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Synthesis and Evaluation of Four Novel Stationary Phases in High Pressure Liquid Chromatography

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SYNTHESIS AND EVALUATION OF FOUR NOVEL STATIONARY PHASES IN

HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

SYNTHESIS AND EVALUATION OF FOUR NOVEL STATIONARY PHASES IN
HPLC

by Niloofar Salehi

High pressure liquid chromatography is one of the most frequently used methods
for the separation of mixtures. Chemically modified silica hydride stationary phases, due
to their unique selectivity and versatility, have been the focus of an increasing number of
studies in the past several years. The objective of fabricating stationary phases with dual
properties is to achieve separation of both polar and non-polar components in a single
analysis. Due to an increasing demand in the fields of drug discovery, proteomics, and
metabolomics to analyze numerous samples with a wide range of polarities, there is a
need for more versatile stationary phases for a wide range of applications. In order to
meet the above objective, in this research four novel silica hydride-based columns were
synthesized using a hydrosilation procedure. The characterization of each column was
done using a series of polar and nonpolar compounds by studying their aqueous normal
phase and reversed-phase chromatographic behavior. Under these conditions, it turned
out that two columns showed both RP and ANP behaviors. Of the remaining columns,
one exhibited ANP behavior only and the other performed only in the reversed-phase
mode.

ACKNOWLEDGMENTS

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I. INTRODUCTION

A. Historical Background

Chromatography is an essential separation technique by which components of a mixture are separated based on their physical and chemical properties. This technique has numerous applications (e.g., separation, purification, and characterization) in various fields such as pharmaceutical, biomedical, industrial, environmental, and more. The technique takes advantage of the physical properties of components and their interactions and affinities with the two different phases available in the system: mobile and stationary phases. The solutes with polarities similar to the polarity of the mobile phase have higher affinity for that phase and vice versa. These solutes tend to leave or elute with the mobile phase, and those that tend to stay behind and interact with the stationary phase elute later. Other factors that could influence these interactions include (but not limited to) the size of the particles, the charge or presence of ions (for ionic solutes), and the various combinations [1]. The analyte's traveling time through the stationary phase is called the retention time. Retention time is characteristic and unique to each compound.

Chromatography exists in various formats. The variety is based on the physical state of the mobile phase, the type of the stationary phase, and the separation method. Separation mechanisms such as size exclusion, adsorption, ion exchange, and affinity have resulted in a variety of chromatographic methods. Historically, chromatography started with paper chromatography (PC) and column chromatography (CC). Further developments led to thin layer chromatography (TLC), followed by gas chromatography (GC), and liquid chromatography (LC) [2]. The terms liquid and gas chromatography

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indicate the physical state of the mobile phase and hence the corresponding

chromatographic names. Gas chromatography is more suitable for solutes that are

volatile but non-decomposable when heated, whereas in liquid chromatography solutes

are dissolved in a solvent and a liquid mobile phase carries them along the stationary

phase [1]. The early reports on liquid chromatography date back to 1903 with Tswett, an

Italian scientist working in a Russian laboratory. He discovered the separation of green

pigments of a plant while working with a column of powdered chalk [3].

B.   High Pressure Liquid Chromatography (HPLC)

Until the mid-1960s, liquid chromatography was a technique with a large column

filled with an adsorbent packing material, mainly silica gel or alumina. The separation

took place by filtering the liquid through the packed material under gravitational force

[3]. However, due to the large scale of the materials and instruments, the analytical

results were accompanied by poor resolution and low efficiency. It involved large

consumption of solvents, generation of a substantial amount of waste, high cost, and low

accuracy and precision in results.

In modern analytical chemistry, accuracy and precision in measurements at very

low concentrations and high resolutions are essential [2]. To address these requirements,

researchers introduced high pressure liquid chromatography (HPLC). HPLC is an

effective tool for rapidly improving the resolution and efficiency of each analysis by

running the samples at a very high pressure, up to 6000 psi [4]. In a modern HPLC

system, there are four major components: solvent delivery, sample delivery, separation, and detection available, as shown in Figure 1.
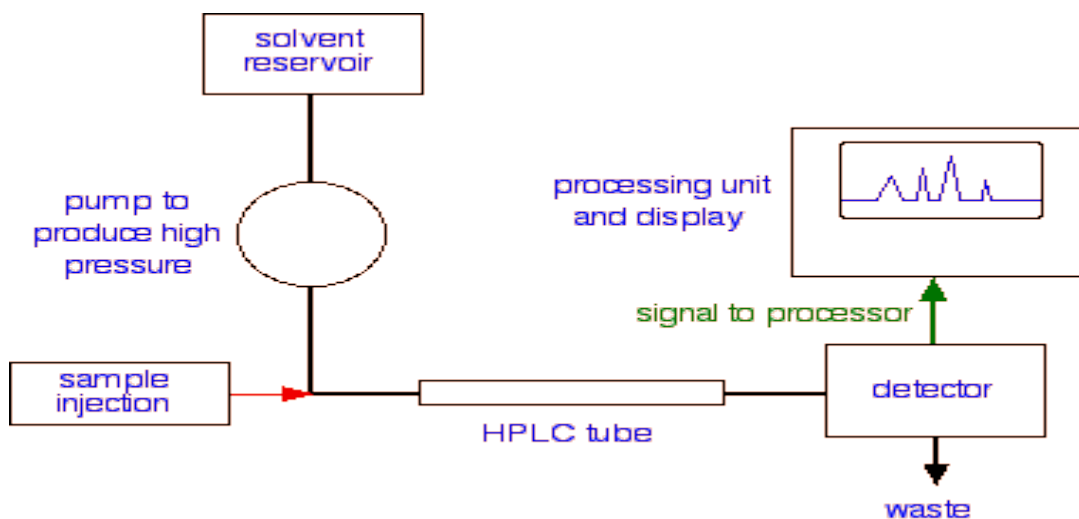
Figure 1. Schematic view of basic components in HPLC

The solvent delivery system is comprised of a pump (the major component), the solvent reservoirs, degassers, mixers, gradient formers, and pulse dampers. The solvent reservoir is specifically beneficial when there is more than one solvent involved in the composition of mobile phase. Generally, solvents have some O_2 and N_2 dissolved in them, and, if they are not removed, the chromatogram could show them as additional peaks interfering with the analyte data. Pumping Helium gas at a constant flow through a sparger can solve this problem. The gradient system is designed for introducing varying concentrations of the mobile phase per time unit. The mobile phase is pumped through

the stationary phase (column) at a very high pressure resulting at a flow rate of 0.1 – 10 mL per min [5].

The injector comprises the sample delivery system. There are two ways to introduce the sample, manually or automatically. If the HPLC instrument is equipped with a manual injector, there is a loop that introduces the sample into the system in a continuous flow from a single switch valve. For HPLC instruments with an autosampler, the sample is injected into the column at preset intervals [1, 4, 6].

Separation takes place in the column or the stationary phase in HPLC. Columns are small stainless steel or polymer tubes with a typical length between 50 and 250 mm and inner diameter of 4.6 mm, or smaller. The stationary phase is immobilized on small sized porous particles, between 1.5–10 µm [1]. The material for the stationary phase is typically silica or silica modified with organic derivatives.

The detection system in HPLC utilizes a property of the analyte or mobile phase to produce an electronic signal. The signal appears in a form of retention time versus concentration of the analyte in the mobile phase. This output is referred to as the chromatogram. There are a variety of different detectors that exploit different physical and chemical properties of the analytes. Ultraviolet-visible and fluorescence absorbance detectors are able to detect chemicals based on how they absorb or emit these types of light. Mass spectrometers (MS) and refractive index (RI) are other commonly used detectors that respond to a much wider selection of analytes [1, 6].

C.   Detectors

1.   The Ultraviolet (UV) detector

Many compounds can absorb UV- visible light in the region of 200 – 800 nm (except for compounds without a chromophore), and this is the principle that allows the application of UV detectors. Upon absorption of UV light by the analyte, the intensity of the light that passes to the detectors decreases, and the extent of absorption is directly related to the concentration of the analyte, as described in Beer's law. UV light sources emit wavelengths over a wide range; in simple detectors and with the aid of a filter or monochromator, the wavelength range can be narrowed considerably. More advanced UV detectors provide multiple wavelengths at the same time. Besides the simplicity, UV detectors are versatile, sensitive, and reliable with high linearity [7].

2.   Diode array detector

The diode array detector (DAD) uses a polychromatic light source, which, after passing through a liquid sensor cell and dispersing by a prism or diffraction grating, collects data on a group of photosensitive diodes. Each diode detects a slightly different wavelength than the other which eventually leads to the absorption spectrum for possible identification of the sample [8].

D.   Modes of Separation

Modes of separation mainly depend on the polarity of the stationary and the mobile phases. As a result, there are two general modes of separation, which will be explained below.

1.   Normal phase high performance liquid Chromatography

In normal phase (NP) chromatography adsorption on the surface of the stationary phase is the principle behind the separation of the analytes. In NP, the stationary phase is relatively polar and the mobile phase is non-polar. As a result, polar analytes tend to interact and stay with the stationary phase longer than the non-polar analytes, hence higher retention time for the polar compounds is expected. Consequently, non-polar analytes tend to elute faster resulting in lower retention time. The mobile phase in NP chromatography usually consists of a non-polar solvent, such as hexane, heptane, chloroform, isopropanol, or ethyl acetate. Alternatively, the stationary phase may have some polar functional groups such as diol, amino, and cyano groups bonded to the surface of the silica [1, 6].

2.   Reversed phase high performance liquid chromatography

Reversed phase (RP) chromatography utilizes a stationary phase that is non-polar and a polar mobile phase. Columns with organic moieties attached to the surface of silica can provide a non-polar stationary phase. The mobile phase consists of polar solvents such as water and soluble organic solvents such as acetonitrile, methanol, and

tetrahydrofuran (THF). The presence of a polar mobile phase causes the analytes with medium to high polarity to elute first, and inversely those of lower polarity are retained on the stationary phase and elute later. In RP chromatography, partition of solutes into the non-polar phase is the main mechanism for the separation of solutes [1, 6].

3.   **Aqueous normal phase high performance liquid chromatography**

When a stationary phase possesses both non-polar and polar retention properties, the mode is called aqueous normal phase (ANP) [9]. A study by Pesek, *et al.* demonstrates that ANP is one of the most versatile separation modes allowing for a wide range of solutes to be analyzed and separated at the same time. More specifically, in ANP, with a binary solvent system, altering the polarity of one of the solvents enables the column to perform in both the NP and the RP retention modes. A higher concentration of a polar solvent such as water allows the column to operate in the RP mode. When there is a higher concentration of organic solvent, the column has the ability to perform in the ANP mode. Moreover, addition of buffers such as formic acid, ammonium formate, or ammonium acetate in small amounts to the mobile phase enhances the retention of acidic or basic polar analytes [9, 10]. Another unique feature of ANP is the composition of the stationary phase. In ANP, silica is replaced by silica hydride. The replacement of ─OH functional group by ─H, which is done via a silanization process, eliminates many unwanted interactions due to hydrogen bonding in the former or due to electrostatic interaction when the silanols are deprotonated. The ANP mode differs from the somewhat similar hydrophilic interaction chromatography (HILIC). ANP and HILIC are

distinguished because the former can retain both polar and non-polar analytes in the stationary phase upon altering the composition of mobile phase, whereas the latter is a technique that only deals with the separation of polar compounds [11].

E.   Column in HPLC

1.   Column efficiency

There are several factors involved in the performance of a column: resolution, selectivity, the sample capacity factor, and column efficiency. According to equation (1), column efficiency (N) is directly related to the number of theoretical plates:

$$N = 5.54 \ (t_R/W)^2 \tag{1}$$

t_R = the retention time of the solute   W = the width of the peak

The height equivalent to a theoretical plate (HETP) is inversely related to the column efficiency and directly related to the length of the column, L, as described by the Van Deemter equation (2) [1,12].

$$HETP = L \ / \ N \tag{2}$$

2.   Column packing

In choosing the right material for the stationary phase, one must consider several important criteria. The durability, inertness, high efficiency, and adequate surface area are some of the physical properties of importance. Some of the packing materials available are silica, zirconia, thoria, alumina, and titania. However, silica (a readily available material with low cost) not only possesses all of the aforementioned properties,

but also comes in different pore sizes and shapes, an important factor for various

industrial applications. The pore size of silica has a direct correlation with the size of the

molecules retained on the stationary phase [13]. Therefore, silica is considered to be one

of the most widely used packing materials for columns in HPLC. There are, however,

some limitations associated with the use of silica. Silica, due to its structure, is somewhat

acidic and tends to interact with basic compounds. Additionally, silica is not stable at a

pH lower than 2.0 or higher than 8.0 due to decomposition or dissolution [14].

Silica is a porous material having a chemical composition of $SiO_2 \cdot H_2O$. The

structure consists of a network of siloxane bonds (Si–O–Si) and terminal silanol groups

(Si–OH). The silanols can exist in geminal or vicinal forms as depicted in Figure 2 [15].
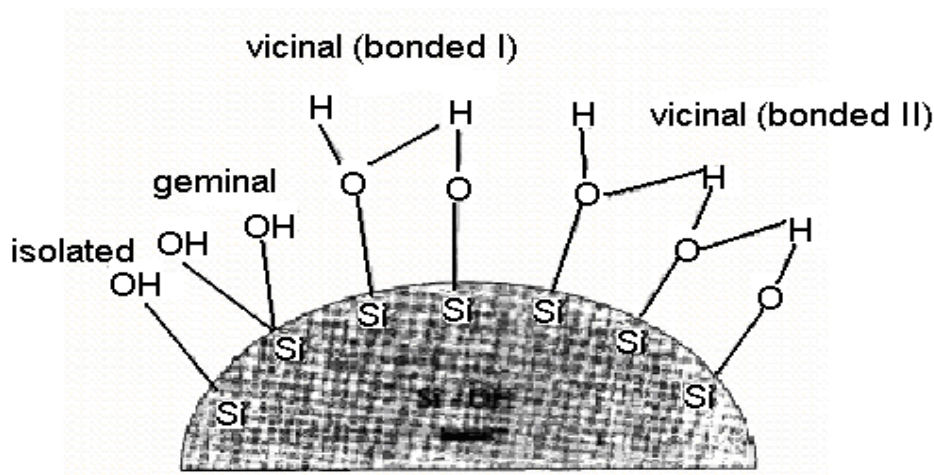
Figure 2. Different types of silanols on the silica surface [15]

It is the presence of the ─OH group that gives silica acidic properties and makes

silica less suitable for certain compounds and at certain pH values. In other words, in RP

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chromatography, when the mobile phase is a binary mixture of organic solvents and H_2O, or in cases when polar compounds are analyzed, there will be some irreversible adsorption of the compounds on the silica surface due to the formation of hydrogen bonds. For instance, the acidic silica can cause unwanted interactions with analytes with basic properties leading to poor efficiency. Moreover, when water is used as part of the mobile phase, it can be adsorbed by the stationary phase (Type B Silica) because of the free ―OH groups on the surface, Figure 3. As the water content on the surface of the stationary phase increases, the retention of analytes often changes [16]. The presence of free silanols also causes peak tailing in the chromatograms.
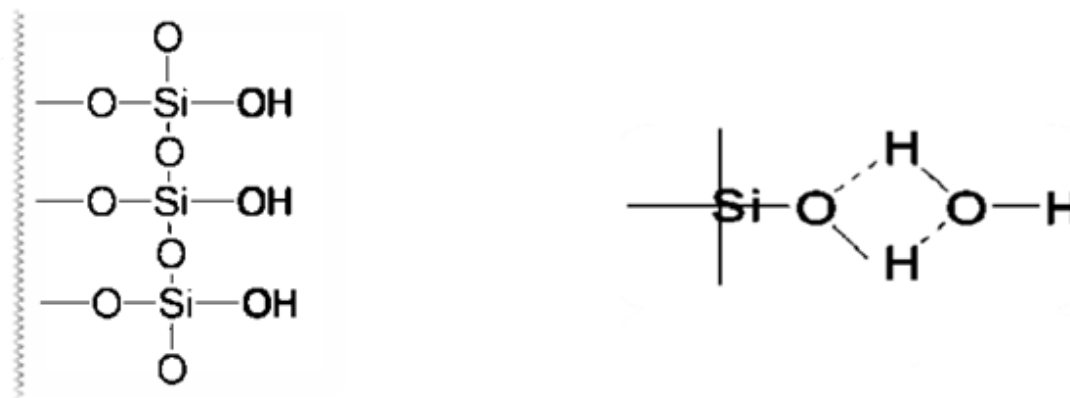
Figure 3. Type B silica and strong water molecule association through hydrogen bonding [16]

3.   Methods to modify silica surface

In order to overcome the problems caused by the silanol groups on the silica surface, one logical approach is the conversion of the ―OH group to a less active

functional group such as —H. The conversion prevents the mobile phase water

adsorption on the surface, as shown in Figure 4.
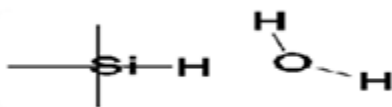
Figure 4. Type C silica and elimination of water molecule [16]

There are several methods proposed to address this problem. Each method comes

with its own advantages and disadvantages. The following are the most commonly used

approaches.

i.   Esterification

This method, which is similar to an organic esterification method, results in

conversion of the —OH in silanol into an —OR using an alcohol as the R-group donor,

as shown in reaction (1). This material undergoes Si–O–R bond breakage, under acidic

hydrolytic conditions, and is not suitable for most chromatographic applications [17].
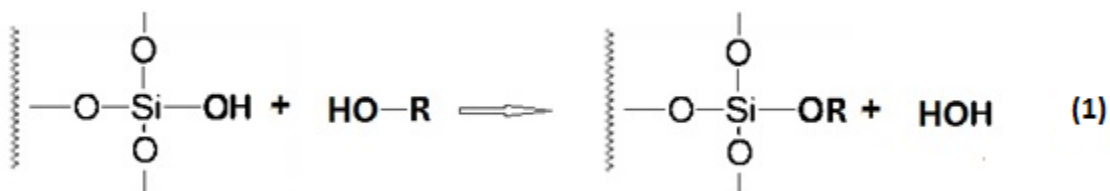
ii.   Organosilanization

Another approach to remove the acidic –OH group of silanol is organosilanization. As shown in reaction (2), a dimethylalkyl silane (X–SiR'$_2$R) replaces the –OH in silanol [18]. The formation of an Si–R bond is very desirable in terms of introducing various R groups; hence, multiple stationary phases can be produced. However, the HX byproduct can cause the hydrolysis of the Si–R bond and unwanted results. Moreover, if the conversion of all Si–O bonds to Si–R is not completely successful the presence of silanols on the surface could cause irreversible adsorption between the analytes and the stationary phase [18].

In the second type of organosilanization, as shown in reaction (3), a trifunctional organosilane reacts with the surface silanols followed by a crosslinking, which results in the formation of a bonded stationary phase [19]. The disadvantage of this approach could be the significant reduction in the interactions between basic solutes and silanols [19].
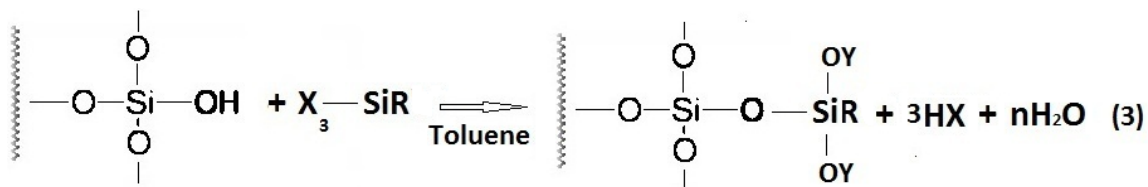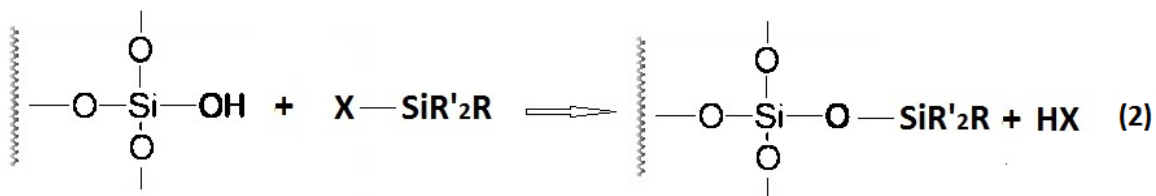
where n = 1 – 3 and Y = H or Si(Osi)$_2$R based on the extent of crosslinking [19].

iii. Grignard Reaction

A Grignard reaction involves addition of thionyl chloride in the presence of toluene to form a silica-halide intermediate, as described in reaction (4). Addition of an organolithium reagent or a Grignard reagent to this intermediate results in the formation of a Si–R bond and removal of the silanol groups, as shown in reaction (5). Although the reaction replaces almost all of the silanol groups, this method suffers from acidic hydrolysis of the intermediate [18].

$$\equiv\text{Si}-\text{OH} + \text{SOCl}_2 \longrightarrow \equiv\text{Si}-\text{Cl} + \text{SO}_2 + \text{HCl} \qquad (4)$$

$$\equiv\text{Si}-\text{Cl} + \text{RMgBr} \longrightarrow \equiv\text{Si}-\text{R} + \text{MgClBr} \qquad (5)$$
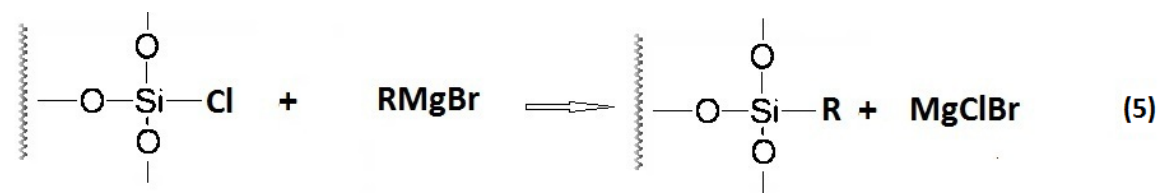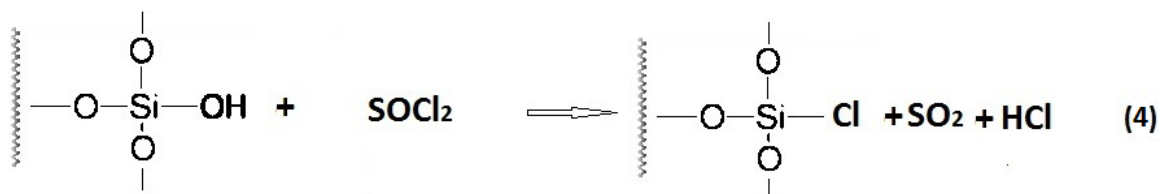
iv. Silanization/ Hydrosilation

Introduced first by Pesek, *et al.*, this novel technique has addressed many of the disadvantages of silica. The technique has also opened the door to the production of a variety of stationary phases for different purposes. There are two major steps involved in this method. The first step is the silanization reaction. Following a successful silanization, hydrosilation, the second step of the procedure, will complete the process.

Silanization involves the removal of the –OH group from silica and replacing it

with hydrogen, hence the formation of silica hydride. This results in the formation of

what is commercially known as Type-C silica instead of Type-B silica. A schematic

comparison of the two types is given in Figure 5. As shown in reaction (6), the Si–OH

bond converts to Si–H in the presence of triethoxy-silane (TES) and an aqueous HCl

catalyst. Studies have shown that the hydride product obtained from silanization is stable

and does not undergo a hydrolysis reaction [20]. Moreover, the modification is efficient

in replacing more than 95% of the silanol groups [14].

$$\underset{|}{\overset{|}{-}}Si-OH + (OEt)_3\,Si-H \; \xrightleftharpoons{\;H^+\;} \; \underset{\underset{O}{|}}{\overset{\overset{O}{|}}{Si}}-O-\underset{\underset{OY}{|}}{\overset{\overset{OY}{|}}{Si}}-H + EtOH \qquad (6)$$
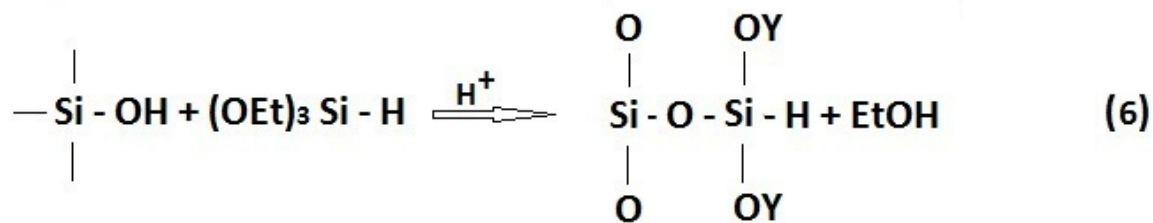
Y = H or Si

Recent studies have shown that the minimal steric hindrance of the small Si–H

group, which could result in a large Si–H population on the surface of silica, could also

cause formation of multilayered SiH groups. The drawbacks were mainly observed when

TES was involved as one of the reactants and water as a catalyst [21].

TYPE-B SILICA **TYPE-C SILICA**
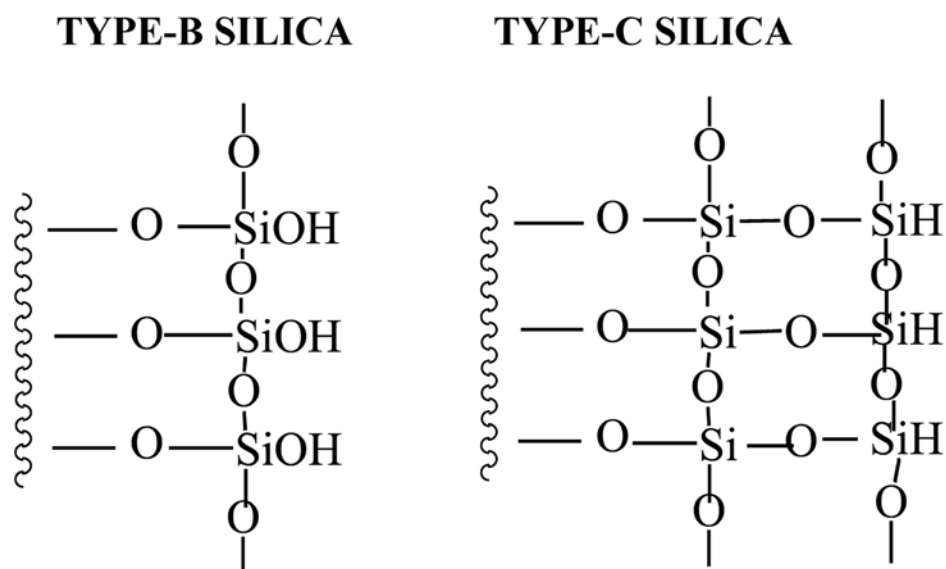
Figure 5. Schematic comparison of Type-B vs. Type-C silica [14]

To resolve this issue, Sandoval has suggested two different methods. In the first approach, silica is reacted with $HSiCl_3$, trichlorosilane (TCS), dissolved in an organic solvent such as toluene [22]. In their second approach, silica is reacted with TES, $HSi(OEt)_3$ in a small amount of aqueous HCl dissolved in THF [21].

Hydrosilation is the introduction of an organic group to the surface of silica hydride from silanization. Depending on the structures and properties of the added groups to the surface, hydrosilation produces stationary phases with various properties suitable for hydrophilic, hydrophobic, or ion exchange chromatography. Through this process, an organic moiety attaches to the surface of silica hydride forming a stable (Si–C) bond. In hydrosilation the silica hydride and a terminal olefin, the most used form of organic compounds, react using hexachloroplatinic acid in 2-propanol, also known as Speier's catalyst, as shown in reaction (7).

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$$\equiv Si-H \ + \ CH_2 {=} CH-R \ \xrightarrow{\text{cat.}} \ \equiv Si-CH_2-CH_2-R \qquad (7)$$

One of the drawbacks of this reaction is perhaps the use of hexachloroplatinic acid in 2-propanol. The Speier's catalyst available in this reaction can produce intermediate species with the organic compound. This reaction could in turn result in lower product yield and efficiency of the reaction. Additionally, platinum metal can deposit on the surface of the silica hydride and reduce the efficiency [23]. There have been studies that have suggested alternative catalysts such as azobisisobutyronitrile (AIBN) for the hydrosilation reactions [23].

Although terminal olefins are the most commonly used organic moieties in hydrosilation, recent studies have led to the development of reactions using alkynes [24], cyano compounds [25], and non-terminal olefins [26]. For instance, structures I and II in reaction (8) can be considered as two proposed bonding structures in the synthesis of a stationary phase from hydrosilation of silica hydride with an alkyne.

$$(8)$$

Structure I   Structure II

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F.   Research Goals

In this study, silica hydride was the base material for the stationary phases used in HPLC. However, attachment of various moieties to the surface of the silica hydride results in a stationary phase better adapted to either reverse phase (RP) or aqueous normal phase (ANP) characteristics. The objective of many studies has been to create columns that are responsive to both conditions, RP and ANP, at the same time. The focus of this study was also to introduce novel organic moieties to the surface of the silica hydride and, therefore, to develop columns that can perform under both reverse phase (RP) and aqueous normal phase (ANP) conditions without the need to exchange columns when switching from one condition to the other. To verify the accomplishment of the goal, each column was then subjected to a variety of hydrophilic and hydryophobic analytes as well as polar and non-polar mobile phases for retention behavior studies.

The first part of the study was to synthesize the stationary phases. To obtain HPLC columns, which are amenable to many applications, silica was initially converted to silica hydride through the silanization method, as explained above. FTIR was used to characterize the material and confirm that Si–H groups were in abundance on the surface. The next phase was hydrosilation through which five carefully chosen organic compounds were introduced onto the silica hydride surface. In the hydrosilation reactions, *m*-dimethylbenzene, ethynylanaline, 1,8-nonadiyene, 6-chloro-1-hexyne, and 5-cyano-1-pentyne were bonded to the surface of silica hydride through the aromatic ring in *m*-dimethylbenzene and alkyne groups in other compounds. Upon completion of each reaction, the newly synthesized stationary phases were subjected to spectroscopic

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characterization and determination of percent surface coverage. To confirm the success

of each hydrosilation, the samples were spectroscopically evaluated using FTIR, ^{13}C-

NMR, and ^{29}Si-NMR. Carbon analyses for each sample measured the percent surface

coverage. Following the evaluations, it was discovered that *m*-dimethylbenzene did not

result in a reasonable surface coverage and therefore was removed from the rest of the

study. Each of the other four compounds was used as a stationary phase and packed in a

stainless steel HPLC column.

The next phase in this research was to evaluate each column for its behavior

towards hydrophilic and hydrophobic analytes under a wide range of polar and non-polar

mobile phases. This study was done by selecting analytes from different categories to

observe the retention behavior of each under RP and ANP conditions.

The goal for this study was to design a stationary phase(s) that contains both RP

and ANP modes simultaneously. When using a mixture of polar and not-polar solvents

as the mobile phase, an ideal retention time graph for such a column has a U-shape curve,

as illustrated in Figure 6. This behavior could be interpreted as the polarity of the mobile

phase decreases the stationary phase shifts toward the ANP mode. This would result in a

longer retention time for hydrophilic compounds and vice versa.

With that objective in mind, four bonded stationary phases were synthesized,

characterized, and evaluated for their RP/ ANP mode using a wide variety of polar and

non-polar analytes.
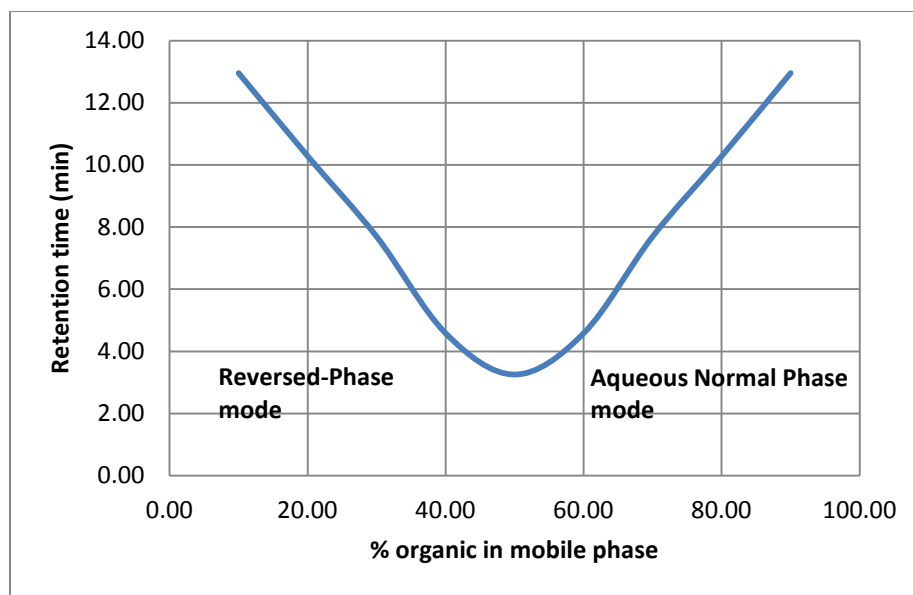
Figure 6. Ideal RP and ANP regions retention map on Si–H stationary phase

II.   EXPERIMENTAL

A.   Materials

1.   Silanization: Preparation of silanol

The materials used for this part are listed in the following tables. The main
compound in the production of stationary phases was silica. AstroSil® Silica was
obtained from Stellar Phases (Langhorne, PA).

Table 1. Specfications of AstroSil® silica.

Lot Number	7-122FA
Pore Size (nitrogen, average pore)	98 Å
Pore Volume	0.9 mL/g
Surface Area (nitrogen, BET multipoint)	345 sq. m/g
Apparent Density (tap density)	0.45 g/mL*
Particle Size (microscopic analysis)	5.2 μ
Particle Distribution dp 90/dp 10	1.6
Metal Content (ICP scan, HF digest)	
Na	<4 ppm
Mg	< 10 ppm
Ca	< 10 ppm
Al	< 2.8 ppm
Fe	< 2.7 ppm

*Tap density is based on 15 µm particles. Values for smaller particles will be higher.

Table 2. Compounds used for the silanization.

Compound	CAS number	Manufacturer
Silica		Stellar Phases
Triethoxysilane (TES)	78-07-09	Sigma-Aldrich
Dioxane	123-91-1	Fisher Chemicals
Hydrochloric acid, 2.3M	7647-01-0	Aldrich
Diethylether	60-29-7	Sigma-Aldrich
Tetrahydrofuran (THF)	142-68-7	Aldrich

2.   Hydrosilation: Preparation of stationary phase

In this step, the silanols were converted into silica hydrides. The conversion was followed by introduction of five organic moieties on the surface of silica hydride for a novel stationary phase. Each organic moiety was bonded individually.

Table 3. Compounds used for hydrosilation.

Compound	CAS number	Manufacturer
Hexachloroplatinic acid	16941-12-1	Sigma-Aldrich
Toluene	108-88-3	Fisher Chemicals
Diethylether	60-29-7	Sigma-Aldrich
AstroSil® hydride		hydrosilation AstroSil® silica
1,8-Nonadiyne		GFS Chemicals, Inc.
m- Diethynylbenzene		GFS Chemicals, Inc.
5-Cyano-1-pentyne		GFS Chemicals, Inc.
6-Chloro-1-hexyne		GFS Chemicals, Inc.
4-Ethynylaniline		GFS Chemicals, Inc.

3.   HPLC: Mobile phase preparation

The chromatographic evaluation of each stationary phase utilized different concentrations of an organic solvent, acetonitrile, and a polar solvent, MilliQ H_2O. In addition to the mentioned solvents, small volumes of an additive, formic acid, was used in some cases.

Table 4. Compounds used in the mobile phase.

Compound	CAS number	Manufacturer
Acetonitrile HPLC grade	75-05-8	Fisher Chemicals
MilliQ DI Water		Millipore
Formic Acid	64-18-6	Spectrum Mfg. Corp.

4. HPLC analysis: Compounds analyzed with the stationary phases

The following tables, 5, 6, 7, 8, and 9 represent the analytes used in this study.

Table 5. Amino acids (hydrophilic/ polar).

Compound	CAS number	Manufacturer
L-(+)-α-Phenylglycine 99%	2935-35-5	Sigma-Aldrich
Phenylalanine	20700-34-3	Sigma-Aldrich

Table 6. Nucleobases (hydrophilic/ polar).

Compound	CAS number	Manufacturer
Adenine	73-24-5	Sigma-Aldrich
Guanine		NBC National Biochemical Corp.

Table 7. Neurotransmitters (hydrophilic/ polar).

Compound	CAS number	Manufacturer
Epinephrine	51-43-4	Sigma
Norepinephrine	51-41-2	Sigma
Dopamine	62-31-7	Sigma

Table 8. Steroids (hydrophobic/ non-polar).

Compound	CAS number	Manufacturer
Adrenosterone	382-45-6	
Δ^4-Androsten-3,17-dione	63-05-8	Sigma-Aldrich
Estrone	53-16-7	Sigma-Aldrich
Estradiol	50-28-2	Sigma-Aldrich
Corticosterone	50-22-6	Sigma-Aldrich

Table 9. Polycyclic aromatic hydrocabons (PAH) (hydrophobic/ non-polar).

Compound	CAS number	Manufacturer
Naphthalene	91-20-3	Sigma-Aldrich
Phenanthrene	85-01-8	Sigma-Aldrich
Pyrene	129-00-0	Aldrich
Fluorene	86-83-7	Aldrich

B.   Structures

1.   Organic compounds used in the synthesis of the bonded stationary phase

There were five organic compounds designed to be introduced to the surface of stationary phases. The structure of these compounds are illustrated below.

1,8-Nonadiyne
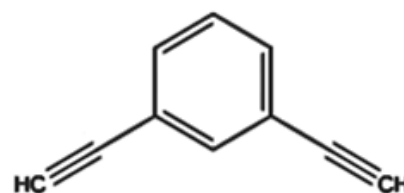
m-diEthynylbenzene
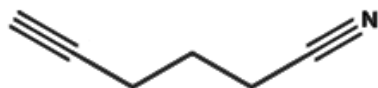
5-Cyano-1-pentyne

6-Chloro-1-hexyne
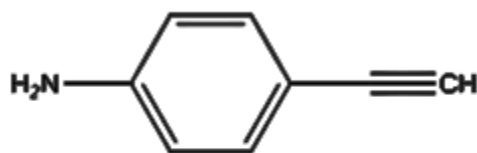
4-Ethynylanaline

2.   Analytes used for the HPLC evaluations

i.   Amino acids

Phenylalanine and Phenylglycine were the two amino acids used as polar anayltes in this study. The structures of the two amino acids are shown below.
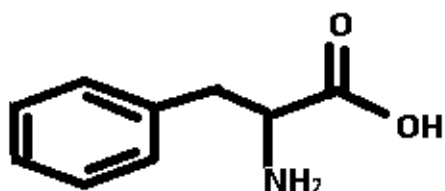
Phenylalanine
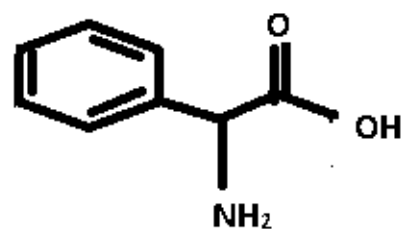
Phenylglycine

ii.   Nucleobases

For the nucleobases, Adenine and Guanine with the structures shown below were used.
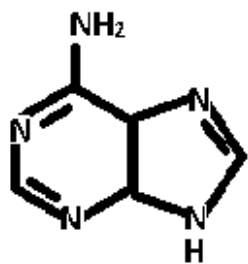
Adenine
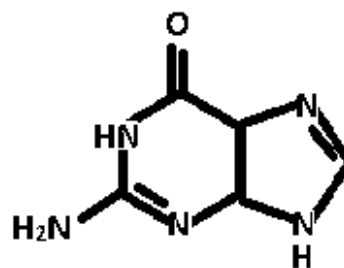
Guanine

iii.   Neurotransmitters

The other set of polar compunds used as analytes were the neurotramsitters, Epinephrine, Norepinephrine, and Dopamin. The structers to these compounds are shown below.
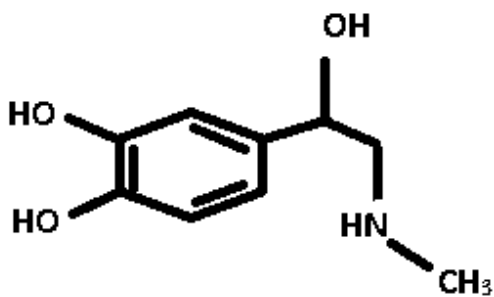
Epinephrine
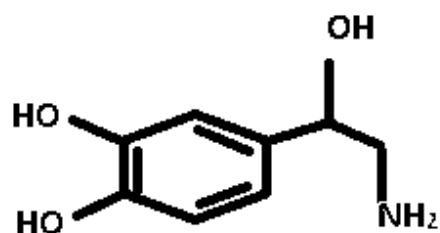
Norepinephrine
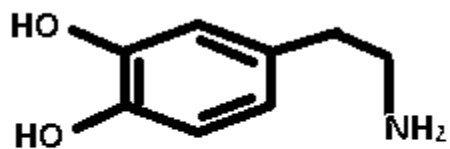
Dopamine

iv.   Steroids

Adrenosterone, Androsten-3,17-dione, Esterone, Estradiol, and Corticosterone were five non-polar analytes from the steroid family. The structre of each analyte is shown below.

Adrenosterone
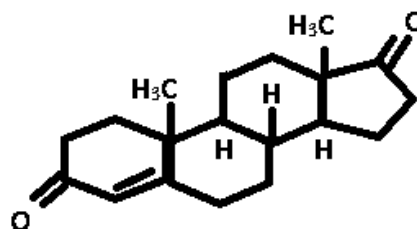
Androsten-3,17-dione
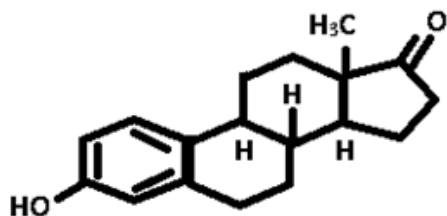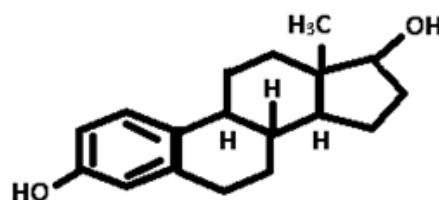
Esterone

Estradiol
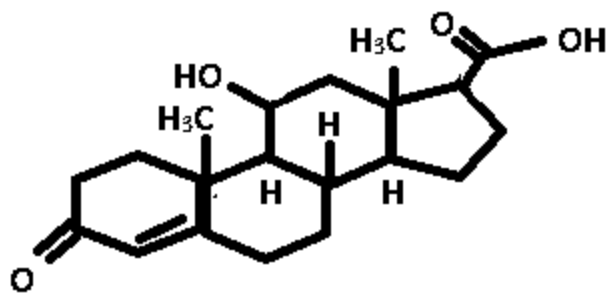
Corticosterone

v.   Polycyclic Aromatic Hydrocarbons

The poly aromatic hydrocarbons, Naphthalene, Phenanthrene, Pyrene, and Flourene were used as another group of non-polar analytes. The structure of each analyte is shown below.
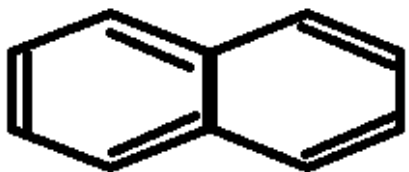
Naphthalene
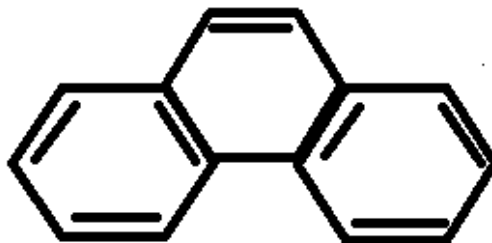
Phenanthrene
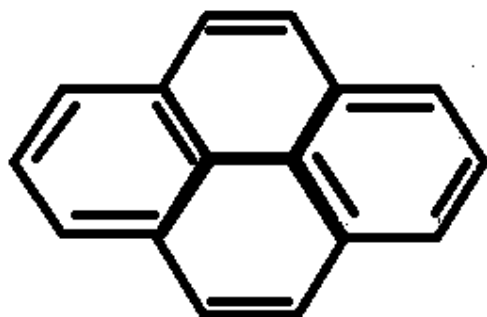
Pyrene
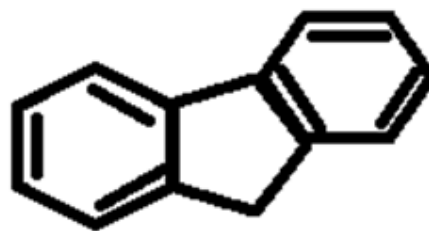
Fluorene

C.   Methods

1.   Silanization: Synthesis of silica hydride

Silanization must be performed under strictly controlled conditions. Therefore, it was necessary to oven-dry all equipment for 24 hr after careful washing and cleaning. In order to synthesize silica hydride, 25 g of Astrosil Silica™, stored and dried overnight in a vacuum oven at 120 ^{o}C, was weighed. The oven's vacuum pump has a controlled pressure of apporoximately -29 inHg or higher. The dried silica was transferred to a 2000 mL three-necked round bottom flask which contained 900 mL dioxane and 32.4 mL of 2.30 M HCl as a catalyst. To ensure complete transfer of silica, 100 mL of dioxane was used to wash the remaining silica into the flask. The Astrosil Silica™, dioxane, and HCl mixture was heated up to 70 ^{o}C. To facilitate this procedure, the round bottom flask was attached to a condenser and placed on top of a heating mantel. The entire setup was connected to a stirring apparatus. The reaction temperature was controlled by a thermometer, placed inside the three-necked flask through a rubber stopper, and stirring was done by a magnet placed inside the flask.

Once the mixture reached and remained constant at 70 ^{o}C, 35.0 mL of pure triethoxysilane, TES, mixed with 150.15 mL dioxane was added to the solution, in a drop-wise manner with constant stirring in the presence of argon gas, using an addition funnel. Upon completion, the temperature of the mixture was raised to 90 ^{o}C in about 30 min and maintained at that temperature for 90 min. The silica hydride product was separated from the solution using a vacuum filtration method with an oven-dried 90 M sintered funnel. The solid silica hyride was washed three times, using 90 mL dioxane, 90

mL DI water, and 27.63 mL tetrahydrofuran per washing. To ensure complete removal

of TES, 90 mL of diethylether was used for final washing. The silica hydride was then

dried and kept in a vaccum oven at a temperature of 110 ^{o}C for 24 hours [20]. The total

weight of the final product was 21.00 grams, and the productwas characterized using the

FTIR method.

2.   Hydrosilation: Synthesis of stationary bonded phase

The hydrosilation reaction consisted of two major steps. The initial step was the

activation of the selected organic moiety designed to be bonded to the stationary phase.

For the second step, the silica hydride and the activated organic moiety were combined to

form a new bonded stationary phase.

The approach for the hydrosilation portion of this study was to introduce five

selected organic compounds to the surface of silica hyrdride in order to synthesize five

unique stationary bonded phases. The reaction procedures for each stationary phase are

described in the following sections.

i.   Preparation of 1,8-nonadiyne bonded phase

The hydrosilation reaction, similar to silinization, required the use of oven-dried

(temperature of 100 ^{o}C) equipment. The organic moiety 1,8-nonadiyne-

HCC(CH$_2$)$_5$CCH- with the formula weight of 120.20 g/mol and structure shown in

section (B) was used for this particular reaction. After setting up a three-necked round

bottom flask and a condenser placed on top of a heating mantel/ transformer, 195 mL

toluene, 0.675 mL of 10 mM H_2PtCl_6 in 2-propanol (Speier's Catalyst), and 4.08 g of 98% pure 1,8-nonadiyne were transferred to the flask. The temperature was set to 70 ^{o}C and the reaction mixture was stirred. To ensure complete actiavtion of the new complex, the reaction was allowed to remain at this temperature for one hr. Completion of the first step resulted in formation of a clear solution.

Addtion of 5 g silica hydride (synthesized from the silanization reaction and stored in a vacuum oven at 120 ^{o}C) to the solution was the beginning of the second step of the hydrosilation reaction. The silica hydride addition to the flask was done gradually and in small portions with steady stirring. The residual silica was washed with 5 mL of toluene into the solution. The temperature was then raised to 100 ^{o}C where it was held for 96 hr. Upon completion of the reaction, the solid product was separated from the rest of the solution using a 90 M oven-dried sintered glass funnel. The final step was to wash the solid product with 25 mL of toluene at 90 ^{o}C three times, bringing the total volume to 75 mL, followed by 2 washes of 25 mL dietheyl ether. Upon completion of the filtration, the remaining washing solvent dietheyl ether was allowed to evaporate from the product by keeping the product at room temperature over night. Complete removal of excess solvents from the product was achieved by vacuum drying at 110 ^{o}C for 24 hr [27].

The characterization of 5.2735 g 1,8-nonadiyne bonded phase was done by three methods: FTIR, carbon analysis, and solid state NMR. The results will be discussed in the following sections. Theoretically, the hydrosilation of silica hydride using 1,8-nonadiyne could yield the following product as shown in reaction (9).

$$ \tag{9} $$

One possible structure
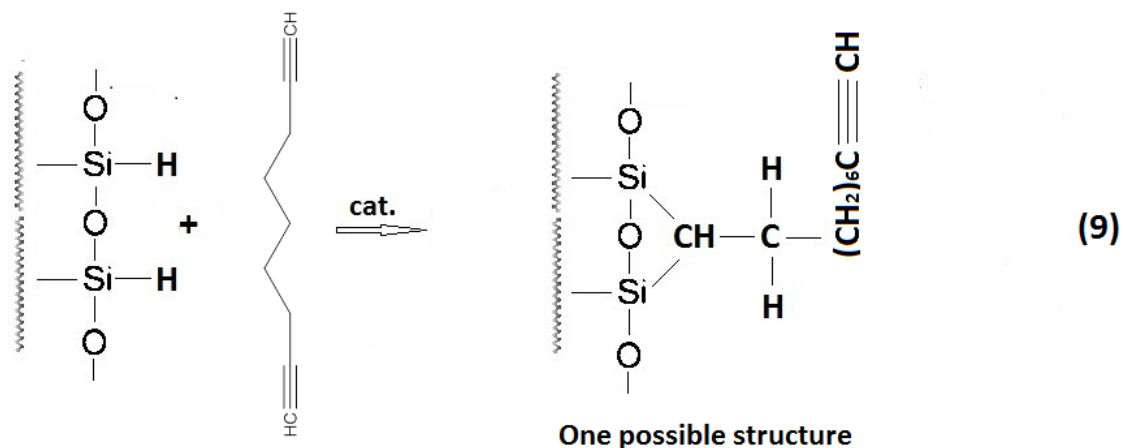
ii.   Preparation of *m*-diethynylbenzene bonded phase

The next organic group selected for a bonded stationary phase was *m*-diethynylbenzene- $C_6H_4(CCH)_2$. This compound with a molecular weight of 126.15 g/mol and the structure shown in Section B, was bonded using a hydrosilation procedure similar to that of 1,8-nonadiyene that was described in the previous section. For the experimental procedure, 5.0365g of *m*-diethynylbenzene was added into a three-necked round bottom flask containing 195 mL toluene and 0.675 mL H_2PtCl_6. The remainder of the *m*-diethynylbenzene was transferred into the flask using 5.0 mL washing toluene. Upon formation of the activated complex, 5.0 g silica hydride was added to the mixture. The solution was allowed to react for 96 hours at 100 $^{\circ}$C followed by washing and filtration [27]. The solid product weighed 5.3424 g. To characterize and confirm the presence of the bonded organic compound on the surface of the stationary phase, FTIR, solid state NMR, and carbon analysis were used.

iii.   Preparation of 5-cyano-1-pentyne bonded phase

5-Cyano-1-pentyne, $HCC(CH_2)_3CN$ with the formula weight of 93.13 g/ mol and structure shown in Section B, was bonded via hydrosilation. This compound (3.7200 g), as well as 0.675 mL Speier's catalyst, 200 mL toluene, and 5.0 g silica hydride were allowed to react for 96 hours at 100 ^{o}C. Following filtration, the solid product was washed three times with 75 mL (total volume) of toluene at 90 ^{o}C and two times with 50 mL (total volume) diethyl ether [27]. The final result was a yellow solid with a total weight of 4.9064 g. The newly synthesized stationary phase was characterized for the presence of 5-cyano-1-pentyne using FTIR, carbon analysis, and solid state NMR spectroscopy. Results will be discussed in the following sections.

iv.   Preparation of 6-chloro-1-hexyne bonded phase

The organic moiety 6-chloro-1-hexyne, $Cl(CH_2)_4CCH$, with a formula weight of 116.59 g/ mol and structure shown in Section B, was chosen to be bonded to the surface of silica hydride. Using 4.6500 g 6-chloro-1-hexyne, 0.675 of mL Speier's catalyst, 200 mL of toluene, and 5.0 g of silica hydride, the hydrosilation reaction proceeded under similar conditions as described in the above sections [27]. The result was the production of 5.3953 g of a new bonded phase material. FTIR, carbon analysis, and solid state NMR spectroscpy were used to characterize the synthesized 6-chloro-1-hexyne bonded phase material. Results will be discussed in the following sections.

v.   Preparation of 4-ethynylanaline bonded phase

The hydrosilation reaction for 4-ethynylanaline, C_8H_7N, an organic compound with a formula weight of 117.15 g/mol and structure shown in Section B, was bonded to the silica hydride according to the standard procedure as described in the earlier sections [27]. For this reaction, 4.68 g of 98% pure ethynylanaline was used and the result was the production of 0.1278 g of a yellow-colored solid. Similar characterization techniques, FTIR, carbon analysis, and solid state NMR spectroscopy were applied for verification of the newly synthesized bonded phase. Results will be discussed in the following sections.

3.   Column packing

Upon characterization and confirmation of the bonded stationary phases, the materials were subsequently utilized for column packing. The results obtained from FTIR spectorscopy and carbon analysis did not provide compelling evidence that *m*-diethynylbenzene had an acceptable surface coverage; hence the material did not appear to be a promising packing material. Column packing involves loading the synthesized bonded phase under high pressure into a stainless steel column for further chromatographic experiments.

The column packing process for 1.8029 g 1,8-nonadiyne bonded phase was performed at San José State University by Dr. Pesek. This process involved a Haskel pneumatic pump and methanol as the driving solvent. The role of methanol was to direct the packing material into the column as a very uniform dispersion of the particles.

1,8-Nonadiyne was suspended into approximately 30 mL CCl_4/ methanol followed by 10 minutes of sonication. The CCl_4 and silica's density are similar, and therefore choosing CCl_4 allowed for an easier particle suspension. The slurry was then added to a reservoir tube, and the remainder of the tube was filled with HPLC grade methanol. The slurry was forced from the reservoir tube into the stainless steel tube under high pressure (6000 psi) in the presence of nitrogen gas. The stainless steel column was purchased from Alltech (Deerfiled, IL). The reservoir was made out of stainless steel for pressure durability. The entire packing procedure took about 15 minutes.

The column packing for 5-cyano-1-pentyne, 6-chloro-1-hexyne, and 4-ethynylanaline was done by Stellar Phases, Inc. (Langhorne, PA). The column 5-cyano-1-pentyne with I.D.# 11-110A was made by packing the synthesized material in to a 75 mm x 4.6 mm stainless steel column under 800 psi at 1.0 mL/ min in 60/ 40 ACN/ H_2O. The number of theoretical plates for this column is 3052. The newly made bonded phase 6-chloro-1-hexyne was packed into a 75 mm x 4.6 mm stainless steel column with the I.D.# 11-110B under ~700 psi and 60/ 40 ACN/ H_2O solution. The number of theoretical plates for this column is 7077. The 4-ethynylanaline bonded phase was packed into a 75 mm x 4.6 mm i.d.column with I.D.# 11-110C stainless steel column using ~600 psi and 60/ 40 ACN/ H_2O. It was reported to have 3583 column theoretical plates with 1.26 asymmetry.

D.   Instrumentation

1.   Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy

DRIFT spectroscopy provides valuable qualitative information on the structure of the compound. The DRIFT characterization is obtained by verifying the characteristic bonds in the compound at a certain wavelength. Infrared radiation in the wavelength region of 4000 - 400 cm^{-1} is directed onto the powdered sample, and the diffusely reflected beam is collected into the spectrometer. The radiation absorption is characteristic of the vibrational modes of the functional groups in the compound. The absorptions are recorded by a detector and interpreted by a Fourier transform function in the form of spectra [28].

The equipment used for the qualitative analysis portion of this study was an ATI Mattson Infinity™ series FTIR with a deuterated triglycine sulfate (DTGS) detector. Each sample was prepared separately by mixing the compound with KBr in 95:5 mg ratio. The mixture was kept in a heated oven (110 ^{o}C) for 24 hours. For the final preparation, the mixture sample was turned into a fine powder using a mortar and pestel for grinding. Each sample was put into a diffuse reflectance cup, a round hollow container 3 mm in diameter and 2 mm depth, and evened out on the surface by using a spatula. Prior to the actual run, the instrument was purged with N_2 gas for approximately 20 minutes which removed unwanted peaks due to the presence of moisture and atmosphoric CO_2. The infrared region at which the spectra were analyzed and recorded was 4000 — 400 cm^{-1}.

2.   Elemental (Carbon) Analysis

It is important to evaluate the percentage of carbon on the surface of the newly synthesized stationary phases since it determines the surface coverage of the bonded phase. The surface coverage provides quantitative information about the organic groups on the surface of the silica hydride. The Berendsen and de Galan equation (3), shown below, provides the direct relationship between the two parameters, carbon percentage and surface coverage of the bonded phase.

$$N = \frac{10^6 P_c}{1200\, N_c - P_c\,(M-1)} \cdot \frac{1}{S} \tag{3}$$

In the above equation, P_c represents the difference in the percent of carbon between the bonded phase and silica hydride, obtainable through carbon analysis. N_c is the number of carbons in the bonded organic group, obtained from the molecular formula of the substrate. M is the molecular mass of the bonded molecule, and S is the Astrosil surface area in m^2/g, a value generally provided by the manufacturer (350 m^2/g for this study) [29].

The carbon analyses of the samples were provided by Columbia Analytical Services, Inc. (Tuscon, AZ). The results will be discussed in the next section.

3.   Nuclear Magnetic Resonance (NMR) Spectroscopy

One of the most powerful techniques in characterization of the compounds is nuclear magnetic resonance spectroscopy. NMR sepctroscopy is a special type of absorption spectrometry. The fundamental mechanism behind the technique relies on

electromagnetic radiation absorption by the sample in an appropriate magnetic field. The absorption patterns of particular nuclei in the sample are characteristic of the compound structure [28].

For this particular study, samples were analyzed using solid-state NMR spectroscopy (CP-MAS ^{29}Si NMR and ^{13}C NMR). Solid-state NMR provides detailed information in characterization of organic- inorganic (hybrid) materials. In addition to identifying the atomic environments, this technique can provide spatial details between various sites [30]. For samples dissolved in a solution (slurry), NMR spectra consist of very sharp peaks due to averaging of anisotropic NMR interactions by rapid random spinning. In solid state NMR, however, similar nuclei are relatively immobilized in many orientations with respect to the magnetic field, hence causing a peak broadening effect. The term chemical shift anisotropy (CSA) refers to orientation dependent interactions in solid-state NMR [31]. In order to mimic the sharp peaks generated from solution NMR in solid-state NMR, samples are spun at a high speed and at a specific angle, 55°, with respect to the magnetic field, called the magic angle. Magic angle spinning, MAS, stimulates the natural tumbling of the solution-phase molecules and results in the formation of sharper peaks. ^{29}Si, with 4.7% natural abundance, allows a more sensitive environment (2.1 times) than ^{13}C but has a longer spin relaxation time [32]. The low abundance of certain nuclei could be the cause for their low sensitivity and therefore their inability to provide structural information when using solid-state NMR. To resolve this problem in solid-state NMR, the cross polarization (CP) technique is found to be helpful. In cross polarization the frequency of the less sensitive nucleus

matches with the sensitive proton. This would increase the nucleus sensitivity

significantly. Applying the CP technique decreases the amount of time taken to achieve a

spectrum [31]. Single pulse ^{29}Si magic angle spinning (MAS) provides information

about Si atoms in the compound [33]. The peak broadening in solid-state NMR can

provide useful information on chemistry, structure, and dynamics of the compound in the

solid state [34].

The solid-state NMR for this study was done by the collaborative efforts of

Professor Klaus Albert, *et al.* at Tubingen University in Germany. The spectra were

obtained by utilizing MAS combined with CP. The characterization required

approximately 150 mg of each sample that was packed into a ZrO_2 rotor and spun at an

angle of 55° with respect to the magnetic field with a CP contact time of 5 ms.

4.   High Performance Liquid Chromatography (HPLC)

The HPLC instrument used initially, and mainly for the 1,8-nonadiyene column,

was a Shimadzu SPD-6A, as pictured in Figure 7. The system was equipped with a

multi-channel interface, a Waters in-line degasser, and a Rheodyne Model 1726 manual

injector with a 20 µL injection loop and a UV detector. The instrument was connected to

an HP AD converter, which in turn was connected to an HP Deskjet printer. The

software for collecting the data on this equipment was Chemstation. The injections were

done manually using a glass syringe. The 25.0 µL glass syringe used in this experiment

was a GASTIGHT® manufactured by Hamilton, Co. (Reno, NV).

Prior to the runs, the instrument was set for a flow rate of 1.0 µL/ min, and the

wavelength was set for 254 nm. The 1,8-nonadiyne column was flushed with pure

methanol for 30 minutes before the experiment. In order to achieve a wide range of

hydrophilicity/ hydrophobicity in the mobile phase, HPLC grade acetonitrile and MilliQ

water were mixed in a wide range of concentrations from a 10:90 to a 90:10 ratio. For

mobile phases with an additive, 0.1 volumetric percent of formic acid was used in the

mobile phases. All mobile phases were filtered using vacuum filtration. The samples

were prepared by dissolving 1 mg of the sample in 1 mL of acetonitrile: H_2O (in a 50:50

ratio) with 0.1 volumetric percent of formic acid. All sample solutions were filtered with

a 20 µm nylon membrane.

Figure 7. A view of the Shimadzu SPD-6A

The liquid chromatographic studies of the rest of the columns for this research were performed using an HP Autosampler 1090 Series II. The instrument was equipped with a cabinet for a three solvent system, heated oven compartment, and an HP-IB interface board. The detector was a UV/Vis scan type or diode array detector (DAD). The instrument had a DR5 solvent delivery system which allowed the system to utilize from one to three solvent channels at any given time. This delivery system consisted of a solvent reservoir and a dual-syringe metering pump with a rotary valve, which allowed chromatographic performance in the isocratic mode (binary or ternary) as well as gradient elution. The HP 1090 had a solvent degassing nitrogen inlet line facilitating the removal of other gases dissolved in the solvents. The sampling system was auto-injection based with a 20 sample tray.

The machine was also equipped with a manual injector which facilitated a controlled single injection, whereas the auto injection increased the speed of the process as it can be programmed to perform up to 99 injections in a sequence. The sample injection valve was a six-port rotary valve designed for a high performance liquid chromatograph. The binary pump in HP 1090 had a very short delay time, comparable with the best pumps in the industry.

Upon an injection, an air actuator rotated the valve, and the solvent moved directly into the column as well as the injector needle which was connected to the syringe [34]. The nitrogen gas tank was from Praxair, UN 1066 N1 861090103. The data-handling device was HP-LC Chemstation software (Pascal series) with HP 3392A

integrator, HP ThinkJet printer, HP PaintJet printer. The HP 1090 used for column characterizations and analyte retention studies is shown in Figure 8.
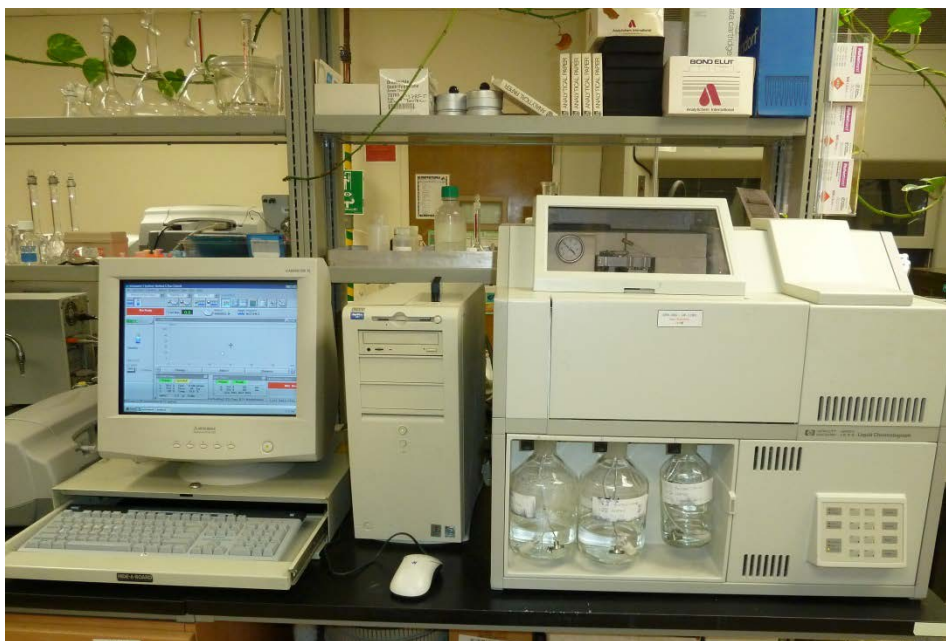
Figure 8. A view of the HP 1090 HPLC system

The mobile phase was vacuum filtered with a 0.2 µm Nylon 66 membrane (Alltech Associates, Inc. Deerfield, IL) to enhance purity of the solvent before entering the instrument. The flow rate when using the autosampler was 0.5 mL/ min.

The columns were flushed with 100% methanol for 1.0 hour as a means of preparation before the actual study. The instrument was set for two wavelengths of 214 nm and 254 nm for optimum detection of all analytes using the HP 1090 system.

III. RESULTS AND DISCUSSIONS

A. DRIFT Spectroscopic Characterization

1. Silica hydride characterization

The first step before performing multiple synthetic processes was to ensure the replacement of the Si—OH bonds by Si—H on the surface of silica via a silanization reaction. DRIFT spectroscopy confirmed the formation of the Si—H bonds on the surface. As shown in Figure 9, DRIFT results showed a prominent peak at 2250 cm^{-1}, a region which represents the Si—H stretching vibration. The presence of a broad peak in the 3000 — 3750 cm^{-1} region can be explained by the presence of H_2O molecules. The presence of a peak in near 3750 cm^{-1} region is due to OH stretching vibrations from the silanol groups. All other peaks pertain to the presence of siloxane backbone in the compound.

2. Moiety surface characterization

i. 1,8-Nonadiyne

Following the silanization reaction, hydrosilation of the 1,8-nonadiyne produced a bonded stationary phase on the surface of silica hydride. DRIFT spectroscopy results for 1,8-nonadiyne, as shown in Figure 10, confirmed the bonding of the organic moiety on the surface of the stationary phase. The significant size reduction in the Si—H stretching bond region, 2200 cm^{-1}, indicated that the surface was not exculsively populated by Si—H, hence confirming the presence of another group on the surface.
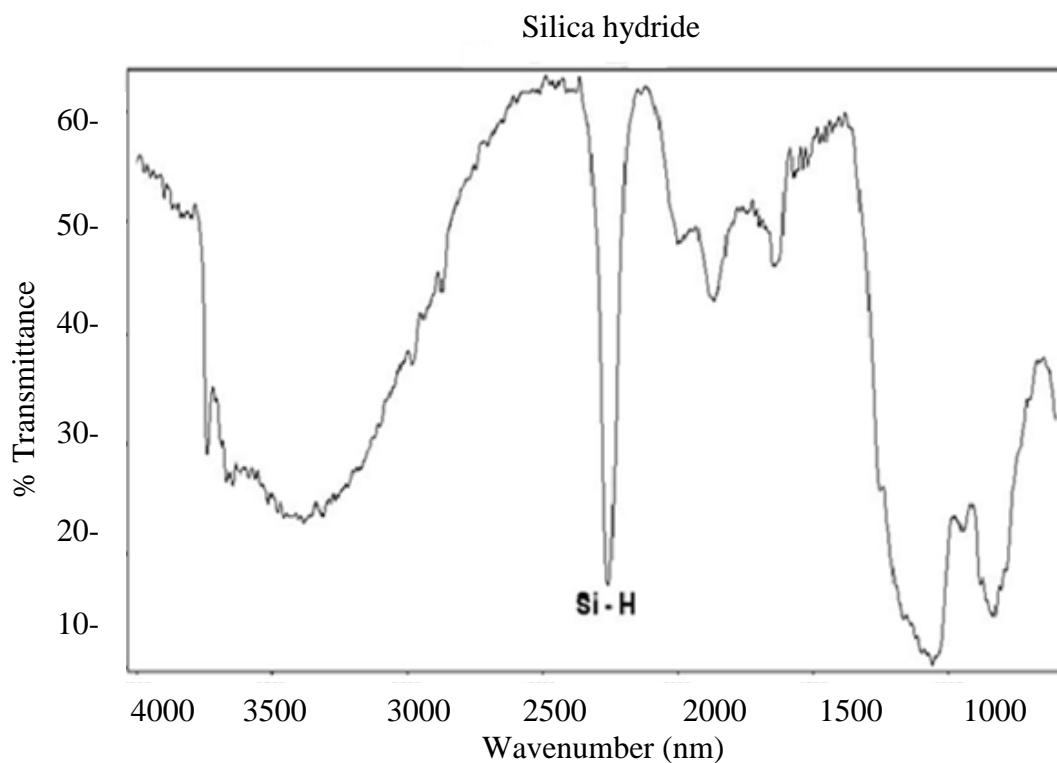
Figure 9. DRIFT spectrum for silica hydride identification

Aditionally, the presence of peaks at approximately 2800-3000 cm^{-1} region represents C—H stretching bands which is indicative of an organic moeity, 1,8-nonadiyne, for this experiment. Moreover, the sharp peak near 1700 cm^{-1} was indicative of the presence of a C≡C bond which is consistent with the organic compound structure.

ii.   *m*-Diethynylbenzene

The DRIFT spectrum for *m*-diethynylbenzene, as shown in Figure 11, showed a Si—H peak size reduction. However, there were no other significant characteristic peaks that could verify either the attachment or significant surface coverage by the moiety.

Further characterization techniques are needed to confirm or deny the hydrosilation of *m*-diethynylbenzene.
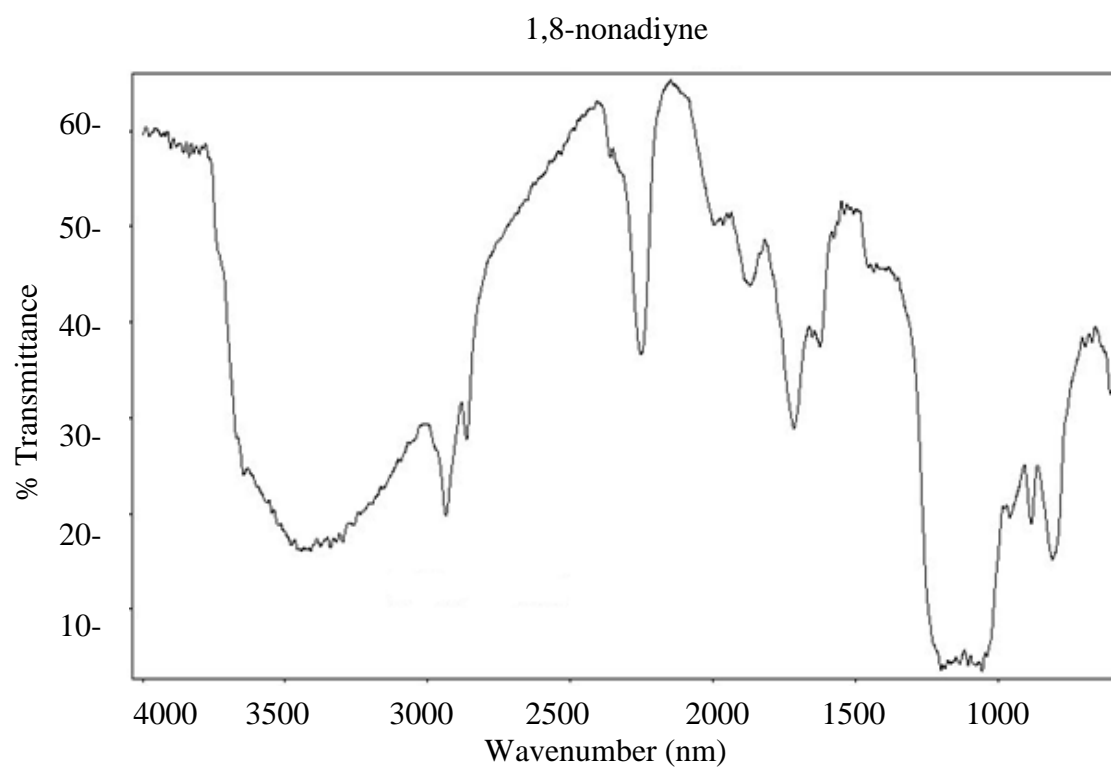
1,8-nonadiyne

Figure 10. DRIFT spectrum of 1,8-nonadiyne bonded phase

m-diethynylbenzene
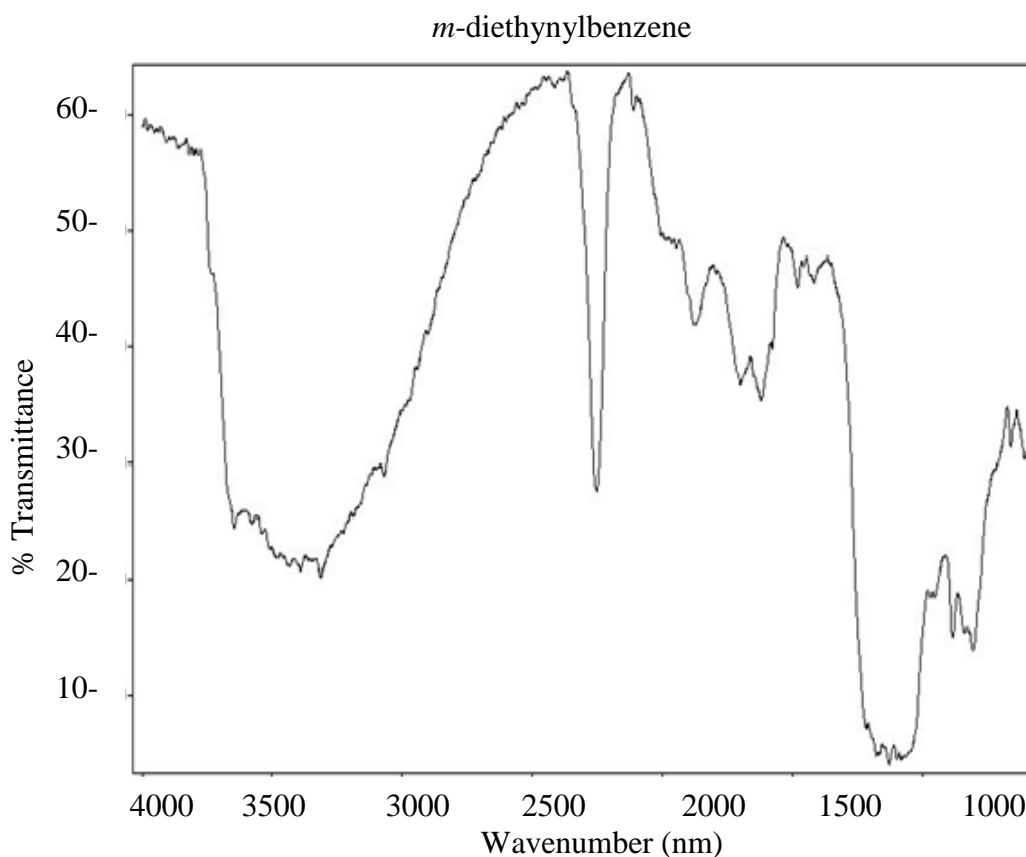
Figure 11. DRIFT spectrum for *m*-diethynylbenzene bonded phase

iii.   5-Cyano-1-pentyne

Figure 12 shows the DRIFT spectrum for the 5-cyano-1-pentyne stationary phase. The insignificant size reduction in the Si—H peak might be interpreted as low to zero surface coverage by the moiety. However, the presence of other characteristic peaks in the spectrum confirmed the formation of the 5-cyano-1-pentyne bonded phase. For instance, the presence of a peak at 1639 cm^{-1} could represent the C=C stretching vibrations for the structures bonded to the silica hydride surface through the C≡N functional group of 5-cyano-1-pentyne. This could also explain the unusually high

intensity peak at 2240 cm^{-1} (the Si—H region), (i.e., the peak was an overlap of the Si—H and C≡N stretching peaks).
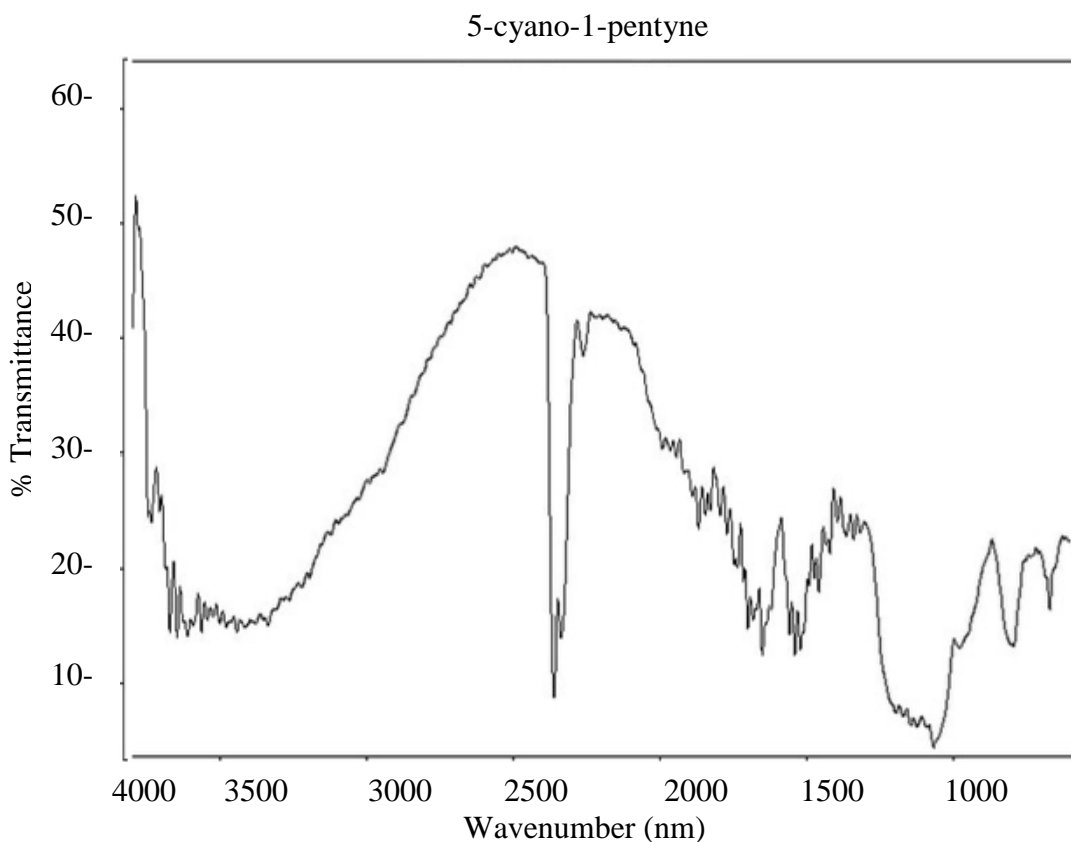
5-cyano-1-pentyne

Figure 12. DRIFT spectrum of 5-cyano-1-pentyne bonded phase

iv.   6-Chloro-1-hexyne

The DRIFT spectrum for 6-chloro-1-hexyne confirmed the attachment of the moiety to the silica hydride surface. As shown in Figure 13, a significant Si—H peak size reduction at 2240 cm^{-1} was indicative of the surface coverage by 6-chloro-1-hexyne. In addition, the presence of peaks at 2800-3000 cm^{-1} are the result of C—H stretching

bands of the organic moiety. Other smaller peaks in the spectrum could be due to the

fundamental vibrations of organic functional groups and the siloxane backbone. A broad

peak in the 3100 – 3700 cm^{-1} region is due to the presence of adsorbed water on the

surface of the solid phase.
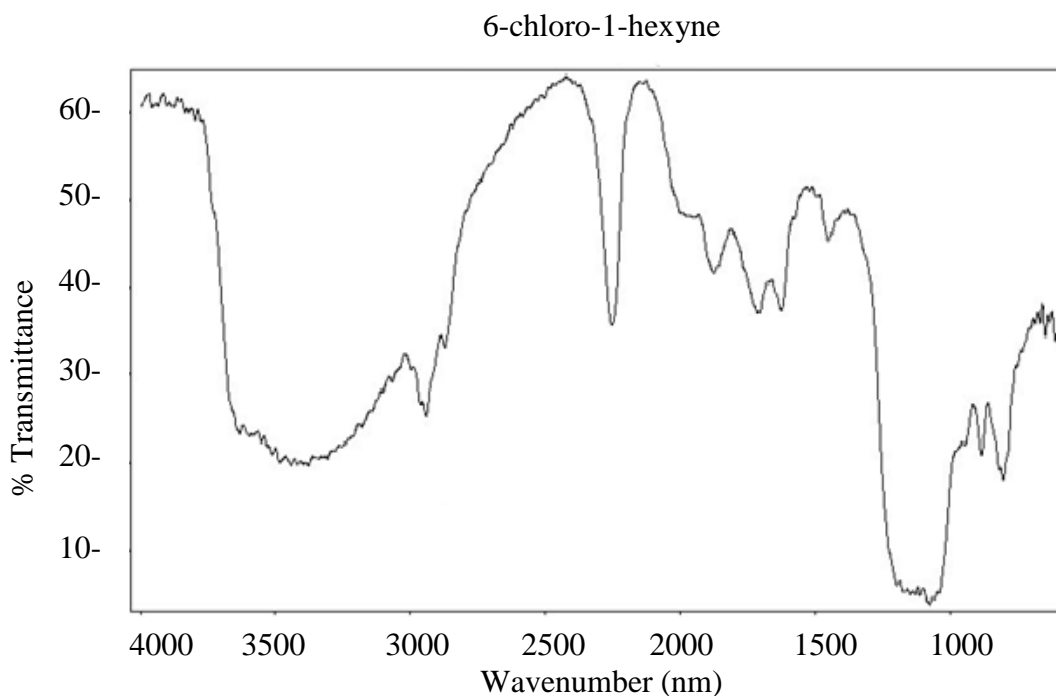
6-chloro-1-hexyne

Figure 13. DRIFT spectrum of 6-chloro-1-hexyne bonded phase

v.   **4-Ethynylanaline**

The IR spectrum for 4-ethynylanaline, as shown in Figure 14, confirmed the

bonding of the organic moiety to the silica hydride surface. The significant reduction in

the Si—H stretching peak intensity near the 2200 cm^{-1} region is indicative of successful

bonding. Additionally, the aromatic C=C stretching vibration appeared as a peak with

medium intensity at 1500 – 1600 cm^{-1}, another important characteristic peak to confirm

the attachment to the silica hydride surface of 4-ethynylanaline.
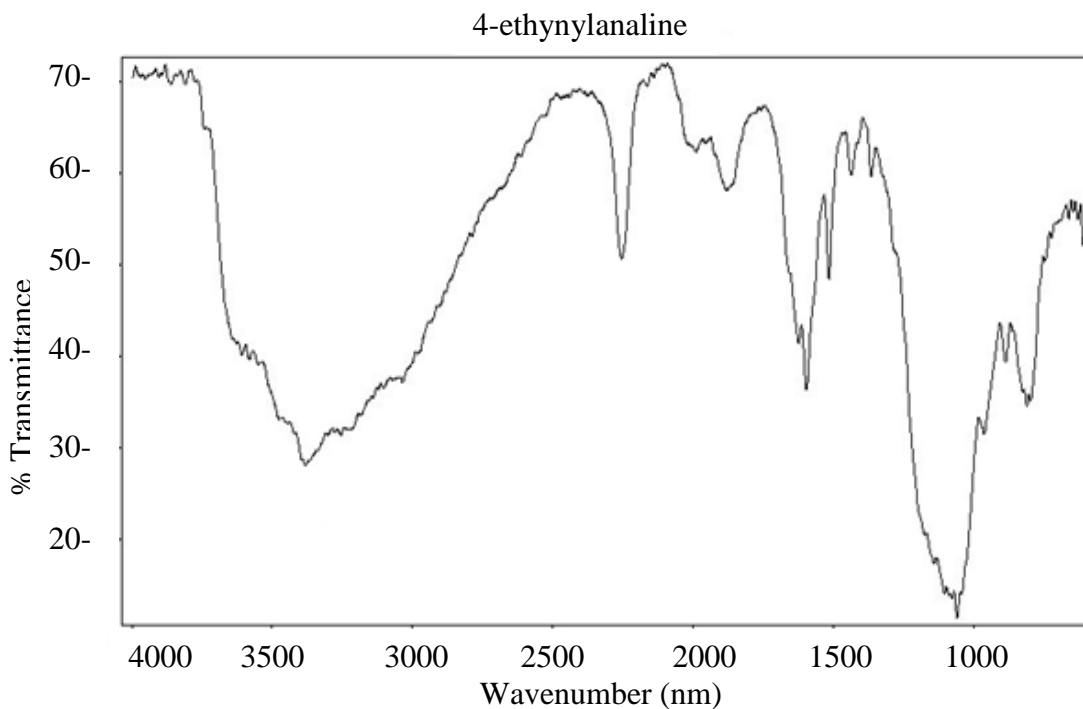
4-ethynylanaline

Figure 14. DRIFT spectrum of 4-ethynylanaline bonded phase

B.   Carbon analysis

Quantitative analysis of organic moieties on the surface of silica hydride can be

obtained through carbon analysis. Carbon analysis provides valuable information about

the carbon load (percentage) and surface coverage of the organic moiety. Surface

coverage plays an important role in the retention time, selectivity, and stability of the

bonded phase. The physical structure of the substrate is a significant factor on the surface

area of the packing material. In addition, the surface area is inversely related to the pore

size of the packing material. Substrates with higher surface area provide greater capacity

and longer analyte retention times. Conversely, substrates with lower surface area can be

used for larger molecules when a faster equilibration time is desired. Surface coverage is

calculated from the carbon load and surface area. Carbon load represents the amount of

the bonded phase on the surface of silica. Higher carbon loads means greater column

capacities and resolution.

The results obtained from the carbon analysis confirmed that *m*-dimethylbenzene

did not show acceptable carbon percentage, and therefore it was removed from the rest of

the study. Table 10 shows the carbon analysis values for all of the other four synthesized

stationary phases. The surface coverage value for each bonded phase was calculated

using the Berendsen and de Galan equation, as explained in the previous chapter.

Table 10. Carbon analysis of the newly synthesized bonded phases.

Compound	Bulk carbon (%)	Surface area (g/m^2)	Surface coverage (μmol/m^2)
1,8-Nonadiyne	7.480	350.00	2.157
5-Cyano-1-pentyne	1.520	350.00	0.615
6-Chloro-1-hexyne	6.180	350.00	3.034
4-Ethynylanaline	12.750	350.00	4.487

C.   NMR Characterization

1.   Solid-state NMR (SS-NMR): Characterization with ^{29}Si CP/ MAS and ^{13}C CP/MAS NMR

i.   1,8-Nonadiyene

Figure 15 is the ^{29}Si CP/ MAS NMR spectrum for the 1,8-nonadiyne bonded phase. The main characteristic in spectra like this one is the Si—H peak size at 85 ppm. The presence of an organic moiety on the surface of a silica hydride base would cause a decrease in the intensity of the peak at 85 ppm when compared with that of a non-bonded stationary phase. Naturally, the more populated the surface, the less free Si—H on the stationary phase and the smaller the 85 ppm peak would be.

The Si—H peak size reduction at 85 ppm observed for 5-cyano-1-pentyne is shown in Figure 17. The same effect is observed for 6-chloro-1-hexyne and 4-ethynylanaline, as shown in Figures 19 and 21, respectively.
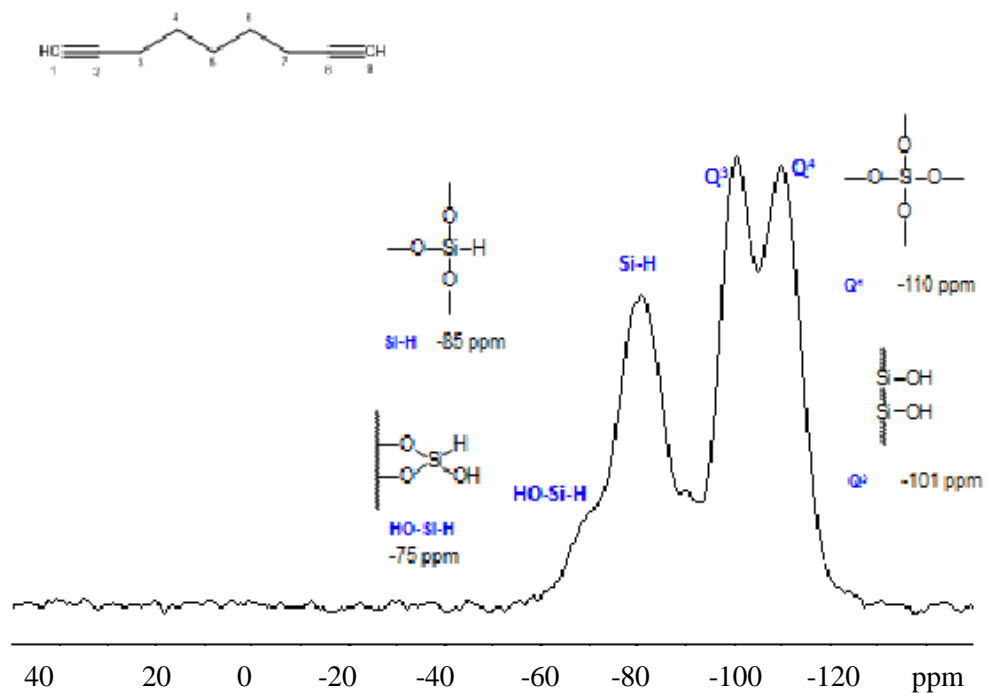
Figure 15. ^{29}Si CP/ MAS NMR spectrum for the 1,8-nonadiyne stationary phase
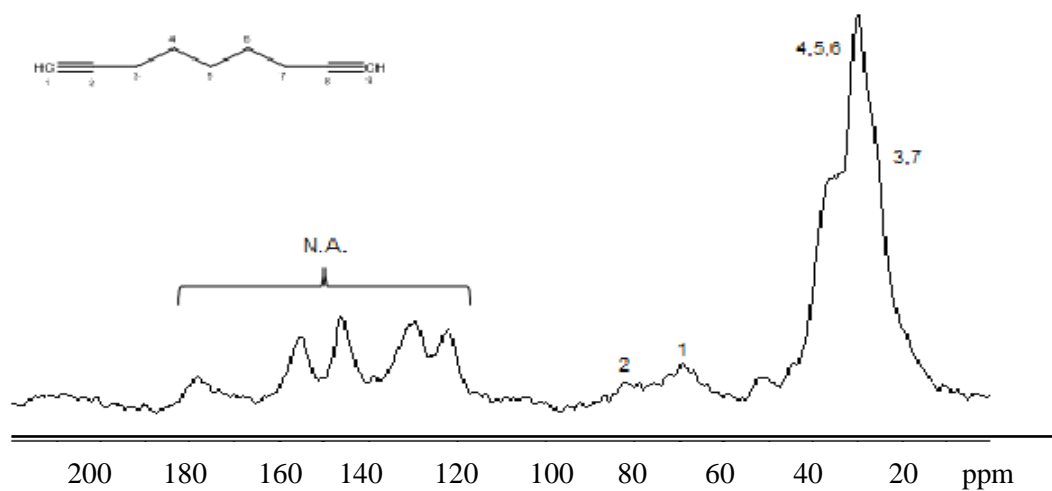
Figure 16. ^{13}C CP/ MAS NMR spectrum for the 1,8-nonadiyne stationary phase

The ^{13}C CP-MAS NMR spectrum is another means to verify the bonding of the organic moiety by hydrosilation. Figure 16 shows the ^{13}C CP-MAS NMR spectrum of the 1,8-nonadiyne bonded phase. The appearance of peaks in the low ppm region (29.5 - 35.5 ppm) is characteristic of the saturated alkane 1,8-nonadiyne, (i.e., carbons 3, 4, 5, 6, and 7 specifically). The non-bonded terminal alkynes (1, 2) appeared further up in the spectrum (69.4- ~85.0 ppm). The ^{13}C CP-MAS-NMR showed an interesting feature about another possible form of attachment of 1,8-nonadiyne to the surface. The presence of certain peaks at higher ppm (121.9 – 178.5 ppm) raises the possibility of formation of a bridge-like structure, where 1,8-nonadiyne could bond to the surface from both ends (terminal alkynes), hence the formation of alkenes in the structure of the bridge.

ii.   5-Cyano-1-pentyne

A significant decrease in the Si—H peak intensity at 85 ppm in ^{29}Si CP-MAS NMR, as well as the presence of a sharp C≡N peak in the high-chemical-shift region (120 ppm) of ^{13}C CP-MAS NMR, confirmed the formation of the 5-cyano-1-pentyne bonded stationary phase. Figures 17 and 18 represent the ^{29}Si and ^{13}C CP-MAS NMR spectra for the cyano bonded moiety, respectively.
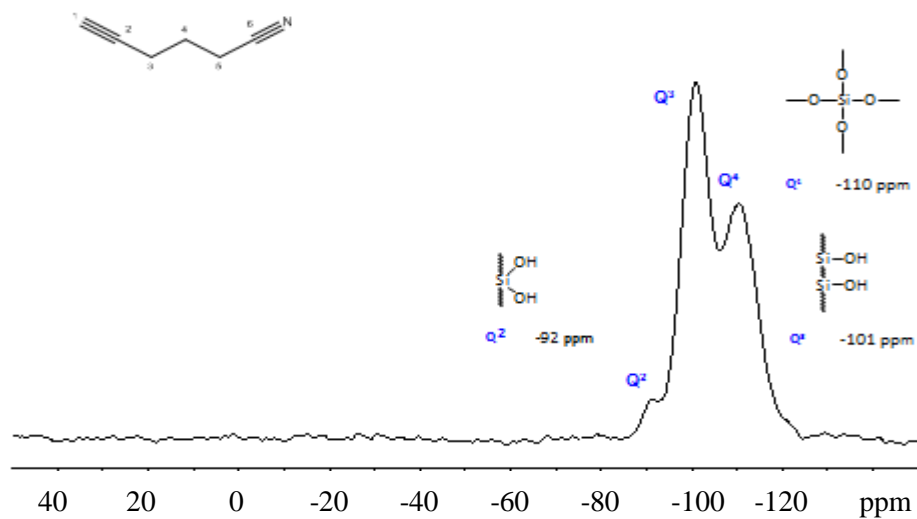
Figure 17. ^{29}Si CP/ MAS NMR spectrum for the 5-cyano-1-pentyne stationary phase
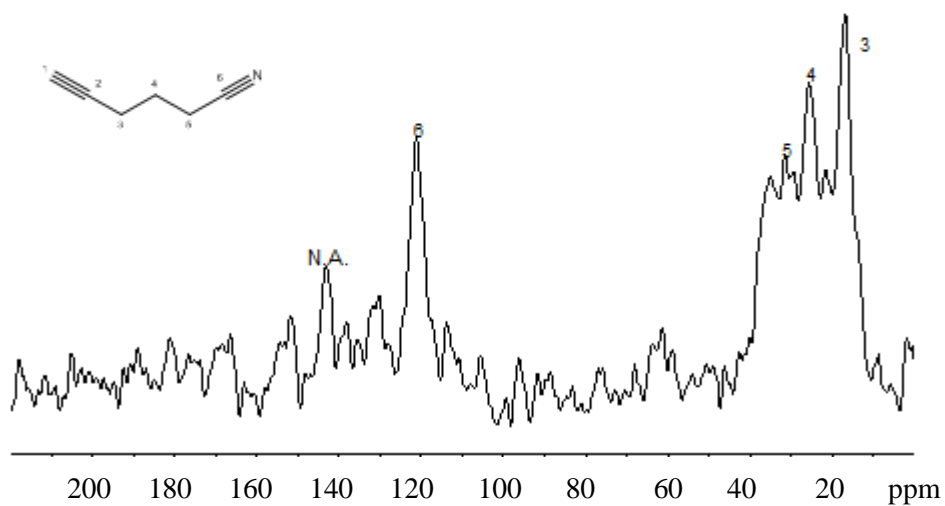
Figure 18. ^{13}C CP/ MAS NMR spectrum for the 5-cyano-1-pentyne stationary phase

iii.   6-Chloro-1-hexyne

The ^{29}Si and ^{13}C CP/ MAS NMR for the 6-chloro-1-hexyne stationary bonded phase confirmed the attachment of the moiety to the surface of silica hydride. Figures 19 and 20 are the ^{29}Si and ^{13}C CP/ MAS NMR spectra, respectively. The lower intensity peak at -85 ppm in ^{29}Si CP/ MAS NMR is indicative of bonding of the organic moiety to the silica hydride surface. The ^{13}C CP/ MAS NMR spectrum shows the presence of peaks at the high-chemical-shift region between 122.2 to 155.2 ppm that represents the olefinic carbons in the 6-chloro-1-hexyne molecule.
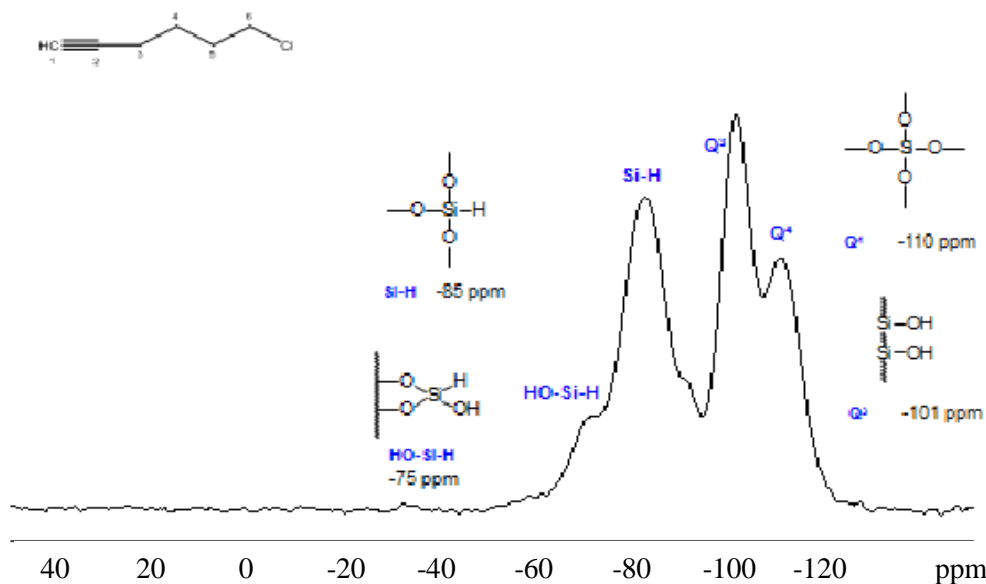
Figure 19. ^{29}Si CP/ MAS NMR spectrum for the 6-chloro-1-hexyne stationary phase
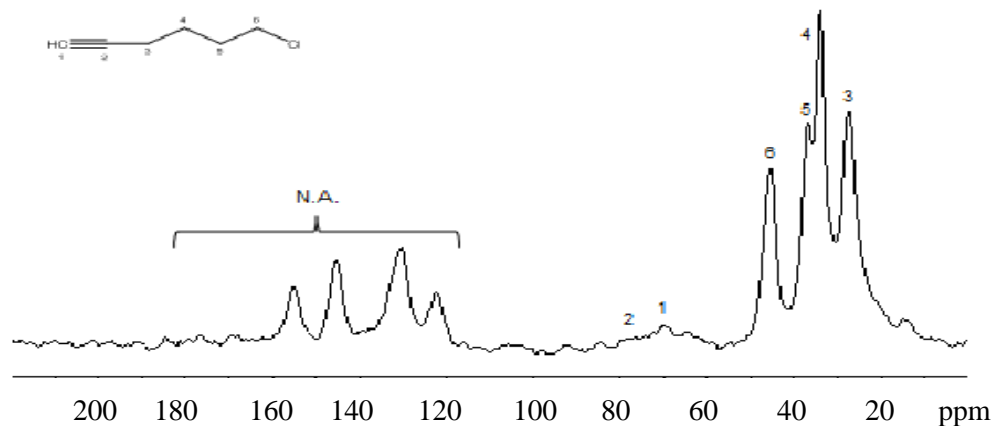
Figure 20. ^{13}C CP/ MAS NMR spectrum for the 6-chloro-1-hexyne stationary phase

iv.   **4-Ethynylanaline**

The peak size decrease at 85 ppm in ^{29}Si CP/ MAS NMR spectrum for

4-ethynylanaline confirmed the bonding of the organic moiety on the silica hydride

surface. The presence of characteristic peaks, i.e. aromatic carbons between 116.3 to

154.3 ppm, as well as unsaturated carbons peaks at the high-chemical-shift region of

200.6 ppm in ^{13}C CP/ MAS NMR, validated the attachment of the organic moiety onto

the silica hydride surface.
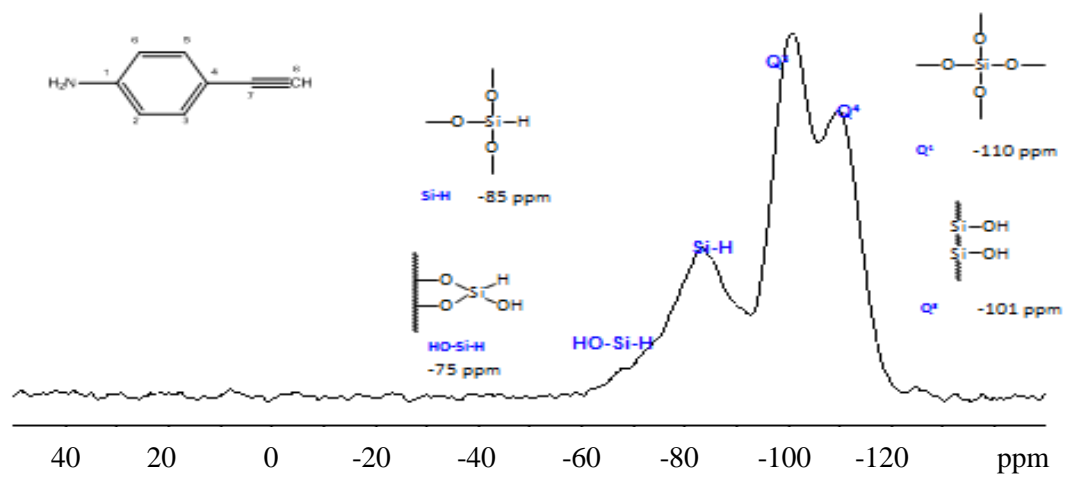
Figure 21. ^{29}Si CP/ MAS NMR spectrum for the 4-ethynylanaline stationary phase
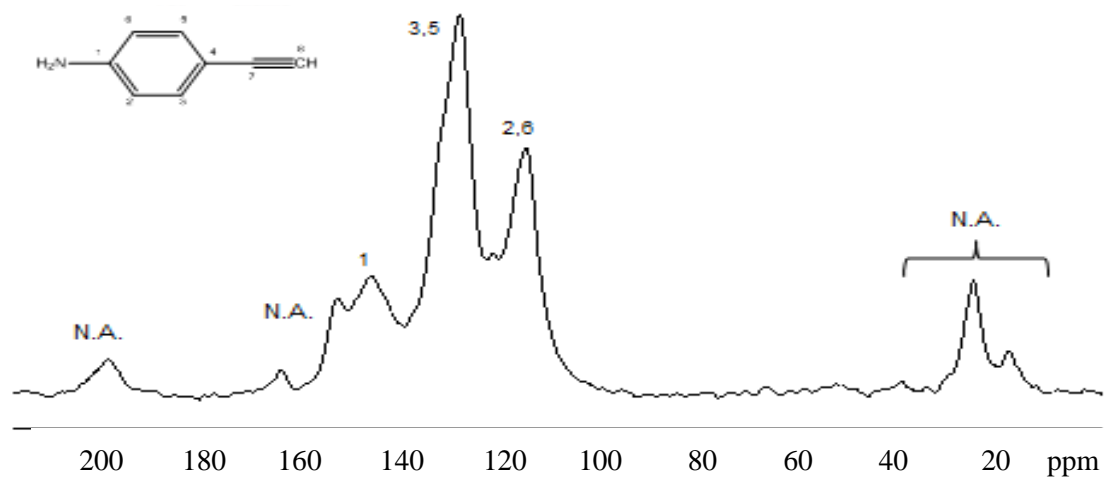
Figure 22. ^{13}C CP/ MAS NMR spectrum for the 4-ethynylanaline stationary phase

D. **HPLC Chromatographic Evaluation of the Columns**

To evaluate the newly synthesized bonded stationary phases and obtain a

retention map for each column, a series of chromatographic evaluations was performed

on the columns. To achieve this goal, a wide selection of analytes ranging from those

that exhibit ANP behavior (hydrophilic molecules such as amino acids, nucleic bases,

neurotransmitters) to analytes with RP characteristics (hydrophobic compounds such as

steroids and polycyclic aromatic hydrocarbons) was chosen for retention behavior

studies. The binary mobile phase for these evaluations was HPLC grade acetonitrile

(ACN)/ MilliQ DI H_2O. The ratio of the two components of the mobile phase was

altered at each step in an isocratic fashion.

1. **1,8-Nonadiyne**

The column was first washed with methanol: water (50 : 50) to remove unwanted

peaks produced by impurities. The flow rate was optimized to 0.5 mL/ min, and the

injection volume was set to 0.2 µL for all the samples.

i. **Hydrophilic analytes**

The retention behaviors of the analytes that exhibit ANP behavior used for the

evaluation of 1,8-nonadiyene are listed in the following tables: 11, 12, 13. The dissolving

solvent was (50:50) acetonitrile : DI H_2O with 0.1% by volume of formic acid. All solid

samples were weighed exactly to maintain the 1.0 mg of analyte per 1.0 mL of solvent

ratio. However, there were several samples with which the column experienced

overloading, an unwanted phenomenon produced by an excessive concentration of the

solute in the solution. Overloading the column could result in peak shape distortions. To

solve this problem the analytes were diluted at least once, and in some cases twice, by a

(10:90) solute to solvent ratio. The chromatographic runs began in the RP mode which

means a high percentage of water at the beginning followed by a gradual increase in the

concentration of the less polar component (ACN). The retentions times for the two

amino acid analytes are listed in Table 11.

Table 11. Retention times of the hydrophilic amino acids using the 1,8-nonadiyne
column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Phenylglycine	Phenylalanine
10	90	2.9000	3.9510
20	80	2.8290	3.6250
30	70	2.6969	3.2900
40	60	2.3200	2.9850
50	50	2.1930	2.9170
60	40	2.2840	3.0690
70	30	2.3430	3.9150
80	20	2.7180	6.1870
90	10	*	*

* peak was not observed

Figure 23 shows the retention maps for the hydrophilic amino acids, phenylalanine and phenylglycine (♦ and ▲, respectively). According to these maps, as the polarity of the mobile phase decreases, i.e. from 50% to 80% ACN, the retention time for polar compounds increases (typical ANP behavior). Inversely, the retention time of the compounds decreases when the mobile phase becomes more hydrophilic, (i.e., above 50% H_2O, showing the RP behavior). The ANP behavior of the analytes is a result of various interactions between the analyte and polar moieties of the stationary phase. The pivotal point for the column, (i.e., switching from RP mode to ANP) is around 50% ACN. Therefore, if the compound carries both hydrophilic and hydrophobic properties, it will be retained on a silica hydride column. Both phenylalanine and phenylglycine are polar and have ANP behavior on the column in the presence of a relatively non-polar mobile phase.

Theoretically, for a column with dual properties (RP and ANP) and a mobile phase composed of binary solvent system (polar and non-polar) the column behaves in RP mode at lower concentrations of non-polar solvent and vice versa. The behaviors were observed for both phenylalanine and phenylglycine amino acids when the %ACN shifts from low to high, forming a curve with a U-shape property.

The next group of hydrophilic compounds to be analyzed under RP/ ANP conditions was the two nucleobases, adenine and guanine. According to Table 12, both guanine and adenine have reasonable retention and show noticeable ANP behavior on this column. The retention for adenine is more significant than that of guanine. On a side note, guanine was very difficult to dissolve in this diluent. In order to dissolve this

compound, an additional amount of water, followed by warming the solution, was required. The actual retention behaviors of adenine and guanine (▲ and ⋈, respectively) with 1,8-nonadiyne column are shown in Figure 24.
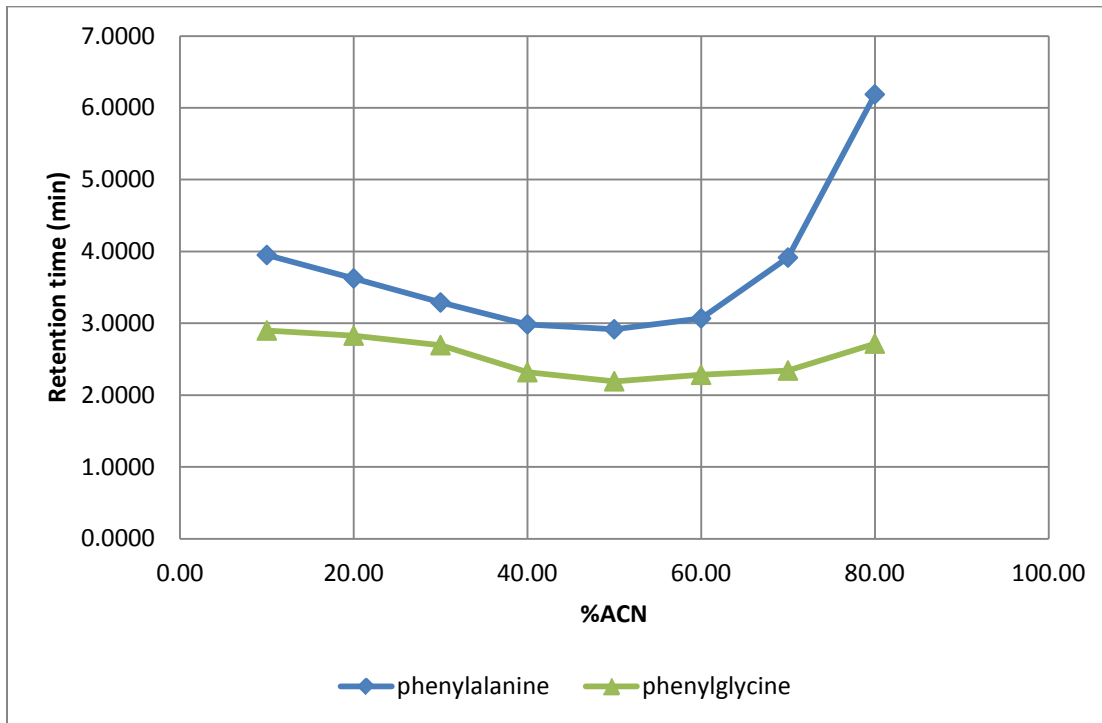
Figure 23. Retention map of the amino acids, phenylalanine and phenylglycine, using the 1,8-nonadiyne column

Table 12. Retention times of the hydrophilic nucleobases using the 1,8-nonadiyne column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	**DI water**	**Adenine**	**Guanine**
10	90	*	*
20	80	*	5.0410
30	70	12.2460	3.9740
40	60	8.2220	3.4120
50	50	5.8610	3.6345
60	40	6.0850	3.9070
70	30	8.3130	4.2811
80	20	16.1518	5.8140
90	10	*	*

* peak was not observed
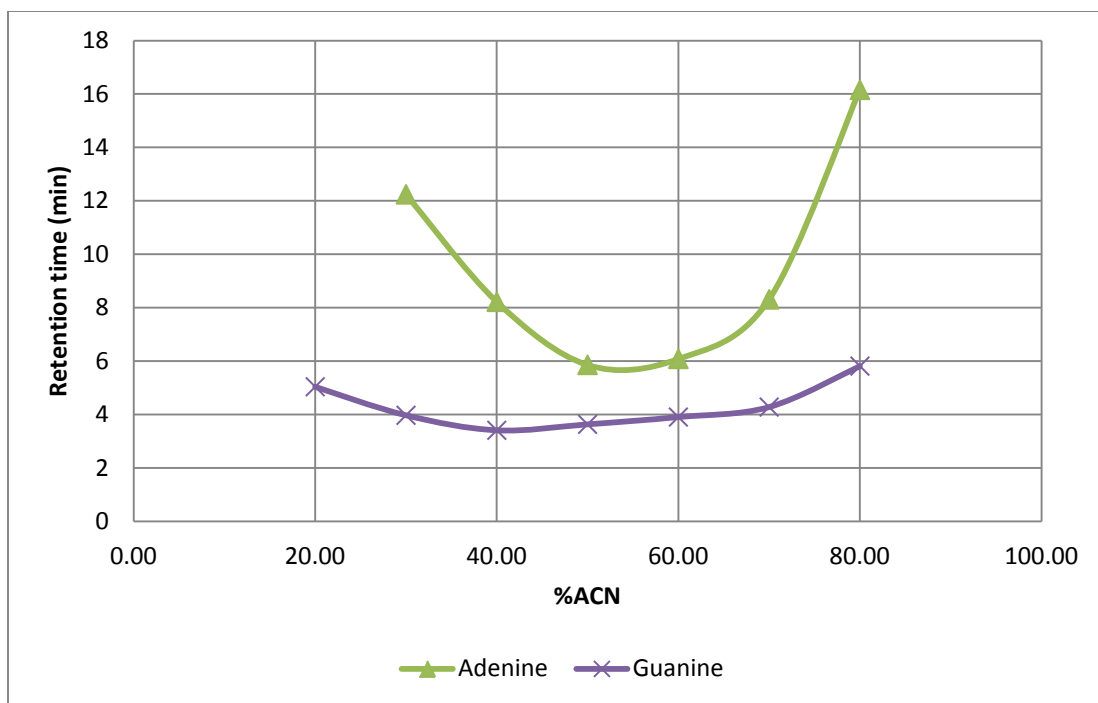
Figure 24. Retention map of the nucleobases, adenine and guanine, using the 1,8-nonadiyne column

According to the retention maps for the two nucleobases, adenine and guanine, the 1,8-nonadiyne bonded stationary phase shows RP and ANP retention for these polar compounds.

Neurotransmitters are the next group of polar analytes used for the characterization of the 1,8-nonadiyne column. Epinephrine, norepinephrine, and dopamine all show some ANP behavior on the column under the less-polar conditions (higher concentrations of ACN) of the mobile phase. Table 13 shows the retention times of these analytes.

Table 13. Retention times of the hydrophilic neurotransmitters using the
1,8-nonadiyne column.

Concentration of the mobile phase (%)		Retention time (min)		
Acetonitrile	DI water	Epinephrine	Norepinephrine	Dopamine
10	90	2.8290	2.3800	2.8490
20	80	2.7330	2.3560	2.7320
30	70	2.4090	2.3070	2.4120
40	60	2.2930	2.3030	2.2920
50	50	2.1760	2.1750	2.1540
60	40	2.2870	2.2800	2.2410
70	30	2.3880	2.3360	2.3370
80	20	3.2320	2.6720	2.3423
90	10	4.0120	*	2.3510

* peak was not observed

a. Reproducibility study (%RSD)

The reproducibility study was conducted for this column using two amino acids,
phenylalanine and phenylglycine, in 70:30 ACN:DI H_2O mobile phase. Ten consecutive
injections of the analytes resulted in retention times with close proximity, as shown in
Table 14. The % RSD values calculated and reported in Table 14, as well. The obtained
%RSD values range for this column is 0.3347-0.6111 which is less than 1.0. The

significance of this range verifies the high degree of precision in the 1,8-nonadiyne

bonded stationary phase performance when obtaining the ANP retention data.

Table 14. Reproducibility for 10 injections of two amino acids using the 1,8-nonadiyne bonded stationary phase.

Number of injections	Retention time (min)	
	Phenylglycine in ACN:DI H_2O (70:30)	Phenylalanine in ACN:DI H_2O (70:30)
1	2.3430	3.9150
2	2.3010	3.8850
3	2.3525	3.9200
4	2.1000	3.9150
5	2.3430	3.9430
6	2.3010	3.9000
7	2.2985	3.9430
8	2.3430	3.9100
9	2.3550	3.8655
10	2.4010	3.9150
%RSD	0.3347	0.6111

ii. Hydrophobic analytes

Analytes listed in Tables 15 and 16 are non-polar compounds. The first group is

from the steroid family. This group shows a small amount of polarity but can be

neglected for the purpose of this study. However, the presence of the ―OH group in the

corticosterone structure could make this compound somewhat more polar than the rest of

the analytes of this family.

Table 15. Retention times of the hydrophobic steroids using the 1,8-nonadiyne column.

Conc. of the mobile phase (%)		Retention time (min)				
ACN	DI H$_2$O	Adrenosterone	Δ^4-Androsten-3,17-dione	Esterone	Estradiol	Corticosterone
10	90	2.8300	16.1050	2.8380	2.3840	*
20	80	2.7500	13.3850	2.7539	2.3510	*
30	70	2.4590	10.6100	2.4500	2.3140	13.3230
40	60	2.3790	6.0760	2.3740	2.3070	6.4830
50	50	2.2320	4.2110	2.2400	2.2410	4.3340
60	40	2.3270	5.8280	2.3330	2.2940	3.3960
70	30	2.3410	*	2.6560	2.3337	*
80	20	2.6190	*	*	2.3390	*
90	10	*	*	*	2.3530	*

* peak was not observed

According to Table 15, neither of the analytes shows considerable ANP behavior as they elute at the early stages of analysis. However, the RP retention behavior for the column with respect to these compounds is more obvious. Figure 25 compares the retention time of three of the studied analytes. The retention maps for corticosterone and adrenosterone (▲ and ♦, respectively) show more RP behavior using 1,8-nonadiyne bonded stationary phase.
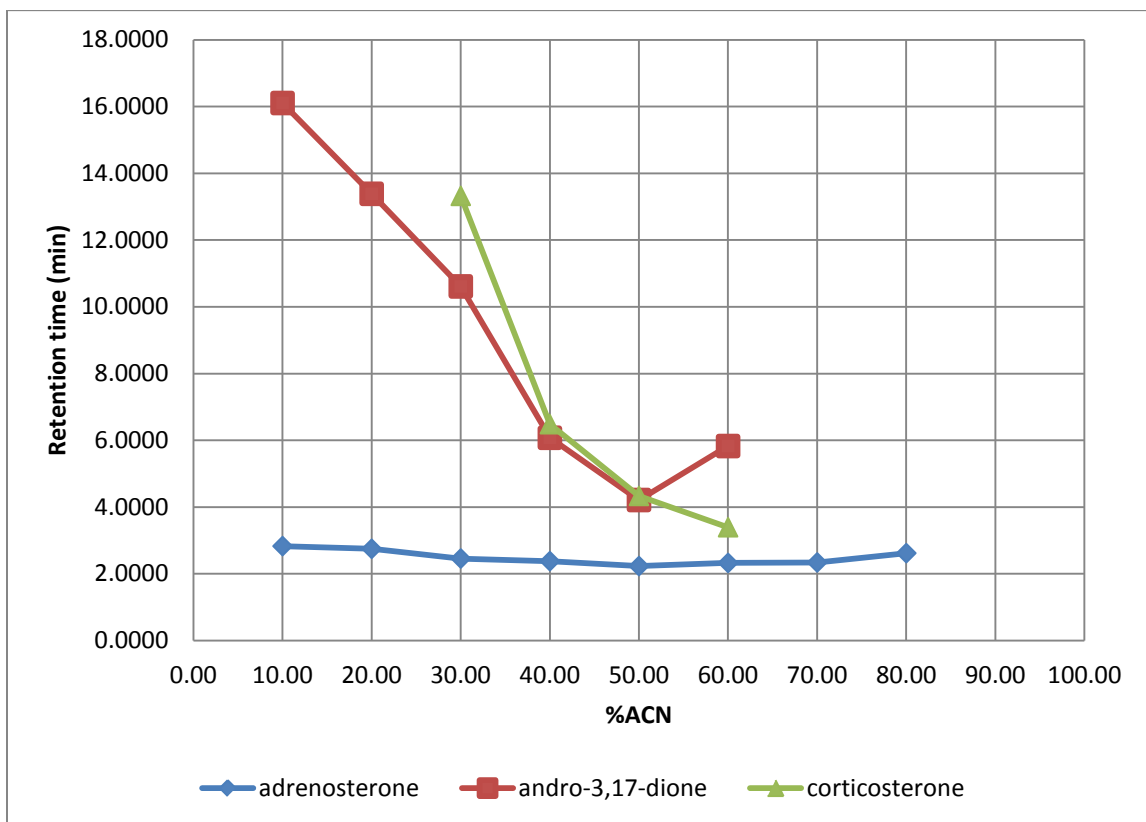
Figure 25. Retention map of the steroids, adrenosterone, Δ^4-androsten-3,17-dione, and corticosterone using the 1,8-nonadiyne column

The next class of hydrophobic compounds for this study was polycyclic aromatic hydrocarbons. As expected, these compounds did not exhibit ANP behavior for this

column. Table 15 reports the retention time of each analyte with the 1,8-nonadiyne column.

Table 16. Retention times of the hydrophobic polycyclic aromatic hydrocarbons using the 1,8-nonadiyne column.

Concentration of the mobile phase (%)		Retention time (min)			
ACN	DI water	Naphthalene	Phenanthrene	Pyrene	Fluorene
10	90	2.3780	*	2.3870	2.3770
20	80	2.3590	*	2.3490	2.3430
30	70	2.3210	*	2.3030	2.3280
40	60	2.3000	*	2.3140	2.2830
50	50	2.1730	10.5930	2.1810	2.1850
60	40	2.3010	5.7980	2.2920	2.2990
70	30	2.3260	5.7880	2.3290	2.4100
80	20	2.6780	4.1620	2.6830	2.6230
90	10	2.7210	6.1020	*	*

* peak was not observed

To better observe the effect of hydrophobicity vs. hydrophilicity of compounds with respect to RP/ ANP conditions, the retention map behaviors of three compounds with various degrees of polarity are plotted in Figure 26. As shown, the polar adenine (♦) shows significant ANP behavior when the mobile phase hydrophobicity increases. In contrast, for a non-polar compound like Δ^4-androsten-3,17-dione (▲), the RP behavior is very significant. Dopamine (■) shows a slight ANP behavior which is, to some extent,

consistent with its mildly polar nature. The elution characteristics of the entire selection

of polar molecules and non-polar molecules using the 1,8-nonadiyne as the bonded

stationary phase for this study are compared in Figure 27.
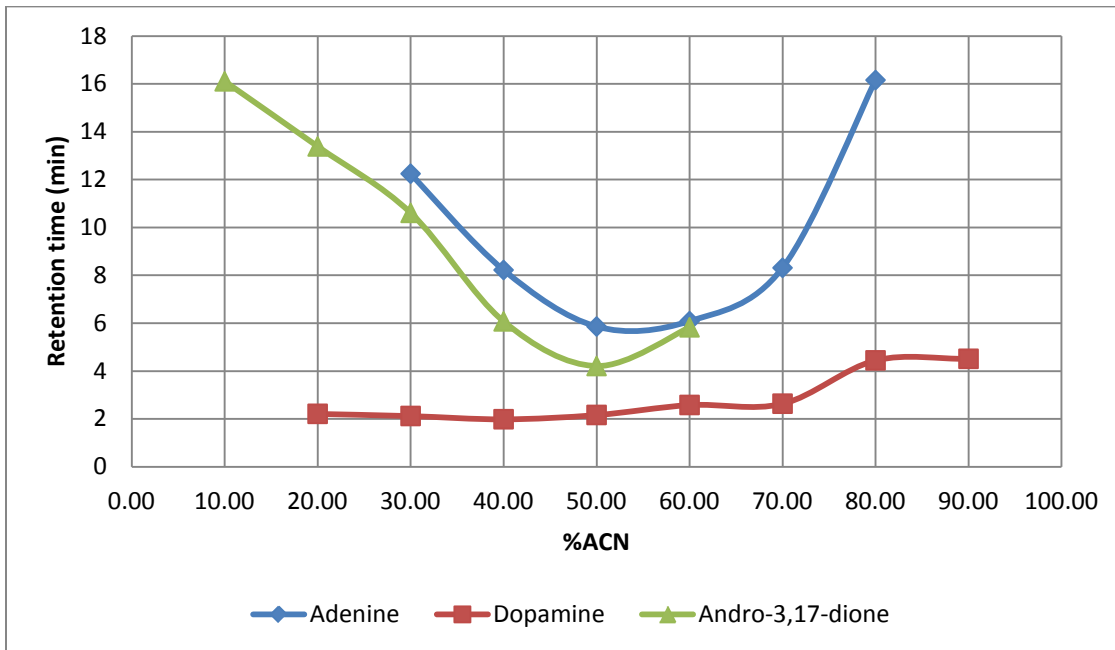
Figure 26. Retention map of adenine, dopamine, and Δ^4-androsten-3,17-dione, using the
1,8-nonadiyne column
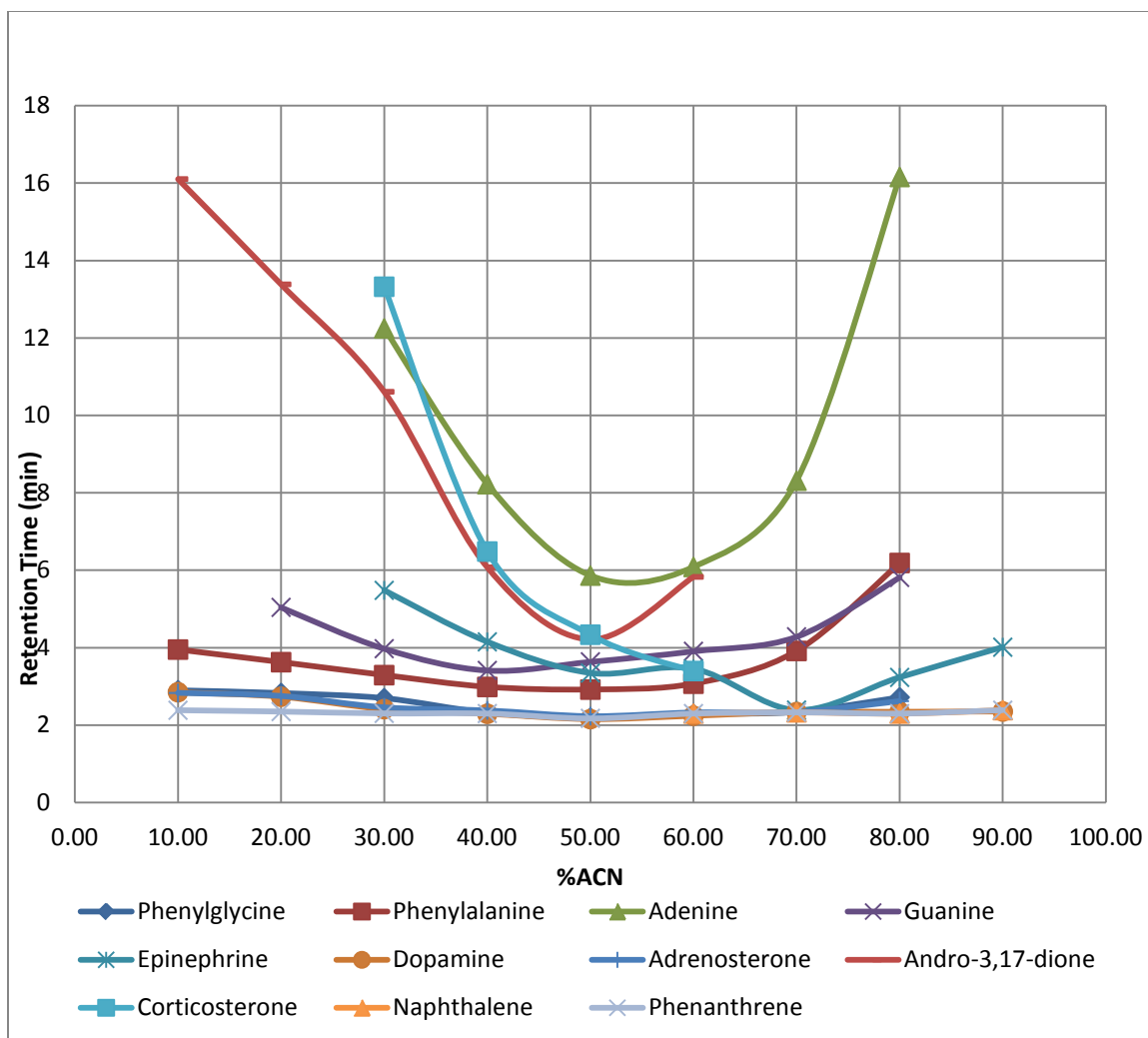
Figure 27. Retention map of the polar and non–polar analytes using the 1,8-nonadiyne column

iii.   Addition of formic acid (buffer)

To further study the retention behavior of the class of analytes with the 1,8-nonadiyne stationary phase, formic acid was used as a buffer. HPLC analysis confirmed that, when 0.1% (v/v) formic acid was added to the binary solvent mobile phase, it enhanced the ANP behavior of the compounds toward the stationary phase. Figure 28 shows the effect of the formic acid (FA) additive on two polar analytes. The effect is

better observed when compared with the similar conditions exclusive of buffer, as shown

in Figure 29. The studies, both with and without formic acid buffer, for the 1,8-

nonadiyne in the presence of a wide range of hydrophilic and hydrophobic analytes show

that the column exhibits both RP and ANP behavior.
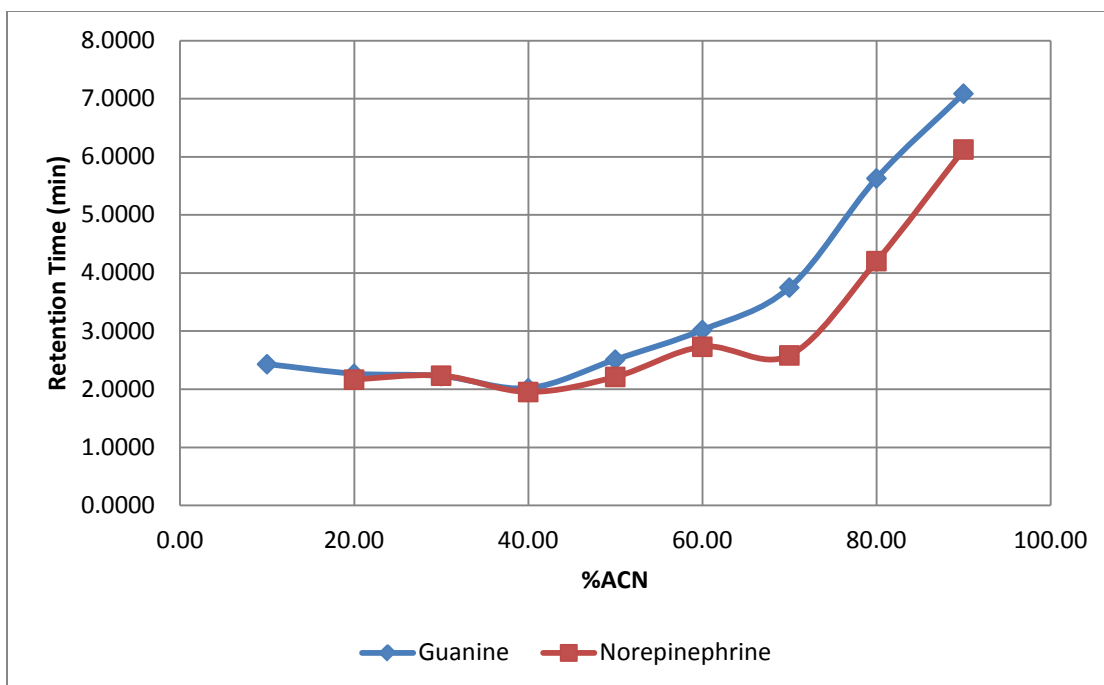
Figure 28. Retention map of two polar analytes using the 1,8-nonadiyne column and
0.1% formic acid in the mobile phase
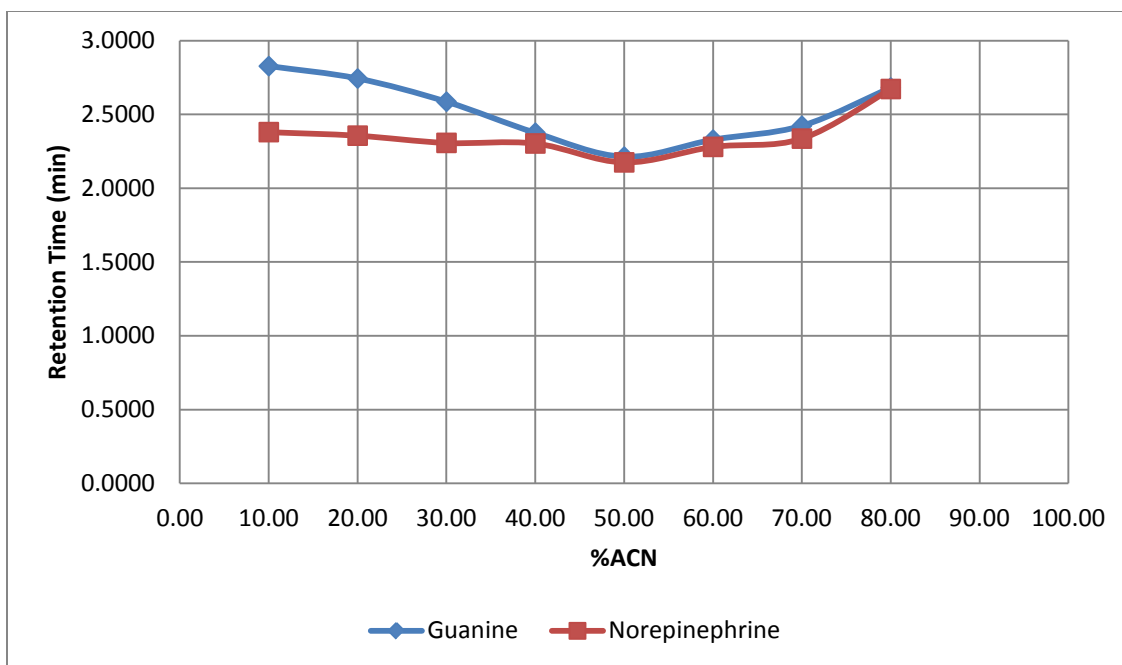
Figure 29. Retention map of two polar analytes using the 1,8-nonadiyne column without buffer
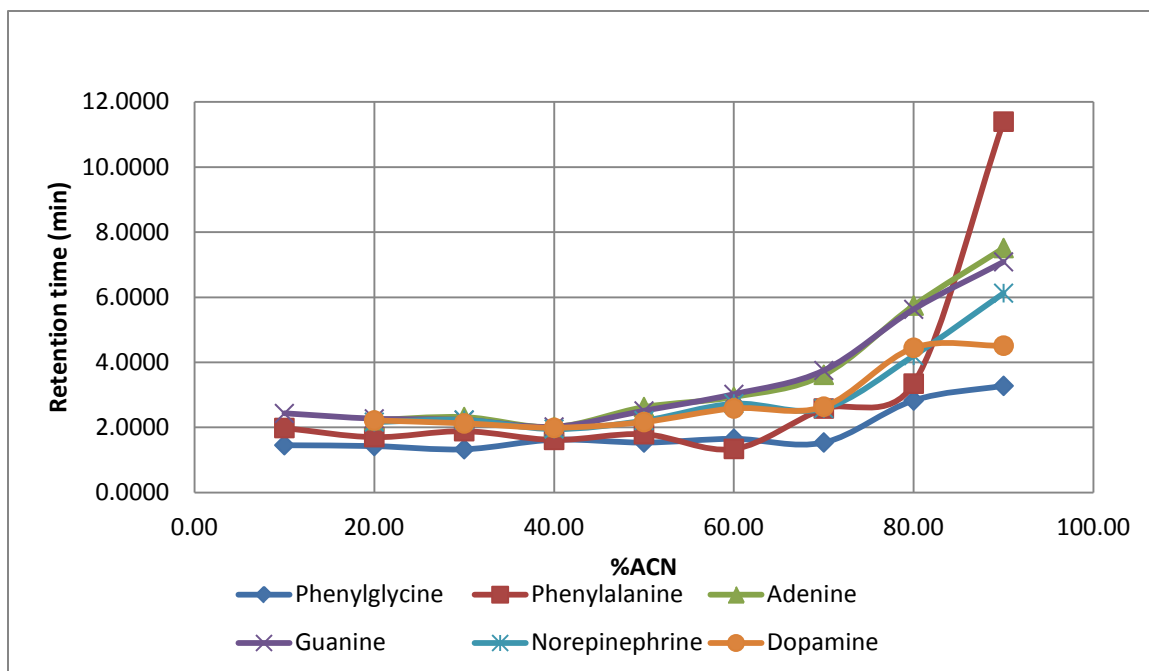
Figure 30. Retention map of the polar analytes using the 1,8-nonadiyne column and 0.1% FA in the mobile phase

Table 17. Retention times of the hydrophilic analytes using the 1,8-nonadiyne column with 0.1% formic acid buffer in the mobile phase.

Conc. of the mobile phase (%)		Retention time (min)						
ACN & 0.1% FA	H$_2$O & 0.1% FA	PhG	PhA	Adn	Gun	Epi	Nepi	Dopa
10	90	1.4550	1.9820	*	2.4300	*	*	*
20	80	1.4290	1.7030	2.2240	2.2650	3.5240	2.1640	2.2130
30	70	1.3310	1.8860	2.3190	2.2240	2.4010	2.2320	2.1230
40	60	1.6240	1.6180	2.0170	2.0170	2.8960	1.9510	1.9880
50	50	1.5370	1.7940	2.6260	2.5090	8.0010	2.2110	2.1660
60	40	1.6510	1.3400	2.9380	3.0180	3.7840	2.7300	2.5830
70	30	1.5380	2.5800	3.6170	3.7490	3.5110	2.5810	2.6370
80	20	2.8260	3.3450	5.7450	5.6280	2.9960	4.2040	4.4460
90	10	3.2770	11.3940	7.5110	7.0860	3.0170	6.1250	4.5120

* peak was not observed

PhG = Phenylglycine	PhA = Phenylalanine	Adn = Adenine	Gun = Guanine
Epi = Epinephrine	Nepi = Norepinephrine	Dopa = Dopamine	FA = Formic acid

2.   5-Cyano-1-pentyne

i.   Hydrophilic analytes

The RP/ ANP behavior of 5-cyano-1-pentyne stationary phase was studied using similar analytes and mobile phases. The results are tabulated, plotted, and presented in the following pages. There was, however, one unusual behavior exclusive to this column. The column, at relatively low concentrations of ACN in the mobile phase, (i.e., $\geq 30\%$, had no observable peaks). In other words, the chromatograms appeared in the form of random noise. This unusual behavior can be explained by possible irreversible adsorption of the analytes on the stationary phase. However, when 0.1% (v/v) formic acid buffer was added, this abnormality disappeared.

Tables 18, 19, and 20 show retention times for the three groups of hydrophilic analytes when testing the 5-cyano-1-pentyne bonded stationary phase with mobile phases ranging from polar to less polar. The collected data, as well as the retention maps, for nucleobases (adenine and guanine) and neurotransmitters (epinephrine, norepinephrine, dopamine) shown in Figures 31 and 32 confirm that 5-cyano-1-pentyne exhibits ANP behavior.

Table 18. Retention times of the hydrophilic amino acids using the 5-cyano-1-pentyne column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Phenylglycine	Phenylalanine
10	90	*	*
20	80	*	*
30	70	*	*
40	60	3.145	3.0850
50	50	2.140	2.2060
60	40	2.5420	2.8670
70	30	3.0370	3.3150
80	20	4.2380	4.7720
90	10	*	*

* peak was not observed

Table 19. Retention times of the hydrophilic nucleobases using the 5-cyano-1-pentyne column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Adenine	Guanine
10	90	*	*
20	80	*	*
30	70	*	*
40	60	3.0260	2.1220
50	50	2.3670	2.0000
60	40	4.9080	2.6030
70	30	5.1990	2.8530
80	20	6.0250	3.3710
90	10	7.9250	4.7140

* peak was not observed
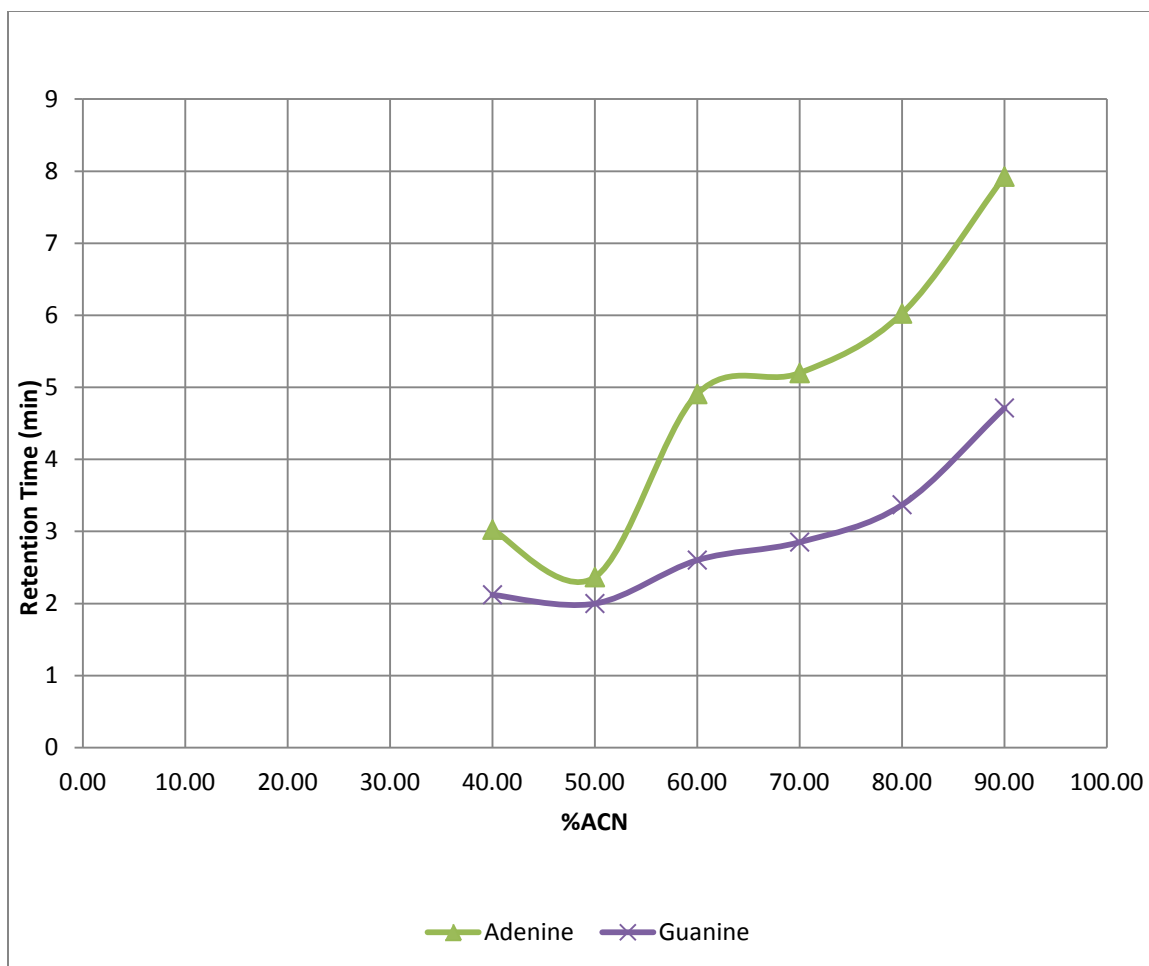
Figure 31. Retention map of the polar nucleobases using the 5-cyano-1-pentyne column

Table 20. Retention times of the hydrophilic neurotransmitters using the 5-cyano-1-pentyne column.

Concentration of the mobile phase (%)		Retention time (min)		
Acetonitrile	DI water	Epinephrine	Norepinephrine	Dopamine
10	90	*	*	*
20	80	*	*	*
30	70	*	*	*
40	60	1.8850	1.8940	1.8980
50	50	1.6660	1.6680	1.8340
60	40	1.8790	1.8790	1.8910
70	30	2.4850	1.8860	1.8970
80	20	2.8600	1.9180	1.9380
90	10	3.6810	1.9350	2.2340

* peak was not observed
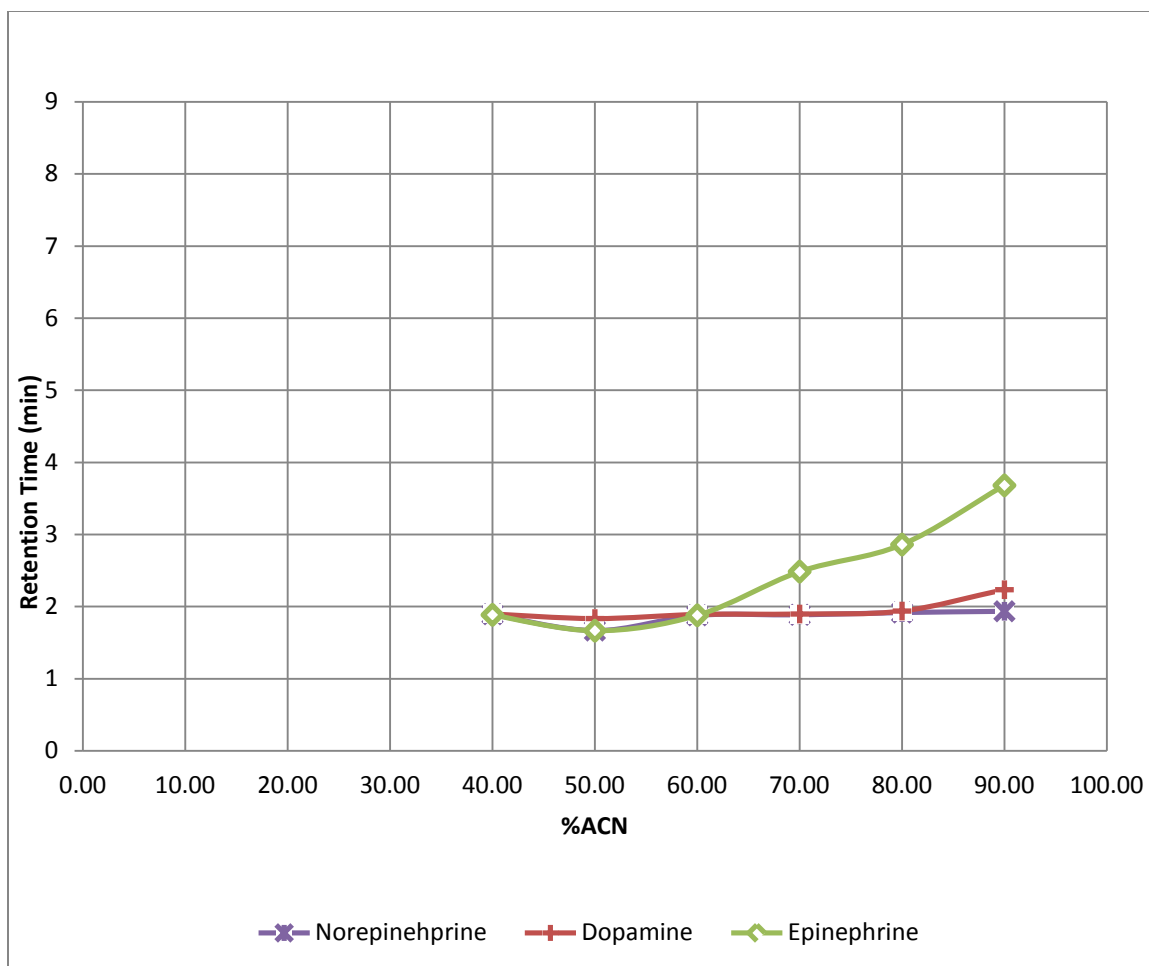
Figure 32. Retention map of the polar neurotransmitters using the 5-cyano-1-pentyne column

ii.   **Hydrophobic analytes**

The retention times for the steroid molecules are short. However, from the retention maps for adrenesterone and estradiol shown in Figure 33 (+ and **-**), one can observe slight ANP behavior for the molecules when testing the 5-cyano-1-pentyne column. The ANP behavior is more substantial for phenanthrene from the polycyclic aromatic hydrocarbons, as shown in Figure 34 (><). Figure 35 compares the retention behavior of some of the non-polar and polar analytes using the 5-cyano-1-pentyne

stationary phase. The retention times of the hydrophobic analytes, steroids, and

polycyclic aromatic hydrocabons, using various concentrations of the mobile phase for

the 5-cyano-1-pentyne bonded stationary phase, are provided in Tables 21 and 22.

Table 21. Retention times of the hydrophobic steroids using the 5-cyano-1-pentyne
column.

mobile phase conc.		Retention time (min)				
ACN	DI H$_2$O	Adreno sterone	Δ^4-Andro 3,17-dione	Esterone	Estradiol	Corticosterone
10	90	*	*	*	*	*
20	80	*	*	*	*	*
30	70	*	*	*	*	*
40	60	1.9140	1.8960	1.8980	1.9020	1.8980
50	50	1.6970	1.6940	1.6800	1.6850	1.6870
60	40	1.9070	1.8830	1.8490	1.5920	1.8810
70	30	1.8990	1.8950	1.8890	1.7960	1.8870
80	20	1.9050	1.9130	1.9130	1.8940	1.9170
90	10	1.9330	1.9200	1.9410	1.9320	1.9340

* peak was not observed
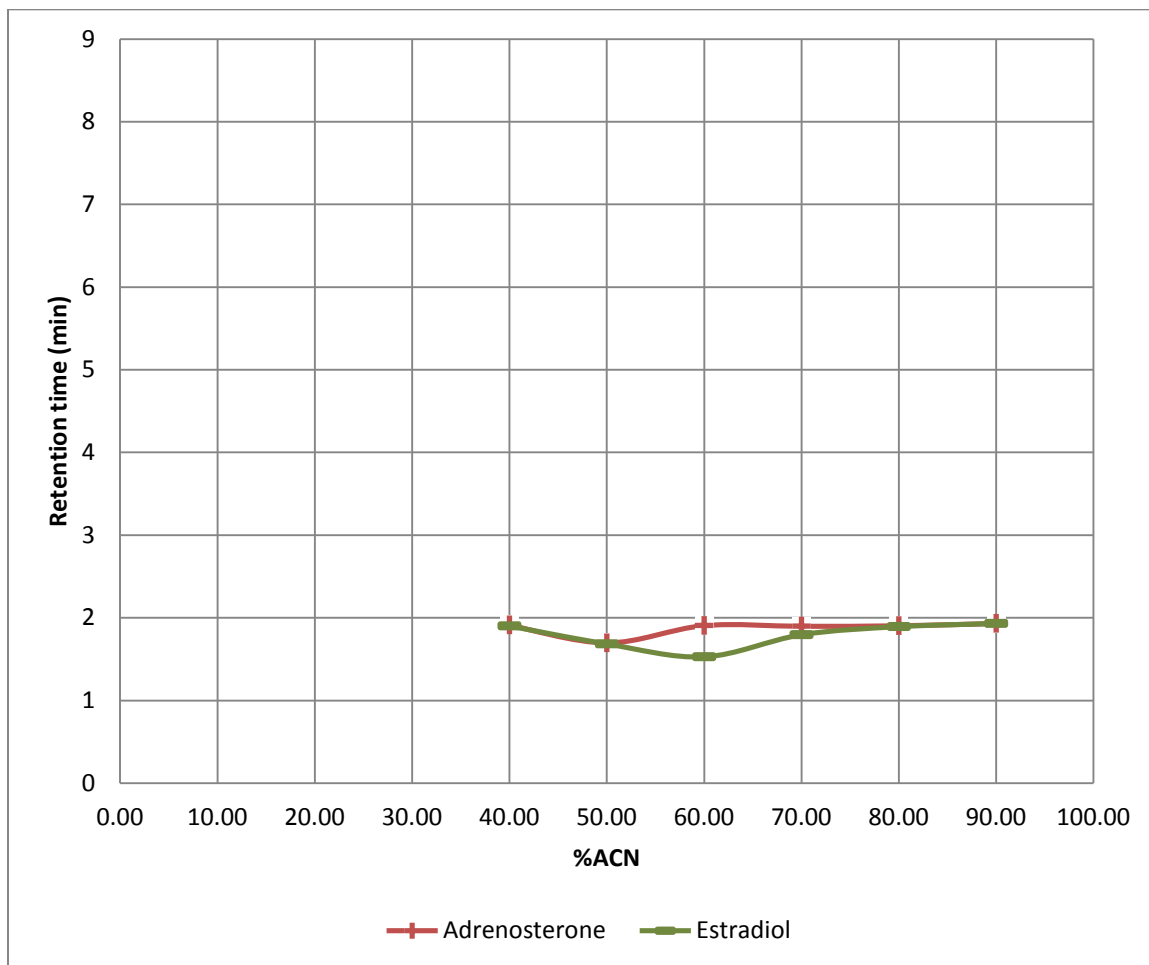
Figure 33. Retention map of the non-polar steroids using the 5-cyano-1-pentyne column

Table 22. Retention times of the hydrophobic polycyclic aromatic hydrocarbons using the 5-cyano-1-pentyne column.

Concentration of the mobile phase (%)		Retention time (min)			
ACN	DI water	Naphthalene	Phenantherene	Pyrene	Flourene
10	90	*	*	*	*
20	80	*	*	*	*
30	70	*	*	*	*
40	60	1.8990	2.1930	2.3310	1.8990
50	50	1.7110	1.97902	1.9770	1.6880
60	40	1.8940	2.7010	1.8230	1.8760
70	30	1.8740	3.2900	1.8850	1.8890
80	20	1.9050	4.6600	1.9180	1.9170
90	10	1.9450	*	1.9410	1.9370

* peak was not observed
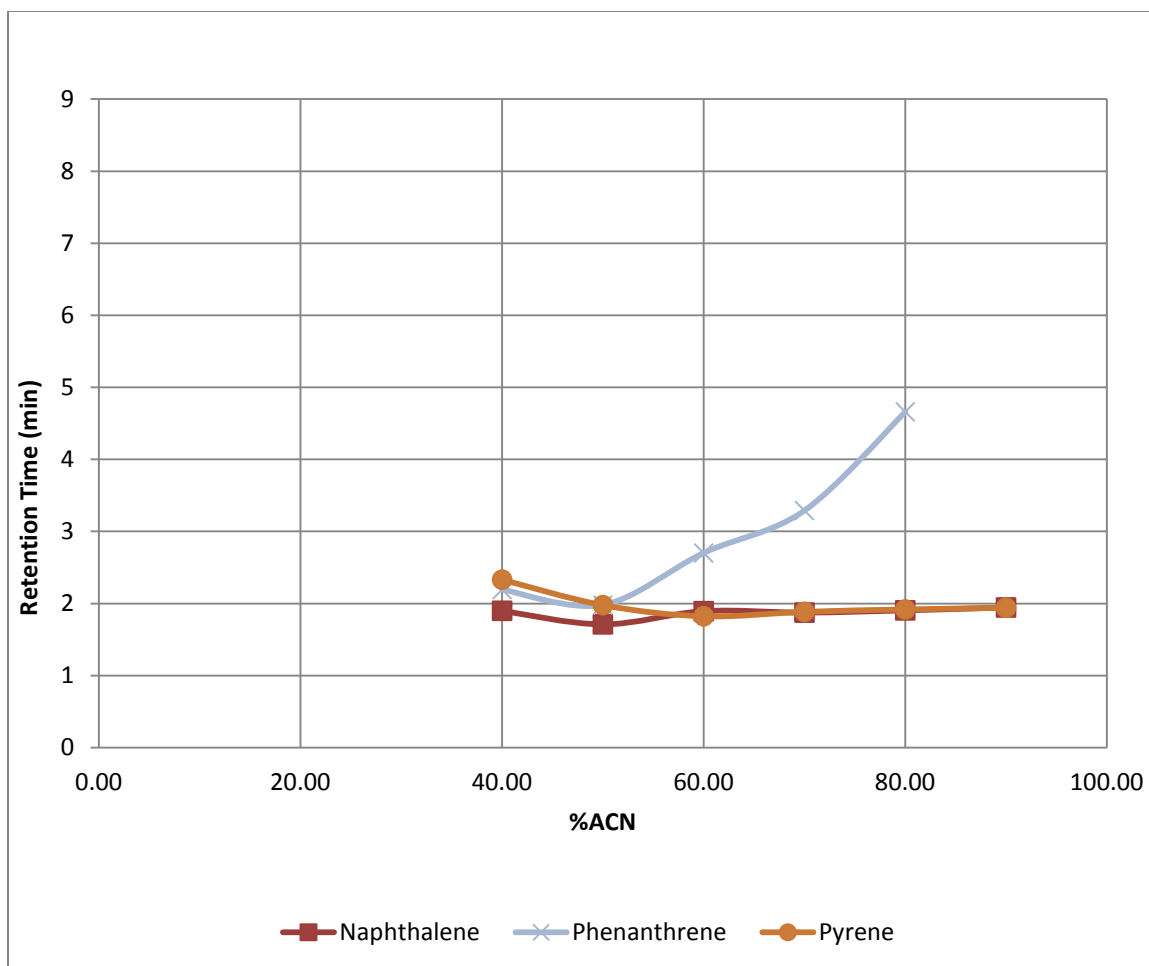
Figure 34. Retention map of the non-polar analytes using the 5-cyano-1-pentyne column
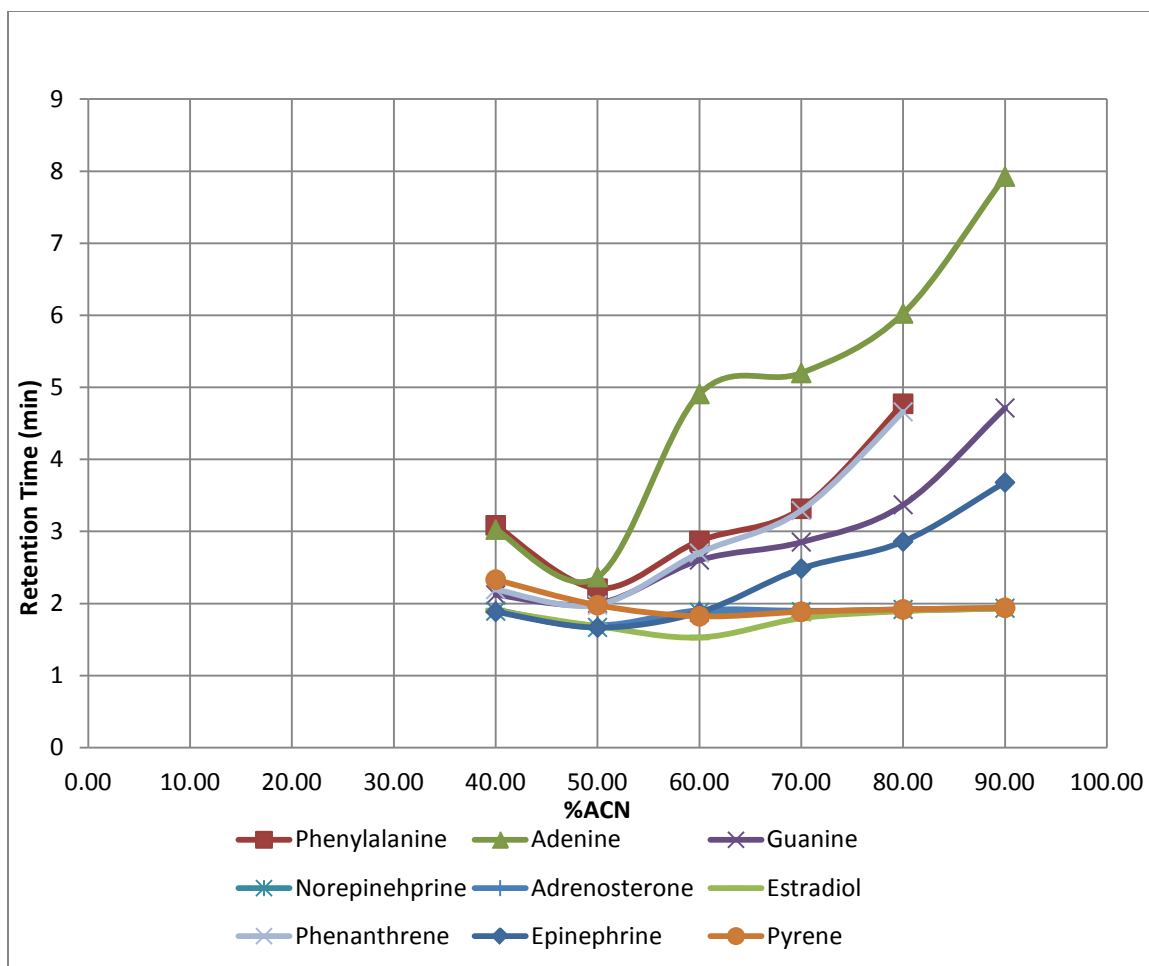
Figure 35. Retention map of the polar and non-polar analytes using the 5-cyano-1-pentyne column

Studying the retention maps of the various analytes, one can determine that the column shows more ANP behavior. One plausible explanation for the 5-cyano-1-pentyne column having ANP behavior only could be the presence of the C≡N bond, which makes the molecule moderately polar.

iii.   Addition of formic acid (buffer)

To observe the effect of buffer on the retention behavior of the compounds on the 5-cyano-1-pentyne bonded stationary phase, two molecules with different properties are chosen. A comparison of a molecule with typical reversed-phase behavior, pyrene, to one with typical ANP behavior, epinephrine, in the presence of 0/1% (v/v) formic acid in the mobile phase is tabulated and plotted in Table 23 and Figure 36. An interesting feature of this plot is that, in the presence of buffer, both molecules exhibit RP and ANP behavior. This phenomenon could be due to the interaction of the buffer with the C≡N functional group on 5-cyano-1-pentyne, the absence of irreversible adsorption of molecules on the surface, or a combination of these possibilities.

Table 23. Retention times of epinephrine and pyrene using the 5-cyano-1-pentyne column with 0.1% formic acid buffer in the mobile phase.

Concentration of the mobile phase (%)		Retention time (min)	
ACN & 0.1% FA	H_2O & 0.1% FA	Epinephrine (hydrophilic)	Pyrene (hydrophobic)
10	90	4.3170	6.1300
20	80	2.7040	3.3070
30	70	2.3090	2.6370
40	60	2.1130	3.41110
50	50	2.3870	2.3600
60	40	2.6970	2.4090
70	30	3.2160	2.6190
80	20	3.5790	3.1260
90	10	6.1970	5.5580
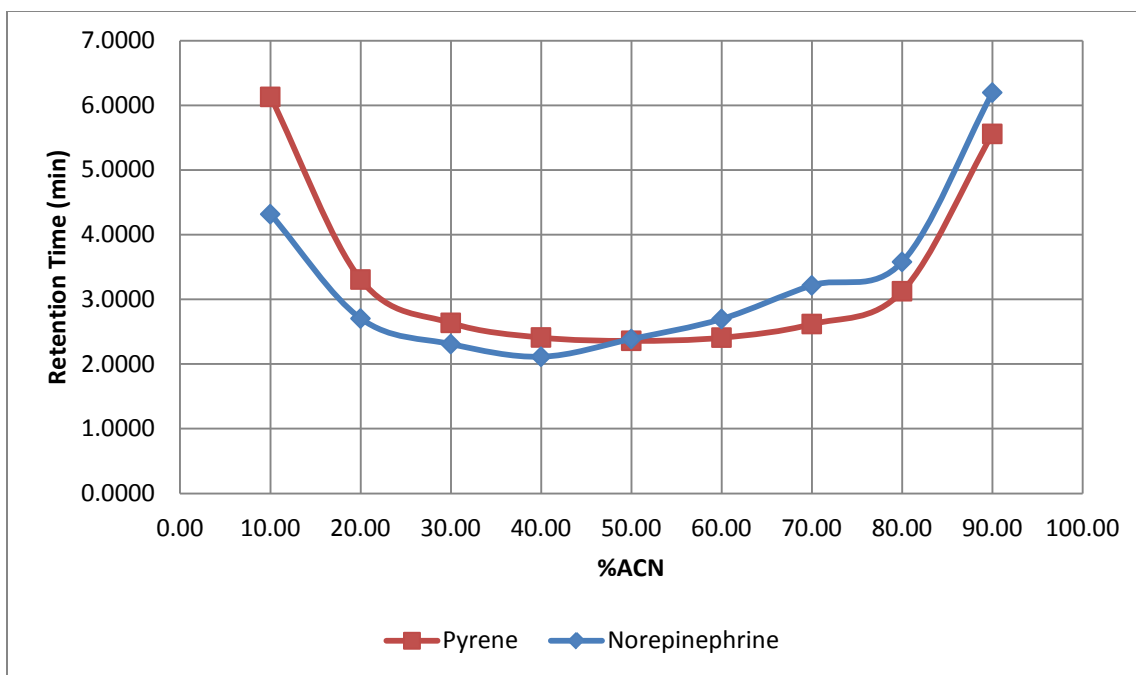
Figure 36. Retention map of a polar and non-polar analytes using the 5-cyano-1-pentyne column and 0.1% formic acid in the mobile phase
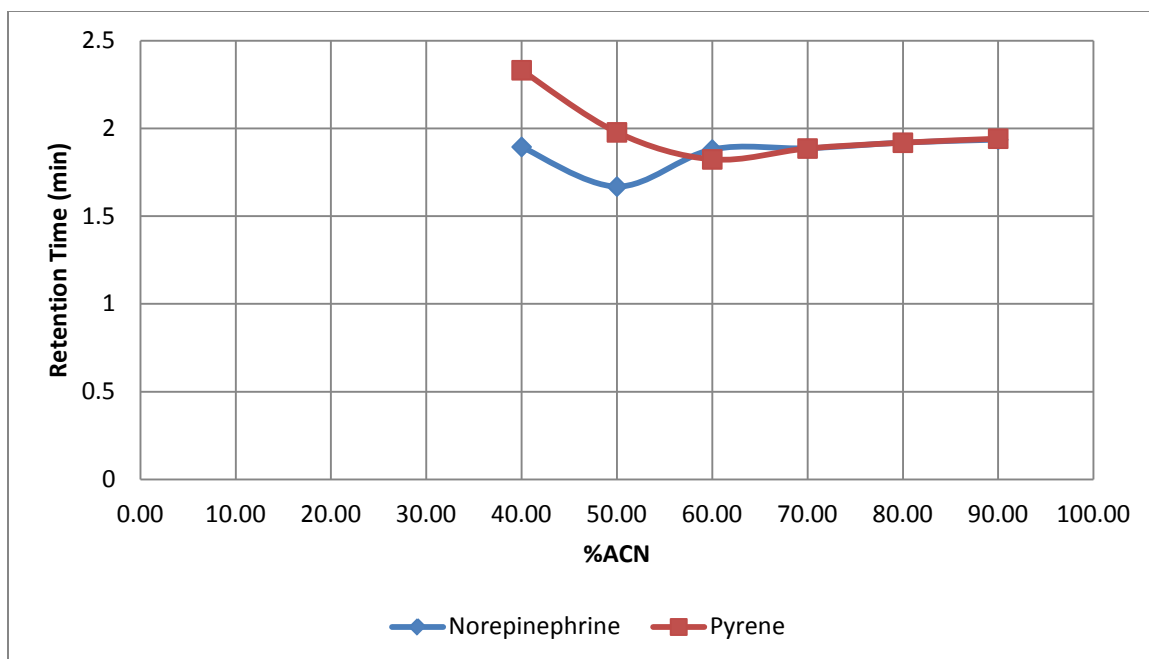
Figure 37. Retention map of a polar and non-polar analytes using the 5-cyano-1-pentyne column without buffer

3. 6-Chloro-1-hexyne

i. Hydrophilic analytes

The next bonded stationary phase to be evaluated was 6-chloro-1-hexyne. The results from running the column with the two amino acids, phenylglycine and phenylalanine, under isocratic condition of the mobile phase of acetornitrile : DI water are in Table 24.

Table 24. Retention times of the hydrophilic amino acids using the 6-chloro-1-hexyne column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Phenylglycine	Phenylalanine
10	90	2.1690	3.2280
20	80	2.0470	2.8390
30	70	2.0010	2.5980
40	60	1.8900	2.2940
50	50	1.7310	2.6970
60	40	2.4390	3.3170
70	30	3.1150	4.2220
80	20	4.3760	6.1650
90	10	*	*

* peak was not observed

As shown in Figure 38, the retention maps for both phenylglycine and phenylalanine (♦ and ■, respectively) are similar in terms of ANP/ RP properties of the column. In other words, the 6-chloro-1-hexyne bonded stationary phase exhibits both ANP and RP retention behavior at the same time. In addition, the column had great reproducibility. The testing of the column with 5 consecutive runs showed the same retention time ± 0.05 min. Similar ANP/ RP results were obtained with adenine and guanine (♦ and ▲, respectively) retention maps as shown in Figure 39.
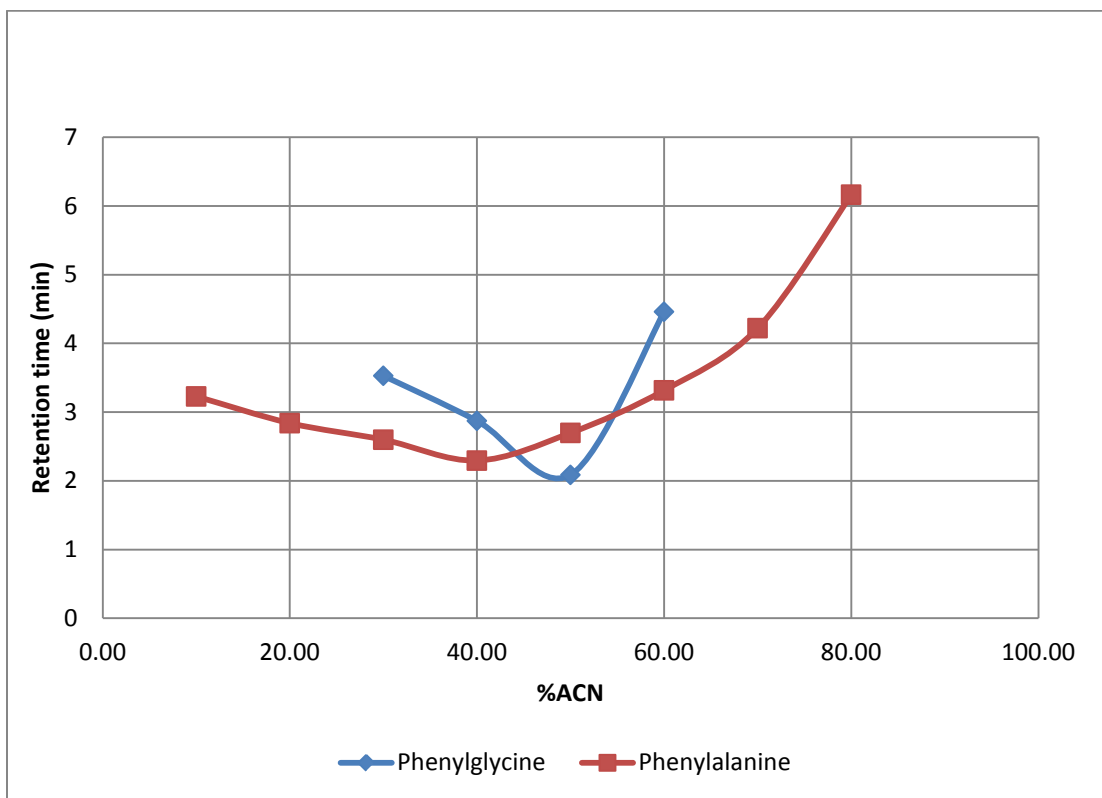
Figure 38. Retention map of the polar amino acids using the 6-chloro-1-hexyne column

Table 25. Retention times of the hydrophilic nucleobases using the 6-chloro-1-hexyne column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	**DI water**	Adenine	Guanine
10	90	*	*
20	80	13.7920	*
30	70	10.5290	4.8220
40	60	9.1750	3.1070
50	50	8.8420	3.2260
60	40	9.3770	4.4430
70	30	10.8570	5.3870
80	20	13.8410	5.8320
90	10	*	6.0260

* peak was not observed
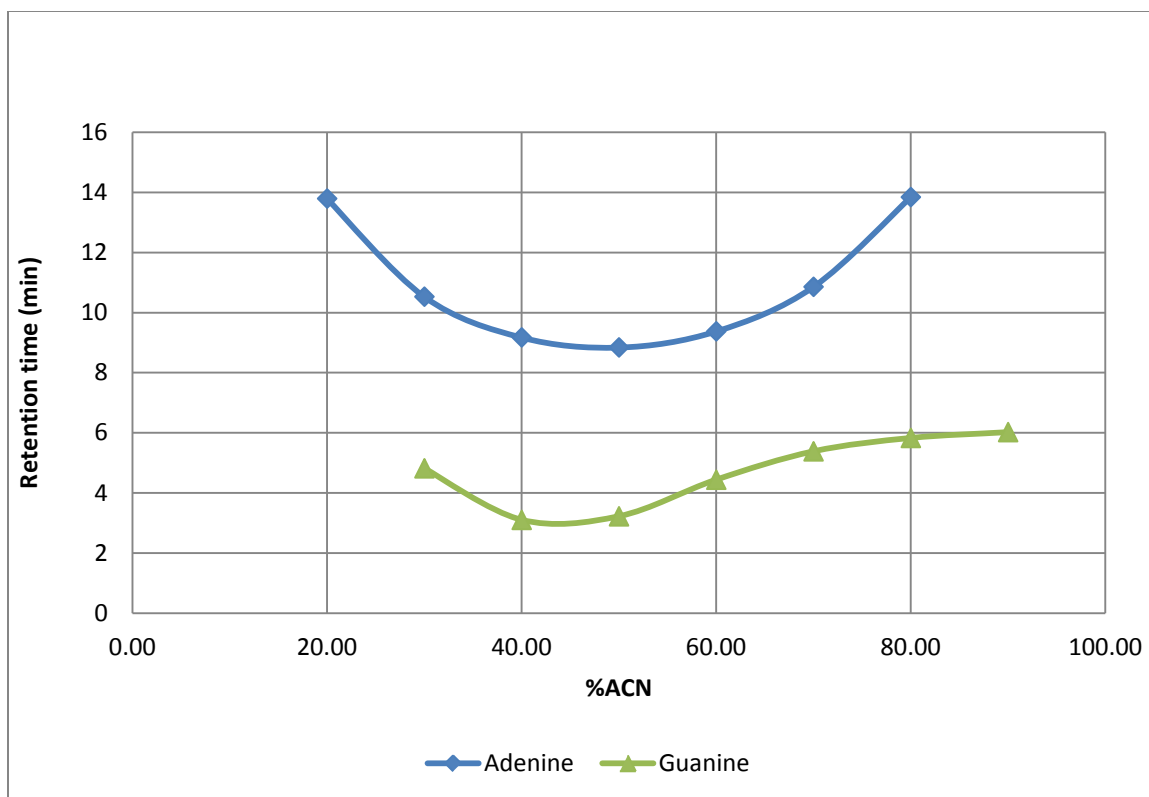
Figure 39. Retention map of the polar nucleobases using the 6-chloro-1-hexyne column

Table 26. Retention times of the hydrophilic neurotransmitters using the 6-chloro-1-hexyne column.

Concentration of the mobile phase (%)		Retention time (min)		
Acetonitrile	DI water	Epinephrine	Norepinephrine	Dopamine
10	90	2.3250		5.2470
20	80	2.2150	3.0120	4.2020
30	70	1.9990	2.5980	3.4050
40	60	4.3670	2.3050	3.0700
50	50	1.9960	2.0930	2.1360
60	40	2.5110	3.5750	2.1620
70	30	2.9960	5.3870	2.1740
80	20	2.5300	*	*
90	10	*	*	*

* peak was not observed

ii.   Hydrophobic analytes

The non-polar compounds for this study when testing the 6-cholor-1-hexyne bonded stationary phase exhibit RP behavior entirely. The ANP behavior is somewhat limited, but molecules such as adrenosterone and corticosterone (♦ and ><, respectively) in Figure 40 and naphthalene and pyrene (♦ and ▲, respectively) in Figure 41, exhibit both RP and ANP behavior. Tables 27 and 28 refer to the retention times for the two groups of hydrophobic molecules. Comparison of the retention times of hydrophilic and

hydrophobic molecules, provided in Figure 42, shows that the 6-chloro-1-hexyne bonded

silica hydride based stationary phase has reversed-phase as well as ANP properties.

Table 27. Retention times of the hydrophobic steroids using the 6-chloro-1-hexyne
column.

Concentration of the mobile phase (%)		Retention time (min)				
ACN	DI water	Adreno sterone	Δ^4-Androsten- 3,17-dione	Esterone	Estradiol	Corticosterone
10	90	*	*	*	*	*
20	80	*	*	*	4.5490	*
30	70	10.2600	*	4.3050	3.7490	7.8380
40	60	5.7390	8.4580	4.0960	3.1260	4.3470
50	50	3.8710	5.0420	3.2200	5.4000	3.0960
60	40	4.2090	3.5230	2.5970	3.5730	2.5090
70	30	5.3890	2.7690	2.2080	*	2.2020
80	20	*	2.3760	1.9860	*	2.0530
90	10	*	*	*	*	2.0130

* peak was not observed

Table 28. Retention times of the hydrophobic polycyclic aromatic hydrocarbons using the 6-chloro-1-hexyne column.

Concentration of the mobile phase (%)		Retention time (min)			
ACN	**DI water**	**Naphthalene**	**Phenanthrene**	**Pyrene**	**Flourene**
10	90	*	3.2410	2.3260	*
20	80	*	2.9430	2.1830	2.1990
30	70	*	2.7170	2.0010	11.5040
40	60	9.0940	2.6560	3.0950	7.8340
50	50	6.9830	2.8190	4.2570	6.9950
60	40	10.3520	3.2440	5.0430	4.1640
70	30	*	3.8830	5.4050	*
80	20	*	4.5280	*	*
90	10	*	5.3210	*	*

* peak was not observed
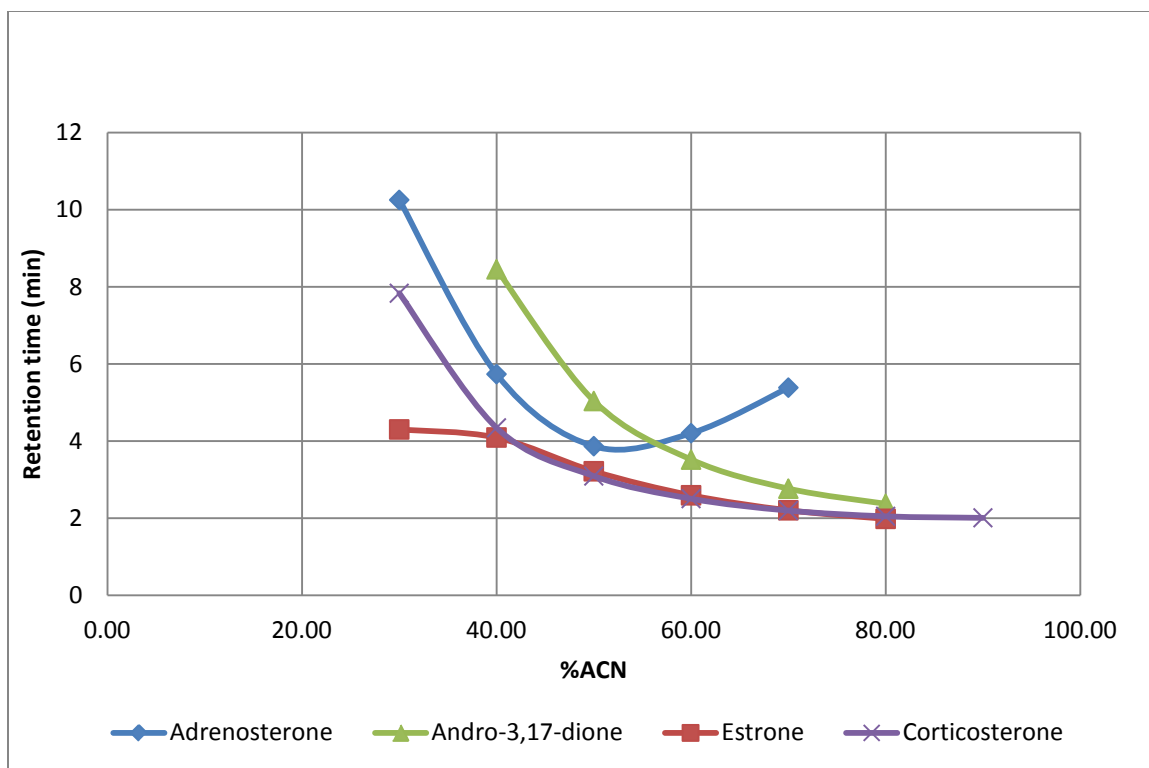
Figure 40. Retention map of the steroid analytes using the 6-chloro-1-hexyne column
without buffer
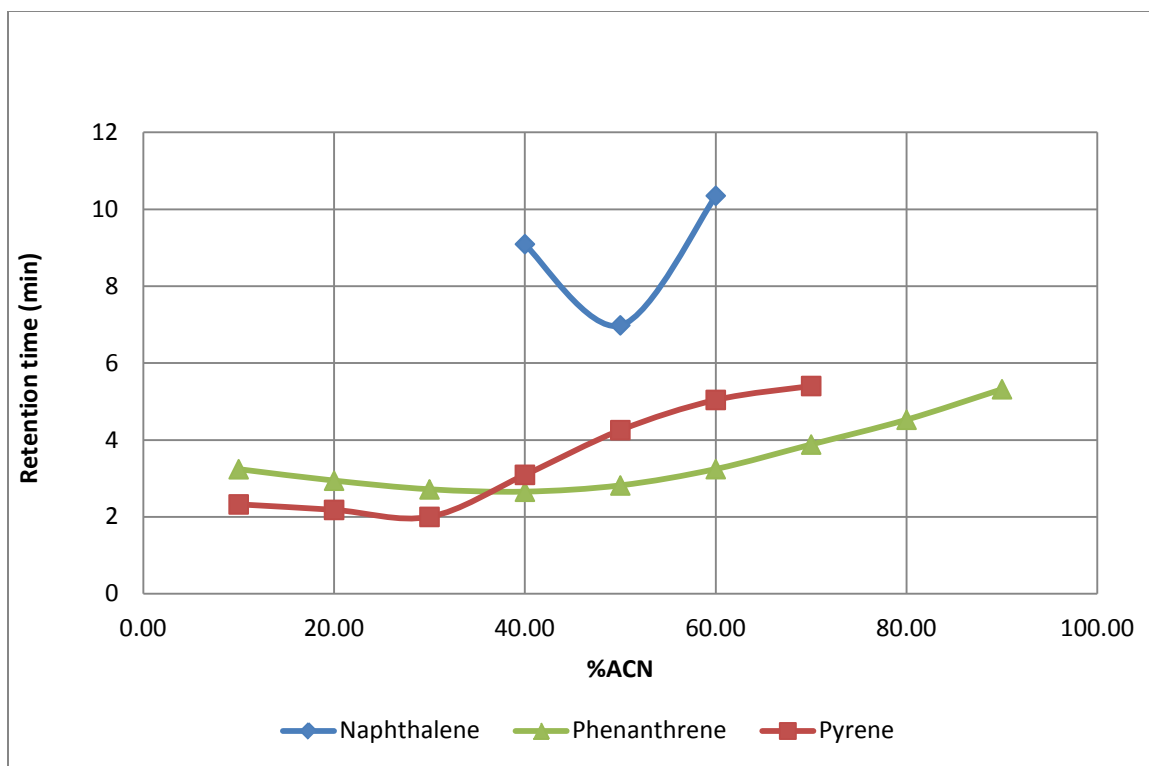
Figure 41. Retention map of the polycyclic aromatic hydrocarbon analytes using the 6-chloro-1-hexyne column without buffer
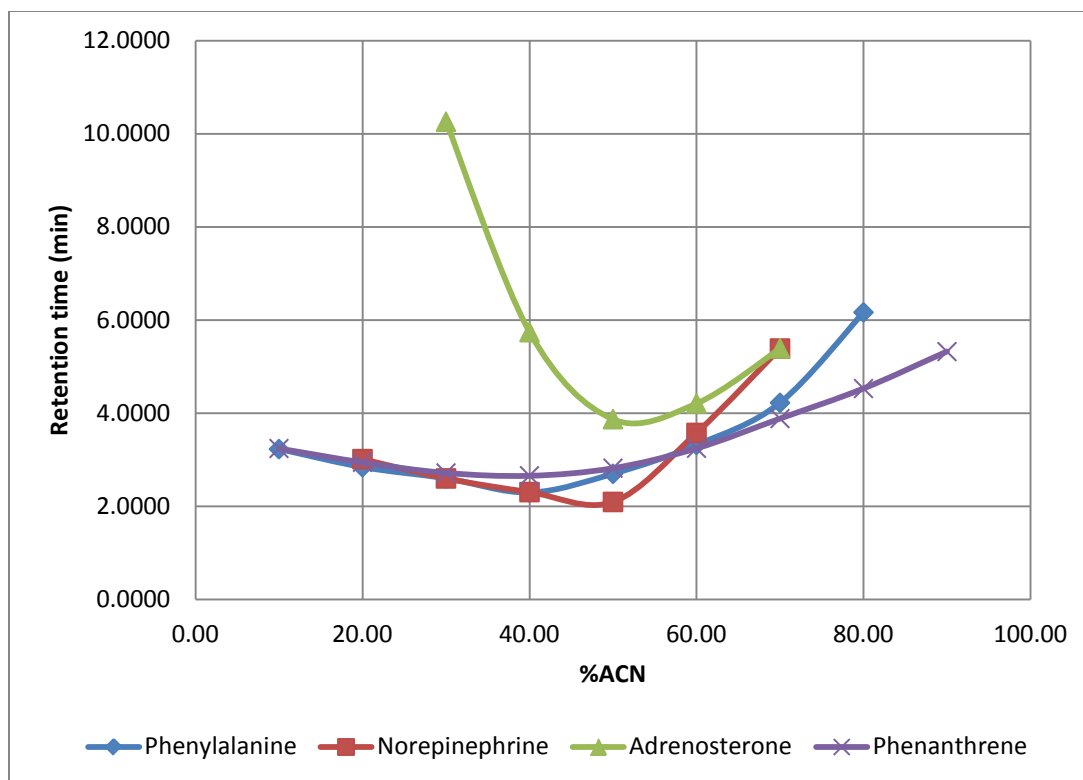
Figure 42. Retention map of the polar and non-polar analytes using the 6-chloro-1-hexyne column without buffer

4. 4-Ethynylanaline

i. Hydrophilic analytes

Three groups of hydrophilic (amino acids, nucleobases, and neurotransmitters) analytes were tested to evaluate RP/ ANP properties of 4-ethynylanaline. The retention times of these analytes for different polarities of the mobile phase are provided in Tables 29, 30, and 31. The results from the retention map plots for three groups of polar molecules in an isocratic mobile phase and 4-ethynylalanine stationary phase indicate that this column shows the RP phase properties and not so much of ANP, as shown in Figure 43.

Table 29. Retention times of the hydrophilic amino acids using the 4-ethynylanaline column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Phenylglycine	Phenylalanine
10	90	*	8.2180
20	80	17.6730	17.8270
30	70	12.5730	12.6520
40	60	9.9460	9.2320
50	50	5.5610	7.4590
60	40	6.2550	6.2660
70	30	5.6090	5.6520
80	20	4.9860	3.9230
90	10	5.0360	4.9620

* peak was not observed

Table 30. Retention times of the hydrophilic nucleobases using the 4-ethynylanaline column.

Concentration of the mobile phase (%)		Retention time (min)	
Acetonitrile	DI water	Adenine	Guanine
10	90	24.6860	21.1390
20	80	17.4390	14.1820
30	70	14.5310	10.0240
40	60	9.0570	8.2360
50	50	7.0860	5.9820
60	40	6.1530	5.3310
70	30	5.7040	4.6530
80	20	4.3550	4.1050
90	10	4.4540	4.0210

Table 31. Retention times of the hydrophilic neurotransmitters using the
4-ethynylanaline column.

Concentration of the mobile phase (%)		Retention time (min)		
Acetonitrile	DI water	Epinephrine	Norepinephrine	Dopamine
10	90	*	*	20.4870
20	80	23.3980	17.2240	14.1930
30	70	20.3290	12.4640	10.4360
40	60	16.1980	9.0760	8.2570
50	50	13.9630	7.5840	7.2750
60	40	12.9630	6.1370	6.6470
70	30	11.0330	5.8240	6.0730
80	20	11.1130	5.2150	5.6460
90	10	*	*	*

* peak was not observed
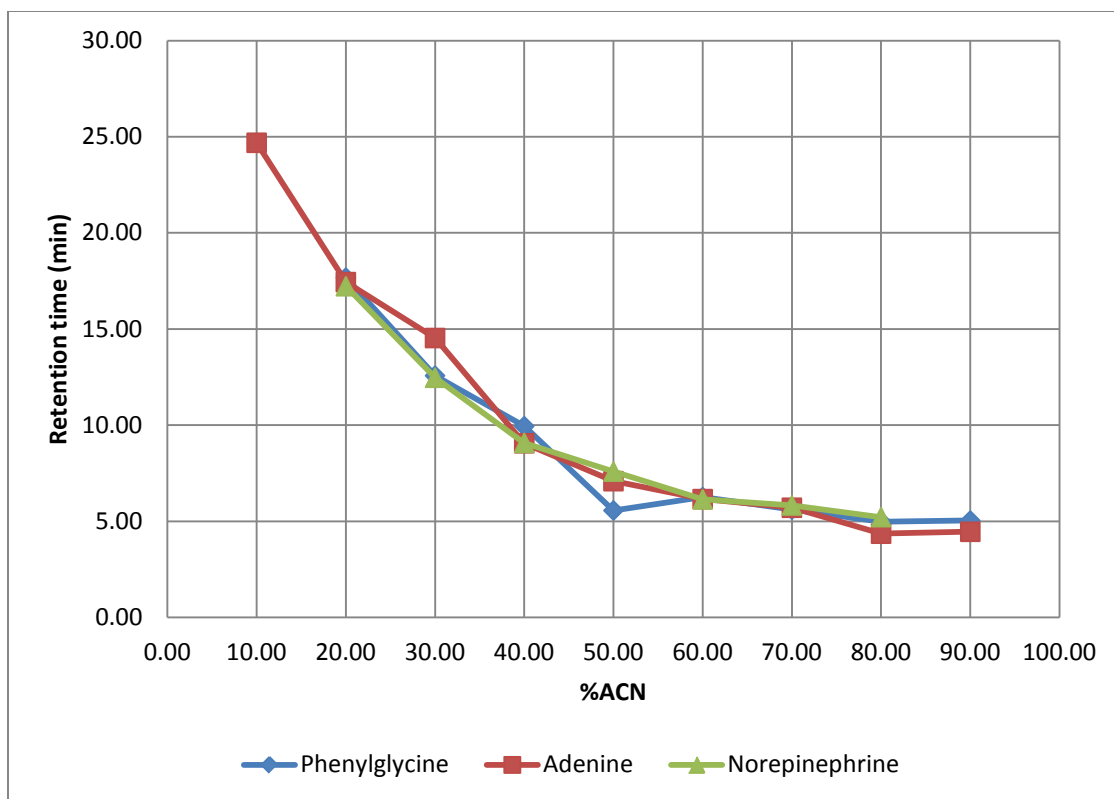
Figure 43. Retention map of the polar analytes using the 4-ethynylalanine column

ii.   Hydrophobic analytes

The RP/ ANP behavior of a 4-ethynylalanine bonded stationary phase was tested using different molecules from two groups of hydrophobic molecules, steroids and polycyclic aromatic hydrocarbons. The retention times in different concentrations of ACN : H_2O mobile phase are provided in Tables 32 and 33.

Table 32. Retention times of the hydrophobic steroids using 4-ethynylanaline column.

Conc. of the mobile phase (%)		Retention time (min)				
ACN	DI H$_2$O	Adreno sterone	Δ^4-Androsten-3,17-dione	Esterone	Estradiol	Corticosterone
10	90	*	*	*	*	*
20	80	17.4190	18.1060	20.0470	19.7910	15.8490
30	70	12.6140	13.2760	14.8860	14.7480	14.4220
40	60	9.4010	9.8430	10.9340	10.8530	10.7150
50	50	7.4530	7.8880	8.7680	8.7550	8.7460
60	40	6.2760	6.4360	7.4520	7.3370	7.3320
70	30	5.7600	5.7970	6.6780	6.6730	6.5590
80	20	5.3740	5.5130	6.2350	6.1730	6.1680
90	10	4.6700	*	6.6290	6.0040	6.4210

* peak was not observed

Table 33. Retention times of the hydrophobic polycyclic aromatic hydrocarbons using the 4-ethynylanaline column.

Concentration of the mobile phase (%)		Retention time (min)			
ACN	DI water	Naphthalene	Phenanthrene	Pyrene	Flourene
10	90	*	*	24.1050	*
20	80	17.4290	18.2920	17.0110	18.6090
30	70	12.3010	15.3710	12.0310	11.2680
40	60	9.1690	9.6610	8.9960	9.6340
50	50	7.6760	7.9790	7.2770	7.8380
60	40	6.1740	6.4990	6.0410	6.6920
70	30	5.5440	5.9240	5.4180	6.1060
80	20	5.2580	5.6650	5.1460	5.7670
90	10	4.8330	5.0580	4.6830	5.3080

* peak was not observed
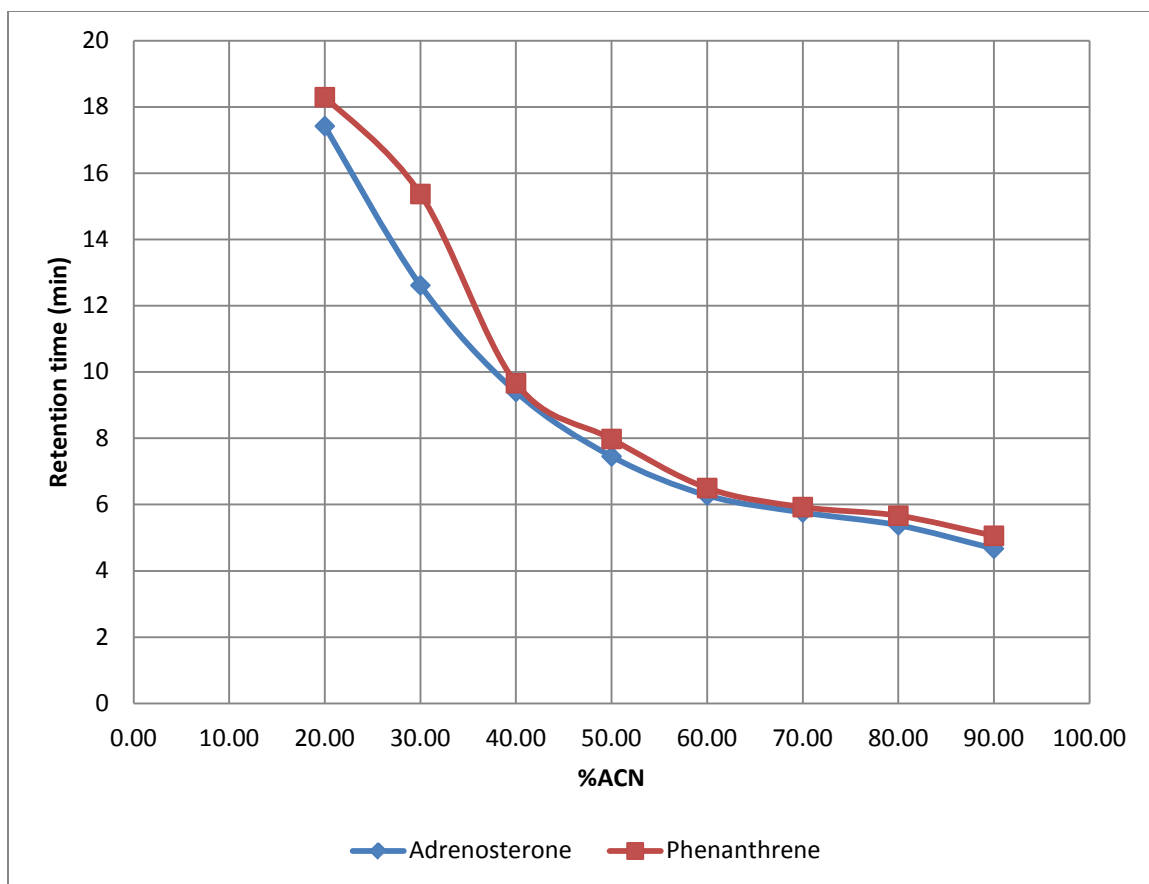
Figure 44. Retention map of the hydrophobic analytes using the 4-ethynylalanine column

Using one of the analytes from each group, both adrenosterone (a steroid) and phenanthrene (a polycyclic aromatic hydrocarbon) show similar results when run on the 4-ethynylanaline column. As shown in Figure 44, adrenosterone and phenanthrene (♦ and ■, respectively) exhibit RP behavior only.

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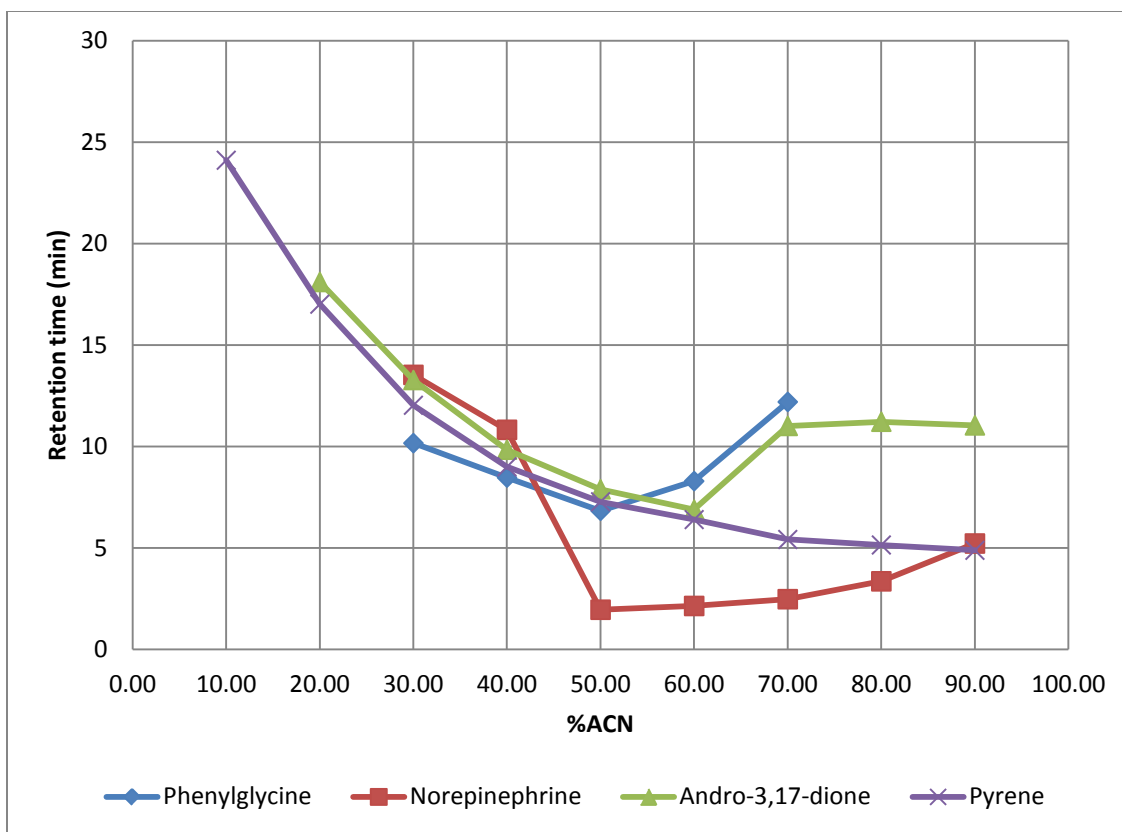
Figure 45. Retention map of the polar and non-polar analytes using the 4-ethynylanaline column without buffer

Figure 45 shows the retention maps for phenylglycine (polar), norepinephrine (polar), 4-androstene-3,17-dione (slightly non-polar) and pyrene (non-polar) using the 4-ethynylalanine column, (♦, ■, ▲, and ><, respectively). A comparison of the analyte retention maps shows that the stationary phase 4- ethynylalanine has reversed-phase properties but does not possess significant ANP properties.

IV.   CONCLUSIONS

The two foci of this study were to synthesize novel stationary phases that could perform in both reversed-phase and aqueous normal phase modes and characterization and identification of each column, in terms of the RP/ANP properties. Four organic moieties were successfully bonded to a silica hydride surface via silanization/ hydrosilation methods. Carbon analysis, DRIFT, and solid-state NMR all confirmed the attachment of four (out of five) organic moieties to the surface of the silica hydride base. HPLC tests on each column with a wide range of hydrophilic and hydrophobic analytes and isocratic conditions of ACN : H_2O mobile phases were designed to evaluate the RP, ANP, or RP/ANP mode of each newly synthesized column. It appeared that under these conditions, the 1,8-nonadiyne and 6-chloro-1-hexyne-bonded stationary phases showed both RP and ANP behaviors. The 5-cyano-1-pentyne-bonded stationary phase exhibited only ANP behavior, whereas the 4-ethynylanaline functioned only in the reversed-phase mode. Furthermore, the reproducibility studies and %RSD values of the columns, the 1,8-nonadiyne-bonded stationary phase in particular, confirmed the high degree of precision in obtaining the retention data.

However, the effect of other variables that were not factored in to this study and that might have shifted the results favorably or unfavorably should not be ignored. For instance, when performing the chromatographic runs with 5-cyano-1-pentyne-bonded stationary phase, there was the appearance of noise across the chromatogram for $\leq 30\%$ ACN: H_2O concentrations of the binary mobile phase.

The study also confirmed that the presence of a buffer (formic acid or other acidic additives) in the mobile phase played a significant role in the appearance or disappearance of RP, ANP, or RP/ANP behavior. The presence of 0.1% (v/ v) formic acid in the binary mobile phase caused an enhancement in the ANP behavior of columns that exhibited this property. In the case of the 5-cyano-1-hexyne-bonded stationary phase, adding buffer (0.1% volumetric) to the binary mobile phase caused the disappearance of noise in the 10% – 30% ACN region, allowing the bonded stationary phase to exhibit RP behavior.

The surface coverage of the bonded phase also played a role in the retention of analytes. The higher the surface coverage, the more likely for the analyte to be retained on the stationary phase, hence producing a longer retention time.

Additional work could be done to consider the effect of temperature on the RP/ANP behavior of stationary phases. Investigating the effect of various buffers could also provide valuable information on the retention properties, since in this study the ANP behavior of stationary phases was enhanced due to the presence of a small amount of acidic buffer in the mobile phase. Expanding the range of the analytes to molecules such as sugars and proteins/ peptides could provide additional information on behavior of newly synthesized stationary phases.

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