ENANTIOMERIC SEPARATION, MICROORGANISM STUDIES AND DETERMINATION OF BINDING CONSTANTS USING CAPILLARY ELECTROPHORESIS

by

CHUNXIA JIANG

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ABSTRACT

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Chunxia Jiang, PhD

The University of Texas at Arlington, 2009

Supervising Professor: Daniel W. Armstrong

Enantiomeric separation of three series of compounds, including β-lactams, synthetic amino acids and ruthenium(II) polypyridyl complexes using different modes of capillary electrophoresis are discussed in this dissertation. Enantiomeric recognition mechanisms are explored by studying the effect of the structures of chiral selectors and analytes. Results indicated that the size correlation between the analyte and the cyclodextrin cavity size play an important role in enantioseparation. Effects of experimental parameters including chiral selector concentration, buffer additive type and concentration, run buffer pH and applied voltage are studied for optimization of the enantioseparations. A new class of chiral selectors, cyclofructans were developed and examined via capillary zone electrophoresis in both normal and reverse polarity mode. Superior enantioselectivity was observed for cationic compounds, especially primary amines and primary amino acids.

Capillary electrophoresis also was studied as a technique for the fast analysis of microorganisms. Online preconcentration approaches in capillaries with greater internal diameters were investigated to improve the sensitivity and reliability for diluted microbial

samples. Rapid detection of *Candida albicans* and/or bacteria in blood plasma by “sample-self focusing” using capillary elelctrophoresis-laser induced fluorescence within 10 mins was successfully achieved.

Finally, a review was prepared on the estimation of apparent binding constants using capillary electrophoresis. It provides information on the fundamentals of binding and a summary of recent applications and advances of CE-based methods.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Capillary electrophoresis is a separation technique based on the analytes' charge and frictional forces in the interior of a small capillary. Typically the capillary is filled with an electrolyte and analytes migrate under the influence of electric field. In the past few decades, capillary electrophoresis has attracted much attention as a separation technique owing to its known advantages including fast analysis, high efficiency and flexibility, low sample requirements, and a generally low cost [1].

1.1 Enantiomeric separation by capillary electrophoresis

An object is chiral if it is not superimposable with its mirror image by translation and rotation. Enantiomers are non-superimposable mirror image steroisomers, which have identical chemical and physical properties in an isotropic environment except for the rotation of plane polarized light. In a chiral environment, such as a living organism that is composed of chiral biomolecules, enantiomers can behave very differently. As a result, enantiomers of drugs can have very distinct biological responses that many translate into marked clinical differences.

The enantioseparation of chiral compounds is of crucial importance to food, medical, pharmaceutical and other industries due to the differences in their biological, pharmacological and toxicological properties [2]. Strict guidelines for chiral drug development were issued by the United States Food and Drug Administration (FDA) in 1992 [3]: "When the drug product is a racemate, and the pharmacokinetic profiles of the isomers are different, manufactures should monitor the effects of the altered metabolic or excretory function and drug-drug interactions." Modern separation techniques, including GC, HPLC, SFC, CE and thin layer chromatography (TLC) are frequently used for enantiomeric separations today.

Ever since the first report of enantiomeric separation of dansyl amino acids using CE by Gassmann *et al.* in 1985 [4], capillary electrophoresis has been used for enantiomeric separations extensively in past two decades [5-9]. The advantages of CE, compared to LC, GC, SFC and TLC, are its simplicity and applicability for the separation of a wide range of compounds using the same instrument and even the same capillary while changing the composition of running buffer [9].

The most simple and frequently used mode is capillary zone electrophoresis (CZE), where the chiral selectors are simply added to the running buffer and chiral recoginition are achieved based on the mobility differences of enantiomers resulting from different association of binding between the analyte and the chiral selector. In this case, the analyte and the chiral selector have different charges to provide a separation window [10-12]. When there is very little mobility difference between the analyte and the chiral selector, micelles can be added to the run buffer to provide a pseudo-stationary phase [13, 14]. The addition of micelles also can improve the solubility of the analyte and the chiral selector.

Chapters 2 and 3 describe the enantiomeric separation of twelve racemic substituted β-lactam compounds and twenty synthetic amino acids via capillary zone electrophoresis, respectively. Three chiral selectors including sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC) and carboxymethyl β-cyclodextrin (CMBC) were used. The effect of experimental parameters, including chiral selector concentrations, buffer pH, applied voltage and addition of organic modifier also were studied for the optimization of separation conditions. The enantiomeric separation of chiral ruthenium(II) polypyridal complexes using capillary zone electrophoresis and micellar capillary electrophoresis are discussed in Chapter 4. Nine cyclodextrin based chiral selectors were examined as run buffer additives. Experimental parameters also were studied in terms of optimization and enantiomeric recognition mechanisms. Sulfated cyclodextrins were always the best chiral selectors for these three series of chiral compounds. It was interesting to note that, the optimal cyclodextrin size increased as

did the analyte size, that is, the best chiral selector for β-lactam was sulfated α-cyclodextrin, sulfated β-cyclodextrin for synthetic amino acid and sulfated γ-cyclodextrin for chiral ruthenium(II) complexes.

Despite the large number of chiral selectors used in other separation techniques such as LC, relatively few classes of selectors have been successfully applied to CE. That is due to the intrinsic requirements of chiral selectors for CE: low UV absorption, high solubility in water, minimum interaction with the fused silica wall, etc. Chiral agents for CE include chiral ligand-exchange materials, cyclodextrins and their derivatives, chiral natural and synthetic surface-active compounds (micelles), macrocyclic antibiotics, peptides, chiral crown ethers, polysaccharides, etc [5]. So far, sulfated cyclodextrins have dominated chiral CE separations [5], while the applications of other chiral selectors are very limited. In Chapter 5, cyclofructans were derivatized and examined as a new class of chiral selectors for CE. Over 200 racemic compounds were examined in both normal and reverse polarities. Sulfated cyclofructans showed enantioselectivity to cationic compounds including primary, secondary, tertiary and quaternary amines and amino acids. The enantioselectivity toward most primary amine compounds are superior to any other existing CE chiral selectors.

1.2 Microbial study by capillary electrophoresis

Microbes, including bacteria and fungi, are frequently the cause of infection and illness in human beings and other organisms. Fast detection and identification of pathogenic microorganisms are important and necessary for safety and quality control in food/beverage [15, 16], pharmaceutical [17], and medical industries [18, 19]. Doctors rely on the information involving identification and quantification of the pathogen to prescribe the proper medicine and devise proper treatments for patients. The traditional standard method is direct inoculation. An aliquot of homogenized sample is placed in a sterile growth media capable of sustaining the microbial growth. After a few days the sample is checked for turbidity or examined under microscopy for the presence of microbes [20]. It usually takes a few days to a few weeks. Great

care needs to be taken to avoid contamination during analysis and only certain microbes capable of growing in the selected media can be detected. To overcome these shortcomings, alternative approaches including hybridization [21], amplification [22], and immunoassay [23] can be used. However, these methods are selective for specific microorganisms, are complex, and require expensive reagents.

Recently, capillary electrophoresis has been explored as an alternative for microbial analysis [24-33]. An effective three injection method was developed by our group for the determination of presence/complete absence of microbes [26, 27, 32]. Coupled with laser-induced fluorescence detections, samples containing as little as a single cell were successfully detected [26].

Although small sample volume injection is an advantage for CE analysis of small molecules, it can be problematic when examining larger microbial entities. On a per-particle basis, microbial solutions generally are a lot more dilute than the solutions of molecules. Chapter 6 discusses the feasibility of online preconcentration approaches using capillaries with larger internal diameters.

In a further CE study, we adapted the current CE method for the microbial detection applications involving real biological samples. In Chapter 7, the fast detection of *Candica albicans* and/or bacteria in blood plasma within 10 mins by “sample-self focusing” using capillary electrophoresis-laser induced fluorescence is presented. Good limits of detection were achieved on samples containing as few as 5 microbial cells.

1.3 Determination of binding constants by capillary electrophoresis

Non-covalent molecular interactions are prevalent in chemical and biological systems. The characterization of intermolecular interactions (such as drug-protein/DNA, antibody-antigen, and peptide-antibiotics interactions) including estimation of binding constants are important in understanding basic biological systems and for the development of new drugs and effective treatments for diseases. Many modern techniques have been used for determination of binding

constants [34], including spectroscopic methods such as NMR, UV-vis and IR, chromatographic techniques such as GC, HPLC and CE as well as other techniques such as potentiometry and calorimetry. These techniques are generally based on monitoring the changes of specific physiochemical properties of the substrate with varying amount of the ligand.

Capillary electrophoresis based techniques, including affinity capillary electrophoresis (ACE), Hummel-Dreyer method (HMD), vancancy peak method, vacancy affinity capillary electrophoresis and frontal analysis as well as direct separation methods and kinetic capillary electrophoresis approaches have been reported for the estimation of the apparent binding constants. Chapter 8 includes a recently prepared review on the use of CE for the determination of binding constants. It provides a fundamental introduction to binding theory followed by a summary of recent applications and advances in the field of CE-based methods for the evaluation of molecular association since 2002 (when our previous review was published).

CHAPTER 2

ENANTIOMERIC SEPARATION OF B-LACTAMS USING CAPILLARY ZONE ELECTROPHORESIS

Twelve racemic substituted β-lactam compounds were examined via capillary zone electrophoresis using three chiral selectors: sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC) and carboxymethyl β-cyclodextrin (CMBC). Ten of the twelve β- lactams are separated and each of the ten compounds is baseline separated by at least one of the chiral run buffer additives under optimized conditions. SAC was found to be the most effective chiral selector, baseline separating seven of the analytes and partially separating another two. The concentration of the chiral selector had a prominent effect on the resolution, generally higher concentrations gave longer migration times and better resolutions. Addition of organic modifier also increased analyses time but gave lower resolution. Decreasing the pH of the run buffer generally decreased analysis times as well as resolution. Decreasing the applied voltage generally improved resolution.

2.1 Introduction

β-Lactams have been a topic of interest in recent years because of their wide application in both pharmaceutical science and synthetic organic chemistry. They are widely used as antibacterial agents and many studies have been published on their antibacterial activity, action mechanism and clinical applications [35-41]. β-lactams and their derivatives also are widely used as intermediates in the organic synthesis of heterocyclic compounds of medical and chemical interest [42], amino acids [43] , short peptide segments [44], alkaloids [45], etc. The synthesis of various β-lactam compounds both chemically [46, 47] and through biosynthetic processes [48] has been reported.

Since the two enantiomers of a compound can have very different behaviors in a chiral environment, as in a physiological matrix, one enantiomer of a drug can have different effects than its antipode. Since many chiral compounds are first synthesized in racemic form, it is necessary to separate enantiomers. Also enantioselective methods are needed for the determination of enantiomeric purities, quality control applications and some pharmaco-kinetic and pharmaco-dynamic studies [3, 49, 50]. However, as two enantiomers have identical chemical and physical properties in nonchiral enviroments, the separation of enantiomers can be challenging. Separation of lactam enantiomers has been reported by HPLC with different CSPs [51-57]. Huang et al. [58]used a β-cyclodextrin based CSP to separate water soluble β-lactams. The β-lactams presented in this paper were separated using HPLC by Péter et al.[59] and Sun et al. [60] using macrocyclic glycopeptide and cyclodextrin CSPs respectively. However, to our knowledge, there has been no report on these separations of the β-lactam enantiomers by capillary electrophoresis (CE).

As is well known, CE provides rapid analyses with high efficiencies and often high resolution. Chiral CE is becoming a popular method for enantiomeric separation [61-63]. Cyclodextrins (CDs) are cyclic oligosacchrides with six (α-CD), seven (β-CD), eight (γ-CD) glucopyranose units forming a hollow truncated cone structure. The open cavity possesses a hydrophobic interior and a hydrophilic external surface with five chiral centers per glucose unit. The idea of using CDs as chiral selectors in CE was borrowed from early LC work [64-67]. Because of their excellent stability over a wide pH range (3-14) and minimal UV absorption, CDs and their derivatives have developed into the most prevalent and widely useful class of chiral selectors in CE [7, 10, 62, 68-70]. In our study, three anionic chiral selectors, sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC) and carboxymethyl β-cyclodextrin (CMBC) were added to the run buffer in order to achieve enantiomeric separations in the capillary zone electrophoresis (CZE) mode. Compared to the results obtained by HPLC on cyclodextrin columns [60], CE achieved higher resolution under optimized conditions.

2.2 Experimental

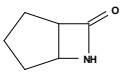
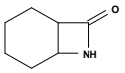
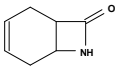
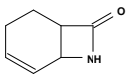
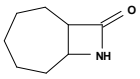
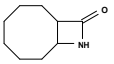
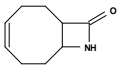
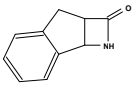
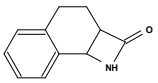
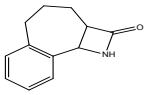
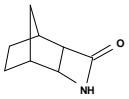
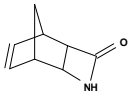
2.2.1 Materials

The 12 racemic β-lactams, cis-6-azabicyclo[3.2.0]heptan-7-one (1), cis-7-azabicyclo[4.2.0]octan-8-one (2), cis-7-azabicyclo[4.2.0]oct-3-en-8-one (3), cis-7-azabicyclo[4.2.0]oct-4-en-8-one (4), cis-8-azabicyclo[5.2.0]nonan-9-one (5), cis-9-azabicyclo[6.2.0]decan-10-one (6), cis-9-azabicyclo[6.2.0]dec-4-en-10-one (7), cis-3,4-benzo-6-azabicyclo[3.2.0]heptan-7-one (8), cis-4,5-benzo-7-azabicyclo[4.2.0]octan-8-one (9), cis-5,6-benzo-8-azabicyclo[5.2.0] nonan-9-one (10), exo-3-azatricyclo[4.2.1.02.5] nonan-4-one (11) and exo-3-azatricyclo [4.2.1.02.5]non-7-en-4-one (12) (Structure refer to Table 2.1) were prepared in our laboratory by cycloaddition of chlorosulfonyl isocyanate to the sorresponding cycloalkanes and cycloalkadienes by Peter *et al* [59, 70, 71]. ; α-cyclodextrin hydrate, sulfated, sodium salt and β-cyclodextrin, sulfated, sodium salt (SAC and SBC) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). SBC is with a degree of substitution of 7-11 moles/mole β-CD. Sodium phosphate, dibasic anhydrous and sodium hydroxide were all obtained from Fisher Scientific (St. Louis, MO, USA). Capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2.2 Equipment

All the separations were performed on a Beckman P/ACE 5000 (Fullerton, CA, USA) or P/ACE 2050 CE instrument (Fullerton, CA, USA) using normal polarity. Capillaries with the dimension of 50μm ID×358 OD, 37cm in length (20 cm to detector) were used. All samples were detected by UV absorbance at 214 nm. All the data were analyzed with Beckman System Gold Software.

Table 2.1 Separations of β-lactams using sulfated α-cyclodextrin (SAC) [a)]

Structure	154 mg/mL SAC		
	t_{m1}(min)	t_{m2}(min)	Rs
1 	33.69	36.30	2.4
2 	33.11	36.25	1.8
3 	22.25	24.72	2.9
4 	29.44	33.42	3.3
5 		——	
6 	24.08	25.90	2.1
7 	15.77	16.55	1.7
8 	19.90	21.04	2.2
9 	22.15	22.96	1.0
10 	21.74	22.36	1.1
11 	30.26	32.66	1.5
12 	22.57	22.84	0.2

a) Separation conditions: 154 mg/mL SAC in 5 mM sodium phosphate buffer, pH 8.0; +6 kV. Other details refer to experimental part.

2.2.3 Method

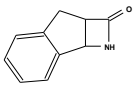
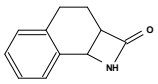
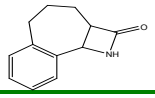
The separation resolution (R_s) was calculated as: $R_s=2(t_{m2}-t_{m1})/(w_1+w_2)$, where t_{m2} and tm1 are the migration times of the second and first observed enantiomers, w_1 and w_2 are the extrapolated peak width at baseline. The apparent mobility μ_{app} was calculated as: $\mu_{app}=L*L_{total}/(t_m*V)$, and the electroosmotic mobility μ_{os} was calculated as: $\mu_{os}=L*L_{total}/(t_{os}*V)$ where L is the length of capillary form the injection end to the window, 30cm, L_{total} is the total length of capillary, 37 cm, t_{os} is the migration time of EOF marker, t_m is the enantiomer migration time and V is the voltage applied across the capillary. The efficiency N was calculated as $N=16*(t_{m1}/w_1)^2$. The selectivity α was calculated as $\alpha=t_{m2}/t_{m1}$. Anhydrous dibasic sodium phosphate was dissolved in deionized, filtered water to make a 5 mM solution and adjusted to the desired pH with 0.1 M hydrochloric acid or 0.1M sodium hydroxide and used as buffer solutions to maintain the pH during running. Sample solutions were made by dissolving samples directly in deionized water (from 1 to 5 mg/mL).

2.3 Results and Discussion

2.3.1 Enantiomeric separation of substituted β-lactams compounds by CZE

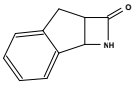
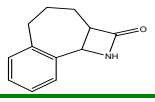
As can be seen from the analyte structures in Table 2.1, each compound consists of a polar lactam moiety fused to a hydrophobic ring system. Hence, these compounds are somewhat soluble in the bulk run buffer solution and can form inclusion complexes with cyclodextrins as well. To obtain an enantiomeric separation, the analyte must have a different mobility than the chiral selectors [68]. Since these chiral analytes have no ionizable groups and are uncharged in buffer solution, the simplest CE system would utilize a charged chiral selector. In the normal polarity mode, the bulk solution moves toward the cathode due to electroosmotic flow (EOF), while the anionic chiral cyclodextrin, moves toward the anode, providing two phases (a bulk solution phase and a cyclodextrin pseudophase) for the analyte distribution.

Table 2.2 Separations of β-lactams using sulfated β-cyclodextrin (SBC) [a)]

Structure	60 mM SBC		
	t_{m1}(min)	t_{m2}(min)	Rs
8 	10.52	10.68	0.7
9 	11.30	14.00	3.4
10 	12.49	15.19	9.5

a) Separation conditions: 60 mM SBC in 5 mM sodium phosphate buffer, pH 8.0; +7 kV. Other details refer to experimental part

Table 2.3 Separations of β-lactams using carboxymethyl β-cyclodextrin (CMBC) [a)]

Structure	20 mM CMBC		
	t_{m1}(min)	t_{m2}(min)	Rs
8 	9.11	9.53	1.5
10 	10.68	10.97	1.3

a) Separation conditions: 20 mM CMBC in 5 mM sodium phosphate buffer, pH 8.0; +10 kV. Other details refer to experimental part

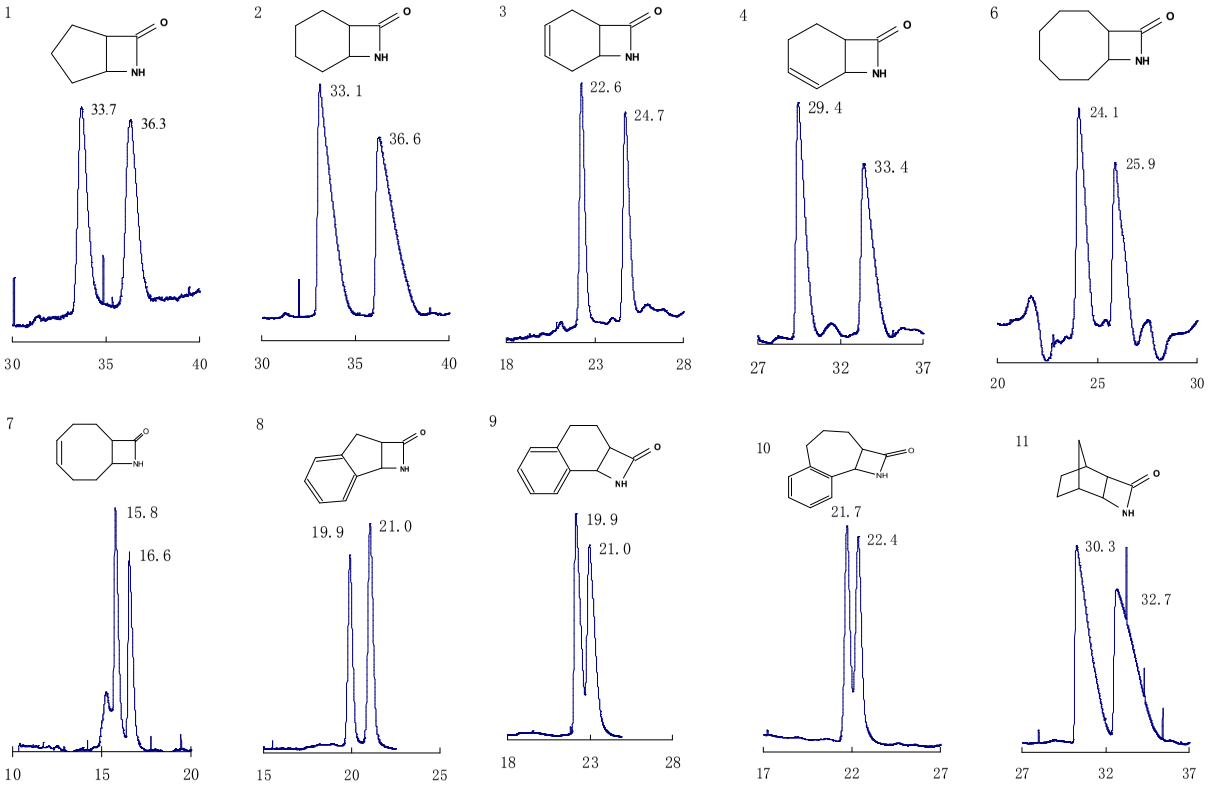


Figure 2.1 Electropherograms of β-lactams 1~4 and 6~11. Experimental conditions: 154 mg/mL sulfated α-cyclodextrin (SAC) in 5 mM sodium phosphate concentration buffer, pH 8.0; +6 kV; Details refer to experimental part

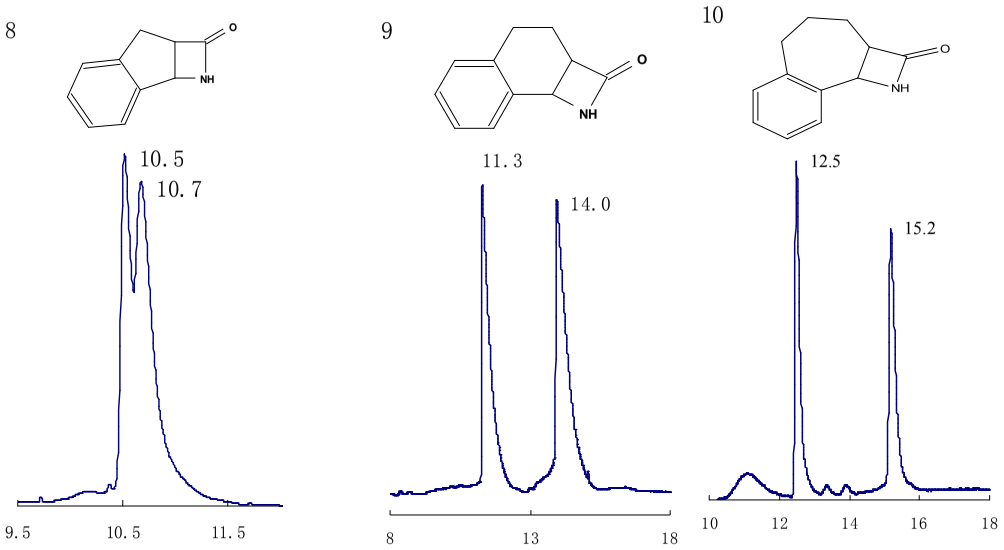


Figure 2.2 Electropherograms of β-lactams 8~10. Experimental conditions: 60 mM sulfated β-cyclodextrin (SBC) in 5 mM sodium phosphate buffer, pH 8.0; +7 kV; Details refer to experimental part

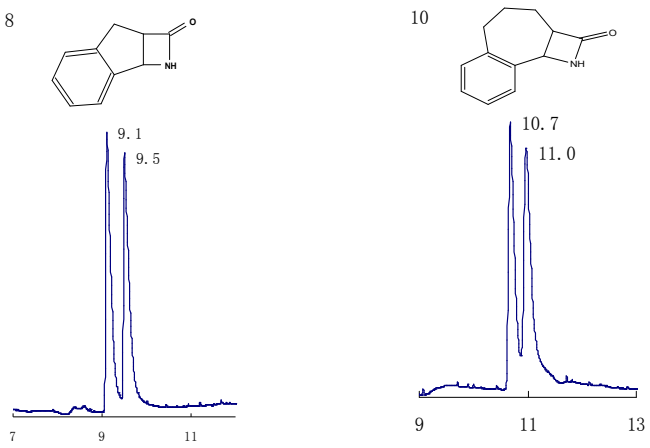


Figure 2.3 Electropherograms of β-lactams 8 and 10. Experimental conditions: 20 mM carboxymethyl β-cyclodextrin in 5 mM sodium phosphate buffer, pH 8.0; +10 kV; Details refer to experimental part

2.3.2 Effect of Chiral Selector Structure and Analyte Structure

The results of the optimized separations are presented in Tables 2.1-2.3. This includes the structures of the β-lactams, migration times, and resolutions under optimized separation conditions. Corresponding electropherograms under optimized conditions using different kinds of chiral selectors are shown in Figures 2.1-2.3 (Fig.2.1 for SAC, Fig.2.2 for SBC and Fig. 2.3 for CMBC).

As shown in Tables 2.1-2.3 and Figures 2.1-2.3, ten of the twelve racemic compounds are separated and each of them is baseline separated, using at least one of the chiral selectors under optimized conditions. SAC is the best overall chiral selector for enatioseparation of the substituted β-lactams, giving 8 baseline separations and 2 partial separations. Among these separations, the highest resolution, Rs=9.5, was achieved for compound 10 using SBC within 16 minutes. SBC produces baseline or partial separations only for 8, 9 and 10, which are tricyclic compounds that possess an aromatic ring. CMBC separates two of the tricyclic aromatic β-lactams (i.e. 9 and 10). Neither SBC nor CMBC separates any compound that does not contain an aromatic ring.

In aqueous solution, hydrophobic analytes can form hydrophobic inclusion complex with cyclodextrins [72, 73]. To obtain enantioselectivity, additional simultaneous interactions such as hydrogen bonding and steric interactions must occur, often at the mouth of the cyclodextrin cavity [65, 72, 73]. Therefore, to obtain enantiomeric separation, it is beneficial for the hydrophobic group of the analyte to have a comparable size with cyclodextrin cavity [65, 72, 73]. This may explain why only the three tricyclic compounds with a rigid aromatic ring (compounds 8, 9 and 10, Table 2.1) are separated with SBC or CMBC. Compounds 1~7 have only a cyclic alkane ring attached to the lactam moiety, compounds 11 and 12 are relatively small tricyclic entities. As observed (results not shown), all the analytes had longer migration time than EOF marker (DMSO) when SBC or CMBC were present. This suggests that all the compounds formed an inclusion complex with SBC and CMBC. However, no

enantioseparations for compounds 1-7, 11-12 were observed. As found previously this is probably due to the fact that they can easily undergo free rotation in the cavity of the CD [65, 72].

2.3.3 Effect of Concentration of Chiral selectors

The concentration of the chiral selector has a pronounced effect on the separation of the twelve chiral lactams. The separation of compound 10 with SBC is used as an example. Electropherograms and other separation data for compound 10 under different SBC concentrations are shown in Figure 2.4. Other factors such as buffer concentration, pH and applied voltage are kept the same in order to focus on the effect of selector concentration. According to the experimental data (Figure. 2.4), higher concentrations of the chiral selector generally increase the migration times. This is due to the fact that, as the concentration of chiral selector increases, a higher percentage of analyte will be included into the CDs. This inclusion complex has a mobility opposite that of the EOF, thereby increasing the migration time of any neutral analyte that forms a dynamic complex [74]. Also, the higher amounts of SBC increase the ionic strength and viscosity of run buffer. This slows the EOF, which also contributes to longer migration times. As can be seen from Figure 2.4, as the concentration of SBC increases from 50 mM to 70 mM, the resolution also increases from 8.1 to 9.0. This trend is consistent with the work of by Wren and Rowe [11, 12].

2.3.4 Effect of Organic Modifier

Organic modifier can affect enantiomeric separations in many ways [7]. As can be seen from Table 2.4, upon addition of ethanol, the EOF is decreased, as are the migration times of analytes. The selectivity α, and resolution, Rs both decrease when higher percentages of ethanol are present. This can be explained by the fact that organic additive can modify the interaction between the CD cavity and analyte [7]. The ethanol tends to compete with analyte for the CD cavity, thus decreasing the binding constants between the analyte and CD [65]. In turn the selectivity and the resolution decrease.

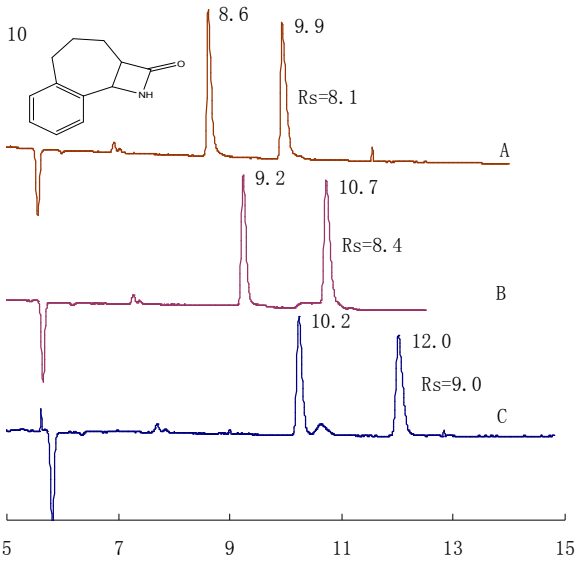


Figure 2.4 Electropherograms of β-lactam 10 separated at different sulfated β-cyclodextrin (SBC) concentration: A: 50 mM SBC; B: 60 mM SBC; C: 70 mM SBC. Experimental conditions: 5 mM sodium phosphate buffer, pH 8.0; +10 kV. Details refer to the experimental part

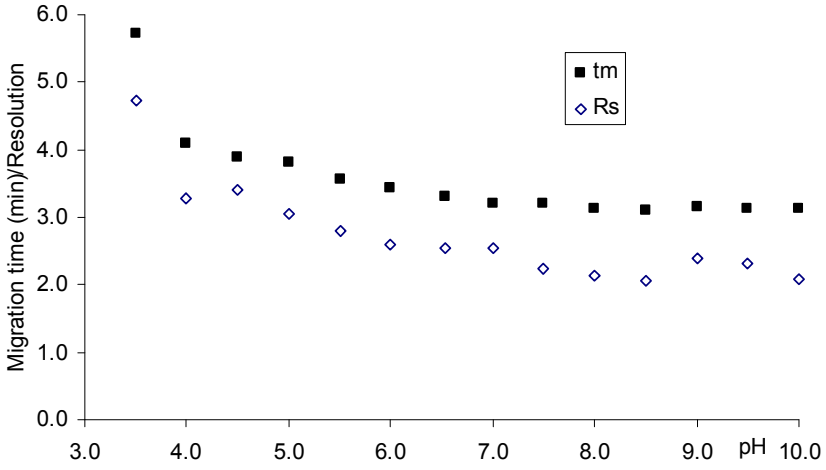


Figure 2.5 Effect of buffer pH when separating β-lactam 10. Conditions: 20 mM sulfated β-cyclodextrin (SBC) in 5 mM phosphate buffer, +10 kV. Details refer to the experimental part

Table 2.4 Effect of addition of organic modifier ethanol on the separation of β-lactam 10 [a)]

EtOH V/V	t_{m1} (min)	t_{m2} (min)	t_{eo} (min)	Rs	N	α
0%	13.60	16.30	10.54	8.9	38000	1.20
5%	15.82	18.10	13.07	7.0	40000	1.14
10%	17.69	19.43	15.43	4.8	41000	1.10

a) Separation conditions: 60 mM SBC in 5 mM sodium phosphate buffer, pH 7.2; +5 kV.

Table 2.5 Effect of applied voltage on the separation of β-lactam 9 [a)]

Voltage (kV)	t_{m1} (min)	t_{m2} (min)	Rs	N	α
16	1.55	1.60	1.2	19000	1.03
15	1.76	1.83	1.3	22000	1.04
14	2.00	2.09	1.6	25000	1.04
12	2.52	2.64	2.0	28000	1.05
11	3.01	3.18	2.3	30000	1.05
10	3.48	3.69	2.6	33000	1.06
9	4.06	4.32	2.8	34000	1.06
8	4.74	5.06	3.0	35000	1.07
7	5.62	6.01	3.0	34000	1.07
6	6.77	7.25	3.1	34000	1.07
5	8.16	8.75	3.1	32000	1.07
4	10.68	11.48	3.1	30000	1.08

a) Separation conditions: 20 mM SBC in 5 mM sodium phosphate buffer, pH 7.2.

2.3.5 Effect of Running Buffer pH

The pH of the run buffer is known to affect the separation in several ways. The pH controls the charge state of ionizable analytes and the chiral selectors [68]. It also controls the magnitude of the EOF [1], which in turn affects the time of the separation. Figure 2.5 shows the effect of pH when SBC is used to separate compound **10**. All other parameters are kept the same while running buffer pH varies from 3.5 to 10.0. As can be seen from the graph, when the pH is increased, generally both the resolution and analyses time decrease. This is due to the fact that, electroosmotic mobility, μ_{os} is increased at higher pH. As reported by Rizzi [75], the selectivity is the ratio of the apparent mobility of the two enantiomers, $\alpha = \mu_{app1}/\mu_{app2}$ (>1), while $\mu_{app}=\mu_e+\mu_{os}$, in which μ_e is the electrophoretic mobility due to its binding to cyclodextrin. Therefore, higher pH increases μ_{os} and thus increases both μ_{app1} and μ_{app2} in same amount. Overall, the enantioselectivity is decreased, as is the resolution.

2.3.6 Effect of Applied Voltage

The applied voltage also affects the enatioseparation by altering the efficiency and selectivity. As the applied voltage decreases, the current will decrease thereby decreasing Joule heating, which in turn suppress the adverse effect of parabolic temperature profile inside the capillary and thus improves the efficiency [1]. At the same time, when the voltage is decreased, the migration time will increase, which allows more time for analyte diffusion which can decrease efficiency [1]. Therefore, there is an optimal applied voltage when the combination of the two factors is minimized. Table 2.5 summarizes the results when using 20 mM SBC to separate compound **9**. As shown in Table 2.5, when voltage is decreased from 16 kV to 4 kV, the efficiency first increases and then decreases, reaching an optimum at a voltage of 8 kV. The selectivity term also increases when the voltage is decreased. Therefore, the resolution, which is the combination of efficiency and selectivity increased as the voltage is decreased, and then reaches a plateau between voltages of 6 kV to 4 kV.

2.4 Conclusion

The separation of twelve racemic, substituted β-lactam compounds were examined via capillary zone electrophoresis using SAC, SBC and CMBC as chiral selectors. Ten of the twelve compounds are separated and optimized to baseline. Overall SAC is the most effective chiral selector, separating the greatest number of compounds as well as giving the greatest number of baseline separation. SBC and CMBC only separate the three tricyclic aromatic lactams but none of the other smaller aliphatic substituted lactams in this group. Increasing chiral selector concentration is the most effective way to improve resolution but it also increases the analysis time. Addition of organic modifier decreases resolution and increases the analysis times. Higher pHs accelerate the analyses but also hurt resolution. Decreasing voltage can affect efficiency and improve enantioselectivity. Generally higher resolution can be obtained at lower voltages.

2.5 Acknowledgements

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CHAPTER 3

ENANTIOMERIC SEPARATION OF SYNTHETIC AMINO ACIDS USING CAPILLARY ZONE ELECTROPHORESIS

Three chiral selectors, sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC) and carboxymethyl β-cyclodextrin (CMBC) were examined as run buffer additives for the separation of sixteen racemic synthetic amino acids and three prepared mixtures of chiral synthetic amino acids, using capillary zone electrophoresis. Seventeen of the nineteen synthetic amino acids were enantiomerically separated and fourteen of them were optimized to baseline using one or more chiral running buffer additives. SAC, with eleven baseline and three partial separations, and SBC, with ten baseline and four partial separations, were found to be the more broadly useful than CMBC. Increasing the chiral selector concentration improved the enantioresolution, but also produced longer analysis times. Addition of organic modifier (ethanol) increased migration times and decreased enantiomeric resolution. Increasing the pH of the run buffer decreased analysis time as well as resolution. Decreasing the applied voltage generally improved resolution.

3.1 Introduction

Enantioseparation of chiral compounds has attracted considerable interest during the past two decades due to the of different biological and pharmaceutical properties of the enantiomers [76]. As building blocks of peptides, proteins and other important biological molecules, amino acids are very important compounds [7]. Amino acids also play an important role in the design of new pharmacons [77, 78]. What's more, unnatural amino acids and their derivatives are commonly used as building blocks in the synthesis of stereochemically pure compounds in pharmaceutical discovery programs [79].

Enantiomeric separation techniques, such as high-performance liquid chromatography (HPLC) [80-86], gas chromatography (GC) [87] and supercritical fluid chromatography (SFC) [88, 89] have been reported for the separation of amino acids enantiomers and their derivatives. Capillary electrophoresis (CE) also is a powerful analytical separation technique due to its high efficiency, low consumption of analytes and buffers [7, 75, 79, 90]. Enantiomeric separations of amino acids by CE using different chiral selectors have reported, including a metal chelate [4, 91-95], cyclodextrins [96-102], and crown ethers [103-108]. Among them, cyclodextrins (CDs) and their derivatives are the most prevalent and broadly useful class of chiral selector in CE [25, 62, 68, 74, 79, 109]. A review on enantioselective separations by CE using CDs has been published [62].

In this work, sixteen racemic synthetic α-amino acids and three prepared mixtures of chiral synthetic α-amino acids were examined via CZE with three anionic chiral selectors: sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC) and carboxymethyl β-cyclodextrin (CMBC). Phenylalanine was also examined as a reference. The elution orders of two of the prepared mixtures were determined by spiking with enantiomers of known absolute configuration into the mixture. The amino acids studied in this paper were also evaluated previously by HPLC using macrocyclic glycopeptide chiral stationary phase (CSP) [77, 110].

3.2 Experimental

3.2.1 Materials

Most of the amino acids were synthesized in our laboratory in Szeged except for compounds **1**, **3** and **13,** which were purchased from Aldrich (Steinheim, Germany). The other seventeen amino acids were either produced as racemates or enantiomerically enriched via asymmetric synthesis [77, 110]. These unusual amino acids are listed as follow**s** (See Table 3.1 for structures): **1**. *m*-tyrosine (*m*-Tyr), **2**. 2',6'-dimethyltyrosine (2',6'-diMeTyr), **3.** α-methyltyrosine (α-MeTyr), **4.** *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine (*erythro*-β-MeTyr), **5.** *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyl-tyrosine (*threo*-β-MeTyr), **6.** 1,2,3,4-tetrahydroisoquinoline-1-

carboxylic acid (Tic1), **7.** 1,2,3,4-tetrahydroisoquino-line-3-carboxylic acid (Tic3), **8.** 5'-methyl-1,2,3,4-tetrahydro- isoquinoline-3-carboxylic acid (5'-MeTic3), **9.** 6'-hydroxy-1,2,3,4-tetrahydroisoquino- line-3-carboxylic acid (6'-HO-Tic3), **10.** 7'-hydroxy -1,2,3,4-tetrahydroisoquinoline- 3-carboxylic acid (7'-HO-Tic3), **11.** *erythro*-(2*S*,3*S* and 2*R*,3*R*)-4-methyl-1,2,3,4-tetra hydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic3), **12.** *threo*-(2S,3S and 2*R*,3*R*)- 4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*threo*-β-MeTic3), **13**. Phenylalanine (Phe) **14.** 2'-methylphenylanaline (2'-MePhe), **15.** 4'-methylphenylanaline (4'-MePhe), **16.** *o*-methyltyrosine **17.** 2',6'-dimethylphenyl- alanine (2',6'-diMePhe), **18.** α-methyl-phenylalanine (α-MePhe) **19.** *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*erythro*-β-MePhe) **20.** *threo*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*threo*-β-MePhe). Among them, **7**, **10**, **17** are artificial mixture of the two enantiomers while the rest of them are obtained as racemates. α-cyclodextrin, hydrate, sulfated, sodium salt (SAC) and β-cyclodextrin, hydrate, sulfated, sodium salt (SBC) with a degree of substitution of 7-11 moles/mole β-cyclodextrin were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Carboxymethyl β-cyclodextrin (CMBC) was obtained from American Maize Products (Hammond, IN, USA). Sodium phosphate, monobasic, sodium phosphate, dibasic, HPLC-grade ethanol, phosphoric acid and sodium hydroxide were all purchased from Fisher Scientific (St. Louis, MO, USA). The fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA).

3.2.2 Method

A Beckman P/ACE 2050 CE instrument (Fullerton, CA, USA) was used for all CE separations with on-column UV detection. The capillary used for all separations had a length of 37 cm (30 cm from inlet to detection window) and a 50 μm inner diameter. The detection window was created by burning off the polyamide coating the desired length of the capillary. Before the first use, the capillary was rinsed with deionized water for 5 minutes and then 1 M sodium hydroxide for 5 min. Before each experiment, the capillary was rinsed with 1 M sodium hydroxide for 30 seconds, deionized water for 1 minutes, then running buffer for 3 minutes to

ensure reproducible EOFs [68]. All the samples were prepared by dissolving 2 mg/mL analyte in deionized water. Samples were injected hydrodynamically. All the analytes were detected by UV absorbance at 214 nm. Sodium phosphate (monobasic) and sodium phosphate (dibasic) were dissolved in deionized water to make 5 mM solutions and then mixed in a 1:1 ratio. This solution was then adjusted to desired pH using 5 mM phosphoric acid or 1 M sodium hydroxide to desired pH. The chiral selectors were added into this mixture and used as running buffer. When utilized, the organic modifier was added on a volume-based ratio prior the addition of chiral selectors.

All the data were analyzed with Beckman System Gold Software. The resolution was calculated as $Rs=2(t_{m2}-t_{m1})/(w_1+w_2)$, the selectivity α was calculated as $\alpha= t_{m2}/t_{m1.}$ The apparent mobility, the efficiency N was calculated as $N=16*(t_{m1}/w_1)^2$, where t_{m1} and t_{m2} are the migration time of the first and second observed peak, while w_1 and w_2 are the extrapolated peak width at baseline.

3.3 Results and discussion

3.3.1 Enantiomeric separation of synthetic amino acids using CZE

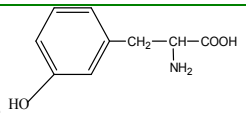
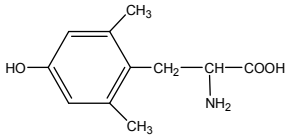
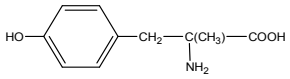
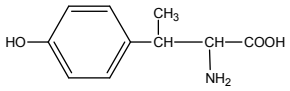
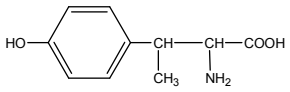
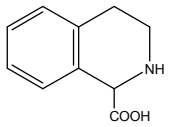
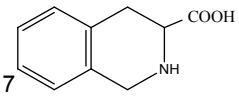
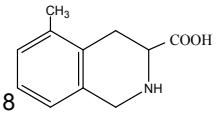
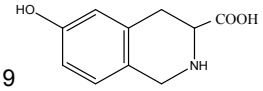
Anionic cyclodextrin derivatives have been one of the most broadly useful types of chiral selectors for CE [25, 62, 68, 74, 110-113]. In the CE normal polarity mode, the bulk solution moves toward the cathode due to electroosmotic flow (EOF) while the anionic cyclodextrin chiral selectors move toward the anode due to electrophoretic movement [68, 111, 113]. Neutral enantiomers (with no electrophoretic mobility themselves) have different distribution**s** between these two countercurrent moving phases, leading to different mobilities and possible enantiomeric separations [25, 62, 68, 74, 111-113]. Synthetic amino acids, which have two ionizable groups with pKas of around 3 and 8, will exist mainly as zwitterions between the two pKas. Therefore, experimental conditions analogous to those used for nonionizable compounds can be used for enantiomeric separation in the appropriate pH range [113].

The structures of all compounds used in this study as well as their migration times and resolutions under optimized conditions, are given in Table 3.1. All of the corresponding electropherograms are given in Figure 3.1-3.3. Among these analytes, compounds **4** and **5**, **11** and **12**, **19** and **20** have two stereogenic centers, and therefore, they can exist as two pairs of enantiomers. However the samples in this study consist of a single pair of enantiomers (see Experimental). Seventeen of the nineteen synthetic amino acids were enantiomerically separated. Among them, fourteen were optimized to baseline using one or more chiral running buffer additives. Eleven baseline and three partial separations were obtained using SAC (Table 3.1). Ten baseline and four partial separations were obtained with SBC (Table 3.1). Many fewer compounds were separated by CMBC, and of these compounds all except compound **19** could be separated by SAC and/or SBC with much higher resolutions. Also, it was found that, the electromigration order of both compounds **7** and **10** were reversed when using SAC and SBC (see electropherograms of **7** and **10** using SAC and SBC in Figures 3.1 and 3.2). Reversal of the migration orders can be important in the determination of enantiomeric impurities [49, 50].

3.3.2 Effect of Chiral Selector Concentration

As reported in many previous studies, the concentration of chiral selector can have a significant effect on enantiomeric separations [11, 12, 68, 74, 111, 113]. The **s**eparation of amino acid **12** using SBC was used as an example to study this effect (see electropherograms and other separation parameters in Figure 3.4). Experimental factors, such buffer concentration, pH and applied voltage were kept the same except for SBC concentration. As shown in Figure 3.4, when the chiral selector (SBC) concentration was increased from 5 mM to 40 mM, a pronounced improvement in the enantiomeric separation was observed**,** from a shoulder (at 5 mM SBC) to a greater than baseline separation with a resolution of 6.1 (at 40 mM SBC).

Table 3.1 Structure and Separations of synthetic amino acids using sulfated α-cyclodextrin (SAC), sulfated β-cyclodextrin (SBC), and carboxymethyl β-cyclodextrin (CMBC)

Structure	90mg/mL SAC [a]			120 mM SBC [b]			65 mM CMBC [c]		
	t_{m1} (min)	t_{m2} (min)	Rs	t_{m1} (min)	t_{m2} (min)	Rs	t_{m1} (min)	t_{m2} (min)	Rs
1. 	Not separated			Not separated			Not separated		
2. 	11.59	11.73	0.4	31.99	38.56	5.9	Not separated		
3. 	Not separated			Not separated			Not separated		
4. 	Not separated			25.87	26.63	1.3	Not separated		
5. 	14.95	16.56	4.7	26.00	27.63	2.8	Not separated		
6. 	13.02	13.75	3.0	Not separated			24.44	25.12	1.0
7 	19.45	20.95	2.8	30.00	31.51	2.1	Not separated		
8 	16.46	16.83	1.0	32.02	36.21	5.2	Not separated		
9 	8.70	8.89	0.9	37.79	38.79	1.1	Not separated		

a) Separation conditions: SAC: 90 mg/mL SAC in 5mM sodium phosphate buffer, pH 8.0; +8 kV except for 15 and 19 at +6 kV with different batch of SAC.
b) SBC: 120 mM SBC in 5 mM sodium phosphate buffer, pH 8.0; +4 kV.
c) CMBC: 65 mM CMBC in 5 mM sodium phosphate buffer, pH 8.0; +7 kV. Other details refer to experimental part

Table 3.1 - *Continued*

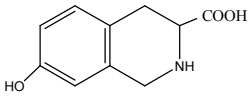
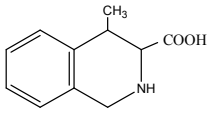
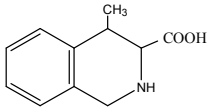
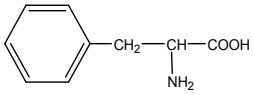
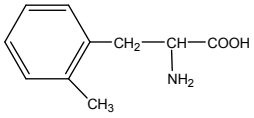
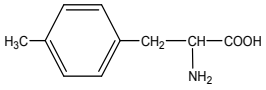
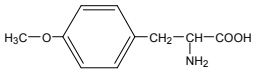
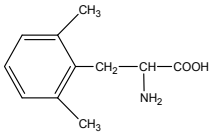
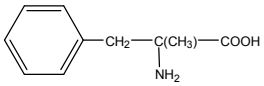
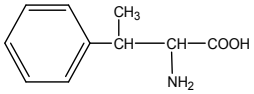
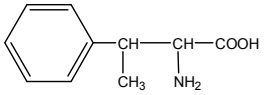
Structure	90mg/mL SAC [a]			120 mM SBC[b]			65 mM CMBC [c]		
	t_{m1} (min)	t_{m2} (min)	Rs	t_{m1} (min)	t_{m2} (min)	Rs	t_{m1} (min)	t_{m2} (min)	Rs
10. 	16.15	17.20	2.1	37.96	48.68	11	Not separated		
11. 	16.92	17.44	1.5	25.09	27.80	4.7	34.7	35.8	1.4
12. 	15.33	19.24	7.5	26.75	36.32	13	40.74	48.01	3.3
13. 	Not separated			Not separated			Not separated		
14. 	17.77	19.02	2.9	32.01	34.74	3.7	27.26	27.83	0.4
15. 	13.44	14.01	2.2	28.48	29.12	1.0	36.8	37.6	0.6
16. 	18.84	20.01	2.9	27.29	27.68	0.6	30.84	31.60	0.5
17. 	Not separated			29.20	31.68	3.6	Not separated		
18. 	14.89	16.27	1.6	Not separated			Not separated		
19. 	Not separated			Not separated			35.40	36.30	0.6
20. 	16.92	17.44	3.3	25.25	26.09	1.5	37.27	38.35	0.6

Table 3.2 Effect of addition of organic modifier ethanol on the separation of amino acid 12 [a)]

EtOH V/V	t_{m1} (min)	t_{m2} (min)	t_{eo} (min)	Rs	N	t_{m2}/t_{m1}
0%	11.97	13.51	10.45	6.6	47000	1.13
5%	14.71	16.35	12.90	5.7	47000	1.11
10%	17.56	19.22	15.50	5.0	47000	1.09

a) Separation conditions: 60 mM sulfated β-cyclodextrin (SBC) in 5 mM sodium phosphate buffer, pH 7.2; +5 kV.

Table 3.3 Effect of the applied voltage on the separation of amino acid 12 [a)]

Voltage (kV)	t_{m1} (min)	t_{m2} (min)	t_{eo} (min)	Rs	N	t_{m2}/t_{m1}
16	1.56	1.59	1.49	0.7	16000	1.02
15	1.62	1.66	1.55	0.7	17000	1.02
14	1.93	1.98	1.84	1.1	28000	1.03
13	2.21	2.27	2.10	1.2	33000	1.03
12	2.56	2.64	2.43	1.4	35000	1.03
11	2.96	3.06	2.82	1.6	36000	1.03
10	3.40	3.53	3.24	1.7	38000	1.04
9	3.94	4.10	3.75	1.8	35000	1.04
8	4.59	4.77	4.36	1.9	36000	1.04
7	5.44	5.67	5.17	1.9	35000	1.04
6	6.45	6.73	6.13	1.9	33000	1.04
5	7.52	7.84	7.14	1.9	32000	1.04
4	10.37	10.85	9.83	2.1	34000	1.05

a) Separation conditions: 20 mM sulfated β-cyclodextrin (SBC) in 5 mM sodium phosphate buffer, pH 7.2

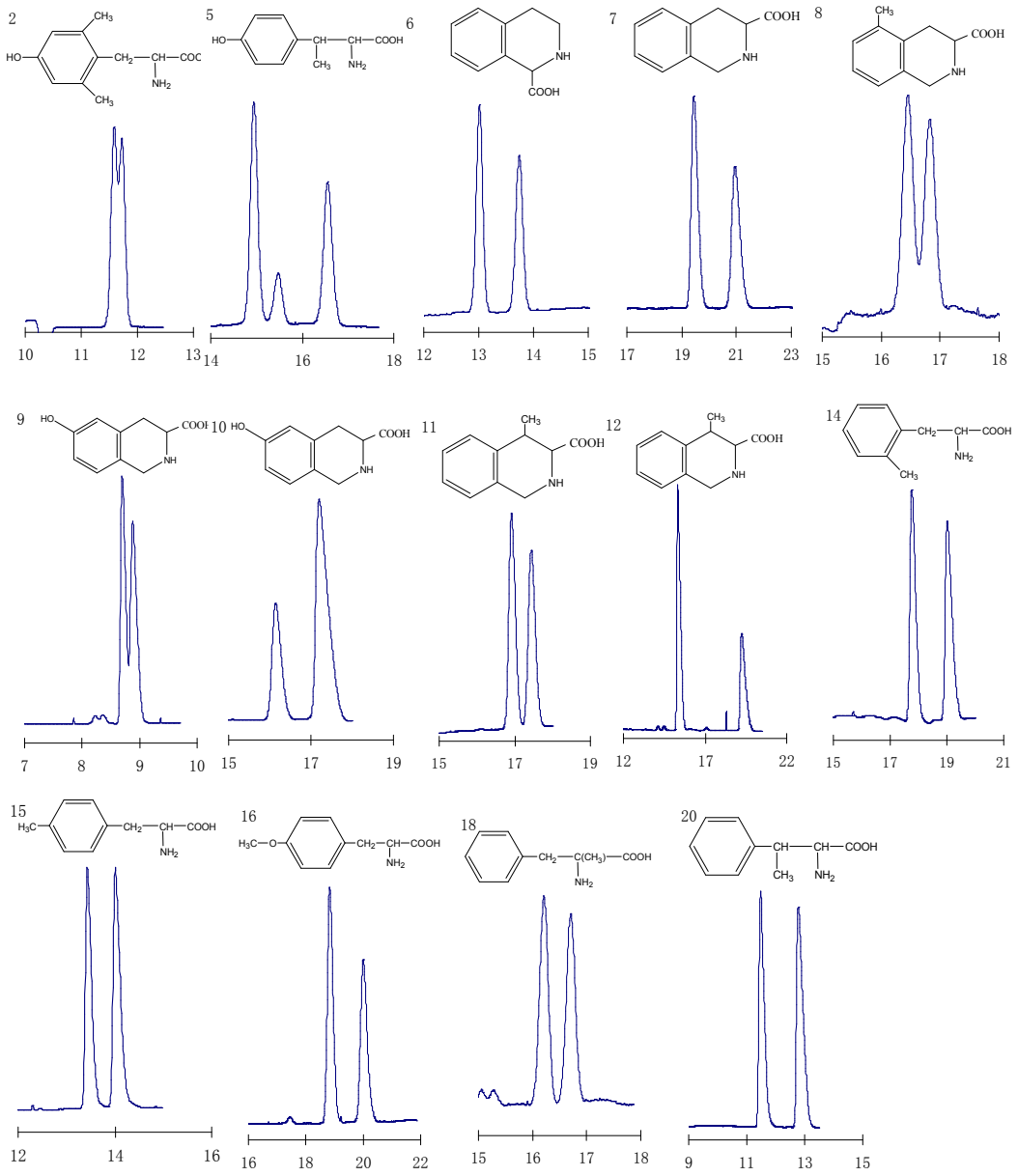


Figure 3.1 Electropherograms of synthetic amino acids that are separated by sulfated α-cyclodextrin (SAC) under optimized conditions. Experimental conditions: 90 mg/mL SAC in 5 mM phosphate buffer, pH 7.2; +8 kV except 15 and 19, +6 kV; Details refer to experimental part

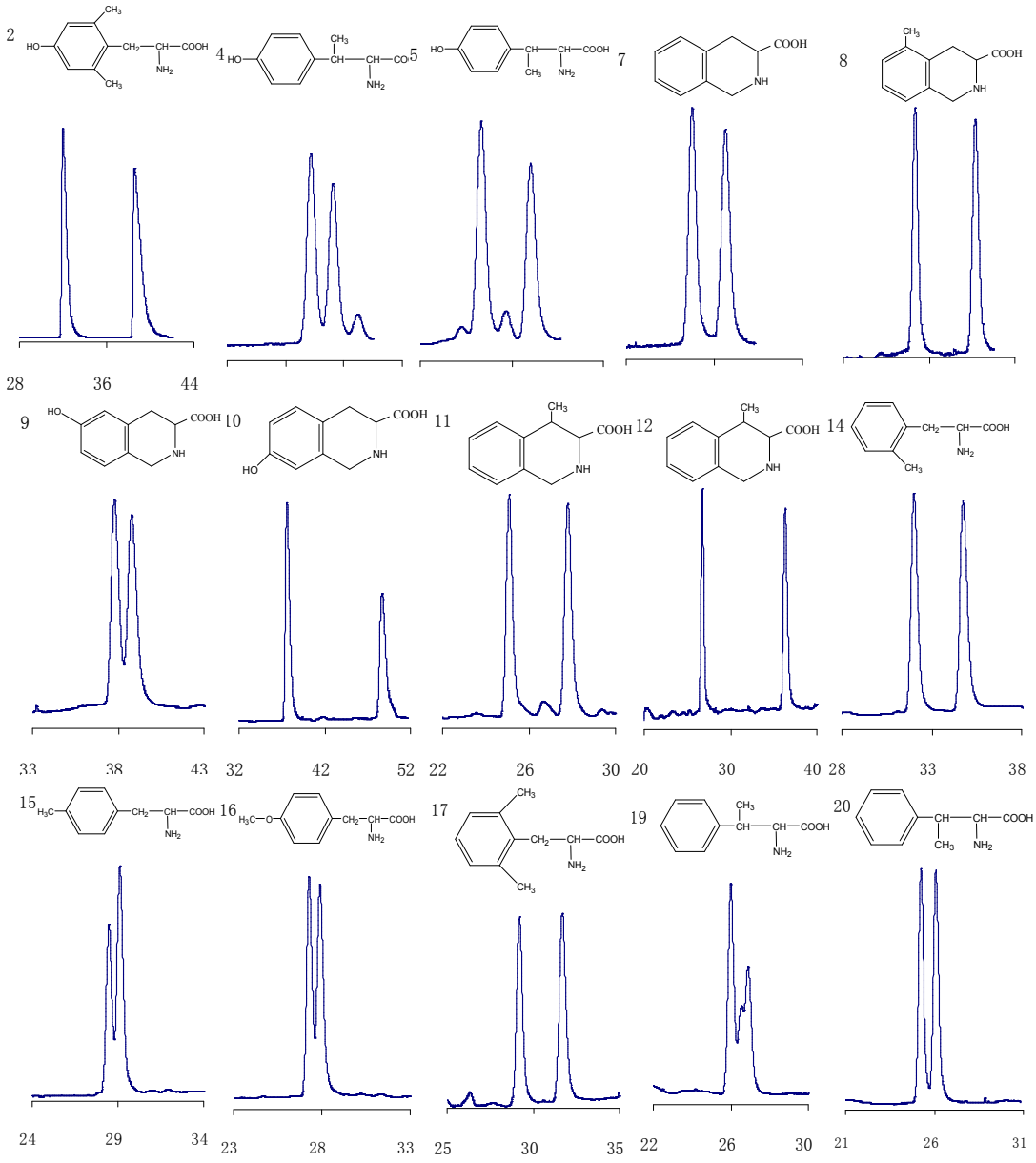


Figure 3.2 Electropherograms of synthetic amino acids that are separated by sulfated β-cyclodextrin (SBC) under optimized conditions. Experimental conditions: 120 mM SBC in 5 mM phosphate buffer, pH 7.2; +4 kV; Details refer to experimental part.

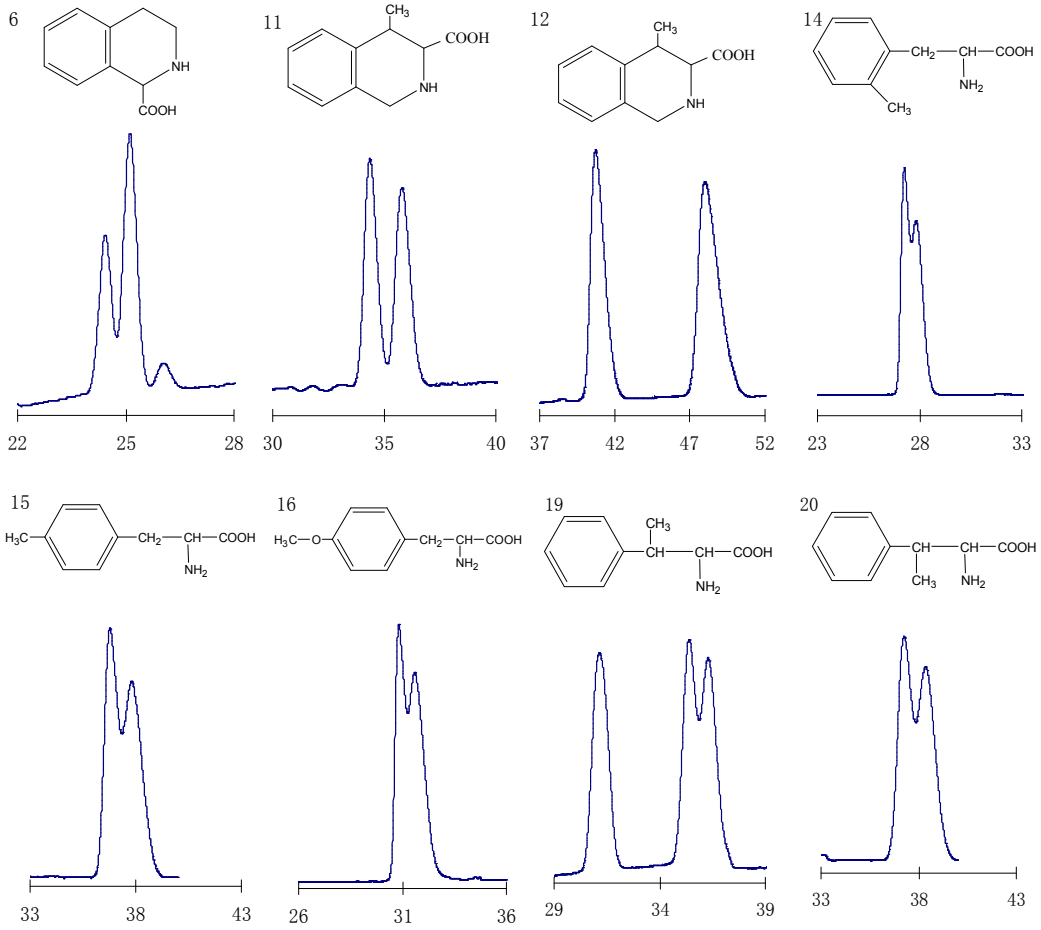


Figure 3.3 Electropherograms of synthetic amino acids that are separated by carboxymethyl β-cyclodextrin (CMBC) under optimized conditions. Experimental conditions: 65 mM CMBC in 5 mM phosphate buffer, pH 7.2; +7 kV; Details refer to experimental part

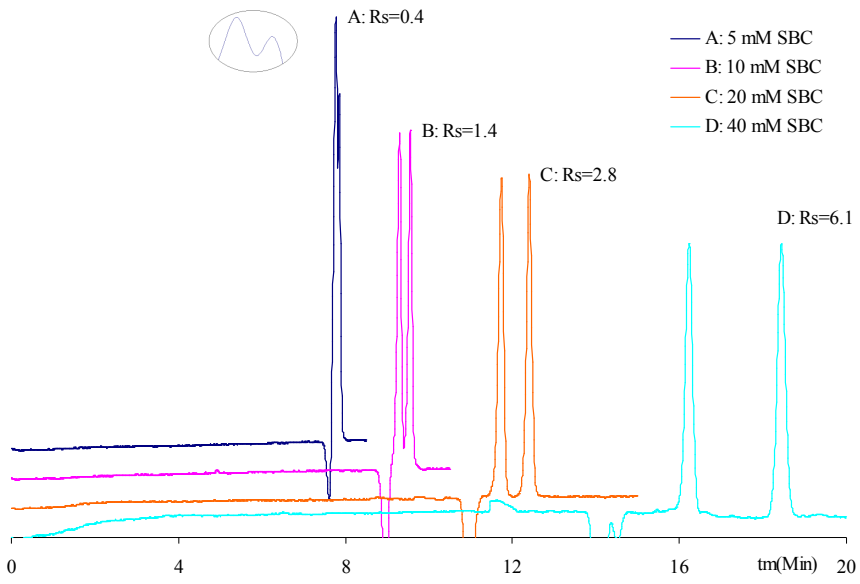


Figure 3.4 Electropherograms of amino acid 12 at different sulfated β-cyclodextrin (SBC) concentrations. Separation conditions: SBC were dissolved in 5 mM phosphate, pH=7.2, +5 kV. Other details refer to experimental part

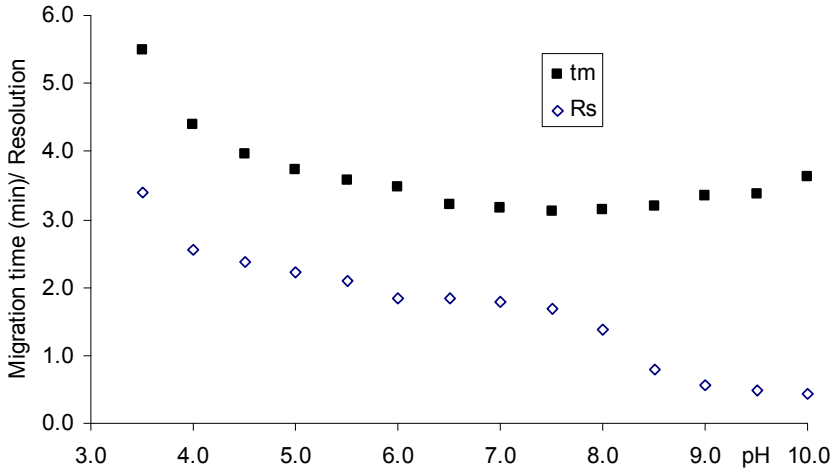


Figure 3.5 Effect of buffer pH when separating amino acid 12 using sulfated β-cyclodextrin (SBC). Conditions: 20 mM SBC in 5 mM phosphate buffer, +10 kV. Details refer to experimental part

3.3.3 Effect of Organic Modifier

The effect of added organic modifiers on the enantioseparations using dissolved cyclodextrin chiral selectors has been reported to be very complicated, as an organic modifier can alter several parameters**.** These include the stability constants for the inclusion complexes, the EOF, the conductivity of the BGE, etc [7, 11, 62]. In some cases, added organic modifiers can improve enantiomeric resolution [114, 115]. However, in most cases when using CDs as chiral selectors, the addition of organic modifier to the running buffer decreases the enantioselectivity [11, 68, 74]. Organic modifier is known to compete with the analyte for binding with the CD cavity. Eventually it decreases the binding constants between the analyte and CD [62, 68, 74]. Table 3.2 shows the experimental data when using 60 mM SBC to separate amino acid **12** at different volume percentage**s** of ethanol. Upon the addition of ethanol, the EOF was slowed and the analysis time was increased. At higher percentage of ethanol, both the selectivity (α) and the resolution (Rs) were decreased.

3.3.4 Effect of Running Buffer pH

Varying the pH of running buffer is known to be an effective way to control the magnitude of the EOF [1]. In the normal polarity mode, higher pHs produce faster EOFs, resulting in shorter analysis time. The running buffer pH also controls the charge state of ionizable analytes and chiral selectors. According to the results shown in Figure 3.**5**, between the pHs of 3.5 and 7.5, higher pHs accelerated analysis at the price of a slight decrease in resolution. As the pH was increased further, the migration times of the two peaks increased while the resolution dropped abruptly. This can be explained by the following facts. The amine group of the analyte starts to be deprotonated at pH 7.5, which gives the analyte a negative charge, and in turn a slower velocity toward the detection window (longer migration time). Also the binding of the anionic amino acid can differ from that of its zwitterionic form.

However, the analysis time also was significantly increased from 8 minutes to 20 minutes. This is due the fact that, as more SBC was present, a higher percentage of analyte

was associated with the cyclodextrin pseudophase**.** This not only gave the analyte an increased electrophoretic mobility toward the anode (which increased analysis time), but also accentuated the mobility difference between the two enantiomers. Also, since the ionic strength was higher when more SBC was present, the EOF magnitude was decreased [1]. This also contributed to longer analysis times.

3.3.5 Effect of Applied Voltage

The effect of applied voltage also was studied using the separation of amino acid **12** as an example. The experimental data is summarized in Table 3.3. When using 20 mM SBC to separate amino acid **12**, as the voltage decreased, the analysis time and migration time of EOF marker increased significantly. The separation also improved from a partial separation with a resolution of 0.7 to a baseline separation with a resolution of 2.1. The efficiency reached an optimum at voltage of 10 kV, while selectivity increased as voltage was decreased in the voltage range studied. These results are consistent with the result we obtained for β-lactams in a previous study [113].

3.4 Conclusion

Nineteen synthetic chiral amino acids were analyzed using CZE and three different anionic cyclodextrins as chiral selectors. SAC and SBC were found to be effective chiral selectors for this series of compounds, each separating about 80% of the analytes with 70% showing a baseline separation. Increasing the chiral selector concentration was found to be the most effective way to improve enantioresolution. Raising pH generally increases analysis time at the expense of a slight loss of resolution**,** when the charge state of both analyte and chiral selector remain the same. Addition of organic modifier usually hurts the resolution and increases the analysis time. Higher resolutions are generally obtained at lower voltages**,** albeit at the expense of longer analysis time**s**.

3.5 Acknowledgements

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CHAPTER 4

ENANTIOMERIC SEPARATION OF CHIRAL RUTHENIUM(II) COMPLEXES USING CAPILLARY ELECTROPHORESIS

Capillary zone electrophoresis (CZE) and micellar capillary electrophoresis (MCE) were applied for the enantiomeric separation of nine mononuclear tris(diimine)ruthenium(II) complexes as well as the separation of all stereoisomers of a dinuclear tris(diimine)ruthenium(II) complex. Nine cyclodextrin (CD) based chiral selectors were examined as run buffer additives to evaluate their effectiveness in the enantiomeric separation of tris(diimine)ruthenium(II) complexes. Seven showed enantioselectivity. Sulfated γ-cyclodextrin (SGC), with four baseline and three partial separations, was found to be the most useful chiral selector. In CZE mode, the derivatized γ-CDs were more effective than β-CDs while sulfated CDs work better than carboxymethyl CDs. In MCE mode, hydroxypropyl β-CD separated the greatest number of tris(diimine)ruthenium(II) complexes. The effects of chiral selector concentration, run buffer pH and concentration, the concentration ratio between chiral selector and other factors were investigated.

4.1 Introduction

Ruthenium(II) tris(diimine) complexes are inherently chiral compounds (see Figure 4.1) [116]. They have been investigated extensively as chiral catalysts [117-120], chiral dopants [121], molecular recognition probe [122-126] and tumor-inhibiting prodrugs [127]. Recently, Ruthenium(II) tris(diimine) complexes have also been developed as chiral mobile phase additives [128] and clay-ruthenium complex adduct chiral stationary phases [129-132] for the enantiomeric separation of chiral compounds on HPLC. As the Δ and Λ enantiomers can have very different properties in these applications, enantiomerically pure compounds are usually preferred and a knowledge of enantiomeric purity is almost always necessary. Unfortunately,

tris(diimine)ruthenium(II) complexes are usually synthesized as racemates or mixtures of moderate enantiomeric excess [133-138].Therefore, effective and efficient methods to evaluate enantiomeric purity are needed.

Traditionally, the enantiomeric separation of tris(diimine)ruthenium(II) complexes has been achieved by diastereoisomeric salt formation and recrystallization [133, 139-143] or chromatographic techniques. Spectroscopic methods, including NMR with chiral shift reagents [142] and linear and circular dichroism [133, 144] are used for enantiomeric recognition.

Cation exchange chromatography with chiral anions as eluent additives was developed by Keene et al as a classical chromatograpic approach for the separation of some metal di(imine) complexes [145]. The Δ and Λ forms of tris(diimine)ruthenium(II) complexes are known to have different affinities to DNA. Therefore calf thymus DNA was immobilized on-column and used for the enantiomeric separation of tris(diimine)ruthenium(II) complexes and other transitional metal complexes [146-148]. A teicoplanin based chiral stationary phase was used for the separation of stereoisomers of dinuclear ruthenium(II) complexes and enantiomers of tris(diimine)ruthenium(II) complexes [149, 150].

Many reviews [5, 7, 8, 76, 151-156] have been published on the extensive use of chiral capillary electrophoresis (CE) in recently years. CE has been used for the enantioseparation of pharmaceutical compounds [76, 154-156], biological samples [7], and asymmetric synthesis products and intermediates [68, 144, 157-162]. There have been relatively few reports on the enantiomeric separation of transition metal poly(diimine) complexes [109, 144, 158-160, 162-164]. Chiral additives used for the CE separation of transition metal complexes are usually chiral anions, including carboxymethyl-β-cyclodextrin [7, 158, 159, 163], tartrate and its derivatives [7, 158, 159, 163], isocitrate and amino acids derivatives [109] and bile salts [159, 163]. In our study, nine cyclodextrin based chiral additives were evaluated by CE and micellar CE for the separation of nine tris(diimine)ruthenium(II) complexes and all stereoisomers of a dinuclear ruthenium(II) tris(diimine) complex (See Figure 4.1 for diimine ligand structures).

Among these ten ruthenium complexes, only $[Ru(phen)_3]^{2+}$, $Ru(bpy)_3]^{2+}$, and cis-$[Ru(bpy)_2py_2]^{2+}$ have been separated by CE using other chiral anions [144, 158, 159] or liquid chromatography [135, 145, 146, 165, 166]. There has been no report on the enantioseparation of the other six ruthenium complexes.

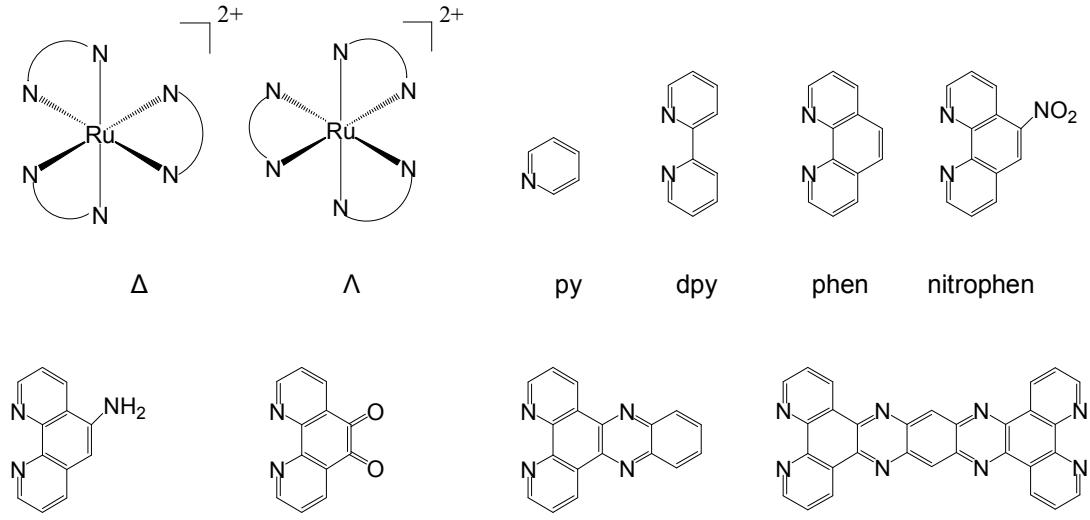


Figure 4.1 Structures of $Ru(diimine)_3^{2+}$ and diimmine ligands; =diimine ligands; py=pyridine; dpy=2,2'-dipyridine; phen=1,10 phenanthroline; nitrophen=5-nitro-1,10 phenanthroline; aminophen =5-amino-1,10 phenanthroline; phendione=1,10 phenanthroline-5,6-dione; dppz= dipyrido[a:3,2 -h:2'3'-c-]phenazine; tatpp=9,10,20,22-tetraaza[3,2-a:2'3'-c:3'',2''-h,2''',3'''-j]tetrapyrido-pentacene

4.2 Material and methods

4.2.1 Materials

Sulfated γ-cyclodextrin (SGC), carboxymethyl γ-cyclodextrin (CMGC) and carboxyethyl γ-cyclodextrin (CEGC) were purchased from Cyclodextrin Technologies Development, Inc. (High Springs, FL, USA). Sulfated β-cyclodextrin (SBC), hydroxypropyl γ-cyclodextrin (HPGC) and hydroxypropyl β-cyclodextrin (HPBC) were purchased from Sigma-Aldrich Company (Saint Louis, MO, USA). Sulfobutyl ether β-cyclodextrin (SBE) was purchased from Advanced Separation Techniques, Inc. (Whippany, NJ, USA). Carboxymethyl β-cyclodextrin (CMBC) was obtained from American Maize Products (Hammond, IN, USA). The tris(diimine)ruthenium(II) complexes were synthesized as previously reported [133-138]. The EOF marker, dimethyl sulfoxide or mesityl oxide, and sodium dodecyl sulfate were purchased from Sigma-Aldrich Corporate (St. Louis, MO, USA). HPLC-grade ethanol and acetonitrile, phosphoric acid and sodium hydroxide were all purchased from Fisher Scientific (St. Louis, MO, USA). The fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA).

4.2.2 Methods

Separations were performed on a Beckman P/ACE 5000 (Fullerton, CA, USA) capillary electrophoresis system equipped with a UV-visible detector or a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with a photodiode array detector and a 50 μm i.d. x 358 μm o.d. capillary. The length of the capillary on P/ACE 5000 system was 37 cm (30 cm to detector) while on P/ACE MDQ the capillary length was 50 cm (40 cm to detector). All the samples were detected at either 214 nm or 254 nm. Sodium phosphate, monobasic or dibasic was dissolved in deionized water and adjusted with concentrated sodium hydroxide or phosphoric acid to the desired pH. Buffer additives (chiral selector and SDS) were added to this buffer solution to make run buffer. Racemic samples or artificial mixtures of enantiomers were dissolved in buffer solution (50 mM SDS in run buffer for MCE samples) to make the sample solutions. Organic modifiers were added by volume percentage prior to the addition of chiral run

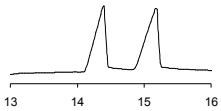
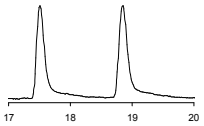
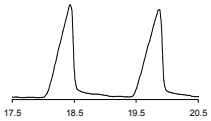
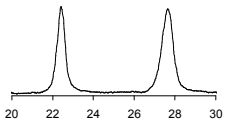
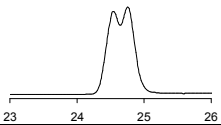
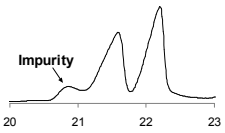
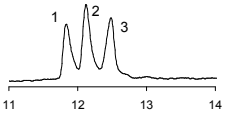
buffer additives. The capillary was rinsed with water for one min, 1 M sodium hydroxide for 5 min and water again for 5 min for conditioning. Between each run, the capillary was rinsed with methanol for 1 min, water for 1 min, 1 M sodium hydroxide for 1 min, and again water for 1 min and followed by run buffer for 2 min. Subsequently the sample solution was injected hydrodynamically at 0.5 psi for 5 seconds. All CZE separations were done in the normal polarity mode with a pH 7.5 buffer P/ACE 5000 system and 6.9 for P/ACE MDQ system. Micellar CE (MCE) separations were completed in the reverse polarity mode with a buffer pH of 2.6. Sample identity was confirmed by UV spectrum obtained with PDA detector in P/ACE MDQ system. The electromigration order was determined by spiking with a pure enantiomer.

The resolution (Rs) was calculated as: $Rs=2(t_{m2}-t_{m1})/(w_1+w_2)$, the apparent mobility (μ_{app}) was calculated as: $\mu_{app}=L^*L_{total}/(t_mV)$, the electroosmotic mobility (μ_{eof}) was calculated as $\mu_{eof}= L^*L_{total}^*/(t_{eof}V)$, the electrophoretic mobility (μ) was calculated as $\mu=L^*L_{total}^*(1/t_m-1/t_{eof})/V$, the mobility difference ($\Delta\mu$) was calculated as $\Delta\mu=\mu_1-\mu_2$, the selectivity (α) was calculated as: $\alpha=\mu_{app1}/\mu_{app2}$, and the number of theoretical plates (N) was calculated as $N=16^*(t_m/w)^2$, where t_{m1} and t_{m2} are the migration times of the first and second peak, t_{eof} is the migration time of EOF marker, and w is the baseline peak width. L is the length of capillary from the injection end to the detection window, L_{total} is the total length of capillary. The resolution (Rs) can also be expressed as: $Rs=\Delta\mu^*N^{1/2}/(4\mu_{app,avg})$.[58] As the selectivity term $\Delta\mu/\mu_{app,avg}=2(\mu_{app2}-\mu_{app1})/(\mu_{app1}+\mu_{app2})=(\alpha-1)/(\alpha+1)$, $Rs=(\alpha-1)/(\alpha+1)N^{1/2}/(2\mu_{app,avg})$.

4.3 Results and discussion

According to Kano's NMR study, only anionic γ -or β- cyclodextrins (per-CO_2-γ-CD and per-CO_2-β-CD) showed chiral recognition for $Ru(phen)_3(ClO_4)_2$ [142]. As the ten ruthenium trisdiimine complexes (Table 4.1 and Figure 4.1) are all positively charged, they will preferably interact with negatively charged chiral selectors. Also it is well known that negatively charged selectors provide the largest separation window for positively charged analytes, which was first reported in 1994 [162]. Therefore, the five anionic chiral cyclodextrin derivatives, SGC, CMGC,

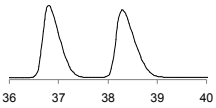
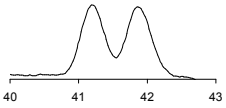
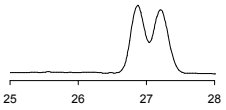
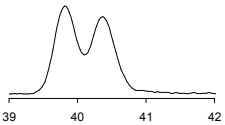
Table 4.1 CZE separations of tris(diimine)ruthenium complexes with sulfated γ-cyclodextrin[a)]

Structure	t_{m1} (min)	[SGC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_3]^{2+}$	14.39	10	1.9	1.06	20000	Λ,Δ	
$[Ru(phen)_2nitrophen]^{2+}$	17.51	10	5.3	1.08	81000	Λ,Δ	
$[Ru(phen)_2aminophen]^{2+}$	18.43	30	2.9	1.08	24000	Δ,Λ	
$[Ru(phen)_2phendione]^{2+}$	22.35	10	5.4	1.24	11000	Δ,Λ	
$[Ru(bpy)_3]^{2+}$	24.54	60	0.6	1.01	31000	Δ,Λ	
Cis-$[Ru(phen)_2py_2]^{2+}$	21.58	110	1.1	1.03	24000	Λ,Δ	
$[Ru(phen)_2dppz]^{2+}$			No analyte peak was observed				
$[Ru(phendione)_3]^{2+}$			No analyte peak was observed				
$[Ru(dppz)_3]^{2+}$			No analyte peak was observed				
[b)] $[Ru_2(phen)_4(tatpp)]^{4+}$	11.83	20	1.2	1.02	41000	NA	
	12.12		1.4	1.03	41000		

a) Data obtained on Beckman P/ACE 5000 CE system with 50 μm ID capillary with a total length of 37 cm (30 cm to detection window). Separation conditions: 60 mM phosphate, pH=7.5, +5 kv, detected at 214 nm.

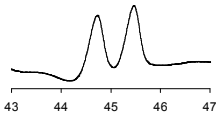
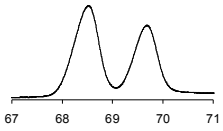
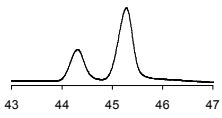
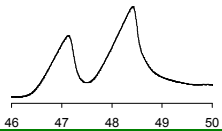
b) Top row is for peak 1, 2, bottom row for peak 2, 3

Table 4.2 CZE separations of tris(diimine)ruthenium complexes with carboxymethyl γ-cyclodextrin [a)]

Structure	t_{m1} (min)	[CMGC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_3]^{2+}$	36.80	260	2.1	1.04	46000	Λ,Δ	
$[Ru(phen)_2nitrophen]^{2+}$	41.17	260	1.0	1.02	57000	Λ,Δ	
$[Ru(bpy)_3]^{2+}$	26.86	260	0.8	1.01	58000	Δ,Λ	
Cis-$[Ru(phen)_2py_2]^{2+}$	39.80	260	0.7	1.01	47000	Δ,Λ	

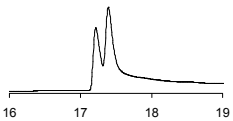
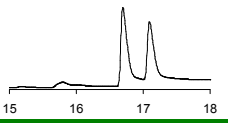
a) Data obtained on Beckman P/ACE 5000 CE system with 50 μm ID capillary with a total length of 37 cm (30 cm to detection window. Separation conditions: 60 mM phosphate, pH=7.5, +5 kv, detected at 214 nm. Analytes with no separation or no observation of peak are not listed.

Table 4.3 CZE separations of ruthenium tris(diimine) complexes with sulfated β-cyclodextrin[a)]

Structure	t_{m1} (min)	[SBC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_3]^{2+}$	44.73	250	1.4	1.02	120000	Λ,Δ	
$[Ru(phen)_2nitrophen]^{2+}$	68.52	250	1.2	1.02	84000	Λ,Δ	
$[Ru(bpy)_3]^{2+}$	44.04	250	1.8	1.02	120000	Λ,Δ	
Cis-$[Ru(phen)_2py_2]^{2+}$	47.13	250	1.2	1.03	31000	Λ,Δ	

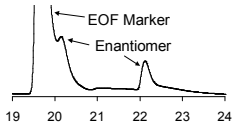
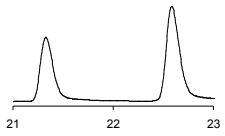
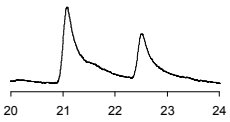
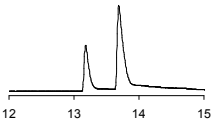
a) Sample solutions are made from artificial mixtures of two enriched enantiomers. Data obtained on Beckman P/ACE MDQ CE system with 50 μm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=6.9, +8kv, detected at 214 nm. Analyte with no separation or no observation of peak are not listed.

Table 4.4 CZE separations of ruthenium tris(diimine) complexes with carboxymethyl β-cyclodextrin [a)]

Structure	t_{m1} (min)	[CMBC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_2nitrophen]^{2+}$	17.22	90	1.0	1.01	130000	Δ,Λ	
Cis-$[Ru(phen)_2py_2]^{2+}$	16.70	90	1.9	1.02	100000	Δ,Λ	

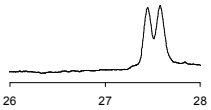
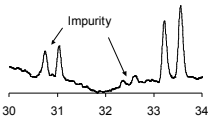
a) Sample solutions are made from artificial mixtures of two enriched enantiomers. Data obtained on Beckman P/ACE MDQ CE system with 50 μm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=6.9, +8kv, detected at 214 nm. Analytes with no separation or no observation of peak are not listed.

Table 4.5 CZE separations of tris(diimine)ruthenium complexes with sulfobutyl ether β-cyclodextrin [a)]

Structure	t_{m1} (min)	[SBE] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_3]^{2+}$	20.18	80	4.7	1.10	42000	Δ,Λ	
$[Ru(phen)_2$nitro-phen$]^{2+}$	21.33	80	5.2	1.06	130000	Δ,Λ	
$[Ru(phen)_2$amino-phen$]^{2+}$	21.08	80	1.2	1.07	6200	Δ,Λ	
$[Ru(bpy)_3]^{2+}$	13.18	50	3.3	1.04	130000	Λ,Δ	

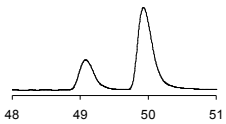
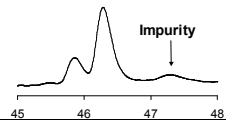
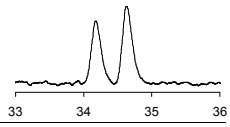
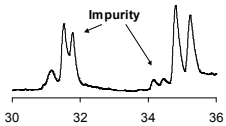
a) Sample solutions are made from artificial mixture of two enriched enantiomers. Data obtained on Beckman P/ACE MDQ CE system with 50 μm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=6.9, +8kv, detected at 214 nm. Analytes with no separation or no observation of peak are not listed.

Table 4.6 Micellar CE separations of tris(diimine)ruthenium complexes with hydroxypropyl γ-cyclodextrin[a)]

Structure	t_{m1} (min)	[SDS] (mM)	[HPGC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_2aminophen]^{2+}$	27.45	210	395	0.8	1.01	390000	Δ,Λ	
$[Ru(phendione)_3]^{2+}$	No analyte peak was observed							
$[Ru(dppz)_3]^{2+}$	33.22	200	395	1.8	1.01	510000	NA	

a) $[Ru(phen)_2aminophen]^{2+}$ is an artificial mixture. Data obtained on Beckman P/ACE MDQ CE system with 50 μm ID capillary with a total length of 50 cm (40 cm to detection window). Analyte with no separation was not listed. Separation conditions: 50 mM phosphate, pH=2.6, +30kv; all samples are dissolved in 50 mM SDS, 50 mM phosphate with pH=2.6, detected at 214 nm

Table 4.7 Micellar CE separations of tris(diimine)ruthenium complexes with hydroxypropyl β-cyclodextrin [a)b)]

Structure	t_{m1} (min)	[SDS] (mM)	[HPBC] (mg/mL)	Rs	α	N_{avg}	EMO	Electropherogram
$[Ru(phen)_3]^{2+}$	49.08	210	365	2.0	1.02	220000	Λ,Δ	
$[Ru(phen)_2$aminophen$]^{2+}$	45.85	210	365	0.9	1.01	150000	Λ,Δ	
$[Ru(bpy)_3]^{2+}$	34.18	200	365	1.6	1.01	240000	Λ,Δ	
$[Ru(dppz)_3]^{2+}$	34.80	200	365	1.1	1.01	130000	NA	

a) Data obtained on Beckman P/ACE MDQ CE system with 50 μm ID capillary with a total length of 50 cm (40 cm to detection window). Analyte with no separation was not listed. Separation conditions: 50 mM phosphate, pH=2.6, +30kv; all samples are dissolved in 50 mM SDS, 50 mM phosphate with pH=2.6, detected at 214 nm

b) $[Ru(phen)_3]^{2+}$,$[Ru(phen)_2$aminophen$]^{2+}$, and $[Ru(bpy)_3]^{2+}$ are all artificial mixtures. No analyte peak was observed for $[Ru(phendione)_3]^{2+}$

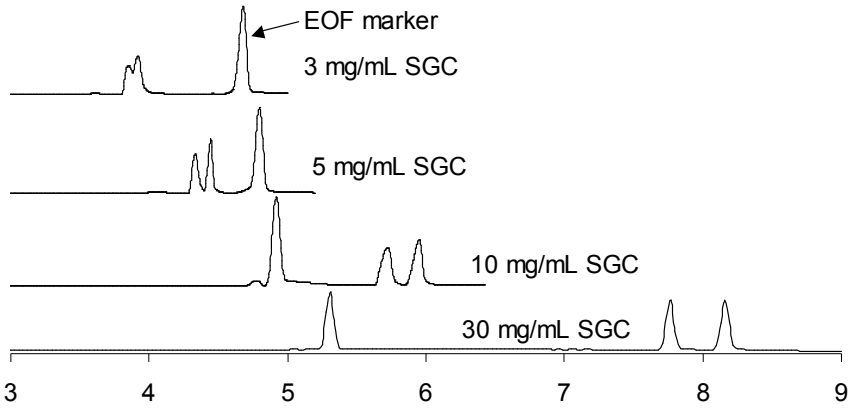


Figure 4.2 Effect of SGC concentration when separating $[Ru(phen)_2nitrophen]^{2+}$. Separation conditions: Data obtained on Beckman P/ACE 5000 CE system with 50 µm ID capillary with a total length of 37 cm (30 cm to detection window). Separation conditions: 60 mM phosphate, pH 8.5, +10 kV, detected at 214 nm

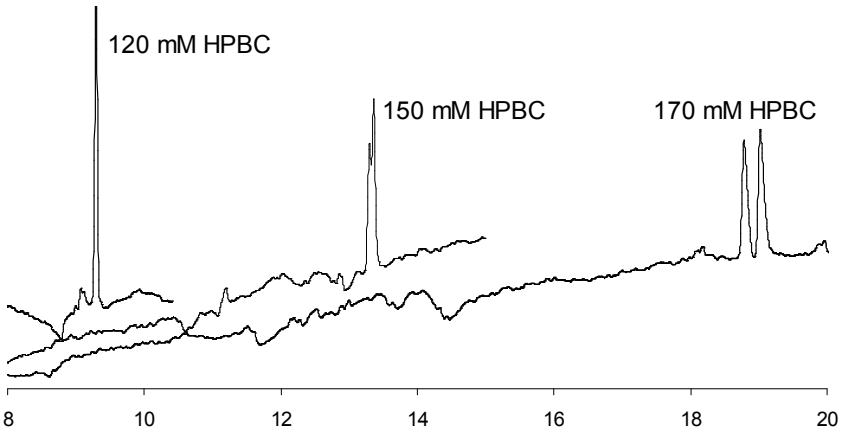


Figure 4.3 Effect of chiral selector hydroxypropyl β-cyclodextrin (HPBC) concentration at Fixed SDS concentration (200 mM) when separating $[Ru(bpy)_3]^{2+}$. Data obtained on Beckman P/ACE MDQ CE system with 50 µm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=2.6, -30kV; all samples are dissolved in 50 mM SDS, 50 mM phosphate with pH=2.6, detected at 214 nm

SBC, CMBC and SBE were initially evaluated as chiral selectors. Tables 4.1-4.5 give the structure of ruthenium complexes, migration times for the first eluted peaks, cyclodextrin concentrations, resolutions, selectivities, efficiencies (the average of the two peaks), electromigration orders and electropherograms of the optimized separations. SGC appeared to be the most powerful chiral selector, giving the largest resolution for the greatest number of racemates, and it did so at lower concentrations. According to our results, sulfated cyclodextrins separated a greater number of ruthenium complexes. Derivatized γ- cyclodextrins also gave a larger number of separations with higher resolutions at lower chiral selector concentrations.

No peaks were observed for the following three ruthenium complexes in CZE mode: $[Ru(phen)_2dppz]^{2+}$, $[Ru(phendione)_3]^{2+}$ and $[Ru(dppz)_3]^{2+}$. Therefore, SDS was added to the running buffer to improve the solubility of these complexes. Neutral cyclodextrins, HPGC, HPBC, DMBC and γ-CD as well as CMGC, CMBC and SBE were tested as chiral selectors in micellar capillary electrophoresis (MCE). In the MCE mode, the pH was adjusted to 2.6 to reduce the EOF [1]. The results are listed in Tables 4.6-4.7. The peak for $[Ru(phendione)_3]^{2+}$ was never observed. Only the neutral chiral selectors HPBC and HPGC produced enantiomeric separations for any ruthenium complexes and then only at very high concentrations of both cyclodextrin and SDS. Also, this approach was characterized by longer analysis times and lower resolutions compared to conventional CZE (except for $[Ru(phendione)_3]^{2+}$). Other cyclodextrin derivatives showed limited solubility in the presence of high SDS concentrations.

Increasing the concentration of chiral selector is known as an effective way to improve enantiomeric separations [8, 62, 68, 112, 113, 157]. The CE separation of $[Ru(phen)_2nitrophen]^{2+}$ enantiomer with SGC is a typical example. All conditions including buffer concentration, pH, voltage and so forth were equivalent for all runs as the SGC concentration was altered (Figure 4.2). As the SGC concentration was increased from 3 mg/mL to 30 mg/mL, the enantioresolution improved from a partial separation (Rs=0.6) to more than a baseline separation (Rs=3.3) due to an improvement in selectivity. The analysis time increased from 5

Table 4.8 The effect of pH on the enantiomeric separation of $[Ru(phen)_2aminophen]^{2+}$

pH	t_{m1} (min)	t_{m2} (min)	t_{eof} (min)	Rs	Δμ	$\mu_{app,avg}$	$\Delta\mu/\mu_{app,avg}$	α	N_{avg}
5.0	16.43	18.58	10.50	5.6	0.98	8.0	0.12	1.13	34000
6.8	10.41	11.23	7.26	4.6	0.98	12.8	0.08	1.08	58000
8.7	9.60	10.30	6.86	4.0	0.97	14.0	0.07	1.07	54000
10.5	8.53	9.10	6.03	3.9	1.04	15.7	0.07	1.07	56000

a) The unit for mobilities are all $cm^2kV^{-1}min^{-1}$
b) Data obtained with compound $[Ru(phen)_2aminophen]^{2+}$ on Beckman P/ACE 5000 CE system with 50 μm ID capillary with a total length of 37 cm (30 cm to detection window. Separation conditions: 40 mg/mL SGC, 60 mM phosphate, +8kv, detected at 214 nm

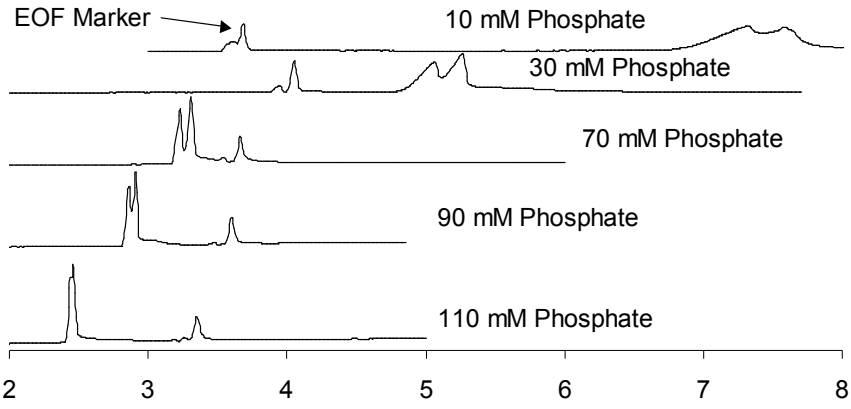


Figure 4.4 Effect of buffer (sodium phosphate) concentration when separating $[Ru(phen)_3]^{2+}$. Data obtained on Beckman P/ACE 5000 CE system with 50 μm ID capillary with a total length of 37 cm (30 cm to detection window). Separation conditions: 9 mg/mL SGC, pH=8.5, +10 kv, detected at 214 nm

min to 9 min. The effect of chiral selector concentration on the enantiomeric separation of $[Ru(bpy)_3]^{2+}$ using micellar CE also was studied and is shown in Figure 4.3. Similar trends were observed. With a fixed SDS concentration and all other conditions the same, a longer analysis time and higher resolution were observed at higher HPBC concentrations.

Run buffer pH can affect the enantioseparation by affecting EOF mobility [1], changing the charge state of the analyte and chiral selector, which in turn affects their association behavior [7, 62]. The effect of pH on CE enantiomeric separations has been reported many times [74, 112, 113, 157, 167]. Four pH values were evaluated for the enantioseparation of $[Ru(phen)_2aminophen]^{2+}$. Results are given in Table 4.8. As the pH increased from 5.0 to 10.5, both the EOF marker and analyte migrated much faster, which reduced the analysis time by 50%. This is because of the deprotonation of a higher percentage of silanol groups on the capillary wall, which greatly increased the EOF mobility. From pH 5.0 to pH 10.5, the average apparent mobility almost doubled, however, mobility differences between the two enantiomers remained essentially the same.

In enantioselective CE separations, the buffer plays an important role [1]. In addition to its buffering capacity, it controls the ionic strength of the solution. It stabilizes the current, which minimizes baseline noise and also helps to maintain a constant EOF [1, 168]. Also, the proper buffer suppresses electromigration dispersion [62, 169] which leads to improved efficiency. However, excessive Joule heating due to high current would be expected at high concentrations of buffer. Buffer may also affect the association chemistry between analytes and the chiral selector. In our study, electrostatic interaction is significant in the complexation of the positively charged analytes and negatively charged chiral selectors. Higher buffer concentration provides higher ionic strength, which suppresses the electrostatic interaction between analytes and chiral selectors. Figure 4.4 shows the electropherograms of the CE separation of $[Ru(phen)_3]^{2+}$ with SGC at different phosphate buffer concentrations, while all other conditions remained the same. At 10 mM sodium phosphate, the analyte peak was very wide. As the phosphate

concentration increased to 70 mM, the efficiency improved significantly. This is because at a higher ionic strength run buffer, there are more buffer ions that can disrupt the electrostatic attraction between analytes and chiral selectors. The analytes migrated faster than the EOF marker at high buffer concentration (above 70 mM) but slower than the EOF marker at low buffer concentration (below 30 mM). This also indicates that higher ionic strength suppresses the binding of analytes and the chiral selector, since longer migration times indicate stronger binding of analyte to the negatively charged chiral selectors. When the buffer concentration was further increased to 110 mM, the enantioresolution was almost lost as the interaction between analyte and chiral selector was greatly suppressed. Optimum resolution was observed around 70 mM phosphate buffer.

Increasing the applied voltage is an effective way to shorten analysis times [1]. Increased voltage greatly decreases analysis time, which in turn suppresses molecular diffusion leading to sharper peaks [1]. Increased voltage also produces higher current and more Joule heating, which hurts the efficiency. The data in Table 4.9 shows the effect of voltage on the separation of $[Ru(phen)phendione]^{2+}$ enantiomers. As expected, analysis time was decreased from 17 min to about 2 min as the voltage was increased from 4 kV to 15 kV. The efficiency reached at a maximum at 12kV and then decreased at higher voltage. The enantioresolution and selectivity decreases as the voltage was increased.

The effect of organic modifier on enantiomeric separations can be very complicated [7, 62]. It can modify several parameters, including the association constants between analyte and the chiral selector, the EOF, the conductivity of run buffer, in turn the Joule heating [7, 62, 170]. The effect of methanol and acetonitrile on the separation of $[Ru(phen)_3]^{2+}$ enantiomers are given in Table 4.10. Both organic modifiers suppressed the EOF, with methanol showing a greater effect. The selectivity, however, was slightly increased by methanol but significantly decreased by higher concentrations of acetonitrile. Both organic modifiers decreased the efficiency, with acetonitrile producing a more pronounced effect. Overall, the addition of methanol increased

Table 4.9 The effect of voltage on the enantiomeric separation of [Ru(phen)2phendione] $^{2+}$ a)b)

Voltage (kV)	t_{m1} (min)	t_{m2} (min)	t_{eof} (min)	Rs	μ_{eof}	μ_1	μ_2	$\Delta\mu$	$\mu_{app,avg}$	$\Delta\mu/\mu_{app,avg}$	N_{avg}
4	15.10	16.92	11.20	4.3	24.8	6.4	8.4	2.0	17.4	0.11	23000
8	6.21	6.77	4.89	3.5	28.4	6.0	7.9	1.8	21.4	0.086	26000
12	3.12	3.29	2.67	2.6	34.6	5.0	6.5	1.5	28.9	0.053	38000
15	1.85	1.90	1.74	1.1	42.5	2.5	3.6	1.1	39.5	0.027	27000

a) The unit for mobilities are all $cm^2kV^{-1}min^{-1}$
b) Data obtained with compound $[Ru(phen)_2phendione]^{2+}$ on Beckman P/ACE 5000 CE system with 50 µm ID capillary with a total length of 37 cm (30 cm to detection window. Separation conditions: 10 mg/mL sulfated γ-cyclodextrin (SGC), 60 mM phosphate, pH=7.5, detected at 214 nm

Table 4.10 The effect of organic modifier on the enantiomeric separation of $[Ru(phen)_3]^{2+}$ a)b)

Organic modifier	t_{m1} (min)	t_{m2} (min)	t_{eof} (min)	Rs	$\Delta\mu$	$\mu_{app,avg}$	$\Delta\mu/\mu_{app,avg}$	α	N_{avg}
0%	4.92	5.07	5.22	1.4	0.83	27.9	0.030	1.03	84000
4% MeOH	5.19	5.37	5.68	1.2	0.90	26.3	0.034	1.04	21000
8% MeOH	6.11	6.32	7.15	1.0	0.76	22.3	0.034	1.03	15000
4% ACN	4.92	5.08	5.51	0.9	0.89	27.8	0.032	1.03	14000
8% ACN	4.68	4.78	5.70	0.2	0.61	29.3	0.021	1.02	3000

a) The unit for mobilities are all $cm^2kV^{-1}min^{-1}$
b) Data obtained with compound $[Ru(phen)_3]^{2+}$ on Beckman P/ACE 5000 CE system with 50 µm ID capillary with a total length of 37 cm (30 cm to detection window. Separation conditions: 8 mg/mL sulfated γ-cyclodextrin (SGC), 60 mM phosphate, +8kv, pH=7.5, detected at 214 nm

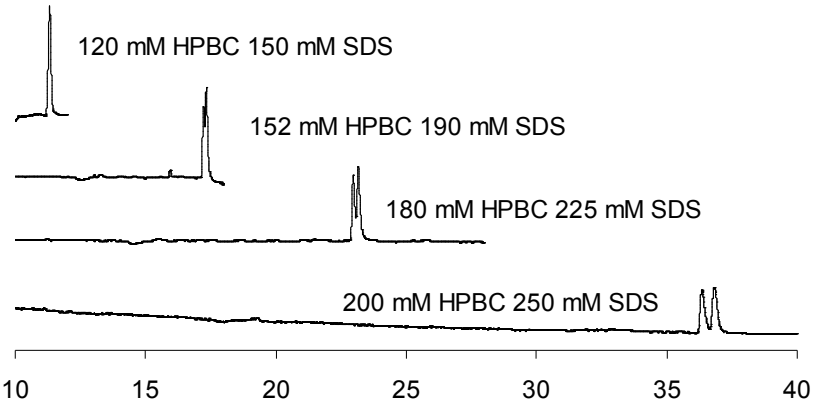


Figure 4.5 Effect of varying the concentration of a fixed ratio of hydroxypropyl-β-cyclodextrin (HPBC) and sodium dodecyl sulfate (SDS) (0.8) when separating $[Ru(bpy)_3]^{2+}$ Data obtained on Beckman P/ACE MDQ CE system with 50 µm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=2.6, -30kv; all samples are dissolved in 50 mM SDS, 50 mM phosphate with pH=2.6, detected at 214 nm

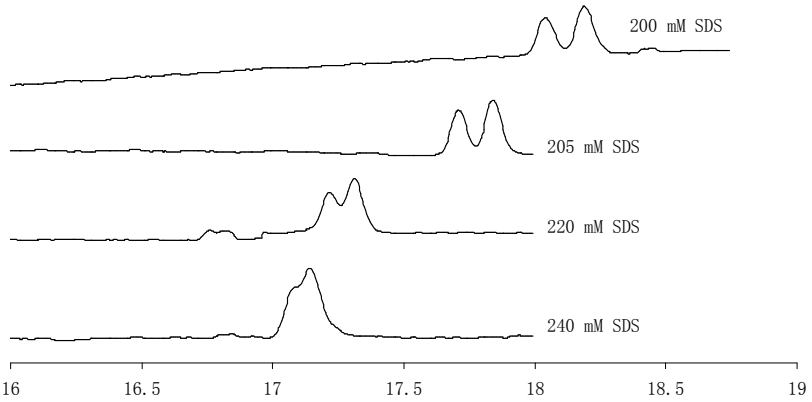


Figure 4.6 Effect of sulfated dodecyl sulfate (SDS) concentration at a fixed hydroxypropyl β-cyclodextrin (HPBC) concentration (160 mM) when separating $[Ru(bpy)3]^{2+}$. Data obtained on Beckman P/ACE MDQ CE system with 50 µm ID capillary with a total length of 50 cm (40 cm to detection window). Separation conditions: 50 mM phosphate, pH=2.6, -30kv; all samples are dissolved in 50 mM SDS, 50 mM phosphate with pH=2.6, detected at 214 nm

analysis times and produced slightly decreased resolution. Acetonitrile produced slightly decreased analysis times, and severely decreased resolution. This behavior is in contrast with most chiral organic compounds, where there is often a beneficial effect with the addition of an optimum level of an organic modifier [25, 68, 74, 112, 113, 157, 170].

In micellar CE, both the concentration of the chiral selector and the surfactant have a significant effect on enantioseparation. Generally, higher chiral selector concentrations improve enantioresolution, but lead to longer analysis times. Higher surfactant concentrations, and therefore micellar concentrations, shorten analysis times and decrease enantioresolution. The effect of these two buffer additive concentrations at a fixed ratio (0.8) was studied for the separation of $[Ru(bpy)_3]^{2+}$. Electropherograms are shown in Figure 4.5. At 120 mM HPBC and 150 mM SDS, no enantioseparation was observed. As the concentrations of HPBC and SDS increased, the analysis time and enantioresolution increased. At 200 mM HPBC and 250 mM SDS, the enantioresolution was greatly improved to 1.4.

The effect of surfactant concentration at fixed chiral selector concentration also was studied on the enantiomeric separation of $[Ru(bpy)_3]^{2+}$. The electropherogram is shown in Figure 4.6. When the surfactant concentrations were increased, the analysis times were shortened, and the enantioresolutions decreased, which is consistent with previous finding [74, 112].

4.4 Conclusions

Nine cyclodextrin based chiral selectors were examined for the CE enantioseparation of nine chiral ruthenium (II) tris(diimine) complexes and the separation of all stereoisomers of one dinuclear tris(diimine)ruthenium(II) complexes. Seven of the chiral organometallic compounds were separated by one or more chiral selectors. $[Ru(phendione)_3]^{2+}$ was not eluted in any mode. In both CZE and MCE modes, enantioresolution can be significantly improved by increasing the chiral selector concentration. In the CZE mode, better resolutions were obtained at lower pHs

and lower applied voltages, but with longer analysis time. In MCE mode, higher surfactant concentrations speed up analysis, but with decreased enantioresolution. Increasing the concentration of a fixed ratio of surfactant and chiral selector helps to optimize an MCE mode enantioseparation but leads to longer analysis times.

4.5 Acknowledgement

We gratefully acknowledge the support of National Institute of Health (NIH 5 ROICM053825-11).

CHAPTER 5

SYNTHESIS AND EXAMINATION OF SULFATED CYCLOFRUCTANS AS A NOVEL CLASS OF CHIRAL SELECTORS FOR CAPILLARY ELECTROPHORESIS

Cyclofructans are a class of cyclic oligosaccharides with a crown ether skeleton. No enantioseparations have previously been reported using this class of chiral oligosaccharides in chromatography or electrophoresis. Cyclofructans and their sulfated derivatives were examined as chiral selectors using capillary electrophoresis. The native cyclofructans showed no enantioselectivity toward any tested compounds, while the sulfated cyclofructan showed exceptional selectivity toward many cationic analytes, including primary, secondary, and tertiary amines and amino acids. Enantiomeric resolution factors (as high as 15.4) were achieved within short analysis times (generally below 10 min). The effect of buffer type, buffer concentration, buffer pH, chiral selector concentration and organic modifier concentration were examined and optimized.

5.1 Introduction

The modern technique of capillary electrophoresis (CE) has several known advantages such as high efficiency, short analysis times, low sample consumption, simple instrumentation and a generally low operation cost [5, 7, 8, 62, 75, 151, 153, 171-173]. In spite of the large number of chiral selectors used in modern enantiomeric separations, relatively few classes (all of which originated from LC) have been as successful in chiral CE [174-177]. This is due to the inherent requirements of CE chiral selectors: low UV absorption, high solubility in water, minimum interaction with the fused silica wall, etc. To date, cyclodextrins (CDs) and especially their derivatives (charged and uncharged) have dominated chiral CE separations. In this work, we introduce cyclofructans (CFs) and their derivatives as a new class of chiral selectors.

Cyclofructans may be the first class of chiral selectors that show comparable suitability to cyclodextrins for CE enantiomeric separations.

Cyclofructans (CFs), also known as cycloinulo-oligosaccharides, are enzymatic digestion products of inuline by the extracellular enzyme, cycloinulo-oligosaccharides transferase [178-183]. As shown in Figure 5.1, cyclofructans consist of a crown ether skeleton and fructofuranose residues that are linked to the crown ether ring in a spiral arrangement [184-186]. Each fructofuranose moiety has three hydroxyl groups, which not only make cyclofructans highly soluble in aqueous solution (>1.2g/ml), but also provide multiple H-bonding sites. In addition, CFs are UV transparent to nearly 200 nm. These unique characteristics make them ideal candidates for chiral selectors in CE. Unlike cyclodextrins, which possess a truncated cone shape [72, 187]. Cyclofrutans are more disc-shaped with central indentation [186]. Since the first report in 1989 [178], cyclofructans have been used in many applications including: hardening accelerators in adhesives [188], silver halide photographic materials [189-191], gelling-prevention agents for frozen eggs [192], complexation agents [186, 193-195], drug carriers and health food additives [196-199] as well as bad taste inhibitors [200, 201]. However, few reports have been found concerning the application of cyclofructan as chiral selectors [202-204]. To our knowledge, there has been no reported use of any cyclofructan as a chiral selector in electrophoresis or chromatography.

5.2 Materials and methods

5.2.1 Materials

Cyclofructan 6 (CF6) and Cyclofructan 7(CF7) were gifts from Dr. Mari Yasuda at the Mitsubishi Chemical Group (Tokyo, Japan). Dimethyl sulfoxide, pyridine, sulfur trioxide pyridine complex, sodium acetate, tetraethylammonium nitrate, tetrabutylammonium nitrate and all chiral analytes tested were purchased from Sigma-Aldrich (Milwaukee, WI, USA). HPLC-grade

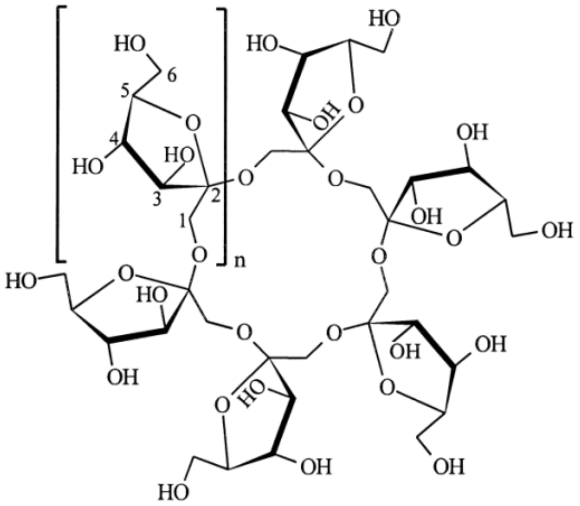


Figure 5.1 Structure of cyclofructan (CF). n=1, CF6; n=2, CF7; n=3, CF8. Reprint from Immel et al. [23]

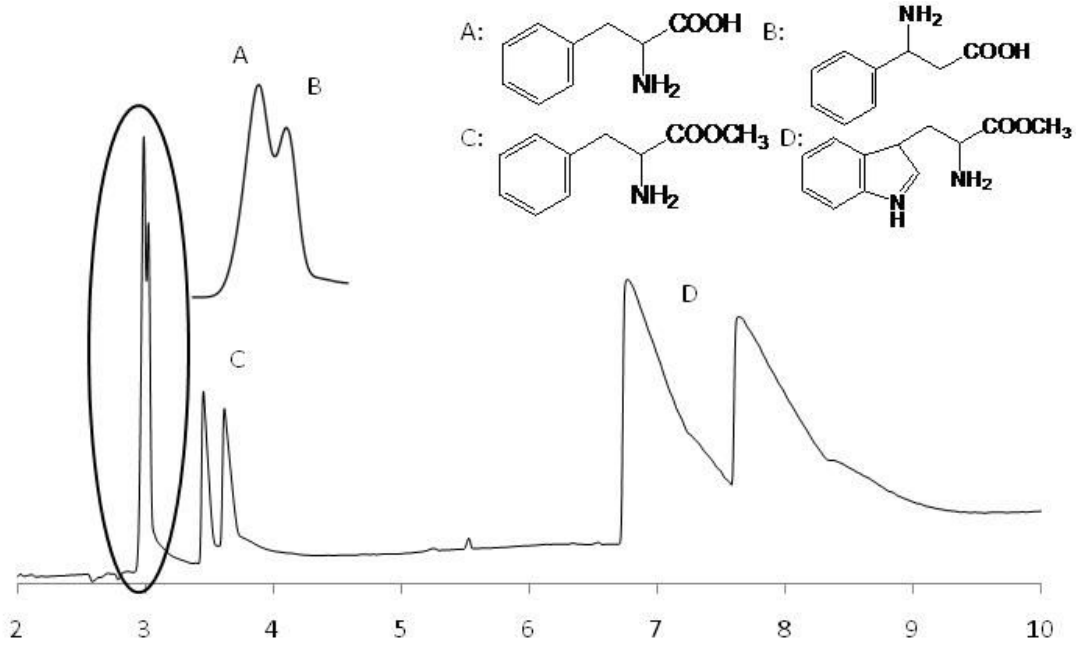


Figure 5.2 Electromigration order of 4 compounds with similar structures. Electropherogram was obtained at 214 nm with 5 mM sulfated cyclofructan 6 (SCF6). Conditions: +25 kV, 30/40 cm 50 µm I.D capillary, 4 mM ammonium acetate, 5%MeOH, pH=4.1.

methanol, phosphoric acid, glacial acetic acid, and sodium hydroxide were purchased from VWR (Bridgeport, NJ, USA). Ammonium acetate was purchased from Fisher Scientific (St. Louis, MO, USA). The fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA) and Beckman Coulter (Fullerton, CA, USA).

5.2.2 Methods

All separations were performed on a Beckman Coulter P/ACE MDQ system capillary electrophoresis system equipped with a photodiode array detector. The capillary (50 μm i.d. ×358 μm o.d.) was used with a total length of 40 cm (30 cm from inlet to detection window). All the electropherograms were obtained with detection at 214 nm and sample identity was confirmed by UV spectrum. Ammonium acetate was dissolved in deionized water and adjusted to desired pH with glacial acetic acid or phosphoric acid and used as the background buffer in normal polarity mode. Phosphoric acid was dissolved in deionized water and adjusted to desired pH with hydrochloric acid to be used as the background buffer in reverse polarity mode. Organic modifiers were added, based on volume percentage, prior to the addition of chiral selectors. Chiral selectors were then added to the background buffer solution to make run buffer. Due to the hydrolysis of the sulfate group on sulfated cyclofructans, fresh run buffer was made every 4 to 6 hours. Racemic samples or artificial mixtures of enantiomers were dissolved in the corresponding background buffer or water to make sample solutions.

When a new capillary was installed, it was rinsed with 1 M sodium hydroxide solution for 5 min, and then deionized water for 5 min for capillary conditioning. Between each run, the capillary was rinsed with 1 M hydrochloric acid solution for 1 min, deionized water for 1 min, 1 M sodium hydroxide solution for 1 min, deionized water for 1 min and then run buffer for 2 min. Subsequently, the sample solution was injected hydrodynamically at 0.5 psi for 3 seconds. All the compounds were first tested and the separation conditions were optimized in the normal polarity mode. Subsequently reverse polarity with low pH buffers was used to minimize the wall

interaction of cationic analytes. The electromigration order was determined by spiking with a pure enantiomer.

The parameters were calculated as follows: Resolution (Rs): $Rs=2(t_{m2}-t_{m1})/(w_1+w_2)$, the apparent mobility (μ_{app}): $\mu_{app}=L*L_{total}/(t_mV)$, mobility difference ($\Delta\mu$): $\Delta\mu=\mu_{app1}-\mu_{app2}$, electroosmotic mobility (μ_{eof}): $\mu_{eof}= L*L_{total}*/(t_{eof}V)$, electrophoretic mobility (μ): $\mu=L*L_{total}*(1/t_m-1/t_{eof})/V$, selectivity ($\alpha$): $\alpha=\mu_{app1}/\mu_{app2}$, and the number of theoretical plates (N): $N=16*(t_m/w)^2$, where t_{m1} and t_{m2} are the migration times of the first and second peak, t_{eof} is the migration time of the EOF marker, and w is the baseline peak width. L represents the length of the capillary from the injection end to the detection window and L_{total} is the total length of capillary. When the separation showed severe tailing, it was difficult to measure w, therefore, Rs was estimated by comparing to computer generated chromatograms.[205] The resolution (Rs) can also be expressed as: $Rs=\Delta\mu*N^{1/2}/(4\mu_{app,avg})$ [206]. As the selectivity term $\Delta\mu/\mu_{app,avg}=2(\mu_{app2}-\mu_{app1})/(\mu_{app1}+\mu_{app2})=2(\alpha-1)/(\alpha+1)$, $Rs=2(\alpha-1)/(\alpha+1)*N^{1/2}$.

5.2.3 Sulfation of cyclofructans

The procedure for the sulfation of CF6 and CF7 was developed by following a previously reported procedure for the sulfation of cyclodextrins [207]. Sodium sulfated cycloinulohexaose (SCF6) and sodium sulfated cycloinuloheptaose (SCF7) were synthesized in an analogous manner. Specifically, sulfur trioxide pyridine complex (6.82 g, 0.043 mols) was dissolved in anhydrous pyridine (12-15 mL) and heated to 80-85°C for 20 min. Next, the native cyclofructan (1.135 g) was added and the mixture was stirred and heated at 80-85°C for 6 hrs. After this time, the reaction mixture was allowed to cool to room temperature. The mixture was then processed with methanol (10 x 100mL); decanting in between washings. One more extraction was performed by allowing the semi-solid product to be stirred in methanol (100 mL) overnight. After decanting the final washing, the brownish semi-solid product was dissolved in 30% sodium acetate (6.5 mL, 0.024 mols). Then, deionized water (7 mL) was added and the solution was stirred at room temperature for 2 hrs. Next, the solution was slowly added to

methanol (100 mL) and stirred for 1 hr., resulting in the precipitation of the product. Suction filtration, washing with ethanol, and drying yielded the pure sulfated cyclofructans in the sodium salt form. The product composition was examined with ESI-MS. The mass spectrum showed that SCF6 is a mixture containing 11-15 sulfate groups and SCF7 is a mixture containing 16-20 sulfate groups.

5.3 Results and discussion

5.3.1 Binding mechanism

Cyclofructans were reported to form complexes with certain metal ions [186, 193, 195], in an analogous manner to crown ethers. It is known that changes in the electrophoretic mobility of an analyte at various chiral selector concentrations can be used to estimate the binding constant between the analyte and chiral selector [208-211]. Since SCFs are highly negatively charged species, analytes will show a lower mobility toward the anode when they associate with SCFs. Among the tested neutral compounds containing no nitrogen, all except catechin showed no binding to SCFs, since all of them coelute with the EOF marker in the normal polarity mode. Other nitrogen containing compounds, including amino acid amides/esters, amino acids and other amine-containning compounds, showed lower mobilities toward the anode in the presence of SCFs. Clearly, electrostatic interactions play an important role in the binding of analytes to SCFs. Generally, cationic analytes, including amines, and amino acid esters, bind more strongly than neutral analytes, such as amides and amino acids (which are zwitterions at the buffer pH used). Analytes with multiple positive charges bind more strongly than singly charged analytes. Figure 5.2 shows the electropherograms obtained for selected compounds with similar structures. The electromigration order was obtained by spiking compounds with known structures. It was interesting that 3-amino-3-phenylpropionic acid, which is a β-amino acid, showed significantly stronger binding to SCF than its corresponding α-amino acid, phenylalanine.

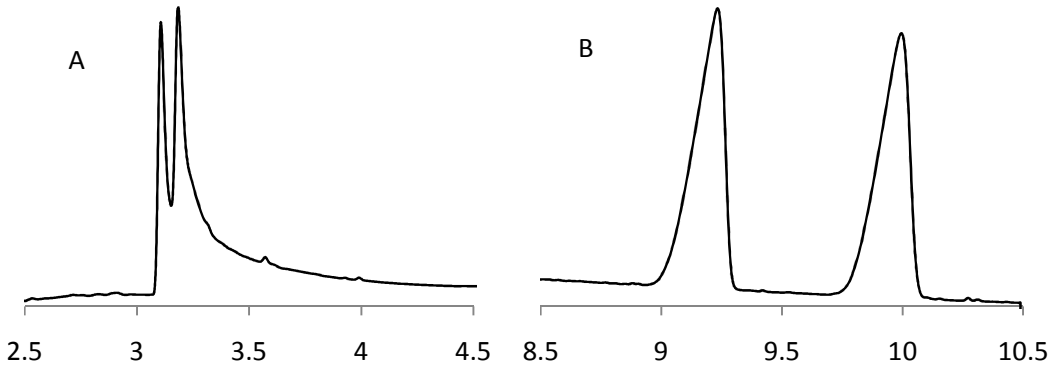


Figure 5.3 Comparison of normal polarity and reverse polarity. Conditions: tyrosine methyl ester, sulfated cyclofructan 6 (SCF6) 15 mM. A: 4 mM ammonium acetate, adjust with 1 M HCl to pH 4.1, +25 kV, 30 cm/40 cm capillary with 50 μm i.d.; B: 4 mM phosphate, 5%MeOH, pH=2.0, -16 kV, 20 cm/30 cm capillary with 50 μm i.d.

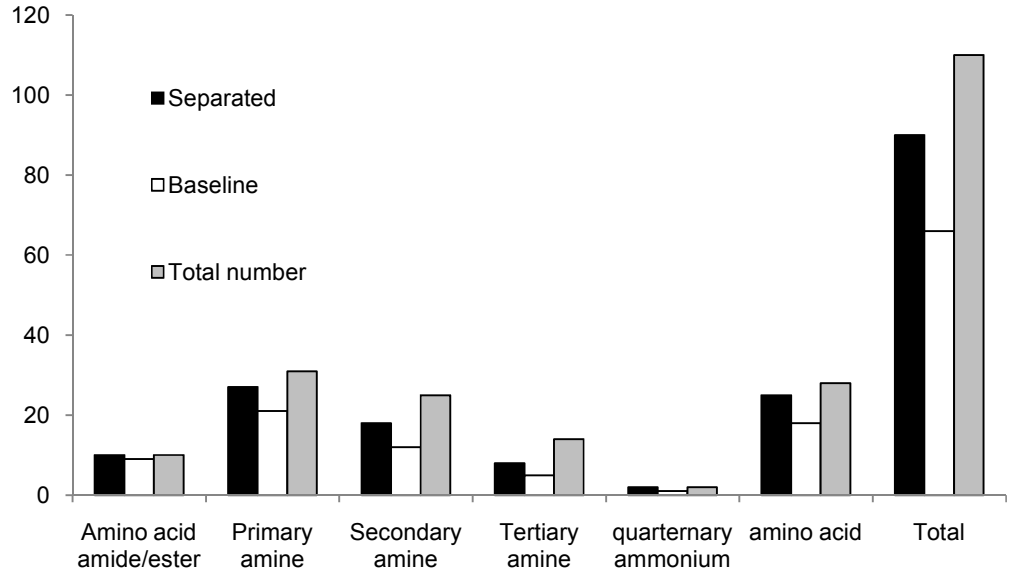
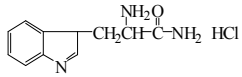
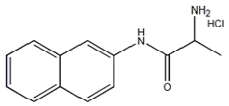
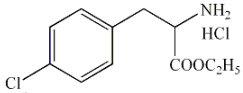
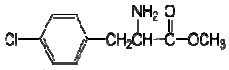
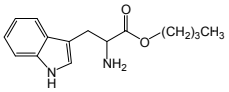
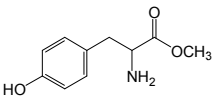
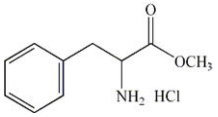


Figure 5.4 Summary of separation of amines and amino acids with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) in both normal and reverse polarity mode.

Table 5.1 Experimental data for enantiomeric separations of amino acid amides and amino acid esters with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) [a)]

#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
1	 Tryptophanamide	8.700	1.18	1.27	1.6	1100	-	SCF6 10 mM Buffer 2
		6.262	0.85	1.12	1.9	4200	-	SCF7 10 mM Buffer 2
2	 DL-alanine-β-naphthylamide hydrochloride	12.354	0.08	1.02	1.5	-	-	SCF6 15 mM Buffer 2
		6.688	0.33	1.05	2.6	42000	-	SCF7 10 mM Buffer 2
3	 DL-4-Chlorophenylalanine ethyl ester hydrochloride	3.933	0.92	1.08	4.3	52000	-	SCF6 15 mM Buffer 2
		6.742	1.21	1.20	8.1	33000	-	SCF7 15 mM Buffer 2
4	 DL-4-Chlorophenylalanine methyl ester hydrochloride	4.037	0.67	1.06	1.3	-	-	SCF6 15 mM Buffer 2
		7.537	0.66	1.12	4.6	30000	-	SCF7 15 mM Buffer 2
5	 DL-Tryptophan butyl ester hydrochloride	5.142	1.66	1.22	5.7	16000	-	SCF6 15 mM Buffer 2
		6.667	1.46	1.25	3.4	3800	-	SCF7 15 mM Buffer 2
6	 Tyrosine methyl ester	3.104	0.38	1.03	0.9	-	-	SCF6 15 mM Buffer 2
		4.829	0.17	1.02	0.7	-	-	SCF7 15 mM Buffer 2
7	 DL-Phenylalanine methyl ester hydrochloride	4.379	0.61	1.06	1.5	-	L>D	SCF6 15 mM Buffer 2
		5.217	0.53	1.06	1.5	-	L>D	SCF7 15 mM Buffer 2

a) Unit for time (tm_1) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Conditions: 30/40 cm 50 μm I.D capillary; +25 kV; buffer 1: 5 mM ammonium acetate, pH=4.1; buffer 2: 4 mM ammonium acetate, 5% MeOH, pH=4.1; buffer 3: 4 mM ammonium acetate, 5% MeOH, pH=3.7; buffer 4: mixture of 20 mM ammonium acetate and 10 mM phosphoric acid, pH=4.7. Buffer 1,2,3 were used with capillary batch 2, buffer 4 was used with capillary batch 1.

Table 5.1 - *Continued*

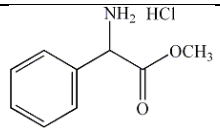
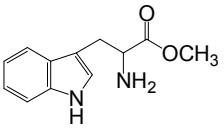
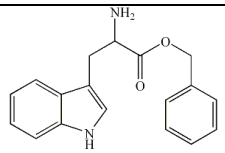
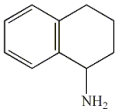
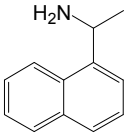
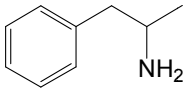
#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
8	 (±)-2-Phenylglycine methyl ester hydrochloride	3.604	0.49	1.04	2.2	59000	L>D	SCF6 15 mM Buffer 2
		4.404	2.29	1.27	7.9	48000	L>D	SCF7 15 mM Buffer 2
9	 DL-Tryptophan methyl ester	8.275	0.59	1.11	1.1	-	L>D	SCF6 15 mM Buffer 2
		10.392	0.32	1.07	1.1	-	L>D	SCF7 15 mM Buffer 2
10	 DL-Tryptophan benzyl ester	6.979	1.75	1.34	4.0	2200	-	SCF6 10 mM Buffer 2
		4.092	1.56	1.15	2.7	6500	-	SCF7 10 mM Buffer 2

Table 5.2 Experimental data for enantiomeric separations of primary amines with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) [a)]

#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
11	 1,2,3,4-Tetrahydro-1-naphthylamine	4.625	0.10	1.01	0.4	-	-	SCF6 15 mM Buffer 2
		5.329	0.40	1.05	1.4	-	-	SCF7 15 mM Buffer 2
12	 1-(1-Naphthyl)ethylamine	7.263	1.63	1.33	3.4	3400	-	SCF6 5 mM Buffer 4
		5.400	1.49	1.20	4.2	9700	-	SCF7 5 mM Buffer 4
13	 DL-Amphetamine sulfate salt	3.413	0.40	1.03	1.0	-	-	SCF6 15 mM Buffer 2
		5.217	0.25	1.03	0.8	-	-	SCF7 15 mM Buffer 1

a) Unit for time (t_{m1}) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Other conditions are same as Table 1

Table 5.2 - *Continued*

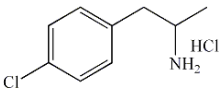
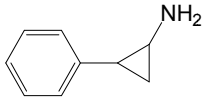
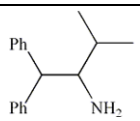
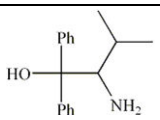
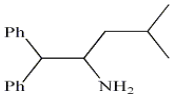
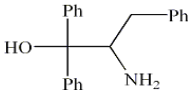
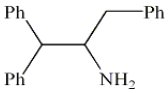
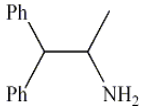
#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
14	 DL-p-Chloroamphetamine HCl	3.096	0.49	1.03	2.1	68000	-	SCF6 15 mM Buffer 2
		6.500	0.25	1.04	1.5	-	-	SCF7 15 mM Buffer 1
15	 trans-2-Phenylcyclopropylamine	4.475	0.84	1.08	1.4	-	-	SCF6 15 mM Buffer 2
		10.254	1.20	1.26	1.5	-	-	SCF7 15 mM Buffer 2
16	 (±)-2-Amino-3-methyl-1,1-diphenylbutane	4.117	0.17	1.02	0.9	-	-	SCF6 15 mM Buffer 2
		3.604	-	-	-	-	-	SCF7 15 mM Buffer 2
17	 (±)-2-Amino-3-methyl-1,1-diphenyl-1-butanol	4.529	0.37	1.04	0.9	-	R>S	SCF6 15 mM Buffer 2
		3.254	0.19	1.01	0.9	-	R>S	SCF7 15 mM Buffer 1
18	 (±)-2-Amino-4-methyl-1,1-diphenylpentane	4.483	0.38	1.04	2.2	58000	R>S	SCF6 15 mM Buffer 2
		3.608	0.70	1.06	1.5	-	R>S	SCF7 15 mM Buffer 2
19	 (±)-2-Amino-1,1,3-triphenyl-1-propanol	4.904	1.37	1.16	9.4	62000	R>S	SCF6 15 mM Buffer 2
		3.879	2.38	1.24	15.4	85000	R>S	SCF7 15 mM Buffer 1
20	 (±)-1-Benzyl-2,2-diphenylethylamine	5.746	0.14	1.02	1.1	-	R>S	SCF6 15 mM Buffer 2
		7.558	0.88	1.16	3.4	12000	R>S	SCF7 15 mM Buffer 1
21	 (±)-1,1-Diphenyl-2-aminopropane	4.579	0.25	1.02	1.1	-	R>S	SCF6 15 mM Buffer 2
		3.850	0.24	1.02	1.3	-	R>S	SCF7 15 mM Buffer 2

Table 5.2 - *Continued*

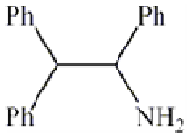
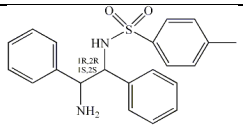
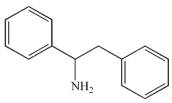
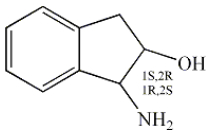
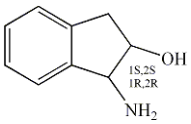
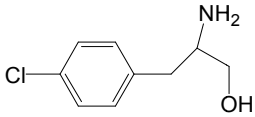
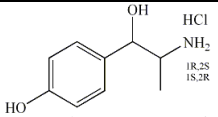
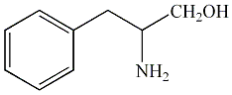
#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
22	 (±)-1,2,2-Triphenylethylamine	4.204	0.83	1.08	2.9	23000	R>S	SCF6 15 mM Buffer 2
		3.438	0.83	1.06	3.3	49000	R>S	SCF7 15 mM Buffer 2
23	 (±)-N-p-Tosyl-1,2-diphenylethylenediamine	3.038	-	-	-	-	-	SCF6 15 mM Buffer 2
		2.783	1.27	1.08	0.9	-	-	SCF7 15 mM Buffer 2
24	 1,2-Diphenylethylamine	3.450	1.25	1.10	6.0	79000	-	SCF6 15 mM Buffer 2
		3.850	1.10	1.10	3.0	27000	-	SCF7 15 mM Buffer 2
25	 (±) cis-1-Amino-2-indanol	4.546	-	-	-	-	-	SCF6 15 mM Buffer 2
		6.400	0.82	1.12	1.5	-	-	SCF7 15 mM Buffer 1
26	 (±) trans-1-Amino-2-indanol	3.330	0.03	1.00	0.6	-	-	SCF6 15 mM Buffer 2
		4.771	0.21	1.02	1.0	-	-	SCF7 15 mM Buffer 1
27	 DL-4-Chlorophenyl alaninol	6.554	0.58	1.09	4.9	57000	-	SCF6 15 mM Buffer 4
		8.525	0.47	1.09	5.2	56000	-	SCF7 15 mM Buffer 4
28	 (±)-alpha-(1-Aminoethyl)-4-hydroxybenzyl alcohol HCl	6.792	0.18	1.03	0.8	-	-	SCF6 18 mM Buffer 3
		4.258	-	-	-	-	-	SCF7 15 mM Buffer 1
29	 2-Amino-3-phenyl-1-propanol	5.092	0.52	1.06	2.4	29000	S>R	SCF6 15 mM Buffer 4
		6.558	0.49	1.07	1.8	10000	S>R	SCF7 15 mM Buffer 4

Table 5.2 - *Continued*

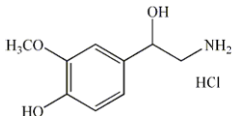
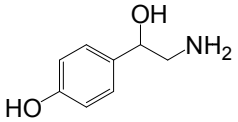
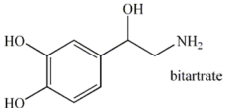
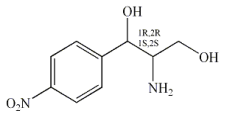
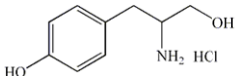
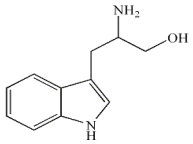
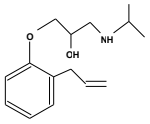
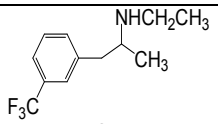
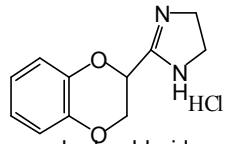
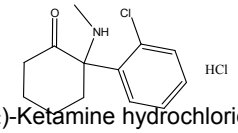
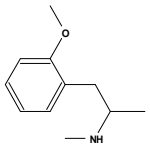
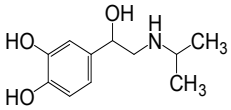
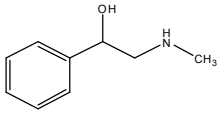
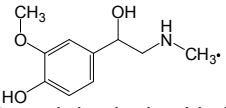
#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
30	 DL-Normetanephrine HCl	5.883	0.19	1.02	0.6	-	-	SCF6 18 mM Buffer 3
		5.263	0.21	1.02	1.1	-	-	SCF7 15 mM Buffer 2
31	 Norphenylephrine HCl	3.817	0.15	1.01	0.5	-	-	SCF6 15 mM Buffer 2
		4.892	0.17	1.02	0.7	-	-	SCF7 15 mM Buffer 2
32	 (+/-)-Norepinephrine L-bitartrate hydrate	7.213	0.13	1.02	0.6	-	-	SCF6 18 mM Buffer 3
		5.875	0.21	1.03	1.2	-	-	SCF7 15 mM Buffer 1
33	 (±)-2-Amino-1-(4-nitrophenyl)-1,3-propanediol	8.208	0.38	1.07	3.7	52000	S,S> R,R	SCF6 15 mM Buffer 4
		6.171	0.44	1.06	1.5	-	S,S> R,R	SCF7 15 mM Buffer 2
34	 DL-Tyrosinol hydrochloride	5.888	0.43	1.06	2.5	33000	L>D	SCF6 15 mM Buffer 4
		4.638	0.33	1.03	1.1	-	L>D	SCF7 15 mM Buffer 2
35	 DL-Tryptophanol	7.021	0.98	1.17	1.5	3400	L>D	SCF6 15 mM Buffer 2
		11.963	1.23	1.44	4.4	18000	L>D	SCF7 15 mM Buffer 2

Table 5.3 Experimental data for enantiomeric separations of secondary amines with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7)[a)]

#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
36	 Alprenolol	9.625	0.16	1.03	0.8	-	-	SCF6 15 mM Buffer 4
		4.517	0.37	1.04	1.1	-	-	SCF7 15 mM Buffer 1
37	 (±)-Fenfluramine hydrochloride	6.333	0.13	1.02	0.6	-	-	SCF6 15 mM Buffer 2
		5.146	0.44	1.05	2.2	33000	-	SCF7 15 mM Buffer 2
38	 Idazoxan hydrochloride	6.104	0.86	1.12	3.0	16000	-	SCF6 15 mM Buffer 2
		7.025	-	-	-	-	-	SCF7 15 mM Buffer 2
39	 (±)-Ketamine hydrochloride	5.358	0.26	1.03	0.8	-	-	SCF6 15 mM Buffer 2
		4.317	0.51	1.05	1.4	-	-	SCF7 15 mM Buffer 1
40	 Methoxyphenamine	4.848	-	-	-	-	-	SCF6 15 mM Buffer 2
		5.296	0.28	1.03	1.1	-	-	SCF7 15 mM Buffer 2
41	 DL-Isoprenaline hydrochloride	4.750	0.12	1.01	0.7	-	-	SCF6 15 mM Buffer 2
		3.625	0.14	1.01	0.6	-	-	SCF7 15 mM Buffer 2
42	 alpha-(Methylaminomethyl) benzyl alcohol	4.338	-	-	-	-	-	SCF6 15 mM Buffer 2
		5.229	0.13	1.01	0.8	-	-	SCF7 15 mM Buffer 2
43	 Metanephrine hydrochloride	5.667	0.38	1.04	0.9	-		SCF6 15 mM Buffer 2
		3.604	0.23	1.02	0.9	-		SCF7 15 mM Buffer 2

a) Unit for time (t_{m1}) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Other conditions are same as Table 1.

Table 5.3 - *Continued*

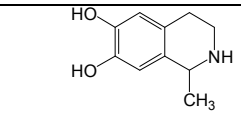
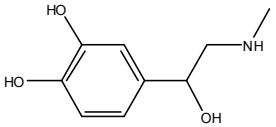
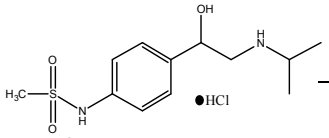
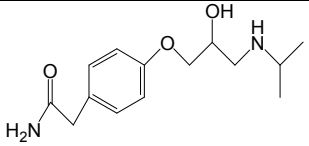
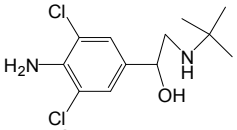
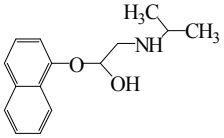
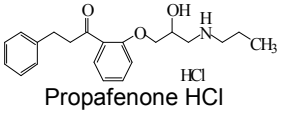
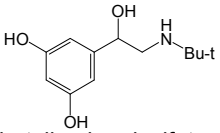
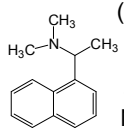
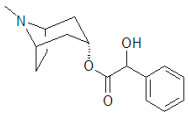
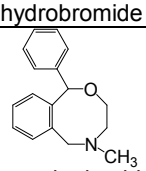
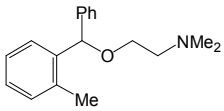
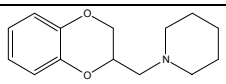
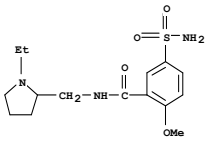
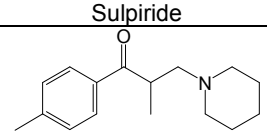
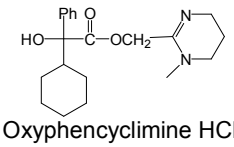
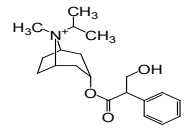
#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
44	 1-Methyl-6,7-dihydroxy-1,2,3,4 - tetrahydroisoquinoline HCl	4.783	0.95	1.10	1.4	-	-	SCF6 15 mM Buffer 2
		4.217	0.19	1.02	1.1	-	-	SCF7 15 mM Buffer 2
45	 Epinephrine	6.350	0.14	1.02	0.6	-	-	SCF6 15 mM Buffer 2
		6.550	0.12	1.02	1.0	-	-	SCF7 15 mM Buffer 2
46	 (±)-Sotalol hydrochloride	8.721	0.18	1.03	0.7	-	-	SCF6 15 mM Buffer 2
		9.271	0.06	1.01	0.4	-	-	SCF7 15 mM Buffer 2
47	 Atenolol	7.646	0.10	1.02	0.5	-	-	SCF6 18 mM Buffer 3
		6.792	0.23	1.03	0.9	-	-	SCF7 15 mM Buffer 1
48	 Clenbuterol	6.092	-	-	0.3	-	-	SCF6 15 mM Buffer 2
		4.787	0.41	1.04	1.8	-	-	SCF7 18 mM Buffer 3
49	 DL-Propranolol hydroxide	8.154	0.53	1.10	2.0	19000	-	SCF6 15 mM Buffer 2
		6.592	0.63	1.10	2.5	12000	-	SCF7 15 mM Buffer 2
50	 Propafenone HCl	3.163	0.29	1.02	0.6	-	-	SCF6 15 mM Buffer 2
		5.067	0.51	1.06	1.8	19000	-	SCF7 15 mM Buffer 1
51	 Terbutaline hemisulfate salt	2.592	-	-	-	-	-	SCF6 15 mM Buffer 2
		3.533	0.13	1.01	0.5	-	-	SCF7 15 mM Buffer 2

Table 5.4 Data for enantiomeric separations of tertiary amines with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) [a)]

#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
52	 (R)-(+)-N,N-Dimeth-yl-1-(1-naphthyl) ethylamine (S)-(−)-N,N-Dimethyl-1-(1-naphthyl) ethylamine	5.633	1.65	1.24	1.7	1600	-	SCF6 10 mM Buffer 2
		3.496	1.71	1.14	1.6	2500	-	SCF7 10 mM Buffer 2
53	 DL-Homatropine hydrobromide	6.300	0.58	1.08	0.5	-	-	SCF6 15 mM Buffer 2
		7.233	0.28	1.04	0.8	-	-	SCF7 15 mM Buffer 2
54	 Nefopam hydrochloride	4.938	0.73	1.08	3.2	28000	-	SCF6 15 mM Buffer 2
		7.371	0.76	1.13	2.2	6600	-	SCF7 15 mM Buffer 2
55	 Orphenadrine citrate salt	3.837	0.24	1.02	1.2	-	-	SCF6 15 mM Buffer 2
		3.733	0.35	1.03	1.2	-	-	SCF7 15 mM Buffer 2
56	 dl-Piperoxan 2-(N-Piperidinomethyl)-1,4-benzodioxane	10.371	0.32	1.07	3.1	32000	-	SCF6 15 mM Buffer 4
		8.950	0.37	1.07	3.7	44000	-	SCF7 15 mM Buffer 4
57	 Sulpiride	7.375	0.14	1.02	1.1	-	-	SCF6 15 mM Buffer 2
		3.671	0.32	1.02	0.7	-	-	SCF7 15 mM Buffer 2
58	 Tolperisone hydrochloride	2.521	-	-	-	-	-	SCF6 15 mM Buffer 2
		6.263	-	-	0.4	-	-	SCF7 15 mM Buffer 2
59	 Oxyphencyclimine HCl	2.929	1.36	1.08	4.1	43000	-	SCF6 15 mM Buffer 2
		5.342	1.12	1.12	5.1	31000	-	SCF7 15 mM Buffer 1

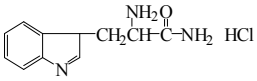
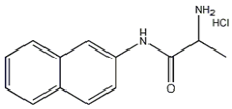
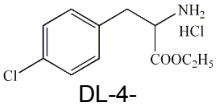
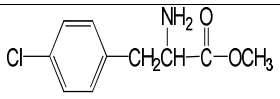
a) Unit for time (t_{m1}) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Other conditions are the same as Table 1

Table 5.5 Data for enantiomeric separations of quantiary amine with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) [a)]

#	Compound	tm_1	Δμ	α	Rs	N	EMO	Condition
60 & 61	 33%Ipratropium bromide monohydrate 67%8-S isomer	6.963	0.19	1.03	1.1	-	-	SCF6 15 mM Buffer 2
		7.713	0.15	1.02	1.1	-	-	
		5.388	0.32	1.04	2.3	68000	-	SCF7 15 mM Buffer 2
		5.779	0.24	1.03	2.2	94000	-	

a) Unit for time (t_{m1}) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Other conditions are the same as Table 1

Table 5.6 Experimental data for enantiomeric separations of amines with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) in the reverse polarity mode [a)]

#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
1	 Tryptophanamide	-18	20	2.150	1.06	1.07	1.6	8100	SCF6 10 mM Buffer 6
		-18	20	1.892	0.81	1.05	0.8	4200	SCF7 10 mM Buffer 6
2	 DL-alanine-β-naphthylamide HCl	-18	20	2.875	0.13	1.01	0.6	40000	SCF6 10 mM Buffer 6
		-18	20	2.467	0.99	1.08	4.3	50000	SCF7 10 mM Buffer 6
3	 DL-4-Chlorophenylalanine ethyl ester HCl	-25	20	3.904	0.96	1.19	7.4	31000	SCF6 10 mM Buffer 7
		-18	20	3.842	0.91	1.12	7.1	68000	SCF7 10 mM Buffer 6
4	 DL-4-Chlorophenylalanine methyl ester hydrochloride	-18	20	3.929	0.92	1.12	5.3	34000	SCF6 10 mM Buffer 6
		-18	20	3.625	0.49	1.06	3.0	47000	SCF7 10 mM Buffer 6

a) Any compound that didn't show peaks or enantioseparation is not listed. Unit for applied voltage (V) is kV, capillary length to detection window (L) is cm, total length Ltot=L+10 cm, time (t_{m1}) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Conditions: 50 μm I.D capillary; buffer 5, 4 mM phosphate with 5%MeOH, adjust with 1 M HCl, pH=1.96; buffer 6: 5 mM phosphoric acid, pH=2.45;buffer 7: 4 mM phosphoric acid, 5%MeOH, pH=2.45

Table 5.6 - *Continued*

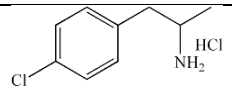
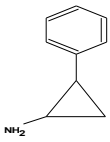
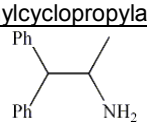
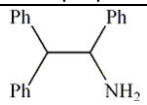
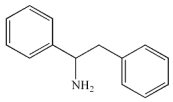
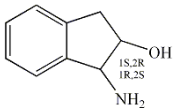
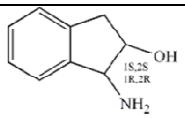
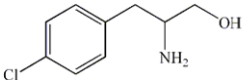
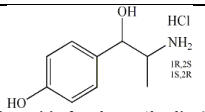
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
14	 DL-p-Chloroamphetamine HCl	-20	20	3.129	1.29	1.16	3.8	12000	SCF6 10 mM Buffer 6
		-18	20	3.658	0.20	1.02	1.4	65000	SCF7 10 mM Buffer 6
15	 trans-2-Phenylcyclopropylamine	-20	20	4.65	0.34	1.06	2.6	38000	SCF6 10 mM Buffer 6
		-18	20	3.754	1.15	1.15	3.5	10000	SCF7 10 mM Buffer 6
21	 (±)-1,1-Diphenyl-2-aminopropane	-18	20	6.283	0.26	1.05	1.3	10000	SCF6 10 mM Buffer 6
		-18	20	5.596	0.25	1.04	2.3	45000	SCF7 10 mM Buffer 6
22	 (±)-1,2,2-Triphenylethylamine	-18	20	6.583	0.90	1.22	9.0	34000	SCF6 10 mM Buffer 6
		-18	20	5.958	0.91	1.19	5.3	22000	SCF7 10 mM Buffer 6
24	 1,2-Diphenylethylamine	-18	20	4.146	1.46	1.22	8.9	35000	SCF6 10 mM Buffer 6
		-18	20	3.846	1.21	1.16	7.3	38000	SCF7 10 mM Buffer 6
25	 (±) cis-1-Amino-2-indanol	-18	20	4.592	0.18	1.03	0.7	13000	SCF6 10 mM Buffer 6
		-12	20	9.033	0.44	1.09	1.6	8100	SCF7 20 mM Buffer 5
26	 (±) trans-1-Amino-2-indanol	-18	20	6.129	0.091	1.02	0.7	33000	SCF6 10 mM Buffer 6
		-12	20	14.329	0.16	1.05	1.8	24000	SCF7 20 mM Buffer 5
27	 DL-P-Chlorophenylalaninol	-25	20	3.954	0.47	1.08	2.5	16000	SCF6 10 mM Buffer 7
		-18	20	3.821	0.35	1.04	2.8	71000	SCF7 10 mM Buffer 6
28	 (±)-α-(1-Aminoethyl)-4-hydroxybenzyl alcohol	-25	20	9.554	0.07	1.03	0.9	21000	SCF6 10 mM Buffer 7
		-12	20	20.104	0.005	1.00	0.6	17000	SCF7 20 mM Buffer 5

Table 5.6 - *Continued*

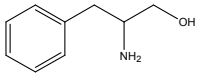
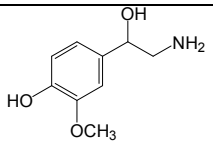
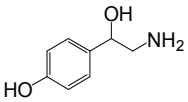
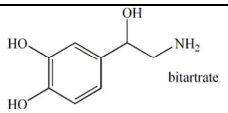
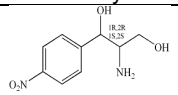
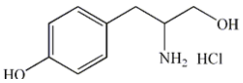
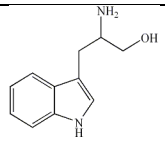
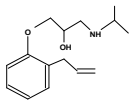
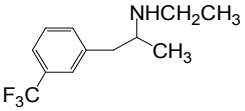
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
29	 2-Amino-3-phenyl-1-propanol	-18	20	4.888	0.68	1.12	2.1	6600	SCF6 10 mM Buffer 6
		-12	20	7.500	0.93	1.16	2.1	3700	SCF7 10 mM Buffer 6
30	 DL-Normetanephrine	-25	20	3.675	0.19	1.03	1.1	23000	SCF6 10 mM Buffer 7
		-12	20	9.146	0.16	1.03	1.7	45000	SCF7 20 mM Buffer 5
31	 Norphenylephrine HCl	-18	20	5.696	0.17	1.03	1.1	24000	SCF6 10 mM Buffer 6
		-18	20	6.354	0.19	1.04	1.2	19000	SCF7 10 mM Buffer 6
32	 (+/-)-Norepinephrine L-bitartrate hydrate	-18	20	5.737	0.16	1.03	0.9	15000	SCF6 10 mM Buffer 6
		-18	20	5.438	0.15	1.03	1.5	58000	SCF7 10 mM Buffer 6
33	 (±)-2-Amino-1-(4-nitrophenyl)-1,3-propanediol	-18	20	4.563	0.26	1.04	2.1	54000	SCF6 10 mM Buffer 6
		-18	20	4.208	0.41	1.05	3.2	60000	SCF7 10 mM Buffer 6
34	 DL-Tyrosinol HCl	-18	20	12.967	0.083	1.03	1.9	55000	SCF6 10 mM Buffer 6
		-18	20	5.162	0.28	1.05	1.8	25000	SCF7 10 mM Buffer 6
35	 DL-Tryptophanol	-18	20	3.563	1.28	1.16	1.7	2200	SCF6 10 mM Buffer 6
		-18	20	2.275	0.88	1.06	0.9	3400	SCF7 10 mM Buffer 6
36	 Alprenolol	-18	20	7.329	0.22	1.05	0.8	4700	SCF6 10 mM Buffer 6
		-12	20	20.721	0.24	1.11	1.7	4200	SCF7 20 mM Buffer 5
37	 (±)-Fenfluramine HCl	-18	20	4.892	-	-	-	17000	SCF6 10 mM Buffer 6
		-18	20	4.371	0.49	1.07	4.2	81000	SCF7 10 mM Buffer 6

Table 5.6-*Continued*

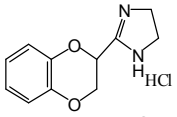
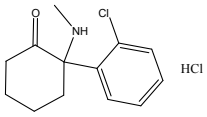
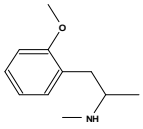
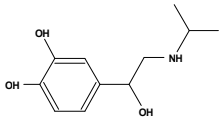
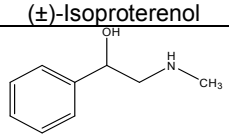
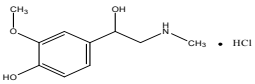
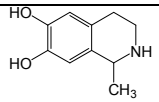
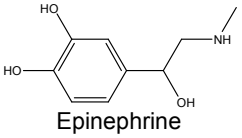
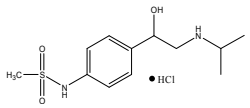
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
38	 Idazoxan HCl	-18	20	3.529	0.83	1.10	4.3	39000	SCF6 10 mM Buffer 6
		-17	20	3.358	-	-	-	38000	SCF7 10 mM Buffer 6
39	 (±)-Ketamine HCl	-18	20	6.671	0.92	1.23	7.8	27000	SCF6 10 mM Buffer 6
		-18	20	5.987	0.26	1.05	1.3	13000	SCF7 10 mM Buffer 6
40	 Methoxyphenamine	-18	20	5.146	0.18	1.03	1.1	25000	SCF6 10 mM Buffer 6
		-12	20	11.979	0.25	1.06	2.2	20000	SCF7 20 mM Buffer 5
41	 (±)-Isoproterenol	-18	20	8.283	0.18	1.05	1.3	19000	SCF6 10 mM Buffer 6
		-18	20	9.838	0.16	1.05	1.4	13000	SCF7 10 mM Buffer 6
42	 α-(Methylaminomethyl) benzyl alcohol	-18	20	5.638	0.20	1.04	1.5	30000	SCF6 10 mM Buffer 6
		-18	20	4.904	0.15	1.02	1.0	32000	SCF7 10 mM Buffer 6
43	 Metanephrine HCl	-25	20	4.404	0.26	1.05	1.6	17000	SCF6 10 mM Buffer 7
		-18	20	4.279	0.28	1.04	1.4	26000	SCF7 10 mM Buffer 6
44	 1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	-18	20	4.213	0.89	1.13	4.1	20000	SCF6 10 mM Buffer 6
		-18	20	4.779	0.69	1.11	3.7	22000	SCF7 10 mM Buffer 6
45	 Epinephrine	-18	20	7.879	0.14	1.04	1.3	22000	SCF6 10 mM Buffer 5
		-18	20	7.912	0.14	1.04	0.8	8500	SCF7 10 mM Buffer 6
46	 (±)-Sotalol HCl	-25	20	5.246	0.071	1.02	0.5	18000	SCF6 10 mM Buffer 7
		-12	20	10.517	-	-	-	22000	SCF7 20 mM Buffer 5

Table 5.6 - *Continued*

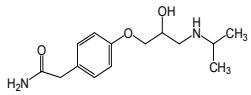
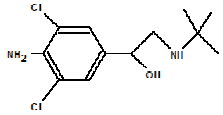
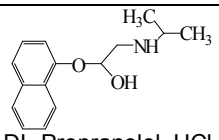
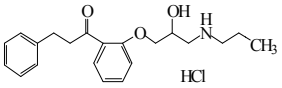
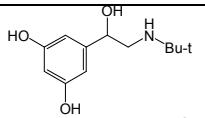
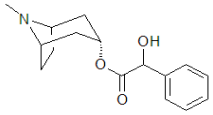
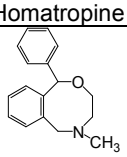
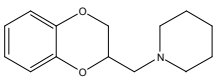
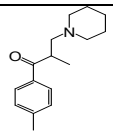
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
47	 Atenolol	-18	20	5.200	0.096	1.02	0.6	32000	SCF6 10 mM Buffer 6
		-18	20	4.825	0.11	1.02	0.9	52000	SCF7 10 mM Buffer 6
48	 Clenbuterol	-18	20	6.287	0.17	1.03	1.4	28000	SCF6 10 mM Buffer 6
		-18	20	5.796	0.36	1.07	3.2	39000	SCF7 10 mM Buffer 6
49	 DL-Propranolol HCl	-20	20	2.95	1.27	1.14	2.9	8100	SCF6 10 mM Buffer 6
		-18	20	3.029	0.66	1.06	2.4	26000	SCF7 10 mM Buffer 6
50	 Propafenone HCl	-18	20	5.129	0.36	1.06	3.0	44000	SCF6 10 mM Buffer 6
		-18	20	3.604	0.35	1.04	2.5	66000	SCF7 10 mM Buffer 6
51	 Terbutaline hemisulfate salt	-18	20	14.788	0.24	1.12	1.2	2000	SCF6 10 mM Buffer 6
		-18	20	13.017	0.18	1.08	1.2	4200	SCF7 10 mM Buffer 6
53	 DL-Homatropine HBr	-18	20	3.579	0.20	1.02	1.1	43000	SCF6 10 mM Buffer 6
		-18	20	3.567	0.17	1.02	0.9	47000	SCF7 10 mM Buffer 6
54	 Nefopam HCl	-18	20	4.075	0.60	1.08	1.8	9200	SCF6 10 mM Buffer 6
		-18	20	3.267	0.48	1.05	1.1	9000	SCF7 10 mM Buffer 6
56	 dl-Piperoxan	-25	20	3.925	0.32	1.05	1.0	7700	SCF6 10 mM Buffer 7
		-18	20	3.542	0.34	1.04	2.2	60000	SCF7 10 mM Buffer 6
58	 Tolperisone HCl	-18	20	4.888	0.22	1.03	1.0	15000	SCF6 10 mM Buffer 6
		-12	20	11.004	-	-	-	11000	SCF7 20 mM Buffer 5

Table 5.6 - *Continued*

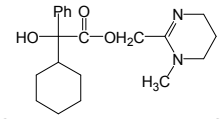
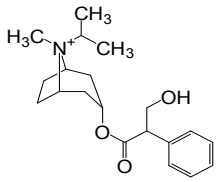
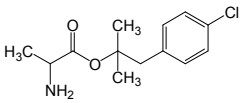
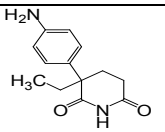
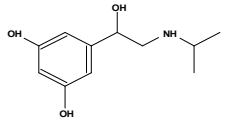
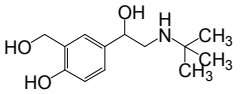
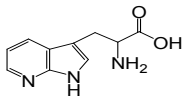
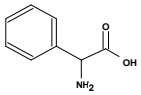
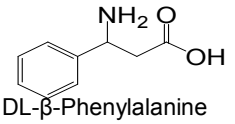
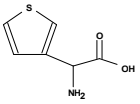
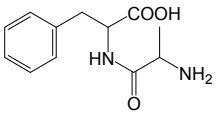
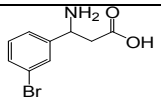
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
59	 Oxyphencyclimine HCl	-20	20	3.304	2.91	1.47	10.0	13000	SCF6 10 mM Buffer 6
		-18	20	4.4	1.34	1.21	7.9	30000	SCF7 10 mM Buffer 6
60 & 61	 Ipratropium bromide monohydrate	-18	20	5.342	0.11	1.02	0.8	33000	SCF6 10 mM Buffer 6
				5.833	0.15	1.03	1.2	34000	SCF6 10 mM Buffer 6
		-18	20	4.704	0.20	1.03	1.6	51000	SCF7 10 mM Buffer 6
				5.079	0.28	1.04	2.4	53000	SCF7 10 mM Buffer 6
62	 Alaproclate HCl	-25	20	9.375	0.11	1.05	0.5	3000	SCF6 10 mM Buffer 7
		-12	20	13.333	0.12	1.03	1.0	17000	SCF7 20 mM Buffer 5
63	 DL-Aminoglutethimide	-18	20	4.033	1.01	1.14	7.7	56000	SCF6 10 mM Buffer 6
		-17	30	10.996	0.23	1.04	2.6	79000	SCF7 20 mM Buffer 5
64	 Metaproterenol	-18	20	11.654	0.20	1.08	1.1	3500	SCF6 10 mM Buffer 6
		-18	20	9.408	0.26	1.08	1.9	10000	SCF7 10 mM Buffer 6
65	 Salbutamol hemisulfate salt	-18	20	11.917	-	-	-	8300	SCF6 10 mM Buffer 6
		-18	20	9.346	0.044	1.01	0.5	2100	SCF7 10 mM Buffer 6

Table 5.7 Experimental data for enantiomeric separations of amino acids with sulfated cyclofructan 6 (SCF6) and sulfated cyclofructan 7 (SCF7) in the reverse polarity mode [a)]

#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Condition
66	 DL-7-Azatryptophan hydrate	-16	20	1.825	-	-	-	16000	SCF6 15 mM Buffer 5
		-17	30	3.308	0.29	1.01	0.8	56000	SCF7 20 mM Buffer5
67	 DL-alpha-Aminophenyl-acetic acid	-16	20	20.488	0.10	1.06	1.3	8500	SCF6 15 mM Buffer5
		-12	20	22.813	0.39	1.21	5.7	16000	SCF7 20 mM Buffer 5
68	 DL-β-Phenylalanine	-16	20	9.692	0.22	1.06	2.5	29000	SCF6 15 mM Buffer 5
		-18	30	17.079	0.16	1.04	2.1	40000	SCF7 20 mM Buffer 5
69	 DL-alpha-Amino-3-thiopheneacetic acid	-16	20	14.088	0.096	1.04	1.4	24000	SCF6 15 mM Buffer 5
		-20	30	17.575	0.43	1.15	6.1	32000	SCF7 15 mM Buffer 5
70 & 71	 DL-Ala-DL-Phe	-16	20	12.183	0.072	1.02	1.0	28000	SCF6 15 mM Buffer 5
				15.054	0.17	1.07	3.0	29000	
		-18	20	7.029	0.050	1.01	0.4	24000	SCF7 10 mM Buffer 6
				8.325	0.082	1.02	1.0	36000	
72	 3-Amino-3-(3-bromophenyl)propionic acid	-16	20	7.654	0.16	1.03	1.5	35000	SCF6 15 mM Buffer 5
		-17	30	12.596	0.23	1.04	2.6	63000	SCF7 20 mM Buffer 5

a) Any amino acid that didn't show peaks or enantioseparation is not listed. Unit for applied voltage (V) is kV, capillary length to detection window (L) is cm, total length L_{tot}=L+10 cm, time (tm_1) is min, mobility (Δμ) is $cm^2kV^{-1}min^{-1}$. Conditions: 50 μm I.D capillary; buffer 5, 4 mM phosphate with 5%MeOH, adjust with 1 M HCl, pH=1.96; buffer 6: 5 mM phosphoric acid, pH=2.45;buffer 7: 4 mM phosphoric acid, 5%MeOH, pH=2.45

Table 5.7 - *Continued*

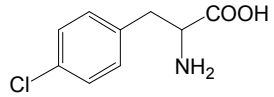
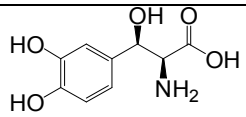
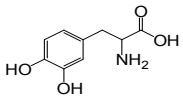
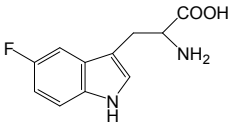
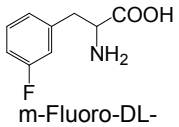
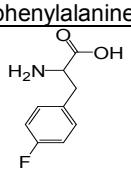
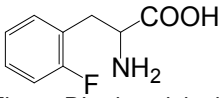
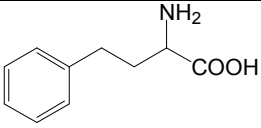
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
73	 4-Chloro-DL-phenylalanine	-16	20	9.779	0.33	1.09	3.4	22000	SCF6 15 mM Buffer 5
		-17	30	17.413	0.35	1.09	4.9	48000	SCF7 20 mM Buffer 5
74	 DL-threo-β-(3,4-Dihydroxyphenyl)serine	-18	20	16.171	0.077	1.04	1.2	17000	SCF6 10 mM Buffer 6
		-18	20	15.729	0.066	1.03	0.7	7400	SCF7 10 mM Buffer 6
75	 3,4-Dihydroxy-DL-Phe	-18	20	13.008	0.075	1.03	1.0	18000	SCF6 10 mM Buffer 6
		-17	30	21.379	0.12	1.04	1.8	40000	SCF7 20 mM Buffer 5
76	 5-Fluoro-DL-tryptophan	-16	20	5.042	0.68	1.10	3.4	21000	SCF6 15 mM Buffer 5
		-17	30	7.933	0.78	1.10	3.4	22000	SCF7 20 mM Buffer 5
77	 m-Fluoro-DL-phenylalanine	-16	20	11.133	0.25	1.08	3.3	29000	SCF6 15 mM Buffer 5
		-17	30	19.104	0.43	1.13	6.4	43000	SCF7 20 mM Buffer 5
78	 p-Fluoro-DL-phenylalanine	-16	20	14.667	0.24	1.10	3.6	22000	SCF6 1 mM Buffer 5
		-17	30	19.654	0.23	1.07	3.4	42000	SCF7 20 mM Buffer 5
79	 o-Fluoro-DL-phenylalanine	-18	20	16.375	0.070	1.04	1.4	24000	SCF6 10 mM Buffer 6
		-18	30	No peak was observed for 42 min					SCF7 10 mM Buffer 6
80	 DL-Homophenylalanine	-16	20	11.271	0.13	1.04	1.8	32000	SCF6 15 mM Buffer 5
		-17	30	22.150	0.065	1.02	0.9	32000	SCF7 20 mM Buffer 5

Table 5.7 - *Continued*

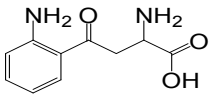
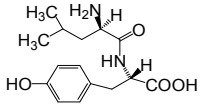
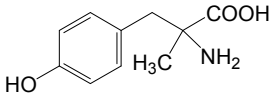
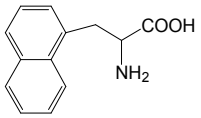
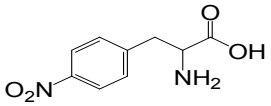
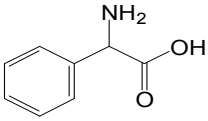
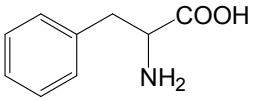
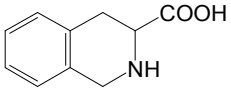
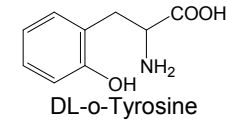
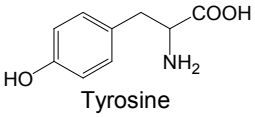
#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
81	 DL-Kynurenine	-16	20	2.862	0.64	1.05	2.7	48000	SCF6 15 mM Buffer 5
		-20	30	3.621	0.69	1.04	1.4	17000	SCF7 15 mM Buffer 5
82	 DL-Leucyl-DL-Tyrosine	-18	20	12.863	0.071	1.03	0.8	14000	SCF6 10 mM Buffer 6
		-12	20	21.404	0.051	1.02	0.8	24000	SCF7 20 mM Buffer 5
83	 α-Methyl-DL-tyrosine	-18	20	13.825	0.043	1.02	0.5	14000	SCF6 10 mM Buffer 6
		-18	20	12.304	0.062	1.02	0.8	18000	SCF7 10 mM Buffer 6
84	 3-(1-Naphthyl)-DL-alanine	-16	20	6.563	0.80	1.16	5.7	24000	SCF6 15 mM Buffer 5
		-12	20	7.175	1.16	1.20	7.7	29000	SCF7 20 mM Buffer 5
85	 4-Nitro-DL-phenylalanine	-16	20	6.429	0.25	1.05	2.0	33000	SCF6 15 mM Buffer 5
		-17	30	11.979	0.22	1.04	2.7	79000	SCF7 20 mM Buffer 5
86	 2-Phenylglycine	16	20	6.100	0.39	1.07	2.8	31000	SCF6 15 mM Buffer 5
		17	30	26.692	0.47	1.22	7.8	29000	SCF7 20 mM Buffer 5
87	 Phenylalanine	16	20	7.575	1.24	1.34	9.8	19000	SCF6 15 mM Buffer 5
		20	30	17.658	0.37	1.12	5.6	37000	SCF7 15 mM Buffer 5
88	 1,2,3,4-Tetrahydro-3-isoquinolinecarboxylic acid	16	20	11.650	0.28	1.10	3.5	25000	SCF6 15 mM Buffer 5
		12	20	11.313	0.87	1.24	6.9	16000	SCF7 20 mM Buffer 5

Table 5.7 - *Continued*

#	Compound	V	L	t_{m1}	Δμ	α	Rs	N	Conditions
89	 DL-o-Tyrosine	18	20	18.229	0.20	1.12	2.9	10000	SCF6 10 mM Buffer 6
		18	20	22.754	0.16	1.12	4.0	22000	SCF7 10 mM Buffer 6
90	 Tyrosine	18	20	12.483	0.11	1.04	1.4	20000	SCF6 10 mM Buffer 6
		20	30	18.329	0.15	1.05	2.7	56000	SCF7 15 mM Buffer 5

5.3.2 Overview of the enantioseparation results

In order to examine the enantioselective capabilities of sulfated cyclofructans, over 200 pairs of enantiomers were tested. A series of amines and amino acids were used to examine optimization parameters. In the normal polarity mode, only cationic amines showed enantioselectivity. However, some analytes showed severe tailing, probably due to adverse wall interactions. Therefore, reverse polarity using a background buffer with pH around 2 was examined. This approach greatly improved the efficiency for compounds with severe tailing (as shown in Figure 5.3). The low pH also allowed for the protonation of the carboxylic group of amino acids, which enabled their subsequent enantioseparation. All results are summarized in Tables 5.1-5.7 and Figure 5.4. Among the 110 amine-containing compounds tested (including two sets of diastereoisomers of which each contains two pairs of enantiomers), 90 of them showed enantioselectivity, with 66 of them being baseline separated by one or both of the SCFs. This is a relatively high percentage. In fact, a resolution of 15.4 was easily achieved for compound #19 (2-amino-1,1,3-tripheyl-1-propanol), within 7 minutes (Table 5.2). All the analytes showed similar migration time under the same SCFs concentration, which indicates similar binding strength of analytes to both SCFs. In addition, it was observed that in all cases in which the electromigration order was determined, the electromigration order was the same for

both SCF6 and SCF7. Among the 90 pairs of enantiomers that showed enantioselectivity, 84 of them were separated by both SCFs. These three facts indicate that similar molecular recognition mechanisms may be operative for both cyclofructans. However, for a few specific analytes, the two SCFs showed significant difference in enantioselectivity. For example, compound #38 (idazoxan) was only separated on SCF6 (Rs=4.3) while compound #37 (fenfluramine) was only separated on SCF7 (Rs=4.2).

With a careful examination of data, certain interesting facts were noticed. First, it was observed that the resolution (Rs) for the separation of amino acid esters increased as the size of the ester group increase. For example, compound #3 (4-chlorophenylalanine ethyl ester) was better separated than compound #4 (4-chlorophenylalanine methyl ester). Another interesting phenomenon is that a chloro- substituent in the *p*-position of phenyl groups (i.e. compounds #4, #14 and #29) can increase the selectivity compared to their non-halogenated counterparts (i.e. compounds #7, #13 and #27, respectively).

5.3.3 Factors affecting enantioseparation

Several factors, such as buffer type and concentration, pH, chiral selector concentration, and organic modifiers, are commonly used to optimize enantiomeric separations [7, 25, 62, 75, 113, 157, 171, 212, 213].

Buffer plays an important role in enantioselective separations. It controls the pH, stabilizes the current, and maintains the EOF [62, 206]. It can also modify the interaction between an analyte and chiral selector. Four types of buffers were tested in this study, and the results are shown in Figure 5.5. Overall, ammonium acetate produced the best enantiomeric resolutions within reasonable analysis times. The buffer concentration effect also was studied and the results are shown in Figure 5.6. The optimum buffer concentration was in the range of 4 mM to 7 mM, which is significantly lower than typical optimum buffer concentration used for sulfated cyclodextrins [7, 62, 153, 157]. The finding that high buffer concentration suppresses the association of analyte and SCFs indicates the importance of electrostatic interactions for

enantioselectivity by SCFs. The buffer pH can affect the charge state of analyte and chiral selector as well as the EOF. Lower pH slows the EOF, which in turn greatly improves selectivity and thus the resolution by decreasing the apparent mobility of the analytes (see Figure 5.7) [6, 206].

Altering the chiral selector concentration has been shown to be an effective way to improve enantioresolution [7, 62]. Electropherograms obtained for the separation of alprenolol using with different SCF7 concentrations (while other conditions remain the same) are shown in Figure 5.8. The optimum concentration was determined to be 15 mM, which is close to the optimum concentration when chiral crown ethers are used as chiral selectors [214], but significantly lower than a typical optimum sulfated cyclodextrin concentration [62].

Another important experimental factor that affects enantioseparations is the organic modifier [62]. Organic modifiers not only increase the solubility of hydrophobic analytes, but also suppress the joule heating by lowering the current and slowing the EOF. These effects can improve selectivity. However, the organic modifier may also compete for the chiral selector, thus disrupting the association between analyte and selector. Figure 5.9 shows the effect of methanol percentage on the separation of p-chloroamphetamine. The observed effects are significantly different than what is observed with sulfated cyclodextrins [62, 157, 215], in that the resolution was not affected to a great degree. This allows for the determination that the chiral recognition mechanism of sulfated cyclofructans is significantly different than sulfated cyclodextrins, in that hydrophobic inclusion complexation is not as important with SCFs.

Different capillary batches can have different surface properties. Therefore, different wall interactions and EOFs can be observed under the same conditions, which may result in different enantioresolutions. Figure 5.10 shows the electropherograms of same compound under identical separation conditions in the normal polarity mode on capillaries of different batches (see experimental). Employment of reverse polarity with low pH (around 2) buffer can minimize this difference (as shown in Figure 5.3)

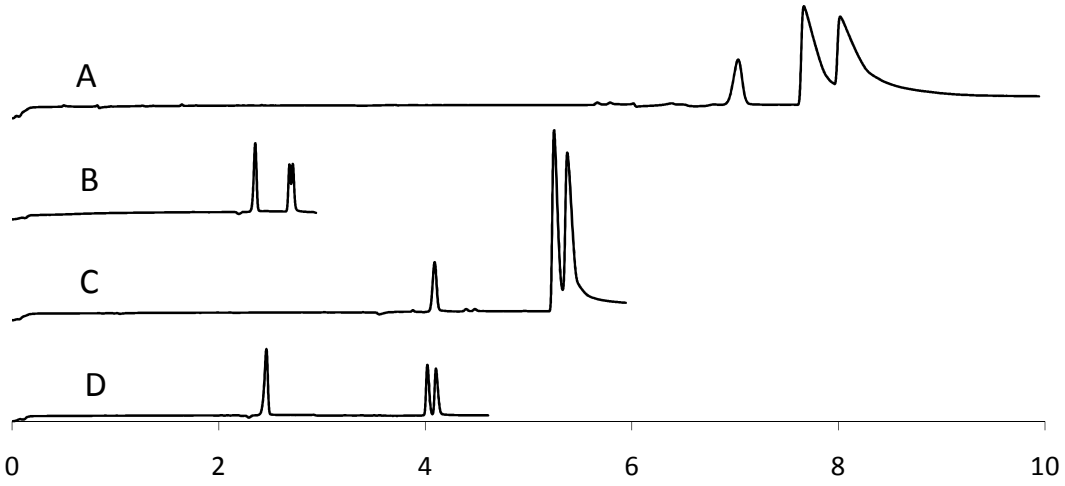


Figure 5.5 Effect of buffer type when using 5 mM sulfated cyclofructan 7 (SCF7) separating p-chloroamphetamine. buffer: 20 mM, pH=4.7, +25 kV, 30/40 cm 50 µm I.D capillary. A: tetrabutylammonium nitrate and sodium acetate; B: phosphate; C: sodium acetate; D: ammonium acetate. First peak is EOF marker.

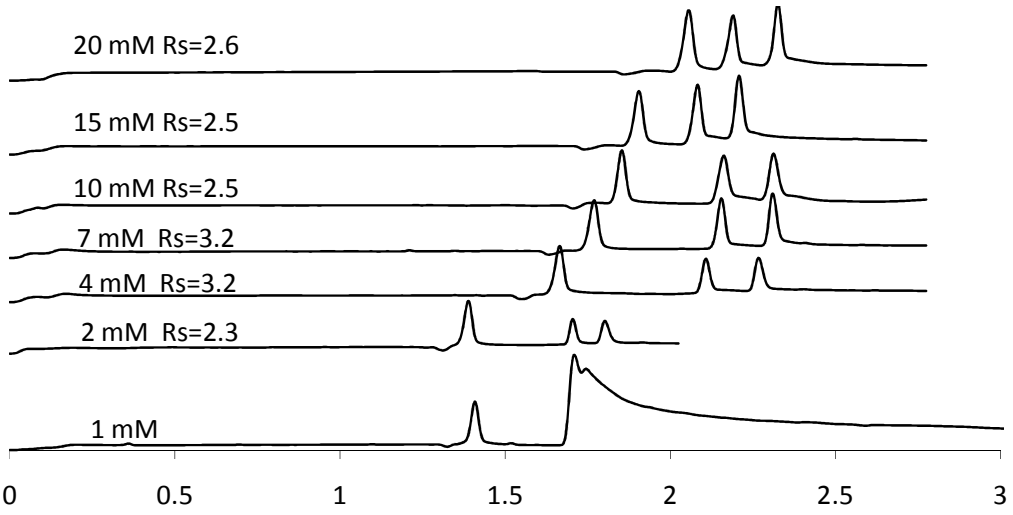


Figure 5.6 Effect of Buffer (ammonium acetate) concentration when using 5 mM sulfated cyclofructan 7 (SCF7) to separate 1,2-diphenylethylamine. pH=4.7, +25 kV, 30/40 cm 50 µm I.D capillary. First peak is EOF marker.

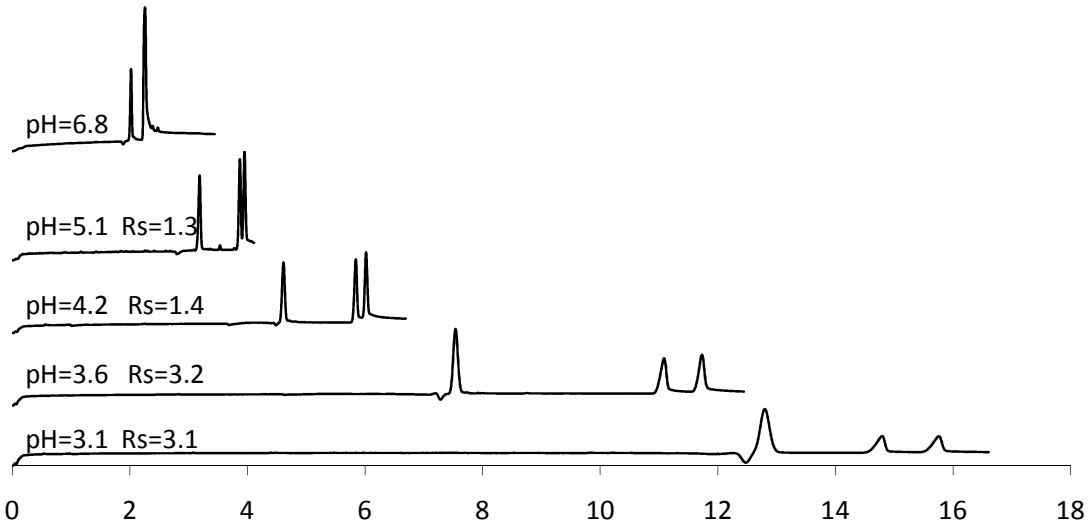


Figure 5.7 Effect of pH when using 5 mM sulfated cyclofructan 7 (SCF7) separating *p*-chloroamphetamine. Buffer: 20 mM ammonium acetate, +25 kV, 30/40 cm 50 µm I.D capillary. First peak is EOF marker.

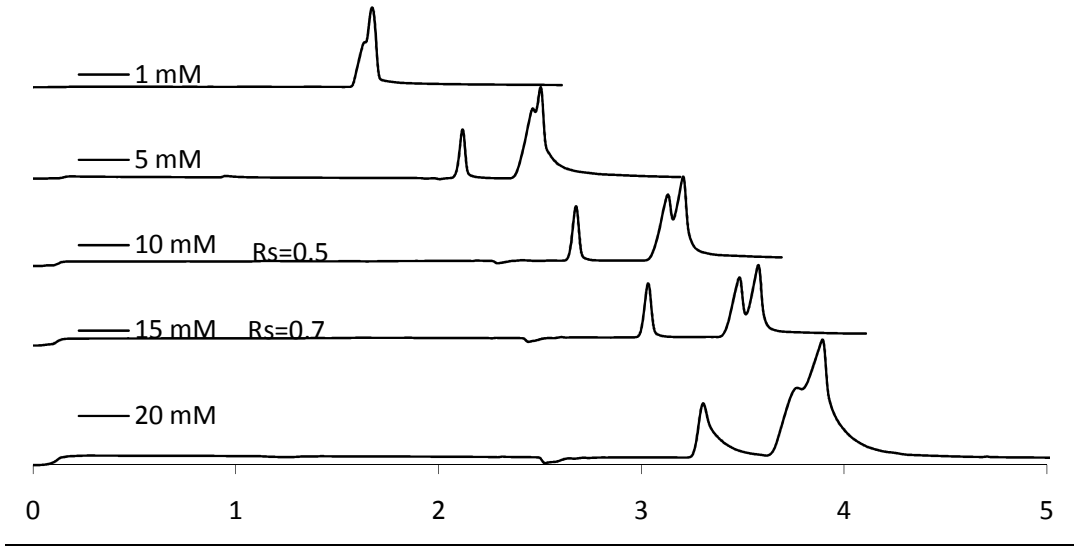


Figure 5.8 Effect of sulfated cyclofructan 7 (SCF7) concentration when separating alprenolol. buffer: 5 mM ammonium acetate, pH=4.7, 30 cm (from inlet to detection window)/37 cm capillary, 50 µm i.d., +25 kV. First peak is EOF marker.

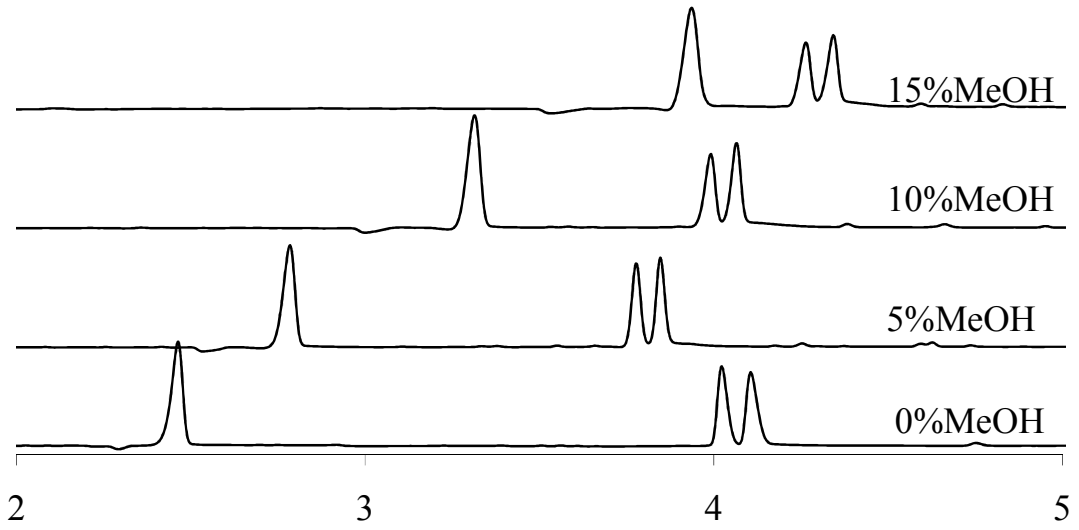


Figure 5.9 Effect of MeOH percentage (v/v) when using 5 mM sulfated cyclofructan 7 (SCF7) separating *p*-chloroamphetamine buffer: 20 mM ammonium acetate, pH=4.7, +25 kV, 30/40 cm 50 µm I.D capillary. First peak is EOF marker.

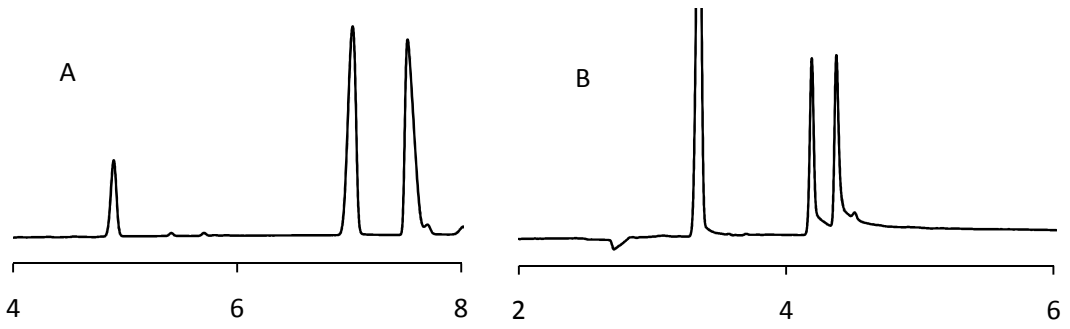


Figure 5.10 Comparison of capillary batches. Conditions: *p*-chloroamphetamine, sulfated cyclofructan 6 (SCF6) 15 mM, 10 mM ammonium acetate,10 mM phosphoric acid, pH=4.7,+25 kV, 30 cm/40 cm capillary with 50 µm i.d. A: capillary batch #1; B: capillary batch #2. First peak is EOF marker

5.4 Concluding remarks

Sulfated cyclofructan 6 and 7 showed high enantioselectivity towards all types of amine containing compounds. Fast separations (<10 min) were achieved for most of the analytes separated. Electrostatic interaction plays an important role in both association and molecular recognition. SCFs showed similarities and differences to both crown ether and sulfated cyclodextrin chiral selectors. While chiral crown ethers showed enantioselectivity to mainly primary amines, SCFs showed good enantioselectivity to all amines. Compared to sulfated cyclodextrins, SCFs showed better selectivities for amine-containing compounds, allowing faster baseline separations. Their high solubility, UV transparency and minimum wall interaction enable them to be useful and competitive chiral selectors for capillary electrophoresis.

5.5 Acknowledgements

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CHAPTER 6

STERILITY TESTING BY CAPILLARY ELECTROPHORESIS: A COMPARISON OF ON-LINE PRECONCENTRATION APPROACHES IN CAPILLARIES WITH GREATER INTERNAL DIAMETERS

Detection of microbial contamination is of critical importance in the medical and the food industry. Rapid tests for the absence or presence of viable microorganisms are in urgent demand. Capillary electrophoresis is a modern analytical technique that can be adapted for rapid screening of microbial contamination. However, the small dimensions of capillaries allow introduction of only a small fraction of the sample, which can be problematic when examining large samples. In this article, we examine the possibilities of introducing larger sample volumes using capillaries with greater internal diameters (i.d.) together with different stacking techniques. The use of 0.32 mm i.d. capillary and the injection of 60 % of the capillary volume led to approximately 120-fold improvement of the injected sample volume over the classical injection 5% of a 0.10 mm i.d. capillary. The setup we described opens new possibilities in sterility testing using capillary electrophoresis.

6.1 Introduction

The detection and identification of microorganisms in samples, especially pathogenic microorganisms, is a crucial and necessary procedure to ensure the safety and quality in the food/beverage [15, 16], pharmaceutical [17] and medical industries [18, 19]. Given the large numbers of diagnostic tests required, there is a strong demand for fast and accurate methods to assess sterility of products/samples. Traditionally, the direct inoculation method and its modifications involve time consuming cultivation in a sterile growth medium with an aliquot of sample, which usually take days to weeks and works only for a defined group of microorganisms [15, 216]. Existing fast approaches such as hybridization, immunoassay and

nucleic acid amplification (PCR) are more complex to carry out, require professional personnel training, and are organism specific rather than general all-encompassing techniques [15, 19, 217, 218].

Recently, capillary electrophoresis (CE) has been established as an approach for the fast separation and identification of microorganisms [19, 26, 27, 32, 219]. Hjertén et al. [28] first showed that microbes and viruses have an ability to migrate in the electric field together with the electroosmotic flow. The actual effect of the orientation of a virus on its electrophoretic mobility was examined by Grossman and Soane [220]. In 1993, Ebersole and McCormick first successfully employed CE in the separation and identification of a series of four bacteria [33]. Under similar conditions, the electrophoretic mobilities of 3 different bacteria populations were determined and their separations were achieved by Pfetsch and Welch. [221]. However, these initial works required 250 cm long capillaries, showed large peak widths, long migration times and small differences in the migration times as compared to the CE of molecules. A method for fast separation of microorganisms with sharp peaks were established by Armstrong and coworkers [29, 222-226] by introducing poly(ethyleneoxide), PEO, to the running buffer. The mechanism was explored [29, 223, 227-229]. Two models of CE behavior were introduced: (i) interaction between the PEO molecules and microbes decreased the zeta potential of the microorganisms and induced aggregation to sharp zones, (ii) non-uniform velocities of non-spherical microorganisms caused collisions and similarly to the previous model the aggregation, This technique was successfully applied in the determination of cell viability, identification of the causative pathogens of urine tract infections and food contamination [224-226]. Covalent coating of the capillary wall was also used to minimize the microorganism-wall interaction and thus obtain good peak efficiencies [228, 230-232]. A “three injection method” for quick sterility test (which is to give a binary answer regarding the presence/absence of a wide variety of microorganisms) using a “blocking agent”, where all the microorganisms are concentrated and “swept” to one single peak was developed [26, 27, 32].

Despite the fact that small sample solution injection volume is an advantage for the CE analysis of small molecules, it can become an intrinsic disadvantage when it comes to analysis of microorganisms. On a per-particle basis, microbial solutions are generally more dilute than solutions of molecules. Typically the injection volume does not exceed a few tens of nanoliters, which raises the problem in real world analysis: Is the sample solution injected representative of the real sample? Supposing the sample concentration is 10^3cfu/ml, which means it contains 1 microorganism in every microliter solution and the injection volume is 25 nL, then the probability of injecting a microbe is only 2.5%. This would lead to false-negative results and inaccurate quantification. One solution is on-line analyte concentration, which is performed by injecting large volumes of sample solutions and then focusing analytes to a narrow zone before analysis (see for example [233]). Relatively few reports are focused on online concentration methods for the analysis of microbes [228, 234].

These facts (*vide supra*) give rise to an important challenge, which is how to introduce larger sample volumes while maintaining all of the other positive features of CE. The injected volume can be enlarged by extending the length of the capillary, like the large volume sample stacking method presented by Yu and Li [234], or by increasing the capillary diameter. Previous reports on CE analysis of bacteria used different capillary diameters: the group of Armstrong (e.g. [26, 27, 32, 222, 235]) employed 0.100 mm i.d. capillaries as well as Hjertén et al.[28], Yu et al. [234] and Ebersole et al.[33]; the group of Buszewski (e.g. [19, 232]) as well as Petr et al. [236] used 0.075 mm i.d. capillaries; and Pfetsch and Welsch [221] believed that the 0.250 mm i.d. capillary was better for determination of the electrophoretic mobilities of bacteria.

Table 6.1 Capillary characteristics

capillary i.d.	0.10 mm	0.25 mm	0.32 mm
capillary volume (length 30 cm)	2.36 µL	14.7 µL	24.1 µL
capillary volume increase	1.0 times	6.3 times	10.2 times
probability of a positive match with 60 % volume injection from 1cfu/50 µL sample	2.8 %	17.7 %	29.0 %
injected volume (5 s by 0.5 psi)[a)]	0.1 µL	4.2 µL	11.4 µL
% of capillary injected[a)]	4.6 %	28.8 %	47.2 %

a) calculated for injection time 5 s, pressure 0.5 psi, capillary length 30 cm and sample viscosity 1.3 mPas

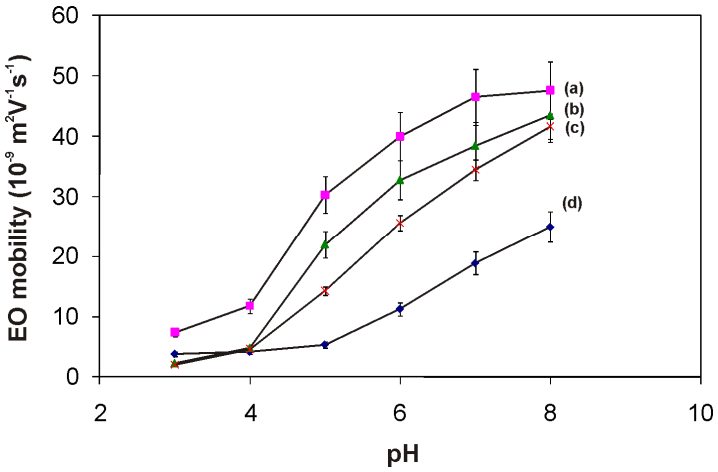

Figure 6.1 A dependence of the EO mobility on the electrolyte pH. (a) 0.25 mm i.d. capillary (Supelco), (b) 0.32 mm i.d. capillary (Supelco), (c) 0.10 mm i.d. capillary (Polymicro), (d) 0.10 mm i.d. capillary (Supelco); BGE: 10 mM citrate/Tris buffer. See section 2 for details.

The influence of a capillary diameter on the CE performance has been studied extensively for molecular compounds [237]. Table 6.1 lists the physical/geometrical characteristics of different i.d. capillaries. Some of the conclusions from these studies mainly for the practical point of view are summarized below:

(i) Poiseuille's equation relates the injection volume (V_c) to the applied pressure (ΔP), inner diameter (d), injection time (t), solution viscosity (η) and capillary length (L):

$$V_c = \frac{\Delta P \pi d^4 t}{128 \eta L}. \quad (1)$$

The biquadrate of the capillary i.d. in equation (1) has a huge effect. The volume injected in the 0.32 mm i.d. capillary is more than 100-times higher than that in the 0.10 mm i.d. capillary while the total capillary volume increased only approximately 10-times. The pressure and injection time should be adjusted for percentage of injection volume over the total capillary volume when the i.d. of capillary is varied. In our study, injection pressure and duration was calculated based on Poiseuille's equation to meet these needs (see Table 6.1). The viscosity could be determined from equation (1) with apparatus by using pressurized drive of the marker (e.g. N,N-dimethylformamide) in the solution of interest [238].

(ii) Ohm's law describes the relationship between voltage (U), the current (I), the length of capillary (L), the inner diameter (d) and the buffer conductivity (κ):

$$U = IR = I\frac{L}{S\kappa} = I\frac{4L}{\pi d^2 \kappa}. \quad (2)$$

If the voltage 30 kV results a current of 20 μA in the 0.10 mm i.d. capillary, the same voltage in the 0.32 mm i.d. capillary will result in a current of 200 μA according to equation (2), which is unacceptable for CE analysis. In order to maintain the current (for reproducible results and to control the Joule heating), the applied voltage needs to be lowered when large i.d. capillary is used, which greatly increase the analysis time.

These facts indicate that capillaries with larger i.d.s are possible to use, but they introduce additional mitigating factors that must be accounted for. The aim of this work was to compare different on-line preconcentration approaches with a possibility of using large volume injection in capillaries with higher i.d.s, mainly for the task of fast sterility testing.

6.2 Materials and methods

Tris(hydroxymethyl)-aminomethane (Tris), cetyltrimethylammonium bromide (CTAB), sodium hydroxide, hydrochloric acid and luria broth were obtained from Sigma Aldrich (Milwaukee, WI). Citric acid was purchased from Fisher Scientific (Fair Lawn, NJ). Nutrient and brain heart infusion broths were products of Difco Laboratories (Franklin Lakes, NJ). Dimethyl sulfoxide (DMSO) was purchased from EM Science (Gibbstown, NJ) and used as EOF marker. *Escherichia coli* (ATCC no. 10798), *Bacillus subtilis* (ATCC no.12695), *Candida albicans* (ATCC no. 10231), *Rhodotorula* (ATCC no. 20254), and *Salmonella subterranea* (ATCC no. BAA-836) were purchased from American Type Culture Collection (Manassas, VA). Uncoated fused silica capillaries with i.d.s of 0.100 mm were purchased from Polymicro Technologies (Phoenix, AZ) and bare silica capillaries with i.d. 0.100 mm, 0.250 mm, 0.320 mm (Supelco brand) were purchased from Sigma Aldrich (St. Louis, MO).

All experiments were performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with photodiode array using capillaries with total length of 30 cm (20 cm to the detector). New capillaries were rinsed with 0.5 M NaOH, deionized water, 0.5 M HCl, and running buffer each for 10 min for conditioning before use. Between runs, the capillaries were washed with 0.5 M NaOH, deionized water, and running buffer for 5 min each. Working citrate/Tris buffers were prepared by dissolving an appropriate amount of citric acid in deionized water and then adjusted by titration with Tris to desired pH. CTAB was added to the final buffer with concentration of 1 mg/mL. All bacteria and fungi were cultured according to the instructions from the supplier. The microorganisms were initially grown in the appropriate liquid broth, and then plated on agar growth media and stored under refrigeration. All broths and agar

were autoclaved (Primus autoclave, Omaha, NE) for 1 h prior to inoculation. For experiments, fresh liquid broth was inoculated with a single microbe colony that was taken from the agar plate. These cells were grown at 37 °C under gentle agitation for approximately 24 h, producing a cellular concentration of 10^8 colony forming units (CFU)/mL. Cell concentrations were approximated by serial dilutions and plate-count methods when necessary. The microorganisms were centrifuged down, and the excess broth was removed. These cells were then washed with working citrate/Tris buffer or water, re-centrifuged, and finally re-suspended in the fresh buffer or water (same volume as the culture broth to maintain the microbe concentration) for analysis. All samples were vortexed for 30 s and sonicated briefly prior to analysis to disperse cellular aggregates. All run buffers, solutions, and vials used in the CE analysis were autoclaved prior to the run, too.

6.3 Results and discussion

6.3.1 Electroosmotic flow

The electroosmotic flow (EOF) has a large effect on the migration of bacteria. Therefore the effect of capillary i.d. on the EOF mobility was first evaluated. Capillaries from Supelco with three different i.d.s (0.10 mm, 0.25 mm and 0.32 mm) and capillaries from Polymicro with an i.d. of 0.10 mm were compared in Figure 6.1. The EO mobility was measured in 10 mM citrate/Tris buffers with pHs that varied from 3.0 to 8.0. In cases when the EOF was weak, the method published by Williams and Vigh [239] was employed. Significant differences in the EO mobilities in all the capillaries were observed. However, all the curves presented a analogous EOF profiles [1] as shown in Figure 6.1. The EO mobility also differed in the capillaries with same i.d. (0.10 mm) from different manufacturers. Similar results were reported previously by Kohr et al [240].

6.3.2 Normal stacking mode

The normal stacking mode is the simplest sample concentration method [241]. It is based on an injection of a long plug of sample in a low conductivity matrix followed by applying

high voltage for analysis. According to Ohm's law, the field strength of the sample zone will be higher than that of the rest of the capillary. As a result, the sample will stack near the interface [242]. The preconcentration effect could be enhanced by using a large volume injection; sometimes a sample is injected to more than 60 % of a capillary volume [243, 244].This technique was explored primarily by using 10 mM citrate/Tris buffer at pH 7.0 (similar results were obtained for buffer with pH 8.0) in our study using an injection of 5 % of capillary volume. The preconcentration effect was first studied with the microbes suspended in deionized water using 0.10 mm i.d. capillaries from Polymicro. The preconcentration effectiveness was evaluated as the ratio of the peak height of microbes when suspended in deionized water versus when suspended in the running buffer. Following results were obtained: 2.2 for *Escherichia coli*, 1.8 for *Bacillus subtilis*, 2.2 for *Candida albicans*, 2.3 for *Rhodotorula*, and 2.5 for *Salmonella subterranea*. Since the purpose of this work was to optimize a method for sterility testing where separation of individual types of microorganisms was not needed, only *Salmonella subterranea* was further studied as a model microorganism.

Next we compared the analysis of the model microbe *Salmonella subterranea* in capillaries from Supelco with different i.d.s, as shown by the electrophoregrams in Figure 6.2. The percentages of injection volumes were kept the same (here, 72 - 75% of the total capillary volume). The peak height significantly increased with higher capillary i.d.s. This is because: 1) a larger sample volume was injected when using the larger i.d. capillaries (i.e. 10 times when the 0.32 mm i.d. capillary was used compared to that when the 0.10 mm i.d. capillary was used); 2) The optical pathlength was longer for larger i.d. capillaries. As a result, the peak height was 20 times higher when the 0.32 mm i.d. capillary was used compared to that obtained with the 0.10 mm i.d. capillary. As capillary i.d. was increased, the applied voltage was also adjusted in order to maintain the current around 60-80 μA. Therefore the migration time of the microbe sample was longer in the larger i.d. capillaries. The apparent mobilities of the microbes were determined to be the same (22 $m^2V^{-1}s^{-1}$), but the relative standard deviations increased with

higher capillary i.d.s (2.2 % for 0.10 the mm i.d. capillary, 6.5 % for the 0.25 mm i.d. capillary, and 9.6 % for the 0.32 mm i.d. capillary).

Figure 6.3 shows the peak height as a function of injection volume percentage (over the total capillary volume). Assuming that the absorbance is directly proportional to the length of the absorbing media, the peak height was normalized in regard to optical path length using an i.d. increment factor (1.0 for 0.10 mm i.d., 2.5 for the 0.25 mm i.d. capillary and 3.2 for the 0.32 mm i.d. capillary), where i.d. increment factor is the ratio of capillary i.d. to 0.10 mm (which is the smallest capillary i.d. used). The preconcentration effect was evaluated as the corrected peak height, which was calculated as peak height divided by the i.d. increment factor. As shown in Figure 6.3A, the corrected peak heights all increased as the injection volume percentage increased for all three capillaries with different i.d.s, while the effect is more significant in larger i.d. capillary. The increase between 0.10 mm i.d. capillary and 0.25 mm i.d. capillary is not as high as was expected. Two explanations are possible. First, the mechanism of aggregation depends on the free movement of bacterial cells and deviations from the flat EOF profile in the larger capillaries could have an additional effect on the aggregation. Second, the aggregation is affected by the electric field strength as described by Zheng and Yeung [1, 27, 32, 245, 246]. In the larger capillaries, current requirements cause a decrease oin the electric field strength and therefore it could affect the aggregation.

6.3.3 Stacking in the reverse EOF mode

Cetyltrimethylammonium bromide (CTAB) has been used to reverse the EOF in CE [1, 27, 32, 245, 246]. Generally, the method with reversed EOF represents a typical option for analysis of anionic species [246, 247]. As with methods that use the normal direction of the EOF (from the anode to the cathode), stacking based on-line preconcentration could be used in the reversed EOF mode as well [245]. In our case, a BGE containing 10 mM citrate/Tris pH 7.0 with 1 mg/mL CTAB was used. This CTAB concentration was found to be sufficient to form the anodic EOF [246]. The EO mobility was measured in all the three Supelco capillaries using

dimethylsulfoxide as the EOF marker. The EO mobility was measured: -42 (SD for 5 runs was 3) x 10^{-9} $m^2V^{-1}s^{-1}$ for the 0.10 mm i.d. capillary, -48 (7) x 10^{-9} $m^2V^{-1}s^{-1}$ for the 0.25 mm i.d. capillary, and -53 (10) x 10^{-9} $m^2V^{-1}s^{-1}$ for the 0.32 mm i.d. capillary. This indicates that EO mobilities and deviations increase with capillary i.d.s. Similarly to stacking with normal EOF conditions, the preconcentration effectiveness in terms of peak height in all the three capillaries was studied as a function of the injection volume percentage (Figure 6.3B). The migration time of *Salmonella subterranea* increased from approximately 6.9 min when using a 0.10 mm i.d. capillary to approximately 15 min for the 0.32 mm i.d. capillary, which means there was a 2.5 times prolongation of the analysis time with a 10-fold increase of the injection volume. However, the peak height in 0.32 mm capillary was 20 times greater than that in 0.10 mm capillary, which is the result of a combination of the larger injection volume, longer optical path length and the stacking effect. Moreover the use of CTAB had additional advantages. The formation of random spikes in CTAB based electrolytes was fully suppressed, probably due to the dynamic coating of a capillary wall by CTAB molecules and the overall equilibrium in the capillary.

6.3.4 Stacking induced by pH

The next method examined for the on-line preconcentration of microorganisms was the use of a junction between electrolytes with different pHs (pH induced stacking) [241, 248, 249]. Generally, two possible setups could be used: 1) the microbes are suspended in acidic buffer solution while the running buffer has basic pH, or 2) the microbes are diluted in the basic buffer solution and the running buffer has an acidic pH. The stacking mechanism is based on the assumption that the mobility of microbes will be different in the acidic BGE than that in the basic BGE. However a side effect of the use of a low pH electrolyte was described in previous papers [32, 249]. The microbes have an increased tendency to form clusters [32], not only composed from single species but also hybrid clusters from more than one species [250]. Nevertheless in the case of sterility testing, there is no need to separate the microbe clusters.

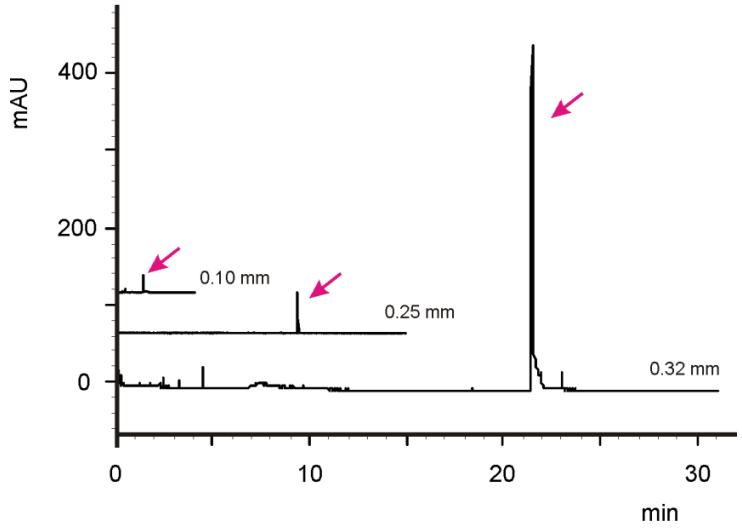

Figure 6.2 Analysis of *Salmonella subterranea* upon stacking conditions in capillaries with different inner diameters. Conditions: 10 mM citrate/Tris pH 7.0; 0.10 mm i.d. capillary: 0.5 psi for 60 s injection (75 % of total volume), 30 kV; 0.25 mm i.d., 0.1 psi for 50 s (72%), 5 kV; 0.32 mm i.d., 0.1 psi for 30s (74%), 2.5 kV. See section 6.2 for details.

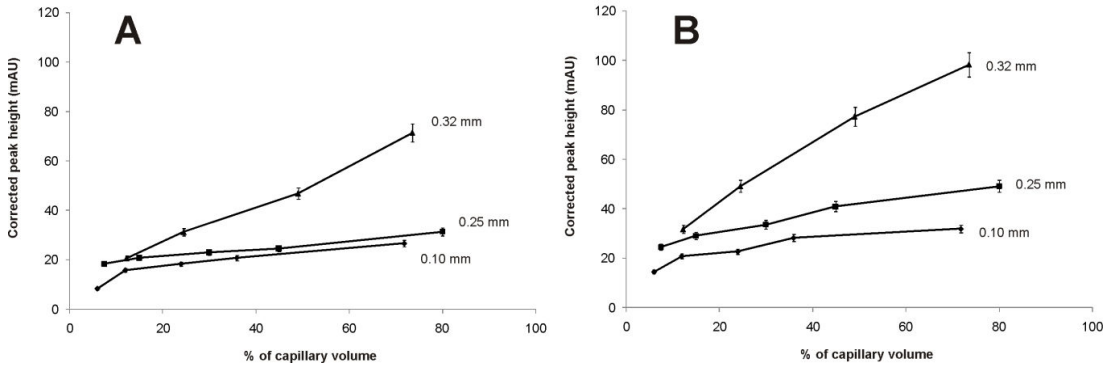

Figure 6.3 The effect of injected volume percentage on corrected peak heights in capillaries with different i.d.s. A: Normal stacking conditions; BGE: 10 mM citrate/Tris pH 7.0, B: Stacking conditions in the reverse polarity mode using CTAB; BGE: 10 mM citrate/Tris pH 7.0 with 1 mg/mL CTAB; Other conditions are the same for both modes: Salmonella subterranea was suspended in water, injection pressure and duration was calculated with Poiseuille's equation (a) 0.32 mm i.d. capillary, (b) 0.25 mm i.d. capillary, (c) 0.10 mm i.d. capillary (all the capillaries are from Supelco). See section 6.2 for details.

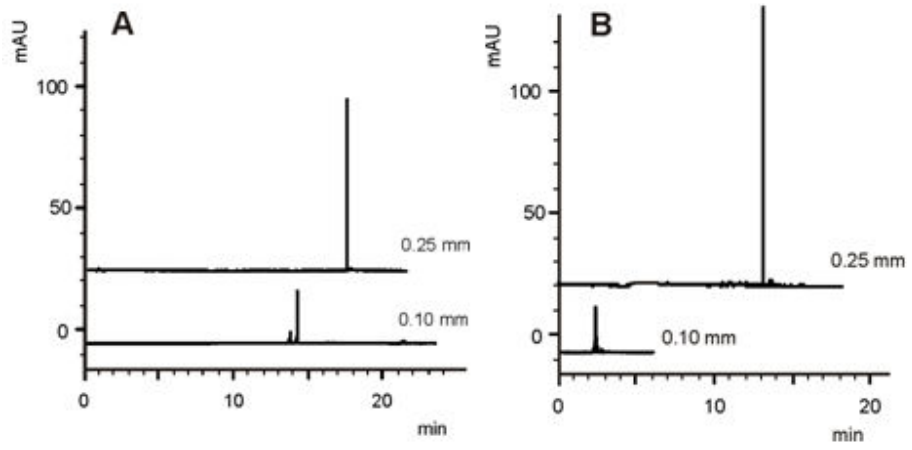

Figure 6.4 Analysis of *Salmonella subterranea* upon pH stacking conditions in capillaries with different inner diameter. Conditions: A: 10 mM citrate/Tris pH 3.0 as BGE, sample was suspended in 10 mM citrate/Tris pH 8.0; B:10 mM citrate/Tris pH 8.0 as BGE, sample was suspended in 10 mM citrate/Tris pH 3.0;voltage: 10 kV for 0.10 mm i.d. capillary, 5 kV for 0.25 mm i.d. capillary. See section 2 for details.

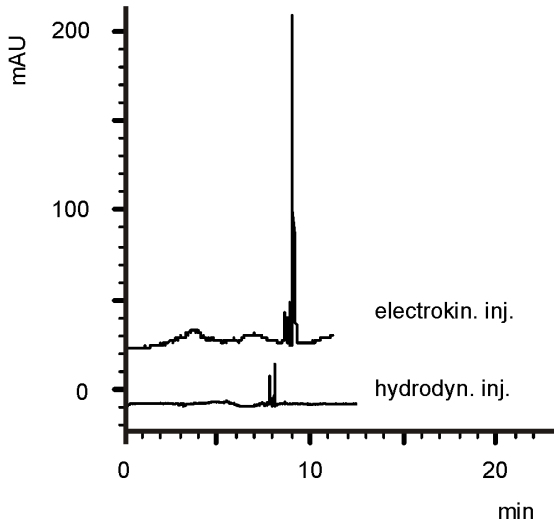

Figure 6.5 A comparison of injection types in the analysis of *Salmonella subterranea.* Conditions: 10 mM citrate/Tris pH 8.0 with 1 mg/mL CTAB as BGE, -10 kV (reverse polarity). Electrokinetic injection: first inject water plug by pressure at 0.5 psi for 2s; then inject *Salmonella subterranean* in1 mM citrate/Tris pH 8.0 by voltage (-10 kV) for 90s; hydrodynamic injection: inject *Salmonella subterranean* in water with pressure 0.5 psi for 5s (6% of the capillary volume). See section 2 for details.

Table 6.2 A Comparison of different on-line preconcentration approaches in terms of relative corrected peak heights[a)] for analysis of Salmonella subterranea in capillaries with different i.d.s

Online preconcentration approaches	0.10 mm	0.25 mm	0.32 mm
Normal CZE mode (injection from BGE, 5 % of the cap. vol.)	1.0	1.3	1.7
Normal stacking mode (injection from water, 5 % of the capillary volume)	2.5	3.1	3.6
Large volume sample stacking mode (60 % of the capillary volume)	5.4	6.0	12.5
Large volume sample stacking in the CTAB mode (60 % of the capillary volume)	5.7	8.1	16.0
pH stacking mode with BGE pH 8.0 (injection from pH 3.0)	3.0	6.3	-
pH stacking mode with BGE pH 3.0 (injection from pH 8.0)	3.2	4.1	-
electrokinetic injection (90 s, -10 kV)	8.6	-	-

a) The relative corrected peak heights were the ratios of the corresponding corrected peak heights over the corrected peak heights obtained in the normal CZE mode in 0.10 mm i.d. capillary

We studied the potential of pH induced stacking with 10 mM citrate/Tris pH 3.0 and 10 mM citrate/Tris pH 8.0 in all three capillary dimensions (of the Supelco brand). Since the difference in EO mobility in those BGEs is approximately 10-fold (Figure 6.1), the analysis time increased in the BGE at pH 3.0 . *Salmonella subterranea* suspended in water gave a peak at approximately 14 min in 10 mM citrate/Tris pH 3.0 while it gave a peak at approximately 3 min in 10 mM citrate/Tris pH 8.0, both in the 0.10 mm i.d. capillary. When the 0.32 mm i.d. capillary was used, the current was not stable and the analysis time was more than two hours, which was not acceptable for a fast and efficient analysis. Figure 6.4A shows an example of the *Salmonella subterranea* analysis where the microbes were re-suspended in the BGE at pH 8.0 and the separation was performed in the BGE with a pH of 3.0. Increasing the injection volume from 5 % to 20 % of the total capillary volume did not affect the preconcentration. The opposite system, where *Salmonella subterranea* was suspended in the BGE at pH 3.0 while the separation in the BGE at pH 8.0, was tested, too (Figure 6.4B). The increase in the injection

volume did not show any effect on the preconcentration. Generally, the difference of the EO mobility or more precisely the mobility of the pH boundary had an important role here. However, in the same manner, decreasing the electric field strength in 0.25 mm i.d. capillaries could affect the formation of aggregates and the separation.

6.3.5 Electrokinetic injection

The last tested on-line preconcentration technique was electrokinetic injection. Electrokinetic injections can achieve from 100 to over 100,000-fold sample preconcentrations [249, 251-253] when combined with different stacking modes, such as field amplified sample stacking (FASS, sometimes called field enhanced sample injection, FESI) and sweeping combined with cation (or anion) selective exhaustive injection. The following equation can be used to estimate the amount of analyte injected (n_i):

$$n_i = Sc_i l_i = Sc_i \mu_{app} E t_{inj} \qquad (3)$$

Where S is the cross-sectional area, c_i is the concentration of the ion species i, μ_{app} is the apparent mobility of the ion species i, E is the electric field and t_{inj} is the injection time.

According to equation (3), the beneficial preconcentration effect from electrokinetic injection using larger capillary i.d.s would be negated by the fact that injection voltage has to be lowered to maintain the current below 80 μA. However, the sensitivity can still benefit from the increased optical path length and the greater injection volume.

These supposed effects were then confirmed experimentally. The FASS technique was tested for the *Salmonella subterranea* standard sample. In this technique, a short water plug was hydrodynamically introduced prior to the electrokinetic injection of the sample solution. A total of 10 mM citrate/Tris (pH 8.0) with 1 mg/mL CTAB was used as the background electrolyte and *Salmonella subterranea* was suspended in buffer solution that was diluted ten times from BGE or in plain water. However, analysis using capillaries with 0.25 mm and 0.32 mm i.d. were not successful due to the long analysis times and unstable currents. A successful analysis was performed only in the 0.10 mm i.d. capillary using an injection voltage of 10 kV in the reverse

polarity mode (Figure 6.5). The influence of injection time was studied in the range of 10 – 90 s. However, generally longer injections resulted in unstable currents and irreproducible results. Analysis obtained with microorganisms re-suspended in diluted BGE was more reproducible than in water. Figure 6.5 compares the electropherograms obtained with electrokinetic injection and hydrodynamic injection.

6.4 Concluding remarks

To improve the sensitivity of CE analysis of microorganisms and the reliability of sterility tests of dilute microorganism solutions, several preconcentration techniques combined with injection volume increases using capillaries with different i.d.s were explored. Possible theoretical benefits were examined experimentally. A comparison of all the studied approaches was made (Table 6.2). The use of large volume sample stacking in the 0.32 mm i.d. capillary with a 60% injection volume gave a 120-fold increase in the injection volume compared to the use of a 0.10 mm i.d. capillary with 5% injection volume. Thus the probability of a positive match between injected sample and real sample can be greatly improved when using very dilute samples. Another advantage of using large i.d. capillaries is the increase in the optical path length, which in turn leads to increased sensitivity and an improvement in the detection limits. Interestingly, the preconcentration effect was also improved when larger i.d. was used. It was shown that a 16-fold increase was observed for the corrected peak height when large volume sample stacking was used with 60% injection volume in the 0.32 mm i.d. capillary compared to regular CZE with 5% injection in a 0.10 mm i.d. capillary.

6.5 Acknowledgement

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CHAPTER 7

FAST DETECTION OF CANDIDA ALBICANS AND/OR BACTERIA IN BLOOD PLASMA BY "SAMPLE-SELF FOCUSING" USING CAPILLARY ELECTROPHORESIS-LASER INDUCED FLUORESCENCE

Detection of microbial contamination in blood plasma is critical and necessary in different medical and research fields. Most of the current standard procedures for the detection of bacteria and fungi can be time-consuming. For example, direct inoculation methods of microbial cultures in respective growth media can take a few days to several weeks. A fast analysis method with high sensitivity output such as CE-laser induced florescence becomes an attractive alternative. Previously, a spacer-injection method with the use of zwitterionic surfactant (SB3-10) as a blocking agent to negate the cells' mobility, and induce aggregation and single microbial peak formation in a buffer solution, was reported. Here, a fast, simple direct method for microbial detection in blood plasma without using the spacer and blocking agent is reported. To compensate for the natural electrophoretic heterogeneity of microbes, a CTAB additive was used to sweep all microbial cells towards the plasma peak where a single sharp microbial peak is formed and detected. With the use of BacLight Green bacterial stain™, the microbial peak, generally, can be detected within 10 min in front of the plasma peak using capillary electrophoresis coupled with laser-induced fluorescence detection. The LOD of microbes detectable were 5 cells per injection. This technique provides a great advantage over traditional, time-consuming microbial inoculation methods.

7.1 Introduction

A rapid detection method for pathogenic microorganisms is an important and necessary component of safety and quality control in many areas of science and technology, including pharmaceutical, food and beverage, and medical products [16-18]. Currently, several methods

are employed to test for microbial contamination. Among them, the simplest and most widely utilized approach procedure is the direct inoculation method [20]. However, there are drawbacks to this technique including the time required for microbial incubation (over several days or weeks), and great care is needed to prevent any contamination during analysis. Furthermore, this approach does not detect all microorganisms of interest, but only those amenable to the growth media and conditions used. Some molecular based detection methods such as hybridization [21], amplification [22], and immunoassay techniques [254] have been developed to shorten the analysis times. Nevertheless, these techniques can be complex and usually requires extensive training. Also, they are used for the identification of specific microorganisms at the species level. Moreover, reagents and materials required for these types of testing can be expensive. Hence, these approaches are not useful as a general contamination test to determine the presence or complete absence of all microorganisms.

Traditionally, capillary electrophoresis (CE) has been used for separations of molecules based on their mass-to-charge ratio. Recently, this technique has been explored as a method for the analysis and characterization of microorganisms and seems to be very promising [30, 219, 220, 222, 255-257]. Also, due to its unique attributes including rapid, high efficiency analysis and small sample requirements, CE becomes an attractive approach for “biocolloid” analysis. However, the main problem of analysis of intact microbial cells is that separation can be degraded by adhesion of the bacteria to the fused silica surface of the capillary causing non-reproducible electroosmotic flow (EOF) and decreasing separation efficiency by band broadening [257]. This could happen when cationic components on a bacterial surface interact with anionic silanol groups of the capillary wall. Armstrong *et. al.* reported the bacterial migration behavior using a CCD camera coupled with LIF [229]. They showed that under certain experimental conditions, self-focusing process of microbes happened inside the capillary as they migrated in an electric field. Buszewski *et. al.* reported that the aggregation of bacteria can decrease the magnitude of electrophoretic mobilities, leading to poor reproducibility of migration

times [231, 232]. Recently, we developed a rapid CE method using either ultraviolet-visible (UV-vis) or laser-induced fluorescence (LIF) detection to indicate the presence or complete absence of microbes in a solution sample [26, 32]. A wide variety of bacteria are compatible with this method and the analysis times are typically under 10 min. Subsequently, we adapted this approach to use an ionic liquid supporting electrolyte for the detection of microbial contamination [27] and specifically for *Candida albicans* (*C. albicans*) in samples by using a capillary electrophoresis-fluorescence *in situ* hybridization (CE-FISH) technique [235]. As yet the determination of microbial contamination in a real biological sample, i.e., whole blood or blood plasma, using CE has not been reported.

Candida albicans is one of the more common fungal pathogens that exists as a commensal of warm-blooded animals including humans. It colonizes on mucosal surfaces of the vagina, inside oral cavities, as well as in the digestive tract [258]. This dimorphic fungus is responsible for the majority of localized fungal infections in human. Patients with impaired immune system, for example those who have had cancer treatments or AIDS infection, more easily develop *C. albicans* infection, which is called Candidasis [259-261]. About 50-70% of nosocomial blood stream infections are caused by *C. albicans* [262]. The most common detection methods for *C. albicans* in blood include: culturing the infected blood sample on Sabouraud glucose or potato dextrose agar followed by germ tube analysis [263]; or increasing the DNA of *C. albicans* using the polymerase chain reaction (PCR) [264] and detecting with peptide nucleic acid – fluorescence *in situ* hybridization (PNA-FISH) [265]. There are several drawbacks to these methods such as the time required for the PCR of *C. albicans* DNA and culturing cells before analysis. Alexander *et. al.* reported that the use of PNA-FISH for detection of *C. albicans* can reduce the cost of treatment approximately $ 1,800 per patient [266]. Three drugs are commonly used to treat C. albicans infections. They are caspofungin, fluconazole and itraconazole [267-269]. Caspofungin and fluconazole are used as a first-line antifungal agent for the treatment of *C. albicans* infections because of its well-known efficacy and safety profile.

However, due to the fact that caspofungin is more expensive than fluconazole, many patients start with caspofungin instead of fluconazole at the beginning of the treatment. If these drugs fail on the treatment, itraconzaole, a wider spectrum antifungal drug, can be used [266]. The length of treatment, depending on the area of infection, is usually from weeks to months [270].

Due to the fact that *C. albicans* has emerged as a significant cause of nosocomial infections, the rapid and direct identification and detection of the presence or complete absence of *C. albicans* and/or other bacteria in blood plasma is necessary. This research is to provide an quick and easy CE based method for the detection of *C. albicans* in blood plasma.

7.2 Materials and Methods

7.2.1 Buffers and stock solutions

Tris(hydroxymethyl)aminomethane (TRIS), citric acid, sodium hydroxide, hydrochloric acid and cetyltrimethylammonium bromide (CTAB), dimethyl sulfoxide (DMSO) were obtained from Aldrich Chemical (Milwaukee, WI). 3-(Decyldimethyl-ammonio)propanesulfonate and caprylyl sulfobetaine (SB3-10) were from Sigma (St. Louis, MO). Yeast and mold (YMB) broth and nutrient broth (NB) were from Difco Laboratories (Franklin Lakes, NJ). Bovine plasma with sodium citrate as anticoagulant was purchased from Innovative Research (Novi, MI). BacLight™ Green bacterial stain (B35000) was purchased from Invitrogen (Carlsbad, CA). Uncoated fused-silica capillaries were with an i.d. of 100 μm and an o.d. of 365 μm were from Polymicro Technologies (Phoenix, AZ).

7.2.2 Bacteria and cell growth

Brevibacterium taipei (ATCC no. 13744), *Bacillus cereus* (ATCC no. 10702), *Bacillus subtilis* (ATCC no. 12695), *Candida albicans* (ATCC no. 10231), *Bacillus megaterium* (ATCC no. 10778) were all purchased from American Type Culture Collection (ATCC, Manassas, VA). *Candida albicans* were grown overnight for 20-24 h at 25˚C in Yeast Malt (YM) Broth. Bacteria were grown overnight for 20-24 h at 30˚C in NB. All microorganisms examined in this study are rated biosafety level one. Standard microbiological practices, therefore, may be employed.

7.2.3 Methods

The CE experiments were performed on a P/ACE MDQ capillary electrophoresis system equipped with photodiode array and 488 nm laser-induced fluorescence detectors (Fullerton, CA). The bare silica capillaries used in this experiment were 30 cm long (20 cm to the detector), with an i.d. of 100 μm and an o.d. of 365 μm. Fluorescence emission from Baclight™ Green bacterial stain cells was detected at 516 nm. New capillaries were initially conditioned with 1 M sodium hydroxide for 5 min, deionized water for 5 min, and running buffer for 5 min. Between each runs, the capillaries were washed with 1 M sodium hydroxide, 1 M deionized water for 1 min each and running buffer for 3 min. The working buffer of 1 mM TRIS, 0.33mM citric acid was prepared from 10 x dilution of 10mM TRIS, 3.3mM Citric acid. pH was adjusted to 7 using 1 M sodium hydroxide or 1M hydrochloric acid. CTAB was added freshly into the working buffer to obtain actual running buffer.

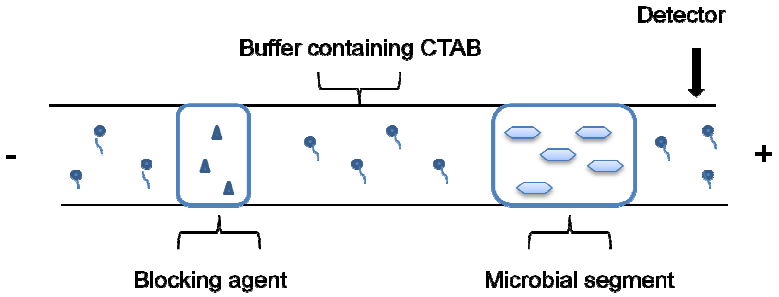


Figure 7.1 A schematic of the three injection method of microbial detection. Three injections are made as follow: (1) a plug of microbial sample; (2) run buffer as a spacer; (3) blocking agent segment.

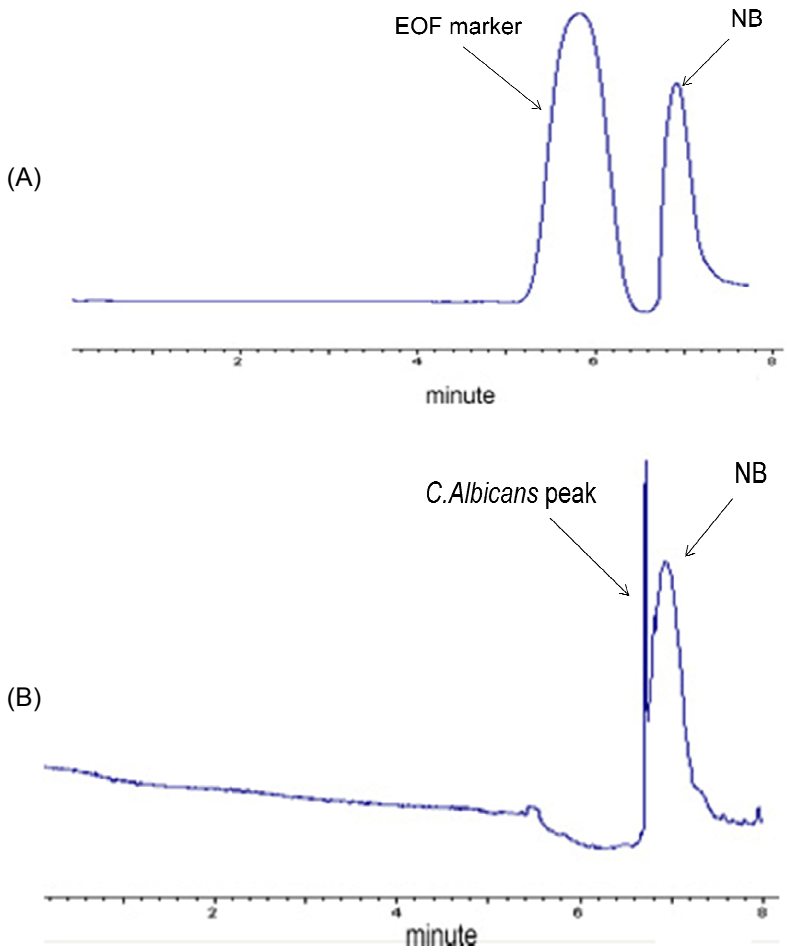


Figure 7.2 Electropherograms obtained using the three injection method. Sample: (A) EOF marker (DMSO) in run buffer; (B) C. albicans in run buffer; Other conditions: run buffer: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; blocking agent: 8 g/L nutrient broth (NB) in run buffer; voltage: -3 kV; detection at 214 nm; see section 7.2.3 for details.

All bacteria and fungi were grown according to the instructions from the manufacturer (see section 7.2.2), which produced a concentration of cells of about 3 x 10^8 colony forming units (CFU)/mL (verified by plate counting method). Serial dilutions of microbial solutions were made with working buffer when necessary. The broth containing microbes were centrifuged for 2 min, and the excess broth was then removed to withdraw the microbes. The microbial cells were washed with working TRIS/citric acid acid buffer, recentrifuged, and finally resuspended in bovine plasma. All samples were vortexed for 30 s and sonicated briefly prior to analysis to prevent cell aggregates. Baclight™ Green bacterial stain was used to stain the cells for fluorescence detection using LIF at 516nm. This dye was dissolved in DMSO to produce 1 mM solution according to the instruction from the manufactures. The cells then were stained by adding 2 µL of dye solution per 1 mL of microbial solution and incubated in the dark for at least 30 min. After incubation, the solution were centrifuged for 2 min and pelleted, then all but the last few microliters of solution was removed. The cells were then washed with working TRIS/citric acid buffer, recentrifuged, pelleted again and all but the last few microliters of the remaining liquid was removed. This washing step was repeated at least 2 times in order to reduce the interference from the plasma peak. The sample was finally resuspended in fresh buffer solution for CE analysis. All run buffers and vials used in the study were autoclaved prior to the experiment. Prior to the separation, the capillary was filled with running buffer. All separations were performed at 3 kV in reverse polarity mode due to reversal of the electroosmotic flow (EOF) by CTAB. All experiments were repeated at least 3 times to ensure reproducibility of the results. Data were analyzed with Beckman System Gold software.

7.3 Results and discussion

7.3.1 Three plug injection method in buffer samples

The goal of this study was to develop a rapid and simple method capable of determining whether any microbial contamination is present or completely absent in a blood plasma sample. As reported previously, a single peak of microbes, regardless of the individual

species and their electrophoretic heterogeneity, was achieved using CTAB as a run buffer additive with a three plug injection method consisting of the microbial sample, running buffer spacer, and blocking agent [26, 32]. Figure 7.1 shows the schematic of this three injection method. Briefly, the capillary was initially filled with running buffer containing CTAB. The sample of bacteria without CTAB was then injected followed by an injection of a spacer containing CTAB. Finally, a segment of SB3-10 which serves as a blocking agent and does not contain CTAB, was injected into the capillary. The run buffer additive, i.e. CTAB, residing in the front of the microbes (on the anode side) migrates towards the cathode while the microbes move towards the anode. As the CTAB passes through the microbial sample zone, it carries the bacteria with it. As the microbes travel through the spacer, they are removed from any contaminants in the sample plug region. Upon reaching in the front of the blocking agent, microbial aggregation occurs and a large marcoparticle is formed, at which point the electrophoretic mobility of microbes is lost and the plug then migrates at the same speed and direction as the EOF. The EOF direction, under these conditions, is reversed as it flows towards the anode, as does the flow of the microbial sample plug and the blocking agent. Figure 7.2 shows the electropherograms obtained using this three-injection method. In Figure 7.2A only DMSO was dissolved in sample solution while in Figure 7.2B only the *C. albicans* were present in sample. It is clear that the *C. albicans* form a sharp peak in the front of blocking agent zone, which is away from the sample plug zone. The microbes can therefore be removed from sample plug that might contain neutral contaminants (e.g. DMSO in figure 7.2A).

7.3.2 Application to blood plasma sample

A similar experiment using the three injection method was performed with a real blood plasma sample spiked with the fungi *C. albicans* used instead of the microbial buffer sample plug of Figure 7.1. Figure 7.3 was a control run with a blank sample, i.e., blood plasma sample without *C. albicans*. The electropherogram shows that a small plasma interference peak was detectable at 6.5 min. The effect of adding Baclight^{TM} Green bacterial stain to the blood plasma

is shown in Figure 7.4. Exactly, 2 µL of Baclight ^{TM} Green bacterial stain dye was added to 1 mL of the blank blood plasma sample prior to the separation. The migration time of the interference peak remained the same. However, the peak width was substantial and the peak area was greatly enhanced (at least 50 times). This indicates that Baclight^{TM} Green bacterial stain could interact with components of the blood plasma. Figure 7.5A and 7.5B show the electropherograms of the three injection method using blood plasma spiked with different *C. albicans* concentrations ($3x10^8$ CFU/mL and $3x10^4$ CFU/mL, respectively). Results show that the plasma-dye interference peak was greatly reduced compared to Figure 7.4 and a single *C. albicans* peak was obtained in front of the interference peak on both electropherograms. These indicate that washing and dilution of blood plasma-dye microbial sample with working buffer in the experimental procedures is able to reduce the effect of interference peak prior to CE analysis (see 7.2.3 methods section).

In order to separate the microbial single peak and the plasma interference peak, varying the injection length of the spacer plug was performed. However, similar results were obtained where a single peak was always detectable in front of a small plasma interference peak with similar migration times. Based on these results, we hypothesized that blood plasma containing different kinds of proteins, albumins and peptides that could potentially be a surface active species was responsible for cellular aggregation. Therefore, another experiment without the use of a spacer plug and blocking agent was performed. Results show that a similar electropherogram was obtained where a single microbial peak was also obtained at about 7 min followed by a small interference peak. Apparently, when the voltage was applied in this situation, the microbial cells migrate towards the anode out of the blood plasma plug while the surfactant, CTAB, residing in the front of the microbe-containing plasma plug migrates towards the cathode. When the microbes encountered the cationic surfactants, they were dynamically coated by the surfactant. The microbes then reverse their migrating direction towards the cathode. Upon reaching the blood plasma segment, the microbes aggregated, lost their

electrophoretic mobility and formed a large marcoparticle in front of the blood plasma peak. The large macroparticle then migrated towards the anode with the EOF.

It is important to note that the actual concentrations of the fungi and bacteria in the original sample after 20-24 h incubation were about 3 x 10^8 CFU/mL. This concentration was higher than that in real infected blood plasma sample. Wain *et. al.* and Werner *et. al.* showed that concentration of bacteria in blood during bacteremia rarely exceeds 10^3 CFU/mL [271, 272]. As a result, the LOD for this method must be evaluated. Figure 7.6 shows the electropherogram of 3 x10^4 CFU/mL (10000x dilution from the original concentration) of *C. ablicans* concentration. With the physical limitation of our CE instrument, it requires a minimal sample volume of 0.5 µL for proper injection. Also, based on our previous studies, the optimal injection of sample (158 nL using 5 sec at 5 psi) was used [26]. Therefore, approximately five cells were injected per each separation.

If this method is to provide an alternative means for determining the presence or complete absence of microbial contamination in blood plasma, this method should be applicable to virtually any microorganism. However, Rodriguez *et. al.* showed that CTAB may lyse bacterial cells when the concentration exceeds 2 mg/mL, resulting in lower peak heights causing inaccurate results [273]. Conversely, higher CTAB concentration has no influence on fungi such as *C. albicans* probably due to protection by its cell wall [27]. Therefore, four different kinds of gram positive bacteria (*Brevibacterium taipei*, *Bacillus cereus*, *Bacillus subtilis,* and *Bacillus megaterium*) were examined with lower CTAB concentrations (1mg/mL) (see Figure 7.7). All bacterial peaks could also be obtained within 10 min using 1mg/mL of CTAB. These results indicate that this method is not only applicable to the detection of fungi but also on the detection of bacteria in blood plasma with the use of low concentration of CTAB. In order to specifically identify *C. albicans* from a blood sample, we, currently, are evaluating the herein described method with the use of capillary electrophoresis-fluorescence *in situ* hybridization (CE-FISH) to determine and quantify the C*. albicans* from a mixed-microbial blood sample.

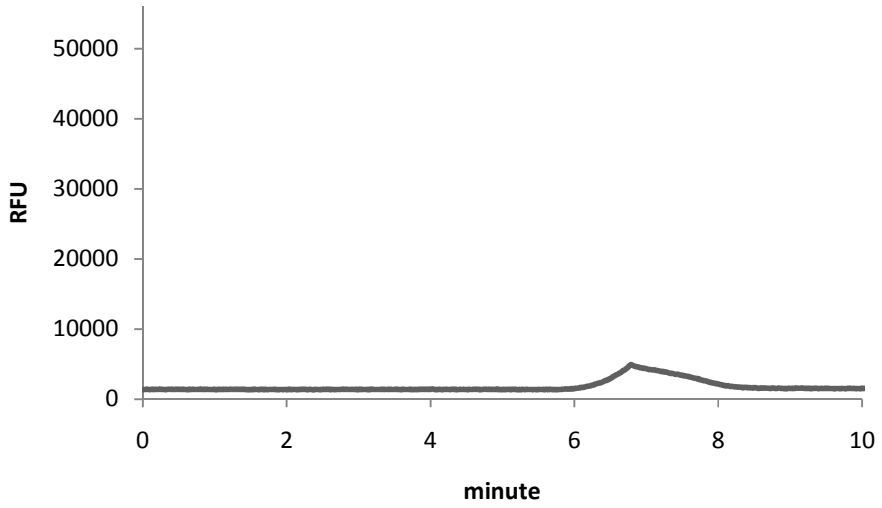


Figure 7.3 Electropherogram of a blank (blood plasma without microbes) using the three injection method. Conditions: run buffer: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; blocking agent: 10 mg/mL SBC-10 in run buffer; voltage: -3 kV; detection at 214 nm; see section 7.2.3 for details.

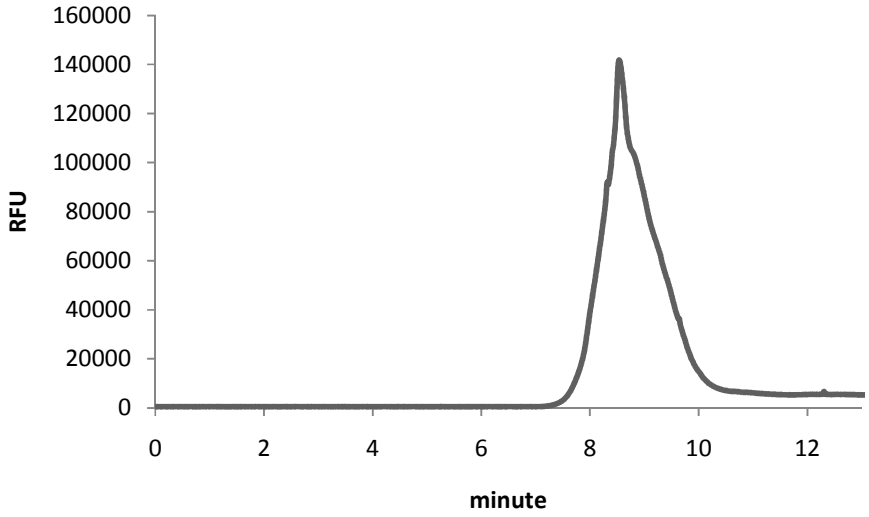
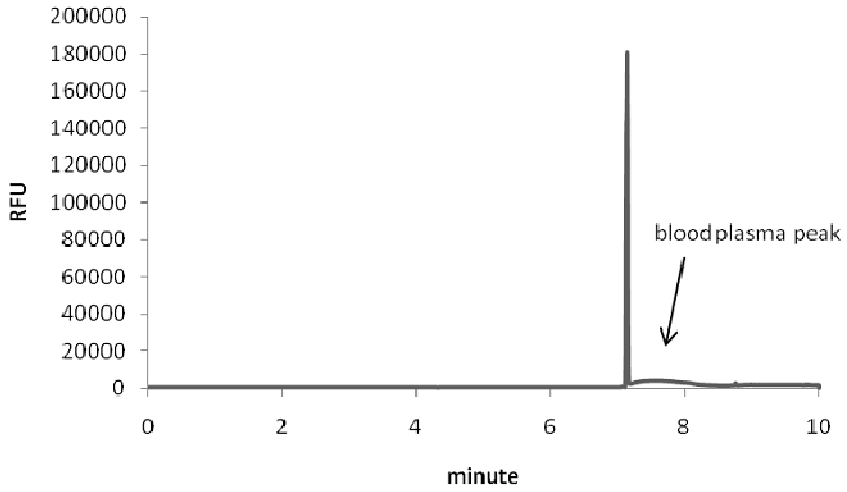


Figure 7.4 Electropherogram obtained for Baclight^{TM} Green stained blood plasma. Conditions: run buffer: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; blocking agent: 10 mg/mL SBC-10 in run buffer; voltage: -3 kV; detection at 214 nm; see section 7.2.3 for details.

(A)



(B)

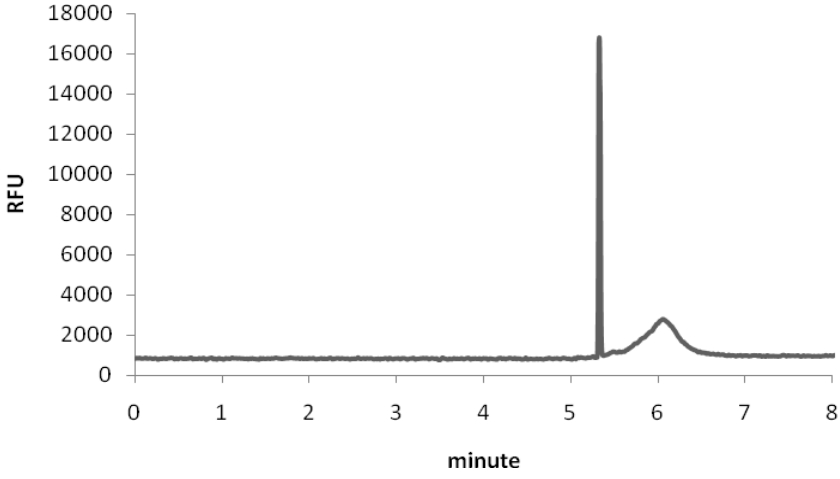


Figure 7.5 The electropherograms obtained with the three injection method for *C. albicans* in blood plasma. Samples contain: (A) $3x10^8$ CFU/mL *C. albicans*, approximate 48,000 cells/injection; (B) $3 x10^4$ CFU/mL *C. ablicans* , approximately 5 cells/injection. Prior to CE analysis, washing and dilutions of dye-interference plasma with working buffer was performed (see section 2.3 for details). Experimental conditions are the same as listed in Figure 7.3.

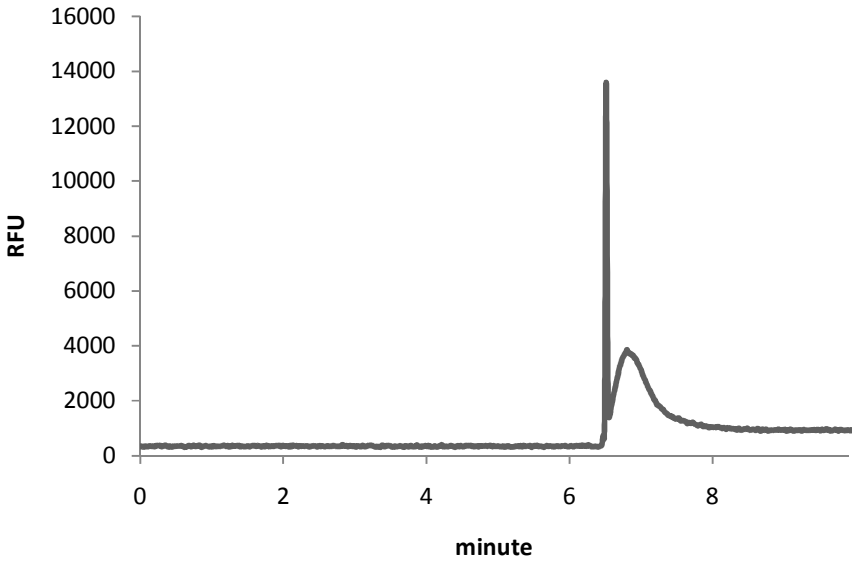


Figure 7.6 The electropherogram of *C. ablicans* in blood plasma (concentration: 3 x10^4 CFU/mL) using the self-focusing method without spacer segment and blocking agent plug. Experimental conditions: 1 mM TRIS/0.33 mM citric acid at pH 7 with 6 mg/mL CTAB; Sample buffer: 1 mM TRIS/0.33 mM citric acid at pH 7. See section 7.2.3 for details.

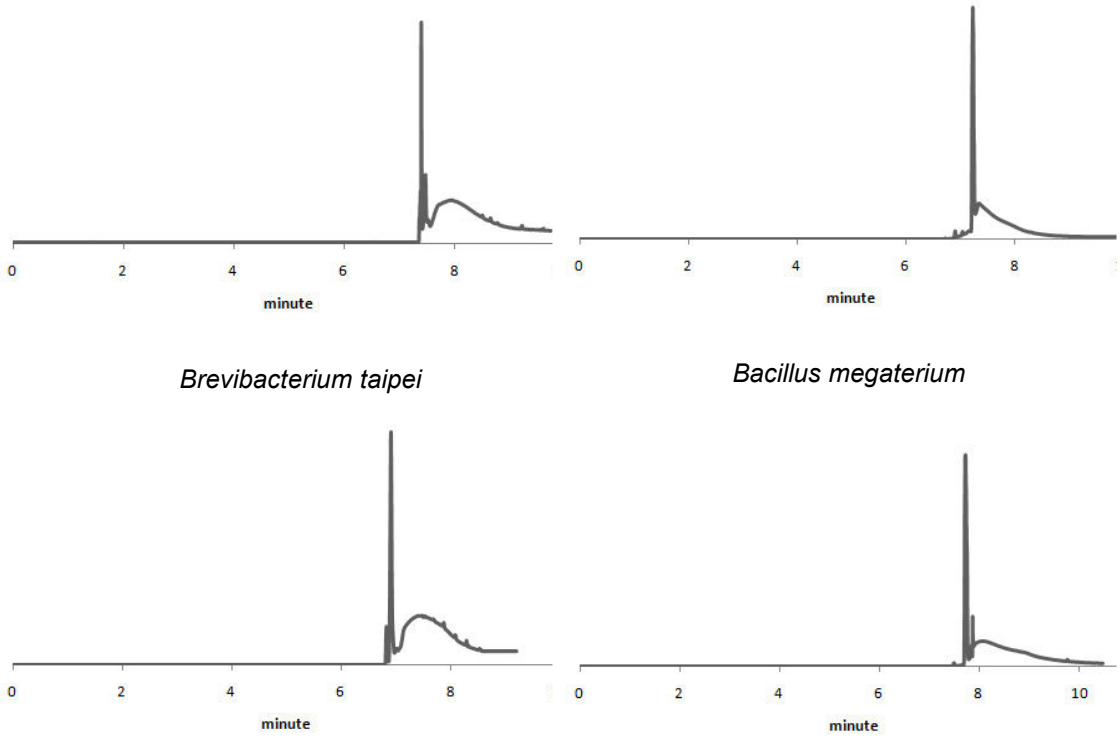


Figure 7.7 Four different bacteria in blood plasma using the self-focusing method without spacer segment and blocking agent plug. Conditions for all electropherograms are the same those listed in Figure 6 except that the CTAB concenration was 1 mg/mL. See section 7.2.3 for details.

7.4 Concluding remarks

A rapid detection method for determining the presence or complete absence of microorganisms in a real biological sample is needed. In this study, a simple, fast detection approach without the use of spacer and blocking agent was examined to provide a quick answer for presence/complete absence of microbes in blood plasma sample within 10 min. Results show that blood plasma containing various kinds of blood plasma proteins and peptides is capable of aggregating microbial cells to form a single sharp peak in front of the plasma interference peak in CE experimental conditions. In order to prevent lysing of cells, CTAB concentration as low as 1mg/mL was also able to sweep all bacterial cells, while fungi cell required at least 5mg/mL, to form a single peak in front of the blood plasma peak. The LOD of approximate 5 cells per injection was able to be detected using this approach.

7.5 Acknowledgements

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CHAPTER 8

USE OF CAPILLARY ELECTROPHORESIS FOR THE DETERMINATION OF BINDING CONSTANTS

In the past two decades, capillary electrophoresis (CE) has been used frequently for the measurement of apparent binding/association constants. CE has numerous advantages, including short analysis times, low sample consumption, simplicity of operation etc. This review provides a fundamental introduction to binding theory and then summarizes recent applications and advances in the field of CE-based methods for the evaluation of molecular association. The time period for this survey is from 2002 (when our previous review was published) to the present.

8.1 Introduction

Non-covalent molecular interactions in solution between molecular substrates and ligands, such as protein/DNA-drug, antibody-antigen, peptide-antibiotics, exist widely in chemical and biological systems. A knowledge of association/dissociation/binding constants is crucial to the understanding of molecular interactions and can be useful in the development of new drugs and effective treatments for diseases [274].

Many techniques have been developed to measure binding constants. A thorough review on the fundamentals of molecular association and techniques to measure binding constants was given by Connors [34] over 20 years ago. Spectroscopic approaches (including UV, NMR, fluorimetry, refractometry, *et al.*), solubility measurement, potentiometry, calorimetry, liquid-liquid partition, dialysis, and chromatography (including gas/liquid chromatography and capillary electrophoresis) are frequently used [34] . They are based on the differences in the properties of bound and unbound ligands/analytes.

Capillary electrophoresis (CE) has well known advantages as a separation technique such as high efficiency, short analysis time, low sample consumption, simple instrumentation and a generally low cost. These benefits can also be advantageous when using CE for the determination of binding constants [209-211, 275]. Generally, a series of experiments are carried out using a fixed amount of the substrate and varying amount of the ligand or vice versa. Binding constants can be determined by monitoring the change in either mobility, peak area, peak height or relative migration time ratio (RMTR). Rundlett and Armstrong first reviewed [209] different CE-based methods for evaluating binding constants [209-211], and explained the fundamentals and different experimental approaches for 1:1 binding systems. A analogous review including mathematical data analysis and experimental considerations was given by Tanaka and Terabe [276]. Several other reviews on evaluation of binding/association constants using CE have been published with different focuses [274, 275, 277-283]. This review gives a basic introduction to binding theory and summarizes different CE-based methods for the determination of binding constants as well as recent studies and advances from 2002 to the present time. It should be understood that the optimal experimental approach for determining binding constants by CE are often dictated by: (a) the kinetics of the binding equilibrium, (b) the size of the binding constants, (c) the detectability of the ligand and substrate, (d) the amount of ligand and/or substrate available for the experiment and (e) the mobility difference between the complex and uncomplexed species. Further it should be noted that the binding constants measured under the conditions discussed are the apparent binding constant derived from apparent substrate and ligand concentrations, not true thermodynamic binding constants. Other hyphenated techniques such as mass spectrometry (MS) coupled with CE [34], where MS was used for separation and quantification, are beyond the scope of this review.

8.2 Binding fundamentals

The theory of binding has been introduced previously [209, 211, 274, 284]. For a 1:1 binding scenario, the binding constant K between the substrate, S and the ligand, L is defined as:

$$K = \frac{[SL]}{[S][L]}$$

where [SL], [S] and [L] represents the equilibrium concentrations of complex SL, free substrate S and free ligand L.

Therefore, the ratio of bound substrate over total substrate r can be expressed as:

$$r = \frac{[SL]}{[S]+[SL]} = \frac{K[L]}{1+K[L]} \qquad (1)$$

Bound substrate ratio r varies from zero, when there is no ligand present to one when the substrate is saturated by the ligand and only exists as complex SL. This bound substrate ratio r can be replaced by an experimental response R, when the system response is weighted response of free substrate and complex. Equation (1) can be expressed as the binding isotherm:

$$\frac{\Delta R}{\Delta R_{max}} = \frac{R - R_f}{R_C - R_f} = \frac{K[L]}{1+K[L]} \qquad (2)$$

where R is the system response, R_f and R_c are the system response of free substrate and complex. The system response can be a mobility shift in affinity capillary electrophoresis (ACE) [209, 285-288], relative migration time ratios in partial filling ACE [289-294], retention factor k in chromatography [295-297], chemical shifts in NMR [298], absorbance shifts in UV-vis [299], etc. However, this response change must only be caused by the association between the substrate and ligand. Some CE system responses, such as mobility, can also be affected by other factors such as viscosity, ionic strength, and interactions with the capillary wall [1]. These factors should be eliminated or quantified so that the mobility can be corrected.

Equation (2) can be rearranged to the following linear forms:

$$\frac{1}{\Delta R} = \frac{1}{\Delta R_{max} K[L]} + \frac{1}{\Delta R_{max}} \qquad (3)$$

$$\frac{[L]}{\Delta R} = \frac{1}{\Delta R_{max} K} + \frac{[L]}{\Delta R_{max}} \qquad (4)$$

$$\frac{\Delta R}{[L]} = -K\Delta R + K\Delta R_{max} \qquad (5)$$

Binding constants can be estimated by plotting with linear equations (3)-(5) as well as fitting the experimental data to the binding isotherm [equation (2)] using nonlinear least-square curve-fitting methods. The linear equations (3)-(5) have been called the double reciprocal, the y reciprocal and the x reciprocal plotting methods respectively. They have been called different names in literature. The double reciprocal plot is also called the Benesi-Hilderbrand plot in spectrophotometry and the Lineweaver-Burk plot [211, 276]. The x reciprocal plot is known as Eadie plot in enzyme kinetics or Scatchard plot in protein binding studies [211, 276]. Although equation (3)-(5) are just algebraic rearrangement of equation (2), they have different statistical weights of data points and may not produce results of equivalent accuracy and/or precision [211, 283, 300, 301]. Monte Carlo simulation was used to assess the accuracy and precision using nonlinear regression and the three linear plotting methods [300, 302]. It was concluded that nonlinear regression of the binding isotherm gives the most accurate and precise estimation[300]. Weighting formulas using the effective variance approach also were derived for the least squares analysis of data by linear equations [301].

An alternative plotting method, which is similar to the titration of acid/base to obtain pK_as / pK_bs, was described by Li *et al.*[303]. Equation (5) can be rearranged to:

$$\frac{\Delta R}{\Delta R_{max} - \Delta R} = K[L] \qquad (6)$$

Taking the logarithm of equation (6) and rearranging gives:

$-\log [L] = \log K - \log \{\Delta R/(\Delta R_{max}-\Delta R)\}$

If $y= \log K - \log \{\Delta R/(\Delta R_{max}-\Delta R)\}$, then the first derivative (y') and the second derivative (y'') can be described as:

$y'=-\Delta R_{max}/\{\Delta R\ (\Delta R_{max}-\Delta R)\}$

$y''=\Delta R_{max}\ (\Delta R_{max}-2\Delta R)\ /\{\Delta R^2\ (\Delta R_{max}-\Delta R)^2\}$

Making $y''=0$ gives

$\Delta R= \Delta R_{max}/2$ (7)

Substituting equation (7) to (6) gives

$K=1 / [L]$ (8)

That is to say, K is the reciprocal of [L] at the inflexion point of the curve which is plotted with ΔR as a function of log [L] [303]. All of the above equations are relevant for 1:1 binding scenarios and the following discussion is mainly focused on these cases. However, in biological systems, such as protein-drug interaction, there are often multiple interaction types and multiple binding sites of same type of interaction [274, 275, 278]. Assuming there are *m* types of binding, n_i binding sites for each type of binding and each binding site does not affect the bindings at the other sites, the binding isotherm of such an interaction can be commonly expressed as [274, 284, 304]:

$$r= \sum_{j=1}^{m} \frac{n_j K_j [L]}{1+K_j [L]} \quad (9)$$

where r is the ratio of bound substrate and total analytical substrate concentration. Again r can be replaced by appropriate experimental responses. Binding constants can be estimated by nonlinear fitting of the binding isotherm equation (9). However, data analysis can be very complicated and a knowledge of the binding stoichiometry is preferred for better fitting to obtain accurate results [305-307].

Among above plotting methods, the nonlinear binding isotherm (equation (9)), x reciprocal plot and the titration curve plot were reported for multivariate binding interactions [305-315].

The binding constant can also be calculated from kinetic parameters. For the association of substrate S and ligand L:

$$S + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SL$$

The binding constant K can be calculated from the ratio of the rate constants k_1 and k_{-1}, of forward and reverse reactions, respectively. The rate constants can be determined by capillary electrophoresis [281, 316-319].

CE can also be used to measure thermodynamic parameters to calculate the binding constants [287]. This topic will not be covered in this review.

8.3 CE-based methods

Based on the kinetics of the equilibrium, CE-based methods can be classified in three modes:

(1) dynamic equilibrium CE with fast kinetics. This is the most common mode as most systems belong to this category (see Table 8.1). In this mode, the relaxation time τ << CE migration time. The relaxation time τ is defined as the time required to the exponential variable (i.e. the concentration of substrate) to decrease to 1/*e* (0.368) of its initial value. CE methods in this mode include ACE, Hummel-Dreyer method (HDM), vacancy affinity CE (VACE), vacancy-peak method (VPM) and frontal analysis (FA) [209, 284].

(2) pre-equilibrated mode with slow kinetics where the relaxation time τ >> migration time. In this mode, the sample is pre-incubated to reach equilibrium before analysis and any formation and dissociation of the complex are assumed to be negligible[275]. Direct separation

method using CZE (also called preincubated CZE)[275] and frontal analysis (FA) are suitable for systems in this mode.

(3) kinetic mode with intermediate kinetics where where the relaxation time τ ~ migration time. This mode is much more complicated and will be discussed briefly in section 8.3.6.

As the principles of ACE, HDM, VACE, VPM, FA and direct separation method were thoroughly discussed in previous reviews [209, 276, 284], only the applications and advances of each method from 2002 to present will be discussed in detail. A summary is given in Table 8.1 and a comparison of CE methods is given in Table 8.2. Table 8.2 can be used as a general guide for the choice of a method.

8.3.1 ACE and partial filling ACE

ACE is the most simple and frequently used method (see Table 8.1) for the estimation of binding constants. In ACE, the sample contains fixed amounts of substrate and the running buffer contains varying amounts of ligand. Upon electrophoresis, the electrophoretic mobility of the substrate is monitored and analyzed as the system response in equation (2)-(5) for 1:1 binding. For best results, data points are fitted to these equations using least squares methods.

Partial filling ACE (PFACE) and its modification were studied extensively by Gomez and co-workers [289-292, 294, 320-328]. These techniques are illustrated clearly by this group's work and in a previous review [209]. In these techniques, only part of the capillary is filled with running buffer that contains ligand. These techniques enable the use of ligands with UV chromophores. In the previous few years, this technique has expanded to include different variants including PFACE, flow-through PFACE (FTPFACE), on-column ligand derivatization ACE (OCLDACE), on-column receptor ACE (OCRDACE), multi-step injection PFACE (MSIPRACE) [292] etc.

In ACE the total ligand concentration, in place of free ligand concentration used in equation (2)-(5), is used for the calculation, which can cause a systematic error. Usually the

ligand concentration is 10 to 100 times higher than that of the substrate to minimize system errors and to cover the entire span of the binding isotherm / linear fitting curve to minimize error propagation from measurements [211]. As discussed previously, mobility also can be affected by other factors such as wall interaction, viscosity and ionic strength. Dribek *et al* [329] used a non-covalent coating of poly(ethylene oxide) (PEO) to suppress the peptide adsorption to the wall. The impact of viscosity can be negated by a correction factor, which is the relative viscosity ν [329-332]:

$$\nu = \eta / \eta^0$$

η and η^0 are the viscosities of the running buffer with and without ligand, respectively. When the ligand is an ionic species, the mobility can be corrected with the extended Debye, Hückel and Onsager (DHO) theory introduced by Falkenhagen *et al.* [333] and Pitts [334]:

$$\mu = \mu_0 - \left[\frac{8.204 \times 10^5}{(\varepsilon_T T)^{2/3}} + \frac{4.275}{\eta(\varepsilon_T T)^{1/2}} \right] X \frac{I^{1/2}}{1 + 50.29\alpha(\varepsilon_T T)^{-1/2} I^{1/2}}$$

Where μ_0 is the mobility at zero ionic strength ($10^{-9} m^2 V^{-1} S^{-1}$), ε_T is the relative permittivity of the solvent, η is the solvent viscosity (Pa s), T is absolute temperature and α (in Å) is the distance of the closest approach between the central ion and the ions of its ionic atmosphere.

The inconsistencies in the electroosmotic flow (EOF) and variations of electrophoretic mobility due to fluctuations in the voltage and current in a single run also can be a problem. A reliable way to minimize these factors is to use reference standards to obtain relative migration time ratios (RMTR)) [289-291, 293, 294, 322, 335-343] instead of mobility. The use of RMTR allows a voltage gradient to be used for accurate estimation of binding constants [326].

Table 8.1 Summary of binding constant studies from 2002 to the present time

System	CE based-method[a)]	Plotting method	System response [b)]	Refer-ence
Phosphinate in phosphnic pseudopeptides with H+	ACE	Titration curve	Mobility	[344]
Procaine with β-CD	ACE	Titration curve	Mobility	[303]
D-Ala-D-Ala peptides with teicoplanin	ACE	Scatchard plot	RMTR	[336]
Enediynes with bovine serum albumin	ACE	Scatchard plot	Mobility	[287]
Four peptides with a poly(lactic-coglycolic acid)-based polymer (PLGA-BP)	ACE	Nonlinear binding isotherm & three linear plots	Mobility	[288]
D-Ala-D-Ala peptides with ristocetin A	ACE	Scatchard plot	RMTR	[337]
Retinoic acid with β-lactoglobulin B (protein)	ACE	Nonlinear binding isotherm & three linear plots	Mobility	[345]

a) ACE: affinity capillary electrophoresis; FA: frontal analysis; FACCE: frontal analysis continuous capillary electrophoresis; PFACE: partial filling affinity capillary electrophoresis; FTPFACE: flow-through partial filling technique; OCLDPFACE: on-column ligand derivatization partial filling affinity capillary electrophoresis; OCRDPFACE: on-column receptor derivatization partial filling capillary electrophoresis; MIACE: multiple-injection affinity capillary electrophoresis; MSLIPFACE: multiple-injection partial filling capillary electrophoresis; VGPFACE: voltage gradient partial filling affinity capillary electrophoresis; HMD: Hummel-Dreyer method

b) RMTR: relative migration time ratio

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Acid herbicide with vancomycin	ACE	Nonlinear binding isotherm Multivariate binding	Mobility	[311]
Macrocyclic dodecapeptides bacteracin A_1 with proton and zinc ion	ACE	Titration curve & nonlinear binding isotherm Multivariate binding	Mobility	[305]
Macrocyclic dodecapeptides bacteracin A_1 with zinc ion in water-2,2,2-trifluoroethanol	ACE	Nonlinear binding isotherm	Mobility	[285]
Green fluorescent protein-labeled almodulin with Ca^{2+} and calmodulin-binding protein	ACE	Scatchard plot	RMTR	[338]
Azidothymidine (AZT) with Yeast baker RNA	ACE	Scatchard plot Multivariate binding	Mobility	[312]
Ni^{2+}, Cu^{2+} and Zn^{2+} (ligand) with mcrocyclic dodecapeptide bacteriacin A_1	ACE	Nonlinear binding isotherm	Mobility	[346]
Enzyme (ADP-glucose pyrophosphorylase) and its altered forms with its substrate and activator	ACE	Scatchard plot	RMTR	[339]
Hydrophobically end-capped poly(ethylene glycol)s with highly sulfated β-cyclodextrin	ACE	Nonlinear binding isotherm & three linear plot	Mobility	[347]
Low-molecular-weight heparin, porcine mucosa heparin and heparin sulfate with amyloid precursor protein	ACE	Scatchard plot & nonlinear binding isotherm	Mobility	[348]
Bovine serum albumin with its antibody; prion proteins and their specific antibody	ACE	Scatchard plot	Mobility	[349]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Aromatic phosphonic acids with ammonium and potassium cations	ACE	Nonlinear binding isotherm	Mobility ratio	[350]
Alprostadil withα -cyclodextrin	ACE	Nonlinear binding isotherm	Mobility	[351]
N,N-diacetyl Lys-D-Ala-D-Ala with vancomycin, teicoplanin, ristocetin	ACE	x-reciprocal plot	Mobility	[286]
Amino compounds with cucurbit[n]uril(n=6,7) complexes	ACE	Nonlinear binding isotherm	Mobility	[352]
Alkylnaphthalene derivatives with carboxymethyl-β-cyclodextrin	ACE	x-reciprocal plot Multivariate binding	Mobility	[313]
Oral malodorous compounds and odor precursors with native and derivatized cyclodextrins	ACE	x-reciprocal plot	Mobility	[31]
Six napthalenesulfonate derivatives with β-cyclodextrin simultaneously	ACE	Nonlinear binding isotherm & three linear plot	Mobility	[332]
Ca2+, Mg2+ and heparin (single site) with human serum amyloid P component	ACE	Nonlinear binding isotherm	Mobility	[353]
Vasoactive intestinal peptide with poly(amidoamine) dendrimers	ACE	Nonlinear binding isotherm	Mobility	[329]
Carbonic anhydrase B with ligand 4-carboxy-benzenesulfonamide	ACE	Nonlinear binding isotherm	Mobility	[354]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Polyethylene glycol vancomycin derivatives with D-Ala-D-Ala terminus peptides	ACE	Scatchard plot	RMTR	[355]
N-acetyl-D-Ala-D-Ala with vancomycin and ristocetin, carbonic anhydrase B with arylsulfonamide	ACE	Nonlinear binding isotherm & three linear plots	Mobility and retention factor k	[356]
Single-stranded nucleic acid and oligonucleotide with metal ions	ACE	Nonlinear binding isotherm; multivariate binding	Mobility	[314]
Several fatty acids with β-cyclodextrin	ACE	Nonlinear binding isotherm & three linear plots	Mobility	[330]
Five polycyclic aromatic hydrocarbons with human serum albumin	ACE	Scatchard plot	Mobility	[357]
Diclofenac sodium and bovine serum albumin	ACE	Scatchard plot	Mobility	[358]
Sulfated polysaccharide 916 with human serum albumin	ACE	The double reciprocal plot	RMTR	[342]
Valinomycin with lithium	ACE	Nonlinear binding isotherm	mobility	[331]
Indomethacin with β-cyclodextrin	ACE	Nonlinear binding isotherm & three linear plots	Mobility	[359]
Antithrombin with heparin fragments	ACE and FACCE	Nonlinear binding isotherm & three linear plots	Mobility & concetration	[360]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Acid-rich diketopiperazine receptors withbasic tripeptides	ACE on a microchip	Nonlinear binding isotherm; multivariate binding	Mobility	[309]
Drugs with sulfated β-cyclodextrin	ACE on microchip	Nonlinear binding isotherm	Mobility	[304]
Ca2+ and Na+ with proteins	Competitive ACE	Titration curve multivariate binding	Mobility	[310]
Folate vs heparin with bovine folate-binding protein; relative binding strength	Competitive ACE	NA	NA	[361]
Neodymium with fulvic acid	Competitive direct separation	Nonlinear binding isotherm	Concentration	[362]
Neutral phenols and 2-nathtalenesulfonate with β-CD	Competitive FACCE in capillary and microchip	Nonlinear binding isotherm	Bound ratio	[363]
Carbonic anhydrase B with neutral arylsulfonamides	competitive FTPFACE	Scatchard plot	RMTR	[289]
Carbonic anhydrase B with neutral arylsulfonamide	Competitive FTPFACE	Scatchard plot	RMTR	[364]
CBH 1-cellubiose with protein Cellulase Cel 7A	Competitive PFACE	Nonlinear binding isotherm (with Kd)	Mobility ratio	[365]
trans-activation response (TAR) element, risidues 1-59 of HIV-1 mRNA with four potential anti-HIV drugs	Direct separation	K calculated from definition equation	Concentration	[366]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Taxol with calf thymus DNA	Direct separation	Scatchard plot	Peak height	[367]
Antitumer drug indazolium [trans-tetrachlorobis(1 H-indazole)ruthenate(III)] with human serum albumin and transferrin	Direct separation	Nonlinear binding isotherm multivariate binding	Bound ratio	[306]
Heparin with programmed cell death 5 (PDCD5)	Direct separation	Scatchard plot	Bound ratio	[368]
Human Gc-globulin (vitamin D-binding protein) and its isoforms with G-acetin	Direct separation	Nonlinear binding isotherm	Bound ratio	[369]
Thrombin (protein) with Thrombin-binding aptamer	Direction separation on a microchip	Nonlinear binding isotherm	Bound ratio	[370]
Drug amlodipine and its enantiomer levamlodipine with human plasma albumin and bovine serum albumin	FA	Scatchard plot and klotz equation	Bound ratio	[371]
Dexamethasone and albumin	FA	Double reciprocal plot	Bound ratio	[372]
Integrin fragments and RGD-based peptides	FA	Transformed x-reciprocal	Bound ratio	[373]
$\alpha_v\beta_3$ integrin with arginin-glycin-aspartic-acid (RGD) containing fluorescently labeled cyclic peptide	FA	Nonlinear binding isotherm multivariate binding	Bound ratio	[307]
Dexamethasone with human serum albumiin	FA	Double reciprocal plot multivariate binding	Bound ratio	[315]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
B-lactoglobulin (protein) with poly(vinylsulfate) (polyanion)	FACCE	Nonlinear binding isotherm, multivariate binding	Bound ratio	[308]
Carbonic anhydrase B with arylsulfonamides and D-Ala-D-Ala terminus peptides with vancomycin and ristocetin	FTPFACE	Scatchard plot	RMTR	[322]
D-Ala-D-Ala peptides with vancomycin; arylsulfonamides with carbonic anhydrase B	FTPFACE, Competitive FTPFACE, OCLDPFACE, MSLIPFACE	Scatchard plot	RMTR	[291]
Carbonic anhydrase B with vancomycin and teicoplanin	MIACE	Scatchard plot	RMTR	[340]
D-Ala-D-Ala peptides with Glycopeptide antibiotics vancomycin, ristocetin and teicoplanin	MIACE	Scatchard plot	RMTR	[293]
Fmoc-(Gly, Ala, Val, and Phe)-D-Ala-D-Ala peptides with vancomycin, ristocetin and teicoplanin	MIACE	Scatchard plot	RMTR	[341]
D-Ala-D-Ala peptides with vancomycin and teicoplanin; Carbonic anhydrase B with arylsulfonamides	MIPFACE	Scatchard plot	RMTR and mobility	[327]
Platinum anticancer drugs with serum transport proteins	Modified HMD	Nonlinear binding isotherm	Bound ratio	[374]
Fmoc-amino acid –D-Ala-D-Ala peptide derivatives with ristocentin and teicoplanin	OCLDACE	Scatchard plot	RMTR	[335]
D-Ala-D-Ala peptides with derivatized teicoplanin and ristocetin	OCRDPFACE	Scatchard plot	RMTR	[290]

Table 8.1- *Continued*

System	CE based-method	Plotting method	System response	Refer-ence
Glycopeptide antibiotics with D-Ala-D-Ala terminus peptides. An overview of the group's previous work on PFACE	PFACE and its variations	Scatchard plot	RMTR	[292]
Carbonic anhydrase B with vancomycin	PFACE on a microchip	Nonlinear binding isotherm	RMTR	[328]
Protein (Human serum albumin and α_1-acid glycoprotein)-drug system	Pressure-assisted FA/FA	Nonlinear binding isotherm	Bound ratio	[375]
Carbonic anhydrase B with arylsulfonamides; vancomycin with D-Ala-D-Ala terminus peptides	Voltage-gradient PFACE MIVGPFACE	Nonlinear binding isotherm	RMTR	[326]

Table 8.2 Comparison of different CE approaches for determining binding constants for fast kinetic systems [a)]

CE methods	Advantages	Requirements and disadvantages
Affinity capillary electrophoresis (ACE)	Simple experimental setup and easy data analysis; most frequently used	Substrate should be UV absorbant and ligand should be UV transparent; significant charge/mobility difference between free substrate and complex; approximation of total ligand concentration to free ligand concentration can introduce error; limited binding constant range (10-104)
Vacancy peak method (VACE)	Similar to ACE	Ligand should have UV absorbance; others requirements are similar to ACE
Partial filling affinity capillary electrophoresis (PFACE)	Suitable for ligands with UV absorbance; elimination of error due to fluctuation or EOF, voltage, temperature between and with runs by using dual marker; can be coupled with online substrate/ligand derivatization	More complicated instrument setup than ACE; additional injection steps require accurate instrument control of pressure; approximation of total ligand concentration to free ligand concentration can introduce error; limited binding constant range
Hummel-Dreyer method (HDM) and vacancy peak method (VPM)	Suitable for ligands with UV absorbance; concentration estimated from calibration curve; no approximation of total and free ligand concentration provide more accuracy results	Estimation of peak area may introduce error; internal or external calibration curve required
Frontal analysis (FA) and frontal analysis continuous capillary electrophoresis (FACCE)	Suitable for systems with high binding affinities (103-108)[376]; concentration estimated from calibration curve; no approximation of total and free ligand concentration which provides more accuracy	Calibration curve required; only applicable to systems (eg. protein-drug) with similar substrate and complex mobilities and significantly different ligand mobility
Competitive CE combined with above methods	Extend the applicability to the systems with similar substrate, ligand and complex charge/mobility	A secondary equilibrium system as a indication of interaction between target substrate and ligand; complicated experimental setup and data analysis

a) Note that the optimal experimental approach for determining the binding constants by CE are often dictated by: (a) the kinetics of the binding equilibrium,(b) the size of the binding constants, (c) the detectability of the ligand and substrate, (d) the amount of ligand and/or substrate available for the experiment and (e) the mobility difference between the complex and uncomplexed species

Multiple injection methods coupled with ACE, PFACE and FTPFACE were described [291, 293, 323, 326-328, 340, 341]. In multiple-injection ACE [340], a sample plug containing a non-interacting standard is injected followed by multiple plugs of sample containing the substrate and then a final injection of sample containing a second standard. Between each injection of sample, a sample plug of buffer containing increasing concentration of ligand is injected. Then the voltage is applied and the samples undergo electrophoresis with buffers containing ligands with increasing concentrations. Multiple injection formats coupled with other ACE variations are operated in a similar manner [291, 293, 323, 326-328, 340, 341]. Using this approach, analysis times are greatly decreased [291, 293, 323, 326-328, 340, 341]and the effects of fluctuations in the EOF and voltage are minimized.

Competitive binding methods with a second substrate or ligand have been reported [289, 291, 326, 363-365] for systems which have small or no system response shifts for the substrate and complex.

An alternative plotting method similar to the titration approach coupled with ACE has been used to determine the dissociation constant between a proton and phosphinate group in phosphinic pseudopeptides [344], the binding constant for the inclusion complex between procaine and β-cyclodextrin [303] and metal ions to high affinity sites of calcium-containing proteins [310].

8.3.2 Hummel-Dreyer method

Hummel-Dreyer method (HMD) is mostly used for protein-drug systems. In the HMD approach, a drug of varying concentrations is added to the running buffer, creating a high background signal. The protein is dissolved in the sample solution before injection. A negative peak corresponding to the bound drug will be observed. The bound drug concentration can be calculated with an external or internal calibration method, which in turn can be fitted into a binding isotherm for estimation of binding constants [209, 276, 284].

A modified HDM was used to determine the binding constants and stoichiometry for a drug-protein system with very slow kinetics and multiple protein binding sites [374]. Sample mixtures containing fixed amounts of proteins and varying concentrations of drug were incubated 48 hours before CE analysis. In order to obtain accurate bound drug concentrations, an internal calibration curve was constructed and a conversion factor was used to calculate the $[D_{bound}]$[284, 374].

8.3.3 Vacancy peak method and vacancy affinity CE

In the vacancy peak method and vacancy affinity CE, the running buffer contains the substrate and varying amount of ligand, resulting in a high background signal [209, 276, 284]. Then a neat buffer plug was injected, causing two negative peaks. One of the negative peaks corresponds to the free ligand. The peak area can be converted to free ligand concentration and fitted into the binding isotherm [209, 276, 284]. For VACE, the mobility shift is used as the system response with equation (2)-(5) [209, 276, 284].

8.3.4 Frontal analysis (FA)

Frontal analysis is both used for dynamic equilibrium CE [274] and pre-equilibrated CE [275] modes. In frontal analysis technique, a long plug of pre-equilibrated mixture of substrate and ligand are injected and electrophoresed with neat buffer [209]. Frontal analysis continuous CE (FACCE) combines the sample injection and separation steps into one [377]. FA and FACCE are also frequently used in protein-drug systems. The electrophoretic mobility of the drug must be different from the protein and the complex. For dynamic equilibrium systems the mobility of the protein and the complex are assumed to be the same. Upon electrophoresis the free drug will migrate out of the sample plug, forming a plateau [209]. The plateau height of the drug with varying concentrations of protein can be treated as the system response or converted to concentration for fitting to a binding isotherm [209]. The advantage of frontal analysis is that it is insensitive to changes or fluctuations in migration times, EOF and applied voltage [275].

External air pressure was used by Jia *et al.* [375] to shorten the analysis time, prevent protein loss and achieve a better drug plateau.

The use of magnetic microbeads and CE for the determination of binding constants between teicoplanin and D-Ala-D-Ala terminus peptides also was reported. In this approach, an aliquot of the peptide was incubated with varying amount of magnetic beads with covalently attached teicoplanin[378]. Then supernatant was separated from the magnetic microbeads with an external magnet and then subjected to CE with setup similar to frontal analysis [378]. The peak height was used to quantify the free peptide concentration in the supernatant, which in turn was analyzed via Scatchard plot [378]. Technically CE coupled with UV detection was only used as a quantification approach. Therefore, it does not fall in the category of frontal analysis.

8.3.5 Direct separation method

The direct separation method is used for systems with slow kinetics, typically with proteins [306, 362, 366, 369, 370] or DNA/RNA [366, 367, 379]. The substrate and ligand are pre-incubated and then subjected to CE separation. The concentrations can be quantified with an external calibration curve. Usually the bound ratio of substrate or ligand is used for data analysis [306, 308, 368-371, 375].

8.3.6 Kinetic methods

A series of kinetic CE methods using different experimental settings and data-analysis strategies, where kinetic parameters are obtained to measure binding constants were developed [281, 316, 317, 380-390].

A capillary zone electrophoresis (CZE) of equilibrium mixture (NECEEM) approach for the determination of binding constant and rate constants of systems with intermediate kinetics was introduced by Krylov and Berezovski [381] in 2003. The experimental setup is the same as with the direct separation method, where a pre-incubated mixture is injected and then electrophoresed with plain buffer. During the separation the complex dissociates, forming an electropherogram similar to the one depicted in Figure 8.1. It is assumed that the equilibrium

fractions of substrate and ligand are separated immediately from the complex zone and the middle section of the electropherogram is only due to the dissociation of complex [381].

8.4 Computer simulation studies

Carefully controlled experimental observations combined with computer simulations were frequently used to explore the theory of CE methods [249, 391-406]. Computer simulation for binding studies are frequently based on the mass transfer of substrates, which can be described by a set of two diffusion-convection-reaction equations or their variations [402-404, 407-409]:

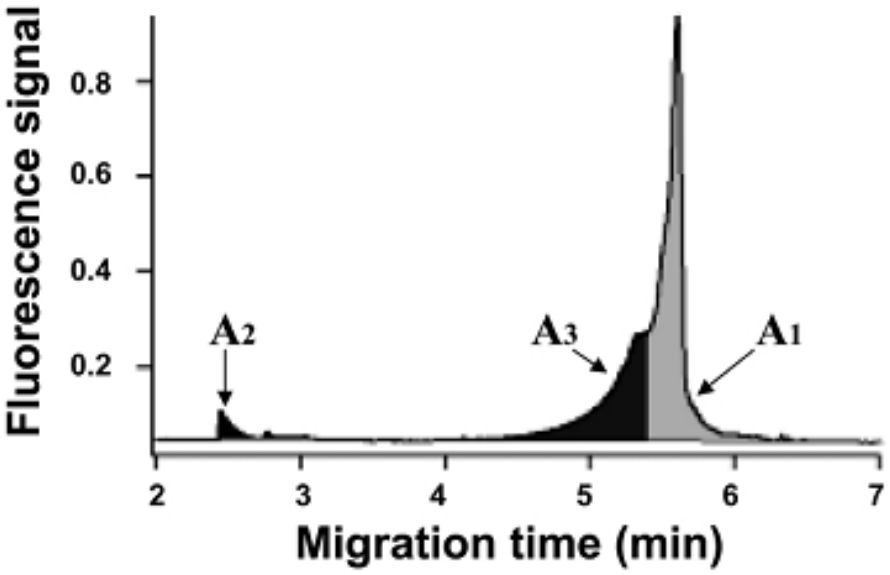

Figure 8.1 Determination of binding constant from determination of rate constants of forward and reverse reactions using non-equilibrium capillary electrophoresis of equilibrium mixture (NECEEM) method. Reprited from [381] with permission.

$$\frac{\partial C_1}{\partial t} = D_1 \frac{\partial^2 C_1}{\partial x^2} - v_1 \frac{\partial C_1}{\partial x} - k_1 C_1 + k_2 C_2$$

$$\frac{\partial C_2}{\partial t} = D_2 \frac{\partial^2 C_2}{\partial x^2} - v_2 \frac{\partial C_2}{\partial x} + k_1 C_1 - k_2 C_2$$

where C designates concentration, D represents the diffusion coefficient, and v is the electrophoretic velocity, k_1 and k_2 are the forward and reverse rate constant, respectively, subscripts 1 and 2 designate the free substrate and complex, respectively. Different assumptions are made under different conditions. The peak shape under different values of parameters [403] and different substrate, complex and additive mobility orders [410] were simulated. Better accuracy for estimation of binding constants was obtained by converging 2-D curves using enumeration algorithm of possible combinations of binding constant and complex mobility for experimental data[409]. Commonly used CE methods including ACE, Hummel-Dreyer method, vacancy affinity CE and frontal analysis were also successfully simulated and showed remarkable resemblance to experimental data.

8.5 Miniaturization

Miniaturization is a continuing goal in analytical instrumentation [411]. The microchip-CE system can offer advantages of low cost, rapid analysis, compactness, and multiplex capabilities. However, due to difficulty with the electrokinetic control of the sample plug by the simple cross, tee or double-tee injectors, as well as the limitation of the detection systems available for planar formats [278], only a few applications in binding constant determination on a chip have been published [304, 309, 328, 360, 378, 412-418]. Rapid direct separation of a protein (thrombin) and aptamer complex from the free aptamer using a microchip achieved within 10 s was reported [370]. Using a microchip, the analysis time for determination of the binding affinity between diketopiperzarine receptors and peptide ligands was shortened by 50 times compared to regular CE [309]. Applications and recent advances in microchip capillary

electrophoresis on both chiral separations and the estimation of binding constants were reviewed recently [278].

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CHAPTER 9

GENERAL CONCLUDIONS

In chapters 2, 3 and 4, the enantiomeric separations of three series of enantiomeric compounds were examined via capillary zone electrophoresis and micellar capillary electrophoresis. The effects of experimental parameters, including chiral selector concentration, buffer concentration and pH, organic modifier content, applied voltage are studied for optimization. Increases in the chiral selector concentration turned out to be the most effective approach for optimization as it can greatly improve the resolution by increasing both selectivity and efficiency. Buffer additives can affect enantioseparations in multiple ways, including: a) modifying the interaction between the chiral selector and the analyte; and b) affecting the EOF mobility. Buffer pH can change the charge state of both chiral selectors and analytes, thus the nature of analyte-chiral selector interaction. Buffer pH also can be used to monitoring the EOF mobility. Organic modifiers can affect the binding chemistry and the EOF while the applied voltage can affect the enantioseparation by changing the Joule heating and altering the analyte/chiral selector mobility.

Generally, if a chiral selector shows enantioselectivity to an analyte, the separation can usually be optimized to a baseline separation. Sulfated cyclodextrins are the best chiral selectors for all three series of analytes in this dissertation. It should be noted that the cavity size of the best chiral selector increase as the size of the chiral analyte increased. That is, the best chiral selectors for β-lactams (smallest), synthetic amino acids (medium) and ruthenium(II) polypyridyl complexex (largest) are sulfated α-cyclodextrin, sulfated β-cyclodextrin and sulfated γ-cyclodextrin, respectively. This phenomenon agrees well with the chiral recognition mechanism of cyclodextrins proposed by Armstrong in 1986 [72]. In aqueous solutions, hydrophobic analytes can form hydrophobic inclusion complexes with cyclodextrins. In order to

obtain enantioselectivity, additional simultaneous interactions such as hydrogen bonding and steric interactions must occur, often at the mouth of the cyclodextrin cavity [72].

In Chapter 5, a new class of chiral selectors, sulfated cyclofructans were successful synthesized (by derivatization of native cyclofructans) and used as chiral selector for CE. They showed great enantioselectivities to cationic compounds (compounds contain primary, secondary, tertiary and quaternary amines). Enantioselectivity to most primary amines are superior to any other existing CE chiral selectors. Extreme fast enantiomeric separation with of some metal complexes that generated large resolutions (as high as 15) also were achieved within 3 mins (results not shown in this dissertation). Subsequently, the cyclofrutans were successfully utilized in HPLC applications by my labmates, which were shown to be very promising and will likely be commercialized soon. In future work on this project, we would like to increase the stability of this class of chiral selector in aqueous solution by substituting the sulfate groups with sulfonate groups.

Chapters 6 and 7 involve the study of microorganism using capillary electrophoresis. To improve the sensitivity of CE analyses of microorganisms and the reliability of sterility tests on dilute solution of microorganism, several preconcentration techniques were examined. Injection volume increases using capillaries with different i.d.s were explored in Chapter 6. Possible theoretical benefits were examined experimentally and a comparison of different approaches was made. The use of large volume sample stacking in a large i.d. capillary can greatly increase the sensitivity (the peak height for a sample with fixed microbe concentration), which, in turn lowers the detection limit. The improvement is a combination of sample stacking, an increase in injection volume and an increase of the optical path length. In Chapter 7, the three-injection method developed previously was successfully used for the detection of *Candida albicans* and/or bacteria in blood plasma. A “sample-self focusing” method also was successfully developed, where the sample solution was simply injected without any spacer or blocking agent.

Both methods are extremely rapid (10 min). Coupled with laser-induced fluorescence, the detection limit was as low as 5 microorganism cells per injection.

APPENDIX A

PUBLICATION INFORMATION FOR CHAPTER 2-8

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BIOGRAPHICAL INFORMATION

Chunxia (Violet) Jiang obtained her Bachelor of Science degree from Zhejiang University majoring in Chemistry. Then she went to study under Dr. Armstrong at Iowa State University and the University of Texas at Arlington. She earned her Doctor of Philosophy in December 2009 working on the research of enantiomeric separations, development and evaluation of chiral selectors, determination of binding constants and microorganism using capillary electrophoresis.