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Evaluation of Silica Hydride Based Amide Stationary Phase for High Performance Liquid Chromatography and Aqueous Normal Phase Separations

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EVALUATION OF SILICA HYDRIDE BASED AMIDE STATIONARY PHASE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND AQUEOUS NORMAL PHASE SEPARATIONS

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ABSTRACT

EVALUATION OF SILICA HYDRIDE BASED AMIDE STATIONARY PHASE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND AQUEOUS NORMAL PHASE SEPARATIONS

by Emma R. Tardiff

With many modern chromatographic advancements, silica hydride based columns (also known commercially as Type-C) represent yet another evolutionary step in stationary phase development. Type-C silica hydride columns have the ability to retain both polar and nonpolar analytes and have the ability to behave in either RP or ANP modes of separation depending on mobile phase composition. A consequence of this increased stability is that Type-C columns can be used over a wide range of mobile phase compositions, from purely aqueous to purely nonpolar organic solvents, and any combination of the two. While the retention behavior of Type-C columns depends highly on the mobile phase environment, it can also be influenced by the organic moiety attached to the surface. This project encompasses the evaluation of a silica hydride-based column with amide functionalities to be used in high performance liquid chromatography. A series of separations were performed using reference standards of varying analyte sizes, functionalities, and polarities. This project assesses the specific column selectivity and identifies classes of compounds which show high potential for effective retention, resolution, and efficiency when using amide functionalized silica hydride columns for RP and ANP separations.

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I. Introduction

Chromatography

Chromatography is a universal technique used across industries and academic fields in a variety of ways in which the physical or chemical characteristics of a molecule effect its speed of travel through a given medium.[1-5] This difference in travel time is used to separate out mixtures of many compounds. After separation of compounds from a mixture, each can be identified and quantified by a paired detection method. [6] Academic use of chromatography as a tool to solve qualitative questions was first recorded in the 1800s when it was used to separate plant pigments by Mikhail Tswett., hence, the base name of chroma, meaning color. [7,8]

Fundamental to chromatography is the existence of a "stationary phase" which interacts with a compound and a "mobile phase" which carries the compound. The more interaction with or affinity a compound has for the stationary phase, the longer it will be retained or associated with that stationary phase, the longer it will take to travel through the medium and the later it will elute from the system. There are two phases primarily through which a chromatographic separation takes place, gas and liquid. Liquid chromatography, the subject of this project, has gone through a series of scientific advancements over the last decade, culminating in the development of high-performance liquid chromatography (HPLC). HPLC has been shown to be a groundbreaking analytical technique that has enabled the advancement of industries far and wide including from environmental, to food and health, pharmaceutical, and materials engineering. [9–15]

High Performance Liquid Chromatography (HPLC)

HPLC was developed in the 1960s, building on interdisciplinary advancements from chemistry to engineering. HPLC is distinguished from earlier liquid chromatography for its ability to maintain a constant flow at pressures of up to 600 bar. Typical particle sizes used in HPLC range from 3-10 µM. Separation columns are the heart of an HPLC system and are typically tubular and packed with these small particles. The classic particle shape of a sphere is used to maximize surface area available for interactions with analytes compared to volume of the particle. As seen in Equation 1, smaller sized particles offer improved interactions because as diameter decreases, the surface area to volume ratio increases. [16]

Equation 1: Surface Area to Volume Ratio of a Sphere

$$\frac{Surface\ Area}{Volume} = \frac{4 * \pi * r^2}{{}^4/_3 * \pi * r^3} = \frac{3}{r}$$

Typical HPLC instrumentation includes a 1) solvent degasser, 2) quaternary pump, 3) sample injector, 4) column, 5) detector, and 6) signal processor and data analysis system. Each component of a modern HPLC system has undergone its own history of advancements which collectively result in the analytical improvements offered by the technique. A simple diagram of an HPLC is shown in Figure 1.

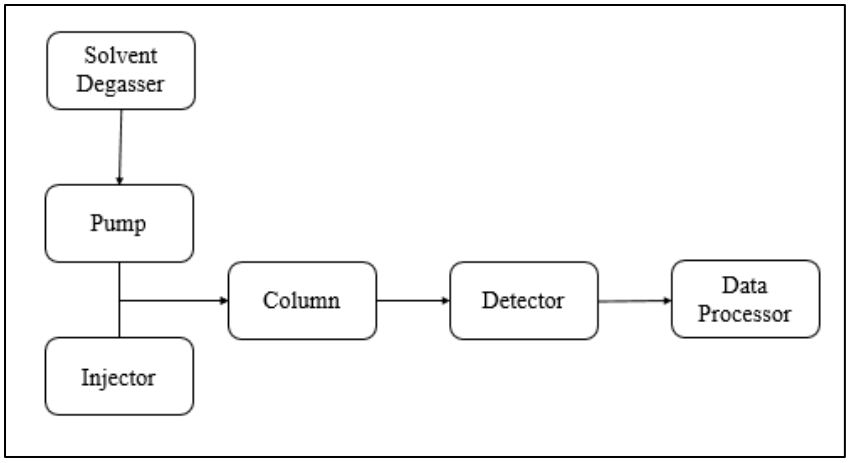

Figure 1: Diagram of HPLC Components

The use of a solvent degasser allows for control of dissolved gasses in the mobile phase and improves repeatability between mobile phase batches and day to day, lab to lab variations. Advancements in pump technology have led to consistent, nonplusing flow of mobile phase into the system with the ability of the operator to vary mobile phase makeup, pulling from multiple solvent components. This allows for matrix matching between more complex samples as well as the ability to perform gradient separations. Improvements in the accuracy and precision of sample injectors directly resulted in elevated abilities to quantify data. [1,3,5] The column is where separation of mixed component samples takes place which can be based on many interactions including adsorption, partitioning, and ion exchange to name a few. After separation and elution of target analytes from the column, the detector provides the key function of identification of intensity. This intensity is quantified by several methods including absorbance, conductance, fluorescence, energy transfer, refractive index, and more. Detection is an area of study in chemistry and physics that is always expanding. Lastly, advancements in

computational and computer sciences have led to process signal intensity and analysis data improvements. Just like all science and engineering, HPLC owes its capabilities to many years of research, testing and boundary pushing from across the spectrum of academic disciplines. Figure 2 shows the HPLC used to collect all data.

Figure 2: A Photo of Agilent LC1200 Series HPLC in the Pesek Lab.

Common Modes of Chromatographic Separations

Normal Phase Chromatography (NP)

NP was the first mode to be invented and widely used. It employs a polar stationary and a non-polar mobile phase. Common stationary phases include end groups such as silica, amino, alcohol, and cyano. Common mobile phases employed include hexane, tetrahydrofuran (THF) and ethyl acetate. In general, the elution sequence can be expected to follow the trend of least polar, more hydrophobic analytes eluting first because of minimal interactions with the polar stationary phase and preference to exist in the nonpolar mobile phase, whereas more polar, hydrophilic analytes are expected to be

retained and elute later. The nonpolar mobile phases used in NP often show poor solubility for polar compounds. [17]

Reversed-Phase Chromatography (RP)

RP chromatography is currently the most popular liquid chromatography method in separation science. In this mode, a nonpolar, hydrophobic stationary phase is employed along with a polar, hydrophilic mobile phase. A common example of this is an alkyl column like a C18, or a phenyl column with an aqueous/organic mobile phase. In general, the elution sequence can be expected to follow the trend of most polar eluting first, having minimal interactions with the stationary phase, to the least polar eluting last. RP also does not retain ionized analytes well for the same reasoning. [17] Figure 3 depicts the structure of common RP stationary phases.

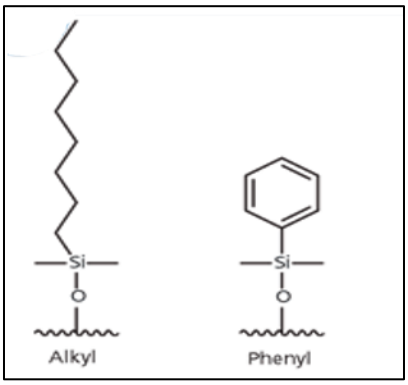

Figure 3: Examples of commonly used stationary phases for RP chromatography.

Aqueous Normal-Phase Chromatography (ANP)

ANP is one of the newest modes of chromatographic separation with a large potential for future studies and industry applications. ANP uses a mobile phase that primarily a relatively nonpolar organic solvent, commonly acetonitrile, along with a small amount of

water to make a mixed system. Polar analyte retention increases as the organic solvent content increases and the hydrophilicity of the mobile phase decreases. [18] This is the same general trend that is seen in hydrophilic interaction liquid chromatography columns as shown in Figure 4.

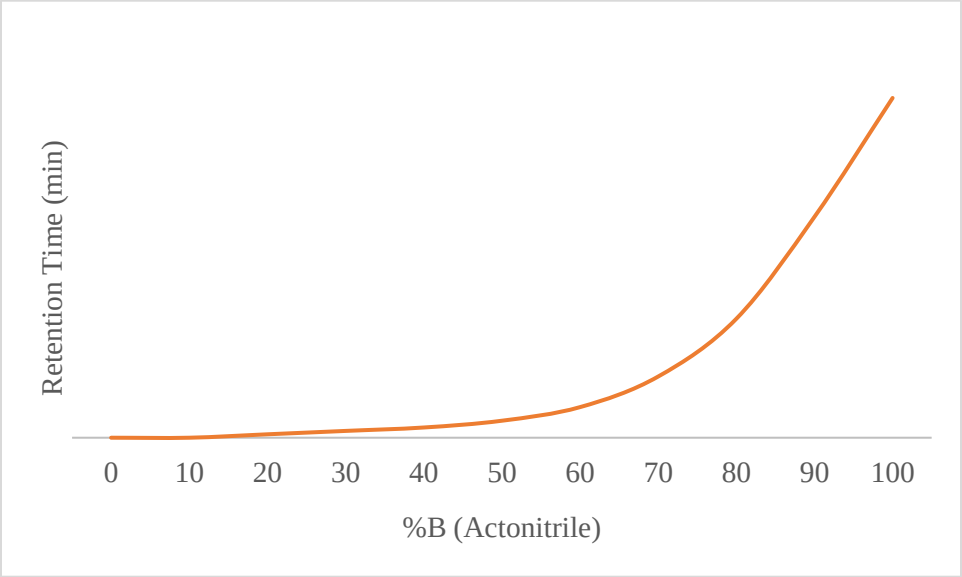

Figure 4: An example of an ideal ANP retention trend on silica hydride stationary phase.

ANP trend is distinct from the other modes mentioned here because it is observed on stationary phase based on silica hydride backbone as opposed to standard silica. Silica hydride particles are commonly referred to as "Type C" silica and will be expanded on further in the next section. [19,20]

Hydrophilic Interaction Liquid Chromatography (HILIC)

HILIC is a recent modification of the classic normal and RP modes which was creating in the 1950's but was not popularized until the early 2000's [21]. It employs a polar stationary phase with a mobile phase that is primarily (>60%) organic solvent and a small

percent water. The water in the mobile phase associates with the polar stationary phase and creates an aqueous rich layer adsorbed to the surface. Although HILIC columns are considered polar, the mobile phase employed is similar to traditional RP methods. For this reason there is wide misunderstanding of how to categorize HILIC by the general public and it is sometimes described as "reversed 'reverse phase'" [22]. It is also mistakenly used interchangeably with "aqueous normal-phase chromatography", the differences of which are key to this project and will be discussed in detail. HILIC separations typically follow an ANP-like trend of nonpolar analytes eluting first and polar eluting last depending on the mobile phase used. It is commonly used to separate polar and ionic species. [23,24]

Silica Stationary Phases

There have been significant advancements within the area of packed columns in the last few decades.

Type B Silica

Type B silica is the most used silica particle base for chromatographic separations today [11]. During its manufacturing it typically undergoes an additional acid washing step to removed metal contaminates which were commonly seen in earlier generations of silica particles. This leads to more consistent pore size and distribution, as well as lower surface acidity resulting in better peak shape for compounds with basic functional groups or those that may complex with metals. Because of improved manufacturing methods, more uniform size and shape of particles can be achieved and stability at intermediate and high pH environments was improved. However, Type B silica, like earlier generations of

silica particles, is based on silanols at the surface. Surface silanols have been shown to produce unwanted electrostatic interactions with some analytes leading to poor retention of nonpolar analytes and peak tailing for basic compounds. [25] Additionally, studies have shown that surface silanols have the ability to bond with adjacent silanols. [26] Figure 5 shows structures known to be on the surfaces of Type B silica.

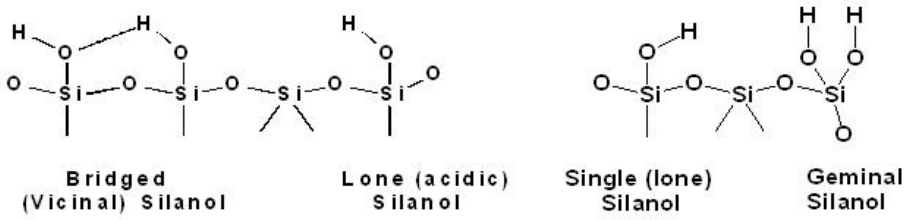

Figure 5: Examples of surface silanols on Type B silica.
(Adapted from Christy, 2014[26])

Aside from the intra particle bonding displayed above, when water is present in the environment it easily associates with silanols at the surface. The figure below depicts water hydrogen bonding with vicinal, geminal, and free silanol on a Type B silica particle. The orientation of the silanol groups impacts the strength of these hydrogen bonds. [26] Figure 6 shows the possible bonding and associations surface silanols may have with water present in the mobile phase.

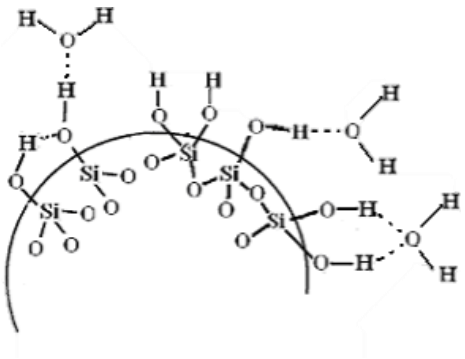

Figure 6: Examples of surface silanols on Type B silica association with water. (Adapted from Christy, 2014[26])

Type C Silica

The next stage in stationary phase advancements comes in the form of Type C silica, originally synthesized and characterized by Dr. Joseph Pesek of San Jose State University. Improved properties and characteristics of Type C silica result from replacement of the surface silanol groups by a non-polar hydride via a simple TES-silanation reaction depicted below in Figure 7.

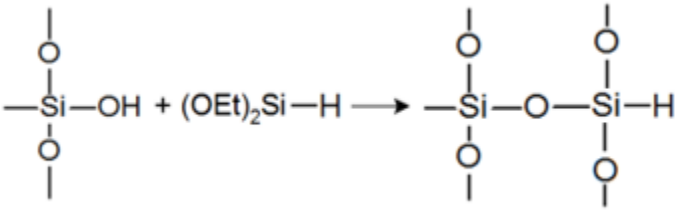

Figure 7: An example of TES-Silanization scheme.

This silica hydride is a hydrolytically stable intermediate which is then reacted with a desired end group in the presence of a metal catalyst, such as hexachloroplatinic acid, or free radial initiator, such as t-butyl peroxide. [27,28] Functional groups used to modify Type C silica are typically alkenes and alkynes. This reaction is depicted in Figure 8.

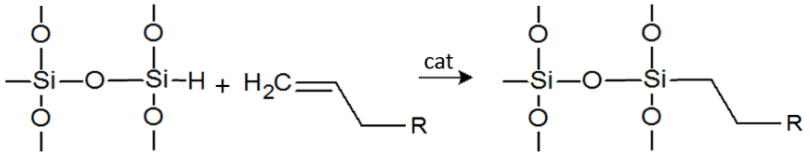

Figure 8: An example of a hydrosilation scheme.

The loading of the R-group is controlled by temperature, solvent, and catalyst. Steric considerations of the group being added will also influence surface coverage. By replacing the silicon-carbon-oxygen bond, which is present at the surface of equivalently functionalized Type B columns, with a silicon-carbon bond which is more stable, the durability of the column increases. By replacing the polar silanol end group with a nonpolar hydride, a marked improvement in many chromatographic properties has been shown, including greater pH stability and diminished ionic interactions with basic compounds. Type C silica columns, unlike classic Type B columns and HILIC columns, are able to participate in both RP and ANP separations which employs the use of a nonpolar, aqueous mobile phase consisting of a small amount of water and a low polarity organic solvent. [20,25]

Separation Modes using Type B Silica vs. Type C Silica

While the modes of separation in research and industry commonly known are normal-phase and RP, both have notable limitations. Because normal-phase separations rely on a nonpolar mobile phase, solubility of hydrophilic compounds in the mobile phase may be a problem, leading to sample preparation issues. Similarly, RP separations rely on a aqueous mobile phase which will have limited retention and separation of polar or

ionized hydrophilic compounds. Highlighting biological applications, these samples typically have a matrix with a high aqueous content. By attempting to matrix match a high aqueous sample by running a high aqueous mobile phase on a classic nonpolar RP column like a Type B C18, dewetting, also known as phase collapse, may be observed. In the case of phase collapse, the hydrophilic mobile phase can no longer penetrate the pores of a column with hydrophobic surface groups. This effectively lowers interactions with the stationary phase and thereby retention because the pores contain the majority surface area of a silica particle. Furthermore, due to a void of solvent, the stationary phase within a pore can collapse in onto itself causing permanent damage to the column and lessening its ability to retain any analyte type. The ability to evaluate polar and hydrophilic compounds is essential to countless areas of research and industry, including most clinical, and pharmaceutical applications. In this respect, Type C columns offer an alternative.

Type C columns can function at both high and low aqueous mobile phase concentrations without damage to the column and do not require long equilibration times between runs or method changes like typical Type B columns would. Unlike previously discussed modes of separation which utilize Type B silica columns, Type C can participate in both ANP and RP retention trends. Larger compounds with both hydrophobic and hydrophilic moieties can show dual retention at both ends. [27] Both ANP and RP overall trends are shown in Figure 9.

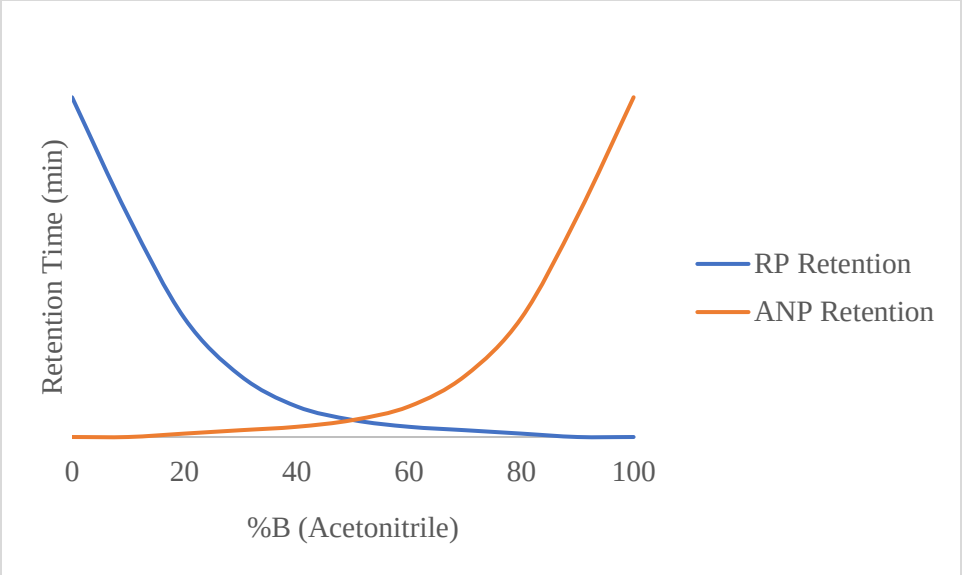

Figure 9: An example of ideal retention trends on silica hydride stationary phase.

When comparing Type C columns to HILIC columns, which are often confused for one another because of similar ANP-like retention trends, it is key to understand the difference in retention mechanisms resulting from the type of silica backbone used. The polar surface of a HILIC stationary phase based on Type B silica attracts water molecules which are adsorbed to create a water-rich layer at the surface of the stationary phase. Because of this, HILIC columns have a liquid-liquid interface through which analytes must participate in a partition mechanism before reaching the stationary phase. For this reason, HILIC columns are unable to retain hydrophobic analytes because they cannot penetrate this solvation layer. Analytes must be soluble in the aqueous solvent layer before they can interact and be retained by the HILIC stationary phase. A depiction of the different between adsorption and partition mechanisms seen on Type C and Type B columns is shown in Figure 10.

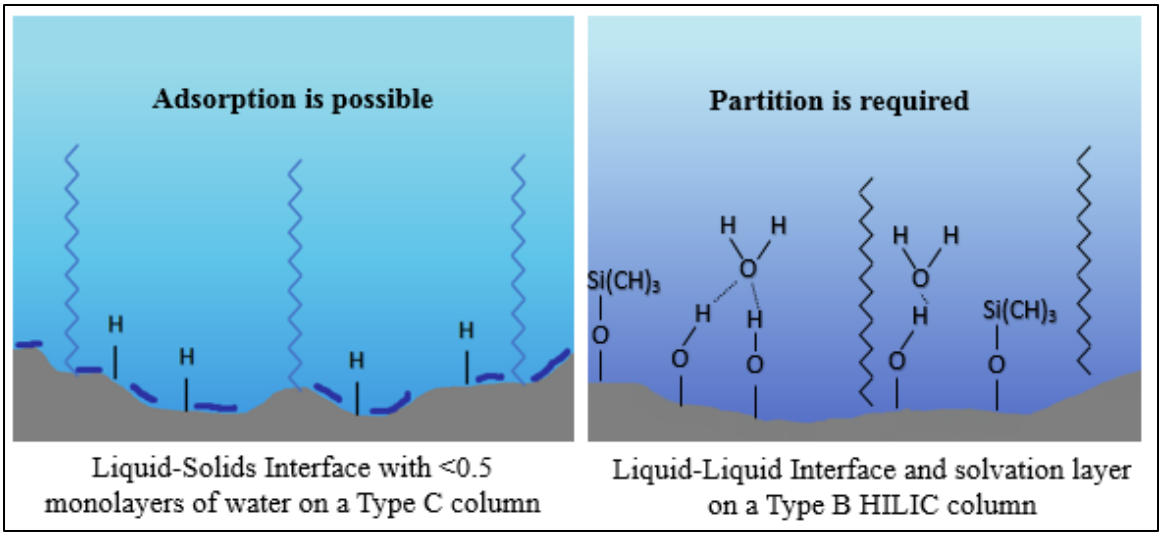

Figure 10: A diagram of retention mechanisms of Type C vs Type B silica.

In contrast, Type C columns do not have this solvation layer because of the absence of silanols on the surface. It has been shown that Type C columns have <0.5 monolayers of water at their surface. This allows both polar and nonpolar, hydrophilic and hydrophobic analytes to access the surface of the stationary phase and interact by adsorption. Significant correlations have been found between the depth of the water solvation layer and a given columns selectivity when separating compounds with large polarity differences. A weaker association with water also results in shorter equilibration times when changing modes or using a gradient mobile phase. [1,24,29–31]

Research Goals

A Cogent 4 micron Amide Hydride column based on Type C silica was provided for evaluation. The primary goal of this research was to evaluate the retention trends displayed by the column and to draw reasonable conclusions as to its appropriate use. Specific goals included 1) confirm ANP properties, 2) confirm RP properties, 3) use a broad range of compounds to empirically assess the given column's selectivity.

Several small molecules were utilized to establish a baseline for retention trends according to functional group, hydrophobicity coefficient, number of sites available to hydrogen bond, and effective polar surface area. Once retention trends were solidified, a handful of more complex molecules were analyzed as examples of potential applications for the given column.

A primary amide group is considered to be polar based on its ability to hydrogen bond and the presence of nitrogen, an electro negative atom. It was hypothesized that an amide functionalized column would interact most with compounds containing similarly polar functional groups, groups which can participate in hydrogen bonding, and compounds with a low hydrophobicity coefficient (Log P). Additionally, it was theorized that there would be minimal ion-exchange interactions in the retention of ionizable compounds as the pH will be controlled through formic acid addition. [32,33]

A series of isocraftic runs were performed and analytes detected by UV analysis. The data was collected, analyzed, and is discussed in the Result and Discussion section below.

II. Experimental

Materials

The analytical column utilized in this project was the Cogent 4 Amide Hydride, manufactured by MicroSolv technologies (Leland, NC), batch number 160405-09, dimensions 10 x 2.1 mm, part number 40036-10P-2, serial number C2588. It was reported to have a standard pore size of 100 Å, a surface area of 390± 30 m^2/gm, a pore volume of 1.00± 0.10 mL/g, a particle size of 4 µm, a calculated carbon load of 2-3 %, a pH range of 2.5-7.5, and a max temperature of 80 °C.

Instrumentation

An Agilent Series 1200 HPLC with a Diode Array Detector (DAD) was provided by the Pesek lab at San Jose State University and used for this project.

Analytes

All compounds analyzed in this study are listed in table 1.

Table 1: A list of compounds used for column evaluation.

Chemical Name	Manufacturing Company
Phenol	Sigma-Aldrich
Anisole	Sigma-Aldrich
Benzylamine	Sigma-Aldrich
1,2 Dimethyl Benzene (o-Xylene)	Sigma-Aldrich
1,3 Dimethyl Benzene (m-Xylene)	Sigma-Aldrich
1,4 Dimethyl Benzene (p-Xylene)	Sigma-Aldrich
Guanidine (CAS	NBC National Biochemical Corp.
Adenosine (CAS 58-61-7)	CALBIOCHEM Co. LA
Adenosine Triphosphate	Sigma Chemical Company
Thymidine	Nutritional Biochemical Corp.
L-Tryptophan	Sigma Chemical Company
Norepinephrine	Sigma-Aldrich
Creatinine	Sigma Chemical Company
Caffeine (CAS 58-08-2)	Sigma Chemical Company

Solvents

All solvents used in this study are listed in Table 2.

Table 2: A list of all solvents used for column evaluation.

Chemical Name	Manufacturing Company
Acetonitrile (CAS 75-05-8)	Fisher Chemicals
Formic Acid (CAS 64-18-6)	Sigma Chemical Company
Deionized Water (CAS 7732-18-5)	Provided by SJSU

All reference to solvent "A" refers to deionized water. All reference to solvent "B" refers to Acetonitrile. Solvents were degassed and spiked with 0.1% formic acid before introduction to the HPLC system.

Sample Preparation

All sampled were prepared in 50% acetonitrile and 50% deionized and degassed H_2O. Formic acid was used as a buffer in all solvents used at a concentration of 0.1%.

Instrument Parameters

A five-minute equilibration time was allowed between each injection using a different mobile phase composition. An injection volume of 1 to 20 µL was used depending on the intensity of signal seen for each compound. A flow rate of 0.3 mL/min was used for all reported results. A column temperature of 22 °C was used for all reported results. All reported results are from a wavelength of either 254 nm or 210 nm.

Data Analysis

All data were analyzed using the Agilent ChemStation software, version B.04.03[16].

III. Results and Discussion

Phenol

While it was theorized that compounds with higher polarity and hydrogen bonding characteristics would have the best retention on the amide column, phenol was tested in an attempt see what impact a phenolic oxygen and pi-pi interactions may have on retention. The structure for phenol is shown in Figure 11 for reference.

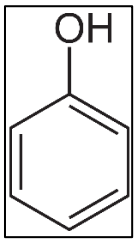

Figure 11: The chemical structure of phenol.

As previously documented, [2] if the column surface were primarily silica hydride, which is less polar than after amide functionalization, notable retention of phenol should be observed in the RP mode. The retention graph shown in Figure 12 suggests little to no retention of phenol on the given column which supports the manufacturer's claim of high amide surface coverage.

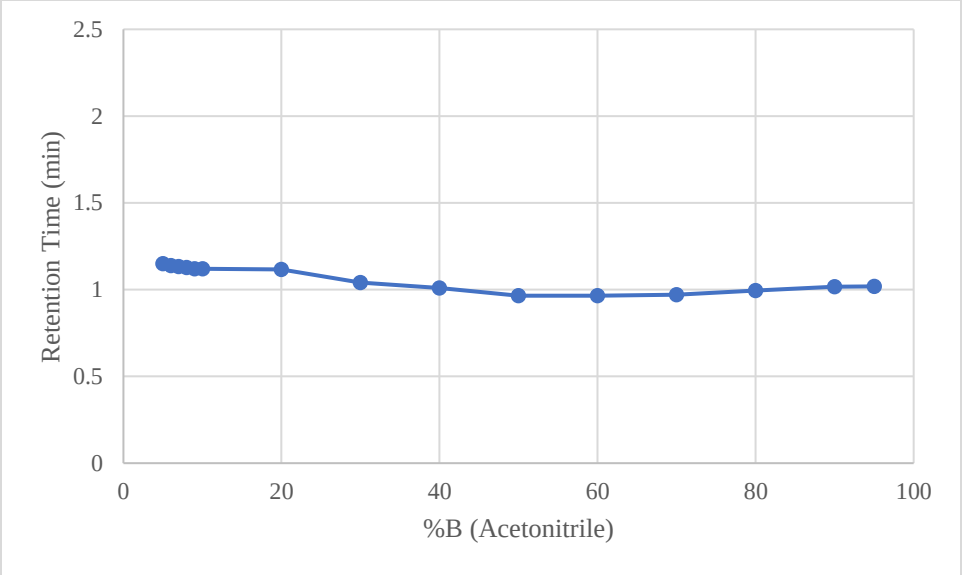

Figure 12: The experimental retention trend of phenol.

Anisole

Anisole's dominating feature is its nonpolar aromatic ring and a single ether functional group. With its low number of hydrogen bond donors (0) and acceptors (1) and relatively low polar surface area (9.2 $Å^2$), and preference to partition into an organic phase over aqueous, it was not expected to participate is extensive interactions with the

amide functional group when the organic portion of mobile was high. The structure of anisole is provided in Figure 13 for reference.

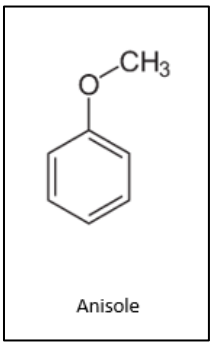

Figure 13: The chemical structure of anisole.

A logP of 2.1 for anisole suggests that anisole is expected to prefer to partition into the acetonitrile organic mobile. Experimentally, it was seen to exhibit RP retention trend and was only retained when interactions with the column were encouraged by limited the concentration of organic solvent in the mobile phase. Results for analysis of anisole are shown in Figure 14.

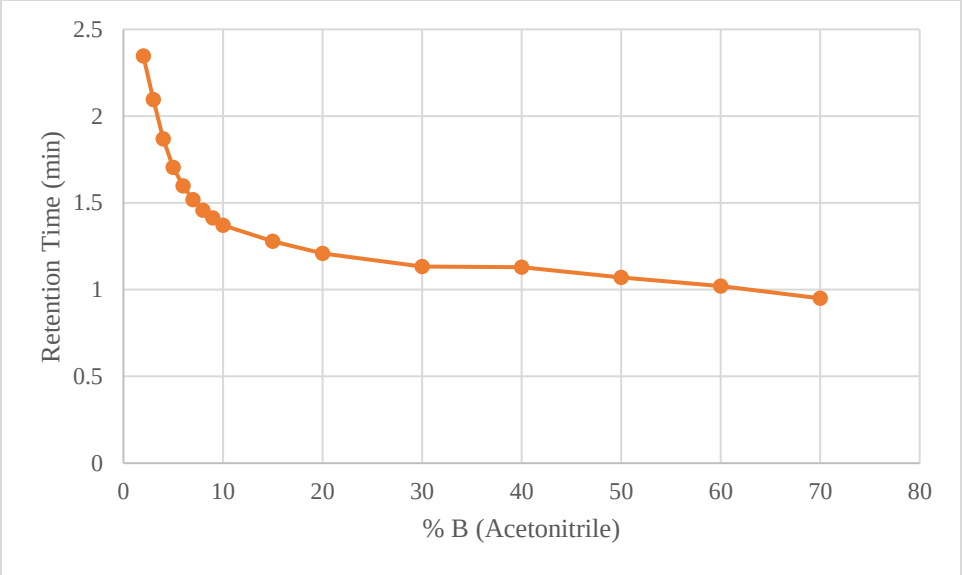

Figure 14: The experimental retention trend of anisole.

By comparing anisole with the previous results for phenol it can be seen that, despite the nonpolar characteristics of both anisole and phenol, the ether group on anisole may have additional interactions with the amide column allowing it to be retained in the RP mode. This suggests that the available electrons on the ether in anisole interact to a greater extent with the amide column than the hydroxyl group on phenol. Although the comparison is not perfect because anisole's ether location allows for additional destabilization of resonance electrons. This example shows that retention on the amide column is not simply a function of polar surface area. A side by side comparison of anisole and phenol suggests that phenol should have more retention because of an advantage in higher polar surface area. A summary of compound characteristics for both anisole and phenol is shown in Table 3 and their experimental retention trends are shown in Figure 15.

Table 3: A comparison of anisole and phenol characteristics.

	Anisole	**Phenol**
Molecular Weight (g/mol)	108.14	94.113
Log P (Hydrophobicity)	2.1	1.5
H bond donors	0	1
H bond acceptors	1	1
Polar Surface Area (Å^2)	9.2	20.2

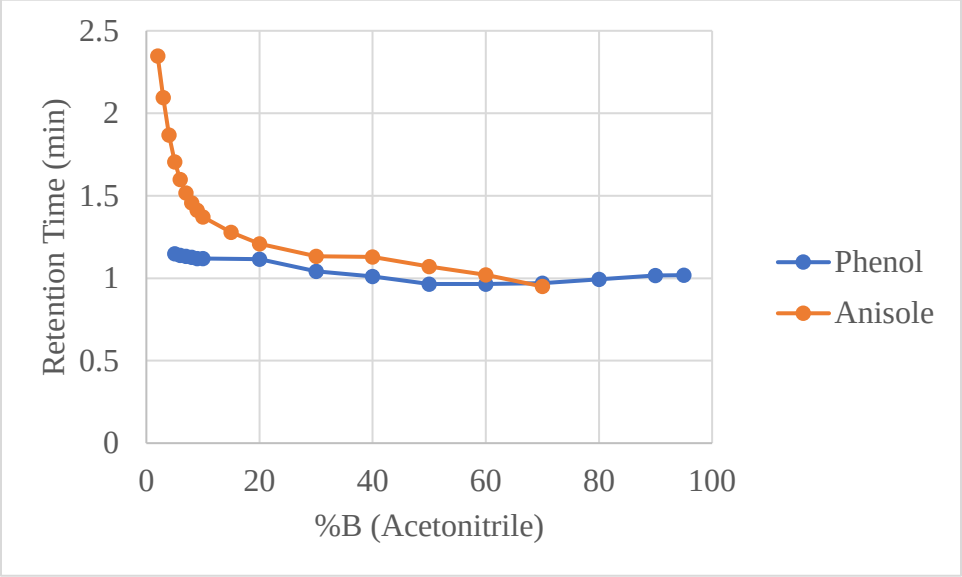

Figure 15: The experimental retention trends of anisole and phenol.

Comparison of Di-Methoxy Benzene Isomers

The interactions between the given silica hydride amide column and simple molecules with two ether groups was explored and compared. Isomers of di-methoxy benzene were analyzed to understand the effect that structure and consequently hydrophobicity and steric hinderance may play in retention on the given column. Structure of methoxy benzene compounds used are provided in Figure 16 for reference.

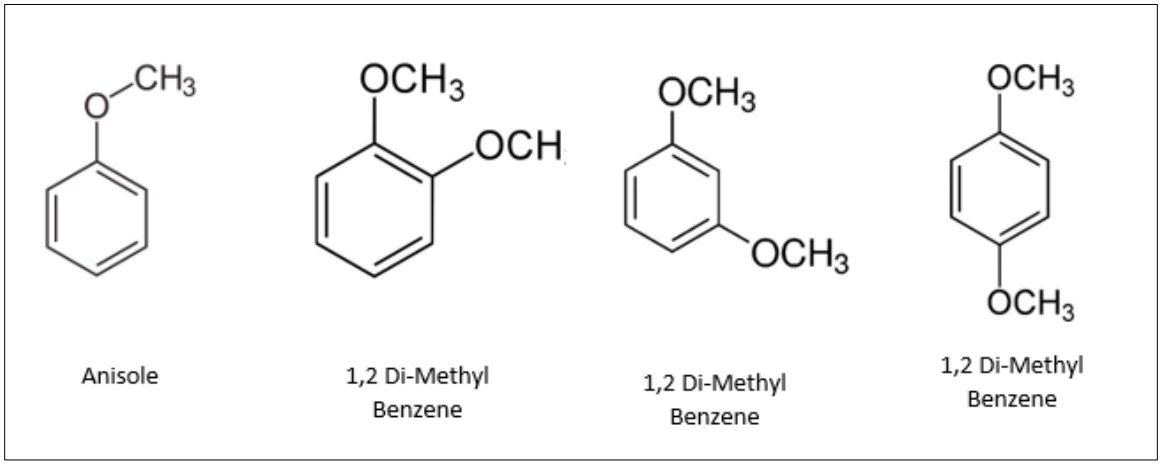

Figure 16: Chemical structures of all di-methoxy benzene isomer analyzed.

All three compounds tested have similar hydrogen bonding interactions, pi-pi interactions and resonance. The primary difference between the three is the placement of the ether functional groups. Location of the ether groups very slightly impacts the overall expected hydrophobicity of the compound and how sterically available the functional groups are for interactions. A summary of compound characteristics for all di-methoxy benzene isomers is shown in Table 4.

Table 4: Anisole and di-methoxy benzene isomer compound characteristics.

	Anisole	**1,2 Di-Methoxy Benzene**	**1,3 Di-Methoxy Benzene**	**1,4 Di-Methoxy Benzene**
Molecular Weight (g/mol)	108.14	138.16	138.16	138.16
Log P (Hydrophobicity)	2.1	1.6	2.2	2.0
H bond donors	0	0	0	0
H bond acceptors	1	2	2	2
Polar Surface Area ($Å^2$)	9.2	18.5	18.5	18.5
Retention with 2% B mobile phase (min)	2.38	2.24	2.86	2.39

Experimentally, overall retention differences were indistinguishable for higher acetonitrile mobile phase tests because of low retention, just as was seen in anisole. Even using a mobile phase of 90% aqueous, 10% acetonitrile did not show significant retention differences. Small differences in retention can be seen however in a very high aqueous mobile phase. Using a mobile phase of 98% aqueous, 2% acetonitrile shows small differences for the isomers, as seen in the below figure. Retention trends suggest that the 1, 3 Di-Methyl Benzene has the most retention on the column, possibly due to greater steric availability of the ether groups. Very slight differences in retention of di-methoxy benzene isomers is shown in Figure 17 and Figure 18.

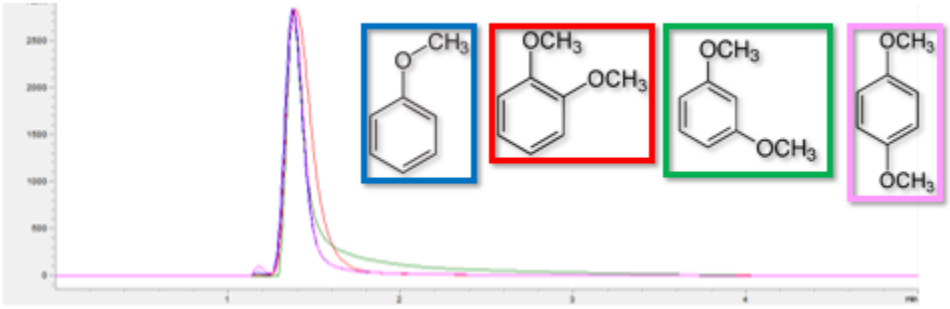

Figure 17: Retention differences of di-methoxy benzene isomers at 90% aqueous, 10% acetonitrile

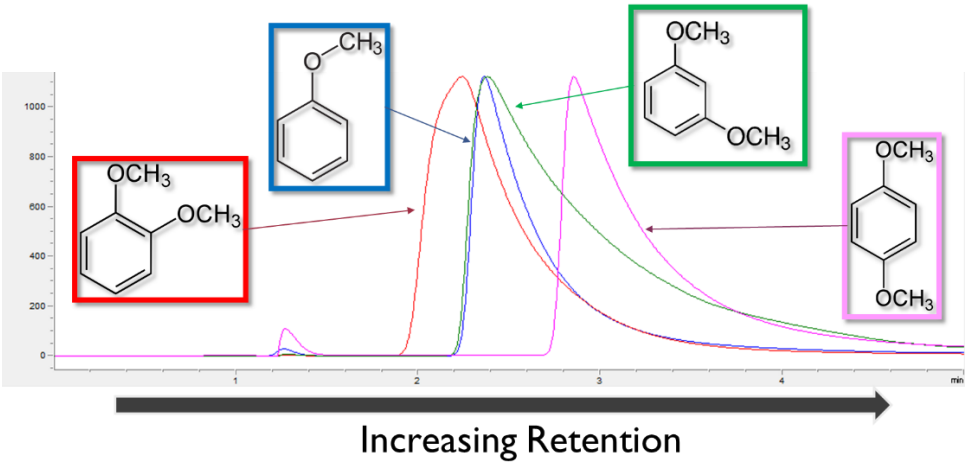

Figure 18: Retention differences of di-methoxy benzene isomers at 98% aqueous, 2% acetonitrile

Benzylamine

A simple primary amine structure was analyzed to gain insight into amine interactions with the amide column. The ability of a primary amine to participate in hydrogen bonding and its effect on compound polarity suggests that there should be retention on the amide column. Benzylamine was chosen because of its simplicity and ease of

detection. Like previous compounds, its aromatic ring is reliably detected in the UV range. The structure of benzylamine is provided in Figure 19 for reference.

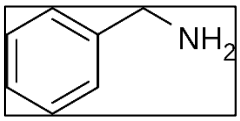

Figure 19: The chemical structure of benzylamine.

Although benzyl amine has similar solvent partition values, hydrogen bonding sites, and polar surface area to phenol, it showed markedly different retention trends on the amide column. Benzylamine showed typical ANP retention. Retention trends of other amine compounds were consequently explored in an effort to understand the given amide column. A side by side summary of compound characteristics for anisole, phenol, and benzylamine is shown in Table 5 and their experimental retention trends are shown in Figure 20.

Table 5: Characteristics of Anisole, Phenol, and Benzylamine.

	Phenol	**Anisole**	**Benzylamine**
Molecular Weight (g/mol)	94.113	108.14	107.16
Log P (Hydrophobicity)	1.5	2.1	1.1
H bond donors	1	0	1
H bond acceptors	1	1	1
Polar Surface Area ($Å^2$)	20.2	9.2	26

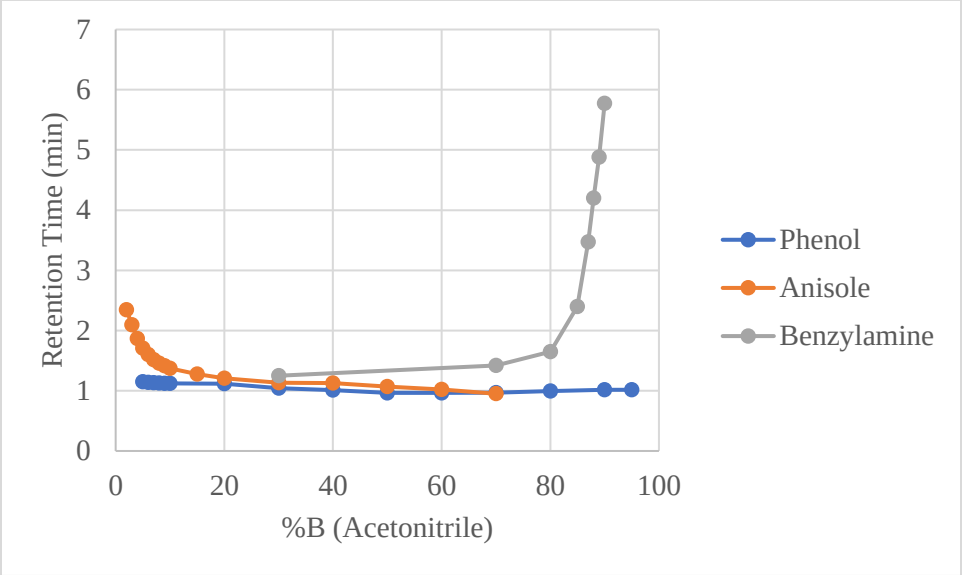

Figure 20: The experimental retention trends of benzylamine, anisole and phenol.

Guanidine

Guanidine is a simple compound with a variety of derivatives and uses across industries including polymer synthesis, high energy fuel, protein purification, and chemotherapy. [35–37] It is a group found on many molecules, including the amino acid arginine. The chemical also results from normal metabolic processes in the body and is excreted in urine and is UV active making it easily detected on a diode array detector. [38] The structure of guanidine is provided in Figure 21 for reference.

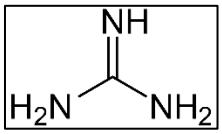

Figure 21: The chemical structure of guanidine.

Although it is not an ideal comparison to benzylamine since the amines in guanidine are participating in resonance in a way that is not present in benzylamine, the compound can be used to confirm amine interactions and retention trends on the given amide column. The observed data allows us to confirm ANP retention for simple molecules with primary amines. The results for evaluation of guanidine analysis are shown in Figure 22.

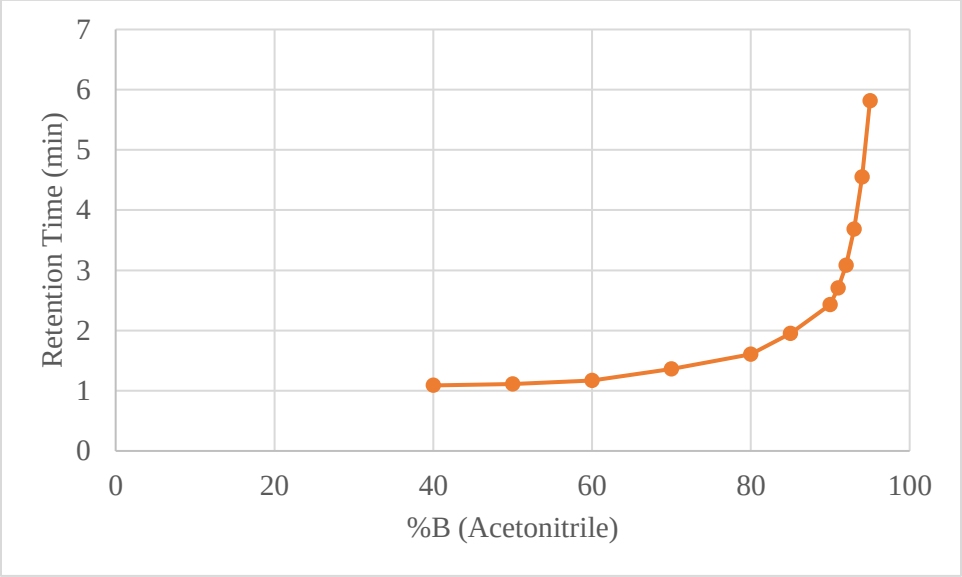

Figure 22: The experimental retention trend of guanidine.

Adenosine and Adenosine Triphosphate

Adenosine is a nucleoside base which has both natural abundance in biochemical processes and uses in pharmaceuticals. It's derivatives also play critical roles biochemical processes. Adenosine consists of the nucleic base adenine and a ribose sugar component. [39–41] The structures of adenosine and adenosine triphosphate are provided in Figure 23 and Figure 24 for reference.

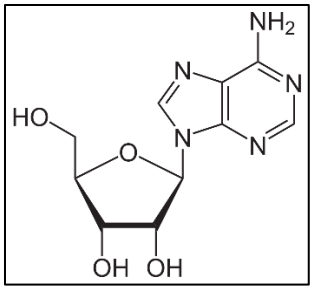

Figure 23: The chemical structure of adenosine.

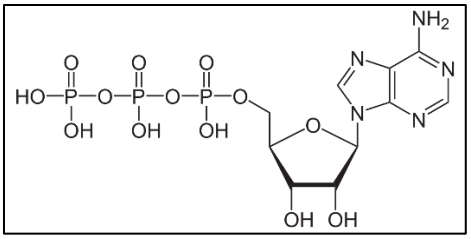

Figure 24: The chemical structure of adenosine triphosphate.

Adenosine triphosphate (ATP) is nucleotide consisting of the same components present in adenosine with three additional phosphate groups bound to the ribose. ATP is best known for its ability to carry and deliver energy throughout the body in the form of its phosphate bonds. [42,43,44] Relative ATP concentration is often monitored by swab and luminometry systems used across industries. Although rapid and reliable methods exist to detect the presence of ATP already [45,46], the compound was analyzed to better understand how additional phosphate groups effected retention on the given amide column. The results of column evaluation using adenosine and ATP are shown in Figure 25.

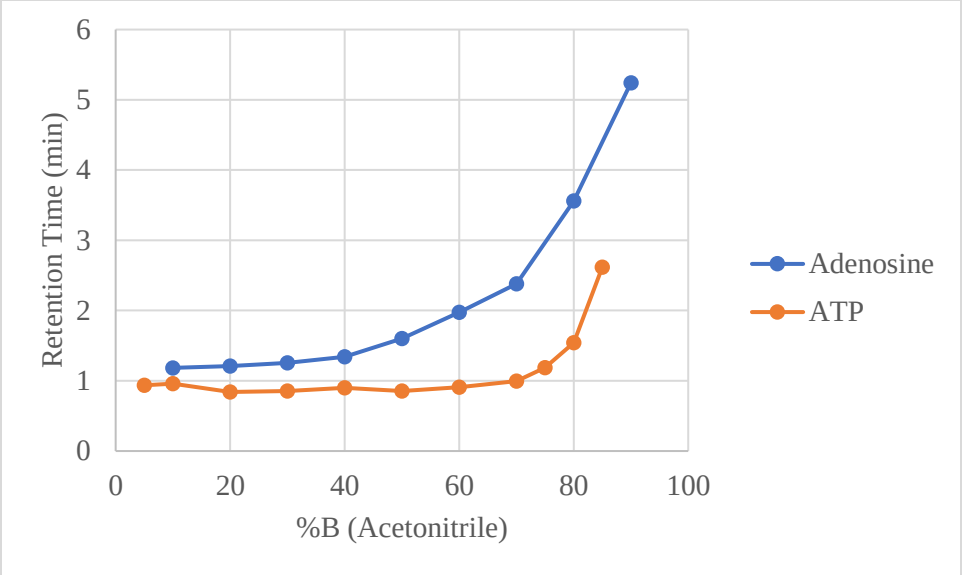

Figure 25: The experimental retention trends of adenosine and adenosine triphosphate.

It can be seen that both adenosine and adenosine triphosphate follow an ANP retention trend, however adenosine looks to retain slightly better than adenosine triphosphate. This suggests that the addition of the phosphate group decreases retention on the amide column. As a consequence of the additional phosphates, overall polar surface area of ATP is much lower than that of adenosine. Despite ATP having more hydrogen bonding sites in the form of hydroxyl groups, those sites are less available to interact with the amide column and have previously been shown to display weaker retention trends on the given column than amines. This comparison also contradicts the idea that a lower hydrophobicity will always result in greater ANP retention. A summary of relevant characteristics for adenosine and ATP is shown in Table 6.

Table 6: Compound characteristics of adenosine and ATP.

	Adenosine	**ATP**
Molecular Weight (g/mol)	267.24	507.18
Log P (Hydrophobicity)	-1.1	-5.7
H bond donors	4	7
H bond acceptors	8	17
Polar Surface Area ($Å^2$)	140	26.3

Thymidine

When comparing adenosine with thymidine, it can be seen that retention trends match with what would be expected. Adenosine had more hydrogen bond donors and acceptors, more amines which have been shown to interact with the amide column, and greater polar surface area. The structure of thymidine is provided in Figure 26 for reference.

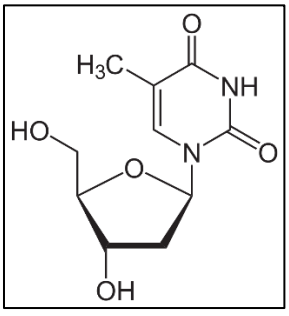

Figure 26: The chemical structure of thymidine.

Based on the characteristics depicted below in Table 7, it was hypothesized that adenosine would have better retention on the given column, which was shown to be true experimentally. Retention data for adenosine and thymidine are shown in Figure 27.

Table 7: Compound characteristics of adenosine and thymidine.

	Adenosine	**Thymidine**
Molecular Weight (g/mol)	267.24	242.23
Log P (Hydrophobicity)	-1.1	-1.2
H bond donors	4	3
H bond acceptors	8	5
Polar Surface Area ($Å^2$)	140	99.1

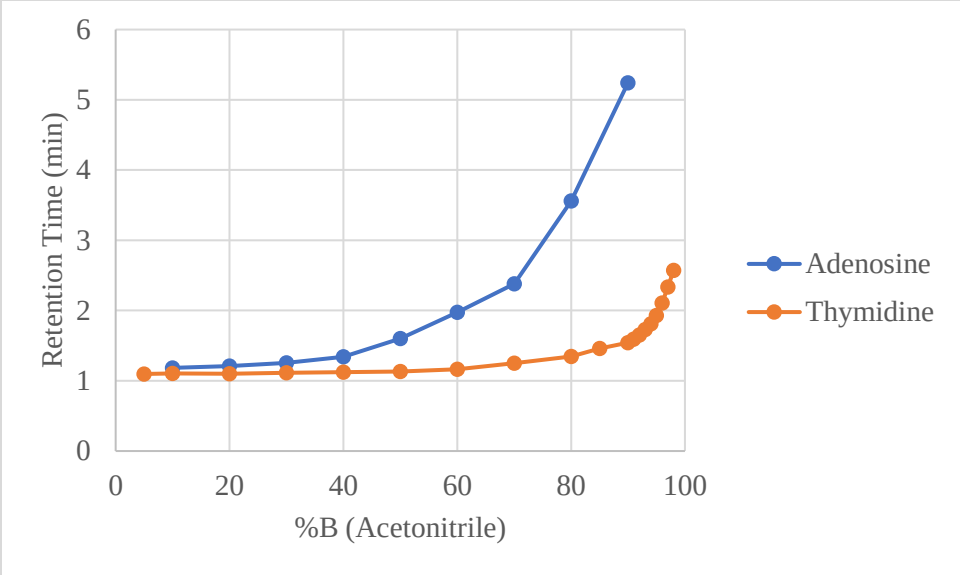

Figure 27: The experimental retention trends of adenosine and thymidine.

Tryptophan

As a potential application, tryptophan was analyzed. It may be found throughout the biological system and is typically obtained through diet. Tryptophan has been shown to be a precursor for serotonin, melatonin, and vitamin B3, is a building block for proteins, and has been used to diagnose chronic kidney disease in patients. [47] The ability to quickly analyze a complex biological sample for tryptophan is a step towards analytical method improvements that could lead to faster and more complete diagnostics in the medical industry. The structure for tryptophan is shown in Figure 28 for reference.

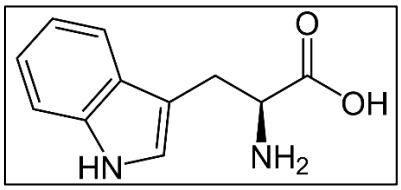

Figure 28: The chemical structure of tryptophan.

Tryptophan's structure includes an amino, a carboxyl, and an indole group. It is considered a relatively nonpolar aromatic amino acid, however the nitrogen in the indole group has the additional ability to participate in influential hydrogen bonding with the given column. [48] It is also one of three aromatic amino acids, meaning that it absorbs light in the UV range making it a prime candidate for analysis on the HPLC-DAD. [49] The retention shown in Figure 29 is for the major peak when analyzing a tryptophan standard.

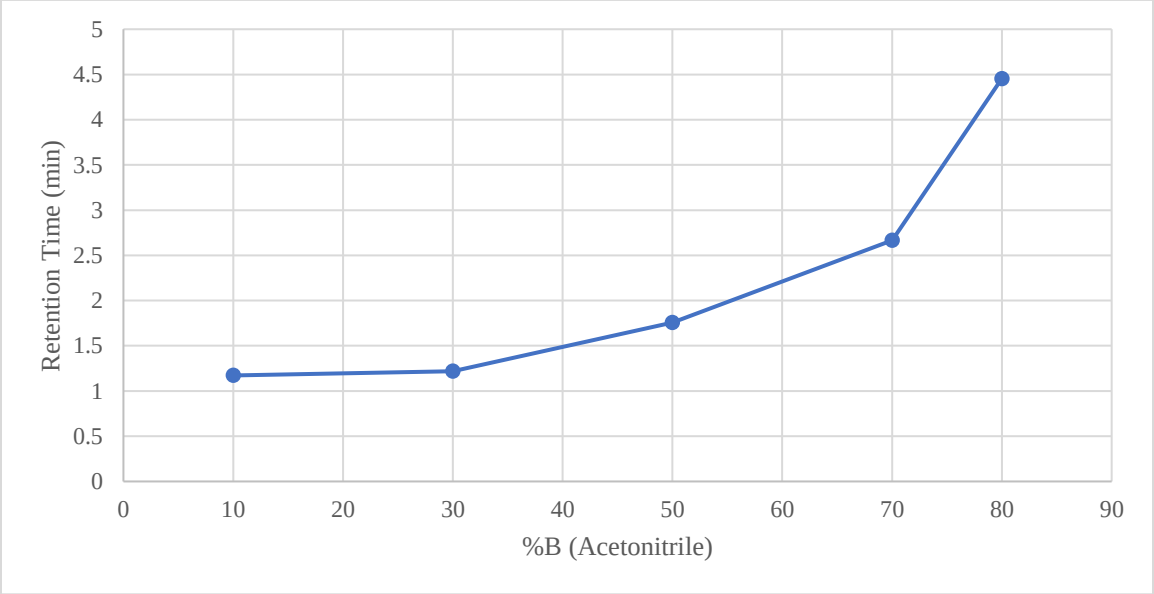

Figure 29: The experimental retention trend of tryptophan.

Norepinephrine

Norepinephrine is a hormone and neurotransmitter produced in the brain. Its presence in biological samples has been shown to increase during periods of stress and correlates with an increase heart rate, blood pressure, and negative cardiovascular consequences. [50] Norepinephrine has previously been quantified by HPLC in a number of studies. [51,52,53] The structure for norepinephrine is provided in Figure 30 for reference.

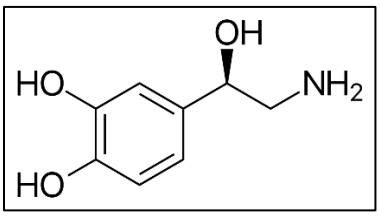

Figure 30: Structure of Norepinephrine

A side by side comparison of tryptophan and norepinephrine may suggest that norepinephrine should be retained slightly better in ANP mode. This hypothesis is based on norepinephrine's greater number of hydrogen bonding sites and higher polar surface area. Relevant characteristics are shown in Table 8, below.

Table 8: Compound characteristics of tryptophan and norepinephrine

	Tryptophan	**Norepinephrine**
Molecular Weight (g/mol)	204.23	169.18
Log P (Hydrophobicity)	-1.1	-1.2
H bond donors	3	4
H bond acceptors	3	4
Polar Surface Area ($Å^2$)	79.1	86.7

In contrast to the original hypothesis, it is seen experimentally that norepinephrine does not have better retention on the given column. Tryptophan may gain its better retention from the presence of its indole and carboxylic groups compared with norepinephrine's primary amine and hydroxyl groups. Retention data is shown in Figure 31.

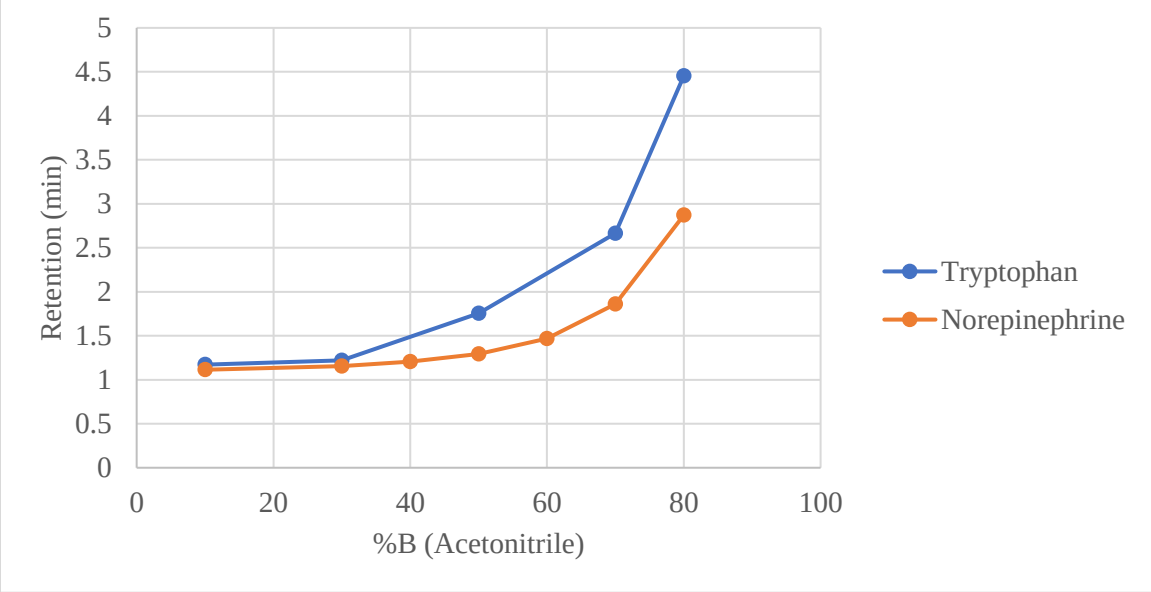

Figure 31: The experimental retention trends of tryptophan and norepinephrine.

Creatinine

Creatinine possesses another potential application. It is a product of the metabolic breakdown of creatinine in muscle tissue, can be found in blood, and is filtered out by the kidney. Creatinine levels in the body are relatively consistent but have been shown to rise when chronic or extensive kidney damage is present. Creatinine concentration in urine and blood is the most widely used measure of kidney function, is associated with

various diseases, and is valuable for identifying patients requiring dialysis. [54,55,56] The structure for creatinine is provided in Figure 32 for reference.

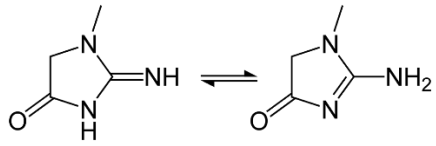

Figure 32: The chemical structure of creatinine.

Although found by colorimetric procedures in many medical labs, complex biological samples are known to commonly have interferences by these methods. [57] For this reason alternative analytical methods offer the opportunity for improved results. A standard of creatinine was analyzed with results shown below. Creatinine retention trends were also compared with that of guanidine to gain further insight into how functional group changes affect retention on the given amide column. Experimental data for creatinine and guanidine is displayed in Figure 33.

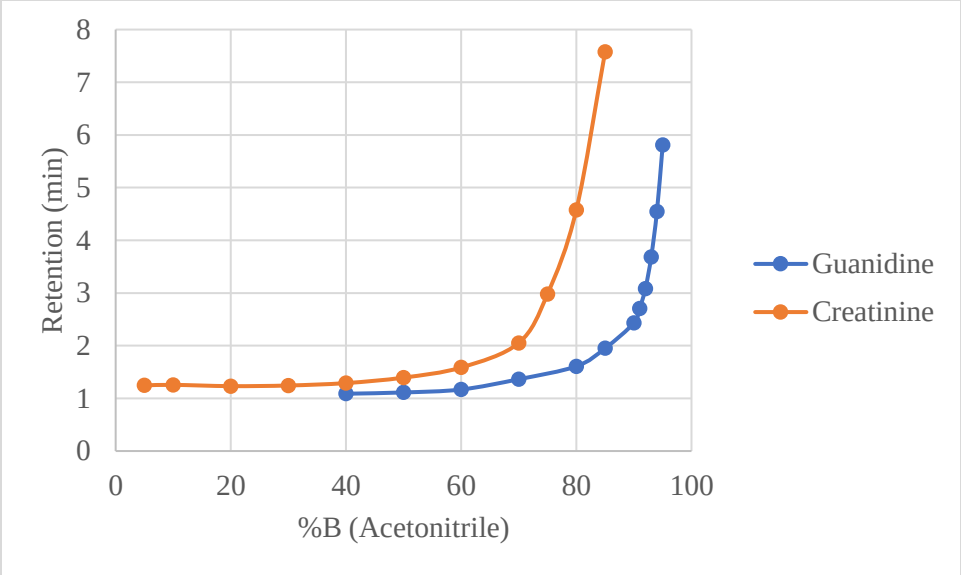

Figure 33: The experimental retention trends of guanidine and creatinine.

In Figure 33 an ANP retention trend for of creatinine can be controlled and the method can be altered and optimized by adjusting the mobile phase composition. By comparison, published chromatographic methods on a non-polar C18 column have limited versatility and can take 25+ minutes per run with an additional equilibration requirement between runs. [58,59] A decrease in sample run time like that displayed above improves the viability of routinely using a chromatographic method in medical labs by increasing sample throughput.

The ANP retention of creatinine is slightly better than the ANP retention of guanidine. Although guanidine has more nitrogen sites available for hydrogen bonding, creatinine's greater polar surface area and the interactions with the additional amide group contribute towards greater interactions with the amide column. A summary of relevant compound characteristics is provided in Table 9.

Table 9: Compound characteristics of guanidine and creatinine.

	Guanidine	**Creatinine**
Molecular Weight (g/mol)	59.18	113.12
Log P (Hydrophobicity)	-1.3	-1.8
H bond donors	3	1
H bond acceptors	1	1
Polar Surface Area ($Å^2$)	26.3	58.7

Caffeine

A standard of caffeine was shown to retain in ANP mode, with retention increasing exponentially at greater than 95% acetonitrile mobile phase compositions. The structure of caffeine is provided in Figure 34 for reference.

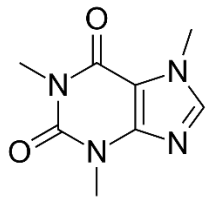

Figure 34: The chemical structure of caffeine.

The ability to run efficiently at the extreme end of mobile phase compositions is a key difference between silica hydride and HILIC columns. Because HILIC columns rely on a

partition mechanism as discussed earlier, the mobile phase composition is limited to a maximum organic percentage of 98% at its most extreme. [60] Running HILIC columns at this high organic phase requires extended equilibration times which render these methods time inefficient. The long equilibration times are required for the water layer on a HILIC column's surface to become established, which is not needed when using a silica hydride column. The retention trend of caffeine on the given amide silica hydride column is shown in Figure 35.

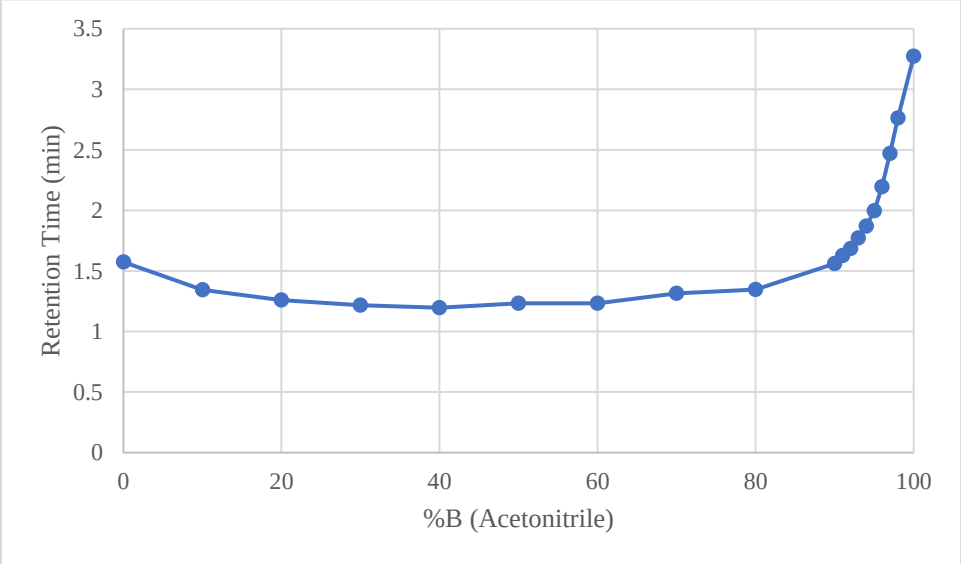

Figure 35: The experimental retention trend of caffeine.

In comparison to the given Type C column used in this study, the HILIC amide column used by Guo et al. did not show significant retention of caffeine with the authors stating that "caffeine (1,3,7-trimethylxanthine) was found to have little retention on the amide phase even in the mobile phase containing 95% acetonitrile due to the presence of three methyl groups". [61] The ability of the silica hydride amide column to retain caffeine

is a direct consequence of its absorption retention mechanism as opposed to the partitioning mechanism utilized by HILIC columns.

IV. Conclusions

The goal of this project was to evaluate the retention trends seen on the given silica hydride amide functionalized column, in particular the ability to participate in an ANP retention trend. This goal was achieved. The known advantages of silica hydride columns were confirmed including dual retention of polar and non-polar compounds, the ability to function in both high organic and high aqueous mobile phase environments, and fast equilibration times.

Results show that compounds with greater polar characteristics and polar surface area that contain groups able to participate in hydrogen bonding have the most retention. Compounds containing nitrogen groups like amides, amines, and indoles resulted in better retention on the amide column compared to others like hydroxyl groups.

In the face of retention trends not aligning flawlessly with hypothesized results based on LogP values, number of hydrogen bonding sites, and surface polarity, comparisons displayed in the results and discussion section confirm the need for column evaluation experiments. A chromatographic separation is a complex system with many interactions occurring simultaneously. Theoretical retention trends do not always match experimental retention trends with the difference offering us learning opportunities.

Further studies should be performed to determine the effect of varying pH with buffers, formic acid content, sample solvent concentration, and column temperature. By analyzing additional molecules more insight can be provided into expected trends on the

amide column. Analyzing similar molecules with and without carboxylic end groups is of interest.

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