THE USE OF LIQUID CHROMATOGRAPHY AND SUBCRITICAL FLUID CHROMATOGRAPHY FOR CHIRAL SEPARATIONS USING MACROCYCLIC CHIRAL STATIONARY PHASES: INSIGHTS INTO MECHANISMS OF RETENTION AND CHIRAL DISCRIMINATION

by

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Abstract

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Enantiomeric separations are an essential component of pharmaceutical drug development, not only at the analytical scale, but also to separate usable quantities for further analysis. The field of asymmetric synthesis is also heavily dependent on chromatographic methods to separate and quantitate the results of asymmetric transformations as well as characterize new ligands and catalysts. This dissertation focuses on the use of macrocyclic chiral stationary phases for use in high performance liquid chromatography as well as subcritical fluid chromatography to separate individual enantiomers of molecules of importance to the scientific community. Optimized separation conditions are provided for many of these important analytes, which will expedite the evaluation of their usefulness in a variety of applications. Particular emphasis is put on elucidating the mechanism of interaction between analyte and stationary phase. In chapters two and three, principle component analysis is applied to the chromatographic data to gain better understanding of the factors contributing to retention and enantioselectivity. It was shown that optimized separation conditions are also provided for newly synthesized isochromene and Tröger base derivatives using

cyclodextrin and cyclofructan based chiral stationary phases. The fourth chapter provides separation conditions for a variety of novel synthetic biaryl atropisomers, which have the potential to serve as useful ligands in asymmetric transformations as well as possessing antibiotic/antimicrobial properties. Preparative scale separation conditions are also provided allowing for these important analytes to be prepared and evaluated in their enantiomerically pure form. Insight into the mechanism of analyte retention is provided indicating that dipolarity/polarizability is the primary retentive interaction between substituted biaryls and derivatized cyclofructans. Chapter five provided a valuable comparison of commonly used chromatographic conditions for the separation of primary amines using cyclofructan based chiral stationary phases. The effect of various addivies and polar modifiers was investigated and the results indicate that a combination of acidic and basic additives is necessary to obtain optimal separations. The advantages of individual chromatographic modes are also provided. Normal phase separations provided the greatest selectivities at the cost of longer analysis times while modified carbon dioxide mobile phases provided excellent peak profiles and short analysis times. Preparative scale separations are also provided using modified carbon dioxide mobile phases allowing for enantiopure compounds to be prepared in an environmentally friendly manner without the use of petroleum based solvents.

Table of Contents

List of Illustrations

List of Tables

Chapter 1

Introduction

1.1 Introduction to Stereoisomerism And Methods For Enantioseparation

The vast majorities of molecules of biological significance are chiral and exist as stereoisomers. The need for effective methods to separate the individual stereoisomers within a mixture is well established. This need was written into FDA guidelines in 1992 with the issue of the *Development of Stereoisomeric Drugs* which states "The stereoisomeric composition of a drug with a chiral center should be known and the quantitative isomeric composition of the material used in pharmacologic, toxicological, and clinical studies known."[1] It is often the case that one drug stereoisomer is responsible for the desired effect(s) while the other isomer(s) are either inactive or contribute to the side effects.[2,3] The consequence of this is twofold. First, it requires pharmaceutical companies to establish the pharmacological effect of the individual stereoisomers present for all drugs intended for commercial production, which in turn, generates a strong impetus to develop enantiomerically pure drugs and starting materials, primarily through chromatography (early stage drug discovery) and asymmetric synthesis (commercial production).[4-6] The percentage of new drugs marketed as single enantiomer formulations has increased year after year and was at 39% in 2002.[3] Thus methods must be developed to characterize and quantitate not only stereoisomers of newly developed pharmaceutical drugs, but also the myriad of chiral catalysts, auxiliaries, synthons, ligands etc. that are developed for asymmetric synthesis and related fields.[7]

While diastereomers (stereoisomers not related through a reflection operation) can often be separated by simple physical or chromatographic methods, enantiomers (stereoisomers related through a reflection operation *i.e.* non-superimposable mirror images) present a much greater challenge due to their identical chemical and physical

properties in an isotropic environment. While some racemates (a 50/50 mixture of enantiomers) undergo spontaneous recrystallization under the proper conditions *i.e.* dissolving in an appropriate solvent, using the proper temperature etc., the vast majority (>85%) cannot be resolved in this way and must be resolved by forming a transient diastereomeric complex.[8] This can be accomplished by introducing another chiral molecule that interacts differentially with the individual enantiomers.[8] While many analytical methods have been used to determine the enantiomeric and diastereomeric composition of molecules interest, chromatography, and in particular, high performance liquid chromatography (HPLC) is the dominant analytical technique in use today.[9] The advantages of HPLC over other methods are many-fold and include a wide variety of commercially available chiral stationary phases (CSPs), excellent accuracy, precision, sensitivity, reproducibility and ease-of-use.[10] Analytical HPLC methods are also easily transferred to semi-preparative and preparative scales, allowing for the isolation of the individual stereoisomers for further study.[5] The method of choice to separate enantiomers by HPLC is by using a CSP that has been either coated or immobilized onto a solid support, usually silica gel.[9] The sample is introduced onto the head of the column and eluted with an appropriate mobile phase. As the individual enantiomers traverse the column, they can interact differentially with the chiral selector and form different transient diastereomeric complexes. The migration of one enantiomer is often retarded relative to the other resulting in it eluting from the end of the column at a later time, allowing for the quantitation and isolation of the individual enantiomers if sufficient differences in migration time are present. Unfortunately, many times the difference in the migration time is not sufficient to successfully separate the individual enantiomers on a given CSP. There is also no current model capable of predicting the appropriate CSP for separating a given analyte of interest, and given the large number of CSPs currently available,

significant trial-and-error work is often necessary to develop a chiral HPLC method capable of separating and quantifying the enantiomers of a newly developed compound. The complexity is compounded significantly by the fact that the identity and composition of the mobile phase used can have an enormous impact on the success of the method.[9,11] Binary mobile phases are typically a requisite and there are any number of mobile phase combinations that could potentially yield a successful separation. Potentially successful chiral HPLC mobile phases fall into three broad categories: normal phase (a nonpolar major component such as hexane and a polar modifier such as ethanol or isopropanol), reversed phase (aqueous major component and a less polar modifier such as methanol or acetonitrile) and the polar organic mode first identified by Armstrong in 1992 (typically acetonitrile as the major component with an alcohol modifier). While these are the predominant chiral HPLC mobile phase modes, a great number of substitutions can be made to one or all the components with potentially tremendous impact on retention and selectivity. To compound the issue further, low percentage acidic and basic additives also can greatly impact both retention and selectivity for a given analyte/CSP/mobile phase combination.[12] Thus it is of great importance to have a strong knowledge pool of the combinations of CSPs, mobile phases, additives etc. that have been successful for various types and classes of analytes to “narrow the field” and increase the likelihood of success when developing new chiral HPLC methods.

Supercritical(subcritical) fluid chromatography (SFC) is a technique that employs a supercritical or subcritical fluid as the major mobile phase component. A supercritical fluid is a substance whose temperature and pressure are above the critical point and thus are neither a liquid or a gas.[13] SFC has consistently increased in popularity over the last few decades as a technique for chiral separations, primarily due to shorter analysis times

relative to HPLC separations as well as lower solvent consumption.[4,9,14,15] Typically, liquid carbon dioxide replaces hexane or heptane in normal phase methods with only minor changes in method development parameters.[4,14] The moniker SFC can be somewhat misleading as the true mobile phase is rarely supercritical but rather modified carbon dioxide due to the presence of a polar modifier such as methanol. This is necessary because liquid CO_2 has a lower polarity than small aliphatic hydrocarbons such as hexane and thus has a low eluotropic strength.[16] Typically, the amount of polar modifier needed to elute analytes from the CSP is large enough to make achieving a supercritical state difficult at normal operating temperatures and pressures.[16] SFC thus can refer to either supercritical or subcritical fluid chromatography. The acronym SFC will be used throughout this dissertation to refer to using liquid carbon dioxide as the major component of the mobile phase for chromatographic separations regardless of whether operating under supercritical or subcritical conditions. While the primary disadvantage of SFC is the significant instrumentation costs relative to HPLC, cost savings are typically achieved over time due to lower solvent consumption and concomitant waste disposal.[14] Regardless of whether using HPLC or SFC for determining enantiomeric compositions or developing preparative scale methods, the majority of the basic chromatographic principles are shared including the use of identical chiral stationary phases to achieve separation.

1.2 Mechanisms of Retention And Selectivity for Commonly Used Chiral Stationary Phases

Many popular CSPs are based upon derivatized polysaccharides developed by Okamoto et al.[17-19] (Figure 1-1). The mechanism of retention and selectivity for these types of CSPs are not fully elucidated; however, experiments using solid state NMR indicate that enantioselectivity may occur within chiral grooves and cavities present on

the polysaccharide backbone (Figure 1-2). Various mechanisms for analyte retention are possible when using polysaccharide CSPs and are strongly analyte dependent. Hydrogen bonding, dipolarity/polarizability and π-π interactions are forces that may contribute to the overall mechanism of specific analyte retention and chiral discrimination.

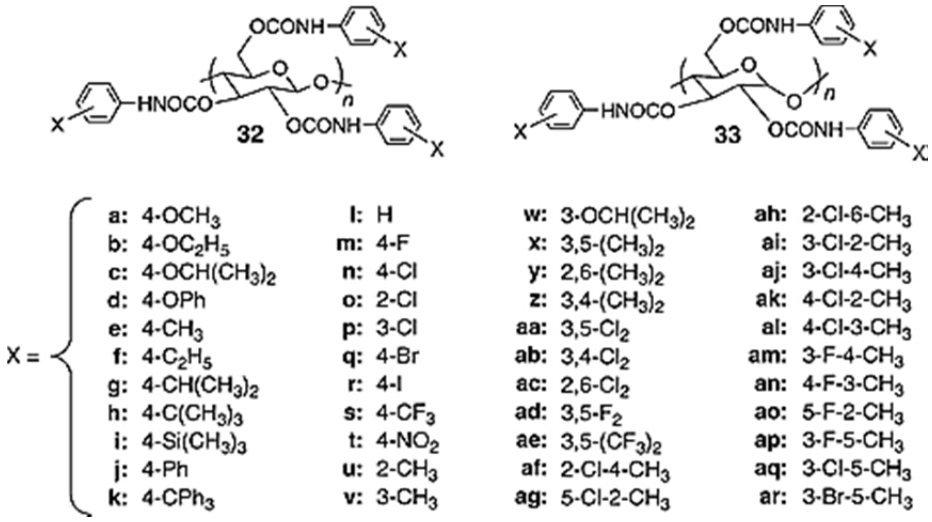

Figure 1-1 Structures of phenylcarbamate derivatives of cellulose (32) and amylose (33). Adapted from Ref. 19 with permission from the Royal Society of Chemistry

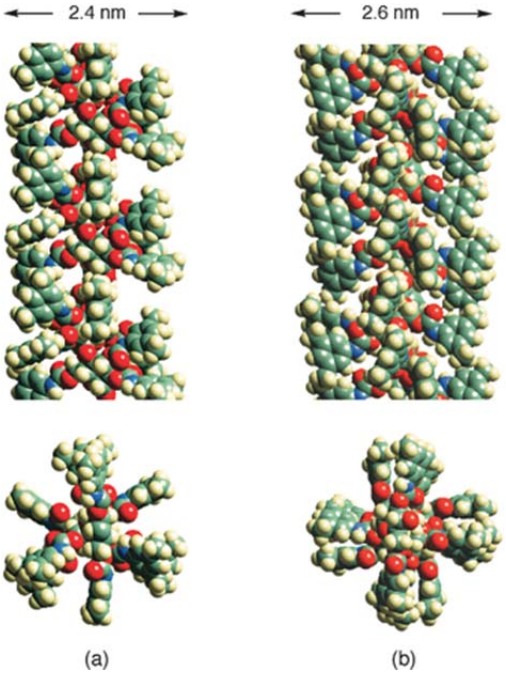

Figure 1-2 Optimized structures of 3,5-dimethylphenylcarbamates of cellulose (a) and amylose (b). Along (top) and perpendicular (bottom) to the helix axis. Reproduced from Ref. 19 with permission from the Royal Society of Chemistry

Other successful CSPs include macrocyclic glycopeptides such as vancomycin, ristocetin A and teicoplanin developed by Armstrong et al. (Figure 1-3).[20,21] Once immobilized on a solid support, these antibiotics have been shown to have broad applicability, can be used in reversed-phase, polar organic and normal phase modes and have excellent loading capacities for preparative separations.[22] These antibiotics contain multiple macrocycles, ionizable groups, aromatic functionalities and chiral centers. For example, vancomycin contains three macrocycles, five aromatic rings, a carbohydrate dimer, nine hydroxyl groups, two amine groups, seven amido groups and eighteen stereogenic centers.[20] With such an array of functionalities, the mechanism of retention and selectivity is not only strongly analyte dependent, but also dependent on the type and

composition of the mobile phase. In fact, for a given analyte, chiral recognition can be obtained by two different mechanisms when operating in either the normal phase or reversed phase modes.[20] Mechanisms of retention and chiral discrimination can include ionic interactions as well as hydrophobic, hydrogen bonding, dipole-dipole, π–π and steric repulsion.[23]

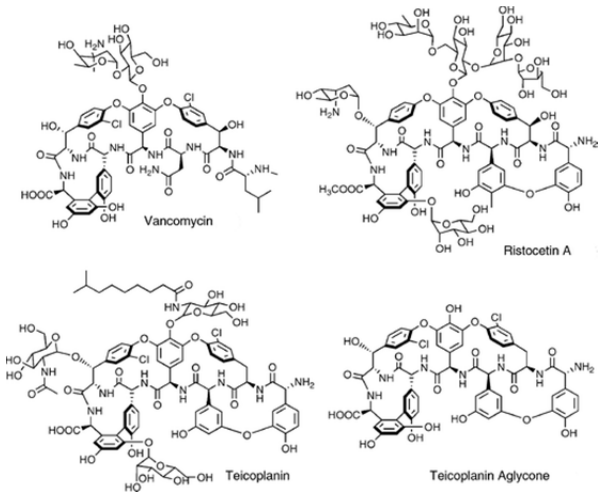

Figure 1-3 Structures of glycopeptide (aglycone)-based CSPs.

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Native and derivatized cyclodextrins (Figure 1-4), also developed by Armstrong, have been shown to separate a variety of racemic mixtures.[24,25] Cyclodextrins are produced using the enzyme cyclodextrin glycosyltransferase with starch as a substrate.[22] While many varieties of cyclodextrins have been characterized, those with 6, 7 and 8 glucose units (α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, respectively) have been well studied as CSPs.[26] Like other CSPs discussed previously, retention and selectivity vary based upon separation mode, *i.e.* reversed phase *vs.* polar organic, and is analyte dependent. Under reversed phase conditions, retention is typically governed by inclusion complexation between a hydrophobic portion of the analyte and the cyclodextrin cavity

with chiral discrimination typically due to secondary interactions at the rim of the cavity (Figure 1-5B).[27] However, when using a high concentration of acetonitrile with a small amount of methanol as a polar modifier (polar organic mode), acetonitrile occupies the cyclodextrin cavity and retention and chiral discrimination occur on the “mouth” of the cavity (Figure 1-5A).[28,29]

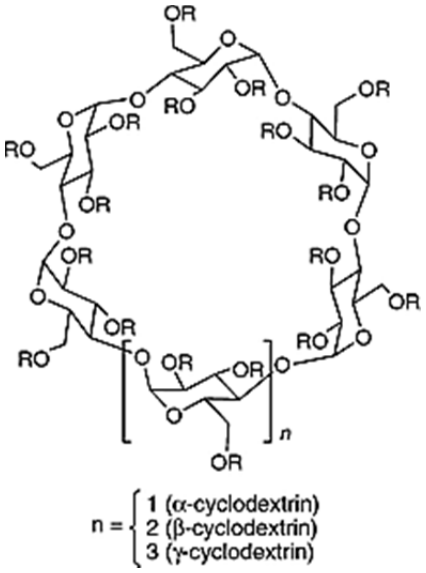

Figure 1-4 Structures of cyclodextrin-based CSPs

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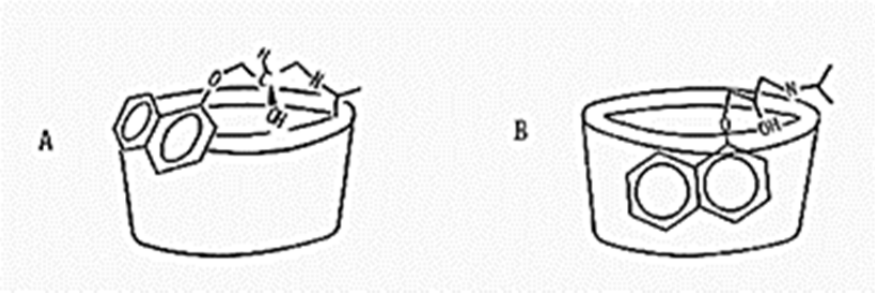

Figure 1-5 Simplified schematic illustrating two different enantioselective retention mechanisms for the native β-cyclodextrin/propanolol system

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Some research efforts have gone into developing “class selective” CSPs, *i.e.* a CSP capable of separating the majority of analytes that possess a specific shape or functional group. One such group of class selective CSPs are the chiral crown-ethers. Pioneered by Cram et al.[30] Chiral crown ether CSPs have been extensively synthesized and evaluated, primarily for the resolution of primary amine containing chiral analytes (Figure 1-6).[31-35] Under acidic reversed phase conditions, retention and enantioselectivity is achieved through host-guest complexation between the protonated amine and the crown ether core through multiple hydrogen bonding interactions.[30]

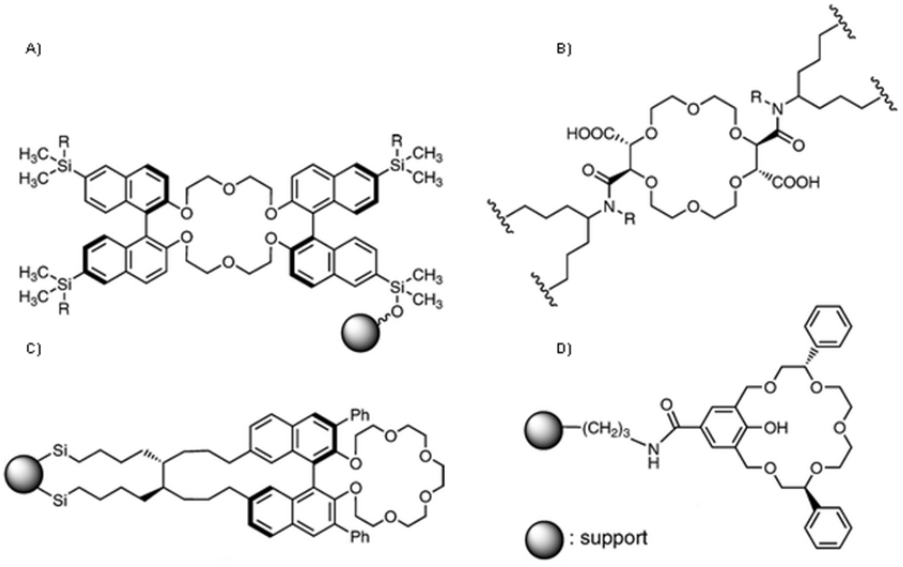


Figure 1-6 Various crown-ether CSPs

Adapted from Ref. 19 with permission from the Royal Society of Chemistry

Recently, a new CSP based upon Cyclofructans (Figure 1-6) has been shown to possess class selectivity for analytes containing a primary amine functional group.[36]

Cyclofructans are cyclic oligosaccharides consisting of six to eight β(2→1)-linked *D*-fructofuranose units and once partially derivatized with aliphatic functional groups, can separate a variety of racemic compounds. Cyclofructans are named based upon the number of fructofuranose units; hence a cyclofructan with six units is called CF6. CF6 possesses an innate 18-crown-6 moiety and once partially derivatized with isopropylcarbamate groups, it can separate primary amines much like other 18-crown-6 CSPs (Figure 1-5).[36] However, unlike other 18-crown-6 CSPs, the isopropylcarbamate CF6 (CF6-P) can separate primary amine containing racemates without the need for aqueous mobile phases that are not convenient for preparative scale separations. It can also separate a variety of other chiral analytes under normal phase and polar organic chromatographic conditions.[37-40]

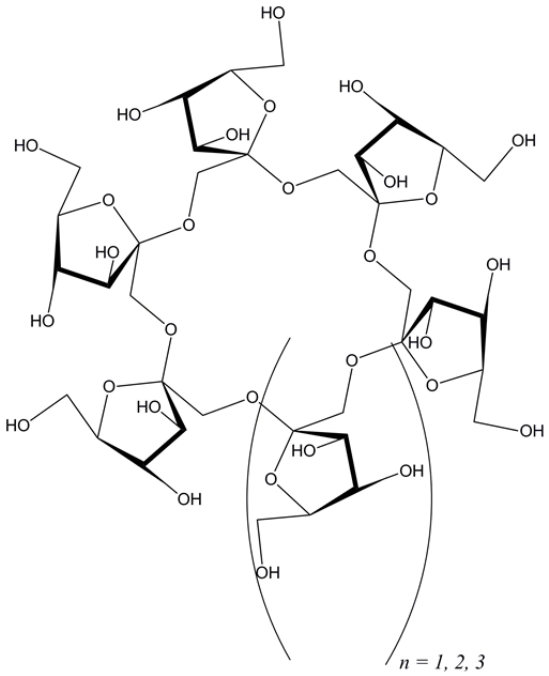


Figure 1-7 Structure of native cyclofructan

1.3 Structure of the Dissertation

The focus of this dissertation is in the development of new chiral HPLC and SFC methods to aid in the characterization of newly developed chiral reagents and pharmaceutical drugs, as well as to further elucidate the mechanisms of retention and chiral discrimination using immobilized macrocyclic chiral selectors.

Chapter two describes the development of chiral HPLC methods to separate various recently synthesized chiral isochromene derivatives and the application of principle component analysis to further understand the factors contributing to retention and chiral discrimination. This is the first published application of principle component analysis that uses simple chromatographic parameters such as retention factors and resolutions.

Chapter three is a similar study in which chiral HPLC methods are developed for Tröger base racemates with a similar application of principle component analysis to the chromatographic results. Further developments into the application of principle component analysis to chiral chromatography are presented.

Chapter four describes the development of chiral HPLC methods for newly synthesized biaryl atropisomers using cyclofructan based chiral stationary phases with insights into the mechanisms of retention and chiral discrimination. This is the first time many of these important analytes have been separated by any means. Preparative scale methods are also presented allowing for these analytes to be studied in their enantiomerically pure form.

Chapter five describes the comparison of SFC, normal phase and polar organic chromatographic conditions to the separation of primary amine containing racemates with particular attention paid to optimizing method parameters for SFC. The effects of acidic and basic additives, various polar modifiers as well as instrument specific parameters are

evaluated and discussed. Optimized mobile phase conditions are presented for screening chiral primary amines by SFC as well as polar organic and normal phase chromatographic conditions.

Chapter 2

Enantiomeric Separation of Isochromene Derivatives by HPLC Using Cyclodextrin Based (Cyclobond) Stationary Phases and Principal Component Analysis of the Retention Data

2.1 Abstract

Isochromene derivatives are very important precursors in the natural products industry. Hence the enantiomeric separations of chiral isochromenes are important in the pharmaceutical industry and for organic asymmetric synthesis. Here we report enantiomeric separations of 21 different chiral isochromene derivatives, which were synthesized using alkynylbenzaldehyde cyclization catalyzed by chiral gold(I) acyclic diaminocarbene complexes. All separations were achieved by HPLC with commercial cyclodextrin based (Cyclobond) CSPs. Retention data of 21 chiral compounds and 14 other previously separated isochromene derivatives was analyzed using principal component analysis (PCA). Effect of the structure of the substituents on the isochromene ring on enantiomeric resolution and other separation properties was analyzed in detail. Using PCA it can be shown that the structural features that contribute to increased retention are different from those that enhance enantiomeric resolution. In addition to that PCA is useful for eliminating redundant factors. Also the chiral recognition mechanism is different for the larger γ-cyclodextrin as compared to the smaller β-cyclodextrin derivatives. Finally this specific system of chiral analytes and cyclodextrin based chiral selectors provide an effective format to examine the application of PCA on enantiomeric separations using basic retention data and structural features.

2.2 Introduction

Isochromene derivatives exist in variety of natural products. Isochromene derivatives have very important biological effects including antitumor properties, hence isochromenes plays vital role in natural products research.[41,42] The isolation of

isochromene based compounds from living systems, such as various types of fungi, is a common practice[43]. Isochromenes also are useful as intermediates in the synthesis of other natural products and pharmaceuticals. Consequently some organic chemists tend to produce isochromene derivatives in bulk quantities using asymmetric synthesis[44,45]. In these cases, it is important to efficiently determine the enantiomeric excess (% ee) as well as to separate chiral products or intermediates in larger quantities. HPLC is the most dominant chiral separation technique available. Here we present chiral HPLC methodologies for the separation of isochromene derivatives using Cyclobond HPLC columns. The Cyclobond line of chiral selectors (cyclodextrins) is made of 1-4 linked α-D-glucopyranosides. The number of glucose units for bonded cyclodextrins are 6, 7 and 8 (named α, β, and γ respectively)[24,25,46-52]. Among the Cyclobond columns used in this study, with the exception of the Cyclobond II column, the cyclodextrin hydroxyls have been partially derivatized with various functional groups to enhance enantioselectivity. Previously, neutral hydrophobic molecules with few polar functional groups have been shown to separate particularly well on Cyclobond chiral stationary phases[48,53-55]. Recently 21 chiral isochromene derivatives were synthesized using a new class of chiral Au^{I}/acyclic diaminocarbene (ADC) catalysts.[56] These analytes were synthesized by asymmetric alkynylbenzaldehyde cyclization.[56] Most of these have not been separated previously on any HPLC chiral stationary phase. We then employed principal component analysis (PCA) to analyze the retention data of these chiral isochromene derivatives along with the retention data of 14 other related compounds that had been reported previously.[57] Principal component analysis is a powerful tool that can be used to understand the differences between calculated and measured retention data, to determine the external variables that significantly affect retention and to reduce the number of chromatography systems/analytes to solve specific practical and theoretical

problems in chromatography[58]. PCA was employed in previous two studies to analyze retention data of chiral selectors/compounds by Camilleri et al. and Montanari et al.[58-60] Camilleri et al. used quantitative structure – property relationships (QSPR) whereas Montanari et al. used molecular interaction fields (MIF). Hence both of those studies had complex molecular modeling and complex electronic property estimation. However, in our study we used common chromatographic variables such as retention factor and resolution and substitution position as well as the volume of the substituted group. Hence, to our knowledge, this is the first report which uses PCA with simple common variables to help understand HPLC chiral separations. Also this is the first report on the use of PCA to analyze the chiral separation data of isochromene derivatives.

2.3 Experimental

HPLC grade acetonitrile, methanol, 2-propanol and heptane were purchased from EMD chemicals (Gibbstown, NJ). Deionized water was prepared using a Millepore© Synergy 185 system (Billerica, MA). Cyclobond columns were obtained from Supelco© (Bellafonte, MA). Larihc columns were obtained from AZYP LLC. (Arlington, TX). The chiral stationary phases used in this study consisted of Cyclobond II, AC, RSP, DM and Larihc CF7-DMP. Figure 2 shows the structures of these CSPs. All column dimensions were 250 x 4.6 mm.

IC1-IC21 samples were dissolved in 2-propanol (1mg mL^{-1}). An Agilent© 1200 series LC equipped with a diode array detector was used as the detector. Analytes were monitored at 254nm UV detection. Separations were performed at ambient temperature unless otherwise noted. All injections were 5 µL and flow rates were 1ml min^{-1} unless otherwise noted.

The dead time (t_0) was determined using the peak caused by the change in refractive index from the injection solvent on each column. The retention factor (k), the enantioselectivity (α) and the resolution factor (R_s) were calculated using following equations: $k = (t_r - t_0)\, t_0^{-1}$, $\alpha = k_2\, k_1^{-1}$, $R_s = 2(t_{r2} - t_{r1})\,(w_1 + w_2)^{-1}$ where, t_{r2}, t_{r1} are retention times of the first and second enantiomers and w_1, w_2 are corresponding base peak widths. The principal component analysis was conducted using Matlab (Mathworks., Natick, Massachusetts, U.S.A.) The retention data for compounds IC22-IC35 were obtained from our previous publication[53]

2.4 Results & Discussion

Figure 4-1 shows the general isochromene core structure plus all of the derivatives used in this study. R1 and R2 can be either aliphatic or aromatic substituents. R3 can be an iodine, a sulfur group, an aliphatic group or an aromatic group. R4 can be either a hydrogen or methoxy group. There are 35 different isochromene derivatives with different combinations of substituents groups (R1-R4). Table 2-1 shows the specific substituents present on IC1-IC35. The 21 newly synthesized isochromene derivatives were screened with Cyclobond DM, RSP, II, and AC CSPs (Figure 2-2). The rest of the previously reported compounds were screened with Cyclobond RSP, DM and II CSPs as reported in a previous study conducted by our group and their retention data were used in this study for the principal component analysis.

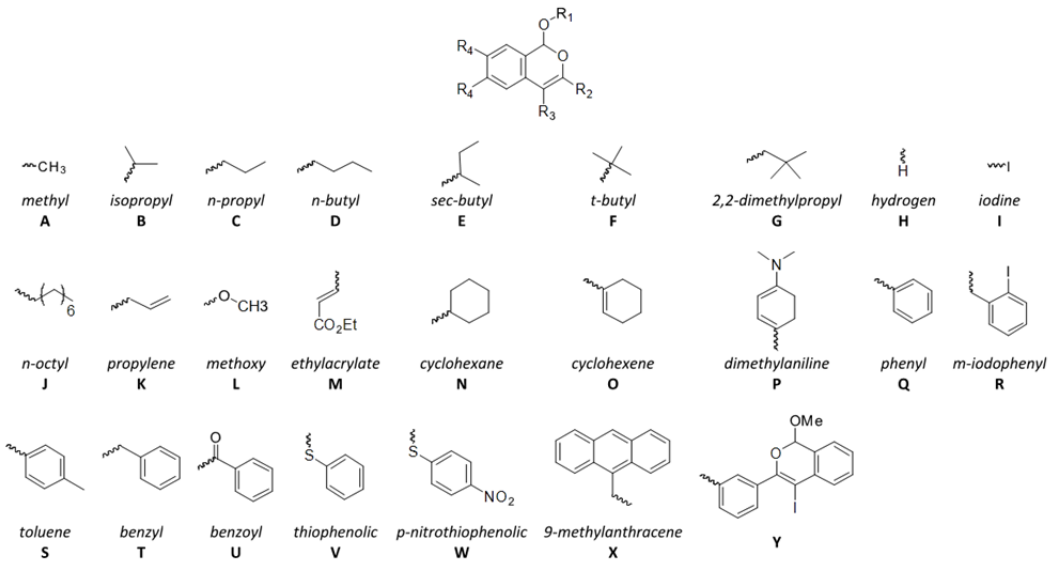


Figure 2-1

General structure and ring numbering conventions for studied chiral compounds

Table 2-1 Type of substituent groups present on IC1-IC35

Compound	Substituted Groups			
	R1	R2	R3	R4
IC1	B	Q	H	H
IC2	C	Q	H	H
IC3	D	Q	H	H
IC4	A	Q	H	H
IC5	B	C	H	H
IC6	D	C	H	H
IC7	J	C	H	H
IC8	A	C	H	H
IC9	X	S	H	H
IC10	X	Q	H	H
IC11	E	Q	H	H
IC12	B	S	H	H

Compound	Substituted Groups			
	R1	R2	R3	R4
IC13	K	S	H	H
IC14	G	S	H	H
IC15	N	Q	H	H
IC16	T	Q	H	H
IC17	F	Q	H	H
IC18	N	S	H	H
IC19	A	S	H	H
IC20	J	S	H	H
IC21	J	Q	H	H
IC22	A	Q	W	H
IC23	D	Q	I	H
IC24	R	Q	I	H

Compound	Substituted Groups			
	R1	R2	R3	R4
IC25	U	Q	I	H
IC26	A	Q	Q	H
IC27	A	O	V	H
IC28	A	D	W	H
IC29	A	Q	M	H
IC30	A	O	I	H
IC31	A	Y	I	H
IC32	P	O	I	H
IC33	F	Q	I	H
IC34	A	Q	I	L
IC35	A	Q	W	L

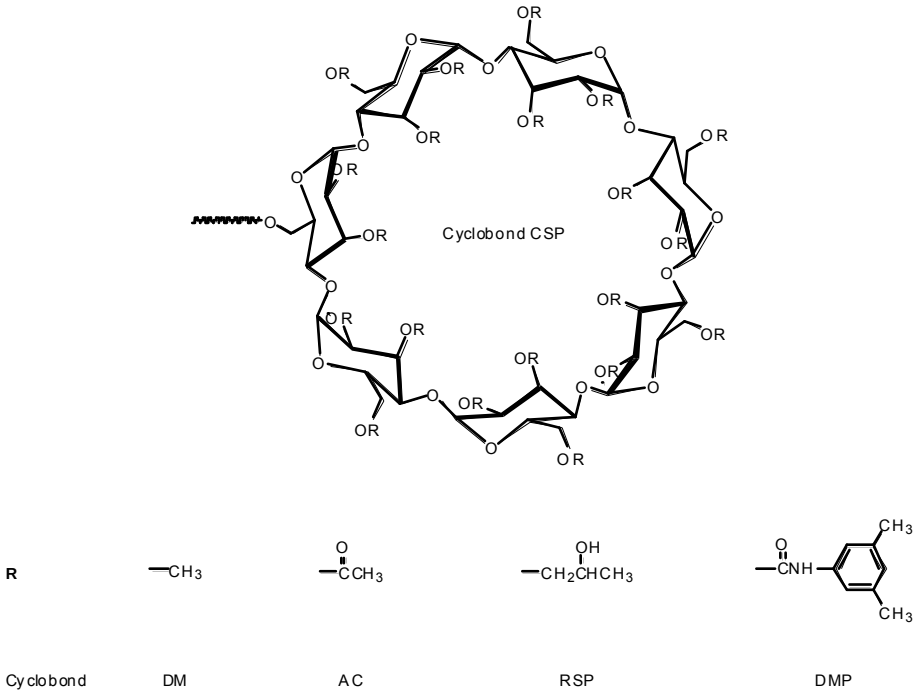


Figure 2-2 General structure of commercial CSP's used in this study.

All Cyclobond CSPs used in this study are made from derivatized β-cyclodextrins except Cyclobond II which is made from native γ-cyclodextrin

2.4.1 Separation of IC1-IC21 Compounds

Table 2-2 lists the retention factors of the first peak (k_1), enantioselectivities (α), and enantioresolutions (R_S) of the IC1-IC35 compounds on all the Cyclobond CSPs in the reverse phase mode. Observable enantioresolutions (Rs ≥ 0.7) for 17 compounds were achieved on the Cyclobond DM column. Out of these 17 observable enantioresolutions, 15 were baseline separated. For the Cyclobond RSP column, observable enantioresolutions of ($R_S \geq 0.5$) were achieved for 16 compounds and 12 compounds were baseline separated ($R_S \geq 1.5$). The Cyclobond II column (native γ cyclodextrin) did not generate as many enantiomeric separations as the Cyclobond RSP and DM. Only 7

enantiomeric separations (Rs $\geq$ 0.5) were achieved none of them were baseline separated ($R_S \geq 1.5$). Table 2-5 lists the best separation conditions achieved for these compounds. Simple solvent systems such as 50:50 water:methanol can be used to separate most of these compounds. It should be noted that three compounds were better separated with cyclofructan based Larihc CF7-DMP CSP[36,37,61]. However since there was not sufficient data for structural analysis these results are not analyzed in this work and will not be discussed further.

Table 2-2 Retention factor of the first peak (k_1), enantioselectivity (α), and enantioresolution (R_S) of all chiral compounds on the Cyclobond DM, RSP and II CSP in the reverse phase mode.

	Cyclobond DM				Cyclobond RSP				Cyclobond II			
	k_1	α	R_s	$H_2O\%^a$	k_1	α	R_s	$H_2O\%^a$	k_1	α	R_s	$H_2O\%^a$
IC1	2.01	1.37	2.8	55	6.19	1.13	1.5	50	1.77	1.02	0.5	70
IC2	2.37	1.54	3.6	55	2.12	1.25	1.9	40	2.45	1.10	0.8	70
IC3	1.44	1.42	2.8	50	2.31	1.13	1.4	40	1.27	1.13	1.0	60
IC4	1.33	1.23	1.8	50	6.46	1.16	1.7	50	1.53	1.00	0	70
IC5	1.01	1.33	1.5	60	1.90	1.31	2.5	50	2.04	1.08	0.8	80
IC6	0.28	1.29	1.4	40	2.70	1.18	1.5	50	3.34	1.03	0.5	80
IC7	0.33	1.18	0.7	30	2.09	1.08	0.8	40	0.88	1.00	0	50
IC8	0.62	1.38	2.7	50	2.21	1.23	1.9	50	0.18	1.17	0.4	50
IC9[#]	0.67	1.39	1.6	50	–	–	–	–	1.12	1.00	0	50
IC10[#]	0.76	1.00	0	50	–	–	–	–	0.95	1.00	0	50
IC11[#]	0.93	1.58	0.9	50	–	–	–	–	0.31	1.00	0	50
IC12	2.22	1.13	1.5	50	3.75	1.11	0.8	50	0.30	1.00	0	50
IC13	2.47	1.15	1.6	50	4.51	1.13	1.58	50	1.99	1.00	0	70
IC14	1.79	1.31	1.5	50	4.71	1.13	1.5	50	1.80	1.00	0	70
IC15	2.70	1.20	1.6	50	6.43	1.00	0	50	2.55	1.00	0	70
IC16	3.70	1.23	1.9	50	6.93	1.06	0.9	50	4.86	1.11	0.9	70
IC17	1.33	1.00	0	50	0.79	1.00	0	50	0.26	1.00	0	50
IC18	3.40	1.26	1.9	50	5.76	1.14	1.5	50	3.20	1.18	1.3	70
IC19	2.90	1.15	1.8	50	4.77	1.17	1.7	50	1.35	1.00	0	70
IC20	2.00	1.15	1.5	50	6.44	1.21	2.1	50	1.71	1.00	0	70
IC21	2.30	1.27	1.9	50	7.61	1.04	0.5	50	3.53	1.00	0	70
IC22	3.69	1.40	1.6	60	3.93	1.25	2.1	50	3.40	1.22	1.0	60
IC23	1.93	1.43	1.1	60	2.48	1.40	2.5	50	3.70	1.00	0	60
IC24	3.26	1.24	0.3	55	4.91	1.32	1.5	50	3.83	1.19	0.8	55
IC25	5.13	1.09	0.3	60	7.50	1.17	1.5	55	3.34	1.19	1.0	55
IC26	1.94	1.00	0	65	4.22	1.00	0	60	2.77	1.00	0	65
IC27	3.34	1.21	0.8	60	10.9	1.24	1.9	50	6.18	1.10	0.3	65
IC28	3.31	1.00	0	60	4.23	1.00	0	50	3.62	1.00	0	65
IC29	2.21	1.00	0	70	2.62	1.00	0	60	2.52	1.33	1.8	75
IC30	4.54	1.35	1.3	60	2.64	1.31	2.3	50	5.79	1.14	1.0	55
IC31	5.80	1.00	0	65	7.53	1.00	0	55	2.30	1.00	0	50
IC32	5.16	1.24	0.7	55	6.36	1.18	1.5	45	12.4	1.21	0.8	60
IC33	3.41	1.00	0	60	6.26	1.00	0	55	6.25	1.00	0	70
IC34	2.12	1.00	0	65	5.11	1.00	0	55	4.58	1.00	0	60
IC35	1.92	1.42	1.1	60	4.10	1.46	3.5	55	3.94	1.00	0	60

[a] $H_2O\%$: percentage of water in the mobile phase H_2O:methanol (v/v)

[#] These compounds had decomposed by the time they were screened on the Cyclobond RSP CSP

2.4.2 Principal Component Analysis (IC1-IC35)

2.4.2.1 Cyclobond DM CSP

Table 2-3 lists volumes of substituent groups (R1- R4). Principal component analysis was employed on the separation data (Table 2-1) using Matlab software. Initially

PCA was performed using k_1, α, R_S and H_2O % as the variables. This resulted in two major principal components (i.e., PC1 and PC2) used to draw a 2D plot (Figure 2-3A).

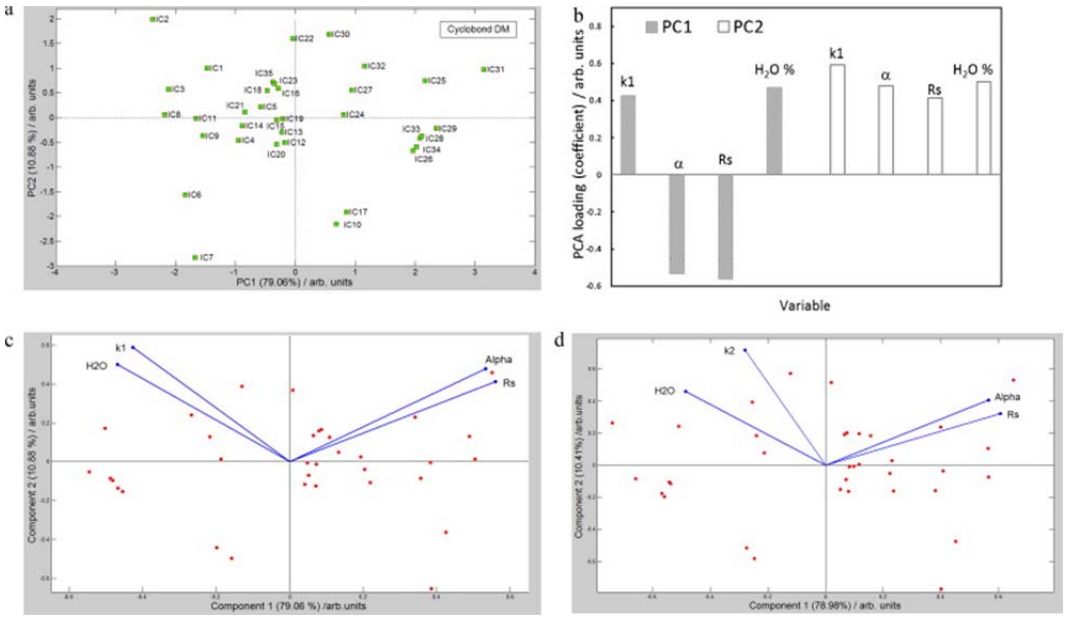


Figure 2-3 Principal component analysis results for Cyclobond DM CSP

a) PCA plot for retention data on Cyclobond DM CSP [only two major principal component axes (PC2 vs PC1) were used], b) PCA loading values PC1 and PC2, c) the biplot of the PCA when retention factor of the first peak (k_1), water percentage in the mobile phase (%H_2O), enantioselectivity (α), and enantioresolution (R_S) are used as variables, d) the biplot of the PCA when retention factor of the second peak (k_2), %H_2O, α and RS are used as the variables. Note that axes are in arbitrary units in all four plots.

Table 2-3 Volume of substituent group ($Å^3$)

	R1	R2	R3	R4
IC1	69.89	98.92	10.48	10.48
IC2	69.89	98.92	10.48	10.48
IC3	88.26	98.92	10.48	10.48
IC4	32.22	98.92	10.48	10.48
IC5	69.89	69.89	10.48	10.48
IC6	88.26	69.89	10.48	10.48
IC7	161.4	69.89	10.48	10.48
IC8	32.22	69.89	10.48	10.48
IC9	218.98	116.91	10.48	10.48
IC10	218.98	98.92	10.48	10.48
IC11	88.18	98.92	10.48	10.48
IC12	69.89	116.91	10.48	10.48
IC13	66.49	116.91	10.48	10.48
IC14	105.68	116.91	10.48	10.48
IC15	111.94	98.92	10.48	10.48
IC16	124.18	98.92	10.48	10.48
IC17	87.81	98.92	10.48	10.48
IC18	111.94	116.91	10.48	10.48
IC19	32.22	116.91	10.48	10.48
IC20	161.4	116.91	10.48	10.48
IC21	161.4	98.92	10.48	10.48
IC22	32.22	98.92	138.6	10.48
IC23	88.26	98.92	37.37	10.48
IC24	141.88	98.92	37.37	10.48
IC25	120.13	98.92	37.37	10.48
IC26	32.22	98.92	98.92	10.48
IC27	32.22	107.78	118.58	10.48
IC28	32.22	88.26	138.6	10.48
IC29	32.22	98.92	114.5	10.48
IC30	32.22	107.78	37.37	10.48
IC31	32.22	277.33	37.37	10.48
IC32	153.75	107.78	37.37	10.48
IC33	87.81	98.92	37.37	10.48
IC34	32.22	98.92	37.37	40.56
IC35	32.22	98.92	138.6	40.56

2.4.2.2 Understanding PCA Plots

The correlation between principal component and the variables, correlation coefficient (*cc),* also called *loadings*, describes how each variable contributes to the principal component. The sum of all squared correlation coefficients (*loadings)* is always equal to 1 ($cc_1^2 + cc_2^2 + cc_3^2 + \ldots + cc_n^2 = 1$)[62]. Figure 2-3b shows the loadings plot for two major principal components. According to Figure 2-3b it is clear that principal component 1 (PC1) is largely composed of enantioselectivity (α) and enantioresolution (R_S). Principal component 2 (PC2), which is orthogonal to PC1, is largely composed of the retention factor of the first peak (k_1) and the water percentage of mobile phase. Figure 2-3c shows the *biplot* of the principal component analysis of retention data when k_1, H_2O percentage, α and R_S are used as the variables. In a biplot, the lines represent variables and the data points represent observations. The length of the line represents the variance of the variable[63]. The direction of the line shows the direction of the variable. The angle between lines gives an estimate as to the correlation between the two variables those lines represent. If the angle is closer to 90, or 270 degrees, then typically the correlation is smaller. If the angle is closer to 0 degrees then it reflects a correlation of 1, if its 180 degrees then correlation is −1 [63]. The volumes of substituent groups in the R1, R2 and R3 positions were introduced as new variables in the PC analysis. Note that volume of the substituted group in the R4 position was not included as a variable since there were limited variations in this substituted group. Figure 2-3d shows the *biplot* of the principal component analysis of the retention data when the retention factor of second peak (k_2), H_2O percentage, α and Rs are used as the variables.

2.4.2.3 Cyclobond DM Biplot Interpretation

Figure 2-3 shows the biplot of compounds screened on the Cyclobond DM CSP. The blue lines represent the magnitude and the direction of variables: retention factor (k), enantioresolution (R_S) and volume of substituents groups (R1,R2, R3). The cosine of each line to the component 1 or 2, represents each variable's contribution to the component 1 or 2, both magnitude and in sign (+ or -). According to Figure 2-3 it is observed that k_1 and the volume of the R2 group are closely related. The retention data in the Table 2-1 confirms that, generally retention factor (k_1) increases with the volume of the R2 (see Figure 2-2). However, sometimes the trend can be disturbed by the effects from R1 and R2 groups.

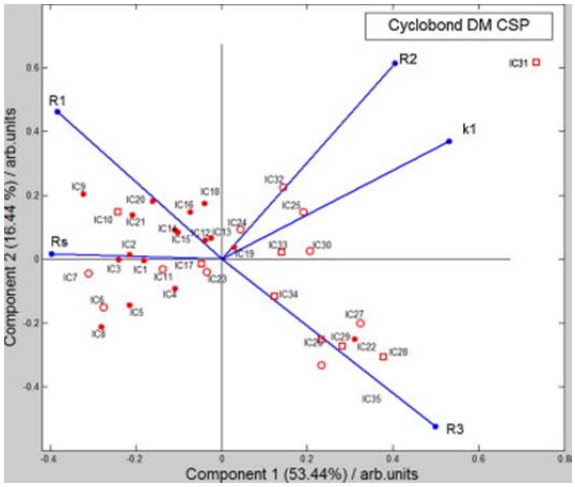


Figure 2-4 The biplot of compounds screened on Cyclobond DM CSP

The blue lines represent the magnitude and direction of the variables: retention factor (k), enantioresolution (RS) and volume of the substituent groups (R1, R2, R3). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

Except for two compounds (IC10 and IC17) all compounds in (-, +) and (-,-) quadrants are separated. The volume of the R1 is the dominant variable in that region. There are 12 compounds which have methyl groups in the R1 position (volume = 32.22 Å3). Out of those 12 compounds 5 of them were not separated. There are 23 compounds which have substituents other than methyl groups at the R1 position, where each of them have volume larger than 32.22 Å3. Out of those 23 compounds only 3 compounds were not separated. If the volume of R1 is larger than 88.18 Å3, then all compounds but one were separated (a total of 14 compounds). Hence it is clear that the size of the R1 substituent group plays a significant role in the enantiomeric separation of isochromene derivatives. It is important to note that the group most responsible for enantiomeric separations (R1) is not the group that is most responsible for retention (R2). Except for 2 compounds all analytes in the (+,-) and (+,+) quadrants are either partially separated (6 compounds) or not separated (6 compounds). R3 and R2 are the major variables in that section. According to Table 2-2, there are 14 compounds which have combined volumes of R2 and R3 that are equal to or larger than 136.9 $Å^3$. Out of 14 compounds only one compound (IC22) was base line separated (Rs ≥ 1.5). Six were partially separated (0<Rs<1.5) and six were not-separated (Rs=0). Therefore it can be concluded that the combined volumes of R2 and R3 also is an important factor for the enantiomeric separation of isochromene derivatives and the larger these groups, the more difficult the separation becomes.

2.4.2.4 Cyclobond RSP CSP

Principal component analysis was employed using the data in Tables 2-1 and 2-2 and the biplot was drawn on the separation data using Matlab software. Figure 2-5 shows the the biplot of compounds screened on Cyclobond RSP CSP.

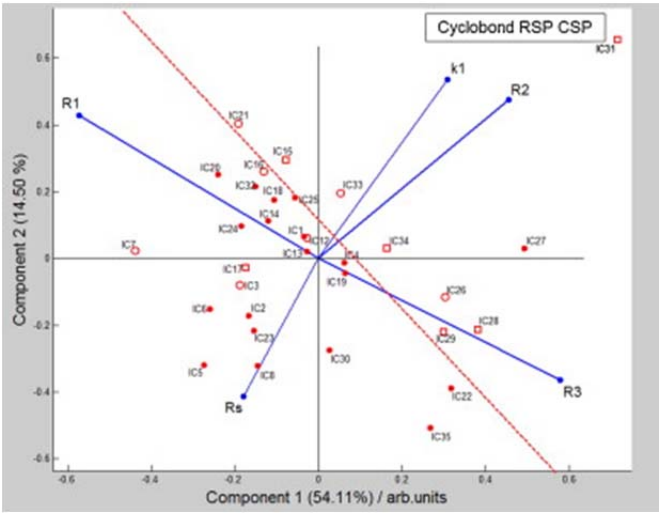


Figure 2-5 The biplot of compounds screened on Cyclobond RSP CSP

The blue lines represent the magnitude and direction of the variables: retention factor (k), enantioresolution (RS) and volume of the substituent groups (R1, R2, R3). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

According to Figure 2-5 the retention factor is closely correlated with the volume of the R2 substituent group both in magnitude and direction. It seems that larger the

volume of R2 the higher the retention factor. However the trend can be affected by volume of the R1 and R_3 groups also.

The angle between R1 and R_S lines is close to 90º (Figure 2-5). According to bipot theory the correlation between R1 and R_S should be small. However, it is observed that a large number of resolved compounds (Rs >0) exist in the R_1 direction. A closer look at Tables 2-1, 2-2 confirmed that if the volume R1 group is equal to or larger than 120.13 Å, then all compounds were separated (7 compounds). In addition, if the volume of R1 is equal to or larger than 66.49 Å, then 17 out of 20 compounds were separated. The three compounds with R1 ≥66.49 Å and which were not separated are IC15, IC17 and IC33. IC 15 has a *cyclohexane* group at the R1 position, while IC 17 and IC 33 have *t-butyl* groups in R1 position. The *t-butyl* group and the *n-butyl* group have approximately same volumes (See Figure 2-1) however *t-butyl* has more spherical structure and *n-butyl* has a more linear structure. If we consider IC3 and IC17, they have similar structures except at the R1 position. IC3 has *n-butyl* group in the R1 position whereas IC17 has *t-butyl* in the same position. IC3 was separated but IC17 was not. Therefore it is clear that, even though the volume is larger than 66.49 Å, the larger diameter of the R1 group hurt the enantioresolution of IC15, IC17 and IC33. There are total of 8 non-separated compounds on the Cyclobond RSP, 5 of the compounds have *methyl* group (volume = 32.22 Å) at the R1 position. Hence it is clear that the R1 position and its volume play a major role in the enantioresolution of isochromene derivatives.

Other than above mentioned five compounds, seven compounds have *methyl* groups at the R1 position. They were separated on the Cylobond RSP column, which means not only the R1 group but also R2 and R3 groups also can positively contribute to the enantioseparation. In the biplot (Figure 2-5), the compounds to the left side of the red dashed line all were separated except one. The R2 and the R3 lines are directed

opposite to the compounds to the left side of the red dashed line. It is observed that except for 2 compounds (IC 15 and IC17) all compounds were separated if the combined volume of R2 and R3 is equal to or smaller than 127.39 $Å^3$ (16 out of 18). The reasons for unusual behavior of IC 15 and IC17 were discussed earlier.

2.4.2.5 Cyclobond II CSP

Figure 2-6 shows the biplot of compounds screened on the Cyclobond II CSP. The blue lines represent the magnitude and the direction of variables: retention factor (k), enantioresolution (R_S) and volume of substituents groups (R1,R2, R3).The cosine of each line to the component 1 or 2, represent each variables contribution to the component 1 or 2, both magnitude and in sign (+ or -). According to Figure 2-6, the retention factor (k_1) and the enantioresolution (R_S) are closely related. In the cases of Cyclobond RSP and DM they are not that closely related. Hence it is clear that separations on these CSPs are governed by different factors than the γ-cyclodextrin phase.

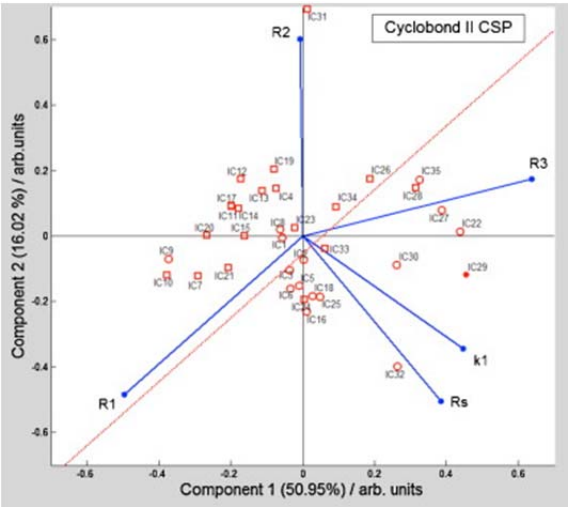


Figure 2-6 The biplot of compounds screened on the Cycolbond II CSP

The blue lines represent the magnitude and direction of the variables: retention factor (k), enantioresolution (RS) and volume of the substituent groups (R1, R2, R3). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

2.4.2.6 CSP Comparison: Cyclobond RSP, DM AND II

Even though IC29 was not separated on either Cyclobond RSP or DM, it was separated on the Cyclobond II CSP. IC22 and IC29 have similar structures except for the groups in the R3 position. IC29 has *ethylacrylate* group in R3 position whereas IC22 has *p-nitrothiophenolic* group. IC22 has separated on all 3 CSPs, which means it is clear that when the *ethylacrylate* group is in the R3 position it hurts the enantiomeric separation on Cyclobond RSP and DM. However same scenario can positively contribute for the enantiomeric separation on Cyclobond II CSP. Also it appears that the R1 group is the

most dominant factor for enantiomeric separation on Cyclobond DM and RSP CSPs. However, on Cyclobond II RSP (which is the larger γ-cyclodextrin), R3 is the most dominant factor for enantiomeric separation. Similar behavior was observed with IC15, where it was not separated in Cyclobond RSP and II, but was separated in Cyclobond DM. According to Figure 2-4, 2-5 and 2-6, it is clear that Cyclobond DM and RSP CSP's behave similarly and effectively when separating enantiomers of isochromene derivatives, where as Cyclobond II behaves less effectively and differently. The optimum conditions for enantiomeric separations of IC1-IC21 compounds were listed in Table 2-4.

2.4.3 Predictions

Based on the results of this study, various predictions can be made. The enantiomers of isochromene derivatives are more likely to separate on Cyclobond DM and RSP CSPs than Cyclobond II CSP. If the volume of the R1 group is larger than 88.18 $Å^3$, then those enantiomers have tendency to separate on Cyclobond DM CSP. In addition to that, if the combined volume of R1 and R2 groups is larger than 136.9 $Å^3$, those enantiomers tend to poorly separate on Cyclobond DM CSP. When the volume of R1 group is larger than 66.49 $Å^3$, those enantiomers are more likely to separate on Cyclobond RSP CSP. Also if R2 and R3 groups have combined volume larger than 127.39 $Å^3$, poor enantiomeric separations on the Cyclobond RSP CSP can be expected.

2.5 Conclusions

The enantiomers of 21 isochromene derivatives were successfully separated on various commercially available HPLC CSPs. The retention data those compounds along with the retention data of other 14 different compounds were used to employ principal component analysis. It was found that Cyclobond DM and RSP CSPs provide better enantiomeric separations of isochromene derivatives than the other CSPs. Also it was

observed that the volume of the R1 group has more effect on the enantiomeric separations on those CSPs than the volumes of R2 and R3 groups. Also the structural factors that lead to increased retentions on these CSPs do not lead to better enantiomeric separations. PCA found to be useful for eliminating redundant factors.

Chapter 3

Enantiomeric Separation of Functionalized Ethano-Bridged Tröger Bases Using Macrocyclic Cyclofructan and Cyclodextrin Chiral Selectors in High-Performance Liquid Chromatography and Capillary Electrophoresis with Application of Principal Component Analysis

3.1 Abstract

The enantiomeric separation of a series of racemic functionalized ethano-bridged Tröger bases compounds was examined by high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Using HPLC and CE the entire set of 14 derivatives was separated by chiral stationary phases (CSPs) and chiral additives composed of cyclodextrin (native and derivatized) and cyclofructan (derivatized). Baseline separations (Rs ≥ 1.5) in HPLC were achieved for 13 of the 14 compounds with resolution values as high as 5.0. CE produced 2 baseline separations. The separations on the cyclodextrin CSPs showed optimum results in the reversed phase mode, and the LARIHC^{TM} cyclofructan CSPs separations showed optimum results in the normal phase mode. HPLC separation data of the compounds was analyzed using principal component analysis (PCA). The PCA biplot analysis showed that retention is governed by the size of the R1 substituent in the case of derivatized cyclofructan and cyclodextrin CSPs, and enantiomeric resolution closely correlated with the size of the R2 group in the case of non-derivatized γ-cyclodextrin CSP. It is clearly shown that chromatographic retention is necessary but not sufficient for the enantiomeric separations of these compounds.

3.2 Introduction

Ethano-Tröger base is a structural analog of methano-Tröger base (2,8-Dimethyl-6H,12H-5,11-methanodibenzo[b,f][1,5]diazocine) both of which are shown in Figure 3-1 as compounds B and A respectively. Tröger base was first synthesized in 1887 by Julius

Tröger via condensation reactions of formaldehyde and aniline [64]. Tröger base exhibits chirality due to two stereogenic nitrogen atoms bridged by a methylene group and maintains a conformation angle of approximately 90 degrees between the planes of the aryl groups[65]. The ridged, angular structure of Tröger base made it an attractive framework for developing related chiral entities to be utilized in molecular recognition studies[66], DNA targeting fluorescent supramolecular scaffolds[67], synthetic receptor systems[68], and self-assembled structures[69]. In all of these studies the enantiomerically pure compounds are preferred.

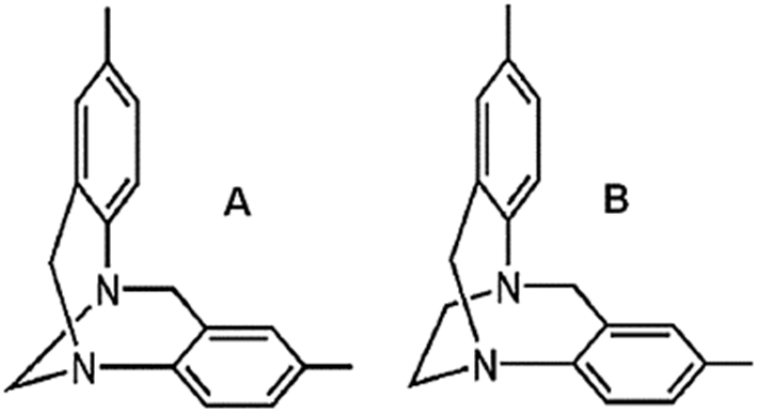


Figure 3-1 Native structure (A) *methano*-Tröger base and (B) *ethano*-Tröger base

In typical acidic reaction conditions the enantiomerically pure methano-Tröger base racemizes via formation of an iminium intermediate which poses a problem in asymmetric synthesis and for its conceivable applications in supramolecular chemistry and asymmetric catalysis. One solution is to transform the methano-Tröger base into an ethano-Tröger base which is stable and avoids racemization in acidic solutions [70]. Recently, the direct transformation of methano-Tröger bases into functionalized ethano-Tröger bases was reported to occur with high diastereoselectivity. In one approach, it was accomplished by the synthesis of quaternary ammonium ions of racemic methano-Tröger bases in conjunction with a ring-expansion Stevens-like rearrangement[71]. In

another approach, single step rhodium or copper catalyzed reactions of aryl diazoesters or aryl diazoketones with enantioenriched methano-Tröger bases(%ee >99%) were performed and yielded enantioenriched ethano-Tröger bases (%ee up to >99%) with excellent levels of chirality transfer[72].

In all cases, effective methods are needed for the separation of enantiomers of the racemic functionalized ethano-Tröger bases. The most common technique for separation of enantiomers is high performance liquid chromatography (HPLC)[10]. Well-established chiral HPLC stationary phases are cyclodextrins (CDs) and their derivatives. Cyclodextrins have been used to separate a tremendous variety of chiral analytes and are widely applicable to rigid aromatic molecules[24,73-77]. Recently, a series of new HPLC chiral stationary phases have been developed based on derivatized cyclofructans (CFs). Derivatized cyclofructans have been shown to separate a variety of enantiomers including most primary amine containing compounds and many chiral pharmacologically active compounds[78-80].

Principal component analysis (PCA) is a tool for extracting applicable information from an unclear data set[58]. It is a process to reduce complex data sets to a lower dimension to reveal relevant unseen trends. PCA is known for finding correlations between variables and in some cases allowing for elimination of redundant variables[58,81]. PCA has not been utilized extensively for the analysis of separation data produced by chiral chromatography[59,60,81]. Camilleri et al. and Montanari et al. used complex molecular modeling and electronic property estimations in their analysis of enantiomeric separation data[59,60]. Chapter 2 utilized common chromatographic variables (e.g. retention factor, resolution, substitution position, and volume of the substituent group) for the analysis[81]. The latter is a more simplistic PCA approach that aids in the analysis of HPLC chiral

separations data. That is the approach used in this work to analyze the separation data of 14 ethano-Tröger bases.

In this study, derivatized cyclofructan and cyclodextrin-based chiral stationary phases (CSPs) were used to evaluate the enantiomeric separations of 14 functionalized ethano-Tröger base compounds. Cyclodextrin chiral run buffer additives were used for the separations in CE. Principal component analysis was then used to analyze the HPLC separation data. This is the first report on the use of PCA to analyze chiral separation data of functionalized ethano-bridged Tröger bases and is only the second time this approach has been used for any chiral separations.

3.3. Material And Methods

3.3.1 Materials

HPLC grade methanol (MeOH), acetonitrile (ACN), and heptane (Hept) were purchased from VWR (West Chester, PA, USA). HPLC grade ethanol (EtOH) and 2-propanol (IPA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Deionized water was produced by a Milli-Q system (Billerica, MA,USA). Ammonium acetate (H4NOAc), diethylamine (DEA), and glacial acetic acid (HOAc) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). All the chiral analytes analyzed in this study were synthesized according to Ref. 41, 42. All analytical HPLC columns used were (250 x 4.6 mm, 5 µm) in dimension and obtained from AZYP, LLC (Arlington, TX, USA) and Supelco / Astec, (Milwaukee, WI, USA). The HPLC columns used were the newly introduced LARIHC^{TM} line manufactured by AZYP, LLC and the Cyclobond series columns manufactured by Supelco / Astec. The cyclofructan based LARIHC^{TM} columns were as follows: CF6-P (isopropylcarbamate functionalized cyclofructan 6), CF6-RN (R-naphthylethyl-functionalized cyclofructan 6), and CF7-DMP (3,5-dimethylphenyl

functionalized cyclofructan 7). The cyclodextrin based columns by Supelco / Astec were as follows: Cyclobond I 2000 (β-cyclodextrin), Cyclobond II 2000 (γ-cyclodextrin), Cyclobond I 2000 AC (acetylated β-cyclodextrin), Cyclobond I 2000 DM (dimethylated β-cyclodextrin), Cyclobond I 2000 DMP (dimethylphenyl carbamate β-cyclodextrin), Cyclobond I 2000 DNP (2,6-dinitro-4-trifluoromethylphenyl ether β-cyclodextrin), Cyclobond I 2000 RN (R-naphthylethyl carbamate β-cyclodextrin), and Cyclobond I 2000 RSP (hydroxypropyl ether β-cyclodextrin).

3.3.2 Equipment And Analysis

Chromatographic separations were carried out with an Agilent 1200 series (Agilent Technologies, Inc., Santa Clara, CA) system. The system consisted of a G1322A degasser, G1311A quaternary pump, G1367B auto sampler, G1315D diode array detector, and Chemstation© software. In this system the mobile phase was degassed by ultra-sonication under vacuum for 5 minutes. All compounds were detected at 254 nm. Flow was set to 1 mL min^{-1}. The buffer component of the reverse phase system is 20 mM ammonium acetate pH 4.1.

3.3.3 Calculations

The HPLC retention factor (k) of the first enantiomer eluted was calculated using the equation $k = (t_r - t_0)/t_r$, where t_r is the retention time, to is the dead time, which is determined by the first baseline perturbation due to unretained solvent . Selectivity (α) was calculated by $\alpha = k_2/k_1$, where k_1 and k_2 are the retention factors of the first and second enantiomers, respectively. The resolution (Rs) was determined using $R_s = 2 \times (tr2 - tr_1)/(w_1 + w_2)$, where w_1 and w_2 is the base peak width of the first and second enantiomers, respectively. Principal component analysis was conducted using Matlab (Mathworks, Natick, Massachusetts, USA). The volumes of substituent groups are

defined as the $Å^3$ areas of the substituent groups. They were determined by Spartan Software by Wavefunction Inc. (Irvine, CA).

3.4 Results & Discussion

3.4.1 Analytes

The 14 ethano-bridged Tröger bases analyzed in this study are shown in Figure 3-2. The functionalized ethano-bridged Tröger bases have multiple chiral centers. The synthesis process described by Michon et al. and Sharma et al. gave rise to enantiomers with a high diastereomeric ratios determined by NMR analysis[70,71]. For example, in the Michon et al. study, NMR analysis of compound T13 shows stereoisomers with a diastereometric ratio greater than 98 : 2, and X-Ray diffraction analysis of racemic compound T13 reveals the enantiomers' configurations to be 5SN,11SN,14RC and 5RN,11RN,14SC[71]. It should be noted that these analytes have poor solubility in reversed phase mobile phases containing greater than 65-70% water.

Ethano-Bridged Tröger Base

Compound	Substituted Group		Compound	Substituted Group	
	R_1	R_2		R_1	R_2
T1	B	A	T8	J	D
T2	C	D	T9	C	L
T3	E	D	T10	D	D
T4	F	D	T11	K	K
T5	G	D	T12	K	L
T6	H	D	T13	M	A
T7	I	D	T14	N	A

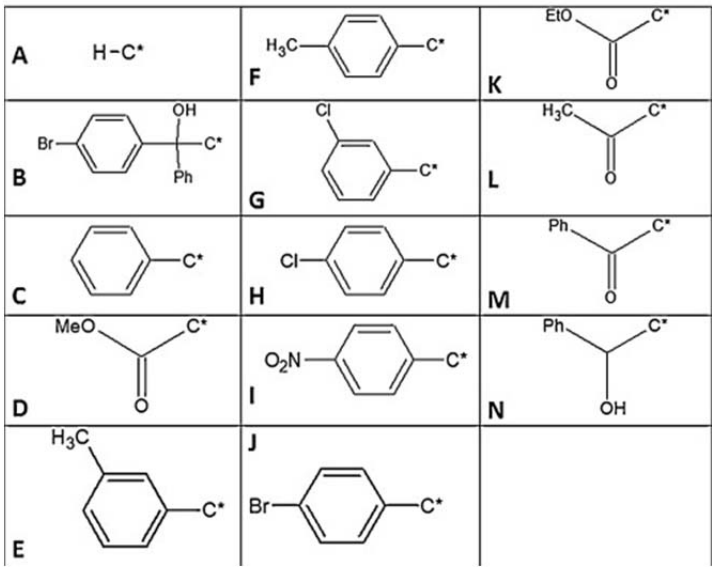


Figure 3-2 General Structure and Substituted Groups of the Fourteen Troger Racemates Structures identified as T1-T14. *C is the carbon where the substituted groups are attached. R1 and R2 are the substituted group attached to *C.

3.4.2 High Performance Liquid Chromatography Separations

Eleven macrocyclic CSPs were screened with a series of structurally related racemic analytes. The native cyclodextrin and derivatized cyclodextrin based CSPs yielded 12 baseline separations ($R_s \geq 1.5$) and 2 partial separations only in the reversed phase mode. The derivatized cyclofructan based CSPs yielded 8 baseline separations (Rs ≥ 1.5) and 2 partial separations in the normal phase mode. Clearly the pronounced structural differences in the cyclodextrin vs. cyclofructan macrocycles lead to their

opposite and unique modes of chiral recognition. There is little possibility of hydrophobic inclusion complexation with cyclofructans, while this mechanism dominates cyclodextrin separations in the reversed phase mode[74]. Among the cyclodextrin CSPs that separated the analytes, the RSP derivatized β-cyclodextrin (Cyclobond RSP) and native γ-cyclodextrin (Cyclobond II) exhibited the best enantioselectivity for these Tröger base compounds. Among the cyclofructan CSPs that separated the analytes, the CF6-RN CSP showed the best overall enantioselectivity. A summary of total, partial, and baseline separations for all analytes on all the columns tested is given in Figure 3-3, and representative chromatograms are shown in Figure 3-4. Table 3-1 shows the optimized separation conditions for each of the compounds in this study.

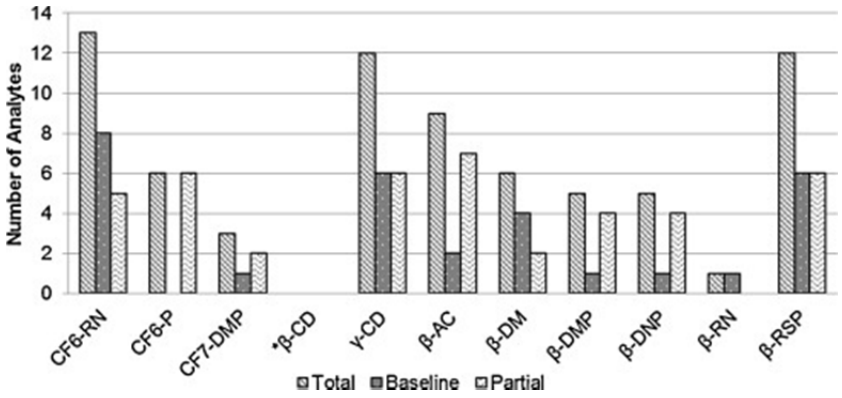


Figure 3-3 Summary of HPLC Total, Partial, and Baseline Separations

*No separations were seen on the β-CD CSP. See experimental for column abbreviations

Table 3-1 Optimized HPLC enantiomeric separation conditions for each compound

Compound	CSP[a]	Mobile Phase		k_1	R_s	α
		Percentage (v/v)	Composition[b]			
T1	β-RN	40/60	20 mM NH_4OAc pH 4.1/ACN	1.3	1.5	1.1
T2	γ-CD	65/35	20 mM NH_4OAc pH 4.1/EtOH	2.1	2.5	1.4
T3	γ-CD	65/35	20 mM NH_4OAc pH 4.1/EtOH	1.7	2.6	2.6
T4	γ-CD	65/35	20 mM NH_4OAc pH 4.1/EtOH	1.9	1.9	1.3
T5	CF6-RN	99.5/0.5	Heptane/2-propanol	1.7	1.5	1.4
T6	CF6-RN	99.5/0.5	Heptane/2-propanol	1.7	1.5	1.4
T7	CF6-RN	97/3	Heptane/2-propanol	1.5	1.6	1.2
T8	CF6-RN	99.5/0.5	Heptane/2-propanol	2.7	1.5	1.2
T9	CF6-RN	99.5/0.5	Heptane/2-propanol	1.4	1.5	1.3
T10	β-RSP	60/40	20 mM NH_4OAc pH 4.1/EtOH	1.2	2.0	1.8
T11	β-RSP	60/40	20 mM NH_4OAc pH 4.1/EtOH	1.1	1.6	1.3
T12	γ-CD	65/35	20 mM NH_4OAc pH 4.1/EtOH	1.4	0.7	1.1
T13	β-RSP	67/33	20 mM NH_4OAc pH 4.1/EtOH	6.3	1.5	1.2
T14	β-RSP	60/40	20 mM NH_4OAc pH 4.1/ACN	1.3	1.5	1.5

[a]See experimental section for description of CSP abbreviations. NH_4OAc: ammonium acetate, EtOH: ethanol

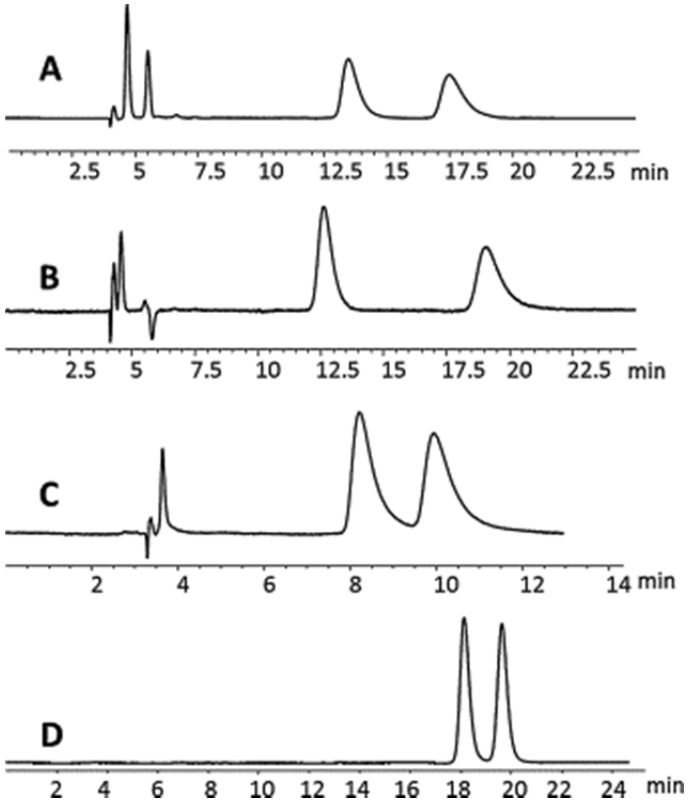


Figure 3-4 Representative chromatograms

(A) Reverse phase HPLC separation of compound T11 with Cyclobond I-RSP. (B) Reverse phase HPLC separation of compound T10 on Cyclobond I-RSP. (C) Reverse phase HPLC separation of compound T6 using Cyclobond II. (D) Normal phase separation of compound T8 with LARIHC CF6-RN. Mobile phase: 65:35 20 mM ammonium acetate pH 4.1:ethanol (A,B,C), 99.5:0.5 heptane:2-propanol (D)

Table 3-2 shows the impact of selectivity and retention of the compounds on native β-cyclodextrin and γ-cyclodextrin CSPs in the reversed phase mode. The compounds showed selectivity on γ-CD, and no selectivity on β-CD. Retention on the γ-CD CSP was significantly lower for most compounds. These are two points of interest concerning these results. First, this is one of the few examples reported in the past 30

years where γ-CD showed superior enantioselectivity for a class of compounds than β-CD[82]. Second, the generally greater retention on the β-CD CSP indicates that the strength of inclusion to a cyclodextrin is not necessarily an important factor for chiral recognition. Only compound 9 was strongly retained by the γ-CD CSP, and it did not separate.

Table 3-2 Comparison of the retention (k) and enantioselectivity (α) of the β and γ-cyclodextrin in the same HPLC reverse phase conditions.

Compound	β-Cyclodextrin		γ-Cyclodextrin	
	k_1	α	k_1	α
T1	7.5	1.0	0.8	1.0
T2	3.2	1.0	2.1	1.3
T3	3.5	1.0	1.7	1.3
T4	3.6	1.0	2.9	1.2
T5	4.2	1.0	1.8	1.2
T6	4.2	1.0	1.7	1.2
T7	3.7	1.0	1.1	1.1
T8	4.6	1.0	1.9	1.2
T9	3.4	1.0	11.0	1.0
T10	1.5	1.0	0.5	1.1
T11	1.7	1.0	0.4	1.0
T12	1.9	1.0	1.7	1.1
T13	3.4	1.0	0.6	1.1
T14	2.6	1.0	0.6	1.0

Chromatographic conditions: mobile phase, 65:35 (v/v) 20 mM ammonium acetate pH 4.1:ethanol; flow, 1 mL min-1. Longer retention is seen on the β-CD CSP with no selectivity. Shorter retention is seen on the γ-CD CSP with greatly improved enantioselectivity.

Combining the results of the native β-CD CSP (Table 3-2) and the results of RSP derivatized β-CD CSP (See Figure 3-3) another interesting trend can be seen by looking at compounds T10 and T11 (Figure 3-4A and 3-4B). Neither retained compound showed enantioselectivity on the native β-cyclodextrin column in the reversed phase mode (Table 3-2). However the hydroxypropyl ether derivatized β-cyclodextrin CSP did show selectivity for these compounds. Again, it appears that inclusion, which is the primary mechanism of retention, on native β-cyclodextrin cannot be directly correlated to enantioselectivity. This could possibly be due to the large size of compounds T10 and T11, thus limiting the amount of inclusion into the cyclodextrin cavity. On the contrary, the extended hydroxypropyl groups of the RSP cyclodextrin CSP allows additional enantioselective interactions. This behavior was seen in the most of the separations done on the native β-cyclodextrin and RSP β-cyclodextrin CSPs. The methyl esters of compound T10 showed greater selectivity and resolution compared to compound T11 on the RSP β-cyclodextrin CSP.

3.5 High Performance Liquid Chromatography Principal Component Analysis

3.5.1 Understanding Principal Component Analysis Plots

PCA two dimensional plots show points as a pattern of similarity between the samples (plotted points) and the variables. The principal components are the x and y axis of the 2D PCA plot. Correlation coefficients describes how each variable contributes to the principle component. The sum of all the squared correlation coefficients is always equal to 1. A biplot of the PCA is an exploratory graph that shows information of both samples and variables of the data matrix of the PCA. The samples are shown as points and the variables are shown as vectors. The direction of the vector shows the direction of the variable, and its magnitude shows the weight of the variable. The correlation of two variables (vectors) is distinguished by the angle between them. If the angle is close to 0˚

then it exhibits a correlation close to 1 (correlation between variables). If the angle is 180˚ the correlation is -1 (opposite correlation between variables). If the angle is orthogonal (90˚) then the correlation is small between the variables. The cosine of each line (vector) to the principle component (x or y axis) represents each variables' contribution to the principle component in both sign and magnitude. However, when using Matlab to create biplots, it forces the variable with the largest magnitude of the correlation coefficient to be positive[83]. This in turn flips some of the vectors in the correlation coefficients to the opposite direction, but makes the biplot easier to interpret. The change of the correlation coefficient vector does not change the meaning of the biplot.

A simplified PCA analysis for the investigation of chiral chromatographic systems was desired, and simple chromatographic and analyte variables were chosen. The variables applied to the PCA analysis were enantiomeric resolution (Rs), retention of first enantiomer (t1), and substituent size (R1 and R2) of the analytes (Table 3-3). Application of PCA was performed on the data of the three of the best chiral HPLC conditions for the set of compounds with the following respective HPLC conditions: CF6-RN, 97/3 (v/v) Heptane/IPA; Cyclodextrin RSP, 50/50 (v/v) 20 mM NH_4OAc pH 4.1/ethanol; and γ-Cyclodextrin, 65/35 (v/v) 20 mM NH_4OAc pH 4.1/ethanol. The chromatographic data applied to the PCA biplot analysis is shown in Table 3-3.

Table 3-3 The 14 racemates (T1-T14) with the list of the volume (Å^3) of substituted groups and the chromatographic data

Compound	Retention (t_1)			Resolution (R_s)			Volume of the Substituted Group (Å^3)	
	CF6-RN	CD-RSP	γ-CD	CF6-RN	CD-RSP	γ-CD	R_1	R_2
T1	10.6	11.8	5.5	0.6	0.7	0.0	227.3	10.5
T2	5.0	7.3	9.4	1.0	1.7	2.5	98.9	63.5
T3	4.8	4.8	8.0	0.5	1.0	2.5	117.4	63.5
T4	4.9	5.8	11.7	0.7	1.1	1.8	117.4	63.5
T5	4.9	5.4	8.3	0.9	0.7	1.9	112.7	63.5
T6	4.9	4.9	8.2	0.9	0.6	1.9	112.7	63.5
T7	7.4	5.7	6.2	1.7	0.0	1.1	121.2	63.5
T8	4.8	4.7	8.8	0.9	0.5	1.7	117.2	63.5
T9	5.2	6.3	36.0	2.9	0.0	0.4	98.9	54.5
T10	8.5	3.8	4.4	1.0	1.9	0.7	63.5	63.5
T11	6.0	3.6	4.2	0.2	1.0	0.3	82.0	82.0
T12	6.0	4.0	8.2	0.0	0.0	0.7	82.0	54.5
T13	6.9	4.0	4.9	0.6	0.5	0.8	120.1	10.5
T14	9.4	9.3	4.8	0.7	0.0	0.0	124.9	10.5

HPLC conditions: mobile phase 97:3 (v/v) heptane:2-propanol (CF6-RN); 50:50 (v/v) 20 mM ammonium acetate pH 4.1:ethanol (CD-RSP); 65:35 (v/v) 20 mM ammonium acetate pH 4.1:ethanol (γ-CD)

3.5.2 Cyclobond Ii Principal Component Analysis Biplot Interpretation

The Cyclobond II PCA biplot is shown in Figure 3-6A. The weight the variables contribute to the principle components (x-axis and y-axis) is shown in Figure 3-6B, and R1 is the largest weighted variable (largest squared correlation coefficient) in both principle components. A cluster of points (circled in red) seen mainly in the (+,-) quadrant shows a grouping of the racemic analytes that are separated. The cluster of compounds reside in the area of the biplot were the variables of resolution and R2 substituent size resides. This shows the contribution of the size of the R2 substituent to the enantioselectivity of these compounds. In addition, the low angle value between the two vectors of the variables shows that there is a correlation between resolution and the size of the R2, which are mainly methyl ester groups.

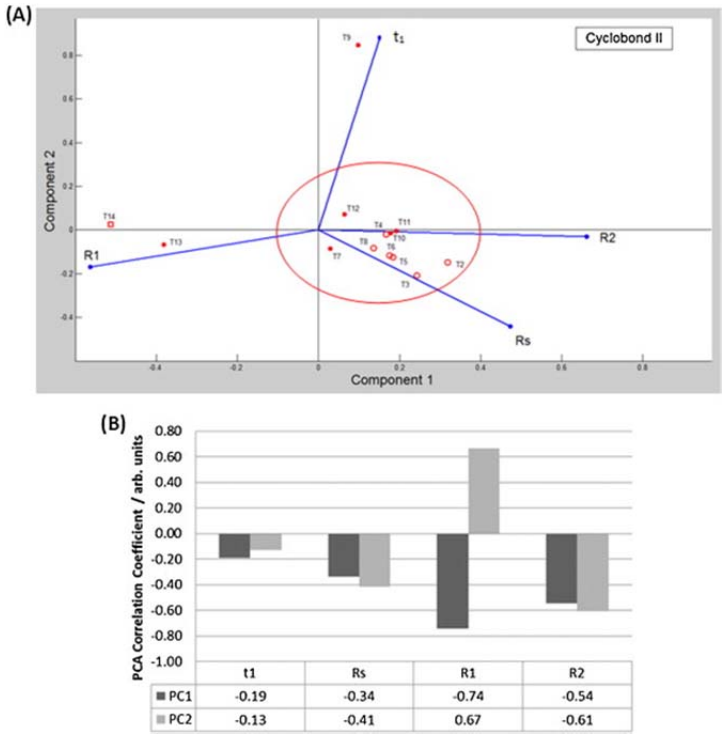


Figure 3-5 PCA Biplots of Tröger Bases Using Cyclobond II and PCA Correlation Coefficient

A) The blue lines represent the magnitude and direction of the variables: retention factor (t_1), enantioresolution (R_S) and volume of the substituent groups (R1 & R2). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

B) PCA correlation coefficient. The contribution of the variable (t_1), 1st enantiomer retention time, (R_s) enantioresolution and (R1 & R2) substituent group volume to the respective principle component.

Compounds T14 and T13 lie outside of the cluster and are weighted near the vector of the size of the substituent R1. Compound T14 and T13 have large substituent groups in the R1 position with small R2 substituents. These compounds are outside of the cluster region that show increased enantioresolution. Compound T9 lies in the domain of vector of the retention variable (t_1) in the biplot. Looking at the chromatographic data of Table 3-1, compound T9 has the highest retention with the γ-CD CSP. There is no clear explanation, with the variables analyzed, for the long retention or the lack of enantioselectivity. Clearly this compound is an outlier.

3.5.3 Cyclobond RSP Principal Component Analysis Biplot Interpretation

The Cyclobond RSP PCA biplot is shown in Figure 3-6A. The weight the variables contribute to the principle components (x-axis and y-axis) is shown in Figure 3-6B. Figure 6B shows R1 is the largest weighted variable (largest squared correlation coefficient) on the x-axis, and R2 is the largest weighted variable on the y-axis. A cluster of points (circled in red) seen mainly in the (-,+) quadrant shows a cluster of samples were the variables of resolution and R2 substituent size resides. The angle between the two vectors of resolution and R2 is large and thus the correlation of the two variables is present but not great.

The Cyclobond RSP PCA biplot analysis (Figure 3-6A) does show a strong correlation between retention and the size of the R1 group. The larger R1 groups are substituted aromatic rings (Figure 3-2), which are well known to include into the cyclodextrin cavity in reversed phase conditions. However, even though retention was seen, none of the variables in the PCA biplot showed a strong correlation to enantioresolution. This is due to the fact that inclusion is necessary but not sufficient for enantioselectivity in this separation system. It is the pendant isopropyl groups on the β-CD that enhance chiral selectivity.

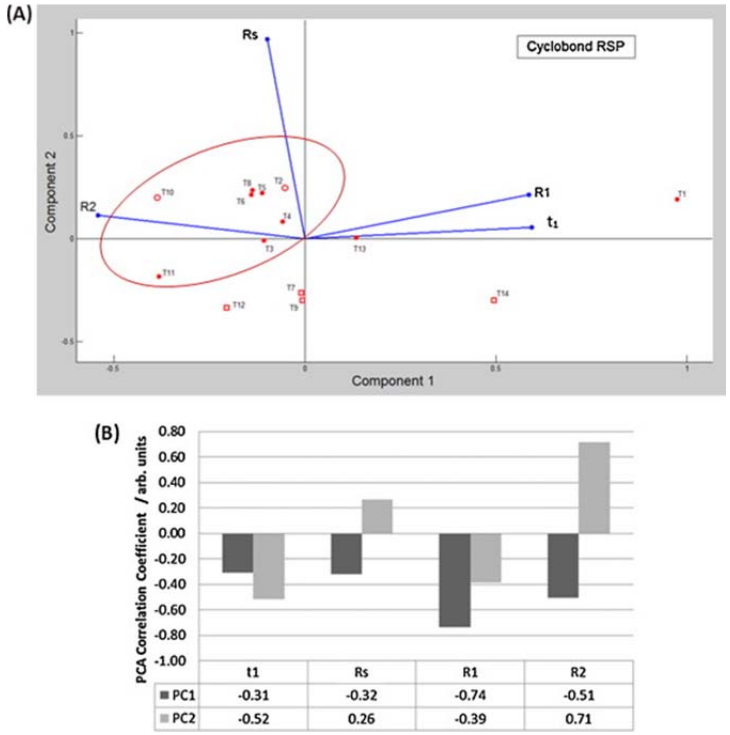


Figure 3-6 PCA Biplots of Tröger Bases Using Cyclobond RSP and PCA Correlation Coefficient

A) The blue lines represent the magnitude and direction of the variables: retention factor (t_1), enantioresolution (R_S) and volume of the substituent groups (R1 & R2). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

B) PCA correlation coefficient. The contribution of the variable (t_1), 1st enantiomer retention time, (R_s) enantioresolution and (R1 & R2) substituent group volume to the respective principle component.

3.5.4 CF6-RN Principal Component Analysis Biplot Interpretation

The CF6-RN PCA biplot is shown in Figure 3-7A. The weight the variables contribute to the principle components (x-axis and y-axis) is shown in Figure 3-7B. Figure 7B shows R1 is the largest weighted variable (largest squared correlation coefficient) on the x-axis, and the R2 is the largest weighted variable on the y-axis. A cluster of points (circled in red) seen mainly in the (+,+) and (+,-) quadrants shows a cluster of samples were the variables of resolution and R2 substituent size resides. The angle value between the two vectors of resolution and R2 is large and the correlation of the two variables is present but not great.

The CF6-RN PCA biplot analysis (Figure 3-7A) shows a very strong correlation between retention and the size of the group R1. The size of the R2 substituent does inversely correlate to retention. The PCA biplot did not show a strong correlation between enantioresolution and the variables analyzed. The chromatographic data does show that the majority of the analytes did display enantioselectivity for CF6-RN CSP in the normal phase mode. In previous work by Sun et al., methano-Tröger base was separated using the CF6-RN column in normal phase conditions and a resolution of 5.2 was observed [84]. This information agrees with the PCA analysis shown here where the variables R1 and R2 do not strongly correlate to enantioresolution. Thus, in this chiral separation system, it is clear that the size and/or presence of the R1 and R2 groups play little role in the chiral recognition of these particular analytes.

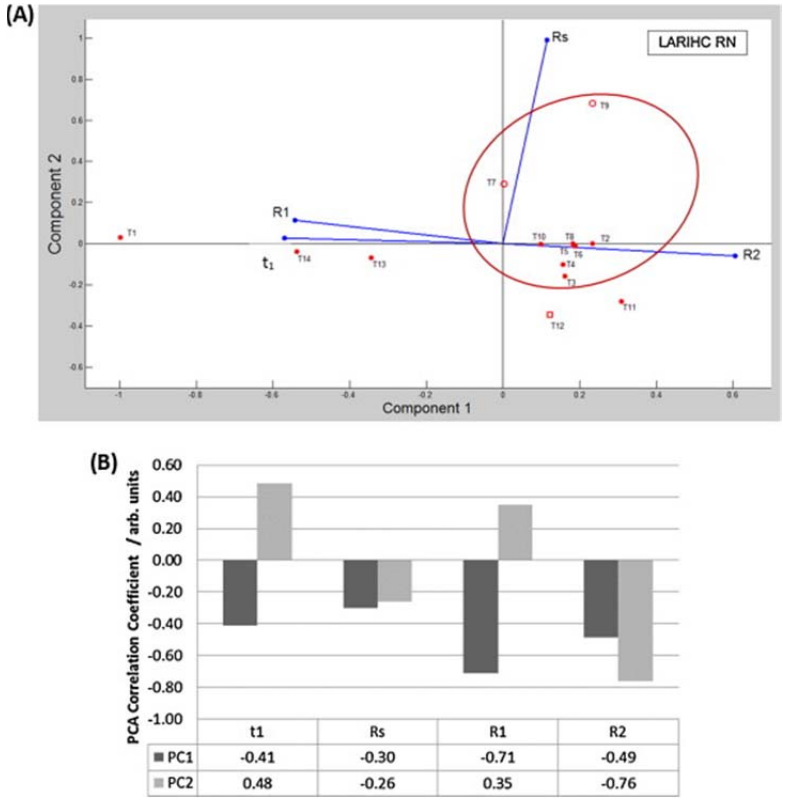


Figure 3-7 PCA Biplots of Troger Bases Using CF6-RN and PCA Correlation Coefficient

A) The blue lines represent the magnitude and direction of the variables: retention factor (t_1), enantioresolution (R_S) and volume of the substituent groups (R1 & R2). The cosine of each line to the component 1 or 2, represent each variables contribution to the component, both magnitude and in sign (+ or -). Data points: (●) baseline separated compounds ($R_s \geq 1.5$), (○) partial separations ($0<R_s < 1.5$) and (□) non-separated compounds ($R_s = 0$). Note that axes are in arbitrary units.

B) PCA correlation coefficient. The contribution of the variable (t_1), 1st enantiomer retention time, (R_s) enantioresolution and (R1 & R2) substituent group volume to the respective principle component.

3.6 Capillary Electrophoresis Separations

While using capillary electrophoresis to examine the efficacy of the larger derivatized γ-cyclodextrins, only 2 of 14 compounds were enantiomerically separated using hydroxypropyl β and γ-cyclodextrin (Figure 3-8). In the CE analysis the examination of the compounds was poorly achieved due to the reduced solubility of the analytes in aqueous solvent. However, compound T14 was separated using β and γ-hydroproxylated cyclodextrins (Figure 3-8a and 3-8b). Comparing the migration time and the enantiomeric resolution values of hydroxypropyl β and γ-cyclodextrin (Figure 8A and 8B) it is seen that γ-hydroproxylated cyclodextrin has shorter migration time (less analyte inclusion) with greater enantioresolution. In addition, adding an organic modifier such as ethanol improved the solubility of these hydrophobic compounds and allowed compound T13 to be separated with γ-hydroproxylated cyclodextrin (Figure 3-8C).

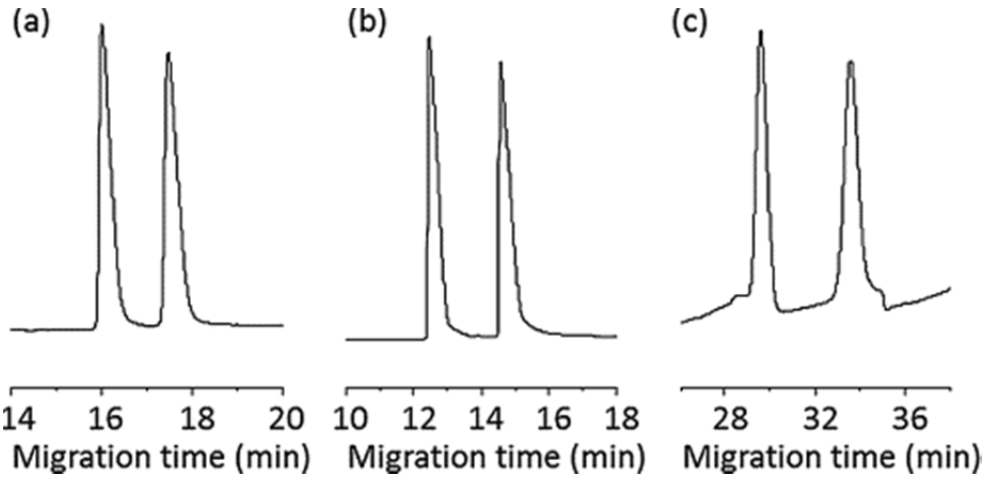


Figure 3-8 Tröger Electropherograms

Electropherograms for (a) compound T14 using 50 mM HP-β-CD, (b) compound T14 using 50 mM HP-γ-CD and (c) compound T13 using 50 mM HP-γ-CD with 20% ethanol modifier. Buffer: 50 mM phosphate pH 2.5, 12 kV (normal polarity). Resolutions values: 2.8 (a), 3.4 (b), 3.7 (c).

3.7 Conclusions

Enantiomeric separation methods have been developed for 14 newly synthesized functional ethano-Tröger base racemates in HPLC and CE. Cyclodextrin based CSPs were most successful under reversed phase conditions in HPLC using RSP derivatized β-cyclodextrin and native γ-cyclodextrin. Cyclofructan based CSPs were most successful under normal phase conditions in HPLC with the CF6-RN column being the most successful. Using CE only two of 14 compounds were enantiomerically separated using hydroxypropyl β and γ-cyclodextrin. In HPLC it was seen that native γ-cyclodextrin CSP separated the ethano-Tröger base racemates whereas the native β-cyclodextrin CSP did not. RSP β-cyclodextrin allowed enhanced interaction of the analytes via its isopropyl ether functional groups on the rim of the cyclodextrin, and this increased enantiomeric resolution. Without the pendant isopropyl ether groups on the β-cyclodextrin, chiral selectivity is lost. The PCA biplot analysis of RSP-cyclodextrin, and CF6-RN showed that retention is governed by the size of the R1 substituent. In all three cases, the PCA biplot analysis of γ-cyclodextrin, RSP-cyclodextrin, and CF6-RN showed enantioresolution is not governed by retention. However, in the case of γ-cyclodextrin resolution was closely correlated with the size of the R2 group.

Chapter 4

Enantiomeric Separation of Biaryl Atropisomers Using Cyclofructan Based Chiral Stationary Phases

4.1 Abstract

Normal phase chiral HPLC methods are presented for the enantiomeric separation of 30 biaryl atropisomers including 18 new compounds recently produced via a novel synthetic approach. Three new cyclofructan based chiral stationary phases were evaluated. Separations were achieved for all but six analytes and the LARIHC^{TM} CF6-P alone provided 15 baseline separations. Effects of polar modifiers and temperature effects also were studied. Apparent thermodynamic parameters were determined by van't Hoff plots. Preparative scale methods were developed and employed resulting in the first ever isolation of these novel atropisomers in their pure enantiomeric form. Insights into the mechanism of retention and chiral discrimination are presented.

4.2 Introduction

Substituted biaryls in which the rotation around the aryl-aryl single bond is hindered are referred to as atropisomers. They represent a major class of axially chiral molecules that have found use in many applications including privileged ligands in asymmetric synthesis[85-88], chiral resolving agents[89], and as pharmaceutical compounds[90,91]. Recently, a variety of novel 2,2`-diamino-1,1`-binaphthalenes[92] as well as 2,2`-aminohydroxy-1,1`-biaryls were synthesized using a transition metal free direct arylation method[93]. These new compounds have the potential to be used as chiral ligands in asymmetric synthesis and may possess unique biological activities including antitumor and antimicrobial activities[94]. The chiral analytes considered herein are 1,1`-biaryls and fall into one of three groups: 2,2`-diol, 2-amino-2`-ol and 2,2`-diamine. Probe analytes also differ in aryl type and type/position of substituents on the aryl groups.

Chiral molecules are often needed as pure enantiomers for evaluation in the aforementioned applications and thus the need for methods to determine the enantiomeric excess (%ee) of newly synthesized molecules is ever present[95-100]. There is also a need to develop preparative HPLC methods to purify milligram to gram scale amounts of enantiomerically pure compounds [5,101]. HPLC combined with chiral stationary phases (CSP's) has proven to be an excellent technique for the separation of axially chiral molecules[102-105]. A wide variety of CSP's have been used to separate biaryl atropisomers including bonded cyclodextrins[73], 1,3,5 triazine based CSP's[106], quaternized brucine-based CSP's[107], derivatized cyclofructans[39,80] and immobilized polysaccharide-based CSP's[108]. Chiral HPLC is also useful for preparing single enantiomers as instrumental methods and HPLC column dimensions are easily scaled from analytical to semi-preparative and preparative capacities[5].

A new class of CSP's based upon derivatized cyclofructans, which are cyclic oligosaccharides consisting of six or more β(2→1)-linked *D*-fructofuranose units has recently been introduced[37]. In this study, three functionalized cyclofructan CSP's were evaluated for use as HPLC CSP's. The first, the LARIHC CF6-P (isopropylcarbamate derivatized cyclofructan-6) has shown exceptional selectivity for racemates with a primary amine moiety[36] while the LARIHC CF6-RN (R-napthyethylcarbamate derivatized cyclofructan-6) and LARIHC CF7-DMP (dimethyphenylcarbamate derivatized cyclofructan-7) CSP's have shown broad selectivity and applicability for a variety of classes of molecules.[38,40,61,109,110]

In this chapter, 30 biaryl atropisomers were screened with three CSP's under normal phase and polar organic HPLC conditions to elucidate potential interactions governing retention and enantioselectivity on cyclofructan based chiral selectors. The three commercially available binapthyl analytes, BINAM, BINOL and NOBIN as well as 1-

(2-aminophenyl)naphthalen-2-amine were selected for further analysis to study the effect of the three different 2,2` substituents and type of aryl groups on retention and enantioselectivity. For normal phase separations, the effects of both the type and composition of the polar modifier was investigated as well as the effect of column temperature on enantioselectivity. The effect of acidic and basic additives also was investigated. A preparative separation of 1-(2-amino-3,4,5-trichlorophenyl)naphthalen-2-ol is presented allowing the pure enantiomers of this novel molecule to be evaluated for antimicrobial/antibiotic activity (data not reported), showing the separations reported herein are both scalable and necessary for future applications involving these new biaryls. This is the first report on the enantiomeric separation of many of these important analytes which, in turn, provides insights into the mechanism of retention and chiral recognition for cyclofructan based CSP's.

4.3 Experimental

4.3.1 Materials

HPLC grade heptane, ethanol, acetonitrile and ACS grade hexanes (5% methylpentanes) were purchased from Fisher Scientific (Waltham, MA). HPLC grade 1-propanol, 2-propanol 1-butanol, ACS grade trifluoroacetic acid and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). 1,1′-binaphthyl-2,2′-diamine (BINAM, Table 1 no. 1), 1,1′-bi-2-naphthol (BINOL, no. 2), , 2′-amino-1,1′-binaphthalen-2-ol (NOBIN, no. 3), 6,6'-dibromo-[1,1'-binaphthalene]-2,2'-diol (no. 23), 3,3'-bis(3,5-dimethylphenyl)-5,5',6,6',7,7',8,8'-octahydro-[1,1'-binaphthalene]-2,2'-diol (no. 24), 3,3'-diphenyl-[2,2'-binaphthalene]-1,1'-diol (VANOL, no. 25), 3,3'-dibromo-5,5',6,6',7,7',8,8'-octahydro-[1,1'-binaphthalene]-2,2'-diol no. 26 , 3,3'-dibromo-[1,1'-binaphthalene]-2,2'-diol (no. 27), 3,3'-bis(triphenylsilyl)-[1,1'-binaphthalene]-2,2'-diol (no. 28), 3,3'-

di(anthracen-9-yl)-[1,1'-binaphthalene]-2,2'-diol (no. 29), and 2,2'-dimethoxy-1,1'-binaphthalene (no. 30) were purchased from Sigma-Aldrich (St. Louis, MO). LARIHC CF6-P, CF6-RN and CF7-DMP were obtained from AZYP L.L.C. (Arlington, TX). Analytes 4-22 (Table 1) were synthesized as reported in Ref. 9.

4.3.2 HPLC Methods

All analytical analyses were performed on an Agilent© 1260 Infinity HPLC system utilizing a degasser, quaternary pump, autosampler, column thermostat and diode array detector. Data analysis was carried out using OpenLAB CDS Chemstation© Edition Rev. C.01.04. Samples were prepared at approximately 0.5 mg mL^{-1} in ethanol. Analytical column dimensions were 250 x 4.6 mm with 5 µm particle diameter. All injections were 5µL unless otherwise noted. Flow rates were held at 1 mL min^{-1} unless otherwise noted. Wavelengths monitored were 254 nm and 280 nm. Separations were performed at ambient temperature unless otherwise noted. Normal phase mobile phases consisted of heptane with a polar modifier. Ethanol, 1-propanol, 2-propanol and 1-butanol were evaluated as polar modifiers in the range of 1-50% (v/v). Polar organic mobile phases consisted of acetonitrile with 0-10% methanol as a modifier. Void volumes were determined by the first disturbance in the baseline resulting from unretained diluent. Resolutions (R_s) and peak symmetries (PAF) were calculated using Chemstation© software.

Thermodynamic experiments were carried out at 25°C, 29°C, 33°C, 37°C and 41°C to determine the enthalpic and entropic contributions using the equation: $\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + ln\phi$ where ΔH° and ΔS° represent the change in standard molar enthalpy and entropy respectively, R is the universal gas constant and T is the absolute temperature (K) of the column, ϕ is the ratio of stationary phase and mobile phase

volumes, V_s and V_m respectively. $\Delta S°*$ is used in place of $\frac{\Delta S°}{R} + \ln \phi$ as the chromatographic phase ratio is not easily determined. All thermodynamic values are stated as apparent rather than absolute due to the inability to distinguish between enantioselective and non-enantioselective interactions. Thermodynamic values were calculated using Microsoft© Excel.

Preparative scale analyses were conducted on a Shimadzu© preparative LC system consisting of an LC-20AP pump, SPD-20AV detector, SIL-10AP autosampler and FRC-10A fraction collector. Data analysis was conducted using LabSolutions© Ver. 5.54 SP1. The LARIHC CF6-P preparative column dimensions were 250 x 21.2 mm with 5µm particle diameter (AZYP, LLC). Sample 19 (Table 1) was dissolved in 50:50 hexanes: ethanol at 60 mg mL^{-1}. The mobile phase consisted of 98:2 hexanes: ethanol with a flow rate of 30 mL min^{-1}. Stacked injections of 200 µL (12mg) were performed at 15 minute intervals. The wavelength used was 254 nm. Fractions containing each enantiomer were pooled and solvent removed under reduced pressure. For determining enantiomeric excess of the collected fractions, detector linearity was confirmed at 0.4 – 20 µg (on column, R^2 = 0.998, n = 5). Samples were prepared at 1 mg mL^{-1}. S/N for the minor enantiomer peak was >100 with the major enantiomer peak < 1 A.U.

4.4 Results & Discussion

4.4.1 Separations Obtained and Insights into Retention and Chiral Recognition

Table 4-1 shows the analyte structures, optimized separation conditions and chromatographic data for 30 biaryl atropisomers. Under normal phase conditions, the LARIHC CF6-P stationary phase showed enantioselectivity towards 22 out of the 30 analytes with 15 baseline separations ($R_s \geq 1.5$). The CF6-RN and CF7-DMP showed enantioselectivity for 15 analytes each with 8 and 10 baseline separations respectively. The CF6-RN column best complemented the CF6-P column in that it was able to provide

two unique separations (Table 4-1, compounds 9, 24) which were not obtained on the CF6-P column. Further, the CF6-RN provided one additional baseline separation (Table 4-1, compound 26) which was only partially separated by the CF6-P. Though the CF7-DMP phase did not provide any unique separations, it did on occasion yield excellent resolutions such as a R_s value of 7.6 (Table 4-1, compound 6). In all, enantioselectivity was observed for 24 of 30 analytes with 17 baseline separations using a heptane mobile phase with ethanol as a polar modifier. Clearly, the CF6-P is the most useful CSP studied in the separation of this set of atropisomers.

The common normal phase additives triethylamine (TEA) and trifluoroacetic acid (TFA) were evaluated at various concentrations. Peak symmetry was improved by using TEA but retention and selectivity were decreased. No significant increase in resolution was observed when TEA concentrations ranged from 0.05-0.2%. A large decrease in retention was observed when using 0.2% TEA. Using TFA in the mobile phase caused a decrease in retention and no significant improvement in resolution.

Both retention and selectivity varied considerably for different analyte and CSP combinations. The lack of aromatic functionality on the CF6-P column indicates that π-π interactions play no role in either retention or enantioselectivity when using this CSP. The potential exists for π-π interactions when using the aromatic functionalized CF6-RN and CF7-DMP CSP's. However, no pronounced trend showing increased retention was observed under similar mobile phase conditions for these CSP's *vs*. the CF6-P. Under polar organic conditions, no retention was observed for any analyte when using 100% acetonitrile indicating that retention due to hydrogen bonding between the analyte and CSP is not substantial in the presence of that solvent. The potential exists for hydrogen bonding to play a role in chiral recognition, but only under conditions that favor analyte retention *i.e.* high heptane content in the mobile phase. Given the apparent lack of

strong π-π and H-bonding interactions, the primary mechanism for analyte retention is proposed to be dipole-dipole interactions between the polar 2,2` moieties of the biaryls and the polar groups present on the derivatized cyclofructans. Previous studies using linear free energy relationship (LFER) models have shown that dipolarity/polarizability plays an important role in both retention and chiral recognition when using derivatized cyclofructans under normal phase conditions.[110] This is further validated by the lack of retention observed for analytes with steric hindrance at the 2,2` positions (Table 4-1, compound 28, 29, 30). This indicates that retention is highly dependent on the ability of the polar 2,2` moieties to form a strong interaction with the CSP.

The retention and selectivities observed for the various analytes varied considerably depending on both the type of aryl groups present as well as the type and location of substituents on the aryl rings. As can be seen in Table 4-1, when using the CF6-P CSP with 10% ethanol in heptane, the binapthyl diamine BINAM retained longer and showed greater enantioselectivity (k_1 = 3.4, α = 1.22) than the 2,2`-diol BINOL ($k_1$ = 2.6, α = 1.12), with the 2-amino-2`-ol (NOBIN) showing intermediate retention and selectivity (k_1 = 2.7, α = 1.14). This indicates that the amine group plays an integral role in both retention and enantioselectivity on this CSP. When comparing BINAM (k_1 = 3.4, α = 1.22) to 1-(2-aminophenyl)naphthalen-2-amine (k_1 = 3.0, α = 1.12), retention and selectivity were also increased indicating that selectivity is greatly improved when both aryl groups are naphthyl.

With respect to the types of substituents on the aryl groups, the general trend was that increasing the number of electron withdrawing substituents had the effect of increasing retention and selectivity. As can be seen in Fig. 4-1, a pronounced increase in selectivity is observed (1.15 *vs*. 1.01) on the CF6-P stationary phase when comparing 1-(2-amino-3,4,5-trichlorophenyl)naphthalen-2`-ol (Table 4-1, compound 19) *vs*. 1-(2-

amino-3-chlorophenyl)naphthalen-2`-ol (Table 4-1, compound 8) with the dichloro substituted analyte showing intermediate selectivity. Possible explanations for the enhanced chiral recognition include altering of the dihedral angle of the aryl-aryl bond as well as induced differences in the hybridization of the amine group. The majority of analytes that showed poor selectivity were either of the napthyl-phenyl type with only a single substituent present on the phenyl ring or had the 2,2` moieties hindered by bulky substituents at the 3,3` positions. Modeling studies to determine the effect of substituents on the dihedral angle and to elucidate the types of interactions between the derivatized cyclofructans and biaryl analytes is ongoing.

Table 4-1 Structures and chromatographic data for chiral separations

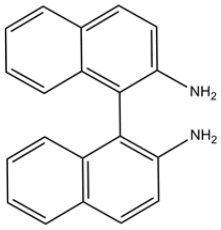
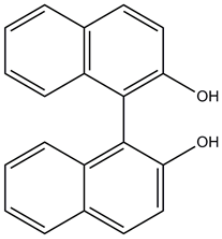
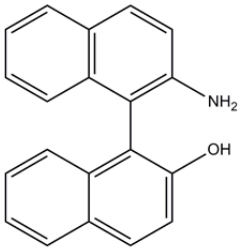
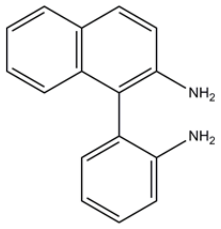
Analyte no.	Structure	Column	% ethanol	α	R_S	k_1	k_2
1	 [1,1'-binaphthalene]-2,2'-diamine	CF6-P	10%	1.26	4.8	4.1	5.2
		CF6-RN	10%	1.21	4.6	5.0	6.1
		CF7-DMP	30%	1.35	6.8	3.1	4.2
2	 [1,1'-binaphthalene]-2,2'-diol	CF6-P	10%	1.10	2.6	2.8	3.1
		CF6-RN	5%	1.06	1.0	5.5	5.9
		CF7-DMP	5%	1.09	1.5	5.2	5.7
3	 2'-amino-[1,1'-binaphthalen]-2-ol	CF6-P	10%	1.10	2.7	3.0	3.4
		CF6-RN	5%	1.14	1.6	5.5	6.3
		CF7-DMP	10%	1.27	5.5	5.8	7.4
4	 1-(2-aminophenyl)naphthalen-2-amine	CF6-P	10%	1.16	2.9	3.5	4.1
		CF6-RN	10%	1.10	2.6	4.0	4.5
		CF7-DMP	5%	1.2	5.3	4.5	5.5

Table 4-1 continued

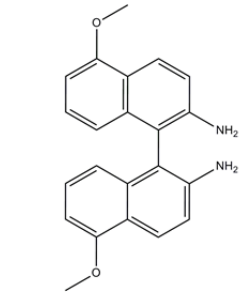
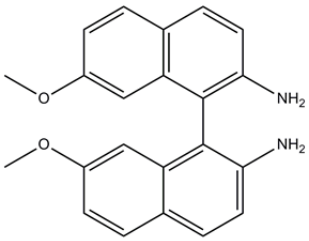
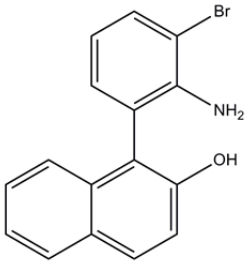
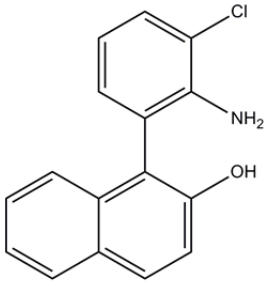
Analyte #	Structure	Column	% ethanol	α	R_S	k`$_1$	k`$_2$
5	 5,5'-dimethoxy-[1,1'-binaphthalene]-2,2'-diamine	CF6-P	10%	1.15	3.0	5.1	5.9
		CF6-RN	10%	1.10	2.7	7.0	7.8
		CF7-DMP	50%	1.27	3.2	2.6	3.3
6	 7,7'-dimethoxy-[1,1'-binaphthalene]-2,2'-diamine	CF6-P	5%	1.1	1.7	8.0	9.0
		CF6-RN	5%	1.1	1.9	8.6	9.4
		CF7-DMP	30%	1.5	7.6	5	7.5
7	 1-(2-amino-3-bromophenyl)naphthalen-2-ol	CF6-P	10%	1.06	1.0	1.5	1.7
		CF6-RN	10%			1.7	
		CF7-DMP	1%			3.7	
8	 1-(2-amino-3-chlorophenyl)naphthalen-2-ol	CF6-P	10%	1.06	1.0	1.55	1.67
		CF6-RN	10%			1.8	
		CF7-DMP	1%			3.7	

Table 4-1 continued

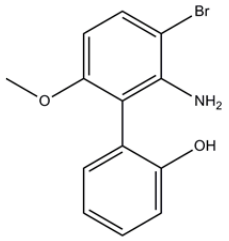
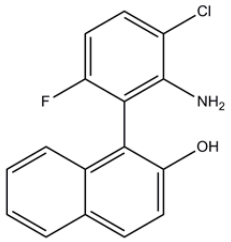
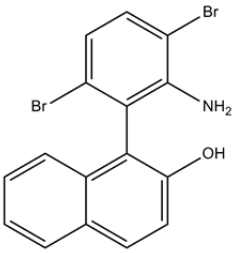
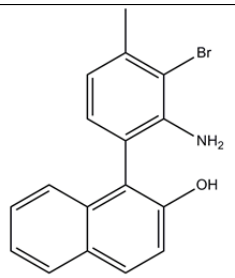
Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
9	 2'-amino-3'-bromo-6'-methoxy-[1,1'-biphenyl]-2-ol	CF6-P	10%			5.1	
		CF6-RN	1%	1.07	1.9	8.3	8.9
		CF7-DMP	1%			5.6	
10	 1-(2-amino-3-chloro-6-fluorophenyl)naphthalen-2-ol	CF6-P	1%	1.03	1.1	17.0	17.6
		CF6-RN	10%			2.7	
		CF7-DMP	5%	1.04	1.0	7.4	7.7
11	 1-(2-amino-3,6-dibromophenyl)naphthalen-2-ol	CF6-P	10%	1.13	2.3	2.1	2.4
		CF6-RN	1%			13.6	
		CF7-DMP	1%	1.04	1.5	7.4	7.8
12	 1-(2-amino-3-bromo-4-methylphenyl)naphthalen-2-ol	CF6-P	1%			4.8	
		CF6-RN	10%			1.5	
		CF7-DMP	1%			3	

Table 4-1 continued

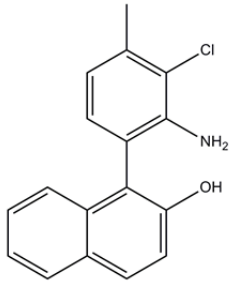
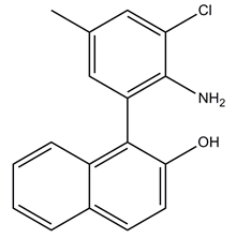
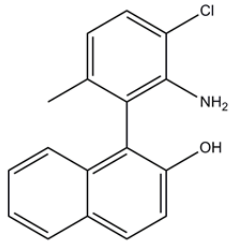
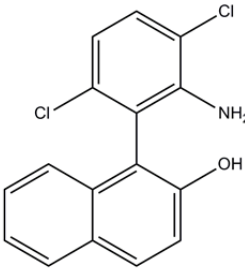
Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
13	 1-(2-amino-3-chloro-4-methylphenyl)naphthalen-2-ol	CF6-P	10%	1.03	0.4	3.5	3.6
		CF6-RN	10%			1.5	
		CF7-DMP	1%			3.0	
14	 1-(2-amino-3-chloro-5-methylphenyl)naphthalen-2-ol	CF6-P	10%			1.2	
		CF6-RN	10%			1.5	
		CF7-DMP	25%			1.9	
15	 1-(2-amino-3-chloro-6-methylphenyl)naphthalen-2-ol	CF6-P	1%	1.01	0.3	5.32	5.39
		CF6-RN	10%			1.5	
		CF7-DMP	1%			3.1	
16	 1-(2-amino-3,6-dichlorophenyl)naphthalen-2-ol	CF6-P	5%	1.10	2.1	4.5	4.9
		CF6-RN	10%			2.5	
		CF7-DMP	1%	1.04	1.2	7.1	7.4

Table 4-1 continued

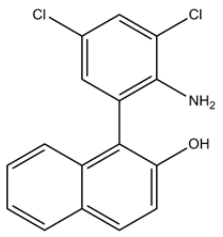
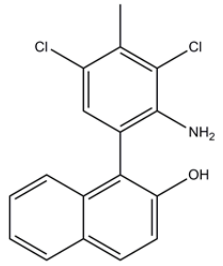
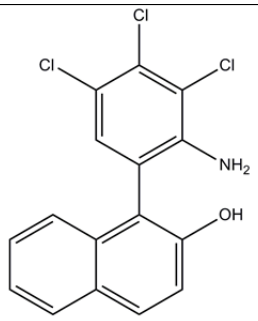
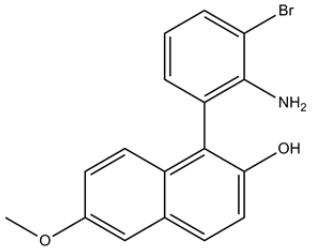
Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
17	 1-(2-amino-3,5-dichlorophenyl)naphthalen-2-ol	CF6-P	5%	1.1	2.3	3.7	4.1
		CF6-RN	10%			2.1	
		CF7-DMP	1%	1.03	1.1	6.5	6.8
18	 1-(2-amino-3,5-dichloro-4-methylphenyl)naphthalen-2-ol	CF6-P	5%	1.09	2.0	3	3.3
		CF6-RN	10%			1.8	
		CF7-DMP	1%	1.05	1.6	5.4	5.7
19	 1-(2-amino-3,4,5-trichlorophenyl)naphthalen-2-ol	CF6-P	5%	1.15	3.6	4.8	5.6
		CF6-RN	10%	1.02	0.5	5.8	5.9
		CF7-DMP	1%	1.09	2.5	9.1	9.9
20	 1-(2-amino-3-bromophenyl)-6-methoxynaphthalen-2-ol	CF6-P	10%			5.4	
		CF6-RN	10%			2.4	
		CF7-DMP	1%			10	

Table 4-1 continued

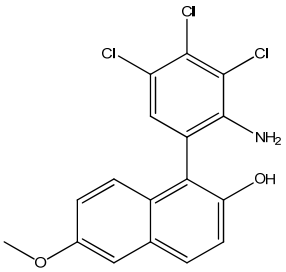
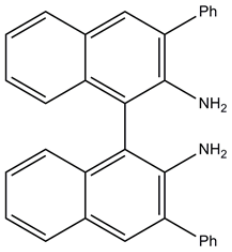
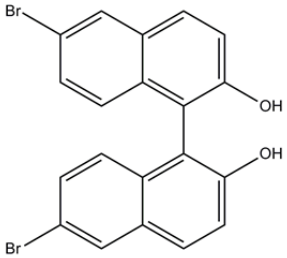
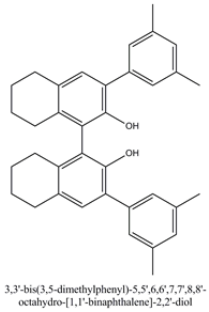
Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
21	 1-(2-amino-3,4,5-trichlorophenyl)-6-methoxynaphthalen-2-ol	CF6-P	10%	1.3	5.8	2.9	3.9
		CF6-RN	10%	1.06	2.2	7.9	8.4
		CF7-DMP	5%	1.2	3.6	4.4	5.0
22	 3,3'-diphenyl-[1,1'-binaphthalene]-2,2'-diamine	CF6-P	1%	1.13	1.5	1.1	1.2
		CF6-RN	5%			0.8	
		CF7-DMP	5%			0.5	
23	 6,6'-dibromo-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%	1.15	2.7	7.3	8.4
		CF6-RN	5%	1.12	2.2	8.3	9.3
		CF7-DMP	5%	1.07	1.5	5.8	6.2
24	 3,3'-bis(3,5-dimethylphenyl)-5,5',6,6',7,7',8,8'-octahydro-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%			0.1	
		CF6-RN	1%	1.07	0.6	0.8	0.9
		CF7-DMP	5%			0.18	

Table 4-1 continued

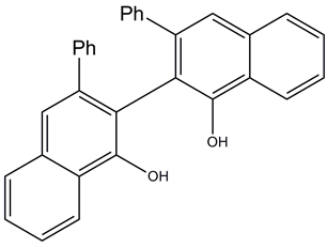
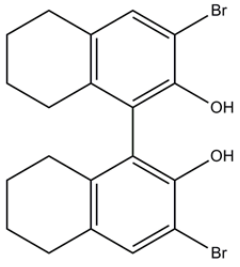
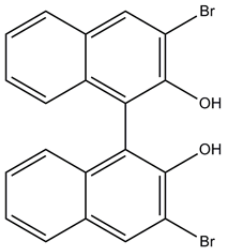
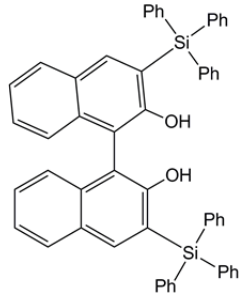
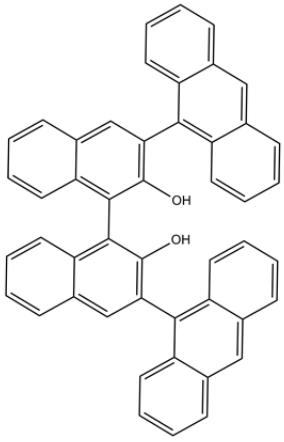
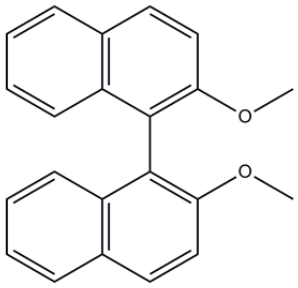
Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
25	 3,3'-diphenyl-[2,2'-binaphthalene]-1,1'-diol	CF6-P	5%	1.18	2.1	1.7	2
		CF6-RN	1%	1.09	1.3	5.6	6.1
		CF7-DMP	5%	1.17	2.0	1.2	1.4
26	 3,3'-dibromo-5,5',6,6',7,7',8,8'-octahydro-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%	1.07	0.8	1.4	1.5
		CF6-RN	1%	1.19	1.7	4.7	5.6
		CF7-DMP	5%			1.14	
27	 3,3'-dibromo-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%	1.05	0.9	4.4	4.6
		CF6-RN	5%	1.06	0.9	4.9	5.2
		CF7-DMP	5%	1.03	0.9	2.9	3.0
28	 3,3'-bis(triphenylsilyl)-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%			0.1	
		CF6-RN	5%			0.95	
		CF7-DMP	5%			0.42	

Table 4-1 continued

Analyte #	Structure	Column	% ethanol	α	R_S	k_1	k_2
29	 3,3'-di(anthracen-9-yl)-[1,1'-binaphthalene]-2,2'-diol	CF6-P	5%			2.3	
		CF6-RN	1%			0.34	
		CF7-DMP	5%			2.0	
30	 2,2'-dimethoxy-1,1'-binaphthalene	CF6-P	5%			0.57	
		CF6-RN	1%			1.5	
		CF7-DMP	5%			0.8	

Conditions: detector, UV 254 nm; column temperature, ambient; flow rate, 2 mL min^{-1}

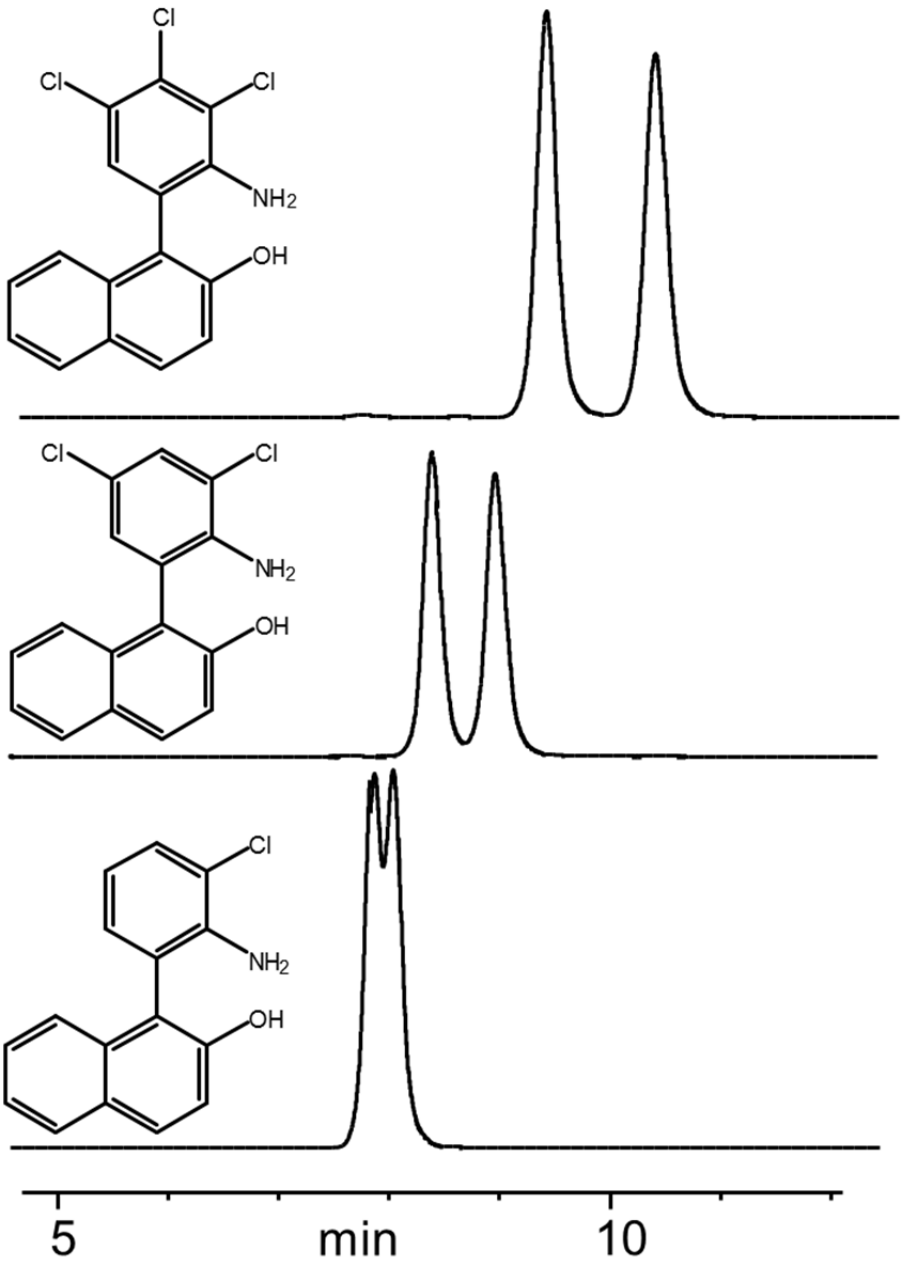


Figure 4-1 Effects of additional halogen substituents on retention and selectivity.

Conditions: column, CF6-P; mobile phase: 95:5 (v/v) heptane: ethanol; detector, UV 254 nm; flow rate, 2 mL min^{-1}

4.4.2 Effect of Polar Modifier

By increasing the alkyl chain length of the polar modifier in the mobile phase, retention was increased. Minor improvements in selectivity were observed when going from ethanol to 1-propanol or 1-butanol (Table 4-2). However, both efficiencies and peak symmetries were diminished. Significant tailing was observed when using 1-butanol as a polar modifier and observable band-broadening occurred when switching away from ethanol in all cases. This provides further evidence for a dipolarity/polarizability mechanism of retention as using lower polarity solvents increased retention and slowed on/off kinetics between analyte and CSP. The effect of varying the composition of polar modifier was an increase in retention and selectivity when the % ethanol was changed from 16% to 4% (Table 4-3). Based upon the combined results, a mobile phase composition of 10% ethanol in heptane is recommended for screening new biaryl atropisomers in the normal phase mode.

Table 4-2 Effect of polar modifier on retention and selectivity

modifier	ethanol				1-propanol				1-butanol			
analyte no.	k_1	α	R_s	PS	k_1	α	R_s	PS	k_1	α	R_s	PS
1	3.4	1.22	4.1	0.82	4.5	1.27	4.2	0.66	5.7	1.27	3.4	0.59
2	2.6	1.12	2.2	0.85	2.8	1.14	2.1	0.77	3.0	1.12	1.6	0.72
3	2.7	1.14	2.6	0.93	3.2	1.21	3.2	0.66	3.9	1.28	3.5	0.60
4	3.0	1.12	2.6	0.76	4.0	1.17	2.5	0.58	5.4	1.17	1.7	0.54

Conditions: column, CF6-P; mobile phase, 90:10 heptane: modifier (v/v); detector, UV 254 nm; flow rate, 2 mL min^{-1}; column temperature, 25 ºC

Table 4-3 Effect of polar modifier composition on retention and selectivity

% ethanol	16%			13%			7%			4%		
analyte no.	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
1	2.1	1.19	3.3	2.6	1.22	3.7	4.6	1.24	4.9	7.1	1.3	6.1
2	1.5	1.11	1.8	1.9	1.12	2.0	3.7	1.13	2.4	6.2	1.1	2.6
3	1.7	1.12	1.9	2.1	1.13	2.2	3.7	1.16	3.2	5.7	1.2	4.2
4	1.9	1.12	1.8	2.3	1.14	2.3	4.0	1.15	3.0	5.8	1.2	4.0

Conditions: column, CF6-P; detector, UV 254 nm; flow rate, 2 mL min^{-1}; column temperature, 25 ºC

4.4.3 Temperature Effect

Excellent linearity was observed for van't Hoff plots in the range of 25-41 °C indicating that the CSP was not altered significantly in this temperature range. All ΔΔS°* values were negative suggesting enthalpy-driven enantioselectivity (Table 4-4). The absolute $\Delta H_2°$ values were in the range of 14-19 kJ mol^{-1} indicating a strong interaction with the CSP. The trend in absolute values for the $\Delta H_2°$ energies of the 1`,1-binapthyls matches the trend in retention and selectivity observed for the three types of 2`,2 substituents, *i.e.* 2,2`-diamine > 2-amino-2`-ol > 2,2`-diol. This indicates that analyte adsorption to the CSP is more exothermic for 2,2`-diamines and 2-amino-2`-ol analytes than for 2,2`-diols. Absolute values for ΔΔH°* ranged from 1.5-2.5 kJ mol^{-1} *vs*. 14-19 kJ mol^{-1} for $\Delta H_2°$, indicating much of the analyte-CSP interaction is common to both

enantiomers. However, the differences in ΔΔH°* were of sufficient magnitude to provide adequate selectivity at room temperature. No significant differences in thermodynamic parameters were observed when switching from ethanol to 1-propanol or 1-butanol (data not shown). Previous studies utilizing the immobilized polysaccharide CSP Chiralpak IA have reported thermodynamic parameters for BINOL using 90:10 hexane: propanol (v/v)[108]. Under those conditions, BINOL showed entropy driven enantioselectivity vs. enthalpy driven for the CF6-P using 90:10 heptane: ethanol. Clearly the driving forces for enantiomeric separation on these two CSP's are different with respect to BINOL, with the CF6-P showing enthalpy driven enantioseparation and the Chiralpak IA showing entropy driven enantioseparation.

Table 4-4 Thermodynamic parameters for enantiomeric separations

Analyte no.	$\Delta H°_1$ (kJ/mol)	$\Delta S°*_1$ (J/mol*K)	R^2	$\Delta H°_2$ (kJ/mol)	$\Delta S°*_2$ (J/mol*K)	R^2	ΔΔH° (kJ/mol)	ΔΔS° (J/mol*K)
1	-16.1	-39.7	0.999	-18.6	-45.9	0.999	-2.5	-6.2
2	-12.1	-31.6	0.999	-14.6	-38.8	0.999	-2.4	-7.2
3	-15.2	-40.3	0.999	-16.9	-44.1	0.999	-1.7	-3.8
4	-17.4	-44.3	0.999	-18.8	-47.6	0.999	-1.4	-3.4

Conditions: column, CF6-P; mobile phase, 90:10 heptane: ethanol; detector, UV 254 nm; flow rate, 2 mL min^{-1}

4.4.4 Preparative Scale Separations

Analyte no. 19 (1-(2-amino-3,4,5-trichlorophenyl)naphthalen-2-ol) was initially selected for antimicrobial/antibiotic activity screening (data reported elsewhere) and was needed in an enantiomerically pure form. A preliminary loading study was conducted on a 250 x 4.6 mm CF6-P column with a flow rate of 2 mL min^{-1} and a resolution of 1.4 was obtained when injecting 100 µg at a concentration of 10 mg mL^{-1} (Figure 4-2). By switching from heptane to hexanes, significant cost savings were realized without a loss

of selectivity. Acceptable selectivity was observed at a flow rate of 30 mL min $^{-1}$ using 2% ethanol in hexanes. Injection volume was increased until baseline resolution was lost. Acceptable resolution was observed on the preparative column when injecting 200 µL at a concentration of 60 mg mL^{-1} (Figure 4-3). By stacking injections every 15 minutes, a total of 24 mg of each enantiomer was collected per hour at a cost of 1.8 L of hexanes. After combining fractions and removing solvent, the %ee of each sample was determined to be ≥ 98%

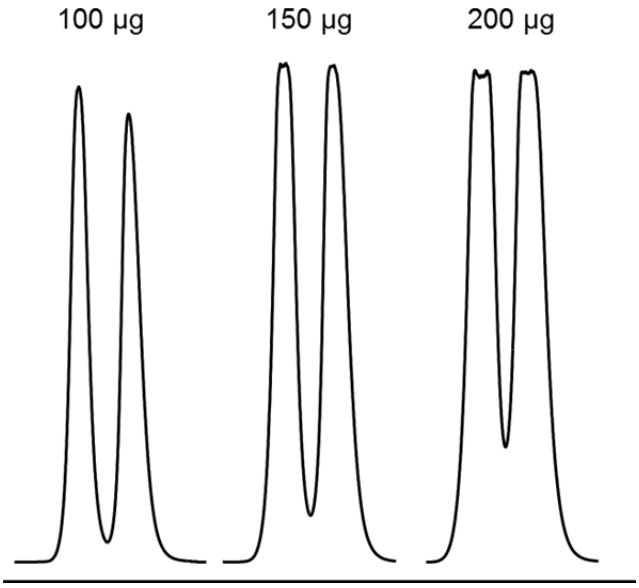


Figure 4-2 Analytical loading study for CF6-P.

Conditions: probe no. 19 prepared in 50:50 heptane: ethanol at 10 mg mL^{-1}, injection volumes, 10, 15, 20 µL; mobile phase, 95:5 heptane: ethanol; flow rate, 2 ml min^{-1}; UV 254 nm

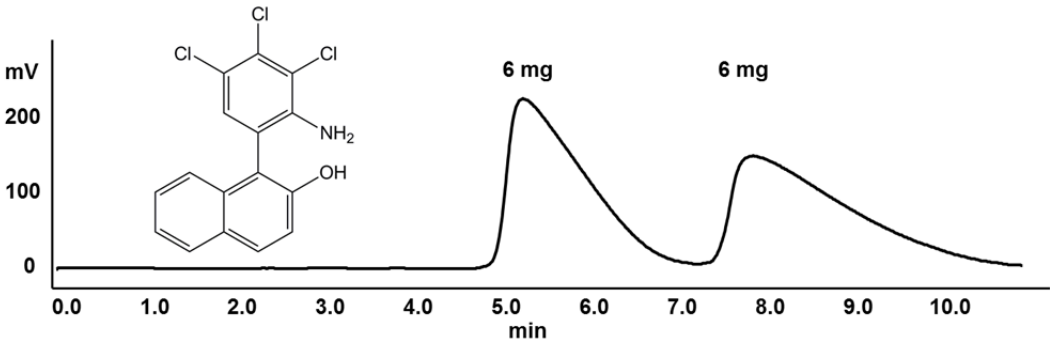


Figure 4-3 Preparative scale enantioseparation on CF6-P.

Conditions: sample no.19 prepared in 50:50 hexanes: ethanol at 60 mg mL^{-1}; column dimensions, 250 x 21.2 mm; mobile phase, 98:2 (v/v) hexanes: ethanol; flow rate, 30 mL min^{-1}; UV 254 nm; stacked injections of 200 µL every 15 minutes.

4.5 Conclusions

New chiral HPLC methods were presented for the enantiomeric separation of a variety of biaryl atropisomers using heptane with ethanol as a polar modifier. The primary mechanism of retention is likely dipolarity/polarizability interactions between the 2,2` functionalities of the biaryls and polar groups present on the derivatized cyclofructans. Selectivity was observed for 24 out of 30 probe analytes with 17 baseline separations using three different CSP's. The CF6-P CSP was the most successful with 15 baseline separations using simple mobile phases without the need for additives. When ethanol was used as a polar modifier, high efficiencies and good peak symmetries were observed. When propanol and butanol were used, selectivity was improved but band broadening and peak tailing were increased. All probes studied showed enthalpy-driven patterns and the trend in absolute enthalpies of the second eluting enantiomer matched the trend in selectivities observed for the three types of 2,2'-binapthyls. Future

work will involve determining the energy barriers to racemization as well as modeling studies to determine the effects of different substituents on molecular hybridization and chiral recognition.

Chapter 5

Comparison of Enantiomeric Separations and Screening Protocols for Chiral Primary Amines by SFC and HPLC

5.1 Abstract

Supercritical(subcritical) fluid chromatography (SFC) was evaluated as an alternative to HPLC for the enantiomeric separation of primary amines on a cyclofructan-based chiral stationary phase. The effect of various organic modifiers, acidic and basic additives as well as instrumentation specific parameters such as column temperature, flow rate and backpressure were evaluated. The results were compared to normal phase and polar organic modes. SFC provided similar performance and showed improved peak symmetries compared to polar organic and normal phase modes. The use of acidic or basic additives individually resulted in poor performance but when used in combination, excellent selectivities and peak symmetries were observed. Insights into the effect of acidic and basic additives as well as additive combinations on retention and selectivity are presented. SFC is demonstrated to be a viable alternative to both normal phase and polar organic HPLC for the separation of racemic primary amines using the cyclofructan based chiral stationary phase. It was determined that optimal screening mobile phases should consist of methanol as a polar modifier with 0.3% (v/v) trifluoroacetic acid and 0.2% (v/v) triethylamine.

5.2 Introduction

Supercritical fluids were introduced as mobile phases in chromatographic separations by Klesper et al. in the 1962 [111]. While capillary-based methods did not become widely utilized, packed column super(sub)critical fluid chromatography (SFC)

has become increasingly utilized over the last 15 years.[112-115] In the last decade, many of the hardware shortcomings have been improved and instrumentation for both analytical and preparative separations are readily available.[116,117] The advantages of SFC are particularly pronounced in the field of chiral separations where many commonly used stationary phases provide optimal separations in the normal phase mode [117-120]. Now, with an impetus for "green separations" and high throughput screening, SFC has become the platform of choice for many pharmaceutical companies, where speed is an essential aspect of method development [116,121,122]. Higher flow rates without concomitant loss of column efficiency as well as lower solvent consumption are two of the major advantages of adopting SFC based separations [115,116]. Despite being commonly referred to as supercritical fluid chromatography, better separations are often obtained under subcritical conditions due to the improvements achieved by using a polar modifier such as methanol in combination with carbon dioxide.[120,123,124] Regardless of the state of the carbon dioxide modified mobile phase, separations utilizing SFC instrumentation with modified carbon dioxide mobile phases are most commonly referred to as SFC separations. Carbon dioxide has a polarity similar to pentane and can replace the nonpolar solvent in normal phase methods thus allowing them to be easily transferred to SFC instrumentation and vice-versa.[120,125,126] Short columns combined with high flow rates allow for rapid evaluation of multiple chiral stationary phases (CSPs) using multiple organic modifiers in a short period of time.[127] When screening multiple CSPs and mobile phase combinations, baseline resolution is not mandatory and analysis times can often be reduced to less than ten minutes.[127] By incorporating column and mobile phase switching systems, what would typically take a technician days can often be reduced to hours. Advantages abound at the preparative scale as well as the major component of the

mobile phase require no evaporative step and the low viscosity of CO_2 allows for high flow rates [128].

The use of crown-ether based chiral stationary phases to separate primary amine racemates was introduced in 1978 by Cram et al. [129]. Since then, several crown ether based CSPs have been developed and evaluated [31,130-133]. These reversed phase CSPs suffer from the need to operate under acidic aqueous conditions and are therefore not suitable for use with carbon dioxide mobile phases and are not advantageous for preparative scale separations.

A relatively new class of immobilized chiral selectors based upon derivatized cyclofructans (CFs) have been shown to provide excellent selectivity towards a variety of racemic compounds [36-40,109,110,134,135]. Cyclofructans also possess crown ether moieties with 6-8 pendant fructofuranose units. Once derivatized with isopropylcarbamate groups, CF6 can separate a variety of primary amines without the need for aqueous mobile phases [37]. However, no comprehensive study on its use under SFC conditions has been performed.

In this work, the LARIHC CF6-P CSP was evaluated as a chiral selector under SFC conditions using 25 chiral primary amine probe analytes. These chromatographic results were compared to normal phase conditions (hexane and ethanol) as well as polar organic conditions (acetonitrile and methanol). These three modes represent the most useful chromatographic conditions for many commonly used CSPs and a comparison of these modes will aid in developing future chiral methods using this CSP.

In SFC, the polar modifiers methanol, ethanol and 2-propanol were evaluated using three probe analytes with short, intermediate and long retention. Various acidic and basic additives and additive combinations were evaluated under similar mobile phase conditions. The advantages of using a combination of additives instead of

individual acid or base additives include improved peak shapes, shorter retention times and suppression of non-specific interactions [136]. Based upon the collective data, recommended screening conditions are provided for SFC, normal phase and polar organic separations.

5.3 Experimental

5.3.1 Materials

HPLC grade hexane, methanol, ethanol, 2-propanol, and acetonitrile were purchased from Fisher Scientific (Waltham, MA). ACS grade acetic acid (AA), ammonium hydroxide (37 % w/w), trifluoroacetic acid (TFA), triethylamine (TEA), butylamine (BA), diisopropylamine (DIPA), and diethylamine (DEA) were purchased from Sigma-Aldrich (St. Louis, MO). LARIHC^{TM} CF6-P HPLC columns 150 x 4.6 mm, 5 µm particle diameter (analytical scale) 250 x 21.1 mm 5 µm particle diameter (preparative scale) were obtained from AZYP L.L.C. (Arlington, TX).

5.3.2 Chiral Test Compounds

(*S*)-(-)-1-(2-naphthyl)ethylamine, (*R*)-(+)-1-(2-naphthyl)ethylamine, (*1R*,*2R*)-(-)-2-amino-1-(4-nitrophenyl)-1,3-propanediol, (1S,2S)-(+)-2-amino-1-(4-nitrophenyl)-1,3-propanediol, (*1R*,*2S*)-(+)-cis-1-amino-2-indanol, (*1S*,*2R*)-(-)-cis-1-amino-2-indanol, (*1R*,*2R*)-(-)-trans-1-amino-2-indanol, (*1S*,*2S*)-(+)-trans-1-amino-2-indanol, α-methyl-4-nitrobenzylamine hydrochloride (±), α-methylbenzylamine (±),1,2-diphenylethylamine (±),norphenylephrine hydrochloride (±), DL-4-chlorophenylalaninol, normetanephrine hydrochloride (±), norephedrine hydrochloride (±), octopamine hydrochloride (±),trans-2-phenylcyclopropylamine hydrochloride (±),(1S,2R)-(+)-phenylpropanolamine, (1R,2S)-(-)-phenylpropanolamine, (R)-(-)-2-phenylglycinol, (S)-(+)-2-phenylglycinol, (S)-(-)-2-amino-3-phenyl-1-propanol, (R)-(+)-2-amino-3-phenyl-1-propanol, 1-(1-naphthyl)ethylamine

(±),(1S,2S)-(+)-2-amino-1-phenyl-1,3-propanediol, (1R,2R)-(-)-2-amino-1-phenyl-1,3-propanediol, (S)-(-)-2-amino-1,1-diphenyl-1-propanol, (R)-(+)-2-amino-1,1-diphenyl-1-propanol, (1R,2S)-(-)-2-amino-1,2-diphenylethanol, (1S,2R)-(+)-2-amino-1,2-diphenylethanol, (S)-(-)-2-amino-3-methyl-1,1-diphenylbutane, (R)-(+)-2-amino-3-methyl-1,1-diphenylbutane, (R)-(+)-2-amino-4-methyl-1,1-diphenylpentane, (S)-(-)-2-amino-4-methyl-1,1-diphenylpentane, α-methyl-DL-phenylalanine methyl ester hydrochloride, (S)-(-)-1,1′-binaphthyl-2,2′-diamine, (R)-(+)-1,1′-binaphthyl-2,2′-diamine, (R)-(+)-2-amino-1,1′-binaphthalen-2`-ol, (S)-(-)-2-amino-1,1′-binaphthalen-2`-ol, (R)-(+)-1,1-diphenyl-2-aminopropane, (S)-(-)-1,1-diphenyl-2-aminopropane, DL-alanine β-naphthylamide hydrochloride, methoxamine hydrochloride (±),1-aminoindan (±) were purchased from Sigma-Aldrich (St. Louis, MO).

5.3.3 HPLC Methods

All HPLC analyses were performed on an Agilent© 1260 Infinity HPLC system utilizing a degasser, quaternary pump, autosampler, column thermostat and diode array detector. Data analysis was carried out using OpenLAB CDS Chemstation© Edition Rev. C.01.04. Flow rates were held at 2 mL min^{-1} unless otherwise noted. Normal phase separations were carried out using hexane with ethanol as a polar modifier in the range of 5-30% (v/v). Polar organic mode separations were carried out using acetonitrile with methanol as a polar modifier in the range of 5-20% (v/v).

5.3.4 SFC Methods

A Jasco 2000 series SFC (SFC-2000-7) equipped with a CO_2 pump (PU-2086), a modifier pump (PU-2086), a back pressure regulator (BP-2080), an autosampler (AS-2059-SFC), a column oven (CO-2060), a variable wavelength detector (UV-2075) and a makeup pump (PU-2080) supplying additional methanol to the backpressure regulator was used for all SFC analyses. The CO_2 pump was chilled to -10 ºC using a Julabo

chiller. The backpressure regulator was maintained at 60 ºC. Instrument operation and data analysis was conducted using ChromNAV via an LC-NET II/ADC. Flow rate was held at 4 mL min^{-1} unless otherwise noted. Methanol, ethanol and 2-propanol were used in the range of 2-40% (v/v). Acidic and basic additives were used in the range of 0.1-3% (v/v).

5.3.5 Universal Parameters

Samples were prepared in ethanol at 1 mg mL^{-1} unless otherwise noted. All injections were 5 µL unless otherwise noted. Column temperature was held at 30 ºC unless otherwise noted. UV detectors were operated at 254 nm.

5.3.6 Preparative Scale Parameters

1,1′-binaphthyl-2,2′-diamine was prepared at 20 mg mL^{-1} in toluene. Injection volumes were 0.5 mL. Flow rate was held at 20 mL min^{-1}. The mobile phase consisted of 90:10 CO_2:methanol. The column oven was turned off and separations were run at ambient temperature.

5.4 Results & Discussion

5.4.1 Effect of Additives

Table 5-1 provides data that allow comparison of the effects of various acidic and basic additives using three probe analytes and otherwise common chromatographic conditions. The common SFC additive, ammonium hydroxide, was evaluated at 0.2% (v/v) in methanol and absolutely no selectivity was observed (Figure 5-1). Indeed it was clear from these studies that ammonium ion negates enantioselectivity and therefore should not be used with these stationary phases. When switching to 0.2% triethyamine, moderate selectivity was observed for two of the probe analytes, but none could be baseline separated. Under acidic conditions when using trifluoroacetic acid at 0.3%,

excellent selectivity was observed but significant peak asymmetry resulted in no baseline separations. By using a combination of trifluoracetic acid and triethylamine at 0.3%, 0.2% (40 mM TFA, 15 mM TEA in methanol, 8 mM TFA, 3 mM TEA overall) respectively, excellent selectivity and peak shapes were observed with two baseline separations. A likely explanation for the need for TFA is that chiral recognition is improved when analytes are ionized and interact more favorably with the crown-ether core and by adding a competitive organic amine (TEA), mass transfer kinetics are improved and sharper peak profiles are obtained. Minor changes in retention and selectivity are observed when using alternate organic amines such as diethylamine, diisopropylamine and butylamine. However, none provided greater resolutions than triethylamine. It appears that the smaller ammonium group most likely complexes too strongly with the chiral selector which inhibits chiral recognition between the derivatized cyclofructan and the probe analytes. Further evidence of this is provided by using a combination of TFA and ammonium hydroxide where retention is strongly attenuated and selectivities are very poor. When using a combination of acetic acid and triethylamine, retention times were increased and selectivities were lower compared to TFA/TEA at the same concentration. Previous studies using polar organic chromatographic conditions show that a 3:2 (v/v) acid:base ratio provides optimal separation conditions.(30) This was confirmed for SFC by also testing 2:2 and 4:2 (v/v) acid:base combinations for the separation of three test analytes. Overall, selectivity and resolution values were always at a maximum value when the acid:base ratio was at 3:2 (v/v). For example, when 1,2-napthylethylamine was screened using the 3:2 acid:base ratio, selectivity and resolution values were 1.13 and 1.5, respectively. However, when 2:2 and 4:2 acid:base ratios were used to separate 1,2-napthylethylamine, selectivity and resolution decreased, in both cases, to 1.11 and 1.2, respectively. In SFC, the effect of holding the ratio constant and varying the total

concentration of the additives was that increasing the amount of additives shortened retention and improved efficiencies but with minimal improvement in selectivity or resolution (Figure 5-2, chromatograms C,D). This is not the case when operating under polar organic conditions where increasing the concentration of the additives improved peak symmetries and reduced analysis time with minimal loss of selectivity (Figure 5-2, chromatograms A,B). This is likely due to the higher diffusivity of CO_2 relative to acetonitrile and overall improved mass transfer kinetics relative to polar organic or normal phase conditions. Based upon the combined results, the recommended additive for screening primary amines in SFC is 0.3/0.2% (v/v) in methanol. When operating under polar organic or normal phase conditions, it is possible to either premix the mobile phases or to put additives in the separate mobile phase reservoirs (e.g. in the hexane and the ethanol for normal phase chromatography or in the acetonitrile and methanol in the polar organic mode) and let the instrument mix the two solvents in the desired proportions. Regardless of one's approach, the overall additive concentration in the eluent should be 0.3/0.2% (v/v) TFA/TEA for normal phase or polar organic mode separations.

Table 5-1 Effect of Additives of Enantiomeric Separations[a]

	1,2-napthylethylamine			α-methyl-4-nitrobenzylamine			2-amino-1-(4-nitrophenyl)-1,3-propanediol		
Additive[b]	t_r^1	α	R_s	t_r^1	α	R_s	t_r^1	α	R_s
TFA	1.9	1.20	1.0	2.53	1.15	1.0	2.48	1.19	0.8
TEA	3.47	1.09	1.0	2.60	1.00	0.0	3.40	1.10	0.5
NH_4OH	2.80	1.00	0.0	2.50	1.00	0.0	5.56	1.00	0.0
TFA, NH_4OH	1.60	1.04	0.0	1.72	1.06	0.0	2.60	1.05	0.0
TFA, TEA	2.7	1.13	1.5	4.00	1.12	1.1	5.75	1.18	1.7
AA, TEA	5.43	1.14	1.0	4.45	1.00	0.0	8.27	1.17	1.1
TFA, DEA	2.54	1.17	1.4	3.36	1.12	1.1	3.82	1.18	1.6
TFA, DIPA	2.40	1.13	1.2	2.96	1.12	1.1	1.40	1.26	1.5
TFA, BA	1.68	1.11	1.0	1.93	1.10	0.8	2.00	1.11	0.8

[a]Conditions: column, 150 x 4.6 mm 5 µm particle diameter; mobile phase, 75:25 CO_2: methanol (v/v); acid concentration, 40 mM in methanol, base concentration, 15 mM in methanol; detector, UV 254 nm; column temperature, 30 ºC; flow, 4 mL min^{-1}. [b]TFA: trifluoroacetic acid, TEA: triethylamine, NH_4OH: ammonium hydroxide (37% w/w), DEA: diethylamine, DIPA: diisopropylamine, BA: butylamine

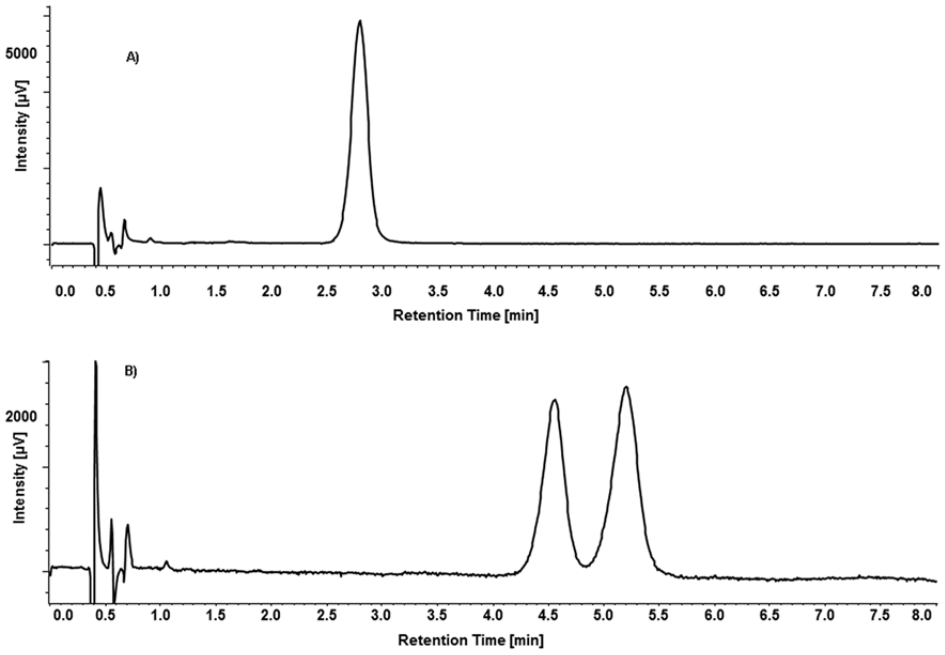


Figure 5-1 Effect of Additives on Retention and Selectivity

Analyte, 1,2-naphthyethyamine; mobile phase: 80:20 CO_2:methanol (v/v); flow 4 mL min^{-1}; A) 0.2% (v/v) NH_4OH; B) 0.3/0.2% (v/v) TFA/TEA

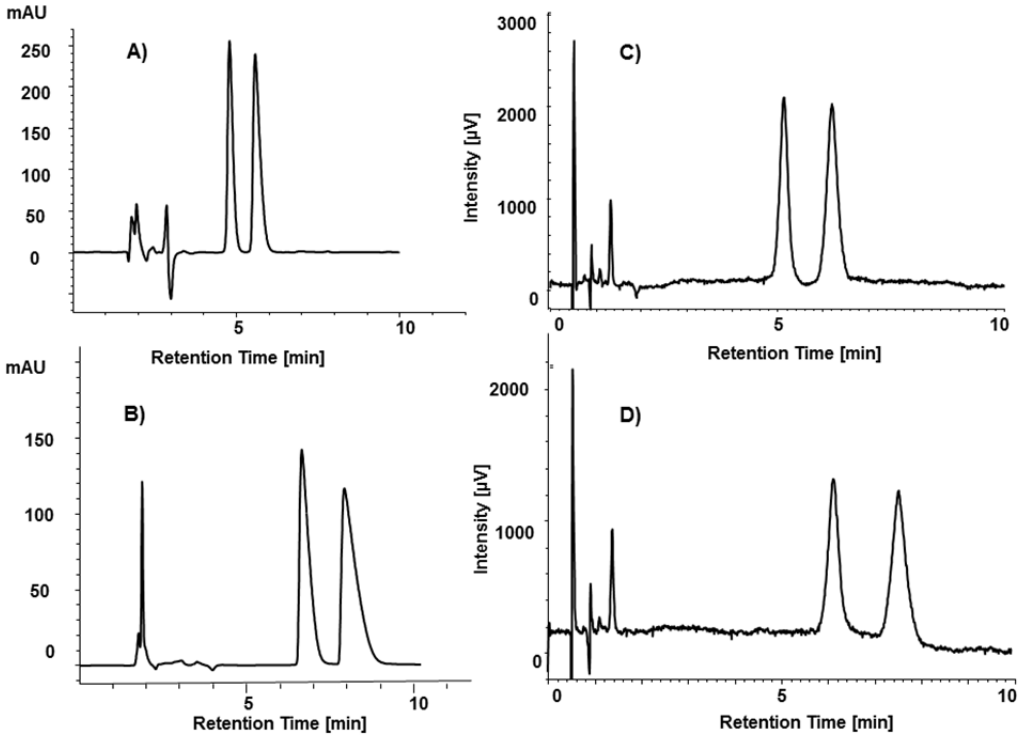


Figure 5-2 Effect of additive concentration on retention, selectivity and peak symmetry.

Analyte (RS/SR) 2-amino-1,2-diphenylethanol; chromatograms A,B 90:10 acetonitrile:methanol; flow 1 mL min^{-1} A) 0.3/0.2% (v/v) TFA/TEA (overall), B) 0.3/0.2% (v/v) TFA/TEA (methanol only); chromatograms C,D) mobile phase 80:20 CO_2:methanol; flow 3 mL min^{-1}; C) 0.3/0.2% (v/v) TFA/TEA (overall), D) 0.3/0.2% (v/v) TFA/TEA (methanol only)

5.4.2 Effect of Polar Modifier

Different commonly used organic modifiers for SFC were evaluated at 25% (v/v) with 0.3, 0.2% TFA, TEA and the results are reported in Table 5-2. The general trend for SFC was that as larger alcohol modifiers were employed, selectivities were often improved but mass transfer kinetics were significantly diminished resulting in low plate numbers and pronounced peak tailing. Resolutions were lower for all probe analytes in all cases when changing the organic co-solvent from methanol (Table 5-2). Plate

numbers dropped precipitously when going from methanol to 2-propanol. Based upon these results, methanol is recommended as the polar modifier of choice when screening primary amines using the LARIHC CF6-P CSP in SFC. The added advantage of increased organic modifier volatility, when dealing with preparative separations, provides additional impetus for using methanol when developing chiral SFC methods.

Table 5-2 Effect of Polar Modifier on Enantiomeric Separations

	1,2-napthylethylamine				α-methyl-4-nitrobenzylamine				2-amino-1-(4-nitrophenyl)-1,3-propanediol			
modifier[a]	tr_1	α	R_S	N	tr_1	α	R_S	N	tr_1	α	R_S	N
MeOH	2.7	1.13	1.5	1800	4.0	1.12	1.1	2060	5.75	1.18	1.7	1700
EtOH	4.7	1.17	1.2	1220	6.1	1.17	1.1	1150	9.1	1.22	1.6	950
2-PrOH	5.4	1.19	0.8	300	10.5	1.13	0.5	500	16.2	1.20	0.5	500

Conditions: column 150 x 4.6 mm 5 µm particle diameter; mobile phase, 75:25 CO_2: modifier (v/v); additive, 0.3/0.2% TFA/TEA in modifier; detector, UV 254 nm; column temperature, 30 ºC; flow, 4 mL min^{-1} . a methanol (MeOH), ethanol (EtOH), 2-propanol (2-PrOH)

5.4.3 Effect of Column Temperature, Flow Rate And Backpressure Under Subcritical Conditions

The same three probe analytes used in the "additive study" were used to evaluate the effect of column temperature at 25 ºC, 30 ºC, 35 ºC and 40 ºC using 25% methanol with 0.3, 0.2 % TFA, TEA (Table 5-3). As the column temperature was increased the selectivity and resolution diminished without significant improvement in the analysis time. Interestingly, the highest temperature did not improve efficiency, indicating that under these mobile phase conditions, mass transfer kinetics are not hindered by operating at subcritical temperatures. The loss of resolution observed by going from 30 ºC to 40 ºC was significant enough to merit operating at 30 ºC. Given the necessity of having considerable polar modifier to elute the analytes from the CSP (15-30% v/v) and

the fact that resolutions were diminished at elevated temperatures, no effort was made operate under true supercritical conditions as chromatographic performance would certainly be compromised at the temperatures and pressures necessary to reach the critical point.

Flow rates of 1, 2, 3 and 4 mL min^{-1} were evaluated to study the effects of operating at higher linear mobile phase velocities. The results also are presented in Table 5-3. When going from 1 mL min^{-1} to 4 mL min^{-1}, plate counts were reduced by approximately 40% without a loss of selectivity. Resolutions were higher at 1 mL min^{-1}, but came with an obvious cost of analysis time. Because selectivities were not lower at higher linear velocities and plate counts were still acceptable, 4 mL min^{-1} is the recommended flow rate for screening.

A similar study was performed by analyzing the three probe analytes with the backpressure regulator set at 80, 100, 120 and 140 bar. The effect was a moderate decrease in retention time at higher pressure with minor loss of selectivity and resolution. Minor losses in plate count were observed by increasing the column backpressure and thus a recommended backpressure of 100 bar seemed acceptable.

Table 5-3 Effect of Instrument Parameters on Enantiomeric Separations

	1,2-naphthylethylamine				α-methyl-4-nitrobenzylamine				2-amino-1-(4-nitrophenyl)-1,3-propanediol			
Parameter	t_r^1	α	R_S	N	t_r^1	α	R_S	N	t_r^1	α	R_S	N
Temp, 25 °C	4.4	1.17	1.5	2000	5.9	1.15	1.5	2900	9.0	1.19	2.0	2000
Temp, 30 °C	4.6	1.17	1.5	1800	5.8	1.13	1.5	2800	9.0	1.18	1.9	1800
Temp, 35 °C	4.3	1.15	1.3	1700	5.7	1.13	1.4	2600	8.9	1.17	1.7	1800
Temp, 40 °C	4.2	1.14	1.2	1500	5.5	1.13	1.3	2600	8.8	1.16	1.5	1700
Flow 1 mL min^{-1}	18.1	1.15	1.6	3000	24.3	1.12	1.8	5200	39.0	1.18	2.4	2900
Flow, 2 mL min^{-1}	8.9	1.15	1.6	2700	11.8	1.13	1.6	3800	18.8	1.18	2.1	2400
Flow, 3 mL min^{-1}	5.9	1.16	1.5	2200	7.9	1.13	1.5	3000	12.4	1.18	2.0	2100
Flow, 4 mL min^{-1}	4.5	1.17	1.5	1800	5.8	1.12	1.5	2400	9.0	1.19	1.9	1900
BPR, 80 BAR	4.6	1.16	1.4	1900	6.0	1.12	1.5	2000	9.3	1.20	1.8	2000
BPR, 100 BAR	4.5	1.17	1.5	1800	5.8	1.12	1.5	1800	9.0	1.19	1.9	1900
BPR 120 BAR	4.1	1.16	1.3	1800	5.7	1.13	1.3	1700	8.8	1.19	1.7	1800
BPR, 140 BAR	3.9	1.15	1.3	1900	5.6	1.13	1.2	1600	8.7	1.20	1.8	1800

For each parameter, the default for parameters not being varied was 30ºC, 4mL min^{-1} and 100 bar.

5.4.4 Comparison Between SFC, Normal Phase and Polar Organic Modes

Table 5-4 gives the chromatographic results for the separation of 25 racemic primary amines under SFC, polar organic and normal phase modes. In general, SFC provided the best peak symmetries, while the polar organic mode provided the shortest analysis times and the normal phase provided the greatest resolutions at a cost of analysis time (Figure 5-3). Under SFC conditions, 16 out of 25 analytes were baseline separated in the screen while the polar organic and normal phase modes provided 13 and 17 baseline separations respectively. In total, the SFC screen showed enantioselectivity for all but three of the tested analytes. It should be noted that, for comparative purposes, TFA and TEA were only added to the polar modifier in the polar organic and normal phase modes. In practice however, it is recommended to make the overall concentration 0.3/0.2% (v/v) TFA/TEA when using either the POM or NP. This will result in more baseline separations when using HPLC due to improved peak symmetries, particularly when operating under polar organic conditions (as discussed earlier and shown in Figure 5-2).

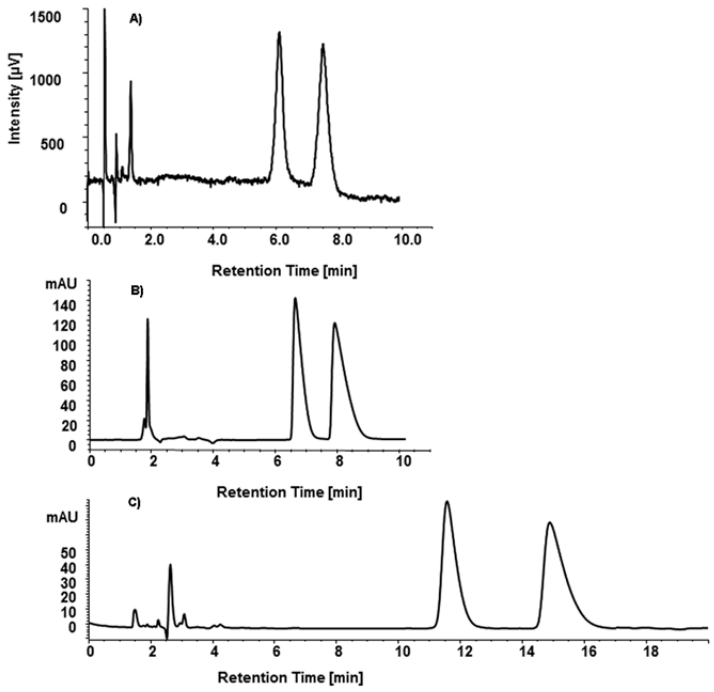


Figure 5-3 Comparison of SFC, Polar Organic and Normal Phase Chromatographic Conditions

Analyte: RS/SR 2-amino-1,2-diphenylethanol, chromatogram A) mobile phase: 80:20 CO2:methanol, flow 3 mL min-1; B) 90:10 acetonitrile:methanol, flow 1 mL min-1 ; C) 80:20 hexane:ethanol, flow 1 mL min-1; all chromatograms 0.3/0.2% (v/v) TFA/TEA (polar modifier only)

Acetonitrile provided the greatest eluotropic strength followed by hexane, with CO_2 having the lowest strength. Retention factors were always highest for SFC separations even when operating at a greater percentage of methanol relative to the polar organic mode. Because the normal phase separations were conducted using ethanol instead of methanol as a polar modifier, direct comparisons of retention factors for normal phase and SFC are not possible, however, normal phase retention factors

under the tested conditions were always intermediate relative to SFC and polar organic modes.

Table 5-4 Chromatographic data for enantiomeric separations under SFC, polar organic and normal phase conditions[a]

Analyte	Mode (% modifier)	t_r[1]	k_1	α	R_s
1,2-naphthylethylamine	SFC (20%)	4.50	10.25	1.17	1.5
	POM (5%)	5.20	4.78	1.09	1.2
	NP (20%)	5.70	5.33	1.17	1.8
2-amino-1-(4-nitrophenyl)-1,3-propanediol	SFC (20%)	9.0	22.38	1.18	1.9
	POM (10%)	3.55	2.94	1.17	1.7
	NP (20%)	4.40	3.89	1.20	1.6
cis-1-Amino-2-indanol	SFC (20%)	5.48	12.69	1.07	0.5
	POM (10%)	3.00	2.33	1.14	0.8
	NP (20%)	8.40	8.33	1.00	0.0
trans-1-Amino-2-indanol	SFC (20%)	5.20	12.00	1.29	2.5
	POM (10%)	4.40	3.89	1.31	2.3
	NP (20%)	7.90	7.78	1.23	2.1
1-aminoindan	SFC (20%)	9.44	22.60	1.11	1.5
	POM (10%)	3.50	2.89	1.19	1.9
	NP (20%)	5.80	5.44	1.12	1.5
α-methyl-4-nitrobenzylamine	SFC (20%)	5.80	13.5	1.15	1.5
	POM (5%)	7.36	7.18	1.11	1.2
	NP (30%)	9.60	9.67	1.15	1.5
α-methylbenzylamine	SFC (20%)	3.22	7.05	1.13	1.5
	POM (5%)	3.80	3.22	1.21	1.9
	NP (20%)	6.30	6.00	1.19	1.5
1,2-diphenylethylamine	SFC (20%)	3.55	7.88	1.24	2.2
	POM (10%)	2.36	1.62	1.25	1.9
	NP (20%)	4.40	3.89	1.29	2.5
norphenylephrine	SFC (25%)	11.10	26.75	1.16	1.5
	POM (10%)	5.80	5.44	1.18	1.5
	NP (30%)	18.95	20.06	1.18	1.5

[a]Conditions: column 150 x 4.6 mm 5 µm particle diameter; polar modifier: methanol (SFC, POM), ethanol (NP); TFA concentration, 0.3% (v/v) in modifier, TEA concentration, 0.2% (v/v) in modifier; flow, 4 mL min^{-1} (SFC), 2 mL min^{-1} (POM, NP)

Table 5-4 continued

Analyte	mobile phase	t_r^1	k_1	α	R_s
DL-p-chlorophenylalaninol	SFC (20%)	7.05	16.63	1.12	1.5
	POM (5%)	6.20	5.89	1.10	1.0
	NP (20%)	12.70	13.11	1.14	1.5
DL-normetanephrine	SFC (40%)	5.40	12.50	1.16	1.5
	POM (10%)	6.10	5.78	1.17	1.6
	NP (30%)	28.30	30.44	1.20	1.5
norephedrine	SFC 20%	5.60	13.00	1.13	1.5
	POM (5%)	6.00	5.67	1.18	1.5
	NP (20%)	8.30	8.22	1.18	1.9
DL-octopamine	SFC (25%)	13.60	33.00	1.15	1.5
	POM (5%)	11.70	12.00	1.15	1.5
	NP (30%)	24.30	26.00	1.16	1.5
trans-2-phenylcyclopropylamine	SFC 20%	10.20	24.50	1.04	0.4
	POM (5%)	9.20	9.22	1.02	0.4
	NP (20%)	10.20	10.33	1.06	0.6
phenylpropanolamine	SFC 20%	4.65	10.63	1.11	1.5
	POM (5%)	6.40	6.11	1.16	1.7
	NP (20%)	8.20	8.11	1.18	1.9
2-phenylglycinol	SFC (20%)	4.99	11.48	1.11	0.0
	POM (5%)	5.50	5.11	1.02	0.4
	NP (20%)	10.20	10.33	1.00	0.0
2-amino-3-phenyl-1-propanol	SFC (20%)	5.70	13.25	1.17	1.5
	POM (10%)	3.90	3.33	1.15	1.5
	NP (20%)	9.70	9.78	1.13	1.6
1-(1-naphthyl)ethylamine	SFC 20%	4.00	9.00	1.22	2.0
	POM (10%)	4.60	4.11	1.16	1.5
	NP (20%)	4.89	4.43	1.23	2.2

Conditions: column 150 x 4.6 mm 5 µm particle diameter; polar modifier: methanol (SFC, POM), ethanol (NP); TFA concentration, 0.3% (v/v) in modifier, TEA concentration, 0.2% (v/v) in modifier; flow, 4 mL min^{-1} (SFC), 2 mL min^{-1} (POM, NP)

Table 5-4 continued

Analyte	mobile phase	t_r[1]	k_1	α	R_s
2-amino-1-phenyl-1,3-propanediol	SFC (20%)	8.30	19.75	1.06	0.5
	POM (5%)	5.90	5.56	1.16	1.2
	NP (20%)	12.60	13.00	1.00	0.0
2-amino-1,2-diphenylethanol	SFC (20%)	4.50	10.25	1.24	2.4
	POM (10%)	2.60	1.89	1.29	2.7
	NP (20%)	5.00	4.56	2.00	2.8
1,1′-binaphthyl-2,2′-diamine	SFC (5%)	10.50	25.25	1.11	1.5
	POM (10%)	1.00	0.11	1.00	0.0
	NP (5%)	7.10	6.89	1.18	1.5
2-amino-1,1′-binaphthalen-2`-ol	SFC (5%)	8.60	20.50	1.09	1.4
	POM (10%)	1.00	0.11	1.00	0.0
	NP (5%)	6.30	6.00	1.11	1.4
1,1-diphenyl-2-aminopropane	SFC (20%)	2.45	5.14	1.06	0.5
	POM (5%)	2.30	1.56	1.11	0.5
	NP (20%)	3.40	2.78	1.12	1.2
DL-alanine-β-naphthylamide	SFC (20%)	10.77	25.93	1.00	0.0
	POM (10%)	4.30	9.75	1.00	0.0
	NP (20%)	9.20	9.22	1.06	0.6
methoxamine	SFC (20%)	5.00	11.50	1.00	0.0
	POM (5%)	4.60	4.11	1.11	0.9
	NP (20%)	8.20	8.11	1.00	0.0

Conditions: column 150 x 4.6 mm 5 µm particle diameter; polar modifier: methanol (SFC, POM), ethanol (NP); TFA concentration, 0.3% (v/v) in modifier, TEA concentration, 0.2% (v/v) in modifier; flow, 4 mL min^{-1} (SFC), 2 mL min^{-1} (POM, NP)

5.4.5 Preparative Scale Separation

In order to demonstrate the applicability of the CF6-P CSP for preparative separations using SFC instrumentation a method was developed to separate 20 mg of 1,1′-binaphthyl-2,2′-diamine racemate using a 250 x 21.1 mm preparative column. By injecting 0.5 mL of analyte at a concentration of 20 mg mL^{-1}, a resolution of 1.4 was observed using 10% methanol in CO_2 as a mobile phase (Figure 5-4). As can be seen, excellent peak profiles and high column capacity is obtained when using SFC, primarily

due to the higher diffusivity of CO^2 relative to other comparable mobile phases. When separating biaryl atropisomers, additives are not necessary for efficient separations unlike other primary amine type analytes.

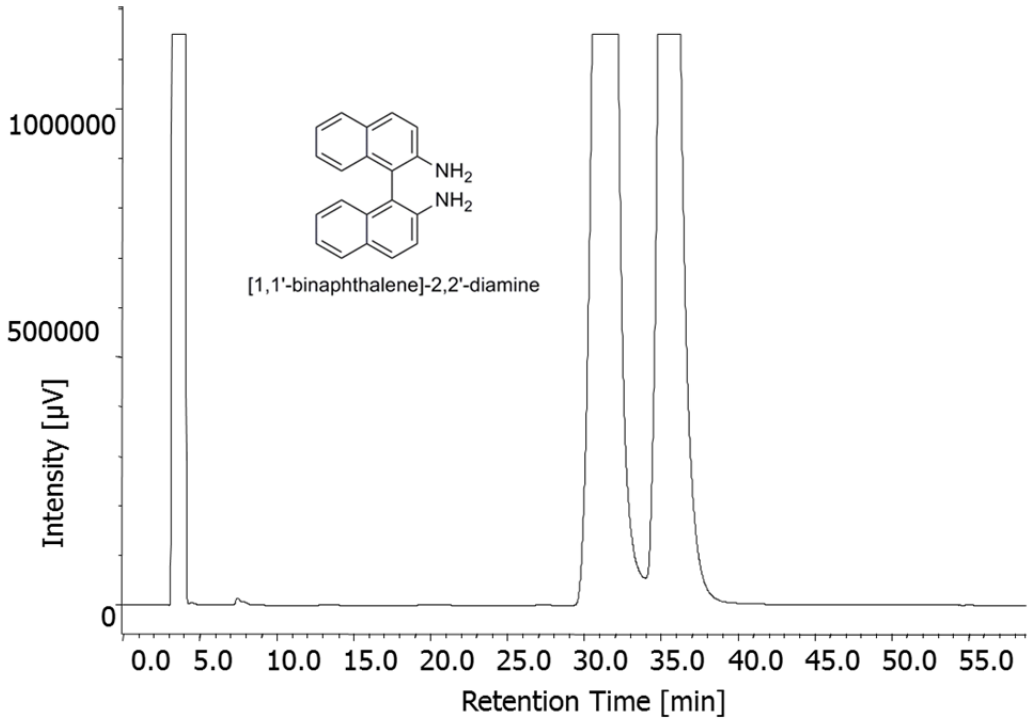


Figure 5-4 Preparative Separation using CF6-P CSP under SFC Conditions

See section 5.3.6 for experimental parameters

5.5 Conclusions

SFC was evaluated as an alternative to HPLC for the separation of primary amines using the CF6-P chiral stationary phase. Using ammonium hydroxide as a basic additive destroys enantioselectivity. However, using methanol as a polar modifier with 0.3/0.2% (v/v) TFA/TEA as additives, excellent selectivity was observed with 18 baseline separations and 22 hits out of 25 probe analytes under general screening conditions. When compared to normal phase chromatography and polar organic mode chromatography using the same CSP, SFC showed comparable selectivities and analysis times and improved peak symmetries. SFC was demonstrated to be useful for preparative scale separation of biaryl type analytes without the need for additives. The recommended mobile phases for screening chiral primary amines in the polar organic and normal phase modes are 90:10 acetonitrile:methanol and 80:20 hexane:ethanol respectively with an overall concentration of 0.3/0.2% (v/v) TFA/TEA. In general, additional optimization of the mobile phase composition further enhances the selectivity and resolution of all analytes as compared to the screening solvent.

Chapter 6

Conclusions

This dissertation presents valuable methods for the enantiomeric separation of biologically and chemically significant molecules as well as insights into the mechanisms of retention and chiral discrimination using macrocyclic chiral stationary phases. Principle component analysis was shown to be a valuable tool to interpret chromatographic data. The second and third chapters of the dissertation focus on the development of chiral HPLC methods for the separation of important newly synthesized isochromene and Trögers base derivatives using PCA techniques. The Cyclobond DM and RSP provided better overall separations compared to other CSPs tested. The results from the principle component analysis studies indicated that the volume of the R1 substituent had a greater effect on enantiomeric separations compared to the R2 and R3 group. Factors related to retention on the CSPs do not correlate with increased enantioselectivity. Different separation mechanisms were observed for the β-cyclodextrin relative to the γ-cyclodextrin. E*thano*-bridged Tröger bases were well separated using cyclodextrin based CSP in the reverse phase mode and cyclofructan based CSPs in the normal phase mode. It was observed that the RSP β-cyclodextrin provided better selectivities than the native β-cyclodextrin indicating that the pendant isopropyl ether groups create enhanced enantioselectivity. PCA biplot analysis indicated that retention on the RSP-β-cyclodextrin and CF6-RN CSPs is governed by the size of the R1 substituent. All PCA biplots indicated that retention is not sufficient for enantioresolution. Enantioresolution was shown to be closely correlated with the size of the R2 group when using the γ-cyclodextrin. Principle component analysis was shown to be a valuable tool for eliminating redundant variables as well as identifying correlation between structural features of the analyte and enantiomeric separation data. Future work in this area should

include applying PCA studies using different structural and chemical properties as variables along with the chromatographic results in order to gain a better understanding of what variables provide useful data. This will aid future investigators wishing to apply PCA to chiral separations.

The fourth chapter focuses on the enantioseparation of biaryl atropisomers using cyclofructan based CSPs. Given a combination of observations, the primary retentive interaction between these analytes and the CSP is likely dipolarity/polarizability between the 2,2` moieties and the polar groups of the derivatized cyclofructans. The isopropylcarbamate derivatized cyclofructan 6 was the most successful CSP screened with 16 baseline separations. Heptane with ethanol as a polar modifier was the best mobile phase for enantioseparation of this group of analytes. No retention was observed in the polar organic mode. Van't Hoff plots indicated that all enantioseparations were enthalpy driven. Future work should include applying PCA to the chromatographic results using the dihedral angles and other physical and chemical properties of the biaryls as variables.

The fifth chapter covers the development and comparison of methods to separate enantiomers containing primary amine moieties under normal phase, polar organic and SFC chromatographic conditions. SFC was shown to be an effective technique with comparable performance to more traditional LC separations. Improved peak profiles were observed under SFC conditions. A preparative separation of 1,1′-binaphthyl-2,2′-diamine was conducted demonstrating the advantages of modified carbon dioxide mobile phases relative to normal phase separations for the isolation of mg to gram quantities of analytes in their enantiomerically pure form. Future work should include preparing and evaluating chiral stationary phases based upon derivatized

cyclofructan 7 and 8. These selectors have not been studied to the same extend and cyclofructan 6 and may provide unique separations.

Overall, the methods presented herein provide valuable information concerning the development of new chiral separations as well as information regarding mechanisms of interaction between analytes and macrocyclic chiral stationary phases.

Appendix A

Publication Information for Chapters 2-5

Chapter 2: A manuscript published in the Journal of Chromatography A. Yasith S. Nanayakkara, Ross M. Woods, Zachary S. Breitbach, Sachin Hada, LeGrande M. Slaughter, Daniel W. Armstrong, 2013, 1305, 94-101. Copyright © 2013 with permission from Elsevier.

Chapter 3: A manuscript published in the Journal of Chromatography B. Choyce A. Weatherly, Yun-Cheol Na, Yasith S. Nanayakkara, Ross M. Woods, Ankit Sharma, Jerome Lacour, Daniel W. Armstrong, 2014, 955-956, 72-80. Copyright © 2014 with permission from Elsevier.

Chapter 4: A manuscript published in the Journal of Chromatography A. Ross M. Woods, Darshan C. Patel, Yeeun Lim, Zachary S. Breitbach, Hongyin Gao, Craig Keene, Gongquiang Li, Laszlo Kurti, Daniel W. Armstrong, 2014, DOI: 10.1016.j.chroma.2014.04.080. Copyright © 2014 with permission from Elsevier

Chapter 5: A manuscript accepted for publication in LC/GC North America. Ross M. Woods, Zachary S. Breitbach, Daniel W. Armstrong.

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Biographical Information

Ross Woods obtained a Bachelor of Science in biological chemistry from the University of Texas at Arlington in 2009. His undergraduate research focused on the characterization of non-heme metalloenzymes. He joined the research group of Professor Daniel Armstrong shortly after graduation where his research efforts focused on characterization of environmental and commercial samples, development of new chiral HPLC methods and synthesis of new derivatization reagents for HPLC-ESI-MS/MS. He graduated with a PhD in chemistry in July, 2014 and began work as an investigator at GlaxoSmithKline in Collegeville PA immediately after graduation.