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Synthesis and characterization of C8 bonded stationary phases for HPLC

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SYNTHESIS AND CHARACTERIZATION OF C8 BONDED STATIONARY PHASES FOR HPLC

A Thesis

Presented to
The Faculty of the Department of Chemistry
San Jose State University

In Partial Fulfillment
of the Requirements of the Degree
Master of Science

by
Xiaofang Pan
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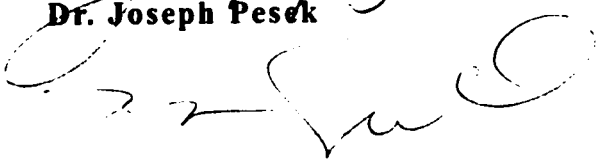
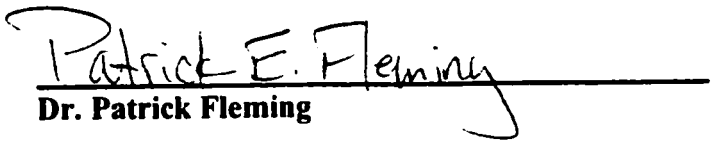


Dr. Roger Terrill



Dr. Patrick Fleming

APPROVED FOR THE UNIVERSITY

Abstract

SYNTHESIS AND CHARACTERIZATION OF C8 BONDED STATIONARY PHASES FOR HPLC

by Xiaofang Pan

C_8 bonded silica-based packings have been synthesized by reaction of Kromasil and Vydac high-performance liquid chromatographic silica with 1-octyne via a hydride intermediate. Surface properties of two stationary phases have been characterized by several physicochemical techniques, such as elemental analysis, diffuse reflectance infrared fourier transform (DRIFT), and chromatography. The novel stationary phases possess direct Si-C bonds and densely bonded surface. Chromatographic separation of a series of structurally diverse solutes was used to characterize the new stationary phase materials. Quantitative structure-retention relationships derived revealed the typical reversed-phase character. Other specificities such as shape selectivity, silanol activity, and bonded phase stability were also evaluated.

Acknowledgments

I would first like to thank Dr. Pesek for his direction of this research project and his personal guidance, advice, and inspiration. I would also like to thank Dr. Maria Matyska for her valuable advice and assistance in time of need. I appreciate her enthusiasm and I am very grateful for having had the opportunity to work with her in the laboratory. In addition, I would like to thank the members of my committee, Dr. Terrill and Dr. Fleming, for their support. Finally, I thank my family for their undying support throughout the course of my studies. They have been understanding and encouraging from the start and I am forever indebted to them.

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CHAPTER I

INTRODUCTION

Modern high performance liquid chromatography, also known as high pressure, high resolution, and high-speed liquid chromatography, and universally referred to as HPLC, originated in the late 1960's, has developed into a generally applicable analytical method. This technique is now accepted as a rapid and versatile separation technique that not only permits preparative separation of different multicomponent mixtures, but also provides a number of highly selective variants to resolve almost every type of sample mixture. The present HPLC instrument meets the high levels of speed, accuracy, precision, and reliability demanded by the special requirement of various analytical tasks. As far from perfect as it is, HPLC is widely used in pharmaceutical, biochemical, biomedical, and environmental analysis.

Although the development of HPLC has been pursued vigorously for many years and the method has reached maturity, there have been a number of examples in which the technique is unable to achieve separation. Scientifically and technologically significant problems will determine the future of HPLC. The recent advances in molecular biology and biotechnological process directly influence the current developments in HPLC methodology and provide extremely powerful incentives for its future. In general there are still numerous challenges remaining and new

applications for HPLC are being developed every day. Among all these studies, innovative approaches to designing silica based column packings with chemically bonded phases are at the center. The fundamentally important steps in creating highly selective phase systems include change of physicochemical properties of the support or the chemical nature of bonded ligands and adjustment of the final stationary phase properties by changing the type and/or composition of the mobile phase, leading to specific and non-specific interactions between the mobile phase, solutes, and the surface of the packing and suppressing undesired side-interactions.

A. The Principle of HPLC

High performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the mixture to be separated is transferred to a column that contains the stationary phase with a solvent or a solvent mixture (mobile phase). An HPLC instrument consists of an injector, a pump, a column, and a detector. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase at different rates and leave the column after different times. The interactions of the stationary phase and the sample with the mobile phase, determine the degree of migration and separation of the components contained in the sample. For example, those

samples that more strongly interact with the stationary phase than with the mobile phase will elute from the column less quickly, and thus have a longer retention time, while the reverse is also true. The eluted solutes are detected by a detector. The signals generated by the detector are sent to the data acquisition system producing a chromatogram. The process is depicted in Figure 1. Since all the molecules of a particular analyte do not behave exactly the same, the peak for that analyte will have a finite width

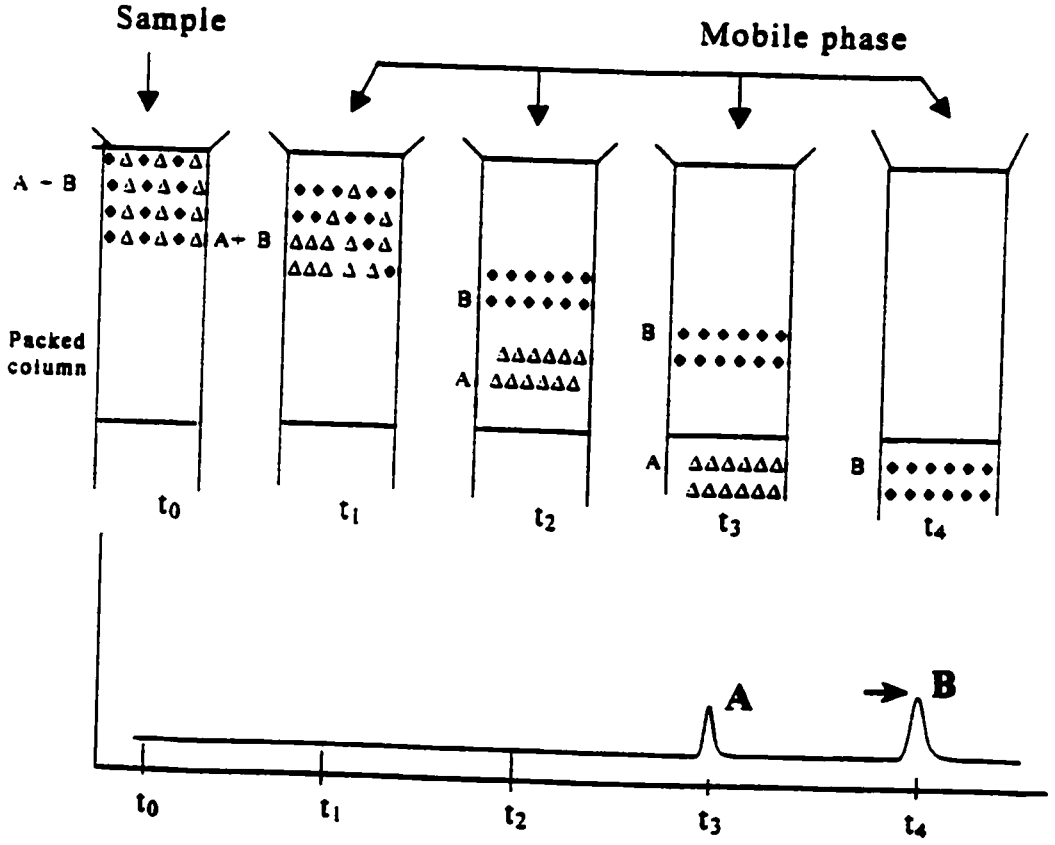


Figure 1 Schematic of HPLC Chromatographic Process.

in the chromatogram. Figure 2 shows the chromatogram and the symbol notation used to describe features of the peaks and the chromatogram.

The chromatogram can be used to provide information on separation efficiency. Here w is the peak width at the baseline, t_0 is the dead time or retention time of an unretained solute. t_0 is identical for every chromatogram on the same column and represents the mobile-phase residence time. t_R is the retention time; this is the period between sample injection and recording of the peak maximum.

The capacity factor (k') is more useful than the retention time (t_R) in comparing chromatograms obtained from columns of dissimilar lengths and under conditions of dissimilar flow rate of the mobile phase.

$$k' = \frac{t_R - t_0}{t_0}$$

k' is independent of the column length and mobile phase flow rate and represents the molar ratio of the compound in the stationary and mobile phase. k' is directly proportional to the volume occupied by the stationary phase or to the specific surface area ($m^2\ g^{-1}$) in the case of adsorbents. Silica with narrow pores produces larger k' values than a wide-pore material since surface area is inversely proportional to the pore diameter. The adjusted retention time, t_R, can be considered to be an approximate measure of the time spent by a component immobilized in and on the bonded layer. Thus its magnitude will depend on the specific surface area

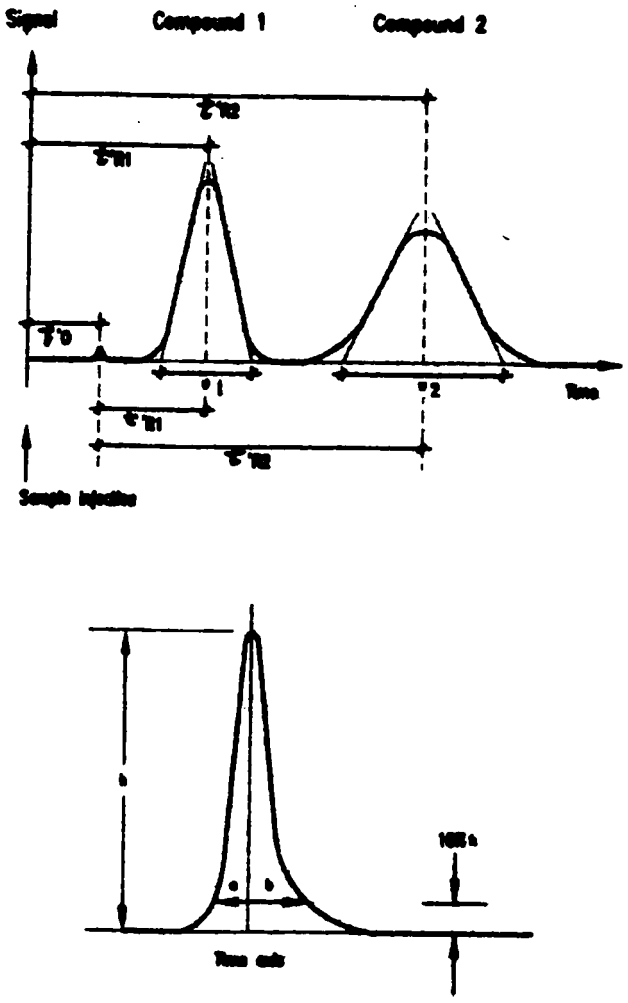


Figure 2. Data and Symbols for Chromatogram

and other properties of the bonded materials such as its hydrophobic character, and the degree of solvation. The overall retention behavior of packed columns depends on the physio-chemical properties of the silica (specific surface area, packing density), the chemical surface properties (type and concentration of surface silanols, surface concentration of metal oxides), the bonding procedure and the surface concentration of bonded groups achieved.

Relative retention, α, also known as the separation factor is determined as follow:

$$\alpha = \frac{t_{R2}-t_0}{t_{R1}-t_0} = \frac{k_2'}{k_1'}$$

with $k_2' > k_1'$. Relative retention is a measure of the chromatographic system's potential for separating two compounds, i.e. its selectivity. Selection of the stationary and mobile phases can affect the value of α.

The chromatogram also can be used to calculate the number of theoretical plates, N, in the column:

$$N = 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2$$

where $w_{1/2}$ is the peak width at half-height. N characterizes the column efficiency. Column efficiency refers to the performance of the stationary phase to accomplish particular separations. The efficiency of a column

can be measured by several methods. One method is based upon measurement of the width at 50% of peak height. This method is more reproducible and convenient since the width at 50% peak height is less prone to variations.[1]

The number of theoretical plates increases with packing density, column length and optimization of the mobile phase flow-rate conditions. A column with a high number of plates can also separate mixtures in which the components have similar relative retention values, α. All of the above equations yield accurate results only if the peak has a Gaussian shape.

The separation of any two bands in the chromatogram can be varied systematically by changing experimental conditions. Resolution R_s can be expressed in terms of three parameters (k, α, and N) which are directly related to experimental conditions:

$$R_s = 1/4\ (\alpha\text{-}1)\ N^{1/2}\ \frac{k}{1+k}$$

where $k = (k_1' + k_2')/2$. To provide efficient separations, R_s values ≥ 1.25 are required.

The peak asymmetry factor, A_s, can be used to measure the peak shape and is very important in method development. As, is determined at

1. P. C. Sadek, Troubleshooting HPLC Systems, John Wiley and Sons, Inc., New York, 2000, p. 45.

10% of the total peak height, h as shown in Figure 2:

$$A_s = \frac{b_{0.1}}{a_{0.1}}$$

where $a_{0.1}$ is the distance between the peak front and the peak maximum, measured at 10% of the total peak height, and $b_{0.1}$ is the distance between the peak maximum and peak end, measured at 10% of the total peak height. Peak asymmetry must be less than 1.2 for a "good" column. Peaks with poor symmetry suggest some problems such as, sample overload, buildup of residue on the column inlet, chemical or secondary retention (silanol effects), wrong solvent for sample, plugged frit or void.

a. **Factors Influencing Retention**

The factors that influence the distribution of the sample between the stationary and mobile phases and hence retention are:

- Composition and properties of the mobile phase;
- Type and properties of the stationary phase;
- Temperature
- The intermolecular forces between the component(s) and the stationary and mobile phase. The operative interactions are mainly of a physical nature, but might also include chemical equilibria. They are:
    - Dispersion forces

- Dipole-dipole interactions
- Hydrogen bonding
- π-π interactions
- Ion exchange

Dispersion forces are the most universal intermolecular interactions. Therefore in non-polar solvents or stationary phase dispersion forces are the main interactions between molecules. The association is the result of induced dipoles formed by molecular electrons and nuclei interacting on the polarizable electronic systems of other molecules to induce coherent dipoles.

Van der Waal's retention forces are a consequence of dipole-dipole interactions between molecules. Dipole-induced dipole interactions arise from the charge on one molecule (component or stationary phase) disturbing the electrons in a second associated molecule, producing a shift in charge which then form the induced dipole.

Hydrogen is able to form an associative or weaker bond which is only one-tenth to one-thirteenth as strong as covalent bonds with electron-rich molecules. Hydrogen bonding may occur via inter-or intramolecular association. Hydrogen bonding between a solute and the stationary phase (or mobile phase) is a relatively strong attractive force which in HPLC can lead to slow equilibrium processes and tailing of peaks. As a result, the retention of solutes is often the sum of several contributions. π-π

interactions can be defined as the interaction between the π-electrons of the chromatographic material and those of the solute species. Usually, several types of interactions together determine the solute retention, but one is dominant.

b. **Types of HPLC**

There are many ways to classify liquid column chromatography. Since the major objective in chromatographic separation is high selectivity arising from specific interactions of sample components in the phase system, it is useful to classify packings and stationary phases according to the type of interactions and the resulting selectivities.

If this classification is based on the nature of the stationary phase and the separation process, the following modes can be specified.

- Adsorption chromatography
  - Normal phase chromatography
  - Reversed-phase chromatography
- Ion-exchange chromatography
- Size exclusion chromatography

Reversed-phase liquid chromatography (RPLC) has rapidly become one of the principal methods for separating molecules in solution. It is estimated that 80-90% of HPLC chromatographic systems currently in use utilize reversed-phase columns. In reversed-phase chromatography, the stationary phase is nonpolar (hydrophobic) in nature, while the mobile

phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Substances are eluted in a general order of decreasing polarity. The high degree of popularity of reversed-phase HPLC can be attributed to its ease of use, flexibility, and wide applicability of these phases to diverse separation problems. Many nonionic, ionic, and ionizable compounds can be separated, often at the same time in the same sample, using a single column and mobile phase.

Eluent polarity plays the dominant role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition and strength is steadily changed during the run.

B. The Silica Surface and Its Structure

Silica is the most widely used packing material in chromatography since silica supports have favorable physical characteristics. Silica is available in a variety of diameters, pore sizes, and surface areas. Specifications can be chosen to meet the chromatographic requirements. A particular advantage of most silica particles is their high mechanical strength. The mechanical strength of silica allows porous particles with very large surface areas to be used at high mobile-phase pressures, which yields fast mass-transfer conditions during chromatographic separations. Silica–based columns can provide the highest column efficiency of any of

the materials used to produce packings for HPLC. Rigid, high strength particles also produce columns that exhibit lower backpressures and longer lifetime.

Silica as used for chromatographic purposes is amorphous in nature and generally is prepared by polymerization of silicic acid. There are several parameters that describe the physical characteristics of silica. They are particle size (in μm), pore size (in nm), specific surface area (m^2/g) and silanol content. Particle size largely determines the pressure drop of a column and in particular its stability, i.e., the lifetime. Column permeability depends on the inverse square of the average particle diameter. Particle diameters of about 5μm represent a good compromise for analytical columns balancing concerns of column efficiency, backpressure, and lifetime.[2] Smaller porous particles (e.g., 3μm) are available for faster separations and give high values of N.

The pore diameter is the parameter, which influences chromatographic properties as well as the corresponding bonded moiety. In general, pore diameter is inversely proportional to surface area. On enlarging the pore size, the specific surface area decreases. The surface area of silica determines its accessibility, the solute retention (in terms of the solute capacity factor k') and the loadability of the column. The surface area of the stationary phase is proportional to the mass

2. L. R. Snyder, J. J. Kirkland, J. L. Glajch, Practical HPLC Method Development, 2nd Edition, John Wiley and Sons, Inc., New York, 1997, p. 176.

loadability. Pore diameters at least four times the hydrodynamic diameter of the solute ensures that restricted diffusion of the solute does not degrade column efficiency.[3] A large pore diameter of the substrate silica is also required, since the attachment of an organic moiety through modification leads to a reduction of both surface area and pore diameter. Larger pore diameters enable a molecule to gain access to all binding sites on the surface of a bonded phase. Typically, support media for the preparation of a chemically bonded phases has the following values: pore diameter, 10 to 30 nm, specific surface area, 150 to 500 m^2g^{-1} and particle size, 3 to 7μm.

Silica used for chromatography can be regarded as polymers of silicic acid, consisting of interlinked SiO_4 tetrahedra. At the surface, the structure terminates in either a siloxane group (≡Si-O-Si≡) with the oxygen on the surface, or one of several forms of silanol groups (≡Si-OH).

The silanol group plays a key role in the chromatographic properties of silica. Most of the properties of silica are determined by their specific surface area and by the type of functional groups that are located on their surface. Silanol groups on the surface of a silica particle can be categorized into the following types:

1. single silanols

3. L. R. Snyder, J. J. Kirkland, J. L. Glajch, Practical HPLC Method Development, 2nd Edition, John Wiley and Sons, Inc., New York, 1997, p. 177.

2. single silanols
3. geminal silanols
4. triple terminal silanols (their existence has been discussed and not universally accepted)
5. hydrogen-bonded (associate) hydroxyls, which may be either isolated silanols or vicinal hydroxyl groups (among them terminal silanols)
6. internal silanols. hydroxyls that are not located on the surface and thus not accessible to some absorbable molecules.

The total number of reactable silanols varies with the silica surface area, but 8 ($\mu mol/m^2$) is assumed as the silanol concentration for all rehydroxylated silicas.[4] Different silanols have different adsorption activity. Free silanols are considered more active as well as undesirable adsorption sites on siliceous HPLC-bonded-phases. The accessible silanol groups localized on the silica surface will react with certain organic reagents to produce chemically bonded phases.

Silanol groups are weakly acidic (pK_a typically 5-7); thus, they are able to undergo hydrogen-bond and dipole-dipole interactions with polar compounds resulting in peak tailing and loss of chromatographic resolution.

Silica gel is also known to be an efficient cationic ion exchanger.

4. G. B. Cox, J. Chromatogr. A 656 (1993) 355.

Protonated amines (80 % of all pharmaceuticals contain basic nitrogen functionalities) might participate in ion-exchange reactions with unreacted silanols as illustrated in the following equation:

Solute ionization:

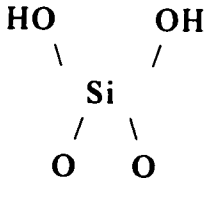
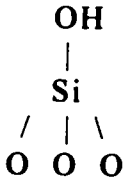
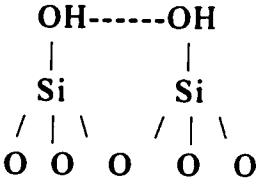
$$NH_2\text{-}R + {}^{+}H \Leftrightarrow {}^{+}H_3N\text{-}R$$

Ion exchange:

$$\equiv Si - OH + {}^{+}H_3N - R \Leftrightarrow \equiv Si - OH_3N\text{-}R + H^{+}$$

The extent of ion exchange depends on the pK_a values (in the mobile phase) of the solutes and the ion-exchange constant.

Table 1. Surface Silanol Types with Their FTIR Peak Positions and Names

			
	Geminal	**Single**	**Hydrogen bonded vicinal**
FTIR			
	3750 cm-1	3750 cm-1	3660 cm-1
	free	free	bridged

The support of silica-based bonded phases will dissolve in high pH mobile phases. The solubility of silica largely depends on pH and

increases drastically above pH 9 at room temperature.

$$\equiv Si\text{-}OH + M^{+} \Leftrightarrow \equiv Si\text{-}OM + H^{+} \text{ at pH} < 8,$$

$$\text{or } \equiv Si\text{-}OH + OH^{-} \Leftrightarrow \equiv Si\text{-}O^{-} + H_2O \text{ at pH} \geq 8.$$

$$(SiO_2)_x + 2H_2O \Leftrightarrow Si(OH)_4 + (SiO_2)_{x-1}$$

$$Si(OH)_4 + OH^{-} \Leftrightarrow Si(OH)_5^{-1}$$

By providing a point of attack for water or other reagents in the mobile phase, unreacted silanols accelerate dissolution of the underlying silica leading to reduced column lifetimes.

C. Silica Based Chemically Bonded Stationary Phases

The polar and heterogeneous surface of silica limited its use for chromatography due to irreversible adsorption, denaturation of biopolymeric solutes and limited pH stability. Chemical modification of the surface can alleviate these problems. Modification has three potential advantages. First, the bonding of functional groups to the silica surface creates a secondary surface structure. In this way the stationary phase can provide optimum and selective interactions with solutes. Second, in the course of bonding of ligands, the most active surface centers are removed or covered by a protective layer. As a result, the matrix effects of silica are drastically reduced. Third, by the attachment of dense layers, the chemical stability of bonded silica toward harsh mobile phases is much improved.

To achieve these objectives, chemists have developed a number

of synthetic approaches to modify the silica surface. The first attempt to bond an organic moiety to the silica surfaces was made by Halasz and Sebestian in 1969[5] who attached aliphatic hydrocarbon chains to the silica gel surface by means of the silicon-oxygen-carbon linkage. In 1973, Gilpin and Burke[6] described the use of chlorosilanes as bonding reagents. When the hydroxyl group of the silica gel reacts with a chlorosilane, hydrogen chloride is released and the organic moiety is attached by means of a silicon-oxygen-silicon (Si-O-Si) bridge. This type of bond became the basis for most contemporary liquid chromatography bonded phases. The reactions proceed according to the following scheme:

$$Si\text{-}OH + R_{(4-n)}SiX_n \rightarrow Si\text{-}O\text{-}SiX_{(n-1)}R_{(4-n)} + HX \qquad (1)$$

where n=1,2,3; R is alkyl or substituted alkyl group; X is halide, alkoxy (-OMe, -OEt), N,N-alkylamino (NMe_2, NEt_2) or acyloxy (RCOO-). The silanes could be trifunctional (n=3), difunctional (n=2) or monofunctional (n=1).

When tri-functional silanes are used in the synthesis, such as octyltrichlorosilane, the reagent reacts with a silanol group and the adsorbed water on the silica surface. The cross-linked multi-layer stationary phase, called a polymeric phase, can be formed. When bi-functional silanes are used in the synthesis, such as methyl-

5. I. Halasz, I. Sebastian, Angew. Chem., 81(1969) 453.
6. R. K. Gilpin, M. F. Burke, Anal Chem., 45 (1973) 1383.

octyldichlorosilane, the dichlorsilane reacts with a silanol group, and can also lead to the formation of cross-linked polymeric structures.

With monofunctional organosilanes, the modification produces a surface where the silane covers the silica as a monolayer (see Equation 2). The corresponding stationary phase sometimes is called a "brush" or monomeric phase, which is the most reproducible and the most commonly used LC stationary phase. However, due to steric reasons, adjacent silanols on the silica surface may be blocked by the bonded species and reaction by-products and therefore may be unavailable for further reaction. So an appreciable amount of the silanol groups on the surface of the silica remain unreacted and the surface coverage can be low. It is these unreacted silanols that can lead to chromatographic problems. This type of stationary phase is usually limited to operating within the narrow pH stable range of approximately 2 ~ 8.

$$\text{Si-OH} + \text{Cl-Si}\,(CH_3)_2R \rightarrow \text{Si-O-}\overset{\displaystyle CH_3}{\overset{|}{\underset{|}{\text{Si}}}}\text{-R} \quad + \text{HCl} \qquad (2)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \underset{\displaystyle CH_3}{|}$$

Polymeric bonded phases are however, more difficult to prepare reproducibly because of the multiple reaction products of the silylation reaction. Furthermore, it is believed that the slower rates of adsorption and desorption of analyte molecules at the surface of such polymeric phases reduces mass transfer rates and consequently column efficiency.

However, it has better chromatographic selectivity than monomer phases toward very hydrophobic compounds such as PAHs.

Recently, several new approaches as well as variations to the existing approaches have attracted more interest with respect to chemical modification of silica surfaces. End capping is one of the effective methods to minimize the number of unreacted, accessible silanols. In this way, the residual silanols can be removed by secondary silanization with short-chain alkyl silanes such as hexamethyldisilazane or trimethylchlorsilane, but it does not affect the remaining bonded groups greatly.[7] Kirkland[8] et al. described column packings with monomeric "sterically protected" bonded silanes containing bulky side groups such as diisopropyl- and diisobutyl-. Monomeric C18 packings with these sterically protected side groups are more resistant to hydrolysis and are highly stable in high temperature environments. Also, polar analytes cannot interact with the non-blocked silanols, and in comparison with the conventional C18 phases, the retention reproducibility is substantially improved. However, these approaches do not increase the carbon coverage of the column packings.

In order to improve the hydrolysis stability and reduce the silanol activity, Akapo and Fatunmbi[9] developed a potential chromatographic

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7. H. H. Freiser, M. P. Nowlan, D. L. Gooding, J. Liq. Chromatogr. 12, (1989), 827.
8. J. J. Kirkland, J. L. Glajch, R. D. Farlee, Anal. Chem. 61, (1989), 2.

stationary phase prepared by horizontal polymerization of mixed trifunctional silanes into dense monolayers which was first suggested by Wirth and Fatunmbi et al.[10] These phases are produced by horizontal polymerization of a mixture of long-chain and short-chain alkyltrichlorosilanes onto porous silica particles under anhydrous conditions, except for a monolayer of water on silica. This procedure results in a new type of polymeric phase.

Khong and Simpson[11] described a fluidized bed technique. In this method, silica particles were treated with silane vapor in a fluidized bed reactor using dry nitrogen as the fluidizing gas. The particles move apart and become suspended in the flow. In this way, uneven contact between the silane reagent and the silica particles is avoided and the bonded phases have a more homogeneous surface coverage. The merit of this technique is that it improves the chromatographic performance and batch to batch reproducibility.

In this research, a new technique, hydride intermediate silanization followed by hydrosilation with an alkyne which was first proposed by Sandoval and Pesek,[12] was employed to produce a direct Si-C bond and a densely bonded surface. The goal was to increase the hydrolytic stability

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9. S. O. Akapo, H. O. Fatunmbi, LC-GC Volume 17, No. 4, April, 1999, 334
10. M. J. Wirth, H. O. Fatunmbi, Anal. Chem. 64, (1992), 2783.
11. T. M. Khong, C. F. Simpson, Chromatographia 24, (1987), 385.
12. J. E. Sandoval, J. J. Pesek, Anal. Chem. 61, (1989) 2067.

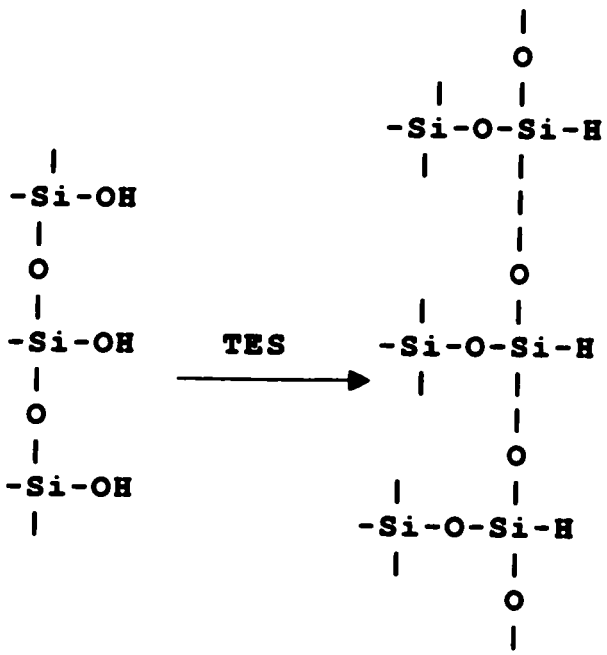
and reduce the silanol activity of the silica-based packing materials. The synthesis can be accomplished by employing a two-stage reaction sequence involving first treatment with triethoxysilane (TES) to form a hydride layer followed by reaction with 1-octyne. The chemistry of the modification of the silica surface developed in this research is shown in Equations 3 and 4.

In such circumstances, the advantage of forming a hydride layer is that very few silanols remain on the silica surface. The Si-H group reacts with unsaturated organic functional groups only in the presence of a transition metal as a catalyst or free radical initiator. The reason to choose 1-octyne as the reactant for the hydrosilation reaction is that an alkyne has more chemical reactivity than an olefin. Thus the corresponding phase is more dense. The high surface coverage of alkyl chains leads to a higher peak capacity and a more versatile column. More importantly, when 1-octyne reacts with silica hydride it may produce two silicon-carbon bonds between the organic moiety and the silica surface. This kind of structure sterically protects the siloxane bond from dissolution. Meanwhile, better peak shape is often observed on shorter chain phases like C8 bonded phases. The effect is possibly due to a better wetting of the surface by the mobile phase that results in better solute mass transfer.[13] The shorter chain also reduces steric hindrance which

13. N. Tanaka, K. Kimata, K. Hosoya, H. Miyanishi, T Araki, J. Chromatogr. A 656 (1993) 267.

increases accessibility of silanol groups in the bonding process. C8 bonded phases are not as hydrophobic as C18 bonded-phases. Strong hydrophobic interactions might cause considerable losses in the bioactivity of proteins. There is a preference to use n-butyl and n-octyl-bonded silicas in RPC of proteins over longer n-alkyl derivatives. The shorter chain length leads to a lower probability of denaturation of the native protein by surface-induced effects. However, RP silicas with very short n-alkyl groups suffer from easier hydrolytic cleavage of the bonded n-alkyl group at both low and high pH.

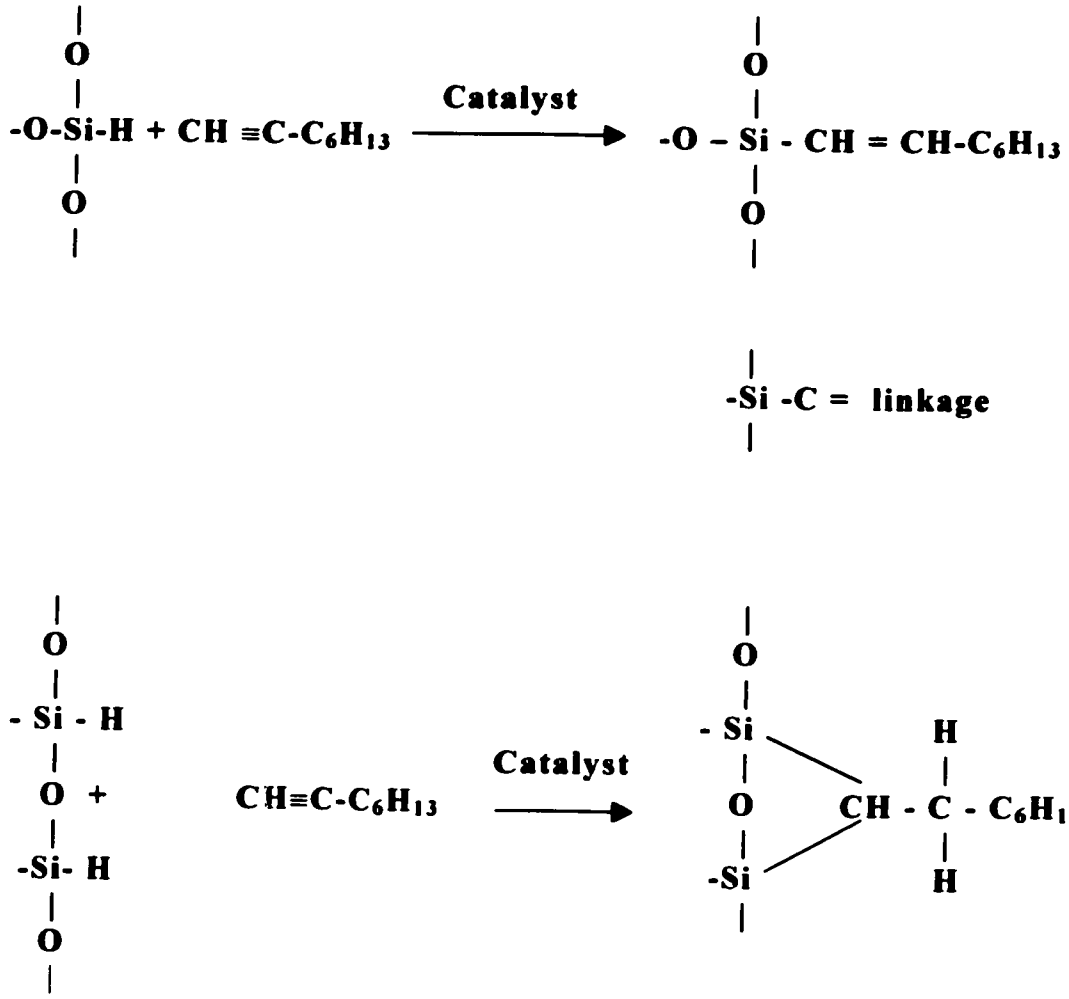
Silanization



(3)

Si-H monolayer

Hydrosilation:



(4)

The stationary phases synthesized in this study consist of silica based packings with octyl chains covalently bound. The stationary phases should be basically reversed-phase in character. The phases operate on the basis of hydrophilicity and lipophilicity.

It is essential to first specifically address the issue of the stability of the bonded phases. In recent years, reversed-phase HPLC has found wide utility for separating many basic compounds such as

pharmaceuticals, agricultural chemicals, and unprotected peptides and proteins. A common approach for achieving good peak shape and retention is to separate these types of samples at lower pH (pH<4) with aqueous/organic phases. A typical procedure utilizes trifluoroacetic acid (TFA) in the mobile phase. TFA is now widely used in the reversed-phase separation of peptides and proteins. However, typical bonded-phase columns used for reversed-phase separations at low pH (<3) are not stable even over relatively short periods of time. The new bonded-phase packings with a more stable Si-C link and double silicon-carbon linkages between the organic moiety and the silica surface should improve stability of bonded-phases at low pH conditions, while maintaining good separation quality for peptides, proteins, and other biomolecules.

Samples containing ionizable compounds, including basic solutes, which are unstable at low pH or are protonated at low pH and thus elute too quickly are usually best separated with mobile phases of pH≥ 9. With high pH mobile phases, basic compounds are in the neutral state, and unreacted silanol groups on the silica support are completely ionized. Therefore, this high pH condition minimizes any unwanted ionic interactions between basic solutes and the silica support that might occur at intermediate pH where partial ionization of solutes and the silica surface can co-exist. However, silica-based columns are not recommended for operating at higher pH, because of potential dissolution of the silica

support, causing collapse of the packed bed. Some column packings have been designed to operate at higher pH (e.g., graphitized carbon, porous polymers, and polymeric phases on alumina supports). However, these materials have not reached a high level of usage because of problems with reproducibility, efficiency, and limitations in mobile phases that can be effectively used. Therefore, silica-based column packings without these limitations would be especially attractive for use at higher pH, providing columns of such materials are adequately stable. It has been found that the stability of silica-based columns at high pH strongly depends on the type of silica support used and the bonding procedures. Greatest resistance to degradation is shown by the densely bonded packings. It is hoped that the C8 bonded silica-based phases developed in this study can also enhance column stability toward harsh high pH mobile phases. The stability of two C8-bonded phases at high pH was determined by chromatographic measurements.

Besides the consideration of the chemical nature of the surface, the design of high performance packings for liquid chromatography must include other criteria. From the point of view of separation efficiency and selectivity, important roles are played by parameters such as particle size and pore diameter. In recent years, attention has been drawn to the chemical and physical properties of the silica matrix. As mentioned before, various factors such as porosity, and concentration of silanol

groups and the surface purity significantly influence processes connected with surface modification and formation of chemically bonded phases. For this reason, two different types of silica - Kromasil and Vydac silica were used for this research. It is desirable to establish some coherent correlation between parameters such as specific area, pore size, particle size, or ligand density and chromatographic performance of the stationary phase. Improvements in understanding of the chemical nature and physical structure of bonded phases will certainly help in future applications. In conclusion, the choice of 1-octyl-bonded silicas with denser organic layers is a compromise between retention, biorecovery, and column stability.

D. Characterization of Alkyl-Bonded Stationary Phases

A variety of methods have been employed to characterize alkyl-bonded stationary phases. The quantity of bonded material is typically calculated from data by carbon elemental analysis and surface area measurement. Elemental analysis gives a measure of the amount of carbon associated with a bonded stationary phase, but this information alone is not a useful descriptor of bonded chain density. Carbon content is only informative if used in conjunction with the surface area of the underivatized support material to give the ligand density on the surface of the support (in $\mu mol\ m^{-2}$). It is important to control and measure functional group content expressed as ligand density. This property may

influence the chromatographic selectivity and stability of a bonded phase. The shielding effect of the non-polar residue is dependent on the degree of coverage and the bulkiness of the carbon chain.

Ligand densities in principal refer to the moles of a specific functional group per unit area on a bonded phase surface. The surface coverage (α_{RP}) is calculated from the total carbon content of the modified sample:

$$\alpha_{RP} = \frac{P_c \times 10^6}{n_c(1200)-P_cM} \times \frac{1}{S_{BET}} \quad [\mu mol/m^2]$$

where: P_c is the percentage of carbon in the modified silica in weight percent, n_c is the number of carbon atoms per bonded hydrocarbon moiety, M is the molar mass of the modifier, and S_{BET} is the specific surface area of unmodified support.

A number of different spectroscopic techniques have been employed to investigate the surface properties of the bonded-phase. By diffuse reflectance infrared fourier transform (DRIFT) spectroscopy, differentiation between the various surface silanols, the CH and SiH absorptions and other functional groups from modification procedures is possible. Spectroscopic studies were carried out to confirm the success of the modification procedures of the silica. DRIFT spectroscopy was also used in this study to provide information about the chemical structure of the chemically bonded-phases.

The chromatographic data reflect the behavior of the whole bonded phase system. In order to obtain an overall characterization of the bonded material, a series of test mixtures must be applied under various conditions. The primary goal of these procedures was the characterization of different bonded-phases in a fundamental way, providing a description for the hydrophobicity, the silanol activity and other characteristics of the modified surface. The elution sequence of simple monofunctional benzene derivatives can be used to describe RP behavior. The Perkin Elmer (PE) mixture was selected for this purpose (Fig. 3). The more hydrophobic the surface matrix is, the greater is the tendency of the column to retain hydrophobic moieties. The PE mixture test can give insight into hydrophobic properties of the two columns used in this study. In addition, the relative retention between any two of these compounds can be used to measure the purely hydrophobic selectivity of the stationary phases. Secondly, it is possible to determine the shape selectivity of the stationary phases. A mixture of three polycyclic aromatic hydrocarbon (PAH) compounds (SRM 869) was selected that contains benzo[α]pyrene (BaP), phenanthro[3,4-c]phenanthrene (PhPh), and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN). BaP is planar, whereas PhPh has an out of plane structure and is the most nonplanar of the three test solutes, and TBN is a nonplanar saddle-shaped solute (Fig. 4). The elution order for monomeric phases is usually BaP ≤ PhPh < TBN, whereas for polymeric

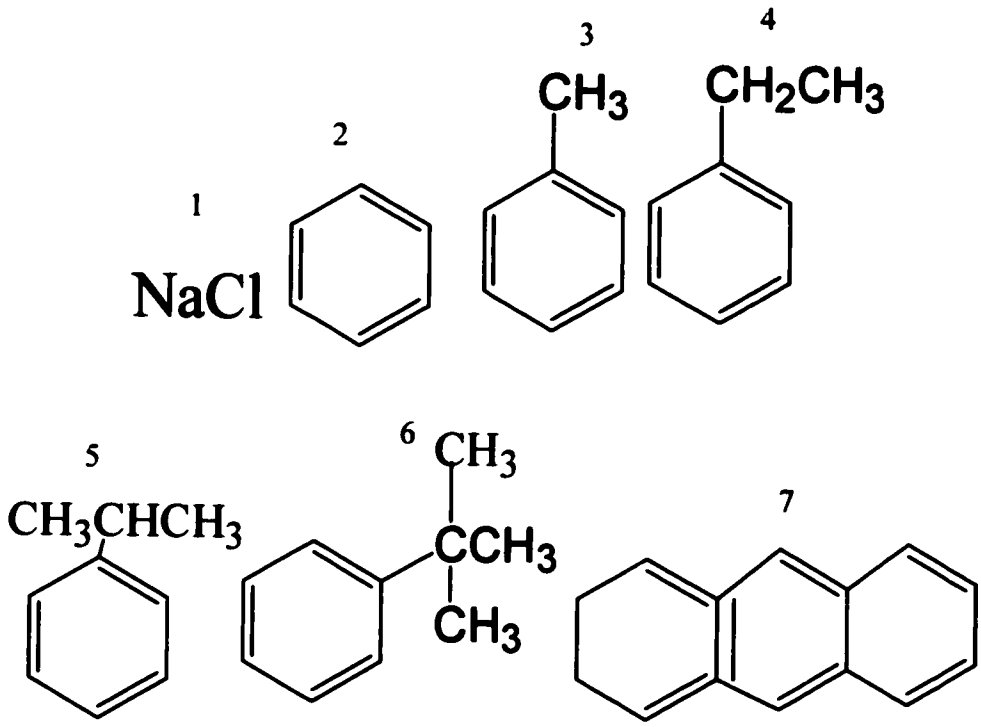


Figure 3. Structures of PE mixture: 1= sodium chloride; 2= benzene; 3= toluene; 4= ethylbenzene; 5= isopropylbenzene; 6= tert-butyl benzene, 7= anthracene.

phases, it is usually PhPh < TBN ≤BaP. The selectivity of TBN to BaP ($\alpha_{TBN/BaP}$) is a convenient means of classifying phase selectivity. Polymeric phases yield $\alpha_{TBN/BaP} \leq 1$ and for monomeric phases $\alpha_{TBN/BaP} \geq 1.7$, whereas phases that give $1 < \alpha_{TBN/BaP} < 1.7$ (elution order of PhPh < BaP < TBN) are termed intermediate and are often either lightly loaded polymer phases or densely loaded monomeric phases. Thirdly, the most interesting parameter of a reversed-phase packing is the interaction of basic analytes with the residual surface silanols of the bonded phase. Once again, a mixture of basic compounds in a non-buffered mobile phase at different temperatures and various compositions were used to measure the silanophilic and ion exchange properties of two bonded phases.

In reversed-phase HPLC, the most important factor for controlling absolute solute retention is mobile phase composition. In order to establish the effect of mobile phase composition on chromatographic retention, the solutes were chromatographed at six ratios of methanol/water in the mobile phase: 100:0, 90:10, 80:20, 70:30, 60:40, and 50:50 (v/v). For a "pure" reversed-phase mechanism, retention decreases with increasing organic composition of the mobile phase, and plots of the natural logarithm k' versus the percentage of organic modifier are linear.

It has been suggested that the type of silica used in the bonded-phase packings can have a strong influence on column stability, as well as

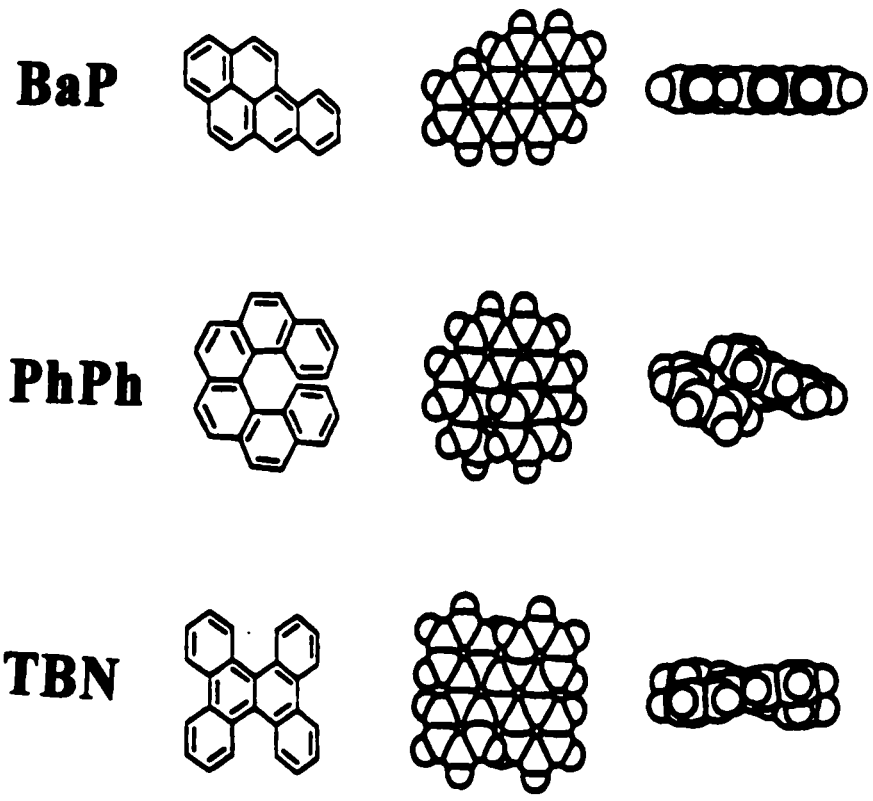
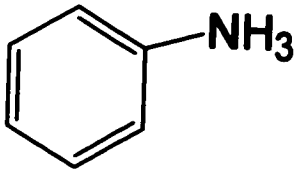
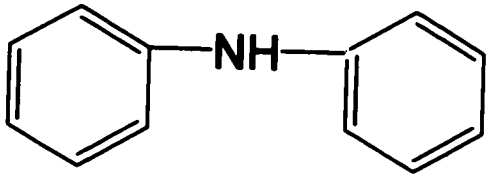


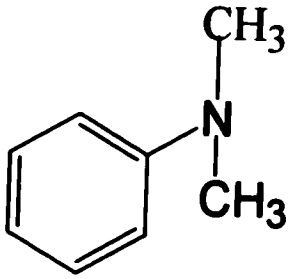
Figure 4. Structures and Space-filling Models for PAHs in SRM 869



aniline



diphenylamine



N, N' - dimethylaniline

Figure 5. Structures of Three Basic Compounds

the type and method of bonded-phase attachment. Here the stability of the two stationary phases in this study is evaluated in higher pH environments. Bonded-phase degradation in this pH range is a result of loss of silica into solution rather than the loss of covalently bound ligands by hydrolysis from silica supports. The stationary phase stability was then determined by chromatographic measurement. Aggressive support dissolution tests were conducted on the two columns using the conditions of an unbuffered sodium hydroxide solution at room temperature.

CHAPTER II

EXPERIMENTAL

A. Materials

a. Chemicals

The chemicals used in this study are listed in Table 2 along with their chemical abstract service (CAS) registry numbers. Water was deionized and purified by means of the Mili-Q SP reagent water system manufactured by Millipore (Bedford, MA, USA) before use. The column

Table2. List of chemicals used in synthesis & characterization

Name	Source	CAS Registry Number
Aniline	Sigma	[62-53-3]
Anthracene	J.T.Baker Chemicals	[120-12-7]
Biphenyl	Aldrich Chemicals	[92-52-4]
Diethyl ether (anhydrous)	Fisher Scientific	[60-29-7]
Dioxane	Fisher Scientific	[17647-74-4]

Diphenylamine	Scientific Products	[106-49-0]
Fluorene	Chem Service	[86-73-7]
Hexachloroplatanic acid (38-40% Pt)	Aldrich Chemicals	[16941-12-1]
Hydrochloric acid	Fischer Scientific	[7647-01-0]
Methanol	General Chemicals	[67-56-1]
Methylene chloride	Fisher Scientific	[74-87-3]
Naphthalene	Allied Chemical	[1146-65-2]
N,N-dimethylaniline	Alfa Products	[121-69-7]
1-Octyne	Aldrich	[629-05-0]
Phenanthrene	J.T.Baker Chemicals	[[85-01-8]
Potassium Bromide	Aldrich Chemicals	[7758-02-3]
Pyrene	Aldrich Chemicals	[129-00-0]

Sodium hydroxide	Fischer Scientific	[1370-73-2]
Tetrahydrofurane (THF) (anhydrous)	J.T.Baker Chemicals	[109-99-8]
Toluene	EM Science	[108-88-36]
Triethoxysilane (TES)	Huls-Petrarch System	[998-30-1]
Trimethylchlorosilane	United Chemical Technologies	[75-77-4]
Triphenylene	Allied Chemical	[217-59-4]

selectivity test mixture for liquid chromatography (Polycyclic Aromatic Hydrocarbons, SRM 869) was obtained from the National Institute of Standards & Technology (Gaithersburg, MD, USA).

b. Silica Used for Synthesis

VydacTP 106 and Kromasil (The Separations Group, Hesperia, CA and Eka Nobel Bohus, Sweden) were used as the support material for the preparation of the chemically bonded phases. Their surface and structural parameters are given in Table 3.

Table 3. Surface and Structural Parameters of the Chromatographic Silica Used in This Research

Silica	Particle Mean diam, μm	Pore Mean diam, nm	Sp surf Area, m^2/g	Silanol content mmol/g	Silanol content μmol/m^2
Kromasil	5	10.4	340	2.16	6.6
Vydac	6.8	33.4	106	4.20	47.3

B. Procedures

a. Diffuse Reflectance Infrared Fourier Transform Spectrometry (DRIFT)

Silica hydride and the synthesized bonded phases were characterized with a Mattson Instruments-Infinity Series FTIR™ (Madison, WI, USA). The collection of light re-emitted by scattering defines the diffuse reflectance (R) of the sample. The reflectance (R_0) of pure KBr, used as a reference, provides the diffuse reflectance spectrum $R_\infty = R/R_0$ of the sample considered as infinitely thick. DRIFT consists of an integrating sphere which is used to collect diffuse light, an ellipsoidal mirror as a collector, and a diffuse-reflectance accessory (Spectra Tech., Stamford, CT, USA). The powder samples diluted in KBr by 10:1 wt/wt were placed in a cup and its surface is leveled with a spatula. Liquid nitrogen was used to supply gaseous nitrogen (55-65 psi) to the sample

compartment of the infrared spectrophotometer (Perkin-Elmer model 1800) to remove moisture.

Each spectrum was scanned 100 times at a resolution of 2 cm^{-1} with an Mercury cadmium telluride (MCT) detector. The spectral range was between 4000 cm^{-1} to 450 cm^{-1}. The data was analyzed by a Venturis FX-2 Computer. The spectra were plotted by a HP 722C printer (Palo Alto, CA).

b. Synthetic Procedures

1. Silica Hydride

Silica samples were dried at 60 ^0C under vacuum overnight to remove physically adsorbed water. All glassware was washed thoroughly and rinsed with distilled deionized water and dried in an oven overnight at 110 ^0C.

A 1.00 mM TES solution was prepared in a nitrogen glove box and was then transferred to a 125-ml pressure-equalizing-addition funnel. This funnel was connected to a three-necked, 3000 mL, round-bottom flask fitted with a thermometer, a 50-cm reflux condenser and a magnetic stirring bar. Cooling water was circulated through the condenser. The flask was then placed on a heating mantle. The heating mantle was connected to an electric heating device for temperature regulation and was placed on top of a magnetic stirrer. Then 11.1 ± 0.01g of Vydac (or 7.5 ± 0.01g of Kromasil silica), 273 mL of distilled 1,4-dioxane and 10.8 mL of 2.3 mM hydrochloric acid were placed in the reaction flask.

The reaction mixture was stirred and heated to 93 ^{0}C, followed by dropwise addition of TES (about two drops per minute) for 25 to 30 minutes. Then the reaction mixture was stirred for an hour at 93 ^{0}C. After this period, the reaction mixture was cooled to room temperature, and the liquid phase was decanted. Then 10 mL of freshly prepared 1:1 THF-deionized water (by volume) solvent was added into the centrifuge tubes. The mixture was washed repeatedly three times with freshly prepared 1:1 THF-deionized water (by volume) solvent (30 mL) followed by three times with diethyl ether (30 mL). The solid was recovered by centrifugation for 10 minutes at 1500 rpm. The supernatant was decanted. The silica hydride was dried in the hood at room temperature overnight, followed by drying in a vacuum oven at 60 ^{0}C for 24 hours.

2. **Synthesis of C8 Bonded Phases From 1-Octyne**

Two stationary phases were synthesized according to the following procedure. Silica hydride was dried at 60 ^{0}C under vacuum overnight. Then 15-mL of toluene was added into a 100-mL 3-neck round-bottom flask equipped with a thermometer, a condenser with a filled drying tube and a glass stopper. Next 15 mL of 1-octyne and 2 mL of 5 mM hexachloroplatinic acid in 2-propanol were introduced. The reactants were stirred at 68 ^{0}C while silica hydride was added slowly to the reactants over 1 hour. Afterwards, the mixture was stirred at 100 ^{0}C for 96 hours. After completion, the mixture was divided into four centrifuge tubes and

was centrifuged for 10 minutes at 1500 rpm and the supernatant was decanted. The solid materials were stirred and washed for 10 minutes with toluene and centrifuged for 10 minutes. The product was stirred and washed three times each with methylene chloride (20 mL) and diethyl ether (20 mL) to give the C8 bonded phase. The final product was dried at room temperature overnight and then dried under vacuum at 60 ^{0}C for 24 hours.

C. Column Packing

The two prepared phases were packed into 150 mm x 4.6 mm i.d. stainless steel tubes purchased from Alltech (Deerfield, Il, USA). Approximately 2.0 g of bonded silica was stirred with 10% (v/v) methanol in chloroform, followed by a 10-minute sonication. The slurry was then packed in the columns using methanol as the driving solvent. Two columns were packed using a Haskel (Burbank, CA, USA) pneumatic pump. The column was allowed to remain on the packer for at least 30 minutes before removal.

D. Experimental Conditions Employed in the Chromatographic Investigations

Methanol was chosen as the organic eluent component because of its lower toxicity compared to acetonitrile. Moreover, methanol should be the first choice at pH 7.0, because it provides better peak shape for many solutes. Columns were operated using a flow rate of 0.7 ml min^{-1}. The

basic compound test was carried out at several temperatures with the column thermostated in a column heater (Model CH-30, Flatron Systems Inc., Oconomowoc, Wisconsin, U.S.A.). At least 10 column volumes were purged through before use with each new mobile phase. Each solute was made up at a concentration of 100 mgL^{-1} in the relevant mobile phase. 0.2 μg of the analyte was injected in each case to prevent significant overloading of the column. The determination of t_0 was carried out using KNO_3. The pH of the mobile phase was measured before adding methanol. Isocratic conditions were used. No buffer or salt solutions were used in the aqueous component. Data analysis was performed on a Microsoft Excel spreadsheeet.

E. Equipment

The HPLC system consisted of a Shimazu LA-6 pump, a Shimadzu SPD-6A UV Spectrophometric Detector operated at 254 nm (Shimadzu, Kyoto, Japan) and a HP 3396A Integrator (Palo Alto, CA).

CHAPTER III

RESULTS AND DISCUSSION

A. Elemental Analysis

The carbon contents of the two bonded phases were obtained from Desert Analytics (Tucson, AZ). Elemental analysis of the materials showed 13.8 and 5.7% (w/w) carbon. From the surface area values provided by the manufacturer, the bonding densities were calculated to be 4.72 and 5.82 $\mu mol/m^2$, respectively.

Table 4. Surface Characteristics of the Chemically Bonded Phases

Type of phase	Type of structure	n_c	P_c in %C	α_{RP} $\mu mol/m^2$
C8 on Kromasil	monomeric	8	13.8	4.72
C8 on Vydac	monomeric	8	5.7	5.82

This result suggests that a high concentration of bonded ligands was obtained leaving only small amount of the starting surface silanols unreacted and with these stationary phases the influence of silanols on the retention of basic solutes should be very negligible. Meanwhile it was found that a wider pore silica resulted in a phase with higher coverage.

B. DRIFT Spectra

The treatment of silica with TES results in a stable Si-H bond. In

Figures 6 and 7, the FTIR spectra reveal a significant decrease in the free Si-OH stretching at ~ 3750 cm^{-1} and the appearance of Si-H stretching near 2250 cm^{-1}. This result confirms the formation of the hydride intermediate.

Silica hydride reacts with the 1-octyne and a stable Si-C bond is formed. In Figures 8 and 9, the infrared band for the Si-H stretching at 2250 cm^{-1} is lower in intensity when compared to the spectrum of the hydride. The FTIR spectra show silicon hydride stretching at 2250 cm^{-1}, the absence of free SiOH stretching at ~ 3750 cm^{-1} and the appearance of C-H stretching at 3000 - 2800 cm^{-1}. These data confirm bonding of the 1-octyne to the hydride intermediate and suggests that the surface below the C8 hydrocarbon consist mainly of Si-H bands. Small peaks above 3100 cm^{-1} indicate the presence of an olefin which may not easily be detected by DRIFT or perhaps only a small amount of –C=C- exists. It can easily be seen from equation 4 that there could be some olefin on the surface which was sterically blocked for the further reaction with silica hydride.

C. Hydrophobic Properties and Selectivity

The hydrophobic properties were determined by measuring the retention of benzene, toluene, ethylbenzene, isopropylbenzene, t-butylbenzene, and anthrancene in methanol-water (65:35). Typical chromatograms of the test solutes obtained are shown in Figures 10 and 11. All the solutes eluted with symmetrical peaks and all the solutes are

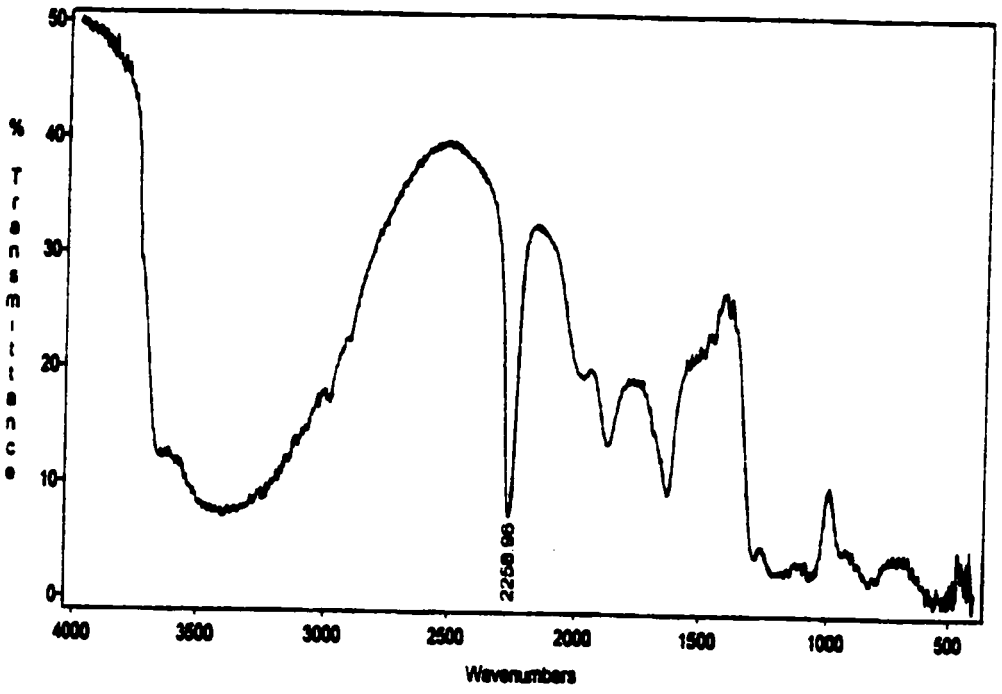


Figure 6. The DRIFT Spectrum of Vydac Silica Hydride

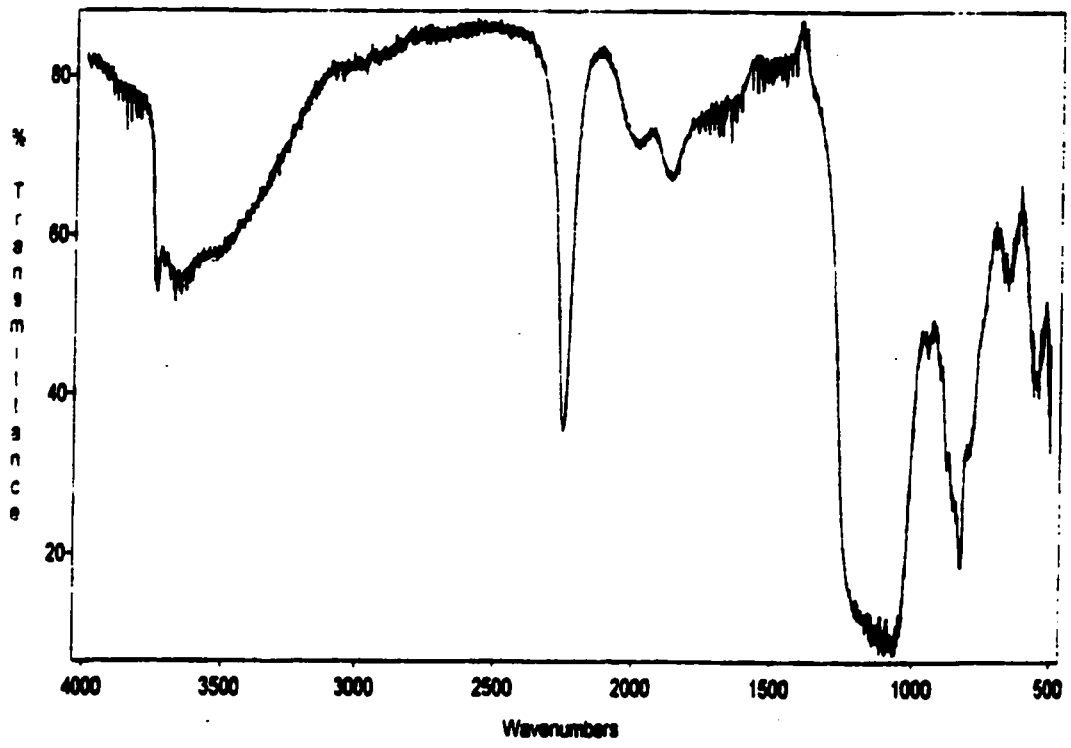


Figure 7. The DRIFT Spectrum of Kromasil Silica Hydride

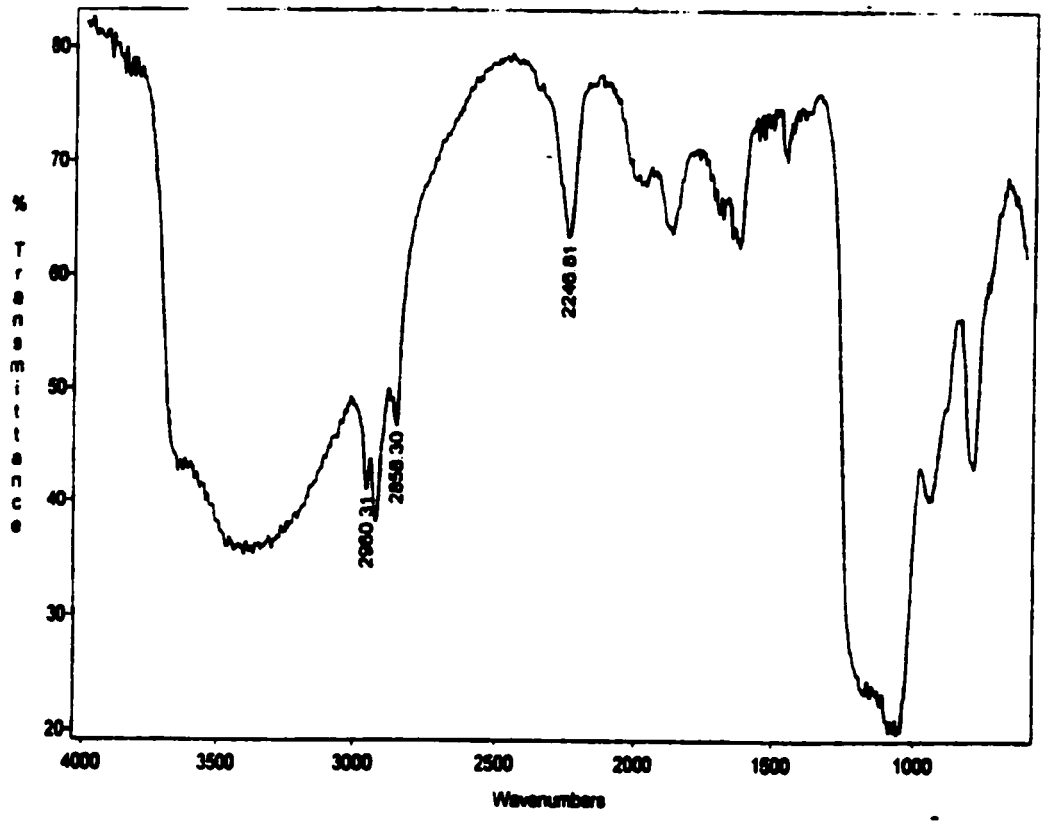


Figure 8. The DRIFT Spectrum of C8 Bonded to Vydac Hydride.

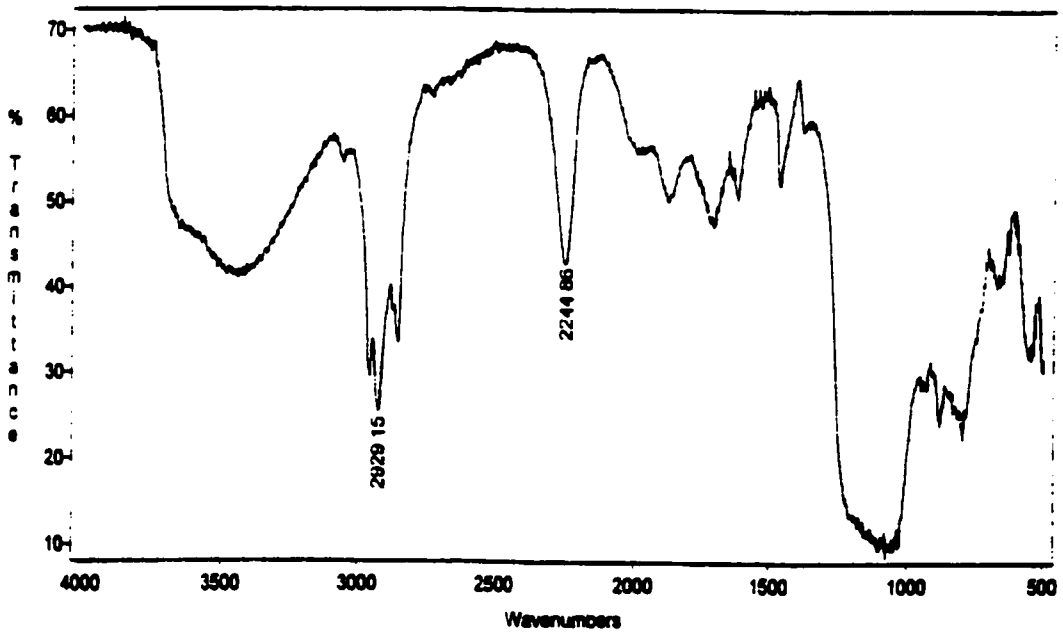


Figure 9. The DRIFT Spectrum of C8 Bonded to Kromasil Hydride.

baseline resolved except benzene and toluene on Vydac C8. Table 5 presents the retention data. It can be seen that the elution orders of the two columns are the same. With 65% methanol, the Kromasil C8 phase gave 1.5-3 times longer retention times than the Vydac C8 phase for the hydrophobic solutes. However, the hydrophobic selectivity of the C8 bonded on Kromasil silica with a larger surface area (340 m^2/g) is identical to that of the C8 bonded on Vydac silica with a smaller surface area (106 m^2/g). These results suggest that the selectivity of these two materials depends solely on the type of modifier employed in the separation and the bonded phases are typical reversed-phase in character. Absolute retentions are different due to differences in carbon content and the silica porosity. It is possible, therefore, to compensate for differences in retention due to variation of carbon content just by altering the methanol-water ratio. With C8 bonded to Kromasil phase usually 10% more organic eluent component is required to achieve similar analysis times as on the C8 bonded on Vydac phase. This result shows clearly that C8 bonded on Kromasil phase merely increases the retention capacity and does not introduce a new retention mechanism compared with the C8 bonded Vydac column. A decrease in retention is caused by the decrease in the specific surface area for silica with large pore diameters.

D. Determination of Stationary Phase Shape Selectivity

Phase selectivity is the ability of a stationary phase to discriminate

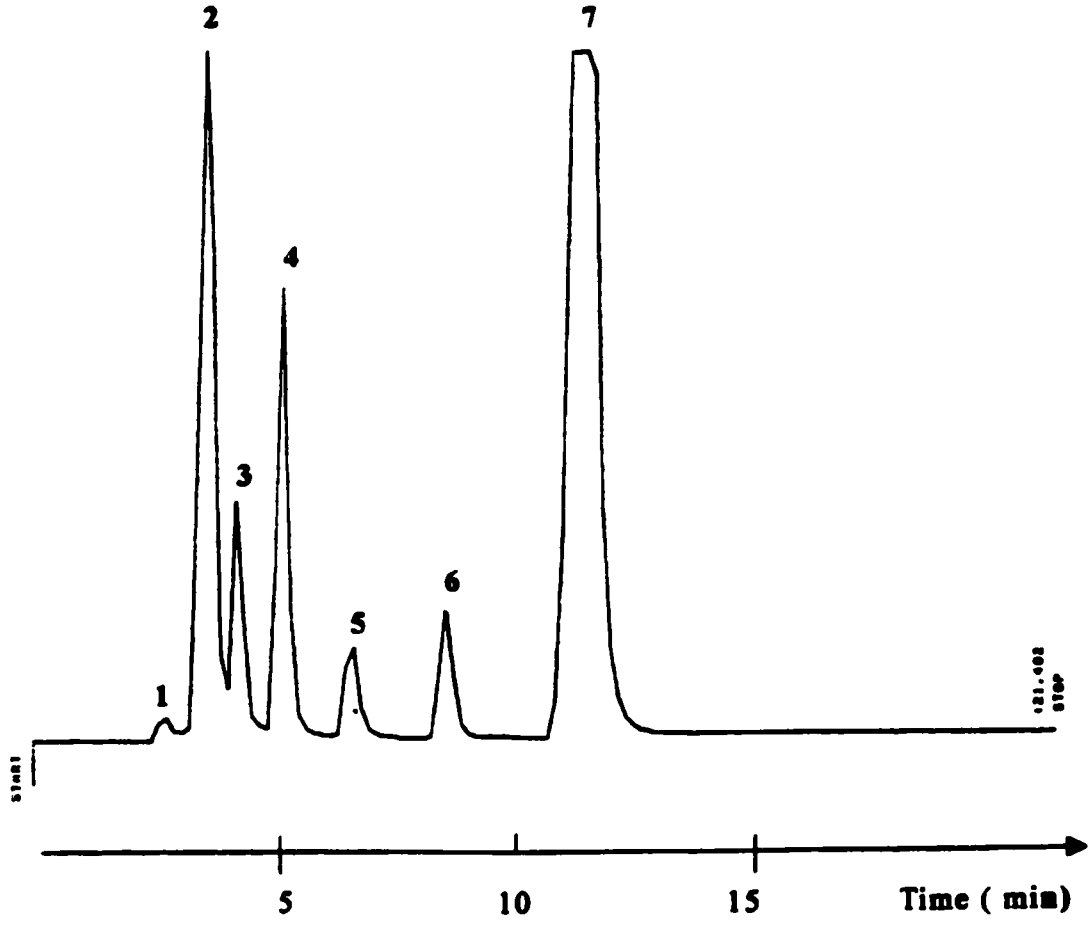


Figure 10. Chromatogram on C8 Bonded to Vydac Stationary Phase of PE Mixture. Mobile phase: 65:35 methanol/water. UV detection at 254 nm and flow rate = 0.7 mL/min. Peaks: 1= sodium chloride; 2= benzene; 3=toluene; 4= ethylbenzene; 5= isopropylbenzene; 6= tert-butyl benzene, 7= anthracene.

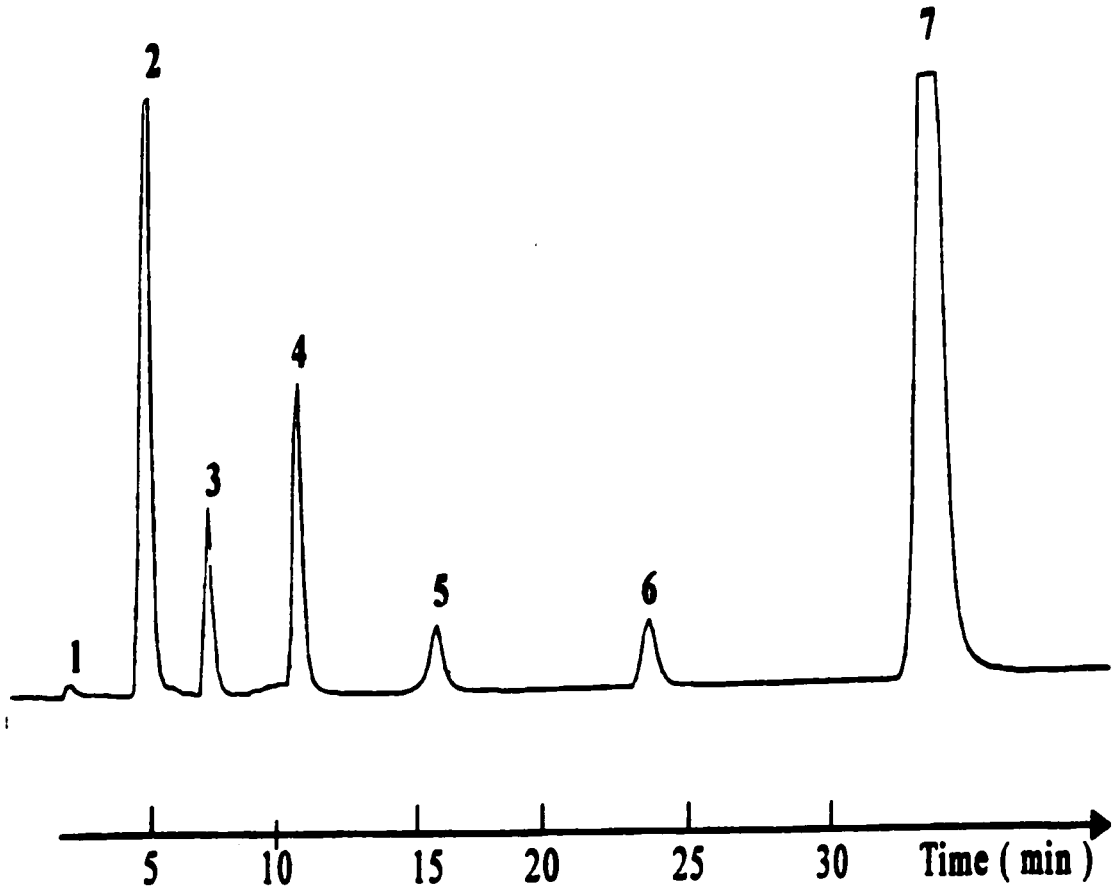


Figure 11. Chromatogram on C8 Bonded to Kromasil Stationary Phase of PE mixture. Mobile phase: 65:35 methanol/water. UV detection at 254 nm and flow rate = 0.7 mL/min. Peaks: 1= sodium chloride; 2= benzene; 3= toluene; 4= ethylbenzene; 5= isopropylbenzene; 6= tert-butyl benzene, 7= anthracene.

Table 5. Retention Properties of PE Mixture*

		Peaks≠					
Column	Property	2	3	4	5	6	7
C8 bonded on Kromasil column							
	t_R	5.215	7.587	11.036	16.395	24. 406	35.269
	k'	1.087	2.036	3.416	5.561	8.766	13.113
	α		1.873	1.678	1.628	1.576	1.496
C8 bonded on Vydac column							
	t_R	3.596	4.351	5.345	6.704	8.825	11.634
	k'	0.269	0.547	0.901	1.384	2.138	3.137
	α		1.963	1.646	1.536	1.545	1.404

*Mobile phase = 65:35 (v/v) methanol-water at 0.7 mL/min. Temperature around 20- 25 ^{0}C. ≠ Peaks: 2= benzene, 3= toluene, 4= ethybenzene, 5= isopropylbenzene, 6= tert-butyl benzene, 7= anthracene.

retention of PAH isomers based on their three-dimensional structure. The most important parameter affecting phase selectivity toward PAHs is phase type, whether a phase was prepared by monomeric or polymeric synthesis chemistry. SRM 869 provides a sensitive measure of the polymeric or monomeric character of the phase. Since these two kind of phases showed significant differences in shape recognition toward PAH isomers, distinguishing a polymeric stationary phase from a monomeric stationary phase is important in method development for PAH separations. From Figure 12 and Table 6 it can be seen that both of the phases showed an elution order BaP<PhPh<TBN with $\alpha_{TBN/BaP} > 1.7$. These phases are

classified as having monomeric-like selectivity. The shape selectivity behavior of the two bonded-phases was essentially unaffected by changes in pore size of the silica support. Shape selectivity depends on the type of bonding chemistry used in the preparation of the stationary phase. Also C8 is short alkyl chain and not as likely as C18 to have good shape selectivity.

Table 6. Elution Order and Column Selectivity toward SRM 869

Packing Materials	Elution Order	$\alpha_{TBN/BaP}$
Kromasil C8	BaP<PhPh<TBN	1.751
Vydac C8	BaP<PhPh<TBN	1.799

E. Dependence of Chromatographic Retention on Eluent Composition

The solutes were chromatographed at six compositions of the methanol/water mobile phase: 100:0, 90:10, 80:20, 70:30, 60:40,and 50:50 (v/v). The plot of logarithm of the capacity factor (log k') versus methanol concentration in the eluent for both columns is shown in Figures 13 and 14. In these figures it can be seen that the dependence of log k' on eluent composition results in non-parallel (with different slopes) and non-linear lines for all solutes on the two stationary phases. The possibility of deviation from linearity can be due to the following. The alkyl chains possess a certain molecular mobility.[14] A disordered "folded" state is

14. A. Tchapla, S. Heron, E. L.Colin, J. Chromatogr. A 656 (1993) 105.

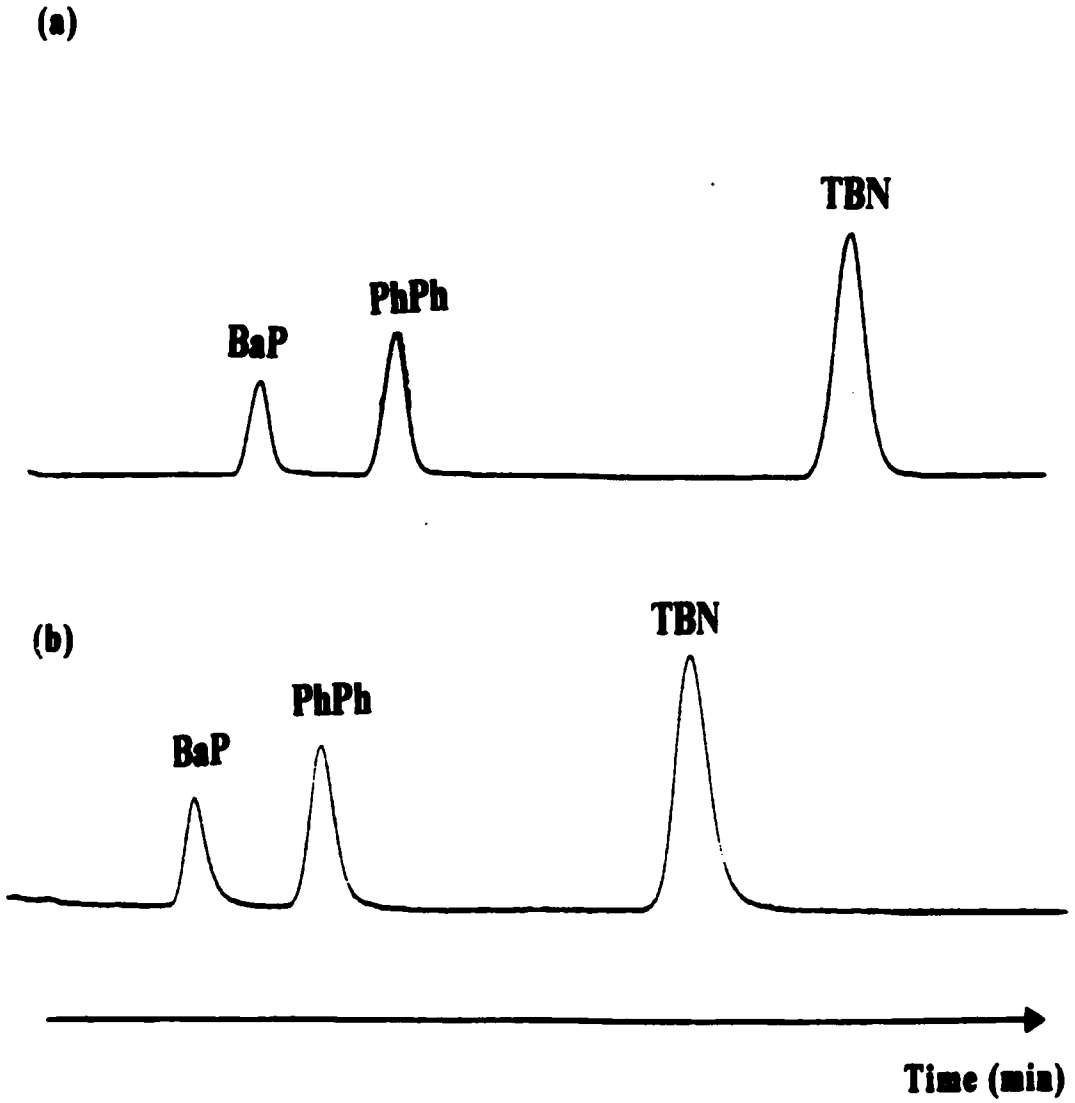


Figure12. Elution Order of PhPh, TBN and BaP on (a) C8 bonded Kromasil column and (b) C8 bonded Vydac column. Mobile phase: methanol-water (75:25), flow rate: 0.5 mL/min; UV absorbance at 254 nm. PhPh = phenabthro[3,4-c]phenanthrene; BaP = benzo[α]pyrene; and TBN=1,2:3,4:5,6:7,8-tetranenzonaphthalene.

favored when the stationary phases are in the presence of a non-wetting mobile phase, and a more ordered "brush" state is favored when the chains are wetted with a mobile phase of high organic content. The surface layer can adjust itself depending on the mobile phase composition. The bonded phase morphology becomes more open in solvents compatible with n-alkanes, i.e., promoting extension of the bonded alkyl chains ("brush" structure) and the chains collapse in fairly polar solvents. It is into this milieu that the solute molecule partitions. The curves in the figures display a change of slope with an obvious deviation from linearity above 80% methonal in the mobile phase. These irregularities could be explained by changes in the conformations of the bonded phase.

Typically, a 10% increase in the organic modifier concentration in the mobile phase will decrease k' for every solute by a factor of 2-3. It was clear that in the isocratic separation mode the overall retention time, the k' range, and the resolution are very sensitive to the mobile-phase composition.

F. Determination of the Retention Behavior of Basic Compounds

The analysis of basic compounds using reversed-phase high-performance liquid chromatography continues to receive much attention, due to problems of poor peak shape, which are generally attributed to detrimental interaction of these analytes with underivatised silanol groups. The considerable interest in this area is partially due to the large

number of important pharmaceuticals and other clinically significant compounds which posses basic groups. The possible interactions between solutes and silanol groups, ion exchange, hydrophobic and silanophilic, all contribute to the overall retention mechanism; the performance problems with basic molecules in reversed-phase chromatography are mainly a consequence of ion exchange on active silanol groups. The retention of this type of solute is greatly affected by the availability of the silica surface and compound stereochemistry and basicity.[15] No detailed study has so far focused on their role in determining retention behavior. Unbuffered methanol-water was selected as the mobile phase for this study. The system pH was between 7 and 8. Many authors report that peak shapes are generally worst around pH 7. At this pH, support silanol groups and basic solutes are often partially ionized. Therefore, this pH often presents the greatest challenge in obtaining good peak shape and high column efficiency. At lower pH, peak shape may be improved due to reduced dissociation of silanols, whereas at higher pH, decreasing protonation of the base may improve peak symmetry. In each case, reduced ion-exchange interaction should result. The solutes chosen for this study were intended to exemplify the different types of basic compounds, which may be expected to interact with silica surfaces. These were N, N- dimethylaniline (a basic tertiary amine), pK_a=5.15, aniline (a

15. I. Jane, J. Chromatogr., 111 (1975) 227.

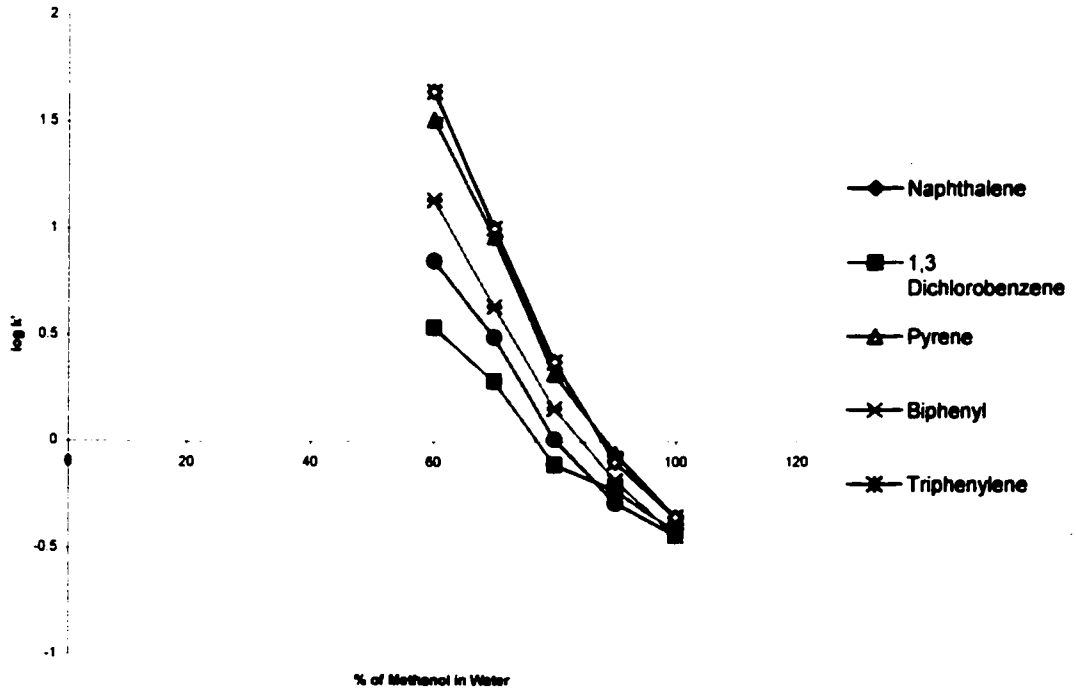


Figure 13. Log k' vs. Methanol Concentration for C8 Bonded Kromasil Silica

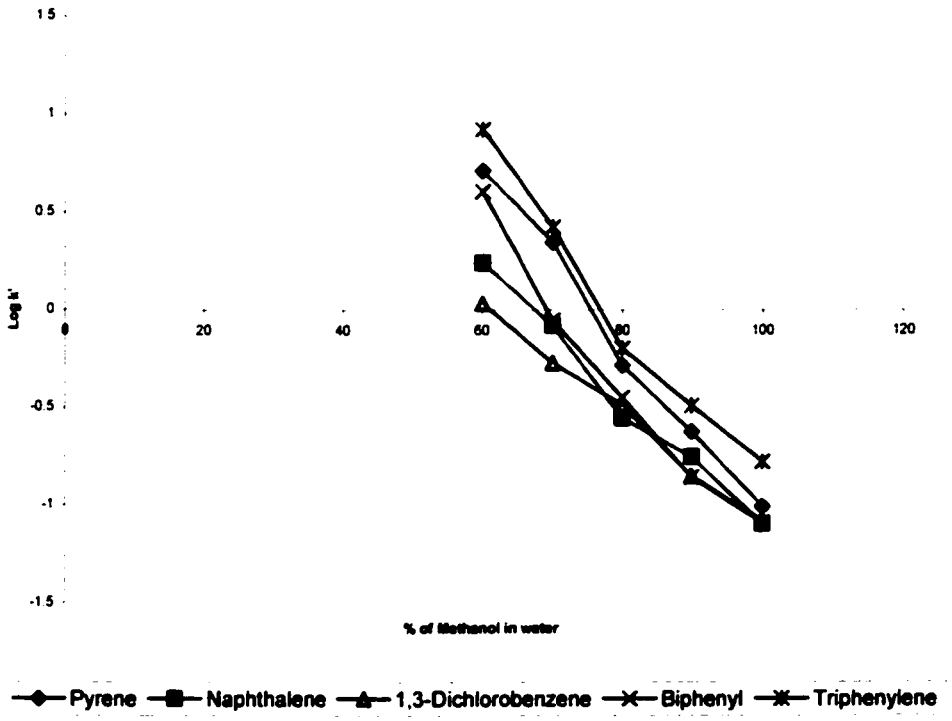


Figure 14. Log k' vs. Methanol Concentration for C8 Bonded Vydac Silica

primary amine), pK_a=4.63, and diphenylamine $(C_6H_5)_2NH$ (secondary amine), pK_a=0.79. In order to investigate the effect of changing the methanol concentration at room temperature, 40 ^{0}C, 50 ^{0}C and 60 ^{0}C on the capacity factors and column performance of the three solutes, an experiment was carried out on the two columns measuring the retention of the three basic compounds as a function of methanol concentration when the mobile phase does not contain buffering components. In this way it was hoped to be able to discover more about the influence of compound properties and the accessibility of the silica surface and the accompanying bonded-hydrocarbon conformation and solvation-layer changes at different temperatures on retention. The influence of pK_a of these compounds on retention must also be considered. The higher the pK_a, the stronger the interactions of the solute with the strongly acidic silanols. In Figures 15 and 16 it can be seen all three solutes eluted as symmetrical peaks and all of them were base-line resolved. In Figures 17, 18, 19, 20, 21, 22, 23 and 24, all of the aniline log *k'* vs. % MeOH plots are not linear indicating that the interaction mechanism is mixed. All of the others do not show a clear linearity except N, N- dimethylaniline at room temperature. The plots of log *k'* vs. methanol concentration at different temperatures show decreased retention as the temperature increases, corresponding to the expected increase in mass transfer. But changing temperature didn't generate a favorable conformation for better

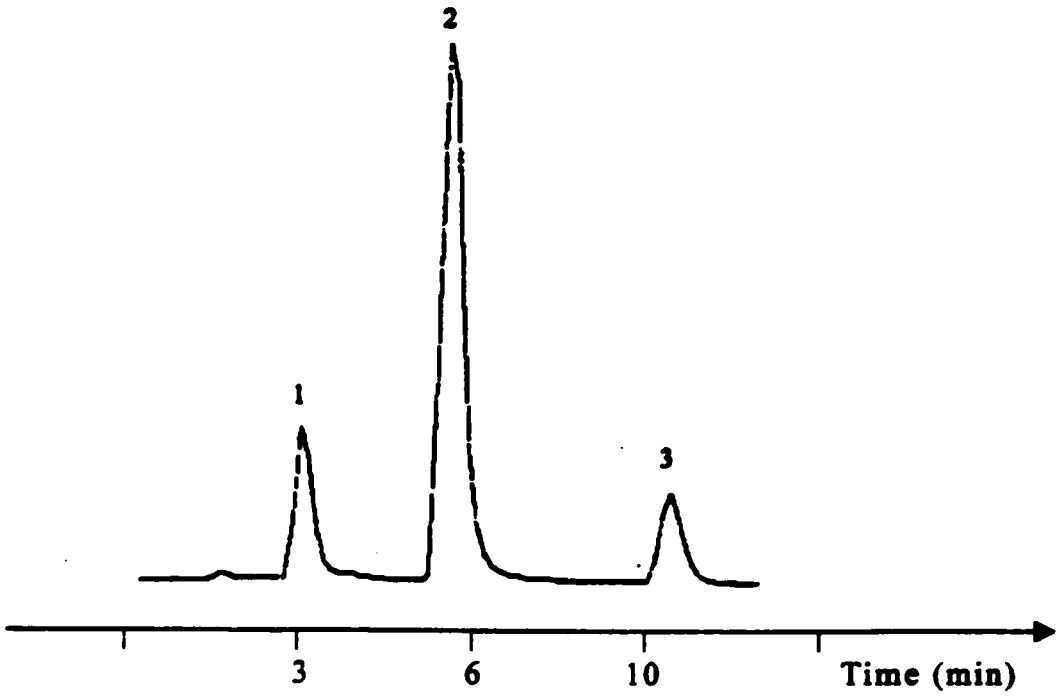


Figure 15. Separation of Three Basic Compounds Using a C8 Bonded to Vydac Phase. Mobile phase: 50:50 (v/v) methanol-water; flow rate: 1.0 mL/min; column temperature: 50 ^{0}C; detection UV at 254 nm. Peaks: 1 = aniline, 2= N, N- dimethylaniline, 3= diphenylamine.

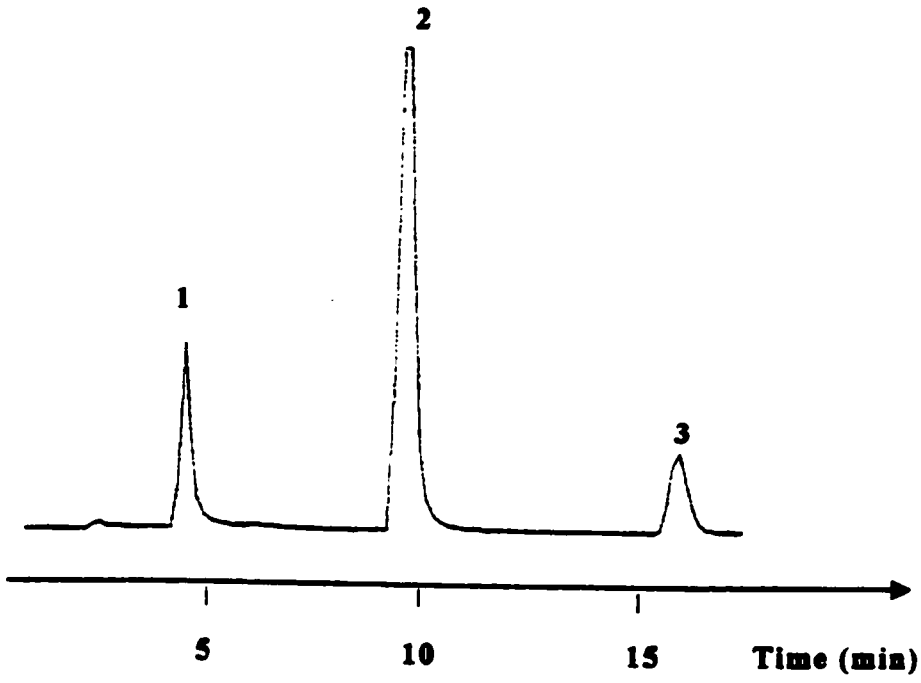


Figure 16. Separation of Three Basic Compounds Using a C8 Bonded to Kromasil Phase. Mobile phase: 60:40 (v/v) methanol-water; flow rate: 0.7 mL/min; column temperature: room temperature; detection UV at 254 nm. Peaks: 1 = aniline, 2= N, N- dimethylaniline, 3= diphenylamine.

interaction. This is logical since the bonded C8 chain is relatively short and the bonding density is reasonably high, so there is minimal variation in the conformation of this ligand and only minor changes of conformational configurations of the alkyl bonded layer with temperature. It appears that steric effects around the basic nitrogen atom are of major importance. The tertiary amine showed smaller deviation from the expected linear relationship relative to the primary and secondary amines. This is reasonable because steric effects of the substituent at the basic center reduces the access of the basic nitrogen atom to column active sites. Also a tertiary amine is more hydrophobic. On the two phases studied here, the effect of pK_a was less important. This is reasonable since identical mobile phases were used for each basic test solute. At pH around 7, all three basic compounds are in an unionized form and the degree of solvation of the stationary phase as well as the degree of ionization of accessible silanols for each test solute is the same. An interesting point to note is the change of the slope of the plots with changes in methanol concentration. The initial increases in methanol concentration from 50 to 80 % results in a decrease in slope. At higher methanol concentrations the slope of the plot increases. This means that the silica surface is increasingly polar at high methanol concentration. A hydrogen-bonding interaction would be expected to decrease with increasing water concentration. At low concentrations of organic solvent, the behavior is

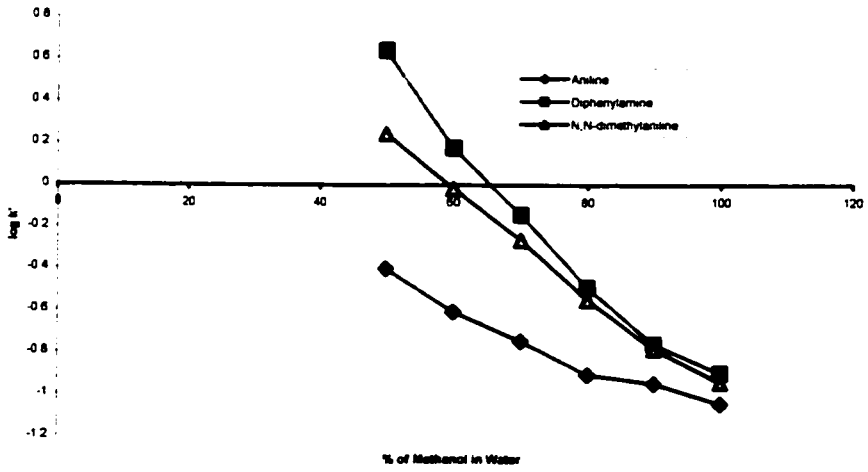


Figure 17. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Vydac Phase (a). Flow rate: 0.7 mL/min; column temperature: room temperature; detection UV at 254 nm.

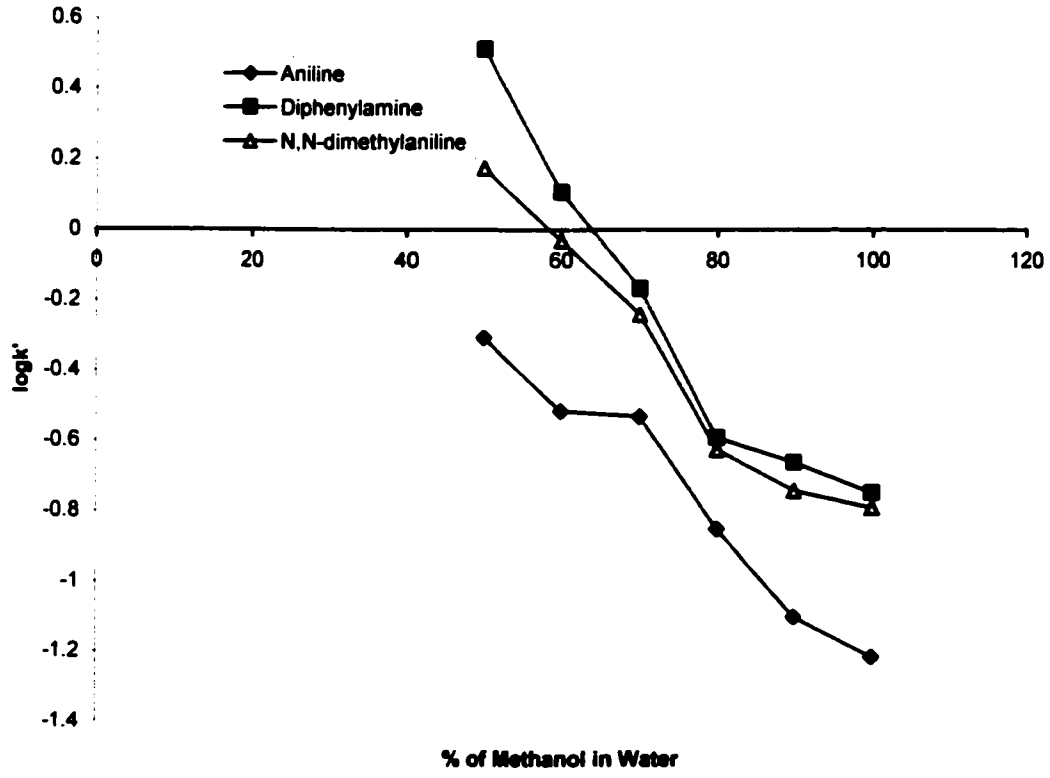


Figure 18. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Vydac Phase (b). Flow rate: 0.7 mL/min; column temperature: 40 ^{0}C; detection UV at 254 nm.

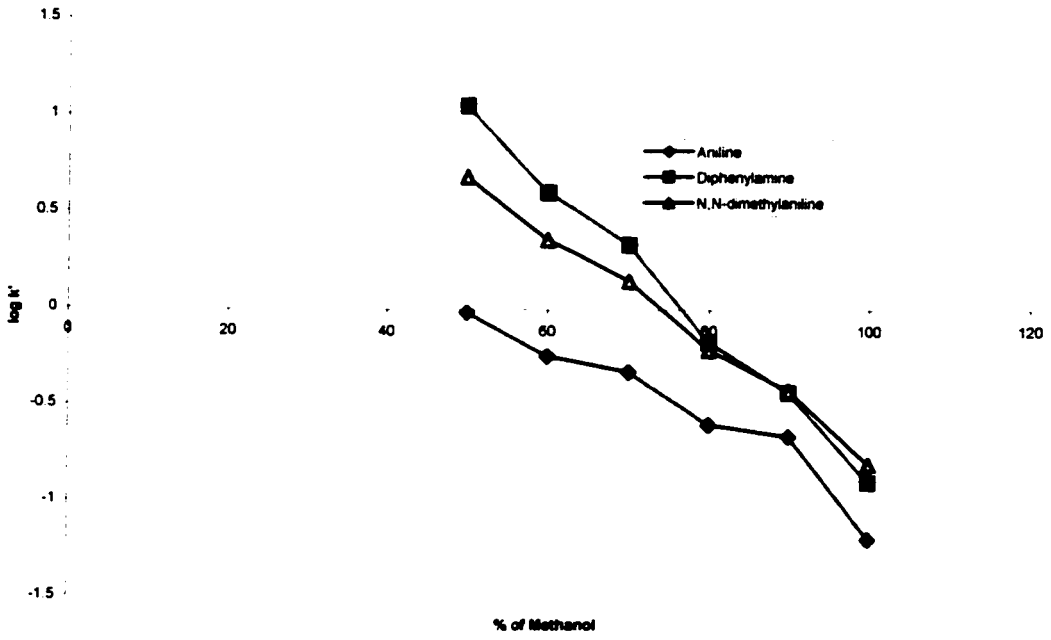


Figure 19. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Vydac Phase (c). Flow rate: 0.7 mL/min; column temperature: 50 ^{0}C; detection UV at 254 nm.

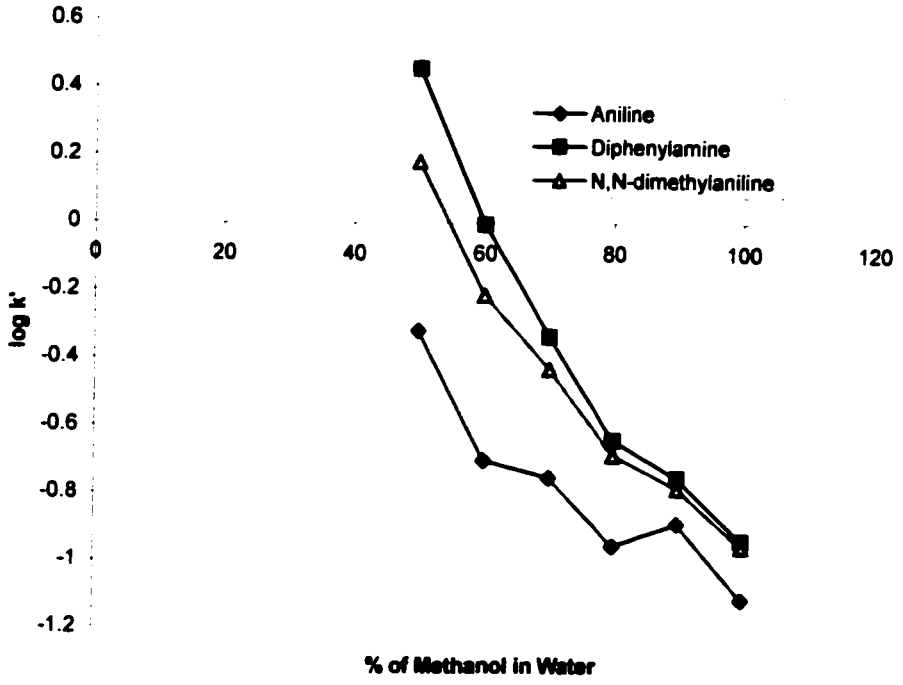


Figure 20. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Vydac Phase (d). Flow rate: 0.7 mL/min; column temperature: 60 ^{0}C; detection UV at 254 nm.

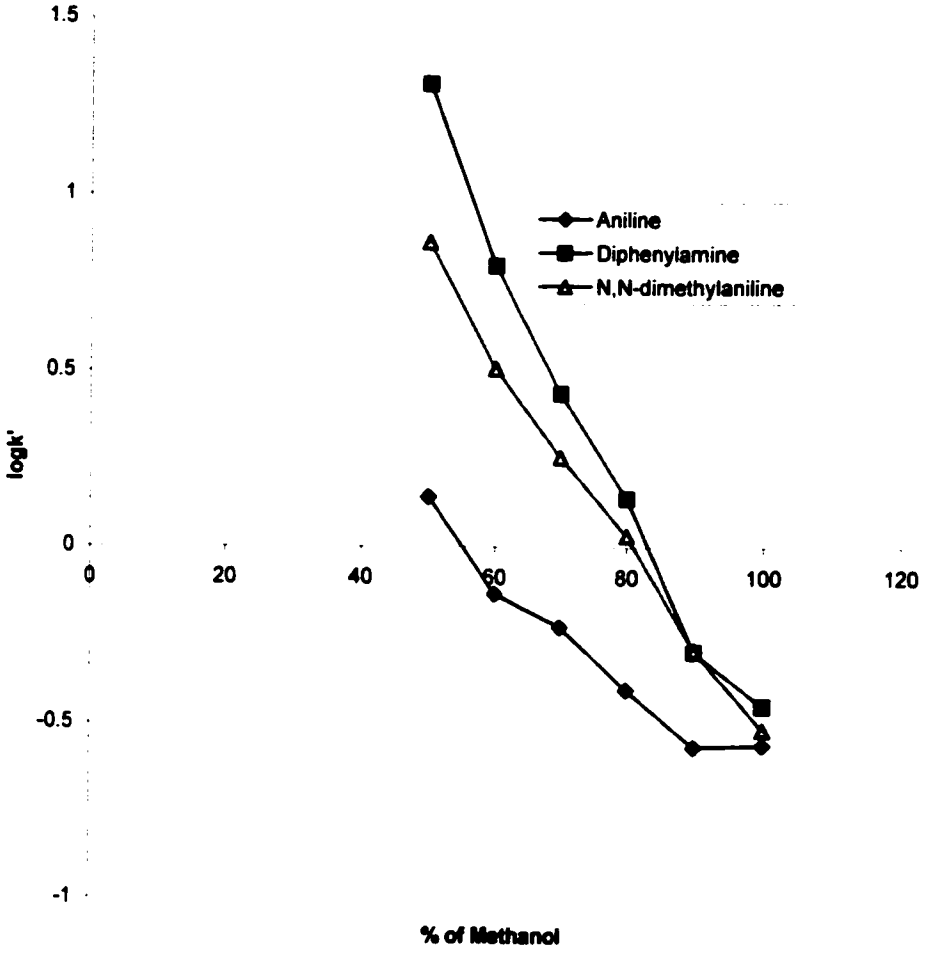


Figure 21. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Kromasil Phase (a). Flow rate: 0.7 mL/min; column temperature: room temperature; detection UV at 254 nm.

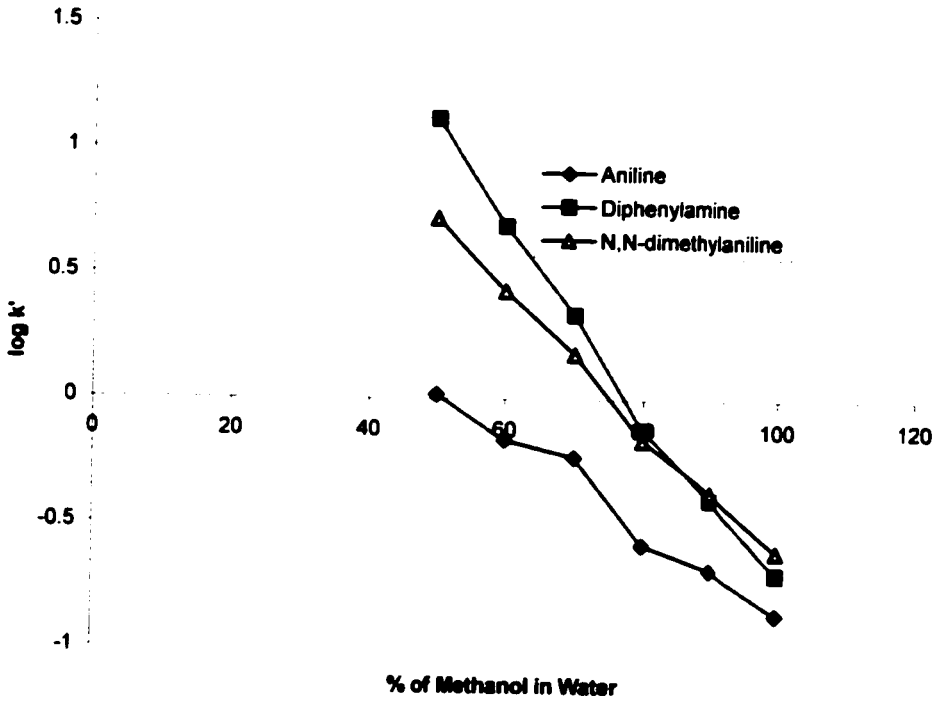


Figure 22. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Kromasil Phase (b). Flow rate: 0.7 mL/min; column temperature: 40 ^{0}C; detection UV at 254 nm.

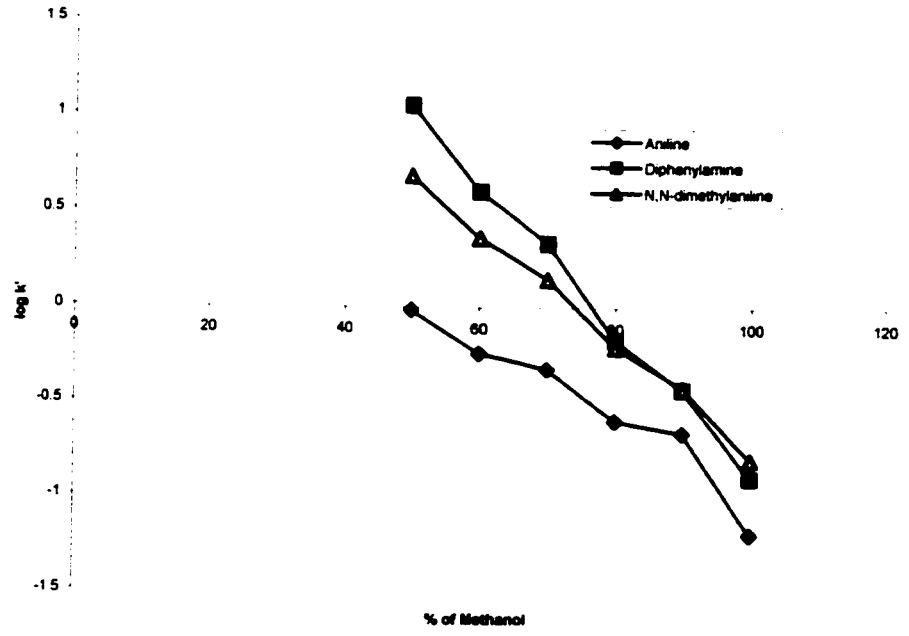


Figure 23. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Kromasil Phase (c). Flow rate: 0.7 mL/min; column temperature: 50 ^{0}C; detection UV at 254 nm.

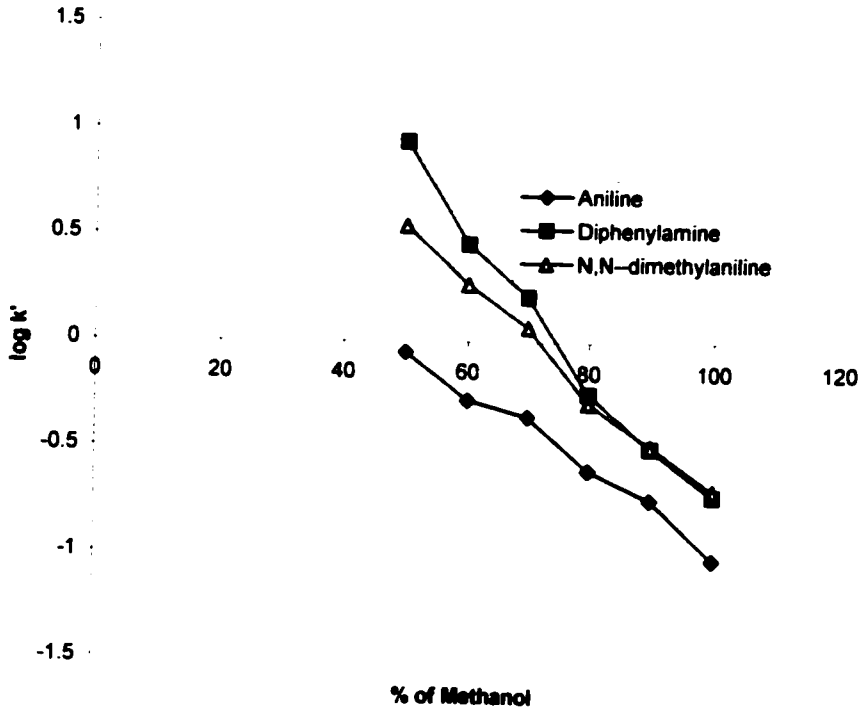


Figure 24. Chromatographic Studies of Three Basic Compounds on C8 Bonded to Kromasil Phase (d). Flow rate: 0.7 mL/min; column temperature: 60 ^{0}C; detection UV at 254 nm.

predominantly reversed-phase, but at higher concentrations the solutes show increasing normal phase behavior. From column performance data in Table 7 it can be seen that two columns separated the basic compounds with good peak shape ratios, which varied from 1.01 to 1.21. This result suggests that the residual silanols were well protected by the bonded ligands. The basic compounds are slightly more retained on the C8 bonded on Kromasil column than on C8 bounded on Vydac column. This result could be because of the high ligand density of C8 bonded on Kromasil column. The column efficiency of C8 on Kromasil column is higher than that of the C8 on Vydac column. This suggests C8 bonded on Kromasil column has better performance for the analysis of these basic compounds than C8 bonded on Vydac column.

G. The Selectivity of Polycyclic Aromatic Hydrocarbon (PAH) with Similar Retention Behavior

The separation of polyaromatic hydrocarbons is one of the most important areas of environmental testing today and reversed-phase HPLC on C_{18} phases has been the most common way for the separation of PAHs. With these two stationary phases, the separation of six of the 17 PAHs which were identified by the U.S. Environmental Protection Agency (EPA) as priority pollutants, benzene, naphthalene, fluorene, phenanthrene, anthracene and pyrene was examined using a

methanol/water mobile phase. In Figures 25 and 26, all of the PAH analytes were separated on the two phases within a reasonable time since

Table 7. Column Performance Data for Three Basic Compounds

Compound	pKa (at 25 °C in water)		Temperature	*k'*	N	As
N, N-dimethyl -aniline	5.15	C8 bonded on Kromasil*	Ambient	2.79	19911	1.07
			40 °C	2.61	18009	1.21
			50 °C	2.38	16503	1.12
			60 °C	2.98	27167	1.04
		C8 Bonded on Vydac♣	Ambient	1.63	17146	1.20
			40 °C	1.48	6615	1.18
			50 °C	1.31	8770	1.05
			60 °C	1.26	9578	1.18
aniline	4.63	C8 bonded on Kromasil	Ambient	0.56	6907	1.13
			40 °C	0.65	5318	1.18
			50 °C	0.61	5756	1.16
			60 °C	0.57	5561	1.17
		C8 Bonded on Vydac	Ambient	0.33	7705	1.17
			40 °C	0.48	5007	1.16
			50 °C	0.35	4895	1.11
			60 °C	0.33	5347	1.19
Diphenyl -amine	0.79	C8 bonded on Kromasil	Ambient	5.55	49131	1.01
			40 °C	4.76	37976	1.17
			50 °C	4.14	36648	1.15
			60 °C	3.59	45712	1.05
		C8 Bonded on Vydac	Ambient	4.13	25778	1.19
			40 °C	3.12	25376	1.17
			50 °C	2.93	31445	1.16
			60 °C	2.48	18389	1.20

*: Mobile phase= 60:40 (v/v) methanol-water at 0.7 mL/min.
♣: Mobile phase= 50:50 (v/v) methanol-water at 0.7 mL/min.

the *k'* of phenanthrene is very similar to that of its isomer with the same number of rings, anthracene, the bonded-phases are promising for use in the analysis of PAH components with similar hydrophobicities. Chromatographic tests showed that the two phases behave like a C18

phase toward the separation of PAHs. Also, using a methanol/water mobile phase instead of an acetonitrile/water mobile phase ensures a less toxic solvent consumption.

H. Stability of Bonded Phases

The stability of the bonded phases was investigated at high pH (9 - 10) by monitoring changes in the retention factor and theoretical plate number of pyrene. The k' was measured before exposure to high pH conditions and after periodically purging the columns at room temperature with a measured number of column volumes of a mobile phase consisting of 70:30 (v/v) methanol -water with unbuffered sodium hydroxide solution delivered at 0.7mL/min. Any significant change in retention and N of this compound suggests the loss of silica stationary phase under this aggressive mobile-phase condition. The silica used for a synthesis can determine the stability of the resultant bonded phase. Whenever possible, the retention factors measured were corrected during the hydrolysis experiments for any changes that occurred for a control column when it was not exposed to hydrolysis conditions. Those corrections compensated for small differences caused by mobile phase preparation, evaporative loss of methanol, temperature variation and manual injection. The retention factor and theoretical plate number N of pyrene are the accepted measures of hydrophobic retention and column efficiency and thus should be a

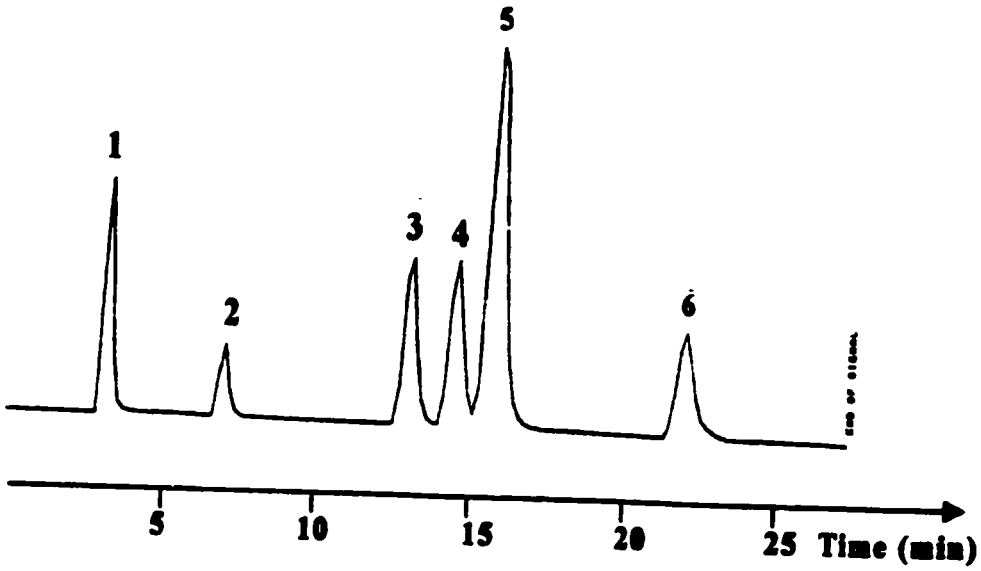


Figure 25. Separation of a Six-component Mixture of PAHs Using a C8 Bonded to Kromasil Phase. Mobile phase: 70:30 (v/v) methanol-water; flow rate: 0.7 ml/min; column temperature: room temperature (around 25 ^{0}C); detection UV at 254 nm. Peaks: 1= benzene, 2 = naphthalene, 3 = fluorene, 4 = phenanthrene, 5 =anthracene, 6 = pyrene.

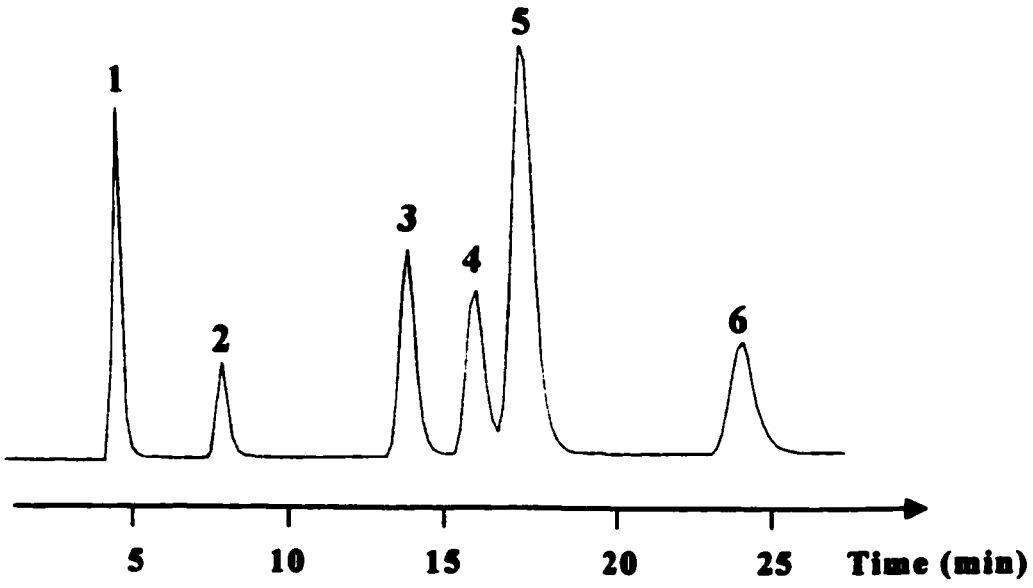


Figure 26. Separation of a Six-component Mixture of PAHs Using a C8 Bonded to Vydac Phase. Mobile phase: 60:40 (v/v) methanol-water; flow rate: 0.7 ml/min; column temperature: room temperature (around 25 ^{0}C); detection UV at 254 nm. Peaks: 1= benzene, 2 = naphthalene, 3 = fluorene, 4 = phenanthrene, 5 =anthracene, 6 = pyrene.

sensitive indicator of silica matrix-bonded phase loss. In Figures 27 and 28 the separation of a six component PAH mixture is shown on a column prior to column aging (upper chromatogram) and the same column after purging with about 4000 column volumes of pH 9 mobile phase (bottom chromatogram). In Figures 29 and 30 the separation of three basic compounds is shown under the same column conditions as the six-component PAH mixture. As seen, the behavior the columns exhibit toward basic compounds was hardly affected by basic hydrolysis, where the absolute and relative retention between phenanthrene and anthracene did change. This result suggests some base attack on the silica matrix. A gradual dissolution of silica matrix in an alkaline mobile phase causes a decrease in the amount of bonded phase but the relative (%C) carbon coverage stays at approximately the same. Meanwhile, the hydrolytic loss of silica matrix did not generate new active silanols on the surface which influences both the retention and peak shape of basic solutes. The resistance of the two C8 bonded silica-based columns to highly alkaline conditions is well illustrated in Figures 31 and 32, which shows that after purging 10000 column volumes of a basic mobile phase, there is a little change in k' and plate numbers. The rate of column degradation is slower. The two columns exhibit resistance to basic hydrolysis through high ligand density. The high stability of the two C8 bonded phases in basic solvent environments favors their use at high pH for increased separation

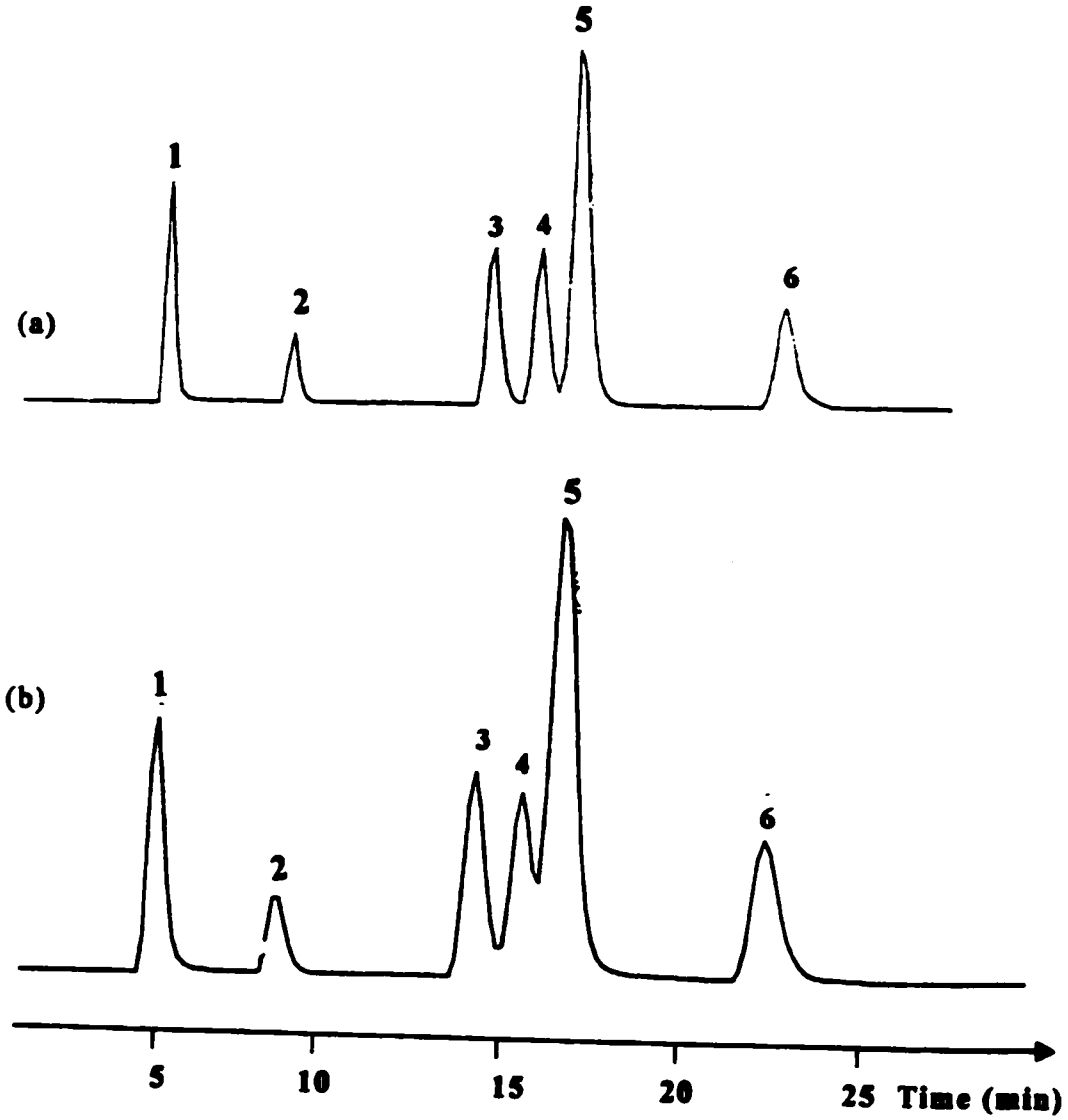


Figure 27. Comparative Separations with Six PAHs Mixture on C8 Bonded to Kromasil Column. (a) Initial-mobile phase: methanol-water 70:30 (v:v); flow rate: 0.7 mL/min; C8 bonded to Kromasil silica column prior to column aging; room temperature. (b) After 4000 column volumes of purge with mobile phase: methanol - sodium hydroxide solution, pH 9.0 (70:30); flow rate: 0.7 mL/min.

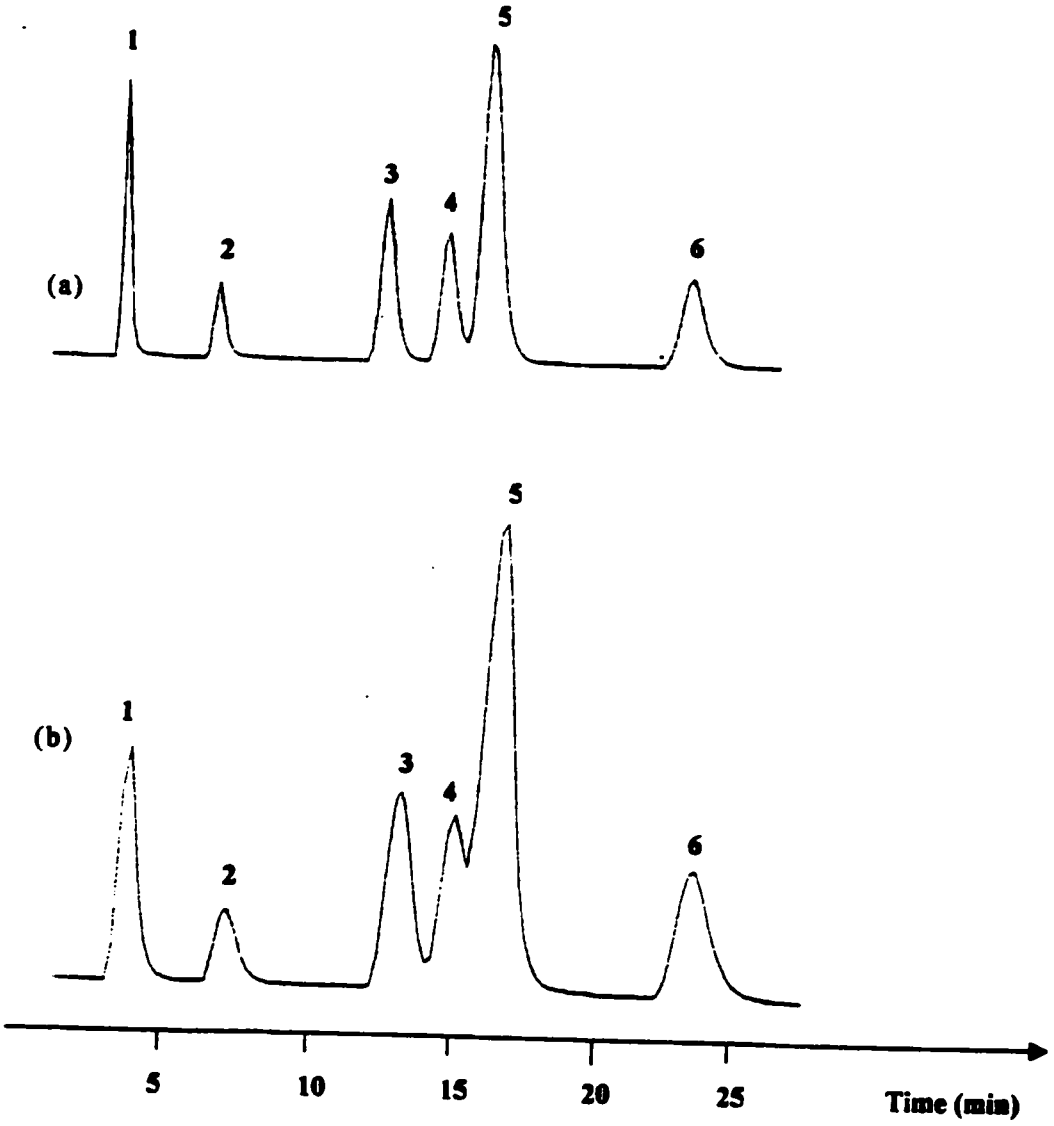


Figure 28. Comparative Separations with Six PAHs Mixture on C8 Bonded to Vydac Column. (a) Initial-mobile phase: methanol-water 60:40 (v:v); flow rate: 0.7 mL/min; C8 bonded to Vydac silica column prior to column aging; room temperature. (b) After 4000 column volumes of purge with mobile phase: methanol-sodium hydroxide solution, pH 9.0 (60:40); flow rate: 0.7 mL/min.

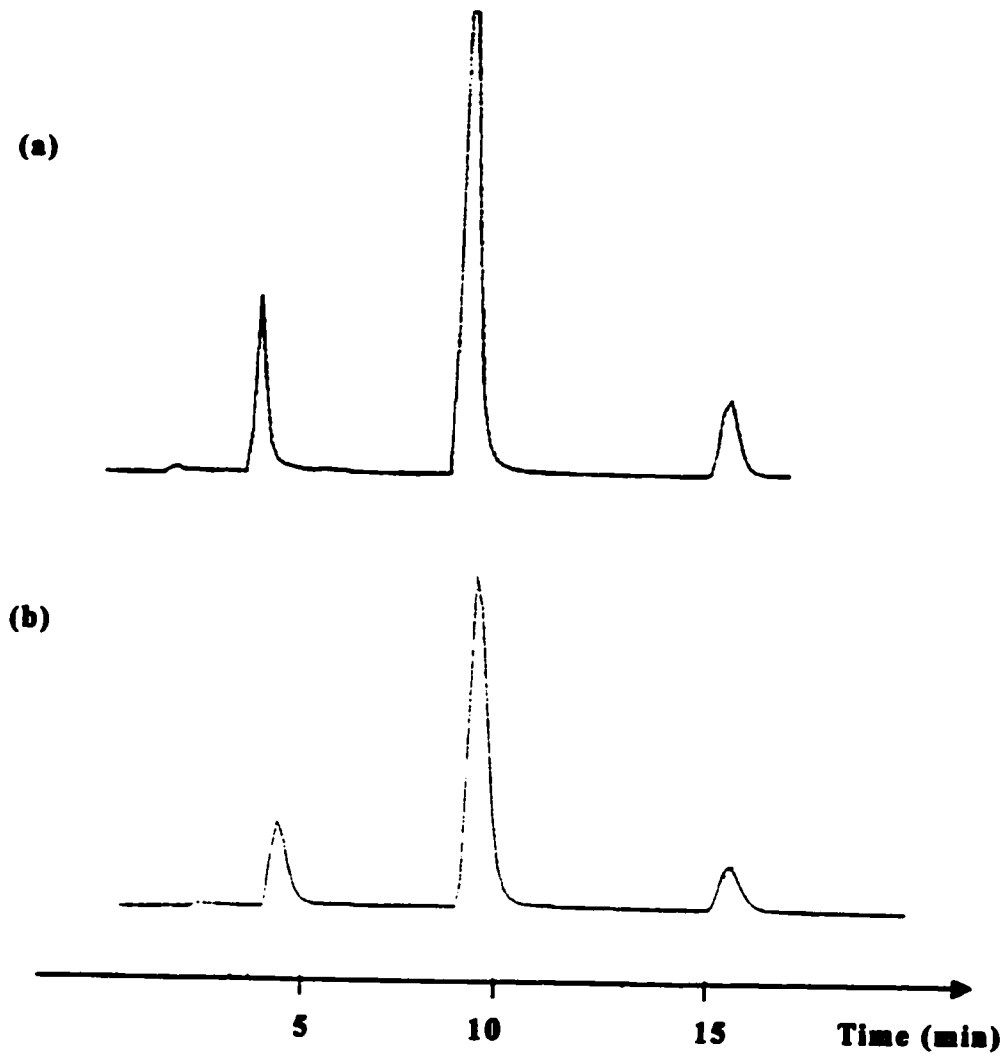


Figure 29. Comparative Separations with Three Basic Compounds on C8 Bonded to Kromasil Column. (a) Initial-mobile phase: methanol-water 60:40 (v:v); flow rate: 0.7 mL/min; C8 bonded to Kromasil silica column prior to column aging; room temperature. (b) After 4000 column volumes of purge with mobile phase: methanol-sodium hydroxide solution, pH 9.0 (60:40); flow rate: 0.7 mL/min.

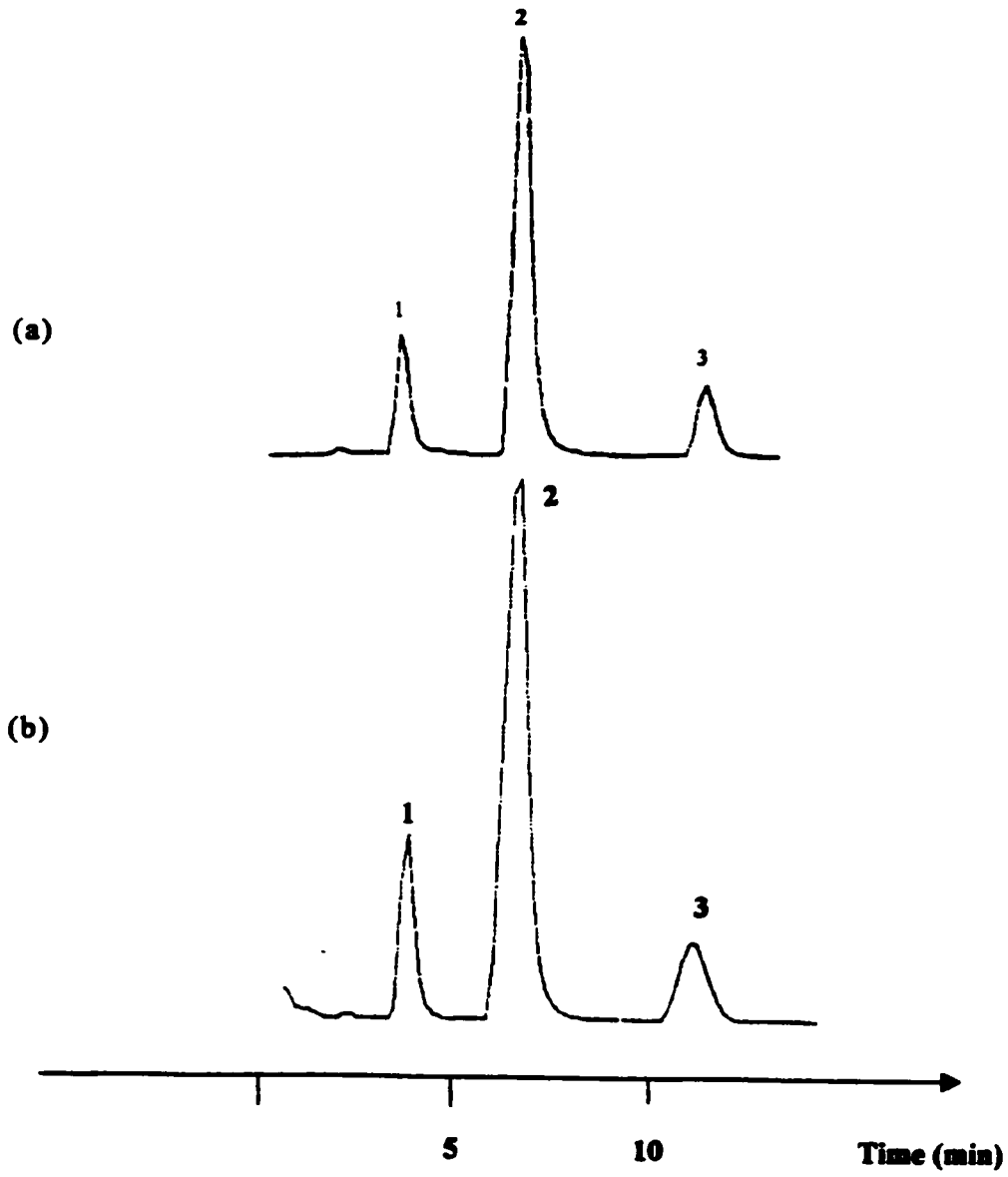


Figure 30. Comparative Separations with Three Basic Compounds on C8 Bonded to Vydac Column. (a) Initial-mobile phase: methanol-water 50:50 (v:v); flow rate: 0.7 mL/min; C8 bonded to Vydac silica column prior to column aging; at 50 ^{0}C. (b) After 4000 column volumes of purge with mobile phase: methanol-sodium hydroxide solution, pH 9.0 (50:50); flow rate: 0.7 mL/min.

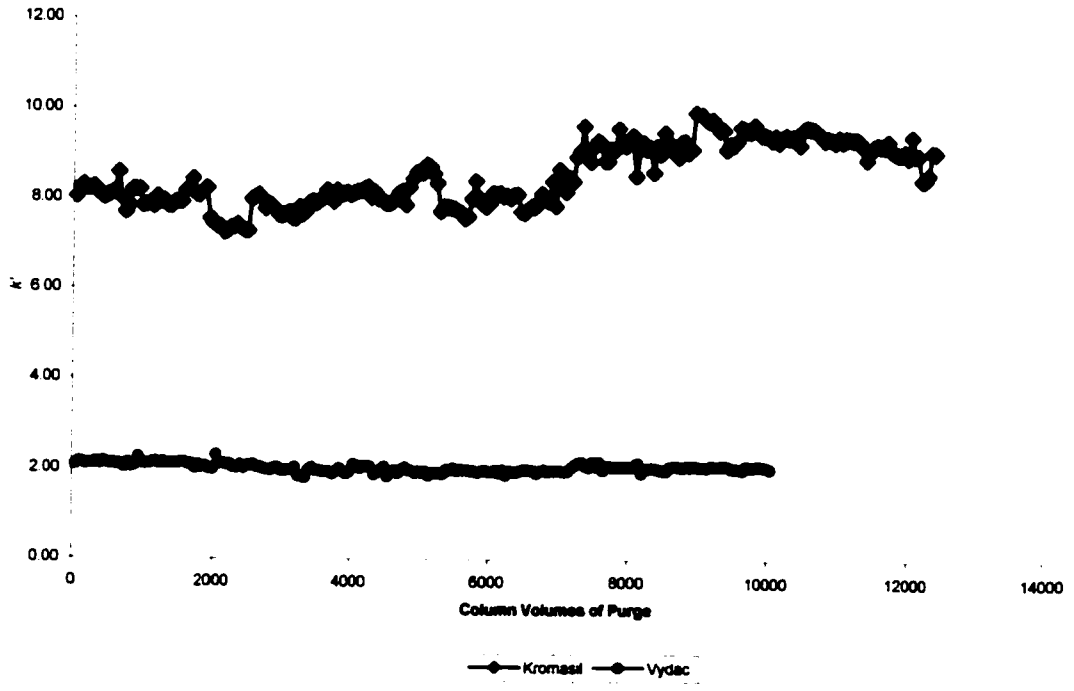


Figure 31. Plots of Pyrene Retention Factor versus Number of Mobile-Phase Volumes at pH 9-10. (After purging 7000-column volumes mobile phase, pH changed to 10). Mobile phase: 70:30 (v:v) methanol-water; flow rate: 0.7 mL/min; column temperature: room temperature (20~25 ^{0}C); detection: UV absorbance at 254 nm; sample volume 20 μL.

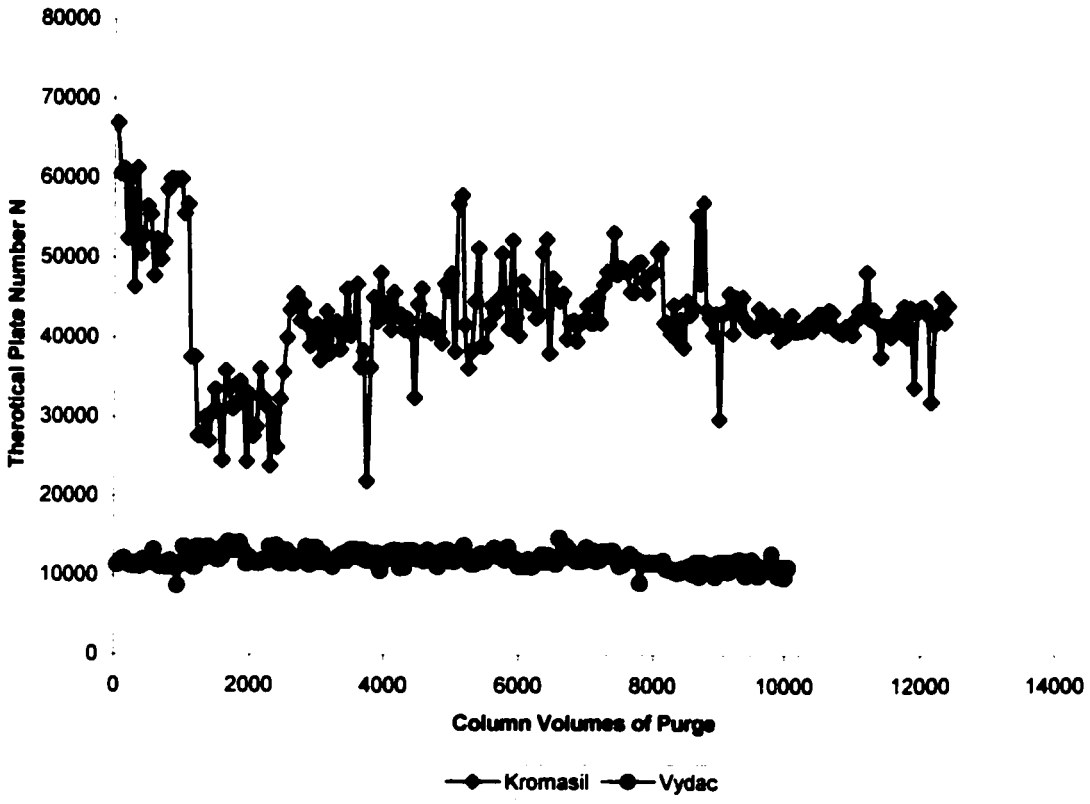


Figure 32. Plots of Pyrene Theoretical Plates versus Number of Mobile-phase Volumes at pH 9- 10. (After purging 7000 column volumes mobile phase, pH changed to 10). Mobile phase: 70:30 (v:v) methanol-water; flow rate: 0.7 mL/min; column temperature: room temperature (20~25 ^{0}C); detection: UV absorbance at 254 nm; sample volume 20 μL.

efficiency. The C8 bonded Vydac column shows slightly less silica support solubility than the C8 bonded on Kromasil column, perhaps because of a lower surface area and higher surface coverage.

CHAPTER IV

CONCLUSIONS

C8 stationary phases bonded to Kromasil and Vydac synthesized by the silanization/hydrosilation method possess high surface coverage and a direct Si-C linkage. The absolute retention of neutral compounds depends on the carbon content of the column. The latter is a function of the silica porosity and amount of carbon bonded. The Kromasil column gives higher k' values than the Vydac column. The two phases provided satisfactory selectivity for mixtures of polycyclic aromatic hydrocarbons which confirms hydrophobic interactions in the HPLC separation process. From the point of view of chromatographic performance, the selectivity of these materials was found to be determined solely by the type of organic moiety employed in the synthesis. The two phases all possess monomeric selectivity toward SRM 869 test mixture. The quantitative structure-retention relationships derived revealed typical reversed-phase character. The strongest interactions of amine or tertiary amino compounds with active silanol are favored by reduced steric hindrance around the nitrogen atom. More favorable interactions were available at low temperature. The variations are less pronounced for the tertiary base (N, N-dimethylaniline) included in the studies. Solutes with primary amino groups seem to be more sensitive to silanophilic interactions. The interactions between residual silanols and solute can play a major role particularly when an

organic-rich mobile phase is used. Unreacted silanols can have a profound influence on the retention of basic compounds even those having maximum surface coverage. The high-density C8 bonded silica phases are remarkably stable in high pH solvent environments, which makes them good candidates for biotechnological applications.

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