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ABSTRACT

HYDROPHILIC INTERACTION AND MICELLAR LIQUID CHROMATOGRAPHY APPROACHES FOR THE SEPARATION OF AROMATIC CARBOXYLIC ACID POSITIONAL ISOMERS PLUS ION EXCHANGE CHROMATOGRAPHY FOR THE SEPARATION OF SULFONATED COMPOUNDS

by

Ashley Elizabeth Richardson

Separation of aromatic positional isomers is typically limited to separating a couple of the isomers in a class, not all of them. A novel HILIC method has been developed to separate ten aromatic carboxylic positional isomers, including the hydroxybenzoic acid isomers, hydroxycinnamic acid isomers, syringic, vanillic, ferulic, and sinapic acids, using a ternary mobile phase comprised of acetonitrile, ammonium acetate buffer, and pentane. This method is compatible with mass spectrometry (MS) detection and can separate the analytes in under 45 minutes with baseline resolution of all isomers.

Terephthalic acid purity is extremely important during the production of polyethylene terephthalate. There are few methods available that can separate terephthalic acid from eight major impurities. Those impurities include two sets of positional isomers. Using micellar liquid chromatography (MLC), a method has been developed using an acidic sodium dodecyl sulfate (SDS) mobile phase, a C18 column, and a flow rate gradient to separate terephthalic acid from the eight impurities in less than 20 minutes with baseline resolution. Detection limits have been improved when compared to previous methods. This method is also inexpensive and environmentally friendly. Using the same set of analytes, the use of SDS and Brij-35 surfactants in MLC under ultra-high performance liquid chromatography (UHPLC) conditions is compared. While the UHPLC method using SDS is a slight improvement over the standard MLC method previously developed, the use of Brij-35 shows slightly longer analysis time. However, the use of Brij-35 could possibly allow for the use of MS detection as it is not entirely necessary to have Brij-35 in the mobile phase.

Due to the adulteration of heparin in 2007 and 2008, quality control testing of heparin has become extremely important. Heparin and other glycosaminoglycans tend to have extremely broad peaks, excessive retention, and poor resolution. Therefore, model compounds will be used to optimize the separation conditions before applying the method to heparin and impurities. This method uses a cation exchange column that is modified with protamine to make an anion exchange column. By keeping protamine in the mobile phase, the column is constantly equilibrated. An acidic mobile phase consisting of protamine and sodium perchlorate has successfully separated naphthalene mono-, di-, and tri-sulfonate. Additionally, it has been able to positively identify a polystyrene sulfonate compound in a wrinkle serum.

HYDROPHILIC INTERACTION AND MICELLAR LIQUID CHROMATOGRAPHY APPROACHES FOR THE SEPARATION OF AROMATIC CARBOXYLIC ACID POSITIONAL ISOMERS PLUS ION EXCHANGE CHROMATOGRAPHY FOR THE SEPARATION OF SULFONATED COMPOUNDS

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LIST OF ABBREVIATIONS

Abbreviation	Technique/Concept
λ	Packing factor
μ	Linear velocity
μ_{opt}	Optimum velocity
ψ	Obstruction factor
A	Eddy diffusion term in the van Deemter equation
B	Longitudinal molecular diffusion term in the van Deemter equation
C	Mass transfer term in the van Deemter equation
C_M	Mass transfer in the mobile phase
CMC	Critical micelle concentration
C_S	Mass transfer in the stationary phase
d	Difference in retention time of two peaks
d_f	Stationary phase thickness
D_M	Diffusion coefficient in the mobile phase
d_p	Particle size
D_S	Diffusion coefficient in the stationary phase
H	Plate height
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IEC	Ion exchange chromatography
IPC	Ion-pair chromatography
k'	Retention factor
K_{AM}	Binding constant between the solute and surfactant monomer
K_{AS}	Binding constant of the solute
K_{MW}	Partition coefficient between the micelle and water
K_{SM}	Partition coefficient between the stationary phase and micelle
K_{SW}	Partition coefficient between the stationary phase and water
L	Column length
LC	Liquid chromatography

M	Micelles in the mobile phase
MALDI	Matrix-assisted laser desorption/ionization
MEEKC	Microemulsion electrokinetic chromatography
MLC	Micellar liquid chromatography
MS	Mass spectrometry
N	Number of theoretical plate
NPLC	Normal phase liquid chromatography
PAGE	Polyacrylamide gel electrophoresis
Pk_A	Acid dissociation constant
RPLC	Reverse phase liquid chromatography
R_s	Peak resolution
S	Stationary phase activity
t_R	Retention time
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
V_0	Void volume
V_e	Volume needed to elute an analyte
V_s	Volume of the stationary phase
w_b	Peak width at the baseline

Abbreviation	Chemical
2-HB	2-Hydroxybenzoic acid
2-HC	2-Hydroxycinnamic acid
3-HB	3-Hydroxybenzoic acid
3-HC	3-Hydroxycinnamic acid
4-HB	4-Hydroxybenzoic acid
4-HC	4-Hydroxycinnamic acid
BA	Benzoic acid
CTAB	Cetyltrimethylammonium bromide
EtOH	Ethanol

FBA	4-Formylbenzoic acid
Fer	Ferulic acid
GAG	Glycosaminoglycan
HMA	Hemimellitic acid
HMDX	Hexamethyldisiloxane
IPA	Isophthalic acid
MeCN	Acetonitrile
NDS	Naphthalene disulfonate
NMS	Naphthalene monosulfonate
NS	Naphthalene sulfonate
NTS	Naphthalene trisulfonate
OPA	o-Phthalic acid
OSCS	Oversulfonated chondroitin sulfate
p-TA	p-Toluic acid
PET	Polyethylene terephthalate
PS	Polystyrene sulfonate
PS-DVB	Polystyrene-divinyl benzene
PSA	Polystyrene sulfonate (75,000 g/mol)
PSB	Polystyrene sulfonate (200,000 g/mol)
PTA	Purified terephthalic acid
SDS	Sodium dodecyl sulfate
Sin	Sinapic acid
Syr	Syringic acid
THF	Tetrahydrofuran
TMA	Trimellitic acid
TPA	Terephthalic acid
TSA	Trimesic acid
Van	Vanillic acid

DEDICATION

I dedicate this dissertation to my parents; I would not be here without their support and unconditional love. I also dedicate this work to my father. Though he isn't here on earth, I know he would be proud of all that I have accomplished.

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I am incredibly grateful for all of the support from so many people during this journey. I would first like to thank my advisor, Dr. Neil Danielson for all of his encouragement and support. I appreciate his emphasis on maintaining a balance between work and life and keeping a positive attitude. His dedication to my projects is inspiring, and his willingness to help out when life goes awry is impressive and incredibly appreciated.

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CHAPTER 1

Introduction

1.1: High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is an analytical technique that is used to separate compounds, particularly those that are polar and high molar mass. HPLC is extremely versatile as it has found use in academia, government, and industry science research, often biological in nature. The goal in HPLC to establish varying degrees of compound retention on a column by their interaction with the solid stationary phase and the liquid mobile phase. The degree of interaction is then determined by a variety of factors including the structure, size, polarity, and ionization state of the analyte. HPLC has many advantages over other modes of separation including efficiency, resolution, sensitivity, and speed.

An HPLC instrument is made up of four major components: a pump, an injector, a column, and a detector. The liquid mobile phase solvent is propelled through the column using the pump. The injector can be a stand-alone injection valve or part of an autosampler, making the analysis of many samples easier. Columns in HPLC vary in functionality, but the majority contain particle sizes between 2 to 5 μm, a length of 50 to 250 mm, and an inner diameter of 2 to 4 mm. The difference in functionality of the columns will be discussed later. The detector is determined by the application, often by the common structure type of the compounds of interest. Ultraviolet (UV) detection is one of the most common detectors with HPLC. UV detection is popular because it is stable, boasts good detection limits, and can detect a wide range of compounds, particularly aromatic hydrocarbons. Mass spectrometry (MS), fluorescence, conductivity, refractive index, and evaporative light scattering are other detectors that are commonly used with HPLC. Mass spectrometry has seen greater use in recent years because it provides lower detection limits than UV while being able to detect compounds that are not UV active.

Large porous particles (approximately 100 μm) were the original particles used in HPLC. Pellicular particle packings were then introduced in the 1960s by Jack Kirkland. These particles were superficially porous meaning the pores don't extend all the way through the particle. They boasted higher efficiency than the large porous particles because of their decreased diffusion capability, but they also had decreased sample capacity due to the small surface area. Small porous particles (<10 μm) were then developed. These particles began as irregular shapes, but eventually spherical particles became standard because they could be packed more

homogeneously, dramatically improving column efficiencies [1,2]. High efficiency is important because it produces better peak resolution in the resultant chromatogram. Silica particles can be functionalized in various ways to provide different separation environments. Reversed phase columns are commonly a silica support functionalized with a nonpolar group such as alkyl or phenyl groups. Normal phase columns are more polar and utilize bare silica, amino, or cyano bonded phases. These silica-based columns come in a variety of particle sizes that range from 2 μm to 10 μm, with sub-2 μm particles being reserved for ultra-high performance liquid chromatography (UHPLC). Apart from silica particles, HPLC columns can also be packed with materials such as zirconia, polystyrene-divinyl benzene (PS-DVB), and other organic polymers. Non-silica particles offer advantages like higher pH stability and larger temperature range stability. Another option besides packed particle columns is the monolithic column. As opposed to being packed, these columns are cast with a continuous solid support inside the tube. This support can be either silica or polymer based, with various functionalities such as reversed phase, hydrophobic interaction, ion exchange, and affinity chromatography. Monolithic columns have been heavily used for the separation of large biomolecules [2].

HPLC columns are manufactured to withstand pressures up to 6,000 psi and are capable of separating up to 20 components in a mixture, commonly using an isocratic (constant composition) mobile phase. Using a gradient mobile phase (increasing solvent strength with time) allows much more complex mixtures to be resolved. As mentioned previously, UHPLC columns contain particles that are below 2 μm. Because column pressure is inversely related to the square of the particle size, these small particles result in pressures up to 15,000 psi. This increase in pressure results in decreased analysis time and an increase in the number of sample components that can be resolved in a mixture without sacrificing peak resolution or efficiency. The fast and efficient separations are beneficial for the chemical industry because it increases the sample throughput when compared to HPLC.

There are various equations used to evaluate chromatographic methods. First, plate number, N, is used to determine efficiency. A plate can be thought of as the minimum cross-section part of the column where equilibrium of the sample component between the mobile and stationary phases is established. The higher the number of theoretical plates, the higher the column efficiency. Based on a Guassian peak, N is defined in Equation 1, where t_r is the retention time and w_b is the peak width at the baseline.

$$N = 16(\frac{t_R}{w_b})^2 \quad (1)$$

From this equation, plate height, the cross-section width, can be calculated using N and the length of the column, L. By factoring in column length, efficiencies can be compared between columns of varying lengths. The equation is shown in Equation 2.

$$H = \frac{L}{N} \quad (2)$$

A different way to measure efficiency is resolution, R_S. This term determines the degree of separation between two peaks. R_S is defined in Equation 3, where d is the difference in retention time of the two peaks, w_{bA} is the width at the base of peak A, and w_{bB} is the width at the base of peak B [1].

$$R_S = \frac{2d}{w_{b_A} + w_{b_B}} \quad (3)$$

Although plate theory is useful and convenient, the only type of broadening assumed is diffusion. Rate theory, developed by van Deemter, describes three terms that affect band broadening within a column: eddy diffusion (A term), longitudinal molecular diffusion (B term), and mass transfer into the mobile and stationary phases (C term). Eddy diffusion, the A term, is equal to two times the packing factor (λ) multiplied by the particle size (d_p). To decrease this term, uniform particles must be packed as tightly as possible. Longitudinal diffusion, the B term, is equal to two times the obstruction factor (ψ) multiplied by the analyte diffusion coefficient in the mobile phase (D_M). To decrease this term, D_M must be minimized. This can be accomplished by using high velocities because this decreases the time an analyte can spend for molecular diffusion. The B term is also divided by the linear velocity (μ), meaning a high velocity will further decrease longitudinal diffusion. The mass transfer term is defined using two variables, mass transfer in the mobile phase, C_M, and mass transfer in the stationary phase, C_S. The particle size (d_p) is directly proportional to C_M, while the analyte diffusion coefficient in the mobile phase (D_M) is inversely proportional to C_M. The thickness of the stationary phase (d_f) is directly proportional to C_S, while the analyte diffusion coefficient in the stationary phase (D_S) is inversely proportional to C_S [1]. The extended van Deemter equation is shown in Equation 4.

$$H = A + \frac{B}{\mu} + (C_M + C_S)\mu \quad (4)$$

Taking the derivative of Equation 4 with respect to μ and setting this to zero, an equation for the optimum velocity (μ_{opt}) can be formed and is shown in Equation 5.

$$\mu_{opt} = \sqrt{\frac{B}{C_M + C_S}} \tag{5}$$

Because particle size is directly proportional to C_M and inversely proportional to μ_{opt}, the optimum velocity increases as the particle size is decreased [1]. A graphical depiction of this is shown in Figure 1.1. Mass transfer effects remain minimal and at a minimum even at high flow rates for small particles.

There are many modes of liquid chromatography that have been developed. Each mode differs in the separation mechanism, and therefore each mode is efficient at separating certain classes of compounds. This chapter will discuss hydrophilic interaction liquid chromatography (HILIC), micellar liquid chromatography (MLC), and ion exchange chromatography (IEC). These three modes are the types researched in this dissertation.

Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a mode of chromatography best suited for the separation of polar analytes. HILIC uses a high organic mobile phase component and has become increasingly popular in recent years because of its compatibility with MS detection [3,4]. The retention mechanism is dependent on analyte partitioning into the partially immobilized water layer on the polar stationary phase. Common stationary phases include plain silica, ethylene bridges, cross-linked diols, and zwitterionic phases. These columns are all hydrophilic, though they vary in the strength of hydrogen bonding and electrostatic interaction capabilities [5,6]. The mobile phase composition has a great effect on the retention of the analytes. The bulk solvent most commonly used is acetonitrile (MeCN). This comprises approximately 80 to 95% of the mobile phase, though when using a mobile phase gradient this can change. The rest of the mobile phase is usually an aqueous buffer such as ammonium acetate and ammonium formate, which have a high compatibility with MS detection. The concentration of the buffer varies, though as the buffer concentration increases, analyte retention tends to increase as well since electrostatic interactions are decreased. The higher salt concentration can affect the thickness of the water layer. If the organic content of the mobile phase is high enough, the buffer salt will partition into the water layer which will increase analyte retention [6,7]. To

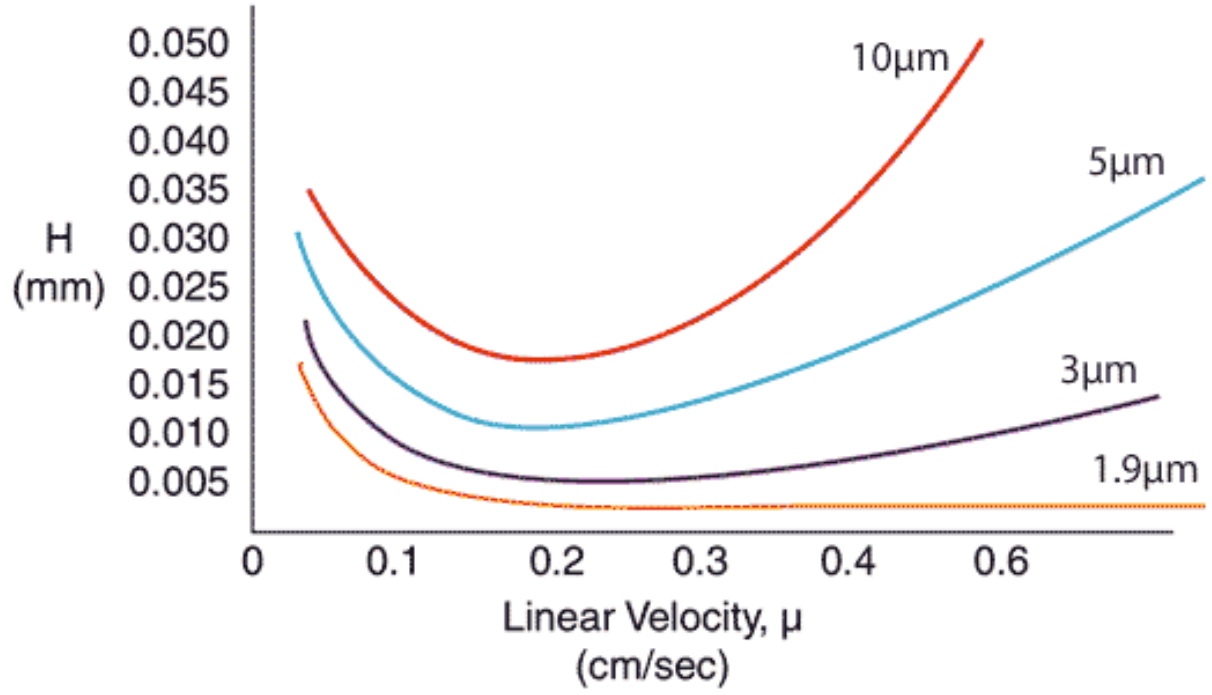

Figure 1.1. The difference in the effect of linear velocity on plate height at various particle sizes [32].

decrease retention of analytes, the aqueous content of the mobile phase can be increased, as shown in Figure 1.2.

A third solvent can be added to the mobile phase to affect the retention and elution order of the analytes. This addition sets up a hydrophilicity gradient between the aqueous and organic layers due to the difference in hydrophilic character. As the steepness of this gradient increases, the retention of polar and hydrophilic analytes is increased. To increase the steepness of the hydrophilicity gradient, the third solvent must become more nonpolar. For example, a nonpolar solvent like hexane causes the largest effect on hydrophilic compound retention [8]. Figure 1.3 shows the difference in organic and aqueous layer distinction, or steeper hydrophilicity gradient, caused by the addition of the nonpolar solvent pentane.

HILIC boasts some advantages over other modes of chromatography. When compared to RPLC, HILIC offers kinetic advantages. Because of the organic rich mobile phase, analyte diffusivity is increased, leading to perceived kinetic performance gains. This can allow for diffusion to happen faster, which enhances mass transfer, thus lowering the C term in the van Deemter equation. This leads to greater column performance at higher mobile phase velocities, leading to faster analysis times [4,9]. HILIC also has some advantages over normal phase LC (NPLC). Though both modes utilize a polar column, the hydro-organic mobile phase of HILIC means there is wider sample solvent compatibility when compared to the non-aqueous mobile phase in NPLC [10].

Due to the polar nature of HILIC columns, this mode is excellent for the separation of polar compounds. This makes HILIC complementary to RPLC, where polar compounds are eluted in the void volume. Charged and non-aromatic analytes are particularly well separated using HILIC. With respect to pharmaceutical drugs, the metabolites tend to be more hydrophilic than the parent drug. This makes separation by RPLC difficult because the more hydrophilic compounds will be less retained [11]. Though metabolites and nucleosides have been separated by ion-pair chromatography (IPC), often MS detection is required, making IPC an unusable mode [12]. As stated previously, HILIC is very compatible with MS detection. The high amount of organic solvent in the mobile phase allows for increased sensitivity and a higher desolvation efficiency [7]. Acylcarnitines have also been well separated using HILIC. Because carnitines are quanternary ammonium compounds, they are scarcely retained under RPLC conditions. However, better retention and separation of a variety of carnitine compounds has been

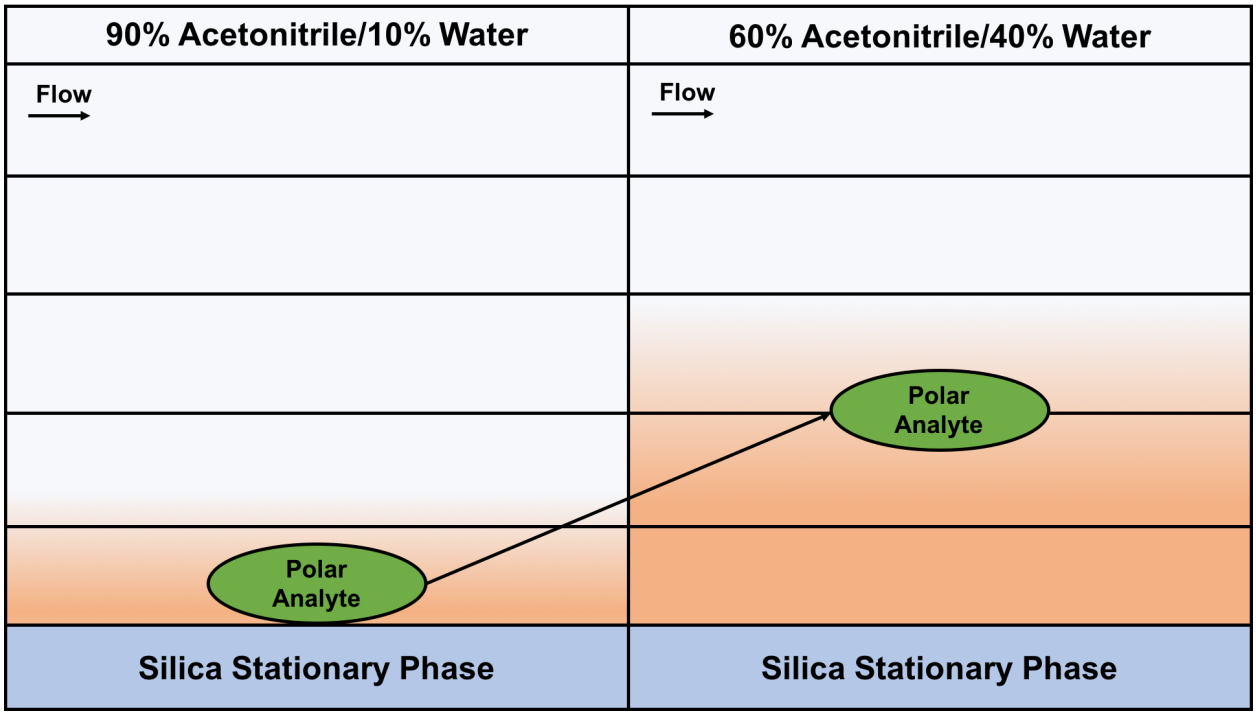

Figure 1.2. Retention of a polar analyte when the aqueous phase is a low percentage vs elution of a polar analyte when the aqueous phase percentage is increased.

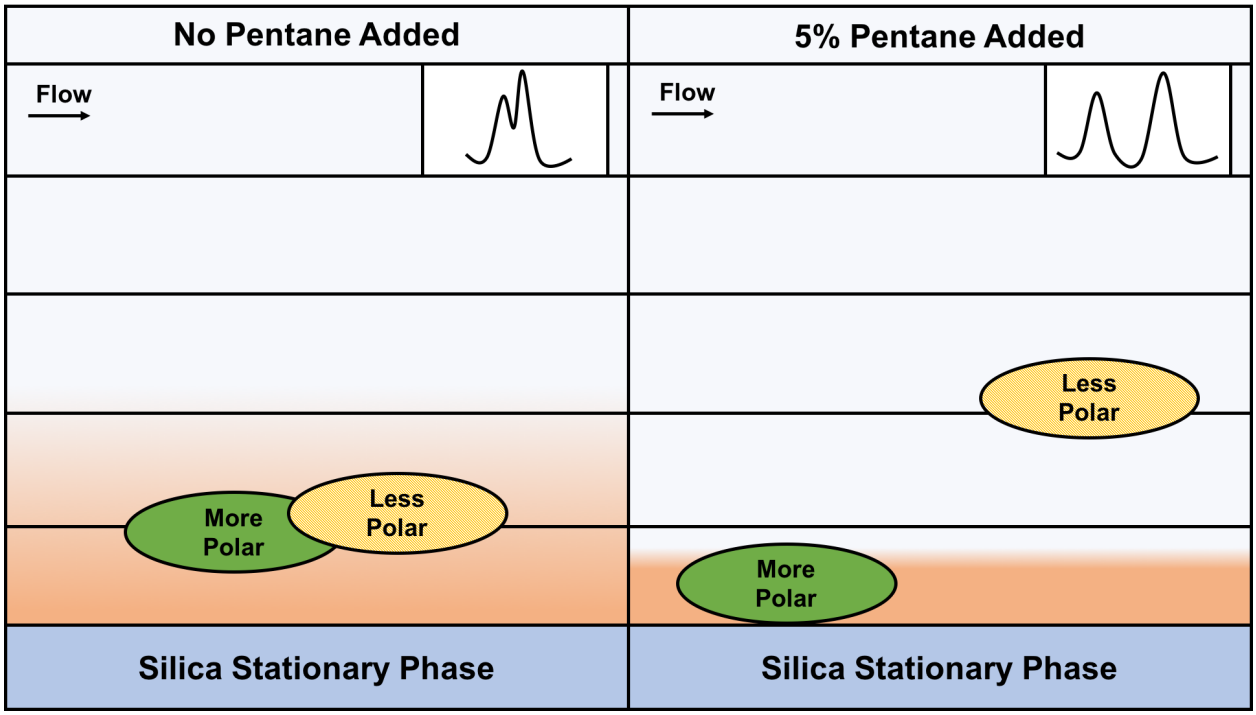

Figure 1.3. Incorporation of a hydrophilicity gradient through the addition of a second organic solvent, usually more nonpolar than acetonitrile.

shown by Onorato showed that the separation, peak shape, and retention of carnitine is improved when using HILIC conditions rather than RPLC conditions [13]. Additionally, Kivilompolo developed a HILIC method using UHPLC conditions to separate carnitine and 11 acylcarnitines in under seven minutes [14].

Micellar Liquid Chromatography

Micellar liquid chromatography (MLC) is a mode of chromatography that utilizes surfactants as mobile phase modifiers. The surfactant can be either below or above its critical micelle concentration (CMC), though it is more common to use the surfactant above its CMC. MLC is often viewed as an alternative to reversed-phase liquid chromatography (RPLC) and is able to separate aromatic acids and bases well [15–18]. The three most common surfactants are the anionic sodium dodecyl sulfate (SDS), the cationic cetyltrimethylammonium bromide (CTAB), and the nonionic Brij-35 [19]. The stationary phase is most often a nonpolar RPLC column like a C8 or C18, though mixed-mode columns, like a cyanopropyl column, have been used as well. With a nonpolar column, the nonpolar chains of the surfactant hydrophobically interact with the nonpolar chains of the stationary phase [17]. Figure 1.4 shows this interaction in an SDS environment. A small amount of organic solvent is sometimes used to help with retention and elution of analytes; acetonitrile and 1-propanol are most commonly used. Though there is a small amount of organic solvent used, this method is still more environmentally-friendly than traditional RPLC due to the low toxicity of the surfactants. The use of aqueous surfactants over bulk organic mobile phases makes MLC a more cost effective option. The one major limitation of MLC is that it is not compatible with MS detection [20–22].

The retention mechanism has been well studied and thoroughly described by Armstrong and Nome. They described three partitioning equilibria: between the micelle and the bulk water, between the bulk water and the stationary phase, and between the micelle and the stationary phase, as shown in Figure 1.4. They also describe anti-binding solutes as solutes that show an increase in retention with increased micelle concentration and non-binding solutes as solutes that do not show a change in retention as the micelle concentration is changed. The following partitioning coefficients describe the equilibria: K_{MS} (between the micelles and stationary phase), K_{WM} (between the bulk water and micelles), and K_{WS} (between the bulk water and stationary phase). Factors such as mobile phase pH, surfactant type and concentration, ionic strength, and the presence of an organic modifier can affect the partitioning equilibria [23,24].

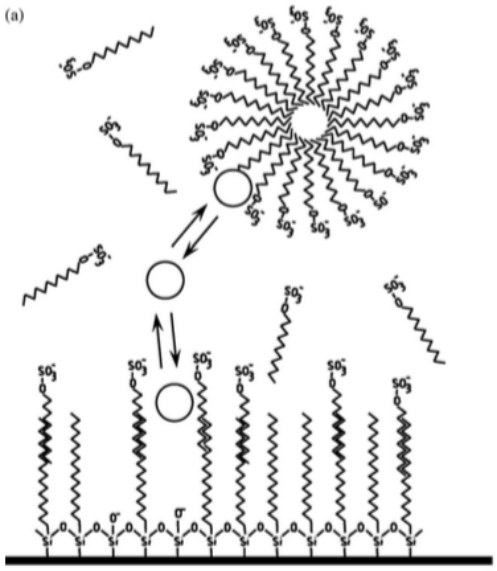

Figure 1.4. Hydrophobic interaction of SDS monomers with a C18 stationary phase. Reprinted from [24] with permission from Elsevier, Copyright 2009.

Armstrong and Nome described the retention behavior in a micellar system by proposing a partitioning model, shown in Equation 6 [23]:

$$\frac{V_S}{V_e - V_0} = \frac{\upsilon(K_{MW} - 1)}{K_{SW}}[M] + \frac{1}{K_{SW}} \quad (6)$$

In this equation, V_s is the volume of the stationary phase, V_e is the volume needed to elute the analyte, V_0 is the void volume, υ is the partial specific volume of the surfactant in the micelle, K_{MW} is the partition coefficient between the micelle and the water, K_{SW} is the partition coefficient between the stationary phase and the water, and [M] is the concentration of the surfactant in the micelle. [M] is calculated by subtracting the CMC from the total micelle concentration. When $\frac{V_S}{V_e - V_0}$ is plotted against [M], there should be a linear fit. From this plot, K_{SW} is equal to the intercept, and K_{MW} is equal to the slope. K_{SM}, the partition coefficient between the stationary phase and the micelle, can be calculated using Equation 7.

$$K_{SM} = \frac{K_{SW}}{K_{MW}} \quad (7)$$

Arunyanart and Cline-Love used binding constants and retention factors to describe the retention mechanism in MLC [25]. By substituting retention factor and the binding constants in Equation 6, the result is Equation 8.

$$\frac{1}{k'} = \frac{1}{\phi K_{AS}[S]} + \frac{K_{AM}}{\phi K_{AS}[S]}[M] \quad (8)$$

In this equation, k' is the retention factor, K_{AS} is the binding constant of the solute, A, with the stationary phase, K_{AM} is the binding constant between the solute and the surfactant monomer of the micelle, and M is the micelle concentration. The phase ratio, ϕ, is equal to $\frac{V_S}{V_0}$, where V_S is the volume of the stationary phase, and V_0 is the void volume of the column. [S] is the stationary phase activity and is usually constant. The micelle concentration is calculated by subtracting the CMC from the total surfactant concentration. The plot of 1/k' versus [M] should result in a straight line. From this plot, K_{AM} can be calculated by dividing the slope by the intercept [25].

One disadvantage of MLC is that it is less efficient than other modes of liquid chromatography due to a lower analyte diffusion coefficient in the mobile phase. To enhance efficiency, an organic modifier is often used. The most common modifiers include propanol, acetonitrile, ethanol, and methanol. The modifiers improve peak shape and reduce long retention

times, though alcohol modifiers reduce retention time the most. While the type and concentration of the organic modifier affects the chromatographic environment, the concentration of the surfactant and hydrophobicity of the analyte is also important to consider when choosing an organic modifier. Micellar conditions, such as the CMC and aggregation number, are altered when an organic modifier is incorporated. While the addition of organic modifiers does enhance efficiency and separation, it also increases the cost of the method as well as make it less environmentally friendly [26,27].

MLC has been used in the separation of basic drugs like β-blockers. Ruiz-Ángel has improved upon the RPLC method for the separation of β-blockers by using a mobile phase comprised of SDS and acetonitrile. Under RPLC conditions, β-blockers interact with free silanols on the nonpolar stationary phase which leads to significant peak deformity. However, using MLC, the surfactant forms an adsorbed layer on the stationary phase which decreases the free silanol interactions. It was found that peak shapes were dramatically improved and analysis time was decreased [28].

Ion Exchange Chromatography

Ion exchange chromatography (IEC) is used to mainly separate ions, though it can be used to separate some charged organic polar molecules as well. There are four classifications for IEC: strong cation exchange, weak cation exchange, strong anion exchange, and weak anion exchange. The charge capacity of the stationary phase for strong ion exchange, whether cationic or anionic, is independent of pH, while that for weak ion exchange is dependent on pH. The solid support, or resin, that is most commonly used in IEC is a PS-DVB copolymer, which is functionalized to suit the type of ion exchange. Strong cation exchange columns are commonly functionalized with sulfonic groups, while weak cation exchange columns are functionalized with carboxylic acid groups. Strong and weak anion exchange columns are usually functionalized with tertiary (R_3N-) and secondary (R_2NH-) alkyl (R) amine groups, respectively. An aqueous mobile phase is used in IEC because ion formation is favored in an aqueous environment. However, the mobile phase is usually buffered to a specific pH to control the charge of the analytes and capacity of the weak ion exchange column (if used) [1,29,30].

To explain the retention mechanism, consider the Equation 9 as an example of cation exchange:

$$R^-A^+ + Y^+ = R^-Y^+ + A^+ \quad (9)$$

R^- is the ion exchange resin, A^+ is a cation present in the mobile phase (also called the counter ion), and Y^+ is the analyte being separated. For retention of Y^+ to occur, the resin must have a selective affinity for that analyte that is different from the resin's affinity for other analytes. Additionally, the choice of A^+ in the mobile phase must be considered carefully because it cannot be too strongly held by the resin. If the resin's affinity for A^+ is too strong, no retention of any analyte will occur because no exchange from R^-A^+ to R^-Y^+ can occur. Figure 1.5 shows a schematic representation of the retention mechanism. The elution order for IEC is determined by ionic charge, polarizability, and the hydrated radius, especially for cations. Factors that can affect the elution of analytes include mobile phase pH, the counter ion choice, and the counter ion concentration. As stated before, the counter ion must be chosen carefully so it is not held too strongly by the resin. Counter ions that are larger and have a larger charge will likely decrease analyte interaction with the resin, resulting in shorter retention times when compared to smaller counter ions with a smaller charge. The pH of the mobile phase can affect the ionization of analytes and the ionization of the resin. The pH of the mobile phase should be between the pK_A of the resin and the pK_A of the analytes. A pH gradient can be used to change the ion exchange capabilities during analysis to aid in analyte elution. Additionally, a salt gradient can be used to elute analytes. The salt gradient is usually set up for an increase in salt concentration during analysis [1,29,30]. For Equation 8, this means increasing A^+, which shifts the equilibrium back to the left side, decreases the retention of Y^+.

Ion exchange has been used in peptide separation, a field largely dominated by RPLC. However, IEC offers different selectivities, especially when looking at differences such as deamidation and acetylation. Often the sample is injected in a low ionic strength mobile phase, and then it is eluted by increasing the ionic strength of the mobile phase. When using cation exchange, an increase in mobile phase pH will decrease the net positive charge on the peptides and decrease retention. For anion exchange, an increase in pH will increase the carboxyl group charge of the peptide and increase retention [31].

1.2: Specific Aims

(1) To develop an innovative HILIC method that is compatible with MS detection for the separation of hydroxy aromatic carboxylic acid positional isomers using a plain silica stationary

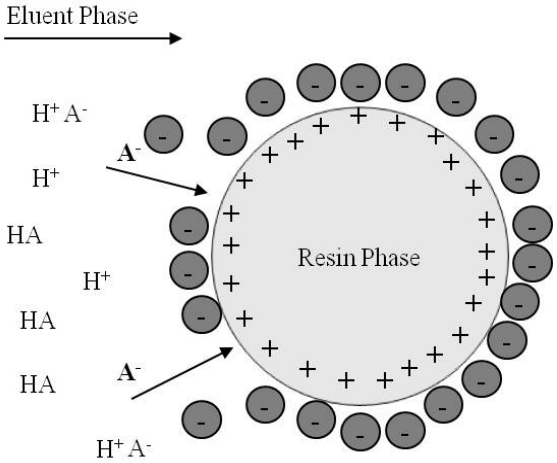

Figure 1.5. Schematic representation of the ion exchange mechanism [33].

phase and a ternary mobile phase comprised of acetonitrile, an ammonium acetate buffer, and pentane. The developed method is intended to overcome the lack of isocratic methods that are MS compatible to separate positional isomers.

(2) To develop a novel MLC method for the separation of terephthalic acid from eight impurities found during the production of polyethylene terephthalic acid (PET) using the anionic surfactant SDS in the mobile phase. The developed method is intended to overcome the lack of green, isocratic, and time-consuming methods that are currently used to separate these acids.

(3) To compare the use of SDS and Brij-35 surfactants under UHPLC conditions for the separation of terephthalic acid from eight impurities found during the production of PET. The developed and compared methods are intended to provide further information on MLC under UHPLC conditions, as well as broaden the comparison of different surfactants in MLC.

(4) To develop a novel ion exchange method for the separation of sulfonated compounds, like polystyrene sulfonate and chondroitin sulfate using a protamine-modified ion exchange column and protamine mobile phase. The developed method is intended to provide an alternative method for the separation of sulfonated compounds with the hope of overcoming the limitations of the separation methods for heparin from other glycosaminoglycans.

In addition, the appendix summarizes a method for the separation of peptides using a surfactant pluronic polymer and wide-bore tube gel electrophoresis. The developed method is intended to provide a green and alternative electrophoresis separation mode for peptides, which are difficult to resolve using standard gels.

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CHAPTER 2

Hydrophilic Interaction Liquid Chromatography of Hydroxy Aromatic Carboxylic Acid Positional Isomers

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2.1: Abstract

Hydrophilic interaction liquid chromatography (HILIC) has become increasingly popular as an alternative to reversed phase LC due to its ease of separating complex polar compound mixtures and the compatibility of the mobile phase with mass spectrometry (MS). Using a plain silica column (150mm x 4.6mm), we have shown a mixture containing three hydroxybenzoic acid isomers plus syringic and vanillic acid and three hydroxycinnamic acid isomers plus ferulic and sinapic acid can be separated using a mobile phase comprised of 90% acetonitrile (MeCN) and 10% 20 mM ammonium acetate at pH 6 in under 45 minutes. This method is appropriate when using UV detection at 275 nm. However, for improved MS compatibility, a buffer concentration of 10 mM is recommended but this greatly decreases the analyte retention factors. A second more nonpolar organic solvent component in the mobile phase is found to offset this loss in retention. The optimum mobile phase is found to be 90% MeCN, 5% 10 mM ammonium acetate pH 6, and 5% pentane with resolution of eight of the ten compounds with a separation time of 30 min. The use of pentane in the HILIC mobile phase has not been previously explored. Using UV detection, we have shown that detection limits range from 36 – 133 pmole and quantitation limits are spread from 94 – 376 pmole for six of the analytes. Upon testing this method on two other silica columns from different manufacturers, it is found that while resolution is similar, further optimization of the mobile phase is recommended.

2.2: Introduction

Since its emergence in the 1990s, hydrophilic interaction liquid chromatography (HILIC) has proven to be a powerful mode of liquid chromatography (LC). For HILIC, the stationary phase is polar while the mobile phase is comprised of water or an aqueous buffer phase and a water-miscible organic solvent like acetonitrile. While this mobile phase is similar in composition to that of reversed-phase liquid chromatography (RPLC), the strong solvent in HILIC is water while the weak solvent is acetonitrile. The retention mechanism in HILIC is primarily partitioning of the analyte between the aqueous layer and the organic layer [1]. Ideally,

HILIC is an excellent approach for the separation of polar or hydrophilic compounds, like neurotransmitters [2], parasitic metabolites [3], and various classes of lipids [4]. Mass spectrometry (MS) has become increasingly popular as the detector of choice for HILIC because of its mobile phase compatibility [5,6].

Many studies have been done that investigate the various HILIC stationary phases available including zwitterionic sulfobetaine groups, diols, diisopropyl-cyanopropylsilane, amide, and amino types however bare silica is also effective. These columns are all hydrophilic, though they vary in the strength of electrostatic interaction and hydrogen bonding capability [7,8]. It has been shown that polar stationary phases extract water from the mobile phase more strongly than less polar stationary phases. When using a binary mobile phase, the extraction of water is stronger when the water concentration is lower. Through comparison of 14 stationary phases, it was found that mainly the stationary phase polarity determines the strength of the water extraction from the mobile phase [9]. The effect of salt, organic solvent, and mobile phase pH are commonly tested parameters for HILIC. A higher salt content tends to lead to higher retention since this increases the water layer thickness, while the pH of the mobile phase should be at a value where most analytes are in their ionized form and therefore more retained due to the increased hydrophilic character. The choice of organic solvent can greatly affect the retention and elution order. Acetonitrile has been found to be one of the weaker eluting solvents, which tends to work best for HILIC as the primary mobile phase component, though methanol, ethanol, and isopropyl alcohol have also been used as co-solvents [7,8].

Additionally, a third solvent can be added to the mobile phase to further affect the retention and elution order of analytes. A hydrophilicity gradient exists between the organic and aqueous layers in HILIC due to the difference in hydrophilic character between the two layers. More hydrophilic and polar analytes will be retained longer with a steeper gradient. The largest effect on retention of hydrophilic compounds should be when a nonpolar solvent, such as hexane, is used [10]. Polar compounds like methacrylic acid, cytosine, nortriptyline, and nicotinic acid showed an increase in retention and resolution as the mobile phase modifier was changed from methanol to ethanol to isopropanol, suggesting that as the polarity of the modifier is decreased, the retention of polar analytes will increase [11,12]. However, to the best of our knowledge, neither a hydrocarbon nor an alcohol has been used as a co-solvent to enhance the HILIC retention of aromatic compounds with polar substituents.

Aromatic hydroxy carboxylic acids, commonly found in wine, beer, and fruit juices, are a well-studied class of compounds. They have been separated using various modes of chromatography including RPLC, ion-pair RPLC, ion exchange chromatography, normal phase liquid chromatography, and thin-layer chromatography [13–21]. Although RPLC methods have advanced to shorter analysis times, they continue to use complex gradients, and these methods tend to leave out one isomer in an isomeric set. Micellar UHPLC has been used to separate aromatic carboxylic acids, including two sets of isomers, with baseline resolution in less than 35 minutes, however the surfactant mobile phase is not compatible with MS [22]. Recent work using UPLC-MS/MS has shown the separation of seventeen compounds with two isomeric sets in about 10 min, although the method requires a complicated five step RPLC acetic acid-acetonitrile mobile phase gradient [19].

Hydroxy aromatic carboxylic acids are naturally occurring compounds that possess anti-oxidative qualities and exist as secondary metabolites in many plants. Because of their antioxidant properties, they are important to the human diet. Apart from appearing in food and beverages, they also play a role in pharmaceuticals and cosmetics. There are two primary groups that make up these hydroxy aromatic carboxylic acids, or phenolic acids: hydroxybenzoic and hydroxycinnamic acids [23–25]. The HILIC separation of isomeric hydroxy- and amino-benzoic acids has been characterized using a zwitterionic sulfobetaine stationary phase [26]. Although no chromatograms were shown and discussion of peak resolution was absent, separation of the isomeric sets of aromatic acids seemed apparent at 90% acetonitrile-10% 15 mM ammonium acetate. Such a study using a plain silica column was not evident in the literature.

In this work, we present an isocratic HILIC method to separate these two previously indicated classes of compounds, including positional isomers (structures represented in Figure 2.1), that is compatible with MS detection. To extend the versatility of silica beyond normal phase LC to separate aromatic positional isomers, a plain silica column is used with a ternary mobile phase comprised of acetonitrile, ammonium acetate buffer, and pentane. Although pentane is volatile ensuring MS compatibility and has the best water solubility of hydrocarbon solvents, it has not been previously considered as a co-solvent for HILIC. Additionally, few isocratic methods are available to separate positional isomers with even fewer methods available that are MS compatible.

2.3: Apparatus and Conditions

Chromatographic separations were conducted on a Dionex UltiMate 3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA) equipped with a ternary pump, online degasser, autosampler, temperature-controlled column oven, and diode array UV detector. Chromeleon 6.8 software (Thermo Scientific, Sunnyvale, CA, USA) was used for instrument control and data acquisition. Mobile phase optimization as well as all separations were done using a plain silica stationary phase involving a Phenomenex Nucleosil silica column (Torrance, CA, USA) (150 x 4.6 mm, 3 μm). Both an Agilent Poroshell 120 HILIC column (Santa Clara, CA, USA) (150 x 4.6 mm, 2.7 μm) and a Grace (Columbia, MD, USA) silica column (150 x 4.6 mm, 3 μm) were compared to the Nucleosil column to determine chromatographic reproducibility between columns with the same dimensions and particle size. A Thermo Scientific Accucore HILIC column (Sunnyvale, CA, USA) (100 x 2.1 mm, 2.6 μm) was used to compare the final method on a dimensionally smaller silica column.

A 2 μL injection size was used. The column was kept at ambient temperature. UV detection was monitored at 254 and 275 nm, however 275 showed greater response for most analytes.

2.4: Chemicals and Procedures

All solutions were made using 18.2 MΩ water that was distilled and de-ionized before being passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA) and a UV photolyzer. Acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) comprised the majority of the mobile phase. The concentration of ammonium acetate (Fisher Scientific, Fair Lawn, NJ, USA) as was its pH using acetic acid (Sigma-Aldrich, St. Louis, MO, USA) was varied in the mobile phase. All analyte chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were made in 90% acetonitrile, 10% water. The column was equilibrated with the mobile phase for 60 minutes prior to the first injection. Before pentane (Fisher Scientific, Fair Lawn, NJ, USA) was added to the mobile phase, mixing of acetonitrile and ammonium acetate was done by the pump. However, the mixing of pentane and ammonium acetate proved to be problematic for the degasser. This was remedied by pre-mixing the mobile phase, particularly the acetonitrile-pentane phase first before the addition of the buffer.

The standard mixture used for method optimization contained three mono-hydroxybenzoic acid isomers at 5 mM concentration as well as three mono-hydroxycinnamic isomers, syringic

acid, vanillic acid, ferulic acid, and sinapic acid at 1 mM concentration. To determine the analytical figures of merit, five standard solutions were made at the following concentrations: 10, 25, 50, 75, and 100 μM. Acid names numbered in order with abbreviations and analyte pKa values can be found in Table 2.1, while Figure 2.1 shows their structures.

2.5: Results and Discussion

Optimization of ammonium acetate concentration

The effect of ammonium acetate concentration in the mobile phase was first investigated. Previous research has shown that buffer concentration can greatly affect the retention factor of analytes in the HILIC mode. Volatile buffers like ammonium acetate or ammonium formate are the most commonly used buffers to permit MS compatibility and decrease electrostatic interactions. If electrostatic attraction is dominant, retention is decreased with increasing salt concentration; if electrostatic repulsion is dominant, retention is increased with increasing salt concentration. Additionally, salt concentration affects the thickness of the water layer. If the organic solvent concentration is high enough, the salt will partition into the water layer resulting in a more extensive immobilized aqueous layer that can increase analyte retention [8,26]. An ammonium acetate concentration range of 5-30 mM adjusted to pH 7 in the mobile phase was initially chosen. However, once the concentration exceeded 20 mM, the negatively charged analytes were retained for an excessive amount of time that is not reasonable for analysis. This can be explained by the buffer salt surrounding the negative silanol groups decreasing analyte electrostatic repulsion. Therefore, the highest concentration of ammonium acetate investigated was 20 mM which did provide near baseline resolution of the ten acids monitored. However, there is a significant decrease in retention factor and loss of resolution below 17.5 mM ammonium acetate. As the concentration of ammonium acetate decreases, there is also less salting out of the analytes from the mobile phase. The previous report using the sulfobetaine HILIC column showed a more gradual decrease in retention factor from 4-8 to 1-2 for mono-hydroxy benzoic acids as well as Van and Syr as the ammonium acetate decreased from 20 to 1 mM [26]. Retention of 2-HB was consistently low and retention of 4-HB was stronger in that

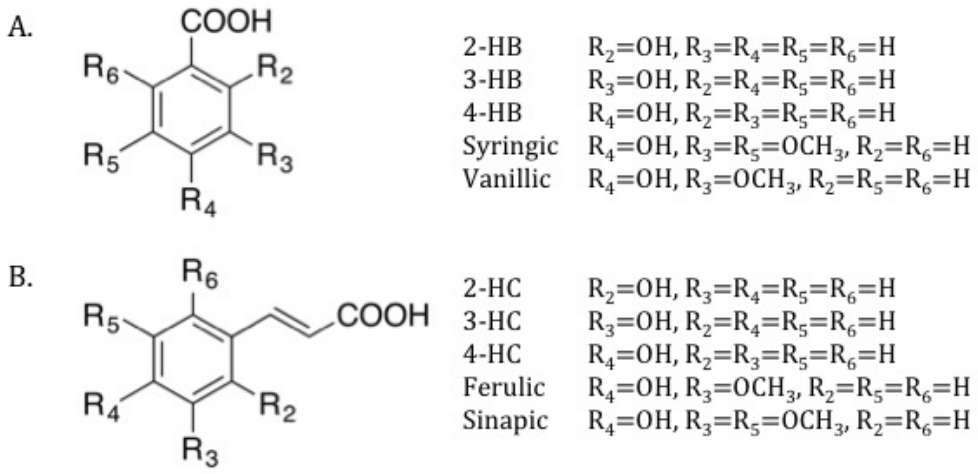

Figure 2.1. Structures of monohydroxybenzoic, syringic, vanillic acids (A) and monohydroxycinnamic, ferulic, and sinapic acids (B).

Table 2.1. List of carboxylic acids with corresponding dissociation constants

Acid Name	Abbreviation	pK_a	Peak #
2-Hydroxybenzoic	2-HB	2.97	1
3-Hydroxybenzoic	3-HB	4.06	2
4-Hydroxybenzoic	4-HB	4.48	3
2-Hydroxycinnamic	2-HC	4.04	4
3-Hydroxycinnamic	3-HC	4.44	5
4-Hydroxycinnamic	4-HC	4.10	6
Syringic	Syr	4.21	7
Vanillic	Van	4.52	8
Ferulic	Fer	4.42	9
Sinapic	Sin	4.25	10

study as well as ours. These trends for ortho and para substituted aromatic acids are expected considering normal phase LC; in our study, 2-HC also showed only modest retention but 4-HC was retained better.

Optimization of mobile phase pH

The pH of the mobile phase has been shown to have an effect on the retention factor of analytes in HILIC. Typically, a pH should be chosen so that all analytes are in their charged form as this increases their hydrophilicity and therefore increases their retention. Additionally, above a pH value of 4 or 5 the silanols on a silica phase will become deprotonated, leading to a strong negative charge on the stationary phase [8]. To investigate the effect of mobile phase pH on the aromatic carboxylic acids, 20 mM ammonium acetate adjusted to pH values 7 and below were chosen. Figure 2.2 displays the effect of mobile phase pH on the retention factor. According to the plot, a pH value of 5.0 shows the most separation, but in the chromatogram, the peaks were broad, and therefore there was little resolution. A pH value of 4 produced a more resolved chromatogram, but the analyte retention factor range is low. Furthermore, a pH value of 4 is very close to the pKa values of the acids, meaning most acids would not be fully protonated or deprotonated. A pH value of 6 provided the most resolution between all ten peaks in the mixture and was therefore chosen as the optimum mobile phase pH for this work. A previous pH comparison involving similar isomeric aromatic carboxylic acids also showed substantially higher retention factors at pH 7 as compared to pH 3 with the sulfobetaine column [26]. Figure 2.3 shows the resulting HPLC chromatogram at this optimized mobile phase of 90% MeCN/10% ammonium acetate, 20 mM, pH 6. With this mobile phase, eight analytes can be separated, though with limited baseline resolution. However, all of the analytes are able to be identified in this mixture. It is worth noting that ferulic and sinapic acids were not present in this chromatogram, however they elute at 12.4 and 15.6 minutes respectively and would therefore be fully resolved from the rest of the analytes (Figure 2.3 insert).

Because HILIC is a commonly used LC-MS mode, a buffer concentration that is considered compatible with MS should be sought. When developing a method that is MS compatible, the buffer concentration is definitely recommended to be below 25 mM and lower if possible for routine work [27]. One exception to this buffer limit is the ESI-MS study of noncovalent complexes of biomolecules [28]. However, for analytical LC-MS, the concentration of the ammonium acetate buffer in the mobile phase is normally 10 mM or less, even in gradient

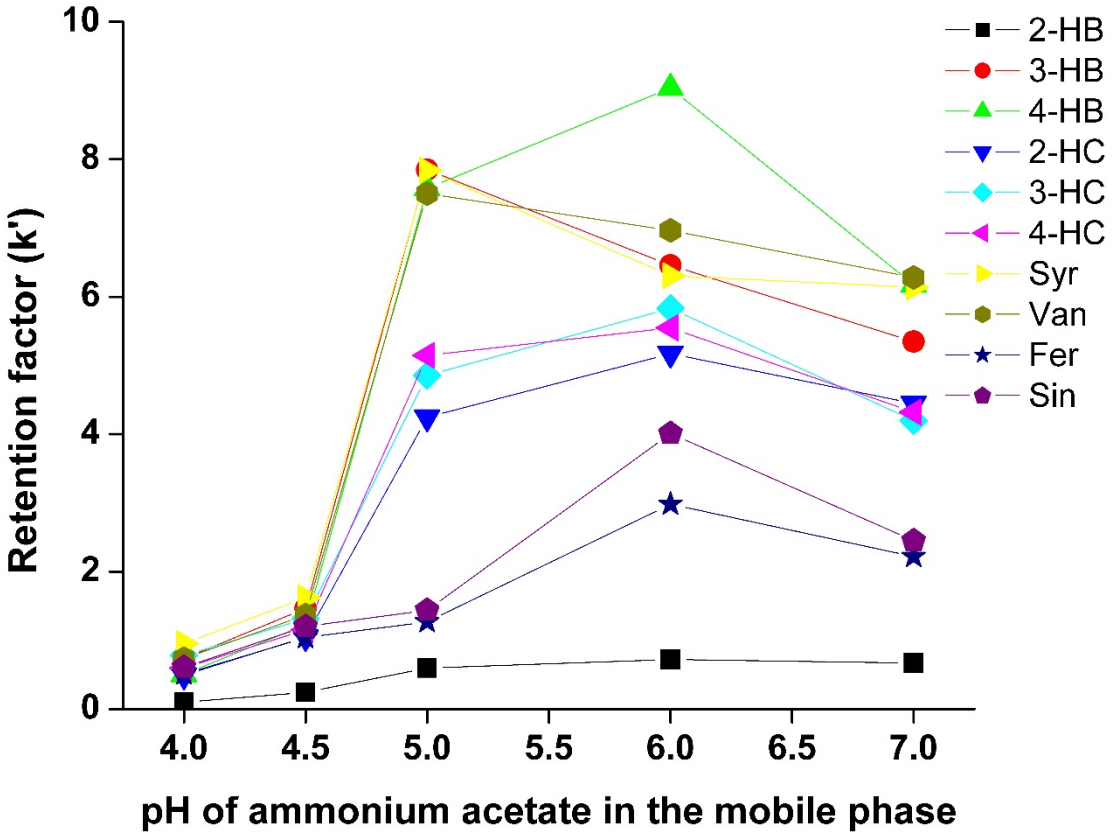

Figure 2.2. The influence of mobile phase pH on retention factor (k') with the Phenomenex Nucleosil silica column. k' values are averages of 3 replicates with % RSD values between 0.04 and 14%. Points are connected for clarity.

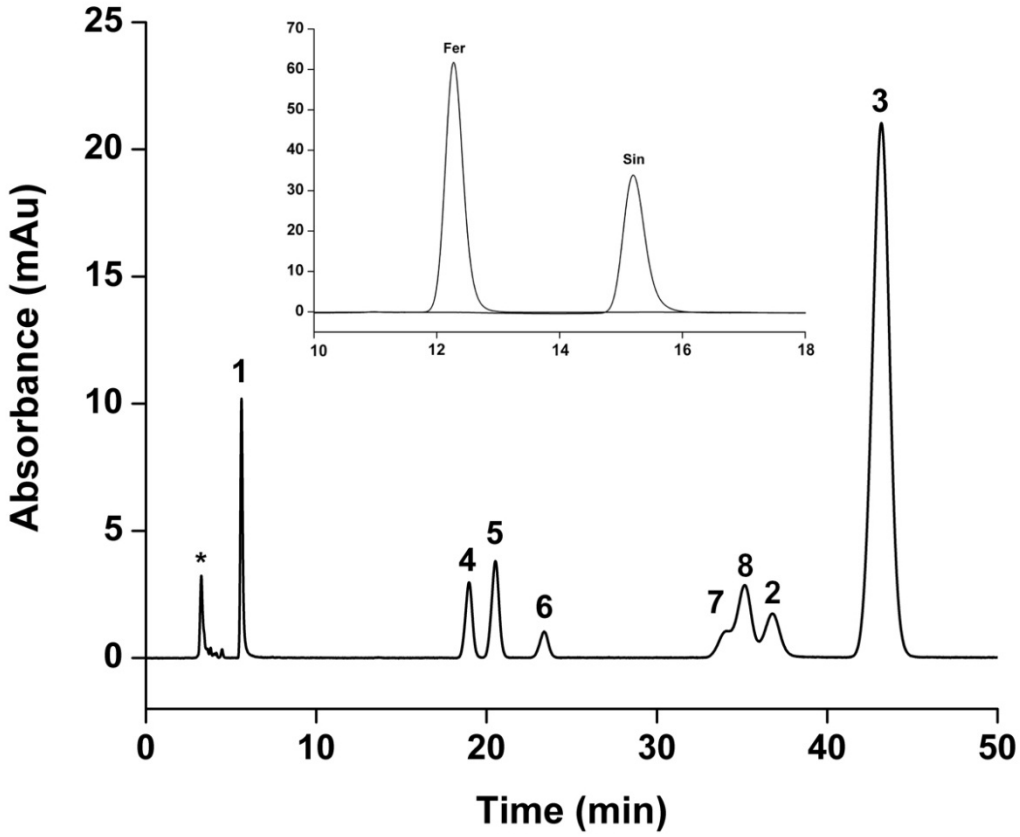

Figure 2.3. HPLC chromatogram of the eight analyte mixture. Fer and Sin were not present in this mixture but have retention times of 12.4 and 15.6 minutes respectively (see insert). The mobile phase was 90% MeCN/10% ammonium acetate, 20 mM, pH 6 with the Phenomenex Nucleosil silica column. Peak assignments are as given in Table 1.

runs [27,29]. When the ammonium acetate concentration was decreased to 10 mM, a similar trend with the retention factor as found with pH variation was indicated. There was increased resolution at pH 4, however this would require the mass spectrometer to be used in both positive and negative ion modes. Because of this, a pH above the pKa values of the analytes was chosen. A pH of 6 still provided some baseline resolution, but due to the decreased salt concentration, more analytes co-eluted. Figure 2.1S shows the HPLC chromatogram for injection of the eight analyte mixture using a mobile phase of 90% MeCN/10% ammonium acetate, 10 mM, pH 6. Because there were only four distinct peaks, further adjustment to the mobile phase is needed in order for this method to be LC-MS compatible.

Addition of pentane to mobile phase

Using a mobile phase that consists only of acetonitrile and 10 mM ammonium acetate was not shown to be sufficient for separation of the ten aromatic carboxylic acids using an ammonium acetate concentration of 10 mM at pH 6. Gritti and Guiochon have shown that the addition of a third solvent to the mobile phase in HILIC can alter the retention time, separation efficiency, and elution order. Solvents investigated included water, acetonitrile, ethanol, tetrahydrofuran (THF), and hexane [10]. The trend observed in reference 10 was confirmed as shown in Figure 2.2S. Three polar compounds were used as test compounds: toluene (as the unretained marker), cytosine, and nicotinic acid. The mobile phase consisted of 90% MeCN, 5% ammonium acetate, 20 mM, pH 6, and 5% of the modifier. The co-solvents investigated included water, ethanol, tetrahydrofuran, acetonitrile, hexamethyldisiloxane (HMDX), and pentane. Overall, it was confirmed that as the polarity of the modifier was decreased, the retention of the polar analytes was increased. HMDX was added to the possible third solvent list due to its unusual modifier behavior for normal phase LC [30] and pentane replaced hexane due to its increased solubility in water. The solubility of pentane in water is 45 mg/L while the solubility of hexane in water is only 6.5 mg/L. Based on Figure 2.2S, pentane was chosen as the third solvent to be investigated in this research because it provided the most resolution for the test compounds used. Since pentane is more nonpolar than acetonitrile, the distinction between the organic and aqueous phases in the mobile phase would likely be increased. This is known as a hydrophilicity gradient, where some compounds will prefer the aqueous layer while others will prefer the organic layer. Analytes that partition more into the aqueous layer will be retained longer while analytes that stay in the organic layer will have shorter retention times [10]. By forcing analytes

into either the organic layer or the water layer, some of the co-eluting analytes could likely be better resolved.

Before beginning the optimization of pentane in the mobile phase, the miscibility limit of the aqueous phase with pentane was determined. While ideally the aqueous phase would be kept at 10%, this was not possible when pentane comprised 3 or more percent of the mobile phase. Therefore, the aqueous phase was decreased to 5%. For an initial comparison, the analytes were separated using a mobile phase of 95% MeCN/5% ammonium acetate, 10 mM, pH 6. The resulting chromatogram showed a very poor resolution and peak shape (Figure 2.3S). The percentage of pentane was varied between 1% and 5% and the 10 mM ammonium acetate percentage was kept constant at 5% while the acetonitrile percentage was adjusted to accommodate the addition of pentane. The retention factor trend showed some not easily explained fluctuation over this % pentane range with the best peak resolution definitely noted at 5%. Figure 2.4A shows the HPLC chromatogram for eight acids with a mobile phase of 90% MeCN/5% 20 mM ammonium acetate, pH 6/5% pentane. Ferulic and sinapic acids eluted at 16.3 and 19.2 minutes respectively so they would still be fully resolved from the rest of the analytes (Figure 2.4A insert). These two acids were not resolved using a gradient RPLC method [24]. While there is not a large difference in resolution between Figure 2.3 and Figure 2.4A, the advantage of using pentane is easily seen when the ammonium acetate concentration is lowered to 10 mM. Figure 2.4B shows the HPLC chromatogram for eight acids with a mobile phase of 90% MeCN/5% 10 mM ammonium acetate, pH 6/5% pentane. Without pentane, there were only four peaks in the chromatogram. Although analysis time was sacrificed, seven of the eight acids are nearly baseline resolved. Again, ferulic and sinapic acid were not in this mixture but they elute at 13.9 and 16.4 minutes respectively (Figure 2.4B insert). The two acids that co-elute are 3-hydroxybenzoic acid and vanillic acid. Since these acids are not isomers, MS would be able to identify both acids in a mixture, though quantitation would be more difficult. Compatibility of this 90% MeCN/5% 10 mM ammonium acetate/5% pentane mobile phase with electrospray mass spectrometry is shown in Figure 2.4S for detection of 2-HB.

Although pentane as the third solvent provided the desired retention of these acids, two other organic solvents were also investigated to determine their effectiveness at separating these analytes. The aprotic solvent tetrahydrofuran (THF) and the protic solvent ethanol were chosen. The buffer component was kept at 10 mM, pH 6 since the lower ammonium acetate

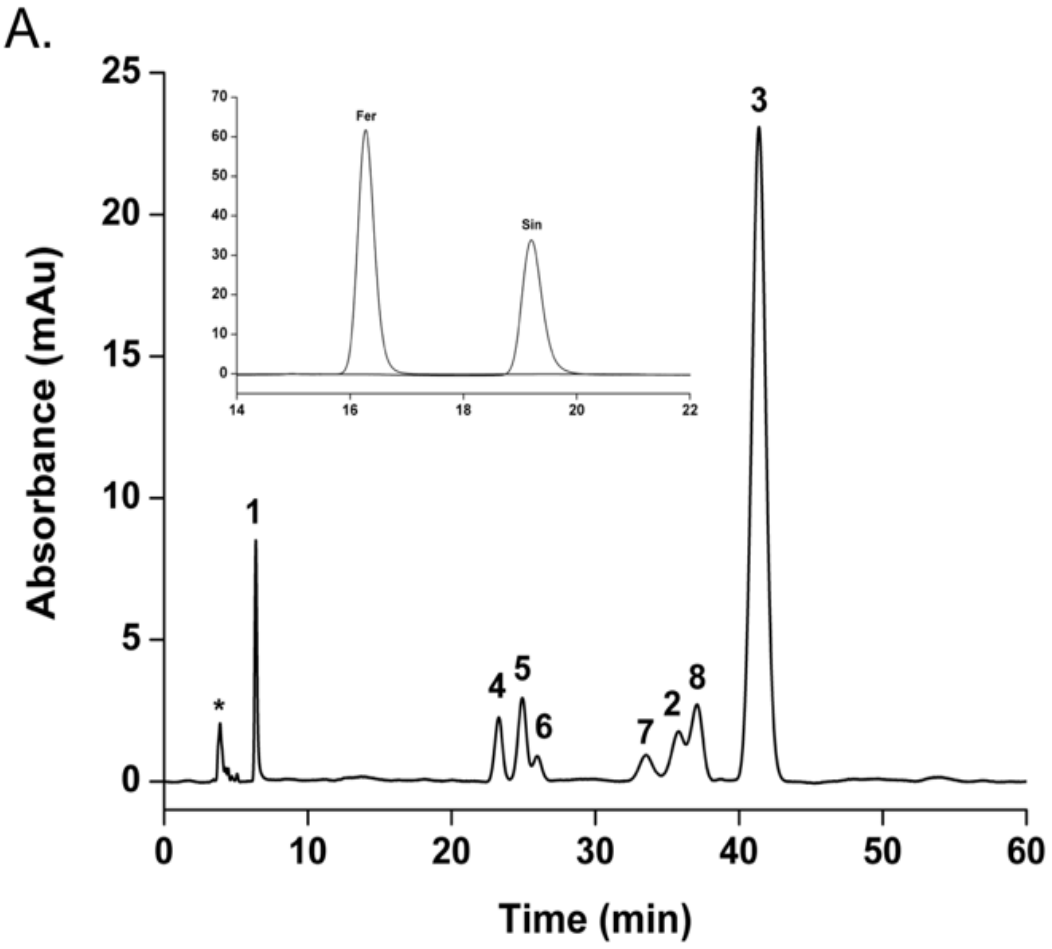

Figure 2.4A. HPLC chromatogram for injection of the eight analyte mixture. Fer and Sin were not present in this mixture but have retention times of 16.3 and 19.2 minutes respectively (see insert). The mobile phase was 90% MeCN/5% ammonium acetate, 20 mM, pH 6/5% pentane with a flow rate of 0.4 mL/min with the Phenomenex Nucleosil silica column. Peak assignments are as given in Table 1.

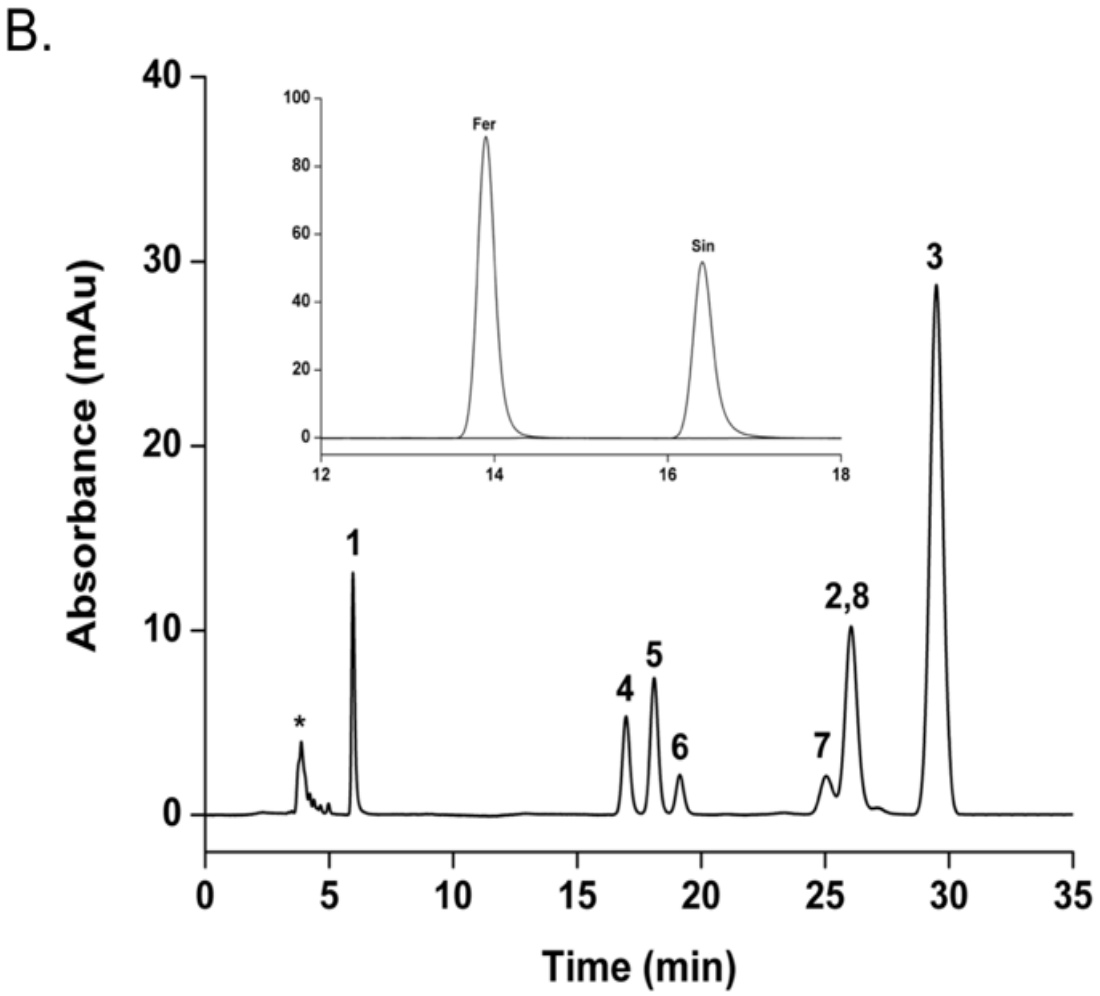

Figure 2.4B. HPLC chromatogram for injection of the eight analyte mixture. Fer and Sin were not present in this mixture but have retention times of 13.9 and 16.4 minutes respectively (see insert). The mobile phase was 90% MeCN/5% ammonium acetate, 10 mM, pH 6/5% pentane with a flow rate of 0.4 mL/min with the Phenomenex Nucleosil silica column. Peak assignments are as given in Table 1.

concentration showed a great difference when a third solvent was used. The HPLC chromatogram when 5% THF was used is shown in Figure 2.5A. The analysis time is similar to that of 5% pentane, though the resolution is slightly decreased. For this analysis, all ten analytes were used, which resulted in three sets of co-eluting pairs. The analytes that co-eluted were 4-hydroxybenzoic acid with ferulic acid, 4-hydroxycinnamic acid with vanillic acid, and 3-hydroxybenzoic acid with 3-hydroxycinnamic acid. The HPLC chromatogram when 5% ethanol was used is shown in Figure 2.5B. Again, all ten analytes were used in this analysis. There are still some resolution issues with the following analytes co-eluting: 2-hydroxycinnamic acid with vanillic acid, 3-hydroxybenzoic acid with sinapic acid, and 3-hydroxycinnamic acid with syringic acid. While it is possible that a different buffer concentration or pH could enhance resolution in these two chromatograms, pentane still showed the most improvement in resolution.

A van Deemter curve was generated using the optimum mobile phase that would be compatible with MS, 90% acetonitrile: 5% 10 mM ammonium acetate, pH 6: 5% pentane. Figure 2.6 shows the effect of mobile phase velocity on plate height for 2-hydroxybenzoic acid and vanillic acid. Peak height, H, was determined from the number of plates, N, calculated using the Foley-Dorsey equation. The flow rate range was 0.1-10 mL/min. The flow rate 2-HB did not show a large change in plate height as flow rate was increased, but it is also the least retained compound. In contrast, the analyte Van is well retained so the shape of the van Deemter curve shows a significant change in plate height at lower flow rates. From this plot, it was determined that a flow rate of 0.4 mL/min was the optimum flow rate. Once the optimum flow rate was confirmed, analytical figures of merit were then determined using the Nucleosil column. Using standards that ranged from 10-100 μM, calibration curves were generated for the following acids: 2-hydroxybenzoic, 2-hydroxycinnamic, ferulic, vanillic, 3-hydroxycinnamic, 3-hydroxybenzoic, and syringic acids. Table 2.2 shows the linearity and correlation values as well as the limits of detection and quantitation. The LOD values ranged from 2.97-7.14 ppm (36-113 pmole), and the LOQ values ranged from 7.72-19.7 ppm (93-376 pmole).

While there are many samples out there that this method could be applied to, the analysis of wine would be straightforward since it would likely only require syringe filtration before injection. Rose' wine in particular contains some of the acids separated in this work including 4-HB, 4-HC, Fer, Van, and Syr. These acids, found in concentrations ranging from 1.24 to 7.6 mg/kg, are all well separated from each other meaning quantitation of these acids in rosé wine

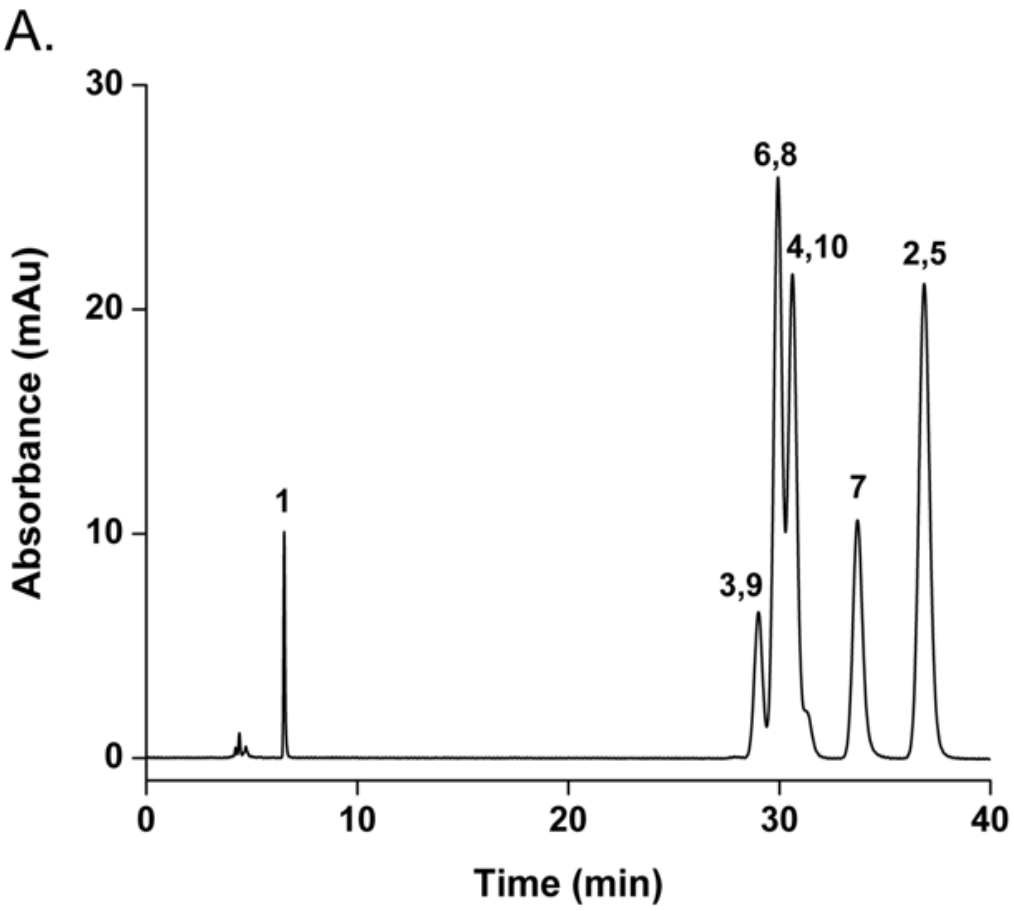

Figure 2.5A. HPLC chromatogram of the ten phenolic acid mixture. The mobile phase was 90% MeCN/5% ammonium acetate, 10 mM, pH 6/5% THF with a flow rate of 0.4 mL/min with the Phenomenex Nucleosil silica column. Peak assignments are as given in Table 1.

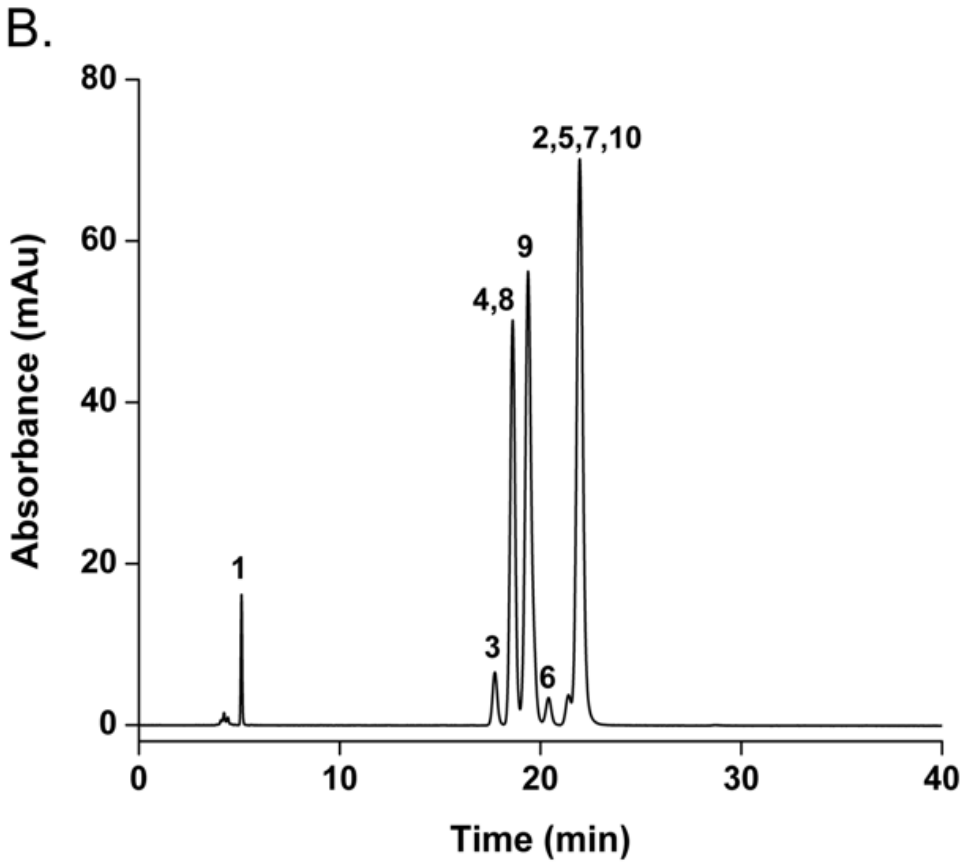

Figure 2.5B. HPLC chromatogram of the ten phenolic acid mixture. The mobile phase was 90% MeCN/5% ammonium acetate, 10 mM, pH 6/5% ethanol with a flow rate of 0.4 mL/min with the Phenomenex Nucleosil silica column. Peak assignments are as given in Table 1.

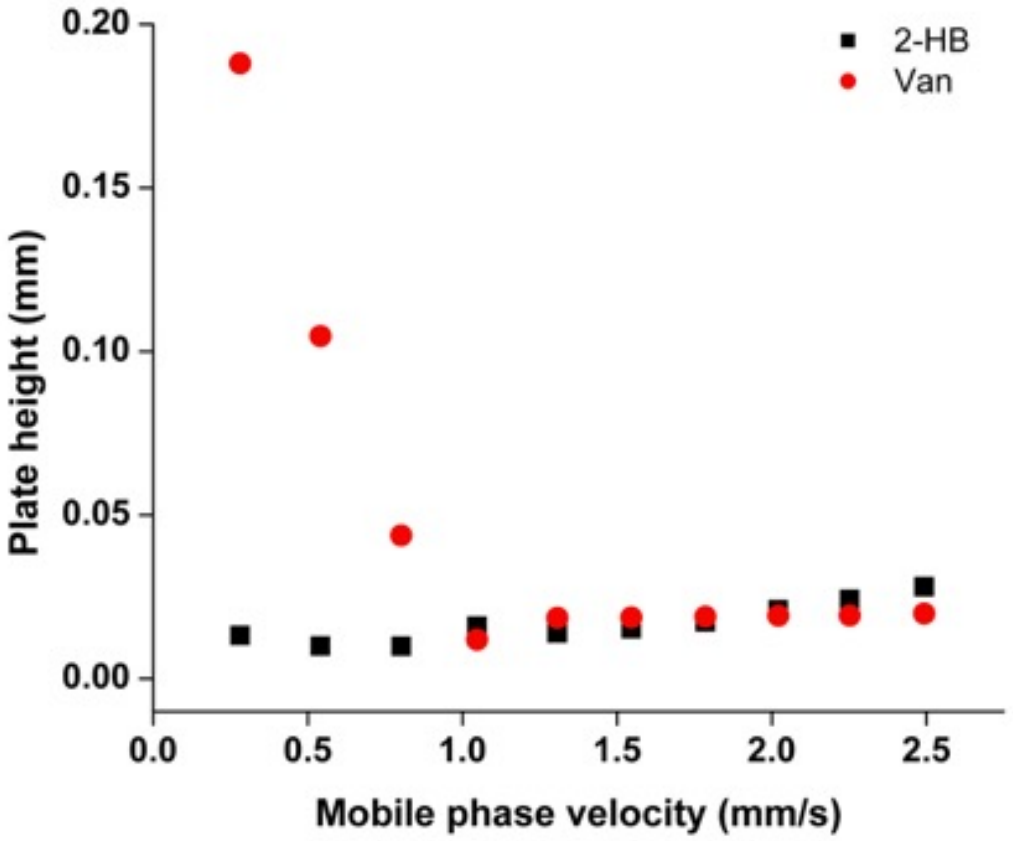

Figure 2.6. Comparison of the van Deemter plots for 2-HB (■) and Van (●) with the Phenomenex Nucleosil silica column.

Table 2.2. Analytical figures of merit including limit of detection, limit of quantitation, linearity, and R^2 values for all monohydroxy acids.

Compound	t_R[a]	A[b]	B[c]	R^2	LOD[d]	LOQ[e]
2-HB	6.12	0.01869	0.007133	0.9996	69.9	144
Fer	13.91	0.08151	0.05972	0.9995	37.9	93.7
3-HC	18.01	0.04507	0.02555	0.9975	86.9	240
4-HC	19.39	0.03495	0.03726	0.9996	36.2	94.1
Syr	24.92	0.06525	0.03995	0.9985	58.2	155
4-HB	29.07	2.440×10^{-5}	0.002821	0.9949	113	376

[a] Retention times are given as the average of three replicates with RSDs between 0% and 0.52%

[b] A values are given as the y-intercept (signal units) from the linear regression

[c] B values are given as the slope (signal/ppm) from the linear regression

[d] Detection limit values are given in pmoles and were calculated using the following equation: $LOD=(A*3*A_\sigma)/B$, where A_σ is the standard deviation of the y-intercept

[e] Quantitation limit values are given in pmoles and were calculated using the following equation: $LOQ=(A*10*A_\sigma)/B$, where A_σ is the standard deviation of the y-intercept

should be feasible. Rye crispbread is another potentially interesting sample that could be easily analyzed using the described method. The solutes Fer and Van appear in this type of bread and are well separated using this HILIC method. The sample preparation for the bread would include pooling and homogenization prior to injection. These acids have been found in concentrations ranging from 8.5 to 1099 mg/kg [24].

Comparison of plain silica columns

Because bare silica columns can vary in retentive character from manufacturer to manufacturer, it was important to compare this method to other bare silica columns from different manufacturers. The optimization of the ammonium acetate concentration and pH as well as the optimization of the percentage of pentane was also completed on a Grace silica column and these data were identical to that collected on the Nucleosil silica column having the same dimensions and particle size. The optimum mobile phase determined on each column was also the same (90% MeCN/5% ammonium acetate, 10 mM, pH 6/5% pentane). Figure 2.5S shows an HPLC chromatogram of eight acids on the Grace silica column using the optimized mobile phase. The effects of ammonium acetate concentration, ammonium acetate pH, and percentage of pentane on retention factor using the Agilent Poroshell 120 HILIC column that had identical dimensions but slightly smaller particles are shown in Figures 2.6SA, 2.6SB, and 2.6SC respectively. The effect of k' with salt concentration was more of a steady rise as compared to that found on the Nucleosil column, with peak resolution on the Agilent column definitely improving at 15 mM. Although pH 6 showed the highest analyte retention, pH 7 provided near baseline resolution of the ten acids with a slightly shorter analysis time. The percentage of pentane was varied from 1% to 5% on the Agilent column as well. While the plot does not show any extreme differences, the chromatogram for 3% pentane provided the best resolution and peak shape. Therefore, the Agilent column yielded an optimum mobile phase of 92% MeCN/5% ammonium acetate, 10 mM, pH 7/3% pentane. A van Deemter curve, shown in Figure 2.7SA, was made. The flow rate range was 0.1 – 1.0 mL /min. The optimum flow rate on the Agilent column was also determined to be 0.4 mL/min. Finally, a Thermo Scientific HILIC column was used to compare this method on a column with smaller dimensions and particle sizes. The optimum mobile phase from the Nucleosil column was used on the Thermo column. A van Deemter curve, shown in Figure 2.7SB, was made between 0.1 and 1.0 mL/min and the optimum flow rate was determined to be 0.3 mL/min. The resulting chromatograms from the Agilent and

Thermo Scientific columns are shown in Figures 2.7A and 2.7B, respectively. The Agilent column showed similar analysis times compared to the Nucleosil column with similar resolution. Out of the ten analytes, eight are almost completely resolved. While the Thermo Scientific column showed a great decrease in analysis time, the resolution was diminished. It is likely that the mobile phase used is not quite the optimum for this column. However, the lack of resolution may be due primarily to the loss of plate count.

2.6: Conclusions

An isocratic HILIC method for separating two classes of hydroxy aromatic carboxylic acids, each containing two sets of isomers, has been developed. Increasing ammonium acetate concentration, ammonium acetate pH, and % pentane in the mobile phase have all tended to increase analyte retention. The method was optimized to be potentially compatible with MS detection by lowering the ammonium acetate concentration and raising the % pentane in the mobile phase. Quantitative linearity data was collected for all ten acids being studied. This method offers a simple, isocratic separation for structurally related polar aromatic acids that may be found at low ppm concentrations. However, when using the same mobile phase, changes in peak resolution between different silica columns was noted. This needs to be addressed in a future study perhaps with an expansion of the type of nonpolar second organic solvent. A similar study to this one using a bonded phase HILIC column should also prove interesting.

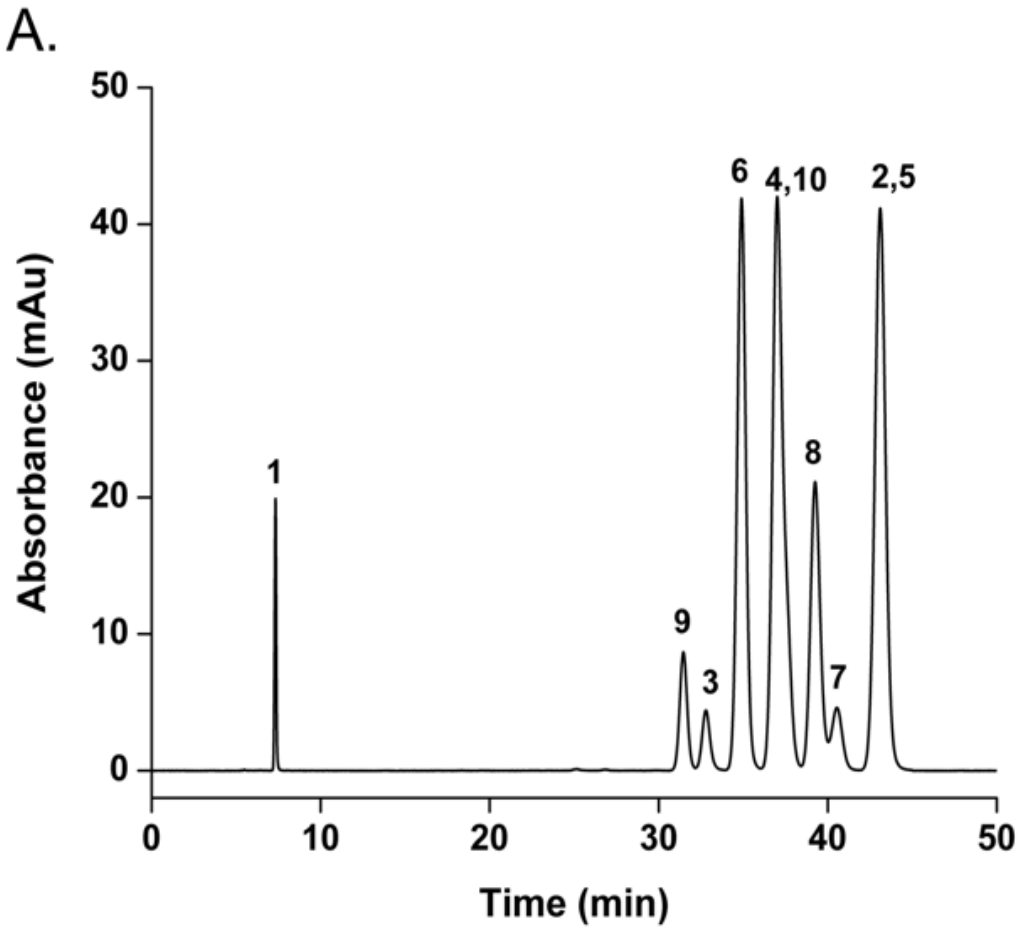

Figure 2.7A. HPLC chromatogram of the ten phenolic acid mixture on the Agilent Poroshell 120 HILIC column. The mobile phase was 92% MeCN/5% ammonium acetate, 10 mM, pH 7/3% pentane with a flow rate of 0.4 mL/min. Peak assignments are as given in Table 1.

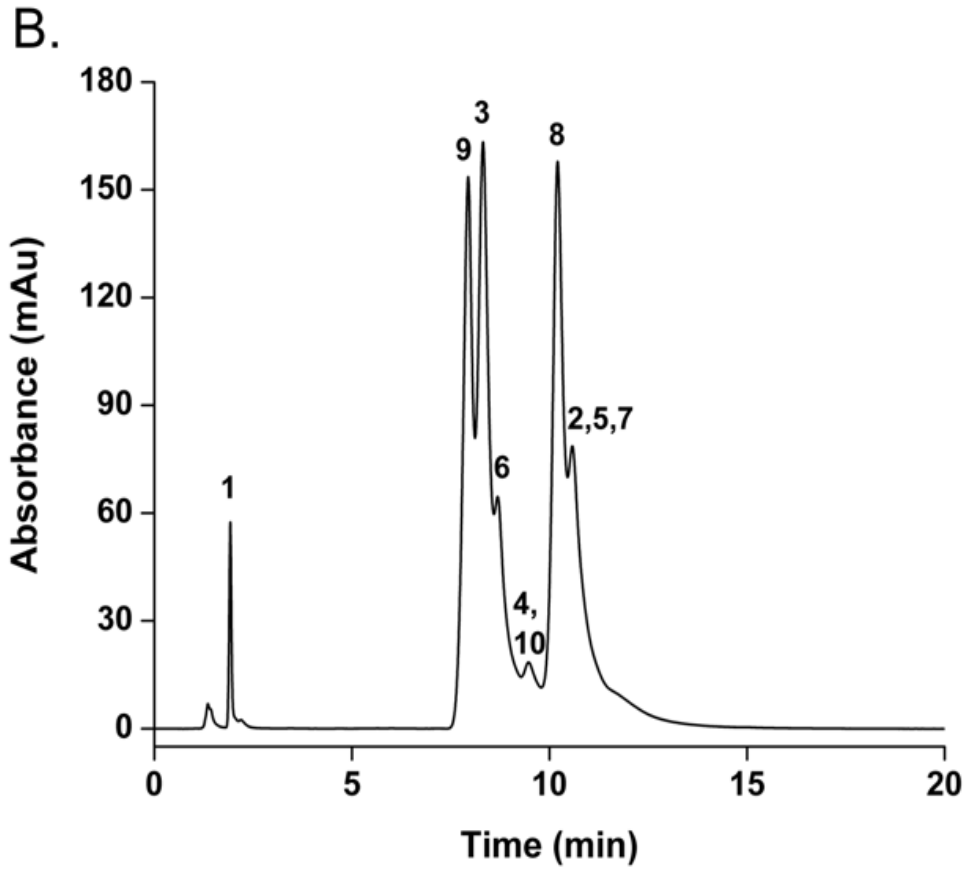

Figure 2.7B. HPLC chromatogram of the ten phenolic acid mixture on the Thermo Scientific Accucore HILIC column. The mobile phase was 90% MeCN/5% ammonium acetate, 10 mM, pH 7/5% pentane with a flow rate of 0.4 mL/min. Peak assignments are as given in Table 1.

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2.8: Supplemental Information

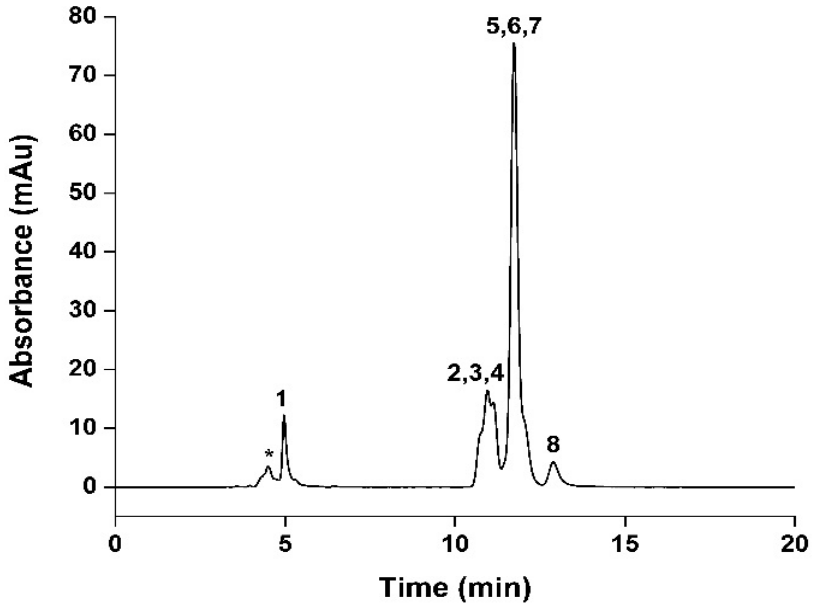

Figure 2.1S. HPLC chromatogram for injection of the eight analyte mixture. Fer and Sin were not present in this mixture but have retention times of 10.0 and 10.8 minutes respectively. The mobile phase was 90% MeCN/10% 10 mM ammonium acetate, pH 6. Peak assignments are as follows: (1) 2-HB, (2) 3-HC, (3) 3-HB, (4) 4-HC, (5) 2-HC, (6) 4-HB, (7) Van, (8) Syr.

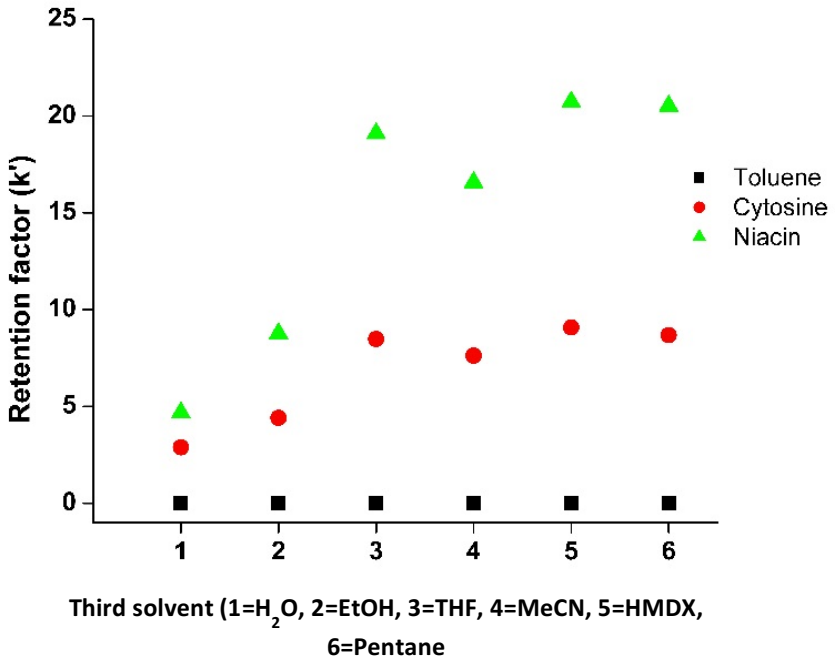

Figure 2.2S. The effect of third solvent in the mobile phase on retention factor for toluene, cytosine, and niacin. The mobile phase was 90% MeCN/5% ammonium acetate, 20 mM, pH 6/5% third solvent.

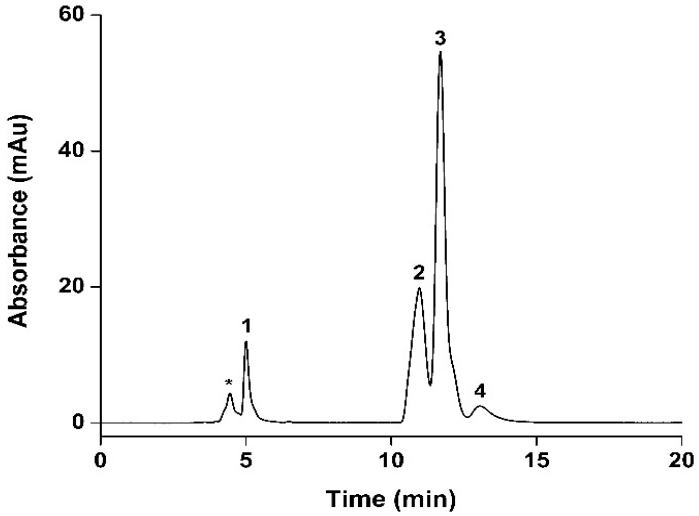

Figure 2.3S. HPLC chromatogram for injection of the eight analyte mixture. The mobile phase was 95% MeCN/5% ammonium acetate, 10 mM, pH 6 with a flow rate of 0.4 mL/min. Peak assignments are as follows: (1) 2-HB, (2) 2-HC, 3-HC, 4-HC, (3) Syr, 3-HB, 4-HB (4) Van.

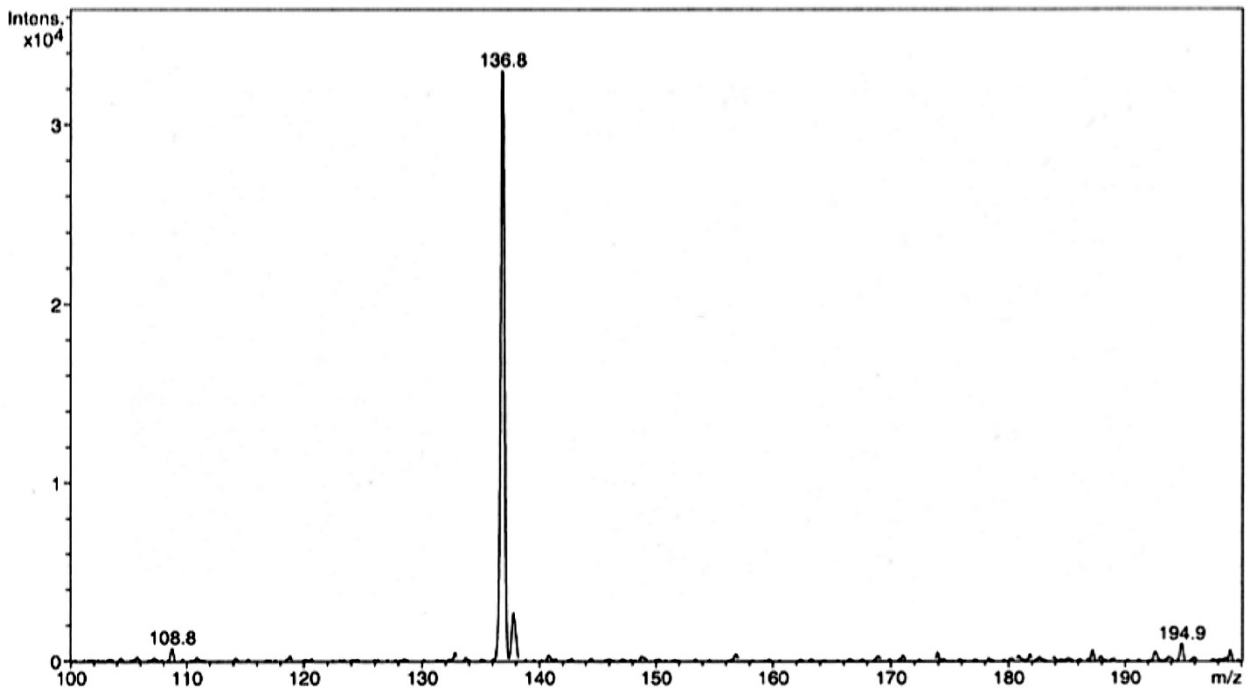

Figure 2.4S. Mass spectrum of o-HB, 0.066 mg/L. This spectrum was taken in the mobile phase 90% MeCN/5% ammonium acetate, 10 mM, pH 4/5% pentane. Mass spectrum was collected on a Bruker Esquire-LC ESI. The following conditions were used: negative ion mode, nebulizer 7.0 psi, dry gas 6.00 L/min, desolvation temperature 310°C, capillary voltage 4 kV, cone voltage 40 V.

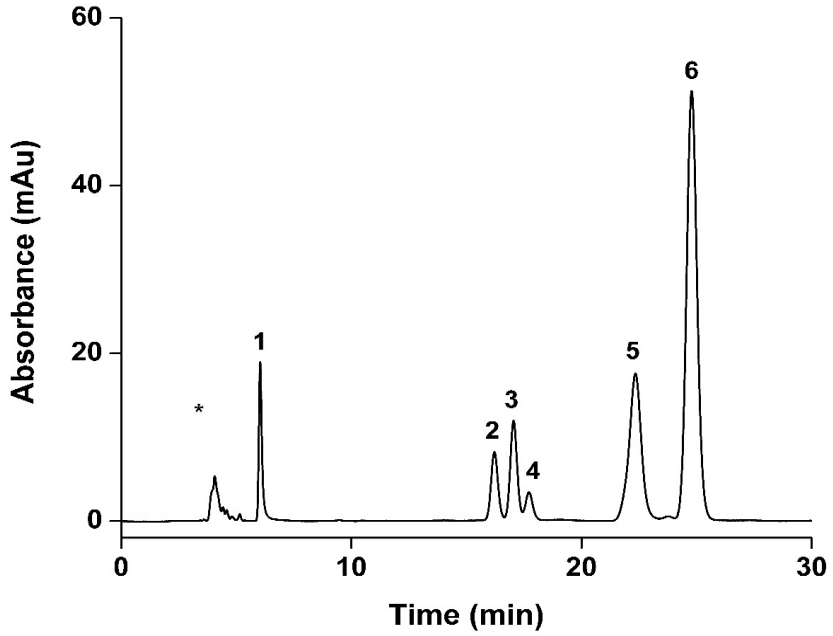

Figure 2.5S. HPLC chromatogram of the eight analyte mixture on the Grace silica column. Fer and Sin were added later and were never tested on this column. The mobile phase was 90% MeCN/5% ammonium acetate, 10 mM, pH 6/5% pentane with a flow rate of 0.4 mL/min. Peak assignments are as follows: (1) 2-HB, (2) 2-HC, (3) 3-HC, (4) 4-HC, (5) Syr, 3-HB, Van, (6) 4-HB).

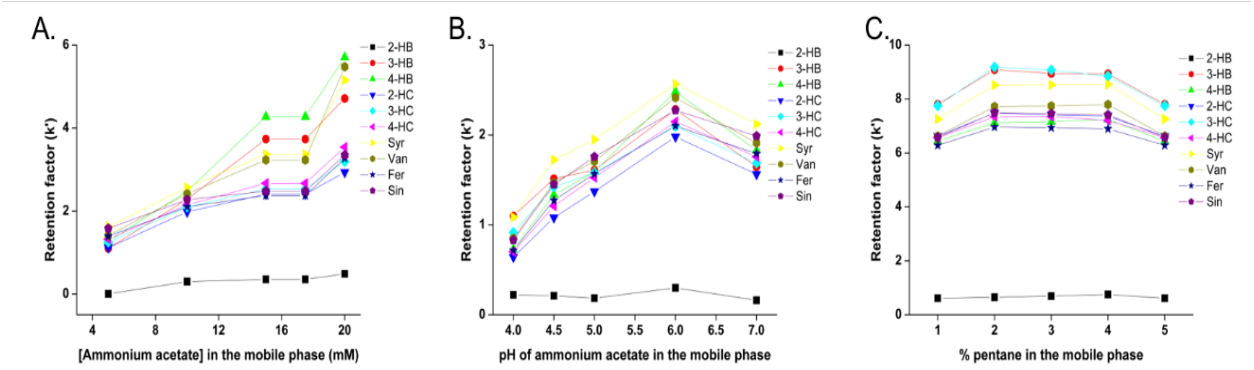

Figure 2.6S. The effect of ammonium acetate concentration (A), pH, 10 mM ammonium acetate (B), and % pentane in the mobile phase (C) on retention factor (k') using the Grace column. k' values given are an average of triplicate measurements with % RSDs between 0% and 15% (A), 0% to 3.4% (B), and 0.68% to 16% (C).

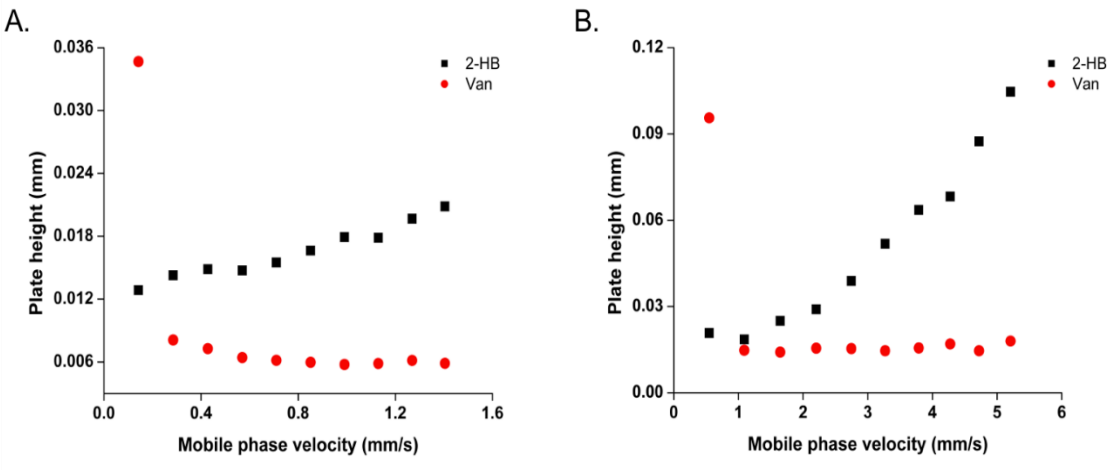

Figure 2.7S. Comparison of the van Deemter plots for 2-HB and Van on the Agilent Poroshell column (A) and the Thermo Accucore column (B).

CHAPTER 3

Micellar Liquid Chromatography of Terephthalic Acid Impurities

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3.1: Abstract

The production of terephthalic acid (TPA) by oxidation of p-xylene is an important industrial process because high purity TPA is required for the synthesis of polyethylene terephthalate, the primary polymer used to make plastic beverage bottles. Few separation methods have been published that aim to separate TPA from eight major aromatic acid impurities. This work describes a "green" micellar liquid chromatography (MLC) method using a C18 column (100 x 4.6 mm, 3 μm), an acidic 1% sodium dodecyl sulfate (SDS) mobile phase, and a simple step flow rate gradient to separate TPA and eight impurities in less than 20 minutes. The resulting chromatogram shows excellent peak shape and baseline resolution of all nine acids, in which there are two sets of isomers. Partition coefficients and equilibrium constants have been calculated for the two sets of isomers by plotting the reciprocal of the retention factor versus micelle concentration. Quantitation of the nine analytes in an actual industrial TPA sample is possible. Limits of detection for all nine acids range from 0.180 to 1.53 ppm (2.16 to 19.3 pmoles) and limits of quantitation range from 0.549 to 3.45 ppm (6.48 to 43.0 pmoles). In addition, the method was tested on two other reversed phase C18 columns of similar dimensions and particle diameter from different companies. Neither column showed quite the same peak resolution as the original column, however slight modifications to the mobile phase could improve the separation.

3.2: Introduction

Pioneered in the late 1970s, micellar liquid chromatography (MLC) has grown to be a well-studied branch of chromatography [1–4]. It gained popularity because the method uses little to no organic solvent. Instead the mobile phase consists of an aqueous surfactant above the surfactant's critical micelle concentration (CMC). Common surfactants used include sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and Brij-35 [5]. The choice

of surfactant is dictated by the compounds being determined, but SDS is most commonly used. Since MLC involves a reversed phase retention mode, the stationary phases commonly used are C18 and C8 [4]. For hydrophobic analytes such as polyaromatic hydrocarbons, shorter chain stationary phases can minimize the problem of excessive retention factors [6]. A C1 column was shown to increase the selectivity of moderately hydrophobic analytes such as phenol, benzene, and p-nitroaniline [7]. The retention mechanism for MLC has been well studied. Armstrong and Nome described the mechanism by using a three-process model. The three processes include partitioning between the stationary phase and the bulk water, the stationary phase and the micelle, and the micelle and the bulk water. Many different factors can affect these equilibria including temperature, pH, ionic strength, type and concentration of the surfactant, and salt or organic modifiers [8,9]. In an aqueous mobile phase, hydrophobic interaction between the alkane (often C18) stationary phase and the alkyl surfactant group is pronounced [10]. This modified stationary phase with the exposed polar group has a major role in the selectivity of the hydrophobic and possible electrostatic or polar retention mechanisms.

To ensure wettability of the stationary phase by the aqueous mobile phase to gain efficiency, both a higher temperature, such as 40°C, and the addition of an organic solvent, such as an alcohol, have been considered [11,12]. Acetonitrile and propanol (3-5%) are considered the most effective solvents but the "green" solvent ethanol has also been used [13–15]. However, there are MLC studies in which the use of an organic solvent in the mobile phase provided no significant advantage. A completely aqueous mobile phase has been used to determine various biological components like melamine [16,17], anticancer compounds [18], antiretroviral drugs [19], and acetaminophen [20]. Additionally, MLC with a completely aqueous mobile phase has been used to model permeability of skin [21] and evaluate biogenic amines in fish sauce [22].

Terephthalic acid (TPA) is polymerized to make polyethylene terephthalate (PET), a polymer commonly used in the production of beverage bottles. TPA is made by reacting p-xylene with dioxygen in acetic acid in the presence of a metal catalyst [23]. Purity of the starting material is crucial to the production of PET because it is difficult to purify once made. The presence of impurities leads to discoloration and degradation of the polymer [24]. The oxidation of p-xylene leads to two impurities: benzoic acid (BA) and p-toluic acid (p-TA) due to excessive oxidation and 4-formylbenzoic acid (FBA) due to partial oxidation. Beyond just the oxidation of p-xylene introducing impurities, the presence of o-xylene and m-xylene may introduce other impurities as

well including isophthalic acid (IPA), o-phthalic acid (OPA), trimesic acid (TSA), trimellitic acid (TMA), and hemimellitic acid (HMA) [25–27]. Currently, there are few methods to separate TPA and these eight impurities found during the production of PET. Huang et al. used microemulsion electrokinetic chromatography (MEEKC) to separate all nine acids. By varying the microemulsion component, SDS, they were able to separate the nine acids in under 22 minutes, though with limited resolution [25]. Yuan et al. used HPLC-UV to separate the nine acids in under 60 minutes through the use of a quite complex methanol-water-buffer gradient on a C18 column [28].

The aim of this research is to develop an isocratic MLC method to separate TPA and eight impurities (structures in Figure 1) found during the production of PET. This method, using a C18 column and a SDS mobile phase, could resolve both standard and industrial samples with baseline peak resolution in under 20 minutes, a definite improvement over the current reversed phase gradient HPLC methods that take nearly an hour to resolve these samples. This "green" method does not use any organic solvent modifier or mobile phase gradient but instead utilizes a flow rate gradient to optimize separation. This method was also checked on two other HPLC columns to determine reproducibility across column manufacturers.

3.3: Apparatus and Conditions

Chromatographic analyses were performed on a Thermo Scientific UltiMate 3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA) equipped with a pump, online degasser, autosampler, temperature-controlled column oven, and multiple wavelength UV detector. Chromeleon 7.2.1 software (Thermo Scientific, Sunnyvale, CA, USA) was used for instrument control and data acquisition. For the method development and optimization, a Waters X-Bridge column (Waters Corporation, Milford, MA, USA) (100 x 2.1 mm, 3.5 μm) was used. This endcapped column has a 18% carbon loading, 130 Å pore size, and a surface area of 185 m^2/g. Once the method had been developed and optimized, two different C18 columns were used to validate the method: a Phenomenex Gemini C18 column (Torrance,
CA, USA) (100 x 2 mm, 3 μm) and a Thermo Scientific Accucore C18 column (Sunnyvale, CA, USA) (100 x 2.1 mm, 2.6 μm). The Phenomenex Gemini C18 column is an endcapped column that has a 14% carbon loading, pore size of 110 Å, and a surface area of 375 m^2/g. The Thermo Scientific Accucore C18 column is also an endcapped column that has an 8% carbon loading, pore size of 80 Å, and a surface area of 130 m^2/g.

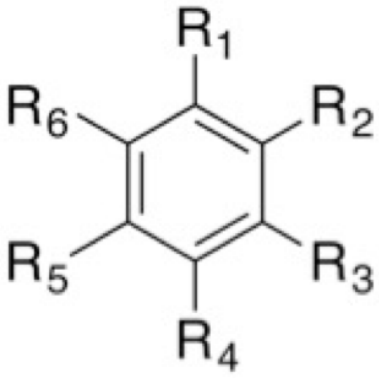
A step gradient was incorporated to fully resolve all nine acids. The flow rate gradient was set to the following for the HPLC methods: 0.1 mL/min for 0.00-1.00 min, 0.3 mL/min for 1.00-7.00 min with a 1.00 min ramp, 0.5 mL/min for 7.00-20.00 min with a 1.00 min ramp. A 2.00 min equilibration step was initially added prior to each injection to ensure the flow rate was consistent throughout the entire column, however it was determined that this is not necessary. A 2 μL injection size was used. The column was kept at ambient temperature. UV detection was collected at 210, 240, and 254 nm, however 240 nm showed greater response for most analytes.

3.4: Chemicals and Procedures

All compounds analyzed were purchased from Sigma Aldrich (St. Louis, MO, USA) and were dissolved in solution using water rated at 18.2 MΩ from a Milli-Q water purification system (Millipore, Bedford, MA, USA) that distilled and deionized the water and enough dilute sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA) to dissolve the acids. Sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA) and sodium dodecyl sulfate (Sigma Aldrich, St. Louis, MO, USA) were used to make the mobile phase. During optimization, mobile phase pH was adjusted using sulfuric acid, citric acid (Fisher Scientific, Fair Lawn, NJ, USA), and sodium hydroxide. Mobile phases were made using water from the Milli-Q water purification system. To generate a mobile phase with a pH of 2, 1.84 mM sulfuric acid (pH 3) was acidified slightly. A mobile phase with a pH of 4 was created using citric acid. To make a mobile phase with a pH of 6, only water was used to make the SDS mobile phase. The optimum mobile phase was 1% SDS or 3.5×10^{-2} M SDS at pH 3.

Mixtures were chosen based on structural similarity (Figure 3.1) and are shown in Table 3.1. The first mixture consisted of isophthalic, phthalic, and terephthalic acids (IPA, OPA, TPA) at approximately 40 ppm concentration. The second mixture consisted of benzoic, p-toluic, and 4-formylbenzoic acids (BA, p-TA, FBA) at approximately 35 ppm concentration. The third mixture consisted of trimesic, trimellitic, and hemimellitic acids (TSA, TMA, HMA) at approximately 50 ppm concentration. The fourth and final mixture combined all nine acids.

Five standard solutions were made so that each analyte had the following concentrations: 1, 10, 25, 50, and 75 ppm. These standards were used to create a calibration curve. Both a simulated real sample and an oxidized TPA mother liquor sample from a commercial purified



Isophthalic	$R_1=R_3=COOH, R_2=R_4=R_5=R_6=H$
Terephthalic	$R_1=R_4=COOH, R_2=R_3=R_5=R_6=H$
o-Phthalic	$R_1=R_2=COOH, R_3=R_4=R_5=R_6=H$
Benzoic	$R_1=COOH, R_2=R_3=R_4=R_5=R_6=H$
p-Toluic	$R_1=COOH, R_4=CH_3, R_2=R_3=R_5=R_6=H$
4-Formylbenzoic	$R_1=COOH, R_4=CHO, R_2=R_3=R_5=R_6=H$
Trimesic	$R_1=R_3=R_5=COOH, R_2=R_4=H$
Trimellitic	$R_1=R_2=R_4=COOH, R_3=R_5=R_6=H$
Hemimellitic	$R_1=R_2=R_3=COOH, R_4=R_5=R_6=H$

Figure 3.1. Structure assignments for aromatic carboxylic acids

Table 4.1. List of aromatic carboxylic acid analytes with corresponding abbreviations and dissociation constants

Mixture	Acid Name	Abbreviation	pK_a
Mix 1	Terephthalic	TPA	3.51, 4.82
	o-Phthalic	OPA	2.98, 5.28
	Isophthalic	IPA	3.46, 4.46
Mix 2	p-Toluic	p-TA	4.36
	4-Formylbenzoic	FBA	3.78
	Benzoic	BA	4.17
Mix 3	Trimesic	TSA	3.12, 3.89, 4.70
	Trimellitic	TMA	2.52, 3.82, 5.20
	Hemimellitic	HMA	2.80, 4.20, 5.87

terephthalic acid (PTA) plant were analyzed. Both samples were filtered using a 0.22 μm nylon syringe filter, diluted by a factor of one hundred, and separated using the step gradient on the HPLC column. The concentration of the acids in the industrial sample were determined using the standard calibration curve data.

3.5: Results and Discussion

Optimization of separation parameters

Optimization of the mobile phase began by varying the concentration of SDS in the mobile phase at pH 3. This pH value was chosen based on previous work to separate aromatic carboxylic acids using micellar UHPLC [29]. Although the C18 columns were coated with SDS prior to use, the addition of SDS to the mobile phase has been shown to stabilize both the stationary phase coating as well as the micelles in the mobile phase [29]. SDS concentrations were chosen to be both below and above its CMC, 0.23% (w/w) or 8.1×10^{-3} M SDS, to determine the effect of this parameter on separation efficiency and retention factor. It has been shown previously that as the percentage of SDS in the mobile phase is increased, retention of analytes decreases.

Figure 3.2 shows the effect that varying percentages of SDS had on the retention factor at pH 2 for Mix 1 (A) and Mix 3 (B) and at pH 3 for Mix 1 (C) and Mix 3 (D). Under both pH values, there is a general downward trend of retention factor with increasing % SDS in the mobile phase. This is consistent with previous work in our lab [29]. While the retention factor data for pH 2 shows much lower retention factors, the peak shapes were much poorer using a mobile phase at pH 2. A pH value of 3 showed longer retention but excellent peak shape and resolution. Figure 3.3 shows the effect that varying percentages of SDS had on the retention factor at pH 4 for Mix 1 (A) and Mix 3 (B) and at pH 6 for Mix 1 (C) and Mix 3 (D). Both of these mobile phases showed poor separation of the analytes. At a pH value above 4, the majority of the analytes are deprotonated, giving them a negative charge. Previously, it has been reported the pKa values of aliphatic carboxylic acids (C3-C6), about 4.7 in aqueous solution, remain about the same in 0.01 M SDS but do increase to 4.8-5.3 with increasing chain length in 0.1 M SDS [30]. Therefore, the decrease in retention for all analytes at pH values of 4 and 6 compared to that observed at lower pH values is likely due to the repulsion of the analytes by the negatively charged sulfate group in SDS. The anionic group is present on the SDS absorbed on to the stationary phase, the outside of the liquid phase SDS micelles, as well as SDS monomer in the

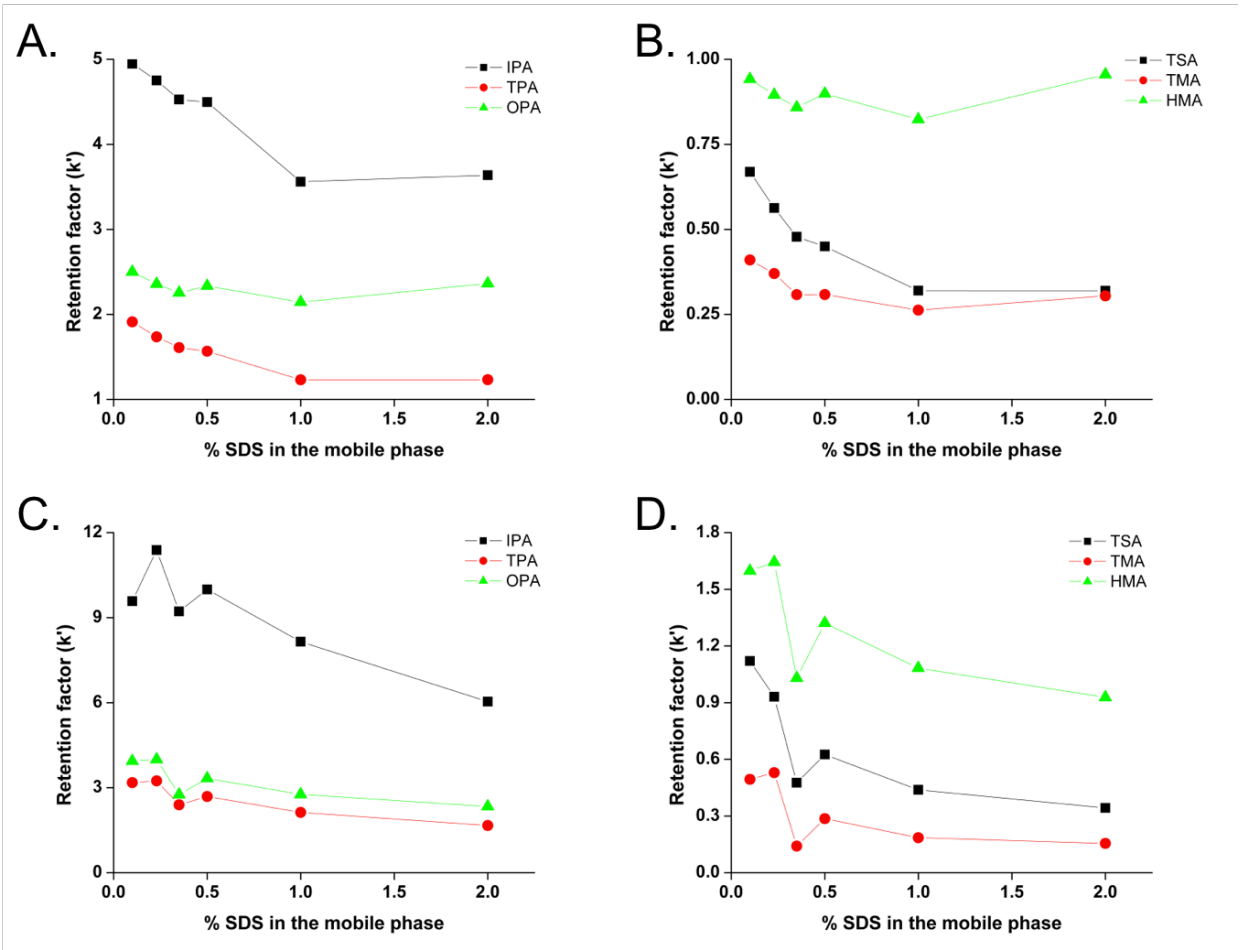


Figure 3.2. The effect of the % SDS in the mobile phase on the retention factor (k') for Mix 1 and Mix 3 at pH 2 (A and B) and pH 3 (C and D). k' values given are an average of 3 replicates with %RSDs between 0 and 1.76% for pH 2 and %RSDs between 0 and 13% for pH 3.

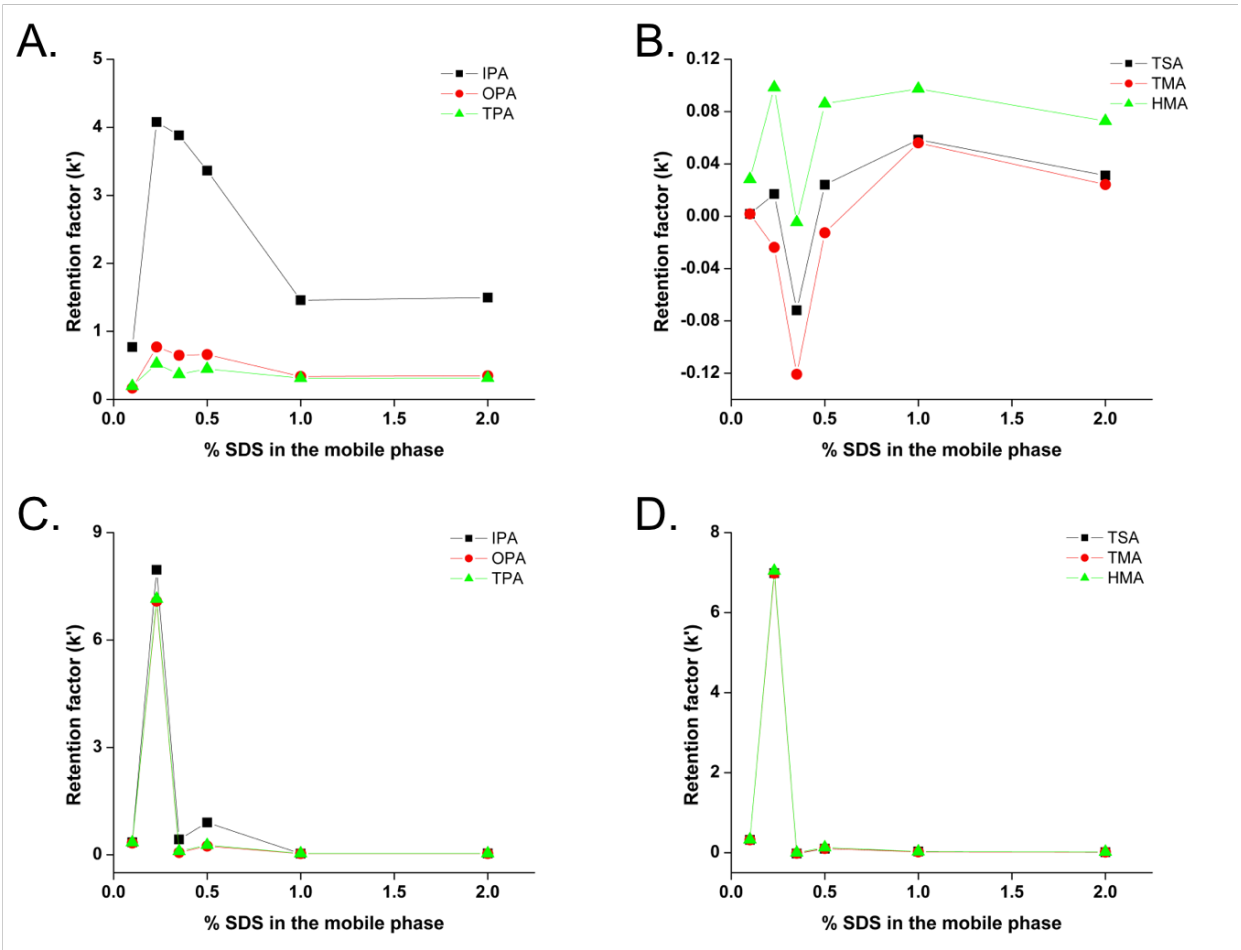


Figure 3.3. The effect of the % SDS in the mobile phase on k' for Mix 1 and Mix 3 at pH 4 (A and B) and pH 6 (C and D). k' values given are an average of 3 replicates with %RSDs between 0 and 14.7% for pH 4 and %RSDs between 0 and 16.4% for pH 6.

aqueous mobile phase. However, when the pH value is 3 or lower, the majority of the analytes are protonated and as a result are present as neutral species. These neutral analytes are not repelled by the micelles or the modified stationary phase, and this allows for interaction with the SDS coated stationary phase and to a lesser extent with the liquid phase SDS micelles. Retention factor data for Mix 2 are not shown because of the excessively long retention times observed for these compounds when the percentage of SDS in the mobile phase was below 1%. Once the % SDS exceeds 1%, the analytes in Mix 2 are eluted in a reasonable time. The long retention at low % SDS is likely due to their increased hydrophobic character (smaller number of acid groups) as compared to the analytes in Mix 1 and 3.

The optimum mobile phase was chosen based on the criteria of minimizing retention times while still obtaining baseline resolution of the analytes. Figure 3.1S displays HPLC chromatograms for Mix 1 (A) and Mix 3 (B) using 1% SDS at pH 3 sulfuric acid. This mobile phase produced short retention times, good resolution, and good peak shape for these components. The selected mobile phase also needed to be able to elute Mix 2 components in a reasonable time. At 1% SDS, Mix 2 analytes had retention times of approximately 30-40 minutes. Therefore 1% SDS at pH 3 was chosen as a suitable mobile phase for the separation of the nine components of interest.

Once a suitable mobile phase was chosen, the next step was to demonstrate acceptable resolution of the components while minimizing overall analysis time. While higher SDS percentages, above 2%, would elute Mix 2 analytes, a mobile phase gradient was not considered because of concerns over the re-equilibration time between each injection. It has been shown that an organic solvent gradient, starting from a completely micellar mobile phase to remove unwanted matrix components, may require only a few minutes of re-equilibration [31]. This has been shown effective for the separation of β-blockers in urine [31], sulphonamides in milk or urine [32], and derivatized biogenic amines [33]. However, our method was developed to be a "green" method. Once an organic solvent like acetonitrile or 1-propanol is incorporated, more consideration must be taken with respect to waste treatment. Instead of a mobile phase gradient, a flow rate gradient was incorporated into the separation in order to minimize analysis times. By eluting the first few acids at 0.100 mL/min, the middle acids at 0.300 mL/min, and the last few acids at 0.500 mL/min, baseline resolution of all nine acids was achieved in under 20 minutes. Figure 3.4 shows a chromatogram of the 50 ppm standard containing all nine acids run on the

Waters X-Bridge HPLC column using 1% SDS at pH 3. Without the flow rate gradient, the total analysis time was approximately twice as long as that shown in Figure 4 with diminished resolution. In general, tri-acids elute prior to di-acids, and these were followed by mono-acids. The notable exception is FBA which elutes earlier than the other di-acids.

Equilibrium and partition constants were calculated based on the acids in Mixes 1 and 3. The classification of these acids as binding solutes was based on the three-process model described by Armstrong and Stine [34]. Overall, these acids displayed a decrease in retention time as the concentration of SDS was increased and can therefore be classified as binding solutes. A graphical representation of micelle concentration as a function of the reciprocal of the retention factor for Mix 1 and Mix 3 is shown in in Figure 3.2SA and 3.2SB respectively. The calculated values for slope, intercept, and correlation for Figure 3.2SA and 3.2SB as well as equilibrium constants and partition coefficients are shown in Table 3.1S. K_{AM}, the equilibrium constant per micelle, determines the strength of an

analyte's interaction with the micelles. Since TPA and HMA have the lowest K_{AM} values, it can be concluded that they have the weakest interaction with the micelles. The other four acids have significantly higher K_{AM} values. Based on structure, we are uncertain why these trends occur. Hydrophobicity can also be concluded from Table 3.1S. The lower the value of $\Phi K_{WS}[S]$, the lower the hydrophobic character of an analyte. In this study of Mix 1 and Mix 3, IPA was substantially most hydrophobic while TMA was the least hydrophobic. This agrees with the retention order trends in Figure 3.2C and 3.2D.

A 2 µL injection size was chosen because it provided excellent peak shape and resolution. Injection volume sizes of 10 and 20 µL were investigated, however for both the standards and the industrial sample these volumes showed more peak asymmetry and tended to cause maximum absorbance response by the UV detector. While the limit of detection tends to improve with injection volume, resolution of peaks generally tends to decrease with an increasing injection volume [35].

Since a flow rate gradient was of interest, it was important to generate the van Deemter plots for the HPLC column to determine the extent of efficiency loss as the flow rate increased. Figure 3.5 shows the effect mobile phase velocity has on plate height (HETP) for TPA and FBA. Plate count N was calculated using the Foley-Dorsey equation, derived to take into account peak asymmetry [36]. The HPLC column showed minimal loss in plate height as flow rate was

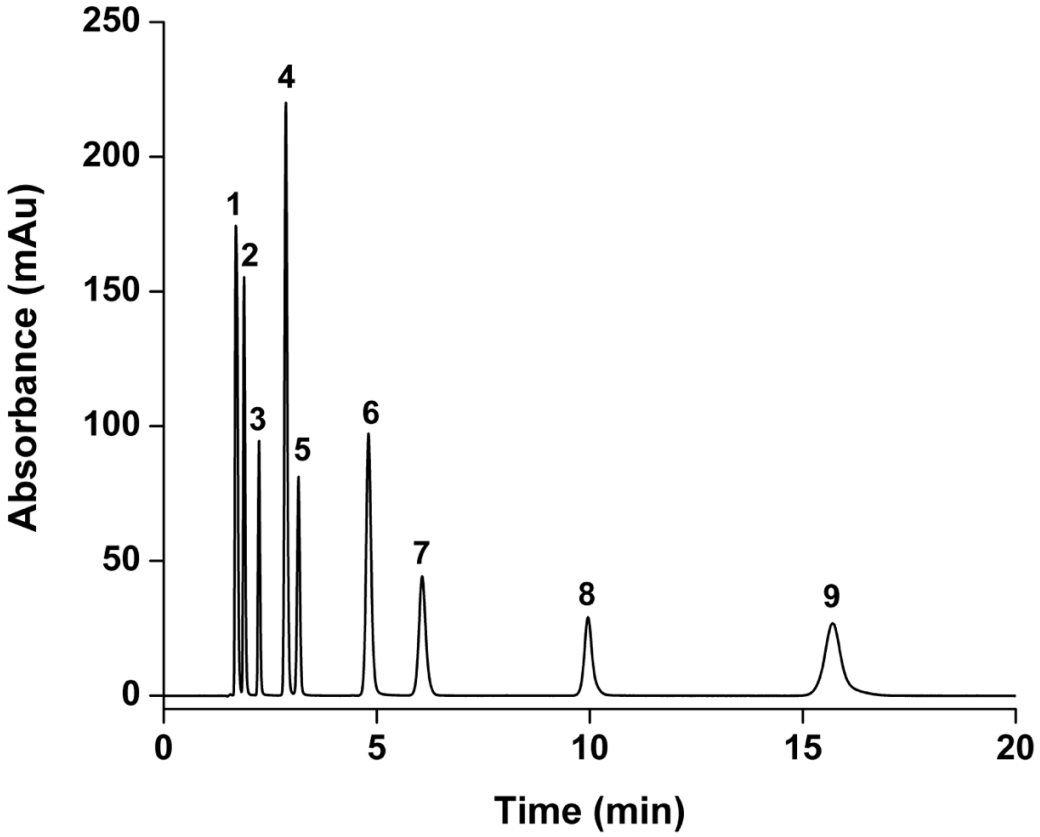


Figure 3.4. HPLC chromatogram of Mix 1, Mix 2, and Mix 3 combined in the 50 ppm standard. The mobile phase was 1% SDS, 1.84 mM H_2SO_4, pH 3 using the step gradient described in the Methods. Peak assignments are as follows: (1) TMA, (2) TSA, (3) HMA, (4) FBA, (5) OPA, (6) TPA, (7) IPA, (8) BA, (9) p-TA.

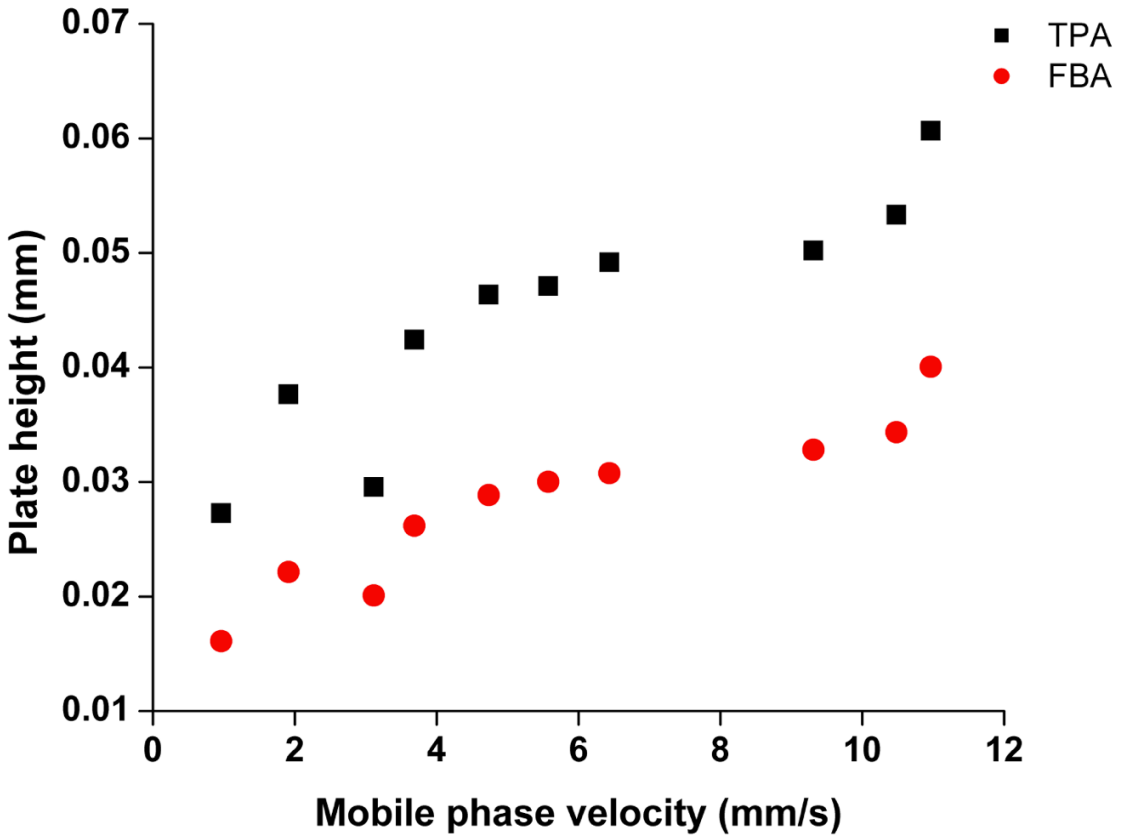


Figure 3.5. Comparison of the van Deemter plots of FBA and TPA.

increased to values as high as 1.0 mL/min (10.96 mm/s). A flow rate of 0.1 or 0.3 mL/min (0.96 mm/s and 3.12 mm/s) provides nearly the same minimum plate height on the HPLC column. While a flow rate of 0.5 mL/min (4.73 mm/s) produced a larger plate height, this higher flow rate was incorporated so that the hydrophobic analytes would elute from the column in a reasonable time.

Industrial sample analysis

Calibration standards were made for all nine analytes at the following concentrations: 1, 10, 25, 50, and 75 ppm. Table 3.2 shows the linear regression data as well as the detection and quantitation limit values. Detection limits ranged from 0.180 to 1.53 ppm (2.16 to 19.3 pmoles), and quantitation limits ranged from 0.549 to 3.45 ppm (6.49 to 43.0 pmoles). Following the determination of detection and quantitation limits as well as the high linearity, a simulated sample was made and separated using the HPLC micellar method. The chromatogram for this sample is shown in Figure 3S. This sample provided confirmation of the peak assignments as well as approximate relative concentrations that would be seen in a commercial sample. However, the simulated sample did not include TSA but there is a peak where TSA elutes. Because the acids were reagent grade, it is likely that the isomers are not totally pure. If there is a small amount of another isomer present, this could cause additional peaks to be seen in a chromatogram. The oxidized TPA mother liquor sample from a commercial plant was then analyzed. The Waters X-Bridge HPLC chromatogram for the industrial sample is shown in Figure 3.6, and all nine acids were found in the sample. There are also other peaks present in the chromatogram that could be other impurities in the sample that are not seen as being major interferences with the production of PET. Since the reaction of p-xylene and dioxygen to make TPA is made in an acetic acid:water solvent, it was important to determine whether or not acetic acid would interfere with the UV signal. Upon inspection, it was found that acetic acid does not produce a peak or affect the baseline at 240 nm. Using peak area and the calibration curves previously generated, the concentration of each acid in the commercial sample was determined. The concentrations are as follows: TMA, 6094 ppm; TSA, 106.8 ppm; HMA, 294.7 ppm; FBA, 457.5 ppm; OPA, 1991 ppm; TPA, 978.5 ppm; IPA, 4000.5 ppm; BA, 15170 ppm; p-TA, 2292 ppm. These concentrations are generally similar to those expected to be found (100-16000 ppm). This agree with the data reported by Huang et al [25].

Table 3.2. Calibration parameters and analytical figures of merit

Compound	t_R[a]	A[b]	B[c]	R^2	LOD[d]	LOQ[e]
TMA	1.792	0.171	-0.0175	0.9999	0.446 (4.25)	1.73 (16.4)
TSA	1.999	0.0229	0.00530	0.9999	0.615 (5.85)	1.51 (14.4)
HMA	2.310	0.0220	0.000188	0.9999	0.264 (2.51)	0.681 (6.48)
FBA	2.985	0.193	0.0548	0.9999	0.594 (7.92)	1.32 (17.6)
OPA	3.305	0.0332	0.0111	0.9999	0.894 (10.8)	2.20 (26.5)
TPA	5.085	0.517	0.0111	0.9999	0.180 (2.16)	0.549 (6.61)
IPA	6.441	0.0188	0.0132	0.9998	1.53 (18.4)	3.45 (41.5)
BA	10.49	0.0143	0.00798	0.9999	1.18 (19.3)	2.63 (43.0)
PTA	16.87	0.0884	0.0364	0.9998	1.16 (17.0)	2.90 (42.6)

[a] Retention times are given in min and as the average of three replicates with RSDs between 0% and 0.52%

[b] A values are given as the y-intercept from the linear regression equation of peak area versus ppm.

[c] B values are given as the slope from the linear regression equation of peak area versus ppm.

[d] Detection limit values are given in ppm (and pmoles) and were calculated using the following equation: $LOD=(A+3*A_\sigma)/B$, where A_σ is the standard deviation of the y-intercept

[e] Quantitation limit values are given in ppm (and pmoles) and were calculated using the following equation: $LOQ=(A+10*A_\sigma)/B$, where A_σ is the standard deviation of the y-intercept

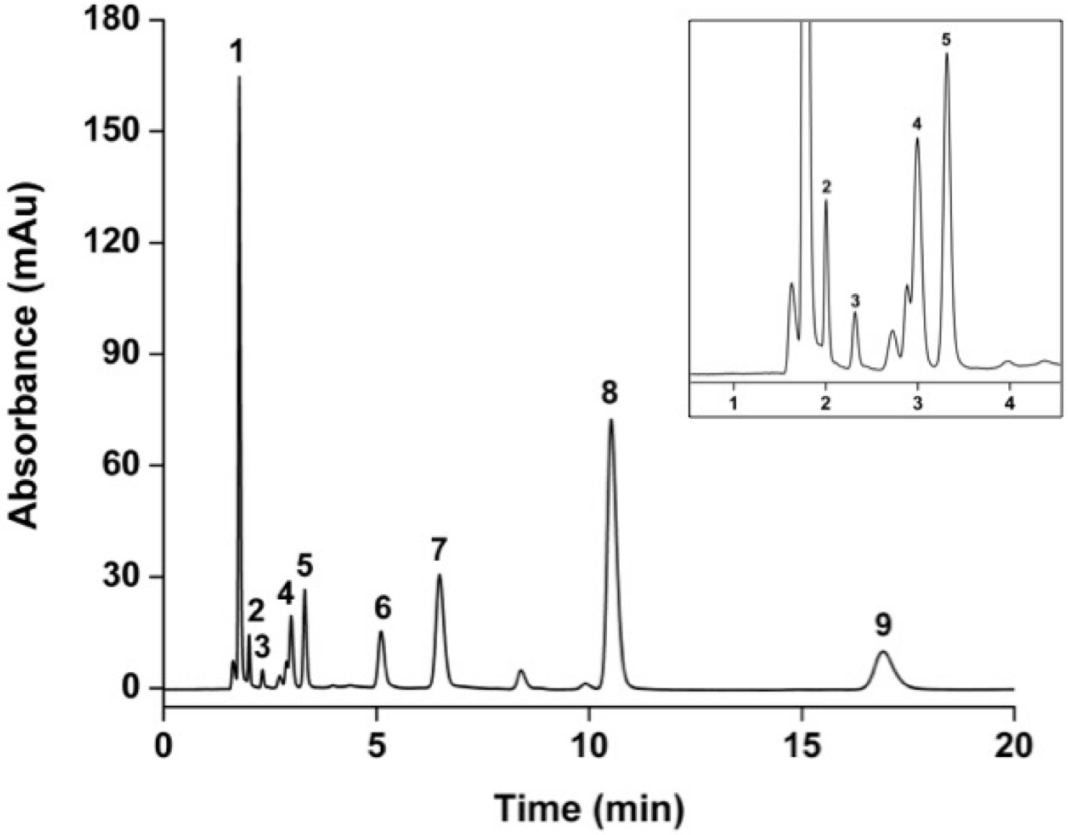


Figure 3.6. HPLC chromatogram of oxidized PTA sample using the optimized mobile phase and step gradient. Peak assignments are as follows: (1) TMA, (2) TSA, (3) HMA, (4) FBA, (5) OPA, (6) TPA, (7) IPA, (8) BA, (9) p-TA.

HPLC Column Comparison

Two columns from different manufacturers were then chosen to compare the method across other reversed phase columns. A Gemini C18 column from Phenomenex and an Accucore C18 column from Thermo Scientific were selected. The columns are extremely similar in dimension with all three being the same length and only varying in inner diameter. The Waters and Phenomenex columns have 3 μm particles while the Thermo Scientific column has 2.6 μm particles. All three columns are endcapped, though they vary in pore size and surface area. Each column was coated with SDS according to the Methods section. The following samples and mixtures were analyzed: each acid individually for k' data and positive assignment, five standards, and the diluted industrial sample. The k' values on the Phenomenex Gemini column were very similar (0.115 to 9.472) to the k' values on the Waters XBridge column. However, the k' values on the Thermo Scientific Accucore column were smaller (0.058 to 7.359) than the k' values on the other two columns. The chromatograms for the 50 ppm standard were chosen as the comparison chromatograms. Figure 3.7A provides the chromatogram from the Phenomenex Gemini column while the chromatogram for the Thermo Scientific Accucore column is indicated in Figure 3.7B. It should be noted that BA was missing from both chromatograms, likely due to some instability of this hydrophobic acid in an acidic solution. However, it appeared between IPA and pTA in the industrial sample as well as when it was analyzed by itself. On the Gemini column, it has a retention time of approximately 12 minutes, while on the Accucore column, it has a retention time of approximately 8 minutes. The peaks on the Gemini column were much broader and resolution was lost between TSA and HMA as well as TBA and FBA. While further optimization would be needed, a combination of a higher % SDS to decrease the overall retention of the analytes as well as a slower flow rate gradient could encourage separation between TSA and HMA as well as TBA and FBA. The Accucore column showed narrow peaks and decreased retention, likely due to the slightly smaller particle size. Again, resolution was slightly decreased between TMA and TSA, but it was completely lost between TPA and FBA. A decrease in % SDS as well as a slightly different flow rate gradient could encourage separation between these acids.

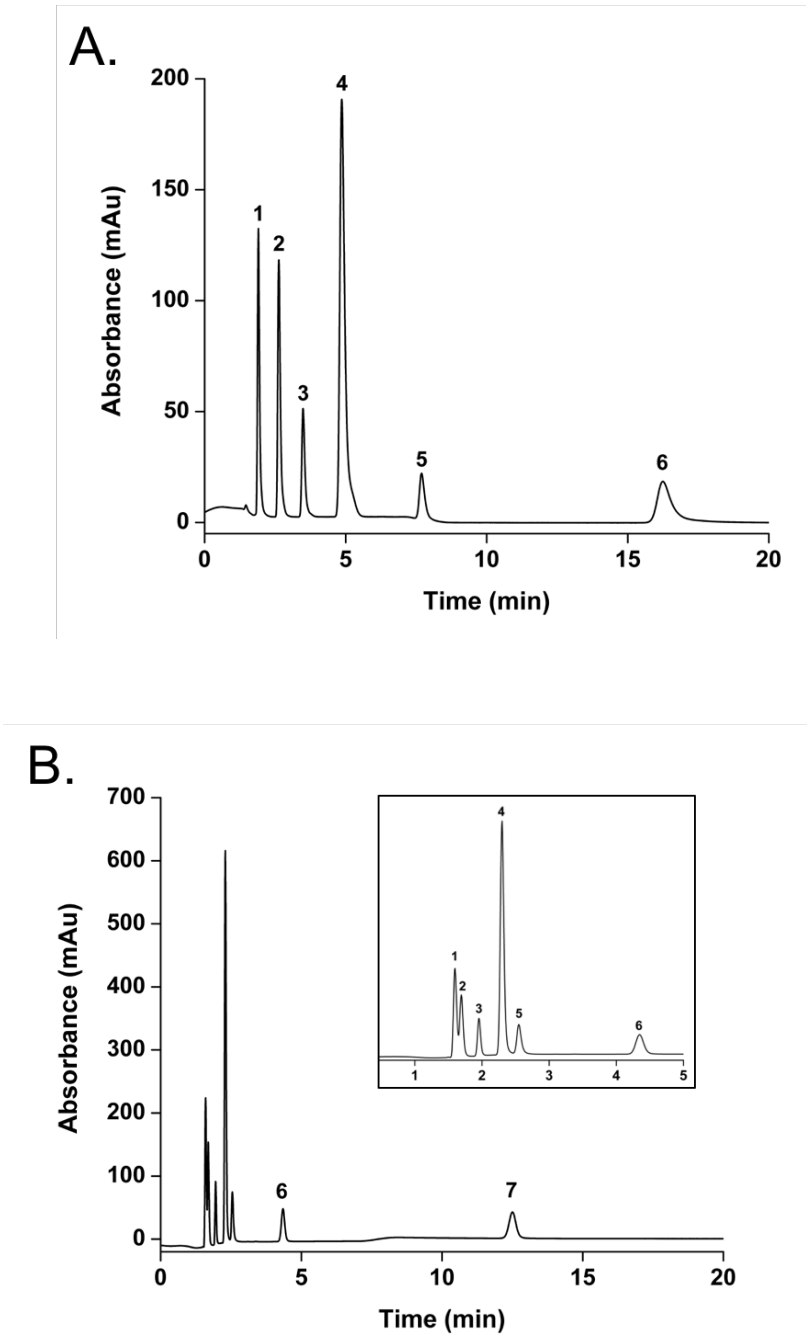


Figure 3.7. HPLC chromatograms of the 50 ppm standard using the optimized mobile phase on the Phenomenex Gemini C18 column (A) and the Thermo Scientific Accucore C18 column (B). Peak assignments for the Gemini column are as follows: (1) TMA, (2) TSA, HMA, (3) OPA, (4) TPA, FBA, (5) IPA, (6) p-TA. Peak assignments for the Accucore column are as follows: (1) TMA, (2) TSA, (3) HMA, (4) TPA, FBA (5) OPA, (6) IPA, (7) p-TA.

Solute retention loss in the presence of a purely aqueous mobile phase has been shown to be problematic when the pore size of the packing material is less than 10 nm [37, 38]. High % carbon loading (15-20%) of the stationary phase also accentuates retention loss in a 95% or greater water- 5% or less methanol mobile phase [38]. The proposed mechanism has been related to the difficulty of a non-wetting liquid like water to penetrate the hydrophobic pore structure of the C18 modified silica. However, the surface tension of the SDS mobile phase at the CMC is about 40, much lower than the surface tension of pure water, about 72 [39]. A mobile phase composition of 80% water-20% methanol has an equivalent surface tension of about 40 [40]. Therefore, it seems more likely the reduced retention observed for the Accucore column is due more to the lower 8% carbon loading and less due to the pore size of 8 nm. Finally, for these two columns, limits of detection and quantitation were determined for the acids that were well resolved. On the Gemini column, this included TMA, OPA, IPA, and p-TA. LOD ranged from 2.12 to 76.5 ppm (20.2 to 921 pmoles), and LOQ ranged from 5.07 to 246 ppm (48.2 to 2960 pmoles). On the Accucore column, TMA, TSA, HMA, OPA, IPA, and p-TA LOD and LOQ were determined. LOD ranged from 0.441 to 6.50 ppm (4.19 to 78.2 pmoles), and LOQ ranged from 1.52 to 32.2 ppm (14.4 to 388 pmoles). These values are similar to the ranges for LOD and LOQ on the Waters XBridge column.

3.6: Conclusion

The chromatograms of TPA and eight aromatic acid impurities showed baseline resolution and good peak shape in the micellar mode using the Waters XBridge C18 column and an acidic SDS mobile phase. When compared to reversed phase HPLC, our method offers a “green”, inexpensive, and faster approach to separate TPA and aromatic acid impurities produced during the industrial production of TPA. Additionally, this method provides better peak shape and resolution when compared to the MEEKC method. Calibration curves for each analyte were generated to permit the acids present in a commercial sample to be quantified. The concentrations of each acid were found to be typical for industrial samples. This method was further compared using two other C18 columns of similar characteristics from two different companies. Both columns were able to resolve seven of the nine analytes in less than 20 minutes using a 1% SDS solution in pH 3 sulfuric acid. Slight changes in the mobile phase would be necessary to fully resolve all nine analytes on each column, however both boast detection and quantitation limits that are comparable to those found using the original column.

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3.8: Supplemental Information

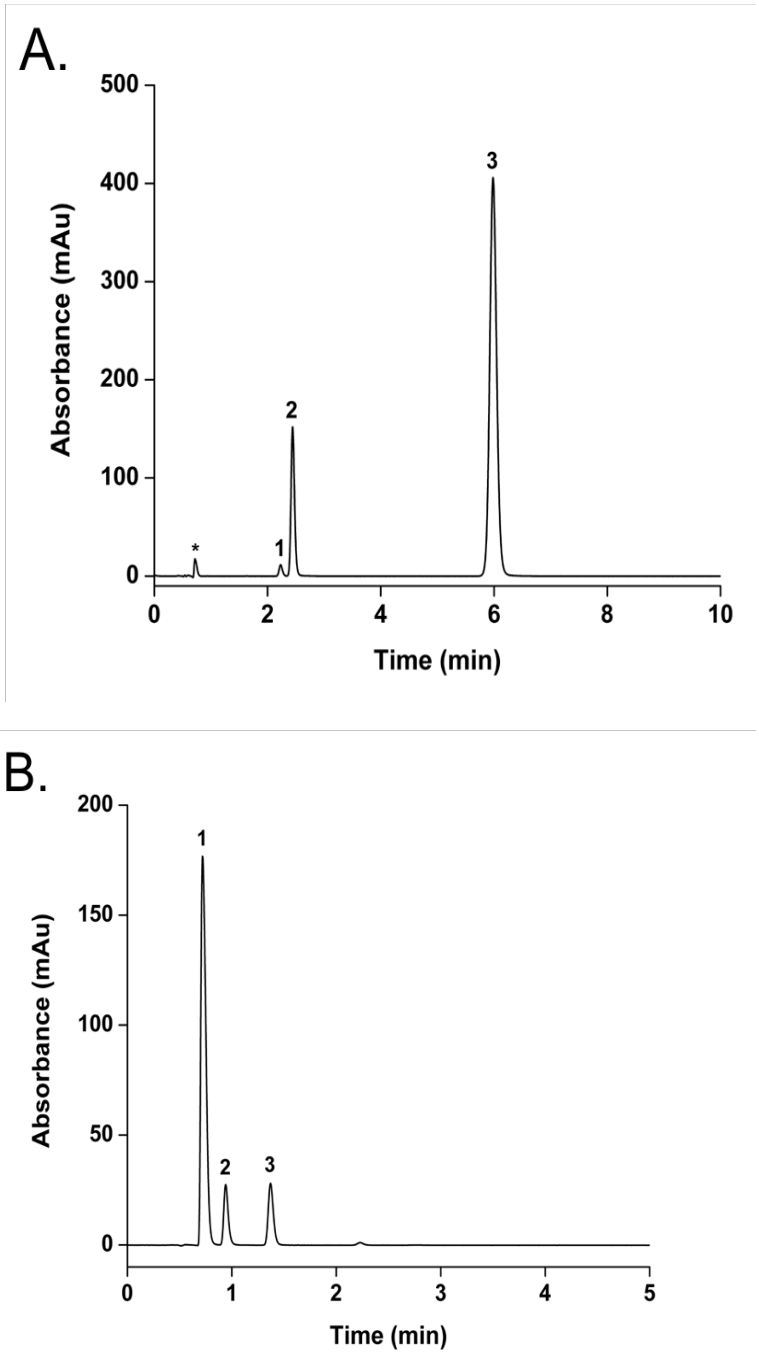

Figure 3.1S. HPLC chromatogram of Mix 1 (A) and Mix 3 (B) using a 1% SDS pH 3 mobile phase. Peak assignments for A are as follows: (1) TPA, (2) OPA, (3) IPA. Peak assignments for B are as follows: (1) TMA, (2) TSA, (3) HMA.

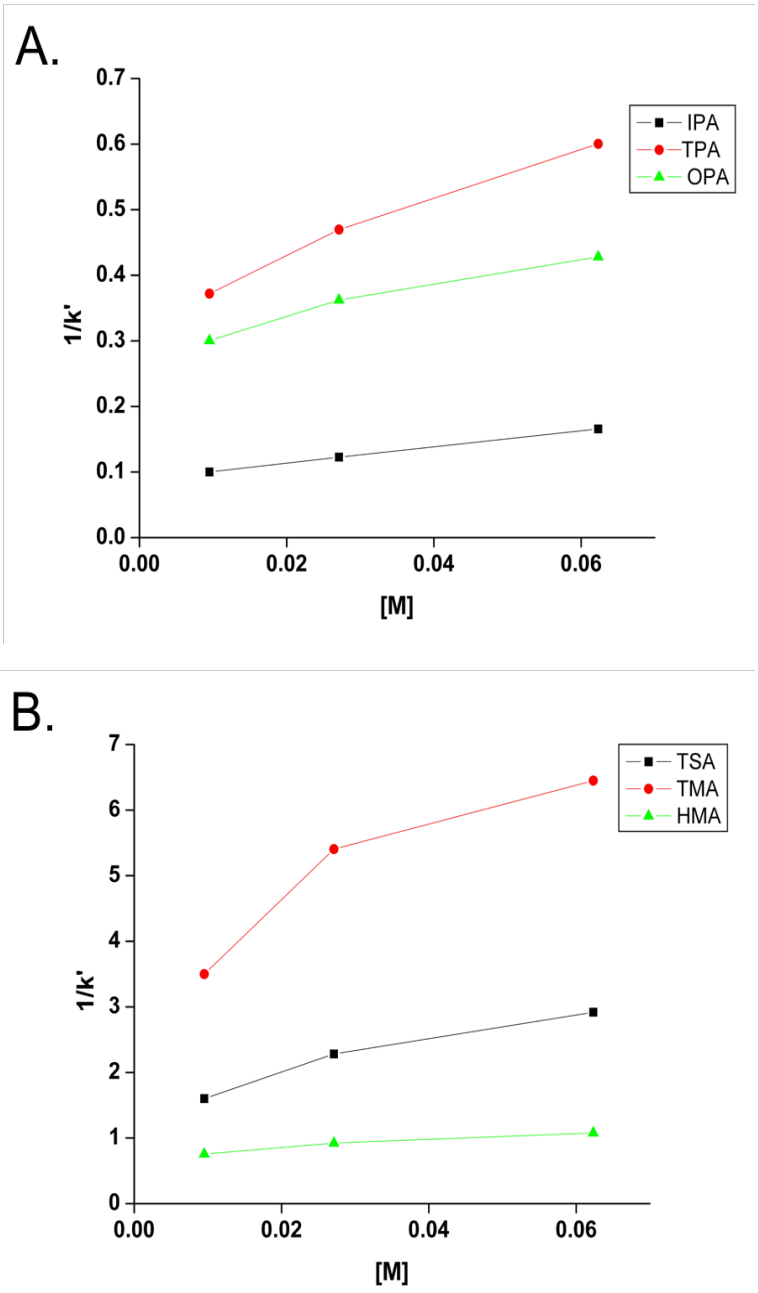

Figure 3.2S. Plot of micelle concentration (x axis) as a function of the reciprocal of the retention factor (y axis) for Mix 1 (A) and Mix 3 (B).

Table 3.1S. Linear regression values and equations based on Figure 3.1S.

Compound	A	B	R^2	K_{AM} (L/mol)[a]	K_{eq}[b]	P_{WM}[c]	$\Phi K_{AS}[S]$[d]
IPA	0.0886	1.24	0.9998	14.0	0.2256	57.9	11.3
OPA	0.341	4.24	0.9886	12.4	0.2004	51.5	2.93
TPA	0.286	2.34	0.9706	8.17	0.1317	34.2	3.49
TSA	1.476	24.0	0.9567	16.2	0.2618	67.0	0.677
TMA	3.399	52.1	0.8775	15.3	0.2471	63.3	0.294
HMA	0.727	5.84	0.9556	8.03	0.1295	33.6	1.38

[a] K_{AM} is the equilibrium constant of the solute with a surfactant monomer in the micelle, where $K_{AM}=B/A$.

[b] K_{eq} is the equilibrium constant per micelle, where $K_{eq}=(K_{AM})(N)$. N is the aggregation number that for SDS in water is equal to 62.

[c] P_{WM} is the partition coefficient between the bulk water and the micelle. $P_{WM}=(K_{AM}/\upsilon)+1$, where υ is the molar volume of the surfactant in the micelle. For SDS, υ is equal to 0.246 L/mol.

[d] K_{AS} is the equilibrium constant of the solute with the stationary phase binding sites, Φ is the phase ratio, and S is the stationary phase binding sites. The equation for this term is $\Phi K_{AS}[S]=1/A$.

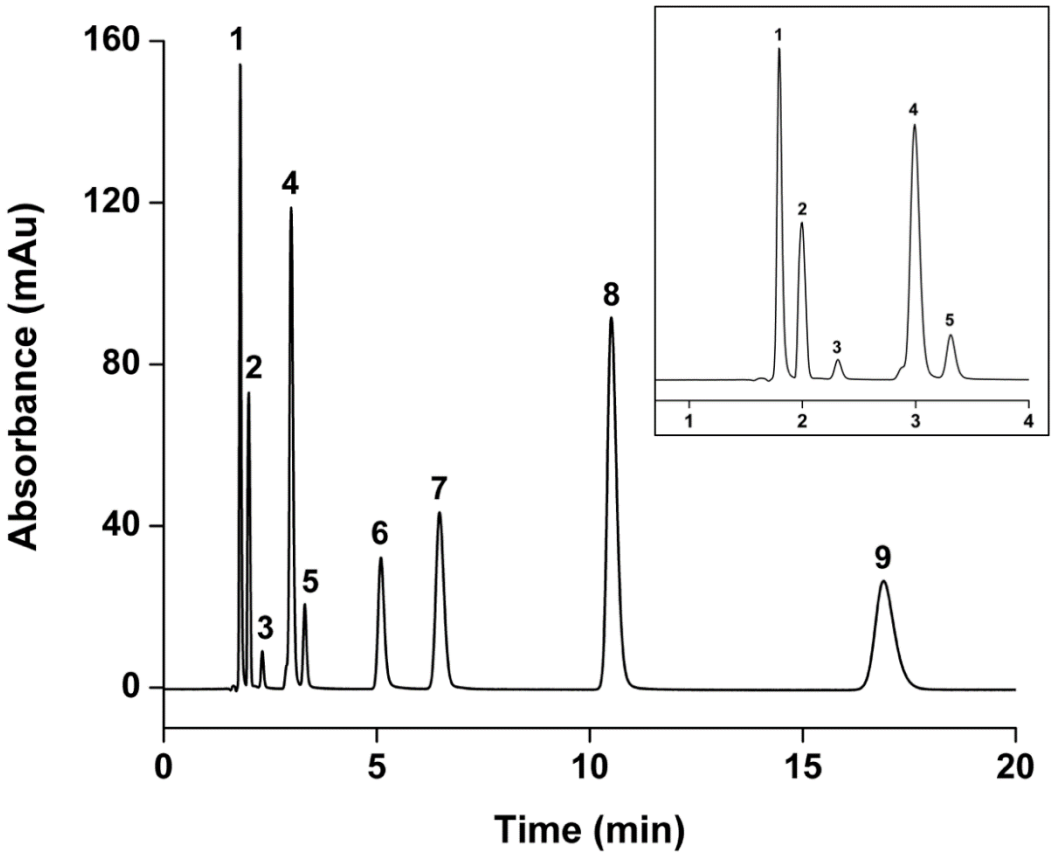

Figure 3.3S. HPLC chromatogram of the simulated PTA sample using the optimized mobile phase and step gradient. Peak assignments are as follows: (1) TMA, (2) TSA, (3) HMA, (4) FBA, (5) OPA, (6) TPA, (7) IPA, (8) BA, (9) p-TA.

CHAPTER 4

Comparison of Sodium Dodecyl Sulfate and Brij-35 Surfactants for Ultra-High Performance Micellar Liquid Chromatography of Aromatic Carboxylic Acids

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4.1: Abstract

Terephthalic acid (TPA), required for the production of polyethylene terephthalate (PET), is made commercially by oxidation of p-xylene. The separation of TPA from eight major acid impurities, which includes two sets of positional isomers, has been compared using SDS and Brij-35 surfactants as mobile phase modifiers with a C18 ultra-high performance liquid chromatography column (100 x 2.1 mm, 1.6 μm) and a simple step flow rate gradient. It is found that a mobile phase of 1% SDS in 1.84 mM H_2SO_4, pH 3 can resolve TPA and its eight major impurities in under 10 minutes with detection limits ranging from 0.294 to 24.2 ppm. A mobile phase of 0.005% Brij-35 in 10 mM ammonium formate, pH 3.5 can resolve TPA and its eight major impurities in under 35 minutes with improved resolution over SDS. Under Brij-35 conditions, detection limits for all nine acids ranged from 0.310 to 8.70 ppm. A potentially mass spectrometry compatible method using no Brij-35 in the mobile phase but still a surfactant coated column showed no loss in peak resolution.

4.2: Introduction

Micellar liquid chromatography (MLC) is a mode of chromatography that was developed in the late 1970s. MLC gained popularity because it uses little to no organic solvent and therefore had a lower impact on the environment as compared to reversed phase liquid chromatography (RPLC). Instead of using an organic solvent to modify mobile phase polarity, MLC utilizes various surfactants like sodium dodecyl sulfate (SDS), cetyltrimethylammonium (CTAB), and polyethylene glycol dodecyl ether (Brij-35), either below or above the critical micelle concentration (CMC), to perform the separation [1–5]. The stationary phase is most commonly a nonpolar phase like C18, though shorter chain columns like C1 or C8 have been used. The surfactant adsorbs to the nonpolar stationary phase, affecting the retention of analytes. Additionally, the more nonpolar the stationary phase, the amount of adsorbed surfactant

increases [4,6–8]. The retention mechanism has been well described by Armstrong and Nome using a three-process model. They state that there are three partitioning processes that can occur: between the stationary phase and the micelle, between the micelle and the bulk water, and between the stationary phase and the bulk water. Factors such as mobile phase pH, ionic strength, column temperature, the presence of mobile phase modifiers like propanol or acetonitrile, and the type and concentration of the surfactant can all affect the partition equilibria [8,9].

SDS tends to be the most commonly used surfactant, however the non-ionic Brij-35 has some advantages. Brij-35 has been used to determine certain active ingredients in cold medicine [10], an ASTM text mixture such as benzyl alcohol, benzaldehyde, acetophenone, methyl benzoate, benzene, and dimethyl terephthalate [11], and various positional isomers such as nitrolaniline, quinolinol, cresol, nitrophenol, and others [12]. These aromatic positional isomers are commonly found in pharmaceutical products and are difficult to separate using RPLC. Each isomer set was considered individually and was separated using a C18 column modified with Brij-35 and a Brij-35/propanol mobile phase. The overall resolution was improved compared to the RPLC methods, however baseline resolution was not achieved for all analytes [12]. A combination of SDS and Brij-35 has also shown to be efficient at separating basic compounds like tricyclic antidepressants and β-blockers [13]. A comparison of SDS and Brij-35 to separate carbamates has also been shown. While both surfactants yielded similar selectivities, Brij-35 provided shorter analysis times than SDS. This method was favorably compared to an EPA method that uses an acetonitrile:water mobile phase gradient [14]. SDS and CTAB have also been compared for the separation of non-steroidal anti-inflammatory drugs. Because of the stronger electrostatic interactions, retention for these aromatic carboxylic acids was longer when CTAB was used [15].

There is great interest in terephthalic acid (TPA) since it is used in the polymer industry to make polyethylene terephthalate (PET), the polymer commonly used in the production of beverage bottles. Prior to polymerization, TPA is synthesized by reacting p-xylene with dioxygen in acetic acid in the presence of a metal catalyst [16]. One of the common impurities is 4-formylbenzoic acid (FBA), also known as 4-carboxybenzaldehyde. This compound is extremely important to remove from terephthalic acid because it co-crystalizes with TPA which leads to incomplete oxidation. However, because FBA is so structurally similar to TPA, it is even

more difficult to purify TPA [17]. It is important to ensure a high purity of TPA before it is polymerized because purification of PET is much more complicated. There are many impurities that can lead to discoloration and degradation of the polymer. Additionally, the use of fluorescence detection has shown that FBA and TPA form a complex, thus adding to the difficulty of separating the two compounds [18]. Benzoic acid (BA) and p-toluic acid (p-TA) (4-methylbenzoic acid) arise from excessive oxidation of p-xylene, while 4-formylbenzoic acid (FBA) is due to partial oxidation of p-xylene. The presence of o- and m-xylene can lead to the production of the other two dicarboxylic acid isomers (o-phthalic acid (OPA), isophthalic acid (IPA)), and the three tricarboxylic acid isomers (trimesic acid (TSA), trimellitic acid (TMA), and hemimellitic acid (HMA)) [19–22]. Capillary electrophoresis has been used to separate such TPA impurities, however this instrument is not commonly found in this industry [20,21]. There are few chromatography methods available to separate these eight impurities from TPA. Yuan et al. used RPLC-UV to separate the nine compounds using a methanol/water/buffer mobile phase gradient [22] These acids have also been fully resolved using MLC with an acidic SDS mobile phase without the use of an organic modifier [23]. Ultra-high performance liquid chromatography (UHPLC) has also been used to separate these impurities. Ultra-high performance liquid chromatography (UHPLC) is a mode of chromatography that typically utilizes columns with sub 2 μm particles that typically generate backpressure above 6,000 psi [24–26]. UHPLC has shown to be advantageous over HPLC because it tends to provide higher selectivity and sensitivity, higher resolving power, and decreased analysis time and solvent consumption [27–30]. TPA has been separated from a few of the eight major impurities using gradient reversed phase UHPLC with both mass spectrometry (MS) detection and ultraviolet (UV) detection. One method was able to separate TPA from FBA and p-TA in under 6 minutes while a second method was able to separate TPA, p-TA, FBA, IPA, OPA, and BA in under 7 minutes, however neither method separates all of the impurities or indicates detection limits achieved [31,32]. While there are RPLC methods available to separate these acids, the current UHPLC methods only separate a fraction of the impurities of interest.

Very little work has been done in ultra-high performance micellar liquid chromatography. To our knowledge, the use of MLC under UHPLC conditions has only been performed using SDS to separate hydroxybenzoic, hydroxycinnamic, and phthalic acid isomers [33]. This work

Table 4.1. Acid names, abbreviations, and pK_A values.

Mixture	Name	Abbreviation	pK_A
Mix 1	Isophthalic	IPA	3.46, 4.46
	o-Phthalic	OPA	2.98, 5.28
	Terephthalic	TPA	3.51, 4.82
Mix 2	Benzoic	BA	4.17
	p-Toluic	pTA	4.36
	4-Formylbenzoic	FBA	3.78
Mix 3	Trimesic	TSA	3.12, 3.89, 4.70
	Trimellitic	TMA	2.52, 3.84, 5.20
	Hemimellitic	HMA	2.80, 4.20, 5.87

aims to investigate the difference between SDS and Brij-35 under isocratic UHPLC conditions to separation TPA from eight major impurities (listed in Table 4.1). We have found SDS above the CMC is required in the mobile phase in contrast to either a low Brij-35 % or no aqueous Brij but still a Brij coated C18 column. The analysis time of 35 minutes for the Brij-35 method is about twice that for the SDS one but the former approach is potentially MS compatible. Both methods are completely green since they do not use an organic modifier, and both utilize a flow rate gradient to minimize analysis time.

4.3: Apparatus and Conditions

Chromatographic separations were performed on a Thermo Scientific UltiMate 3000 UHPLC (Sunnyvale, CA, USA) instrument, equipped with a pump, online degasser, autosampler, temperature-controlled column oven, and variable wavelength UV detector. Instrument control and data acquisition was performed using Chromeleon 7.2.1 software (Thermo Scientific, Sunnyvale, CA, USA). A Phenomenex Luna Omega C18 column (Torrance, CA, USA) (100 x 2.1 mm, 1.6 μm) was used for separation. This endcapped column has a pore size of 100 Å, a surface area of 260 m^2/g, and a % carbon load of 11%. Before use, the column was pre-coated with 10 mM SDS in water (or 10 mM Brij-35) for 3 hr at 0.1 mL/min and then rinsed with water for 3 hr at 0.1 mL/min.

A step flow rate gradient was incorporated to fully resolve the analytes as well as decrease overall analysis time. The gradient was set to the following: 0.1 mL/min for 0.00-1.00 min, 0.3 mL/min for 1.00-7.00 min with a 1.00 min ramp, and 0.5 mL/min for 7.00-35.00 min with a 1.00 min ramp. An injection size of 2 μL was used, the column was kept at ambient temperature, and the UV detector set to 240 nm.

4.4: Chemicals and Procedures

All of the acids analyzed were purchased from Sigma Aldrich (St. Louis, MO, USA) and were dissolved using water distilled and purified with a rating at 18.2 MΩ by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Small volumes of a dilute solution of sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA) were used to dissolve the acids. Sulfuric acid (Fisher Scientific, Fair Lawn, NJ, USA) and sodium dodecyl sulfate (ACS reagent ≥99.0%) (Sigma Aldrich, St. Louis, MO, USA) were used to make the SDS mobile phase. Brij-35, ammonium formate, and formic acid (Sigma Aldrich, St. Louis, MO, USA) were used to make the Brij-35 mobile phase at pH 3.5, while Brij-35 and sulfuric acid were used to make the

Brij-35 mobile phase at pH 3. Brij-35 mobile phases with a pH of 4 and 4.5 were made using a sodium acetate-acetic acid buffer (Sigma Aldrich, St. Louis, MO, USA), while the Brij-35 mobile phase at pH 6 was made using water.

For retention factor data collection, each acid was made at a concentration of 0.25 mM. Five standard solutions were made so that each analyte was present at the following concentrations: 1, 10, 25, 50, and 75 ppm. These standards were then used to construct a calibration curve. An industrial sample from a commercial plant was analyzed by filtering the sample using a 0.22 μm syringe filter before dilution by a factor of one hundred. This sample was analyzed using the optimum SDS and Brij-35 mobile phases along with the flow rate gradient. Finally, the concentration of the analytes was determined using the constructed calibration curves.

4.5: Results and Discussion

Optimization of SDS

Optimization of the SDS mobile phase began by varying the concentration of SDS in the mobile phase at pH 3. Previously, it was determined that a pH value of 3 provided the best peak shapes and overall resolution for those same analytes under MLC HPLC conditions [23]. The concentrations of SDS were chosen based on this previous work as well, providing k' data both below and above the CMC for SDS, 0.23% (w/w) or 8.1 x 10^{-3} M. The advantage of having the surfactant in the mobile phase is that it should stabilize the column modification as well as create a mobile phase-analyte partitioning mobile phase [33].

Figure 4.1A and 4.1B shows the effect of % SDS on the retention factor of the analytes in Mix 1 (A) and Mix 3 (B). The pH value of 3 was chosen because the acids are partially protonated and therefore present in a more neutral state. Because SDS is an anionic surfactant, the acids are repelled by the modified stationary phase causing decreased retention and little to no resolution [23]. At a pH value below 3, the acids in fact also display shorter retention times, due to likely enhanced interaction with the micellar mobile phase; however the peaks shapes are much worse and therefore resolution is again lost [23]. Retention is weaker for the tri-carboxylic acids as compared to the di- and mono-carboxylic acids as expected for RPLC. The downward trend in retention factor as the micelle concentration is increased means that the analytes are preferentially interacting with the micelles in the column. This would classify these acids as binding solutes based on the three-process model explained by Armstrong and Stine [34]. Overall, a concentration of 1% SDS was chosen to be the optimum mobile phase. Below 1%, the

overall analysis time is longer, while above 1% there is some loss in resolution. This optimum mobile phase is the same as previously found under HPLC conditions. The retention order is nearly identical, with the exception of the order of TPA, FBA, and OPA. Under UHPLC conditions, the order is TPA, FBA, then OPA. However, under HPLC conditions the order is FBA, OPA, then TPA. The reason for the change in retention order for these three compounds is not obvious. Possibly mass transfer of the these analytes between the mobile and stationary pahse is affected by the significant differences in particle size and column pressure between HPLC and UHPLC.

Previously we have shown that utilizing a flow rate gradient with MLC on a HPLC column was necessary to elute highly retained compounds (the mono-carboxylic acids) while preserving resolution. Since the mobile phase composition is not changing, there is no need for re-equilibration between injections. A van Deemter plot was constructed using the optimum SDS mobile phase of 1% SDS in 1.84 mM H_2SO_4 at pH 3 to determine the loss in efficiency as the flow rate is increased. Figure 4.2A displays the effect of mobile phase velocity on the plate height (HETP) for TPA. The Foley-Dorsey equation was used to calculate plate number N since this equation will account for peak asymmetry [35]. The minimum plate height occurs at 0.05 mL/min (0.490 mm/s). There is a slight increase in plate height as the flow rate is increased to 0.5 mL/min (5.81 mm/s). However, this slight loss in plate height was sacrificed to incorporate the flow rate gradient. By using a higher

flow rate to elute the strongly retained compounds, the overall analysis time is significantly shortened. Without the mobile phase gradient, the overall analysis time was nearly 60 min. Figure 4.2B shows the UHPLC chromatogram of the 10 ppm standard run using the optimum 1% SDS mobile phase and flow rate gradient. There is an unusual loss in signal for only TPA when injected as part of the mixture under these UHPLC conditions. To determine the possible source of this signal loss, the column was moved to be between the pump and the autosampler, and a short (3 mm) union was placed where the column would normally be to connect the autosampler and the detector. This provided the same backpressure observed during analysis without having to increase the flow rate or change the diameter of the instrument tubing. TPA was injected and the UV signal was collected at a flow rate of 0.3 and 0.5 mL/min. It was found that the peak area, height, and width did not change with a change in pressure. Using the

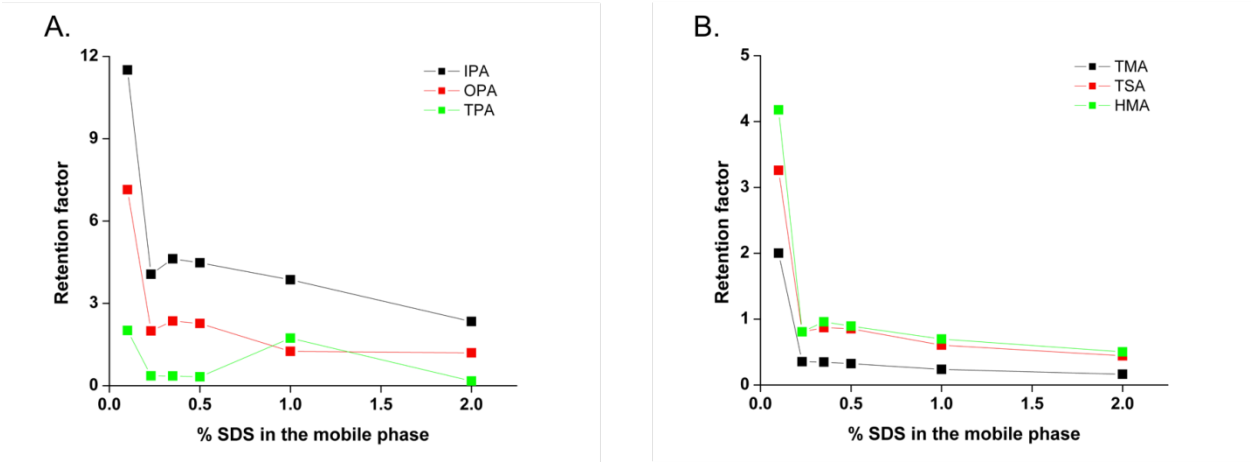

Figure 4.1. The effect of the % SDS on the retention factor (k') for Mix 1 (A) and Mix 3 (B). Each mobile phase percentage of SDS was made in 1.84 mM H_2SO_4, pH 3.0. k' values given are an average of triplicate measurements with % RSDs between 0 and 5.6%.

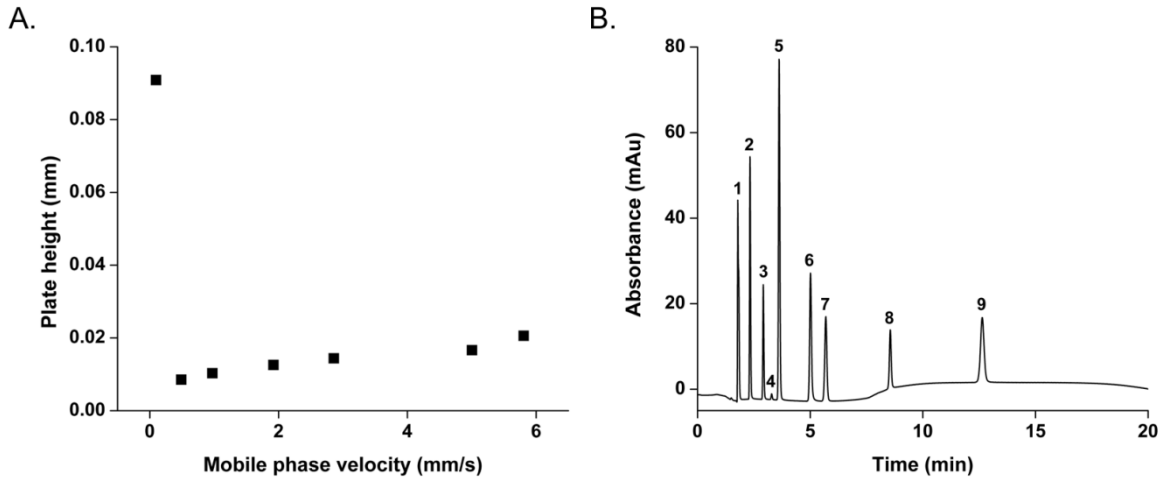

Figure 4.2. The van Deemter plot for TPA (A) and a UHPLC chromatogram for the 10 ppm standard using a mobile phase of 1% SDS in 1.83 mM H_2SO_4, pH 3.0 (B). The standard was run using the flow rate gradient. Peak assignments are as follows: (1) TMA, (2) TSA, (3) HMA, (4) TPA, (5) FBA, (6) OPA, (7) IPA, (8) BA, (9) pTA.

standard HPLC instrument configuration, the 10 ppm standard was injected and analyzed using a mobile phase of more dilute (0.1%) SDS in 1.84 mM H_2SO_4. This was to determine whether or not the high concentration of SDS (above the CMC) had an effect on the peak area loss. It was found that even under sub-CMC conditions the peak area for TPA was lost. This anomaly, only observed for TPA, was not observed using the same mobile phase and a standard HPLC column [23]. We are unsure what is the reason, but it appears related to the use of a small particle UHPLC column.

Optimization of Brij-35

Optimization of the Brij-35 mobile phase began by observing the effect of the percentage of Brij-35 in the mobile phase on the retention factor of the analytes. First, the SDS was stripped from the column by rinsing it with methanol for 3 hr at 0.1 mL/min. Once the column was then modified with Brij-35, the first pH value tested was pH 3 based on the optimum mobile phase pH observed using an SDS modified column and mobile phase. Figure 4.3A shows the effect of % Brij-35 on the retention factor at a mobile phase pH of 3. The percentages of Brij-35 were chosen to be both below and above the CMC, 0.011% or 0.09 mM. Interestingly, there is no significant decrease in retention as the percentage of Brij-35 is increased. Retention order for Mix 1 showed a switch in order for TPA and OPA when compared to SDS, while retention order for Mix 3 also showed a switch in order for TSA and HMA when compared to SDS. Just like with SDS, Mix 2 is retained preferentially using Brij-35. However, using the partition coefficient equations defined by Armstrong and Stine, the analytes do display a slightly positive slope when 1/k' is plotted again the concentration of the micelle in the mobile phase. This positive slope indicates that the analytes are in fact binding solutes [34]. Although the acids were well resolved at this pH, the analysis time is nearly 60 minutes even when incorporating the flow rate gradient previously used. When using an SDS environment, it was observed that as the mobile phase pH was increased, overall retention of the analytes decreased [23]. Therefore, the mobile phase pH was increased to 3.5, 4, 4.5, and 6 to determine the effect of pH on the retention (Figure 4.1S). Although the overall analysis time is significantly shorter, once the mobile phase pH was increased to pH 4 and above, there is noticeable loss in resolution of many of the analytes. Based on Table 1, further ionization of the di- and tri-carboxylic acids will be significant. Therefore, it was determined that a pH of 3.5 would be the best mobile phase pH to use with a Brij-35 mobile phase. Figure 4.3B shows the retention factor data at various percentages of Brij-35 at a mobile

phase pH of 3.5. Again, there is no significant decrease in retention as the percentage of Brij-35 is increased. At this pH, the overall analysis time is under 35 minutes when using the flow rate gradient. When comparing SDS and Brij-35 UHPLC chromatograms, TSA is more retained using Brij-35. TSA elutes between TPA and IPA, placing it among the diacids group on the chromatogram. This increase in retention was seen under every Brij-35 mobile phase tested and could possibly be attributed to some preferential interaction with the ethylene oxide chain of Brij-35. Likewise, as when HPLC and UHPLC SDS chromatograms were compared, the trio of TPA, FBA, and OPA are eluted in a different order using Brij-35. In a Brij-35 environment their retention order is OPA, FBA, then TPA. Possibly, the carboxylic groups of FBA and TPA in the para position could result in better interaction with the ethylene oxide chain of Brij-35.

Because analyte retention did not change significantly as % Brij-35 was increased, a mobile phase that did not contain any Brij-35 was tested. It was found that a simple buffered mobile phase was sufficient to resolve the analytes while keeping the analysis time below 35 minutes. In particular, resolution of FBA, TPA, and TSA was improved slightly with a non-surfactant mobile phase. This mobile phase is advantageous because micellar chromatography is not compatible with mass spectrometry (MS) detection. However, if the mobile phase does not contain any surfactant, then the method potentially becomes more compatible with MS. To determine the stability of the column modification, 12 injections of the 10 ppm standard were made onto the column every 2 hours. The data was collected and compared to determine if there is a loss of efficiency after so much usage time. Plate count (N) was determined using the following equation: $N = 16 \times (\frac{t_R}{w})^2$, where t_R is retention time and w is peak width at the baseline. Plots of plate count versus time for Mix 1, Mix 2, and Mix 3 are shown in Figure 4.2SA, 4.2SB, and 4.2SC respectively. Data for TPA and FBA is not shown because the analytes were not resolved enough to accurately determine peak width. Overall, there is no decrease in plate count over time. Although this mobile phase provided excellent results, it would be wise when using UV detection to include a small amount of Brij-35 in the mobile phase to ensure a stable column modification. Therefore, a mobile phase of 0.005% Brij-35 in 10 mM ammonium formate at pH 3.5 was determined to be the optimum mobile phase.

Since the flow rate gradient helped decrease the overall analysis time, the effect of mobile phase velocity on the plate height was determined for the Brij-35 modified column. Figure 4.4A displays the van Deemter curve for TPA. Again, the Foley-Dorsey equation was

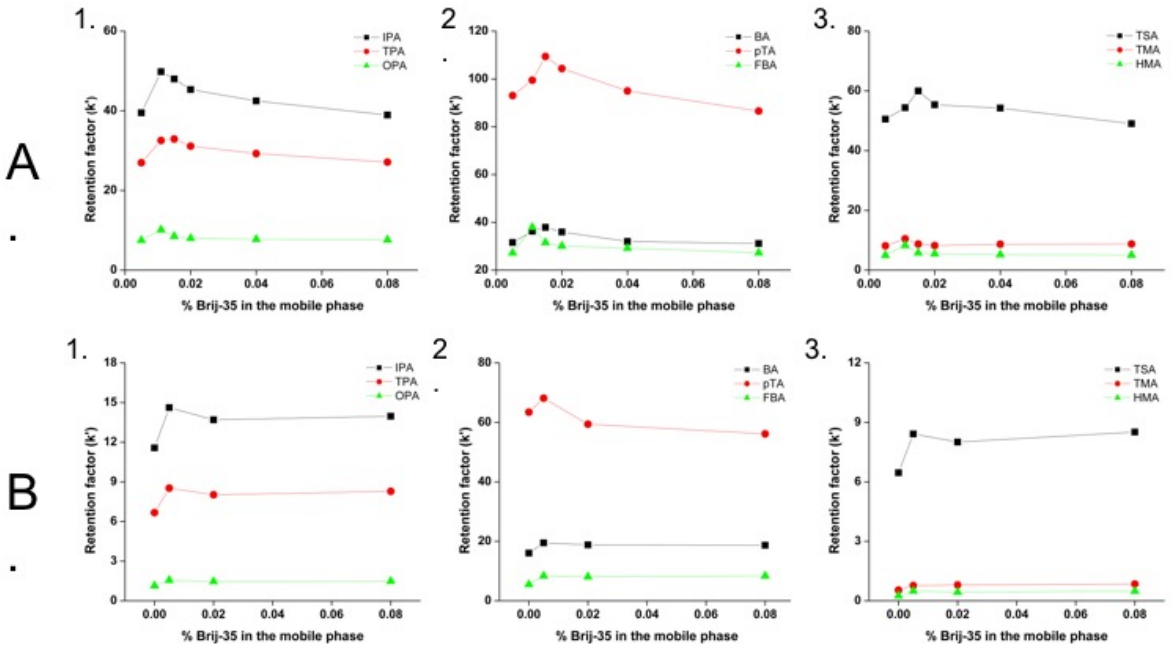

Figure 4.3. The effect of the % Brij-35 on the retention factor (k') using a mobile phase pH of 3.0 (A) for Mix 1 (1), Mix 2 (2), and Mix 3 (3) and a mobile phase pH of 3.5 (B) for Mix 1 (1), Mix 2 (2), and Mix 3 (3). k' values given are an average of triplicate measurements with % RSDs between 0.20 and 5.4% (A) and 0 and 5.8% (B).

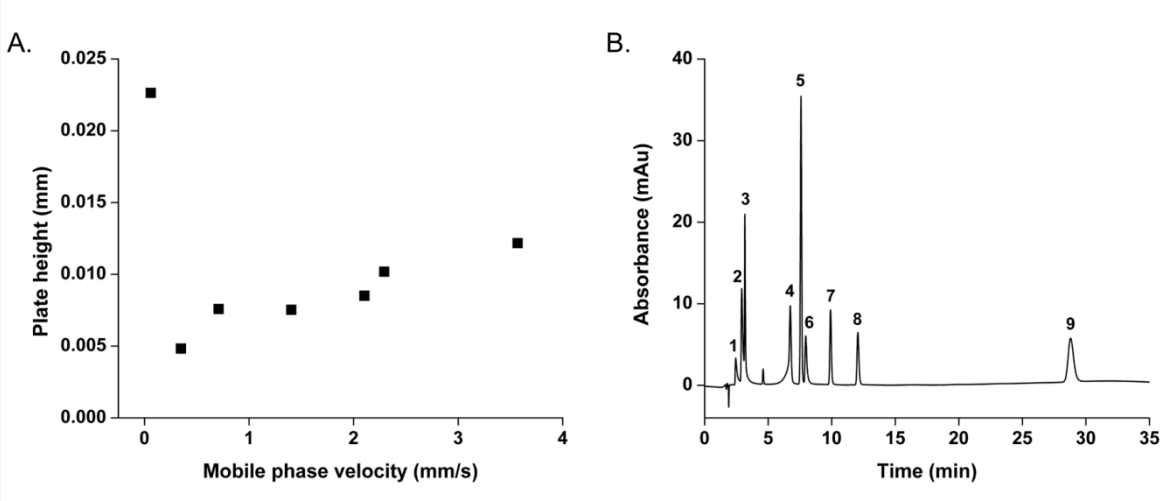

Figure 4.4. van Deemter plot of TPA (A) and UHPLC chromatogram of the 10 ppm standard (B) in a mobile phase of 0.005% Brij-35, 10 mM ammonium formate, pH 3.50. The standard was run using the flow rate gradient. Peak assignments are as follows: (1) TMA, (2) HMA, (3) OPA, (4) FBA, (5) TPA, (6) TSA, (7) IPA, (8) BA, (9) pTA.

used to determine plate number N because it takes peak asymmetry into account [35]. The minimum plate height occurs at a flow rate of 0.05 mL/min (0.350 mm/s). However, there is not much increase in plate height as the flow rate is increased up to 0.5 mL/min (3.57 mm/s). Because of more facile mass transport in such a low % Brij aqueous mobile phase as compared to a micellar one, the overall profile for H is lower than that for SDS. Similar to the SDS environment, without a flow rate gradient, the overall analysis time was over 60 minutes. Again, this minimal change in plate height meant that a flow rate gradient could be incorporated without sacrificing much efficiency so that the overall analysis time is not excessively long. Figure 4.4B shows the UHPLC chromatogram for the 10 ppm standard analyzed using the Brij-35 coated column and a mobile phase of 0.005% Brij-35 in 10 mM ammonium formate, pH 3.5. Although there is some significant peak fronting on FBA, this is diminished when using a smaller injection size of 1 μL. However, when using a smaller injection size, detection limit is often sacrificed [36]. In contrast to the SDS method, no loss in peak area for TPA was observed when injected as part of the mixture.

Industrial sample

Five calibration standards were made at the following concentrations for all nine analytes: 1, 10, 25, 50, and 75 ppm. These standards were analyzed on both the SDS modified column and the Brij-35 modified column. The mobile phase for the SDS modified column was 1% SDS in 1.84 mM H_2SO_4, pH 3, and the mobile phase for the Brij-35 modified column was 0.005% Brij-35 in 10 mM ammonium formate, pH 3.5. The upper half of Table 4.2 also summarizes the linear regression data, detection limits, and quantitation limits of the analytes in an SDS separation environment. All of the data was collected at 240 nm. When comparing the maximum wavelength for each analyte, 240 nm was the closest to the maximum for all nine acids since the maximum wavelengths ranged from 210 to 260 nm. Detection limits ranged from 1.77 to 53.1 ppm (16.9 to 870 pmoles), and quantitation limits ranged from 4.34 to 139 ppm (52.3 to 2278 pmoles). The lower half of Table 4.2 also summarizes the linear regression data, detection limits, and quantitation limits of the analytes in a Brij-35 separation environment. Detection limits ranged from 0.310 to 8.70 ppm (3.74 to 82.8 pmoles), and quantitation limits ranged from 0.993 to 30.2 ppm (11.9 to 288 pmoles). Because the UV wavelength used was not always the maximum wavelength for each analyte, this may affect the LOD and LOQ values. For example,

the maximum wavelength for pTA is 240 nm which could help with the low LOD and LOQ values determined. However, the maximum wavelength for TPA is 240 nm as well, but the LOD and LOQ values are much higher. This could be caused by the broader peak observed for TPA. The peak observed for FBA was also very broad and the maximum wavelength is above 240 nm which might explain the higher LOD and LOQ values determined.

Once the linearity, detection limits, and quantitation limits had been determined, an industrial sample was prepared and analyzed under both SDS and Brij-35 conditions. Figure 4.5A shows the UHPLC chromatogram for the industrial sample analyzed on the SDS modified column. The mobile phase was 1% SDS in 1.84 mM H_2SO_4, pH 3 and used the flow rate gradient described in the Materials and Methods. Using peak area and the calibration curves generated using the calibration standards, the acids concentrations were found to be as follows: IPA, 2954 ppm; OPA, 1843 ppm; TPA, 14670 ppm; BA, 15370 ppm; pTA, 3393 ppm FBA, 189.1 ppm; TSA, 177.9 ppm; TMA, 5151 ppm; HMA, 3753 ppm. Figure 4.5B shows the UHPLC chromatogram for the industrial sample analyzed on the Brij-35 modified column. The mobile phase was 0.005% Brij-35 in 10 mM ammonium formate, pH 3.5 and used the flow rate gradient as well. The acid concentrations were found to be as follows: IPA, 2986 ppm; OPA, 4393 ppm; TPA, 230.5 ppm; BA, 16330 ppm; pTA, 3290 ppm; FBA, 675.2 ppm; TSA, 365.7 ppm; TMA, 4588 ppm; HMA, 528.8 ppm. In our previous work using standard MLC with SDS, we reported the concentrations of these acids in the 106.8 to 15170 ppm range for the same type of industrial sample but a different lot [23]. It's interesting to note the difference in peak height in Figure 4.5 for nearly every acid. This peak height difference between SDS and Brij-35 environments affected the analyte concentrations found. When comparing the concentrations for the acids between the two methods, TPA, FBA, OPA, and HMA are the only acids that differ significantly. This difference was calculated using a student t-test. The other five acids do not differ significantly. These results were also compared to the previous MLC method published, and it was found that all nine acids differ significantly between the HPLC and UHPLC method in an SDS environment. Additionally, all of the analytes except HMA differed significantly when the SDS HPLC method was compared to the Brij-35 UHPLC method.

4.6: Conclusion

There are few comparisons of different surfactants for MLC in the literature, and there are even fewer examples of MLC under UHPLC conditions. Using a C18 UHPLC column

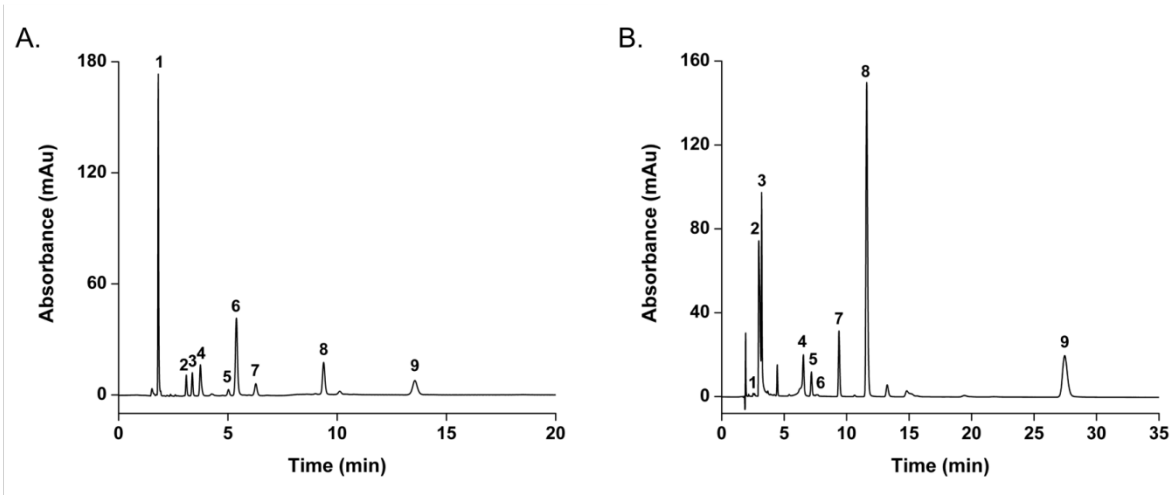

Figure 4.5. UHPLC chromatogram of the industrial sample under the optimized SDS conditions (A) and Brij-35 conditions (B). The mobile phase for A was 1% SDS, 1.84 mM H_2SO_4, pH 3. The mobile phase for B was 0.005% Brij-35, 10 mM ammonium formate, pH 3.50. Peak assignments for A are as follows: (1) TMA, (2) TSA, (3) HMA, (4) TPA, (5) FBA, (6) OPA, (7) IPA, (8) BA, (9) pTA. Peak assignments for B are as follows: (1) TMA, (2) HMA, (3) OPA, (4) FBA, (5) TPA, (6) TSA, (7) IPA, (8) BA, (9) pTA.

Table 4.2. Calibration parameters and figures of merit under SDS conditions and Brij-35 conditions.

SDS	Compound	A[a]	B[b]	R^2	LOD[c]	LOQ[d]
	IPA	0.0428	0.2152	1.000	0.294	0.514
	OPA	-1.80	0.323	0.9764	9.93	46.1
	TPA	0.0264	0.00579	0.9043	24.2	69.9
	BA	-0.635	0.167	0.9883	7.04	32.3
	pTA	-0.0347	0.289	0.9999	0.478	1.87
	FBA	2.03	0.494	0.9770	15.3	41.4
	TSA	0.0798	0.276	1.000	0.725	1.74
	TMA	-0.0230	0.299	1.000	0.326	1.27
	HMA	0.00492	0.143	1.000	0.442	1.39
Brij-35	Compound	A[a]	B[b]	R^2	LOD[c]	LOQ[d]
	IPA	0.00232	0.129	1.000	0.310	0.993
	OPA	-0.0659	0.143	0.9993	1.46	5.93
	TPA	-0.675	0.167	0.9847	5.03	26.2
	BA	0.0159	0.147	1.000	0.446	1.24
	pTA	0.0575	0.278	1.000	0.443	0.994
	FBA	0.0415	0.256	0.9982	3.26	10.5
	TSA	-0.0200	0.362	1.000	0.874	3.04
	TMA	0.0786	0.183	0.9873	8.70	28.0
	HMA	-0.313	0.0816	0.9807	6.38	30.2

[a] A values are given as the y-intercept from the linear regression equation of peak area versus concentration (ppm).

[b] B values are given as the slope from the linear regression equation of peak area versus concentration (ppm).

[c] Detection limit values are given in ppm and were calculated using the following equation: $LOD=(A+3*A_\sigma)/B$ where A_σ is the standard deviation of the y-intercept.

[d] Quantitation limit values are given in ppm and were calculated using the following equation: $LOD=(A+10*A_\sigma)/B$ where A_σ is the standard deviation of the y-intercept.

modified with SDS and an acidic SDS mobile phase, analysis time of TPA and eight impurities is under 15 minutes with baseline resolution. However, there is a loss in UV signal for TPA under these conditions. Using the same C18 column modified with Brij-35 and an acidic ammonium formate mobile phase, the same analytes are resolved in under 35 minutes. Though the analysis time is sacrificed, there does not seem to be a loss in UV signal for any analyte using Brij-35. Overall, using a Brij-35 modified column and a very dilute Brij mobile phase produced better detection and quantitation limits than using a SDS modified column and a 1% SDS mobile phase. In addition, a MS compatible UHPLC separation of these aromatic positional isomers was possible using simply a Brij coated C18 column and just the buffered (no surfactant) mobile phase.

4.7: References

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4.7: Supplementary Information

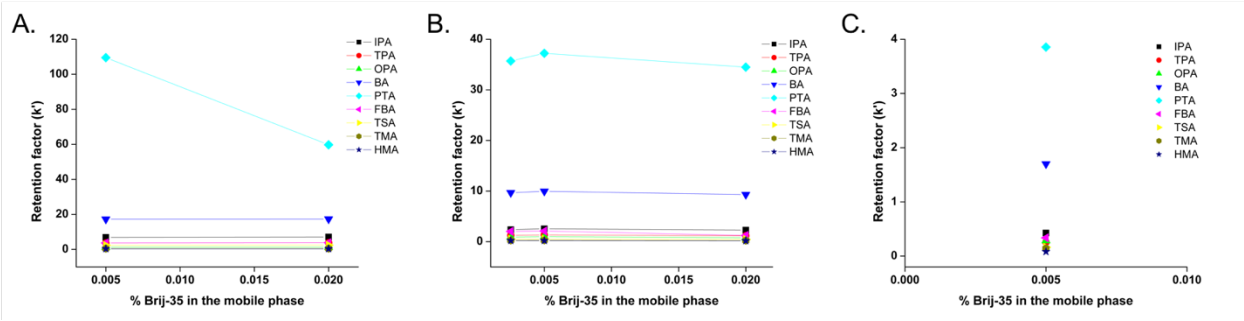

Figure 4.1S. The effect of % Brij-35 on retention factor at pH 4 (A), pH 4.5 (B), and pH 6 (C). The peak shapes at pH 4 were worse than at pH 4.5. Therefore, a smaller amount of Brij-35 was used at pH 4.5 to determine if the retention could be extended enough to gain more resolution. Once the pH was increased to 6, the analytes were nearly unretained. k' values given are an average of triplicate measurements with % RSDs between 0% and 8.9%.

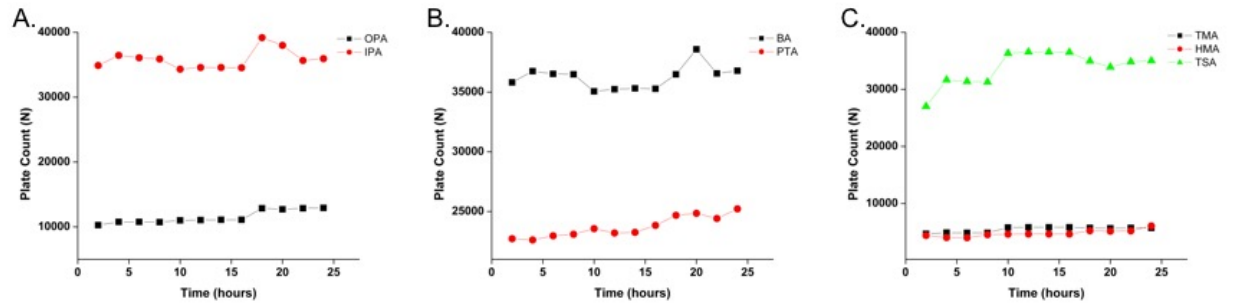

Figure 4.2S. The effect of plate count over time for Mix 1 (A), Mix 2 (B), and Mix 3 (C). An injection of the 10 ppm standard was made once every two hours over a 24-hour period. The mobile phase was 0% Brij-35 in 10 mM ammonium formate, pH 3.50.

CHAPTER 5

Anion Exchange Chromatography of Sulfonated Compounds using a Protamine as the Mobile Phase and Stationary Phase Modifier

Ashley E. Richardson, Elise M. Leonard, Matthew T. Webb, and Neil D. Danielson

5.1: Abstract

Since the heparin crisis 2007, the separation of heparin from other glycosaminoglycans, like oversulfonated chondroitin sulfate, is extremely important. The standard methods are commonly strong ion exchange chromatography, though these methods yield long analysis times, broad peaks, and poor resolution. A strong cation exchange column has been modified with protamine, a commonly used compound for heparin reversal, to make an anion exchange column. Protamine is also included in the mobile phase. Various model compounds have been used like naphthalene mono-, di-, and trisulfonate, polystyrene sulfonate, and chondroitin sulfate. The mobile phase, comprised of small amount of protamine and perchlorate in phosphoric acid, has been successfully optimized for the naphthalene sulfonate compounds and polystyrene sulfonate. A polystyrene sulfonate compound has been identified in a wrinkle serum sample using the optimized method. Further optimization is needed for chondroitin sulfate and similar glycosaminoglycans.

5.2: Introduction

Ion exchange chromatography (IEC) is a mode of chromatography that is useful for the separation of inorganic ions and high molar mass biological molecules [1,2]. The retention mechanism for IEC is based on the analyte - counter ion equilibrium with the charged resin. The counter ion cannot be held too strongly by the resin or else the analyte will not be able to exchange places with the counter ion and be retained by the resin and therefore be retained. There are multiple factors that affect the retention and elution of analytes that include counter ion choice, counter ion concentration, and mobile phase pH (in the case of weak ion exchange). A counter ion that is large in size and has a high charge tends to interact more with the resin than a counter ion that is smaller (more hydrated) with a lower charge [1].

Sulfonated compounds can be separated using IEC or ion-pair chromatography. Ion pair chromatography has been commonly used for low molar mass charged aromatic organic compounds because of the effectiveness of the dynamic ion exchange/ion-pair retention mechanism [3]. If little is known about the sulfonated compounds in question [4], IEC can be

coupled to mass spectrometry detection, not an easy option for ion-pair chromatography. For some reason, IEC has not been commonly used to separate polymeric aromatic sulfonated compounds, like polystyrene sulfonates. However, IEC has been applied for the separation of more aliphatic polymeric sulfonates such as glycosaminoglycans (GAGs). Figure 5.1 shows the structure for important GAGs such as heparin, OSCS, chondroitin sulfate, dermatan sulfate, and hyaluronic acid. Current methods available to analyze chondroitin sulfate are mostly strong anion exchange methods. These tend to be long in analysis time and produce broad peaks for chondroitin sulfate [5,6]. However, one important application is the separation of heparin from oversulfonated chondroitin sulfate (OSCS). This separation is extremely important due to the heparin crisis of 2007/2008 [7,8]. Today, there is a great amount of care taken to ensure that heparin is not adulterated with OSCS. Strong anion exchange is the FDA-approved method for the separation of heparin in a pharmaceutical quality control setting [9]. The detection method is most commonly absorbance detection at a low UV wavelength [10–13]. However, many IEC methods require long analysis times that can be up to 90 minutes. They tend to use a high salt gradient which can cause the retention times to drift. The high salt concentration can also affect the column stability over time [13,14]. Other methods using IEC to separate heparin from other GAGs produce extremely broad peaks that can be up to 20 minutes wide. When the peaks are extremely broad, the resolution is sacrificed significantly as well [15,16]. Weak anion exchange has alleviated partially some of these limitations of strong anion exchange [17]. However, there is still need for a new approach that has an ion-pair component that may overcome the disadvantages of the standard ion exchange methods.

In this research, the retention behavior of strong anion exchange mediated by the ion-pair reagent protamine will be modeled using small and polymeric aromatic sulfonates before testing with a GAG. By incorporating protamine (structure and UV spectrum shown in Figure 5.2), a common antidote for heparin in the medical field, into the mobile phase, the large negative charge on heparin or the model compounds will be shielded by the positive charge of protamine (due to the multiple arginine amino acids) [15]. In addition, the protamine will electrostatically interact with a cation exchange resin turning it into an anion exchanger. The low UV absorbance of protamine should able to be baseline corrected by the HPLC detector permitting a wide range of wavelengths to be selected. The model compounds will include naphthalene monosulfonate,

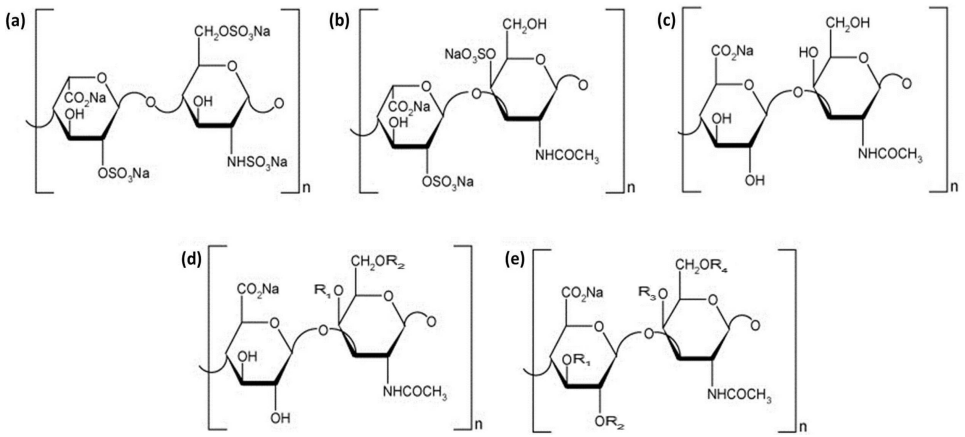

Figure 5.1. The structure of heparin (A), dermatan sulfate (B), hyaluronic acid (C), chondroitin sulfate (D), and oversulfonated chondroitin sulfate (E). For D, R is H and for E, R is SO_3Na.

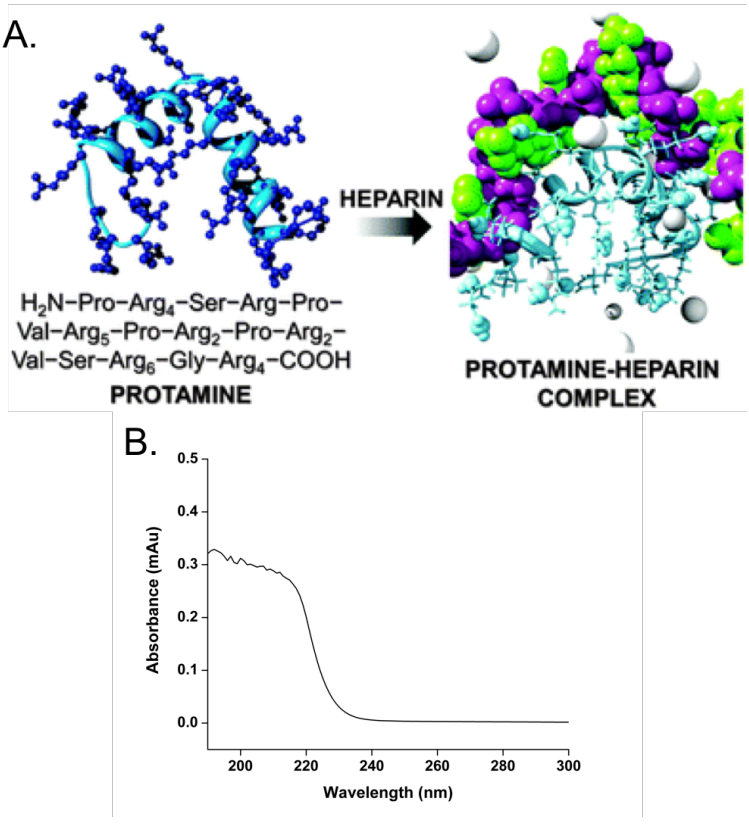

Figure 5.2. The structure of protamine and the complex formed by protamine and heparin (A) and the UV spectrum for protamine (B).

naphthalene disulfonate, naphthalene trisulfonate, and polystyrene sulfonate. Once the conditions have been optimized using these model compounds, we plan to apply this method to the separation of the GAG chondroitin sulfate in a skin care sample. In the future, we plan to use this method to show that it is possible to achieve narrower peaks and improved resolution in the separation of heparin from oversulfonated chondroitin sulfate. To the best of our knowledge, the use of protamine as an ion-pair/ion exchange retention control reagent has never been explored in liquid chromatography.

5.3: Apparatus and Conditions

Chromatographic experiments for the naphthalene sulfonate and polystyrene sulfate compounds were first carried out on a HPLC instrument consisting of a Dionex LPG-2400A HPLC pump (Thermo Scientific, Sunnyvale, CA, USA) equipped with an Agilent 1100 degasser (Santa Clara, CA, USA), a Waters 717 autosampler (Milford, MA, USA), and Hewlett-Packard Series 1050 UV detector (Wilmington, DE, USA). Chromatographic experiments for chondroitin sulfate and the wrinkle serum were performed on a Dionex UltiMate 3000 HPLC instrument (Sunnyvale, CA, USA) that was equipped with a diode array detector. Data acquisition was carried out using Chromeleon software (Thermo Scientific, Sunnyvale, CA, USA) for both instruments. A Tosohaas TSK-gel SP-5PW column (7.5 mm i.d. x 7.5 cm, 10 μm) (King of Prussia, PA, USA) that was packed with polymethylmethacrylate-based strong cation exchange resin particles in the sulfonic acid-form was used for chromatographic separations.

A 20 μL injection size was used with a flow rate of 1.0 mL/min. The column was kept at room temperature, and UV data was collected at 280 nm for the naphthalene sulfonate and polystyrene sulfonate compounds and 205 and 260 nm for the chondroitin sulfate samples. The UV spectra for the polystyrene sulfonate compounds and chondroitin sulfate are shown in Figure 5.3.

5.4: Chemicals and Procedures

Naphthalene monosulfonate (NMS), naphthalene disulfonate (NDS), naphthalene trisulfonate (NTS), protamine, and sodium perchlorate were purchased from Sigma Aldrich (St. Louis, MO, USA). Two polystyrene sulfonate compounds were used: one had an approximate molecular weight of 75,000 g/mol (PSA), and the other had an approximate molecular weight of 200,000 g/mol (PSB). Both polystyrene sulfonate compounds were purchased from Sigma Aldrich (St. Louis, MO, USA). Phosphoric acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Chondroitin sulfate (BulkSupplements.com, Henderson, NV, USA) was purchased from Amazon. Reactivator Wrinkle Serum was purchased from Amazon. The list of ingredients is shown in Table A.1. The naphthalene sulfonate compounds, polystyrene compounds, and chondroitin sulfate were all made using
18.2 MΩ water that was passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA) that distilled and deionized the water.

The mobile phase was made in 0.01 M phosphoric acid, pH 2.3. The concentration of perchlorate and protamine were optimized by variation studies between 0.001 M and 0.5 M for perchlorate and 0.05% and 0.5% for protamine. The column was equilibrated for 30 minutes prior to the first injection. The naphthalene sulfonate compounds were made at a concentration of 0.5 g/L. The polystyrene sulfate compounds were made at the following concentrations: 0.5 g/L, 1.0 g/L, and 1.5 g/L to confirm peak assignment. The chondroitin sulfate standards were made at the following concentrations: 0.1 g/L, 0.5 g/L, 1.0 g/L, and 1.5 g/L. The wrinkle serum was diluted by a factor of 4 using water.

5.5: Results and Discussion

Optimization of the mobile phase for the naphthalene sulfonates separation

The optimization of the mobile phase began by monitoring the retention time of the naphthalene sulfonate (NS) compounds. It had previously been shown that a mobile phase of 0.01 M phosphoric acid has the potential to separate larger glycosaminoglycans (GAGs), like heparin, chondroitin sulfate, oversulfonated chondroitin sulfate, and hyaluronic acid using the same column in the ion exclusion mode. However, these results do not seem to be easy to reproduce and analyte retention tends to be weak [18]. A counter ion is traditionally required in IEC for ionic strength control. Sodium perchlorate was chosen
because it provides an extremely low UV cutoff, which is important for the detection of GAGs. Although increasing the counter ion concentration should promote shorter retention times, the effect is not always as great as it needs to be with large, negatively charged compounds like GAGs. Therefore, it was hypothesized that the addition of a large, positively charged compound, like protamine, could help alleviate the issues of excessive retention of large, negatively charged compounds in IEC. Because the column is a cation exchange column, protamine should electrostatically interact with the sulfonic acid groups and convert it to an anion exchanger. However, working with large compounds like the GAGs listed above proves to be challenging

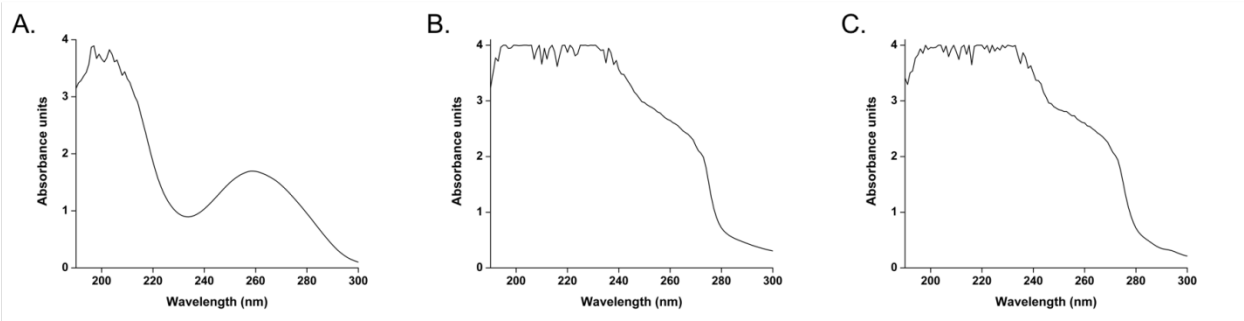

Figure 5.3. UV spectra for chondroitin sulfate (A), polystyrene A (B), and polystyrene B (C). Each compound had a concentration of 1.0 g/L.

Table 5.1. List of ingredients in Reactivator Wrinkle Serum

Ingredient No.	Name
1	Purified Water
2	Acetyl Hexapeptide-8
3	Sodium Polystyrene Sulfonate
4	Acrylates/ceteth-20 Itaconate Copolymer
5	Hydrolyzed Wheat Protein
6	Propylene Glycol
7	Sodium Hydroxide
8	Aloe Barbadensis Leaf Juice
9	Carbomer
10	Soluble Collagen
11	Sodium Hyaluronate
12	Chamomila Recutita (Matricaria) Flower Extract
13	Cucumis Sativus (Cucumber) Fruit Extract
14	Rosa Canina Fruit Extract
15	Hamamelis Virginiana (Witch Hazel) Extract
16	Tetrasodium EDTA
17	Prophylparaben
18	Methylparaben
19	Adenosine
20	Human Oligopeptide-1

due to their difficulty in UV detection and high charge. Therefore, model compounds were chosen to optimize the mobile phase. The NS compounds are like an aromatic representative of a single heparin unit and would therefore be an ideal first model. The phosphoric acid concentration was kept constant at 0.01 M based on previous research [18] but the perchlorate and protamine concentrations were varied to determine the optimum concentration of each.

The concentration of perchlorate first tested was 0.01 M, and the concentration of protamine first tested was 0.1%. From there, each component was decreased and increased to create a profile for the effect that it has on the retention of the naphthalene sulfonate compounds. Figure 5.4 shows a plot of the effect that the concentration of perchlorate has on these compounds (Figure 5.4A) and the effect that the concentration of protamine has on these compounds (Figure 5.4B). However, as expected an increase in protamine concentration produced an increase in retention for these analytes. From these plots, it was determined that a mobile phase of 0.01 M perchlorate+0.3% protamine in 0.01 M phosphoric acid was sufficient to separate these three compounds. Figure 5.5 shows the chromatogram of the mixture of the three naphthahlene sulfonate compounds. The peaks are broader than anticipated, in part due to the wide column diameter (7.5 mm) which allows for pressure stability at a 1 mL/min flowrate.

Polystyrene sulfonate retention factor and analysis of wrinkle serum

The next step was to apply the optimized mobile phase to polystyrene sulfonate (PS) compounds, which are bulky in structure and highly charged like GAGs. However, the PS compounds are easily detectable. Three concentrations, 0.5 g/L, 1.0 g/L, and 1.5 g/L, were made for each compound. By using three different concentrations, more confidence would be had in the peak assignment because the peak area and height should increase as the concentration of the analyte increases. Initially the optimized mobile phase determined using NS compounds was tested, however it was found that this was not an ideal mobile phase for these PS compounds. PSA and PSB were both mixed with two concentrations of protamine, 0.05% and 0.5%. Both polystyrene sulfonate compounds had a concentration of 1.0 g/L. PSA showed a significant turbidity light scattering reading when mixed with 0.5% protamine, though the reading dropped to zero when PSA was mixed with 0.05% protamine. When PSB was mixed with both concentration of protamine, the turbidity readings were zero or close to zero. Mobile phase optimization with respect to the perchlorate concentration and then the protamine concentration was repeated to determine their effect on retention factor. PSA was found to be excessively

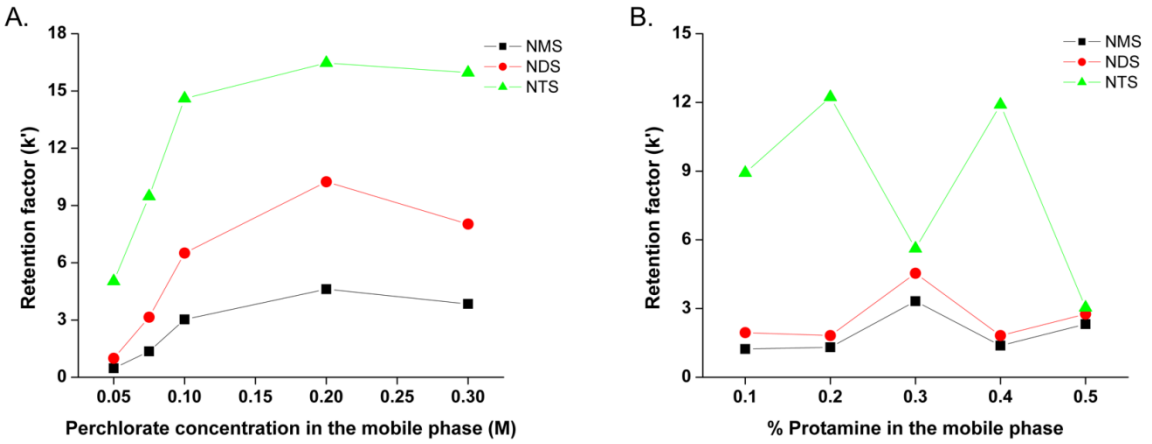

Figure 5.4. The effect of perchlorate concentration (A) and protamine percentage (B) on the retention factor of naphthalene monosulfonate, naphthalene disulfonate, and naphthalene trisulfonate.

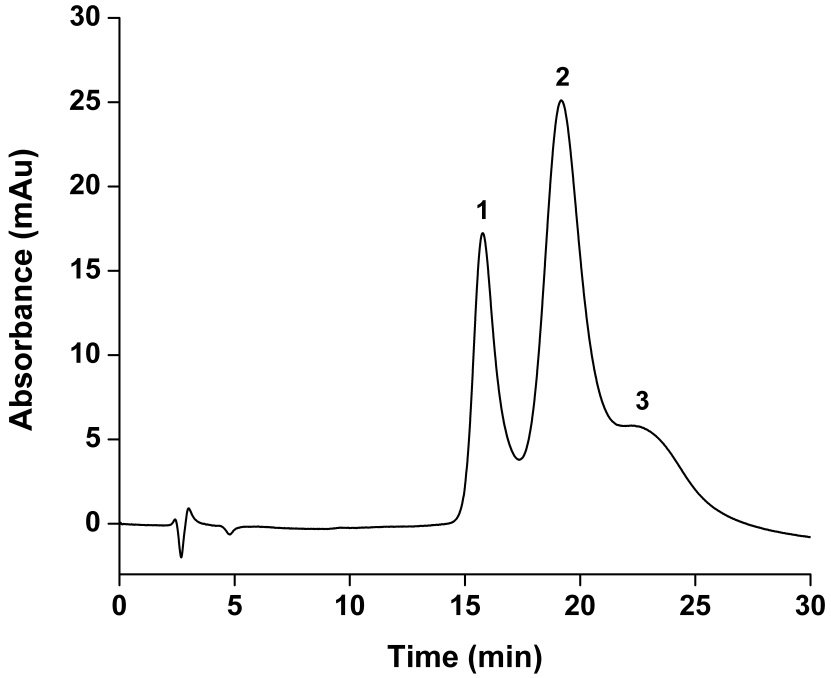

Figure 5.5. HPLC chromatogram of NMS (1), NDS (2), and NTS (3) in a mixture. The mobile phase consisted of 0.01 M perchlorate + 0.3% protamine in 0.01 M phosphoric acid. The flow rate was 1.0 mL/min.

retained when compared to PSB, regardless of the mobile phase. The extensive retention of PSA compared to PSB is somewhat surprising because of its lower molar mass of 75,000 as compared to 200,000 for PSB. However, based on the light scattering data collected, it is possible that PSA forming particles with protamine at the majority of the protamine concentrations tested. Figure 5.6A shows how retention factor for PSB changed with varying concentrations of perchlorate, while Figure 5.6B shows how retention factor for PSB changed with varying concentrations of protamine. As the concentration of perchlorate was increased, PSB was retained less until it reached a relatively stable plateau. Because perchlorate was used as the counter ion, a high concentration of perchlorate should reduce the retention of PSB so this was as expected. An increase in retention as the protamine concentration was increased was as hypothesized. The column used is a cation exchange column that is modified with a sulfonic acid group. Therefore, the more positive charge there is around the analyte, the more it will be retained. Alternatively, the protamine can electrostatically interact with the cation exchange resin, converting it to the anion exchange form which would retain the PS analytes. Based on these two plots, the optimum mobile phase was determined to be 0.01 M perchlorate + 0.1% protamine in 0.01 M phosphoric acid. Figure 5.7 shows a chromatogram of PSB in this mobile phase. PSA appeared to be much more retained than PSB because the majority of the chromatograms using each possible mobile phase showed no peak. A 1.0 g/L sample of PSA was mixed with 0.05% and 0.5% protamine to determine whether or not particles would form. There was significant turbidity value given when the PSA sample was mixed with 0.5% protamine, however when mixed with 0.05% protamine the turbidity reading was zero. Further light scattering testing of PSA with this mobile phase containing 0.1% protamine to ensure turbidity formation is not an issue is warranted.

A wrinkle serum sample was tested that had sodium polystyrene sulfonate listed as the third ingredient (Table 5.1). Because ingredient lists are usually made with the highest concentration at the top of the list, it is likely that polystyrene sulfonate would appear as a major peak in the chromatogram. The serum was diluted by a factor of four using water and injected onto the column. The mobile phase used was 0.01 M perchlorate + 0.1% protamine in 0.01 M phosphoric acid. The resulting chromatogram is shown in Figure 5.8. There are a couple other peaks present in the chromatogram, though based on retention time assignment of PSB, it is likely that the second peak is the polystyrene sulfonate in the wrinkle serum. When PSB was injected by itself using the same mobile phase, it had a retention time of 6.87 min. The second

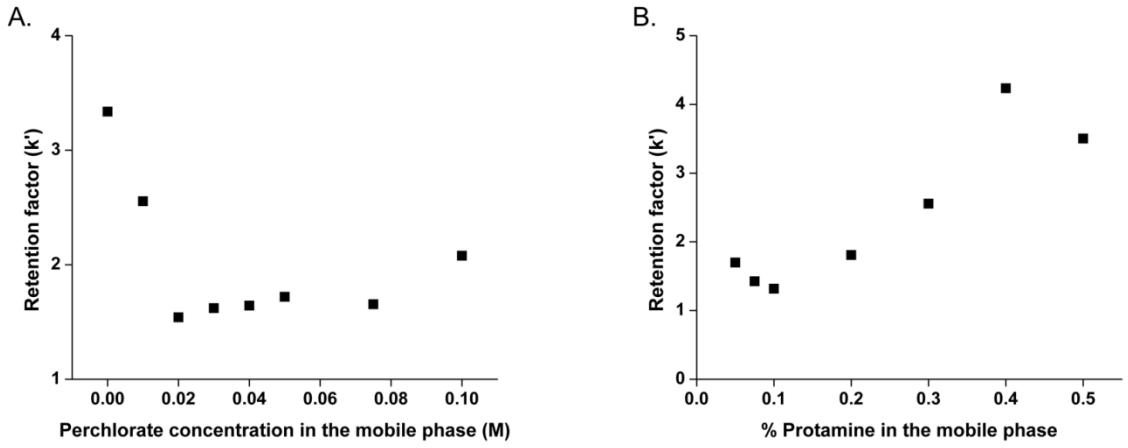

Figure 5.6. The effect of perchlorate concentration (A) and protamine percentage (B) on the retention factor of polystyrene sulfonate B (MW ~ 200,000).

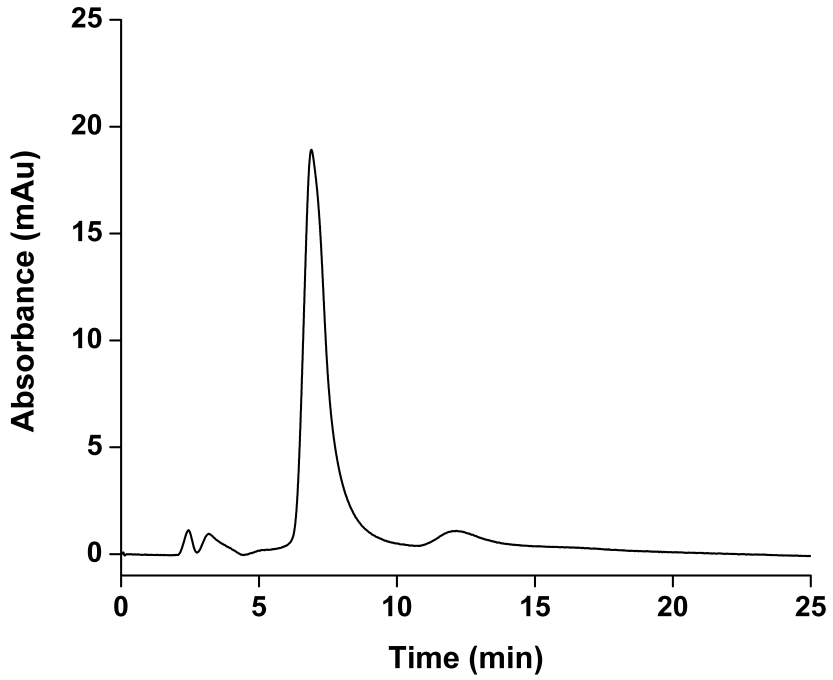

Figure 5.7. HPLC chromatogram of polystyrene sulfonate B (MW ~ 200,000) at 1.0 g/L. The mobile phase consisted of 0.01 M perchlorate + 0.1% protamine in 0.01 M phosphoric acid. The flow rate was 1.0 mL/min.

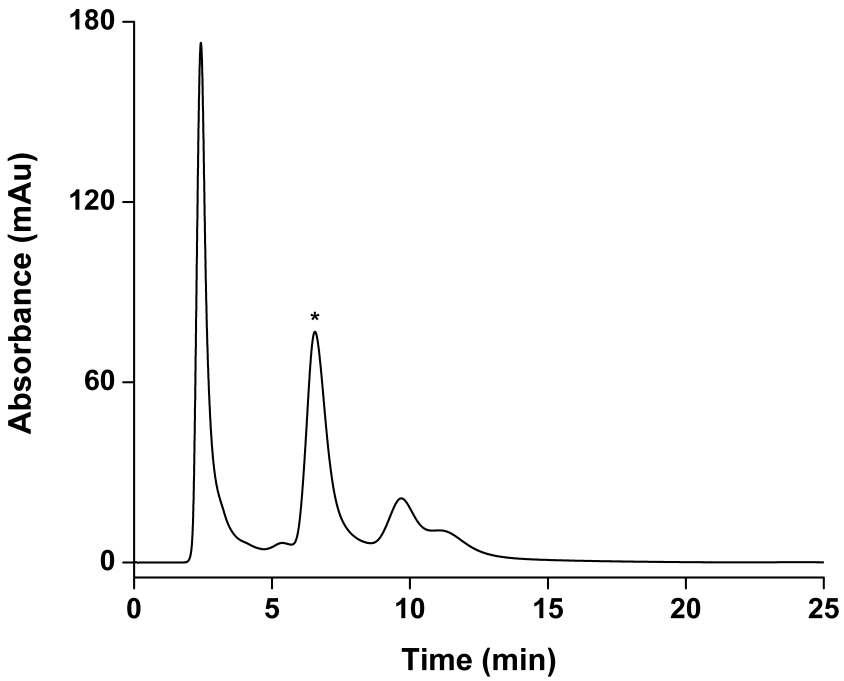

Figure 5.8. HPLC chromatogram of the wrinkle serum (diluted by a factor of 4). Peak 2 has been positively identified as the polystyrene sulfonate component in the serum. The mobile phase consisted of 0.01 M perchlorate + 0.1% protamine in 0.01 M phosphoric acid. The flow rate was 1.0 mL/min.

peak in the wrinkle serum had a retention time of 6.83 min. While it is difficult to confirm if this the exact polystyrene sulfonate used in the serum, this was a promising result. Based on a peak area ratio calculation and taking into account the dilution factor, the concentration of polystyrene sulfonate in the wrinkle serum is 13.9 g/L. A label value for comparison is unfortunately not available.

Retention of chondroitin sulfate

A literature search did not find any HPLC methods for the direct determination of native chondroitin sulfate. The primary HPLC approach is strong anion exchange separation with UV detection at 230 nm of the disaccharide fragments formed after enzymatic hydrolysis of chondroitin sulfate [5]. Using our approach, native chondroitin sulfate was tried as a test compound to determine the viability of this method for larger GAGs like heparin and oversulfonated chondroitin sulfate. The optimized mobile phase described in the previous section was used initially, though it was later changed. The data was also collected at 205 and 260 nm instead of 280 nm. These two wavelengths were determined after taking a full spectrum of chondroitin sulfate (Figure 5.3C). It seems structurally surprising that the peak at 260 nm on the spectrum is truly due to the chondroitin sulfate; other GAGs do not absorb at a wavelength higher than about 210 nm. However, a similar spectrum for chondroitin sulfate has been shown in the literature [19,20]. We do not expect there would be that significant an impurity in the commercial sample (although it is derived from an animal source [6]) and therefore were confident in collecting data at 260 nm as well as at 205 nm. The mobile phase used for PSB and the wrinkle serum was also chosen for the chondroitin sulfate. Unfortunately, the retention was extremely excessive with no peak evident. Based on the retention factor data for PSB, a higher concentration of perchlorate should decrease retention. However, when this was tried, it did not result in lower retention. Additionally, a lower percentage of protamine was also used, but the retention did not decrease under these conditions. A 1.0 g/L sample of chondroitin sulfate was mixed with 0.3% protamine to determine whether or not particles would form. It was found that the turbidity reading of this concentration of chondroitin sulfate with 0.3% protamine was nearly zero. It is likely that a much higher ionic strength mobile phase would be necessary to elute chondroitin sulfate in a reasonable time.

5.6: Conclusion

While the data collected using naphthalene sulfonate and polystyrene sulfonate compounds appeared promising, application of this method to chondroitin sulfate unfortunately needs further research. It appears that chondroitin sulfate is extremely well retained, meaning the mobile phase likely needs additional optimization. A mobile phase with a high concentration of perchlorate or a different, preferably multi-charged, anion like sulfate and low percentage of protamine should be tested. Based on the retention factor plots of PSB, these two conditions should promote shorter retention. However, polystyrene sulfonate was positively identified and its concentration estimated in a wrinkle serum sample. Spectra comparison of the PSB standard and the matching wrinkle serum peak using a photodiode array detector could confirm whether there are any other components being co-eluted.

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APPENDIX

Surfactant pluronic gel phases for electrophoresis

Ashley E. Richardson, Elise M. Leonard, Yudan Chen, Junyi Wang, Wenjun Wei, Neil D. Danielson

A.1: Introduction

The most common way to separate proteins is through the use of electrophoresis involving a gel, either agarose or polyacrylamide. These gels form pores, and the separation is based on a sieving effect of the proteins by size and/or charge. When sodium dodecyl sulfate (SDS) is added to a polyacrylamide gel, the proteins separate based on size only because the SDS gives each protein the same charge. However, the separation of peptides using these gels is much more difficult. The pores of an agarose or polyacrylamide gel can only be made so small, meaning peptides tend to migrate the entire distance of the separation area [1–3].

Pluronic polymers have primarily been investigated as a possible gel phase for the separation of DNA. For example, You and Winkle used Pluronic F127 in capillary gel electrophoresis to separate supercoiled plasmid DNA that consisted of 2,000 to 10,000 base pairs [4]. These polymers are tri-block, uncrosslinked, and uncharged polymers consisting of [poly(ethylene oxide)(EO)]$_x$[poly(propylene oxide)(PO)]$_y$[poly(ethylene oxide)(EO)]$_x$. These are also thermo-responsive polymers. Interestingly, below room temperature (0 to 5°C), these polymers are a free-flowing liquid when their concentration is less than 30%. Once the polymer is brought to room temperature, it becomes a gel. This process occurs because the hydrophobic propylene units form a hydrophobic micelle core surrounded by the hydrophilic ethylene units. This interesting characteristic means Pluronic polymer gels are much easier to prepare than traditional gels and analyte recovery is much simpler as well [5–7]. The Pluronic polymer forms pores like polyacrylamide but the core of the micelle is about 9 nm in diameter and the total micelle is about 18 nm in diameter. The interstitial space between the micelles is 4-9 nm in diameter [8].

This polymer has previously been used in slab gel electrophoresis to separate myoglobin tryptic peptides. Using a 24% Pluronic F-127 gel, they compared their results when a 15% polyacrylamide gel was used and found that the use of a Pluronic polymer yielded comparable data. This work also investigated two-dimensional electrophoresis using the Pluronic polymer gel and found that the separation mechanism using Pluronic F-127 for peptides is similar to the

separation mechanism using polyacrylamide for peptides [3]. To the best of our knowledge, the use of a Pluronic gel to separate peptides is uncommon.

The aim of this work is to develop a method to separate pre-stained peptides by using wide-bore tube gel electrophoresis where the tubes are filled with a Pluronic polymer gel. The gel is made in a buffer that consists of Tris-HCl, tricine, and an optimized concentration of sodium dodecyl sulfate. Before peptides were used, three small organic dyes, Alizarin Red, Eosin Y, and Tartrazine, were used as model compounds to optimize the gel before separating five peptides. Matrix-assisted laser desorption/ionization will be used to confirm the contents of the peptides bands.

A.2: Materials and Methods

The tube gel electrophoresis instrument was purchased from Nyx Technik (San Diego, CA, USA) and was comprised of two components. The electrophoresis experiments were carried out using an Electronyx MiniPage 2D tube setup with a 70 mm separation distance. This electrophoresis box can hold up to ten 1.0 mm ID capillary tubes at one time. Electrical parameters were controlled by a programmable Voltronyx Reactor 330 EPSU. This electrophoresis voltage control has a possible maximum voltage of 300 V and maximum current of 3000 mA. The MALDI experiments were carried out on a Bruker Autoflex III (Billerica, MA, USA).

Sodium dodecyl sulfate (SDS) and glycine were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pluronic F-127, Tris-HCl and tricine were purchased from Sigma Aldrich (St. Louis, MO, USA). The anode buffer consisted of 0.2 M Tris-HCl at pH 8.9. The pH was adjusted using sodium hydroxide, purchased from Fisher Scientific. The cathode buffer consisted of 0.1 M Tris-HCl, 0.1 M tricine, and 0.2% SDS. The pH was left unadjusted. The gel buffer consisted of 0.25 M Tris-HCl, 0.19 M glycine, and varying percentages of SDS (0, 0.1, 0.23, 0.35, and 0.5%) and a pH of 8. The gel buffer was used to dissolve the Pluronic F-127 to form the gel. The percentage of Pluronic F-127 in the gel buffer that would form a gel at room temperature was investigated. The following electrical parameters were used: voltage, 300 V; current, 50 mA; power, 10 W; time, 60 min. All water used was distilled and purified with a rating at 18.2 MΩ by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Solutions of three organic dyes, Alizarin Red, Eosin Y, and Tartrazine, were made at a concentration of 1 g/L so that the color was distinguishable. The following peptides were

purchased from Sigma Aldrich (St. Louis, MO, USA): angiontensin I, angiotensin II, aprotinin, oxytocin, and neurotensin. They were dissolved in dilute acetic acid and diluted with water. The pre-stain compound, Remazol Brilliant Blue, was purchased from Sigma Aldrich (St. Louis, MO, USA) and made by making a 1 mg/mL solution in 1% sodium bicarbonate.

A.3: Results and Discussion

Optimization of the Pluronic F-127 gel

In order to determine the optimum percentage of polymer required to form a gel, the Pluronic F-127 polymer was varied between 10% and 40%. Each percentage was dissolved in 25 mL of water and left to dissolve at 4°C overnight. The solution was not stirred prior to chilling because the mixture of water and polymer was extremely sticky. However, by letting the polymer rest in water overnight at 4°C, the polymer will go into solution. This happens because the lower temperature favors the hydrophilic ethylene oxide chains dissolving in the water [7]. The next day, the solution was then left to warm to room temperature, approximately 25°C. The results of the findings are summarized in Table A.1. From this table, it was determined that 30% Pluronic F-127 was the optimum percentage needed to form a gel at room temperature. Because Pluronic polymers are not crosslinked polymers, they boast a semi-flow property which allows them to take the shape of their container. This is because the polymer builds the gel by forming layers of cubic micelles without covalent interactions. Instead, the interactions are simply hydrophilic and hydrophobic interactions [8]. This property allows the cooled gel (in a liquid phase) to be pulled into a syringe and when left at room temperature will form a gel within the syringe. Then, the fully formed gel can be pushed into the capillary tubes used for electrophoresis. This results in multiple capillary tubes being filled from the same gel batch which increases gel reproducibility between capillary tubes. The gel can also be cooled to 4°C and be stored until it is used next without diminishing the separation capabilities of the gel. This is a benefit over a polyacrylamide gel which must be prepared immediately before use. Another benefit of a Pluronic polymer over polyacrylamide gel is the casting time. Polyacrylamide generally needs at least 30 minutes for polymerization. Pluronic F-127 only needs approximately 5 minutes before the gel phase is formed. Even though the capillary tubes must be filled, this process takes only a couple minutes per tube so even filling 10 capillary tubes would take no more than 20 minutes to complete.

During an electrophoresis experiment, however, the gel was made in a buffer instead of water. A common gel buffer for an SDS-PAGE experiment in a biochemistry laboratory course

was used as the starting point for optimization. The gel buffer consisted of 0.25 M Tris-HCl, 0.19 M glycine, and x% of SDS initially. However, it was found that varying the percentage of SDS would affect the migration of organic dyes. Therefore, the next step was to optimize the % SDS in the gel buffer using three small organic dyes.

Separation of organic dyes

Three small organic dyes were initially used to optimize the gel buffer. The dyes were Alizarin Red (342.25 g/mol), Eosin Y (647.89 g/mol), and Tartrazine (534.36 g/mol), and their structures are shown in Figure A.1. At a pH of 8, Alizarin Red should be doubly charged (pK_A values for the hydroxyls are about 5.5 and 10 [9]), Eosin Y should be doubly charged (pK_A values of 2 and 3.8 [10]), and Tartrazine should be triply charged (pK_A of the hydroxyl is 9.4 [11] or pK_A of the azo hydrogen-carbonyl is 10.9 [12]). Due to their differences in charge and size, each organic dye to should have a different migration length that can be optimized by changing the gel buffer. The dyes were made at relatively high concentrations to ensure the color could be easily seen. Each dye was made at 1 g/L. To help keep the dye in a tight band, 5% glycerol was added to each dye mixture. By ensuring a tight band, the separation could be more easily determined.

The anode and cathode buffers were chosen based on previous work [13], while the gel buffer was chosen based on a common gel buffer from a Miami biochemistry research laboratory. The first gel buffer tested had 0.5% SDS in it. This percentage is above the critical micelle concentration (CMC) for SDS, meaning the surfactant will form micelles. However, according to Svingen, the Pluronic polymer is comprised of three liquid crystal and micelle domains: cubic-phase domains (crystallites), the blocking domain of the cubic crystal, and the grain boundary between domains [7]. Generally, there is less resistance along the grain boundary between domains, but there is high resistance within the blocking domain of the cubic crystal. Therefore, an analyte migrating against the blocking domain will move the slowest. Because the Pluronic polymer is kept at a constant percentage, these domains will shouldn't change significantly as the gel buffer is optimized.

For the optimization of % SDS, values were chosen both above and below the CMC of SDS. Figure A.2 shows how the concentration of SDS affected the migration distance of each dye. The larger and more highly negatively charged compound like tartrazine tended to migrate further down the capillary tube. Alizarin being less bulky than the other two dyes also migrated

Table A.1. The change in the state of the gel at varying percentages of Pluronic F-127 below room temperature and at room temperature.

% Pluronic F-127	4°C	25°C
10	Liquid	Liquid
15	Liquid	Liquid
20	Liquid	Liquid
25	Liquid	Liquid + Gel
30	Liquid	Gel
35	Gel	Gel + Solid
40	Gel	Gel + Solid

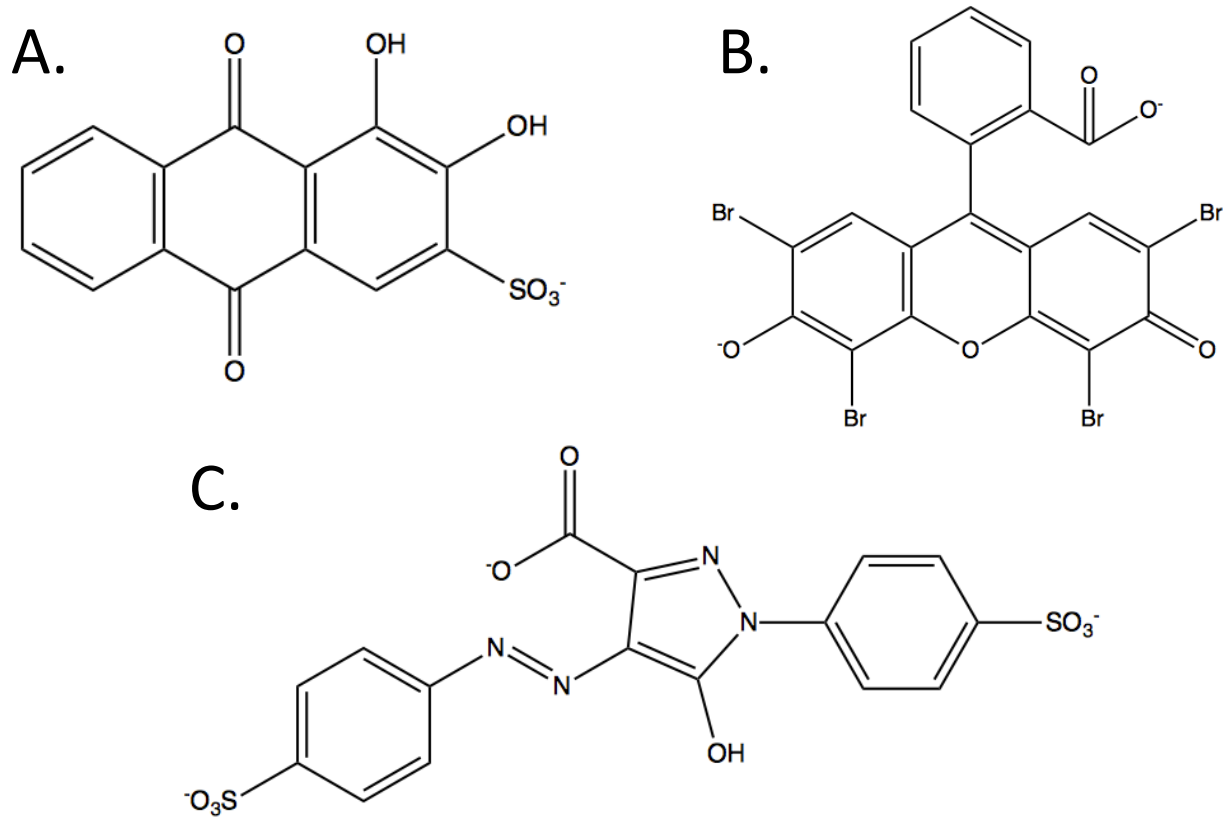

Figure A.1. The chemical structure for Alizarin Red (A), Eosin Y (B), and Tartrazine (C).

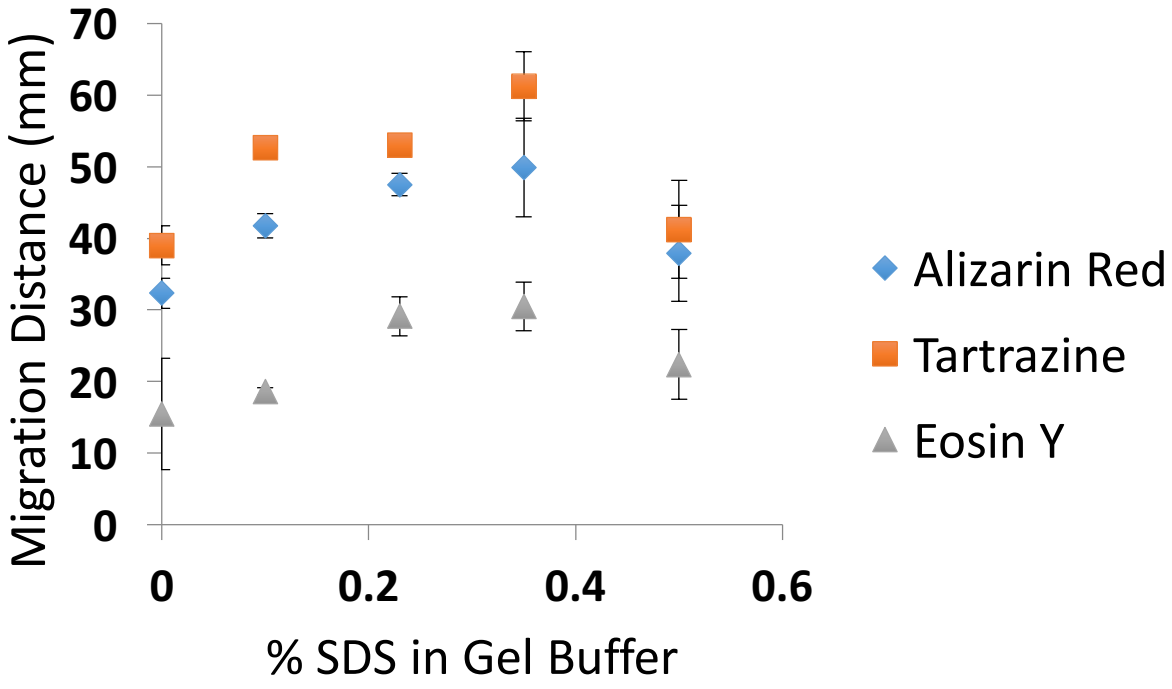

Figure A.2. The effect of % SDS on the migration distance of Alizarin Red, Eosin Y, and Tartrazine. The standard deviation of the points is based on n=3.

quite far. A 0.1% SDS composition boasted the best resolution between the three bands while also having the least amount of deviation between experiments. Therefore, 0.1% SDS was chosen as the optimum percentage for the gel buffer. It is possible that when the concentration of SDS is above the CMC that the SDS micelles interfere with the Pluronic micelles which produces poorly replicated experiments.

Separation of peptide mixture

Once the gel buffer had been optimized using organic dyes, a peptide mixture was tested. The mixture consisted of angiontensin I, angiotensin II, aprotinin, oxytocin, and neurotensin. These peptides were dissolved individually at first using water and enough formic acid to ensure everything was in solution. Based on previous research, Remazol Brilliant Blue was used to pre-stain the peptides because Coomassie Blue diffused into the gel and made it difficult to determine where the peptide band was located [13]. The pre-stain dye and peptides were mixed in a 1:1 ratio and lightly vortexed to ensure complete mixing. Prior to loading each peptide in a capillary and subjecting it to the electrophoresis, each peptide was spotted on a MALDI plate in the presence of the gel to ensure that the signal would not be hindered. According to prior research, the Pluronic polymer gel did not interfere with the MALDI signal for a peptide. The viability of ionizing the peptides using MALDI was determined to see if the Remazol Brilliant Blue or Pluronic F127 would interfere with the peptide signal. The samples were prepared by adding 250 uL of acetonitrile to dissolve the gel, and the MALDI matrix used was sinapinic acid according to previous research [13]. Aprotinin has a molecular weight of 6511 Da, and oxytocin has a molecular weight of 1007 Da. According to the MALDI spectra, neither of these peaks appear. It is possible that the gel itself does interfere with the signal by suppressing the peptide signal. If this is true, a simple extraction method could be performed to enhance the peptide signal.

Each peptide was loaded onto a capillary and subjected to the electrophoresis individually, however these data are unable to be found. Moving forward, the migration distance and band width of each peptide should be tested. The ideal gel buffer would be one where each peptide has a different migration distance. Then, the peptides can be mixed together and loaded into a capillary and be separated using the electrophoresis conditions.

A.4: Conclusion

The concentration of SDS in the gel buffer was shown to affect the migration of three small organic dyes. A concentration of 0.1% SDS was found to separate the three dyes sufficiently while also being reproducible. The viability of detecting peptides in the presence of the Pluronic polymer gel using MALDI was tested. However, it appears that the gel suppresses the MALDI signal for the two peptides tested. The next steps include determining the best gel buffer to separate the five peptides. By looking at the migration order of those peptides, it might be possible to determine the best way to predict the order of any peptide separated under these conditions.

A.5: References

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CHAPTER 6
Conclusions

The separation of aromatic hydroxy carboxylic acids by HILIC has been developed. A silica column and ternary mobile phase that consists of acetonitrile, an ammonium acetate buffer, and pentane was used to separate ten acids and quantify eight. This method improves current available methods that are unable to separate multiple groups of positional isomers. An important advantage of this HILIC method is its compatibility with mass spectrometry detection. Moving forward, this HILIC method could be applied to other mixtures, like the dihydroxy aromatic carboxylic acids investigated previously in our research group (1). Although the buffer component might need to be altered slightly, the use of a third solvent like pentane or HMDX could be helpful in completely resolving these acids. This would provide a MS-compatible method to the polymer industry.

Through the use of micellar liquid chromatography, eight aromatic carboxylic acid impurities (which includes two groups of positional isomers) found from the industrial synthesis of terephthalic acid have been separated. A reversed-phase C18 column with an acidic sodium dodecyl sulfate (SDS) mobile phase was used to separate these nine acids in under 20 minutes. Both a simulated and an actual industrial sample were analyzed and all nine acids were able to be quantified. This method is environmentally friendly and improves upon the lack of resolution and long analysis times when other chromatography methods are used. Moving forward, this green method could be used to separate the acids investigated in Chapter 2. This method showed short retention times, which would be an improvement on the outcome of Chapter 2.

A comparison of SDS and Brij-35 in ultra-high performance micellar liquid chromatography for the separation of terephthalic acid from eight impurities was performed. A C18 column was used along with either an acidic SDS or Brij-35 mobile phase. Again, all nine acids were able to be fully resolved, including the two sets of positional isomers. Another industrial sample was analyzed under both conditions and the acids were quantified. Though there is some discrepancy between the final concentrations, each method provides some advantage. The analysis time was decreased using the SDS mobile phase when compared to the method run under standard high performance conditions. Although the Brij-35 sub-micellar mobile phase produced a longer analysis time, there was improved resolution and detection of certain analytes. In addition, use of

only a Brij-35 coated C-18 column with no surfactant in the mobile phase was also a viable method that should be MS compatible. This research could benefit from additional investigation as to why the Brij-35 mobile phase boasts such a long analysis time, why TPA disappears when the mixture is run under SDS conditions, and why there are discrepancies between the limits of detection and quantitation between SDS and Brij-35. Additionally, these methods could be compared to standard reversed phase methods on the same column. Preliminary data generated in our research group has shown reversed phase separation of the terephthalic acid impurities can be done almost isocratically with just one mobile phase step gradient. It would be interesting to have a complete comparison of the same acids under SDS micellar, Brij-35 micellar, RPLC, and HILIC methods.

The use of ion exchange chromatography is being investigated for the separation of heparin from large glycosaminoglycans (GAGs) like oversulfonated chondroitin sulfate. Protamine, a peptide structurally dominated by the positively charged amino acid arginine, complexes strongly with GAGs which should lower the extensive negative charge of this sulfonated class of compounds. By using a cation exchange column with a mobile phase that contains protamine and sodium perchlorate for ionic strength control, a dynamic cation exchange column is formed in addition to the complexation of the sulfonated polymers with protamine. The separation of three small model compounds, naphthalene mono-, di-, and trisulfonate, was optimized by testing varying perchlorate and protamine concentrations. The next model compound tested was a polystyrene sulfonate compound with an average molecular weight of 200,000 g/mol. The mobile phase used to separate possibly this particular polymer from others in a wrinkle serum sample was optimized. Chondroitin sulfate, a more easily detectable GAG, was used as the last model before testing heparin. However, no peak was noted in the chromatogram indicating chondroitin sulfate was extremely well retained under the previously optimized mobile phases. Continued optimization of the mobile phase will need to be completed before heparin can be tested. The optimization of both the protamine and the perchlorate would be the best place to begin. A calibration curve of polystyrene sulfonate can be made to ensure linearity of the separation method. If an optimum mobile phase can be found for polystyrene sulfonate, that might lend itself to be more helpful at finding an optimum mobile phase for the separation of the GAGs.

A Pluronic polymer has been used in wide-bore tube gel electrophoresis for the separation of small organic dyes, Alizarin Red, Eosin Y, and Tartrazine, with the hope of separating peptides. The addition of SDS to the gel buffer was found to alter the migration distance of three dyes so it was optimized within the gel buffer. It was found that 0.1% SDS separated the dyes the best while also having the most reproducible migration distances. Five peptides were purchased, angiontensin I, angiotensin II, oxytocin, neurotensin, and aprotinin, and work has just begun on determining the applicability of separating these peptides. Moving forward, these peptides could be analyzed using MALDI first to determine a standard spectrum for each peptide. The peptides will need to be pre-stained with RBB prior to separation so it would be beneficial to also collect MALDI spectra for the peptides with RBB present. Once that has been collected, the peptides with RBB should be analyzed in the presence of the gel. It had been previously reported that the gel did not interfere with the MALDI signal for peptides; however, this should be confirmed first. If the gel does not interfere, an optimum separation environment can be determined. However, there are simple methods available to remove the gel if it interferes with the peptide+RBB signal.

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