APPLICATION OF CAPILLARY ELECTROPHORESIS FOR DETERMINATION OF
GLYCOSAMINOGLYCANS IN BIOLOGICAL AND COSMETIC SAMPLES

Miss Kanokporn Chindaphan

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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การประยุกต์คะพิลลารีอิเล็กโทรฟอริซิสสำหรับการหาปริมาณของ
ไกลโคซามิโนไกลแคนในตัวอย่างชีวภาพและเครื่องสำอาง

น.ส.กนกพร จินดาพรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

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By          Miss Kanokporn Chindaphan

Field of Study          Chemistry

Thesis Advisor          Assistant Professor MONPICHAR SRISA-ART, Ph.D.

Thesis Co Advisor          Associate Professor THUMNOON NHUJAK, Ph.D.

Assistant Professor THASINAS DISSAYABUTRA, Ph.D., M.D.

---

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..................................................... Dean of the Faculty of Science

(Professor POLKIT SANGVANICH, Ph.D.)

DISSERTATION COMMITTEE

..................................................... Chairman

(Associate Professor VUDHICHAI PARASUK, Ph.D.)

..................................................... Thesis Advisor

(Assistant Professor MONPICHAR SRISA-ART, Ph.D.)

..................................................... Thesis Co-Advisor

(Associate Professor THUMNOON NHUJAK, Ph.D.)

..................................................... Thesis Co-Advisor

(Assistant Professor THASINAS DISSAYABUTRA, Ph.D., M.D.)

..................................................... Examiner

(Assistant Professor PUTTARUKSA VARANUSUPAKUL, Ph.D.)

..................................................... Examiner

(Assistant Professor KANET WONGRAVEE, Ph.D.)

..................................................... External Examiner

(Assistant Professor Somsak Sirichai, Ph.D.)

กนกพร จินดาพรรณ : การประยุกต์คะพิลลารีอิเล็กโทรฟอริซิสสำหรับการหาปริมาณของ ไกลโคซามิโนไกลแคนในตัวอย่างชีวภาพและเครื่องสำอาง. ( APPLICATION OF CAPILLARY ELECTROPHORESIS FOR DETERMINATION OF GLYCOSAMINOGLYCANS IN BIOLOGICAL AND COSMETIC SAMPLES) อ.ที่ปรึกษาหลัก : ผศ. ดร.มนพิชา ศรีสะอาด, อ.ที่ปรึกษาร่วม : รศ. ดร.ธรรมนูญ หนูจักร,ผศ. ดร. นพ.ฐสิณัส ดิษยบุตร

งานวิจัยนี้ได้พัฒนาเทคนิคคะพิลลารีอิเล็กโทรฟอริซิสสำหรับการเพิ่มปริมาณแบบออนไลน์ และการหาปริมาณของไกลโคซามิโนไกลแคน โดยเฉพาะอย่างยิ่งคอนดรอยทินซัลเฟต เดอมาแทนซัลเฟต และกรดไฮยาลูโรนิก เทคนิคการบรรจุสารตัวอย่างปริมาณมากและทำให้สารตัวอย่างเคลื่อนที่ชิดติดกันโดยใช้แรงอิเล็กโทรออสโมซิสได้ถูกใช้สำหรับการเพิ่มความเข้มข้นของสารตัวอย่าง และเพิ่มสภาพไวในการตรวจวัดของเทคนิคคะพิลลารีอิเล็กโทรฟอริซิส การออกแบบการทดลองแบบเซ็นทรัลคอมโพสิทได้ถูกนำมาใช้ในการหาภาวะที่เหมาะสมของพารามิเตอร์ทั้งหมดของเทคนิคคะพิลลารีอิเล็กโทรฟอริซิส โดยภาวะที่เหมาะสมคือ สารละลายอิเล็กโทรไลต์ที่ประกอบด้วยโซเดียมไดไฮโดรเจนฟอสเฟตเข้มข้น 200 มิลลิโมลาร์ บิวทิลลามีนเข้มข้น 200 มิลลิโมลาร์ และพอลิเอธิลีนไกลคอลเข้มข้น 0.5 ร้อยละโดยมวลต่อปริมาตร ที่พีเอช 4.0 และศักย์ไฟฟ้าที่ใช้ในการแยกที่ -16 กิโลโวลต์ ขีดจำกัดการตรวจวัด และขีดจำกัดการหาปริมาณสำหรับคอนดรอยทินซัลเฟต เดอมาแทนซัลเฟต และกรดไฮยาลูโรนิกคือ 3.0, 5.0 และ 1.0 และ 10.0, 15.0 และ 3.0 มิลลิกรัมต่อลิตร ตามลำดับ ความเที่ยงของวิธีที่พัฒนาขึ้นได้ศึกษาจากร้อยละของส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์ของพื้นที่ใต้พีกแก้ไขของสารมาตรฐานที่ทราบความเข้มข้นที่แน่นอนที่เติมลงในสารตัวอย่าง พบว่าค่าร้อยละของส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์มีค่าน้อยกว่าร้อยละ 7 และ 8 สำหรับการวัดแบบภายในวันเดียวกันและระหว่างวัน ตามลำดับ นอกจากนี้ร้อยละการได้กลับคืนของคอนดรอยทินซัลเฟต และกรดไฮยาลูโรนิกมีค่าอยู่ในช่วงร้อยละ 84-104 และ 73-120 ตามลำดับ ดังนั้นเทคนิคที่ถูกพัฒนาขึ้นนี้มีความน่าเชื่อถือ ง่าย มีการเตรียมตัวอย่างเพียงเล็กน้อย จึงเหมาะที่จะนำไปประยุกต์ใช้ในการหาปริมาณของคอนดรอยทินซัลเฟต และกรดไฮยาลูโรนิกในตัวอย่างทางชีวภาพ (น้ำไขสันหลัง น้ำเลี้ยงเซลล์ พลาสมา ปัสสาวะ) เพื่อเป็นประโยชน์สำหรับการพยากรณ์หรือวินิจฉัยโรค การรักษาทางการแพทย์ และในตัวอย่างเครื่องสำอาง และผลิตภัณฑ์อาหารเสริม ซึ่งเป็นประโยชน์สำหรับการควบคุมคุณภาพของผลิตภัณฑ์ได้ในอนาคต

สาขาวิชา        เคมี                                 ลายมือชื่อนิสิต ................................................

ปีการศึกษา     2561                              ลายมือชื่อ อ.ที่ปรึกษาหลัก ............................

                                                          ลายมือชื่อ อ.ที่ปรึกษาร่วม .............................

                                                          ลายมือชื่อ อ.ที่ปรึกษาร่วม .............................

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Kanokporn Chindaphan : APPLICATION OF CAPILLARY ELECTROPHORESIS FOR DETERMINATION OF GLYCOSAMINOGLYCANS IN BIOLOGICAL AND COSMETIC SAMPLES. Advisor: Asst. Prof. MONPICHAR SRISA-ART, Ph.D. Co-advisor: Assoc. Prof. THUMNOON NHUJAK, Ph.D.,Asst. Prof. THASINAS DISSAYABUTRA, Ph.D., M.D.

In this research, on-line preconcentration and determination of glycosaminoglycans (GAGs), especially chondroitin sulfate (CS), dermatan sulfate (DS) and hyaluronic acid (HA) using capillary electrophoresis (CE) was developed. Large-volume sample stacking using an electroosmotic flow (EOF) pump (LVSEP) technique was employed for on-line preconcentration and improvement of the detection sensitivity in CE method. Central composite design (CCD) was used to simultaneously optimize all parameters for CE separation. The optimized CE conditions were background electrolyte consisting of 200 mM sodium dihydrogen phosphate, 200 mM buthylamine and 0.5% w/v polyethylene glycol at pH 4.0 and separation voltage of -16 kV. Limit of detections (LODs) and the limit of quantitations (LOQs) were 3.0, 5.0 and 1.0 and 10.0, 15.0 and 3.0 mg $L^{-1}$ for CS, DS and HA, respectively. Precisions of the method were investigated from the percent relative standard deviation (%RSD) of corrected peak area of spiked standards in real samples, which were lower than 7% and 8% for intra-day and inter-day precisions, respectively. In addition, recovery percentages of CS and HA in various samples were found in the range of 84-104% and 73-120%., respectively. The developed method was reliable, simple and minimal sample pretreatment. Therefore, the proposed CE method could be applied to determine the amounts of CS and HA in biological samples (cerebrospinal fluid, cell culture media, plasma, urine) for prognostic diseases and medical pretreatment and in cosmetic and supplement samples for product quality control in the future.

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| Field of Study: | Chemistry | Student's Signature ............................... |
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| | | Co-advisor's Signature ......................... |
| | | Co-advisor's Signature ......................... |

# ACKNOWLEDGEMENTS

Kanokporn  Chindaphan

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# LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AOAC | Association of Official Analytical Chemists |
| BGE | background electrolyte |
| $C_1$ | the determined amount of the analyte |
| $C_2$ | the known concentration of spiked analyte |
| CCD | central composite design |
| CE | capillary electrophoresis |
| CEC | capillary electrochromatography |
| CGE | capillary gel electrophoresis |
| CIEF | capillary isoelectric focusing |
| CITP | capillary isotachophoresis |
| CS | chondroitin sulfate |
| CSF | cerebrospinal fluid |
| CZE | capillary zone electrophoresis |
| D | design matrix |
| DS | dermatan sulfate |
| $E$ | electric field strength |
| EOF | electroosmotic flow |
| FL | fluorescence |
| GAGs | glycosaminoglycans |
| Gal | galactose |
| HA | hyaluronic acid or hyaluronan |
| HaCat | human keratinocyte immortal cells |
| HDF | human dermal fibroblasts cells |
| HP | heparin |
| HPLC | high performance liquid chromatography |
| HS | heparin sulfate |
| i.d. | inner diameter |
| k | the number of experimental factors |

| | |
|---|---|
| KS | keratan sulfate |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| LVSEP | large-volume sample stacking using an electroosmotic flow pump |
| M | factor |
| MEKC | micellar electrokinetic chromatography |
| MS | mass spectrometry |
| N | the amount of samples |
| NACE | nonaqueous capillary electrophoresis |
| o.d. | outer diameter |
| OFAT | one-factor at a time |
| OSCS | oversulfated chondroitin sulfate |
| PDL | human periodontal ligament cells |
| PEG | polyethylene glycol |
| $r_h$ | size of an ion or hydrodynamic radius |
| Rs | resolution |
| RSD | relative standard deviation |
| RSM | response surface methodology |
| SF | semisolid formulations |
| T | transpose of a matrix |
| Tris | tris (hydroxymethyl) aminomethane |
| UV | ultraviolet |
| $v_{eo}$ | electroosmotic velocity |
| $v_{ep}$ | electrophoretic velocity |
| $x_i$ | dependent variable of i factor |
| $x_j$ | dependent variable of j factor |
| y | response |
| $z$ | charge of an ion |
| α | rotatable variable |
| β | coefficient |

$\beta_0$ — intercept or constant coefficient

$\beta_i$ — regression coefficient of i factor

$\beta_{ii}$ — regression coefficient of i × i factor

$\beta_{ij}$ — regression coefficient of i × j factor

$\epsilon$ — dielectric constant of the electrolyte

$\eta$ — viscosity

$\mu$ — electrophoretic mobility

$\mu_{eo}$ — electroosmotic mobility

$\zeta$ — zeta potential

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# CHAPTER I

# INTRODUCTION

## 1.1  Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides consisting of repetitive disaccharide units of uronic acid (acid sugar) and hexosamine (amine sugar). GAGs can be divided into six groups, including chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA), heparin (HP), heparin sulfate (HS), and keratan sulfate (KS) [1]. The differences of GAGs are attributed to the components consisting of uronic acid, hexosamine and sulfate pattern. GAGs encompass a crucial component of extracellular matrix [2]. They have been found in biological fluids, such as synovial fluid, cerebrospinal fluid, blood plasma and urine. The levels of GAGs in biological samples can be used as prognostic markers for many diseases, such as osteoarthritis, arthritis and brain injury diseases [3, 4]. Therefore, quantitative analysis of GAGs is necessary to prevent these diseases at early stage and follow-up treatment outcome. Additionally, HA is normally used in cosmetic formulation due to its anti-aging properties (radical scavenging and hydrating properties). Accordingly, a method for quality control of HA in cosmetic products is also needed.

GAGs could be separated and quantified by several separation techniques, such as high-performance liquid chromatography (HPLC), which uses an expensive column, requires sample preparation and consumes large amount of samples and reagents [4-6], gel electrophoresis and cellulose acetate membrane electrophoresis which are laborious and time consuming [7, 8]. Additionally, capillary electrophoresis (CE) is one of the separation techniques that can separate charged and uncharged species migrating through electrolyte solution in a small capillary column under an applied voltage [9]. CE has several similarities to HPLC, such as quantitative analysis, on-line detection and compatible with a variety of detectors; however, CE offers no sample preparation except for filtration [10]. Additionally, CE has high separation efficiency and requires low consumption of samples and reagents. According to these advantages, CE has been applied in analysis of many types of analytes, such as

biomolecules (carbohydrates, nucleic acids, proteins, peptides, amino acids and metabolites) [11-16], inorganic ions [12, 17], biological fluids (serum, plasma, urine, cerebrospinal fluid (CSF)) [10-12, 15, 18-20], nanoparticles [21] and pharmaceutical products [22, 23]. Thus, CE is an alternative and powerful technique that has been used for separation and determination of GAGs and GAG-derived oligosaccharides, which they are negatively, charged molecules. Moreover, CE is a good choice for analysis of GAGs due to rapid, simple, high efficiency, no sample preparation except for filtration and low consumption of samples and reagents [14].

However, few studies have been reported for simultaneous determination of GAGs in biological fluids and cosmetic samples using CE. For examples, Alkrad et al. [24] developed a CE method for determination of HA in pharmaceutical semisolid formulations (SF) samples, which required sample preparations using an enzyme and concentrated sulfuric acid to digest HA. Similar to this study, Teodor et al. [25] reported a CE method for determination of intact and hydrolyzed HA using different conditions with requirement of sample pretreatment for natural extract samples. Furthermore, there have been published studies for determination of CS, DS, HS and oversulfated chondroitin sulfate (OSCS) using CE methods in pharmaceutical samples [26-28]. The use of high buffer concentration, chondroitinase ABC enzyme and adding resolving agents into buffer solution can improve the sensitivity of CE detection. The results showed good separation of these GAGs, but these methods were complicated for preparation of samples. In addition, GAGs can be found in biological samples, such as synovial fluids from osteoarthritic rabbits and mouse plasma [20, 29]. From these previous studies, sample pretreatments, such as enzymatic digestion and derivatization were required for eliminating interferences and improving the limit of detection. Although some studies proposed CE methods for determination of GAGs without sample pretreatment, such as depolymerization [20], but a long run time over 33 min was required to determine these GAGs. Furthermore, the results showed a poor resolution between HP and CS and also low detection sensitivity. Thus, in this work a simple CE method for determination of GAGs was developed to resolve the problems associated with a long run time and complicated sample preparation and to improve the limits of detection and quantification.

Due to low UV absorption and high negatively charged of GAGs molecules, it is challenging to develop a CE method to improve the sensitivity of detection. Because the level of GAGs could be a possible biomarker for several diseases, a preconcentration technique is the most important for analysis of GAGs. In this research, an on-line preconcentration technique was required before CE separation for improvement the sensitivity of GAGs detection. Large volume sample stacking using an electroosmotic flow (EOF) pump or LVSEP was applied. LVSEP is suitable for analysis of anionic analytes, which migrate in the opposite direction of EOF. The basic principle of LVSEP is loading large amount of sample into a capillary column by pressure. Under the reversed polarity condition, the sample matrix zone is removed to the cathodic end by the EOF and then the concentrated anionic analytes can be separated and migrate to the detector [30]. The main advantage of LVSEP is easy experimental procedure without polarity switching. Since GAGs are negatively charged molecules, a LVSEP method was used for on-line preconcentration of GAGs before CE separation to enhance the limits of detection of CS, DS and HA. Moreover, it is the first time to determine the levels of GAGs in biological (cerebrospinal fluid, cell culture media, plasma or serum, urine) and cosmetic (serum) samples without sample preparation except for dilution and filtration.

## 1.2 Objective of this research

The objective of this work was to develop a capillary electrophoresis method with large volume sample stacking using an electroosmotic flow pump for on-line preconcentration and determination of glycosaminoglycans in biological and cosmetic samples.

## 1.3 Scope of this research

Capillary electrophoresis (CE) with large volume sample stacking using an electroosmotic flow pump (LVSEP) for on-line preconcentration and determination of glycosaminoglycans in biological and cosmetic samples was developed.

For on-line preconcentration technique using LVSEP, sample injection time was investigated. The CE parameters were studied to obtain the best condition for

separation and determination of glycosaminoglycans (GAGs). One-factor at a time (OFAT) approach was used for preliminary studies to find the optimal ranges of each parameter e.g. buffer concentration, pH and separation voltage before central composite design (CCD) optimization. After that, CCD approach was also used for optimization CE parameters simultaneously in the optimal ranges, which are received from the preliminary studies.

In addition, the analytical performance of this method, including accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ) was studied. Finally, the proposed LVSEP-CE method was applied to determine glycosaminoglycans in real samples, e.g. cerebrospinal fluid, plasma or serum, cell culture media, urine, cosmetic products and supplement.

# CHAPTER II

# THEORY

## 2.1 Basic principle of capillary electrophoresis (CE) [31]

Capillary electrophoresis (CE) is a powerful separation technique that separates analytes which migrate in a narrow capillary column filled with background electrolyte (BGE) under the influence of electric field. The separation mechanism is based on the difference in electrophoretic mobility ($\mu$) of charged analytes, which depends on charge to size ratio [12, 14, 16, 32, 33].

### 2.1.1 Electrophoretic mobility [31, 32]

The electrophoretic mobility, $\mu$ ($m^2\ V^{-1}\ s^{-1}$) is a measure of the velocity of analytes or electrophoretic velocity, $v_{ep}$ ($m\ s^{-1}$) through the medium under the influence of electric field strength, $E$ ($V\ m^{-1}$). The relationship among these factors is shown in **Equation 2.1**.

$$\mu = \frac{v_{ep}}{E} = \frac{ze}{6\pi r_h \eta} \tag{2.1}$$

From **Equation 2.1**, it shows that the electrophoretic mobility depends on charge, $z$ *(coulomb)* and size or hydrodynamic radius, $r_h$ (m). Other factors that affect the electrophoretic mobility are charge density of analytes, temperature, voltage, organic solvent, pH, viscosity, $\eta$ (Poise, $N\ s\ m^{-2}$), and ionic strength of the background electrolyte.

### 2.1.2 Electroosmotic flow (EOF) [31, 34-36]

EOF, also called electroendosmotic flow, is the movement of medium in a capillary column under the applied voltage. In CE, the inner capillary surface consists of silanol group that can be ionized to negative charges when the pH of buffer solution is higher than 2, resulting in high number of positive ions in the buffer solution. Positive ions or cations in the buffer solution will migrate and be attracted

to the negative charges on the capillary, forming the electrical double layer as shown in **Figure 2.1**. When applying voltage, the excess cations that are hydrated by the buffer solution and water molecules will migrate towards the cathodic end, pulling water and creating a pumping action simultaneously. The electroosmotic velocity is proportional to the zeta potential, as revealed by **Equation 2.2**;

$$v_{eo} = -\frac{\varepsilon\zeta}{4\pi\eta} E \qquad (2.2)$$

where $\varepsilon$ is the dielectric constant of the electrolyte, $\zeta$ is the zeta potential (V), $\eta$ is the viscosity (Poise, N s m$^{-2}$), and $E$ is electric field strength (V m$^{-1}$). From **Equation 2.2**, dividing $v_{eo}$ by $E$ gives electroosmotic mobility, $\mu_{eo}$ (m$^2$ V$^{-1}$s$^{-1}$), as shown by **Equation 2.3**.

$$\mu_{eo} = -\frac{\varepsilon\zeta}{4\pi\eta} \qquad (2.3)$$

Normally, bare fused silica is a weak acid with pKa of 6.25. At high pH, the silanol groups are deprotonated. It means that the net charge of the surface is highly negative charges. Thus, the EOF is more important at high pH than at low pH. The relationship among EOF, pH and percent ionized to negatively charged surface is shown in **Figure 2.2**.

The factors that affect the EOF are voltage, size of capillary column, temperature, organic solvent, pH, viscosity and ionic strength of the BGE.
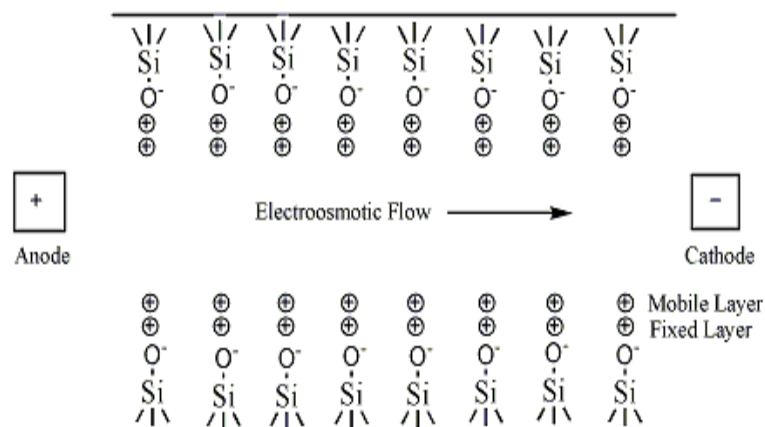
**Figure 2.1** The models of the electroosmotic flow. Reproduced from reference [34, 35].
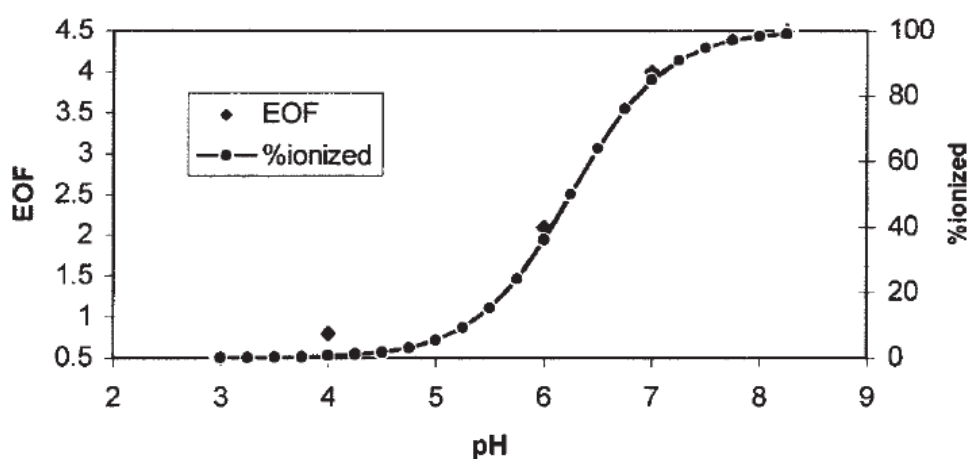


**Figure 2.2** The relationship between EOF and percent ionized as a function of pH. Reproduced from reference [36].

Moreover, the direction of EOF depends on the sign of the charge on the wall of the capillary. Generally, EOF is always towards the electrode that has the same charge as the capillary wall. The velocity of liquid or medium in the narrow capillary is nearly uniform across the inner diameter of the capillary column occurring in a "plug or flat flow" profile. Meanwhile, the parabolic or laminar flow exhibited by pumped systems in HPLC can create a velocity profile across the diameter of the

tube. The peak width of analyte in CE is narrower than that in HPLC. As a result, the peak efficiency in CE is better than that in HPLC. The flow profiles and peaks in CE and HPLC are shown in **Figure 2.3**.
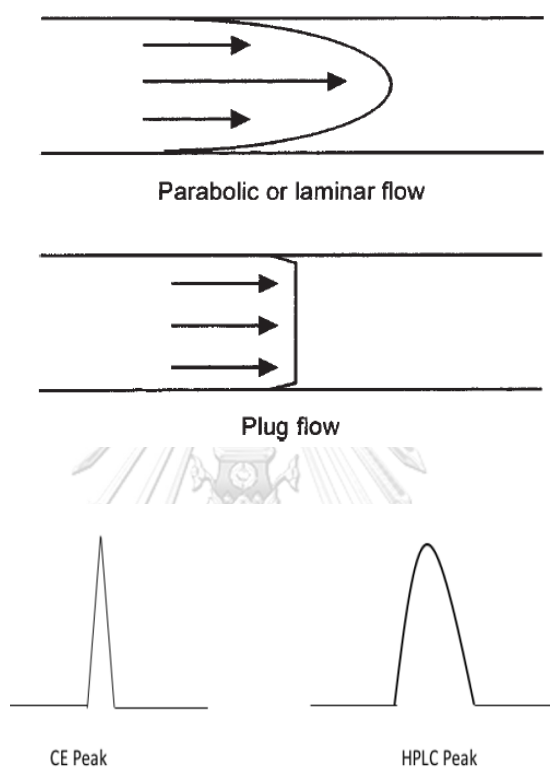


**Figure 2.3** Flow profiles and peaks in CE and HPLC. Reproduced from reference [36].

CE has many advantages, including high separation efficiency, ease of use, short analysis time, minimal sample preparation, and low consumption of samples and reagents.

## 2.2 Instrumentation of CE [9]

### 2.2.1 Basic instrument

The basic CE instrument is shown in **Figure 2.4**. A CE system consists of reservoirs for buffer and sample, electrodes (normally platinum (Pt) wire), a high voltage power supply (up to 30 kV), a capillary column with 10-200 μm internal

diameter and 20-100 cm in length, a detector (such as ultraviolet (UV), fluorescence (FL) and mass spectrometry (MS)) and a computer for controlling the instrument and collecting data.
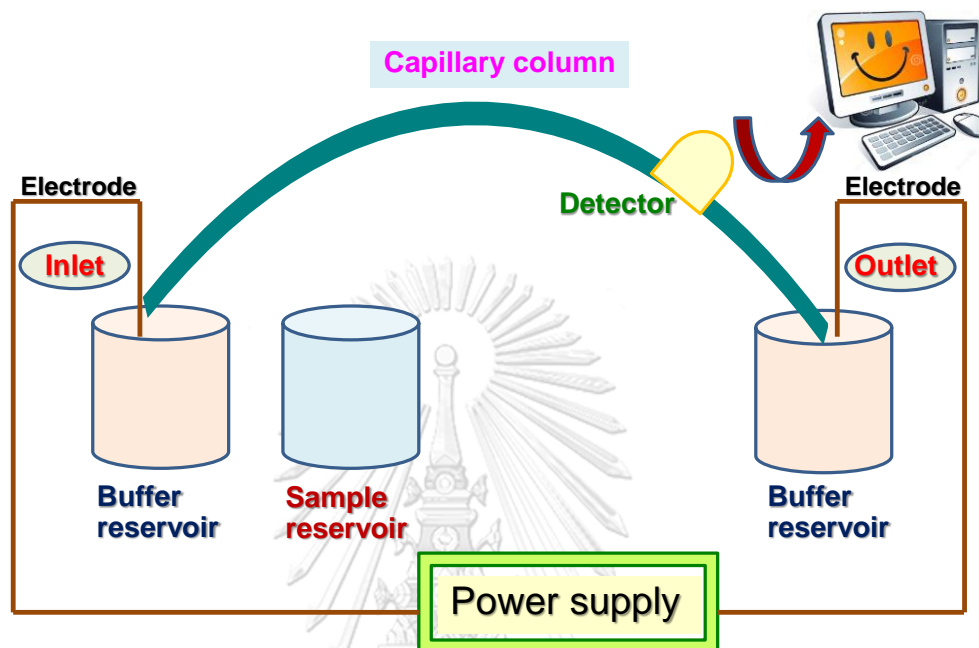


**Figure 2.4** Basic CE instrument. Adapted from reference [37].

For basic procedure, firstly, the buffer solution is loaded into a capillary column. After that, the inlet buffer solution vial is replaced by the sample vial, and the sample solution is injected into a capillary by pressure or electrokinetic injection. Then, a high voltage is applied to the capillary column. Finally, the charged analytes can be separated and migrate to the detector according to the difference in electrophoretic mobilities of analytes.

### 2.2.2   Sample introduction [31, 38]
#### 2.2.2.1   Sample injection method [39, 40]

Two methods that are widely used for sample introduction to a capillary column are hydrodynamic and electrokinetic injections.

*- Hydrodynamic injection*

Hydrodynamic injection is the most widely used method because it is free of injection bias due to the difference in sort of samples. This mode introduces a small sample volume (few nanoliters) into the capillary column. Hydrodynamic injection can be divided into gravity or siphoning (inserting the inlet end of the capillary into the sample reservoir and raising the reservoir and capillary relative to the outlet end), pressure (applying a positive pressure to the sample reservoir at the inlet end) and vacuum injection (applying a vacuum to the buffer reservoir at the outlet end). The types of sample injection are shown in **Figure 2.5**.
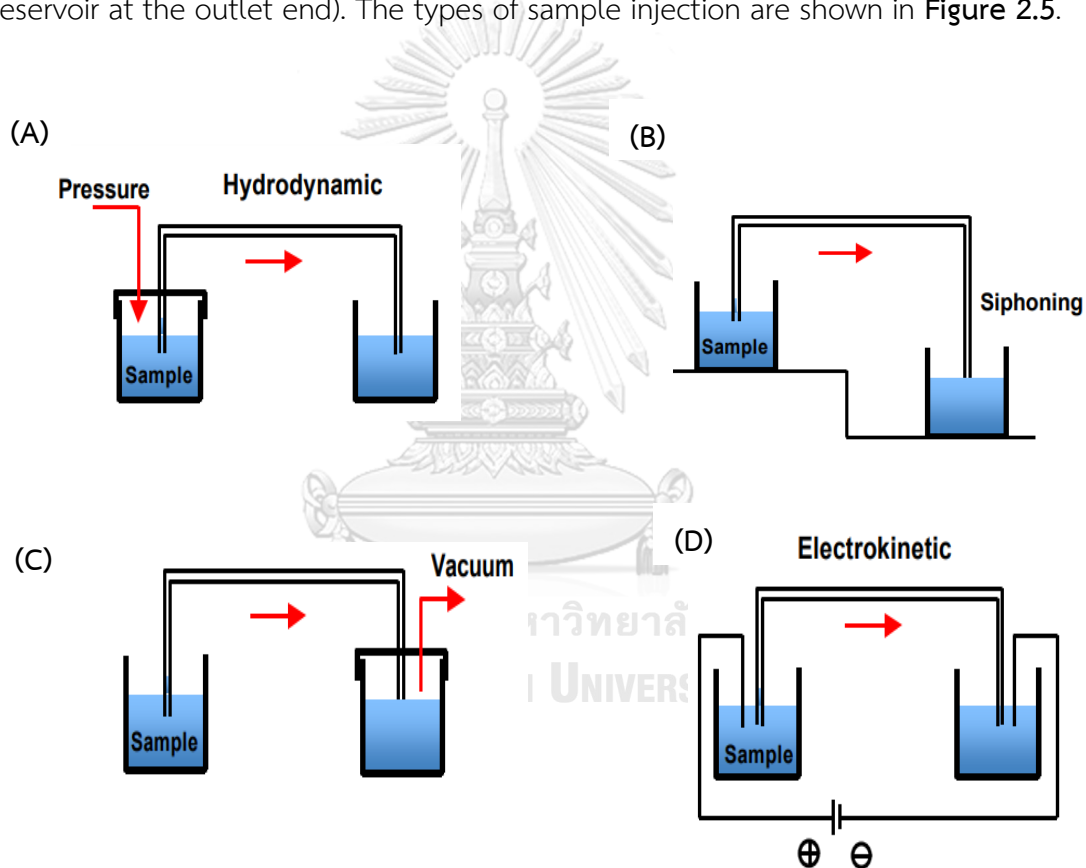


**Figure 2.5** Modes of sample injection in CE. (A) hydrodynamic injection by pressure, (B) hydrodynamic injection by gravity, (C) hydrodynamic injection by vacuum, and (D) electrokinetic injection, Reproduced from reference [41].

A pressure injection is the most frequently used approach in hydrodynamic injection and it was employed in this work.

*- Electrokinetic injection*

Electrokinetic injection, also known as electrophoretic or electromigration injection, is easier to be set up than hydrodynamic injection because no air pumping is required. The inlet of capillary column is inserted into the sample reservoir and the outlet into a buffer reservoir. The injection voltage for introduction of the sample into a capillary is approximately 3 to 5 times lower than the separation voltage. In addition, the sample is drawn into a capillary column through a combination of both electrophoretic migration and the pumping action of EOF. A unique property of electrokinetic injection is that the amount of an analyte injected depends on the electrophoretic mobility of the individual analytes, so discrimination occurs for ionic species because an analyte with a higher electrophoretic mobility is injected in a higher amount than an analyte with a lower mobility. Thus, this injection method is prone to injection bias. However, electrokinetic injection is suitable for samples or media with high viscosity, such as gels in capillary gel electrophoresis, and it can be utilized when hydrodynamic flow is suppressed or ineffective.

### 2.2.2.2  On-line sample preconcentration [42, 43]

Since small amount of sample is injected into a capillary column having a narrow optical path length for UV detection, the sensitivity of CE is relatively poorer than that of HPLC. Moreover, quantitative analysis at low concentration levels is not correct for the low UV absorption compounds. To improve the limits of detection and the response of detector for capillary electrophoresis, an on-line preconcentration has been utilized and developed, such as large volume sample stacking, field-amplified sample stacking, pH-mediated sample stacking, and sweeping. Basic concept for on-line sample preconcentration is loading large amount of sample into a capillary column. After the voltage is applied, the sample matrices are removed and the analytes are stacked and concentrated, simultaneously.

Large-volume sample stacking using an electroosmotic flow pump or LVSEP is another on-line preconcentration technique. For LVSEP approach, the analytes should migrate in the opposite direction to EOF, so the anionic analytes are

proper. Low pH of buffer solution is required for LVSEP in order to reduce the EOF, so anionic analytes can move to the detector against the EOF. Basic procedure of this technique is loading large amount of sample into a capillary column by pressure. After the high voltage is applied, anionic analytes move in the opposite direction to EOF. The sample matrices are removed by EOF into the inlet end, whilst the analytes are stacked and concentrated. The advantages of this technique are simple to use and short analysis time. However, the limitation of this technique is only positive or negative analytes can be concentrated at a time.

In this research, LVSEP was used for on-line preconcentration of analytes. Since glycosaminoglycans (GAGs) are low UV absorption and high negatively charged molecules. Moreover, GAGs move in the opposite direction to EOF. Therefore, it is possible to improve the detection sensitivity of GAGs using LVSEP. A LVSEP process for on-line preconcentration of anionic analytes is divided into four steps, as shown in **Figure 2.6**. First step (sample injection), a large amount of sample is loaded into a capillary column by pressure. Step 2 (start of anions stacking and removal of sample matrix under a negative voltage), the electroosmotic velocity in buffer solution is less than the electroosmotic velocity in sample zone when applying the negative voltage. Thus, anionic analytes quickly migrate to the anodic end in the opposite direction to EOF. Next in step 3 (process of anions stacking and removal of sample matrix), the anionic analytes migrate against the lower EOF, so they moved backward to the cathodic end. After that, anionic analytes are stacked in a narrow band in front of the sample zone, resulting in the reduction of sample zone length, whilst the matrices as the neutral, positive or anionic analytes that cannot migrate against EOF will be simultaneously removed to cathodic end by EOF. Finally in step 4 (complete removal of sample matrix and CZE separation), when matrices are removed completely, the concentrated anionic analytes can be separated and migrate to the detector according to their own electrophoretic mobilities.
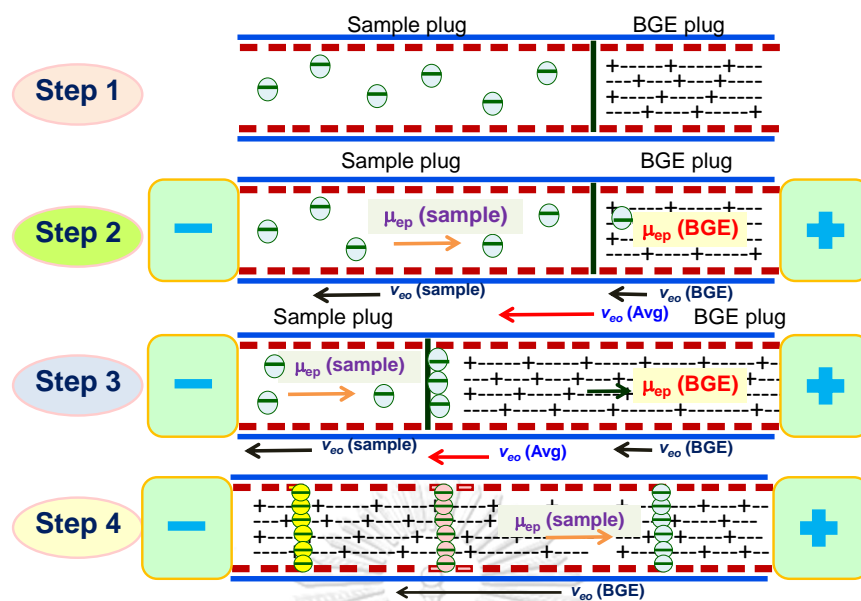
**Figure 2.6** On-line preconcentration steps of large-volume sample stacking using an EOF pump. Adapted from reference [30].

## 2.3 Mode of CE

CE can be categorized into different modes [16]:

1. Capillary zone electrophoresis (CZE) is the simplest mode of CE. The separation mechanism is based on the difference in electrophoretic mobility of charged analytes, which depends on charge-to-mass ratio.

2. Micellar electrokinetic chromatography (MEKC) is a mode of CE separation in which a surfactant is added to the buffer solution at a concentration that forms micelles. This mode is useful to resolve both charged and neutral compounds.

3. Capillary gel electrophoresis (CGE) is adapted from traditional gel electrophoresis by using soluble polymers to create a replaceable molecular sieve in a capillary column to allow for size separation.

4. Capillary electrochromatography (CEC) is a hybrid separation method that couples the high separation efficiency of CZE with HPLC and uses an electric field rather than hydraulic pressure to drive the mobile phase through a packed bed.

5. Capillary isoelectric focusing (CIEF) allows amphoteric molecules, proteins, to be separated in a pH gradient generated in a capillary column between cathode and anode.

6. Capillary isotachophoresis (CITP) is a focusing technique based on the migration of compounds between leading and terminating electrolytes.

7. Nonaqueous capillary electrophoresis (NACE) involves the separation of analytes in nonaqueous media that allows additional selectivity options in method development.

CZE was used in this work. Additionally, CZE is the simplest and most widely used mode in CE. The separation mechanism is based on the difference in electrophoretic mobility of charged analytes, which depends on charge to size ratio of analyte (as shown in **Equation 2.1**). A wide variety of buffers can be employed in CZE, such as phosphate, acetate and borate with or without an organic solvent. When applying voltage, a mixture in a solution can be separated into its individual components quickly and easily. It means that each species of analyte ions migrates in discrete zones towards the electrode of the opposite charge due to the electrophoretic mobility. Under the high EOF condition, both anions and cations migrate to the detector according to individual electrophoretic mobility, whilst neutral molecules migrate due to the EOF.

In this work, LVSEP combined with CZE was used for on-line preconcentration and determination of GAGs in various samples. Because CZE is useful for separation of ions and large molecules, such as proteins and peptides, GAGs as anionic analytes can be also separated by this method. Moreover, LVSEP combined with CZE method is simple, fast, and low consumption of samples, which is suitable for analysis of GAGs in biological samples.

## 2.4 Glycosaminoglycans

GAGs are linear polysaccharide chains consisting of repeating disaccharide units having negative charges [20]. They encompass a crucial component of extracellular matrix [21]. The differences of GAGs are attributed to the components consisting of hexuronic acid, hexosamine and sulfate pattern. Accordingly, GAGs can

be divided into four structural groups: heparin/heparan sulfate (HP/HS), keratan sulfate (KS), dermatan/chondroitin sulfate (DS/CS) and hyaluronic acid (HA) [20]. The structures of these GAGs are illustrated in **Figure 2.7**.

Chondroitin sulfate (CS) comprises *D*-glucuronic acid and *N*-acetylated *D*-galactosamine [22]. CS can be extracted from cartilage, such as pig laryngeal cartilage, shark cartilage and bovine nasal cartilage [20]. Moreover, CS is a major component in articular fluid and has been used for curing of arthritis because it does not cause toxicity or side-effects after long-term dosage.

Dermatan sulfate (DS) has a similar structure to chondroitin sulfate with *D*-glucuronic acid being replaced with *L*-iduronic acid [22]. DS is found in fibroblasts/skin, bone, cartilage, connective tissue [23]. DS is implicated in cardiovascular diseases, tumorigenesis, infection, wound repair, and fibrosis [23].

Hyaluronic acid or hyaluronan (HA) is an anionic and non-sulfated acidic GAG containing *N*-acetyl-*D*-glucosamine and *D*-glucuronic acid. Generally, HA has the largest molecular weight. Since HA is found in animal tissues, it is used as an ingredient of eye drops, artificial synovial fluids in pharmaceuticals and clinical applications, respectively. Moreover, HA is a viscoelastic agent, which is added in skin care products because of the ability to replenish moisture [22, 24, 25].

Heparin (HP) and heparan sulfate (HS) are alternating *L*-iduronic or *D*-glucuronic acid and *D*-glucosamine [22]. HS is a highly-sulfated GAG and highly negatively charged molecule. Basically, HS is extracted from porcine tissue [26]. It is used as anticoagulant medicines for the treatment and prevention of thrombosis and cardiovascular diseases. In addition, HS can be used in cosmetic surgery to enhance subcutaneous microcirculation [20, 23].

Keratan sulfate (KS) is a disaccharide backbone consists of *β-D*-galactose (Gal) and *N*-acetylgalactosamine. It is found in cornea, bone and cartilage [44].

The levels of GAGs can indicate several diseases, such as osteoarthritis, arthritis, hereditary multiple exostoses, vascular dementia, Alzheimer, subarachnoid hemorrhage, and mucopolysaccharidosis [2-5, 45-49]. **Table 2.1** showed the amounts of GAGs found in biological fluid and various samples.

GAGs have been separated and quantified by different separation techniques, such as high-performance liquid chromatography (HPLC), which use an expensive column, require sample preparation and consume large amount of samples and reagents [4-6], gel electrophoresis and cellulose acetate membrane electrophoresis which are laborious and time consuming [7, 8]. Therefore, CE, as an alternative technique that has been used for separation and determination of GAGs and GAG-derived oligosaccharides due to rapid, simple, high efficiency, minimal sample preparation and low consumption of samples and reagents.
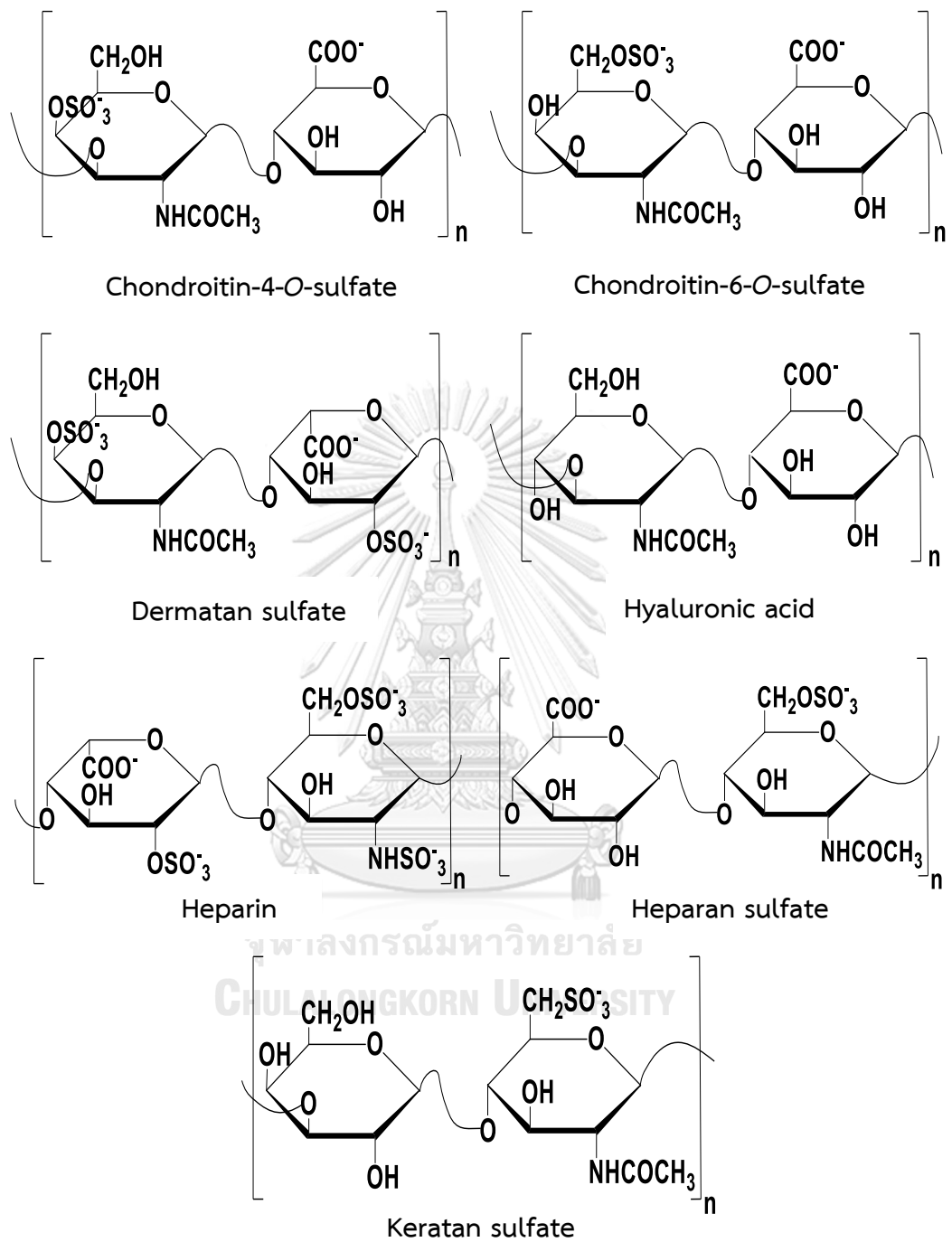
**Figure 2.7** The structures of major disaccharide repeating units found in each GAG. Adapted from reference [50].

Table 2.1 The amounts of GAGs found in various samples from the previous studies.

| Types of GAGs | Sample | Amount | References |
|---|---|---|---|
| CS | Synovial fluid | *Controls*: 21.0 µg L$^{-1}$ | [3] |
| | | *Patients with osteoarthritis*: 13.2 µg L$^{-1}$ | |
| | | *Patients with rheumatoid arthritis*: 5.9 µg L$^{-1}$ | |
| | Plasma fraction | *Controls*: 12.5 ± 3.1 mg L$^{-1}$ | [49] |
| | | *Patients*: 14.8 ± 4.5 mg L$^{-1}$ | |
| HA | CSF | *Controls without Alzheimer* : 142.7 ± 68.7 ng L$^{-1}$ | [46] |
| | | *Patients with Alzheimer*: 177.6 ± 131.5 ng L$^{-1}$ | |
| | | *Controls without vascular dementia* : 156.4 ± 109.6 ng L$^{-1}$ | |
| | | *Patients with vascular dementia*: 192.6 ± 89.7 ng L$^{-1}$ | |
| | Lumbar CSF | *Controls*: 164.0 µg L$^{-1}$ | [2] |
| | | *Patients with subarachnoid hemorrhage*: 246 µg L$^{-1}$ | |
| | Serum | *Controls*: 6.9 µg L$^{-1}$ | [2] |
| | | *Patients with subarachnoid hemorrhage*: 12.8 µg L$^{-1}$ | |
| | Human umbilical cord | 4,100 mg L$^{-1}$ | [45] |
| | Human joint synovial fluid | 1,400-3,600 mg L$^{-1}$ | [45] |
| | Human dermis | 200-500 mg L$^{-1}$ | [45] |
| | Human epidermis | 100 mg L$^{-1}$ | [45] |

## 2.5 Central composite design (CCD) [51-56]

Experimental design or Designed experiments is a laying out of a detailed experimental plan in advance of doing the experiment. The approach to experimental design is shown in **Figure 2.8.** In Chemistry, there are four main reasons why the chemists can be more effective if they understand the basic of experimental design. The first is screening; the experiments involve seeing which factors are important for the success of a process. The second is optimization, which is one of the commonest applications in chemistry to find the best optimum condition that results in a minimum or maximum response. The third is saving time that is possibly the major motivation for experimental design, especially in industry applications. The final reason is the quantitative modelling, in which a series of observations is required to obtain a mathematical model of the system and to predict mathematically how a response of the experiment relates to the values of various factors. The purpose of experimental design is to characterize, predict, improve the behavior of the system or process, and deliver maximum attended information with the fewer of experimentation.
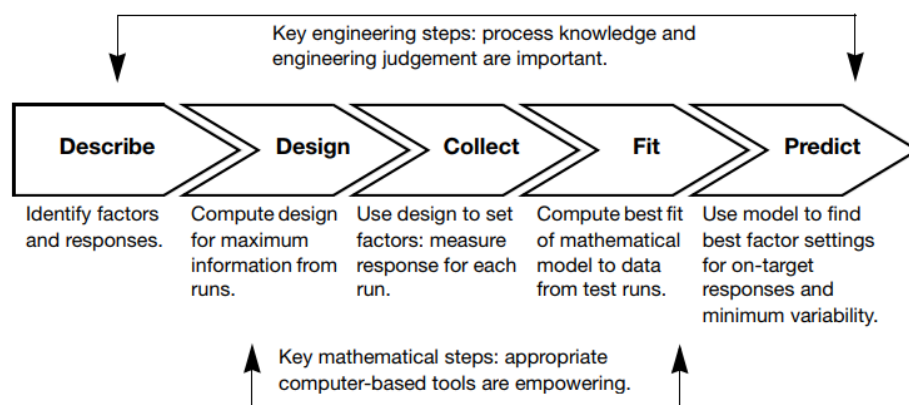


**Figure 2.8** An approach to experimental design. Reproduced from reference [54].

The components of experimental design are *"factors"* (controllable or uncontrollable variables) or inputs to the process, *"levels"* or settings of each factor in the study, and *"responses"* or output of the experiment.

Generally, the scientists find the optimal factors by varying only a single factor at a time, whilst the other factors are fixed at the specific conditions. This approach is called "one-factor at a time". The optimized condition might be accomplished in case of no interaction or no correlation between factors in experiments. Actually, the effects of each factor are correlated. Therefore, one-factor at a time is not proper for optimization. Moreover, this approach is time consuming and not providing the polynomial, interaction and also the error or stability of the study system. To get the interaction of each factor with the fewer number of experimentations, the experimental design can be utilized.

An experimental design that has been widely used is central composite design or CCD because multi-parameters can be optimized simultaneously. CCD contains three blocks, as depicted in **Table 2.2.** In this work, the rotatable CCD was used for optimization of three parameters. The weight of rotatable CCD is equal for all design points (except the center points), so the rotatable is important in determining the response surface. The rotatable variable (α) is calculated by **Equation 2.7**;

$$\alpha = \sqrt[4]{2^k} \tag{2.7}$$

where k is the number of experimental factors.

The CCD design with a sphere corresponding to an experimental run with three parameters is illustrated in **Figure 2.9.**

**Table 2.2** The rotatable central composite design with three factors and each factors
consisted of two levels.

| Experiment | $x_1$ | $x_2$ | $x_3$ | |
|---|---|---|---|---|
| 1 | -1 | -1 | -1 | Full factorial |
| 2 | 1 | -1 | -1 | |
| 3 | -1 | 1 | -1 | |
| 4 | 1 | 1 | -1 | |
| 5 | -1 | -1 | 1 | |
| 6 | 1 | -1 | 1 | |
| 7 | -1 | 1 | 1 | |
| 8 | 1 | 1 | 1 | |
| 9 | -1.682 | 0 | 0 | Star |
| 10 | 0 | -1.682 | 0 | |
| 11 | 0 | 0 | -1.682 | |
| 12 | 1.682 | 0 | 0 | |
| 13 | 0 | 1.682 | 0 | |
| 14 | 0 | 0 | 1.682 | |
| 15 | 0 | 0 | 0 | Replication |
| 16 | 0 | 0 | 0 | |
| 17 | 0 | 0 | 0 | |
| 18 | 0 | 0 | 0 | |
| 19 | 0 | 0 | 0 | |
| 20 | 0 | 0 | 0 | |

**Figure 2.9** The rotatable CCD models with three factors. Reproduced from
reference [57].

For three main factors, CCD consists of full factorial design; a star design and five replicates, which result in twenty experiments. An explanation for these data in **Table 2.2** and **Figure 2.9** is as follows:

- The first block, experiment number 1 to 8, is the full factorial experiment consisting of $2^3$ experiments for three factors with two levels. It is used to estimate all of the interaction terms. These experiments are placed on the eight corners of the cube.

- The second block, experiment number 9 to 15, is star design consisting of the center point and the point in the middle of each of six faces of the cube. There are seven experiments which are employed to estimate the squared or quadratic terms. In this design, at least three levels are required for each factor, denoted by +1.682, 0, and -1.682, with level "0" being at the center.

- The third block, experiment number 16 to 20, is replication design when is important to estimate the error or stability of the system. This is typically performed by repeating the same experiment for five times at the center of the design.

**2.6  Response Surfaces Methodology (RSM) [55]**

After the experimentations using CCD optimization, the relationships between responses and effects of each factor were quantified and interpreted using response surfaces methodology (RSM).

RSM is a set of advanced experimental design. The aims of RSM are to find the optimum values of each factor and to see how the factors perform over the whole experimental domain, including the interaction between two factors correlated to the response.

A response surface is a k-dimensional surface in k+1 dimensional space, where k is the number of factors. The regression model of parameters with the individual, interaction and second order polynomial terms can be used in order to find an approximation of the function as shown in **Equation 2.8**;

$$y = \beta_0 + \Sigma \beta_i x_i + \Sigma \beta_{ii} x_i^2 + \Sigma \beta_{ij} x_i x_j \qquad (2.8)$$

where y is the response, $\beta_0$ is an intercept or constant coefficient, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are the regression coefficients, $x_i$, $x_j$ are the dependent variables, $\beta_i x_i$ is linear term, $\beta_{ii} x_i^2$ is the quadratic term, and $\beta_{ij} x_i x_j$ is the interaction term.

In addition, multiple linear regression (MLR) was used to calculate the coefficients (β) of each term in the regression model as given in **Equation 2.9**;

$$\beta = \left(D^T \cdot D\right)^{-1} \cdot D^T \cdot y \qquad (2.9)$$

where all factors are square matrix, β is the coefficient (M×1), D is the design matrix (N×M), T is the transpose of a matrix, y is the response (N×1), M is the factor, and N is the amount of samples.

The difference between a response surface equation and the equation for factorial design is the addition of the quadratic or squared term in $x^2$ to allow for model curvature in the response. A response surface plot is a three-dimension surface, which is plotted between responses (y) and factors ($x_i$). For example, if there

are three factors, the response surface is a 3-*D* surface in 3-*D* space, which has three pairs of graphs with one of the variables being held constant. Generally, the method to examine this surface is to hold one of the variables constant (usually at its center point) and examine y as a function of the other two. This procedure generates three graphs which are y *vs* $x_1$, $x_2$ ($x_3$ constant), y *vs* $x_1$, $x_3$ ($x_2$ constant) and y *vs* $x_2$, $x_3$ ($x_1$ constant). An example of response surface plot is shown in **Figure 2.10**.
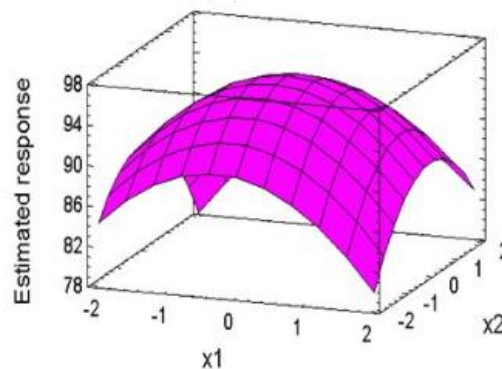


**Figure 2.10** An example of response surface plot for estimated response vs $x_1$, $x_2$ ($x_3$ constant). Reproduced from reference [55].

The optimal conditions as a range for each parameter were obtained from the response surface plot. To find out the optimal condition for each parameter as an exact value, the first derivative of an obtained equation could be used.

# CHAPTER III

# EXPERIMENTAL

## 3.1 Instruments and apparatus

**Table 3.1** List of instruments and apparatus.

| Instruments and apparatus | Companies |
|---|---|
| 1. Capillary electrophoresis (P/ACE MDQ CE System equipped with a photodiode array detector (PDA)) | Beckman Coulter, USA |
| 2. Fused silica capillary tubing (50 μm i.d., 375 μm o.d.) | Polymicro Technologies, USA |
| 3. Milli-Q ultra pure water system | Merck millipore, Germany |
| 4. pH meter | Mettler Toledo, USA |
| 5. Ultrasonic bath | Ultrasonic steri-cleaner, Taiwan |
| 6. Balance (5 digits) | Mettler Toledo, USA |
| 7. Syringe filter (0.22 μm nylon membrane) | Filtrex, Thailand |
| 8. Syringe (1 and 3 mL plastic syringe) | Nipro, USA |
| 9. Centrifuge | |
| - UNIVERSAL 320 R, | Hettich Centrifuge, Germany |
| - Sorvall ST 16 R | Thermo Fisher Scientific, Thailand |

## 3.2   Chemicals

**Table 3.2** List of chemicals.

| Chemicals | Companies |
| --- | --- |
| 1. Chondroitin sulfate sodium salt (CS) from shark cartilage | Sigma-Aldrich, USA |
| 2. Dermatan sulfate sodium salt (DS) | TCI Europe N.V., Japan |
| 3. Sodium hyaluronic acid (HA) from cockscomb | TCI Europe N.V., Japan |
| 4. Sodium phosphate monobasic monohydrate | Carlo-Erba, Italy |
| 5. Tris (hydroxymethyl) aminomethane (Tris) (99.8%) | Aldrich, USA |
| 6. Butylamine (98%) | Sigma-Aldrich, USA |
| 7. Methanol | Merck, USA |
| 8. Phosphoric acid | Merck, USA |
| 9. Polyethylene glycol (PEG) | Merck, USA |
| 10. Sodium hydroxide | Merck, USA |
| 11. Acetonitrile | Merck, USA |
| 12. Ethanol | Merck, USA |
| 13. Hydrochloric acid | Merck, USA |
| 14. Sodium acetate | Merck, USA |
| 15. Bovine serum albumin | Sigma-Aldrich, USA |
| 16. Chondroitinase ABC enzyme | Sigma-Aldrich, USA |

## 3.3   Preparation of standard solutions

Standard stock solutions of CS, DS and HA at 500 and 1,000 mg $L^{-1}$ each were separately prepared by dissolving 5 and 10 mg of each standard with water in 10 mL volumetric flasks, respectively.

In addition, GAGs standard working solutions at the concentration range of 10 to 400 mg $L^{-1}$ were prepared in 10% v/v BGE. The total volume was adjusted to 1 mL in 1.5 mL microcentrifuge tubes, as shown in **Table 3.3**.

**Table 3.3** Preparation of GAGs standard working solutions at different concentrations.

| GAGs (mg L$^{-1}$) | GAGs stock standard solution | | BGE (μL) |
| --- | --- | --- | --- |
| | Concentration (mg L$^{-1}$) | Volume (μL) | |
| 10 | 500 | 20 | 100 |
| 20 | 500 | 40 | 100 |
| 50 | 500 | 100 | 100 |
| 100 | 500 | 200 | 100 |
| 150 | 500 | 300 | 100 |
| 200 | 500 | 400 | 100 |
| 250 | 1,000 | 250 | 100 |
| 300 | 1,000 | 300 | 100 |
| 350 | 1,000 | 350 | 100 |
| 400 | 1,000 | 400 | 100 |

## 3.4 Preparation of samples

### 3.4.1 Biological samples

Biological samples, such as cell culture media, cerebrospinal fluid (CSF), urine, plasma and serum were obtained from the Faculty of Medicine, Chulalongkorn University (IRB No. 466/60). Cell culture media consisted of (i) human keratinocyte immortal cells (HaCat) (ii) human dermal fibroblasts cells (HDF) (iii) human periodontal ligament cells (PDL). The biological samples, except for plasma and serum, were diluted in 10% v/v BGE and filtered using 0.22 μm nylon membrane filters.

*- Protein precipitation*

Protein precipitation was used for sample pretreatment of plasma and serum. The volume ratio of organic solvent to sample was 1.5 to 1. Plasma or serum and organic solvent (acetonitrile, methanol or ethanol) were prepared by pipetting 500 μL and 750 μL, respectively into a 1.5 mL microcentrifuge tube. After mixing, shaking and centrifugation at 10,000 ppm for 10 min at 4 °C, protein precipitated out

of the sample solution and then the supernatant was pipetted into a new microcentrifuge tube. Then, the organic solvent was eliminated by blowing out with nitrogen gas. Dried plasma or serum was kept at -20 °C until use. Before analysis of dried plasma or serum using CE, Milli-Q water was added into the dried samples. In order to preconcentrate of analytes, the added volume was half of the supernatant volume of the sample. The treated plasma or serum sample was filtered using 0.22 µm nylon membrane filters before CE analysis.

  *- Enzymatic digestion*

  The sample preparation using an enzymatic digestion was additionally employed for analysis of CS in real samples (PDL and urine). In this work, CS in PDL cell culture media and urine samples was digested by chondroitinase ABC. Briefly, samples were filtered using 0.2 µm nylon syringe filter. The samples were prepared by pipetting 250 µL of each sample into 1.5 mL microcentrifuge tubes. After that, 25 µL of 1 M Tris, 30 µL of 1 M sodium acetate, 10 µL of 1% BSA and 1 µL of chondroitinase ABC enzyme were added, respectively. Finally, the volume of samples was made up to 500 µL by Milli-Q water. The sample solutions were incubated at 37 °C for 24 hours and then analyzed using CE method.

### 3.4.2  Cosmetic samples

  Cosmetic samples (serum) were purchased from a local convenience store. All samples were filtered through 0.22 µm nylon membrane filters and then appropriately diluted with 10% v/v BGE in the 0.2 mL microcentrifuge tubes prior to CE analysis.

### 3.4.3  Supplement sample

  A supplement sample (Glucosamine sulphate (750 mg) + Chondroitin sulphate (600 mg)) was supplied from a local pharmaceutical store. The sample was appropriately diluted with 10% v/v BGE in a 1.5 mL microcentrifuge tube and then filtered through 0.22 µm nylon membrane filters prior to CE analysis.

### 3.5  CE conditions

The general CE conditions for determination of GAGs are shown in **Table 3.4**. Conditions for rinsing capillary column are shown in **Table 3.5**.

**Table 3.4** General conditions of CE system for determination of GAGs.

| CE conditions | Details |
|---|---|
| CE instrument | P/ACE MDQ CE System (Beckman Coulter) equipped with a photodiode array detector (PDA) |
| Capillary column | Uncoated fused-silica capillary with 50 µm i.d., 375 µm o.d.,  total length of 40.2 cm (30 cm to detector) |
| Sample introduction | Hydrodynamic injection (0.5 psi for 60 s) |
| Detection | 195 nm |
| Applied voltage | -16 kV |
| Temperature | 25 °C |

**Table 3.5** The conditions for rinsing the capillary column. All solutions were driven through the capillary column using pressure at 20 psi.

| Rinse | Reagent | Time (min) |
|---|---|---|
| A new capillary | - MeOH | 20 |
| | - 1 M NaOH | |
| | - 0.1 M NaOH | |
| | - Milli-Q water | |
| | - 1 M $H_3PO_4$ | |
| | - 0.1 M $H_3PO_4$ | |
| | - BGE (200 mM $NaH_2PO_4$, 0.5% w/v PEG and 200 mM butylamine at pH 4.0) | |
| Before analysis of each day | - 0.1 M $H_3PO_4$ | 15 |
| | - BGE | |
| Before each injection | - MeOH | 1 |
| | - 0.1 M $H_3PO_4$ | |
| | - BGE | 2 |
| After the end of each run | - 0.1 M NaOH | 3 |
| | - Milli-Q water | |
| | - 0.1 M $H_3PO_4$ | |
| | - BGE | |
| After analysis of each day | - MeOH | 5 |
| | - 0.1 M NaOH | 15 |
| | - Milli-Q water | |

### 3.6  CE optimizations

#### 3.6.1  Ion pair reagent concentration

Effect of ion pair reagent concentration on the CS and DS separation was investigated by varying butylamine concentration in the range of 100 to 300 mM. Results are shown in Section 4.1.2.1.

#### 3.6.2  On-line sample preconcentration

This work employed large-volume sample stacking using an electroosmotic flow (EOF) pump (LVSEP) to perform on-line sample preconcentration. To optimize injection time for LVSEP, a standard mixture containing CS, DS and HA (333 mg L$^{-1}$ each) prepared in 10% v/v BGE was hydrodynamically injected using 0.5 psi with different injection times from 5 to 99 s and then separated using BGE containing 50 mM NaH$_2$PO$_4$, 200 mM butylamine, pH 3.0, -15 kV at 25 °C. Results are shown in Section 4.1.2.2.

#### 3.6.3  Preliminary study for CE conditions

Type and concentration of buffer, pH and an applied voltage were initially optimized using one-factor at a time (OFAT) method for preliminary investigation. All of the ranges of each parameter for CE optimizations were suggested by literature review.

- *Type of buffer*

NaH$_2$PO$_4$, Tris-base and Tris-HCl were investigated for GAGs separation. Results are shown in Section 4.1.2.3.

- *Concentration of buffer*

Concentration of buffer was studied in the range of 50-200 mM. Results are shown in Section 4.1.2.3.

- *pH of buffer*

pH of buffer was investigated in the range of 3.0-6.0. Results are shown in Section 4.1.2.3.

*- Applied voltage*

Applied voltage for CE analysis was investigated in the range of -10 to -25 kV. Results are shown in Section 4.1.2.3.

### 3.6.4 Central composite design (CCD)

Central composite design (CCD) was chosen for simultaneously optimizing the parameters for CE separation of GAGs. The actual and coded values for three main parameters: concentration of $NaH_2PO_4$ ($X_1$), pH ($X_2$) and applied voltage ($X_3$) investigated in this work are shown in **Table 3.6**. In addition, the orthogonal designs for CCD in 20 experiments are shown in **Table 3.7**, in which the ranges of the three parameters were selected based on the preliminary results. Statistical analysis of results was performed using Microsoft Excel and MATLAB (The MathWorks, Inc.: Version R2014a) as shown in Section 4.1.2.4.

**Table 3.6** The actual and coded values of three main factors.

| Factor | Symbol | Actual and coded values | | | | |
|---|---|---|---|---|---|---|
| | | -1.682 | -1 | 0 | 1 | 1.682 |
| conc. of $NaH_2PO_4$ (mM) | $X_1$ | 72.8 | 100 | 140 | 180 | 207.2 |
| pH | $X_2$ | 3.7 | 3.8 | 4.0 | 4.2 | 4.3 |
| Voltage (-kV) | $X_3$ | 14.2 | 14.5 | 15.0 | 15.5 | 15.8 |

Table 3.7 The orthogonal design for CCD in 20 experiments.

| Experiment | $x_1$ | $x_2$ | $x_3$ |
|---|---|---|---|
| 1 | -1 | -1 | -1 |
| 2 | 1 | -1 | -1 |
| 3 | -1 | 1 | -1 |
| 4 | 1 | 1 | -1 |
| 5 | -1 | -1 | 1 |
| 6 | 1 | -1 | 1 |
| 7 | -1 | 1 | 1 |
| 8 | 1 | 1 | 1 |
| 9 | -1.682 | 0 | 0 |
| 10 | 0 | -1.682 | 0 |
| 11 | 0 | 0 | -1.682 |
| 12 | 1.682 | 0 | 0 |
| 13 | 0 | 1.682 | 0 |
| 14 | 0 | 0 | 1.682 |
| 15 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 |
| 19 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 |

## 3.7 Validation of CE method

### 3.7.1 Standard addition curves

The sample matrices affected the analysis of GAGs. To avoid the sample matrices effect, standard addition method was used for quantitative analysis of the real samples. Standard addition curves were constructed using five concentration levels of GAGs standards spiked into the blank sample as shown in **Table 3.8**. The standard addition curves were plotted using corrected peak areas (peak area divided by migration time) of the analyte against the spiked standard concentrations. Results are shown in Section 4.2.1.

**Table 3.8** Spiked concentration ranges of standard addition curves.

| Analyte | Spiked concentration (mg $L^{-1}$) | | | | |
|---------|---------|---------|---------|---------|---------|
|         | Level 1 | Level 2 | Level 3 | Level 4 | Level 5 |
| CS      | 100     | 200     | 300     | 400     | 500     |
| HA      | 10      | 25      | 50      | 75      | 100     |
|         | 50      | 100     | 150     | 200     | 250     |

### 3.7.2 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were evaluated by injecting each diluted GAGs standard solution until the signal-to-noise (S/N) ratios were 3 and 10 for LOD and LOQ, respectively. Results are shown in Section 4.2.2.

### 3.7.3 Accuracy and precision

Accuracy of the developed CE method was investigated using percent recovery of CS and/or HA standard spiked into water and real samples of biological, cosmetic or pharmaceutical samples at three concentration levels, as shown in **Table 3.9** and **3.10**, respectively. The amounts of CS and HA were determined by CE analysis using standard addition curves. Results are shown in Section 4.2.3 and 4.2.4.

The accuracy is expressed in terms of the percentage of recovery as shown in **Equation 3.1**;

$$\%Recovery \quad = \quad \frac{C_1}{C_2} \times 100 \qquad (3.1)$$

where $C_1$ refers to the determined amount of the analyte and

$C_2$ refers to the known concentration of spiked analyte.

**Table 3.9** Concentrations of CS, DS and HA added in water for accuracy study.

| Analyte | Concentration level (mg L$^{-1}$) | | |
|---------|------|--------|------|
|         | Low  | Medium | High |
| CS      | 100  | 250    | 400  |
| DS      | 150  | 350    | 450  |
| HA      | 60   | 90     | 140  |

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**Table 3.10** Concentrations of CS and HA added in various samples for accuracy study.

| Analyte | Sample | Concentration level (mg L$^{-1}$) | | |
|---|---|---|---|---|
| | | Low | Medium | High |
| CS | PDL | 150 | 250 | 350 |
| | Urine | 150 | 250 | 350 |
| | Supplement | 100 | 200 | 400 |
| HA | HaCaT | 60 | 120 | 180 |
| | HDF | 60 | 120 | 180 |
| | CSF | 20 | 40 | 60 |
| | Plasma | 20 | 40 | 60 |
| | Cosmetic I | 60 | 120 | 180 |
| | Cosmetic II | 40 | 60 | 90 |
| | Cosmetic III | 20 | 40 | 60 |

## 3.8 Applications to real samples

The developed and validated CE method was used for quantitation of GAGs in biological, pharmaceutical and cosmetic samples. All of samples were prepared using the procedures previously mentioned in Section 3.4. Results are shown in Section 4.3.

# CHAPTER IV
# RESULTS AND DISSCUSION

## 4.1 Overview and optimization of CE

### 4.1.1 Overview of this work

GAGs (CS, DS, HA) are linear polysaccharide, highly negative charges and acidic compounds with p$K_a$ 3.38, 3.82 and 3.00 for CS, DS and HA, respectively [58-61]. Since the migration of GAGs is opposite to the direction of EOF, the determination of GAGs should be performed in a reversed polarity mode. Moreover, GAGs are low UV absorption molecules causing low detection sensitivity in the CE-UV method. An on-line preconcentration technique was required to improve the detection sensitivity. In this research, large volume sample stacking using an electroosmotic flow pump or LVSEP was selected for online preconcentration. All of parameters that affected the resolution between CS and DS and also the GAGs separation in CE-UV method were optimized using the central composite design or CCD approach.

### 4.1.2 Optimization of CE

#### 4.1.2.1 Effect of ion pair reagent concentration

Since the structures of CS and DS are relatively similar, addition of a polyamine as an ion-pairing or resolving reagent, such as ethylenediamine, trimethylamine, trimethylamine and butylamine into BGE was reported to improve the separation efficiency of GAGs [26]. The polyamines are positively charged compounds, which can interact with the negative charges of GAGs [26]. Therefore, butylamine was selected and added into the BGE for GAGs separation according to the previous study [62]. Separation of CS and DS was found to be problematic in this study because these two compounds share similar migration properties, such as overall charges with the pKa values of 3.38 for CS and 3.82 for DS [58, 60, 61] and the structurally similar compound, which resulted in their co-eluting peaks (**Figure 4.1** (a)). Butylamine reacted with CS and DS at different extents, leading to CS-butylamine and DS-butylamine complexes with more significant difference in

migration properties compared with the analytes in their free forms [26]. It should be noted that the addition of butylamine could also increase pH of the BGE. Accordingly, the pH of BGE was re-adjusted with 1 M $H_3PO_4$. Moreover, the concentration of butylamine affected the resolution (Rs) between CS and DS **(Figure 4.2)** and also the migration time. The resolution of CS and DS at 200 mM butylamine provided acceptable resolution with short analysis time when compared with other concentrations **(Figure 4.3)**. Thus, 200 mM butylamine was chosen for further analysis.



**Figure 4.1** Overlaid electropherograms of the standard mixture (CS, DS and HA at 333 mg $L^{-1}$ each) obtained from CE separations using 50 mM $NaH_2PO_4$ at pH 3.0; (a) without butylamine and (b) with 200 mM butylamine. Other CE conditions: uncoated fused silica capillary 50 μm i.d. × 40.2 cm (30 cm to detector), separation voltage of -15.0 kV, 0.5 psi pressure injection for 60 s, temperature of 25 °C and UV detection at 195 nm. Peak identification: (1) CS, (2) DS and (3) HA.

**Figure 4.2** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 50 mM NaH$_2$PO$_4$ at pH 3.0 and butylamine in the concentration range of 100-300 mM. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
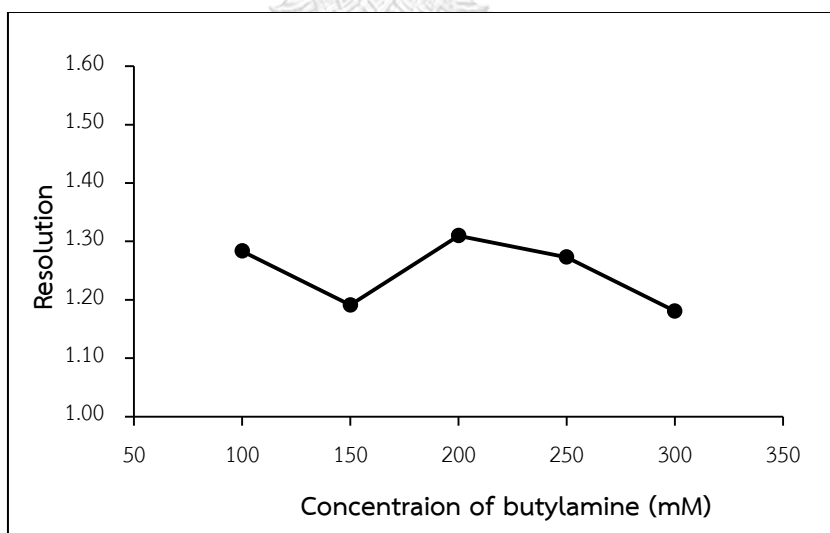


**Figure 4.3** Effect of butylamine concentration on the resolution between CS and DS. Other CE conditions are shown in **Figure 4.2**.

### 4.1.2.2 On-line sample preconcentration

From the structures, GAGs are negatively charged and weakly absorb UV light. Thus, determination of these GAGs present in small quantities is problematic for CE measurements using UV detection. To improve sensitivity, large-volume sample stacking using an EOF pump (LVSEP) was applied to perform on-line sample preconcentration [30]. LVSEP is suitable for analytes migrating in the opposite direction to EOF. LVSEP preconcentrates anionic analytes by means of large volume sample injection and matrix removal from the sample zone using EOF, thus making the sample zone narrower and more concentrated. Typically, the LVSEP technique is suitable for analysis of anionic analytes under a reversed polarity. To perform LVSEP, BGE with low pH is required to minimize EOF and a large volume of sample is hydrodynamically injected. After application of voltage, EOF moving in the opposite direction to anions removes matrix from the sample zone. As a result, the sample plug is focused into a narrow zone, thus enhancing the concentration of the analytes. In this study, GAGs are negatively charged, which can possibly be preconcentrated using LVSEP under a reversed polarity condition. Accordingly, the standard mixture of CS, DS and HA was introduced into the CE system using 0.5 psi hydrodynamic injection. Injection time between 5 to 99 s was applied to investigate the concentration enhancement when using the LVSEP method. The separation was then performed using CE conditions from the previous work: BGE containing 50 mM $NaH_2PO_4$ and 200 mM butylamine at pH 3.0 and a separation voltage of -15 kV [62]. The use of low pH BGE was to suppress the EOF and ensure that the negatively charged GAGs moved to the detector without being swept backwards. Under these conditions, EOF moved towards the inlet (cathode) end, while the sample plug migrated against the EOF towards the outlet (anode) end. As a result, EOF removed the matrix from the sample zone. Subsequently, the negatively charged GAGs were stacked into a narrow zone and preconcentrated before migrating and being separated according to their electrophoretic mobilities. The LVSEP for GAGs separation is shown in **Figure 4.4**.

For on-line preconcentration using LVSEP, the effect of injection time on the preconcentration efficiency of LVSEP was studied, as shown in **Figure 4.5.** As

expected, the larger injection volume (by increasing the injection time) resulted in the higher peak heights/areas of GAGs. In addition, it was observed that when injection time was increased, the migration times of all analytes were also slightly increased. Anionic analytes in the sample zone migrated against EOF, which could cause slightly backward migration of the analytes during matrix removal by EOF. After the end of matrix removal, the analytes migrated towards the detector. The longer the injection time was introduced to CE system, the longer the backward distance of the analytes was observed. This resulted in longer migration distance, causing longer analysis time when increasing the injection time. In addition, broader peaks were also observed when using longer injection time because the large volumes of sample were loaded to a capillary column, suppressing resolution of the critical pair (CS and DS). Thus, for further optimization of CE conditions, the injection time of 60 s was selected to compensate between sensitivity and resolution.
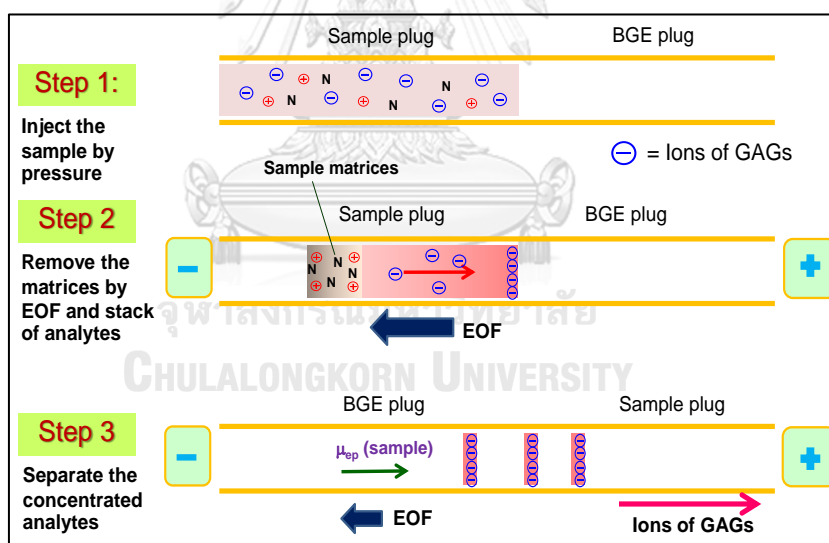


**Figure 4.4** Simple schematic of the developed LVSEP-CE for GAGs separation. **Step 1** is hydrodynamic injection of the sample. **Step 2** is LVSEP process by exploiting conductivity difference between sample and buffer zone. The sample matrices are removed by EOF, whilst GAGs are stacked and concentrated. Finally, **step 3** is separation and detection of the GAGs.
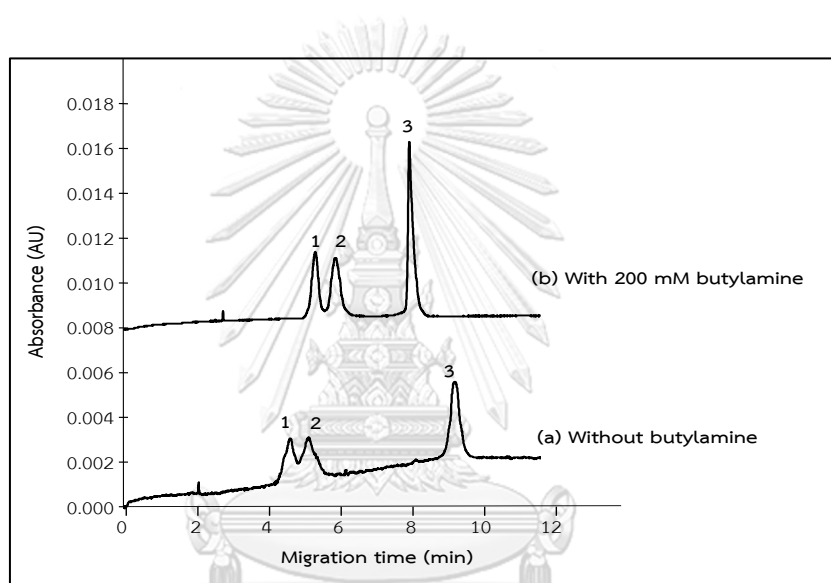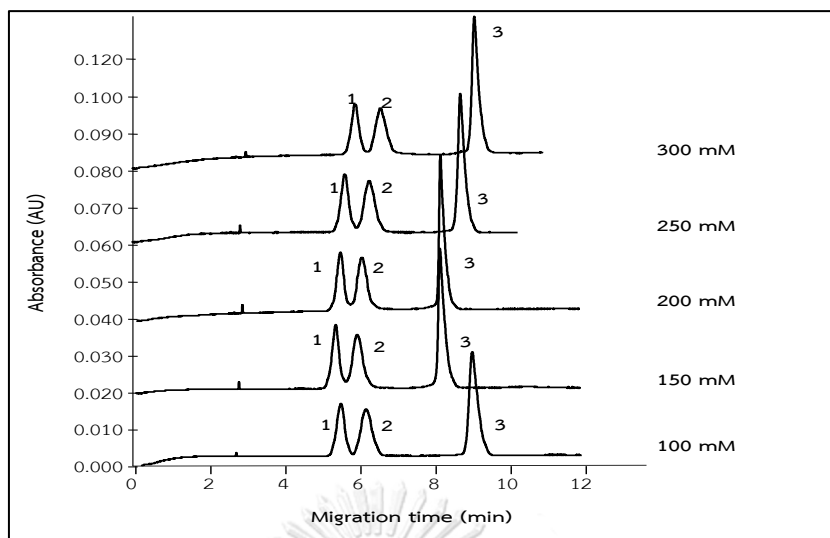
**Figure 4.5** Overlaid electropherograms of the standard mixture (CS, DS and HA at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 50 mM NaH$_2$PO$_4$, 200 mM butylamine at pH 3.0 with different sample injection times in the range of 5 to 99 s. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.

### 4.1.2.3 Preliminary study

For preliminary experiments for CE optimization, type and concentration of buffer, pH and applied voltage were optimized using the one-factor at a time (OFAT) method. Generally, this approach is performed by varying one factor at a time, while other factors are fixed [63], which is a fast and simple approach.

- *Effect of buffer type*

Three different types of buffer, NaH$_2$PO$_4$, Tris-base and Tris-HCl were investigated for the CE separations of GAGs. Results showed that 50 mM sodium phosphate (NaH$_2$PO$_4$) buffer provided better resolution between CS and DS and faster separation when compared to Tri-base and Tris-HCl (as shown in **Figure 4.6** and **Figure 4.7**). In addition, sodium phosphate buffer was suitable for GAGs separation because of its good buffer capacity at low pH and optical clarity in UV spectrum [64]. Thus, NaH$_2$PO$_4$ buffer was selected for further experiments.

**Figure 4.6** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 200 mM butylamine at pH 3.0 and different types of buffer; (a) 50 mM NaH$_2$PO$_4$, (b) 50 mM Tris-base and (c) 50 mM Tris-HCl. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
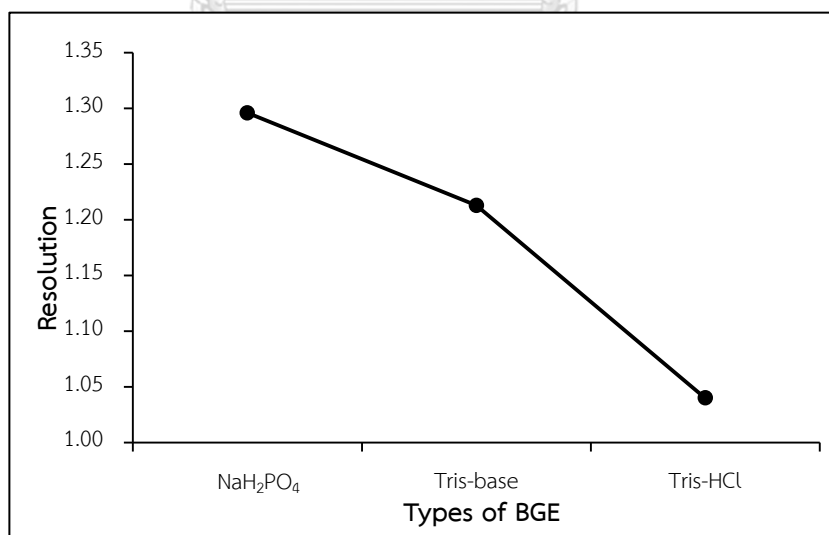


**Figure 4.7** Effect of types of BGE on the resolution between CS and DS. Other CE conditions are shown in **Figure 4.6**.

*- Effect of NaH$_2$PO$_4$ concentration*

       In this work, when increasing NaH$_2$PO$_4$ buffer concentration, the migration times of all analytes, especially CS and DS, were not significantly different because the sodium ion from the buffer solution did not affect the structure of CS and DS. For HA, the migration time was slightly shifted due to self-association of HA segments in the presence of sodium ion from the buffer solution [65, 66], which resulted in a slightly increase of the viscosity and migration time **(Figure 4.8)**. However, the resolution between CS and DS tended to gradually increase with buffer concentration **(Figure 4.9)**. Accordingly, a high buffer concentration was suitable for GAGs separation. The benefits of high buffer concentration are to reduce the EOF and band broadening from electromigration dispersion [20, 27], and also improve the sample stacking process [64]. Moreover, when increasing the buffer concentration, Joule heating was increased, affecting the repeatability and resolution of separations. In order to solve the problem, fresh buffer is recommended.
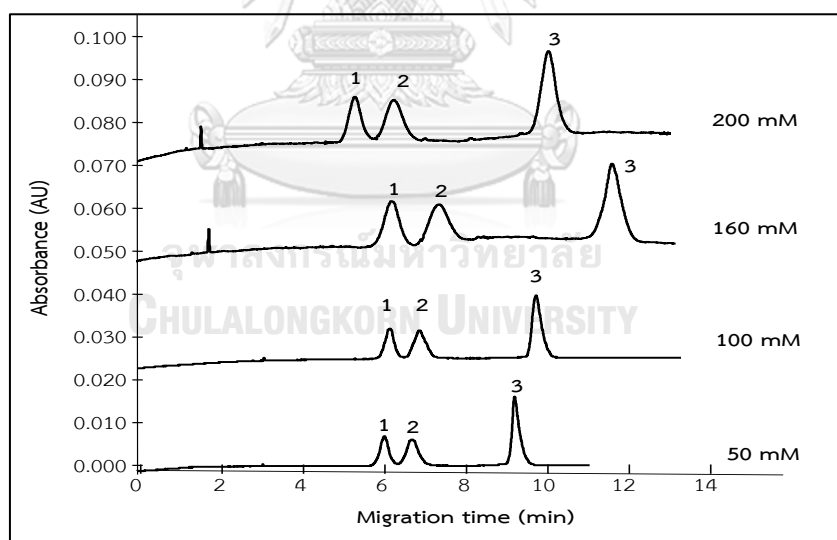


**Figure 4.8** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 200 mM butylamine at pH 3.0 and NaH$_2$PO$_4$ concentration in the range of 50-200 mM. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
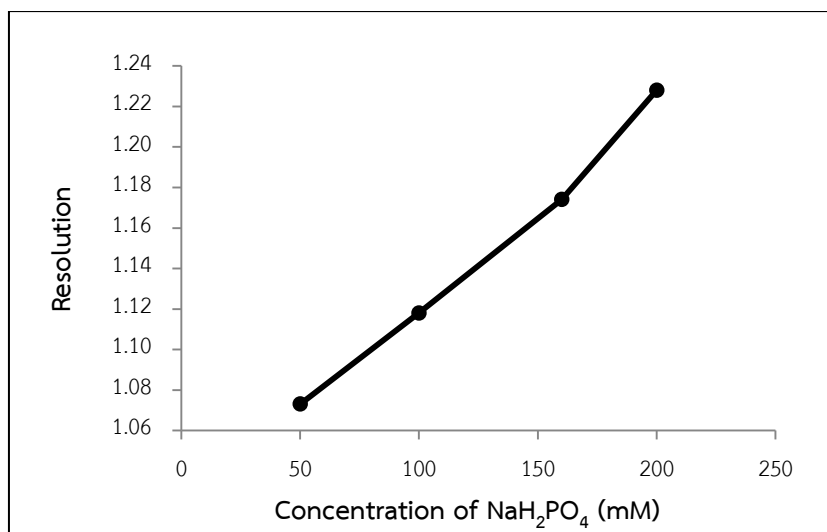
**Figure 4.9** Effect of NaH$_2$PO$_4$ concentration on the resolution between CS and DS. Other CE conditions are shown in **Figure 4.8**.

- *Effect of pH*

According to LVSEP, low pH is required for online preconcentration and separation. Accordingly, a pH range of 3.0 to 6.0 was investigated. Results in **Figure 4.10** and **Figure 4.11** showed that when increasing pH from 3.0 to 4.5, migration times of CS and DS were decreased, thus decreasing Rs between CS and DS. This is because higher pH values caused more ionization of the analytes (pK$_a$ values of CS and DS are 3.38 and 3.82, respectively [58, 60, 61]). However, when the pH of BGE was further increased to pH 5.0 and 6.0, it was found that the migrations of CS and DS were slower than those at lower pH values. This was due to stronger EOF at higher pH values, which resisted the migrations of analytes. The best Rs between CS and DS was obtained when using BGE at pH 3.5 which was also suitable for on-line preconcentration using LVSEP.
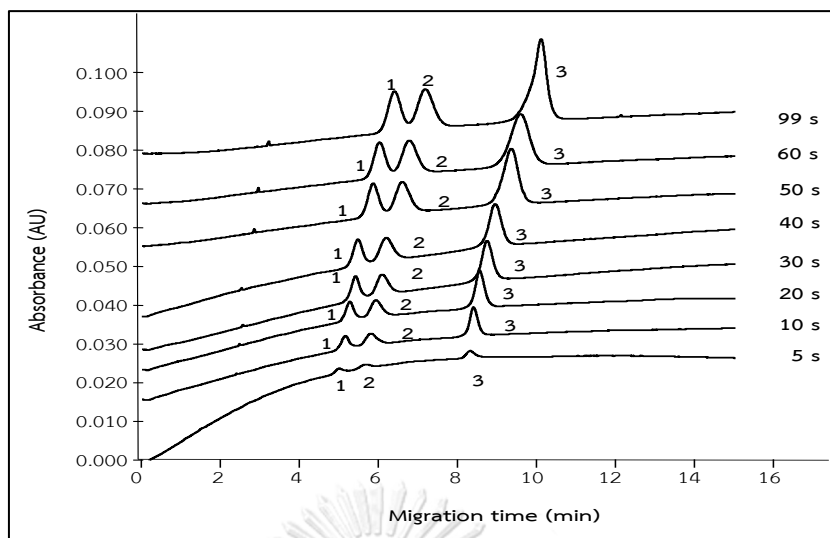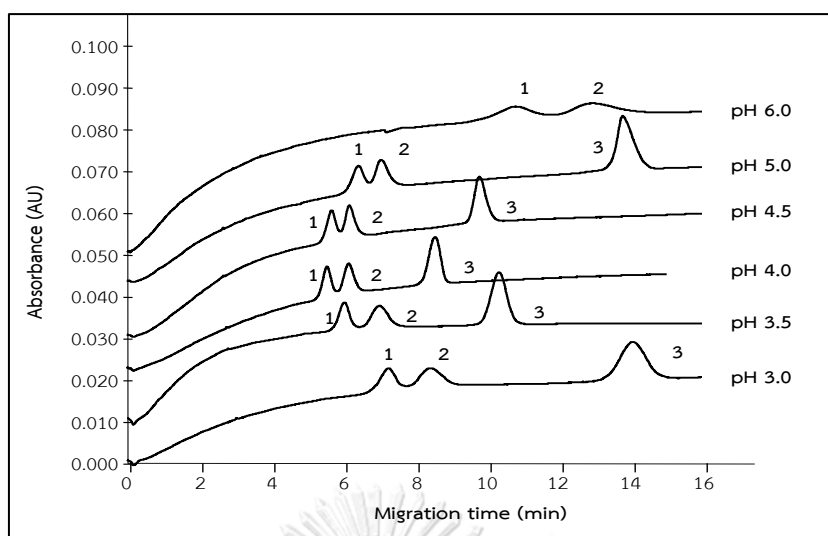
**Figure 4.10** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 200 mM butylamine and 50 mM NaH$_2$PO$_4$ with different pHs from 3.0 to 6.0. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
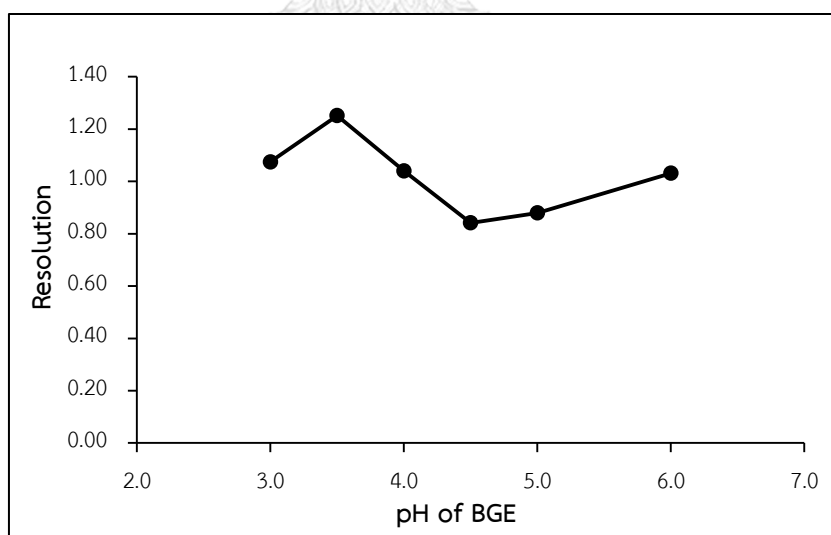


**Figure 4.11** Effect of pH of BGE on the resolution between CS and DS. Other CE conditions are shown in **Figure 4.10**.

*-Effect of applied voltage*

As expected, the higher the separation voltage, the shorter the migration time causing the decrease in Rs between CS and DS, as shown in **Figures 4.12** and **4.13**. At -10 kV, the migration time of all analytes was slow with the analysis time up to 20 min, but the resolution between CS and DS was higher than those of -15 and -20 kV, respectively. In the case of -15 and -20 kV, the resolution at -15 kV was higher than that of -20 kV and the Joule heating was lower.
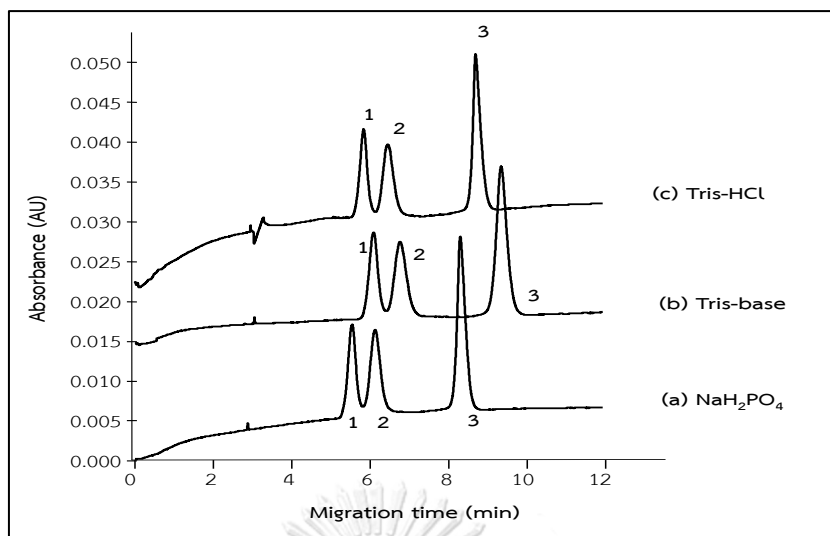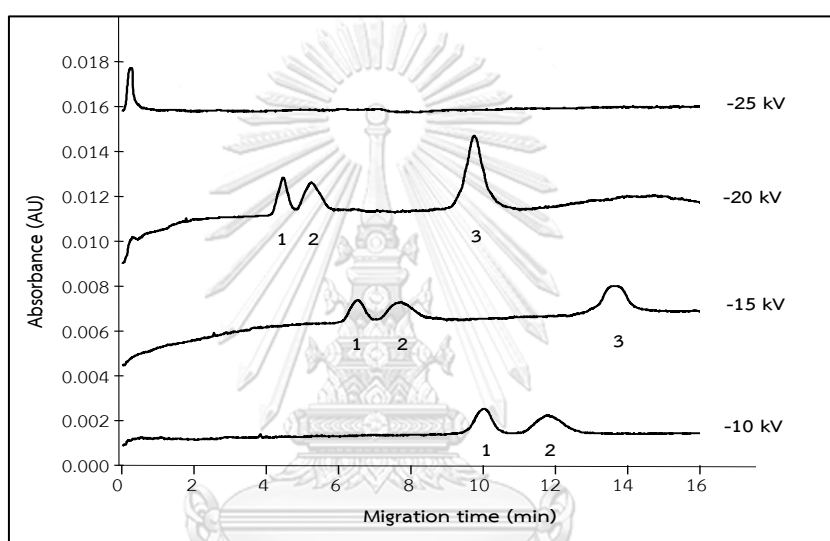


**Figure 4.12** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 333 mg L$^{-1}$ each) obtained from CE separations using buffer containing 50 mM NaH$_2$PO$_4$, 200 mM butylamine at pH 4.0 and applied voltage in the range of -10 to -25 kV. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
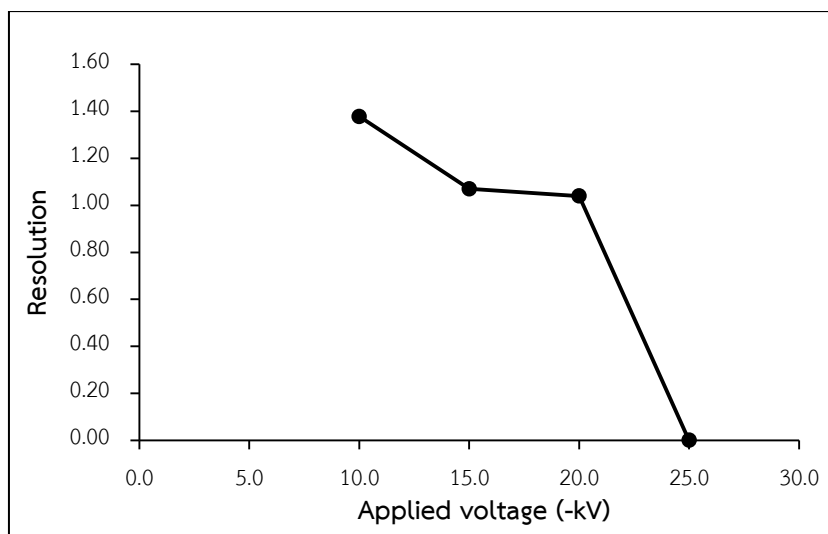
**Figure 4.13** Effect of applied voltage on the resolution between CS and DS.
Other CE conditions are shown in **Figure 4.12.**

For the optimization of parameters using one-factor at a time, it is carried out based on the assumption that these factors are not correlated. However, it is rarely found that individual factors do not affect each other. Therefore, it is time consuming to try many experiments for optimization of all parameters. Moreover, the correlation between factors is neglected when using one-factor at a time, so that the best optimization cannot be true [67]. Therefore, one-factor at a time is not an actually suitable approach to determine the optimal conditions of all parameters, but it is useful as a guideline for the ranges of factors for further optimization using an experimental design, such as a central composite design (CCD), which was used in this work.

From the preliminary studies, three parameters consisting of concentration of $NaH_2PO_4$, pH and applied voltage were selected for CCD experiments because these parameters demonstrated the important effects on the resolution between CS and DS. Therefore, other parameters were fixed as follows; 200 mM buthylamine and 60 s injection time.

### 4.1.2.4 Central composite design (CCD) investigations

CCD is a multivariate approach based on the variation of several parameters at the same time, which shows the interactions among experimental parameters in analytical chemistry [51, 68, 69]. Since the separation of GAGs especially, CS and DS depended on several experimental parameters, an experimental design was performed in order to simultaneously select several optimized parameters. From the preliminary studies, three parameters including concentrations of $NaH_2PO_4$, pH and applied voltage were selected for CCD experiments because these parameters demonstrated the important effects on the resolution between CS and DS. Accordingly, the three main factors: concentration of $NaH_2PO_4$ ($X_1$) ranging from 72.8 to 207.2 mM, pH ($X_2$) ranging from 3.7 to 4.3 and applied voltage ($X_3$) ranging from -14.2 to -15.8 kV were investigated for the resolution between CS and DS. It should be noted that the narrow pH range for CCD optimization was applied because LVSEP required low pH of the buffer solution. Twenty experiments were designed and carried out in random order to avoid systematic error. Experimental conditions for CCD and responses from the experiments presented in terms of the resolution between CS and DS are shown in **Table 4.1**.

Multiple linear regression (MLR) was used to calculate the linear, interactive and quadratic parameters as a regression model which correlated to the resolution between CS and DS. Using MS excel and MATLAB programs, the coded values were used to construct a regression model described as follows:

$$y_{resolution} = 1.22 + 0.03X_1 - 0.10X_2 + 0.02 X_3 - 0.02X_1^2 - 0.06X_2^2 - 0.02X_3^2 + 0.002X_1X_2 + 0.02X_1X_3 + 0.04X_2X_3$$

where $X_1$, $X_2$ and $X_3$ are linear terms corresponding to the concentration of $NaH_2PO_4$, pH of BGE and applied voltage, respectively. $X_1^2$, $X_2^2$, $X_3^2$ and $X_1X_2$, $X_1X_3$, $X_2X_3$ are quadratic terms and interaction terms, respectively, of these parameters. The size and sign (positive or negative) of the coefficients indicated the magnitude and

direction of each parameter, respectively. Results show that these parameters were correlated and affected the resolution between CS and DS. Therefore, the separation conditions optimized using the one-factor at a time approach was not satisfied. The response surface methodology (RSM) was employed to construct response surfaces to investigate the optimum values providing the best resolution between CS and DS.

Three-dimensional (3D) response surface plots are depicted in **Figure 4.14 (a)-(c)**. From the response surface plots, the optimum values of each parameter for CE separations were 207.2 mM $NaH_2PO_4$, pH 4.0 and an applied voltage of -15.8 kV. However, in practice, 200 mM $NaH_2PO_4$ at pH 4.0 and -16.0 kV were used instead.

**Table 4.1** The resolution (Rs) between CS and DS of 20 experiments for determination of GAGs.

| Experiment | $NaH_2PO_4$ (mM) | pH | Applied voltage (-kV) | Resolution (Rs) |
|:---:|:---:|:---:|:---:|:---:|
| 1 | 100 | 3.8 | 14.5 | 1.19 |
| 2 | 180 | 3.8 | 14.5 | 1.23 |
| 3 | 100 | 4.2 | 14.5 | 0.94 |
| 4 | 180 | 4.2 | 14.5 | 1.00 |
| 5 | 100 | 3.8 | 15.5 | 1.15 |
| 6 | 180 | 3.8 | 15.5 | 1.29 |
| 7 | 100 | 4.2 | 15.5 | 1.07 |
| 8 | 180 | 4.2 | 15.5 | 1.19 |
| 9 | 72.8 | 4.0 | 15.0 | 1.11 |
| 10 | 140 | 3.7 | 15.0 | 1.22 |
| 11 | 140 | 4.0 | 14.2 | 1.15 |
| 12 | 207.2 | 4.0 | 15.0 | 1.17 |
| 13 | 140 | 4.3 | 15.0 | 0.81 |
| 14 | 140 | 4.0 | 15.8 | 1.14 |
| 15 | 140 | 4.0 | 15.0 | 1.23 |
| 16 | 140 | 4.0 | 15.0 | 1.24 |
| 17 | 140 | 4.0 | 15.0 | 1.23 |
| 18 | 140 | 4.0 | 15.0 | 1.27 |
| 19 | 140 | 4.0 | 15.0 | 1.26 |
| 20 | 140 | 4.0 | 15.0 | 1.20 |
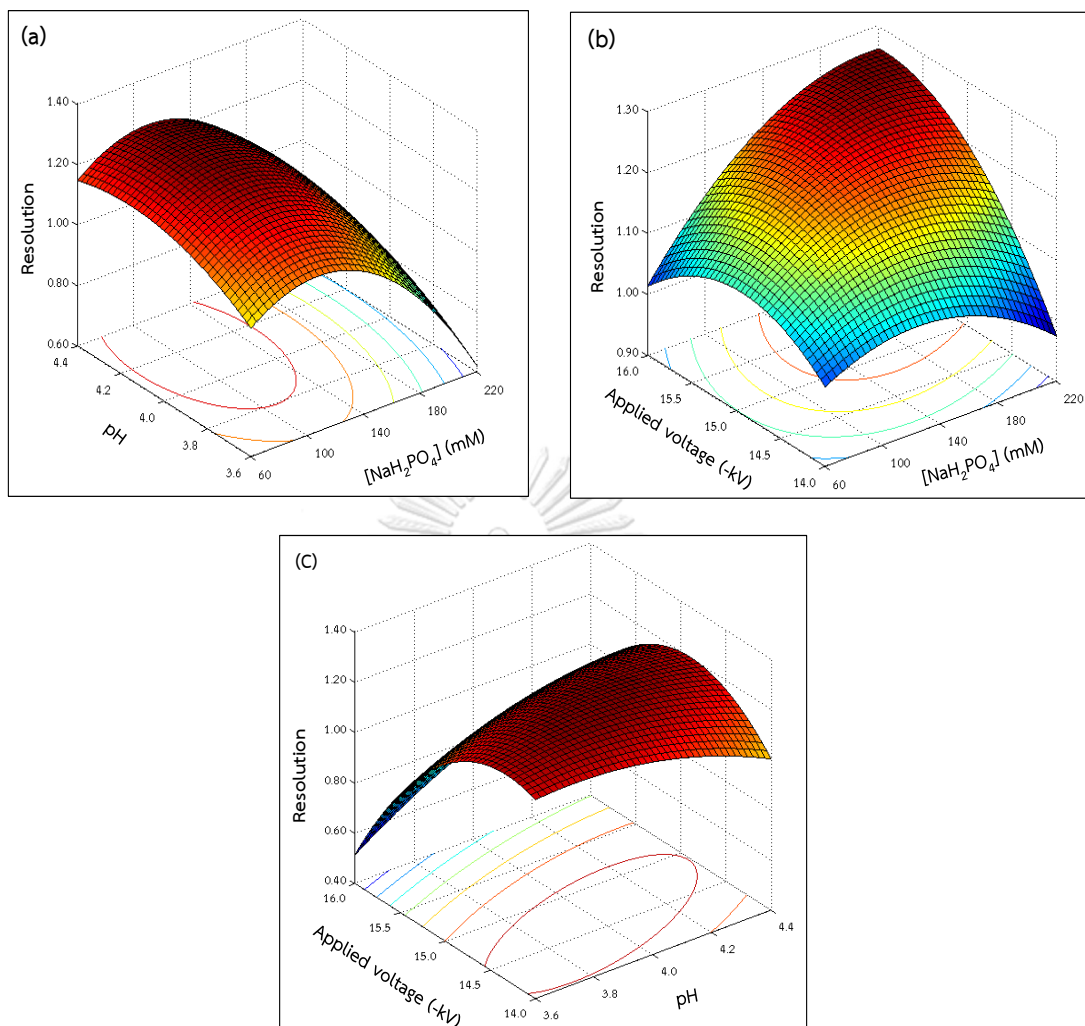
**Figure 4.14** Response surface and contour plots for the resolution between CS and DS; (a) a plot between concentration of $NaH_2PO_4$ and pH of buffer solution, (b) a plot between concentration of $NaH_2PO_4$ and applied voltage and (c) a plot between pH of buffer solution and applied voltage.

### 4.1.2.5  Effect of PEG concentration

Since GAGs are polysaccharides with large molecules that could easily adsorb on the capillary surface [70], the separation and quantitation of GAGs are often not precise. Accordingly, polyethylene glycol (PEG) is normally added into the BGE in order to minimize sample adsorption onto the capillary surface, thus improving the repeatability of the CE analysis [30, 71]. **Figures 4.15** and **4.16** showed the HA peaks for three replicates in the buffer solution without and with PEG, respectively. Results showed that PEG can reduce the adsorption of GAGs on the capillary surface, thus improving the repeatability of the analysis. In this experiment, the molecular weight of PEG was investigated between 4,000 and 10,000 g mol$^{-1}$. Results are shown in **Figure 4.17**. The use of 10,000 g mol$^{-1}$ PEG gave the higher resolution between CS and DS when compared to the use of 4,000 g mol$^{-1}$ PEG. Moreover, the peak shapes of CS, DS and HA showed good symmetry and no peak broadening. Therefore, the molecular weight of 10,000 g mol$^{-1}$ was chosen for further experiments.

The concentration of PEG (M.W. 10,000 g mol$^{-1}$) was studied in the range of 0.1-1.0% w/v. Results are shown in **Figure 4.18**. At 0.1% w/v of PEG, the peak broadening of HA and peak distortion of DS were observed. Meanwhile, 1.0% w/v of PEG caused peak broadening of analytes and long analysis time because the high percent or concentration of PEG increased the viscosity of buffer solution, which affected the migration time and peak shapes of all analytes. Therefore, PEG at 0.5% w/v was selected because of its compromises between peak shape and analysis time for GAGs separation. In summary, the BGE used in this work for GAGs analysis consisted of 200 mM NaH$_2$PO$_4$, 200 mM butylamine and 0.5% w/v PEG at pH 4.0.
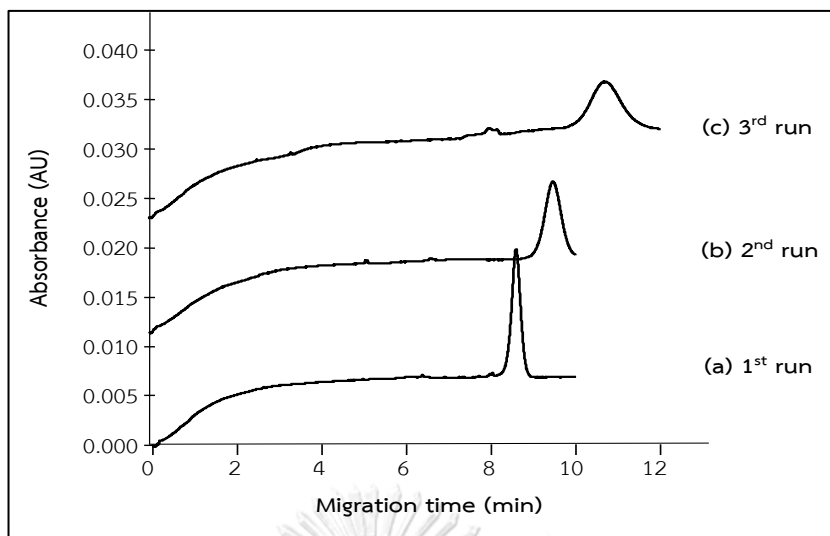
**Figure 4.15** Overlaid electropherograms of 200 mg L$^{-1}$ HA; (a) 1$^{st}$ run, (b) 2$^{nd}$ run and (c) 3$^{rd}$ run. The CE separations using buffer containing 200 mM NaH$_2$PO$_4$, 200 mM butylamine at pH 4.0 and separation voltage of -16 kV. Other CE conditions are shown in **Figure 4.1**.
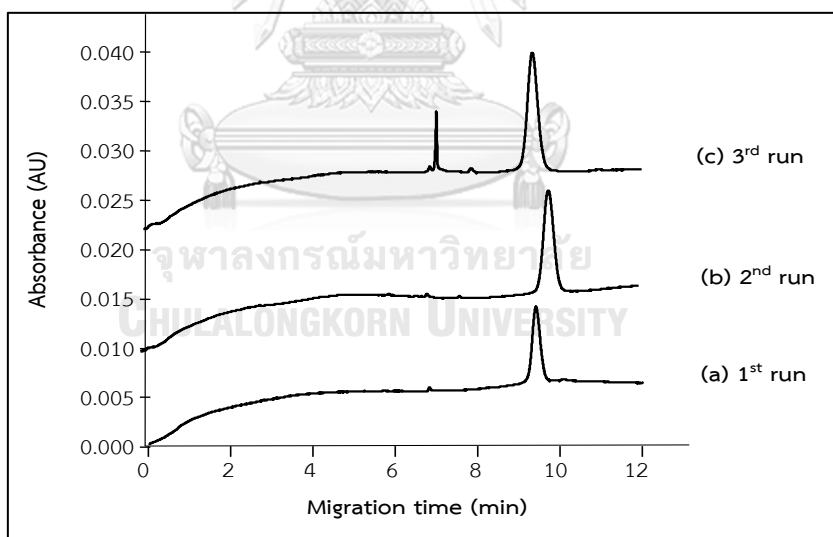


**Figure 4.16** Overlaid electropherograms of 200 mg L$^{-1}$ HA; (a) 1$^{st}$ run, (b) 2$^{nd}$ run and (c) 3$^{rd}$ run. The CE separations using buffer containing 200 mM NaH$_2$PO$_4$, 200 mM butylamine at pH 4.0, separation voltage of -16 kV and 0.5% w/v PEG. Other CE conditions are shown in **Figure 4.1**.
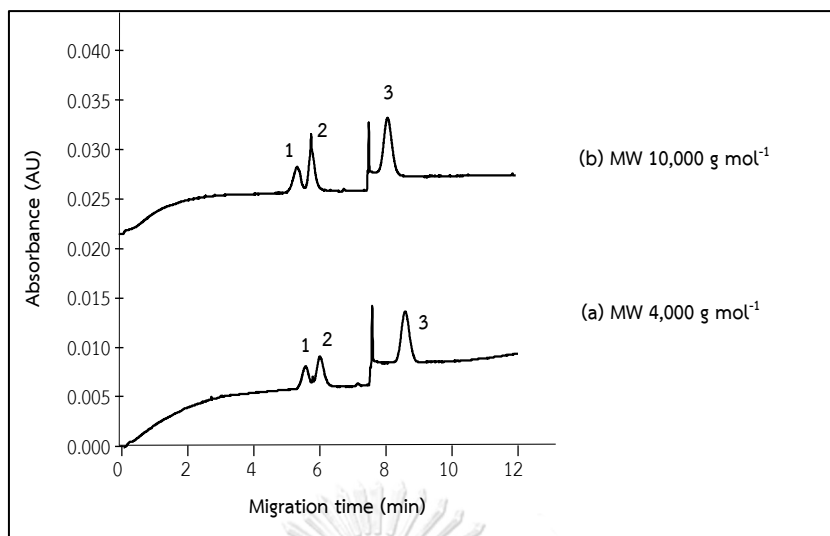
**Figure 4.17** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 100 mg $L^{-1}$ each) obtained from CE separations using buffer containing 200 mM $NaH_2PO_4$, 200 mM butylamine at pH 4.0, separation voltage of -16 kV and 0.5% w/v PEG with different molecular weights of 4,000 and 10,000 g $mol^{-1}$. Other CE conditions are shown in **Figure 4.1**. Peak identification: (1) CS, (2) DS and (3) HA.
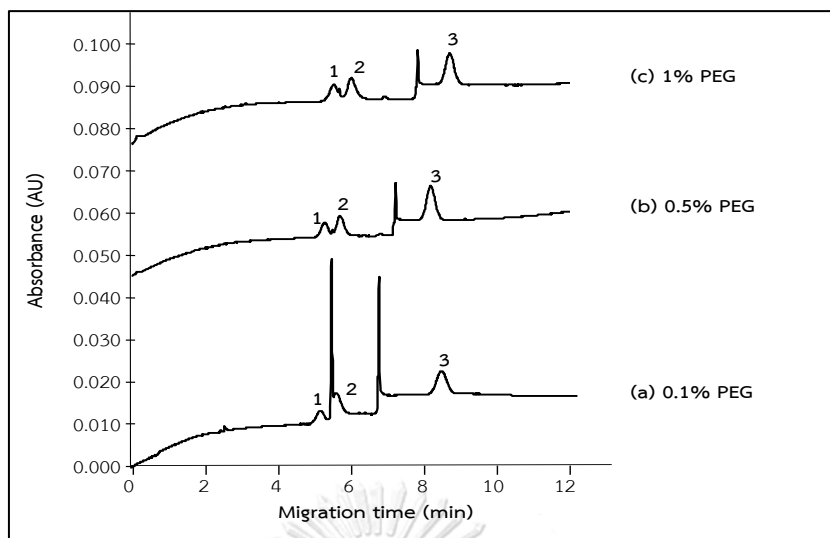
**Figure 4.18** Overlaid electropherograms of the standard mixture (CS, DS and HA, at 100 mg L$^{-1}$ each) obtained from CE separations using PEG (M.W. 10,000 g mol$^{-1}$) with different concentrations of 0.1, 0.5 and 1.0% w/v. Other CE conditions are shown in **Figure 4.1** and **4.17**. Peak identification: (1) CS, (2) DS and (3) HA.

Under the optimal conditions, the standard GAGs and standard mixture of GAGs were analyzed as shown in **Figure 4.19**. From the overlaid electropherograms, the standard of each GAGs showed good separation in standard mixture with short analysis time within 10 min and the resolution between CS and DS was 1.30.
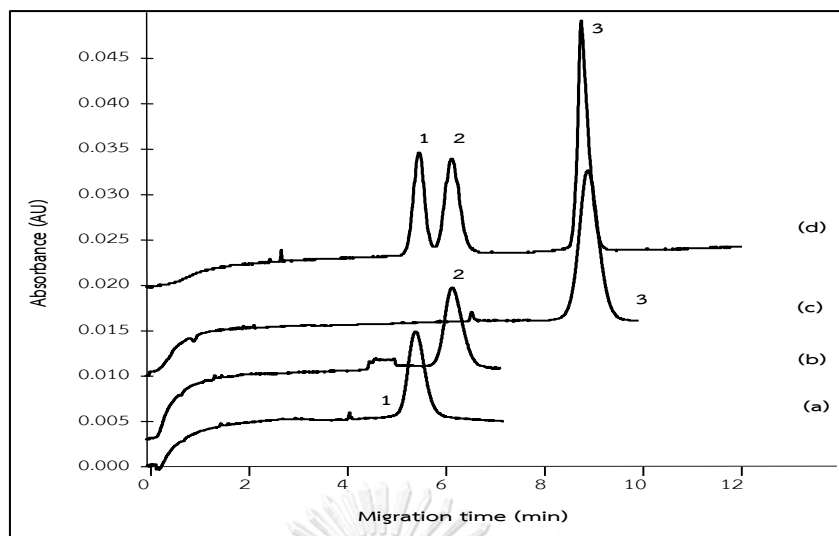
**Figure 4.19** Overlaid electropherograms of (a) CS, (b) DS, (c) HA and (d) standard mixture at a concentration of 333 mg $L^{-1}$ each. CE conditions: uncoated fused silica capillary 50 μm i.d. × 40.2 cm (30 cm to detector), buffer containing 200 mM $NaH_2PO_4$, 200 mM butylamine and 0.5% w/v PEG at pH 4.0, a separation voltage of -16.0 kV, 0.5 psi pressure injection for 60 s, temperature of 25°C and UV detection at 195 nm. Peak identification: (1) CS, (2) DS and (3) HA.

## 4.2 Validation of CE method

Initially, external calibration curves were constructed for determination of GAGs. An example of calibration curve for determination of HA is shown in **Figure 4.20**. The linear relationship between corrected peak area and concentration of HA was good. However, quantitative analysis of HA in real samples was not accurate when using the external calibration curve. For example, the percent recoveries of spiked HA in a cosmetic sample was found in the range of 55-229%. It implied that sample matrix and viscosity of the cosmetic sample affected the peak area of HA. Therefore, the standard addition method was more suitable for quantitation of GAGs in real samples.
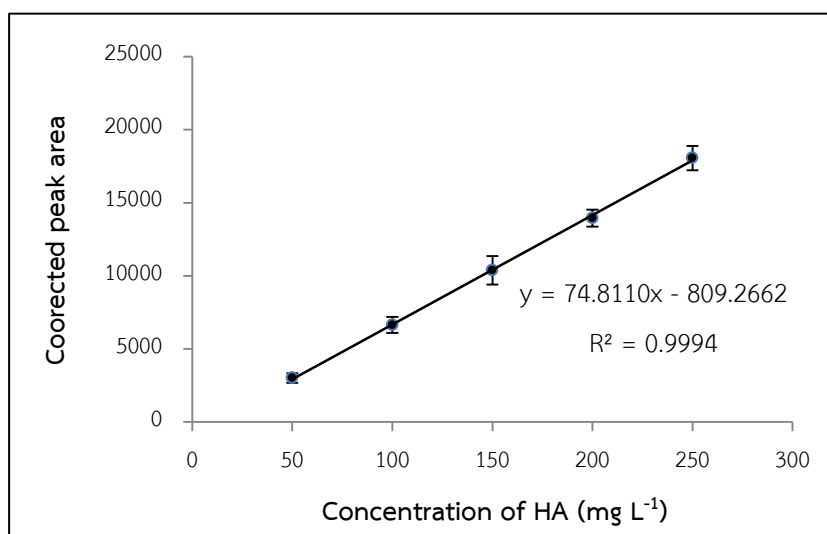
**Figure 4.20** An external calibration plot between corrected peak area and HA
concentration.

### 4.2.1 Standard addition curve

The standard addition method was used to construct calibration curves to
avoid sample matrix for quantitative analysis of the samples. For CS, the standard
addition curve of supplement and PDL samples spiked with chondroitin sulfate were
plotted, as shown in **Figures 4.21** and **4.22**, respectively. For HA, the standard
addition curve of CSF, plasma, HDF and cosmetic samples spiked with HA, as shown
in **Figures 4.23-4.26**, respectively. All of standard addition curves showed good
linearity with high coefficient of determination ($R^2$) more than 0.99.
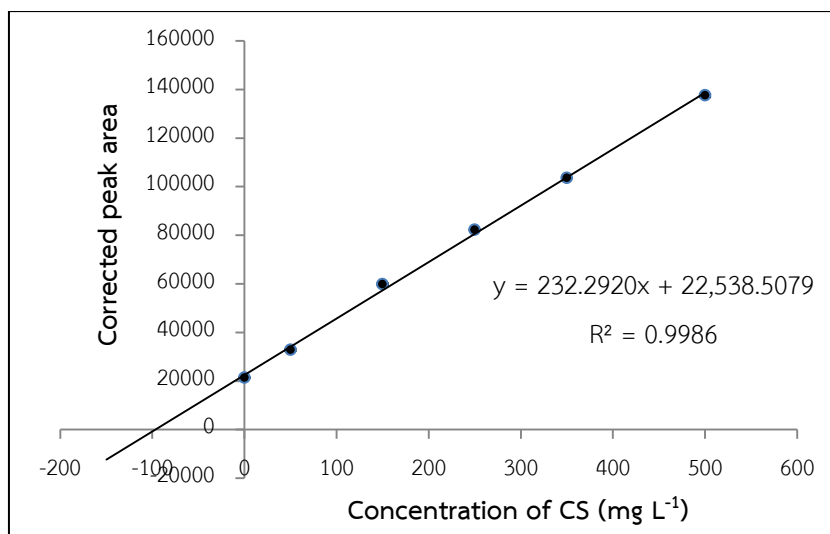
**Figure 4.21** A standard addition plot between corrected peak areas and CS concentration added in a supplement sample.
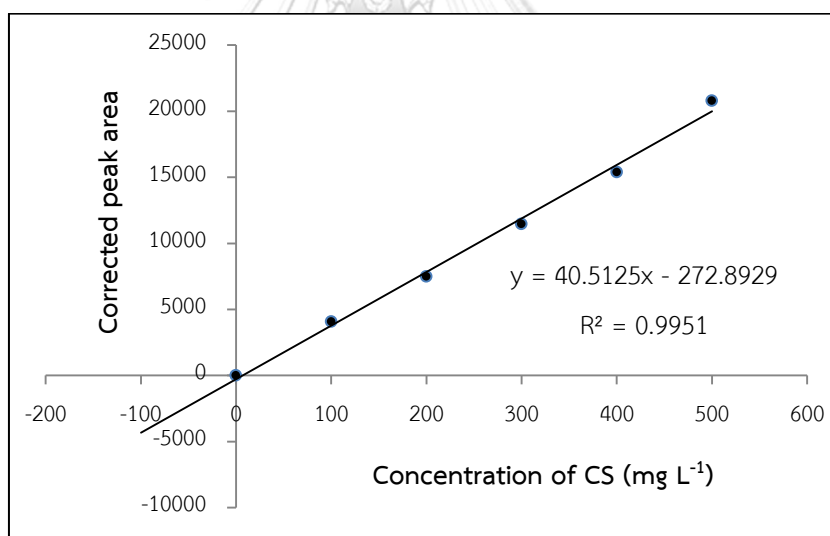


**Figure 4.22** A standard addition plot between corrected peak areas and CS concentration added in a PDL sample.
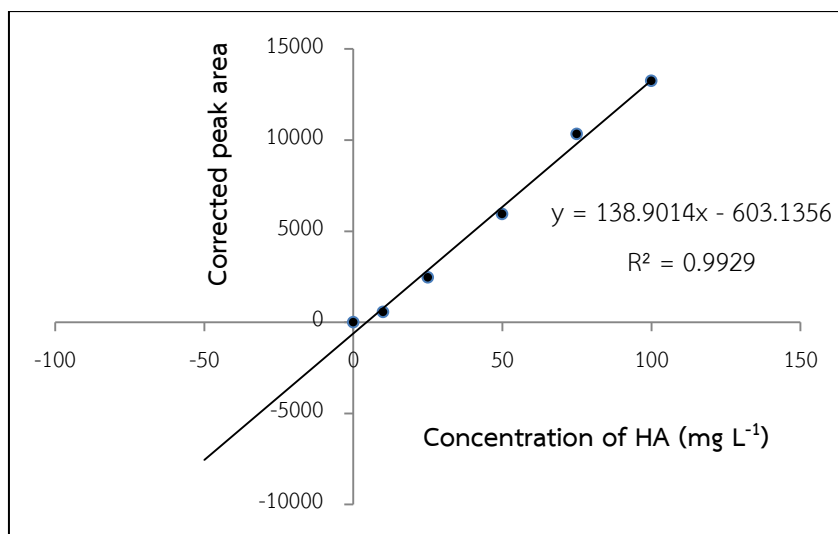
**Figure 4.23** A standard addition plots between corrected peak areas and HA concentration added in a CSF sample.
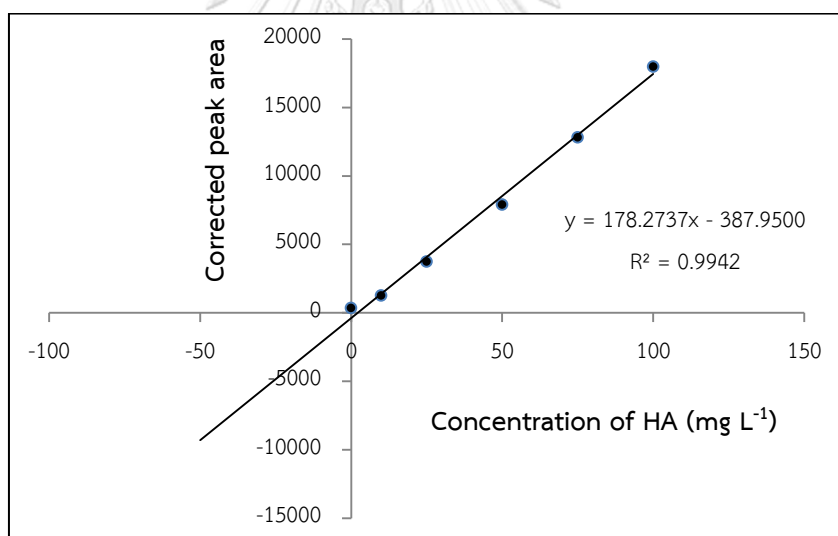


**Figure 4.24** A standard addition plots between corrected peak areas and HA concentration added in a plasma sample.
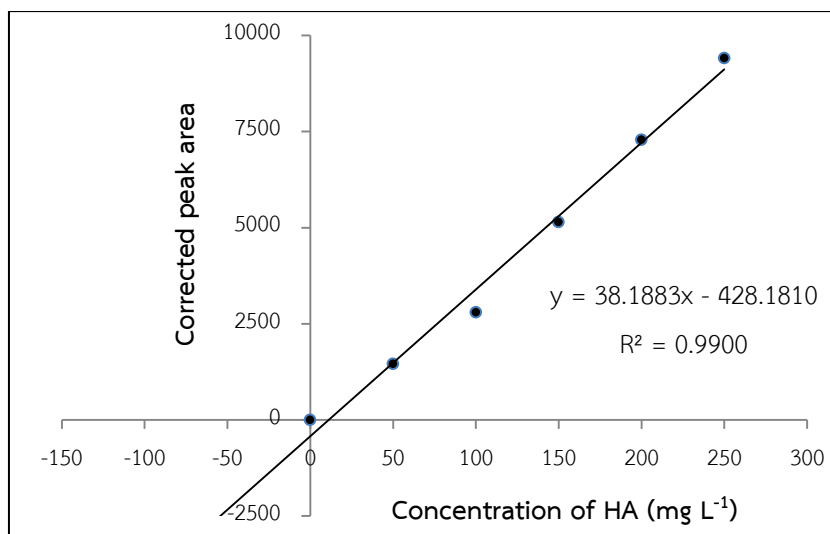
**Figure 4.25** A standard addition plots between corrected peak areas and HA concentration added in an HDF sample.
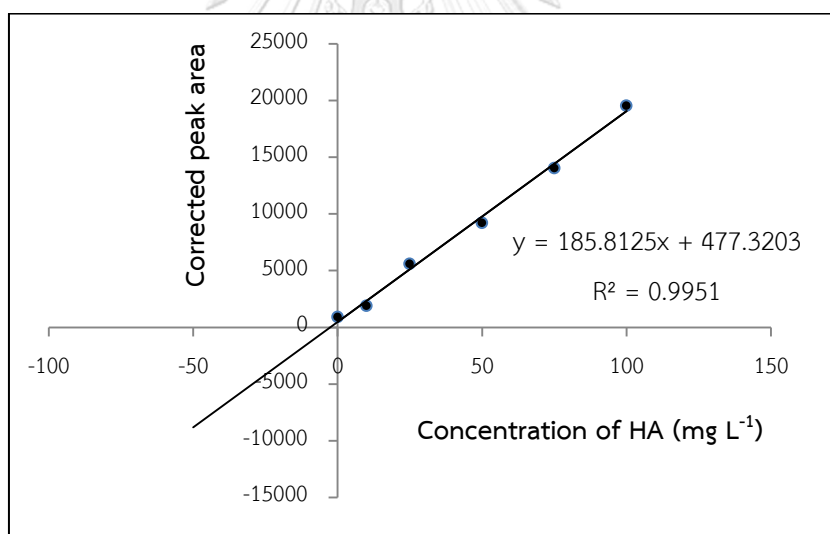


**Figure 4.26** A standard addition plots between corrected peak areas and HA concentration added in a cosmetic sample.

### 4.2.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The sensitivity of this method was investigated and shown in terms of limit of detection (LOD) and limit of quantitation (LOQ), calculated using 3 and 10 times of signal to baseline ratio, respectively. Results in **Table 4.2** showed that LOD and LOQ

of this work were 3.0, 5.0, 1.0 and 10, 15, 3.0 for CS, DS and HA, respectively. A comparison of LOD values of the developed method and those of the previous studies is presented in **Table 4.3**. Results showed that almost all of the LOD values of the developed method are better than those of the previous studies [20, 24, 25, 27, 29, 72-74]. This should be due to the effect of LVSEP applied in this work, which improved the sensitivity of the proposed CE approach. The sensitivity of detection can be further improved by using longer injection times, but the resolution between CS and DS and also the total run time should be considered.

**Table 4.2** LOD and LOQ of the developed CE method for determination of GAGs.

| Analyte | LOD (mg L$^{-1}$) | LOQ (mg L$^{-1}$) |
|---------|-------------------|-------------------|
| CS | 3.0 | 10.0 |
| DS | 5.0 | 15.0 |
| HA | 1.0 | 3.0 |

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**Table 4.3** Comparison of LOD between the developed CE and previous studies.

| Analyte | LOD (mg L$^{-1}$) | References |
|---------|-------------------|------------|
| CS | 3.0 | [72] |
| | 17.0 | [74] |
| | 120.0 | [20] |
| | 150.0 | [29] |
| | 3.0 | this work |
| DS | 3.0 | [72] |
| | 260.0 | [27] |
| | 5.0 | this work |
| HA | 1.0 | [73] |
| | 2.0 | [74] |
| | 5.0 | [24] |
| | 9.0 | [20] |
| | 15.0 | [29] |
| | 19.0 ± 0.4 | [25] |
| | 1.0 | this work |

### 4.2.3 Accuracy

Accuracy of the developed method was studied using percent recovery of standard solutions spiked in *(1) water* and *(2) real samples*.

*(1) Spike standard in water*

Percent recovery of spiked standard solutions in water was calculated from an **Equation 3.1** to evaluate the accuracy of developed method for determination of GAGs. Results showed that percent recoveries of CS, DS and HA standard solutions analyzed at three concentration levels were in the range of 97-109%, 98-102% and 106-109%, respectively, as shown in **Table 4.4**. It implied that the developed method was reliable and accurate for determination of GAGs.

**Table 4.4** The recovery percentages of spiked CS, DS and HA standards in water.

| Analyte | Concentration (mg L$^{-1}$) | | %Recovery |
|---------|--------|--------|-----------|
|         | Spiked | Found  |           |
|         | 100    | 97     | 97        |
| CS      | 250    | 271    | 109       |
|         | 400    | 438    | 109       |
|         | 150    | 153    | 102       |
| DS      | 350    | 357    | 102       |
|         | 450    | 443    | 98        |
|         | 60     | 65     | 109       |
| HA      | 90     | 95     | 106       |
|         | 140    | 153    | 109       |

*(2) Spike standard in real samples*

In addition, the accuracy of developed method is expressed in terms of percent recoveries of standard solutions spiked into real samples. The amounts of analytes originally found in the samples and recoveries of spiked standards are displayed in **Table 4.5**. The recoveries of HA standard spiked in cosmetic samples were found in the range of 92-120%. Moreover, the recovery of CS standard spiked in supplement samples was found in the range of 97-102%. For biological samples, recoveries of HA standard spiked in different samples were found in the range of 81-132%.

**Table 4.5** The amounts of CS and HA found in different biological, cosmetic and supplement samples and recoveries of spiked standards.

| Sample | Analyte | Content (mg L$^{-1}$) | Recovery (%) | | |
|---|---|---|---|---|---|
| | | | Low | Medium | High |
| HaCaT | HA | ND | 72.7 ± 8.1 | 79.8 ± 10.0 | 99.9 ± 5.7 |
| HDF I | HA | 9.2 | 80.3 ± 3.6 | 91.9 ± 21.0 | 97.1 ± 7.3 |
| HDF II | HA | ND | 111.7 ± 1.6 | 103.7 ± 2.5 | 101.8 ± 0.5 |
| PDL I | CS | ND | 104.4 ± 1.2 | 96.9 ± 1.6 | 84.0 ± 0.6 |
| | HA | ND | 116.7 ± 3.9 | 74.5 ± 0.5 | 105.9 ± 4.8 |
| PDL II | CS | ND | 105.0 ± 1.2 | 100.3 ± 1.8 | 106.7 ± 1.5 |
| CSF I | HA | 8.6 | 81.1 ± 8.3 | 86.2 ± 0.9 | 87.3 ± 1.2 |
| CSF II | HA | 5.8 | 111.8 ± 8.8 | 103.9 ± 1.0 | 100.7 ± 1.3 |
| CSF III | HA | ND | 96.4 ± 0.1 | 91.9 ± 0.6 | 103.6 ± 0.2 |
| Plasma I | HA | 4.4 | 103.1 ± 1.9 | 99.1 ± 1.1 | 101.9 ± 0.2 |
| Plasma II | HA | 15.9 | 132.1 ± 0.7 | 105.9 ± 1.0 | 97.6 ± 1.7 |
| Plasma III | HA | 2.2 | 104.1 ± 1.0 | 99.7 ± 3.3 | 101.2 ± 1.7 |
| Plasma IV | HA | 5.3 | 93.8 ± 0.7 | 92.2 ± 0.4 | 97.7 ± 2.6 |
| Plasma V | HA | 4.7 | 104.9 ± 1.3 | 99.7 ± 1.0 | 97.1 ± 0.2 |
| Urine | CS | 3.0 | 101.9 ± 4.0 | 101.4 ± 1.6 | 100.2 ± 4.3 |
| Cosmetic I | HA | 1,067.0 | 98.3 ± 0.4 | 100.5 ± 0.6 | 91.8 ± 1.8 |
| Cosmetic II | HA | 257.0 | 97.5 ± 1.0 | 106.6 ± 0.7 | 103.3 ± 0.5 |
| Cosmetic III | HA | 93.4 | 120.5 ± 2.6 | 116.7 ± 2.7 | 106.7 ± 5.8 |
| Supplement | CS | 97.0 | 102.0 ± 0.8 | 99.9 ± 0.5 | 96.7 ± 0.5 |

* ND: Not detected

### 4.2.4 Precision

The precisions of developed method were studied using percent relative standard deviation (%RSD) of corrected peak areas of GAGs standards spiked in water and real samples.

Intra-day and inter-day precisions of the developed method were investigated from the analysis of GAGs standard solutions and real samples, such as CSF, HaCaT, HDF, PDL and cosmetic samples, spiked with standard solutions. Intra-day precision was observed from analysis of the GAGs within the same day for five replicates (n=5). For inter-day precision, %RSD was investigated from CE analysis of both GAGs standard solutions and each sample spiked with HA standard in three consecutive days and five replicates for each day (n=3×5). Results in **Tables 4.6** and **4.7** revealed high precision for determination of GAGs in the standard solutions and real samples, according to the AOAC setting a guideline for repeatability and reproducibility of 4% and 8%, respectively, at a concentration level of 100 mg $L^{-1}$ [75].

**Table 4.6** Precisions of corrected peak areas of GAGs standard solutions in water.

| Analyte | Concentration (mg $L^{-1}$) | Intra-day precision (n=5), %RSD | Inter-day precision (n=3), %RSD | | | |
|---------|------------------------------|----------------------------------|--------|--------|--------|--------|
| | | | Day 1 | Day 2 | Day 3 | mean |
| CS | 100 | 2.0 | 1.8 | 2.5 | 1.3 | 1.9 |
| | 250 | 0.5 | 1.6 | 5.0 | 1.3 | 2.6 |
| | 400 | 0.8 | 0.8 | 5.4 | 5.7 | 3.9 |
| DS | 150 | 2.2 | 6.4 | 6.7 | 2.7 | 5.2 |
| | 350 | 2.1 | 0.6 | 9.4 | 5.8 | 5.2 |
| | 450 | 1.6 | 1.6 | 8.7 | 4.2 | 4.8 |
| HA | 60 | 1.5 | 2.2 | 3.6 | 3.9 | 3.2 |
| | 90 | 2.4 | 0.9 | 4.0 | 4.9 | 3.3 |
| | 140 | 2.2 | 2.7 | 5.4 | 8.4 | 5.5 |

**Table 4.7** Precisions of corrected peak area of HA spiked in various samples.

| Sample | Concentration of spiked HA (mg L$^{-1}$) | Intra-day precision (n=5), %RSD | Inter-day precision (n=3), %RSD | | | |
|---|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 | mean |
| CSF | 20 | 3.0 | 8.7 | 5.1 | 3.0 | 5.6 |
| | 40 | 3.3 | 1.0 | 2.8 | 3.3 | 2.4 |
| | 60 | 1.4 | 6.6 | 1.4 | 1.4 | 3.1 |
| HaCaT | 60 | 4.1 | 3.2 | 1.2 | 4.1 | 2.8 |
| | 120 | 2.4 | 5.1 | 2.3 | 2.4 | 3.3 |
| | 180 | 3.0 | 0.9 | 4.6 | 3.0 | 2.8 |
| HDF | 60 | 3.4 | 2.9 | 2.0 | 3.4 | 2.8 |
| | 120 | 3.5 | 2.1 | 4.5 | 3.5 | 3.4 |
| | 180 | 1.7 | 5.0 | 3.6 | 1.7 | 3.4 |
| PDL | 20 | 4.8 | 5.1 | 7.3 | 4.8 | 5.7 |
| | 40 | 1.2 | 3.2 | 3.4 | 1.2 | 2.6 |
| | 60 | 7.0 | 9.6 | 5.5 | 7.0 | 7.4 |
| Cosmetic I (serum) | 60 | 0.6 | 1.4 | 0.6 | 1.3 | 1.1 |
| | 120 | 0.9 | 4.2 | 0.9 | 0.5 | 1.8 |
| | 180 | 1.8 | 2.6 | 1.8 | 2.4 | 2.3 |

## 4.3 Application to real samples

The proposed method was applied for determination of GAGs in biological samples and cosmetic products. The reasons for selection of samples were that GAGs are prognostic markers for diseases, such as brain injuries [46, 76] and subarachnoid hemorrhage [2]. Therefore, monitoring the amount of GAGs could help medical diagnostics, treatment and prevention. In addition, since HA is normally included in cosmetic formulations due to its radical scavenging, anti-aging and hydrating properties, determination of HA can be beneficial for controlling product quality in the manufacturing process and can guarantee the consumers high quality

products. The biological samples used in this work as shown in **Table 4.8**. In addition, three cosmetic samples (cosmetics I, II and III) were determined for the amount of HA. The supplement sample was quantified for the amount of CS. The standard addition method was used for determination of GAGs because sample matrix affected the CE analysis of GAGs.

Examples of electropherograms of biological, cosmetic, supplement and cell culture media samples are shown in **Figures 4.27-4.34** and the amounts of each GAGs found in different samples are presented in **Table 4.5**. The amounts of CS determined using the developed CE method were 3.0 and 97.0 mg $L^{-1}$ in urine and supplement samples, respectively. For the supplement sample, the determined amount of CS (97.0 mg $L^{-1}$ corresponding to 582 mg $sachet^{-1}$) was close to the real value (600 mg) labelled on the package with an error percentage of 3.0%. It confirmed that the developed CE method was accurate. The quantitation of HA in various samples can be divided into two groups; first group is biological samples and second group is cosmetic samples. For biological samples, the amounts of HA were found in the range of 2.2-15.9 mg $L^{-1}$ in HDF I, CSF I and II, and plasma I-V samples. For cosmetic samples, the amounts of HA were found in the range of 93-1,067 mg $L^{-1}$. From the results, the amounts of HA found in various samples can be detected. Therefore, the developed method could be used as the tool to follow up the levels of GAGs of patients for treatment in medical applications. In addition, the results guaranteed that the cosmetic products had high amounts of HA. However, CS and HA were not detected in HaCaT, HDF II, PDL, and CSF III samples. Releasing of GAGs from cells into the media could be diluted, which affected the quantitation of GAGs in cell culture media because the amounts of GAGs in cell culture are low. In addition, the developed method was not able to detect the amounts of GAGs at very low concentrations (lower than LODs). Therefore, preconcentration method could be applied to improve the analysis. For the urine sample (**Figure 4.31**), it was found that sample matrices affected the migration time of CS peak (around 5-6 min). Therefore, to improve the performance of the proposed CE method for determination of CS in real samples, an enzymatic digestion of CS to cleave the complex linear polysaccharides into smaller molecules could be another option to improve the

separation efficiency. However, the proposed CE with LVSEP method has been developed to be an easy, fast and reliable approach for determination of GAGs in real samples without sample preparation (except for filtration and dilution).

**Table 4.8** The biological samples used in this work.

| Type of sample | Expected analyte | Prognostic disease |
| --- | --- | --- |
| Cell culture media | | |
| *(i) HaCaT cells* | HA | Skin diseases |
| *(ii) HDF cells* | HA | Skin diseases |
| *(iii) PDL cells* | CS or HA | Periodontal diseases |
| *(iv) Chondrocyte cells* | CS or DS | Osteoarthritis diseases |
| Cerebrospinal fluid | HA | Brain diseases |
| Plasma | HA | Kidney diseases |
| Urine | CS or HA | Kidney and urinary system diseases |
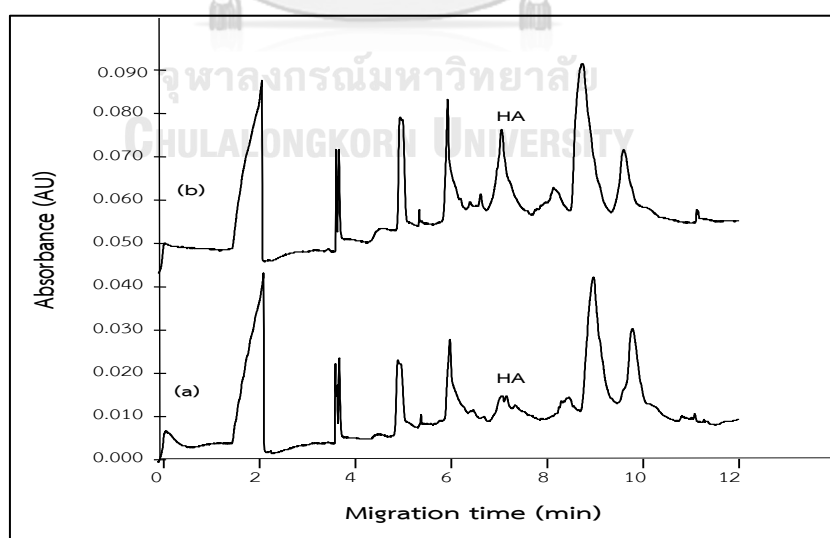


**Figure 4.27** Overlaid electropherograms of HDF I (a) without spiking HA and (b) with spiking 60 mg L$^{-1}$ HA. CE conditions are shown in **Figure 4.19**.
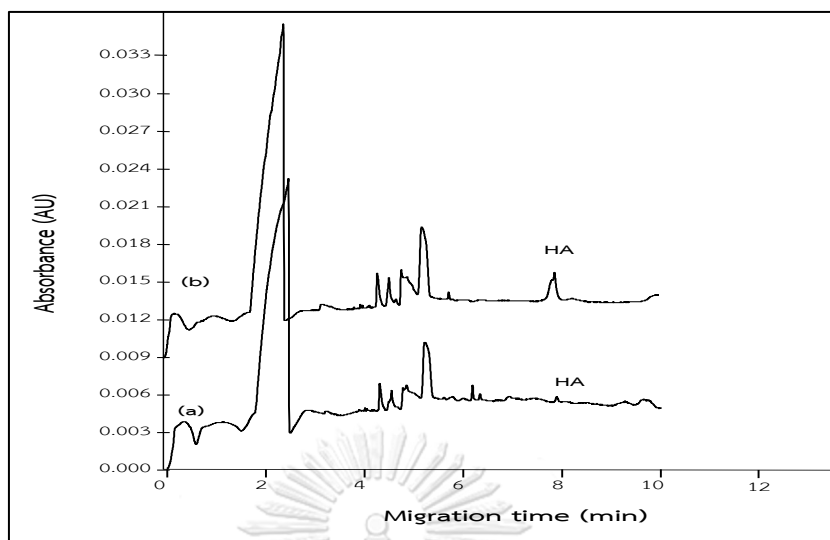
**Figure 4.28** Overlaid electropherograms of CSF I (a) without spiking HA and (b) with spiking 20 mg L$^{-1}$ HA. CE conditions are shown in **Figure 4.19**.
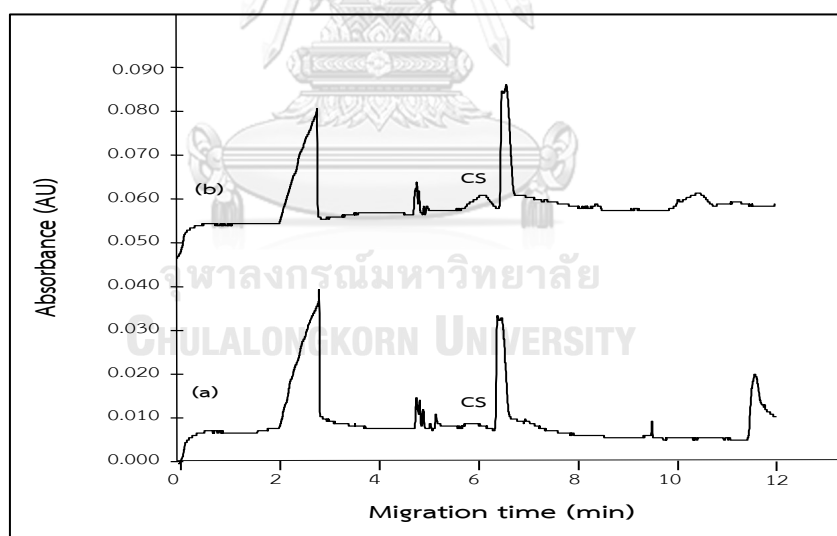


**Figure 4.29** Overlaid electropherograms of PDL (a) without spiking CS and (b) with spiking 130 mg L$^{-1}$ CS. CE conditions are shown in **Figure 4.19**.
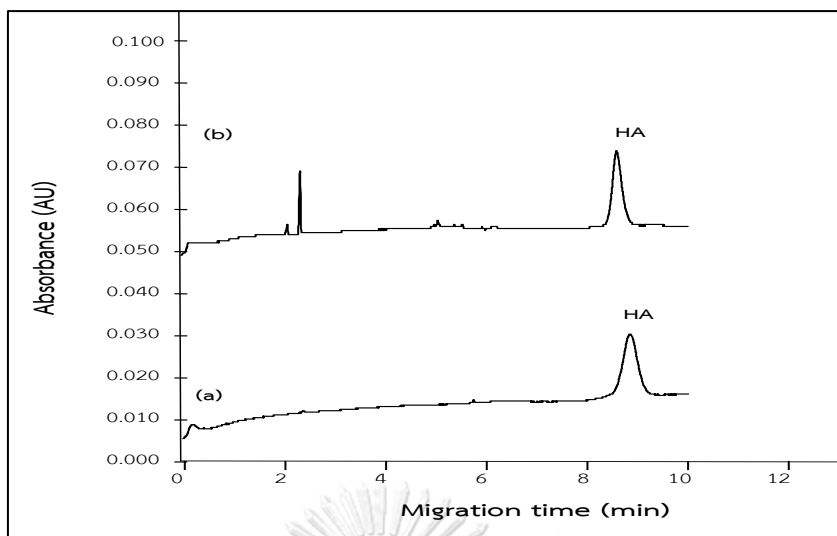
**Figure 4.30** Overlaid electropherograms of cosmetic samples; (a) cosmetic I diluted
100 times and (b) cosmetic II diluted 5 times. The cosmetic samples
were spiked with 100 mg L$^{-1}$ HA. CE conditions are shown in **Figure 4.19**.
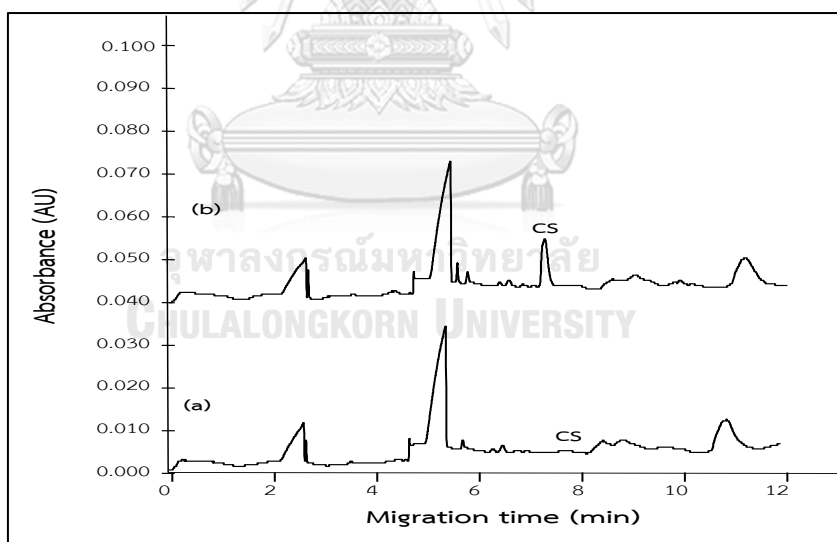


**Figure 4.31** Overlaid electropherograms of urine samples digested by chondroitinase
ABC enzyme (a) without spiking CS and (b) with spiking 300 mg L$^{-1}$ CS.
conditions are shown in **Figure 4.19**.
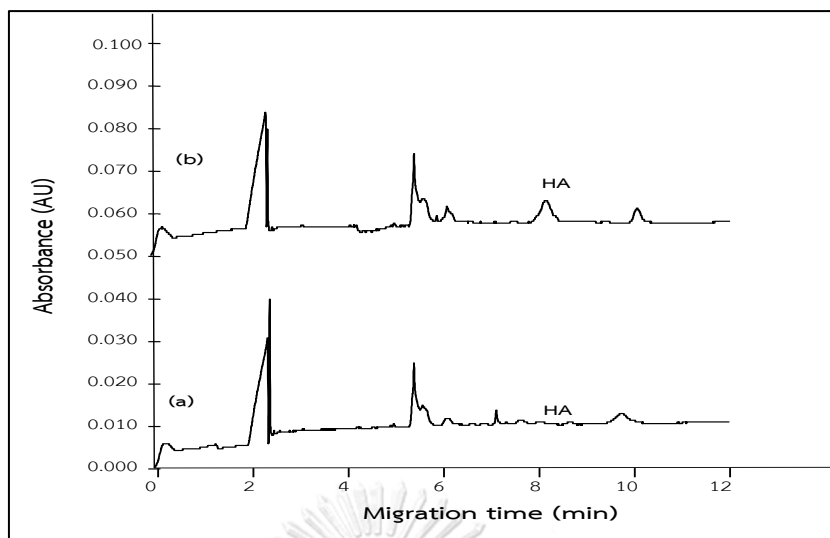
**Figure 4.32** Overlaid electropherograms of plasma I samples (a) without spiking HA and (b) with spiking 25 mg $L^{-1}$ HA. CE conditions are shown in **Figure 4.19**.
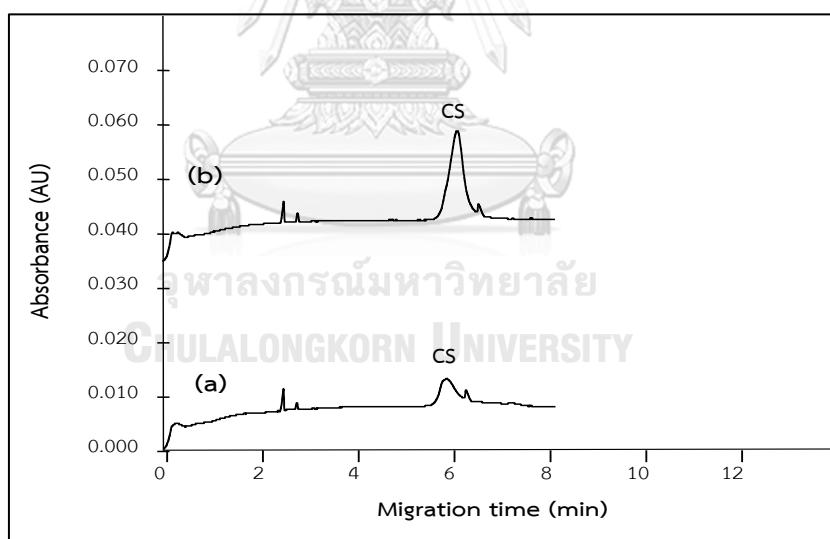


**Figure 4.33** Overlaid electropherograms of supplement samples (a) without spiking CS and (b) with spiking 200 mg $L^{-1}$ CS. CE conditions are shown in **Figure 4.19.**
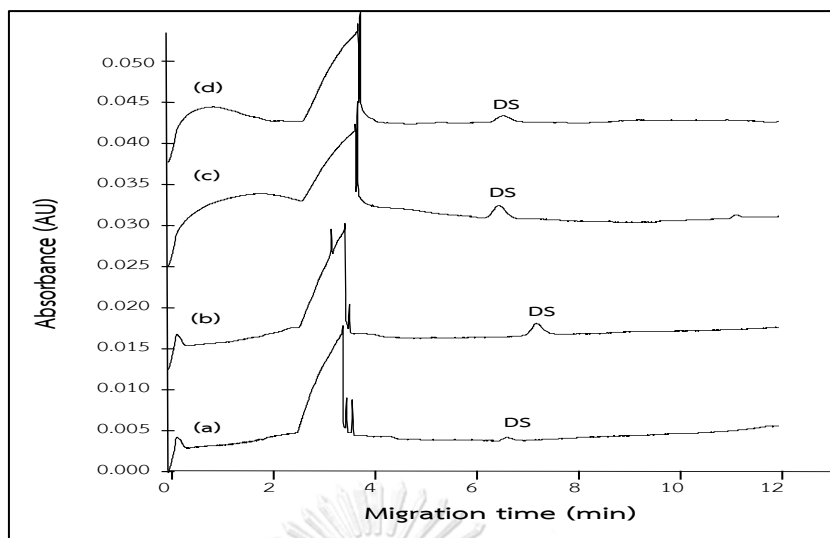
**Figure 4.34** Overlaid electropherograms of chondrocyte cell culture media samples (a) without hydrogen peroxide ($H_2O_2$) and bupivacaine, (b) with 0.4 mM $H_2O_2$, (c) with 0.125% of bupivacaine, and (d) with 0.25% of bupivacaine. CE conditions are shown in **Figure 4.19**.

# CHAPTER V

# CONCLUSIONS AND FUTURE WORK

In this work, a simple capillary electrophoresis (CE) method with large-volume sample stacking using an electroosmotic flow pump (LVSEP) was developed for on-line preconcentration and determination of glycosaminoglycans (GAGs) in biological and cosmetic samples. For on-line preconcentration using LVSEP, the sample injection time of 60 s was employed for sample injection into a capillary column. The peak height was improved around ten times when compared to a normal injection time of 5 s. The optimal values of CE parameters for GAGs separation using CCD approach were 200 mM of sodium dihydrogen phosphate ($NaH_2PO_4$) at pH 4.0 and separation voltage of -16.0 kV. Moreover, 200 mM of butylamine and 0.5% w/v polyethylene glycol (PEG) were also added to the buffer solution to improve the resolution between CS and DS and to reduce the adsorption of GAGs molecules on the capillary wall, respectively. In addition, the higher concentration of buffer solution can cause the Joule heating in the between runs that affecting repeatability and resolution of separations. In order to solve this problem, the fresh buffer must be used by changing the BGE vials prior to next runs.

For method validation, the quantitative analysis of GAGs was performed using the standard addition method to avoid sample matrices in real samples. The standard addition plot was constructed using the corrected peak area of GAGs and GAGs concentrations added in real samples. High linearity of the curves was obtained, with the coefficient of determination ($R^2$) more than 0.99. The LODs and LOQs of the developed method were 3.0, 5.0 and 1.0 and 10.0, 15.0 and 3.0 mg $L^{-1}$ for CS, DS and HA, respectively. Almost all of the values for the recoveries of the known amounts of standard GAGs spiked in water and real samples (biological and cosmetic samples) were found to be close to 100%. However, the percent recoveries of spiked standard in few samples were not satisfied according to AOAC method (85-110% at 100 mg $L^{-1}$). The sample matrices, viscosity of samples, and also the spiked standard into the samples at a low concentration level affected the percent

recoveries in real samples. In addition, the precision of the developed method was investigated using percent relative standard deviation (%RSD) of the corrected peak area. From the analysis of GAGs standard solution and real samples (cerebrospinal fluid, cell culture media and cosmetic) spiked with three concentration levels of standard GAGs, %RSD of corrected peak areas were found to be lower than 7% and 8% for intra- and inter-day precision, respectively.

From the experiments, we would suggest that a sample preparation technique, such as protein precipitation could be employed prior CE analysis to minimize sample matrix effects and to improve the separation performance. In addition, the use of an enzymatic method to cleave the complex linear polysaccharides into smaller molecules could be another option to improve the separation efficiency. However, the proposed CE approach for separation of GAGs in biological samples was easy, rapid and acceptable without sample preparation. Therefore, this developed CE method could be further applied in clinical applications for prognostic diseases, such as joint diseases, bone diseases, kidney diseases, brain diseases and metabolic disorder. Moreover, it could be used for product quality control in cosmetic and pharmaceutical industry involving analysis of GAGs in complex samples in the future.

# REFERENCES

[1]     Xiong, S., Li, A., and Hou, D. Review: Progress in Structural Analysis of Glycosaminoglycans and Their Applications in Biomaterials. <u>African Journal of Biotechnology</u> 11(19) (2012): 4311-4316.

[2]     Heula, A.L., Sajanti, J., and Majamaa, K. Glycosaminoglycans in Subdural Fluid and CSF After Meningeal Injury. <u>Acta Neurochirurgica</u> 157 (2015): 2105-2110.

[3]     Belcher, C., Yaqub, R., Fawthrop, F., Bayliss, M., and Doherty, M. Synovial Fluid Chondroitin and Keratan Sulphate Epitopes, Glycosaminoglycans, and Hyaluronan in Arthritic and Normal Knees. <u>Annals of the Rheumatic Diseases</u> 56 (1997): 299-307.

[4]     Huang, Y., Toyoda, H., Toida, T., and Imanari, T. Determination of Chondroitin Sulphates in Human Whole Blood, Plasma and Blood Cells by High-Performance Liquid Chromatography. <u>Biomedical Chromatography</u> 9(2) (1995): 102-105.

[5]     Plaas, A.H.K., West, L.A., Wong-Palms, S., and Nelson, F.R.T. Glycosaminoglycan Sulfation in Human Osteoarthritis. <u>The Journal of Biological Chemistry</u> 273(20) (1998): 12642-12649.

[6]     Upreti, V.V., Krurana, M., Cox, D.S., and Eddington, N.D. Determination of Endogeneous Glycosaminoglycans Derived Disaccharides in Human Plasma by HPLC: Validation and Application in a Clinical Study. <u>Journal of Chromatography B</u> 831 (2006): 156-162.

[7]     Seno, N., Anno, K., Kondo, K., Nagase, S., and Saito, S. Improved Method for Electrophoretic Separation and Rapid Quantitation of Isomeric Chondroitin Sulfates on Cellulose Acetate Strips. <u>Analytical Biochemistry</u> 37 (1970): 197–202.

[8]     Turnbull, J.E. and Gallagher, J.T. Oligosaccharide Mapping of Heparan Sulphate by Polyacrylamide-Gradient-Gel Electrophoresis and Electrotransfer to Nylon Membrane. <u>Biochemical Journal</u> 251 (1988): 597–608.

[9]     Grossman, P.D. and Colburn, J.C. <u>Capillary Electrophoresis: Theory and Practice</u>. San Diego: Academic Press, 1992.

[10]     Kitagishi, K. and Shintani, H. Review: Analysis of Compounds Containing Carboxyl Groups in Biological Fluids by Capillary Electrophoresis. Journal of Chromatography B 717 (1998): 327-339.

[11]     Deyl, Z., Tagliaro, F., and Miksik, I. Review: Biomedical Applications of Capillary Electrophoresis Journal of Chromatography B 656 (1994): 3-27.

[12]     Geiger, M., Hogerton, A.L., and Bowser, M.T. Review: Capillary Electrophoresis. Analytical Chemistry 84 (2012): 577-596.

[13]     Gllges, M., Kleemiss, M.H., and Schomburg, G. Capillary Zone Electrophoresis Separations of Basic and Acidic Proteins Using Poly (vinyl alcohol) Coatings in Fused Silica Capillaries Analytical Chemistry 66(13) (1994): 2038-2046.

[14]     Harstad, R.K., Johnson, A.C., Weisenberger, M.M., and Bowser, M.T. Review: Capillary Electrophoresis. Analytical Chemistry 88 (2016): 299-319.

[15]     Munoz, J.A., Lopez-Mesas, M., and Valiente, M. Development and Validation of a Simple Determination of Urine Metabolites (Oxalate, Citrate, Uric Acid and Creatinine) by Capillary Zone Electrophoresis. Talanta 81 (2010): 392-397.

[16]     Volpi, N. and Maccari, F. Capillary Electrophoresis of Biomolecules: Methods and Protocols. Humana Press, 2013.

[17]     Himeno, S., Inazuma, N., and Kitano, E. Simultaneous Determination of Phosphonate, Phosphate, and Diphosphate by Capillary Elecrophoresis Using in-Capillary Complexation with Mo(VI). Journal of Separation Science 30 (2007): 1077-1081.

[18]     Cubas, J.J.M., Simoes, R.S., Oliveira-Filho, R.M., Simoes, M.J., Baracat, E.C., and Soares-Jr, J.M. Glycosaminoglycan Distribution in the Rat Uterine Cervix During the Estrous Cycle. Clinics 65(7) (2010): 703-708.

[19]     Kubalczyk, P., Bald, E., Furmaniak, P., and Glowacki, R. Simultaneous Determination of Total Homocysteine and Cysteine in Human Plasma by Capillary Zone Electrophoresis with pH-Mediated Sample Stacking. Analytical Methods 6 (2014): 4138-4143.

[20] Liu, X., Sun, C., Zang, H., Wang, W., Guo, R., and Wang, F. Capillary Electrophoresis for Simultaneous Analysis of Heparin, Chondroitin Sulfate and Hyaluronic Acid and Its Application in Preparations and Synovial Fluid. Journal of Chromatographic Science 50(5) (2012): 373-379.

[21] Ban, E., Yoo, Y.S., and Song, E.J. Analysis and Applications of Nanoparticles in Capillary Electrophoresis. Talanta 141 (2015): 15-20.

[22] Smith, N.W. and Evans, M.B. Capillary Zone Electrophoresis in Pharmaceutical and Biomedical Analysis Journal of Pharmaceutical and Biomedical Analysis 12(5) (1994): 579-611.

[23] Tamizi, E. and Jouyban, A. Development and Validation of a Simple and Rapid Capillary Zone Electrophoresis Method for Quantification of Heparin in a Pharmaceutical Product and Stability Studies Pharmaceutica Analytica Acta 3(8) (2012): 1-6.

[24] Alkrad, J.A., Merstani, Y., and Neubert, R.H.H. New Approaches for Quantifying Hyaluronic Acid in Pharmaceutical Semisolid Formulations Using HPLC and CZE. Journal of Pharmaceutical and Biomedical Analysis 30 (2002): 913-919.

[25] Teodor, E.D., Truica, G., and Radu, G.L. Hyaluronic Acid Detection from Natural Extract by Diode Array-Capillary Electrophoresis Methods. Revue Roumaine de Chimie 57(3) (2012): 223-227.

[26] Leogel, T.N., Trombley, J.D., Taylor, R.T., and Danielson, N.D. Capillary Electrophoresis of Heparin and Other Glycosaminoglycans Using a Polyamine Running Electrolyte. Analytica Chimica Acta 753 (2012): 90-96.

[27] Somsen, G.W., Tak, Y.H., Torano, J.S., Jongen, P.M.J.M., and de Jong, G.J. Determination of Oversulfated Chondroitin Sulfate and Dermatan Sulfate Impurities in Heparin by Capillary Electrophoresis. Journal of Chromatography A 1216 (2009): 4107-4112.

[28] Volpi, N., Maccari, F., and Linhardt, R.J. Quantitative Capillary Electrophoresis Determination of Oversulfated Chondroitin Sulfate as a Contaminant in Heparin Preparations. Analytical Biochemistry 388 (2009): 140-145.

[29]    Zhao, T., et al. Development of a Rapid Method for Simultaneous Separation of Hyaluronic Acid, Chondroitin Sulfate, Dermatan Sulfate and Heparin by Capillary Electrophoresis. Carbohydrate Polymers 141 (2016): 197-203.

[30]    Kitagawa, F., Kawai, T., and Otsuka, K. On-line Sample Preconcentration by Large-volume Sample Stacking with an Electroosmotic Flow Pump (LVSEP) in Microscale Electrophoresis. Analytical Sciences 29 (2013): 1129-1139.

[31]    James, N. Capillary electrophoresis [Online]. 2017. Available from: https://www.whitman.edu/chemistry/edusolns_software/GC_LC_CE_MS_2017/CH%204%202017.pdf [September 10, 2018]

[32]    Beckman Coulter. Introduction to Capillary Electrophoresis [Online]. 2018. Available from: https://sciex.com/Documents/manuals/IntroductiontoCapillaryElectrophoresisVol-I.pdf [September 10, 2018]

[33]    Precissi, J. Capillary Electrophoresis [Online]. 2015. Available from: https://chem.libretexts.org/Core/Analytical_Chemistry/Instrumental_Analysis/Capillary_Electrophoresis [July 10, 2016]

[34]    Capillary electrophoresis [Online]. 2018. Available from: https://en.wikipedia.org/wiki/Capillary_electrophoresis [October 10, 2018]

[35]    Fairhurst, D. An Overview of the Zeta Potential - Part 1: The Concept [Online]. 2013. Available from: https://www.americanpharmaceuticalreview.com/Featured-Articles/133232-An-Overview-of-the-Zeta-Potential-Part-1-The-Concept/ [October 5, 2018]

[36]    Whatley, H. Chapter 2: Basic Principles and Modes of Capillary Electrophoresis in Petersen, J.R. and Mohammad, A.A. (eds.), Clinical and Forensic Applications of Capillary Electrophoresis, pp. 21-58. New York city, United state: Humana Press, Totowa, NJ, 2001.

[37]    Weinberger, R. Practical Capillary Electrophoresis. San Diego: Academic Press, 1993.

[38]    Foret, F., Krivankova, L., and Bocek, P. Capillary Zone Electrophoresis. Weintheim: Wiley-VCH, 1993.

[39]    Barbooti, M.M. Environmental Applications of Instrumental Chemical Analysis. Canada: Apple Acedamic Press, Inc., 2015.

[40]   Poole, C.F. <u>Capillary Electromigration Separation Methods</u>, ed. Poole, C.F. Netherlands: Elsevier, 2018.

[41]   Ronald, E. <u>Basics of Capillary Electrophoresis</u> [Online]. 2018. Available from: http://matematicas.udea.edu.co/~carlopez/cnq533/CE_Basics.pdf [November 4, 2018]

[42]   Burgi, D.S. and Chien, R.L. Optimization in Sample Stacking for High-Performance Capillary Electrophoresis. <u>Analytical Chemistry</u> 63(18) (1991): 2042-2047.

[43]   Osbourn, D.M., Weiss, D.J., and Lunte, C.E. On-line Preconcentration Methods for Capillary Electrophoresis. <u>Electrophoresis</u> 21(14) (2000): 2768–2779.

[44]   <u>Glycosaminoglycans and Proteoglycans</u> [Online]. Available from: http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/glycosaminoglycans-and-proteoglycans.html [July 15, 2016]

[45]   Kogan, G., Soltes, L., Stern, R., and Mendichi, R. Chapter 31: Hyaluronic Acid: A Biopolymer with Versatile Physico-Chemical and Biological Properties. in Pethrick, R.A.e.a. (ed.) <u>In: Handbook of Polymer Research: Monomers, Oligomers</u>, pp. 393-439: Nova Science Publishers, Inc., 2007.

[46]   Nagga, K., Hansson, O., Westen, D.V., Minthon, L., and Wennstrom, M. Increased Levels of Hyaluronic Acid in Cerebrospinal Fluid in Patients with Vascular Dementia. <u>Journal of Alzheimer's Disease</u> 42 (2014): 1435-1441.

[47]   Qteishat, A.A., et al. Changes in hyaluronan production and metabolism following ischaemic stroke in man. <u>Brain</u> 129 (2006): 2158–2176.

[48]   Vonk, R.J. <u>Determination of Glycosaminoglycans in Human Serum: Development of a Method for the Size-Characterization of Glycosaminoglycans</u>. Master degree, Analytical Chemistry Universiteit van Amsterdam, 2011.

[49]   Anower-E-Khuda, F., et al. Glycosaminoglycans in the Blood of Hereditary Multiple Exostoses Patients: Half Reduction of Heparan Sulfate to Chondroitin Sulfate Ratio and the Possible Diagnostic Application. <u>Glycobiology</u> 23(7) (2013): 865-876.

[50]   Mao, W., Thanawiroon, C., and Linhardt, R.J. Review: Capillary Electrophoresis for the Analysis of Glycosaminoglycans and Glycosaminoglycan-Derived Oligosaccharides. <u>Biomedical Chromatography</u> 16 (2002): 77-94.

[51] Akesolo, U., Magurequi, M.I., Gonzalez, L., Jimenez, R.M., and Alonso, R.M. Experimental Design Optimization of a Capillary Zone Electrophoresis Method for the Screening of Several Diuretics and ACE Inhibitors. <u>Journal of Chromatographic Science</u> 42 (2004): 74-79.

[52] Cavazzuti, M. <u>Optimization Methods: From Theory to Design Scientific and Technological Aspects in Mechanics</u>. Springer-Verlag Berlin Heidelberg, 2013.

[53] Design Institute for Six Sigma. <u>Concepts of Experimental Design</u> [Online]. Available from: http://support.sas.com/resources/papers/sixsigma1.pdf [October 5, 2018]

[54] Proust, M. <u>Design of Experiments Guide</u>. SAS Institute Inc., Cary, NC, USA, 2012.

[55] <u>Chemometrics Unit 4: Response Surface Methodology</u> [Online]. 2018. Available from: http://mams.rmit.edu.au/ve1pbehqj7e5.pdf [October 1, 2018]

[56] Brereton, R.G. <u>Applied Chemometrics for Scientists</u>. England: John Wiley & Sons, Ltd, 2007.

[57] Chigbu, P.E. and Ukaegbu, E.C. Recent Developments on Partial Replications of Response Surface Central Composite Designs: A Review. <u>Journal of Statistics Applications & Probability</u> 6(1) (2017): 91-104.

[58] Franks, F. <u>Water Science Reviews 4: Hydration Phenomena in Colloidal Systems</u>. Vol. 4. Cambridge: Cambridge University Press, 1989.

[59] Liao, L.H., Jones, S.A., Forbes, B., Martin, G.P., and Brown, M.B. Hyaluronan: Pharmaceutical Characterization and Drug Delivery. <u>Drug Delivery</u> 12(6) (2005): 327-342.

[60] Tømmeraas, K. and Wahlund, P.O. Poly-Acid Properties of Biosynthetic Hyaluronan Studied by Titration. <u>Carbohydrate Polymers</u> 77 (2009): 194-200.

[61] Wang, H.M., Loganathan, D., and Linhardt, R.J. Determination of the $pK_a$ of Glucuronic acid and the Carboxy Groups of Heparin by $^{13}$C-Nuclear-Magnetic-Resonance Spectroscopy. <u>Biochemical Journal</u> 278 (1991): 689-695.

[62] Kalpongnukul, N. <u>Urinary Glycosaminoglycan Levels and Supersaturation in Kidney Stone Familial Members</u>. Master degree, Biochemistry Chulalongkorn University, 2013.

[63]    Czitrom, V. Teacher's Corner: One-Factor-at-a-Time Versus Designed Experiments. The American Statistician 53(2) (1999): 126-131.

[64]    Wielgos, T., Havel, K., Ivanova, N., and Weinberger, R. Determination of Impurities in Heparin by Capillary Electrophoresis Using High Molarity Phosphate Buffers. Journal of Pharmaceutical and Biomedical Analysis 49 (2009): 319-326.

[65]    Sheehan, J.K., Arundel, C., and Phelps, C.F. Effect of the Cations Sodium, Potassium and Calcium on the Interaction of Hyaluronate Chains: a Light Scattering and Viscometric Study. International Journal of Biological Macromolecules 5 (1983): 222-228.

[66]    Turner, R.E., Lin, P., and Cowman, M.K. Self-Association of Hyaluronate Segments in Aqueous NaCl Solution. Archives of Biochemistry and Biophysics 265(2) (1988): 484-495.

[67]    Wittayarak, I., Imyim, A., and Wongravee, K. Simultaneous Removal of As(III) and As(V) from Wastewater by Co-precipitation Using an Experimental Design Approach. Desalination and Water Treatment (2015): 1-12.

[68]    Capella-Peiró, M.E., Bose, D., Rubert, M.F., and Esteve-Romero, J. Optimization of a Capillary Zone Electrophoresis Method by Using a Central Composite Factorial Design for the Determination of Codeine and Paracetamol in Pharmaceuticals. Journal of Chromatography B 839(1-2) (2006): 95-101.

[69]    Chen, Y.L., Jliang, S.J., Feng, C.H., Wang, S., W.,, Lin, Y.H., and Liu, P.Y. Application of Central Composite Design for the Determination of Exfoliating Agents in Cosmetics by Capillary Electrophoresis with Electroosmotic Flow Modulation. Electrophoresis 34(11) (2014): 1670-1682.

[70]    Fermas, S., et al. Sulfated Oligosaccharides (Heparin and Fucoidan) Binding and Dimerization of Stromal Cell-Derived Factor-1 (SDF-1/CXCL 12) are Coupled as Evidenced by Affinity CE-MS Analysis. Glycobiology 18(12) (2008): 1054-1064.

[71]    Gilges, M., Kleemiss, M.H., and Schomburg, G. Capillary Zone Electrophoresis Separations of Basic and Acidic Proteins Using Poly (vinyl alcohol) Coatings in Fused Silica Capillaries Analytical Chemistry 66(13) (1994): 2038-2046.

[72]    Bendazzoli, C., Liverani, L., Spelta, F., Prandi, M., Fiori, J., and Gotti, R. Determination of Dermatan Sulfate and Chondroitin Sulfate as Related Substances in Heparin by Capillary Elecrophoresis. Journal of Pharmaceutical and Biomedical Analysis 53 (2010): 1193-1200.

[73]    Hayase, S., Oda, Y., Honda, S., and Kakehi, K. High-performance Capillary Electrophoresis of Hyaluronic Acid: Determination of its Amount and Molecular Mass. Journal of Chromatography A 768 (1997): 295-305.

[74]    Malavaki, C.J., Asimakopoulou, A.P., Lamari, F.N., Theocharis, A.D., Tzanakakis, G.N., and Karamanos, N.K. Capillary Electrophoresis for the Quality Control of Chondroitin Sulfates in Raw Materials and Formulations. Analytical Biochemistry 374 (2008): 213-220.

[75]    AOAC. AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [Online]. 2002. Available from: https://www.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/SLV_ Guidelines_Dietary_Supplements.pdf [May 15, 2018]

[76]    Rauch, U. Review: Extracellular Matrix Components Associated with Remodeling Processes in Brain. Cellular and Molecular Life Sciences 61 (2004): 2031-2045.

# VITA

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|---|---|
| **NAME** | Miss Kanokporn Chindaphan |
| **DATE OF BIRTH** | 29 August 1988 |
| **PLACE OF BIRTH** | Chanthaburi |
| **INSTITUTIONS ATTENDED** | Bachelor's degree of Science in Chemistry from the Faculty of Science, Burapha University in 2010. Master's degree in Analytical Chemistry from the Faculty of Science, Burapha University in 2013. |
| **HOME ADDRESS** | 6/1 Moo.1 Nayai-arm Chanthaburi 22160 |
| **PUBLICATION** | Poster presentation |

"Capillary Electrophoresis for Determination of Hyaluronic Acid in Cosmetic Samples" Kanokporn Chindaphan, Thumnoon Nhujak, Thasinas Dissayabutra, Kanet Wongravee, Monpichar Srisa-Art. Annual Conference on Engineering and Applied Science (ACEAT), on 27-29 November 2018, Osaka International Convention Center, Osaka, Japan

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