions were prepared in synthetic urine.

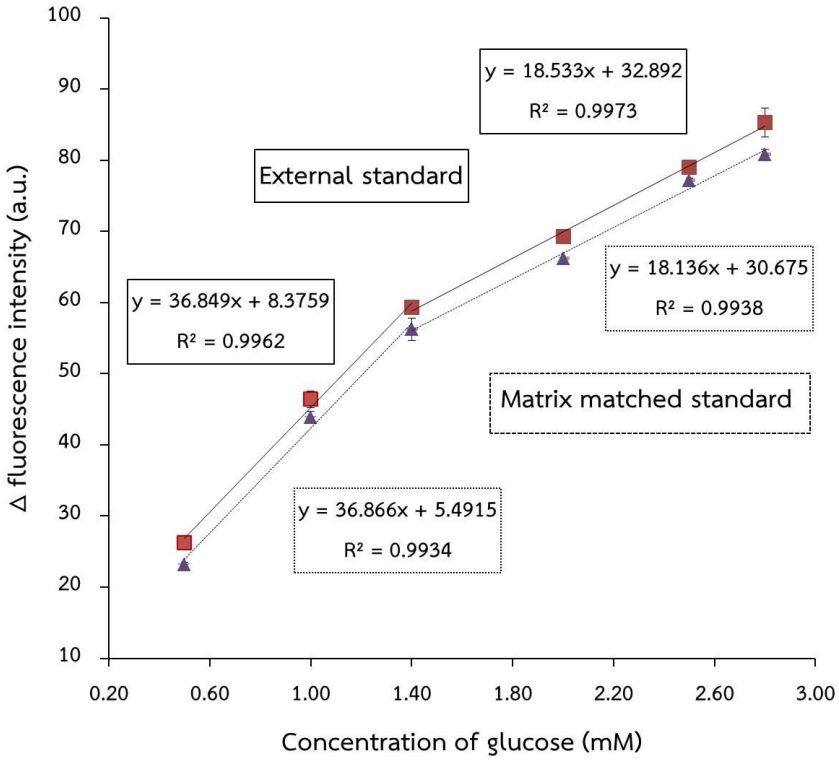


Figure 4.8 External standards calibration curve and matrix matched standards calibration curve for glucose determination.

The results show that the extent of florescence quenching observed in the analysis of external standard solutions were slightly higher than the values obtained from the analysis of glucose in matrix matched standards, while the slope of these calibration curves were the same. The differences deficit was probably due to the matrix effect. Apart from curcumin, the urine matrix probably competitively reacted with hydroxyl radicals, resulting in a loss of certain quantity of hydroxyl radicals. In order to reduce the matrix effect, either a sample preparation technique or a dilution of sample is

required. In this study, the sample dilution was adopted and glucose in the synthetic urine was diluted at least two folds. In this part, the highest concentration studied was 1.40 mM glucose which represented 2.80 mM glucose after a 2-fold dilution. The delta fluorescence intensity of curcumin in the analysis of glucose in external and matrix matched standards are shown in Figure 4.9.

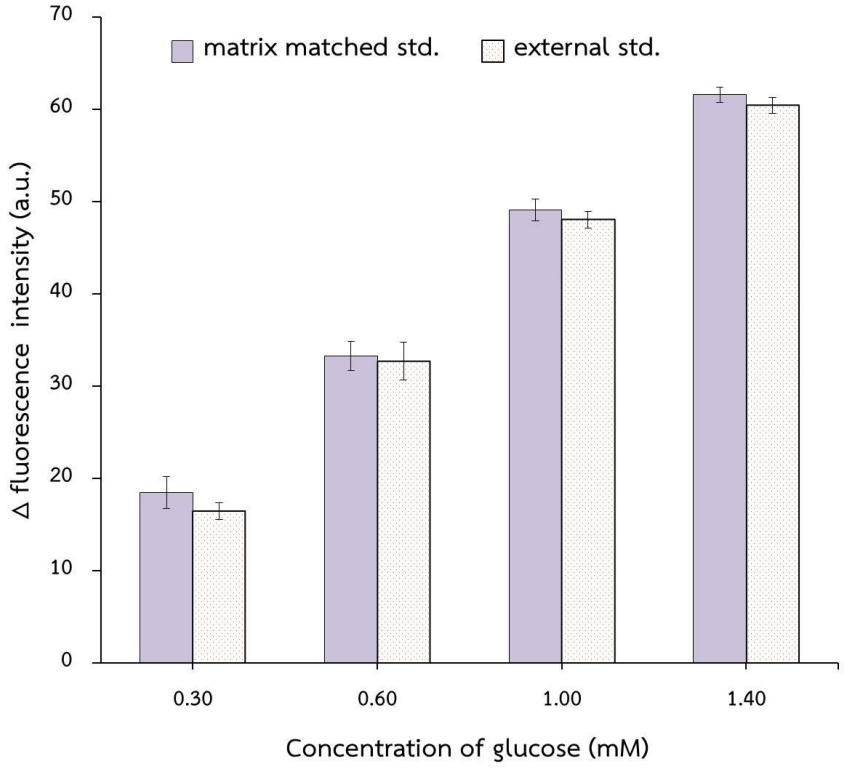


Figure 4.9 The delta fluorescence of curcumin in the analysis of glucose in external and matrix matched standards with a two-fold dilution.

With a sample dilution, the fluorescence quenching observed in the analysis of glucose in standard solution and diluted synthetic urine solution were not significantly different at 95% confidence level by pair t-test. These results demonstrated that the matrix effect was minimized due to the matrix

dilution and the external standard calibration could be used for the quantification of glucose instead of matrix matched standard calibration.

4.3.2 Effect of potential interfering species

The interfering species chosen for this study were creatinine, bovine serum albumin (BSA) and ascorbic acid, all of which are commonly found in human urine. These interference species may react with analyte instead of curcumin, which affects glucose determination in urine. In this study, the concentration levels of BSA and creatinine were measured at their maximum concentrations which can be found in human urine. While, ascorbic acid was the only interference species which had impacts on the determination of urinary glucose, thus it was studied to find the tolerance value which is not irrelevant to the determination of urinary glucose.

The effect of the presence of potential interfering species on the determination of glucose was investigated by adding the species of interest in synthetic urine to prepare binary mixtures. Each binary mixture contained glucose standard and 2590 mg/L creatinine, 300 mg/L BSA or ascorbic acid of different concentrations (630, 310, 150, 50, 25 mg/L). The concentrations of glucose in the mixtures were 0.70, 1.60 or 2.50 mM. Then the mixtures were 2-fold diluted before generating hydrogen peroxide with glucose oxidase reaction and mixing with reagents. The fluorescence quenching observed in synthetic urine sample with and without BSA and creatinine were shown in Figure 4.10.

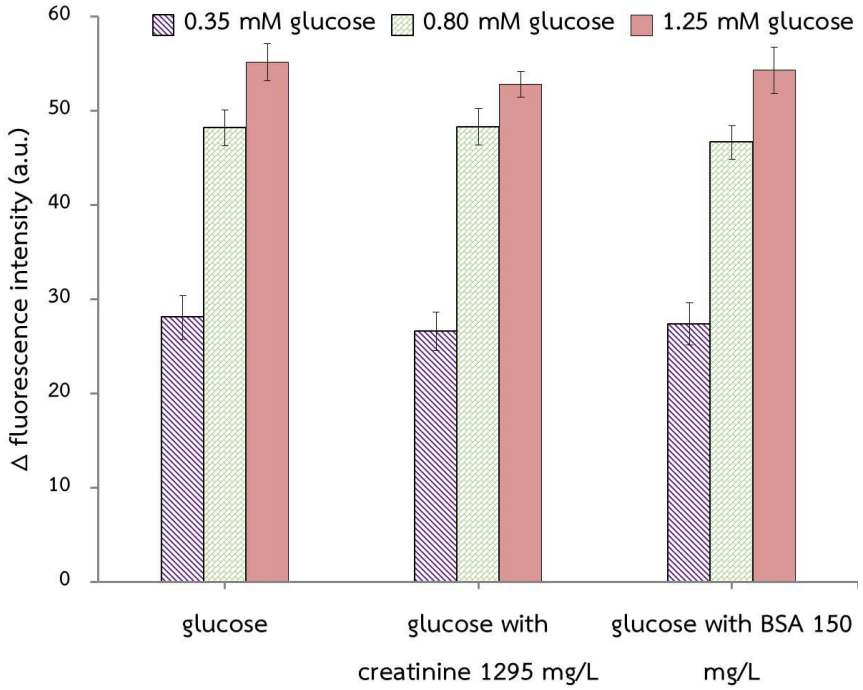


Figure 4.10 The fluorescence quenching observed in the analysis of glucose standard solutions without and with 1259 mg/L creatinine and 150 mg/L BSA.

The results of fluorescence quenching observed in the analysis of glucose in solutions containing 1295 mg/L creatinine or 150 mg/L BSA were not significantly different from those observed in solutions without these species at 95% confidence level by t-test. Thus, the presence of creatinine and BSA of high level did not have impact on glucose detection. On the other hand, ascorbic acid which is a reducing agent strongly affected the determination of urinary glucose by the proposed method because ascorbic acid could competitively react with hydroxyl radicals and cause negative error. Therefore, the ascorbic acid was presented in terms of tolerance value. The tolerance value is the highest quantity of a co-existing species that did not affect the determination of glucose in this proposed method. The tolerance value of ascorbic acid was 12.5 mg/L.

4.3.3 Effect of time period for glucose oxidase reaction

In the previous experiments, a time period of 60 minutes was applied in the glucose oxidase reaction to ensure the completeness of the reaction. To find the shortest time for the quantitative generation of hydrogen peroxide, the glucose oxidase reaction was performed in different periods of time for the detection of 0.50 and 1.40 mM glucose which were concentration of glucose after diluting sample. The results are presented in Figure 4.11.

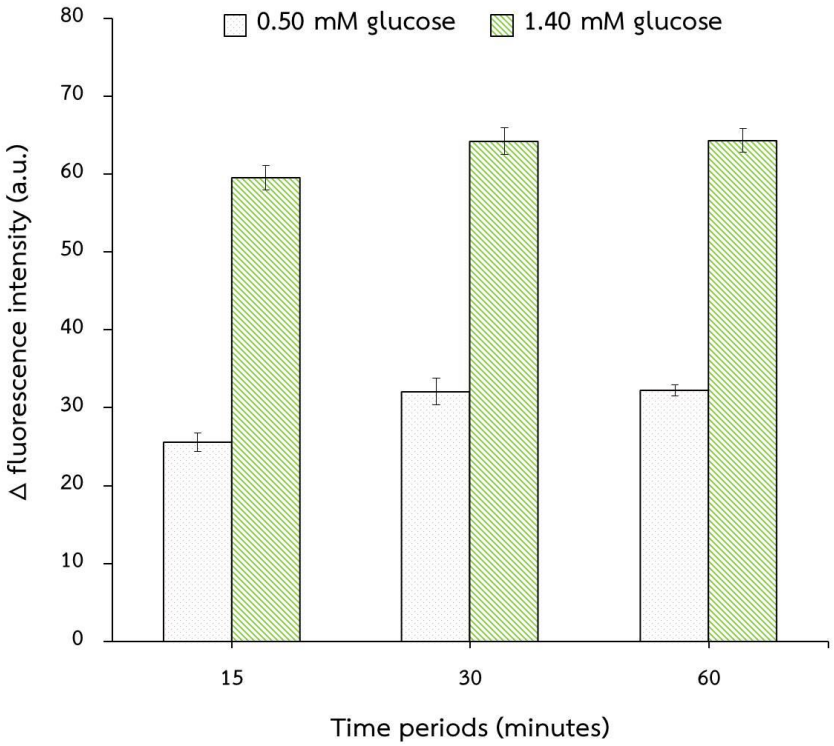


Figure 4.11 The effect of time periods of glucose oxidase reaction on the fluorescence quenching of curcumin in the detection of glucose.

The extents of fluorescence quenching observed in the analysis of glucose using 30 and 60 minutes in glucose oxidase reaction were not

significantly different at 95% confidence level by t-test. On the other hand, a comparatively lower extent of fluorescence quenching was obtained when 15-minute time period was used, revealing that the generation of hydrogen peroxide from glucose oxidase reaction was not complete. Hence, the time period of 30 minutes was chosen to generate hydrogen peroxide quantitatively to reduce the analysis time.

4.4 Method performance

The performance of the proposed method was evaluated under the selected condition described as follows. A 1.00 mL of glucose standard solution or human urine diluted at least 2-fold was mixed with acetate buffer (0.20 mL) and glucose oxidase (0.020 mL) for 30 minutes. Then, 0.10 mL of the mixture was taken and mixed with 6 mL of 0.145 mM ferrous ammonium sulfate and 1.50 mL of 0.382 mM curcumin solution for 30 minutes before the detection by fluorescence spectroscopy. The excitation wavelength was 419 nm and the intensity of fluorescence was observed at 555 nm. Under this condition, the limit of detection (LOD), the limit of quantification (LOQ) and the linear range were determined.

4.4.1 Limit of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is the lowest concentration which gives reliable signal differentiated from the background noise while the limit of quantification (LOQ) is the smallest concentration of analyte that gives an accurate signal and acceptable calibration point. In this work, a signal of the concentration that was the limit of detection (LOD) and limit of quantification (LOQ) was delta fluorescence intensity of blank plus three and ten times of standard deviation of delta fluorescence intensity of blank, respectively. The LOD and LOQ values were found to be 0.10 and 0.30 mM, respectively.

4.4.2 Linearity and working range

The calibration curve was plotted between the delta fluorescence intensity of curcumin observed at 555 nm and the concentrations of glucose as illustrated in Figure 4.12.

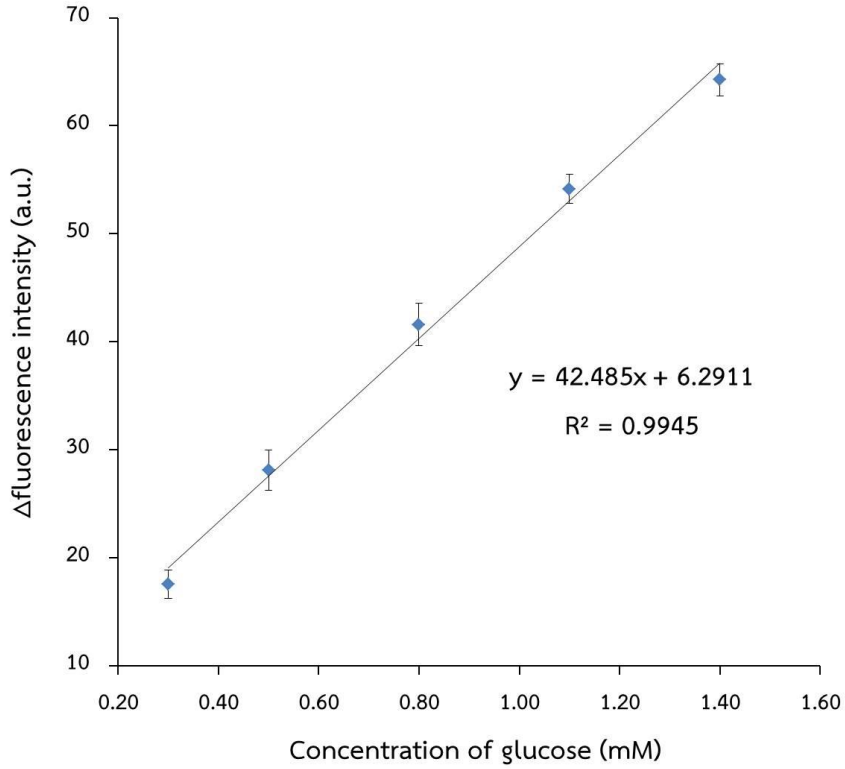


Figure 4.12 The calibration for glucose determination.

The LOQ was used as the lowest concentration of the working range. The working range of the determination of urinary glucose by the proposed method was 0.30-1.40 mM with a correlation coefficient of 0.9907. As the urine sample was diluted at least two folds, the concentration of glucose in urine sample in normal and abnormal range lay in this working range.

4.5 Application in real human urine sample

4.5.1 Accuracy and precision

The proposed method was used for determining glucose level in urine samples and glucose spiked urine samples (1.00 or 2.30 mM glucose). As recommended in the method, the samples were diluted at least two folds prior to the analysis. The accuracy of this method was evaluated by comparing the obtained test results with the results observed by using the hexokinase glucose-6-phospate dehydrogenase method as summarized in Table 4.1.

Table 4.1 The results of glucose determination in human urine samples using the proposed method and hexokinase glucose-6-phospate dehydrogenase method

Sample	Glucose concentration (mM)	Hexokinase method		Fluorescence method	
		Glucose concentration (mM)	%RSD	Glucose concentration (mM)	%RSD
	Added	Found		Found	
urine 1	0.00	0.21±0.03		N.D.	-
	1.00	1.27±0.06	4.3	1.25±0.01	0.9
	2.30	2.60±0.00	0.0	2.62±0.10	3.9
urine 2	0.00	0.11±0.00		N.D.	-
	1.00	1.17±0.06	4.8	1.13±0.03	2.5
	2.30	2.48±0.06	2.6	2.53±0.05	1.9
urine 3	0.00	0.28±0.00		N.D.	-
	1.00	1.32±0.03	2.4	1.37±0.03	2.3
	2.30	2.72±0.00	0.0	2.69±0.07	2.7

N.D. = non detectable

The concentrations of glucose in human urine samples determined by the proposed method and the hexokinase glucose-6-phospate dehydrogenase standard method were not significantly different at 95% confidence level by pair t-test, indicating that the accuracy of the proposed method was acceptable. The relative standard deviation (%RSD) of the results obtained lay between 0.9-3.9% when the proposed method was used (Table 4.1). These results demonstrate that the proposed method can be used to detect glucose in human urine samples with acceptable precision according to the criteria of %RSD of analytical results obtained in the analysis of target species of different concentration levels (Table 4.2) [59].

Table 4.2 Criteria of recovery and %RSD of the results obtained in the analysis of analytes of different concentrations according to AOAC international

Analyte (%)	Analyte fraction	Unit	Recovery range (%)	RSD (%)
100	1	100%	98-102	1.3
10	10^{-1}	10%	98-102	2.8
1	10^{-2}	1%	97-103	2.7
0.1	10^{-3}	0.10%	95-105	3.7
0.01	10^{-4}	100ppm	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

CHAPTER V
CONCLUSION

5.1 Conclusion

This work presents an alternative method to detect glucose in human urine based on glucose oxidase reaction, Fenton reaction and the reaction of curcumin. The fluorescence quenching of curcumin as a result of the presence of glucose was monitored. An excitation wavelength at 419 nm was applied and the intensity of fluorescence was observed at 555 nm. The reagents in this work consisted of ferrous ions and curcumin while hydrogen peroxide produced from glucose oxidase was mixed with these reagents. The optimal condition for the determination of glucose was obtained by studying various parameters. The ferrous ammonium sulfate was prepared in 0.10 M HCl to avoid positive error from oxidation of ferrous ions to ferric ions. The time period required in glucose oxidase reaction and reaction between reagents and analyte were both 30 minutes. To perform the reactions, the mole ratio of curcumin : ferrous ion : glucose at 2.80 mM of 2:3:1 was used. The dilution human urine sample at least two folds before the analysis was crucial to overcome the matrix effect. The potential interfering species in human urine did not affect the analysis of glucose except the ascorbic acid which had the tolerance value of 12.5 mg/L.

Based on the optimal condition and dilution of human urine sample, the working range of the determination of urinary glucose was 0.30-1.40 mM. . The limit of detection and limit of quantification were 0.10 and 0.30 mM, respectively. Moreover, the results of glucose analysis by the proposed method and a standard method were not significantly different at 95% confidence level by pair t-test, while the percent relative standard deviation of analytical results lay in the range of 0.9-3.9%. It was demonstrated that the proposed method can be used with an acceptable precision and accuracy.

5.2 Suggestion for future work

The method can be developed as glucose biosensor due to the distinct change of color of these reagents.

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APPENDIX

VITA

Miss Sutthida Ruamarm was born on March 9, 1991 in Nakhon Prathom, Thailand. She received a Bachelor degree of Science from Chulalongkorn University in 2012. After that, she has been a graduate student at Department of Chemistry, Faculty of Science, Chulalongkorn University and a member of Environmental Analysis Research Unit under the supervision of Assistant Professor Dr. Fuangfa Unob and Assistant Professor Dr. Narong Praphairaksit. She received a proceeding of the 41st congress on science and technology of Thailand, Suranaree University of Technology, Nakhon Ratchasima, Thailand, November 6-8, 2015. She completed Master's degree of Science in 2015.