CHARACTERIZATION OF CALIX[N]ARENES AS STATIONARY AND MOBILE PHASE MODIFIERS FOR RP-HPLC

By

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

YOUNGSTOWN STATE UNIVERSITY
August, 2004

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Abstract

The bowl-shaped class of macrocyclic molecules known as calixarenes has become an increasingly important area of interest and research in the chromatographic sciences. Due to their ability to form host-guest complexes with a variety of analyte molecules, calixarenes have been successfully used as modifiers in both the stationary phases and mobile phases of reversed phase high performance liquid chromatography. Some calixarenes have also proven useful in performing chiral separations using HPLC.

This research was a study of 4-*tert*-butylcalix[n]arenes (n=4, 6, 8) as stationary phase modifiers when covalently bound to a silica stationary phase substrate and also when physically absorbed into a C18 stationary phase. 4-*tert*-butylcalix[4]arene and 4-sulfonic calix[4]arene were also studied as mobile phase additives for modifying RP-HPLC separations using a number of solute probes.

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Symbol	Definition	Units
HPLC	High Performance Liquid Chromatography	
psi	Pounds per Square Inch	
C18	Octadecyl	
UV	Ultraviolet	
μ, μm	micron	1×10^{-6} meter
mm	millimeter	1×10^{-3} meter
nm	nanometer	1×10^{-9} meter
PDA	PhotoDiode Array	
g	gram	
mL	milliliter	1×10^{-3} liter
ODS	Octadecylsilane	
μL	microliter	1×10^{-6} liter
min	minute	
°C	degrees Celsius	
L	liter	
M	Molar	moles per liter
HETP	Height Equivalent Theoretical Plate	
MeOH	Methanol	
A	Peak Asymmetry Value	

CHAPTER I

Introduction

Calixarenes

Calixarenes are macrocyclic, bowl-shaped molecules similar in structure to the crown ethers and cyclodextrins. Most commonly, they are comprised of repeating *para*-substituted phenolic units linked by methylene bridges. Generally, variations to the base structure are made by substitution of functional groups at either the upper or lower rim of the molecule, although other types of modification are possible as well. One of the more common calixarenes, 4-*tert*-butylcalix[4]arene is shown in Figure 1. As a result of their circular structure, calixarenes are able to form inclusion complexes with certain guest molecules. The relatively wide availability and low cost of calixarenes, along with their host-guest complexation properties, have made them valuable tools in the separation sciences.[1]

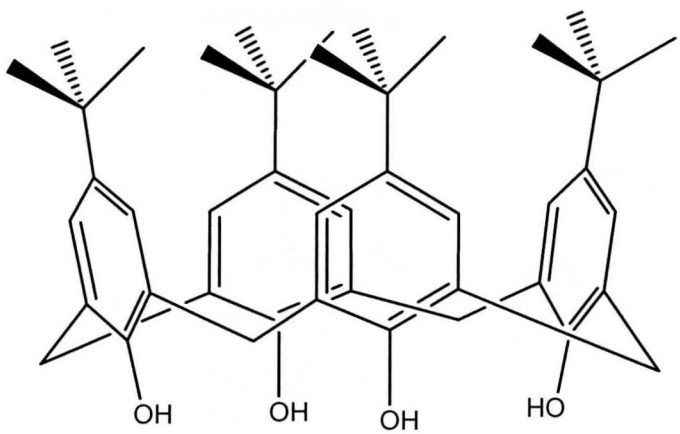


Figure 1. 4-*tert*-butylcalix[4]arene

The most common method of calixarene synthesis is the alkaline condensation reaction of formaldehyde and *tert*-butylphenol. Although this reaction is well known, leading to the *tert*-butylcalix[n]arene (n = 4, 6, or 8) product, the reaction mechanism is not well understood. Also, this synthetic 'one pot' method requires reflux conditions at a high temperature in irritating solvents.[1]

Recently, however, a one pot, solventless synthetic route to the *tert*-butylcalix[n]arenes was reported.[2] This method involves heating the formaldehyde and *tert*-butylphenol under alkaline conditions to a temperature of 50 °C for 45 hours, followed by workup of the product. This method offers the environmental and monetary advantages of being solventless while still producing similar yields to the more traditional synthetic method.[2] While these and other synthetic methods are well known and commonly used, calixarenes are also available for purchase, which can save time and money when studying the synthetic pathway is not the focus of the research.

As previously mentioned, calixarenes can be modified by adding various substituents to either the upper or lower rim of the molecule. One particular example of this is 4-sufonic calix[4]arene hydrate in which the functional group is substituted at the upper rim of the basket (Figure 2).

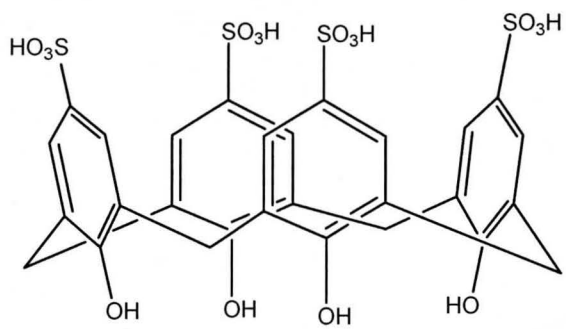
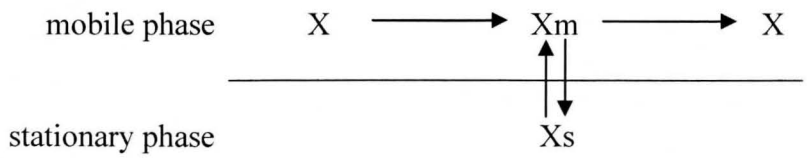


Figure 2. 4-sulfonic calix[4]arene hydrate

High Performance Liquid Chromatography

High Performance Liquid Chromatography, or HPLC, is a technique that, when used on an analytical scale, can separate, identify, and quantify a wide variety of solutes. HPLC can also be used on a preparative scale to separate and purify substances. Separations are based on the same principle as other forms of chromatography, which is the partitioning of the solute molecules between a stationary phase and a mobile phase. The characteristics unique to HPLC are a uniformly sized solid or immobilized liquid (or pseudo liquid) stationary phase, a liquid mobile phase, and operating pressures typically in the range of 1000 – 4000 psi.

In HPLC, as a sample is injected onto the head of the column, the mobile phase is pumped through the column and carries the sample with it. The level of interaction between the solute molecules, the mobile phase, and the stationary phase determines the amount of time the solute will take to elute from the column and be carried to the detector. Analytes that have stronger interactions with the mobile phase will elute earlier than analytes that have stronger interactions with the stationary phase. As a result of these interactions, a primary steady-state, which under ideal conditions approaches the equilibrium state, is established inside the HPLC column. This is illustrated below in Figure 3:



X = Analyte molecule

Figure 3. Primary Steady State/Equilibrium Process

For the ideal system depicted above, the equilibrium is characterized by the equilibrium constant, k, which is defined as ratio of the concentration of analyte in the mobile phase to the concentration of analyte in the stationary phase (Equation 1).

$$k = \frac{[Xm]}{[Xs]}$$ **Equation 1**

Ion Pair Chromatography

Ion pair chromatography is considered here as a useful analogy to the host-guest interactions of the calixarenes when used as mobile phase modifiers in chromatography. Ion pair chromatography is a technique useful for the separation of strong acids and bases (i.e., charged molecules) using a conventional reversed-phase HPLC column. This is done by adding a bulky organic molecule of opposite charge from the analyte to the mobile phase. Ion pairs are formed between the organic molecules in the mobile phase and the analyte molecules. These ion pairs act as nonionic polar molecules that are soluble in organic solvents, and are therefore soluble in the stationary phases used for reversed-phase chromatography.[3]

As the ion pairs are formed and carried through the column during a separation, a secondary equilibrium is established, which is illustrated by Figure 4. The equilibrium expression is then defined as in Equation 2.[4]

$$A^+_{mp} + B^-_{mp} \rightleftharpoons A^+B^-_{sp}$$

A^+_{mp} = Analyte ion in mobile phase

B^-_{mp} = Ion pairing agent in mobile phase

$A^+B^-_{sp}$ = Ion pair in stationary phase

Figure 4. Ion Pair Formation Equilibrium

$$k = \frac{[A^+B^-_{sp}]}{[A^+_{mp}][B^-_{mp}]}$$ **Equation 2**

where: k = equilibrium constant
$[A^+B^-_{sp}]$ = concentration of ion pair in the stationary phase
$[A^+_{mp}]$ = concentration of A^+ in the mobile phase
$[B^-_{mp}]$ = concentration of B^- in the mobile phase

Factors that affect the value of the equilibrium constant, and thus the separation results, are mobile phase pH, mobile phase ionic strength, organic content of the mobile phase, and temperature. Any change to the identity of the pairing agent typically has the most drastic effect on the separation results, which can be somewhat difficult to predict.[3] However, changing the concentration of the pairing agent is usually the easiest way to adjust the mobile phase strength while keeping the selectivity of the separation constant.

Due to the large number of pairing agents used, and the ability to easily modify the concentration of the pairing agent, it is possible to design an optimal ion pair separation for almost any desired charged analytes.

Host-Guest Complexation

As previously described, calixarenes are circular and can have thermodynamically stable conformations that give them the shape of a bowl or a cup, which allows them to act as host molecules (Figure 5). Certain solutes, or guest molecules, are able to interact with calixarenes, and the strength of the interaction depends upon a combination of several factors. The first factor is calixarene cavity size. The more phenolic units contained in the calixarene, the larger the cup, and the calixarene can host larger guest molecules.

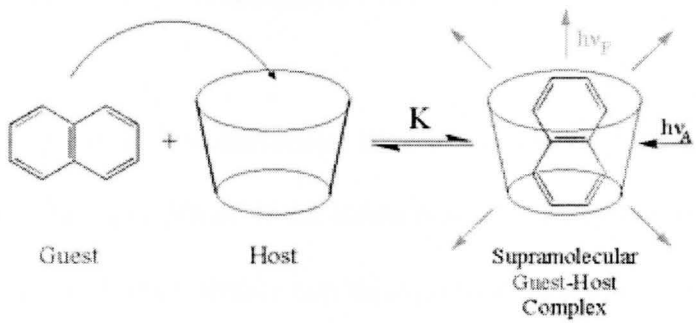
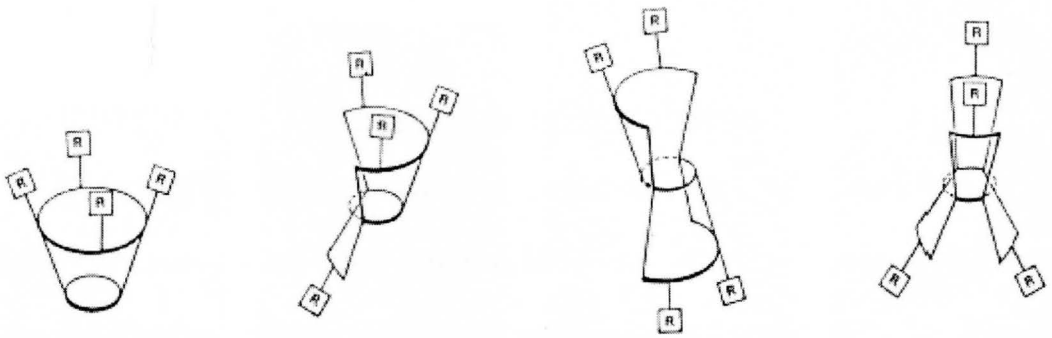


Figure 5. Illustration of Host-Guest Complexation

Related to the size of the calixarene cup is the conformation of the calixarene (Figure 6). The calix[4]arenes and calix[8]arenes are very similar in their conformational interconversions. They have higher conformational inversion barriers, and as a result prefer to exist in the characteristic 'cone' conformation. Calix[6]arene has a lower conformational inversion barrier and also a higher number of possible conformers.

Therefore, a more flattened out cone conformation is favored.[5] As a result, it is hypothesized that the calix[4]arenes and calix[8]arenes may be able to form stronger, more stable host-guest complexes than the calix[6]arenes.



Cone Partial Cone 1, 2-Alternate 1,3-Alternate

Figure 6. Illustration of Common Conformations of Calix[4]arenes[5]

Once the proper size guest molecules have fit themselves into the calixarene cavity, other interactions take place. If the solute molecule contains a hydrophobic region, it will interact with the phenolic cup through London forces and π-π interactions. Hydrogen bonding can also occur between regions of the solute molecules and the –OH groups on the basket of the calixarene.

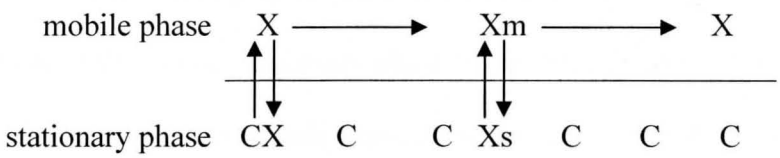
The strength of the host-guest complex can be estimated using the mathematical equation derived by Kalchenko et. al (Equation 3). It relates the capacity factor of the analyte of interest to the concentration of calixarene host present in the separation.[6]

$$1/k' = 1/k'_o + [Host]/K_D \times k'_o \qquad \textbf{Equation 3}$$

where: k' = capacity factor for peak of interest in presence of the host
k'_o = capacity factor for peak of interest in absence of the host
[Host] = concentration of calixarene mobile phase additive
K_D = dissociation constant for the host-guest complex

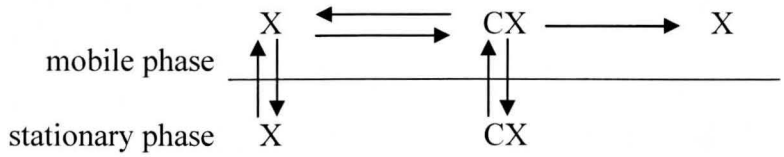
Calixarenes and Chromatography

As mentioned in the discussion of ion pair chromatography, secondary equilibrium processes can occur within an HPLC column. Other examples of these are metal complexation, micelle interactions, and host-guest complexation. The focus of this research is on host-guest complexation. Addition of host molecules to the stationary phase, the mobile phase, or both increases the levels and types of interactions that can occur with guest solute molecules during a separation. If host molecules are dissolved or immobilized primarily in the stationary phase, the retention of guest molecules is expected to increase (Figure 7). Conversely, if the host molecules are primarily dissolved in the mobile phase, the retention of guest molecules is expected to decrease (Figure 8).



X = Analyte molecule
C = Calixarene
CX = Calixarene/Analyte complex

Figure 7. Secondary Equilibrium Process with Calixarene in the Stationary Phase



X = Analyte molecule
CX = Calixarene/Analyte complex

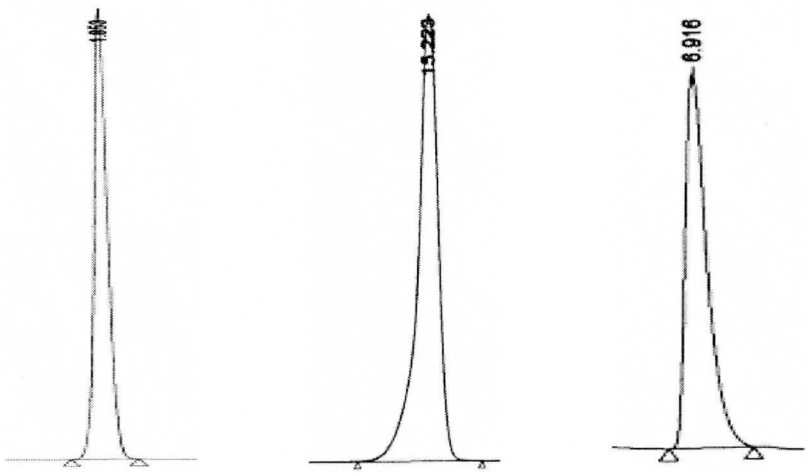
Figure 8. Secondary Equilibrium Process with Calixarene in the Mobile Phase

These secondary equilibrium processes add to the complexity of the separation, and make it more difficult to place a numerical value on the equilibrium constant.

Two of the most apparent chromatographic indicators of host-guest interactions are peak asymmetry and deviation from linearity in a graph of the log of the retention time versus the homologue number of a component of a homologous series of compounds. First for separations performed where only typical reversed-phase interactions occur, the resulting peaks are expected to be relatively Gaussian in shape. However when additional levels of interactions occur, the peak shapes become increasingly asymmetrical, observed as either peak fronting or peak tailing (Figure 9). More specifically, ideal chromatographic peaks are Gaussian in shape due to the concentration of the analyte in the stationary phase being directly proportional to the concentration of the analyte in the mobile phase throughout the separation. If additional levels of interactions occur that cause the concentration of analyte in the stationary phase to be greater than the concentration of analyte in the mobile phase, then peak tailing is observed and termed Langmuir behavior. Conversely, if additional interactions occur that cause the concentration of the analyte in the stationary phase to be less than the

concentration of the analyte in the mobile phase, then peak fronting is observed and termed Anti-Langmuir behavior.

Second, for separations of a homologous series of compounds performed under typical reversed-phase conditions on an alkyl column, a plot of the log of the retention time of each component versus the homologue number of that component is linear or nearly linear in nature. Therefore, any deviation from linearity in this plot is a strong indication of higher levels of interaction, in this case host-guest complex formation.[13]



(a) (b) (c)

Figure 9. Illustration of Peak Shapes in Chromatography
(a) Ideal Gaussian Peak
(b) Peak Fronting
(c) Peak Tailing

Another indication of host-guest interactions in a separation is a shift in the retention time of the solutes from pre-modification to post-modification conditions. This

direct comparison can only be made if the strength of the mobile phase remains constant throughout the experiments. If the strength of the mobile phase is changed, then the capacity factor for each solute pre-modification and post-modification can be used for the comparison. Capacity factor (k') is defined as the retention time of the component minus the column dead time divided by the column dead time (Equation 4).

$$k' = \frac{t_r - t_0}{t_0} \qquad \textbf{Equation 4}$$

where: k' = capacity factor
t_r = retention time of the component of interest
t_0 = column dead time

CHAPTER II

Statement of the Problem

There are numerous research reports utilizing a wide variety of calixarenes as stationary and mobile phase modifiers for HPLC. The purpose of this research is to provide a comprehensive examination of the most common types of calixarenes used as both stationary and mobile phase modifiers for HPLC. Within this research, there are four areas of focus. First, the synthetic method of chemically binding 4-*tert*-butylcalix[n]arenes to silica and the resulting effect of using this modified stationary phase for HPLC analysis were examined. Second, the method of absorbing the 4-*tert*-butylcalix[n]arenes to the stationary phase and the resulting effect of using this type of modified stationary phase for HPLC analysis was studied. Third, the use of 4-*tert*-butylcalix[n]arenes as additives to modify the mobile phase and the effect of these modifiers on separation results was examined. Fourth, the use of the water soluble 4-sulfonic calix[4]arene hydrate as a mobile phase modifier for HPLC analysis was probed. Tying all of this information together allows for a better understanding of the use of calixarenes in HPLC, both in the ease of use of the methods and also in the levels of interactions that occur in each of the methods.

CHAPTER III

Historical

The host-guest complexation characteristics, widespread availability, and relative inexpense of calixarenes make them an interesting and increasingly popular area of research, especially for the separation sciences. Their popularity has also led to an increasing amount of research focused on utilizing calixarenes in the chromatographic process.

One application in which calixarenes have been used is capillary gas chromatography. Chiral calixarenes and *p-tert*-octyl calix[5]arene have been researched as stationary phase modifiers and shown to successfully form host-guest complexes in capillary gas chromatography.[7,8] Also, in 1999, Park et al. utilized calixarene stationary phases in capillary gas chromatography to successfully resolve positional isomers of numerous compounds.[9] Similar results were obtained by Zhang et al. when they used polysiloxane calixarene stationary phases to resolve aromatic isomers in capillary gas chromatography.[10]

Calixarenes have also been utilized for performing enantiomeric separations by electrokinetic chromatography. In this research, four amino acid residues were attached to the lower rim of calix[4]arene. The product was then used in electrokinetic capillary chromatography to separate enantiomers of a racemic mixture of binaphthyl derivatives.[11]

One major difference between calixarenes and cyclodextrins, which are also known to form host-guest complexes with a variety of analytes, is that calixarenes absorb light strongly in the UV region of the spectrum. This property of the calixarenes, in

addition to their host-guest complexation ability, makes them useful in capillary electrophoresis for separation and detection of analytes that are transparent in the UV region. Using the technique proposed by Arce et al., sulfonated calixarene mobile phases can be used in capillary electrophoresis to quickly separate amino acids, amines, alkaline earth cations, and anions.[12]

Several studies have been conducted using silica-bonded calixarene stationary phases in liquid chromatography. Brindle et al. developed two different methods for synthesizing these bonded stationary phases. The first method involved the reaction of triethoxysilyl calix[4]arene with silica, which resulted in the bonded calix[4]arene tetraamide stationary phase. The second method utilized a hydrosilylation reaction of hydride-derivatized silica with *p*-allylcalix[6]arene hexaester, which resulted in a calix[6]arene hexaester stationary phase. Characterization studies provided proof that the calixarenes were indeed chemically bonded to the silica.[13] Similarly, Xu et al. synthesized a hydrophobic *p-tert*-butyl-calix[6]arene silica bonded stationary phase. Using this stationary phase and a methanol based mobile phase, they were able to separate isomers of aminophenol and nitroaniline and also positional isomers of PAHs by HPLC.[14] These separations suggest the presence of different host-guest interactions between the calixarene stationary phase and the isomers.

A method of immobilizing calixarenes onto a silica substrate for use in an HPLC column has been studied and reported, and has recently been patented. This method involves refluxing silica with a polyhalide tether such as 1,7-dichlorooctamethyltetrasiloxane in an appropriate solvent, adding the *tert*-butylcalix[n]arene, and refluxing again. In this reaction, a base is needed to consume the

HCl that is produced as a byproduct. When using a reflux solvent such as toluene, pyrazine can be added to consume the HCl. An alternative to the toluene/pyrazine system is substituting triethylamine as a basic solvent for the reflux reaction.[15,16]

Chemical immobilization of calixarenes onto the HPLC stationary phases offers a relatively high percent coverage of the stationary phase. However in order to avoid the time involved and problems associated with this synthetic method, absorption is an alternate method of placing the calixarenes onto the stationary phase. By dissolving a hydrolyzed 4-*tert*-butylcalix[4]arene O, O', O'',O'''*tetra*-L-serine derivative in an appropriate solvent and pumping it over a C18 column for a predetermined length of time, it has been shown that the calixarene absorbs onto the C18 stationary phase. This is determined by reduction of capacity factors and tailing peak shapes of a phenyl ring homologous series when run on the calixarene modified column versus a C18 column.[17]

Calixarene bonded stationary phases in HPLC have shown varying selectivities toward a variety of analytes. Many factors have been proposed that influence the separations of mixtures of these analytes such as: host-guest interactions, hydrogen bonding, hydrophobic interactions, ring size of the calixarene, substitution at the upper rim, and absorption of the solvent into the stationary phase or calixarene cavity. This variety of influences can make it difficult to predict separation results for mixtures of analytes.[18]

As previously stated, calixarenes are able to form host-guest complexes due to their annular shape and bowl-like conformation. Related to this property is the ability of the molecule to discriminate between isomers of chiral substances. Recently, the synthesis of chiral calixarenes and their ability to be used as chiral recognition molecules

has been reported. Once the chiral calixarenes were synthesized and their structures confirmed using NMR spectroscopy, it was shown that the calixarenes were able to discriminate between the isomers of α-phenylethylamine. More specifically, the (-)-receptor readily formed a host-guest complex with the (R) isomer, and did not with the (S) isomer of α-phenylethylamine.[19]

Similarly, the synthesis of inherently chiral water soluble calix[4]arenes was reported by Tairov et. al. By placing achiral groups asymmetrically on the calixarene rim, the molecule becomes chiral. Since chiral calixarenes are known to be effective hosts for chiral guest molecules, this water solubility property makes them even more valuable for use in chiral chromatography.[20]

Still another method for using calixarenes as modifiers for chromatography has been to dissolve them in the mobile phase of an HPLC separation. 4-sulfonic calix[6]arene and *p*-(N,N-diallylaminomethyl)calix[6]arene were used as mobile phase additives for HPLC and showed significant reduction in the capacity factors of a series of aminonapthol compounds. This indicates that host-guest complexation is occurring between the analytes and the calixarene additive in the mobile phase.[21]

Similarly, *p*-sulfonatocalix[4]arene was used by Kalchenko et. al. as a mobile phase additive for the separation of amino acids. Again, the capacity factors for the amino acids are reduced upon addition of the calixarene to the mobile phase, indicating host-guest interactions. Taking this system one step further, the researchers used their data to study the stability constants of the host-guest complexes that formed between the amino acids and the calixarenes. They were able to derive an equation that related the

capacity factors and concentration of the host in the mobile phase to the dissociation constant for the complex.[6]

The research that has been conducted on calixarenes has resulted in a better understanding of the molecules and their possible applications. The purpose of the current research is to characterize the interactions of calix[n]arenes as mobile and stationary phase modifiers for reversed-phase high performance liquid chromatography.

CHAPTER IV

Materials and Apparatus

Reagents for HPLC

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (Milwaukee, WI). HPLC grade THF was purchased from JT Baker (Phillipsburg, NJ). Uracil, phenol, N,N-diethyl-m-toluamide, toluene, benzene, naphthalene, anthracene, aniline, 2-aminoanthracene, 2-aminonaphthalene, 1,5-naphthalenediamine, 1,8-naphthalenediamine, and 2,3-naphthalenediamine were from Aldrich Chemical Company (Milwaukee, WI). Ethylbenzene, propylbenzene, butylbenzene, and 4-sulfonic calix[4]arene hydrate were purchased from Acros Organics (Morris Plains, NJ).

Reagents for Synthesis

Triethylamine was purchased from Atofina (Philadelphia, PA). Nucleosil® silica, 7 μm, 1000Å pore size, was purchased from Macherey-Nagel (Easton, PA). The 4-*tert*-butylcalix[4]arene, 4-*tert*-butylcalix[6]arene, 4-*tert*-butylcalix[8]arene, chloroform and 1,7-dichlorooctamethyltetrasiloxane were purchased from Aldrich Chemical Company (Milwaukee, WI). THF and methanol were purchased from Fisher Scientific (Pittsburgh, PA).

Apparatus for HPLC

The synthesized calixarene stationary phases were packed into clean, empty stainless steel HPLC columns using an Alltech Standard Slurry Packer, PN 1666. Commercially available pre-packed columns used in this research included: Alltech Adsorbosphere C18 5 μm, 250 mm x 4.6 mm column, Phenomenex Sphereclone ODS 5μ, 250 mm x 4.6 mm column, and Alltech Nucleosil® C18 5μm, 150 mm x 4.6 mm column.

A Waters 2695 Separations Module was used for all HPLC analyses. The Separations Module was equipped with an autosampler, an inline solvent degasser, a column and sample chamber heater, and a gradient proportioning valve capable of blending up to four solvents. The system was run using a 100 μL sample loop and a 250 μL syringe. Detection was performed with a 996 PhotoDiode Array detector tuned to 254 nm. The system was controlled with a Waters Millennium software package.

CHAPTER V

Experimental

Sample Preparation of Standard Test Mixture

The standard test mixture consisted of 4 components: uracil, phenol, N,N-diethyl-m-toluamide, and toluene (Figure 10). This mixture was prepared by weighing out 0.01 g of uracil, 0.02 g of phenol, 0.03 g of N-N-diethyl-m-toluamide, and 0.03 g of toluene and diluting to a total volume of 20 mL with HPLC grade methanol. The individual components were prepared in the same manner.

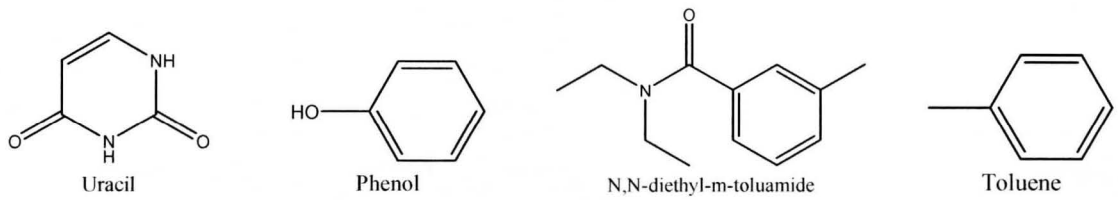


Figure 10. Standard Test Mixture Components

HPLC Analysis of Standard Test Mixture

The separation of the components of the standard test mixture was performed on all C18 columns using a mobile phase composition of 65/35 methanol/water. Using the bound 4-*tert*-butylcalix[6]arene modified stationary phase, a mobile phase of 10/90 methanol/water was used to separate the standard test mixture components. When 4-*tert*-butylcalix[4]arene was used as a mobile phase modifier with the Phenomenex ODS column, the composition of the mobile phase was 50/50 THF/water. A flow rate of 1.0

mL/min was used with a column and sample temperature of 25 °C. The injection volume was 15 μL in all cases except the experiments with the sulfonic calix[4]arene mobile phase additive, in which the injection volume was 5 μL. Detection was performed using a PDA at 254 nm.

Sample Preparation of Alkylbenzene Homologous Series

The alkylbenzene homologous series contained toluene, ethylbenzene, propylbenzene and butylbenzene (Figure 11). This mixture was prepared by measuring 0.5 mL of each of the components and diluting to a final volume of 20 mL with HPLC grade methanol. The individual components were prepared in the same manner.

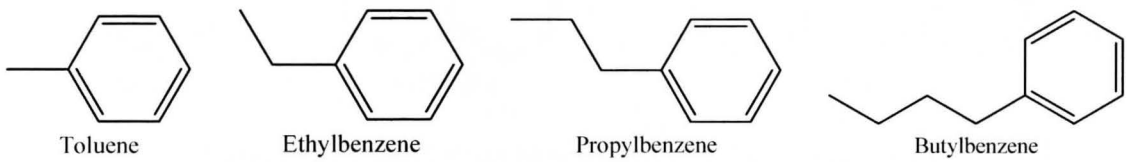


Figure 11. Alkylbenzene Homologous Series Components

HPLC Analysis of Alkylbenzene Homologous Series

The alkylbenzene homologous series was separated on all C18 columns using a mobile phase composition of 75/25 methanol/water. When using the bound 4-*tert*-butylcalix[6]arene modified stationary phase, the mobile phase composition was 30/70 methanol/water. The mobile phase composition was 75/25 methanol/water when the 4-*tert*-butylcalix[n]arenes were absorbed on the stationary phase of the Adsorbosphere C18

columns. When 4-*tert*-butylcalix[4]arene was used as a mobile phase additive with the Phenomenex ODS column, the mobile phase composition was 50/50 THF/water. The flow rate was 1.0 mL/min, the injection volume was 15 µL, and the sample and column temperatures were set at 25 °C. Detection was performed by a PDA at 254 nm.

Sample Preparation of Phenyl Ring Homologous Series

The phenyl ring homologous series consisted of benzene, naphthalene, and anthracene (Figure 12). This was prepared by mixing 0.02 g of benzene, 0.01 g of naphthalene, and 0.01 g of anthracene and diluting to a final volume of 20 mL with HPLC grade methanol. The individual components were prepared in the same way.

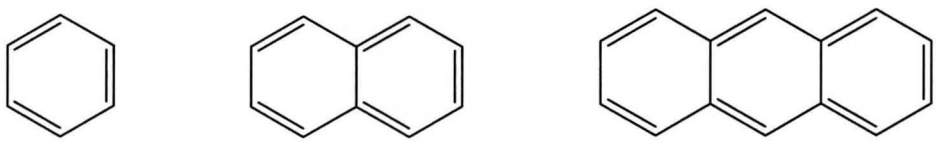


Figure 12. Phenyl Ring Homologous Series Components

HPLC Analysis of Phenyl Ring Homologous Series

The phenyl ring homologous series components were separated on all C18 columns using a mobile phase of 75/25 methanol/water, an injection volume of 15 µL, and a flow rate of 1.0 mL/min. When using the bound 4-*tert*-butylcalix[6]arene modified stationary phase, the mobile phase composition was 35/65 methanol/water, the injection volume was 15 µL, and the flow rate was 1.0 mL/min. When the 4-*tert*-

butylcalix[4,6]arenes were absorbed onto the stationary phase of the Adsorbosphere C18 columns, the mobile phase composition, injection volume, and flow rate were the same at 75/25 methanol/water, 15 μL, and 1.0 mL/min. When 4-*tert*-butylcalix[4]arene was used as a mobile phase additive with the Phenomenex ODS column, the composition of the mobile phase was 50/50 THF/water, the injection volume was 15 μL, and the flow rate was reduced to 0.25 mL/min. When the 4-sulfonic calix[4]arene was used as a mobile phase additive with the Nucleosil® column, the mobile phase composition was 75/25 methanol/water, the injection volume was 5 μL, and the flow rate was 1.0 mL/min. In all cases, the sample and column temperatures were 25 °C, and detection was performed with a PDA at 254 nm.

Sample Preparation of Amino Phenyl Ring Homologous Series

The amino phenyl ring homologous series contained aniline, 2-aminonaphthalene, and 2-aminoanthracene (Figure 13). It was prepared by weighing out 0.015 g of aniline, 0.007 g of 2-aminonaphthalene, and 0.004 g of 2-aminoanthracene and diluting to a final volume of 4 mL with HPLC grade methanol. The individual components were prepared in the same way.

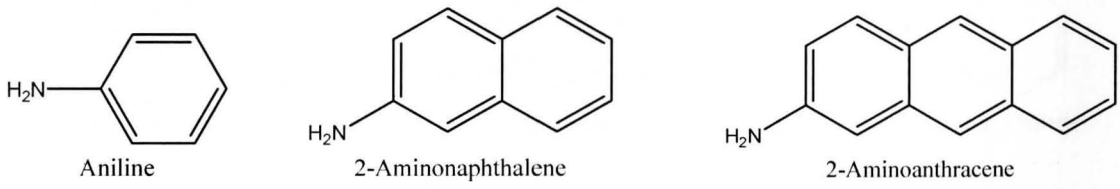


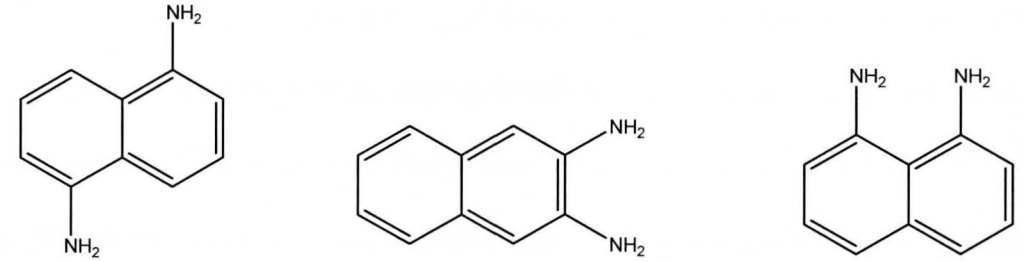
Figure 13. Amino Phenyl Ring Homologous Series Components

HPLC Analysis of Amino Phenyl Ring Homologous Series

The amino phenyl ring homologous series was separated on the Nucleosil® C18 column using a mobile phase composition of 60/40 methanol/water and 55/45 methanol/water. When the 4-sulfonic calix[4]arene was used as a mobile phase modifier with the Nucleosil® column, the composition was 55/45 4-sulfonic calix[4]arene in methanol/water. In both cases, the flow rate was 1.0 mL/min, the injection volume was 5 µL, the sample and column temperatures were 25 °C, and detection was performed with a PDA at 254 nm.

Sample Preparation of Diaminonaphthalene Series

The diaminonaphthalene mixture contained 1,5-diaminonaphthalene, 2,3-diaminonaphthalene, and 1,8-diaminonaphthalene (Figure 14). It was prepared by weighing out 0.004 g of 1,5-diaminonaphthalene, 0.004 g of 2,3-diaminonaphthalene, and 0.005 g of 1,8-diaminonaphthalene and diluting to a final volume of 4 mL with HPLC grade methanol. The individual components were prepared in the same way.



1,5-Diaminonaphthalene 2,3-Diaminonaphthalene 1,8-Diaminonaphthalene

Figure 14. Diaminonaphthalene Series Components

HPLC Analysis of Diaminonaphthalene Series

The components of the diaminonaphthalene mixture were separated on the Nucleosil® C18 column using a mobile phase composition of 50/50 methanol/water and 45/55 methanol/water. When the 4-sulfonic calix[4]arene was used as a mobile phase modifier, the mobile phase composition was 45/55 4-sulfonic calix[4]arene in methanol/water. In both cases, the flow rate was 1.0 mL/min, the injection volume was 5 μL, the sample and column temperatures were 25 °C, and detection was performed with a PDA at 254 nm.

Preparation of Bound 4-*tert*-butylcalix[6]arene Modified Stationary Phase

The chemically bound 4-*tert*-butylcalix[6]arene stationary phase was synthesized using the following procedure, which was previously researched by this group and patented during the course of this research.[13,14] Nucleosil® silica was dried at 200 °C for 16 hours. A reflux apparatus was thoroughly purged with nitrogen. Approximately 1.83 g of the dried silica, 100 mL of dry triethylamine, and 2 mL of 1,7-dichlorooctamethyltetrasiloxane were added to the round bottom flask. The mixture was refluxed for 8 hours. After cooling, 5 g of 4-*tert*-butylcalix[6]arene was added to the mixture in the round bottom flask, and again refluxed for 8 hours. The mixture was filtered over a 0.45 μm nylon membrane filter. The product was washed with 100 mL of warm THF, 50 mL of chloroform, and then 100 mL of warm methanol. The isolated product was dried at 120 °C for 5 hours.

A slurry of the bound 4-*tert*-butylcalix[6]arene stationary phase was prepared in methanol and packed into a stainless steel, 150 x 4.6 mm HPLC column using an Alltech air pump. The column was packed at 5000 psi for approximately 20 minutes.

Preparation of Absorbed 4-*tert*-butylcalix[n]arene Modified Stationary Phases

The 4-*tert*-butylcalix[4]arene modified stationary phase was prepared in the following manner. A 0.001 M solution of 4-tert-butylcalix[4]arene in THF was prepared by weighing 0.32 g of 4-*tert*-butylcalix[4]arene and diluting to 500 mL with THF. The solution was pumped over the Phenomenex Sphereclone ODS column at 0.25 mL/min for 4 hours. The mobile phase was then switched to 65/35 methanol/water and pumped at 1.0 mL/min for 1 hour.

The 4-*tert*-butylcalix[6]arene modified stationary phase was prepared in a similar way. A 0.001 M 4-*tert*-butylcalix[6]arene solution in THF was prepared by weighing 0.50 g of 4-*tert*-butylcalix[6]arene and diluting to 500 mL with THF. This solution was pumped over an Adsorbosphere C18 column at 1.0 mL/min for 1 hour. The mobile phase was then switched to 65/35 methanol water and pumped at 1.0 mL/min for 30 minutes.

Preparation of Calixarene Modified Mobile Phases

The 0.001M 4-*tert*-butylcalix[4]arene modified mobile phase was prepared by weighing 0.32 g of 4-*tert*-butylcalix[4]arene and diluting to 500 mL with THF. The 5×10^{-4} M 4-sulfonic calix[4]arene modified mobile phase was prepared by weighing 0.37 g of 4-sulfonic calix[4]arene hydrate and diluting to 1 L with methanol.

CHAPTER VI

Results and Discussion

Use of the Standard Test Mixture

The standard test mixture used as a reference in this work was chosen because of its common use to probe and monitor the performance of traditional reversed-phase C18 columns. The four components of the mixture, uracil, phenol, N,N-diethyl-m-toluamide, and toluene, each provided insights regarding different types of interactions occurring between the mobile and stationary phases.

Because of its relatively high polarity, uracil is commonly considered unretained by a C18 stationary phase. Thus it is widely used to mark the dead time of the column, which is the time it takes for a solute to travel in the mobile phase from the injector, through the column, and to the detector. The dead time of each column was used in the calculation of the corrected retention time of each solute analyzed. This was done by subtracting the dead time from the retention time of each solute. Phenol, which is a weak proton donor, provided insights about the basicity of the stationary phase. N,N-diethyl-m-toluamide, a weak proton acceptor, provided information about the acidity of the stationary phase, which is a good indicator of the availability of any free surface silanol groups for solute interaction. Finally the non-polar hydrocarbon, toluene, is used to monitor the overall non-polarity of the stationary phase, and its peak shape can help to indicate column degradation via hydrolysis or packing defects.

This standard test mixture can also be used to monitor characteristics of the column such as the number of theoretical plates (Equation 5) and the height equivalent

theoretical plate, or HETP (Equation 6). These two pieces of data are useful to track over time to assess the general quality of the stationary phase and condition of the column packing. Drastic changes in these values, either suddenly or over a period of time, may indicate that significant physical and or chemical changes have occurred to alter the nature of the stationary phase and its physical packing, or they may indicate that the column's maximum lifetime is reached and the column should be replaced. Tracking such data is a quality control in monitoring solute interactions as was done in this work.

$$N = 5.54(t_r/w_{1/2})^2 \qquad \textbf{Equation 5}$$

where: N = number of theoretical plates
t_r = retention time
$w_{1/2}$ = peak width at half height

$$HETP = l/N \qquad \textbf{Equation 6}$$

where: HETP = height equivalent theoretical plate
l = length of the column
N = number of theoretical plates

The standard test mixture was used as a reference point throughout the course of this research. It was separated on each column used, both with and without the stationary or mobile phase modifiers. Figure 15 shows a chromatogram of the standard test mixture separated on the Nucleosil® C18 column with a mobile phase composition of 65/35 methanol/water. Table 1 provides an example of the data used to track N for the Nucleosil® C18 column, and table 2 provides an example of the data used to track HETP for the Nucleosil® C18 column throughout the course of its use in this research. Only the

N,N-diethyl-m-toluamide and toluene peaks were chosen to monitor for this column, and the standard test mixture was run in triplicate about once every two weeks.

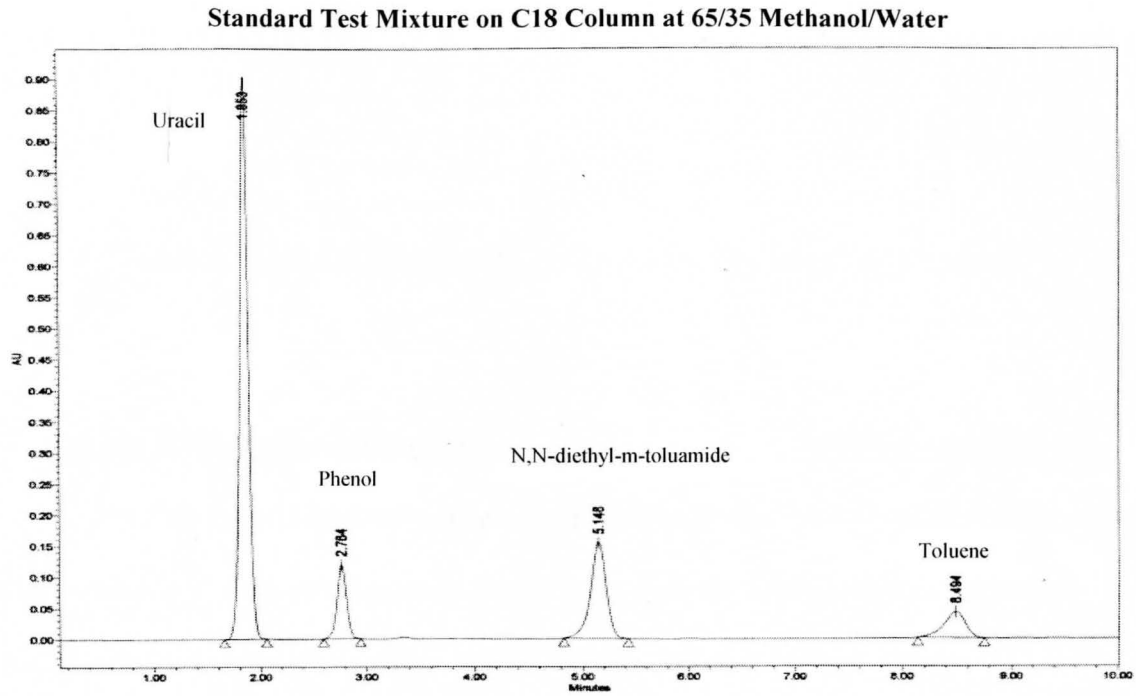


Figure 15. Standard Test Mixture on Nucleosil® C18 Column at 65/35 Methanol/Water

Run #	N,N-diethyl-m-toluamide	Toluene
1	6495	9306
	6468	9289
	6504	9282
2	6753	10196
	6826	10013
	6802	9811
3	6237	10244
	6216	9650
	6153	9857

Table 1. Table for Tracking the Number of Theoretical Plates for the Nucleosil® C18 Column

Run #	N,N-diethyl-m-toluamide	Toluene
1	0.02309	0.01612
	0.02319	0.01615
	0.02306	0.01616
2	0.02221	0.01471
	0.02197	0.01498
	0.02205	0.01529
3	0.02405	0.00146
	0.02413	0.01554
	0.02438	0.01522

Table 2. Table for Tracking the HETP for the Nucleosil® C18 Column

Studies With the Bound 4-*tert*-butylcalix[n]arene Modified Stationary Phases

The first type of stationary phase modification studied was covalent attachment of a calixarene to a silica substrate as outlined in prior work.[15] The synthetic method for binding the calixarenes to the silica seemed straightforward, but did prove to be somewhat problematic (Figure 16).

The first step was to dry the silica at a temperature of 200 °C for 16 hours. This was done to drive off water that may have been bound to silanol groups on the surface of the silica. This helped to maximize coverage by the tether used to bind the calixarene, and therefore maximize the surface coverage of the calixarene. Water can be tenaciously bound by the silica, so long drying times at high temperatures were a necessity.

The second step was to attach the 1,7-dichlorooctamethyltetrasiloxane tether to the silica. This was done by refluxing the two in triethylamine under a nitrogen environment. Because the attachment of the tether to the silica liberated HCl, a base was needed to consume the HCl. Previous research on this synthetic method utilized toluene as the reflux solvent, and pyrazine was added as the base to consume the HCl.[15] In this

research, the use of the basic reflux solvent triethylamine eliminated the need to add additional base to the reaction. Once the tether was successfully attached to the silica, the 4-*tert*-butylcalix[n]arene was added to the mixture and again refluxed in a nitrogen environment. This was done to attach the calixarene to the tether.

Three separate syntheses were completed: one with 4-*tert*-butylcalix[4]arene, one with 4-*tert*-butylcalix[6]arene, and one with 4-*tert*-butylcalix[8]arene. In each case, the product was filtered using a 0.45 μm nylon membrane filter. Because the filtration was very slow and the products tended to clog the filter apparatus, the product washes to remove excess reactants (i.e., calixarene, triethylamine, and tether) were done with warm THF and methanol. Chloroform was also used as a wash solvent.

The synthesis of the bound 4-*tert*-butylcalix[4]arene modified stationary phase initially appeared successful. The product was white and homogeneous in appearance. It was packed into a 150 mm x 4.6 mm stainless steel column, and initial separations of the standard test mixture, alkylbenzene homologous series, and phenyl ring homologous series were completed. Backpressure problems were encountered during these separations, and it was suspected that excess unbound 4-*tert*-butylcalix[4]arene was present inside the column. The column was disconnected from the detector and rinsed with chloroform at 1.0 mL/min. After doing this, the column packing was inspected and a void had formed at the head of the column.

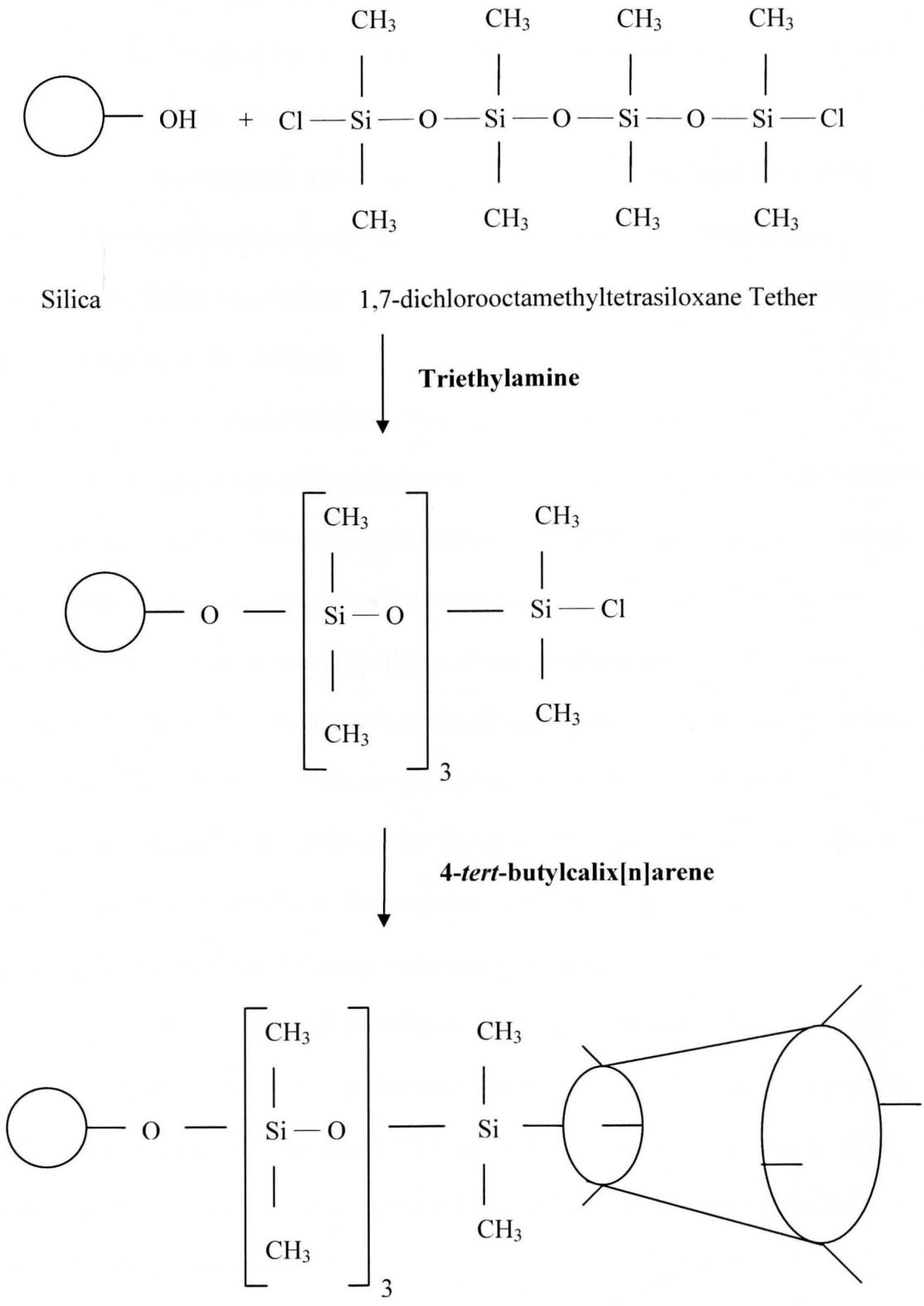
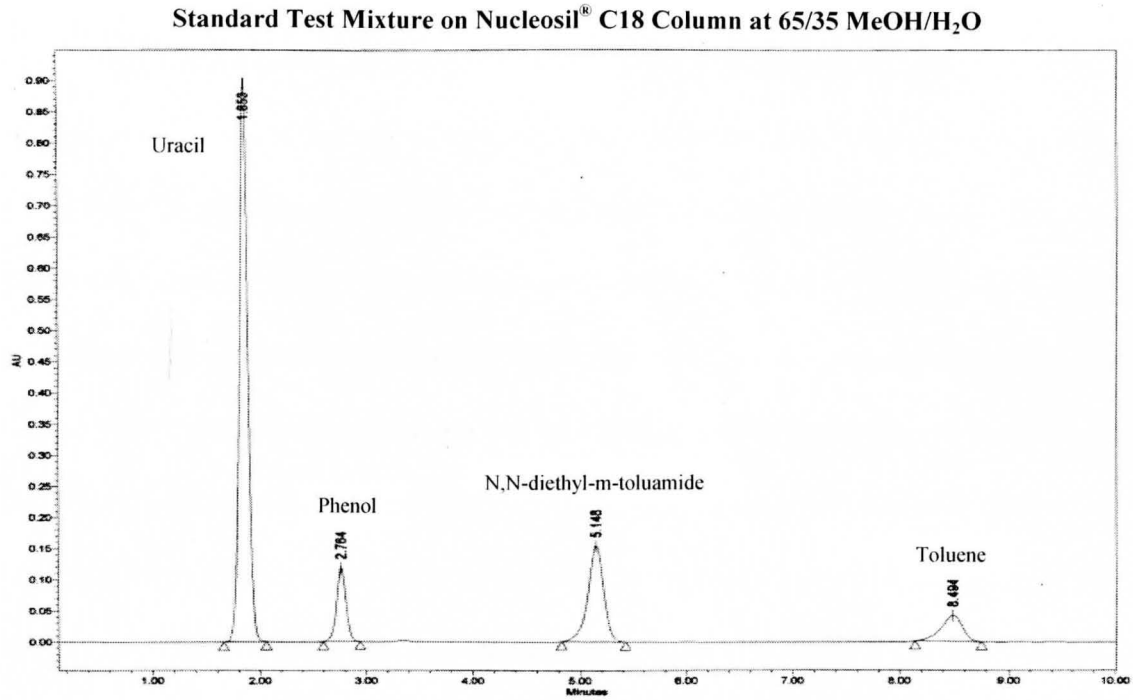


Figure 16. Reaction Scheme for Synthesis of the Covalently Bound 4-*tert*-butylcalix[n]arene Modified Stationary Phases

The column was unpacked, and the remaining stationary phase was again slurried with methanol and repacked into a 75 mm x 4.6 mm stainless steel column. Again, when analyzing the same three solute series, backpressure problems were encountered. The chloroform wash was repeated, which again resulted in a void at the head of the column. At this point there was not enough synthesized stationary phase remaining to pack another column, so the experiments with the bound 4-*tert*-butylcalix[4]arene modified stationary phase were discontinued.

The synthesis of the bound 4-*tert*-butylcalix[8]arene also proved to be problematic. The attachment of the tether to the silica and the 4-*tert*-butylcalix[8]arene to the tether went smoothly. However, upon filtering and washing the product it was noted that the product was brown in color and inhomogeneous in appearance. Despite this initial indication of an unsuccessful synthesis, it was decided to proceed with column packing. This is when the second problem was encountered. The column did not appear to pack 'normally', which was evidenced by the inability of the air pump to hold pressure. Upon inspection it was found that the column was only packed approximately half full with the stationary phase. At this point, work with the bound 4-*tert*-butylcalix[8]arene modified stationary phase was discontinued.

The synthesis of the bound 4-*tert*-butylcalix[6]arene stationary phase was more successful than the previous two experiments. The product was white and homogeneous in appearance, and packed easily into a 150 mm x 4.6 mm stainless steel column. The standard test mixture was separated on this column, and the results compared to the Nucleosil® C18 column (Figure 17).



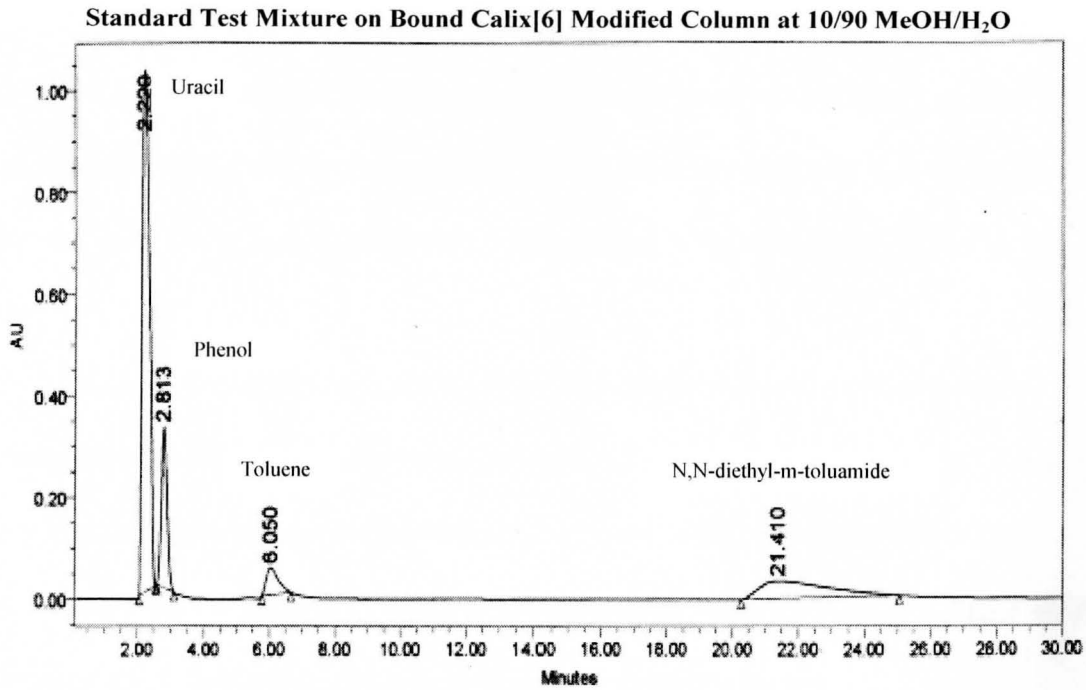
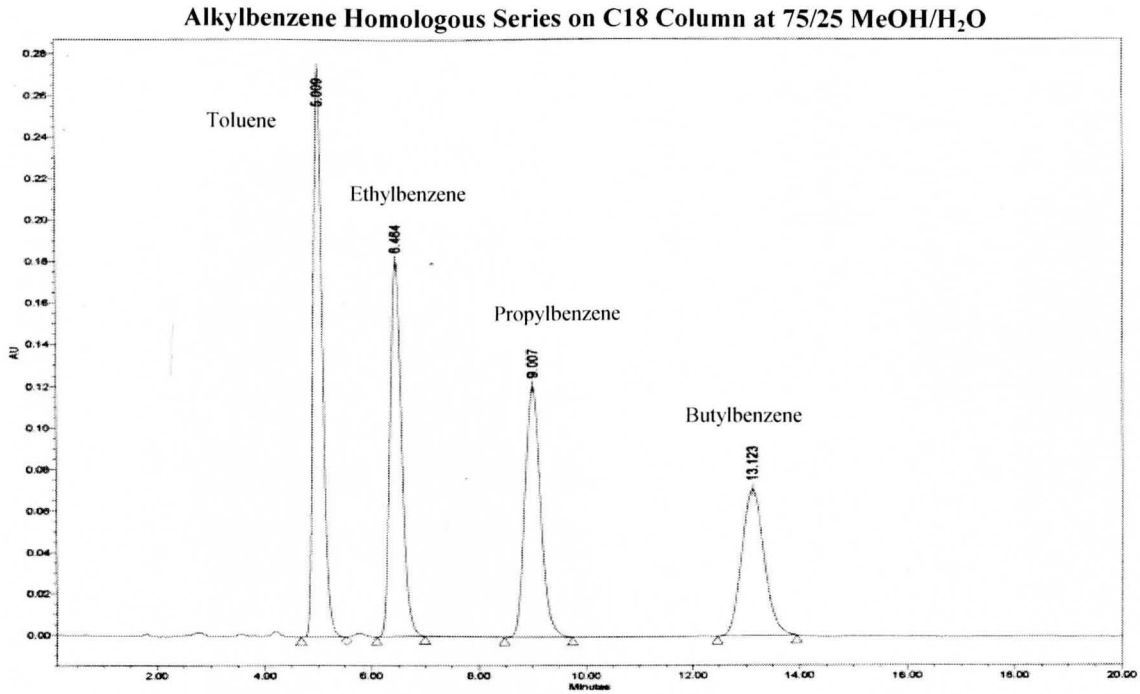


Figure 17. Comparison of Standard Test Mixture on C18 Column and Bound 4-*tert*-butylcalix[6]arene Modified Column

The first noticeable difference between the two columns was a change in the order of elution of the peaks. On the C18 column, the order was uracil, phenol, N,N-diethyl-m-toluamide, toluene. On the bound 4-*tert*-butylcalix[6]arene modified column, the order was uracil, phenol, toluene, N,N-diethyl-m-toluamide. This behavior had been noted in prior work and was attributed to strong interaction of N,N-diethyl-m-toluamide with free silanol groups on the surface causing it to be retained longer.[15] Another observation was that because toluene is an indicator of column polarity, the earlier elution of toluene indicated that adding the calixarene to the stationary phase increased the polarity of the stationary phase as compared to a C18 stationary phase. This caused the toluene to be retained less and elute earlier.

Another major difference between the two columns was a necessary change in the strength of the mobile phase between the C18 stationary phase and the bound 4-*tert*-butylcalix[6]arene stationary phase; a much weaker solvent was necessary in order to get peak retention on the calixarene stationary phase. This also indicated that the calixarene stationary phase was significantly more polar relative to the reference C18 stationary phase.

Because it is known that phenyl ring compounds tend to form host-guest complexes with calixarenes, a homologous series of alkylbenzenes (Figure 18) and a homologous series of phenyl ring compounds (Figure 19) were separated on both columns to assess whether the calixarenes were actually bound to the silica, and if so, if they were in a suitable conformation to form host-guest complexes.



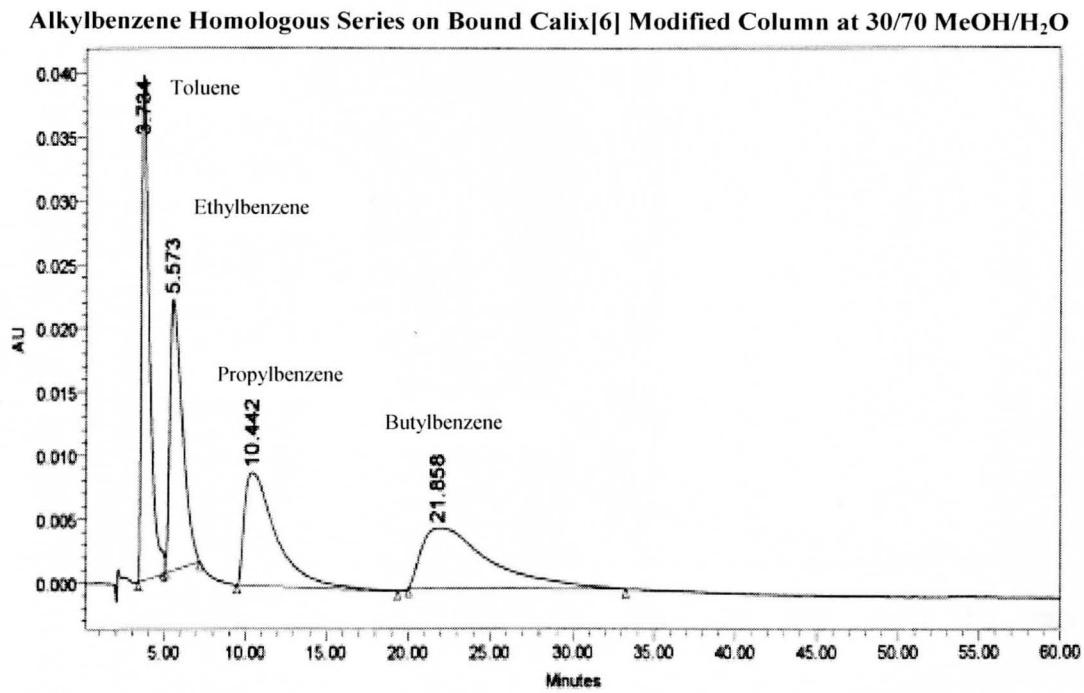
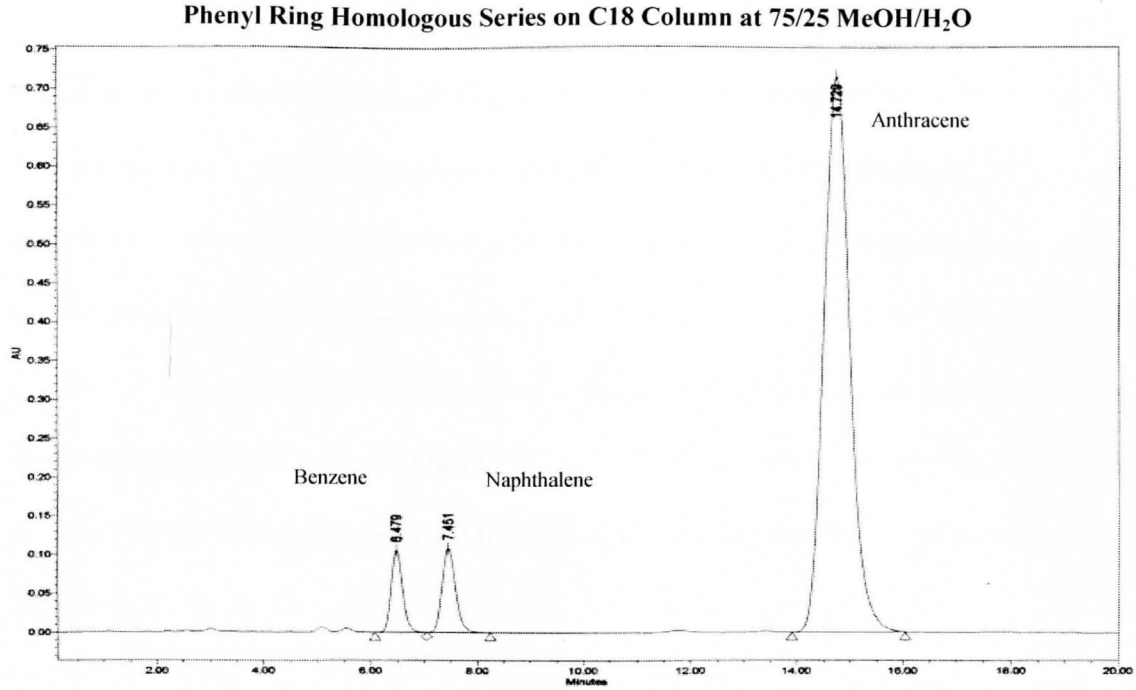


Figure 18. Comparison of Alkylbenzene Homologous Series on C18 Column and Bound 4-*tert*-butylcalix[6]arene Modified Column



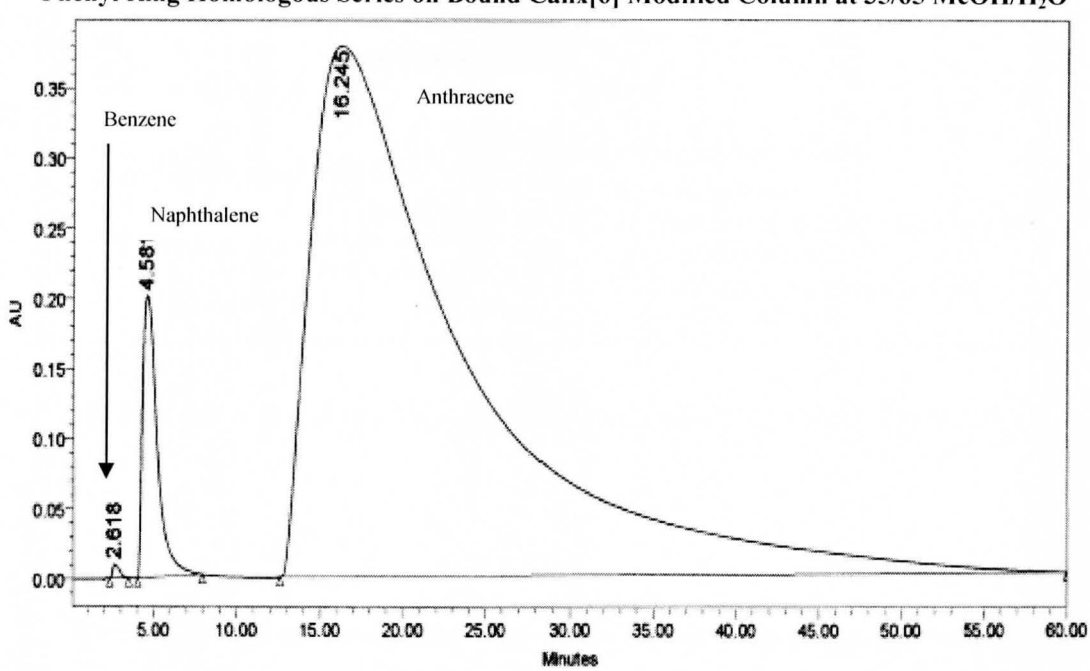


Figure 19. Comparison of Phenyl Ring Homologous Series on C18 Column and Bound 4-*tert*-butylcalix[6]arene Modified Column

Comparison of the two homologous series separations on the C18 column and the modified 4-*tert*-butylcalix[6]arene stationary phase showed indications of host-guest interactions on the modified stationary phases. A difference in peak shapes between the separations was an indication of host-guest interactions. Peak asymmetry was assessed by dropping a vertical line from the peak apex, and measuring from that line to the side of the peak at 10% peak height. The ratio of these measurements was used to assign a numerical value to the peak asymmetry (Figure 20).[22] A perfectly Gaussian peak has an A value of 1.0. Values less than 1.0 indicate peak fronting, and values greater than 1.0 indicate peak tailing.

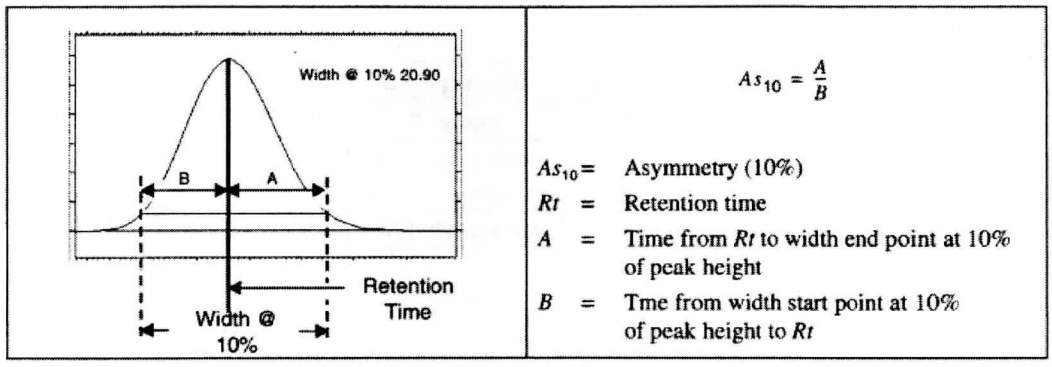


Figure 20. Illustration of the Method Used to Calculate Peak Symmetry[22]

On the C18 column, the peaks were nearly Gaussian in shape. However, on the bound 4-*tert*-butylcalix[6]arene modified column, the peaks shapes were very asymmetrical with significant tailing on the later eluting peaks. This change in peak symmetry likely indicated that higher levels of interactions were occurring between the

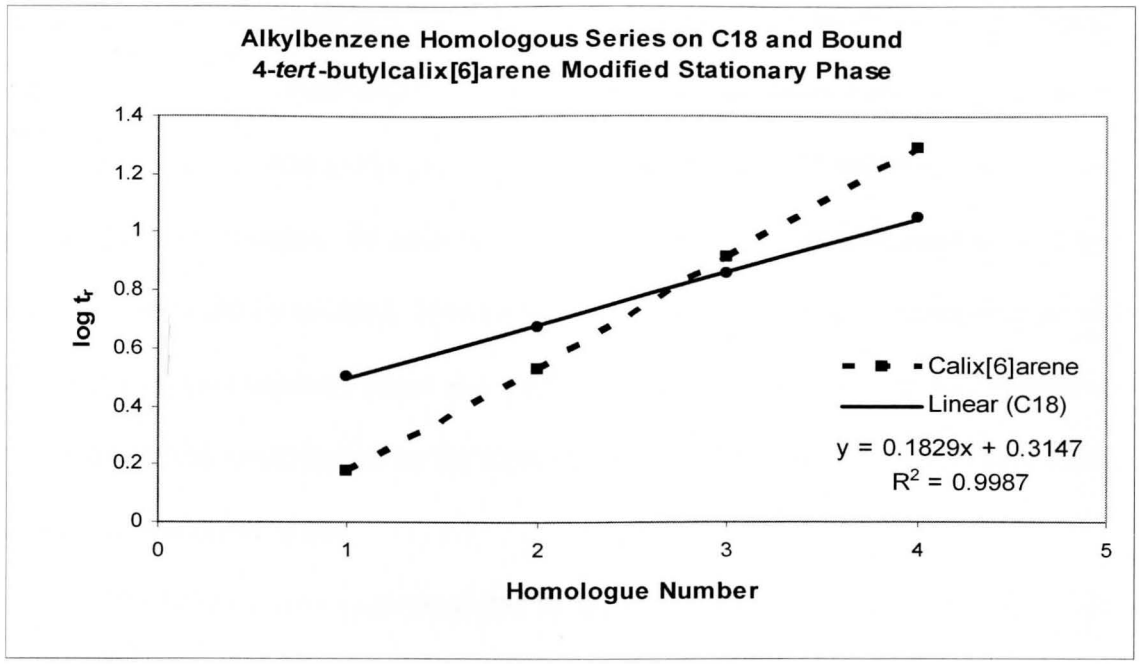
solutes and the stationary phase in the modified column (Table 3).[13] While not the only cause of peak tailing, slow kinetics in the exchange of solutes between the mobile and stationary phases will cause peaks to tail significantly. Because of the nature of the host-guest interaction, it was expected that the kinetics of the host-guest complexation would be slow relative to other partitioning mechanisms dependent only upon phase solubility.

Also, indicative of host-guest complexation were the deviations in linearity observed in plots of the log of the retention time for each component versus the homologue number of the solutes (Figure 21), the cause of which has been outlined in previous work.[15] These plots were compared against those of the alkylbenzene and phenyl ring homologous series separated on an unmodified C18 column.

Component	A Value
Toluene	2.7
Ethylbenzene	3.9
Propylbenzene	4.2
Butylbenzene	4.5
Benzene	2.9
Naphthalene	3.0
Anthracene	6.1

Table 3. A Values for Peaks on the Bound 4-*tert*-butylcalix[6]arene Column

At this point, due to the significant difficulties encountered with the synthetic method, it was decided to suspend this portion of the research and pursue alternate and potentially more user-friendly methods of modifying the stationary phase.



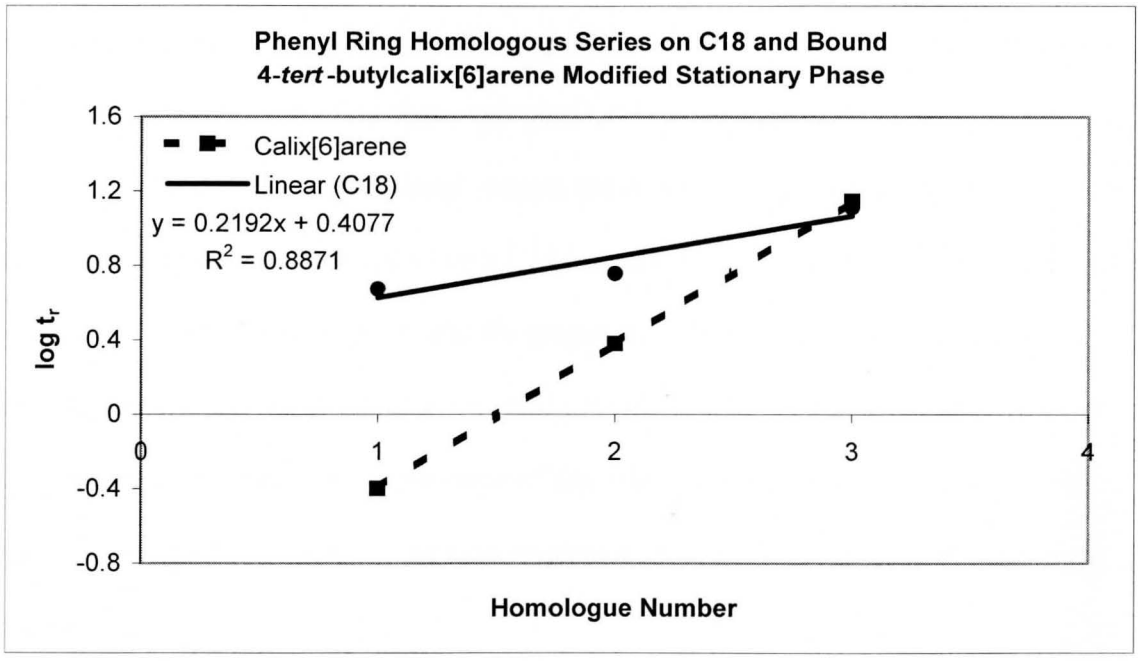


Figure 21. Graph of log t_r Versus Homologue Number for the Alkylbenzene and Phenyl Ring Homologous Series on a C18 and the Bound 4-*tert*-butylcalix[6]arene Modified Stationary Phase

Studies With the Absorbed 4-*tert*-butylcalix[n]arene Modified Stationary Phases

An alternate modification to covalently binding the 4-*tert*-butylcalix[n]arenes to the stationary phase was to physically absorb them onto the C18 stationary phase of a premanufactured column. By doing this, the cost, time, and problems encountered with the synthesis could be avoided. However, this resulted in a lower percent coverage of the calixarene on the stationary phase and a different mechanism of separation. Therefore, the results of the modification on the separations were not as drastic as with the bound calixarene stationary phase.

This behavior was evidenced first by the separation of the standard test mixture. On the covalently bound calixarene modified stationary phase, the presence of free silanol groups changed the polarity of the stationary phase so drastically that the N,N-diethyl-m-toluamide was found to change positions in the order of elution. Using the absorbed calixarene modified stationary phase, which was based on an endcapped C18 stationary phase with no free silanol groups, the order of elution of the components of the test mixture remained the same as on a C18 column because the two columns had similar polarities. Despite these facts, under the proper conditions an absorbed calixarene modified stationary phase column may still provide separation enhancements. This was also advantageous because the presence of free silanol groups during separations can cause problems due to mixed-mode retention mechanisms, especially when separating amines.

The first issue addressed in this portion of the research was the solubility of the 4-*tert*-butylcalix[n]arenes in a variety of solvents. It was found that 4-*tert*-butylcalix[4]arene was slightly soluble in THF and insoluble in water, methanol, and

acetonitrile. Both 4-*tert*-butylcalix[6]arene and 4-*tert*-butylcalix[8]arene were very soluble in THF and insoluble in water, methanol, and acetonitrile. Considering this data, along with the knowledge that acetonitrile has been shown to occupy the cavity of calixarenes through host-guest complexation, THF was chosen as the absorption solvent, and a methanol/water solution was chosen as the mobile phase for the separations involving the absorbed 4-*tert*-butylcalix[n]arene modified stationary phases.[17]

For the 4-*tert*-butylcalix[4]arene studies, a Phenomenex Sphereclone C18 column was used. An approximately 0.001M solution of 4-*tert*-butylcalix[4]arene in THF solution was pumped over the column at 0.25 mL/min for 4 hours. A slow flow rate and long absorption time were chosen due to the only slight solubility of 4-*tert*-butylcalix[4]arene in the THF. After the 4 hour absorption, 65/35 methanol/water was pumped over the column at 1.0 mL/min for 1 hour to immobilize the calixarene onto the stationary phase and rinse away any unabsorbed 4-*tert*-butylcalix[4]arene. At that point, the stationary phase was considered saturated with 4-*tert*-butylcalix[4]arene.

Comparison of the alkylbenzene homologous series and the phenyl ring homologous series separated three times each pre- and post-calixarene absorption showed significant differences in the retention times of all components of both homologous series. This was determined by performing an independent samples t-test on the pre- and post-absorption retention times for each component. A p value of less than 0.05 was obtained for all components of both homologous series, indicating statistically significant differences in the results (Table 4).

Component	Avg. t_r pre-calixarene absorption	Avg. corrected t_r pre-calixarene absorption	Avg. t_r post-calixarene absorption	Avg. corrected t_r post-calixarene absorption	p value
Toluene	5.353	2.553	5.516	2.716	<0.05
Ethylbenzene	6.384	3.584	6.615	3.815	<0.05
Propylbenzene	8.100	5.300	8.464	5.664	<0.05
Butylbenzene	10.699	7.899	11.329	8.529	<0.05
Benzene	4.432	1.632	4.529	1.729	<0.05
Naphthalene	6.511	3.711	6.776	3.976	<0.05
Anthracene	12.728	9.928	13.534	10.734	<0.05

Table 4. Comparison of Retention Times Pre- and Post- 4-*tert*-butylcalix[4]arene Absorption

The presence of the 4-*tert*-butylcalix[4]arene on the stationary phase increased the relative retention times of the components of the alkylbenzene and phenyl ring homologous series due to the host-guest interactions occurring between the solutes and the modified stationary phase. Addition of the calixarene to the stationary phase increased the overall polarity of the stationary phase, which resulted in the polar solutes interacting more with the stationary phase than the mobile phase as they partition through the column. Also, the formation of the host-guest complexes between the solute probes and the calixarenes in the stationary phase resulted in a stronger level of interaction between the solutes and the stationary phase by host-guest complex formation with the calixarenes. These two factors led to increased retention of the solutes on the calixarene modified stationary phase, and thus increased retention times. This shift in retention times can be easily seen in an overlay of chromatograms pre- and post-absorption (Figures 22 and 23).

As previously discussed, two other indications of host-guest interactions were changes in peak symmetry and deviation from linearity in a graph of the log of the

retention time versus the homologue number of the solute.[13] Table 5 provides the data for peak symmetry pre- and post-calixarene absorption. The changes in peak shape for this type of modification were negligible. Graphs for both the alkylbenzene and phenyl ring homologous series showed only slight deviations from linearity between the pre- and post-calixarene absorption conditions (Figure 24).

Component	**Avg. A pre-calixarene absorption**	**Avg. A post-calixarene absorption**
Toluene	1.2	1.2
Ethylbenzene	1.0	1.2
Propylbenzene	0.8	0.9
Butylbenzene	0.6	0.5
Benzene	1.3	1.2
Naphthalene	1.3	1.2
Anthracene	1.3	1.4

Table 5. A Values Pre- and Post- 4-*tert*-butylcalix[4]arene Absorption

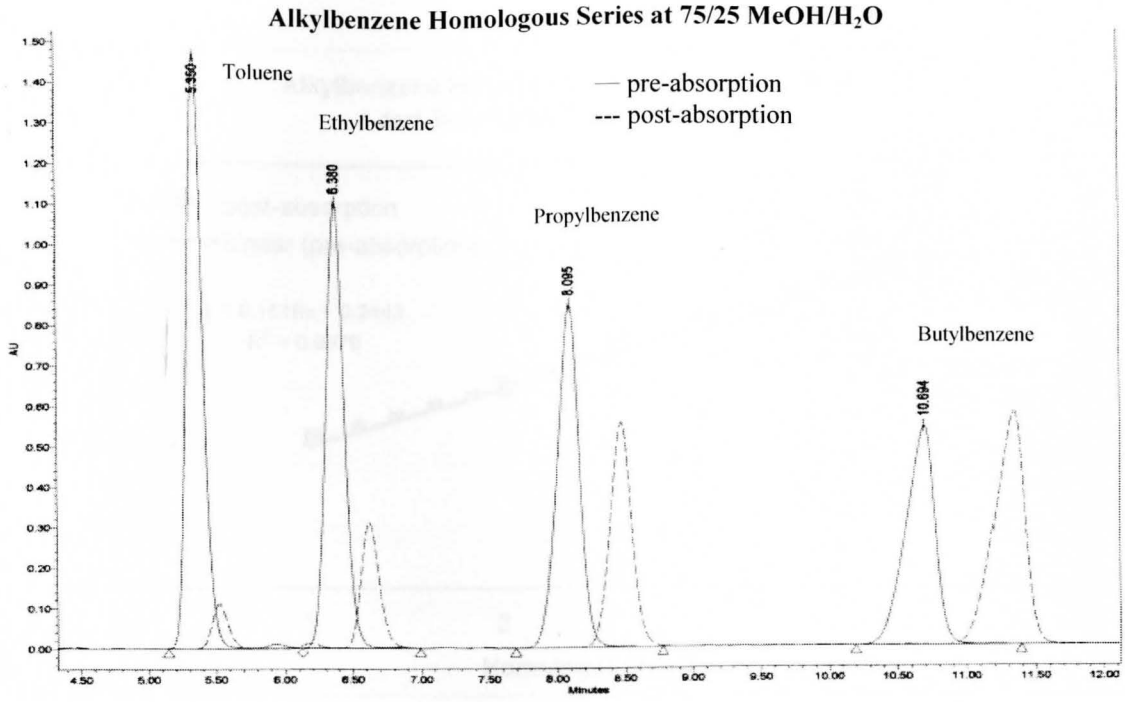


Figure 22. Comparison of Alkylbenzene Homologous Series Separated Pre- and Post- 4-*tert*-butylcalix[4]arene Absorption

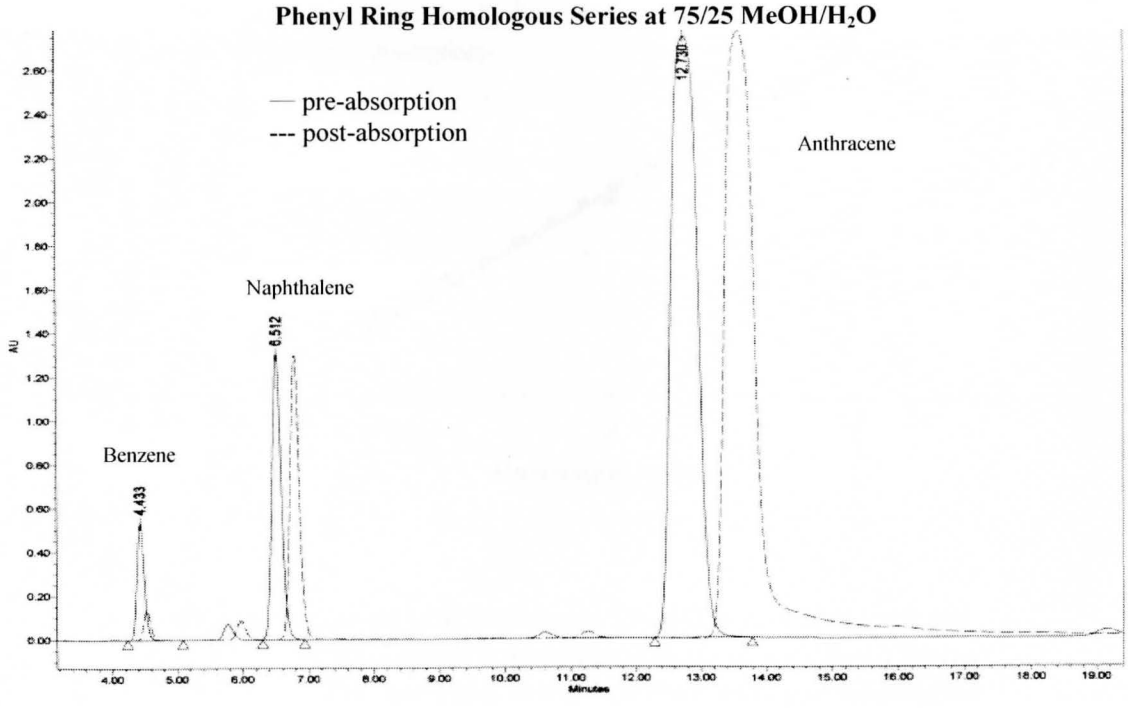
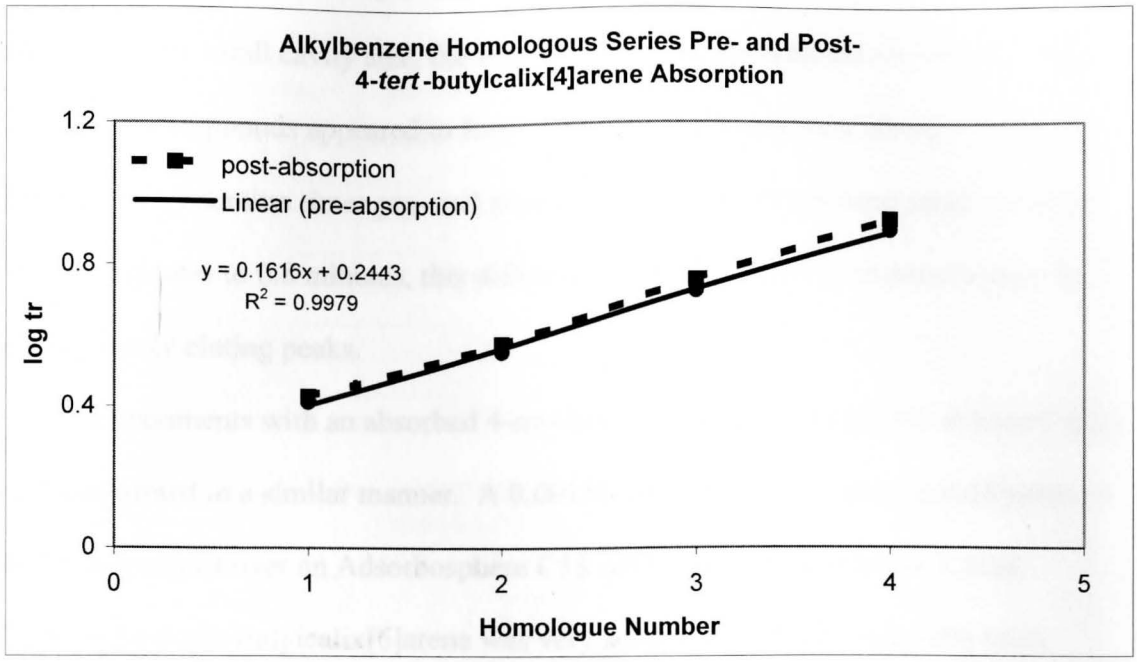


Figure 23. Comparison of Phenyl Ring Homologous Series Separated Pre- and Post- 4-*tert*-butylcalix[4]arene Absorption



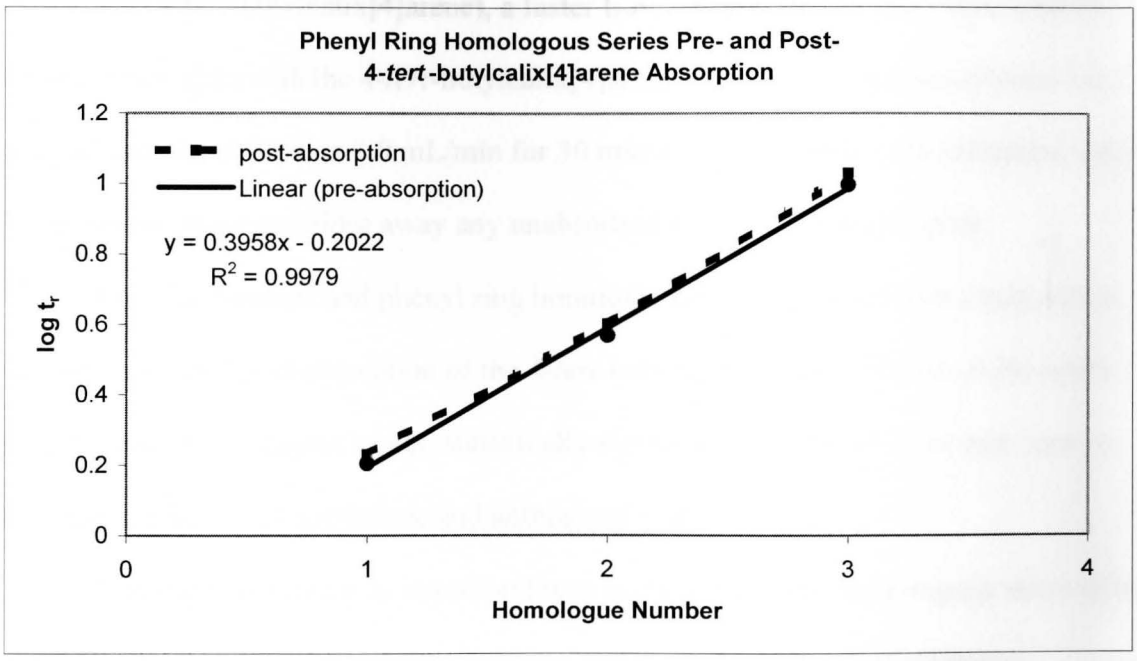


Figure 24. Graph of log t_r Versus Homologue Number for the Alkylbenzene and Phenyl Ring Homologous Series Pre- and Post- 4-*tert*-butylcalix[4]arene Absorption

Because the 4-*tert*-butylcalix[4]arenes tended to prefer the cone conformation and had a relatively small cavity size, the host-guest complexes with the alkylbenzenes and phenyl ring compounds appeared to form relatively easily and were strong. Although the differences in retention times pre- and post-calixarene absorption were small, ranging from 0.2 minutes to 0.8 minutes, this difference may be enough to improve a separation of two closely eluting peaks.

Experiments with an absorbed 4-*tert*-butylcalix[6]arene modified stationary phase were performed in a similar manner. A 0.001M solution of 4-*tert*-butylcalix[6]arene in THF was pumped over an Adsorbosphere C18 column at 1.0 mL/min for 1 hour. Because the 4-*tert*-butylcalix[6]arene was very soluble in THF (considerably more soluble than 4-*tert*-butylcalix[4]arene), a faster flow rate and shorter time were used for the absorption than with the 4-*tert*-butylcalix[4]arene. Then, 65/35 methanol/water was pumped over the column at 1.0 mL/min for 30 minutes to immobilize the calixarene onto the stationary phase and rinse away any unabsorbed 4-*tert*-butylcalix[6]arene.

The alkylbenzene and phenyl ring homologous series were separated three times each both pre- and post-absorption of the 4-*tert*-butylcalix[6]arene. Based on the results of the independent samples t-tests, statistically significant differences were only seen in the retention times of naphthalene and anthracene (Table 6).

This slight difference in retention times in the phenyl ring homologous series was seen in an overlay of the chromatograms pre- and post- 4-*tert*-butylcalix[6]arene absorption (Figure 25).

Component	Avg. t_r pre-calixarene absorption	Avg. corrected t_r pre-calixarene absorption	Avg. t_r post-calixarene absorption	Avg. corrected t_r post-calixarene absorption	p value
Toluene	6.505	3.905	6.497	3.897	>0.05
Ethylbenzene	8.313	5.713	8.297	5.697	>0.05
Propylbenzene	11.526	8.926	11.491	8.891	>0.05
Butylbenzene	16.757	14.157	16.688	14.088	>0.05
Benzene	4.996	2.396	4.998	2.398	>0.05
Naphthalene	8.066	5.466	8.083	5.483	<0.05
Anthracene	17.610	15.010	17.711	15.111	<0.05

Table 6. Comparison of Retention Times Pre- and Post- 4-*tert*-butylcalix[6]arene Absorption

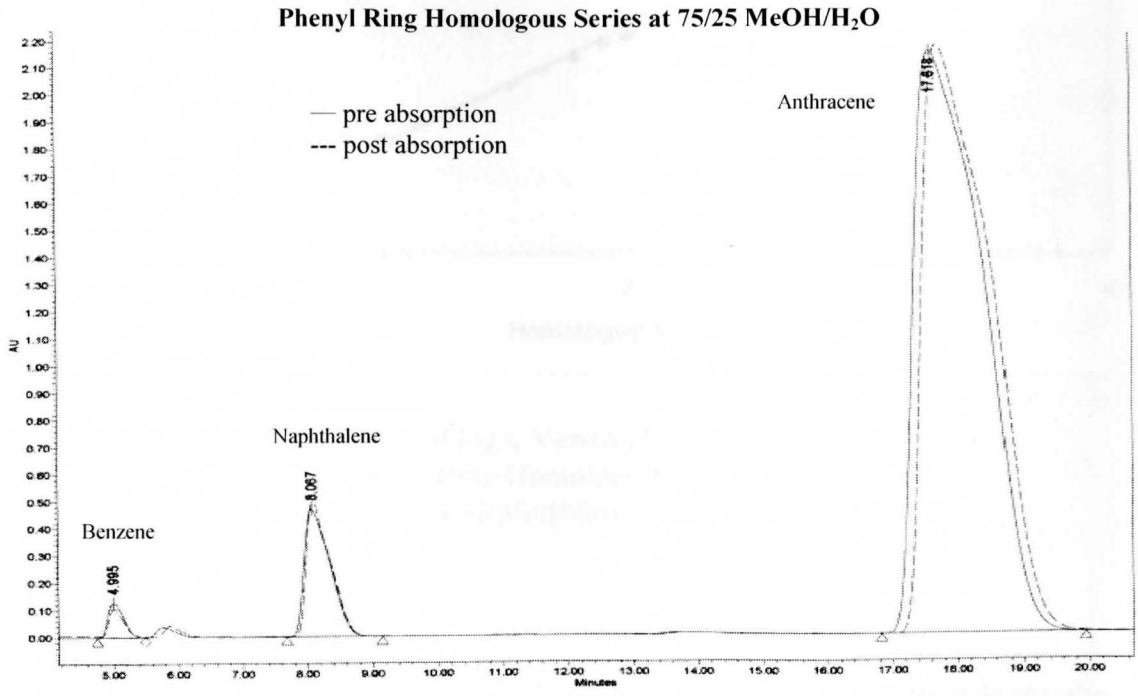


Figure 25. Comparison of Phenyl Ring Homologous Series Separated Pre- and Post- 4-*tert*-butylcalix[6]arene Absorption

In a plot of the log of the retention time versus the homologue number, the lines for pre- and post-calixarene absorption conditions exactly overlap (Figure 26). Also, there were no significant differences noted in the peak shapes pre- and post-calixarene absorption. This did not indicate that host-guest interactions were not occurring with the calixarene modified stationary phase, but simply showed that the interactions were not strong enough to have any effect on this plot.

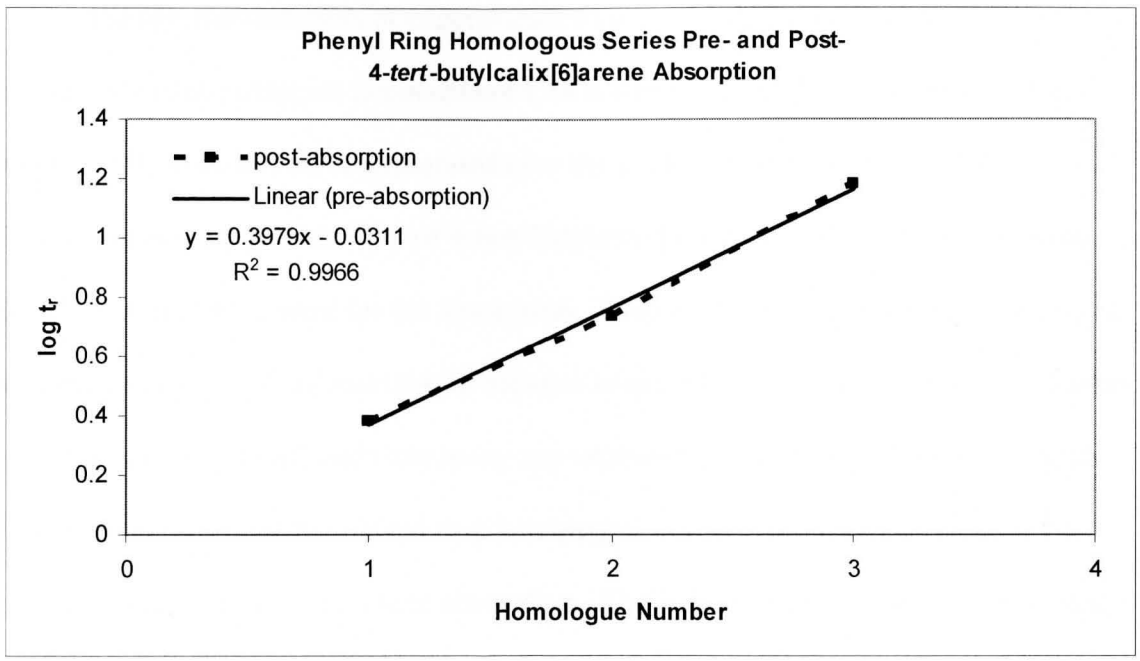


Figure 26. Graph of log t_r Versus Homologue Number for the Phenyl Ring Homologous Series Pre- and Post-4-*tert*-butylcalix[6]arene absorption

Although there were indications that the 4-*tert*-butylcalix[6]arene was absorbed onto the stationary phase and host-guest interactions were occurring, the retention time differences were not large enough to offer any significant improvements for this type of

separation. Because 4-*tert*-butylcalix[6]arene is known to spend more time in a flattened out cone conformation, and the size of the cavity is larger than that of 4-*tert*-butylcalix[4]arene, it was expected that the magnitude of host-guest interactions with these particular solute probes would be less than with the 4-*tert*-butylcalix[4]arene, accounting for the observed differences. Supporting this contention is the fact that the larger cavity size was most likely the major reason why significant host-guest interactions were only observed with the two largest compounds in the homologous series.

Finally, the same sets of experiments were performed with 4-*tert*-butylcalix[8]arene using an Econosphere C18 column. A 0.001M solution of 4-*tert*-butylcalix[8]arene in THF was pumped over the column at 1.0 mL/min for 1 hour. Again, due to the high solubility of 4-*tert*-butylcalix[8]arene in THF, a faster flow rate and shorter time were used for the absorption. Then 65/35 methanol/water was pumped over the column at 1.0 mL/min for 30 minutes to immobilize the 4-*tert*-butylcalix[8]arene onto the stationary phase and rinse away any unabsorbed calixarene. The alkylbenzene homologous series and the phenyl ring homologous series were separated three times each both pre- and post-calixarene absorption. The independent samples t-test on these retention times showed no significant differences in retention of any components of the homologous series.

Like the 4-*tert*-butylcalix[4]arenes, the 4-*tert*-butylcalix[8]arenes prefer the cone conformation, which made their cavities more available for host-guest interactions. However, the cavity size is again increased over the 4-tert-butylcalix[6]arenes. Therefore, the solutes may have been able to interact as guests with the 4-*tert*-butylcalix[8]arenes by moving in and out of the cavity, but the interactions were not at a

strong enough level to cause a shift in the retention times of the solutes. From this, it was determined that the 4-tert-butylcalix[8]arenes were not useful as absorbed stationary phase modifiers for smaller solute molecules.

The common problem encountered with all three absorption experiments was cleaning the calixarenes off the column to restore the stationary phase to its original C18 state. THF was the first rinse solvent used because the calixarenes should theoretically be dissolved by the THF and washed through the column. Rinsing with THF at 1.0 mL/min for 1 hour and reanalyzing the homologous series did not shift the retention times back to the pre-calixarene absorption conditions.

The second rinse solvent used was chloroform, which proved useful in rinsing away unbound calixarene from the chemically bound calixarene stationary phase studies. A chloroform rinse at 1.0 mL/min for 1 hour still did not bring the retention times of the homologous series solutes back to their initial conditions, and a 2 hour isopropanol rinse at 1.0 mL/min was also unsuccessful.

Finally, a 0.001M solution of anthracene in methanol was pumped over the column at 1.0 mL/min for 1 hour. The reasoning behind this rinse solution was that the anthracene could form a strong host-guest complex with the absorbed calixarenes and 'pull' them off the column. Again, this was not successful in restoring the stationary phase to the pre-absorption conditions.

Studies With the 4-*tert*-butylcalix[4]arene Modified Mobile Phase

Because 4-*tert*-butylcalix[4]arene showed the strongest indication of host-guest complex formation with the solute probes used in this work for examining the modified

stationary phases, it was chosen as the mobile phase modifier for this portion of the research.

Using a Phenomenex Sphereclone C18 column, the alkylbenzene and phenyl ring homologous series were separated using a THF/water mobile phase and also a 0.001M 4-*tert*-butylcalix[4]arene in THF/water mobile phase. Only one separation was completed with each homologous series. With the alkylbenzene homologous series, the most noticeable difference between the THF mobile phase and the modified calixarene mobile phase was peak splitting when the modified calixarene mobile phase was used (Figure 27). This peak splitting may have been an indication of secondary host-guest interactions occurring between the solutes and the 4-*tert*-butylcalix[4]arenes. However, because peak splitting is undesirable in an HPLC separation, these conditions were not useful for modifying this type of separation.

When the phenyl ring homologous series was separated, backpressure problems were encountered with the 4-*tert*-butylcalix[4]arene modified stationary phase. The flow rate was reduced from 1.0 mL/min to 0.25 mL/min, and the backpressure was still an issue. Because the 4-*tert*-butylcalix[4]arene was only slightly soluble in THF and was insoluble in water, it was hypothesized that the 4-*tert*-butylcalix[4]arene was precipitating from the mobile phase and clogging the column, thereby increasing the backpressure on the HPLC system.

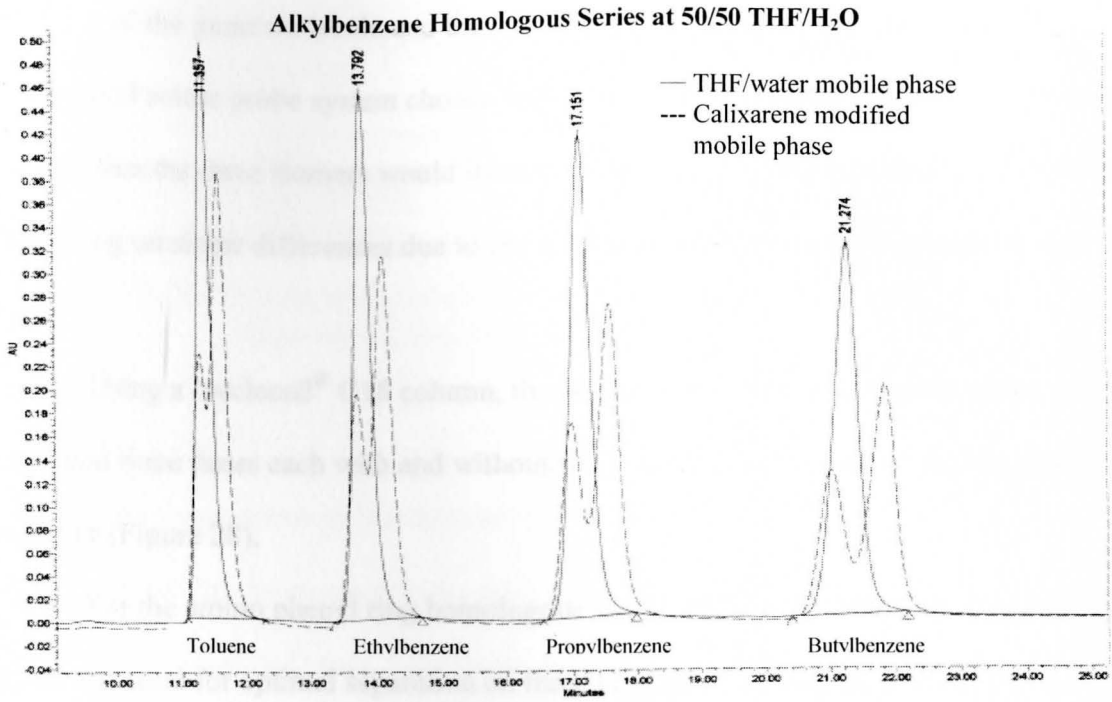


Figure 27. Comparison of Phenyl Ring Homologous Series with THF/water Mobile Phase and 4-*tert*-butylcalix[4]arene Modified Mobile Phase

Studies With the 4-sulfonic calix[4]arene Modified Mobile Phase

In order to avoid the solubility issues encountered with the 4-*tert*-butylcalix[4]arene in the previous studies, the mobile phase modifier was switched to the water soluble 4-sulfonic calix[4]arene. These experiments were performed on a Nucleosil® C18 column with a methanol/water mobile phase and a 5.0 x 10^{-4} M 4-sulfonic calix[4]arene in methanol/water mobile phase.

Another change made between these and the previous experiments were the solute probes used in the separations. One solute probe system chosen was similar to the phenyl ring homologous series, but with an amino group substituted on the ring system. Based upon prior work, it was thought that the amino group would add to the strength of the interactions of the host-guest complex with the calixarene by hydrogen bonding of the

amino N of the guest molecule and the hydroxyl H on the rim of the calixarene cup.[19] The second solute probe system chosen was a diaminonaphthalene isomer series. It was thought that the three isomers would interact differently with the 4-sulfonic calix[4]arene depending on shape differences due to the placement of the two amino groups on the rings.

Using a Nucleosil® C18 column, the amino phenyl ring homologous series was separated three times each with and without the 4-sulfonic calix[4]arene mobile phase modifier (Figure 28).

For the amino phenyl ring homologous series, a 60/40 methanol/water mobile phase was used for optimal separation on the C18 column. Using the modified mobile phase which was 5.0×10^{-4} M in 4-sulfonic calix[4]arene, the mobile phase strength had to be decreased to 55/45 methanol/water. Because a change in the mobile phase strength was necessary to achieve satisfactory separation, the initial method used for comparing retention of the solutes was examination of the capacity factors for each component.

The capacity factor for each component of the amino phenyl ring homologous series decreased in the presence of the 4-sulfonic calix[4]arene mobile phase modifier (Table 7). This decrease in capacity factor, especially when observed under conditions of a weaker eluent, is indicative of host-guest interactions with the 4-sulfonic calix[4]arene modifier.

Component	Avg. k' with 60/40 methanol/water mobile phase	Avg. k' with 55/45 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase
Aniline	0.5728	0.1059
2-Aminonaphthalene	1.8678	0.7376
2-Aminoanthracene	7.2084	4.3568

Table 7. Comparison of the Amino Phenyl Ring Homologous Series Capacity Factors Pre- and Post-Mobile Phase Modification

Also, comparison of the chromatograms using the non-modified and modified mobile phases showed changes in the peak asymmetry for all components.[13] In the non-modified separation the peaks were more Gaussian in shape, and were actually slightly fronting, whereas in the calixarene modified separation, the peaks showed tailing characteristics (Table 8). When the fronting peaks were observed on the C18 column, it was suspected that overloading of the column was the cause. The samples were all diluted in concentration by half and reanalyzed on the C18 column. The peaks were still fronting, so overloading was ruled out as the cause.

Component	Average A Value with 60/40 MeOH/H_2O mobile phase	Average A Value with 55/44 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase
Aniline	1.2	0.6
2-Aminonaphthalene	0.8	1.1
2-Aminoanthracene	0.6	1.5

Table 8. A Values for Components of the Amino Phenyl Ring Homologous Series

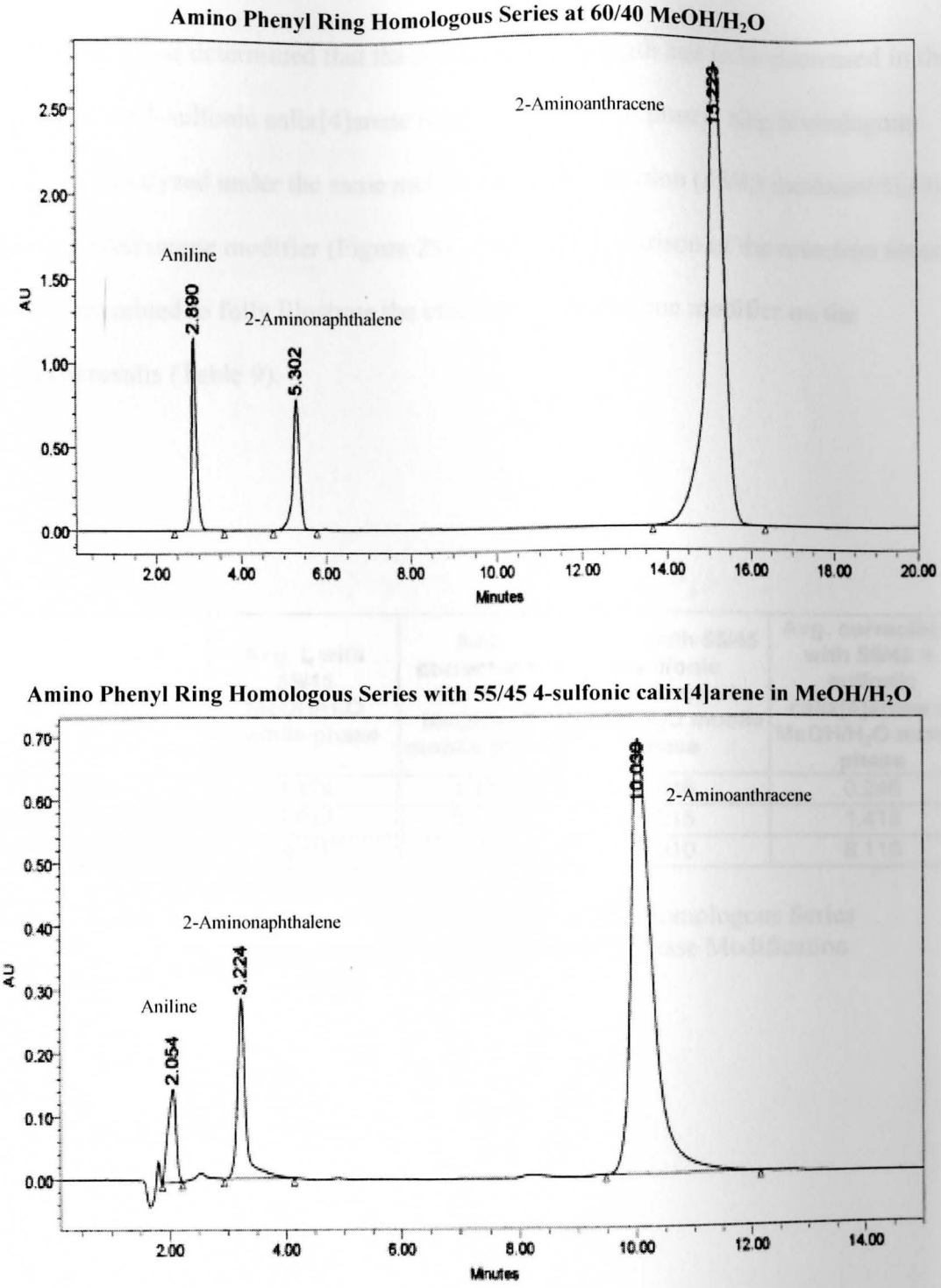
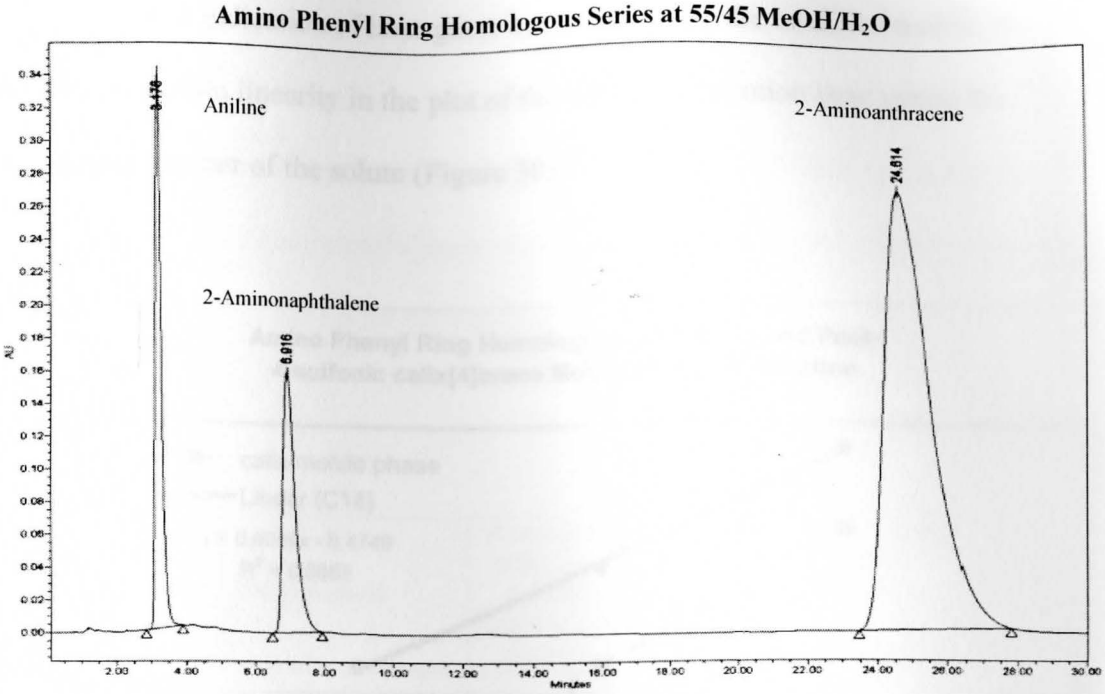


Figure 28. Initial Comparison of Amino Phenyl Ring Homologous Series Separated with Non-modified and 4-sulfonic calix[4]arene Modified Mobile Phase

Once it was determined that the mobile phase strength had to be decreased in the presence of the 4-sulfonic calix[4]arene modifier, the amino phenyl ring homologous series was reanalyzed under the same mobile phase composition (55/45 methanol/H_2O) without the calixarene modifier (Figure 29). A direct comparison of the retention times was then examined to fully illustrate the effect of the calixarene modifier on the separation results (Table 9).

Component	Avg. t_r with 55/45 MeOH/H_2O mobile phase	Avg. corrected t_r with 55/45 MeOH/H_2O mobile phase	Avg. t_r with 55/45 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase	Avg. corrected t_r with 55/45 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase
Aniline	3.174	1.374	2.046	0.246
2-Aminonaphthalene	6.912	5.112	3.215	1.415
2-Aminoanthracene	24.597	22.797	9.910	8.110

Table 9. Comparison of the Amino Phenyl Ring Homologous Series Retention Times Pre- and Post-Mobile Phase Modification



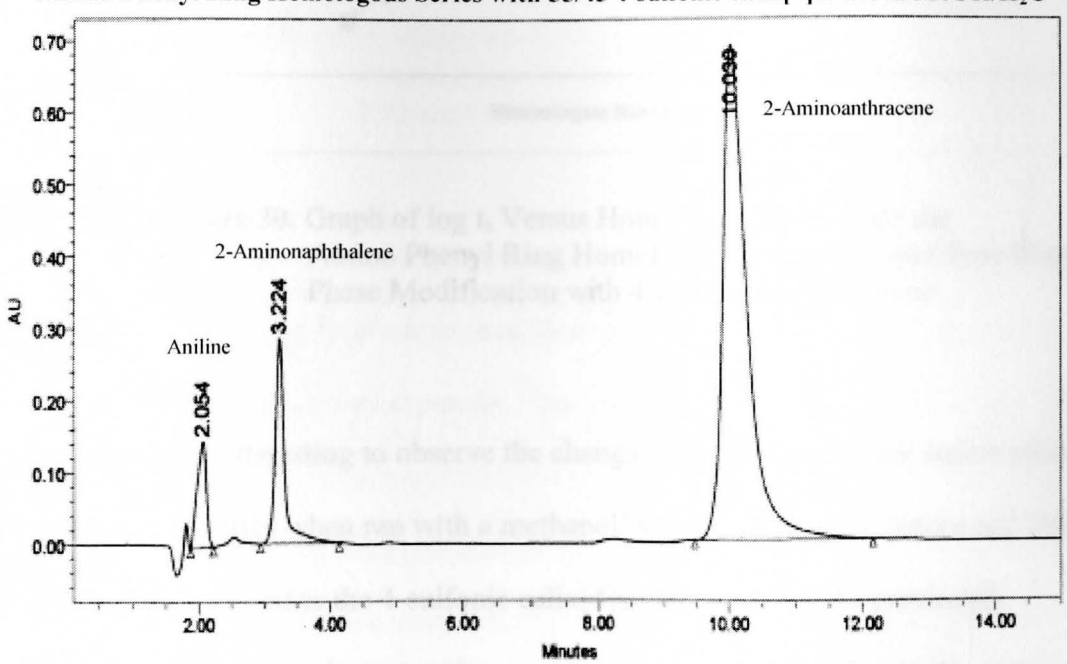


Figure 29. Direct Comparison of Amino Phenyl Ring Homologous Series Separated with Non-modified and 4-sulfonic calix[4]arene Modified Mobile Phase

Another indication of host-guest interactions with the modified mobile phase was the deviation from linearity in the plot of the log of the retention time versus the homologue number of the solute (Figure 30).[15]

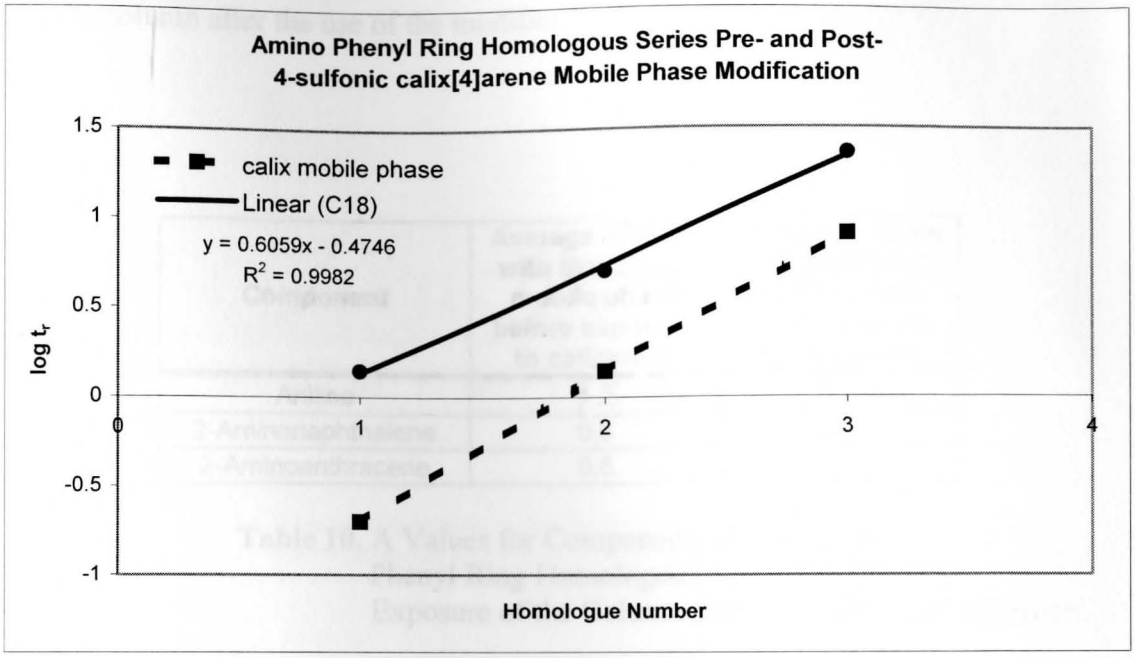


Figure 30. Graph of log t_r Versus Homologue Number for the Amino Phenyl Ring Homologous Series Pre- and Post-Mobile Phase Modification with 4-sulfonic calix[4]arene

It was also interesting to observe the change in peak shape for the amino phenyl ring homologous series when run with a methanol/water mobile phase before and after the column was exposed to the 4-sulfonic calix[4]arene modifier. As previously mentioned, when the homologous series was run on the new C18 column, the peaks were actually fronting slightly. Because overloading of the column was ruled out, this type of peak shape was an indication of Anti-Langmuir behavior within the column during the separation. After the column was exposed to the 4-sulfonic calix[4]arene modifier, the

peaks of the homologous series run with a methanol/water mobile phase were tailing, which was indicative of Langmuir behavior (Table 10). This change in peak shape was a good indicator that a change had occurred to the surface of the stationary phase by exposure to the 4-sulfonic calix[4]arene, even though attempts were made to completely rinse the column after the use of the modifier.

Component	Average A Value with MeOH/H_2O mobile phase before exposure to calixarene	Average A Value with MeOH/H_2O mobile phase after exposure to calixarene
Aniline	1.2	2.0
2-Aminonaphthalene	0.8	2.3
2-Aminoanthracene	0.6	2.7

Table 10. A Values for Components of the Amino Phenyl Ring Homologous Series Pre- and Post-Exposure of the Column to the 4-sulfonic calix[4]arene

From these results, it was apparent that the 4-sulfonic calix[4]arene in the mobile phase was indeed forming host-guest complexes with the solute probe components and drastically affecting the separation results. This type of mobile phase modification could be useful in shortening the time required for separation of these types of compounds, or for selectively affecting one retention time in a set of two coeluting peaks.

The diaminonaphthalene isomer series was separated on the Nucleosil® C18 column three times each using a 50/50 methanol/water mobile phase and a 45/55 methanol/water mobile phase that was 5.0×10^{-4} M in 4-sulfonic calix[4]arene modifier (Figure 31).

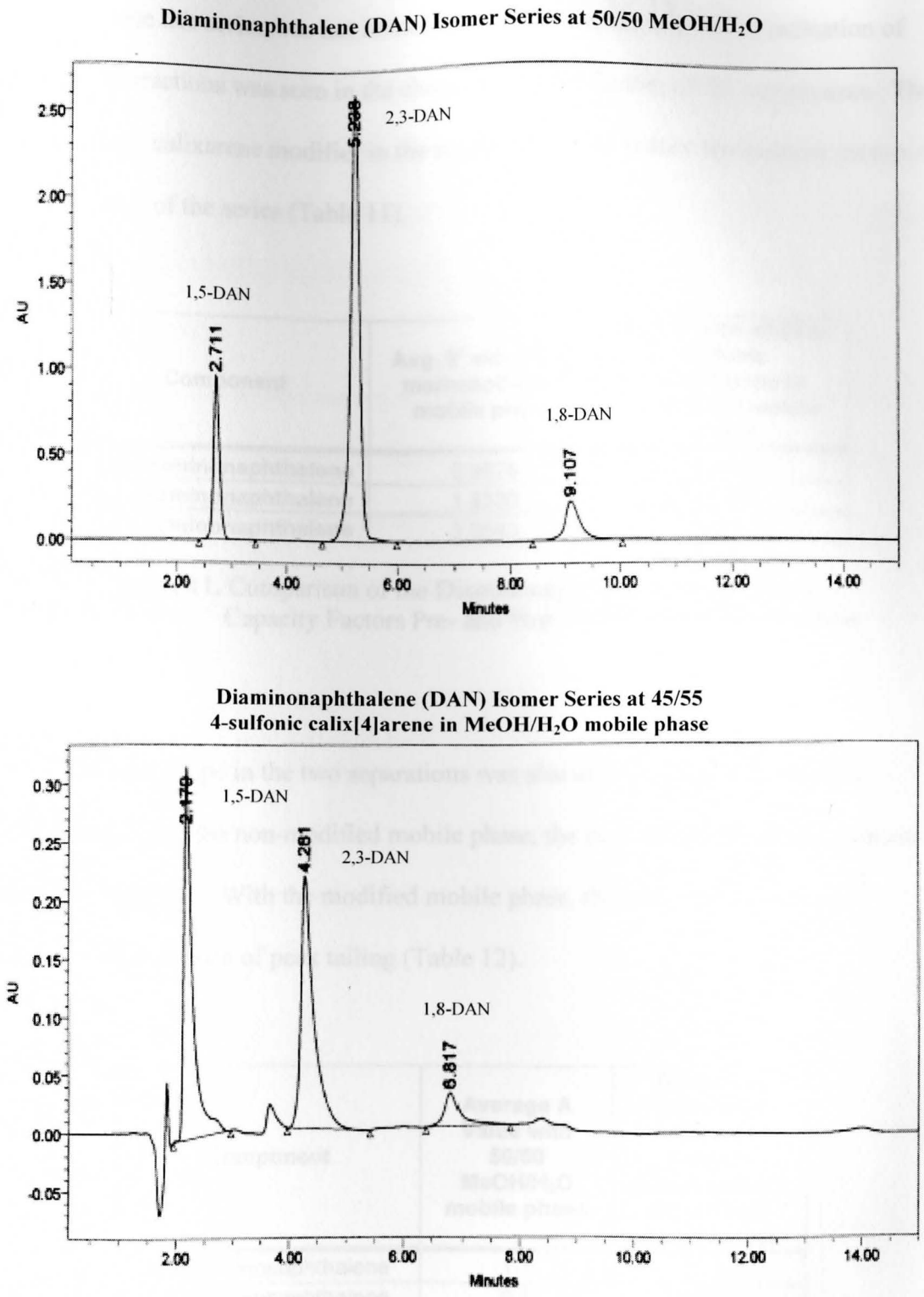


Figure 31. Initial Comparison of Diaminonaphthalene Isomer Series Separated with Non-modified and 4-sulfonic calix[4]arene Modified Mobile Phase

Because this series was not homologous in nature, the best initial indication of host-guest interactions was seen in the changed capacity factors of the components. The presence of the calixarene modifier in the mobile phase did reduce the capacity factors of all components of the series (Table 11).

Component	Avg. k' with 50/50 methanol/water mobile phase	Avg. k' with 45/55 4-sulfonic calix[4]arene in $MeOH/H_2O$ mobile phase
1,5-Diaminonaphthalene	0.4675	0.1859
2,3-Diaminonaphthalene	1.8330	1.3370
1,8-Diaminonaphthalene	3.9543	2.6967

Table 11. Comparison of the Diaminonaphthalene Isomer Series Capacity Factors Pre- and Post-Mobile Phase Modification

The peak shape in the two separations was also an indication of host-guest interactions. Using the non-modified mobile phase, the peak shapes for all components were fairly Gaussian. With the modified mobile phase, the peak shapes were more irregular, with evidence of peak tailing (Table 12).

Component	Average A Value with 50/50 $MeOH/H_2O$ mobile phase	Average A Value with 45/55 4-sulfonic calix[4]arene in $MeOH/H_2O$ mobile phase
1,5-Diaminonaphthalene	1.1	3.3
2,3-Diaminonaphthalene	0.8	3.2
1,8-Diaminonaphthalene	1.3	1.9

Table 12. A Values for Components of the Diaminonaphthalene Isomer Series

Also, as with the amino phenyl ring homologous series, the need to reduce the strength of the mobile phase from 50/50 methanol/water to 45/55 methanol/water with calixarene modifier was an indication of host-guest complexation. Once this was determined, the diaminonaphthalene isomer series was run using the same mobile phase composition (45/55 MeOH/H_2O) but in the absence of the 4-sulfonic calix[4]arene modifier (Figure 32).

Using this additional data, a direct comparison of the retention times with and without the calixarene modifier was drawn (Table 13). It was readily apparent that the presence of the 4-sulfonic calix[4]arene in the mobile phase drastically reduced the retention times of all 3 components of the isomer series.

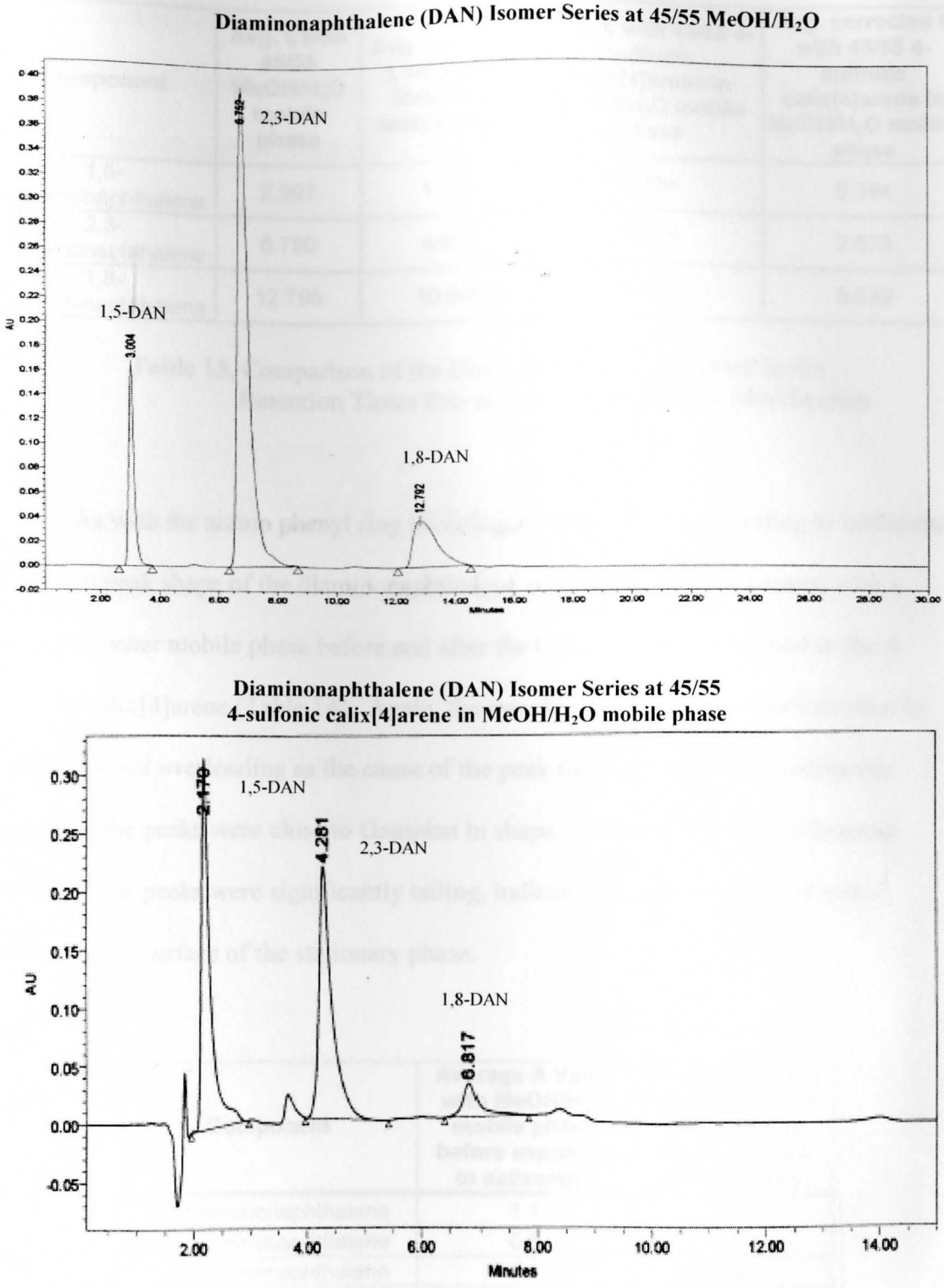


Figure 32. Direct Comparison of Diaminonaphthalene Isomer Series Separated with Non-modified and 4-sulfonic calix[4]arene Modified Mobile Phase

Component	Avg. t_r with 45/55 MeOH/H_2O mobile phase	Avg. corrected t_r with 45/55 MeOH/H_2O mobile phase	Avg. t_r with 45/55 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase	Avg. corrected t_r with 45/55 4-sulfonic calix[4]arene in MeOH/H_2O mobile phase
1,5-Diaminonaphthalene	2.997	1.197	2.194	0.394
2,3-Diaminonaphthalene	6.750	4.950	4.323	2.523
1,8-Diaminonaphthalene	12.795	10.995	6.839	5.039

Table 13. Comparison of the Diaminonaphthalene Isomer Series Retention Times Pre- and Post-Mobile Phase Modification

As with the amino phenyl ring homologous series, it was interesting to notice the change in peak shape of the diaminonaphthalene components when separated with a methanol/water mobile phase before and after the C18 column was exposed to the 4-sulfonic calix[4]arene (Table 14). Again, the samples were diluted in concentration by half to rule out overloading as the cause of the peak fronting. Before the calixarene exposure, the peaks were close to Gaussian in shape. However, after the calixarene exposure, the peaks were significantly tailing, indicating Langmuir behavior and a change in the surface of the stationary phase.

Component	Average A Value with MeOH/H_2O mobile phase before exposure to calixarene	Average A Value with MeOH/H_2O mobile phase after exposure to calixarene
1,5-Diaminonaphthalene	1.1	2.1
2,3-Diaminonaphthalene	0.8	2.8
1,8-Diaminonaphthalene	1.3	3.2

Table 14. Peak A Values for Components of the Diaminonaphthalene Isomer Series Pre- and Post-Exposure of the Column to the 4-sulfonic calix[4]arene

These results indicated that the three diaminonaphthalene isomers did form host-guest complexes with the 4-sulfonic calix[4]arenes, and the strength of the complexes was large enough to affect the separation results.

CHAPTER VII

Conclusions and Future Work

The synthetic method used to covalently bond 4-*tert*-butylcalix[n]arenes to the silica stationary phases has been researched by this group,[15] and a patent has been recently filed by another group for a similar process.[16] This modification method appeared to provide a high percent coverage of the stationary phase with the calixarene, as evidenced by the large changes in peak shape and necessary drastic change in mobile phase strength needed to achieve separation.[15] As a result, this type of modification gave way to the largest changes in the results of the chromatography. It would therefore be beneficial to further investigate and probe this modification method.

First, each step of the synthetic method could be studied individually to determine the effect of material changes on the quality of the stationary phase product. This would include alternate types of base silica, reflux solvents, and tethers. In fact, some preliminary work has already been performed and reported on varying tether length.[15] Calixarenes with various substituents on the upper or lower rim could also be investigated with this type of system. These changes in the starting materials may provide better separation results, and therefore the conditions of the synthetic method could be optimized for different types of separations.

Another possibility is to study the use of this synthetic method of binding a chiral calixarene to the stationary phase. The solute probe used would then be a homologous series of chiral compounds. Chiral chromatography is very important to the pharmaceutical industry, and is typically performed under normal phase conditions.

Normal phase chromatography tends to be more problematic than reverse phase chromatography due to difficulties pumping and degassing the solvents used in the mobile phase. Therefore, if this synthetic method proves to be viable with chiral calixarenes, then a modified chiral stationary phase could be used under reverse phase conditions for separation of drug enantiomers. Again, some preliminary work with chiral calixarene modifiers has been reported.[17]

Another possibility for studying the interactions between a covalently bound calixarene stationary phase and various solute probes is to purchase a commercially prepared calixarene column and repeat the homologous series studies. Comparison of the data between the synthesized stationary phase and the premanufactured stationary phase would provide information on the quality, ease, and repeatability of the synthetic method.

The second method of stationary phase modification was the absorption of the 4-*tert*-butylcalix[n]arenes. This method showed some indications of successful stationary phase modification and host-guest complex formation with the solute probes. This was evidenced by the reduction in retention times for some of the homologous series components when using the same strength mobile phase. However, the changes were not very dramatic and significant problems were encountered with the solubility of the 4-*tert*-butylcalix[n]arenes.

Future work on this type of modification would need to begin with solubility studies on the 4-*tert*-butylcalix[n]arenes. Doing this would allow for optimization of the absorption solvent, rinse solvent, and mobile phase composition.

The next possible area for investigation would be to examine the effect of solute molecule size in forming host-guest complexes with the 4-*tert*-butylcalix[6]arene and 4-

tert-butylcalix[8]arene. Since both of these calixarenes did not form strong host-guest complexes with the smaller solute probes used in this work, it may be possible that larger polymers or biomolecules may form stronger host-guest complexes. Series of either of these types of molecules could be separated using the absorbed calixarene modified stationary phase to determine if the size of the calixarene cavity in relation to the size of the solute molecules has an effect on the interactions.

Another option is to investigate other types of calixarenes for absorption onto the stationary phase. Adding various substituents to the upper or lower rim of the calixarene basket alters their properties and may make them more or less efficient at modifying the chromatography. Two possibilities are the 4-sulfonic calix[n]arenes, since they showed strong indications of host-guest interactions as mobile phase modifiers, and an inherently chiral calixarene in an attempt to perform chiral separations under reverse phase conditions.[17]

In order to further study the success of absorbing the calixarenes onto the stationary phase, fluorescence spectroscopy could be used to analyze the modified stationary phases.[15] Once the absorption of the calixarenes is complete and the chromatography data collected, the column could be unpacked and the stationary phase analyzed by fluorescence spectroscopy. This may provide confirmation of the presence of the calixarene on the stationary phase, and could potentially be further developed as a method to quantify the percent coverage of the stationary phase.

One problem encountered with the absorption studies was removal of the calixarene at the completion of the study to restore the stationary phase to its original C18 state. Again, fluorescence spectroscopy could be used to investigate this issue. Once the

presence of the calixarene on the stationary phase is confirmed by fluorescence spectroscopy, aliquots of the stationary phase could be washed with various solvents and analyzed again by fluorescence spectroscopy. This may provide information as to an effective wash solvent for restoring the stationary phase in the column.

The studies involving the 4-*tert*-butylcalix[4]arene modified mobile phase were somewhat inconclusive. Peak splitting with the alkylbenzene homologous series was an initial indication that secondary interactions were occurring between the stationary phase, mobile phase, and solute probes. However, no significant differences were seen in the retention times of the components. Again, solubility of the calixarenes was the biggest problem, causing backpressure on the chromatography system. Perhaps alternate solute probes could be chosen that would have stronger interactions with the calixarenes, thus providing more solid evidence of host-guest interactions. Also alternate mobile phases could be investigated, depending on the solubility of the 4-*tert*-butylcalix[n]arenes in the organic/water combinations.

For all of the modification studies using the 4-*tert*-butylcalix[n]arenes, it would be beneficial to investigate alternate solute probes. As stated previously, compounds that have stronger interactions with the 4-*tert*-butylcalix[n]arenes would provide more drastic and definitive results. Two possibilities are the amino phenyl ring homologous series and the diaminonaphthalene isomer series used in the latter parts of this research. Both showed evidence of strong interactions with the 4-sulfonic calix[4]arenes, so similar results may be seen with the 4-*tert*-butylcalix[n]arenes. Again, this would make it easier to draw solid conclusions regarding the host-guest interaction levels.

Studies involving the 4-sulfonic calix[4]arene modified mobile phase showed the highest degree of host-guest complex formation with the solute probes chosen. This was seen in a reduction of capacity factors and changes in peak shape for all components of both solute probe systems used.[15] To elaborate on these results, the concentration of the mobile phase modifier or the solute probe components could be varied. Using this data and the equation proposed by Kalchenko et al., the strength of the host-guest complexes could be estimated.[6] This information would be useful in determining which types of compounds are most affected by the presence of the 4-sulfonic calix[4]arene modifier in the mobile phase.

Another possibility is to repeat the same experiments performed with the 4-sulfonic calix[4]arene modified mobile phase using an alternate organic solvent in the mobile phase. It may be possible to further affect the separation results using an alternate mobile phase. Since acetonitrile is know to occupy the cavity of calixarenes, it would also be interesting to investigate whether, when used as part of the mobile phase, the acetonitrile competes with the guest compounds in forming complexes with the 4-sulfonic calix[4]arenes.[17]

One final possible area of research would be studies to improve the peak shapes in the calixarene modified systems. Although changes in peak shape are positive indications of host-guest interactions, symmetrical peaks are always desired in chromatography. In some chromatographic separations, a modifier can be added to the mobile phase to improve the peak shape. One example is adding methyl-*tert*-butyl ether in a very small amount, typically 0.5% – 2.0%, to reduce peak tailing in chiral

chromatography. It may be possible to identify a modifier that would effectively improve the peak shape in the presence of the calixarenes.

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