การวิเคราะห์สารประกอบไทออลด้วยพอลิไดเมทิลไซลอกเซนไมโครชิพคะพิลลารีอิเล็กโทรฟอรีซิสร่วมกับการตรวจวัดทางเคมีไฟฟ้า

นางสาวสุกัญญา วิริยะธนวิโรจน์

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ปีการศึกษา 2552

ANALYSIS OF THIOL COMPOUNDS BY POLY(DIMETHYLSILOXANE) MICROCHIP CAPILLARY ELECTROPHORESIS COUPLED WITH ELECTROCHEMICAL DETECTION

Miss Sukanya Viriyatanavirote

A Thesis Submitted in Partial Fulfillment of the Requirements
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งานวิจัยนี้มุ่งเน้นพัฒนาเทคนิคการแยกและวิเคราะห์สารประกอบไทออลหลายชนิดพร้อมกันโดยสารประกอบไทออลที่สนใจ เช่น โฮโมซิสเตอีน, กลูทาไทโอน และ เอ็น-อะซีทิล แอล-ซิสเทอีน เป็นต้น ด้วยระบบไมโครชิพคะพิลลารีอิเล็กโทรฟอรีซิสร่วมกับตัวตรวจวัดทางเคมีไฟฟ้า ซึ่งเป็นวิธีที่มีประโยชน์และประสบความสำเร็จโดยการวัดค่ากระแสไฟฟ้าที่เกิดขึ้นจากปฏิกิริยาออกซิเดชันของสารประกอบไทออลกลุ่มนี้ด้วยเทคนิคพัลส์แอมเพอโรเมทรี ภายหลังจากที่สารประกอบไทออลเหล่านี้ผ่านการแยกด้วยระบบไมโครชิพ อิทธิพลของสนามไฟฟ้าที่ใช้แยกสาร ศักย์ไฟฟ้าที่ใช้ในการตรวจวัด ตลอดจนความเข้มข้นและค่าพีเอชของสารละลายบัฟเฟอร์ เป็นปัจจัยสำคัญที่มีผลต่อการทดลองที่ต้องทำการหาภาวะที่เหมาะสม ผลการวิเคราะห์แสดงให้เห็นอย่างชัดเจนถึงความเชื่อถือได้สำหรับการแยกและวิเคราะห์โฮโมซิสเตอีน, กลูทาไทโอน, และ เอ็น-อะซีทิล แอล-ซิสเทอีน ซึ่งสามารถแยกจากกันได้ภายในเวลาน้อยกว่า 2 นาที โดยใช้ระบบการแยกและตรวจวัดดังนี้คือ ใช้สารละลายเอ็มอีเอสบัฟเฟอร์ (MES) (pH 6.0, ความเข้มข้น 25 มิลลิโมลาร์) และ 3 มิลลิโมลาร์ เอสดีเอส เป็นสารละลายตัวพา ทำการแยกด้วยสนามไฟฟ้า 1200 โวลต์ และ ศักย์ไฟฟ้าที่ใช้ตรวจวัดคือ +0.8 โวลต์ ค่าขีดต่ำสุดของการตรวจวัดของโฮโมซิสเตอีน, กลูทาไทโอน, และเอ็น-อะซีทิล แอล-ซิสเทอีน เป็น 0.129, 0.224 และ 0.232 ไมโครโมลาร์ ตามลำดับ (ค่าสัญญาณกระแสต่อสัญญาณรบกวนมีค่ามากกว่าเท่ากับ 3) ในงานวิจัยนี้ได้แสดงให้เห็นถึงบทบาทและประสิทธิภาพของระบบไมโครชิพคะพิลลารีอิเล็กโทรฟอรีซิส จากผลการวิเคราะห์คาดว่าระบบไมโครชิพที่มีตัวตรวจวัดแบบเคมีไฟฟ้าที่เสนอนี้จะเป็นที่ยอมรับและสามารถนำมาเป็นวิธีทางเลือกใหม่สำหรับการวิเคราะห์ทางคลินิกได้

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต สุกัญญา วิริยะธนวิโรจน์

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

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SUKANYA VIRIYATANAVIROTE : ANALYSIS OF THIOL COMPOUNDS BY POLY(DIMETHYLSILOXANE) MICROCHIP CAPILLARY ELECTROPHORESIS COUPLED WITH ELECTROCHEMICAL DETECTION. THESIS ADVISOR: ASSOC. PROF. ORAWON CHAILAPAKUL, Ph.D., 93 pp.

The goal of this research is to develop the simultaneous separation and detection of biomarkers including homocysteine (Hcy), glutathione (GSH), and N-acetyl-L-cysteine (NAC) using Poly (dimethylsiloxane) (PDMS) microchip capillary electrophoresis coupled with electrochemical detection. Pulse amperometric detection mode for microchip CE was applied to detect these biomarkers. The influences of separation voltage, detection potential, as well as the concentration and pH value of the running buffer on the response of the detector were carefully assayed and optimized. The results clearly show that the determination of homocysteine, glutathione, and N-acetyl-L-cysteine by the degree of electrophoretic separation was performed in less than 2 min using a MES buffer (pH 6.0, 25 mM) and 3 mM SDS, with 1200 V separation voltage and +0.8 V detection potential. The detection limits for Hcy, GSH, and NAC were 0.129, 0.224, and 0.232 μM (S/N ≥ 3), respectively. The results obtained should be a step towards developing an automated and reliable microchip CE-ED for clinical analysis.

Field of Study : Petrochemistry and Polymer science Student's Signature Sukanya Viriyatan
Academic Year : 2009 Advisor's Signature

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LIST OF ABBREVIATIONS

i_{pa}	anodic peak current
E_{pa}	anodic peak potential
B	buffer reservoir
CEC	capillary electrochromatography
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CZE	capillary zone electrophoresis
i_{pc}	cathodic peak current
E_{pc}	cathodic peak potential
cmc	critical micelle concentration
Cys	cysteine
Cys2	cystine
t_{del}	delay time
E_{det}	detection potential
t_{det}	detection time
ϵ	dielectric constant
E	electric field strength
ECD	electrochemical detection
μ_e	electrophoretic mobility
ν	electrophoretic velocity
EOF	electroosmotic flow
GSH	glutathione
GSSG	glutathione disulfide
g	gram
Hcy	homocysteine
t_{int}	integration time
LIF	laser-induced fluorescence
LOD	limit of detection

MS	mass spectrometry
Met	methionine
MEKC	micellar electrokinetic chromatography
MCE	microchip capillary electrophoresis
μL	microliter
μm	micrometer
μM	micromolar
t	migration time
mL	milliliter
mm	millimeter
mM	millimolar
MES	2-Morpholinoethanesulfonic acid
NAC	N-acetyl-l-cysteine
nA	nanoampere
E_{oxd}	oxidation potential
t_{oxd}	oxidation time
σ^2	peak dispersion
PC	polycarbonate
PDMS	poly(dimethylsiloxane)
PETG	poly(ethyleneterephthalate) glycol
PMMA	polymethylmethacrylate
PS	polystyrene
PUR	polyurethane
PAD	pulsed amperometric detection
r	radius of the molecule
E_{red}	reduction potential
t_{red}	reduction time
S	sample reservoir
SW	sample waste reservoir
SDS	sodium dodecyl sulfate
q	the charge of the ionized solute
L	the length of capillary to the detector

N	the number of theoretical plates
D	the solute's diffusion coefficient
$w_{0.5}$	the width of the peak at half height
V	voltage
η	viscosity of the mobile phase
W	waste reservoir
ζ	zeta potential

CHAPTER I

INTRODUCTION

1.1 Introduction

Thiol compounds such as homocysteine (Hcy), glutathione (GSH), and N-acetyl-L-cysteine (NAC) significantly relate to metabolism and biological systems. The individual health can identify from the concentrations of these compounds that found in biological fluids such as plasma and urine. For example, increasing the levels of Hcy is a risk factor of cardiovascular disease [1]. Normally, the concentration of Hcy in several studies of healthy humans varies from 5 to 15 μM. For GSH, it has an important role because it acts as scavenger of reactive oxygen species (ROS) and free radicals [2, 3]. The last analyte is NAC, which has been shown to be the ideal precursor of GSH. Moreover, NAC is used for treatment of paracetamol overdose, treatment of congestive and obstructive heart diseases [4]. Thus, the method for determination of these compounds is importance for diagnosis. Conventional methods including high-performance liquid chromatography, gas chromatography, capillary electrophoresis have been used to determine these compounds. However, these methods require expensive instruments and time consuming analysis.

Microchip capillary electrophoresis (MCE) based on separation devices, which have emerged in recent years. MCE system (cross-section dimentions of channel 10-100 μm) has become an interesting tool for determination of various chemical and biological species. MCE has the benefits of small size, low cost, reduces use of reagent and sample, generate lower waste generation, fast analysis time, and portability. In general, material used to make microchip is glass or quartz. Unfortunately, these substrates are expensive, fragile, and highly thermal bonding. From mentioned disadvantage, a variety of polymeric materials such as poly(methylmethacrylate) (PMMA), poly(dimethylsiloxane) (PDMS), and polycarbonate (PC) has been proposed. In particular, PDMS becomes more popular

material due to its good optical transparency, easy fabrication, using low temperature for curing, and good adhesion.

For detection methods, there are many detection schemes coupled with MCE including laser induced fluorescence (LIF), Mass spectroscopy (MS), and electrochemical detection (ECD). Among these methods, ECD is an attractive for microchip devices because it is inexpensive and does not require analyze derivatization when compared to those of optical detection. Even though, this method provides many advantages over other methods such as high sensitivity, selectivity, and compatible with micro fabrication technique. The big problem of this technique is to foul of electrode surface that occurred when direct current (DC) amperometry or a constant potential is applied for detection. This effect usually found when measuring thiols, phenol, and carbohydrates. Therefore, to overcome electrode fouling, the pulsed amperometric detection (PAD) is proposed. For PAD technique, a highly positive potential is applied to oxidize and clean the electrode surface, followed by a negative potential step to reduce and reactivate the electrode surface. Finally, the moderate potential is applied for detection.

In this work, the thiol compounds including Hcy, GSH and NAC were determined simultaneously. A MCE device is utilized for the determination of these compounds with PAD by gold microwire working electrode. The performance of PDMS microchip is characterized by the separation of dopamine and catechol before use. For determination of thiol compounds, the effects of detection potential, separation potential, buffer concentration, pH buffer injection time were studied.

1.2 Research objective

There are two targets for this work

- To develop and optimize the conditions of PDMS microchip capillary electrophoresis system coupled with electrochemical detection for determination of thiol compounds (homocysteine, glutathione, and N-acetyl-L-cysteine)

- To apply an optimized and sensitive PDMS microchip capillary electrophoresis system coupled with electrochemical detection for analysis of thiol compounds.

1.3 Scope of research

To achieve the research objectives, the following scope was set:

- The PDMS microchip was fabricated from our preparation.
- The separation and detection of thiol compounds was accomplished using PDMS microchip capillary electrophoresis with pulsed amperometric detection.
- The effect of detection potential, separation voltage, concentration of buffer, pH buffer, and injection time were studied.
- Analytical parameter including LOD, linear range, and calibration curve were investigated.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Theory of capillary electrophoresis

2.1.1 Background theory [5, 6]

Capillary electrophoresis (CE) is an analytical technique that allows rapid and efficient separations of charged components presented in small volumes. Separation system is based on the differences in electrophoretic mobilities of ions in electrophoretic media within a small capillary. The basic CE system consists of a fused silica capillary, two buffer reservoirs, high-voltage power supply, electrode, detector and data processor. CE separation is usually performed in fused silica tube that filled with buffer solution. When an electrical potential is applied, a small volume of sample is introduced into the channel. Next, the separation of charges species are occurred inside the channel. Moreover, detection mode such as laser induced fluorescence (LIF), electrochemistry and mass spectrometry (MS) is chosen with suitable for each work.

In addition, CE system is usually used for application of chemical, biomedical and pharmaceutical targets. The examples include the separations of proteins and peptides, DNA sequencing, serum analysis, analysis of neurotransmitters in single cells, determination of organic and inorganic ions, and chiral separations.

The advantages of capillary electrophoresis are:

- fast analysis
- high separation efficiency
- reduced use of sample and reagents
- low waste generation
- flexibility of design
- reproducibility

- providing many application to wider selection of analytes when compared to other analytical separation techniques

2.1.2 Fundamental of capillary electrophoresis [7-9]

For capillary electrophoresis, the fundamental terms that ralated to the velocity of analyte species are the electrophoretic mobility (μ), the electrophoretic velocity (ν), and the electric field strength (E). The relationship of these terms is shown in Eq. 2.1.

$$\mu = \nu/E \qquad (2.1)$$

Where ν is the velocity of the analyte (ms^{-1}), μ is electrophoretic mobility of the analyte ($m^2v^{-1}s^{-1}$) and E is electric field strength ($V\ m^{-1}$)

The electric field strength can be calculated using the applied voltage (V) divided by the length of the capillary to the detector (L) as shown in Eq. 2.2.

$$E = V/L \qquad (2.2)$$

Thus; the relationship between electrophoretic mobility, electric field strength, and migration time is shown in Eq. 2.3.

$$\mu = l/tE = lL/tV \qquad (2.3)$$

Where l is the distance from the injection zone to the detector zone, and t is the time taken for the species to migrate to the region of the detector.

The difference in the speed of movement (migration) of ions or solutes is important factor for the separation by electrophoresis. Cations are attracted toward the negatively charged electrode (cathode). At the same time, anions are movement toward the positively charged electrode (anode).

For CE, the fluid is move from the starting point of the capillary to the detection zone by applying either an electric potential or an external pressure. However, an attractive method in driven fluid for separation is applying voltage because it provided the flat flow profile. When the voltage is applied, the analyte species are migrated due to the electrophoretic mobility of the analyte (μ_e) and the mobility of the electroosmotic flow (μ_{eof}) as shown in Eq. 2.4 that μ is the apparent mobility.

$$\mu = \mu_e + \mu_{eof} \qquad (2.4)$$

2.1.3 Electrophoretic mobility (μ_e) [8,9]

Electrophoretic mobility is a factor that identifies the motion of ions or solute through a given medium (for example a buffer solution). The charged electric species migrates under the influence of an electric field that characterized by its electrophoretic mobility. Mobility is dependent on the charge density of the solute (the overall valence and size of the solute molecule), the dielectric constant, and viscosity of the electrolyte. Electrophoretic mobility can be calculated following the Eq. 2.5.

$$\mu_e = \frac{q}{6\pi\eta r} \qquad (2.5)$$

Where q is the charge of the ionized solute, η is the viscosity of the mobile phase, and r is the radius of the molecule, which is related to its mass.

From Eq. 2.5, it is found that the migration time of the analytes depended on charge-to-size ratio. For the same charge, a smaller ion moves to the detection zone faster than a larger ion. At the same time, a higher charge ion will move toward the detector faster than a lower charge of the same size. The electrophoretic mobility is the rather important factor in electrophoresis because that is characteristic property of

the analytes and will always be constant. Therefore, due to the difference in electrophoretic mobility that is the possible to separation the mixture of several ions and solutes by using electrophoresis.

2.1.4 Electroosmotic flow (EOF) [5-7,9]

The buffer solution usually moves through the capillary under the influence of an electric field. An important phenomenon occurred in capillary electrophoresis is electroosmotic flow (EOF). EOF is the motion of liquid inside the capillary influenced by applied potential across a capillary. In addition, EOF is an electrically driven pump towards the detector. Recently, the most capillaries are made of fused silica, which contains surface silanol groups (pK_a 3-5) that ionized as a function of the pH of the separation buffer. When a buffer is flushed inside a capillary, the inner surface of a capillary was created as a charge. This may be due to the ionization of the capillary surface or adsorption of ions from the buffer onto the capillary wall. An uncoated fused-silica, the surface silanol (Si-OH) groups are ionized to negatively charged silanoate (Si-O-) groups at pH above three, as show in Eq.2.6.

$$\text{-Si} - \text{OH} + \text{OH}^- \longrightarrow \text{-Si-O}^- + \text{H}_2\text{O}$$

$$\text{-Si} - \text{OH} + \text{H}_2\text{O} \longrightarrow \text{-Si-O}^- + \text{H}_3\text{O}^+ \qquad (2.6)$$

The silanoate groups attract cations from the buffer, which form an inner layer of cations at the capillary wall. These cations are not sufficient density to neutralize all the negative charges, then, outer layer of cations forms. The inner layer is tightly held by the Si-O- group, which is referred to as the fixed layer. The outer layer of cations is not tightly held because it is further away from the silanoate groups, and it is referred to as the mobile layer, as shown in Figure 2.1.

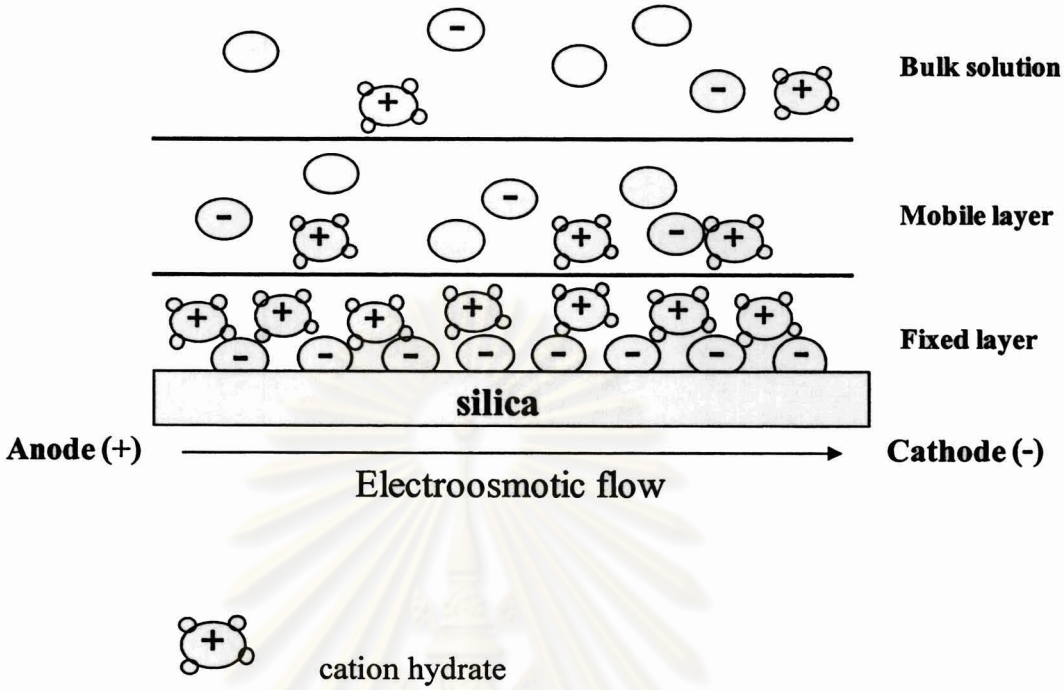

Figure 2.1 Schematic of electroosmotic flow.

The EOF is used for the simultaneous determination of neutral, cations, and anions species in the same analysis. The negatively-charged wall attracts positively-charged ions from the buffer, creating an electrical double layer. When a voltage is applied across the capillary, cations in the diffuse portion of the double layer migrate in the direction of the cathode, carrying water with them. The result is a net flow of buffer solution in the direction of the negative electrode. The direction of the movement of ions with EOF is shown in Figure 2.2. The order of migration is cations, neutrals, and anions as show in Figure 2.3.

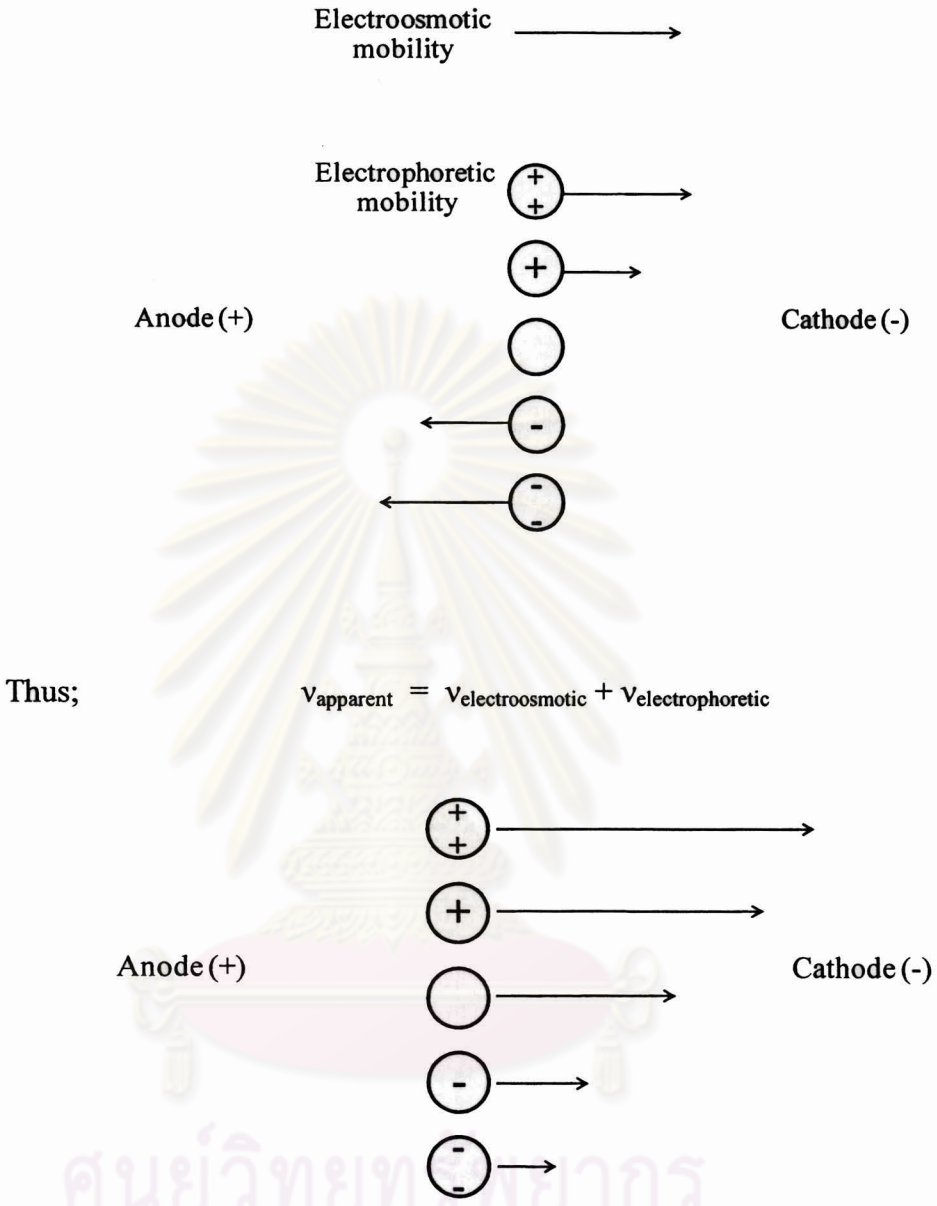

Figure 2.2 Direction of the movement of ions with the electroosmotic flow [10].

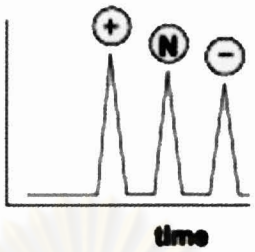

Figure 2.3 Migration order of the ions with electroosmotic flow [11].

The electroosmotic flow is identified by

$$\nu_{eo} = \frac{\epsilon \zeta E}{4\pi\eta} \qquad (2.7)$$

Where ν_{eo} is the electroosmotic flow mobility, ϵ is the dielectric constant, η is the viscosity of the buffer, and ζ is the zeta potential measured at the plane of shear close to the liquid-solid interface.

The zeta potential is related to the inverse of the charge per unit surface area, the number of valence electrons, and the square root concentration of the electrolyte. Since this is an inverse relationship, increasing the concentration of the electrolyte decreases the EOF.

2.1.5 Separation efficiency [7,8]

Both the separation efficiency and resolution are related to the direction and flow of the EOF, because the electroosmotic flow affects the amount of time that a solute resides in the capillary. The flow profile of EOF and laminar flow are shown in Figure 2.4. Unlike the laminar flow that is characteristic of pressure-driven fluids, the EOF has minimal effect on resistance to mass transfer. As a result, the plate count in a capillary is far larger than that of a chromatography column of comparable length.

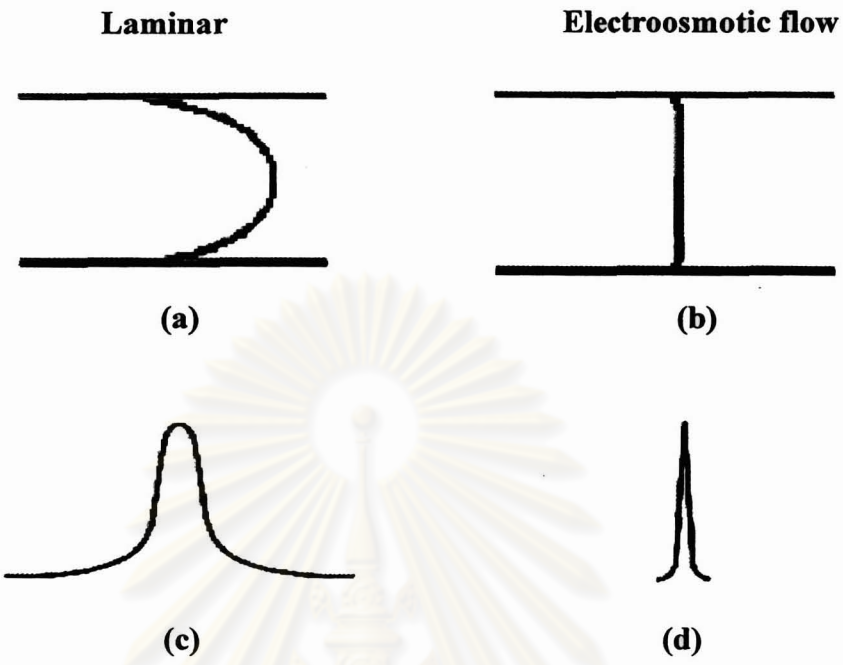

Figure 2.4 Flow profiles of (a) laminar and (b) electroosmotic flow, peak from (c) laminar and (d) electroosmotic flow [9].

The migration time of a solute, or the time t required for a zone to migrate from the point of injection to the point of detection, is given by

$$t = l/v = l/\mu E = lL/\mu V \qquad (2.8)$$

Where v is the migration velocity, t is time to diffuse

During migration through the capillary, molecular diffusion occurs leading to peak dispersion, σ^2, calculated as

$$\sigma^2 = 2Dt = 2DL^2/\mu V \qquad (2.9)$$

where D is the solute's diffusion coefficient (cm^2/s). The separation efficiency of an electrophoretic system may be expressed in terms of the number of theoretical plates, N, where

$$N = L^2/\sigma^2 \qquad (2.10)$$

and, by substituting Eq. (2.9) into Eq. (2.10),

$$N = \mu V/2D \qquad (2.11)$$

From Eq. 2.11, there are three points that can be drawn. First, the most direct approach to high separation efficiencies in zone electrophoresis is the use of very high voltages (V); second, large molecules, such as DNA and proteins, which have low diffusion coefficients (D), will give high efficiencies because they exhibit less dispersion than small molecules; and, third, highly mobile species (μ) will produce high plate counts because the rapid velocity through the capillary minimizes the time for diffusion. Although the last two points appear contradictory, Eq. 2.11 illustrates the wide range of molecular weights across which high-efficiency separations are possible in CE. As long as heat dissipation is adequate, capillary length plays no direct role in separation efficiency.

In addition, the use of high voltages will also provide the greatest efficiency by decreasing the separation time. The practical limit of voltage generally used with today's technology is about 30 kV. The practical limit of field strength (one could use very short capillaries to generate high field strength) is called Joule heating that is a consequence of the resistance of the buffer to the flow of current. The problems of heat generation/dissipation will be covered shortly.

The efficiency may be determined experimentally using

$$N = 5.54\,(t/w_{0.5})^2 \qquad (2.12)$$

Where t is the migration time and $w_{0.5}$ is the width of the peak at half height. Eq. 2.12 is strictly valid only for Gaussian peaks, and any peak asymmetry should be taken into account, for example, by the use of central moments.

2.1.6 Separation modes

Capillary electrophoresis technique is introduced in the analysis of biomolecules from charged species to macromolecules. CE technique consists of many modes that can be classified by the different separation characteristics. The modes include;

- Capillary zone electrophoresis (CZE)
- Micellar electrokinetic chromatography (MEKC)
- Capillary electrochromatography (CEC)
- Capillary gel electrophoresis (CGE)
- Capillary isoelectric focusing (CIEF)
- Capillary isotachophoresis (CITP)

In this research, the capillary zone electrophoresis (CZE) and micellare electrokinetic chromtrography (MEKC) mode were used for separation of thiol compounds. Thus, the principle of separation of CZE and MEKC is presented herein in brief.

2.1.6.1 Capillary zone electrophoresis (CZE) [5,6,12]

Capillary zone electrophoresis (CZE) or free-solution capillary electrophoresis is the most commonly mode of CE because it can be applied to separate of cations and anions. The neutrals compounds can not be separated in this mode. The separation mechanism is based on the differences of the electrophoretic mobilities that is relative in the charge-to-mass ratio of the analytes at a given pH. The mechanism of separation with CZE is shown in Figure 2.5. The capillary is filled with a homogenous buffer solution. The order of migration in CZE is cations, neutrals and anions. The polarity setup of the anode being at the injection inlet and the cathode at the outlet, neutral sample will move towards the detector with the velocity of the EOF; cations will move to the cathode first with a higher apparent

velocity (apparently faster) and anions will move against the EOF with a reduced apparent velocity (apparently slower) as shown in Figure 2.5.

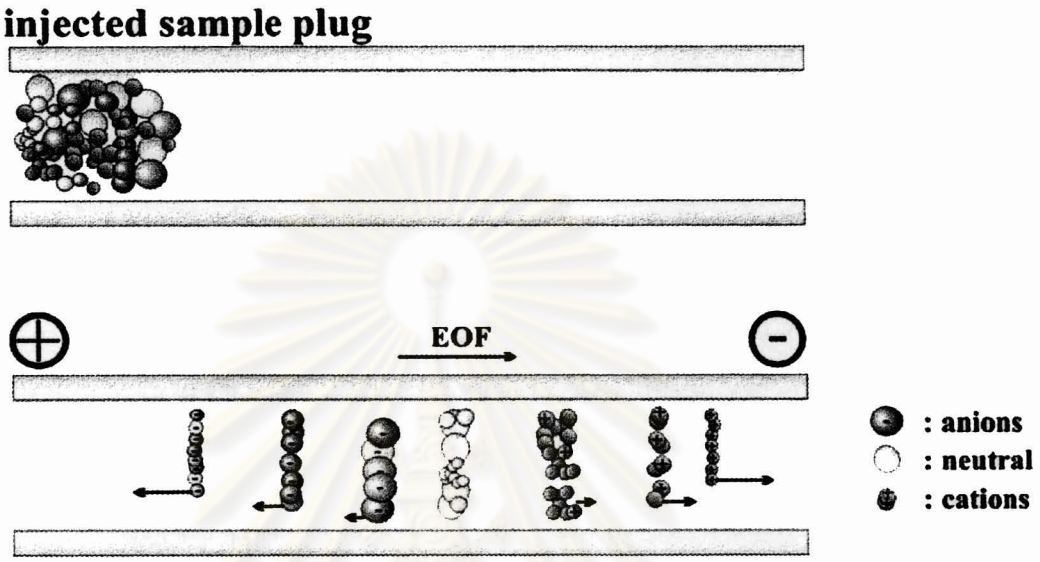

Figure 2.5 Mechanism of separation in CZE.

2.1.6.2 Micellar electrokinetic chromatography (MEKC) [5,6]

The mechanism of separation with MEKC is shown in Figure 2.6. An important development in CE is the introduction of micellar electrokinetic capillary chromatography (MEKC or MECC) by Terabe and co-workers in 1984 [13,14]. The main separation mechanism is based on solute partitioning between the micellar phase and the solution phase. This technique provides a way to resolve neutral molecules as well as charged molecules by CE.

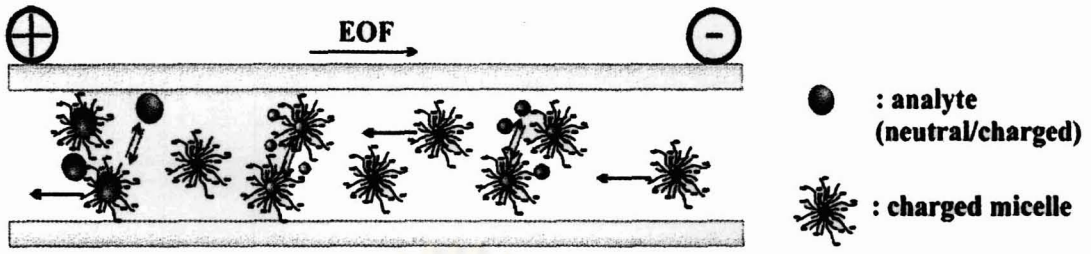

Figure 2.6 Mechanism of separation in MEKC.

Micelles form in solution when a surfactant is added to water in concentration above its critical micelle concentration (cmc). The most commonly used surfactant in MEKC is sodium dodecyl sulfate (cmc = 0.008 M at 25 ^{O}C), which is an anionic surfactant. Even though these anionic micelles are attracted toward the anode, in an uncoated fused silica capillary they still migrate toward the cathode because of electroosmotic flow. However, the micelles move towards the cathode at a slower rate than the bulk of the liquid because of their attraction towards the anode. Neutral molecules partition in and out of the micelles is based on the hydrophobicity of each analyte. Consequently the micelles of MEKC are often referred to as a pseudo (or moving) stationary phase. A very hydrophilic neutral molecule will spend almost no time inside the micelle and will therefore migrate essentially at the same rate as the bulk flow and eluted earlier. On the other hand, a very hydrophobic neutral molecule will spend nearly all the time inside the micelles and will therefore elute later, together with the micelles. All other solutes with intermediate hydrophobicity will migrate within this migration window. MEKC can be used with ionic substances as well as neutral compounds.

2.2 Microchip capillary electrophoresis

Microchip capillary electrophoresis (MCE) system was established in the early 1990s. The concept of conventional capillary electrophoresis (CE) was proved to be excellent match for MCE system. An interesting system is application for miniaturization of chemical and biological field, including genetic analysis, clinical diagnostics, drug screening and environmental monitoring. Since the initially establishment, the numerous of application and fundamental development have been published.

MCE is great potential over conventional CE because of many benefits. The benefits of MCE include better flexibility of design, reduced consumption of samples and reagents, lower waste generation, portability, fast analysis speed, and inexpensive.

2.2.1 Material for microchip capillary electrophoresis

Initially, glass (or quartz) is a substrate material for fabrication of microchips. Surface chemistry of glass is similar to the fused silica capillary used in classical CE. Thus, the surface modification can be applied. This substrate provides many advantages including excellent optical transparency, high chemical and mechanical stability. However, there are several also disadvantages for glass microchip. First, process of fabrication glass microchip needs clean-room accessory and time-consuming of bonding process. Second, glass is fragile and can be easily broken and too expensive. Third, this microchip is probably blocked up and difficult to clean. Recently, polymer substrate has become an alternative material to construct microchip devices. The variety of polymers are poly(dimethylsiloxane) (PDMS), polymethylmethacrylate (PMMA), poly(ethyleneterephthalate) glycol (PETG), polycarbonate (PC), polyurethane (PUR), polystyrene (PS), polyacrylamide.

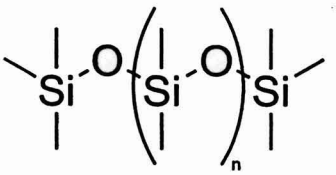

Figure 2.7 The chemical structure of poly(dimethylsiloxane) (PDMS).

Figure 2.7 shows the chemical structure of PDMS. PDMS is one of the most attractive of polymer material because there are several advantages. PDMS can easily to seal with other materials, and the casting step does not require the clean room. Moreover, it is inexpensive, easier to fabricate than glass, excellent for optical transparency. It also leave the master intact, cure at low temperature, and is ready to produce another device and non-toxicity. The chemical and physical properties of PDMS are shown in Table 2.1.

Table 2.1 Physical and Chemical properties of PDMS [15].

property	characteristic	consequence
optical	transparent; UV cutoff, 240 nm	optical detection from 240 to 1100 nm
electrical	insulating; breakdown voltage, 2×10^7 V/m	allows embedded circuits; intentional breakdown to open connections
mechanical	elastomeric; tunable Young's modulus, typical value of ~750 kPa	comforms to surfaces; allows actuation by reversible deformation; facilitates release from molds
thermal	insulating; thermal conductivity 0.2 W/(m·K); coefficient of thermal expansion, 310 μm/(m·^{O}C)	can be used to insulate heated solutions; dose not allow dissipation of resistive heating from electrophoretic separation

Table 2.1 (continue) Physical and Chemical properties of PDMS [15].

property	characteristic	consequence
interfacial	low surface free energy ~ 20 erg/cm^2	replicas release easily from molds; can be reversibly sealed to materials
permeability	impermeable to liquid water; permeable to gases and nonpolar organic solvents	contains aqueous solutions in channels; allows gas transport through the bulk material; incompatible with many organic solvents
reactivity	inert; can be oxidized by exposure to a plasma	unreactive toward most reagents; surface can be etched; can be modified to be hydrophilic and also reactive toward silanes; etching with (TBA)F can alter topography of surfaces
toxicity	nontoxic	can be implanted in vivo; mammalian cell growth

The most common PDMS elastomer (Sylgard® 184 from Dow Corning) was used for this work. Sylgard have two part resins containing vinyl groups (PDMS oligomer) and hydrosiloxane groups (cross-linker) as shown in Figure 2.8. The mixing of two resins induces to a cross-linked network of dimetylsiloxane groups.

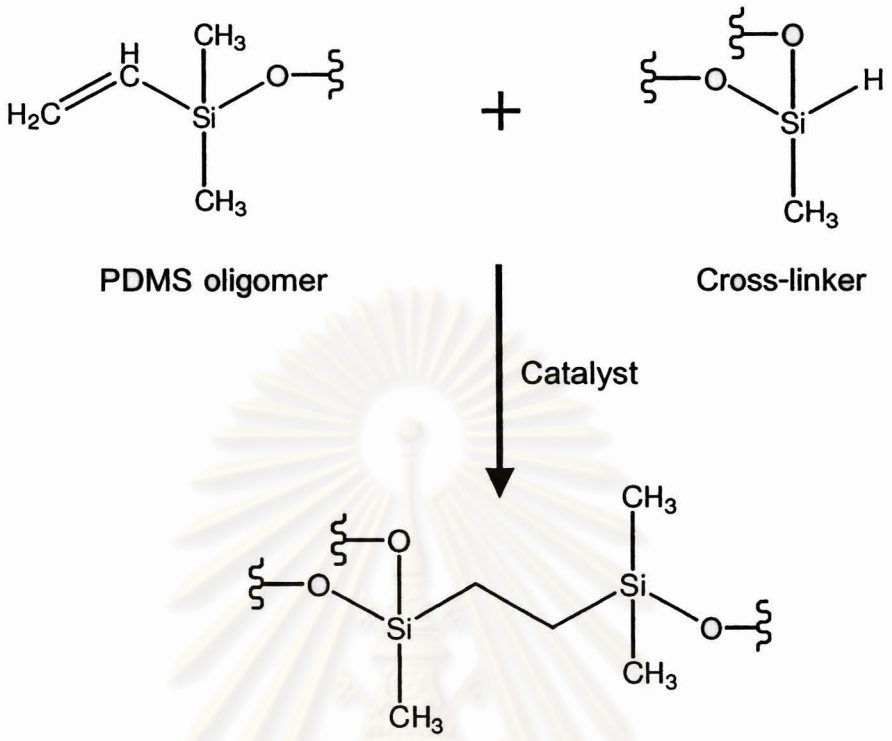

Figure 2.8 PDMS crosslinking [16].

2.2.2 Injection mode [17-19]

The gated injection and pinched injection are two common forms of injection modes that are used in microchip CE. Therefore, two of these modes of injection are described in this part.

2.2.2.1 Gated injection [20]

Principle of the gated injection is shown as Figure 2.9. The waste reservoir (W) at the end of the injection channel is set to ground all the time. So, it

performs as the anode of the electroosmotic flow direction. Other reservoirs are set at higher positive potentials. The sample reservoir (S) is set at positive potentials higher than the sample waste reservoir (SW). The buffer reservoir (B) is filled with buffer and is set at slightly higher voltage than S to prevent a part of sample from "bleeding" into the separation channel. Furthermore, pre-injection and run step are set at the same voltage.

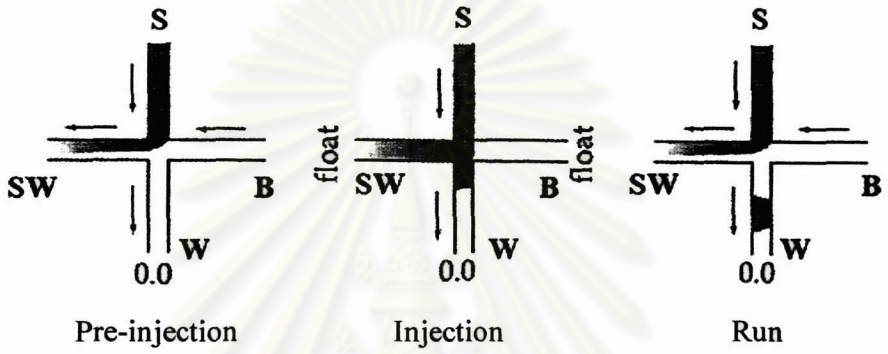

Figure 2.9 Principle of gated injection. Sample reservoir (S), sample waste reservoir (SW), buffer reservoir (B), waste reservoir (W).

In the injection step, both B and SW are set as float. The high voltage is set as S. The sample will flow from S to SW as before, and buffer will flow into the separation channel. Thus, sample will flow into the separation channel, where individual components will be separated and detected. The injection step is finished when the sample flow into the separation channel and releasing a plug of sample flowing towards the anode. The injected volume is mainly determined with timing of the injection sequence and sample can flow into the separation channel as long as this electrokinetic value is open, this injection procedure is called "gated injection".

The advantage of gated injection is very simple to perform, and it allows for injection of a plug into the separation channel. Furthermore, a new sample plug can be injected into the separation channel at any time, which means that a new sample can even be injected into the separation channel before the previous sample reaches the detector.

Disadvantage of gated injection is a bias in the injection. The species with the highest electrokinetic velocity are injected at a larger extent than those with lower electrokinetic velocities. This means that in regular EOF, a sample plug injected into the cation sample plug will be slightly longer than that for neutral species and the anion sample plug will be slightly shorter than that for neutrals. This will provide lower the limit of detection somewhat for negatively charged species, but the effect is usually not large enough to be significant. If the gated injection is used to make a calibration curve for the species, this bias will even out since the same bias will apply for the calibration and sample injection.

2.2.2.2 Pinched injection [20]

Principle of the pinched injection is described as Figure 2.10. The sample reservoir is on one of the side-arms, and prior to injection, sample is continuously pumped across the intersection to the waste reservoir. Additionally, the voltages are arranged so that there is also flow from the B to the reservoir at the end of the separation channel towards the intersection SW. This is again to avoid premature bleeding of sample into the separation channel. By adjusting the voltages one can pinch the sample stream more or less on its passage through the intersection. Therefore, this technique is often called "pinched injection".

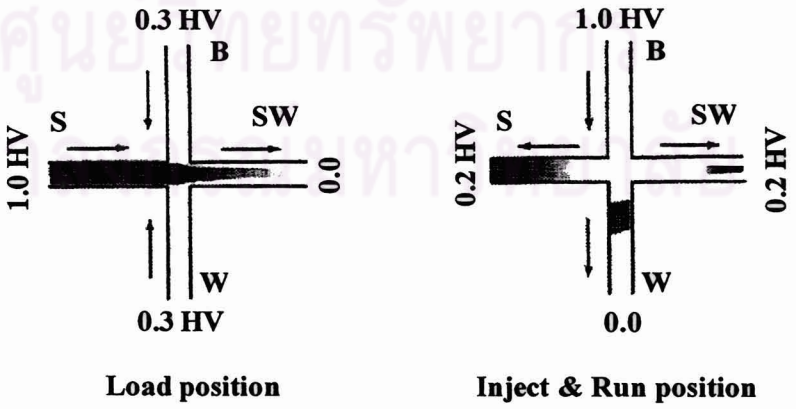

Figure 2.10 Principle of pinched injection. Sample reservoir (S), sample waste reservoir (SW), buffer reservoir (B), waste reservoir (W).

The main differences with respect to gated injection are:

- The injection volume is pre-defined and fixed. Only the volume defined by the junction geometry and the pinching is injected once the voltages are switched accordingly.
- If the loading step is given sufficient time, then the composition of the sample solution present at the intersection is the same as the composition of the original sample solution, and consequently there is no electrokinetic bias upon injection.

2.2.3 Detection for microchip capillary electrophoresis

A detection is very important for microchip separation. Several detection methods have been applied in CE system such as mass spectrometry (MS), laser-induced fluorescence (LIF), electrochemical detection (ECD), and ultraviolet detection (UV). In this report, the detections including MS, LIF and ECD are described because they are popular for analysis in CE system.

2.2.3.1 Laser induced fluorescence (LIF) [21]

LIF is the most widely used and the first adapted to detect analytes for microchip CE. LIF is very sensitive for detection of the small mass quantities of analytes. It has been applied with microchip CE such as the analysis of DNA and protein. Nevertheless, there are disadvantages such as relatively large and expensive lasers and optics system. The most of all limitations is only applicable for fluorescent samples and their derivatives. However, many analytes are not fluorescent in the usable wavelengths.

2.2.3.2 Mass spectroscopy (MS) [21]

MS has been coupled to microchip CE for a variety of applications especially in protein sequencing (proteomics). MS is the ultimate identification tool in chemical analysis as it can provide molecular weight information and fragmentation patterns. However, the disadvantages of MS include high-instrument

cost, a high level of technical expertise needed for operation, and large instrument size.

2.2.3.3 Electrochemical detection (ECD) [22-25]

Electrochemical detection (ECD) is an ideal, attractive candidate for miniaturized analytical systems, as there are several advantages including remarkable sensitivity, portability, independence of optical path length, low cost and power requirements, inherent miniaturization, and high compatibility with modern microfabrication technologies. Moreover, the analytical sensitivity is difficult to obtain by miniaturization, and the associated instrumentation is relatively inexpensive and simple compared to optical detectors or mass spectrometry. Furthermore, this method does not require analyte derivatization.

2.3 Fundamentals of electrochemistry

2.3.1 Amperometry [26]

Amperometric detection was the measurement of current at a fixed potential. Either an oxidation or reduction was forced to occur by judicious selection of the potential applied to an electrode by a controlling potentioststst. A typical waveform of amperometry is shown in Figure 2.11.

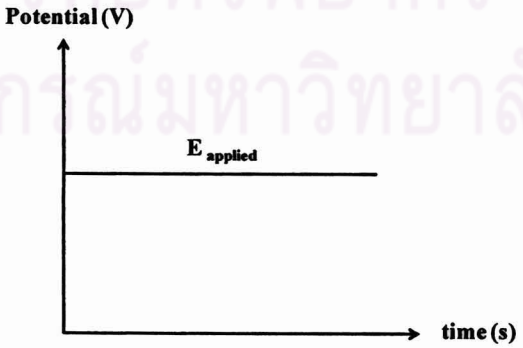

Figure 2.11 A typical of amperometry waveform.

The electrode acts as an oxidizing or reducing agent of variable power. In order to use amperometric measurements effectively, it was important to recognize that electrochemical detection was a surface technique, which means molecules not adjacent to the electrode must be moved to the surface to react.

The advantage of amperometry over most analytical detection techniques involves a direct conversion of chemical information to an electrical signal without the use of optical or magnetic carriers. If a reduction takes place, electrons flow from the electrode to the molecule in a heterogeneous transfer; conversely, an oxidation was the transfer of electrons in the opposite direction. Under steady-state condition, the current measured is contributed from three sources: the background electrolyte, the electrode material itself, and the analyte.

2.3.2 Pulsed amperometry [27-29]

Pulsed amperometric detection (PAD) is the amperometric detection under the control of a multistep potential-time waveform. The typical waveform for PAD is the triple step waveform. This waveform consists of three potential steps, which are detection, cleaning, and reactivation steps. In detection step, the oxidation reaction of the electroactive species of analyte interest occurrs. The potential applied in this step is called the detection potential (E_{det}). The time duration for the application of this potential is called a detection time (t_{det}). This time duration consists of two timing parameters, namely, delay time (t_{del}) and integration time (t_{int}). The delay time is necessary to overcome the double-layer charging currents. The current response is sampled during a short integration time period after a delay of delay time. The adsorption of the adsorbed detection products and/or solution impurities occurs in the step of detection. These adsorbed species are necessary desorbed from the electrode surface before the next detection process. The desorption process is performed at the oxidation step (cleaning step). In cleaning step, the electrode potential is set at the potential value more positive than the detection potential. The timing parameter for the application of this potential is oxidation time, t_{oxd}. Besides the removal of the adsorbed molecule from the electrode surface, the oxide layer is also developed in this cleaning step. This oxide layer covering the electrode surface can be deactivated the

electrode activity. Thus, the electrode activity must be regenerated by a subsequent negative potential to reduction potential, E_{red} for a duration of reduction time, t_{red}. This last step is called the reactivation step. In the last step, the oxide film is dissolved from the electrode surface and the active surface is ready for the next cycle of PAD waveform. The typical PAD waveform is shown in Figure 2.12. From the schematic diagram of PAD waveform displayed in Figure 2.12, the PAD waveform parameters can be divided into two categories; potential and timing parameters. The overall PAD waveform parameters are E_{det}, t_{det} (t_{del} and t_{int}), E_{oxd}, t_{oxd}, E_{red}, and t_{red}. Therefore, there are seven waveform parameters which the optimization must be performed.

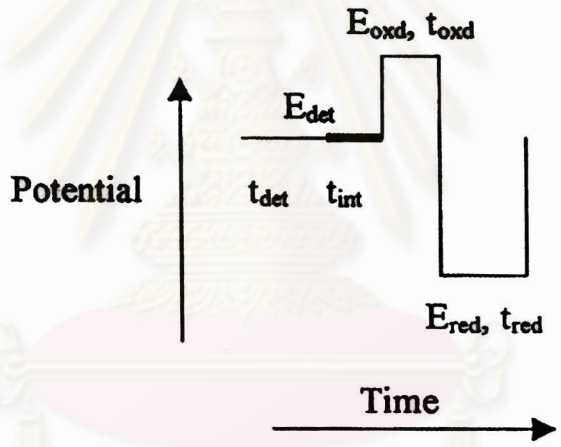

Figure 2.12 The PAD waveform.

2.3.3 Voltammetry [30]

Voltammetry is one of the electroanalytical methods. The information of the anaylte is derived from the measurement of current under conditions of concentration of polarization of working electrode. The potential of the working electrode is varied and the resulting current is recorded as a function of applied potential. In the presence of the electroactive (reducible or oxidizable) species, a current will be recorded when the applied potential becomes sufficiently negative or positive for it to be electrolyze.

The recording result is called a voltammogram. The potential excitation signal is imposed on an electrochemical cell containing an electrode. The waveforms of four of the most common excitation signals used in voltammetry are shown in Figure 2.13. The classical voltammetric excitation signal is a linear scan as shown in Figure 2.13a, in which the dc voltage applied to the cell increases linearly as a function of time. The current that develops in the cell is then measured as a function of the applied voltage. The two-pulse excitation signals are shown in Figure 2.13b and Figure 2.13c. Currents are measured at various times during the lifetimes of these pulses. With the triangular waveform shown in Figure 2.13d, the potential is varied linearly between a maximum and a minimum value. This process may be repeated numerous times while the current is recorded as a function of potential.

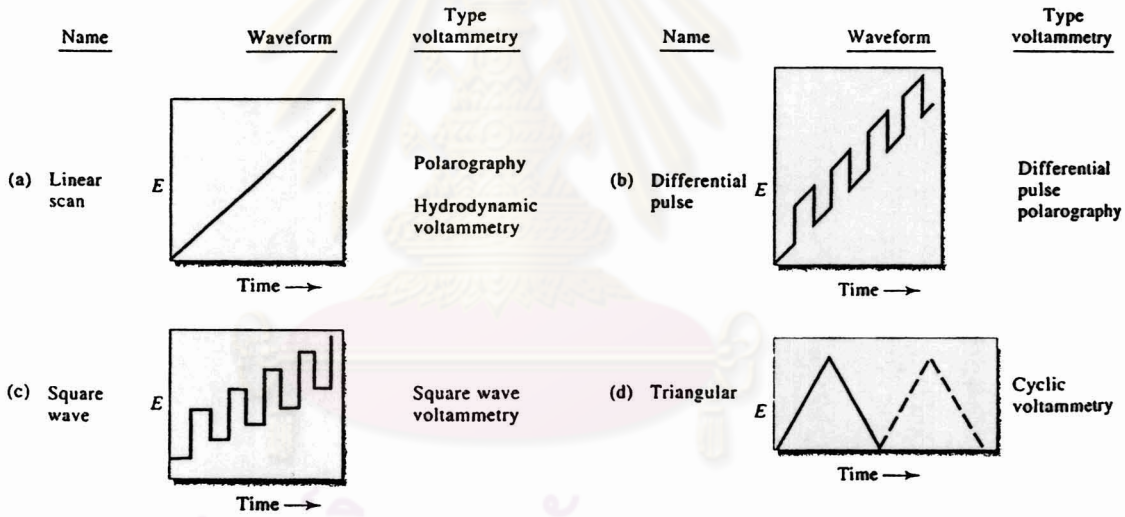

Figure 2.13 Voltage versus time excitation signals used in voltammetry.

2.3.3.1 Cyclic Voltammetry [31]

Cyclic voltammetry is the most widely used technique for acquiring qualitative information of the electrochemical reactions. The power of cyclic voltammetry result from its ability to rapidly provide considerable information on the thermodynamics of redox processes, on the kinetics of heterogeneous electron-transfer reactions, and on coupled chemical reaction or adsorption processes. Cyclic

voltammetry is often the first experiment performed in an electroanalytical study. In particular, it offers a rapid location of redox potentials of the electroactive species, and convenient evaluation of the effect of media upon the redox process.

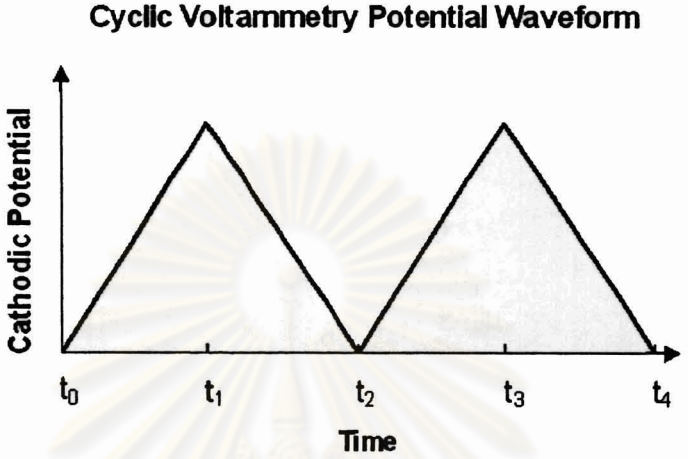

Figure 2.14 Schematic of cyclic voltammetry triangular potential waveform.

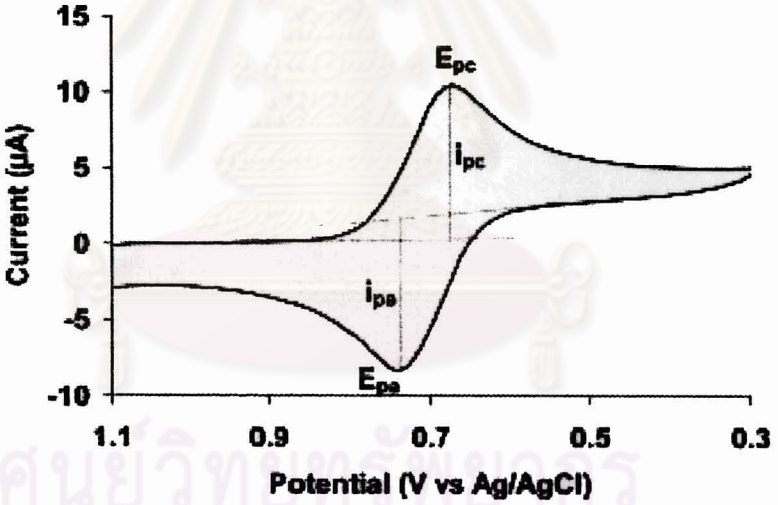

Figure 2.15 Schematic of typical cyclic voltammogram.

Cyclic voltammetry is a technique that employs a triangular potential waveform (Figure 2.14) to scan linearly the potential at a stationary working electrode in an unstirred solution. Depending on the information sought, single or multiple cycles can be used. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting plot of current versus potential is termed a cyclic voltammogram (Figure 2.15). The significant parameters in cyclic voltammogram are the cathodic peak potential (E_{pc}), the anodic peak potential (E_{pa}),

the cathodic peak current (i_{pc}), and the anodic peak current (i_{pa}). The cyclic voltammogram is a complicated, time-dependent function of a large number of physical and chemical parameters.

2.4 Thiol compounds [32]

The thiol compounds plays an important role with chemically and biochemically. Thiol compounds such as homocysteine (Hcy), glutathione (GSH), and *N*-acetyl-l-cysteine (NAC) significantly relate to metabolism and biological systems. Therefore, the method for determination of Hcy, GSH and NAC would be effective with diagnosis at the early stages of disease. The concentrations of these compounds in biological fluids are significant biomarkers for a variety of diseases. Thus, this research studied the determination of Hcy, GSH and NAC.

2.4.1 Homocysteine (Hcy)

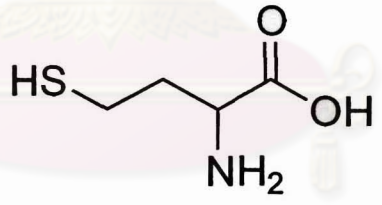

Figure 2.16 Chemical structure of homocysteine.

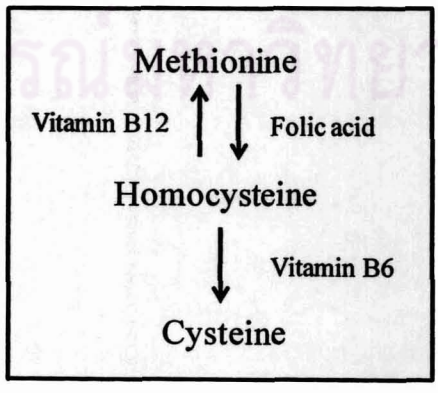

Figure 2.17 Homocysteine metabolic pathways.

Homocysteine (2-amino-4-mercaptobutyric acid) (Hcy) is a sulfur amino acid in blood. The chemical structure of Hcy shows in Figure 2.16. Homocysteine can be remethylated back to methionine by the remethylation pathway or converted to cysteine by the transsulfuration pathway (Figure 2.17). This thiol compound is not found directly in the diet, but it is an intermediate formed of transulfiration pathway. The nutrients with greatest influence are vitamin B6, vitamin B12 and folic acid, which are concern with metabolic pathway. The decrease levels of these nutrients effect an accumulation of homocysteine.

Normally, the concentration of Hcy in healthy humans ranges between 5 to 15 μM. The level of Hcy are referred to the hyperhomocysteinemia including the moderate range 15-30 μM, the intermediate range 30-100 μM and the severe range > 100 μM.

Hyperhomocysteinemia associates too much homocysteine, which is present in fluid body. It has been related to increased risk of cardiovascular disease and stroke, pregnancy loss, pregnancy complications, Alzheimer's disease, mental disorders and some tumors.

For these reasons, the monitoring of Hcy in human fluid is important for diagnosis. Several determination methods have been developed to determine Hcy in biological. Table 2.2 shows various methods for the determination of Hcy.

Table 2.2 The various methods for determination of Hcy.

year	Analytes	Sample	Separation	Detection	Linear reange (μM)	LOD (μM)	Ref.
1995	Hcy, GSH, Cys	blood	HPLC	ECD	6.25-100 μM	0.02	33
1997	Hcy, GSH, Cys	Human plasma	CE	UV	-	0.5	34
1999	Hcy	Human plasma	GC	MS	-	0.17	35
1999	Hcy, GSH, NAC	Human plasma	CE	LIF	-	3	36
2000	Hcy	Human plasma	HPLC	UV	-	0.1	37
2000	Hcy	blood	HPLC	ECD	> 0-500 μM	0.14	38
2001	Hcy	Human plasma	HPLC	fluorescence	3.9-62.5 μM	0.12	39
2002	Hcy	Human plasma	CE	ECD	1-100 μM	0.5	40
2002	Hcy, Cys, NAC, GSH	Human urine	CE	ECD	-	0.011	41
2002	Hcy, Cys2, Cys, GSH, Met, Hcy2, GSSG	standards	HPLC	ECD	0.05-100 μM	1	42
2003	Hcy, GSH, Cys, GSSG	Rat tissues	LC	MS	-	0.75	43
2004	Hcy,Cys,GSH,NAC	Standards	Glass Microchip capillary electrophoresis (μCE)	Electro chemical (EC)	5-50 μM	0.75	44
2004	Hcy	Human plasma	Chemilumines cence immunoassay (ICL)	fluorescence	2.4-58.8 μmol/l	< 0.9	45
2005	Hcy, Cys, Met	human plasma	HPLC	ECD	0.2-100 μM	0.1	46
2006	Hcy	Human serum, plasma	LC	MS-MS	> 61.6 μmol/l	1.0	47
2007	Hcy	Standard	HPLC	Electro chemical (EC)	0.1-5 μM	0.03	48
2007	Hcy, GSH	Human serum	PDMS Microchip capillary electrophoresis (μCE)	LIF	2.5-20 mM	1.25	49

* Homocysteine (Hcy), Glutathione (GSH), N-acetyl-l-cysteine (NAC), Cysteine (Cys), Cystine (Cys2), Methionine (Met), glutathione disulfide (GSSG)

2.4.2 Glutathione (GSH)

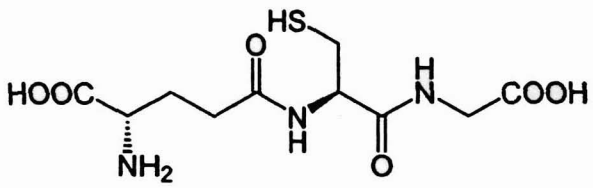

Figure 2.18 Chemical structure of glutathione.

Glutathione consists of three amino acid including glutamine, cysteine and glycine (Figure 2.18). In the low molecular weight thiols, glutathione is a significant antioxidant. It plays an important role in biological function such as a free radical scavenger (antioxidant), detoxification agent of xenobiotics and heavy metals, enzyme cofactor, thiol reducing equivalence, and control the signaling pathway. GSH involves with the regeneration of other antioxidants such as vitamin E and ascorbic acid.

The concentration of glutathione has found in plasma about 4.5 μM, in the liver about 4 to 8 μM. Lower concentrations of GSH may link to other diseases like inherited deficiency, immune deficiency, liver inflammation, pulmonary disease, neurological system. Furthermore, this condition indicated the risk of cardiovascular disease, diabetes mellitus, aging and cancer.

The numerous methods for determination GSH are developed in recent years. Table 2.3 shows various methods for the determination of GSH.

Table 2.3 The various methods for determination of GSH.

year	Analytes	Sample	Separation	Detection	Linear reange (μM)	LOD (μM)	Ref.
1993	GSH, Met, Cys, Cys_2	Human blood	LC	Pulsed Electro-chemical Detection (PED)	0.5-20	0.036	50
1997	GSH	-	CE	Pulsed electro-chemical (PED)	2.5 – 250	0.5	51
1997	GSH, Hcy, Cys	Human plasma	CE	UV	-	1	34
1999	GSH, Hcy, NAC	Human plasma	CE	LIF	-	3	36
2001	GSH, GSSG	Mouse liver tissue	HPLC	MS-MS	-	0.2 pmol	52
2002	GSH, GSSG, Hcy, Cys, AAs, peptides	Human plasma	CE	UV	-	2.35	53
2002	GSH, Cys, NAC, Hcy	Human urine	CE	ECD	-	134 nM	41
2002	GSH, Hcy, Cys2, Cys, Met, Hcy2, GSSG		HPLC	ECD	0.05-100	1	42
2003	GSH, GSSG	Human plasma	CE	UV	1-90	0.5	54
2003	GSH	Human hepatocar cinoma cells	CE	ECD	-	1.7	55
2003	GSH, Hcy, Cys, GSSG	Rat tissues	LC	MS	-	0.16	43
2004	Hcy,Cys, GSH,NAC	-	Glass Microchip capillary electrophoresis (μCE)	ECD	5-50	2.9	44
2007	GSH	-	HPLC	ECD	0.1-2.5	0.09	48
2007	GSH	Human serum	PDMS Microchip capillary electrophoresis	LIF	2.5-20 mM	1.04	49
2004	GSH, GSSG, GSSC, Cys	standards	HPLC	UV		5.0 nmol	56
2008	GSH, GSSG, Hcy, Cys, AAs	Standard, human whole blood	CE	UV	0-200	50	57

* Homocysteine (Hcy), Glutathione (GSH), N-acetyl-l-cysteine (NAC), Cysteine (Cys), Cystine (Cys2), Methionine (Met), glutathione disulfide (GSSG)

2.4.3 N-acetyl-L-cysteine (NAC)

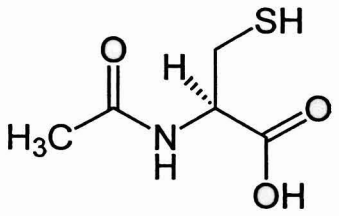

Figure 2.19 Chemical structure of N-acetyl-L-cysteine.

N-acetyl-L-cysteine is an amino acid which an acetylated derivative attracted to the amino group. The chemical structure of NAC shows in Figure 2.19. NAC is a necessary precursor for the synthesis of GSH within the body. NAC has performed as a free radical scavenger (antioxidant) and reactive oxygen species (ROS), that this activity makes NAC to a powerful antioxidant. NAC is used for treatment of paracetamol (acetaminophen) overdose and mucolytic agent. In addition, NAC may be useful for potentially therapeutic agent in treatment of cardiovascular diseases, cancer, human immunodeficiency virus (HIV) infection. Moreover, NAC is an important mucolytic agent that used to decrease the viscosity of pulmonary secretions for respiratory diseases. Several methods for determination NAC are shown in Table 2.4.

Table 2.4 The various methods for determination of NAC.

year	Analytes	Sample	Separation	Detection	Linear reange	LOD	Ref.
1984	Non-protein-bound N-acetyl cysteine	plasma	HPLC	-	-	-	58
1986	NAC	Human serum	HPLC	UV	-	3 µg/ml	59
1990	NAC, Cys, $(NAC)_2$, $(Cys)_2$	Human urine	LC	ECD	-	500 fmol	60
1991	NAC	Human plasma	GC	MS	0.1-2.0 µg/ml	-	61
1999	NAC, Hcy, GSH	Human plasma	CE	LIF	-	3 µM	36
2002	GSH, Cys, NAC, Hcy	Human urine	CE	ECD	-	104 nM	41
2004	Hcy,Cys, GSH,NAC	Standards	Glass Microchip capillary electrophoresis (µCE)	Electro-chemical (EC)	5-50 µM	3.3 µM	44
2006	NAC	Pharmaceutical sample	-	Sweep linear voltam-metry	1.2×10^{-4} – 8.3×10^{-4} mol/L^{-1}	6.3×10^{-5} mol/L^{-1}	62

* Homocysteine (Hcy), Glutathione (GSH), N-acetyl-l-cysteine (NAC), Cysteine (Cys), Cystine (Cys2), Methionine (Met), glutathione disulfide (GSSG)

CHAPTER III
EXPERIMENTAL

Electrochemical instruments set-up, chemicals, materials, and PDMS microchip fabrication are explained thoroughly in this chapter.

3.1 Instruments and equipments

3.1.1 Microchip fabrication

The instruments and equipments for PDMS microchip fabrication are listed in Table 3.1.

Table 3.1 List of instruments and equipments for PDMS microchip fabrication.

Instruments, equipments and chemicals	Details
Silicon wafer	University wafer, South Boston, MA.
Analytical balance	AB204-S, Mettle, Toledo
Vacuum pump	GAST manufacturing corporation
Oven	Model UFB 400, MEMMERT
Gold microwire	99.9%, diameter 25 μM, Good fellow, USA
Microscope	Model SZ-PT, Olympus, USA
Air plasma cleaner	Model PDG-32G, Harrick plasma cleaner/sterilizer
Silicone glue	Sparko high tech glass silicone, sparko USA, Inc., USA

Table 3.1 (continue) List of instruments and equipments for PDMS microchip fabrication.

Instruments, equipments and chemicals	Details
Conductive silver paint	SPI supplies, structure probe, Inc., USA
Electrical wire	ALPS industrial CO., LTD.

3.1.2 Electrochemical Analysis

The instruments and equipments for electrochemical analysis are listed in Table 3.2.

Table 3.2 List of instruments and equipments for electrochemical analysis.

Instruments and equipments	Details
Autolab Potentiostat	Model PG-30, Methrom
High-voltage power supply	Homemade
Platinum microwire	Good fellow, USA

3.1.3 Preparation of solutions

The instruments and equipments for preparation of solution are listed in Table 3.3.

Table 3.3 List of instruments and equipments for preparation of solution.

Instruments and equipments	Details
Millipore Milli-Q purification system	Model ZMQS 5 VOOY, Millipore, USA, R ≥ 18.2 MΩcm
pH meter	Metrohm 744 pH meter, Metrohm, Switzerland
Micropipette (20, 100, 1000, and 5000 µL) and tip	Eppendrof, Germany
Syringe filters PTFE	13 mm, 0.22 µm, RESTEK Corporation

3.2 Chemicals

All chemicals were an analytical grade or better, that were used as received without further purification. Aqueous solutions were prepared using deionized-distilled water obtained from a Milli-Q-system. Table 3.4 shows the chemicals, molecular formula and their suppliers.

Table 3.4 Chemicals, molecular formula, and corporation.

Chemicals	Molecular formula	Corporation
Sylgard 184 silicone elastomer	-	Dow Corning, USA
Curing agent	-	Dow Corning, USA
Methanol	CH_3OH	MERCK, Germany
Sodium hydroxide	NaOH	Fluka
Hydrochloric acid	HCl	MERCK, USA
2-Morpholinoethanesulfonic acid monohydrate (MES)	$C_6H_{13}NO_4S.H_2O$	Fluka, USA
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}O_4S$ Na	SIGMA, USA
Boric acid	H_3BO_3	SIGMA, USA
Dopamine hydrochloride	$C_8H_{11}NO_2.HCl$	SIGMA, USA
Pyrocatechol	$C_6H_6O_2$	Fluka, USA
L-Glutathione reduced	$C_{10}H_{17}N_3O_6S$	SIGMA, USA
N-Acetyl-l-cysteine	$C_{15}H_9NO_3S$	SIGMA, USA
Glucose	$CH_2OHCH(CHOH)_3CHOH$	CARLO ERBA

3.3 Preparation of solutions

This part mentions of the preparation procedures for the supporting electrolyte and the standard solutions that employed in this experimental.

1) Preparation of 0.1 M sodium hydroxide solution

0.400 g of sodium hydroxide was dissolved to 100 mL total volume with deionized water in a volumetric flask.

2) Preparation of 0.1 M hydrochloric acid

0.98 mL of 37%w/v hydrochloric acid was pipetted into a 100 mL volumetric flask and diluted with deionized water.

3) Preparation of 20 mM MES buffer

0.4265 g of MES was dissolved in deionized water. The resulting solution was adjusted to the required pH with either 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, and was diluted to 100 mL total volume with deionized water in a volumetric flask.

4) Preparation of boric acid buffer 20 mM

0.1237 g of boric acid was dissolved in deionized water. The resulting solution was adjusted to the required pH with either 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, and was diluted to 100 mL total volume with deionized water in a volumetric flask.

5) Preparation of 20 mM MES containing 1 mM SDS

0.425 g of MES and 0.0288 g of SDS were dissolved in deionized water. The resulting solution was adjusted to the required pH with either 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, and was diluted to 100 mL total volume with deionized water in a volumetric flask.

6) Preparation of 25 mM MES containing 3 mM SDS

0.5331 g of MES and 0.0865 g of SDS were dissolved in deionized water. The resulting solution was adjusted to the required pH with either 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, and was diluted to 100 mL total volume with deionized water in a volumetric flask.

7) Preparation of 0.01 M stock standard solution of dopamine

0.0190 g of dopamine hydrochloride was dissolved with 10.00 mL of 0.1 M hydrochloric acid.

8) Preparation of 0.01 M stock standard solution of catechol

0.0110 g of catechol was dissolved with 10.00 mL of 0.1 M hydrochloric acid.

9) Preparation of 10 mM stock standard solution of homocysteine

0.0068 g of homocysteine was dissolved with 5.00 mL of deionized water.

10) Preparation of 10 mM stock standard solution of glutathione

0.0153 g of glutathione was dissolved with 5.00 mL of deionized water.

11) Preparation of 10 mM stock standard solution of N-acetyl-L-cysteine

0.0082 g of N-acetyl-L-cysteine was dissolved with 5.00 mL of deionized water.

12) Preparation of 10 mM stock standard solution of glucose

0.0090 g of glucose was dissolved with 5.00 mL of deionized water.

3.4 PDMS microchip fabrication

The fabrication of poly(dimethylsiloxane) (PDMS) microchip was briefly performed in this part with modified from [63].

1. Sylgard 184 silicone elastomer and curing agent were mixed in the ratio of 10:1 (w/w). (Figure 3.1)

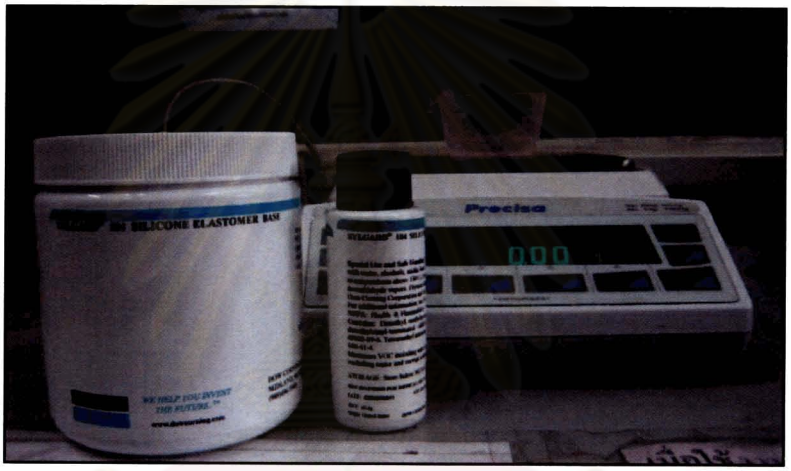

Figure 3.1 Sylgard 184 silicone elastomer and curing agent.

2. These mixture was degassed by vacuum. (Figure 3.2)

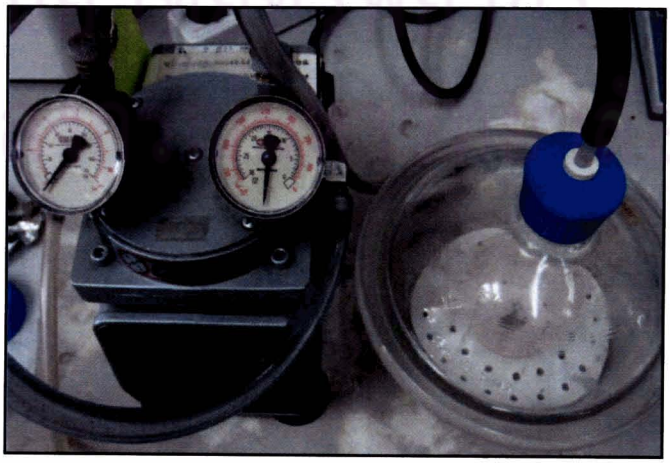

Figure 3.2 Vacuum pump for degassing.

3. The mixture was poured onto a molding master and a blank wafer and then was cured in an oven at least 2 h at 65 °C (Figure 3.3).

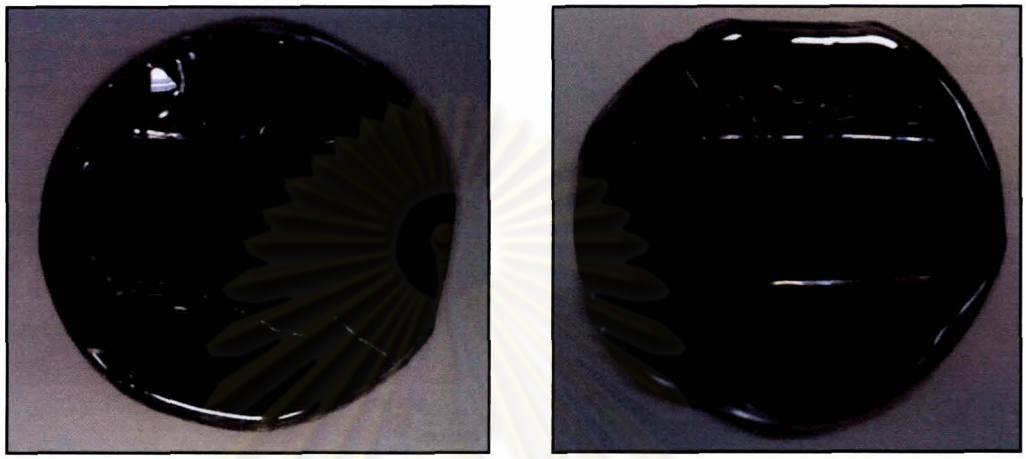

Figure 3.3 The molding master and a blank silicon wafer, respectively.

4. The cured PDMS were cut to separate from silicon wafer and then punched the holds (6 mm. of inner diameter) at the end of each channels (Figure 3.4).

Figure 3.4 The cured PDMS.

5. The gold wire (25 μm of inner diameter) was placed in the electrode channel as shown in Figure 3.5.

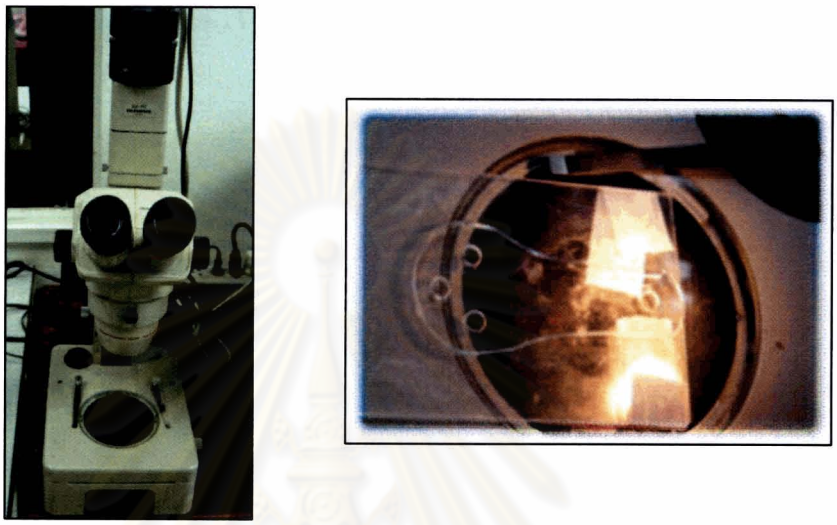

Figure 3.5 The microscope and Au microwire electrode placed in the electrode channel.

6. Then, the two PDMS layers were placed in air plasma cleaner (Figure 3.6) to oxidized for 45 s and immediately attached together to form an irreversible bond.

Figure 3.6 The air plasma cleaner.

7. The extremities of the electrode channel were sealed with super-glue. Finally, an electrical connection of the working electrode was achieved by silver paint and a silver wire as shown in Figure 3.7.

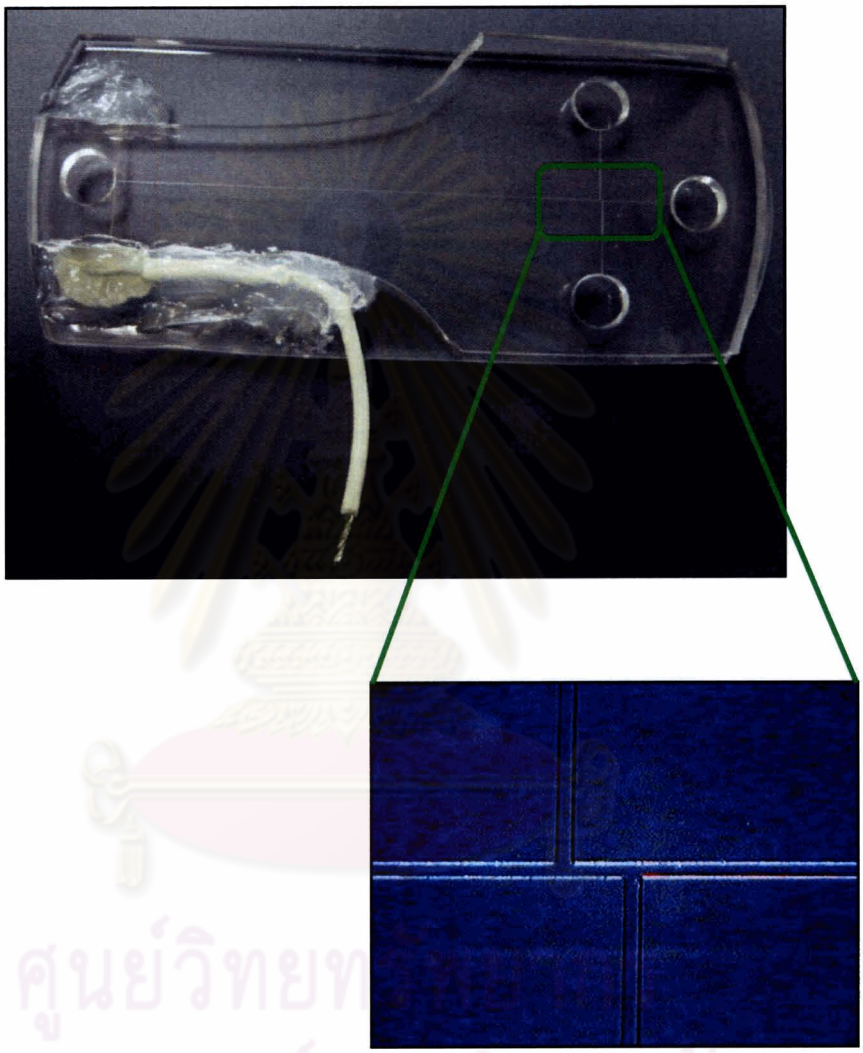

Figure 3.7 PDMS microchip and double-T injection channel.

3.5 Configuration of PDMS microchip

The configuration of PDMS microchip prepared by our fabrication is shown in Figure 3.8.

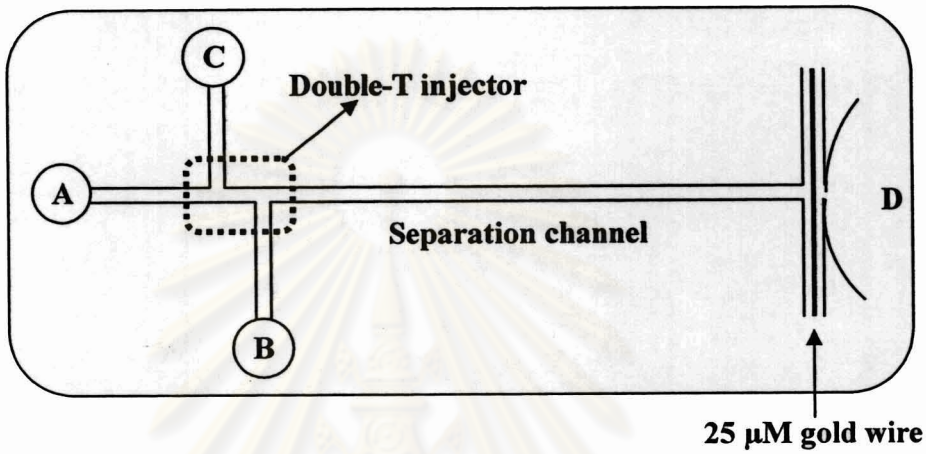

Figure 3.8 Pattern of PDMS microchip: (A) buffer reservoir, (B) sample reservoir, (C) sample waste reservoir, and (D) waste reservoir. All channels are 50 μm of width and depth. The length of separation and Double-T injection channel is 50 mm and 250 μm, respectively.

This microchip consists of two layers of PDMS that sealed together with an irreversible bond. A bottom layer was a blank PDMS layer. A top layer consists of four reservoirs including buffer, sample, sample waste and waste reservoir with a diameter of 6 mm, double-T injection channel, separation channel and working electrode channel (50 μm of width and depth). A length of separation channel and double-T injection channel were 50 mm and 250 μm, respectively. The double-T injector with a volume of 250 pL was used for our experiments.

A gold microwire that used as working electrode was placed in an electrode channel at the end of the separation channel. The electrode alignment in PDMS microchip is shown in Figure 3.9.

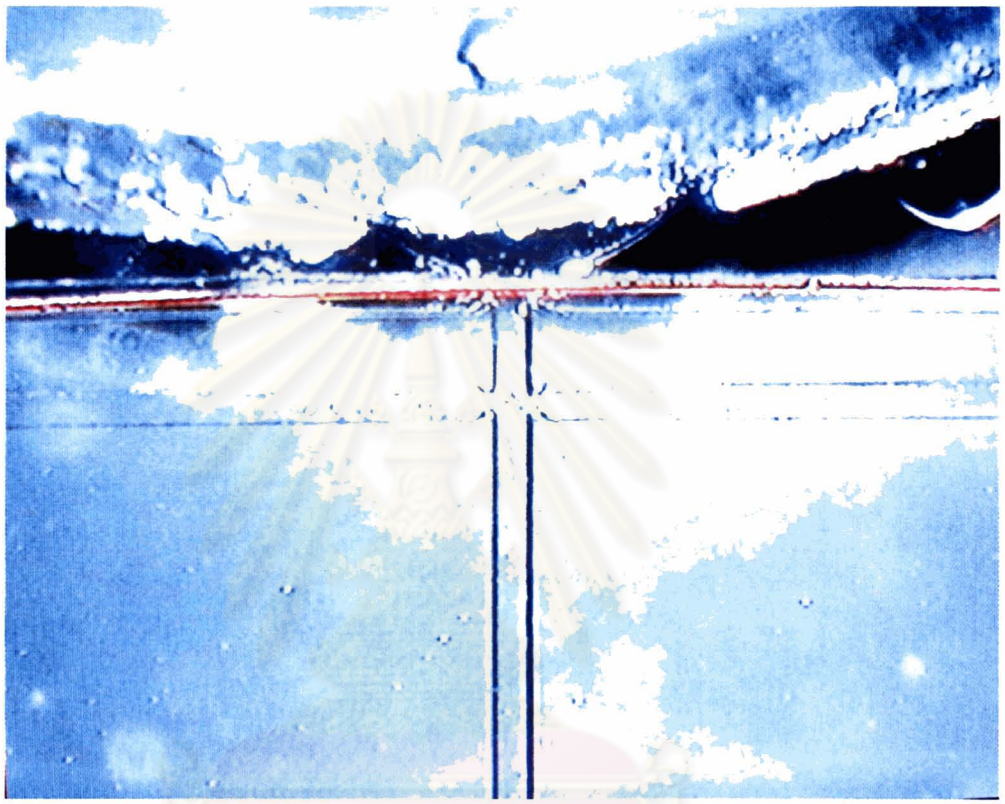

Figure 3.9 A gold microwire electrode in PDMS microchip.

3.6 Procedures of microchip capillary electrophoresis (microchip CE)

3.6.1 Microchip CE layout

The microchip CE system that used for these experiments is showed in Figure 3.10. This system consists of PDMS microchip, high voltage power supply, and electrochemical detector.

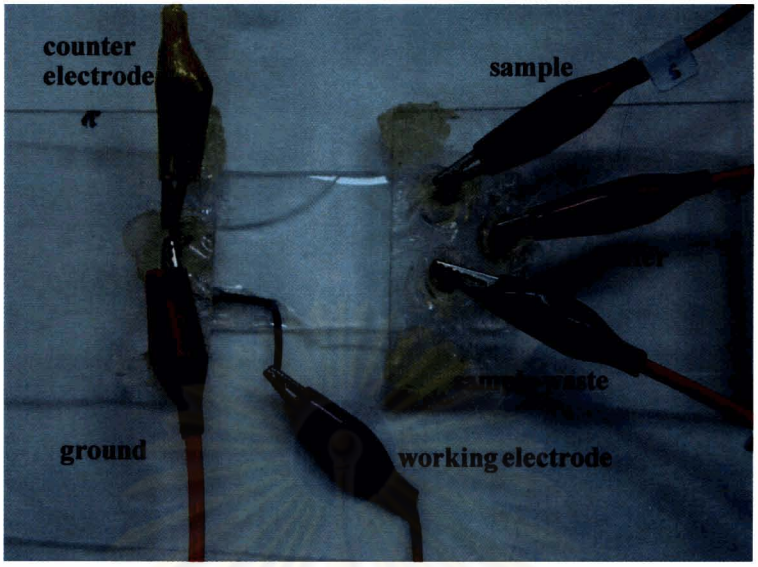

Figure 3.10 Microchip CE with electrochemical detection system.

CE system, a high-voltage DC power supply provides that electrical field that necessary to produce the EOF of the bulk solution inside a channel as well as electromigration of the charged analytes. Two positive high-voltage power supplies which adjustable voltage rang of 0 and +4000 V and a negative high-voltage power supply which adjustable voltage rang of 0 and -4000 V was used for supplying the high voltage to microchip CE. High voltage power supply shown in Figure 3.11 was used in this work.

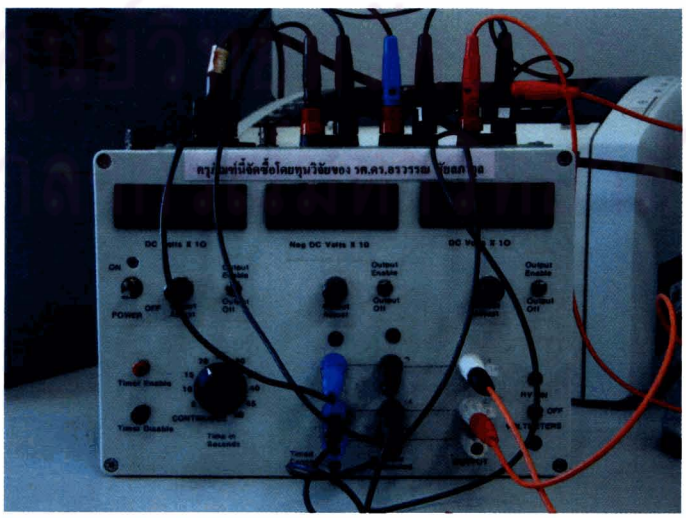

Figure 3.11 High voltage power supply.

3.6.2 Electrophoresis procedure

Before using PDMS microchip, a 0.1 M sodium hydroxide as filled through the channels for at least 30 min in order to generated negative charge at the PDMS surface and then rinsed with dionized water. After that, the running buffer was flushed into the channels at high voltage for 10 min. Next, the buffer at sample reservoir was removed and then the sample was replaced. Afterwards, a sample was injected into the separation channel at high voltage with pinched injection mode. The typical of potentials that used during the injection and separation procedure are shown in Figure 3.12 and summarized in Table 3.5. Injection step was performed by applying high voltage at the sample reservoir (+450 V) and a lower high voltage (-160 V) at the sample waste reservoir. Separation step was performed by applying high voltage (+1200 V) at the buffer reservoir and a lower high voltage (+450 V) at the sample and sample waste reservoirs. During injection and separation step, the waste reservoir maintained constantly at ground. The current signal related to the migration time of each analytes was monitored in real time.

Table 3.5 Potentials voltage applied during injection and separation for this experiment.

Reservoir	Injection (V)	Separation (V)
Sample	+450	+450
Sample waste	−160	+450
Buffer	+450	+1200
Waste	ground	ground

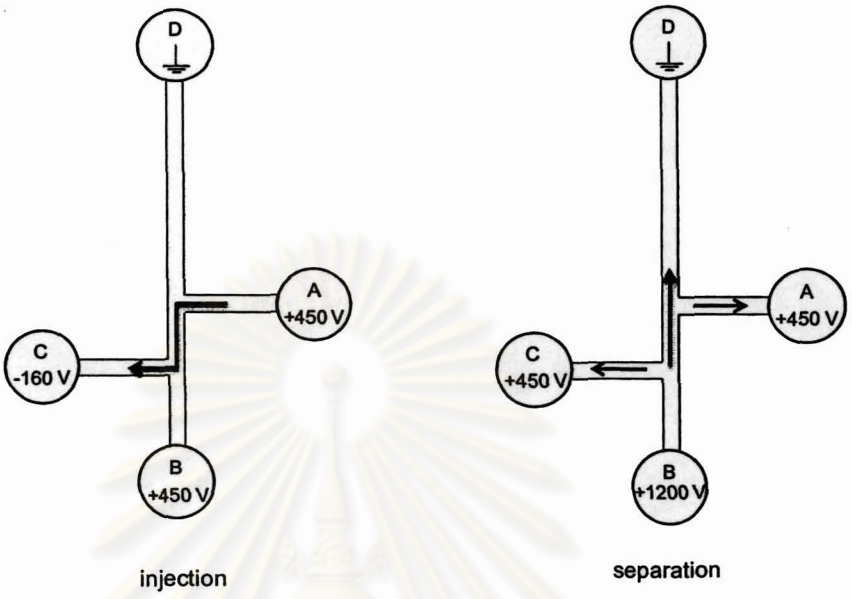

Figure 3.12 Format of potentials voltage applied during injection and separation for this experiment. (A) sample reservoir, (B) buffer reservoir, (C) sample waste reservoir, and (D) waste reservoir.

3.6.3 Electrochemical detection [64]

Electrochemical detection was performed by using amperometric detection and pulsed amperometric detection (PAD). Amperometric detection was used for the detection of dopamine and catechol compound. These compounds were chosen to characterize the efficiency of PDMS microchip capillary electrophoresis system. For PAD, it was used to analyze thiol compounds. Both amperometric detection and PAD were conducted by a commercially available potentiostat (Autolab Potentiostat, PG-30, Methrom) as shown in Figure 3.13. Result signals were reported in the form of "i-t curve".

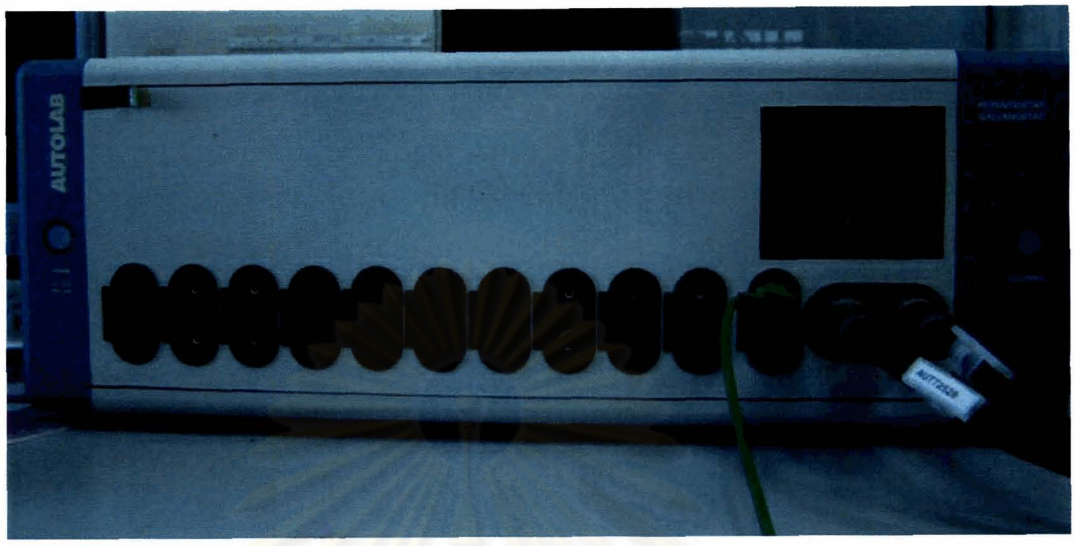

Figure 3.13 The potentiostat that was used for this work (Autolab Potentiostat, PG-30, Methrom, USA).

Both amperometric detection and PAD were run in a three electrode configuration. A gold microwire (25μm diameter) was used as the working electrode. The second electrode, a Pt wire (1.6 mm diameter) was used as counter and reference electrode that placed in the waste reservoir. For amperometric detection, a constant potential at 0.8 V was applied to the working electrode. For PAD waveform, the cleaning/oxidation and the reduction/regeneration potential was held +1.6 V and -0.5 V for 0.05 s, respectively. Finally, the detection potential was applied at +0.8 V for 0.2 s. The parameters of PAD waveform is summarized in Table 3.6.

Table 3.6 Pulsed amperometric detection parameter for the detection of thiol compounds.

step of PAD	Potential (V)	Time (s)
Clean	+1.6	0.05
Reactivate	-0.5	0.05
Detect	+0.8	0.2

3.6.4 Safety consideration

The high voltage power supply was used for this experiment. Therefore, the high voltage power supply and associated open electrical connection should be touched with extremely care to prevent an electrical shock. Metal ions, volatile, acid, and base are toxic, irritant, and dangerous for the environment that should be handled in the fume hood carefully. All accident including skin or eye contact, ingestion, and inhalation should be avoided. The waste solutions of acid or base must be stored in a closed small glass container, and isolated from any chemicals or reagents.

3.7 Conditions optimization of Microchip CE

The determination of thiol compounds was studies in this work. Therefore, the conditions of microchip CE with electrochemical detection for thiol compounds detection were optimized. These parameters were investigated such as separation voltage, detection potential, injection time, pH of buffer, concentration of buffer, and concentration of SDS. A single variable parameter was varied while all other parameters were constant until the optimal result was obtained. The features including the migration time, the current, resolution, and separation efficiency were considered during the optimization of conditions.

3.7.1 Effect of separation potential

The optimization of separation potential in the range of 500 – 1300 V was required to improve the detection sensitivity and the resolution of separation.

3.7.2 Effect of detection potential

The effect of detection potential of each analytes was studied in this work between +0.6V and +1.1V. The maximum current of thiol compound detection obtained from the optimization of detection potential will be selected for the next experiments.

3.7.3 Effect of pH buffer

The influence of pH buffer was investigated under the various pH of buffer to obtain the good separation of the analytes at the optimum pH buffer. The optimization of pH buffer was investigated between pH 5 and 7.5.

3.7.4 Effect of buffer concentration

The buffer concentration has effected on analysis time and sensitivity so it was studied using various concentration of buffer. The effect of buffer concentration was optimized in the range of 5 - 30 mM

3.7.5 Effect of injection time

The effect of injection time was optimized in the range of 10 – 30 s to get the maximum current response and resolution of thiol compounds.

3.7.6 Linear range

10 mM stock solutions of each analytes were freshly prepared prior to use and then diluted to final concentrations of range from 2.5 to 400 μM. For this experiment, each concentration of the analytes was determined at three replicates. Next, the results were used to plot the calibration curve of each analytes. Then, the linear range of these analytes was obtained.

3.7.7 Limit of detection (LOD)

Limit of detection (LOD) was carried out by calculating the concentration of analytes for three times standard deviation of background signal under the optimal condition. A definition of LOD is the concentration that provide a signal response three times higher than the noise ($S/N \geq 3$).

3.7.8 Limit of quantitation (LOQ)

Limit of quantitation (LOQ) was carried out by calculating the concentration of analytes for three times standard deviation of background under the optimal condition. A definition of LOQ is the concentration that provided a signal response ten times higher than the noise ($S/N \geq 10$).

3.7.9 Repeatability

The repeatability was determined by injection three times of each analyte solution in the same PDMS microchip CE. The repeatability is estimated in term of the relative standard deviation (%RSD), follow by this formula (Eq. 3.1):

$$\%\,RSD = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \qquad (3.1)$$

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Microchip capillary electrophoresis characterization

The PDMS microchip was characterized before it was used to detect the sample. Dopamine and catechol were chosen to study the performance of PDMS microchip using amperometry.

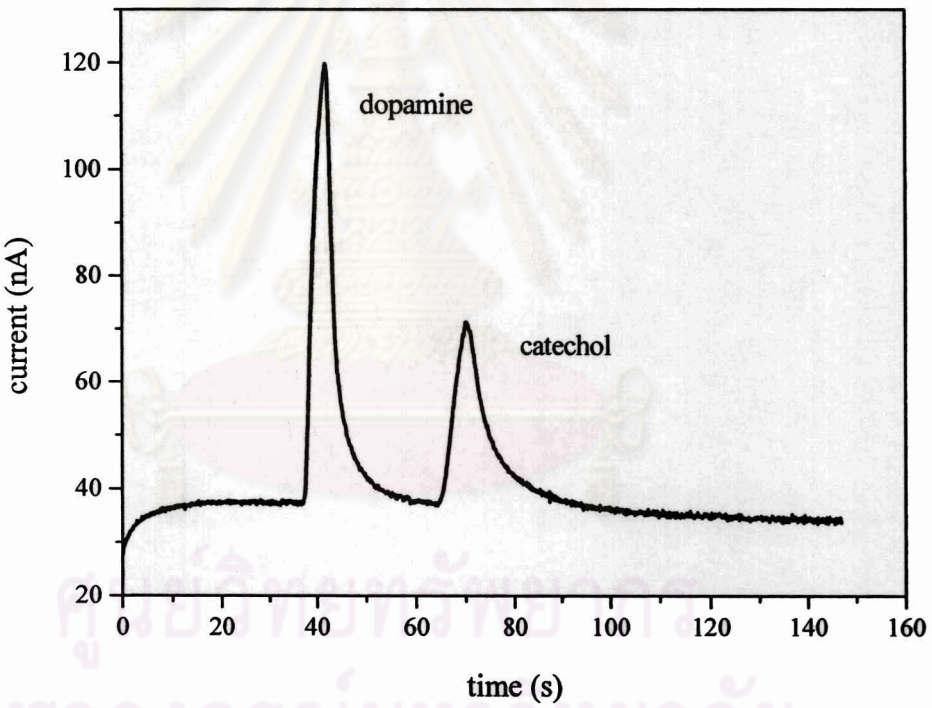

Figure 4.1 Electropherogram for the separation of dopamine and catechol. Conditions: 250 μM dopamine and 250 μM catechol; buffer = 20 mM MES buffer (pH 7.0); separation voltage = 1300 V; detection potential = +0.8 V; injection time = 15 s.; working electrode; 25 μm Au wire.

The electropherogram obtained from separating the mixture of 250 μM dopamine and 250 μM catechol was shown in Figure 4.1. The conditions for the separation were 20 mM MES buffer (pH 7.0) as running buffer, separation potential of 1300 V, detection potential of 0.8 V and injection time of 15 s. As can be observed, two of these analytes can be well separated and the migration times of dopamine and catechol were 42 and 73 s, respectively. This indicated that the PDMS microchip can be next used to determine analytes. For well separation of dopamine and catechol demonstrated that the PDMS microchip can be used for the separation of other analytes.

4.2 Determination of glucose and homocysteine

4.2.1 Determination of glucose and homocysteine using sodium hydroxide as running electrolyte

Du et.al. (2004) [65] reported the detection of glucose in human plasma on microchip capillary electrophoresis with electrochemical detection. The microchip was made from hybrid poly(dimethylsiloxane) (PDMS) / glass chip. The running electrolyte was sodium hydroxide. Thus, sodium hydroxide was used as running electrolyte for the determination of glucose and homocysteine in this experiment.

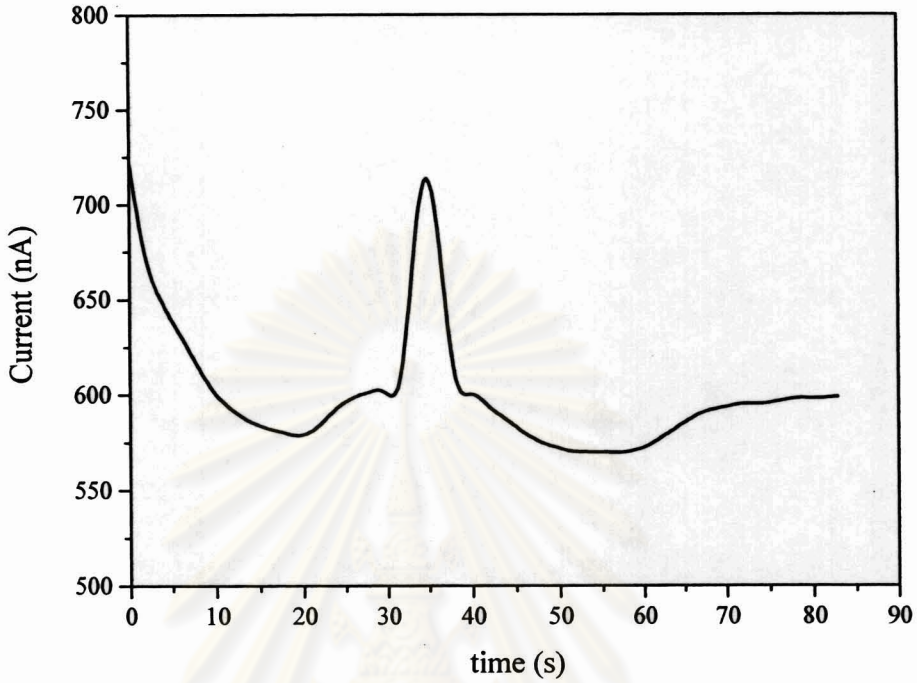

Figure 4.2 The electropherogram of glucose. Conditions: 500 μM glucose; running electrolyte = 10 mM NaOH; separation voltage = 1400 V; detection potential 1.0 V; pinched injection time 15 s; working electrode; 25 μm Au wire.

From Figure 4.2, glucose could be detected at migration time of 35 s using sodium hydroxide as the running electrolyte.

Then, this running electrolyte was also used to test the separation of homocysteine and glucose. Unfortunately, as can be noticed, the mixture compounds could not be separated at these conditions.

Chen et.al. (2004) [44], has studied the determination of thiol compounds (homocysteine, cysteine, glutathione and N-acetyl-l-cysteine) using miniaturized fused silica capillary with phosphate buffer. Carbon nanotube microelectrode was used as working electrode. Therefore, the electrolyte was changed from sodium hydroxide to phosphate buffer and was applied for the next experiment (4.2.2).

4.2.2 Determination of glucose and homocysteine using phosphate buffer with sodium dodecyl sulfate (SDS) as running electrolyte

Using only phosphate buffer to separation the mixture of glucose and homocysteine, it was found that the mixture of homocysteine and glucose could not be separated.

To adjust the condition, the phosphate was modified by adding SDS to obtain better separation. SDS is an anionic surfactant. The concentration of SDS that was added in buffer is higher than the critical micelle concentration (CMC) to form nanosized structures (micelles). The CMC of SDS in pure water at 25 °C is 0.0082 M [66]. Micelles were characterized as pseudo-stationary phase in micellar electrokinetic chromatography (MECK) inside the microchannels. The solutes are separated based on the different partition of analytes between aqueous phase and micelles phase. Therefore, the different solutes can be separated.

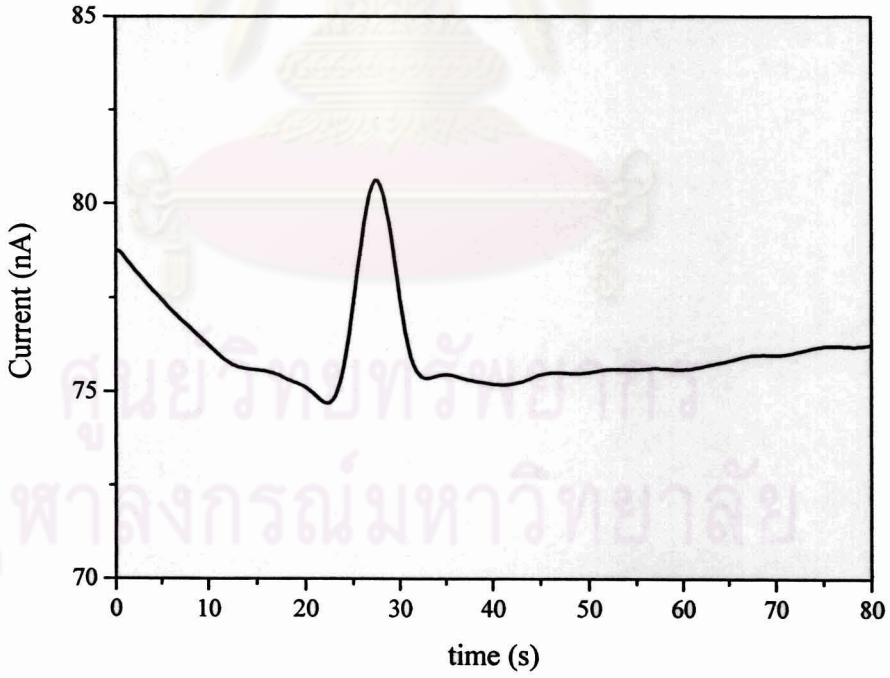

Figure 4.3 The electropherogram of glucose. Conditions: 250 μM glucose; running electrolyte = 20 mM Phosphate + 10 mM SDS buffer pH 7.4; separation voltage = 1300 V; detection potential 0.7 V; pinched injection time 15 s; working electrode; 25 μm Au wire.

Figure 4.3 shows the standard glucose detected at migration time of 27 s when using phosphate buffer with SDS.

In these conditions, the standard homocysteine was also studied.

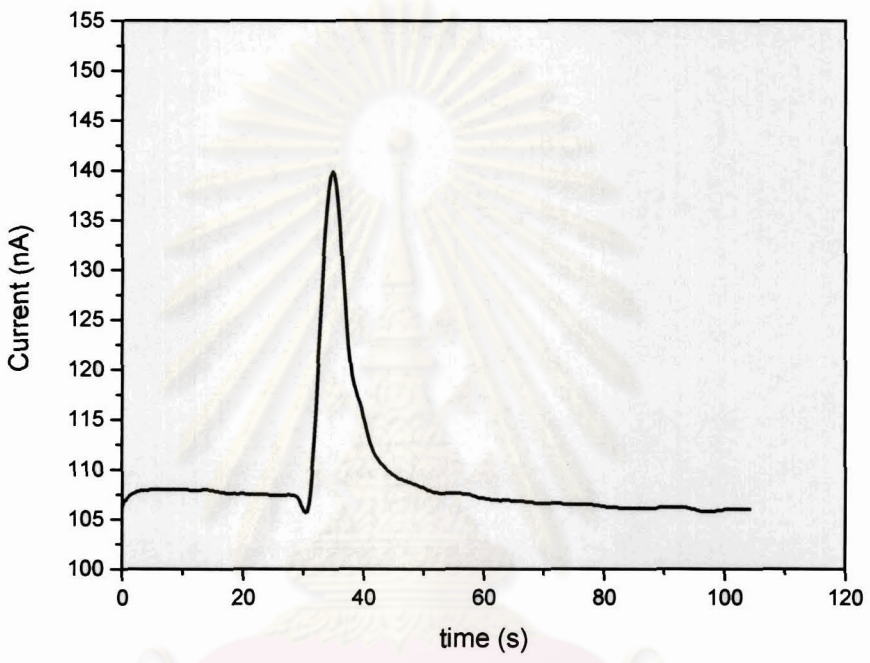

Figure 4.4 The electropherogram of homocysteine. Conditions: 250 μM homocysteine; running electrolyte = 20 mM phosphate + 10 mM SDS buffer pH 7.4; separation voltage = 1300 V; detection potential 0.7 V; pinched injection time 15 s; working electrode; 25 μm Au wire.

Figure 4.4 show that homocysteine could be detected at migration time of 36 s when using phosphate buffer.

Then, the mixture of homocysteine and glucose were analyzed using phosphate buffer with SDS (Figure 4.5).

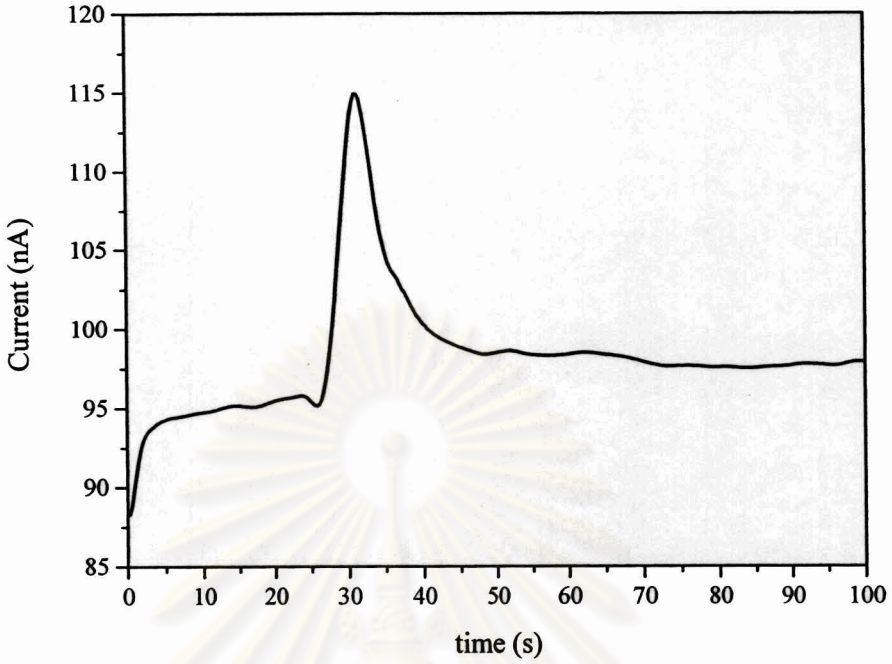

Figure 4.5 The electropherogram of glucose and homocysteine. Conditions: 250 μM glucose + 150 μM homocysteine ; running buffer 20 mM phosphate + 10 mM SDS buffer pH 7.4 ; separation voltage 1300 V ; detection potential 0.7 V; pinched injection time 15 s ; working electrode 25 μm Au wire.

From the results, even though the standard of glucose or homocysteine could be detected at 27 and 36 s, respectively (Figure 4.3, 4.4). However, the mixture solution could not be separated in these conditions (Figure 4.5).

Furthermore, the various concentrations of SDS in the ranging from 10 mM to 40 mM were investigated but these mixture solutions were still not separated. Therefore, the pH of buffer solution was varied to pH 7.4, 7.5 and 8.0. However, glucose and homocysteine still could not separate. From results obtained, the use of weak base of phosphate can not be used to separate glucose and homocysteine.

According to the results that the glucose and homocysteine could not be separated in phosphate buffer with SDS, the electrolyte was changed from phosphate to boric acid for the separation of glucose and homocysteine. The next effort is using boric acid as the electrolyte.

4.2.3 Determination of glucose and homocysteine using boric acid with sodium dodecyl sulfate (SDS) as running electrolyte

Vickers (2005) [64] reported a method of incorporating a functional Pd microwire decoupler with Au or Pt working electrodes in microchip CE-EC devices. The separation was performed using boric acid with SDS buffer solution. This system was applied to detect catecholamines, carbohydrates and thiols compounds. Thus, the boric acid solution was selected to analyze glucose and homocysteine in this work.

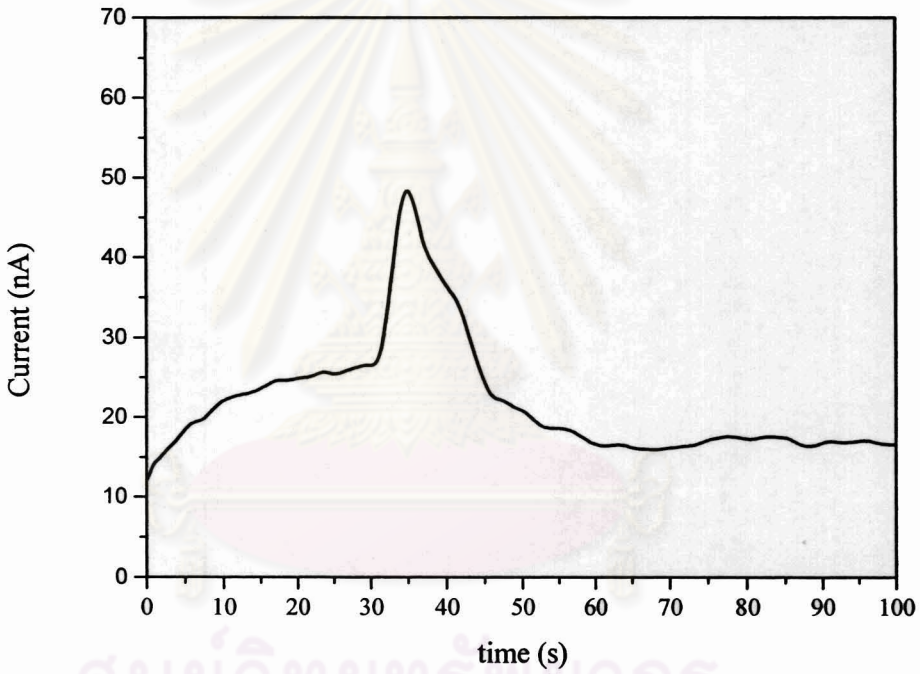

Figure 4.6 The electropherogram of glucose. conditions : 300 μM glucose ; running buffer 20 mM boric acid + 20 mM SDS buffer pH 9.0 ; separation voltage 1000 V ; detection potential 0.7 V; pinched injection time 15 s ; working electrode 25 μm Au wire.

An electropherogram for the detection of glucose was shown in Figure 4.6. The standard glucose solution was detected at migration time of 35 s.

Next, the standard homocysteine was determined by these conditions (Figure 4.7).

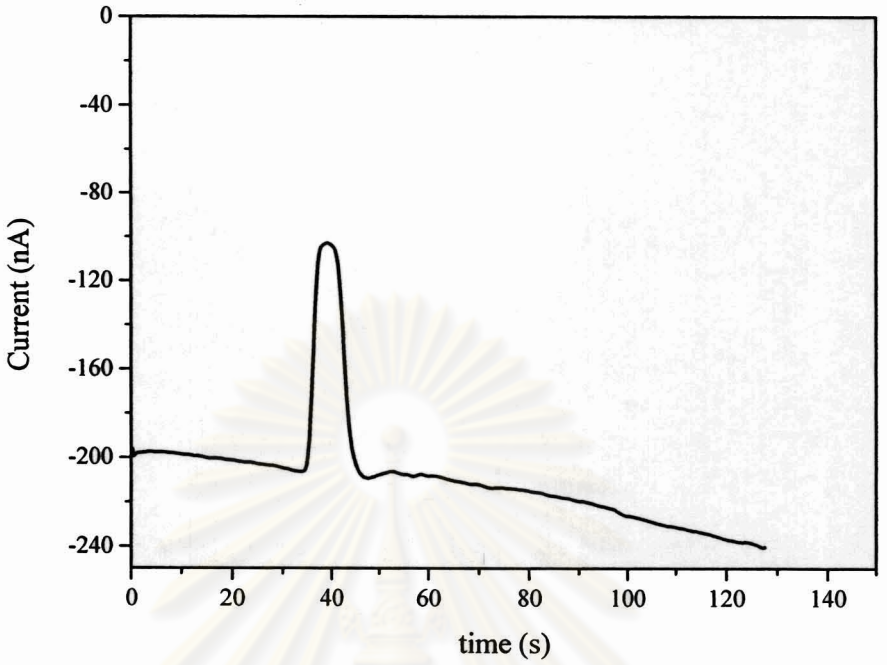

Figure 4.7 The electropherogram of homocysteine. conditions: 250 μM homocysteine ; running buffer 20 mM boric acid + 20 mM SDS buffer pH 9.0 ; separation voltage 1000 V ; detection potential 0.7 V; pinched injection time 15 s ; working electrode 25 μm Au wire.

An electropherogram for the detection of homocysteine was shown in Figure 4.7. The standard homocysteine solution was also detected at migration time of 39 s.

Then, the mixture of homocysteine and glucose were analyzed using boric acid buffer with SDS (Figure 4.8).

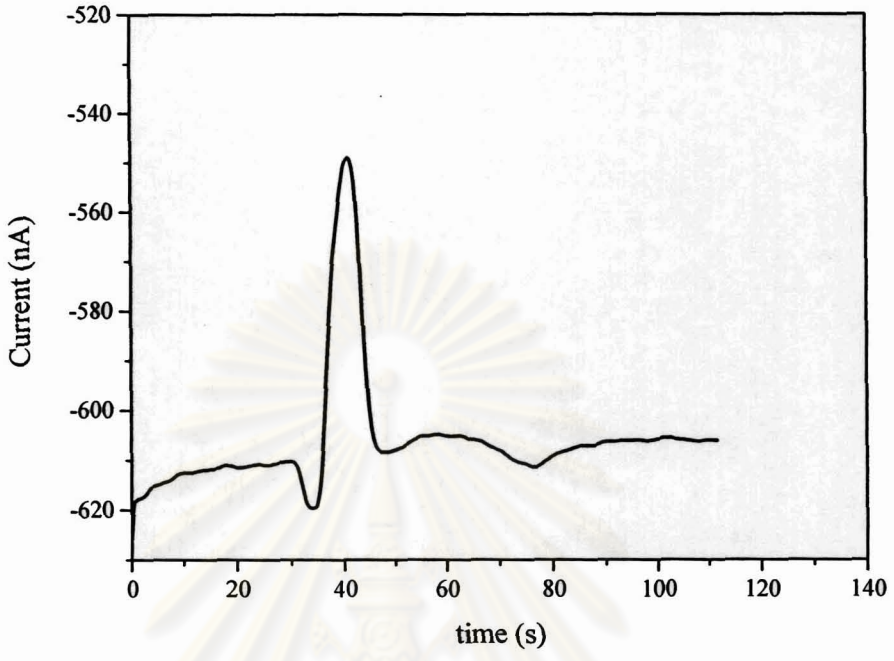

Figure 4.8 The electropherogram of glucose and homocysteine. Conditions : 300 μM glucose + 300 μM homocysteine ; running buffer 20 mM boric acid + 20 mM SDS buffer pH 9.0 ; separation voltage 1000 V ; detection potential 0.7 V ; pinched injection time 15 s ; working electrode 25 μm Au wire.

The electropherogram for the mixture of glucose and homocysteine solution is shown in Figure 4.8. However, these mixtures could not be separated using boric acid as electrolyte due to the only one peak was observed at migration time of 41 s. Thus, the alteration of electrolyte to boric acid with SDS does not successfully provided the separation of the mixture of glucose and homocysteine.

It can be seen that the standard of homocysteine and glucose can be detected separately but the mixture of these solutions can not be separated. This indicated that the thiol and glucose compounds can not be separated.

After that, the rest of the experiments was done to determine thiol compounds such as homocysteine and glutathione (see in 4.3).

4.3 Determination of homocysteine and glutathione

4.3.1 Determination of homocysteine and glutathione using boric acid as running electrolyte

From 4.2.3, the detection of standard homocysteine solution was performed using boric acid buffer. Therefore, boric acid buffer was first used for analysis of homocysteine and glutathione because these compounds were in the same family.

The effect of detection potential on the signal was determined between 0.8 and 1.6 V for analysis the mixture of homocysteine and glutathione. Figure 4.9 shows the electropherograms for homocysteine and glutathione using boric acid buffer. The detection order is homocysteine and glutathione, respectively. The mixture solutions are not absolutely separation at the detection potential lower than 1.1 V. Meanwhile, the peaks were broadening and noise level was high at the detection potential upper than 1.1 V because when current flow inside the channel induces joule heating. Thus, the optimal detection potential is +1.1 V was used for the determination of homocysteine and glutathione.

From these results (Figure 4.9), the mixture of homocysteine and glutathione can be completely separated by the use of boric acid as the buffer solution. To increase the performance of method, another thiol, N-acetyl-L-cysteine was added into the mixture.

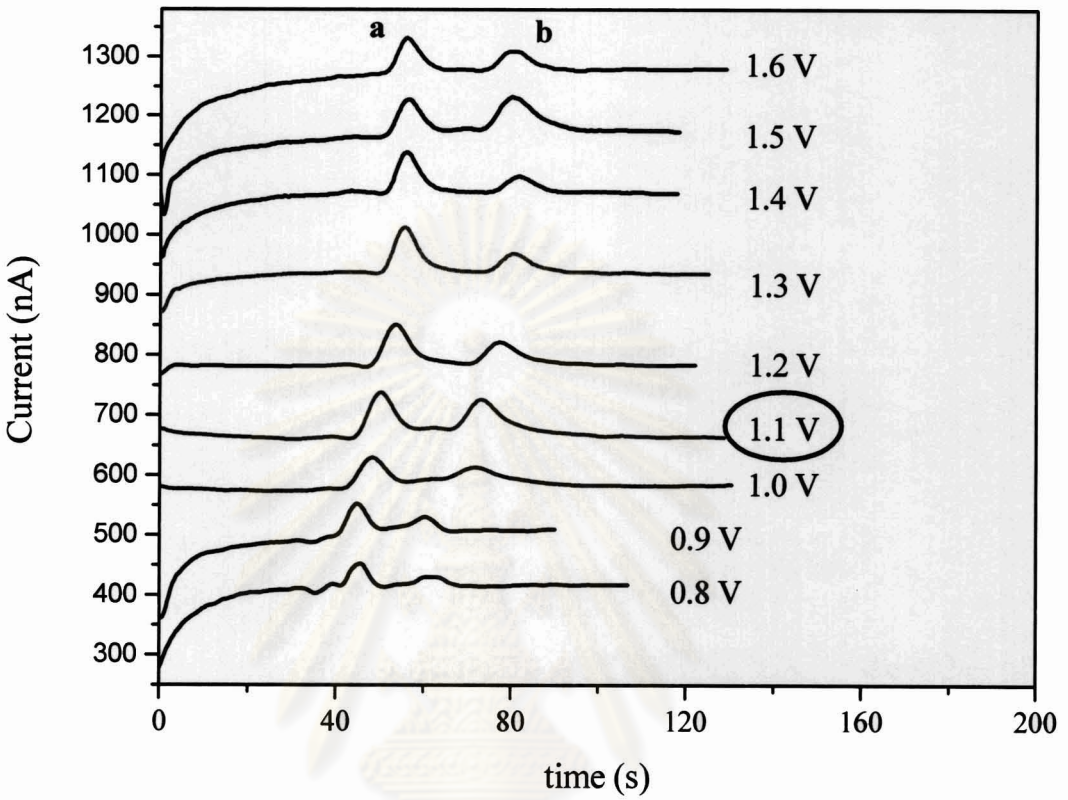

Figure 4.9 The electropherogram of homocysteine and glutathione. Conditions : 300 μM homocysteine (a) + 500 μM glutathione (b); running buffer 20 mM boric acid pH 9.0 ; separation voltage 1300 V ; detection potential 0.8 – 1.6 V ; pinched injection time 20 s ; working electrode 25 μm Au wire.

4.4 Determination of homocysteine, glutathione and N-acetyl-L-cysteine

4.4.1 Determination of homocysteine, glutathione and N-acetyl-L-cysteine using boric acid as running electrolyte

4.4.1.1 Effect of injection time

The injection time affects the sample volume, and then also affects the peak current and peak shape. Thus, time required for sample injection was investigated. The effect of injection time was studied between 15 and 30 s as shown in Figure 4.10. From Figure 4.10, the migration time of each analytes decreased but peak shape was broad after increasing the injection time. If injection time was more than 15 s, the peak current increased but the separation was not good. From these results, 15 s was chosen as the injection time for determination of Hcy, GSH, and NAC using boric acid as buffer.

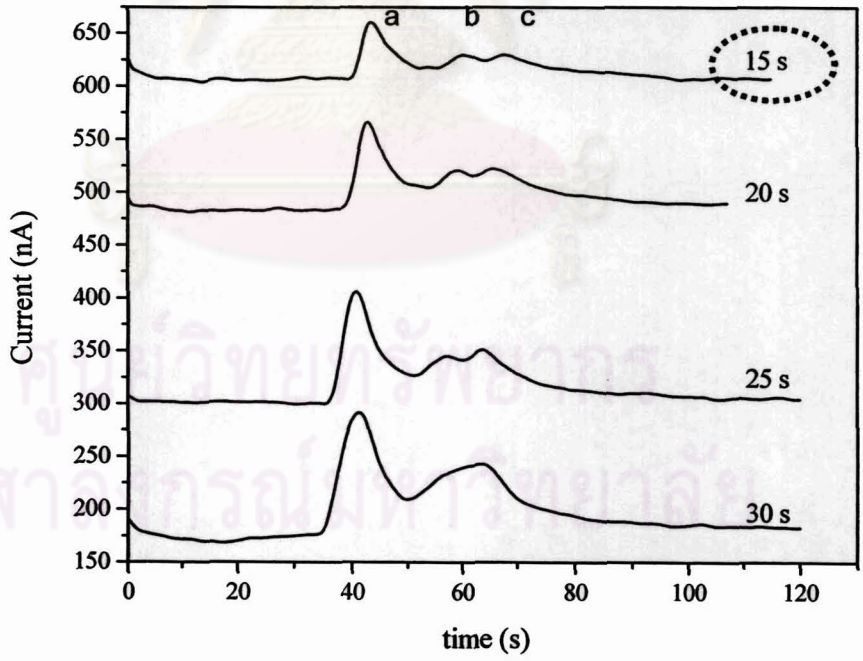

Figure 4.10 The electropherograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions : 250 μM (a) homocysteine (b) glutathione and (c) N-acetyl-L-cysteine ; running buffer 20 mM boric acid (pH 9.0) ; separation voltage 1300 V ; detection potential 1.4 V ; working electrode 25 μm Au wire.

4.4.1.2 Effect of separation potential

The effect of separation potential was investigated between 1,000 and 1,400 V. Figure 4.11 shows the effect of separation potential on the separation efficiency including migration time and peak shape. Migration time of each analytes decreased when the separation potential increased. However, the current responses were not different for every separation potentials. Therefore, at 1,400 V was selected as the separation potential for determination of Hcy, GSH, and NAC using boric acid as buffer.

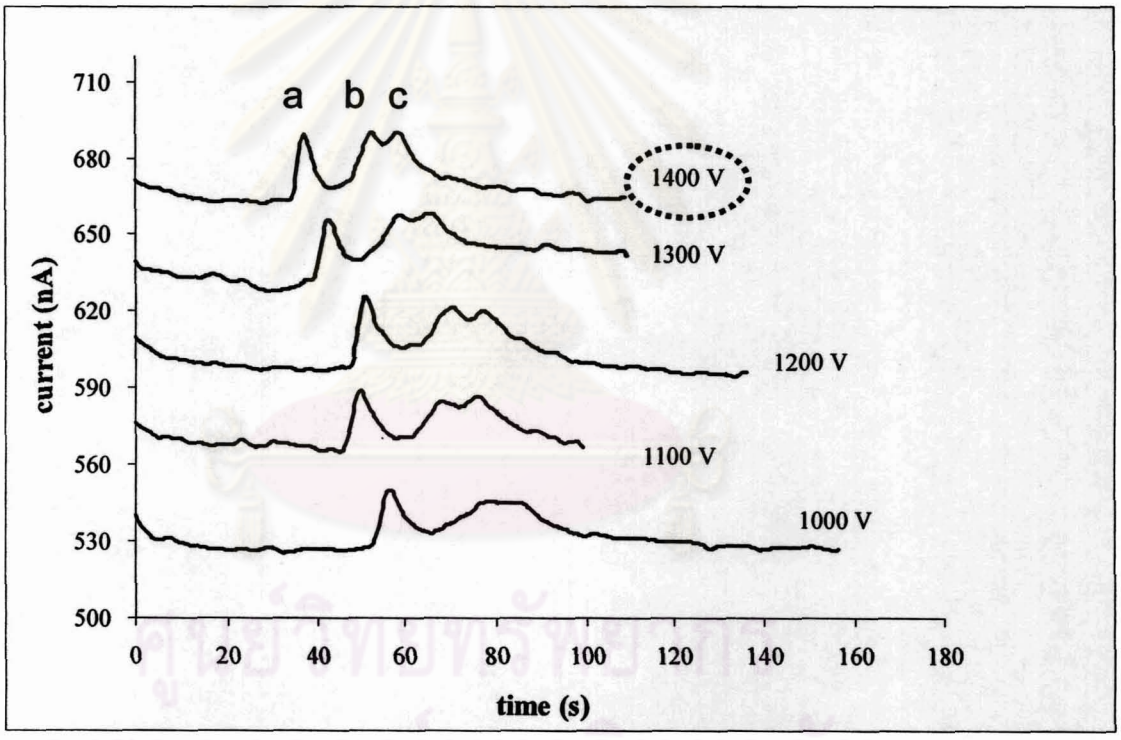

Figure 4.11 The electropherograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions : 250 μM (a) homocysteine (b) glutathione and (c) N-acetyl-L-cysteine ; running buffer 20 mM boric acid (pH 9.0) ; detection potential 1.4 V ; pinched injection time 15 s ; working electrode 25 μm Au wire.

4.4.1.3 Effect of pH buffer

For PDMS microchip CE, pH of running buffer is important for controlling the EOF and separation efficiency. The effect of pH buffer on separation efficiency was determined between pH 7.0 and 9.0. The relationship between peak current and pH of buffer is shown in Figure 4.12. The currents of Hcy, GSH, and NAC decreased with the decreasing of pH. At the same time, the resolutions of Hcy, GSH, and NAC increased with the decreasing of pH. It was found that the analytes can be well separated at pH 8.0. Therefore, pH 8.0 was chosen as the optimal value for the determination of Hcy, GSH, and NAC using boric acid as buffer because the analytes can be best separated at this buffer pH.

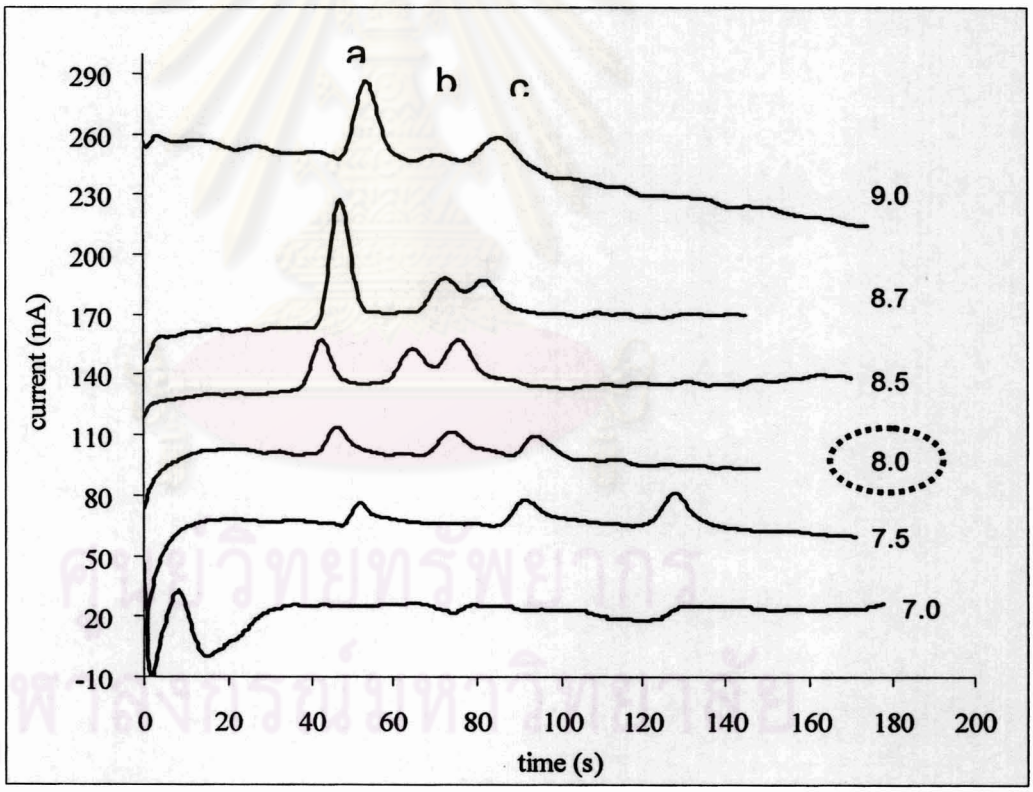

Figure 4.12 The electropherograms of homocysteine, glutathione and N-acetyl-L cysteine. Conditions : 250 μM (a) homocysteine (b) glutathione and (c) N-acetyl-L-cysteine ; running buffer 20 mM boric acid; detection potential 1.4 V ; separation voltage 1400 V; pinched injection time 15 s ; working electrode 25 μm Au wire.

From the results, three thiol compounds can be separated with boric acid buffer. However, the use of boric acid buffer at higher pH results yielded the disulfide bond between these thiol compounds. From this effect, the thiol compounds are very difficult to determine. Therefore, the buffer is changed from boric acid to MES buffer. MES buffer is used as running buffer in the microchip capillary electrophoresis system.

4.4.2 Determination of homocysteine, glutathione and N-acetyl-L-cysteine using MES as running electrolyte

MES is a common name of the 2-(*N*-morpholino)ethanesulfonic acid compound. As well known, MES is used as buffering agent in biochemical analysis [67-69]. In addition, this buffer is rather safe. Therefore, MES was selected to use as buffer agent for analysis of homocysteine, glutathione and N-acetyl-L-cysteine.

4.4.2.1 Effect of injection time

The injection time required for sample loading was studied. The separation efficiency can be controlled by injection time. In addition, the injection time effects to the sample plug, that also affects the peak current and peak shape.

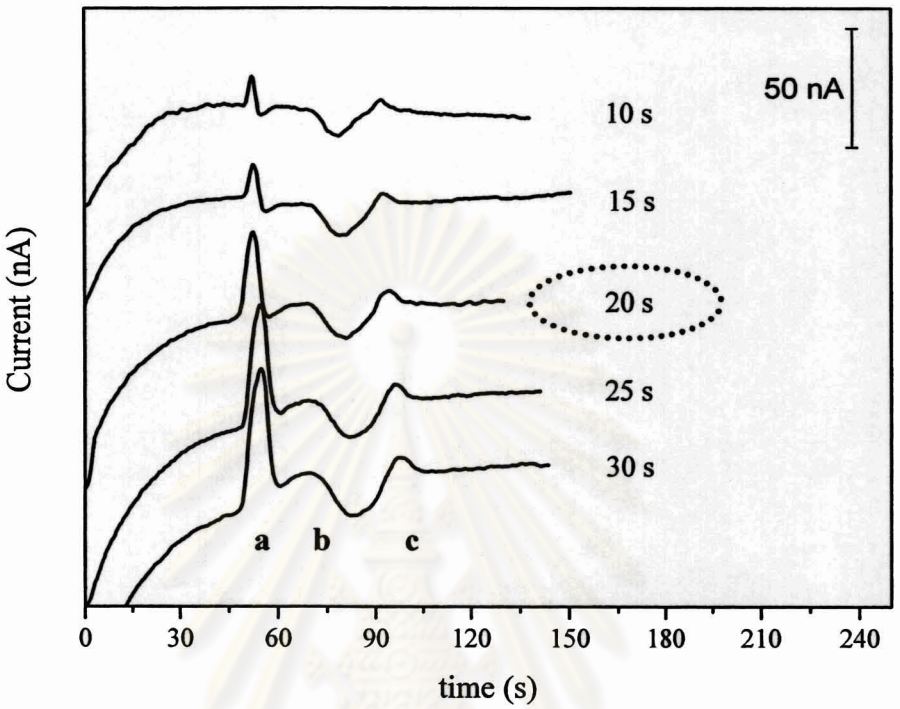

Figure 4.13 The electropherograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; 250 μM homocysteine (a), 250 μM glutathione (b), 250 μM N-acetyl-L-cysteine (c) ; running buffer 20 mM MES + 1 mM SDS pH 6.0 ; detection potential 1.0 V ; separation voltage 1000 V ; working electrode 25 μm Au wire.

The injection time was studied between 10 s and 30 s as shown in Figure 4.13. The mixture of thiol compounds was determined using these conditions (running buffer of 20 mM MES + 1 mM SDS pH 6.0, detection potential of 1.0 V, separation voltage of 1000 V). The injection time affects the peak current and peak shape. As can be seen, when the injection time increased, the peak current also increased but the peak shape was broadened. If the injection time was more than 20 s, the signal was increased gradually. In the following experiments, 20 s was selected as the optimal injection time for the determination of these thiol compounds.

4.4.2.2 Effect of detection potential

For microchip CE with electrochemical detection system, the detection potential strongly affects on the sensitivity. The relationship between the detection potential and the peak current (hydrodynamic voltammogram) was studied. The detection potentials of Hcy, GSH, and NAC were measured from +0.6 V to + 1.1 V as shown in Figure 4.14. As can be observed, the signal response of Hcy is higher than the GSH and NAC at the same condition. The peak current increased when the potential increased. Then, maximum of the current was reached, after that the level off. However, the signal decreased when higher the potentials was applied. It can be explained that the oxide is formed on the working electrode surface during the measurement. On the other hand, the oxidation of the analytes is inhibited [70,71]. The maximum current of Hcy, GSH, and NAC were obtained at +0.8 V. Thus, this potential was chosen as the optimal detection potential.

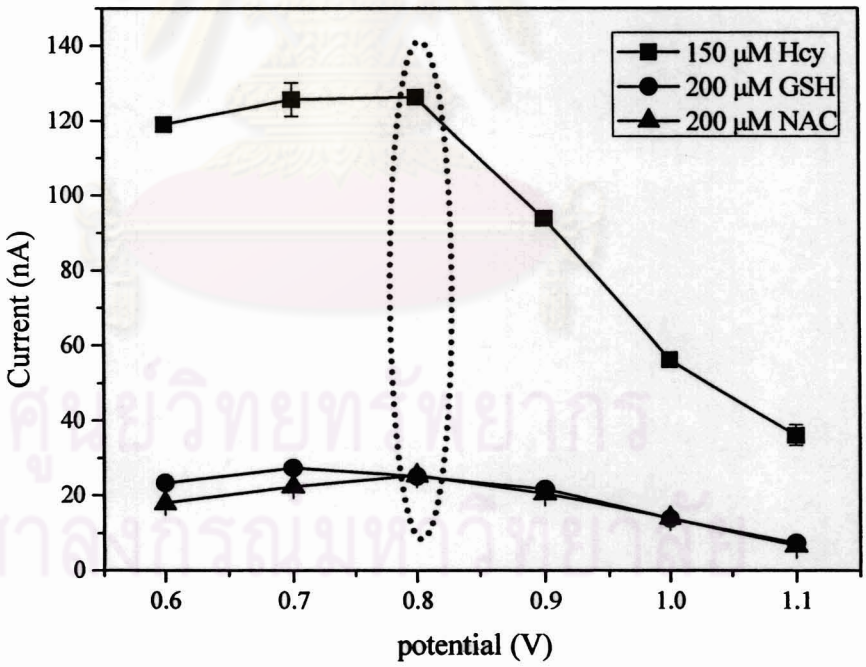

Figure 4.14 Hydrodynamic voltammograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; 150 μM homocysteine (■), 200 μM glutathione (●), 200 μM N-acetyl-L-cysteine (▲) ; running buffer 20 mM MES + 1 mM SDS pH 6.0 ; separation voltage 1200 V ; pinched injection time 20 s ; working electrode 25 μm Au wire.

4.4.2.3 Effect of separation potential

For microchip CE system, the separation potential affected on the electric field strength, EOF, separation resolution, migration time and migration velocity of each analytes within the channel. The electrochemical response was resulted from the coupling of the electric field for separation and the electrochemical detector, so optimizing separation voltage was needed to improve detection sensitivity. When higher separation voltage was applied, it produced an air bubble that caused of clogging in the channel and caused of higher joule heating. The relationship between current signals and migration time obtained from mixture of Hcy, GSH, and NAC. The separation potential of Hcy, GSH, and NAC were examined between 500 V to 1300 V as shown in Figure 4.15. It was found that the migration times of each analytes decrease after increasing the separation potential between 500 and 1,300 V. In this experiment, the 1,200 V was selected as the optimal separation voltage in order to compromise between sensitivity and analysis time.

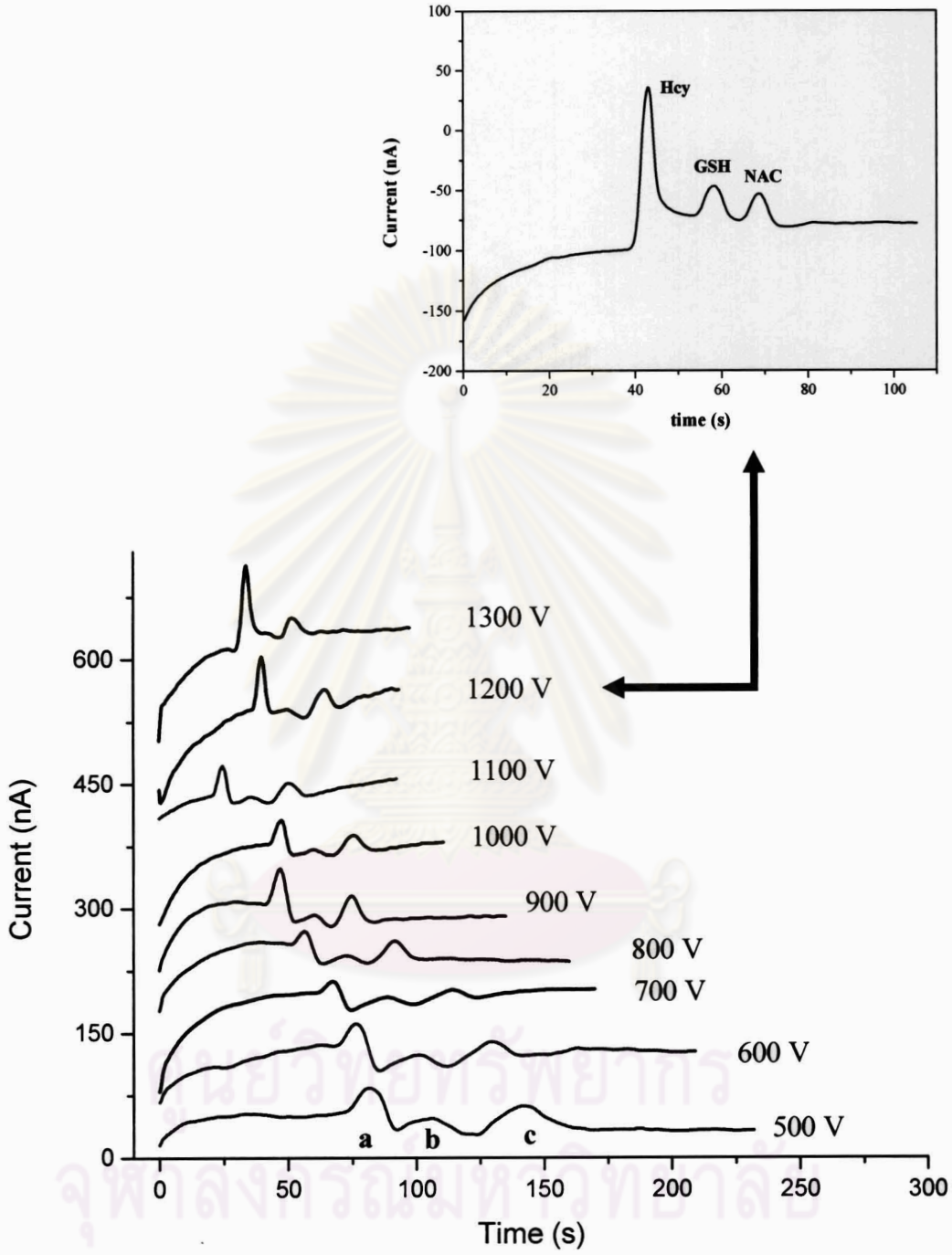

Figure 4.15 The electropherograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; (a) 150 μM homocysteine, (b) 200 μM glutathione, (c) 200 μM N-acetyl-L-cysteine; running buffer 20 mM MES + 1 mM SDS pH 6.0 ; detection voltage 0.8 V ; pinched injection time 20 s ; working electrode 25 μm Au wire.

4.4.2.4 Effect of pH of buffer

In CE system, the pH of buffer is the other important parameter because pH of buffer influences on the migration time, the separation efficiency of the analytes, and the EOF. The effect of pH of buffer on the migration times of Hcy, GSH, and NAC was investigated. The pH of buffer was investigated between pH 5 and 7.5 using 25 mM MES add 1 mM SDS buffer. A plot of migration time as a function of pH of buffer is shown in Figure 4.16. All thiol compounds were separated within 80 s. The shorter migration times with good separation were observed at pH 6.

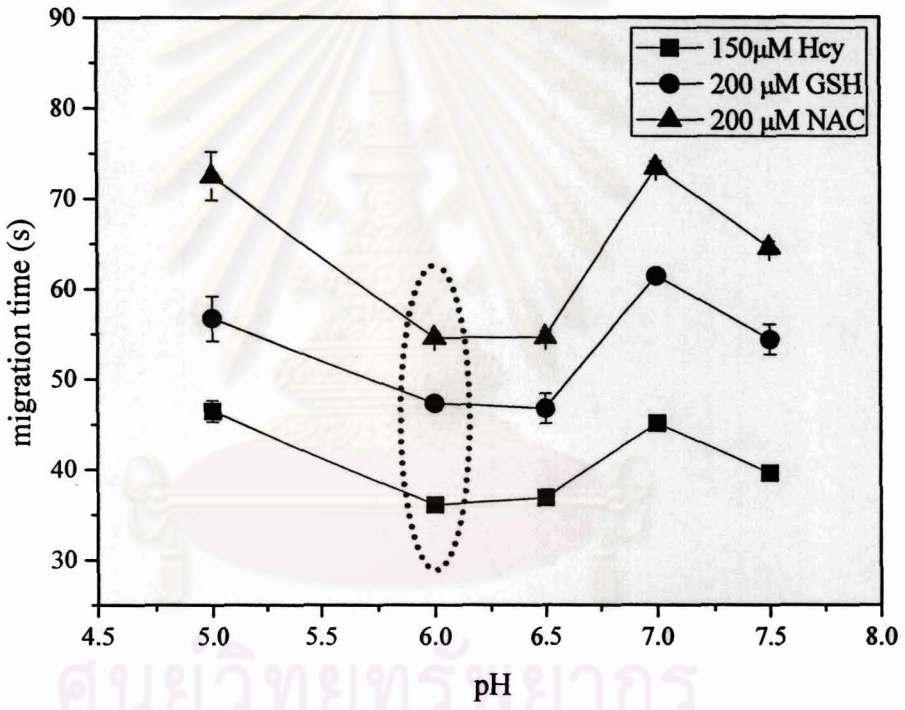

Figure 4.16 The effect of pH of buffer for homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; 150 μM homocysteine (■), 200 μM glutathione (●), 200 μM N-acetyl-L-cysteine (▲) ; running buffer 20 mM MES + 1 mM SDS ; detection potential 0.8 V ; separation voltage 1200 V ; pinched injection time 20 s ; working electrode 25 μm Au wire.

Table 4.1 Resolution (Rs), standard deviation (SD), and relative standard deviation (%RSD) of three thiol compounds (n=3), other condition as same in Figure 4.16.

pH	Rs (Hcy,GSH)	SD	%RSD	Rs (GSH,NAC)	SD	%RSD
5	1.09	0.01	1.27	1.25	0.15	12.40
6	**1.83**	**0.09**	**4.67**	**1.44**	**0.18**	**12.29**
6.5	1.14	0.08	6.65	1.24	0.19	15.61
7	2.26	0.12	5.37	1.48	0.14	9.29
7.5	1.69	0.17	9.97	1.24	0.14	11.59

Table 4.1 summarizes the three parameters including resolution (Rs), standard deviation (SD), and relative standard deviation (%RSD) of three thiol compounds that obtained from pH study. It can be seen that when the pH of buffer was set at pH 6 and pH 7, the three thiol compounds could be well separated. However, the migration time of the three thiol compounds at pH 7 is longer than at pH 6. Therefore, at pH 6 was chosen as the optimal value for this experiment.

4.4.2.5 Effect of buffer concentration

The buffer concentration affects on the peak shape and separation efficiency. Figure 4.17 shows the electropherogram of Hcy, GSH, and NAC. The buffer concentration containing 5, 10, 15, 20, 25, and 30 mM MES (pH 6.0) were investigated. As shown in Figure 4.17, the migration time and resolution of Hcy, GSH, and NAC increased after increasing the buffer concentration. The higher buffer concentration led to slower of EOF. This influence resulted with longer separation times and peak shapes became broad.

The relation between the current and migration time of the three thiol compounds is shown in Figure 4.18. As can be seen, the maximum current of NAC was obtained at concentration of 25 mM. Furthermore, the current of Hcy and GSH gradually decrease after the concentration of 20 mM. Threrfore, the buffer concentration 25 mM was selected as the optimal value for this work to compromise the analysis time and resolution of these three compounds.

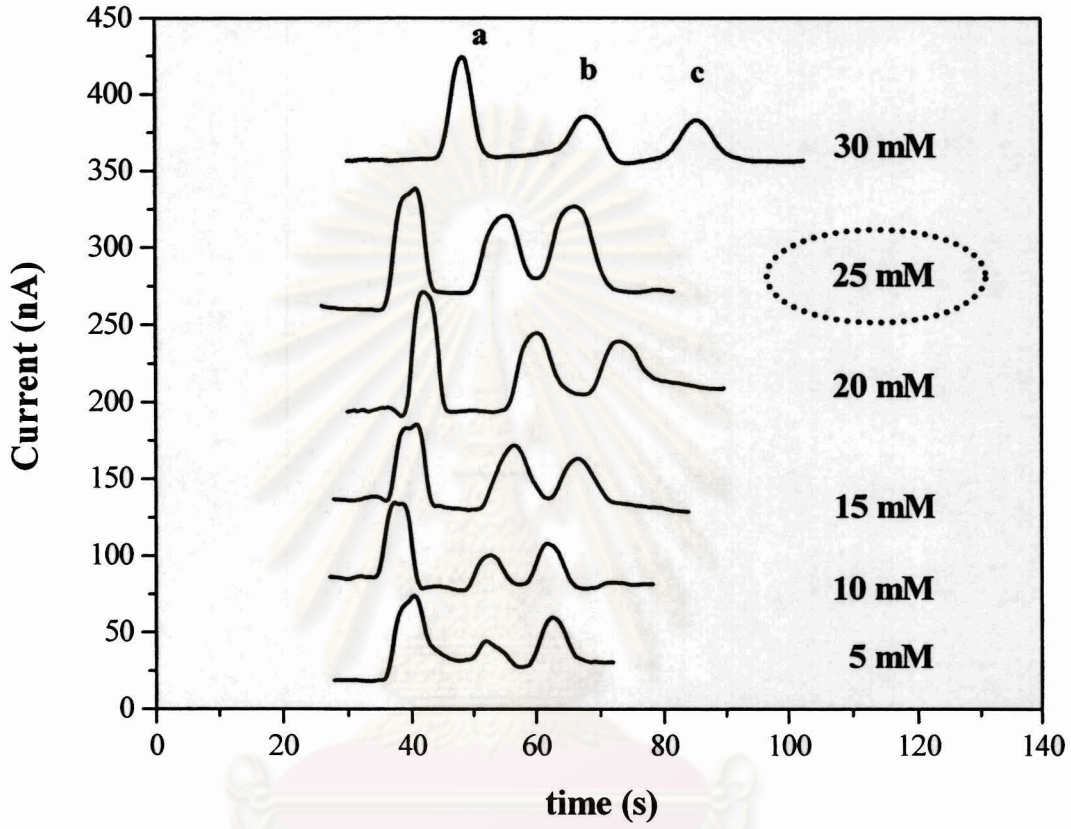

Figure 4.17 The electropherograms of homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; (a) 150 μM homocysteine, (b) 200 μM glutathione, (c) 200 μM N-acetyl-L-cysteine; running buffer MES + 1 mM SDS pH 6.0 ; detection voltage 0.8 V ; pinched injection time 20 s ; working electrode 25 μm Au wire.

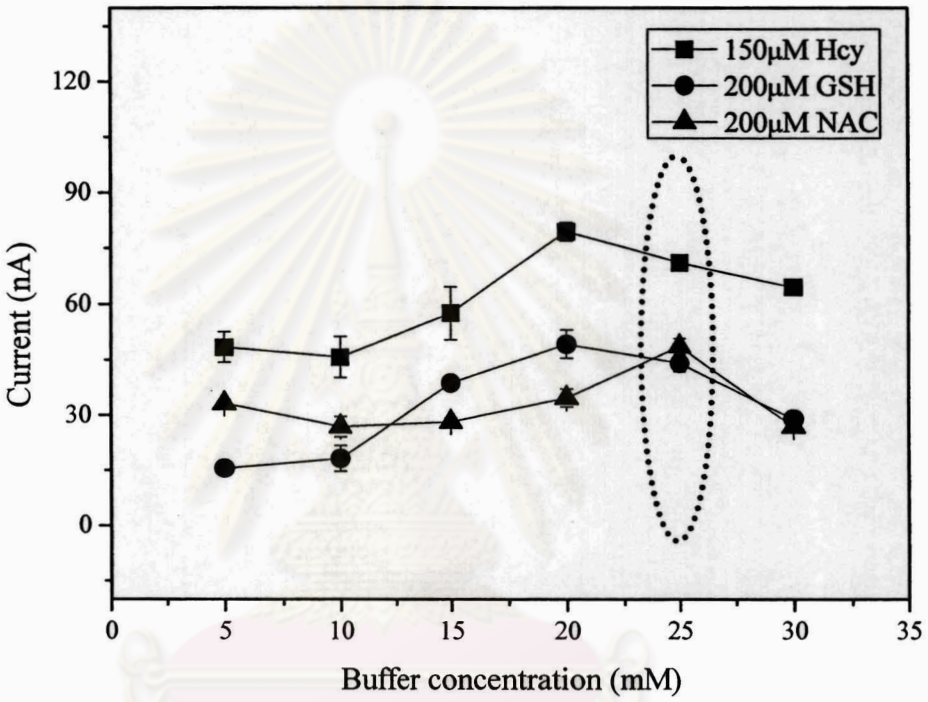

Figure 4.18 The effect of buffer concentration for homocysteine, glutathione and N-acetyl-L-cysteine. Other conditions are the same as in Figure 4.17.

4.4.2.6 Effect of SDS concentration

To control and stabilize the EOF using surfactants has been studied for PDMS microchip system [72]. In this work, SDS was chosen to add into the MES buffer to stabilize EOF. A plot of SDS concentration as a function of current of Hcy, GSH, and NAC is shown in Figure 4.19. The SDS concentration was investigated between 0 and 5 mM. It was found that, the maximum currents of the three thiol compounds were obtained at 3 mM SDS. Thus, 3 mM was chosen as the optimal SDS concentration for this experiment to stabilize EOF only.

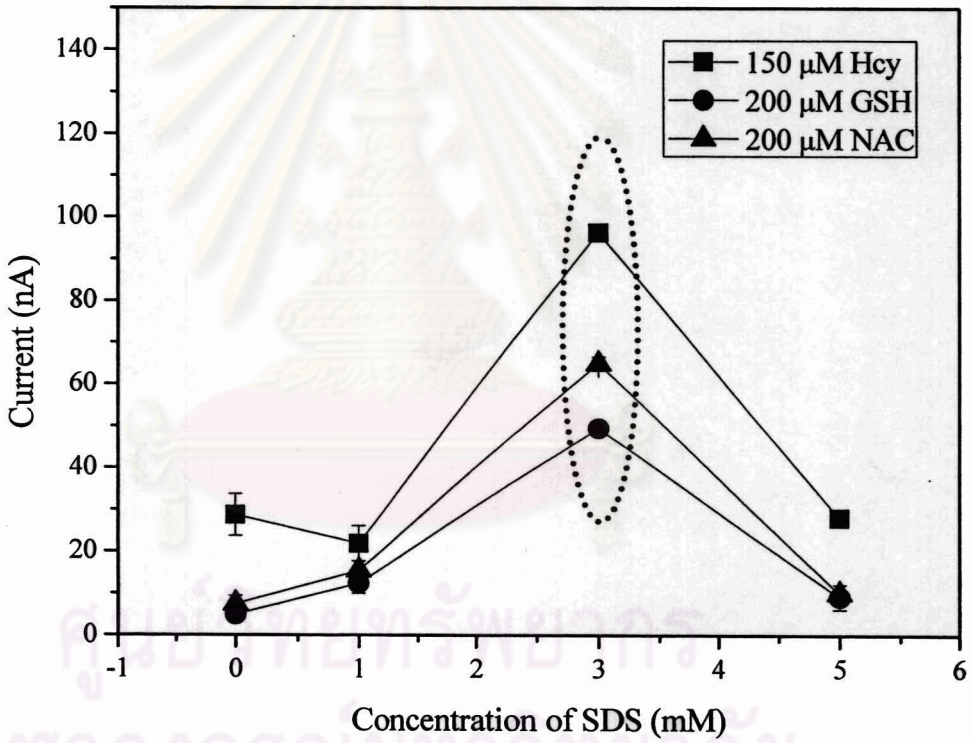

Figure 4.19 The effect of SDS concentration for homocysteine, glutathione and N-acetyl-L-cysteine. Conditions ; 150 µM homocysteine (■), 200 µM glutathione (●), 200 µM N-acetyl-L-cysteine (▲) ; running buffer 25 mM MES pH 6.0 ; detection potential 0.8 V ; separation voltage 1200 V ; pinched injection time 20 s ; working electrode 25 µm Au wire.

4.4.2.7 Linear range, limit of detection, and limit of quantitation

From the previous study of the optimized condition, the optimal conditions for separation and detection Hcy, GSH, and NAC (detection potential +0.8 V, separation potential 1200 V, injection time 20 s, and buffer MES 25 mM added 3 mM SDS (pH6.0)) were obtained. Under these optimal conditions, the linear relationship between the peak current and concentration for the three thiol compounds were obtained (Figure 4.20 – 4.22). The LOD of Hcy, GSH, and NAC were 0.129 μM, 0.224 μM, and 0.232 μM, respectively.

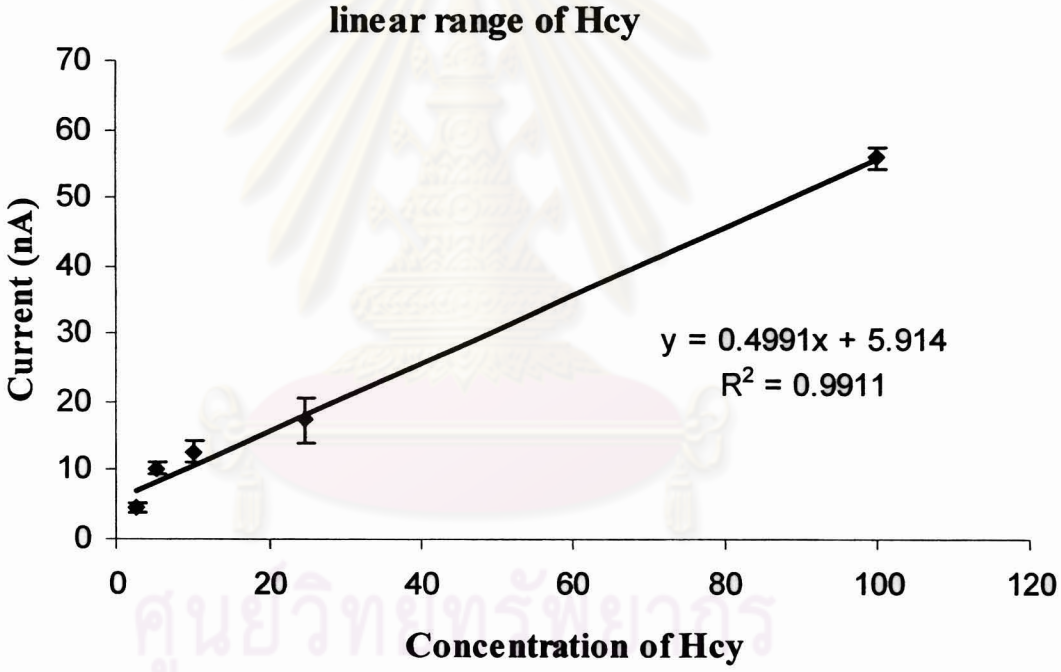

Figure 4.20 Linear relationship between peak current and concentration for 2.5 – 100 μM homocysteine. Experimental conditions: detection potential +0.8 V, separation potential 1200 V, injection time 20 s, and buffer MES 25 mM added 3 mM SDS (pH6.0).

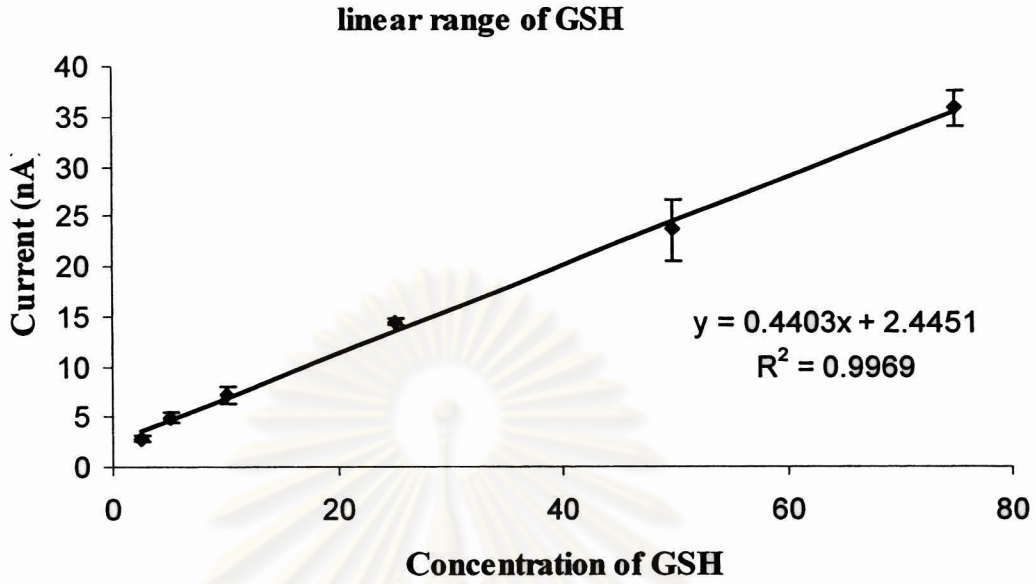

Figure 4.21 Linear relationship between peak current and concentration for 2.5 – 75 μM glutathione. Other conditions same as in Figure 4.20.

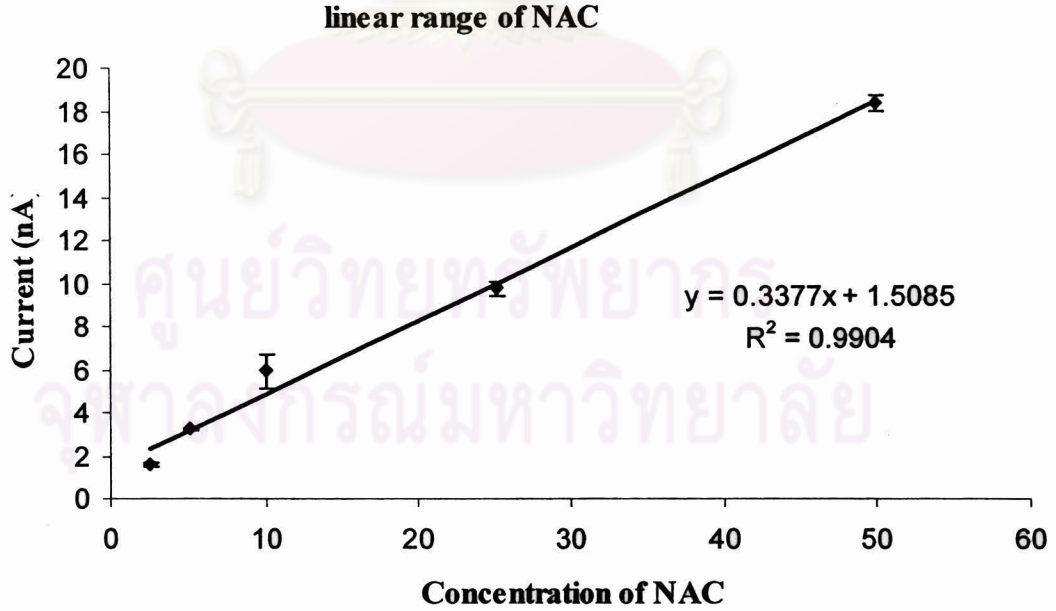

Figure 4.22 Linear relationship between peak current and concentration for 2.5 – 50 μM N-acetyl-L-cysteine. Other conditions same as in Figure 4.20.

The analytical parameters including regression equation, corresponding correlation coefficient, linear range, limit of detection, and limit of quantitation for the three thiol compounds are summarized in Table 4.2.

Table 4.2 The analytical parameters for Hcy, GSH, and NAC.

parameter	**homocysteine**	**glutathione**	**N-acetyl-L-cysteine**
Regression equation	y = 0.4991x + 5.914	y = 0.4403x + 2.4451	y = 0.3377x + 1.5085
R^2	0.9911	0.9969	0.9904
Linear range	2.5 – 100 μM	2.5 – 75 μM	2.5 – 50 μM
Limit of detection (LOD)	0.129 μM	0.224 μM	0.232 μM
Limit of quantitation (LOQ)	0.432 μM	0.747 μM	0.775 μM
Analysis time (s)	36 s	50 s	60 s

4.4.2.8 Repeatability

The repeatability is evaluated in term of the relative standard deviation (%RSD). In this experiment, the repeatability was determined with three times of injection in the same microchip. %RSD was calculated followed by equation 3.1. Table 4.3 shows the relative standard deviation of peak current for the PDMS microchip. %RSD of Hcy, GSH, and NAC are 2.65%, 3.19%, and 2.47%, respectively.

Table 4.3 The reproducibility of the three thiol compounds.

Thiol compounds (50μM)	Peak current (nA)	SD	%RSD (n=3)
Hcy	25.15	0.667	2.65
GSH	12.92	0.412	3.19
NAC	10.22	0.252	2.47

CHAPTER V

CONCLUSIONS

In this work, a sensitive microchip capillary electrophoresis coupled with electrochemical detection was presented. This method was used for determination of three thiol compounds including homocysteine (Hcy), glutathione (GSH), and N-acetyl-L-cysteine (NAC). Microchip was fabricated from poly(dimethylsiloxane) (PDMS) for this experiments.

To test the performance of the PDMS microchip system, dopamine and catechol were detected using amperometry. The well separation of dopamine and catechol suggested that this PDMS microchip can be used for the separation of other analytes. The conditions for the separation are:

- Dopamine and catechol concentration of 250 μM
- Separation potential of 1300 V
- MES buffer concentration of 20 mM (pH 7.00)
- Detection potential of +0.8 V
- Injection time 15 sec.

The determination of Hcy, GSH, and NAC has been demonstrated using PAD mode. The 25 mM of MES and 3 mM of SDS (pH 6.00) was used as buffer solution for this experiment. The optimal conditions for determination of these thiol compounds were:

- 25 mM of MES added 3 mM of SDS (pH 6.00) buffer solution
- Injection time of 20 s
- Separation potential of 1200 V

- Detection potential of +0.8 V

From these optimal conditions, the three thiol comounds can be well separated and detected. The analytical parameters for these compounds are summarized in Table 5.1.

Table 5.1 The analytical parameters for the determination of the three thiol compounds.

parameter	homocysteine	glutathione	N-acetyl-L-cysteine
Regression equation	y = 0.4991x + 5.914	y = 0.4403x + 2.4451	y = 0.3377x + 1.5085
R^2	0.9911	0.9969	0.9904
Linear range	2.5 – 100 μM	2.5 – 75 μM	2.5 – 50 μM
Limit of detection (LOD)	0.129 μM	0.224 μM	0.232 μM
Limit of quantitation (LOQ)	0.432 μM	0.747 μM	0.775 μM
Analysis time (s)	36 s	50 s	60 s
% RSD	2.65	3.19	2.47

These thiol compounds can be well separated rapidly. The result indicated that PDMS microchip CE is suitable for detection of three thiol compounds. Therefore, the microchip CE format offers an attractive method for detection thiol compounds in clinical diagnosis.

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