Synthesis and Characterization of an HPLC Stationary Phase Based on tert-Butyl-tetra-[2-aminoethoxy]calix[4]arene

By

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Abstract

Calixarenes are basket-shaped macromolecules comprised of phenolic units arranged in a "cup" shaped conformation. Calixarenes have become the interest in many areas of chromatography, specifically in reversed-phase HPLC. The reason calixarenes are preferred is due to their ability to form host-guest interactions with solute molecules during HPLC. When performing HPLC with a calixarene stationary phase there is a different selectivity compared to a normal C_{18} column.

An amino derived calix[4]arene was successfully attached to a silica-gel stationary phase. This new reversed phase material demonstrated the expected host-guest interactions as evidenced by the non-linear relationship of the log k' versus number of carbon atoms generated plots. To ensure that these were in fact host-guest interactions and not only mixed-mode retention, which also is shown by tailing peaks, comparison data of before and after endcapping was presented. The endcapping step was necessary to cover the reactive free silanol groups because of the known ability for the free silanol groups to cause mixed-mode retention. The data from the calix-derived silica was compared to a commercial C_{18} bonded phase. This research shows that amines can easily be linked to aminopropyl silica gels using glutaraldehyde as a linker.

Acknowledgements

I would like to give my deep and sincere thanks to Dr. Mike for not only being a great advisor but also being there as a friend. He is the reason I have gone as far as I did. He helped me every step of the way whenever I had questions on any subject. I have known and have been receiving guidance from Dr. Mike for the six years I attended Y.S.U. and he has truly been a blessing.

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List of Abbreviations and Symbols

Symbol	Definition	Units or Reference
$\overline{\Delta G}$	Gibbs free energy contribution of a homologue	-
°C	Degrees Celsius	-
Å	Angstrom	1×10^{-10} meter
amu	Atomic Mass Unit	-
CP/MAS	Cross Polar Magic Angle Spin	-
FTIR	Fourier Transform Infrared Spectroscopy	-
g	Gram	-
HETP	Height equivalent to a theoretical plate	-
HPLC	High Performance Liquid Chromatography	-
Hz	Hertz	s^{-1}
IR	Infrared Spectroscopy	-
K	Thermodynamic equilibrium constant	-
k'	Capacity factor	-
LC/MS	Liquid Chromatography/Mass Spectroscopy	-
m	Meter	-
m/z	Mass to charge ratio	-
mg	Milligram	1×10^{-3} gram
MHz	Megahertz	1×10^{6} hertz
mL	Milliliter	1×10^{-3} liter
mm	Millimeter	1×10^{-3} meter
mol	Mole	6.022×10^{23} molecules
nm	Nanometer	1×10^{-9} meter
NMR	Nuclear Magnetic Resonance	-
PAH	Polyaromatic hydrocarbon	-
ppm	Parts per million	-
psi	Pounds per square inch	-
R	Universal gas constant	8.314 J/mole*K
s	Second	-
SIMS	Secondary Ion Mass Spectroscopy	-
TLC	Thin Layer Chromatography	-

u	Linear flow rate	cm/min
ΔG°	Gibbs free energy	J
ΔH	Change in enthalpy	Cal/mol
ΔS	Change in entropy	J/mole * K
Φ	Volume ratio of the stationary phase to the mobile phase	-

Chapter I

Introduction

High Performance Liquid Chromatography

HPLC (High performance liquid chromatography) is a powerful and widely used separation technique that depends upon the interaction of solutes with two phases: a liquid mobile phase and a solid stationary phase, which usually has a modified surface. The mobile phase is pumped at relatively high pressures (up to 5000 psi) through a column, which is packed with small, uniform porous particles of the stationary phase.

In HPLC the solid stationary phase is packed into a stainless steel column and usually consists of silica gel, although alumina and other support materials have also been used. The stationary phase is rigid and only the surface or modified surface of the stationary phase actually interacts with the analyte in retention processes known as adsorption, which is a surface phenomenon, and absorption, which is a bulk phenomenon. The mobile phase is usually composed of two components, but can be a single component or several.

The elution time from the column depends upon how each sample component interacts with the mobile and stationary phases. The sample to be separated and analyzed is injected into the column and carried by the mobile phase. If the sample does not significantly interact with the stationary phase, it will elute more quickly because it favors and is more soluble in the mobile phase. If the sample strongly interacts with the stationary phase it will take longer to elute from the column and favors the stationary phase. Molecules that favor the

stationary phase are being adsorbed onto the surface or absorbed into the material because of their strong interactions and are going to remain longer. Thus, the analyte will move through the column slower and elute at a longer retention time.

HPLC instrumentation includes a pump, injector, column, detector and data system (1) (see Figure 1). The column is where separation occurs and, because the stationary phase is composed of such small size porous particles, a high-pressure pump is required to move the mobile phase through the column. Separation of components occurs as the components and mobile phases are pumped through the column. Eventually, each component will elute from the column, which is shown as a peak on the chromatogram.

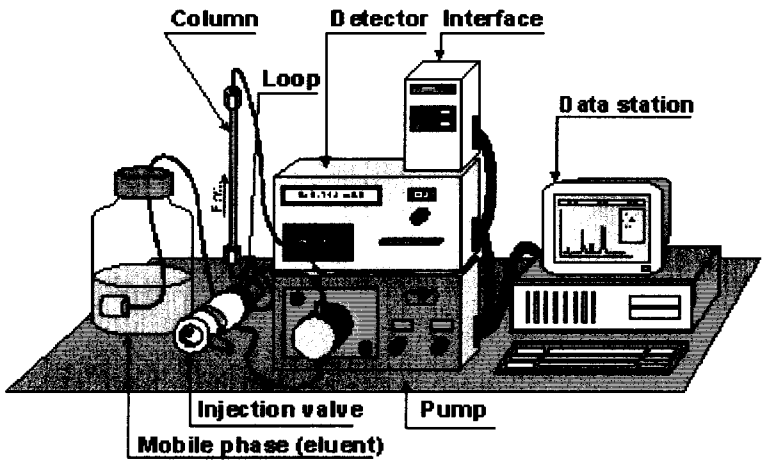


Figure 1: Diagram of an HPLC (1)

Types of HPLC

In both normal and reversed phase HPLC there are different types of interactions occurring. Normal phase HPLC utilizes polar interactions: dipole-dipole and dipole-induced dipole. RP HPLC utilizes London dispersion interactions and intermolecular interaction based on weak van der Waals forces. RP uses non-specific hydrophobic interactions and some analytes might have specificity because of the interactions with the residual silanols or any active groups that are attached to the ligands (2).

Stationary phases

Stationary phases are the materials (e.g., silica gel) that are packed into the column and actually absorb or adsorb and retain the components desired for separation. Silica is made up of silicon and oxygen atoms and is the most widely used stationary phase base material due to its physical stability and its reactivity toward derivatizing agents because of the silanol groups on the surface. Silanol groups are composed of hydroxyl groups and can be arranged in different ways.

The stability of the silica surface is dependent on the types of silanols present and on their reactivity. Figure 2 (1) shows different types of adsorption sites on the surface of hydroxylated silica. Silanols can be termed free silanols, in which only one hydroxyl group is attached or geminal and triol silanols that contain two and three hydroxyl groups respectively. Following the geminal and triol silanol examples, silanol groups with physically adsorbed water are shown. Next, siloxane bonds are prepared by reacting the silanol group of the silica with

an organochlorosilane. Hydrogen-bonded silanol groups are also given, which is defined by the interaction of hydrogen with oxygen, nitrogen, or fluorine.

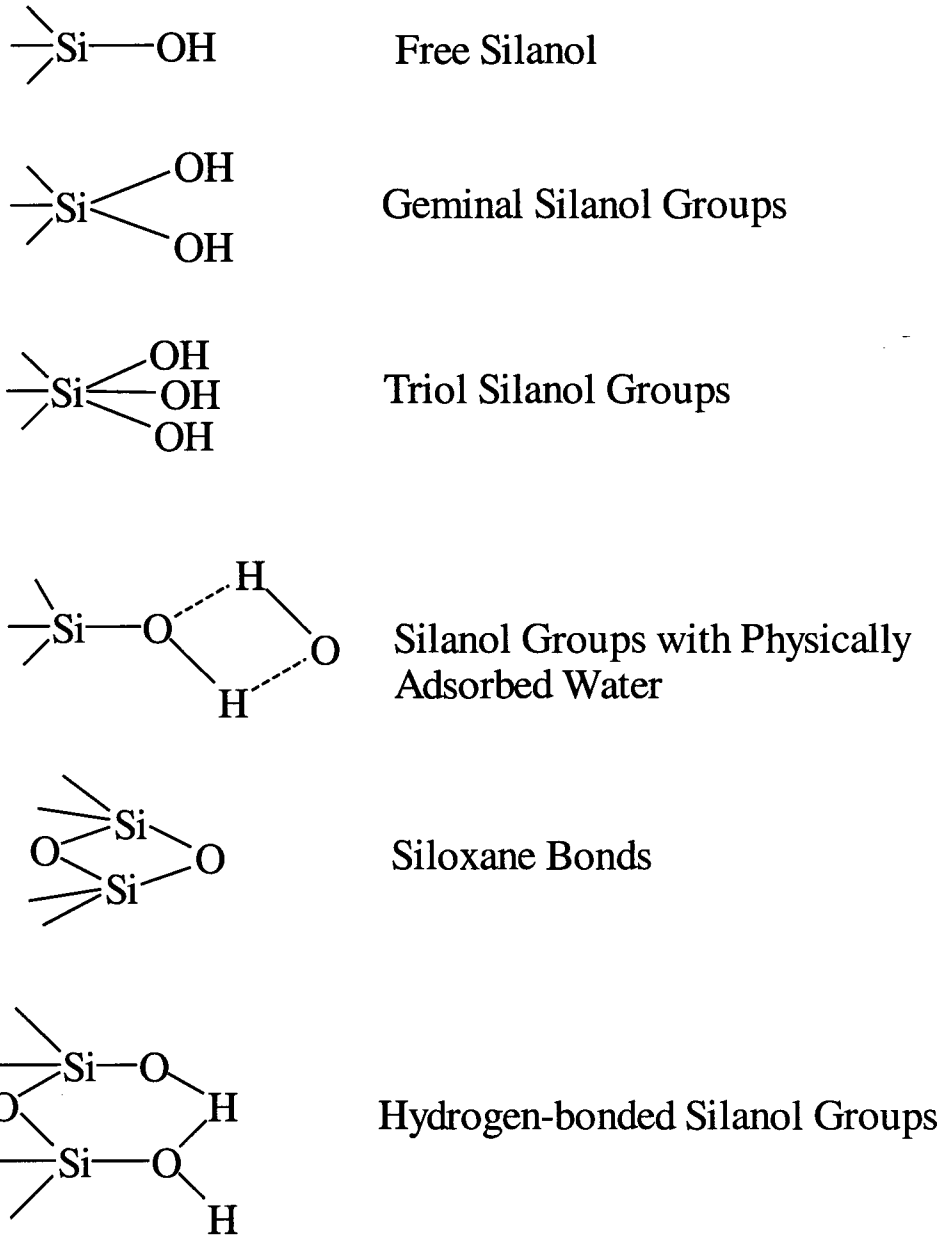


Figure 2: Examples of Silanol Groups

Atoms other than oxygen can be tethered to the silica surface (see Table 1). Organofunctional groups can have different substituents like amines, phenolic hydroxyls, alcohols, or carbonyls. The functional groups can be represented by **R** and there are three common ways **R** can be tethered to the silica. In Si-R, **R** is bonded directly to the surface of the silica and the elimination of the original hydroxyl group is done by chlorinating the silica and then treating it with organometallic compounds. This approach does not usually give stable surface modification.

In Si-O-R the bond is an ester bond and it is formed with a reaction of an alcohol and a hydroxyl group. Treating hydroxylated silica surfaces with organosilanes of R_nSiX_{4-n} brings about Si-O-Si-R, where X is the reactive group such as ethoxy or methoxy (3) (see Figure 3).

Functional Group	*Illustration*
Organofunctional Group	Si-R
Ester Bond	Si-O-R
Organosilanes of R_nSiX_{4-n}	Si-O-Si-R

Table 1: Functional Groups

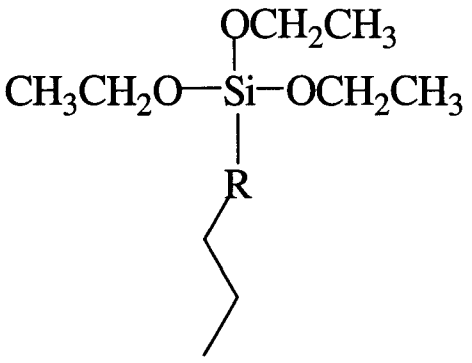
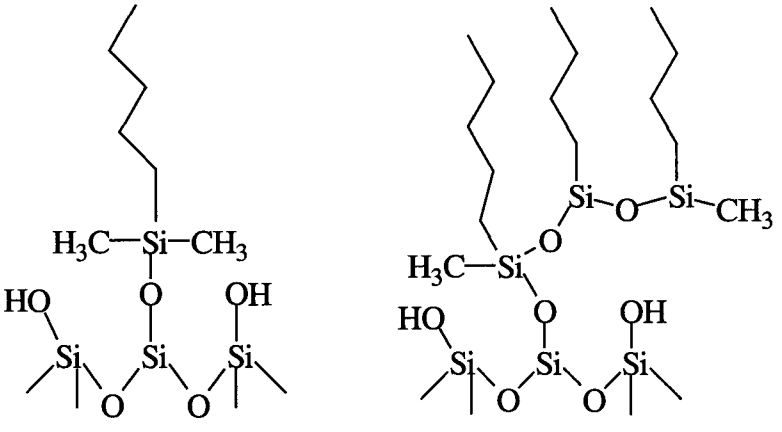


Figure 3: Triethoxy Silane

When the hydroxylated silica surface is reacted with monochlorosilane, it leads to attachment of only one alkylsilane ligand for each silanol group. This type of bonding is called monomeric bonding. Polymeric bonding can be done by reacting the hydroxylated silica surface with either a dichlorosilane or a trichlorosilane, which will lead to tree-like structures situated on one reacted silanol group. Figure 4 shows examples of monomeric bonding and polymeric bonding, respectively. Due to steric hindrance of the monomeric and polymeric bonded layers some of the available silanols groups will not be able to react.



Monomeric Bonding Polymeric Bonding

Figure 4: Monomeric and Polymeric Bonding

The free silanol groups that were not able to react can cause significant problems in RP HPLC separations, even with long substituent chains hindering the way to the free silanols at the surface. Free silanol groups are polar and can interact with components in the solute adding to undesired side effects on the solute distribution within the solute band causing skewed peak shapes on the chromatogram. To minimize the effects, sometimes called mixed-mode retention, endcapping was introduced as a way to cover-up unreacted silanol groups on the silica surface. Endcapping is the process of reacting a small molecule, trimethylchlorosilane, with the free silanol groups left on the silica.

Trimethylchlorosilane is a small molecule and is not readily obstructed by the presence of large chains on the silica surface; it penetrates them with relative ease. Once it reaches the silica surface underneath, it will react with the free silanol groups and will "cover" them so they are no longer reactive. This minimizes undesired interactions between the solute molecules and the reactive free silanols on the stationary phase.

Band Broadening and Retention

Separation of solutes in a chromatographic column is based on interactions between the stationary phase with the solute and mobile phase. Between 5 and 20 μL of sample are usually injected as a relatively narrow band. During the chromatographic run the narrow band will be spread due to the interactions that are occurring with the stationary phase or porous particles. Slow adsorption kinetics, longitudinal diffusion, and other factors are responsible for this effect, which is called band broadening (4). The longer the component is

retained on the column, the broader will be its peak on the chromatogram. The example below shows this phenomenon of a sample containing the solutes **A** and **B.**

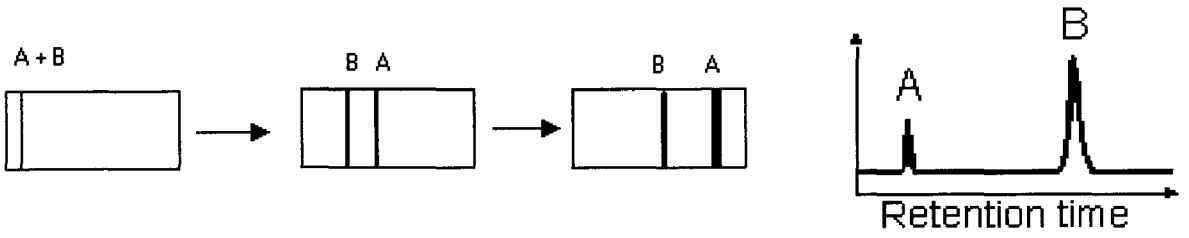


Figure 5: The phenomenon of band broadening

The performance of the separation, or how well the components of the sample are separated from one another, depends on band broadening and the extent of solute retention. The solute retention time is the time elapsed between sample introduction and when the detector recognizes the maximum of the retained peak. Band broadening is a kinetic parameter and helps to determine chromatographic efficiency. It is a function of the peak width, which will suggest the efficiency of the column and it is dependence on different items including column length, flow rate, and particle size. The van Deemter equation can help interpret these ideas:

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

Where u is the linear velocity of the mobile phase; A is the term which is related to the flow path effects called eddy diffusion; B is the related to longitudinal diffusion; and C is the term related to the rate of solute mass transfer between the mobile and stationary phases. J. J. Van Deemter introduced this equation because it combines the three phenomena (e.g., eddy diffusion, longitudinal

diffusion, and mass transfer) (5). It represents them as the dependence of h or the height equivalent to a theoretical plate (HETP) on u, which is the linear velocity of the mobile phase (see Figure 6).

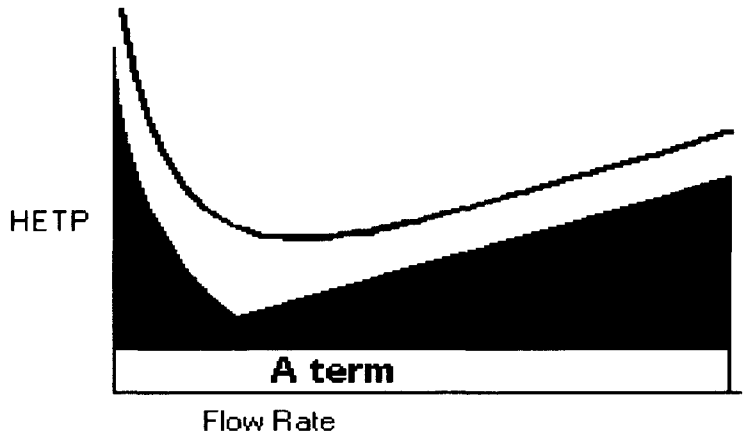


Figure 6: HETP vs. Flow Rate per the Van Deemter Equation

Term A, or eddy diffusion, occurs in packed columns because of the lack of homogeneity of packing. Eddy diffusion arises from the different solvent flow streams within the column. Term B or longitudinal diffusion occurs due to diffusion in the mobile phase, which is the molecular movement from a greater concentration to a lesser concentration. Mass transfer or term C is the most complicated because it occurs across phase boundaries. Mass transfer is due to the lack of equilibrium between the stationary and mobile phases and it refers to the differing partitioning rates between the mobile and stationary phases (see Figure 7).

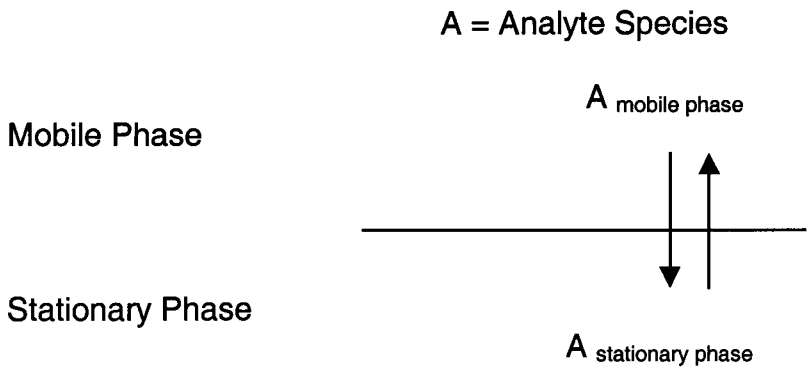


Figure 7: The Partitioning Process

Thermodynamics

The rate that solutes elute off the column deals with equilibrium and is a thermodynamic phenomenon. The two basic descriptions of thermodynamics for HPLC retention are based on adsorption and partitioning. Adsorption is a surface phenomenon and is chemical in nature. Adsorption describes how solute molecules can interact with the stationary phase, which is usually underived. Partitioning is a bulk phenomenon that depends on concentration changes in the system and is due to the dispersal of the solute components between the mobile and stationary phases. Stationary phases are usually derived in order to allow partitioning to occur.

For the partitioning process a common thermodynamic description can be expressed using the van't Hoff equation in terms of the systems free energy. The van't Hoff equation is:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = - RT \ln K$$

and:

$$K = k' / \phi$$

therefore:

$$\ln k' = - \Delta G^\circ / RT + \ln \phi$$

where K is the equilibrium constant, k' is the solute capacity factor, $\Delta G°$ is the free energy of partitioning, ΔH and ΔS are enthalpy and entropy of the partitioning between the mobile and stationary phases, *R* is the universal gas constant, *T* is the temperature in Kelvin, and ϕ is the phase to volume ratio of the volume of stationary phase to the volume of the mobile phase.

If:
$$\Delta G° = \eta\overline{\Delta G} + b \qquad (6)$$

Where $\overline{\Delta G}$ is the free energy contribution for each of η carbon atoms in a molecule or chain and *b* corresponds to ϕ. Therefore a plot of ln k' versus η at a constant temperature will have a slope of:

$$d \ln k' / d\eta = - \overline{\Delta G} / RT$$

If the ln k' versus η is a straight line there is said to only be one partitioning process or interaction taking place. This relationship is crucial when interpreting data especially when interactions like host-guest are expected.

Calixarenes

Calixarenes are basket-shaped macromolecules comprised of *para*-substituted phenolic monomers linked by methylene bridges. The phenolic monomers frequently arrange themselves in a "cup" or cone-shaped conformation. Other conformations are possible, but the cup shape is frequently favored sterically and thermodynamically because of hydrogen bonding of the phenolic hydroxyls at the bottom of the cup and because of the bulkiness of groups attached to the top of the cup. Often, the cup shaped can be locked into place by the introduction of a guest into the calixarene.

To prepare 4-t-butylcalix[n]arenes, condensing formaldehyde onto a p-substituted phenol in the presence of a base is an easy approach (7). Depending on the reaction conditions, the products of the synthetic reaction are mainly composed of 4, 6, or 8 repeating phenolic units. The calixarenes with an even number of phenyl groups are more favored and form the “cup” shape whereas calixarenes with an odd number of groups tend to form planar structures. The tetramer is the most stable product so it is the most favored conformation. The “bottom” of the cup consists of hydrogen-bonded hydroxyls from the phenolic functional groups, and the "rim" is the *p*-phenolic substituent. See Figures 8 and 9 for example structures of calix[4]arene and *p-tert*-butyl-calix[4]arene.

Calixarenes have only recently become the interest in many areas of chromatography, specifically reverse-phase HPLC. The interest has arisen because of the ability of calixarenes to form host-guest complexes (see Figure 10). Figure 10 represents an uncharged host (*p-tert*-butyl-calix[4]arene) containing an uncharged guest molecule (anisole). The complex is shown as an enclosed caged structure. The interaction facilitating host-guest complexation is the interaction of π electrons of the guest molecule (anisole) in contact with the π electrons of the calixarene (8).

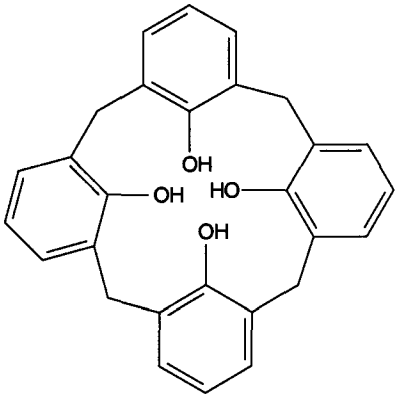


Figure 8: Calix[4]arene

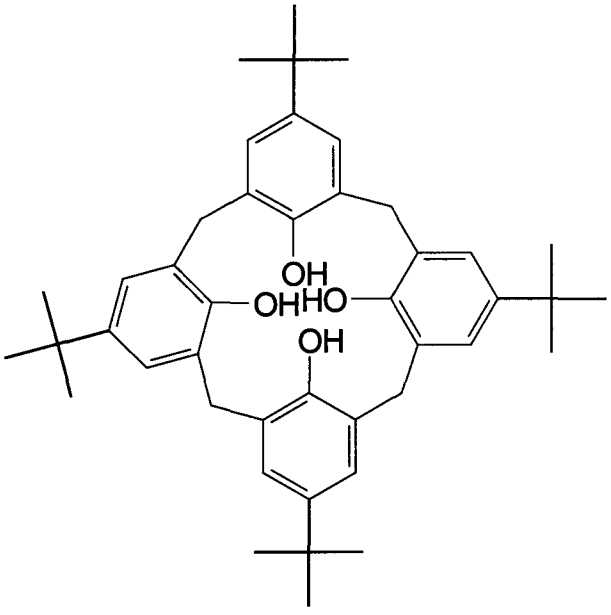


Figure 9: *p-tert*-butyl-calix[4]arene

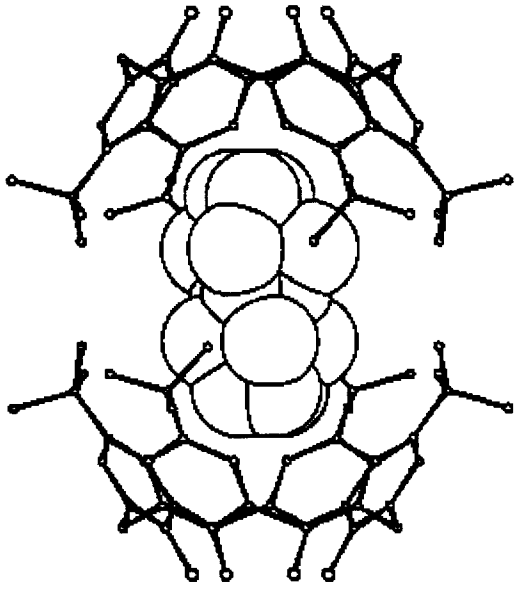


Figure 10: Complex of *p-tert*-butyl-calix[4]arene and Anisole (8).

The interest in calixarenes as bonded stationary phases began because of their close resemblance to cyclodextrins and crown ethers (see figure 11), which are naturally occurring macrocyclic compounds similar in structure to the calixarenes. Cyclodextrins are also known to form host-guest interactions (7). Crown ethers tend to be unappealing as bonded stationary phase materials because their simplest form are loops rather than cavities. Cyclodextrins have been used extensively for chromatographic separations, but tend to be less exciting because they are natural occurring products that cannot handle high temperatures and can degenerate. Calixarenes, on the other hand, are more robust than cyclodextrins and form host-guest complexes in much the same way. Calixarenes are stronger due to their methylene linkage whereas cyclodextrins have ether links, which can more easily be cleaved.

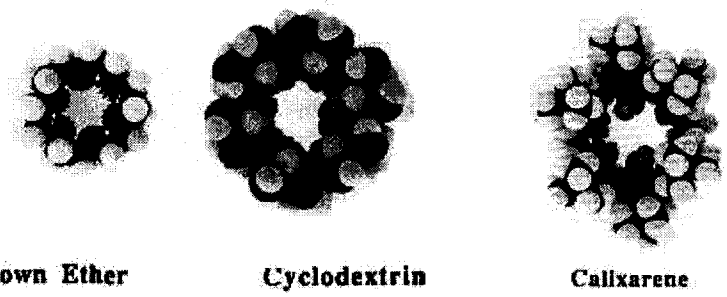


Figure 11: Models of a Crown Ether (18-crown-6), a Cyclodextrin (α-cyclodextrin), and a Calixarene *(p-tert-*butylcalix[6]arene) (7).

One reason that calixarenes are so potentially useful is because they can be modified in many different ways for whatever specific separation might be needed. They can be neutral, positively or negatively charged, or have the substituents attached to the rings altered in some meaningful way (e.g., enantiomers). The molecular weights of the calixarenes are high (around 1000 AMU and higher) and because of this they have high melting points. Modification of the calixarene structure can be done at the "reactive sites", which include sites on the aromatic rings and sites on the hydroxyl groups.

In general, calixarenes are understood to be vase shaped (as in the cyclic tetramer). It is known that cyclic tetramers are changeable in character and can exist in several shapes other than the vase shape. The four possible conformers in the solid state are the cone, the partial cone, the 1,2 alternate, and the 1,3 alternate are shown in figure 12 (7). The tendency of the calixarenes to take on various forms and the ability to stay in one form or the other is one of the reasons

calixarenes have become so interesting.

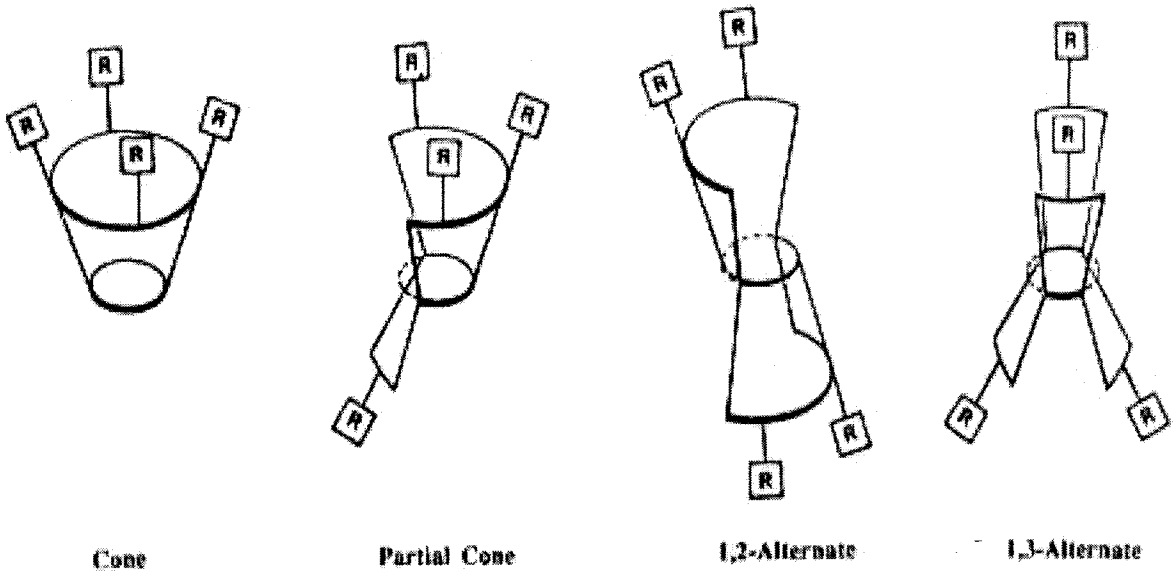


Figure 12: Shapes of the Baskets – Conformers of Calixarenes (7)

The conformation of the basket is very important in the arrangement of the calixarene because host-guest interactions depend on the shape as well as functionality. Calixarenes are known to form host-guest interactions with different types of molecules. The host-guest interaction is determined by the overall macrocyclic structure, the cavity size, and most importantly, by the nature of the functional groups on the host molecule, which can act at the binding sites. Much of the previous research on modification of silica using calixarenes has synthesized stationary phases through the "top" of the cup in which interactions would occur through the "bottom" phenolic groups of the cup.

The use of calixarene derived stationary phases is developing at a slower pace than was seen with cyclodextrin derived phases. It is developing, however,

and their easy accessibility is helping to spur further development. Using base-induced condensation provides high yields of phenol-derived calixarenes in a variety of cavity sizes and acid-catalyzed condensations provide high yields of the resorcinol-derived calix[4]arenes. These methods provide a way for making these “basket shaped” molecules readily available, which is essential for the progress of calixarenes. These methods also allow for the preparation of functionalized calixarenes, which contain groups that allow for their attachment to stationary phases. The functional groups can also serve as points for the attachment or introduction of chiral groups into the calixarene "basket". The functionalized calixarenes allow for a wide variety of molecules to be attached, which makes the calixarenes accommodating to difficult or complicated separations.

Chapter II

Historical

Initial Investigation into Calixarenes

The history of calixarenes starts with Adolph Von Baeyer(7), who performed research on the reaction of phenols with formaldehyde. Baeyer was not able to characterize the complex product from the phenol and formaldehyde mixture because in 1867, analytical tools were somewhat limited in their ability to separate complex mixtures of substances.

Lederer and Manasse (7) independently studied the base-induced reactions between formaldehyde and phenol. The success of their research depended on carefully controlled conditions. When the conditions were not well controlled, the base-induced reaction made a resinous tar that resisted any characterization whatsoever.

The early polymeric material, Bakelite (7), came along soon after and it was realized that methylene groups were the most likely types of linkages between a pair of aromatic rings in a formaldehyde-phenol product. Then Alois Zinke, a University professor, and his coworker Ziegler, decided to study the *p*-substituted phenols in condensation reactions with formaldehyde. Niederl and Vogel (7) had proposed a cyclic tetrameric structure for compounds obtained from the acid-catalyzed treatment of aldehydes and phenols.

This was the first mention of calixarenes, which are basket-shaped macromolecules comprised of phenolic units arranged in a "cup" shape. There are other conformations possible but the cup-shape can be locked using host-

guest interactions. In general, calixarenes are a class of macrocyclic compounds composed of a series of para-substituted monomers linked by methylene bridges.

D. C. Gutsche (7) investigated the benefits of calixarenes in the 1980's. The name "calixarene" is derived from the name of a Greek cup called calix crater, which the calixarene resembles, as well as the arene functionality. Depending on the reaction conditions, the products may be composed of 4, 6, or 8 repeating phenolic units. Because the structure of a calixarene can be cup-like, the "bottom" of the cup-like structure consists of intramolecularly hydrogen-bonded hydroxyls from the phenolic functional group and the "tops" of the cup-like structure are the para-substituents of the phenols.

Host-guest Interactions

The host-guest interactions of calix[n]arenes with a variety of neutral and ionic species have been widely reported (9). The extent of host-guest interaction is determined by the overall macrocyclic structure of the calixarene, most importantly by the cavity size, but also by the nature of the functional groups, which may act as binding sites.

Before calixarenes were introduced to chromatography, separations that utilized host-guest interactions as a mode of interaction were possible by using cyclodextrins or crown ethers in liquid and gas chromatography. One reason that calixarenes may be better suited for this process is because cyclodextrins are natural products composed of dextrose and can undergo degradation from naturally occurring processes (mold, bacteria, etc.) Also the ether linkages that

hold the cyclodextrins together are more easily broken than the methylene bridges in the calixarenes.

The benefits of cyclodextrins are they are inherently chiral whereas calixarenes must be synthesized with a chiral center. Research on separating enantiomeric compounds with chiral calixarene compounds has been done and examples are outlined in the following section.

Chiral Calixarenes

Buschmann et al. (10) investigated the use of chiral crown ethers, chiral calixarenes, and cyclodextrins in enantiomeric identification of various amine compounds using liquid membranes. Factors influencing enantiomeric recognition of some amine compounds by chiral macrocyclic compounds have been reported. Hamilton et al. (11) also investigated chiral separations using capillary electrophoresis by using calixarenes as buffer additives due to their ability to form inclusion complexes. Functional groups were added to the rims of the calixarenes in order to enhance their chiral selectivity and parameters such as pH were changed and compared.

Healy et al. (12) synthesized a new type of chiral stationary phase from triethoxysilyl derivatives by immobilization. A silica bonded calix[4]arene was functionalized at the lower rim with L-(-) ephedrine. Enantiomeric separations using reversed phase conditions were done with a high flow rate. The new functionalized calixarenes were applicable to chiral aromatic and nonaromatic solutes and were capable of resolving enantiomers.

Warner (13) examined the advantages and disadvantages of combining capillary electrophoresis with micelle polymers and chiral calixarenes. He compared calixarenes and cyclodextrins by separating four enantiomeric pairs while using two new types of mobile phase additives, micelle polymers, and chiral calixarene derivatives.

Y. Zhang et al. (14) investigated chiral aminocalixarene derivatives with amino acid groups attached to the lower rim of the p-tert-butylcalix[4]arene. Separation was done utilizing capillary electrophoresis after p-pentoxyl sulfonated calix[6]arene was synthesized. The water soluble chiral calixarenes act as pseudostationary phases to produce chiral separations.

Xiao et al. (15) reviewed and reported on calixarenes in chromatographic separations with emphasis on GC, as well as on the use of calixarenes as HPLC mobile phase additives. They reviewed 22 references on application of calixarenes in chromatography. Hefley (6) researched the ability of calix[4]arene to participate in host-guest interactions and new types of stationary phases for performing enantiomeric separation in capillary GC. Chirality was introduced through the addition of L-phenylalanine to the bottom of the cup-like structure.

L. Zhang et al. (16) studied stationary phases prepared by hydrosilylation with methyldichlorosilane followed by a condensation reaction with silanol-terminated polydimethylsiloxane. Characteristics such as column efficiency, polarity, and selectivity were examined utilizing gas chromatography. The mechanism of specific selectivity for position isomers based on the calix[4]crown ether ring, the molecular size and shape of the solute were discussed.

M. Sullenberger (17) also worked with separating chiral compounds by two HPLC methods. First the use of synthesized chiral calixarene as a modifier of a reverse phase stationary phase in HPLC and the second is the use of the same calixarene modifier of the mobile phase. Characterization included comparing retention times, capacity factors, and asymmetry factors when the synthesized calixarene was used as a modifier.

Thermodynamic Properties

Zeng et al. (18) researched the thermodynamic properties and recognition mechanism of solutes on two different kind of calix[4]arene derived stationary phases. The thermodynamic parameters and inclusion properties were discussed and conclusions dealing with steric hindrance were drawn.

Wang et al. (19) also investigated the thermodynamic properties of molecular recognition on three different calixarene columns. Thermodynamic parameters Δ H, ΔS, and ΔG were reported and superthermodynamic aspects were discussed.

Synergistic effects of liquid crystal calixarenes were studied on mixed stationary phases by Yuan et al. (20) using GC. The mechanisms of the effect were discussed along with the effects of temperature.

Recent Calixarene Research

Glennon et al. (21) have attempted to use calixarenes for the separation of alkali and alkaline earth ions. The separation of alkali and alkaline earth ions can be done on chemically bonded crown ether stationary phases using simple

water-methanol mobile phases. A silica-bonded macrocyclic calix[4]arene tetradiethylamide phase was synthesized and characterized.

Brindle et al. (22) synthesized silica-bonded calixarenes using two different methods. The first was reaction of triethoxysilyl calix[4]arene with the silica to produce a bonded calix[4]arene tetramide phase. The second method used a hydride-derivatized silica, which underwent a hydrosilylation reaction with a *p*-allylcalix[6]arene hexaester phase. After the synthesis was performed and characterization of the resulting products was achieved with cross polarization/magic angle spinning (CP/MAS) NMR spectroscopy. Brindle et al. investigated new ways of synthesizing and bonding calixarenes because little work has been reported on the characterization of silica bonded stationary phases.

Sokoließ et al. (23) reported the chromatographic behavior of six calixarene-bonded stationary phases. While trying to investigate a new theory on the retention mechanisms, he looked to the effects of several organic modifiers. The different materials included calix[4]-, calix[6]-, and calix[8]arenes and its derivatives substituted with *p-tert.*-butyl-groups bonded on silica gel. Those were then compared with a reversed phase C_{18} column, a phenyl and a phenyl ether stationary phase. The calixarene phases and phenyl ether phase contained silica-bonded calix[n]arenes attached via a propyl spacer on Polygosil® using a patented procedure.

Meynes et al. (24) efficiently separated polycyclic aromatic hydrocarbons by using calixarene-modified stationary phases. Applications of stationary phase supports in HPLC are suggested.

Lin et al. (25) discussed the applications of calixarene derivatives in analytical chemistry. Because the calixarenes possess a "cavity", conformational and functional groups are the ideal hosts suited for the purpose of the complexation and inclusion guest. The fact that the calixarenes can receive ions or neutral molecules shows their high selectivity and complexing abilities opposed to that of crown ethers.

C. Lowe (26) previously investigated soluble calixarenes as mobile phase additives. After successfully synthesizing these new stationary phases he was able to compare and investigate other methods of detection other than UV absorbance such as fluorescence and visible spectroscopy. Capacity factors of the solutes were then compared when no calixarene additives were added to the mobile phase to that when a calixarene additive was used.

R. Hirschl (27) covalently bonded 4-*t*-butylcalix[8]arene and 4-*t*-butylphenol to porous silica. The attachment utilized 1,2-dichorotetramethyldisilane as a difunctional tether in dry toluene. The silanated silica was then reacted with t-butylcalix[8]arene. Characterization included making a variety of different length columns in which capacity factors were calculated and compared.

Chapter III

Statement of Problem

Calixarenes are interesting compounds because of their high melting points and complex properties. The initial interest was because of their close resemblance to cyclodextrins, naturally occurring macrocyclic compounds with a similar structure to calixarenes, which were known to form host-guest interactions. Host-guest interactions of calix[n]arenes with a variety of neutral and ionic species have been widely reported. The belief is that calixarenes will form significant host/guest interactions with solute molecules during chromatography. The interaction is determined by the overall macrocyclic structure but most importantly the cavity size and the nature of the rim or functional groups, which act at the binding sites.

The goal of the research is to synthesize a new stationary phase for HPLC using a 4-tertbutylcalix[4]arene derivative. This stationary phase will be covalently attached to an HPLC column consisting of Nucleosil® (Macherey-Nagel) silica. It will then be characterized using different test solutions with HPLC. Finally the chromatograms will be analyzed to ensure the stationary phase is chemically attached to the silica. New columns are essential in HPLC to achieve better resolution and selectivity.

Chapter IV

Materials and Methods

Reagents

Mobile phase solvents were HPLC grade (Fisher Scientific, Pittsburgh, PA) and were filtered through a 0.45μm nylon filter before using. All reagents were ACS grade or better. N,N-diethyl-*m*-toluamide, 1- and 2-aminonapthalene, 1- and 2-aminoanthracene, K_2CO_3, $CDCl_3$, bromoacetamide, chlorotrimethyl silane, ethylene diamine, sodium borohydride, ethylene glycol, 5, 11, 17, 23-*tert*-butylcalix[4]arene, borane in THF (sure-seal® bottle), and glutaraldehyde were obtained from Aldrich Chemical Company (Milwaukee, WI). Nucleosil® (Macherey-Nagel) 5μm, 1000 Å pore size, toluene, methyl-, ethyl-, propyl-, and butyl-benzenes were obtained from Alltech Associates (Deerfield, IL). Disodium phosphate, benzene, and napthalene were obtained from J.T. Baker Chemical Company (Phillipsburg, NJ). 3-Aminopropyldimethylethoxysilane was obtained from United Chemical (Bristol, PA). Anthracene and uracil were purchased from Eastman Kodak Company (Rochester, NY). Anhydrous ethanol was obtained from Quantum Chemical Corporation (Tuscola, IL). Trichloroethylene and phenol were obtained from Mallinckrodt. Acetone, tetrahydrofuran, and chloroform were obtained from Fisher Scientific. Acetanilide was purchased from Carlo Erba (Milano, Italy). Deionized water was obtained from an in-house deionizer.

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 166 UV-Vis single wavelength detector, an Altex Model 210A injector with a 20 µL loop, and Beckman System Gold Software (V. 5.10). The mobile phases consisted of methanol/water premixed at various concentrations with a flow rate of 1.00 mL/min and detection of 254 nm.

NMR

NMR spectra were performed using a Varian Gemini 2000 400 MHz spectrometer at Youngstown State University. $CDCl_3$ was the solvent used and the chemical shifts were reported in parts per million downfield from the TMS reference.

Fluorescence and UV Spectroscopy

Fluorescence measurements were made using a Shimadzu Model RF 5000U scanning spectrofluorophotometer at Youngstown State University. Quartz glass fluorescence cuvettes were Fisher Brand catalogue number 14-385-918A. The samples were dissolved in ethylene glycol for Fluorescence and UV for measurement. For UV data an in house Hewlett Packard 8453 UV/Vis spectrophotometer was used.

LS/MS and MS

LS/MS and MS experiments were performed on a Bruker Daltonik Esquire LC with HP 1100 HPLC Mass Spectrometer at Youngstown State University. The samples were dissolved in methanol with a concentration of 1 mg/mL.

Elemental Analysis

Elemental analyses were performed on a Carlo Erba NA 1500 at Youngstown State University, which analyzed the percent nitrogen and carbon. The samples were weighed on a Cahn (Model 22) electronic microbalance. Acetanilide was used as a standard for the analysis.

- The K factor was determined as follows:

  K = (percent theoretical standard) * (weight of sample in mg)/Peak area

- The determination of the percentage of carbon and nitrogen elements was determined as follows:

  Elemental Percentage = (K factor (standard) * Sample Area))/ Weight (mg)

Synthesis of 5,11,17,23-tert-butyl-35,37-(carbamoylmethoxy)calix[4]arene **(1)** (As described in reference 28 with modification)

- 5, 11, 17, 23-*tert*-Butylcalix[4]arene (6.48 g, 15.3 mmol) was added to K_2CO_3 (10.12 grams, 73.3 mmol) and 2-bromoacetamide (12.62 g and 91.45 mmol) was refluxed under nitrogen for 6 days in dry acetone (250 mL).
- The mixture, after cooling, was poured into 400 mL of .1 M H_2SO_4.
- After one hour the precipitate was filtered, washed with water, and dried *in vacuo* at 70°C for 24 hours.

Synthesis of tert-butyl-tetra-[2-aminoethoxy]calix[4]arene **(2) (see figure 13)** (As described in reference 28 with modification)

- A solution of borane in THF (40 mL of 1 M) was added to **1** (4.55 g, 6.07mmol) in THF (80 mL).

- The mixture was stirred for an hour at room temperature and then refluxed for an hour and an additional 30 mL of the borane THF solution was added.
- The mixture was refluxed overnight and allowed to cool. A 6 N HCL solution (120 mL) was added and the mixture was refluxed for an additional hour.
- The THF was removed on the rotary evaporator and the colorless suspension was kept at 0°C for 12 hours and was then filtered, washed with water, and dried *in vacuo* at ambient temperature for 24 hours.

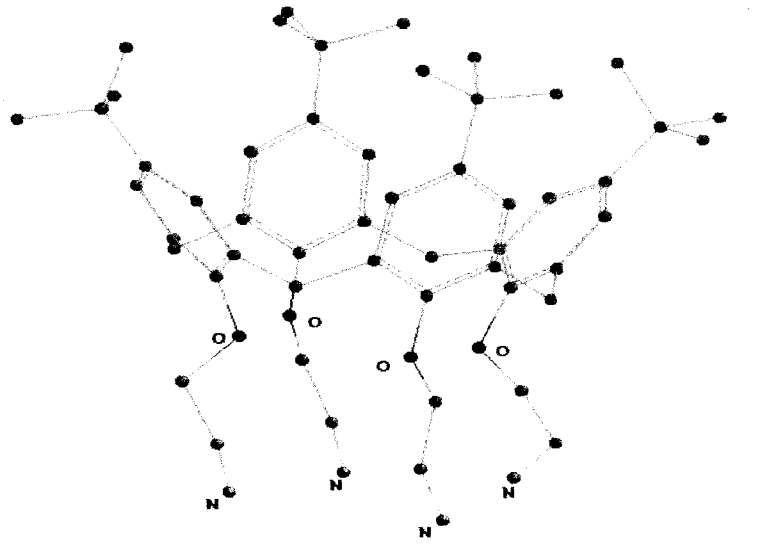


Figure 13 – Tert-butyl-tetra(2-aminoethoxy)calix[4]arene (2)

Attachment to the Silica (see Figure 14)

- To prepare the silica, 2 g of Nucleosil® Macherey-Nagel, 5µm, 1000 Å pore size was put into a 250 mL RB in 100ml of .1MHCL and it was then placed in oil bath for 24 hours at 90 degrees.

- The silica was cooled and filtered on a .45 μm nylon frit. The silica was then washed with 100 mL of DI water.
- It was then placed in beaker and in the oven at 130°C overnight.
- 15 mL of glutaraldehyde and 25 mL of a disodium phosphate buffer (pH 7.0) was added to 2.13 g of the amino-derived Nucleosil®.
- After two hours 0.5377 g of **2** dissolved in chloroform was added to the amino-derived silica glutaraldehyde solution. This was left for 48 hours to intensify surface coverage.
- After 48 hours the solution was filtered (.45μm nylon frit) and washed with anhydrous ethanol and DI water.
- 10 mL of anhydrous ethanol was then added to the treated silica along with 0.35 g of 99% sodium borohydride.
- After 2 h the solution was filtered with anhydrous ethanol and DI water.
- The product was placed in a vacuum dessicator for 1 h and it was then used to pack the before endcapping HPLC column.

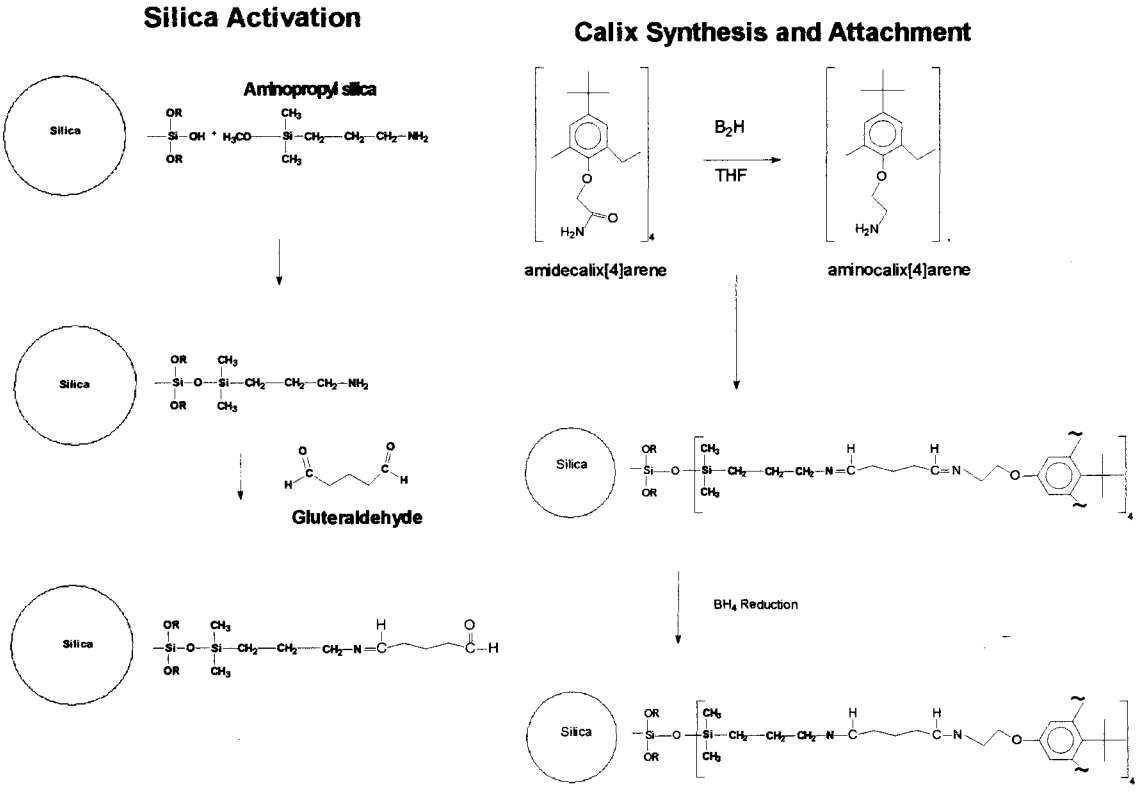


Figure 14 – Step by Step Synthesis

Endcapping

After getting the desired data the column was unpacked, the packing material washed with anhydrous ethanol and dried at 110°C for 30 minutes. The sample was then added to 50 mL of dry MeCl and 5 mL of chlorotrimethyl silane, 98% was added slowly. After 2 hours under nitrogen 150 mL of anhydrous ethanol was added to neutralize the chlorotrimethyl silane and the product was filtered with anhydrous ethanol. 10 mL of trichlorotheylene was added to the product and the column was then repacked for further analysis.

Packing the Column

To pack the column a 4.5 mm x 50 mm threaded stainless steel column (Alltech Associates, Inc.) and an Alltech air-driven slurry column packer was

used. Treated stationary phase (2 g, Nucleosil® from Macherey-Nagel, 5µm, 1000 Å pore size) was suspended in 10 mL trichloroethylene driven into the column at a pressure of 3000 psi using 300 mL of 100% methanol.

Test Mixtures

There were five different test mixtures that were used to test the stationary phases before and after endcapping columns. They were the reversed phase test mixture, the phenyl ring homologous series, the alkyl benzene homologous series, and the ENE series (see Figure 15). The reversed phase test mixture contained uracil, phenol, N,N-diethyl-m-toluamide, and toluene. The phenyl ring homologous series contained benzene, napthalene, and anthracene. The alkyl benzene homologous series was made up of methyl-, ethyl-, propyl-, and n-butylbenzene. The ENE series contained 1- and 2-aminonapthalene, napthalene, and 1- and 2-aminoanthracene. All compounds were dissolved in methanol and individual solutions were also made for individual runs of each.

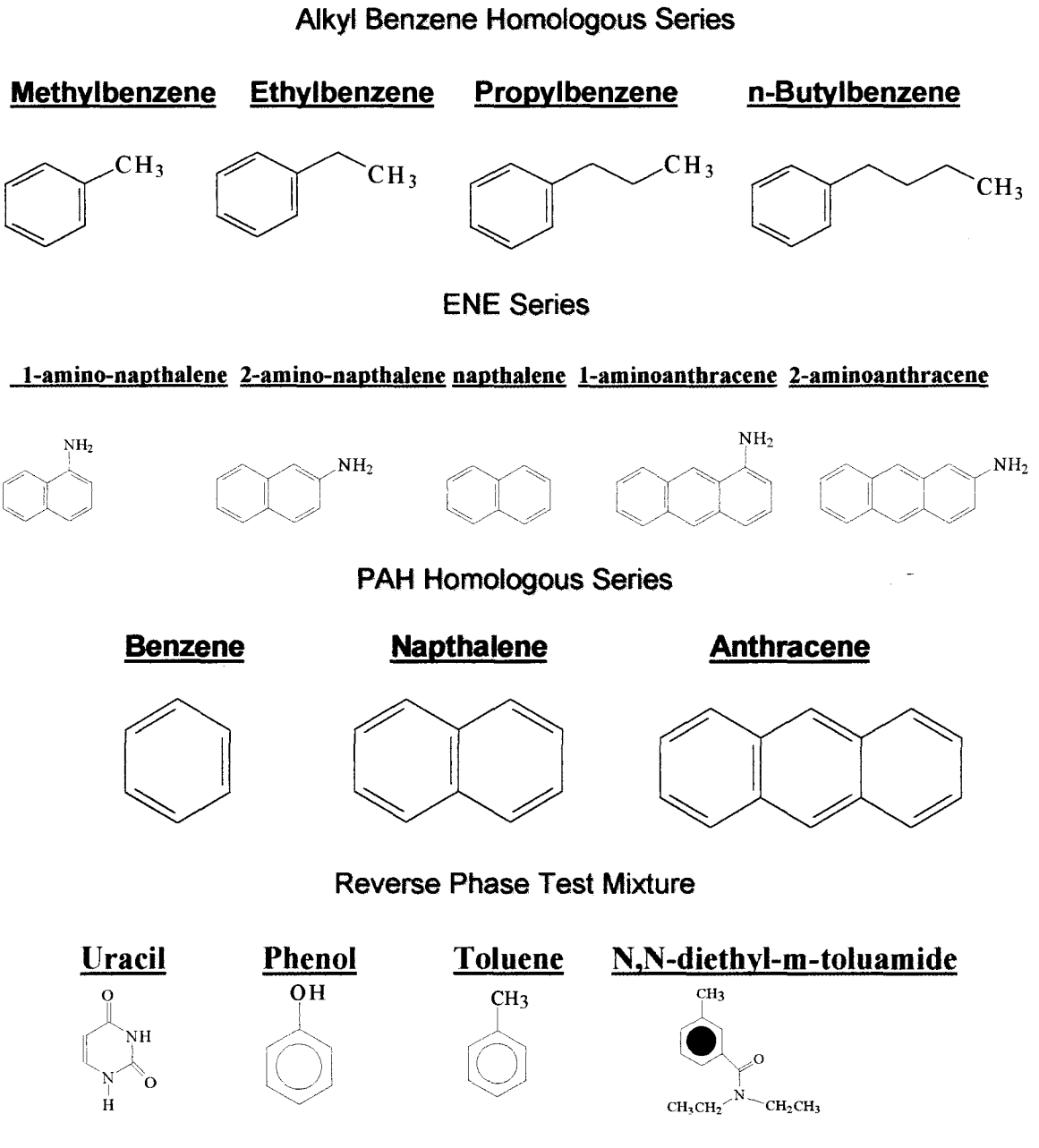


Figure 15 – Series for HPLC columns before and after endcapping

Knox Test (27)

The Knox test requires a dry non-polar mobile phase and a polar solute. A Knox test was performed on the before and after endcapping columns using dry hexane as the mobile phase and methanol as the solute. First pumping 100

mL of dry methanol, 100 mL of anhydrous ethanol, and then 100 mL of dry hexane prepared the column before actually injecting the solute. Methanol is then injected while dry 100 % hexane is acclimated in the column.

The Knox test is a test that probes the stationary phase for free silanols by performing chromatography on reversed phase columns while using a normal phase method. The Knox test is performed to see if there are large amounts of free silanols. If the solute is unretained on the stationary phase then a large number of free silanols are not present.

Chapter V

Results and Discussion

Characterization of 5,11,17,23-tert-butyl-35,37-(carbamoylmethoxy)calix[4]arene (1)

The literature procedure as outlined in Chapter IV (28), was followed and the product was characterized using NMR, LC/MS, fluorescence spectroscopy, and UV spectroscopy. TLC plates were run in 35% acetone and 65% hexane and showed a single product.

- NMR

The product was dissolved in deuterated chloroform and an ^1H NMR was performed. The ^1H NMR spectrum (Figure 16) in d-chloroform showed some impurities. The singlet at 1.206 ppm represented the tert-butyl groups at position 3. The peak at 1.764 ppm represented the NH_2 group at position 1. At 2.619 ppm the methyl group was represented by a singlet at position 5. The methylene bridge was represented by two doublets at 3.542 ppm and 4.211 ppm at position 4. Finally the protons from the ring located at position 2 were represented at 7.047 ppm.

- LC/MS

The product was analyzed using mass spectroscopy and is shown in Figure 17. The product was ionized by an atmospheric pressure chemical ionization (APCI) and the ions were mass analyzed with an ion trap. A scan range of 50-1500 m/z was used with an accumulation time of 71 μs and the polarity positive. The formula weight of **1** was 877.13, which corresponds to the LC/MS result of the [M + 1] peak of 877.61.

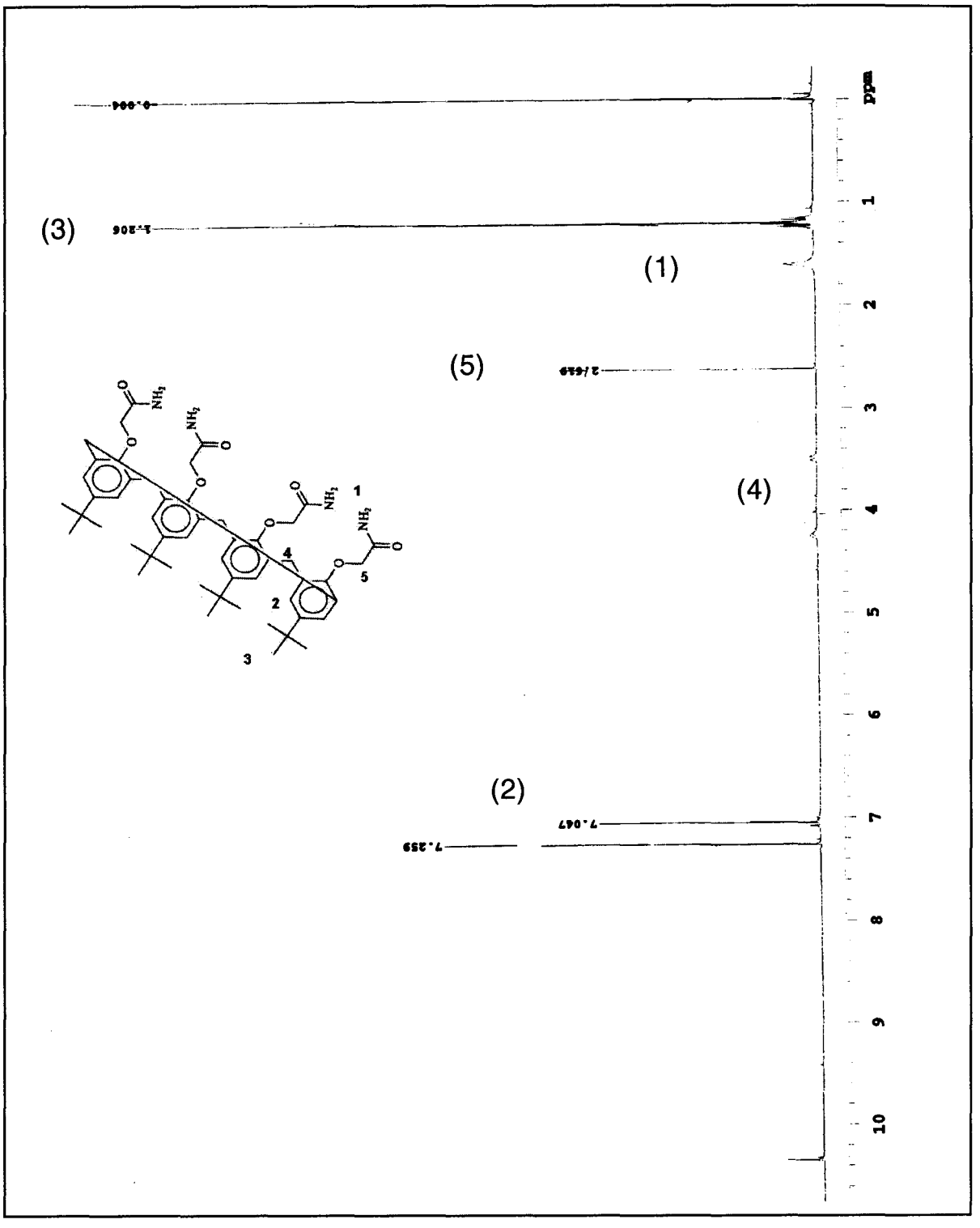


Figure 16: H' NMR of **1**

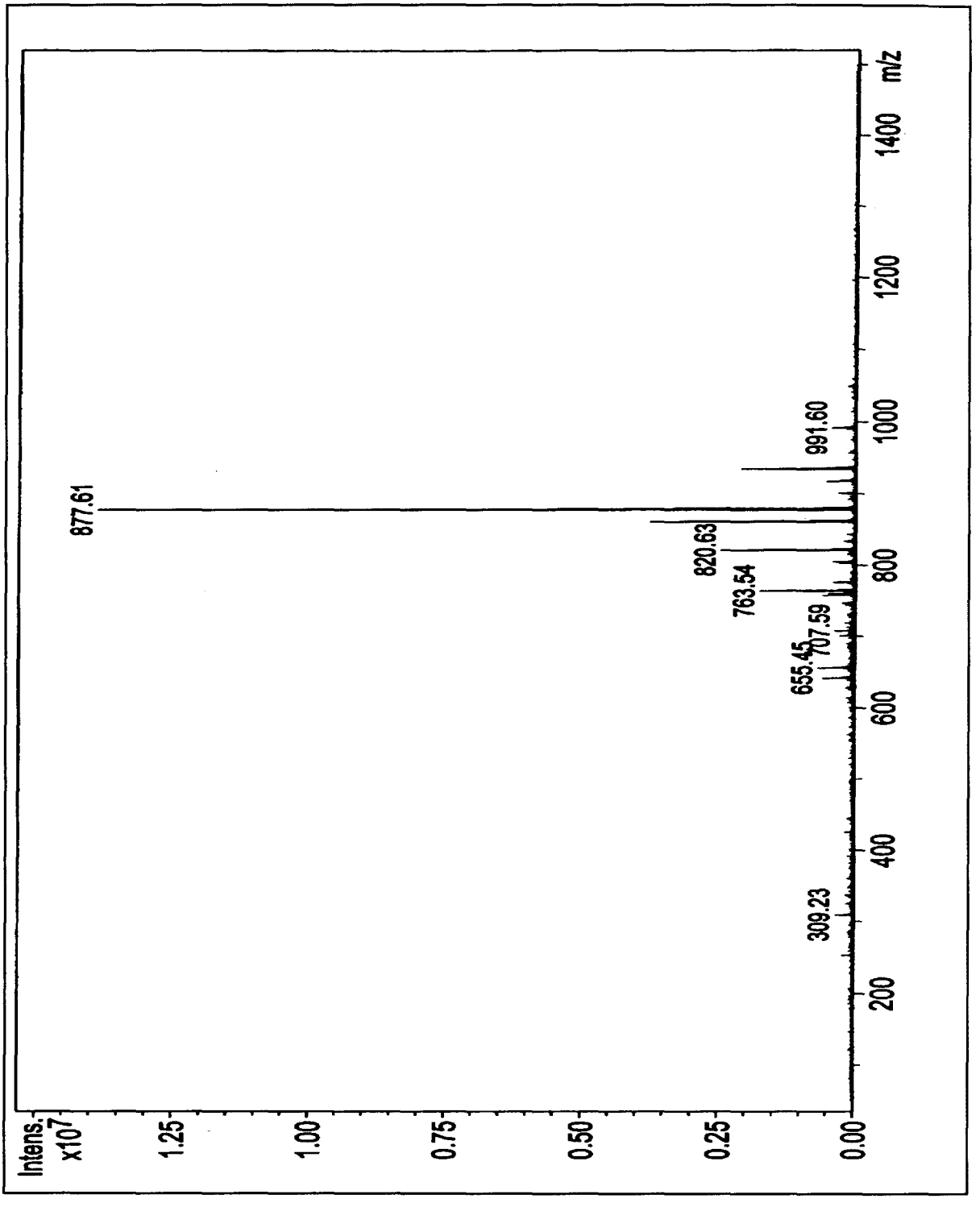


Figure 17: Mass Spectrum of **1**

Characterization of tert-butyl-tetra-[2-aminoethoxy]calix[4]arene (2)

TLC plates were run in 3/2 isopropanol/hexane. The product was run against the starting material and the product showed two spots. The spots were detected from an UV lamp. The R_f value of the starting material was 0.59 and the spot was relatively intense. The product had R_f values of 0.57 and 0.42. The spot located at 0.57 was not very intense and it was concluded that the spot correlated to the starting material. The spot with the R_f value of 0.42 represented the product and was shown intensely. It was concluded that a small amount of starting material was present after further characterization.

- NMR

The product was dissolved in deuterated chloroform and an ^1H NMR was performed. The ^1H NMR spectrum (Figure 18) in d-chloroform showed some impurities. The singlet at 1.151 ppm represented the tert-butyl groups at position 3. The peak at 1.264 ppm represented the CH_2 group at position 5. The peak at 1.512 ppm represented the CH_2 group at position 6. The peak at 2.291 ppm represented the NH_2 group at position 1. The methylene bridge was represented by two doublets at 3.465 ppm and 4.194 ppm at position 4. Finally the protons located at position 2 from the ring were represented at 6.894 ppm.

- LC/MS

The product was also analyzed by mass spectroscopy shown in Figure 19. The product was ionized by an atmospheric pressure chemical ionization (APCI) and the ions were mass analyzed with an ion trap. A scan range of 50-1500 m/z was used with an accumulation time of 779 μs and the polarity positive. The formula weight of **2** was 821.19, which corresponds to the LC/MS result of the [M + 1] peak of 822.02.

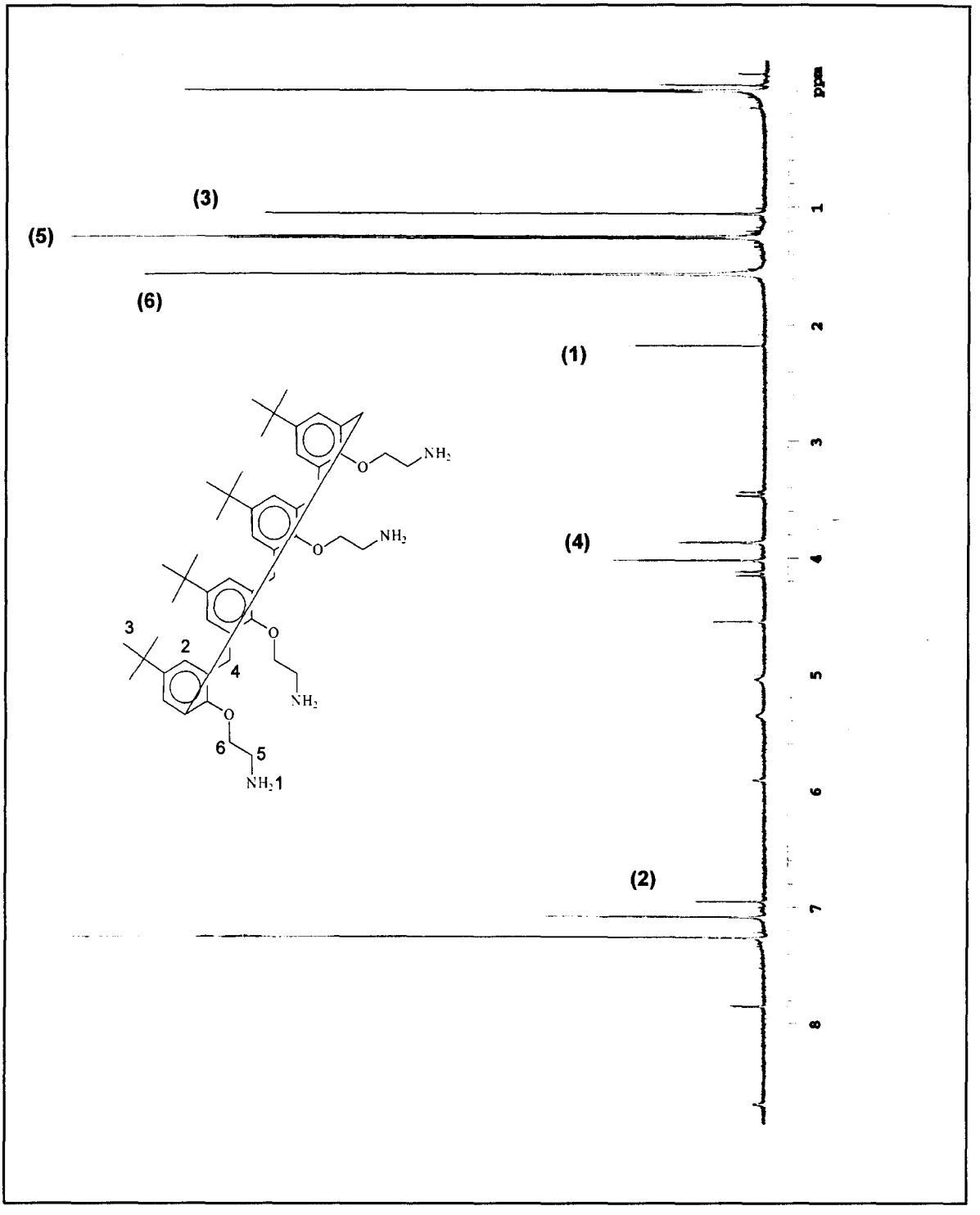


Figure 18: H' NMR of **2**

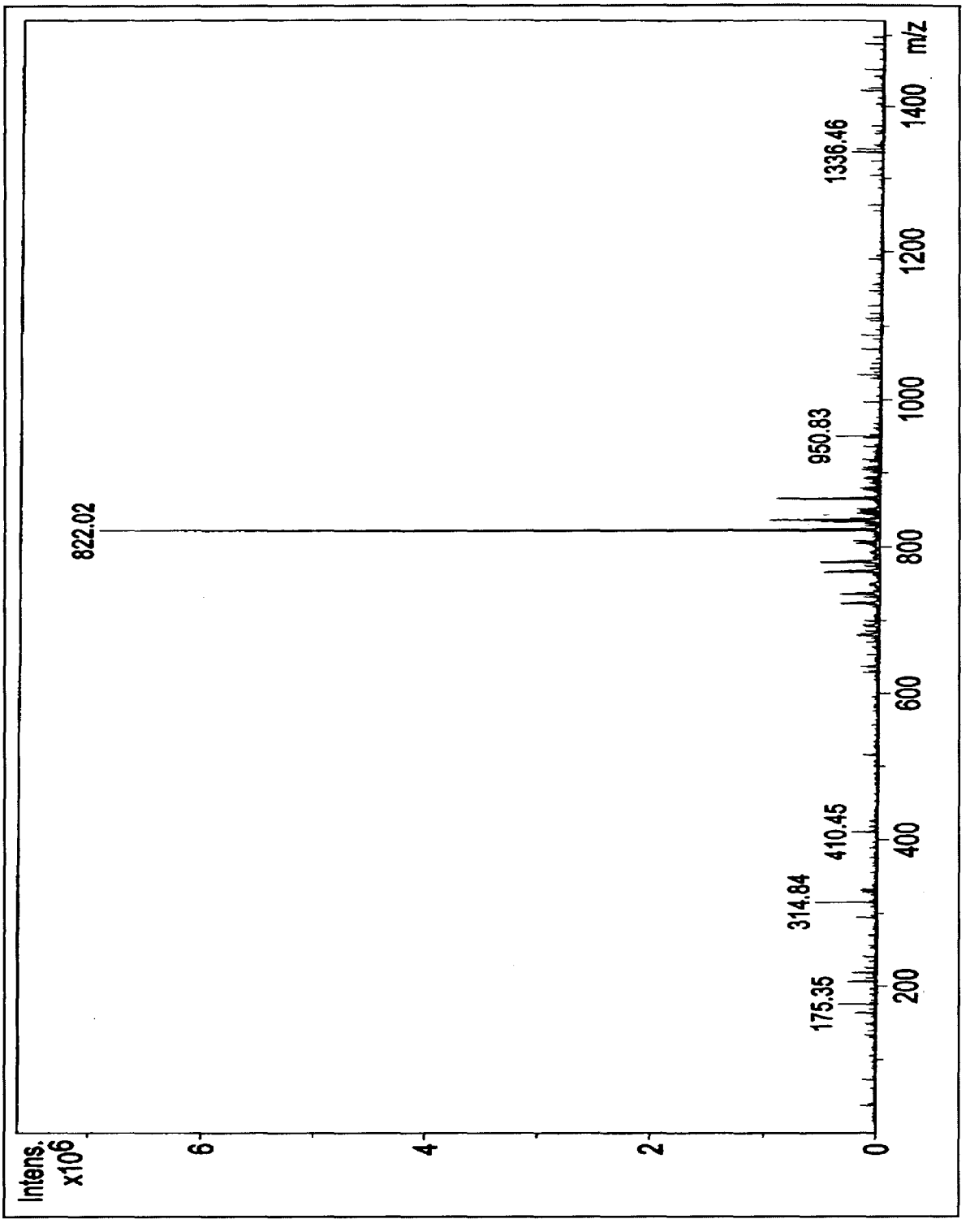


Figure 19: Mass Spectrum of **2**

➢ UV Spectroscopy and Fluorescence Spectroscopy

Silica does not have the ability to absorb photons above 200 nm so any absorption or emission above this should indicate successful synthesis and attachment of the amino derived calix[4]arene. The calix[4]arene has the ability to fluoresce due to the conjugated ring structure so any fluorescence must be due to the presence of the calix[4]arene.

The spectrum was scanned from 200 to 600 nm for the UV spectroscopy. The UV spectrum of the calix as it was taken through various steps of the synthesis and attachment was shown in Figures 20-25. Figure 20 showed the UV spectrum of tert-butyl-tetra-[2-aminoethoxy]calix[4]arene before any attachment to the silica was performed. Absorption occurred at 208, 273 and 278 nm. Figure 21 represented tert-butyl-tetra-[2-aminoethoxy]calix[4]arene after the glutaraldehyde attachment, in which excitation shifted to 272, 388, and 393 nm. Figure 22 showed the next step in the synthesis step, the borohydride reduction. The peaks were at wavelengths of 205, 281, and 387 nm.

Finally Figures 23 and 24 represented the before and after endcapping steps of tert-butyl-tetra-[2-aminoethoxy]calix[4]arene. The before endcapping spectra showed excitation at 284, 421, and 485 nm and the after endcapping spectra had peaks at 204, 229, and 281 nm.

Fluorescence of calix[4]arene was performed with an excitation of 333 nm. This was represented in Figure 25 and shows peaks at 410 and 680 nm. Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene attached to Nucleosil® was represented in Figure 26. With excitation of 250 nm, peaks were shifted to 290, 415, 490, and 610 nm.

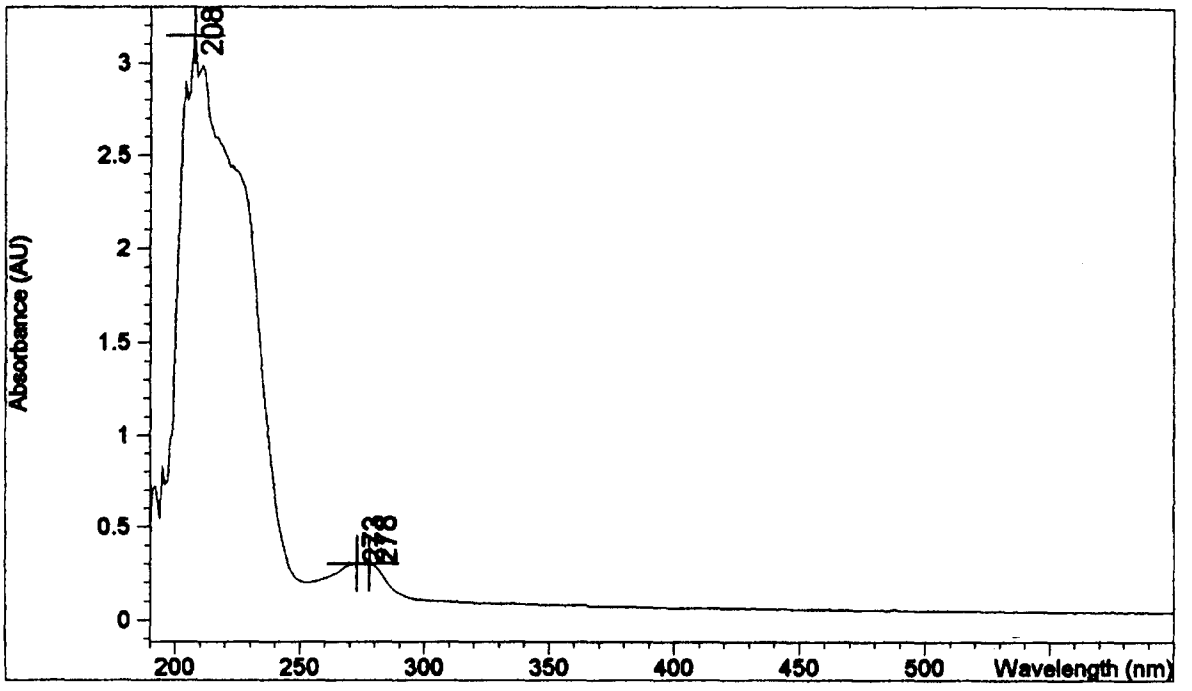


Figure 20: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene Before Attachment to Nucleosil®

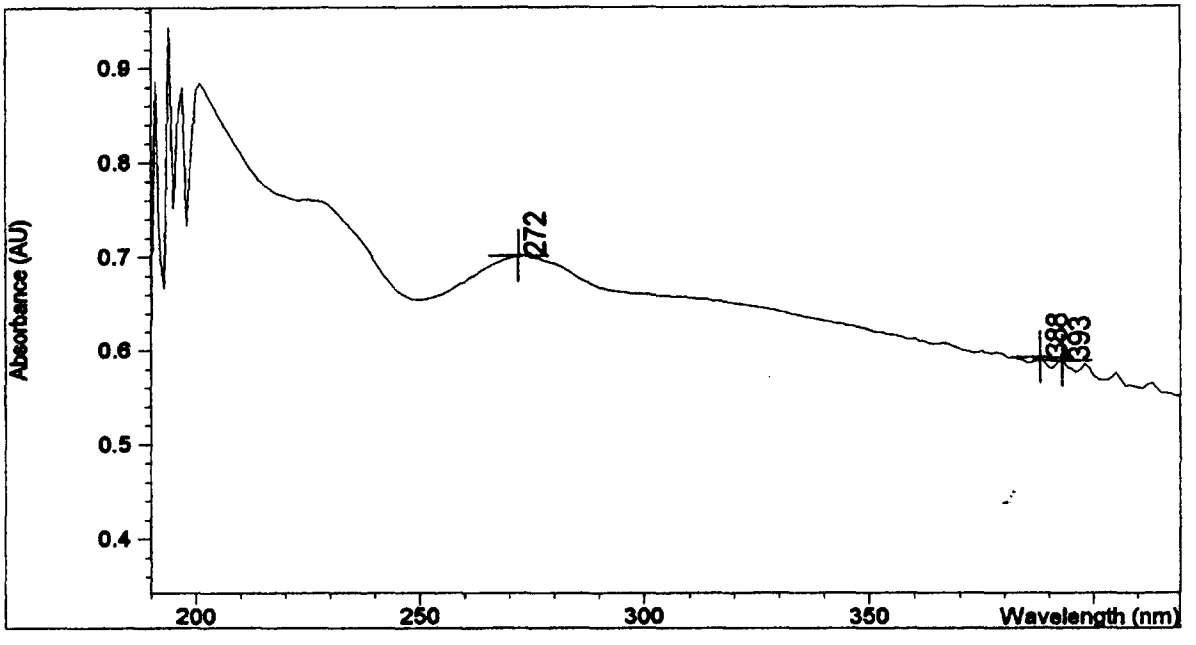


Figure 21: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene Before Glutaraldehyde Step

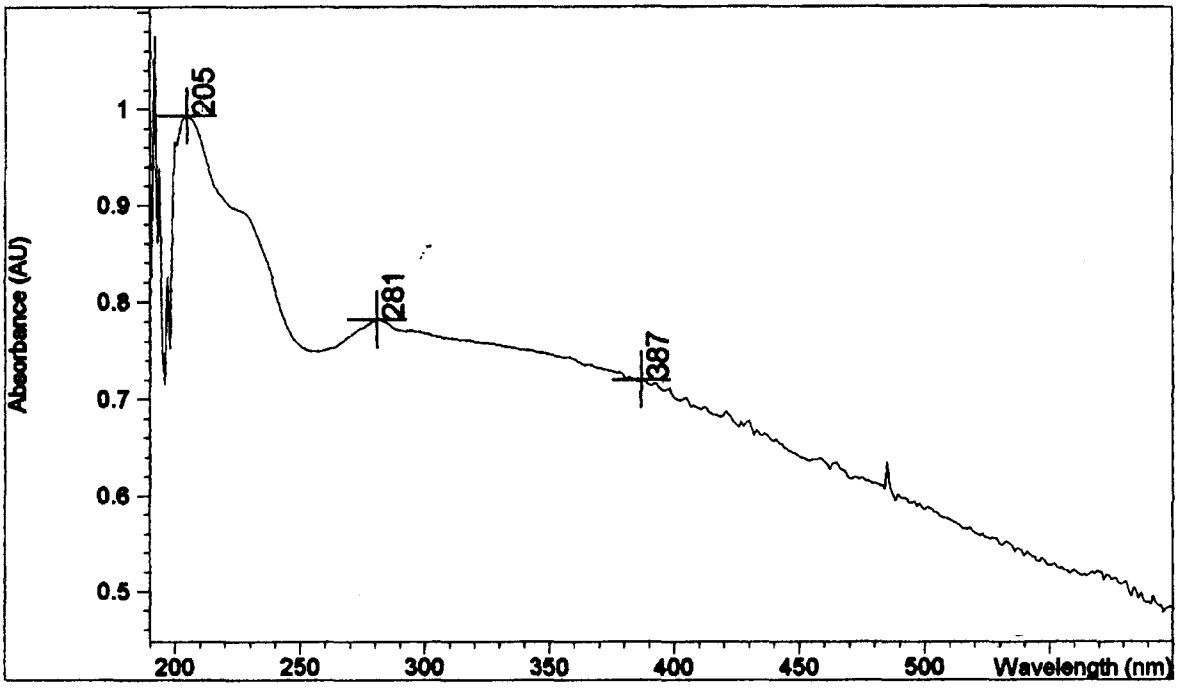


Figure 22: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene After Borohydride Reduction

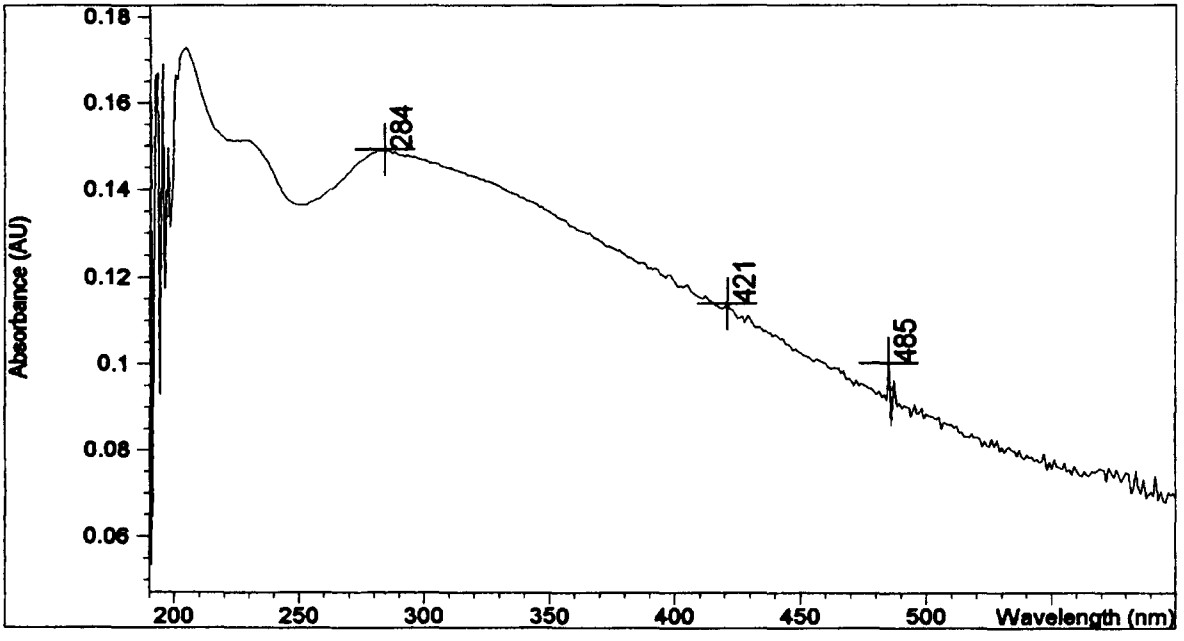


Figure 23: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene Fully Reacted with Nucleosil® (Before Endcapping)

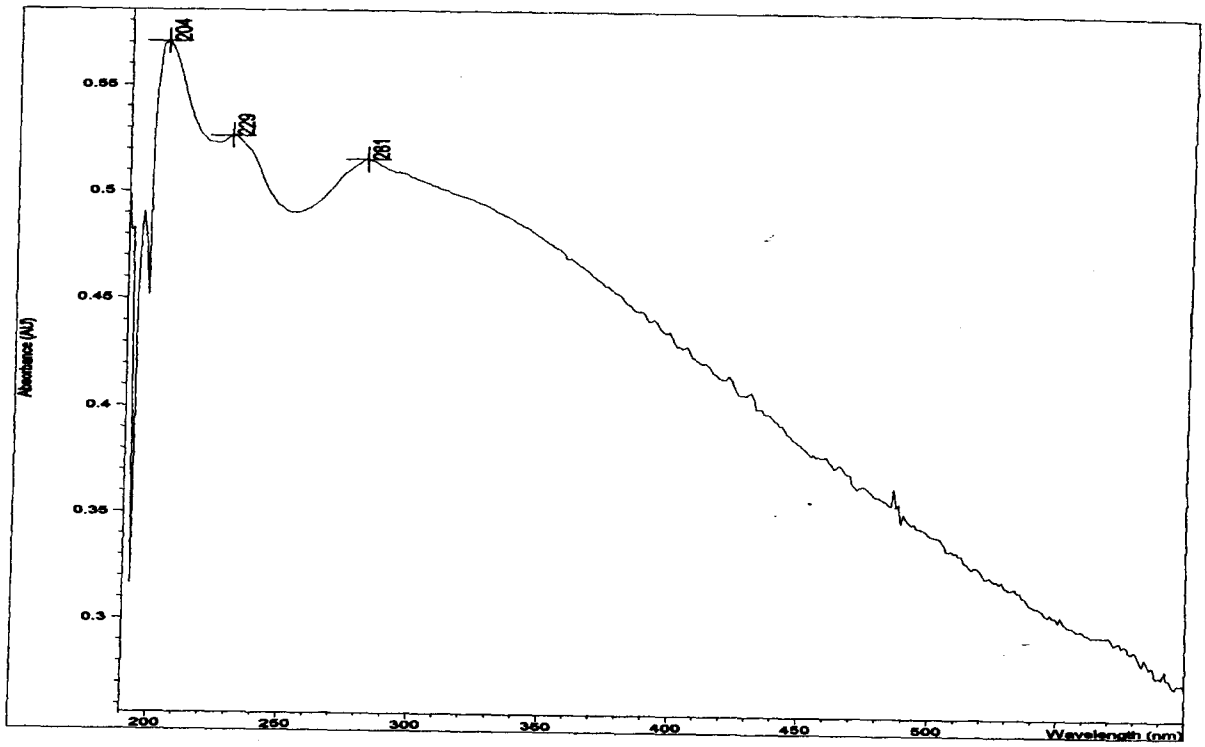


Figure 24: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene Fully Reacted with Nucleosil® (After Endcapping)

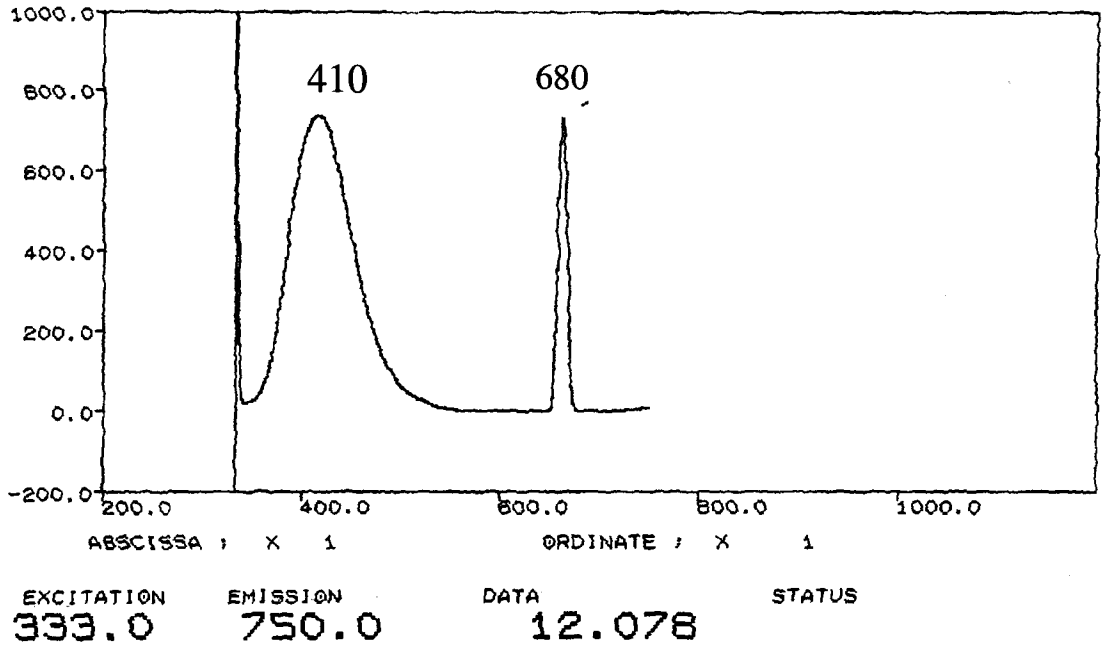


Figure 25: Calix[4]arene

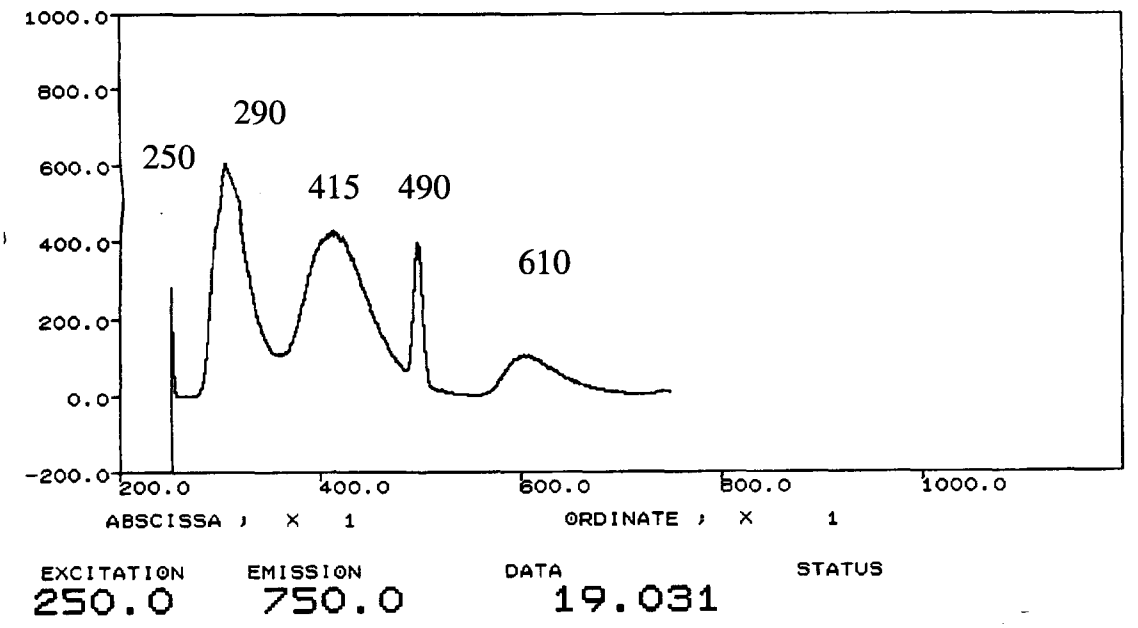


Figure 26: Tert-butyl-tetra-[2-aminoethoxy]calix[4]arene Attached to Nucleosil®

HPLC Characterization of the Stationary Phase

HPLC was performed on the amino derived calix[4]arene reversed phase column. Data was taken before and after endcapping with the five test mixtures. The mobile phases were varied until the ideal methanol to water mixture was found.

The test mixtures consisted of the reversed phase test mixture, the phenyl ring homologous series or PAH homologous series, the alkyl benzene homologous series, and the ENE series. The data was then compared to a high quality C_{18} Nucleosil® Macherey-Nagel, 5µm, 1000 Å pore size column using the PAH and the alkyl benzene series. The mixtures were selected to investigate how the stationary phases behave with regard to polarity, mixed mode retention, and host-guest complexation.

Peak tailing and broadening occurs from mixed mode retention and host-guest interactions. If the interactions were in fact host-guest, significant amounts of band broadening are expected. Mixed mode retention is interaction due to free silanol groups on the stationary phase. When endcapping is performed, chlorotrimethyl reacts with the free silanol groups left on the stationary phase. With the silanol groups "covered", any peak broadening can be narrowed to host-guest interactions and not mixed mode retention.

- Alkyl Benzene Homologous Series

The first homologous series to be separated was the alkyl benzene homologous series, which was made up of methyl-, ethyl-, propyl-, and n-butylbenzene. The purpose of separating this homologous mixture was to determine if more than one type of interaction was taking place. If only one interaction was in fact taking place then the plot of the log of the retention time versus the number of carbon atoms should produce a linear line. If the line was not linear this could be caused by host-guest interactions or mixed-mode retention.

As seen in Figure 27, the log of k' versus the number of carbon atoms, showed linearity with the C_{18} column. From the linearity of the line it was concluded that secondary interactions, like host-guest interactions, were not taking place with the C_{18} phase. The before and after endcapping runs showed some non-linearity but it can not be concluded with certainty that host-guest interactions were taking place.

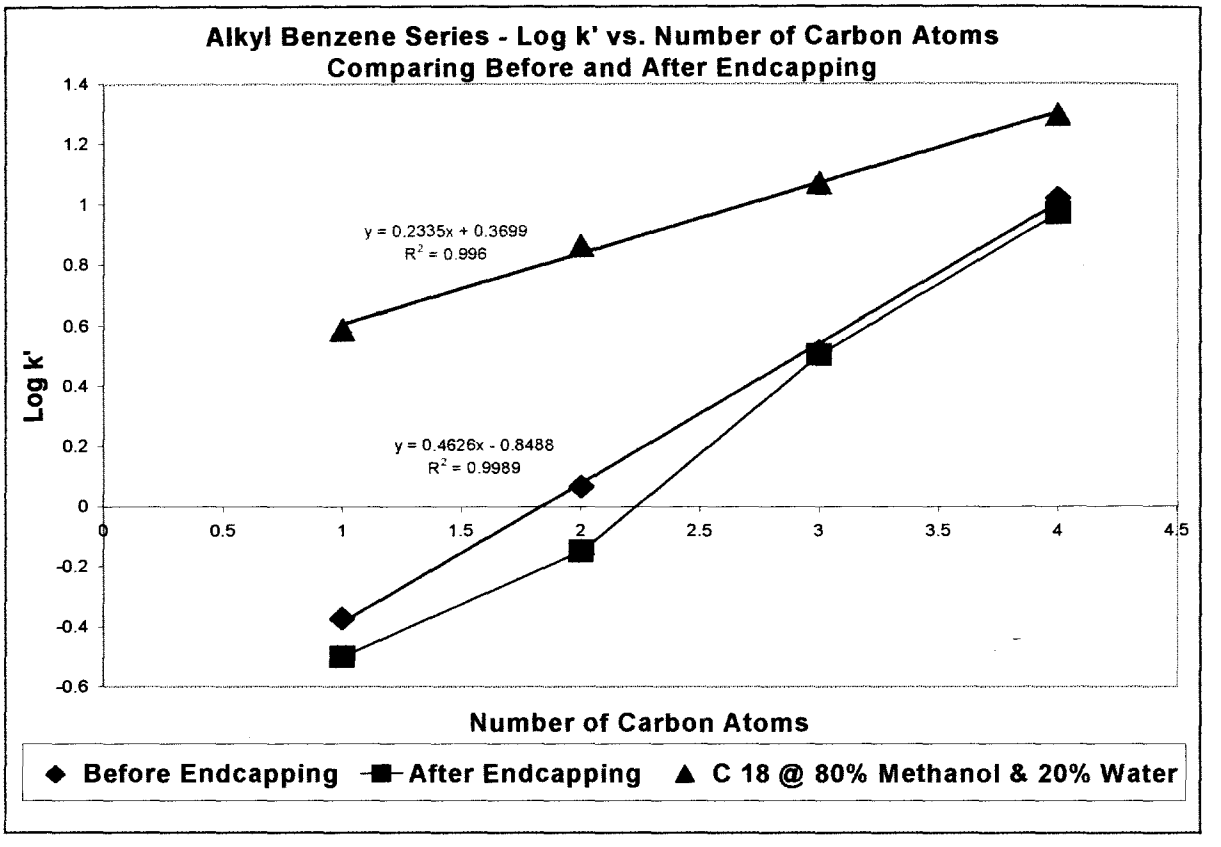


Figure 27: Log k' vs. Number of Carbon Atoms for the Alkyl Benzene Homologous Series Comparing Before and After Endcapping

This particular series was based on the phenyl ring and then methyl groups are added (-methyl, -ethyl, -propyl, and –n-butyl). With only the methyl groups differing, the molecules sizes were not dramatically different. This means that the increase in retention was due to London interactions first and then to host-guest interactions. As seen in Figure 28, the retention times in the chromatogram of the before and after endcapping columns do not differ significantly. The mobile phase was 90% water and 10% methanol.

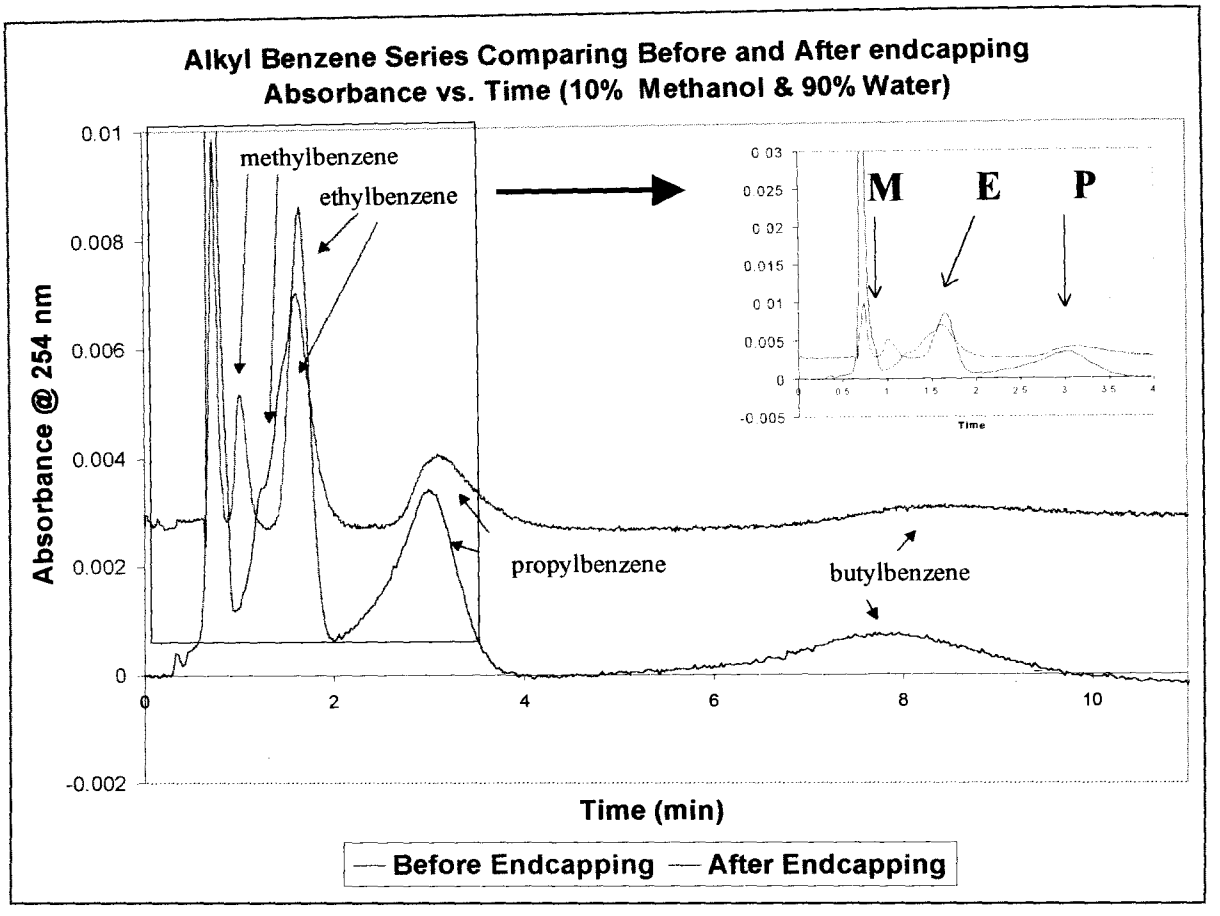


Figure 28: HPLC Data for the Alkyl Benzene Homologous Series Comparing Before and After Endcapping (10% Methanol & 90% Water)

Table 2 and 3 summarized the results from the chromatogram separating the alkyl benzene homologous series before and after endcapping. The retention times, t_r, were reported where t_r was the observed retention time in minutes. The capacity factor is represented as k' where:

$$k' = (t_r - t_m) / t_m$$

where t_m is the dead time in minutes. Figure 29 graphically compares k' before and after endcapping.

Asymmetry factors were also calculated from the formula:

$$A_s = b / a$$

where *b* is the width from the center of the peak to the end of the peak at half of the peak height and *a* is the width from the center of the peak to the beginning of the peak at half of the peak height.

An asymmetry factor can help conclude whether or not host-guest interactions were present. If the asymmetry factor of the peak is one, the peak is perfectly symmetrical and secondary interactions are not occurring. Asymmetry factors greater than one shows peak tailing and asymmetry factors less than one shows peak fronting. Non-symmetrical peaks occur due to different reasons like host-guest interactions.

When host-guest interactions are present, the kinetics of the guest molecule entering the host can cause non-symmetrical peaks, therefore when the peaks are intensely non-symmetrical there is a good chance that host-guest interactions occurred (17).

Analyte	t_r	k'	A_s
Methylbenzene	1.04	0.4247	0.80
Ethylbenzene	1.58	1.1644	1.42
Propylbenzene	3.13	3.2877	1.2
n-Butylbenzene	8.37	10.4658	1.1

Table 2: Alkyl Benzene Series Before Endcapping

Analyte	t_r	k'	A_s
Methylbenzene	1	0.3158	1.0
Ethylbenzene	1.3	0.7105	1.1
Propylbenzene	3.18	3.1842	0.46
n-Butylbenzene	7.85	9.3289	0.71

Table 3: Alkyl Benzene Series After Endcapping

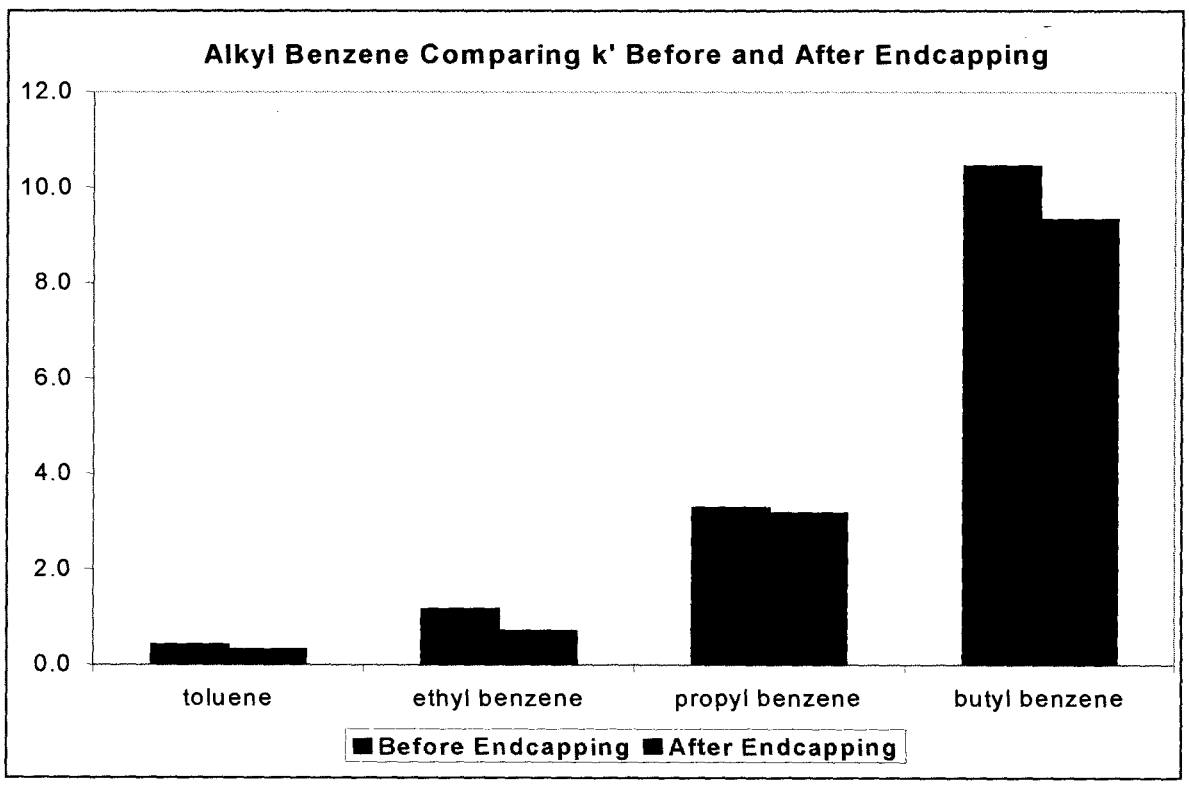


Figure 29: Capacity factors for the Alkyl Benzene Homologous Series Comparing Before and After Endcapping

➢ PAH Homologous Series

The phenyl ring homologous series contained benzene, napthalene, and anthracene. For the PAH homologous series non-polarity was added as the additional benzene rings were added. Due to the increase in the number of phenyl rings, host-guest interactions were more likely to occur.

Figure 30 showed the plot of the log k' versus the number of benzene rings. As with the alkyl benzene series, the PAH series on the C_{18} column was linear and it was shown that host-guest interactions are not present. The amino-derived Nucleosil® before and after endcapping column do not show significant non-linearity and it can not be concluded with confidence that host-guest interactions were taking place. Figure 31 showed the chromatograms using a mobile phase of 35% methanol and 65% water. As in the alkyl benzene series, retention times of the analytes on the before and after endcapping columns does not differ much.

Tables 4 and 5 showed the corrected retention times, the capacity factor, and the asymmetry factor, respectively, for the before and after endcapping columns. The asymmetry factors do not stray too far from 1, which means that there was not severe tailing or fronting of peaks. The k' of before and after endcapping was shown graphically in Figure 32.

Non formation of host-guest complexation was also indicated by the asymmetry factor, which were very close to the after endcapping values. This indicated that the tailing peaks were likely due to mixed mode retention from the free silanol groups.

Analyte	t_r	k'	A_s
Benzene	0.87	0.2582	1.0
Napthalene	1.77	1.5782	1.0
Anthracene	11.06	15.0836	1.05

Table 4: PAH Series Before Endcapping

Analyte	t_r	k'	A_s
Benzene	0.89	0.1266	1.0
Napthalene	1.80	1.2785	0.74
Anthracene	10.82	12.6962	0.83

Table 5: PAH Series After Endcapping

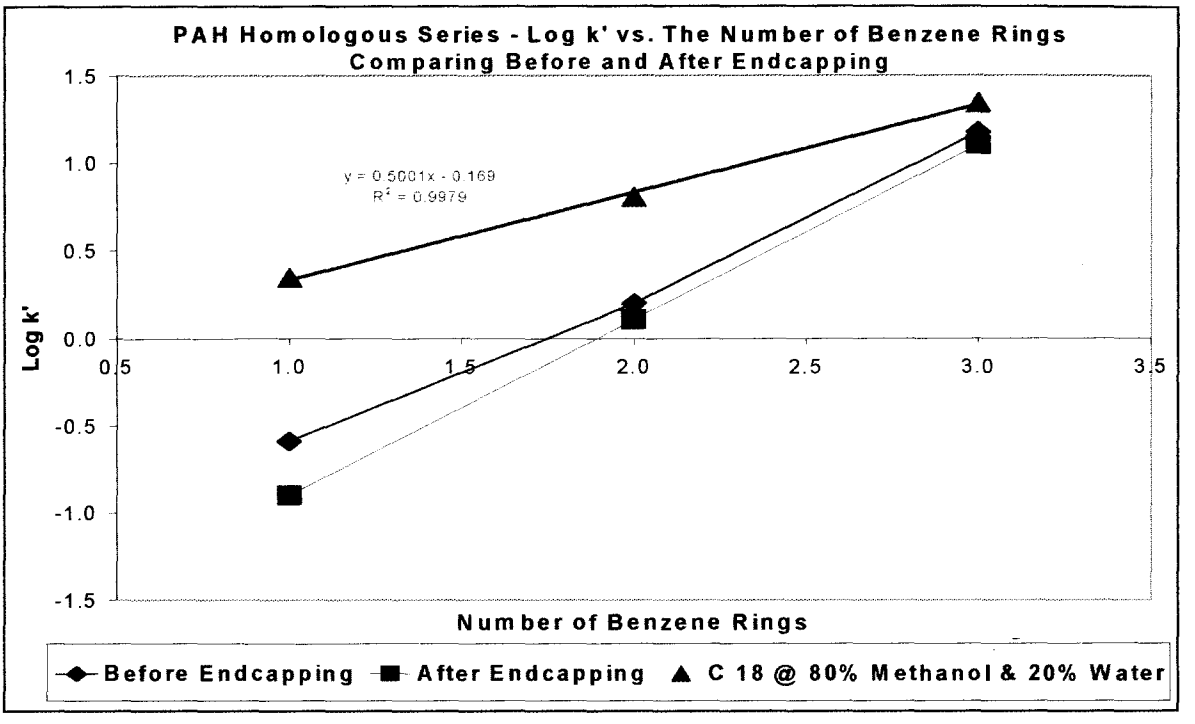


Figure 30: Log k' vs. Number of Carbon Atoms for the PAH Homologous Series Comparing Before and After Endcapping

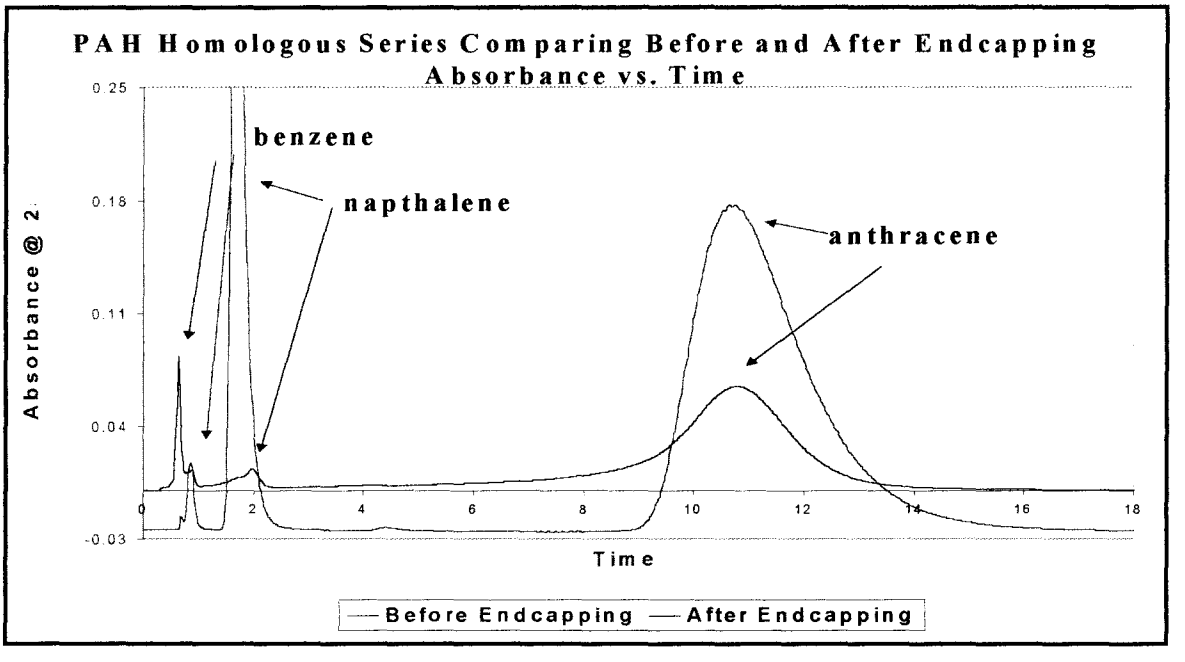


Figure 31: HPLC Data for the PAH Homologous Series Comparing Before and After Endcapping (35% Methanol & 65% Water)

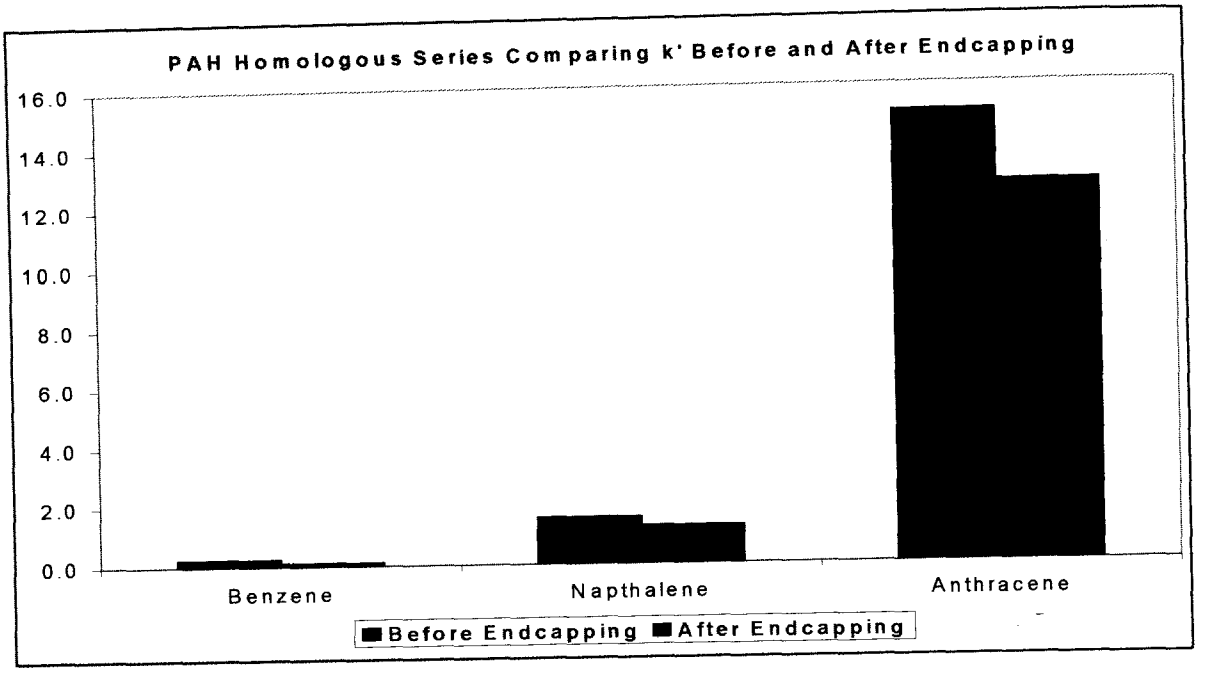


Figure 32: Capacity factors for the PAH Homologous Series Comparing Before and After Endcapping

- The Reversed Phase Test mixture

The reversed phase test mixture contained uracil, phenol, N,N-diethyl-m-toluamide, and toluene. This mixture was necessary to show the reversed nature of the column. If the solutes demonstrate retention when using relatively polar mobile phase mixtures then the stationary phases can be said to be non-polar and exhibits reversed phase behavior. If the solutes are close to the dead time it can be concluded that the stationary phase was somewhat polar and exhibits normal phased behavior.

Normally, uracil is the dead time marker because it is water-soluble and usually unretained. Toluene generally shows non-polar interactions whereas

N,N-diethyl-m-toluamide is prone to strong interactions with free silanol groups. Phenol is a weak acid and can be used to examine proton acceptor attributes.

Tables 6 and 7 showed t_r, k', and A_s respectively for the before and after endcapping columns. The results were interesting for toluene and N,N-diethyl-m-toluamide. Before endcapping the toluene and N,N-diethyl-m-toluamide eluted together and had an asymmetry factor of 1.85, indicating tailing. After the column was endcapped, in which the free silanol groups are covered, the asymmetry factors are close to one. Toluene and N,N-diethyl-m-toluamide separated on the endcapped column nicely. This concluded that after the column was endcapped it was made more non-polar or reversed phase.

Figure 33 compared the chromatograms before and after endcapping. The peaks were labeled and note the retention after endcapping was performed. The before endcapping column did not separate any of the solutes nicely. The after endcapping column showed better separation of all of the solutes. Figure 34 compared the capacity factors before and after endcapping.

Analyte	t_r	k'	A_s
uracil	0.8	0.1429	1.0
phenol	0.86	0.2286	0.81
toluene	1.288	0.8400	1.85
N,N-diethyl-m-toluamide	1.288	0.8400	1.85

Table 6: Reversed Phase Test Mixture Before Endcapping

Analyte	t_r	k'	A_s
uracil	0.880	0.158	1.0
phenol	1.030	0.355	1.1
toluene	1.940	1.553	0.86
N,N-diethyl-m-toluamide	2.380	2.132	1.29

Table 7: Reversed Phase Test Mixture After Endcapping

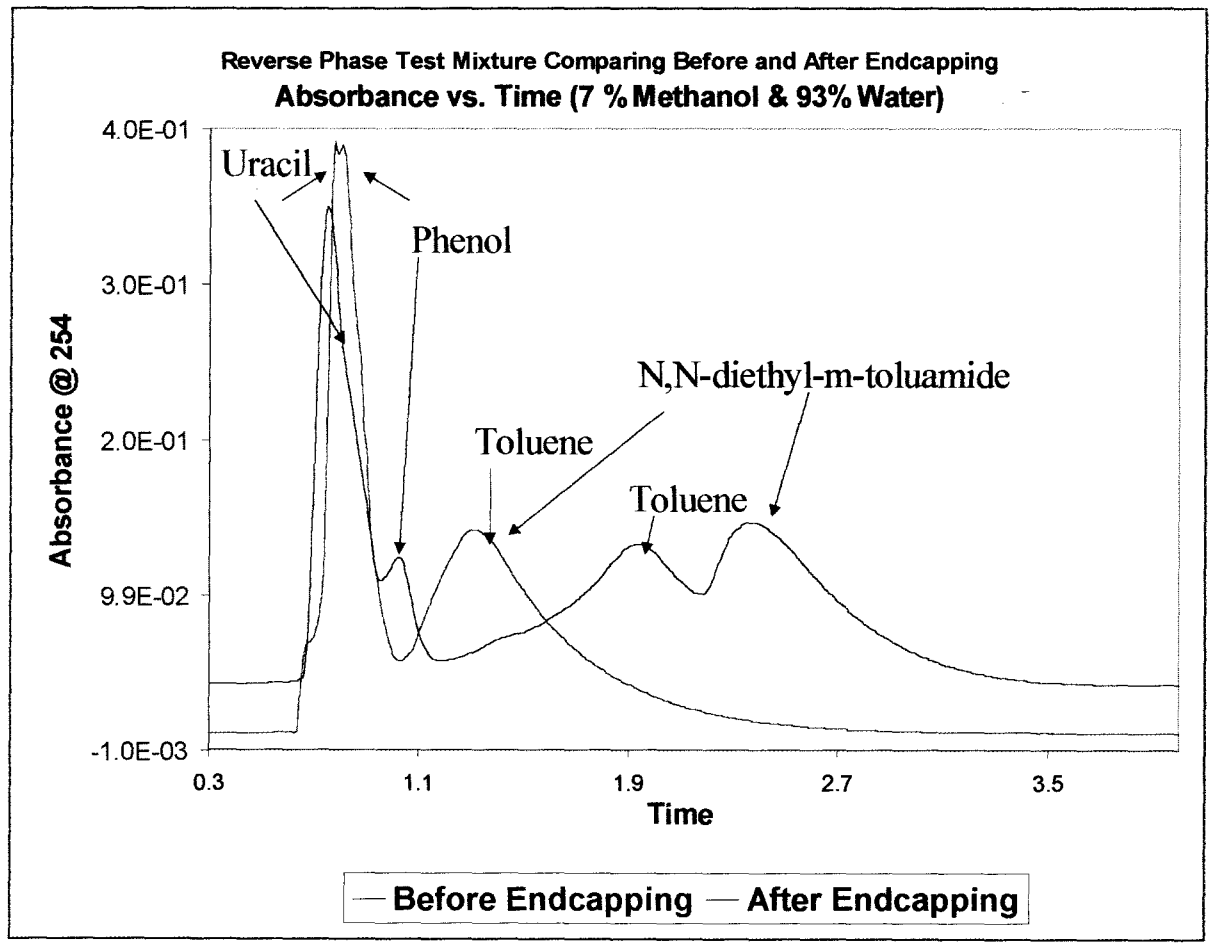


Figure 33: HPLC Data for the Reversed Test Mixture Comparing Before and After Endcapping (7% Methanol & 93% Water)

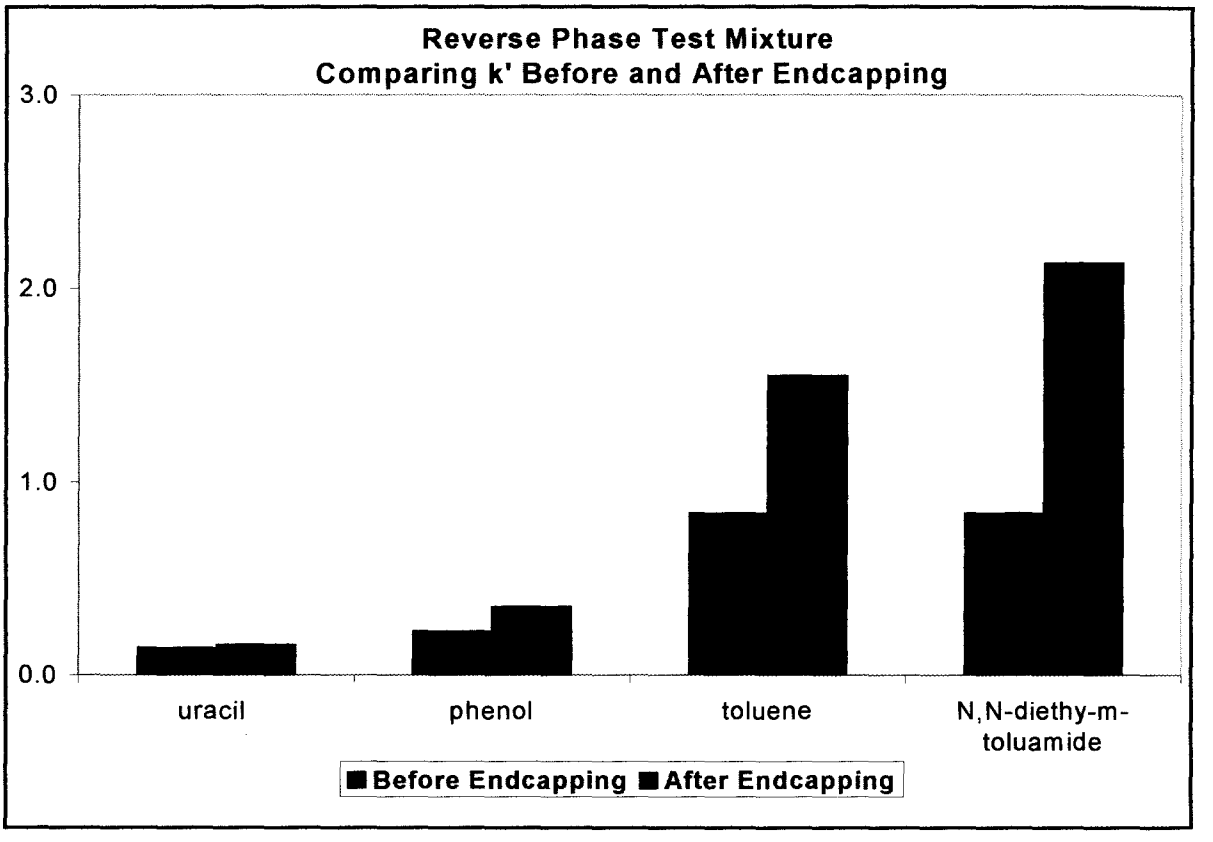


Figure 34: Capacity factors for the Reversed Phase Test Mixture Comparing Before and After Endcapping

- The ENE Series

The ENE series contained 1- and 2-aminonapthalene, napthalene, and 1- and 2-aminoanthracene. This series contains benzene rings and amino groups were added to the benzene rings. The napthalenes have two benzene rings and the anthracenes have three benzene rings. Amino groups are added to the benzene rings in different orientations thus resulting in different retention.

Table 8 showed the asymmetry factors for the before endcapping column and the 1- and 2-aminoanthracene were the only peaks that were significantly

tailing. Comparing those with the after endcapping data, Table 9, the 1- and 2-aminoanthracene were not tailing as significantly.

Figure 35 showed the before and after chromatograms with a mobile phase of 12% methanol and 88% water. The after endcapping column retains all of the solutes longer. The endcapping step covered the free silanols and this eliminated most of the mixed mode retention, leaving host-guest interactions the main reason for the longer retention.

The reason for the elution order in the series of 1- and 2-aminonapthalene was due to the way the amino groups were oriented on the benzene molecules. The 1-aminonapthalene interacted more with the stationary phase than the 2-aminonapthalene thus retaining longer on the column. The aminoanthracenes showed the same pattern. When just looking at the aminonapthalenes and napthalene, the aminonapthalenes eluted faster due to the polarity that the amino groups added. Figure 36 compares the k' before and after endcapping.

Analyte	t_r	k'	A_s
1-amino-napthalene	1.09	0.4544	1.25
2-amino-napthalene	1.64	1.1889	1.0
napthalene	4.50	5.0071	1.20
1-amino-anthracene	21.40	27.5732	2.15
2-amino-anthracene	27.63	35.8723	2.65

Table 8: ENE Series Before Endcapping

Analyte	t_r	k'	A_s
1-amino-napthalene	1.39	0.9857	1.40
2-amino-napthalene	1.72	1.4571	1.0
napthalene	5.83	7.3286	1.35
1-amino-anthracene	23.01	31.8714	1.85
2-amino-anthracene	28.76	40.0857	1.45

Table 9: ENE Series After Endcapping

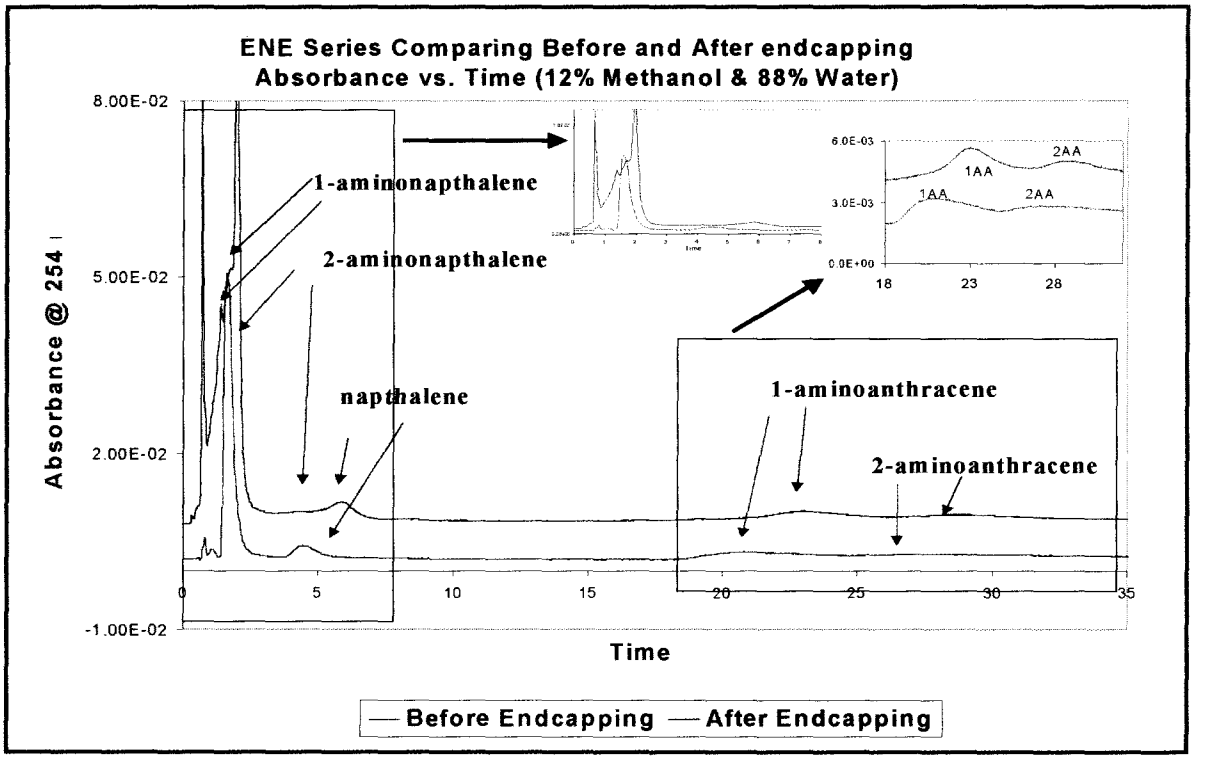


Figure 35: HPLC Data for the ENE Series Comparing Before and After Endcapping (12% Methanol & 88% Water)

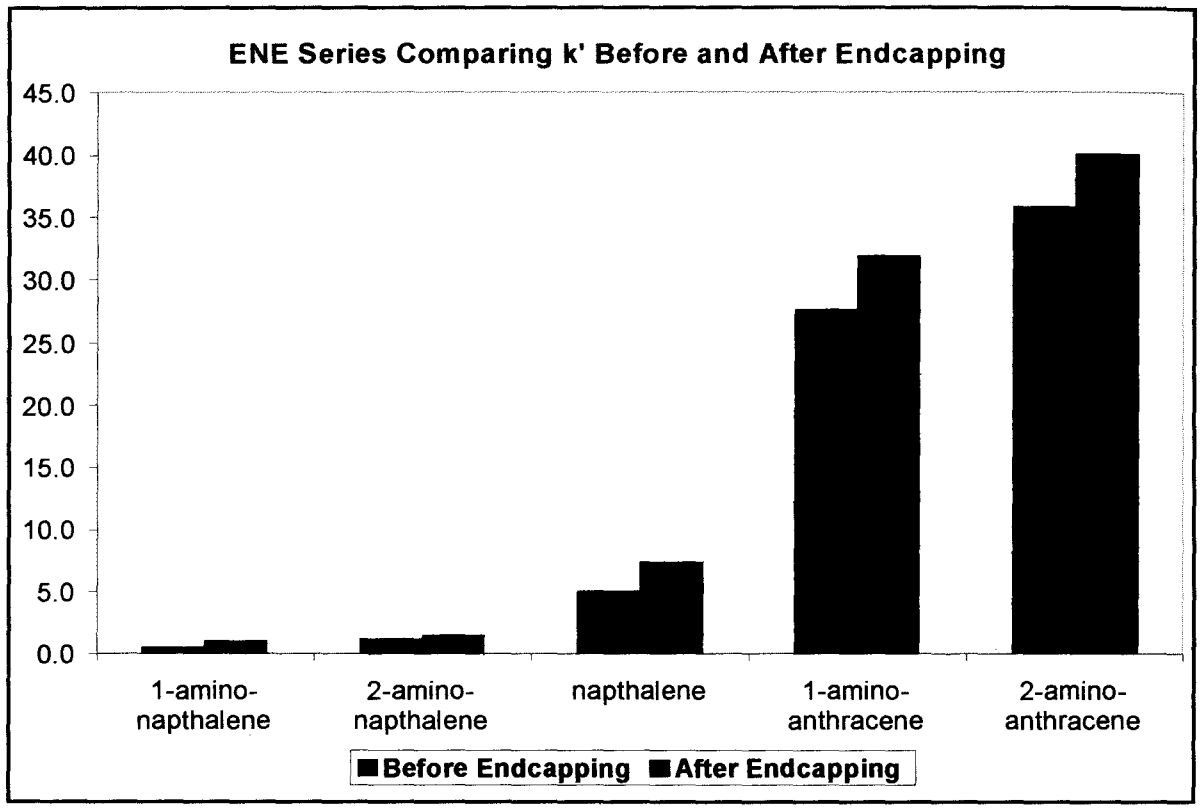


Figure 36: Capacity factors for the ENE Series Comparing Before and After Endcapping

The Knox Test (27)

The Knox test was performed to observe if in fact there were abundant amounts of free silanols left on the amino-derived silica. In the Knox test dry hexane was the non-polar mobile phase with methanol as the solute. The Knox test investigated stationary phases for residual silanols by performing the chromatography on a reversed phased column with a normal phase method.

The dry hexane was very non-polar and it allowed polar solutes (methanol) to partition with free silanol groups. If there were not a significant amount of silanol groups there will be no retention, but if a number of free silanol

groups are still left on the amino-derived silica, a significant amount of peak tailing will take place. The asymmetry factor can display qualitatively the presence of these types of interactions that will lead to peak tailing.

The results for the Knox test are shown in Table 10. The corrected retention times, t_r' were calculated from the formula:

$$t_r' = t_r - t_m$$

where t_r was the observed retention time in minutes and t_m was the dead time in minutes. The determination if there was a significant amount of peak tailing was observed based on the capacity factor. If k' was less than 0.5 there is no significant peak tailing. If peak tailing was significant the capacity factor will be greater than 0.5 and the result was a large number of free silanols still on the surface of the silica.

Conditions	t_r'	k'	A_s
Before Endcapping	0.80	1.14	1.0
After Endcapping	0.29	0.41	1.2

Table 10: The Knox Test with 100% Dry Hexane and Methanol as the Solute Comparing Before and After Endcapping

The results of the Knox test summarized in Table 10 indicates that the methanol solute was retained before endcapping, which was a sure indication that there were free silanols on the surface. The k' before endcapping was 1.14, which is greater than 0.5 and it was concluded that the endcapping step was necessary. After endcapping the k' was 0.41, which means the methanol was not retained and no interactions due to free silanols are taking place. The results

concluded that the surface of the derived silica was covered during the initial synthesis and left some free silanols on the surface. Post endcapping the results show interactions occurring on the column were due to host-guest interactions and not mixed mode retention.

The asymmetry factors showed symmetrical peaks because of the way the peaks are calculated. The distance was measured from half of the peak height and as shown in Figure 37, the peaks are close to one at that distance even though looking at the graph it may seem otherwise.

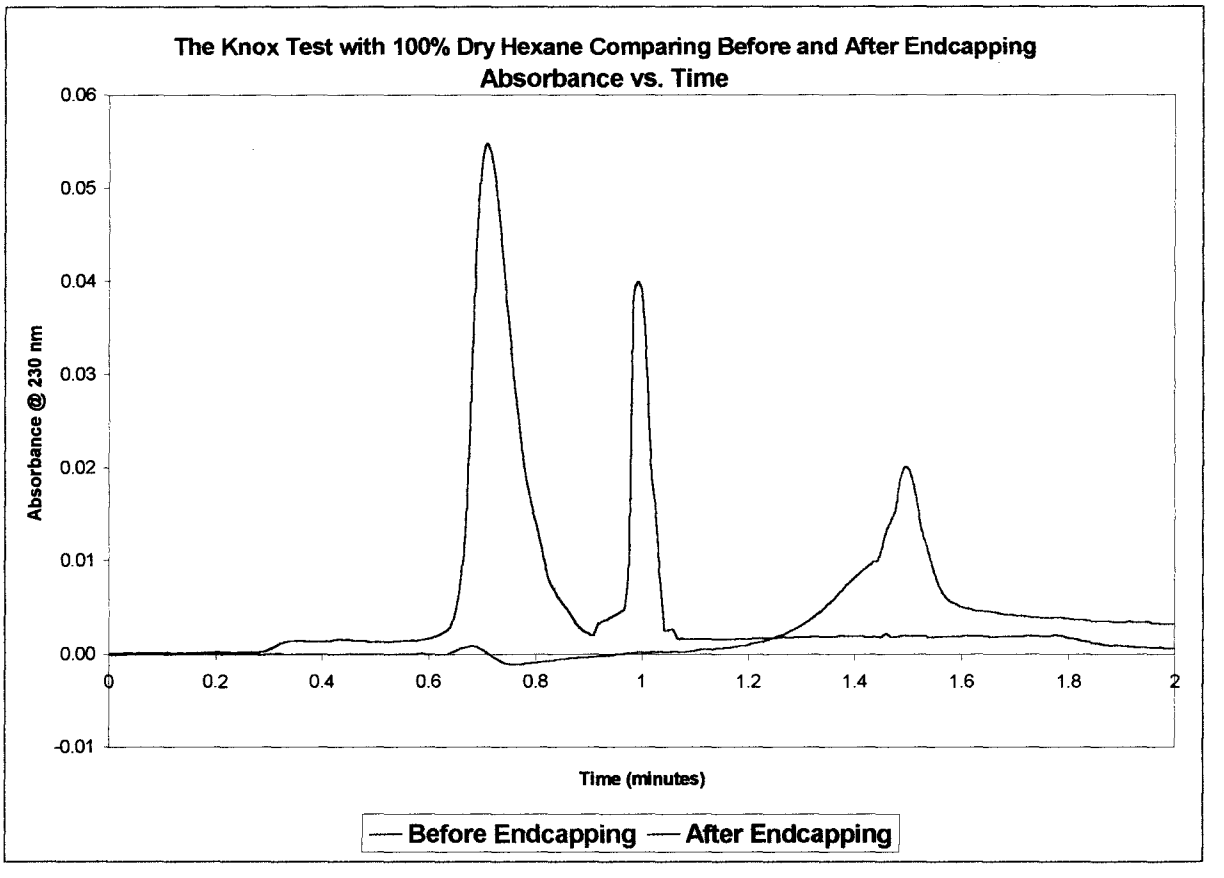


Figure 37: HPLC Data for the Knox Test with 100% Dry Hexane and Methanol as the Solute Comparing Before and After Endcapping

Elemental Analysis

Elemental analysis was performed to determine the surface coverage on the silica. Table 11 showed the % nitrogen and carbon after each step. Each step was analyzed to ensure coverage was accomplished. Starting with the Nucleosil® (Macherey-Nagel), the initial coverage was 0.0862% carbon coverage and 0.00492% nitrogen coverage. The aminopropyldimethylethoxysilane was attached to the Nucleosil® and the % carbon was then 0.0345 and the % nitrogen was 0.881.

When the tert-butyl-tetra-[2-aminoethoxy]calix[4]arene was reacted with glutaraldehyde and aminopropyl silica gel the % carbon significantly increased to 1.6% and the nitrogen to 0.12%. This indicates the desired coverage was accomplished. There was not a significant increase after the reduction of the product or after endcapping was performed. Increased nitrogen or carbon percentages were not expected to increase after these steps. This indicates that although there were some free silanols, as we saw in the chromatographic characterization section, they were not significant enough to increase the carbon or nitrogen percentages.

Sample	*% Nitrogen*	*% Carbon*
Nucleosil® (Macherey-Nagel)	0.00492	0.0862
3-aminopropyldimethylethoxysilane attached to the Nucleosil® (aminopropyl silica gel)	0.0881	0.345
tert-butyl-tetra-[2-aminoethoxy]calix[4]arene with glutaraldehyde and aminopropyl silica gel	0.12	1.60
tert-butyl-tetra-[2-aminoethoxy]calix[4]arene with glutaraldehyde and aminopropyl silica gel after reduction	0.12	1.54
tert-butyl-tetra-[2-aminoethoxy]calix[4]arene with glutaraldehyde and aminopropyl silica gel after endcapping	0.14	1.56

Table 11: Elemental Analysis Comparing the % Attachment of Nitrogen and Carbon

Chapter VI

Conclusions

The amino derived calix[4]arene reversed phase stationary phase was successfully prepared and characterized. The attachment of the tert-butyl-tetra-[2-aminoethoxy]calix[4]arene with the glutaraldehyde and aminopropyl silica gel was confirmed by elemental analysis. By spectroscopically comparing different steps of the synthesis and attachment, fluorescence and UV spectroscopy confirmed that the tert-butyl-tetra-[2-aminoethoxy]calix[4]arene was present. NMR and LC/MS also confirmed the structure of the tert-butyl-tetra-[2-aminoethoxy]calix[4]arene before attachment to the silica step was performed.

The attachment of the tert-butyl-tetra-[2-aminoethoxy]calix[4]arene with the glutaraldehyde to the aminopropyl Nucleosil® was confirmed by the resulting chromatograms, which is shown by host-guest complexation. Host-guest complexation was not indicated by the log k' versus the number of carbon atoms plot for the alkyl benzene series and the log k' versus the number of benzene rings in the PAH series. Host-guest interaction was suggested by the significant tailing of the 1- and 2-aminoanthracene peaks in the ENE series and the N,N-diethyltoluamide in the Reversed Phase test mixture.

To distinguish between host-guest interactions and mixed-mode retention, which also is shown by tailing peaks, comparison data of before and after endcapping was presented. Mixed-mode retention was due to free silanol groups left unreacted on the silica. The endcapping step was necessary to cover

the reactive free silanols due to the ability of mixed-mode retention interactions to take place.

Endcapping had a large effect on the Alkyl Benzene and PAH series as indicated by the asymmetry factors and plots of the log k' versus *n*. The endcapping process did not particularly effect the ENE series. The major effect in endcapping were observed in the Reversed Phase test mixture series leading to better separation because of the elimination of mixed-mode interaction with the free silanol groups.

Chapter VII

Future Work

Although many methods were utilized to prove attachment of the glutaraldehyde to the aminopropyl Nucleosil® other characterization methods could be used. A very useful method of characterization would be solid state NMR or CP/MAS NMR. This is a useful way to look at the surface of the silica and determine attachment of the amino derive calix[4]arene. Secondary – ion Mass Spectrometry (SIMS) would be another way to investigate the surface of the amino derived silica. With SIMS a beam of cesium ions of sufficiently high energy are directed on the surface and secondary ions are generated from the surface. This technique is a powerful way of looking at larger organic molecules (29).

Using the amino derived column synthesized in this research different types of series can be attempted to be separated. Different detection methods would have to come into play with some of the various series to be separated. For example if separating normal chained alcohols, a refractive index detector would be used. Separation with various chained sugars could take place using an electrochemical detector. With an increase in varied chromatographic data, stronger supported conclusions can be made.

To utilize the stationary phases synthesized in this research in industry, chirality must come into play. One method (30) would be to condense an amino protected chiral amino acid directly to the secondary amine already on the synthesized stationary phase. Protecting groups like t-butyloxycarbonyl or

fluorenylmethoxycarbonyl allow the carboxylic acid group of the amino acid to be selectively bonded to an amine without interference from the amino group that is already present on the amino acid.

The amino group on the new chiral center can then be produced at a later time by different methods. For example, t-butyloxycarbonyl is removable with an acid like 1% TFA in dichloromethane. Fluorenylmethoxycarbonyl is base labile with 20% piperdine in dimethylformamide. This situation tests if the chirality needs to be part of the calix cup structure to effect a chiral separation since the chiral groups would only be on the outside of the anchoring groups.

Another approach would be to use a chiral derivative that contains the bromoacetamide structure within it and add it to the bottom of the calix rings. Thus compounds like R or S 2-phenyl-2-bromoacetamide or R or S 2-bromoproprionamide could simply be used in place of the bromoacetamide the same synthesis. This would give a chiral phase where the chiral center is part of the bottom of the cup structure.

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