Retention Characteristics of Water-Soluble Calixarene Modified Mobile Phases in HPLC

by

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Abstract

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Modified Mobile phase in HPLC

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In recent years a class of compounds classified as calixarenes, basket-shaped macromolecules comprised of phenolic units arranged in a cup, have become of interest in many areas of chromatography. A specific area of interest for this work, is their use as mobile phase modifiers in reversed-phase HPLC. This interest is due principally to similarities in host/guest complex formation between the calixarenes and the cyclodextrins. These similarities have led to the belief that calixarenes will form significant host/guest interactions with solute molecules during chromatography. Interactions have been determined to take place by the reduction of capacity factors of solutes when a calixarene additive was introduced to the mobile phase.

One problem associated with use of calixarenes as mobile phase modifiers is their strong absorbance in the UV region of the spectrum. This problem has been circumvented by fluorescence and Vis absorbance detection of solutes has been successfully employed here to get around this problem.

Acknowledgments

I would like to express my sincere gratitude to Dr. James Mike for all that he has done for me in the past two years. I would not be where I am today without his guidance and encouragement. I would also like to thank Dr. Peter Norris for all his assistance on synthetic matters, and his time spent review this manuscript. Finally I would like to thank Dr. Daryl W. Mincey for his time spent review this manuscript.

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SYMBOL	Definition	Units of Reference
mL	Milliliter	1 x 10^{-3} liters
µL	Microliter	1 x 10^{-6} liters
mg	Milligram	1 x 10^{-3} grams
µg	Microgram	1 x 10^{-6} meters
nm	Nanometer	1 x 10^{-9} meters
mM	Millimolar	1 x 10^{-3} molar
M	Molar	mol per liter
mol	Mole	
L	Liter	
g	Gram	
min	Minute	
°C	Degrees Celsius	
HPLC	High Performance Liquid Chromatography	
TLC	Thin Layer Chromatography	
NMR	Nuclear Magnetic Resonance	
^{1}H	Hydrogen-1	
UV	Ultraviolet	
Hz	Hertz	
Vis	Visible	
I.D.	Internal Diameter	
C_{18}	Octadecyle	

CHAPTER I

Introduction

Calixarenes and their Synthesis

Calixarenes are a class of macrocyclic compounds composed of a series of *para* substituted phenolic monomers linked by methlyene bridges. The term calixarene was coined by D. C. Gutsche (1). The name is derived from the name of a Greek cup called a *calix crater*, which the calixarene resembles, and also the arene functionality. They are of interest due to their reported ability to form host/guest complexes with a variety of compounds in a manner similar to the cyclodextrins, which are used extensively in a variety of chromatographic systems (2-6).

The synthesis of *p*-substituted calixarenes is achieved by the base-induced condensation of *p*-tert-butylphenol and formaldehyde as depicted in Figure 1 (7). The initial reaction of the *p*-tert-butylphenol and formaldehyde results in the formation of a precursor mixture as shown in Figure 2 (7). The initial step of the formation of calixarenes is fairly well understood, and involves hydroxymethylation forming an *o*-hydroxymethylphenol, which then undergoes arylation by means of an intermediate *o*-quinonemethide. While the following steps are proceeding, simultaneous dehydration between pairs of hydroxylmethyl compounds takes place to form dibenzyl ethers. This results in a mixture of about three dozen different diphenylmethane-type and dibenzyl ether-type compounds called the “precursor”, from which the calixarene arises. The

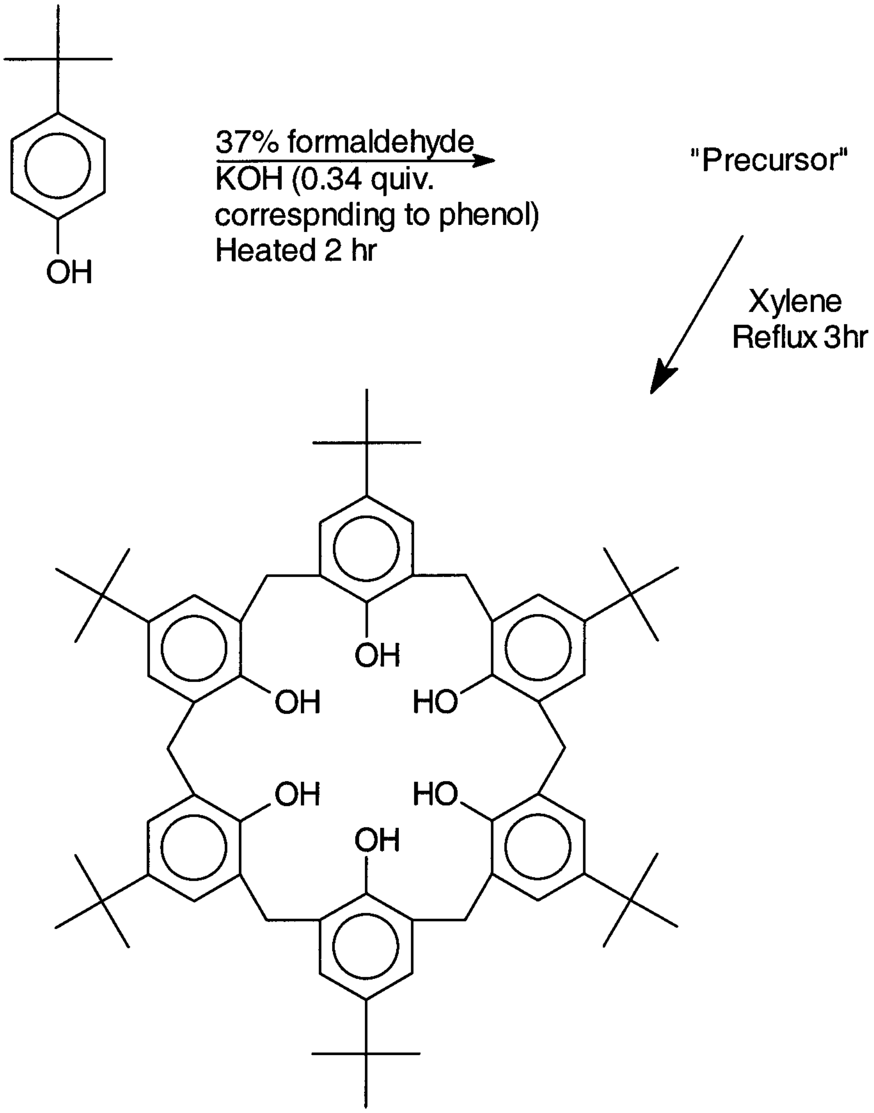


Figure 1: Synthesis of *p*-tert-butylcalix[6]arene (**1**)

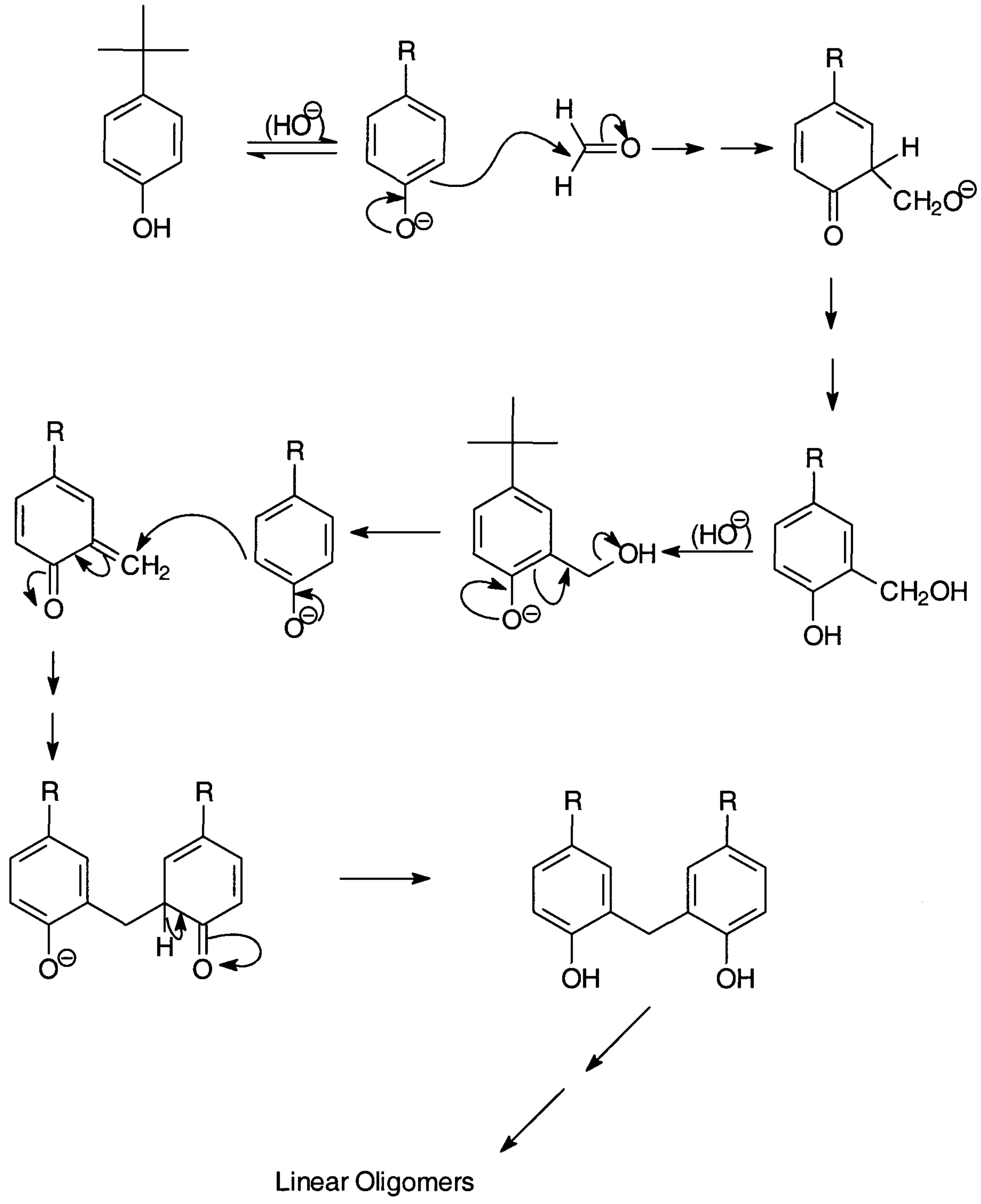


Figure 2:Mechanism for the synthesis of the “precursor”

formation of the calixarene takes place using xylenes as the solvent. The process of linear oligomers transforming into the calixarene is not completely understood, but is theorized to be a result of a process in which hydrogen bonding between the linear oligomers takes place (7).

The method used in this work to functionalize *p*-tert-butylcalix[6]arene was a Mannich base/quinonemethide route of functionalization (Figure 3). This method was chosen over other methods in the literature, such as *p*-Claisen Rearrangement (7), because of the need for fewer steps, higher yields, and the simplicity of the method.

The first step in this route was the removal of the tert-butyl group at the *para* position, which was easily facilitated by dealkylation using an $AlCl_3$ catalyzed reaction in the presence of an acceptor solvent such as toluene. This was followed by the addition of a diallylamine at the *para* position of the phenol to form a Mannich base. The Mannich base was then easily quaternized, which allowed for the removal of the amino functionality and substitution by two equivalents of the nucleophilic sodium diethylmalonate (7).

High performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a technique used to separate solutes, and then quantify them. HPLC is based on the physical partitioning of solutes between a mobile phase and a stationary phase. The mobile phase is a liquid, which is passed through a packed bed of particulate

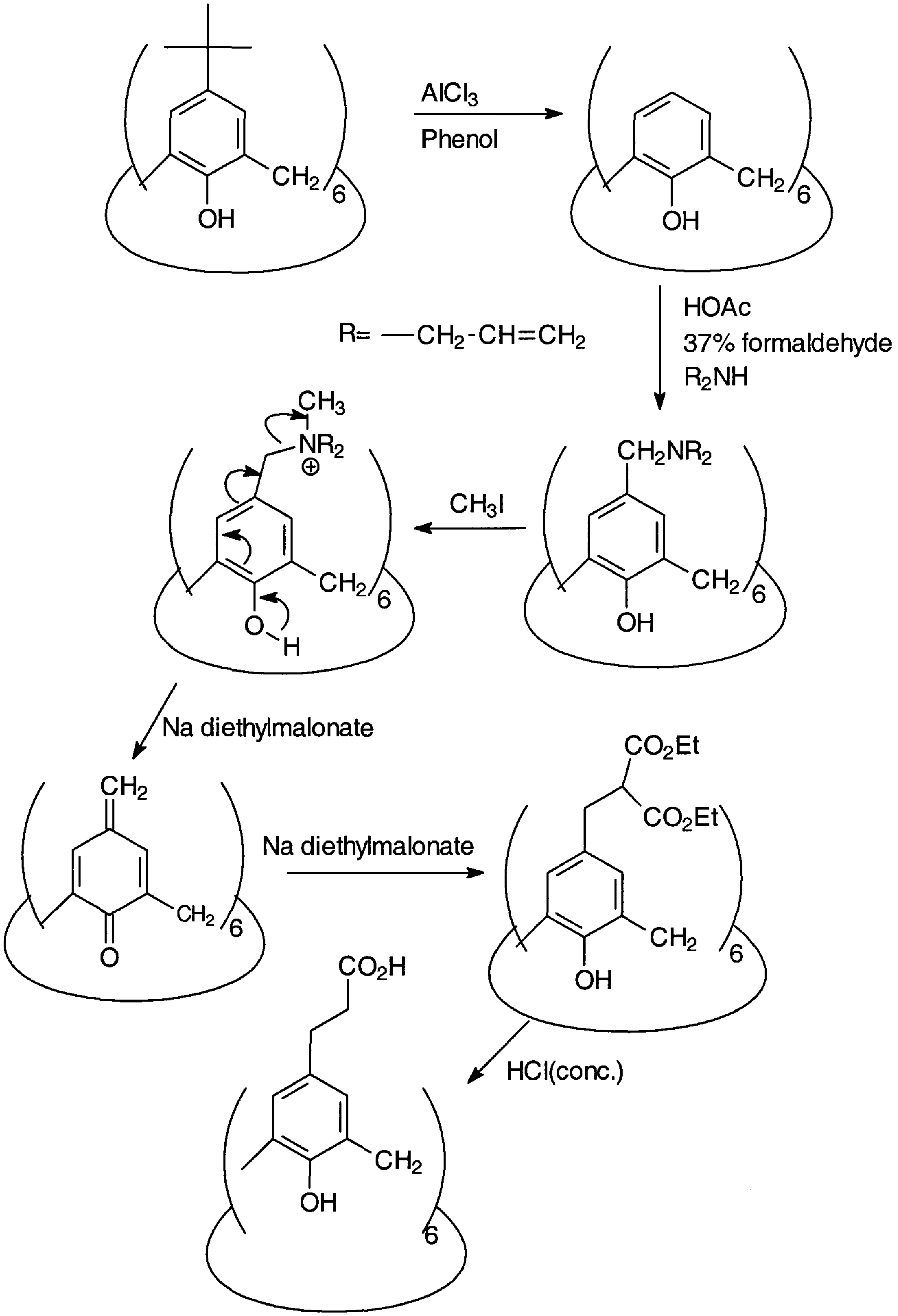


Figure 3: Mannich Base Route of functionalization of *p*-tert-butylcalix[6]arene

solid stationary phase, contained in a column. The mobile phase is pumped over the stationary phase at moderately high pressures (2000-5000 psi). Solutes placed at the head of the column are thus carried through the stationary phase by the mobile phase. Separation of solutes occurs as a result of the varying equilibrium between the mobile and stationary phases for an individual solute. All solutes of an assay interact with the stationary phase for different amounts of time. As a result, they elute from the column at different times and are separated from one another.

Secondary Equilibrium

The equilibrium of the solute that is established between the stationary and mobile phases is referred to as the primary equilibrium. Other equilibrium processes that occur in addition to the primary equilibrium are referred to as secondary chemical equilibria. An example of this is the equilibrium of a weak acid between it's fully protonated and ionized forms in the mobile phase by the adjustment of the pH (8). The variation of the weak acid's ionization, which is a function of pH will result in a variation of retention time. The variation of the pH to favor the non-ionized form with a weak acid or base is called ion suppression chromatography. It is only one of many different modes of chromatography that employ secondary equilibrium as an additional means to adjust a chromatographic system for optimum separation of solutes. Some other modes include ion pairing, metal complexation, and solute micelle association. As a result of this utilization of secondary equilibria, additional modes of varying the retention of solutes are obtained.

The secondary chemical equilibrium process is attributed to the addition of an equilibrant (X), which interacts with the analyte (A) to form a complex (AX). This complex subsequently forms its own equilibrium with the mobile and stationary phases along with the analyte as depicted in Figure 4 (8-10). A and AX can both exist at the same time with different retention times in a chromatographic system without causing band broadening and multiple peaks due the fast equilibrium time in reversed phase HPLC (11). The equilibrant is assumed not to be retained by the stationary phase. In many cases the distribution coefficient established for an analyte in the presence of an equilibrant is not defined by the distribution coefficient of A or AX individually, but by a combination of both. This is also true for the capacity factor of the analyte. The capacity factor observed in the presence of an equilibrant is defined as the combined weighted averages of the capacity factor of both A and AX as in equation 1.

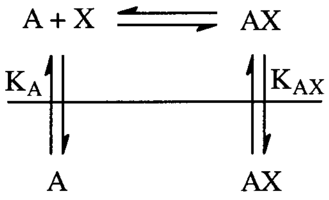


Figure 4: Secondary Chemical Equilibrium

$$k_{obs}= F_A k_A + F_{AX} k_{AX}$$

Equation 1

Here k_{obs} is the capacity factor of the analyte eluted under experimental conditions, and F_A and F_{AX} are the stoichiometeric factors of each analyte in the forms A and AX. F_A and F_{AX} can be expressed in terms of the equilibrium constants of A, X and AX and substituted into equation 1. From Equation 2 it can be seen that k_{obs} is defined by the concentration of the equilibrant, the

$k_{obs}= K_{SCE}/([X] +K_{SCE})k_A + [X]/([X] + K_{SCE})k_{AX}$ **Equation 2**

secondary chemical equilibrium constant K_{SCE} and the limiting capacity factor of the two possible forms of the analyte.

The simplest form of a secondary chemical equilibrium is ion suppression. The buffering of the mobile phase pH can be used to control a weak acid's or base's state of ionization. This method can be used with both ionizable and neutral solutes because only the ionizable solute's retention will be affected. In the situation where strong acids and bases are involved, ion suppression cannot be used because silica-based stationary phases are only stable within a pH range of 2-8 (11).

Equation 2 can be rewritten in the situation where the equilibrant is $[H^+]$ and the complex formed with X^- is the acidic form of a weak acid. This rewritten form is similar to that of the dissociation of an acid. An example is depicted Figure 5 where salicylic acid has replaced A with the conjugate base and X is the proton donated by the buffer resulting in the formation of the unionized form of salicylic acid (HX). In agreement with Equation 3, the values for k_{HX} (the intercept) and k_{X-} (the

$$k_{obs} = k_{HX}([H^+] / ([H^+] + K_a)) + k_{X^-}(K_a /([H^+] + K_a))$$ **Equation 3**

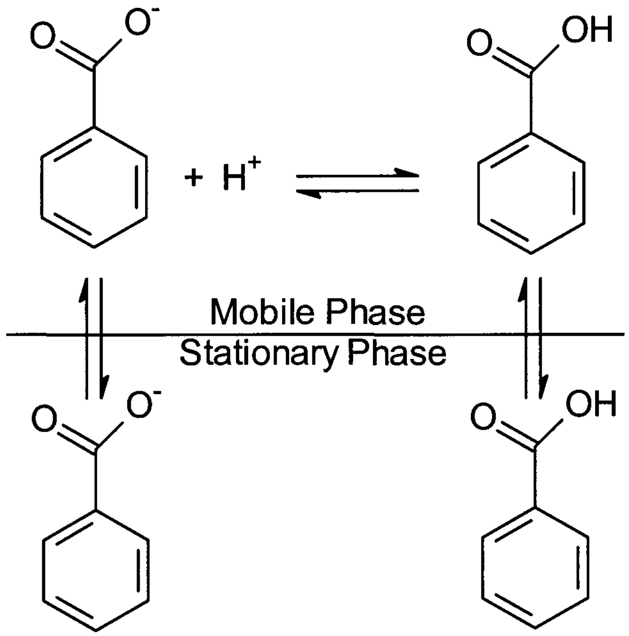


Figure 5: Salicylic Acid Secondary Chemical Equilibrium

slope) are obtained from the plots of $k_{obs}(1+K_a/[H^+])$ vs. $K_a/[H^+]$, which is linear (12). Careful investigation of equation 3 reveals that k_{obs} and pH have a sigmoidal relationship in which the pH, where [A] and [AX] equal one another, corresponds to the pK_a of the acid. This is very similar to a titration curve of an acid as seen in figure 6. A small change in the pH near the pK_a results in the large change in K_{obs}, but at the extreme ends of the curve where the curve flattens out, the effects of changes in pH are severely reduced.

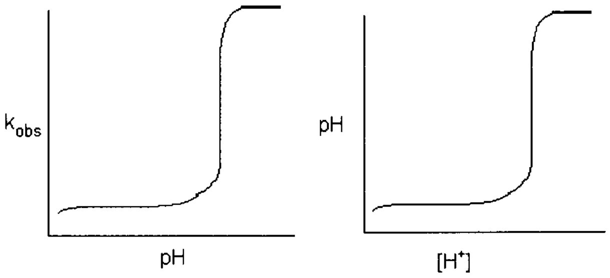


Figure 6: Sigmoidal Relationship of k_{obs} vs. pH compared to Titration Curve

Micellar HPLC

The secondary chemical equilibrium process of interest for this research is that of micellular interaction with solutes. Surfactants are molecules with two distinctly different regions. One region is charged or highly polar and represents only a small fraction of the total molecular volume. The other region is typically hydrocarbon-like and is thus very non-polar. Micelles are structures formed as the result of self-aggregation of surfactants. This takes place when the concentration of a surfactant has been increased above the critical micelle concentration for that individual surfactant. The micelle structure is microscopically inhomogeneous, and presents a microenvironment that clearly differs from that of the mobile phase (12).

In aqueous, or largely aqueous phases, the polar or charged group forces the solvent and the non-polar portions are forced to turn inward and associate together (Figure 7). As a result of this micelles greatly increase the solubility of hydrophobic compounds in the aqueous environments of reverse phase HPLC by means of surface adsorption, pseudophase extraction (partitioning), and solute-surfactant coassembly (comicellization). The concentration of micelles in the mobile phase can be increased above the critical micelle concentration due to their very low affinity for the stationary phase, thus allowing for greater interaction of the solutes with the micelles resulting in reduction of retention times (12).

Micellar liquid chromatography is of interest in this work due the similar interactions of micelle and water-soluble calixarenes with solutes. The calixarenes that were used in this project do not act as traditional micelles forming a structure of self-aggregated calixarenes. They act instead as a unimolecular micelle. Even though the calixarenes are unimolecular miceller they provide a microenvironment different from the mobile phase. The Increasing

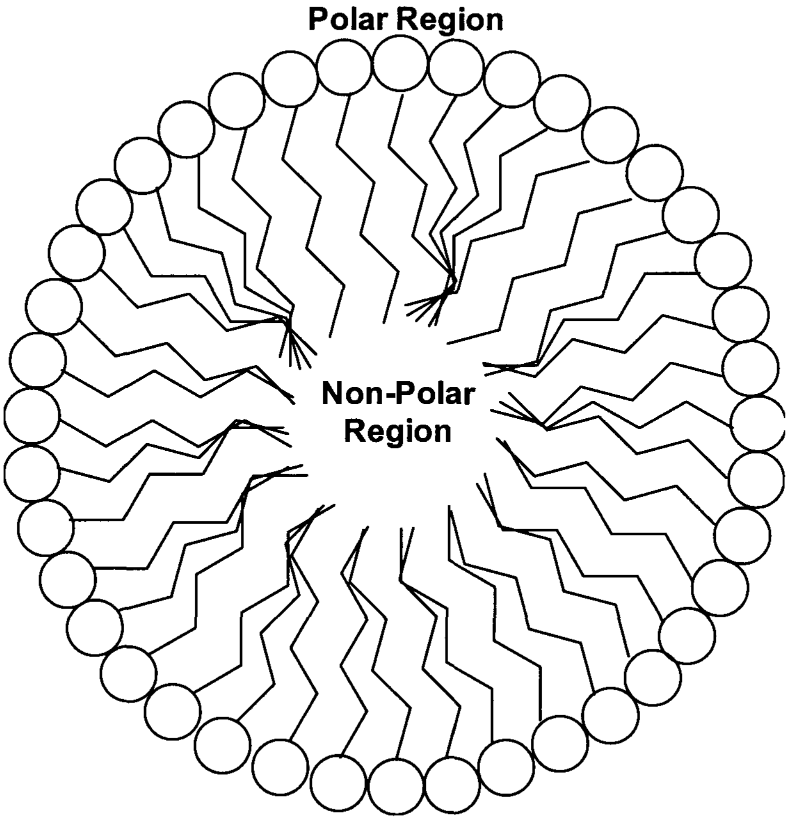


Figure 7: Micelle

solubility of hydrophobic compounds and the resulting reduction of the retention times of solutes is similar to the behavior seen with micelles. The solutes interact with the calixarenes by fitting themselves into the cavity of the calixarene in such a way that the hydrophobic regions of the solutes can interact with the hydrophobic phenolic basket of the calixarene by means of π–π interactions and London forces. The polar regions of the solutes can interact with the polar regions of the calixarenes by means of hydrogen bonding, as with the amino and hydroxyl functionality of 5-amino-1-naphthalene (Figure 8).

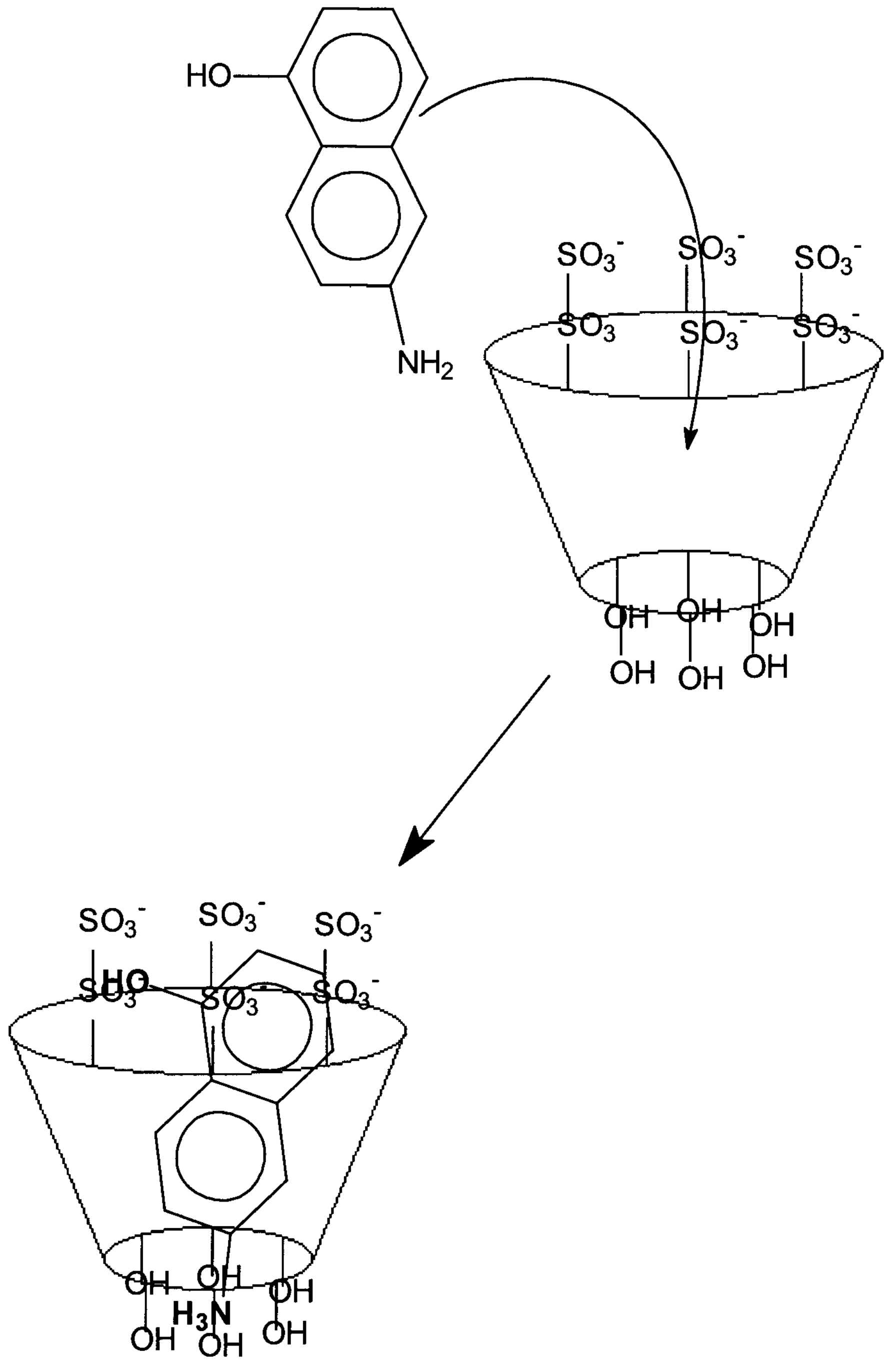


Figure 8 Host/guest interaction of the solute with 4-sulfonic calixarene. The basket represents a hydrophobic region of phenol rings.

CHAPTER II

Statement of the Problem

There have been few investigations of calixarenes as mobile phase additives. This has been due to the limited availability of water-soluble calixarenes and their ability to be strong ultraviolet absorbers. This leads to the purpose of this research. There are three focus points. The first was the determination of complexation between water-soluble calixarenes and solutes in the mobile phase of High Performance Liquid Chromatography (HPLC). The second was to determine qualitatively what effect the host/guest complex has on the retention of the solutes. The final purpose was to investigate other methods of detection other than ultraviolet absorbance such as fluorescence and visible spectroscopy. Observation in the differences of capacity factors of solutes when no calixarene additive was used compared to that when a calixarene additive was used was an indication of complexation.

CHAPTER III

Historical

A review has appeared by Bressille *et. al.* on the use of cyclodextrins (CD's) in HPLC and capillary electrophoresis (13). CD's are cyclic structures composed of D-glycopyranose units bonded through α-1,4-linkages. There are three main types: α-CD, which is composed of six glucose units; β-CD composed of 7 units, and γ-CD composed of 8 units. The glucose units in CD's are reported to take a $^4C_1$ chair conformation and position themselves so that the molecule can form a toroidal/hollow truncated cone structure. This structure allows for a variety of water soluble and insoluble compounds to form inclusion complexes by fitting themselves into the cavity of the CD's.

The biggest problem with the use of native CD's was indicated to be their low solubility in an aqueous-organic environment. However, a beneficial trait of the CD structure was it's ease of modification to increase solubility and more importantly, to introduce chirality into the structure. Introduction of chirality to the CD facilitates the ability to perform enantiomeric separations.

CD's may be used as HPLC mobile phase modifiers to allow enantiomeric separations (13). The use of CD's as HPLC mobile phase modifiers for enantiomeric separations is heavily based on the selectivity of the formation of the inclusion complexes. The stability of these complexes is the foundation of the chromatographic properties of CD's.

The two properties acting synergistically and directly toward the properties of solutes and their interactions with the CD's are selectivity and stability.

Separations of enantiomers comes from the different inclusion complex stabilities based on differences in hydrophobic and hydrogen bond interactions with the CD's. In order to obtain reasonable separations with CD's high concentrations, near or at the solubility limit, are needed. This presents a large problem that has been resolved by the use of more soluble CD's.

The inclusion complex equilibria of β-CD as a chiral mobile phase additive has been described by Walhagen and Edholm in Equation 4 (14).

$$1/k = 1/k_o + K_f[CD]_T^N/k_0$$ **Equation 4**

The capacity factor of the solute in k; the total concentration of the CD in the mobile phase is $[CD]_T$; k_o is the capacity factor of solute not in the presence of the CD; K_f is the formation constant of the inclusion complex; and N is the ratio of CD to solute forming the complex. This was determined under the assumption that the CD and the complex formed were unretained by the stationary phase.

D.C. Gutsche has reported the use of *p*-(*N,N*-diallylaminomethyl)calix[6]arene and *p*-(2-carboxyethyl)calix[6]arene to form complexes with polycyclic aromatic hydrocarbons (PAH) (3). The *p*-quinonemethide route of functionalization was used to synthesize a series of water-soluble calixarene containing diallyamino and carboxyl groups at the *para* position. The magnitude of the host/guest complexes were calculated for nine PAH's varying in size from the smallest, durene, to decacyclene with functionalized calixarenes ranging in size from calix[5]arene to calix[8]arene. A solid-liquid extraction procedure was used. This procedure involved the addition of a small amount of guest molecule to an aqueous solution containing one of the calixarenes. The solution was stirred vigorously, centrifuged, filtered, and the aqueous solution was extracted with an organic solvent. UV-VIS spectroscopy was used to determine the amount of hydrocarbon remaining. The distribution constant was determined using equation 5:

$$H_{water} + G \rightleftharpoons HG_{water}$$ **Equation 5**

Here G is the amount of the guest able to dissolve in water, HG_{water} is equal to the amount of guest molecule minus the value for G determined by UV-VIS and finally the value for the host is equal to the amount originally added minus the value for HG_{water}.

At the time of this writing, there was only one reported use of calixarenes as a mobile phase additive for HPLC. The water soluble calixarene, 4-sulfonic calix[6]arene, was investigated with mono-substituted phenols by Park *et. al* (2). The capacity factors for methoxy, amino, and nitrophenols were obtained with and without 4-sulfonic calix[6]arene. Capacity factors were observed to be reduced upon the addition of 4-sulfonic calixarene to the mobile phase. The reduction of capacity factors was the indication of the formation of host/guest complexation between the solutes and the calixarene additive. Host/guest complexation was shown to form in the mobile phase but the use of UV detection sensitivity was significantly reduced by interference from the 4-sulfonic calix[6]arene.

CHAPTER IV

Materials and Apparatus

Reagents for HPLC Analysis

Solvents for HPLC were of HPLC guality from Fisher Scientic (New Jersey). 4-sulfonic calix[6]arene was obtained from Acros Organic (New Jersey). Glacial Acetic acid was acquired from Malllinckrodt Chemical Works (St. Louis). Standard solutes (naphthalene, phenanthrene, anthracene, fluoranthene, pryene, 3-amino-2-naphtol, 5-amino-1naphthol, 1-aminonaphthalene, Acid Blue 45, Acid Red 8, Acid Red 88, and Acid Red 114) were acquired from Aldrich Chemicals (Milwaukee, WI).

Reagents for Synthesis of Calixarenes

Toluene was dried over calcium hydride (Fisher Scientific, New Jersey) under a nitrogen atmosphere, and tetrahydrofuran (THF) (Fisher Scientific, New Jersey) was dried over sodium under an argon atmosphere. All other solvents were used without further purification and were obtained from Fisher Scientific (New Jersey). Potassium hydroxide was obtained from EM Sciences (Gibbstown, NJ). Potassium carbonate (anhydrous) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Acetic acid and phenol were acquired from Malllinckrodt Chemical Works (St. Louis). Aluminum chloride was from Spectrum Chemical Co (Gardena, CA). Formaldehyde (37% solution) was from Fisher Scientific (New Jersey, NJ). Diallyamine and *p*-tert-butylphenol were obtained from Aldrich Chemicals (Milwaukee, WI). Analytical thin layer chromatography (TLC) was conducted on aluminum backed silica plates (Whatman, Kent, England). Visualization was accomplished with an ultraviolet lamp and/or by staining with 5% (w/v) phosphomolybdic acid (PMA) in ethanol, which was obtained from Aldrich Chemicals (Milwaukee, WI).

Apparatus For HLPC Analysis

A Beckman System Gold reverse-phase HPLC was used for this project. The system was comprised of a Beckman 406 analog interface module, two model 110B solvent delivery modules, module 166 UV-VIS detector, injector with a 20 μL injection loop, and a Shimadzu RF-535 Fluorometer. The column used was 4.6 mm id x 250 mm, 5 μm, and Nucleosil C_{18} packed in-house. The system was controlled by, and data was collected by Beckman's System Gold software version 5.10.

Apparatus For Synthesis of Calixarenes

NMR spectra (^1H) were recorded with a Varian Gemini 2000, 400 MHz spectrometer, with $CDCl_3$, as the solvent. The ^1H chemical shifts are reported in parts per million downfield from $(CH_3)_4Si$.

CHAPTER V

Experimental

Sample Preparation of Aromatic Hydrocarbons.

The polycyclic aromatic hydrocarbons samples where prepared by diluting stock solutions of naphthalene, phenanthrene, anthracene, fluoranthene and pyrene containing 200 µg/µL to the concentrations listed in Table 1 using HPLC grade acetone. These concentrations were used because they were determined to give the maximum fluorescence response without over-ranging the detector.

Compound	Concentration(mM)
napthalene	6.24E-02
phenathrene	2.24E-02
anthracene	4.48E-03
fluoranthene	1.38E-03
pyrene	3.95E-04

Table 1: Concentration of PAH solutes

HPLC Analysis of PAH'S

The separation of the PAH's without the calixarene additive was accomplished with a mobile phase experimentally determined to be 73/27 acetonitrile/ water. The separation of the PAH's with the calixarene additive was accomplished with a mobile phase of 73/27 acetonitrile/water with 0.005M 4-sulfonic calix[6]arene. A flow rate of 1.0 mL/min was used. Injection volumes were 20µL of sample. Detection was performed in both cases by fluorescence spectroscopy with an excitation wavelength of 250 nm and an emission wavelength of 389 nm.

Sample Preparation of Amino-Naphthols and 1-Aminonaphthalene

The samples containing 5-amino-1-naphthol, 3-amino-2-naphthol, and 1-aminonaphthalene concentrations were prepared in a similar fashion to that of the PAH sample. The concentrations used were determined to give the best fluorescence response without over-ranging. These concentrations were obtained by diluting a stock solution of 0.001M for each solute to the concentrations of the solutes listed in Table 2, with HPLC grade acetone.

Compound	Concentration (M)
5-amino-1-naphthol	5.00E-06
3-amino-2-naphthol	5.00E-06
1-aminonaphthalene	1.50E-05

Table 2: Concentration of Amino-Naphthols and 1-Aminonaphthalene solutes

HPLC Analysis of Amino-Naphthols and 1-Aminonaphthalene

The separation of 5-amino-1-naphthol, 3-amino-2-naphthol, and 1-aminonapthalene was accomplished with mobile phases determined experimentally to be 40/60 acetonitrile/water and 50/50 methanol/water when no calixarene additive was employed. The mobile phases that were found to give the best separations when the calixarene additive was used were 25/75 acetonitrile/water with 0.0005M 4-sulfonic calix[6]arene and 40/60 methanol/water with 0.0005M 4-sulfonic calix[6]arene. A flow rate of 1.0 mL/min was used. Injection volumes were 20 µL of sample. Detection was performed in both cases by fluorescence spectroscopy with an excitation wavelength of 220 nm and an emission wavelength of 400 nm.

Preparation of Sulfonated Azo Dyes Samples and Mobile Phases

The samples containing Acid Blue 45, Acid Red 8, Acid Red 88, and Acid Red 114 were prepared by the dilution of 0.001M stock solutions to the concentrations listed in Table 3. The concentrations used for the samples were those that gave the best response without over-ranging. The mobile phase containing tetratbutylammonium hydrogen suflate was prepared at a concentration of 0.002M in 55/45 acetonitrile/aqueous 1% acetic acid. The acetic acid was used in order to be consistent with the mobile phase containing the calixarene. The mobile phase containing *p*-(*N,N* diallylaminomethyl)calix[6]arene was prepared at a concentration of 0.002M in 55/45 acetonitrile/aqueous 1% acetic acid. The acetic acid was used to facilitate the solvation of the calixarene additive.

Solute	Concentration (mM)
Acid Blue45	0.25
Acid Red 8	0.25
Acid Red 88	0.25
Acid Red 114	0.25

Figure 3: Concentration of sulfonated azo dyes solutes

HPLC Analysis of Sulfonated Azo Dyes

The separation of the mixture containing Acid Blue 45, Acid Red 8, Acid Red 88, and Acid Red 114 was accomplished with mobile phases determined experimentally of tetratbutylammonium hydrogen sulfate 0.002M in 55/45 acetonitrile/aqueous 1% acetic acid and, *p*-(*N,N* diallylaminomethyl)calix[6]arene

0.002M in 55/45 acetonitrile/aqueous 1% acetic acid. A flow rate of 1.0 mL/min was used. Injection volumes were 20 µL of sample. Detection was performed in both cases by Vis absorbance spectroscopy at 552 nm.

Synthesis *p*-tert-butylcalix[6]arene

To a slurry of *p*-tert-butylphenol (0.655 mol, 100 g) and 37% formaldehyde solution (1.8 mol, 135 mL) potassium hydroxide (0.27mol, 15 g) was added. For two hours this was stirred and heated to reflux under a nitrogen atmosphere. Water from the formaldehyde solution was collected in a Dean Stark trap. The reaction was cooled to room temperature after two hours, xylenes were added and the mixture was quickly brought to boiling under nitrogen atmosphere and refluxed for three hours. Product was removed by suction filtration yielding 116 g of crude material. The product was then dissolved in chloroform (2.5 L) and 1M HCL (0.8 L). Aqueous and organic layers were separated. The organic layers were washed with successively with three 350 mL aliquots of deionized water. The product was dried over $MgSO_4$. The product was triturated with hot chloroform (1 L) and acetone (1 L) yielding 75 g of pure product. Melting point: 359-361 °C

^{1}H NMR: δ 10.44 (s, 6 H), 7.14 (s, 12 H), 3.87 (br s, 12 H), 1.25 (s, 54 H)

Synthesis of Calix[6]arene

To a slurry of toluene (375 mL) and *p*-tert-butylcalix[6]arene (33 mmol, 31.62g) were added phenol (198 mmol, 18.57 g) and $AlCl_3$ (264 mmol, 35.25 g) were added. This mixture was stirred for 24 hours in a nitrogen atmosphere at room temperature. The reaction was quenched with deionized ice water (300 mL). The organic layer and aqueous layers were separated. The toluene was removed by evaporation. The product was dissolved in chloroform (1 L) and washed with three 200 ml aliquots of deionized water. The chloroform was removed by evaporated leaving a residue, which was triturated with MeOH (210 mL) yielding 14.63 g of pure product.

^{1}H NMR: δ 10.4(s, 1H, ArOH), 7.15 (d, 2H, ArH), 6.83 (dd, 1H, ArH), 3.9 (br s, 2H, $ArCH_2Ar$)

Synthesis of p-(N,N-Diallylaminomethyl)calix[6]arene

To a solution of THF (60 mL) and calix[6]arene (2.91 mmol, 1.86g) was added glacial acetic acid (30 mL). After 5 minutes 37% formaldehyde (33.9 mmol, 2.76 g) and diallylamine (12.3 mmol, 4.2 g) was added and the mixture stirred at room temperature for 24 hours. The mixture was poured into 300 mL of deionized water and K_2CO_3 was added until the entire product was precipitated out of solution. The product was removed from the solution by suction filtration and recrystallized with Et_2O/*n*-hexane yielding 2.0 g of pure product.

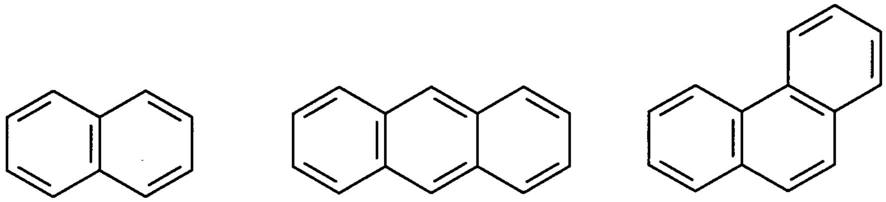
^{1}H NMR: δ 7.09 (s, 2H, ArH), 5.85 (m, 2H, CH=C), 5.15 (m, 4H, $C=CH_2$), 4.3-3.5 (br s, 2H, $ArCH_2Ar$), 3.43 (s, 2H, $ArCH_2N$), 3.03 (d, 4H, NCH_2C).

CHAPTER V

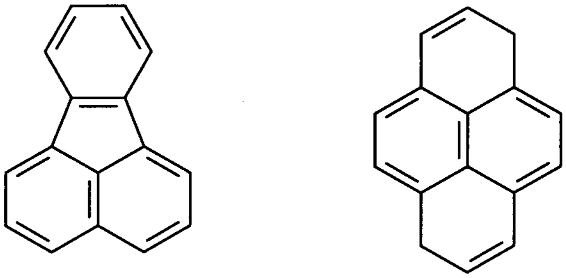
Results and Discussion

Initial Studies with Polycyclic Aromatic Hydrocarbons (PAH's)

A mixture of five PAH's (Figure 9) was separated using different mobile phases. The initial mobile phase composition was a mixture of 73/27 acetonitrile/water and the second was 73/27 acetonitrile/aqueous 0.005M 4-sulfonic calix[6]arene. The main focus of these experiments was to examine the retention characteristics on addition of 4-sulfonic calixarene to the mobile phase by means of fluorescence spectroscopy, which was used for detection, was chosen because of the highly ultraviolet absorptive nature of the calixarene molecules.



Naphthalene Anthracene Phenanthrene



Fluoranthene Pyrene

Figure 9: PAH solutes

The resulting chromatograms shown in Figure 10 and 11 where obtained from the use of mobile phases with and without the 4-sulfonic calix[6]arene additive. The peak at the dead time of approximately 3.4 minutes was from the abrupt change in the refractive index as the sample solvent of the mixture passed through the flow cell.

The order of elution of the solutes is listed in Tables 4 and 5 along with the retention times, arranged in order of increasing time. The increase in retention times roughly correlated with the increase in the number of phenyl rings added to each solute, but was also dependent on their shape. Capacity factors of the solutes in table 6 were calculated from retention times listed in tables 4 and 5. Inspection of the capacity factors in Table 5 and the chromatograms in Figure 10 and 11 revealed what appeared to be a trend towards reduction of the capacity factor for each solute upon the addition of the 4-sulfonic calix[6]arene to the mobile phase. This trend was expected as an indication of host/guest inclusion complexation between the solutes and the calixarene additive.

Statistical single factor analysis of variances (ANOVA) was performed to see if the observed trend was significant. The results of this are located in Table 7. The F values determined from the ANOVA for the solutes naphthalene, phenathrene, and pryene do not surpass the F critical values at a confidence interval of 90%, thus indicating that their capacity factors cannot be said to significantly differ from those when no calixarene additive was used. However, F values did surpass the F critical values for the solutes anthracene and fluoranthene, thus indicating that the capacity factors of the solute in the

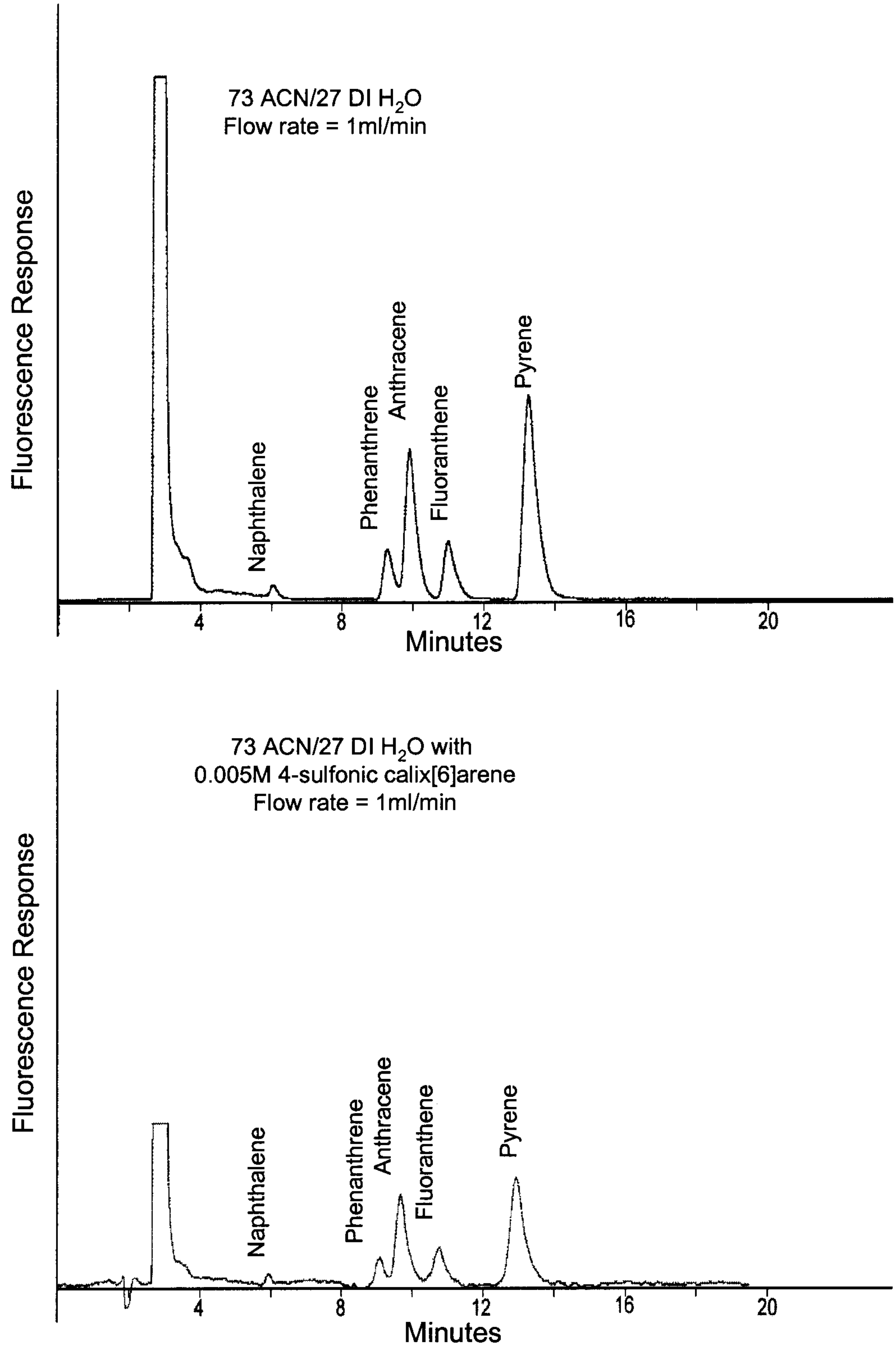


Figure 10 Chromatographs of the separation of PAH's with and without 4-sulfonic calix[6]arene

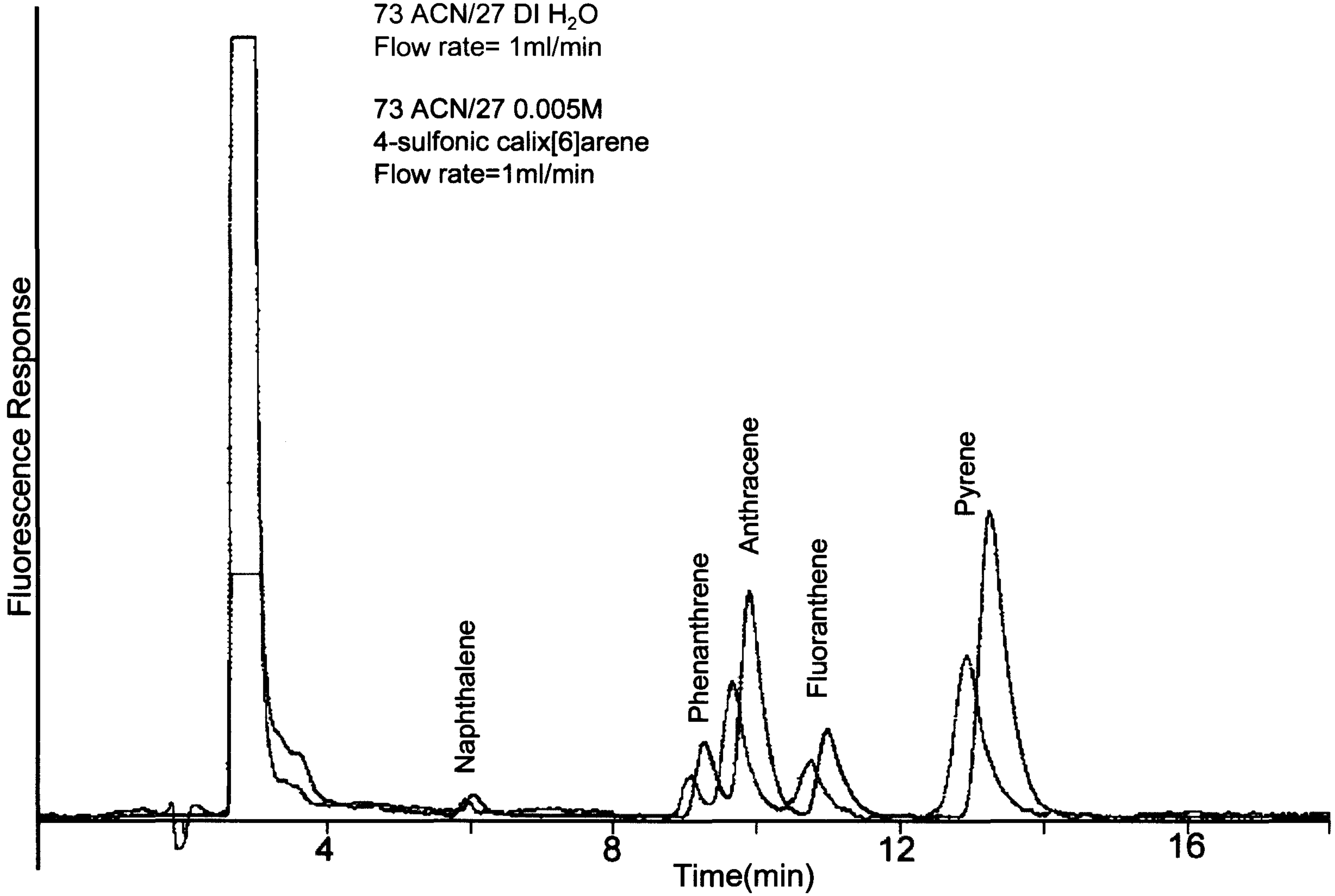


Figure 11:Comparison of Chromatographs with and without 0.005M 4-sulfonic calix[6]arene mobile phase without additives did significantly differ from the capacity factors of those particular solutes without the use of the calixarene mobile phase additive.

Solute	Assay #1	Assay #2	Assay #3	Average Retention time
naphthalene	7.797	7.744	7.788	7.776
phenathrene	12.072	11.886	11.716	11.891
anthracene	12.917	12.689	12.830	12.812
fluoranthene	14.363	14.067	14.126	14.185
pyrene	17.396	16.972	16.703	17.024
Dead Time	3.422	3.429	3.439	3.430

Table 4: Retention times of solutes without the use of 4-sulfonic calix[6]arene as a mobile phase additive

Solute	Assay #1	Assay #2	Assay #3	Average Retention time
naphthalene	7.615	7.700	7.550	7.622
phenathrene	11.678	11.667	11.550	11.632
anthracene	12.424	12.422	12.320	12.389
fluoranthene	13.818	13.782	13.658	13.753
pyrene	16.580	16.563	16.470	16.538
Dead Time	3.452	3.435	3.342	3.410

Table 5: Retention times of solutes with the use of 4-sulfonic calix[6]arene as a mobile phase additive

Compound	k' without calix	k' with calix
naphthalene	1.26	1.23
phenanthrene	2.47	2.41
anthracene	2.73	2.63
fluoranthene	3.13	3.03
pyrene	3.96	3.85

Table 6 Capacity factors of chromatograms without and with the use of 4-sulfonic calix[6]arene

Compound	F values	F critical values (alpha=0.10)
naphthalene	3.540	4.545
phenathrene	1.724	4.545
anthracene	8.664	4.545
fluoranthene	6.124	4.545
pyrene	2.111	4.545

Table 7 F values and F critical values form the single factors ANOVA

Study of Amino-Naphthols and Naphthalene as Analytes

A mixture of amino-naphthols and aminonaphthalene (Figure 12) was separated using two types of organic mobile phase modifiers, both with and without a calixarene additive. The initial mobile phase compositions without the calixarene additive were mixtures of 40/60 acetonitrile/water, and 50/50 methanol/water. The compositions of the mobile phases with the calixarene additive were mixtures of 25/75 acetonitrile/aqueous 0.0005M 4-sulfonic calix[6]arene and 40/60 methanol/aqueous 0.0005M 4-sulfonic calix[6]arene. Again, the main focus of these studies was to examine the qualitative effects, if any, that the addition of 4-sulfonic calixarene had on the capacity factors/retention of the amino-naphthols and 1- aminonaphthalene upon it's addition to the mobile phase in HLPC with fluorescence detection.

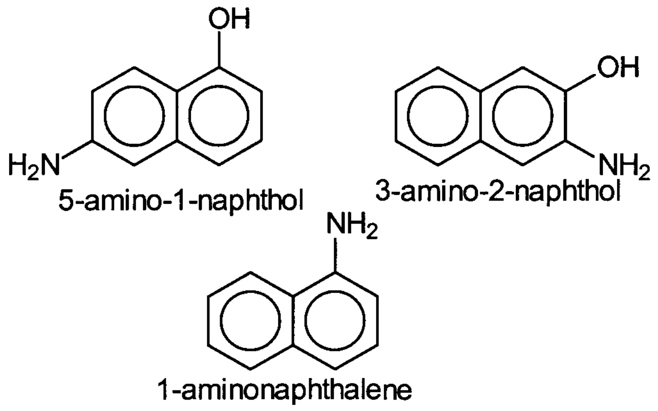


Figure 12: Amino-naphthols and aminonaphthalene Solutes

Figures 13 and 14 are the resulting chromatograms of the separation of 5-amino-1-naphthol, 3-amino-2-naphthol and 1-aminonaphthlaene with and without the addition of 4-sulfonic calix[6]arene to the mobile phase additive. The elution order for all of the chromatograms was 5-amino-1-naphthol, 3-amino-2naphthol,

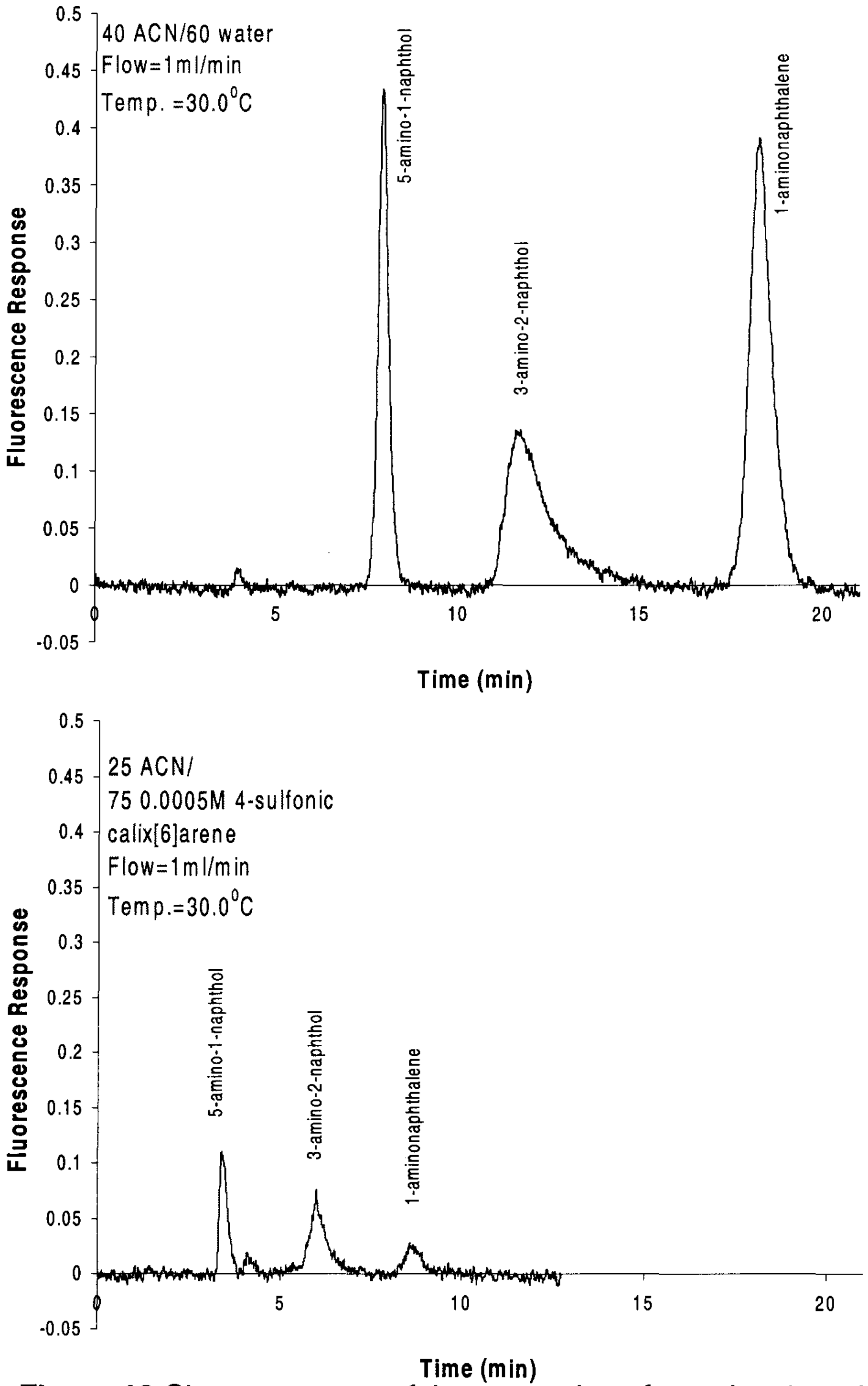


Figure 13 Chromatograms of the separation of 5-amino-1-naphthol, 3-amino-2-naphthol, and 1-aminonaphthalene using acetonitrile as the organic modifier without (above) and with (below) 4-sulfonic calix[6]arene as a mobile phase additive

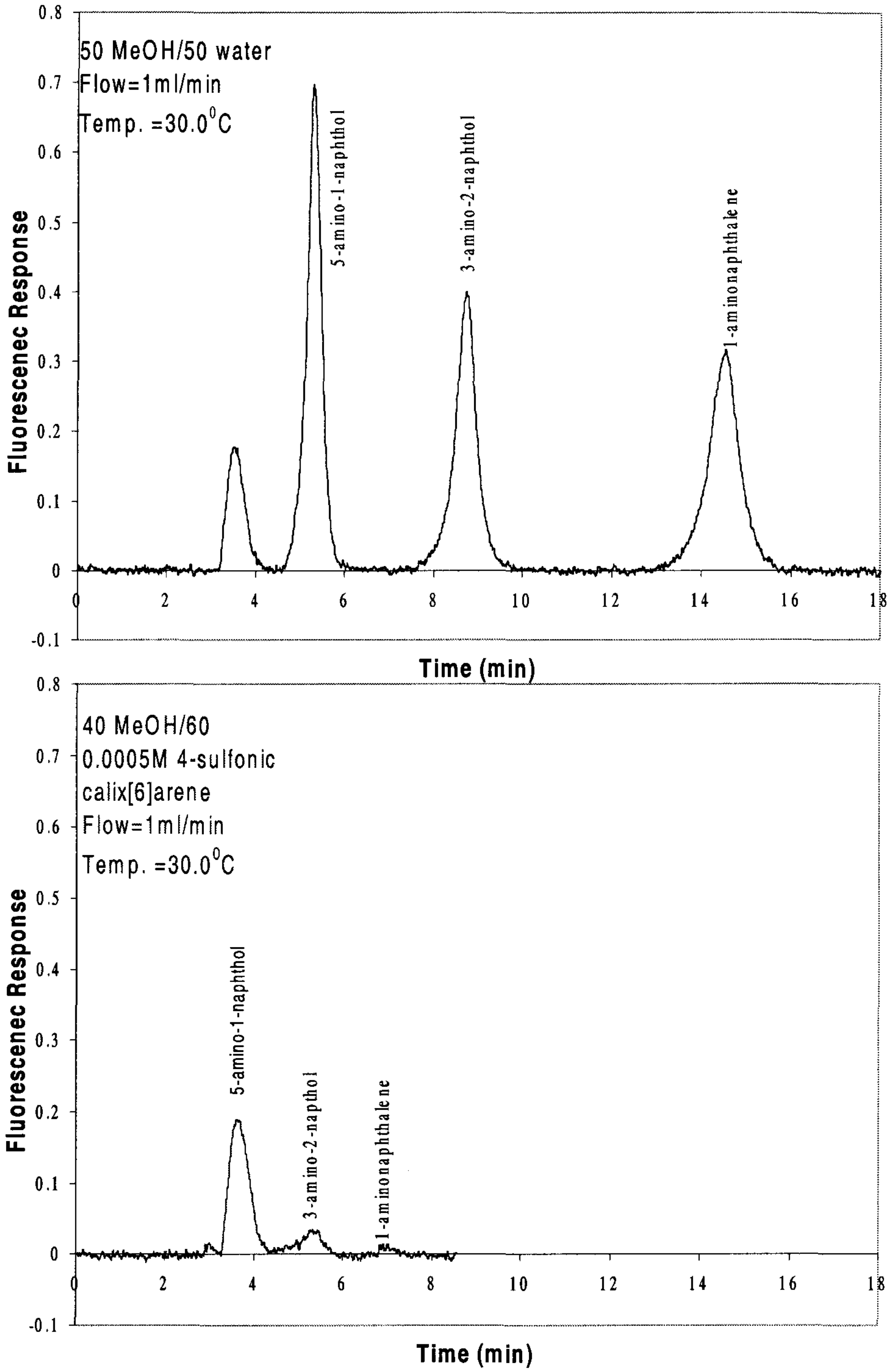


Figure 14 Chromatograms of the separation of 5-amino-1-naphthol, 3-amino-2naphthol, and 1-aminonaphthalene using methanol as the organic modifier without (above) and with(below) 4-sulfonic calix[6]arene as a mobile phase additive

and 1-aminonaphthalene. This corresponded to the decreasing hydrophobicty of each compound.

The hydrophilic (polar) functional groups were "spread out" over the naphthalene base for the 5-amino-naphthol resulting in a more hydrophilic nature when compared to 3-amino-2-naphthol, which had hydrophilic functional groups located at one end only of the naphthalene base. This caused the 3-amino-2-naphthol to have to a hydrophilic and hydrophobic end. In the case of 1-aminonaphthalene there was only one hydrophilic functionality resulting in it being the least hydrophilic solute. The retention times for the assays of each solute are listed in Tables 9 to 11. They were used to calculate the average capacity factors for each solute in the various mobile phases. Visual analysis of both the chromatograms in Figures 13 and 14 and the capacity factors in Tables 12 and 13 revealed a great reduction in the capacity factors of the solutes upon the addition the 4-sulfonic calix[6]arene to the mobile phase.

Compound	Assay #1	Assay #2	Assay #3	Average Retention Time
5-amino-1-naphthol	8.200	8.040	7.920	8.053
3-amino-2-naphthol	12.080	11.850	11.720	11.883
1-aminonaphthalene	18.470	18.270	18.240	18.327
Dead Time	3.970	3.910	3.930	3.937

Table 8: Retention times of solutes with methanol organic modifier and without the use of 4-sulfonic calix[6]aren additive

Compound	Assay #1	Assay #2	Assay #3	Average Retention Time
5-amino-1-naphthol	0.000	0.000	0.000	0.000
3-amino-2-naphthol	5.320	5.280	5.250	5.283
1-aminonaphthalene	7.030	6.920	6.890	6.947
Dead Time	3.000	2.960	2.970	2.977

Table 9: Retention times of solutes with methanol organic modifier and with the use of 4-sulfonic calix[6]arene additive

Compound	Assay #1	Assay #2	Assay #3	Average Retention Time
5-amino-1-naphthol	8.200	8.040	7.920	8.053
3-amino-2-naphthol	12.080	11.850	11.720	11.883
1-aminonaphthalene	18.470	18.270	18.240	18.327
Dead Time	3.970	3.910	3.930	3.937

Table 10: Retention times of solutes with acetonitrile organic modifier and without the use of 4-sulfonic calix[6]arene additive

Compound	Assay #1	Assay #2	Assay #3	Average Retention Time
5-amino-1-naphthol	0.000	0.000	0.000	0.000
3-amino-2-naphthol	5.990	5.940	6.020	5.983
1-aminonaphthalene	8.670	8.670	8.600	8.647
Dead Time	3.450	3.410	3.420	3.427

Table 11: Retention times of solutes with acetonitrile organic modifier and with the use of 4-sulfonic calix[6]arene additive

Compound	K' without Calix	k'with Calix
5-amino-1-naphthol	0.535	0.000
3-amino-2-naphthol	1.571	0.582
1-aminonaphthalene	3.300	1.080

Table 12: Capacity factors of solutes with methanol organic modifier with and without 4-sulfonic calix[6]arene mobile phase additive.

Compound	k' without Calix	k' with Calix
5-amino-1-naphthol	1.046	0.000
3-amino-2-naphthol	2.019	0.746
1-aminonaphthalene	3.655	1.523

Table 13: Capacity factors of solutes with acetonitrile organic modifier with and without 4-sulfonic calix[6]arene mobile phase additive

In Tables, 13 and 14 variances between the capacity factors for when the calixarene was used and was not used have been calculated in terms of F values by means of single factor ANOVA. Comparison of these values against the F critical values at a confidence level of 95% located in tables 13 and 14 confirm that the difference of the capacity factors are greatly significant.

Compound	F values	F critical values (alpha=0.05)
5-amino-1-naphthol	132.145	7.709
3-amino-2-naphthol	42.639	7.709
1-aminonaphthalene	63.422	7.709

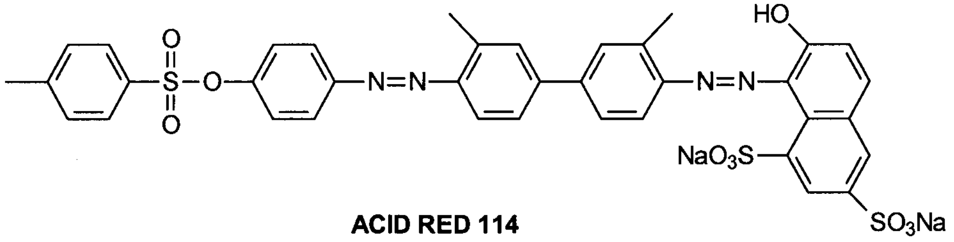
Table 14: Methanol organic mobile phase F values and F critical values from the single factor ANOVA

Compound	F values	F critical values (alpha=0.05)
5-amino-1-naphthol	4589.492	7.709
3-amino-2-naphthol	4093.524	7.709
1-aminonaphthalene	25800.062	7.709

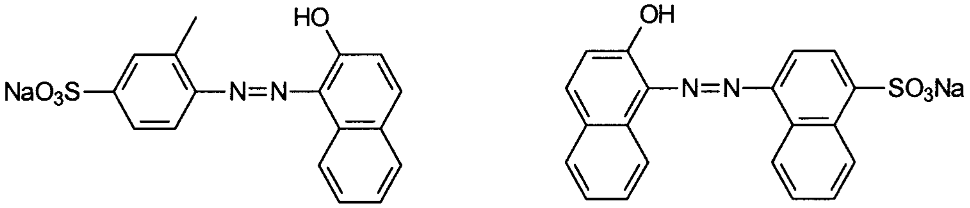
Table 15: Acetonitrile organic mobile phase F values and F critical values from the single factor ANOVA

Preliminary Studies with *p*-(*N,N*-diallylaminomethyl)calix[6]arene as mobile phase Additive

A mixture of four sulfonated azo dyes (Figure 14) was separated using a conventional ion-pairing agent for the comparison with *p*-(*N,N*-diallylaminomethyl)calix[6]arene as mobile phase additive. The initial mobile phase composition of the ion pairing agent mixture was 0.002M-tetrabutyl ammonium hydrogen sulfate in 55/45 acetonitrile/aqueous 1% acetic acid solution. The composition of the mobile phases with the calixarene additive was 0.002M *p*-(*N,N*-diallylaminomethyl)calix[6]arene hydrogen sulfate in 55/45 acetonitrile/aqueous 1% acetic acid solution. Detection was performed by means of visible absorbance at a wavelength of 552 nm. A comparison of the effects of *p*-(*N*,*N*-diallylaminomethyl)calix[6]arene had on the capacity factors/retention of the solutes to that of tetrabutylamonium hydrogen sulfate were made.

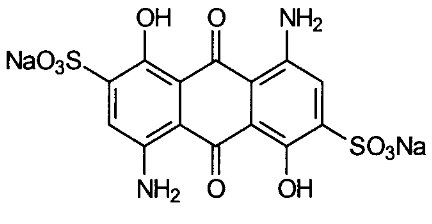


ACID RED 114



ACID RED 8

ACID RED 88



ACID BLUE 45

Figure 15: Sufonated Azo Dye Solutes

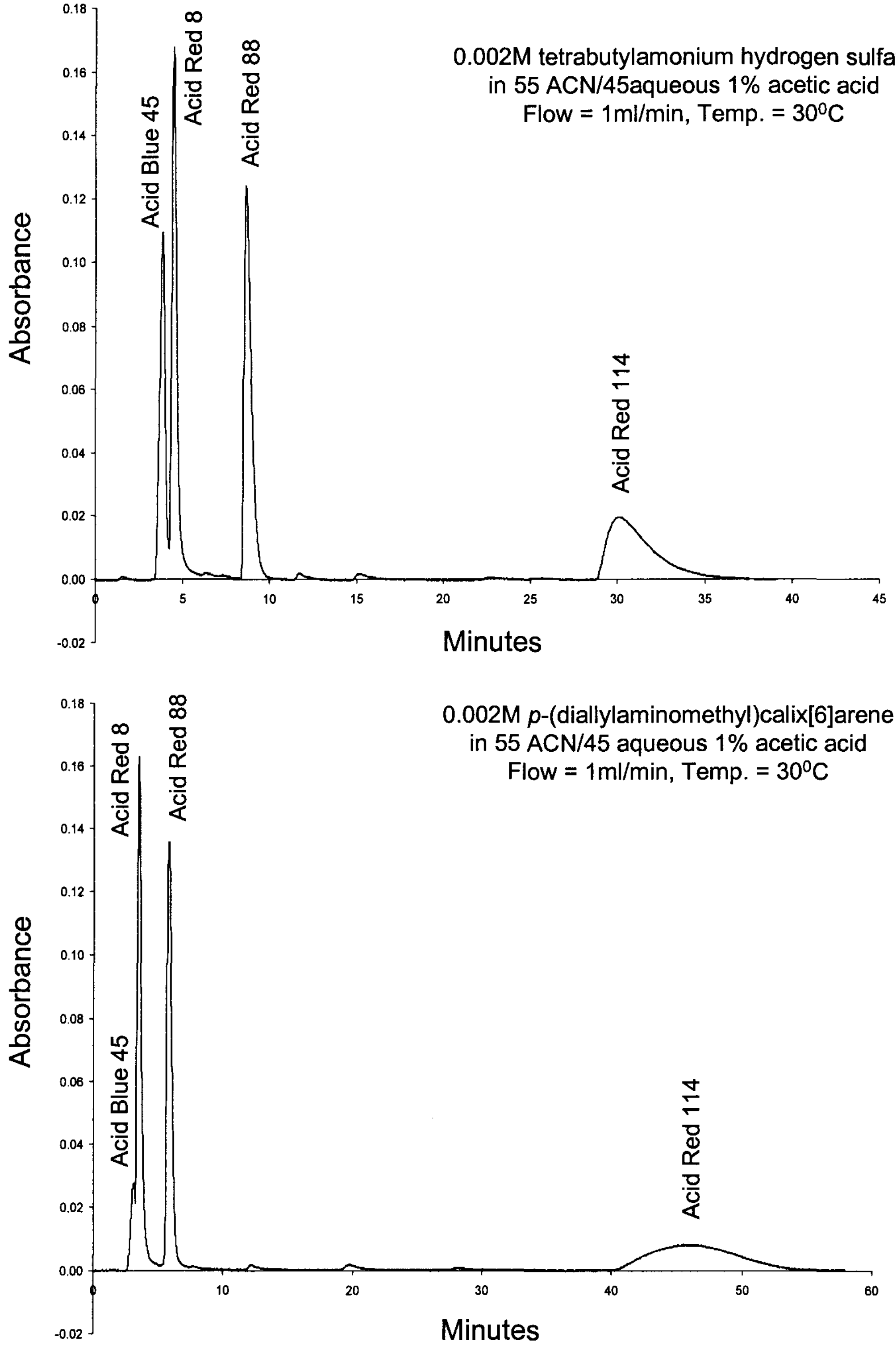
The dead time was marked by the retention of uracil in a column test mixture. In Table 16, the retention times of solutes in their order of elution are listed. The elution order was also determined to not change upon addition of the calixarene additive. The elution order corresponded to the increasing mass of the solutes. Capacity factors (Table 17) for each mobile phase composition were determined from the single assay of a test mixture containing the dyes Acid Blue 45, Acid Red 8, Acid Red 88, and Acid Red 114. Analysis of the capacity factors of the solutes in Table 17 and the chromatograms in Figure 16 show a radical change from the use of tetrabutylammonium hydrogen sulfate to the use of *p*-(*N,N*-diallylaminomethyl) calix[6]arene. Acid blue 45, Acid Red 8, and Acid Red 88 all had decreased capacity factors, while Acid Red 114 had an increase in capacity factor.

Solute	**t_m with ion paring agent**	**t_m with calixarene**
Acid Blue 45	3.85	3.13
Acid Red 8	4.49	3.49
Acid Red 88	8.66	5.80
Acid Red 114	30.15	45.80
Dead Time	2.75	2.75

Table 16 Retention time of solute in the presence of tetrabutylammonium hydrogen sulfate and *p*-(*N,N*-diallyaminomethyl)calix[6]arene

Solute	**k' ion paring agent**	**k' with calixarene**
Acid Blue 45	0.40	0.14
Acid Red 8	0.63	0.27
Acid Red 88	2.15	1.11
Acid Red 114	9.96	15.65

Table 17 Capacity factors of solute in the presence of tetrabutylamonium hydrogen sulfate and *p*-(*N,N*-diallyaminomethyl)calix[6]arene



0.002M tetrabutylamonium hydrogen sulfate
in 55 ACN/45aqueous 1% acetic acid
Flow = 1ml/min, Temp. = 30^0C

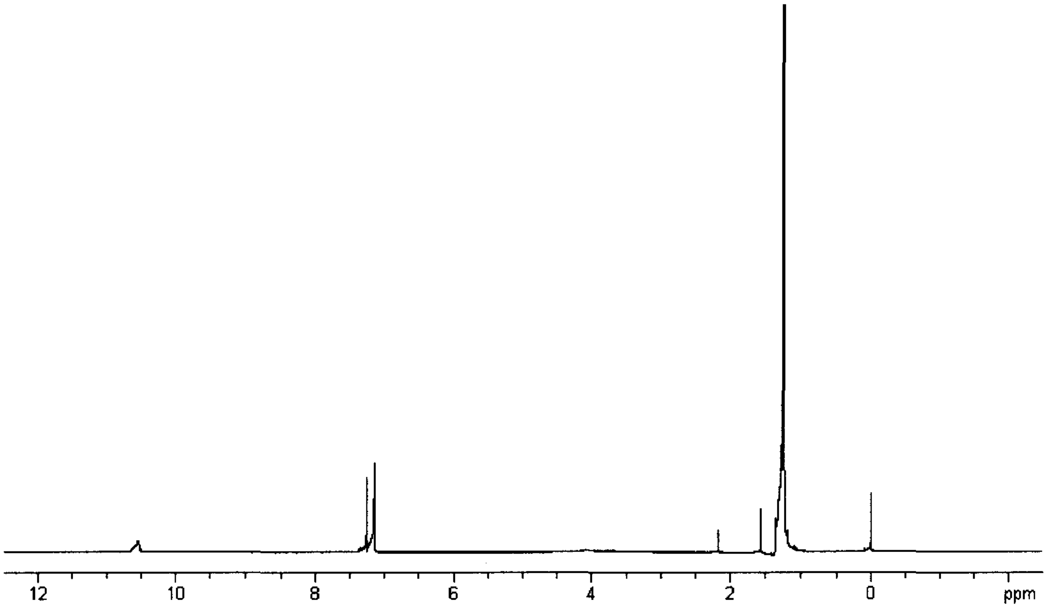
0.002M *p*-(diallylaminomethyl)calix[6]arene
in 55 ACN/45 aqueous 1% acetic acid
Flow = 1ml/min, Temp. = 30^0C

Figure 16: Chromatograms of the separation of sulfonate azo dyes with tetrabutylamonium hydrogen sulfate (above) and *p*-(*N,N*-diallylaminomethyl)calix[6]arene (below)

Synthesis of *p*-tert-butylcalix[6]arene

The Modified Petrolite Procedure described by Gutsche (16) went very smoothly, and no modifications were needed. A yield of around 75% was obtained for the product, which was slightly less than the literature value of 85%. This was easily attributed to the fact this procedure was only performed once. Analysis for purity could not be performed by gas chromatography due to the high melting point of the compound. However, TLC analysis showed the presence of only one spot upon trituration of the product though, and the ^{1}H NMR data coincided with the literature values for this compound (Figure17).

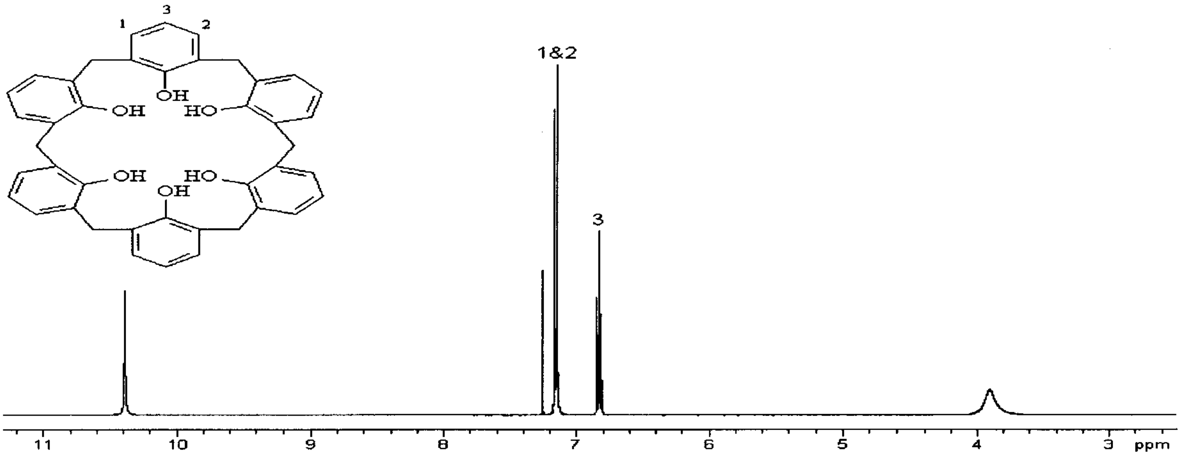
Figure 17: ^{1}H NMR: of *p*-tert-butylcalix[6]arene



Synthesis of calix[6]arene

The dealkylation step described here was also from Gutsche (16). This procedure also went very smoothly, but there was the need to make a minor modification. The modification centered on the product and the toluene forming a slurry. Upon addition of the final reaction mixture to the ice water to quench it, the product remained in the organic layer as a slurry, but byproducts of the $AlCl_3$ were retained with the product in the organic phase yielding an impure product. This was avoided by removal of the toluene by evaporation, after separation from the aqueous layer. The resulting residue was dissolved in chloroform and washed with three aliquots of water, successively. This modification of the procedure resulted in a yield of 70% which was about ten percent lower than the literature value. Analysis by TLC showed a single product, and ^{1}H NMR showed the presence of only minor amounts of impurities. The ^{1}H NMR spectrum (Figure 17) showed some deviation from the reported spectrum. A multiplet was reported at 6.7-7.4 ppm; this was not obtained. A doublet at 7.15 (1) was obtained for the protons at positions 1 and 2, and a doublet of doublets at 6.83 (2 H) was obtained for the proton at position 3 (Figure 18). This was a mostly likely the result of increased resolution from the use of a higher field NMR than the one used to determine the spectrum in the literature.

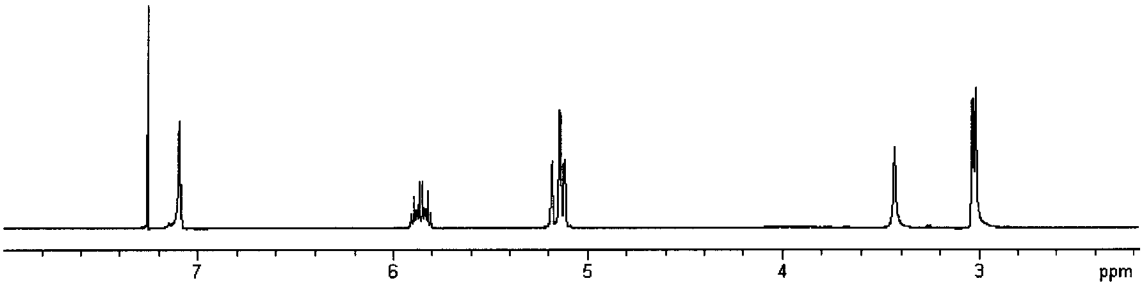
Figure 18^{1}HNMR: of calix[6]arene



Synthesis of *p*-(*N,N*-Diallylaminomethyl)calix[6]arene

Following the route described by Gutsche (3), the synthesis went very smoothly and no modifications were needed. Yields of around 50% were obtained for the product. This was slightly less than the literature value of 57%. This is easily attributed to the fact that the literature value is the best out of many reaction trials. Again, analysis for the purity could not be performed by gas chromatography due to the high melting point of the compound. TLC also could not be performed for this product. Because no solvent could be found to elute the product up the TLC plate. The ^{1}H NMR spectrum agreed with the literature spectrum (Figure 19).

Figure 19: ^{1}H NMR: of *p*-(*N,N*-diallylaminomethyl)calix[6]arene



CHAPTER VII

Conclusion

HPLC Analyses

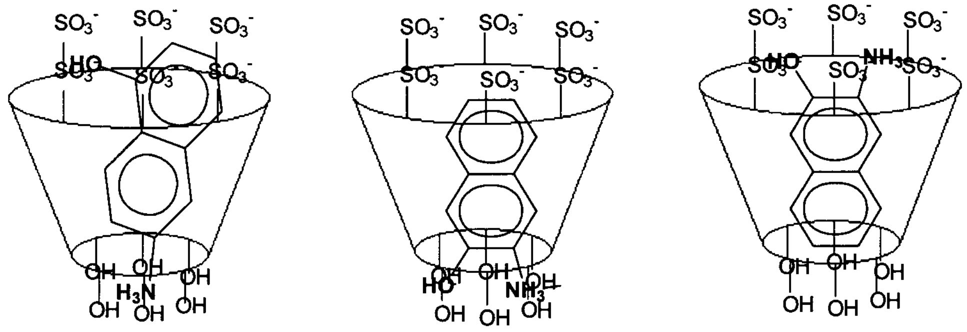
In the studies involving the PAH's the variation of the capacity factors was not reflective of the host/guest complexation that was believed to take place as in work done by Gutsche (3) with PAH's and *p*-(2-carboxyethyl)calixarenes. The lack of significant host/guest complexation may have been the result of the more highly polar sulfonated calixarene.

The reason for the variance between the solute statistics was most likely the result of the inconsistent temperature of the column. However, the possibility of it being the result of a real difference in the formation of host/guest complexes of the solutes with the calixarene additive may also exist.

Even though statistical evidence of a host/guest complexation was not evident with all of the solutes, a qualitative variance between the capacity factors and chromatographs can be seen upon visual inspection of the chromatograms in Figures 10 and 11 (chapter 6) and capacity factors in Table 6 (chapter 6). In addition to signs of host/guest complexation between the solutes and calixarenes, the use fluorescence spectroscopy proved to be a method of detection that could be used with little interference from the calixarene additive. This gave respectable detection limits as seen from the chromatographs in Figure 10 and 11 (chapter 6).

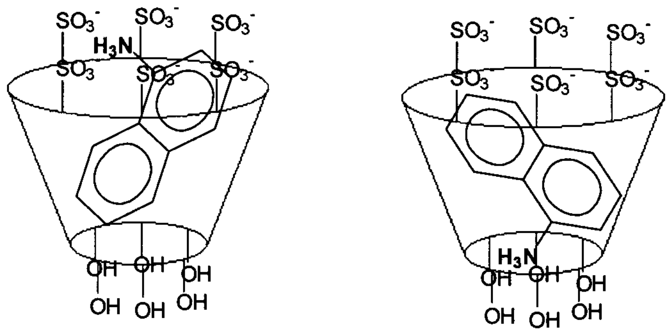
A study in the future may be beneficial to investigate whether or not the lack of a consistent column temperature was the reason for the variance between solutes. This could easily be achieved be redoing the assays using a column oven to control the column temperature at 30°C. In addition to the following a series of studies using *p*-(*N,N*-diallylaminomethyl)calix[6]arene and *p*-(2-carboxyethyl)calix[6]arene may also be beneficial.

From the studies dealing with the amino-naphthol and 1-aminonaphthalene there were three significant signs of the formation of host/gust inclusion complexes between these three solutes and the calixarene additive. The first was the necessity of increasing the aqueous portion of the mobile phases upon the addition of the 4-sulfonic calix[6]arene additive in order to obtain the retention of solutes. In addition to that, retention of only two of the solutes, 3-amino-2-naphthol and 1-aminonaphthalene, with both acetonitrile and methanol was obtained. The retention of 5-amino-1-napthol with either organic modifier was unobtainable due to the strong nature of the host/guest complex formed. The strength of this complex was directly related to the structure of 5-amino-1-naphthol and how it fit into the cavity of the calixarene. The complex envisioned with the calixarene and 5-amino-1-naphthol is depicted in Figure 19. The amino functionality associated itself with the hydroxyl functionalities at the bottom of the calixarene's cavity, and the hydroxyl functionalities formed H-bonds with the sulfonic functionality of the calixarene. The envisioned complexes of 3-amino-2-naphthol and 1-aminonaphthalene are depicted in Figure 19. The complexes of these solutes, as with 5-amino-1-naphthol, involved the H-bonding of the amino and hydroxyl functionalities to the sulfonic or hydroxyl functionalities of the calixarene with the conjugated phenol structure located in the hydrophobic cavity of the calixarene. The complexes of 3 amino-2-naphthol and 1-aminonaphthalene did not appear to be as strong as the complex of 5-amino-1-naphthol with the calixarene. This could be due to 5-amino-1-naphthol forming a bond at the top of the cup and the bottom trapping it in the cavity where with the solutes can form H-bonds at only the top or bottom of the cavity. This would result in weaker complexes.



5-amino-1-naphthol

3-amino-2-naphthol



1-aminonaphthalene

Figure 20: Predicted Host/Guest Complexes

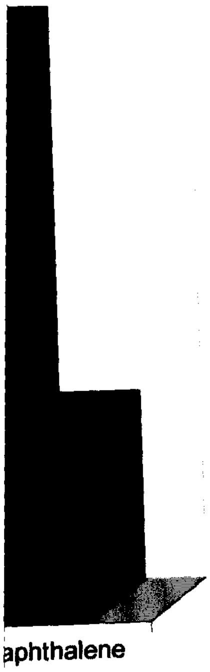
The drastic reduction of the fluorescence response seen in the chromatograms when 4-sulfonic calix[6]arene was added to the mobile phase was another significant sign of the formation of host/guest complexes. This was explained as the result of the calixarene shielding the solutes from the excitation

while they were located within the cavity of the calixarene (figure 19).
karene, being a strong ultraviolet absorber, absorbs the excitation and/or
ı energy before it can reach the solute or detector.

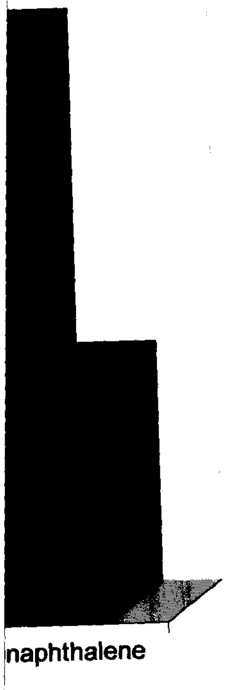
ıe third significant sign of the formation of host/guest complexes, and the
ortant, was the major reduction in capacity factors of the solutes when
onic calixarene mobile phase additive was used. This was seen in the
grams (Figures 12 and 13 chapter 6) and is demonstrated in the
depiction of the capacity factors in Figures 20 and 21 (chapter 6).

ne of the additional studies that need to be performed with *p*-(*N,N*-
omethyl) calix[6]arene and sulfonated azo dye are concentration
here the effects of the concentration of 4-sulfonic calixarene on the
f the solutes is investigated. Studies investigating the fluorescence
eduction when the calixarene is used need to be performed. These
reveal the nature of the host/guest complex that is forming.

preliminary study of the use of *p*-(*N,N*-diallylaminomethyl)
e as an additive in the mobile phase with the sulfonated azo dye
revealed that in the case of the first three solutes in Table15 (chapter
/guest complex was not readily formed because of the shape or
ıe solutes, and/or the host/guest complexes formed were not as
as the complexes formed with tetrabutylammonium hydrogen
complexes formed might have been similar to those postulated for
ohthols and aminonapthalene, where the solutes were envisioned to
resulting in an affinity for the mobile phase. Acid 114 had a



and with calixarene



) and with calixarene