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Synthesis and evaluation of a short chain alkyl stationary phase using ELSD

Jayasree Pindi Venkat
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SYNTHESIS AND EVALUATION OF A SHORT CHAIN ALKYL STATIONARY
PHASE USING ELSD

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In partial fulfillment

of the requirement for the Degree

Master of Science

by

Jayasree Pindi Venkat

December 2007

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A handwritten signature in cursive script, appearing to read "J. J. Pesek", written over a horizontal line.

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ABSTRACT

SYNTHESIS AND EVALUATION OF A SHORT CHAIN ALKYL STATIONARY PHASE USING ELSD

by Jayasree Pindi Venkat

Evaluation of newly synthesized stationary phases using high performance liquid chromatography (HPLC) connected to an evaporative light scattering detector (ELSD) is the focus of this work. Bare silica was modified using the silanization/hydrosilation method, in which a terminal alkyne, 1-pentyne, was attached to the silica hydride surface. Modified silica stationary phases (C5) were then characterized using infrared spectroscopy, elemental analysis, and HPLC techniques. The efficiency of these newly synthesized stationary phases was evaluated using test solutes such as proteins, peptides, amino acids and sugars. The performance of the C5 columns was then compared to a C18 column prepared by the same technique. The newly synthesized columns worked better for protein and peptide separations than the C18 column. Sugars did not show much retention under the conditions used in this research.

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I INTRODUCTION

A. Chromatography

Chromatography is the most common technique for the separation of closely related components of a complex mixture. In a typical high performance liquid chromatography system, a column holds the stationary phase, and the mobile phase, which carries the sample, is pumped through the column. The components of the sample which have higher affinity for the stationary phase will spend more time in the column as opposed to those with higher affinities for the mobile phase. This difference in retention is the basis for separation of compounds in a mixture.

Different types of chromatography have been explored since its inception in 1906 by the Russian botanist, Mikhail Tswett. A fundamental classification of chromatographic techniques is the one based on the type of mobile phase and the type of stationary phase used. The three categories can be listed as: (1) gas chromatography (GC), (2) liquid chromatography, and (3) supercritical fluid chromatography [1]. As the names suggest, the mobile phases are a gas, a liquid, and a supercritical fluid respectively. It is well known that gas chromatography can only be used for volatile samples whereas liquid chromatography can be used for a variety of volatile, non-volatile and ionic samples [1].

Early liquid chromatography was carried out in glass columns under gravity-flow and separation times were often several hours. To overcome the difficulties associated with the use of conventional chromatography, researchers introduced high performance liquid chromatography in the late 1960's [1]. Since then HPLC has dramatically

improved with the invention of different system components and a variety of column packing materials. Different types of detectors and data handling devices have paved the way for a system with high levels of performance and sensitivity.

Based on the separation mechanism and the nature of the stationary phase, HPLC can be classified into five basic types: adsorption chromatography, partition chromatography, ion-exchange chromatography, size-exclusion chromatography and affinity chromatography. Partition chromatography can be further divided into (1) normal phase chromatography, where a non-polar mobile phase (hexane, ethyl acetate, etc.) and a polar stationary phase (cyano, amino bonded phases, etc.) are used and, (2) reverse phase (RP) chromatography, where a non-polar stationary phase (C18, C8, C5, etc.) and a polar mobile phase (acetonitrile-water, methanol-water, etc.) are used [1]. Aqueous normal phase (ANP) is a relatively new addition to the list and is unique to the columns prepared by silanization/hydrosilation procedure. In this mode, the retention of polar compounds increases as the amount of least polar component of the mobile phase increases [2]. For a water-acetonitrile system, the retention increases as the percentage of acetonitrile increases. The water component in this mobile phase is an important aspect because of the specific interactions that play a role in the retention. The columns based on this behavior can operate in both reversed phase and aqueous normal phase modes with a variation in the amount of water in the mobile phase. In this research both RP and ANP behavior have been observed with the columns used.

It can be deduced from the above description that the stationary phase and the mobile phase form the heart of the HPLC system and manipulation of these two

components can lead to the desired results. The mobile phase is also an important part of the chromatographic system and based on its use there are two different elution modes: isocratic and gradient. In isocratic elution, a constant mobile phase composition is pumped into the column throughout the separation, whereas in the gradient elution the composition of the mobile phase is changed throughout the separation. Based on the design, the shape of a gradient profile can be step or linear. A linear gradient is the simplest and most used design. In a linear gradient the percentage change of the organic component in the mobile phase is constant throughout the separation [3]. In gradient elution factors such as the percentage change of organic phase over time (steepness), the starting and ending compositions of the organic phase, the overall gradient time and the re-equilibration time play an important role. Gradient elution can be designed in the reverse phase mode or the normal phase mode depending on the nature of the solute. It is used more often when biological samples of high molecular weight or complex mixtures are to be separated using HPLC. Biological samples such as proteins and peptides are complex in nature and different parts of a single molecule may vary widely in their relative affinity for the stationary phase making it difficult to use isocratic elution. At the beginning of a gradient run, the retention of the sample will be strong, i.e., the retention factor (k') will be large and hence the sample remains near the column inlet. As the percentage of the organic solvent increases (reverse phase gradient), the k' value of the compound decreases and the sample moves through the column to the detector [3].

Some of the disadvantages of gradient elution are that there are several parameters to be controlled during a gradient run and the column has to be re-equilibrated after every

run in order to get reproducible retention times. Apart from these some of the traditional detectors show a prominent baseline drift as the gradient proceeds. Despite these disadvantages a gradient is almost always used for the analysis of most biological macromolecules [4].

The stationary phase on the other hand is usually rigid and impermeable. A myriad of variations are possible with this component of the HPLC system. Materials like silica, alumina, zirconia, polystyrene-divinylbenzene synthetic resin or an ion-exchange resin have been traditionally used as stationary phase materials [1]. Of all the materials, silica has been the most commonly used material due to its desirable properties which are described below.

B. Silica Surface

Silica particles are available in different pore sizes and surface areas and are compatible with both aqueous and organic mobile phases. They can operate between pH 2.00 to 7.50 and are stable under the high pressure conditions used for the separation [4]. Two types of silica have been used in this research: Astrosil and Vydac. Astrosil is narrow pore size silica (100 Å) whereas Vydac is wider pore material (300 Å). The pore size of silica is important since much of the interactive surface lies inside the pores of the stationary phase. In general, large pore silica is considered to be advantageous over narrow pore for the analysis of macromolecules since it provides sufficient room for interaction [3]. The structure of silica mainly consists of core siloxane moieties and chemically reactive surface silanol groups. Typically, a fully hydroxylated silica surface contains about 8 $\mu\text{mol}/\text{m}^2$ of surface silanol groups [1]. However, all the silanol groups

cannot be converted in bonded phase reactions due to steric effects and several other factors. The types of groups that form the structure of silica are shown in Figure 1 [5]. The silanol groups confer weak acidity to the material and can interact with the polar and basic compounds through hydrogen bonding and dipole-dipole interactions resulting in peak tailing and loss of chromatographic resolution. However these silanol groups also react with silane reagents to form bonded phases which greatly increase the applicability of silica.

C. Silica Modification

Silica is an extensively used stationary phase material, but when used in the native form it shows limited pH stability and peak tailing with the use of basic solutes. Also prolonged exposure to aqueous solutions decreases the specific surface area of silica. To overcome the disadvantages of bare silica, chemical modification of the surface of silica has been used. The silanols on the silica surface interact with the bonding moiety to form the bonded phase. Geminol and associated silanols are less acidic than free silanols and are considered “friendly” for separating basic compounds. Fully hydroxylated packings with high concentrations of geminol and associated silanols are the most desired types for HPLC applications [4].

Several methods have been traditionally used to modify the surface of silica by covalent bonding of an organic moiety. Some of the methods are described below.

1. Esterification

This method involves a reaction between an alcohol and the silanol. The reaction can be illustrated as follows:

This is a simple coupling reaction and results in a silicon-oxygen-carbon (Si-O-C) linkage between the silica surface and the organic moiety. Although the process is simple, the bonded phase is unstable in the presence of water and hence does not offer a significant advantage for routine HPLC applications. This process results in a thermally stable phase, but it cannot be used with aqueous mobile phases [6].

Figure 1. Types of silanols.

2. Organosilanization

Another process most commonly used for synthesizing several commercial columns is organosilanization [7, 8]. Silica based reversed phase packings are typically made by covalently bonding an organosilane on the silica surface. Organosilanization can be used to produce a monomeric phase or a polymeric phase depending on the silane used. Monochlorosilanes yield monomeric phases whereas trichlorosilanes yield polymeric phases.

Monomeric phase

X-halide and R-alkyl

Polymeric phase

Both the reactions yield a Si-O-Si-C linkage but differ in the properties of the bonded phase. The monomeric phase has good separation efficiency and is a reproducible reaction, but has poor hydrolytic stability under moderately acidic and moderately basic conditions. The polymeric phase on the other hand has better stability but is an irreproducible reaction [7, 8].

3. Chlorination followed by reaction with a Grignard's Reagent or an Organolithium Compound

This method involves chlorination of silica using a compound such as thionyl chloride leading to the formation of an unstable chlorinated intermediate. The second step involves reaction of this intermediate with an organometallic compound leading to the

formation of Si-C linkage which is stable but the by-product of this step is a metal salt which is difficult to wash off and can be a potential contaminant of the material [6, 8].

The reactions can be illustrated as follows:

OR

These limitations have paved way to develop a new kind of stationary phase which can overcome most of the limitations of the previous procedures and yet be simple.

4. Silanization/Hydrosilation

Silanization is a two step process, introduced by Pesek et al., [9]. It is characterized by the simplicity of the procedure and stability of the final product. The first step involves the formation of a stable intermediate called silica hydride which is then modified to form the organic bonded moiety. The reactions can be illustrated as follows:

Silanization:

Hydrosilation:

Cat. = Catalyst, metal complex such as hexachloroplatinic acid or free radical initiator such as t-butyl peroxide

The first step, silanization, involves formation of a silica hydride intermediate by reacting silica with triethoxysilane (TES) ($\text{HSi}(\text{OCH}_2\text{CH}_3)_3$) in the presence of trace amounts of water, an appropriate solvent, and an acid catalyst. This reaction results in a surface coverage of more than 95% with non-polar Si-H monolayer [9]. The second step, hydrosilation, involves the formation of an Si-C linkage using the hydride intermediate. The hydrosilation reaction is carried out either in the presence of a transition metal complex such as Speier's catalyst or a free radical compound such as t-butyl peroxide. Speier's catalyst is a solution of chloroplatinic acid hexahydrate ($\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$) dissolved in 2-propanol. The reaction between the metal (Pt) from the catalyst and silica hydride can result in the deposition of Pt on the surface. Free radical catalyst on the other hand is free of such deposition. The hydrosilation reaction results in the replacement of the surface hydrides with the organic group and results in the formation of thermodynamically and hydrolytically stable Si-C linkage [8, 9]. One of the important advantages of this method is the stable hydride intermediate which itself has been found to be useful in several chromatographic applications.

D. Alkyne Bonded Phase

Olefins have most commonly been used for the synthesis of bonded phases, but a variety of compounds ranging from simple alkynes to complex steroids have been used to produce bonded phases for different applications.

The hydrosilation reaction of an alkyne with the silica hydride results in the formation of a double Si-C linkage which has higher degree of stability [10]. Studies have shown that there are several possibilities for this attachment and the structures in Figure 2 illustrate a few possibilities [10]. In this research a terminal alkyne compound, 1-pentyne was used to synthesize the bonded phase.

E. Types of Samples

1. Proteins and Peptides

Separation of proteins and peptides is an important and challenging task in the biotechnological and pharmaceutical industries. Due to the complex structure of these proteins and peptides, more often gradient elution is required for their analysis [3]. Typically proteins have a hydrophobic core and a hydrophilic coating and hence the gradient method should be developed such that the initial conditions are hydrophilic to retain the protein in the column and then gradually increase the organic percentage to elute the protein. A low pH organic-buffer mobile phase is recommended, for reverse phase separations, to suppress ionization of any residual silanols that can interact with the basic amino acid side chains in the molecule and result in poor peak shape. Ion-pairing reagents such as trifluoroacetic acid are commonly used to increase the retention of small and poorly retained peptides and proteins by ion-pairing with the free amino terminus of

basic amino acids. Reverse phase chromatography often leads to the unfolding of the protein to a certain extent due to the acidic conditions used in the analysis [4].

Figure 2. Possible structures of alkyne bonded silica stationary phase.

2. Amino Acids

These are the building blocks of proteins and peptides and their analysis has been a challenging task in the field of chemistry. By definition, an amino acid is a molecule that contains both amine and carboxylic acid functional groups. The difference between the amino acids stems from the difference in their side chain. Depending on the polarity of the side chain the hydrophobic character of the molecule changes [3]. Amino acids form short polymer chains called peptides and long chains called proteins via condensation reactions. The distribution of hydrophilic and hydrophobic amino acids in a protein determines its tertiary structure which in turn determines the interactions of the proteins with the stationary phase. Most of the amino acids cannot be detected with the traditionally used ultraviolet (UV) detector and hence require derivatization, a time consuming process, before they can be detected. However, with the evaporative light scattering detector (ELSD), amino acids can be detected without any derivatization [3]. One of the goals of this research was to record the behavior of amino acids using a short chain alkyl stationary phase prepared by the silanization/hydrosilation procedure using an HPLC instrument in conjunction with an ELSD. The results were then compared to a C18 column prepared via the same process.

3. Sugars

These form a class of compounds which have weak chromophores and are difficult to detect using a traditional UV detector. ELSD provides a useful means for the detection of these carbohydrates without derivatization. Separation of sugars is very important in

the food industry [3]. This research attempts to separate mono, di, and tri saccharides using three columns under aqueous normal phase conditions.

F. Characterization Techniques

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFT), elemental analysis, the Neue test and high performance liquid chromatography techniques have been used to evaluate the newly synthesized stationary phase. A general description of these evaluation methods is given below.

1. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT)

Infrared spectroscopy is based on the vibrations of the atoms of a molecule along chemical bonds and the spectra obtained are due to the transitions of atoms from one vibrational state to another [1]. Hence it provides qualitative information about the structure of the molecules and is often considered the finger print of the molecule. The infrared region of the spectrum ranges from $12,800\text{ cm}^{-1}$ to 10 cm^{-1} . Within this range the region is divided into near, mid, and far infrared regions. The mid-infrared region which ranges from 4000 cm^{-1} to 200 cm^{-1} is most often used to obtain the structural information for molecules [1]. When a molecule absorbs infrared radiation, there is a net change in the dipole moment as a result of its vibrational and rotational motions. When the natural vibrational frequency of the molecule matches the frequency of the incident infrared radiation, the radiation is absorbed. Diffuse reflectance occurs when radiation impinging on the surface is partially reflected and partially transmitted. As the light passes into the material it can again be reflected or transmitted. Hence the sum of the surface reflected and the bulk re-emitted radiation constitute the diffuse reflectance of the sample [11].

The Fourier transform mode allows for signal averaging thereby increasing the sensitivity and hence allowing the characterization of samples with weak absorption bands [1].

DRIFT spectroscopy is used in this research to study the bonding characteristics at the surface of silica.

2. Elemental Analysis

Elemental analysis is used to determine the percentages of certain elements in the sample [12]. This method was used in this research to determine the amount of carbon in the surface modified silica. Elemental analysis involves the combustion of the material to carbon dioxide and water for determining the amounts of carbon and hydrogen. The surface coverage estimation gives an idea of the amount of organic group bonded to the silica surface. Surface coverage can be calculated using an equation proposed by Berendson and De Galan [12]:

$$\alpha_R (\mu \text{ mol/m}^2) = \frac{10^6 P_c}{(100M_c n_c - P_c M_R) S_{\text{BET}}}$$

P_c - carbon percentage of the bonded material

M_c - atomic weight of the carbon

n_c - number of carbon atoms in the bonded organic group

M_R - molecular weight of the organic compound

S_{BET} - specific surface area (m^2/g)

3. Neue Test

This test is used to determine the hydrophilicity and silanophilicity of the bonded stationary phase. Neue and co-workers [13] have developed a method by which a new

column can be characterized to give an idea of the type of compounds it would retain.

Based on the values of the hydrophobicity and silanophilicity, the relative behavior of the column can be predicted.

3.1. Hydrophobicity Parameter (v)

Hydrophobicity Parameter indicates how well the column can retain non-polar solutes. A higher value indicates that the column has greater affinity towards non-polar solutes.

Naphthalene and acenaphthene are used as test solutes for this test. The hydrophobicity parameter can be calculated using the following equation:

$\ln(k) = v \times V - 3.068$ where V is the molar volume of the analyte (acenaphthene or naphthalene), k is the capacity factor, v is the hydrophobicity parameter [13].

3.2. Silanophilic Interaction Parameter (S)

Silanophilic Interaction Parameter measures strong polar/adsorptive interactions with the surface. A Lower value is an indication of less surface adsorption which results in better peak shapes for polar analytes [13]. Amitriptyline is used as the test solute and uracil (unretained compound) is used to determine the dead volume of the column.

Silanophilicity can be determined using the following equation:

$$S = \ln(k_{\text{amitriptyline}}) - 0.7124 \times \ln(k_{\text{acenaphthene}}) + 1.9748$$

4. High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is the most commonly used technique by separation scientists in resolving complex mixtures of compounds. A schematic of an HPLC system is shown in Figure 3. HPLC utilizes a mobile phase (pumped under high

pressure) and a stationary phase to resolve the components of a mixture. The sample to be analyzed is first dissolved in an appropriate solvent and then injected into the stream of mobile phase which is pumped at high pressure into the column. The eluent then passes into the detector and the output can be obtained in the form of a chromatogram with the help of data handling devices. Resolution of sample components is an important consideration and is dependant upon the extent of interaction between the solute components and stationary phase. The extent of interaction depends on the type of column and the type of mobile phase used.

Apart from the stationary phase and mobile phase, the detector is also an important part of the HPLC system. There are several types of detectors, like UV, fluorescence, refractive index, evaporative light scattering, and a mass spectrometer. The selection of the detector partly depends on the purpose of the analysis and the nature of the sample. A mass spectrometer is the most sensitive of all the detectors listed above and is considered a universal method. It can also provide the molecular information of the sample. UV detectors are the most widely used detectors but the compound should have a chromophore to absorb the radiation. Use of a fluorescence detector requires the presence of a fluorophore in the sample to be detected. Samples which do not have these radiation absorbing moieties must be derivatized before they can be detected. The refractive index detector is considered a universal system as it gives a signal based on the changes in the refractive index of the mobile phase composition. The main disadvantage of this type of detector is that it cannot be used under gradient elution conditions [3].

An evaporative light scattering detector has good sensitivity, can be used with gradient elution and does not require sample derivatization. The relationship of concentration of sample to peak area is non-linear since the light scattering process is a function of the particle size rather than the concentration of the sample [14].

Figure 3. Schematic of an HPLC system.

The ELSD operates based on three simple steps: nebulization, evaporation and detection. In the first step, the column effluent passes through a needle and mixes with the nitrogen gas to form an aerosol. This aerosol then passes through a heated drift tube where the mobile phase evaporates leaving a fine, uniform mist of sample particles in solvent vapor. These sample particles in the solvent vapor pass through a cell and scatter light from a

laser beam. The scattered light is detected with the help of a photodiode generating a signal. This type of detector is especially useful for the detection of solutes like amino acids since they are difficult to handle with a traditional UV detector [15].

ELSD detectors require a means to divert part of the aerosol to be able to effectively operate under the high flow rates commonly encountered in HPLC. One of the earliest designs involved a nebulization chamber with flow restriction caused by a sharp turn. In this design larger particles fail to make the turn and smaller particles would successfully move towards the detection cell. While this design works well for high flow rates and difficult to evaporate mobile phases, much of the sample is sent to the waste under less severe operating conditions compromising the detector sensitivity. One of the relatively recent solutions to this problem is the plate impactor where larger particles hit the plate and condense into the drain while smaller particles make it through the annular spaces of the impactor. In this design, the plate impactor is either on or off and there is no in between setting for moderate flow rates and moderate to evaporate mobile phases [16].

The detector used in this research is the SofTA Model 400 ELSD. It uses a patent pending thermo-split technology which allows a smooth variation of the aerosol cloud over a wide range [16]. The aerosol splitter (spray chamber) combines a gentle bend with temperature controlled walls to vary the split ratio of the aerosol. For a mobile phase with high organic content (easily evaporated mobile phase), the walls are heated. Under increased temperature, the solvent rapidly evaporates and all the particles negotiate the bend. For mobile phases with high aqueous content (difficult to evaporate mobile phase),

or for high flow rates, the walls of the chamber are cooled. Under sub-ambient conditions, a part of the aerosol condenses to form larger particles which cannot negotiate the bend and are sent down the drain. The main advantage of this system is that the split ratio can be controlled in a smooth analog fashion by simply varying the temperature. The temperature of the spray chamber can be varied from 0° C to 80° C in 1° C increments [16]. Apart from these features, the detector also has a user-friendly touch screen which allows for convenient on-line monitoring. A schematic representation of the thermo-split technology can be seen in Figure 4. Figure 4A represents the heated surface and 4B represents the cooled surface where the solvent condenses down the drain [16].

Advantages of ELSD:

- a. It is a near universal detector as it can detect almost everything that is less volatile than the mobile phase.
- b. It gives a more accurate representation of sample mass than most commonly used detectors.
- c. It simplifies sample preparation by eliminating the sample derivatization step.
- d. It has detection limits in the low nanogram range.
- e. It gives a very stable baseline even with rapid gradients.
- f. Lower investment and lower operating costs than a mass spectrometer.

Limitations of ELSD:

- a. Only samples which are less volatile than the mobile phase can be detected.
- b. Any mobile phase modifiers used should be volatile. Non-volatile mobile phase modifiers will lead to noise in the detector.

A

B

Figure 4. A schematic representation of thermo-split technology. A. Heated surface when using an easy to evaporate mobile phase. B. Cooled surface when using a difficult to evaporate mobile phase.

G. Research Goals

One major goal of this research work is to synthesize a short chain alkyl stationary phase and test its applicability for the separation of biological macromolecules using an HPLC-ELSD system. To obtain the bonded phase, a silica hydride intermediate was initially synthesized using the silanization method. Then a 5 carbon terminal alkyne was used for the synthesis of the C5 bonded phase via the hydrosilation method. The reaction was carried out in the presence of both Speier's catalyst and t-butyl peroxide catalyst at different temperatures to determine the optimal conditions. This modified silica was characterized using elemental analysis, spectroscopic and chromatographic techniques. These methods were used to determine the type and extent of bonding of the organic compound on silica. Chromatographic evaluation was used to determine the performance of the newly synthesized bonded phase and to compare the performance with respect to a C18 column.

The other goal of this work is to compare the performance of the ELSD detector with the conventional UV detector for the detection of proteins and peptides. The behavior of the columns with four different types of solutes (proteins, peptides, amino acids and sugars) comprise the main focus of this research work. For proteins and peptides, gradient elution was used due to the complexity of the samples. Amino acids were studied using different mobile phase compositions of acetonitrile, water (with formic acid) and acetonitrile, ammonium acetate in the isocratic mode. Separation of sugars was studied in the ANP mode using different mobile phase compositions of acetonitrile and water.

II Experimental

A. Materials

Table 1. Types of silica used in the synthesis of bonded phases

Type of Silica	Astrosil	Vydac
Surface Area	320 m ² /g	106 m ² /g
Pore Size	100 Å ^o	300 Å ^o
Particle Size	4.5 µm	6.5 µm

Table 2. Chemicals used in synthesis and characterization

Chemical Name	Manufacturing Company
1-Pentyne	GFS Chemicals, Columbus, OH
Hexachloroplatinic acid	Aldrich Chemical Co., St Louis, MO
t-butyl peroxide	Aldrich Chemical Co., Milwaukee, WI
Dioxane	Fisher Chemicals, Fair Lawn, NJ
Triethoxysilane	Aldrich Chemical Co., Milwaukee, WI
Toulene	Fisher Chemicals, Fair Lawn, NJ
Diethyl ether	Fisher Chemicals, Fair Lawn, NJ
Chloroform	J.T.Baker Chemical Co., Phillipsburg, NJ

Table 3. Chemicals used in HPLC separations

A. Chemicals used for Neue test

Chemical	Manufacturing Company
Uracil	Matheson Coleman & Bell, Cincinnati, Ohio
Amitriptyline	J.T.Baker Chemical Co., Phillipsburg, NJ
Naphthalene	Packard Instrument Company, Downers Grove, ILL
Acenaphthene	J.T.Baker Chemical Co., Phillipsburg, NJ
Methanol	J.T.Baker Chemical Co., Phillipsburg, NJ
Potassium dihydrogen phosphate	Matheson Coleman & Bell, Cincinnati, Ohio
Potassium monohydrogen phosphate	Matheson Coleman & Bell, Cincinnati, Ohio

B. Chemicals used for protein, peptide, amino acid and sugar separations

Chemical	Manufacturing Company
Acetonitrile	Spectrum Mfg. Corp., Gardena, CA
Methanol	J.T.Baker Chemical Co., Phillipsburg, NJ
Formic acid	Spectrum Mfg. Corp., Gardena, CA
Trifluoroacetic acid	Spectrum Mfg. Corp., Gardena, CA
Ammonium acetate	EM Science, Gibbstown, NJ

Table 4. Samples used for characterization of the column

A. Proteins used in the analysis

Protein Sample	Source	Molecular Weight (KDa)	Manufacturing Company
Hemoglobin	Human	64.5	Sigma Chemical Co., St Louis, MO
Cytochrome C	Equine heart	12.4	Sigma Chemical Co., St Louis, MO
Apotransferrin	Human	76	Sigma Chemical Co., St Louis, MO
Alpha-casein	Bovine milk	23.6	Sigma Chemical Co., St Louis, MO
Albumin	Bovine Serum	66	Sigma Chemical Co., St Louis, MO
Albumin	Chicken egg white	45	Sigma Chemical Co., St Louis, MO
Myoglobin	Horse skeletal muscle	17.6	Sigma Chemical Co., St Louis, MO
Alpha-lactalbumin	Bovine Milk	14.2	Sigma Chemical Co., St Louis, MO

B. Peptides used in analysis

Name	Amino Acid Sequence	Manufacturing Company
Peptide YY Human	Tyr-Pro-Ile-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro-Glu-Glu-Leu-Asn-Arg-Tyr-Tyr-Ala-Ser-Leu-Arg-His-Tyr-Leu-Asn-Leu-Val-Thr-Arg-Gln-Arg-Tyr-NH ₂	Sigma-Aldrich Inc., St. Louis, MO
Bradykinin acetate	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Sigma-Aldrich Inc., St. Louis, MO
Angiotensin II human acetate	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	Sigma-Aldrich Inc., St. Louis, MO
5-[Methionine] Enkephalin acetate salt hydrate	Tyr-Gly-Gly-Phe-Met	Sigma-Aldrich Inc., St. Louis, MO
[5-Leucine] Enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH	Sigma-Aldrich Inc., St. Louis, MO
[Ala ⁹²] - Peptide 6 Human	Asp-Ala-Ala-Arg-Glu-Gly-Phe-Leu-Ala-Thr-Leu-Val-Val-Leu-His-Arg-Ala-Gly-Ala-Arg	Sigma-Aldrich Inc., St. Louis, MO
[D-Ala ² , D-Met ⁵]-Enkephalin acetate salt	Tyr-D-Ala-Gly-Phe-D-Met	Sigma-Aldrich Inc., St. Louis, MO

C. Amino acids used in the analysis

Name	Manufacturing Company
Histidine	Sigma Chemical Co., St Louis, MO
Arginine	Matheson Coleman & Bell, Cincinnati, Ohio
Lysine	Sigma Chemical Co., St Louis, MO
Leucine	Cal Biochem, Los Angeles, CA
Isoleucine	Sigma Chemical Co., St Louis, MO
Phenylalanine	Matheson Coleman & Bell, Cincinnati, Ohio
Tyrosine	Cal Biochem, Los Angeles, CA
Tryptophan	Sigma Chemical Co., St Louis, MO

D. Sugars used in the analysis

Name	Manufacturing Company
Glucose	Sigma Chemical Co., St Louis, MO
Fructose	Sigma Chemical Co., St Louis, MO
Xylose	Sigma Chemical Co., St Louis, MO
Maltose	Fisher Chemicals, Fair Lawn, NJ
Lactose	Fisher Chemicals, Fair Lawn, NJ
Sucrose	Fisher Chemicals, Fair Lawn, NJ
Raffinose	Fisher Chemicals, Fair Lawn, NJ
Melzitose	Sigma Chemical Co., St Louis, MO

B. Synthetic Procedures

1. Silanization

Prior to the synthesis, all the required glassware and 15.00 g of Astrosil silica were dried overnight at 100° C in a vacuum oven. This dried silica was transferred to a 1000 mL, 3-neck round bottomed (RB) flask. The RB flask was equipped with a thermometer, a condenser with a drying tube, an addition funnel with an equalizing tube, a stopper, a magnetic stirrer, a magnetic stir bar, and a heating mantle. 600 mL of dioxane was added to the RB flask followed by the dried silica and 7.35 mL of 2.3 M HCl solution (catalyst) and the reaction mixture was heated to 70° C. TES (triethoxysilane) mixture was prepared by placing 90.15 mL of dioxane in the addition funnel equipped with a stopper and then adding 20.85 mL of TES to the dioxane under argon. After the temperature remained constant at 70° C, the TES mixture was added drop wise by using an addition funnel over a period of 25-30 min with constant stirring. The temperature was then raised to 90° C and the mixture was allowed to reflux for 90 min. After a cooling period, the product was transferred to a medium sized filter crucible, attached to a filtering flask. The product was filtered using vacuum suction leaving a solid silica hydride layer. It was then washed two times with dioxane (50 mL each time), three times with toluene (50 mL each time) and three times with diethyl ether (50 mL each time). The final product was then dried at room temperature overnight to evaporate the ether used for washing and finally the finished product was dried in the vacuum oven overnight at 110° C.

2. Hydrosilation

2.1. Reaction with Free Radical Catalyst

The bonded phase synthesis was carried out first on a small scale using 0.5 g of Astrosil silica hydride at 100° C and 60° C final temperatures in two separate experimental setups. The optimized conditions were then scaled up. The experimental setup was similar to the one used for silanization, i.e., the RB flask was fitted with a thermometer, a condenser with a drying tube, a glass stopper, a magnetic stirrer, a magnetic stir bar, and a heating mantle. Prior to hydrosilation, all the glassware was dried in a vacuum oven, overnight at 110° C. A mixture of 57.5 mL of toluene, 1.85 mL of 1-pentyne and 116.05 µL of t-butyl peroxide (free radical catalyst) were placed in a 250 mL RB flask and heated to 50° C. The reaction was allowed to continue with constant stirring at a stable temperature of 50° C for 1 hour and this time period is called the induction period. Then 2.5 g of silica hydride was added slowly to the flask through the open neck by carefully removing the glass stopper. The flask was flushed with nitrogen and the temperature of the reaction was raised to 100° C. This reaction was allowed to proceed for 110 hours with continuous stirring. After this time period the reaction mixture is allowed to return to room temperature and was transferred to a filter crucible to remove the solvent with vacuum suction and the solid was washed with five 30 mL portions of toluene, followed by similar washings of diethyl ether. The washed final product was left in the fume hood overnight to evaporate ether and then dried overnight in a vacuum oven at 110° C.

2.2. Reaction with Speier's Catalyst

The bonded phase synthesis was carried out first on a small scale using 0.5 g of Astrosil silica hydride at 100° C final temperature. The optimized reaction was then scaled up. This optimized experimental procedure was also used to prepare bonded phase material using Vydac silica hydride. The experimental setup consisted of an RB flask equipped with a thermometer, a condenser with a drying tube, a glass stopper, a magnetic stirrer, a magnetic stir bar, and a heating mantle. In a 100 mL RB flask, 25 mL of toluene, 5 mL of 1-pentyne and 312.5 µL of platinum catalyst were placed and the reaction mixture was heated to 50° C for 1 hour with constant stirring. After this induction period, 2.5 g of Astrosil silica hydride was added to the RB flask and the temperature was raised to 100° C. The reaction was allowed to proceed for 110 hours at this temperature with constant stirring. After this time period the reaction mixture is allowed to return to room temperature and was transferred to a filter crucible to remove the solvent with vacuum suction and the solid was washed with five 30 mL portions of toluene, followed by similar washings of diethyl ether. The washed final product was left in the fume hood overnight to evaporate ether and then dried overnight in a vacuum oven at 110° C.

C. Instrumental Procedures

1. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT)

After the synthesis, DRIFT spectra were obtained using an ATI Mattson Infinity series FTIR spectrometer. The spectrometer was equipped with a deuterated triglycine sulfate (DTGS) detector and controlled by WinFIRST™ software. To handle the samples, an EasiDiff diffuse-reflectance accessory with a 1.6 mm deep sampling cup was used.

Before the start of the analysis, the instrument was purged with nitrogen gas from a liquid nitrogen tank to provide moisture free samples. The samples for the analysis were prepared by mixing 95% of bonded silica with 5% (by weight) KBr and finely grinding the mixture. This mixture was placed in the sampling cup and the surface was smoothed with the help of a spatula. The sampling cup was placed inside the instrument to obtain the IR spectrum characteristic of the modified surface. The spectra were collected in the mid-infrared region ($4000\text{-}450\text{ cm}^{-1}$) averaged over 100 sample scans. All the sample scans were referenced to KBr.

2. Carbon Elemental Analysis

Small amounts (5 mg) of the bonded phases which were obtained after the hydrosilation step were sent to Desert Analytics (Tucson, AZ) for the determination of the carbon content. A conventional combustion method was used for the analysis of each sample. The results were obtained as the percentage carbon in the bonded organic molecule from which the surface coverage values were calculated.

3. Column Packing

The columns used in this research were packed in-house by a slurry method using a CCl_4 -methanol (9:1 v/v) mixture. Stainless steel tubes of dimensions (150 x 4.6 mm i.d) provided by Alltech, Deerfield, IL were used to pack the bonded phase material. A pneumatic pump operated at 6000 psi pressure, with HPLC grade methanol as the driving solvent, was used to pack the columns. After completion the column was held in position for about 30 minutes before removal.

4. High Performance Liquid Chromatography (HPLC)

Two different HPLC systems were used in this research. The first system was equipped with a SoftA corporation model 400 Evaporative Light Scattering Detector (ELSD), a LDC MiltonRoy CM4000 multiple solvent delivery system, a rheodyne model 7126 manual injector with 20 μ L injection loop, a waters in-line degasser. The detector required a continuous supply of nitrogen at 65 psi pressure (with approximately 2.5 SLPM gas consumption rate) for nebulization of the column eluent. It uses a 670 nm laser diode and hermetically sealed photodiode for the light scattering and detecting processes. The data collection system consisted of Chemstation software, a HP 35900C multichannel interface, and a HP Deskjet 693C printer. Mobile phases used in the analysis were vacuum filtered prior to the analysis using a 0.2 μ m Nylon 66 membrane (Alltech Associates, Inc. Deerfield, IL). For all HPLC separations a 0.5 mL/min mobile phase flow rate was used. Deionized water was prepared on a Milli-Q^{TM} purification system (Millipore Corp., Bedford, MA).

The second system was equipped with a HP series 1050 variable wavelength detector, an HP 1050 quaternary pump, HP 1050 autosampler and a Waters in-line degasser. Chemstation software was used to obtain the chromatograms after the analysis. Mobile phases used in the analysis were vacuum filtered prior to the analysis using a 0.2 μ m Nylon 66 membrane (Alltech Associates, Inc. Deerfield, IL). For all HPLC separations a 0.5 mL/min mobile phase flow rate was used. Deionized water was prepared on a Milli-Q^{TM} purification system (Millipore Corp., Bedford, MA).

III Results and Discussion

The main goal of this research was to synthesize a short chain alkyl stationary phase and investigate its properties both spectroscopically and chromatographically. The following sections will start with a discussion of the synthesis results followed by chromatographic data. For the confirmation of the bonding, DRIFT and elemental analysis techniques were used. The respective data will be discussed in detail in the following sections. To determine the efficiency of this newly synthesized material, HPLC technique was used. Finally behavior of four types of solutes (proteins, peptides, amino acids and sugars) on three columns will be discussed.

A. DRIFT Spectroscopic Evaluation

1. Confirmation of formation of silica hydride

The DRIFT technique provides important information regarding the functional groups present in a compound. In this research, this technique was used to provide valuable information about the molecules that are chemically bonded to the surface. The DRIFT spectra of silica hydride using Astrosil silica and Vydac silica are shown in Figures 5 and 6 respectively. The most intense band at 2250 cm^{-1} , in both the spectra, represents the Si-H stretching band. This confirms the successful formation of Si-H intermediate after the initial silanization reaction with TES. The broad band between 3000 and 3700 cm^{-1} in both the spectra represents the adsorbed water and H-bonded OH groups (silanols) from the silica. The spectra show similar characteristics, indicating that the same reaction has taken place in both the cases.

Figure 5. DRIFT spectrum of Astrosil silica hydride.

Figure 6. DRIFT spectrum of Vydac silica hydride.

2. Confirmation of Formation of Bonded Phase

After the silanization step, the silica hydride intermediate was subjected to hydrosilation which led to the formation of the organic bonded phase. Figures 7 and 8 show the DRIFT spectra of the 1-pentyne bonded phase using Astrosil hydride and Vydac hydride respectively. In both the cases hexachloroplatinic acid was used as the catalyst at 100° C. The following are the common features in both spectra:

- a. A loss of intensity of the Si-H band at 2250 cm^{-1} indicating that the hydride monolayer has participated in the hydrosilation reaction.
- b. The appearance of strong C-H stretching vibration bands in the range of $2800\text{--}3100\text{ cm}^{-1}$ indicative of the formation of the organic (pentyl) bonded phase.

These common characteristics are indicative that a similar reaction has taken place in both cases.

B. Elemental Analysis

Surface coverage values are primarily used as indicators of the extent of the bonded phase reaction. Table 5 shows the surface coverage values of 1-pentyne bonded Astrosil and Vydac hydrides. The results showed a higher surface coverage value for the Vydac bonded phase compared to the Astrosil bonded phase. This indicates a greater amount of bonding in the Vydac bonded phase although the experimental conditions for both reactions were the same. Since the Vydac hydride used in the experiment was obtained from an earlier synthetic procedure which was not completely optimized, there is a possibility that the reaction might have led to the formation of more than a monolayer. This might have led to a higher degree of bonding of the organic moiety which is

reflected in the higher surface coverage value. There is also a possibility that due to the wider pore size of Vydac silica, there was less steric hindrance leading to higher surface coverage value. Nevertheless, the surface coverage values of both silicas indicated that the bonding was good so that chromatographic evaluation was possible.

Figure 7. DRIFT spectrum of 1-pentyne bonded to Astrosil silica hydride using Speier's catalyst.

Figure 8. DRIFT spectrum of 1-pentyne bonded to Vydac silica hydride using Speier's catalyst.

Table 5. Elemental analysis results and surface coverage values

Type of Silica	Compound	Percentage Carbon (% C)	Surface Coverage (α) $\mu\text{mol/m}^2$
Astrosil	1-Pentyne	6.45	3.62
Vydac	1-Pentyne	4.59	7.61

C. Chromatographic Evaluation

Chromatographic performance of the columns has been tested by analyzing a variety of solutes. Initially the hydrophobicity and silanophilicity of the columns were determined using four types of test solutes. This determination can provide information about the type of compounds retained by the column.

1. Neue Test

This test is used to determine the hydrophobic and silanophilic interactions of the column. The test solutes used are shown in Figure 9. The results of this test can be used to compare column characteristics based on a standard set of conditions. Acenaphthene and naphthalene are used as test solutes for determining the hydrophobicity of the column. The retention time of these solutes is a measure of the retaining capacity of the column for non-polar compounds. Uracil is used to determine the dead volume of the column. The silanophilic test which uses amitriptyline, gives a measure of the polar interactions of the column.

Uracil

Naphthalene

Acenaphthene

Amitriptyline

Figure 9. Structures of solutes used for Neue test.

The separation of the test solutes was carried out in the isocratic mode using 35:65 20 mM phosphate buffer/ methanol mobile phase at pH 7. The concentrations of the samples are as follows: uracil 16 mg/L, naphthalene 60 mg/L, acenaphthene 200 mg/L and amitriptyline 100 mg/L in methanol. The flow rate was set at 0.5 mL/min at ambient temperature. The wavelength of the detector was set at 214 nm. Table 6 shows the results obtained in this test. From the results, it is evident that the column shows typical reverse phase behavior with a greater retention of the most non-polar analyte and the least retention for the most polar analyte. Although both the columns were prepared by a similar technique, the retention times are slightly different due to the difference in the underlying silica type. Wider pore silica (Vydac) usually shows lower retention values than narrow pore silica (Astrosil) under similar experimental conditions due to lower surface area of the former type of silica.

Table 6. Neue Test Results

Compound	Astrosil C5 Column Retention Time(min)	Vydac C5 Column RetentionTime(min)
Uracil	3.66	3.64
Naphthalene	5.91	5.51
Acenaphthene	7.09	6.88
Amitriptyline	3.59	3.56

2. Protein Analysis

Analysis of proteins in this study involved eight compounds with two gradient time tables on three columns. The newly synthesized stationary phases were packed into two 150 x 4.6 mm i.d columns. A C18 column (75 x 4.6 mm i.d) was used for comparative purposes. The main goal of this work was to determine the qualitative separation efficiency of the columns. The flow rate was set at 0.5 mL/min, at ambient temperature with a 20 μ L injection volume for all separations. The concentration of the protein samples was 1 mg/mL in DI water. The parameters for the ELSD detector were adjusted according to the initial conditions of the gradient. The temperatures of the drift tube and the spray chamber were adjusted until a smooth baseline was observed. The spray chamber temperature for most analyses was maintained at ambient or sub-ambient temperature (25° C). The drift tube temperature was set high enough to evaporate the mobile phase (45° C or 55° C). At the start of each day, a blank run was performed to ensure that the column was free of contamination from previous runs. In addition to the light scattering detector, a UV detector was used with the same gradient conditions and flow rate as for the ELSD. The wavelength used for all the separations was 214 nm as it is the wavelength where the peptide bonds absorb radiation. All the runs were performed at ambient temperature with an injection volume of 5 μ L. The gradient programs used in this work are shown in Table 7. The main goal of this work is to study the behavior of proteins on C5 stationary phase and the following discussion will provide some detail regarding this goal.

Table 7. Mobile phase gradients used for the separation of proteins and peptides

Gradient A			Gradient B		
Time	A%	B%	Time	A%	B%
0.0 min-	70	30	0.0 min -	80	20
15.0 min-	30	70	15.0 min -	20	80
20.0 min-	30	70	30.0 min -	80	20
30.0 min-	70	30	40.0 min-	80	20
40.0 min-	70	30			

Where A% - 0.1% Trifluoroacetic acid in DI water

B% - Acetonitrile

2.1. Effect of Column

With a difference in the hydrophobicity of the column, the behavior of proteins changes significantly. A mixture of myoglobin, cytochrome C and alpha-lactalbumin was prepared and analyzed using the three columns with the HPLC system connected to the ELSD. Figure 10 shows the separation on the Astrosil C5, Vydac C5 and C18 columns respectively using the same gradient conditions. For all the columns the proteins eluted in the same order but with different retention times. The smallest of the three proteins, cytochrome C eluted first followed by alpha-lactalbumin and finally myoglobin in all the columns. With the Astrosil C5 column it can be seen that alpha-lactalbumin and

myoglobin elute very closely whereas on the Vydac C5 column the proteins are baseline separated. This is because Vydac silica provides a greater pore volume for the proteins and hence each protein has the freedom to diffuse in and out of the pores. In Astrosil silica (narrow pore silica) there is more restricted diffusion of the macromolecules, and hence limited resolution is obtained. With the C18 column it can be seen that the later two peaks are not well separated and also the peaks show slight splitting when compared to the C5 columns. Since the C18 column is more hydrophobic than the C5 column, the proteins might have unfolded partially in the column leading to a distortion in the peak shape.

Figure 11 shows the separation of apotransferrin and hemoglobin on the three columns. With all the columns the proteins eluted in the same order but with different retention times. Apotransferrin (molecular weight 76 KDa) eluted first followed by hemoglobin (molecular weight 64.5 KDa). The reason for this is that the retention of the proteins on a reverse phase column is based predominantly on the interactions of the bonded phase with the hydrophobic sites of the protein. Hence it can be deduced that more hydrophobic moieties in the protein, result in a longer retention time. As with the previous example, in this example too, the Vydac column has performed better than the Astrosil C5 and the C18 columns. In the C18 column there are prominent small peaks immediately after the main peaks indicating a possibility that some of the hydrophobic moieties have adsorbed strongly onto the phase leading to partial unfolding.

Figure 10. Separation of cytochrome C, alpha-lactalbumin and myoglobin on the three columns. Peak identification: 1. Cytochrome C 2. Alphalactalbumin 3. Myoglobin. Mobile phase gradient A. Drift tube temperature 55° C and spray chamber temperature 25° C.

Figure 11. Separation of apotransferrin and hemoglobin on the three columns. Peak identification: 1. Apotransferrin 2. Hemoglobin. Mobile phase gradient B. Drift tube temperature 55° C and spray chamber temperature 25° C.

2.2. Effect of Detector

With a change in the detector, the chromatographic detail differs to a certain extent. The traditional UV detector shows a drift in the baseline due to the gradient conditions used, whereas the ELSD shows a stable baseline. Figure 12 shows a comparison of the chromatograms obtained with UV and ELSD detectors on the Astrosil C5 column. The chromatogram obtained using the UV detector, does not show a prominent peak and the baseline drift makes it difficult to quantify the peak. In the chromatogram obtained by the ELSD detector, the baseline is stable with a prominent myoglobin peak and it can be used for quantitative measurements. The UV data does not show a prominent peak for myoglobin under similar conditions. In order to have a successful analysis, multiple runs using a different gradient or a different wavelength are required.

Figure 13 shows the separation of two types of albumin (bovine serum and chicken egg white) on the C18 column. From the chromatograms, it can be seen that both the separations are almost comparable. The peak shapes are similar and the elution order is also the same. A small peak in between the two peaks is more prominently observed with the ELSD than with the UV detector. The peak might be an impurity in the sample. However, it is noteworthy that with the ELSD detector, it was possible to see this minor component in contrast to the UV method.

Figure 14 shows the comparison of the chromatograms obtained with UV and ELSD detectors on the Vydac C5 column. The chromatogram obtained using the UV detector shows a prominent baseline drift making it difficult to quantify the peak. In the chromatogram obtained by the ELSD detector, the baseline is stable with a prominent

alpha-casein peak. A small peak associated with the main peak is observed in both chromatograms. This peak appears to be well separated from the main peak in the ELSD data.

Figure 12. Chromatograms of myoglobin on the Astrosil C5 column using UV and ELSD detectors. Mobile phase gradient A. Drift tube temperature 55° C and spray chamber temperature 25° C.

Figure 13. Separation of two types of albumin on the Astrosil C18 column using UV and ELSD detectors. Peak identification: 1. Bovine serum albumin 2. Chicken egg white albumin. Mobile phase gradient A. Drift tube temperature 55° C and spray chamber temperature 25° C.

Figure 14. Chromatograms of alpha-casein on the Vydac C5 column using UV and ELSD detectors. Mobile phase gradient B. Drift tube temperature 55° C and spray chamber temperature 25° C.

3. Peptide Analysis

Peptide samples have been analyzed on all the three columns. Separation of three different peptides, bradykinin, angiotensin II and, 5-leucine enkephalin on the three columns is shown in Figure 15. The resolution of the peaks was most prominent on the Astrosil column. The resolution on the Vydac and C18 columns was comparable. Also the retention time of the peptides was greater on the Astrosil C5 column, followed by the Vydac C5 column and the C18 column respectively. In general the retention time on wider pore silica is less than narrow pore silica and the separation complies with this general concept. The C18 column being the most hydrophobic surface of all the three columns minimally retains the peptides, since these are relatively hydrophilic compounds. Figure 16 shows the separation of 5-leucine enkephalin and ala-peptide 6 on the Vydac C5 column. A solvent front peak occurs before the actual peaks at about 3.5 minutes. As it can be seen from the figure, the peaks are baseline separated and 5-leucine enkephalin elutes earlier since it is a smaller peptide. Figure 17 shows the separation of 5-leucine and ala-peptide 6 on Astrosil C5 column. In this case also 5-leucine enkephalin elutes earlier than ala-peptide 6 since it is a smaller peptide. A solvent front peak was observed at 3.5 minutes. All the solutes showed good retention and are well separated on both the C5 columns.

Figure 18 shows the separation of peptide YY human on all three columns. The peptide was prepared as a 250 $\mu\text{g/mL}$ solution in DI water. It can be observed from the figure that at low concentrations, the ELSD detector shows a very good signal-to-noise ratio.

Figure 15. Separation of bradykinin, angiotensin II and 5-leucine enkephalin on the three columns. Peak identification: 1. Bradykinin 2. Angiotensin II 3. 5- Leucine enkephalin. Mobile phase gradient A. Drift tube temperature 60° C and spray chamber temperature 15° C.

The chromatogram from C18 column shows two small peaks after the main peak which might be due to a sample impurity adsorbed to the column. Table 8 shows the retention times of all the proteins and peptides analyzed on the three columns.

Figure 16. Separation of 5-leucine enkephalin and ala-peptide 6 on the Vydac C5 column. Peak identification: 1. 5-leucine enkephalin 2. Ala-peptide 6. Mobile phase gradient A. Drift tube temperature 55⁰ C and spray chamber temperature 25⁰ C.

Figure 17. Separation of 5-leucine enkephalin and ala-peptide 6 on the Astrosil C5 column. Peak identification 1. 5-leucine enkephalin 2. Ala-peptide 6. Mobile phase gradient A. Drift tube temperature 55° C and spray chamber temperature 25° C.

Figure 18. Chromatograms of peptide YY human on the three columns.
Mobile phase gradient B. Drift tube temperature 60° C and spray chamber
temperature 15° C.

Table 8   Retention times of proteins and peptides on the three columns

A.   Retention times on Astrosil C5 column

Protein	Retention time	Gradient	Peptide	Retention time	Gradient
Hemoglobin	15.54	B	Peptide YY Human	14.12	B
Cytochrome C	12.10	A	Bradykinin	4.89	A
Apotransferrin	14.12	B	Angiotensin II human	5.28	A
Alpha-casein	15.41	B	5-[Methionine]Enkephalin	12.52	A
Albumin BS	14.69	B	[5-Leucine]Enkephalin	6.60	A
Albumin CEW	17.42	B	[Ala92]-Peptide 6 Human	14.20	B
Myoglobin	14.47	A	[D-Ala2, D-Met5]-Enkephalin	12.52	A
Alpha-lactalbumin	13.69	A			

B. Retention times on Vydac C5 column

Protein	Retention time	Gradient	Peptide	Retention time	Gradient
Hemoglobin	16.03	B	Peptide YY Human	9.98	A
Cytochrome C	11.84	A	Bradykinin	4.23	A
Apotransferrin	14.55	B	Angiotensin II human	4.44	A
Alpha-casein	15.53	B	5-[Methionine]Enkephalin	4.63	A
Albumin BS	13.50	A	[5-Leucine]Enkephalin	4.93	A
Albumin CEW	18.47	A	[Ala92]-Peptide 6 Human	6.40	A
Myoglobin	15.03	A	[D-Ala2, D-Met5]-Enkephalin	5.24	A
Alpha-lactalbumin	13.67	A			

C. Retention times on Astrosil C18 column

Protein	Retention time	Gradient	Peptide	Retention time	Gradient
Hemoglobin	11.12	A	Peptide YY Human	11.13	B
Cytochrome C	8.78	A	Bradykinin	9.03	B
Apotransferrin	10.03	A	Angiotensin II human	9.86	B
Alpha-casein	11.79	B	5-[Methionine]Enkephalin	2.17	B
Albumin BS	10.72	A	[5-Leucine]Enkephalin	10.20	B
Albumin CEW	14.34	A	[Ala92]-Peptide 6 Human	11.28	B
Myoglobin	11.75	A	[D-Ala2, D-Met5]-Enkephalin	3.69	A
Alpha-lactalbumin	11.55	A			

4. Amino Acids

Underivatized amino acids were analyzed under isocratic conditions using the ELSD detector to study their retention characteristics on all three columns. Structures of the eight amino acids used in this research are shown in Figure 19. Lysine, arginine and histidine are relatively hydrophilic and basic amino acids. Leucine and iso-leucine are the aliphatic amino acids. Phenylalanine, tryptophan and tyrosine are the aromatic amino acids. All the samples were dissolved in 50:50 acetonitrile/ water at a concentration of 0.5 mg/mL. The temperatures of the drift tube and spray chamber were varied according to the mobile phase composition to obtain a stable baseline. A small percentage of formic acid (FA) was used in the mobile phase compositions since it is volatile and hence compatible with the light scattering detector. Amino acids were initially analyzed using different mobile phase compositions of acetonitrile (0.1% FA)/ DI water (0.1% FA).

The retention map of the amino acids on the Astrosil column is shown in Figure 20. It can be seen that from 20% to 50% acetonitrile concentration, there was not much retention of the amino acids. The retention time of uracil (unretained compound) on this column was about 3.4 minutes which further clarifies that these solutes have minimal retention under these conditions. However from 60% to 80% acetonitrile concentration, there was an increase in retention which is an indication of ANP behavior. Furthermore, this behavior was more prominent in the relatively hydrophilic, basic amino acids (lysine, arginine, histidine). All these observations indicate that relatively polar amino acids have been retained on this column in ANP mode. Figure 21 shows the separation of leucine and arginine on Astrosil column at 80% acetonitrile concentration. It can be seen that

both the amino acids show good retention and are well resolved. Arginine, although a relatively polar amino acid than leucine shows a greater retention since it is being retained under ANP conditions. Figure 22 shows the retention map of amino acids on the Vydac C5 column. From 20% acetonitrile to 50% acetonitrile, the retention time of the amino acids did not change significantly and there was not much difference in their retention time at a particular composition. Since this is also a 150 mm column, the unretained compound took about 3.3 minutes to elute from the column. At 60% and 70% acetonitrile, retention time increased somewhat for all the amino acids. The retention times were lower when compared to those on the Astrosil column since Vydac offers a lower surface area. Hence, large molecules are best separated on wider pore silica and small molecules are best separated on narrow pore silica. Figure 23 shows the retention map of the amino acids on the C18 column. It is evident from the plot that there is minimal retention in this case too. The C18 column has a length of 75 mm, and the unretained compound on this column elutes at about 1.2 minutes. It can be seen from the graph that there is not much difference in the retention time of different amino acids at a particular mobile phase composition. From 20% to 70% acetonitrile there was not much change in the retention time, but at 80%, the retention times have slightly increased. At 80% acetonitrile concentration, lysine, arginine and histidine showed an increase in retention which is due to the ANP behavior.

One common observation for all the analyses involving amino acids was that the peak shape was distorted. The peaks showed splitting and were broad particularly at high concentrations of acetonitrile. To improve the peak shape and retention time, two

approaches have been employed. In the first approach, 70:30 and 80:20 ratios of acetonitrile (0.5% FA)/ DI water (0.5% FA) were used on all the columns. There was a slight change in the peak shape, but there was no change in the retention time. In the second approach, 50:50, 60:40 and 70:30 ratios of acetonitrile/ 20 mM ammonium acetate was used on the three columns. 20 mM ammonium acetate has a pH of 6.8 and often the selectivity of the column differs when going from a lower pH to a higher pH. However, there was not much difference in the retention characteristics for most of the amino acids and the peak shape also did not improve significantly. Tables 9, 10 and 11 show the retention times using both the approaches for Astrosil C5, Vydac C5 and Astrosil C18 columns respectively.

From Table 9, with Astrosil column, it can be seen that for arginine and lysine, at higher pH (ammonium acetate buffer) the retention time has increased when compared to the analysis at a lower pH with formic acid. However at 70:30 acetonitrile/ 20 mM ammonium acetate, a broad ill-defined peak was observed for both the solutes. It might be because these solutes are being retained by ANP behavior of the column and at that particular mobile phase composition are irreversibly adsorbed to the column. From Table 10, with the Vydac column, it can be seen that there was not much improvement in the retention time with either of the approaches. Table 11 shows the retention times for the two approaches on the Astrosil C18 column and it can be seen that at 70:30 acetonitrile/ 20 mM ammonium acetate, there was a significant increase in the retention time of arginine and lysine, but the peaks were broad and ill-defined. An increase in retention time with an increase in acetonitrile concentration indicates that these solutes are being

retained in the ANP mode at this particular mobile phase composition. Figure 24, shows a comparison of the peak shapes with three different approaches using phenylalanine as the test solute on the Vydac column. It can be deduced that there was not much improvement in the peak shape. As none of the approaches were successful in improving the peak shape it appeared that there might be some influence of the detector temperature used for the analysis. To understand the influence of the temperature on the peak shape, lysine was selected randomly and three different temperatures were used with an 80:20 acetonitrile/ 0.5% formic acid mobile phase composition on the Astrosil C5 column. The results are shown in Figure 25. It can be seen that there is a difference in the peak shape with a change in the temperature, but further analysis is required to determine the effect of temperature on different solutes.

5. Sugars

Analysis of sugars was carried out using 80:20, 90:10 and 95:05 ratios of acetonitrile/ water mobile phase compositions. Sugars being polar molecules are expected to be retained in the ANP mode on columns synthesized by the silanization/hydrosilation under suitable conditions. Monosaccharides (glucose, fructose, xylose), disaccharides (sucrose, maltose, lactose) and trisaccharides (raffinose, melezitose) have been analyzed on all three columns. Sugar molecules showed minimal retention on all the columns. Furthermore, multiple peaks, peak splitting and broad peaks were observed with all the solutes. Figure 26 shows the chromatograms of xylose and sucrose on Astrosil column at a mobile phase composition of 90:10 acetonitrile/ water. It can be seen that there is some peak splitting in both the cases.

Lysine

Arginine

Histidine

Tryptophan

Leucine

Isoleucine

Phenylalanine

Tyrosine

Figure 19. Structures of Amino Acids.

Figure 20. Retention map of amino acids on Astrosil C5 column.

Figure 21. Separation of leucine and arginine on Astrosil C5 column. Peak identification: 1. Leucine 2. Arginine. Mobile phase 20:80 DI water (0.1% FA)/ acetonitrile (0.1% FA). Drift tube temperature 55⁰ C and spray chamber temperature 25⁰ C.

Figure 22. Retention map of amino acids on Vydac C5 column.

Figure 23. Retention map of amino acids on C18 column.

Table 9. Retention times on Astrosil C5 column

Samples	% Acetonitrile with DI water+0.5% formic acid Retention time (min)		% Acetonitrile with 20 mM Ammonium acetate Retention time (min)		
	70%	80 %	50 %	60 %	70 %
Isoleucine	3.89	5.04	3.23	3.44	3.85
Leucine	3.88	5.03	3.26	3.48	3.87
Phenylalanine	3.85	4.94	3.42	3.53	3.85
Tryptophan	3.94	4.94	3.55	3.55	3.87
Tyrosine	3.66	4.62	3.10	3.30	3.22
Histidine	3.35	6.22	3.71	3.76	3.76
Arginine	3.84	6.13	6.86	10.19	19.5
Lysine	3.80	6.24	6.27	10.24	17.5

Table 10. Retention times on Vydac C5 column

Samples	% Acetonitrile with DI water+0.5% formic acid Retention time (min)		% Acetonitrile with 20 mM Ammonium acetate Retention time (min)		
	70%	80 %	50 %	60 %	70 %
Isoleucine	3.83	4.38	3.33	3.41	3.44
Leucine	3.94	4.44	3.34	3.45	3.40
Phenylalanine	3.94	4.32	3.39	3.48	3.44
Tryptophan	4.09	4.74	3.46	3.57	3.70
Tyrosine	4.06	4.65	3.31	3.26	3.30
Histidine	3.82	4.36	3.33	3.32	3.52
Arginine	4.05	4.91	3.82	4.11	4.93
Lysine	3.81	4.11	3.75	4.03	4.88

Table 11. Retention times on Astrosil C18 column

Samples	% Acetonitrile with DI water+0.5% formic acid Retention time (min)		% Acetonitrile with 20 mM Ammonium acetate Retention time (min)		
	70%	80 %	50 %	60 %	70 %
Isoleucine	1.44	1.63	1.28	1.45	1.58
Leucine	1.46	1.68	1.29	1.35	1.61
Phenylalanine	1.46	1.70	1.33	1.35	1.64
Tryptophan	1.47	1.72	1.38	1.50	1.63
Tyrosine	1.36	1.39	1.22	1.23	1.39
Histidine	1.24	1.27	1.28	1.29	1.63
Arginine	1.30	1.38	2.35	2.93	8.5
Lysine	1.28	1.33	2.13	2.18	8.5

Figure 24. Peak shape comparison. Sample: phenylalanine, Column: Vydac C5. Mobile phase composition 70% acetonitrile with the respective concentrations of formic acid and ammonium acetate.

Figure 25. Temperature effect on lysine using Astrosil C5 Column. Mobile phase composition 80:20 Acetonitrile/ 0.5% Formic Acid.

Figure 26. Chromatograms of xylose and sucrose on Astrosil C5 column.

A. chromatogram of xylose obtained using Astrosil C5 column with 90:10 acetonitrile/ water mobile phase. B. Chromatogram of sucrose obtained using Astrosil C5 column with 90:10 acetonitrile/ water mobile phase.

Figure 27 shows the chromatograms obtained from the Astrosil column at 95:05 acetonitrile/ water mobile phase composition. It can be observed that there is not much retention under these conditions. Figure 28 shows the chromatogram of glucose, lactose and melizitose on the C18 column at three different mobile phase compositions. As can be seen in the figure, the mixture elutes from the column very close to the dead volume with significant peak splitting.

Figure 29 shows the elution of the same mixture on the Astrosil C5 column with two different mobile phase compositions. It can be seen that the mixture elutes very close to the dead volume and there is noticeable splitting at 90% acetonitrile concentration. Figure 30 shows the chromatograms of mixture of sucrose-lactose-maltose using two different aqueous normal phase gradients. It can be seen that all the components of the mixture elute as a single peak and the retention time is close to the dead volume.

Figure 27. Chromatograms of sugars. A. chromatogram of glucose obtained using Astrosil C5 column with 95:05 acetonitrile/ water mobile phase. B. Separation of glucose-fructose-xylose obtained using Astrosil C5 column with 95:05 acetonitrile/ water mobile phase.

Figure 28. Chromatograms of a mixture of glucose-lactose-melizitose on C18 Column at 80, 90 and 95% acetonitrile.

Figure 29. Mixture of glucose-lactose-melizitose on Astrosil C5 Column at 90 and 95 % acetonitrile.

Figure 30. Chromatograms of sucrose-maltose-lactose obtained using aqueous normal phase gradients on Astrosil C5 column. A. mobile phase gradient: 0.00 - 05.00 min 90% acetonitrile (hold), 05.00 - 10.00 min 90% to 80% acetonitrile, 10.00 - 15.00 min 80% to 50% acetonitrile. B. mobile phase gradient: 0.00 - 05.00 min 95% acetonitrile (hold), 05.00 - 10.00 min 95% to 80% acetonitrile, 10.00 - 15.00 min 80% to 50% acetonitrile.

IV. CONCLUSIONS

Silanization/hydrosilation is a suitable method to prepare short chain alkyl stationary phase for protein and peptide separations. Results from DRIFT spectra, the Neue test and elemental analysis showed that the C5 bonded phase synthesized using hexachloroplatinic acid catalyst bonded well to silica hydride. Also higher temperature yielded better bonding which is due to accelerated reaction rates. From the results of the Neue test it was observed that the columns have good hydrophobicity to retain non-polar solutes which is required for the retention of proteins and peptides on a reversed phase column. It was also seen that the newly synthesized columns performed better compared to a C18 column due to their lower hydrophobicity which is ideal for protein separations.

The ELSD can provide useful information alone or in conjunction with the UV detector. There was no baseline drift and the signal-to-noise ratio was good even at low concentrations of the sample. For protein and peptide separations gradient elution was used with both the ELSD and UV detectors. The chromatograms obtained from the UV system showed baseline drift and in some cases a low intensity peak. On the other hand, the chromatograms obtained from the ELSD system showed stable baseline indicating that this system is gradient compatible. For amino acids and sugars under isocratic conditions, the chromatograms showed some peak splitting. The solutes did not show much retention under the conditions used in this research. To investigate the cause of peak splitting, different mobile phase compositions were used. However, there was not much change in the peak shape indicating that the detector rather than the column might be the cause of peak splitting. The temperature of the ELSD seems to depend not only on

the mobile phase but also on the solute of interest. This was evident with the example of lysine where the peak shape changed with a change in the temperature. However, it cannot be concluded definitively at this point that the temperature of the drift tube is the cause of the peak splitting. Hence further work is required in this direction.

The study suggests that proteins and peptides can be effectively separated and detected with the C5 column using an ELSD detector. Future work can focus on gradient elution of amino acids and sugars to improve retention. Also the effect of temperature on the peak shape should be further studied to ascertain the underlying cause of peak splitting.

REFERENCES

1. Skoog, A. D.; Holler, J. F.; Niemann, A. T. *Principles of Instrumental Analysis*, 5th ed.; Saunders College Publishing: Florida, 1998; Chapters 26, 28.
2. Pesek, J. J.; Matyska, M. T. *LC/GC*. **2006**, 24, 296-303.
3. Snyder, L. R.; Kirkland, J. J.; Glajch, J. L. *Practical HPLC Method Development*, 2nd ed.; John Wiley and Sons: New York, 1997; Chapters 3, 5, 8, 11.
4. *HPLC of Biological Macromolecules*; Karen, M. G., Fred E. R., Eds.; Marcel Dekker, Inc.: New York, 1990; Chapters 6, 9, 10, 11.
5. Nawrocki, J. J. *Chromatogr A*. **1997**, 779, 29-71.
6. Pesek, J. J.; Sandoval, J. E.; Matyska, M. T. *J. Liq. Chrom. & Rel. Technol.* **1996**, 19, 2843-2865.
7. Kirkland, J. J.; Glajch, J. L.; Farlee, R. D. *Anal. Chem.* **1986**, 61, 2-11.
8. Sandoval, E. J.; Pesek, J.J. *Anal. Chem.* **1991**, 63, 2634-2641.
9. Chu, C. H.; Jonsson, E.; Auvinen, M.; Pesek, J. J.; Sandoval, E. J. *Anal. Chem.* **1993**, 65, 808-816.
10. Pesek, J. J.; Matyska, M. T.; Oliva, M.; Evanchic, M. J. *Chromatogr. A*. **1998**, 818, 145-154.
11. Pere, E.; Cardy, H.; Cairon, O.; Simon, M.; Lacombe, S. *Vib. Spectrosc.* **2001**, 25, 163- 175.
12. Berendsen, G. E.; De Galan, L. *J.Liq. Chromatogr.* **1978**, 1, 561-568.
13. Neue, U. D.; Tran, K.V.; Iraneta, P. C.; Alden, B. A. *J. Sep. Sci.* **2003**, 26, 174-186.
14. Bongers, J.; Chen, T. K. *J. Liq. Chrom. & Rel. Technol.* **2000**, 23, 925-933.
15. Yan, D.; Li, G.; Xiao, X. H.; Dong, X. P.; Li, Z. L. *J. Chromatogr. A*. **2007**, 1138, 301-304.
16. Superior HPLC Detection. <http://www.softacorporation.com/> (accessed July 18, 2007).