DEVELOPMENT, EVALUATION AND APPLICATION OF CYCLOFRUCTANS AS SEPARATION AGENTS

by

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This dissertation is dedicated to my beloved husband Milan, my loving son Nidun and daughter Sathni.

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ABSTRACT

DEVELOPMENT, EVALUATION AND APPLICATION OF

CYCLOFRUCTANS AS SEPARATING AGENTS

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Hydrophilic interaction chromatography (HILIC) is now considered as a viable and established analytical tool for the separation of very polar and hydrophilic compounds, which are not sufficiently retained under Reversed phase liquid chromatography (RPLC). HILIC is becoming a routine technique in the areas of proteomic and metabolomic research and it is steadily increasing in importance in the fields of pharmaceutical, biolological, environmental and food chemistry. HILIC shows a complementary selectivity to RPLC. The use of water-miscible organic rich mobile phases (generally a mixture of acetonitrile and water (1-40 % of water in the mobile phase) is compatible with atmospheric pressure mass spectrometric (MS) analysis. In this chromatographic technique, the polar analytes interact with a hydrophilic stationary phase via a combination of hydrogen bonding, dipolar interactions, electrostatic interactions and sometimes partitioning between the mobile phase and the water-rich layer that is solvating the surface of the stationary phase.

There are an increasing number of HILIC stationary phases commercially available. Understanding the principles that drive the separation and selection of both stationary phase and the chromatographic conditions enhance the number and quality of the applications in the HILIC mode. In part one of this thesis, we present new approaches for the HILIC separation of very important biological compounds using a recently developed silica based native cyclofructan 6 (FRULIC-N) stationary phase. This stationary phase provided excellent selectivity towards mononucleotides. Traditional hydrogen bonding/ dipolar interactions can be supplemented by dynamic ion interaction effects for the separation of these anionic analytes. Also, we present the development of new HILIC stationary phase based on sulfonated cyclofructan 6, and its applications. The new columns successfully separates polar and hydrophilic compounds including beta blockers, xanthines, salicylic acid related compounds, nucleic acid bases, nucleosides, maltooligosaccharides, water soluble vitamins and amino acids.

Today, chirality is an important concern for biological processes because asymmetry dominates biological processes. Chirality is of fundamental interest in large number of areas including chemistry, biochemistry, pharmacology and so forth. In particular, the separation and characterization of the desired enantiomer in active pharmaceutical ingredients is critical in drug discovery and development. High performance liquid chromatography (HPLC) is ubiquitous as a highly efficient and selective technique in enantiomeric separation and analysis. However, the efficient development of enantiomeric separation methods is still challenging and time consuming. In part two of this thesis, we present the development of new chiral

stationary phases (CSPs) based on cyclofructans; evaluation and applications of these newly synthesized CSPs. The isopropyl-functionalized cyclofructan 6 chiral stationary phases (LARIHC CF6-P) provided remarkable enantiomeric selectivity for the primary amines while R-naphthylethyl-carbamate cyclofructan 6 (LARIHC CF6-RN) and dimethylphenyl-carbamate cyclofructan 7 (LARIHC CF7-DMP provided enantioselectivity toward a broad range of compounds, including chiral acids, amines, metal complexes, and neutral compounds. Also, we present new classes of chiral selectors based on cationic and basic derivatives of cyclofructan 6 for the first time as bonded chiral stationary phases for high performance liquid chromatography (HPLC).

Cyclofructans, cyclic fructofuranose oligomers, have unique crown ether cores. They tend to form complexes with a variety of metal cations in solution. This special character of cyclofructans prompted NMR studies in order to understand the selective complexation ability of CFs for metal cations. In the last part of this thesis, we examine the host-guest complexation between cyclofructans and large numbers of metal ions. This study showed the specific binding ability of native CF6 with Ba^{2+} and Pb^{2+} cations, while the traditional synthetic crown ether form preferential complexes with the alkali metal ions.

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LIST OF ACRONYMS

HPLC	:	High performance liquid chromatography
RPLC	:	Reversed phase liquid chromatography
HILIC	:	Hydrophilic interaction chromatography
GC	:	Gas chromatography
SFC	:	Supercritical Fluid Chromatography
CE	:	Capillary Electrophoresis
NMR	:	Nuclear magnetic resonance spectroscopy
CSP	:	Chiral stationary phase
CF6	:	Cyclofructan6
CF7	:	Cyclofructan 7

CHAPTER 1

INTRODUCTION

1.1 Hydrophilic Interaction Chromatography

The separation of hydrophilic polar compounds is of great importance in many fields including, pharmaceutical, clinical and biological research and forensic analysis. Reversed phase liquid chromatography (RPLC) using hydrophobic stationary phases and polar mobile phases is one of the most widely used chromatographic techniques for separating these analytes. However, the usual RPLC stationary phases have been problematic for the separation of highly polar compounds because of their lack of retention and/or selectivity. Often, very low organic solvent contents are used to obtain some retention. Inadequate phase wetting and expulsion of the analyte from the pore space often accompanied with highly aqueous eluents in RPLC. This is known to cause problems such as irreproducible retention times and low separation efficiencies.[1] Ion-exchange chromatography is an obvious choice for separating polar compounds that have one or more charged moieties and show lack of retention in RPLC.

An alternative to ion exchange is ion pairing, which allows the use of RP columns, which usually provide better separation efficiencies compared to ion exchange phases. The increased retention is mediated by an ion pairing agent, which is an ionic compound of opposite charge to the solute, capable of entering

into an ion pairing equilibrium.[2] However, occasional use of ion pairing agents on a multipurpose instrument is undesirable due to potential contamination. In addition, diminished column life time and long equilibration times can be problematic when using gradient ion-pair techniques. Also, typical ion pairing agents (e. g. trifluoroacetate or tetrabutylammonium hydrogen sulfate etc) have shown to significantly reduce signal intensity in mass spectrometry with ESI (ESI-MS).[3] Normal phase liquid chromatography (NPLC) which involves a polar stationary phase and an apolar mobile phase is another alternative for separating polar analytes. The retention increases as the polarity of the mobile phase decreases, and thus polar analytes are more strongly retained than nonpolar ones. However, it has not been an option for separating highly hydrophilic polar compounds (e.g: charged pharmaceuticals) due to poor solubility in the apolar solvent. [4]

The most rational way to address very hydrophilic and polar compounds in high performance liquid chromatography (HPLC) is hydrophilic interaction chromatography (HILIC). HILIC is a special sub-type of normal phase liquid chromatography (NPLC) with a polar stationary phase and an aqueous–organic mobile phase. This specific separation mode has been used in polar analyte separations for many years, [5-9] the name HILIC was coined in 1990.[10] Since then, HILIC has been steadily gaining popularity for the separation of hydrophilic polar analytes and has become an established and consolidated chromatographic

technique for the separation of polar compounds in many scientific areas.[2,5-7,9,11-14] As shown in Figure 1.1, the number of scientific publications on HILIC separations has substantially increased from 2003 to 2012.

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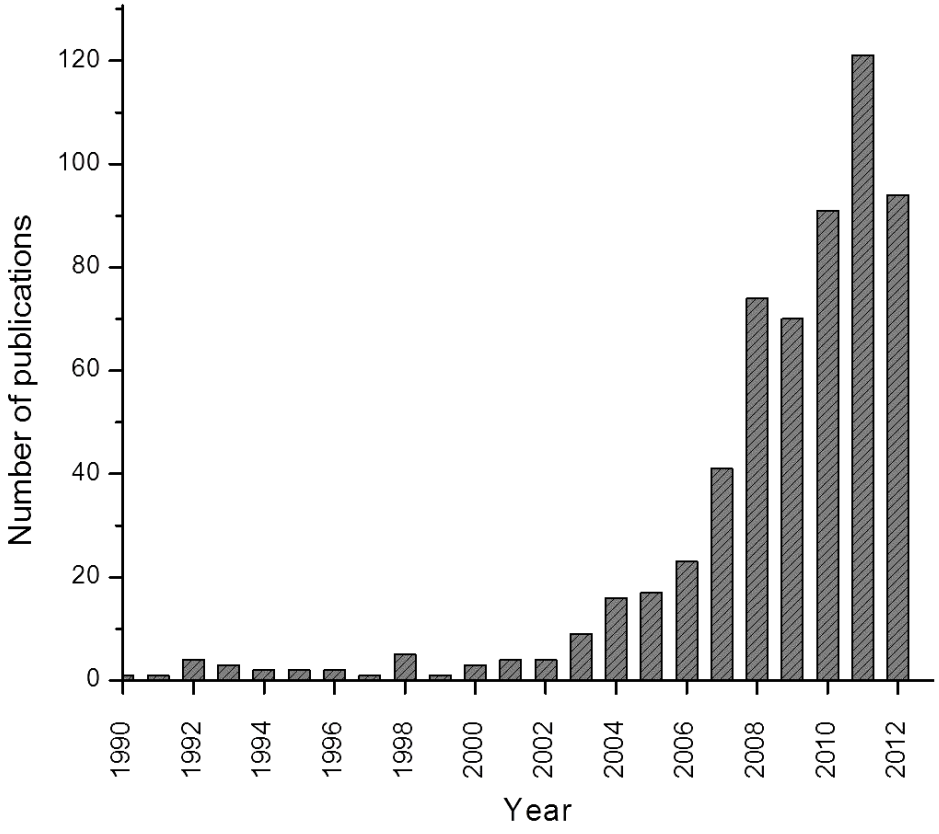
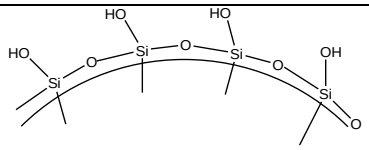
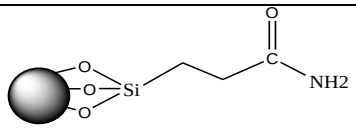
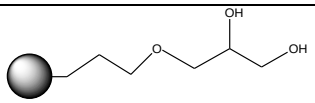
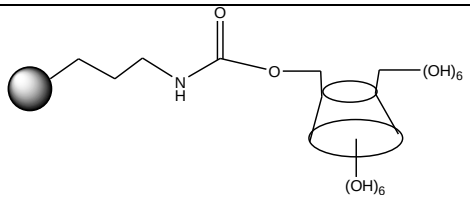
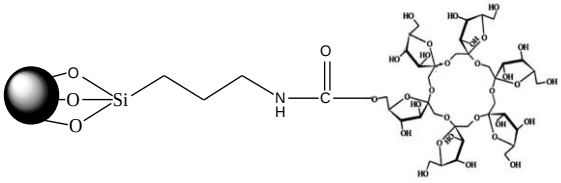
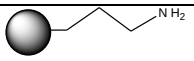
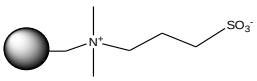
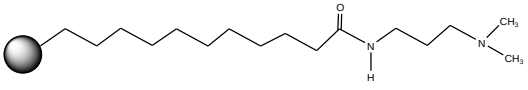


Figure 1.1 SciFinder Scholar search results documenting the research area of HILIC, based on the numbers of publications per year

In this chromatographic technique, the polar analytes interact with a hydrophilic stationary phase via a combination of hydrogen bonding and other dipolar effects and, in some cases, some partitioning between the mobile phase and the water-rich layer that is solvating the surface of the stationary phase.[2,6,7,10,15,16] However, the predominant retention mechanism in a HILIC separation is not well understood and is complicated by the fact that the mechanism can vary from one stationary phase to another, especially when different analytes, mobile phases are applied. In general, the analytes are eluted with a binary eluent, which consists mainly of a large amount of acetonitrile (usually >60%) and a smaller amount of aqueous solvent (usually 1- 40%). In particular hydrogen bonding between analytes and appropriate stationary phases are enhanced by acetonitrile; a poor hydrogen bonding solvent. In addition, the high acetonitrile content also gives HILIC two additional advantages; high sensitivity in ESI-MS[17,18] and faster separations due to the lower viscosity of HILIC eluents compared to standard RP eluents.[19] In contrast to NPLC, the use of water as the strong eluting solvent also gives enhanced compatibility for mass spectroscopic detection and better dissolving power for polar solutes.[2,19,20]

Table 1.1 Selected stationary phases used in HILIC mode separations

	Packing Material	Structures of Stationary Phases
1	Underivatized silica	
2	Amide	
3	DIOL bonded phases	
4	ß-cyclodextrin	
5	Cyclofructan bonded phases	
6	Amino bonded phases	
7	zwitterionic sulfobetaine bonded phases	
8	Mixed-mode phases	

1.1.1 Stationary phases for HILIC

Considering the growing importance of HILIC mode separations, there have been a number of stationary phases introduced in the past few years.

In general, they can be categorized as silica bonded, neutral stationary phases (e.g. amide and diol phases and carbohydrate phases), charged and zwitterionic stationary phases (e.g. amine and sulfobetaine phases) and mixed mode phases (long carbon chains with polar groups). A summary of these stationary phases that have been used for dedicated HILIC separations is shown in Table 1. It should be noted that, stationary phases will be referred to by their chemistry and not by brand names in order to facilitate an understanding of the concepts (or scientifically significant).

1.1.1.1 Silica gel as a HILIC phase

The first generation of stationary phases for HILIC included bare silica and a large fraction of the recently published works is still using conventional nonmodified (naked) silica.[20-31] It should be noted that performance of bare silica columns differ between various types of silica. Type A silica gels, prepared by precipitation from the solutions of silicates, are acidic and form complexes with some chelating solutes, causing strong retention or asymmetric peaks. Type B silica gels are formed by the aggregation of silica sols in air, contain very low amounts of metals, and are more stable at intermediate and higher pH values (up to pH 9). At higher pH values, silanol groups are ionized and cation exchange

plays a important role in retention, especially for positively charged basic compounds to provide better separations.[32] Silica gel type C with a hydrosilated surface populated with nonpolar silicon hydride Si–H groups instead of silanol groups show a unique selectivity for many polar compounds in high organic eluents.[33] Recently, un-derivatized 1.7 μm bridged ethylene hybrid organic silica (BEH) particles and fused-core particles were used in HILIC mode separations and they also showed improved separation capabilities. Also, silica-based monolithic columns were reported in HILIC mode applications.[34,35] However, bare silica columns might cause considerable problems such as irreproducible results, strong adsorption and tailing peaks for some analytes (e.g. carbohydrates, amines) due to interactions with ionized silanol groups and overloading effects. [2,25]

1.1.1.2 Neutral stationary phases

A popular type of neutral bonded phase is amide bonded silica columns. They have been used for the HILIC mode separations of peptides.[36,37] Retention on these columns is less sensitive to eluent pH and less prone to irreversible chemisorption due to the low reactivity of amide groups and these columns shows good and stability.[36] Diol silica is another neutral bonded phase that most closely resembles naked silica in overall polarity.[2,38] These stationary phases demonstrate high polarity and hydrogen bonding properties, along with a relative absence of dissociable moieties and they should be nearly ideal for HILIC applications.[2]

Polyhydroxyl based phases originated from oligosaccharides (carbohydrate bonded stationary phases) have been also successfully used in the HILIC mode.[5-7]

1.1.1.2.1 Carbohydrate bonded stationary phases

There are a large number of reports appeared on the effective separation of polar analytes on the carbohydrate bonded stationary phases in the HILIC mode.[6,7,15,39] Silica bonded cyclodextrin (CD) phases that composed of five or more 1→4 linked α-D-glucopyranoside units are known to be effective for both chiral and other isomeric separations in HPLC. Also, they are attractive in HILIC mode separations due to the high density of exposed hydroxyl groups.[36] They have been used as effective materials for the separation of saccharides.[5-7] CD is also an intriguing phase when it is used in chiral separations in HILIC mode.[40,41] It has been shown that HILIC is a promising separation mode for polar chiral compounds, as the enantiomers of a compound that separated well in HILIC mode failed to resolve under more conventional, nonaqueous NP conditions on CD functionalized silicas.[40]

Recently, Armstrong *et.al*. introduced cyclofructans (CF)s as new HILIC stationary phase. CFs are cyclic oligosaccharides which consist of six or more β-(2→ 1) linked D-fructofuranose units (commonly abbreviated as CF6, CF7, CF8, etc., which indicate the number of fructose units in the macrocyclic ring). Their central skeleton has the same structure as the respective crown ethers. Each fructofuranose unit contains one primary hydroxyl group and two secondary

hydroxyl groups.[42] Kawamura and Uchiyama first reported CFs produced by fermentation of inulin using an extracellular enzyme from a strain of *Bacillus circulans* OKUMZ31B.[43] In 1994, Kushibe et al. reported a different strain of Bacillus circulans (MCI-2554), which enabled more efficient cyclofructan production.[44] There are a number of applications for CFs. They have been suggested as bulk additives in various industrial formulations such as ink formulation agents,[45] as browning preventative agents,[46] as food additives,[47] excipients in pharmaceutical applications[48] and ion trapping reagents due to their ability to form complexes with many metal cations.[49,50] Recently, Armstrong *et al.* employed derivatized cyclofructans as chiral selectors for capillary electrophoresis (CE),[51] gas chromatography (GC)[52] and HPLC.[53-55] Also, native CF6 and sulfonated CF6 have been introduced as HILIC stationary phases and they showed exceptional selectivity for the separation of achiral polar analytes.[56,57]

1.1.1.3 Charged and zwitterionic stationary phases

Aminopropyl-bonded silica was the first bonded stationary phase to be routinely used for carbohydrate separations in HILIC mode.[2,8,9] These columns provide an increased rate of mutarotation when compared to the underivertized silica columns, which prevents formation of double peaks due to anomer resolution. However, significant concerns with aminopropyl silica are irreversible adsorption[8] and the required long equilibration times.

Zwitterionic sulfoalkylbetaine stationary phases which are grafted onto silica gel or a polymer support have also been introduced more recently for HILIC separations.[2] These stationary phases contain both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups that are separated by a short alkyl spacer. Recently, new zwitterionic stationary phases were introduced by covalently bonding 3-P,P-diphenyl phosphonium-propylsulfonate to silica gel.[58] These stationary phases possess both a negatively charged sulfonate group and a positively charged quaternary phosphonium group. A combination of the water-retaining properties of the (zwitter)ionic environment, and a low surface charge that does not promote strong ion exchange interactions makes them ideal for HILIC mode separations.

1.1.1.4 Mixed mode HILIC phases

Over the last two decades, HILIC phases based on mixed mode retention mechanisms were introduced. When ion exchange groups are present, mixed-mode HILIC/ion-exchange mechanism controls the retention and it may cause specific selectivity effects. Also, the mixed mode stationary phases included RP interactions as these phases frequently have hydrophobic character. In the 1990s, various poly(succinimide)-based phases have been used in numerous works with HILIC and mixed mode separations.[59-61] However, these stationary phases show a limited longtime stability and/or column bleeding.[62,63]

1.2 Enantiomeric separations

Enantiomeric separations have attracted great attention in areas of chemistry, biochemistry, pharmacology, etc. In particular, chirality is a subject of intense interest in the modern pharmaceutical industry since many drug molecules have asymmetric centers and can exist as enantiomers on which have different pharmacokinetic and pharmacodynamic effects.[64,65] The Food and Drug Administration (FDA) has recognized the importance of chirality and issued a policy statement concerning the development of new stereogenic drugs.[66] Thus, the ability to analyze and/or separate enantiomers is usually a prerequisite for almost all areas of research involving chirality.

Chromatography is a highly sensitive and rapid method for enantiomeric separations and it has become the method of choice due to the wide availability of chromatographic instruments, high extent of automation, as well as its high reproducibility and transferability. The various chromatographic methods including gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SFC) and chiral HPLC methods have been used to separate enantiomers. Among them, HPLC with chiral stationary phases is most successful in the field of enantiomeric separation showing good reproducibility, wide selectivity and capability for using both analytical and preparative scale separations. The success of enantiomeric separation using HPLC is also due to the

discovery of a tremendous number of CSPs and effectively adapting them to modern separation technologies.[67]

There are a few types of CSPs that dominate enantiomeric separations, including polysaccharide stationary phases,[68-71] macrocyclic antibiotic stationary phases[72-81] and cyclodextrin phases.[39,82-85] Even with those CSPs, it is a challenging task to choose the right CSP and chromatographic conditions for certain compounds. Therefore, researchers are continuing to develop new HPLC CSPs in order to (a) find broader applicability than existing CSPs, (b) superior separations for specific groups of compounds, or (c) fill an important unfulfilled separation niche.[55] However, not only the CSP, but also analyte and mobile phase must be taken into consideration when developing a chiral separation method. Thus, the understanding of the possible chiral recognition mechanisms on a given CSP is the key for successful enantiomeric separation of a particular class of compounds in addition to considering some practical factors including the solubility of analyte in the mobile phase, analysis time, column cost, column robustness, column capacity, etc.

1.3 Cyclofructan 6 as host guest complex with cations/metals

Among CFs, CF6 possesses an 18-crown-6 core. It consists of six fructofuranose rings which are arranged in spiral fashion around the central crown ether and they are positioned either up or down toward the mean plane of the crown ether.[86,87] However, CF6 is distinct from typical synthetic crown ethers in

that they have unique structural characteristics: (1) the ether ring of CF6 is surrounded by hydrophilic, chiral fructofuranose units (2) there is restricted internal motion of the ring due to the large substituents (3) there a different conformational arrangement of the ring in the crystalline state.[88] The ether ring of CF6 has gtgtgt conformation (g and t stand for gauche and trans, respectively) around the -C-CH_2- bonds in the uncomplexed crystalline state[87] whereas 18-crown-6 has gtḡḡtg conformation for the uncomplexed state.[89]

The crown ether core in CF6 is somewhat different from the synthetic crown ether. For example, when synthetic crown ethers are complexed with potassium ion, the six -O-CC-O- units adopt a gḡgḡgḡ configuration,[89] and are evenly distributed above and below the crown ether mean plane. However, the six oxygen atoms in CF6 are all aligned towards one side of the macrocycle due to the gtgtgt configuration of the six -O-C-C-O- units in the center 18-crown-6 core.[87] Native and derivatized CF6 have been found to complex with some metal cations[50,88,90-92] and have been used as ion trapping agents in various applications.[49,93] Because there have been only a few ions considered in published reports on host-guest studies for native CFs and their derivatives, [50,88,90-92] further research in this field is needed.

1.4 Research Objectives and Organization of the Thesis

My general research goal was to develop HPLC methods for both achiral and chiral separations using silica bonded native/derivatized CF6 and to do this via three approaches, which correspond to the three parts of this thesis. The first part of my research focuses on (1) expanding the general knowledge of HILIC mode separations and developing new HILIC stationary phases based on silica bonded derivatized CF6. Chapter two discusses (2) the separation of nucleotides using a silica based native CF6 column (FRULIC-N) in the HILIC mode. The developed HILIC method for separating these important molecules showed greater selectivity and efficiency when compared to the previously published methods under HILIC conditions. Chapter three presents (3) the development of a sulfonated CF6 based stationary phase for HILIC. This phase was evaluated for the separation of a large variety of polar compounds; successfully separating beta blockers, xanthines, salicylic acid related compounds, nucleic acid bases, nucleosides, maltooligosaccharides, water soluble vitamins and amino acids. It showed broad applicability for HILIC mode separations and it is competitive with and often superior to some popular commercial columns.

The second part of my research involves the development, evaluation and application of new CSPs based on derivatized CFs. Chapter four presents the evaluation of aromatic-derivatized cyclofructan 6 and 7 as HPLC chiral selectors. These selectors showed different chiral recognition ability when derivatized with

different groups. Among the synthesized CSPs, R-naphthylethyl-carbamate CF6 (RN-CF6) and dimethylphenyl-carbamate CF7 (DMP-CF7) showed broad selectivity toward a variety of compounds, including chiral acids, amines, metal complexes, and neutral compounds. Chapter five discusses the synthesis of cationic and basic derivatives of CF6 (with nitrogen atom-containing functionalities that can carry positive charges) and the enantioseparation capability of silica bonded cationic CSPs. Chapter six presents applicability of the most successful CFs based CSPs (LARIHC series) for the enantiomeric separation of chiral illicit drugs and controlled substances.

The last part of my research was dedicated to understanding the ability to make host-guest complexes of CF6 with different metals ions. Although there are a few metal-based host-guest studies of CFs reported, additional research is this area is needed for a better understanding of complexation ability of CF6s. Chapter seven outlines the binding ability of native, isopropylated and permethylated cyclofructan 6 with various cations, including alkali metals, alkali earth metals, transition metals and lanthanides. The binding ability was characterized under various solvent conditions and association constants (K_{ass}) were measured using NMR titrations and a nonlinear least square treatment of the data.

CHAPTER 2

SEPARATION OF NUCLEOTIDES BY HYDROPHILIC INTERACTION CHROMATOGRAPHY USING THE FRULIC-N COLUMN

2.1 Abstract

A stationary phase composed of silica-bonded cyclofructan 6 (FRULIC-N) was evaluated for the separation of four cyclic nucleotides, six nucleoside monophosphates, four nucleoside diphosphates and five nucleoside triphosphates via hydrophilic interaction chromatography (HILIC) in both isocratic and gradient conditions. The gradient conditions gave significantly better separations, by narrowing peak widths. Sixteen out of nineteen nucleotides were baseline separated on the FRULIC-N column in one run. Unlike other known HILIC stationary phases, there can be dual retention mechanisms unique to this media. Traditional hydrogen bonding/ dipolar interactions can be supplemented by dynamic ion interaction effects for anionic analytes. This occurs because the FRULIC-N stationary phase is able to bind certain buffer cations. The extent of the ion interaction is tunable, in comparison to stationary phases with embedded charged groups, where the inherent ionic properties are fixed. Optimization approaches were examined by varying the organic modifier (acetonitrile) content, as well as salt type/concentration and electrolyte pH. The thermodynamic characteristic of the FRULIC-N column was investigated by evaluating the

column temperature effect on retention and utilizing van't Hoff plots. This study shows that the retention of nucleotide di and tri phosphates is entropically and the cyclic nucleotides are enthalphically driven on the FRULIC-N column.

2.2 Introduction

Nucleotides are a class of molecules that play an important role in biological systems. They form the building blocks of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA).[94] Nucleotides also play very important roles in cell signaling, different metabolic pathways and many enzymatic reactions. Single nucleotide units are present in many important biological compounds such as coenzyme A, flavin adenine dinucleotide, nicotinamide adenine dinucleotide, and many others.[94,95] Nucleotides also are found in many biological food products such as milk and meat and have been identified as conditionally essential nutrients and are widely found in infant formulae.[96] Since these molecules are very important in biological systems, their analysis and determination is of great importance to the fields of chemistry, biochemistry, medicine, genetics, metabolomics and environmental reseach.[97-105]

Many analytical methods have been developed to determine nucleotides in complex matrices.[99-104,106] Reversed phase liquid chromatography (RPLC) and ion exchange chromatography (IEC) are the most widely used techniques for the determination of nucleotides.[107-111] Conventional ion exchange methods seem to be more suitable for the analysis of nucleotides, however these

methods are often not compatible with mass spectrometric detection as the high salt concentrations are used in mobile phases to elute nucleotides.[112,113] In general, RPLC methods have been considered better alternatives to separate nucleotides given their versatility and relative ease of operation.[98,101,102,114] However, the most useful solvent systems for nucleotide analysis in RPLC often contain nonvolatile modifiers (e.g. ammonium phosphate) which restrict their use with ESI-MS detection.

The separation of nucleotides by means of conventional RPLC can be enhanced by means of using specially prepared columns such as polar embedded C18 phases, hybrid columns, polymer grafted silica columns,[115,116] porous carbon columns,[117] polymer based monolithic columns such as hydroxymethylmethacrylate-based columns[118] and columns based on glycidyl methacrylate.[119] These types of columns offer advantageous over conventional C18 phases, such as suppressed silanol activity, reduced metal ion (chelation) interactions, phase stability under highly aqueous conditions and unique selectivities.

Another widely used approach for nucleotide analysis is RPLC with ion pairing agents. The ion pairing reagent associates with the phosphate groups and retention increases as the number of phosphate groups increase.[109] Volatile ion-pairing reagents have been applied in ion pair RPLC-mass spectrometric combinations.[120,121] However, occasional use of ion pairing agents on a

multipurpose instruments is undesirable due to contamination by the ion pairing agents. Typical ion pairing agents (e.g., trifluoroacetate, tetrabutylammonium hydrogen sulfate, etc.) have been shown to significantly reduce signal intensity in mass spectrometry with ESI-MS. In addition, diminished column life time and long equilibration times when using gradient ion-pair techniques are inherent disadvantages of this method.

Hydrophilic interaction chromatography (HILIC) represents an alternative chromatographic approach to effectively separate small polar compounds on polar stationary phases. HILIC is a special sub-type of normal phase liquid chromatography (NPLC) that uses an aqueous–organic mobile phase.[5-7,15] In this chromatographic technique, the polar analytes interact with a hydrophilic stationary phase via a combination of hydrogen bonding, dipolar interactions, electrostatic interactions and sometimes partitioning between the mobile phase and the water-rich layer that is solvating the surface of the stationary phase.[5,7,10,15] HILIC has been steadily gaining popularity for the separation of hydrophilic polar analytes including a wide variety of different areas.[2,4] There have been a few reported HILIC studies on the separation of nucleotides[122-126] using different types of stationary phases including underivatized silica (Atlantis –HILIC), aminopropyl bonded silica (Luna NH2, TSK-gel NH2)[96], cyanopropyl bonded silica (Luna CN), amide bonded silica (TSK Gel Amide 80)[127], ZIC HILIC[128] and the polymer based ZIC-*p*HILIC.[129] Recently, the term Electrostatic repulsion

hydrophilic interaction chromatography (ERILIC) was proposed as a combination of electrostatic and hydrophilic interaction. In ERILIC, solutes can be retained through hydrophilic interactions even if they have same charge as the stationary phase.[130] This mixed-mode technique was used to separate nucleotides using a cation-exchange column.[130,131] It is of great importance to explore new separation material for facile resolutions of nucleotides.

Native cyclofrutan 6 (CF6) and derivatized CF6 based stationary phases have been reported as effective media for the separation of polar analytes in the HILIC mode.[56,57] Cyclofructans are new types of macrocyclic oligosaccharides[132] which consist of six or more β-(2→1) linked D-fructofuranose units. Each unit contains one primary hydroxyl group and two secondary hydroxyl groups, which account for the hydrophilic character of these molecules. Although native cyclofructans have limited capabilities as chiral selectors,[53,55] they showed exceptional selectivity for the separations of achiral polar analytes in the HILIC mode.[57] However, to date, there have been no published reports focusing on nucleotide separations in the HILIC mode using the silica bonded native CF6 stationary phase (FRULIC-N). In the present work, we present a comprehensive study on the separation of 19 nucleotides using the FRULIC-N column.

2.3 Experimental

2.3.1 Reagents

Acetonitrile (ACN) of HPLC grade was obtained from VWR (Sugarland, TX). Ammonium acetate, ammonium nitrate, ammonium trifluoroacetate, acetic acid, triethyl amine and all the nucleotides tested in this study were purchased from Sigma-Aldrich (St.Luis, MO). Water was purified by a Milli-Q-water purification system (Millipore, Billerica, MA). The native cyclofructan 6 column (FRULIC-N) (25 cm × 4.6 mm) was obtained from AZYP (Arlington, TX).

2.3.2 HPLC method

The Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA, USA) was used in this study. It consists of a diode array detector and a temperature controlled column chamber, auto sampler and quaternary pump. For data acquisition and analysis, the Chemstation software version Rev. B.01.03 was used on the system in Microsoft Windows XP environment. All separations were carried out at room temperature (˜20 ºC) unless stated otherwise. For all HPLC experiments, the injection volume was 5 µL and flow rate was 1.0 ml/min in isocratic mode. The UV wavelengths of 195, 210, 254 and 280 nm were employed.

The separation conditions were optimized by varying mobile phase composition and pH. The first investigation was carried out by varying the mobile phase composition in the range of 60-90% ACN (pH and amount of

ammonium acetate buffer kept constant (100 mM and pH 6.6). The pH of the 100 mM ammonium acetate solution was adjusted with using 99.7 % acetic acid, acetic acid and ammonium hydroxide. The ammonium acetate buffer/salt concentration and pH were varied from 0 to 400 mM and 4.0-7.0, respectively. All analytes were dissolved in water or ACN / water mixtures. The mobile phase was degassed by ultrasonication under vacuum for five minutes. Each sample was analyzed in duplicate. For the calculation of chromatographic data, t_0 was determined by the refractive index change caused by the sample solvent.

2.4 Results and discussion

2.4.1 Separation of nucleotides mixtures

For this study, four cyclic nucleotides, six nucleoside monophosphates, four nucleoside diphosphates and five nucleoside triphosphates were selected. Figure 2.1 shows the structures of these analytes. Each analyte (nucleotide) consists of three main components, a nitrogenous base (either purine or pyrimidine), a pentose sugar (ribose or deoxy ribose), and one, two or three phosphate groups. The phosphate groups form bonds with either the 2', 3' or 5'-carbon of the sugar, with the 5'-carbon site being the most common. When the phosphate group is bound to two of the sugar's hydroxyl groups, cyclic nucleotides are formed.[133] Considering the excellent performance of the FRULIC-N column for separating other polar analytes,[57] the feasibility of using this approach for the selective separation of nucleotides in the HILIC mode was

thought to be promising. The chromatographic conditions were optimized by changing the composition, buffer/salt type and the pH of the mobile phase as well as operating with both isocratic and gradient elution. The amount of ACN markedly affected the retention time of these analytes and it was used to fine tune the separations. The optimum separation conditions were selected in terms of selectivity, resolution, efficiency and analysis time.

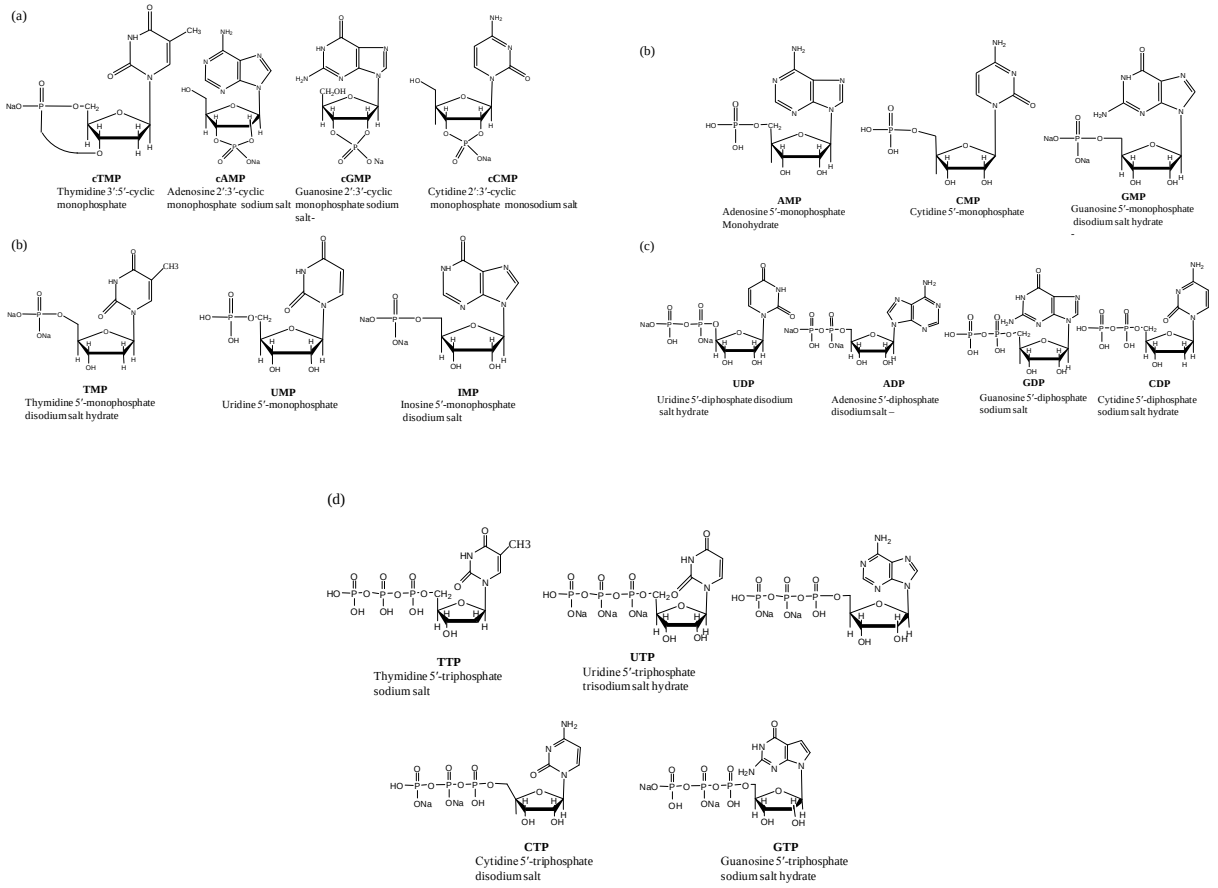


Figure 2.1 Structures of (a) nucleoside monophosphates (b) nucleosidediphosphates (c) nucleoside triphosphates

Figure 2.2 shows the isocratic separations of cyclic nucleotides, nucleoside monophosphates, nucleoside diphosphates and nucleoside triphosphates using a mobile phase containing different amounts of ACN and 100 mM ammonium acetate at pH 6.6. The shortest retention times were observed for the cyclic nucleotides which are least polar analytes among all nucleotides studied. They were baseline separated within 10 min using a mobile phase containing ACN and 100mM ammonium acetate at pH 6.6(70:30 v/v).Nucleoside monophosphates also were well separated under the same mobile phase conditions. The use of same mobile phase for the separation of nucleoside diphosphates and nucleoside triphosphates required long retention times and resulted asymmetric and very broad peaks. (e.g. retention times for ATP and GTP were 91.6 min and 103.1 min). Therefore, a stronger mobile phase containing ACN and 100mM ammonium acetate at pH 6.6 (65:35 v/v) was used for the separation of nucleoside diphosphates and nucleoside triphosphates.

As shown in Figure 2.2 (c) and (d), ADP, GDP and CDP were partially separated while the nucleoside triphosphates were nicely separated under this condition. From Figure 2.2, it is clear that under the same mobile phase conditions, these analytes elute in the following order: cyclic nucleotides < nucleoside monophosphates < nucleoside diphosphates < nucleoside triphosphates. This is expected as an increase in the number of phosphate groups of the analytes increases their hydrophilicity.

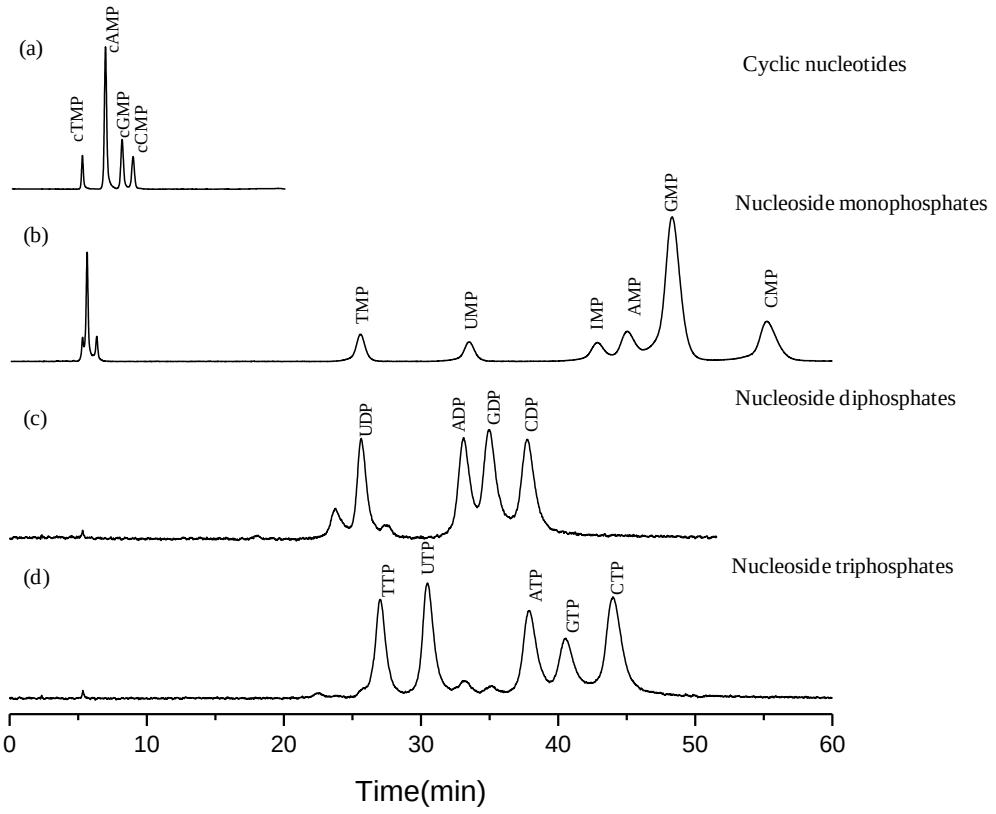


Figure 2.2 Isocratic separation of nucleotides on FRULIC-N column

Conditions for (a) and (b): phase: ACN/100 mM ammonium acetate pH = 6.6 (70/30,v/v), Conditions for (c) and (d): Mobile phase: ACN/100 mM Ammonium acetate pH = 6.6(65/35,v/v),Flow rate : 1.0 ml/min, Detection: UV at 254 nm.

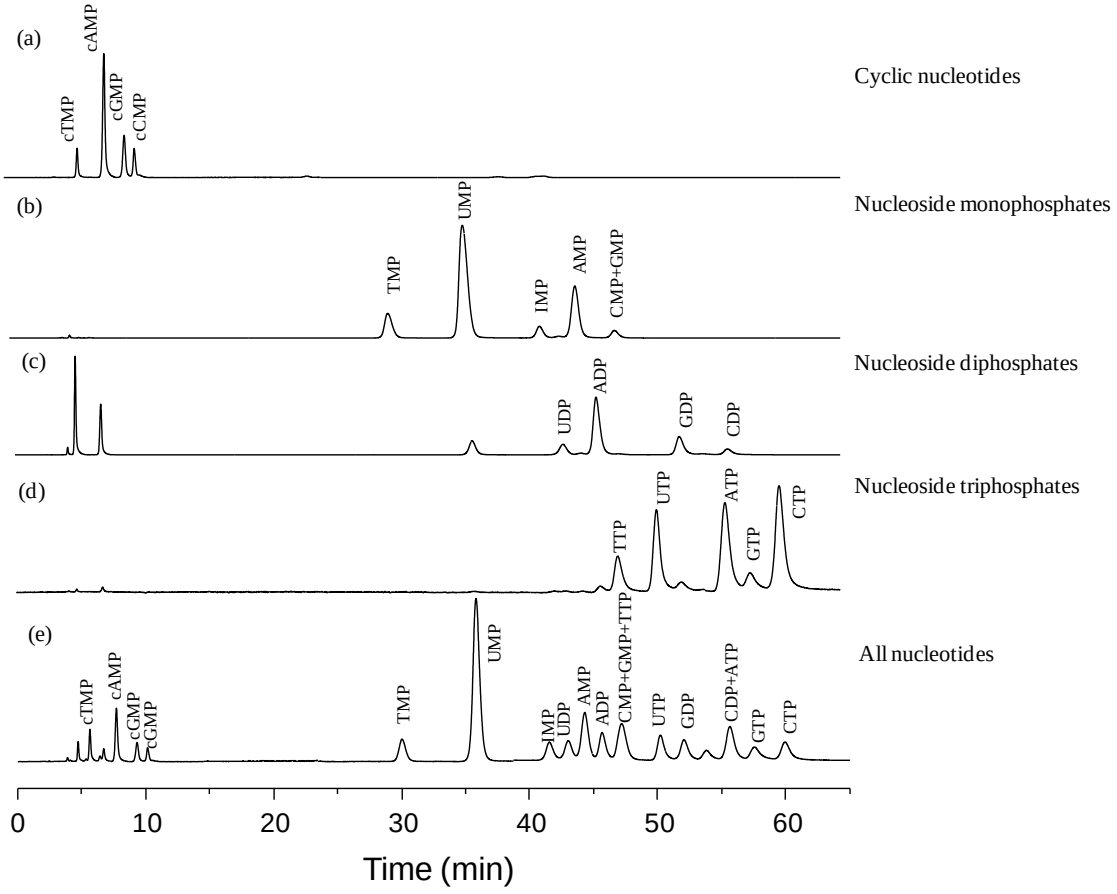


Figure 2.3 Gradient separation of nucleotides on FRULIC-N column

Time 0-10 min ACN/100 mM Ammonium acetate pH = 6.6 (72/28,v/v),

Time 10-50 min ACN/100 mM Ammonium acetate pH = 6.6 (62/38,v/v),

Time 50-60 min ACN/100 mM Ammonium acetate pH = 6.6 (62/38,v/v),

Time 60-65 min ACN/100 mM Ammonium acetate pH = 6.6 (50/50,v/v),

Time 65-70 min ACN/100 mM Ammonium acetate pH = 6.6 (72/28,v/v)

Flow rate : 1.0 ml/min, Detection: UV at 254 nm

As expected, the FRULIC-N column provided significantly better separations (in terms of band broadening) with gradient elution compared to isocratic elution. Typically, a HILIC gradient involves decreasing the organic solvent, although increasing the additional salt concentration also can be used. In the case of nucleotide separations on the FRULIC-N column, a gradient condition was used with decreasing organic solvent in order to increase the polarity of the mobile phase. Figure 2.3 shows the chromatograms of cyclic nucleotides, nucleoside monophosphates, nucleoside diphosphates, nucleoside triphosphates and a mixture of all the nucleotides under the gradient conditions.

Cyclic nucleotides and nucleoside diphosphates were all baseline separated (Figure 2.3 (a) and 3 (c)). According to Figure 2.3 (b), nucleoside monophosphates were also well separated under the same conditions with the exception of GMP and CMP. The selectivity and the efficiency of nucleoside triphosphates were greatly improved under the step gradient conditions (Figure 2.3 (d)). It has been reported that the elution order of analytes under gradient conditions may change as a function of the ACN mobile phase content depending on the prominence of the various interactions involved in the HILIC mode.[118] However, the FRULIC-N column showed similar elution order for all analytes in gradient as compared to isocratic conditions (Figure.2.2). For comparison purposes, a silica based ZIC-HILIC stationary phase was also evaluated for the separation of nucleotides. Although this column resulted in partial separations of

the selected nucleotide mixtures with a mobile phase containing ACN and 100mM ammonium acetate at pH 6.6 (70:30 v/v), broad peaks were observed (results not shown). The analytes were not satisfactorily separated on this column even with weaker mobile phases which caused significantly longer run times. The broad and asymmetrical peak shapes observed on the ZIC-HILIC were consistent with a previously reported attempt to separate nucleotides on the ZIC-HILIC.[129]

2.4.2 Impact of mobile phase variables on retention and selectivity

Experimental parameters such as the amount of organic modifier, salt type and concentration and buffer/salt pH, and temperature were evaluated to study their effects on retention and selectivity.

2.4.2.1 Effects of organic modifier

In this study, ACN was investigated as the organic modifier, as it is the most commonly used solvent in HILIC. At high percentages of acetonitrile in the mobile phase, an analyte's retention is governed by a combination of hydrogen bonding, dipolar and hydrophilic interactions between the solute and the stationary phase.

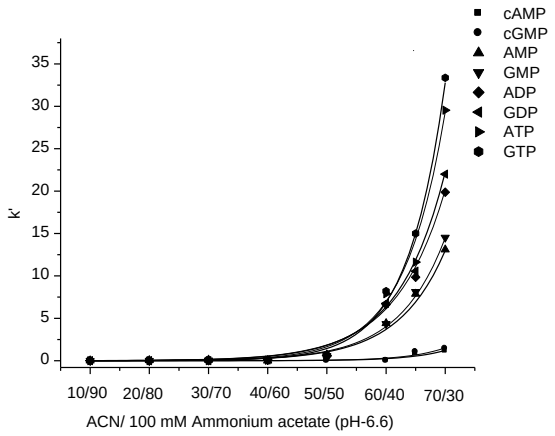


Figure 2.4 The effect of ACN % on nucleotide retention factors on the FRULIC-N

Conditions: Mobile phase: varying amounts of ACN/100 mM ammonium acetate

pH = 6.6.Flow rate: 1.0 ml/min, Detection: UV at 254 nm

Figure 2.4 shows a typical dependence of retention factors (k') on ACN content in the mobile phase for adenosine and guanosine based nucleotides on the FRULIC-N column. When the mobile phase composition is in the range of 10–60% ACN, retention is minimal as all solutes elute near the dead volume. However, retention factors increased steeply when the acetonitrile content exceeded ~60% by volume and the HILIC separation mechanism becomes predominant. It should be noted that all analytes except the cyclic nucleotides produced broad peaks shapes or did not elute when the ACN amount was higher than 70%. Overall, the amount of ACN in the mobile phase was the factor that had the greatest influence on all analyte retention.

2.4.2.2 Buffer/salt effects

2.4.2.2.1 Salt/buffer type

Although phosphate or carbonate salts are typically used in RPLC, they were not evaluated for this HILIC study due to the poor solubility in the mobile phases containing high amounts of acetonitrile.[37] Ammonium acetate and ammonium formate are the most commonly used HILIC buffers due to their solubility in the HILIC mobile phases and their compatibility with MS detection. Initially, ammonium salts with different anions (e.g. ammonium acetate, trifluoroacetate, formate and nitrate) were used for the separation of nucleotides on the FRULIC-N column. Table 2.1 lists the retention parameters for the adenosine and guanosine nucleotides (which showed adjacent peaks), using different ammonium salts. Considering the data for cyclic nucleotides (cAMP and cGMP) in Table 2.1, the highest selectivity was obtained using a mobile phase containing ammonium acetate. However, the selectivity and resolution were slightly improved for nucleotide monophosphates (AMP and GMP) in a mobile phase containing ammonium trifluoroacetate. Also using ammonium trifluoroacetate, nucleoside diphosphates and triphosphates showed slightly increased selectivity with significant increases in retention factors. The use of ammonium nitrate also produced long retention times without significant improvements in the selectivity and resolution. In some cases, very broad and asymmetric peaks were obtained using ammonium nitrate for the long retained

compounds (i.e. ATP and GTP). It was determined that ammonium acetate is the optimum salt for use with the FRULIC-N column.

Table 2.1 The effect of different ammonium salts on retention parameters for adenosine and guanosine nucleotides using the FRULIC-N column

	Ammonium acetate				Ammonium			
	k_1	k_2	α	R_s	k_1	k_2	α	R_s
cAMP	0.66	1.01	1.53	1.3	1.71	1.83	1.07	0.7
cGMP								
AMP	7.06	7.06	1.00	0.0	5.19	5.60	1.07	0.4
GMP								
ADP	10.4	10.67	1.06	1.0	12.91	14.04	10.9	0.8
GDP								
ATP	11.60	12.51	1.08	1.2	21.56	23.77	1.10	0.8
GTP								
	Ammonium formate				Ammonium nitrate			
	k_1	k_2	α	R_s	k_1	k_2	α	R_s
cAMP	.96	1.15	1.20	1.2	1.72	1.93	1.12	1
cGMP								
AMP	5.24	5.24	1.00	0.0	5.4	5.4	1.00	0.0
GMP								
ADP	10.84	10.84	1.00	0.0	13.02	13.70	1.05	0.8
GDP								
ATP	13.81	13.85	1.04	0.4	19.34	20.48	1.06	0.8
GTP								

2.4.2.2.2 Salt amount

The effect of the concentration of ammonium acetate in the mobile phase also was evaluated. The investigation was carried out by varying the salt concentrations in the range of 0 – 400 mM (acetonitrile percentage was kept

constant). Figure 2.5 shows plots of retention factors (k') versus ammonium acetate concentration in the mobile phase for the representative adenine and guanosine nucleotides (i.e. cAMP, cGMP, AMP, GMP, ADP, GDP, ATP, GTP) on the FRULIC-N column.

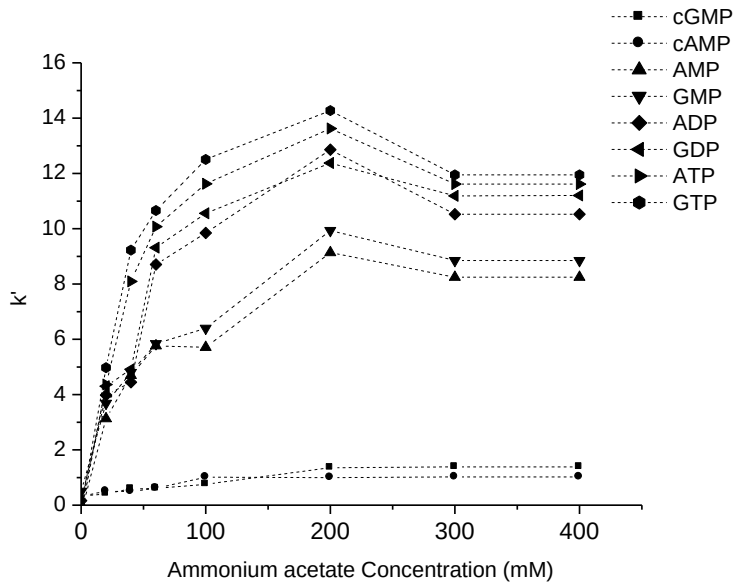


Figure 2.5 The effect of ammonium acetate concentration on retention using on the FRULIC-N column.Conditions: Mobile phase: ACN/ ammonium acetate pH = 6.6 (65/35,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm.

There was a substantial influence of the ionic strength on retention. The retention factors increase with increasing ammonium acetate content up to about 200 mM, after which retention decreased slightly. Interestingly, this observation is in keeping with the affinity of macrocyclicligands such as crown ethers for

cations and metals.[134,135] Crown ethers are well known for their ability to selectively complex cations and this unique selectivity has been used to perform chromatographic and other types of separations.[136] In general, the cations that fit more closely into the macrocycle cavity are bound more strongly. Thus, aqueous eluents containing cations that can bind to such macrocycle-based columns will form positively charged exchange sites. These types of stationary phases have been used to separate anions chromatographically.[134,137]

FRULIC-N (native CF6 bonded to silica) has a crown ether core. Previous studies showed that, alkali metals and ammonium salts make complexes with CF6 by directly comparing the complex ion intensities in ESI mass spectra.[92] The relative order of binding affinity for CF6 was $Na^+ > K^+ > Rb^+ > Li^+ > Cs^+ > NH_4^+$. In addition, it has been reported that cavity inner diameter of CF6 (i.e. distance between opposing oxygen atoms in the molecular core) is 2.30 Å[55] which certainly facilitate the complexation with an ammonium cation with an ionic radius of 1.75Å. Thus, it can be postulated that although CF6 is uncharged, the ammonium ion in the mobile phase can complex with the stationary phase making it positively charged. This cationic CF6 complex can associate with the anionic phosphate groups on nucleotides.

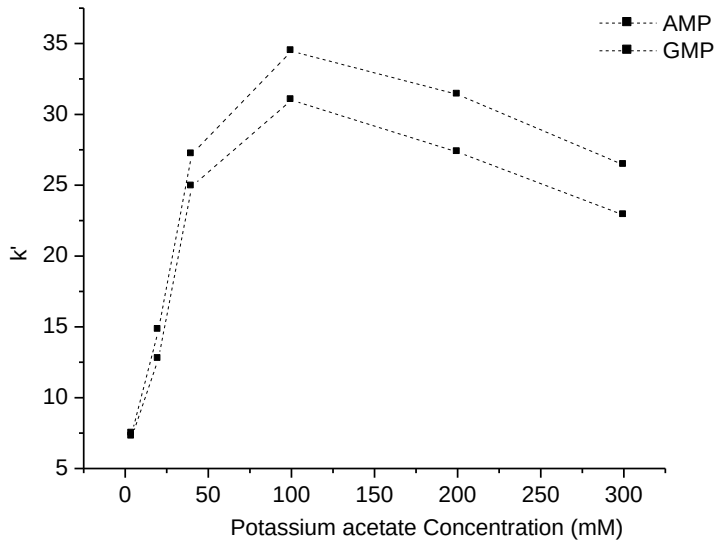


Figure 2.6 The effect of potassium acetate concentration on retention using on the FRULIC-N. Conditions: Mobile phase:ACN/ potassium acetate pH = 6.6 (65/35,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm.

The results in Figure 2.5 show maximum retention factors when using 200 mM ammonium acetate. Below this level, the column capacity to make cationic sites continues to increase. It must be noted, however that, the eluting power of the mobile phase also increases. Yet, in this case (before the saturation of CF6 sites with ammonium ions), the effect of the increasing the population of cationic sites outweighs the effect of increasing the eluting power of the mobile phase. After the available sites of CF6 are saturated with ammonium ions, the retention factors of the analytes gradually decreased. In this case, the amount of ammonium

salt increased the eluting strength of the mobile phase, thus resulting in less retention of anionic nucleotides.

Overall, it can be proposed that not only hydrophilic interaction but also ionic interaction retention mechanisms play important roles on the FRULIC-N column's ability to separate nucleotides. Similar behavior was observed with the use of potassium ions instead of ammonium ions (Figure 2.6). However, retention was consistently higher with potassium than with ammonium, due to potassium's stronger affinity for the CF6.[92] As expected, the stationary phase was saturated by lower concentrations of potassium (100 mM) compared to ammonium. Ultimately, the optimum condition for the nucleotide separation was determined to be 100 mM ammonium acetate in the mobile phase.

2.4.2.2.3 Buffer pH

This investigation was carried out by varying the buffer/salt pH from 4.0 to 7.0 at a fixed buffer concentration and ACN content in the mobile phase. Figure 2.7 shows the effect of the retention on nucleotides by varying pH. In contrast to the other nucleotides, the retention factors for cyclic nucleotides slightly decreased when changing the buffer/salt pH up to 6.0. However, other analytes showed increased retention up to pH 6.0. Slightly low retention was observed at pH 6.6 and it increased at pH 7.0. The pKa values for the first and second protons of the phosphoric acid moiety are ~1 or less and 6 ~7 respectively.[138] Therefore, at neutral pH or below, the net charge on nucleotide is

-1. At low pH, the interaction of the monovalent anion with the stationary phase is relatively low compared to high pH.

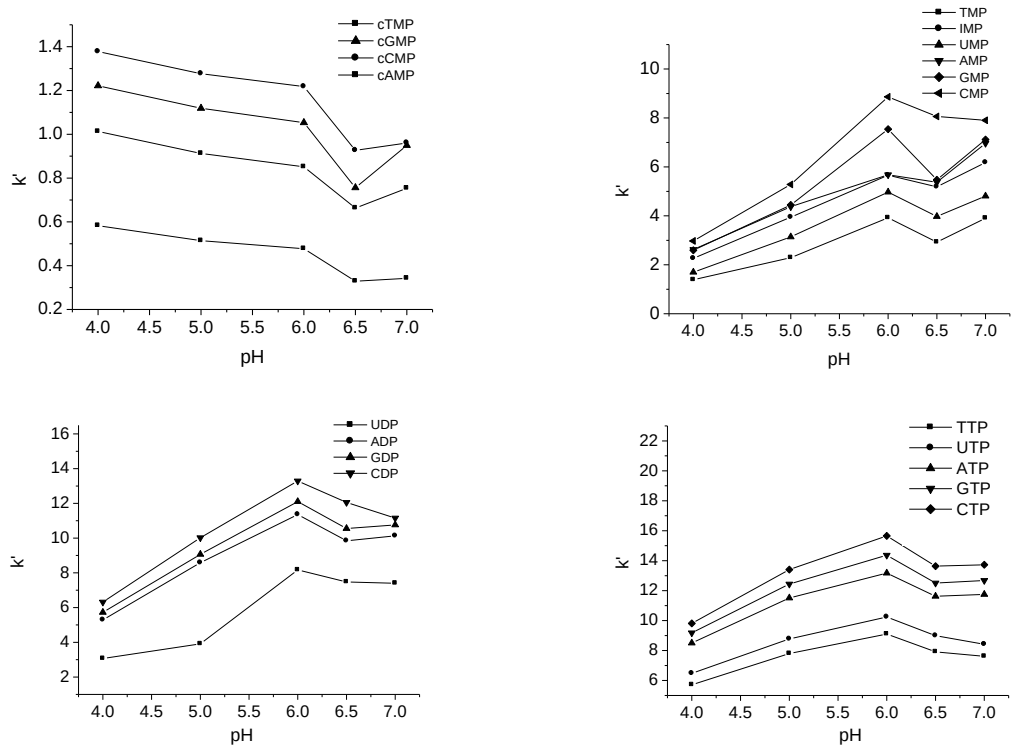


Figure 2.7 The effect of pH on nucleotide retention factors on the FRULIC-N column .Conditions: Mobile phase: ACN/100 mM ammonium acetate (65/35,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm..

This is most likely the reason for the observed low retention factors for the mono, di and tri phosphate nucleosides at low pH. However, a significant drop in retention factors was observed for all analytes at pH 6.6, where phosphate groups are beginning to acquire their second negative charge.Although, the phosphate

groups increase in negative charge at higher pH, the amino groups on the nucleobases also are neutralized. In addition, apparent pH values in the water/organic mixtures vary from the initial pH values of the buffer in the mobile phase.[139] These are possible reasons for the slight difference of retention factors of nucleotides at pH 6.5 and 7.0.

2.4.3 Thermodynamic study

High temperatures can significantly affect an analytes diffusivity, mobile phase viscosity and the enthalpy of transfer between mobile and stationary phases.[140] From a kinetic stand-point, tempersture has been used for improvement of HPLC performance.[141] The dependence of the natural logarithm of retention factors (ln k) on the inverse of temperature (1/T) is routinely used to determine thermodynamic data that may relate to the separation mechanism (Figure 2.8).

The dependence of analyte retention on temperature can be expressed by the van't Hoff equation (eq1).

$$\ln k = \frac{-\Delta H_i}{RT} + \frac{\Delta S_i}{R} + ln\emptyset \qquad \text{(eq1)}$$

Where, k is the retention factor of a solute, ΔHi is the partial molar enthalpy of transfer in the chromatographic system, ΔSi is the partial molar entropy of the transfer, and Ø is the phase ratio of the chromatographic column ($\emptyset = V_S/V_M$)

where, V_M is the dead volume and V_S is by the geometric internal volume of the column minus V_M).

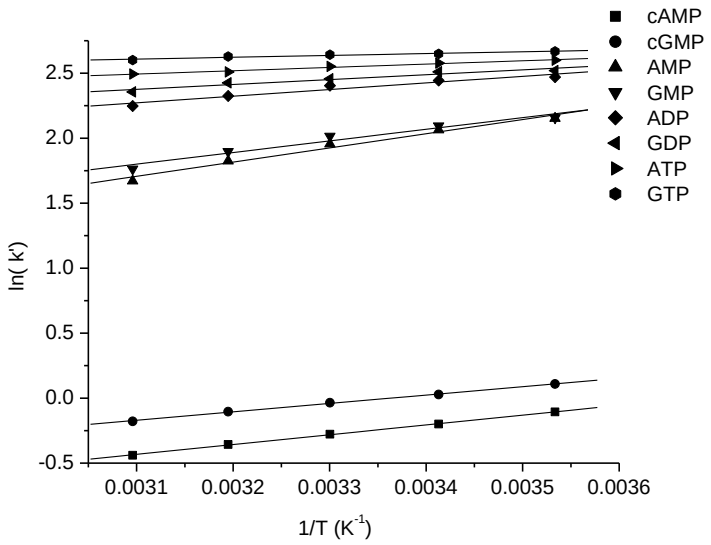


Figure 2.8 Dependence of logarithms of retention factors (ln k) on the inverse of temperature for nucleotides on the Frulic-N column.Conditions: Mobile phase: ACN/100 mM ammonium acetate pH = 6.6 (65/35,v/v); Flow rate: 1.0 ml/min, Detection: UV at 254 nm.

The effect of temperature on the separation of nucleotides was studied over a temperature range of 10 ºC to 50 ºC at 10 ºC intervals. High temperatures produced the expected decrease in retention of the analytes and narrow peak widths. The van't Hoff plots showed linear behavior within the studied temperature interval for the selected nucleotides. Linear dependencies indicate no

change of interaction mechanism within the measured temperature range. The thermodynamic data for nucleotides are summarized in Table 2.2. Partial molar enthalpies (ΔH_i) were calculated based on the slopes of the van't Hoff plots which were negative for nucleotides. This indicates transferring solutes from mobile phase to the stationary phases is exothermic and the association between these analytes on the stationary phase is more favorable.

Table 2.2 The thermodynamic parameters resulting from linear regression for nucleotides on the FRULIC-N column

Compound	Correlation Coefficient (R^2)	ΔH_i/ (kJ/mol)	ΔS_i/ (J/mol K)	ΔG (J/mol)
cAMP	0.9996	-6271	-15	-1878
cGMP	0.9989	-5373	-10	-2435
AMP	0.9894	-9089	-6.0	-7348
GMP	0.9809	-6553	3.0	-7427
ADP	0.9675	-4.240	14	-8281
GDP	0.9657	-3091	18	-8445
ATP	0.9910	-1467	24	-8624
GTP	0.9698	-1.175	26	-8820

Mobile phase: ACN/100mm Ammonium acetate pH = 6.6 (65/35,v/v), Flow rate;1.0 ml/min; Detection: UV at 254 nm.

Moreover, from Table 2.2, it was seen that the entropy values were negative for cAMP, cGMP, AMP, while they were positive for GMP, ADP, GDP, ATP and GTP. Clearly there is a greater entropic contribution for the GMP, ADP, GDP, ATP and GTP with the FRULIC-N column. Also, the entropy values of the selected analytes increased in the following order: cyclic nucleotides < nucleoside monophosphates < nucleoside diphosphates < nucleoside triphosphates. In fact the entropic contributions to the Gibbs free energy of transfer to the stationary phase increased with the number of phosphate groups. Clearly, the more phosphate moieties that are present on an analyte, the greater the hydration "shell". These highly ordered waters of hydration are released (desolvation) upon association with the stationary phase providing the observed increase in ΔS. Also, the degree of freedom may contribute for the increase in ΔS. Thus, it appears that the retention of the cyclic nucleotides is enthalphically driven while the nucleotide di and triphosphates are entropically driven.

2.5 Conclusions

In this study, a silica bonded cyclofructan 6 (FRULIC-N) column was evaluated for its ability to separate nucleotides in the HILIC mode. This column baseline separated 16 out of 19 nucleotides in a single run. Both isocratic and step gradient conditions were examined and the best separation of all anlytes was achieved with the step gradient condition. Because of the cyclofructan's ability to bind buffer cations, FRULIC-N has proven to be the only "neutral" HILIC phase

that can show a combination of ion interaction separations behavior as well as the more common HILIC modes of retention. Furthermore, unlike stationary phases with embedded charged groups, this effect is tunable with the FRULIC column. Also, a thermodynamic study showed that the retention of most nucleotides is entropically driven on this stationary phase. The mobile phases used are mass spectrometry compatible, thus this method could be directly applied to LC-MS techniques which could be useful for sensitive detection in biological matrixes. It is anticipated that this column will prove useful in further analyses of nucleotides in biological samples.

CHAPTER 3

SULFONATED CYCLOFRUCTAN 6 BASED STATIONARY PHASE FOR HYDROPHILIC INTERACTION CHROMATOGRAPHY

3.1 Abstract

A stationary phase composed of silica-bonded sulfonated cyclofructan 6 (SCF6) was synthesized and evaluated for hydrophilic interaction chromatography (HILIC). The separation of a large variety of polar compounds was evaluated on different versions of the stationary phase and compared to the same separations obtained with commercially available HILIC columns. The new columns successfully separates polar and hydrophilic compounds including beta blockers, xanthines, salicylic acid related compounds, nucleic acid bases, nucleosides, maltooligosaccharides, water soluble vitamins and amino acids. The separation conditions were optimized by changing the composition and pH of the mobile phase. The dependence of analyte retention on temperature was studied using van't Hoff plots. The newly synthesized stationary phase showed broad applicability for HILIC mode separations.

3.2 Introduction

The separation of hydrophilic compounds is of great importance in many fields including, pharmaceutical, clinical and biological research and forensic analysis. Reversed phase liquid chromatography (RPLC) sometimes plays a role in the separation of polar analytes, however it is not the best choice for the

separation of highly polar compounds because of their poor retention and selectivity.[15] In the early 1950s, a somewhat different approach for the separation of polar analytes was suggested. [142] The use of polar stationary phases and an organic mobile phase that also contained water was referred to as aqueous normal phase. From the 1970s to 1990s, several reports appeared on the effective separation of saccharides on bonded cyclodextrin stationary phases and aminopropyl silanized silica gel using either an acetonitrile (ACN) or acetone mobile phase containing small amounts of water.[5-9]In 1990, the term hydrophilic interaction chromatography (HILIC) was coined for these type of separations.[10] HILIC has been steadily gaining in popularity for the separation of hydrophilic polar analytes including a wide variety of carbohydrates,[5-7] peptides, [11,12] and pharmaceutical products.[13,14]

Some regarded HILIC as a special sub-type of normal phase liquid chromatography (NPLC) with an aqueous–organic mobile phase. In the HILIC mode, acetonitrile-water mixtures are the most commonly used mobile phases, with the water content typically varying from 1 to 40 %. In contrast to normal phase liquid chromatography, the use of water as the strong eluting solvent gives enhanced compatibility for mass spectroscopic detection and better dissolving power for polar solutes.[2,19,20,143]

The retention mechanism for HILIC has received considerable attention. Alpert suggested that the retention mechanism for HILIC is governed by a

partitioning mechanism arising from the preferential adsorption of water onto the polar stationary phase which results in a relatively higher water content in the stagnant liquid phase of the stationary phase support than in the mobile phase.[2,10,15] However, it was concluded that this phenomenon was obscure and some dipole-dipole interactions may be involved. Previously it was suggested and later supported that many polar analytes interact with the stationary phase via hydrogen bonding, dipole-dipole and in appropriate cases, charge-dipole interactions. [6,7,16,143] In particular hydrogen bonding between analytes and appropriate stationary phases are enhanced by acetonitrile – a poor hydrogen bonding solvent.

A variety of different types of stationary phases has been employed for HILIC separations. They can be categorized as bare silica, [20,21] amine, [9] amide, [36] Diol, [144] cyclodextrin, [6,7,16,39] zwitterionic phases such as sulfoalkylbetaine silica[145-147] and other polar chemically bonded stationary phases such as cyano and poly(succinimide) phases. [2,148] Although there are different varieties of HILIC columns that are commercially available, large number of papers appeared on conventional non modified silica stationary phases such as Betasil, [20-23] Hypersil, [24] Kromasil[25,26] and Atlantis.[27-31] However, bare silica columns might cause considerable problems such as irreproducible results, strong adsorption[2,25] and tailing peaks for some analytes such as carbohydrates[25] that might be due to interactions with ionized silinol groups and overloading effects. Therefore,

stationary phases that consist of functionalized molecules on silica supports have been receiving more attention.

Recently, HILIC columns packed with small particles (around 2 μm) have been successfully used in ultra high pressure liquid chromatography in order to achieve fast and efficient separations.[149] Un-derivatized 1.7 μm bridged ethylene hybrid organic silica (BEH) particles show improved chemical resistance when compare to un-derivatized silica for polar compounds in the HILIC mode. Fountain and co-workers studied the influence of mobile phase pH and stationary phase chemistry on retention and selectivity in HILIC mode separations using BEH stationary phases.[150] Nováková *et al.* evaluated that BEH and BEH amide stationary phases for the separation of polar basic analytes and showed that the BEH amide column provides sufficient selectivity for separation of pteridine derivatives.[151]

Fused-core particles are used in HILIC mode separations as an alternative to 2 μm particles (Halo HILIC or Kinetex HIlIC). Although these particles offer lower backpressure than 2 μm particles, they produce slightly lower efficiencies in columns of the same dimensions.[152] McCalley proposed the coupling of various HILIC fused-core columns in series in order to reach the highest possible efficiencies under pressures compatible with conventional instrumentation.[153] Several studies have been done in order to investigate kinetic and thermodynamic

performances on fused-core columns[152,154] and these two strategies were applied to achieve fast separation of various substrates and their metabolites.

There are large number of reports appeared on sugar bonded stationary phases in HILIC mode separations.[2,6,10,39] Sugar stationary phases that were prepared using the click chemistry approach were reported as effective materials for HILIC mode separations.[155-161] Other types of HILIC stationary phases consists of organic polymer materials such as both cation-exchange and anion-exchange styrene-divinylbenzene resins,[162,163] zwitterionic stationary phases such as sulfobetaine[145,164-166] and phosphorycholine bonded phases,[167] silica-based monolithic columns,[168-170] organic polymer HILIC monolithic columns[34,35] were also reported.

Although the family of HILIC stationary phases has continuously enlarged, researchers continue to investigate new HILIC stationary phases in order to broaden the applications of HILIC mode separations. The stationary phases based on sulfonated cyclofructan 6 (SCF6) are presented here. Cyclofructans (CF)s are cyclic oligosaccharides which consist of six or more β-(2→ 1) linked D-fructofuranose units. Each fructofuranose unit contains one primary hydroxyl group and two secondary hydroxyl groups making this molecule very hydrophilic.[42] Kawamura and Uchiyama first reported CFs produced by fermentation of inulin using an extracellular enzyme from a strain of *Bacillus circulans* OKUMZ31B.[43] There are a number of applications for CFs.

They have been used as bulk additives in various industrial formulations such as ink formulation agents, [45] as browning preventative agents, [46] as food additives.[47,171,172] Recently, Armstrong *et al.* first employed native and/or derivatized cyclofructans as chiral selectors for capillary electrophoresis(CE),[51] gas chromatography (GC)[52] and high performance liquid chromatography (HPLC).[53-55] Although native cyclofructans have limited capabilities as chiral selectors,[55] they showed exceptional selectivity for the separations of achiral polar analytes in HILIC mode.[58] However, to date and to our knowledge, there have been no published reports on HILIC separations using derivatized sulfonated CFs bonded to silica gel. In the present work, we outline the synthesis of sulfonated CF6 (SCF6), its linkage to silica gel and the separation of a large variety of polar compounds. Also the synthesized sulfonated cyclofructan 6 columns are compared to existing commercially available HILIC columns: ZIC-HILIC, Diol and Cyclobond I 2000.

3.3 Experimental

3.3.1 Materials

CFs were produced by fermentation and crystallization as previously reported.[42,86,87] CF6 was used for the present study due to its highly defined geometry. Daiso silica of 5 μm spherical diameter (100 Å, 440 m^2/g) was utilized. Acetonitrile (ACN) of HPLC grade was obtained from VWR (Sugarland, TX). Anhydrous N-N-dimethylformamide (DMF), anhydrous pyridine, anhydrous

toluene, sodium hydride, 1,3 propane sultone, 3-(triethoxysilyl)propyl isocyanate, ammonium acetate and all other analytes tested in this study were purchased from Sigma-Aldrich (St.Luis, MO). Water was purified by a Milli-Q-water purification system (Millipore, Billerica, MA). The ZIC HILIC column was obtained from Merck Sequent (Darmstadt, Germany) and Diol and Cyclobond I 2000 columns were obtained from Supelco (Bellefonte, PA). The native cyclofructan 6 column (Frulic-N) was obtained from AZYP (Arlington, TX).

3.3.2 Synthesis of sulfonated CF6 (SCF6) (Frulic-C)

In this work, sulfonated cyclofructan 6 (SCF6) was synthesized. CF6 was dried in the vacuum oven over night at 40 ºC. The reactions were carried out at room temperature under nitrogen. 1.00 g of CF6 was dissolved in 100 ml of anhydrous DMF and the mixture was stirred for about 30 minutes. Then, 0.25 g of anhydrous sodium hydride was slowly added. The resultant slurry was then stirred for about four hours. After that, 1.26 g of 1,3 propane sultone in 5.0 ml of anhydrous DMF was added dropwise to the reaction mixture (30 minutes). Next, the mixture was stirred for 12 hours. Finally, methanol was slowly added to the flask (0.0 º C) to quench unreacted sodium hydride. The sodium salt of SCF6 was recovered by suction filtration and drying under vacuum oven. The molecular mass distribution and degree of sulfonate substitution of the product were determined by electrospray ionization mass spectrometry (ESI-MS). The mass

spectrum showed that SCF6 is a mixture containing 1-6 sulfonate groups with the average degree of substitution of two (SCF6-L). Sequential addition of sodium hydride and 1,3 propane sultone afforded the highly substituted SCF6 containing 3-11 sulfonate groups with the average degree of substitution of six (SCF6-H) with the 90 % yield.

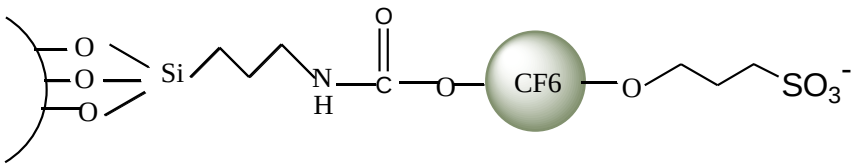


Figure 3.1 Structure of chemically bonded sulfonated cyclofructan 6 stationary phase.

3.3.3 Synthesis of SCF6 based stationary phase

SCF6 was chemically bonded to silica gel via carbamate linkage.[55] 1.88 g of dried SCF6 was dissolved in 20.0 ml of anhydrous pyridine at 75 ºC for two hours. A solution of 1.6 ml of 3-(triethoxysilyl)propyl isocyanate in 10.0 ml of pyridine was added dropwise to the above solution under dry nitrogen. The mixture was then heated to 100.0 ºC for 3.5 hours. Simultaneously, a slurry of 3.00 g of dried silica in 60 ml of anhydrous toluene was refluxed and ≈ 10.0 ml of toluene with residual water was azeotropically removed using a Dean-Stark trap. After reaction mixtures were cooled to room temperature, SCF6 reaction mixture was added to the silica-toluene slurry. The combined mixture was heated to reflux

at 110 ºC overnight. The final mixture was cooled, filtered through sintered glass crucible and washed with toluene, pyridine, DMF, methanol and water mixture, acetone and dichloromethane. Finally, the mixture was dried in the vacuum oven overnight (40.0 ºC). Fig.3.1 represents the structures of newly prepared stationary phases. Table 3.1 shows elemental analysis data for the SCF6 stationary phases. The synthesized stationary phase was slurry packed into 25 cm × 0.46 cm (i.d) stainless steel columns.

Table 3.1 Elemental analysis data for SCF6 columns

SCF6 – bonded to silica gel	Degrees of substitution	C %	H %	N %	S %
SCF6-L	1-6	10.6	1.8	1.3	0.7
SCF6-H	3-11	11.1	2.2	1.1	2.8

3.3.4 HPLC method

Agilent 1200 and Agilent 1050 HPLC (Agilent Technologies, Palo Alto, CA, USA) were used in this study. The 1200 HPLC consists of a diode array detector and a temperature controlled column chamber, auto sampler and quaternary pump. All separations were carried out at room temperature (˜20 ºC) unless stated otherwise. For all HPLC experiments, the injection volume was 5 µL and flow rate was 1.0 ml/min in isocratic mode. The UV wavelengths of 195, 210, 254 and 280 nm were employed. A Shimadzu RID-10A refractive index detector was used to detect sugars.

The separation conditions were optimized by varying mobile phase composition and pH. The first investigation was carried out by varying the mobile phase composition in the range of 60-90% ACN (amount and pH of ammonium acetate buffer kept constant (20 mM and pH 4.0). The 20 mM ammonium acetate solution was prepared by dissolving 1.56 g of ammonium acetate in 200.0 ml of purified water and transferred into a 1000.0 ml of volumetric flask. The stock solution was diluted to just below the mark with water. The desired pH was adjusted by using 99.7 % acetic acid. The ammonium acetate buffer/salt concentration and pH were changed from 0 to 20 mM and 3.7-6.5, respectively. All analytes were dissolved in ACN / water or ACN / water / methanol mixtures or appropriate mobile phase. The mobile phase was degassed by ultrasonication under vacuum for five minutes. Each sample was analysed in duplicate. The dead time t_0 was determined by injecting toluene as void volume marker. The peak efficiency was determined by injecting uracil and cytosine as standards in ACN/20 mM ammonium acetate buffer at pH 4.0 (90/10(v/v)). Table 3.2 shows the data for the peak efficiency for all tested columns.

Three commercial columns were selected for the comparison purpose on the basis of different polar stationary phases and applicability for the separation of polar analytes. All the columns have similar dimensions (250 mm × 4.6 mm). ZIC-HILIC carries covalently bonded, zwitterionic sulfobetain type functional groups whereas Cyclobond I 2000 and Diol columns have hydroxyl functional

groups.[2] Diol column is very close to bare silica column when comparing overall polarity.

The separation of a large variety of polar compounds has been evaluated on this novel SCF6 stationary phase including beta blockers, purine related compounds, salicylic acid and related compounds, nucleic acid bases and nucleosides, maltooligosaccharides, water soluble vitamins and amino acids. The column showed no significant deterioration after more than 1000 injections and it provided the same retention for the same analytes in the same mobile phase. It indicates that the SCF6 columns are stable and the analysis of polar analytes using SCF6 columns is reproducible. The effect of column temperature was investigated for the retention of polar compounds. The column temperature was changed from 10.0 ºC to 50.0 ºC at 10 ºC intervals in the mobile phase of ACN/20 mM ammonium acetate buffer at pH 4.0 (90/10(v/v)).

3.4 Results and Discussion

Table 3.1 shows the elemental analysis data for the new stationary phases. SCF6-H has a slightly higher carbon loading than SCF6-L. Also, SCF6-H has the higher average degree of substitution of sulfonate groups. Table 3.2 shows the data for column efficiency as determined with a mobile phase containing ACN and 20 mM ammonium acetate buffer at pH 4.0 (90/10(v/v)). Uracil and cytosine, the test analytes were used because they had been used previously to measure efficiency of the commercial ZIC-HILIC column. The efficiencies of the

synthesized stationary phases are competitive with the commercial columns and the SCF6-H stationary phase has higher efficiencies than SCF6-L with the same mobile phase.

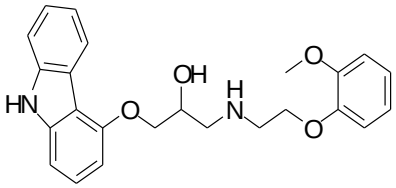
Table 3.2 Column efficiency data for all tested columns.

	Column efficiency(plates/m)				
Compound	SCF6-H	SCF6-L	ZIC-HILIC	Diol	Cyclobond I 2000
Uracil	65000	54000	54000	58000	72000
Cytosine	64000	52000	48000	56000	62000

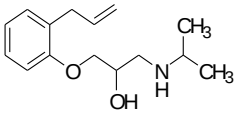
3.4.1 Separation of polar mixtures

3.4.1.1 Separation of beta blockers

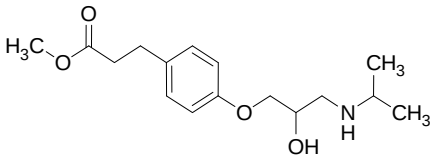
In this study, ten beta blockers were selected and used as probe analytes to evaluate the HILIC stationary phases. Figure 3.2 gives the structures of these compounds and shows that they are hydroxylamine-containing compounds and contain at least one aromatic ring. Figure . 3.3 shows the chromatograms of beta blockers on all columns using a mobile phase containing ACN and 20 mM ammonium acetate at pH 4.0 (70/30(v/v)). Mobile phases containing only ACN/water produced poor peak shapes, however the presence of ammonium acetate in the mobile phase resulted in more symmetrical peaks of narrower peak widths. The separation window of beta blockers on SCF6-H is around 35 minutes which is slightly greater than for the SCF6-L stationary phase.



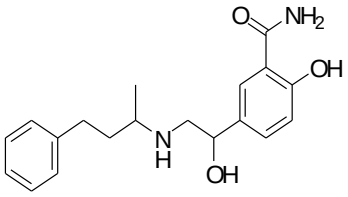
(1) Carvedilol



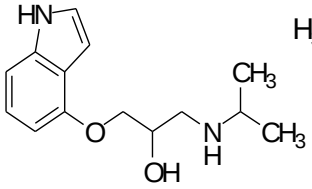
(2) Alprenolol



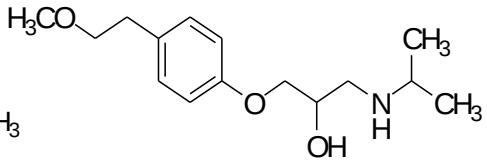
(3) Esmolol



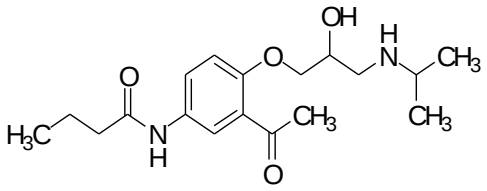
(4) Labetalol



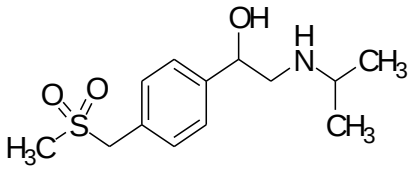
(5) Pindolol



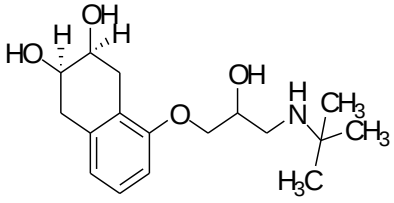
(6) Metoprolol



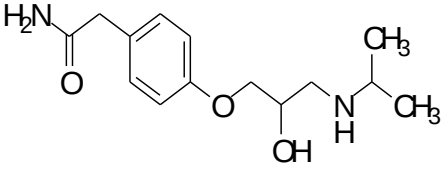
(7) Acebutolol



(8) Sotalol



(9) Nadolol



(10) Atenolol

Figure 3.2 Structures of beta blocker

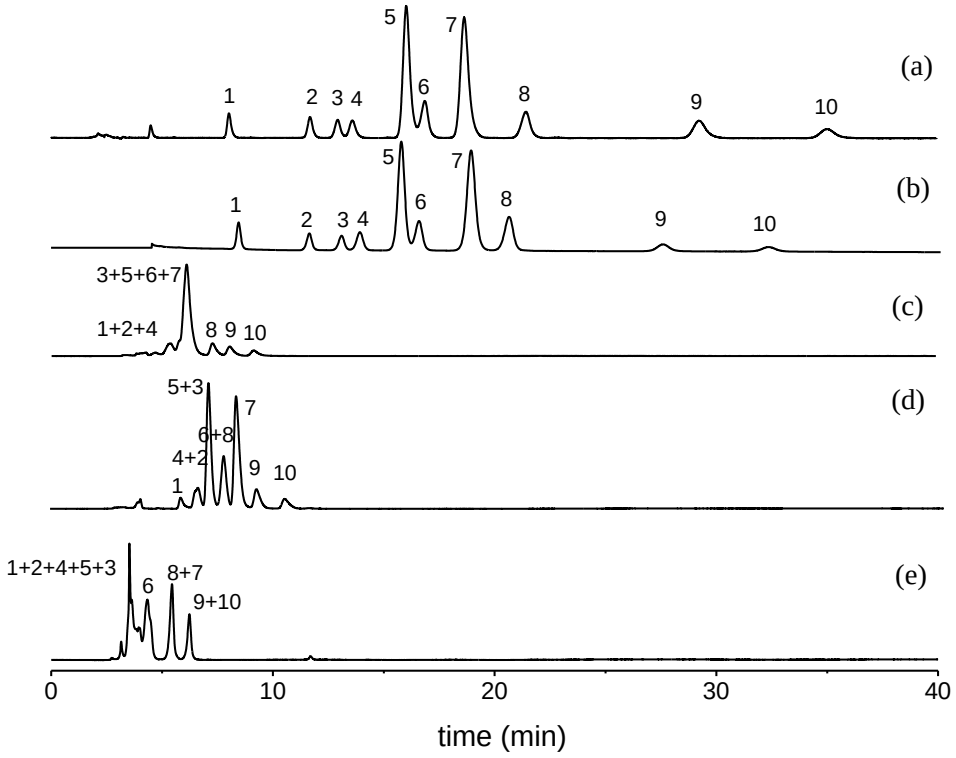


Figure 3.3 Separation of beta blockers

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (70/30,v/v), Flow rate : 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Carvedilol, (2) Alprenolol, (3) Esmolol, (4) Labetalol, (5) Pindolol, (6) Metoprolol, (7) Acebutolol, (8) Sotalol, (9) Nadolol, (10) Atenolol.

All the compounds remain in their protonated form with this mobile phase and longer retention on SCF6-H indicates that there are strong interactions of protonated analytes with the higher number of sulfonate groups on the stationary phase. All these analytes were baseline separated on both sulfonated cyclofructans columns except pindolol and metoproplol which were partially separated (α = 1.06 and Rs = 1.0 on SCF6-H and α = 1.04 and Rs = 0.9). As shown in figure 3.3, the separation windows for all commercial coloum are below 15 minutes and they exhibited severe coelution with the same mobile phase. In order to obtain a more fair comparison, mobile phase conditions were optimized for the commercial columns. ACN/20 mM ammonium acetate at pH 4.0 (90/10(v/v)) was the optimized mobile phase condition for these columns. Figure 3.4 shows the separation of beta blockers on all the columns using each column with their optimized mobile phase. This clearly indicates that both SCF6-H and SCF6-L columns afforded better selectivity when compared to the commercial columns at their optimized conditions. Optimum conditions were selected in terms of both selectivity and resolution. Both SCF6 columns produced the same retention orders while the other stationary phases show different elution orders due to their different functionalities. Among the five tested columns, sulfonated cyclofructans ones were the most effective columns in terms of selectivity, resolution and having shorter analysis times.

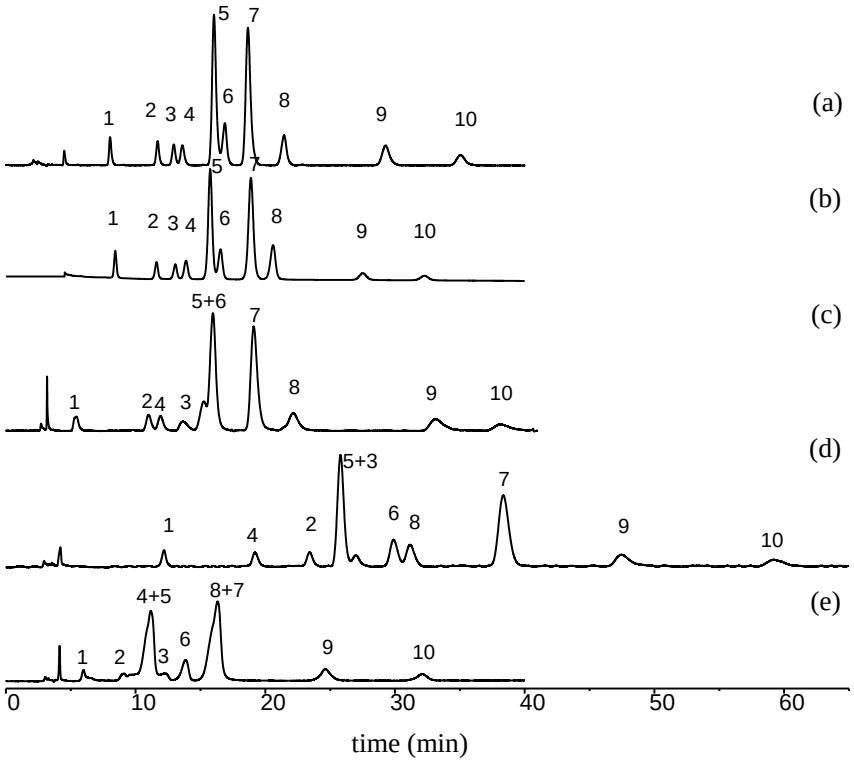


Figure 3.4 Separation of beta blockers

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase for (a) and (b): ACN/20 mM Ammonium acetate pH = 4.0 (70/30,v/v), Mobile phase for (c), (d) and (e): ACN/20 mM Ammonium acetate pH = 4.0 (90/10,v/v) Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Carvedilol, (2) Alprenolol, (3) Esmolol, (4) Labetalol, (5) Pindolol, (6) Metoprolol, (7) Acebutolol, (8) Sotalol, (9) Nadolol, (10) Atenolol.

The Diol column was the most retentive and it also is an effective column for the separation of beta blockers. Pindolol and esmolol were partially separated (α = 1.05 and Rs = 0.9) on the Diol column. The elution order of beta blockers on ZIC-HILIC is comparable with the SCF6 stationary phases. However, the retention order for esmolol and labetalol was reversed on the ZIC-HILIC when compared to the SCF6 columns. Although the ZIC-HILIC has sulfonate groups at the end of the alkyl chains, they also have an embedded quaternary ammonium group. This gives a dual possibility for the interaction of analytes. The Cyclobond I 2000 column did not perform as well for the separation of beta blockers. This was due to the partial separation of two analyte pairs (labetalol and pindolol, sotalol and acebutolol).

3.4.1.2 Separation of xanthenes

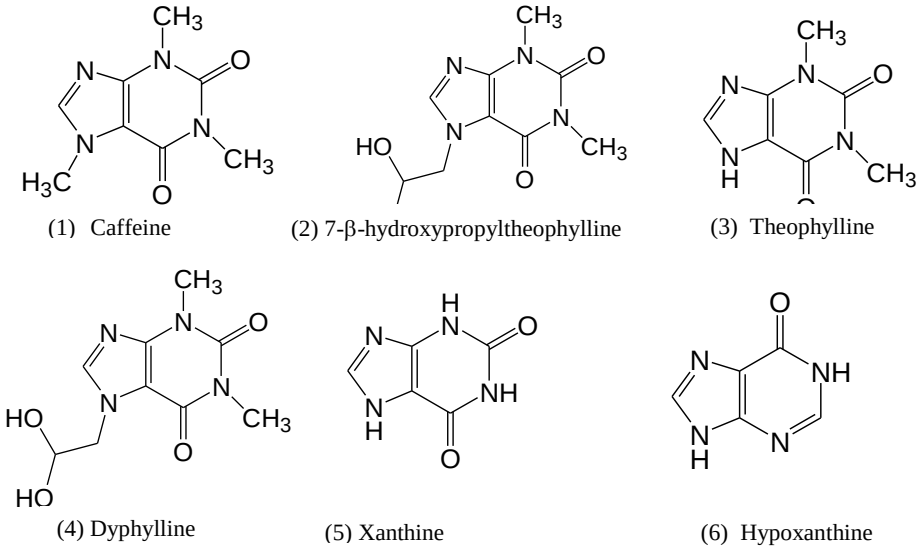


Figure 3.5 Stuctures of xanthines

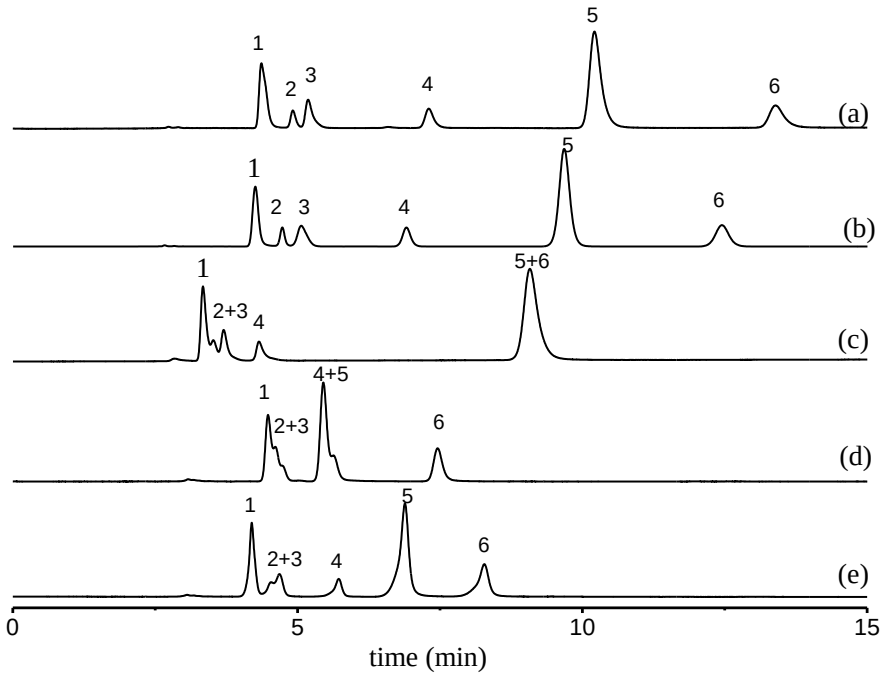


Figure 3.6 Separation of xanthenes

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (90/10,v/v), Flow rate : 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Caffeine, (2) 7-β-hydroxypropyltheophylline, (3) Theophylline, (4) Dyphylline,(5) Xanthine, (6) Hypoxanthine.

Figure. 3.5 shows the structures of selected xanthine compounds and Figure. 3.6 shows the separation of these analytes on all columns. All analytes were baseline separated within 15 minutes on both SCF6 stationary phases. As previously

reported, xanthines remain in their neutral form under aqueous-organic mobile phase condition.[173] As shown in Figure. 3.6, SCF6 columns exhibited higher selectivity and resolution when compared to the commercial columns. There were coeluting peaks observed for commercial columns, and the Cyclobond I 2000 column showed slightly fronting peaks for xanthine and hypoxanthine. However, the Cyclobond I 2000 column showed fairly good separation selectivity when compared to the Diol and ZIC HILIC columns The same retention order was observed for all columns.

3.4.1.3 Separation of salicylic acid and related acidic compounds

A group of small polar acidic compounds (Figure. 3.7) was examined since it is known that they are poorly retained in RPLC.[37,174] Shorter retention times were observed for these compounds when compared to the other classes of compounds tested.

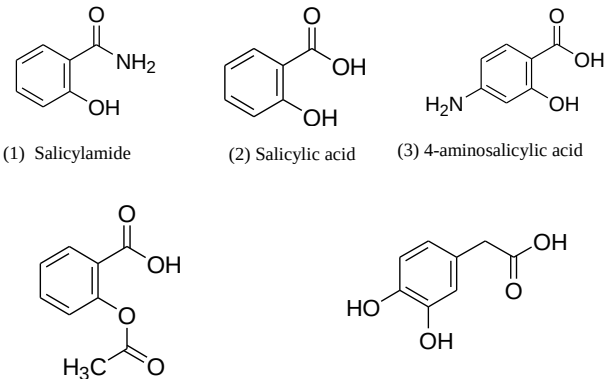


Figure 3.7 Structures of salicylic acid and related acidic compounds

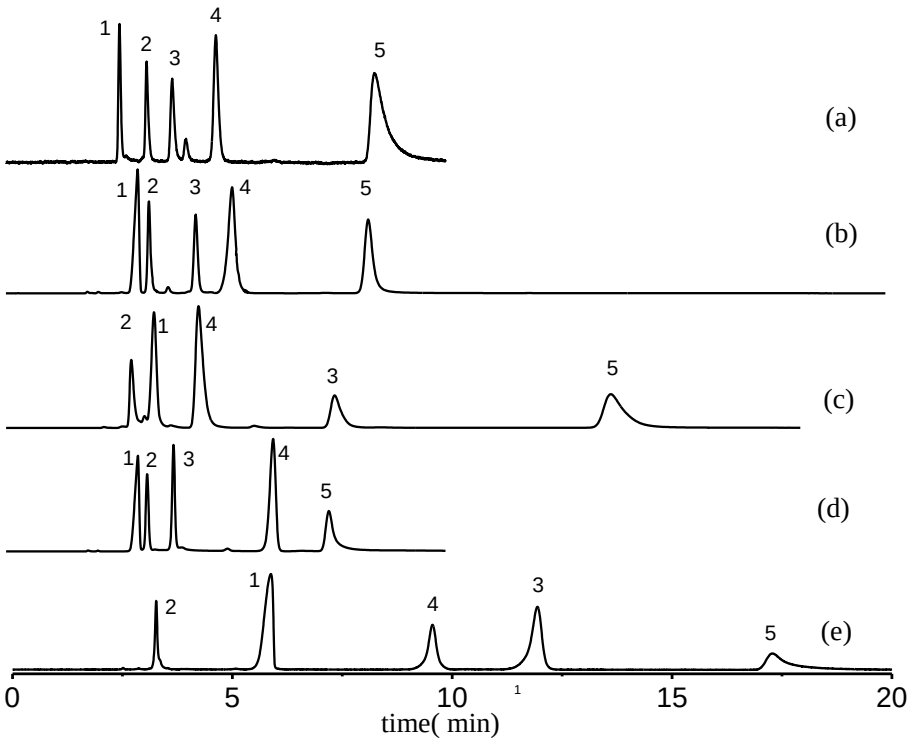


Figure 3.8 Separation of salicylic acid and related acidic compounds

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (90/10,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Salicylamide, (2) Salicylic acid, (3) 4-aminosalicylic acid, (4) Acetylsalicylic acid, (5) 3,4-dihydroxyphenylacetic acid.

This group of compounds was well separated on all the tested columns (Figure. 3.8). Good peak shapes and efficiencies were observed for all the analytes except for 3,4-dihydroxyphenylacetic acid on SCF6 columns. 3,4-dihydroxyphenylacetic acid had greater retention and some tailing on all the columns. However, a severe tailing peak was observed for this particular compound on SCF6-H when compared to the SCF6-L. Among all the tested columns, the shortest analysis time was observed for the Diol column. Cyclobond I 2000 column exhibited both fronting and tailing peaks.

3.4.1.4 Separation of nucleic acid bases and nucleosides

Figure. 3.9 shows the structures of nucleic acid bases and nucleosides used as test analytes. As shown in Figure. 3.10, the separation window of these ten analytes is around 37 minutes on the SCF6-H column that is slightly greater than that for the SCF6-L column. In this case, the higher number of sulfonate groups could attribute to the stronger interaction between these analytes and the stationary phase (as was the case for beta blockers). Most of the analytes were baseline separated on the new synthesized columns except for adenine and adenosine. They were partially separated on both SCF6-H and SCF-L columns (α = 1.04, Rs = 0.7 for SCF6-H and α = 1.03, Rs =0.5 for SCF6-L). The opposite elution order also was observed for adenine and adenosine on SCF6-H and SCF6-L. Among all the columns examined, both SCF6 and ZIC-HILIC columns

performed best in separating nucleic acid bases and nucleosides. The Diol and Cyclobond I 2000 columns displayed short retention times and poor selectivities compared to the SCF6 columns and ZIC HILIC column.

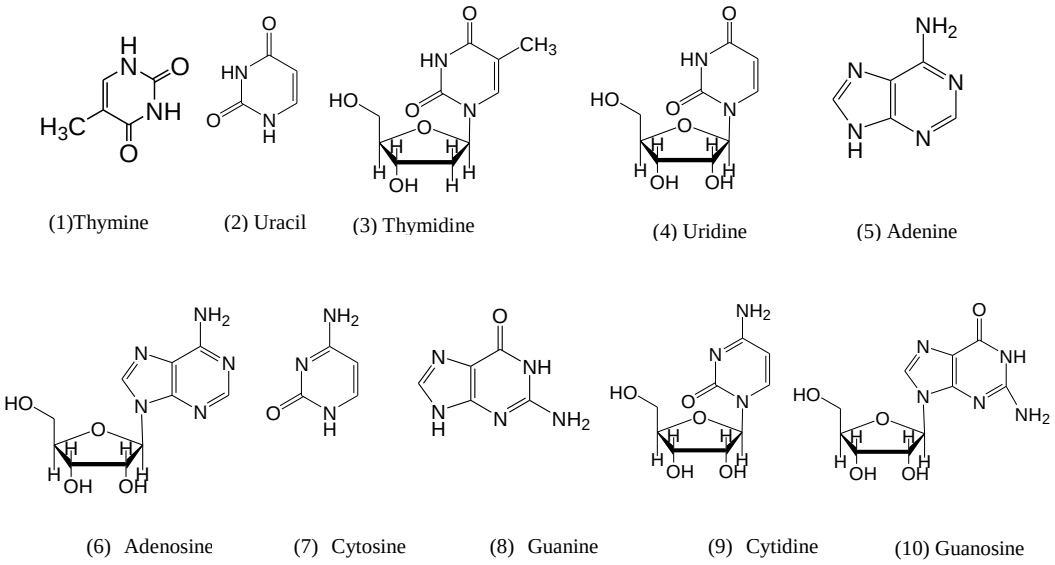


Figure 3.9 Structures of nucleic acid bases and nucleosides.

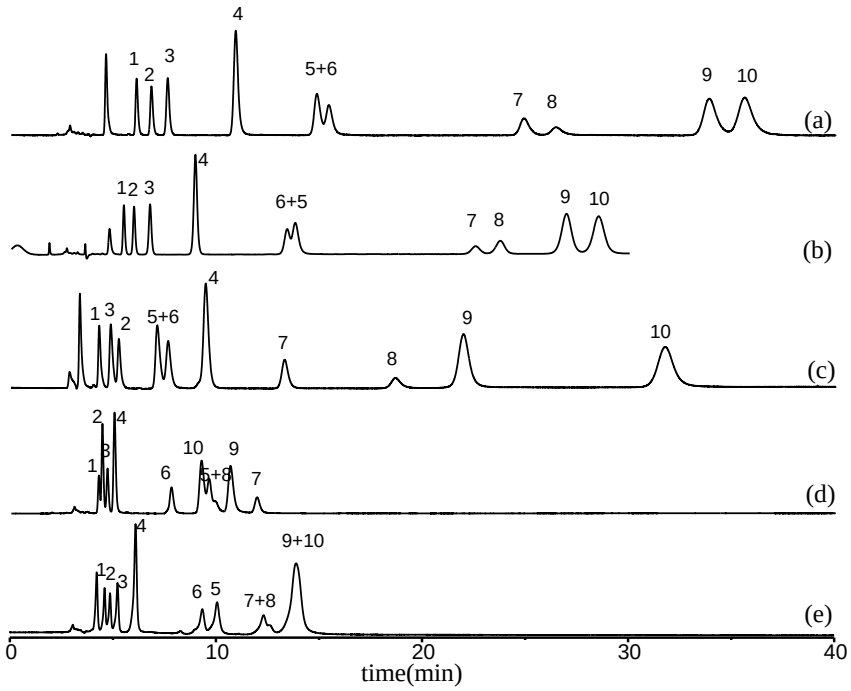


Figure 3.10 Separation of nucleic acid bases and nucleosides

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (90/10,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Thymine, (2) Uracil, (3) Thymidine, (4) Uridine, (5) Adenine, (6) Adenosine, (7) Cytosine, (8) Guanine, (9) Cytidine, (10) Guanosine.

3.4.1.5 Separation of maltooligosaccharides

A homologous series of six native maltooligosaccharides (two to seven glucose units) ((Figgure. 3.11) was separated. As previously reported, native

cyclodextrin columns provide excellent separations of oligosaccharides due to the hydrogen bonding interactions between the hydroxyl groups on the oligosaccharides and the cyclodextrin stationary phase.[6,7] Acetonitrile and water mobile phase (65/35(v/v)) provided good peak shapes and selectivity on the SCF6 columns.

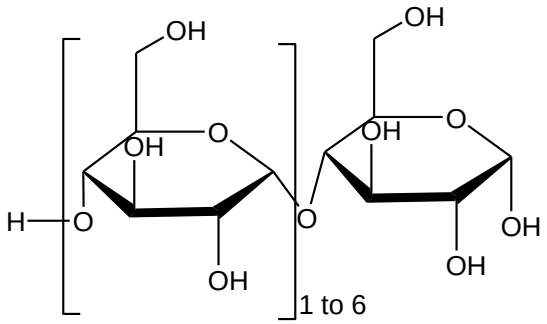


Figure 3.11 Structures of maltooligosaccharides

Figure. 3.12 shows the chromatograms for the separation of these analytes on all the tested columns using a RI detector. Note that both SCF6-H and SCF6-L produced baseline separation of these analytes. The separation window of maltooligosaccharides on SCF6-H is around ten minutes while it is around 15 minutes for SCF6-L. The greater retention was observed on SCF6-L due to the higher number of available hydroxyl groups which provided the additional hydrogen bond interactions with the analytes. Further, retention times increased with the number of available hydroxyl groups on the analytes. The retention order of these analytes indicates that hydrogen bond interactions are dominant in the retention of the analyte. The Cyclobond I 2000 also showed excellent selectivity.

There was no separation of these analytes observed on the Diol column. Formation of double peaks on the ZIC-HILIC column indicates that anomer formation and detection occurred. Split peaks due to anomer formation also could be observed on the SCF6 columns when the mobile phase composition was more than 85 % ACN. Among all the tested columns, the SCF6-H and the Cyclobond I 2000 columns produced the best separation.

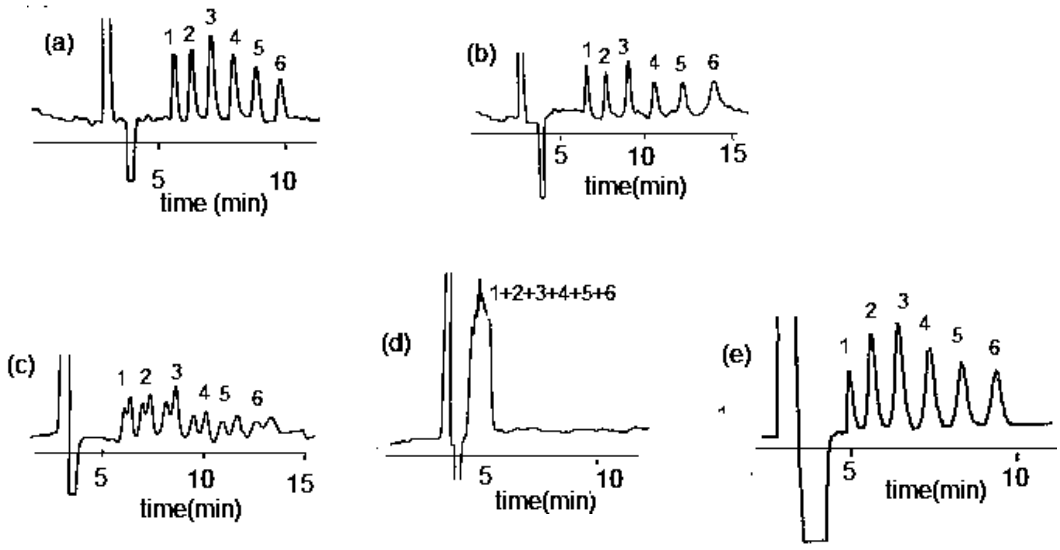


Figure 3.12 Separation of maltooligosaccharides

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/water(65/35(v/v); Flow rate: 1.0 ml/min, RI detection. Compounds: (1) Maltose, (2) Maltotriose, (3) Maltotetraose, (4) Maltopentaose, (5) Maltohexaose, (6) Maltohepatose.

3.4.1.6 Separation of water soluble vitamins

Initially, six water soluble vitamins were selected as test analytes. However, thiamine was strongly adsorbed onto the SCF6-H and SCF6-L stationary phases and did not elute even after 90 min. It carries a positive charge and therefore can interact strongly with the negatively charged sulfonate groups. Figure. 3.13 shows the selected water soluble vitamins.

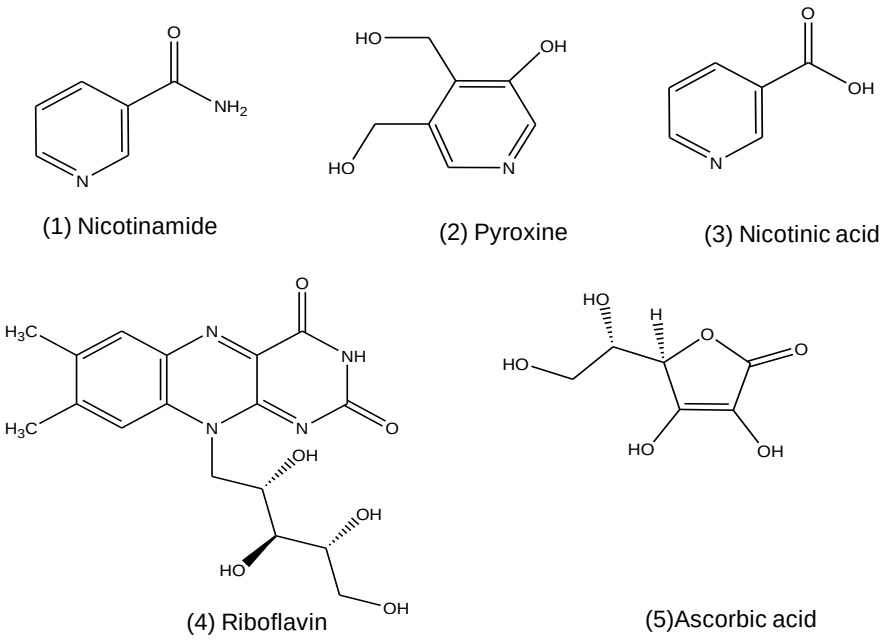


Figure 3.13Structures of water soluble vitamins

Ascorbic acid was not stable in water hence its stock solutions had to be prepared daily. The optimum mobile phase condition was ACN/20 mM ammonium acetate

solution (90/10 (v/v)) at pH 5.7 on the basis of better peak shapes and selectivity. Figure. 3.14 shows the chromatograms of water soluble vitamins using all five columns. All analytes were baseline separated while riboflavin and ascorbic acid were nearly baseline separated on SCF6-H column (α = 1.08 and Rs =1.2). Under the same mobile phase condition, the SCF6-L was less successful in separating these types of analytes, since a coeluting peak was observed. The main difference between the SCF6-H and SCF6-L is the number of sulfonate groups on the stationary phase. In this case, the difference in selectivity depends only on the nature of the stationary phase. SCF6-H was the most retentive column and effective column for this type of analytes. The Diol column also was effective in separating these analytes. All the analytes were separated on this column under the same mobile phase in a short time (around 15 minutes). Riboflavin and ascorbic acid were coeluted on SCF6-L, ZIC-HILIC and Cyclobond I 2000 columns.

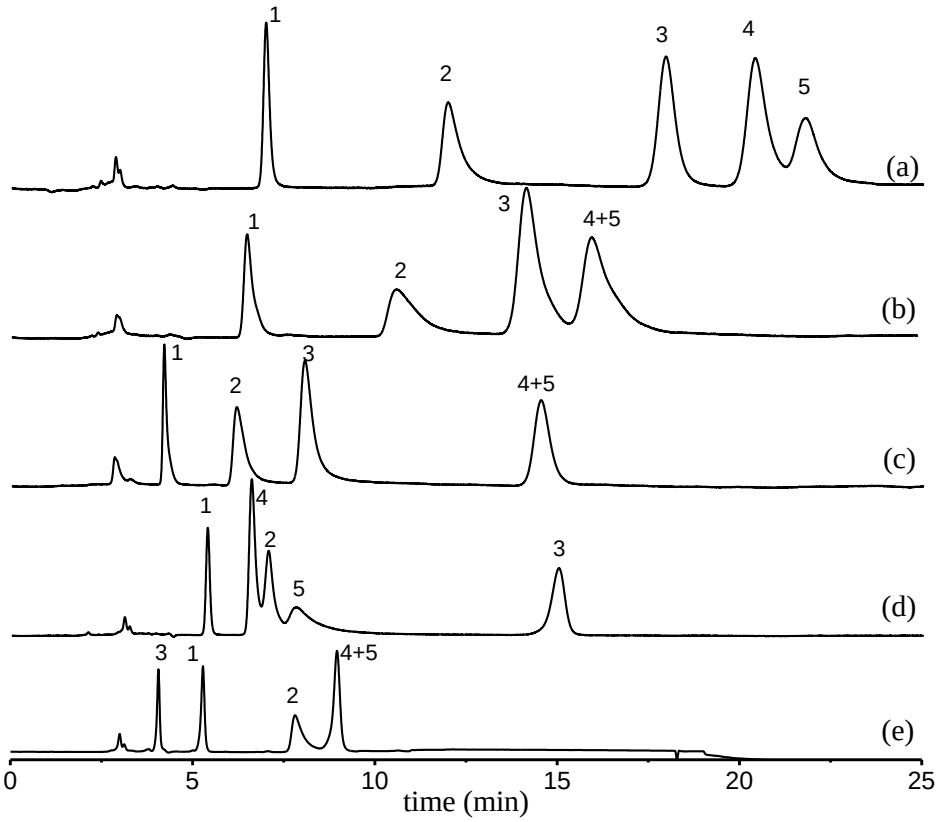


Figure 3.14 Separation of water soluble vitamins

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 5.7 (90/10,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Nicotinamide, (2) Pyridoxine, (3) Nicotinic acid, (4) Riboflavin, (5) Ascorbic acid.

3.4.1.7 Separation of amino acids

In this work, fifteen standard amino acids ((Figure. 3.15) that did not have basic and acidic side chains were selected to evaluate all columns. Figure. 3.16 shows the chromatograms for the separation of these native amino acids. There were observed three coeluted peaks for both SCF6-H and SCF-L columns. Methionine and tyrosine, proline and cystine and serine, glycine and glutamine were coeluted on SCF6-H column. However, isoleucine was baseline separated on the SCF6-H column, while it was coeluted with methionine and tyrosine on SCF6-L column. Hence, the selectivity was improved for the SCF6-H column compared to the SCF6-L. Poor selectivities and poor peak shapes were observed for all other commercial columns. ZIC HILIC provided relatively better peak shapes when compared to the Diol and Cyclobond I 2000 columns. It was also the least retentive column for these analytes. The Cyclobond I 2000 column gave the worst peak shapes for amino acids among all the tested columns.

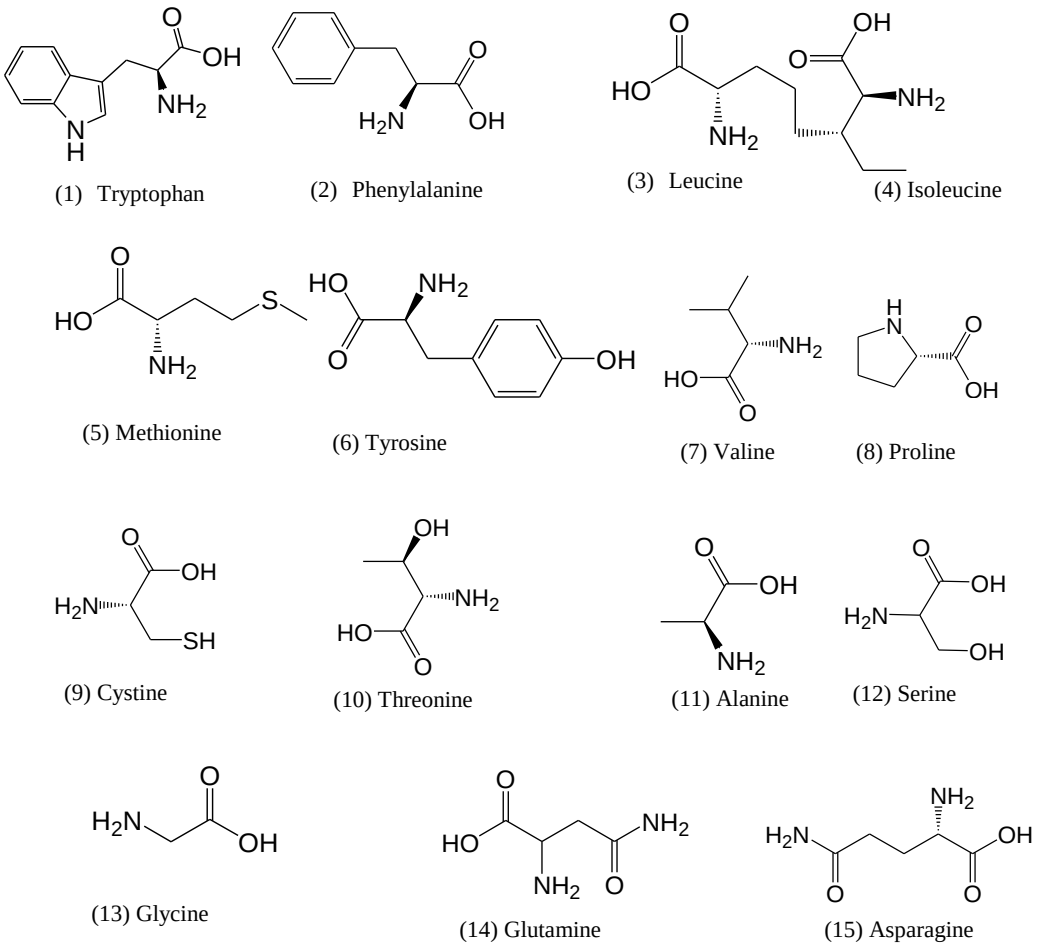


Figure 3.15 Structures of amino acids

A negative peak was observed within the analysis window for all columns. It was due to the slight mismatch of the injection solvent and the eluent. The peaks were generally quite broad when compared to the other classes of compounds tested. Much higher concentrations of these samples were used to detect these amino acids at 210 nm and this likely contributed to the band broadening. performed

substantially better. SCF6-H constantly produced higher selectivity among the tested columns.

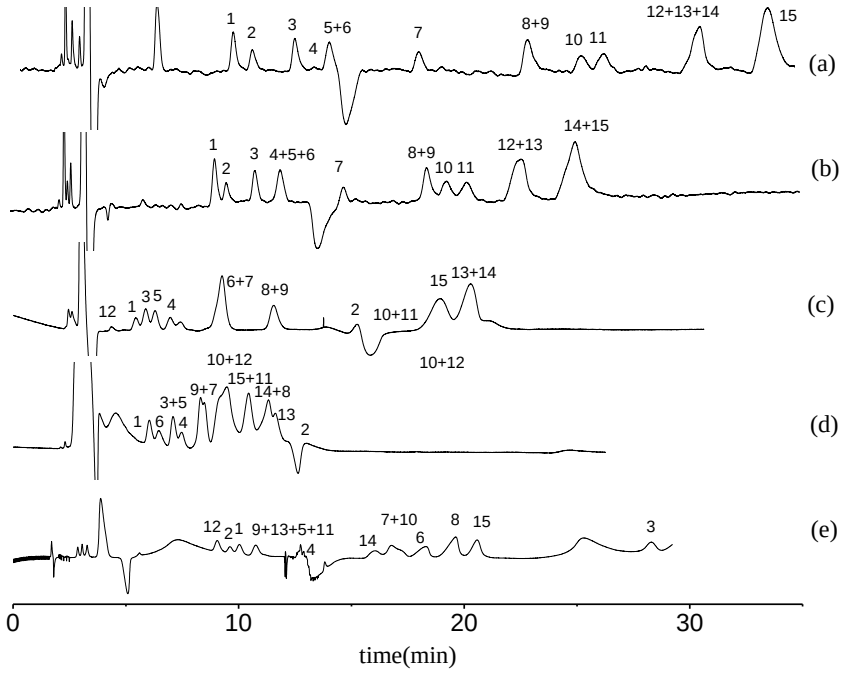


Figure 3.16 Separation of amino acids

(a) SCF6-H column, (b) SCF6-L column, (c) ZIC-HILIC column, (d) Diol column, (e) Cyclobond I 2000 column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (80/20,v/v), Flow rate: 1.0 ml/min, Detection: UV at 210 nm. Compounds:(1) Tryptophan, (2) Phenylalanine, (3) Leucine, (4) Isoleucine, (5) Methionine, (6) Tyrosine, (7) Valine, (8) Proline, (9) Cystine, (10) Threonine, (11) Alanine, (12) Serine, (13) Glycine, (14) Glutamine, (15) Asparagine.

When compared to the commercial columns, both SCF6-L and SCF6-H. Attempts have been made to separate five amino acids which have basic and acidic groups separately. (results not shown) However, Lysine was observed to strongly retain on both sulfonated columns even after 90 min. It is positively charged at neutral pH and there is a possibility for the strong electrostatic attraction with the sulfonate groups on these stationary phases.

3.4.2 Impact of mobile phase variables on retention and selectivity

3.4.2.1 Effects of organic modifier

There are numerous reports have been published in order to investigate mobile phase variables such as the type of protic modifier, the amount of protic modifier, the amount of salt and the temperature as well as stationary phase variables such as different ligand surface density to obtain improved selectivity and efficiency.[175,176] In this study, only ACN was investigated as the organic modifier, as it is the most commonly used solvent in HILIC. ACN cannot effectively compete with polar active sites on the surface of the stationary phase and on the polar analytes. Hence, analytes can be sufficiently retained on the stationary phase to achieve a separation. Further, ACN is miscible in all proportions with water and ammonium acetate.

When the mobile phase composition was changed from 60-95 % ACN in 20 mM ammonium acetate buffer at pH 4.0, broad peak shapes or even irreversible retention of most of the analytes occurred at the higher ACN

concentrations. This may be due to the solubility limitations of many of the analytes in the ACN rich mobile phase. Plots of the retention factors (k) versus ACN content in the mobile phase for the three representative xanthines on the SCF6 stationary phases are shown in Figure 3.17. These analytes elute near the dead volume at acetonitrile concentrations less than 60-70 % (by volume). Their retention increases dramatically at higher ACN concentrations as is usual for HILIC separations. The amount of ACN in the mobile phase was the factor that had the greatest influence on all analyte retention. Similar behavior was observed for all the analytes tested. (results not shown).

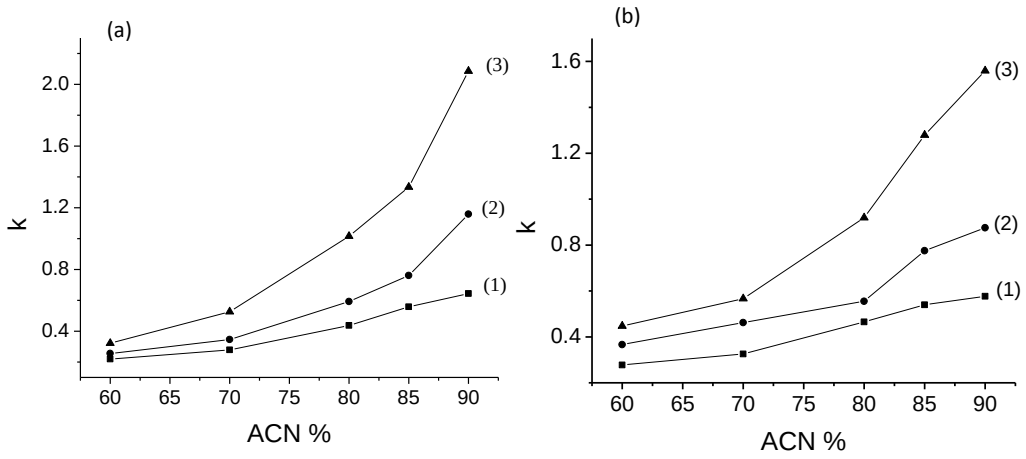


Figure 3.17 The effect of ACN % on the retention factors.

(a) SCF6-H column, (b) SCF6-L column, Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0, Flow rate: 1.0 ml/min, Detection: UV at 254 nm.

Compounds: (1) Caffeine, (2) Theophylline, (3) Dyphylline

3.4.2.2 Effects of buffer concentration

Ammonium acetate is by far the most commonly used HILIC buffer due to its high solubility in the organic modifier and its compatibility with MS detection. The ammonium acetate buffer concentration was changed from 0 to 20 mM at pH 4.0. Figure 3.18 shows plots of retention factors (k) versus ammonium acetate concentration in the mobile phase for the four representative beta blockers on the SCF6 columns. (ACN content was kept constant).

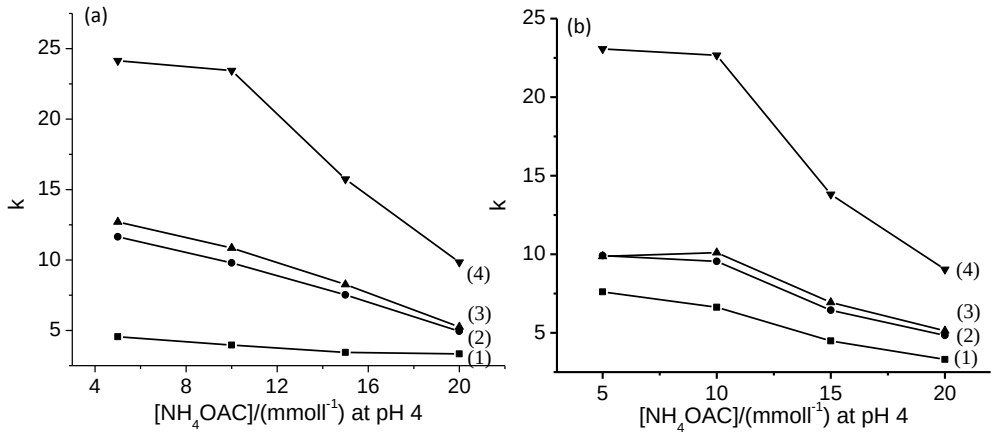


Figure 3.18 The effect of ammonium acetate buffer on the retention factors. (a) SCF6-H column, (b) SCF6-L column. Conditions: Mobile phase: ACN/Ammonium acetate pH = 4.0 (70/30,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds: (1) Alprenolol, (2) Esmolol, (3) Metoprolol, (4) Nadolol.

A reduction in the retention factors was observed on both SCF6 columns as the ammonium acetate concentration was increased from 5.0 mM to 20.0 mM. Poor

peak shapes and longer retention were observed for ionizable analytes when no buffer was used. Also, sharper peaks were observed when increasing the buffer concentration.

3.4.2.3 Effects of buffer pH

The effect of mobile-phase pH on the ionization of analytes is an important factor in analyte retention. Since the mobile phase containing ammonium acetate continuously produced sharper peak shapes for ionizable compounds (but not for the maltooligosaccharides), the pH of the ammonium acetate aqueous solution was adjusted from 3.7 - 6.5 to investigate its effect (both ACN content and ammonium acetate concentrations were fixed). Table 3.3 shows the effect of pH on the retention parameters of beta blockers using the SCF6-H column. Retention factors increased when changing the buffer pH in the working range. However, this did not improve the selectivity for many analytes. Alprenolol can be taken as example. It shows a selectivity (α) of 1.67 at pH 4.0 and an α of 1.58 at pH 6.5. As a general trend, the selectivity and resolution improved somewhat at pH 4.0. The selectivity and resolution were not greatly affected for the acid compounds, the xanthines or the nucleic acid bases and nucleosides.

Table 3.3 The effect of ammonium acetate pH on retention parameters for beta blockers using the SCF6-H column

Compound	pH 3.7		pH 4.0		pH 5.0		pH 6.5	
	α	Rs	α	Rs	α	Rs	α	Rs
Carvedilol	-	-	-	-	-	-	-	-
Alprenolol	1.57	4.9	1.67	6.4	1.78	4.2	1.58	1.6
Esmolol	1.15	2.1	1.25	2.5	1.0	2.3	1.14	1.3
Labetalol	1.08	1.1	1.05	1.5	0.92	1.3	1.10	1.4
Pindolol	1.09	1.5	1.13	1.5	0.94	1.2	1.21	1.3
Metoprolol	1.11	1.0	1.09	1.0	1.11	1.0	1.03	1.0
Acebutolol	1.60	3.0	1.70	2.7	1.50	2.7	1.83	2.6
Sotalol	1.30	1.5	1.30	4.0	1.27	3.8	1.31	3.7
Nadolol	6.60	6.0	7.01	6.0	6.83	5.9	4.61	4.8
Atenolol	4.00	4.0	5.02	5.1	4.82	5.0	4.05	3.8

a) Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (70/30,v/v), Flow rate: 1.0 ml/min, Detection: UV at 254 nm.

3.4.2.4 Thermodynamic study

The effect of column temperature on analyte retention was investigated since it has been recognized as an important parameter in HILIC mode.[37,177,178] It is generally accepted that column temperature can affect analyte retention and selectivity based on thermodynamic considerations. In HILIC mode separations, the retention of the analyte is based on the formation of reversible associates by polar analytes interacting with the stationary phase via hydrogen bonding, dipole-dipole and in appropriate cases, charge-dipole interactions.[6,7,16,143] In this study,

thermodynamic data (ΔG_i, ΔH_i, ΔS_i) were calculated according to the Gibbs-Helmholtzequation(eq1).

$$\Delta G_i = \Delta H_i - T\Delta S_i = -RT \ln K_i \qquad \text{(eq1)}$$

Where, ΔG_i is the molar Gibbs free energy, K_i is the solute partition coefficient, R is the universal gas constant and T is the absolute temperature. The dependence of analyte retention on temperature can be expressed by the van't Hoff equation (eq2).

$$\ln k = \frac{-\Delta H_i}{RT} + \frac{\Delta S_i}{R} + \ln \emptyset \qquad \text{(eq2)}$$

Where, k is the retention factor of a solute, ΔH_i is the partial molar enthalpy of transfer in the chromatographic system. ΔS_i is the partial molar entropy of the transfer, and Ø is the phase ratio of the chromatographic column ($\emptyset = V_S/V_M$) where, V_M is the dead volume and V_S is by the geometric internal volume of the column minus V_M).

In this study, the effect of temperature on the separation of nucleic acid bases and nucleosides was studied over a temperature range of 10 ºC - 50 ºC at 10 ºC intervals. Higher temperatures produced the expected decrease in retention of the analytes (results not shown). The van't Hoff plots (ln k vs 1/T) were constructed and Figure. 3.19 shows the plots of ln k vs 1/T on SCF6 columns. The correlation coefficients of the van't Hoff plots for the tested analytes indicated

good linearity (correlation coefficients were higher than 0.9811 and 0.9822 for SCF6-L and SCF6-H respectively, see Table 3.4 and Figure. 3.19. This means that the retention mechanism does not change with the temperature within the measured temperature range.[140]

The thermodynamic data for nucleic acid bases and nucleosides are summarized in Table 3.4. Partial molar enthalpies (ΔH_i) were calculated based on the slopes of the van't Hoff plots were negative for nucleic acid bases. This indicates transferring solutes from mobile phase to the stationary phases is exothermic. The ΔH_i values are in the range of -2.80 to -6.14 (kJ/mol) for SCF6-H column and -3.25 to -6.03 (kJ/mol) for SCF6-L column. The enthalpy change for uracil, thimidine, uridine, cytosine, guanine and guanosine are more negative on the SCF6-L column. This means, that the association between these analytes on SCF6-L stationary phase is more favorable. Moreover, from Table 3.4, it is seen that the entropy values were positive in all cases except thymidine on the SCF6-L column. The ΔS_i value obtained on SCF6-H column varied from 0.97 to 22.32 (J/mol K) and they were more positive than the values obtained from SCF6-L column. Clearly there is a greater entropic contribution with the SCF6-H column.

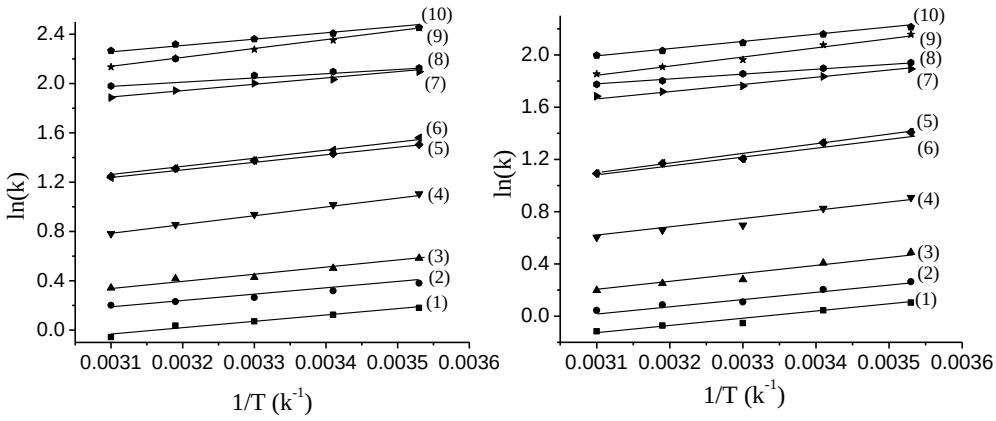


Figure 3.19 Dependence of logarithms of retention factors (ln k) on the inverse of temperature for nucleic acid bases and nucleosides.

(a) SCF6-H column (b) SCF6-L column. Conditions: Mobile phase: ACN/20 mM Ammonium acetate pH = 4.0 (90/10,v/v); Flow rate: 1.0 ml/min, Detection: UV at 254 nm. Compounds (1) Thymine, (2) Uracil, (3) Thymidine, (4) Uridine, (5) Adenine, (6) Adenosine, (7) Cytosine, (8) Guanine, (9) Cytidine, (10) Guanosine

Table 3.4 The thermodynamic parameters resulting from linear regression for nucleic acid bases and nucleosides on SCF6 columns

	SCF6-H			SCF6-L		
Compound	Correlation Coefficient (R^2)	ΔH_i/ (kJ/mol)	ΔS_i/ /(J/mol K)	Correlation Coefficient (R^2)	ΔH_i/ (kJ/mol)	ΔS_i/ (J/mol K)
Thymine	0.9847	-4.26	0.97	0.9811	-4.25	0.23
Uracil	0.9922	-3.40	5.51	0.9828	-4.24	1.62
Thymidine	0.9822	-4.34	3.90	0.9818	-5.63	-1.46
Uridine	0.9998	-4.84	1.98	0.9850	-5.92	1.01
Adenine	0.9990	-6.14	9.88	0.9907	-6.03	4.82
Adenosine	0.9988	-6.12	5.76	0.9905	-6.02	4.87
Cytosine	0.9954	-3.92	18.04	0.9951	-4.08	15.75
Guanine	0.9908	-2.80	22.32	0.9972	-3.25	19.12
Cytidine	0.9990	-5.96	13.74	0.3331	-5.91	11.48
Guanosine	0.9990	-3.56	22.29	0.9973	-4.27	17.79

3.4.3 Comparison of SCF6 Stationary phases to CF6 stationary phases.

SCF6 columns are useful for the analysis of wide range of polar analytes. These columns particularly provided greater selectivity and retention for the beta blockers when compared to native CF6 columns which have no charge.[58] This result most likely is due to the possible ionic interactions of the positively charged amine groups of the beta blockers in a mobile phase consisting of ACN/ 20 mM ammonium acetate at pH 4.0 (70/30,v/v). Consequently, shorter retention times were observed for the acidic analytes on SCF6 columns when compared to the

other types of analytes tested. The acidic analytes also were eluted within nine minutes on the native CF6 stationary phases with a mobile phase consisting of ACN/ 20 mM ammonium acetate at pH 4.1 (85/15,v/v).[58] With this mobile phase, these analytes did not retain well and coelutions were observed on the SCF6 columns. This result is due to the electrostatic repulsion between the negatively charged sulfonate groups on the stationary phase and the ionized acidic compounds. Clearly, there are obvious ionic interactions in the separation of β-blockers and ionized acids on the SCF6 stationary phases. The previously published high load CF6 column produced greater separation selectivity for the nucleic acid bases and nucleosides compared to the SCF6 columns.[58] All the analytes were baseline separated on CF6 column while adenine and adenosine coeluted on the SCF6 columns. The baseline separations of maltooligosaccharides were observed on both native and sulfonated CF6 columns in a mobile phase consisting of ACN/water (65/35,v/v). Separation windows of these analytes are 10 minutes on the SCF6-H columns and approximately 12 minutes on the CF6 columns. This result again indicates that the retention of sugars/carbohydrates is almost entirely dependent on the number of available hydrogen bonding groups on the stationary phases.

Comparing the capability of separating polar analytes, the SCF6 produced the best separation selectivity for the beta blockers and also afforded better or equal separation capability for other classes of analytes. However, a better

separation of nucleic acid bases and nucleosides was achieved on the native CF6 columns.[58] The difference in selectivity and retention on both columns depends not only in the nature of stationary phase but also nature of the analytes and the separation environment. Both native and sulfonated CF6 columns provide complimentary selectivity and show potential applications for the separation of polar analytes. Finally, thermodynamic studies showed linear dependencies of ln k vs 1/T for nucleic acid bases and nucleosides on both stationary phases. Also, negative values of ΔH_i were observed for both columns while more positive ΔS_i values were obtained on the SCF6 columns. Clearly, entropic contributions are somewhat more significant on the SCF6 columns when compared to the native CF6 columns.[58]

3.5 Concluding remarks

In this work, silica-based sulfonated cyclofructan 6 HILIC stationary phases were successfully synthesized and evaluated. They appear to be exceptional HILIC stationary phases for the separation of beta blockers, xanthines, salicylic acid related compounds, nucleic acid bases and their nucleosides, maltooligosaccharides, water soluble vitamins and amino acids. The highly sulfonated cyclofructan 6 HILIC stationary phase is competitive with and often superior to popular commercial columns. Additionally, SCF6 columns are stable and reproducible. Based on high success rate for the separation of polar

compounds, it is believed that this type of stationary phase will have a significant impact in HILIC mode separations.

CHAPTER 4

EVALUATION OF AROMATIC-DERIVATIZED CYCLOFRUCTANS 6 AND 7 AS HPLC CHIRAL SELECTORS

4.1 Summary

The two best aromatic-functionalized cyclofrucan chiral stationary phases, R-naphthylethyl-carbamate cyclofructan 6 (RN-CF6) and dimethylphenyl-carbamate cyclofructan 7 (DMP-CF7), were synthesized and evaluated by injecting various classes of chiral analytes. They provided enantioselectivity toward a broad range of compounds, including chiral acids, amines, metal complexes, and neutral compounds. It is interesting that they exhibited complementary selectivities and the combination of two columns provided enantiomeric separations for 43% of the test analytes. These extensive chromatographic results provided useful information about method development of specific analytes, and also gave some insight as to the enantioseparation mechanism.

4.2 Introduction

In the past two and a half decades, enantiomeric separations have developed from a highly challenging method into a routine laboratory technique.[179] This is mainly because of the rapid development of a plethora of HPLC chiral stationary phases (CSPs). Given the large number and types of CSPs, method development for a specific enantiomeric compound can involve

time-consuming screening of a large number of CSPs. Therefore, researchers continue to investigate new chiral stationary phases, in hopes of finding either a more universal column, which is widely effective for different classes of compounds, or columns with a well defined (and therefore predictable) selectivity.

Developing a new chiral selector with a broad range of enantioselectivies has been a challenge, and derivatization of a naturally-occurring molecule has proven to be a successful strategy, which can broaden the application range of the original chiral selector.[68,71,83-85,180-194] For example, aromatic derivatization (dimethylphenyl, R- and S-naphthylethyl, dinitrophenyl) of β-cyclodextrin greatly extended its utility in the normal phase mode.[84,85,180-183] Native cellulose and amylose are known to be poor chiral selectors, while dimethylphenyl-substituted ones (Chiralpak AD and OD) are among the most widely useful CSPs.[68,71,184-186] Aided by additional π-π interactions and dipolar interactions, the aromatic-derivatized chiral selector may provide totally different mechanisms of chiral recognition, compared to the original one.

A new class of macrocyclic oligosaccharides, cyclofructans, has recently received considerable attention in the area of enantiomeric separations. Cyclofructans are composed of six or more β-(2→1) D-fructofuranose units, name as CF6, CF7, CF8, and etc.[195] They were first reported in 1989 and they have been used in a variety of industrial applications, such as moderators of food

and drink bitterness and astringency, inhibitors for odor and taste of iron.[45-47]However, its application as a chiral selector for HPLC, CE and GC has been investigated only recently by our group.[51,52,54,196] Both LC and CE studies have demonstrated that native cyclofructan 6 (CF6) has limited capabilities for chiral recognition.[51,55]However, additional studies indicated that optimally derivatized-CF6 had considerable promise as a chiral selector.[55] By varying the nature and degree of substitution of the substituent(s), the functionalized cyclofructans could be "tuned" to separate different classes of molecules. While aliphatic-substituted CF6 has been thoroughly examined,[54] aromatic derivatives have not.

Therefore, the purpose of the present work is to examine the potential and overall chiral selectivities of aromatic-derivatized CF6 and CF7. In order to evaluate their applicability, a large number of racemic compounds, including chiral acids, amines, metal complexes, and neutral compounds, were injected on the two best chiral stationary phases, R-naphthylethyl-carbamate CF6 (RN-CF6) and dimethylphenyl carbamate CF7 (DMP-CF7). Also, effects of the chiral selector structure on enantioseparations are discussed.

4.3 Experimental

4.3.1 Materials

Anhydrous toluene, anhydrous pyridine, trifluoroacetic acid (TFA), ammonium nitrate, 3-(triethoxysilyl)propyl isocyanate, 1,6-diisocyanatohexane, (3-aminopropyl)dimethylethoxysilane, R-1-(1-naphthyl)ethyl isocyanate, 3,5-

dimethylphenyl isocyanate, and most of racemic analytes tested in this study were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Ru(II) complexes were donated by Dr. Fred (Department of Chemistry and Biochemistry, the University of Texas at Arlington). CF6 was obtained by fermentation and crystallization as described previously.[42,86,87]

CF7 was purified as reported previously.[197] Acetonitrile (ACN), isopropanol (IPA), heptane, ethanol (ETOH), and methanol (MEOH) of HPLC grade were obtained from EMD (Gibbstown, NJ). Water was obtained form Millipore (Billerica, MA). Kromasil and Daiso silica (5μm spherical diameter, 100 Å, 120 Å, 200 Å pore size) were obtained from Supelco (Bellefonte, PA). The aromatic-substituted CF6 and CF7 chiral stationary phases were synthesized, according to the previous paper.[55] Then it was slurry packed into a 25cm×0.46cm (i.d.) stainless steel column.

4.3.2 HPLC method

The chromatographic system used was an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA), consisting of a diode array detector, an autosampler, a binary pump and a temperature-controlled column chamber. For all HPLC experiments, the injection volume and the flow rate were 5 μL, 1mL/min, respectively. The column temperature was 20 ^{o}C, if not specified otherwise. The mobile phase was degassed by ultrasonication under vacuum for 5 min. The analytes were dissolved in ethanol, or the appropriate mobile phases. In

the normal phase mode, heptane/ethanol (or isopropanol) with/without trifluoroactic acid was used as the mobile phase. The mobile phase of the polar organic mode was composed of acetonitrile/methanol with 0.2% ammonium nitrate (weight percentage). For the calculation of retention factors (k_1, k_2), t_0 was determined by the peak of the refractive index change due to the sample solvent or by injecting 1,3,5-tri-*tert*-butylbenzene in the normal phase mode.

4.4 Results and Discussions

4.4.1 Screening chromatographic results of the RN-CF6 CSP and the DMP-CF7 CSP

Preliminary studies showed that aromatic functionalization of cyclofructans significantly broadened the application range of these CSPs.[55] Therefore, different aromatic-substitution groups on cyclofructan6 (CF6) and cyclofructan7 (CF7), including dimethylphenyl, methylphenyl, naphthylethyl, dichlorophenyl, and chlorophenyl, were tested. It was found that two of these CSPs provided the best performance and they showed the broadest selectivity for the tested racemic analytes. They are the R-naphthylethyl-carbamate CF6 (RN-CF6) and dimethylphenyl-carbamate CF7 (DMP-CF7). It was also found that better selectivity and resolution were often obtained in the normal phase mode on the aromatic functionalized cyclofructan stationary phases.[55] Therefore, the current study focuses on a systematic chromatographic evaluation of the RN-CF6 and DMP-CF7 stationary phases, mainly in the normal phase mode.

A set of 220 racemic compounds with various functionalities were used to evaluate these two columns. Racemic analytes were divided into five groups: acids, amines (including primary, secondary and tertiary amines), alcohols, ruthenium complexes, and other neutral compounds. It was reported that enantiomers of ruthenium complexes were quickly separated with high selectivities by the R-naphthylethyl β-cyclodextrin column (Cyclobond RN),[198] which is closely related to the structure of the RN-CF6 stationary phase. Therefore, these Ru(II) complexes were tested in order to compare the performance of cyclofructan- and cyclodextrin-based stationary phases. Earlier studies indicated that the RN-CF6 CSP provided enantioselectivities for a few unique primary amines, which the isopropyl-CF6 column could not easily separate.[54] Therefore, in this study, several primary-amine containing compounds (grouped into "amines") were selected to investigate the capability of CF6- and CF7-based CSPs for separating primary amines. Table 4.1 lists the chromatographic data for all compounds separated by the RN-CF6 and DMP-CF7 CSPs. The chromatographic data include retention factor (k_1), selectivity (α), and resolution (Rs). In the case of Ru complexes (group D in Table 4.1), it was necessary to use the polar organic mode plus salt addition (such as ammonium nitrate) to obtain elution in a reasonable time. Optimized separations of all other compounds were achieved in the normal phase mode. The chromatograms in

Figure 4.1 (20 ˚C vs 0 ˚C) demonstrate effects of temperature on these enantioseparations.

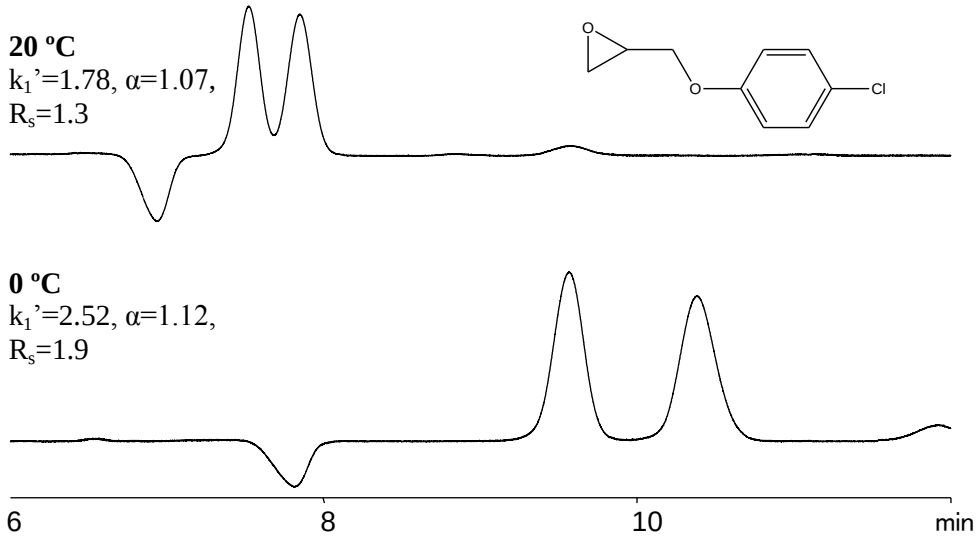


Figure 4.1Effects of the column temperature on enantioseparations by the DMP-CF7 CSP.

The analyte and mobile phase are 4-chlorophenyl 2,3-epoxypropyl ether and 95Hep5EtOH0.1TFA, respectively.

The selectivity of separating 4-chlorophenyl 2,3-epoxypropyl ether was improved from 1.07 to 1.12 when decreasing the column temperature. Usually, decreasing the column temperature increases enantioselectivity, retention, and resolution. Therefore, decreasing the column temperate to 0 ˚C can be an effective strategy during the optimization process.

The data in Table4. 1 indicates that the number of separations for each class of analyte obtained on two columns is 8, 15, 10, 7, 54, respectively. The total number of separations achieved on the combination of RN-CF6 and DMP-CF7 are 94.

Table 4.1 Summary of optimized chromatographic data achieved on RN-CF6 and DMP-CF7 CSPs

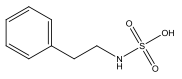
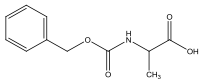
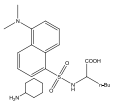
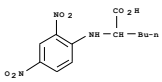
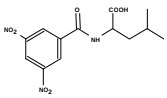
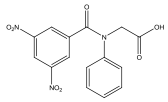
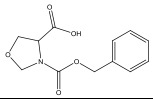
#	*Compound name*	*Structure*	*CSP*	k_1	α	*Rs*	*Mobile phase*[a]
A. Chiral acids							
1	Phenethylsulfamic acid		RN-CF6	1.52	1.20	1.6	60H40E0.1TFA
2	Carbobenzyloxy alanine		DMP-CF7	9.10	1.10	1.7	95H5E0.1TFA0C
3	Dansyl-norleucine cyclohexylammonium salt		DMP-CF7	2.42	2.03	8.6	80H20E0.1TFA
4	N-2,4-DNP-DL-norleucine		DMP-CF7	9.93	1.10	1.5	95H5E0.1TFA
5	N-(3,5-dinitrobenzoyl)-DL-leucine		RN-CF6	0.84	1.49	4.5	50H50E0.1TFA
			DMP-CF7	16.90	1.13	3.0	95H5E0.1TFA
6	N-(3,5-Dinitrobenzoyl)-DL-phenylglycine		RN-CF6	2.50	1.16	2.2	50H50E0.1TFA
			DMP-CF7	21.66	1.07	0.8	95H5E0.1TFA
7	3-(Benzyloxycarbonyl)-4-oxazolidinecarboxylic acid		DMP-CF7	4.11	1.05	0.9	95H5E0.1TFA

Table 4.1 - Continued

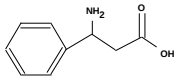
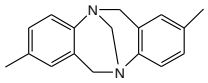
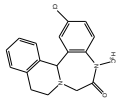
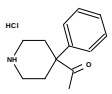
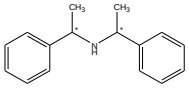
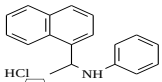
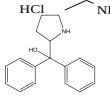
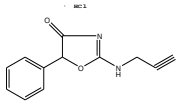
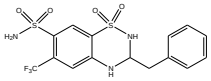
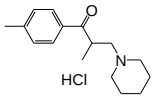
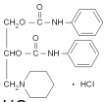
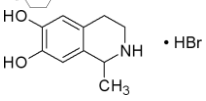
8	DL-3-Amino-3-phenylpropionic acid		DMP-CF7	8.25	1.04	0.7	80H20E0.1TFA
B. Amines							
1	Tröger's base		RN-CF6	0.79	1.50	5.2	70H30E
			DMP-CF7	3.19	1.29	3.7	80H20E0.1TFA
2	2-Chloro-5,9,10,14B-tetrahydro- 5-Me-		RN-CF6	2.58	1.02	0.4	60H40E0.1TFA
3	4-Acetyl-4-phenylpiperidine hydrochloride		DMP-CF7	5.14	1.05	0.7	80H20E0.1TFA
4	Bis-[(R/S)-1-phenylethyl] amine hydrochloride		RN-CF6	3.59	1.15	2.4	90H10E0.1TFA
5	N-Benzyl-1-(1-naphthyl) ethylamine hydrochloride		RN-CF6	8.92	1.02	0.6	95H5E0.1TFA
6	α,α-Diphenylprolinol		RN-CF6	15.03	1.12	1.7	90H10E0.1TFA
7	Oxyphencyclimine hydrochloride		RN-CF6	13.68	1.12	1.5	60H40E0.1TFA
8	5-Phenyl-2-(2-propynyl-amino)-2-oxazolin-4-one		DMP-CF7	5.21	1.08	1.5	80H20E0.1TFA
9	Bendroflumethiazide		RN-CF6	1.81	1.16	2.0	60H40E0.1TFA
10	Tolperisone hydrochloride		RN-CF6	5.52	1.12	1.8	70H30E0.1TFA
11	Diperodon hydrochloride		RN-CF6	7.42	1.11	1.2	70H30E0.1TFA
12	1-Methyl-6,7-dihydroxy-1,2,3,4-		RN-CF6	4.33	1.17	2.0	60H40E0.1TFA

Table 4.1- Continued

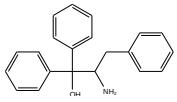
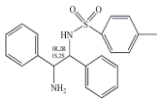
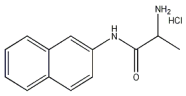
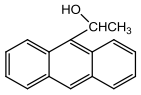
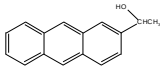
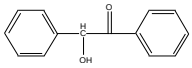
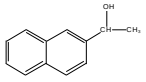
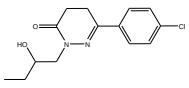
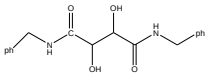
			DMP-CF7	12.12	1.05	0.6	80H20E0.1TFA
13	2-Amino-1,1,3-triphenyl-1-propanol		RN-CF6	3.40	1.10	1.5	80H20E0.1TFA
			DMP-CF7	3.12	1.02	0.5	80H20E0.1TFA
14	N-p-Tosyl-1,2-diphenylethylenediamine		RN-CF6	8.28	1.24	2.9	80H20E0.1TFA
			DMP-CF7	5.43	1.13	1.4	80H20E0.1TFA
15	DL-alanine-β-naphthylamide hydrochloride		RN-CF6	10.03	1.10	1.5	80H20E0.1TFA
C. Alcohols							
1	α-Methyl-9-anthracenemethanol		RN-CF6	11.30	1.06	1.3	99H1I0.1TFA0C
2	1-Anthracen-2-yl-ethanol		DMP-CF7	14.59	1.05	1.0	99H1E
3	Benzoin		RN-CF6	9.30	1.06	1.5	99H1I0.1TFA0C
			DMP-CF7	7.79	1.09	1.5	99H1E
4	α-Methyl-2-naphthalenemethanol		DMP-CF7	9.90	1.02	0.5	99H1E
5	6-(4-chlorophenyl)-4,5-dihydro-2-(2-		DMP-CF7	13.63	1.03	0.6	95H5E0.1TFA
6	N,N'-Dibenzyl-tartramide		RN-CF6	8.21	1.04	0.9	90H10E0.1TFA
			DMP-CF7	22.76	1.03	0.8	95H5E0.1TFA

Table 4.1- Continued

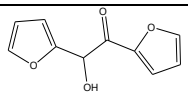
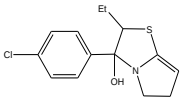
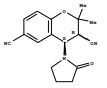
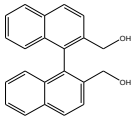
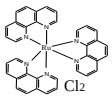
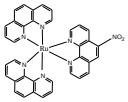
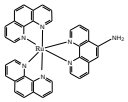
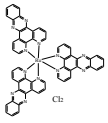
7	Furoin		RN-CF6	5.24	1.02	0.5	90H10E0.1TFA
			DMP-CF7	17.46	1.04	0.7	98H2E0.1TFA
8	3-(4-Chlorophenyl)-2-ethyl-2,3,5,6-		DMP-CF7	5.05	1.10	0.7	80H20E0.1TFA
9	Cromakalim		DMP-CF7	17.24	1.08	1.5	95H5E0.1TFA
10	1,1'-Binaphthyl-2,2'-dimethanol		RN-CF6	6.37	1.02	0.6	95H5E0.1TFA
			DMP-CF7	7.44	1.05	0.7	80H20E0.1TFA
D. Ru(II) complexes							
1	$[Ru(phen)_3](Cl_2)$		RN-CF6	0.62	1.49	3.8	60M/40A/0.2 NH_4NO_3[b]
			DMP-CF7	3.81	1.14	1.3	80M/20A/0.2 NH_4NO_3
2	$[Ru(phen)_2$nitrophen$](Cl_2)$		RN-CF6	0.46	1.65	4.1	60M/40A/0.2 NH_4NO_3
			DMP-CF7	3.52	1.25	1.8	80M/20A/0.2 NH_4NO_3
3	$[Ru(phen)_2$aminophen$](Cl_2)$		RN-CF6	0.54	1.53	3.9	60M/40A/0.2 NH_4NO_3
			DMP-CF7	2.81	1.15	1.3	80M/20A/0.2 NH_4NO_3
4	$[Ru(dppz)_3](Cl_2)$		RN-CF6	0.78	2.90	11.4	60M/40A/0.2 NH_4NO_3
			DMP-CF7	2.63	1.44	2.7	80M/20A/0.2 NH_4NO_3

Table 4.1- Continued

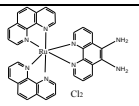
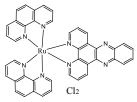
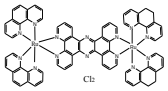
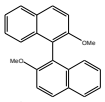
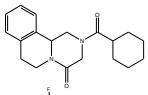
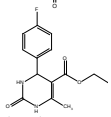
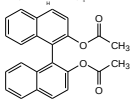
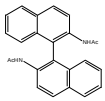
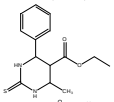
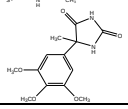
5	[Ru(phen)$_2$phendiamine] (Cl$_2$)		RN-CF6	0.59	1.51	3.3	60M/40A/0.2 NH_4NO_3
			DMP-CF7	4.02	1.16	1.2	80M/20A/0.2 NH_4NO_3
6	[Ru(phen)$_2$dppz](Cl$_2$)		RN-CF6	0.67	1.80	5.8	60M/40A/0.2 NH_4NO_3
			DMP-CF7	6.25	1.32	2.2	80M/20A/0.2 NH_4NO_3
7	[Ru$_2$(phen)$_4$(tpphz)](Cl$_4$)		RN-CF6	2.24	2.67	6.9	60M/40A/0.2 NH_4NO_3
			DMP-CF7	16.24	1.30	0.9	80M/20A/0.2 NH_4NO_3
E. Others							
1	2,2'-Dimethoxyl-1,1'-binaphthyl		RN-CF6	6.20	1.02	0.5	95H5E0.1TFA
2	Praziquantel		RN-CF6	9.69	1.05	1.0	95H5E0.1TFA
3	4-(4-Fluoro-phenyl)-6-methyl-2-oxo-1,2,3,4-		DMP-CF7	14.85	1.12	2.6	95H5E0.1TFA
4	1,1'-Bi(2-naphthyl diacetate)		RN-CF6	0.83	1.12	1.5	70H30E0.1TFA
			DMP-CF7	4.18	1.13	2.2	99H1E
5	N-2'-Acetylamino-[1,1']binaphthalenyl-2-yl)-		DMP-CF7	23.45	1.04	0.9	95H5E0.1TFA
6	6-Methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydro-		DMP-CF7	12.92	1.11	1.5	98H2E0.1TFA
7	5-Methyl-5-(3,4,5-trimethoxyphenyl)hydantoi		RN-CF6	13.08	1.04	1.1	90H10E0.1TFA

Table 4.1- Continued

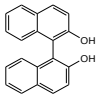
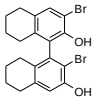
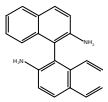
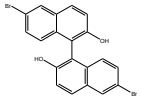
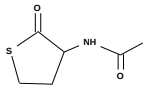
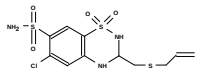
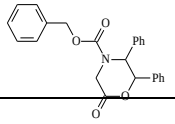
			DMP-CF7	13.36	1.05	1.5	90H10E0.1TFA
8	5,5',6,6',7,7',8,8'-Octahydro(1,1'-		RN-CF6	6.80	1.20	4.9	99H1I0.1TFA
			DMP-CF7	5.13	1.05	0.7	80H20E0.1TFA
9	3,3'-Dibromo-5,5',6,6',7,7',8,8'-		RN-CF6	4.89	1.28	5.2	99H1I0.1TFA
			DMP-CF7	5.13	1.04	0.7	80H20E0.1TFA
10	2,2'-Diamino-1,1'-binaphthalene		RN-CF6	2.03	1.18	2.7	70H30E
			DMP-CF7	4.36	1.48	5.0	80H20E0.1TFA
11	6,6'-Dibromo-1,1'-bi-2-naphthol		RN-CF6	3.67	1.11	2.3	90H10E0.1TFA
			DMP-CF7	3.70	1.17	2.6	90H10E0.1TFA
12	1,1'-Bi-2-naphthol		RN-CF6	3.33	1.07	1.7	90H10E0.1TFA
			DMP-CF7	3.91	1.23	5.0	90H10E0.1TFA
13	DL-N-Acetylhomocysteine thiolactone		RN-CF6	12.08	1.03	0.8	90H10E0.1TFA
14	Althiazide		RN-CF6	3.04	1.20	2.7	60H40E0.1TFA
			DMP-CF7	12.42	1.09	1.5	80H20E0.1TFA
15	Benzyl-6-oxo-2,3-diphenyl-4-morpholine carboxylate		RN-CF6	7.08	1.05	1.6	95H5E0.1TFA

Table 4.1- Continued

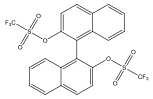
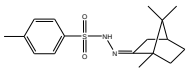
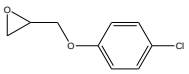
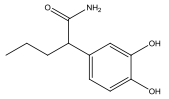
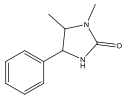
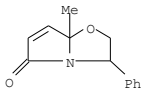
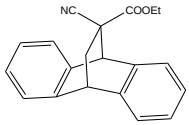
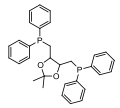
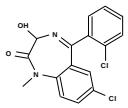
			DMP-CF7	6.26	1.06	1.5	95H5E0.1TFA
16	1.1'-Bi-2-naphthol bis(trifluoromethanesulfona		RN-CF6	7.80	1.07	1.2	100HEP0C
	Table 4.1 continued						
17	Camphor p-tosyl hydrazon		DMP-CF7	7.53	1.18	3.4	95H5E0.1TFA
18	4-Chlorophenyl 2,3-epoxypropyl ether		DMP-CF7	2.52	1.12	1.9	95H5E0.1TFA0C
19	3,4-dihydroxyphenyl-alfa-propylacetamide		DMP-CF7	6.15	1.06	1.0	80H20E0.1TFA
20	1,5-Dimethyl-4-phenyl-2-imidazolidinone		RN-CF6	11.17	1.06	1.5	95H5I0.1TFA
			DMP-CF7	11.10	1.07	1.5	95H5E0.1TFA
21	2,3-Dihydro-7a-methyl-3-phenylpyrrolo[2,1-		RN-CF6	2.19	1.15	2.1	90H10E0.1TFA
			DMP-CF7	8.26	1.07	1.6	99H1E
22	Ethyl 11-cyano-9,10-dihydro-endo-9,10 -		RN-CF6	3.07	1.08	1.7	99H1E0.1TFA
			DMP-CF7	1.94	1.57	5.2	95H5E0.1TFA
23	2,3-O-Isopropylidene 2,3-dihydroxy-1,4-		RN-CF6	10.89	1.05	0.8	90H10E0.1TFA
			DMP-CF7	5.80	1.02	0.5	80H20E0.1TFA
24	Lormetazepam		RN-CF6	9.57	1.04	0.8	90H10E0.1TFA

Table 4.1- Continued

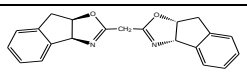
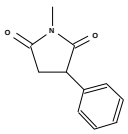
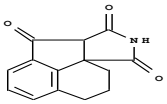
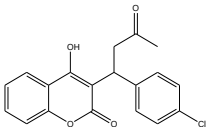
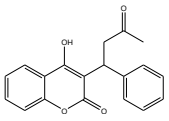
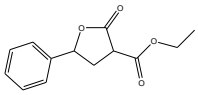
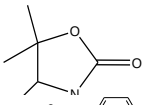
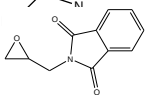
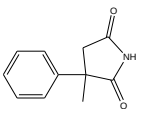
25	[3aS/R-[2(3'aR*,8'aS*),3'aα/β,		RN-CF6	9.84	1.21	1.2	70H30E0.1TFA
26	Phensuximide		RN-CF6	6.12	1.05	1.5	95H5E0.1TFA
			DMP-CF7	15.98	1.09	1.2	98H2E0.1TFA
27	3a,4,5,6-Tetrahydro-succininido[3,4-		RN-CF6	4.31	1.08	1.5	80H20E0.1TFA
			DMP-CF7	12.60	1.10	2.1	90H10E0.1TFA
28	3-(α-Acetonyl-4-chlorobenzyl)-4-		RN-CF6	11.54	1.10	1.8	95H5E0.1TFA
			DMP-CF7	12.16	1.30	4.5	95H5E0.1TFA
29	Warfarin		RN-CF6	12.20	1.10	1.9	95H5I0.1TFA
			DMP-CF7	11.54	1.17	2.6	95H5E0.1TFA
30	2-Carbethoxy-gamma-phenyl-gamma-		RN-CF6	12.79	1.09	1.0	99H1I0.1TFA0C
			DMP-CF7	11.79	1.12	2.4	98H2E0.1TFA0C
31	5,5-dimethyl-4-phenyl-2-oxazolidinone		DMP-CF7	8.26	1.08	1.8	95H5E0.1TFA
32	N-(2,3-Epoxypropyl)-phthalimide		DMP-CF7	14.59	1.08	1.7	98H2E0.1TFA
33	α-Methyl-alpha-phenyl-succinimide		RN-CF6	10.28	1.05	1.5	95H5E0.1TFA0C
			DMP-CF7	9.77	1.09	2.0	95H5E0.1TFA

Table 4.1- Continued

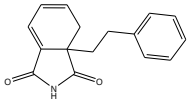
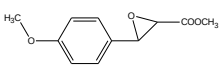
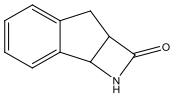
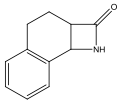
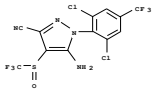
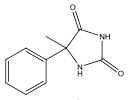
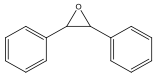
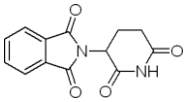
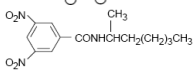
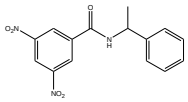
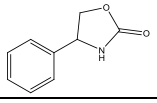
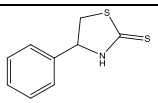
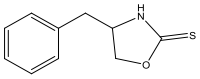
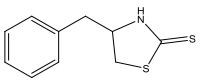
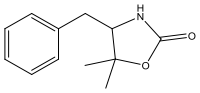
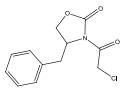
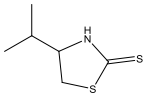
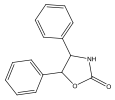
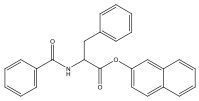
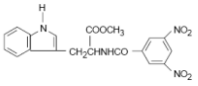
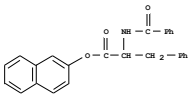
34	Phenethylphthalimide		RN-CF6	4.07	1.08	1.3	99H1I0.1TFA0C
			DMP-CF7	3.82	1.25	3.2	99H1E
35	Methyl trans-3-(4-methoxyphenyl)glycidate		RN-CF6	7.22	1.02	0.6	90H10E0.1TFA
			DMP-CF7	14.07	1.09	1.9	95H5E0.1TFA
36	cis-3,4-benzo-6-azabicyclo[3.2.0]heptan-7-		RN-CF6	5.31	1.07	1.5	90H10E0.1TFA0C
			DMP-CF7	22.03	1.04	0.8	98H2E0.1TFA
37	cis-4,5-benzo-7-azabicyclo [4.2.0]octan-8-one		RN-CF6	8.27	1.05	1.3	95H5E0.1TFA0C
			DMP-CF7	17.70	1.03	0.6	98H2E0.1TFA
38	Fipronil		RN-CF6	17.42	1.07	1.7	98H2E0.1TFA0C
39	5-Methyl-5-phenyl hydantoin		DMP-CF7	14.73	1.16	5.0	95H5E0.1TFA
40	trans-Stilbene oxide		RN-CF6	1.70	1.05	1.1	100HEP0C
41	Thalidomide		RN-CF6	12.65	1.05	1.1	80H20E0.1TFA
42	3,5-DNB-2-aminoheptane		RN-CF6	0.86	1.13	1.5	70H30E0.1TFA
43	3,5-Dinitro-N-(1-phenylethyl)benzamide		RN-CF6	0.96	2.05	9.8	50H50E0.1TFA
44	4-Phenyl-2-oxazolidinone		DMP-CF7	7.12	1.05	1.0	90H10E0.1TFA

Table 4.1- Continued

45	4-Phenylthiazolidine-2-thione		RN-CF6	13.27	1.04	1.3	95H5E0.1TFA0C
46	4-Benzyloxazolidine-2-thione		RN-CF6	6.98	1.02	0.5	90H10E0.1TFA
			DMP-CF7	6.90	1.15	2.9	90H10E0.1TFA
47	4-Benzylthiazolidine-2-thione		DMP-CF7	4.25	1.09	1.9	90H10E0.1TFA
48	4-Benzyl-5,5-dimethyl-2-oxazolidinone		RN-CF6	7.06	1.02	0.6	90H10E0.1TFA
49	4-Benzyl-3-chloroacetyl-2-oxazolidinone		RN-CF6	3.73	1.02	0.5	90H10E0.1TFA
50	4-Isopropylthiazolidine-2-thione		DMP-CF7	5.65	1.08	1.8	95H5E0.1TFA
51	cis-4,5-Diphenyl-2-oxazolidinone		RN-CF6	14.80	1.05	1.5	95H5E0.1TFA0C
			DMP-CF7	13.63	1.09	1.9	95H5E0.1TFA
52	N-Benzoyl-DL-phenylalanine beta-		RN-CF6	5.92	1.06	0.5	90H10E0.1TFA0C
			DMP-CF7	14.80	1.08	1.5	95H5E0.1TFA0C
53	3,5-DNB-Tryptophan methyl ester		RN-CF6	5.52	1.12	1.8	70H30E0.1TFA
			DMP-CF7	5.75	1.14	5.0	80H20E0.1TFA
54	N-Benzoyl-DL-phenylalanine beta-		DMP-CF7	8.52	1.06	1.1	95H5E0.1TFA

Enantiomers of 63 compounds out of 94 were baseline separated. 43% of the tested compounds, which were randomly chosen, were separated on these two CSPs. The representative chromatograms of a chiral acid, secondary amine, tertiary amine, alcohol, Ru(II) complex, and a neutral compound, are shown in Figure 4.2.

The data in Table 4. 1 and Figure 4. 2 indicates that the RN-CF6 and DMP-CF7 CSPs provide excellent enantioselectivity toward a wide range of analytes. Compared to native CF6 and CF7, their capabilities for chiral recognition were significantly improved. This can be explained by the fact that derivatization of native cyclofructans effectively disrupts internal hydrogen bonding, relaxing (denaturing) the molecule and exposing its entire surface.[27, 28] In addition, the presence of the aromatic and carbonyl groups provide ample opportunities for π-π interactions and dipolar interactions, as well as additional steric interaction sites. These are the type of interactions that play a pronounced role in chiral recognition in nonpolar solvents (the normal phase mode) rather than in polar solvents. It should be also noted that the CF-based chiral stationary phases were very robust and not irreversibly altered when changing from one mobile phase mode to another.These results also indicate that the performance of cyclofructan stationary phases was significantly different from other known crown-ether based CSPs, although cyclofructans also contain crown-ether cores. CF-based CSPs provided chiral recognition toward a variety of compounds in the normal phase mode,

while other known crown-ether CSPs show limited selectivity except for primary amines and they are preferably used with acidic aqueous mobile phases.[199-203] The analytes, stationary phases, and d mobile phases are: (A) Dansyl-norleucine cyclohexylammonium salt, DMP-CF7, 80H20E0.1TFA; (B) 1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide, RN-CF6, 60H40E0.1TFA; (C) Tröger's base, DMP-CF7, 80H20E0.1TFA; (D) Benzoin, RN-CF6, 99H1I0.1TFA (0˚C); (E) $[Ru(phen)_3]Cl_2$, RN-CF6, 60M/40A/0.2 NH_4NO_3 ; (F) Ethyl 11-cyano-9,10-dihydro-endo-9,10 - ethanoanthracene-11-carboxylate, DMP-CF7, 95H5E0.1TFA.

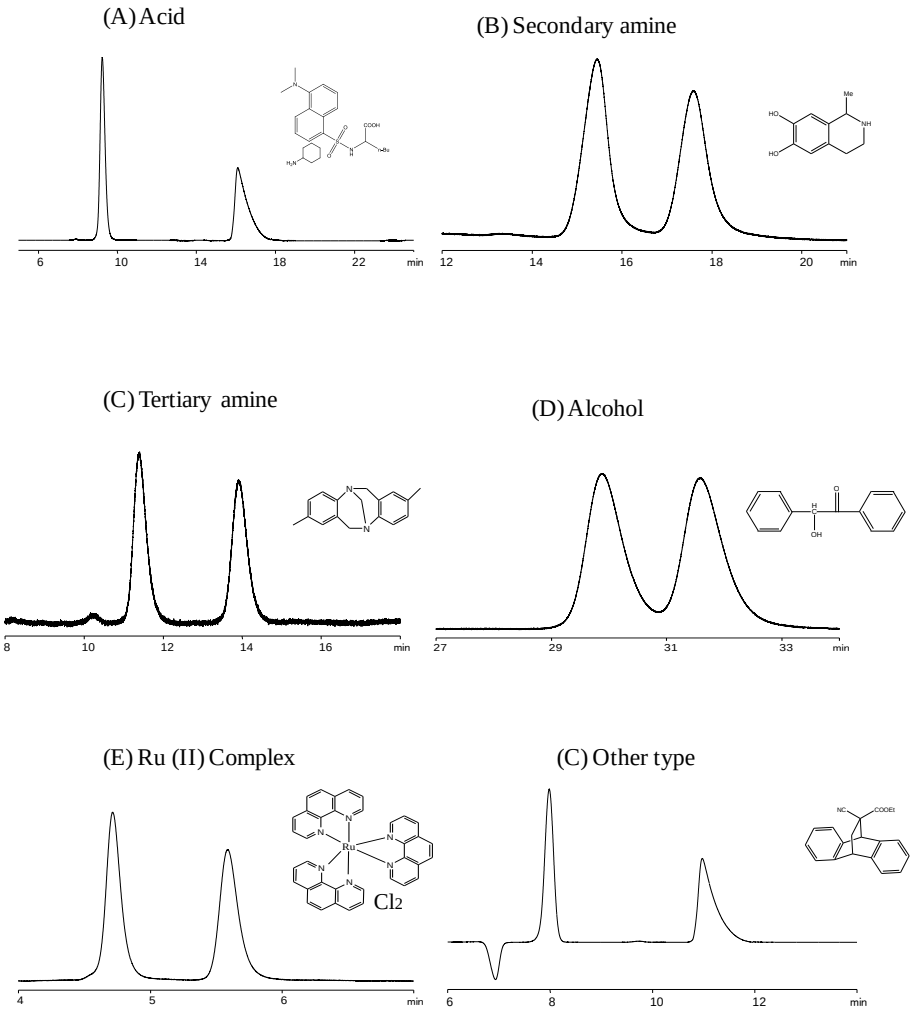


Figure 4.2 Representative chromatograms showing enantioseparations of various types of compounds.

4.4.2 Complementary selectivity provided by RN-CF6 and DMP-CF7

Figure 4.3 summarizes the separations obtained on the RN-CF6 CSP, the DMP-CF7 CSP and a combination of the two columns. The total number of separations achieved on the RN-CF6 CSP and the DMP-CF7 CSP are 69, and 69, respectively. Of these, there were 43 baseline separations on the RN-CF6 column

and 41 on the DMP-CF7 column. Enantiomers of 25 compounds were separated only by the RN-CF6 CSP, while another 25 were separated only with the DMP-CF7 CSP. It was often observed that some analytes were baseline separated on the RN-CF6 column, while only a partial separation or no separation was observed on the DMP-CF7 CSP, and vice versa. Therefore, the RN-CF6 and DMP-CF7 CSPs demonstrated complementary enantioselectivities.

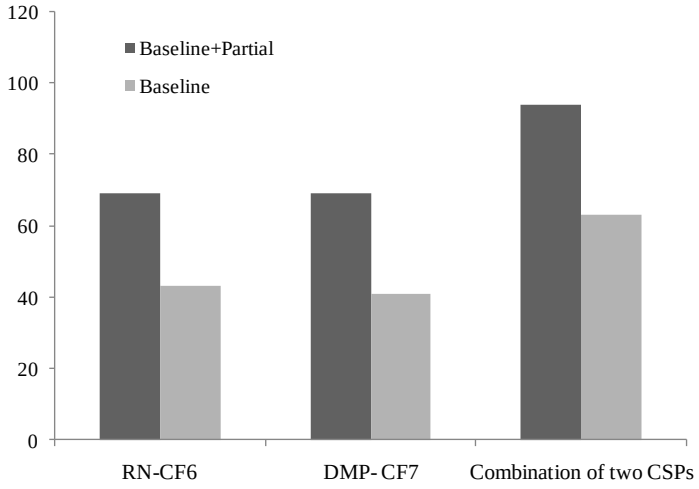


Figure 4.3 Summary of enantioseparations obtained on RN-CF6 and DMP-CF7 based chiral stationary phases.

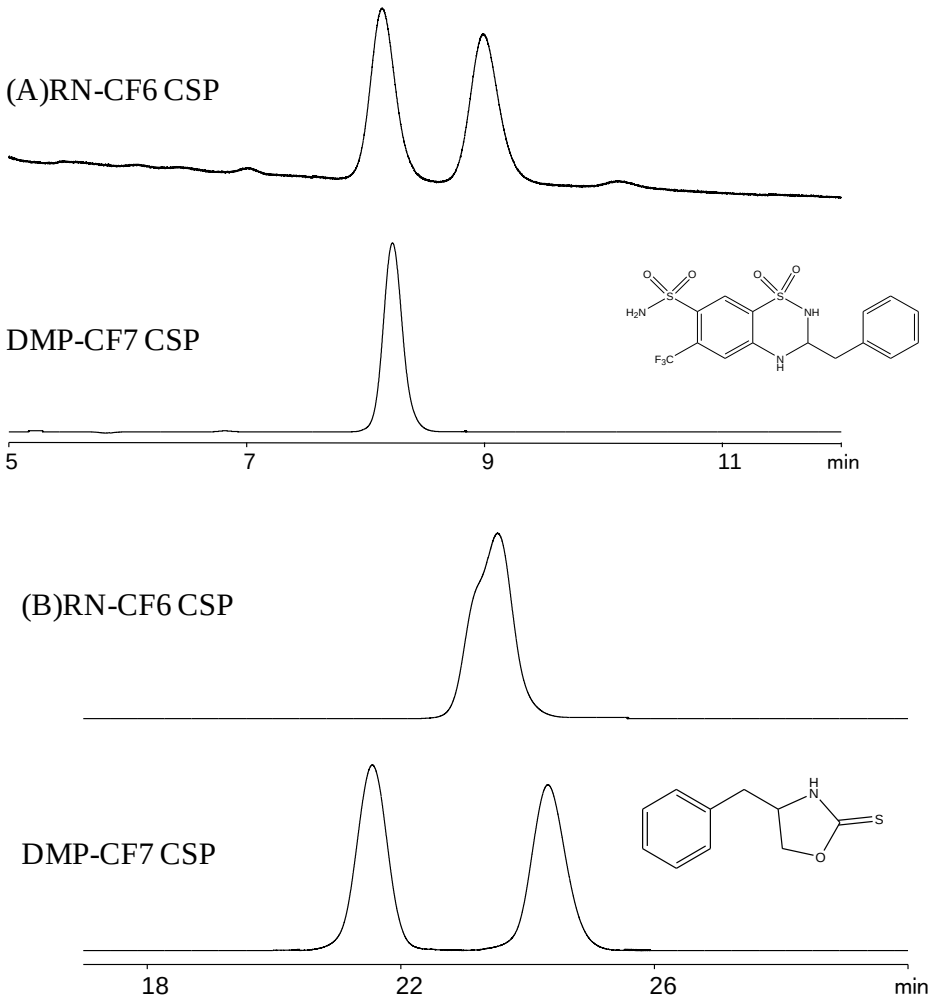


Figure 4.4 Comparison between RN-CF6 and DMP-CF7 stationary phases

The analyte and mobile phase are (A) bendroflumethiazide, 60heptane/40ethanol/0.1TFA;(B)4-benzyloxazolidine-2-thione, 90heptane/10ethanol/0.1TFA.

Figure 4.4 gives two examples of the complementary selectivity provided by these columns. Enantiomers of bendroflumethiazide were baseline separated with the RN-CF6 CSP with a high selectivity (α=1.16) while no separation was

observed on the DMP-CF7 CSP (Fig.4A). The RN-CF6 column gave a partial separation of 4-benzyloxazolidine-2-thione with a tiny shoulder (α=1.02) while the DMP-CF7 column provided high selectivity (α=1.15) and a baseline separation (Fig.4B).

Although the overall success rates of the RN-CF6 and DMP-CF7 CSPs are the same (31%), their capabilities for chiral recognition for various classes of compounds are different. For example, only 2 out of 8 in group A (chiral acids), 4 out of 15 in group B (amines), and 4 out of 10 in group C (alcohols) were separated by both columns.

The others compounds (over 60%) in these three groups were only separated by one column (either the RN-CF6 or the DMP-CF7 CSP). However, for group E (others), these two stationary phases shared a bigger overlap and about 50% analytes in group E (Table 4.1) were separated by both columns. For Ru complexes (group D), both columns provided enantiomeric separation, although the RN-CF6 CSP gave a much higher selectivity than the DMP-CF7 CSP.

Considering the success rate of two columns for different groups of compounds, the DMP-CF7 CSP obtained greater success than the RN-CF6 CSP when separating chiral acids, based on the fact that 8 analytes were separated by the DMP-CF7 CSP compared to 3 by the RN-CF6 CSP. It was also found that the RN-CF6 CSP provided a higher enantioselectivity toward chiral amines (group B)

than the DMP-CF7 CSP in most of cases. The characteristic that the RN-CF6 and DMP-CF7 columns offered complementary selectivities for different groups of compounds is advantageous when selecting columns for separating a large number of different compounds.

4.4.3 Effects of the size of the cyclofructan ring, i. e., CF6 vs CF7 on enantioseparations

The effects of the size of the central crown ether core (i. e., 18-crown-6 vs 21-crown-7) in CF6 and CF7 can be examined by comparing DMP-CF6 and DMP-CF7 as LC chiral selectors. The capabilities of chiral recognition provided by DMP-CF6 and DMP-CF7 CSPs are quite different. The DMP-CF7 column provided significantly different resolution for over 60 % of analytes separated by the DMP-CF6 CSP. Generally, the DMP-CF7 CSP gave better performance than the DMP-CF6 CSP for separating acidic and neutral analytes. For example, enantiomers of dansyl-norleucine cyclohexylammonium salt were well separated with an extremely high selectivity (α=2.03) by the DMP-CF7 CSP while only a partial separation was obtained on the DMP-CF6 CSP (shown in Figure 4.5). For amine containing compounds, the DMP-CF6 column gave higher selectivity than the larger DMP-CF7 based CSP. Also, this was true for all derivatized-CF6 stationary phases and respective CF7 columns. Therefore, the size of the cyclofructan ring plays a significant role in interactions between amine containing analytes and the chiral selector.

The selectivity of the RN-CF6 and RN-CF7 stationary phases also were compared. The relative performance of the RN-CF6 and RN-CF7 CSPs was opposite to that found for the DMP functionalized cyclofructans. Indeed, it was the smaller RN-CF6 CSP that usually provided better resolution than the larger RN-CF7 CSP. Also, it is noted that the chromatographic differences between the RN-CF6 and RN-CF7 CSPs are much smaller compared to the more substantial difference between the DMP-CF6 and DMP-CF7 columns.

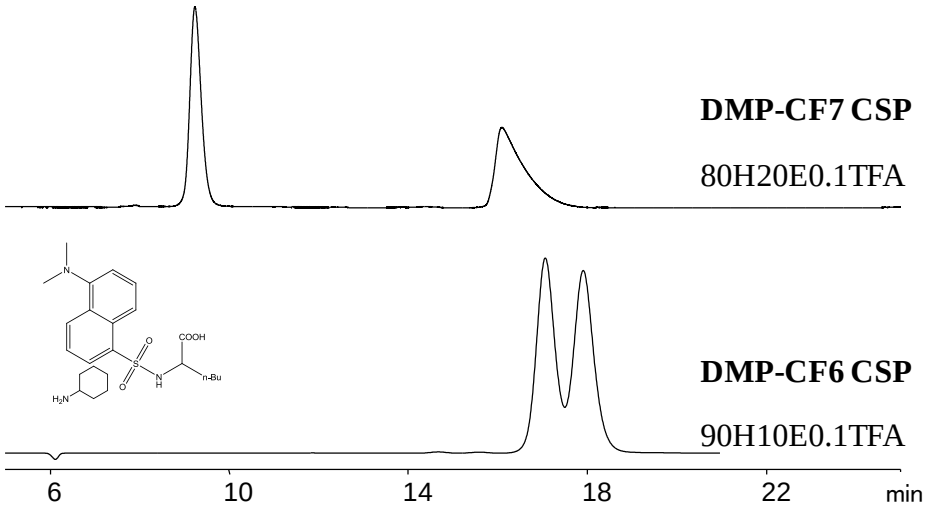


Figure 4.5Comparison between DMP-CF6 and DMP-CF7 CSPs. The analyte is dansyl-norleucine cyclohexylammonium salt.

The difference between CF6 and CF7 is the central crown ether size and the number of available hydroxyl groups. Although CF6 and CF7 differ only by one fructose unit, their spatial structures are quite different according to

computational modeling studies.[42] Also, the separation of CF6 and CF7 on a HILIC stationary phase showed that their number of available hydroxyl groups was quite different.[197] There is a much bigger difference in the separation of CF6 and CF7, than is found for the comparable separation of α-, β-, and γ-cyclodextrin. This may be due to the extensive internal hydrogen bonding of cyclofructans.[197]

Currently, there is no reported crystal structure of CF7 and it is difficult to give more detailed explanations about the chromatographic differences between CF6 and CF7-based stationary phases without further study. However, the chromatographic results clearly indicated that the size and geometry of cyclofructan is an important factor in interactions between cyclofructans and chiral analytes.

4.4.4 Effects of the nature of the derivatization group on enantioseparations

The nature of the derivatization group also contributes to chiral recognition. Our earlier studies of various aliphatic- and aromatic-derivatized CF6 stationary phases support this contention.[55] The nature of the aromatic group plays an important role since the derivatizing groups change the interactions between analyte and stationary phase (i.e., steric repulsion, π-π interaction, dipolar interactions, etc). Also, the R-naphthylethyl moiety itself is chiral, and its defined stereogenic configuration may contribute additional interaction sites.

Special attention was paid to the separations of racemic ruthenium(II) complexes. The RN-CF6 stationary phase provided significantly better selectivity toward Ru complexes than other derivatized-CF6 stationary phases, such as DMP-CF6, R/S-methylphenyl-CF6.[55] This fact indicated that the nature of the derivative plays a major role in the chiral recognition process between the Ru complex and the chiral selector. Comparisons between RN-CF6 and SN-CF6 provide further insights about the chiral recognition mechanism. The configuration of the naphthylethyl carbamate moieties is opposite for the "RN" (R-naphthylethyl) and "SN"-CF6 (S-naphthylethyl) stationary phases. RN-CF6 easily separates enantiomers of Ru(II) complexes, with enantioselectivities ranging from 1.49-2.90. The SN-CF6 column can also baseline separate all of the tested complexes, with enantioselectivities ranging from 1.17-1.97 (data not shown). The most important fact is that enantiomers of these metal complexes were separated on RN- and SN-CF6 columns with opposite elution orders. This indicates that the substituent significantly contributed to the chiral recognition. The stereogenic configuration of the naphthylethyl carbamate group is a major factor for chiral recognition, and the cyclofructan6 plays a secondary role. If CF6 played no role in the enantiomeric selectivity for these complexes, then the SN-CF6 CSP would produce exactly opposite enantiomeric separations. However, this is not the case.

Although the exact orientation of the aromatic groups of derivatized-CFs is not known, it is likely that chiral recognition is the sum of many different interactions arising from different parts of the bonded chiral selector. Both the derivative group and the core cyclofructan play an important role in enantiomeric separations.

4.4.5 Effects of the analyte structure

To try to understand the separation mechanism, it helps to look at closely related compounds, where slight structural changes greatly alter enantioselectivity. In Group E, compounds 44-51 are a series of structurally related compounds, which all contain a five-member ring, having -O-CO-NH-, or -S-CO-NH-, or -S-CS-NH- units.

For example, 4-phenyl-2-oxazolidinone (E44) and 4-phenylthiazolidine-2-thione (E45) have similar chemical structures, in which the only difference is that the -O-CO-NH- moiety in compound E44 is replaced by -S-CS-NH- in E45. Chromatographic results indicate that the RN-CF6 stationary phase provided enantioselectivity toward E45, but not E44, while the DMP-CF7 column separated racemic E44, but not E45. Another analogous example is the comparison of 4-benzyloxazolidine-2-thione (E46) and 4-benzylthiazolidine-2-thione (E47). E46 contains a -O-CS-NH- unit, while E47 has a -S-CS-NH unit. The other functionalities are the same. The RN-CF6 CSP provided enantioseparation only for E46. The DMP-CF6 column showed significantly

different selectivity toward E46 and E47 (1.15 vs 1.09). The structural difference between E44/E45, and E46/47 mainly affected dipolar interactions or hydrogen bonding interactions. The facts that the CSPs provided hugely different selectivities indicated that dipolar interactions or hydrogen bonding interactions play an import role in chiral recognition of cyclofructan chiral selctors.

Π-π interactions and steric interactions may significantly contribute to enantiomeric separations on cyclofructan-based stationary phases as well. For example, compared with E44, compound E51 has an additional phenyl group, which can provide additional π-π interactions and increase the steric bulkiness of the analyte. The fact that the DMP-CF7 stationary phase only separated racemic E44 while the RN-CF6 CSP exhibited chiral recognition only for E51, demonstrated that π-π and/or steric interactions can significantly affect chiral recognition interactions between the chiral selector and analyte.

4.5 Conclusions

The RN-CF6 and DMP-CF7 stationary phases provided chiral recognition toward a variety of compounds, including acidic, basic, neutral organic compounds, and metal complexes. It is interesting that they exhibited complementary selectivity and the combination of two columns provided enantioseparations for 43% of test analytes. Generally, better separation of amine containing compounds was obtained on the RN-CF6 stationary phases, while the DMP-CF7 column worked more effectively for acidic compounds in most cases.

It was found that both the crown ether ring and the nature of the derivative group on the cyclofructans affected selectivity and enantiomeric separations.

These extensive chromatographic results offered useful information for method development of specific chiral analytes and a better knowledge of enantioseparation mechanism. Also, they provide insight concerning design of new chiral stationary phases. Currently, the studies in which both ionic groups (anionic or cationic) and aromatic groups are inserted in one CF molecule, are underway, in hopes of finding more widely-applicable chiral selectors.

CHAPTER 5

ENANTIOMERIC SEPARATIONS OF ILLICIT DRUGS AND CONTROLLED SUBSTANCES USING CYCLOFRUCTAN BASED (LARIHC) AND CYCLOBOND I 2000 RSP HPLC CHIRAL STATIONARY PHASES

5.1 Abstract

Recently a novel class of chiral stationary phases (CSPs) based on cyclofructan (CF) has been developed. Cyclofructans are cyclic oligosaccharides that possess a crown ether core and pendent fructofuranose moieties. Aliphatic and aromatic functionalized cyclofructan 6 and 7 have been recently introduced as new chiral selectors which possess unique enantiomeric selectivities. Herein, we evaluate the applicability of these novel CSPs for the enantiomeric separation of chiral illicit drugs and controlled substances directly without any derivatization.

A set of 21 racemic compounds were used to evaluate these columns including 8 primary amines, 6 secondary amines, and 7 tertiary amines. Of the new cyclofructan based LARIHC columns, 15 enantiomeric separations were obtained including seven baseline and eight partial separations. The LARIHC CF6-P column proved to be the most useful in separating illicit drugs and controlled substances accounting for 12 of the 15 optimized separations. The polar organic mode containing small amounts of methanol in acetonitrile was the most useful solvent system for the LARIHC CF6-P CSP. Furthermore, the LARIHC CF7-DMP CSP proved to be valuable for the separation of the tested

chiral drugs resulting four of the optimized enatiomeric separations, whereas the CF6-RN did not yield any optimum separations. The broad selectivity of the LARIHC CF7-DMP CSP is evident as it separated primary, secondary and tertiary amine containing chiral drugs. The compounds that were partially or un-separated using the cyclofructan based columns were screened with a Cyclobond I 2000 RSP column. This CSP provided four baseline and six partial separations. The chiral analysis of an actual pharmaceutical product using the developed method was done.

5.2 Introduction

Many illicit drugs and controlled substances are chiral and occur as enantiomers (nonsuperimposable mirror image isomers). Enantiomers can have different pharmacological and/or psychotropic activities. They can have one or more chiral centers, and it is well known that one enantiomer of particular drug can be accepted for its medicinal usage while the other enantiomer may be a drug of abuse.[204] For example (S)-(+) methamphetamine is a widely abused, DEA schedule II, controlled substance whereas (R)-(-) methamphetamine is used in an over-the-counter nasal decongestant.[204,205] Amphetamine is one of the most frequently abused drugs. (S)-(+)-amphetamine is several times more potent than the R-(-)-enantiomer in eliciting central nervous system effects.[204,205] Conversely, R-(-)-amphetamine has somewhat more potent cardiovascular effects. Both methamphetamine and amphetamine are basic compounds that stimulate the

central nervous system and provide increased alertness, diminished fatigue, and cardiovascular activation etc.

In sports, drugs of abuse can provide unfair advantages, thus they are prohibited by the World Anti-Doping Agency.[206] These performance enhancing drugs can be produced as racemates (i.e., equimolar mixture of two enantiomers) or enantiomerically enriched compounds depending on the method of synthesis and purification. Thus the ability to separate and determine the enantiomeric ratios of drugs provides valuable intelligence for determining drug potency and for tracking its synthetic origin.[207-210] For example, the enantiomeric ratio of methamphetamine is closely related with the optical activity of its precursors (most commonly, ephedrine or pseudoephedrine).[209,210] This information is important and can be utilized for the investigation of manufacturing sources of methamphetamine starting materials. Lee *et al*. investigated the enantiomeric ratios of 433 crystalline methamphetamine samples seized in Korea from 1994 to 2005. They found that, most of the seizures were the pure S(+) enantiomer, but 21 % contained the R(-) enantiomer above the 1 % level and concluded that alternative precursor sources may have been used in these cases. To obtain this information, rapid and effective analytical techniques are required. In addition, separating enantiomers of these types of drugs is important for pharmacological, toxicological, and forensic studies, as well as production quality control.

There have been reports on a variety of analytical methodologies for separating enantiomers of illicit drugs and controlled substances, including: gas chromatography (GC),[204,211-213] high performance liquid chromatography (HPLC),[204,214-220] capillary electrophoresis (CE)[206,221], GC/MS,[222,223] LC/MS,[224-226] and CE-ESI-MS.[227] One approach may be superior to another depending on the goal of the investigator and the nature of the problems to be solved. Clearly, one method may be useful for biological assays while another approach is better suited for forensic assays or quality control of pharmaceutical products. For example, CE may be the best approach for the rapid analysis of pure compounds; however GC and LC may be more appropriate when analyzing low levels of a compound in physiological samples. Usually, GC using derivatized cyclodextrin chiral stationary phases is a highly effective approach in terms of efficiency, sensitivity and peak capacity for separating these enantiomers.[204,228] However in the case of physiological and biological studies on extremely small amounts of analyte, sensitivity and isolation from a complex biological matrix is problematic. Often, a prederivatization step is necessary in order to reach sufficiently low limits of detection in both GC and HPLC techniques.[215]

In this work, we report the enantiomeric separation of 21 racemic illicit drugs and controlled substances using new cyclofructan based chiral stationary phases and without the use of derivatizing agents. Cyclofructans are cyclic oligosaccharides that possess a crown ether core and pendent fructofuranose

moieties. Aliphatic and aromatic functionalized cyclofructan 6 and 7 have been recently introduced as novel chiral selectors[53-55] while native and sulfonated cyclofructan 6 were introduced as hydrophilic interaction liquid chromatographic stationary phases.[56,57] The chiral stationary phases based on aliphatic and aromatic functionalized cyclofructan 6 and 7 possess unique enantiomeric selectivities for chiral primary amines (alkyl derivatives) and broad selectivities for a wide variety of all other racemates. Herein, the applicability of these new CSPs for the enantiomeric separation of selected chiral illicit drugs and controlled substances is evaluated. Also, the Cyclobond I 2000 RSP column[83,229] was evaluated for the compounds that were not adequately separated using the cyclofructan based columns.

5.3 Experimental

5.3.1 Materials

LARIHC CF6-P, LARIHC CF6-RN and LARIHC CF7-DMP columns (25 cm × 0.46 cm (i.d)) were obtained from AZYP, LLC. (Arlington, TX, USA). The cyclobond I 2000 RSP column was obtained from Supelco (Bellefonte, PA, USA). All the analytes were purchased as racemates from Cerilliant Corporation (Round Rock, TX, USA) with the exception of cathinone and pseudoephedrine which were obtained as pure enantiomers and mixed together to produce racemates. Chloroephedrine was produced in house by Dr. Morrison. Acetonitrile (ACN), 2-propanol (IPA), n-heptane, ethanol (EtOH), and methanol (MeOH) of

HPLC grade were obtained from EMD (Gibbstown, NJ). Trifluoroacetic acid (TFA), acetic acid (AA), triethylamine (TEA), sodium carbonate and dichloromethane were purchased from Sigma-Aldrich (St. Louis, MO,USA). Water was purified by a Milli-Q water purification system (Millipore, Billerica, MA, USA). The over-the-counter drug (Mucinex D) was obtained from a local pharmacy.

5.3.2 Chromatographic Conditions

An Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) was used in this study. It consisted of a 1200 diode array detector, autosampler and quaternary pump. All separations were carried out at room temperature (20 ^{o}C) unless stated otherwise. For all HPLC experiments, the injection volume was 5 µL and the flow rate was 1.0 mL/min in isocratic mode. The UV wavelengths 195, 210, 254 and 280 nm were employed for detection. All analytes were dissolved in methanol or methanol/water mixtures. The mobile phase was degassed by ultrasonication under vacuum for 5min. Each sample was analyzed in duplicate.

Three operation modes (the normal phase, polar organic mode, and reversed phase) were tested. In the normal phase, heptane with ethanol or isopropanol was used as the mobile phase. In some cases, TFA and TEA were used as additives to optimize/improve separations. The mobile phase for the polar organic mode was composed of acetonitrile/methanol and small amounts of acetic

acid and triethylamine. Water/acetonitrile or acetonitrile/triethylammonium acetate buffer (0.5 %, pH 4-5) was used as the mobile phase in the reversed phase. The "dead time" t_0 was determined by the peak of the refractive index change due to the unretained sample solvent.

5.3.3 Extraction of the over-the-counter drug

One tablet of Mucinex D (600 mg guaifensin & 60 mg pseudoephedrine HCl) was ground well and dissolved in 20 ml of water. The pH of the sample was adjusted to 10.0 with 0.1 M sodium carbonate and the solution was stirred for 2 hours. The sample was extracted with 3 volumes of dichloromethane. The organic layer was concentrated using a rotorevaporator and the dried product (0.1 g) was dissolved in 1 ml of methanol for HPLC injection.

5.4 Results and Discussion

5.4.1 Enantiomeric separations of illicit drugs and controlled substances on LARIHC chiral stationary phases (CSPs)

Previous studies showed that LARIHC CF6-P, LARIHC CF6-RN and LARIHC CF7-DMP columns provide excellent selectivities for racemic compounds containing different functionalities.[53-55] In general, the LARIHC CF6-P shows unique selectivity and broad applicability for racemates containing primary amines.[54] Considering the ubiquity of amine groups in drugs of abuse, the current study focuses on a systematic chromatographic evaluation of these chiral stationary phases, mainly for chiral drugs containing amines. A set of 21 racemic

compounds were used to evaluate these columns including 8 primary amines, 6 secondary amines, and 7 tertiary amines. (Structures shown in Table 5.1 and 5.4

Table5.1 lists the chromatographic data for all compounds separated by the LARIHC columns. The data in Table 5.1 indicates the number of successful separations for compounds containing primary amines on LARIHC CF6-P. This stationary phase provided better separations in the polar organic mode compared to the normal phase and reverse phase modes. Seven out of eight primary amine-containing drugs were separated on the LARIHC CF6-P stationary phase. Clearly, this stationary phase provides excellent enantioselectivity toward a variety of primary amines with diverse structures. However, clenbuterol which contains both a primary and a secondary amine was not separated using this stationary phase, while it was separated on the LARIHC CF7-DMP column. The primary amine group on clenbuterol is five atoms away from the stereogenic center and is sterically hindered (by two neighboring Cl atoms). Further, it is an aromatic amine, all of which render this particular primary amine containing drug unseparable in the polar organic mode. A thorough review of the literature reports of polar organic enantiomeric separations of primary amines reveals that no aniline type primary amine has ever been separated on LARIHC CF6-P.[54]

Table 5.1 Chromatographic data of chiral illicit drugs and controlled substances separated on the LARIHC CF6-P and LARIHC CF7-DMP columns in the optimized conditions.

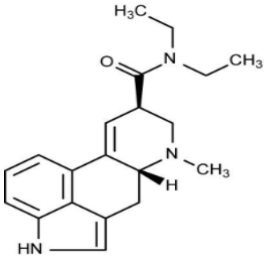
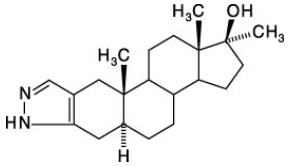
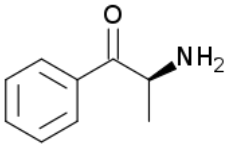
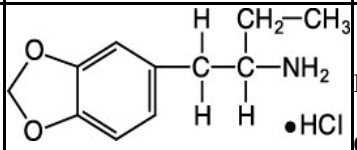
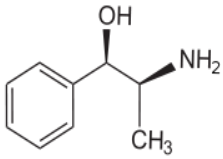
	Drug	**Structure**	**CSP**	**Mobile Phase**	**k'**	**α**	**R_s**
1	Lysergic acid diethylamide		LARIHC CF6-P	90ACN/ 10MeOH/0.3AA/ 0.2TEA	18.7	1.15	2.5
2	Stanozolol		LARIHC CF6-P	80HEP/20EtOH/ 0.1TFA	7.0	1.51	2.0
3	Cathinone		LARIHC CF6-P	70HEP/30EtOH/ 0.1TFA	15.9	1.09	1.5
4	3,4-Methylenedioxyphenyl-2-butanamine hydrochloride		LARIHC CF6-P	60ACN/40MeOH /0.3AA/0.2TEA	2.3	1.08	1.5
5	Phenylpropanolamine		LARIHC CF6-P	60ACN/40MeOH /0.3AA/0.2TEA	3.6	1.08	1.5

Table 5.1- Continued

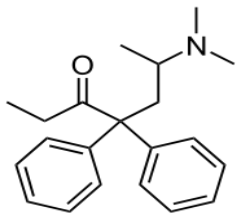
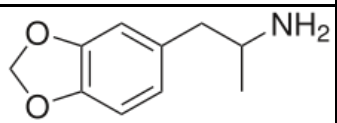
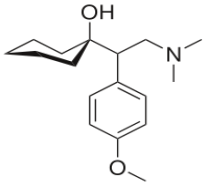
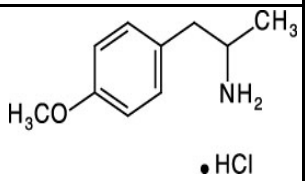
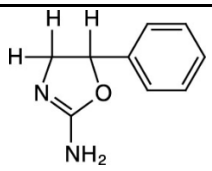
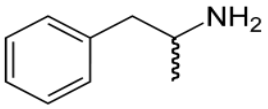
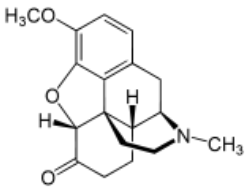
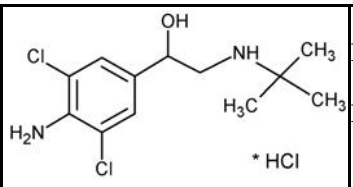
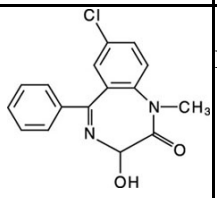
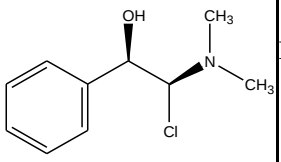
6	Methadone		LARIHC CF6-P	85ACN/15MeOH /0.3AA/0.2TEA	4.5	1.13	1.3
7	3,4-Methylenedioxyamphetamine		LARIHC CF6-P	80ACN/20MeOH /0.3AA/0.2TEA	14.5	1.05	1.3
8	Venlafaxine		LARIHC CF6-P	80ACN/20MeOH /0.3AA/0.2TEA	2.2	1.05	1.0
9	p-Methoxyamphetamine HCl		LARIHC CF6-P	80ACN/20MeOH /0.3AA/0.2TEA	14.2	1.02	0.8
10	Aminorex		LARIHC CF6-P	80ACN/20MeOH /0.3AA/0.2TEA	3.6	1.03	0.6
11	Amphetamine		LARIHC CF6-P	80ACN/20MeOH /0.3AA/0.2TEA	13.7	1.01	0.4
12	Hydrocodone		LARIHC -P	80ACN/20MeOH /0.3AA/0.2TEA	2.7	1.03	0.5

Table 5.1- Continued

13	Clenbuterol		LARIHC CF7-DMP	80Hep/20EtOH/0.1TFA	3.3	1.19	2.0
14	Temazepam		LARIHC CF7-DMP	80Hep/20EtOH/0.1TFA	3.5	1.10	1.8
15	Chloroephedrine		LARIHC CF7-DMP	90ACN/10M/0.3AA/0.2TEA	1.5	1.08	1.3

Another interesting obseravation can be made involving the enantiomeric separation mechanism of LARIHC CF6-P CSP by analyzing the separation data (Table 1) for cathinone, phenylpropanolamine and amphetamine. As shown in Figure 5.1, cathinone contains a ketone group and phenylpropanolamine contains an alcohol group next to the sterogenic center. These additional hydrogen-bonding functional groups (ketone and alcohol) contributed to the enantiomeric separation of cathinone (R_s = 1.1) and phenylpropanolamine (R_s = 1.5) respectively in the polar organic mode. However, amphetamine does not possess a neighboring hydrogen-bonding group next to the stereogenic center, and it is only partially separated (shoulder (R_s = 0.4) Further, it appears that the beneficial hydrogen-bonding contribution of the ketone in cathinone is not as great as the contribution

of the hydroxy group on phenylpropanolamine . Yet, it should be noted that primary amines without secondary hydrogen-bonding interactions have been separated on the LARIHC CF6-P.[54] Sun et.al reported the separation of α-methylbenzylamine with a R_s of 1.8.[54]

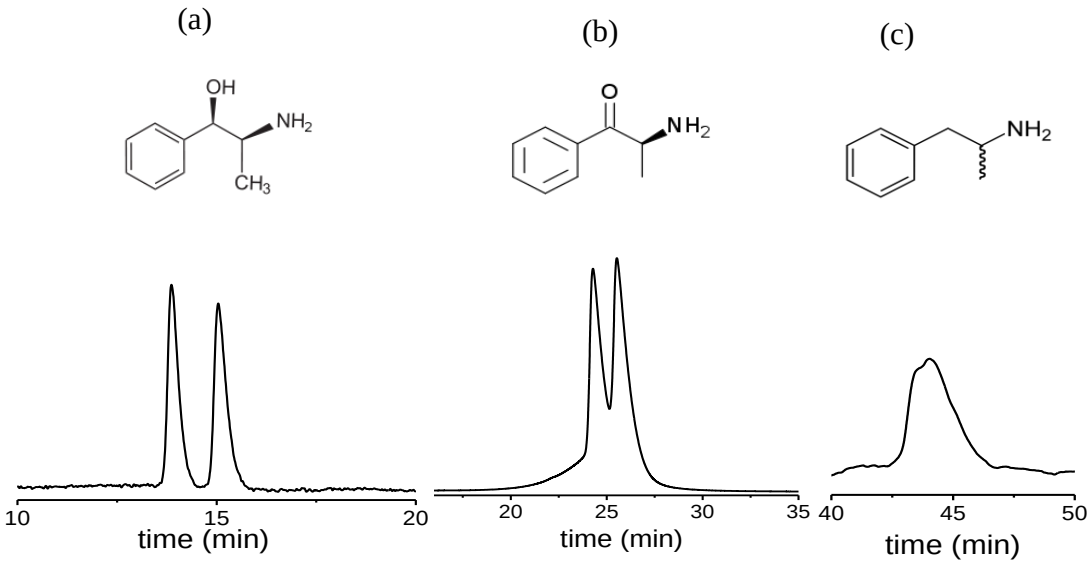


Figure 5.1Enantiomeric separation of (a) phenylpropanolamine (b) Cathinone (c) amphetamine

Mobile phase: (a) 60ACN/40MeOH/0.3AA/0.2TEA (b)90A/10M/0.3AA/0.2TEA (c): 80A/20M/0.3AA/0.2TEA on the LARIHCCF6-P column. UV detection at 254 nm, flow rate:1 ml/min

Considering α-methylbenzylamine (R_s=1.8) only differs from amphetamine (R_s=0.4) by a single -CH_2 group which separates the aromatic ring

from the stereogenic center, it can be postulated that when there is no hydrogen-bonding group present in the chiral primary amine drug, a ring structure alpha to the stereogenic center is required for complete enantiomeric separation.

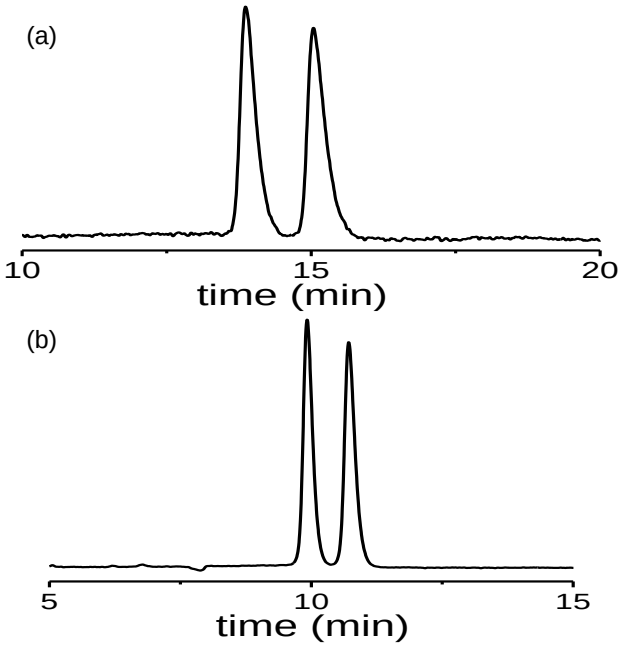


Figure 5.2 Enantiomeric separation of (a) phenylpropanolamine and (b) 3,4-methylenedioxyphenyl-2-butanamine hydrochloride on the LARIHC-CF6-P column.

Conditions: Mobile phase: 60ACN/40MeOH/0.3AA/0.2TEA, UV detection at 254 nm, flow rate:1 ml/min

Representative chromatograms of the separation of phenylpropanolamine and methylenedioxyphenyl-2-butanamine hydrochloride and on the LARIHC CF6-P column are shown in Figure 5.2. Enantiomers of both drugs were separated in the polar organic mode with a resolution of 1.5. The LARIHC CF6-RN and LARIHC CF7-DMP columns were not as effective in separating these drugs. Pihlainen *et. al* studied the enantiomeric separation of similar controlled substances using vancomycin and native β-cyclodextrin CSPs.[224] They reported that β-cyclodextrin was more suitable than vancomycin for the separation of phenylethylamines yielding partial separations (R_s < 1.5) for amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, *p*-methoxy-methamphetamine, *p*-methoxy-amphetamine and baseline separations ($R_s \geq 1.5$) of 3,4-methylenedioxyethylamphetamine and 3,4-methylenedioxyethylmethamphetamine).[224] Phenylpropanolamine which could not be separated on the cyclodextrin column was baseline separated (R_s =1.5) on the LARIHC CF6-P column in the polar organic mode (Figure 5.2).

LARIHC CF6-P was also able to separate lysergic acid diethylamide (LSD), stanozolol, cathinone, 3,4-methylenedioxyphenyl-2-butanamine hydrochloride, methadone, phenylpropanolamine, 3,4-methylenedioxyamphetamine, venlafaxine methoxyamphetamine, aminorex amphetamine and hydrocodone, which cannot be effectively separated using the other stationary phases tested. Enantiomers of methadone and hydrocodone which

contain tertiary amines were partially separated on LARIHC CF6-P (Table 5.1). LSD, which contains both a tertiary and a secondary amine was baseline separated. Surprisingly, other analytes that contained only secondary amines were not separated on this stationary phase (e.g. 3,4-methylenedioxy methamphetamine and 3,4-methylenedioxyethylamphetamine). Overall, the LARIHC CF6-P exhibited enantioselectivity toward 73% of the chiral drugs evaluated, proving this stationary phase is broadly applicable for these types of compounds.

Among the aromatic derivatized-cyclofructan CSPs (LARIHC CF6-RN and LARIHC CF7-DMP), the LARIHC CF7-DMP CSP produced better separation results. Four of the optimized enantiomeric separations listed in Table 5.1 were obtained on the CF7-DMP phase, whereas the CF6-RN did not yield any optimum separations. Phenobarbital, clenbuterol and temazepam were baseline separated while chloroephedrine was partially separated (R_s = 1.3) on the LARIHC CF7-DMP phase. The broad selectivity of the LARIHC CF7-DMP CSP is evident as it showed enantioselectivity toward analytes which include primary, secondary and tertiary amines. Compared to LARIHC CF6-P, the LARIHC CF7-DMP column is not as effective in separating the tested enantiomers, however, it demonstrates some complementary enantiomeric selectivities. For example, clenbuterol (CLB) which was well separated (R_s = 2.0) on the LARIHC CF7-DMP column with good selectivity (α = 1.19), was not separated on the LARIHC CF6-P or LARIHC CF6-RN columns.

The LARIHC CSPs showed better selectivity and resolution in the polar organic mode as compared to the reversed phase mode. Water in the reversed phase system may compete too effectively for hydrogen bonding sites on the chiral stationary phase, and thus it has a negative effect on the enantiomeric separation. The LARIHC CF6-P CSP performed best in the polar organic mode, while the LARIHC CF7-DMP CSP excelled under normal phase conditions. It is well-known that certain interactions such as π-π, n-π, dipolar, and hydrogen bonding interactions are enhanced in less polar solvents.[55] Thus, these must be the dominant associative interactions in these enantiomeric separations.

5.4.2 Effect of additives on mobile phase

Enantiomeric separations of amine-containing drugs were observed mainly in the polar organic mode. However, two analytes (stanozolol and cathinone) were separated more effectively on LARIHC CF6-P in the normal phase mode. Yet, higher efficiencies and better resolutions usually were observed in the polar organic mode which also offered short analysis times and better analyte solubility in the mobile phase. It has been shown that the highest enantioselectivity in the polar organic mode was obtained using the combination of triethylamine (TEA) and acetic acid (AA) as additives on CF6 based CSPs.[53-55] In order to evaluate the effects of the ratio of TEA and AA in the polar organic mode, the separation of 3,4-methylenedioxyphenyl-2-butanamine hydrochloride and phenylpropanolamine which are baseline separated in the mobile phase

containing 60/40 ACN/MeOH were investigated. Table 5.2 shows the retention factors, selectivity and resolution with respect to different amounts of TEA and AA in the mobile phase (60/40 ACN/MeOH). These analytes were barely retained when no additives were used. Similar behavior was observed in the presence of 0.1 % (v/v) TEA where the analytes were in their neutral form. Also, more symmetrical peaks were observed when 0.1 % TEA was used, due to the interaction of TEA with free silanol groups. The retention time of these analytes increased dramatically when 0.1 % (v/v) AA was added to the mobile phase and the analytes were in their protonated form. Under these conditions tailing peaks were observed for most of the analytes, and neither resolution nor selectivity improved significantly.

Retention times were decreased when equimolar amounts of TEA and AA were used. In this case the resolution improved significantly. When higher molar amounts of TEA were used higher efficiencies were observed. However, resolution and selectivity were maximized when higher amounts of AA were used compared to the TEA amount. Ultimately, the combination of 0.3% AA and 0.2% TEA (by volume) was determined to be the optimal polar organic mobile phase additive ratio.

Table 5.2 Chromatographic data of 3,4-Methylenedioxyphenyl-2-butanamine hydrochloride and phenylpropanolamine on the LARIHC CF6-P column with different additive ratios.

Conditions:Mobile phase: 60ACN/40MeOH, UV detection at 254 nm, flow rate:1 ml/min

	Additive percentage (v/v) in the Mobile Phase		**3,4-Methylenedioxyphenyl-2-butanamine hydrochloride**			**Phenylpropanolamine**		
	%	**% AA**	**k'**	**α**	**R_s**	**k'**	**α**	**R_s**
1	0.0	0.0	0.35	1.00	0.0	0.10	1.02	0.5
2	0.10	0.0	0.62	1.00	0.4	1.00	1.02	0.6
3	0.0	0.10	5.42	1.06	0.8	7.00	1.08	0.8
4	0.36	0.15	1.44	1.05	1.3	2.43	1.05	1.4
5	0.89	0.10	0.82	1.03	1.2	1.43	1.02	1.2
6	0.20	0.30	2.31	1.08	1.5	3.62	1.08	1.5

5.4.3 Effects of mobile phase composition

It has been reported that the use of acetonitrile as a modifier produces the best resolution in the polar organic mode.[54] Therefore, the effect of the percentage of acetonitrile was examined. The percentage of acetonitrile was increased from 0% to 100% when the acid/base additive concentrations were kept constant (0.3% AA and 0.2 % TEA).

Table 5.3 Chromatographic data of 3,4-Methylenedioxyphenyl-2-butanamine hydrochloride and phenylpropanolamine on the LARIHC CF6-P column with different amounts of ACN in the mobile phase (0.3AA/0.2TEA), UV detection at 254 nm, flow rate:1 ml/min

	Mobile Phase (0.3AA/0.2TEA)		**3,4-Methylenedioxyphenyl-2-butanamine hydrochloride**			**Phenylpropanolamine**		
	%	**%**	**k'**	**α**	**R_s**	**k'**	**α**	**R_s**
1	10	90	0.50	1.04	1.2	0.60	1.04	1.2
2	20	80	0.52	1.04	1.3	0.66	1.04	1.3
3	40	60	0.10	1.05	1.4	1.40	1.06	1.4
4	60	40	2.31	1.08	1.5	3.62	1.08	1.5
5	85	15	3.55	1.09	2.0	6.05	1.10	2.0

Table 5.3 shows the retention factors, selectivity and resolution of two probe compounds (the same compounds used to investigate additive effects) with varying ACN concentration. Increasing the concentration of acetonitrile increases the retention considerably. This is due to the competition of the methanol with the analyte for hydrogen bonding sites on the stationary phase. Also, both selectivity and resolution were increased when increasing the acetonitrile concentration. The concentration of acetonitrile has a significant effect on the enantiomeric separation of the selected drugs. Therefore, optimization can be easily achieved by varying the ACN percentage.

5.4.4 Separations on Cyclobond I 2000 RSP column

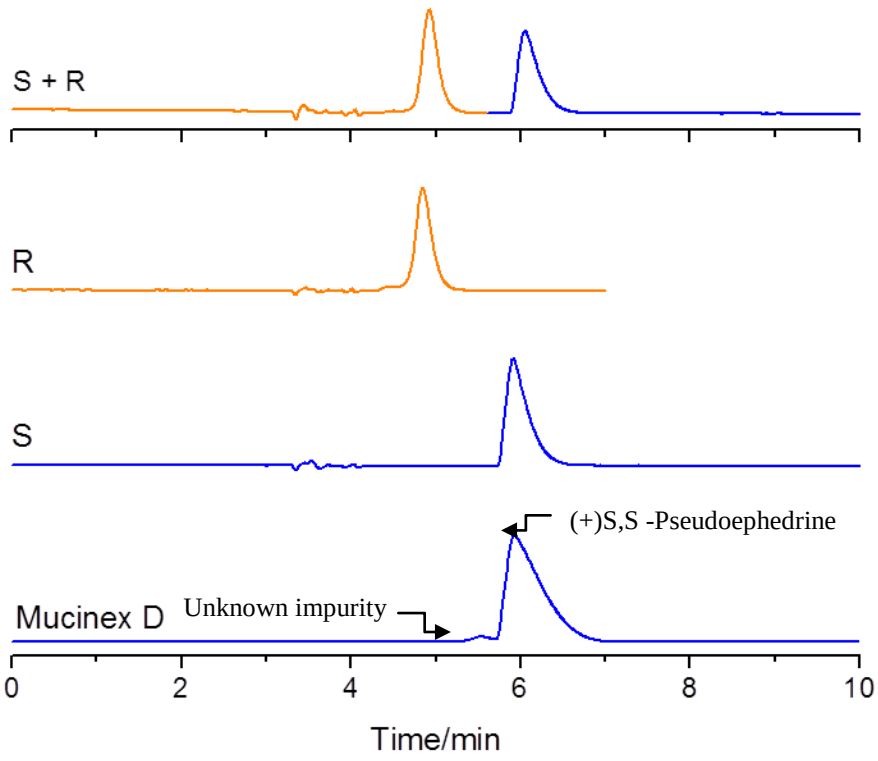


Figure 5.3Separation of (a) Pseudoephedrine racemate (b) (-) (R,R)-pseudoephedrine (c) (+) (S,S)-pseudoephedrine (d) Mucinex D (600 mg guaifensin & 60 mg pseudoephedrine HCl) on the Cyclobond I 2000 RSP column. Conditions: Mobile phase: 95 TEAA(0.5 %, pH=4.2)/ 2.5 MeOH/ 2.5 ACN, UV detection at 254 nm, flow rate: 1ml/min

The compounds that were partially or not separated using the cyclofructan based columns were screened with a Cyclobond I 2000 RSP column. Table 5.4 summarizes the optimized separations of the drugs on the Cyclobond column. Pseudoephedrine, methylephedrine, methadone and chloroephedrine were baseline separated on this stationary phase ($R_s \geq 1.5$). 3,4-methylenedioxyethylamphetamine, 3,4-methylenedioxy methamphetamine, methamphetamine and methoxymethamphetamine were partially separated on this column whereas they were not separated on the LARIHC columns. However, 3,4-methylenedioxyamphetamine, venlafaxine and p-methoxyamphetamine HCl were partially separated on the both LARIHC CF6-P and Cyclobond I 2000 RSP columns and the LARIHC CF6-P column and provided better resolutions compared to the Cyclobond I 2000 RSP column.

The Cyclobond I 2000 RSP column was applied to the enantiomeric resolution of pseudoephedrine in a Mucinex D over-the-counter pill. Only (+)-Pseudoephedrine was detected for Mucinex D, as shown in Figure 5.3. In order to confirm whether low levels of enantiomeric impurities (i.e. <0.1%) were present in this drug, a high concentration of the analyte was injected (the cause for the broadened peak seen in Figure 3 (d)), yet no (-)-Pseudoephedrine was detected.

Table 5.4 Chromatographic data of chiral illicit drugs and controlled substances separated on the Cyclobond I 2000 RSP column the optimized condition.

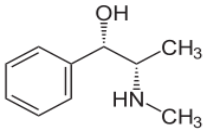
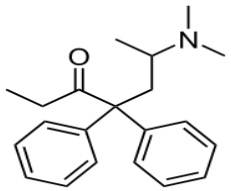
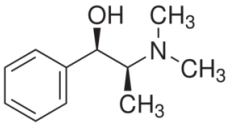
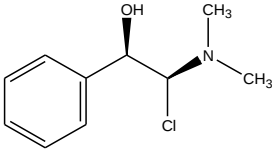
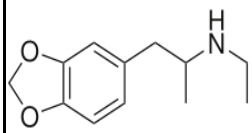
	Drug	Structure	Mobile Phase	k'	α	R_s
1	(Pseudoephedrine		95 (0.5 %TEAA, pH=4.2)/ 2.5 MeOH/ 2.5 ACN	1.5	1.20	2.0
2	Methadone		95 (0.5 %TEAA, pH=4.2)/ 5 % MeOH	3.92	1.28	2.0
3	Methylephedrine		99 (0.5 % TEAA pH=4.2)/ 0.5 MeOH/ 0.5 ACN	1.0	1.15	1.5
4	Chloroephedrine		98 (0.5 % TEAA pH=4.2)/ 1 MeOH/ 1ACN	2.9	1.24	1.5
5	3,4Methylenedioxy ethylamphetamine		98 (0.5 % TEAA pH=4.2)/ 1 MeOH/ 1ACN	7.0	1.10	1.3

Table 5.4 – Continued

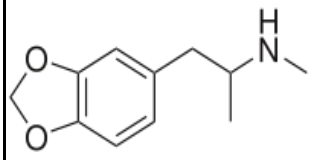
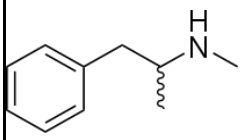
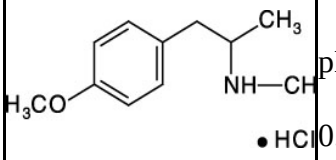
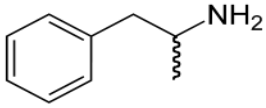
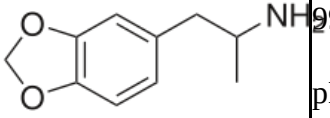
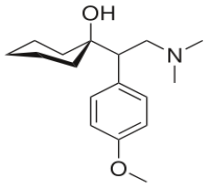
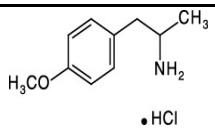
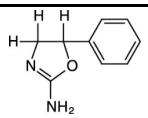
6	3,4-Methylenedioxy Methamphetamine		95 (0.5 % TEAA pH=4.2)/ 2.5 MeOH/ 2.5 ACN	3.5	1.08	1.3
7	Methamphetamine		99 (0.5 % TEAA pH=4.4)/ 0.5 MeOH/ 0.5 ACN	2.6	1.08	1.0
8	p-Methoxymethamphetamine		99 (0.5 % TEAA pH=4.1)/ 0.5 MeOH/ 0.5 ACN	1.56	1.04	1.0
9	Amphetamine		95 (0.5 % TEAA pH=4.4)/ 2.5 MeOH/ 2.5 ACN	1.0	1.03	0.8
10	3,4-Methylenedioxyamphetamine		99 (0.5 % TEAA pH=4.7)/ 1 MeOH	1.82	1.06	1.0
11	Venlafaxine		99 (0.5 % TEAA pH=4.7)/ 1 MeOH	3.43	1.00	No

Table 5.4 - Continued

12	p-Methoxyamphetamine HCl		99 (0.5 % TEAA pH=4.7)/ 1 MeOH	1.28	1.04	0.7
13	Aminorex		99 (0.5 % TEAA pH=4.7)/ 1 MeOH	1.15	1.04	0.8

5.5 Conclusion

In this study, three new cyclofructan based chiral selectors were evaluated for their ability to separate enantiomers of 21 chiral illicit drugs and controlled substances. Eight baseline separations and eight partial separations were obtained. The LARIHC CF6-P CSP resulted in the greatest number of enantiomeric separations and yielded resolution values as high as 2.5. In general, the polar organic mode was the most useful solvent system, were small amounts of methanol in acetonitrile was employed. It should be noted that this mobile phase composition is mass spectrometry compatible, thus these methods could be directly applied to LC-MS techniques which could be useful for sensitive drug detection in biological matrices. It is anticipated that this novel line of CSPs will prove useful in further analyses of chiral illicit drugs and controlled substances and will aid in gathering intelligence on the production origin of these drugs of abuse.

CHAPTER 6

DEVELOPMENT OF NEW CHIRAL STATIONARY PHASES BASED ON BASIC/CATIONIC DERIVATIVES OF CYCLOFRUCTAN 6 FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

6.1 Abstract

New classes of chiral selectors based on cyclofructan 6 (CF6) derivatized with imidazole, benzimidazole, pyridine, aliphatic and aromatic amines were introduced for the first time as bonded chiral stationary phases for high performance liquid chromatography (HPLC). Cyclofructans are cyclic oligosaccharides that possess a crown ether core which have attached pendent fructofuranose moieties. Aliphatic and aromatic functionalized cyclofructan 6 and 7 have been recently introduced as new chiral selectors which possess unique enantiomeric selectivities. Herein, basic/cationic derivatives were synthesized of CF6 and they were covalently bonded to silica. The separation performances of the newly synthesized chiral stationary phases were examined with a broad range of racemic compounds including acids, neutral compounds, secondary and tertiary amines, amino alcohols and amino esters. Enantiomeric separations were achieved for many chiral analytes. Additionally, these stationary phases can be protonated creating positively charged pyridinium, imidazolium and ammonium groups which provide electrostatic interactions and strong hydrogen bonding with

the analytes. Among the synthesized CSPs, imidazole functionalized CF6 shows better selectivity than other cationic derivatives.

6.2 Introduction

Enantiomeric separations have attracted great attention in areas of chemistry, biochemistry, pharmacology, etc. In particular, chirality is a major concern in the pharmaceutical industry. Separation and isolation of enantiomers in active pharmaceutical ingredients is critical in this field since they may have different pharmacological, pharmacokinetic and pharmacodynamic effects.[64,65] Enantioseperation is achieved by the use of chiral selectors in the form of i) chiral derivatization reagents, ii) chiral stationary phases (CSPs) or iii) chiral mobile phase additives.[52] All these methods find application in high performance liquid chromatography (HPLC) as a highly efficient and selective technique in chiral analysis.

There are a growing number of CSPs that are commercially available today i.e. polysaccharide-based CSPs,[69-71] macrocyclic antibiotic CSPs,[72-74,76,77,81] and cyclodextrins (CD) CSPs.[39,82-85] CDs are macrocylic oligosaccharides with a hydrophilic outer surface and a hydrophobic cavity and they have been used extensively for enantioseparations. Many useful chiral selectors based on cyclodextrins including native-CDs (α-CD, β-CD,γ-CD),[230,231] neutral CDs (acetyl β-CD, hydroxypropl-β-CD, 2,3-dimethyl-β-CD[16,232] have been reported. Also,

there are few reports on the application of anionic and cationic CD chiral selectors. Stalcup et al. reported that sulfated β-CD as a chiral mobile phase additive in capillary electrophoresis (CE) or capillary zone electrophoresis (CZE)[233] and as CSP in HPLC.[234] These negatively charged β-CD CSPs have shown enantioselectivity towards a broad range of chiral analytes. Both electrostatic interactions and inclusion into the hydrophobic cavity contributed to the enantioselectivity. Cationic β-CD chiral selectors have also been reported for CE.[235,236] A few cationic β-CD CSPs (e.g. alkyl imidazole, alkyl amine, vinyl pyridine) were successfully used for enantioseparations of different types of analytes (e.g. phenyl alcohols, flavanones and thiazides) in HPLC[237-240] and CE.[236,241-243]

Recently, Armstrong, et al. introduced a novel class of CSPs based on cyclofructans (CFs).[53-55] Cyclofructans are cyclic oligosaccharides that are quite different from CDs in both their structure and behavior. CFs possess a crown ether core with six or more (2 →1) linked D-fructofuranose units (commonly abbreviated CF6, CF7, CF8). Aliphatic and aromatic functionalized cyclofructan 6 and 7 have been introduced as new chiral selectors which possess unique enantiomeric selectivities for chiral primary amines (alkyl derivatives) and broad selectivities for a wide range of other racemates. In addition, native and sulfonated CF6 have been introduced as stationary phases for hydrophilic interaction chromatography.[56,57] Over the past several years, chiral selectors

bearing charged groups have been proposed because they are more soluble in water and are able to establish ion pair interactions with charged analytes. These derivatives are commonly used as chiral additives for separation, particularly in capillary electrophoresis (CE). The main advantage of charged selectors is that they can provide higher mobility differences between enantiomers.[244] Recently, Jiang et al. reported that the application of sulfate functionalized CFs as chiral selectors in CE and showed exceptional selectivity toward many cationic analytes, including primary, secondary, and tertiary amines and amino acids.[51] Electrostatic interaction between the sulfated CFs and the ammonium group of the analytes played a dominant role in enantioselectivity.

The correlation of the cationic moieties with the enantioseparation of CSPs in HPLC is intriguing and the use of cationic functionalized CF6 CSPs covalently bonded to silica gel in HPLC is still unexplored. Additionally these charged groups could contribute hydrogen bonding sites, π-π interactions and/or steric hindrance to chiral recognition. The purpose of the present work is to evaluate basic derivatized CF6 CSPs for enantioselectivity. To accomplish this goal, we synthesized three types of imidazole, benzimidazole, pyridine, alkyl and aromatic amine (3-(dimethylamino)-1-propylamine and 4-(dimethylamino) phenyl resectively) derivatized CF6s and covalently bonded them to silica gel. The enantioseparation performance of these seven CSPs was investigated by

using 13 chiral acids, 7 chiral neutral compounds, 14 chiral bases including secondary and tertiary amines, amino alcohols and esters.

6.3 Experimental

6.3.1 Reagents

CF6 was used for the present study due to its highly defined geometry and obtained from AZYP (Arlington, Daiso silica of 5 μm spherical diameter (100 Å, 440 m^2/g) was utilized and the synthesized stationary phases were slurry packed into 25 cm × 0.46 cm (i.d) stainless steel columns. Anhydrous toluene, anhydrous pyridine, anhydrous dimethylformamide (DMF), ethyl acetate, acetic acid (AA), triethylamine (TEA), trifluoroacetic acid (TFA), ammonium acetate, 3-(triethoxysilyl)propyl isocyanate, 1,1′-carbonyldiimidazole (CDI), 1-(3-aminopropyl)imidazole, 2-(aminomethyl)benzimidazoledihydrochloride, 3-(dimethylamino)-1-propylamine, 4-(dimethylamino)phenyl isocyanate and 3-pyridyl isothiocyanatewere purchased from Sigma-Aldrich (St.Luis, MO). All of 34 racemic analytes tested in this study were purchased from Sigma–Aldrich, Anichem (North Brunswick, NJ, USA).Acetonitrile (ACN), methanol (MeOH), n-heptane (Hep), isopropyl alcohol (IPA) of HPLC grade were obtained from VWR (Sugarland, TX). Water was purified by a Milli-Q-water purification system (Millipore, Billerica, MA).

6.3.2 Preparation of CF6 derivatives

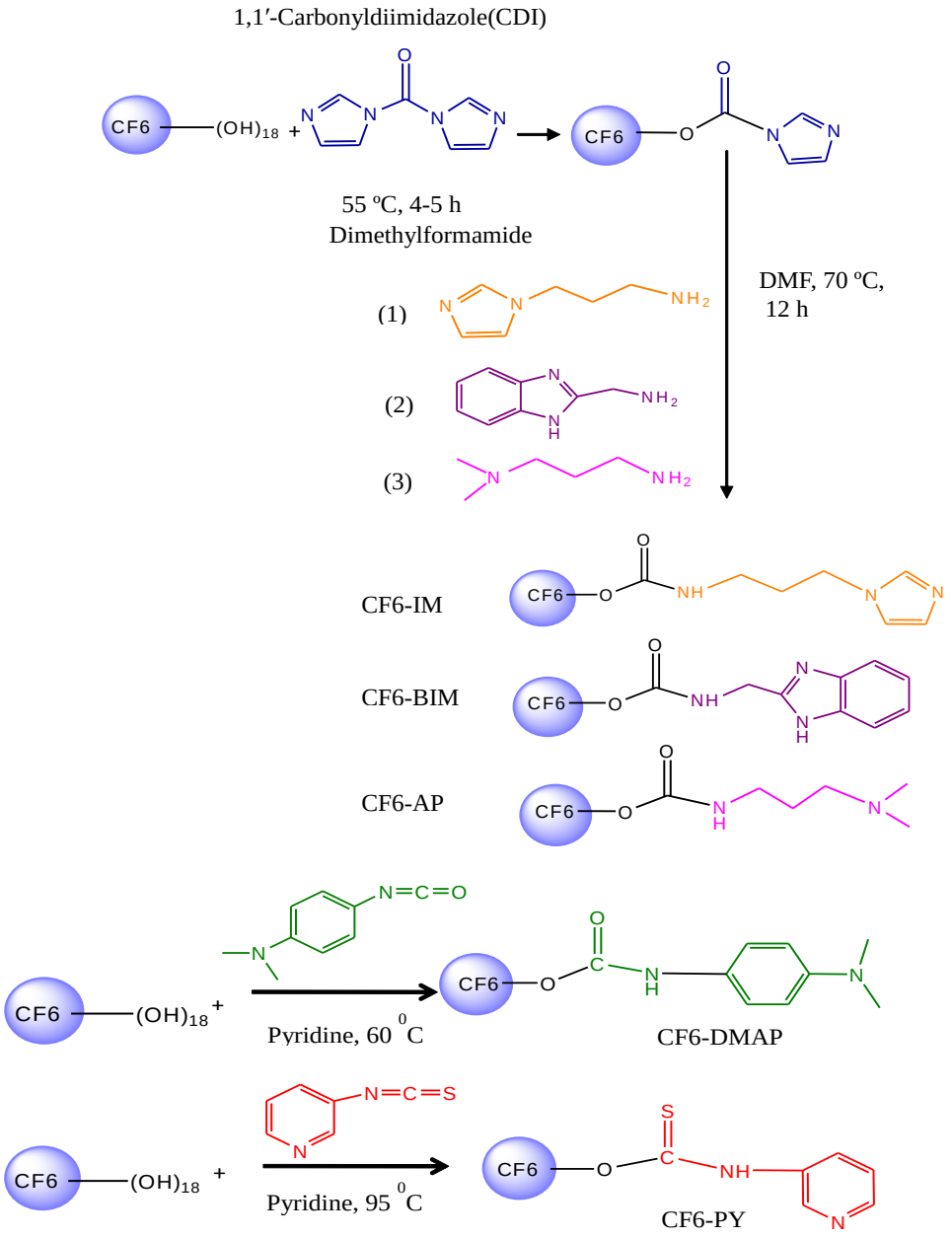


Figure 6.1Synthesis of cationic derivatives of CF6

The synthetic procedure for basic derivatives of CF6 are depicted Figure 6.1. Imidazole, benzimidazole and 3-(dimethylamino)-1-propylamine functionalized CF6s were prepared using 1,1′-Carbonyldiimidazole (CDI) reagent. The advantages of this reagent includes its ease of use and relatively low toxicity when compared to the other possible reagents (e.g. phosgene) and simple by products (imidazole).[245,246]The acyl imidazole moiety formed in situ by reacting CDI with the hydroxyl groups on CF6. These reactive acyl imidazole groups were then reacted with the corresponding primary amine (one pot). Attempts to isolate the acyl imidazole intermediate resulted in an insoluble gel. The other derivatization groups were also bonded to CF6 via a carbamate or a thiocarbamate linkage. Detailed procedures are provided in the following sections. All the reactions were carried out under dry nitrogen atmosphere protection.

6.3.2.1 Preparation of imidazole functionalized CF6 (CF6-Im-1 – 3)

1.00 g (1.03 mmol) of CF6 was dissolved in anhydrous DMF (40 ml) in a 100 mL round bottom flask (RBF)using a heat gun (~ 1 min) and then sonication (~1-2 mins). 1.017 g of CDI (6.27 mmol) was added to this solution and then placed in an oil bath. The temperature of the oil bath was slowly raised to 55°C and held there for five and half hours. By this time the CDI was entirely consumed and the acyl imidazole intermediate was confirmed by electrospray ionization mass spectrometry (ESI-MS) (not shown). 0.982 ml of 1-(3-

aminopropyl) imidazole (8.23 mmol) was added to the same pot and the temperature rose to 70°C and the reaction continued overnight. The following day the mixture was dumped into a large volume of toluene. A white precipitate formed and the product (CF6-IM-1 and CF6-IM-2) was recovered by suction filtration and dried in a vacuum oven (P_2O_5) overnight. The molecular mass distribution of the product was determined by ESI-MS(3-8imidazole groups with the average degree of substitution of six). Yield = 90 %. The amounts of reagents were doubled to afford the more highly substituted derivative (CF6-IM-3) which contained 8-14 imidazole groups with the average degree of substitution of ten (90 % yield).

6.3.3 Preparation of benzimidazole functionalized CF6 (CF6-Bim)

Procedure (6.3.2.1) was followed up to the point where the acyl imidazole intermediate was formed. Thereafter, 3.4 g of 2-(aminomethyl)benzimidazole dihydrochloride (15.45 mmol) was added to the mixture and the temperature of the oil bath was raised to 70 °C. The reaction continued overnight and was purified according to the 6.3.2.1. The mass spectrum showed that CF6-BIM is a mixture containing 7-11benzimidazole groups with the average degree of substitution of nine (90 % yield).

6.3.4 Preparation of 3-(dimethylamino)-1-propylamine functionalized CF6 (CF6-AP)

Procedure (6.3.2.1) was followed up to the point where the acyl imidazole intermediate was formed. Thereafter, 1.683 mL of 3-(dimethylamino)-1-propylamine (13.4 mmol) and allowed to react overnight at 70 ^{o}C. Purification was performed as above. The mass spectrum showed that CF6-AP is a mixture containing eight to fourteen 3-(dimethylamino)-1-propylamine groups with the average degree of substitution of eleven (90 % yield).

6.3.4.1 Preparation of 4-(dimethylamino)phenylfunctionalized CF6 (CF6-DMAP)

1.00 g (1.03 mmol) of CF6 was dissolved in 30 mL anhydrous pyridine (100 mL RBF) at 60°C (oil bath) under nitrogen protection for two hours. Then, 1.335 g of 4-(dimethylamino)phenyl isocyanate (8.23 mmol) was dissolved in 10 ml of anhydrous pyridine and added drop wise over 30 min with stirring. The reaction was carried out an additional five hours. The reaction mixture became slightly yellow and the solution was rotary evaporated under reduced pressure. The crude product was washed with ethyl acetate to remove impurities. The purified, final product was recovered by suction filtration and dried in a vacuum oven (P_2O_5) overnight. The mass spectrum showed that CF6-DMAP is a mixture containing four to eight 4-(dimethylamino)phenyl groups with the average degree of substitution of six (88 % yield).

6.3.4.2 Preparation of pyridine functionalized CF6 (CF6-PY)

1.00 g (1.03 mmol) of CF6 was dissolved in 30 mL anhydrous pyridine (100 mL RBF) at 60°C under nitrogen protection for two hours. Then, 0.918 ml of 3-pyridyl isothiocyanate (8.22 mmol) was dissolved in 10 ml of anhydrous pyridine and added drop wise over 30 min with stirring. The temperature of the oil bath was raised to 90ºC and allowed to react for an additional five hours. The reaction mixture became slightly brown and the solution was rotary evaporated under reduced pressure. The crude product was purified as in 6.3.4.1. The mass spectrum showed that CF6-PY is a mixture containing three to seven pyridyl groups with the average degree of substitution of five (85 % yield).

6.3.5 Preparation of CSPs

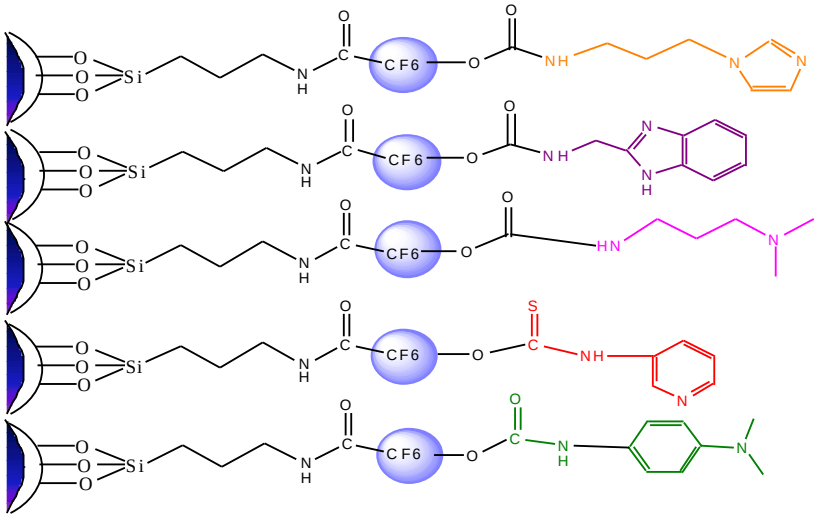


Figure 6.2 Sationary phase based on cationic derivatives of CF6

Figure 6.2 shows the structures of covalently bonded cationic derivatives of CF6 CSPs. Derivatized CF6 (2 g) was dissolved in 40 mL of anhydrous DMF in a 100 mL RBF (oil bath). To this solution, 0.536 mL of 3-(triethoxysilyl)propylisocyanate (2.06 mmol) in 10 ml of anhydrous pyridine was added drop wise and the oil bath heated to 90 °C and held there for 5 h. Next, residual water was removed from oven-dried silica gel (3 g in a three neck 250 mL RBF) using a Dean-Stark trap and 150 mL of anhydrous toluene (~20 mL removed). After the two reaction mixtures were cooled to room temperature, the derivatized CF6 reaction mixture was added to the silica-toluene slurry and heated to 110 °C and allowed to react overnight.

Table 6.1 Elemental analysis data for synthesized chiral stationary phases

Name	**C %**	**N %**	**Ratio CF6:Linker**	**Average degrees of derivatives on CF6**
SP-CF6-IM-1	19.4	5.6	1:4	6
SP-CF6-IM-2	14.8	3.5	1:2	6
SP-CF6-IM-3	12.2	3.8	1:2	10
SP-CF6-BIM	16.6	4.2	1:2	9
SP-CF6-AP	11.3	2.7	1:2	11
SP-CF6-PY	12.6	1.8	1:2	6
SP-CF6-DMAP	12.1	2.5	1:2	5

The reaction mixture was cooled and the CSP filtered and washed with toluene, DMF, pyridine, water/methanol mixture, acetone and DCM. After the product was dried in vacuum overnight (P_2O_5), the desired CSP was obtained. Table 6.1 shows elemental analysis data for the all CSPs, indicating that significant amounts of derivatized CF6 were attached to the silica surface (carbon loading > 11 % for all the CSPs). A 1:4 molar ratio of CF6: linker was used for the SP-CF6-IM1 and 19.4 % carbon loading was obtained for this particular CSP.

6.3.6 HPLC method

An Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA, USA) was used in this study. It consists of a diode array detector and a temperature controlled column chamber, auto sampler and quaternary pump. For data acquisition and analysis, the Chemstation software version Rev. B.01.03 was used on the system in Microsoft Windows XP OS. All separations were carried out at room temperature (20 – 23 ºC) unless stated otherwise. For all HPLC experiments, the injection volume was 5 µL and flow rate was 1.0 mL/min in isocratic mode. The following UV wavelengths were monitored: 195, 210, 254 and 280 nm.

Enantioseparations were evaluated and optimized in the normal phase, polar organic and reverse phase modes. The normal phase mode consisted of heptane/ethanol/TFA mobile phase compositions. Acetonitrile/methanol mixtures

containing acetic acid and triethylamine additives were used in the polar organic mode while the reversed phase mode consisted of acetonitrile/ammonium acetate buffer (pH range- 4-6.5) mobile phase compositions. The "deadtime," t_0, was estimated by the refractive index changed by the solvent peak. All analytes were dissolved in ethanol or ethanol/water mixtures. All mobile phases were degassed by sonication under vacuum for five minutes. Each sample was analysed in duplicate.

6.4 Results and discussion

Previous studies showed that aliphatic and aromatic functionalization of cyclofructans significantly broadened the application range of these CSPs.[53-55] Therefore, basic/cationic substitution groups on cyclofructan 6 (CF6), including imidazole, benzimidazole, pyridine, aliphatic and aromatic amines were tested. Chiral recognition of these stationary phases may be attributed to the presence of hydrogen bonding sites, π-π interactions, electrostatic interactions (for ionizable analytes) and/or steric hindrance which are mainly provided by the cationic substutions on the CF6. Table 6.2 lists the chromatographic data for all compounds examined by the newly synthesized CSPs. The data in Table 2 indicates the number of separations for compounds containing different types of functional groups such as acidic groups, neutral, amines, etc. The cationic CF6 stationary phases exhibited better enantioselectivities in the normal phase mode when compared to the polar organic and reverse phase mode. Thus, interactions

such as hydrogen bonding and dipole-dipole stacking in NPLC are important towards the retention and enantioseparation of the analytes. This is in agreement with the former observations for CD CSPs for HPLC and confirms that similar recognition mechanisms are active under NPLC.[239] In the normal phase mode, 22 out of 34 analytes were separated on the combined CSPs (eight baseline ($R_s \geq 1.5$) and fourteen partial separations ($R_s < 1.5$).

Table 6.2 Chromatographic data for all analytes examined on the basic CSPs

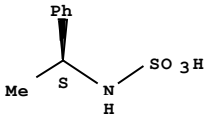
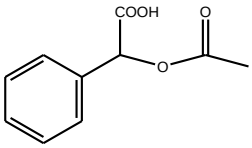
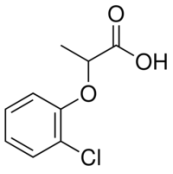
	Compound/Structure	**CSP**	**Mobile Phase**	**k'**	**α**	**R_s**
	Phenethylsulfamic acid 	SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	6.64 6.58	1.05 1.02	1.0 0.8
2	Acetylmandelic acid 	SP-CF6-PY SP-CF6-IM-2 SP-CF6-DMAP	80ACN/20MeOH/0.3AA /0.2TEA 90HEP/10EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	0.62 5.71 0.53	1.10 1.00 1.01	1.0 0.4 0.5
3	DL-2-(2-Chlorophenoxy)-propionic acid 	SP-CF6-DMAP	60ACN/40MeOH/0.3AA /0.2TEA	3.06	1.03	0.6

Table 6.2 - Continued

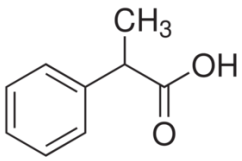
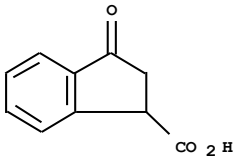
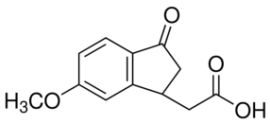
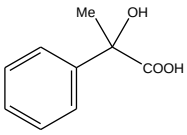
4	2-Phenylpropionic acid 	SP-CF6-PY	60ACN/40MeOH/0.3AA /0.2TEA	0.78	1.01	0.5
5	3-Oxo- 1-indancarboxylic acid 	SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	3.74 14.3	1.02 1.05	0.5 0.5
6	5-Methoxy-1-indanone-3- acetic acid 	SP-CF6-IM-2	98ACN/2MeOH/0.3AA/ 0.2TEA	6.19	1.06	0.5
7	Atrolactic acid hemihydrate 	SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA	2.76 1.61 5.79	1.00 1.00 1.00	- - -

Table 6.2- Continued

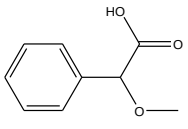
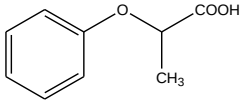
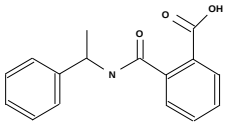
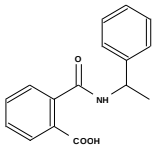
8	alpha-Methoxyphenylacetic acid 	SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA	1.94 0.68 4.53	1.00 1.00 1.00	- - -
9	2-Phenoxypropionic 	SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA	1.24 4.60 2.82	1.00 1.00 1.00	- - -
10	Phenethylphtalamic acid 	SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3 SP-CF6-BIM	80HEP/20EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	7.75 3.33 5.20 7.10	1.00 1.00 1.00 1.00	- - - -
11	alpha-Methylbenzyl)phthalic acid monoamide 	SP-CF6-IM-1	95HEP/5EtOH/0.1TFA	11.58	1.00	-

Table 6.2- Continued

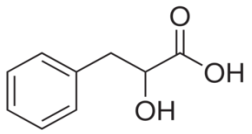
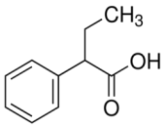
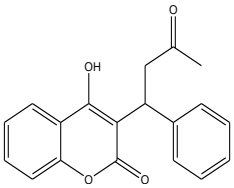
12	DL-beta-Phenyllactic acid 	SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3	80HEP/20EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA	4.51 4.53 11.59	-1.00 1.00 1.00	- - -
13	2-Phenylbutyric acid 	SP-CF6-IM-1 SP-CF6-BIM	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	6.97 8.96	1.00 1.00	- -
14	Warfarin (3-(alpha-Acetonylbenzyl-4- 	SP-CF6-IM-1 SP-CF6-BIM SP-CF6-IM-2 SP-CF6-IM-3 SP-CF6-AP SP-CF6-PY SP-CF6-DMAP	90HEP/10EtOH/0.1TFA 80 HEP/20EtOH/ 0.1TFA 85 HEP/15EtOH/ 0.1TFA 70 HEP/30EtOH/ 0.1TFA 80 HEP/20EtOH/ 0.1TFA 80 HEP/20EtOH/ 0.1TFA 90HEP/10EtOH/0.1TFA	1.38 0.88 1.12 1.30 0.67 0.93 8.26	1.30 1.28 1.17 1.17 1.13 1.19 1.05	2.5 2.0 1.5 1.6 1.5 1.5 1.3

Table 6.2- Continued

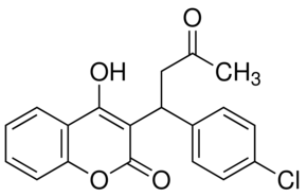
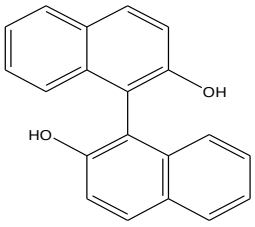
15	3-(alpha-Acetonyl-4-chlorobenzyl) hydroxycoumarin 	SP-CF6-DMAP SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3 SP-CF6-AP SP-CF6-PY SP-CF6-BIM	90HEP/10EtOH/0.1TFA 70 HEP/30EtOH/ 0.1TFA 85 HEP/15EtOH/ 0.1TFA 70 HEP/30EtOH/ 0.1TFA 80 HEP/20EtOH/ 0.1TFA 80 HEP/20EtOH/ 0.1TFA 80 HEP/20EtOH/ 0.1TFA	2.75 2.91 2.60 2.82 2.60 2.51 2.42	1.11 1.17 1.17 1.16 1.17 1.03 1.01	2.0 1.5 1.5 1.5 1.5 1.0 1.0
16	1,1'-Bi-2-naphthol 	SP-CF6-DMAP SP-CF6-IM-1 SP-CF6-IM-2 SP-CF6-IM-3 SP-CF6-AP	90HEP/10EtOH/0.1TFA 70 HEP/30EtOH/ 0.1TFA 90HEP/10EtOH/0.1TFA 80 HEP/20EtOH/ 0.1TFA 70 HEP/30EtOH/ 0.1TFA	6.88 7.05 6.67 5.36 5.21	1.08 1.07 1.04 1.03 1.05	1.5 1.3 1.0 0.8 1.0

Table 6.2- Continued

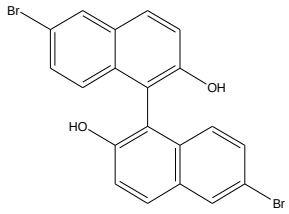
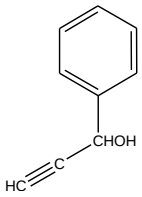
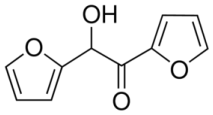
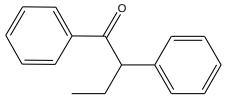
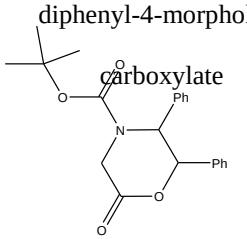
17	6,6'-Dibromo-1,1'-bi-2-naphthol 	SP-CF6-DMAP SP-CF6-IM-2 SP-CF6-IM-3	90HEP/10EtOH/0.1TFA 90HEP/10EtOH/0.1TFA 80 HEP/20EtOH/ 0.1TFA	5.70 1.94 1.65	1.04 1.17 1.13	1.0 0.8 0.8
18	1-Phenyl-2-propyn-1-ol 	SP-CF6-DMAP	90HEP/10EtOH/0.1TFA	7.98	1.03	0.8
19	Furoin 	SP-CF6-IM-2	70ACN/30MeOH/0.3AA /0.2TEA	0.67	1.02	0.6
20	Phenylbutyrophenone 	SP-CF6-IM-2	90HEP/10EtOH/0.1TFA	0.39	1.02	0.5
21	tert-Butyl-6- oxo-2,3-diphenyl-4-morpholine carboxylate 	SP-CF6-IM-2 SP-CF6-IM-3	85HEP/15EtOH/0.1TFA 85HEP/15EtOH/0.1TFA	1.48 0.89	1.12 1.08	2.0 1.5

Table 6.2- Continued

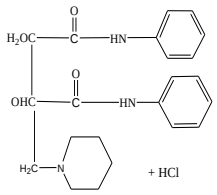
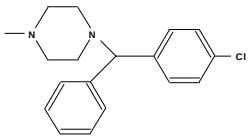
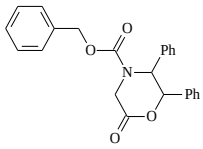
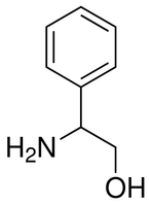
22	Diperodon hydrochloride 	SP-CF6-BIM	95HEP/5EtOH/0.1TFA	18.6	1.16	2.0
23	Chlorcyclizine 	SPCF6-IM-3 SP-CF6-BIM SP-CF6-IM-2	80HEP/20EtOH/0.1TFA 98ACN/2MeOH/0.3AA/ 0.2TEA 98ACN/2MeOH/0.3AA/ 0.2TEA	1.25 1.25 2.34	1.18 1.18 1.17	1.7 1.7 1.0
24	Benzyl-6-oxo-2,3-diphenyl-4-morpholine carboxylate 	SP-CF6-IM-2 SP-CF6-IM-3 SP-CF6-BIM-1	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	1.25 1.21 1.29	1.14 1.14 1.14	1.5 1.5 1.5
25	2-Phenylglycinol 	SP-CF6-IM-2	80HEP/20EtOH/0.1TFA	0.96	1.18	1.6

Table 6.2- Continued

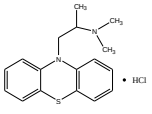
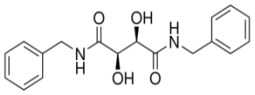
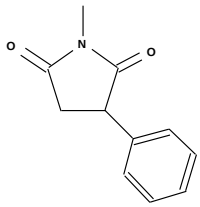
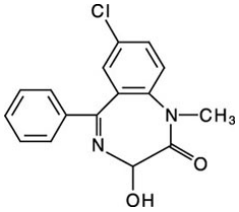
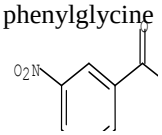
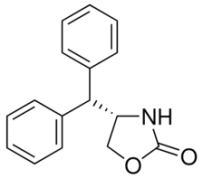
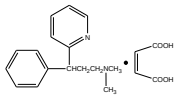
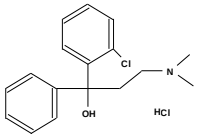
26	Promethazine hydrochloride 	SP-CF6-BIM SP-CF6-IM-2	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	7.82 7.21	1.08 1.03	1.4 0.8
27	N,N'-Dibenzyl-tartramide 	SP-CF6-PY SP-CF6-DMAP	80HEP/20EtOH/0.1TFA 80HEP/20EtOH/0.1TFA	6.92 7.02	1.05 1.01	0.8 0.5
28	Phensuximide 	SP-CF6-IM-2	90HEP/10EtOH/0.1TFA	3.01	1.02	0.7
29	Temazepam 	SP-CF6-IM-2	80HEP/20EtOH/0.1TFA	3.63	1.03	0.8

Table 6.2- Continued

30	5-(alpha-Phenethyl)semioxamazide	SP-CF6-IM-1	80HEP/20EtOH/0.1TFA	1.32	1.02	0.6
31	N-(3,5-Dinitrobenzoyl)-DL-phenylglycine 	SP-CF6-DMAP	80HEP/20EtOH/0.1TFA	6.72	1.01	0.5
32	4-(Diphenylmethyl)-2-oxazolidinone 	SP-CF6-DMAP	80HEP/20EtOH/0.1TFA	5.63	1.01	0.5
33	Pheniramine maleate salt 	SP-CF6-PY	60ACN/40MeOH/0.3AA/0.2TEA	6.12	1.01	0.5
34	Chlophedianol 	SP-CF6-IM-2	98ACN/2MeOH/0.3AA/0.2TEA	2.46	1.04	0.5

Representative chromatograms of the normal phase mode separations are shown in Figure 6.3 ($R_s \geq 1.5$). Warfarin (3-(alpha-Acetonylbenzyl-4-hydroxycoumarin) was separated on all the CSPs. Figure 6.3 (a), Figure 6.3 (f) and Figure 6.3 (g) show the separation of this compound on the SP-CF6-IM-1, SP-CF6-AP and SP-CF6-PY CSPs. SP-CF6-IM-1 exhibited very sharp, symmetrical peak shapes (with $R_s = 2.5$, $\alpha = 1.30$) compared to the both SP-CF6-AP and SP-CF6-PY CSPs. The separation of benzyl-6-oxo-2,3-diphenyl-4-morpholine carboxylate and tert-butyl-6- oxo-2,3-diphenyl-4-morpholine carboxylate were not reported previously on the neutral derivatives of CF6 stationary phases.[54,55] However, these analytes were successfully separated on the both SP-CF6-IM-2 ($R_s = 1.5$, $\alpha = 1.14$; Figure6.3(b)) and SP-CF6-IM-3 ($R_s = 1.5$, $\alpha = 1.08$; Figure6.3(c) respectively). Diperodon hydrochloride was baseline separated only on the SP-CF6-BIM CSP ($R_s = 2.0$, $\alpha = 1.16$; Figure 6.3 (d)) with a long retention time (k' = 18.6).

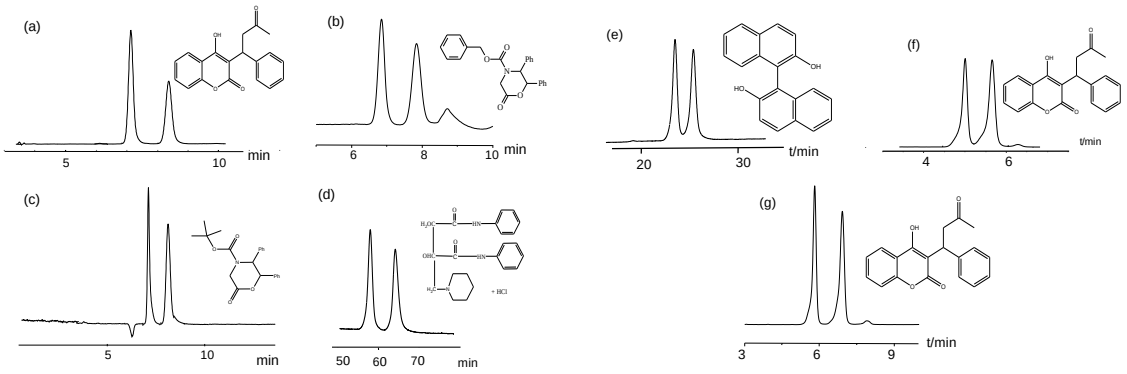


Figure 6.3 Enantiomeric separation of selected analytes on the new CSPs

(a) Warfarin on the SP-CF6-IM-1, mobile phase: 80HEP20EtOH0.1TFA

(b) Benzyl-6-oxo-2,3-diphenyl-4-morpholine carboxylate on the SP-CF6-IM-2, mobile phase: 70HEP30EtOH0.1TFA

(c) tert-Butyl-6- oxo-2,3-diphenyl-4-morpholine carboxylate on the SP-CF6-IM-3, mobile phase: 70HEP30EtOH0.1TFA

(d) Diperodon hydrochloride on the SP-CF6-BIM, mobile phase: 95HEP5EtOH0.1TFA

(e) 1,1'-Bi-2-naphthol on the SP-CF6-DMAP, mobile phase: 95HEP5EtOH0.1TFA

(f) Warfarin on the SP-CF6-AP, mobile phase: 80HEP20EtOH0.1TFA

(g) Warfarin on the SP-CF6-PY, mobile phase: 80HEP20EtOH0.1TFA

UV detection at 254 nm, flow rate

6.4.1 Comparison of enantioseparation capability of cationic functionalized CF6 CSPs

Cationic CSPs based on CF6 are a new development for HPLC and there are no comparative studies on these stationary phases. Figure 6.4 shows the number of separations achieved for the different CSPs. The highest number of separations was obtained on SP-CF6-IM-2 CSP which has an average degree of substitution (A.D.S.) of six. Interestingly, SP-CF6-IM-1 has a similar A.D.S. but a higher carbon loading (see Table 1) and yet shows poor enantioselectivity. The higher nitrogen and carbon loading on SP-CF6-IM1 is due to the increased amount of carbamate linker used (2.2). SP-CF6-IM-3 (A.D.S. = 10) also exhibited poor enantioselectivity. Clearly the imidazole selector does contribute to the enantioselectivity of CF6 towards the selected racemates, however a high A.D.S. has a deleterious effect. This may be attributed to the steric crowding of CF6 by the achiral portion of the selector and less availability of hydroxyl groups to interact with the analytes. This result is similar to previously reported observations for highly aromatic derivatized CF6.[55] They reported that a highly aromatic derivatized CF6 selector has a sterically crowded structure that hinders access to CF6's molecular core but provides other ample interaction sites about its periphery. The benzimidazole CSP (SP-CF6-BIM) exhibited lower enantioselectivity compared to the imidazole bonded stationary phases (SP-CF6-IM-2 and SP-CF6-IM-3). Although the benzimidazolium selector is rigid, closer

to the CF6 molecule and has more π-π interaction ability, the high A.D.S. again resulted in diminished enantioselectivity.

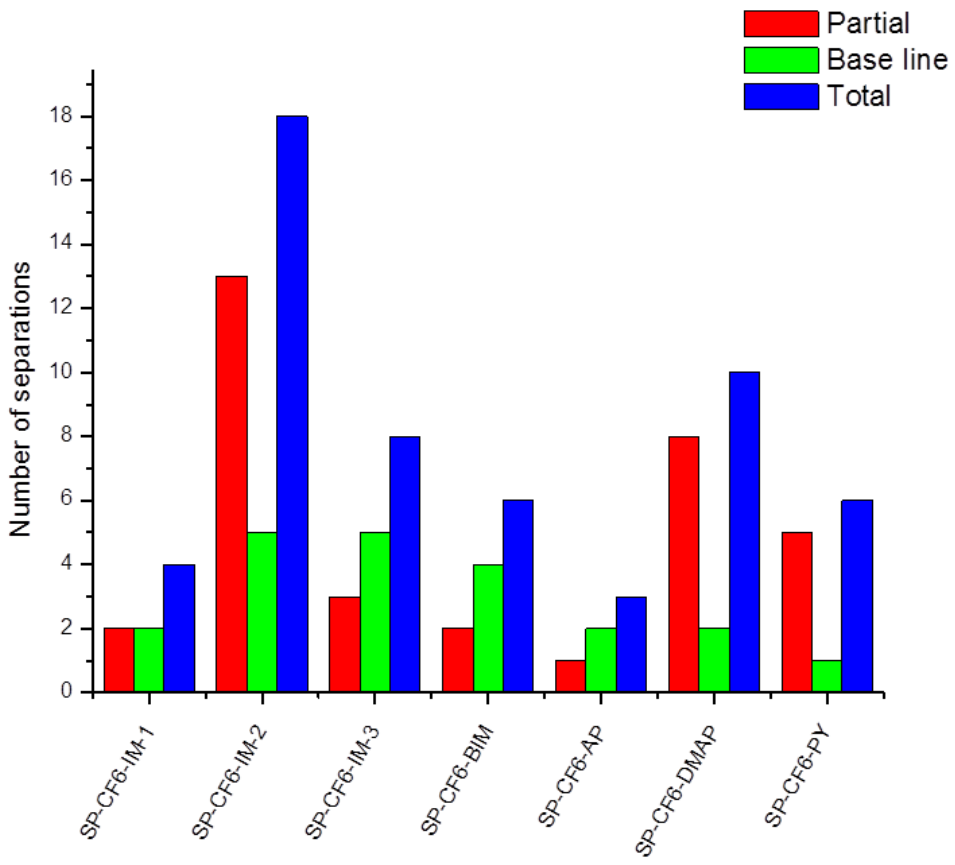


Figure 6.4 Number of enantiomeric separations on the synthesized CSPs.

The 4-(dimethylamino)phenyl CSP (SP-CF6-DMAP) was the next most successful CSP. A total of 10 separations including two baseline separations (R_s > 1.5) were achieved on this stationary phase. The 4-(dimethylamino)phenyl groups, in addition to having the same modes of interaction as the above selectors,

is even closer to the CF6 macrocycles. The 3-(dimethylamino)-1-propylamine functionalized CF6 stationary phase (SP-CF6-AP) was able to separate the least number of racemates (two baseline and one partial) and also had the highest A.D.S. (11). In contrast to the other synthesized CSPs, this CSP is unable to afford π-π stacking although this is not essential considering the commercialized LARICH-CF6-P which has an isopropyl carbamate selector. Pyridine bonded CF6 CSP (SP-CF6-PY) provided moderate enantioselectivity towards the selected analytes and this CSP was the only one containing a thiocarbamate linker which is a poorer hydrogen bond acceptor compared to the carbamate linker.

6.4.2 Evaluation of cationic CF6 CSPs for acidic analytes

Six partial separations out of 13 acidic analytes were obtained using all the CSPs (Table 6.2). The chain length between the cationic moieties and the CF6 core plays a role in the separation of enantiomers on these CSPs. For example, 2-phenylpropionic acid was partially separated (R_s = 0.5) on the SP-CF6-PY CSP while it was not separated on any other columns. The pyridine group on this stationary phase was closely attached to the CF6 when compared to the imidazole, benzimidazole and alkyl amine derivatives. Consequently, the carboxylic acid group on 2-phenylpropionic acid can better interact with the pyridine moiety and the CF6 macrocycle than the other CSPs where the cationic moieties are further away.

The other interesting observation can be made considering the partial separation of 2-phenylpropionic acid when compared to the no selectivity for 2-phenylbutyric acid and atrolactic acid. As shown in Table 6.2, 2-phenylbutyric acid contains an ethyl group while 2-phenylpropionic acid contains a methyl group at the stereogenic center. The stereogenic center is thus slightly more crowded in the former case making chiral recognition more difficult. Also, the stereogenic center of atrolactic acid is substituted with both methyl and hydroxyl groups. Although it contains a hydrogen bonding group, lack of selectivity towards atrolactic acid could be attributed to the increased steric hindrance exerted by methyl group. It should be noted that the selectivity of 2-phenylpropionic acid could not be improved by using a weaker mobile phase in the polar organic mode (ACN/MeOH/AA/TEA:95/5/0.3/0.2) despite longer retention. Meanwhile, the separation of, phenethylsulfamic acid and acetylmandelic acid were not reported in previously published papers.[54,55] and partial separations were obtained on the cationic CSPs. Overall, these CSPs have exhibited poor enantioselectivity for chiral acids.

6.4.3 Evaluation of cationic CF6 CSPs for neutral analytes

Seven neutral analytes were selected and all of them showed enanatioselectivity with one or more CSPs (three baseline and four patial separations). For example, warfarin (3-(alpha-acetonylbenzyl)-4-

hydroxycoumarin) and 3-(alpha-Acetonyl-4-chlorobenzyl)-4-hydroxycoumarin provided excellent separation selectivity for all the cationic CSPs (most cases Rs $\geq$1.5). Interestingly, the site of the halogen substituent had a significant impact on the retention factor, can be seen for these two compounds. The latter compound is simply the 4-chlorobenzyl substituted analogue of warfarin. The retention factors increased significantly with the latter compound on all the CSPs. Clearly, the presence of the chloro group in the para position of the benzyl moiety provides stronger interactions with all the CSPs.

6.4.4 Evaluation of cationic CF6 CSPs for other analytes

All the selected analytes (fourteen analytes) were separated with synthesized CSPs (five base line and nine partial separations). Interestingly, tert-butyl-6- oxo-2,3-diphenyl-4-morpholine carboxylate and benzyl-6-oxo-2,3-diphenyl-4-morpholine carboxylate were baseline separated (Rs $\geq$1.5) on the imidazole based columns (SP-CF6-IM-2 and SP-CF6-IM-3). The tert-butyl versus benzyl carbamate on the morpholine nitrogen made little difference in enantioselectivity on these CSPs. However on SP-CF6-BIM showed enantioselectivity only towards the benzyl carbamate. Here the increased steric bulk of the tert-butyl carbamate impeded chiral recognition.

6.5 Conclusions

Seven basic derivatives of cyclofrucatan 6 (CF6) were prepared and chemically bonded to silica gel. The separation performances of these newly synthesized CSPs were examined with 34 racemic compounds including acids, neutral analytes and other basic analytes such as secondary and tertiary amines, amino alcohols and amino esters etc. Enantiomeric separations were achieved for some chiral analytes. Additionally, these stationary phases can be protonated creating positively charged pyridinium, imidazolium and ammonium groups which can provide electrostatic interactions and strong hydrogen bonding sites. Unfortunately the electrostatic interaction ability was insignificant. The imidazole based CSPs showed better selectivity than the rest. All, CF6 CSPs having a A.D.S. > 10 consistently exhibited lower enantioselectivites. Cyclofructan based chiral selectors are still in their infancy and it is clear that they will need to be further developed by different types of derivatives to make even greater impact in future enantiomeric separations.

CHAPTER 7

BINDING CHARACTERISTICS OF NATIVE CYCLOFRUCTAN 6 AND ITS DERIVATIVES WITH METAL IONS

7.1 Abstract

Cyclofructan 6(CF6) is a cyclic oligosaccharide that possesses an 18-crown-6 ether core and six pendent fructofuranose moieties. Both the external hydroxyl-groups and the oxygen atoms in 18-crown-6 core of native cyclofructan 6 and its derivatives have the potential of forming complexes with metal cations. In this study, the binding ability of native, isopropylated and permethylated CF6 with various cations was characterized in water and hydro - organic solvents. Association constants (K_{ass}) of 24 cations including alkali metals, transition metal and lanthanides were measured using NMR titrations and a nonlinear least square treatment of the data. Metal ion binding increased with increasing methanol content. The weaker binding of cations with CF6 in acetonitrile-d_3 compared to those of methanolic solvents was noted and indicated the importance of dehydration of the host and guest to binding affinity. The effect of counter-anions such as chloride, nitrate, perchlorate and acetate on binding examined. Isopropyl-CF6 provided slightly higher binding affinities with cations than native CF6 in D_2O and 40% methanol-d_4, whereas permethylated CF6 only associated with K^+

cation in 80% methanol-d_4. There are distinctly different metal ion binding selectivities between native and derivatized CF6.

7.2 Introduction

Cyclofructans (CFs) are cyclic oligosaccharides which consist of six or more β-(2→1) linked D-fructofuranose units. Each fructofuranose unit contains one primary and two secondary hydroxyl groups making this molecule very hydrophilic.[42] Kawamura and Uchiyama first reported CFs produced by fermentation of inulin using an extracellular enzyme from a strain of *Bacillus circulans* OKUMZ31B.[43] There are a number of applications for CFs. They have been suggested as bulk additives in various industrial formulations such as ink formulation agents,[45] as browning preventative agents,[46] as food additives.[47] Recently, Armstrong *et al.* first employed native and/or derivatized cyclofructans as chiral selectors for capillary electrophoresis (CE),[51] gas chromatography (GC)[52] and high performance liquid chromatography (HPLC). [53-57]

Among CFs, CF6 has attracted the most attention due to its availability in pure form and its well defined geometry. In CF6, six fructofuranose rings are arranged in spiral fashion around the crown ether skeleton, either up or down from the mean plane of the crown ether.[86,87] However, CF6 is distinct from typical synthetic crown ethers in that they have unique structural characteristics: (1) the ether ring of CF6 is surrounded by hydrophilic, chiral fructofuranose units, (2) there is restricted internal motion of the ring due to the large substituents and (3)

there are different conformational arrangements of the ring in the crystalline state.[88] The ether ring of CF6 has gtgtgt conformation (g and t stand for *gauche* and *trans*, respectively) around the -C-CH_2- bonds in the uncomplexed crystalline state[87] whereas 18-crown-6 has gt$\bar{g}\bar{g}$tg conformation for the uncomplexed state.[89] The crown ether core in CF6 is somewhat different from the synthetic crown ether. For example, when synthetic crown ethers are complexed with potassium ion, the six -O-C-C-O- units adopt a g$\bar{g}$g$\bar{g}$g$\bar{g}$,[89] and are evenly distributed above and below the crown ether mean plane. However, the six oxygen atoms in CF6 are all aligned towards one side of the macrocycle due to the gtgtgt configuration of the six -O-C-C-O- units in the center 18-crown-6 core.[87] CF6 also has the potential for forming complexes with metal ions as do synthetic crown ethers. Native and derivatized CF6 have been found to complex with a variety of metal cations,[50,88,90-92,247-249] and have been used as ion trapping agents in various applications.[49,93] The complexation of permethylated CFs with various metal cation has been also characterized by means of FAB mass, NMR spectrometry and X-ray crystallography.[91] It has been shown that the selectivities of permethylated CFs for alkali metals are identical to their counterpart crown ethers: i.e., permethylated CFs binds to alkali metals in the decreasing order of $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$ in acetone.[91,248] There have been only a few published reports on host-guest studies for native CFs and their derivatives,[50,88,90-92,247-249] and further development of this field is needed.

NMR spectrometry has been widely used for the host-guest studies of many crown ethers and their related complexes with alkali and alkaline-earth metal ions, transition metal ions, lanthanide ions and small organic molecules.[250,251] The NMR technique provided information on the stoichiometry and the interaction of complexes with metals. Especially, curve fitting methods using non-linear square tool through NMR titration have been used to calculate binding constants because it is widely applicable and has fewer experimental constraints compared to graphical methods.[252,253]

In the present study, we study the complexation of native CF6 and its isopropylated, permethylated and butylated derivatives with a greater variety of metal cations using the NMR titration method. In addition, the binding of wide variety of metal cations is characterized in different solvents. Also the effect of the metal counter anion is considered.

7.3 Experimental

7.3.1 Chemicals

LiCl, NaCl, KCl, $CrCl_3$, $MnCl_2$, $CoCl_2$, $NiCl_2$, RbCl, $SrCl_2$, YCl_3, $RbCl_3$ trihydrate, $Pd(NO_3)_2$ hydrate, $AgNO_3$, $CdCl_2$, CsCl, $BaCl_2$, $Ba(NO_3)_2$, $Ba(ClO_4)_2$, $Ba(CO_2CH_3)_2$, $CeCl_3$, $NdCl_3$, $EuCl_3$, $GdCl_3$, $IrCl_3$ hydrate, AuCl hydrate, $Pb(NO_3)_2$, $Pb(ClO_4)_2$ hydrate, $Pb(CO_2CH_3)_2$ trihydrate, and $Bi(CF_3SO_3)_3$ were obtained from Sigma-Aldrich (St. Louis, MO). The deuterated water (D_2O),

methanol (methanol-d_4) and acetonitrile (acetonitrile-d_3) used in the NMR titration were also purchased from Sigma-Aldrich.

7.3.2 Synthesis of CF6 derivatives

CFs are produced by fermentation and purified by crystallization and chromatographically as previously reported.[42,86,87] CF6 was used for the present study and it was obtained from AZYP, LLC (Arlington, Texas).

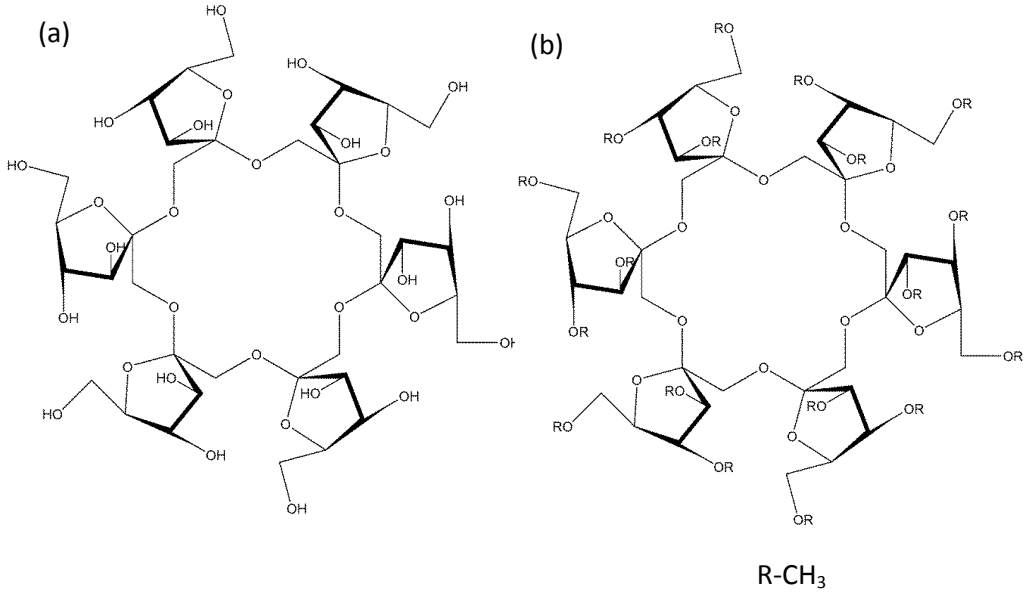


Figure 7.1 The chemical structures of (a) Cyclofructan 6 (b) permethylated cyclofructan 6

All derivatization moieties were bonded to CF6 via a carbamate linkage. Partially derivatized isopropyl CF6 (IP-CF6, average degree of substitution ~ 4, confirmed by ESI-MS) and fully derivartized permethylated CF6 (PM-CF6, average degree of substitution ~ 18, confirmed by ESI-MS) were synthesized in

house, according to the previously reported methods.[52,55] In addition, butylated CF6 (PB-CF6, average degree of substitution ~ 16, confirmed by ESI-MS) synthesized using butyl isocyanate was provided by Dr. Zachary S. Breitbach,[1] (AZYP, LLC). The chemical structures of native CF6 and permethylated CF6 are shown in Figure 7.1.

7.3.3 NMR measurement

The ^{1}H NMR experiments for measurements of binding constants were carried out on a JEOL ECX 300 MHz (Tokyo, Japan). The chemical shifts of all spectra were referenced to the internal deuterated H_2O signal at 4.65 ppm except deuterated methanol signal at 4.78 ppm for 80% methanol-d_4 solution and deuterated acetonitrile signal at 1.94 ppm for 80% acetonitrile-d_3. The peak assignments of native CF6 and permethylated CF6 (PM-CF6) were referred from the result of Yoshie *et. al*.[88] and Takai *et. al*.[91] respectively.

7.3.4 Stoichiometry and binding constant (K_{ass})

The stoichiometry of the complexes of the native CF6 with Ba^{2+} cation was determined by the continuous variation method of Job's plot.[254,255] The total concentration of the CF6 with $BaCl_2$ in D_2O was kept constant at 10 mM and the molar fraction of the CF6 was varied from 0.2 to 0.8.

For the determination of the binding constants by NMR titration method, 5 mM of native CF6 and IP-CF6 as hosts were prepared with 0%, 10%, 20%, 40% or 60% methanol-d_4 in D_2O depending on desired experiments. Stock solution of

1 M cation as guests was prepared in the same deuterated solvent used for hosts. Each 1 mL of hosts was filled into NMR tube, which was titrated by the addition of the guest stock solution. The guests added up to 160 mM in consecutive order to get minimum ten chemical shift values. For the use of 80% methanol-d_4 and 60% acetonitrile-d_3 in D_2O, the stock solution was diluted 10 times because of low solubility of the cations in them.

The chemical shift changes were recorded according to the metal ion concentrations added. The binding constant (K_{ass}) and maximum chemical shift ($\Delta\delta_{max}$) values by cation-complex were calculated by the non-linear least square method[253,256] using the Solver function in the Excel program

7.4 Results and discussion

7.4.1 Binding ability of CF6 with cations

The binding ability of native CF6 as a ligand for some metal ions in D_2O have been reported with the same order of K_{ass} compared as synthetic 18-crown-6.[88] The observed metals were limited to K^+, Na^+, Ca^{2+}, Pb^{2+} and Ba^{2+}. For a broader understanding of the metal ion affinity of CF6, comprehensively, 24 cations including alkali metals, transition metals and lanthanides were examined in this study. The NMR spectrum of CF6 in D_2O is shown in Figure 1a. All of the proton peaks representing CF6 were observed between 3.5 and 4.1 ppm. The chemical shift values changed if the cations were complexed with CF6. In the case of the Ba^{2+} complex, H1, H3, H4, H6 and H6′ proton peaks shifted down-

field whereas those of H1′ and H5 shifted to up-field (Figure. 1b-e). The induced ppm shift of H3 was more pronounced than those of H4 and H6, but all maintained their peak shapes. However, the H1, H1′, H5 and H6′ peaks began to overlap with increased Ba^{2+} concentration. Therefore, the selection of H3 peaks for calculation of the binding constants was reasonable and gave the most reliable results.

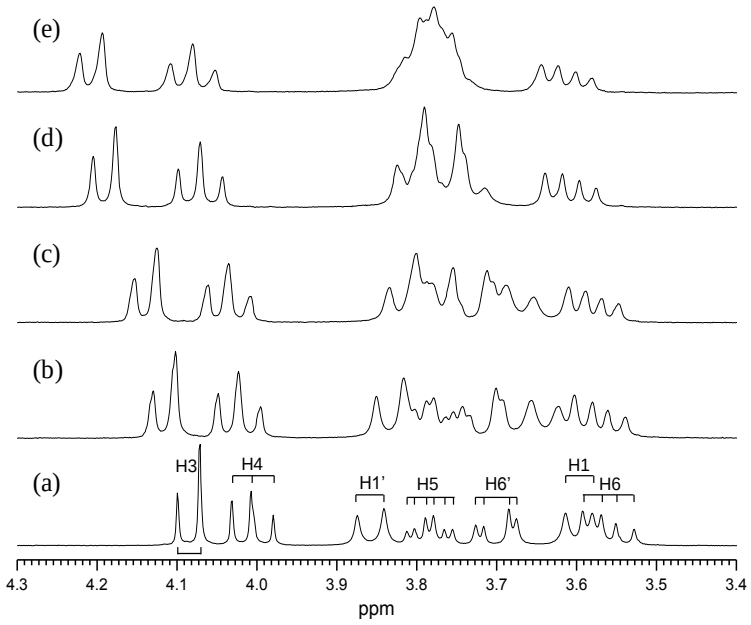


Figure 7.2 ^{1}H-NMR (300 MHz) spectra of (a) free CF6 (5 mM), (b) CF6 (5 mM) + $BaCl_2$ (10 mM), (c) CF6 (5 mM) + $BaCl_2$ (20 mM), (d) CF6 (5 mM) + $BaCl_2$ (50 mM), and (e) CF6 (5 mM) + $BaCl_2$ (80 mM) in D_2O

A Job's plot was constructed in order to determine the stoichiometry of the complex between native CF6 and Ba^{2+}. As shown in Figure 7.2, the stoichiometry was found to be 1:1 under the experimental conditions employed. Other cation complexes were assumed to be the same stoichiometry as the barium complexes for the sake of the binding constant calculations. According to types of cations, the resonance signals of each proton on CF6 by complexes showed different behaviors in the direction of shift (ppm) or the peak shapes. The peak shift trends of complexes with K^+, Rb^+, Ag^+, Cs^+ and Pb^{2+} ions were similar with those of Ba^{2+}. All peaks of Y^{3+}, Pd^{2+}, Nd^{3+}, Eu^{3+} and Bi^{3+} shifted up-field whereas Sr^{2+} shifted all peaks down-field. The Ni^{2+} complexes produced mainly broadened and overlapped signals which made binding constant measurement difficult.

The chemical shift of the Co^{2+} complex were larger ($\Delta\delta_{max}$ = 14.29) and were up-field even though, it shows very low binding affinity (K_{ass} = 0.5 M^{-1}). In addition, the chemical shifts of Cr^{3+} (d^3), Mn^{2+} (d^5) and Gd^{3+}(f^7) complexes with CF6 showed random behavior. It is most likely due to the paramagnetism of these complexes in solution.

All of the 24 cation-complexes with CF6 indicated fast exchange rates compared to the NMR time scale. Table 7.1 gives all binding constants and $\Delta\delta_{max}$ values for 24 metal cations and it includes values of K_{ass} < 10 M^{-1} as long as clear signal shifts were observed. The order of the K_{ass} values in D_2O was Ba^{2+} (16.7 M^{-1}) > Pb^{2+} (13.9 M^{-1}) > Cs^+ (6.9 M^{-1}) > Rb^+ (5.8 M^{-1}) > Sr^{2+} (3.4 M^{-1}) > K^+ = Ag^{2+}

(1.9 M^{-1}) > the rest of the cations. The binding of Ba^{+2} and Pb^{+2} was noted previously.[15] In contrast to synthetic 18-crown-6, the association of metal ions to CF6 did not show a relationship between the size of the 18-crown-6 cavity and the ionic diameters of the guest. Rather these seemed to be a correspondence between the increases in K_{ass} to the increasing atomic number of the metal group.

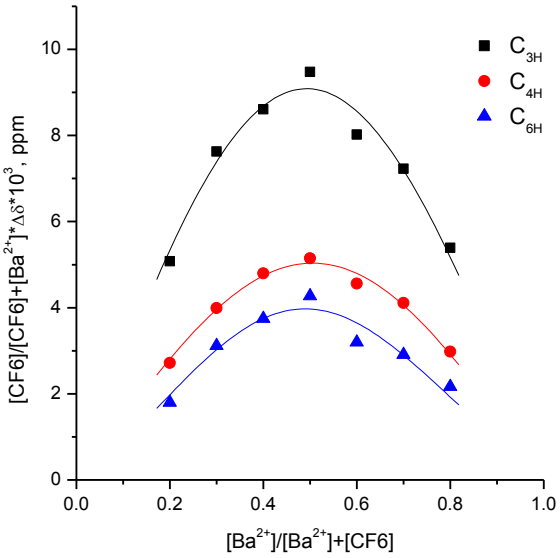


Figure 7.3Job's plot of CF6 with Ba^{2+}. The concentration was kept constant at 10 mM, i.e. $[Ba^{2+}]+[CF6]=10$ mM.

7.4.2 Binding Effect of CF6 in methanol-water mixtures

Ba^{2+} and Pb^{2+} had the largest K_{ass} with CF6 in D_2O. Thus, they were selected to examine the effect of different organic modifier content of 0, 10, 20,

40 and 60% methanol-d_4 (v/v) on the association behaviors. The results are shown in Figure 3. The binding constants (log K_{ass}) increased substantially up to 3.5 for Ba^{2+} and 2.7 for Pb^{2+} in 60% methanol-d_4. In common with the results of metal binding of macrocycles, the binding ability of CF6 with cations also increased with decreasing solvent polarity.

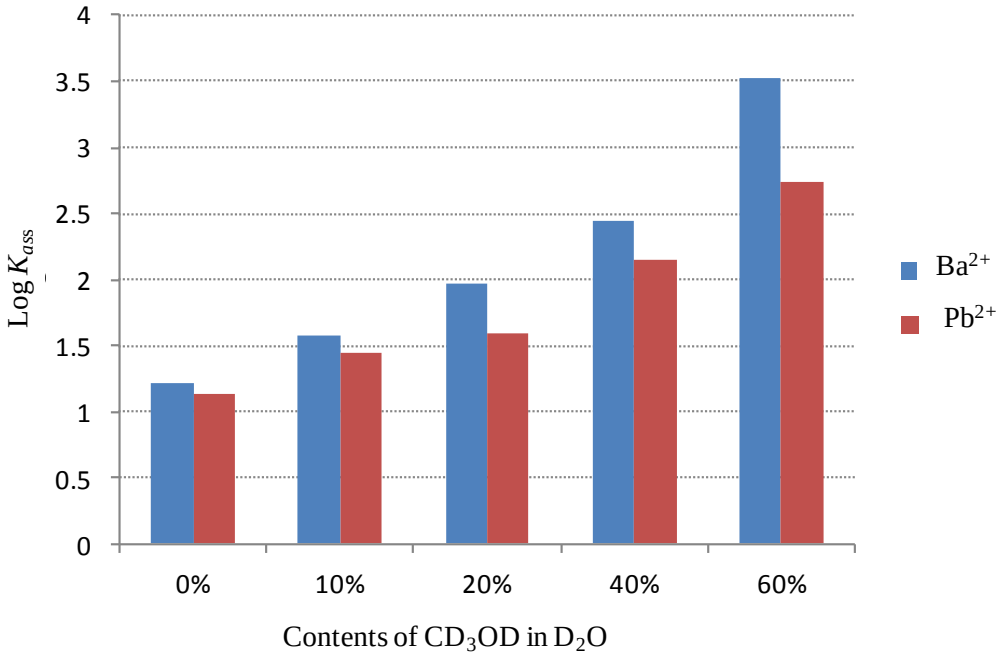


Figure 7.4 Comparison of log K_{ass} values of CF6 with Ba^{2+} and Pb^{2+} cations in different methanol contents

All cations used for determination of K_{ass} in D_2O were applied to obtain the K_{ass} in 40% MeOH. Representative spectra of Ba^{+2} complexes are shown in Figure 4. Overall, binding constants were increased compared to those of complexes in D_2O (Table 7.1).

Table 7.1 Binding constants (K_{ass}) of native CF6 with 24 cations

Atomic No.	Cations	Ionic radii (pm)	D_2O			40% methanol-d_4		
			K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2
3	Li^+	90	0.4	2.42	1.000	0	-	-
11	Na^+	116	0	-	-	0	-	-
19	K^+	152	1.9	0.13	0.959	16.7	0.05	0.991
24	Cr^{3+}	76	-	-	-	-	-	-
25	Mn^{2+}	81	-	-	-	-	-	-
27	Co^{2+}	79	0.5	14.29	0.999	2.3	5.34	1.000
28	Ni^{2+}	83	0.3	2.11	0.997	9.9	0.28	0.999
37	Rb^+	166	5.8	0.05	0.983	11.1	0.09	0.996
38	Sr^{2+}	132	3.4	0.06	0.991	4.1	0.08	0.908
39	Y^{3+}	104	0.8	0.30	0.992	8.2	0.13	0.990
44	Ru^{3+}	82	0.1	3.17	0.991	7.0	0.22	0.987
46	Pd^{2+}	100	0.1	2.39	0.985	4.0	0.16	0.992
47	Ag^+	115	1.9	0.06	0.980	18.2	0.04	0.989
48	Cd^{2+}	109	0.0	-	-	0.1	1.54	0.888
55	Cs^+	181	6.9	0.03	0.984	9.2	0.04	0.985
56	Ba^{2+}	149	16.7	0.22	0.999	281.2	0.14	0.999
58	Ce^{3+}	115	0.0	-	-	4.4	0.19	0.997
60	Nd^{3+}	98	1.1	0.25	0.998	0.7	0.32	0.985
63	Eu^{3+}	109	1.3	0.30	0.966	4.8	0.19	0.997
64	Gd^{3+}	108	-	-	-	-	-	-
77	Ir^{3+}	68	0	-	-	0	-	-
79	Au^+	151	0.1	2.36	0.982	4.3	0.14	0.990
82	Pb^{2+}	133	13.9	0.18	0.998	140.8	0.21	1.000
83	Bi^{3+}	117	0.2	2.01	0.995	-	-	-

Especially for Ba^{2+} and Pb^{2+} which form the strongest complexes with CF6. The order of the K_{ass} values was Ba^{2+} (281.2 M^{-1}) > Pb^{2+} (140.8 M^{-1}) >> Ag^{+} (18.2 M^{-1}) > K^{+} (16.7 M^{-1}) > Rb^{+} (11.1 M^{-1}) > Cs^{+} (9.2 M^{-1}) > Y^{3+} (8.2 M^{-1}) > the rest cations. Nevertheless, some metals, such as Li^{+}, Na^{+}, Cd^{2+}, Gd^{3+} and Bi^{3+}, did not appear to bind with CF6 in these methanolic solutions.

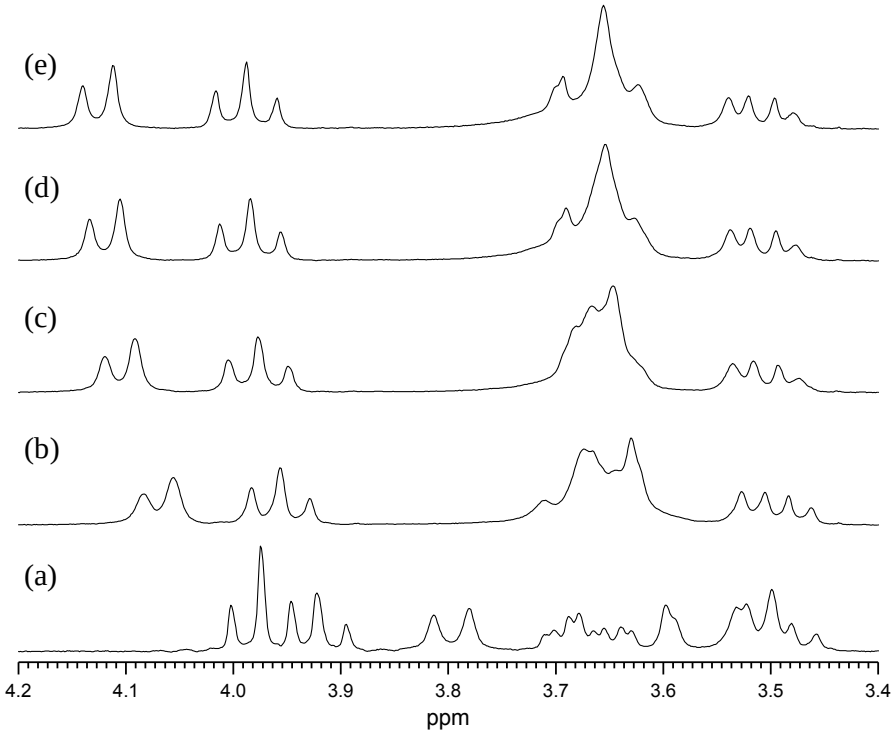


Figure 7.5 ^{1}H-NMR (300 MHz) spectra of (a) free CF6 (5 mM), (b) CF6 (5 mM) + $BaCl_2$ (5 mM), (c) CF6 (5 mM) + $BaCl_2$ (10 mM), (d) CF6 (5 mM) + $BaCl_2$ (15 mM), and (e) CF6 (5 mM) + $BaCl_2$ (20 mM) in 40% methanol-d_4.

7.4.3 Effect of counter-anion in binding with native CF6

Most of metal ions studied in this study were used as their chloride salts. Chloride salts of some cations, Pd^{2+}, Au^{+}, Pb^{2+}, and Bi^{3+} have low solubilities in D_2O so that alternative counter-anions were used for measuring binding constants. The size or hydration of anions in the solvent system used may affect the interaction between the cations and CF6.[257,258] Four types of anions including chloride, nitrate, perchlorate and acetate, and two cations (Ba^{+2} and Pb^{+2}) were chosen and their binding affinities (K_{ass}) were measured in both of D_2O and 40% methanol-d_4 (Table7. 2). It appeared that the nature of these anions has very little effect on the CF6-Ba^{2+} interaction in both solvent conditions. Nitrate and perchlorate anions for Pb^{2+} also provided similar K_{ass} values showing the absence of a significant anion effect. Unusually, adding acetate anion as the counter ion of Pb^{2+}, into CF6 solution, produced a broadend peak by merging the H3 and H1 peaks. (results not shown) However, the K_{ass} value was measured by using the shifted ppm values of H1′ in D_2O. In addition, the measurement in 40% methanol-d_4 could not be achieved due to distorted spectra. The $PbCl_2$ salt could not be measured because of lack solubility in each solvent.

Table 7.2 Effect of anions as the binding constants (K_{ass}) of metal ions to native CF6.

Anions	D_2O						40% methanol-d_4					
	Ba^{2+}			Pb^{2+}			Ba^{2+}			Pb^{2+}		
	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2
NO_3^-	14.4	0.188	0.991	13.9	0.181	0.998	351	0.143	1.000	140.8	0.209	1.000
Cl^-	16.7	0.220	0.999	-	-	-	281	0.143	0.999	-	-	-
ClO_4^-	21.7	0.195	0.998	13.9	0.242	0.998	337	0.157	0.997	117.0	0.238	0.998
$CH_3CO_2^-$	20.6	0.140	0.997	6.6[a]	0.174	0.995	327	0.173	0.999	-	-	-

[a] The K_{ass} value was calculated using the ppm shift of H1′ peak.

7.4.4 Effect of different organic solvents on K_{ass} -value

Complexation of native CF6 with cations is clearly affected by cation-solvent interaction as well as CF6- solvent interactions. Polar solvents such as water provided hydrogen bonding and ion-dipole interactions with the crown ether core.[259,260] In the case of CF6, the entire molecule is highly hydrated. As previously described, the effect of increasing methanol content was to enhance the binding ability of CF6 with cations. However, the binding constants of CF6 in 60% acetonitrile, having somewhat similar polarity with methanol, were significantly reduced although the values were calculated with the H1′ peak due to overlapping of H3 peaks with the D_2O signal (Table 3). The reason for this is not clear, although it may be that the hydrogen bonding solvent, methanol is more effective at displacing the water which hydrates both the cation and CF6.

Table 7.3 Binding constants (K_{ass}) of CF6 according to different solvent system

Cations	CF6 (60% methanol-d_4)			CF6 (60% acetonitrile-d_3)[a]		
	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2
Na^+	0	-	-	0	-	-
K^+	155	0.048	0.999	37	0.155	1.000
Cs^+	117	0.010	0.978	62	0.182	0.999
Ba^{2+}	3347	0.154	0.999	1120	0.147	0.999
Pb^{2+}	540	0.243	1.000	456	0.146	0.996

[a] The K_{ass} value was calculated using the ppm shift of H1′ peak.

7.4.5 Binding ability of CF6 derivatives

Derivatization of CF6 has been known to disrupt the hydrogen bonding between 3-OH groups on inward-inclined fructofuranose units. The opened cavity conformation may allow easier access of cations to the 18-crown-6 core.[55] This would be expected to provide different binding interactions with the CF6 derivatives as compared to native CF6. Figure 7.5a shows the NMR spectrum of IP-CF6 in D_2O. Intense peaks at 1.137 ppm from the three protons of the methyl group of isopropyl-substituted are indicated. A quartet of peaks from the proton of -CH- proton on isopropyl and the proton peaks of fructofuranose units of CF6 were observed as overlapped and broaden peaks between 3.5 and 4.3 ppm in the spectrum. Although the peaks in the spectra were not assigned completely, NMR peak shifts were observed when adding some cations into IP-CF6 solutions (Figure 7.5b and7. 5c). The binding constants calculated by a selected peak

(marked as * in Figure 7.5a) are shown in Table 4. The K_{ass} value of Na^+-IP-CF6 complex in D_2O was 6.5 M^{-1} although native-CF6 did not provide any measurable interaction with Na^+ cation. K^+, Ba^{2+}, and Pb^{2+} cations had slightly higher K_{ass} values, 12.0, 20.0 and 21.0 M^{-1}, respectively, than those of CF6. In 40% methanol, the K_{ass} of the Na^+-complex was almost the same as it was in D_2O, whereas other cation-complexes provided considerably higher K_{ass} with IP-CF6. Comparing native CF6 in 40% methanol-d3, the K_{ass} values of K^+, Ba^{2+} in IP-CF6 were about twice as large. (42.8 M^{-1} and 530.5 M^{-1}, respectively).

Table 7.4 Binding constants (K_{ass}) of metals to isopropyl-CF6 and permethyl-CF6

Cations	IP-CF6 (D_2O)			IP-CF6 (40% CD_3OD)			PM-CF6 (80% CD_3OD)		
	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2	K_{ass} (M^{-1})	$\Delta\delta_{max}$	r^2
Na^+	6.5	0.044	0.879	7.0	0.042	0.964	0	-	-
K^+	12.0	0.052	0.946	42.8	0.068	0.997	1600	0.116	1.000
Ba^{2+}	20.0	0.179	0.996	530.5	0.171	0.996	0	-	-
Pb^{2+}	21.0	0.167	0.977	132.9	0.202	0.996	0	-	-

NMR spectra changes of permethylated (PM-CF6) are shown in Figure 6a. The spectrum shows the intense methyl proton peaks of the substituted CF6. Interestingly, K^+ was only cation to bind with PM-CF6 in 80% methanol-d4, which showed the significant spectral changes for the Me-3, 4, 6 and H3 peaks. All other peaks were merged and broaden as the metal was added (Figure 6b). The value of K_{ass} for the K^+-complex was 1.6×10^3 M^{-1}. It was comparable with

the results of Takai *et.al* [91] who reported a K_{ass} value in 70% MeOH of 2.5×10^2 M^{-1}. PM-CF6 did not show any induced shift with the other guest cations.

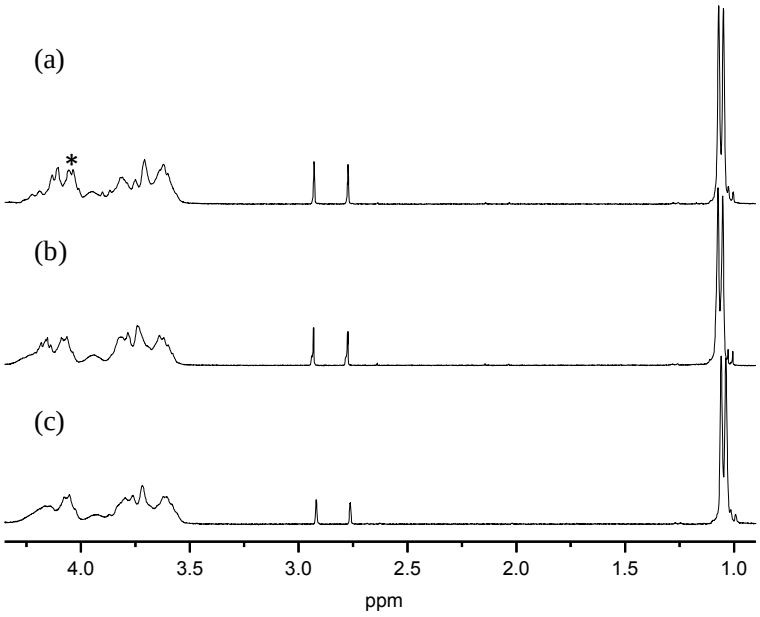


Figure 7.6 ^{1}H-NMR (300 MHz) spectra of (a) free IP-CF6 (5 mM), (b) IP-CF6 (5 mM) + $PbCl_2$ (20 mM), and (c) IP-CF6 (5 mM) + $BaCl_2$ (20 mM) in D_2O.

It is more likely that the K^+ ion can complex with the crown core while the other metals (Na^+, Ba^{2+} and Pb^{2+}) may interact with the external hydroxyl groups on CF6. Perbutylated-CF6 was used to compare the binding ability to other CF6 derivatives. However, there was no binding affinity with PB-CF6 by the cations studied.

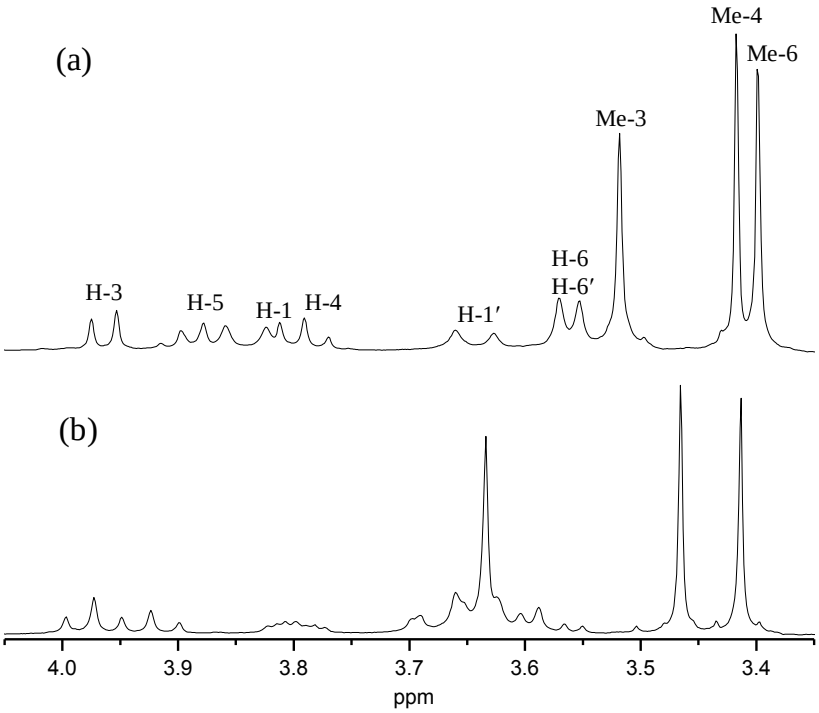


Figure 7.7 ^{1}H-NMR (300 MHz) spectra of (a) free PM-CF6 (5 mM), (b) PM-CF6 (5 mM) + KCl (30 mM) in 80% methanol-d_4.

7.5 Conclusions

This study showed the specific binding ability of native CF6 with Ba^{2+} and Pb^{2+} cations, while the traditional synthetic crown ether form preferential complexes with the alkali metal ions. Especially, the distinct binding properties of native CF6 was more notable in aqueous solvent mixtures with a higher content of organic solvent. There appeared to the little effect of counter-anions on the formations of cation-CF6 complexes in solution. The cation binding ability of

CF6 was altered by different organic solvents. The effect seemed to be governed by the solvent polarity and its ability to compete with water in solvating both the CF6 and metal ion. Exposing the crown cavity of CF6 by blocking in its hydroxyl groups alters its selectivity towards cations. Indeed only certain functionalized CF6s appeared to more closely mimic some traditional synthetic 18-crown-6 macrocycles. For example, permethylated CF6 showed specific binding ability with K^+ ion while it was not capable of binding with other cations.

CHAPTER 8

GENERAL CONCLUSIONS

8.1 Part 1 (Chapter 2 and 3)

Cyclofructans are a novel class of HILIC and chiral selectors. Although the native cyclofructan 6 has limited capability as a chiral selector, it showed exceptional selectivities for hydrophilic polar compounds in the HILIC mode. A stationary phase composed of silica-bonded cyclofructan 6 (FRULIC-N) was evaluated for the separation of nucleotides in the HILIC mode and a method was developed with remarkable selectivity for these compounds in both isocratic and gradient conditions. Because of the cyclofructan's ability to bind buffer cations, FRULIC-N has proven to be the only "neutral" HILIC phase that can show a combination of ion interaction separations behavior as well as the more common HILIC modes of retention. Furthermore, unlike stationary phases with embedded charged groups, this effect is tunable with the FRULIC column. Also, a thermodynamic study showed that the retention of most nucleotides is entropically driven on this stationary phase. In addition, the used mobile phase composition is compatible with mass spectrometry, thus the developed method can be directly applied to LC-MS techniques which is useful for the sensitive detection of polar analytes in biological matrixes.

Because of the steadily growing interest in the separation of hydrophilic polar analytes in the HILIC mode, a new stationary phase composed of silica-bonded

sulfonated cyclofructan 6 (SCF6) was synthesized and evaluated . The separation of a large variety of polar compounds including beta blockers, xanthines, salicylic acid-related compounds, nucleic acid bases, nucleosides, maltooligosaccharides, water soluble vitamins and amino acids were evaluated on different analogues of the stationary phase and compared to the same separations obtained with commercially available HILIC columns. The highly sulfonated cyclofructan 6 HILIC stationary phase is competitive with and often superior to popular commercial columns. Based on high success rate for the separation of polar compounds, it is believed that these types of stationary phase will have a significant impact in HILIC mode separations.

8.2 Part 2 (Chapter 4-6)

While native CF6 showed exceptional selectivity in the HILIC mode, derivatized cyclofructans showed excellent selectivities for enantiomeric separations. By varying the nature and degree of substitution of the substituent(s), the functionalized cyclofructans were used to separate different classes of chiral molecules. Aliphatic functionalized cyclofructan 6 molecules have been recently introduced (2009) as new chiral selectors which possess a niche enantioselectivity for primary amines. Aromatic derivatives of CF6 and CF7 were thoroughly examined for a broad range of compounds, including chiral acids, amines, metal complexes, and neutral compounds. R-naphthylethyl-carbamate CF6 (RN-CF6) and dimethylphenyl carbamate CF7 (DMP-CF7) showed complementary

enantioselectivities to each other. It was found that both the crown ether ring and the nature of the functional group on the cyclofructan affected enantioselectivity.

The high success rate of separating a wide variety of chiral compounds on the silica bonded aliphatic and aromatic CFs CSPs prompted us to evaluate their applicability in separating the racemates of chiral illicit drugs and other controlled substances. The isopropyl functionalized CF6 stationary phase (LARIHC CF6-P column) proved to be the most useful in separating illicit drugs and controlled substances which contained primary amines. The 3,5 dimethyl phenyl derivatized CF7 stationary phase (LARIHC CF7-DMP column) also displayed enantioselectivity towards analytes containing different functional groups. Knowledge of the enantiomeric ratio of illegally synthesized chiral drugs can often provide insight into the synthesis employed by the criminal chemist. Due to the importance of determining enantiomeric ratios of these chiral drugs, a method was developed for the chiral analysis of an actual pharmaceutical product.

Extensive chromatographic evaluation of the CF based CSPs offered useful information for the method development of specific chiral analytes and a better knowledge of mechanism of chiral recognition. Also, they provided insight into the design of new chiral stationary phases. Therefore, new chiral stationary phases based on cationic/basic derivatives (five different types) of CF6 were synthesized in hopes of finding either a more universal column, which is widely effective for different classes of compounds, or columns with a well defined

selectivity. The separation performances of newly synthesized basic chiral stationary phases were examined with a broad range of racemic compounds including acids, neutral compounds and other types of basic analytes including secondary and tertiary amines, amino alcohols and amino esters. Enantiomeric separations were achieved for many chiral analytes. Imidazole functionalized CF6 showed better selectivity than other cationic type derivatives. However, cyclofructan based chiral selectors are still in their infancy. It is clear that they will be further developed by different types of derivatives and play a substantial role in future enantiomeric separations.

8.3 Part 3 (Chapter 7)

Part three of the thesis studied the host-guest chemistry of native and derivatized CFs using NMR titrations and a non-linear least squares treatment of the data. Association constants (K_{ass}) of 24 cations including alkali metals, transition metal and lanthanides were measured in different solvents. This study showed the specific binding action of native CF6 with Ba^{2+} and Pb^{2+} cations. The cation binding abilitiy of CF6 was affected by different organic solvents and the binding constants increased with increasing methanol content. There were distinct differences in metal ion binding selectivities between native and derivatized cyclofructan 6. Indeed, certain functionalized CF6s more closely mimic some of the traditional synthetic 18-crown-6 macrocycles.

APPENDIX A

PUBLICATION INFORMATION OF THE DISSERTATION

Chapter 2: “Separation of Nucleotides by Hydrophilic Interaction Chromatography using the FRULIC-N Column.” Nilusha L. T. Padivitage, Milan K. Dissanayake and Daniel W. Armstrong, A manuscript submitted to *Journal of Analytical and Bioanalytical Chemistry*, **2013**

Chapter 3: “Sulfonated cyclofructan 6 based stationary phase for hydrophilic interaction chromatography” Nilusha L. T. Padivitage and Daniel W. Armstrong, *Journal of Separation Science*, **2011**, 34, 1636–1647.

CHAPTER 4: “EVALUATION OF AROMATIC-DERIVATIZED CYCLOFRUCTANS 6 AND 7 AS HPLC CHIRAL SELECTORS”PING SUN, CHUNLEI WANG, NILUSHA LASANTHI THILAKARATHNA PADIVITAGE, YASITH S. NANAYAKKARA, SIRANTHA PERERA, HAIXIAO QIU, YING ZHANG AND DANIEL W. ARMSTRONG, *ANALYST*, **2011**, 136, 787-800.

Chapter 4: “Enantiomeric Separations of Illicit Drugs and Controlled Substances Using Cyclofructan Based (LARIHC) and Cyclobond I 2000 RSP HPLC Chiral Stationary Phases” Nilusha L.T. Padivitage, Edra Dodbiba, Zachary S. Breitbach,Daniel W. Armstrong,A manuscript submitted to *Journal of Drug testing and Analysis*, **2013.**

CHAPTER 5: “DEVELOPMENT OF NEW CHIRAL STATIONARY PHASES BASED ON BASIC/CATIONIC DERIVATIVES OF CYCLOFRUCTAN 6 FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY” NILUSHA L.T.

PADIVITAGE, JONATHAN SMUTS, ZACHARY S. BREITBACH, MILAN K. DISSANAYAKE, EDRA DODBIBA AND DANIEL W. ARMSTRONG, A MANUSCRIPT NEED TO BE SUBMIT, 2013.

Chapter **6**: "Binding Characteristics of Native Cyclofructan 6 and its Derivatives with Metal Ions,Yun-Cheol Na, Nilusha L. T. Padivitage, Milan K. Dissanayake and Daniel W. Armstrong,A manuscript submitted to *Journal of Organic Chemistry*, **2013.**

APPENDIX B

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