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Normal phase chromatography with hydride-based stationary phases

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NORMAL PHASE CHROMATOGRAPHY
WITH HYDRIDE-BASED STATIONARY PHASES

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Anushka Sharma

May 2007

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ABSTRACT

NORMAL PHASE CHROMATOGRAPHY WITH HYDRIDE-BASED STATIONARY PHASES

by Anushka Sharma

Silanization and hydrosilation of silica leads to highly stable silica hydride stationary phases where silicon hydride groups replace 95% of the silanols, thus imparting unique properties to the stationary phase. These phases were originally developed for reversed-phase chromatographic applications. Because some preliminary tests indicated that they could be used for normal phase applications, an effort to study their normal phase retention behavior was undertaken in this study.

Ten columns were evaluated by using them for the separations of some model compounds that are used to make catalysts. Out of ten columns, seven were hydride-based and three were commercially available silica and C18 columns used as controls for comparison. All the columns were evaluated using liquid chromatography-mass spectrometry. The hydride-based columns displayed typical normal phase behavior for all the compounds.

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I. Introduction

A. Chromatography

Chromatography is a technique that allows separation of different components in a mixture and facilitates identification based on their affinity for the two phases involved in the separation, the stationary phase, and the mobile phase. The stationary phase can be a solid, liquid, or gel (1) and remains fixed in a place as the name implies while the mobile phase, which can be a gas, liquid, or supercritical fluid, is forced to flow through the stationary phase in a particular direction. A mixture is introduced into the mobile phase and when the latter flows through the stationary phase, the various components of the mixture interact to different degrees with both the phases. Some compounds that prefer the mobile phase flow along with it and do not interact with the stationary phase while some are retained because they prefer the stationary phase. Depending on their affinities for both the phases, the compounds are separated into several bands or zones that can be analyzed. Chromatography can be classified in two ways, based upon the nature of physical contact between the two phases and by their physical state (2). Figure 1 shows the general classification of the various chromatographic methods.

1. High Performance Liquid Chromatography

The chromatographic method utilized in this study is high performance liquid chromatography (HPLC). The stationary phase is packed in a cylindrical stainless steel

column through which the mobile phase is forced under high pressures. The basic components of the system are shown in Figure 2.

Figure 1. General classification of chromatography.

Figure 2. High performance liquid chromatography set-up.

A pump is used to deliver the mobile phase to the stationary phase, which in this case is a column. Typically, the sample to be analyzed is injected into the mobile phase that is continuously flowing through the system. Once the sample reaches the column, different components in the mixture interact differently with the stationary phase because of which they are separated in the column. Since the mobile phase is continuously flowing, the separated components eventually elute out at different times and the detector, which in this case is a photodiode array, measures the UV absorbance of the sample and gives a readout in the form of a chromatogram. The chromatogram thus is a plot of sample concentration vs. time. An example is shown in Figure 3.

Figure 3. Chromatogram. (From Introduction to Modern Liquid Chromatography 2nd edition by Snyder and Kirkland. 1979. Reprinted with permission of John Wiley & Sons, Inc.)

A bell-shaped peak (3) represents each compound that elutes from the column and so different compounds after separation appear as different peaks in the chromatogram. The time at which a peak appears is characteristic of the compound for that particular stationary-mobile phase combination. This retention time t_R , the time taken for a peak to

appear after the sample has been injected, can be used to identify compounds. In addition, the area under the peak can be used for measuring the amount of a particular compound present in a sample mixture.

Different stationary and mobile phase combinations can be used and based upon their relative polarities (2) the separations are classified as reversed-phase (RPC) and normal-phase chromatography (NPC). In reversed-phase chromatography, the stationary phase is relatively less polar than the mobile phase. Some examples of the different kinds of stationary phase that can be used are derivitized silica like C8 or C18 columns formed by the modification of silica surface by covalently attaching 1-octane or 1-octadecane respectively (4). The mobile phase consists of a solvent mixture that is more polar than the column packing; one of the components is water while the other is an organic solvent like acetonitrile, methanol, THF etc.

Compounds typically partition between the two phases, the non-polar analytes are retained strongly, and the polar analytes elute faster. The retention properties of compounds can be varied by changing the mobile phase composition. Sometimes when the compounds are too polar they are not retained at all under reversed-phase conditions, and at the other extreme, if they are too non-polar they are too strongly retained (4). For such compounds, it can be advantageous to perform normal-phase chromatography. Because this study utilizes only normal phase separations, this type of chromatography is explained in detail in the following section.

a) Normal Phase Chromatography

In normal phase separations, the mobile phase is relatively less polar than the stationary phase. The mobile phase consists of a mixture of organic solvents, most commonly a combination of a non-polar solvent is used with a polar solvent like hexane/ethyl acetate, hexane/isopropanol etc. Silica, cyano, diol, and amino columns are the most commonly used stationary phases in normal phase chromatography (4). The compounds are retained due to adsorption processes occurring at the column surface. The silanols in the case of silica columns and the ligands and residual silanols on the cyano, amino, and diol columns are the adsorption sites. These sites attract the solvent molecules when the mobile phase is flowing through the system, thus resulting in a layer of solvent adsorbed on the column surface. When sample molecules in the mobile phase reach the column, they displace the solvent molecules and are adsorbed on the surface. Both solvent molecules and the polar sample molecules compete for the polar sites on the stationary phase. Thus changing the amount or type of polar component in the mobile phase composition allows more control over selectivity in case of NPC when compared to RPC (4). In normal phase separations, as the polarity of the mobile phase composition increases, the compounds elute faster.

B. Mass Spectrometry

This analytical technique allows identification of molecules based on the mass-to-charge ratio (m/z) of their ions. M/z for an ion can be calculated by dividing the mass of the ion 'm' by its charge 'z'. (2). Figure 4 shows the different components of a mass spectrometer.

Figure 4. Mass spectrometer set-up.

All the components except the sample inlet and atmospheric pressure chemical ionization (APCI) source (a type of ionization source) operate in a vacuum environment. The pressure maintained varies from 10^{-4} to 10^{-8} torr. The vacuum environment not only facilitates sample transfer from one region to another but also prevents collisions between ions and adjacent atoms or molecules that might neutralize them (2). During analysis, the molecules of interest are introduced into the system through a sample inlet (5). They can be in the solid, liquid, or gaseous state. After introduction, the sample needs to be ionized. For this purpose, there are several ionization sources available, some of which will be described later. The ionization chamber is maintained at slightly lower pressure and this facilitates sample transfer from the inlet into the chamber. Here ionization takes place by bombardment of the sample with a beam of high energy electrons, ions, atoms or photons depending on the type of source used. Both positive and negative ions can form depending on the ionization method.

The entire sample is not ionized and so the vacuum pumps extract the unionized portion continuously. The ions formed in the chamber are drawn into the mass analyzer under the influence of electric fields. Here, the ions are accelerated in the mass analyzer using different magnetic or electric fields. Several types of mass analyzers are available.

In the case of a quadrupole mass analyzer, for a particular field applied only ions of a certain mass/charge (mass of the ion divided by the charge) can follow a stable path and reach the detector. Thus by varying the fields in this region, ions can be separated according to their mass/charge. A transducer produces a current proportional to the number of ions reaching the detector. An interface connecting the detector to the computer converts the current into a comprehensible output, the mass spectrum. The information in the mass spectrum allows identification of compounds. It is a graph of the number of ions of each m/z ratio produced when a sample is ionized (1). Figure 5 shows a mass spectrum of ethyl benzene (2).

Figure 5. Mass spectrum of ethyl benzene. (From Principles of Instrumental Analysis 5th edition by SKOOG. 1998. Reprinted with permission of Brooks/Cole, a division of Thomson Learning: www.thomsonrights.com. Fax 800 730-2215)

The abscissa indicates 'm/z' and the ordinate indicates the relative abundance of ions. The tallest peak in the plot referred to as the 'base peak' is the most abundant ion produced upon ionization; in this case, it is indicated at m/z 91. The other peaks in the spectrum are normalized to this peak. As mentioned earlier, positive ions can form in the ionization chamber. An important ion formed in many cases is the radical cation formed by the removal of a single electron known as the molecular ion peak, and represented by the symbol M^+ (5). Because the ion is missing only one electron, it is assumed to represent the molecular weight of the molecule and so it can be used to identify molecules by their molecular weight. For ethyl benzene, M^+ occurs at m/z 106. During ionization, the high-energy source imparts enough energy to the sample that causes fragmentation. Sometimes excessive fragmentation leads to loss of M^+ peak and so several small peaks appear in the spectrum at m/z corresponding to their individual masses. Thus in situations where the M^+ peak is missing, molecules can be identified by the fragmentation patterns. In addition, isotopic peaks for molecules can also be observed in the spectrum usually at one or two mass units above the M^+ peak as M+1 or M+2 peaks. Thus, mass spectrometry is a very useful tool for structural determination of compounds.

C. Liquid Chromatography-Mass Spectrometry

Coupling liquid chromatography with mass spectrometry allows overcoming identification issues that are hard or almost impossible to resolve if the individual techniques are used independently. For instance in liquid chromatography, identification is based entirely upon retention characteristics (*I*). The retention time of the unknown

sample is compared to a reference material and thus identified. As mentioned earlier, retention behavior depends on the nature of the stationary and mobile phases involved in the separation. Because there are so many different combinations that can be used, the probability of two compounds having the same retention characteristics increases. In such cases, only one peak might appear in the chromatogram instead of two and this will lead to wrong identification. In addition, despite the broad range of phases that can be used for a separation, a successful separation is not always achieved. Thus by chromatography alone it is hard to unambiguously identify compounds based upon retention characteristics.

Mass spectrometry has some drawbacks too when used alone. It is easier to identify one compound by MS; however, when it is present in a mixture the analysis can be difficult (*1*). The mass spectrum for a mixture will show ions formed for all the compounds in the mixture. If the compound of interest is present in small quantities then the M^+ peak might be difficult to observe. Sometimes due to excessive fragmentation, the low intensity of the molecular ion peak makes it harder to identify the compound if it is part of a mixture. In such cases, prior to identification via MS, if the mixture is purified using a liquid chromatographic system, then the analysis will be easier. Even if partial purification is obtained, at least the mass spectrum will not appear so complex.

When chromatography fails to separate two compounds because of their similar retention characteristics and results in overlapping peaks in the chromatogram, mass spectrometry aids as a secondary detector by allowing identification based upon the molecular ions or fragmentation patterns observed in the mass spectrum. Thus coupling

both the techniques allows utilizing the advantages of both and provides an excellent analytical tool for identification and structural determination of compounds.

In order to couple both techniques an interface is required that will overcome the following incompatibility. The systems cannot be connected directly because the effluent coming out of the LC system has solvent and this will compromise the vacuum requirements of the MS system. Thus, an interface is required to remove the mobile phase prior to sample introduction into the mass spectrometer (1). There are several kinds of interfaces available for different kinds of analysis and they all need to fulfill these basic requirements. The interface should not compromise the performance of either of the systems, i.e. sample introduction should be such that the purified fractions eluting out of the LC should remain pure while being transferred. Additionally, removal of the mobile phase, during the transfer should not compromise the vacuum requirements, allowing both the systems to carry out their respective functions effectively. In addition, the interface should not contribute to the background signal and no chemical modifications in the sample should occur during transfer (1).

1. Ionization Methods and Interface Technology

Many ionization methods are available to facilitate sample ionization. The ion sources that are used can be classified into two types: gas-phase sources and desorption sources (2). In the case of gas-phase sources, the sample has to be in the vapor phase prior to ionization, while with desorption sources the sample can either be in a solid or liquid form and is directly converted to gaseous ions. Selecting the right ionization method is important because the technique chosen should preserve the sample properties

during ionization in order to obtain useful information (6). The following Table 1 lists some common types of sources and the ionizing agent involved in the process (2). As mentioned earlier, an interface is required for sample introduction into the mass spectrometer, subsequent to which the ionization process takes place. Thus, depending on the applications, in many cases the ionization methods are integrated with the different interfaces. A brief summary of some of the common ionization methods and interfaces that can be used is included in the following section.

Table 1. Most common ion sources used in mass spectrometry

Type of Source	Name	Ionizing agent
Gas Phase	Electron ionization (EI)	Energetic electrons
	Chemical ionization (CI)	Reagent gaseous ions
	Atmospheric pressure chemical ionization (APCI)	Vaporized reagent ions
Desorption	Electrospray ionization (ESI)	High electrical field
	Fast-atom bombardment (FAB)	Energetic atom beam
	Thermospray ionization (TS)	High temperature
	Matrix-assisted laser desorption ionization (MALDI)	Laser beam

Source: From Principles of Instrumental Analysis 5th edition by SKOOG. 1998.

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a) Electron Ionization (EI)

In this technique, high-energy electrons are used to bombard the sample in vapor phase. Consequently, sample molecules absorb the energy and undergo ionization and fragmentation. In most cases, excessive fragmentation occurs because of the high-energy impact and so a molecular ion is not observed. This method is mostly used for small organic molecules having molecular weights less than 10^3 daltons (2). A moving-belt or particle-beam interface can be used with this ionization method (1). In the case of a moving belt interface, the LC eluent is deposited on a continuously moving belt, which passes under an infrared heated region equipped with several vacuum pumps to remove the mobile phase. Once the sample is vaporized, EI ionization is induced and the belt is cleaned by heating or washing so further sample depositions can be made (1).

In the case of a particle beam interface, the LC eluent is converted into droplets by passing it through a pneumatic and thermal nebulizer. These droplets are then introduced via a carrier gas into a desolvation chamber, where by heating the droplets are vaporized. From this chamber the vaporized mixture is introduced into a 'two-stage momentum (jet) separator', where as the vapor passes through, the vacuum pumps aligned at different regions separates the lower momentum carrier gas particles from the higher momentum sample particles. These particles are then pumped into the mass spectrometer where ionization takes place (1).

b) Chemical Ionization (CI)

CI is an improvement on the previous technique where the only difference is that the sample is mixed with a reagent gas like methane, ammonia, or isobutane, and this

mixture is bombarded with high-energy electrons as in EI. Since the reagent gas is in excess, it is ionized exclusively. Intermolecular reactions between the reagent gas and sample molecules result in sample ionization. Because the reagent gas is ionized first, there is less fragmentation and the molecular ion is preserved in most cases. Moving-belt, particle-beam, and direct-liquid-introduction (DLI) interfaces can be used with this ionization method (1). The DLI consists of a probe, which has a pinhole at one end. Adjacent to the pinhole is a desolvation chamber that is connected to the ion source of the mass spectrometer. Vacuum pumps are aligned at the pinhole and near the ion source. Basically, the LC eluent flows through the probe and at the pin hole the vacuum pump draws the eluent into the desolvation chamber and consequently into the ion source where ionization takes place (1). The ionization technique used in this study is atmospheric pressure chemical ionization (APCI). Since this method involves ionization of sample in solution form, it is hard to separate the interface properties from the ionization method. A detailed description of this ionization method is provided in the following section.

2. Atmospheric Pressure Chemical Ionization

As the name implies, this type of ionization occurs at atmospheric pressure. An advantage of this technique is that the liquid part of the LC eluent is vaporized while only the ionized sample enters the mass spectrometer (6). Figure 6 shows the APCI interface that was used for this study (7).

Figure 6. Atmospheric pressure chemical ionization interface. (Courtesy of Waters Corporation, Milford, Mass., USA)

The HPLC line carrying the eluent is connected to the ion source of the mass spectrometer, which is the APCI probe, a capillary in this case. There is a constant flow of nitrogen (nebulizing gas) around the probe that aids in mobilizing the sample and mobile phase molecules. An APCI heater adjacent to the probe along with the nitrogen flow helps in vaporizing the eluent when it exits the probe. A corona discharge needle is placed adjacent to the probe tip. When a high potential is applied to this needle, it ionizes the surrounding environment and the phenomenon is known as the 'corona discharge'. Since the needle is surrounded by the vapor generated from the LC eluent, the sample and solvent vapor are ionized when a potential is applied to the needle. Because the solvent vapor is in excess, it is ionized exclusively. Interactions between ionized solvent vapor and sample molecules result in sample ionization. The sampling cone placed orthogonal to the probe attracts only sample ions that have a particular velocity only to pass through

into the mass spectrometer (1). Thus because this Z-spray interface does not allow the solvent molecules and unionized sample to pass through, it helps in preventing contamination and clogging of the sampling cone. Because the sample ions are charged, electric potential can be applied to direct them through the sample cone, extraction cone, and hexapole RF lens into the mass analyzer for further analysis.

In addition, there is a roughing pump and a split flow turbomolecular pump that creates a vacuum environment, which helps in ion transmission into the mass analyzer, prevents ion collisions, and aids in transferring solvent and unionized sample molecules to the waste. Different pressure regions are created in the mass spectrometer, a map is shown in Figure 7 (7).

Figure 7. Vacuum system in the mass spectrometer. (Courtesy of Waters Corporation, Milford, Mass., USA)

During ionization, positive ions can be formed when the protonated solvent transfers a proton to the sample forming MH^+ ions as shown in equation 1 and negative

ions can be formed when the sample donates a proton to the deprotonated solvent as shown in equation 2.

S: solvent molecules; M: sample molecules

In this study, methanol was introduced into the APCI interface in order to aid in ionization. For ionization to occur, the mobile phase needs to be protic; however the normal phase solvents that were used for the chromatographic runs were aprotic and do not undergo ionization. Thus at the interface another capillary introduced methanol into the LC eluent and so the mixture upon ionization consisted of methanol and sample molecules. The solvent molecules were pulled away by the vacuum pumps. Reactions between ionized methanol and sample molecules resulted in the formation of sample ions. Post column addition of methanol was done because the samples used in the study were insoluble in methanol and so if the methanol were added to the mobile phase prior to separation then the compounds would precipitate on the column. Because APCI is a soft ionization technique, molecular ion is preserved. Some advantages of this technique are that it can accommodate high flow rates and so is compatible with HPLC. It can be used to ionize compounds with low to reasonably high polarities. It is extremely sensitive, almost the entire sample introduced is ionized, and it gives good results in both positive and negative modes (1). This interface is suitable for neutral molecules but not thermally labile or nonvolatile analytes (6).

3. Mass Analyzer

The different types of ionization methods available have led to the development of several kinds of mass analyzers. A mass analyzer is chosen depending on the application and the kind of information required from the sample of interest. Some important characteristics that are considered during selection are mass resolving power, mass accuracy, mass range, linear dynamic range, efficiency, and compatibility with ionizer (8). The most common analyzer used in mass spectrometry is the quadrupole mass analyzer. Because it was also used in this study, a detailed description is provided in the following section.

a) Quadrupole Mass Analyzer

After ionization, the sample ions pass through the sample cone and the extraction cone into the hexapole RF lens assembly, which guides the ion beam towards the quadrupole mass analyzer. Figure 8 shows the quadrupole set-up.

Figure 8. Quadrupole mass analyzer set-up. (Courtesy of Waters Corporation, Milford, Mass., USA)

The quadrupole consists of four parallel cylindrical rods (2). Each opposing pair is connected electrically and a RF voltage is applied between the two pairs. A direct current is superimposed on the RF voltage. The applied voltages affect the trajectory of the ions traveling towards the detector between the four rods. Only ions of a certain m/z reach the detector for a given ratio of RF/DC voltages applied and the other ions undergo unstable oscillations and collide with the rods. Thus, a mass spectrum can be obtained by monitoring the ions passing through the quadrupole as the voltages of the rods are varied. The quadrupole can be used to obtain full scan spectrum where ions with masses from low to high range are monitored (1) or it can be used to monitor a fraction of masses only, in which case the technique is known as selected-ion monitoring (SIM). For this study, the full scan function of the quadrupole was utilized.

Quadrupole mass analyzers have fast scanning rates and their working range is between 10 and 4000 m/z (2). Ions differing by one m/z unit can be distinguished in the mass spectrum. In addition, almost all the ions formed reach the detector. The detector used in this study was a dynolite photomultiplier tube that consisted of a conversion dynode, a phosphor, and a photomultiplier tube (7). The conversion dynode converted the ion beam exiting out of the quadrupole mass analyzer into an electron beam. The electrons on striking the phosphor resulted in excitation and emission of photons. The emitted photons on striking the photocathode at the front of the photomultiplier tube in turn produced electrons and the multiplier amplified the electric signal, which could be measured.

D. Hydride-based Stationary Phases

Silica is the most common support for HPLC stationary phases (4). Some properties that make silica-based columns desirable are high mechanical strength, high column efficiency, and ease of fabrication. Another advantage is that several bonded phases can be developed via silica derivitization with a variety of compounds having different functional groups. The adsorption sites for silica are the silanols. These can be measured by magic-angle-spinning (MAS) ^{29}Si NMR and diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy with (4). These acidic silanols interact with basic analytes and lead to peak tailing, poor separation and resolution. In addition to the silanols, another feature of silica that contributes to peak tailing is the metal content. Metals like, Fe, Al, Zn, etc. that are found in silica have been found to affect retention properties during separations (4). The older materials known as Type-A silicas are highly acidic and less pure, i.e. they contain small amounts of metals (4). Type-B silicas, an improvement over these are less acidic and contain lower amounts of metals. Moreover, they offer better separation capabilities especially for basic solutes.

In addition to having all the advantages of Type-B silicas, a new kind of silica phase marketed as Cogent “Type-C silica” by Microsolv Technology Corporation offers reduced surface acidity and improved pH stability (9). The development of this silica phase started in Dr. Joseph Pesek’s laboratory at San Jose State University, California. A two-step procedure, the silanization/hydrosilation process was developed to overcome some drawbacks of the organosilanization process (10, 11). The silanization process allows for removal of almost 95% of its silanols by replacing them with nonpolar Si-H

groups (11). Subsequent hydrosilation allows further modification of the surface imparting different properties like hydrophilicity, hydrophobicity, chirality etc. to the stationary phase (12). The generic reactions are shown in Figure 9.

When these column packings were developed, preliminary tests indicated that silanization provided SiH coverage efficiencies of about $100\pm 20\%$ with respect to silanols (11). C₈ stationary phases prepared via the silanization and hydrosilation process were found to have high surface coverage with a direct Si-C linkage (13). In the same study, chromatographic studies indicated typical reverse phase behavior, absence of interaction between basic solutes, and residual silanols and high stability towards alkaline pH mobile phases. Another study demonstrated good alkyl coverage and improved hydrolytic stability towards mobile phases containing trifluoroacetic acid (10). In all the studies mentioned above, Si-H coverage was determined by ²⁹Si cross polarizing-MAS NMR and alkyl coverage was determined by elemental analysis along with DRIFT spectroscopy, which was also used for hydrolytic stability tests. The key features in DRIFT spectra that were used to determine properties like coverage and stability were the carbon-hydrogen stretching bands between 2,800- 3, 000 cm⁻¹ and the Si-H band at 2,250 cm⁻¹.

Figure 9. Silanization-Hydrosilation reactions.

E. Project Goals

Subsequent to the development of the hydride-based columns, preliminary tests indicated that they could be used under normal phase conditions as well. However, this property was not explored until now. Thus, an effort to study the normal phase behavior of these stationary phases has now been undertaken in this research work. Four groups of model compounds belonging to different classes of organic molecules as mentioned in the experimental section below were being used to evaluate the normal phase retention behavior of these columns under various mobile phase conditions. The model compounds tested are representative synthons used to access molecular diversity for

Symyx's homogeneous catalyst discovery programs (14). Within these programs, synthesis is a significant bottleneck and improved methods of separation are desired. Some catalysts that are screened are water sensitive and thus non-aqueous normal phase chromatography is an attractive option. Since previous attempts of normal phase separation at Symyx, with bare silica columns led to poor reproducibility and broad peaks, they were interested in an alternative stationary phase. Thus by trying to separate these model compounds under normal phase conditions, an attempt understand the nature of the hydride-based stationary phases is being made. In addition, the viability of these columns for normal phase applications is being explored.

II. Experimental

A. Materials

1. Columns

Three hydride-based non-commercial columns that were used in this study were developed in Dr. Pesek's lab at San Jose State University, California. Two of them were silica hydride columns fabricated by the silanization process only, so the surface was populated predominantly by Si-H groups. The difference between the two columns silica hydride-batch A and silica hydride-batch B was the amount of catalyst (HCl) used during the silanization reaction. For batch A, the amount of catalyst used was 5.6 mL of (aqueous) 2.3M HCl. For batch B, the amount of catalyst used was 7 mL of (aqueous) 2.3M HCl. After many trial and error experiments, 7mL of (aqueous) 2.3M HCl seemed to be the right amount of catalyst that gave maximum hydride coverage. Catalyst amount below 5.6mL and above 7mL was found to increase the silanol concentration on the surface (11). When the reaction took place in a shorter period of time or in the presence of low catalyst amounts, the surface had more silanols left. When the amount of the catalyst was very high then polymerization occurred leading to Si-O-Si coupling or cross-linking. Instead of linear polymerization, the molecules polymerized in all direction and so some silanols were left uncrosslinked on the molecule. When the amount of catalyst was in the optimum range (5.6mL to 7mL) maximum coverage of hydride was observed. Thus, both batches of silica hydride columns should give approximately similar data because they fall in the optimum catalyst range. The third non-commercial column was

synthesized by the silanization - hydrosilation process where the Si-H groups formed in the first step were reacted with 1-octadecyne giving rise to a non-polar bidentate (two points of attachment to the surface) C-18 column. A commercial silica column (Zorbax Rx-Sil) purchased from Agilent technologies that had already been used for other normal phase applications was used as a control for comparison purposes. A commercial C18 (C18) phase also used as a control in this study was purchased from Agilent Technologies. The remaining five commercial columns used for the study were purchased from MicroSolv Technology Corporation (Eatontown, NJ). Out of these, two were silica hydride columns (SiH-I and SiH-II), two were bidentate C18 columns (BC18-I and BC18-II, 16.5% C), and one was unmodified silica (e-Si), used as a control. The unmodified silica was the same type of silica that was used for making the hydride-based columns and this was different from the silica used in Zorbax Rx-Sil column. The specifications for these columns are listed in Table 2.

2. Compounds

Samples used for the study are proprietary and were provided by Symyx Technologies. Four different groups of model compounds were used for the study. The generic structures of the compounds are listed in Figure 10. The first group consisted of two phenolic compounds labeled as A and B. These compounds were used because they gave strong UV and MS signals. The second group consisted of four phenolic compounds with different functionalities labeled as C, D, E, and F. The first three compounds were similar in polarity and difficult to separate with a bare silica commercial column. The third group was a reaction mixture of a heterocyclic-aryl starting material G

and product H. The fourth group was another reaction mixture consisting of the same heterocyclic-aryl starting material G but with a different product I. Since these model compounds were representative of those that were difficult to separate with bare silica columns under normal phase conditions they seemed appropriate for evaluating the hydride-based phases.

3. Solvents

Solvents used for the chromatographic runs (for preparing sample stock solutions, mobile phases, wash solvents) were hexane, ethyl acetate, methylene chloride, diethyl ether, tetrahydrofuran, and methanol. These were purchased from commercial suppliers in the highest purity available.

B. Instrumentation

Chromatographic runs were performed on a Waters LC system interfaced with a Micromass ZQ detector. The LC component consisted of a two Waters pumps, LC 515 used to deliver methanol to the interface and LC 600 controller used to deliver mobile phases to the column. Columns used for the study have been cited above. A Waters 2996 photo diode array served as the primary detector. This LC detector was interfaced with a Micromass ZQ that served as the secondary detector. The ZQ consisting of a quadrupole mass analyzer and a photomultiplier tube operated under high vacuum conditions that were created by turbomolecular pumps. The mass spectrum was obtained using the atmospheric pressure chemical ionization (APCI) mode. A schematic of the instrument setup is shown in Figure 11.

Table 2. Different columns evaluated in this study.

Column packing	Column size	Particle size	Pore	Supplier
Non-commercial silica hydride-batch A (SiH-A)	4.6 mm i.d. x 75 mm	4 μm	-	Dr. Pesek's lab
Non-commercial silica hydride-batch A (SiH-B)	4.6 mm i.d. x 75 mm	4 μm	-	Dr. Pesek's lab
Non-commercial bidentate C18 (BC18)	4.6 mm i.d. x 75 mm	4.2 μm	-	Dr. Pesek's lab
Commercial silica (Zorbax Rx-Sil)	4.6 mm i.d. x 150 mm	5 μm	80 $\text{\AA}$	Agilent Technologies
Commercial C18 (Eclipse XDB-C18, C18)	4.6 mm i.d. x 75 mm	3.5 μm	-	Agilent Technologies
Commercial silica hydride I (Type-C Silica, SiH-I)	4.6 mm i.d. x 75 mm	4 μm	100 $\text{\AA}$	Microsolv Technology Corporation
Commercial silica hydride II (Type-C Silica, SiH-II)	4.6 mm i.d. x 75 mm	4 μm	100 $\text{\AA}$	Microsolv Technology Corporation
Commercial bidentate C18 I (BC18-I)	4.6 mm i.d. x 75 mm	4 μm	100 $\text{\AA}$	Microsolv Technology Corporation
Commercial bidentate C18 II (BC18-II)	4.6 mm i.d. x 75 mm	4 μm	100 $\text{\AA}$	Microsolv Technology Corporation
Commercial Astrosil silica (Silica e-column, eSi)	4.6 mm i.d. x 75 mm	4 μm	100 $\text{\AA}$	Microsolv Technology Corporation

“-, data not available.”

Figure 10. Model compounds used for evaluating the hydride based stationary phases in this study.

Figure 11. Liquid chromatography mass spectrometry set-up.

C. Sample Preparation

Stock solutions of all the samples were made by dissolving approximately 20 mgs of each compound in 20 mL of 5% ethyl acetate in hexane. These solutions were filtered using 0.2 micron syringe filters prior to use. For analyzing mixtures, equal amounts of compounds were mixed together.

D. Chromatographic Procedures

1. Column Cleaning Procedures

The three hydride-based non-commercial columns had been previously used for extensive reverse phase applications. In order to remove any traces of water retained in the column the following cleaning procedure was followed prior to use. Each test column was first washed with 100% methanol overnight at a flow-rate of 0.2 mL/min. Successive washes were performed with 100% ethyl acetate, 100% methylene chloride

and 100% hexane for a half an hour each, in the order mentioned and at a flow rate of 1 mL/min. In order to perform a fair comparison all the other columns used in the study were washed with the same procedure prior to use.

2. Chromatographic Runs

Chromatographic separations were performed using the following mobile phase compositions: 3%, 4%, 5%, and 10% ethyl acetate (EtOAc) in hexane; 100% hexane; 10% and 20% methylene chloride (CH_2Cl_2) in hexane; 5% and 10% diethyl ether ($(\text{C}_2\text{H}_5)_2\text{O}$) in hexane; 5% and 10% tetrahydrofuran (THF) in hexane. The mobile phase flow-rate used was 1 mL/min. The sample injection volume was set at 10 μL and the concentration used was 1 mg/mL for each run. For most analyses, the run time was ten minutes with a few exceptions of thirty minutes. The equilibration time between different mobile phase compositions used varied between twenty to thirty minutes. All the experiments were performed at room temperature.

3. Conditioning Procedure

The column was washed with 10% methanol in toluene for twenty minutes and allowed to equilibrate with the appropriate mobile phase for the same amount of time. This conditioning procedure was performed only once with column SiH-I.

III. Results and Discussion

A. LCMS Data Format

LCMS separation of the model compounds resulted in three kinds of data sets. For each run, a UV chromatogram, a total ion current chromatogram, and a mass spectrum were obtained. During the separation, the mass spectrometer was set to scan repetitively over a 10 to 1000 mass range. At the end of each scan, a spectrum of the sum of intensities of ions present at each m/z , the total ion current (TIC), was stored (*I*). The run time was set at ten minutes for most of the runs and so after this time, the spectrometer stopped scanning. In addition, it was set to scan alternatively in the positive and negative modes, i.e., one scan was monitoring the number of positive ions formed while the subsequent scan was monitoring the number of negative ions formed. Because APCI was the ionization source used, the TIC chromatogram consisted of an APCI+ chromatogram and an APCI- chromatogram. The results included in this section show mostly UV chromatograms and in some cases TIC with only the APCI+ chromatogram. The APCI+ chromatograms were used to analyze the mass spectra of the model compounds.

B. Molecular Weight Determination of the Compounds using LC-MS

All the compounds were first analyzed individually on the LC-MS using 5% EtOAc in hexane. Figure 12 shows the TIC chromatograms for compounds A & B run on the non-commercial bidentate C18 column. There are two plots in the figure; the first one on top shows the chromatogram for compound A and the second plot shows the

chromatogram for compound B. The abscissa for each plot indicates retention time in minutes and the ordinate indicates the relative abundance of ions. The mass spectrum obtained using APCI+/- mode as shown in Figure 13 was used to identify both the compounds by their molecular ions. The first plot in the figure shows the mass spectrum for compound A with the molecular ion at m/z 324 and the second plot shows the mass spectrum for compound B with the molecular ion at m/z 228. The four plots in Figure 14 show the TIC chromatograms for compounds C, D, E, and F respectively, run on the non-commercial bidentate C18 column.

Figure 12. Total ion current chromatograms for compounds A and B on non-commercial bidentate C18 column.

Figure 13. APCI (+) mass spectra of compounds A and B.

Figure 14. Total ion current chromatograms for compounds C, D, E, and F on non-commercial bidentate C18 column.

The first three plots in the figure show the elution times of compounds C, D, and E respectively, all three elute within two minutes. Compound F has the longest retention time of all ~ 4.28 minutes as seen in the fourth plot, which is expected because it has an acidic functional group and is the most polar compound in this group. The four plots in Figure 15 show the mass spectra of all the four compounds.

Figure 15. APCI (+) mass spectra of compounds C, D, E, and F.

The first plot shows the mass spectrum for compound C having a molecular ion at m/z 255. The consecutive plots show mass spectra of the other three compounds; D with a molecular ion at m/z 407; E with a molecular ion at m/z 277 and F with a molecular ion at m/z 321. Figure 16 shows three TIC chromatograms for compounds G, H, and I run on the commercial silica hydride column-I.

Figure 16. Total ion current chromatograms for compounds G, H, and I on commercial silica hydride column-I.

The TIC chromatograms shown in the three plots indicate the different retention times of all three compounds. Figure 17 shows the mass spectra of these three compounds. The first plot in the figure shows the mass spectrum for compound G with a characteristic molecular ion at m/z 356. The second and third plots show mass spectra of compounds H and I with molecular ions at m/z 600 and m/z 530 respectively. Because the molecular weights of all the compounds were determined using the mass spectrometer, it was easier to identify peaks and their elution order in the chromatogram obtained from the LC. Only the UV chromatograms from the chromatographic results are shown for all the compounds in the following sections.

Figure 17. APCI (+) mass spectra of compounds G, H, and I.

C. Chromatographic Evaluation of Non-commercial Hydride-based Columns

In order to test if the hydride-based columns would work under normal phase conditions or not, preliminary work involved evaluating only the three non-commercial hydride-based columns (SiH-A, SiH-B and BC18) that had been previously developed and characterized in Dr. Pesek's lab. These columns had previously been used for reverse phase applications. When the first few runs led to successful normal phase separations, a commercial silica column (Zorbax Rx-Sil) and a commercial C18 (C18) column were also evaluated as controls for comparison purposes. Only the first two groups of compounds were analyzed during this preliminary work. Results for the evaluation of the non-commercial columns have been categorized according to the

different columns. Thus for each type of column, data for both groups of compounds is listed according to the different mobile phase conditions.

1. Non-commercial Silica Hydride-batch A Column (SiH-A)

a) Group I Data

A mixture of compounds A and B was separated using different mobile phase conditions as shown in Figure 18. The figure shows four UV chromatograms for the runs performed using 5% EtOAc in hexane, 100% hexane, 10%, and 20% CH₂Cl₂ in hexane. In each run, compound A eluted before compound B. Δ RT listed on each plot is the difference in retention times in minutes for both the compounds. Good peak resolution and narrow baseline separations were achieved for all four runs. Peaks were symmetrical in all cases except for the 100% hexane run where fronting was observed for compound B. Compounds were retained longer when 100% hexane was used as the mobile phase compared to 5%EtOAc in hexane indicating normal phase behavior. However, the compounds seemed to undergo a maximum retention at 10% CH₂Cl₂ in hexane and the retention time decreased as the percentage of CH₂Cl₂ increased in the mobile phase. This maximum retention behavior is not typical. Perhaps this behavior is due to solvation effects on the hydride surface, the site that controls the normal phase retention mechanism.

Figure 18. LC separation of compounds A and B on non-commercial silica hydride-batch A column, mobile phase: 5% EtOAc in hexane, 100% Hexane, 10%, and 20% CH₂Cl₂ in hexane.

b) Group II Data

Compounds C, D, E, and F were also analyzed as a mixture, using different mobile phase conditions. Figure 19 shows three UV chromatograms for the solvent conditions 3%, 4%, and 5% EtOAc in hexane. The elution order for the runs was C<D<E<F. Peaks for compounds C, D, and E are well resolved and symmetric as shown in the three plots; however, compound F did not elute in the first two cases and showed up as a very broad peak under the conditions of 5% EtOAc in hexane. Very high retention at low amounts of EtOAc is probably due to the acidic functional group on the

molecule. As shown in Figure 20, with 5% ((C₂H₅)₂O) in hexane, compounds C, D, and E were completely resolved but F did not elute. When 10% (C₂H₅)₂O in hexane was used, compounds C and D co-eluted, compound E was baseline separated, and peak fronting was observed with compound F which eluted as a broad peak. Peak resolution obtained with 5% and 10% (C₂H₅)₂O in hexane was not as good compared to the EtOAc mobile phase run. However, it slightly improved when THF was used as the modifier.

Figure 19. LC separation of compounds C, D, E, and F on non-commercial silica hydride-batch A column, mobile phase: 3%, 4%, and 5% EtOAc in hexane.

Figure 20. LC separation of compounds C, D, E, and F on non-commercial silica hydride-batch A column, mobile phase: 5% and 10% (C₂H₅)₂O in hexane, 5% and 10% THF in hexane.

In the case of 5% THF in hexane, narrow symmetric peaks were observed for compounds C, D, and E. They were baseline separated, the fourth compound F was strongly retained and did not elute during the ten minute run. When the percentage of THF was increased from 5% to 10%, peaks were observed for all four compounds; however the peak intensity for compound F was very low and the peak shape was very broad. Typical normal phase behavior was observed for all the chromatographic runs i.e. as the polarity of the mobile was increased, the retention times of all the compounds decreased.

2. Non-commercial Silica Hydride-batch B Column (SiH-B)

a) Group I Data

Similar experiments were performed on this column. A mixture of compounds A and B was analyzed using different mobile phases as shown in Figure 21. The four mobile phase conditions used were 5%EtOAc in hexane, 100% hexane, 10%, and 20% CH₂Cl₂ in hexane. The elution order observed was A<B. Good peak resolution and narrow baseline separations were achieved for all four runs.

Results obtained were similar to those of SiH-A, where compounds were retained longer when 100% hexane was used as a mobile phase compared to 5%EtOAc in hexane. With this column also, the compounds seemed to undergo a maximum retention at 10% CH₂Cl₂ in hexane and then the retention time decreased as the percentage of CH₂Cl₂ increased in the mobile phase; however, this time the ΔRT value also decreased.

The surface morphology might be slightly different between both batches of non-commercial silica hydride columns and this could account for the change in ΔRT . Since ΔRT for compounds A and B was almost similar in the case of both non-commercial silica hydride columns (SiH-A and SiH-B), under all conditions except 20% CH₂Cl₂ in hexane, further characterization for the SiH-B column was limited due to time constraints. Only Group II compounds were separated on this column using the EtOAc in hexane mobile phase to verify if the column displayed normal phase behavior or not.

Figure 21. LC separation of compounds A and B on non-commercial silica hydride-batch B column, mobile phase: 5% EtOAc in hexane, 100% Hexane, 10%, and 20% CH_2Cl_2 in hexane.

b) Group II Data

A mixture of compounds C, D, E, and F were separated in 3%, 4%, and 5% EtOAc in hexane mobile phases. Figure 22 shows three chromatographic plots under the different mobile phase conditions. The elution order of $\text{C} < \text{D} < \text{E}$ was observed in all the cases and compound F was retained longer than the run. As the amount of EtOAc was increased in the mobile phase, the retention times of the compounds decreased indicating typical normal phase behavior. Peaks were narrow and symmetric, in the case of the 3%

and 4% in hexane runs. With 5% EtOAc modifier, the peak resolution deteriorated slightly. Compounds C and D were not baseline separated.

Figure 22. LC separation of compounds C, D, E, and F on non-commercial silica hydride-batch B column, mobile phase: 3%, 4%, and 5% EtOAc in hexane.

3. Non-commercial Bidentate C18 Column (BC18) Vs Commercial C18 Column (C18)

a) Group I Data

A mixture of compounds A and B was analyzed using different mobile phases as shown in Figure 23. The three mobile phase conditions used were 5%EtOAc in hexane, 100% hexane, and 10% CH₂Cl₂ in hexane.

Figure 23. LC separation of compounds A and B on non-commercial bidentate C18 column, mobile phase: 5% EtOAc in hexane, 100% hexane, and 10% CH₂Cl₂ in hexane.

In each run, compound A eluted before compound B. Baseline separations were achieved for all the runs. The maximum ΔRT was observed with 100% hexane as the mobile phase and it decreased in the case of 10% CH₂Cl₂ in hexane as expected so the mixture was not run in 20% CH₂Cl₂ since it would only give lower retention time. High column efficiency was observed in case of the 5% EtOAc in hexane run while 100% hexane run led to peak tailing. Figure 24 shows that the same A+B mixture could not be separated on a commercial C18 (C18) column under any of the four mobile phase conditions.

Figure 24. LC separation of compounds A and B on commercial C18 column, mobile phase: 5% EtOAc in hexane, 100% Hexane, 10%, and 20% CH₂Cl₂ in hexane.

b) Group II Data

A mixture of compounds C, D, E, and F was analyzed on the BC18 column using the following mobile phase conditions: 3%, 4%, and 5% EtOAc in hexane, 5%, and 10% ((C₂H₅)₂O) in hexane, 5 %, and 10% THF in hexane. Figure 25 shows the separation under EtOAc conditions, the elution order being C<D<E<F. The first three peaks are narrow and the fourth peak is broad but baseline separated. This is a good example illustrating typical normal phase behavior where compounds eluted faster as the polarity of the mobile phase was increased. Good peak resolution and symmetric peak shapes were observed for compounds C, D, and E. Good resolution was observed even in the case of diethyl ether as the modifier as shown in the first two plots of Figure 26. When

THF was used as the mobile phase, compounds E and F co-eluted during the 5% THF run as shown in the third plot in the same figure. During 10% THF run, baseline separations could not be achieved however; four peaks were observed representing the four compounds as shown in the fourth plot in the same figure. Even though narrow peak shapes were maintained, peak resolution deteriorated. For all the chromatographic runs, as the amount of hexane decreased in the mobile phase composition the retention time of all the compounds decreased as well resulting in typical normal phase behavior. Because completely resolved peaks were observed for the solvent conditions 5% EtOAc in hexane and 10% ((C₂H₅)₂O) in hexane, the same mobile phases were used for testing the mixture on the commercial C18 column. Figure 27 shows that only one peak was observed in both the cases and no separation could be achieved.

Figure 25. LC separation of compounds C, D, E, and F on non-commercial bidentate C18 column, mobile phase: 3%, 4%, and 5% EtOAc in hexane.

Figure 26. LC separation of compounds C, D, E, and F on non-commercial bidentate C18 column, mobile phase: 5% and 10% (C₂H₅)₂O in hexane, 5% and 10% THF in hexane.

Figure 27. LC separation of compounds C, D, E, and F on commercial C18 column, mobile phase: 5% EtOAc in hexane and 10% (C₂H₅)₂O, in hexane.

4. Commercial Silica Column (Zorbax Rx-Sil)

a) Group I Data

The separations for this mixture under the different mobile phase conditions are shown in Figure 28.

Figure 28. LC separation of compounds A and B on commercial silica column, mobile phase: 5% EtOAc in hexane, 100% Hexane, 10%, and 20% CH₂Cl₂ in hexane.

ΔRT is maximum for this group of compounds under all the mobile phase conditions tested except 20% CH₂Cl₂ in hexane in comparison to the non-commercial hydride-based columns. The longer retention times could be due to the length of the column, which is twice the length of the other columns used in the study. Thus, the length of this column should be considered while comparing data between different

columns. Slight tailing was observed in all cases, this might not be visible in the figure shown because compared to the other figures the time scale is different. The chromatographic run for 100% hexane mobile phase was thirty minutes long instead of the typical ten-minute run.

b) Group II Data

Figure 29 consists of six plots showing the separation for this group of compounds using six different mobile phase conditions.

Figure 29. LC separation of compounds C, D, E, and F on commercial silica column, mobile phase: 3% and 5% EtOAc in hexane, 5% and 10% (C₂H₅)₂O in hexane, 5% and 10% THF in hexane.

With 3% EtOAc only compound E eluted as shown in the first plot on top and with 5% EtOAc compounds C and D co-eluted, a separate peak was observed for compound E while F was strongly retained as shown in the second plot. A similar situation was observed in the case of 5% and 10% ((C₂H₅)₂O) in hexane, where compounds C and D co-eluted, compound E was baseline separated and compound F was strongly retained as shown in the third and fourth plots. Compounds C, D, and E were better separated in the 5% and 10% THF runs as shown in the fifth and sixth plots, while compound F was still strongly retained. The peaks were broad and resolution was not very high.

In comparison to the non-commercial hydride-based columns, considering the length of the Zorbax Rx-Sil, the performance of the latter seemed inferior. Since successful separations for the two groups of compounds tested were achieved with the non-commercial columns it was concluded that these hydride-based columns could be used for normal phase separations. In some cases, they showed improved performance over the bare silica and commercial C18 columns used in the study. Tables 3, 4, 5, and 6 show the optimum separation results for compounds in Groups I and II obtained with the different columns that were studied.

Table 3. Optimum separation results obtained on non-commercial silica hydride-batch A column.

Separation results for fast analysis						
Compounds	Favorable mobile phase in hexane	Retention time t _R (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	1.13	0.51	$\alpha_{AB} = 1.35$	940	12500
B	5% EtOAc	1.53	1.04		1700	22600
C	10% THF	1.37	0.83	$\alpha_{CD} = 1.53$	1330	17700
D	10% THF	1.7	1.27	$\alpha_{DE} = 1.66$	2000	26700
E	10% THF	2.33	2.11	$\alpha_{EF} = 1.78$	3890	51900
F	10% THF	3.57	3.76		112	1500
Separation results for longer retention						
Compounds	Favorable mobile phase in hexane	Retention time t _R (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	100% Hexane	2.57	2.43	$\alpha_{AB} = 1.28$	2070	27600
B	100% Hexane	3.3	3.4		7790	104000
C	3% EtOAc	1.78	1.37	$\alpha_{CD} = 1.52$	2330	31000
D	3% EtOAc	2.32	2.09	$\alpha_{DE} = 1.97$	2500	3300
E	3% EtOAc	3.85	4.13		4660	62100
F	3% EtOAc	strongly retained				

Table 4. Optimum separation results obtained on non-commercial silica hydride-batch B column.

Separation results for fast analysis						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	1.13	0.51	$\alpha_{AB} = 1.26$	936	12500
B	5% EtOAc	1.42	0.89		1420	18900
C	3% EtOAc	1.57	1.09	$\alpha_{CD} = 1.37$	1900	25300
D	3% EtOAc	1.87	1.49	$\alpha_{DE} = 1.71$	2560	34100
E	3% EtOAc	2.67	2.56		5150	68700
F	3% EtOAc	strongly retained				
Separation results for longer retention						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	10% CH ₂ Cl ₂	2.97	2.96	$\alpha_{AB} = 1.46$	2930	39100
B	10% CH ₂ Cl ₂	4.33	4.77		5910	78800
C	3% EtOAc	1.57	1.09	$\alpha_{CD} = 1.37$	1900	25300
D	3% EtOAc	1.87	1.49	$\alpha_{DE} = 1.71$	2560	34100
E	3% EtOAc	2.67	2.56		5150	68700
F	3% EtOAc	strongly retained				

Table 5. Optimum separation results obtained on non-commercial bidentate C18 column.

Separation results for fast analysis						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	1.02	0.36	$\alpha_{AB} = 1.52$	2930	39100
B	5% EtOAc	1.55	1.07		866	11500
C	10% (C ₂ H ₅) ₂ O	1.22	0.63	$\alpha_{CD} = 1.74$	1250	16600
D	10% (C ₂ H ₅) ₂ O	1.57	1.09	$\alpha_{DE} = 1.37$	1900	25300
E	10% (C ₂ H ₅) ₂ O	1.87	1.49	$\alpha_{EF} = 1.36$	2560	34100
F	10% (C ₂ H ₅) ₂ O	2.27	2.03		3890	51900
Separation results for longer retention						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	10% CH ₂ Cl ₂	1.1	0.47	$\alpha_{AB} = 1.75$	1200	16000
B	10% CH ₂ Cl ₂	1.92	1.56		2320	31000
C	3% EtOAc	1.37	0.83	$\alpha_{CD} = 2.15$	1330	17700
D	3% EtOAc	2.08	1.77	$\alpha_{DE} = 1.37$	1420	18900
E	3% EtOAc	2.57	2.43	$\alpha_{EF} = 4.52$	3090	41100
F	10% (C ₂ H ₅) ₂ O	8.97	10.96		2330	31000

Table 6. Optimum separation results obtained on the commercial silica column.

Separation results for fast analysis						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	20% CH ₂ Cl ₂	1.3	0.73	$\alpha_{AB} = 1.63$	73	485
B	20% CH ₂ Cl ₂	2.12	1.83		355	2360
C	10% THF	2.65	2.53	$\alpha_{CD} = 1.29$	3190	21300
D	10% THF	3.2	3.27	$\alpha_{DE} = 1.49$	7240	48300
E	10% THF	4.4	4.87		6160	41000
F	10% THF	strongly retained				
Separation results for longer retention						
Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	2.18	1.91	$\alpha_{AB} = 1.32$	6400	42700
B	5% EtOAc	2.88	2.84		11000	73100
C	5% THF	2.98	2.97	$\alpha_{CD} = 1.42$	20200	135000
D	5% THF	3.92	4.23	$\alpha_{DE} = 1.61$	9070	60400
E	5% THF	5.85	6.80		10900	72600
F	5% THF	strongly retained				

D. Chromatographic Evaluation of Commercial Hydride-based Columns

Since the results for the non-commercial hydride-based columns were encouraging, new hydride-based columns were purchased from a commercial supplier and were evaluated using the same groups of compounds. In addition two other groups of compounds, groups III and IV were used for evaluation. Compound F from Group II was not analyzed further because it was being strongly retained on all the non-commercial columns under most of the mobile phase conditions that were used. As it was very polar, its retention behavior under normal phase conditions was typical however because no drastic changes in its retention was observed under different mobile phase conditions, further evaluation with similar stationary phases seemed meaningless and so it was eliminated from the group. The second group of compounds will be referred to as “Modified group II” for the results presented in the following sections. The three solvent conditions used for all the tests were 5% EtOAc in hexane, 10% CH₂Cl₂ in hexane, and 10% THF in hexane. Results for the commercial columns have been categorized by its type of stationary phase. Compounds in each group were first analyzed individually and then as mixtures, in order to check batch to batch reproducibility of these columns. Thus for each type of column, data for four groups of compounds is listed according to the different mobile phase conditions.

1. Commercial Silica Hydride Column I (Type-C Silica, SiH-I)

a) Group I Data

Figure 30 shows the UV chromatograms obtained after both compounds A and B were analyzed separately and as a mixture using 5% EtOAc in hexane as mobile phase.

Almost baseline separations were achieved. Slight tailing was observed for compound B while peak for compound A was narrow and symmetric. Similar results were obtained with the 10% CH₂Cl₂ in hexane and 10% THF in hexane runs as shown in Figures 31 and 32. Peak tailing increased in the case of compound B when CH₂Cl₂ was used as the modifier. In addition, peak resolution improved and maximum ΔRT was observed. Separation obtained when 10% THF was used as the modifier was similar to that obtained with 5% EtOAc in hexane run. Narrow, almost baseline-separated peaks were observed for this the run.

Figure 30. LC separation of compounds A and B on commercial silica hydride column I using mobile phase 5% EtOAc in hexane.

Figure 31. LC separation of compounds A and B on commercial silica hydride column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 32. LC separation of compounds A and B on commercial silica hydride column I using mobile phase 10% THF in hexane.

Compared to the non-commercial silica hydride columns the retention times of compounds A and B were shorter with the new columns. With the non-commercial columns, these compounds had a maximum retention with 10% CH₂Cl₂, so in order to check if the retention behavior was reproducible with the new hydride columns the runs were performed again using similar solvent conditions as before. Figure 33 shows that typical normal phase behavior resulted without any evidence of a retention maxima occurring at 10% CH₂Cl₂. Good peak resolution was observed in all cases. Peak tailing for compound B was observed in all cases.

Figure 33. LC separation of compounds A and B on commercial silica hydride column I, mobile phase: 5% EtOAc in hexane, 100% Hexane, 10%, and 20% CH₂Cl₂ in hexane.

This difference in behavior could be because of slight differences in the stationary phase surface of the non-commercial and the commercial columns arising because the non-commercial columns were prepared on a small scale in the laboratory while the new columns were manufactured commercially on a large scale. Large scale synthetic procedures may have been further optimized and thus resulted in a slightly different column surface.

b) Modified Group II Data

With 5% EtOAc in hexane, compounds C and D co-eluted while compound E was retained slightly longer as shown in Figure 34. Narrow, symmetric peaks were observed when the compounds were run individually. However, with this mobile phase, the retention times of compounds C and D were similar and thus they co-eluted in the mixture. Figure 35 shows that retention time of the compounds increased when 10% CH₂Cl₂ was used as the modifier. Narrow, symmetric peaks were observed for compounds C and D while peak shape deteriorated for compound E resulting in a broad peak. Compounds C and D were almost baseline separated. Compounds were least retained in 10% THF in hexane as shown in Figure 36. The peak shapes were narrow and symmetric with the exception of compound D that showed slight tailing when run individually. Despite of good peak shapes resolution did not improve. The retention times for compounds C and D were very close to each other so they co-eluted again in the mixture. In comparison to the non-commercial columns, retention times for all the compounds were shorter with this commercial column.

Figure 34. LC separation of compounds C, D, and E on commercial silica hydride column I using mobile phase 5% EtOAc in hexane.

Figure 35. LC separation of compounds C, D, and E on commercial silica hydride column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 36. LC separation of compounds C, D, and E on commercial silica hydride column I using mobile phase 10% THF in hexane.

c) Group III Data

Compounds G and H were run separately and as a mixture with the three different solvent conditions and the best separation was achieved with 5% EtOAc in hexane as shown in Figure 37. Narrow symmetric peaks were observed for the starting material G as well as the product H. Both compounds were baseline separated. With 10% CH_2Cl_2 in hexane, an unusual peak shape for compound H was observed as shown in Figure 38. Even though peak shape for compound H deteriorated, resolution improved for the separation.

Figure 37. LC separation of compounds G and H on commercial silica hydride column I using mobile phase 5% EtOAc in hexane.

Figure 38. LC separation of compounds G and H on commercial silica hydride column I using mobile phase 10% CH₂Cl₂ in hexane.

The longest retention occurred with 10% CH₂Cl₂ in hexane while the shortest retention was observed with 10% THF in hexane as shown in Figure 39. Since Compound G is a regioisomeric product of a reaction, in some cases, a less intense, small second peak appeared for the minor regioisomeric product. The m/z for this peak was also 356 (not shown). With 5% EtOAc in hexane, the minor peak was evident at 1.72 minutes, with 10% CH₂Cl₂ in hexane it appeared at 3.25 minutes and with 10% THF in hexane, it appeared at 1.27 minutes. Peaks obtained with THF as the modifier were narrow and symmetric. Baseline separations were achieved in all the cases.

Figure 39. LC separation of compounds G and H on commercial silica hydride column I using mobile phase 10% THF in hexane.

d) Group IV Data

For this reaction mixture also, 5% EtOAc in hexane mobile phase gave the best separation as shown in Figure 40.

Figure 40. LC separation of compounds G and I on commercial silica hydride column I using mobile phase 5% EtOAc in hexane.

Good peak resolution and narrow baseline separations were achieved. Similar to the first reaction mixture, retention times increased with 10% CH_2Cl_2 in hexane as shown in Figure 41. With CH_2Cl_2 as the modifier, peak shape for I was unusual. The peak shape improved when the modifier was changed to THF as shown in Figure 42. Symmetric peaks and high column efficiency were observed with this modifier.

Figure 41. LC separation of compounds G and I on commercial silica hydride column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 42. LC separation of compounds G and I on commercial silica hydride column I using mobile phase 10% THF in hexane.

2. Commercial Silica Hydride Column II (Type-C Silica, SiH-II)

a) Group I Data

Narrow baseline separations were achieved for this group under all three mobile phase conditions as shown in Figures 43, 44, and 45. The retention behavior with 5% EtOAc in hexane and 10% CH_2Cl_2 in hexane was similar while 10% THF in hexane yielded the longest retention. In comparison to the SiH-I column, compounds were retained slightly longer on this column. Such differences could be because these columns were synthesized from different batches of silica. It is difficult to achieve identical retention for a set of compounds using two columns that have been synthesized from different batches of silica at different times. As long as the separation capabilities remain the same, such changes can be ignored.

Figure 43. LC separation of compounds A and B on commercial silica hydride column II using mobile phase 5% EtOAc in hexane.

Figure 44. LC separation of compounds A and B on commercial silica hydride column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 45. LC separation of compounds A and B on commercial silica hydride column II using mobile phase 10% THF in hexane.

b) Modified Group II Data

Compounds C, D, and E were retained longer with 5% EtOAc in hexane compared to SiH-I. Better peak resolution was also observed as shown in Figure 46. The peak shapes were narrow and symmetric. With 10% CH₂Cl₂ in hexane, compounds C and D eluted at the same time and so only two peaks were observed for the mixture as shown in Figure 47. Maximum retention was observed with 10% THF in hexane (Figure 48), which was not the case with SiH-I column. Compounds C and D co-eluted and only compound E was baseline separated.

Figure 46. LC separation of compounds C, D, and E on commercial silica hydride column II using mobile phase 5% EtOAc in hexane.

Figure 47. LC separation of compounds C, D, and E on commercial silica hydride column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 48. LC separation of compounds C, D, and E on commercial silica hydride column II using mobile phase 10% THF in hexane.

c) Group III Data

Figure 49 shows UV chromatograms for this reaction mixture with 5% EtOAc in hexane. Both the peaks were well resolved and symmetric. A small peak for the minor regioisomeric product of G was observed at 1.9 minutes. This peak shifted to 4.13 minutes with 10% CH₂Cl₂ in hexane as shown in Figure 50. The separation improved with CH₂Cl₂ modifier. On this column, the peak shape for compound H was typical, unlike the one that was observed with column SiH-I. Compounds were retained longest with 10% THF in hexane as shown in Figure 51. The minor regioisomeric peak of compound G was observed at 2.37 minutes. Compared to the previous separation achieved on SiH-I, Δ RT decreased slightly, however narrow baseline separations were still achieved.

Figure 49. LC separation of compounds G and H on commercial silica hydride column II using mobile phase 5% EtOAc in hexane.

Figure 50. LC separation of compounds G and H on commercial silica hydride column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 51. LC separation of compounds G and H on commercial silica hydride column II using mobile phase 10% THF in hexane.

d) Group IV Data

Narrow intense peaks were observed for this reaction mixture with 5% EtOAc in hexane as shown in Figure 52. Both the peaks were well resolved and symmetric. Retention times did not change much with 10% CH₂Cl₂ (Figure 53) and peak shape for compound I was normal compared to that obtained with SiH-I column. Slight tailing was observed for compound I. Δ RT remained constant compared to the previous run where EtOAc was the modifier. The retention times for both compounds increased with 10% THF in hexane as shown in Figure 54. In this separation, even though Δ RT decreased slightly, the compounds were baseline separated. The minor regioisomeric peak of compound G was observed at 2.37 minutes.

Figure 52. LC separation of compounds G and I on commercial silica hydride column II using mobile phase 5% EtOAc in hexane.

Figure 53. LC-MS separation of compounds G and I on commercial silica hydride column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 54. LC separation of compounds G and I on commercial silica hydride column II using mobile phase 10% THF in hexane.

3. Commercial Bidentate C18 Column I (BC18-I)

a) Group I Data

Baseline separations were achieved for this mixture with 5% EtOAc and 10% CH₂Cl₂ in hexane as shown in Figures 55 and 56. The peak obtained for compound B was broad in the 5% EtOAc in hexane run. Peak tailing was observed for both the compounds. With 10% CH₂Cl₂ in hexane conditions, asymmetric peaks were observed. Peak fronting occurred for compound A while peak tailing was observed for compound B when they were both run as a mixture. In addition, compounds were retained longer with this modifier. When 10% THF in hexane was used, the retention time for all the compounds decreased as shown in Figure 57. A narrow peak for compound A and peak fronting for compound B were observed when the compounds were run individually. Baseline separations could not be achieved when the compounds were run as a mixture.

In order to confirm whether the separation behavior of this commercial bidentate C18 column I was similar to that observed with the non-commercial bidentate C18 column, separations that had previously been performed on the latter were repeated on this column. Figure 58 shows the separation of compounds A and B with the different mobile phase conditions, 5% EtOAc, 100% hexane, 10%, and 20% CH₂Cl₂ in hexane. Typical normal phase behavior was observed, i.e. retention times of the compounds decreased with increasing polarity of the mobile phase. In comparison to the non-commercial bidentate C18 columns, the retention times were slightly lower on this column. Peak fronting and tailing were observed when CH₂Cl₂ was used as the modifier.

Figure 55. LC separation of compounds A and B on commercial bidentate C18 column I using mobile phase 5% EtOAc in hexane.

Figure 56. LC separation of compounds A and B on commercial bidentate C18 column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 57. LC separation of compounds A and B on commercial bidentate C18 column I using mobile phase 10% THF in hexane.

Figure 58. LC separation of compounds A and B on commercial bidentate C18 column I, mobile phase: 5% EtOAc, 100% Hexane, 10%, and 20% CH_2Cl_2 in hexane.

b) Modified Group II Data

Peaks obtained for compounds C, D, and E were somewhat broad with 5% EtOAc in hexane as shown in Figure 59. Compounds C and D had similar retention times so baseline separations could not be achieved when they were analyzed as a mixture. In contrast, the non-commercial bidentate C18 column gave narrow baseline separations under the same conditions. With this commercial column, peak resolution was worse in the case of 10% CH₂Cl₂ in hexane as shown in Figure 60. Peak tailing was observed for all the compounds and no separation could be achieved. When 10% THF was used as modifier, the retention times decreased and there was no improvement in peak resolution as shown in Figure 61.

Results obtained for this group of compounds on the commercial bidentate C18 column I, were significantly different from those observed with the non-commercial bidentate C18 column. With the latter, separations could be achieved under identical conditions. The different results can be explained based on the following assumption. The synthetic procedure for the commercial columns may have been optimized for a large scale synthesis. These column packings were developed originally for reverse phase applications and so it is possible that the optimization that was suitable for reverse-phase applications is not appropriate for normal phase separations and thus is leading to differences in separation behavior of the non-commercial bidentate C18 and the commercial BC18-I columns.

Figure 59. LC separation of compounds C, D, and E on commercial bidentate C18 column I using mobile phase 5% EtOAc in hexane.

Figure 60. LC separation of compounds C, D, and E on commercial bidentate C18 column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 61. LC separation of compounds C, D, and E on commercial bidentate C18 column I using mobile phase 10% THF in hexane.

c) Group III Data

For this group of compounds, no separations could be achieved with 5% EtOAc in hexane (Figure 62) as the retention times for the starting material and the product were same. With 10% CH_2Cl_2 in hexane, the peaks obtained were broad as shown in Figure 63. The retention times were similar, however when the mixture was analyzed, two peaks for the respective compounds appeared in the chromatogram. A tiny broad peak for the minor regioisomer of compound G appeared at 3.53 minutes. Since the peak for H was very broad, it probably overlapped this tiny peak and thus it was not observed in the UV chromatogram of the mixture.

In the case of the 10% THF in hexane run, retention times obtained were similar to those achieved with 5% EtOAc as shown in Figure 64. Since the retention times of both the compounds were similar, no separation was achieved. A less intense minor regioisomeric peak of compound G was observed at 2.3 minutes, which probably was overlapped by the broad peak for compound H during the run when the mixture was analyzed.

Figure 62. LC separation of compounds G and H on commercial bidentate C18 column I using mobile phase 5% EtOAc in hexane.

Figure 63. LC separation of compounds G and H on commercial bidentate C18 column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 64. LC separation of compounds G and H on commercial bidentate C18 column I using mobile phase 10% THF in hexane.

d) Group IV Data

A bimodal peak was observed for compound I with 5% EtOAc in hexane conditions. No peak for the minor regioisomeric product of G was observed. Perhaps the signal at 1.72 minutes for G (Figure 65) contains two overlapping peaks, one of which is participating in the reaction to form a regioisomeric product of compound I. This assumption is being made because two peaks appeared for compound I. The molecular ion for both the peaks in the spectrum was same for compound I. Results were similar for 10% CH₂Cl₂ and 10% THF in hexane mobile phase conditions as shown in Figures 66 and 67. Peak resolution was the worst with the 10% CH₂Cl₂ in hexane run. Peak fronting was observed in the case of compound G and unusual broad peak shape was observed for compound I. With 10 % THF as the modifier, peak shapes improved but a separation could not be achieved.

Figure 65. LC separation of compounds G and I on commercial bidentate C18 column I using mobile phase 5% EtOAc in hexane.

Figure 66. LC separation of compounds G and I on commercial bidentate C18 column I using mobile phase 10% CH₂Cl₂ in hexane.

Figure 67. LC separation of compounds G and I on commercial bidentate C18 column I using mobile phase 10% THF in hexane.

4. Commercial Bidentate C18 Column II (BC18-II)

a) Group I Data

A baseline separation was achieved for this mixture with 5% EtOAc in hexane as shown in Figure 68. Slight tailing was observed in case of compound B. The retention time for both the compounds was quite similar in the case of 10% CH₂Cl₂ in hexane as shown in Figure 69 so they could not be completely separated. Retention was longer with CH₂Cl₂ as the modifier. Even though the retention times were slightly different for both the compounds in the case of 10% THF in hexane as shown in Figure 70, due to peak tailing and fronting, a baseline separation could not be achieved. Retention time decreased with this modifier, it was similar to that observed with 5% EtOAc in hexane.

Figure 68. LC separation of compounds A and B on commercial bidentate C18 column II using mobile phase 5% EtOAc in hexane.

Figure 69. LC separation of compounds A and B on commercial bidentate C18 column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 70. LC separation of compounds A and B on commercial bidentate C18 column II using mobile phase 10% THF in hexane.

b) Modified Group II Data

The retention times obtained for compounds C and D were virtually the same in the 5% EtOAc in hexane run as shown in Figure 71. Compound E was retained slightly longer, but not long enough for a separation to be achieved. Peaks obtained were narrow and symmetric. In the case of 10% CH₂Cl₂ in hexane, the retention times for all the compounds were around 1.7 minutes (Figure 72) so a separation could not be achieved. The retention time increased with this modifier but peak shapes deteriorated for compounds D and E. Similarly, with the 10% THF in hexane run, due to similar retention times of all the compounds, a separation could not be achieved as shown in Figure 73. The retention time decreased with this modifier and peak shapes deteriorated for all the compounds.

Figure 71. LC-MS separation of compounds C, D, and E on commercial bidentate C18 column II using mobile phase 5% EtOAc in hexane.

Figure 72. LC separation of compounds C, D, and E on commercial bidentate C18 column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 73. LC separation of compounds C, D, and E on commercial bidentate C18 column II using mobile phase 10% THF in hexane.

c) Group III Data

Compounds G and H could not be separated in 5% EtOAc in hexane conditions as shown in Figure 74. Narrow peaks were obtained for both the compounds but the retention times were similar. In the case of the 10% CH₂Cl₂ in hexane run, the peak for compound H was very broad. The minor regioisomeric peak of G appeared at 2.10 minutes, but it was not separated from the parent peak. A separation was achieved in this case as shown in Figure 75. Compounds were retained longer with this modifier. In the case of 10% THF in hexane run, the regioisomeric peak of G appeared at 2.05 minutes and it was separate from the parent peak. Because the retention time for H was the same, no separations could be achieved under these conditions as shown in Figure 76. Retention decreased slightly with this modifier and the peak shape for H remained asymmetric.

Figure 74. LC separation of compounds G and H on commercial bidentate C18 column II using mobile phase 5% EtOAc in hexane.

Figure 75. LC separation of compounds G and H on commercial bidentate C18 column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 76. LC separation of compounds G and H on commercial bidentate C18 column II using mobile phase 10% THF in hexane.

d) Group IV Data

The retention times and trends for this mixture were almost identical to those obtained for the Group III mixture as shown in Figures 77, 78, and 79. Peaks obtained were narrow and symmetric with 5% EtOAc modifier. Because the retention times were similar, compounds could not be separated. In the case of the 10% CH₂Cl₂ in hexane run, the peak for compound H was very broad as shown in Figure 78. The minor regioisomeric peak of G appeared at 2.10 minutes, but it was not separated from the parent peak. A separation was achieved with this modifier, retention decreased slightly and the peak shape for H remained asymmetric. In comparison to BC18-I, retention times for all the compounds were shorter on this column. It is assumed that these slight variations in retention times arise due to the different batches of silica used during column manufacturing.

Figure 77. LC separation of compounds G and I on commercial bidentate C18 column II using mobile phase 5% EtOAc in hexane.

Figure 78. LC separation of compounds G and I on commercial bidentate C18 column II using mobile phase 10% CH₂Cl₂ in hexane.

Figure 79. LC separation of compounds G and I on commercial bidentate C18 column II using mobile phase 10% THF in hexane.

5. Commercial Astrosil Silica (Silica e-column, eSi)

a) Group I Data

Narrow baseline separations were achieved for this mixture using 5% EtOAc in hexane as shown in Figure 80. The retention times increased with 10% CH₂Cl₂ in hexane and resulted in a successful separation as shown in Figure 81. Peak tailing was observed for compound B with this modifier. Maximum retention was observed with 10% THF in hexane. Peak symmetry and resolution deteriorated since the peaks were broader, however they remained separated as shown in Figure 82. Compared to the other commercial hydride-based columns, the retention for this mixture was longest in the case of this column, with the exception of BC18-I.

Figure 80. LC separation of compounds A and B on commercial Astrosil silica column using mobile phase 5% EtOAc in hexane.

Figure 81. LC separation of compounds A and B on commercial Astrosil silica column using mobile phase 10% CH₂Cl₂ in hexane.

Figure 82. LC separation of compounds A and B on commercial Astrosil silica column using mobile phase 10% THF in hexane.

b) Modified Group II Data

For this mixture, a separation could be achieved only with 5% EtOAc in hexane as shown in Figure 83. In the case of 10% CH₂Cl₂ in hexane, compounds C and D co-eluted as shown in Figure 84. A separate peak for compound E was observed. Peak resolution deteriorated in case of 10% THF in hexane. The retention times obtained for the compounds were longer when they were analyzed separately as compared to that of the mixture as shown in Figure 85. For this mixture, the results obtained on this silica column were comparable to those obtained on the commercial silica hydride columns. Their performance was superior when compared to the results obtained on the commercial bidentate C18 columns.

Figure 83. LC separation of compounds C, D, and E on commercial Astrosil silica column using mobile phase 5% EtOAc in hexane.

Figure 84. LC separation of compounds C, D, and E on commercial Astrosil silica column using mobile phase 10% CH₂Cl₂ in hexane.

Figure 85. LC separation of compounds C, D, and E on commercial Astrosil silica column using mobile phase 10% THF in hexane.

c) Group III Data

For this mixture, narrow baseline separations were achieved with both 5% EtOAc and 10% CH₂Cl₂ in hexane as shown in Figures 86 and 87. The minor regioisomeric peak of compound G appeared at 1.77 minutes during the 5% EtOAc in hexane run. Peak tailing was observed for compound H when CH₂Cl₂ was used as the modifier. The minor regioisomeric peak of compound G appeared at 1.62 minutes during the 10% CH₂Cl₂ in hexane run. The minor regioisomeric peak of compound G appeared at 1.48 minutes for the 10% THF in hexane run as shown in Figure 88. The peak shape for compound H was unusual and no separation could be achieved for this mixture under these conditions. For this mixture, the silica hydride columns SiH-I and SiH-II were superior in performance compared to this bare silica column.

Figure 86. LC separation of compounds G and H on commercial Astrosil silica column using mobile phase 5% EtOAc in hexane.

Figure 87. LC separation of compounds G and H on commercial Astrosil silica column using mobile phase 10% CH₂Cl₂ in hexane.

Figure 88. LC separation of compounds G and H on commercial Astrosil silica column using mobile phase 10% THF in hexane.

d) Group IV Data

Narrow baseline separations were achieved with 5% EtOAc in hexane as shown in Figure 89. Peak fronting was observed for compound I when EtOAc was used as the modifier. The minor regioisomeric peak of compound G appeared at 1.77 minutes during the 5% EtOAc in hexane run. This peak shifted to 1.62 minutes in the case of the 10% CH₂Cl₂ in hexane as shown in Figure 90. The peak for compound I was very broad and low in intensity when the mixture was analyzed. In the case of 10% THF in hexane, the minor regioisomeric product of compound G appeared at 1.48 minutes as shown in Figure 91. Perhaps the minor regioisomer of compound G also participated in the reaction to form compound I, thus resulting in two regioisomer peaks for I as observed in the figure. Both the peaks had the same molecular ion in the mass spectrum. This peak was very intense and appeared in the chromatogram even when the mixture was analyzed. Baseline separations could be achieved with this modifier. For this mixture, in some cases the results obtained on this silica column were better than those obtained on the commercial hydride-based columns. Tables 7, 8, 9, 10, and 11 show the optimum separation results for compounds in all the four groups obtained with the different commercial columns that were studied.

Figure 89. LC separation of compounds G and I on commercial Astrosil silica column using mobile phase 5% EtOAc in hexane.

Figure 90. LC separation of compounds G and I on commercial Astrosil silica column using mobile phase 10% CH₂Cl₂ in hexane.

Figure 91. LC separation of compounds G and I on commercial Astrosil silica column using mobile phase 10% THF in hexane.

Table 7. Optimum separation results obtained on commercial silica hydride column I.

Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	10% THF	1.07	0.43	$\alpha_{AB} = 1.15$	3460	46200
B	10% THF	1.23	0.64		4040	53800
C	10% CH ₂ Cl ₂	1.72	1.29	$\alpha_{CD} = 1.26$	2000	26700
D	10% CH ₂ Cl ₂	1.97	1.63	$\alpha_{DE} = 1.68$	2810	37400
E	10% CH ₂ Cl ₂	2.8	2.73		463	6180
G	5% EtOAc	1.1	0.47	$\alpha_{GH} = 1.3$	1200	16000
H	5% EtOAc	1.43	0.91		1420	18900
G	5% EtOAc	1.1	0.47	$\alpha_{GI} = 1.31$	1200	16000
I	5% EtOAc	1.87	1.49		2560	34100

Table 8. Optimum separation results obtained on commercial silica hydride column

II.

Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	10% THF	2.12	1.83	$\alpha_{AB} = 1.14$	1750	23300
B	10% THF	2.42	2.23		2300	30700
C	5% EtOAc	1.53	1.04	$\alpha_{CD} = 1.24$	1700	22600
D	5% EtOAc	1.72	1.29	$\alpha_{DE} = 1.22$	2000	26700
E	5% EtOAc	1.93	1.57		2560	34100
G	10% CH ₂ Cl ₂	1.43	0.91	$\alpha_{GH} = 1.5$	1420	18900
H	10% CH ₂ Cl ₂	2.15	1.87		1480	19700
G	10% THF	2.12	1.83	$\alpha_{GI} = 1.31$	3190	42500
I	10% THF	2.77	2.69		5320	71000

Table 9. Optimum separation results obtained on commercial bidentate C18 column I.

Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	1.78	1.37	$\alpha_{AB} = 1.4$	1090	14500
B	5% EtOAc	2.5	2.33		720	9600

Table 10. Optimum separation results obtained on commercial bidentate C18 column II.

Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	5% EtOAc	0.82	0.09	$\alpha_{AB} = 1.34$	449	5980
B	5% EtOAc	1.1	0.47		866	11500

Table 11. Optimum separation results obtained on commercial Astrosil silica column.

Compounds	Favorable mobile phase in hexane	Retention time tR (min)	Capacity factor (k)	Selectivity α	Column efficiency N	# plates/meter
A	10% CH ₂ Cl ₂	1.8	1.40	$\alpha_{AB} = 1.31$	2330	31000
B	10% CH ₂ Cl ₂	2.35	2.13		4040	53800
C	5% EtOAc	1.5	1	$\alpha_{CD} = 1.33$	1600	21300
D	5% EtOAc	1.75	1.33	$\alpha_{DE} = 1.88$	2220	29500
E	5% EtOAc	2.63	2.51		2140	28600
G	5% EtOAc	1.17	0.56	$\alpha_{GH} = 1.44$	1010	13500
H	5% EtOAc	1.68	1.24		936	12500
G	5% EtOAc	1.17	0.56	$\alpha_{GI} = 2.11$	1010	13500
I	5% EtOAc	3.55	3.73		2270	30300

E. Reproducibility Data

An important aspect that was also studied was whether the commercial hydride-based columns gave reproducible data or not. Successive injections made on a single day and some runs performed on different days indicated that the same retention behavior could be achieved reproducibly. Figure 92 shows separation of compounds C, D, E, and F on the SiH-I column using 10% (C₂H₅)₂O in hexane. Three consecutive injections of the sample mixture were made and the same retention behavior was observed in all the cases. Four more successive injections of the same mixture on the same column using a different mobile phase led to similar observations as shown in Figure 93.

Figure 92. Reproducibility data for Group II mixture on SiH-I column with 10% (C₂H₅)₂O in hexane.

Figure 93. Reproducibility data for Group II mixture on SiH-I column with 5% EtOAc in hexane.

The above two experiments indicated that chromatographic runs performed on the commercial silica hydride columns were reproducible. Similar experiments were performed with one of the commercial bidentate C18 columns; BC18-I also indicated good reproducibility as shown in Figure 94. Four successive injections of Group II mixture resulted in similar retention times for all the four compounds. Based on the experiments performed, it was concluded that the columns gave reproducible data.

Figure 94. Reproducibility data for Group II mixture on BC18-I column with 5% EtOAc in hexane.

F. Conditioning Studies with Commercial Silica Hydride Column I

In order to improve peak shape quality and separation, one of the columns SiH-I was conditioned using 10% methanol in toluene as mentioned earlier in the conditioning procedure of the Experimental section. After the column was conditioned, only mixtures from all the groups of compounds were tested using 5% EtOAc, 10% CH₂Cl₂, and 10% THF in hexane mobile phase conditions. The results obtained after conditioning were different, in addition the retention properties and peak resolution varied significantly. In order to check if the surface of the column was modified or not, the column was washed

with the appropriate mobile phase for 5-7 hours and the runs were repeated. The results are categorized by reaction mixtures as shown in the following figures. Each figure has three plots; the first one on top shows the separation obtained prior to conditioning; the second plot shows the result obtained after conditioning; and the third plot shows the result obtained after the column was washed with the mobile phase for a longer period of time.

1. Group I Data

Figure 95 shows the results obtained after a mixture of compounds A and B was run on the column using 5% EtOAc in hexane. Retention times for both the compounds doubled after conditioning. ΔRT was doubled as well and the peak shapes were broader. Subsequent to washing the retention properties of both the compounds returned to the original values. With 10% CH_2Cl_2 in hexane, compounds were retained longer and ΔRT was doubled after conditioning as shown in Figure 96. The chromatographic run following the wash did not reverse the retention properties, and the peak shapes obtained were very broad and distorted. With 10% THF in hexane, compounds were retained longer, ΔRT was tripled, and peaks were broader after conditioning as shown in Figure 97. Subsequent wash and run yielded lower retention times similar to those obtained prior to conditioning.

Figure 95. Conditioning results for Group I mixture on SiH-I column using 5% EtOAc in hexane.

Figure 96. Conditioning results for Group I mixture on SiH-I column using 10% CH₂Cl₂ in hexane.

Figure 97. Conditioning results for Group I mixture on SiH-I column using 10% THF in hexane.

2. Modified Group II Data

For the second mixture consisting of compounds C, D, and E conditioning had a negative effect leading to poorly resolved broad peaks with all three solvent conditions as shown in Figures 98, 99, and 100. With 5% EtOAc modifier, prior to conditioning a separation could not be achieved. Compounds C and D co-eluted. The separation results after conditioning yielded similar results with C and D co-eluting together. The retention times for all the three compounds increased. Even though ΔRT for the co-eluted peak and compound E increased, the compounds were not baseline separated. The peaks were broader and subsequent washing did not improve peak shape. The retention time shifted

back to the original, but this time only one broad peak was observed for the three compounds.

With 10% CH₂Cl₂ in hexane mobile phase condition, baseline separation was achieved for the three compounds prior to conditioning. After conditioning, a separation could not be achieved and the retention time did not change for any of the compounds resulting in one broad tailing peak. Subsequent washing increased retention, unlike the other cases and did not improve peak shape. Results obtained with THF modifier were similar to those observed with 5% EtOAc in hexane. Conditioning increased retention time, but did not yield a separation. Subsequent washing decreased retention time and resulted in a broad tailing peak.

Figure 98. Conditioning results for Modified Group II mixture on SiH-I column using 5% EtOAc in hexane.

Figure 99. Conditioning results for Modified Group II mixture on SiH-I column using 10% CH₂Cl₂ in hexane.

Figure 100. Conditioning results for Modified Group II mixture on SiH-I column using 10% THF in hexane.

3. Group III Data

Figure 101 shows the results for the third mixture consisting of compounds G and H, obtained using 5% EtOAc in hexane. After conditioning the retention times and ΔRT doubled, but the compounds were not baseline separated. Subsequent washing reversed the retention behavior but did not result in a separation either. With 10% CH_2Cl_2 in hexane also, no separation could be obtained after conditioning as shown in Figure 102. Conditioning resulted in a broad tailing peak for the mixture. Subsequent washing did not change the results. With 10% THF in hexane, longer retention times were observed after conditioning as shown in Figure 103. The compounds were almost baseline separated after conditioning, but the peaks were very broad. Subsequent washing decreased retention but did not result in a baseline separation.

Figure 101. Conditioning results for Group III mixture on SiH-I column using 5% EtOAc in hexane.

Figure 102. Conditioning results for Group III mixture on SiH-I column using 10% CH₂Cl₂ in hexane.

Figure 103. Conditioning results for Group III mixture on SiH-I column using 10% CH₂Cl₂ in hexane.

4. Group IV Data

For the last mixture, consisting of compounds G and I, separations could be achieved with 5% EtOAc and 10% THF in hexane mobile phase conditions but not with 10% CH₂Cl₂ in hexane as shown in Figures 104, 105, and 106. In the case of 5% EtOAc and 10% THF modifiers, the retention times increased significantly with conditioning, decreased with subsequent washing resulting in broad separated peaks. Peak quality did not improve in either of the cases. With 10% CH₂Cl₂ modifier, conditioning resulted in a broad tailing peak for the mixture, which had a retention time somewhere in between those obtained for the individual compounds. Subsequent washing increased retention but resulted in a broad tailing peak.

Figure 104. Conditioning results for Group IV mixture on SiH-I column using 5% EtOAc in hexane.

Figure 105. Conditioning results for Group IV mixture on SiH-I column using 10% CH₂Cl₂ in hexane.

Figure 106. Conditioning results for Group IV mixture on SiH-I column using 10% THF in hexane.

For all the mixtures, the conditioning experiment did affect the retention properties. In most cases, after conditioning the retention times for all the compounds increased. The peaks became broader and resolution deteriorated. Subsequent washing also affected retention behavior, in some cases the retention times decreased, and in some situations, it remained the same. Peak quality did not improve with the washing step. It seems like minor changes in the mobile phase composition adsorbed to the surface of the stationary phase was affecting the retention properties of compounds that were analyzed. Maybe the toluene used during conditioning, was being retained on the slightly hydrophobic stationary phase (Si-H groups) forming a layer of solvent on the hydride surface. It is also possible that the underlying silanols that did not get silanized during the silanization step (tests have indicated that almost 95% of silanols are replaced by Si-H groups) were close enough to the surface in order to attract some methanol molecules. It is assumed that the twenty-minute equilibration time was insufficient to wash off this newly formed layer of solvent and so when compounds were injected on the column, they interacted differently with the new solvent mixture adsorbed to the column surface and hence underwent a change in retention.

Another factor that could explain this change in retention behavior is the presence of water in one of the organic mobile phases. Methanol has a strong affinity for water and even though organic solvents purchased are in a dry state, quite often they contain a certain amount of water (3). Depending on the humidity and the water content of the starting mobile phase, the solvent can pick up water from the atmosphere. This variation in the mobile phase water content can lead to variations in retention times of compounds

from run to run (3). In the case of the conditioning experiments, perhaps the water content in methanol was high or increased with time and so it was either being irreversibly adsorbed to the unsilanized silanols and affecting retention or it was affecting the solubility and adsorption properties of the solutes. In order to test if this hypothesis is true, water balance experiments can be performed to determine the degree of water adsorption on the hydride-based stationary phases as a function of increasing water content in the mobile phase (15). Since further studies are required to investigate the conditioning results, no substantial conclusion can be offered explaining the change in retention behavior. Further work investigating this aspect needs to be pursued in order to understand the influence of column conditioning on normal phase chromatography.

IV. Conclusions

For the model compounds tested, separation results with the non-commercial columns indicated that these hydride-based columns are suitable for normal phase applications. The optimum separation results for all the non-commercial columns have been summarized in Tables 3, 4, 5, and 6. Evaluation of the commercial hydride-based stationary phases available led to the same conclusion. The commercial columns showed different results than the non-commercial columns, specifically for bidentate C18 columns. The conclusions for the different commercial hydride-based columns have been categorized below.

A. Commercial Silica Hydride Columns

SiH-I and SiH-II demonstrated similar separation performance under all mobile phase conditions. For Group I mixture, the three mobile phase compositions used, was appropriate for separation, with 10% CH₂Cl₂ in hexane yielding slightly better results. Because the modified Group II mixture separation could be achieved only under one mobile phase condition, it can be assumed that this stationary phase is not suitable for separation of such compounds. Perhaps some polar modification of the hydride-based phases will enhance separation. In the case of Group III and IV mixtures, baseline separations were achieved with all the mobile phase conditions with 10% THF in hexane giving the maximum retention. In some cases, separations achieved were better when compared to those obtained on the commercial Astrosil silica column. The optimum

separation results for commercial silica hydride columns summarized in Tables 7 and 8 indicate that these columns can operate under normal phase conditions.

B. Bidentate C-18 Columns

Only Group I mixture could be separated on these columns when the mobile phase was 5% EtOAc in hexane. The optimum results are summarized in Tables 9 and 10. Other compounds mostly co-eluted even though different mobile phases were used. This behavior is not surprising because the bidentate C18 columns are non-polar. These non polar columns are typically used for reversed phase applications and this is the first time they were studied under normal phase conditions. Based on the separation results for all the four groups of compounds tested, it can be concluded that these columns are not suitable for such normal phase applications.

Reproducibility studies performed indicated that separations were reproducible on the commercial hydride-based columns. Conditioning studies indicated that column conditioning affected retention behavior of compounds and did not change the surface chemistry of the stationary phase. Thus, chromatographic evaluation of the hydride-based stationary phases performed in this study indicated that there might be some industrial normal phase applications for these columns; however, more research is required to improve retention and optimize separations on these columns in order to make them highly applicable for normal phase chromatography.

References

1. Ardrey, B. *Liquid Chromatography-Mass Spectrometry: An Introduction*; Wiley: England, 2003; pp 7, 40, Chapter 1, 36, 77, 90 f, 82 f, 13, 125, 54, 58.
2. Skoog, D.A.; Holler, J. F.; Nieman, T.A. *Principles of Instrumental Analysis*; Brooks/Cole: United States, 1998; pp 675, 254, 512, 500, 499, 505, 258, 260.
3. Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*; Wiley-Interscience: New York, 1979; pp 20 f, 376, 393.
4. Snyder, L. R.; Kirkland, J. J.; Glajch, J. L. *Practical HPLC Method Development*; Wiley-Interscience: New York, 1997; pp 235, 268-270, 178-181.
5. Pavia, D.; Lampman, G; Kriz, G. *Introduction to Spectroscopy*. Brooks/Cole: United States, 2001; pp 390-392, 394.
6. Vestal, M. L. *Chem.Rev.* **2001**, 101, 361-375.
7. Waters Corporation. *Operation and Maintenance of the ZQ with MassLynx v.4.0*; United States, 2002; Vol. 1, Chapters 2-3.
8. McLuckey, S. A.; Wells, J. M. *Chem. Rev.* **2001**, 101, 571-606.
9. About TYPE-C SilicaTM based HPLC products?
http://www.microsolvtech.com/hplc/typec_about.asp#3, (25 September 2006).
10. Sandoval, J.E.; Pesek, J. J. *Anal. Chem.* **1991**, 63, 2634-2641.
11. Chu, C. H.; Jonsson, E.; Auvinen, M.; Pesek, J. J.; Sandoval, J. E. *Anal. Chem.* **1993**, 65, 808-816.
12. Pesek, J. J.; Matyska, M. T.; Oliva, M.; Evanchic, M. *J. Chromatogra A.* **1998**, 818, 145-147.

13. Pesek, J. J.; Matyska, M. T.; Pan, X. *J. Chromatogra A.* **2003**, 992, 57-65.
14. Boussie, T. R.; Diamond, G. M.; Goh, C.; Hall, K. A.; LaPointe, A. M.; Leclerc, M.; Lund, C.; Murphy, V.; Shoemaker, J. A. W.; Tracht, U.; Turner, H.; Zhang, J.; Uno, T.; Rosen, R. K.; Stevens, J.C. *J. Am. Chem. Soc.* **2003**, 125, 4306-4317.
15. Roush, D. J.; Hwang, L. Y.; Antia, F. D. *J. Chromatogra A.* **2005**, 1098, 55-65.