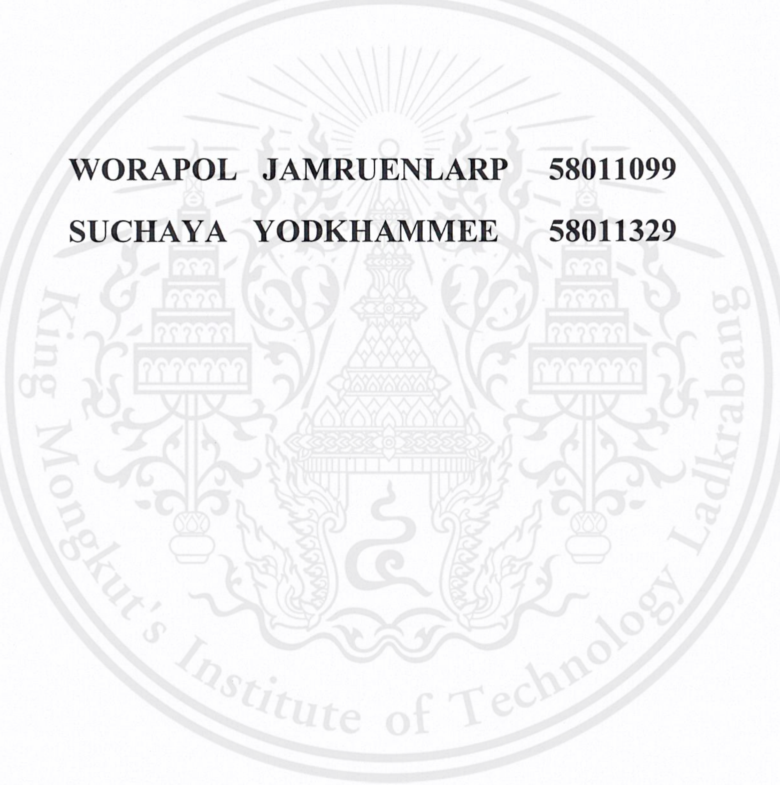
DEVELOPMENT OF A BIOSENSOR TO DETECT SODIUM CONCENTRATION IN URINE USING MOLECULARLY IMPRINTED POLYMERS



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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF ENGINEERING IN BIOMEDICAL ENGINEERING FACULTY OF ENGINEERING KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG ACADEMIC YEAR 2018

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การพัฒนาไบโอเซนเซอร์โดยการใช้โพลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลสำหรับการตรวจวัดความเข้มข้นของปริมาณโซเดียมในปัสสาวะ

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Thesis title	Development of a biosensor to detect sodium concentration in urine using molecularly imprinted polymers
Students	Mr. Worapol Jamruenlarp
	Miss Suchaya Yodkhammee
Field of Study	Biomedical Engineering

Accepted by the Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang in Partial Fulfillment of the Requirements for the Degree of Bachelor of Engineering (Biomedical Engineering).

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ABSTRACT

This research is concerned with the development of a polymer biosensor made using molecularly imprinted polymers methods (MIPS) to measure the concentration of sodium in the urine. This is achieved by measuring the electrical current from the redox reaction that occurs between sodium ions and iron(II) ions on the surface of the sensor which can be used to determine the sodium concentration in the urine. The accuracy of measurement results was found to be suboptimal due to the fact that the results from normal urine samples were found, to lie outside of the expected normal measurement range as calibrated using synthetic urine samples of varying concentration. As a result, this research tool is currently not suitable for real-world use with high accuracy (i.e. P>0.001) need to indicate whether the urine of a patient is abnormal or not. Future work is therefore needed to improve the accuracy of the sensor.

Keywords: Biosensor, Cyclic voltammetry, Electrochemical sensor, Molecularly imprinted polymers(MIPs), Real urine, Sodium ions

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หัวข้อปริญญานิพนธ์	การพัฒนาไบโอเซนเซอร์โดยการใช้โพลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลสำหรับการตรวจวัดความเข้มข้นของปริมาณโซเดียมในปัสสาวะ
นักศึกษา	นายวรพล จำเริญลาภ นางสาวสุชญา ยอดคำมี
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บทคัดย่อ

งานวิจัยชิ้นนี้เกี่ยวข้องกับการพัฒนาไบโอเซนเซอร์โดยการทำโพลิเมอร์ด้วยวิธีรอยพิมพ์ประทับโมเลกุลสำหรับการตรวจวัดความเข้มข้นของปริมาณโซเดียมในปัสสาวะ โดยวัดกระแสไฟฟ้าจากปฏิกิริยารีดอกซ์ที่เกิดขึ้นระหว่างโซเดียมไอออนและเหล็ก(II)ไอออนบนพื้นผิวของเซนเซอร์ และสามารถประเมินได้ว่ามีปริมาณความเข้มข้นของโซเดียมเท่าใดในปัสสาวะ จากผลการดำเนินงานวิจัยพบว่า ค่าความถูกต้องของผลการวัดยังไม่สามารถยอมรับได้เนื่องจากผลการวัดในปัสสาวะอาสาสมัครปกติ มีค่ากระแสต่ำกว่าค่าจากการสอบเทียบ ส่งผลให้เครื่องมือวิจัยนี้ยังไม่เหมาะสมสำหรับการนำไปใช้ระบุความเข้มข้นในปัสสาวะของผู้ใช้งานได้ ดังนั้นจึงจำเป็นต้องมีการปรับปรุงความแม่นยำของเซนเซอร์เพื่อแก้ไขปัญหาดังกล่าวและพัฒนาเครื่องมือให้สามารถนำไปใช้งานได้จริงในอนาคต

Keywords: ไบโอเซนเซอร์, ไซคลิกโวลแทมเมตทรี, อิเล็กโทรเคมิคอลเซนเซอร์, อนุภาคพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุล, ปัสสาวะ, โซเดียมไอออน

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CHAPTER I
INTRODUCTION

1.1 Background

Sodium is an essential nutrient of the human body. It plays a critical role in the body's ability to control blood pressure and blood volume. However, the body needs it in relatively small quantities because of add much sodium, it will increase health risks such as high blood pressure increase. Monitoring and maintaining healthy blood pressure levels reduces the risk of cardiovascular disease, congestive heart failure, and kidney disease. The major source of our daily sodium intake is from table salt (sodium chloride). The Institute of Medicine states[1] that for individuals aged 9 to 50, the Adequate Intake (AI) level for sodium is 2,300 milligrams per day.

Approximately 8 million Thai people suffer from chronic kidney disease[2]. Most individuals did not know they had the disease, nor did they undergo testing and diagnosis within a medical facility. We therefore decided to development and produce a prototype device to detect sodium concentration in aqueous samples by using molecularly-imprinted polymers (MIPs) coated on an electronic circuit to allow the measurement of current derived from sodium in synthetic urine followed by further analysis of real urine. The electronic prototype device could then be used to convert the current obtained from an unknown sample to a concentration of sodium in urine.

1.2 Objectives

1) To develop a biosensor to detect sodium ions concentration in urine by using the molecularly imprinted polymers (MIPs) method coated on a sensor.
2) Make a sensor for access to everyone, it will take short detection time, cheap cost and easy to use.

1.3 Scope of Work

1) Study methods to detect sodium in urine: molecularly-imprinted polymers (MIPs).
2) Analyze sodium concentration in synthetic urine and real urine.

1.4 Benefits of Research

1) Can develop biosensor to detect sodium ion concentration in urine that has high sensitivity and accuracy.
2) Obtain new knowledge about Chemistry and experience about the experiment all laboratory.

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1.5 Work plan

Table 1.1 Work plan in August to November.

Activity	August				September				October				November			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Find the research support.			✓													
Discuss with advisor about the way to conduct project.				✓												
Produce sensor with selectivity for the required chemical to be detected by use MIPs method.					✓	✓	✓	✓								
Detect Na+ in solution by measuring current (use potentiostat and Nova 1.11 program).									✓	✓						
Choose the best monomer that can detect sodium well.										✓						
Test the endurance of sensor.											✓	✓				
Test the performance of sensor in synthetic urine and real urine.													✓	✓	✓	✓

Table 1.2 Work plan in January to March.

Activity	January				February				March			
	1	2	3	4	1	2	3	4	1	2	3	4
Test performance of sensor in synthetic urine and real urine.			✓	✓	✓	✓	✓	✓				
Summarize data.									✓	✓	✓	✓
Design the electronic part.											✓	✓
Do the report and presentation.											✓	✓

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CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 The Chemical Composition of Urine

Urine is a liquid from the body it is filtered in the kidneys to remove waste products from the bloodstream. Excretion is achieved through the urinary tract, from the kidney ureter and into the urinary bladder and urethra then excretion out of the body. Each person's urine varies in color according to the chemical composition.

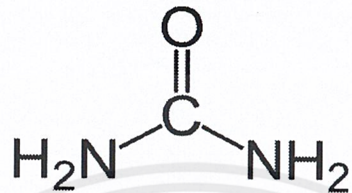

Figure 2.1 Structure of Urine.

2.1.1 A Representative Chemical Composition of urine

- Water (H_2O): 95%
- Urea (H_2NCONH_2): 9.3 g/l to 23.3 g/l (2%)
- Chloride (Cl^-): 1.87 g/l to 8.4 g/l (0.6%)
- Sodium (Na^+): 1.17 g/l to 4.39 g/l (0.6%)
- Potassium (K^+): 0.750 g/l to 2.61 g/l (0.15%)
- Creatinine ($C_4H_7N_3O$): 0.670 g/l to 2.15 g/l (0.1%)

2.1.2 Chemical Elements in Human Urine[3]

The element abundance depends on diet, health, and hydration level, but human urine consists of approximately:

- Oxygen (O): 8.25 g/l
- Nitrogen (N): 8/12 g/l
- Carbon (C): 6.87 g/l
- Hydrogen (H): 1.51 g/l

2.1.3 Chemicals That Affect Urine Color

Human urine ranges in color from nearly clear to dark amber, depending largely on the amount of water that is present. A variety of drugs, natural chemicals from foods, and diseases can alter the color. For example, eating beets can turn urine red or pink (harmlessly). Blood in the urine may also turn it red. Green urine may result from drinking highly colored beverages or from a urinary tract infection. The color of urine indicates chemical differences relative to normal urine but there aren't always an indication of illness. [3]

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2.2 Chronic kidney disease

Chronic kidney disease or chronic kidney failure is a condition where kidney function is reduced and cannot be restored to normal because the kidney was permanently destroyed. It often goes undetected and undiagnosed until the disease is well advanced. It is not unusual for people to realize they have chronic kidney failure only when their kidney function is down to 25 percent of normal. Causes of chronic kidney disease include high blood pressure, nephrotic syndrome, diabetes and Urethral Obstruction.

2.2.1 Chronic kidney disease diagnosis[4]

1) Tests for albumin (a type of protein) and/or blood in urine.
2) A blood test to find out the level of waste products in the blood and calculate glomerular filtration rate.
3) A blood pressure test as kidney disease causes high blood pressure, which can damage the small blood vessels in the kidneys. High blood pressure can also cause kidney disease.
4) An ultrasound or Computed Tomography scan (CT scan) to take a picture of the kidneys and urinary tract. These tests show the size of the kidneys, locate kidney stones or tumors and find any problems in the structure of the kidneys and urinary tract.

2.2.2 The kidney test results[5]

2.2.2.1 Glomerular filtration rate (GFR)

Glomerular filtration rate (GFR) is the best measure of the kidney function and helps decide the stage of kidney disease. It shows how well the kidneys are cleaning the blood. The GFR is usually estimated (eGFR) from the results of the creatinine blood test. eGFR is reported in milliliters per minute per $1.73m^2$ ($mL/min/1.73m^2$).

2.2.2.2 Albuminuria

Albuminuria can mean that the kidneys are damaged so albumin, an abundant blood protein, leaks into the urine. A small or ‘micro’ amount of albumin in the urine is called microalbuminuria, and a larger ‘macro’ amount is called macroalbuminuria. Albuminuria is often an early warning of kidney disease but can also be present for other reasons. Albuminuria can be detected by a special urine test called an albumin: creatinine ratio (ACR). An ACR is performed on a single sample of urine.

2.2.2.3 Haematuria

Haematuria or blood in the urine occurs when red blood cells leak into the urine. It can turn urine a red or dark cola color. Sometimes the blood in the urine is not visible to the eye but may be found on a urine test. This is called microscopic haematuria. Blood in the urine is a common sign of urinary tract infections but can also be the first sign of a problem with the kidneys or the bladder.

2.2.2.4 Creatinine

Creatinine is a waste product made by the muscles. It is usually removed from the blood by the kidneys and passes out in the urine. When the kidneys aren’t working well, creatinine stays in the blood. A blood test helps to work out how quickly your kidneys remove or ‘clear’ creatinine from the blood.

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2.2.2.5 Urea

Urea is a waste product made by the body as it processes protein from the food that had eat. If people have lost some kidney function, the kidneys may not be able to remove all the urea from the blood.

2.2.2.6 Potassium

Potassium is a mineral found in many foods. If the kidneys are healthy, they can remove extra potassium from the blood. If the kidneys are damaged, the potassium level can rise and affect the heart. A low or high potassium level can cause an irregular heartbeat.

2.2.3 Stages of chronic kidney disease[6]

Changes in the Glomerular Filtration Rate (GFR) can assess how advanced the kidney disease is. In the UK, and many other countries, kidney disease stages are classified as follows:

- **Stage 1** GFR rate is normal. However, evidence of kidney disease has been detected.
- **Stage 2** GFR rate is lower than 90 milliliters, and evidence of kidney disease has been detected.
- **Stage 3** GFR rate is lower than 60 milliliters, regardless of whether evidence of kidney disease has been detected.
- **Stage 4** GRF rate is lower than 30 milliliters, regardless of whether evidence of kidney disease has been detected.
- **Stage 5** GFR rate is lower than 15 milliliters. Renal failure has occurred.

2.3 Urine Sodium Level Test

A urine sodium test determines can evaluate kidney function, particularly in terms of its sodium regulation capability. There are two types of sodium urine test. A random test looks at sodium in a single urine sample. A 24-hour test looks at urine sodium over the course of a 24-hour period.[7] The normal serum sodium level is 135-145 mmol/L.[8]

2.3.1 Hyponatremia

Low levels of sodium in urine may indicate kidney problems or hyponatremia. Hyponatremia is a serum sodium level of less than 135 mmol/L.[9] Symptoms include fatigue, nausea, and vomiting, headache, loss of appetite, confusion or disorientation, hallucinations, loss of consciousness or coma.

2.3.1.1 Causes of Hyponatremia[10]

Many possible conditions and lifestyle factors can lead to hyponatremia, including

- **Certain medications.** Some medications, such as some water pills (diuretics), antidepressants and pain medications, can interfere with the normal hormonal and kidney processes that keep sodium concentrations within the healthy normal range.

- **Heart, kidney and liver problems.** Congestive heart failure and certain diseases affecting the kidneys or liver can cause fluids to accumulate in your body, which dilutes the sodium in your body, lowering the overall level.

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- **Syndrome of inappropriate anti-diuretic hormone (SIADH)** In this condition, high levels of the anti-diuretic hormone (ADH) are produced, causing your body to retain water instead of excreting it normally in your urine.

- **Chronic, severe vomiting or diarrhea and other causes of dehydration.** This causes your body to lose electrolytes, such as sodium, and also increases ADH levels.

- **Drinking too much water.** Drinking excessive amounts of water can cause low sodium by overwhelming the kidneys' ability to excrete water. Because you lose sodium through sweat, drinking too much water during endurance activities, such as marathons and triathlons, can also dilute the sodium content of your blood.

- **Hormonal changes.** Adrenal gland insufficiency (Addison's disease) affects your adrenal glands' ability to produce hormones that help maintain your body's balance of sodium, potassium and water. Low levels of thyroid hormone also can cause a low blood-sodium level.

- **The recreational drug Ecstasy.** This amphetamine increases the risk of severe and even fatal cases of hyponatremia.

2.3.1.2 Risk factors

- **Age:** Older adults may have more contributing factors for hyponatremia, including age-related body changes, taking certain medications and a greater likelihood of developing a chronic disease that alters the body's sodium balance.

- **Certain drugs:** Medications that increase your risk of hyponatremia include thiazide diuretics as well as some antidepressants and pain medications. In addition, the recreational drug Ecstasy has been linked to fatal cases of hyponatremia.

- **Conditions that decrease your body's water excretion:** Medical conditions that may increase your risk of hyponatremia include kidney disease, syndrome of inappropriate anti-diuretic hormone (SIADH) and heart failure, among others.

- **Intensive physical activities:** People who drink too much water while taking part in marathons, ultramarathons, triathlons and other long-distance, high-intensity activities are at an increased risk of hyponatremia.

2.3.2 Hypernatremia

High levels of sodium in the urine may be due to diet, kidney problems, or hypernatremia. Hypernatremia is a serum sodium level greater than 145 mmol/ L.[11] Symptoms include thirst, fatigue, swelling in hands and feet, weakness, insomnia, rapid heartbeat, coma.[12]

2.3.2.1 Causes of Hypernatremia

1) Low volume

- Inadequate intake of free water associated with total body sodium depletion. Typically, in elderly or otherwise disabled patients who are unable to take in water as their thirst dictates and also are sodium depleted. This is the most common cause of hypernatremia.

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2.4.1 Molecular Imprinting Technique

MIP is prepared by mixing four compositions: template, monomers, cross-linking and initiator in a solvent. This pre-polymerization mixture is irradiated with UV light or subjected to heat in order to initiate polymerization.[14]

There are two methods for creating polymers. The first is use self-assembly, method which involves the formation of the polymer by mixing all elements of the MIP and providing the molecular interactions to form the cross-linked polymer with the template bound. The second method involves covalently linking the imprinted molecule to the monomer.

After the polymerization process is complete, the template is removed from the polymer by washing, leaving specific recognition sites complementary in shape, size and chemical functionality to the template molecule. The resultant polymer recognizes and binds selectively only the template molecules. (Fig 2.2)

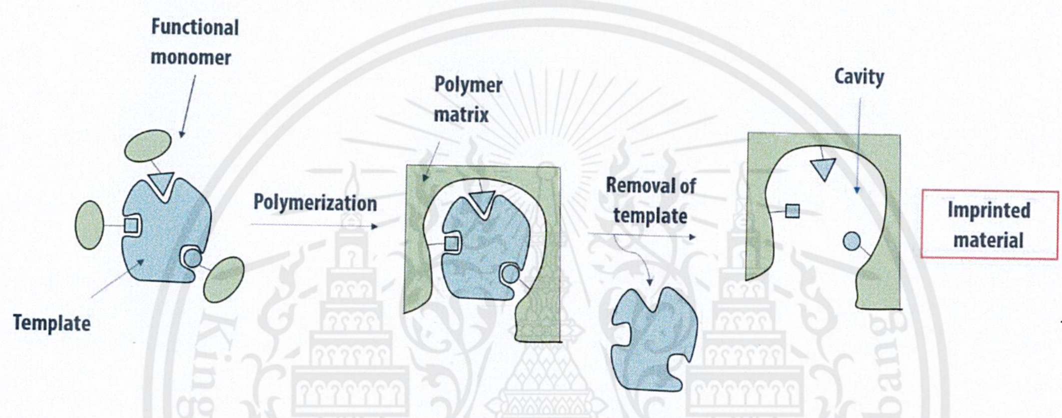

Figure 2.2 Molecular imprinting process.

Molecular identification is dependent on various factors, such as shape complementarity, functional complementarity, contributions from the surrounding environment. Moreover, the quantity and quality of MIP recognition sites is a direct function of the mechanisms and extent the monomer- interactions present in the pre-polymerization mixture. The recognition of the polymer constitutes an induced molecular memory, which makes the recognition sites capable of selectively recognizing the imprint species.

2.4.2 Category of MIP[15]

Molecular imprinting procedures have two kind based on covalent bonds or non-covalent. The special binding sites are formed by covalent or non-covalent interaction between of template and monomers, followed by a crosslinkers polymerization.

1. **Covalent interactions**

Covalent imprinting, the templates are covalently bound to one or more polymerizable groups. After polymerization, the template is cleaved, and the functionality left in the binding site is capable of binding the target molecule by re-establishment of the covalent bond.

2. **Non-covalent interactions**

In non-covalent imprinting, the interactions between monomer and template during polymerization are the same as those between polymer and template in

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the rebinding step. These are based on non-covalent forces such as H-bonding, ion-pairing and dipole-dipole interactions. This method was first introduced in organic polymers by the group of Mosbach.

2.4.3 Reagents for molecular imprinting[16]

The functioning of an imprinted polymer is controlled by the type of reagents for molecular imprinting such as template, ligand, crosslinkers.

2.4.3.1 Templates

The template is central importance as it directs the organization of the functional groups in all molecular imprinting processes.

The template should satisfy the following three requirements.

- It should not contain groups involved in or preventing polymerization.
- It should exhibit excellent chemical stability during the polymerization reaction.
- It should contain functional groups well adapted to assemble with functional monomers.

Most templates applied in molecular imprinting are derived from widespread environmental pollutants, which can be grouped into three broad categories, including endocrine disrupting chemicals (EDC), pharmaceuticals and toxic metal ions. Endocrine disrupting chemicals have attracted extensive attention due to global environmental problems as a result of disturbances of central regulatory functions in humans and wildlife. Worse still, some endocrine disrupting chemicals are even suspected to cause human infertility or stunt growth of children. Among those endocrine disrupting chemicals, hormone drugs, triazine pesticides, and bisphenol are the most widely concerning pollutants used in preparation of imprinted polymers. Pharmaceuticals have gained increasing attention and interest in the last few years due to their continuous release in the environment. Water compatible imprinted polymers have been a significant development in the field of molecular imprinting technology. At present, water compatible imprinted polymers for determination of quinolones in urine, milk, river water have been synthesized. Heavy metal ions are always the focus of attention owing to their difficulty of degradation and ease of bio enrichment.

2.4.3.2 Monomers

The careful choice of functional monomer is the utmost importance in order to provide complementary interactions between the template and substrates. For covalent molecular imprinting, the effects of changing the template to functional monomer ratio is not necessary because the template dictates the number of functional monomers that can be covalently attached; furthermore, the functional monomers are attached in a stoichiometric manner. For non-covalent imprinting, the optimal template monomer ratio is achieved empirically by evaluating several polymers made with different formulations with increasing template. The underlying reason for this is thought to originate with the solution complex between functional monomers and template, which is governed by Le Chatelier's principle. Applying Le Chatelier's principle to the complex formed prior to polymerization, increasing the concentration of components or binding affinity of the complex in the pre-polymerization mixture

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would predict an increase in the pre-polymer complex. Correspondingly, there is an increase the number of final binding sites in the imprinted polymer, resulting in an increased binding or selectivity factor per gram of polymer.

From the general mechanism of formation of MIP binding sites, functional monomers are responsible for the binding interactions in the imprinted binding sites, and for non-covalent molecular imprinting protocols, are normally used in excess relative to the number of moles of template to favor the formation of template-functional monomer assemblies. It is very important to match the functionality of the template with the functionality of the functional monomer in a complementary fashion (e.g. H-bond donor with H-bond acceptor) in order to maximize complex formation and thus the imprinting effect. Higher retention and resolution can be by the two-co-monomer imprinting polymer than the single monomer imprinting polymer. This indicates an increase in the affinity of the MIP with the sample as a result of the cooperation effect of the binding sites. However, it's important to note thats the reactivity ratios of the monomers to ensure those co-polymerisations are feasible.

Figure 2.3 Common functional monomers used in non-covalent molecular imprinting procedures.

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2.4.3.3 Cross-linkers

The selectivity is greatly influenced by the kind and amount of cross- linking agent used in the synthesis of the imprinted polymer. The careful choice of functional monomer is another importance choice to provide complementary interactions with the template and substrates. In an imprinted polymer, the cross-linker fulfils three major functions: First of all, the cross-linker is important in controlling the morphology of the polymer matrix, whether it is gel-type, macroporous or a microgel powder. Secondly, it serves to stabilize the imprinted binding site. Finally, it imparts mechanical stability to the polymer matrix. From a polymerization point of view, high cross-link ratios are generally preferred in order to access permanently porous (macroporous) materials and in order to be able to generate materials with adequate mechanical stability. So, the amount of cross-linker should be high enough to maintain the stability of the recognition sites. This may be because the high degree of cross-linking enables the microcavities to maintain three-dimensional structure complementary in both shape and chemical functionality to that of the template after removal of the template, and thus, the functional groups are held in an optimal configuration for rebinding the template, allowing the receptor to `recognize' the original substrate. Polymers with cross-link ratios in excess of 80% are often be used. Quite a number of cross-linkers compatible with molecular imprinting are known, and a few of which are capable of simultaneously complexing with the template and thus acting as functional monomers.

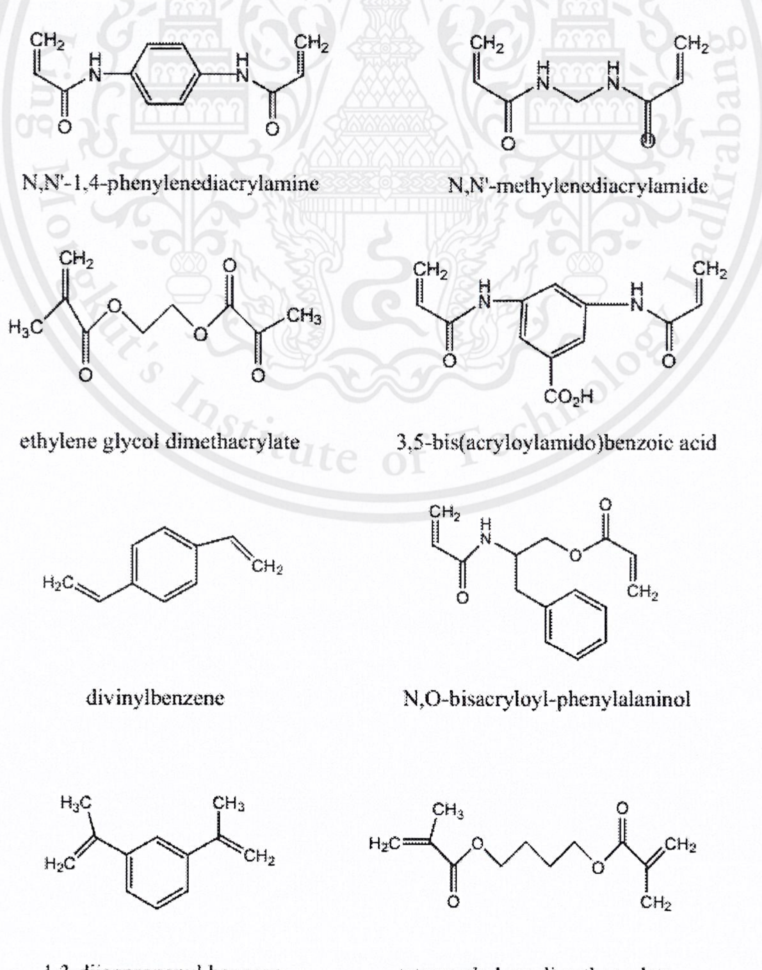

Figure 2.4 Chemical structure of common cross-linkers used in non-covalent molecular imprinting.

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2.4.3.4 Solvent

Porogenic solvents play an important role in formation of the porous structure of MIP, also known as macroporous polymers. It is known that the nature and level of porogenic solvents determines the strength of non-covalent interactions and influences polymer morphology which, obviously, directly affects the performance of MIP. Firstly, the template molecule, initiator, monomer and cross-linker have to be soluble in the porogenic solvents. Secondly, the porogenic solvents should produce large pores, in order to assure the good flow-through properties of the resulting polymer. Thirdly, the porogenic solvents should be relatively low polarity, in order to reduce the interferences during complex formation between the imprint molecule and the monomer, as the latter is very important to obtain high selectivity MIP.

Porogenic solvents with low solubility phase separate early and tend to form larger pores and materials with lower surface areas. Conversely, porogenic solvents with higher solubility phase separate later in the polymerization provide materials with smaller pore size distributions and greater surface area. More specifically, use of a thermodynamically good solvent tends to lead to polymers with well-developed pore structures and high specific surface areas, use of a thermodynamically poor solvent leads to polymers with poorly developed pore structures and low specific surface areas. However, the binding and selectivity in MIP is not appeared to dependent on a particular porosity.

Although the results of molecular recognition weaken with polar porogenic solvents, it is important to stress that in some cases sufficiently strong template: monomer interactions have been observed in rather polar solvents (e.g. methanol/water). Increasing the volume of porogenic solvents increases the pore volume. Besides its dual roles as a solvent and as a pore forming agent, the solvent in a non-covalent imprinting polymerization must also be judiciously chosen such that it simultaneously maximizes the likelihood of template, functional monomer complex formation. Normally, this implies that apolar, non-protic solvents, e.g. toluene, are preferred as such solvents stabilize hydrogen bonds, however if hydrophobic forces are being used to drive the complexation then water could well be the solvent of choice.

2.4.3.5 Initiators

The role of the initiator in radical polymerization is well-known as it is responsible for the creation of monomer radicals in order to propagate the polymer formation. The rate of radical polymerization depends on the nature of the concentration of the initiator. 2,2’-azo-bis-isobutyronitrile (AIBN) and potassium persulphate are the most efficient initiators. The choice of the initiator depends on the nature of the template.

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azobisisobutyronitrile   dimethylacetal of benzil   benzoylperoxide

azobisdimethylvaleronitrile   4,4'-azo(4-cyanovaleric acid)

Figure 2.5 Chemical structure of common initiators used in non-covalent molecular imprinting.

2.4.4 Preparation Methods of MIP

Ion imprinted polymers are generally synthesized by polycondensation, while organic ion imprinted polymers are mainly prepared by free radical polymerization. The formats of these ion imprinted polymers can vary according to the polymerization process. Bulk polymerization will lead to monolithic materials, whereas well-defined particles can be produced by heterogeneous (suspension or emulsion) or homogeneous (dispersion or precipitation) polymerization.

2.4.4.1 Bulk polymerization

Molecularly imprinted polymers can be prepared in a variety of physical forms to suit the final application desired. The conventional method for preparing MIP is via solution polymerization followed by mechanical grinding of the resulting bulk polymer generated to give small particles and sieve the particles into the desired size ranges, which diameters usually in the micrometer range. This method, by far the most popular, presents many attractive properties, especially to newcomers. In fact, it is fast and simple in its practical execution and it does not require particular operator skills or sophisticated instrumentation. Particle sizes <25 μm are usually used in chromatographic studies. Such ground and sieved particles have been packed into conventional HPLC columns, immobilized on TLC plates, and entrapped in capillary columns using acrylamide gels or silicate matrices.

Although bulk polymerization is simple, and optimization of imprinting conditions is relatively straightforward, bulk polymerization method presents many drawbacks. First of all, the particles obtained after the last sieving step have a highly irregular in size and shape, some interaction sites are destroyed during grinding, and thus lead to a negative impact on chromatographic performance and lower MIP loading capacity with respect to theoretical values. Moreover, the procedure of grinding and sieving is cumbersome, and it causes a substantial loss of useful polymer, that can be estimated between 50 and 75% of the initial amount of bulk material. Since a portion of polymer can only be used as packing material, this method suffers from high consumption of the template molecules. Last, but not least, due to its exothermical nature, bulk polymerization cannot be scaled-up without danger of sample overheating.

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2.4.4.2 Suspension Polymerization

A rather simple method for the preparation of imprinted supports what does not require mechanical grinding is called suspension polymerization. This yields aggregates of spherical particles, if the system is sufficiently dilute, uniformly sized microspheres. To avoid above interference in multi-step swelling method, suspension polymerization in perfluorocarbon solvents has been studied. In two-phase systems, the use of liquid perfluorocarbons instead of water as the continuous phase might be preferred since water may have a detrimental effect on the non-covalent complex between monomers and imprint molecule. Although regular molecularly imprinted microspheres have been prepared and excellent chromatographic performance was obtained from polymer beads produced by using these methods and selectivity was good even at high flow rates, unfortunately, the specialized perfluorocarbon solvent and fluorinated surfactant impose limits on the applicability and practicality of this method.

Bovine serum albumin-imprinted polyacrylamide gel beads were synthesized via inverse-phase seed suspension polymerization, using high-density crosslinked gel beads as core, low-density crosslinked polyacrylamide gel as imprinting shell. The selectivity test showed that imprinting gel beads exhibited good recognition for template proteins, as compared to the control protein. The imprinting beads had quick adsorption rate and possessed improved regeneration property in comparison with those prepared directly via inverse-phase suspension polymerization. They consider the formation of multiple hydrogen bonds and complementary shape between the imprinting cavities and the template proteins are the two factors that lead to the imprinting effect.

A MIP that uses a stable isotope labeled compound as the template molecule, the so-called IMIP, was developed by suspension polymerization. The selectivity factors of MIP and IMIP for bisphenol A (BPA) were 4.45 and 4.43, respectively. Therefore, IMIP was found to have the same molecular recognition ability as MIP. When MI-SPE with IMIP was used and followed by LC–MS in the analysis of river water sample, the detection limit of BPA was 1 ppt with high sensitivity.

2.4.4.3 Multi-step Swelling Polymerization

In recent years, much effort has been dedicated to developing alternative methods to prepare imprinted stationary phases, which are superior in terms of efficiency and mass transfer properties. Micrometer-sized spherical imprinted polymers with narrow size distribution have been prepared through several techniques. Uniformed spherical particles have been obtained by using multi-step swelling method. Particles can be prepared directly in the form of spherical beads of controlled diameter. Beads synthesized in this way can be rendered magnetic through inclusion of iron oxide particles. Although particles obtained using this technique are comparatively monodisperse in size and shape and well suited for chromatographic applications, however, fairly complicated procedures and reaction conditions are required, and the aqueous suspensions used in this technique could interfere with the imprinting and thus lead to a decrease in selectivity. The requirement for aqueous emulsions can interfere with the imprinting process and the selectivity of these particles is still not completely satisfactory.

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Uniformly sized MIP for d-chlorpheniramine (CP) and bromphenira-mine (BP) prepared by a multi-step swelling polymerization method and evaluated using a mixture of phosphate buffer and acetonitrile as mobile phase· CP and BP enantiomers were retained the most as a monovalent cation on MAA-co-EDMA polymers and a divalent cation on TFMAA-co-EDMA polymers. Ion exchange and hydrophobic interactions could mainly work for the retention and enantioseparation of CP and BP on both MAA-co-EDMA and TFMAA-co-EDMA polymers in hydro-organic mobile phases.

2.4.4.4 Precipitation Polymerization

MIP microspherical shapes with more uniform size can be obtained by the method of precipitation polymerization, which offers a higher active surface area by manipulating its compositions. As regards precipitation polymerization, this technique involves coagulation of nano-gel beads followed by ordered particle growth due to capture of oligomers from surrounding solution. In this manner, near-monodispersed spherical beads can be prepared, and size and porosity can be fine-tuned thereby changing the polymerization conditions. This technique has been reported in MIP-based competition assays and capillary electrochromatography[16], but only recently works have been published, in which it is clearly shown that precipitation polymerization can be a potentially fruitful technique for preparing chromatography-grade molecularly imprinted beads.

Recently, precipitation polymerization has been employed for the production of molecularly imprinted microspheres by Ye and Puoci et al [17-18]. Despite the higher yields, a large amount of template molecules is needed for the preparation process, because of the high dilution factor. The feasibility of preparing highly selective morphine imprinting polymer particles using precipitation polymerization is demonstrated by Ho et al. [19] Since the template, morphine hydrochloride, is dilute in the polymerization solution, it is assumed that the pH effect can be neglected in the system. The MIP prepared by precipitation polymerization gave uniform particles, and this proved to be a feasible method for fabricating MIP. By controlling the separation point during the cross-linking polymerization process, starting with a dilute monomer solution, uniform molecular imprinted microspheres were obtained in good yield. Compared with the traditional method, these particles exhibit greater recognition binding ability in the sensing of morphine without a time-consuming process of grinding and sieving. The rebinding of MIP by the precipitation polymerization in a solution containing morphine exhibited better performance than that of NMIP.

Baggiani[20] prepare near monodispersed polystyrene beads by precipitation polymerization in acetonitrile, and these polymeric beads cheap and easy-to-make, represent a convenient alternative to the expensive chromatography-grade silica in the infester-mediated grafting of MIP. The chromatographic behavior of a column packed with these imprinted beads was compared with another column packed with irregular particles obtained by grinding of a bulk pyrimethanil-imprinted polymer. These results are consistent with an influence of the polymerization method on the morphology of the resulting polymer and not on the molecular recognition properties due to the molecular imprinting process.

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2.4.4.5 Surface Imprinting Polymerization

Surface grafting of MIP layers onto preformed beads has been recently proposed as attractive and apparently general techniques to obtain chromatography-grade imprinted materials. In this method, thin imprinted layers have been successfully used as coatings on chromatography-grade porous silica using several techniques to restrain the radical polymerization at the surface of the beads.

An imprinted layer selective to specific molecules on the surface of widely used polymers without affecting the bulk features was prepared by Sreenivasan. The methodology is simple and modified surface could be used in applications as diverse as separation, sensing, medical uses, etc. Say et al. [21] prepared phosphotriesterase mimic surface imprinted polymeric microbeads using MAH–Cu(II) as a new metal–chelating monomer. The paraoxon hydrolytic activity results showed that hydrolytic activity of PIBs was higher than NIBs. The preparation of the polymer is simple, inexpensive and results demonstrated that the catalytic activity of microbeads has decreased by only 17% after several uses.

The surface imprinting technique utilizing water-in-oil (W/O) emulsions was applied to the preparation of a metal ion-imprinted membrane by Arak The zinc(II) ion imprinted membrane was successfully prepared by emulsion polymerization with 1,12-dodecanediol-O,O'-diphenylphos-phonic acid (functional host molecule), l-glutamic acid dioleylester ribitol (emulsion stabilizer), and divinylbenzene (polymer matrix-forming monomer). To obtain flexible and mechanically stable membranes for practical applications, the polymerization was conducted in the presence of acrylonitrile-butadiene rubber and hydrophilized poly(tetrafluoroethylene) membranes. The use of acrylonitrile-butadiene rubber and a porous solid support in the polymer matrix resulted in improved flexibility and mechanical strength of the imprinted membrane. The permeation mechanism of the metal ions was considered to be hopping of metal ions on the bindin sites in the membranes.

Piacham et al. [22] prepare ultra-thin MIP films using surface initiated radical polymerization. Polymer films are directly formed on gold-coated quartz crystal resonator, which offers easy monitoring of polymer growth. With this approach its easy control the thickness of the MIP film to be below 50 nm, where the selective recognition of target analytes can be easily detected by the underlying quartz crystal resonator. When used in a flow injection analysis system, the assembled QCM sensor generated a large frequency change (>30 Hz) upon encountering a small amount of analyte (0.19 mM). The sensor had a very short response time (<1 min), and displayed certain chiral selectivity towards the original template, (S)-propranolol at a concentration higher than 0.38mM in aqueous solution.

2.4.4.6 Monolithic Imprinted Polymerization

Monolithic molecularly imprinted technology combined the advantage of monolithic column and molecular imprinted technology, which was prepared by a simple, one-step, in-situ, free-radical polymerization ''molding'' process directly within a chromatographic column without the tedious procedures of grinding, sieving, and column packing. Monolithic MIP is expected to improve the separation and enable direct analysis with high-speed and high performance after in–situ

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polymerization. Matsui et al. [23] first used the in-situ polymerization technique for preparation of molecularly imprinted monoliths. Template, functional monomer, cross-linker and initiator were dissolved in mixture porogenic solvents (cyclohexanol and 1-dodecanol) and the mixture was degassed and poured into a stainless steel column. After polymerization, the template and porogenic solvents were removed by exhaustive washing with methanol-acetic acid. The monolithic molecularly imprinted technology has attracted significant interest because of their ease of preparation, high reproducibility, high selectivity and sensitivity, and rapid mass transport. Furthermore, the preparation of this type of MIP is more cost-efficient, because the amount of template molecules required is much lower. Moreover, their greater porosity, and hence good permeability, and high surface area are well suited for both small molecules and large biopolymers. Monolithic molecularly imprinted stationary phases have become a rapidly expanding field in chromatographic stationary phase preparation in recent years.

The proportion of mixture composition and polymerization temperature defines the monolithic structure and separation characteristic without further processing. The key to successful column preparation of MIP monolith is both choice of the composition of the pre-polymerization mixture and porogenic solvents and careful timing of the polymerization reaction. In order to compare the different polymerization methods, Mayes et al. prepared three types of MIP by bulk, multistep-swelling and grafting methods when some β-blockers were used as the template molecules. In that study, ground monolithic imprinted polymer was thought to be the best all-round performer for enantiomeric separations of drugs by HPLC. In-situ polymerization has similar recognition ability and possesses the advantages of a one-step preparation procedure and high yield. Matsui and Huang prepared a set of monolithic molecularly imprinted polymers with cinchonine and amino acid derivatives as the template molecules. Separation of the corresponding enantiomers was achieved but the separation mechanism was not mentioned. In recent years, the uses of monolithic media for superior chromatographic separation in high-performance liquid chromatography and capillary electrochromatography have attracted considerable attention.

Imprinted monolithic membranes by polymerizing mixtures of methacrylic acid and dimethacrylate crosslinkers within microporous support of filtered paper is described by Kiełczynski. These membranes are selectively permeable for template molecules but transport of others species is mostly limited. The measured transport stereoselectivity varied from 1.1 to 3.7 depending on the system used and the presence of cinchonine in the monomer mixture made membrane more permeable while cinchonidine reduced its permeability. Optimization of the imprinted polymer membrane in terms of the number of binding sites and their selectivity for the template enantiomer ought to be connected with optimization of membrane porosity and template interactions with functional groups. When EDMA and EDMA:MAA monolithic membranes behave in the predicable way, i.e. transport of cinchona alkaloids follows membrane templating, TEGDMA-family membranes show abnormal preference to transport cinchonidine more effectively.

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Table 2.1 Summary of MIP prepared by different methods.[16]

MIP format	Benefits	Limitations
Bulk polymerization	- Polymerization simplicity and universality - No require skills or sophisticated instrumentation	- Tedious procedures of grinding - Sieving - Column packing - Irregular particle in size and shape - Low performance
Suspension polymerization	- Spherical particles - Highly reproducible results - Large scale possible	- Phase partitioning of complicates system - Water is incompatible with most imprinted procedures - Specialist surfactant polymers required
Multi-step swelling polymerization	- Monodisperse beads of controlled diameter - Excellent particle for HPLC	- Complicated procedures - Reaction conditions - Need for aqueous emulsions
Precipitation polymerization	- Imprinted microspheres - Uniform size and high yields	- Large amount of template - High dilution factor
Surface polymerization	- Monodisperse product - Thin imprinted layers	- Complicated system - Time consuming
In-situ polymerization	- One-step - In-situ preparation - Cost-efficient - good porosity	- Extensive optimization required for each new template system

2.4.5 Polymerization[24]

Polymerization is the reaction of combining these monomers to form long chains or three-dimensional networks. Polymerization can be classified into two categories.

- Step-Growth or Condensation Polymerization
- Chain-Growth or Addition Polymerization

2.4.5.1 Condensation Polymerization

Condensation polymers form from the step growth polymerization. Here when molecules of monomers react to form a bond, they replace certain molecules. These molecules are the by-product of the reaction. In most cases, this by-product is a water molecule.

The type of polymers that result from a condensation polymerization depends on the monomers. If the monomer has only one reactive group, the polymers that form have low molecular weight. When monomers have two reactive ends groups, we get linear polymers. And monomers with higher than two reactive groups result in a polymer with a three-dimensional network.

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2.4.5.2 Addition Polymerization

The repeating monomers form a linear or branch structure depending on the type of monomer. During addition polymerization, the monomers rearrange themselves to form a new structure. But there is no loss of an atom or a molecule.

Addition polymerizations can be classified into four types are

- **Free Radical Polymerization:** The addition polymer-forms by addition of atoms with a free electron in its valence shells. These are known as free radicals. They join in a successive chain during free radical polymerization.
- **Cationic polymerization:** A polymerization where a cation is formed causing a chain reaction. It results in forming a long chain of repeating monomers.
- **Anionic Vinyl Polymerization:** Involves the polymerization of particularly vinyl polymers with a strong electronegative group to form a chain reaction
- **Coordination Polymerization:** This method was invented by two scientists Ziegler and Natta who won a Nobel Prize for their work.[82] They developed a catalyst which control the free radical polymerization. It produces a polymer which has more density and strength.

Table 2.2 Types of Addition Polymerization Undergone by Various Unsaturated Monomers.[25]

Monomers	Type of Initiation		
	Radical	Cationic	Anionic
Ethylene	+	-	+
1-Alkyl alkenes (α-olefins)	-	-	+
1,1-Dialkyl alkenes	-	+	-
1,3-Dienes	+	+	+
Styrene, α -methyl styrene	+	+	+
Halogenated alkenes	+	-	-
Vinyl esters (CH_2=CHOCOR)	+	-	-
Acrylates, methacrylates	+	-	+
Acrylonitrile, methacrylonitrile	+	-	+
Acrylamide, methacrylamide	+	-	+
Vinyl ethers	-	+	-
N-Vinyl carbazole	+	+	-
N-Vinyl pyrrolidone	+	+	-
Aldehydes, ketones	-	+	+

Addition Polymerization have 3 steps are[26]

1) **Initiation:** In the first stage, a substance is split into two identical parts, each with an unpaired electron. A molecule with an unpaired electron is called a free radical. The free radical then initiates the reaction sequence by forming a bond with one of the carbon atoms in the double bond of the monomer. One electron for this new bond comes from the free radical, and the second electron for the new bond comes from one of the two bonds between the carbon atoms. The remaining electron from the broken bond shifts to the carbon atom on the far side of the molecule, away from the newly formed bond, forming a new free radical. Each half-headed arrow

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indicates the shift of one electron. Typical initiators include organic compounds with a labile group: e.g. azo(-N=N-), disulfide(-S-S-), or peroxide (-O-O-). Two examples are benzoyl peroxide and AIBN.

2) Propagation: when the new free radical formed in the initiation stage reacts with another monomer to add two more carbon atoms. This process repeats repeatedly to form chains containing thousands to millions of carbon atoms.

3) Termination: when any two free radicals combine, thus pairing their unpaired electrons and forming a covalent bond that links two chains together.

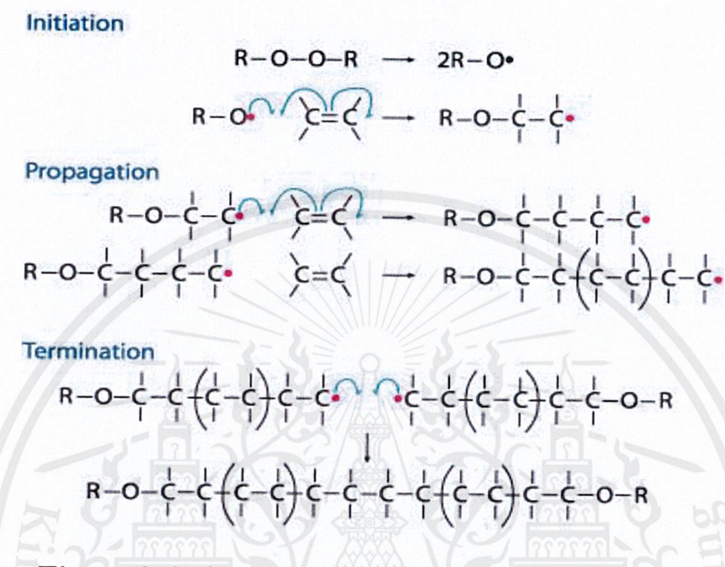

Figure 2.6 Three steps of Addition Polymerization.

2.4.6 Components of molecular imprinting process

2.4.6.1 Template

1) Sodium Chloride

An ionic compound with the chemical formula NaCl, representing a 1:1 ratio of sodium and chloride ions. With molar masses of 22.99 and 35.45 g/mol respectively, 100 g of NaCl contains 39.34 g Na and 60.66 g Cl. Sodium chloride is the salt most responsible for the salinity of seawater and of the extracellular fluid of many multicellular organisms. In its edible form of table salt, it is commonly used as a condiment and food preservative. Large quantities of sodium chloride are used in many industrial processes, and it is a major source of sodium and chlorine compounds used as feedstocks for further chemical syntheses. A second major application of sodium chloride is de-icing of roadways in sub-freezing weather.

Na — Cl

Figure 2.7 Structure of Sodium Chloride.

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2) 18-Crown-6

An organic compound with the formula $[C_2H_4O]_6$ and the IUPAC name of 1,4,7,10,13,16 hexaoxacycloocta-decane. It is a white, hygroscopic crystalline solid with a low melting point. Like other crown ethers, 18-crown-6 functions as a ligand for some metal cations with a particular affinity for potassium cations (binding constant in methanol: 106 M−1). The point group of 18-crown-6 is S6. The dipole moment of 18-crown-6 varies in different solvent and under different temperature. Under 25 °C, the dipole moment of 18-crown-6 is 2.76 ± 0.06 D in cyclohexane and 2.73 ± 0.02 in benzene. The synthesis of the crown ethers led to the awarding of the Nobel Prize in Chemistry to Charles J. Pedersen.

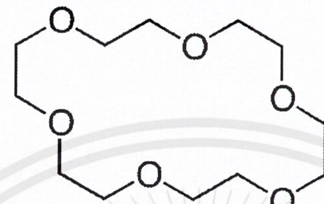

Figure 2.8 Structure of 18-Crown-6.

2.4.6.2 Functional monomer(s)

1) Methacrylic acid (MAA)

Methacrylic acid is a clear colorless liquid (or low-melting solid) with a pungent odor. Corrosive to metals and tissue. Flash point 170°F. Melting point 61°F. May polymerize exothermically if heated or contaminated. If the polymerization takes place inside a container, the container may rupture violently. Less dense than water. Vapors heavier than air. Used to make plastics.

Figure 2.9 Structure of MAA.

2) Acrylamide (AAM)

Acrylamide is a colorless, odorless, crystalline amide that polymerizes rapidly and can form as a byproduct during the heating of starch-rich foods to high temperatures. Acrylamide is used in the production of polymers mainly in the water treatment industry, pulp and paper industry and textile treatment industry and is used as a laboratory reagent. The polymer is nontoxic, but exposure to the monomer can cause central and peripheral nervous system damage resulting in hallucinations, drowsiness and numbness in the hands and legs. Acrylamide is reasonably anticipated to be a human carcinogen.

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Figure 2.10 Structure of AMM.

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3) N-Vinylpyrrolidone (NVP)

An organic compound consisting of a 5-membered lactam linked to a vinyl group. It is a colorless liquid although commercial samples can appear yellowish. It is produced industrially by vinylation of 2-pyrrolidone, i.e. the base-catalyzed reaction with acetylene. It is the precursor to polyvinylpyrrolidone (PVP), an important synthetic material. The NVP monomer is commonly used as a reactive diluent in ultraviolet and electron-beam curable polymers applied as inks, coatings or adhesives.

N O CH_2

Figure 2.11 Structure of NVP.

2.5.6.3 Cross-linker

N,N′-(1,2-Dihydroxyethylene)bis-acrylamide

H_2C H N OH O N H CH_2 O OH

Figure 2.12 Structure of DHEBA.

2.4.6.4 Initiator

2,2'-Azobisisobutyronitrile

A common reagent for the initiation of radical reactions. AIBN decomposes at temperatures above 60 C to form isobutyronitrile radicals, which function to initiate radical chemistry. Another very common radical initiator is benzoyl peroxide.

N C H_3C CH_3 N N H_3C CH_3 C N

Figure 2.13 Structure of AIBN.

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2.4.6.5 Solvent

Dimethyl sulfoxide (DMSO)

An organosulfur compound with the formula $(CH_3)_2SO$. This colorless liquid is an important polar aprotic solvent that dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water. It has a relatively high melting point. DMSO has the unusual property that many individuals perceive a garlic-like taste in the mouth after contact with the skin.

In terms of chemical structure, the molecule has idealized Cs symmetry. It has a trigonal pyramidal molecular geometry consistent with other three-coordinate S(IV) compounds, with a nonbonded electron pair on the approximately tetrahedral sulfur atom.

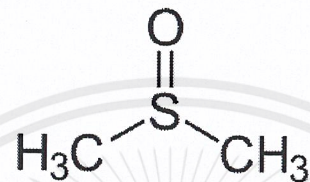

Figure 2.14 Structure of DMSO.

2.4.7 Advantages of molecular imprinted polymers

The main advantages of molecularly imprinted polymers are their high selectivity and affinity for the target molecule used in the imprinting procedure. Imprinted polymers compared to biological systems such as proteins and nucleic acids, have higher physical robustness, strength, resistance to elevated temperature and pressure, and inertness towards acids, bases, metal ions and organic solvents. In addition, they are also less expensive to be synthesized, and the storage life of the polymers is very high, keeping their recognition capacity for several years at room temperature.[27]

2.4.8 Disadvantages of molecular imprinted polymers

Design of a new MIP system suitable for a specific template molecule often requires a lot of time and work for synthesis, washing and testing, attempts to changing various experimental parameters, before finding the optimum conditions.

2.4.9 Example of MIP in biosensor

Hui Peng et al. successfully develop a sulfate bulk acoustic wave sensor based on a molecularly imprinted to determine atropine concentrations in human serum and urine. They are the first case of using the co-polymerize o-PD with aniline (An) to imprint the template atropine and selected bulk acoustic wave (BAW) as the transducer. A direct communication between the imprinted polymer and the transducer has been obtained by using one of the gold electrodes of the BAW device as the working electrode during the electro-polymerization stage. The sensor showed good analytical performance and successfully applied to the determination of atropine sulfate in media of serum and urine. The results are in good agreement with those obtained by spectrophotometry.[28]

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2.5 Cyclic Voltammetry

Cyclic voltammetry (CV) is a powerful and popular electrochemical technique commonly employed to investigate the reduction and oxidation processes of molecular species. CV is also invaluable to study electron transfer-initiated chemical reactions, which includes catalysis. The x-axis represents a parameter that is imposed on the system, here the applied potential (E), while the y-axis is the response, here the resulting current (i) passed.[29]

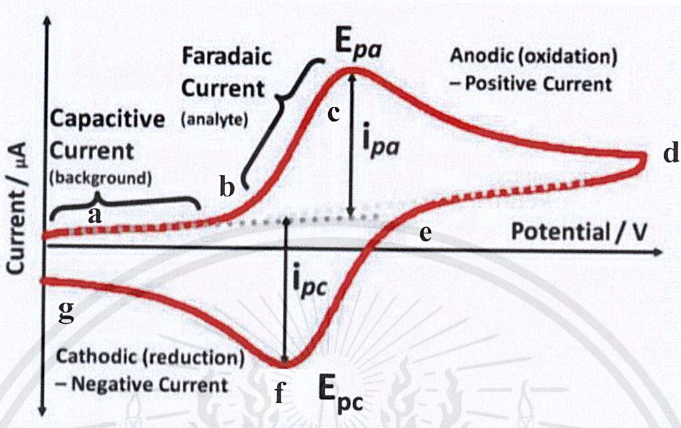

Figure 2.15 Cyclic Voltammetry.

In Figure 2.4, the potential scans positively from (a) to (d). This results in anodic current (i_{pa}) and oxidation to occur. The peak potential at (c) is called the anodic peak potential (E_{pa}) and is reached when all the substrate at the surface of the electrode has been oxidized. After the switching potential has been reached (d), the potential is scanned negatively to cause a reduction. The resulting current is called cathodic current (i_{pc}). The corresponding peak potential occurs at (f) and is called the cathodic peak potential (E_{pc}). The E_{pc} is reached when all of the substrate at the surface of the electrode has been reduced.[30]

2.6 Potentiostat Fundamentals

A potentiostat is an electronic instrument that controls the voltage difference between a Working Electrode and a Reference Electrode. Both electrodes are contained in an electrochemical cell. The potentiostat implements this control by injecting current into the cell through an Auxiliary, or Counter, electrode. In almost all applications, the potentiostat measures the current flow between the Working and Counter electrodes. The controlled variable in a potentiostat is the cell potential and the measured variable is the cell current.

This equipment is fundamental to modern electrochemical studies using three electrode systems for investigations of reaction mechanisms related to redox chemistry and other chemical phenomena. The dimensions of the resulting data depend on the experiment. In voltammetry, electric current in amps is plotted against electric potential in voltage. In a bulk electrolysis total coulomb passed (total electric charge) is plotted against time in seconds even though the experiment measures electric current (amperes) over time. This is done to show that the experiment is approaching an expected number of coulombs.

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Most early potentiostats could function independently, providing data output through a physical data trace. Modern potentiostats are designed to interface with a personal computer and operate through a dedicated software package. The automated software allows the user rapidly to shift between experiments and experimental conditions. The computer allows data to be stored and analyzed more effectively, rapidly, and accurately than historic methods.[31]

2.6.1 The Electrometer

The electrometer circuit measures the voltage difference between the reference and working electrodes. Its output has two major functions: it is the feedback signal in the potentiostat circuit, and it is the signal that is measured whenever the cell voltage is needed. An ideal electrometer has zero input current and an infinite input impedance. Current flow through the reference electrode can change its potential. In practice, all modern electrometers have input currents close enough to zero that this effect can usually be ignored.

Two important electrometer characteristics are its bandwidth and its input capacitance. The electrometer bandwidth characterizes the AC frequencies the electrometer can measure when it is driven from a low-impedance source. The electrometer bandwidth must be higher than the bandwidth of the other electronic components in the potentiostat. The electrometer input capacitance and the reference electrode resistance form an RC-filter. If this filter's time constant is too large, it can limit the effective bandwidth of the electrometer and cause system instabilities. Smaller input capacitance translates into more stable operation and greater tolerance for high impedance reference electrodes.

2.6.2 The I/E Converter

The current to voltage converter measures the cell current. The cell current is forced through a current measurement resistor, Rm. The resulting voltage across this resistor is a measure of cell current. During an experiment, cell current can change by several orders of magnitude. Such a wide range of current cannot be accurately measured by a single resistor. Modern potentiostats have several Rm resistors and an "I/E autoranging" algorithm that selects the appropriate resistor and switches it into the I/E circuit under computer control.

The bandwidth of the I/E converter depends strongly on its sensitivity. Unwanted capacitance in the I/E converter along with Rm forms an RC circuit. In order to measure small currents, Rm must be sufficiently large. This larger resistance, however, increases the RC time constant of the circuit limits the I/E bandwidth. For instance, no potentiostat can measure 10nA at 100 kHz.

Table 2.3 Technical Specifications of Potentiostat.[32]

Analog integrator	false
Compliance voltage range in volt	30
Current resolution	0.0003 % of current range
Dimensions in mm (W/H/D)	520/160/420

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Dimensions remark	without cables
Input impedance in Ohm	1 T
Maximum bandwidth in Hz	100 k
Maximum current in ampère	2
Maximum number of channels	1
Maximum number of modules	1
Modular instrument	true
Multichannel instrument	false
Number of current ranges	9.0
Number of current ranges remarks	10 nA to 1 A
Potential and current accuracy	0.2 % and 0.2 % of current range
Potential range in volt	10
Potential resolution	3 μV (gain 100)
Weight of the instrument in kg	18

2.7 Screen-printed Gold Electrodes

In this project electrode purchased from Metrohm dropSens company have been used. Screen-printed Gold Electrodes 220AT are screen-printed with high temperature curing inks. The working electrode is 4 mm. The working and counter electrodes are made of gold, whereas the reference electrode and electric contacts are made of silver. Ideal for working with 50 μL volume Dimensions: 3.4 x 1.0 x 0.05 cm (Length x Width x Height).[33]

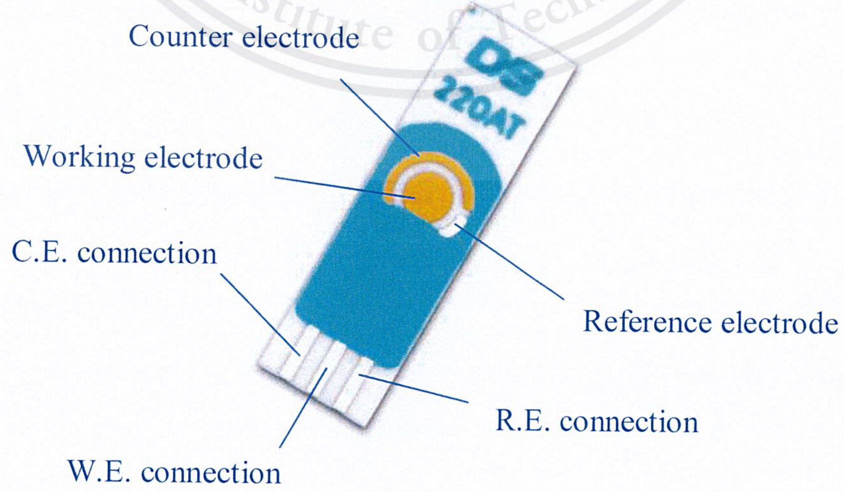

Figure 2.16 Screen-printed Gold Electrodes.

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CHAPTER III
RESEARCH METHODOLOGY

Research methodology are divided into 5 parts: Preparing the sensor, Choose the best monomer, Improve the sensitivity of the sensor, Test the sensor in urine and statics analysist.

3.1 Preparing the sensor

3.1.1 Preparing of polymer

MIP is prepared by mixing four compositions: template, monomers, cross-linking and initiator in a solvent. This pre-polymerization mixture is subjected to heat in order to initiate polymerization.

3.1.1.1 Equipment

1) Beaker 100 ml
2) Hotplate stirrer
3) Eppendorf tube 1.5 ml
4) 20-200 μl autopipette

3.1.1.2 Chemicals

1) Template: Sodium chloride (NaCl)
2) Monomer
3) Solvent: Dimethyl sulfoxide (DMSO)
4) Initiator: Azobisisobutyronitrile (AIBN)
5) Cross-linker: N,N′-(1,2-Dihydroxyethylene) bis-acrylamide
6) Distilled water

3.1.1.3 Procedure

1) Weighing 0.1 mmol NaCl in three Eppendorf tube tubes.
2) Added DMSO in 300 μl to NaCl and stirred the mixture at 25°C for 15 minutes.
3) Added varying of monomer 0.2 mmol then add 1.00 mg AIBN and 0.3 mmol DHEBA to the mixture NaCl-DMSO and stirred them at 25 °C for 15 minutes.
4) Boiling the mixture at 65-70 °C in 100 ml Beaker and observed carefully when the mixture becomes to pre-polymer state.

3.1.2 Coating polymer on the sensor

3.1.2.1 Equipment

1) sensor
2) Well-plate
3) 0.5-2 μl autopipette
4) Sonicator

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3.1.2.2 Chemicals

1) 0.15 mg/ml Graphene oxide (GO)
2) Pre-polymer (from 3.1.1)

3.1.2.3 Procedure

1) Sonicated GO for 5 minutes by sonicator.
2) Mixed the pre-polymer with GO ratio 2:3 in well-plate.
3) Dropped 1.5 μl of the mixture on active area in sensor.
4) Heated the sensor at 65 °C for 15 hr. by oven.

3.1.3 Procedure for use the sensor

3.1.3.1 Equipment

1) Beaker 100 ml
2) Potentiostat and Nova1.1.1 program.
3) 20-200 μl autopipette

3.1.3.2 Chemicals

1) 5 mM of $K_4Fe(CN)_6{\cdot}3H_2O$
2) 0.1 M of HCl
3) Sample of solution that needs to be measured

3.1.3.3 Procedure

1) Washed the coating sensor at 25 °C for 30 min. in 0.1 M HCl.
2) Test sensor by using potentiostat and Nova1.1.1 program.
3) Take coating sensor with probe to connect potentiostat.
4) Dropped 80 μl of $K_4Fe(CN)_6{\cdot}3H_2O$ analyte into sensor for referent concentration.
5) Dropped 80 μl of sample of solution that needs to be measured.
6) Observed the peak of Cyclic Voltammogram (CV) curve.

3.2 Make the Non-Imprint sensor

To compare the response of sodium ions between non-imprint template and imprint sensor with template.

3.2.1 Procedure

1) Prepare the sensor followed step 3.1.1 - 3.1.2 by choose one of monomer and do not add template to make the Non-Imprint sensor.
2) Use sample of syntactic urine at various concentrations (1, 100 mM) and followed step 3.1.3

3.3 Choose the best monomer

Test sensor by using potentiostat and Nova1.1.1 program to observe the peak of Cyclic Voltammogram (CV) curve and measure the current (I) of various Na^+ concentration to find a best monomer and Test endurance of the sensor with Synthetic Urine.

3.3.1 Condition for coated on a sensor

We have three conditions for making a MIP as shown in table 3.1-3.3

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Table 3.1 Condition 1

Name	Quantity	Structure
Methacrylic acid (MAA)	0.2 mol or 16.96 µL	
Sodium chloride (NaCl)	0.1 mol or 5.84 mg	Na — Cl
Dimethyl sulfoxide (DMSO)	300 µL	
Azobisisobutyronitrile (AIBN)	1 mg	
N,N′-(1,2-Dihydroxyethylene)bis-acrylamide (DHEBA)	0.3 mol or 60.06 mg	

Table 3.2 Condition 2

Name	Quantity	Structure
Acrylamide (AAM)	0.2 mol or 14.22 mg	
Sodium chloride (NaCl)	0.1 mol or 5.84 mg	Na — Cl
Dimethyl sulfoxide (DMSO)	300 µL	
Azobisisobutyronitrile (AIBN)	1 mg	
N,N′-(1,2-Dihydroxyethylene)bis-acrylamide (DHEBA)	0.3 mol or 60.06 mg	

Table 3.3 Condition 3

Name	Quantity	Structure
N-Vinylpyrrolidone (NVP)	0.2 mol or 21.31 µL	
Sodium chloride (NaCl)	0.1 mol or 5.84 mg	Na — Cl
Dimethyl sulfoxide (DMSO)	300 µL	
Azobisisobutyronitrile (AIBN)	1 mg	
N,N′-(1,2-Dihydroxyethylene)bis-acrylamide (DHEBA)	0.3 mol or 60.06 mg	

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3.3.2 Procedure

1) Prepare three sensors followed step 3.1.1 - 3.1.2 by use three monomers.
2) Use NaCl solution at various concentrations (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 0.01, 0.1, 1, 10 and 100 g/l) and followed step 3.1.3
3) Measure in three sensors to compare the response.

3.3.3 Test endurance of the sensor

Test endurance of the sensor to find the maximum number of used that can be accepted.

3.2.3.1 Chemicals

1) Mixture of syntactic urine consist of
   - 270 mM of Urea
   - 20mM of KCl
   - 5 mM of $K_4Fe(CN)_6 \cdot 3H_2O$

3.3.3.2 Procedure

1) Prepare the sensor followed step 3.1.1 - 3.1.2 by use the best monomer from previously step 3.2.2
2) Use NaCl solution at various concentrations (40, 130, 200 mM) and followed step 3.1.3
3) Test the sensor five time to compare the response.

3.4 Improve the sensitivity of the sensor

Urine has many chemical compositions, one of that similar to sodium is potassium. So, it important to test the sensitivity of the sensor to compare the response between sodium and potassium to improve the sensor.

3.4.1 Compare sensitivity of sensor between NaCl and KCl solution.

3.4.1.1 Procedure

1) Prepare the sensor followed step 3.1.1 - 3.1.2 by use the best monomer from step 3.2.2
2) Use NaCl solution at various concentrations (0.1, 1, 10, 100, 200 mM) and followed step 3.1.3
3) Then change NaCl to KCl solution at the same concentrations and measure in another sensor to compare the response.

3.4.2 Improve the sensitivity of sensor by add 18-Crown-6-sodium as a template

18-Crown-6 selective for sodium over than potassium to some degree[34]. Try using this additive to chelate the sodium ions in solution and have the MIP recognize this complex, not only imprint only. Add 18-Crown-6-sodium as a template of sensor to see the response of a sensor by use Potentiostat and Nova1.1.1 program.

3.4.2.1 Procedure

1) Prepare the sensor followed step 3.1.1 - 3.1.2 by use 18-Crown-6-sodium as template and use the best monomer from step 3.2.2
2) Use three solutions :18-Crown-6,Nacl , KCl at various concentrations (0.1, 1, 10, 100 mM) and followed step 3.1.3
3) Measure sensor in three solutions to compare the response.

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3.4.3 Use UV-VIS Spectrophotometer

Use UV-VIS Spectrophotometer to see the ability of absorbance between 18-Crown-6 bind with Sodium and 18-Crown-6 bind with Potassium.

3.4.3.1 Equipment

1) UV-VIS Spectrophotometer
2) Starna Spectrophotometer Cells

3.4.3.2 Chemicals

1) Dimethyl sulfoxide (DMSO)
2) 0.1, 1, 10 mM of 18-Crown-6
3) 0.1, 1, 10 mM of Sodium
4) 0.1, 1, 10 mM of Potassium

3.4.3.3 Procedure

1) Fill DMSO in two Starna Spectrophotometer Cells to measure the baseline.
2) Change the one of Starna Spectrophotometer Cells to fill a mixture of 18-Crown-6 and Sodium with ratio 1:1
3) Put two of Starna Spectrophotometer Cells in the UV-VIS Spectrophotometer.
4) Observed the absorbance and the wavelength.
5) Repeat step 2-4 by change Sodium to Potassium.

3.5 Test the sensor in urine

3.5.1 Test a sensor in syntactic urine

Test a sensor in syntactic urine before test in real urine to study whether the sensor can respond with sodium ions.

3.5.1.1 Chemicals

1) Mixture of syntactic urine consist of
   - 270 mM of Urea
   - 20mM of KCl
   - 5 mM of $K_4Fe(CN)_6 \cdot 3H_2O$

3.5.1.1 Procedure

1) Prepare three sensors followed step 3.1.1 - 3.1.2 by use the best monomer from step 3.2.2
2) Use syntactic urine at various concentrations (1, 5, 8, 12, 16, 19, 23 mM) and followed step 3.1.3
3) Repeat in other sensors to compare the response.

3.5.2 Test a sensor in real urine.

Test a sensor in real urine from 20 presumed healthy volunteers.

3.5.2.1 Procedure

1) Prepare the sensor followed step 3.1.1 - 3.1.2 by use the best monomer from step 3.2.2
2) Dilute 20 sample of real urine and followed step 3.1.3

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3.6 Statistical Data Analysis

Statistics is basically a science that involves data collection, data interpretation and finally, data validation. Statistical data analysis is a procedure for performing various statistical operations. It is a kind of quantitative research, which seeks to quantify the data, and typically, applies some form of statistical analysis. Quantitative data basically involves descriptive data, such as survey data and observational data.

3.6.1 Mean

The mean is the average of all numbers and is sometimes called the arithmetic mean. To calculate mean, add together all the numbers in a set and then divide the sum by the total count of numbers.

$$\bar{x} = \frac{\sum x_i}{n} \tag{3.1}$$

3.6.2 Standard Deviation

The standard deviation (SD) is a measure that is used to quantify the amount of variation or dispersion of a set of data values. A low standard deviation indicates that the data points tend to be close to the mean (also called the expected value) of the set, while a high standard deviation indicates that the data points are spread out over a wider range of values.

$$S.D. = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \tag{3.2}$$

3.6.3 Least-squares method

In statistics, linear regression is a linear approach to modeling the relationship between a scalar response (or dependent variable) and one or more explanatory variables (or independent variables). The case of one explanatory variable is called a simple linear regression. For more than one explanatory variable, the process is called multiple linear regression.

$$y_i = c + mx_i + e \quad \text{,Where} \tag{3.3}$$

m : slope
c : Intersection in the y axis
e : the error from the experiment

3.6.4 Coefficient of determination

The coefficient of determination (denoted by R^2) is a key output of regression analysis. It is interpreted as the proportion of the variance in the dependent variable that is predictable from the independent variable. The coefficient of determination is the square of the correlation (r) between predicted y scores and actual y scores; thus, it ranges from 0 to 1. With linear regression, the coefficient of determination is also equal to the square of the correlation between x and y scores.

- **An R^2 of 0** means that the dependent variable cannot be predicted from the independent variable.

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- **An R^2 of 1** means the dependent variable can be predicted without error from the independent variable.

- **An R^2 between 0 and 1** indicates the extent to which the dependent variable is predictable. An R2 of 0.10 means that 10 percent of the variance in Y is predictable from X; an R^2 of 0.20 means that 20 percent is predictable; and so on.

$$R, r = \frac{\sum[(x_i-\bar{x})(y_i-\bar{y})]}{\sqrt{[\sum(x_i-\bar{x})^2][\sum(y_i-\bar{y})^2]}} = \frac{n(\sum x_i y_i) - (\sum x_i)(\sum y_i)}{\sqrt{[n\sum x_i^2 - \sum(x_i)^2][n\sum y_i^2 - \sum(y_i)^2]}} \quad (3.4)$$

R, r : Correlation coefficient

So, R^2 (Coefficient of determination) = (Correlation coefficient)^2 $0 \leq R^2 \leq 1$

3.6.5 Coefficient of Variation

The coefficient of variation (CV), also known as relative standard deviation (RSD), is a standardized measure of dispersion of a probability distribution or frequency distribution. It is often expressed as a percentage and is defined as the ratio of the standard deviation σ to the mean μ (or its absolute value |μ|. The CV or RSD is widely used in analytical chemistry to express the precision and repeatability of an assay. It is also commonly used in fields such as engineering or physics when doing quality assurance studies and ANOVA gauge R&R. In addition, CV is utilized by economists and investors in economic models and in determining the volatility of a security.

$$c_v = \frac{\sigma}{\mu} \quad (3.5)$$

3.6.6 Normal distribution

The normal distribution is the most important probability distribution in statistics because it fits many natural phenomena. For example, heights, blood pressure, measurement error, and IQ scores follow the normal distribution. It is also known as the Gaussian distribution and the bell curve.

The normal distribution is a probability function that describes how the values of a variable are distributed. It is a symmetric distribution where most of the observations cluster around the central peak and the probabilities for values further away from the mean taper off equally in both directions. Extreme values in both tails of the distribution are similarly unlikely.

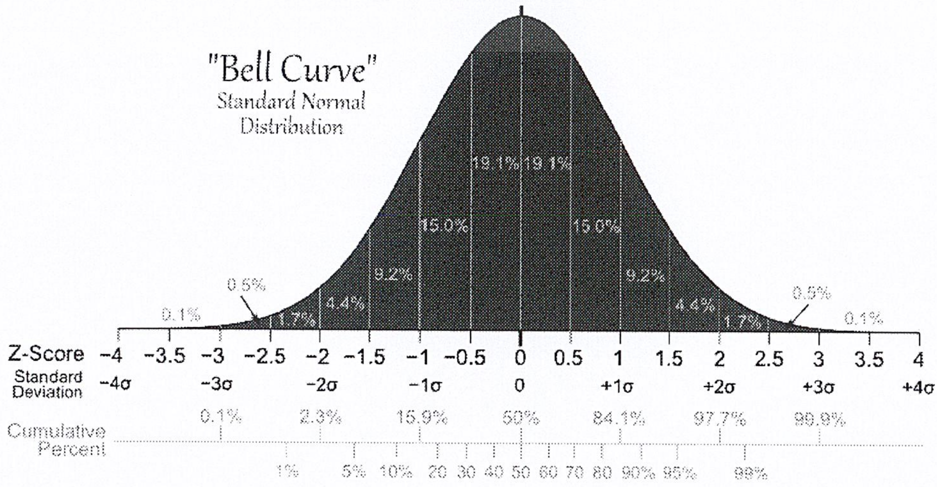

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Figure 3.1 Bell Curve Standard Normal Distribution.

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3.6.7 Kruskal Wallis test

The Kruskal Wallis test is the nonparametric alternative to the One-Way ANOVA. Nonparametric means that the test doesn't assume your data comes from a distribution. The H test is used when the assumptions for ANOVA are not met (like the assumption of normality). It is sometimes called the one-way ANOVA on ranks, as the ranks of the data values are used in the test rather than the actual data points.

3.6.7.1 Method

1) Rank all data from all groups together; i.e., rank the data from 1 to N ignoring group membership. Assign any tied values the average of the ranks they would have received had they not been tied.

2) The test statistic is given by:

$$H = (N-1)\frac{\sum_{i=1}^{g} n_i(\bar{r}_i-\bar{r})^2}{\sum_{i=1}^{g}\sum_{j=1}^{n_i}(r_{ij}-\bar{r})^2} \quad \text{,where} \tag{3.6}$$

n_i is the number of observations in group i

r_{ij} is the rank (among all observations) of observation j from group i

N is the total number of observations across all groups

$\bar{r}_i = \sum_{j=1}^{n_i}\frac{r_{ij}}{n_i}$ is the average rank of all observations in group i

$\bar{r}_i = \frac{1}{2}(N+1)$ is the average of all the r_{ij}

3) If the data contain no ties the denominator of the expression for H is exactly

$$H = \frac{12}{N(N+1)}\sum_{i=1}^{g} n_i\bar{r}_i^2 - 3(N+1) \tag{3.7}$$

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CHAPTER IV
RESULTS

4.1 Comparing between non-imprinted and imprinted methods

To compare the current(I) value in the non-imprinted method at the concentration of sodium chloride solution in 1 and 100 mM. From figure 4.1 which show the result when increasing the concentration of sodium chloride solution, there is very little change in electricity. But when consider figure 4.2 which show the comparing between non-imprinted method and imprinted method at 100mM of NaCl concentration, the imprinted method is the response of sodium current more than the non-imprinted method.

Therefore, it can be concluded that the imprinted method is specific to sodium ions because the imprinted method has the binding site on a surface of sensor that allow sodium ions can bind the hole and change the current of electricity.

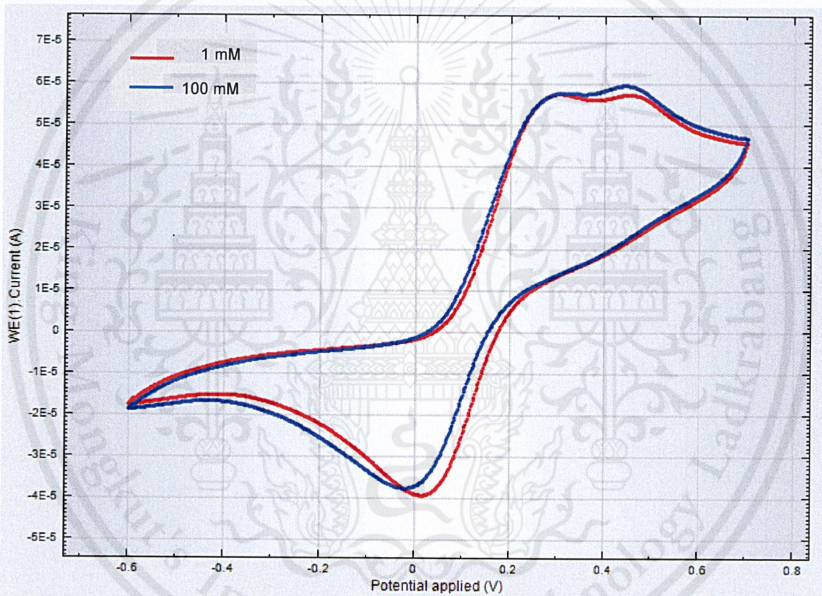

Figure 4.1 Cyclic Voltammogram (CV) graph of the non-imprinted method.

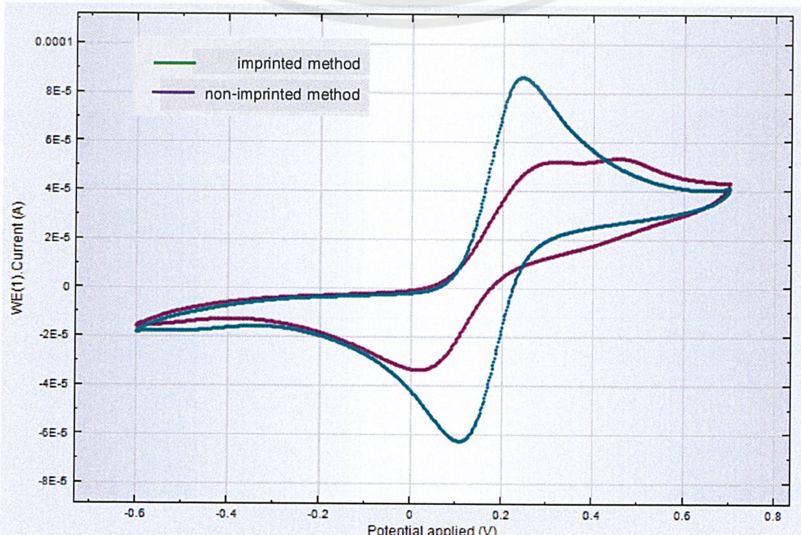

Figure 4.2 CV graph between non-imprinted and imprinted method at 100mM of NaCl concentration.

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4.2 Choose the best monomer

4.2.1 The result of Testing in three monomers

The current(I) obtain various NaCl concentration in three monomers that are Methacrylic acid (MAA), Acrylamide (AAM) and N-Vinylpyrrolidone (NVP). The aim is to find the best monomer. Table 4.1 shows the current (I) of various NaCl concentration in three monomers by increase the concentration 10 times from original concentration. The current in the three monomers is increased when increased the NaCl concentration. This leads to conclude that the increase in sodium concentration is in direct relation to the electric current, but when consider figure 4.3 that shows the relationship between ΔI and log concentration are non-linear curve(S-curve). Concentrations at -7 to -2 are the lower limit of detection, and at -1 to 1 show an ideal linear range and more than 2 is limit of linearity. In this analysis, we used -1 to 1 as the range in consideration.

Table 4.1 The current(I) of various NaCl concentration in three monomers.

NaCl conc. (g/L)	NaCl conc. (mol/L)	Log (conc.)	MAA		AAM		NVP	
			I (μA)	ΔI (μA)	I (μA)	ΔI (μA)	I (μA)	ΔI (μA)
0	0	0	8.65	0.00	6.33	0.00	12.22	0.00
0.000001	0.0000000171	-6.00	19.92	11.27	16.57	10.24	47.49	35.27
0.00001	0.000000171	-5.00	25.48	16.83	29.60	23.27	55.89	43.67
0.0001	0.00000171	-4.00	26.93	18.28	30.57	24.24	46.23	34.01
0.001	0.0000171	-3.00	28.25	19.60	25.40	19.07	48.02	35.80
0.01	0.000171	-2.00	28.54	19.89	24.84	18.51	52.58	40.36
0.1	0.00171	-1.00	29.52	20.87	24.95	18.62	48.09	35.87
1	0.0171	0.00	46.99	38.34	38.29	31.96	70.22	58.00
10	0.171	1.00	66.40	57.75	72.47	66.14	78.12	65.90
100	1.71	2.00	69.34	60.69	73.50	67.17	80.34	68.12

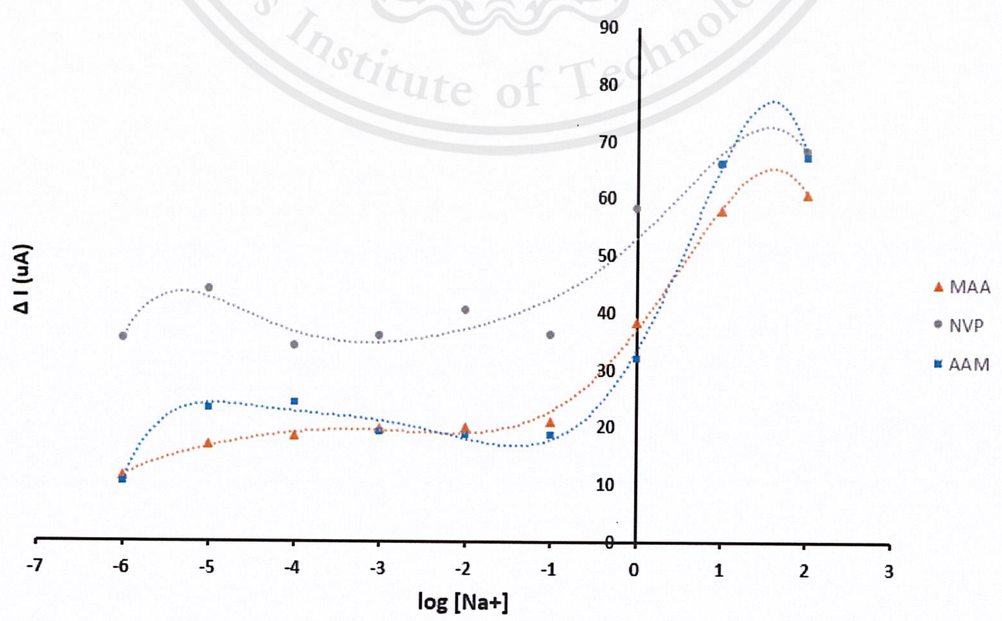

Figure 4.3 S-curve of various NaCl concentration in three monomers.

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Table 4.2 which shows the results of repeated tests of sodium concentration at 0.1-10 g/l in three monomers. As the result, the average difference current at 0.1 g/l of MAA, AAM, and NVP are 13.46, 13.25 and 18.36 μA. The average difference current at 1 g/l of MAA, AAM and NVP are 29.72, 28.45 and 32.26 μA. The average difference current at 10 g/l of MAA, AAM and NVP are 49.15, 59.06 and 56.26 μA. From observed figure 4.4, the r-square of the MAA, AAM and NVP are 0.9974, 0.9636 and 0.9769. It was obviously that when test the electrode more than one time, the electrical current was reduced. But also, the linearity.

Table 4.2 Repeated tests of sodium concentration at 0.1-10 g/l in three monomers.

Monomer	NaCl conc. (g/L)	Log Conc.	ΔI (μA)					Y = mx + C	
			1st	2nd	3rd	$\bar{X}$	*S.D.*	*m*	R^2
MAA	0.1	-1	20.87	14.16	5.34	13.46	7.79		
	1	0	38.34	33.05	17.78	29.72	10.68	17.845	0.9974
	10	1	57.75	47.04	42.65	49.15	7.77		
AAM	0.1	-1	18.62	15.46	5.66	13.25	6.76		
	1	0	31.96	29.67	23.71	28.45	4.26	22.905	0.9637
	10	1	66.14	59.37	51.68	59.06	7.24		
NVP	0.1	-1	35.87	14.01	5.19	18.36	15.80		
	1	0	48.14	25.36	23.28	32.26	13.79	18.950	0.9769
	10	1	65.90	56.45	46.42	56.26	9.74		

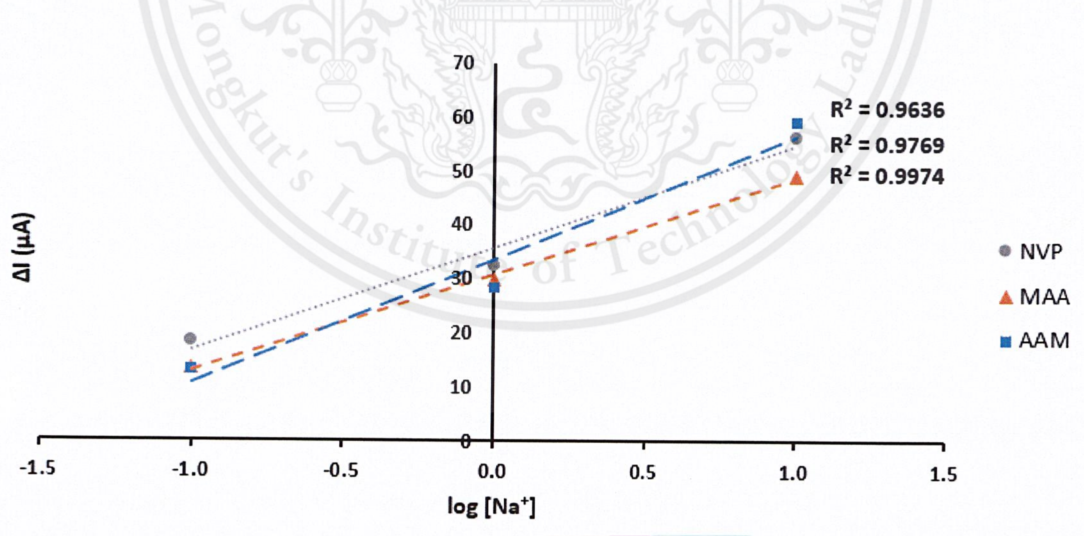

Figure 4.4 Linear graph of three monomer.

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We have performed statistical analysis of the data using the Kruskal-Wallis test. Table 4.3, compares the H and critical value from the x^2 distribution table ($P<0.05$) it was found that there is not a statistically significant difference between three concentrations of NaCl in each monomer ($P<0.05$). So, we need to use other methods to determine the best monomer.

Table 4.3 The comparing between H and critical value from the X^2 distribution table.

Na^+ conc.	*Sum of Rank*			*df*	*H value*	$\alpha = 0.05$
	MAA	AAM	NVP			*Critical value*(X^2)
0.1	15	16	14	3	0.089	5.991
1	16	14	15	3	0.089	5.991
10	10	20	15	3	2.222	5.991

Therefore, if consider the value of R^2, MAA has the highest R^2 (0.9974), the next one is NVP (0.9769) and the last one is AAM (0.9636). In the experiment, NVP usually became solid-state polymer when leaving it around 2 days. we need to repeat it to make a new gel and the system cannot be controlled. The best monomer that has highest R^2 and least of the problem is MAA.

4.2.2 Test endurance of sensor

The endurance was accessed by the current(I) of various Na^+ concentration in NaCl solution. The aim is to test performance of electrode that can be detect sodium ion in solution. Table 4.4 shows the current (I) of various Na^+ concentration by testing at 40, 130 and 200 mM that represent wide range concentration in 5 times. The difference current (ΔI) of 1st to 5th test at 40 mM are 17.54, 15.47, 14.98, 11.77 and 12.20 µA, the next one at 130 mM are 27.54, 25.19, 25.70, 20.63 and 19.48 µA. The last measurements at 200 mM are 37.52, 32.23, 30.06, 25.66 and 25.22 µA. From figure 4.5, It was obviously that when test the electrode more than one time, the electrical current was reduced. But also, the linearity.

Table 4.4 The current (I) of various Na^+ concentration in synthetic urine by testing at 40, 130 and 200 mM in 5 times.

Number of testing	**NaCl conc.**			**y = mx + C**	
	40	**130**	**200**	**m**	$\mathbf{R^2}$
1st	17.54	27.54	37.52	0.1242	0.9949
2nd	15.47	25.19	32.23	0.1049	0.9996
3rd	14.98	25.70	30.06	0.0954	0.9724
4th	11.77	20.63	25.66	0.0874	0.9927
5th	12.20	19.48	25.22	0.0814	1.0000

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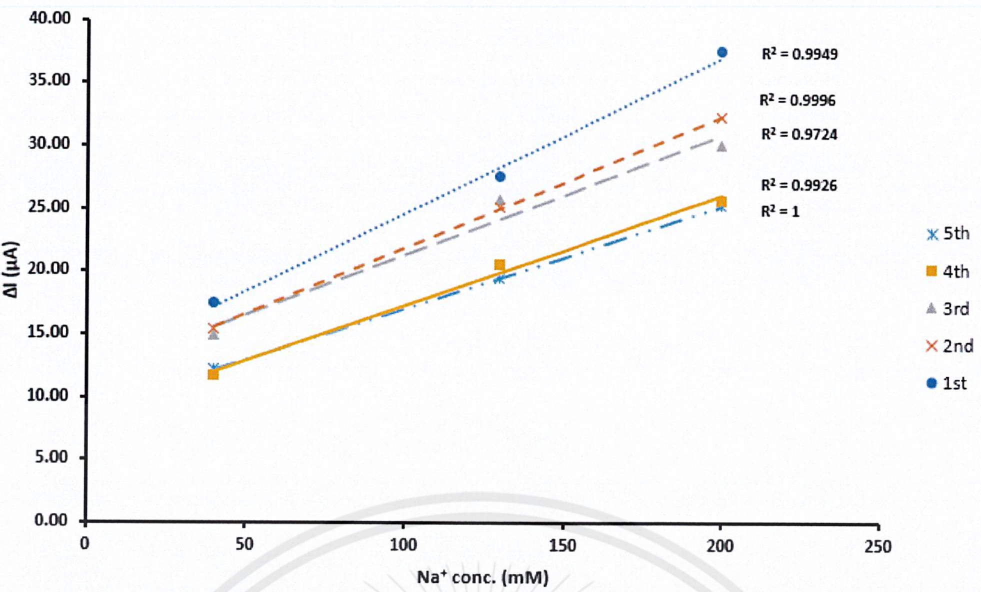

Figure 4.5 The current (I) decreases according to the number of testing.

Table 4.5 shows the average value ($\bar{X}$), the standard deviation (S.D.) and the coefficient of variation (CV) of the difference current (ΔI) in 3, 4 and 5 times of testing for determining the number of the sensor using. A coefficient of variation (CV) of three times is within the acceptable range (% CV <10).

Table 4.5 The average value ($\bar{X}$), the standard deviation (S.D.) and the coefficient of variation (CV) of the difference current (ΔI) in 3,4 and 5 times of testing.

NaCl conc. (mM)	ΔI (μA)					$\bar{X}_3$	$\bar{X}_4$	$\bar{X}_5$	$S.D._3$	$S.D._4$	$S.D._5$	CV_3	CV_4	CV_5
	1st	2nd	3rd	4th	5th									
40	17.54	15.47	14.98	11.77	12.20	16.00	14.94	14.39	1.11	2.07	2.15	6.92	13.84	14.93
130	27.54	25.19	25.70	20.63	19.48	26.14	24.77	23.71	1.01	2.54	3.10	3.87	10.27	13.10
200	35.52	32.23	30.06	25.66	25.22	32.60	30.87	29.74	2.24	3.58	3.92	6.88	11.60	13.18

4.3 Improve sensitivity of sensor

4.3.1 Comparison of NaCl and KCl

The measured current in KCl and NaCl solution were 0.1, 1,10,100,200 mM. The aim is to test the sensitivity of the sensor with other ions that present in the urine. From table 4.6, found that the current(I) value in NaCl less than KCl solution in all concentrations. So, the sensor not only sensitive with sodium ions but also sensitive in potassium ions too. This might be the effect of the preparation of a polymer gel that uses high concentrations of sodium and the physical properties of sodium ions and potassium ions are similar.

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Table 4.6 The current(I) obtained in NaCl and KCl solution.

Solution conc. (mmol/L)	Log (Solution conc.)	NaCl		KCl	
		I (μA)	ΔI (μA)	I (μA)	ΔI (μA)
0	-	4.45	0.00	4.21	0.00
0.1	-1	9.79	5.34	15.18	10.97
1	0	22.23	17.78	22.55	18.34
10	1	47.10	42.65	50.60	46.39
100	2	57.32	52.87	61.32	57.11
200	2.30103	46.11	41.66	59.38	55.17

4.3.2 Efficiency of 18-Crown-6-sodium

The current(I) in 18-Crown-6, KCl and NaCl solution was measured at 0.1, 1, 10, 100, 200 mM. The purpose is to test the sensitivity of the sensor that imprinted 18-Crown-6-sodium with other ions in the urine. From table 4.7, when test in 18-Crown-6 solution, there is no significant increase or decrease concentration. we determined that the sensors develop a greater current(I) for sodium ions compared to potassium but has concentration dependence was very poor (r>0). We therefore decided to employ the previous conditions despite the suboptimal response to potassium ions, because in real urine the amount of potassium ions is considerably less than sodium ions (~10%).

Table 4.7 The current(I) value in 18-Crown-6, KCl and NaCl solution.

Solution conc. (mMol/L)	Log (Solution conc.)	18-Crown-6		NaCl		KCl	
		I (μA)	ΔI (μA)	I (μA)	ΔI (μA)	I (μA)	ΔI (μA)
0	-	6.66	0.00	9.63	0.00	12.74	0.00
0.1	-1	9.42	2.76	15.23	5.59	12.59	-0.15
1	0	10.93	4.28	14.04	4.41	14.06	1.32
10	1	0.99	-5.67	13.95	4.32	15.13	2.40

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4.3.3 Use UV-VIS Spectrophotometer

Use UV-VIS Spectrophotometer to see the ability of absorbance between 18-Crown-6 bind with Sodium and 18-Crown-6 bind with Potassium. From Figure 4.6 found that the absorbance value in NaCl(green) less than KCl(blue) solution in all concentrations.

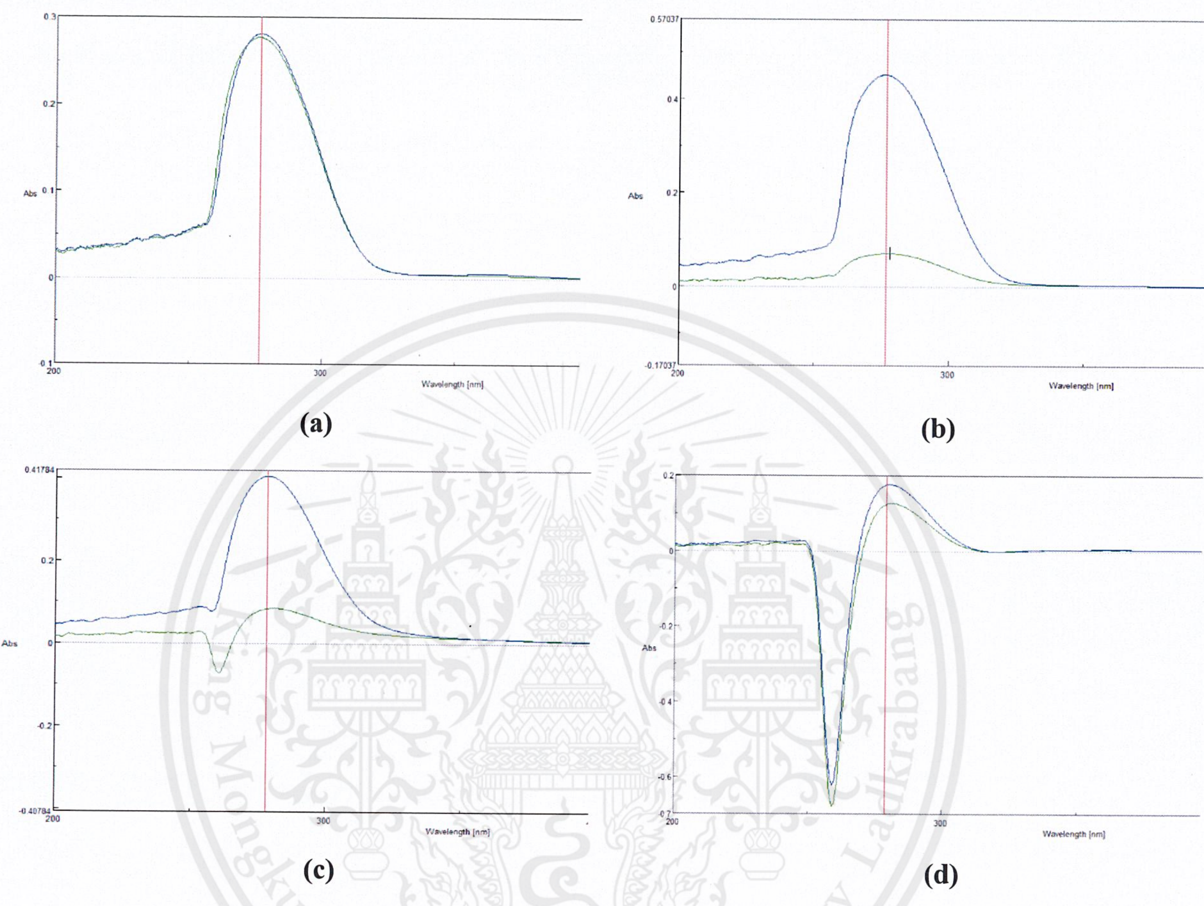

Figure 4.6 The UV-VIS graph shows the ability of absorbance between 18-Crown-6 bind with Sodium and bind with Potassium, at **(a)**0.01mM, **(b)**0.1mM, **(c)**1mM, **(d)**10mM.

4.4 Test sensor in urine

4.4.1 Efficiency of sensor in synthetic urine

The current(I) of various Na^+ concentration in synthetic urine that have NaCl, 20 mM KCl, 270 mM Urea, 5 mM $K_4Fe(CN)_6.3H_2O$. The aim is to test performance of electrode to detect sodium ion in mixing solution. From table 4.8, increasing of current(I) value when increased the sodium concentration. A plot the relationship between the current value and the concentration of sodium solution (figure 4.7) showed a linear relationship(r~1). Therefore, we were able to create a mathematical equation to calculate the sodium ion concentration from the measured current value, that is

$$\hat{Y} = 1.0179x + 16.439 \qquad (4.1)$$

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Table 4.8 Shows the increasing of current(I) value when increased the sodium concentration.

NaCl conc.	ΔI (μA)			Value
	1st	2nd	3rd	
1	12.27	20.77	13.56	15.53±4.58
5	22.27	21.46	21.26	21.67±0.53
8	30.27	24.86	23.73	26.29±3.50
12	34.89	27.51	29.68	30.69±3.79
16	35.99	26.11	33.98	32.03±5.22
19	39.10	28.76	37.08	34.98±5.48
23	42.47	36.71	38.99	39.39±2.90

*value = mean ± standard deviation

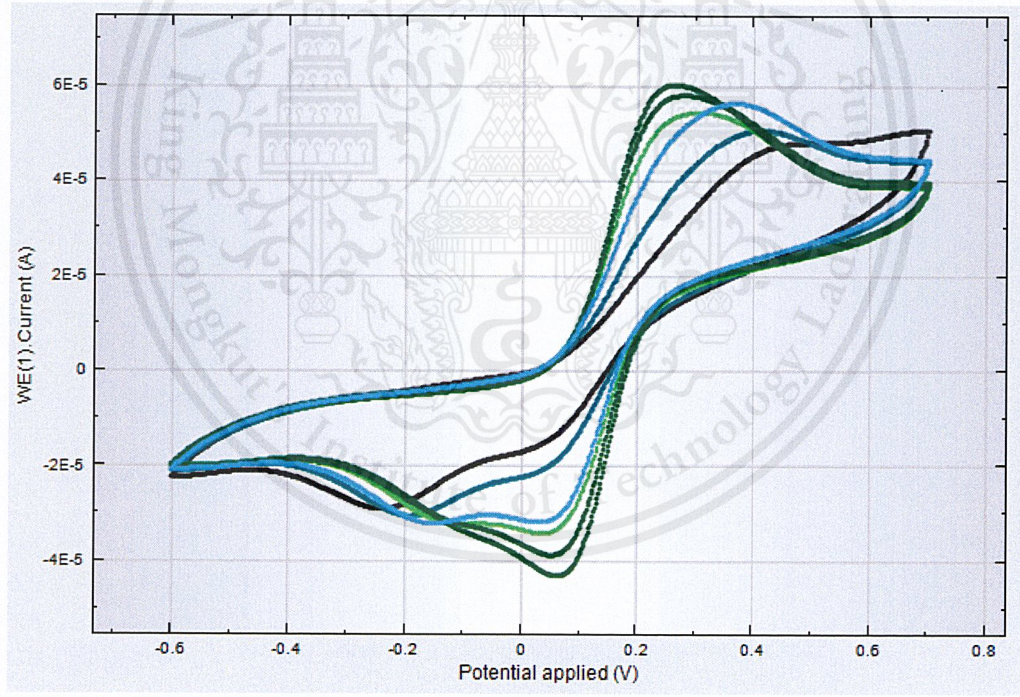

Figure 4.7 CV graph show the increasing of current(I) value when increased the sodium concentration in synthetic urine.

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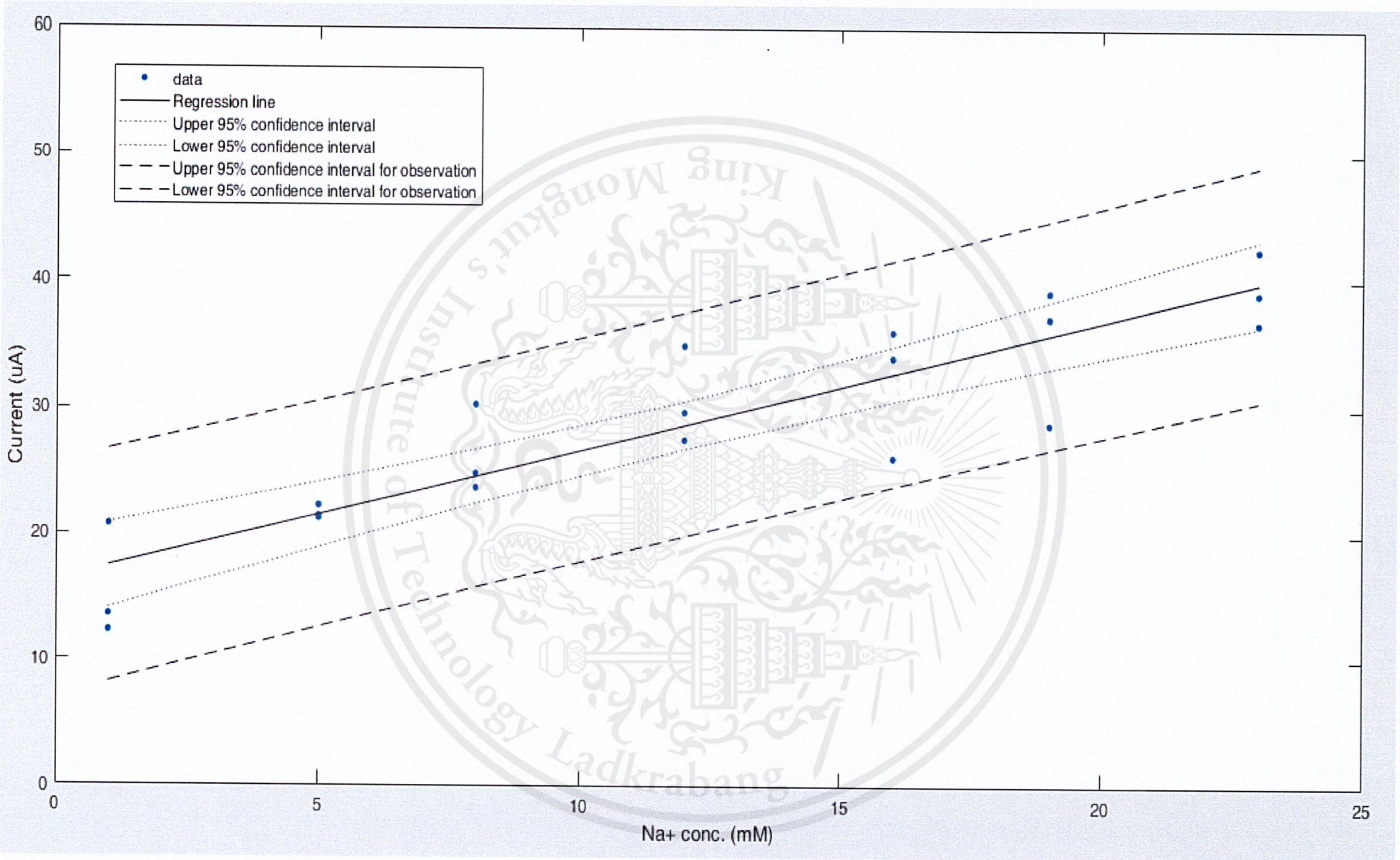

Figure 4.8 Regression line of the current(I) value in synthetic urine.

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4.4.2 Efficiency of sensor in real urine

4.4.2.1 Comparison between 10% real urine and 100% real urine

The current(I) in 10% real urine and 100% real urine. The aim is to test the response of sensor in real urine. Figure 4.9 shows the 100% of real urine graph has two oxidation peaks while 10% of real urine has one oxidation peak, that similar when tested in synthetic urine. We can conclude that 2nd peak in 100% of real urine is not peak response of sodium, but they are interference in real urine. From figure 4.10 the increasing of current(I) value when increased the sodium concentration (1, 5, 8, 12, 16, 19 and 23 mM) in 10% of real urine.

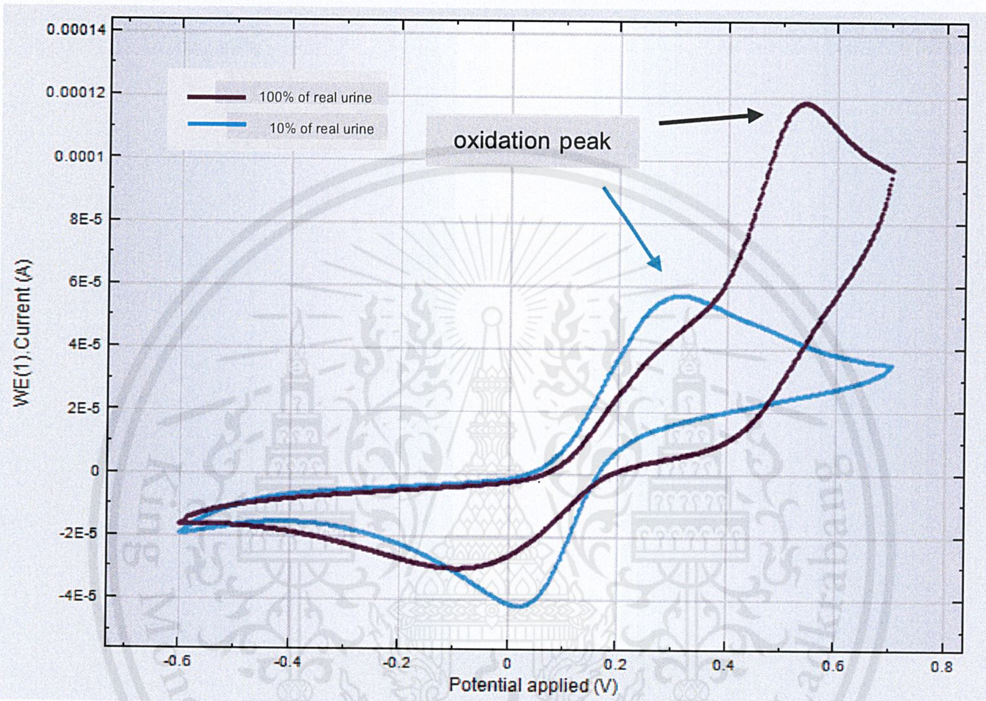

Figure 4.9 CV graph show oxidation peak in 10% and 100% real urine.

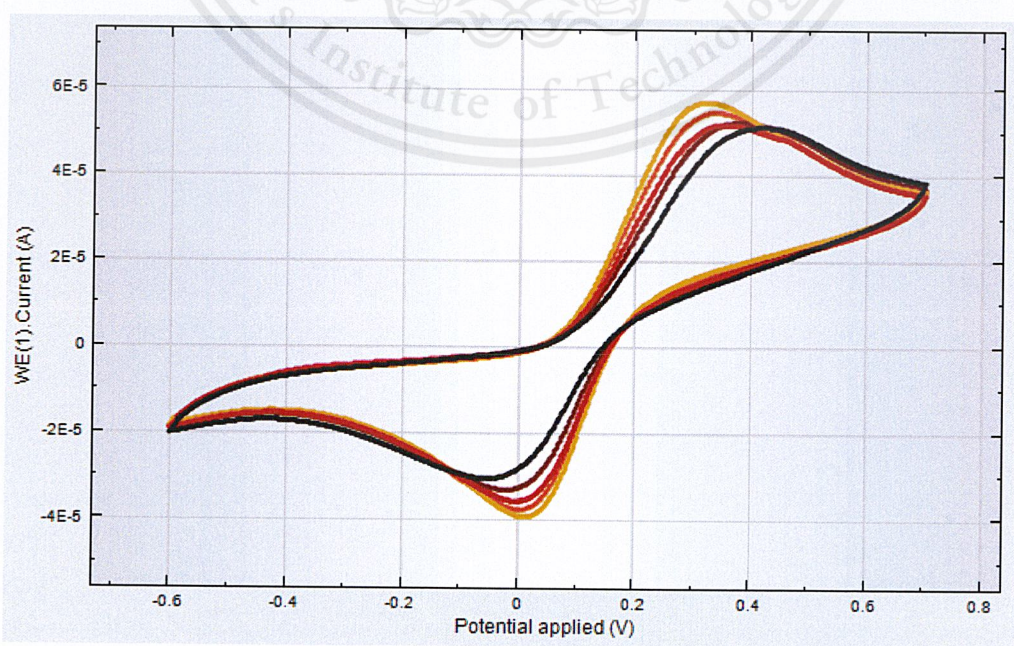

Figure 4.10 CV graph show the increasing of current(I) value when increased the sodium concentration in 10% real urine.

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4.4.2.2 Experiment of real urine in 20 presumed healthy volunteers

The current (I) of 10% a real urine in 20 presumed healthy volunteers and used to calculate the concentration of sodium in urine from (4.1) equation. The aim is to test the sensor in real urine. figure 4.11 which show data distribution of sodium concentration of twenty presumed healthy volunteers that have the cut-off range to be less than 95% confident interval, because the amount of data used is small (N<50) the resulting in a normal distribution that does not meet the hypothesis.

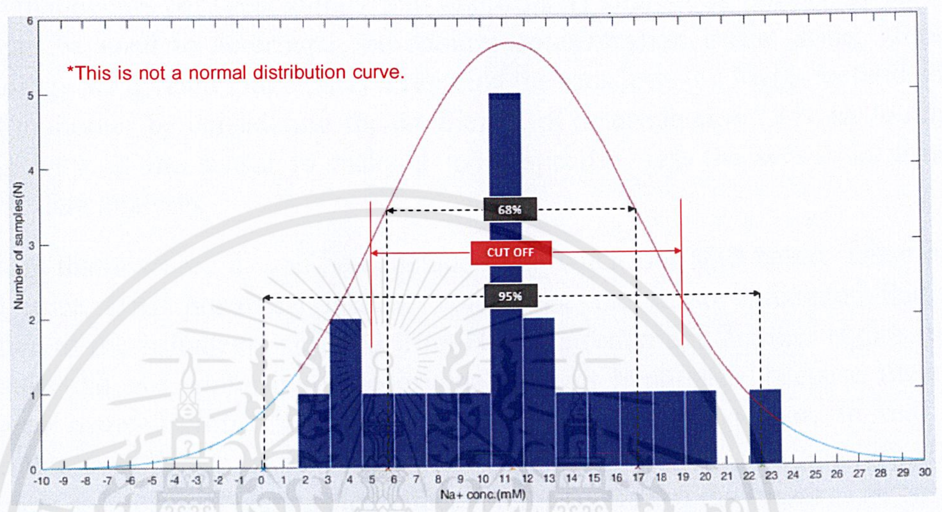

Figure 4.11 Graph show the data distribution of sodium concentration of 20 presumed healthy volunteers.

Figure 4.12 shows the current(I) obtained from a random 10% real urine in 3 presumed healthy volunteers that represent of hyponatremia(Na$^+$ conc. $<$ 5 mM), normal (5 mM $\leq$ Na$^+$ conc. $\leq$ 19 mM) and hypernatremia(Na$^+$ conc. $>$ 19 mM) and compare with synthetic urine. The current(I) from real samples is unexpectedly reduced compared to the calibration curve from synthetic urine. This results in the concentration derived from the calculation showing much greater variability. As the result of real urine have many interferences that effect to the measured current.

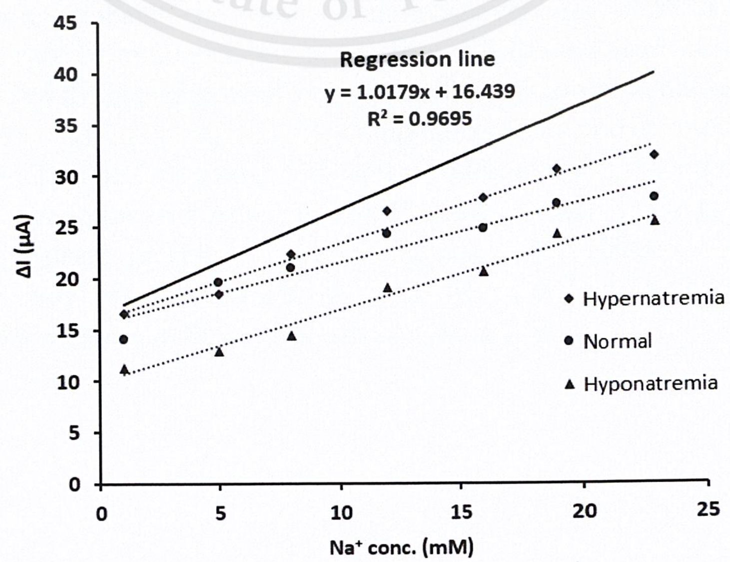

Figure 4.12 Comparing between synthetic urine and three conditions of real urine.

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3) When finish doubleclick your data then Add windower > Scan > Select 2

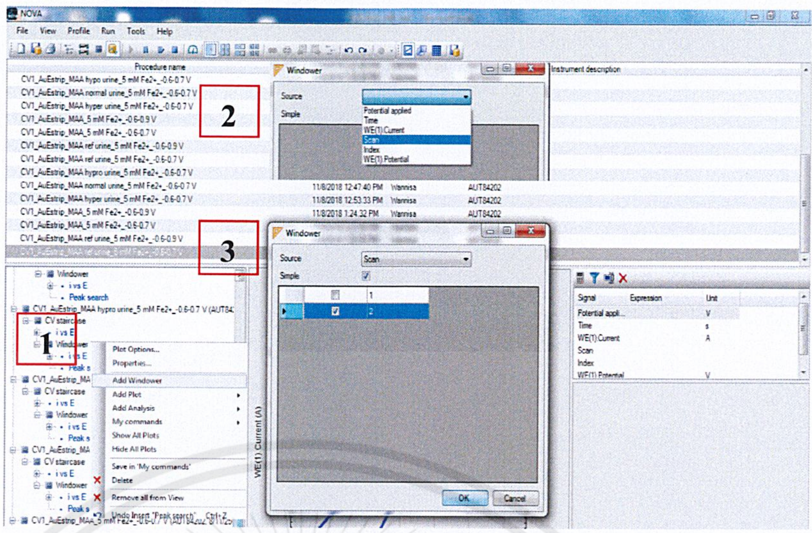

Figure A.3 How to Set widower.

4) Analysis data and find peak by doubleclick your data >Add Plot > i vs E then doubleclick your data again >Add Analysis Peak search.

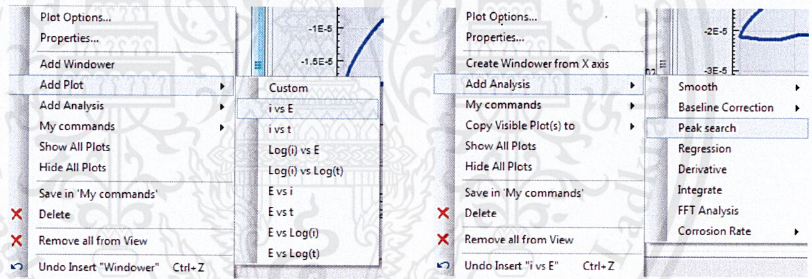

Figure A.4 Analysis data and find peak.

5) Peak position and peak height are in the right side. You can Search peak by use automatic or manual.

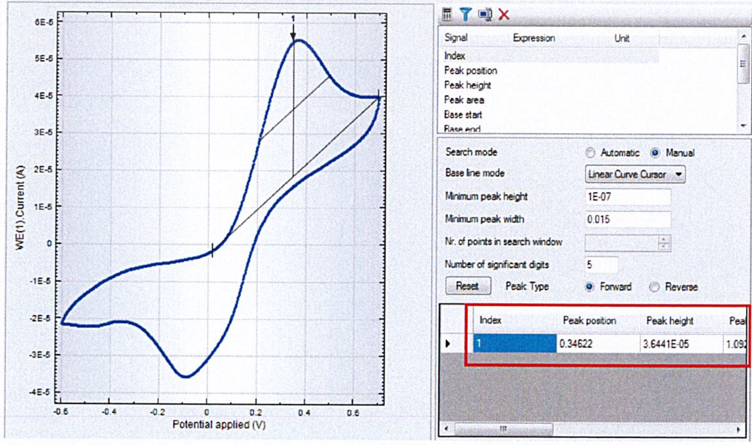

Figure A.5 Peak position and peak height.

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APPENDIX B
SAMPLE OF CALCULATION

B.1 Kruskal Wallis test

The Kruskal Wallis test is used for comparing three groups of monomers that have equal samples size for finding the best monomer.

Example: Following the table B.1 which show the measured current in 3 times of testing at 0.1 mM of sodium chloride in three monomers. Is there is a difference in measured current on the three different monomers at $\sigma = 0.05$?

Table B.1 show the measured current in 3 times of testing at 0.1 mM of sodium chloride in three monomers.

n	MAA	AAM	NVP
1	20.87	18.62	35.87
2	14.16	15.46	14.01
3	5.34	5.66	5.19

Solution

1) First, state two mutually exclusive and exhaustive hypotheses with regard to group medians:

(a) H_0: MAA = AAM = NVP
(b) H_1: MAA ≠ AAM ≠ NVP

2) All the data needs to be put together in one column as shown below:

Table B.2 show the measured current in one column.

Sample	Value
1	20.87
1	14.16
1	5.34
2	18.62
2	15.46
2	5.66
3	35.87
3	14.01
3	5.19

3) The data needs to be organized in ascending order by value (keeping track of what sample the values belongs to). The results are shown below:

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APPENDIX C
EQUIPMENT USED IN THE EXPERIMENT

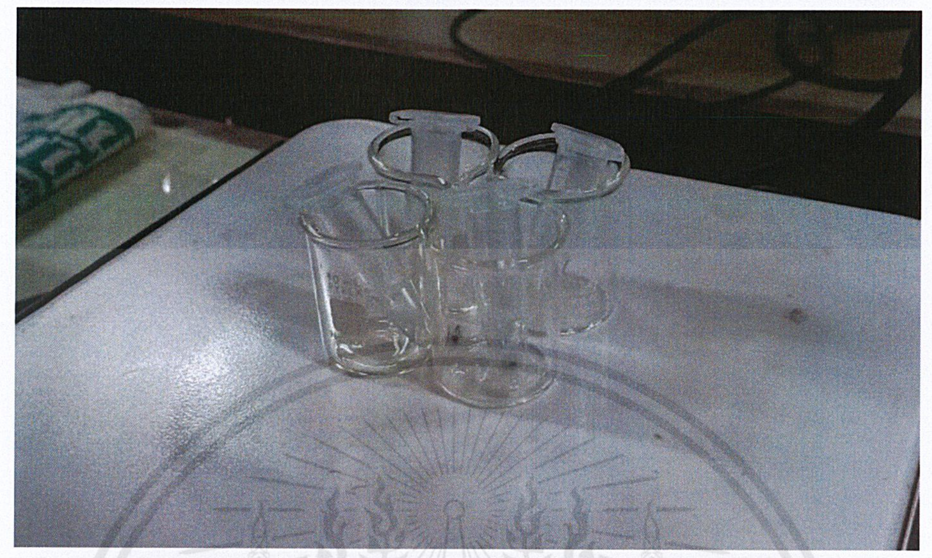

Figure C.1 Stirred them at 25 ºC for 15 minutes.

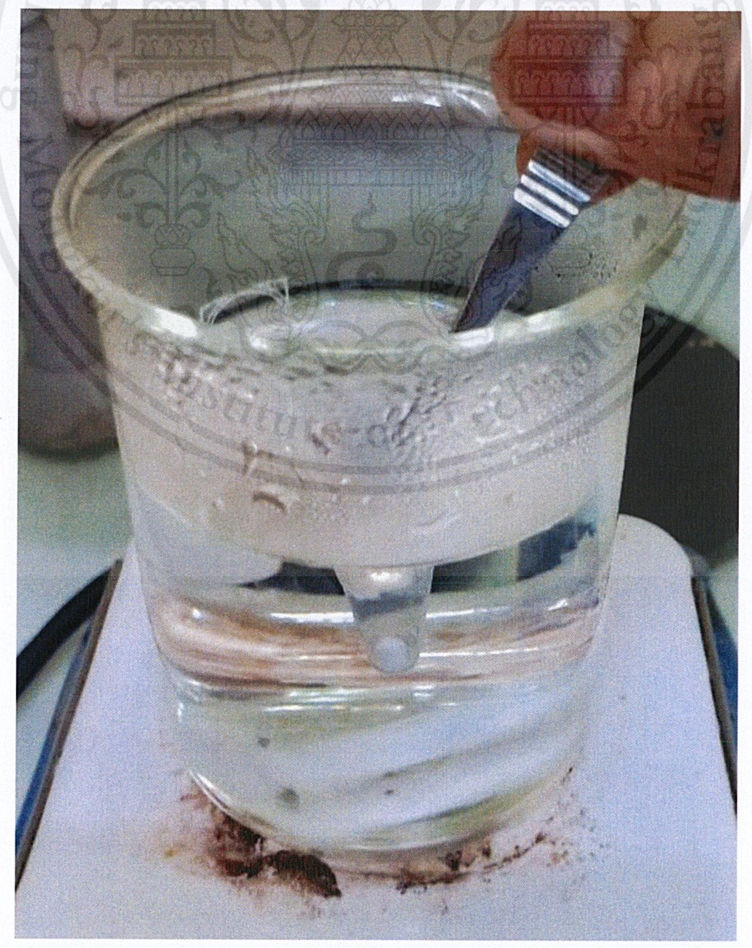

Figure C.2 Boiling the mixture at 65-70 ºC in 100 ml Beaker.

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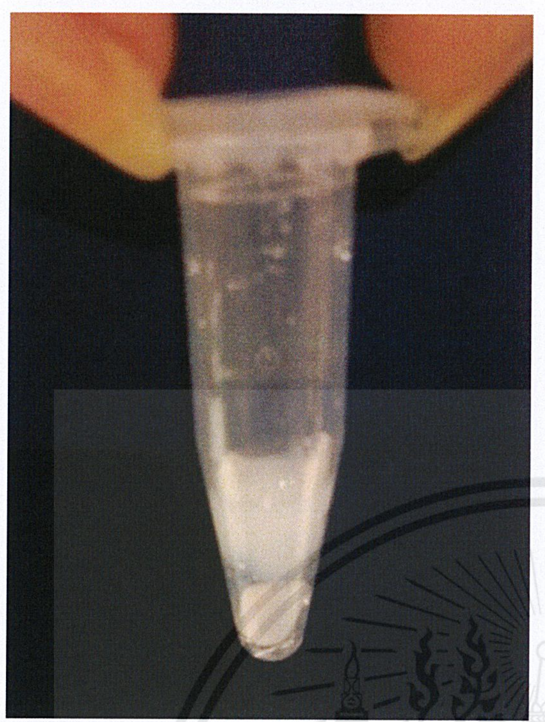
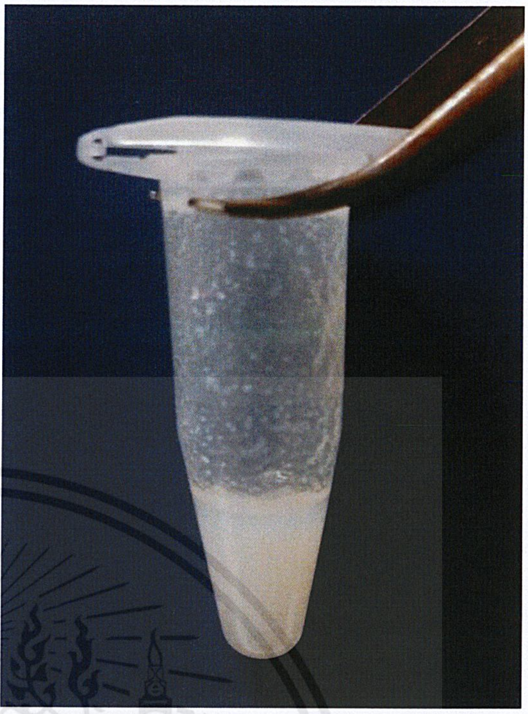

Figure C.3 Solid-stage of polymer gel.

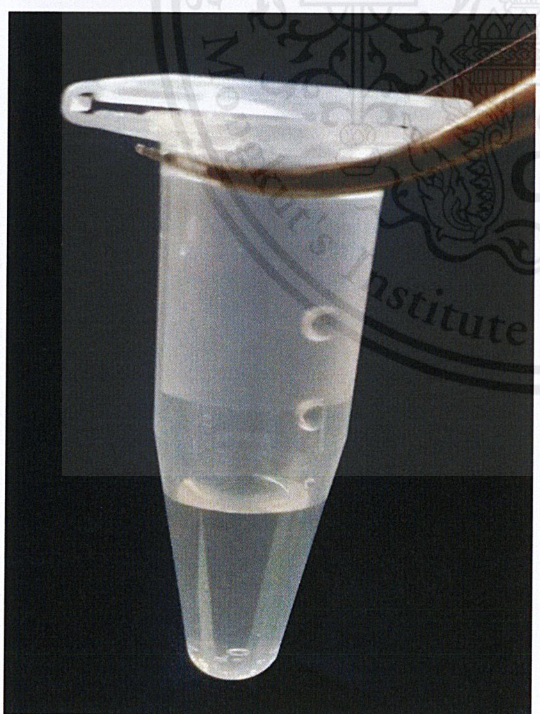
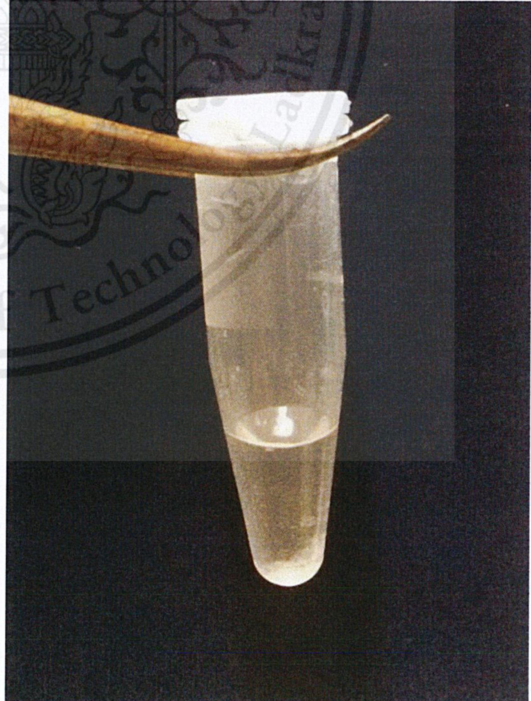

Figure C.4 Pre-polymer gel stage.

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Figure C.5 Prepare for coating polymer on the sensor

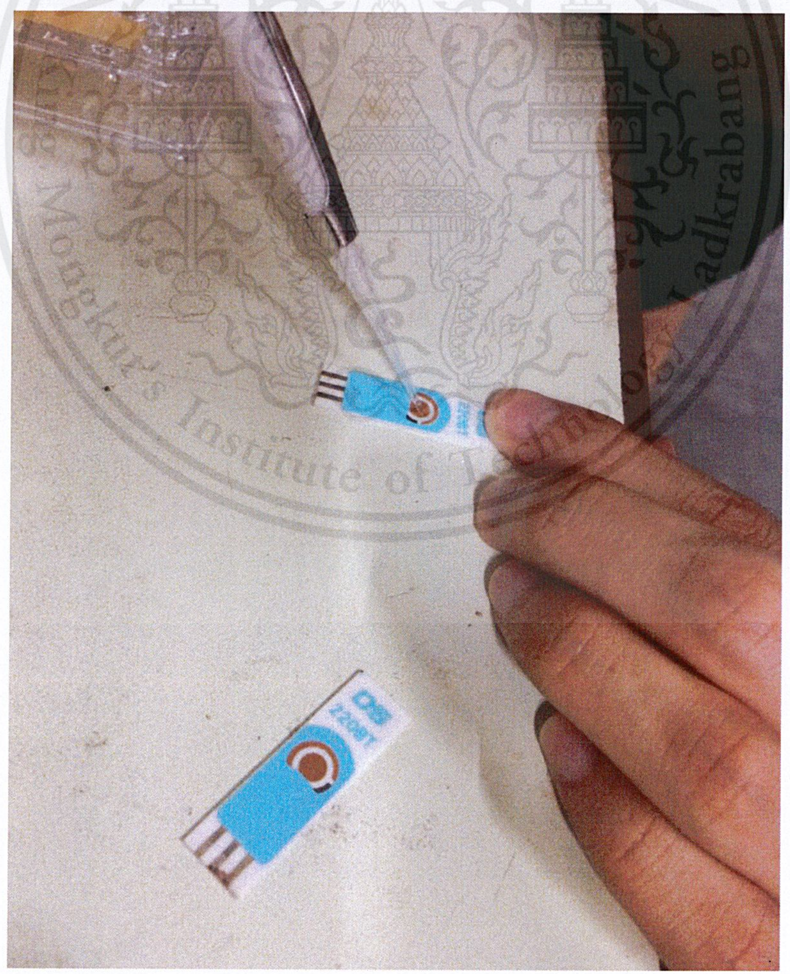

Figure C.6 Dropped 1.5 µl of the mixture on active area in sensor.

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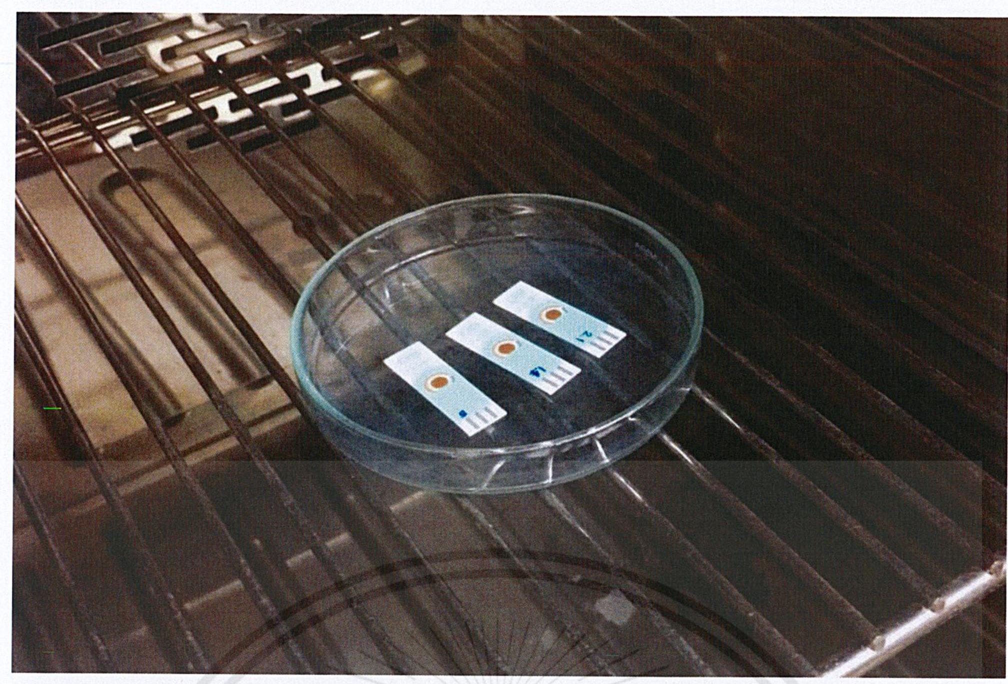

Figure C.7 Heated the sensor at 65 °C for 15 hr. by oven.

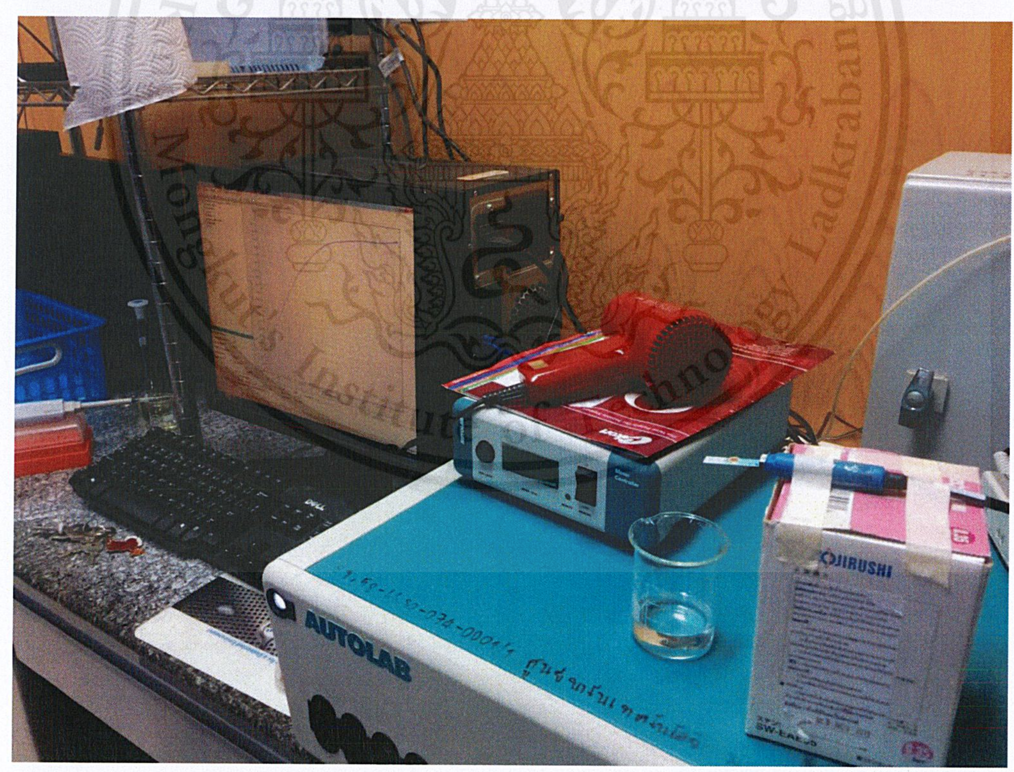

Figure C.8 Procedure for use the sensor.

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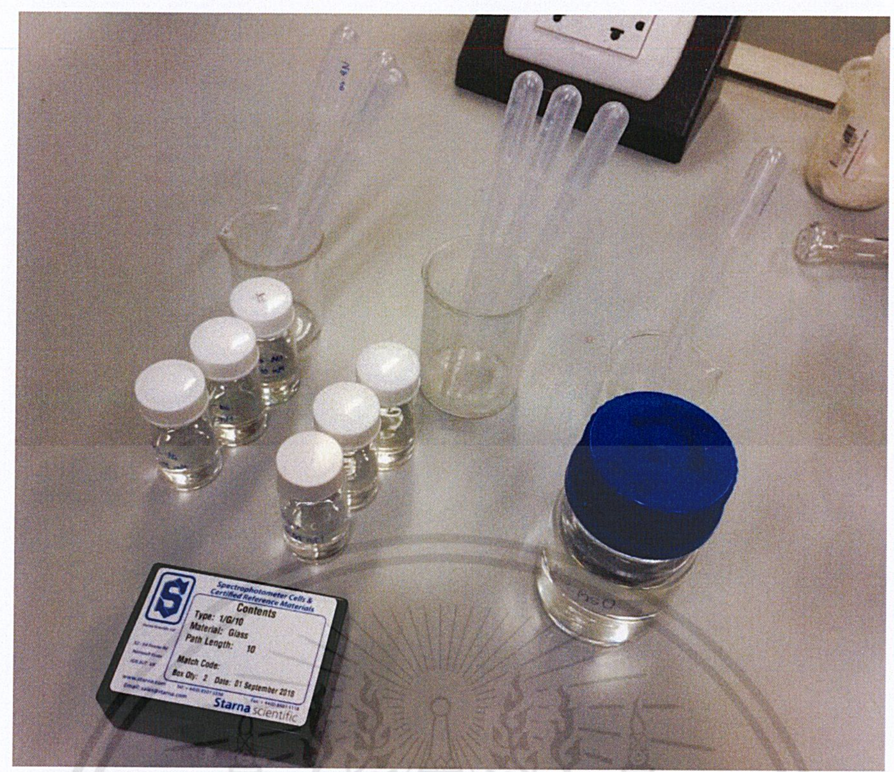

Figure C.9 Prepare equipment and chemicals for UV-VIS.

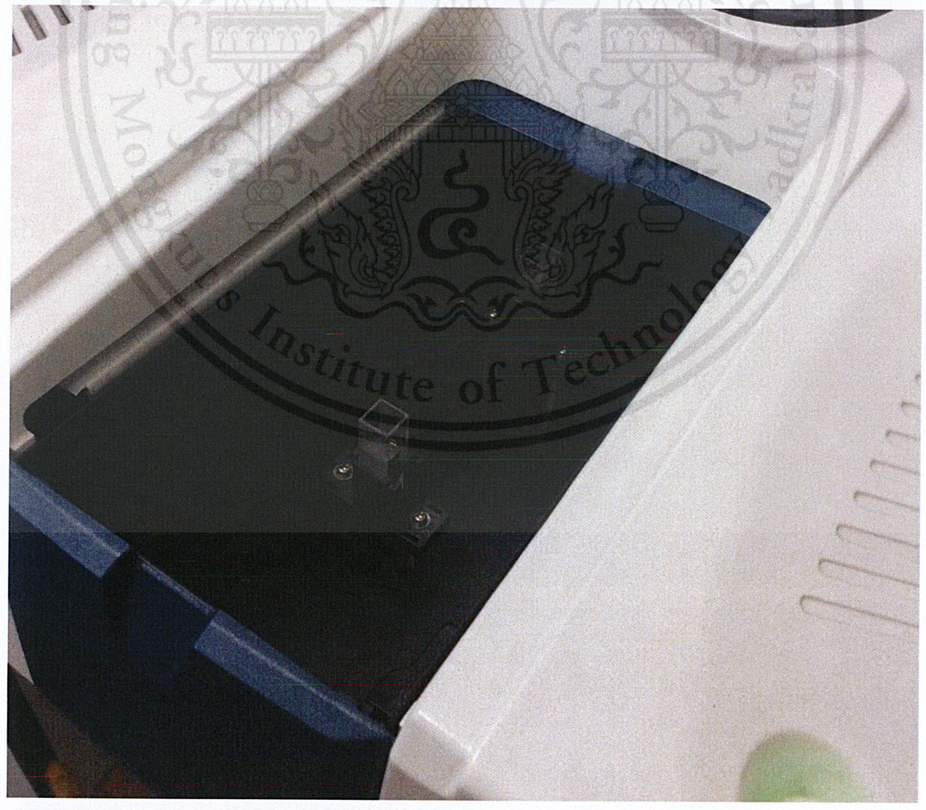

Figure C.10 Put two of Starna Spectrophotometer Cells in the UV-VIS Spectrophotometer.

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