Chemically Modified Cyclofructans for Sub/supercritical Fluid Chromatography and Hydrophilic Interaction Liquid Chromatography

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I dedicate this dissertation to my mother, in memory of my late father, and to my husband.

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Abstract

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Given the importance of supercritical fluid chromatography, several works focused on the application use different SFC stationary phases. In this work, two new derivatized cyclofructan bonded phases with superficially porous particles are introduced in SFC. Sulfonic acid and benzoic acid groups were covalently linked to the cyclofructan molecule to make them polar and hydrophilic. These derivatives were then bonded to superficially porous particles for high-efficiency separations. This work also shows the effect of adding a small amount of water to the modified mobile phases in SFC. Traces of water in the co-solvent enhances the peak symmetry and efficiency of these new column chemistries. Moreover, we also illustrate the advantages of employing tetramethylammonium acetate for fast SFC. The new columns effectively separate polar and hydrophilic compounds, including acidic compounds, β blockers, and basic compounds of pharmaceutical and biological interest.

HPLC using chiral stationary phases (CSPs) has proven to be the most widely employed method to separate enantiomers. This work presents HPLC method developments based on the existing CSPs for several chiral analytes. Chiral analytes were separated on six columns containing different chiral stationary phases (CSPs). The CSPs included with superficially porous particles bonded with two macrocyclic glycopeptides (Vancomycin, NicoShell), a cyclodextrin derivative

(CD Shell-RSP: (hydroxypropyl-β-CD), a cyclofructan derivative (Isopropyl-derivatized CF6). And a cellulose derivative (IC: tris (3, 5-dichlorophenylcarbamate), and a derivatized amylose (IG: tris (3-chloro-5 methylphenyl carbamate), both bonded to fully porous particles were evaluated. The effects of various chiral selector structures, mobile phase compositions, and analyte structures have been studied.

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CHAPTER 1

Introduction

The term stereoisomer describes a class of molecules distinguished only by the atom's orientation in space. Stereoisomers include diastereoisomers, enantiomers, conformational isomers, geometric isomers. Chirality is an important concept in the pharmaceutical industry as many drug molecules have asymmetric centers and exist as enantiomers [1]. Several enantioselective stationary phases are available for HPLC chromatography. In the absence of a universal stationary phase that is capable of resolving all enantiomeric pairs, it is necessary to work with a diversity of stationary phases. Method development usually involves column screening using a variety of mobile phase compositions to provide adequate elution and resolution [2]. The structures of chiral selectors which applied in chapter 3 are shown in Figure 1-1. Armstrong and coworkers developed cyclic oligosaccharide cyclodextrins and functionalized cyclodextrins which facilitated the separation of a wide range of analytes in the early 1980's [3]. Then, a stationary phase of macrocyclic glycopeptides was developed in 1994 as a chiral selector for HPLC [4]. Okamoto and coworkers developed polysaccharide stationary phases by 1980s [5].

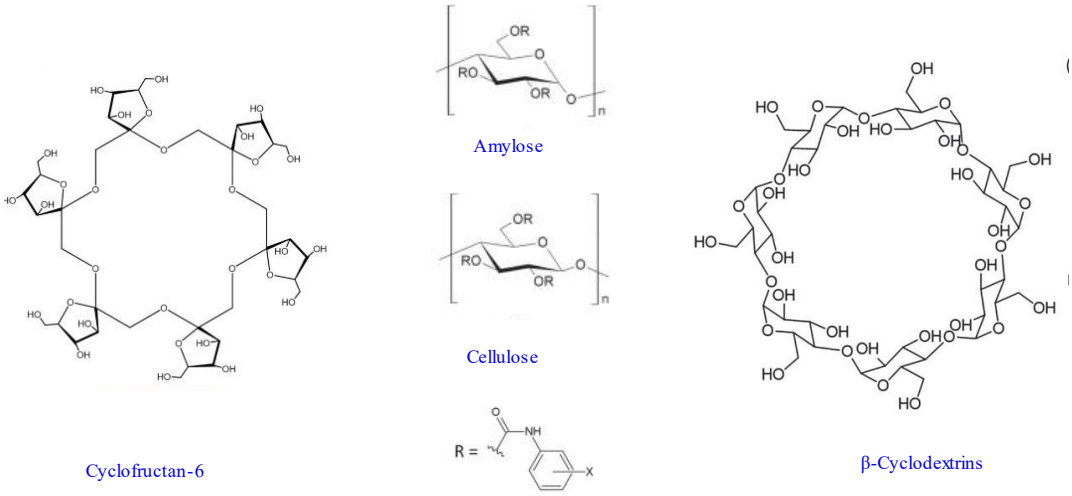

Figure 1-1. Structure of β-cyclodextrin, cyclofructan-6, and polysaccharide selector

The diffusion of the analyte in the liquid mobile phase is slower than in the gaseous phase. According to Giddings, in order to achieve LC performance comparable to GC, it is necessary to use small particles and high pressure throughout the column [6]. An objective of any chiral/achiral separation is to achieve resolution in the shortest amount of time. The equation 1-1 can be used to evaluate and understand the factors affecting the resolution (R_s) of a separation. The peak efficiency ,N; selectivity ,α; retention factor ,k; and, the retention time ,t_R of the analyte all affected R_s [7]:

$$R_s = \left[\frac{\alpha+1}{\alpha-1}\right] \frac{\sqrt{N}}{2} \left[\frac{k}{k+1}\right] \qquad (1\text{-}1)$$

The selectivity (α) and efficiency (N) are calculated from equation 1-2 and 1-3: where the retention factor, $k = t_r - t_0 / t_0$ and W_h is the width of the peak at half height.

$$\alpha = k_2/k_1 \quad (1\text{-}2)$$

$$N = 5.54 (t_R / W_h)^2 \quad (1\text{-}3)$$

Among the key variables, the most important is selectivity, followed by efficiency, and then the retention factor. The selectivity is calculated by the chemistry of the stationary phase, the mobile phase composition, as well as the temperature. The efficiency, N, is another critical variable which is influenced by the nature of the packed bed, particle size and shape, and sorption-desorption kinetics. When the chiral selector is bonded to a stationary phase (fully porous or superficially porous), the material must be packed into chromatographic columns for separation.

The column packing procedure, in addition to optimal synthesis and mass loading of the chiral selector, dictates the peak width and peak shape of chiral separations. The performance of superficially porous particles (SPPs) made in the 2000s were compared with fully porous particles (FPPs) [8]. Several papers have discussed the advantages of using SPPs over FPPs [9-14]. In accordance with the van Deemter equation 1-4 [15] , SPPs exhibit superior performance due to their smaller eddy dispersion term, improved packing homogeneity and an advantage involving the mass transfer terms, based on particle morphology.

$$H = A + B/ u_0 + C \times u_0 \quad (1\text{-}4)$$

The A, B and C terms were referred to eddy diffusion, longitudinal diffusion, and mass transfer resistance respectively, while the u_0 is linear velocity.

The sub-5µm FPPs do not show the same plates as the SPPs. The larger particle size distribution and experimental difficulties associated with packing sub-5 micrometer FPPs could account for this. Additionally, the backpressure of the 2.7 mm SPP is within the pressure limits of typical HPLCs (400 bar). By using SPPs as packing material, it is possible to increase efficiency, and

subsequently R, in short columns. Figure 1-2 shows conventional HPLC with the same mobile phase, for separation of binaphthyl diamine BINAM compound using different particle sizes of FPPs and SPPs. When compared to all FPPs, including the 2.1 µm particles, the CSP based on SPPs provided both the greatest efficiency and quickest analysis time [16].

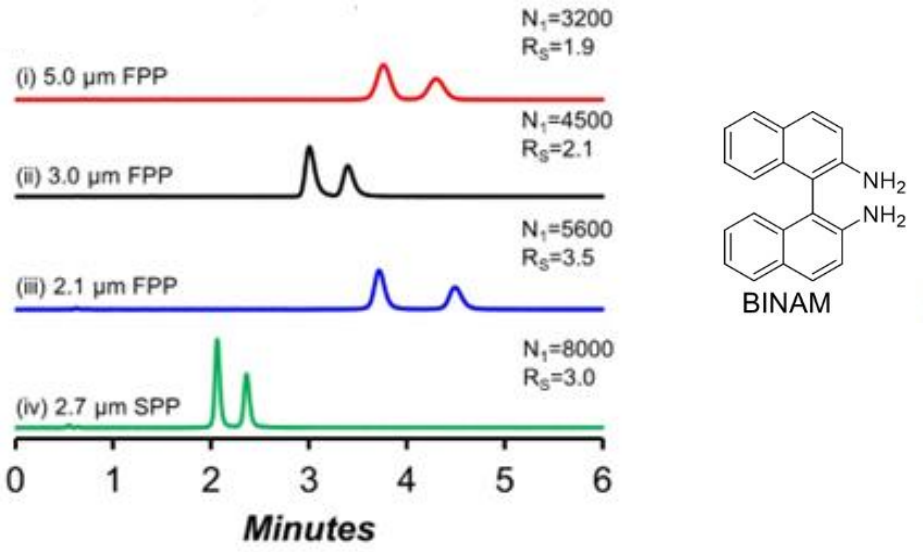

Figure 1-2. Enantiomeric separations of BINAM on CF6-P bonded to SPPs and FPPs at 1.0 mL/min, Tcol = 25 °C. All columns were 5 cm × 0.46 cm in dimensions. (A) Constant MP mode, MP = 92:8 heptane−ethanol. Adapted with permission from Ref [16].

Besides HPLC, an SPP-CSP provides faster separations via SFC, where the viscosity of CO_2 is lower than that of HPLC solvents and improved resistance to mass transfer occurs [17] [18]. According to van Deemter plots in Figure 1-3, SPPs provide significant advantages over FPPs when operating at high flow rates in SFC [19]. The small particle diameter of the superficially porous particles led to higher efficiencies or lower theoretical plate heights (H) when compared to the 5 m FPPs indicating a smaller A term and faster mass transfer as expected.

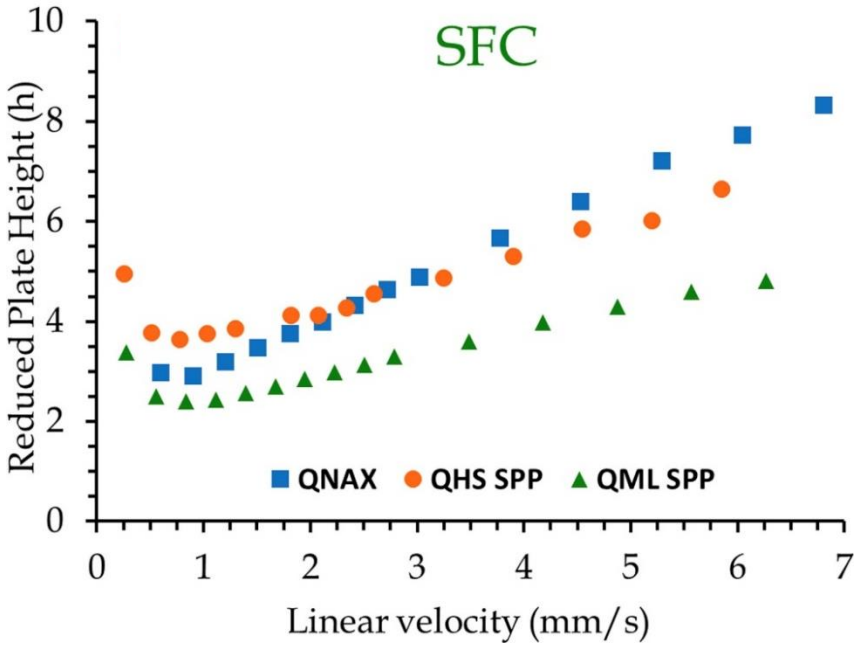

Figure 1-3 The van Deemter plots showing reduced plate height (h) against linear velocity u_0 (mm/s) for chiral analytes in SFC on quinine mercapto linker SPP (QML SPP, 5 × 0.46 cm i.d., 2.7 µm SPP, green triangle, ▲), quinine hydrosilated SPP (QHS SPP, 5 × 0.46 cm i.d., 2.7 µm SPP, orange circle, ●), and Chiralpak QNAX (QNAX, 15 × 0.46 cm i.d., 5 µm FPP, blue square, ■). SFC van Deemter with analyte: FMOC-alanine (1st enantiomer), mobile phase: 60% CO_2 – 40% 100/0.6/0.5 MeOH/FA/$HCOONH_4$ (v/v/w), 254 nm. Backpressure regulator maintained at 78 bar and temperature was ambient. Adapted with permission from Ref [19].

As a result of the combination of non-polar solvent (CO_2) and a polar co-solvent [20], the mobile phase is similar to that of normal phase high performance liquid chromatography (HPLC). Other components referred to as "additives" are used in small proportions in the co-solvent to enhance the elution of most polar and ionizable species. Based on this, several applications have been published including, drug discovery [21-23], metabolomics [24, 25] and plant derived natural dyes, which are used in cosmetics, pharmaceutical and food products [26].

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Chapter 2

High efficiency functionalized hydrophilic cyclofructans as stationary phases in sub/supercritical fluid chromatography

2.1. A B S T R A C T

Packed column SFC has become very popular for preparative and analytical separations due to the low cost of CO_2, its accessible critical temperature, and pressure, with the additional benefit of a low environmental burden. Currently, there is a shortage of new polar stationary phase chemistries for SFC. In this work, two new functionalized cyclofructan columns are introduced and evaluated for their performance in achiral SFC separations for the first time. Cyclofructan (CF6), a macrocyclic oligosaccharide, was covalently linked with benzoic acid (BCF6) and propyl sulfonic acid (SCF6) groups by ether bonds. Superficially porous particles (2.7 μm) bonded with modified CF6 showed markedly different selectivity than native CF6. In SFC, peak shapes of amines and basic compounds are often compromised. We show that small quantities (~5.7% v/v) of water added to the methanol modifier in CO_2 improves peak symmetries of primary, secondary, and tertiary amines.

Efficiencies as high as 200,000 plates/m (reduced plate height ~ 1.8) were observed for benzamide and amitriptyline on the BCF6 column. The relative standard deviations (RSDs) of retention times on BCF6 were about 1.4%, and on SCF6 were less than 1%. Amines on the SCF6 column showed plate counts as high as 170,000 plates/m. Tetramethylammonium acetate is examined as an alternative to water in MeOH. A run time of 36 min with methanol, trifluoroacetic acid, triethylamine mobile phase was reduced to <5 min with complete baseline

resolution for a set of amines. The new stationary phases allow greener approaches towards solving separation problems.

2.2. Introduction

With the widespread acceptance of sub/supercritical fluid chromatography (SFC) in solving academic and pharmaceutical research problems [1–3], the development of new stationary phase chemistries and mobile phase compositions are always attractive to chromatographers. Currently, SFC has a limited choice of stationary phases for small molecule separations. The available surface chemistries include bare silica, bonded phenyl, C18, cyanopropyl, and diol for achiral separations [4,5], whereas polysaccharides, cyclodextrins, macrocyclic glycopeptides, or oligosaccharide derivatives bonded to silica are feasible for chiral separations [5,6]. It has been suggested by pharmaceutical chemists that regular users of SFC are unaware of the power of chiral stationary phases for separating difficult to separate achiral mixtures of drugs and their metabolites [7]. Recently, there has been an increased interest in applying the same silica bonding chemistry used on fully porous particles (FPPs) to superficially porous particles (SPPs). The latter particle morphology maintains the chromatographic selectivity while increasing chromatographic efficiency, which allows a reduction in analysis times [8,12].

Cyclofructan-6 (CF6) belongs to a small class of macrocyclic oligo- saccharides, which include the well-known cyclodextrins [13]. The CF6 unit consists of 6 β (2 → 1) linked D-fructofuranose units connected via ether linkages (Figure 2-1(A)). It was demonstrated that derivatized CF6 bonded to SPPs serve as high efficiency chiral phases and as well as hydrophilic interaction liquid chromatography (HILIC) phases with unique selectivity among 36 commercial columns. Two CF6 derivatives that showed unique selectivity in the HILIC mode

were benzoic acid or propyl sulfonic acid covalently linked to CF6 via ether bond, referred to as BCF6 and SCF6 respectively [14–16]. Since SFC requires polar/- hydrophilic stationary phases, it was natural to investigate the performance of CF6 bonded SPPs with sub/supercritical CO_2, especially in the presence of aqueous methanol [17,18]. Recently the application of water as a co-solvent additive has been explored by several different groups for chiral and achiral SFC [9,17,18].

Sub/supercritical CO_2 has the appealing property of behaving like a "different" solvent depending on the experimental parameters [19,20]. Pure supercritical CO_2 is close to hexane in its elution strength with bare or bonded silica phases in SFC experiments [21]. Precipitation and solubility issues in SFC are well-known among experienced practitioners [1,22]. Highly polar or basic compounds, such as aliphatic/aromatic amines or polyfunctional carboxylic acids, and even many neutral molecules, suffer from irreversible retention or elute with tailing peak shapes due to their limited solubility in the mobile phase. In the SFC literature, spanning several decades, more than 40 additives or co-solvents have been tried in order to solve the above-mentioned problems, ranging from simple and branched alcohols, acetonitrile, alkanoic acids (formic and acetic), alkyl amines, ammonium hydroxide, halogenated hydrocarbons, linear and cyclic ethers, esters, nitro- methane, sulfur hexafluoride, *N*,*N*-dimethylacetamide, *N*,*N*-dimethylformamide, *N*,*N*-dimethyl sulfoxide, hexafluoro isopropanol, propylene carbonate, and water [23,24]. Among this list, water is the least environmentally harmful additive, where the rest have a negative environmental impact. Water also provides additional solubility for polar compounds, dipole interaction, and hydrogen bonding capability to the SFC mobile phase [24]. A combination of a polar stationary phase with sub/supercritical CO_2 containing aqueous methanol can serve as an excellent platform for the simultaneous analysis of hydrophilic and lipophilic substances in

SFC.

In this work, two new high efficiency 2.7 µm SPP stationary phases (BCF6 and SCF6) were investigated for SFC applications for the first time. BCF6 is a weak cation exchanger, and SCF6 is a strong cation exchanger with its hydroxyl rich cyclofructan unit. It is expected that these successful HILIC phases would show promising performance in SFC as well. A high percentage of carbon dioxide with aqueous methanol and tetramethylammonium acetate as additives is employed to keep the methods as "green" as possible. The hydrophilic and ion-exchange character of the stationary phases is examined with four probe analytes since water is an essential component of the modifier in these SFC applications. These stationary phases are hydrophilic, and they should be suitable for small molecule separations such as organic amines, beta- blockers, carboxylic acids, and various multifunctional molecules.

In addition, the role of SFC mobile phase modifiers is investigated for 35 compounds in terms of their chromatographic efficiency and the reduction in their run times. These test compounds have a wide range of polarity and molecular structures ranging from amides, anilines, benzoic acids, aliphatic and aromatic amines. Unique selectivities, high efficiencies, and high stabilities are demonstrated and discussed.

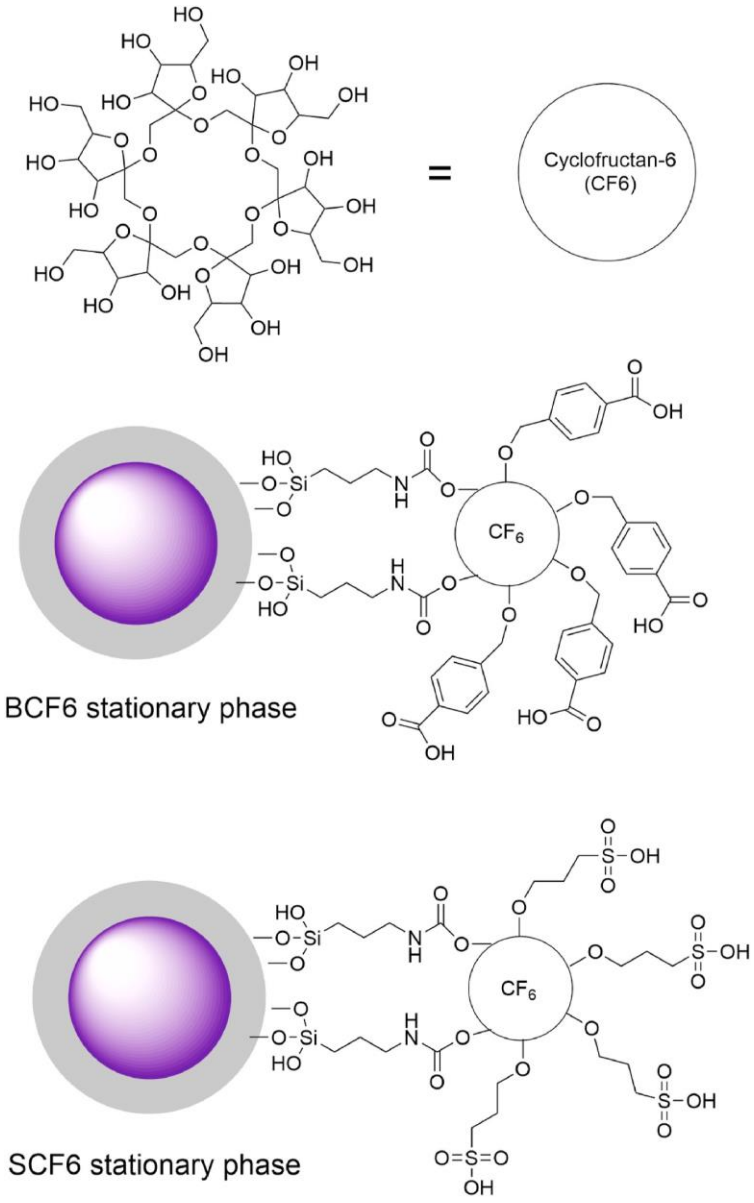


Figure 2-1. The structures of benzoic acid cyclofructan 6 (BCF6) and sulfonated cyclofructan 6 (SCF6) bonded to spp using a triethoxysilane with an isocyanategroup. The isocyanate group reacts with the alcohol functional group to form a carbamate group linkage.

2.3. Experimental

2.3.1. Reagents and standards

All analytes, reagents, and solvents used in this study were obtained from Sigma Aldrich (St. Loius, MO, USA) or Cerilliant Corporation (Round Rock, TX, USA). HPLC grade methanol was used to dissolve all the compounds listed in Table 1, in which the concentrations were ~ 1 mg/mL. Sample concentrations were changed as needed to stay within the linear range of the detector. Cyclofructan-6 was obtained from AZYP LLC, Arlington, TX, USA. Distilled deionized water was purified with a Milli-Q water purification system to 18 MΩ (Millipore, Billerica, MA, USA). Liquified CO_2 was purchased from Airgas (Texas, USA) with a cylinder having a siphon tube.

2.3.2. Preparation of columns

2.3.2.1. Benzoic acid cyclofructan 6 (BCF6)

The benzoic group was introduced to the CF6 molecule by nucleophilic substitution of 4-bromomethyl benzoate, which results in an ether linkage between the benzoic acid group and primary alcohols of CF6 [16,25]. From MS spectrometry analysis, an average of 3.5 4-methyl benzoic acid groups has been substituted in the structure of CF6. The elemental analysis of the BCF6 stationary phase showed a carbon loading of 4.01% and 0.45% N by weight. The CHNS analysis was car- ried out by Quantitative Technologies Inc. (QTI) Whitehouse NJ.

2.3.2.2. Sulfonated cyclofructan 6 (SCF6)

CF6 was sulfopropylated with 1,3-propane sultone to yield sulfonic acid groups covalently attached to CF6. The synthetic detail is outlined in Ref. [16]. The derivatives sugars are bonded to silica via carbamate linkage on a propyl silane, as shown in Fig. 2-1. From the MS results, an

average of 6 of the hydroxyl groups reacted with and were thus modified with the propane sulfonic acid. The prepared SCF6 stationary phase's elemental analysis showed: 5.72% C, 0.68% N and 1.0% S by weight [16].

2.3.3. Packing

The stationary phases were packed into 10 0.46 cm i.d. stainless steel HPLC columns. The columns were pressurized to 13,000 psi using CP-class-pump from SSI (PA, USA).

2.3.4. Instrumental parameters and conditions

A Jasco semi-prep SFC was used for all separations. The SFC carbon dioxide pump (PU-2086) was chilled by a Julabo chiller at —10 °C, and the mobile phase modifier was mixed with CO_2 via another identical pump and a mixing chamber. The pumps can deliver up to 20 mL/min at 50 MPa. The safety valve limiting the pressure to 34 MPa was bypassed.

This plumbing is followed by an autosampler (AS-2059-SFC) with a 5 μL loop, UV detector (UV-2075), backpressure regulator (BP-2080), and a modifier pump (PU-2086). The backpressure is adjusted to 8 MPa and its heat controller remained at 60 °C. The thermostat was bypassed to reduce extra-column broadening due to the small column size. The in- strument is controlled by ChromNAV (1.17.01 Build 8) connected by an LC-NET II/ADS for data analysis. The data sampling rate was 100 Hz. Response time was set 0.05 s (the fastest available), and the type of digital filter to reduce noise was a time accumulation filter (moving average).

2.3.4.Mobile phase preparation

The reported percentage of water signifies the percentage of water added to a given volume of modifier before mixing, e.g., 5.7% v/v H_2O in MeOH was made by adding 6 mL of H_2O to

100 mL of MeOH. Additives such as 0.1 v/v% triethylamine (TEA) and trifluoroacetic acid (TFA) were added to MeOH. Water was transferred to the mobile phase using a 50 mL Class-A burette. The solid additives were weighed, and the liquid additives were delivered via micropipette. 0.1% tetramethylammonium acetate was weighed and added to methanol. Efficiency was calculated using the half-height method, and the symmetry factor or the tailing factor was calculated by $w/2f$, where w is the peak width at 5% height, and f is the width at 5% height between the left tangent and the vertical line dropped from the peak maximum.

2.4. Results and discussions

In a previous study utilizing native CF6 bonded to silica for SFC applications, it was shown that CF6 bonded silica outperformed native silica, C18 silica, and ethyl pyridine bonded silica in terms of efficiency gains for achiral molecules in the presence of water in the SFC modifier [18,26,27]. Consequently, it was desired to modify CF6 with polar functional groups viz. carboxylate and sulfonic acid groups to make the stationary phase more hydrophilic. The embedded carbamate groups and the other polar groups make the stationary phase more hydrophilic (Figure 2-1). Such bonding chemistry is exceptionally resistant to hydrolysis, and the stationary phase is chemically stable compared to current stationary phases, including bare silica [14]. It has been demonstrated that superficially porous silica can generate reduced plate heights ($h = H/d_p$) less than 2 when bonded with chiral cyclofructan derivatives in liquid chromatography [8,14]. The logical next step was to investigate the performance of derivatized CF6 in SFC for achiral separations.

2.4.1. Ion exchange and hydrophilic properties of BCF6 and SCF6 columns

Researchers engaged in stationary phase synthesis are interested in designing materials with new selectivities, high chemical stabilities, and higher efficiencies than the existing stationary phases.

Previous results with native CF6 phase bonded to core shell particles in SFC with water-containing mobile phase additives showed dramatic improvements in basic and neutral compounds' peak shapes. It was expected that introducing a strong acid or an aromatic weak acid moiety into the CF6 structure will alter the surface charge and selectivity of neutral and charged compounds after bonding to silica. Additionally, a highly hydrophilic stationary phase will show affinity towards water, which can benefit the SFC separation of hydrophilic/polar molecules. The selectivity characteristic of the two new stationary phases employed in this work can be determined using four different probes: uracil, cytosine, benzyltriyltrimethylammonium chloride (BTMA), and p-toluene sulfonic acid (pTSA). The retention ratio of cytosine/uracil shows the hydrophilicity of stationary phases [28].

Uracil is a neutral compound, BTMA is a quaternary amine with a permanent positive charge, and the relative retention of BTMA and uracil indicates the cation exchange characteristic of the stationary phase. Higher retention ratios imply stronger cation exchange behavior. pTSA also probes the stationary phase's electrostatic properties [29], but it has a permanent negative charge due to its low pK_a value of -2.8. The retention factor ratio of pTSA/uracil shows the negative surface charge repulsion characteristic of the stationary phases. Figure 2-2 shows a 3D selectivity plot of hydrophilicity ($\alpha_{\text{cytosine/uracil}}$) on the z-axis, surface charge ($\alpha_{\text{pTSA/uracil}}$) on the y-axis, and ion exchange characteristics ($\alpha_{\text{BTMA/uracil}}$) on the x-axis using a mobile phase of 80/20 acetonitrile-5mM ammonium acetate (total concentration) [11]. The chosen dead time marker was acetone because toluene and other hydrocarbons were excluded from the hydrophilic stationary phase and eluted before the expected void volume. As shown in Figure 2-2, $\alpha_{\text{cytosine/uracil}}$ values are larger than native CF6, confirming the expectation that the new stationary phases are more hydrophilic than native CF6. The $\alpha_{\text{pTSA/uracil}}$ is negative for both

derivatized CF6s because of electrostatic repulsion from the stationary phase ($k < 0$ for pTSA), whereas $\alpha_{pTSA/uracil}$ for the native CF6 is positive. The cation exchange behavior is indicated by the large $\alpha_{BTMA/uracil}$ values. Both BCF6 and SCF6 show similar cation exchange characteristics, whereas the native CF6 do not retain BTMA appreciably as expected from the lack of cation exchange sites on the native CF6 stationary phase. The $k_{cytosine}/k_{uracil}$ ratio demonstrates the HILIC behavior of the stationary phase, and one can predict the relative efficiencies of the new columns compared to other HILIC stationary phases. Based on the 3D plot, the HILIC character of BCF6 and SCF6 are close to each other and therefore promising for SFC with aqueous modifiers.

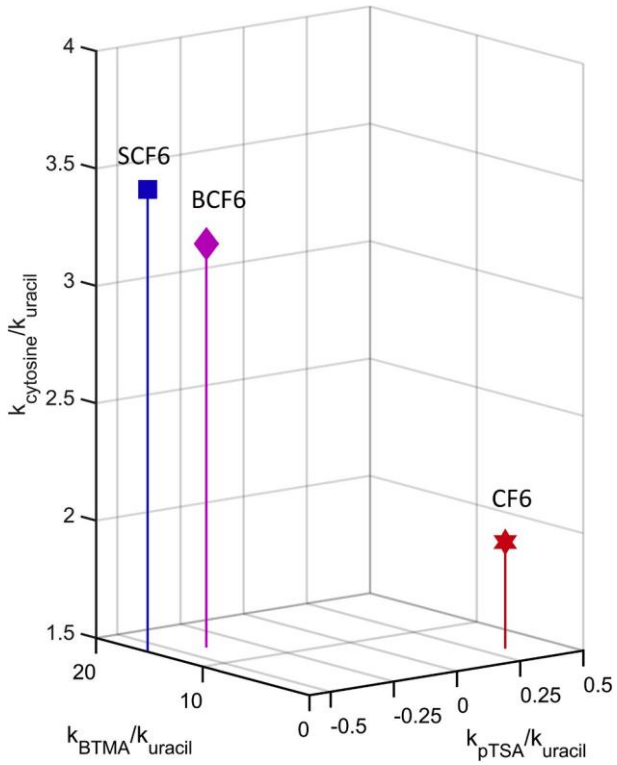


Figure 2-2. 3D plot showing the hydrophilicity vs. ion exchanges vs. surface charge of BCF6, SCF6, and CF6. Conditions: BCF6, SCF6, CF6 (2.7 µm particle, 10 ×0.46 cm); 80/20 acetonitrile/5 mM ammonium acetate pH 6.8 (v/v); flow rate 1.0 mL/min; UV detection at 254/220 nm; Each point is a triplicate with acetone as a dead time marker.

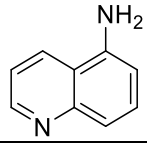
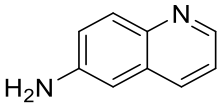
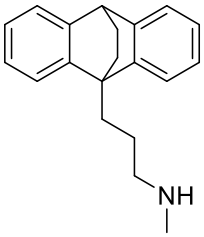
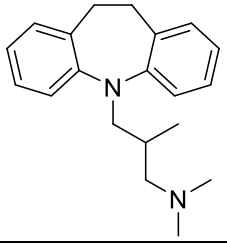
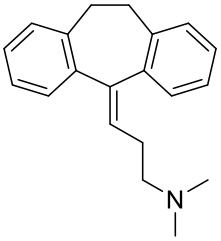
2.4.2 Effect of water as an additive in SFC

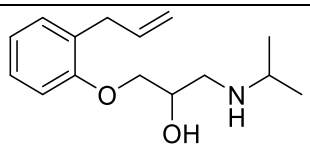
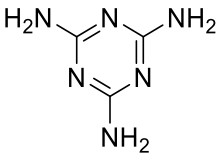
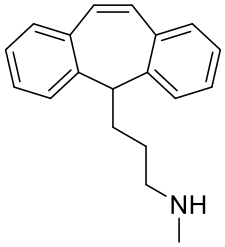
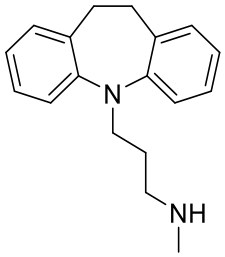
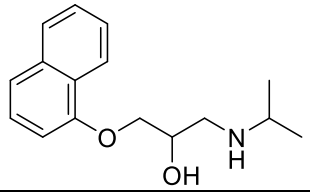
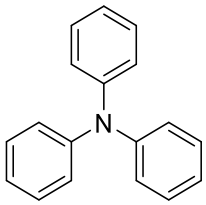
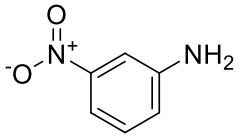
The typical methanol concentrations in SFC range from 5 to 40% [9, 30]. In this study, mobile phase composition of 10% and 20% MeOH with CO_2 was tested. The effect of a small amount of water as an additional additive in SFC with these new column chemistries was also investigated. Table 2-1 summarizes the results of 35 representative acids, bases and neutral compounds on both BCF6 and SCF6 with 80/20 CO_2/MeOH (containing 0.1% TEA + 0.1% TFA) and 80/20 CO_2/MeOH (containing 5.7% H_2O+ 0.1% TEA + 0.1% TFA). Herein, we use two parameters with a or - sign, i.e., ΔN and Δt, which reflect the difference in efficiency and retention time after and before the addition of water, respectively. With the BCF6 stationary phase, most of the basic compounds show a remarkable increase in efficiency (up to 8200) with a small decrease in retention (see Table 2-1).

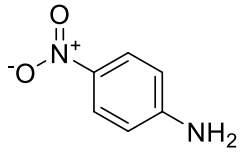
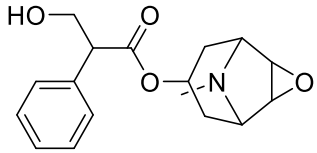
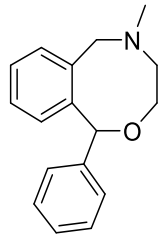
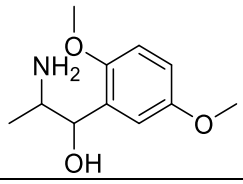
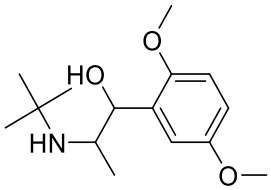
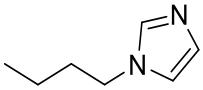
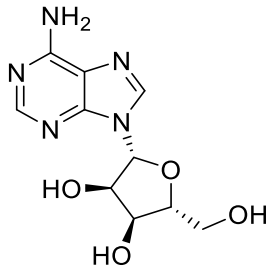
Similarly, neutral compounds showed an efficiency gain of up to 7700 and a retention time loss of ~0.4 min. In contrast, many acids show a loss of efficiency up to 1300 and a negligible increase in retention time except for phenyl succinic acid, which had an increased efficiency of 6100. Similar behavior was observed with native CF6 in the case of acids [18]. In general, longer retained compounds showed a positive change in plate counts as seen for bases on BCF6, e.g., k 14 for scopolamine without water, and with water k ~10 with a 6000 gain in N. The sulfonated cyclofructan stationary phase (SCF6) shows similar trends in increasing plate count in the presence of water. As expected, the SCF6 column, being a strong cation exchanger, had a very high affinity towards amines, e.g., k 14.4 for scopolamine without water. Still, adding water benefited the efficiency by 2200, and the k was reduced to 11. Nefopam is another tertiary amine with a k 43 without water on SCF6. In the presence of water, k decreases to 39 with a parallel increase in plate count from 9100. Neutral compounds showed a variable pattern in gaining and losing retention time and efficiency. The low retention times of acidic com- pounds

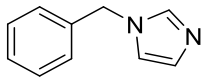
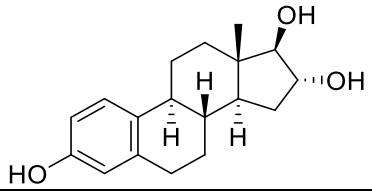
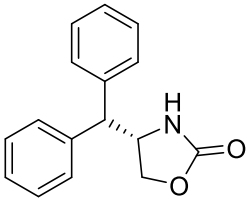
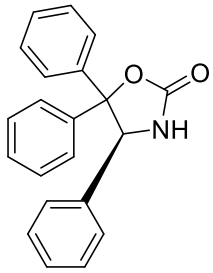
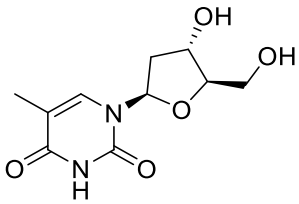
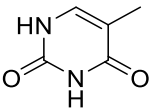
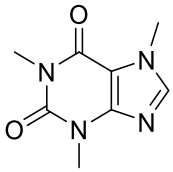
on both stationary phases (k ~1) are consistent with the 3D selectivity chart (Figure 2-2) because the acids are repelled by both stationary phases. Therefore, both BCF6 and SCF6 are very promising column chemistries for basic and neutral compounds and many acidic ones (*vide infra*).

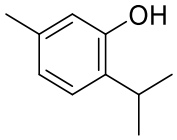
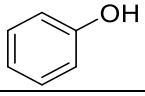
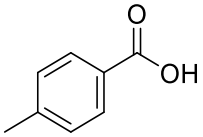
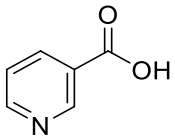
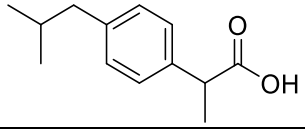
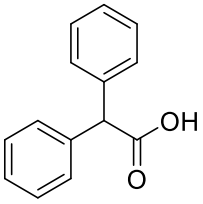
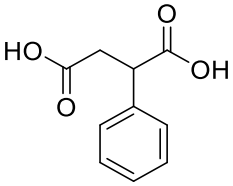
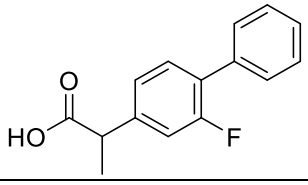
Table 2-1. Change in Efficiency and Retention time by the addition of water in the mobile phase Conditions: BCF6, SCF6 (2.7 µm particle, 10 × 0.46 cm); 80/20 CO_2 / methanol, 0.1% TEA (triethylamine), 0.1% TFA (trifluoroacetic acid) (v/v) without water and with 5.7% water added; flow rate 4.0 mL/min, back pressure 8 MPa; UV detection 220 nm; ambient temperature.

Compound	ΔN_{BCF6}	ΔT_{BCF6}	ΔN_{SCF6}	Δt_{SCF6}
5-aminoquinoline 	+817	-0.34	+825	-3.8
6-aminoquinoline 	+620	-0.7	+443	-6.7
Maprotiline 	+6680	-0.44	+4300	-14.5
Trimipramine 	+4500	-0.17	+1690	-4.5
Amitriptyline 	+8260	-0.5	+3590	-8.3
Alpranolol	+2690	+0.07	+2453	-1.6

				
Melamine 	+7750	+0.01	+1900	-6.4
Protriptyline 	+4150	-0.3	+2240	-10.3
Desipramine 	-3490	-0.25	+2930	-11.2
Propranolol 	+4543	-0.04	+1520	-7.2
Triphenylamine 	+4664	-0.24	-2507	-0.04
3-nitroaniline 	-2000	-0.01	-1950	-0.7
4-nitroaniline	+2795	0.0	+543	-0.05

				
Scopolamine 	+6000	-1.08	+2170	+0.6
Nefopam 	+5640	-0.14	+6700	-0.91
Methoxamine 	+7450	-0.24	+4350	-9.3
Butaxamine 	+3380	-0.2	+2750	-2.7
1-butylimidazole 	+7745	-0.14	+110	-3.3
Adenosine 	+6582	-0.4	-850	+0.2
1,2-dimethylimidazole 	+3800	-0.013	+1332	-10.6
1-benzyl imidazole	+5360	-0.36	-190	-5.9

				
Estriol 	+2050	-0.02	-66	+0.03
(S)-4-diphenylmethyl-2-oxazolidinone 	-4280	-0.03	-3680	-0.07
(S)-4,5,5-triphenyl-2-oxazolidinone 	-2156	-0.04	-6130	-0.18
Thymidine 	+1990	+0.2	+560	+0.4
Thymine 	+1330	+0.1	+520	+1.1
Caffeine 	+95	+0.03	-90	+0.18
Thymol	-1500	-0.003	-2500	-0.02

				
Phenol 	+4380	+0.019	-920	+0.02
p-toluic acid 	-950	+0.013	-272	+0.064
Nicotinic acid 	-210	+0.08	-512	+0.32
Ibuprofen 	-1300	-0.007	+205	-0.02
Diphenylacetic acid 	-1180	+0.01	-1900	-0.01
Phenylsuccinic acid 	+6100	+0.07	+2360	+0.572
Flurbiprofen 	-1980	-0.007	-2760	-0.06

2.4.3. Changing the efficiency and symmetry with the addition of water to the methanolic modifier on the benzoic acid-derivatized cyclofructan column

In Figure 2-3(a), a separation of 12 different compounds consisting of neutral, basic, and acidic analytes is shown using an 80/20/0.1%/0.1% CO_2/MeOH/TEA/TFA system without water. The mixture consists of an acidic compound (4-nitrobenzoic acid), xanthine (caffeine), amide derivatives of benzoic acid (nicotinamide, benzamide), tricyclic antidepressants (desipramine, amitriptyline), tropane alkaloids, namely scopolamine and atropine from the Belladonna plant (Solanaceae) and β-blockers (pindolol, carvedilol, atenolol). Most of the compounds are of pharmaceutical interest as they are used to treat diabetes, cancer, low heart rate (bradycardia), irregular heartbeat, and high blood pressure [31–33]. The separation takes about 10 min to complete without water. As expected from a weak cation exchanger column, Figure 2-3(a) shows that the elution pattern is: first acidic, followed by neutral, and then basic compounds. Only acidic and neutral compounds have acceptable symmetry in the range of (1.02–1.40), whereas amines show severe tailing (1.34– 3.75). 4-nitrobenzoic acid (pK_a 3.42) elutes the earliest. Compounds such as caffeine, benzamide, nicotinamide elute earlier than amines because they do not have electrostatic interactions with the stationary phase. After that, desipramine (pK_a 10.4) and amitriptyline (pK_a 9.18) elute from the column.

Any separation experiment aims to resolve a given mixture in the shortest possible time while avoiding peak overlap issues and without complicated method development. With this aim in consideration, the amount of water was varied in MeOH from 0.99% to 5.7%. The latter is the maximum amount of water in the co-solvent that can be added to subcritical CO_2, otherwise, phase separation occurs [9], and this can be observed from the appearance of a noisy baseline. As shown in Figure 2-3, a small increase in methanol's water content decreases the total run

time from 10 min to 6 min with all compounds resolved baseline (Figure 2-3 (b–d)). With 5.7% added water, the tailing factor improves from 3.75 to 1.87 for scopolamine, 2.50 to 1.34 for atropine, and 1.88 to 1.1 for carvedilol and atenolol. Table 2-2 summarizes the efficiency changes and peak symmetry for the twelve compounds separated in Figure 2-3.

The efficiencies ranged from 7400 to 19,800, with the highest efficiency for benzamide and lowest efficiency for 4-nitrobenzoic acid. The effect of adding 5.7% water was that the efficiency increased by about 160% for scopolamine, 77% for amitriptyline, 80% atenolol, and 74% carvedilol. The highest efficiency of benzamide corresponds to a reduced plate height of 1.8, which is considered to be exceptional [34] and comparable to high-efficiency liquid chromatography [35]. Similarly, all the secondary and tertiary amines such as amitriptyline, atropine, scopolamine, atenolol, and carvedilol show a substantial decrease in retention with a simultaneous improvement in plate count well as peak symmetry (Fig. 2-3(d)).

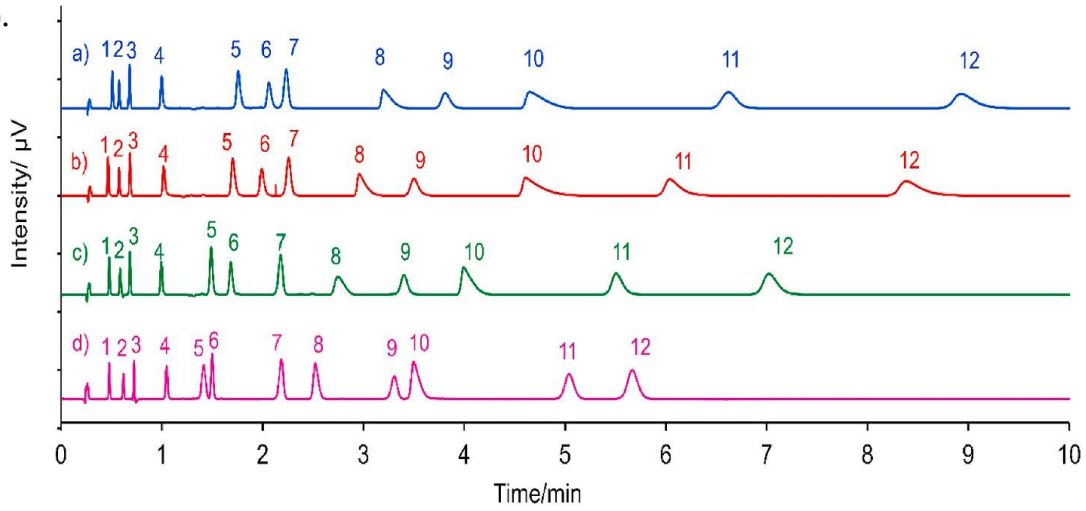


Figure 2-3. Separation of acidic, neutral, basic com- pounds and modified peak shapes and symmetries by adding different amount of water to modifier (1) 4- nitrobenzoic acid (2) caffeine (3) benzamide (4) nicotinamide (5) desipramine hydrochloride (6) amitriptyline hydrochloride (7) estriol (8) atropine (9) pindolol (10) scopolamine (11) atenolol (12) carvedilol. Conditions: BCF6 (2.7 µm particle, 10 ×0.46 cm), a) 80/20 CO_2/methanol, 0.1%TEA (triethylamine), 0.1% TFA (trifluoroacetic acid) with no added water b) with 0.99% water c) with 2.9% water d) with 5.7% water all with respect to MeOH flow rate 4.0 mL/min, UV detection at 220 nm, back pressure 8 MPa, ambient temperature 25 °C.

The performance of BCF6 chemistry in the HILIC mode was studied previously for beta-blockers [14,16]. In the SFC mode, pindalol elutes earlier than carvedilol and in HILIC, this selectivity was reversed with 75/25acetonitrile/20 mM ammonium acetate pH 4.2 (150 4.6 mm i. d.). A similar case appears with the atenolol/carvedilol pair [14]. Hence, SFC and HILIC modes with the same columns could serve well in two-dimensional chromatography, where orthogonality of selectivity is highly desirable. We note that the current SFC instrument may show some degree of tailing. This tail is partially originating from the extra column band broadening. We deliberately show the tailing chromatogram without water and then present improvement in peak shape and efficiency by adding a small amount of water into the modifier (see Table 2-2) even with extra column effects included.

Figure 2-4 shows a linear trend between retention factors of all the acidic, basic, and neutral compounds in Figure 2-3, as a function of % water content in the methanol modifier. Interestingly, the slope of 4-nitrobenzoic acid, caffeine, benzamide, nicotinamide, and estriol is close to zero, showing that the retention factor of neutral compounds does not change appreciably with or without water. It is clear from the large slopes of compounds 6,8,10, 11, and 12, that the amine-containing molecules are most affected by small amounts of added water. Increased efficiency contributes to a higher signal to noise ratio and hence a lower limit of detection. Also, it is known that the quantitation accuracy of tailing peaks diminishes because the integrator cannot determine the start and the end of the peak reliably. It is desirable to have tailing factors <2 for method validation purposes [36]. With the maximum water content in the co-solvent, most peaks are within this acceptable limit of tailing factors <2.

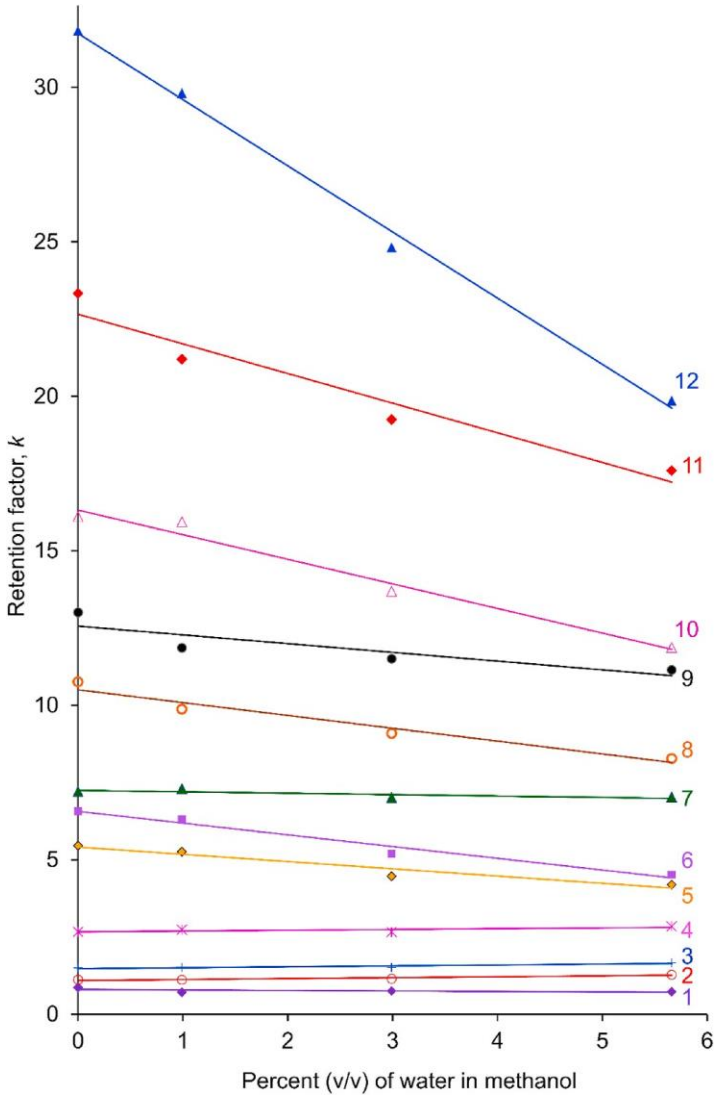


Figure 2-4. Plot of retention factor vs. the percentage (v/v) of water in the mobile phase for the BCF6 column. (1) 4-nitrobenzoic acid (2) caffeine (3) benzamide (4) nicotinamide (5) desipramine hydrochloride (6) amitriptyline hydrochloride (7) estriol (8) atropine (9) pindolol (10) scopolamine (11) atenolol (12) carvedilol. Conditions: BCF6 (2.7 μm particles, 10 × 0.46 cm), 80/20 CO_2/ methanol, 0.1%TEA (triethylamine), 0.1% TFA (trifluoroacetic acid), flow rate 4.0 mL/min, UV detection at 220 nm, back pressure 8 MPa, ambient temperature. A fixed dead time of 0.272 min was used for all conditions.

In Figure 2-5 (a), fast separation of a mixture of three compounds, namely 1-benzyl imidazole, tamoxifen, and atropine, is shown without water. 1- Benzyl imidazole is an inducer of various

cytochrome P-450 isozymes, tamoxifen is used to reduce the risk of new cancer developing in breast cancers, and atropine belongs to a class of drugs called anticholinergic and antispasmodic agents [37–39]. The three molecules contain tertiary amine groups.

Without water, 1-benzyl imidazole (pK_a 6.79) eluted first, followed by atropine (pK_a 14.12), and then tamoxifen (pK_a 8.69). The efficiency ranges from 2700 to 6100 plates, in the absence of water, in which the lowest value is for atropine. The tailing factor for 1-benzyl imid-azole is 3.45, atropine 5.02, and for tamoxifen 2.21. Adding 0.99% water (Figure 2- 5(b)) to the modifier causes the merging of atropine and tamoxifen peaks. Optimizing the amount of water (Figure 2-5(c)) reduces the run time and alters the elution order and with an 85%, 47%, 97% in- crease in N for compounds 1, 2, and 3, respectively. Tamoxifen shows the highest efficiency of 12,000, even under 2.5 min. Therefore, water acts as a tunable solvent component in SFC separations. Further addition of 5.7% water in MeOH results in a selectivity change but with some resolution loss.

2.4.4. An alternative modifier, in lieu of water, in SFC with BCF6 and SCF6 columns

The results listed in Table 1 indicated that while the sulfonated cyclofructan column produced enhanced separation efficiencies in the presence of water, it was even greater for the benzoic acid analog. The improvement was mainly for basic compounds (i.e., amines). The SCF6 column chemistry also showed very strong retention of basic compounds even in the presence of water in the mobile phase, as expected from a strong cation exchanger stationary phase. Consequently, another additive, tetramethylammonium acetate (TMAA), was sought to elute the highly retained bases (free or in hydrochloride form).

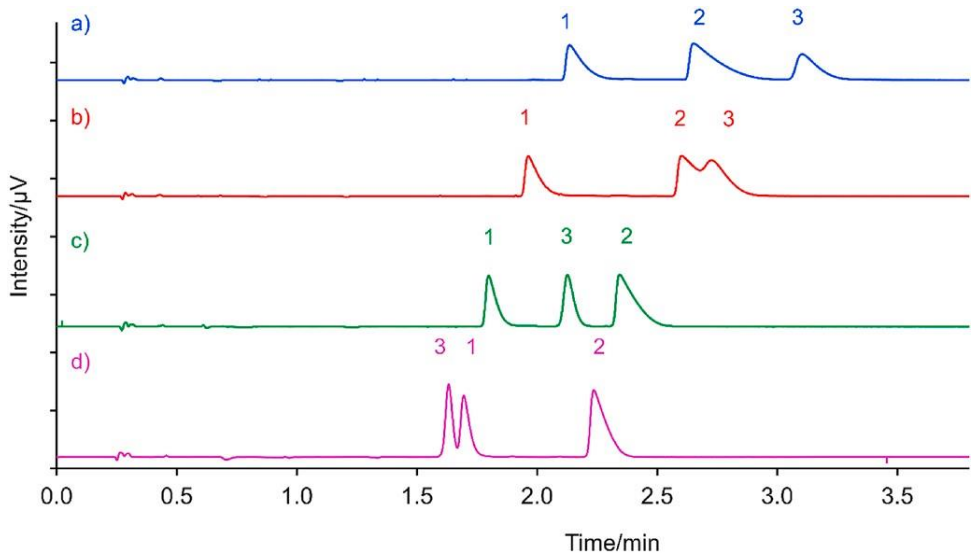


Figure 2-5. Separation of three compounds on the BCF6 (2.7 μm particles, 10 ×0.46 cm) with different amount of water added to modifier (1) 1-benzyl imidazole (2) atropine (3) tamoxifen. Conditions: a) 80/20 CO_2/methanol, 0.1% TEA (triethylamine), 0.1% TFA (trifluoroacetic acid) (v/v); b) with 0.99% water c) with 2.9% water d) with 5.7% water. flow rate 4.0 mL/min, back pressure 8 MPa; UV detection at 220 nm; ambient temperature.

In Figure 2-6(a), a sample of five different primary, secondary and tertiary amines, namely, amitriptyline, desipramine, protriptyline, maprotiline hydrochlorides, and 6-aminoquinoline as a free base, was examined. When there was no additive in the mobile phase, the retention time was more than 30 min for 6-aminoquinoline, and the other secondary/tertiary amines eluted between 18 and 25 min. Figure 2-6(b) shows the effect of adding water in methanol so that the composition was 80/20 (5.7%) CO_2/MeOH (H_2O). Again, the addition of water decreased, the retention factor for all compounds, and improved peak symmetry. The retention time for 6-aminoquinoline was 23 min, and the other amines eluted about 8–13 min. Alternatively, adding 0.05 w/v % TMAA into the MeOH produces even more interesting and enhanced results in the absence of water. Note that no other additive than TMAA and methanol was used. The retention times of all the compounds were dramatically decreased, and the selectivity was changed as well. The retention time for 6-aminoquinoline decreased to 1 min,

and the other amines eluted between 2 and 5.5 min, all baseline separated (Figure 2-6(c)). Further increasing the amount of TMAA to 0.1% w/v, the retention time of the components decreased to less than 5 min, as shown in Figure 2-6(d).

Four more free primary amines were investigated to see if this large selectivity shift was unique to the 6-aminoquinoline free base. The result showed that the effect of TMAA as an additive in MeOH was similar for all primary amines such as 5-aminoquinoline (pK_a 5.54), *R*-1,2-naphthyl ethyl amine (pK_a 9.36), S-1,2,3-triphenylethylene amine (pK_a 8.52), and 3-phenylpropylamine (pK_a 10.29). The primary amine retention times ranged from 29 to 34 min on the 10 cm SCF6 column at 4 mL/min. With 0.05% w/v TMAA, the retention times were reduced to between 1 and 5.5 min. Further investigation was done to confirm if the other secondary and tertiary amines' hydrochloride form had any effect on their retention. The free base form amitriptyline was obtained from the hydrochloride salt by treating with NaOH, followed by ether extraction.

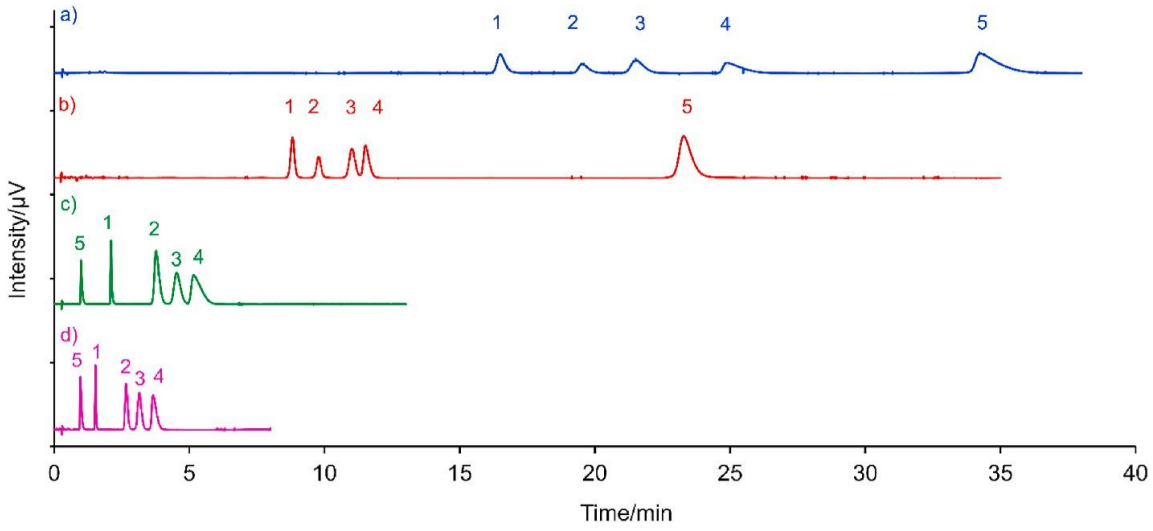


Figure 2-6. Separation of basic compounds by using different additives and moderating the retention time a) without water, b) adding 5.7% water to modifier, 0.1% TEA (triethylamine), 0.1% TFA(trifluoroacetic acid), c) 0.05% TMAA (tetramethylammonium acetate), d) 0.1% TMAA on SCF6 column (2.7 μm particles, 10×0.46cm), (1) amitriptyline hydrochloride (2) desipramine hydrochloride (3) protriptyline hydrochloride (4) maprotiline hydrochloride (5) 6-aminoquinoline. Conditions: flow rate 4.0 mL/min, UV detection at 220 nm, back pressure 8 MPa, ambient temperature.

The same set of compounds (amitriptyline, desipramine, protriptyline and maprotiline hydrochlorides, and 6-aminoquinoline) were run on the BCF6 column under identical conditions, see Figure 2-7. Two key observations are worth noting. There is a selectivity change if we compare Figure 2-6(a) and 7(a). The total run time is very short (4 min) compared to SCF6 (35 min) because the carboxylic group is a weak cation exchanger without water. Adding 5.7% water to the MeOH modifier causes protriptyline and maprotiline coelution (Figure 2-7(b)).

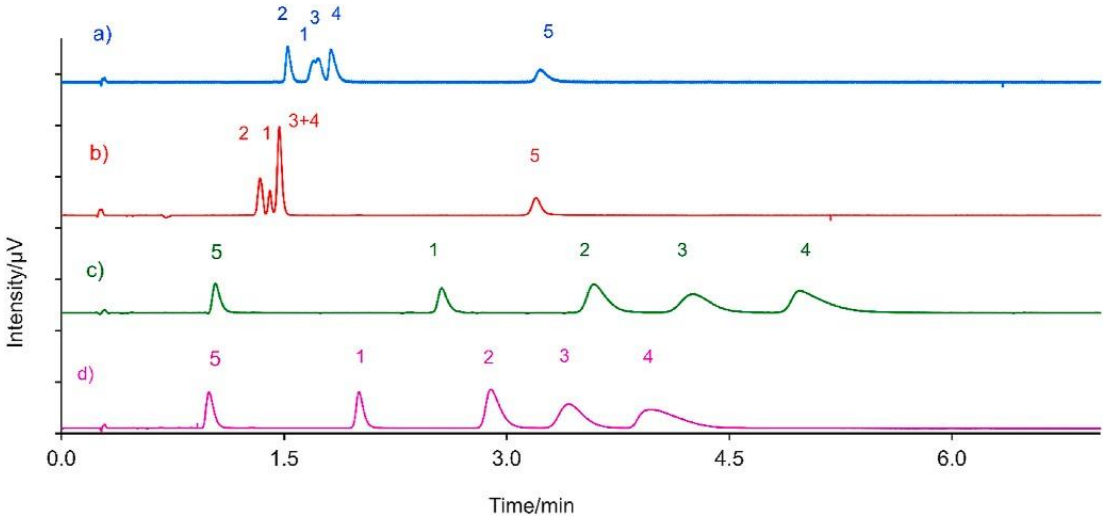


Figure 2-**7.** Separation of basic compounds by using different additives and moderating the retention time a) without water, b) adding 5.7% water to modifier, 0.1% TEA (triethylamine), 0.1% TFA (trifluoroacetic acid), c) 0.05% TMAA (tetramethylammonium acetate), d) 0.1% TMAA on BCF6 column (2.7μm particles, 10 × 0.46 cm), (1) amitriptyline hydrochloride (2) desipramine hydrochloride (3) protriptyline hydrochloride (4) maprotiline hydrochloride (4) 6-aminoquinoline. Conditions: 80/20 CO_2/MeOH with the above-mentioned additives, flow rate 4.0 mL/min, UV detection at 220 nm, back pressure 8 MPa, ambient temperature.

Interestingly, when TMAA is added, 6-aminoquinoline shows the same major elution order shift, as seen in Figure 2-6(b) and 7(b). However, this time the retention of all other compounds has increased, and the peaks are fully baseline resolved with $R_s > 1.5$. The order of elution of amitriptyline, desipramine, protriptyline, and maprotiline hydrochlorides is the

same as SCF6. Therefore, TMAA is a useful additive in SFC to obtain baseline separation of pharmaceutically important amines. TMAA does not require 0.1%TEA and 0.1% TFA to obtain satisfactory peak shapes and retention factors.

2.5. Conclusions

In this work, two new stationary phases with different hydrophilicity were developed for SFC and shown to provide the separation of clinically and pharmaceutically important bases and neutral compounds. Sulfonated cyclofructan-6 and benzoic acid functionalized cyclofructan- 6 (BCF6) were bonded to superficially porous particles using improved synthetic methodologies. Such stationary phases are highly beneficial in SFC for separating polar and basic compounds. The effect of adding water and tetramethylammonium acetate to tune the peak shapes of pharmaceutically important compounds and especially those of amines was found to be very beneficial. Trends were discussed in terms of efficiency gain and retention time changes. In most cases, especially for basic compounds, a small amount of water led to plate count enhancements. It was also demonstrated that tetramethylammonium acetate is very useful for tuning the elution strength of the polar co-solvent in SFC, and it did not need any other acid or base additive in the co-solvent, even with primary amines. Both water and TMAA show pronounced beneficial effects in separating basic compounds via SFC. TMAA shows the shortest retention time for the separation of basic compounds when using SCF6 stationary phases. Water shows a significant effect on the retention time, efficiency, and peak symmetry for separating basic compounds with the BCF6 stationary phase. This study demonstrates that selectivity is based on a combination of favorable features that vary depending on the column and the compound. Both BCF6 and SCF6 stationary phases are hydrophilic, but the effect of adding TMAA additive in the modified mobile phase was different in each case.

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Chapter 3

Liquid chromatography enantiomeric separation of chiral ethanolamine substituted compounds

3.1. Abstract

Eleven racemic ethanolamine derivatives were prepared, and their enantiomers were separated using liquid chromatography with various chiral columns. These derivatives included chiral vicinal amino alcohols, β-hydroxy ureas, β-hydroxy thioureas, and β-hydroxy guanidines, all of which are present in many active pharmaceutical ingredients. The screening study was performed with six chiral columns including four recently introduced superficially porous particles bonded with two macrocyclic glycopeptides, a cyclodextrin derivative, and a cyclofructan derivative. The two remaining columns were based on Okamoto's chiral stationary phases, one being a cellulose derivative and the other a derivatized amylose, both bonded to fully porous particles. The cyclodextrin and cellulose-based chiral stationary phases proved to be the most effective selectors and were able to separate 7 of the 11 tested compounds. With respect to analyte structural features, marked differences in enantiorecognition were observed between compounds containing phenyl and cyclohexyl groups adjacent to the asymmetric center. Additionally, replacing a small and electronegative oxygen atom by a larger and less electronegative sulfur atom induced a significant difference in chiral recognition by the cellulose derivative as well as by the vancomycin-based chiral selectors.

3.2. *Introduction*

The ethanolamine derived molecules described herein were prepared while investigating synthetic methods to be used in the total synthesis of 2-aminoimidazole (2-AI) marine natural products (Figure 3-1). These alkaloids are structurally intriguing targets for total synthesis because of their structural complexity and display a range of biological effects including anticancer and antimicrobial activity[1]. The original intent was to develop synthetic methods for the construction of linear ureas, thioureas, and guanidines which in turn would serve as surrogates for C2-functionalized imidazoles upon dehydrative cyclization. However, the synthesis of this small library of compounds also provided an opportunity to probe the enantiorecognition capabilities of a variety of chiral columns for molecules containing functionalities present in many pharmaceuticals[3], catalysts[4], and natural products[1] (Figure 3-2).

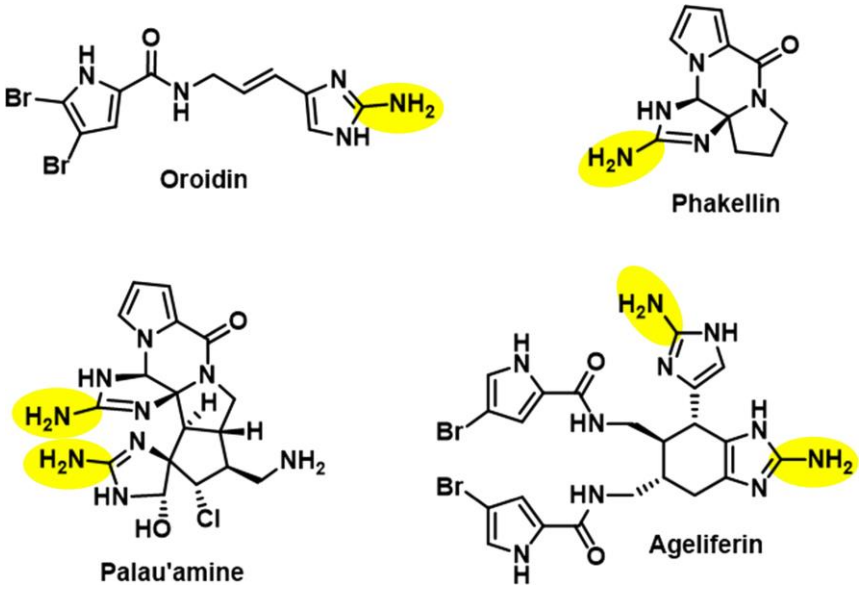
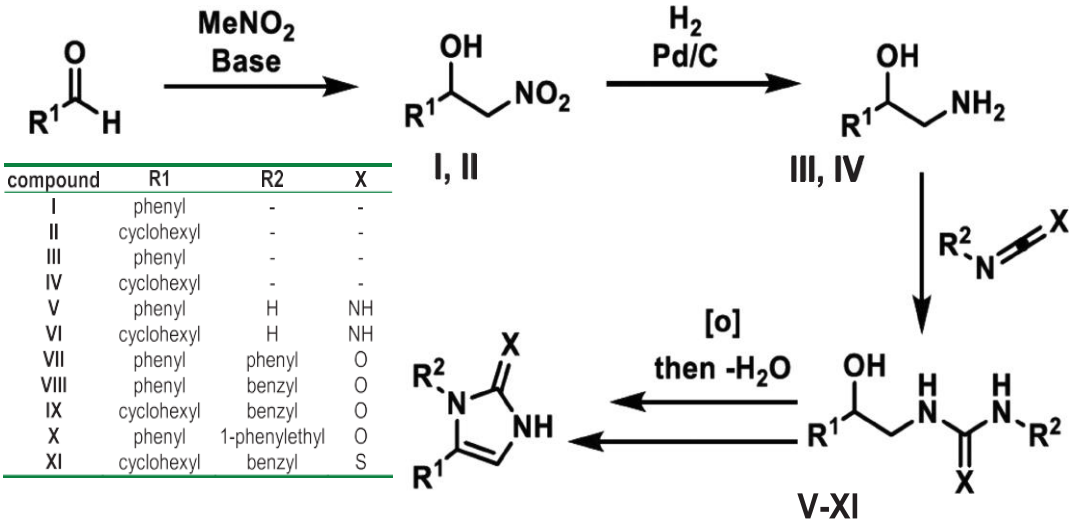


Figure 3-1 Examples of pharmaceutically active 2-aminoimidazolium-based (yellow highlight) marine natural compounds. Oroidin: $C_{11}H_{11}Br_2N_5O$, an antimalarial drug; phakellin: $C_{11}H_{13}N_5O$, an antibiotic; Palau 'amine: $C_{17}H_{22}ClN_9O_2$, a proteasome inhibitor (anticancer); ageliferin: $C_{22}H_{24}Br_2N_{10}O_2$, an actomyosin ATPase activator (muscular/cardiac contraction)

In regard to their usefulness in approaches to 2-AI natural products, the synthesized species provide an indirect method for introducing functionality at the imidazole C2 position. The addition of an amino group at the C2 position is often a significant hurdle in the synthesis of 2-AI natural products, as this renders the molecules highly polar and can make purification difficult using normal phase chromatographic methods. The majority of methods used to synthesize 2-AI alkaloids involve either direct functionalization of imidazole at the C2 position using a strong base and an electrophilic nitrogen source (such as an azide, followed by reduction), or de novo imidazole syntheses such as condensation of α–functionalized ketones with cyanamides. In an effort to provide a solution to a hurdle in a synthetic project and to add to available methods for synthesizing C2 functionalized imidazoles, a pathway for transforming aldehydes into C2-functionalized 1,3-azoles has been developed.

In this pathway, all compounds preceding imidazole formation retain a secondary alcohol asymmetric center. Consequently, all of the intermediate urea, thiourea, and guanidine derivatives obtained are chiral (but racemic mixtures). The importance of enantiomeric separations for the pharmaceutical industry is highlighted by the thalidomide tragedy. The underlying cause was shown to be the result of differing biological action between the two enantiomers of thalidomide[5]. In 1992, the Food and Drug Administration (FDA) issued a policy statement indicating that no active pharmaceutical ingredient (API) can be used as a racemic mixture unless both enantiomers are tested for biological activity, toxicity and whether or not fast enantiomer interconversion occurs.[6] As a result, both enantiomers of potential APIs are needed for testing to determine which enantiomer displays the desired biological activity and to determine if metabolic racemization occurs.



compound	R1	R2	X
I	phenyl	-	-
II	cyclohexyl	-	-
III	phenyl	-	-
IV	cyclohexyl	-	-
V	phenyl	H	NH
VI	cyclohexyl	H	NH
VII	phenyl	phenyl	O
VIII	phenyl	benzyl	O
IX	cyclohexyl	benzyl	O
X	phenyl	1-phenylethyl	O
XI	cyclohexyl	benzyl	S

Figure 3-2. General synthetic scheme using chiral ethanolamines III and IV for the synthesis of C2-heteroatom-functionalized 1,3-azoles (V–VI), cyclic ureas (VII–X), or cyclic thioureas (XI)

To separate the ethanolamine derivatives presented here, liquid chromatography (HPLC) was deemed most appropriate due to the samples' solubility. The enantiomeric separation was performed using chiral stationary phases (CSPs) in which the chiral selector is not consumed but is attached to the stationary phase support[7-16]. It is often difficult to predict which selector will be most efficient to separate a given pair of enantiomers[17-22], so several CSPs must be systematically tested with a set of chiral compounds. The results of such an investigation are presented below.

3.3. Experimental

3.3.1. Chemicals

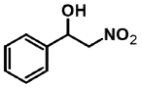
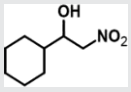
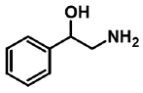
HPLC grade heptane, hexane, methanol, ethanol, isopropyl alcohol and acetonitrile solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Benzaldehyde, cyclohexane carboxaldehyde, nitromethane, isocyanate and isothiocyanate were obtained from Sigma-Aldrich (Millipore-Sigma, St Louis, MO, USA) and used as received except benzaldehyde which was washed with 10% sodium carbonate to remove the organic acid possibly formed by oxidation

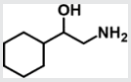
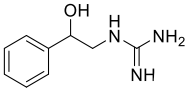
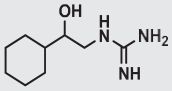
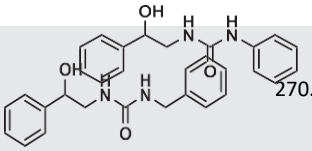
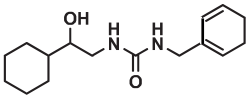
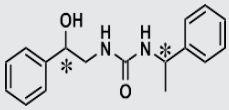
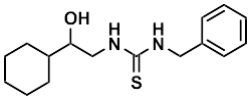
during storage. Analytical grade ammonium formate, triethylamine and acetic acid were obtained from Sigma-Aldrich.

3.3.2. Synthesis pathway

The synthesis of the racemic ethanolamine derivatives is outlined in Figure 3-3. The twelve chiral compounds **I-XII** studied in this work are listed in Table 3-1. The synthesis (Figure 3-3) involves the addition of nitromethane to an aldehyde via Henry chemistry and subsequent reduction of the nitro group to an amine via catalytic hydrogenation using Pd-C and a hydrogen-filled balloon. The resulting ethanolamine derivatives were obtained with yields ranging between 51-85%. These ethanolamine derivatives were then reacted with N, N'-di-Boc-S-methylisothiourea in the presence of mercury (II) chloride to form guanidines, isocyanates to form ureas, or isothiocyanates to form thioureas. The Boc-protected guanidine derivatives then underwent Boc deprotection using trifluoroacetic acid to give the free guanidines in quantitative yield.

TABLE 3-1 Nitroethanol precursors and chiral ethanolamine derivatives studied in this work

#	Lovely code & name	Formula	Structure	m.w.	Log
I	2-Nitro-1-phenylethanol	$C_8H_9NO_3$		167.16	0.9
II	2-Nitro-1-cyclohexylethanol	$C_8H_{15}NO_3$		173.21	1.8
III	2-Amino-1-phenylethanol	$C_8H_{11}NO$		137.18	0.1

IV	2-Amino-1-cyclohexylethanol	$C_8H_{17}NO$		143.23	1.0
V	2-(2-Hydroxy-2-phenylethyl) guanidine	$C_9H_{13}N_3O$		179.22	—0.5
VI	2-(2-Hydroxy-2-cyclohexylethyl) guanidine	$C_9H_{19}N_3O$		185.27	—0.1
VII	1-(2-Hydroxy-2-phenylethyl)-3-phenylurea	$C_{15}H_{16}N_2O_2$		256.30	2.1
VIII	1-(2-Hydroxy-2-phenylethyl)-3-benzylurea	$C_{16}H_{18}N_2O_2$		270.33	2.4
IX	1-(2-Hydroxy-2-cyclohexylethyl)-3-benzylurea	$C_{16}H_{24}N_2O_2$		276.3 7	2.6
X[b]	1-(2-Hydroxy-2-phenylethyl)-3-(1-phenylethyl)urea	$C_{17}H_{20}N_2O_2$		284.34	2.5
XI	1-(2-Hydroxy-2-cyclohexyl ethyl)-3-benzylthiourea	$C_{16}H_{24}N_2OS$		292.38	2.8

Computed by XLogP PubChem version 3.0.

[b]Compound X has two chiral centers (*), hence four stereoisomers, two diasteomers referred as X-1 and X-2, each as a racemate

3.3.3. Chiral chromatography

The liquid chromatography system used was the 1100 Infinity set from Agilent (Santa Clara. CA. USA) including a binary pump, mobile phase degasser, 96 vial sample injector, column thermostat, and diode array UV detector. A personal computer drove the chromatographic system and handled data with the OpenLab CDS ChemStation software (Agilent). Acetonitrile solutions of all racemic samples were made at a concentration of ~1 mg/mL One microliter of each individual solution was injected for each analysis. Since Compounds **II, IV** and **VI** lacked UV chromophores (Table 1) they were assayed on a Shimadzu Prominence HPLC with a 8040 triple quad mass spectrometer (Shimadzu, Columbia, MD, USA) using similar experimental conditions but detected by multiple reaction monitoring MS.

Table 3-2 lists the characteristics of the six chiral columns used. The first three columns were 10 cm long and the other three columns were 15 cm long. The tubing internal diameter was 4.6 mm except for the ChiralPack IC-3 column that was 3 mm. The VancoShell and NicoShell CSPs are based on macrocyclic glycopeptide natural chiral selectors which have differing enantiomeric affinities for a wide variety of chiral compounds.[7-10] The LarihcShell-P column is based on isopropyl derivatized cyclofructan-6 with a unique enantioselectivity for chiral primary amines.[11] The CD-Shell-RSP column contains a hydroxypropyl derivatized β-cyclodextrin selector.[7] These four columns were filled with recently introduced 2.7 µm spherical superficially porous particles (SPPs).[12-15] They were provided by AZYP, LLC (Arlington, TX, USA). The last two columns are based on two different derivatized carbohydrates bonded to 3 µm classical spherical fully porous particles (FPP). They were obtained from Daicel (Chiral Technologies, Inc, Illkirch, France).

Table 3-2 Characteristics of the chiral stationary phases used

Code	Trade name & manufacturer	Chiral selector	Column geometry (cm)	Particle type & diameter
Vanco2	VancoShell AZYP[a]	Vancomycin	10 × 0.46	SPP[b]
2.7 µm Nico	NicoShell AZYP[a]	Macrocyclic glycopeptide	10 × 0.46	SPP[b]
2.7 µm LP	LarihcShell-P AZYP[a]	Isopropyl cyclofructan	10 × 0.46	SPP[b]
2.7 µm RSP	CDShell-RSP AZYP[a]	Hydroxypropyl β-cyclodextrin	15 × 0.46	SPP[b]
2.7 µm IC	ChiralPak IC-3 Daicel[c]	3,5-Dichlorophenyl cellulose	15 × 0.3	FPP[d]
3 µm IG	ChiralPak IG Daicel[c]	3-Methyl, 5-chlorophenyl amylose	15 × 0.46	FPP[d]
3 µm				

[a]AZYP, LLC, Arlington, TX 76019, USA.

[b]SPP: superficially porous particles.

[c]Daicel Chiral Technologies, Inc., Illkirch, France.

[d]FPP: fully porous particles.

Table 3-3 Chromatographic results

Compound #	tr_1[a] (min)	Flow rate (mL/min)	α[b]	Rs[c]	Column[d]	Elution mode[e]
I	7.7	0.6	1.04	0.4	RSP	RP
	20.4	0.4	1.10	2.0	IG	NP
II	23.6	0.6	1.04	1.8	RSP	RP
	4.1	1.0	1.11	1.8	IC	NP
III	20.3 (R)[f]	0.3	1.07	3.4	Vanco	RP
	11.7 (R)[f]	0.6	1.06	2.0	Nico	RP
	4.2 (R)[f]	1.0	1.10	1.8	Nico	POM
	6.0 (S)[f]	1.0	1.13	2.8	LP	POM
	14.9 (R)[f]	0.3	1.05	1.7	RSP	RP
IV	15.1	0.8	1.06	1.3	Nico	RP
	7.9	0.8	1.13	2.6	Nico	POM
	14.1	0.8	1.06	0.6	RSP	RP
V	26.5 (R)[f]	0.25	1.04	2.0	Vanco	RP
	10.2 (R)[f]	0.6	1.06	1.9	RSP	RP
VI	11.4	0.8	1.04	0.8	Nico	RP
	6.1	0.8	1.05	1.4	Nico	POM
	13.3	0.8	1.04	0.8	RSP	RP
VII	17.2 (R)[f]	0.6	1.07	2.1	RSP	RP
	12.5 (R)[f]	0.6	1.15	2.6	IC	NP
	10.0 (R)[f]	0.4	1.33	6.4	IG	POM
VIII	8.6	0.6	1.06	1.7	RSP	RP
	7.7	0.5	1.19	3.5	IC	NP
IX	7.1	0.4	1.01	0.4	Vanco	RP

	7.0	1.0	1.31	4.3	IC	NP
X-1 (1st pair)	51.4	0.3	1.29	5.0	IC	NP
X-2 (2nd pair)	76.1	0.3	1.26	4.7	IC	NP
XI	7.8	0.4	1.06	1.0	Vanco	RP
	3.8	1.0	1.07	0.7	IC	NP

[a]tr_1: retention time of the first enantiomers.

[e]Elution mode: RP = reversed phase with formate buffer and methanol; NP = normal phase with heptane: isopropyl alcohol 95/5% v/v; POM = polar organic

waterless mode with acetonitrile and methanol.

[f]Absolute configuration of the 1st eluted enantiomer. Compound X has two pairs of enantiomers: X-1 and X-2 (see Figure 5C).

3.4. Results and Discussions

In this work a number of different CSPs and mobile phase combinations were evaluated for enantiomeric separations of the entire set of ethanolamine substituted compounds. Initial testing began using standard conditions. Variations in the methods were needed, including detection approaches for specific synthetic intermediates, as will be discussed. The normal phase mode with apolar mobile phases was not tested with the polar CSPs, VancoShell, NicoShell, LarihcShell-P and CDShell-RSP columns even though it can be efficient in particular cases.[13] Similarly, the reversed phase mode is not recommended with amylose or cellulose-based CSPs that were assayed only in the normal phase mode.

3.4.1. Chiral ethanolamine analogue enantioseparations

Table 3-1 shows that there is a noteworthy similarity between the chiral compounds of the studied set. All of the compounds are secondary alcohols. In every case, the stereogenic center bears a hydrogen atom, a hydroxyl group, and either a cyclohexyl or phenyl moiety. The fourth substituent is a methylene moiety bearing simple or complex nitrogen containing groups. Compound **X** has a second stereogenic center in its "nitrogen containing group" which makes it the only compound of this group that has four different stereoisomers, i.e., two pairs of enantiomers (Table 3-1).

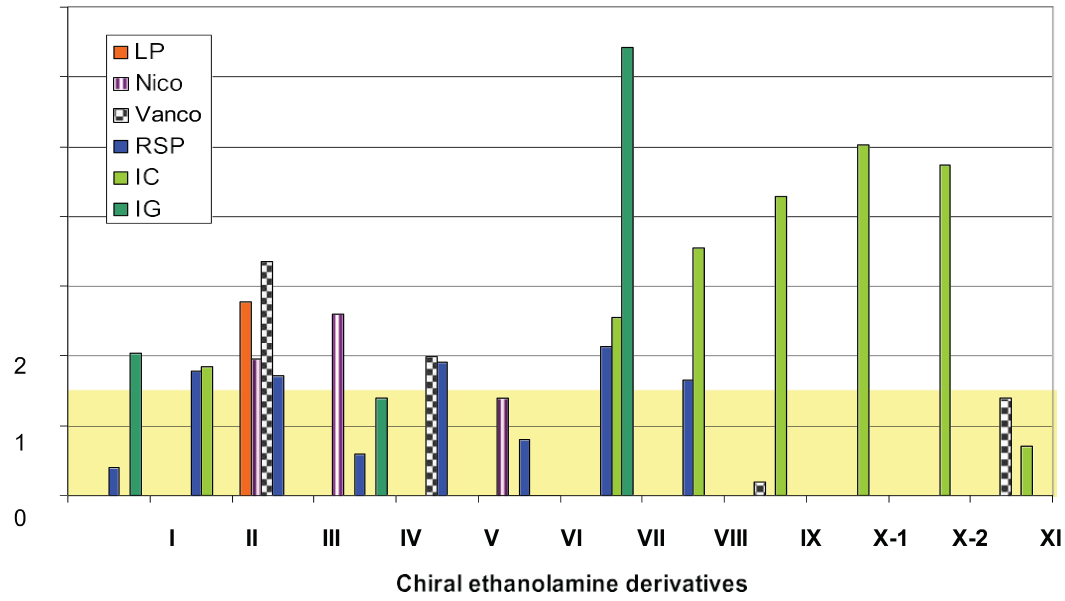


Figure 3-3 Separation of the enantiomers of the Table 3-1 compounds on the six Table 3-2 chiral stationary phase using various elution modes. The chromatographic data is listed in Table 3-3. The yellow area corresponds to a partial separation of the enantiomers with Rs < 1.5

Table 3-3 shows that all 11 enantiomeric pairs could be separated by at least one column of the tested CSP set (Table 3-2). Figure 3-4 shows that the enantiomers of compound **III,** the simple phenylethanolamine compound, were the most easily separated. The four SPP based columns gave baseline separations (Rs > 1.5). The two carbohydrate based FPP columns were unable to differentiate these enantiomers. The most overall effective column was the cyclodextrin-based RSP CSP separating eight compounds of the eleven Table 3-1 ethanolamine derivatives. The cellulose-based IC CSP is next being able to separate seven of the analytes. These two columns are complementary for the separation of this set of compounds: the RSP column separating compounds **I** to **VIII** and the IC column separating compounds **VII** to **XI** (Figure 3-4)**.** The least effective column for this family of compounds was the cyclofructan-based LP column separating only Compound **III**.

3.4.2. Phenyl versus cyclohexyl substituents

Though the phenyl and cyclohexyl substituent are both 6-carbon cyclic groups, they are structurally very different. The phenyl group is flat with 6 sp^2 hybridized carbon atoms containing a ring of π electrons, while the cyclohexyl group is conformationally dynamic with six sp^3 hybridized carbon atoms and only σ bonds. It is interesting to compare the enantioresolution of the phenyl substituted compounds with their cyclohexyl substituted counterparts. Compound **III**, the simple 1-phenylethanolamine, was enantioseparated with the four SPP CSPs. The enantiomers of compound **IV**, 1-cyclohexyl-ethanolamine, were separated by the NicoShell and CDShell-RSP CSPs only. Fig. 5 shows the chromatograms obtained for these two compounds on these two chiral stationary phases in the reversed phase mode. Note that the phenyl substituted molecules have better enantioresolutions and higher chromatographic efficiencies than the cyclohexyl substituted analogues. This observation is not true in the polar organic mode where the same NicoShell CSP provided a better enantioseparated compound **IV** (cyclohexyl) with Rs = 2.6 than compound **III** (phenyl and Rs = 1.8, Table 3-3). It can be seen that, in the reversed phase mode, enantiomers containing a rigid phenyl ring are more easily differentiated than those with the more flexible cyclohexyl substituent. Compounds **V** and **VI**, also differing only by the phenyl or cyclohexyl ring, were both separated by the CDShell-RSP column in the reversed phase mode. The phenyl substituted compound **V** showed full enantioresolution (Rs = 1.9), but the cyclohexyl substituted compound **VI** showed only partial resolution with Rs = 0.8 (Table 3-3). Compounds **VIII** and **XI** also only differ by the phenyl or cyclohexyl substituent on the stereogenic center. Again, the phenyl substituted compound exhibited better enantioseparation in the reversed phase mode (Table 3-3). This trend also was seen in the normal phase mode, where compounds **VIII** and **XI** were

both resolved by the IC column. The enantioresolution factor was 3.5 for the phenyl Compound **VIII** and only 0.7 for its cyclohexyl counterpart Compound **XI** (Table 3-3).

3.4.3. Oxygen versus sulfur moieties

Compounds **IX** and **XI** differ only by their respective oxygen or sulfur atom (Table 3-1). Only the IC column, containing the 3,5-dichlorophenyl derivatized cellulose, first introduced by Okamoto et al.[22], could fully separate these enantiomers in the normal phase mode. Figure 3-5a and 5b shows the obtained chromatograms. The VancoShell column could partially separate these compounds in the reversed phase mode (Table 3-3). This result would be expected considering that the difference between the two compounds is the O or S atoms located relatively far from the stereogenic center. However, on the IC chiral column, there is a large difference in enantiorecognition. It seems that the size difference between the O (120 pm diameter), and S (200 pm) atoms, and/or electronegativity difference (O = 3.44; S= 2.48) completely changes the enantiomeric interaction or access to the helically oriented chiral carbohydrate selector. This also is indicated by the significant retention difference between the two pairs of enantiomers. compounds **IX** (urea) and **XI** (thiourea) have similar polarity (with similar Log P_{oct}, Table 3-1) but very different retention times, respectively 7 min and 3.8 min for the first enantiomers (Figure 3-6a and 6b), eluted by the same heptane/isopropanol 95:5 v/v mobile phase. The enantioselective interactions of the smaller compound **IX** with the helicoidal carbohydrate CSP increase the retention of the two enantiomers differently inducing excellent separation (Figure 3-6a). The interaction of these two compounds with the VancoShell SPP CSP in the reversed phase mode is completely different: the S atom of Compound XI enhances the enantiorecognition (Rs = 1.0) compared to the Compound IX (O atom and Rs = 0.4, Table 3-3)

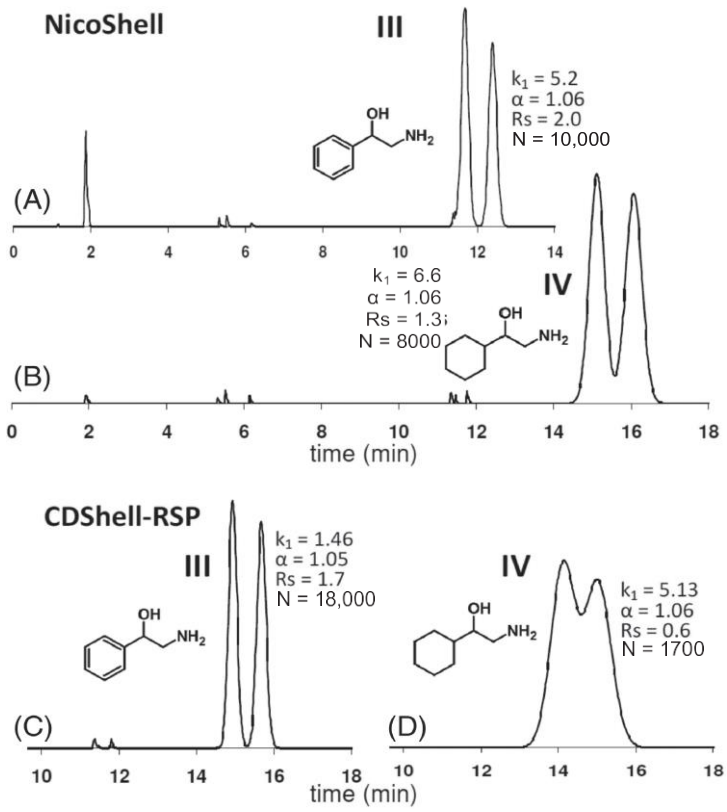


Figure 3-4 Chromatograms of compounds III and IV on the NicoShell (A, B) and CDShell-RSP (C, D) columns in the reversed phase mode. (A) Mobile phase: methanol/formate buffer 90:10 (v:v), 0.6 mL/min; (B) mobile phase: ethanol/ formate buffer 85:15 (v:v), 0.8 mL/min; (C) mobile phase: acetonitrile/buffer 30:70 (v:v), 0.3 mL/min; (D) mobile phase: acetonitrile/buffer 10:90 (v:v), 0.8 mL/min. Detection A and C: ultraviolet (UV) 220 nm.; B and D: mass spectrometer (m/z 144 ion source; m/z 126 product, collision energy 18 kV)

3.4.4. Two stereogenic centers

The phenylethyl urea, used to prepare Compound **X**, was chiral and racemic. Therefore, Compound **X** has four stereoisomers (two pairs of enantiomers). All stereoisomers were baseline separated using the IC column in the normal phase mode (heptane/isopropyl alcohol 95:5 %v/v), as shown in Figure 3-6 c. We were unable to identify the individual enantiomers so Table 3-3 lists values for the stereoisomeric pairs **X-1** and **X-2** which corresponds, respectively, to the first and second peaks, and third and last peaks (Figure 3-6 c). It should be noted that if the first enantiomeric pair was actually peaks 1 and 3, and the second pair was peaks 2 and 4, the enantioselectivity and resolution factors would be even higher. Regardless, this result shows that

this specific derivatized cellulose chiral selector is highly selective for all of the phenyl substituted stereoisomers of Compound **X**.

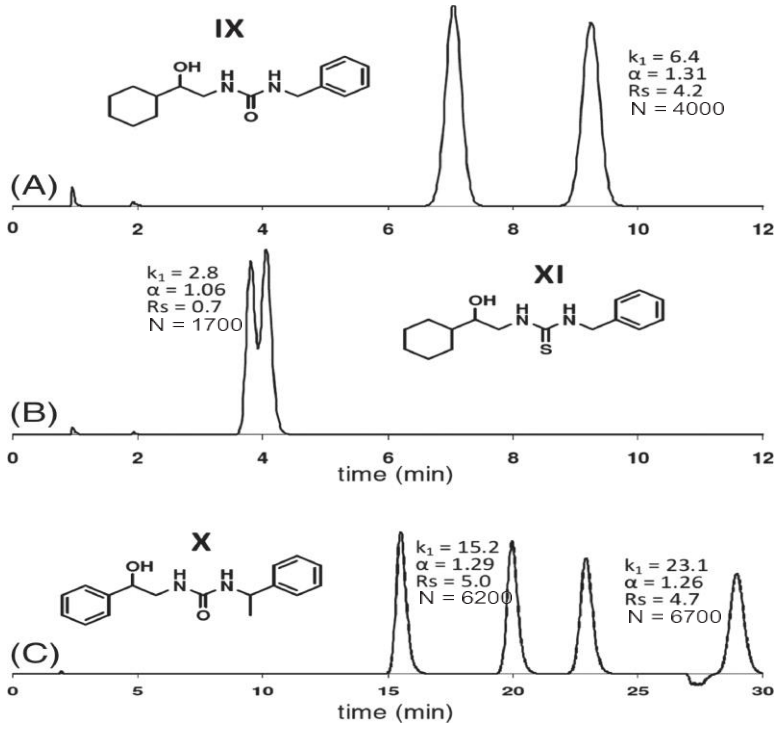


Figure 3-5 Chromatograms of Compounds IX, X, and XI on the ChiralPack IC-3 column. (A,B) Effect of the oxygen or sulfur atom. (C) Separation of the four enantiomers of compound X. The first two peaks are considered as pair X-1 and the last two peaks as enantiomeric pair X-2 in Table 3. Mobile phase: heptane/ isopropanol 95:5 (v:v), 1 mL/min. Detection: ultraviolet (UV) 220 nm

3.5. Conclusions

In a screening study, it is important to be able to test a wide variety of different chiral stationary phases using the three different elution modes: reversed phase, polar organic, and normal phase. When the enantiomers are separated, even partially, the solvent proportions can be optimized to improve the enantioresolution. In this screening study of chiral ethanolamine derivatives, the two most effective CSPs were the derivatized cellulose (ChiralPack IC-3) and the derivatized cyclodextrin (CDShell RSP), both based on carbohydrate structures. Each of these two CSPs were able to partly or fully separate seven and eight out of a set of 11 synthetic ethanolamine

substituted compounds, respectively. The next most effective column separating four enantiomeric pairs was the VancoShell column based on a macrocyclic glycopeptide chiral selector. Examining the structure of the chiral compounds, a rigid phenyl ring attached to the stereogenic center was clearly beneficial, making it easier to differentiate enantiomers by all chiral selectors. It was found that the sulfur atom may induce steric hindrance and/or electrostatic interaction in thiourea chiral derivatives interacting differently with the helicoidal cellulosic selectors while enhancing chiral recognition by the vancomycin based CSP.

3.6. References

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Appendix A

List of Co-authors and Citations

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