SYNTHESIS AND CHARACTERIZATION OF HPLC STATIONARY PHASES USING 4-TERT-BUTYLCALIX[N]ARENES

by

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Synthesis and Characterization of HPLC Stationary Phases Using
4-Tert-Butylcalix[n]arenes

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ABSTRACT

HPLC stationary phases of various types are always in demand. The evolution of stationary phases is continuous because of the widely applied techniques of HPLC. The work presented here is a method used to synthesize new stationary phases. The method is based on typical silane chemistry and uses silica as the support material for attachment of 4-t-butylcalix[n]arenes to the surface of the silica.

In addition to evaluating the behavior of different calixarenes, different silica pore sizes were also examined. Chromatographic studies include reversed phase behavior, and the formation of host/guest complexes. Spectrometric studies include solid state NMR and fluorescence.

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LIST OF SYMBOLS

Symbol	Definition	Units or Reference
mg	Milligram	1×10^{-3} gram
cm	Centimeter	1×10^{-2} meter
mm	Milimeter	1×10^{-3} meter
mL	Milliliter	1×10^{-3} liter
Å	Angstrom	1×10^{-10} meter
µL	Microliter	1×10^{-6} liter
nm	Nanometer	1×10^{-9} meter
mol/L	Molarity	moles per liter
R	Gas Constant	8.314 J/mole K
ΔH	Change in Enthalpy	Cal/mol
ΔS	Change in Entropy	J/mole K
min	minutes	--
°C	Degrees Celcius	--
%	Percent	--
UV	Ultraviolet	--
NMR	Nuclear Magnetic Resonance	--
CP/MAS	Cross Polar Magic Angle Spin	--
HPLC	High Performance Liquid Chromatography	--
LC	Liquid Chromatography	--
LLC	Liquid-Liquid Chromatography	--
GC	Gas Chromatography	--

LIST OF SYMBOLS (CONTINUED)

Symbol	Definition	Units or Reference
G	Gram	--
L	Liter	--
M	Meter	--
PAH	Polyaromatic Hydrocarbon	--
k'	Capacity Factor	--
amu	Atomic Mass Unit	--
ODS	Octadecyl Silane	--

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Chapter I

Statement of the Problem

HPLC is one of the most widely used analytical techniques because of its wide applicability for the analysis of complex mixtures. In today's society the separation of formulations such as cough syrup, and the analysis of environmental samples for toxic compounds, is extremely important. The best method to perform many of these analyses is HPLC.

For a separation to be possible, the correct combination of mobile phase and stationary phase must be obtained. The closer to the "absolute" correct combination of phases, the better the separation becomes. Based upon the relative strengths of solvents and the peak shape, one can usually find the correct mobile phase, which can be changed in small or large increments by simply changing the percent of the organic modifier by an appropriate amount.

The stationary phase, however, is much harder to correct, primarily because there are a limited number of stationary phases available. As a result stationary phases generally cannot be changed in small increments in a manner similar to that used for mobile phases. The commercially available stationary phases are of high quality in terms of materials and workmanship and thus give reproducible results. The problem with most stationary phases, however, is that they only offer minimal solute selectivity. It is not always possible to obtain the correct stationary phase because it may not be commercially available or may not have even been synthesized.

To improve their selectivity, stationary phases need to be synthesized with a wider range of molecules attached to silica. As this concept has become better understood, new stationary phases have begun to be developed. For example, previous work has shown that cyclodextrins were attached to silica allowing enantiomeric separations. Cyclodextrins were used because of their ability to form host/guest complexes differently with different enantiomers.

4-t-Butylcalixarenes are known to form host/guest complexes in a manner similar to the cyclodextrins. 4-t-Butylcalixarenes were thus chosen for this work because they have the potential to form useful stationary phases. In addition, the structure of 4-t-butylcalixarenes is well known as are synthetic methods to modify them. Attaching modified 4-t-butylcalixarenes to silica would allow the stationary phase to interact with various solute molecules that would not interact with commercial phases or with unmodified 4-t-butylcalixarene phases.

In conclusion, the work presented here describes a method to attach 4-t-butylcalixarenes to silica allowing them to be used as stationary phases in HPLC. The potential that lies within this research is wide open and there are several examples of how these stationary phases can be beneficial, such as improving the separation of PAH's. The overall goal of this part of the work was to develop a method for synthesizing 4-t-butylcalixarene stationary phases and once developed, to characterize the material.

Chapter II

Introduction

High Performance Liquid Chromatography:

High performance liquid chromatography (HPLC) is a powerful separation technique that depends upon the interaction of two immiscible phases: a mobile phase and a stationary phase. The mobile phase is pumped at relatively high pressures through a column that is packed with small, uniform porous particles of the stationary phase. The sample components to be separated and analyzed are injected into the column and carried through by the mobile phase. While the sample is in the column, the individual components interact with both the mobile and stationary phases differently, causing them to separate from one another. The more a component interacts with (likes) the stationary phase, the longer it will take to elute off the column.

Similar to liquid-liquid chromatography (LLC), covalently-bonded stationary phase chromatography separates compounds on the basis of type and number of functional groups on the solute molecule.

Stationary phases in HPLC are typically made from either silica or alumina supports. The most widely used stationary phase material is porous microparticles of silica. These particles are very small, with a diameter ranging from 3-10 μm, and contain a large concentration of surface hydroxyl groups (1). Because these particles are porous, they have an extremely high surface area per unit weight, around 300 m^2/g.

The types of stationary phases in HPLC include both non-bonded and bonded phases. Non-bonded phases work through adsorption, which is a surface phenomenon and is more or less chemical in nature. Examples of non-bonded phases include untreated silica and alumina particles. Bonded phases work through absorption, which is a bulk phenomenon and is a physical process.

There are two basic types of bonded stationary phases. One is termed normal-phase and the other is reversed-phase. Normal-phase chromatography uses more polar stationary phases with less polar mobile phases. Samples that have strong polarity are easily separated with normal-phase chromatography. Examples of normal-phase chromatography stationary phases are cyano, amino, and hydroxyl. Reversed-phase chromatography uses less polar stationary phases along with more polar mobile phases to elute less polar solutes. Examples of reversed-phase stationary phases are hydrocarbons, such as C-8, C-18, and phenyl.

Retention and Peak Broadening:

Resolution and separation are controlled by the flow rate, temperature, pressure, and mobile phase composition. However, some samples still will not separate completely under the optimum conditions. Optimizing all conditions is the key to resolving all solutes in a mixture. Resolution is dependent upon factors governing the rate of migration of the solutes down a column and their simultaneous band broadening. While both are determined by thermodynamic and kinetic parameters, the important parameter differs under normal circumstances. The migration rate of the solutes are governed largely by

thermodynamics (equilibrium), while band broadening is controlled largely by kinetics (diffusion). Using the van Deemter equation the mathematical interpretation below is obtained:

$$h = A + B/u + Cu$$

Where u is the linear velocity of the mobile phase; A is the term related to flow path effects (eddy diffusion); B is the term related to simple, longitudinal diffusion; and C is the term related to the rate of solute mass transfer between the mobile and stationary phases. This equation successfully relates column efficiency (h) to the processes responsible for band broadening. It was first developed for the use with GC, but later was found to be useful for HPLC (1).

From this equation it can be seen that as the linear velocity increases, band broadening caused by longitudinal diffusion is decreased while broadening from mass transfer increases. It is also true that as flow rate decreases, broadening from longitudinal diffusion increases whereas band broadening from mass transfer decreases.

Peaks, under normal circumstances, should be narrow and symmetrical. The broader the peak, the less resolution is possible between solutes. In cases where the peaks are very broad and unsymmetrical, interaction between the stationary phase and the solute are extremely strong and more than one type of interaction may be occurring.

Chemically Bonded Stationary Phases:

In liquid chromatography, the most common stationary phases are those that have organic molecules chemically bonded to an inorganic support, such as silica. These stationary phases were developed in order to eliminate the disadvantages that occurred with LLC columns based on mechanically held stationary liquids. Chemically bonded stationary phases are much more stable than the LLC stationary phases, and have unique characteristics that allow separations to be performed with greater ease.

Since chemically bonded stationary phases are more stable than the stationary phases used in LLC, the stationary phase cannot be easily lost during use. This eliminates the need for precolumns and the presaturation of the two phases with the mechanically held stationary phase. There is a large variety of functional groups that allow both normal- and reversed-phase chromatography to be carried out easily on chemically bonded stationary phases.

The most important advancement in modern liquid chromatography (LC) has been the development of chemically bonded stationary phases. During the 1970's, LC was somewhat of an unfavorable technique because manufacturers were making chemically bonded phases with poor lot-to-lot reproducibility. The irreproducibility was a result of many factors, including (and most importantly) poor control of both the chemical and physical properties of the silica support and the bonding reactions. By realizing how important the silica starting material was, manufacturers were able to overcome these problems. Therefore, columns may differ in both retention and selectivity from company to company, but within

the same company, reproducible data is easily obtained. Such irreproducibility is one major drawback of chemically bonded phases. However, if the same company's stationary phase is used, the problem is virtually eliminated. Nonetheless, since this problem can be overcome, chemically bonded phases remain the recommended phases because of their flexibility. Since there are so many choices among chemically bonded stationary phases and mobile phases, almost any separation is possible.

Preparation of Chemically Bonded Phases:

Chemically bonded stationary phases are prepared by various syntheses with most methods using silica as the support compound. Silica is used because it is relatively stable and is easily reactive because there are many silanol groups on the surface. The silanol groups react with various molecules to form a wide variety of chemically bonded stationary phases.

There are three reactions that are commonly used in the synthesis of chemically bonded stationary phases. The first is the formation of silicate esters, which are among the first reported types of chemically bonded stationary phases (2). Silicate esters are prepared by the direct esterification of the silanol groups with alcohols. They are also prepared by first chlorinating the silica and then reacting it with an alcohol. These stationary phases are limited because they cannot be used with mobile phases that contain water or alcohol, which can hydrolyze or break the ester linkage.

The second type of reaction is the formation of silica-carbon and the silica-nitrogen linkages. These stationary phases are prepared by chlorinating

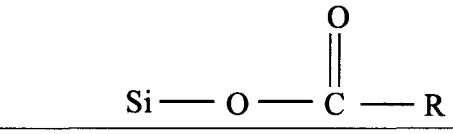
the silica and then reacting it with a Grignard reagent for the Si-C bonds (3,4) or amines for the Si-N bonds (5,6). The organic molecule is directly bonded to the silica in these types of reactions.

These types of stationary phases are more stable than the silicate esters, but still have problems. The drawback of the Si-C stationary phases is that the Grignard reaction is not convenient, and often leaves undesired residues on the surface of the silica. The drawback of the Si-N stationary phases is that they are only useful in the pH range of 4-8.

The third type of reaction, the formation of siloxanes, produces the most useful types of stationary phases. These stationary phases are prepared by reacting the silanol group of the silica with an organochlorosilane. These bonded phases are hydrolytically stable throughout the pH range of 2-8.5, which is why they are the most useful of the three. Table 2.1 illustrates the functional groups utilized in reactions that produce stationary phases.

Table 2.1

Functional Groups

Functional Group	Illustration
Silanol	Si — OH
Silicate Ester	
Siloxane	Si — O — Si — R

These stationary phases contain an organic bonded-phase coating that can be prepared as a monomolecular layer or as a polymerized multilayer

coating. The monomolecular organic layers are made by reacting the surface of the silica (the silanol groups) with a mono-, di-, or tri-functional silane. The stoichiometry of surface reactions with modifiers has been studied extensively and Figure 2.1 summarizes these results (7).

Stoichiometry predicts that a monofunctional silane will react in a one-to-one ratio. Experimental data supports this and that the ratio of the number of moles of silanol groups reacted to the number of moles of modifier reacted, F, is one. Data shows that for di- and trifunctional modifiers, the F values are between one and two. This data shows that by using di- and trifunctional reactants for surface modification there will be up to two unreacted Si-X (X = Cl, OH, OCH_3, or OC_2H_5) groups per functional group unreacted. When these products come into contact with water, the unreacted groups hydrolyze, causing additional silanol groups to form in about the same concentration as the bonded organic functional groups in the product. This occurs when the di- or trifunctional reactants have -OH groups attached to the ends of the molecule. Most of the reactions use these types of reactants.

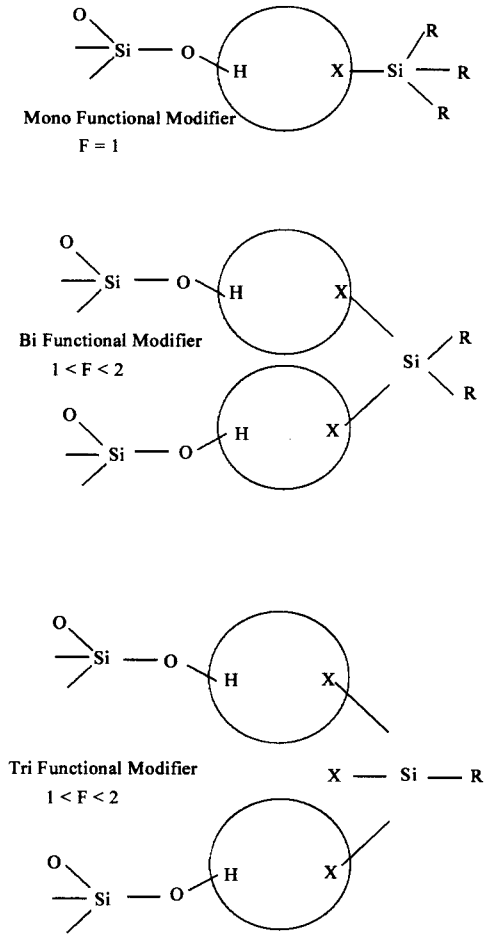


Figure 2.1

The formation of "new" Si-OH groups on the silica surface results in peak tailing and a low sample capacity. To get around these problems, two different techniques have been tried. One is to have a dense monolayer of functional groups instead of a partial coverage of the functional groups. The second is to use monofunctional dimethylsilanes in order to create a homogeneous organic coating with minimal residual Si-OH groups left on the surface (7,8).

Properties of Bonded Phases:

There are several ways to characterize chemically bonded stationary phases. One is by transmission infrared spectroscopy. Another is attenuated

total reflectance infrared spectroscopy (9). A third is by pyrolysis mass spectrometry (10). Cross polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) can also be used in the characterization process (11) Elemental analysis can be done, but the carbon content alone is insufficient in characterizing the stationary phase. A better method (than elemental analysis) in characterization of the stationary phase, is to calculate the surface concentration of the bonded groups, a_{exp} (7). The following is the calculation:

$$a_{\exp} = W/(M)S_{bet}$$

where W is the weight of the functional groups as grams per gram absorbent, M is molar weight of the bonded functional group (g/mmole), and S_{bet} is the specific surface area of the support, corrected for the weight increase due to the modification (m^2/g).

The maximum surface concentration is dependent upon the bulk volume and chain length of the bonded phase modifier. The determination of whether the desired amount of dense coverage of the organic functional groups has been accomplished is easily performed and calculated. For example, reversed-phase packing material can be tested as an adsorbent by using dry n-heptane as a mobile phase. Polar solutes will show symmetrical peaks and $k' << 0.5$ if a dense coverage has been achieved. In addition, if a capping reaction is performed using trimethylchlorosilane, the stationary phase should not increase in carbon content or change the chromatographic properties as stated in the previous test (12).

If a monomolecular layer is chemically attached to porous silica, the mean pore diameter is reduced by twice the thickness of the monolayer. Therefore, it is likely that the surface area and pore volume are also decreased. The longer the modified group chain length, the more noticeable the effect. However, the longer the alkyl chain length, the greater will be the retention of solutes, which indicates that the volume of the bonded phase, rather than surface area, is the determining factor in retention of chemically bonded phases, which is supporting evidence for the principle mode of partitioning is absorption rather than adsorption.

It has been shown that supports with relatively larger mean pore diameters make more efficient columns (13). It would be expected that supports with small pore sizes could be easily blocked by the bonded phase, creating less-rapid solute equilibration. Hence, column separating efficiency is affected by the bonded phase chain length.

Retention Mechanisms:

With the development of bonded-phase chromatography came an interest in a fundamental understanding of the retention mechanism in LC. The first rigorous attempt at relating LC retention to physical chemistry principles involved explaining reversed phase retention was explained using solvophobic theory. This theory attributed the retention process to the mobile phase, and completely ignored any contributions from the bonded stationary phase. An early attempt at describing retention mechanisms using the contributions of the stationary phases focused on the carbon content of the bonded phases (14).

There are many reasons as to why the exact chemistry of the retention process is being studied. One important reason is that until the retention process is clearly understood, attempts to develop expert systems for LC will only be empirically based. If these systems can be converted from an empirical basis to a theoretical basis, a rapid computer-based system for method development will result. This computer-based method system will greatly simplify what is now often a trial-and-error process.

More importantly, understanding the exact nature of the driving force for retention will result in other important developments. Firstly, understanding the driving force will allow the predictive development of unique stationary phase materials for improved separations. Secondly, by understanding the driving force, chromatography columns can be used for measuring physical parameters that are difficult to obtain using any other methods. Some of these goals have been obtained, giving promise that all the goals can eventually be attained (14).

The use of silica as the medium for chromatographic stationary phases is directly related to the observed interactions between solutes dissolved in the mobile phase and its surface active sites. There is also an indirect role that silica plays with the retention. This indirect role is due to the characteristics of bonded phases that are dependent upon the nature of the underlying silica support. The surface-active sites on silica are termed silanols (i.e., they are Si-OH groups). The silanols are the attachment points for the covalent silyl ether bonds that anchor bonded phases to the silica support. Due to steric hindrance, the density of silanols on chromatographic-grade silica is much greater than the maximum

possible concentration of alkyl groups in a bonded phase. Hence, after the silica has been modified, many unreacted silanols are left within the bonded phase. These residual silanols are weakly acidic and can therefore interact with polar compounds through strong hydrogen bonds and dipole-dipole interactions. This results in heterogeneous surfaces, which leads to mixed retention mechanisms, peak tailing, and loss of chromatographic resolution. Since residual silanols create problems for chromatographers, there have been and continue to be attempts to prepare bonded stationary phases with as few as possible active silanol sites (14).

One approach to preparing silica bonded phases with silanol-free silica is to pretreat the silica before any bonding reactions occur. One way of pretreating the silica is to selectively rehydroxylate the surface of the silica. Another pretreatment approach deals with the number of silanols on the silica surface and attempts to reduce the number of silanols by a deactivation process that then allows them all to be reacted during the attachment of the bonded phase. This can be done in one of two ways. One way is to selectively deactivate some of the silanols (15). For example, treating silica with trimethylchlorosilane will selectively deactivate the more reactive silanol groups (16). The other is to heat the silica to a temperature between 200 and 500°C.

One final problem with silica is the presence of trace metals in the silica gel matrix. Even chromatographic-grade silica contains a small (0.1-0.3%) amount of metallic impurities. These impurities increase the adsorptivity of silica

gels. A pretreatment with acid is generally used to get rid of the metal impurities (14).

As mentioned above, retention has traditionally been described in terms of hydrophobic driving forces, and the solvophobic theory is still used in the description of retention in reversed-phase chromatography. However, the solvophobic theory ignores the stationary phase, having no role in the separation process except providing a site for retention. Moreover, hydrophobicity plays a small role in the energetics of a typical reversed-phase partitioning process. Theoretical and experimental studies of the retention process have allowed for these conclusions and have resulted in improved separation processes (14).

Chemists now know that retention is governed by a partitioning process instead of by an adsorption process. Partitioning is when a solute particle becomes embedded between the chains of the stationary phase while adsorption is the specific interaction between a solute particle and the surface of the stationary phase. Partitioning is thus regulated by the chemical potential difference of the solute between the mobile and stationary phases much like solvent extraction, while adsorption depends upon specific types of electronic interaction between the solute and the stationary phase surface. Partitioning is thus a bulk phenomenon that works through absorption. A good way to visualize the difference between the two processes is to view absorption as a physical process and adsorption as a chemical process. The energetics of the solute in both phases are also important. The word hydrophobic is often referred to in describing the driving force for retention. However, the strict meaning of

hydrophobic implies a certain temperature dependence of solubility that is often not encountered with reversed-phase mobile phases.

There are two pieces of evidence that prove that stationary phases play an active role in the retention process. The first is that the retention characteristics of polyaromatic hydrocarbons varies widely from column to column (17,18). The second is the fact that partitioning and selectivity are both highly dependent on the chain density of the bonded hydrocarbon phase (19,20). Since solvophobic theory relies on an incorrect model of the relevant solution processes, it fails to account for these differences.

Another theory has been introduced to describe the retention process (21-23). This theory proposes that two driving forces dominate the retention process. The first is the difference in the free energy attributable to contact interactions of the solute with surrounding molecular neighbors in the stationary and mobile phases as measured by their binary interaction constants. The second governing principle is the partial ordering of the grafted stationary-phase chains, which at very high bonding density leads to an entropic expulsion of solute from the stationary phases relative to what would be expected from a simpler amorphous oil phase/water partitioning process (e.g., octanol/water).

In this theory, the stationary phase is viewed as having both order and an ordered gradient, with disorder increasing with increasing distance from the silica surface. This theory has been tested many times proving that the stationary phase is an ordered system and that if partitioning to occur between a solute particle and the stationary phase, a cavity must be present in the stationary

phase. This induces further chain ordering, making the partitioning process an entropically expensive one.

There are three major areas of research presently being done to study retention mechanisms in reversed-phase chromatography. They are chromatographic experiments, NMR spectroscopy, and solution thermodynamics. Chromatographic experiments have been the most used method for investigating retention mechanisms. These experiments can be done relatively easily and provide useful information only if careful controls are run and the conditions are accurately and carefully described. The spectroscopic experiments are more recent techniques used in determining retention mechanisms by probing the structure of the stationary phase. Information on the conformation of the bonded alkyl chains has been obtained using NMR spectroscopy, while optical experiments have looked at the environment of a sorbed probe molecule. Solution thermodynamic experiments have given unambiguous data that has shown that the predominate means of association of small molecules with the stationary phase is by a partitioning process.

These recent experiments have led to the conclusion that the solvophobic theory, which states that most of the free energy of retention comes from the mobile phase, is incorrect.

The chromatographic experiments are useful for chemical analysis as well as for studying the chromatographic process itself. These experiments range from simple retention versus mobile phase concentration studies to complicated

experiments done to examine the thermodynamics of the partitioning process. For example, through these types of experiments, the value of the partitioning coefficient of a solute from the mobile to the stationary phase is seen to be dependent on the chain density of the bonded alkyl groups, and it is further seen that the chain density affects the shape selectivity (19,20). In addition, and perhaps more importantly, these studies have also shown that the partition coefficient goes through a maximum as a function of chain density and that the predominant driving force changes from an enthalpic to an entropic mechanism as the chain density increases (14).

Most chromatographers use the carbon content of the stationary phases to describe the density of the stationary phase. However, when reported alone, uninterpretable data is obtained. The only useful information is the chain density on the surface, which is reported in units of $\mu mol/m^2$, because it takes into consideration both the carbon content and the surface area of the underivatized silica.

It has been found that van't Hoff analysis can be used to probe the thermodynamics of the partitioning process and to investigate possible phases transitions of the bonded, aligned alkyl chains. The van't Hoff equation for chromatography is

$$\ln k' = (\Delta H/RT) + (-\Delta S/R) + \ln \Phi$$

where k' is the capacity factor, ΔH and ΔS represent the enthalpy and entropy of transfer of the solute from the mobile to the stationary phase, R is the gas

constant, T is the absolute temperature, and Φ is the phase volume ratio (stationary/mobile). A plot of ln k' versus 1/T has a slope of $-\Delta H/R$, and an intercept that contains the entropy and the phase volume ratio. Results obtained from these experiments have shown that hydrophobicity is not the driving force for retention with most mixed aqueous-organic mobile phases (24,25). The results have also shown that the change in entropy during the transfer process increases with increasing chain density of the bonded alkyl groups. This too is evidence that points directly to a partitioning mechanism and has implications for the choice of stationary phases to model other partitioning processes.

The first spectroscopic experiments were done on stationary phases that were derivatized with groups that would be sensitive to analyze by spectroscopy. These experiments showed the power of spectroscopy for interpreting features on the stationary phases, but were not relevant to traditional reversed-phase materials. Therefore, recent developments have included spectroscopic probes that have been intercalated into traditional reversed-phase stationary phases and have provided useful information about the organization of the alkyl chains and the environment of sorbed solutes.

Other spectroscopic experiments have included small angle neutron scattering to measure the thickness of alkyl-bonded silica surfaces (26). These experiments showed that the average thickness for a monomeric C-18 phase is thinner than the fully extended length of octadecane, proving that the stationary

phases are bent, or distorted, resulting in a reduction of phase thickness compared to the extended conformation.

NMR spectroscopy is another tool that has been used to study the conformation of the alkyl chains. Using magnetic resonance imaging on a reversed-phase LC column, it has been found that it is possible to observe band profiles in the column, non invasively (27). Thermal effects within the column have thus been directly revealed.

More recent studies have shown a developed method for wetting the stationary phase under column pressures and then performing NMR experiments (28). This has permitted investigation of the alkyl chain solvation under realistic conditions. This has advanced the understanding of the interfacial region. Previous NMR studies have been done by wetting the stationary phase at ambient pressures. Since capillary action is dependent on pressure, it is likely that these phases did not have the same amount of solvent associated with the stationary phase chains as in the high pressure experiments.

Retention measurements done in chromatographic studies have provided information about the combined role of the mobile and stationary phases during separation. Solution measurements, however, have been completely the opposite. Solution measurements were made independently of the other phase, which allowed the effect of changes in a single-phase composition to be examined independently. Most of these experiments were done using solvatochromism, which is spectral shifts induced by solvent effects, and allows study of forces from both the mobile and stationary phases. This concept has

been reviewed for mechanistic chromatographic studies (29). In one study (30) headspace GC was used to measure gas-liquid partition coefficients. This study demonstrated that over the entire range of solvent composition, most of the free energy of retention in reversed-phase chromatography comes from attractive-dispersive interactions between the solute and the stationary phase, not from the net repulsive interactions in the mobile phase. This also provides further evidence for the partition mechanism and agrees with other studies.

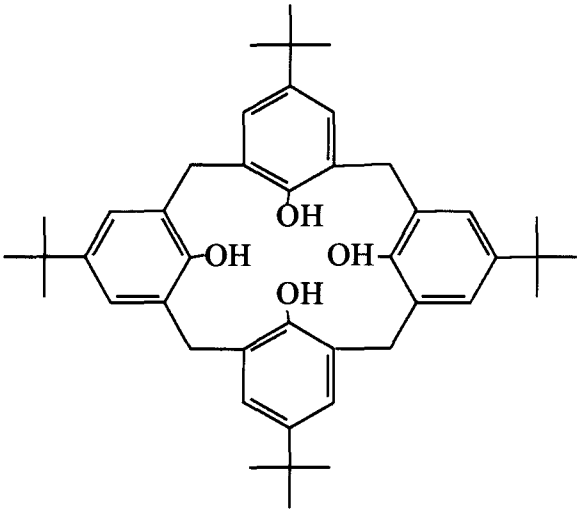
From the comparison of experimental and computed data, activity coefficients showed that regular solution theory is incorrect and should no longer be used for any quantitative predictions involving aqueous systems. It has clearly been found that the thermodynamics of solute transfer from the mobile to the stationary phase are much more complex than once thought.

In conclusion, it is important to mention that advances in reversed-phase LC methods are small but important steps to the understanding of retention mechanisms. Although there are still many questions that remain and improvements that are still necessary, recent studies have been mini-breakthroughs in the understanding of retention mechanisms. For example, stability of bonded phases is not yet ideal. In order to eliminate these problems, new approaches to the synthesis of bonded phases are now being attempted. As techniques develop, chromatography columns have the potential to become a physical chemistry "laboratory" because of their ability to determine physical and thermodynamic constants. There has already been some progress and many people continue working toward complete understanding of the driving force

behind retention mechanisms. The understanding of the structure of the bonded-phases will help lead us in the right direction and help establish further applications.

Calixarenes:

Calixarenes are macrocyclic molecules that contain phenolic units that are linked via methylene bridges in a position meta to the t-butyl groups.



Gutsche coined the term calixarenes because they resemble a chalice, which is the Greek word meaning calix. The suffix arene comes from the presence of aryl rings (31). They have a "basket-like" cavity that allows other compounds to interact with them, as long as they fit into the cavity. This cavity allows inclusion complexes to form host/guest interactions.

The easiest, and most useful way to prepare 4-t-butylcalix [4],[6], and [8]arenes is to condense formaldehyde onto p-substituted phenols in the presence of a base (32). Modifications can be made on the calixarenes using various reactions.

The molecular weights of calixarenes are generally around 1000 a.m.u. and they generally have high melting point temperatures. For example, the smallest common substituted calixarene, 4-t-butylcalix[4]arene has a melting point of 344-346°C.

Calixarenes can be water soluble, depending on the -R groups attached. However, most calixarenes are not soluble in water even though the phenolic functions are free and not substituted. 4-t-Butylcalixarenes are soluble in organic solvents. Calixarenes tend to be stable and non toxic (33).

Calixarenes can be chemically modified by reacting any of the three reactive sites on the molecule. These sites include the hydroxyl groups, the aromatic rings, and the methylene bridges.

The phenolic units that make up calixarenes bond together with methylene bridges to form a cavity. Since a cavity exists, inclusion complexes can form between the cavity of the calixarene and a substrate molecule. The exact properties, such as size of the cavity, depends on the number of phenyl rings and the functional groups attached to the calixarene. Inclusion complexes have been found with neutral molecules and metallic cations in both the liquid and solid states (32).

Chapter III

Historical

Chemically Bonded Stationary Phases:

Chemically bonded stationary phases have been synthesized for many years. The first stationary phases with organic compounds permanently attached to silica were introduced to chromatography in 1969 (34). They were originally synthesized (and are still today) because they are significantly more stable and can handle a wider range of variables (i.e., pressure, solvents, pH, etc.) than non-bonded stationary phases. Another important feature of chemically bonded stationary phases is that they have high separation selectivities.

As early as 1973, Majors and coworkers worked on the development of chemically bonded stationary phases. Their work demonstrated the advantages of stable siloxane phases chemically bonded to silica gel particles that had diameters less than 10μm. The small diameter gave both high efficiency and sufficient coverage for high sample capacity (35).

Previous reaction schemes to synthesize chemically bonded stationary phases have been discussed in the literature. One reaction scheme developed by Gilpin and coworkers was a direct reaction, which consisted of bonding an organochlorosilane directly to the silica under anhydrous conditions (36). Direct polymerization was developed by Abel and coworkers. This consisted of the intentional addition of water vapor to the silica, dry toluene, and organosilane

synthetic mixture. This procedure caused the organosilanes with two or three reactive bonds to polymerize (37).

A third reaction scheme, developed by Aue and coworkers, is a multiple step process. Step one is silyation under anhydrous conditions followed by the conversion of the remaining Si-Cl or Si-O-R to Si-OH by the addition of water vapor. Finally, a second silyation reaction is performed (38).

Majors and coworkers (39) used the previously published methods in order to find the optimum and most reproducible technique for bonding stable siloxane phases to silica. The one step reaction between the organosilane and the silica without intentionally causing polymerization was found to be the best method. They also determined that it was possible to use silanes with both polar and non-polar functional groups to achieve successful attachment. Bonded phase coverage for high surface area silica was dependent upon the type of organosilane and varied between 7 and 17 weight percent. Majors also noted that residual silanols remained even after many treatments were attempted in order to eliminate them (39).

With the emergence of chemically bonded stationary phases and HPLC came the need for more ideal stationary phases. The major problem with the existing methods for synthesizing chemically bonded stationary phases was the fact that too many residual hydroxyl groups remained accessible to solutes, causing mix-mode retention and significant band broadening. In 1980, experiments that decreased the residual hydroxyl group concentration without lowering the concentration of the bonded organic molecules were developed.

Drying the silica before any chemical modifications resulted in a decrease in the residual silanol concentration without decreasing the carbon content of the stationary phase (40).

Another approach to improving chemically bonded stationary phases was to allow for a more uniform coverage of the bonded material. Sandoval and coworkers (41) created an olefin hydrosilation method that provided a suitable method for synthesizing silica based bonded stationary phases. This method improved the hydrolytic stability of stationary phases. This method allowed the hydrolytically advantageous direct formation of Si-C linkages to occur without the difficulties that arise when using the sequential reaction of a chlorinating reagent and an alkylating reagent.

Further work enabled Sandoval and coworkers (42) to develop a synthetic route to improve their method of synthesizing chemically bonded stationary phases. This work involved silanating the silica with triethoxysilane, which provided a simpler method to prepare a hydride modified silica substrate. This method improved the silane coverage as well as eliminated the strictly dry conditions required by the chlorination method.

The number of published methods for synthesizing new chemically bonded stationary phases is a continuing endeavor. This is due to the ever-increasing need for highly selective stationary phases in HPLC. Other published material consists of specific compounds that have been successfully bonded to silica to form stationary phases. These published methods are thus modified versions of what is already available, with the addition of a specific compound. A

number of review articles on chemically bonded stationary phases are available in the literature. For example, Locke published a comprehensive review of advances in bonded phases for LC in the journal of Chromatographic Science in 1973 (43). A more recent review was done by Nawrocki (44).

Calixarenes:

Calixarenes are relatively old compounds but have been popular for a relatively short time. Adolph von Baeyer carried out syntheses in 1872 by reacting phenols with aldehydes. His products, however, went virtually uncharacterized until the 1940's. Upon characterization, they were found to be calixarenes, (45) and have since gained popularity. This is because they are readily accessible, and development of calixarenes is similar to that of crown ethers and cyclodextrins (46).

In the 1940's Zinke and Ziegler performed a base-induced reaction using p-substituted phenols and formaldehyde (47) while Niederl and Vogel performed an acid-catalyzed synthesis with resorcinol and formaldehyde (48). More recently, Gutsche and Högberg have provided more information about both base- and acid-induced syntheses. Their work has provided useful methods for synthesizing calixarenes (49-52).

Calixarenes in HPLC:

Up to this point, Glennon and coworkers have been the only group working on development of calixarenes for use as stationary phases in HPLC. Others, such as Mangia and coworkers, are working on calixarenes for use in

gas chromatography (53) and Park is using calixarenes as mobile phase additives (54).

Using immobilization techniques Glennon and coworkers attached calixarene esters to silica. The work was carried out by the immobilization of tetrameric and hexameric calixarene ethyl esters using the triethoxysilane derivatives of p-allylcalix[n]arene ethyl esters. The purpose was to attach the calixarene esters to silica for the use of separating alkali metal ions by HPLC (55). Further studies included baseline resolution of a standard test mixture and chromatographic studies of the separation of amino acid esters. The standard test mixture contained benzamide, benzophenone, and biphenyl and used methanol/water mixtures as the mobile phases. The results of the amino acid ester separation appeared to be that retention was related to the hydrophobicity of the esters studied (56).

The Glennon group performed more experiments, this time with an emphasis on the synthetic method to attach the calixarene esters to the silica support rather than applications of the prepared phases. Two methods were tried. The first was to react triethoxysilylcalix[4]arene with silica to produce a bonded calix[4]arene tetraamide phase. The second method was a hydrosilation reaction using p-allylcalix[6]arene hexaester with hydride-derivatized silica to yield a calix[6]arene hexaester stationary phase (11). Using a prepared calix[4]arene tetradiethylamide stationary phase, the chromatographic behavior of alkali and alkaline earth metals was reported. Sodium ion was found to possess selective retention over other alkali metal ions and Ca^{2+} over Mg^{2+} ions

using water as the mobile phase with conductivity detection. In a study done with a series of amino acid ester hydrochlorides, it was found that they retained in order of their hydrophobicity on a silica bonded calix[4]arene tetraester phase (57).

Chapter IV

Materials and Methods

Mobile phase solvents were HPLC grade (Fisher Scientific, Pittsburgh, PA). All reagents were of HPLC grade or of the highest grade available. 4-t-Butylcalix[6]arene (95%), napthalene, anthracene, 4-t-butylcalix[4]arene, 1,7-dichlorooctamethyltetrasiloxane (95%), 1,2-dichlorotetramethyldisilane (95%), phenol, and N,N-diethyl-m-toluamide were obtained from Aldrich chemical company (Milwaukee,WI). Uracil was obtained through Nutritional Biochemicals Corporation (Cleveland, OH). The toluene was purchased from Fisher Scientific (Pittsburgh, PA.) Phenol was obtained from Mallinckrodt Chemical Works (St. Louis, MO.). Stationary phases were developed from Adsorbosphere silica (5μm particle size, 80Å pore size) and Machery-Nagel Nucleosil silica (7μm particle size, 1000Å pore size) (Alltech Associates, Inc. Deerfield, IL). Machery-Nagel packing material (C-18, 5μm particle size) was also purchased through Alltech Associates, Inc. (Deerfield, IL). Methyl-, ethyl-, propyl-, and butyl-benzenes were obtained from Alltech Associates (Deerfield, IL). HPLC grade acetone was used for column packing (Fisher Scientific). Benzene was obtained from J. T. Baker Chemical Company (Phillipsburg, N. J.). The methylene chloride used for the fluorescence experiments was spectrophotometric grade (Burdick & Jackson Laboratories Inc. Muskegon, MI). Deionized water was obtained from an in-house deionizer.

Six columns were synthesized and used in this project. A seventh column, C-18, was also used for comparison. Table 4.1 summarizes the different columns synthesized. Three different mixtures were separated on the columns. They were reversed phase test mixture, alkyl benzene homologous series, and phenyl ring homologous series. The reversed phase test mixture contained uracil, phenol, N,N-diethyl-m-toluamide, and toluene. Methyl-, ethyl-, propyl-, and butylbenzenes made up the benzene homologous series. The phenyl ring homologous series contained benzene, napthalene, and anthracene.

Table 4.1

Columns Synthesized

Column I.D.	Tether	Calixarene	Silica
001A1	Long	4-t-butylcalix[6]arene	80 Å
002A1	Long	4-t-butylcalix[6]arene	80 Å
001B1	Short	4-t-butylcalix[6]arene	80 Å
002B1	Short	4-t-butylcalix[6]arene	80 Å
001C1	Short	4-t-butylcalix[4]arene	80 Å
001D1	Short	4-t-butylcalix[6]arene	1000 Å

The long tether refers to the 1,7-dichlorooctamethyltetrasiloxane because it has seven molecules between the reactive Cl groups. The short tether refers to 1,2-dichlorotetramethyldisilane because it only has two molecules between the reacting Cl molecules.

Sample Preparation for HPLC:

Reversed Phase test mixture:

In a 100 mL volumetric flask, 0.5 g of uracil, 400 mg of toluene, 700 mg of phenol, and 100 mg of N,N-diethyl-m-toluamide were added along with 30 mL

of a 65% acetonitrile-water solution. After dissolution, the mixture was diluted to the mark with the acetonitrile/water solution. Individual components of reversed phase test mix were prepared in a similar manner, only in individual flasks.

Alkyl benzene homologous series:

In a 100 mL volumetric flask, 10 mL each of methyl, ethyl, propyl, and butyl benzene were added. The mixture was diluted to the mark with methanol. Individual components of the benzene homologous series were made the same way as in the mixture, only in individual flasks.

Phenyl ring homologous series:

In a 100 mL volumetric flask, 10 mg of benzene, 10 mg of napthalene, and 2.5 mg of anthracene were diluted to the mark with methanol. Individual components of the phenyl ring homologous series were made the same way as in the mixture, only in individual flasks.

Synthesis of Stationary Phase Procedure:

The following reaction scheme (Figure 4.1) is a general scheme used for all the reactions. The reaction scheme remained the same throughout, only the chemicals changed.

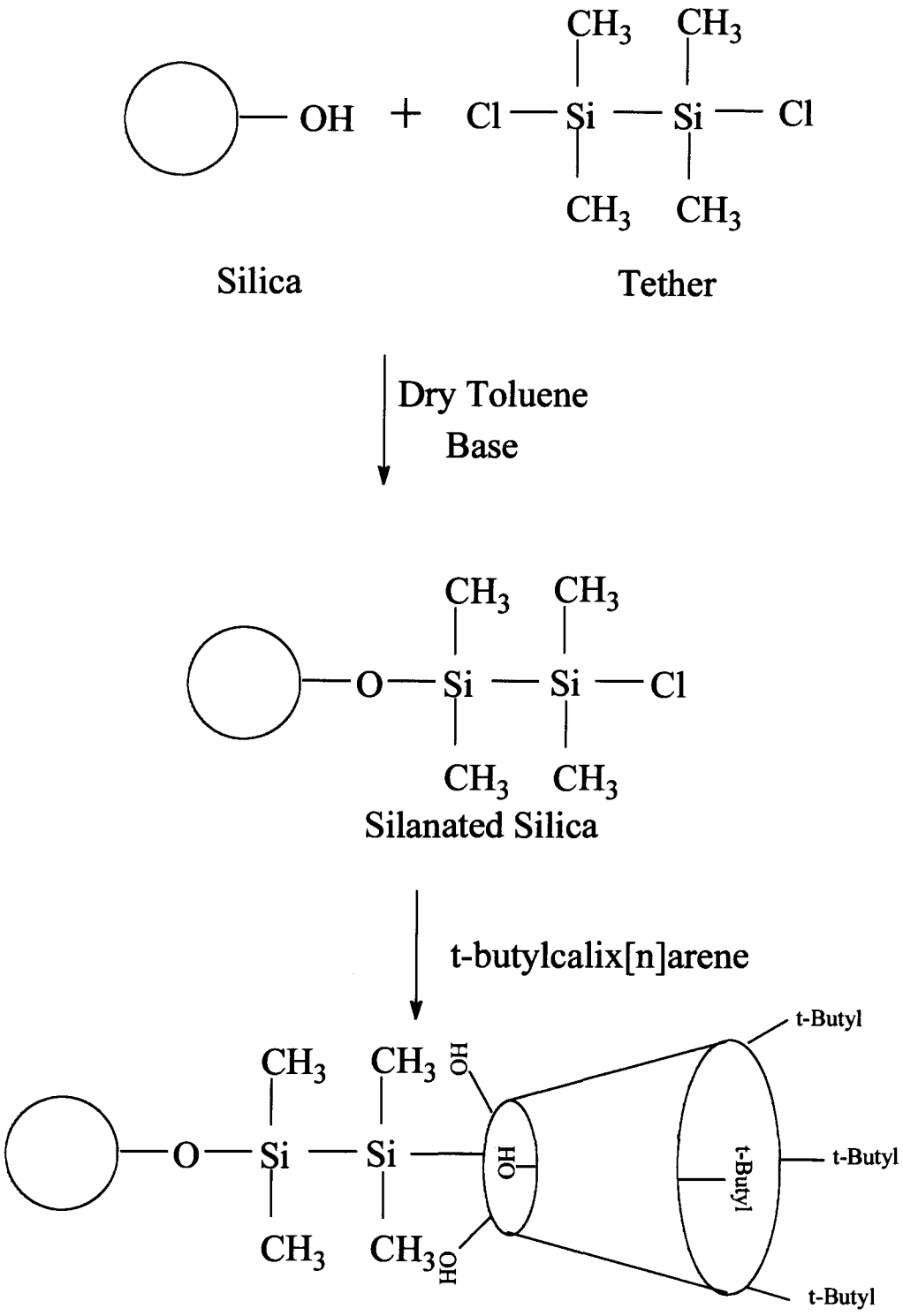


Figure 4.1: Reaction Scheme

Chromatographic grade silica was acid-washed in 0.10 M HCl for 18 hours at 95°C. After acid washing, the silica was filtered using a 0.45 μm pore nylon filter and dried under vacuum to ensure maximum reactivity of the hydroxyl groups. The cleaned and dried silica (3.5 g) was refluxed overnight (18-24 hours) in dry toluene with the tether (2 mL) under anhydrous conditions. After the silanizing reaction was complete, 5.0 g of 4-t-butylcalixarene was dissolved in about 300 mL of dry toluene and added to the silanated silica, also under anhydrous conditions, and refluxed overnight to form the 4-t-butylcalix[n]arene derivatized silica. The product (4-t-butylcalixarene derivatized silica) was suction filtered and rinsed sequentially with 300 mL of toluene, 300 mL of methylene chloride, and 500 mL of methanol to ensure only covalently bound and not adsorbed 4-t-butylcalixarene would be on the surface of the silica. The material was then dried 110°C for 24 hours before packing into a column. The only problem encountered with this method was when 4-t-butylcalix[4]arene was used. The stationary phase would not filter well because the 4-t-butylcalix[4]arene that was unbound would not dissolve. Hence, the filter clogged allowing very little to pass through. Therefore, hot toluene was used to rinse the stationary phase. The following Table (Table 4.2) illustrates the exact materials used in each reaction.

Table 4.2

Materials Used in Synthesis

Method	Silica	Calix[n]arene	Tether
A	Adsorbosphere	4-t-butylcalix[6]arene	1,7-dichlorooctamethyltetrasiloxane
B	Adsorbosphere	4-t-butylcalix[6]arene	1,2-diclorotetramethyldisilane
C	Adsorbosphere	4-t-butylcalix[4]arene	1,2-diclorotetramethyldisilane
D	Nucleosil®	4-t-butylcalix[6]arene	1,2-diclorotetramethyldisilane

Both methods A and B were performed twice to examine reproducibility.

While synthesizing the stationary phases, 3 g of silica was used. This amount was chosen because approximately 2.5 g of stationary phase is required to pack a 15 cm x 4 mm column. This would allow for enough material to pack into the column with additional material for spectroscopic characterizations. After the 3 g of silica was chosen the amount of silane and 4-t-butylcalixarene had to be calculated.

The surface area of the silica was approximately 300 m^2/g. Fully hydrolyzed silica (i.e., acid washed and dried at 200°C) contained approximately 8×10^{-6} moles of Si-OH/m^2. According to Snyder (12), due to steric effects, there were at best 4.5×10^{-6} moles/m^2 of Si-OH available for reaction with the dichlorosilane. For 3 grams of silica this meant that 0.00405 moles of the tether would be required for a 1:1 stoichiometric ratio. For the short tether, 0.75 g and for the long tether, 1.3 g would be required. To ensure an excess of silane, 2.0 g of each was used, assuming a density of 1 g/mL.

The amount of calixarene was chosen based upon an approximate 1:1 stoichiometric ratio of calixarene to available reactive silane, or 0.00405 moles. Assuming an average molecular weight of 1000 g/mol, 5 g of material (0.005 moles) was used.

Column Packing:

Using an air-driven slurry column packer (Alltech Associates, Deerfield, IL), the stationary phases were packed into 4.6 mm x 15 cm type 316 stainless steel HPLC columns. Additional hardware included: column end fittings, frits, ferrules, and solvent spreaders (Alltech Associates, Inc. Deerfield,IL). Slurries were prepared by placing 2.3 g stationary phase into 15 mL acetone and mixing thoroughly. The empty column was placed onto the slurry chamber and pre-filled with methanol. The slurry was placed as quickly as possible after mixing into the slurry chamber, the chamber was topped off with acetone and the columns were packed for 20 minutes at 7000 psi using methanol as a solvent. After 20 minutes, the air pressure was turned off and the column was allowed to "relax" overnight before removing it from the packer and tightening the end fitting.

High Performance Liquid Chromatography:

Chromatographic experiments were performed using a Beckman Instruments System Gold Liquid Chromatograph. It consisted of two Model 110B pumps, a Model 168 UV/VIS diode array detector, and an Altex Model 210A injector with a 20 μL loop. Injections were made using the full loop. Data

was collected using System Gold Software Version 5.10. The mobile phases consisted of acetonitrile/water and methanol/water at various concentrations with a flow rate of 1.0 mL/min and detection at 254 nm, or as noted in the chromatograms.

Reversed phase test mixture was run on each column (C-18, t-butylcalix[4]arene, 4-t-butylcalix[6]arene long silane, 4-t-butylcalix[6]arene short silane, and 4-t-butylcalix[6]arene short silane 1000Å silica). These were run for various reasons but most importantly to see reversed phase behavior, and for comparison later to see if the columns underwent degradation. Test mixture was separated using 65/35 acetonitrile/water as the mobile phase.

Reversed phase test mixture was run on the 4-t-butylcalixarene columns using various mobile phases (acetonitrile/water and methanol/water) until desired retention was obtained. The C-18 column was then run using the same mobile phase for comparison. The C-18 column was also used to see which mobile phase was required to obtain similar retention as on the 4-t-butylcalixarene columns.

The alkyl benzene homologous series was separated on all of the columns. This was done by injecting the sample onto the 4-t-butylcalixarene columns with a mobile phase of 45/55 acetonitrile/water and 55/45 methanol/water. The same homologous series was separated on the C-18 column using mobile phases of 60/40 acetonitrile/water and 70/30 methanol/water.

The phenyl ring homologous series was separated on the 4-t-butylcalixarene columns with mobile phases of 40/60 acetonitrile/water and 55/45 methanol/water. Mobile phases of 60/40 acetonitrile/water and 60/40 methanol/water were used to separate this mixture on the C-18 column.

Temperature studies were done on the 4-t-butylcalix[4]arene column. Reversed phase test mixture, alkyl benzene homologous series, and the phenyl ring homologous series were all separated under temperature control. The reversed phase test mixture was separated using 65/35 acetonitrile/water as the mobile phase and temperatures of 40, 50, and 60°C.

The alkyl benzene homologous series was separated using 30/70 acetonitrile/water as the mobile phase at temperatures ranging from 35-65°C at 10°C increments.

The phenyl ring homologous series was separated using a 40/60 acetonitrile/water mobile phase at the following temperatures: 0°C , 35°C , 45°C , 55°C , and 65°C.

A Knox test was performed using dry heptane as the mobile phase and methanol dissolved in hexane as the solute. The Knox test required a dry non-polar mobile phase and a polar solute. If the solute was unretained, no residual silanols are present on the stationary phase. This test was performed on all of the 4-t-butylcalixarene columns.

Fluorescence Measurements:

Fluorescence experiments were performed using a Shimadzu Instruments, Model RF 5000U scanning spectrofluorophotometer. The

excitation and emission monochromators were each set to 1.5 nm slit width. Sensitivity was set to high and the scan speed was medium. The excitation wavelength was experimentally determined to be 282 nm. Samples were prepared by suspending an accurately weight amount of the material to be examined in spectral grade methylene chloride. As a reference, an accurately weighed amount of 4-t-butylcalix[6]arene and 4-t-butylcalix[4]arene were dissolved in spectral grade methylene chloride. Table 4.3 shows a summary of the sample preparation

TABLE 4.3

Fluorescence Sample Preparation

Tube Number	Tube Contents	Mass (mg)	CH_2Cl_2 (mL)
1	80Å Adsorbosphere Silica	25	5
2	80Å Adsorbosphere Silica + 4-t-butylCalix [6]arene	25+2	5
3	4-t-butylCalix[6]arene	2	5
4	4-t-butylCalix[6]arene/80Å Adsorbosphere Conjugate	25	5
5	4-t-butylCalix[4]arene	25	5
6	4-t-butylCalix[4]arene/80Å Adsorbosphere Conjugate	25	5
7	4-t-butylCalix[6]arene/1000Å Nucleosil® Conjugate	25	5

Organic Content of the Stationary Phase:

The surface coverage of both of the 4-t-butylcalix[6]arene, 4-t-butylcalix[4]arene stationary phases was determined using the heating and weighing method of Verzele and co-workers (58). They showed that there was a very close correlation between the percentage of bonded material as determined using thermogravimetry and the percentage of bonded material determined using the heat and weigh method. The method was demonstrated for several

types of stationary phases, with hydrocarbon (octadecyl) phases showing the best correlation. The octadecyl (C-18) column was also used for comparison reasons.

In this method, the material was first accurately weighed and then heated at 110°C for 2 hours to drive off water and any solvents. The material was then reweighed, followed by heating at 600°C for 1 hour to remove all bonded organic material. The material was weighed a final time and the percentage organic content was calculated from the loss of weight between the 110°C heating and the 600°C heating. The percent organic was obtained from the following equation:

$$\frac{\text{Weight of material after heating to 600 - Weight of material after heating to 110}}{\text{Weight of material after heating to 110}} \times 100\,\%$$

Chapter V

Results and Discussion

Chromatographic separations were performed on three different mixtures of solutes using the various types of 4-t-butylcalix[n]arene stationary phases and a high quality C-18 phase. The solutes chosen were a standard type of reversed phase test mixture, a homologous series of alkyl benzenes, and a series of compounds that was homologous with respect to the number of phenyl rings. Each mixture of solutes was specifically chosen to probe how the stationary phases behaved with regard to polarity, the coverage of the surface with 4-t-butylcalix[n]arene, and to determine if host/guest complexes were formed between some or all of the solutes and the bound 4-t-butylcalix[n]arene.

Reversed Phase Test Mixture:

The reversed phase test mixture was the same mixture that had been used in the laboratory for characterization of bonded alkyl reversed phase columns after in-house packing. The mixture consisted of uracil, phenol, N,N-diethyl-m-toluamide, and toluene and was separated for several purposes. First it was used to determine the dead time of each stationary phase column since uracil was unretained when using acetonitrile/water mobile phases. As a group, the other three components (i.e., phenol, N,N-diethyl-m-toluamide, and toluene) were used to help verify that the columns were operating in a reversed phase mode. If they all demonstrated significant retention when using relatively polar mobile phase mixtures then the stationary phases could be deemed relatively

non-polar and were showing reversed phase behavior. If all of the solutes were largely unretained and eluted at or near the dead time, it could be deduced that stationary phases in the columns were relatively polar and were showing normal phase behavior.

For each stationary phase tested using acetonitrile/water mobile phases, uracil eluted at essentially the same time every time and was thus confirmed as a dead time marker. Using the same mobile phases, the rest of the mixture was retained to various extents with the less polar components eluting after the more polar component. When using methanol/water mobile phases, the same type of behavior was observed, with the exception of uracil, which appeared to also retain on the 4-t-butylcalix[n]arene stationary phases. The stationary phases were thus confirmed to behave as relatively non-polar, reversed-phase type materials.

When considered individually, the components of the reversed phase test mixture (other than uracil) also probed different general characteristics for each stationary phase tested. Retention behavior, peak width, and peak asymmetry were examined to help assess these characteristics. N,N-Diethyl-m-toluamide, as a weak base, was used to examine proton donor (acidic) characteristics while phenol, as a weak acid, was used to examine proton acceptor (basic) characteristics. Toluene, as a hydrocarbon, was expected to show only non-polar interactions and could thus be used to reliably determine the efficiency of the column through calculation of the number of theoretical plates. Assuming no

other interactions with the stationary phase, in a perfectly packed column, the peak shape of toluene should be perfect with an asymmetry factor equal to one.

The asymmetry factors for phenol and N,N-diethyl-m-toluamide as compared to the toluene peak shape, were indicative of the acidic and basic character of the column. The presence of significantly fronting (i.e., asymmetry factor < 1) or tailing (i.e., asymmetry factor > 1) peaks was considered to be indicative of such interactions, since more than one type of strong interaction significantly alters the shape of the solute absorption isotherm between the stationary and mobile phases. The peak asymmetry factors calculated from the test chromatograms are shown in Tables 5.1-5.6.

Table 5.1 shows the asymmetry factors for toluene separated on all seven columns using acetonitrile/water mobile phases. From this table, it can be seen that all of the columns, excluding 001D1, separate toluene with an asymmetry factor of approximately one. The separation of toluene using the 001D1 column demonstrated a factor very much greater than one, indicating that the peak has significant tailing. This difference was attributed to the difference in the pore size of the silica support materials. Acetonitrile has a relatively high binding affinity with 4-t-butylcalixarenes, and therefore strongly competes with the solute molecules for sites on the stationary phases. Since 001D1 had larger pores, more 4-t-butylcalixarenes were available to bind. In addition, the significant tailing meant that other significant interactions besides London interactions were occurring, thus implicating host/guest formation.

Table 5.1

Toluene

Reversed phase test mixture using acetonitrile/water mobile phases

Column	Asymmetry Factor
001A1	0.99
002A1	1.15
001B1	1.01
002B1	1.11
001C1	0.89
001D1	1.82
C-18	1.31

Table 5.2

N,N-Diethyl-m-Toluamide

Reversed phase test mixture using acetonitrile/water mobile phases

Column	Asymmetry Factor
001A1	2.00
002A1	1.80
001B1	2.23
002B1	2.26
001C1	1.13
001D1	2.01
C-18	1.07

Table 5.3

Phenol

Reversed phase test mixture using acetonitrile/water mobile phases

Column	Asymmetry Factor
001A1	1.62
002A1	1.86
001B1	1.43
002B1	2.24
001C1	1.02
001D1	1.31
C-18	1.06

Table 5.4

Toluene

Reversed phase test mixture using methanol/water mobile phases

Column	Asymmetry Factor
001A1	2.00
002A1	2.61
001B1	2.06
002B1	3.60
001C1	2.37
001D1	1.42
C-18	1.44

Table 5.5

N,N-Diethyl-m-Toluamide

Reversed phase test mixture using methanol/water mobile phases

Column	Asymmetry Factor
001A1	1.13
002A1	1.51
001B1	3.33
002B1	2.74
001C1	2.28
001D1	4.98
C-18	1.08

Table 5.6

Phenol

Reversed phase test mixture using methanol/water mobile phases

Column	Asymmetry Factor
001A1	1.52
002A1	1.40
001B1	0.968
002B1	1.28
001C1	0.966
001D1	1.05
C-18	0.718

The asymmetry factors for N,N-diethyl-m-toluamide separated using acetonitrile/water mobile phases are listed in Table 5.2. From this table, it can be seen that all the columns except 001C1 and C-18 had asymmetry factors much greater than one. Again, this indicated peak tailing that in turn indicated significant amount of mixed-mode retention occurred with the phenolic hydroxyls. Since the 001C1 column was the only column synthesized using 4-t-butylcalix[4]arene, it apparently had less phenolic hydroxyls available to the solute molecule. Because they were unavailable, no mixed-mode retention occurred. It may have been sterically impossible for the N,N-diethyl-m-toluamide to fit into the smaller cavity, or it may have been that all of the available hydroxyls were bound to the silica surface.

Asymmetry factors for phenol separated using acetonitrile/water are outlined in Table 5.3. All six 4-t-butylcalix[6]arene columns (again, all columns except 001C1 and C-18) showed significant peak tailing, and thus had asymmetry factors greater than one. It was likely that these columns had π-π phenolic interaction (i.e., host/guest formation) and the unavailability of a suitable complexation site on the C-18 and 4-t-butylcalix[4]arene resulted in non-skewed peaks on these columns.

As seen from Tables 5.4-5.6, the asymmetry factors for methanol/water mobile phases were significantly different than those seen with acetonitrile/water mobile phases, but were consistent with the competitive behavior of the binding between the solute, the solvent, and the bound4-t-butylcalixarene, as outlined above.

Table 5.4 shows the asymmetry factors for toluene using methanol/water mobile phases. Their greater magnitude was attributed to the fact that methanol did not compete as strongly with toluene as acetonitrile to bind onto the 4-t-butylcalixarene sites. In other words, the greater host-guest formation was due to the difference in binding strength between methanol and calixarene (weaker) and acetonitrile and calixarene (stronger).

Table 5.5 shows the asymmetry factors for N,N-diethyl-m-toluamide using methanol/water mobile phases. The only significant difference observed here was the fact that the 001D1 column demonstrated a very strong affinity for the solute and had an asymmetry factor much greater than one. In each case, the N,N-diethyl-m-toluamide showed strong interactions with the acidic phenolic functionality, but apparently the larger pore size in the 001D1 column allowed for more interaction to occur between the solute and the stationary phase.

Table 5.6 shows the asymmetry factors for phenol with methanol water mobile phases. There appears to be little interaction in addition to the London forces acting between the phenol and the stationary phases. The asymmetry factors hover around one for each column.

Tables 5.7 and 5.8 show the determination of plate number for each column using toluene as a reference. These figures mirror the observations made with asymmetry factor. As the host-guest interactions between the toluene and bound 4-t-butylcalixarene get larger, the number of plates on the column decrease significantly. This was due to the increased amount band broadening within the column associated with the mixed mode retention of toluene.

Table 5.7

Number of theoretical plates

Reversed Phase Test Mixture Using Acetonitrile/Water

Column	Theoretical Plates (n)
001A1	957
002A1	838
001B1	1016
002B1	1066
001C1	909
001D1	723
C-18	1426

Table 5.8

Number of Theoretical Plates

Reversed Phase Test Mixture Using Methanol/Water

Column	Theoretical Plates (n)
001A1	958
002A1	833
001B1	1017
002B1	1066
001C1	909
001D1	5
C-18	1426

Also significant in terms of looking at the reversed phase test mixture was the order of elution for the compounds. All of the stationary phases, with the exception of the 1000Å Nucleosil®/4-t-butylcalix[6]arene phase (001D1) showed identical behavior. Using C-18 as a reference, the elution orders given in Tables 5.9 and 5.10 can be seen to be the same for acetonitrile/water mobile phases with the exception of 001D1, where the toluene and N,N-diethyl-m-toluamide can be seen to reverse order. With methanol/water mobile phases, all of the 4-t-

butylcalixarene stationary phases showed a reversal of order for toluene and N,N-diethyl-m-toluamide. In Tables 5.9 and 5.10, the numbers represent the elution order of the compounds on the C-18 reference phase.

Table 5.9

Elution Order for Acetonitrile/Water Mobile Phases

	Solutes			
Stationary Phase	**Uracil**	**Phenol**	**N,N-diethyl-m-toluamide**	**Toluene**
C-18	1	2	3	4
001A1	1	2	3	4
002A1	1	2	3	4
001B1	1	2	3	4
002B1	1	2	3	4
001C1	1	2	3	4
001D1	1	2	4	3

Table 5.10

Elution Order for Methanol/Water Mobile Phases

	Solutes			
Stationary Phase	**Uracil**	**Phenol**	**N,N-diethyl-m-toluamide**	**Toluene**
C-18	1	2	3	4
001A1	1	2	4	3
002A1	1	2	4	3
001B1	1	2	4	3
002B1	1	2	4	3
001C1	1	2	4	3
001D1	1	2	4	3

Figures 5.1–5.4 are the chromatograms of reversed phase test mixture performed on each column.

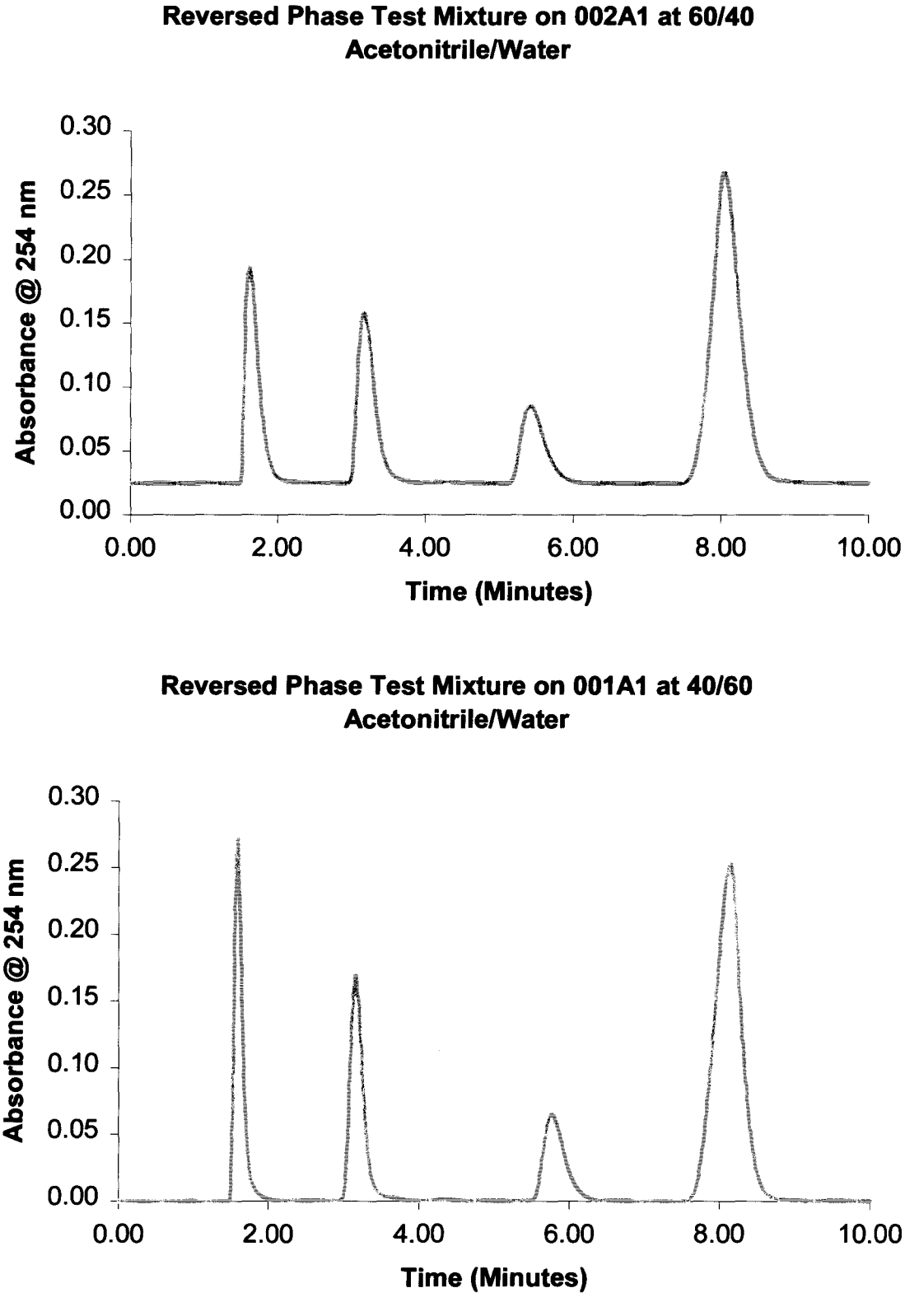


Figure 5.1: Chromatograms on 001A1 and 002A1

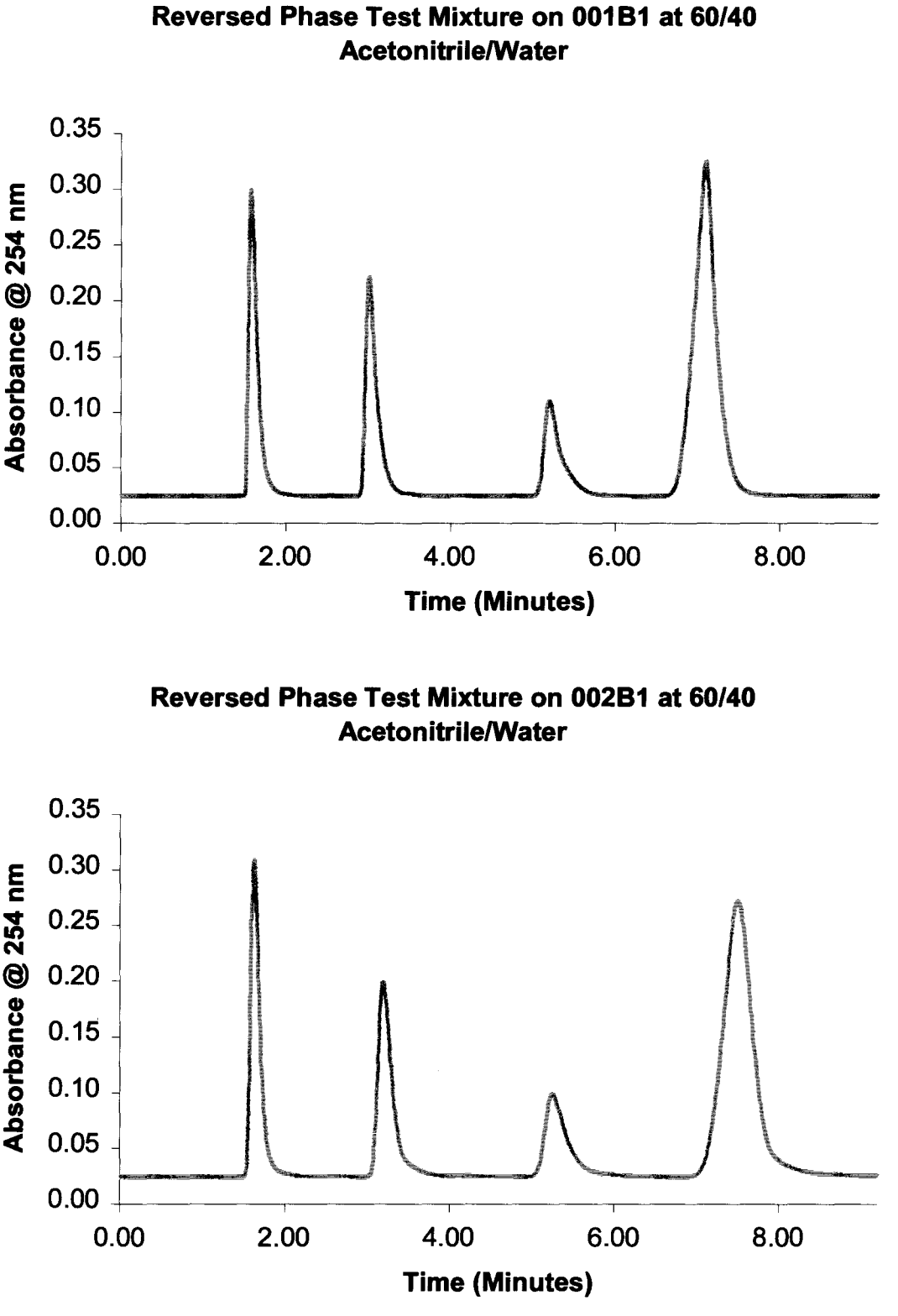


Figure 5.2: Chromatograms on 001B1 and 002B1

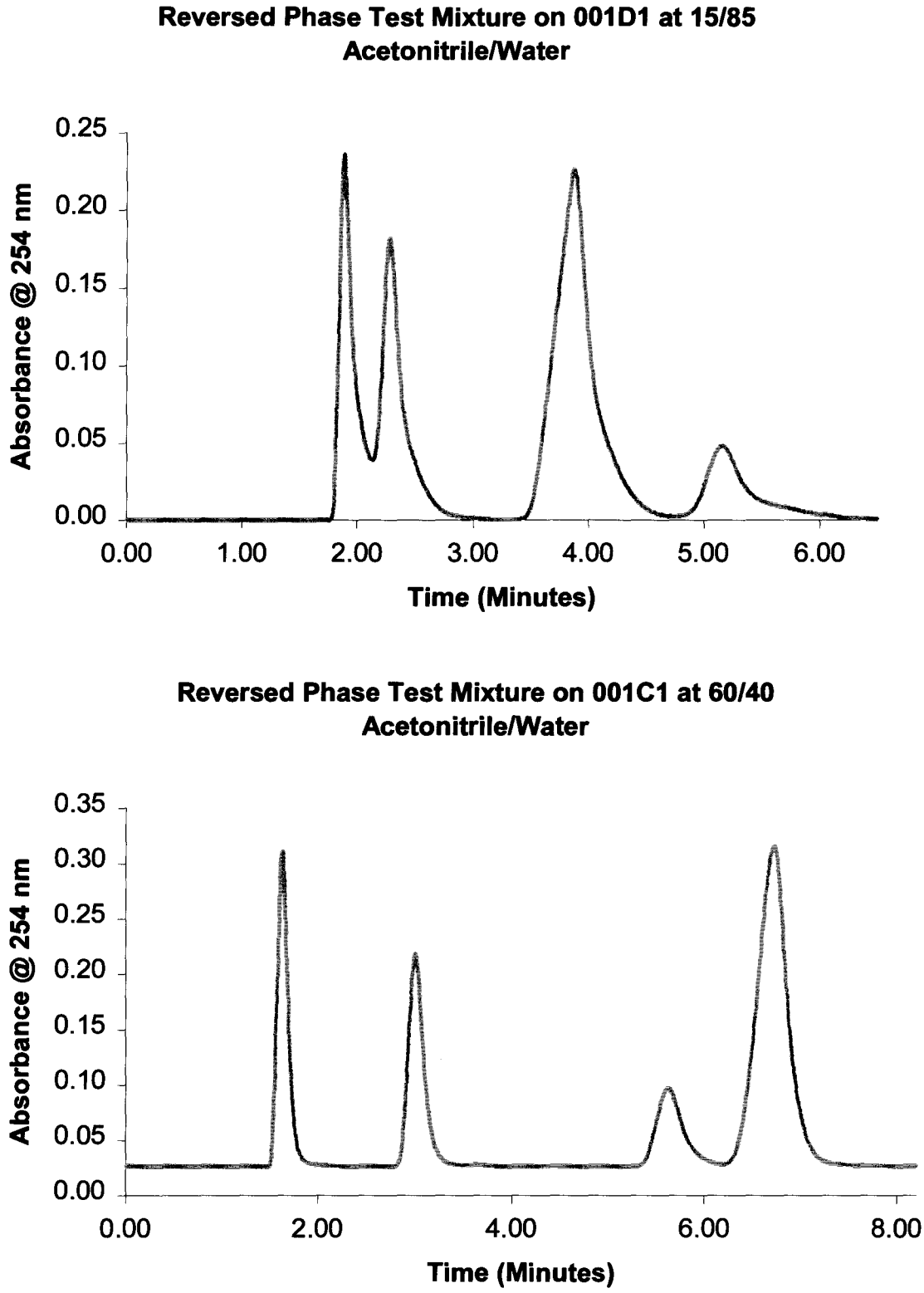


Figure 5.3: Chromatograms on 001C1 and 001D1

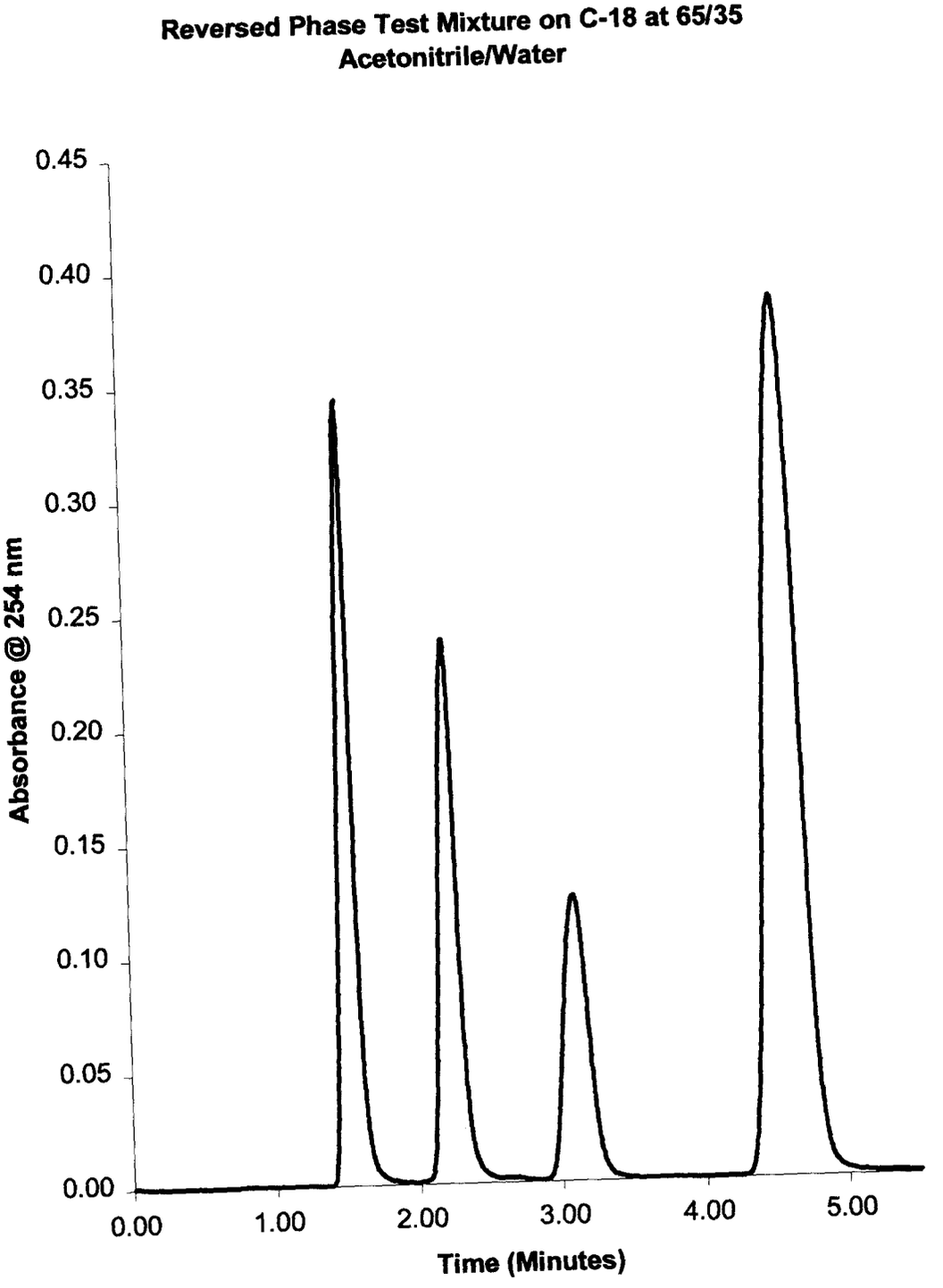


Figure 5.4: Chromatogram on C-18

Homologous Series Determinations:

As outlined earlier, chromatography was also performed using two different types of homologous series. One purpose of separating homologous series was to determine if more than one type of intermolecular interaction was occurring as each of the solutes passed down each column. Normally, with only one major type of interaction occurring between the solute molecules, the mobile phase, and the stationary phase, a plot of the *log of the retention time* versus *the number of carbon* atoms should produce a straight or nearly straight line. Such behavior has been frequently observed for gas chromatography and has been observed for separations of homologous series of solutes on alkyl columns (59). If more than one type of significant interaction were to occur, the graphical description should become non-linear.

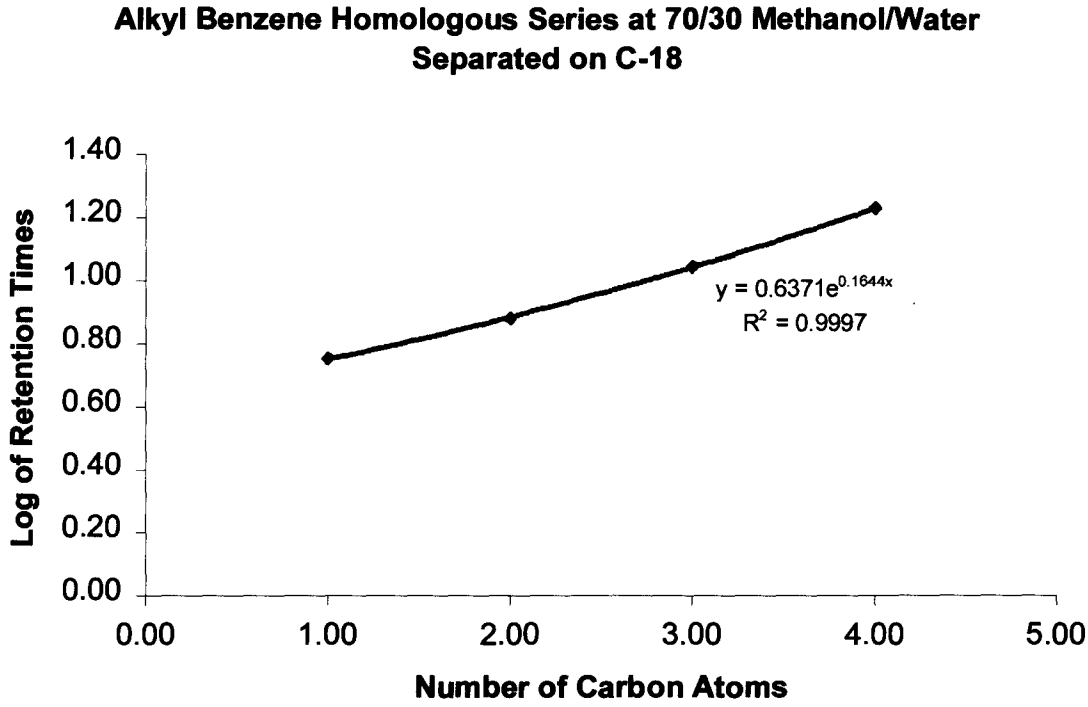
The homologous series used in this work were chosen because phenyl compounds were known to form host/guest complexes in varying degrees with 4-t-butylcalix[n]arenes. The separation of two types of homologous series examined any dependence of solute size and/or shape on the strength of the complex, where more complexation was represented by skewing of the peaks and longer than expected (from extrapolation from C-18 data) retention times.

Longer retention times indicated more time spent in the stationary phase. Using *log* t_r versus *homologue number* plots, a linear, or near linear relationship was expected if the interactions affecting retention were consistent from homologue to homologue. Significant deviation from the expected retention, therefore, represented significant other interactions. In the case of the 4-t-

butylcalixarenes, this was assumed to indicate host/guest complex formation during the separation.

Peak tailing and broadening can occur from two processes: diffusional band broadening and a mixed mode retention mechanism. If host/guest complexation were occurring, it would be expected to be accompanied by significant amounts of band broadening and tailing because of the mixed mode nature of the London and complexation interactions. Also, diffusional band broadening from the relatively slow kinetics that has been associated with host/guest complexation (as compared to the other diffusional processes that occur during separation) would be expected as well. As outlined in the data presented below, both of these effects were observed.

The first homologous series to be separated was one composed of sequential alkyl benzenes. The series chosen consisted of methyl-, ethyl-, propyl-, and n-butylbenzene and was used because the shape of the molecules remained the same with respect to the phenyl ring, hence the size of the molecules were approximately the same. Any increase in retention time was expected to be due primarily to London interactions and secondarily to host/guest interactions with the bound 4-t-butylcalixarenes. It was assumed that if complexation were to occur, it would be relatively constant across the series. The data are summarized in Figures 5.5 and 5.6 and in Tables 5.11 and 5.12. While only plots from C-18 and 001D1 are shown, similar data was obtained from each column. Tables 5.11 and 5.12 do however show the data obtained from all of the columns.



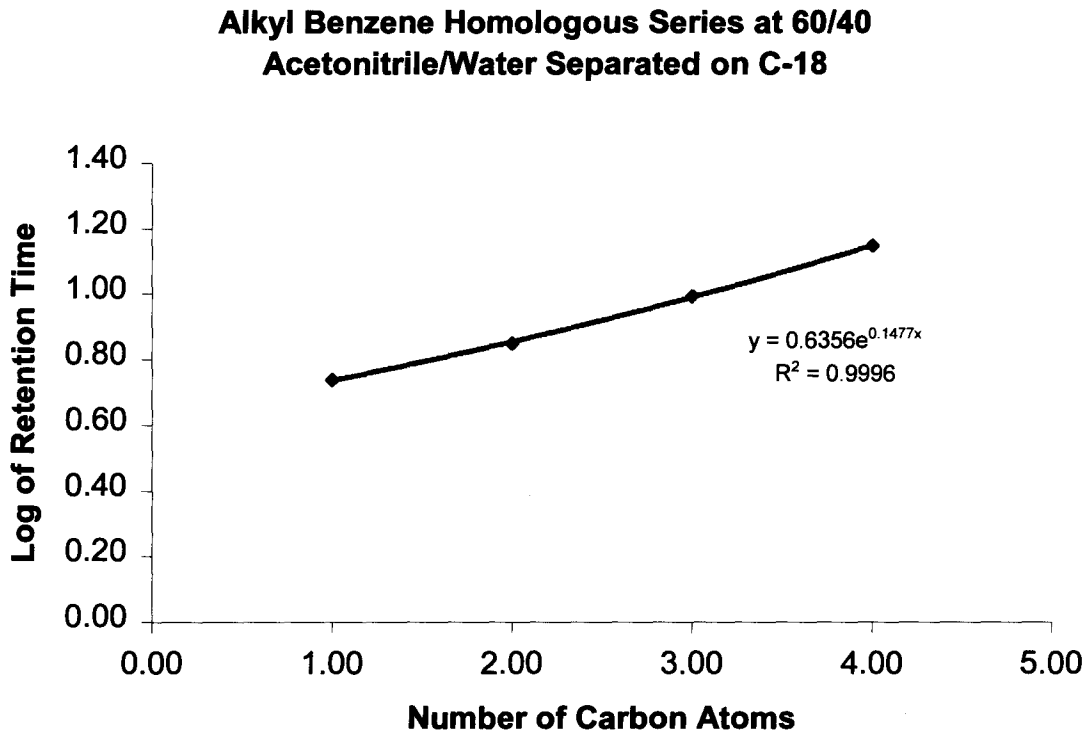
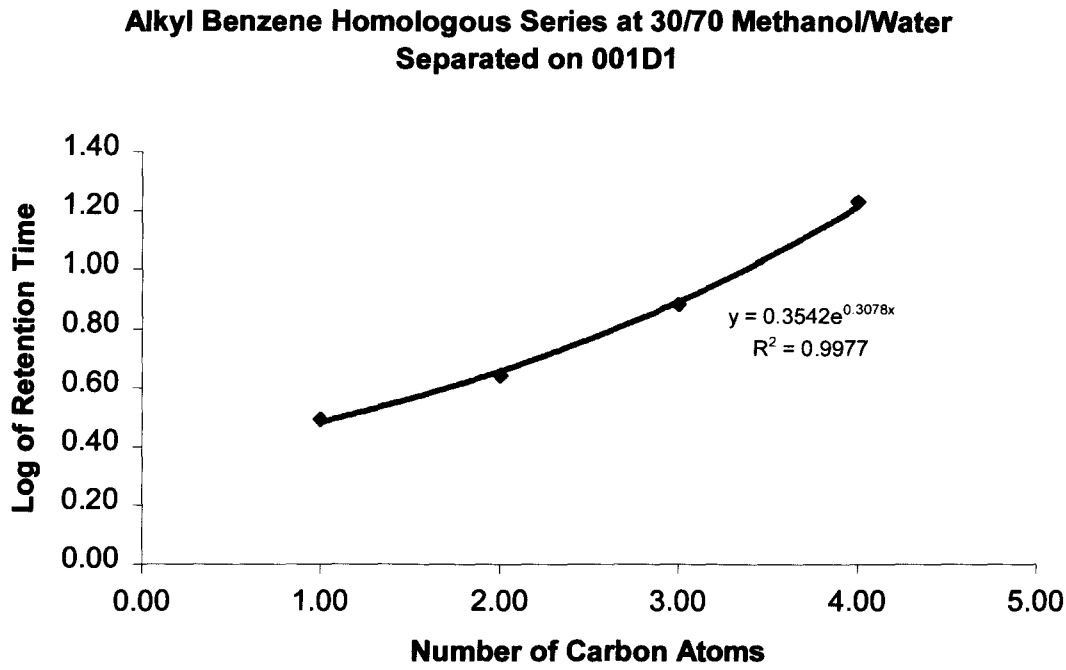


Figure 5.5: Graphs of Alkyl Benzene Homologous Series Separated on C-18



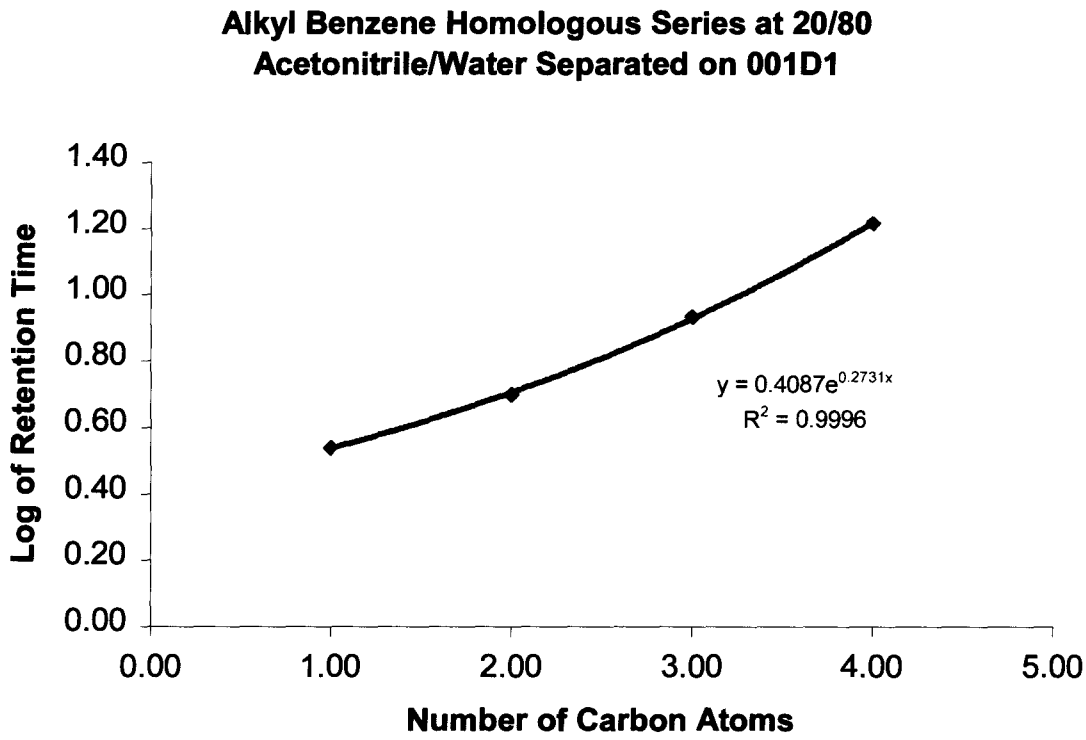


Figure 5.6: Alkyl Benzene Homologous Series Separated on 001D1

Table 5.11

Coefficients and Exponents from the Alkyl Benzene Homologous Series Using Acetonitrile/Water

Column	Coefficient	Exponent
001A1	0.6837	0.1315
002A1	0.672	0.1358
001B1	0.626	0.1322
002B1	0.6542	0.1328
001C1	0.6273	0.1264
001D1	0.4087	0.2731
C-18	0.6356	0.1477

Table 5.12

Coefficients and Exponents from the Alkyl Benzene Homologous Series Using Methanol/Water

Column	Coefficient	Exponent
001A1	0.5392	0.1548
002A1	0.5167	0.1586
001B1	0.4794	0.1509
002B1	0.484	0.1509
001C1	0.4862	0.1502
001D1	0.3542	0.3078
C-18	0.6371	0.1644

From this data, it can be seen that, generally, all of the phases, including C-18, deviated from linearity. While the C-18 retention was distinctly non-linear, it was the most linear of all. Thus, using C-18 as a reference, further deviation from linearity was attributed to increased interaction of the increasingly non-polar homologues with the bound 4-t-butylcalixarenes. This was further demonstrated by the peak shapes observed with the 4-t-butylcalixarene columns as compared to the C-18 column. The observation of tailing peaks was consistent with mixed mode retention. In other words, it seemed apparent that an additional type of

interaction was beginning to play a significant role in the retention of these homologues. As the homologue became more non-polar, the additional interaction became slightly more important. Examples of these chromatograms are in Figures 5.7-5.11.

The last series to be separated was a phenyl ring homologous series that consisted of benzene, napthalene, and anthracene. For this series, not only did the non-polarity increase as additional rings were added, but the size and shape of the solutes changed as well. Additionally, the presence of more phenyl rings was thought to be conducive to the holding of the solute in the 4-t-butylcalixarene cavity (i.e., host/guest complexation). The data are summarized in Figures 5.12 and 5.13 and Tables 5.13 and 5.14. Similar to the alkyl benzene homologous series, example chromatograms are displayed in Figures 5.14-5.18.

Table 5.13

Coefficients and Exponents from the Phenyl Ring Homologous Series Using Acetonitrile/Water

Column	Coefficient	Exponent
001A1	0.4706	0.0735
002A1	0.4961	0.0682
001B1	0.4595	0.0707
002B1	0.481	0.0698
001C1	0.451	0.0681
001D1	0.1778	0.1482
C-18	0.3998	0.0738

Table 5.14

Coefficients and Exponents from the Phenyl Ring Homologous Series Using Acetonitrile/Water

Column	Coefficient	Exponent
001A1	0.3444	0.0716
002A1	0.3255	0.0728
001B1	0.299	0.0751
002B1	0.312	0.0733
001C1	0.3004	0.0737
001D1	0.1278	0.1836
C-18	0.4349	0.1006

From this data, it became quite apparent that a level of other interaction was occurring. This was attributed to an increasing amount of host/guest complexation as the size of the phenyl ring complex was increased. Peak tailing was measurably increased as each phenyl ring was added and, relative to the expected retention from homologue linearity plots, anthracene was retained significantly longer on the 4-t-butylcalixarene stationary phases than on the C-18 phase.

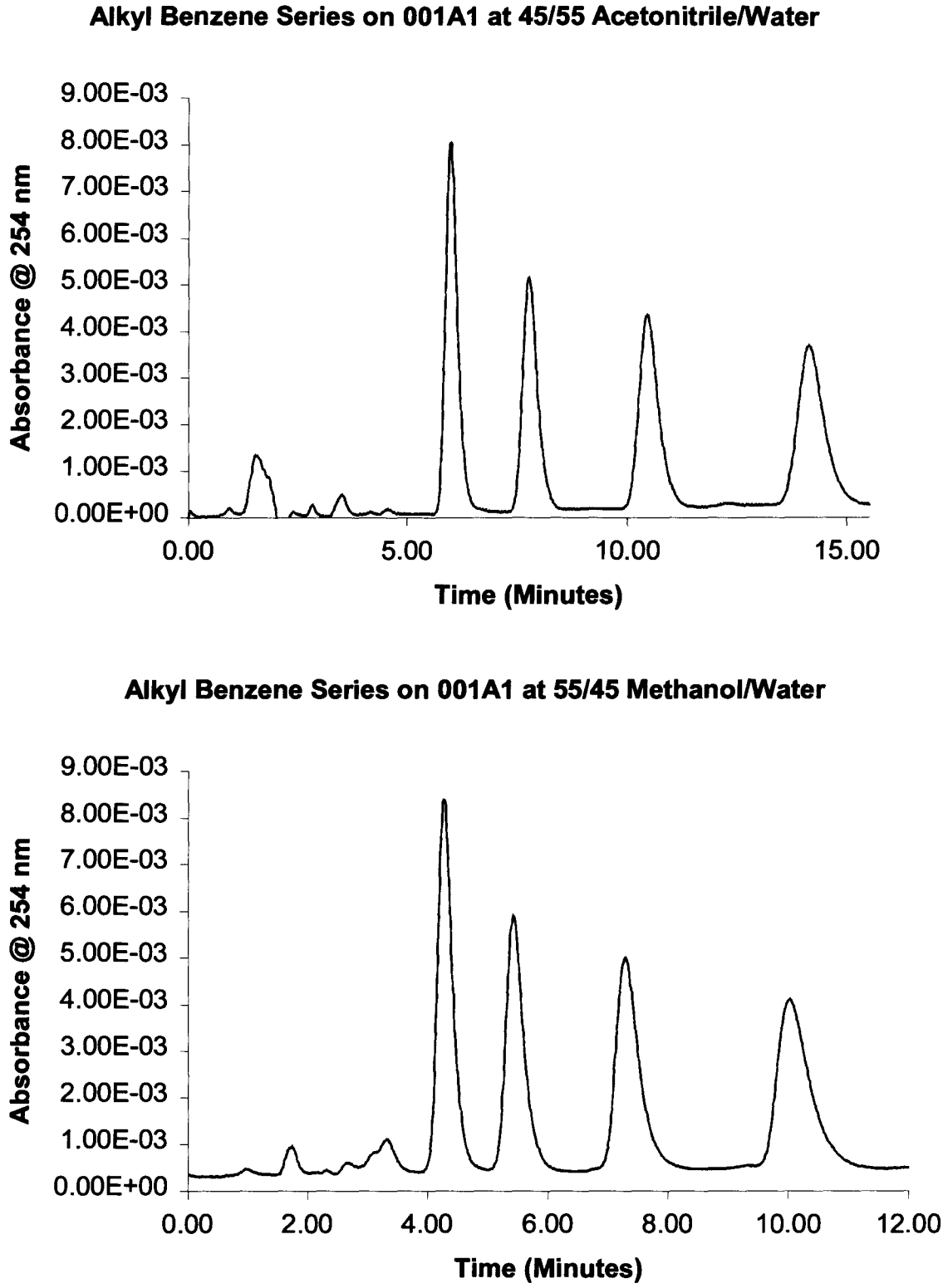


Figure 5.7: Alkyl Benzene on 002A1

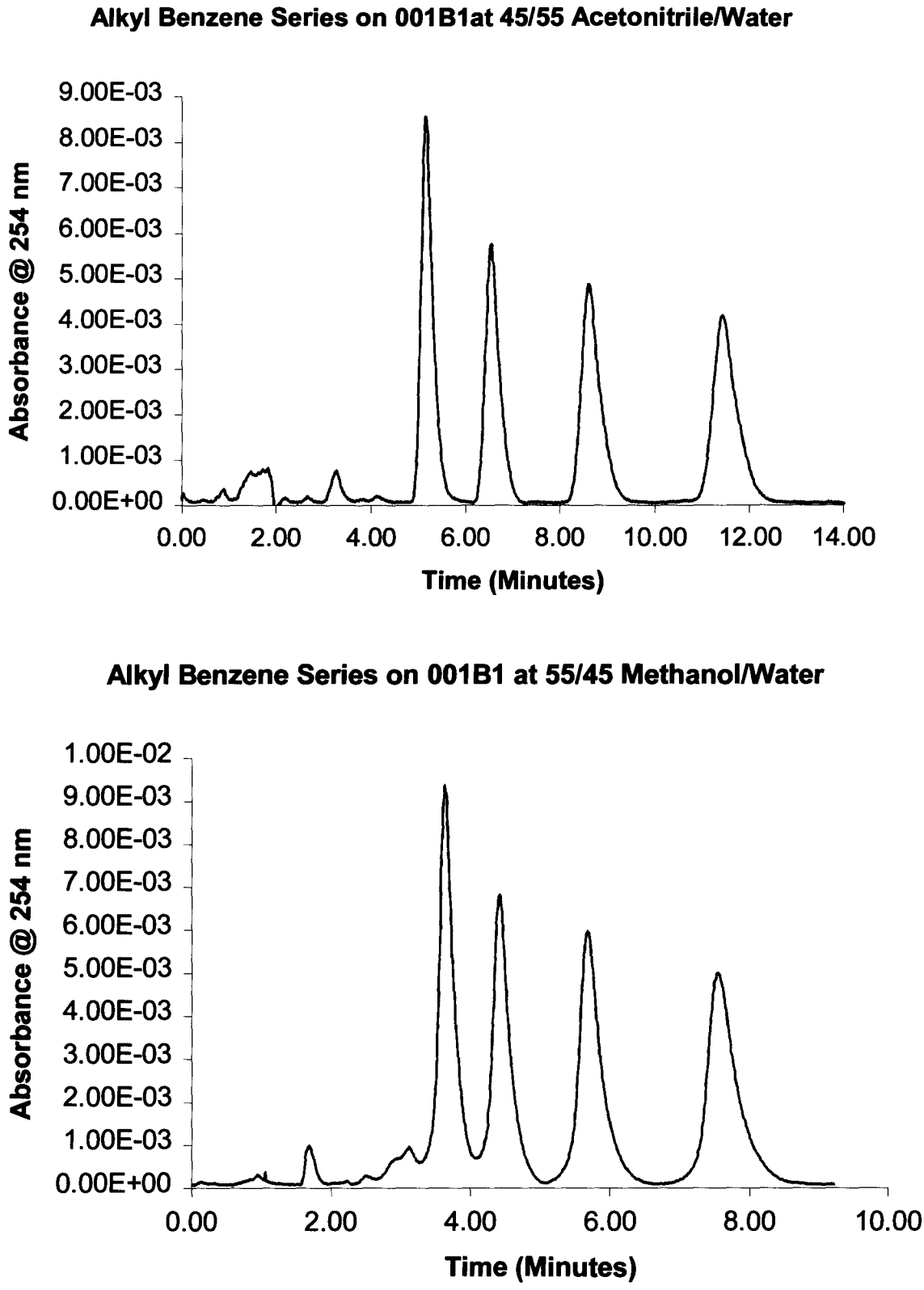


Figure 5.8: Alkyl Homologous Series on 001B1

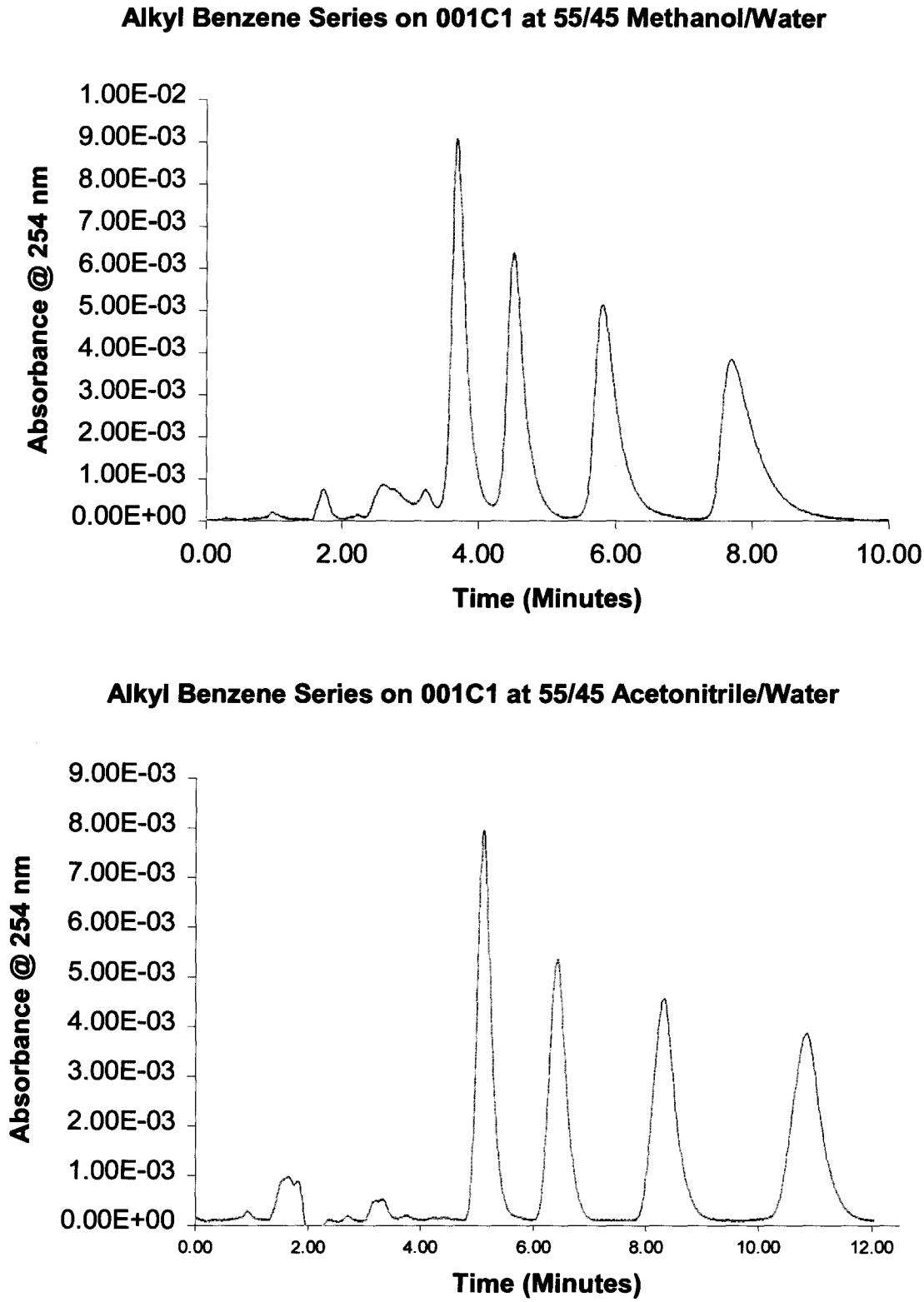


Figure 5.9: Alkyl Benzene Homologous Series on 001C1

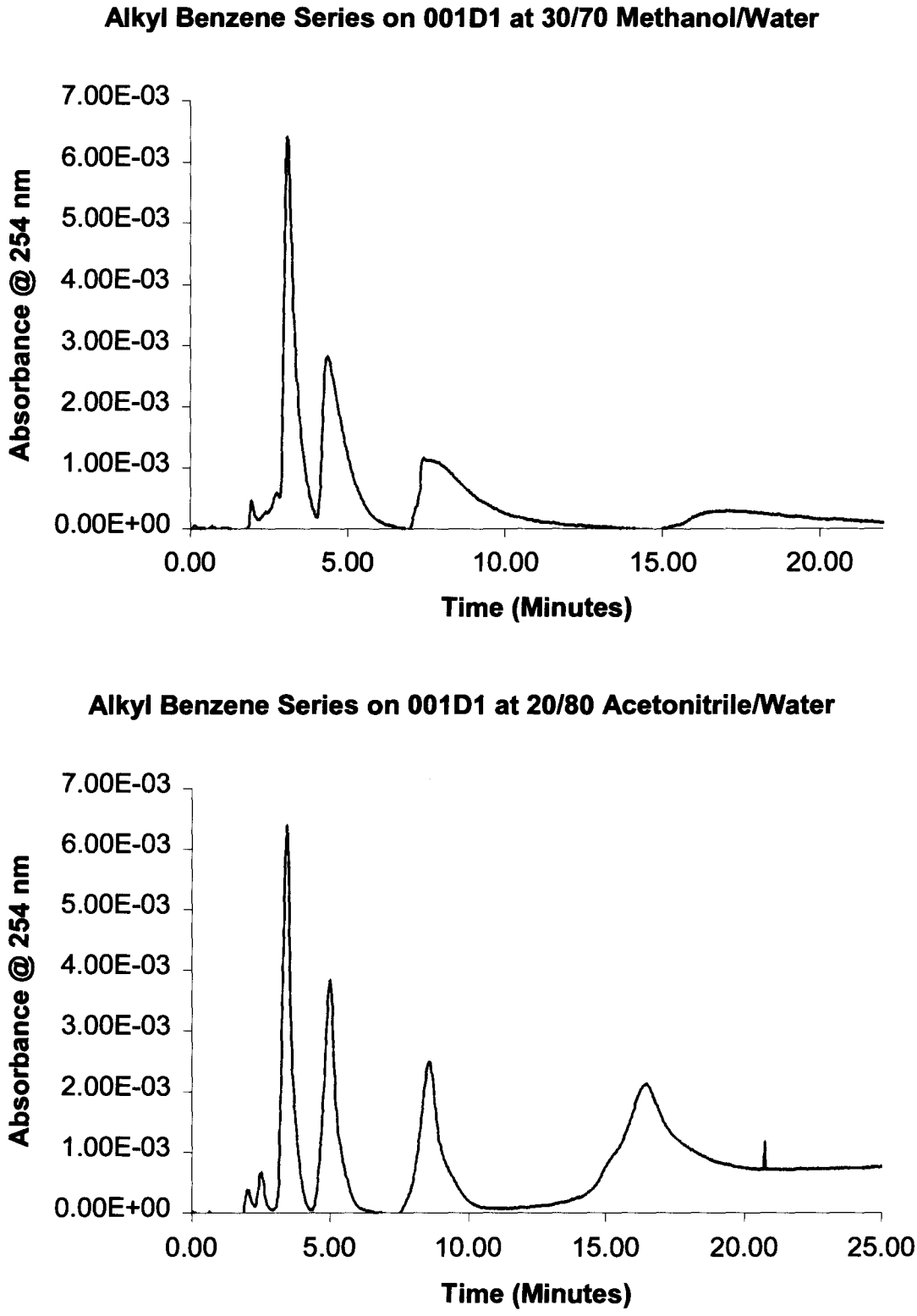


Figure 5.10: Alkyl Benzene Homologous Series on 001D1

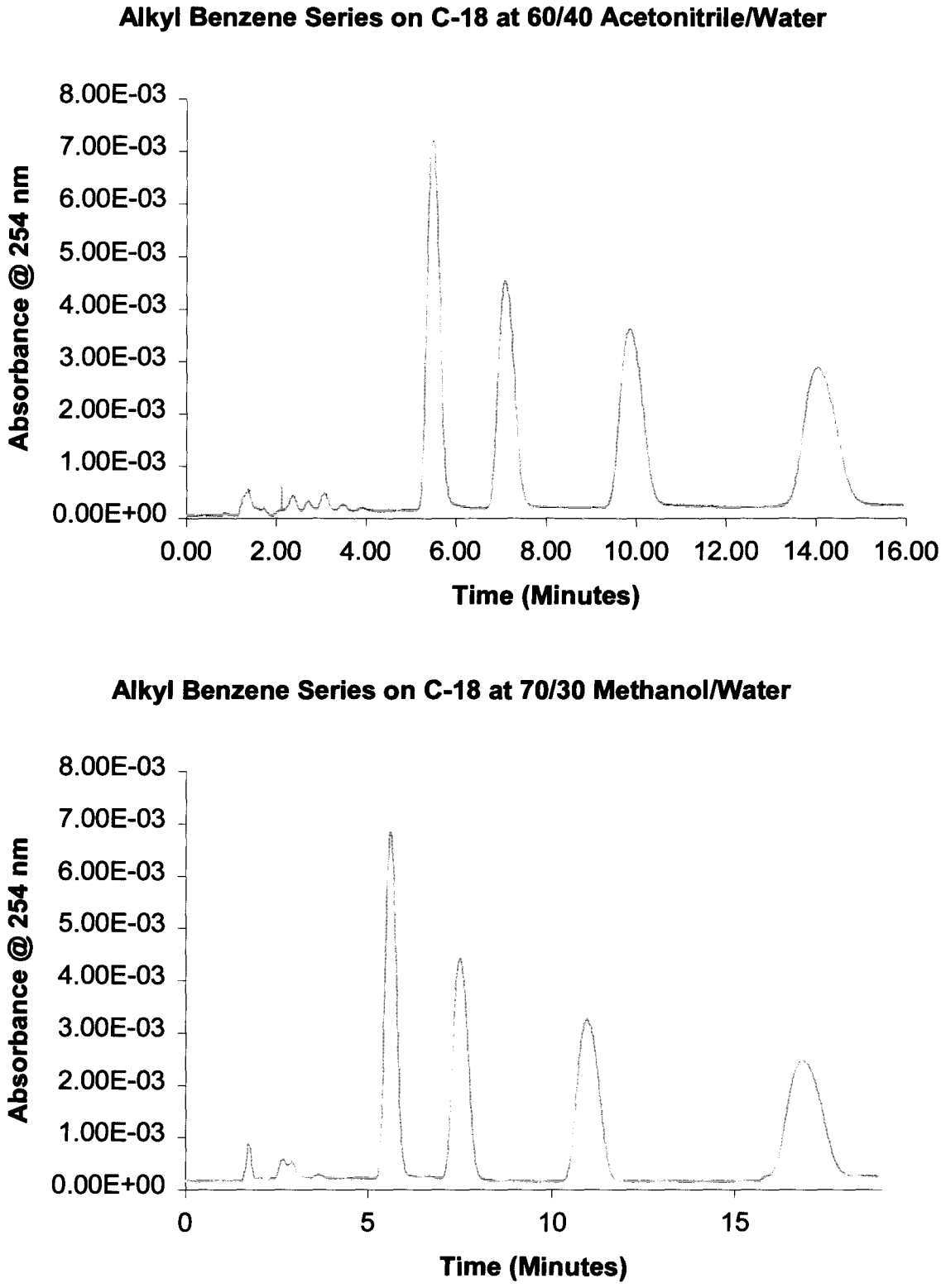
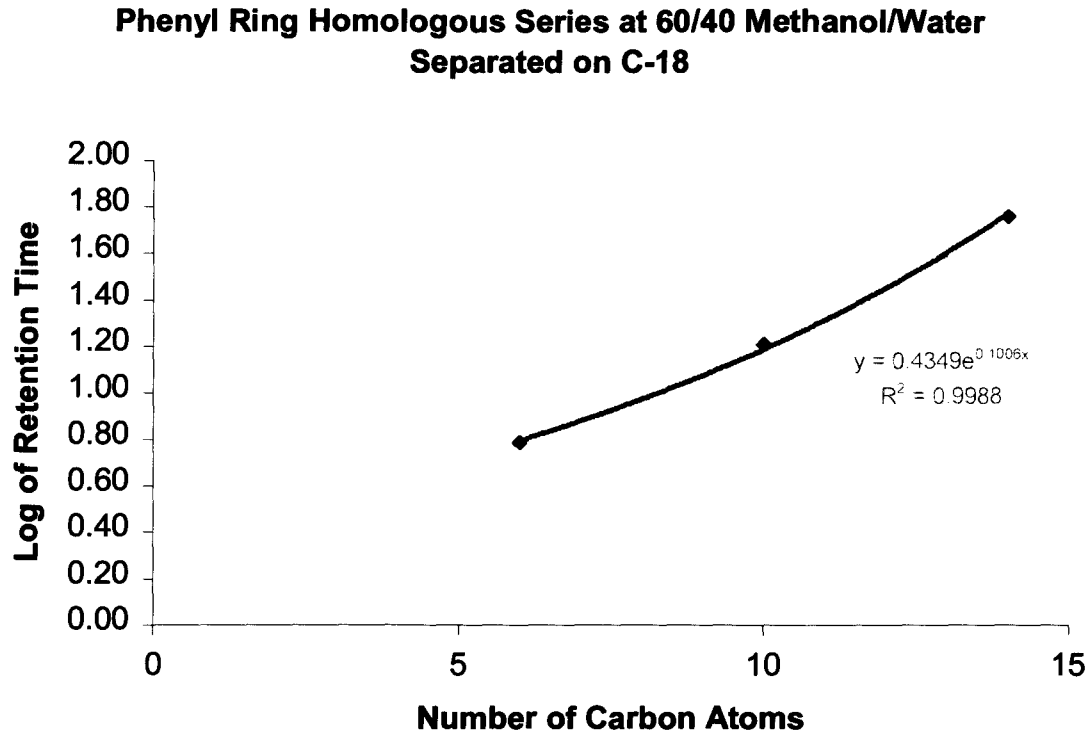


Figure 5.11: Alkyl Benzene Homologous Series on C-18



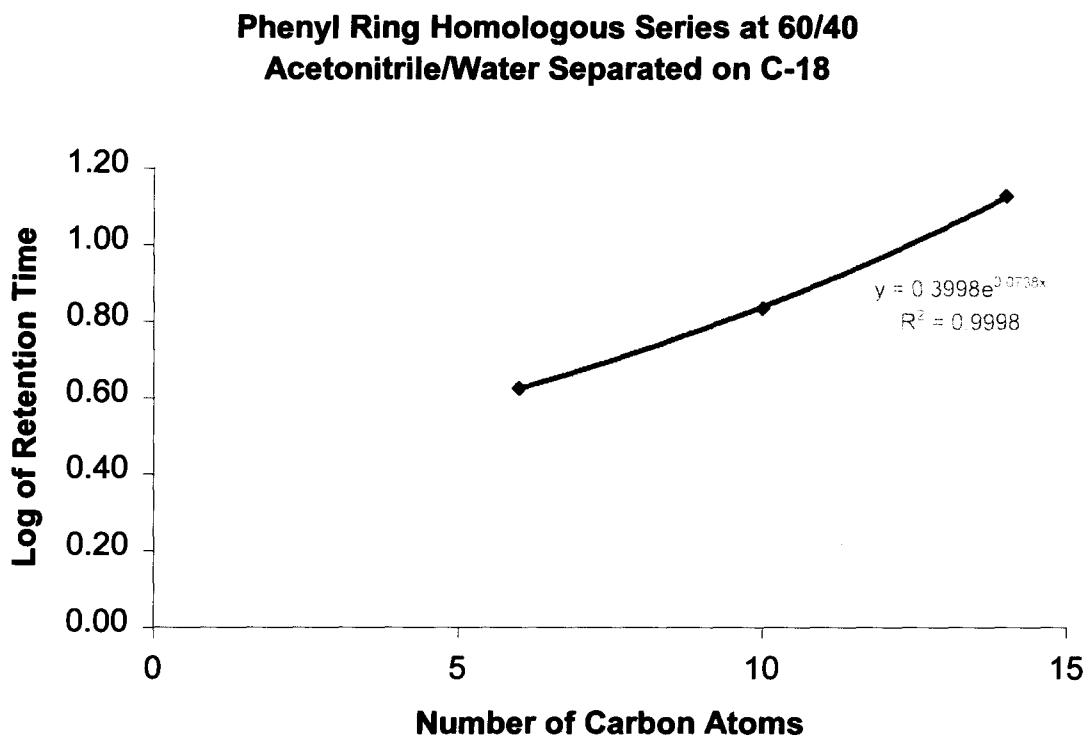


Figure 5.12: Phenyl Ring Homologous Series Separated on C-18

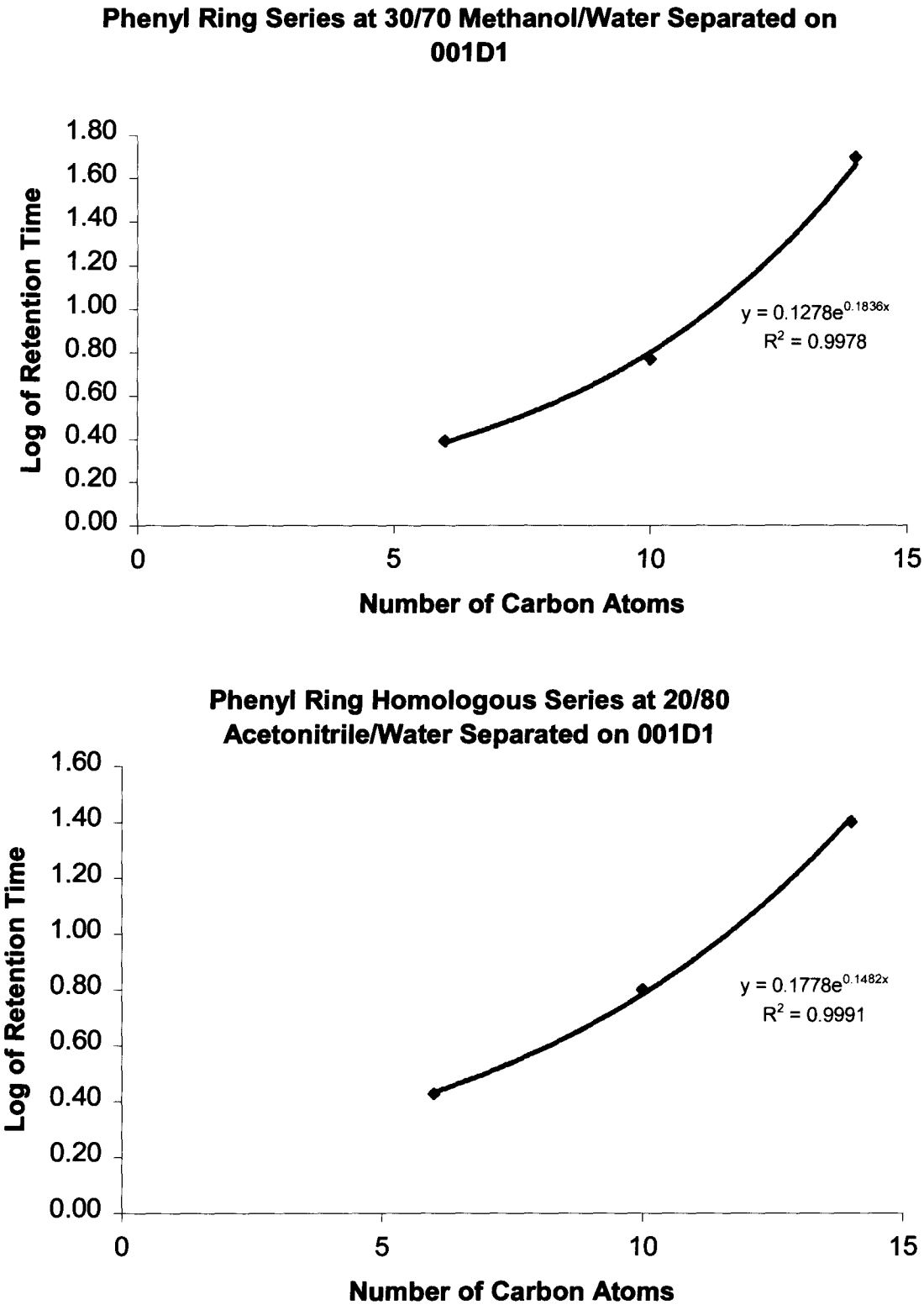


Figure 5.13: Phenyl Ring Homologous Series Separated on 001D1

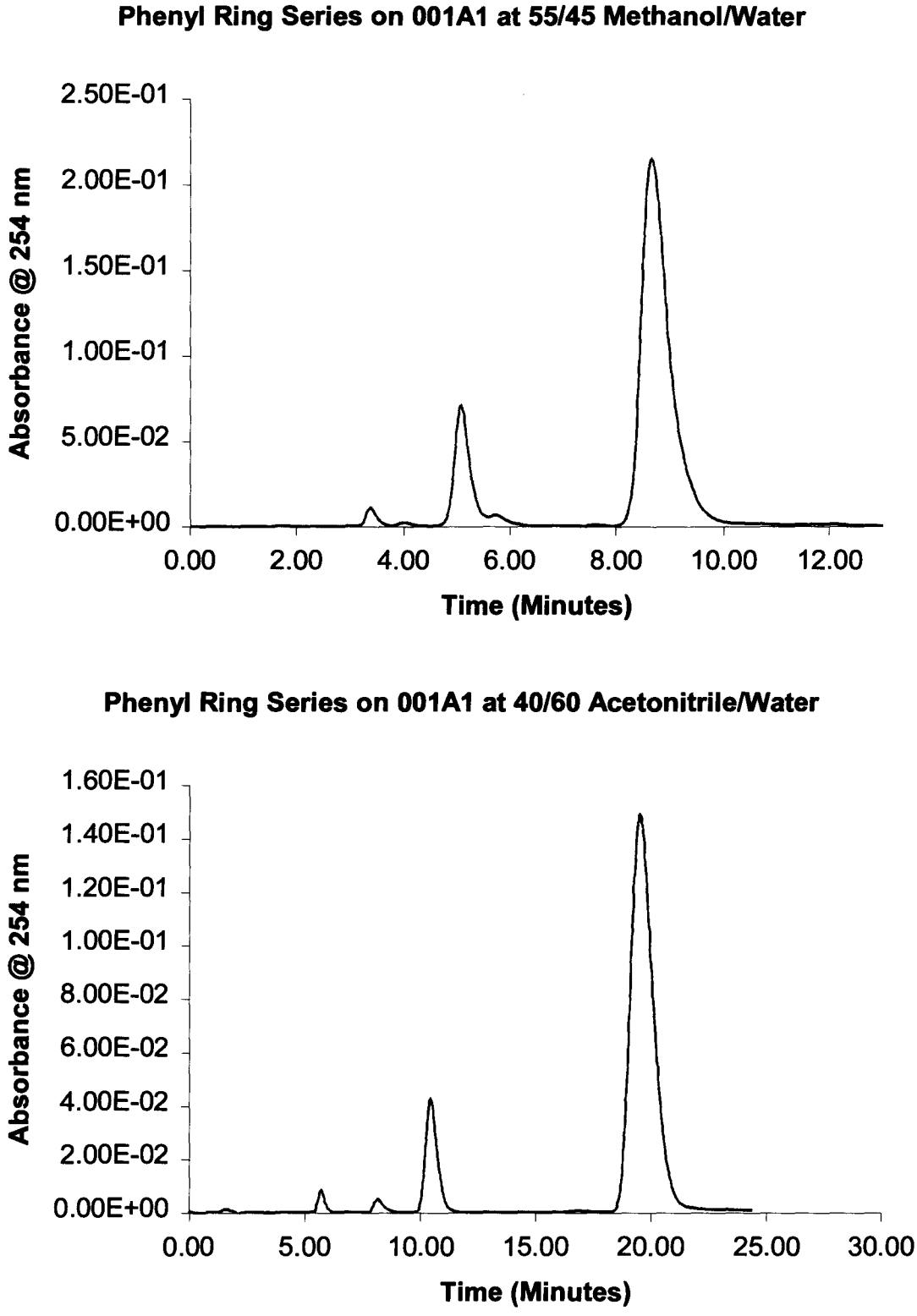


Figure 5.14: Phenyl Ring Series Separated on 001A1

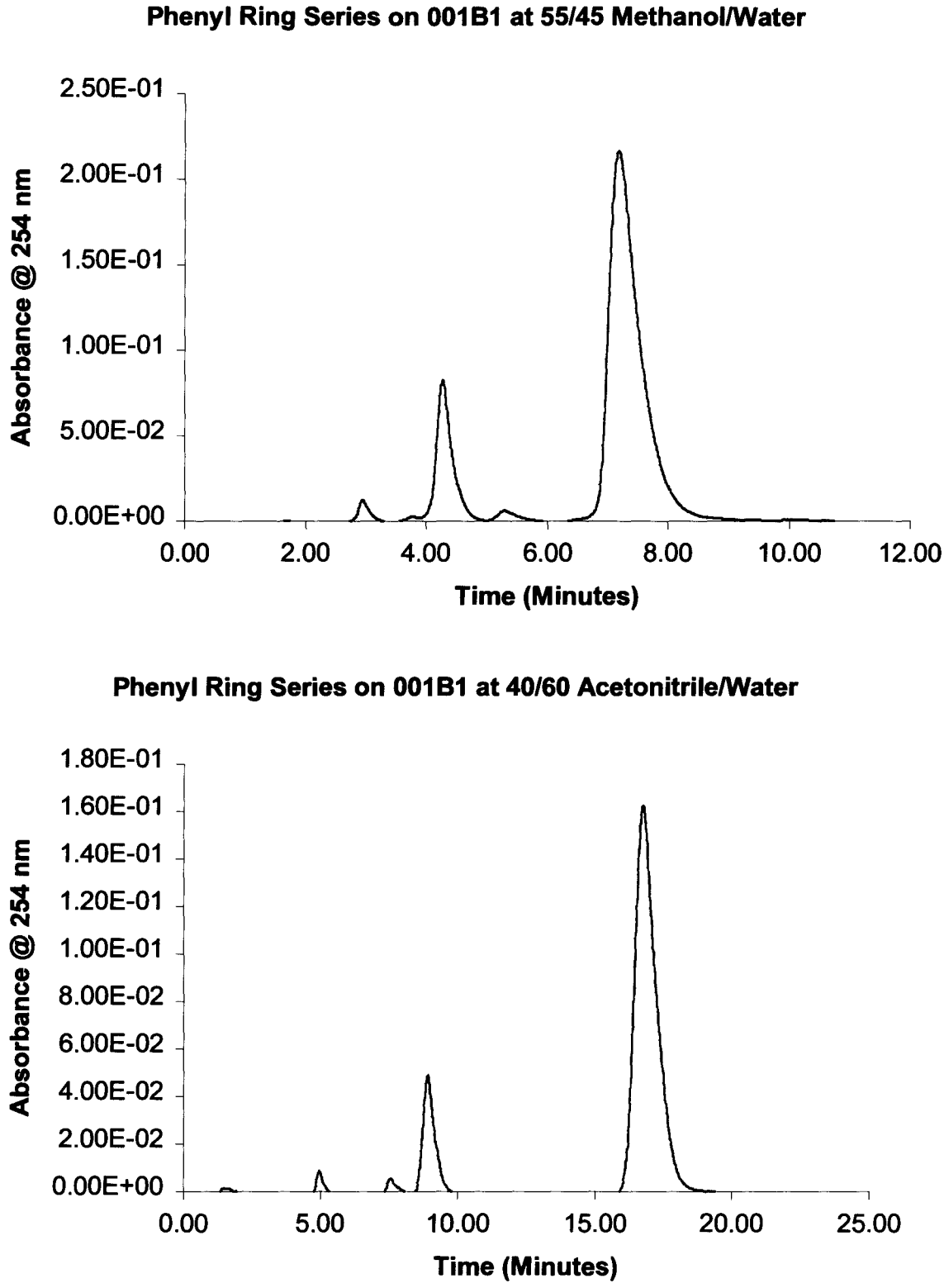


Figure 5.15: Phenyl Ring Homologous Series Separated on 001B1

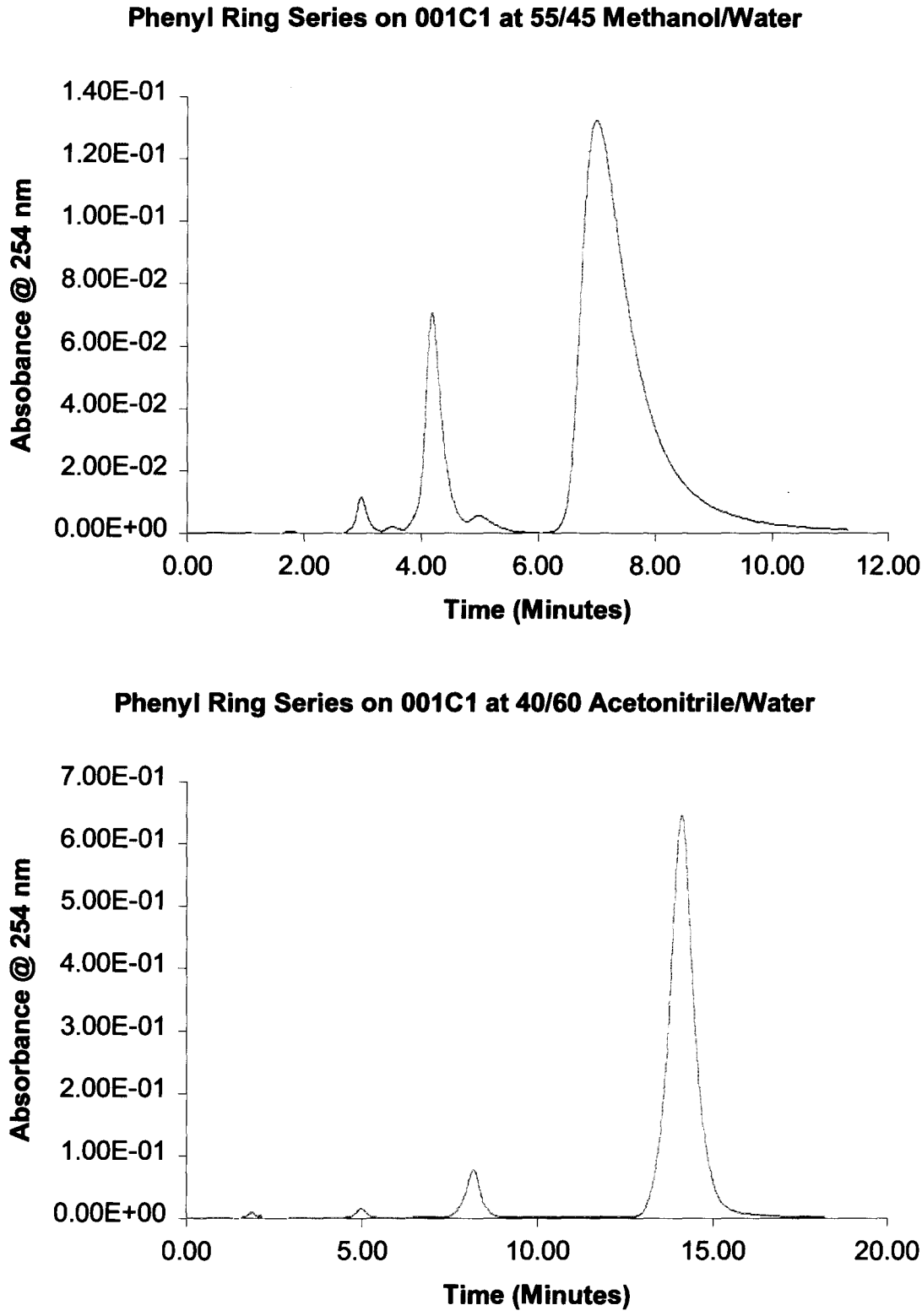


Figure 5.16: Phenyl Ring Homologous Series Separated on 001C1

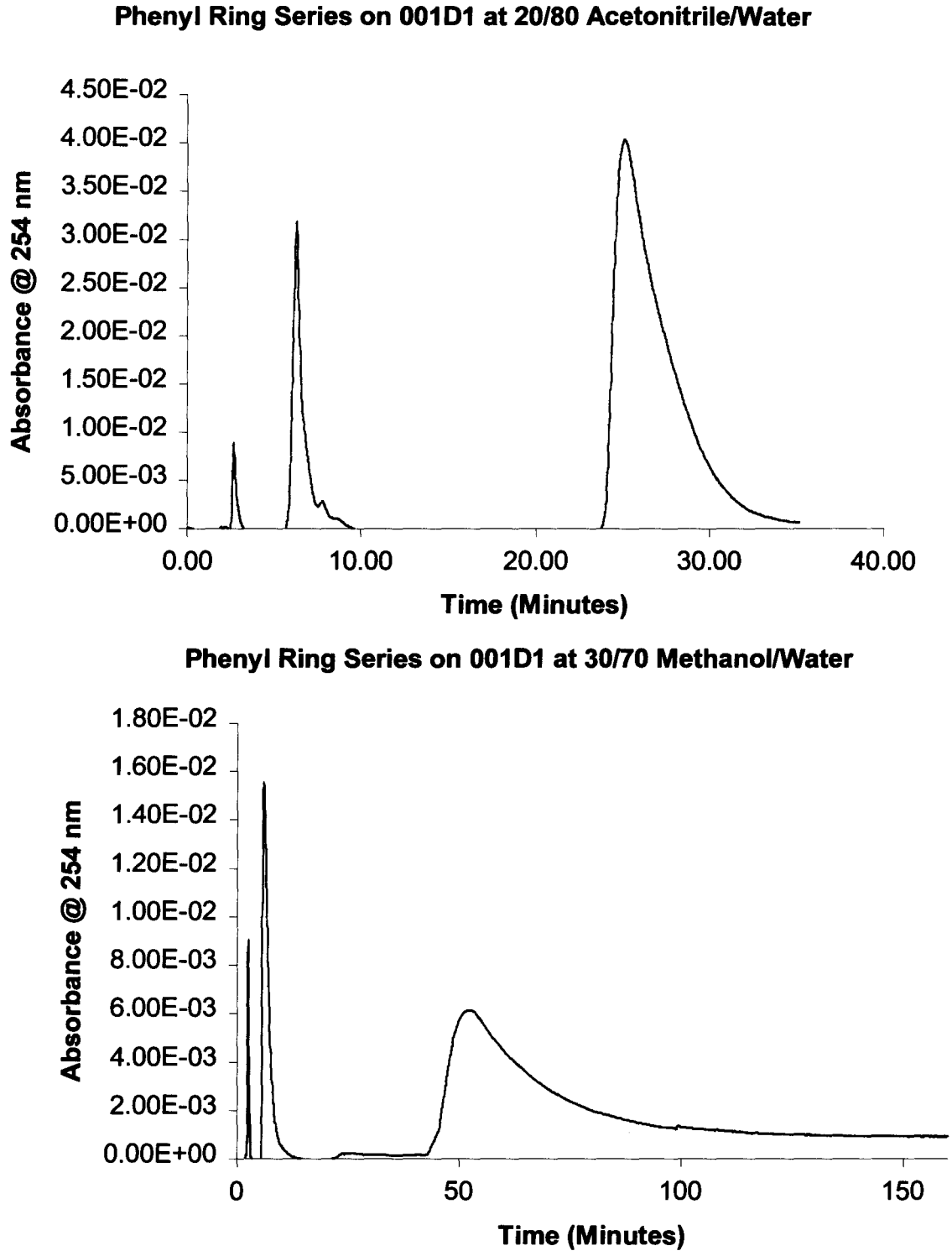


Figure 4.17: Phenyl Ring Homologous Series Separated on 001D1

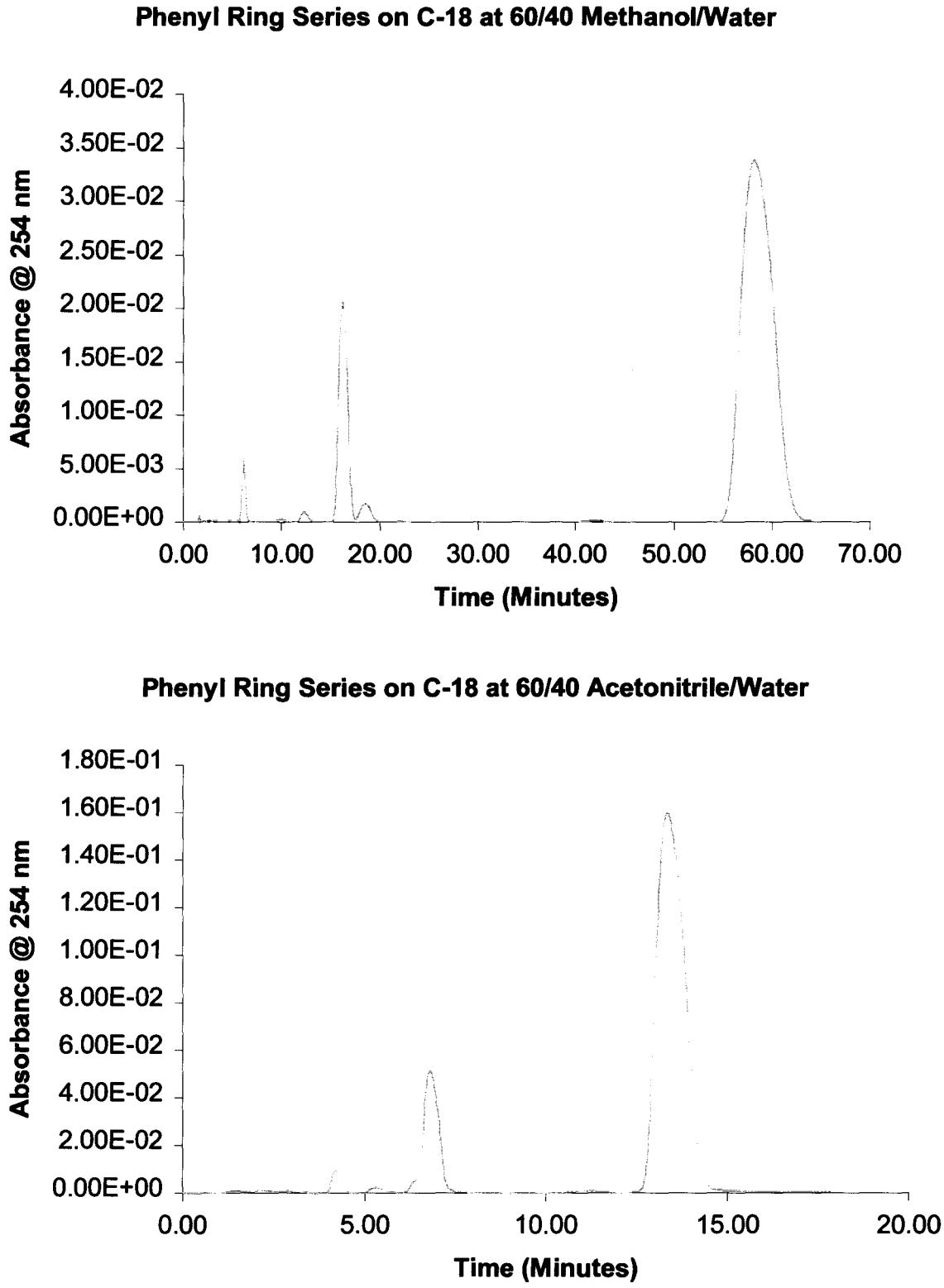


Figure 5.18: Phenyl Ring Homologous Series Separated on C-18

The Effect of Tether Length on Chromatography:

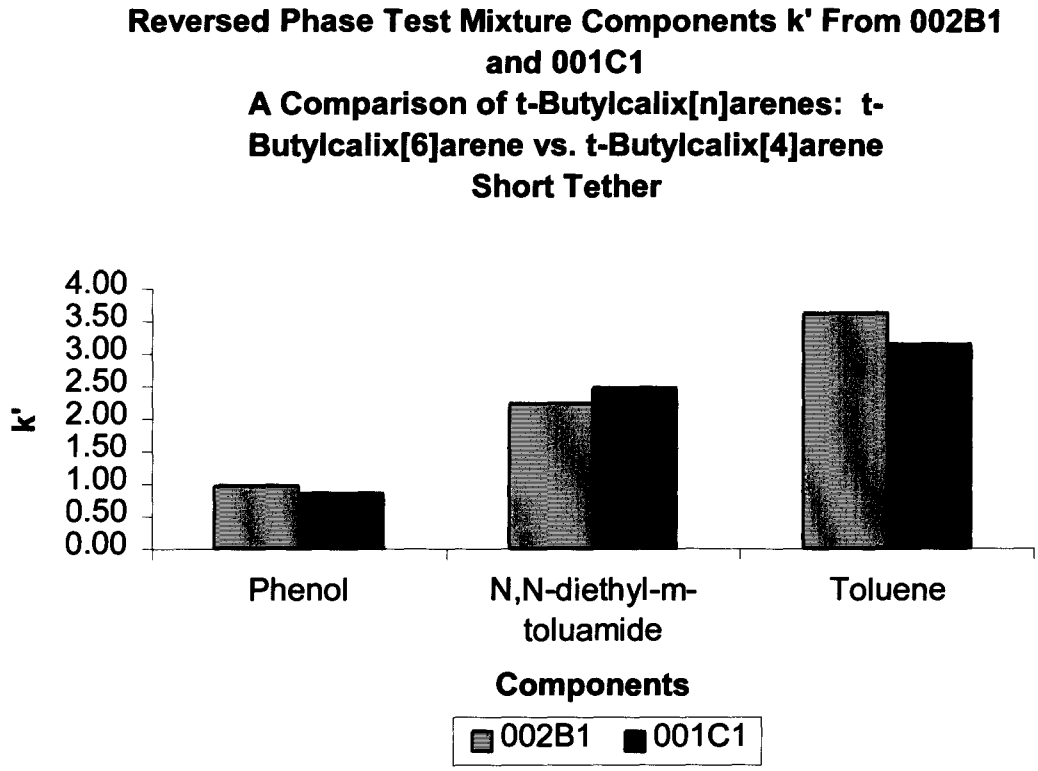
It was initially thought that the length of the tether attaching the 4-t-butylcalixarene to the silica substrate would have a significant effect on the behavior of the stationary phase. While the length did make some difference on the retention, it was not extremely significant until the retention times were large. As the retention increases, the effect of the tether increases. Chromatograms of reversed phase test mixture separated using the long-tether material showed the apparent difference from separations of the same mixture using the short tether material. This is illustrated in Figure 5.19 by comparing the average k' values from reversed phase test mixture performed on 4-t-butylcalix[6]arene columns, one prepared with the long tether (001A1 and 002A1) and one with the short tether (001B1 and 002B1). This graphical interpretation shows the increased significance of the tether length as the retention times increased.

The reversed phase test mixture chromatograms show that the synthesized 4-t-butylcalixarene stationary phases behave in a reversed phase manner. However, the behavior they possess was similar to a hydrocarbon shorter than that of a C-18 column. As can be seen from the previous figures (Figures 5.1-5.4), the retention times of the individual components are greater for the C-18 column than for the 4-t-butylcalixarene columns. This effect can be best explained by the differing chain length of the hydrocarbon-like tether attaching the 4-t-butylcalixarene to the silica substrate as compared to the chain length of the C-18 hydrocarbon. Since the C-18 is a longer and straighter chain molecule than any of the 4-t-butylcalixarenes, it was expected to show a greater

non-polar character. As such, it would take longer for the reversed phase test mixture components to elute off of the C-18 column.

In order to make the retention times relatively equal for the reversed phase test mixture components, the amount of acetonitrile in the mobile phase had to be reduced when separated on the C-18 column. This is an indication that, in terms of polarity, the 4-t-butylcalixarene columns acted as shorter hydrocarbons than did the C-18 column. The fact that the 4-t-butylcalixarene columns retain the test mixture components was sufficient evidence to claim that the calixarene columns had reversed phase behavior.

The 4-t-butylcalix[4]arene and 4-t-butylcalix[6]arene stationary phases did show differences in retention behavior. Figure 5.19 demonstrates the difference in the two 4-t-butylcalixarenes when reversed phase test mixture was separated on the different columns. The size of the cavity the different 4-t-butylcalixarenes have contributed to the difference in selectivity.



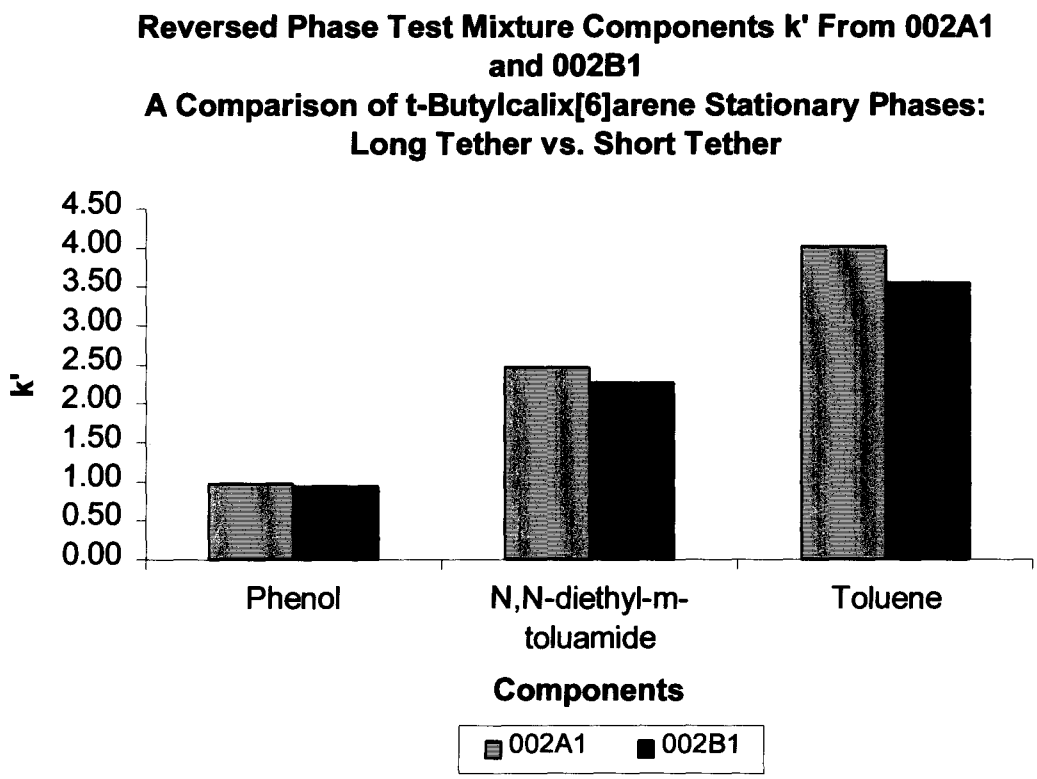


Figure 5.19: Comparison graphs of tether length and calix[n]arene

Spectroscopic Characterization:

Solid State (CP/MAS) NMR :

Due to lack of suitable solid-state NMR equipment within the Chemistry Department at Youngstown State University, as well as the extremely long analysis times (>200 hours per sample) that were apparently required to obtain a suitable signal/noise, it was not possible to have the stationary phase samples fully analyzed using CP/MAS NMR. It was possible, however, to have two samples analyzed for several hours each courtesy of the Chemistry Department at the University of Akron. Both samples were 4-t-butylcalix[6]arene stationary phases, one synthesized using the long tether (002A1) and one using the short tether (001B1). The NMR spectra shown in Figures 5.20 and 5.21 gave inconclusive evidence of successful attachment of the 4-t-butylcalixarene to the silica. The concentration of the 4-t-butylcalixarene on the surface of the silica was too close to the detection limits of the NMR in the time frame of the experiment. Therefore, at best, only extremely weak ^{13}C peaks from the t-butyl groups *might* be seen (with suitably good imagination). It therefore could not be conclusively determined that attachment of the 4-t-butylcalixarenes to the silica surface occurred. The NMR spectra did however, show strong evidence of Si-$^{13}CH_3$ groups, which did give positive evidence for the attachment of the silane tether. Figures 5.20 and 5.21 are the NMR spectra that were obtained from the 002A1 and 001B1 stationary phases.

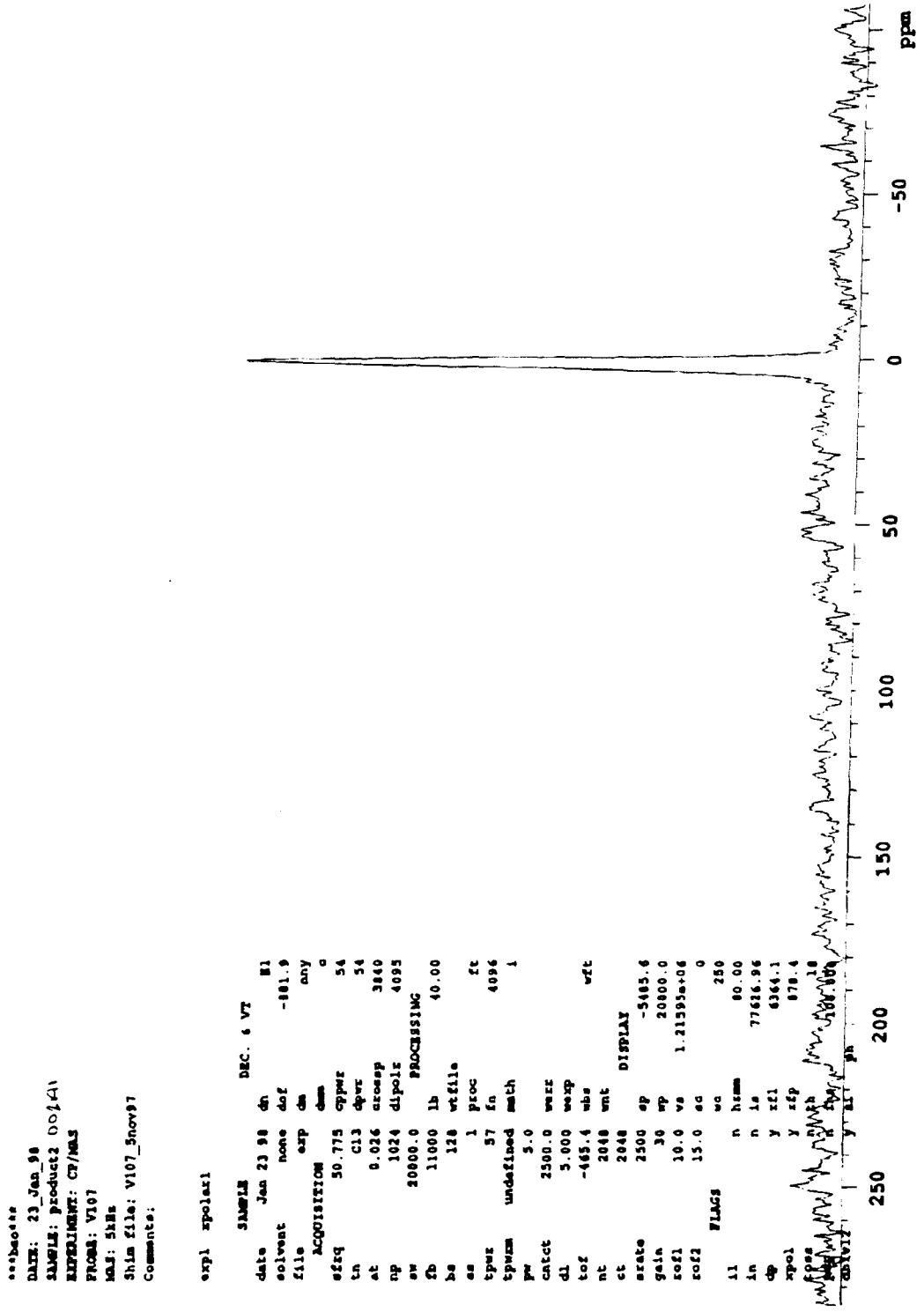


Figure 5.20: NMR Spectra of 002A1

bac
DATE: 22_Jan_98
SAMPLE: 001B1
EXPERIMENT: CP/MAS
PROBE: V107
MAS: 5kHz
Shim file: V107_5nov97
Comments:

expl xpolar1

SAMPLE		DEC. & VT	
date	Jan 22 98	dn	H1
solvent	none	dof	-881.9
file	/service/YSU/-	dm	nny
	Solid/001B1_YSU_CP-	dmm	c
	MAS_S249	cppwr	54
ACQUISITION		dpwr	54
sfrq	50.775	crosep	3840
tn	C13	dipolr	4095
at	0.026	PROCESSING	
np	1024	lb	40.00
sw	20000.0	wtfile	
fb	11000	proc	ft
bs	128	fn	4096
ss	1	wnth	i
tpwr	57		
tpwrm	undefined	werr	
pw	5.0	wexp	
cntct	2500.0	wbs	wft
dl	5.000	wnt	
tof	-465.4	DISPLAY	
nt	2048	sp	-5485.6
ct	2048	wp	20000.0
srate	2500	vs	1.21595e+06
gain	30	sc	0
rof1	10.0	wc	250
rof2	15.0	hzmm	80.00
FLAGS		is	77626.96
il	n	rfl	6364.1
in	n	rfp	878.4
dp	y	th	18
wbs	y	ins	100.000
tomb	n	al	ph
pdp	n		
dblvl2	y		

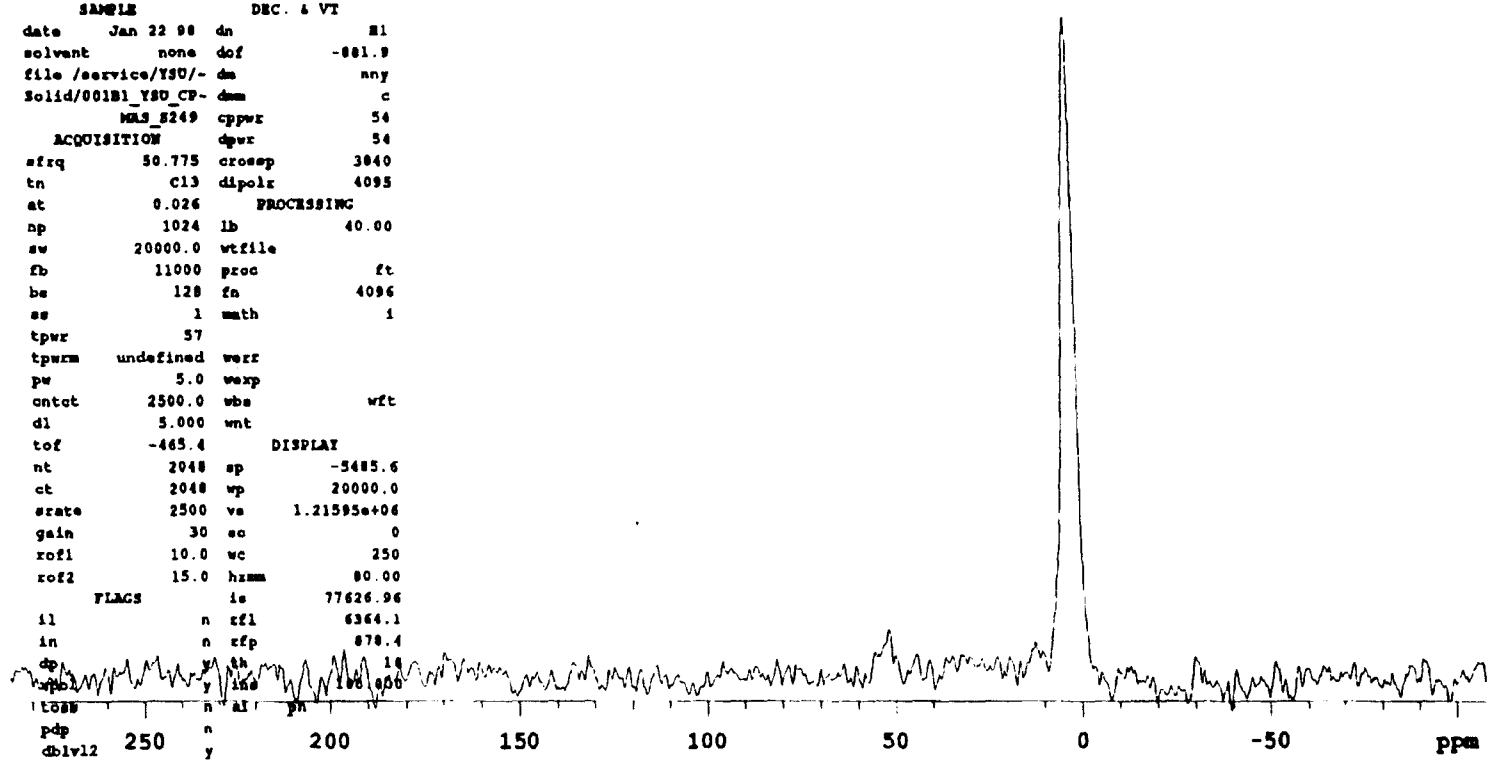


Figure 5.21: NMR Spectra of 001B1

Fluorescence Spectroscopy:

The prepared samples were run on the spectrofluorophotometer. Silica, C-18, and the tethers do not have the ability to fluoresce significantly above an excitation of 200 nm. 4-t-Butylcalixarenes, on the other hand, do exhibit fluorescence due to their conjugated ring structure. As a result, in each sample, 4-t-butylcalixarenes were the only molecules that should fluoresce. Thus, any fluorescence emitted (with proper excitation) by the samples must be due to the presence of 4-t-butylcalixarenes. Furthermore, the samples were washed exhaustively to ensure that only covalently bound 4-t-butylcalixarene would be present on the silica surface. As described in the next paragraph, this gave convincing results that the 4-t-butylcalixarenes had been successfully covalently attached to silica support.

In addition, the stationary phases were also compared to the fluorescence spectra of 4-t-butylcalixarenes in a solution of methylene chloride. This was performed as a security test. The 4-t-butylcalixarene samples fluoresced in a manner similar to the stationary phases under identical conditions. This helped to give additional confirmation that covalent attachment of the 4-t-butylcalixarenes was successful. Figures 5.22-5.27 (fluorescence spectra) show the fluorescence of the 4-t-butylcalix[n]arene stationary phases, the lone 4-t-butylcalix[n]arenes, bare silica substrate, and a stationary phase spiked with additional 4-t-butylcalix[n]arene. It can be seen from the fluorescence spectra that the stationary phases fluoresced in a manner similar to the lone calix[n]arenes. This led to the conclusion that the attachment was successful.

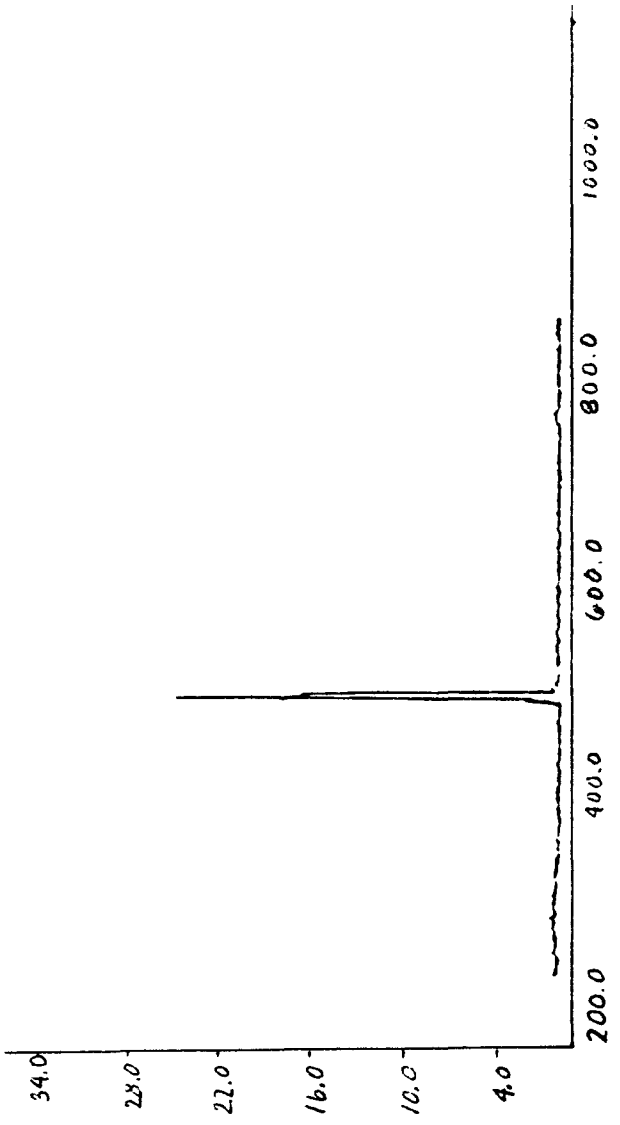


Figure 5.22: Fluorescence Spectra of Silica

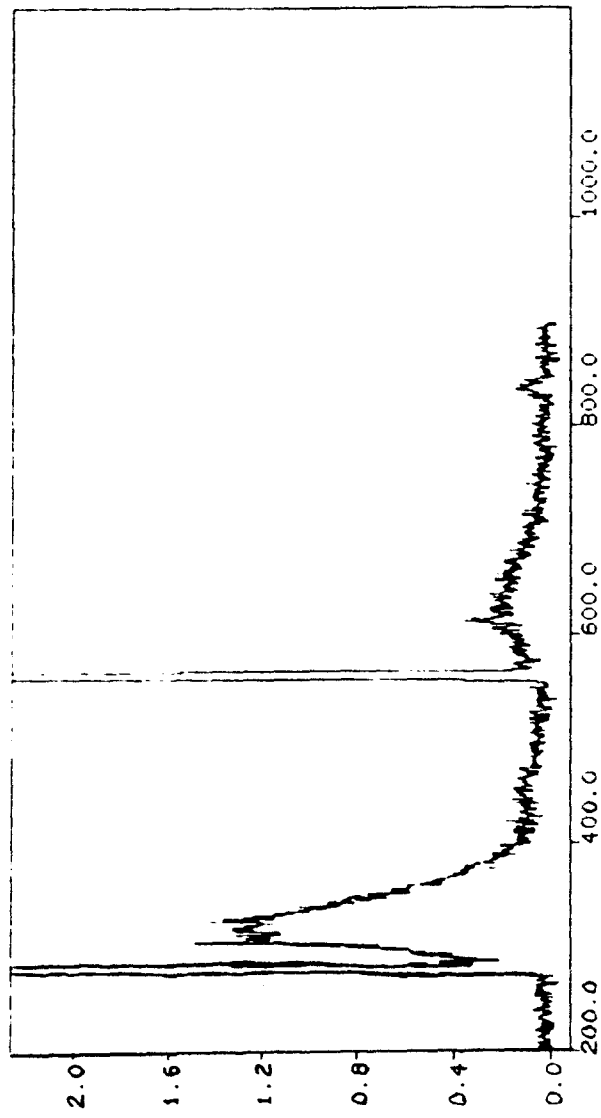


Figure 5.23: Fluorescence Spectra of 4-t-butylcalix[6]arene

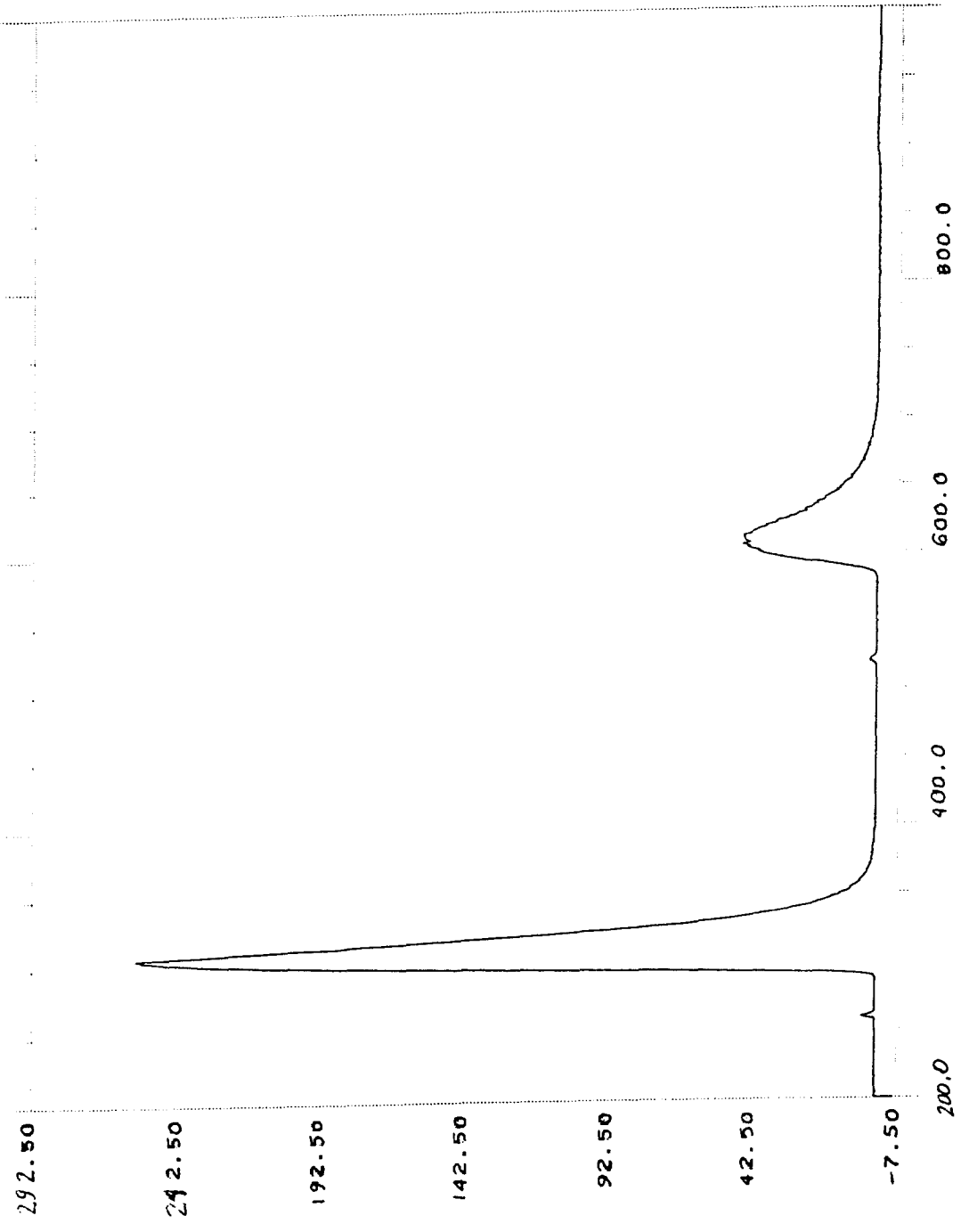


Figure 5.24: Fluorescence Spectra of 4-t-butylcailx[4]arene

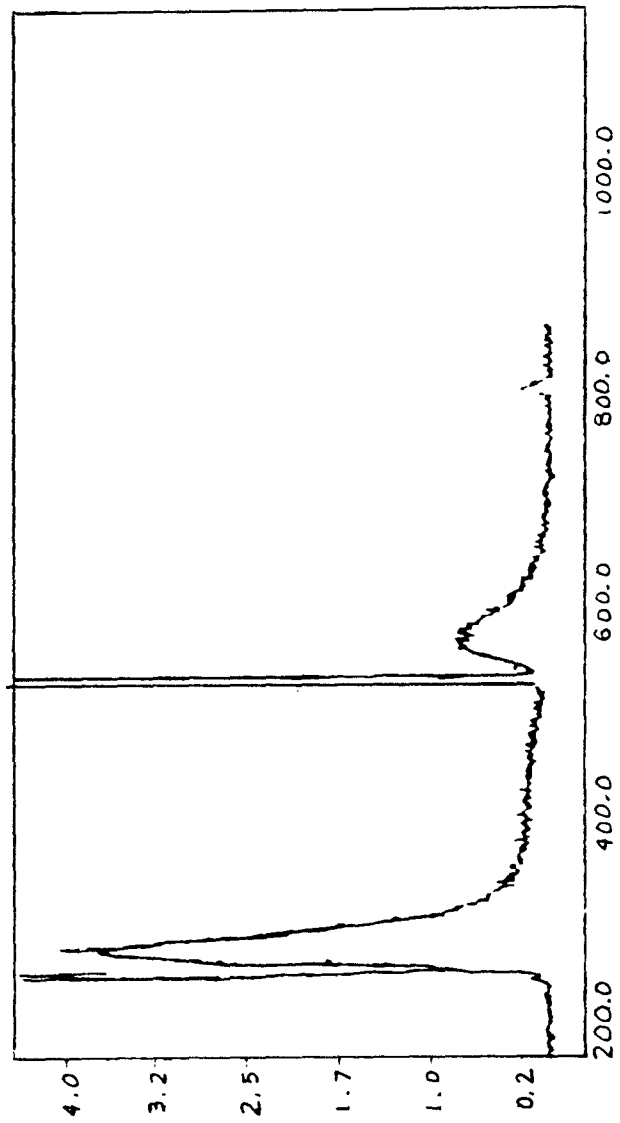


Figure 5.25: Fluorescence Spectra of Spiked Stationary Phase

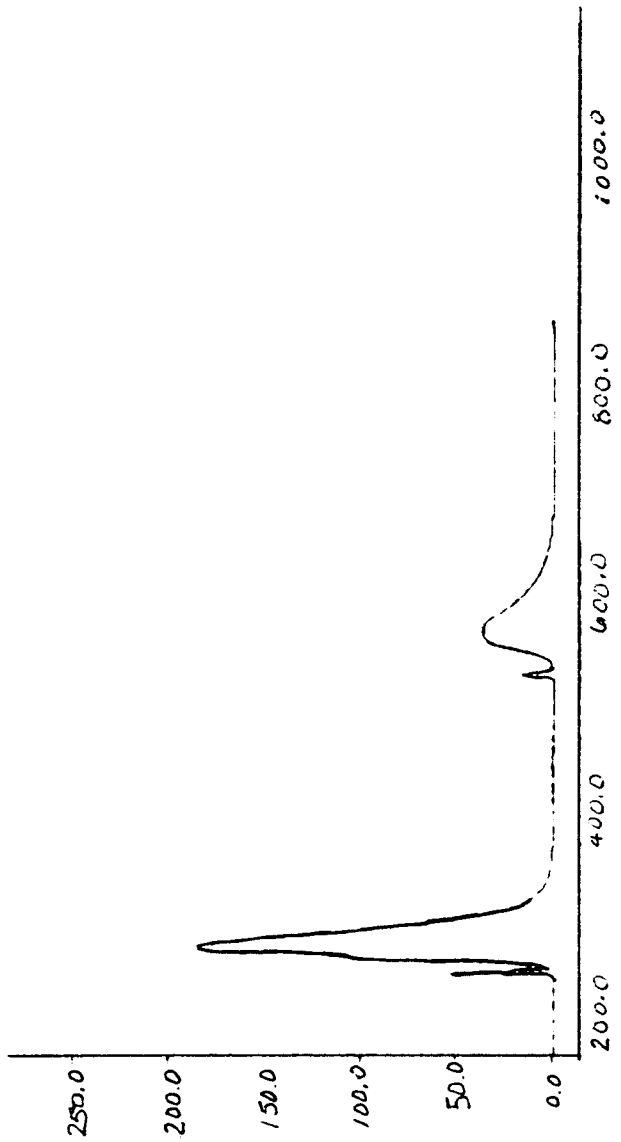


Figure 5.26: Fluorescence Spectra of 002B1

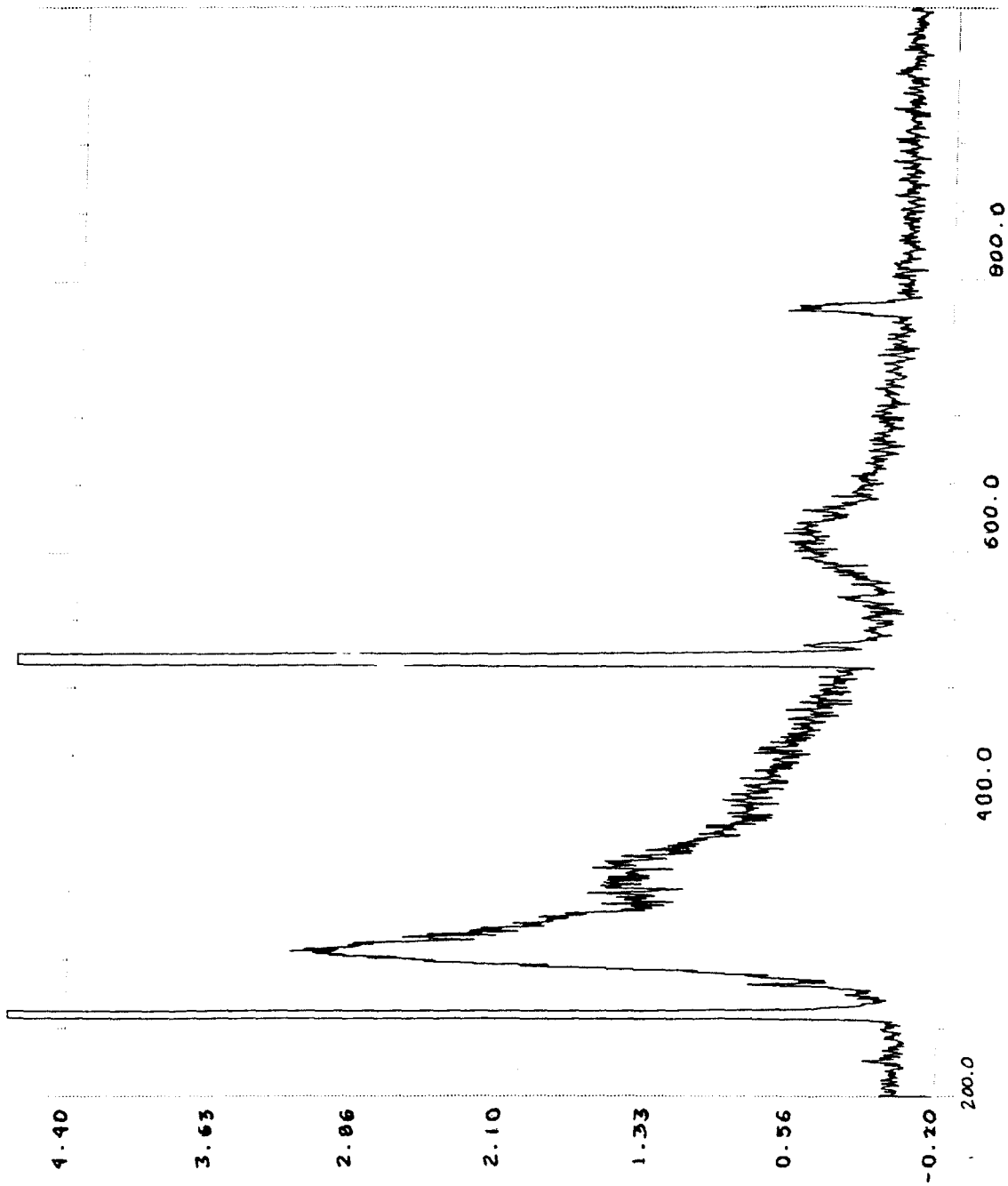


Figure 5.27: Fluorescence Spectra of 001C1

All the 4-t-butylcalixarene columns demonstrated similar results for the fluorescence experiments. From the data it appeared that the various stationary phases each had similar amounts of 4-t-butylcalixarene bound to the silica surface. This helped to confirm the reproducibility of the organosilane tethering reaction scheme.

Percent Organic Content:

The results of the organic content experiment showed that the surface coverage concentration of the stationary phases are around 1%. Table 5.15 shows the results from the percent organic experiment. Results from all of the stationary phases was not possible due to lack of sample amount. Only a few milligrams of sample was available and the analytical balance was not sensitive enough to accurately weigh the samples. As can be seen, the C-18 stationary phase has the largest percentage content of organic material. This was expected since the Nucleosil® C-18 stationary phase is composed of a long (18 carbons) straight molecular chains, most of which exist in a tangle on the silica surface. Also, the reactions utilized to produce commercially competitive alkyl stationary phases are extremely efficient at packing functional groups (per unit area) on the silica surface. Sterically, 4-t-butylcalix[n]arenes are much bulkier than octadecane molecules. It would therefore be expected that not as many would fit (per unit area) onto the surface of a single silica particle as with octadecane. This leads to less 4-t-butylcalixarene molecules per silica particle than the C-18, causing the overall surface concentration (per unit area) of 4-t-butylcalixarene to be considerably less than octadecane. For this reason the

stationary phases containing 4-t-butylcalixarenes were expected to have a smaller organic content than the C-18 column. The stationary phases prepared with the long tether were expected to be slightly higher in organic content than the stationary phases prepared with the short tether. This was due to the long tether containing twice as many methyl groups as the short tether (8 vs. 4).

Table 5.15

Percent Organic Content

Stationary Phase	% Organic
001A1	2.08
002A1	2.45
002B1	0.771
001C1	0.701
C-18	16.5

Temperature Studies:

Temperature studies performed on the 001C1 column showed that as the temperature of the column decreased, the linearity of the log plots increased. As temperature decreases, the kinetics of reactions also decreases. Because of this, the interactions in the column slowed down as the temperature decreased, not allowing mixed mode retention to occur. Figure 5.28 is a plot of the log of retention times versus the number of carbon atoms for the phenyl ring homologous series separated at various temperatures. The equation for each line shows the increase of linearity as the temperature decreases. In addition, Table 5.16 indicates the coefficients and exponents for the exponential curves that fit the lines better as the temperature increases.

Table 5.16

Coefficient and Exponents from Temperature Study

Temperature	Coefficient	Exponent
0	0.6105	0.0581
25	0.4804	0.0628
35	0.4568	0.0631
45	0.4422	0.0627
55	0.4286	0.0624
65	0.4142	0.0619

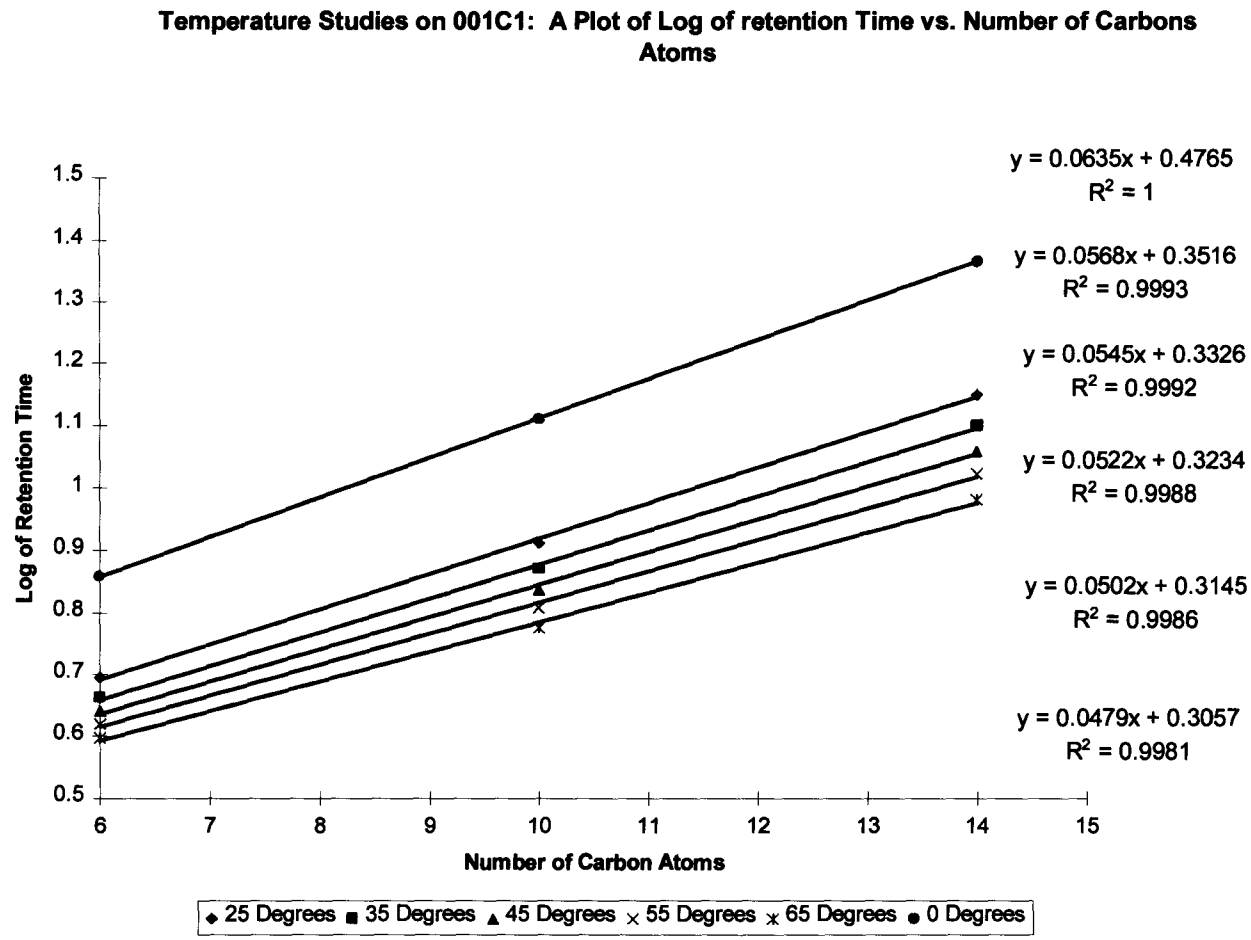


Figure 5.28: Temperature Study on 001C1 Using Acetonitrile/Water to Separated The Phenyl Ring Series

Knox Test:

The Knox test examines stationary phases for residual silanols by performing chromatography on reversed phase columns in a normal phase mode. The very non-polar mobile phase (heptane) will allow polar solutes to partition with any free silanol groups. If there are very few free silanols, there will be no retention. Also, the presence of free silanol groups with the bonded phase can lead to a significant amount of peak tailing. Thus, by looking at retention and peak tailing (i.e., asymmetry factor) one can tell qualitatively whether or not a significant number of silanol groups remain on the derivatized surface.

The figure of merit for few free silanols is retention with a capacity factor less than 0.5 and no significant peak tailing. If significant retention ($k' > 0.5$) and/or peak tailing are observed, the bonding reactions have resulted in incomplete coverage of the surface and the presence of large numbers of free silanols.

The results obtained from the 4-t-butylcalixarene stationary phases were indicative that the synthesis was successful. Figures 5.29-5.31 are the chromatograms from the Knox experiment. As can be seen from the chromatograms, the methanol solute was largely unretained when using dry heptane as the mobile phase, which indicated that there were few residual silanols available to the solute. These results mean that the silica surface was completely covered during the synthesis of the stationary phases. The k' values are summarized in Table 5.17.

The results also showed that the peaks were fairly symmetrical. Table 5.17 summarizes the asymmetry factors from the Knox test. From this table, it can be seen that the asymmetry factors are close to one, indicating little interaction. The reasons that they were not exactly one was because the columns were not end-capped. Although there were no freely available residual silanols on the surface of the stationary phases, there were silanols buried in the surface coverage of calixarene and tether molecules. These buried silanols were expected because of crowding of the large molecules at the silica surface. These may be reacted by "end-capping" with a small chlorosilane reagent such as trimethylchlorosilane.

Table 5.17

Capacity Factors and Asymmetry Factors

Column	Capacity Factor	Asymmetry Factor
001A1	0.17	1.89
002A1	0.11	1.34
001B1	0.17	1.62
002B1	0.20	1.44
001C1	0.16	1.30
001D1	0.09	1.28

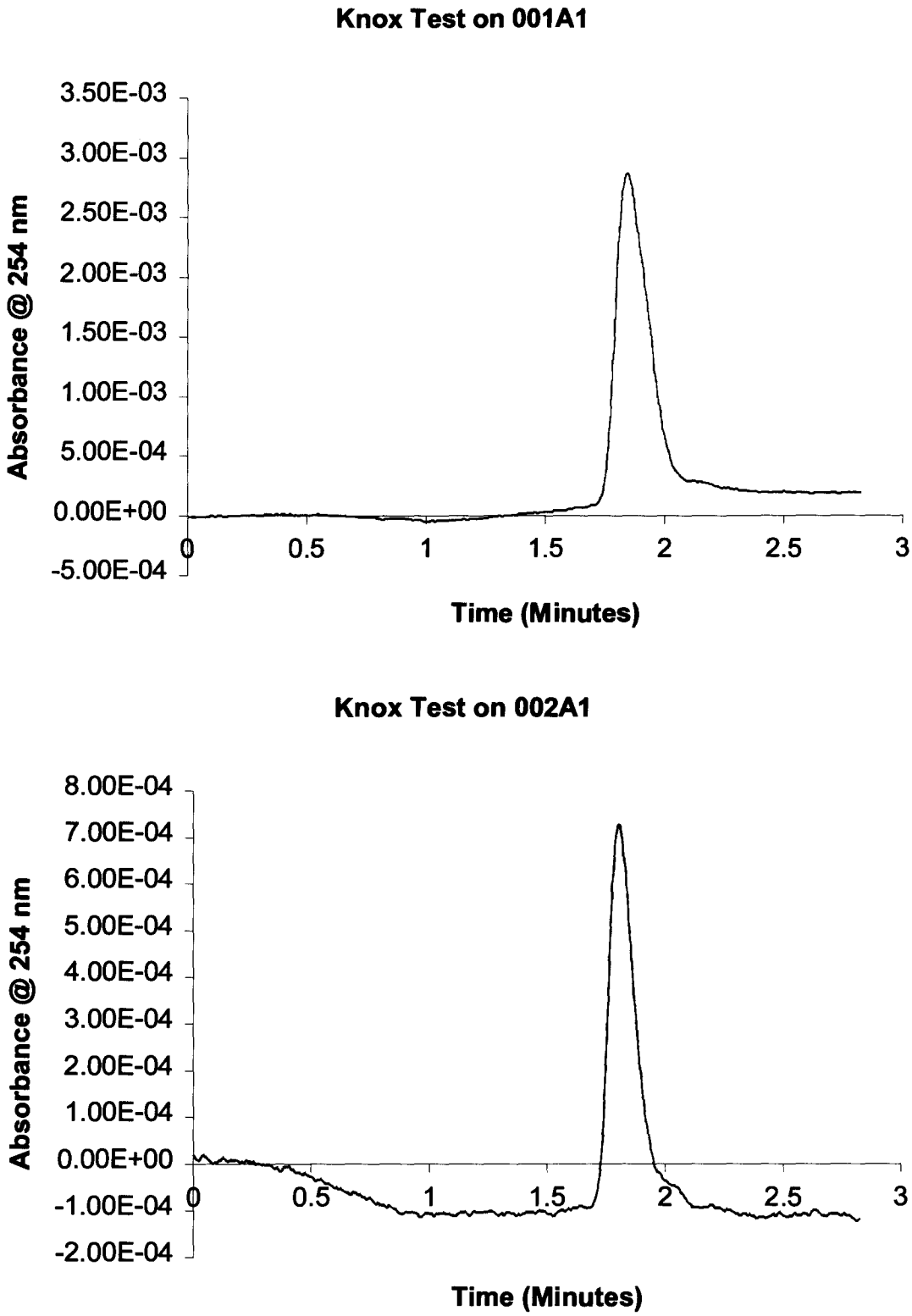


Figure 5.29: Knox Test on 001A1 and 002A1

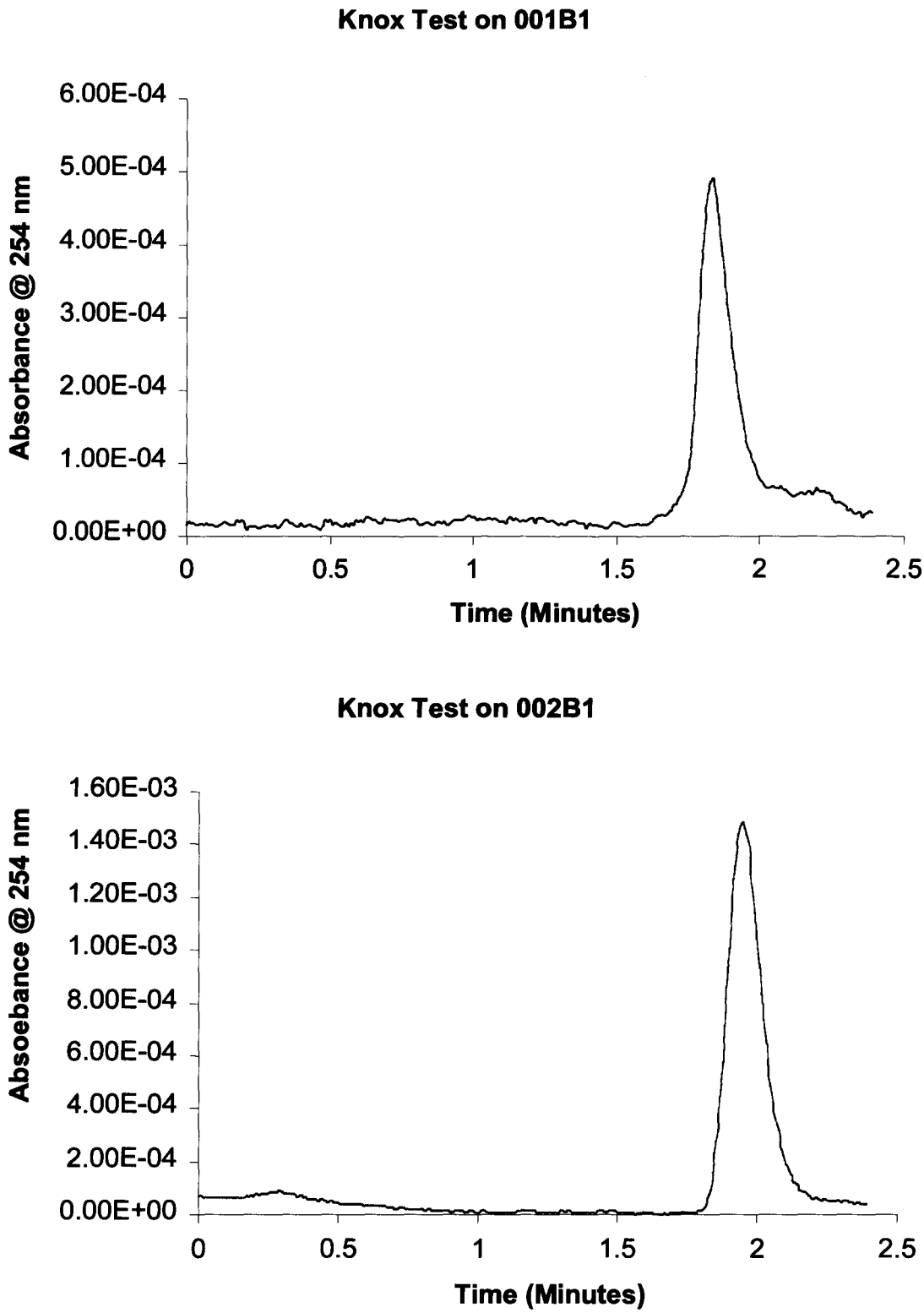


Figure 5.30: Knox Test on 001B1 and 002B1

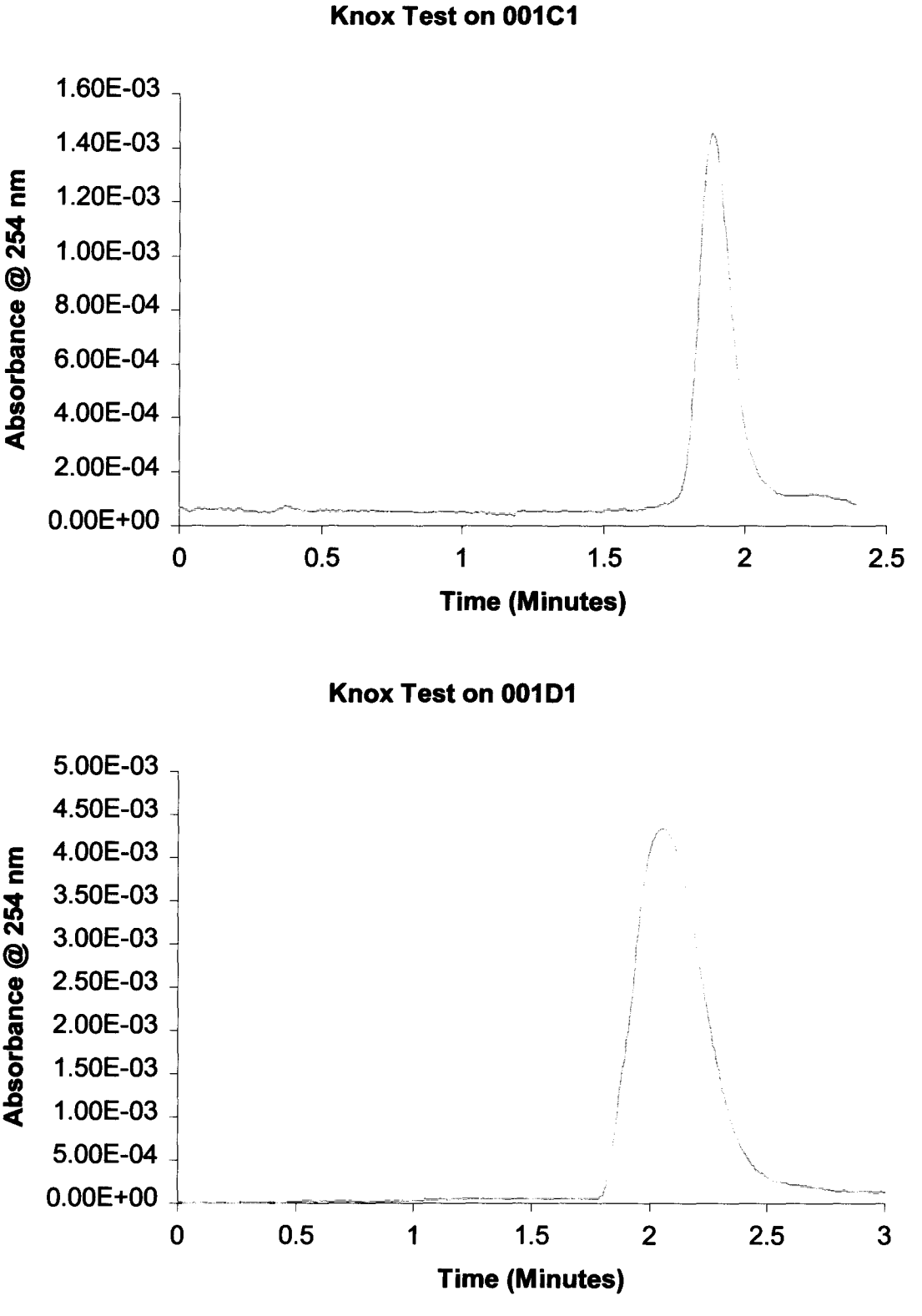


Figure 5.31: Knox Test on 001C1 and 001D1

Chapter VI

Conclusions

The course of the reported work was dictated by several factors, including cost, availability of materials, and serendipity. The work was initiated using 4-t-butylcalix[6]arene because of its wide availability and low cost. After the chromatographic and spectroscopic experiments were performed it appeared that the bonding reactions were successful. The fluorescence spectrum of 4-t-butylcalix[6]arene could be demonstrated on the surface of the silica, even after extensive washing with several solvents, and reversed phase activity could be demonstrated with the test mixtures using the resulting materials. The evidence for host/guest formation, based on retention times, however, was at best only cursory.

Since the immobilization results looked promising, the work was continued with the materials synthesized from 4-t-butylcalix[6]arene. However, there was not much literature available on the host/guest formation properties of 4-t-butylcalix[6]arene and it was possible that the choice of calixarene had been hasty. Extensive review of the literature led to the conclusion that the study of host/guest behaviors of 4-t-butylcalix[4]arene had been more adequately studied and characterized than had similar behaviors for 4-t-butylcalix[6]arene. Thus, a 4-t-butylcalix[4]arene stationary phase material was synthesized and examined chromatographically and spectroscopically.

The 4-t-butylcalix[6]arene materials were utilized for an examination of the effect of the tether length on the observed chromatographic behavior. This was because it was initially uncertain if either tether, especially the longer one, would "fall over" blocking a significant number of reactive binding sites for the 4-t-butylcalix[n]arene attachment. Although it was likely that some "fall over" must have occurred, both tethers successfully bound the calixarenes to the silica surface.

There appeared to be only slight polarity differences in the retention behaviors of the test mixtures using either tether. It was concluded that for the most part, there was little variation between the materials synthesized using either one. The only difference observed in terms of polarity was that the longer tether showed slightly longer retention times for the more non-polar components of the reversed phase test mixture. No changes in retention order or band shape were observed. As a result, the remaining studies were done using the short tether silane (i.e., 1,2-dichlorotetramethyldisilane) because of its more ready availability.

After the tether length and different 4-t-butylcalixarene experiments were completed, work being done simultaneously by Mike (60) using larger pore size (1000 Å) silica gave more interesting results. Although Mike saw results similar to those expressed within this work, it appeared that a greater percentage of 4-t-butylcalixarene was attached to the silica surface using larger pore material. More importantly, it also appeared that the chromatographic behavior in terms of polarity, retention order, and band broadening was significantly altered by

(and dependent upon) variation of the pore size. Therefore, a 4-t-butylcalix[6]arene column was synthesized using 1000Å pore size silica.

Chromatographic Characterization of the Stationary Phases:

Chromatograms obtained from the reversed phase test mixture showed that the newly synthesized columns had reversed phase character. Such character was determined by retention of nonpolar compounds using polar mobile phases such as mixtures of methanol, acetonitrile, and water. This behavior was expected, due simply to the largely hydrocarbon nature of the 4-t-butylcalixarenes. This was, in fact, among the first evidence that the experiment had actually worked.

The reversed phase test mixture components should have eluted from a "standard" C-18 column in the following order: uracil, phenol, N,N-diethyl-m-toluamide, toluene. This was found to be true experimentally, with both types of mobile phases (acetonitrile/water and methanol/water) showing this elution order. The 4-t-butylcalixarene columns, except for column 001D1 (synthesized using 1000 Å pore silica material), also showed this elution order when the mobile phase was acetonitrile/water. However, when methanol/water mobile phases were used, the elution order changed to the following: uracil, phenol, toluene, N,N-diethyl-m-toluamide. The 001D1 material showed this elution order with both mobile phases.

This was originally thought to have been caused from host/guest interaction of the N,N-diethyl-m-toluamide with the bound 4-t-butylcalix[6]arene. Further research by Mike (60) showed that it was, in fact, due to the presence

of t-butyl phenolic groups within the structure of the 4-t-butylcalixarene. In that study, similar behavior had been demonstrated using stationary phases synthesized from 4-t-butylphenol. It is worth noting that since a change in elution order did occur, and t-butyl phenolic units were a part of the 4-t-butylcalixarenes, additional evidence was lent for proof of the attachment.

There may also have been indication of the occurrence of host/guest complexation since the reversal appeared with methanol/water mobile phases and not with acetonitrile/water mobile phases. 4-t-Butylcalix[n]arenes have been known to form host/guest complexes with both methanol and acetonitrile. It may simply have been that the interactions between acetonitrile and the bound 4-t-butylcalix[n]arenes was stronger than the interactions between methanol and the bound 4-t-butylcalix[n]arenes. Acetonitrile would thus have a much stronger tendency than methanol to exclude solutes from the cavity of the bound 4-t-butylcalixarene. As a result, the interactions allowed between the solutes and the bound 4-t-butylcalixarenes by the mobile phase solvent would be more pronounced for methanol/water mobile phases than for acetonitrile/water mobile phases.

The alkyl benzene homologous series chromatograms showed that the solute retention by the 4-t-butylcalix[n]arene columns included not only London force interactions, but host/guest complexation as well. Retention on the C-18 column appeared to be limited to London force interactions only. This was illustrated by the non-linear plots of log retention time vs. number of carbon atoms on the alkyl side chain. The C-18 column produced a relatively straight

line plot, which was the behavior expected from a homologous series where only one predominate force of interaction was occurring. The 4-t-butylcalixarene columns, however, had non-linear plots. This was indicative of complexation occurring, since the addition of complexation interactions to the existing London interactions allowed for a non-constant parameter in the Van't Hoff equation. It was also noted that at the same mobile phase compositions, it took much longer for the solutes to elute from the C-18 column.

The magnitude of the complexation interactions between the solute molecules and the stationary phases were the largest with the phenyl ring homologous series. The greater curvature of the trendline connecting the homologues, as compared to the alkyl benzene homologous series indicated a significantly stronger magnitude of host/guest complex formation. Anthracene showed the greatest complexation of the three homologues, probably because of its better fit with the 4-t-butylcalixarene when compared to the other solutes.

The size of the pores in the silica appeared to play a role in the formation of host/guest complexes as well, since order reversal (vs. C-18) was seen for both methanol/water and acetonitrile/water mobile phases for the stationary phase prepared using 1000Å pore size silica (Column 001D1). Given the larger pore size, it seemed likely that the cavity of the bound 4-t-butylcalixarenes would be more accessible to the solutes.

The relative consistency of the chromatograms from visual comparison, as well as from comparison of capacity factors, demonstrated that the process used to immobilize the 4-t-butylcalixarenes on the silica surface was

reproducible. Figure 6.1, which visually demonstrated the reproducibility of the reaction, shows superimposed chromatograms of reversed phase test mixture separated on columns 001A1 and 002A1 using a 40/60 acetonitrile/water mobile phase. The packing materials in these columns were synthesized at different times using the same chemicals under the same conditions. The superimposed chromatograms confirmed that the reaction was reproducible. The differences in the peak heights were due to partial evaporation of the solutes from the sample. The slight differences in retention were due simply to the fact that they were two different columns. In addition, previous to the chromatograms shown in Figure 6.1, column 002A1 was cleaned using methylene chloride, thereby decreasing its efficiency.

Another means of evaluating reproducibility was to compare the k' values calculated from the chromatograms of the reversed phase test mixture separated on the same stationary phase (i.e., 001A1 vs. 002A1 and 001B1 vs. 002B1). This data is summarized in figure 6.2. Only slight variation was observed.

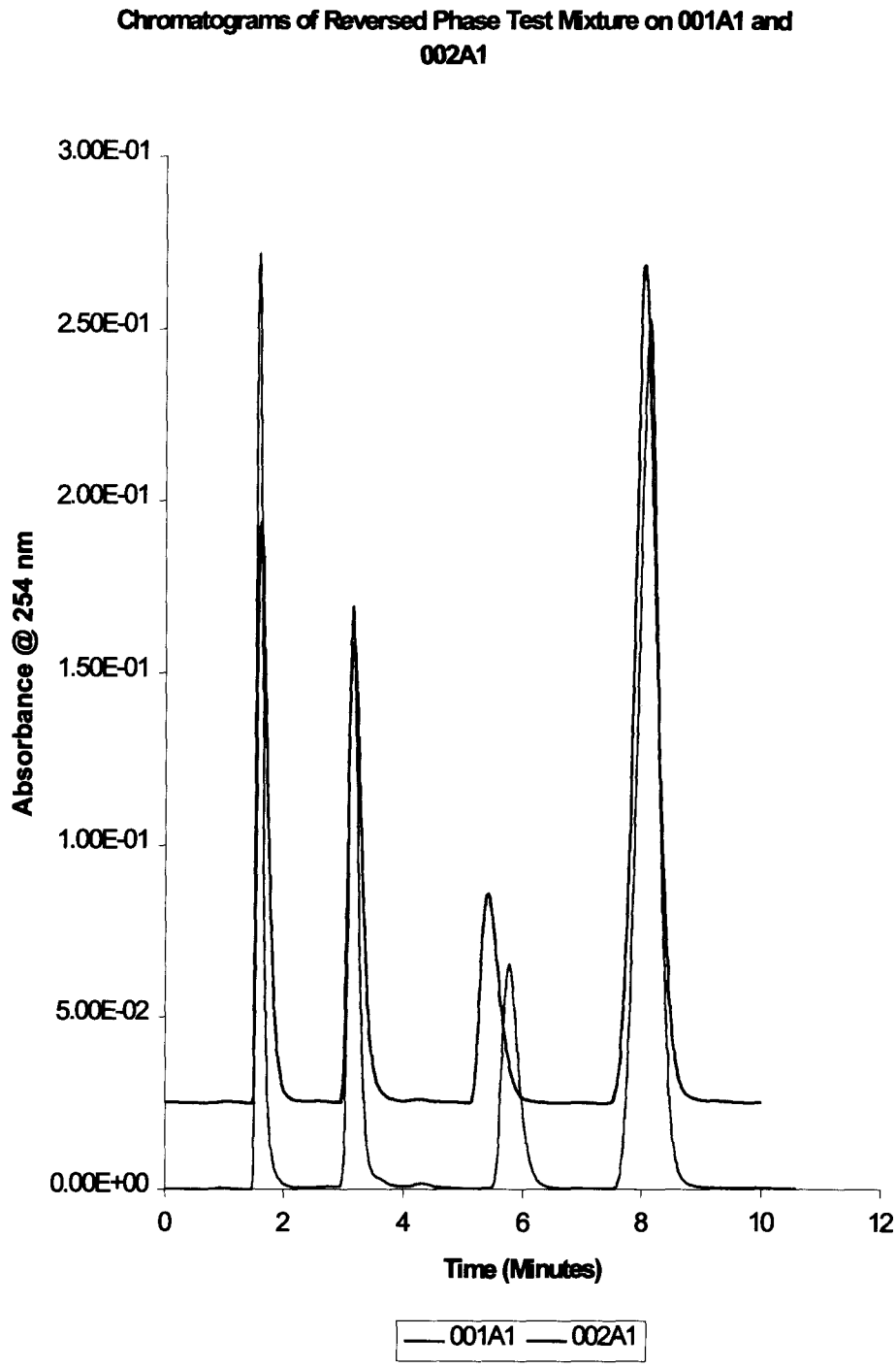
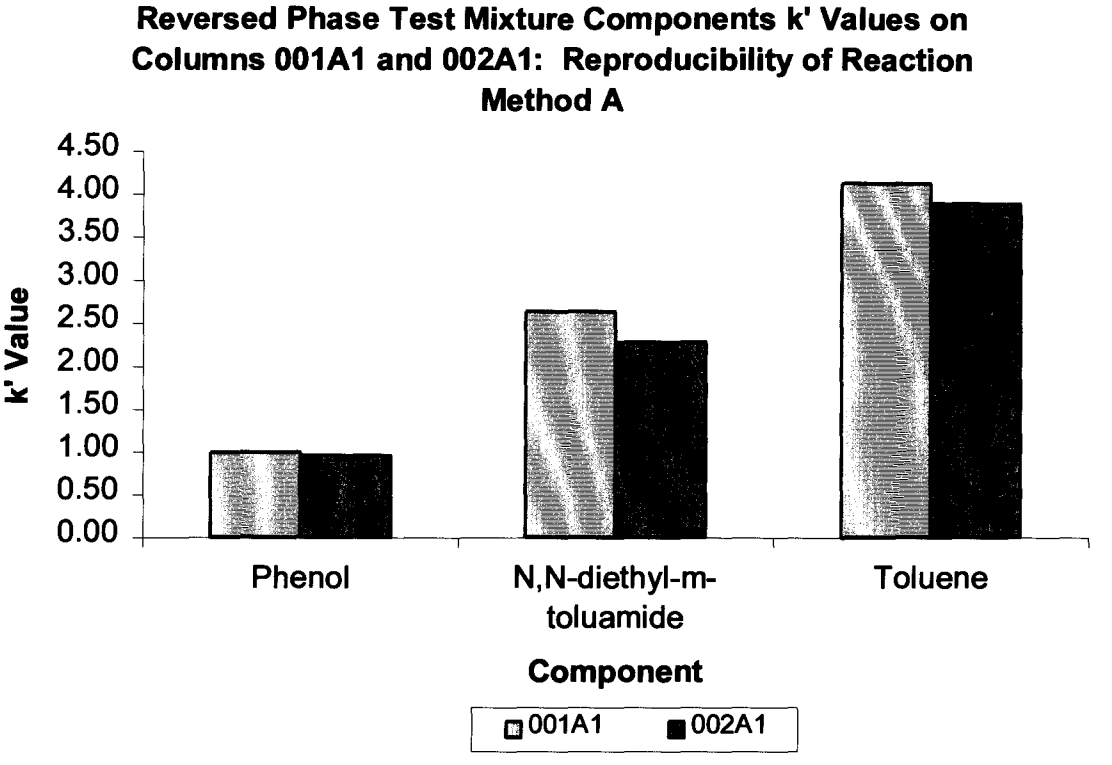


Figure 6.1: Chromatograms of Reversed Phase Test Mixture to Show Reproducibility of Reaction Method



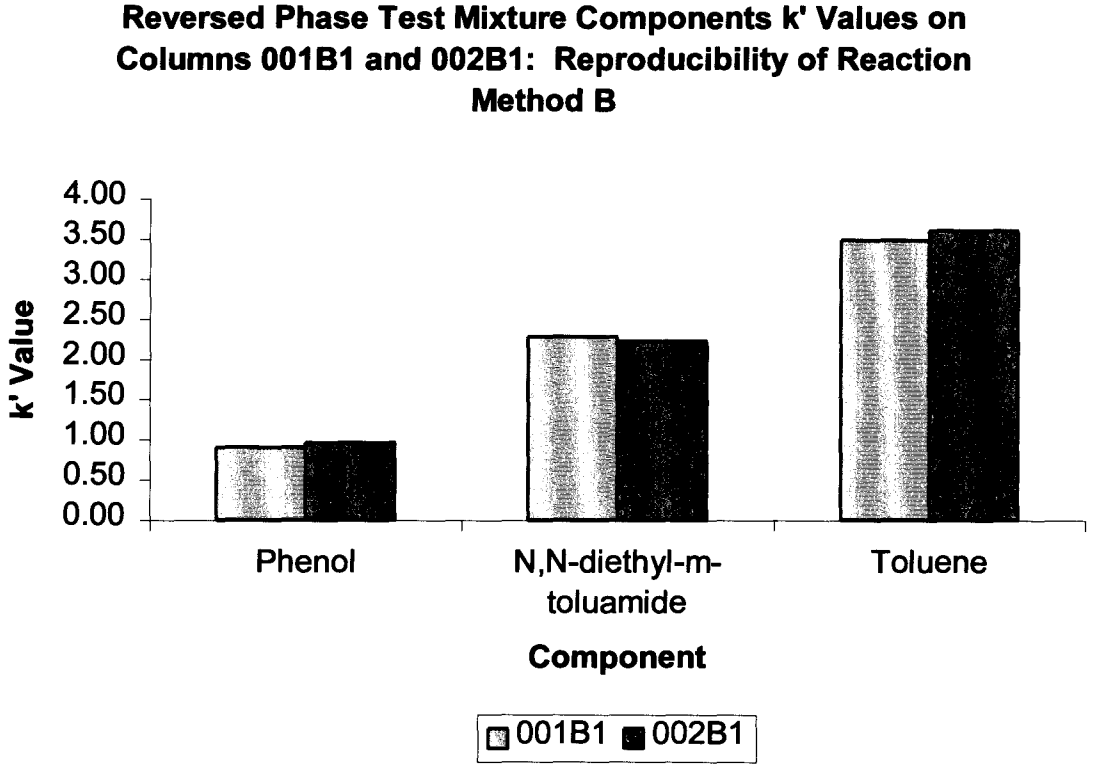


Figure 6.2: Reproducibility of Methods A and B

Spectroscopic Characterization of the Stationary Phases:

Solid State NMR (CP/MAS):

One way to characterize stationary phases was with solid state NMR. This technique had been frequently used to show what was on the surface of chromatographic silica. The results obtained from the stationary phases showed peaks where the t-butyl groups were expected. However, the concentration of the 4-t-butylcalixarene was very close to the detection limits of the NMR. Because of this, conclusive evidence of the 4-t-butylcalixarene on the surface could not be obtained.

Fluorescence:

Fluorescence spectroscopy was used in the characterization process of the newly synthesized stationary phases because of the fluorescing characteristic of 4-t-butylcalixarenes. Since silica does not fluoresce, seeing fluorescence from the stationary phase is a positive indication of a successful reaction.

The fluorescence experiments performed gave strong evidence that the 4-t-butylcalixarene was attached to the silica. At an excitation maximum of 282 nm, the samples gave a peak around 600 nm. The presence of the peak gave conclusive evidence that the 4-t-butylcalixarene was attached to silica.

Chapter VII

Future Work

Recommendations for Future Work:

To extend this research project, many avenues can be taken. One avenue is to do the synthesis using the hydrosilation approach. Sandoval and coworkers (41) have done extensive hydrosilation research and many papers can be found for additional information.

Once this synthesis approach is completed, the product could then be packed into an HPLC column, rinsing well with methanol. Reversed-phase test mixture should be separated first using the recommended mobile phase of 65/35 acetonitrile/water. If the test mixture does not separate, the acetonitrile concentration should be varied until separation is achieved. A reversed-phase test mixture could also be separated with methanol/water mobile phases to see if the order changes as it did in the silanization reaction of 4-t-butylcalix[4]and[6]arene stationary phases test mixture chromatograms.

Chromatograms could also be obtained from a mixture of the alkyl benzene homologous series, and the mixture containing the phenyl ring homologous series in order to determine the presence of and magnitude of any host/guest interactions. These chromatograms should be compared to those obtained from the silanated silica-4-t-butylcalixarene stationary phases that are contained within this document.

Characterization methods could also be performed to compare the surface concentration of the 4-t-butylcalixarene to the surface concentration obtained from the same characterization methods performed on the stationary phases discussed within. This should be done to ensure that maximal coverage of the silica surface by the 4-t-butylcalixarene is occurring.

The purpose of performing the hydrosilation reaction is to compare the two synthetic methods to see if one reaction produces better surface coverage than the other reaction. Performing chromatographic and spectroscopic experiments will indicate which reaction, if any, is better. If one reaction yields higher coverage and shows better results from chromatographic and spectrometric experiments, that synthetic experiment should be adopted. If the results show negligible differences, the easier experiment or the one the chemicals are readily available for should be the reaction performed.

A large amount of work, not reported here, was done to immobilize calixarenes onto the silica surface. Since the problems with the reaction pointed to the aminated 4-t-butylcalix[6]arene being unstable in the atmosphere, the reaction should be performed under inert atmospheric conditions. By completing the reaction under inert atmospheric conditions, the intermediate product should not degrade as it did in the atmospheric conditions. This is caused by the fact that amino groups are not stable in the atmosphere since they break down in the presence of oxygen. Once the aminated 4-t-butylcalixarene is attached to the aminated silica, the stationary phase should

be synthesized and stable to the atmosphere. The product can then be used as a stationary phase in HPLC.

To perform this method, a round bottom flask needs to be attached to a reflux condenser and have a constant purge of argon or nitrogen gas. The 4-t-butylcalixarene, along with dry toluene and 3-aminopropyltriethoxysilane should be added to the round bottom flask. The reaction should then reflux overnight. Another round bottom flask should be set up with dry toluene, silica, and the 3-aminopropyltriethoxysilane, and refluxed overnight. This reaction is not air sensitive and therefore does not need the inert atmospheric conditions as the 4-t-butylcalixarene reaction. These reactions should be done simultaneously. After the reactions are complete, the aminated 4-t-butylcalixarene should be added to the aminated silica through a reaction using the difunctional aldehyde, glutaraldehyde. Similar chromatograms and characterization methods should be performed on this stationary phase.

The above work consists of finding the best method for attaching 4-t-butylcalixarenes to silica in terms of concentration of 4-t-butylcalixarenes on the surface of silica. The higher the concentration, the greater the number of 4-t-butylcalixarenes that will be available to interact with the analytes in samples. This is important because the more interaction the easier it is to see the formation of the host/guest interactions. After the best method is determined, there are experiments that could be done to further this research, as discussed below.

There are several published methods that give procedures to change the functional groups on the top of the 4-t-butylcalixarenes. If 4-t-butylcalixarenes are modified to specifically interact with solute molecules of interest, those 4-t-butylcalixarenes could be attached to silica making just about any separation possible. Real world problems exist that could be alleviated if these stationary phases were available. For example, since it is known that 4-t-butylcalixarenes form host/guest interactions with polyaromatic hydrocarbons (PAHs), 4-t-butylcalixarene stationary phases could be used to separate them. Since PAHs should not be taken into the body, it is sometimes necessary to do soil analysis where food will be planted. If 4-t-butylcalixarene stationary phases were readily available, any organics could be extracted from the soil and analyzed for PAHs. This method will allow people to know if the soil is any good for farming.

Additionally, many drugs are enantiomeric and it is sometimes the case that one enantiomer may be harmful to humans. Frequently one of the enantiomers has no effect on humans. In order for drugs to give maximum potency along with positive effects it is necessary to be able to separate and quantify enantiomers. Unfortunately, due to their nature, enantiomers are difficult to separate. With 4-t-butylcalixarene stationary phases, enantiomeric separation should be much simpler and easier to perform. 4-t-butylcalixarenes are very symmetrical compounds, which must be made chiral in order to perform enantiomeric separations. Fortunately, the 4-t-butylcalixarenes are relatively easy to modify.

Finally, another area of interest is that of pore size. Silica is commercially available with many different pore sizes. It appears in the presented work that pore size made a difference in the amount of 4-t-butylcalixarene that was attached to silica. The 80Å pore size silica seems to have been blocked which would not allow many 4-t-butylcalixarene particles to be attached to the silica. The 1000Å pore silica did not have its pores blocked (or at least not as many) allowing more 4-t-butylcalixarene particles to attach. It would therefore be interesting to see exactly what effect pore size has on this sort of project.

Appendix

The capacity factors for the individual components of reversed phase test mixture, alkyl benzene homologous series, and the phenyl ring homologous series are listed below. Each mixture was separated in duplicate and the k' values given are an average of the two separations.

Table A.1

Reversed Phase Test Mixture
Column 001A1
40/60 Acetonitrile/Water

Component	Average k'
Phenol	0.991786
N,N-diethyl-m-toluamide	2.6376
Toluene	4.127966

Table A.2

Reversed Phase Test Mixture
Column 002A1
40/60 Acetonitrile/Water

Component	Average k'
Phenol	0.952233
N,N-diethyl-m-toluamide	2.27443
Toluene	3.895588

Table A.3

Reversed Phase Test Mixture
Column 001B1
40/60 Acetonitrile/Water

Component	Average k'
Phenol	0.902725
N,N-diethyl-m-toluamide	2.284221
Toluene	3.484474

Table A.4

Reversed Phase Test Mixture
Column 002B1
40/60 Acetonitrile/Water

Component	Average k'
Phenol	0.961916
N,N-diethyl-m-toluamide	2.228501
Toluene	3.603194

Table A.5

Reversed Phase Test Mixture
Column 001C1
40/60 Acetonitrile/Water

Component	Average k'
Phenol	0.84587
N,N-diethyl-m-toluamide	2.458703
Toluene	3.128645

Table A.6

Reversed Phase Test Mixture
Column 001D1
15/85 Acetonitrile/Water

Component	Average k'
Phenol	0.211887
Toluene	1.061034
N,N-diethyl-m-toluamide	1.740405

Table A.7

Reversed Phase Test Mixture
Column 001A1
50/50 Methanol/Water

Component	Average k'
Uracil	0.114692
Phenol	0.746288
Toluene	3.186414
N,N-diethyl-m-toluamide	8.429384

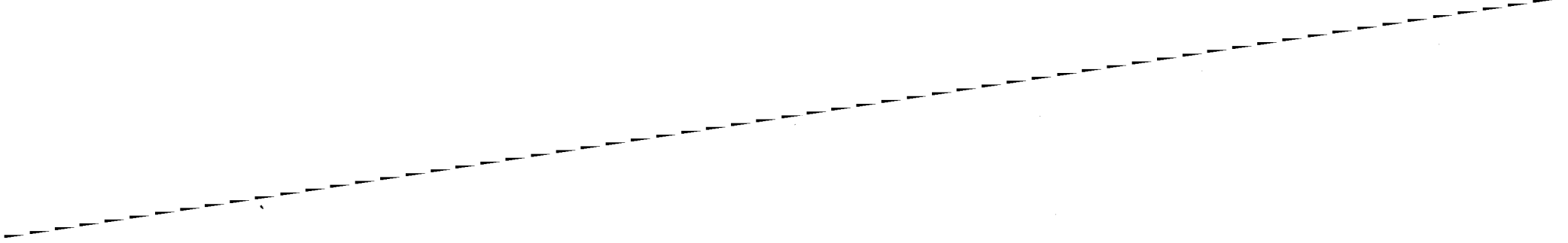


Table A.11

Reversed Phase Test Mixture
Column 001C1
50/50 Methanol/Water

Component	Average k'
Uracil	0.103162
Phenol	0.602702
Toluene	2.314707
N,N-diethyl-m-toluamide	8.25852

Table A.12

Reversed Phase Test Mixture
Column 001D1
20/80 Methanol/Water

Component	Average k'
Uracil	0.015192
Phenol	0.198294
Toluene	0.740938
N,N-diethyl-m-toluamide	3.817964

Table A.25

Phenyl Ring Homologous Series
Column 001A1
40/60 Acetonitrile/Water

Component	Average k'
Benzene	2.299842
Napthalene	5.618641
Anthracene	11.40126

Table A.26

Phenyl Ring Homologous Series
Column 002A1
40/60 Acetonitrile/Water

Component	Average k'
Benzene	2.354326
Napthalene	5.130541
Anthracene	10.52889

Table A.27

Phenyl Ring Homologous Series
Column 001B1
40/60 Acetonitrile/Water

Component	Average k'
Benzene	2.143536
Napthalene	4.653042
Anthracene	9.629278

Table A.28

Phenyl Ring Homologous Series
Column 002B1
40/60 Acetonitrile/Water

Component	Average k'
Benzene	2.254914
Napthalene	4.94226
Anthracene	10.32832

Table A.33

Phenyl Ring Homologous Series
Column 001B1
55/45 Methanol/Water

Component	Average k'
Benzene	0.870722
Napthalene	1.708809
Anthracene	3.56147

Table A.34

Phenyl Ring Homologous Series
Column 002B1
55/45 Methanol/Water

Component	Average k'
Benzene	0.881757
Napthalene	1.710381
Anthracene	3.597666

Table A.35

Phenyl Ring Homologous Series
Column 001C1
55/45 Methanol/Water

Component	Average k'
Benzene	0.812711
Napthalene	1.559718
Anthracene	3.321461

Table A.36

Phenyl Ring Homologous Series
Column 001D1
30/70 Methanol/Water

Component	Average k'
Benzene	0.315032
Napthalene	2.136194
Anthracene	25.91658

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