Supercritical Fluid Chromatography of Ionic Compounds

by

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ABSTRACT

Addition of a small amount of polar solvent (i.e. modifier) which contains an ionic component (i.e. additive) to a CO_2 mobile phase has shown major improvement in the elution of ionic analytes via packed column supercritical fluid chromatography (SFC).

Firstly, we focused on the elution of sodium arylsulfonate analytes by using various ionic additives, such as lithium acetate, ammonium acetate, tetramethylammonium acetate, tetrabutylammonium acetate, and ammonium chloride. The analytes were successfully eluted with all additives with good peak shape under isocratic/isobaric/isothermal conditions. Three stationary phases with different degrees of deactivation were considered. They were conventional Cyanopropyl, Deltabond Cyanopropyl, and non-chemically bonded silica. The effect of additive concentration and additive functionality on retention was also investigated.

Secondly, solid state NMR of the silica packing material before and after being flushed with supercritical CO_2 modified by methanol containing the ionic additives was performed to gain some insight into the retention mechanism(s). A fraction of silanol protons were undetected after being treated with the mobile phase which suggested replacement by the cationic component of the additive. CaChe calculations were carried out on several of the additives in an attempt to explain why different ionic additives produce different effects on chromatographic retention. Modification of the stationary phase and ion pairing with the analyte are two possible retention mechanisms being considered.

As ion-pair formation was considered to be one of the retention mechanisms, the use of sodium sulfonates as mobile phase additives to elute secondary and quaternary ammonium salts was then studied. Propranolol HCl, benzyltrimethylammonium chloride, and cetylpyridium chloride were chosen as the probe analytes. Sodium ethansulfonate, sodium 1-heptanesulfonate, and sodium 1-decanesulfonate were studied as mobile phase additives. The analytes were successfully eluted from Deltabond Cyano phase within 5 minutes, but were retained strongly without additive or with ammonium acetate as the additive. An Ethylpyridine column showed dramatic advantages on the elution of these ammonium analytes. No additive was required to elute these ionic compounds. Protonation of some fraction of the pyridine functional groups and the deactivation of active silanol sites were believed to be the major mechanisms responsible for this behavior.

Lastly, we successfully eluted large peptides (up to 40 mers) containing a variety of acidic and basic residues in SFC. We used trifluoroacetic acid as additive in a CO_2/methanol mobile phase to suppress deprotonation of peptide carboxylic acid groups and to protonate peptide amino groups. The Ethylpyridine column was used for the majority of this work. The relatively simple mobile phase was compatible with mass spectrometric (MS) detection. To our knowledge, this is the first report of the elution of peptides of this size with a simple, MS-compatible mobile phase. Fast analysis speed, the possibility of coupling multiple columns to achieve desired resolution, a normal-phase retention mechanism, and less use of organic solvents are the advantages of SFC approach for peptide separation.

This dissertation is dedicated to

Sheng, Dad, and Mom

Personal Acknowledgements

I would like to thank my families for their support through my entire education. I would like to extend my deepest thanks to my husband, Sheng Tu for his love, encouragement, and inspiration. Without him I couldn't overcome difficulties during the tough times.

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CHAPTER 1

Introduction

A supercritical fluid is any substance above its critical temperature and critical pressure (see **Figure 1**). Supercritical fluids possess both gas- and liquid-like properties. Mass transfer is rapid with supercritical fluids. They have relatively high diffusivities, which are more than ten times those of liquids. Their dynamic viscosities are nearer to those of the gaseous state. On the other hand, their densities allow much greater solvating power than gases [1].

Supercritical fluid chromatography (SFC) is an intermediate technique between gas chromatography (GC) and liquid chromatography (LC), using a supercritical fluid as the mobile phase. In this dissertation, no differentiation will be made between what is sometimes called subcritical fluid chromatography (subSFC, or sSFC) and SFC. Many of the same advantages apply to subSFC and SFC, and the same instrumentation is required. As long as the mobile phase remains in the one-phase region, good chromatographic performance can be achieved [2].

Supercritical carbon dioxide has a relatively low critical pressure and critical temperature (73 atm and 31°C). Because of these factors and because of its chemical inertness and non-flammability, supercritical CO_2 has been the most commonly used mobile phase in SFC. The low viscosity of supercritical carbon dioxide results in: (a) the use of increased flow rate, providing higher column efficiency per unit time, thus shortening analysis time; (b) relatively low backpressure, which allows the use of longer columns and the coupling of columns in series to increase both selectivity and efficiency. From the environmental and economical point of view, much less organic solvent is used

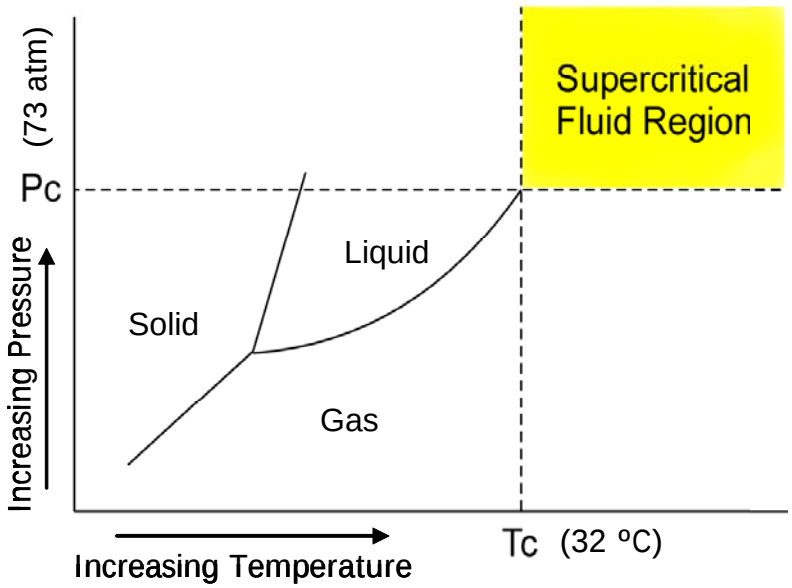


Figure 1. Phase diagram of a pure fluid. (32 °C and 73 atm are the critical temperature and pressure for carbon dioxide.)

in SFC than in traditional LC. This provides major ecomomic advantages in preparative SFC separations. [3]

Solutes must have some solubility in a fluid for that fluid to be an effective chromatographic mobile phase. Unfortunately, the solvating strength of carbon dioxide has been shown to be in the same range as liquid hexane [4,5]. In 1978, Stahl et al.[6,7] demonstrated the solubility of several amino acids and sugars at extremely high pressures. With carbon dioxide at 40 °C and 2000 bar, solubilities were as follows: glycine, 0.40 μg/L; L-leucine, 0.55 μg/L; sucrose, 1.5 μg/L; glucose 2 μg/L, and xylose, 3.0 μg/L. These very low solubilities suggested that successful chromatography of biomolecules with carbon dioxide alone would not be practical. The weak solvating power of pure CO_2 has thus limited the application of SFC to intermediate polar and non-polar compounds. [6-8] Furthermore, conventional HPLC packed columns are not sufficiently deactivated for the separation of polar analytes with non-polar CO_2 as the mobile phase.

Deactivated stationary phases have been studied to extend the application of packed column SFC [9-11]. Ashraf-Khorassani et al. [11] and Berger et al. [12] reported that less active phases provided a significant improvement in peak shape compared to similar, but non-deactivated, phases for the elution of phenols, anilines, and benzylamines. Not only was deactivation of the stationary phases important, but the right polarity match between the stationary phase, solute, and mobile phase was also thought to be critical.

To increase the solvating power of carbon dioxide, researchers have explored the use of polar modifiers. Both inorganic and organic polar solvents have been studied.

When water was used as a CO_2 mobile phase modifier, retention time was shortened and a significant improvement of peak shape was achieved for the elution of weak acids and bases from various stationary phases [13-15]. The low solubility of water in supercritical carbon dioxide, however, limited its further application as a mobile phase modifier. On the other hand, methanol is miscible with liquid CO_2. Solvatochromic dye studies showed that a small amount of methanol added to CO_2 can produce a dramatic increase in mobile phase solvent strength [1]. Many papers have been published on the effects of methanol as a secondary mobile phase component, especially with respect to retention characteristics, selectivity, and peak shape of various test solutes. The separation of clevidipine, a new dihydropyridine drug, and its by-products [16]; a mixture of lenbuterol, salbutamol, terbutaline and fenoterol [17]; Pd-β-diketonates of pentane-2,4-dione, 6-methylheptane-2,4-dione and 2,2,7-trimethyloctane-3,5-dione [18]; and various sulphonamides [19] have all been reported.

The binary mobile phase system is an improvement over pure CO_2. But binary fluids are often still not sufficient to elute highly polar or even ionic compounds because modifiers that are miscible with carbon dioxide are also only moderately polar solvents. Berger et al. [20] conducted solvatochromic dye studies and showed that very polar compounds, such as trifluoroacetic acid (TFA), when added to modifiers could significantly increase the polarity of modified mobile phases. Small concentrations (i.e., 10^{-4} M) of such very polar compounds improved chromatographic peak shapes and eluted solutes that were normally retained. For example, the elution of strong organic bases was investigated with first pure carbon dioxide, pure Freon-23, or pure Freon-13, then with methanol-modified carbon dioxide containing a basic additive [21]. The solutes did not

elute with pure solvent from a packed column (**Figure 2A**). They were eluted but with poor peak shapes when only methanol was added (**Figure 2B**) to the CO_2. The addition of a base to the mobile phase dramatically improved the peak shapes of the solutes that included three benzylamines (**Figure 2C**). Ion suppression of the analytes by the basic additive was believed to be the retention mechanism involved in this work. Other workers have reported the application of various organic acids and bases as mobile phase additives in SFC [20,22-32].

Generally, acidic additives are needed to improve the peak shapes of acidic solutes. Basic additives are needed to improve the peak shapes of basic solutes. Berger has speculated, "Additives will provide a key to the separation of more polar solutes by SFC." [1]

The purpose of this research is to extend the application of SFC to ionic, fully ionic, and even to biologically important, amphoteric polypeptides with up to 4500 Da molecular weight by introducing ionizable additives and novel stationary phases. The retention mechanisms of ionic analytes with ionic additives has also been studied. Chapter 2 deals with the elution of sodium *para*-normal dodecylbenzene sulfonate, sodium 4-octylbenzene sulfonate, and sodium *p*-toluene sulfonate from three stationary phases that vary in degrees of deactivation by using various ammonium salts as mobile phase additives. The ammonium salts that were studied include ammonium acetate, tetramethylammonium acetate, tetrabutylammonium acetate, ammonium chloride and lithium acetate. The three stationary phases applied were bare silica, end-capped Cyanopropyl silica phase, and deactivated, polymer-coated Deltabond Cyano silica phase. In Chapter 3, the retention mechanism of these sodium aryl sulfonates with

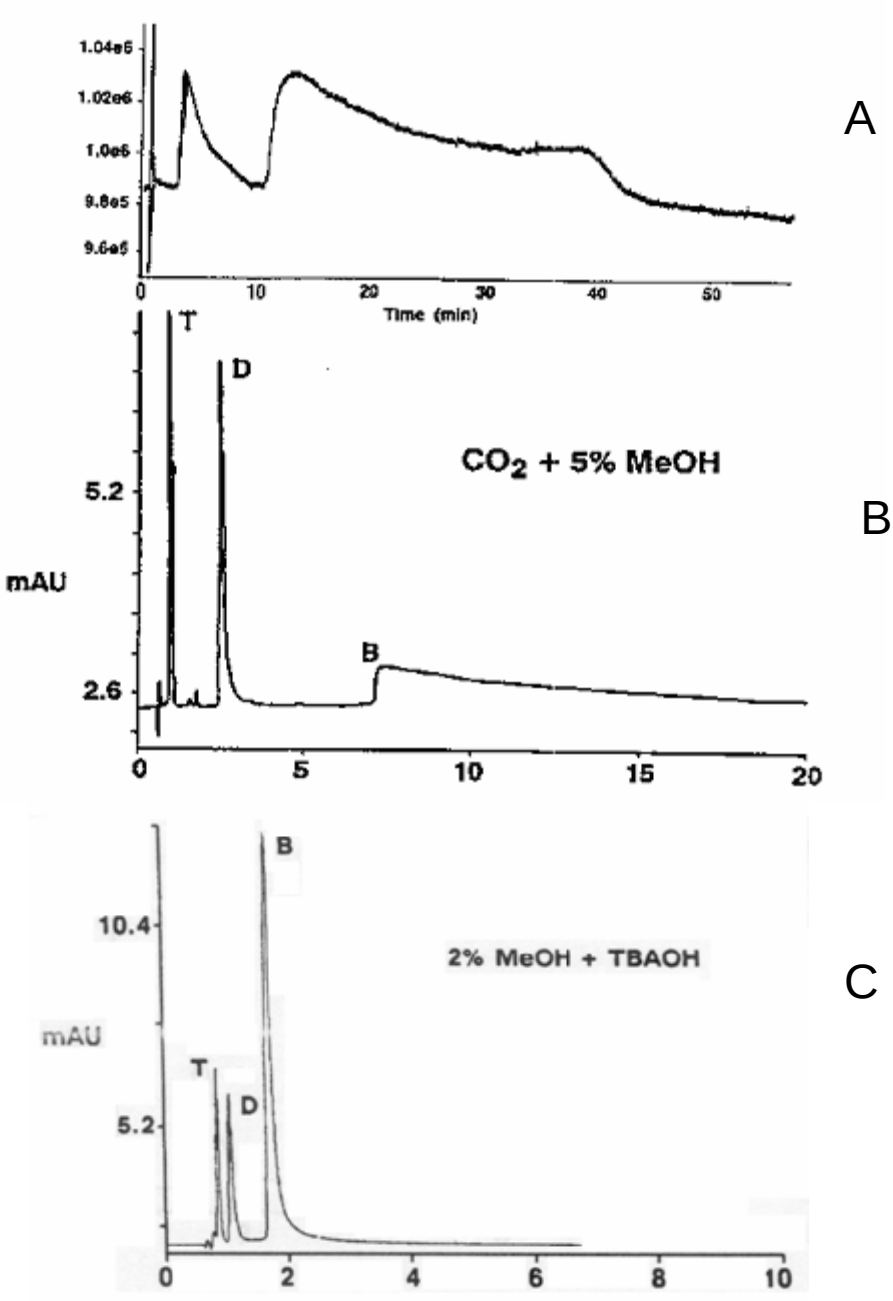


Figure 2. (A) Benzylamines eluted from a Deltabond octyl column using pure carbon dioxide as the mobile phase. 100×2mm, 5μm, 0.5mL/min, 40°C, 182 bar. (B) Separation of three benzylamines on Diol column, using 5% methanol in carbon dioxide; Nucleosil Diol 100×2mm, 7μm, 0.5mL/min, 40 °C, 182 bar. (C) Separation of the same three benzylamines using a ternary mobile phase of 2% methanol (containing 0.1% *t*-butylammonium hydroxide) in carbon dioxide; Deltabond CN column 100×2mm, 5μm, 0.5 mL/min, 40 °C, 182 bar. Solutes in order of retention are: (T) tribenzylamine, (D) dibenzylamine, and (B) benzylamine. [21]

various ammonium additives were investigated using solid state ^{29}Si NMR and computational calculations. We focused on the different behavior of the ammonium additives on the active bare silica column and the deactivated Deltabond Cyano stationary phase. Ammonium acetate, tetramethylammonium acetate, and tetrabutylammonium acetate were evaluated. The success of eluting ionic sulfonate salts by using ammonium salt additives suggested the use of sulfonate salts as mobile phase additives to elute ammonium salt analytes. Chapter 4 demonstrates the successful application of chromatographic reciprocity. Propranolol hydrochloride and two quaternary ammonium salts (cetylpyridinium chloride and benzyltrimethylammonium chloride) were successfully eluted from a Deltabond Cyano column using either sodium ethanesulfonate, sodium 1-heptanesulfonate, or sodium 1-decanesulfonate as additive. The retention mechanisms were also discussed. Beyond the the Deltabond Cyano column, three silica-based stationary phases, an Ethylpyridine column, a strong anion exchange column, and an aminopropyl column, were also evaluated. Finally in Chapter 5, four groups of polypeptides that contain a large variety of amino acid groups, with up to 40 mers and with more than 4500 Dalton, have been investigated using CO_2/methanol based mobile phase. The compatibility of the SFC mobile phase with mass spectrometry was also discussed.

CHAPTER 2

Effect of Ionic Additives on the Elution of Sodium Aryl Sulfonates in Supercritical Fluid Chromatography

2.1 INTRODUCTION

Many papers [13-15] have been published on the effects of methanol as a secondary mobile phase component, especially with respect to retention characteristics, selectivity, and peak shapes of various test solutes [16-19]. Lee et al. [33] for example resolved free bases of rac-propranolol, rac-pindolol, and rac-metoprolol as well as HCl salts of rac-betaxolol and rac-cicloprolol with Chiralcel OD and CH_3OH/CO_2 (20/80, v/v). Direct, preparative, enantioselective chromatography of rac-propranolol hydrochloride was later reported [34] using a Chiralpak AD stationary phase and CH_3OH/CO_2 mobile phase without the use of basic or acidic additives. After the separation, isolated fractions of the hydrochloride salts were positively identified by mass spectrometry. More recently these investigators have demonstrated [35] on-line polarimetric detection with SFC instrumentation for the enantio-separation of the same HCl salts.

While common binary mobile phases significantly improve the elution of polar analytes in SFC, in general, highly polar or ionic compounds are still not eluted because the organic modifiers that are miscible with liquid carbon dioxide are also only moderately polar cosolvents. Berger et al.[20] conducted solvatochromic dye studies and showed that very polar compounds, such as trifluoroacetic acid (TFA), when added to

SFC modifiers, could significantly increase the solvating power of modified mobile phases. Small concentrations (i.e., 10^{-4} M) of such very polar compounds, called additives, improved chromatographic peak shapes and allowed the elution of solutes that were normally very strongly retained. Berger speculated that "additives will provide a key to the separation of more polar solutes by SFC". [20]

Various investigations in this regard have been reported in which weak organic acids and bases have been employed as mobile phase additives. Generally, acidic additives such as trifluoroacetic acid are needed to improve the peak shapes of acidic solutes. Basic additives such as isopropylamine are needed to improve the peak shapes of basic solutes. Berger and Deye [12,21] believed that, in most cases, the mechanism of action involved suppression of analyte ionization by the additive. A less conventional additive, tetramethylammonium hydroxide (TMAOH), was later studied by other workers [22,23] that may suggest a role for the additive other than ion suppression. Specifically, the SFC separation of 24 PTH-amino acids was facilitated with a mobile phase of supercritical CO_2, the additive, and methanol. No modifier was required for the elution of neutral PTH – amino acid derivatives, but the addition of TMAOH and methanol to the mobile phase played a major role in the elution of both acidic and basic PTH amino acids. Peak tailing was minimized and the elution order of several peaks was altered by incorporation of this additive into the mobile phase. The base was thought to interact with, or block active sites on, the stationary phase to significantly improve peak tailing.

The use of ion-pairing principles in SFC has been demonstrated to a limited degree. [24,25] The influence on the selectivity of sodium heptane sulfonate and dimethyloctyl amine (DMOA) with cyano and diol bonded phase columns has been

investigated. The limited solubility of ion-pairing agents in CO_2-modifier mixtures was noted as being a problem in ion-pair SFC. Elution of propranolol with 25 mM sodium heptane sulfonate in CH_3OH/CO_2 was reported; whereas, the analyte failed to elute from the cyano packed column without sodium heptane sulfonate. As a rule of thumb, the paper suggested that the best choice of initial conditions when starting an optimization of the separation of ionizable compounds is to use a diol phase, tributylamine, and acetate ion in methanol as the ion-pairing agent.

Pinkston et al. recently reported the application of mass-spectrometry compatible, volatile ammonium salts as mobile phase additives in SFC. [36] Ammonium acetate, ammonium formate, and ammonium carbonate were used to elute several cationic (quaternary ammonium salts) and anionic (sulfonic acid salts) organic ions under SFC conditions. With the addition of 1.1 mM ammonium acetate in methanol as mobile phase modifier, analytes that were very strongly or irreversibly retained without additive (at the same percentage of methanol) were successfully eluted from a Deltabond Cyano stationary phase. A three-descriptor model was developed in this study where one descriptor, the "relative negative charged surface", explained 61% of the variance in the retention value.

In this work, we have systematically studied the effect of the nature and concentration of mobile phase ionic additives on the elution of sodium 4-dodecylbenzene sulfonate from two cyano bonded silica phases and bare silica itself. Two other sulfonates, sodium 4-octylbenzene sulfonate, and sodium *p*-toluene sulfonate have also been studied in this work.

2.2 EXPERIMENTAL

2.2.1 Chemicals

Methanol was HPLC grade, (EMD, Durham, NC, USA). The carbon dioxide was SFE/SFC grade (Air Products and Chemicals, Inc., Allentown, PA, USA) with no helium head pressure. Lithium acetate (99+%, ACS grade), ammonium acetate (99%, ACS grade), tetramethylammonium acetate (90%, tech. grade), and tetrabutylammonium acetate (97%) were obtained from Sigma Aldrich (Milwaukee,WI, USA). Ammonium chloride (ACS grade) was obtained from J.T.Baker (Phillipsburg, NJ, USA).

Sodium *para*-normal dodecylbenzene sulfonate (tech. grade) (**I**), 4-octylbenzene sulfonic acid sodium salt (97%) (**II**), and sodium *p*-toluene sulfonate (95%) (**III**) were purchased from Sigma Aldrich (Milwaukee,WI), **Figure 3**. A solution of each sample was prepared in methanol at a concentration of approximately 0.5 mg/mL.

2.2.2 SFC/UV Instrumentation

The SFC system was a Berger MiniGram SFC with a Varian 320 Variable Wavelength UV/VIS Detector (Varian,Inc., Walnut Creek, CA), and Berger Instruments SFC ProNTo^{TM} MiniGram software, running on a Dell Dimension 2350 computer. In the middle of the experiment, due to the failure of the detector, we switched to a Berger Instruments Analytical SFC Instrument (Berger Instruments, Newark, DE) with a Hewlett Packard Model 1050 Diode Array Detector (DAD), which employed a 13 μL high pressure flow cell (10 mm path length) and Berger Instruments 3D SFC ChemStation software, version 3.4.

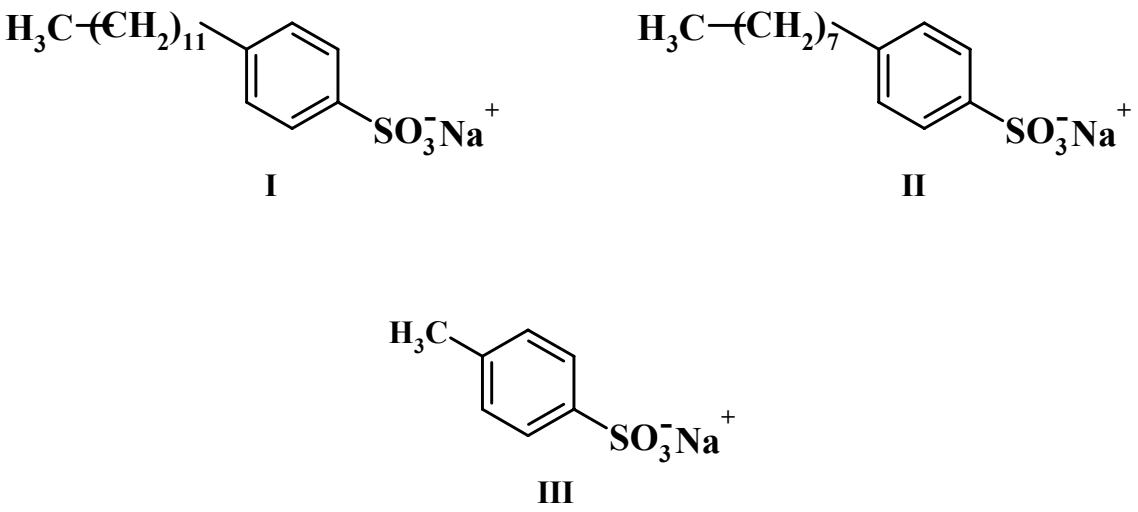


Figure 3. Structures of (I) sodium *para*-normal 4-dodecylbenzene sulfonate; (II) sodium 4-octylbezene sulfonate; (III) sodium *p*-toluene sulfonate

The chromatographic columns were Deltabond Cyano (Thermo Hypersil-Keystone, Bellefonte, PA)*, Supelcosil LC-PCN, and Supelcosil LC-Si (both Supelco; Bellefonte, PA). The column dimensions were 25 cm length and 4.6 mm ID, with a particle size of 5 μm for all three stationary phases. Supelcosil LC-PCN and Supelcosil LC-Si had a pore size of 120 Å, while Deltabond Cyano had a pore size of 200 Å.

Unless otherwise specified, the standard chromatographic conditions were: solution injection volume 10 μL, mobile phase flow rate 2mL/min (measured in the liquid state), column outlet pressure 120 bar, and column oven temperature 40°C. The isocratic mobile phase composition was 15% modifier in CO_2, unless specified. The modifier consisted of either pure methanol, or methanol with 2.5 mM lithium acetate, ammonium acetate, tetramethylammonium acetate, tetrabutylammonium acetate, or ammonium chloride.

Between each switch of the mobile phase additive, the modifier line leading from the modifier bottle to the pump was first purged with the next additive solution. The system was then equilibrated for about 10 minutes by pumping the new mobile phase though the column. Finally, a blank injection of 10 μL of pure methanol through the column was made with the new mobile phase modifier composition, in order to make sure that no analyte was retained on the stationary phase from previous injections.

2.2.3 SFC/MS instrumentation

The SFC-MS system consisted of a Model G1205A (Agilent, Wilmington, DE USA) fluid control module upgraded to a Model FCM-1200, an autosampler, and Version

* The vendor no longer supplies this stationary phase.

3.4 Chemstation SFC control software (all three components from Mettler-Toledo Autochem Berger SFC, Newark, DE USA). A zero-dead-volume chromatographic tee was installed just before the outlet pressure regulator in the fluid control module for the addition of 100 μL/min of 1 mM ammonium acetate in methanol delivered by a Model D Series 260 Isco syringe pump (Isco, Lincoln, NE USA).

From the outlet pressure regulator of the SFC system, 100% of the flow was directed to the TurboIonSpray source of a PE Sciex API-365 Triple-Quadrupole Mass Spectrometer controlled by LC2Tune v1.4 acquisition software (Applied Biosystems, Foster City, CA). The TurboIonSpray source was operated with a Turbo gas flow of 8 L/min at 450 deg C. Nitrogen gas for the nebulizer was set at 60 psi. The electrospray capillary and orifice were held at a potential of -4500 and -50 V respectively; while the multiplier potential was set at 2200 V. MS data were acquired via a Q1 scan from *m/z* 50 to 500 using a 0.1-u step value, a 0.300-ms dwell time, and a 5-ms interscan delay. Product ion scans of *m/z* 325 were obtained at a collision energy of 40 V with nitrogen collision gas. Q3 was scanned from *m/z* 25 to 400 with a 0.1-u step value, a 0.3-ms dwell time, and a 5-ms interscan delay for the product ion scans.

2.3 RESULTS AND DISCUSSION

The initial goal of this work was to study the effect of various mobile phase salt additives on packed column supercritical fluid chromatographic elution of sodium 4-dodecylbenzene sulfonate from three different stationary phases: Deltabond Cyano, conventional cyano, and bare silica. With 100% CO_2 or even 15% (v/v) pure methanol as CO_2 modifier, technical grade sodium 4-dodecylbenzene sulfonate did not elute from any

of the three stationary phases. Introduction of 2.5 mM ammonium acetate into the 15% methanol mobile phase modifier with each of the three stationary phases, however, had a dramatic effect on the chromatography. **Figure 4** shows SFC/UV chromatograms of sodium 4-dodecylbenzene sulfonate elution with 15 % 2.5 mM NH_4OAc in methanol as the CO_2 modifier on Deltabond Cyano, conventional cyanopropyl, and bare silica stationary phases. When ammonium acetate was used as additive, the analyte eluted within 6 minutes with reasonable peak shape under isocratic conditions from each properly conditioned stationary phase. With just methanol modifier, no analyte peaks were detected; thus only a noisy baseline was observed.

A shoulder or split peak is apparent with all three stationary phases. This is likely due to the presence of alkyl chains that are shorter/longer than twelve carbons in the technical grade sodium 4-dodecylbenzene sulfonate [37]. To gain a better perspective on the nature of the split peak, mass spectrometric detection was incorporated into the SFC experiment with a new bare silica stationary phase, **Figure 5**. The UV trace with the new column gave a broader peak with a longer retention time than previously observed, **Figure 5A**. A second new bare silica column was then tested to confirm the earlier results. The initial injection indeed yielded a broad peak with retention time in excess of 10 minutes. Subsequent injections gave a much narrower peak with retention time less than 10 minutes that was clearly split. The MS contour plot **(Figure 5B)** shows clear evidence that the technical grade sulfonate we had been using was in fact a distribution of alkyl groups on the aryl portion ranging from C7 to C15. Thus, this homologous series of sulfonates was only partially separated on the silica phase**.** Next, an analogous experiment was conducted with the Deltabond Cyano phase. **Figure 6** shows the UV

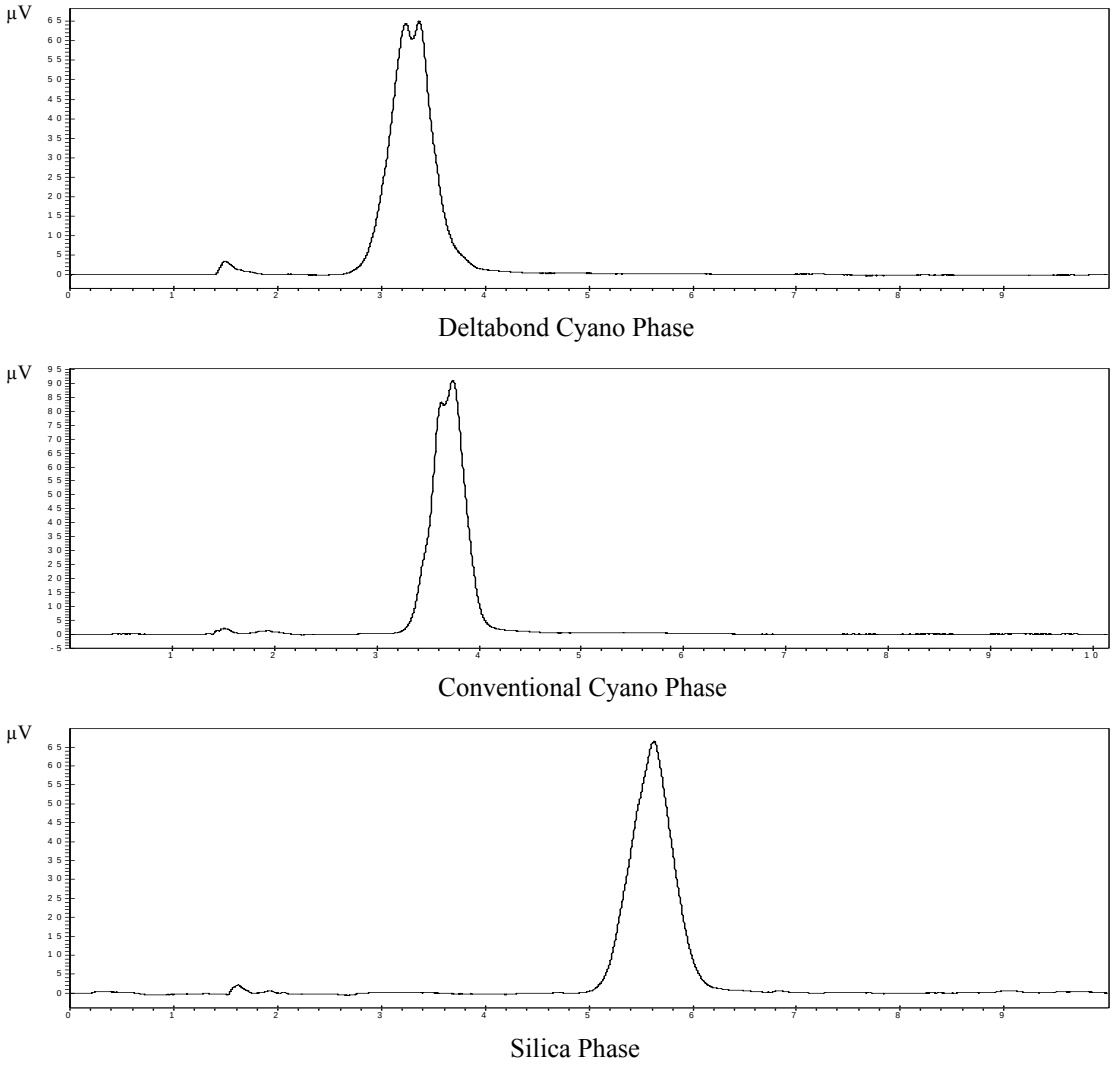


Figure 4. SFC/UV chromatograms (λ = 230 nm) of sodium 4-dodecylbenzene sulfonate with 15% 2.5 mM NH_4OAc in methanol as mobile phase modifier modified CO_2 employing various stationary phases

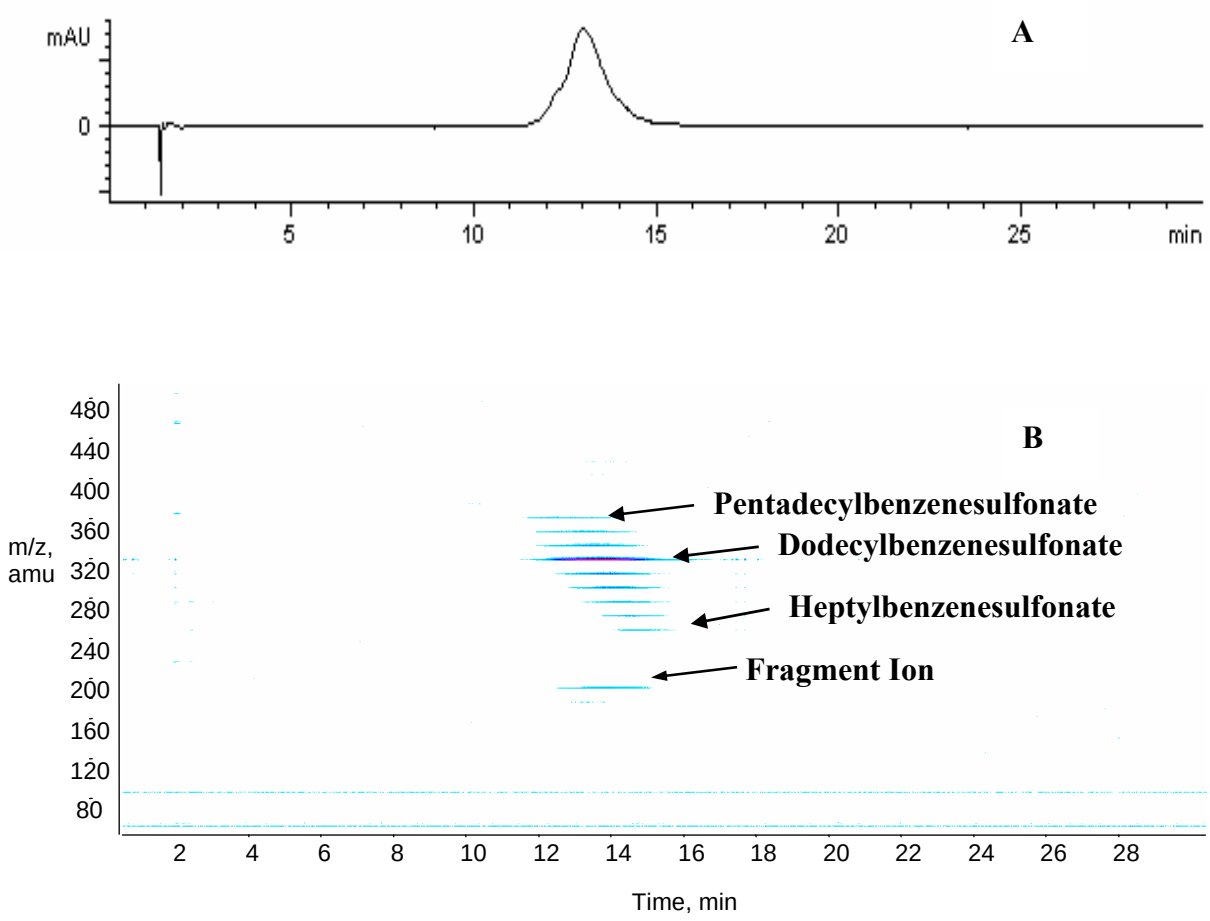


Figure 5. SFC/UV (230nm) trace (A) and SFC/MS contour plot (B) for the elution of sodium 4-dodecylbenzene sulfonate on a silica column. Additive concentration in methanol was 2.5 mM. See Experimental Section for other chromatographic conditions

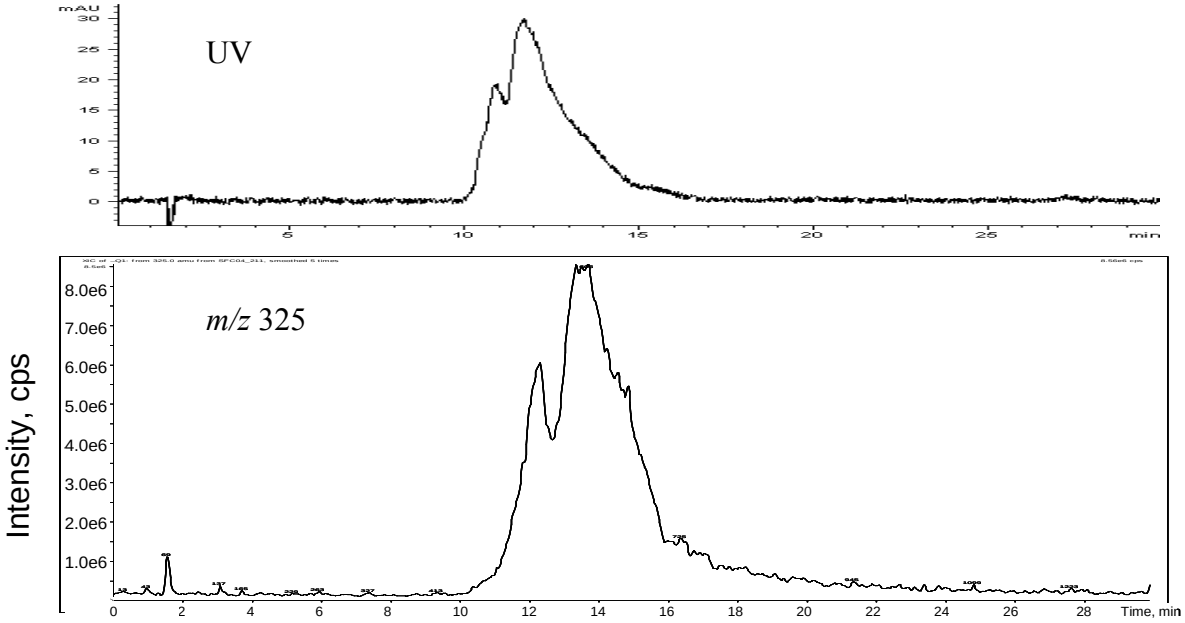


Figure 6. SFC/UV trace (230 nm) and mass chromatogram of *m/z* 325 $[M-H]^-$ ion for the elution of sodium 4-dodecylbenzene sulfonate on Deltabond Cyano column. Additive concentration in methanol was 2.5 mM. See Experimental Section for other chromatographic conditions

absorbance chromatogram at 230 nm and the mass chromatogram for *m/z* 325, which corresponds to the $[M-H]^-$ ion for the major component of the mixture, 4-dodecylbenzenesulfonic acid. The UV trace again clearly indicates a mixture of components. The MS data, however, expanded the suspected multiplicity of components because two peaks with the same mass were observed which suggested the presence of isomeric components. Slightly different product ion spectra for different isomers of sodium 4-dodecylbenzenesulfonate might be expected. **Figure 7** shows that, in this case, the differences are apparent but subtle. Specifically, only a small change in the relative abundance of one of the products of *m/z* 325 was evident. The data discussed above help confirm that the chromatographic peak characteristics we have observed are originating from various components in the sample rather than an undesirable chromatographic event (or mechanism).

Good reproducibility for three injections onto each properly conditioned stationary phase was observed. The highest relative standard deviation (n=3) for retention time was less than 1%. The analyte eluted fastest from the most deactivated stationary phase, Deltabond Cyano, and was retained the longest on the most active stationary phase, silica.

Tetramethylammonium acetate (TMAA), tetrabutylammonium acetate (TBAA), ammonium chloride, and lithium acetate were chosen to augment and evaluate the effect of different salts as mobile phase additives. The same isobaric and isocratic conditions were applied as in the ammonium acetate case to provide clearer information concerning the effect of various additives and stationary phases. **Figure 8** shows the effect of different additives on the elution from the Deltabond Cyano column. While the positive

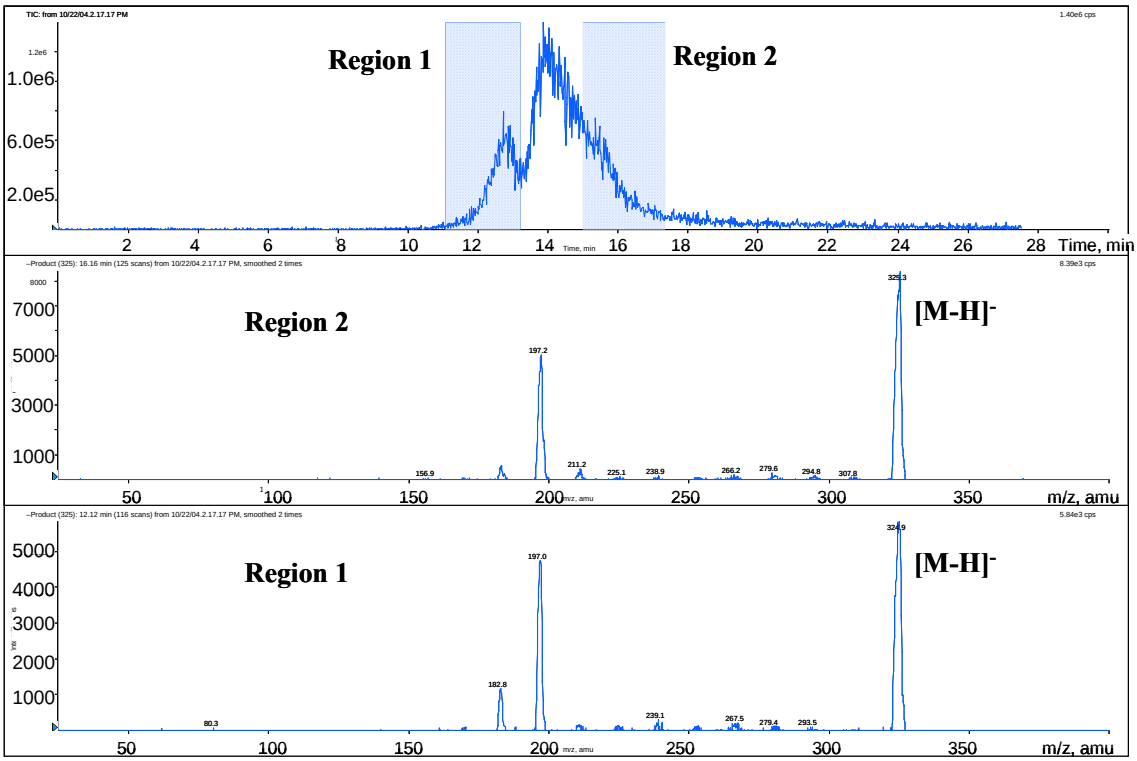


Figure 7. MS/MS reconstructed-total-ion-current chromatogram and product ion spectra of different peak components. Analyte is sodium 4-dodecylbenzene sulfonate. Additive concentration in methanol was 2.5 mM. See Experimental Section for other chromatographic conditions

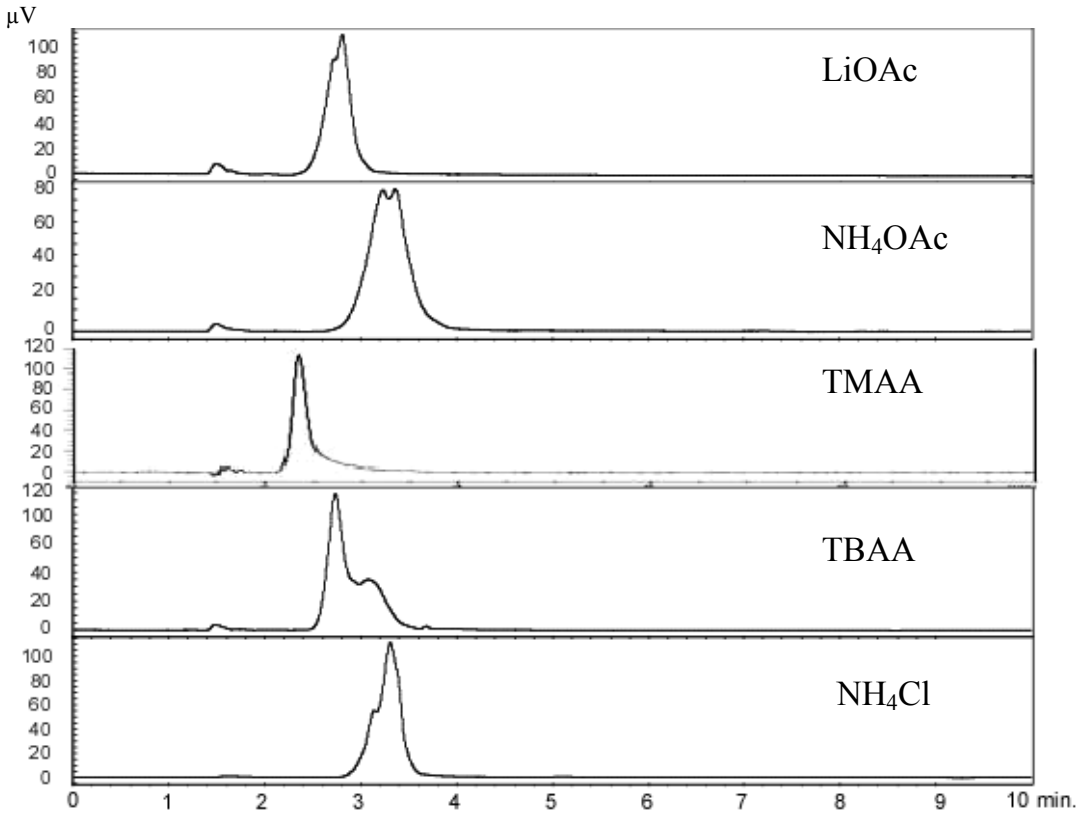


Figure 8. Effect of different mobile phase additives for the elution of sodium 4-dodecylbenzene sulfonate with Deltabond Cyano column. Additive concentration in methanol: 2.5 mM. See Experimental Section for other chromatographic conditions.

effect of ammonium acetate was expected from previous reports, results for the other additive salts were not expected and suggested a general phenomenon that seems to be neither cation (e.g. ammonium, tetraalkylammonium, and lithium) nor anion (e.g. acetate and chloride) specific. A similar effect on elution of the sulfonate from the conventional cyanopropyl and silica columns with each additive was observed, **Figures 9 and 10**. With the exception of TMAA on the silica column, the analyte was successfully and isocratically eluted within 10 minutes in all situations. Interestingly, TMAA provided the fastest elution on the Deltabond stationary phase among the five additives, but the longest retention on the silica phase. Evidence for split peaks was observed in some instances as was the case with ammonium acetate. The Deltabond Cyano phase and TBAA showed the best resolution of the target analyte and its congeners under isocratic conditions.

The fact that bare silica yielded analogous results to the two bonded phases suggests some alteration of the stationary phase by the additive is strategically involved. **Table 1** shows the average retention time and peak area of each analyte for each additive on the three stationary phases with percent relative standard deviation (RSD) in parenthesis. Good reproducibility was achieved since the highest RSD for retention time was 2.35%. Although not shown, reproducibility with tetramethylammonium acetate was equally good. From the highly deactivated Deltabond Cyano phase to the highly active silica phase, the sulfonate was retained longer on the more active phase with the same additive present. This is probably due to interaction between the negatively charged sulfonate ion and the partial positive proton charge of residual, active silanol sites on the solid support of the stationary phase.

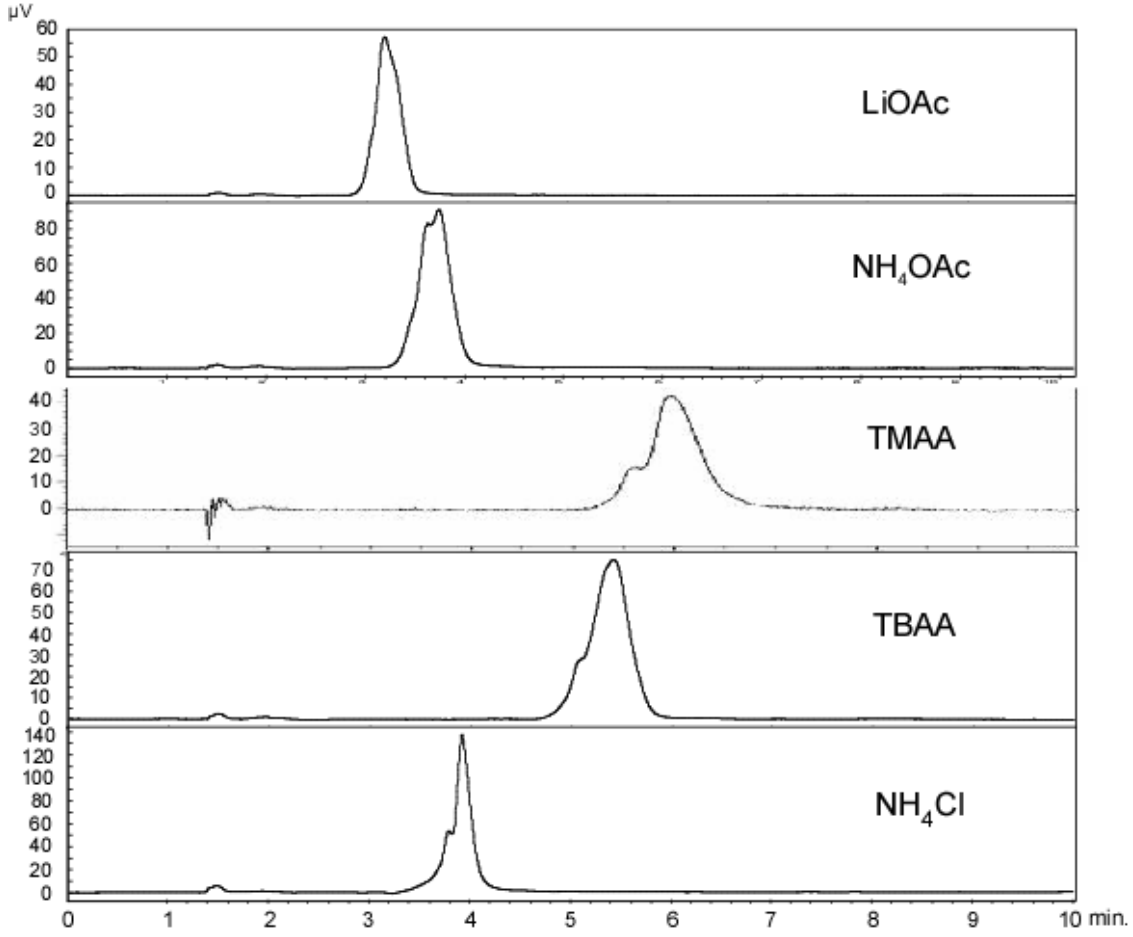


Figure 9. Effect of different mobile phase additives for the elution of sodium 4-dodecylbenzene sulfonate with conventional cyano column. Additive concentration in methanol: 2.5 mM. See Experimental Section for other chromatographic conditions.

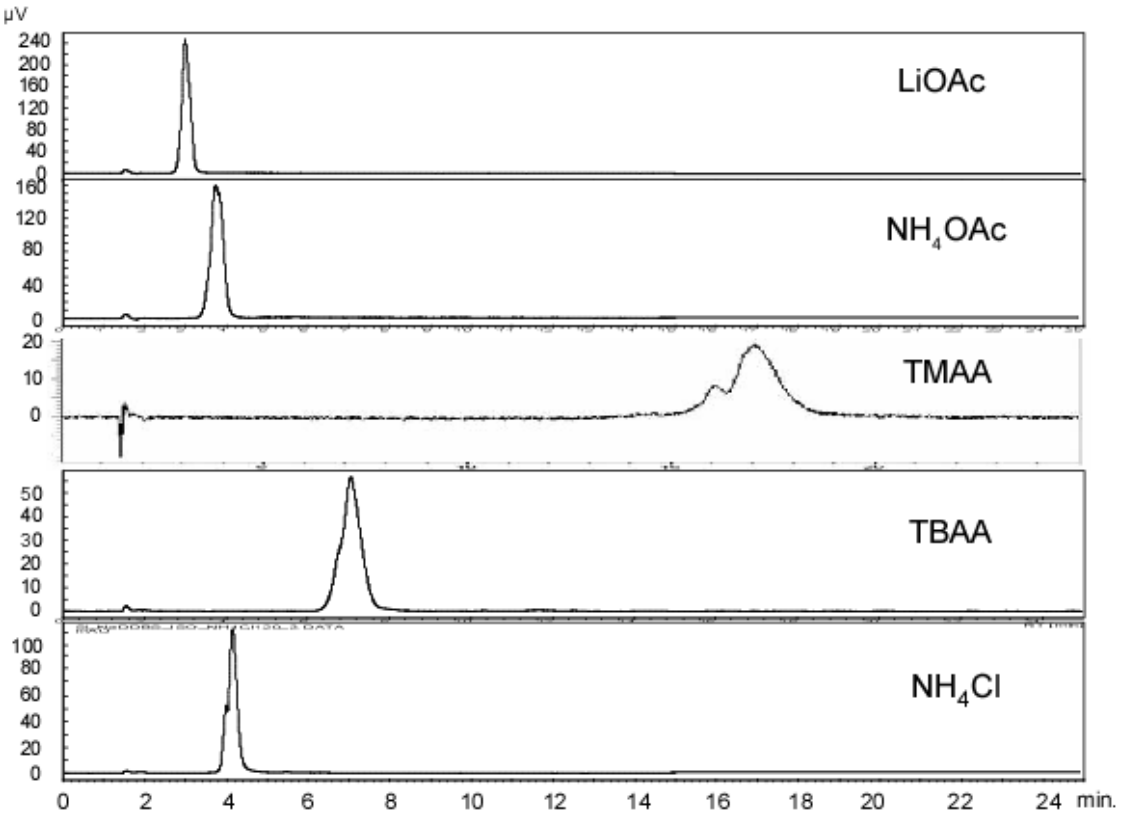


Figure 10. Effect of different mobile phase additives for the elution of sodium 4-dodecylbenzene sulfonate with silica column. Additive concentration in methanol: 2.5 mM. See Experimental Section for other chromatographic conditions.

Table 1. Retention Time/Peak Area Reproducibility Data Versus Stationary Phase and Additive (2.5 mM in 15% methanol modified CO_2).

Stationary Phase	LiOAc		NH_4OAc		TBAA		NH_4Cl	
	RT[a]	PA[b]	RT	PA	RT	PA	RT	PA
Deltabond Cyano	2.81 (0.00)[c]	32.0 (1.11)	3.36 (0.21)	31.4 (1.80)	2.74 (0.26)	32.9 (0.64)	3.32 (0.21)	33.5 (0.84)
Conventional Cyano	3.16 (0.22)	32.8 (1.08)	3.73 (0.57)	34.3 (0.62)	5.41 (0.13)	34.9 (0.81)	3.93 (0.18)	31.5 (1.12)
Bare Silica	5.09 (1.12)	31.5 (1.12)	5.60 (0.88)	32.1 (0.66)	7.01 (0.91)	32.2 (3.07)	4.21 (2.35)	31.2 (1.14)

[a]RT = Retention Time (min.)
PA = Peak Area (μV·min)
[c]Percent Relative Standard Deviation (n=3)

We also studied the effect of column outlet pressure on the elution of sodium 4-dodecylbenzene sulfonate with the three stationary phases and five additives. In each case, the analyte eluted slightly earlier at higher pressure than it did at lower pressure. We believe this may be due to the greater solvating power of the mobile phase at higher pressure.

In Pinkston's previous work [36], 1.1 mM ammonium acetate was dissolved in the modifier in order to elute highly polar compounds. We therefore decided to use ammonium acetate to investigate any additive concentration effect with the Deltabond column. Sodium 4-dodecylbenzene sulfonate did not elute with 0.01 mM or with 0.1 mM NH_4OAc in 25 minutes. The analyte, however, started to elute with methanol modifier containing 0.25 mM NH_4OAc as a very broad peak at about 11 minutes. With increased concentration of additive in methanol, the analyte eluted faster and with a sharper peak shape. At 2.5 mM, the analyte eluted at about 3 minutes compared to 11 minutes with 0.25 mM. We thought that it would be interesting to do the same concentration study with bare silica. The analyte again did not elute with mobile phase modifier containing 0.1 mM NH_4OAc, but started to elute with 0.25 mM NH_4OAc. Interestingly, we found that the sulfonate was retained more strongly on the silica column when the concentration of NH_4OAc in methanol was increased, which was the inverse of the trend we observed with the Deltabond Cyano phase. These results suggested that different elution mechanisms might dominate with the Deltabond Cyano and the silica phases.

When ionic salts are introduced into the primary modifier which is then added to the nonpolar CO_2, it is very important that the salts remain dissolved in the resulting ternary mobile phase. Among the five additives we studied, ammonium chloride had the

worst solubility in methanol. When we tried, for example, to introduce methanol containing 10.0 mM NH_4Cl into the CO_2 mobile phase, a significant increase in inlet pressure was observed, which suggested the precipitation of the salt in column.

The effect of salt additives on the elution of two much purer congeners of sodium 4-dodecylbenzene sulfonate (e.g. sodium 4-octylbenzene sulfonate (OSNa) and sodium *p*-toluene sulfonate (TSNa) was studied with the silica phase. The less complex sulfonates either did not elute (OSNa) or eluted with poor peak shape (TSNa) when pure methanol (15%) was used as the CO_2 modifier, but both compounds eluted with good peak shape when ammonium salts (2.5 mM) were added to the methanol. Each sulfonate sodium salt eluted readily from the silica phase with either ammonium acetate, TMAA, or TBAA as the mobile phase additive. The trend was very similar to the findings obtained with sodium 4-dodecylbenzene sulfonate. TMAA provided the longest retention time among the three ammonium salts; whereas both sulfonates eluted fastest with ammonium acetate. Since these two sodium sulfonates were more pure than sodium 4-dodecylsulfonate, as might be expected, the former two components yielded more narrow chromatographic peaks. The results for TSNa are illustrated in **Figure 11**, for example.

The elution mechanisms envisioned in this study involve (1) modification of the stationary phase by the ionic additive and (2) ion-pair formation between additive and analyte. On the silica surface modification of the stationary phase may actually convert the silica to an ion-exchange phase. This observation is prompted by the fact that when higher concentrations of ammonium acetate were introduced (e.g. more sites were modified by ammonium cations), the sulfonate was retained longer. On the other hand, ion-pairing formation may be the dominating elution mechanism on the Deltabond Cyano

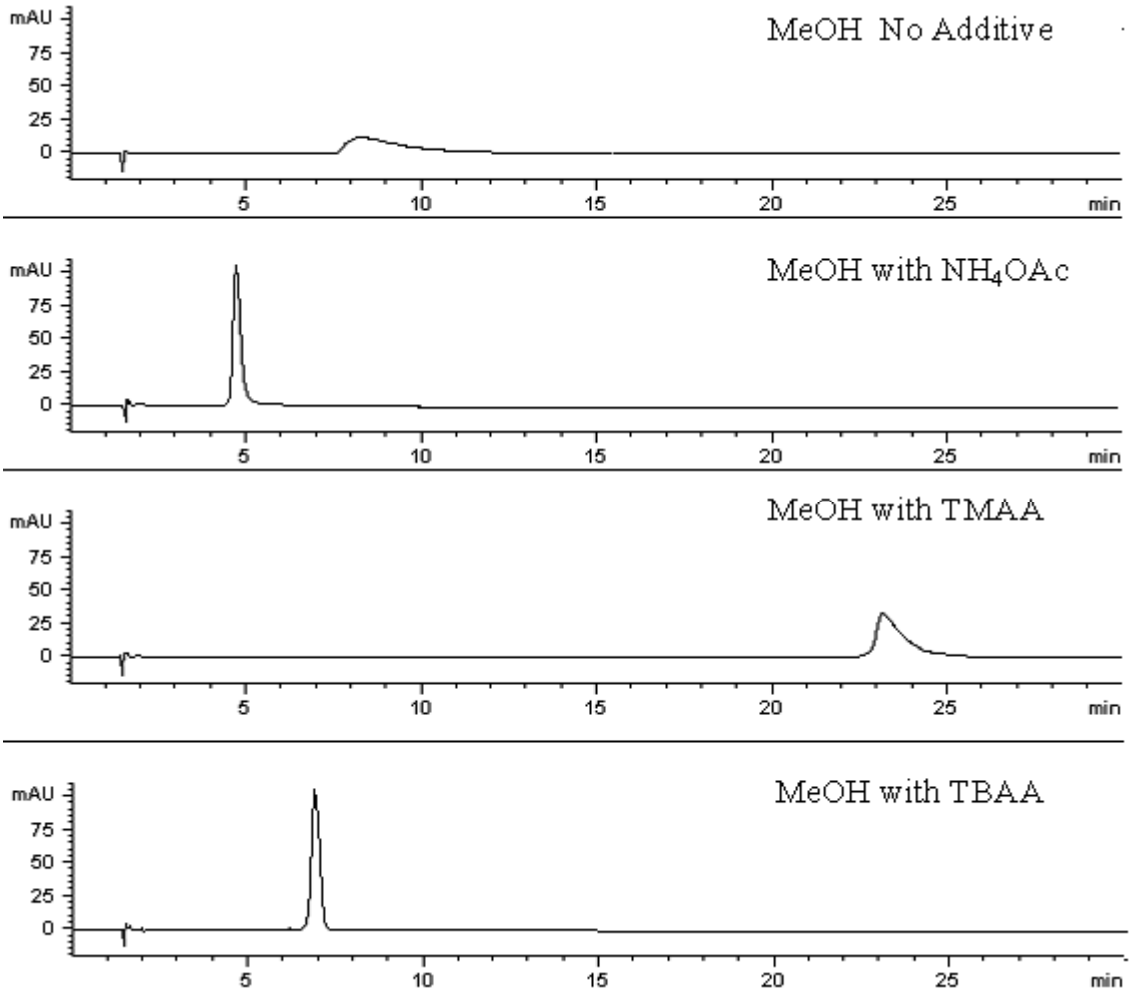


Figure 11. Effect of ammonium salts as mobile phase additives on the elution of sodium *p*-toluene sulfonate on silica column. Additive concentration in methanol: 2.5 mM. See Experimental Section for other chromatographic conditions.

phase since there should be fewer accessible silanol sites on the particle surface. In this case, higher concentrations of ammonium acetate should result in more ion-pairing between ammonium cation and sulfonate anions. Our results with the bonded phase indicated that the analyte eluted faster at a higher concentration of additive. This bimodal behaviour is most dramatically seen with the results afforded by tetramethylammonium acetate.

To gain a more thorough understanding of the general effect of salt additives in the mobile phase, the elution of a secondary ammonium hydrochloride salt, propranolol hydrochloride, on a silica phase was also studied. The cationic amine did not elute with pure methanol as modifier even at concentrations as high as 40% methanol. Elution, albeit with peak tailing, was achieved with 30% methanol containing 2.5 mM ammonium acetate in CO_2. The effect of various salts (2.5 mM) on the elution of the amine hydrochloride on the silica phase is shown in **Figure 12**. In this case, TMAA provided the fastest elution, and lithium acetate provided the longest retention of the analyte. Ammonium acetate provided the strongest retention among the three quaternary ammonium acetate salts. Importantly, the retention trend was opposite to that observed for negatively charged sulfonate analytes in that TMAA gave the shortest retention time for the amine hydrochloride. Unfortunately, this analyte, unlike the sulfonate salts, did not yield sharp peaks on silica with any of the additives under isocratic conditions which suggests that a different type of ionic additive may be required for elution of cationic species.

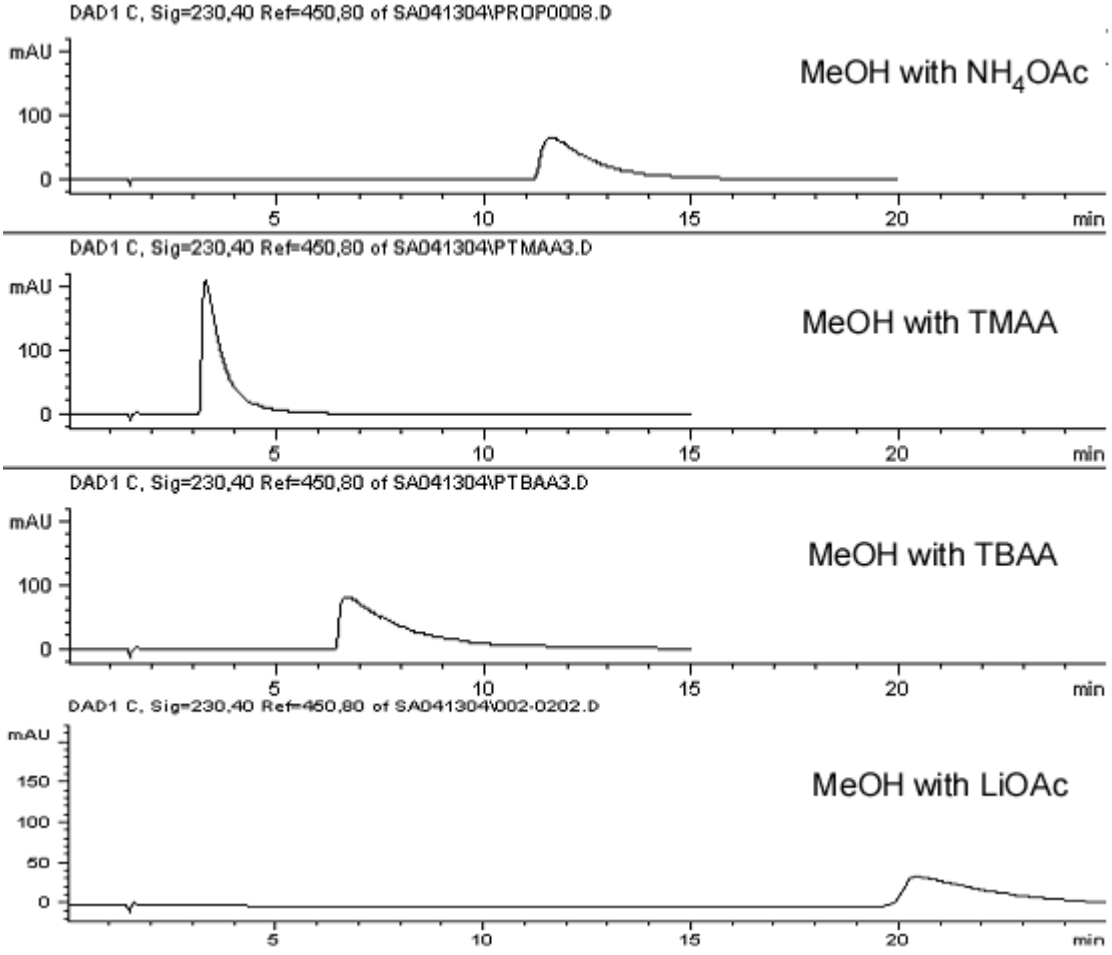


Figure 12. Effect of different salt mobile phase additives on the elution of propranolol hydrochloride from silica column. Mobile phase composition: 30% methanol containing 2.5 mM additive in CO_2. See Experimental Section for other chromatographic conditions.

2.4 CONCLUSIONS

In future work, we plan to use NMR and other spectroscopic methods to study the interaction between additives and the stationary phases. We will also compare the various salt additives using computational modelling methods. These methods, in combination with the data presented here, may help shed more light on the retention and elution mechanisms which dominate the CO_2/modifier/salt additive/silica stationary phase system.

The elution mechanisms envisioned in this study involve modification of the stationary phase by the ionic additive and/or ion-pair formation between additive and analyte. The exact elution mechanism for this study remains uncertain at this time. Nevertheless, the data reported in this work are noteworthy: the ionic analytes were eluted with a CO_2-based mobile phase from a highly active packed column stationary phase. Up to now, this achievement under these conditions would have been thought to be highly unlikely. The exact role of the additive appears critical since each of the ionic analytes was irreversibly retained on each of the same stationary phases with only methanol as the modifier.

CHAPTER 3

Study of the Elution Mechanism of Sodium Aryl Sulfonates on Bare Silica and a Cyano Bonded Phase with Methanol-Modified Carbon Dioxide Containing an Ionic Additive

3.1 INTRODUCTION

Traditionally, the application of packed column supercritical fluid chromatography (SFC) was thought to be limited to non-polar and moderately polar analytes because of (a) the weak solvating power of the mobile phase, which typically was supercritical carbon dioxide, and (b) the strong irreversible interaction between analytes and residual active silanol groups associated with silica-based bonded stationary phases.

The active silanol sites on silica-based stationary phases can irreversibly adsorb highly polar solutes, especially when the mobile phase does not have sufficient solvating power. End-capping with trimethylchlorosilane has been used to reduce the number of residual silanol sites. Due primarily to steric hindrance and a highly porous solid support, all silanol sites on the silica surface cannot be removed by this treatment. Polymer-coated, bonded stationary phases have been shown to provide a higher degree of deactivation. Berger et al.[12] and Ashraf-Khorassani et al.[11], for example, reported that a polymer-coated cyanopropyl (e.g. Deltabond) phase significantly improved chromatographic peak shapes of eluted phenols, anilines, and benzylamines compared to

a non-deactivated cyanopropyl phase. This was thought to be due to the presence of fewer accessible active silanol sites on the polymer-coated phase.

An alternative strategy in this chromatographic area has been to increase the solvating power of supercritical CO_2 by the addition of small amounts of polar organic mobile phase modifiers, such as methanol, 1-propanol, and acetonitrile [16-19,38]. While these binary mobile phases significantly improved the elution of polar analytes in SFC, they are still not sufficiently solvating to elute highly polar or ionic compounds due to the fact that the organic solvents that are miscible with carbon dioxide are not sufficiently polar.

The addition of a small amount of a very polar component to the mobile phase modifier has been shown to further enhance chromatographic performance. More specifically, solvatochromic studies showed that, for example, a low concentration of trifluoroacetic acid (TFA) dramatically improved the solvating power of methanol-modified CO_2 [20]. Such very polar, low molecular weight compounds, called additives, improved chromatographic peak shape and allowed the elution of solutes that would otherwise be very strongly retained by silica-based packed column stationary phases. Both low molecular weight organic acids and bases have been utilized as mobile phase additives in SFC to facilitate elution of moderately acidic and basic analytes [20-23,27-31]. In addition to enhancing the solvating strength of the mobile phase, suppression of analyte ionization by the polar additive was believed to be a competing mechanism of action [12,21].

In addition to (1) enhancing the solvating power of the mobile phase and (2) deactivating the stationary phase, ion-pairing of the ionic analyte with the additive in the

supercritical fluid mobile phase has been successfully demonstrated to a limited degree [24,25]. Successful elution of propranolol hydrochloride from a cyano packed column with 25 mM sodium heptane sulfonate (HSNa) in methanol-modified CO_2 has been reported. Without HSNa in the mobile phase, the analyte failed to elute. Solubility of the ion-pairing reagent (i.e. additive) in the mobile phase was suggested to be a critical feature in the success of the ion-pair SFC elution mechanism.

In another study, ammonium acetate, ammonium formate, and ammonium carbonate were each recently reported to be effective SFC mobile phase additives for the successful elution of several organic ions from packed columns, which up to that point were either very strongly or irreversibly retained on the stationary phase [36]. The authors suggested two possible roles for the ammonium salts: (1) charge neutralization via ion-pairing formation between the solute and additive and (2) charge introduction to the stationary phase by exchange of silanol hydrogen for ammonium ion followed by anion exchange of the analyte.

In our previous research [39], we successfully eluted three sodium aryl sulfonates with various ammonium salts added to the primary modifier at a concentration of 2.5 mM from Deltabond cyanopropyl, conventional cyanopropyl, and bare silica phases. In some cases, we found that lithium acetate as an additive was even effective in promoting elution. We discovered that different stationary phases yielded different analyte retention properties when the same mobile phase additive was used. Moreover, with the same analyte and stationary phase, various ammonium salts gave rise to different analyte retention times. This study addresses our desire to understand more thoroughly the

retention and elution mechanisms of ionic sulfonates on both a deactivated cyano phase and a bare silica phase with ionic mobile phase additives.

Solid state nuclear magnetic resonance (NMR) spectroscopy, with the help of cross polarization and magic angle spinning (CP-MAS), has been widely used to provide valuable structural information concerning the surface of bare and bonded silica stationary phases [40-44]. Three types of silicon atoms can be identified on the bare silica surface by solid state ^{29}Si NMR (i.e., silicon bonded to two, one, or no hydroxyls). Thus, we decided to use this technique to investigate the interaction between ionic mobile phase additives and the silica stationary phase.

In this work, we have studied the elution mechanism of sodium sulfonates on both Deltabond cyanopropyl and bare silica phases with three ammonium salts as the mobile phase additive. Solid state ^{29}Si NMR spectroscopy provided some insight concerning the interaction of the mobile phase additive with the silica-based stationary phase. Computational calculations concerning the charge distribution on various ammonium salts were also performed in an effort to explain the elution behavior of ionic sulfonates observed with the various additives. To support our hypothesis of the retention mechanism, the acidity of CO_2 and methanol mixture solvent was also discussed.

3.2 EXPERIMENTAL

3.2.1 Chemicals

Methanol was HPLC grade, (EMD, Durham, NC USA). The carbon dioxide was SFE/SFC grade (Air Products and Chemicals, Inc., Allentown, PA USA) with no helium

head pressure. Ammonium acetate (AA) (99%, ACS grade), tetramethylammonium acetate (TMAA) (90%, tech. grade), and tetrabutylammonium acetate (TBAA) (97%) were obtained from Sigma Aldrich (Milwaukee,WI USA). Ammonium chloride was purchase from J.T. Baker Inc. (Phillipsburg, NJ USA). Silica packing material with a particle size of 5 μm was donated by Supelco (Bellefonte, PA USA). Universal indicator solution with pH range 4-10 (It consists with four indicators: phenolphthalein disodium salt, bromothymol blue sodium salt, methyl red sodium salt, and thymol blue sodium salt.) and bromocresol green solution (0.04%, aqueous) with pH range of 4.0-5.4 were obtained from Fisher Scientific (Fair Lawn, NJ USA). Methyl red sodium salt solution (0.1%, aqueous) with pH range of 3.1-4.4 was obtained from LabChem, Inc. (Pittsburgh, PA USA).

Sodium *para*-normal dodecylbenzene sulfonate (tech. grade), sodium 4-octylbenzene sulfonate (97%), and sodium para-toluene sulfonate were purchased from Sigma Aldrich. A solution of each sample was prepared in methanol at a concentration of approximately 0.5 mg/mL.

3.2.2 SFC/UV Instrumentation

The SFC system was a Berger Instruments Analytical SFC Instrument (Newark, DE USA) with a Hewlett Packard (Little Creek, DE USA) Model 1050 Diode Array Detector, which employed a 13-μL high pressure flow cell (10-mm path length) and Berger Instruments 3D SFC ChemStation software, version 3.4.

The chromatographic columns were Deltabond Cyano (Thermo Hypersil-Keystone, Bellefonte, PA USA), and Supelcosil LC-Si. The column dimensions were 25

cm in length and 4.6 mm ID, with a particle size of 5 μm for each stationary phase. Supelcosil LC-Si had a pore size of 120 Å, while Deltabond Cyano had a pore size of 200 Å.

Unless otherwise specified, chromatographic conditions were: injection volume 10 μL, mobile phase flow rate 2 mL/min (measured in the liquid state), column outlet pressure 120 bar, and column oven temperature 40°C. The isocratic mobile phase composition was 15% modifier in CO_2, unless specified. The modifier consisted of either pure methanol or methanol with 2.5 mM ammonium salt.

Between each change of mobile phase additive, the stationary phase was regenerated with 90% pure methanol for about fifteen minutes (about nine column volumes) in order to purge previous additive solution from the system, or to remove analyte that had interacted with the stationary phase. After this time period, the next additive solution was introduced to the SFC system, and the column was equilibrated again for 30 minutes prior to injection.

3.2.3 Solid State CP-MAS NMR Spectroscopy

^{29}Si CP-MAS spectroscopy was performed on a Bruker MSL300 operating at 59.601 MHz. Magic Angle Spinning parameters were 45° pulse with proton decoupling and a recycle delay of 15 s. For CP-MAS, a recycle delay of 5 s and a contact time of 4 ms were used.

The weights of samples placed in the rotor were as follows: 143.1 mg for untreated silica gel; 169.3 mg of silica gel treated with methanol/NH_4OAc; 205.8 mg of

silica gel treated with methanol/TMAA, and 227.5 mg of silica gel treated with methanol/TBAA. All NMR spectra employed a fixed number of scans (27 000).

The supercritical fluid treated samples were prepared by packing a 2.5 mL extraction vessel about 40 % full with silica packing material. The rest of the space was filled with a glass rod. The vessel was flushed for one hour with supercritical CO_2 containing 15% or 20% modifier (e.g. pure methanol or methanol containing 2.5 mM of additive) with an outlet pressure of 120 bar and an oven temperature of 40 ºC.

3.2.4 Computational Chemistry

The structures of three ammonium additives, AA, TMAA, and TBAA, were sketched into the CAChe Worksystem Pro Version 6.1 (Fujitsu CAChe Group, Beaverton, OR USA) on a Dell Dimension 2350 computer running Microsoft Windows 2000 operating system. The optimized geometry of the molecules was calculated based on the postulate that the molecules take the form with the lowest energy. The optimized geometry of individual atom was generated by three different methods: AM1 geometry; AM1 geometry including solvent (water) effect; and B88-LYP DFT geometry. The first two methods are semi-empirical methods and the third one is a DGauss calculation that provides an approximate solution to the Schrödinger equation using only mathematical approximation [45].

3.2.5 Study of the Acidity of CO_2/methanol Mixed Solvent

A stable flow of CO_2 gas at atmospheric pressure was provided by the waste outlet of the Berger SFC system. Before this source of CO_2 was used, the SFC system

was flushed with CO_2/methanol (v/v 10/90) at 40 ºC and 60 bar for about 15 minutes to remove any possible residue of additives or analytes from the SFC system, then pure CO_2 was pumped through the system at 2 mL/min (measured as a liquid at the pump) for sufficient time to remove the methanol.

Three pH indicators were used: a "universal indicator", methyl red, and bromocresol green. They were in aqueous solution, and we knew that CO_2 would react with the water from the indicator solution to give a color shift to the acidic range. After dropping about 0.5 mL of each indicator into three flasks, water was removed by mild heating on a hot plate and a stream of N_2 gas. One hundred milliliters of methanol was then added to two flasks containing the dried pH indicator. One hundred milliliters of water was placed in the third flask with the indicator solution. One of the methanol solutions served as a blank to compare to the methanol mixed with CO_2 and water mixed with CO_2.

Gaseous CO_2 was then bubbled into one flask containing the methanolic solution and the flask containing the aqueous solution. Sufficient time was allowed for the color to stabilize. Once the color stabilized, we assumed that the solvents were saturated with CO_2.

To give a better estimation of the pH value of CO_2/methanol, an ammonium chloride aqueous solution was made with universal indicator to match the color of CO_2-saturated methanol. A Corning pH meter 320 (Corning, New York USA) was used to measure the pH value of the NH_4Cl aqueous solution.

3.3 RESULTS AND DISCUSSION

3.3.1 Additive Concentration Study

Due to the fact that ionic compounds have been shown not to elute in SFC even with methanol-modified CO_2 as the mobile phase [36], we wanted to determine the lowest concentration of ionic additive whereby the ionic sulfonates would elute. We chose sodium dodecylbenzene sulfonate as the probe analyte and ammonium acetate as the probe additive.

A systematic study concerning the elution of the probe analyte with various concentrations of ammonium acetate in methanol was performed on both Deltabond Cyano and bare silica columns. Sodium dodecylbenzene sulfonate did not elute in 25 minutes from either stationary phase with 15% modifier containing either 0.01 mM or 0.1 mM NH_4OAc. The analyte, however, eluted isocratically from the Deltabond Cyano column with 15% methanol modifier containing 0.25 mM NH_4OAc as a very broad peak at about 11 minutes, **Figure 13**. With increased concentration of additive in 15% methanol, the analyte eluted even earlier from the bonded phase column and with a sharper peak shape. For example, at 2.5 mM additive the analyte eluted in about 3 minutes compared to 11 minutes with 0.25 mM additive. Interestingly, we found that the sulfonate analyte eluted much earlier with 0.25 mM additive on the silica column than on the bonded-phase column. In contrast on silica, retention actually increased to a point and levelled off when the concentration of NH_4OAc in methanol was increased. For example at 15% methanol containing 0.25 mM additive, elution from the bare silica column was slightly less than four minutes. While at 15% methanol containing 2.5 mM additive, elution of the analyte increased to approximately six minutes.

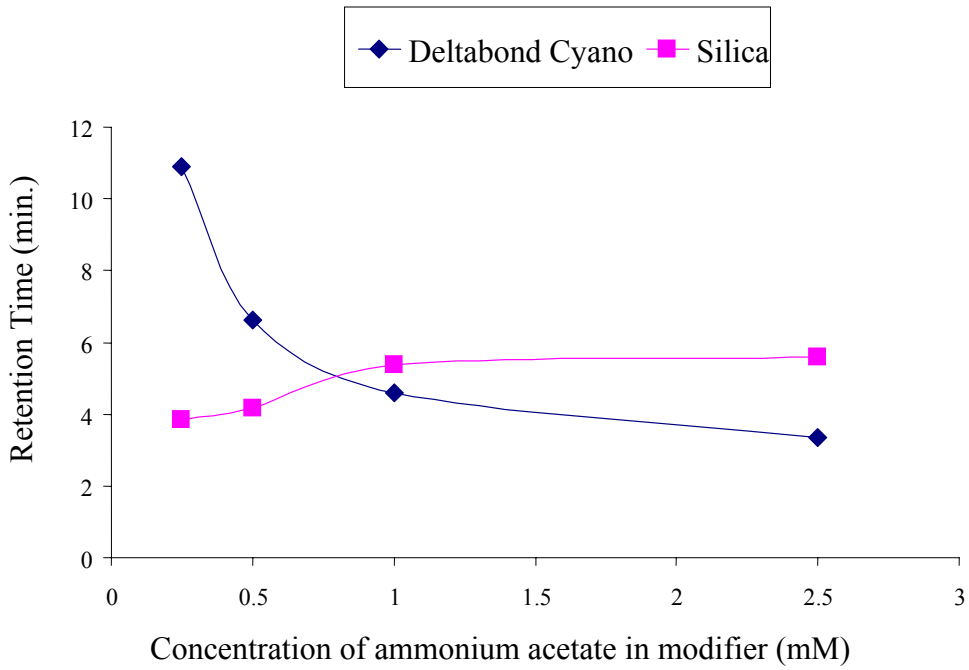


Figure 13. Retention time of sodium dodecylbenzenesulfonate on bare silica and Deltabond Cyano columns with different concentrations of ammonium acetate as mobile phase additive. Outlet pressure: 120 bar; oven temperature: 40 ºC; flow rate: 2 mL/min.

In order to attempt an explanation of these findings a consideration of the factors that influence retention in the absence of ammonium acetate should be initially made. We envision at least two contributors to retention: (1) an irreversible interaction of the sulfonate analyte with the bare silica or the silica support in the Deltabond column case and (2) a dispersion (nonpolar-nonpolar/dipole-induced dipole) interaction of the alkyl chain and/or aryl ring of the analyte with the cyano groups and the siloxane polymer of the cyano-modified polysiloxane coating on the Deltabond phase.

On the Deltabond Cyano column after all the silanol protons that would cause irreversible retention are exchanged for ammonium salts, ion-pairing formation between the sulfonate and ammonium cation may be the dominant mechanism. In this case, higher additive concentrations may lead to formation of more ion-pairs and thus faster elution would be achieved. Alternatively, as the ammonium acetate concentration in the mobile phase rises, it increasingly interacts with the cyano-modified polysiloxane coating. Cyano groups (e.g. permanent dipoles) would interact with ammonium cations, which then would interact with acetate ions. From the analyte's perspective, the stationary phase would present fewer and fewer bare cyano groups and more and more cyano units that are associated with ammonium cations. The cyano groups would be less available to interact directly with the analyte and thus cause retention via the alkyl/aryl part of the sulfonate to decrease.

On the other hand, with a bare silica phase, modification of the stationary phase by the additive may be the dominant mechanism, **Figure 14**. In other words, an ammonium cation might displace a proton and attach itself to the silanol oxygen thereby deactivating the silica stationary phase. We then speculate that sulfonate anion would

then form weaker ion pairs with the partially charged ammonium-modified stationary phase than with the fully protonated silanol site. As the ammonium acetate concentration increases in the mobile phase, one soon reaches the point where all the silanol protons that would cause irreversible retention are exchanged for ammonium salts. Irreversible retention no longer takes place and analytes elute with good peak shape. The ammonium ions bound to the silanols still retain a partial positive charge, and likely interact with acetate and/or sulfonate analyte ions in the mobile phase. The relatively large amount of acetate anion in the mobile phase is then hypothesized to replace any sulfonate from the ammonium-modified stationary phase resulting in elution of the sulfonate as shown again in **Figure 14**. Unlike the irreversible interaction that probably exists between the sulfonate and the bare silica surface silanols, the interaction between NH_4^+ (or tetraalkylammonium ion) that is attached to the silica stationary phase and the sulfonate is more reversible.

In the silica study, the higher concentration of ammonium acetate in the mobile phase led to a longer retention time for the sulfonate in contrast to the similar study just discussed with the Deltabond Cyano phase. An explanation for this phenomenon is not straightforward. As the ammonium acetate concentration increases further, we likely form a pseudo-bonded phase (or layer) of acetates that interact with stationary phase "bound" ammonium ions. The nonpolar acetate tails would interact with the analytes by dispersion. This would cause retention to increase slightly until all the silica surface is covered by this pseudo-phase. An alternate hypothesis is that more than a monolayer of ammonium ion coverage on the silica is possible via interaction with the layer of acetates that cover the pseudo-stationary phase. Subsequent layers of ammonium ion would carry

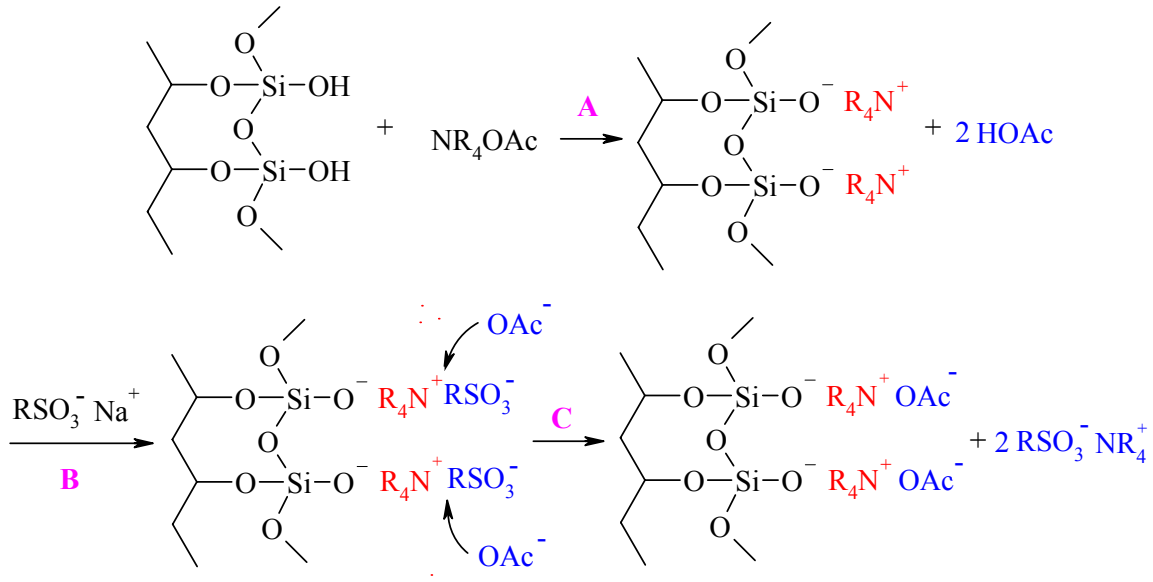


Figure 14. Proposed mechanism for modification of bare silica phase by ionic additive.

a greater positive charge than the cation immediately attached to the silanol oxygen with the consequence that sulfonate would be retained longer with higher concentrations of ammonium in the mobile phase.

Uptake and adsorption of the mobile phase by the stationary phase in supercritical fluid chromatography has been known for some time. Strubinger and Parcher [46] investigated this phenomenon using mass spectrometric tracer pulse chromatography. Below the critical point, an increase in the adsorption of CO_2 is observed with increasing pressure. Above the critical point, the reverse is true. At supercritical pressures, the excess adsorbed CO_2 increases with temperature at constant pressure. This is the reverse of what is observed for subcritical pressures. Modifiers have been found [47] to have a more pronounced effect on the stationary phase than CO_2. Polar additives are expected to exert a strong influence as well.

3.3.2 Memory Effect on Silica Phase

Modification of the bare silica phase by ammonium cations was proposed to be an important step in the overall elution process. In an effort to test this hypothesis, repeated injections of sodium *p*-toluene sulfonate on the silica column were performed. Prior to the first injection, pure methanol-modified carbon dioxide was allowed to flow through the SFC system and column. At the first of fourteen injections, the modifier pump was prompted to deliver 15% 2.5 mM (additive) modifier into the SFC system. **Figure 15** shows the retention time measured for each of the fourteen repeated injections with ammonium acetate as the probe additive. Several chromatograms corresponding to selected injections are presented alongside the retention time versus injection plot. The

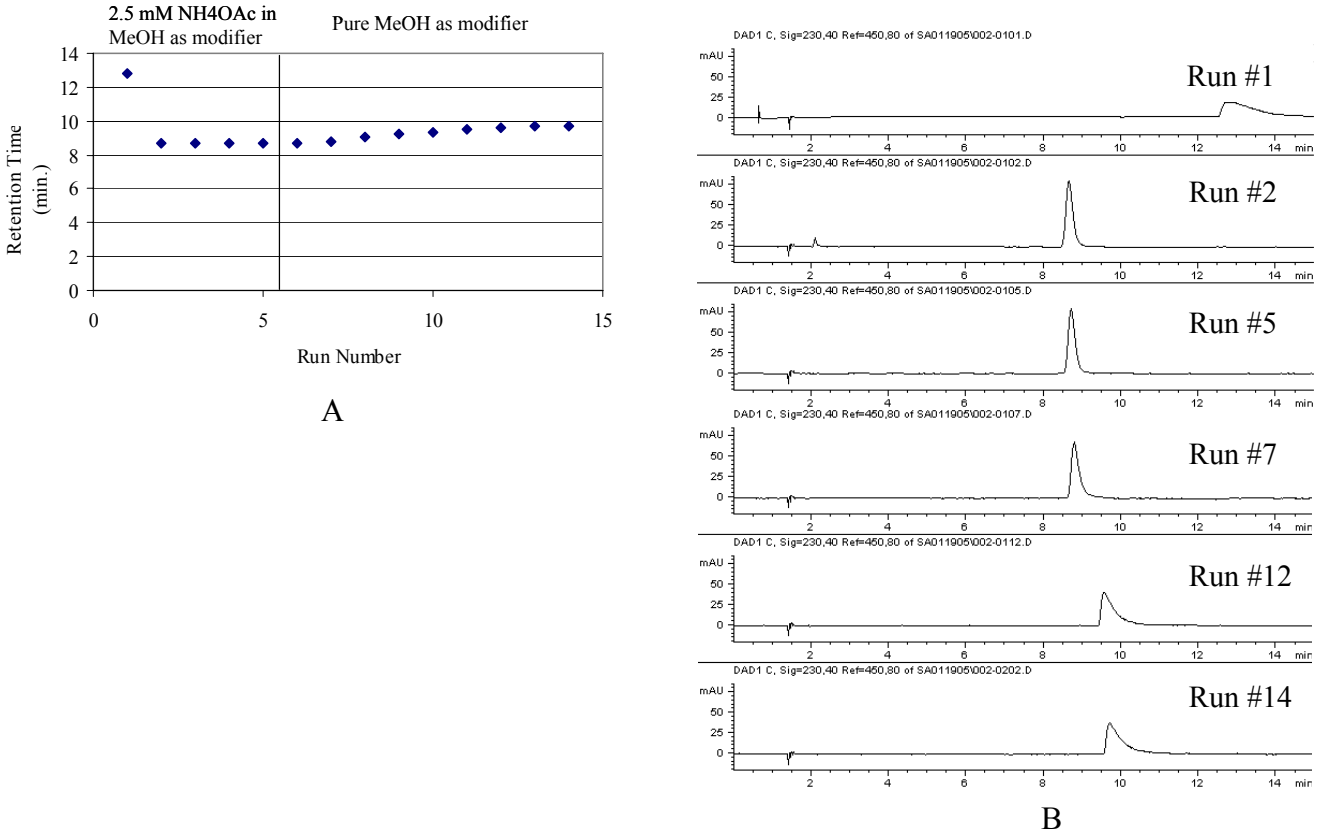


Figure 15. (A) Retention times for fourteen repeated injections of a sodium *p*-toluene sulfonate solution on a silica column. Injections 1-5: modifier contained 2.5 mM ammonium acetate; Injections 6-14: modifier was pure methanol. (B) Selected chromatograms from the fourteen separations.

retention time quickly decreased from 12.79 minutes for the first injection to 8.66 minutes for injections 2-5. This suggested that it took about 15 minutes (i.e. run time of one injection) for the column and mobile phase to reach equilibrium. At the beginning of the sixth injection, pure methanol modifier was re-introduced to the SFC system. When we changed the modifier, the tubing that connected modifier bottle and the system was not filled with new modifier. Thus, it took about 8 minutes for the new modifier (i.e. pure methanol) to get into the column under the experimental conditions. This means that when the 6^{th} injection was made, the mobile phase in the column was still 15% 2.5 mM (additive) methanol, therefore the sulfonate still eluted around eight minutes at the sixth injection. With subsequent injections, retention time increased to approximately ten minutes and levelled off. Even though the retention time did not change appreciably after ammonium acetate was removed, the peak shapes were degraded and tailed badly without the additive in the mobile phase. Failure of the retention time to return to its original near 13 minutes suggested that NH_4OAc not only modified the silica surface, but was retained on it. If necessary the silica column could be regenerated with 90% methanol/10% carbon dioxide for 15 minutes. After this treatment, however, the sulfonate failed to elute with pure methanol as the modifier, which suggested that the additive eventually could be displaced. At lower concentrations of methanol-modifier, the additive probably can be removed, but more column volumes are needed.

Similar experiments were performed on the silica column with the tetraalkylammonium acetate salts as mobile phase additives, TBAA and TMAA. **Figure 16** shows the measured retention time for the 14 repeated injections with TBAA as probe additive. As in the previous case, TBAA was initially introduced to the modifier pump at

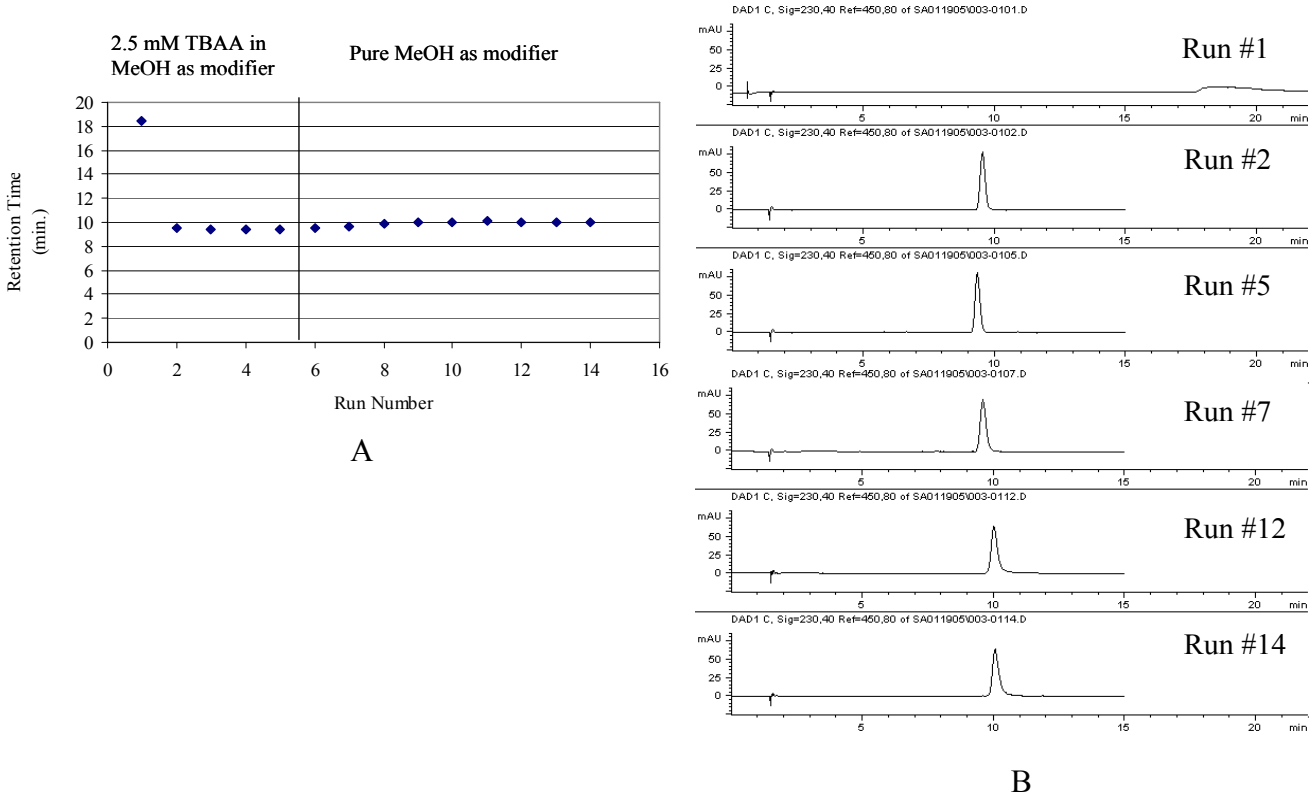


Figure 16. (A) Retention times for fourteen repeated injections of a sodium *p*-toluene sulfonate solution on a silica column. Injections 1-5: the modifier contained 2.5 mM TBAA; Injections 6-14: modifier was pure methanol. (B) Selected chromatograms from the fourteen injections.

the first injection and was removed at the sixth injection. The *p*-toluene sulfonate analyte isocratically eluted at about 18 minutes for the first injection. The retention time was shortened to about 9.5 minutes and fairly good reproducibility was achieved with the third, fourth, and fifth injections. This may suggest that it takes about thirty minutes for the column and the mobile phase to reach equilibrium. After TBAA was removed from the mobile phase, a strong memory effect similar to that found with ammonium acetate was observed. Retention time slightly increased from near 9.3 minutes to only 10 minutes for the subsequent nine injections. The greatest change in retention time after the removal of the TBAA additive was between injections 6 and 8 (e.g. 9.35 to 9.92 minutes).

Figure 17 shows the memory effect observed with TMAA as mobile phase additive on the silica column. From our previous study [39] , TMAA was observed to provide the longest retention time for selected sulfonates on the silica column. This could be due to the greater residual surface charge on TMAA as opposed to TBAA and the ammonium ion, *vide infra*. A greater surface charge on the stationary phase would result in a slightly longer sulfonate retention time. When equilibrium was reached in this case, retention time for *p*-toluene sulfonate was about 16.6 minutes. However, it took a much longer time for the column and mobile phase to reach equilibrium with TMAA as the additive than with ammonium acetate or TBAA. When TMAA was removed from the modifier after the fifth injection, a memory effect was again observed as with the other two additives such that sulfonate analyte could be eluted with pure methanol as the modifier.

Similar experiments involving repeated injections were performed on the Deltabond Cyano column with ammonium acetate as the ionic additive (**Figure 18**).

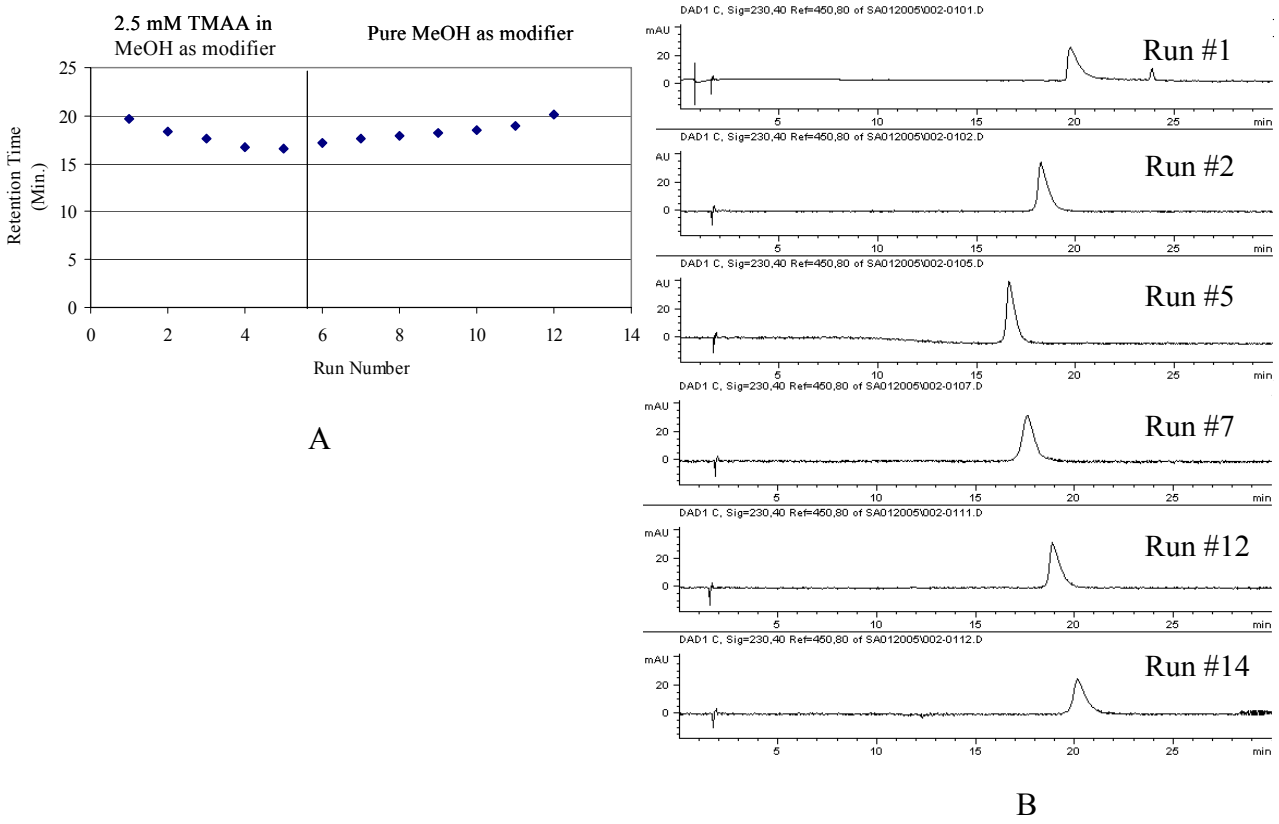


Figure 17. (A) Retention times for twelve repeated injections of a sodium *p*-toluene sulfonate solution on a silica column. Injections1-5: the modifier contained 2.5 mM TMAA; Injections 6-12: modifier was pure methanol. (B) Selected chromatograms from the fourteen injections.

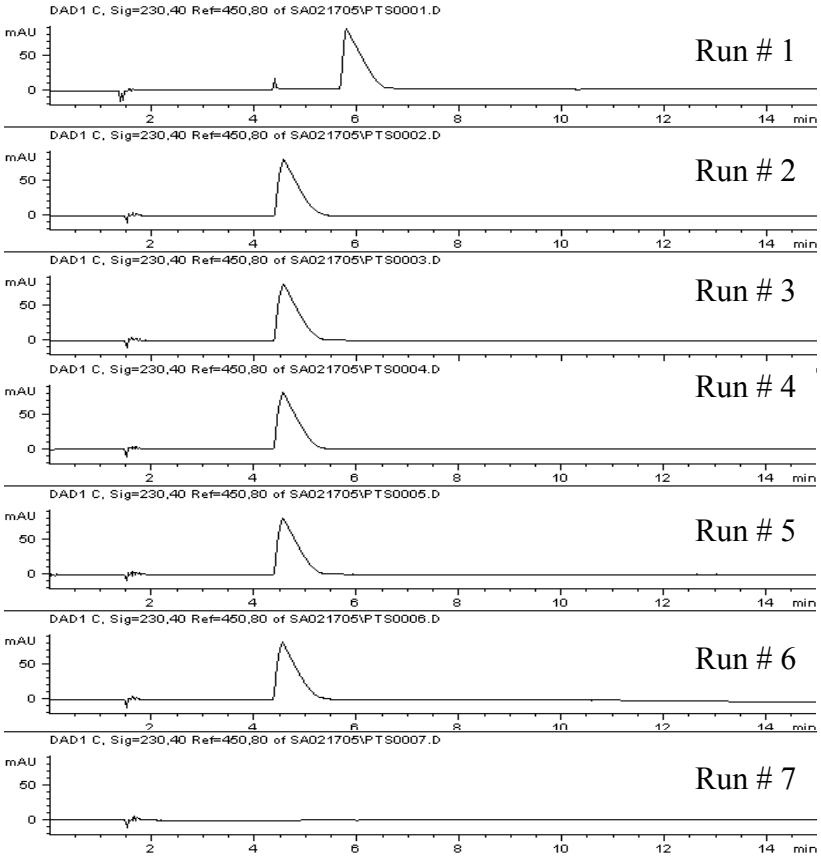


Figure 18. Repeated injections of sodium p-toluene sulfonate on a Deltabond Cyano column. Injections 1-5: the modifier contained 2.5 mM ammonium acetate; Injections 6-14: modifier was pure methanol.

After purging the modifier pump with methanol, 15% 2.5 mM NH_4OAc in methanol modifier was introduced as soon as the first injection was initiated. Once reproducible chromatograms were achieved, the mobile phase modifier was changed back to pure methanol (after the fifth run). Again, due to the modifier delay time, the mobile phase in the column contained NH_4OAc such that the sulfonate eluted in run 6 in similar fashion to the previous injection. By the seventh injection, however, the mobile phase on the column no longer contained NH_4OAc, and sulfonate no longer eluted. Thus, no memory effect was observed with the Deltabond Cyano phase, in striking contrast to the bare silica phase. In this case, ion-pairing formation between the analyte and NH_4OAc is a reasonable elution mechanism.

3.3.3 Solid State NMR Study of Silica Phase

Solid state NMR of bare silica was studied to provide some insight concerning the additive-stationary phase interaction. By using magic angle spinning (MAS) with cross polarization, proton magnetization can be transferred to diluted hetero nuclei such as ^{29}Si. This allows various surface species on silica to be distinguished. Structural elements of bare silica consist of Q^2, Q^3, Q^4 species where the superscripts indicate the number of Si-O-Si linkages (**Figure 19**). These three species can be easily distinguished by their corresponding chemical shifts at -92, -101, and -110 ppm, respectively [42]. Since all of the spectra were obtained using a fixed number of scans, the peak intensity can be used to compare the abundance of each group. The S/N ratio of Q^3 (-Si-OH groups) was measured and was normalized to the peak intensity per mg of each sample. The result is found in **Table 2**. After being flushed with supercritical CO_2 modified by 15% methanol

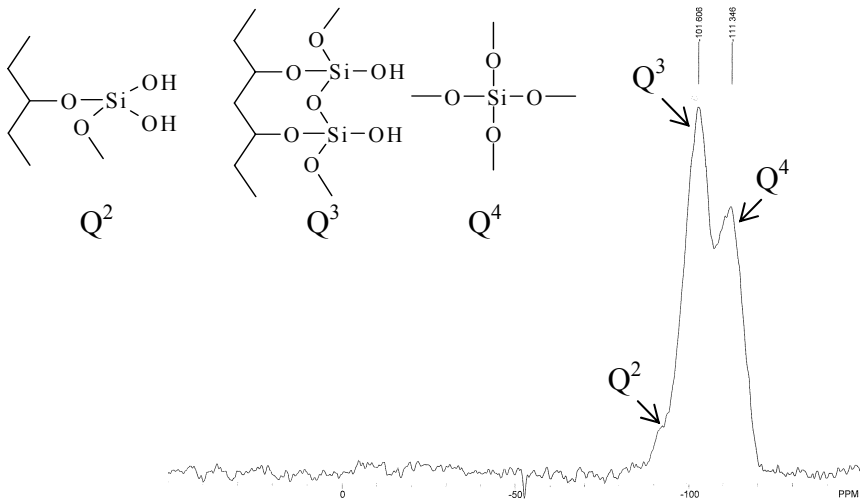


Figure 19. ^{29}Si CP-MAS NMR spectrum of bare silica packing material.

Table 2. Q^3 peak intensity per mg of silica and the percentage of free silanol groups covered by ammonium additive.

	Q^3 peak intensity I=S/N	Q^3 peak intensity/mg of sample ($\times 10^{-2}$)	% of modified Si-OH groups
Untreated silica	31.1	21.7	--
Silica treated with AA	26.4	15.6	28%
Silica treated with TMAA	33.3	16.2	25%
Silica treated with TBAA	38.5	16.9	22%

containing 2.5 mM ammonium acetate for about 60 minutes, the protons on about 28% of total Si-OH groups were no longer detected. The corresponding values for tetramethylammonium acetate and tetrabutylammonium acetate were 25% and 22%, respectively. We presume that the protons were no longer detected because they were exchanged with ammonium ions. All other chromatographic conditions were the same as previously mentioned. Ammonium acetate modified the largest percentage of free silanol groups and TBAA modified the smallest percentage. This might be explained by steric effects since TBAA is the largest cation, and ammonium the smallest.

3.3.4 Acidity of Supercritical CO_2/methanol Mobile Phase

As methyl alcohol is methyl substituted H_2O, it has some similarity to water. The pk_a of methanol in water at 25 °C has been reported to be 15.5 [48], compared to 14 for H_2O. This indicated that methanol should have similar acid-base properties as water. It is known that when CO_2 dissolves in water it reacts to form carbonic acid. Lancaster [49] has studied the extraction of an iron complex with H_2O-modified supercritical CO_2. The author suggested that the acidity of the extracting fluid breaks down the complex and thus provided a low extraction recovery. Methanol was then used as modifier instead of water in this study. Higher extraction recovery was achieved, however, it was still lower than 50%, which indicated that methanol-modified supercritical CO_2 was also acidic, but less acidic than H_2O/CO_2.

Wen et al. [50] studied the pH of methanol/H_2O/CO_2 liquid mixtures under high pressure via UV/visible absorption spectra of several pH indicators. The pH was reported to be 4.73 at methanol/H_2O/CO_2 mole ratio of 55.7/25.1/19.2.

The direct pH measurement of a methanol/supercritical CO_2 mixture has not been reported, to our knowledge. Pinkston and Chester [51] attempted to observe the effect of high pressure CO_2 on a pH indicator, sudan III, dissolved in methanol. The methanol solution was placed in a high pressure view cell. A CO_2 tank was attached to the bottom of the cell, and air was purged by bubbling gaseous CO_2 through the cell. The top of the cell was then sealed. The cell was pressurized with CO_2 from the tank. The color of the indicator changed to a more acidic color as the CO_2 was introduced. As the pressure of CO_2 rose to 60 bar, the color of the fluid switched to an even more acidic range. The exact pH value was not measured.

Due to the instrumental limitation, we were not able to carry out Pinkston and Chester's experiment. We performed somewhat similar experiments at room temperature and atmospheric pressure.

The three pH indicator solutions used were 1) "universal pH indicator", which consisted of four indicators: phenolphthalein disodium salt, bromothymol blue sodium salt, methyl red sodium salt, and thymol blue sodium salt, 2) methyl red sodium salt solution, and 3) bromocresol green sodium salt solution. The color change range for these indicators are 6.2-7.6 for bromothymol blue, 4.2-6.3 for methyl red, 8.0-9.6 for thymol blue, 8.3-10.0 for phenolphthalein, and 3.8-5.4 for bromocresol green [52]. **Figure 20** shows the change of color with universal indicator before, during, and after CO_2 was introduced to methanol and to water. We allowed sufficient time to saturate the CO_2/methanol and CO_2/H_2O mixtures with CO_2 until the color remained stable. The shift to a more acidic color range is clearly evident for both methanol and water. As we had

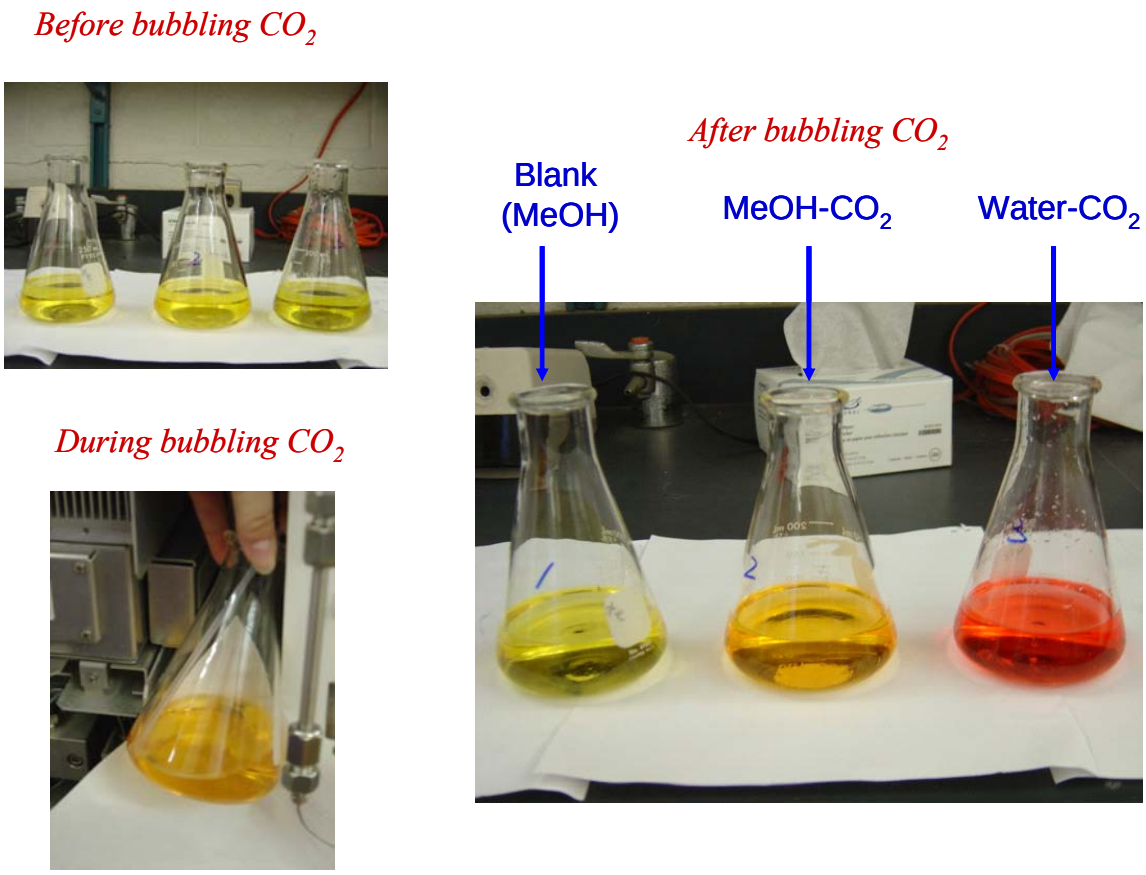


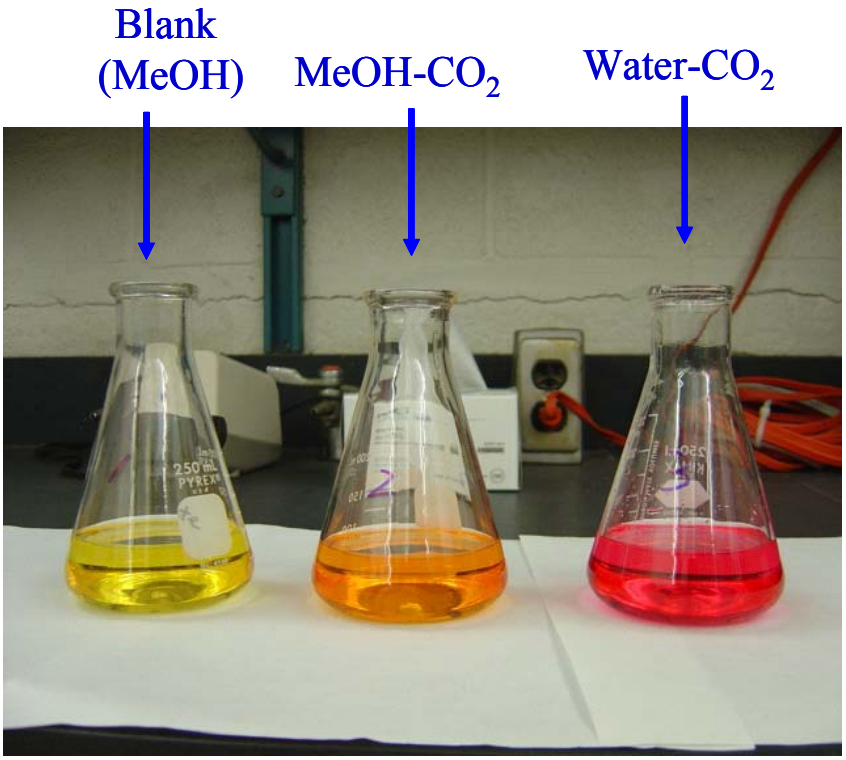
Figure 20. Color change of methanol and water containing universal pH indicator when bubbling CO_2 into the solutions.

expected, the color shift of the indicator was not as dramatic with the CO_2/methanol mixture as with the CO_2/H_2O solvent.

Assuming the dissociation constants (pK_I) of the indicators are similar in water and in methanol, and that the absorbance properties of the indicators in the two solvents are similar, the pH of saturated CO_2/methanol under ambient conditions was estimated to be 5.5 with the color chart of the universal indicator. An ammonium chloride aqueous solution was made with the same indicator to match the color of saturated CO_2/methanol solution. The measured pH value of the NH_4Cl aqueous solution gave a value of 5.32 which was close to what we estimated visually.

The other two indicators supported the conclusions that were drawn from our work with the universal indicator. When either methyl red or bromocresol green were used as indicators, the color change of the methanol and water solutions after saturation with CO_2 gas was visually distinguishable. We therefore estimated the pH value for CO_2/methanol under atmospheric pressure to be 5.5, which falls in the color change range of methyl red (pH 4.2-6.3) [52]. Various colors were observed for the three solutions: bright yellow for pure methanol, orange for CO_2/methanol, and red for CO_2/water, as shown in **Figure 21**. These distinct differences in color clearly support a shift of the methanol solution to a more acidic pH when it is saturated with CO_2.

We believe that high pressure CO_2/methanol would be even more acidic than atmospheric pressure CO_2/methanol, as more CO_2 will dissolve in methanol at high pressure. As the silanol group has a pK_a <2 in an aqueous environment [53], it might have a larger pK_a under methanol/CO_2 environment, but it would still be lower than 5. Therefore it is not surprising that silanol groups would dissociate in the methanol-



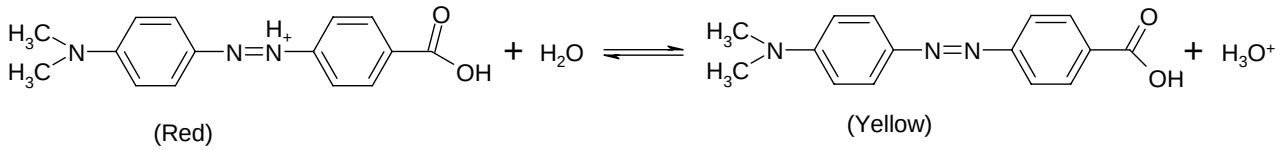


Figure 21. Color change of methanol and water after saturated with CO_2 gas with methyl red as pH indicator (pH 4.2-6.3). Reaction of methyl red when gaining a proton.

modified supercritical CO_2 mobile phase. On the other hand, due to the acidity of the mobile phase, ammonium cations would preferentially exist as NH_4^+, which makes it likely that NH_4^+ would interact with negatively charged SiO^- groups on the silica surface.

3.3.5 Computational Calculation of Charge Distribution

From our previous research [39], the behaviour of TMAA was quite different from that of AA or TBAA. When negatively charged sulfonates were the probe analyte, they were retained much longer on the silica column with TMAA than with the other two ammonium salts. On the other hand, TMAA provided more rapid elution of the same sulfonates on the Deltabond Cyano column. We believe that modification of the stationary phase is the dominant elution mechanism on the silica column and ion-pair formation is the dominant elution mechanism on the deactivated Deltabond Cyano column. The observed elution behaviours with the various ammonium salts cannot be explained by lipophilic interaction between the analyte and additive-modified-stationary phase or between the ion-pairs and the cyanopropyl stationary phase. It might be explained by different charge distribution on the individual ammonium ion. CaChe is a computer-aided chemistry modelling program for predicting molecular structure, properties, spectra, reactions, thermodynamics and kinetics [54]. CaChe allows calculations based on different theories and various methods. The CaChe MOPAC application is a semi-empirical, quantum mechanics package. It determines both an optimum geometry and the electronic properties of molecules by solving the Schrödinger equation using AM1 (Austin Model 1) parameters, MNDO, PM3 and PM5 parameters [45,54] Molecular geometry and partial charge distribution on the probe ammonium salts

were thus calculated using CaChe MOPAC with AM1 parameters, **Figure 22**. This calculation is based on gas state parameters. Another method that also uses MOPAC with AM1 parameters but in aqueous solution was also used to calculate the charge distribution on AA, TMAA, and TBAA. This might be a better approximation than the gas-state-based calculations. The use of AM1 parameters neglects the diatomic differential overlap. To gain a more accurate calculation, a DGauss/B88-LYP calculation was performed. DGauss calculations use DFT (density functional theory) methods that provide an approximate solution to the Schrödinger equation using only mathematical approximations. This method is more computationally intensive than MOPAC. The results of the calculations of charge distribution on the probe ammonium cations are listed in **Table 3**.

All three methods indicated that the tetramethylammonium cation has the largest surface charge of the three ammonium salts. These results helped to explain the chromatographic behaviour. On the silica column, ammonium cations may cause the stationary phase to become positively charged. Since TMAA has the largest surface charge, TMAA-modified silica may retain negatively charged sulfonate analytes longer than silica modified with the other ammonium salts. On the deactivated Deltabond Cyano phase, TMAA may form stronger ion-pairs with sulfonate analytes and help to elute them more rapidly.

The above chromatographic experiments, ^{29}Si solid state NMR on the pressurized fluid treated stationary phase, and computational calculations on the three ammonium salts have shed light on the elution and retention mechanisms of, specifically, sodium *p*-

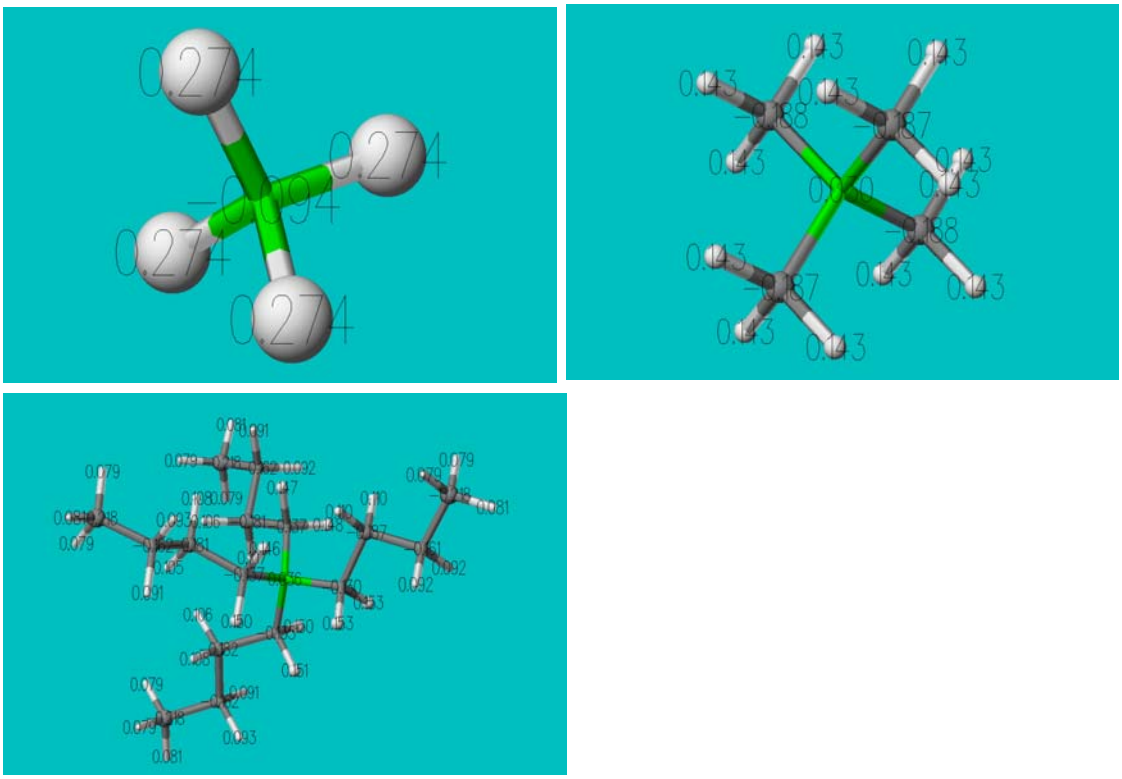


Figure 22. Optimized geometry of AA, TMAA, and TBAA molecules and partial charges on each atom.

Table 3. Partial charge distribution on the three ammonium salts calculated with different theories.

Additive	MOPAC/AM1 gas phase	MOPAC/AM1 in water	DGAUSS/B88-LYP
Ammonium	1.096*	1.076*	1.792*
Tetramethylammonium	1.716	1.732	3.144
Tetrabutylammonium	1.067	0.956	2.562

* Sum of the charges on the terminal hydrogen atoms.

toluene sulfonate with ammonium salts as CO_2-based mobile phase additives. The adsorption of ammonium cations on the active silanol sites of the stationary phase has been demonstrated to be a major contributor to the overall mechanism. On bare silica, where abundant silanol sites are present, modification of the stationary phase may be the dominant effect, while ammonium ion-induced dipole interaction with the cyano function may be dominant with the more deactivated Deltabond Cyano phase.

On the other hand, it is a different situation for the elution of propranolol hydrochloride from the silica column with different ammonium additives. As propranolol HCl is a secondary ammonium salt, it would interact with the active silanol sites in the same manner as the ammonium additives do, which means that the cationic analyte might compete with the additive cations for silanol sites. As the TMAA cation carries a larger positive charge than the other ammonium additive cations, it was not surprising that propranolol hydrochloride eluted faster with TMAA than with AA or TBAA, as shown in **Figure 12**. Therefore, when ammonium additives were used to elute ammonium analytes from bare silica phase, an ion-exchange mechanism was taking place on the active silanol sites.

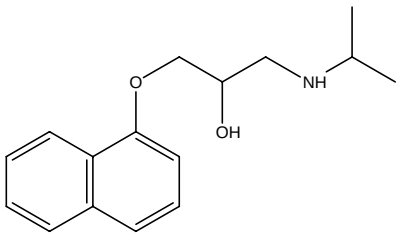
CHAPTER 4

Elution of Amine Salts with and without Sulfonate Additives by Packed Column Supercritical Fluid Chromatography

4.1 INTRODUCTION

Historically, to elute highly polar, potentially ionizable analytes by supercritical fluid chromatography (SFC), a small amount of a highly polar organic acid or base has been added to the CO_2-based mobile phase modifier which has most often been methanol. As suppression of ionization was believed to the role of the secondary modifier (i.e. additive), organic acids were used to elute acidic analytes and organic bases were used to elute basic analytes [20-23,28,29]. Only recently has much attention been directed toward fully ionic analytes such as sodium sulfonates and alkylammonium halides. No doubt the lack of chromatographic study in this area for so many years was due to the long-held opinion that CO_2–based mobile phases would have little solvating power for cationic or anionic organic salts.

Even though an ion-pairing principle has been recognized for some time in HPLC, it has not been fully realized in the SFC field [25,32,36,55] as a strategy for elution of fully ionic analytes. The notion of ion-pairing was first introduced to SFC by Steuer et al. many years ago [25]. In this study, 25 mM sodium heptanesulfonate in 20% methanol-modified, pressurized CO_2 (225 bar, 60^{o}C) was used to achieve fast elution of propranolol[a], IV, from a cyano stationary phase (100 mm x 4.6 mm i.d.), which otherwise failed to elute from this column without the mobile phase additive. See **Figure 23** for



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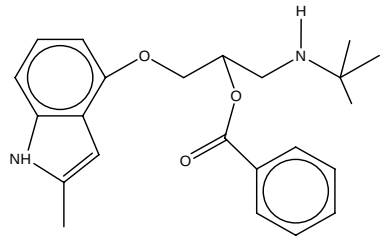

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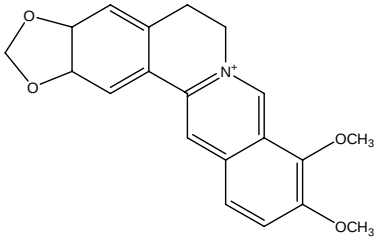

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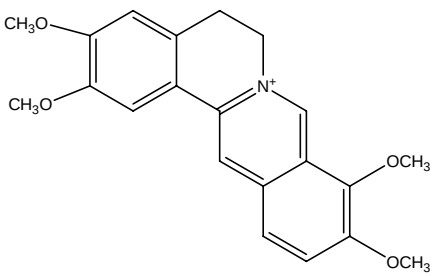

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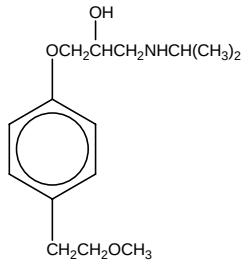

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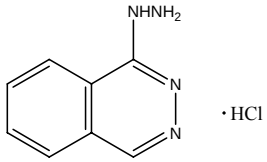

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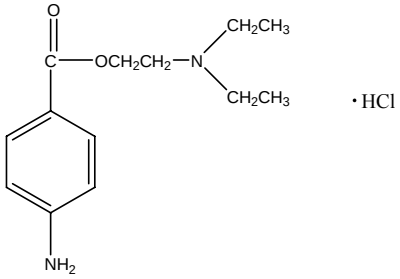

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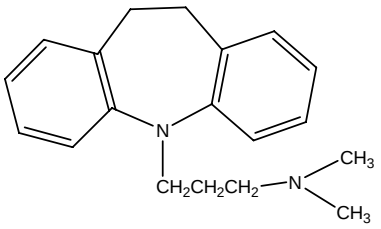

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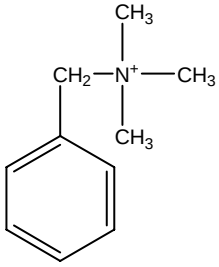

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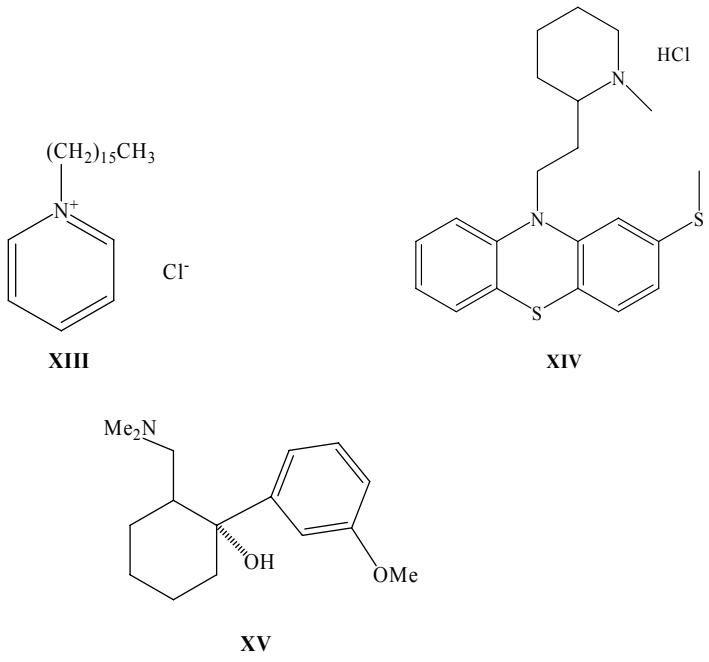


Figure 23. Molecular structure of analytes that are discussed in this chapter.

molecular structures. †Bopindolol, V, and its precursor were also successfully separated on a diol column when 20 mM of both tributylamine and acetic acid (e.g. tributylammonium acetate) were present in the modifier (methanol). The solubility of ion-pairing reagents in the carbon dioxide-modifier mixture was suggested to be a critical limitation concerning the success of the ion-pair SFC elution mechanism.

Some years later, another ion-pair reagent, dioctyl sodium sulfosuccinate, was used to extract (IP-SFE) and analyze (IP-SFC) cationic berberine, VI, and palmatine, VII, from *Phellodendri Cortex* in an on-line coupled experiment [55]. The mobile phase composition for IP-SFC was 100 mM dioctyl sodium sulfosuccinate in 15% (v/v) methanol-modified pressurized CO_2. A bare silica phase yielded satisfactory trapping after SFE and successful chromatographic separation via SFC of analytes with a comparatively short retention time; while an amino column did not retain the alkaloids under the test conditions.

In another study, an SFC method has been reported for separation of both metoprolol tartrate, VIII, and metoprolol succinate from their analogous hydrochlorides in both the bulk drug substance and the finished tablet on a 4 mm i.d. Diol-bonded silica column with UV detection (273 nm) [32]. The mobile phase modifier was 10% (v/v) methanol containing 0.35 M acetic acid and 0.07 M triethylamine. The excess acid was added to promote the stability of the silica-based column. The main advantage of the SFC method was that it was possible to separate and detect isomers that otherwise eluted close to the metoprolol peak in liquid chromatographic systems.

† Although the paper never confirmed it, we assume that propranolol and bopindolol are each the hydrochloride salt.

More recently, ammonium acetate, ammonium formate, and ammonium carbonate were reported to be effective as mobile phase additives in SFC to separate a group of polar and ionic compounds, including alkaloids, acids, di-acids, hydroxyl- and amino-acids, and nitro-compounds [36]. Amine hydrochlorides and quaternary amines that were studied included hydralazine HCl, IX, procaine HCl, X, imipramine HCl, XI, and benzotrimethylammonium chloride, XII. The authors suggested that ion-pairing formation between the ionic solute and additive was one of the possible roles of the ammonium additives.

In our previous research, [39,56] we successfully eluted via SFC three sodium aryl sulfonates with various alkylammonium acetate additives incorporated into the primary modifier at a concentration of 2.5 mM. Ammonium chloride and lithium acetate were also found to be effective ionic additives. Elution could be successfully carried out from Deltabond cyanopropyl, conventional cyanopropyl, and, surprisingly, bare silica stationary phases. The elution mechanism was suggested to be a combination of (1) ion-pairing formation between the cationic additives and the anionic solutes and (2) a dispersive interaction of the analyte and bonded stationary phase. In the case of bare silica, exchange of silanol hydrogen for alkylammonium cation was suggested to be important. While ion-pairing formation would be predicted to take place in the mobile phase no matter what stationary phase was used, on more active stationary phases the modification of silanol groups by the alkylammonium additives would prevent the expected strong interaction between the solutes (or the ion-pairs formed) and the active stationary phase silanol sites. The ion-pairing formation might be more dominant on the deactivated Deltabond cyano phase, since there are anticipated to be less active silanol

sites on the stationary phase. On the other hand, the modification of silanol groups would be more dominant on the active bare silica phase. These interpretations were supported by ^{29}Si solid state NMR and the observation that memory effects on the silica phase were more dominant than on the Deltabond cyano phase.

Many amine salts have been shown to be active pharmaceutical ingredients. Unfortunately, many of them were thought in the past to be too polar to be analyzed by SFC, especially for quaternary ammonium salts. Cetylpyridinium chloride is a quaternary ammonium salt which has been analyzed by IP-HPLC [57,58]. Tomlinson et al., however, have preliminarily reported a packed column SFC/MS/MS method for cetylpyridinium chloride, on a Deltabond Cyano column, with 35% 2.5 mM ammonium acetate/methanol as modifier. [59] Since we have demonstrated that ion-pairing formation is an effective means to elute sodium aryl sulfonates with ammonium salts as an additive in SFC, we have tested herein the use of sulfonate salts in the mobile phase to provide elution of ammonium analytes in SFC by the principle of reciprocity. In this paper, we have explored the elution of propranolol hydrochloride, IV, benzyltrimethylammonium chloride, XII, and cetylpyridinium chloride, XIII, in conjunction with sodium alkylsulfonates as mobile phase additives in methanol-modified pressurized CO_2.

4.2 EXPERIMENTAL

4.2.1 Chemicals

Methanol was HPLC grade, (EMD, Durham, NC, USA). The carbon dioxide was SFE/SFC grade (Air Products and Chemicals, Inc., Allentown, PA, USA) with no helium head pressure. Ammonium acetate (99%, ACS grade), sodium methanesulfonate (98%), sodium ethanesulfonate (97%), sodium 1-decanesulfonate (98%), ethanesulfonic acid (95%), and isopropylamine (99.5+%) were obtained from Sigma Aldrich (Milwaukee,WI, USA). Sodium 1-heptanesulfonate (≥99%) was purchased from Fluka (Milwaukee,WI, USA). Propranolol hydrochloride (99%), benzyltrimethylammonium chloride (97%), and cetylpyridinium chloride (99%) were purchased from Sigma Aldrich. A solution of each sample for chromatographic injection was prepared in methanol at a concentration of approximately 0.5 mg/mL.

4.2.2 SFC/UV Instrumentation

The SFC system was a Berger Instruments Analytical SFC (Newark, DE, USA) with a Hewlett Packard (Little Creek, DE, USA) Model 1050 Diode Array Detector, which employed a 13-μL high pressure flow cell (10-mm path length) and Berger Instruments 3D SFC ChemStation software, version 3.4.

The chromatographic columns were Deltabond Cyano (Thermo Hypersil-Keystone, Bellefonte, PA, USA), Supelcosil LC-Silica, Supelcosil LC-SAX1 (Supelco; Bellefonte, PA, USA), Berger Pyridine (Berger Instruments Inc.) and Princeton SFC Amino (Princeton Chromatography Inc., Cranburry, NJ, USA). The column dimensions were 25 cm in length and 4.6 mm i.d., with a particle size of 5 μm for each stationary phase. Supelcosil LC-Silica and Supelcosil LC-SAX1 both had a pore size of 120 Å;

Berger Pyridine had a pore size of 60 Å, while Deltabond Cyano had a pore size of 200 Å.

Unless otherwise specified, chromatographic conditions were as follows: injection volume 10 μL, mobile phase flow rate 2 mL/min (measured in the liquid state), column outlet pressure 120 bar, and column oven temperature 40°C. An isocratic mobile phase composition was employed: 15% modifier in CO_2 for propranolol hydrochloride and 30% for the other hydrochloride analytes, unless specified. The modifier consisted of either pure methanol or methanol with 2.5 mM additive. Each column was equilibrated with the modified mobile phase which contained the additive under study for 30 minutes at a flow rate of 2 mL/min prior to injection of the ammonium analyte. Between each change of mobile phase additive, the stationary phase was regenerated with 90% pure methanol at 1 mL/min for fifteen minutes (e.g. approximately nine column volumes) in order to purge the previous additive solution from the system and/or to remove analyte that had interacted with the stationary phase.

4.3 RESULTS AND DISCUSSION

4.3.1 Deltabond Cyano Stationary Phase

This study concerns the elution of positively charged nitrogen-containing analytes from silica-derived stationary phases using CO_2-based mobile phases that contain an ionic additive. One analyte was a dialkylammonium chloride (Structure IV, **Figure 20**), one was a tetra-alkylammonium chloride (Structure XII), and one analyte was an alkylpyridinium chloride (Structure XIII). Each of these analytes failed to elute from a

Deltabond Cyano column with 40% methanol-modified CO_2. The addition of a sodium alkylsulfonate to the methanol modifier, however, allowed the elution of each analyte.

Figure 24 shows the elution trace for propranolol hydrochloride on a Deltabond Cyano column with pressurized CO_2 modified with 15% methanol that contained either 2.5 mM sodium ethanesulfonate, sodium 1-heptanesulfonate, sodium 1-decanesulfonate, ethanesulfonic acid, or ammonium acetate. Each additive facilitated elution of propranolol hydrochloride with varying degrees of efficiency. The analyte eluted fastest and with the best peak shape with the three sodium alkyl sulfonate additives. Ethanesulfonic acid as an additive afforded a much later eluting, considerably broader chromatographic peak. This observation suggested the operation of an ion-pairing elution mechanism with the Deltabond Cyano column for the three organo ammonium salts when sulfonate salts were used as mobile phase additives. Since the sodium sulfonate salts should give rise to more sulfonate ions than the neutral sulfonic acid, at the same concentration, more ion-pairs would be expected to form between the nitrogen-containing cationic analyte and the sulfonate anion, and thus give rise to a sharper, earlier eluting peak. A comparison of the three equal molar sulfonate additives revealed that sodium 1-decanesulfonate provided the fastest elution time/sharpest peak and sodium ethanesulfonate provided the latest elution/broadest peak. The ion-pairs formed by sodium 1-decanesulfonate with the propranolol cation would be expected to have the greatest solubility in the mobile phase since this additive has the greatest non-polar character. Thus, the cationic analyte (ion paired with the decanesulfonate) would be predicted to elute from the column in the shortest time. With sodium ethanesulfonate as the additive, which has the least hydrophobicity, propranolol ion eluted last. This

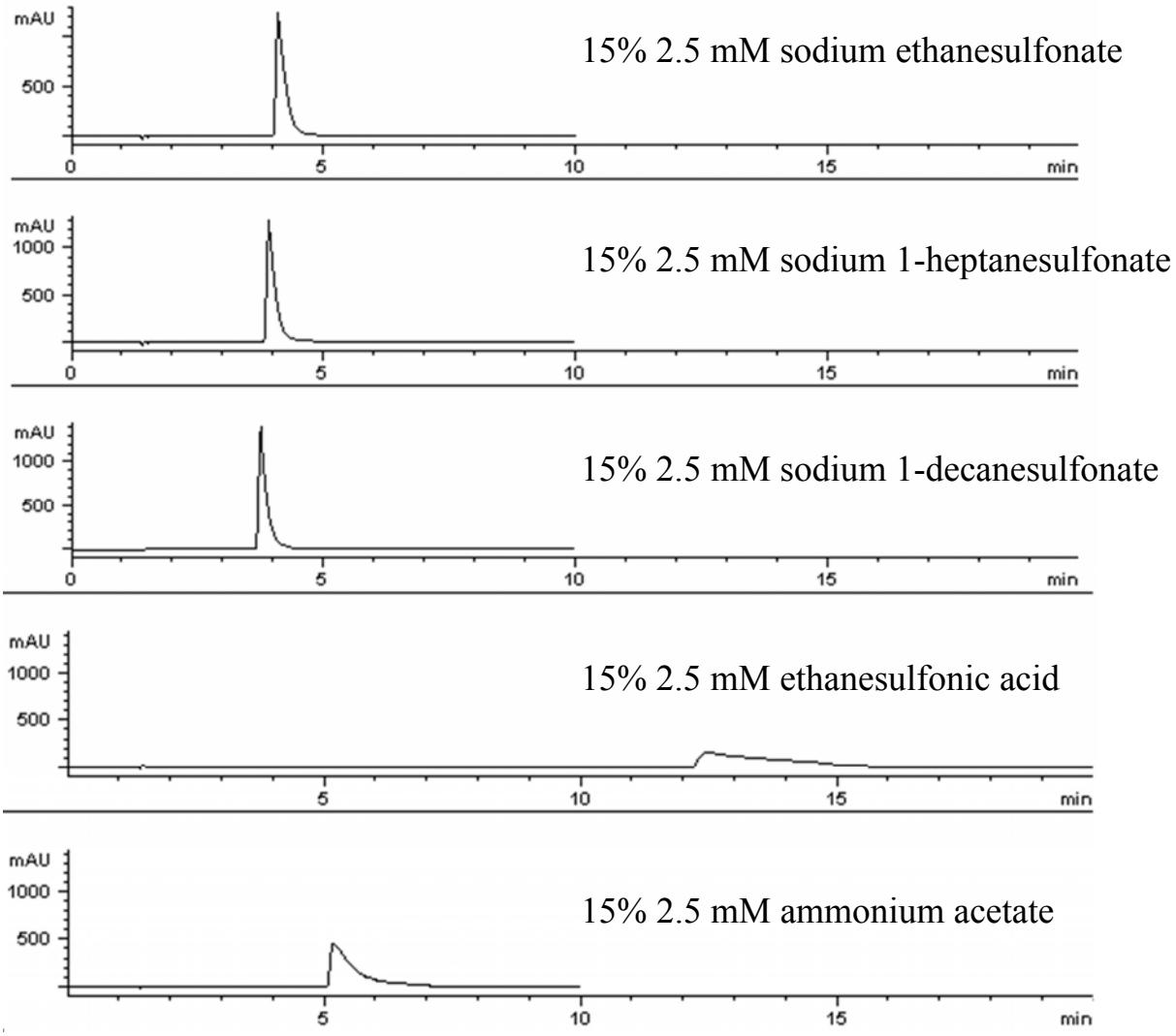


Figure 24. Elution of propranolol hydrochloride from a Deltabond Cyano column with different mobile phase additives. Outlet pressure: 120 bar; oven temperature: 40 ºC; flow rate: 2 mL/min. UV detector: 215 nm.

hypothesis was tested further by using sodium methanesulfonate as a mobile phase additive. Unfortunately, when this additive was incorporated into the mobile phase at 2.5 mM concentration in methanol (i.e. 15% modifier in mobile phase), the inlet pressure dramatically increased, and the analyte failed to elute. Poor solubility of the methanesulfonate in the mobile phase is believed to account for this chromatographic result.

Propranolol hydrochloride could also be successfully eluted from the Deltabond Cyano column with ammonium acetate incorporated into the methanol modifier. The peak, however, tailed badly, **Figure 24**. This result was somewhat surprising as an ammonium additive was used to elute an ammonium analyte. One explanation for this observation is that ion-pairing between the ammonium analyte and the acetate anions is occurring. The peak eluted later and was broader than the peaks obtained with the sodium sulfonate additives. We suggest that these two results with ionic additives are the result of two different elution mechanisms. With sulfonate additives the mechanism is dominated by ion pairing, while with ammonium additives the stationary phase becomes modified. Ion-pair formation is believed to be more effective than modification of the stationary phase.

Benzyltrimethylammonium chloride failed to elute from the Deltabond Cyano column within 20 minutes with 15% of modifier containing 2.5 mM sodium ethanesulfonate as additive. Cetylpyridinium chloride, however, eluted at about 11 minutes with a broad peak shape under the same condition. These analytes, however, eluted around 3.5 minutes when the concentration of modifier increased to 30% with 2.5 mM additive. As these two analytes are quaternary ammonium salts, they would be

expected to have a stronger interaction with the polar bonded Cyano phase via ion-dipole interaction. A higher concentration of modifier (30%) thus was necessary to achieve acceptable retention times and peak shapes for these two analytes.

The elution traces of cetylpyridinium chloride with various sulfonate mobile phase additives are shown in **Figure 25**. Again, sodium 1-decanesulfonate provided the fastest elution among all the additives that were studied due to the higher solubility of the ion-pairs formed by the analyte and the sulfonate additive with a longer aliphatic carbon chain. Again, ammonium acetate afforded analyte elution, but the peak shape was poor. Similar behaviour with various sulfonate additives was observed for elution of benzyltrimethylammonium chloride from the Deltabond Cyano column.

Reproducibility of the chromatographic method for each of the analytes was also investigated, **Table 4**. Good precision in retention factor was achieved with the highest RSD being 2.18%.

4.3.2 Bare Silica Stationary Phase

In our previous research [56], we found that cationic ammonium mobile phase additives covered a fraction of the surface active silanol groups on the silica support. We, consequently, wondered if sulfonate anionic additives could interact with the stationary phase in a somewhat analogous manner. First, we studied the elution of propranolol hydrochloride from a bare silica column with pure methanol, then with sodium ethanesulfonate in methanol, and finally with ethanesulfonic acid in methanol as mobile phase modifier, **Figure 26**. The analyte did not elute with 30% pure methanol as modifier. It did, on the other hand, elute with 2.5 mM sodium ethanesulfonate as the

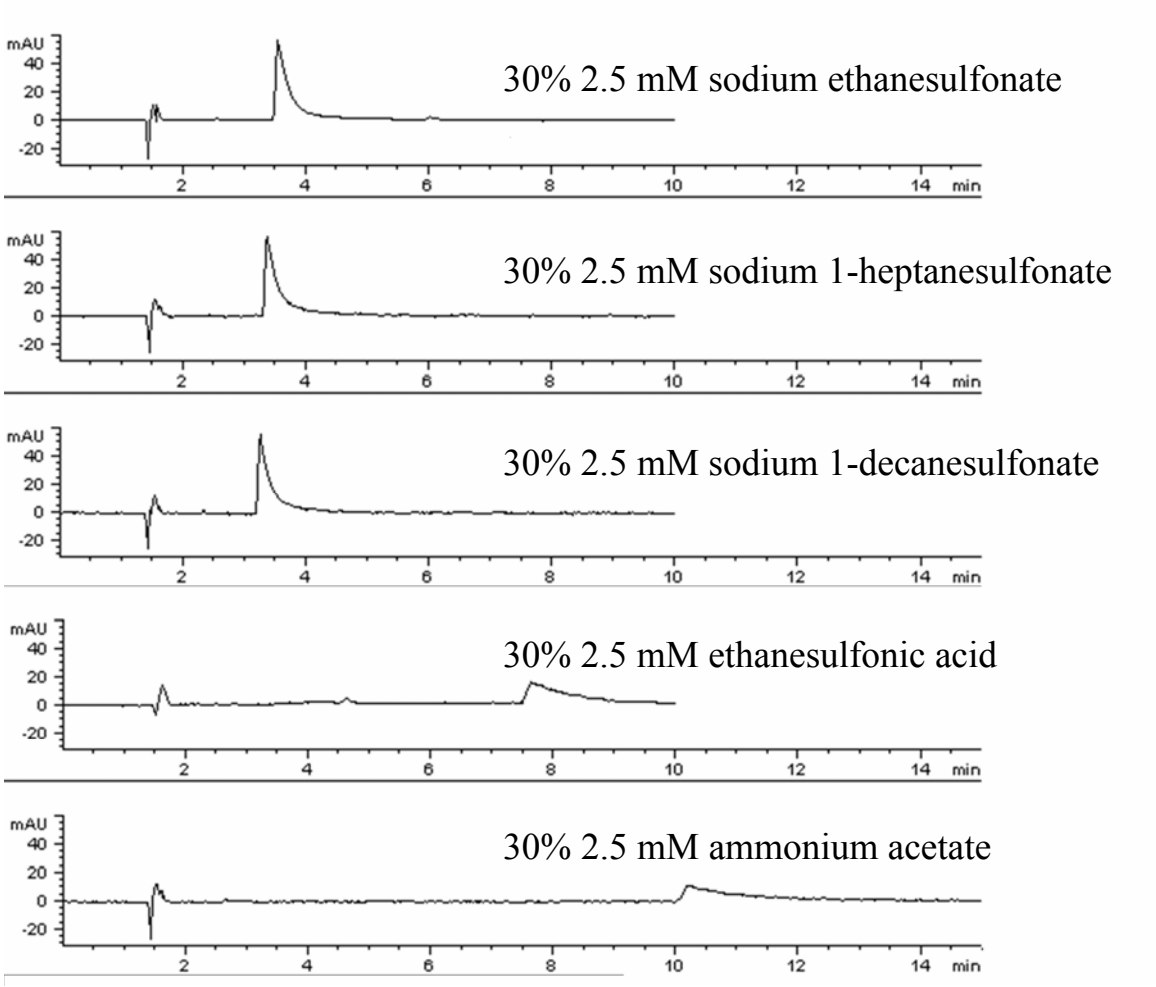


Figure 25. Elution of cetylpyridinium chloride on Deltabond Cyano column with different mobile phase additives. See Figure 24 for chromatographic conditions

Table 4. Retention factor of probe analytes with different mobile phase additives on Deltabond Cyano stationary phase.

	$C_2H_5SO_3Na$	$C_7H_{15}SO_3Na$	$C_{10}H_{21}SO_3Na$	$C_2H_5SO_3H$	AA
Propranolol HCl	1.84 (1.35)[a]	1.71 (0.25)	1.43 (0.40)	7.67 (1.86)	2.47 (1.28)
Cetylpyridinium Chloride	1.27 (1.22)	1.18 (0.13)	1.12 (0.47)[b]	3.69 (0.62)	5.59 (2.18)
Benzyltrimethyl-ammonium chloride	1.33 (1.19)	1.33 (0.64)[b]	1.24 (0.76)	4.05 (0.39)	5.44 (3.46)

[a] Percent RSD (n=3)
[b] n=4

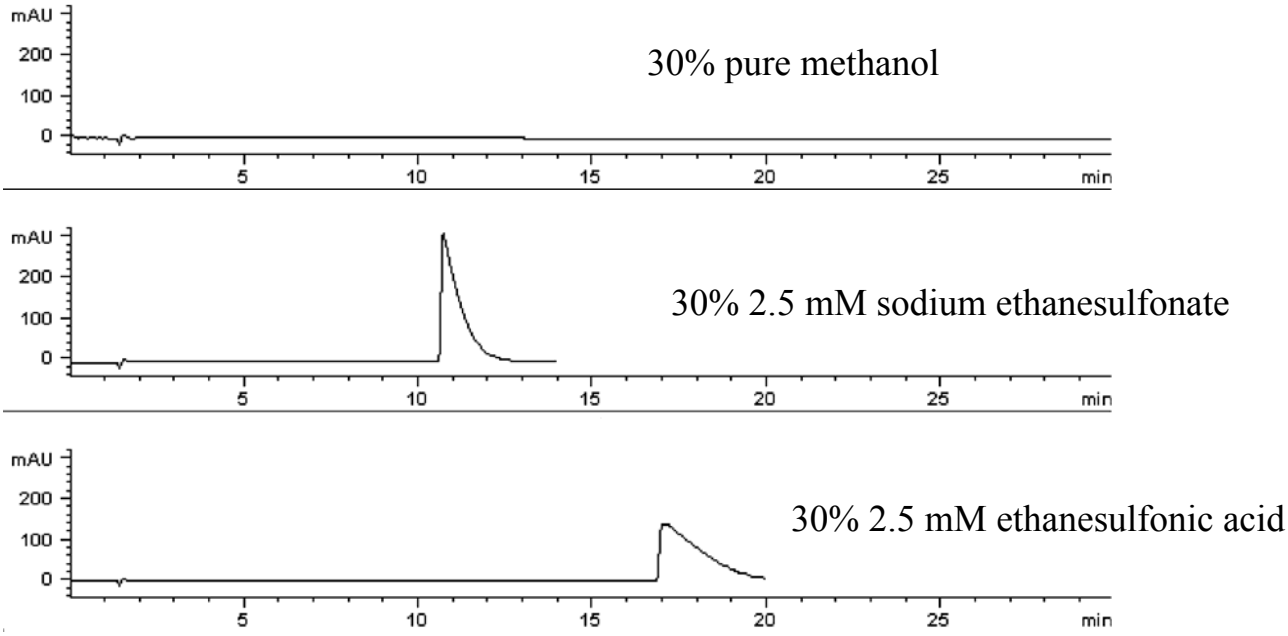


Figure 26. Elution of propranolol hydrochloride on a silica column. See Figure 24 for chromatographic conditions

mobile phase modifier. We feel that this is probably due to ion-pairing between the analyte and the sulfonate additive rather than modification of the stationary phase by the sulfonate ion, *vide infra.* On the silica column, propranolol hydrochloride eluted in about eleven minutes, with 30% modifier (methanol containing 2.5 mM sodium ethanesulfonate) in the mobile phase, compared to a four-minute elution from Deltabond Cyano, with 15% modifier in the mobile phase. This later elution from silica indicated a relatively strong interaction between the ion-pairs formed by the analyte and the sulfonate and the active bare silica stationary phase. We, thus, were not surprised by the fact that with ethanesulfonic acid, the propranolol hydrochloride eluted even later and tailed more than with sodium ethanesulfonte. In other words, it was expected that a greater number of active silanol sites should exist on the bare silica phase with the more acidic mobile phase additive.

In our previous SFC study of sulfonate with ammonium salt mobile phase additives [56], probe aryl sulfonate analytes continued to elute from the bare silica phase even after the additive was removed from the mobile phase, which indicated a certain degree of silica phase modification by the ammonium additive. We have performed a series of repeated injections of propranolol hydrochloride on the bare silica phase with first sodium ethanesulfonate additive in the mobile phase and then with it removed in order to ascertain if a memory effect was operational. Pure methanol was introduced to (or additive was removed from) the mobile phase at the beginning of run #5. Due to a slight delay of modifier reaching the column, the sulfonate additive remained in the mobile phase during run #5. Thus, the sulfonate was completely replaced with pure methanol prior to run # 6. Immediately, propranolol hydrochloride failed to elute. This

observation suggested again that sodium ethanesulfonate probably did not modify the silica stationary phase. Alternatively, these results may indicate that the analyte formed an ion-pair with the sulfonate additive, the solubility of the ion-pair in the mobile phase thus increased, and the analyte was thereafter eluted.

Elution of the three probe analytes with different concentrations of sodium 1-heptanesulfonate in methanol was also studied. The experimental retention factors are plotted in **Figure 27**. All the organo ammonium analytes eluted faster with increasing concentration of additive in methanol up to 2.5 mM. Since a higher concentration of additive could lead to more ion-pairs and these ion-pairs would have greater solubility in the mobile phase than the original organo ammonium salts, it is therefore reasoned that the analytes would be expected to elute faster with higher concentrations of additives.

4.3.3 Ethylpyridine Silica Stationary Phase

Elution of amine hydrochloride salts from polysaccharide coated silica stationary phases without additives was reported. Geiser, et al. [34] have described the direct, preparative enantioselective chromatography of racemic hydrochlorides of propranolol, IV, and thioridazine, XIV, using a rather expensive Chiralpak AD stationary phase and CO_2 modified with only methanol. Isolated fractions of propranolol hydrochloride were positively identified. More recently, this investigation has been extended to the resolution of tramadol, XV, hydrochloride with polarimetric detection on a Chiralpak AD-H stationary phase without the use of either basic or acidic additives [34,35].

We were interested to know if a newly developed ethyl pyridine-bonded, silica-supported phase would afford elution of amine salts without mobile phase ionic additive.

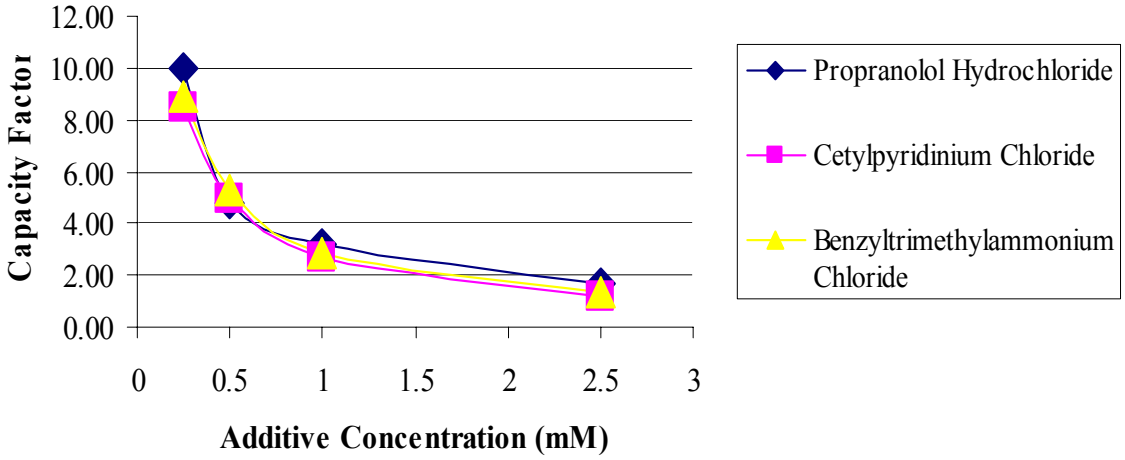


Figure 27. Retention factors for three organo ammonium analytes on Deltabond Cyano column with different concentrations of sodium 1-heptanesulfonate as additive.

Successful elution of all three organic amine salts that were studied earlier in this manuscript was achieved on this phase without the use of sulfonate additives, **Figure 28**. Surprisingly, cetylpyridinium chloride and benzyltrimethylammonium chloride were eluted faster than propranolol hydrochloride with only 20% methanol modifier. On the Deltabond Cyano phase, they either did not elute or eluted with very broad peak shape under the same chromatographic mobile phase conditions as described earlier. If the modifier composition was increased to 30%, all three ammonium salts eluted without retention from the pyridine column. Initially, we thought that the surface silanol groups on the stationary phase had been internally deactivated by the basic pyridine functional group. However, anionic analytes, such as sodium dodecylbenzene sulfonate and sodium p-toluene sulfonate, did not elute from the ethylpyridine column even with 30% pure methanol as the modifier. This suggested to us that a fraction of the pyridine functional groups associated with the stationary phase were possibly protonated and positively charged during the chromatography. If so, the stationary phase would be less attractive to the positively charged amine salt cation than the anionic sulfonate (i.e. elution of the cationic amine and retention of the sulfonate anion). This hypothesis may also explain why the permanently charged quaternary ammonium analytes would be eluted from the stationary phase faster than propranolol hydrochloride which is in equilibrium with its neutral form. Some support for protonation of the pyridine phase is found in a recent report by Jessop, et al.[60], where in it was reported that exposure of a 1:1 mixture of two non-ionic liquids, namely 1,8-diazabicyclo-[5.4.0]-undec-7-ene and 1-hexanol, to gaseous CO_2 at one atmosphere and at room temperature causes conversion of the liquid mixture to an ionic liquid. The equilibrium shown in **Figure 29** is readily converted back

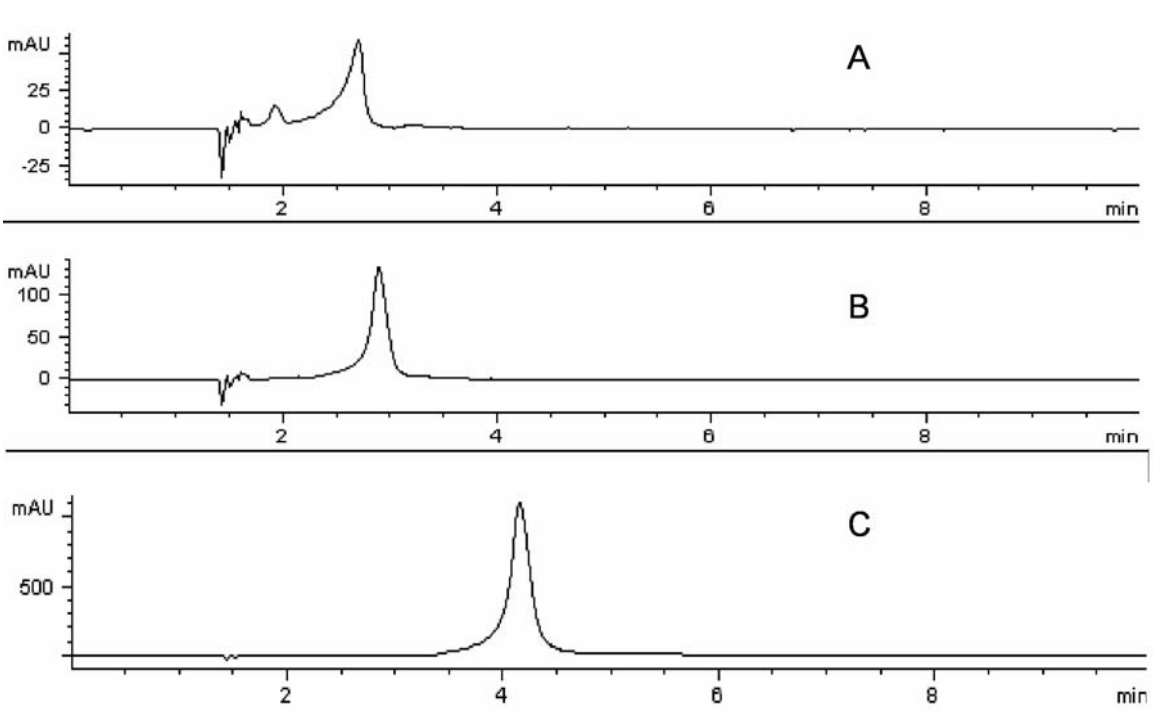


Figure 28. Elution of (A) cetylpyridinium chloride; (B) benzyltrimethylammonium chloride; (C) propranolol hydrochloride on 2-ethyl pyridine column with 20% pure methanol-modified pressurized CO_2. Other chromatographic conditions are the same as in Figure 21.

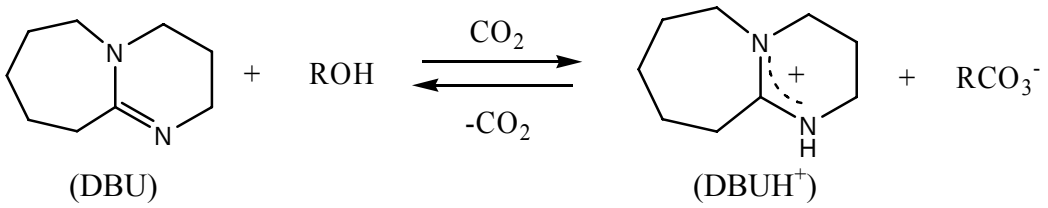


Figure 29. The 'switching' of a switchable solvent. Protonation of DBU (1,8-diazabicyclo-[5.4.0]-undec-7-ene) in the presence of an alcohol and carbon dioxide is reversed when CO_2 is removed. [60]

into a non-ionic liquid by bubbling N_2 or argon through the liquid at room temperature.

We observed fronting peak shapes for all three analytes, **Figure 30,** which usually indicates column overloading. In an attempt to eliminate the peak fronting, we decreased the sample concentration to 0.25 mg/mL and kept the injection volume at 10 µL. The same peak shapes were however obtained. It is difficult to imagine overloading of the column under these conditions as the column dimensions were 250 in length × 4.6mm i.d. Peak fronting here, we believe, indicates repulsion between the protonated pyridine stationary phase (promoted by the high pressure CO_2/methanol) and the organo-amine salt analytes.

To test our hypothesis, a basic additive, isopropylamine, was used as a mobile phase additive. **Figure 30** shows the elution of the three ammonium analytes on the pyridine column with 20% methanol containing 0.5% isopropylamine (A) and 20% methanol containing 2.5 mM ammonium acetate (B) as modifier. The neutral basic additive was believed to suppress the ionization of basic analytes and provide a less charged active stationary phase. Interestingly, isopropylamine (IPA) did not improve the peak shape of the two quaternary ammonium analytes. Cetylpyridinium chloride and benzyltrimethylammonium chloride eluted with broad peak shapes and tailed badly. An ionic additive, ammonium acetate, provided better peak shapes than IPA for these two quaternary ammonium salts. This observation suggested that when IPA is present in the mobile phase, since it is a stronger base than pyridine, it suppresses the protonation of the pyridine functional groups on the stationary phase. Thus, there is less electrostatic repulsion between the stationary phase and the cationic analytes, so that the analytes eluted with tailing peak shapes. Alternatively, ammonium acetate would not prevent the

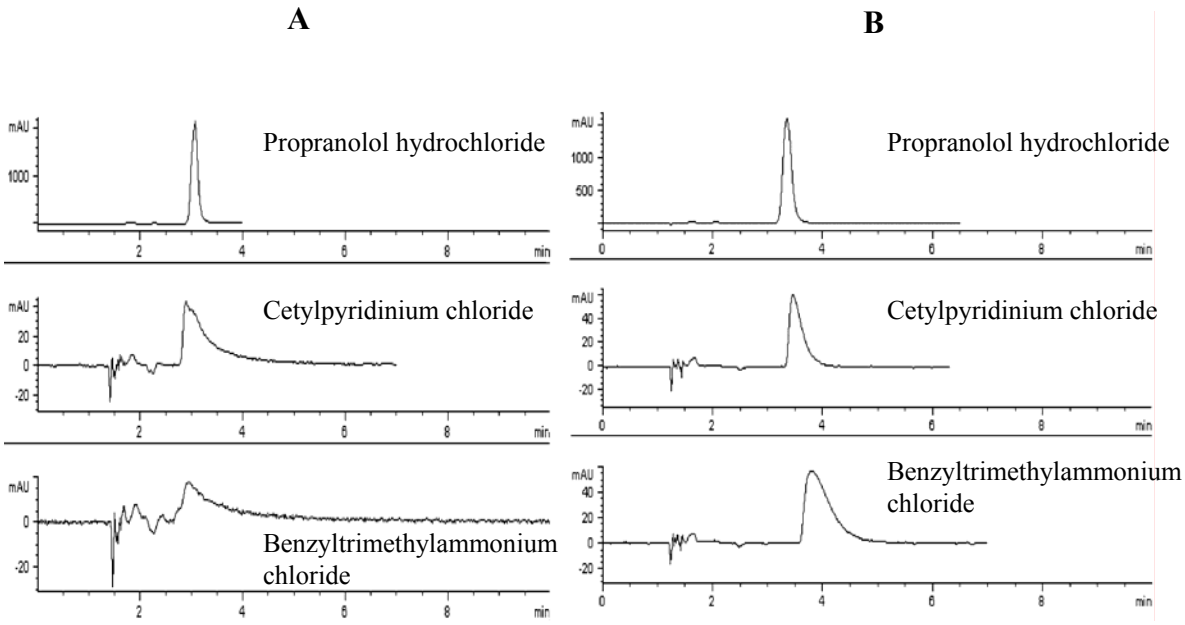


Figure 30. Elution of the three ammonium analytes on 2-ethylpyridine column with (A) 20% methanol containing 0.5% IPA; (B) 20% methanol containing 2.5 mM ammonium acetate as the mobile phase modifier with 80% CO_2. Other chromatographic conditions are the same as in Figure 24.

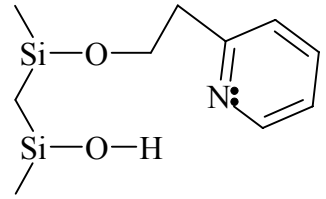
protonation of pyridine and thus it provided better peak shapes for these two quaternary ammonium salts.

These observations supported our hypothesis that the ethylpyridine stationary phase may be partially protonated in the presence of a CO_2/methanol mobile phase, and the positively charged ammonium salt analytes were eluted by electrostatic repulsion between the partially protonated stationary phase and the analytes.

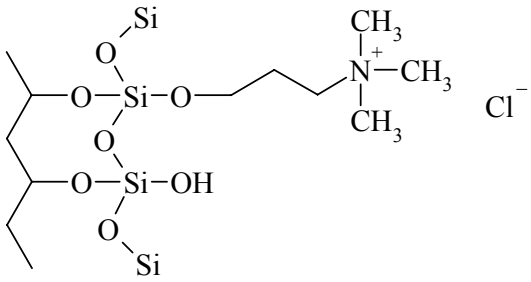
4.3.4 Strong Anion Exchange Stationary Phase

Suspected protonation of the ethylpyridine column in the CO_2/methanol based mobile phase, prompted us to study a strong anion ion exchange (SAX) stationary phase with SFC. The structures of ethylpyridine, SAX and aminopropyl stationary phases are shown in **Figure 31**. This SAX phase carried a permanent positively charged propyltrimethylammonium cationic functional group. While ion exchange columns have been widely used in HPLC, this is the first report of using a SAX column in SFC of which we are aware. Surprisingly, the three amine salts were successfully eluted from the SAX phase without the need of a mobile phase additive (e.g. 30 - 40% methanol), **Figure 32**. The peak shapes for cetylpyridinium chloride and benzyltrimethylammonium chloride again exhibited fronting. These two quaternary ammonium salts eluted faster than propranolol hydrochloride, as was the case on the ethylpyridine column. These observations are consistent with repulsive interaction between the positively charged SAX stationary phase and the positively charged analytes. When 2.5 mM ammonium acetate in methanol was used as a mobile phase modifier, the analytes were again eluted from the SAX phase with good peak shape, however there was little change in retention

A



B



C

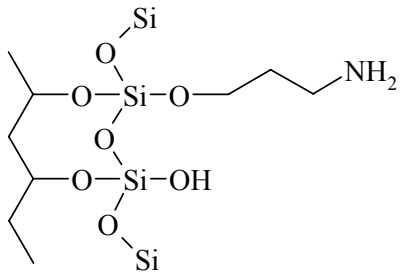


Figure 31. Structures of (A) ethylpyridine; (B) SAX; (C) aminopropyl stationary phases.

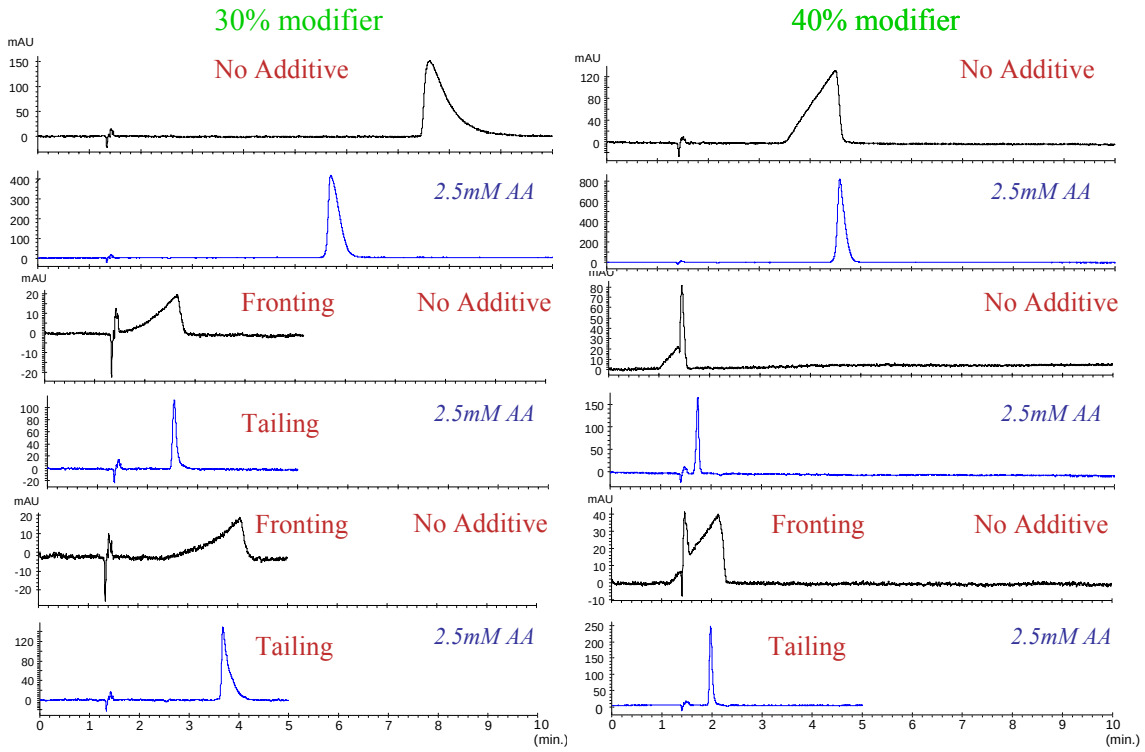


Figure 32. Elution of three ammonium salt analytes on SAX column. (A) propranolol hydrochloride; (B) cetylpyridinium chloride; (C) benzyltrimethylammonium chloride.

time, **Figure 32**. This is probably due to interaction of ammonium acetate with both the active silanol sites present on the ethylpyridine stationary phase and also with the propyltrimethylammonium groups on the SAX column. Compared to the ethylpyridine column, the SAX column seemed to be a more active stationary phase, thus accounting for the poorer peak shapes without the ammonium acetate additive. Peak fronting and the elution order of the three analytes on both columns support our hypothesis of protonation of the ethylpyridine functional group in the presence of the CO_2/methanol mobile phase.

4.3.5 Aminopropyl Silica Stationary Phase

If it is true that the pyridine functional groups on the stationary phase are protonated by methanol-modified CO_2, we would expect to observe a similar effect with an aminopropyl silica phase as the primary amino groups provide a more basic site than the pyridine moiety. The three cationic organic amines were studied on the amino column with 30% methanol and 2.5 mM ammonium acetate in methanol as mobile phase modifiers, **Figure 33**. All three analytes eluted without additive and all yielded fronting peak shapes as was observed with the ethylpyridine and SAX columns. Introduction of ammonium acetate to the mobile phase, afforded much less peak fronting. More symmetric peak shapes indicates a greater interaction between analytes and the stationary phase. These phenomena support our hypothesis that basic functional groups on the stationary phase may be protonated by a mixture of supercritical CO_2 and methanol. These positively charged stationary phases showed advantages for the elution of basic (or positively charged) analytes in SFC.

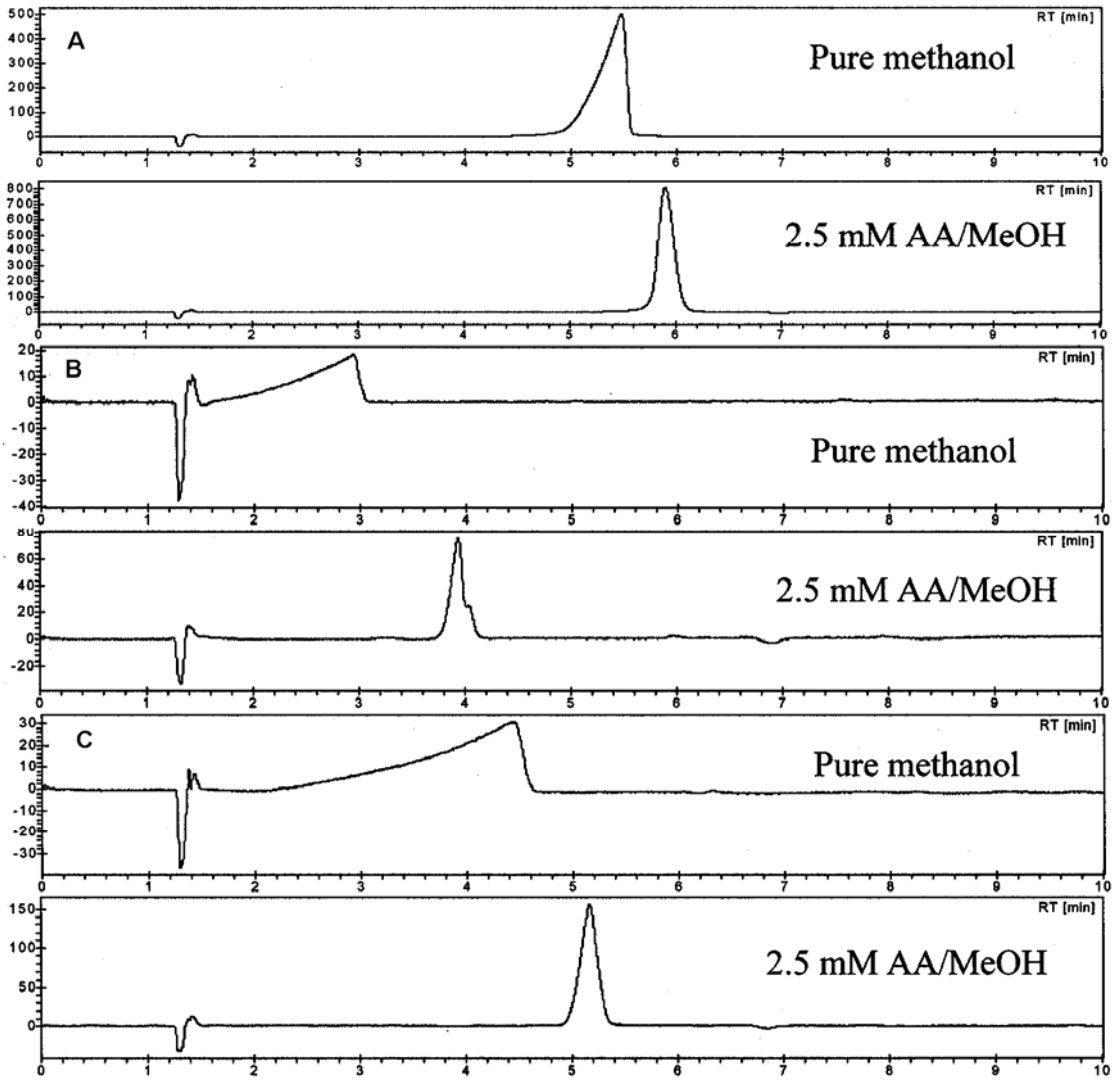


Figure 33. Elution of three ammonium salt analytes on amino column with 30% mobile phase modifier. (A) propranolol hydrochloride; (B) cetylpyridinium chloride; (C) benzyltrimethylammonium chloride.

4.4 CONCLUSIONS

An ion-pair principle was utilized to elute ionizable ammonium analytes from a Deltabond cyano stationary phase with sodium sulfonate additives. The lack of any stationary phase memory effect suggested that there was little or no modification of the stationary phase. Analytes eluted faster as the additive concentration increased from 0.25 mM to 2.5 mM. Ethylpyridine silica, aminopropyl silica, and SAX stationary phases were found to be effective for the elution of the same cationic analytes but without the need of mobile phase additives. A series of experiments provided evidence for the protonation of at lease some of the pyridine (or amino) functional groups on the stationary phases thereby generating a positively charged stationary phase in each case within the CO_2/methanol mixture. A similar electrostatic repulsion mechanism was also observed with the strong anion exchange column. The three probe analytes were eluted from the SAX column without the need of additive. The addition of ammonium acetate, however, helped to achieve better peak shapes for the analytes with each stationary phase.

CHAPTER 5

Advances in the Elution of Polypeptides with SFC/MS

5.1 INTRODUCTION

The low viscosity and high diffusivity of the mobile phase in supercritical fluid chromatography (SFC) affords higher flow rates and lower pressure drops along a packed chromatographic column than is possible in traditional high performance liquid chromatography (HPLC). SFC is a "green" technology since it requires a smaller amount of organic solvent than is required in LC. This is especially appealing for preparative scale chromatography since less time and energy are required to remove solvent and isolate the analytes. Fluids that are commonly used in SFC, such as CO_2, are relatively non-polar. As a result, SFC has been generally limited to separations of non-polar and relatively low polarity compounds.

Researchers have sought to extend the applicability of SFC to a wider range of polar analytes. These strategies included: (1) using more polar pure fluids, such as ammonia [61], sulfur dioxide [62], nitrous oxide [63], and Freon-23 [12], (2) adding a polar organic solvent (i.e. "modifier") to CO_2 to improve the solvating power of the mobile phase [64], and (3) adding a highly polar or ionic component to the modifier (i.e. "additive") to further increase the solvating power of supercritical CO_2 and/or to deactivate the stationary phase [20,21].

Mass spectrometry is an informative and universal detection method for chromatographic separation. LC/MS has, thus, been widely adopted in the analysis of organic molecules.

Various interfaces between SFC and atmospheric pressure ionization (API) mass spectrometry have been studied [65]. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been widely used in SFC/MS [66,67]. The SFC mobile phase is more volatile than an aqueous-based HPLC mobile phase, which allows a substantially higher proportion of the column effluent to be directed to the API interface. Fast, accurate, and quantitative results have been achieved using bioanalytical SFC/MS [68,69]

Kalinoski et al. provided one of the earliest reports of the characterization of high molecular weight, biologically active compounds using SFC/chemical ionization-MS. [70] In work specifically related to the analysis of peptides, these authors analyzed a cyclic undecapeptide, Cyclosporin A, using a packed microbore C-18 column using methanol-modified CO_2 mobile phase or using a fused-silica capillary column with isopropanol as modifier. Cyclosporin A was also purified from a mycelial extract via preparative SFC [71]. Use of far less organic solvent in SFC than in LC provided a dramatic economic advantage in this preparative scale SFC work. It should be noted that cyclosporin A is a hydrophobic peptide. Bolanos et al. [72] later reported the separation of three forms of hydrophobic gramicidin (B, C, and A) by both SFC/ UV and SFC/ESI-MS with a modifier of methanol containing either 2% water, 0.01 M acetic acid (AA), and 0.4% isopropylamine or 0.5% trifluoroethanol on a Phenomenex Luna Cyano column. However, under these conditions, more hydrophilic peptides, such as angiotensin II, did not provide good peak shapes. The mobile phase composition was complex, and not very user friendly for positive-ion mode mass spectrometry when isopropylamine was used as the additive. Blackwell et al. [73] studied the elution of a group of polypeptides

containing 4-9 amino acid units on a divinylbenzene polymeric column with a modifier of either ethanol, or 2-methoxyethanol, containing 50 mM of either methanesulfonic acid, trifluoromethanesulfonic acid, or heptadecafluorooctane-sulfonic acid. The authors claimed that the acidity of the additive was critical and that an acid stronger than trifluoroacidic acid would be required for elution of these peptides. Bradykinin, which contained the largest number of amino acid residues (two of which were basic arginine groups), was retained the longest and gave the broadest peak among the chosen peptides using the same chromatographic conditions.

The goal of our work was to develop supercritical fluid chromatographic methods which could elute a wide variety of polypeptides, both larger than those eluted in previous reports, and containing a wider variety of hydrophilic, basic, and acidic amino acid residues. Furthermore, it was important that the mobile phase be simple and compatible with mass spectrometric detection. Our ultimate goal is to bring the advantages of SFC/MS to the characterization of all peptides.

5.2 EXPERIMENTAL

5.2.1 Chemicals

Methanol was HPLC grade, (Sigma-Aldrich, Milwaukee, WI USA). The carbon dioxide was SFC grade (Matheson Tri-Gas, Morrow, GA USA) with no helium head pressure. Ammonium acetate (AA) (97+%, ACS grade) and ammonium trifluoroacetate (ATFA) were obtained from Sigma-Aldrich. Formic acid (FA) (96+%, ACS grade) was

obtained from Alfa Aesar (Ward Hill, MA USA). Trifluoroacetic acid (TFA) was purchased from Perbio (Rockford, IL USA).

Pro-Leu-Gly amide, leupeptin hydrochloride, methionine enkephalin, bradykinin, bradykinin fragment 1-8, bradykinin fragment 2-9, lys-bradykinin, angiotensin I, human (acetate salt), angiotensin II, human (acetate salt), and [Val^4] angiotensin III[‡], human (acetate salt) were purchased from Sigma-Aldrich. Sauvagine and urotensin II, human (hydrochloride salt) were purchased from AnaSpec, Inc. (San Jose, CA USA). **Figure 34** shows the sequences and monoisotopic mass of each peptide. A solution of each sample was prepared in methanol at a concentration of approximately 0.5 mg/mL, unless specified otherwise.

5.2.2 SFC-UV Instrumentation

The analytical SFC system was from Berger Instruments (Newark, DE USA) with an Agilent (Palo Alto, CA USA) Model 1100 Diode Array Detector (DAD), which employed a 1.7-μL high pressure flow cell (6-mm path length) and SFC Pronto software. UV detection was at 220 nm.

The chromatographic columns were Supelcosil LC-SAX1 (Supelco, Bellefonte, PA USA), SFC Ethylpyridine-bonded silica, and SFC Amino-bonded silica (Princeton Chromatography Inc., Cranbury, NJ USA). The Ethylpyridine and Amino columns were not end-capped. The column dimensions were 25 cm in length and 4.6-mm i.d., with a particle size of 5 μm for each stationary phase. The Supelcosil LC-SAX1 column had a pore size of 120 Å; while both Princeton columns had a pore size of 60 Å.

[‡] [Val^4] Angiotensin III will be referred to as angiotensin III in future text.

Pro-Leu-Gly amide	HPro-Leu-GlyNH2 M.M. = 284.2 Da
Leupeptin hydrochloride	Acetyl-Leu-Leu-Arg-al•HCl M.M. = 426.3 Da
Methionine Enkephalin	HTyr-Gly-Gly-Phe-MetOH M.M. = 573.2 Da
Bradykinin	HArg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-ArgOH M.M. = 1059.6 Da
Bradykinin fragment 1-8	HArg-Pro-Pro-Gly-Phe-Ser-Pro-PheOH M.M. = 903.5 Da
Bradykinin fragment 2-9	HPro-Pro-Gly-Phe-Ser-Pro-Phe-ArgOH M.M. = 903.5 Da
Lys-Bradykinin	HLys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-ArgOH M.M. = 1187.7 Da
Angiotensin I, human (acetate salt)	HAsp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-LeuOH M.M. =1295.7 Da
Angiotensin II, human (acetate salt)	HAsp-Arg-Val-Tyr-Ile-His-Pro-PheOH M.M. = 1045.5 Da
[Val^4] Angiotensin III, human (acetate salt)	*H*Arg-Val-Tyr-Val-His-Pro-PheOH M.M. = 916.5 Da
Sauvagine	PyrGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH2 M.M. = 4599.4 Da
Urotensin II, human (hydrochloride salt)	H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH•HCl M.M. = 1388.6 Da

Figure 34. Sequence and monoisotopic molecular mass of each probe peptide.

Unless otherwise specified, chromatographic conditions were: injection volume 10 μL, mobile phase flow rate 2 mL/min (measured in the liquid state), column outlet pressure 120 bar, and column oven temperature 40°C. The mobile phase composition gradient was: 5% modifier in CO_2 for 1 minute after injection, modifier concentration increased to 50% at 5% per minute, and held for 5 minutes at 50%. The modifier consisted of either pure methanol or methanol with 5 mM or 13 mM additive.

Between each change of mobile phase additive, the stationary phase was washed with pure methanol at 1 mL/min for sixty minutes (about 20 column volumes) in order to either purge previous additive solution from the column or to remove analyte that was strongly retained on the stationary phase. After this time period, the next additive solution was introduced to the SFC system, and the column was equilibrated again for 30 minutes prior to injection.

5.2.3 SFC/MS instrumentation

A zero-dead-volume chromatographic tee was installed immediately after the analytical column and before the inlet to the UV-DAD detector. A 15-cm long, 50-μm i.d. Peaksil tube (Upchurch Scientific, Oak Harbor, WA USA) was installed in the tee and used as a flow splitter before the UV-DAD detector. The tube maintained the pressure at the end of the column and it directed approximately 1/10 of the flow to the TurboIonSpray source of a PE Sciex API-165 Single Quadrupole Mass Spectrometer (Thornhill, Ontario, Canada). The remainder of the effluent was directed to the UV-DAD detector. The mass spectrometer was controlled by LC2Tune version 1.4 acquisition software (Applied Biosystems, Foster City, CA). The TurboIonSpray source was

operated with a Turbo gas flow of 8 L/min at 400 ºC. The nitrogen gas supply for the TurboIonSpray nebulizer and curtain gases was set at 50 psi, with Control Panel settings of 10 and 7 arbitrary units, respectively. The electrospray capillary, orifice, and ring voltages were held at +4000 V, +35 V, and +240 V, respectively. The electron multiplier was held at 2700 V. Scan rage was different for each analyte, and is indicated in the appropriate Figure caption. In most cases, Q1 scans were acquired using a 0.2 u step value, a 0.5 ms dwell time, and a 5 ms interscan delay.

5.3 RESULTS AND DISCUSSION

5.3.1 Separation of Simple Peptides

Four peptides that have amino acid residues within the range of 3-9 mers were investigated first. Mixture 1 was composed of methionine enkephalin and bradykinin. Mixture 2 was composed of Pro-Leu-Gly amide and leupeptin hydrochloride. **Figure 35** shows the SFC-UV traces for the separation of these two mixtures with pure methanol, with 5-mM TFA in methanol, and with 5-mM ammonium acetate in methanol on an Ethylpyridine column. The modifier gradient was: 5% modifier in CO_2 for 1 minute after injection, modifier concentration increased to 50% at 5% per minute, and held for 5 minutes at 50%. Both mixtures were baseline separated with pure methanol as the mobile phase modifier. When either TFA or ammonium acetate was used as an additive, Pro-Leu-Gly amide and leupeptin co-eluted. Changing the modifier (5 mM TFA in methanol) gradient to a 5% hold for 1 min. followed by a 2%/min. increase to 30%, followed by a 5-minute hold, improved chromatographic resolution between this pair, as shown in **Figure**

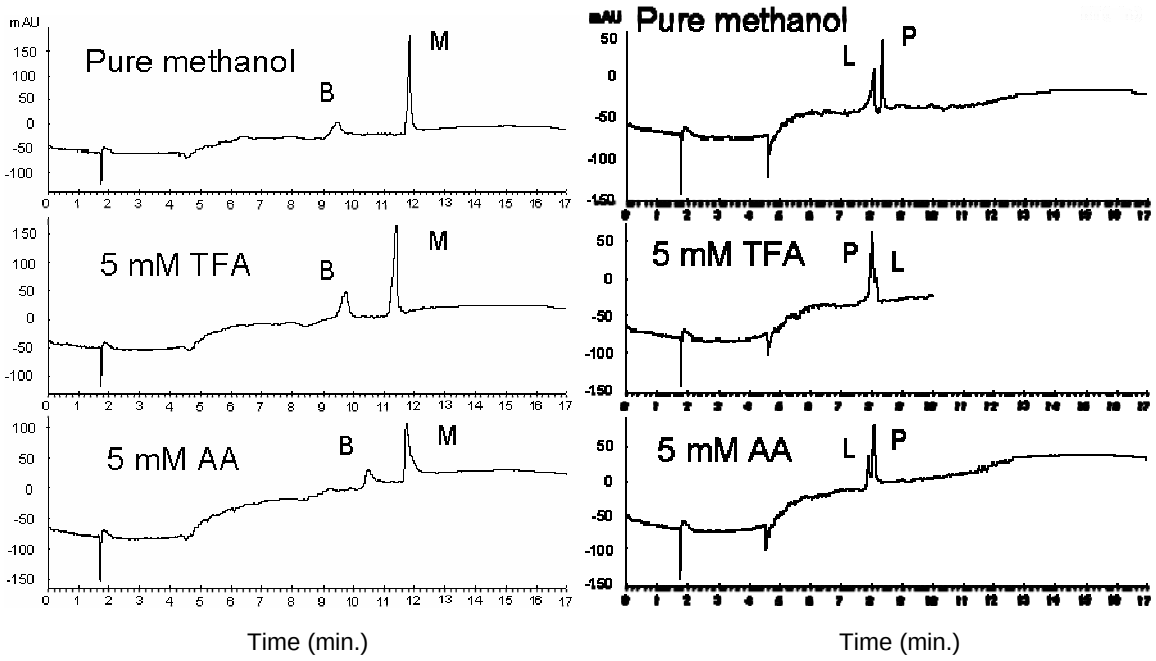


Figure 35. SFC-UV separation of simple peptide mixtures Key: (B) bradykinin; (M) methionine enkephalin; (P) Pro-Leu-Gly amide; (L) leupeptin. See section 5.2 for chromatographic conditions.

36. Selected ion chromatograms are also shown for Pro-Leu-Gly amide and leupeptin under the original modifier gradient. Fronting peak shapes are clearly demonstrated in both mass chromatograms. Note that "leupeptin hydrochloride" likely dissociated during the separation. The component ions, therefore, are believed to have associated with other counter ions available in abundance in the mobile phase. Consequently, we have used the term "leupeptin" rather than "leupeptin hydrochloride" in this report.

An interesting observation was the change in peak shape of bradykinin, methionine enkephalin, and leupeptin with changing mobile phase composition. The bradykinin and methionine enkephalin peaks fronted with 5 mM TFA/MeOH as modifier, but tailed with pure methanol or with 5 mM ammonium acetate/ MeOH as modifier. On the other hand, leupeptin gave fronting peak shapes with both pure methanol and 5 mM TFA/MeOH (as the mass chromatograms showed in **Figure 36**), but a tailing peak with 5 mM AA/MeOH. These observations were not apparent with Pro-Leu-Gly amide. Symmetric peak shapes were obtained with all three modifiers. This is probably because Pro-Leu-Gly amide is a small (3 residues), relatively hydrophobic peptide. Even with pure methanol, it eluted with good peak shape.

In our previous study concerning the elution of quaternary ammonium salt analytes [74] we proposed that there might be at least three retention mechanisms operating in conjunction with the Ethylpyridine phase: (1) the nitrogen on the pyridine functional group attached to the stationary phase might form hydrogen bonds with silanol sites and provide a less active phase; (2) the pyridine aromatic ring might sterically hinder access to active sites on the stationary phase, as illustrated in **Figure 37A**; and (3) a fraction of the pyridine functional group might be protonated by the $MeOH/CO_2$ mobile

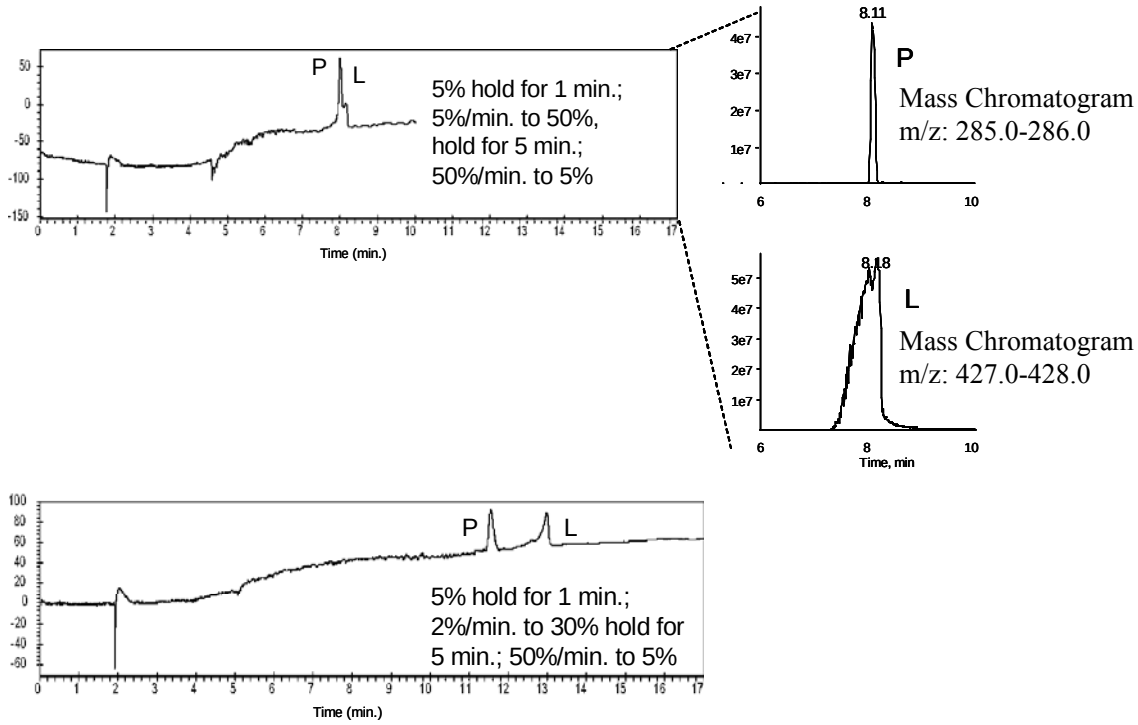


Figure 36. Separations of mixture of Pro-Leu-Gly amide and leupeptin hydrochloride using two different modifier gradients with UV and mass spectrometric detector of (L) leupeptine and (P) Pro-Leu-Gly amide corresponding to the upper separation. Scan range *m/z*: 250-450. See section 5.2 for chromatographic conditions.

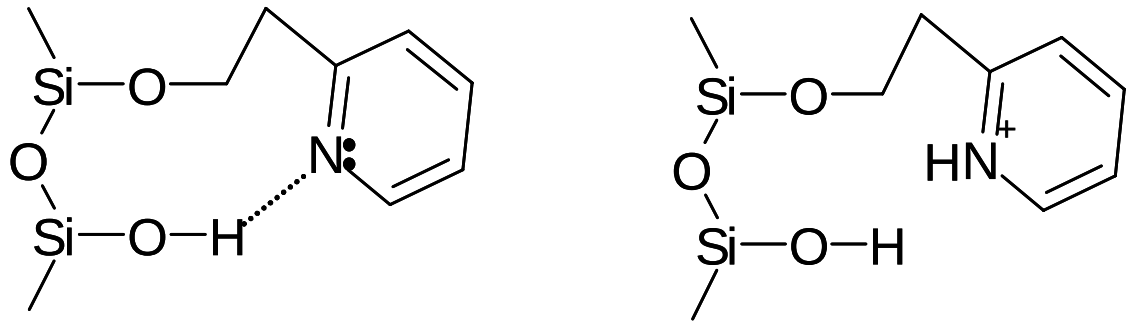


Figure 37. Illustration of proposed interactions on Ethylpyridine stationary phase.

phase, as shown in **Figure 37B**. If the analyte carried a positive charge, for example, there would tend to be less interaction between the analyte and a protonated pyridine bound to the solid support. This lack of interaction between the analyte and stationary phase would result in fronting, "overloaded" peaks. This could possibly explain why leupeptin provided fronting peak shapes with pure methanol and with 5 mM TFA/MeOH as modifier. In the acidic environment (5 mM TFA/MeOH), bradykinin and methionine enkephalin could also be protonated. They were also eluted with a fronting peak shape. On the other hand, ammonium acetate as an additive would not result in protonation of the peptides. On the other hand, ammonium acetate ions would likely interact with the active sites on the solid support, as we have discussed in a previous research report [75], as well as with the peptides by forming ion pairs, thus allowing more interaction between the stationary phase and the analytes. This might be the reason that no fronting peak shapes were observed with ammonium acetate as additive.

5.3.2 Separation of Basic Polypeptides

Bradykinin, bradykinin fragment 1-8, bradykinin fragment 2-9, and lys-bradykinin contain one or more basic amino acid residues, such as lysine and arginine. These four basic bradykinins were studied with the Ethylpyridine column using pure methanol, 5 mM TFA/MeOH, 13 mM TFA/MeOH, 13 mM AA/MeOH, and 13 mM formic acid/MeOH as modifier. **Table 5** shows the retention times and the peak widths at half height after UV detection at 220 nm. Bradykinin appeared to not elute with 13 mM AA/MeOH and 13 mM FA/MeOH. As it was detected by ESI-MS, we believe that the signal was under the limit of detection (LOD) of UV detection. For the same reason, lys-bradykinin was only detected with UV absorbance detection when 13 mM TFA/MeOH

Table 5. Average retention time (min.) and the $W_{1/2h}$ (min.) in parenthesis of the four Bradykinin peptides with different modifier composition. ND: not detected. UV detection at 220 nm (n=2)

Sample Solvent	Bradykinin	Bradykinin F 1-8	Bradykinin F 2-9	Lys-bradykinin
Pure methanol	9.17 (0.49)	9.13 (0.53)	9.58 (0.39)	ND
5 mM TFA/methanol	9.31 (0.36)	9.22 (0.45)	9.56 (0.28)	ND
13 mM TFA/methanol	9.63 (0.23)	9.76 (0.26)	9.68 (0.23)	10.13 (0.14)
13 mM AA/methanol	ND	9.73 (0.31)	9.80 (0.13)	ND
13 mM FA/methanol	ND	9.07 (0.60)	9.35 (0.70)	ND

was used as modifier. These observations indicated that the bradykinin and lys-bradykinin were strongly retained with the other mobile phase compositions. Brakykinin fragment 2-9 was eluted with the sharpest peak shape in most of the cases.

Figure 38 shows SFC/MS mass chromatograms for the elution of bradykinin, its fragment 1-8, and 2-9 from the Ethylpyridine column with pure methanol (**Figure 38A**), 5 mM TFA/MeOH (**Figure 38B**), and 13 mM TFA/MeOH (**Figure 38C**) as modifier. The peak shapes of the bradykinins became sharper and displayed more fronting asymmetry with increasing concentration of TFA.

Bradykinin has two basic amino acid residues, two arginines. It exhibited tailing peak shape with pure methanol containing 5 mM TFA and fronting peak shape with methanol containing 13 mM TFA. Both bradykinin fragment 1-8 and bradykinin fragment 2-9 contain a single arginine. They were less strongly retained by the stationary phase, and exhibited more symmetric peak shapes, than bradykinin, even with pure methanol.

Even though fragment 1-8 and fragment 2-9 are structural isomers, they displayed different retention behavior. Fragment 2-9 gave a sharper peak than the other three peptides with pure methanol and with 5 mM TFA in methanol as modifier (**Table 5**). This indicated that the terminal amino acid group played a role in the retention mechanism. Proline, containing a secondary α-amino group, is on the amino end of fragment 2-9. The secondary amino group is less basic than a primary group, therefore, the interaction between proline's amino group and the column's silanol groups should be weaker. When a more concentrated TFA/methanol solution was used, there was not much difference in peak width among the four peptides.

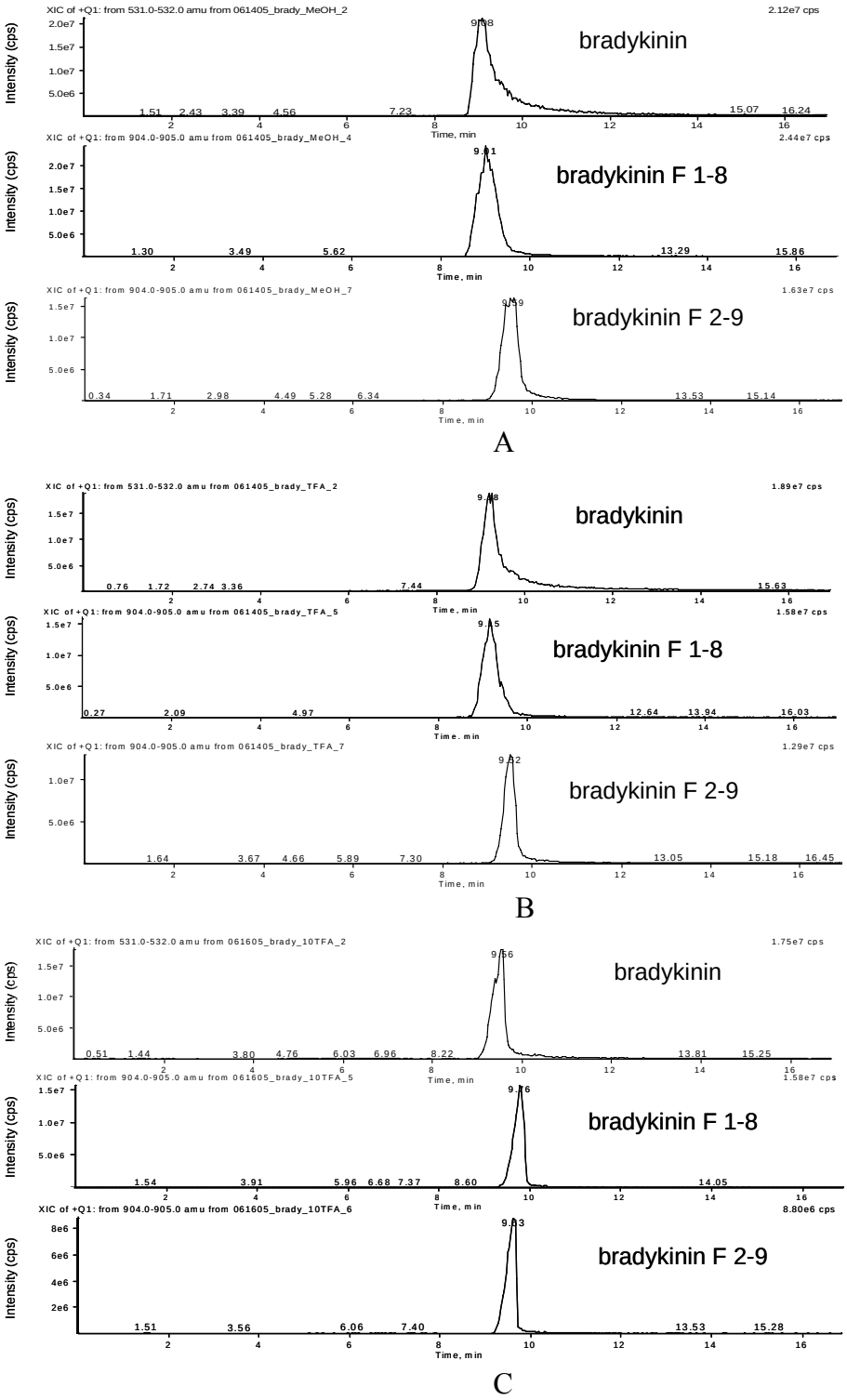


Figure 38. SFC/MS mass chromatograms for the elution of bradykinin, its fragment 1-8, and fragment 2-9 from the Ethylpyridine column. (A) pure methanol as modifier; (B) 5-mM TFA in methanol as modifier; (C) 13-mM TFA in methanol as modifier. Scan range *m/z*: 400-1220. See section 5.2 for chromatographic conditions.

Mass chromatograms of doubly charged lys-bradykinin with various mobile phase compositions are shown in **Figure 39**. The addition of TFA as additive improved the peak shapes of the peptide. Again, the sharpest peak shape was obtained at the highest concentration of TFA (13 mM). The other two additives, 13 mM AA in methanol and 13 mM formic acid in methanol, provided longer retention time and strongly tailing peak shapes for lys-bradykinin, compared to 13 mM TFA in methanol as modifier. These two additives were also studied for bradykinin and its two fragments. AA and FA gave poor peak shapes and long retention time for bradykinin. On the other hand, acceptable, yet tailing peak shapes were achieved for the two fragments with 13 mM AA and FA.

Among the four bradykinins, lys-bradykinin was retained the longest with 13 mM TFA. This is reasonable because it has the largest number of basic residues: one lysine and two arginines. Without the presence of mobile phase additive, these basic amino acid residues would tend to interact strongly with active silanol groups on the solid support, resulting in strong retention. With the presence of 13 mM TFA as additive, a sharper and slightly fronted peak shape was observed compared with results seen with a lower concentration (5 mM) or with other additives, such as ammonium acetate and formic acid. This observation suggested that the acidity of the SFC mobile phase is crucial in obtaining good peak shapes.

Again, fronting peak shapes were observed with 13 mM TFA in methanol, but not with pure methanol or other modifiers. This also provided evidence to support our hypothesis that TFA might protonate the four peptides, as well as some fraction of the pyridine functional groups on the stationary phase. Repulsion between any protonated pyridine groups on the stationary phase and the protonated analyte would reduce

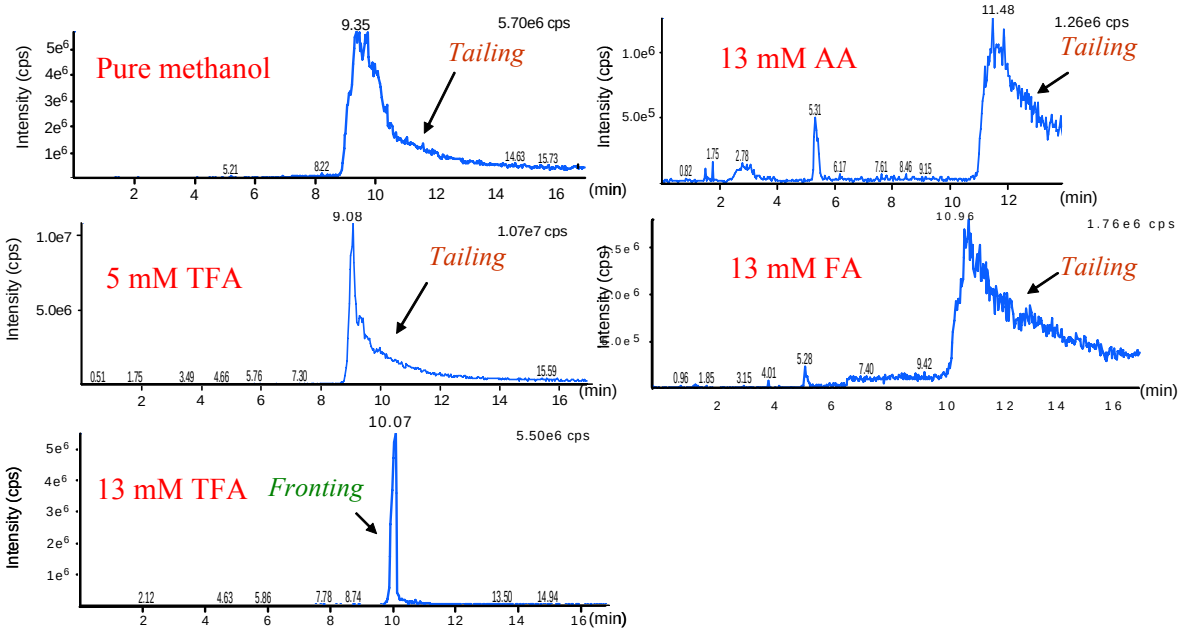


Figure 39. Mass chromatograms of lys-bradykinin on the Ethylpyridine column. Scan range *m/z*: 400-1220. Mass chromatogram selected at *m/z* 594.5-595.5. See section 5.2 for chromatographic conditions.

retention, and result in fronting peak shapes. Without TFA, unwanted interactions between the basic groups of the peptides and the stationary-phase silanol groups came into play and caused peak tailing.

Note that the SFC/MS peak intensity with 13-mM TFA was lower than with 5-mM TFA, even though a sharper peak shape was obtained in the former case. This indicated that higher concentrations of TFA result in ionization suppression of lys-bradykinin. This is a well-known phenomenon in ESI mass spectrometry [76].

A preliminary limit-of-detection (LOD) study with lys-bradykinin was performed. **Figure 40** shows the mass chromatograms for different concentrations of lys-bradykinin injected on column. Initially, the study was conducted in full-scan mode. The scan range was from mass-to-charge ratio (m/z) 500 to 1300 with 0.2-u step size, 0.5-ms dwell time, and 5-ms pause time between each scan. The time required for each scan under these conditions was 2.01 s. Peaks became narrower as lower concentrations were injected, but the full-scan acquisition rate was too low to adequately capture the peak shape. Therefore, we changed from full-scan to single ion monitoring (SIM) mode. We monitored two m/z ranges during SIM operation: m/z 594.8-595.2 and m/z 1188.6-1189.0, with a step size of 0.1 u, a dwell time of 20.0 ms, and a 5-ms pause time. Under these SIM conditions, the scan cycle time was 0.21 s, which provided far better definition of peak shape than in the full-scan mode. The extracted mass chromatograms displayed in **Figure 40** for injection of 0.5 µg/µL and 0.1 µg/µL were obtained under full-scan mode. Injections of 0.05 µg/µL and 0.01 µg/µL were in the SIM mode. Sharper peak shapes at the lower concentrations suggested that overload of the column was achieved for injection at the highest concentration. We estimated the LOD in full-scan mode to be

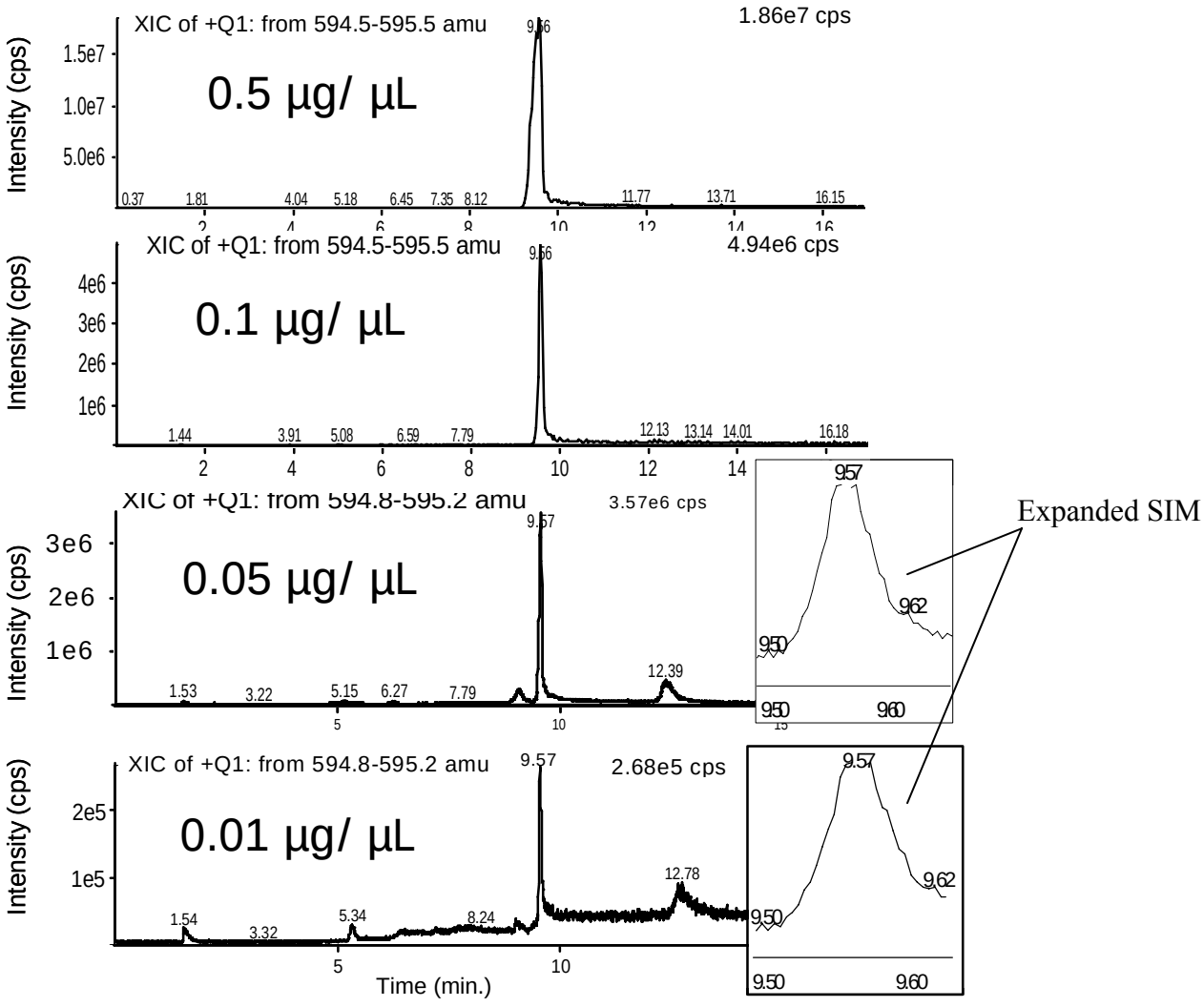


Figure 40. Mass chromatograms of lys-bradykinin at different concentrations injected on column. See section 5.2 for chromatographic conditions.

approximately 0.01-µg/µL (for a signal-to-noise ratio (S/N) of ~10). This corresponds to 100 ng for a 10-µL injection volume with about 10 ng going to the TurboIonSpray source of mass spectrometer. In SIM mode, the LOD was estimated to be 50 ng of lys-bradykinin. Note that we used the mass chromatogram of the doubly-charged ion to estimate these values. Our results again show 13-mM TFA in methanol suppresses the ionization of the analyte. This is not surprising given previous results with electrospray ionization [76]. However, the improved peak shape with 13-mM TFA in methanol compensated for the ionization suppression, and still provided the best results.

Some of our earlier results with quaternary amine salts suggest some similarity between the propylamino and strong anion exchange (SAX) columns and the Ethylpyridine column [74]. Therefore, we tried to elute the bradykinin analogues from these two columns under the same conditions. However, none of the bradykinins eluted from either phase, even with 13-mM TFA in methanol, under the same chromatographic conditions as described previously. This might be explained by the hypothesized deactivation of active silanol sites by the pyridine functional groups on the Ethylpyridine stationary phase.

5.3.3 Separation of Larger Polypeptides

A group of larger polypeptides, with up to 40 amino acid residues, was also studied. The probe peptides were angiotensin I, human (acetate salt), angiotensin II, human (acetate salt), angiotensin III, human (acetate salt), sauvagine, and urotensin II, human (hydrochloride salt) (sequences shown in **Figure 34**). Angiotensin I and angiotensin II do not dissolve in methanol. Angiotensin I, however, does dissolve if 10

μL of either acetic acid or TFA is added to 8 mL of a 0.5-mg/mL methanol solution. Angiotensin II, human was dissolved by adding 10 μL of TFA to 8 mL of a 0.5-mg/mL methanol suspension. No problems were encountered in dissolving the other three peptides in methanol.

Sample solutions of angiotensin III, angiotensin III plus acetic acid, angiotensin I plus acetic acid, angiotensin I plus TFA, angiotensin II plus TFA, urotensin II and sauvagine were prepared. The acid concentration in the sample solutions was approximately 0.1% (v/v) where applicable. **Figure 41** shows mass chromatograms of the above five polypeptides with various mobile phase modifiers. Adding acetic acid to the sample solution did not significantly affect the elution of angiotensin III. Although not shown in the Figure, there were no significant differences between the chromatograms of solutions of angiotensin I containing either acetic acid or TFA. Therefore, while addition of the acids helped dissolve some of the larger peptides, it had little effect on the chromatography. All three angiotensins gave the sharpest peak shapes with 13 mM TFA in methanol as modifier. As angiotensin I and II required 0.1% TFA in methanol for dissolution, it was not surprising that this level of TFA would be required in the mobile phase for good chromatographic behavior. With 13 mM ATFA as additive, better peak shapes were obtained compared to 5 mM TFA in methanol. But 13 mM TFA in methanol modifier still provided the best peak shape.

Urotensin II eluted the fastest and provided the best peak shape with 13 mM TFA in methanol as modifier. 5 mM TFA in methanol modifier provided better peak shape than pure methanol. With the salt of TFA, ATFA, the peptide was retained strongly.

Sauvagine did not elute from the Ethylpyridine column with either pure methanol

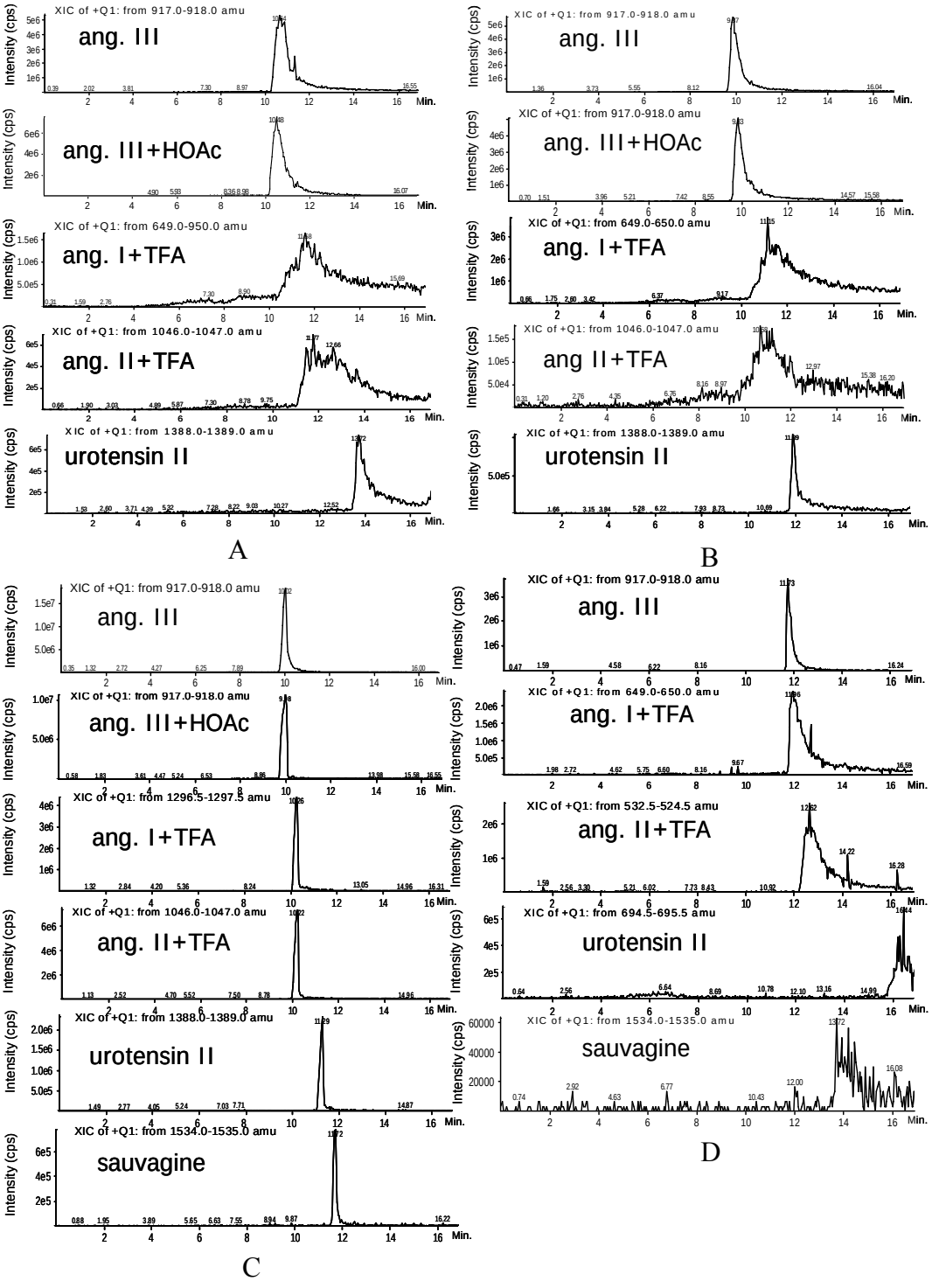


Figure 41. SFC/MS of larger polypeptides with (A) pure methanol; (B) 5 mM TFA/Methanol; (C) 13 mM TFA/Methanol; and (D) 13 mM ammonium trifluoroacetate (ATFA)/Methanol as mobile phase modifier. Scan range *m/z*: 400-1330 for angiotensins, 400-1420 for urotensins, and 500-2350 for sauvagine. See section 5.2 for chromatographic conditions.

or 5-mM TFA in methanol as modifier. We were not surprised by these results due to the fact that it contains 40 residues, having a molecular mass of over 4500 Da, including four basic amino acid groups and seven acidic amino acid groups. Only when 13-mM (~0.1%) TFA in methanol was used as modifier, did sauvagine elute with sharp, symmetrical peak shape. Sauvagine did elute with 13 mM ATFA in methanol as modifier, but it exhibited broad, tailing peak shape.

Thirteen millimolar ammonium trifluoroacetate (ATFA) provided better peak shapes in some cases, but did not provide the good chromatographic behavior provided by 13 mM TFA additive. This indicated that ion-pair formation was not the only player here. The acidity of TFA was also important in the elution of these polypeptides.

Fronting peak shapes were generated with 13 mM TFA/MeOH, but not with less acidic or salt additives. These observations strengthen our hypothesis that suppressing the dissociation of carboxylic acid groups of the peptides and protonating the amino groups on the basic amino acid residues is necessary to elute the polypeptides with good peak shape.

5.3.4 Synthetic Polypeptides

Five synthetic polypeptides were also studied via the SFC/MS method. The monoisotopic mass, the number of residues, and the number of acidic and basic residues are listed in **Table 6**. All five of them have more than 30 residues, molecular mass of more than 3000 Da, and have a variety of acidic and basic amino acid residues. Due to the large size and the high polarity of these peptides, they were strongly retained on the 250×4.6-mm i.d., 5-µm Ethylpyridine column. A shorter, 5-cm-long column was

Table 6. Structural information of the synthetic peptides.

	Monoisotopic Mass (Da)	Number of residues	Number of basic residues	Number of acidic residues
Peptide A	4577.6	40	7	5
Peptide B	3166.8	30	3	4
Peptide C	4047.1	34	6	5
Peptide D	4576.6	40	7	5
Peptide E	4161.2	36	5	3

therefore used to enable a greater number of chromatographic conditions to be studied.

The on-line mass spectra of peptide A and peptide B are shown in **Figure 42**, with 13 mM TFA/methanol as modifier. Ions carrying four and three charges are the most intense peaks in the spectra, respectively. The mass chromatograms of the +4 (m/z 1146) and +3 (m/z 1057) ions for peptide A and B, respectively, are shown in **Figure 43**. Modifiers of pure methanol, 5 mM TFA/methanol, and 13 mM TFA/methanol were used. Again, the fastest elution and the sharpest peak shape were achieved with 13 mM TFA/methanol. The peaks exhibited fronting at the higher concentration of TFA, which is not a surprise based upon our previous results. Nevertheless, these data are impressive when one realizes that the peptides have molecular masses ranging to over 4500 Da and a CO_2-based mobile phase is being employed.

The on-line mass spectra of peptide C and peptide D are shown in **Figure 44**, with 5 mM TFA/methanol as the modifier. Mass chromatograms of the peptides (m/z 1013 for C and m/z 916 for D) using the 5-cm Ethylpyridine column, with pure methanol or 5 mM TFA/methanol as modifier are shown in **Figure 45**. The peptides were retained strongly, with broad peak shapes when pure methanol was used, but eluted with nice peak shapes with 5 mM TFA/methanol. When the TFA concentration in the modifier was increased beyond 5 mM, no significant improvement in chromatographic behavior was observed for these peptides.

Peptide E was first studied by RP-HPLC/UV and LC/MS. Some impurity peaks were observed to co-elute with the major peptide. A monolithic C18 column (100×4.6 mm i.d.) provided the best resolution the researchers (see Acknowledgements) could obtain between peptide E and its oxidation products, as shown in **Figure 46**. LC/MS

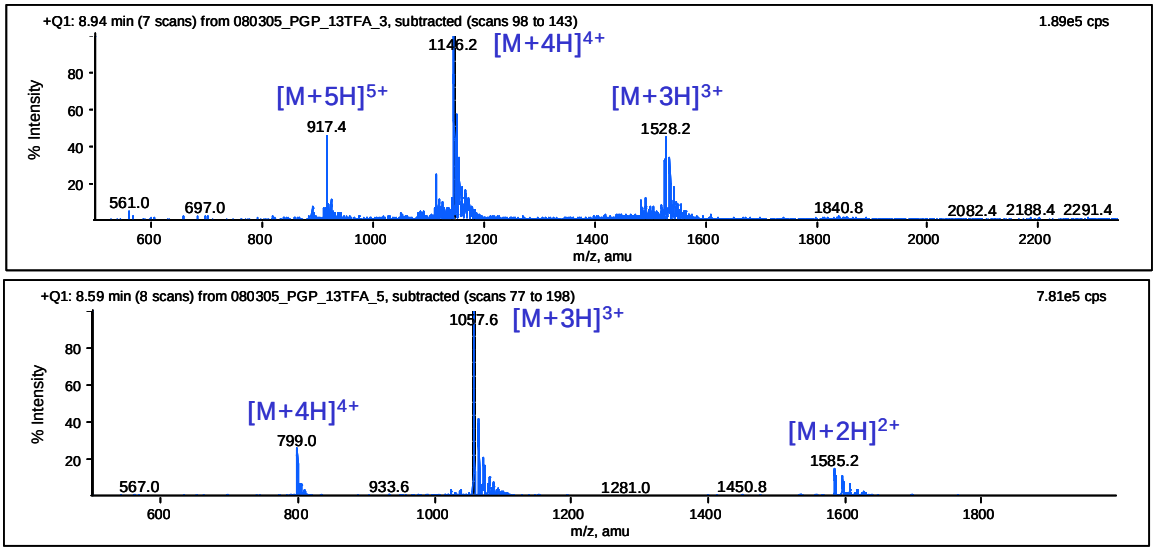


Figure 42. Mass spectra of peptide A and peptide B with scan range from *m/z* 500-2300 for peptide A and *m/z* 500-2000 for peptide B. Modifier: 13-mM TFA/methanol.

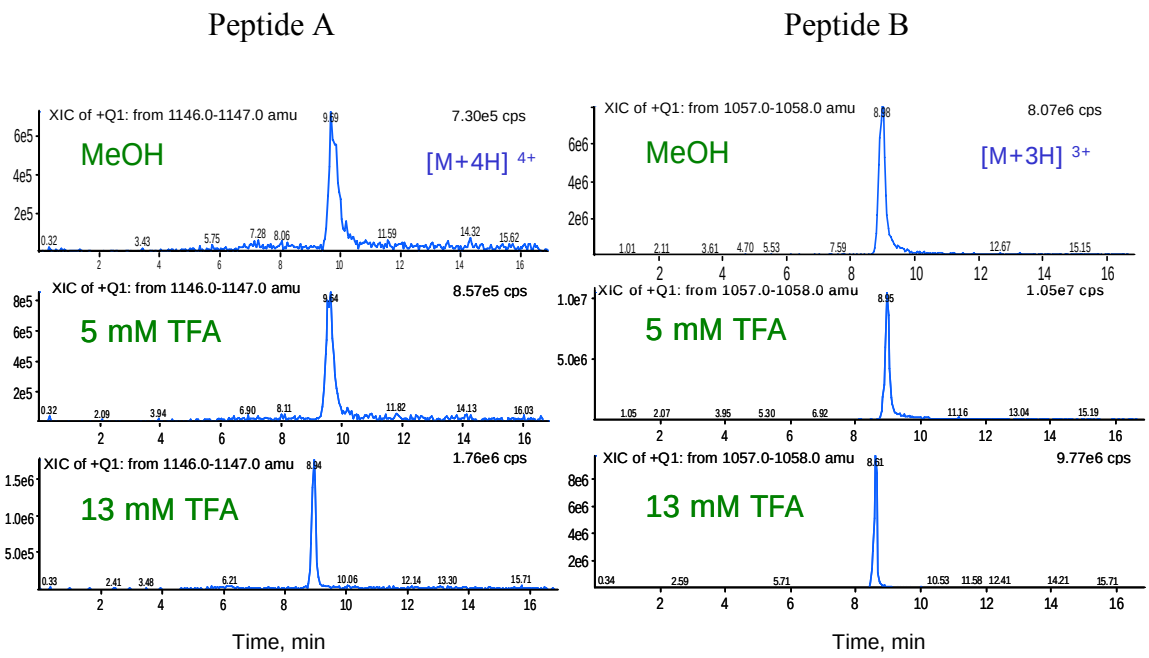


Figure 43. Mass chromatograms of peptide A and peptide B with modifier variation with 5 cm Ethylpyridine column. See section 2 for other chromatographic conditions.

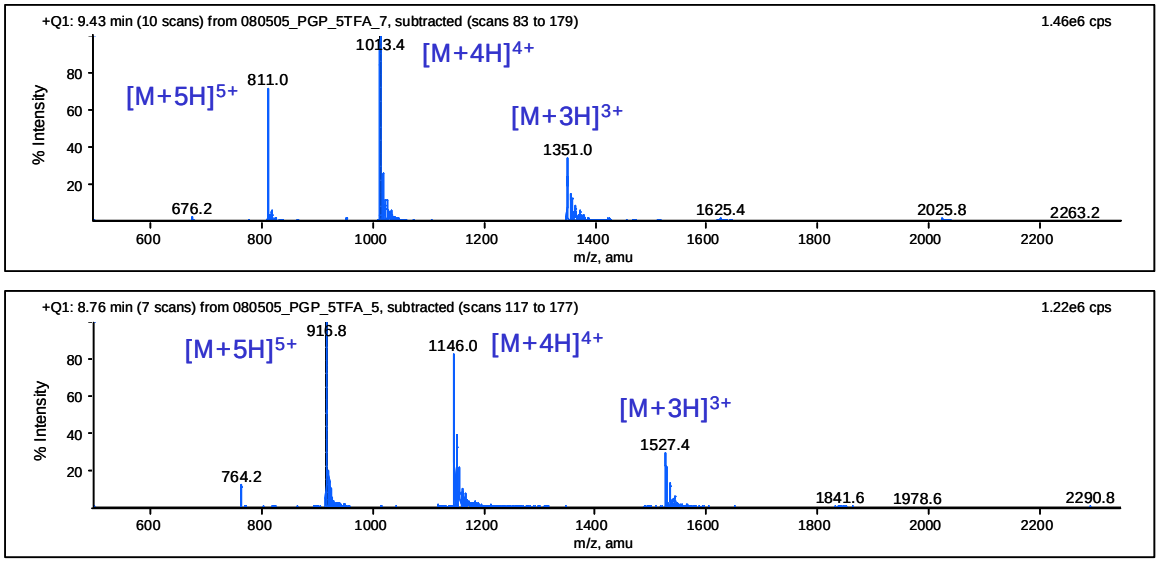


Figure 44. Mass spectra of peptide C and peptide D with scan range from *m/z* 500-2300. Modifier: 5 mM TFA/methanol.

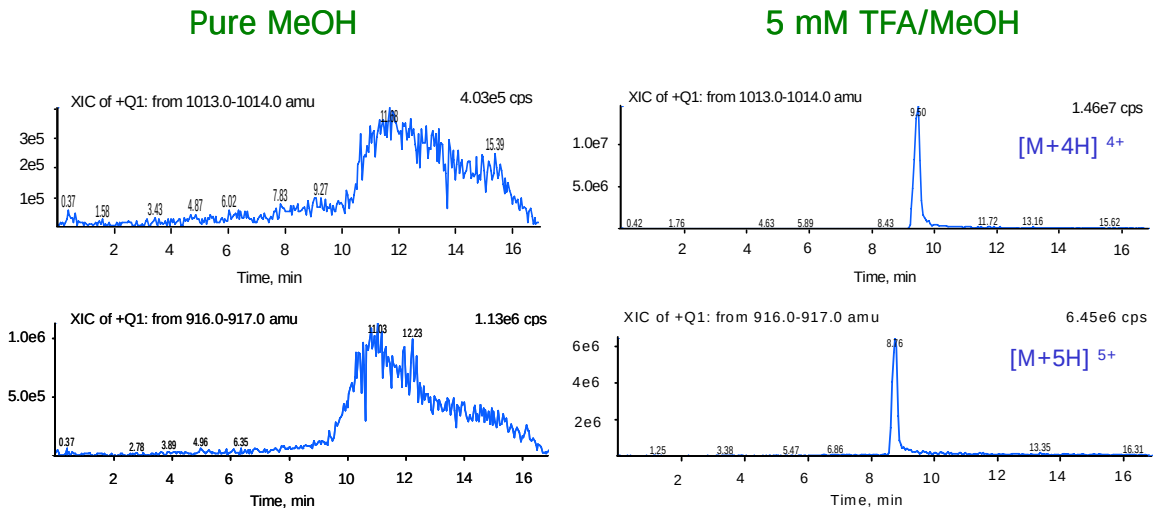


Figure 45: Mass chromatograms of peptide C and peptide D with modifier variation with 5 cm Ethlpyridine column. See section 5.2 for other chromatographic conditions.

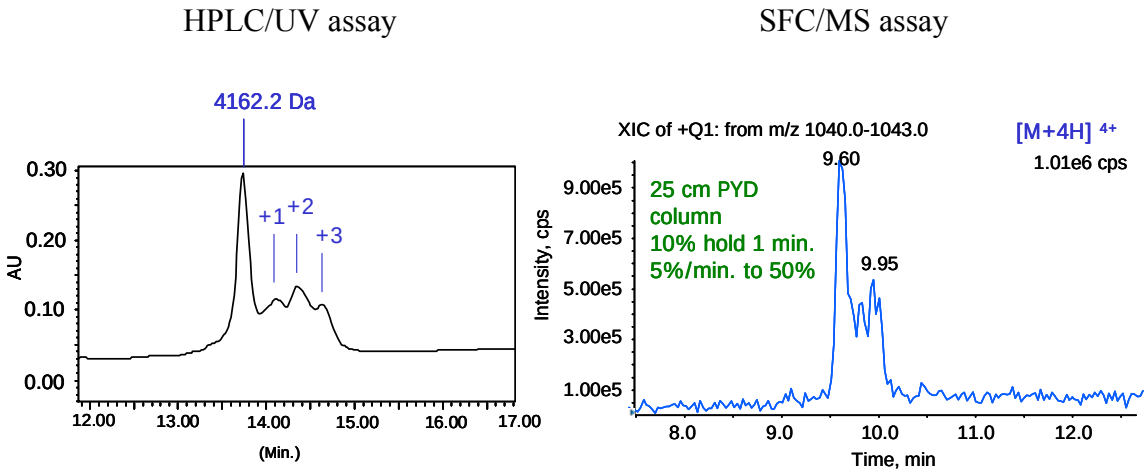


Figure 46. HPLC/UV and SFC/MS of peptide E and its deamination products (Oven temperature was 50 ºC for 25-cm Ethylpyridine column) HPLC-UV conditions: A = 95% water, 5% ACN with 0.1% TFA; B = 5 % water, 95% ACN with 0.1%TFA ; 75% A →50% A in 30 min, 31-36 min 75%A; Phosphate buffer pH 7; sample concentration: 0.45 mg/mL; injection volume: 20 µl; flow rate: 1 mL/min; UV detection wavelength: 210 nm. SFC-MS scan range *m/z*: 800-1500.

substantiated that these impurity peaks contained species with one, two, and three mass units more than the target peptide. This could be explained by the deamination of asparagines or glutamines as the target peptide contains two asparagines and three glutamines. It is not clear at this point which residues might be de-aminated.

We decided to see whether our SFC/MS system might provide a better separation of these closely-related impurities from the main target peptide. Using the 5-cm Ethylpyridine column, the reconstructed-total-ion-current chromatogram simply showed a shoulder after the major peak. Then we decided to use a longer column (25 cm) and, by optimizing the modifier gradient and the oven temperature, we achieved a separation similar to that provided by HPLC. Three impurity peaks were clearly distinguished from the target peptide. With SFC, the separation was achieved in a shorter analysis time than with HPLC (**Figure 46**). The use of long, coupled columns and high flow rates in SFC is a practical approach to achieve chromatographic resolution of tough-to-separate mixtures [77]. We believed this would be a reasonable approach to achieve better resolution of peptides mixtures.

5.4 CONCLUSIONS

Using relatively simple and mass-spectrometry-compatible mobile phases, we were able to extend SFC to the elution of polypeptides. The polypeptides were up to 40 residues in length, and contained a variety of acidic and basic residues. We used trifluoroacetic acid, a relatively strong acid, as additive in the CO_2/methanol mobile phase to suppress ionization of the peptides' carboxylic acid groups and to protonate the

peptides' amino groups. The TFA likely protonates some fraction of the pyridine functional groups on the Ethylpyridine stationary phase. We observed fronting peak shapes and lower retention when higher concentrations of TFA were used. This behavior supports our hypothesis that electrostatic repulsion between the protonated peptides and some fraction of the stationary-phase functional groups is occurring.

We investigated three silica-based stationary phases: a strong anion exchange, an Amino, and an Ethylpyridine stationary phase. The Ethylpyridine was the only one from which we were able to successfully elute the peptides. This was probably due to deactivation of the active silanol groups (as the stationary phase was not end capped) by hydrogen bonding between the pyridine functional groups on the stationary phase and the silanols, as well limited access to the silica surface due to steric hindrance caused by the pyridine aromatic ring.

We believe that SFC and SFC/MS may hold great promise for the characterization of peptide mixtures. Peptide separations are of great economic, human, and environmental importance in the pharmaceutical and other industries. SFC/MS may allow faster determinations of targeted "biologics" (i.e., peptide-based pharmaceuticals) in physiological fluids. Protein degradation studies often lead to complex mixtures of peptides which are studied using high resolution or 2-dimensional separations. The long-column capabilities of SFC may be useful in resolving these complex mixtures. In addition, coupled with reversed-phase HPLC, the more normal-phase elution characteristics of SFC may provide a rapid, orthogonal second dimension in 2-D separations. Finally, and of perhaps far greater economic impact, preparatory scale SFC may provide a more cost-effective route to the purification of important peptides.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The ionic sodium aryl sulfonate salts were successfully eluted from bare silica, conventional Cyano, and Deltabond Cyano stationary phases using various ammonium salts as additives employing methanol modified-mobile phase. Sodium dodecylbenzene sulfonate was retained longer with the more active stationary phases. This was true with each ionic additive that was studied. This observation can be explained by more irreversible interaction between the anionic analyte and the active silanol sites present on more active stationary phases. An interesting observation was tetramethylammonium acetate provided the fastest elution on Deltabond Cyano column and the longest retention on the conventional cyano and bare silica columns among the ionic additives that have been studied. The same statement was true with the other two sodium aryl sulfonate analytes. When a cationic analyte—Propranolol hydrochloride—was studied on silica phase with AA, TMAA, TBAA, or LiOAc as additive, the TMAA eluted the analyte the fastest.

Solid state ^{29}Si NMR and computational calculation were applied to help us further understand the interactions involved when ammonium salts were used as additives. The decreasing intensity of the ^{29}Si signal under CP-MAS NMR suggested that some of the protons of the silanol groups were removed after the silica packing material was flushed under the same chromatographic conditions as used in the separation. Most likely they were replaced by the ammonium cations that were present in the mobile phase as additives. We sought a deeper understanding of the unique behavior of TMAA, compared to the other two ammonium additives, AA and TBAA. The surface charge of

each ammonium cation was calculated by CaChe software, using three different methods. All the three methods gave a consistent result: TMAA carries a larger positive charge on its surface than AA and TBAA do. This would lead one to expect that TMAA-modified silica phase has a stronger interaction with anionic sulfonate analytes, therefore providing a stronger retention. On the other hand, due to its stronger interaction with silanol sites, TMAA provided faster elution of cationic analytes – Propranolol HCl –than did the other two ammonium additives on silica phase by an ion-exchange type mechanism. Additionally, the memory effect of ammonium salt additives on the silica phase indicated strong interactions of the additives and the silica stationary phase. The lack of memory effect on the Deltabond Cyano phase, on the other hand, indicated that the interaction was weaker on a more deactivated column. Ion-pair formation was believed to be the dominant retention mechanism involved with the Deltabond Cyano phase.

Due to the reciprocity of ion pair mechanism, three ammonium analytes – Propranolol HCl, cetylpyridine chloride, and benzyltrimethyl chloride – were successfully eluted with fast elution time and good peak shapes with sodium sulfonate salts as mobile phase additives. The sulfonate salts used were sodium ethanesulfonate, sodium 1-heptanesulfonate, and sodium 1-decanesulfonate. The long chain sulfonate provided slightly faster elution than the ones with shorter chains. We believed that it is due to greatest solubility of the ion-pairs formed by sodium 1-decanesulfonate and the analyte in CO_2 based mobile phase. A higher concentration of modifier was necessary to achieve acceptable retention times and peak shapes for cetylpyridine chloride and benzyltrimethyl chloride than for Propranolol HCl. This is probably because these two analytes are quaternary ammonium salts. They would be expected to have a stronger

interaction with the polar bonded Cyano phase via ion-dipole interactions. A memory effect was not observed on the bare silica phase with sodium ethanesulfonate as additive, which suggested that the interaction between the sulfonate additives and active silanol sites are not as significant as those with ammonium additives.

A novel stationary phase, 2-ethylpyridine bonded silica column, successfully eluted the three ammonium salt analytes with pure methanol as modifier. The fronting peak shapes and faster elution of the two quaternary ammonium salts compared to Propranolol HCl suggests that a fraction of the pyridine functional groups is protonated. Therefore, the stationary phase might carry a fractional positive charge. Electrostatic repulsion between the stationary phase and the cationic analytes yielded the results we observed. Our hypothesis was strengthened by similar observations with the SAX and aminopropyl phases. The addition of ammonium acetate as additive helped to suppress the fronting of the peaks by providing more interaction between the stationary phases and the analytes.

Four groups of polypeptides were successfully eluted from the Ethylpyridine stationary phase by using 0.1% TFA in methanol-modified CO_2. This was the first report of which we are aware that polypeptides with up to 40 mers, with more than 4500 Da in molecular mass, and containing several hydrophilic amino acid groups, were eluted using a mobile phase containing a significant majority of CO_2. The mobile phase composition in this work was simple and also compatible with mass spectrometry. We observed fronting peak shapes and less retention when higher concentrations of TFA were used. This observation supported our hypothesis that TFA likely protonates some fraction of the pyridine functional groups on the Ethylpyridine stationary phase, suppresses the

protonation of the carboxylic acid groups on the polypeptides, and protonates the basic amino groups on the peptides. Electrostatic repulsion between the protonated peptides and some fraction of the stationary-phase functional groups helps to elute the peptides from the stationary phase.

Elution of the peptides from the strong anion exchange and the aminopropyl stationary phase was also studied. The Ethylpyridine was the only one from which we were able to successfully elute the peptides. This was probably due to deactivation of the active silanol groups by hydrogen bonding between the pyridine functional groups on the stationary phase and the silanols, as well limited access to the silica surface due to steric hindrance caused by the pyridine aromatic ring.

In the past two decades, there has been much discussion concerning the promise of supercritical fluid chromatography. The speed and the environmental benefits of SFC are well documented for the analysis of non-polar compounds. The limitation of polarity of compounds that can be analyzed by SFC was considered to be one of the major drawbacks of SFC. Our research has dramatically extended the application of SFC to highly polar and ionic compounds, as well as relatively large biologically active polypeptides. These compounds were generally considered to be impossible for analysis by SFC before we started this project. We have seen a breakthrough in the application of SFC to a much wider range of analytes by choosing the right mobile phase compositions and stationary phases. One of the more significant findings of this work is evidence for the existence of the stationary phase's ionic interactions with CO_2/methanol mobile phase. It is reasonable to consider extending the research to ion chromatography in supercritical mobile phases.

A more complete fundamental understanding of the factors responsible for these separations is desirable. As the 2-ethylpyridine phase has exhibited advantages in analysis of amine salt analytes, it would be interesting to study the 3- or 4- ethylpyridine phase to gain a better understanding of the role of the functional group. Novel stationary phases with various polar functional groups, such as benzamide, propyl acetamide, et al. are promising in providing different selectivity than conventional SFC columns.

Peptide separations are of great importance in pharmaceutical and other industries. As the analysis of polypeptides by SFC has not been thoroughly studied, there is great potential in this area. By adjusting chromatographic conditions, better resolution between similar peptides may be achieved with the result that peptides with larger size may be successfully analyzed. The exploration of other stationary phases in the separation of peptides is also worthwhile.

Scale-up of the separations and extension to chiral stationary phases are two obvious topics for future work. We are confident that with the right stationary phase and suitable mobile phase composition, SFC has a bright future for analysis and purification of a wide range of analytes.

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