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Assessment of Energetic Heterogeneity of Reversed-Phase Surfaces Using Excess Adsorption Isotherms for HPLC Column Characterization

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Assessment of Energetic Heterogeneity of Reversed-Phase Surfaces Using Excess Adsorption Isotherms for HPLC Column Characterization

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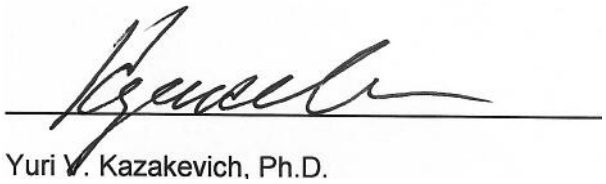
We certify that we have read this dissertation and that, in our opinion, it is adequate in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

Approved



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Abstract

This research is to explore a more general column categorization method using the test attributes in alignment with the common mobile phase components. As we know, the primary driving force for solute retention on a reversed-phase surface is hydrophobic interaction, thus hydrophobicity of the column will directly affect the analyte retention. This research describes a method to determine the column hydrophobicity by the ratio of adsorbed acetonitrile and methanol to water on the column surface using excess adsorption isotherm estimation. An excess adsorption isotherm for a binary mobile phase system represents a competitive interaction of both solvent components with the adsorption sites. In the presence of two distinct types of adsorption sites on the surface, an overall isotherm may be represented as a superposition of two isotherms on the different types of surfaces. Assuming complete independence of surface energy on each type of adsorption site, it is possible to mathematically describe this superposition as a sum of two independent isotherms, where coefficient of each individual term represents a relative amount of surface that is responsible for a particular interaction.

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Abstract

In the past thirty years, High Performance Liquid Chromatography (HPLC) has been widely accepted as one of the major analytical tool in the environmental, pharmaceutical, polymer and food industries. The majority of the recently developed test methods applied Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) techniques. Hundreds of different kinds of reversed-phase columns are also commercially available. Despite the benefit from a large number of column choices, it also leads to difficulties in column selection. Common column categorization methods are usually performed by gathering information from the retention factors of some arbitrarily selected standard solute compounds. Each solute is associated with a specific column property, such as hydrophobicity, silanol activity and molecular shape discrimination. Essentially, these chromatographic methods rely on the selectivity of a pre-selected set of analytes under a pre-selected mobile phase system, therefore are subjective and lack of generality.

The main goal of this research is to explore a more general column categorization method using the test attributes in alignment with the common mobile phase components. As we know, the primary driving force for solute retention on a reversed-phase surface is hydrophobic interaction, thus hydrophobicity of the column will directly affect the analyte retention. This research describes a method to determine the column hydrophobicity by the ratio of adsorbed acetonitrile and methanol to water on the column surface using excess adsorption isotherm estimation. An excess adsorption isotherm for a binary mobile phase system represents a competitive interaction of both solvent components with the adsorption sites. In the presence of two distinct types of adsorption sites on the surface, an overall isotherm may be

represented as a superposition of two isotherms on the different types of surfaces. Assuming complete independence of surface energy on each type of adsorption site, it is possible to mathematically describe this superposition as a sum of two independent isotherms, where coefficient of each individual term represents a relative amount of surface that is responsible for a particular interaction. The test method has been verified with four custom made alkyl bonded columns and four other types of commercially available columns.

Fundamentally, complete demonstration of a chromatographic retention process need to be supported by thermodynamic assessment. In general, HPLC retention factor k' can be related to the thermodynamic equilibrium constant of the system by $k' = \emptyset K$, where $\emptyset = V_S/v_m$ stands for phase ratio of stationary phase volume (V_S) to mobile phase volume (v_m). The Gibbs free energy (ΔG) for the chromatographic system can be then calculated using the Arrhenius correlation $K = e^{-\Delta G/RT}$. The arrived problem is how to define the boundary of the stationary phase and how to determine its volume. In this research, we applied a combined partition and adsorption model where the analyte molecules are partitioned between the mobile phase and an adsorbed layer of solvents with same components but different composition to the bulk mobile phase. This adsorbed solvent layer is taken as the stationary phase.

Acknowledgements

I would like to give my greatest thanks to my research mentor Dr. Yuri Kazakevich for his guidance, encouragement, patience and unwavering support throughout my graduate studies. I am sure that what I have learned from him has been invaluably advancing my knowledge in chromatography and certainly beneficial to my career.

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Dissertation Structure

The research described within this thesis contains the assessment of energetic heterogeneity of reversed-phase surfaces using excess adsorption and its application for HPLC column characterization.

High performance (or high pressure) liquid chromatography (HPLC) is one of the major separation technique for many chemical analysis fields such as environmental, polymer, pharmaceutical and food processing. Rapid expanding of its applications inspired the development of explosive variety of stationary phases which lead to difficult in analytical column selection. Common column characterization methods categorize the columns by gathering information from the retention factors of some arbitrarily selected standard solute compounds. Essentially, these chromatographic methods rely on the selectivity of a pre-selected set of analytes under a pre-selected mobile phase system, thus are subjective and lack of generality. The studies described in this research suggest a more general column characterization method by using excess adsorption model with common HPLC mobile phase solvents.

Section 1 of the study shows the history of chromatography and current approaches undertaken to study the retention behavior and characterization of different types of stationary phases. Most of the popular reversed-phase high performance liquid chromatographic columns usually contain a stationary phase with non-polar ligands bonded on silica surface. General retention models and column characterization methods are discussed in this section.

Section 2 of the study introduces a new method to characterize reversed-phase HPLC columns according to their hydrophobicity, represented by the ratio of non-polar and polar solvents adsorbed on the surface. This ratio is estimated from the excess

adsorption isotherm. Detailed theoretical approaches and experiment results are discussed in this section. Test results are also compared to the legacy test methods using alkylbenzene homologous.

Section 3 of the study cover the estimation of chromatographic Gibbs free energy using excess adsorption isotherm for reversed-phase high performance liquid chromatography. Fundamentally, complete demonstration of a chromatographic retention process need to be supported by thermodynamic assessment. Common retention models often come across difficulty in stationary phase volume determination, thus lead to problem in thermodynamic parameter calculation. By applying the excess adsorption interpretation on a partition-adsorption chromatographic model, we can avoid the trap of stationary phase volume determination.

Chapter 1:

Introduction

1.1. History of Chromatography

Chromatographic adsorption method was first proposed by a Russian botanist Mikhail Semenovich Tswett at the Warsaw Society of Natural Sciences in 1903 [1]. He published two papers in 1906 [2] and discovered that if a solution contains a mixture of colored solutes is allowed to pass through a glass tube filled with powdered adsorbing material, the solutes will adsorb on the powder and separate into a series of colored segment bands. He called these bands a chromatogram and the separation method chromatography. 20 years later, a very important adsorbing material, silica gel was brought into the chromatographic world by Holmes and Anderson [3]. Since then, chromatography has been widely used in industries such as environmental, flavor, fragrance, pharmaceutical, petroleum, polymer and quickly expanded to be one of the most widely used analytical technique. In the 1940's, two major advancements in chromatographic theory was introduced. Wilson and DeVault proposed their mass balance equations in 1941 [4] and 1943 [5] based on the mass-balance for the rectification process. At the same time, Martin and Synge carried out a large number of research and published a paper in partition chromatography [6] which finally led to a Nobel Prize award in 1952. In their research, a concept of theoretical plates was proposed in analogy with distillation where they neglected the solute diffusion from one plate to another plate. The theory assumed that the chromatographic column is divided into a number of zones called theoretical plates. Solutes are in equilibrium between the

gas and liquid phases within each plate. The efficiency of the solutes separation is dependent on the number of theoretical plates of the column and expressed as the height equivalent to a theoretical plate (HETP). Based on this concept, the numerical Van Deemter equation [7] for gas chromatographic pack column and Golay equation [8] for capillary open tubular column were developed in 1956 and 1958, respectively.
In the chromatography history, the beginning 50 years of development was the era of gas chromatography. Until the 1960s, Giddings started using high pressure liquid chromatography (HPLC) with small particle size silica. A major development in liquid chromatography was proceeded by Horvath in the 1960s and 1970s [9] [10] [11]. The majority of the HPLC columns are based on silica. Almost all silica-based HPLC packing materials are very uniform spherical porous particles with narrow particle and pore size distributions. Silica gel possesses many particular properties that makes it an excellent packing material. (1) It provides high mechanical strength to withstand high pressure. (2) Its chemically active surface can be easily modified. (3) It can be manufactured with controllable particle diameter, pore size and surface area.

Recently, small particle size partially porous columns and monolithic columns were also available to improve column efficiency. The partially porous column is specially designed to provide very high column efficiency [12]. It is made with a solid core and covered with a thin porous shell which allow high mobile phase flow rate for fast separations. In contrast to conventional HPLC columns, monolithic columns are formed from a single piece of porous silica gel [13] [14]. It can be considered as a single large particle that fills the entire column without any inter-particle voids. Since the stream of mobile phase do not bypass any significant length of the bed but just percolate through it, the resulting column back-pressure is therefore much lower and allow high mobile phase velocity.

No doubt, the major breakthrough was the invention of a chemically modified surface of small diameter silica particles (3 to 10 um). Today, numerous bonded phases from traditional alkyl chains to ion exchange and chiral surfaces are widely used. Moreover, the chromatographic technique has been diversified to many modern types such as supercritical fluid chromatography (SFC), capillary zone electrophoresis CZE), capillary electrochromatography and tandem with other spectroscopic equipment such as mass spectrometer (LC-MS) and nuclear magnetic resonance (LC-NMR).

1.2. Current High Performance Liquid Chromatographic Technology

Today, high performance (or high pressure) liquid chromatography (HPLC) has been widely accepted as one of the key analytical technique in many fields including environmental, polymer, pharmaceutical and food industry due to its uncomplicate instrumentation and easy to handle. Especially in pharmaceutical industry, majority of the assay, degradation products and other related impurities are determined by HPLC methods. In fact, some of the pharmaceutical active ingredients are even manufactured by large scale preparative liquid chromatography. In these analyses, a complex sample containing multi-components is continuously pumped through a column filled with adsorbents (or absorbents) called stationary phase by a stream of solvent called mobile phase under high pressure. Separation is achieved by selectively retaining the sample components according to their relative strength of interaction with the stationary phase and mobile phase. This interaction determines the time length that the compound will retain in the column. Generally, HPLC may be further categorized by its separation mode as follows:

- Normal-Phase Liquid Chromatography (NPLC) uses a non-polar mobile phase to elute solutes that are retained by a hydrophilic stationary phase. Common organic solvents including hexane, heptane, octane, chloroform, tetrahydrofuran, methanol and acetonitrile are used in this mode.
- Reversed-Phase Liquid Chromatography (RPLC), in contrast, uses a polar mobile phase to elute solutes that are retained by a hydrophobic stationary phase. The term reversed-phase was named after the normal phase as an opposite mode. Common solvents in the mobile phase of this mode are water, acetonitrile, methanol and tetrahydrofuran.
- Ion Exchange Chromatography (IEC) achieves separation by means of the ionic interaction among ionized analytes and charged stationary phase. In practical, Ion Exchange Chromatography is further categorized into cationic and anionic ion exchange modes.
- Ion Pairing Chromatography (IPC) applies a layer of dynamically coated ionic fatty acid salt on a hydrophobic stationary phase where the ionic head can provide ion exchange action. Hence ion pairing chromatography is also called dynamic ion exchange chromatography. Alternatively, Ion Pairing Chromatography may be also viewed as the formation of ion-pairs between the ionic analyte and ion pairing agent in the mobile phase, thus changes the retention due to the introduction of a secondary analyte equilibria in the system (i.e. changes to a more non-polar ion pair).
- Size Exclusion Chromatography (SEC) separates molecules according to their physical dimension. Larger molecules will be eluted faster than the small molecules due to exclusion from entering into the small pores.
- Chiral chromatography separate enantiomers with chiral selective stationary phase or mobile phase.

Among these various modes of separation, reversed-phase liquid chromatography is far more popular than the others. Hundreds of different kinds of reversed-phase columns are commercially available, covering from narrow pore (6-15 nm) for small molecules analyses to wide-pore (30 - 40 nm) for large molecule biopolymers analyses. In addition, various types of bonded phases have been developed including the popular alkyl types such as butyl (C_4), octyl (C_8), octadecyl (C_{18}), phenyl, cyano, amino and the lately developed polar embedded columns. Consequently, almost any sample of organic mixtures may be separated by reversed-phase liquid chromatography.

Despite of the advantage from advance technology, the wide spreading reversed-phase high performance liquid chromatographic application inspired intensive stationary phases development during the past thirty years. This in turn, made column selection a serious problem. Usually, venders only provide limited information using the test results from their own test methods. At the academic side, development of methods for column classification has been carried out since mid-70's. Today, large number of chromatographic methods have been published to help in column selection. Details of these methods are described in Section 1.6.3. These methods generally categorize the columns by gathering information from the retention factors of some arbitrarily selected standard solute compounds. Each solute is associated with a specific column property, such as hydrophobicity, silanol activity and molecular shape discrimination. However, association of a solute to the specific column property is usually a voluntary decision of the method author and rarely supported by any physico-chemical verification. Essentially, these chromatographic methods are relying on the selectivity of a pre-selected set of analytes under a pre-selected mobile phase system, thus are subjective

and lack of generality. In order to establish a more objective way for comparison, a more general column characterization method is needed for column screening.

As we know, the primary driving force for solute retention on a reversed-phase surface is the non-specific hydrophobic interaction. In general, hydrophobicity of a surface can be defined as the strength of water repellence by the surface. Based on the concept of like attracts like, a surface with higher hydrophobicity will exert stronger attraction force to hydrophobic materials and stronger repulsion to water. General column properties such as the type of bonded phase, bonded ligand functional group, bond density, adsorbent surface area, surface coverage and surface end capping directly impact the strength of hydrophobic interaction. As a fact, hydrophobicity becomes an important parameter for preliminary screening of reversed-phase columns. Additional characteristics such as polar interaction, π- π interaction and Other specific molecular attractions can be then added for further categorization.

Currently, several methods have been developed to determine the column hydrophobicity. Walters [15], Tanaka [16] and Engelhardt [17] expressed column hydrophobicity in terms of their selectivity to a homologue of benzene derivatives. Carr characterize columns by hydrophobic subtraction [18]. Abraham and Snyder used Linear Solvation Energy Relationships (LSER) model. Again, all these methods rely on a set of subjectively selected test solutes and mobile phases combination. In order to categorize the columns in a more general way, we proposed a method to determine column hydrophobicity by the ratio of adsorbed organic solvent to water on the column surface using excess adsorption isotherm estimation.

1.3. Structure of Stationary Phase

1.3.1. Silica Substrate

Although compounds are separated by relative affinity to both stationary phase and mobile phase in chromatography, the stationary phase often plays a major role in selectivity. One of the most important stationary phase material used in chromatography is silica.

In the past five decades, Silica (SiO_2) has been the major backbone for chromatographic column supporting material. Most of the bonded phase columns are built on silica substrate. Although many other supporting materials have also been developed, silica continues to be the most common choice due to its good mechanical stability, easy particle size and porosity control [19]. Furthermore, the surface chemistry of silica allows a large variety of functional groups to covalently bond on its surface at high coverage.

Despite the success, persistent problems still exist, especially in the analysis of basic compounds. Undesirable chromatographic effects such as peak asymmetry, low column efficiency, limited pH stability and poor reproducibility are generally attributed to the energetic heterogeneity of the surface due to co-existing of strong and weak adsorption sites. This unfavorable strong adsorption sites are usually unbonded free silanol groups on the surface. Several methods have been applied to suppress this residual silanol activity.

- A short chain silane such as trimethyl silane is often used to endcap or mask the free silanols.
- Bond the silica surface with alkyl ligands containing bulky side groups, e.g. isopropyl or isobutyl alkanes.
- Synthesize a bridged hybrid silica surface by poly-condensation of tetraethoxysilane (TEOS) with 1,2 bis(triethoxylyl)ethane (BTEE)

1.3.1.1. Types of Silica Substrate

Three types of silica are commonly used for chromatographic stationary phase preparation. **Figure 1.1** shows a 2-dimensional skeleton Structure of Type A and Type B Silica. The oxygen atoms at the left of Si atoms are attached to the bulk silica. **Figure 1.2** shows the amorphous structure of the Silica.

So-called Type A silica with lower concentration of silanol groups are sol- gels made by aggregating silica-sol particles. This type of silica gel contains higher amount of impurity metal oxides at 1000 – 3000 ppm, mainly Na, Ca, Al, Mg, Ti, Ni, Fe. It has been suggested that the indirect influence of the matrix incorporated metal impurities on adjacent silanol groups will also enhance the silanol acidity. Type B silica is a high purity silica prepared with a highly-hydroxylated surface containing low concentration of impurities (< 35 ppm of metal ions) [19] [20]. As shown in the figures, both type A and Type B silica surface are covered with a large number of silanol functional groups (Si-OH) [21] [22]. The high reactivity of these silanol groups enable it to bond with alkylsilanes which are the basis to generate the reversed-phase surface. Common silica gels used in chromatography are amorphous, non-crystalline materials which do not produce X-ray diffraction pattern.

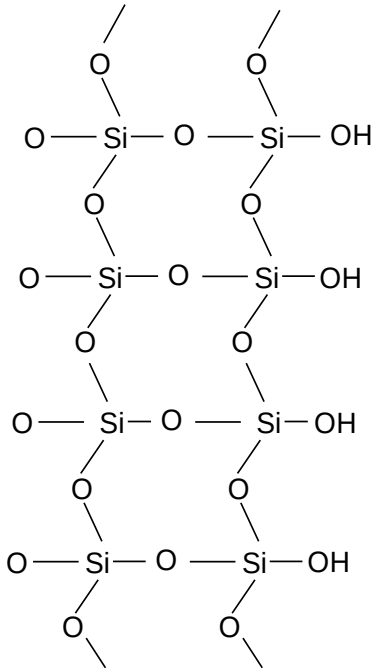


Figure 1.1 *2-dimensional structure of type A and type B silica*

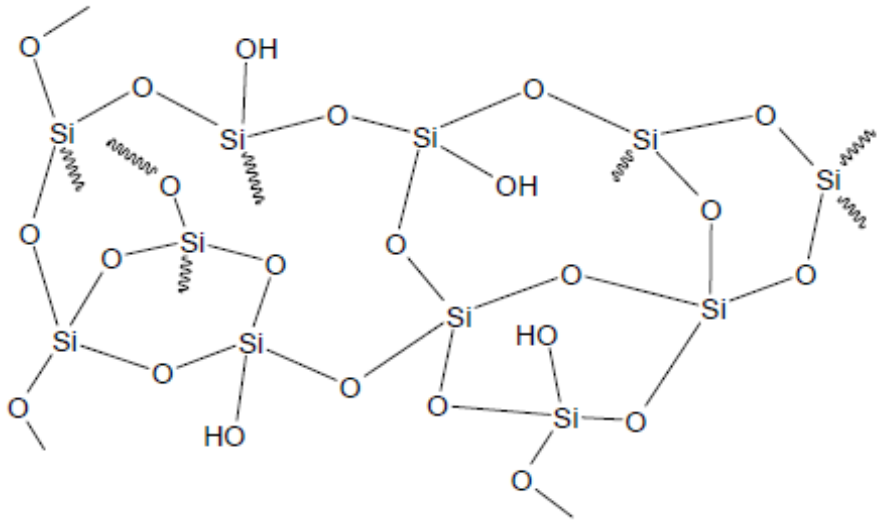


Figure 1.2. *Amorphous structure of silica*

1.3.1.2 Synthetic Process of Silica Substrate

Common silica used in HPLC columns is an amorphous, porous solid which can be synthesized by the following sol-gel methods.

1.3.1.2.1. Xerolgel Formation [23] [24]

Silica gel is synthesized by releasing silicic acid ($Si(OH)_4$) from a strong solution of sodium silicate, with hydrochloric acid as shown in the equation below. The free acid is then polymerized to a colloidal solution called silica-sol and condensed to form soft hydrogel. After being washed and dried at about 120ºC for few hours, a hard, amorphous mass Xerogel is formed. The product prepared in this way is called irregular silica gel, to differentiate it from spherical silica gel. The mass is then ground and sieved. Irregular Xerolgel usually has higher porosity and hence higher specific surface area. It also contains irregular wall thickness and pore shapes.

$$Na_2SiO_3 + H_2O + 2HCl \rightarrow Si(OH)_4 + 2NaCl$$

If the silica-sol is sprayed into fine droplets and dried in a stream of hot air before gelling, small spherical particles can be obtained. This process is known as the spray dry method. Alternatively, the spherical particles may be obtained by dispersing the silica-sol in an organic solvent in the form of emulsion. These particles are then dried at 400 ºC to 800 ºC to obtain sol-gel. This type of silica gel usually contains more uniform pores but with lower porosity and specific surface area.

1.3.1.2.2. Silica Hybrid

Figure 1.3 shows the reaction for *bridged hybrid silica* formation. Recently, hybrid silica containing organic bridge is also introduced for chromatographic supporting material. One of these synthetic processes is carried out by polycondensation of 1,2-

bis(triethoxysilyl)ethane (BTEE) with tetraethoxysilane (TEOS). The resulting bis-polyethoxysilane (BPEOS) silica shows better pH stability because the Si-C bonds are less prone to hydrolysis than Si-O-Si bonds [25].

1.3.1.3. Silanols on Silica Surface

The surface of amorphous silica with a porous structure is composed with highly polar silanol groups and non-polar siloxane bridges. Three kinds of silanols and one kind of siloxane are usually present on the silica surface as shown in **Figure 1.4**.

(i) Single silanol:

The major portion of the surface is covered with isolated single silanols. This kind of silanol contains one hydroxyl group and has the other three bonds attached into the bulk structure.

(ii) Vicinal silanol:

Vicinal silanols are formed by hydrogen bond between adjacent silanols.

(iii) Geminal silanol:

Some silicon atoms on the surface are silane diols containing two hydroxyl groups. They are termed geminal silanol.

(iv) Siloxane:

The calcination process at high temperature (800ºC) can remove water molecules among adjacent silanol groups, resulted in forming a hydrophobic siloxane bridge.

Silanols are usually acidic in nature with pKa values at about 3 to 4. They are active in nature and play two important roles in chromatography. (1) As a high energy hydrophilic site, it can interact with polar solutes, hence provide retention to these

solutes in normal phase liquid chromatography (NPLC). (2) Due to its high reactivity, silanol groups can be bonded with various types of alkylsilanes with or without additional functional group to form a layer of hydrophobic molecules. This layer provides hydrophobic interactions with non-polar solutes, hence facilitate the well-known reversed-phase liquid chromatography (RPLC). Silica particles used in chromatography are usually prepare in 2 – 10 um of particle sizes with specific surface areas at 100 – 600 m^2/g depending on the application requirement and synthetic process. Many attempts have been made to measure the surface silanol bond density. In general, approximately 4.6 to 5 silanol groups/nm^2 will appear on these surfaces [26]. Recently, sub 2-micron particle size silica substrates are also introduced. This kind of packing is mainly used for fast chromatography. Columns packed with these packing materials require ultra-high pressure chromatographic equipment to run at 4000 to 18000 psi.

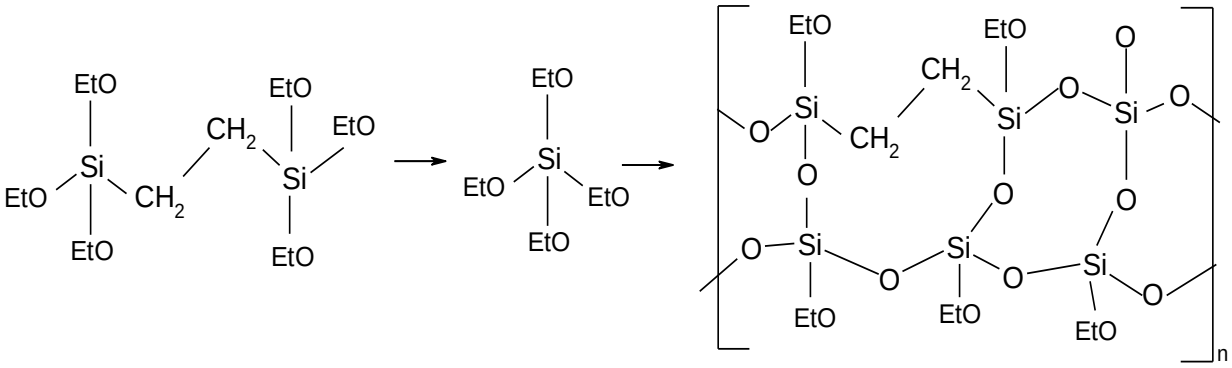
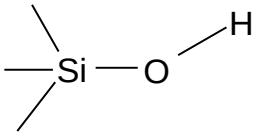
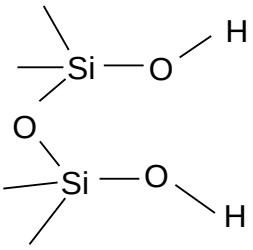


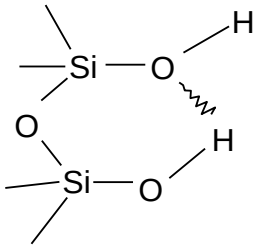
Figure 1.3. *Schematic of the formation of bridged hybrid silica*



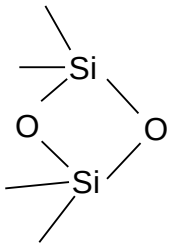
(i) Single



(ii) Geminal



(iii) Vicinal



(iv) Siloxane

***Figure 1.4**. Types of surface silanol*

1.3.1.4. Bonding Mechanism of Organic Ligands on Silica

Figure 1.5 Shows the formation of monomeric, dimeric and oligomeric bonded phases by alkylsilanization with monofunctional and difunctional modifiers. **Figure 1.6** shows the Formation of monomeric, dimeric and polymeric bonded phases by alkylsilanization with trifunctional modifiers

A fully hydroxylated silica surface contains approximately 8 μmole/m^2 of silanol groups (4.8 silanol group/nm^2). Some porous silica for liquid chromatography that is not fully hydroxylated may have a surface silanol concentration at only 5 to 7 μmole/m^2 depending on the preparation process [27]. Silanol groups are considered to be an active and strong adsorption site and hydrophilic in nature with strong tendency to form hydrogen bonds with both hydrogen bond donor and acceptor compounds. Its pH and activity depend on the type it exists (isolated, geminal or vicinal) [28]. A number of studies have been performed in order to determine which type of silanol group dominates as the primary reaction and adsorption site, yet no definite answer has been obtained. In addition, silica is soluble in water at high pH. The equilibrium concentration of amorphous silica at room temperature is about 100 ppm [29], This value does not change much between pH 2 – 7. However, increases exponentially above pH 8 due to the formation of silicate anions. Therefore, a common silica surface usually behaves highly polar, active and non-homogeneous. Its chromatographic application is only limited to polar adsorption with non-polar mobile phases. In order to stabilize and homogenize the surface, silica is often bonded with a layer of hydrophobic material such as alkanes or its derivatives. Silanols present on the surface serve as anchors for the alkyl groups through organosilanization. Typically, porous silica is reacted with organosilanes to yield -Si-R attachment through a Si-O-Si-R (siloxane) linkage [30]:

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

Where n = 1 to 3, R is an alkyl or substituted alkyl group, X is an easily hydrolysable group such as halide, amine, alkoxy or acyloxy. The most popular leaving group is chlorine. Since 1970, Kirkland and De Stefano produced the first bonded phase using a chlorinated alkylsilane to attach alkyl chains to the silica surface [31], the commercially available reversed-phase HPLC stationary phases are mainly manufactured by reacting with alkylchlorosilane type modifiers. The reaction is usually catalyzed by a base such as 2,6-lutidine, imidazole, quinuclidine, or pyridine which at the same time acts as a scavenger base to neutralize the hydrochloric acid by-product.

Generally, three physical forms of bonded phases may be formed depending on the number of bonds per alkylsilane ligand contains [32]. Named brush phase, oligomeric phase, and bulk phase that are formed by monofunctional, difunctional and trifunctional alkylsilanization [33].

When monofunctional modifier alkylsilane such as dimethylchloroalkylsilane is used, only one single surface-silane linkage is possible, and consequently a monomeric brush type phase will be formed as shown in type (i) of **Figure 1.5**

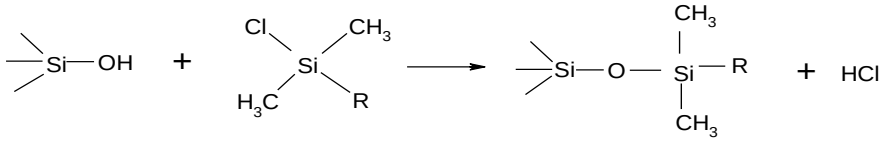
When difunctional alkylsilane such as dichloromethylalkylsilane is bonded to the silica surface, monomeric or dimeric bonded phase may be formed. For the monomeric bonded phase, the silanol groups on the silica surface are reacted first with dichloromethylalkylsilane to link chloromethylalkylsilyl groups to the surface through elimination of one of the chlorine group. Then treated with water which hydrolyses the chloromethylalkylsilyl groups to hydroxymethylalkylsilyl groups with the elimination of one hydrochloric acid molecule. In the case of the dimeric bonded phase, chlorine groups are reacted with the silanols on the silica surface to release two hydrochloric acid molecules. Alternatively, one of the chlorine group may react with the silanols on the silica surface. The remaining chlorine groups are then hydrolyses to hydroxyl group.

These hydroxyl products may further react with more dichloromethylalkylsilane and water to introduce additional hydroxymethylalkylsilane. Accumulating of these hydroxymethylalkylsilane groups will result in the formation of an oligomeric phase. as shown in type (ii) to type (v) of **Figure 1.5**.

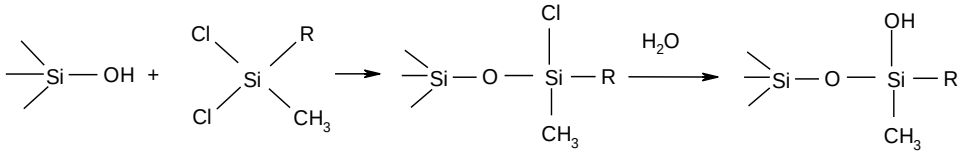
When trifunctional alkylsilane such as trichloroalkylsilane is reacted with silica, monomeric or dimeric bonded phases may be formed. the remaining unreacted chlorine groups are hydroxylated with water to form additional silanol groups that may further cross-link with the other silanol groups to form a polymeric structure. Examples of these alkylsilanization reactions are shown in **Figure 1.6**.

Due to the nature of trichloro function, extensive cross-linking can occur. As a result, the stationary phase has a chemically cross-linked multi-layer character, thus is termed bulk phase. The thickness of these layers may vary according to the reaction conditions. Due to steric hindrance, trimeric bonding is unlikely to happen.

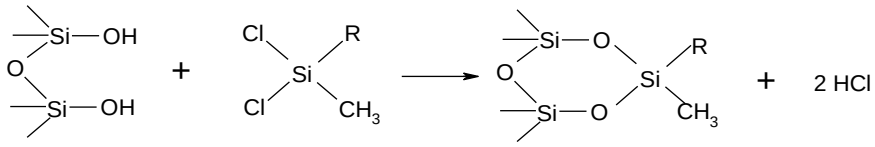
Among the above discussed synthetic procedures, monomeric bonded phases are straightforward to prepare and the reaction conditions should be more reproducible. The resulting monolayer coverage provides excellent mass transfer and high column efficiency for most analyte molecules. The drawback of this type of bonded phases is that it is only stable in pH 2 – 8. On the other hand, polymeric bonded phases are more stable towards hydrolysis, but the preparation process is not as reproducible and may also exhibit lower column efficiency.



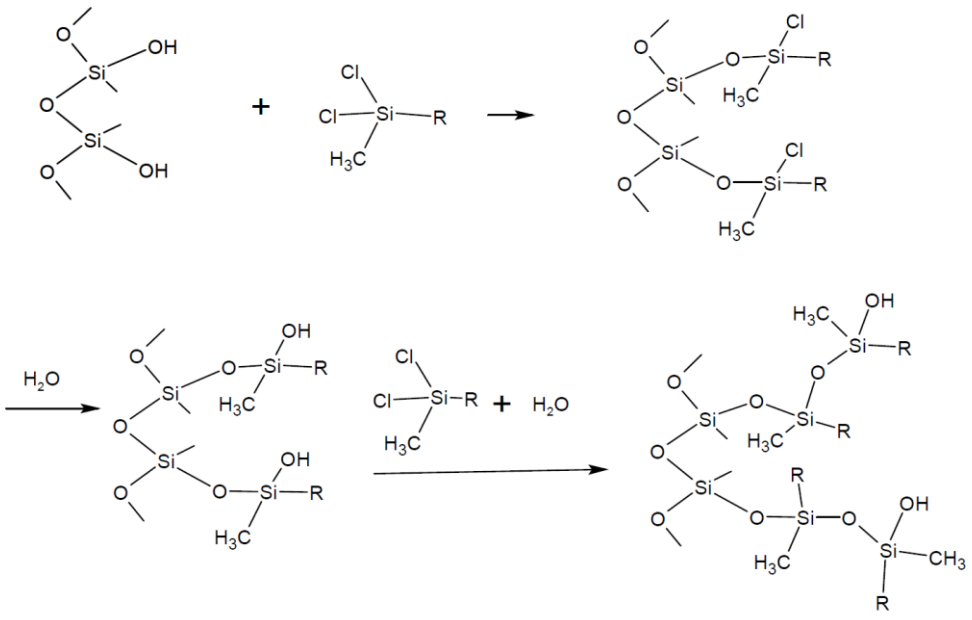
(i) Formation of monomeric bonded phase with monofunctional modifier



(ii) Formation of monomeric bonded phase with difunctional modifier

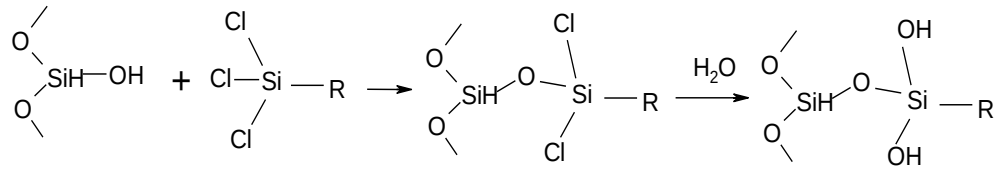


(iii) Formation of dimeric bonded phase with difunctional modifier

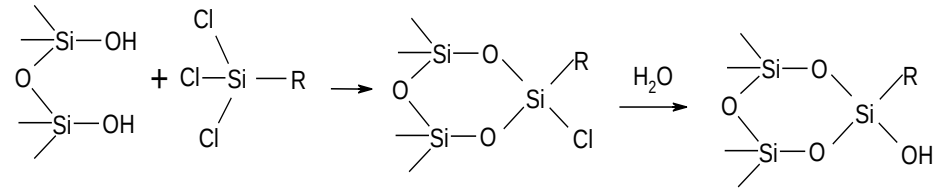


(iv) Formation of oligomeric phase with difunctional modifier

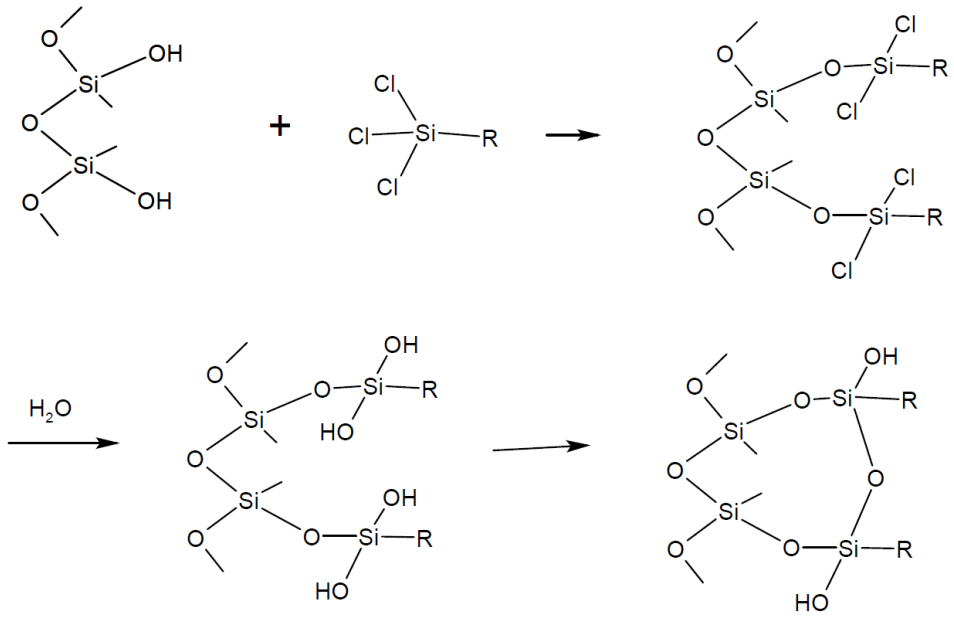
Figure 1.5. *Formation of monomeric, dimeric bonded phases and oligomeric phase by alkylsilanization with* monofunctional *and difunctional modifiers*



(i) Formation of monomeric bonded Phase with trifunctional modifier



(ii) Formation of dimeric bonded phase with trifunctional modifier



(iii) Formation of polymeric bonded phase with trifunctional modifier

Figure 1.6*. Formation of monomeric, dimeric and polymeric bonded phases by alkylsilanization with trifunctional modifiers*

As mentioned above, during the production of a monomeric bonded phase, the monofunctional modifiers decrease the surface silanol concentration by approximately 50% depending on the alkyl chain length of the ligand. When difunctional modifiers are used, it mainly reacts with only one surface silanol group. The remaining chlorine group is mostly hydroxylated to become another silanol group by the residual water in the solvent media, or water used in the adsorbent washing process, thus can only slightly reduce the surface silanol concentration by approximately 12%. When a trifunctional modifier is used, each surface silanol group is substituted with one of the three chlorine groups. The remaining two chlorine groups are then hydroxylated to additional silanol groups in the presence of water, resulted in increasing the total number of silanol groups. These free silanol groups may finally become the site for further reactions such as polymerization.

It has been shown by isotopic studies that access to all surface silanols is sterically hindered to different extents by the dense graft and its protecting alkyl groups as well as the polymeric bulk structure [34]. Furthermore, the bonding density also depends on the pore structure of the silica. On passing through from a flat to concave surface, the bonding density no longer solely depends on the size of the anchor groups, but also on the space decreasing at the tip of the grafts due to the curvature of the pore. This effect becomes more obvious with longer alkyl chain anchored to the pore surface. Silica modified with Chlorotrimethylsilane (alkyl chain C1) has bonding density of 4.2 μmole/m^2, chlorodimethyloctadecylsilane (alkyl chain C_{18}) bonded phase has a bonding density of 2.5 μmole/m^2, these values are translated to the approximate linear distance between anchor of 4.3 Å for C_1 and 7 Å for C_{18} on the surface [35]. For pore diameters less than 120 Á, assume cylindrical pore shape, the pore curvature will significantly decrease the bonding density of dimethyloctadecylsiloxane ligands.

The amount of surface coverage or bonding density directly affects the magnitude of hydrophobicity of a reversed-phase surface and hence is very important to the chromatographer. However, column venders seldom provide bonding density but surface area and percentage of carbon load only. In practice, percentage of carbon load can be determined by elemental analysis. Berendsen and de Galan derived an expression for the calculation of surface coverage values in μmole/m^2 accounting for the weight increase of silica due to the attachment of the boned phase ligands and the loss of hydrogen in the silanization reaction [36].

$$d_b[\mu mol/m^2] = \left[\frac{10^6 C\%}{1200 n_C - C\%(MW - 1)}\right].\frac{1}{S}$$

Where:

d_b	Bond density in μmole/m^2
C%	Percentage of carbon load by weight
n_C	Number of carbon atoms in the bonded phase ligand
MW	Molecular weight of the bonded phase ligand
S	Specific surface area of the silica

For instant, a C_{18} stationary phase was prepared by bonding $C_{18}H_{37}Si(CH_3)_2$-X on a silica surface with 300 m^2/g of specific surface area. The percentage carbon load was found to be 8.4% by weight. Molecular weight of octadecyldimethylsilane is 311 g/mole and number of carbon atom = 20. The calculated bond density is

$$d_b[\mu mol/m^2] = \left[\frac{10^6 \mu mole/mole \times 8.4}{1200 \times 20 - 8.4 \times (311 - 1) g/mole}\right] \times \frac{1}{300 m^2/g} = 1.31[\mu mol/m^2]$$

1.3.1.5. Common Bonded Phases of RPLC

Organic ligands are chemically linked to the silanol groups on the silica surface by reacting with different reagents. **Figure 1.7** shows the chemical structure of alkyl - bonded surfaces and the silica surfaces bonded with different alkylsilane containing different phenyl functional groups. **Figure 1.8** shows the chemical structure of silica surfaces bonded with other types of functional groups. **Table 1.1** gives the main functional interaction of some common types of stationary phase.

The nature of the organic moiety will determine the type of interaction that will take place between the solute and the surface. For reversed-phase chromatography, the fundamental driven force for retention is hydrophobic interaction. The strength of the interaction mainly depends on the hydrophobicity of the surface where the bonded hydrocarbon ligands play a major role. Generally, alkyl bonded surface will provide strong hydrophobic interaction. If a polar functional group such as cyano, phenyl, or amino is attached to the hydrocarbon chain, hydrophobicity of the surface will be reduced. Another important polar source is the existing of residual free silanols. Due to steric restriction, only half or less of the available silanols (4.8 groups/nm^2) on the surface can react with the bonded ligands. The other silanol groups remain unbonded. the column hydrophobicity and hydrophobic interaction will be discussed in detail in chapter 2. Although alkyl bonded stationary phases C_{18} is still the most popular type of column which can adequately separate many compounds, many other bonded phases containing different functional groups are also commercially available today. These functionally diverse stationary phases provide additional separation selectivity to traditional C_{18} columns through different chemical interaction with the analytes. Thus, provide more varieties of choices for chromatographers to achieve their goal for particular separations. This is especially useful when the choices of mobile phase are limited such as for LC-MS method and large scale preparative chromatography. In fact,

chromatographers often start with C_{18} column for initial trials to develop chromatographic method. Then fine tune their method with other types of columns as needed. Some common reversed-phase type of HPLC stationary phases are introduced below.

1.3.1.5.1. Alkyl-Bonded Stationary Phase

Alkyl bonded stationary phase contain an alkyl chain (usually between C_1 and C_{18}). It is the most popular type of stationary phase. Almost 80% of today's HPLC methods separate analytes by using alkyl bonded surfaces. Numerous column characterization studies on this type of surfaces have been published. They are widely spreading over physical, chemical, spectroscopic and chromatographic methods. The majority of the studies are carried out by chromatographic approach. Test results obtained from selected analytes indicate that the retention (usually expressed with capacity factor k') of non-polar solutes on alkyl bonded surface are increasing with increasing alkyl chain length [37] [38] and percent carbon load [39]. This phenomenon evidenced that the retention mechanism is predominated by hydrophobic interaction.

I.3.1.5.2. Phenyl-Bonded Stationary Phase

Although the main research on bonded phases has been focused on alkyl type. Stationary phases prepared from aromatic ligands in which alkyl phenyl with or without additional functional groups [40] have also gained popularity for reversed-phase liquid chromatography. In 1985, Den and Kettrup prepared a series of alkyl phenyl modified substrates using mono, di, and trifunctional silanes. These silanes of different alkyl chain length were synthesized from phenyl-substituted alkenes through hydrosilylation reaction. Phenyl bonded phases have been successfully used to resolve positional isomers [41] [42] and flavonoids [43] [44]. By applying the π-π electron interaction of

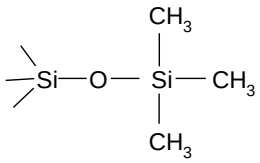
the aromatic ring, phenyl surfaces also introduce additional retention to the solutes that are capable of π-π interaction, therefore provide additional selectivity. Compare to alkyl bonded phases, phenyl surfaces are usually considered as less polar and have lower methylene selectivity due to the reduction of hydrophobic interaction caused by the attached phenyl group. This property may make the separation of alkane homologous series less selective.

I.3.1.5.3. Cyano-Bonded Stationary Phase

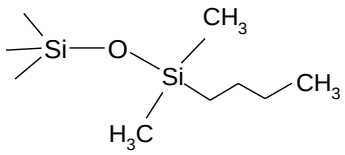
Usually, cyanoalkyl (-$[CH_2]_n$-CN) modifiers are used to prepare this type of column. Compared to alkyl bonded phases, cyano columns are less commonly used due to the general concern of column stability [45] and reproducibility [46]. However, its pronounced difference in analyte retention and selectivity mode often make cyano columns a desired alternative choice for chromatographic method development. Studies by Marchand et al [47] using linear salvation energy relationships (LSERs) showed that cyano columns are much less hydrophobic compared to alkyl column with similar ligand chain length (C4 and C5) primarily due to the greater polarity of cyano group.

I.3.1.5.4. Other Reversed-phase Stationary Phases

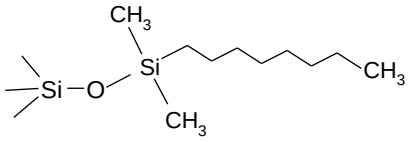
Other types of bonded phases including amino, diol, fluoro and ion exchange column are also available. Recently, many specialty columns such as bidentate alkyl, cholesterol as well as columns for chiral separations have also been created. With such large varieties of columns, plus various combinations of mobile phases, almost any compound can be separated with chromatography.



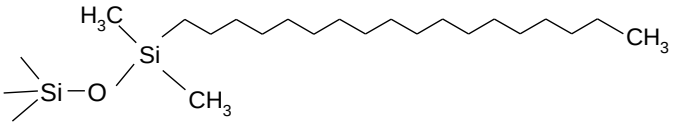
Trimethylsiloxane (C1)



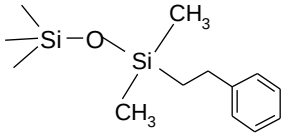
Butyldimethylsiloxane (C4)



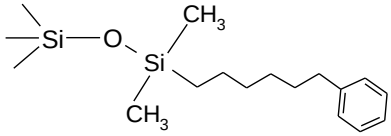
Octadimethylsiloxane (C_8)



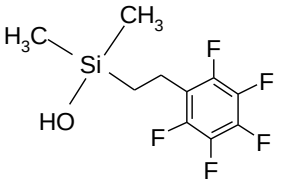
Octadecyldimethylsiloxane (C_{18})



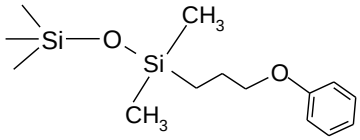
Ethylphenyldimethylsiloxane



Hexylphenyldimethylsiloxane

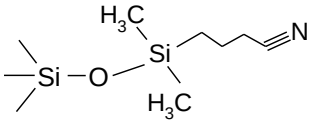


Perfluorophenylethyldimethylsiloxane

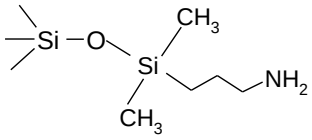


Phenylpropyletherdimethylsiloxane

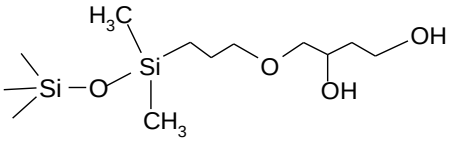
Figure 1.7. Chemical structure of alkyl bonded surfaces and silica surfaces bonded with different phenyl functional groups.



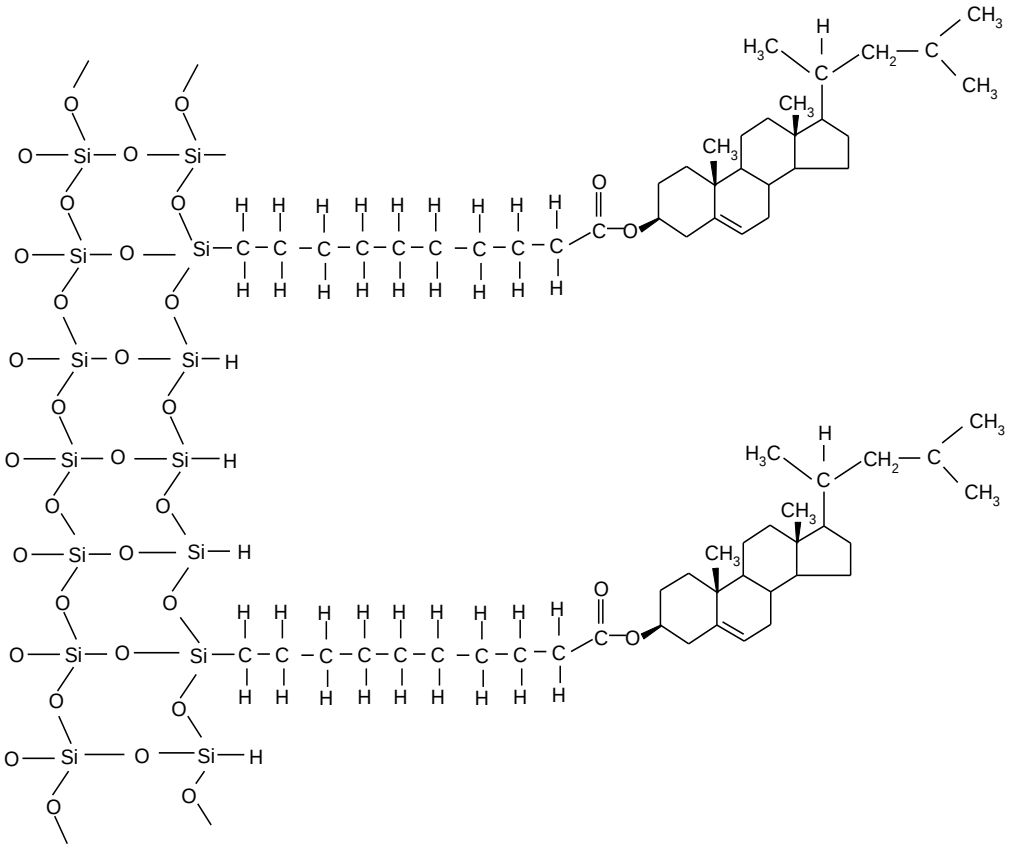
Nitrile (Cyano)



Aminopropyldimethylsiloxane (Amino)



Diol



UDC-Cholesterol silicon hydride (MicroSolv Colgent column)

Figure 1.8. Chemical structure of silica surfaces bonded with cyano, amino, diol and cholesterol functional groups.

Table 1.1 *Chemical interactivity of stationary phases bonded with different functional groups*

Stationary phase	Chemical Interaction
Alkyl C_{18} and C_8	Hydrophobic (dispersion) interaction
Amino	Basic interaction
Cyano (CN)	Dipolar interaction
Phenyl	π - π interaction
Amide	Basic and dipolar interaction
Ether	Largely basic, some H-bonding
Nitro	Strongly dipolar interaction
Diol	H-bonding, basic-acidic interaction
Fluoroalcohol	Acidic interaction
Cholesterol	H-bonding, shape discrimination

1.3.2. Other Inorganic Oxide and Polymer Supporting Materials

Despite the successful application of porous silica in chromatography, persistent problems still exist, mainly due to the poor stability beyond pH 2 - 8 and the existence of residual silanols which often lead to asymmetric peak shapes and the reduction of the column efficiency for basic analytes. Vast research has been pursued to solve these problems. One of the alternative is using other inorganic oxides. Alumina (pH2 – 12), zirconia and titania (pH1 – 14) are well known to be stable in extreme pH environment. They also possess comparable mechanical strength and mass transfer capability like silica that may be prepared with similar synthetic process. While porous silica is amorphous, these oxides often also exist in crystal forms in addition to amorphous. The degree of crystallinity and phase composition significantly affect its chromatographic and physical-chemical surface properties. Silica shows only weak Bronsted acidity, hydrogen bonding and cation exchange ability provided by the free silanols. The surface structures of alumina, zirconia and titania contain both oxygen and metal atoms. The accumulation of negative charge on the oxygen atoms and positive charge on the metal ion lead to their ion exchange, Lewis acid and basic properties, in addition to hydrogen bonding. However, these complex retention mechanisms created by complex surface properties often result in irreproducible analyte retention. Therefore, these types of columns are not popular and practically only used for a limited number of special applications where silica based columns are not appropriate. Among alumina, zirconia and titania, only aluminum and zirconia base columns are commercially available.

1.3.2.1. Alumina Substrate

The common alumina used in liquid chromatography are prepared by dehydration of alumina trihydrate. Porous alumina exists with different pH. The most

widely used form is in neutral pH (~pH7). Basic alumina (~pH10) is used to separate acid labile compounds and used as a cationic exchanger in aqueous solution. Acid alumina (pH3.5 -4.5) is mainly used for the separation of acidic analytes and anionic exchange separation [48].

1.3.2.2. Zirconia Substrate

Zirconia is a crystalline zirconium dioxide compound with high thermal stability. It is completely stable from pH 1 to 14 [49]. The porous zirconia microspheres used for HPLC column packings can be synthesized by means of polymerization-induced colloid aggregation (PICA) method [50] or a sol-gel process [51]. Modification of zirconia surface with polybutadiene and octadecyl-polybutadiene ligands can be found in reference [52] and [53].

1.3.2.3. Polymer based Supporting materials

Majority of the currently used polymer-supporting material for HPLC is polystyrene divinylbenzene copolymer base. Other polymers including polyvinyl alcohol, polyacrylate and polymethacrylate are also used. Unlike silica base, polystyrene divinylbenzene is stable in wide range of pH (pH1 – 14) without hydrolytic problem. Its electron-rich benzene ring is capable of further modification with reversed-phase alkyl chains such as octyl and octadecyl. The polymer can be made in a wide range of particle sizes from 5 to 20 µm, pore diameters from 2 to 400 nm and surface area from 50 to 500 m^2/g [54] [55] [56]. The disadvantages of polymer supporting material are low compression resistance, may shrink or swell up in some organic solvents and show lower chromatographic efficiency compare to silica base columns. The spaces among polymer chains also allow small analyte molecules to diffuse into the polymer matrix

which contain both mesopores and macropores. The resulting eddy diffusion and mass transfer hindrance lead to noticeable increase in band broadening [57].

1.3.2.4. Porous Carbon Supporting materials

Graphitized carbon has been successfully used in gas chromatography for many years. However, it came across quite many hurdles on the road diversifying to liquid chromatography. These materials exhibit poor mechanical strength and often show poor peak shapes due to strong analyte interaction with the mineral, oxygen and nitrogen-containing impurities on the surface. Until late 1980s, a breakthrough reproducible template replication method to produce rigid, mesoporous graphitized carbon particles was invented. The first commercially available porous graphitic HPLC column was made under the name “Hypercarb“ [58].

Porous graphitized carbon surface is more hydrophobic than conventional octadecylsiloxane surface and provide higher methylene selectivity. Its strong polarizable lone-pair electron interaction provides unique selectivity for the separation of polar analyte. The flat planar carbon surface structure also made this type of surface one of the primary choice for separation of conformational isomers. The drawback of graphitized carbon surface is chemically non-reactive and make it hard to further modify directly.

1.3.3. Configuration of Bonded Phase

Figure 1.9 schematically shows the possible configurations of covalently bonded alkyl ligands on silica surface.

In addition to the chemical properties of the bonded phases, ligand configuration is another major factor directly affecting the retention model. In 2001, Kazakevich et al used low temperature nitrogen adsorption (LTNA) and chromatographic methods to

study the alkyl ligand configuration in the bonded silica pore. Their results proved that alkyl chains attached on the porous silica surface are densely packed at the top part of the grafts due to hydrophobic attraction, particularly in high aqueous ratio mobile phase [35]. Thus prevents the analyte molecules penetrate into the bonded phase.

Legacy models described the stationary phase in four possible configurations depending on the ligand chain length and mobile phase composition. They are “picket fence”, “fur”, “stack” and “collapsed surface”.

1.3.3.1. Picket Fence Model

If a very dense layer of alkyl ligands is bonded to the surface, the bonded grafts will closely pack with each other and behave like rigid rods with no internal degree of freedom [59]. In fact, mobile phase and analyte molecules with dimensions encountered in usual HPLC analyses cannot fit between the alkyl chains. These molecules are only adsorbed on the tip of the bonded layer. Under this model, the accessibility of mobile phase and analyte molecules into stationary phases bonded with different chain lengths of alkyl ligands are similar. Essentially, this configuration of stationary phase will only lead to a relatively small change in phase ratio (stationary phase to mobile phase) with different alkyl chain lengths.

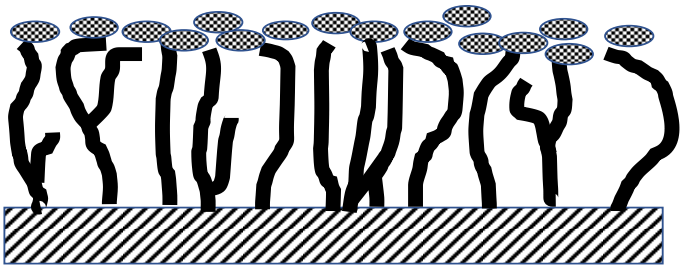
Practically, picket fence model is unlikely to be formed. Common silica surfaces used in HPLC column packing usually contain about 8 μmole/m^2 of silanol bonding sites which about 50% (4 μmole/m^2) of these sites are able to be boned to commonly used alkyl ligands such as C_4, C_8 and C_{18}. This bond density is much smaller than the required surface concentration to form a condensed monolayer.

1.3.3.2. Fur Model

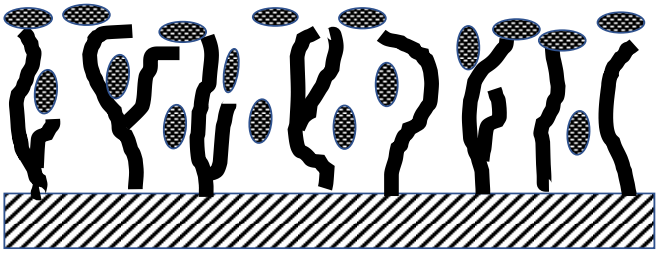
If the mobile phase contains high proportion of non-polar components and the bonded ligand density on the stationary phase surface is moderate, the organic-rich mobile phase will be capable of wetting the alkyl chain, and the ligands will have enough room to stand up on the bonded surface to form a “fur” like configuration. This model implies that the distance among ligand chains is sufficiently large for solute and mobile phase molecules to actually penetrate into the bonded phase and partition between the ligand chains laterally. Lower carbon loading and higher stationary-to-mobile phase ratio than the corresponding “picket fence” model would be expected because the inter-ligand space is part of the stationary phase. However, it may be worth to note that due to pore curvature, alkyl bonded silica surfaces with 4 μmole/m^2 or lower bond density may be still possible to form a closely packed configuration at the top part of the ligands which may become similar to a stack model [35].

1.3.3.3. Stack and Collapsed Model

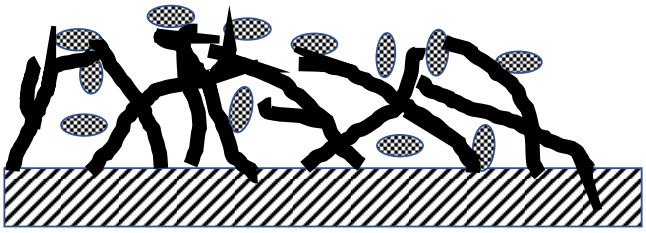
In contrast, when the hydrophobic bonded layer is exposed to a hydro-organic mobile phase containing insufficient among of organic solvent, the ligand chains may not be thoroughly wetted and tend to stick with each other due to strong hydrophobic interaction, resulting in forming greasy patches on the silica surface. In fact, the configuration of a bonded surface described in the partition model is highly dependent on the polarity of the mobile phase. At low organic ratio, the bonded ligands are not fully wetted and stay in a stack-type configuration. In an extreme case, the bonded surface will exist in a collapsed form. When the organic component of the mobile phase increases to a ratio that is high enough to fully wet the ligand chain, the bonded phase will become a fur-type configuration.



Picket fence



Fur



Stack

Collapsed

Figure 1.9. *Schematic illustration of the possible arrangement of alkyl ligand chains on the silica surface and distribution of the solutes.*

=Solute molecules *=Ligand chain*

1.4. Retention Mechanism

The understanding of the retention mechanism is one of the most important fundamentals for the progression of chromatographic technique. Although numerous of research papers have been published with large amount of supporting data, none of their proposed mechanisms are able to satisfactorily explain all retention phenomena. Following are the common retention mechanisms widely discussed in literature.

1.4.1. Solvophobic

Solvophobic mechanism is a mobile phase driven retention model developed by Horvath in 1976 [60]. **Figure 1.10** schematically illustrates the interaction path of the solvophobic mechanism. According to this model the analytes are driven towards the stationary phase depending on their repellence to the aqueous component of the mobile phase. The stationary phase is just taken as a passive accepting surface with no interaction with the analytes. Sovolphobic theory explains the retention mechanism in reversed-phase chromatography as a combined cycle of two conceptual thermodynamic processes. (1) Binding of the analyte to the stationary phase ligands in the gas phase. (2) Transfer of the participating species into the mobile phase. The standard free energy change associated with the retention can be expressed by the following thermodynamic equilibrium equation [61]:

$$\Delta G_R^0 = \Delta G_{solv}^0 + \Delta G_{gas}^0 \qquad \text{Eqn. 1.1}$$

and

$$\Delta G_{solv}^0 = \Delta G_3^0 + \Delta G_1^0 - \Delta G_2^0 \qquad \text{Eqn. 1.2}$$

Where:

ΔG_R^0 = Standard free energy for retention

ΔG_{solv}^0 = The net standard free energy changes due to solvent effect

ΔG_{gas}^0 = The standard free energy change for species binding in gas phase.

ΔG_1^0, ΔG_2^0, ΔG_3^0 = The standard free energy of salvation for the participated species.

In the cycles, the salvation process for each species is considered to proceed in two steps. (1) The mobile phase forms a cavity of sufficient size and shape to accommodate the analyte molecule. (2) The analyte molecule enters into the cavity and interact with the surrounding mobile phase molecules. The net standard free energy change for salvation can be expressed as:

$$\Delta G_{solv}^0 = \left(\Delta G_{cav,AL}^0 - \Delta G_{cav,A}^0 - \Delta G_{cav,L}^0\right) + \left(\Delta G_{int,AL}^0 - \Delta G_{int,A}^0 - \Delta G_{int,L}^0\right) + \Delta\Delta G_{Mix} + \Delta G_{Red} - RT\left(ln\frac{RT}{V_E}\right) \qquad \text{Eqn. 1.3}$$

Where

$\Delta G_{cav,AL}^0$, $\Delta G_{cav,A}^0$, $\Delta G_{cav,L}^0$ = The free energy of cavity formation of the species

$\Delta G_{int,AL}^0$, $\Delta G_{int,A,}^0$ $\Delta G_{int,L}^0$ = The free energy of eluent-species interaction,

$\Delta\Delta G_{Mix}$ = The net free energy of mixing of eluent and species molecules

ΔG_{Red} = The reduction in ΔG_{Gas} due to the presence of eluent

V_E = Molar volume of the eluent molecule

R = Gas constant

T= Absolute temperature (K)

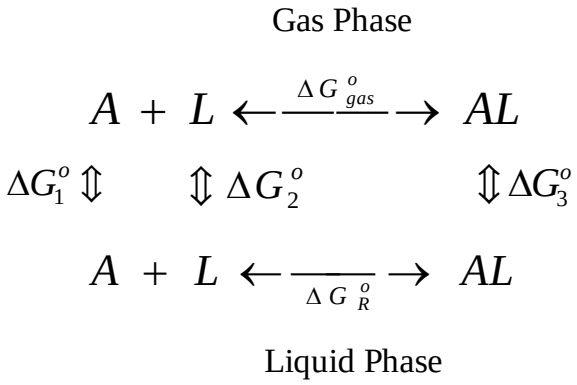


Figure 1.10. *Schematic illustration for the thermodynamic cycle of hypothetical gas phase association and liquid phase salvation process in reversed-phase liquid chromatography.*

Where A, L and AL represent Analyte, ligand and the associated species, respectively.

In summary, solvophobic theory considers RPLC retention and selectivity mainly as a function of the volume change, the surface tension and molecular interaction energies in the mobile phase. A major shortcoming of this model is lack of accounting for the stationary phase influence. Retention is solely a solubility process in the mobile phase rather than a transfer process between the stationary phase and the mobile phase. In fact, many experiments showed that stationary phase does play a role in solute selectivity.

1.4.2. Partition vs. Adsorption Mechanism

Figure 1.11 Schematically Demonstrates the partition and adsorption mechanism.

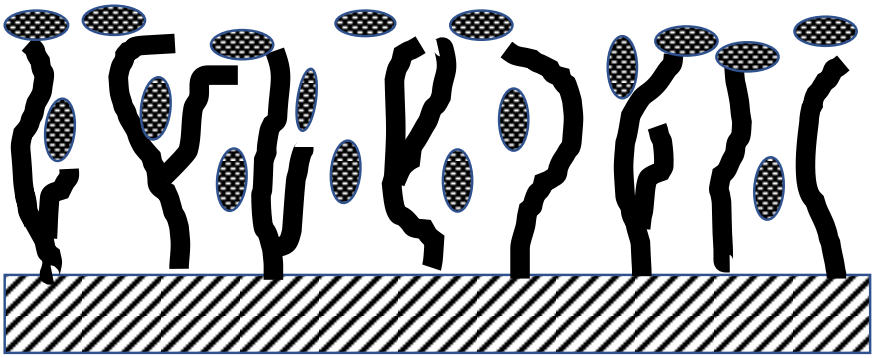
Today, chromatographic scientists generally accept that both mobile phase and stationary phase play a role in retention and selectivity. Retention involves a process of solute transfer from mobile phase to stationary phase through one or more steps. However, whether an analyte is physically partitioned into the interstitial space of the bonded phase grafts or adsorbed at the interface located between the bonded phase and the adjacent mobile phase is still an on-going debate. Although many retention models anticipating both partition and adsorption mechanism have been proposed [62] [63] [64] [65], no set model has been generally accepted. Even the definitions of partition and adsorption are inconsistent. Based on Dorsey and Dill's definition, the distinction is that partition implies that the analyte molecules are approximately fully embedded within the stationary phase, whereas adsorption implies that the analyte molecules are just in surface contact with the stationary phase, but are not embedded [62]. In either case, the analyte molecules are switched from an environment of

surrounded by neighboring mobile phase molecules to another environment of surrounded fully or partially by neighboring molecules of the stationary phase.

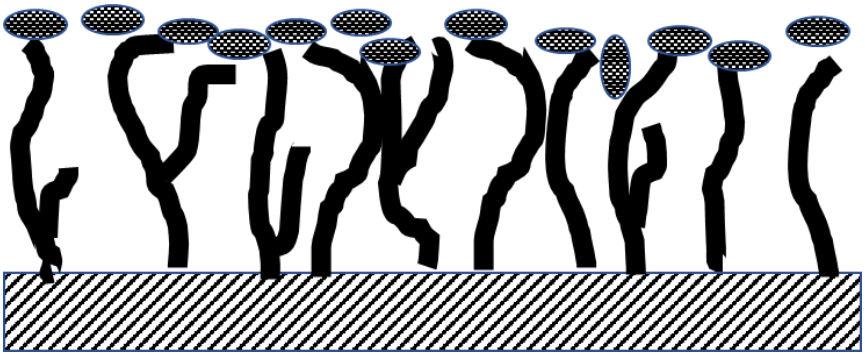
According to Dorsey and Dill, analyte retention for either model is driven by the deferential chemical affinity of the analyte to the mobile phase and stationary phase. The equilibrium constant of transferring an analyte molecule from the mobile phase to the associated stationary phase can be expressed as a difference in standard state chemical potential $\Delta\mu^0$(a) for the analyte "a":

$$lnK = -\left(\frac{\mu^0_{sta}(a) - \mu^0_{mobile}(a)}{RT}\right) = \frac{-\Delta\mu^0(a)}{RT} \qquad \text{Eqn. 1.4}$$

The value of a solute's standard state chemical potential depends strongly on molecular interaction with the stationary or mobile phase molecules. At thermodynamic equilibrium, the chemical potential of the solute in the mobile phase and stationary phases are equal i.e. $\Delta\mu^o = 0$, no chemical shift will occur. If the solute has a higher chemical potential in stationary phase than mobile phase, $\Delta\mu^o$ will shift to a higher value and - $\Delta\mu^o$ represents a release of energy in the system, thus build up solute retention in the stationary phase.



Partition Mechanism



Adsorption Mechanism

Figure 1.11. *Schematic illustration of the partition and adsorption mechanism*

=Solute molecules *=Ligand chain*

1.4.2.1. Full Adsorption Mechanism

If the analyte transfer process is proceeded under the adsorption mechanism, only a fraction of the analyte-mobile phase molecular interactions is replaced by the analyte-stationary phase molecular interactions.

Adsorption is a process of the analyte accumulated on the adsorbent surface under the influence of the surface force which leads to a variation in concentration at the interface. Unlike partition, adsorption process is a surface phenomenon which occurs at the solid-liquid interface. The solute molecules or adsorbates migrate from the liquid phase to the interface (the surface adsorbed layer) and displace the physically adsorbed molecules of the solvent. Interpretation of the adsorption mechanism needs one to define the volume or thickness of the surface adsorbed layer. Many studies have been carried out pertaining to this adsorbed layer [66] [67] [68]. However, there is still no uniform definition for the volume or thickness of the layer. The most popular model for this approach should be the Gibb's model. He defined an imaginary dividing plane at a position above the adsorbent surface. The dividing plane is considered as a delimiter of the adsorption action. Above this plane, there is no adsorption activity anticipated by the adsorbent and the concentration of the analyte will stay constant throughout the bulk liquid phase. The area below this plane is considered to be under the influence of adsorption exerted by the adsorbent, thus the analyte concentration in this area is higher than the bulk liquid phase.

Kiselev was the pioneer in correlating adsorption isotherms to gas chromatography in the 60's [69]. He developed a series of method to measure the surface adsorption isotherm with gas solid chromatography which is known as inversed gas chromatography (IGC). Kovats [68] strengthened the necessity of its application to HPLC. The analyte retention volume for this approach can be expressed as

$$v_R = v_0 + SK_H \qquad \text{Eqn. 1.9}$$

Where v_R is the chromatographic retention volume, S is the total adsorbent surface area and v_0 is the total volume of the liquid phase in the column. K_H is the analyte adsorption constant (Henry constant) or more specifically the slope of the adsorption isotherm. Therefore, the surface specific retention factor defined below is directly related to the Henry constant.

$$k = \frac{v_R - v_o}{S} = K_H \qquad \text{Eqn. 1.10}$$

Note that K_H as well as the surface specific retention factor k in equation 1.10 is not dimensionless. It is expressed in mL/m^2 which may be reduced to a length unit. Since analyte retention by adsorption is a displacement process, K_H can be positive or negative. If the analyte interaction with the adsorbent surface is weaker than the eluent interaction with the adsorbent surface, the analyte molecules will not be able to replace the adsorbed eluent molecules and its retention volume will be smaller than v_o. This indicates that K_H is not a real thermodynamic equilibrium constant which has no dimension. The basic retention equation for a binary mobile phase system can be expressed as

$$v_R = v_o + S\frac{d\Gamma}{dC} \qquad \text{Eqn. 1.11}$$

Where $\frac{d\Gamma}{dC}$ represents the slope of the adsorption isotherm.

1.4.2.2. Excess Adsorption Mechanism

Another adsorption approach is interpreted by the amount of analyte adsorbed on the stationary phase surface in excess to the equilibrium concentration of the same analyte in the bulk liquid (mobile phase) [70] [71]. **Figure 1.12** schematically describes the solvent distribution of the excess adsorption process. The advantage of this approach is it does not need to define a model of adsorption layer a priori, therefore, largely reducing the complexity of experimental measurement. For the scope of this dissertation, the theory of excess adsorption will be given in detail in chapter 2.

Excess adsorption model is also based on the adsorption displacement mechanism, where the analyte is accumulated at a close proximity of the adsorbent surface under the influence of physical interaction force exerted by the surface. Essentially, the mathematical expression for adsorption models may only apply to binary liquid system containing two components. Component 1 is taken as the solute and component 2 is taken as the solvent. For a binary liquid system, the accumulation of one of the liquid component (component 1 as solute) is accompanied by the corresponding displacement of another component (component 2 as solvent) from the surface region to the bulk solution. At equilibrium, the concentration of the accumulated component 1 on the surface will exceed its equilibrium concentration in the bulk solution. This phenomenon can be graphically explained by a static adsorption experiment of two binary liquid systems at constant temperature. These systems contain same liquid volume (v_0), adsorbent surface area (S) and initial solute (component 1) concentration (C_0). In the first system, the adsorbent surface is considered to be inert and does not exert surface force to the solution molecules. The amount of solute measured in the bulk solution will be equal to $v_o C_{0.}$ In the second system, the adsorbent surface is considered to be active, thus the solute is preferentially adsorbed on the absorbent surface and lead to a decrease of solute in the

bulk solution to an equilibrium concentration of C_e. The amount of solute measured in the bulk solution is now equal to $v_o C_e$. The excess amount of solute accumulated on the absorbent surface will be equal to $v_o C_0 - v_o C_e$. If an excess adsorption term Γ is defined as the excess amount of solute adsorbed per unit surface area which is a function of C_e, then the following equation 1.12 can be obtained [72].

$$\Gamma(C_e) = \frac{v_o}{S}(C_0 - C_e) \qquad \text{Eqn. 1.12}$$

Note, here that the amount of excess solute in the adsorbed layer is directly calculated by the difference of the bulk solution concentration before and after the adsorption is occurred. A model of adsorbed layer anticipating boundary concept does not needed to be defined. By applying equation 1.12 to the mass balance calculation (refer to section 1.5), the basic retention equation based on excess adsorption mechanism can be obtained as

$$v_R(C) = v_o + S\frac{d\Gamma(C)}{dC} \qquad \text{Eqn. 1.13}$$

Note that $\frac{d\Gamma(c)}{dc}$ is actually the slope of the adsorption isotherm which is defined as the Henry constant K_H.

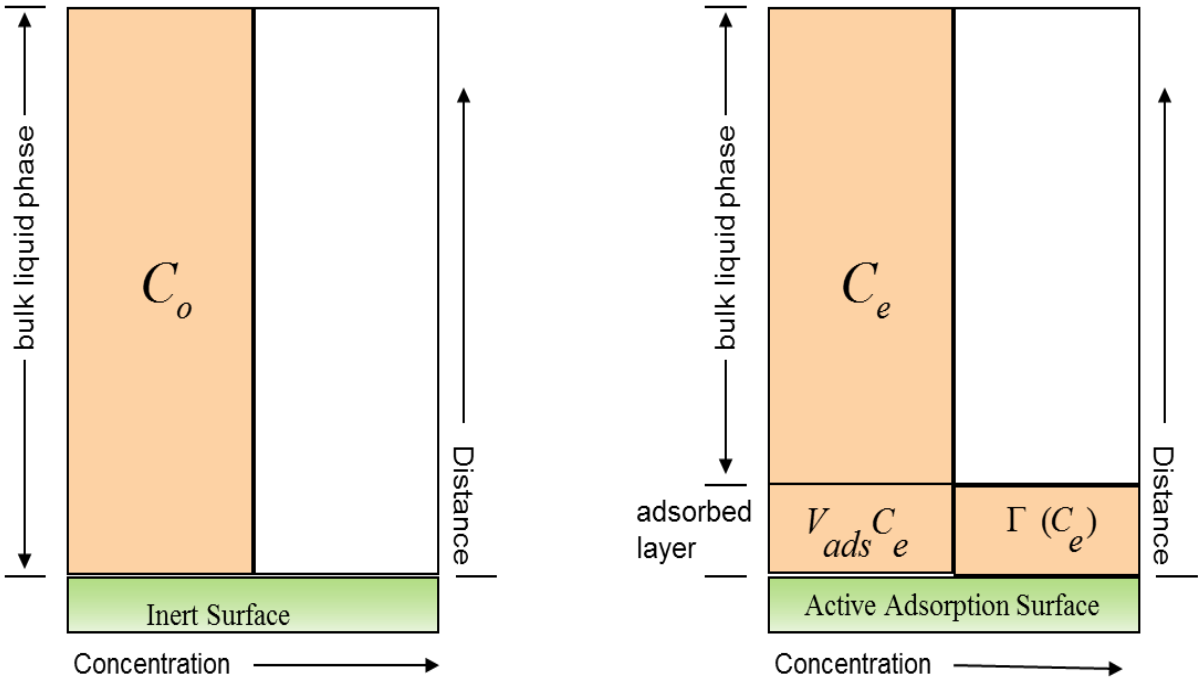


Figure 1.12. *Schematic of excess adsorption.*

Where X-axis represents the solute (component 1) concentration of a binary solution system. Y-axis represents the distance from the adsorbent surface. C_0 and C_e are initial concentration and equilibrium concentration of the solute in the bulk solution respectively. V_0 is the volume of the bulk solution. V_{ads} is the hypothetical adsorbed layer volume. Γ is the surface excess concentration of the solute.

1.4.2.3. Partition Mechanism

Alternatively, if the analyte transfer is processed under a partition mechanism, then the simplest model of retention is resembling the bulk-phase partition between two immiscible liquids where the reversed-phase stationary phase is considered as an amorphous bulk fluid medium. The analytes will partition between the mobile phase and stationary phase. In this case, all the analyte-mobile phase molecular interactions are replaced by the analyte-stationary molecular interactions. The principal driving force for the transfer of an analyte molecule is simply the relative chemical affinity to the mobile and stationary phases. Its chromatographic retention process can be mathematically expressed by equation 1.5.

$$v_R = v_m + V_S K \qquad \text{Eqn. 1.5}$$

Where v_R represents the retention volume of the analyte which is the volume of mobile phase needed to elute the analyte from inlet to outlet of the column, v_m is the volume of the mobile phase in the column, V_s is the volume of the stationary phase, K is a thermodynamic equilibrium constant which can be expressed as an exponential function of the Gibbs free energy for the analyte partitioning between the mobile phase and stationary phase.

On the other hand, the commonly used empirical equation for retention factor (k) may be expressed by the ratio of the adjusted retention volume $(v_R - v_0)$ to the column void volume v_0 as shown by equation 1.6.

$$k = \frac{v_R - v_0}{v_0} \qquad \text{Eqn. 1.6}$$

If we combine equation 1.5 and equation 1.6 together, a straight forward relationship of chromatographically measurable retention factor (k) to the thermodynamic energetic parameter (K) can be obtained.

$$k = \frac{v_m}{v_0} - 1 + \frac{v_S}{v_0} K \qquad \text{Eqn. 1.7}$$

Apparently, this equation contains three different volume parameters, mobile phase volume v_m, stationary phase v_s and void volume v_0. Only taking assumption of $v_m = v_0$ will lead to the commonly used relationship:

$$k = K \frac{v_s}{v_m} \text{ or } ln(k) = ln(K) + ln(\varphi) \qquad \text{Eqn. 1.8}$$

Where φ = v_s / v_m represents the apparent phase ratio.

The assumption of $v_m = v_0$ needs to define a boundary between the mobile phase and stationary phase in the column. However, this boundary is not well defined in RP-HPLC. It is generally accepted that the volume of the stationary phase in partition mechanism is totally built up by the bonded phase [5] [65]. In fact, the stationary phase composition and volume vary with the type and length of the alkyl chain, as well as the type and concentration of the organic solvent used in the mobile phase. Taking assumption of $v_m = v_0$ implies that the void volume of the column is the mobile (moving) phase only, not the total liquid volume in the column. In order to make comparison of the thermodynamic quantities among columns for a chromatographic process, the determination of void volume is also critical.

1.4.2.4. Partition-Adsorption Mechanism

A partition-adsorption retention model is a mix mode chromatography model in which analyte retention involves a combination of analyte partition between the mobile phase and the adsorbed liquid layer, followed by analyte distribution onto the bonded phase surface via adsorption [73]. **Figure 1.13** contains a diagram of the partition-adsorption model. When an aqueous-organic binary mobile phase is passing through a reversed-phase HPLC column. At equilibrium, preferential adsorption of the organic solvent by the surface lead to accumulating of a layer of solvents richer in the organic component adsorbed on the stationary phase surface. This layer contains a different organic to water ratio as compared to the bulk mobile phase. The analyte injected into the column will migrate from the bulk mobile phase into this adsorbed liquid layer through liquid-liquid partition, as well as adsorption by the stationary phase surface. The analyte distribution process of this model may be described by a combination of two thermodynamic equilibriums.

(1) Equilibrium between mobile phase and the adsorbed liquid layer.

(2) Equilibrium between the adsorbed layer and the stationary phase.

Nevertheless, this retention model described by partition–adsorption mechanism is formulated under ideal condition with the following assumptions:

(1) The column has been equilibrated at a constant eluent composition which allows the formation of a stable adsorbed liquid layer with different composition to the bulk liquid phase.

(2) A small volume of analyte solution at dilute concentration is injected onto the column. The small amount of injected analyte does not disturb the equilibrium of the binary solvent system.

The partition-adsorption retention equation can be then expressed as:

$$v_R(C_e) = v_0 - V_S(C_e) + K_p(C_e)[V_S(C_e) + SK_H] \quad \text{Eqn. 1.14}$$

Where

v_R = Retention volume of the analyte as a function of the eluent composition

v_0 = Total volume of the liquid phase within the column

V_S = Volume of the adsorbed liquid layer on the bonded phase surface

K_p = Partition constant of the analyte between the bulk mobile phase and the adsorbed liquid layer

K_H = Henry constant of the analyte adsorption from the adsorbed liquid layer to the stationary phase surface.

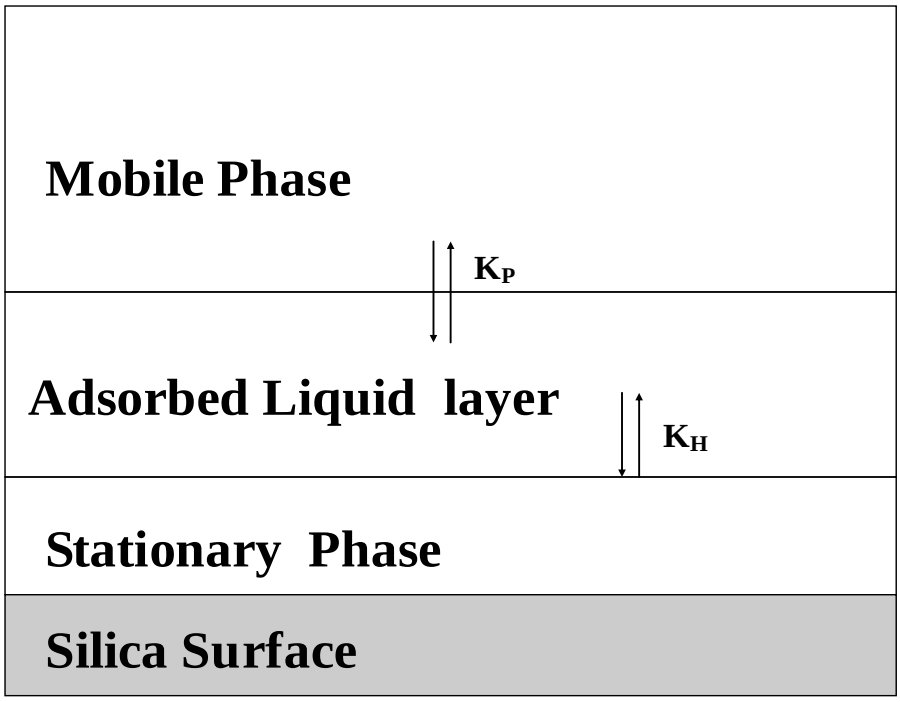


Figure 1.13. *Schematic expression of the partition-adsorption model for reversed-phase HPLC retention.*

1.5. Mathematical Expression for HPLC Retention

1.5.1. Mass Balance Equation

The commonly used retention factor k (capacity factor) for partition mechanism is defined by an empirically generated equation $k = (v_R - v_0)/v_0$. Its validity has to be examined by thermodynamic experiments. In order to achieve a deeper understanding of a chromatographic model, we often connect the retention factor to the mass balance equation applied on it. In gas chromatography, the gas mobile phase and the liquid stationary phase are well distinguished and their volumes are well defined. However, in liquid chromatography the argument of stationary phase volume definition is still on-going. Today, several retention models have been proposed. Therefore, the associated mathematical interpretation need to be applicable to each model. Wilson [74] was the first to use the solution of differential mass balance equation for partition mechanism and Wang et al. [75] applied it to adsorption mechanism using excess adsorption quantitation. Kazakevich summarized the general concept and derivation of the mass balance equation applicable to common HPLC models. Detail derivation can be found in his book “HPLC for Pharmaceutical Scientists”, p37-39 [76]. The concept is based on the following assumptions.

- Molar volumes of the analyte and mobile phase components are constant and compressibility of the liquid phase is negligible.
- The adsorbent is a rigid material impermeable for the analyte and mobile phase molecules.
- The adsorbent is characterized by its specific surface area and pore volume, that are evenly distributed axially and radially in the column.

- The thermal effects to the system are negligible (constant temperature).
- The system is at instant thermodynamic equilibrium.

A final form of the mass balance equation can be then obtained as following equation 1.15. [76]

$$V_R(C) = L\frac{d\Psi(C)}{dC} \qquad \text{Eqn. 1.15}$$

Where:

$V_R(C)$ = Retention volume of the analyte

L = Length of the column

$\Psi(C)$ = Chromatographic distribution function per unit of the column length

Based on this equation, we can see that the correlation of the analyte retention and the retention model is actually determined by its representing distribution function $\Psi(C)$.

1.5.2. Mass Balance Equation Applied to the Partition Model

In this model, the total amount of analyte is distributed between the mobile phase and stationary phase having volumes v_m and V_s, respectively. Thus, the distribution function can be written as:

$$\Psi(C) = v_S C_S + v_m C_m \qquad \text{Eqn. 1.16}$$

Where v_S and v_m are the volume of stationary phase and mobile phase per unit length of the column. C_S and C_m are the equilibrium concentrations of the analyte in stationary phase and mobile phase respectively. Because the analyte concentration in the stationary phase is a function of its concentration in the mobile phase (i.e. $C_S = f(C_m)$). Substitute into equation.1.16 and 1.15, the following equation of retention can be obtained.

$$v_R(C_m) = L\frac{d[v_S f(C_m) + v_m C_m]}{dC_m} \qquad \text{Eqn.1.17}$$

Since v_S = V_S / L and v_m =V_m / L, where V_S and V_m represent the volumes of the stationary and mobile phase. Substitute into equation 1.17, a general equation of retention for partition model eqt.1.18 can be obtained.

$$V_R(C) = V_m + V_s\frac{df(C)}{dC} \qquad \text{Eqn.1.18}$$

In this equation, $\frac{df(C)}{dC}$ is the derivative of the analyte partition distribution function. At low concentration, the distribution function is assumed to be linear to the analyte concentration in the mobile phase and its slope (derivative) is equal to the analyte distribution constant K. Hence, equation 1.18 can be written in its common form.

$$V_R = V_m + V_s K \qquad \text{Eqn.1.19}$$

1.5.3. Mass Balance Equation Applied to the Adsorption Model

Alternatively, calculation of analyte retention using the mass balance equation may be based on adsorption model where the analyte is accumulated on the surface of the stationary phase. Here the stationary phase is considered as impermeable. All retention processes are occurred in the liquid phase. By using surface concentrations and the Gibb's concept of excess adsorption, it is possible to describe the adsorption from a binary solvent system without the definition of the adsorbed phase volume. At equilibrium, a certain amount of the solute will be accumulated on the surface in excess of its equilibrium concentration in the bulk solution. In this model, the total amount of analyte is distributed between the mobile phase and the surface of the stationary phase with a surface area S. Thus, the distribution function can be written as:

$$\Psi(C) = v_0 C_e + s\Gamma(C_e) \qquad \text{Eqn. 1.20}$$

Where:

s is the absorbent surface area and v_0 is the total liquid phase per unit length of the column respectively. C_e is the equilibrium concentration of the analyte in bulk liquid phase and Γ is the excess adsorption per unit of area.

Substitute equation 1.20 into equation 1.15, the analyte retention equation based on excess adsorption mechanism can be written as:

$$\boldsymbol{V_R(C) = V_0 + S\frac{d\Gamma(C)}{dC}} \qquad \text{Eqn.1.21}$$

Where:

S is the total surface area of the stationary phase and V_0 is the total liquid volume in the column.

Usually, the injection volume in HPLC is small that the analyte concentration will be in the linear region of the excess adsorption isotherm (i.e. Henry region). Thus, the

derivative may be substituted by the slope of the excess adsorption isotherm which is known as Henry constant K_H. The retention equation becomes:

$$V_R(C) = V_0 + SK_H \qquad \text{Eqn.1.22}$$

1.5.4. Mass Balance Equation Applied to the Partition-Adsorption Model

The partition-adsorption model assumes formation of an adsorbed liquid layer with different composition to the bulk liquid phase on the adsorbent surface. The analyte distribution process involves a combination of two thermodynamic equilibrium. (1) Equilibrium between mobile phase and the adsorbed layer. (2) Equilibrium between the adsorbed layer and the stationary phase as shown in **Figure 1.13**. The model also assumes the absence of any disturbance to the mobile-stationary phase equilibrium by the small amount of injected analyte (at the Henry region). In isocratic elution, the analyte distribution function may be expressed as [73]:

$$\Psi(C_e) = v_m C_e + v_S C_S + s\Gamma(C_S) \qquad \text{Eqn.1.23}$$

Since $C_S = K_P C_e$

$\Gamma(C_S) = K_H C_S \Rightarrow \Gamma(C_S) = K_H K_P C_e$

and $v_0 = v_m + v_S$

Hence,

$$\Psi(C_e) = \left[v_o + \left(K_p - 1\right)v_s + sK_H K_p\right]C_e \qquad \text{Eqn.1.24}$$

Where:

v_0	Total liquid volume per unit length of the column
v_S	Volume of the adsorbed liquid layer per unit length of the column
C_S	Analyte concentration in the adsorbed liquid layer
C_e	Analyte concentration in the mobile phase
s	Surface area of the adsorbent per unit length of the column
K_P	Partition equilibrium constant of the analyte between the bulk mobile phase and the adsorbed liquid layer
K_H	Henry constant of the analyte adsorption from the adsorbed liquid layer to the stationary phase surface.

Substitute equation 1.24 into equation 1.15. The analyte retention equation based on partition-adsorption mechanism can be written as:

$$V_R(C_{el}) = V_o - V_s(C_{el}) + K_p(C_{el})[V_s(C_{el}) + SK_H] \qquad \text{Eqn. 1.25}$$

Where:

$V_R(C_{el})$	Analyte retention volume as a function of the eluent composition
V_o	Total volume of the liquid phase in the column
$V_s(C_{el})$	Total Volume of the adsorbed layer as a function of the eluent composition
S	Surface area of the adsorbent
$K_p(C_{el})$	Partition equilibrium constant of the analyte between the bulk mobile phase and the adsorbed layer
K_H	Henry constant of the analyte adsorption from the adsorbed liquid layer to the stationary phase surface.

Among these retention equations, equation 1.21 based on excess adsorption mechanism will be used to generate excess adsorption isotherms in this research. There are two advantages for this approach. (1) The amount of excess adsorption can be determined by experimental measurement without a priori assumption of an adsorption model. (2) Since surface area used in equation 1.21 can be accurately measured by Low Temperature Nitrogen Adsorption (LTNA), it can avoid the arguable determination of stationary phase volume.

1.6. Currently Available Common Column Characterization Methods

In the past three decades hundreds of new HPLC columns have been pushed into the market. Most of these columns are based on reversed-phase bonded surface. In fact, many of these columns are very similar with little difference that the vender will claim for particular function or performance. Usually, venders only provide limited information using the test results from their own test methods. These methods are subjective and not enough to adequately categorize the columns. In order to build a more effective strategy to select columns for analytical method development, more efficient and representative methods are needed to allocate different types of columns into repertory. Many methods have been developed to categorize columns using the relative retention of selected solutes such as alkyl benzenes [77], or compare the theoretical retention of characteristic solutes eluted by water [78]. However most of these methods are test compound and mobile phase dependent. They are essentially lack of generality. Traditional column characterization methods can be divided into three major types:

(1) Physical-chemical bulk properties determination on stationary phases.

(2) Using spectroscopic methods such as infrared (IR) and solid-state NMR.

(3) Chromatographic method testing using pre-selected analytes.

1.6.1. Determine by Physical-Chemical Bulk Properties

Bulk properties such as particle size, particle shape, pore size, porosity, specific surface area, bond density and carbon load are usually determined by physical or chemical methods such as low temperature nitrogen adsorption (LTNA), scanning electronic microscope (SEM) and elemental analysis. Most of the time, the column vender will provide some of this data. The dimensional physical parameters mainly determine the column efficiency and to the less extend, selectivity. Bond density and carbon load are related to retention.

Daily chromatographic tests usually required to meet certain system suitability parameters. Four basic parameters are commonly used to monitor the column performance:

(1) Retention factor or capacity factor (k)

(2) Selectivity (α)

(3) Efficiency (N)

(4) Resolution (R)

Retention factor (k) measures the retention of a compound on a particular chromatographic system under a particular eluent and defined as:

$$k=\frac{V_R-V_o}{V_o}=\frac{t_R-t_o}{t_o} \qquad \text{Eqn. 1.26}$$

Where V_R is the analyte retention volume, V_o is the volume of the liquid phase in the column, t_R is the analyte retention time, and t_o is the retention time of a non-retained

analyte. The retention factor is independent of the column dimension and flow rate. Small value of k indicates that the compound is poorly retained by the stationary phase. It is not recommended that analyte retention be too close to the void volume.

Selectivity (α) is a measure of the relative retention of two analytes. Its value indicates the ability of the chromatographic system to discriminate the two analytes and defined as:

$$\alpha = \frac{k_2}{k_1} \qquad \text{Eqn. 1.27}$$

Efficiency (N) of an HPLC system is a measure of the number of theoretical plates that the system can provide. Early chromatography theorized the stationary phase in the column as a stack of N theoretical plates. A thermodynamic equilibrium of the analytes between the mobile and stationary phases occurs within each plate. Thus, the efficiency of the column can be expressed as the number of theoretical plates that the column contains.

$$N = \frac{L}{H} \qquad \text{Eqn. 1.28}$$

Where L is the length of the column and H is the height equivalent to a theoretical plate. Because the dispersion of a peak is a measure of its peak width, N can be considered as a measure of how much is a given solute band will spread during its time in the column. Poor column efficiency will result in band broadening. N can be determined experimentally from a chromatographic system by the following equation 1.29.

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad \text{Eqn. 1.29}$$

Where W is the peak width at the baseline and t_R is the analyte retention time.

Resolution factor (R) provides a measurement on the separation power of a chromatographic column. It is a combined measure of the separation of two analytes by peak dispersion and selectivity. The resolution factor is defined as

$$R = 2\left(\frac{t_2 - t_1}{W_2 - W_1}\right) \qquad \text{Eqn. 1.30}$$

Where t_1 and t_2 are the retention times of compound 1 and compound 2, w_1 and w_2 are the peak widths of compound 1 and compound 2.

1.6.2. Spectroscopic Method

Spectroscopic techniques provide a more direct means to obtain bonded phase structural information. The most popular spectroscopic methods for column categorization are Infrared (IR) and solid state Nuclear Magnetic Resonance (NMR). Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTs) can qualitatively provide evidence for the existence of silanol and silane on the surface [79]. Types of silanols (isolated, geminal or vicinal) can be determined by using ^{29}Si solid state NMR. Type of bonding (mono, di and trifunctional) and end capping can be determined by using ^{13}C solid state NMR [80]. C. R. Silva used CP-MAS-NMR to characterize common alkoxysilane columns [81]. J. Abia used the CP-MAS-NMR to characterize the Cogent bidentate C_{18} ligands bonded to type C silica. Results showed that the surface is densely populated with hydride groups (Si-H), [82].

1.6.3. Chromatographic Method

The disadvantage of physical-chemical methods and spectroscopic techniques is that they can only determine the bulk characteristic of the stationary phase, but are incapable of testing for a particular type of retention associated with the analyte-surface

interaction. Obviously, column categorization must include chromatographic methods. Numbers of these methods have been proposed during the last two decades. These methods may be subdivided into the following two approaches.

(1) By Empirical methods

(2) By Retention model

1.6.3.1. Empirical Methods Based on Selected Test Compounds

These methods categorize the reversed-phase columns by the information collected from some arbitrarily selected test compounds, each compound is supposed to reflect a specific column property such as hydrophobicity, silanol activity and metal activity. These properties together will establish the overall polarity or hydrophobicity of the stationary phase. Representatives of this group are the methods proposed by Tanaka [16], Engelhardt [17], Eyman [83], Walters [84], Gonnet [85], Daldrup [86] and Neue [87].

With respect to the determination of hydrophobicity of the column, most of the tests described in the literature are based on the retentions of benzene derivatives. Engelhardt, Tanaka and Walters defined and calculated hydrophobicity from the relative retention of ethyl benzene / Toluene, amyl benzene / butyl benzene and anthracene / benzene, respectively. In fact, hydrophobicity calculated in this way is actually the hydrophobic selectivity or methylene (CH_2) selectivity since the relative retention is calculated with the retention of two adjacent benzene derivatives from a homologue series. Nevertheless, many results reported by researchers using this kind of experiment showed that the relative retention of alkyl bonded column with different chain lengths do not show significant differences. A large set of reversed phase columns have been tested by Cruz et al using methylene selectivity [88]. Similar results of rather constant hydrophobicity were found. Furthermore, Tanaka [16] reported a

linear dependence of methylene selectivity versus percentage carbon load on a bonded silica column. However in contrast, Engelhadt [17] found a partly non-linear relationship between methylene selectivity and percentage carbon load from several manufacturers. These finding indicated that the hydrophobic selectivity measured by selected analytes is not sufficient to reflect revered phase column hydrophobicity. Testing with orthogonal methods should also be performed for cross-examination and supplement.

1.6.3.2. Evaluation Method Based on Retention Model

These types of method characterize reversed phase columns based on a specific chromatographic model. The representing method among them is linear solvation energy relationships (LSER) model which was defined by Abraham from the solvatochromic method [89].

In late 1970s, Kamlet, Taft [90] [91] and their co-workers developed the solvatochromic model to characterize solute-solvent interaction in different distribution processes such as solute dissolving in solvent, solute distributing between two immiscible solvents as well as distributing between a gas and its condensed phase. The solute's solvatochromic parameters derived by spectroscopic measurements described the different molecular interactions such as cavity formation, dispersion, dipolarity, polarizability, hydrogen bond accepter (basicity) and hydrogen bond donor (acidity). These solvatochromic parameters have been used to study distribution processes including GC and HPLC separations. However, the lack of solvotachromic parameters for less common solvents and a large number of solute parameters needed to be estimated from a very small solvent data base became the hindrance for further development by this model.

In 1993, Abraham introduced a set of new Gibbs thermodynamic energy related solute descriptors called solvation parameters. These parameters were derived from

the equilibrium measurements of the solutes themselves, such as GC data, water-solvent partition coefficients and data related to molecular structure [92] [93]. The quantities were then used in his LSER equation and co-related to chromatographic retention as shown below.

$$\log k = C_1 + rR_2 + s\pi_2^* + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + vV_x \qquad \text{Eqn. 1.31}$$

Where the intercept C_1 is a solute independent constant related to the stationary and mobile phase ratio. rR_2, $s\pi_2^*$, $a\Sigma\alpha_2^H$, $b\Sigma\beta_2^H$ and vV_x account for intermolecular interaction between the solute and the mobile phase as well as stationary phase. The subscripted Greek letter symbols represent the solute properties as following which have been estimated for a large number of simple compounds.

R_2 = Excess molar refraction

π_2^* = Dipolarity / polarizability

$\sum\alpha_2^H$ = Overall effective hydrogen bond donor (acidity)

$\sum\alpha\beta_2^H$ = Overall effective hydrogen bond acceptor (basicity)

V_x = McGowan characteristic volume

Given a representative set of test solutes with known properties, the corresponding parameters r, s, a, b, and v can be determined with multivariate regression analysis for a given reversed phase column under a fixed set of chromatographic conditions. Hence the parameters r, s, a, b, and v can be used to characterize and categorize the column using the same fixed set of chromatographic conditions.

Recently, Jandera and Vynuchalova [94] tested 17 common reversed-phase columns using classical hydrophobicity, silanol activity, alkylbenzene homologous and LSER classifications. Most of the columns tested show certain different selectivity and retention in acetonitrile-water and methanol-water mobile phases.

Although large number of studies have been carried out to improve the LSER characterization methods and more test solute descriptors have been characterized, persistent limitations still exist:

- LSER characterization rely on selected test compounds. Different column may be characterized with different set of solutes. Hence the method is lack of generality. Furthermore, some compounds with multiple properties may lead to replicate count for stationary phase characterization.
- Columns are characterized by chromatograph the test solutes with a specific composition of mobile phase, hence the method is considered to be local only.
- The model assumes a linear relationship between the free energy and the chromatographic system. This assumption may not be true because the solute properties were measured by different methods such as spectroscopic, other than reversed-phase chromatography.
- The LSER intercept C_1 is very difficult to interpret since they contain effects such as phase ratio and the other complex properties of the test solute which may be significant. Thus, the intercepts certainly contain chemical information but are almost never interpreted.

1.6.4. Categorization of Reversed-Phase Liquid Chromatographic Column

By applying the column characterization methods, a data base containing numerous of data will be generated. Meaningful results have to be extracted from these data. In recent years, chemometric methods such as cluster analysis (CA), factor analysis (FA) and the most widely used principal component analysis (PCA) have been successfully applied for the interpretation of chromatographic data and categorization of stationary phases [95 - 98].

PCA is a general tool for interpretation of large data base. The principal is to reduce the large number of variables that are representing different column properties by projecting them onto a smaller number of new variables called principal components (PC). The number of original variables included in a principal component is called loadings of the PC. The value projected onto this PC is called the score. By plotting the scores of the PCs, it is possible to graphically show the similarity and difference among the columns.

In summary, High Performance Liquid Chromatography (HPLC) is one of the analytical technique widely used in environmental, pharmaceutical, polymer and food industries. General HPLC methods can be categorized into normal-phase, reversed-phase, ion exchange, ion pairing, size exclusion and chiral separations. The most popular one is reversed-phase liquid chromatography. Although compounds are separated by their relative affinity to both stationary and mobile phases in chromatography, the stationary phase often plays a major role in selectivity. Most of the HPLC stationary phases are made with silica due to its rigidity, easy particle size and porosity control. Also, the silanol groups on silica surface can be covalently bonded to other functional groups at high coverage. Several retention mechanisms have been introduced to explain solute distribution between the stationary phase and mobile phase. For our purpose, we will apply excess adsorption mechanism in our research.

Chapter 2:

Estimation of Reversed-Phase HPLC Column Hydrophobicity by Non-polar to Polar Solvents Adsorption Ratio Using Excess Adsorption Isotherms

2.1 Introduction

In the past thirty years, a large number of reversed-phase HPLC stationary phases have been developed to fulfil the market need. This rapid column development makes column selection a serious problem for chromatographic method development. As mentioned in Section 1.2, venders only provide limited information using the test results from their own test methods. Similarly, the currently available methods generally categorize columns by gathering information from the retention factors of some arbitrarily selected standard solute compounds [99]. These methods rely on the selectivity of a pre-selected set of analytes under a pre-selected mobile phase system are subjective and lack of generality. In order to assist column selection, we need a more general method to categorize these columns in our repertory.

As we know, the primary driving force for solute retention on a reversed-phase surface is hydrophobic interaction. General column properties such as the type of bonded phase, bond density, surface coverage and bonded surface end capping directly impact the strength of hydrophobic interaction. As a fact, hydrophobicity becomes a major parameter for initial categorization of reversed-phase columns. Other

specific characteristics such as π- π interaction may be also added for auxiliary selectivity evaluation. Currently, several common methods have been developed to determine the column hydrophobicity. Tanaka et al estimated hydrophobicity through deuterium isotope exchange [100]. Buszewsky [101], Tanaka [16] and Engelhardt [17] expressed column hydrophobicity in terms of their selectivity to a homologue of benzene derivatives. Again, all these methods are relying on a set of particularly selected test solutes and mobile phases. In order to pre-screen the columns in a more general way, we proposed a method to determine column hydrophobicity by the ratio of adsorbed organic solvent to water on the column surface using excess adsorption isotherm estimation.

2.2 Experimental

2.2.1. Volume Change Test for Acetonitrile and Methanol Mixed with Water

Acetonitrile and methanol are the most commonly used organic modifiers for reversed-phase HPLC. Using these solvents for HPLC column characterization will better match the mobile phase components for general liquid chromatography. However, mixing of acetonitrile or methanol with water might lead to slight volume contraction. The impact of volume change after mixing of these organic solvents with water were investigated. Experiments were carried out by mixing 0, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 190, and 200 mL of acetonitrile with 200, 190, 180, 160, 140, 120, 100, 80, 60, 40, 20, 10, and 0 mL of water as well as methanol with water in stoppered graduated cylinders. The volumes of the mixed liquids were then measured and their % change in volume were then calculated.

2.2.2. HPLC Systems

Two HPLC systems were used. HPLC System (I) for excess adsorption isotherm estimation was an Agilent 1050 system (Hewlett Packard, New Castle, DE, USA) equipped with an Erma Optical, ERC 1570 RI Detector (ERMA, Kingston, MA, USA) maintained at 25 °C. Experiments for these studies were run in isocratic mode at 1.0 mL/minute flow rate. The mobile phase systems used in the experiments contain 0% to 100% of (1) acetonitrile in water and (2) methanol in water. Column temperatures were maintained at 25ºC. For the experiments using acetonitrile / water as mobile phase, 1 uL of deuterated acetonitrile and deuterated water was injected. For the experiments using methanol / water as mobile phase, 1 uL of deuterated methanol and deuterated water was injected. Column void volumes and excess adsorption isotherms were calculated using the retention volumes of minor disturbance peaks obtained from the injection of deuterated organic solvents and confirmed with the injection of deuterated water.

HPLC system (II) for column surface hydrophobicity tests with alkyl benzene homologues and phenol was an Agilent 1100 system (Agilent Technologies, Palo Alto, Ca. USA) equipped with an UV detector at 260 nm wavelength. Column temperature was maintained at 25ºC. For each experiment, 1 uL of benzene, toluene, ethyl benzene, propyl benzene, butyl benzene and phenol solutions at 1000 ppm in acetonitrile and methanol were injected. All sample solutions were run in isocratic mode at 1.0 mL/minute flow rate via mobile phases containing 50% acetonitrile and 60% methanol in water. All eluents were degassed with the built-in HPLC degasser.

2.2.3. Columns

Four custom made alkyl bonded columns and four other types of commercially available columns were studied. **Table 2.1** contains the physical parameters of these columns. All four custom made alkyl columns were bonded on the same lot of silica and packed into 150 mm x 4.6 mm stainless steel columns (Phenomenex Inc. Torrance, Ca.). Phenomenex also supplied the geometric parameters [35]. The chemical structures of bonded phases are shown in **Figure 2.1**.

2.2.4. Solvents and Chemicals

Acetonitrile (ACN) and methanol (MeOH) were HPLC grade and purchased from Pharmco (Philipsburg, PA, USA). Water was purified by a Milli-Q system from Millipore (Milford, MA. USA). Alkyl benzenes, phenol, deuterated acetonitrile, deuterated methanol and deuterated water were purchased from Sigma–Aldrich (St. Louis, MO, USA).

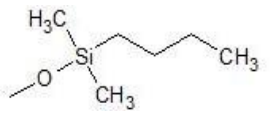
Table 2.1. *Physical parameters of the columns used in the experiments*

Brand	**Column**	**Column Dia. & Length (mm)**	**Particle Size (µm)**	**Specific Surface Area (m^2/g)**	**Pore Size (Å)**	**Bond Density (µmole/ m^2)**	**End cap**	**Carbon Load (%)**
Custom Made	Column A Alkyl C_4	4.6x150	-	[3]374	100	4.15	N	9.5
Custom Made	Column B Alkyl C_8	4.6x150	-	[3]374	100	3.35	N	12.4
Custom Made	Column C Alkyl C_{12}	4.6x150	-	[3]374	100	3.22	N	15.9
Custom Made	Column D Alkyl C_{18}	4.6x150	-	[3]374	100	3.13	N	20.6
Agilent	Zorbax SB-C_{18}	4.6x150	5	180	80	2.04	N	10.0
Phenomenex	[1]Curosil-PFP	4.6x150	5	263	100	2.2	Y	5.7
Phenomenex	Luna - Phenyl Hexyl	4.6x150	5	400	100	4.0	Y	17.5
MicroSolv	[2]Cogent-UDC	4.6x150	4	350	100	1.5	N	7.5

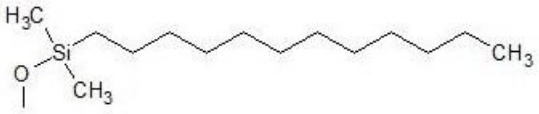
[1] Curosil-PFP: Perfluorophenyl column

[2] Cogent-UDC: Cholesterol column

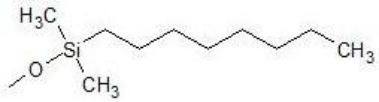
[3] Specific surface area of the bare silica



Alkyl C_4

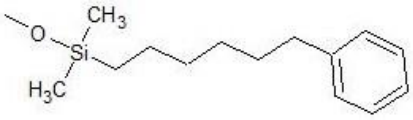


Alkyl C_{12}

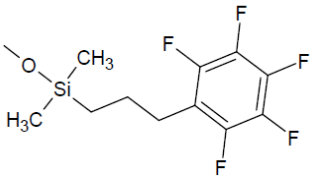


Alkyl C_8

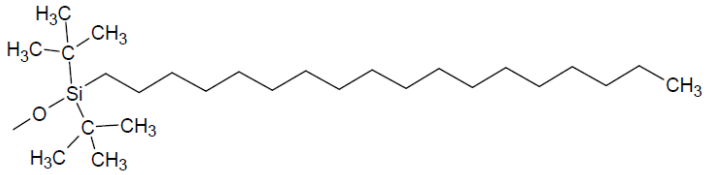
Alkyl C_{18}



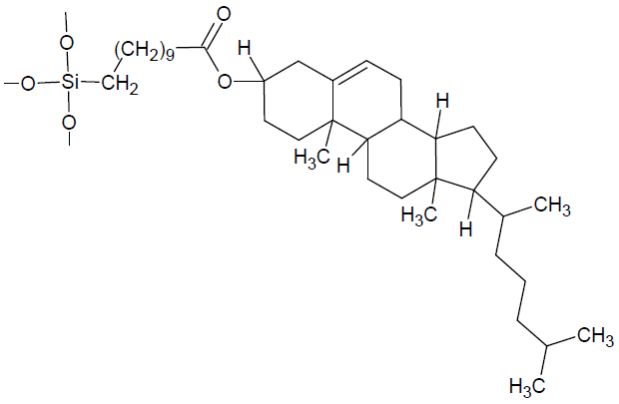
Phenyl Hexyl



Perfluorophenyl



Zorbax SB-C_{18}



Cholesterol

Figure 2.1. *Ligand structures of the columns used in this research*

2.3. Results

Table 2.2 and **Table 2.3** contains the volume change data of acetonitrile and methanol mixed with water. The test results are also plotted in **Figure 2.2**.

Figure 2.3 to **Figure 2.6** show the plot of retention volume versus the percentage of acetonitrile and methanol in the mobile phase. Four custom made alkyl bonded phase columns from C_4 to C_{18} were tested to evaluate the impact of alkyl chain length on the surface hydrophobicity. Since the alkyl ligands of these columns were bonded on the same silica base, the substrate effect can be eliminated. In addition, a commercially available Zorbax SB-C_{18} column and columns with other types of bonded phases including two phenyl columns and a cholesterol column were also tested for comparison. The excess adsorption isotherms of acetonitrile and methanol on these columns are shown in **Figure 2.7** to **Figure 2.10**. **Figure 2.11** shows the representative excess adsorption isotherms of the custom made alkyl C_{18} column with its corresponding linear lines for acetonitrile and methanol in water. The volumes of organic component and water adsorbed on the surface is calculated by equation 2.15 using the slope and intercept of the straight line plotted according to the linear region of the isotherm. **Table 2.4** contains the slope and intercept data of this straight line. The calculated volume and volume ratio of the adsorbed solvents on each column are shown in **Table 2.5.** Alkylbenzene homologous series are often used for HPLC column hydrophobicity estimation. For convenient comparison purpose, we used the alkyl selectivity (α) which is the capacity factor ratio of each alkyl benzene / benzene and the capacity factor ratio of phenol / benzene in this experiment. The natural logarithm of alkyl selectivity (ln(α)) for alkyl benzenes and phenol are presented in **Table 2.6** and **Table 2.7** to compare with the volume ratio of the adsorbed solvents. Test results are also graphically presented in **Figure 2.12** to **Figure 2.15**.

Table 2.2. *The total liquid volume contraction for mixing acetonitrile with water expressed in percent of theoretical total volume (200 mL)*

Acetonitrile Volume (mL)	**Water Volume (mL)**	**Acetonitrile (%V/V)**	**Water (%V/V)**	**Measured Final Volume (mL)**	**Volume Contraction (%)**
0	200	0	100	200	0
10	190	5	95	200	0
20	180	10	90	199	0.5
40	160	20	80	198	1
60	140	30	70	198	1
80	120	40	60	198	1
100	100	50	50	198	1
120	80	60	40	198	1
140	60	70	30	199	0.5
160	40	80	20	199	0.5
180	20	90	10	200	0
190	10	95	5	200	0
200	0	100	0	200	0
Note: Theoretical Total Volume = 200 mL					

Table 2.3. *The total liquid volume contraction for mixing methanol with water expressed in percent of theoretical total volume (200mL)*

Methanol Volume (mL)	**Water Volume (mL)**	**Methanol (%V/V)**	**Water (%V/V)**	**Measured Final Volume (mL)**	**Volume Contraction (%)**
0	200	0	100	200	0
10	190	5	95	199	0.5
20	180	10	90	198	1
40	160	20	80	197	1.5
60	140	30	70	195	2.5
80	120	40	60	194	3
100	100	50	50	195	2.5
120	80	60	40	196	2
140	60	70	30	196	2
160	40	80	20	198	1
180	20	90	10	199	0.5
190	10	95	5	200	0
200	0	100	0	200	0
Note: Theoretical Total Volume = 200 mL					

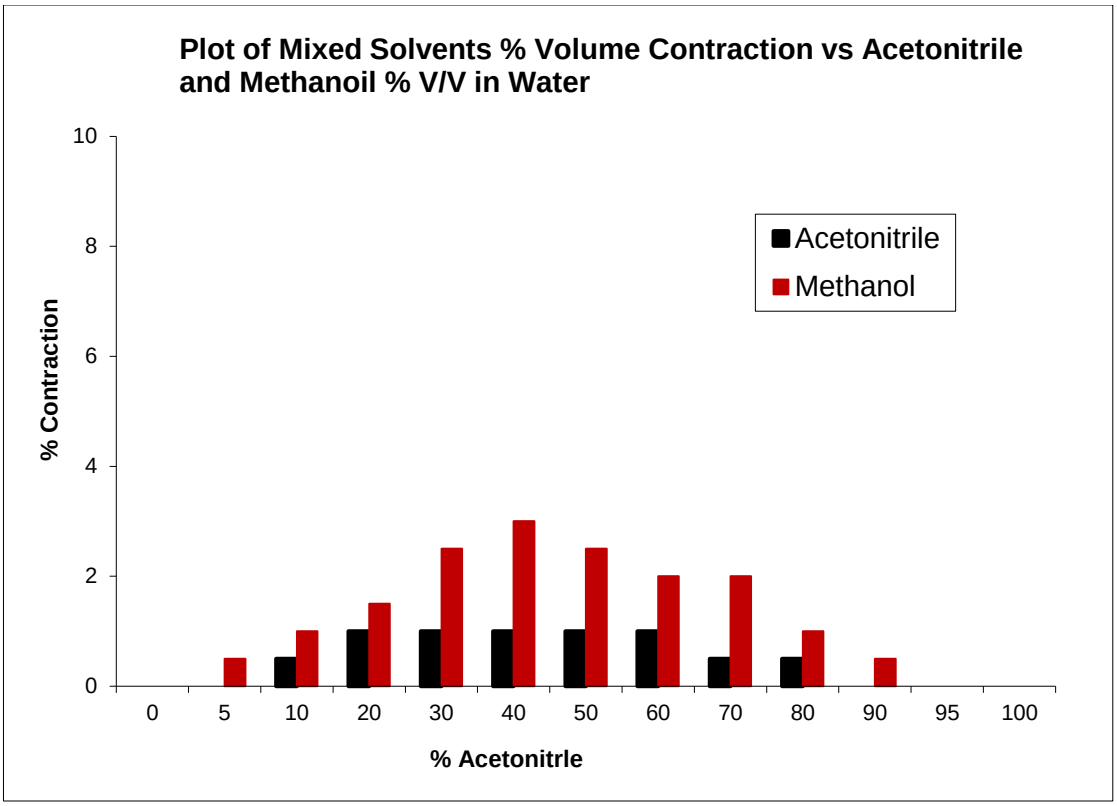


Figure 2.2. *Plot of mixed solvents % volume contraction versus acetonitrile and methanol % V/V in water*

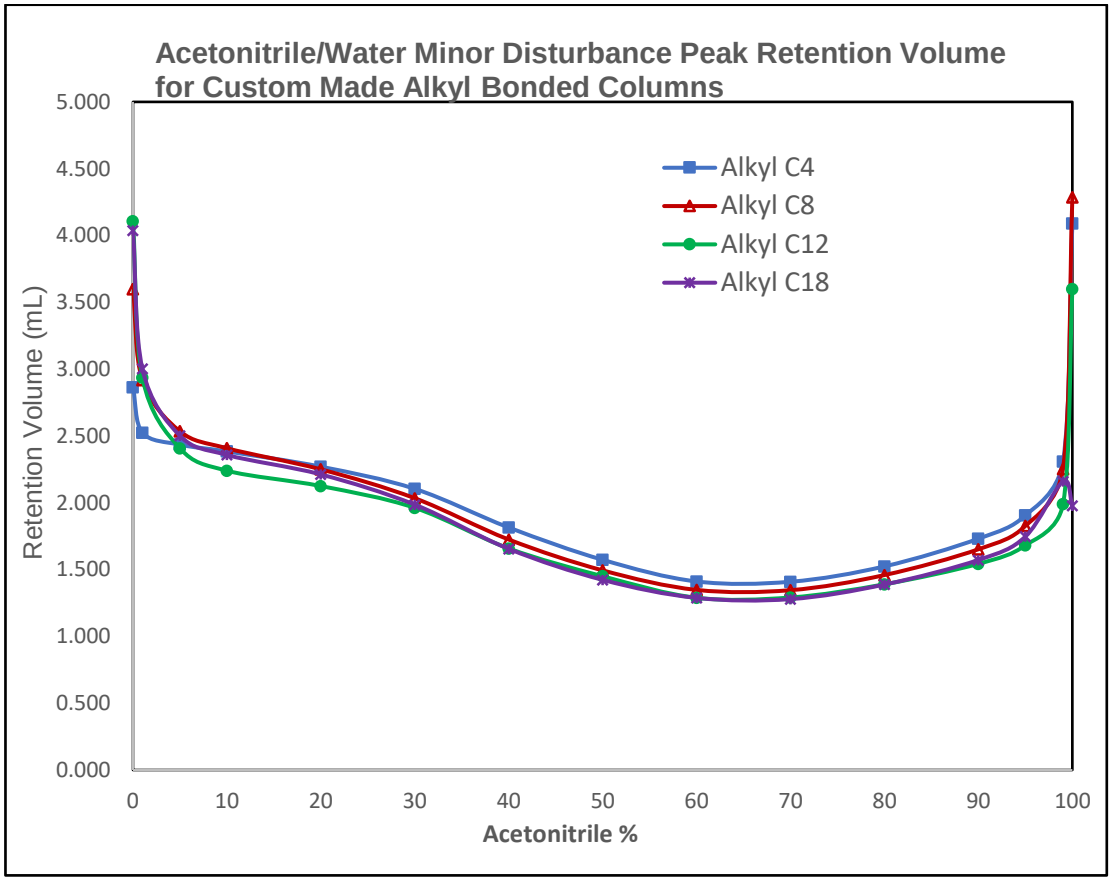


Figure 2.3. *Retention volume (mL) of the minor disturbance peak using mobile phases containing 0 to 100 % acetonitrile in water on custom made alkyl C_4, C_8, C_{12} and C_{18} columns.*

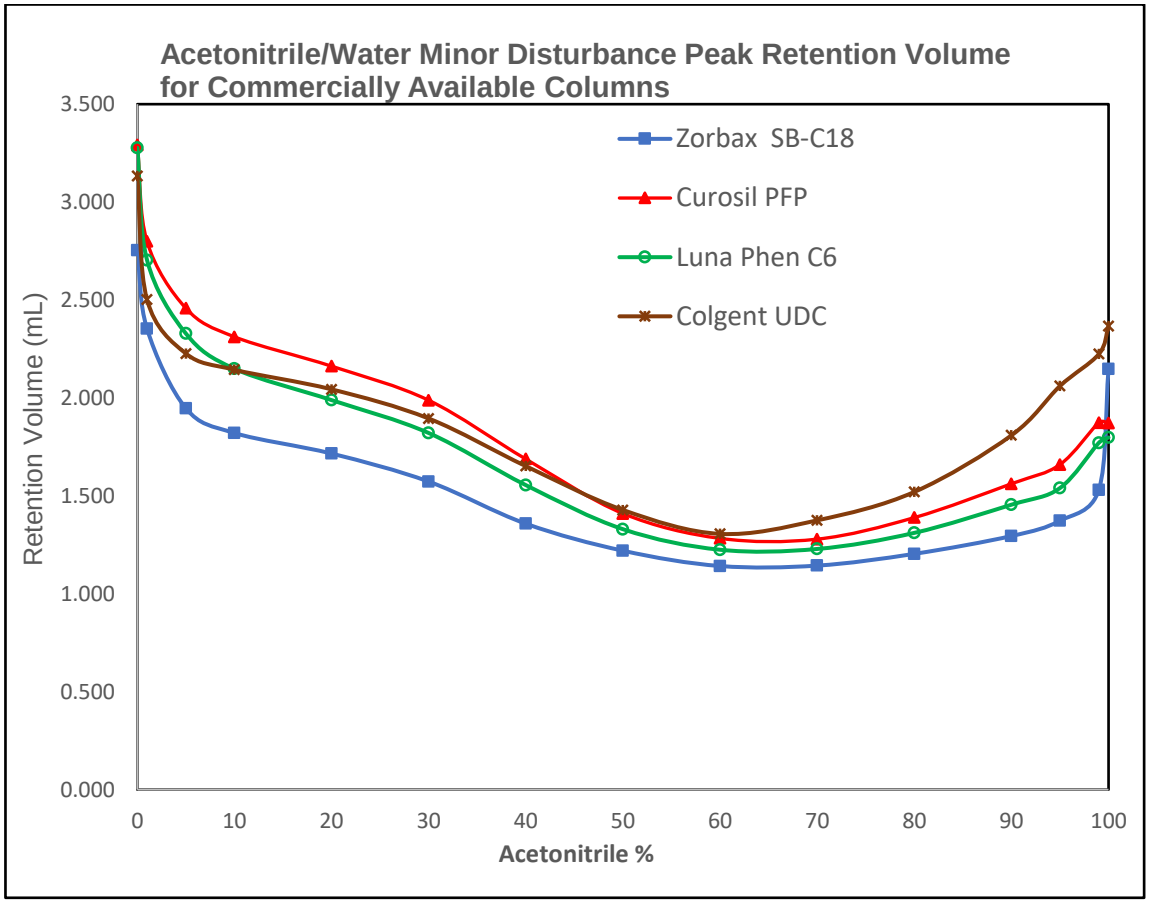


Figure 2.4. *Retention volume (mL) of the minor disturbance peak using mobile phases containing 0 to 100 % acetonitrile in water on Zorbax SB-C_{18}, Curosil* Perfluorophenyl*, Luna Phenyl Hexyl and Cogent UDC* Cholesterol *columns.*

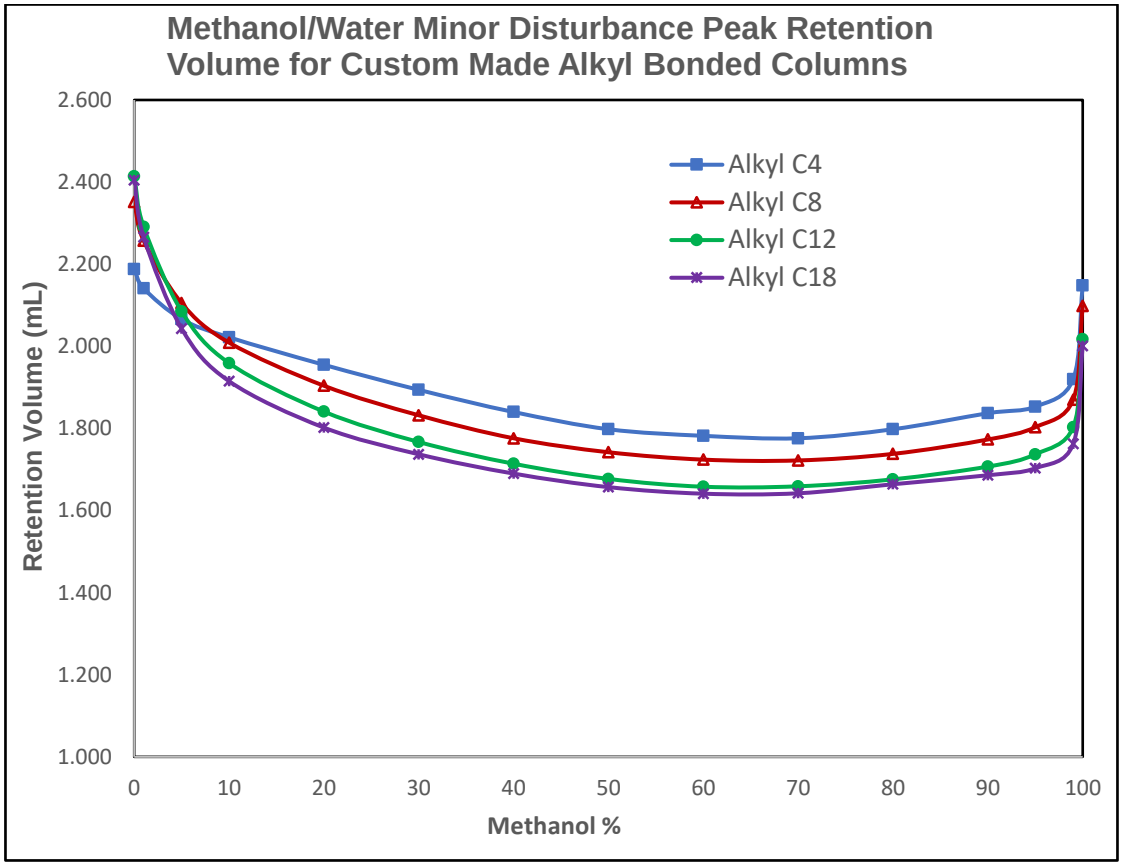


Figure 2.5. *Retention volume (mL) of the minor disturbance peak using mobile phases containing 0 to 100 % methanol in water on custom made alkyl C_4, C_8, C_{12} and C_{18} columns.*

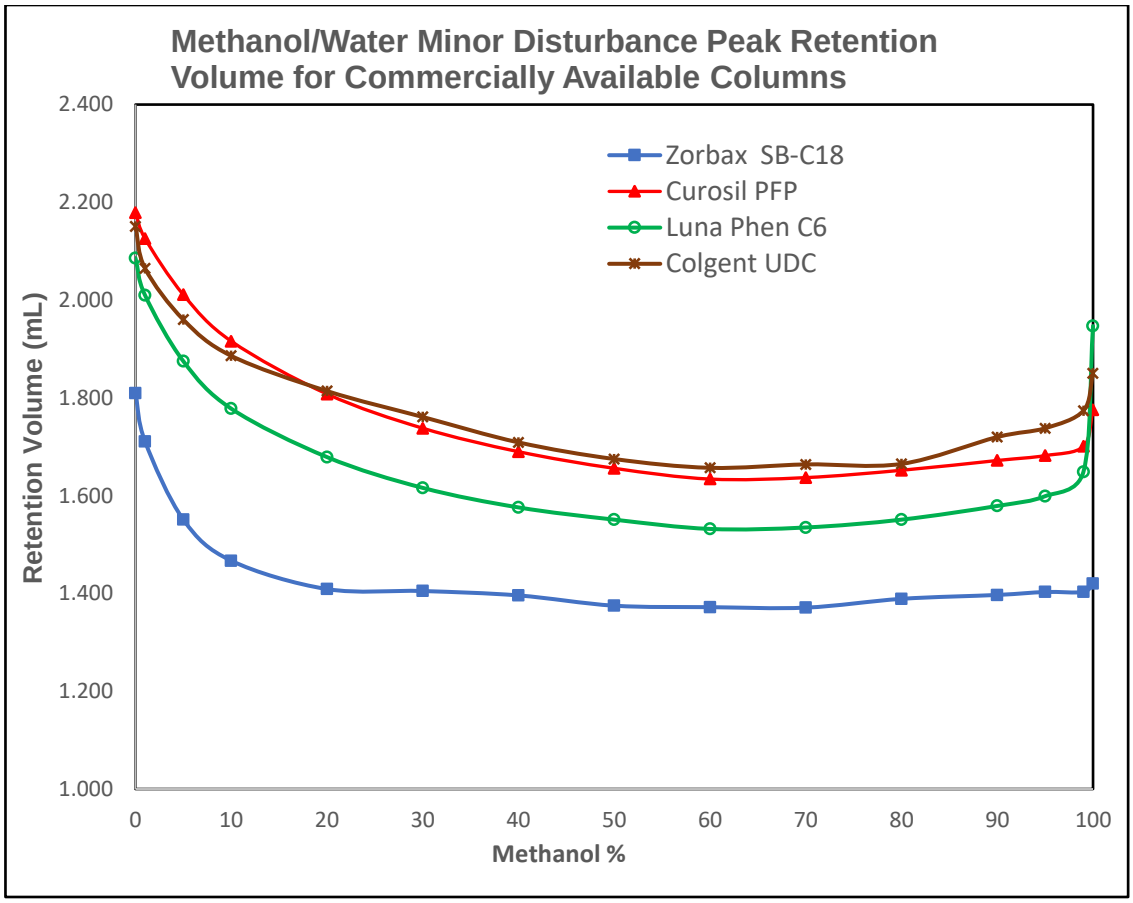


Figure 2.6. *Retention volume (mL) of the minor disturbance peak using mobile phases containing 0 to 100 % methanol in water on Zorbax SB-*C_{18}*, Curosil* Perfluorophenyl*, Luna Phenyl Hexyl and Cogent UDC* Cholesterol *columns.*

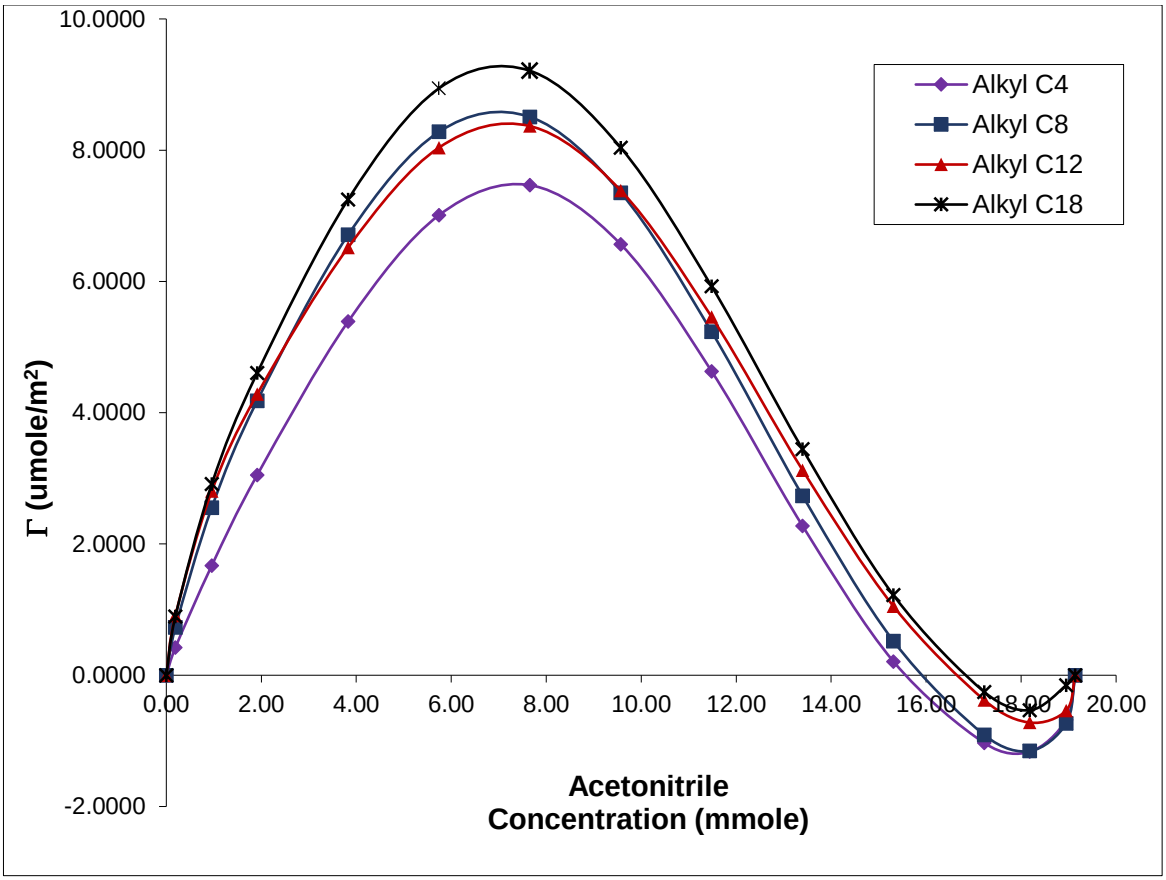


Figure 2.7. *Overlaid excess adsorption isotherms of acetonitrile in water on custom made alkyl C_4, C_8, C_{12} and C_{18} columns.*

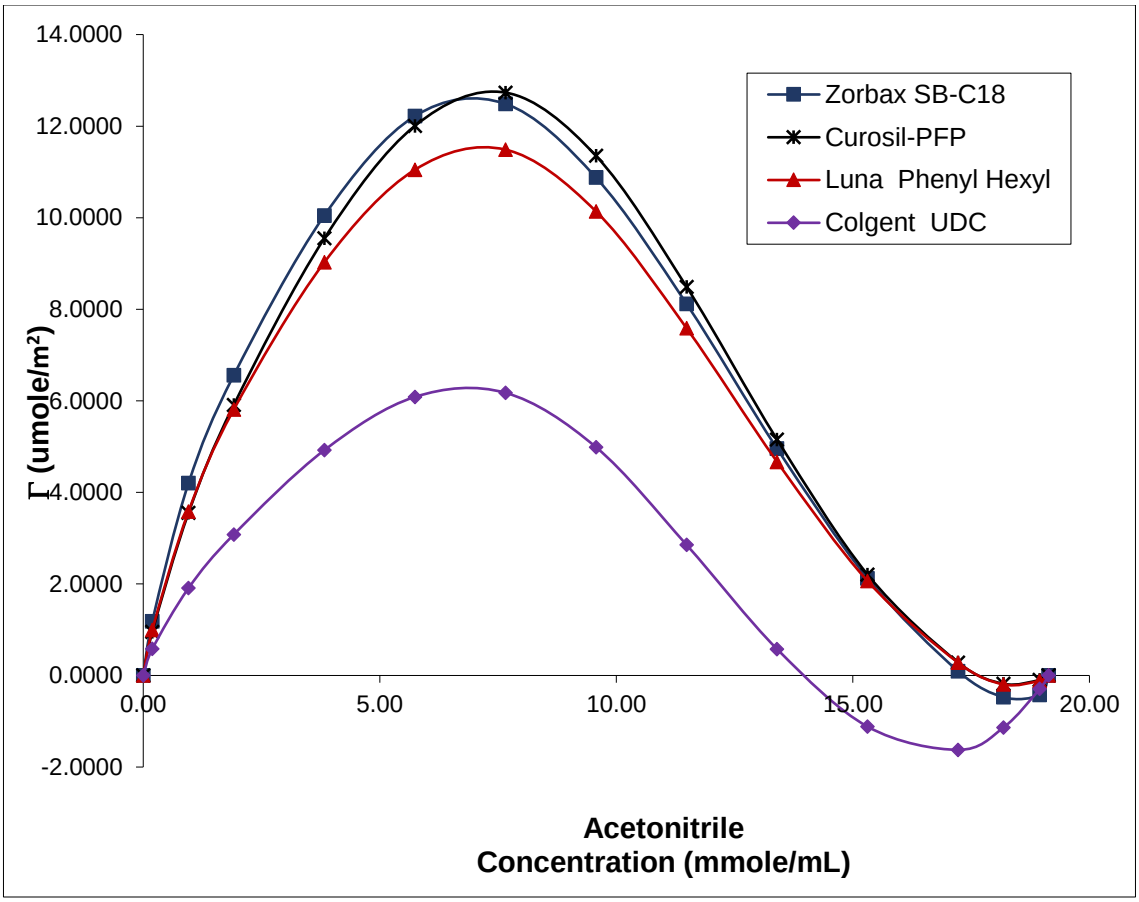


Figure 2.8. *Overlaid excess adsorption isotherms of acetonitrile in water on Zorbax SB-C_{18}, Curosil-PFP, Luna Phenyl Hexyl, Cogent-UDC columns.*

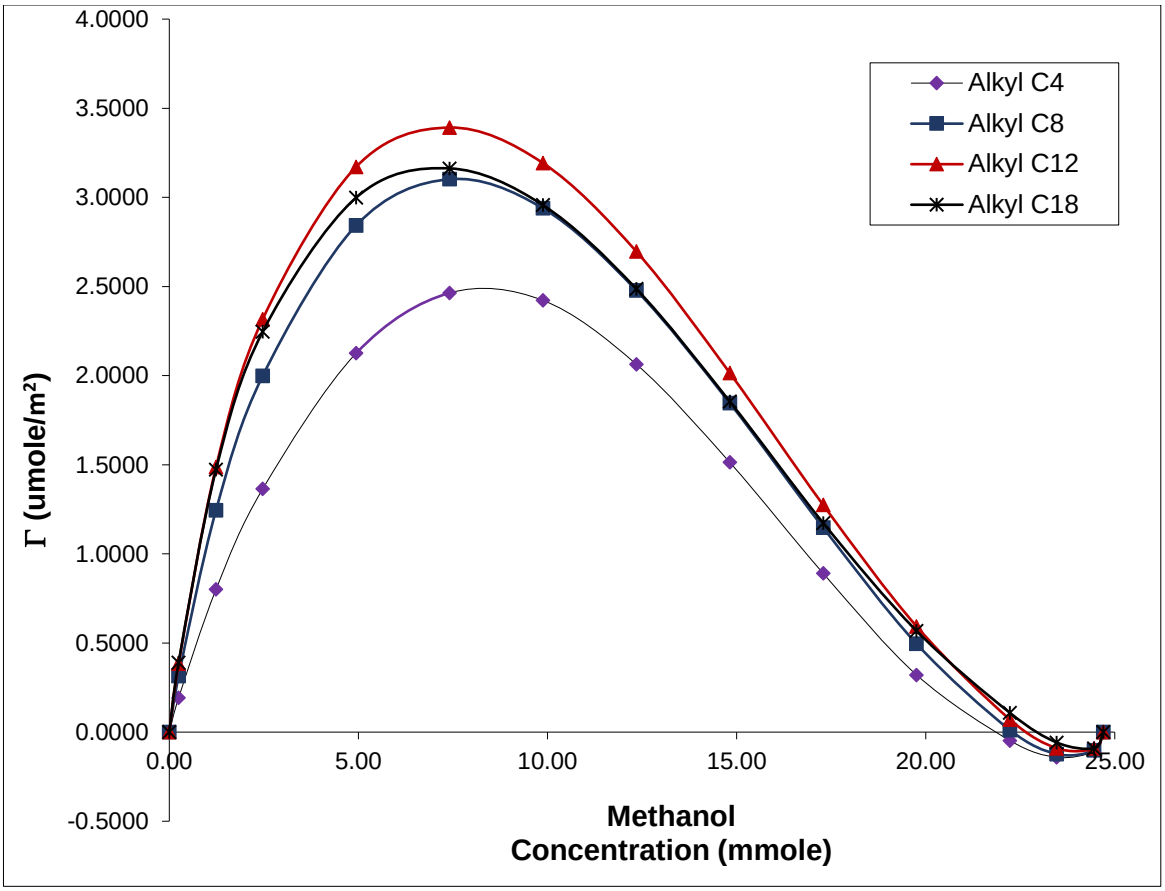


Figure 2.9. *Overlaid excess adsorption isotherms of methanol in water on custom made alkyl C_4, C_8, C_{12} and C_{18} columns.*

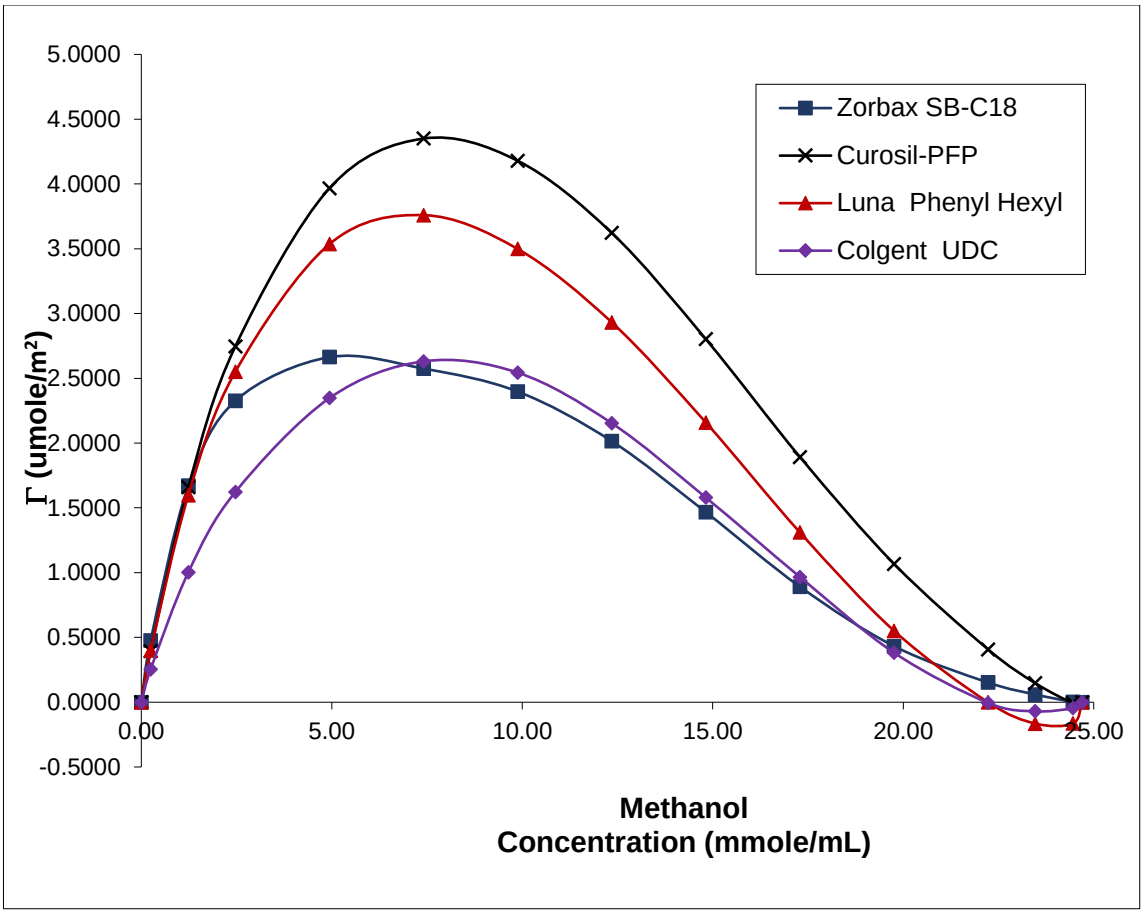


Figure 2.10. *Overlaid excess adsorption isotherms of methanol in water on Zorbax SB-C_{18}, Curosil-PFP, Luna Phenyl Hexyl, Cogent-UDC columns.*

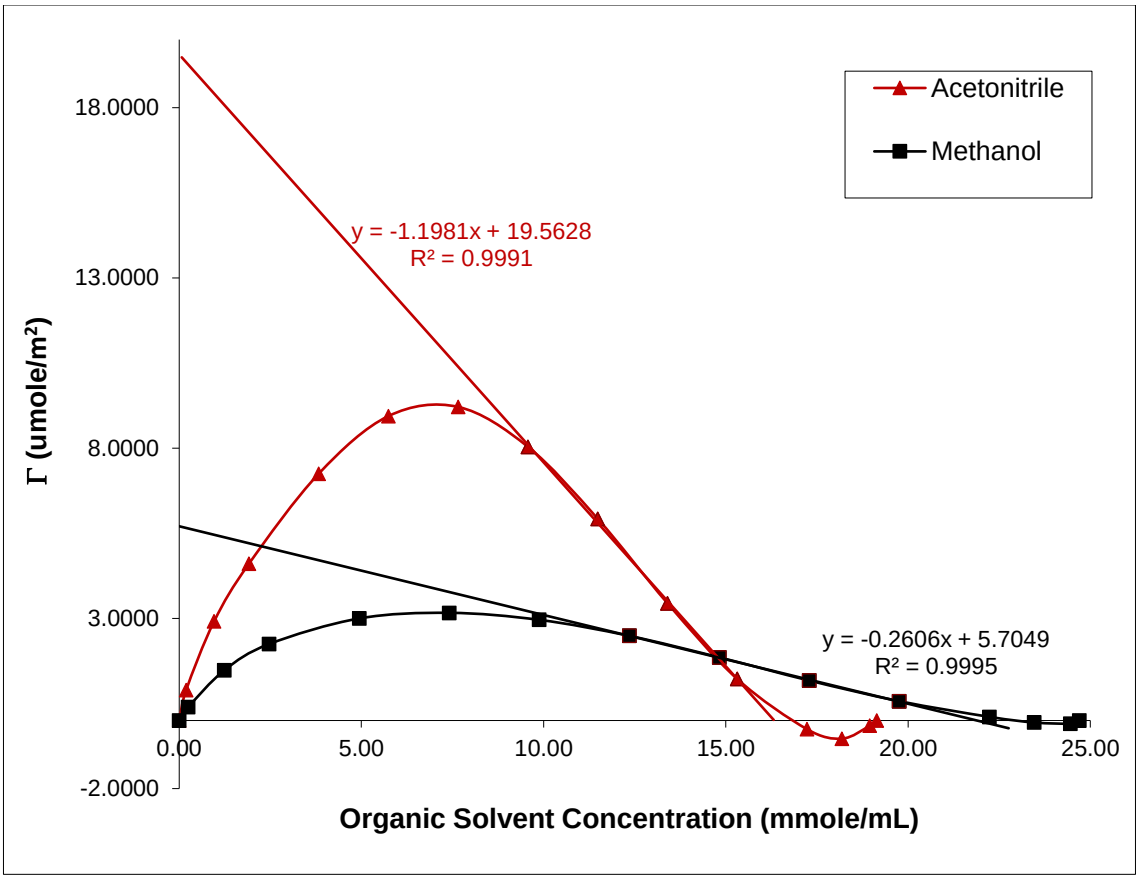


Figure 2.11. *Representative excess adsorption isotherms of acetonitrile and methanol in water on alkyl C_{18} column with extrapolated straight lines at the linear region.*

Table 2.4. *Slope and intercept of the linear plot at the linear region of the excess adsorption isotherms*

Column	Acetonitrile-Water Mobile Phase			Methanol-Water Mobile Phase		
	Slope	**Intercept**	**Correlation Coeff. (R^2)**	**slope**	**Intercept**	**Correlation Coeff. (R^2)**
Column A-Alkyl C_4	1.1193	17.3453	0.9987	0.2369	4.9998	0.9995
Column B-Alkyl C_8	1.2013	18.9052	0.9991	0.2691	5.8115	0.9997
Column C-Alkyl C_{12}	1.1148	18.1240	0.9988	0.2854	6.2269	0.9997
Column D-Alkyl C_{18}	1.1981	19.5628	0.9991	0.2606	5.7049	0.9995
Zorbax SB C-$_{18}$	1.5373	25.6429	0.9994	0.2154	4.6601	0.9979
Curosil PFP	1.6058	26.8081	0.9992	0.3471	7.9188	0.9996
Luna Phenyl hexyl	1.4184	23.7960	0.9994	0.3235	6.9325	0.9997
Cogent UDC	1.0758	15.2087	0.9965	0.2400	5.1229	0.9999

Table 2.5. *Volume of solvents adsorbed on the surface calculated from the linear region of the excess adsorption isotherm (mL/m^2)*

Column	Acetonitrile-Water Mobile Phase			Methanol-Water Mobile Phase		
	Acetonitrile Volume	**Water Volume**	**Volume Ratio***	**Methanol Volume**	**Water Volume**	**Volume Ratio***
Column A-Alkyl C_4	0.3389	0.0797	4.253	0.0757	0.0129	5.878
Column B-Alkyl C_8	0.3694	0.0799	4.625	0.0880	0.0126	6.962
Column C-Alkyl C_{12}	0.3541	0.0628	5.640	0.0943	0.0125	7.567
Column D-Alkyl C_{18}	0.3823	0.0658	5.806	0.0864	0.0111	7.786
Zorbax SB C_{18}	0.2412	0.0356	6.783	0.0352	0.0058	7.046
Curosil PFP	0.3674	0.0545	6.743	0.0842	0.0070	12.092
Luna Phenyl hexyl	0.3261	0.0465	7.011	0.0737	0.0113	6.545
Cogent UDC	0.2781	0.0984	2.826	0.0726	0.0114	6.368

*Organic solvent volume / Water volume

Table 2.6. *Comparison of acetonitrile / water adsorption volume ratio and alkyl benzenes selectivity (α) eluted by acetonitrile / water (50% v/v)*

Column	Ln(α)					Vol. Ratio
	Toluene/ Benzene	Ethyl benzene/ Benzene	Propyl benzene/ Benzene	Butyl benzene/ Benzene	Phenol/ Benzene	Acetonitrile / Water
Column A-Alkyl C_4	0.271	0.544	0.836	1.117	-0.817	4.253
Column B-Alkyl C_8	0.338	0.669	1.034	1.393	-0.999	4.625
Column C-Alkyl C_{12}	0.393	0.769	1.193	1.614	-1.138	5.640
Column D-Alkyl C_{18}	0.439	0.849	1.322	1.793	-1.247	5.805
Zorbax SB C_{18}	0.507	0.991	1.539	2.080	-1.409	6.783
Curosil PFP	0.384	0.717	1.090	1.464	-1.060	6.743
Luna Phenyl hexyl	0.386	0.784	1.207	1.623	-1.227	7.011
Cogent UDC	0.357	0.671	1.041	1.422	-0.974	2.826

Table 2.7. *Comparison of methanol / water adsorption ratio and alkyl benzenes selectivity (α) eluted by methanol / water (60% v/v)*

Column	Ln(α)					Vol. Ratio
	Toluene/ Benzene	Ethyl benzene/ Benzene	Propyl benzene/ Benzene	Butyl benzene/ Benzene	Phenol/ Benzene	Methanol / Water
Column A- Alkyl C_4	0.363	0.720	1.132	1.550	-0.750	5.878
Column B- Alkyl C_8	0.477	0.913	1.414	1.929	-0.937	6.962
Column C- Alkyl C_{12}	0.547	1.029	1.589	2.164	-1.097	7.567
Column D- Alkyl C_{18}	0.598	1.111	1.711	2.325	-1.279	7.785
Zorbax SB C_{18}	0.653	1.231	1.890	2.560	-1.341	7.046
Curosil PFP	0.579	1.017	1.525	2.069	-0.777	12.092
Luna Phenyl hexyl	0.557	1.090	1.667	2.265	-1.133	6.545
Cogent UDC	0.492	0.881	1.348	1.853	-0.970	6.368

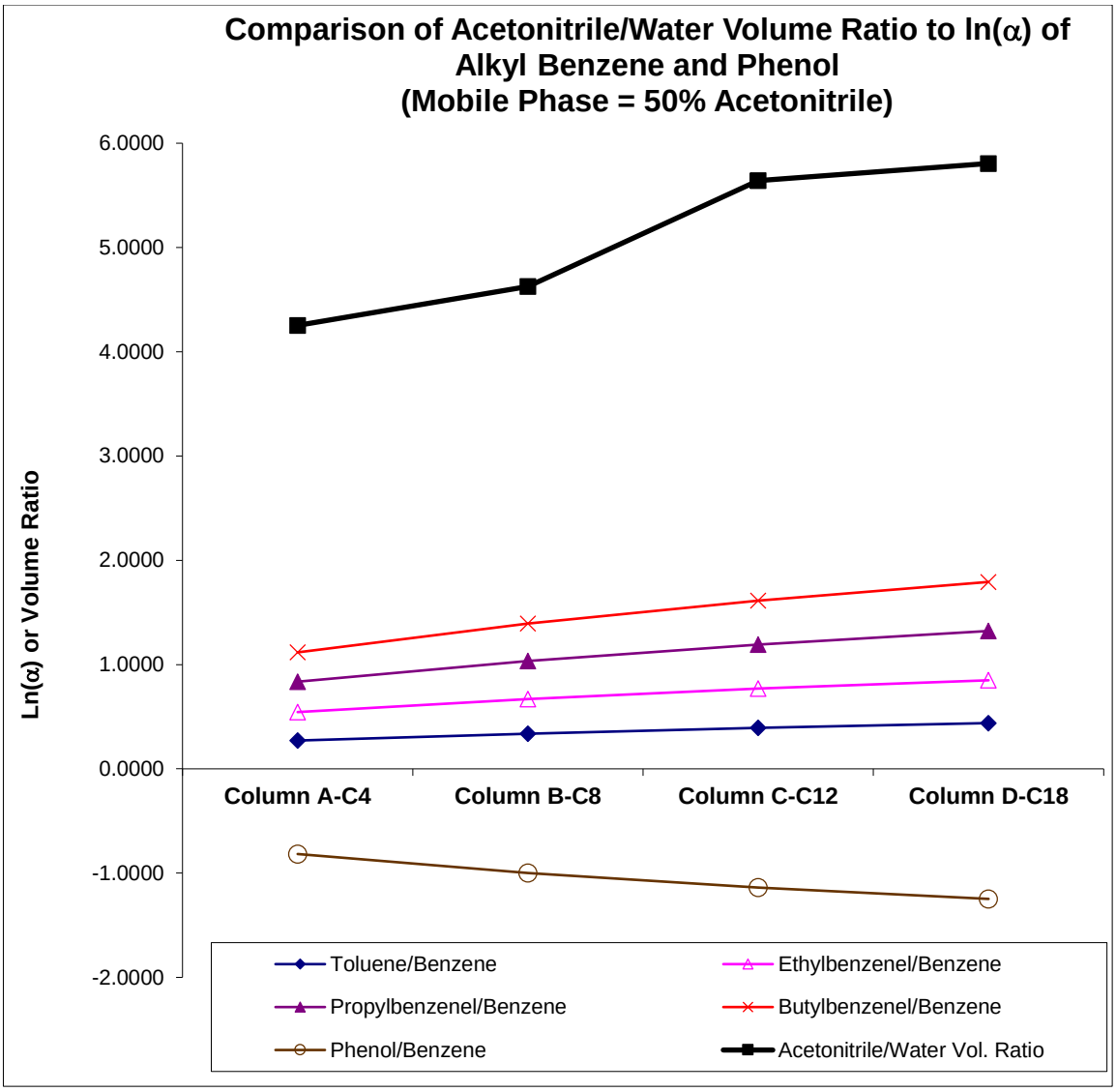


Figure 2.12 *Comparison of hydrophobicity determined by volume ratio (acetonitrile / water) to ln(α) of alkyl benzene and phenol for custom made alkyl columns (mobile phase = 50% acetonitrile in water)*

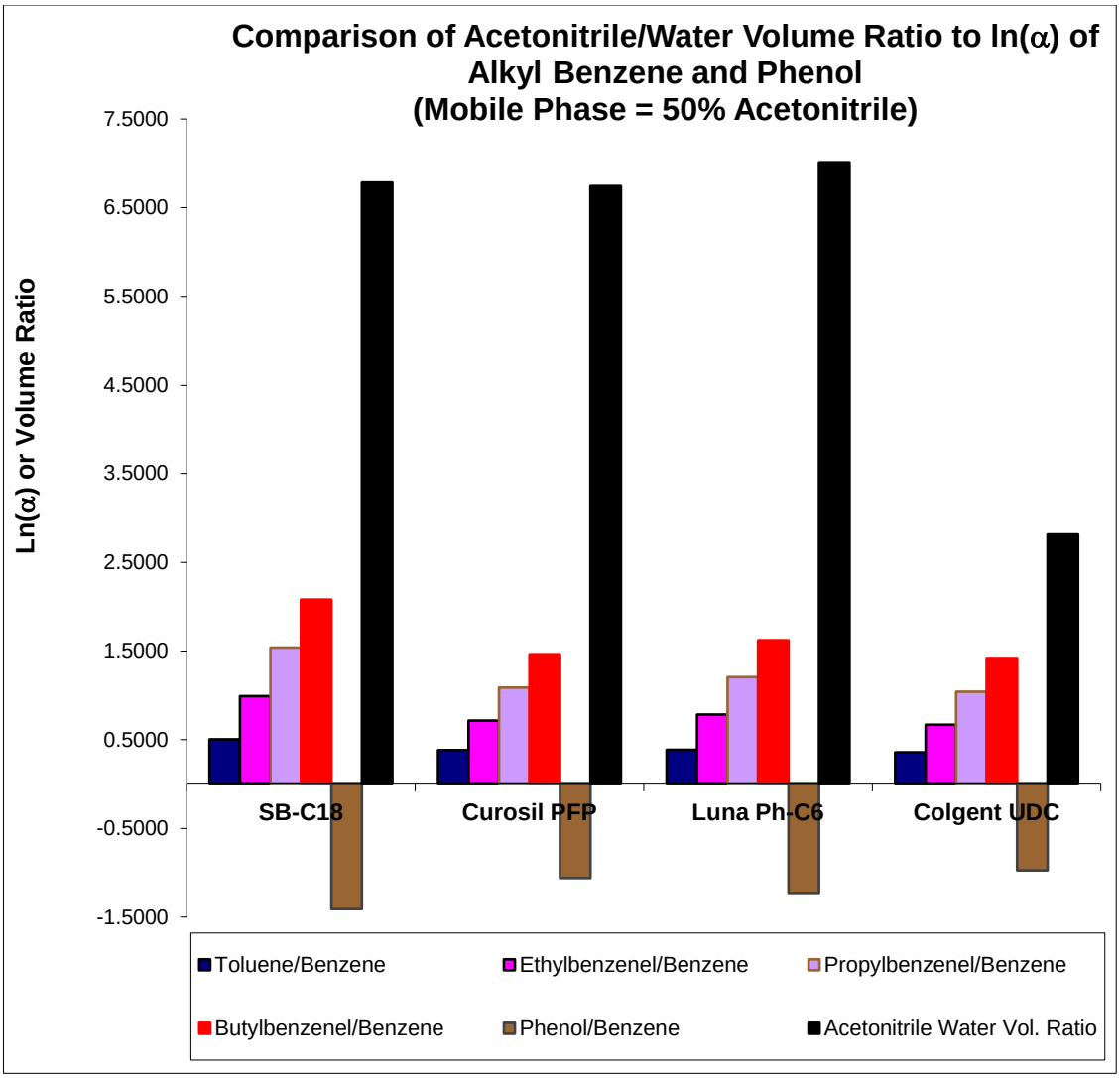


Figure 2.13. *Comparison of hydrophobicity determined by volume ratio (acetonitrile / water) to ln(α) of alkyl benzene and phenol for commercial columns (mobile phase = 50% acetonitrile in water)*

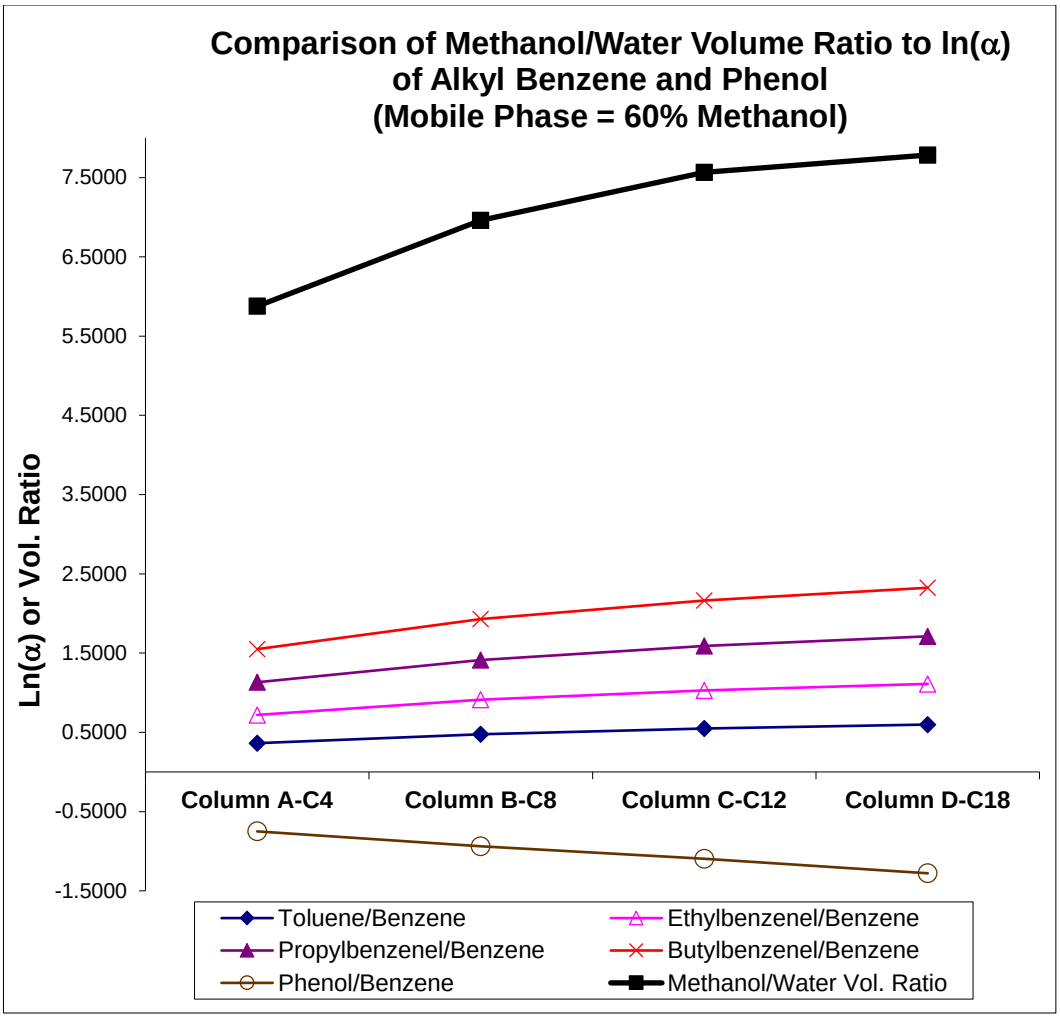


Figure 2.14. *Comparison of hydrophobicity determined by volume ratio (methanol / water) to ln(α) of alkyl benzene and phenol for custom made alkyl columns (mobile phase = 60% methanol in water)*

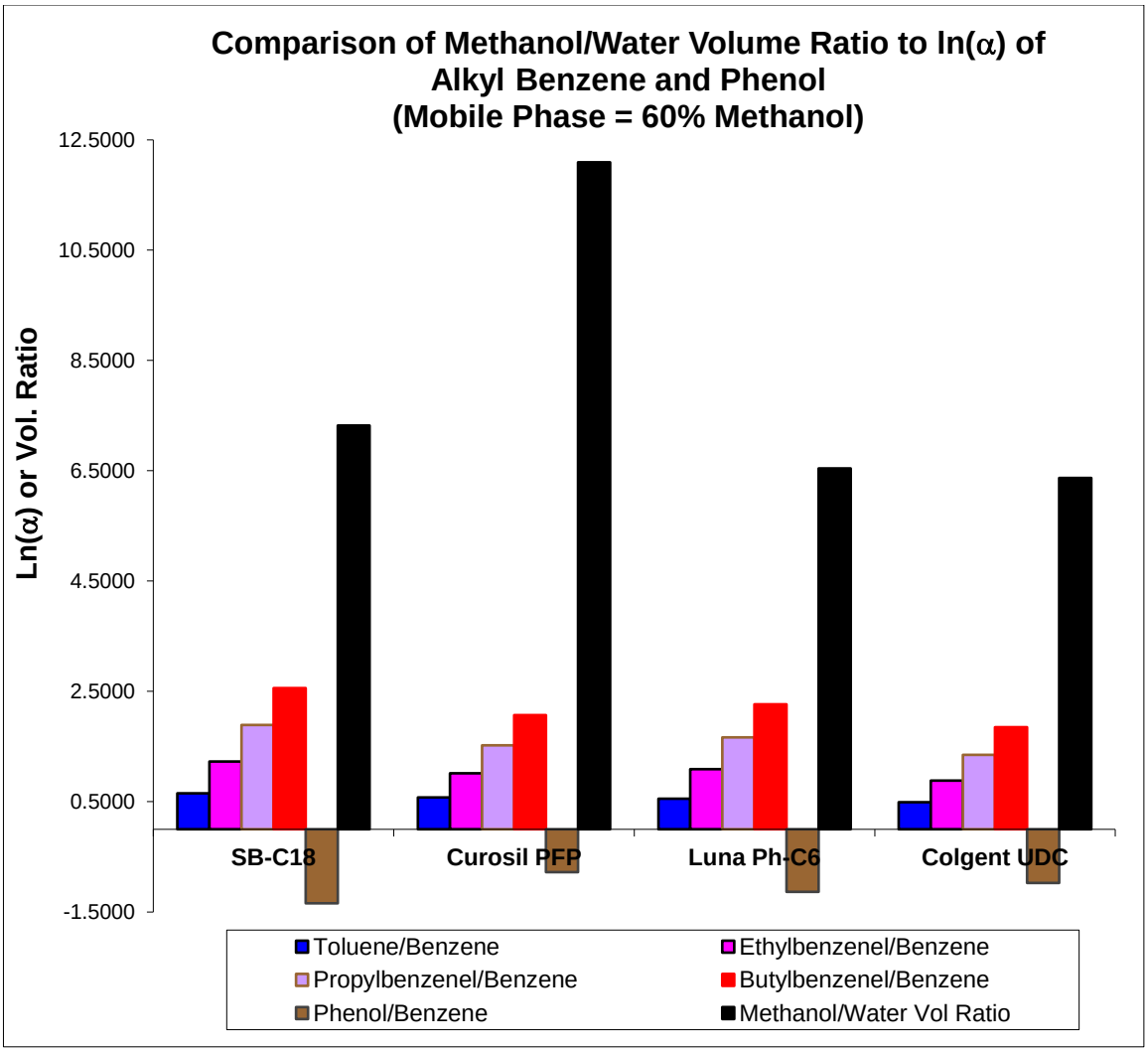


Figure 2.15. *Comparison of hydrophobicity determined by volume ratio (methanol / water) to ln(α) of alkyl benzene and phenol for commercial columns (mobile phase = 60% methanol in water)*

2.4. Discussion

2.4.1. Volume Change of Acetonitrile and Methanol Mixed with Water

Both solvents showed a volume contraction when mixed with water. However, the maximum volume change for acetonitrile-water mix did not exceed 1 % and maximum volume change for methanol-water mix did not exceed 2.5% of the theoretically calculated total volume. Practically, this amount of volume variation in mobile phase will not lead to a significant impact on HPLC determination, therefore volume correction is unnecessary.

2.4.2. Assumption

- The surface adsorption process is isochoric, i.e. molecular volumes of the solution components are constant on the adsorbent surface and in the bulk liquid.
- Adsorbent surface is impermeable and exerts adsorption forces to the liquid phase adjacent to that surface.
- Adsorption process is at instant equilibrium.
- The surface energy is completely independent for different adsorption sites, so they do not interfere with each other.
- A constant thickness adsorption model at the linear region of the isotherm is applied to this experiment so that the liquid layer adsorbed on the adsorbent surface is at constant volume.

2.4.3. Determination of Excess Adsorption Isotherm

In the 1960's, Everett [102] [103], Kiselev and Pavlova [104] [105] established the fundamental definition of excess adsorption based on its experimentally measurable properties. As shown in **Figure 2.16**. If a binary solvent system at constant total volume containing a relatively non-polar solvent B in a polar solvent water is in contact with a reversed-phase surface, at constant temperature, solvent B will be attracted and selectively adsorbed to the surface. Hence a layer of solvent B in excess to the bulk solvent system will be gathered on the surface.

Assuming the molar volume of both solvents on the adsorbent surface and in the bulk liquid phase remain unchanged, at equilibrium, the excess amount of solvent B absorbed on the surface can be experimentally determined by equation 2.1 shown below, where η_B represents the number of mole of solvent B adsorbed on the surface in excess to the bulk liquid above the adsorbent surface, V^l is the volume of the binary solvent system, C_B^o is the concentration of solvent B in the bulk liquid before adding adsorbent into the vessel. C_B^l is the concentration of solvent B in the bulk liquid after adding adsorbent into the vessel and equilibrated with the adsorption surface. If we define a term Γ_B^V as the excess adsorption of solvent B per unit of surface area (A) at constant total solvent volume, this excess adsorption term may be further expressed by equation 2.2.

$$\eta_B = V^l\left(C_B^o - C_B^l\right) \qquad \text{Eqn. 2.1}$$

and

$$\Gamma_B^V = \frac{\eta_B}{A} = \frac{V^l\left(C_B^o - C_B^l\right)}{A} \qquad \text{Eqn. 2.2}$$

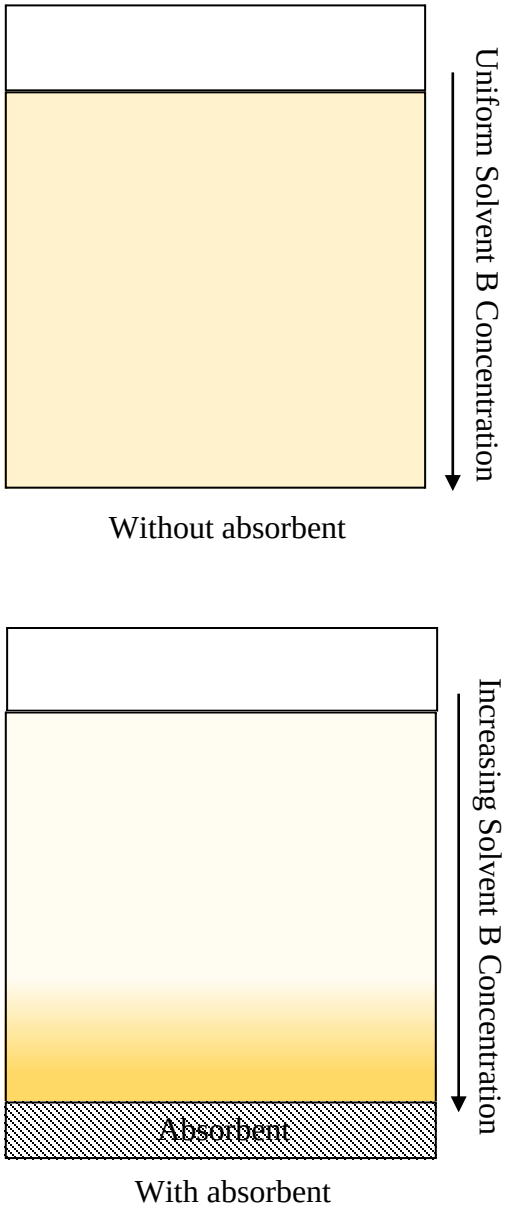


Figure. 2.16. *Schematic expression of a binary solvents system at constant volume containing a relatively non-polar solvent B in water.*

Where the top figure represents the solvent system in a vessel without adsorbent. The bottom figure represents the same system with a hydrophobic adsorbent added to the bottom of the vessel. The color of the shade represents the concentration of solvent B where darker color represents a higher concentration of solvent B.

Essentially, equation 2.2 is established from a static adsorption process. However, the experiment with this approach is very tedious and time consuming to perform, and therefore, is not practical to apply for HPLC column characterization. In order to experimentally generate excess adsorption isotherms by a more convenient method, a connection between HPLC and excess adsorption is needed. In our experiment, we apply equation 2.3 and equation 2.4 below using a minor disturbance peak determination method described by Kazakevich. Detail derivation of the equations can be found in reference [106]. The advantage of this approach is the amount of excess adsorption can be determined by experimental measurement without a priori assumption of an adsorption model. A model is only needed for the interpretation of the excess adsorption isotherm.

$$v_R = v_m + A\frac{d\Gamma_B^V}{dC_B^l} \qquad \text{Eqn. 2.3}$$

and

$$d\Gamma_B^V = \left(\frac{v_R - v_m}{A}\right) dC_B^l \qquad \text{Eqn. 2.4}$$

Where v_R is the retention volume of the minor disturbance peak. C_B^l is the equilibrium concentration of solvent B in the binary solvent mobile phase system. Γ_B^V is the constant volume excess adsorption of solvent B. A is the surface area which can be determined by Low Temperature Nitrogen Adsorption (LTNA) and the adsorbent weight. Integrating equation 2.4 across the whole range of C_B^l from 0% to 100% as shown by equation 2.5 can obtain the whole excess adsorption isotherm.

$$\int_0^{C_B^l} d\Gamma_B^V = \int_0^{C_B^l} \left(\frac{v_R - v_m}{A}\right) dC_B^l \qquad \text{Eqn. 2.5}$$

The void volume v_m in equation 2.4 can be determined by the average area integrated over the whole range of the excess adsorption isotherm using equation 2.6 as derived below:

$$v_R \, dC_B^l = v_m dC_B^l + A \, d\Gamma_B^V$$

Integrating from C_B^l = 0% to 100%:

$$\int_{0\%}^{100\%} v_R \, dC_B^l = v_m \int_{0\%}^{100\%} dC_B^l + A \int_{0\%}^{100\%} d\Gamma_B^V$$

Since the excess adsorption of pure solvent equals to 0 (i.e. Γ(0%) = Γ(100%) = 0), the term $A \int_{0\%}^{100\%} d\Gamma_B^V$ can be eliminated:

$$v_m = \frac{\int_{0\%}^{100\%} v_R \, dC_B^l}{\int_{0\%}^{100\%} dC_B^l} = \frac{\int_{0\%}^{100\%} v_R \, dC_B^l}{\left(C_B^{100\%} - C_B^{0\%}\right)}$$

Thus,

$$v_m = \frac{\int_{0\%}^{100\%} v_R \, dC_B^l}{C_B^{100\%}} \qquad \text{Eqn. 2.6}$$

In fact, this equation represents an integral average of the dependence of the retention volume on solvent B concentration for a binary solvents system. The void volume v_m calculated in this way is independent of which mobile phase it is used in.

2.4.4 Interpretation of Excess Adsorption Isotherm

The excess adsorption isotherm plotted by equation 2.5 contains a linear region with constant negative slope at approximately 50% to 90% of organic solvent B in the

bulk liquid phase, where increasing of C_B^l leads to linearly decreasing of excess adsorption Γ_B^V. This region is interpreted as complete filling of the reversed-phase surface by a layer of solvent B, as shown in **Figure 2.17.** Consequently, no further increase of solvent B can occur on the surface through adsorption, although the concentration of solvent B in the bulk liquid phase keeps increasing.

The total amount of solvent B adsorbed per unit surface (η_B) may be calculated by the following equation 2.7, where V_{ads} is the volume of the adsorbed layer per unit surface.

$$\eta_B = \Gamma_B^V + V_{ads} C_B^l \qquad \text{Eqn. 2.7}$$

As previously mentioned, excess adsorption Γ_B^V can be measured experimentally versus an established bulk liquid concentration C_B^l. Note that we do not need any chromatographic or adsorption model to calculate this experimentally assessable quantity. However, the interpretation of the isotherm itself will need a specific retention model. In fact, this point is mathematically reflected by two undefined unknowns η_B and V_{ads} present in a single equation 2.7. In order to interpret the excess adsorption isotherm, we need to introduce a mathematical model such as constant thickness adsorption model with a Gibbs-defined boundary at its linear region. Thus equation 2.7 is only valid in the linear region of the excess adsorption isotherm where the volume of the adsorbed liquid layer V_{ads} is at maximum. The excess adsorption status at this region is schematically presented in **Figure 2.18**. The Gibbs dividing plane for this model is located between the top surface of the adsorbed layer and the bulk liquid phase.

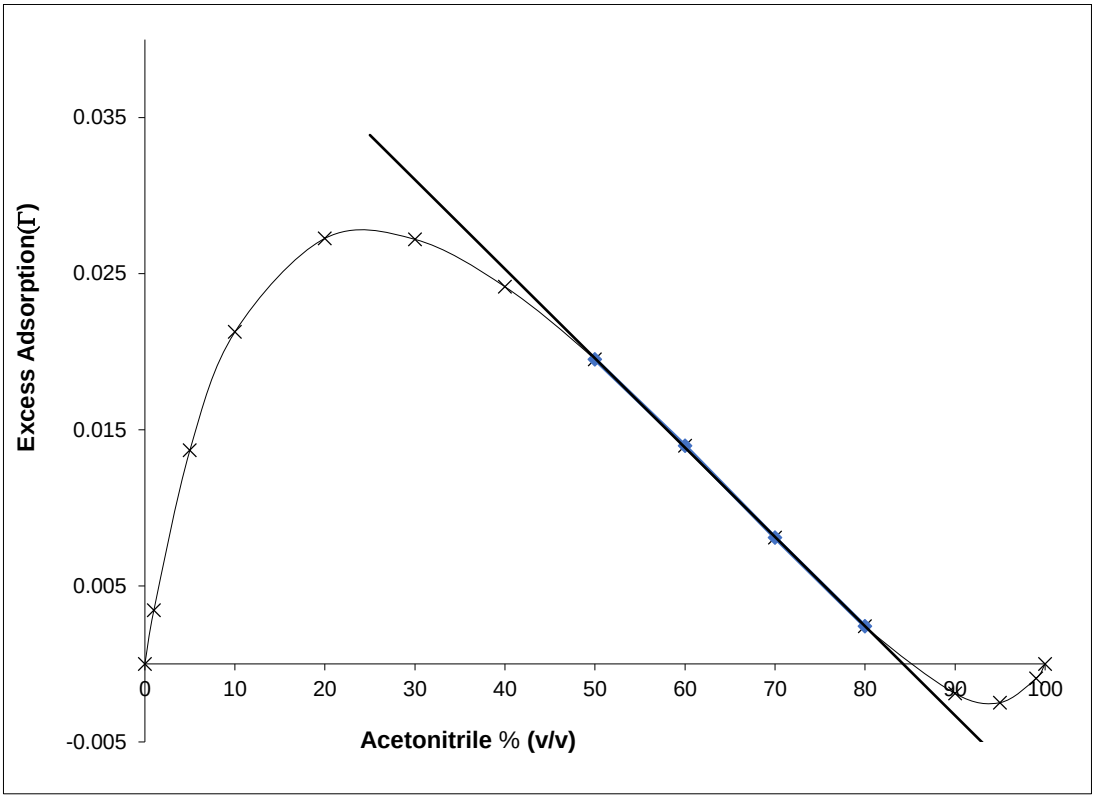
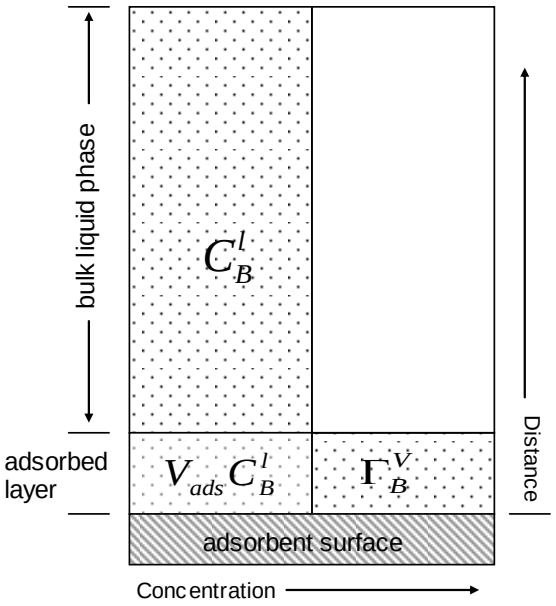


Figure 2.17. *Linear region of the excess adsorption isotherm for a binary solvents system containing acetonitrile in water.*



Γ_B^V = Constant volume surface excess adsorption of solvent B

V_{ads} = volume of the adsorbed layer per unit surface.

C_B^l = Equilibrium concentration of solvent B in bulk liquid phase

Figure 2.18. *Schematic expression of the surface status at the linear region of the excess adsorption isotherm of solvent B.*

Where the bottom left block of the adsorbed layer labeled $V_{ads}C_B^l$ represents the amount contributed from the bulk liquid. The bottom right block labeled Γ_B^V represents the excess amount accumulated on the surface due to adsorption.

2.4.5. Excess Adsorption Isotherm on a Heterogeneous Composite Surface

An excess adsorption isotherm for a binary mobile phase system represents a competitive interaction of both solvent components with the adsorption sites. So far, our discussion was based on an assumption of homogeneous surface. However, in practice, adsorption surfaces are seldom homogeneous. In the presence of two distinct types of adsorption sites on the surface, an overall isotherm may be represented as a superposition of two isotherms on the different types of surfaces. Assuming complete independence of surface energy on each type of adsorption site, it is possible to mathematically describe this superposition as a sum of two independent isotherms, where coefficient of each individual term represents a relative amount of surface that is responsible for a particular interaction. Kazakevich et al [66] indicated that common S-shaped excess adsorption isotherm of a binary system containing a polar (water) and a relatively non-polar (organic) solvents on a reversed-phase surface may be interpreted as the superposition of a polar component and a non-polar component adsorbed on the hydrophilic surface and hydrophobic surface, respectively. This interpretation can be further used to categorize reversed-phase HPLC columns in terms of their relative amount of hydrophilic and hydrophobic surfaces. To eliminate eluent composite effect, the whole excess adsorption isotherm should be used.

Common reversed-phase surface is usually heterogeneous covered by adsorption sites with different affinity to polar and non-polar compounds. The type of adsorption is basically related to the hydrophobicity of their bonded phase and the amount of unbounded hydrophilic residual silanols. **Figure 2.19** demonstrates a heterogeneous surface containing both hydrophobic and hydrophilic sites.

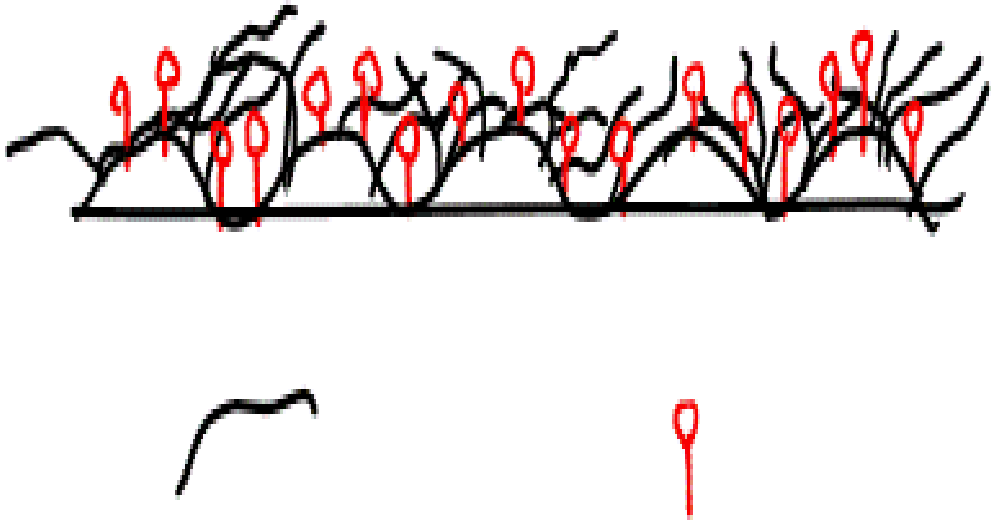


Figure 2.19. *Schematic of a heterogeneous surface containing hydrophobic alkyl chain and hydrophilic unbonded silanol group*

Macroscopically, these adsorption sites can be viewed as a uniform distribution of two general types of surfaces each contributing to a hydrophobic or a hydrophilic interaction as shown in **Figure 2.20.**

For a binary solvents system containing water (polar solvent W) and an organic solvent (non-polar solvent B) such as acetonitrile on a heterogeneous reversed-phase surface, if we imagine viewing the surface as a composite of two distinct parts of a hydrophilic (polar) and a hydrophobic (non-polar) surface, due to their polarity difference, water will be preferentially adsorbed by the hydrophilic part of the surface. On the other hand, acetonitrile will be also preferentially adsorbed by the hydrophobic part of the surface. At equilibrium, when we take water as the accumulating component over the composite surface, the attraction from the hydrophilic surface will create a layer of adsorbed solvent on the composite surface with water ratio exceeding the bulk liquid phase (i.e. the adsorbed layer contains more water than the bulk liquid phase). On the other hand, when we take acetonitrile as the accumulating component over the composite surface, the attraction from the hydrophobic surface will create a layer of adsorbed solvent on the composite surface with acetonitrile ratio exceeding the bulk liquid phase (i.e. the adsorbed layer contains more acetonitrile than the bulk liquid phase). This excess amount of water and acetonitrile vary as their concentration varied in the bulk liquid phase. Assuming complete independence of surface energy for different adsorption sites, two excess adsorption isotherms can be independently generated for each of these two adsorption phenomena. An overall composite excess adsorption isotherm can be then generated by the superposition of these two independent excess adsorption isotherms. **Figure 2.21** demonstrates the surface concentration of both components on a hypothetical composite surface.

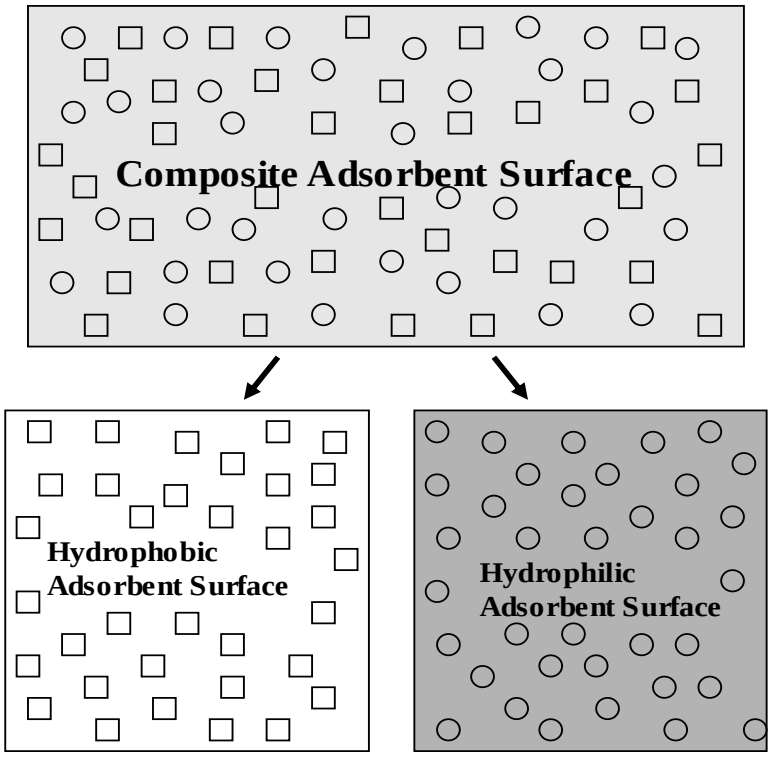


Figure 2.20. *Schematic expression for viewing a surface as the composite of two different types of surface.*

Where: o = *Hydrophilic site;* □ = *Hydrophobic site.*

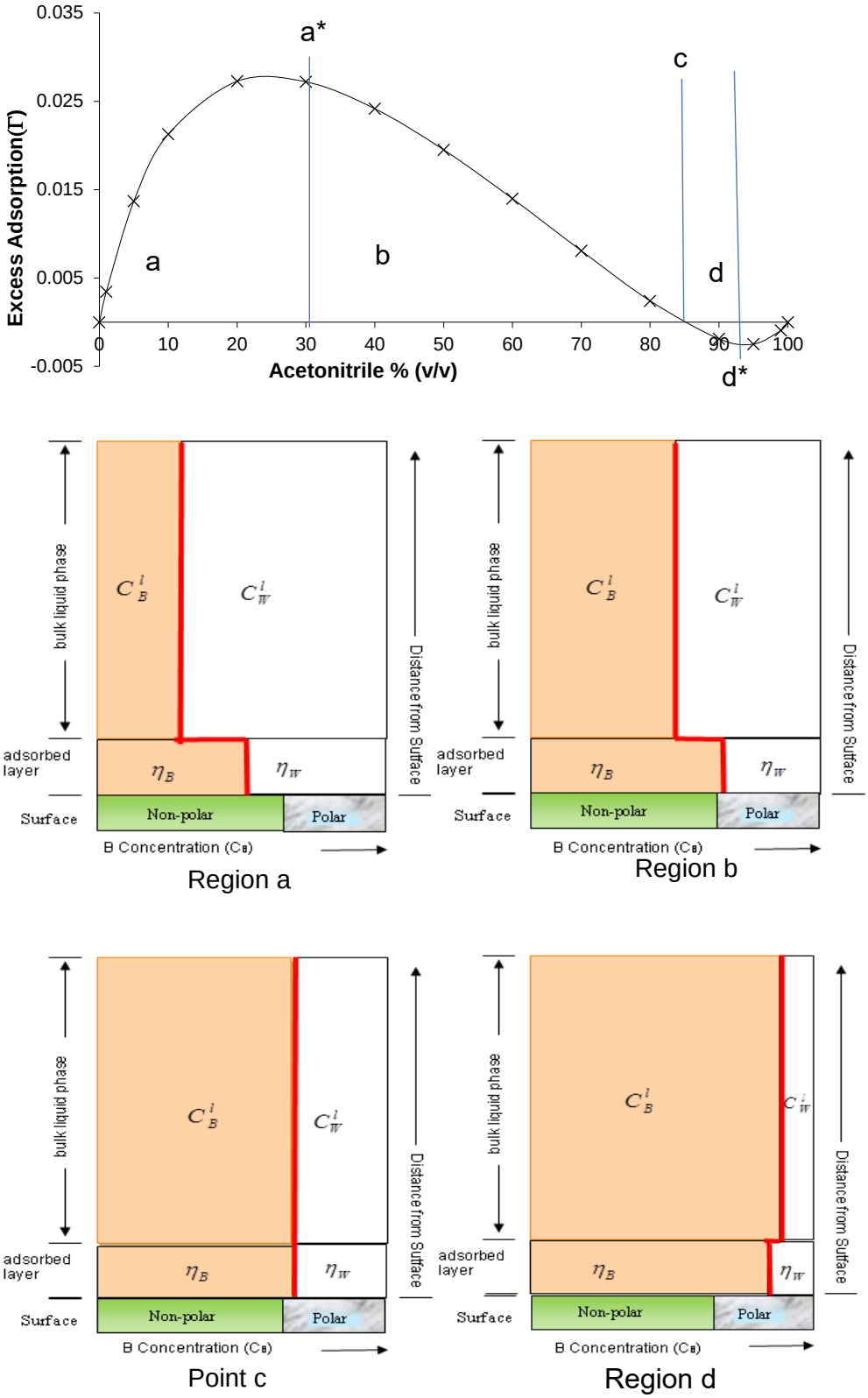


***Figure 2.21**. Schematic expression of a binary solvent system adsorbed on a composite surface at different regions of the excess adsorption isotherm*

The excess adsorption isotherm of solvent B shown in **Figure 2.21** may be explained by four consecutive regions.

(1) At region “a”: Concentration of organic solvent B is low. Any Increase of solvent B in the bulk liquid phase will rapidly increase the excess adsorption of solvent B on the adsorbent surface until point “a*”, where solvent B reaches its maximum concentration on the adsorbent surface.

(2) At region “b”: The adsorbent surface is completely filled. Surface concentration of solvent B is at the maximum. Further increasing of solvent B will only raise its concentration in the bulk liquid phase. Thus, lead to linearly decreasing of excess adsorption on the adsorbent surface.

(3) At point “c”: Concentration of solvent B in the bulk liquid phase is equal to the concentration on the adsorbent surface. Thus, the excess adsorption is equal to zero.

(4) At region “d”: The excess adsorption of solvent B continues to decrease below zero until point “d*” where the concentration of solvent B in bulk liquid phase becomes very high that eventually replace some of the water molecule attracted by the hydrophilic part of the surface, thus, again showing an increase in solvent B excess adsorption.

Mathematically, the excess amount of organic solvent B and water adsorbed on unit surface area created by hydrophobic (non-polar) and hydrophilic (polar) surface attraction, respectively, can be expressed by two analog equations of equation 2.7 as following equations 2.9 and 2.11:

(1) Total amount of organic solvent B adsorbed per unit surface due to attraction by the hydrophobic part of the surface:

$$\eta_B = \Gamma_B^N + V^N C_B^l$$

and

$$\Gamma_B^N = \eta_B - V^N C_B^l \qquad \text{Eqn. 2.8}$$

Since the organic solvent B molecules are mainly accumulated on the hydrophobic part of the surface, thus

$$\eta_B = V^N C_B^N$$

and

$$\Gamma_B^N = V^N \left(C_B^N - C_B^l\right) \qquad \text{Eqn. 2.9}$$

(2) Similarly, total amount of water adsorbed per unit surface due to the attraction by the hydrophilic part of the surface:

$$\eta_W = \Gamma_W^P + V^P C_W^l$$

and

$$\Gamma_W^P = \eta_W - V^P C_W^l \qquad \text{Eqn. 2.10}$$

Since the water molecules are mainly accumulated on the hydrophilic part of the surface, thus

$$\eta_W = V^P C_W^P$$

and

$$\Gamma_W^P = V^P \left(C_W^P - C_W^l\right) \qquad \text{Eqn. 2.11}$$

Where η_B and η_W are the total amount of solvent B and water adsorbed per unit surfaces area. $V^N$and $V^P$are the volume of liquids adsorbed on the hydrophobic and hydrophilic surfaces per unit of surface area, Γ_B^N and Γ_W^P are the excess adsorption of solvent B and water per unit surface area due to attraction by hydrophobic and hydrophilic surfaces. C_B^l and C_W^l are the equilibrium concentration of solvent B and water in the bulk liquid phase. C_B^N and C_W^P are the equilibrium concentration of solvent B and water in the adsorbed liquid layer due to hydrophobic and hydrophilic surface attraction.

If we view the absorbent surface as a composite of two distinct surfaces, mainly one providing hydrophobic interaction and the other hydrophilic interaction. For a binary aqueous-organic solvent system containing an organic solvent (B) and water (W), increase of one component implies a decrease of its complementary component in same volume. Therefore, the excess adsorption of organic solvent (B) may be also expressed by the excess adsorption of water corrected by their molar volume ratio. The total excess adsorption of organic solvent B, (Γ_B^T) may be expressed by the sum of the excess adsorption of solvent B due to hydrophobic surface attraction (Γ_B^N) and hydrophilic surface attraction (Γ_B^P) as following:

$$\Gamma_B^T = \Gamma_B^N + \Gamma_B^P$$

Since

$$\Gamma_B^P = -\Gamma_W^P \frac{D_B}{D_W}$$

Therefore

$$\Gamma_B^T = \Gamma_B^N - \Gamma_W^P \frac{D_B}{D_W}$$ Eqn. 2.12

Substituting equation 2.9 and equation 2.11 for Γ_B^N and Γ_W^P into equation 2.12, we can obtain equation 2.13 below.

$$\Gamma_B^T = V^N\left(C_B^N - C_B^l\right) - V^P\left(C_W^P - C_W^l\right)\frac{D_B}{D_W}$$ Eqn. 2.13

From the definition of mole fraction, if X_W and X_B represent the mole fraction of water and solvent B in the binary solvent system, then $X_W = 1 - X_B$

Since $X_W = \frac{C_W^l}{D_W}$ and $X_B = \frac{C_B^l}{D_B}$

Hence

$$\frac{C_W^l}{D_W} = 1 - \frac{C_B^l}{D_B}$$

and

$$C_W^l = D_W - D_W\left(\frac{C_B^l}{D_B}\right)$$

Substituting into equation 2.13, we can obtain equation 2.14

$$\Gamma_B^T = V^N\left(C_B^N - C_B^l\right) - V^P\left(C_W^P\frac{D_B}{D_W} - D_B + C_B^l\right)$$ Eqn. 2.14

Nomenclature:

η_B	Total amount of organic solvent B adsorbed per unit surfaces area
η_W	Total amount of water adsorbed per unit surfaces area
Γ_B^T	Total excess adsorption of organic solvent B per unit surface area
Γ_B^N	Excess adsorption of organic solvent B per unit surface area due to attraction by the hydrophobic surface.
Γ_B^P	Excess adsorption of organic solvent B per unit surface area due to attraction by the hydrophilic surfaces
Γ_W^P	Excess adsorption of water per unit surface area due to attraction by the hydrophilic surface.
D_B	Molar density of solvent B
D_W	Molar density of water
V^N	Volume of liquid adsorbed on the hydrophobic part of surfaces per unit of total surface area
V^P	Volume of liquid adsorbed on the hydrophilic part of surfaces per unit of total surface area
C_B^N	Concentration of organic solvent B in the adsorbed liquid layer due to hydrophobic surface attraction.
C_W^P	Concentration of water in the adsorbed liquid layer due to hydrophilic surface attraction.
C_B^l	Equilibrium concentration of solvent B in bulk liquid phase (or mobile phase)
C_W^l	Equilibrium concentration of water in bulk liquid phase (or mobile phase)
X_W	Mole fraction of water
X_B	Mole fraction of solvent B

The mathematical correlations for the superposition of the above binary excess adsorptions may be graphically demonstrated by **Figure 2.22**. Essentially, equation 2.14 is an extension of equation 2.7 for a composite surface containing two different types of adsorption sites, where Γ_B^T and C_B^l can be experimentally measured, D_B and D_W are known constants. However, V^N, V^P, C_B^N and C_W^P are undefined unknowns. In order to define these parameters, we need to introduce a physical model. Therefore equation 2.14 is valid only at the linear region of the superposed excess adsorption isotherm by applying a constant thickness adsorption layer model with a Gibbs dividing plane allocated between the adsorbed layer surface and the bulk liquid. This region represents a complete filling of the adsorbent surface where the hydrophobic part of the surface is fully covered by a layer of organic solvent B molecules and the hydrophilic part of the surface is fully covered by a layer of water molecules. Under this condition, the composition and the volume of the adsorbed layer on unit surface area of the composite surface are constant. Thus,

$V^N = V_B;\ C_B^N = D_B;\ V^P = V_W;\ C_W^P = D_W$

Where V_B and V_W represent the volumes of pure solvent B and pure water adsorbed on a unit of hydrophobic and hydrophilic surfaces, respectively. Substituting these terms into equation 2.14 can reduce it to the following final linear equation 2.15,

$$\Gamma_B^T = V_B D_B - (V_B + V_W) C_B^l \qquad \text{Eqn. 2.15}$$

Plotting Γ_B^T versus C_B^l based on equation 2.15 can obtain V_B and V_W from its intercept and slope. The sum of V_B and V_W represents the total volume of the adsorbed layer per unit surface in this region as shown in **Figure. 2.23.**

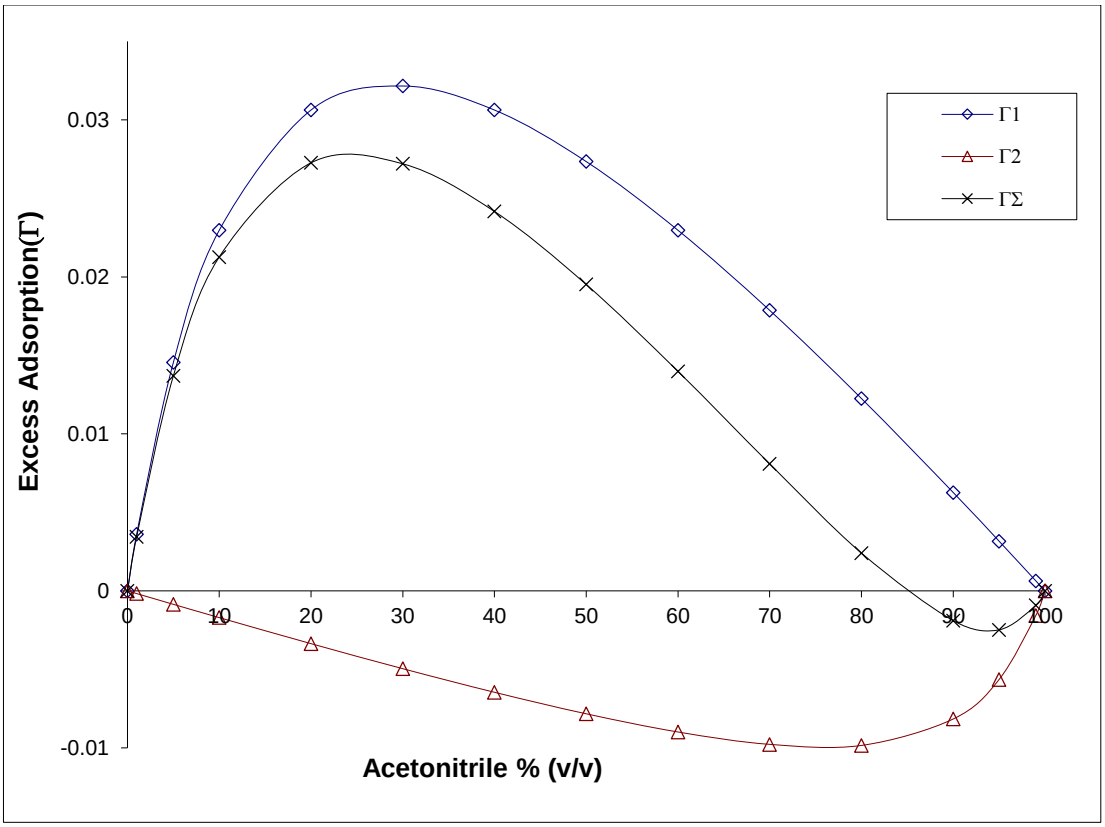


Figure. 2.22. *Schematic expression of the superimposed excess adsorption isotherm for a binary solvent system adsorbed on a composite surface.*

Where the □ curve represents the excess adsorption isotherm of acetonitrile (Γ1). The Δ curve represents the excess adsorption isotherm of water (Γ2) expressed in acetonitrile (complementary component) concentration. The x curve represents the superimposed excess adsorption isotherm on the composite surface (ΓΣ).

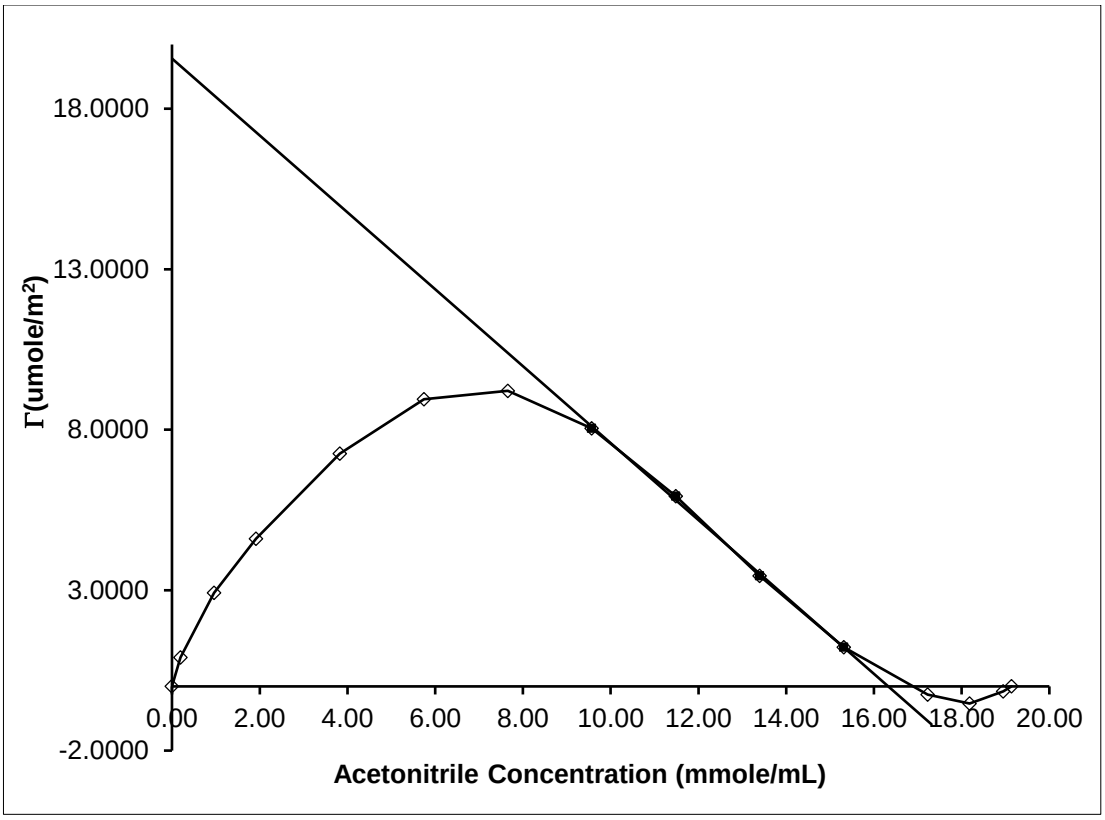


Figure. 2.23. *Linear region of the isotherm of a binary solvents system containing solvent B (acetonitrile) and water*

Since V_B and V_W are directly calculated from the hydrophobic and hydrophilic interactions exerted by the hydrophobic and hydrophilic parts of the surface, their relative volume is therefore directly proportional to the strength of the interactions. For a given set of binary solvent system at constant temperature, the ratio of V_B and V_W on a reversed-phase surface can be used to measure the strength of its hydrophobicity and quantitatively compare to another reversed-phase surface. Again, note that both variable terms Γ_B^T and C_B^l in equation 2.15 can be experimentally measured without relying on any thermodynamic or adsorption model, although a model is still needed to interpret the isotherm. Practically, Γ_B^T and C_B^l can be determined by HPLC method.

In general, a 150 mm column with 4.6 mm diameter column may be filled with approximately 1 gram of column packing materials. Note that taking assumption of 1 gram column packing weight will not affect the column hydrophobicity estimation results because the calculated final hydrophobicity expression is presented in adsorbed solvents volume ratio which is essentially independent of the column physical dimensions, i.e. V_B / V_W is a value of ratio without a physical unit.

In our experiment, the excess adsorption Γ_B^T (in μmole/m^2) was calculated by applying numerical approach with equation 2.5 using the retention volume v_R of the minor disturbance peak. The column void volume v_m was calculated using equation 2.6. Excess adsorption isotherms of acetonitrile and methanol were then generated by plotting Γ_B^T versus C_B^l. By applying a constant thickness adsorption model and a Gibb's dividing plane located between the adsorbed liquid layer on the surface and the bulk liquid phase. The volume of non-polar and polar solvents adsorbed on the surface are then estimated from the linear region of the excess adsorption isotherm. For a reversed-phase surface, according to Everett's definition [102] [103], this region represents the maximum amount of organic modifier co-existing with water on the

adsorbent surface. The volume of the adsorbed layer and compositional ratio of these two mobile phase components adsorbed on the surface stays constant throughout the whole region. Since the main attraction force between a reversed-phase surface and the organic component is hydrophobic interaction, the relative polarity of this solvent combination is directly proportional to the hydrophobic interaction strength between the solvents and the adsorbent surface, therefore the volume ratio of the non-polar and polar solvents adsorbed on the surface can be used to measure the hydrophobicity of the absorbent surface.

2.4.6. Minor Disturbance Peak Retention Volume Profiles

Retention profiles of the minor disturbance peaks of acetonitrile and methanol on the custom-made alkyl C_4, C_8, C_{12} and C_{18} columns are show in **Figure 2.3** and **Figure 2.5.** Retention profiles of the Zorbax SB- C_{18}, Curosil Perfluorophenyl, Luna Phenyl-Hexyl and Cogent UDC cholesterol columns are shown in **Figure 2.4** and **Figure 2.6.**

These retention profiles were used to generate excess adsorption isotherms using equation 2.5. The retention volume profiles versus the eluent composition for the same eluent are quite similar, but the shape of acetonitrile and methanol profiles are slightly different.

2.4.7. Excess Adsorption Isotherm of Tested Columns

Figure 2.7 and **Figure 2.9** show the excess adsorption isotherms of acetonitrile and methanol on the custom made C_4, $C_{8,}$ $C_{12,}$ C_{18} columns, respectively. **Figure 2.8** and **Figure 2.10** show the excess adsorption isotherms of acetonitrile and methanol on the commercially available Zorbax SB-C18, Curosil-PFP, Luna Phenyl Hexyl, Cogent-

UDC cholesterol columns, respectively. The linear region of these isotherms will be used to calculate the organic solvent to water ratio adsorbed on the column surface. Visual results show that excess adsorption of acetonitrile is significantly higher than methanol for all columns. Apparently, the amount of acetonitrile adsorbed on the surface is significantly higher than methanol. In fact, Kazakevich et. al. found that methanol forms a monolayer adsorption where acetonitrile forms a multilayer adsorption on a reversed-phase surface [66].

All isotherms also show a S-shape curve with a small negative region at the high organic ratio end. This indicates that the adsorption sites on the surface are not homogeneous. The existing hydrophilic sites (mainly residual silanols) among the hydrophobic reversed-phase sites preferentially attract water on the surface. In a binary solvent system, this excess adsorption of water in turn leads to a deficit of organic solvent in the adsorbed layer and shown as a negative adsorption. The figures also show that the negative region in each methanol excess adsorption isotherm is generally smaller than its acetonitrile excess adsorption isotherm on the same column. This phenomenon is mainly due to the competitive hydrogen bonding interaction of methanol and water molecules to the uncovered free silanols on the silica surface. On the other hand, acetonitrile with much weaker hydrogen bonding capability shows a more unique hydrophobic interaction with the bonded surface.

2.4.8. Volume Ratio of Organic Solvent to Water Adsorbed on Surface

Figure 2.11 gives an example of the excess adsorption isotherms of acetonitrile and methanol in water (custom made alkyl C_{18} column) with its corresponding linear lines. **Table 2.4** contains the slope and intercept data of these straight lines on different columns. The volume of organic component and water adsorbed on the surface is

calculated by equation 2.15 using the slope and intercept of these straight lines. **Table 2.5** contains the calculated volume and volume ratio of the adsorbed solvents on each column.

In general, each excess adsorption isotherm shows an increase of excess adsorption on the surface with an increase of organic component concentration in the bulk liquid phase until it reaches maximum at about 40% v/v. Further increase of the organic component leads to a steady decrease of the excess adsorption until it passes zero and finally reaches a minimum negative excess adsorption, then increases back to zero at 100% v/v organic component. In the isotherm, there exists a linear region with negative slope from approximately 50% v/v to 90% v/v (9.6 to 17.2 mmole/mL for acetonitrile and 12.4 to 22.2 mmole/mL for methanol) of organic component in the bulk liquid phase. This region represents a complete filling of the adsorbent surface, where the hydrophobic part of the surface is fully covered by a layer of organic solvent B molecules and the hydrophilic part of the surface is fully covered by a layer of water molecules. Further increase of organic solvent concentration in the system will not increase the organic solvent concentration on the surface but merely in the bulk liquid phase only. The adsorbed organic solvent and water volumes can be calculated from the slope and intercept of a linear plot of the excess adsorption versus solvent concentration at this region. The volume ratio of organic solvent to water directly reflects the hydrophobicity of the column. In general, a more hydrophobic column will give higher retention to nonpolar compounds.

As shown in **Table 2.5**, the adsorbed volume ratios for both acetonitrile / water and methanol / water mobile phases basically increase with increasing alkyl chain length of the bonded phase. Theoretically, longer alkyl chain provides higher hydrophobicity. The obtained experimental data match well with the theoretical prediction. However, the rate of increasing in hydrophobic character with increasing

bonded phase alkyl chain length is moderate, indicating that the change of alkyl chain length will not dramatically change the analyte selectivity by hydrophobic interaction alone. As Kazakevich et al pointed out in reference [66], due to the hydrophobic attraction among alkyl chains on the reversed-phase surface, all chains generally stay in their collapsed conformation under general HPLC conditions. The adsorbed solvent and analyte molecules are not able to penetrate into the bonded phase but lie on the top of the alkyl chains. Therefore, majority of the hydrophobic character should be contributed by the upper part of the alkyl chains. This explains the small hydrophobicity difference among alkyl columns with carbon number above C_{12}. Similar tests were also performed by Gritti [107]. He found that the excess adsorption of alkyl alcohols increased with increasing the number of carbon atoms. His experiments focused on the effect of alkyl bonded surfaces with different surface coverage to excess adsorption where we focus on the hydrophobic and hydrophilic interaction of solvents with different types of surfaces.

For the commercial columns, test results showed that the adsorbed volume ratios of acetonitrile to water are in the order of Luna Phenyl Hexyl > Zorbax SB-C_{18} > Curosil-PFP > Cogent UDC. The adsorbed volume ratios of methanol to water are in the order of Curosil-PFP > Zorbax SB-C_{18} > Luna Phenyl Hexyl > Cogent UDC. It is worth to note that methanol is essentially more polar than acetonitrile and has hydrogen bonding capability. This makes the binary mobile phase containing methanol and water not preferred for the determination of column hydrophobicity by solvent adsorption ratio. As shown in **Table 2.5**, methanol will form hydrogen bond with the fluorine atoms of the perfluorophenyl ligands and oxygen atoms of the cholesterol ligands, thus increasing the surface adsorption of methanol and consequently the methanol / water volume ratio. In fact, methanol also forms hydrogen bond with the accessible residual silanols on the silica surface and creates competition to water adsorption, making the mobile phase

system with acetonitrile and water more preferable for hydrophobicity determination by solvent adsorption. The methanol and water system should be used for supporting information only.

2.4.9. Comparison of Hydrophobic / Hydrophilic Adsorption Volume Ratio to Alkylbenzene Selectivity

Alkyl benzenes and phenol selectivity data in the form of ln(α) are presented in **Table 2.6** and **Table 2.7** to compare with the volume ratio of the adsorbed solvents. Since the comparison is performed on the solvent adsorption volume ratios and alkyl benzene to benzene capacity factor ratios, these data are independent of the physical dimensions of the columns but solely on interaction free energy, hence avoid the requirement of accurate surface area and phase ratio measurement. The comparisons of custom made alkyl bonded columns and other commercially available columns using acetonitrile and methanol adsorptions are also graphically presented in **Figure 2.12** to **Figure 2.15**.

Alkylbenzene homologous is often used for HPLC column hydrophobicity estimation. In chromatography, the natural logarithm of the capacity factor (k') of a solute can be correlated to the interaction energy as $\ln(k') = -\Delta G_{mob/stat}/RT + \ln(\Phi)$, where $\Delta G_{mob/stat}$ represents the standard Gibbs free energy for transferring one mole of the solute from mobile phase to stationary phase and Φ is the phase ratio. Generally, an alkyl benzene molecule with longer alkyl chain will release a higher amount of energy when transferring from mobile phase to stationary phase, and therefore has a larger capacity factor. For convenience purpose, we used the comparison of alkyl selectivity (α) which is the capacity factor ratio of each alkyl benzene / benzene and the capacity factor ratio of phenol / benzene in this experiment. The natural logarithm of the

alkylbenzene / benzene ratio ln(α) represents a direct measure of its hydrophobic interaction with the stationary phase. The ln(α) of phenol is used to monitor the retention behavior of a polar analyte. Results were then compared to the ratio of adsorbed non-polar solvent B to polar water volumes determined by excess adsorption.

Since acetonitrile without hydrogen bonding capability can provide purer hydrophobic interaction, surface hydrophobicity estimated from its excess adsorption is considered to be more reliable. Our comparison is mainly based on these results. The surface hydrophobicity estimated by excess adsorption of methanol under the influence of hydrogen bonding is provided for supplementary information only.

As shown in **Figure 2.12** and **Figure 2.14**, the hydrophobic to hydrophilic adsorbed volume ratios for both acetonitrile / water and methanol / water mobile phases are basically increasing with increasing alkyl chain length of the bonded phase, directly comparable to ln(α) of alkyl benzenes and inversely comparable to ln(α) of phenol.

Figure 2.13 and **Figure 2.15** are comparisons for different types of commercially available columns. According to the results obtained from the acetonitrile / water mobile phase, phenyl columns show similar hydrophobicity to the C_{18} column by solvent adsorption ratio but lower alkyl benzene and higher phenol selectivity, reflecting other types of interaction involved. Retention of phenyl columns are heavily influence by π-π interaction of the phenyl rings. Since capacity factor ratios of alkyl benzenes to benzene and phenol to benzene are used in the comparison, essentially, they only account for the hydrophobicity comparison of their alkyl chain interaction and the interaction of hydroxyl group in phenol molecule with the bonded surface. Higher phenol retention can be explained by the additional π-π interaction between the π electron of oxygen in the phenol analyte and the π electron of the phenyl ring in the stationary phase. The reason for lower alkyl benzene selectivity is more ambiguous, possibly due to shorter alkyl chain length of the phenyl columns (propyl on the perfluorophenyl

column and hexyl on the phenyl hexyl column) and the presence of electron cloud in the phenyl ring as well as the fluorine atoms of the bonded phase, makes the molecules more polarizable, therefore showing less affinity to the alkyl chain of an aromatic alkyl analyte as compared to the aliphatic C_{18} bonded phase.

Not surprising, the cholesterol column shows significantly less hydrophobic by solvent adsorption ratio than C_{18} and phenyl columns due to its polar functional groups and lower surface coverage. Low bonding density of the bonded phase makes the unbonded free silanols more accessible to the solvent molecules. Free silanol is well known to be hydrogen bond donor and acceptor. The carboxylate and ether oxygen of the cholesterol molecule can also provide hydrogen bond acceptor property. The alkyl benzene and phenol selectivity of this column are also lower and higher than the other columns in comparison, respectively, indicating that other than π-π interaction, the hydrogen bonding capability of the surface also plays a role.

2.5. Conclusion

This study described a chromatographic method to determine the hydrophobicity of reversed-phase HPLC columns by excess adsorption isotherms. Experimental results showed that the reversed-phase column hydrophobicity can be estimated by surface adsorption volume of an aqueous-organic binary mobile phase. The adsorption volume ratio of its organic component to water represents the hydrophobicity of the column. Common HPLC solvents including acetonitrile and methanol may be used in the experiments. Acetonitrile is considered to be a better candidate due to its stronger interaction with the hydrophobic ligands and negligibly weak interaction with residual free silanols and other polar functional groups of the ligand. On the other hand,

methanol can interact with the hydrophilic free silanols on the surface and polar functional groups of the reversed-phase through hydrogen bonding, hence competes with water molecules to adsorb on the hydrophilic part of the surface and consequently create erroneous estimation. This method may be used to build a repertory of columns scrutinized by their hydrophobicity. Since columns are characterized by adsorbate volume ratio using common HPLC solvents, the comparison is independent of column dimension and does not rely on particularly selected analytes, hence is more objective. As a general rule, a more hydrophobic column will have a longer retention for more nonpolar analyte compounds. Nevertheless, this is only an initial proposal of the approach, more types of columns should be examined in the future, so that a more complete picture of the versatility for this method can be explored.

Chapter 3.

Estimation of Gibbs Free Energy Using Excess Adsorption Isotherm for Reversed-Phase High Performance Liquid Chromatography

3.1. Introduction

In the past thirty years, high Performance Liquid Chromatography (HPLC) has been widely accepted as one of the major analytical tool in many fields such as environmental, pharmaceutical, polymer and food industries. Among various modes of separation, reversed-phase liquid chromatography (RPLC) is far more popular than the others. Various types of bonded phases containing different ligands have been developed. Despite its wide applications, the retention mechanism of chromatography is still controversial. Most of the early retention models focused on the role of mobile phase, mainly due to the technology limitations. Study of retention mechanism by mobile phase variations are experimentally more convenient to perform. The concept of mobile phase driven retention mechanism may be traced back to Horvath's solvophobic theory [108]. According to the model, reversed-phase retention is solely governed by the solubility of analytes in mobile phase. The stationary phase does not participate in any selectivity of the analytes. However, many studies showed that the stationary phase in fact plays an important role. The alkyl chain length [109] [110] [111] [112], surface coverage [38] [113] and functional groups [99] [114] [115] of the bonded phase, all impact the analyte retention. Thereafter, numerous researches have been focused

on the role of solute distribution between mobile phase and stationary phase. Generally, these studies can be summarized into three models.

(1) Analytes are partitioned between mobile phase and stationary phase [116] [117] [118].
(2) Analytes are adsorbed on the bonded reversed-phase surface [75] [119] [64].
(3) The organic component of an aqueous-organic mobile phase is preferentially adsorbed by the bonded reversed-phase, thus form a layer of liquid with different organic to aqueous ratio on the surface. The analytes are partitioned between the mobile phase and this adsorbed layer [120] [66].

Fundamentally, complete demonstration of a chromatographic retention process needs to be supported by thermodynamic assessment, or more specifically, to determine the associated energy changes for analyte molecules transferring between the mobile phase and stationary phase. The main difficulty for estimation of these parameters is how to define the boundary of the stationary phase and how to quantitatively measure its accessible volume. Commonly used HPLC retention factor k' can be related to the thermodynamic equilibrium constant of the system by $k' = \emptyset K$, where $\emptyset = V_S/v_m$ stands for phase ratio of stationary phase volume (V_S) to mobile phase volume (v_m). The Gibbs free energy (ΔG) can be then estimated by the Arrhenius correlation of equilibrium constant K to adsorption free energy change, i.e. $K = e^{-\Delta G/RT}$.

For partition mechanism of a binary mobile phase system, solving the mass balance equation leads to the following basic retention equation 3.1 [121]:

$v_R = v_m + V_S K$ Eqn. 3.1

Where v_R is the retention volume of the analyte. v_m is the volume of the mobile phase. K is the equilibrium constant of the system which can be expressed as an exponential function of Gibbs free energy. Unlike gas chromatography where the volumes of both phases are well defined and can be experimentally measured, the stationary phase volume of an HPLC system is more ambiguous. RP-HPLC studies based on partition theory often accept the bonded phase volume as the stationary phase volume [118] [62]. This definition at the first glance seems working well for long chain bonded phases such as C_{18}. However, for short chain bonded phases, due to high bonding density and lack of conformational freedom, there will be no room for analyte partition. Furthermore, Kazakevich et al [35] found that alkyl chains of reversed-phase stay in their collapsed conformation under general HPLC conditions. The adsorbed solvent and analyte molecules are not able to penetrate into the bonded phases but only adsorbed on the top of the alkyl chains. In fact, the retention of analyte is actually proportional to the surface area of the stationary phase [122]. These phenomena lead to difficulties in performing thermodynamic evaluation with equation 3.1. Another shortage of this approach is the assumption of $v_m = v_0$, where v_0 is the void volume and defined as the total volume of liquid phase in the column. Common chromatography defines retention factor k'as

$k' = \frac{v_R - v_0}{v_0}$ or $v_R = v_0(k' + 1)$

Substitute into equation 3.1 can obtain

$k' = \frac{v_m}{v_0} - 1 + \frac{V_S}{v_0} K$ Eqn. 3.2

Only assuming $v_m = v_0$ in equation 3.2 can lead to $k' = \frac{V_S}{v_0} K = \emptyset K$

This assumption needs to define a dividing plane located on the bonded phase surface and again, fall into the trap of stationary phase volume determination.

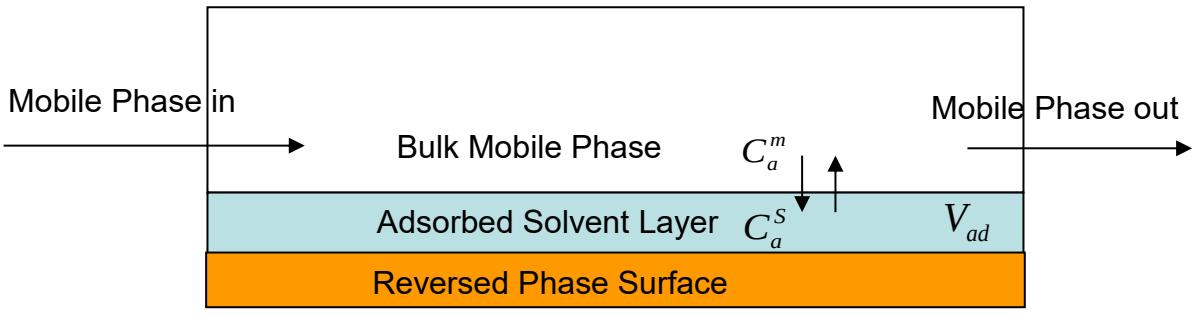
The adsorption theory was first introduced by Kiselev [123] and further explored by Foti et al [124]. Analyte retention volume derived from excess adsorption approach can be expressed as:

$$v_R = v_0 + AK_H \qquad \text{Eqn. 3.3}$$

Where A is the total adsorbent surface area, K_H is the analyte adsorption constant at Henry's region or more specifically, the slope of the analyte excess adsorption isotherm at infinitely small concentration. In this equation, K_H contains a length unit and is not a conventional thermodynamic equilibrium constant. Thus, leads to difficulty for performing thermodynamic assessments.

In this research we proposed a more assessible approach to estimate the Gibbs free energy for a reversed-phase HPLC process based on adsorption theory by using surface excess estimation. By applying the interpretation in Chapter 2, Section 2.4.4 and Section 2.4.5 regarding the linear region of the excess adsorption isotherm of an organic-aqueous binary solvent system. This region represents an adsorbed layer of solvents with same components but different composition to the bulk mobile phase. As shown in **Figure 3.1**, the analyte molecules are transferred into this layer through adsorption by the stationary phase and displace an equal volume of the adsorbed liquid molecules, thus build a distribution between the mobile phase and this adsorbed

solvent layer. The chromatographic free energy is determined through the equilibrium concentrations of the analyte in the mobile phase and this adsorbed solvent layer.



C_a^m = Analyte concentration in mobile phase

C_a^S = Analyte concentration in the adsorbed solvent layer

V_{ad} = Adsorbed solvent layer volume

Figure 3.1. *Schematic of an analyte "a" distributed between the Bulk mobile phase and adsorbed solvent layer*

3.2 Experimental

3.2.1. HPLC System

Column:	Phenomenex Luna C_{18}(2), 5µm, 4.6 x 150 mm, specific surface area = 400 m^2/g
HPLC System:	HP1050 pump with inline degasser and auto injector, Erma Optical ERC 1570 RI Detector
Detector Temperature:	45°C
Column Temperature:	45°C maintained by a circulating water bath.
Flow rate:	0.5 mL/minute for excess adsorption isotherm estimation; 1.0 mL/minute for alkyl benzene and alkane tests

3.2.2. Mobile Phase and Samples

For excess adsorption isotherm estimation:

Isocratic at 0% to 100% of acetonitrile in water. All mobile phases were degassed with an inline degasser. Inject 0.5 µL of deuterated acetonitrile and 0.5 uL of deuterated water. Column void volume and excess adsorption isotherms were calculated using the retention volumes of minor disturbance peaks obtained from the injection of deuterated acetonitrile and confirmed with the injection of deuterated water.

For alkyl benzene test:

Isocratic at 60% to 85% of acetonitrile in water. All mobile phases were degassed with an inline degasser. Inject 0.1 µL each of benzene, toluene, ethyl benzene, propyl benzene, butyl benzene and pentyl benzene.

For alkane test:

Isocratic at 60% to 90% of acetonitrile in water. All mobile phases were degassed with an inline degasser. Inject 0.1 µL each of hexane, heptane, octane and nonane.

3.2.3. Chemicals

Acetonitrile was HPLC grade purchased from Pharmco (Philipsberg, PA USA). Deuterated acetonitrile, deuterated water and alkyl benzenes were purchased from Sigma-Aldrich (St. Louis. Mo., USA). Alkanes were purchased from Fluka (Ronkonkoma, NY, USA). Water was purified by Milli-Q system from Millipore (Milford, MA. USA).

3.3. Results

Figure 3.2 shows the minor disturbance peak retention volume at increasing concentration of acetonitrile in the mobile phase. The generated excess adsorption isotherm is shown in **Figure 3.3** with a linear regression line plotted at the linear region of the isotherm. **Table 3.1** contains the adsorbed liquid volume data obtained from the excess adsorption isotherm.

Alkyl homologues are often used to verify chromatographic process. Their corresponding Gibbs free energies determined from the experiments are shown in **Table 3.2** and **Table 3.4.** The so-called methylene selectivity is the Gibbs free energy difference between each pair of analytes with adjacent carbon number. This data is shown in **Table 3.3 and Table 3.5**. Correlation of ΔG to alkyl benzene and alkane homologues at different organic / water ratios are also plotted in **Figure 3.4** and **Figure 3.5**.

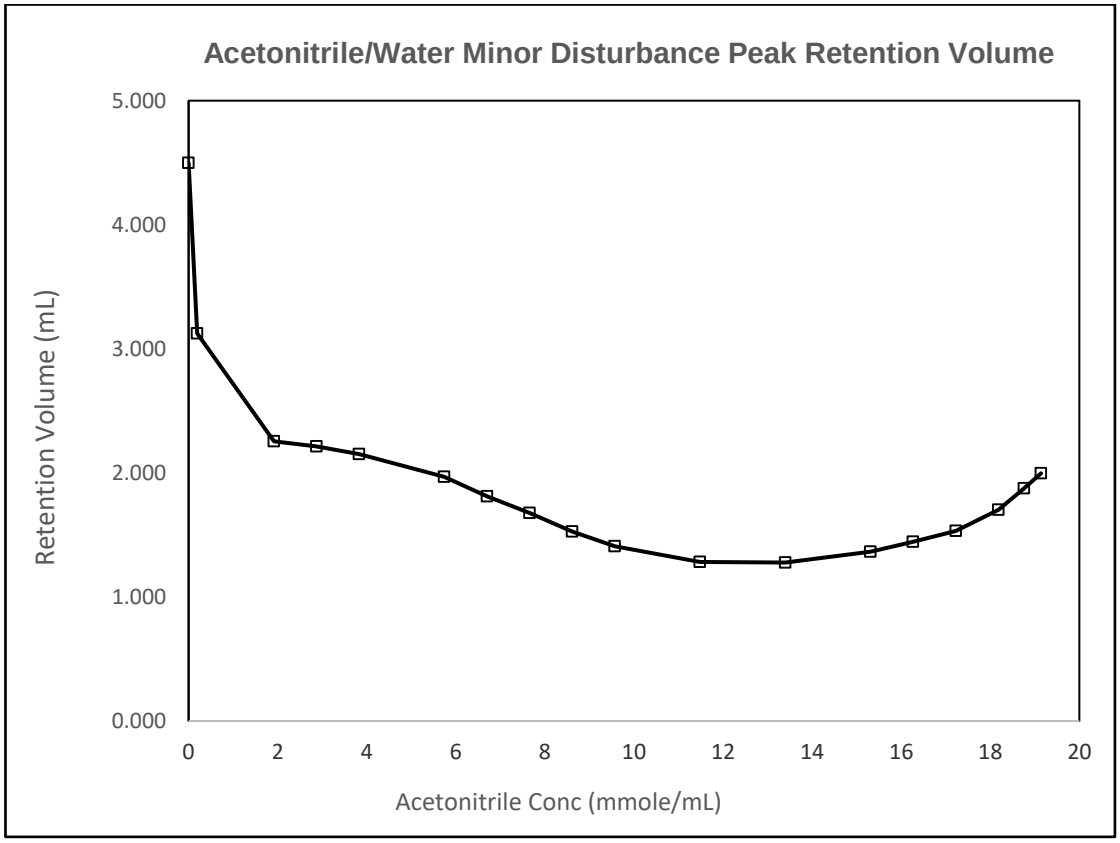


Figure 3.2. *Retention volume (mL) of the minor disturbance peak using mobile phases containing 0 to 100 % acetonitrile in water on a* Luna C_{18}(2), 5µm, 4.6 x 150 mm *column.*

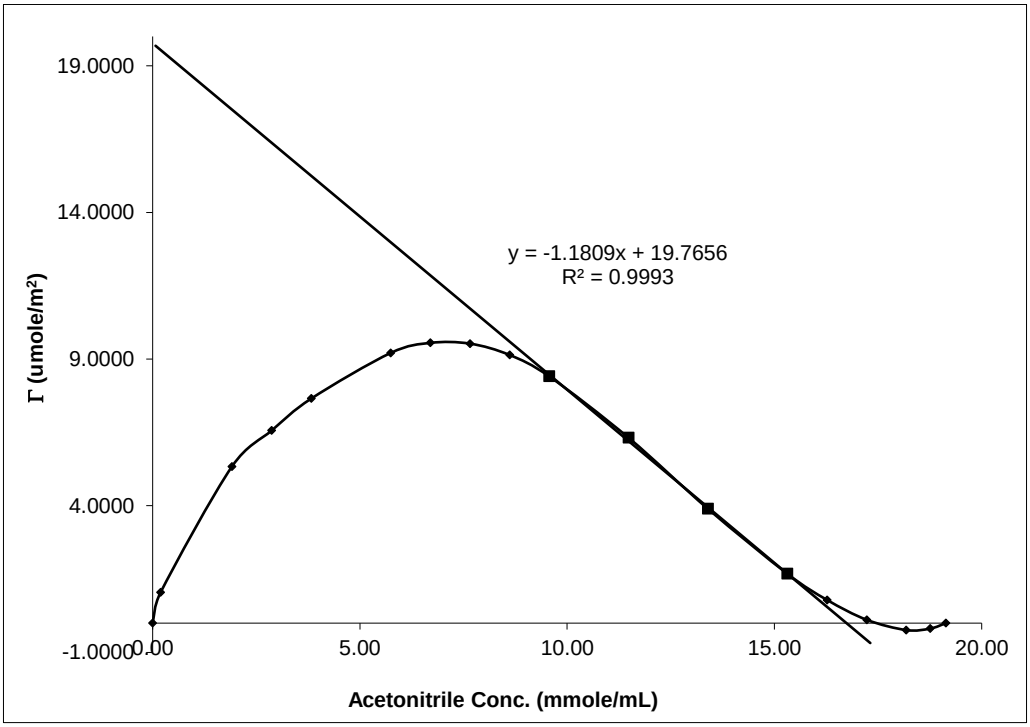


Figure 3.3. *Excess adsorption isotherm of acetonitrile in water on* Luna C_{18}(2), 5µm, 4.6 x 150 mm *column with linear regress line at the linear region of the curve.*

Table 3.1. *Adsorption isotherm test results on Luna C_{18}(2), 5µm, 4.6 x 150 mm column (acetonitrile-water mobile phase)*

Slope	Intercept	Adsorbed Liquid Volume Per Square Meter (Specific Volume)		
		Acetonitrile Volume ($\mu L/m^2$)	Water Volume ($\mu L/m^2$)	Total Volume ($\mu L/m^2$)
1.1809	19.7656	1.0327	0.1482	1.1809

Table 3.2. *Gibbs free energy (ΔG) released for alkylbenzenes in adsorption chromatographic process at 45ºC (Joul/mole)*

Analyte	Acetonitrile % in Mobile Phase					
	60%	**65%**	**70%**	**75%**	**80%**	**85%**
Benzene (Ben C0)	4874	4272	3734	3222	2697	2277
Toluene (Ben C1)	5883	5202	4574	3957	3360	2843
Ethyl benzene (Ben C2)	6837	6058	5348	4657	3984	3393
Propyl benzene (Ben C3)	7948	7069	6272	5487	4728	4049
Butyl benzene (Ben C4)	9066	8091	7209	6344	5487	4708
Pentyl benzene (BenC5)	10196	9131	8155	7202	6261	5403

Table 3.3. *Gibbs free energy (ΔG) released per methylene group (CH_2) for alkylbenzenes in adsorption chromatographic process at 45ºC (Joul/mole)*

Analytes Ratio	Acetonitrile % in Mobile Phase					
	60%	**65%**	**70%**	**75%**	**80%**	**85%**
Ben C1-Ben C0	1009	931	840	735	663	566
Ben C2-Ben C1	954	855	774	700	624	550
Ben C3-Ben C2	1111	1011	924	829	745	656
Ben C4-Ben C3	1118	1022	937	857	759	659
Ben C5-Ben C4	1131	1039	946	858	774	695

Table 3.4. *Gibbs free energy (ΔG) released for alkanes in adsorption chromatographic process at 45ºC (Joul/mole)*

Analyte (Cn)	**Acetonitrile % in Mobile Phase**						
	60%	**65%**	**70%**	**75%**	**80%**	**85%**	**90%**
Hexane (C6)	9554	8638	7805	7036	6289	5487	4657
Heptane (C7)	10737	9724	8816	7965	7130	6238	5309
Octane (C8)	11917	10887	9829	8896	7983	6989	5978
Nonane (C9)	13138	11912	10841	9888	8846	7764	6655

Table 3.5. *Gibbs free energy (ΔG) released per methylene group (CH_2) for alkanes in adsorption chromatographic process at 45ºC (Joul/mole)*

Analytes Ratio	Acetonitrile % in Mobile Phase						
	60%	**65%**	**70%**	**75%**	**80%**	**85%**	**90%**
C7-C6	1183	1086	1011	929	841	752	651
C8-C7	1180	1163	1013	931	853	751	669
C9-C8	1221	1026	1012	992	863	775	677

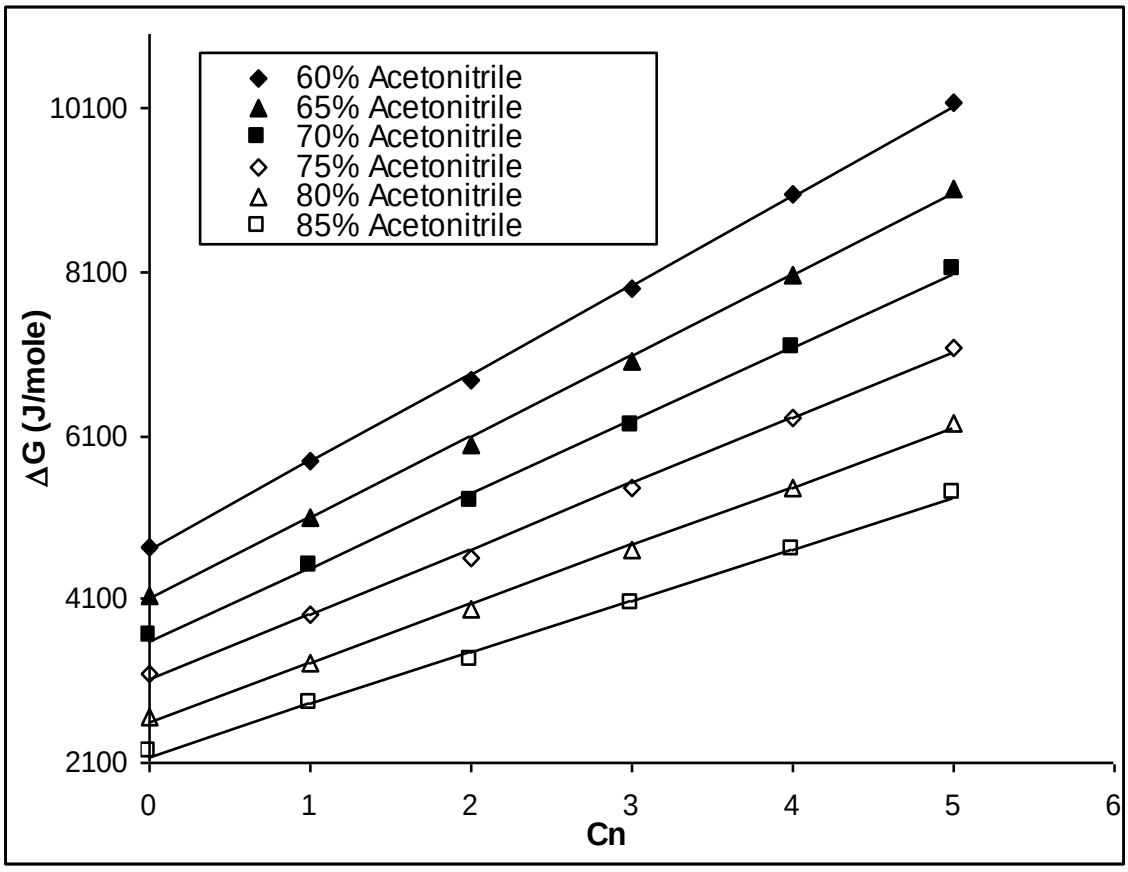


Figure 3.4. *Plot of Gibbs free energy (ΔG) released vs. alkyl chain length (Cn) of alkylbenzenes in adsorption chromatographic process at 45ºC. Mobile phases contain 60% to 85% acetonitrile in water.*

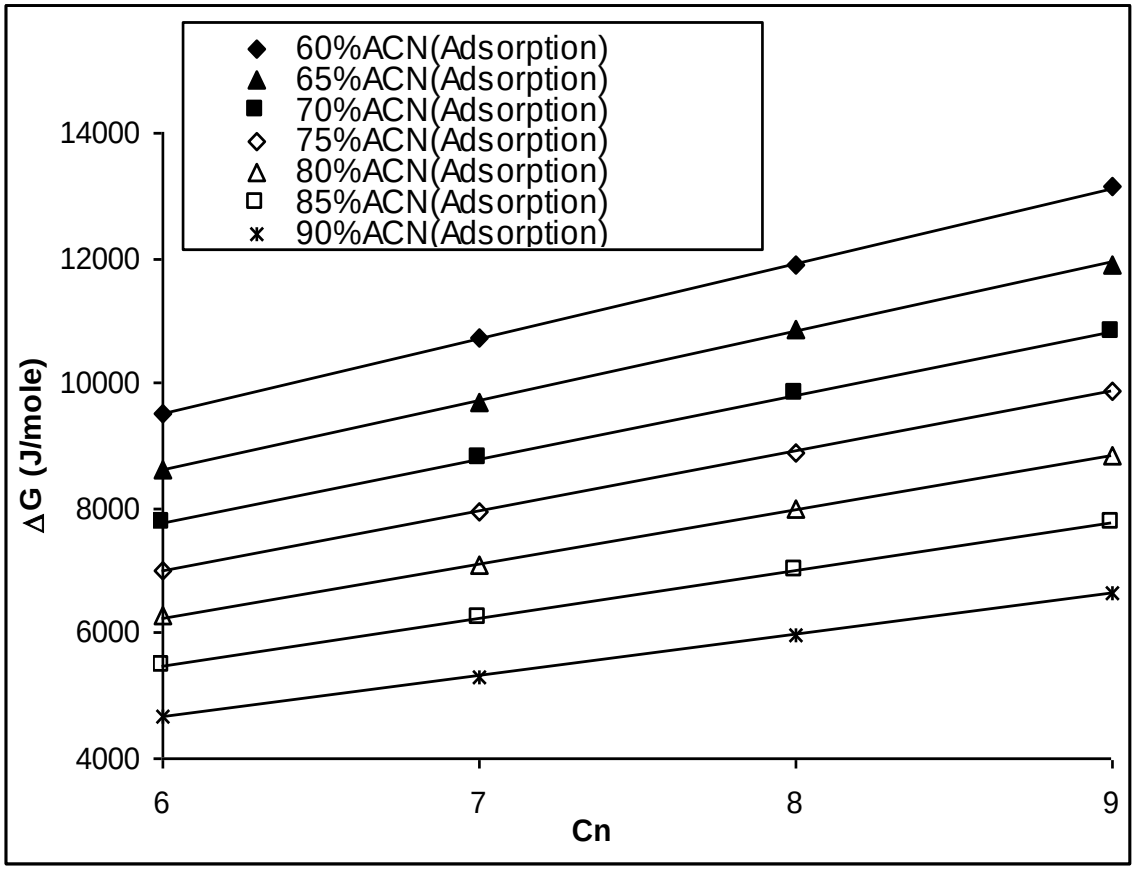


Figure 3.5. *Plot of Gibbs free energy (ΔG) released vs. alkyl chain length (Cn) of alkanes in adsorption chromatographic process at 45ºC. Mobile phases contain 60% to 90% acetonitrile in water*.

3.4. Discussion

3.4.1. Theory of Determination

Practically, injection volume for HPLC analyses are negligibly small that essentially do not affect the mobile phase configuration. De Vault [5] and Kovats [119] discussed the general differential mass balance in the column for a multicomponent system and concluded that a mathematical solution is only available for a binary system. Most of the common chromatographic systems are comprised of three-components where two components of a binary eluent are presented in significantly high concentrations. The third analyte component at a low concentration (several orders of magnitude lower in concentration) is usually injected at very low volume. This allows the assumption that the injection of the infinitesimally small quantity of the analyte does not disturb the adsorption equilibrium of the eluent components, thus it is possible to first describe their adsorption equilibrium and then use it to independently describe the analyte retention. Based on this assumption, when an analyte (a) is injected into a binary aqueous-organic system such as water and acetonitrile in equilibrium with a reversed-phase column, the composition and volume of the mobile phase remain unchanged. If we also apply a constant thickness adsorption layer model as proposed in Chapter 2, Section 2.3.5, the adsorbed solvent layer indicated in Section 3.1 will also remain unchanged as well. The following mathematical derivation introduces a convenient way to estimate the chromatographic free energy by surface excess adsorption.

Nomenclature:

Γ_a	Surface excess adsorption of analyte.
C_a^m	Concentration of analyte in the mobile phase
C_a^S	Concentration of analyte in the Surface adsorbed liquid layer
V_{ads}^S	Volume of adsorbed liquid layer (acetonitrile plus water) on the surface
v_R	Retention volume of the analyte
v_0	Void volume of the column
ΔG	Gibbs free energy of transferring one mole of analyte from mobile phase to stationary phase
R	Gas constant (= 8.314 Joul/mole/°K)
T	Absolute temperature (°K)

According to Everett's definition for excess adsorption [102], The surface excess of the analyte (a) can be expressed by the following equations.

$$\Gamma_a = \frac{V_{ads}^S}{A}(C_a^S - C_a^m) \qquad \text{Eqn. 3.4}$$

Assume instant equilibrium, the Gibbs free energy of this HPLC process can be calculated from the equilibrium constant K as follows, where superscript s and m denote "in the surface adsorbed liquid layer" and "in the mobile phase", respectively for the process.

$$K = \frac{C_a^S}{C_a^m} \text{ and } C_a^S = KC_a^{Sm} \qquad \text{Eqn. 3.5}$$

Substitute equation 3.5 into equation 3.4:

$$\Gamma_a = \frac{V_{ads}^S}{A}(KC_a^m - C_a^m)$$

and

$$\Gamma_a = \frac{V_{ads}^S}{A} C_a^m (K-1) \qquad \text{Eqn. 3.6}$$

In a general HPLC process, the injection volume of the analyte solution is negligibly small compared to the adsorbed layer volume V_{ad}^S , therefor it will not affect the composition as well as the total volume of the adsorbed solvent layer. The derivative of Γ_a can be expressed by the following equation 3.8

$$\frac{d\Gamma_a}{dC_a^m} = \frac{V_{ads}^S}{A}(K-1) \qquad \text{Eqn. 3.8}$$

Substitute into the following analyte retention equation (Eqn. 3.9) which is derived from the excess adsorption model [72], the equilibrium constant K can be calculated by equation 3.10.

$$v_R = v_0 + A\frac{d\Gamma_a}{dC_a^m} \qquad \text{Eqn. 3.9}$$

and

$$v_R = v_0 + A\frac{V_{ads}^S}{A}(K-1)$$

Hence

$$K = \frac{v_R - v_0 + V_{ads}^S}{V_{ads}^S} \qquad \text{Eqn. 3.10}$$

By taking natural log on the Arrhenius correlation of equilibrium constant K to adsorption free energy change ΔG, ln(K) = -ΔG/RT, ΔG can be calculated by the following equation 3.11. This ΔG value represents the molar Gibbs free energy change of the chromatographic system for transferring one mole of analyte molecules from mobile phase to the surface adsorbed liquid layer. Therefore, avoid the involvement of stationary phase volume or surface area specific Henry constant K_H.

Substitute equation 3.10 into $ln(K) = \frac{\Delta G}{RT}$

$$ln\left(\frac{v_R - v_0 + V_{ads}^S}{V_{ads}^S}\right) = -\frac{\Delta G}{RT}$$

and

$$\Delta G = -RT\left[ln\left(\frac{v_R - v_0 + V_{ads}^S}{V_{ads}^S}\right)\right] \qquad \text{Eqn. 3.11}$$

In equation 3.11, v_R and v_0 are chromatographically measurable values. V_{ads}^S can be obtained from the linear part of the binary solvent excess adsorption isotherm using equation 2.15 and determine as stated in chapter 2, section 2.3.5. The following equation 3.12 is a copy of equation 2.15.

$$\Gamma_B^T = V_B^S D_B - (V_B^S + V_W^S)C_B^l \qquad \text{Eqn. 3.12}$$

By plotting Γ_B^T versus C_B^l from equation 3.12, the adsorbed volume of solvent B (V_B^S) and water (V_W^S) can be estimated from the slope and intercept. The sum of V_B^S and V_W^S represents the total volume of the adsorbed layer V_{ads}^S which is the part of the

stationary phase that is physically anticipated in the distribution of an injected analyte. These two volumes are specific volumes expressed in volume per surface area. If the surface area can be determined by physical measurement method such as Low Temperature Nitrogen Adsorption (LTNA) and the adsorbent weight, the total volume of the adsorbed layer on the adsorbent surface can be calculated.

Equation 3.11 is derived from the thermodynamic equilibrium aspect based on the concept of excess adsorption of analyte molecules on the bonded phase surface [121]. It does not need to determine the bonded ligand stationary phase volume. The distribution equilibrium constant of the analyte is determined directly from the retention volume of the analyte and the volume of the adsorbed liquid layer on the stationary phase surface. The only boundary is the mobile phase of the chromatographic system must be a binary solvent mix and the estimation of Gibbs free energy must be performed at the linear region of the excess adsorption isotherm.

3.4.2. Determination of Adsorbed Liquid Layer Volume

Excess adsorption isotherm was generated using minor disturbance method by following the procedure described in Chapter 2, Section 2.4.3, “Determination of Excess Adsorption Isotherm”. The excess adsorption Γ_B^T (in µmole/m^2) was calculated by applying numerical approach with equation 2.5. The volume of the adsorbed liquid layer was then calculated using equation 2.15

Excellent linearity was obtained with R^2=0.9993 (**Figure 3.3**). As explained in Chapter 2, Section 2.3.5, this region represents a complete filling of adsorbed acetonitrile and water. No further accumulation can occur on the surface. Thus, the composition and the volume of the adsorbed layer on unit surface area of the adsorbent are constant.

The column packing material was then emptied and dried in a 60 ºC oven under 1 mm Hg vacuum for 24 hours. The total packing material was weighed and the total surface area was calculated using the vender provided specific area. The total volume of the adsorbed liquid layer was then calculated as following:

Column packing material weight = 0.9375 g

Packing material specific area (Provided by vender) = 400 m^2/g

Total surface area calculated = 0.9375 g x 400 m^2/g = 375 m^2

Volume of adsorbed liquid layer = 1.1809 $\mu L/m^2$ x 375 m^2 = 442.84 µL or 0.4428 mL

3.4.3. Determination of The Chromatographic Gibbs Free Energy

Alkyl homologues are often used to verify chromatographic process. In our experiment, we injected alkyl benzenes with alkyl chain lengths from C_1 to C_5 and alkanes with chain lengths from C_6 to C_9 into the Luna $C_{18}(2)$ column which has been equilibrated with binary mobile phases containing acetonitrile in water at 60% to 90%. In order to obtain reasonable retention time and maintain solubility for all analytes, column temperature was maintained at 45 ºC. The Gibbs free energies (ΔG) for adsorption process were then calculated with equation 3.11. The Gibbs free energy difference between each pair of analytes with adjacent carbon number were also calculated for comparison. This is the so-called methylene selectivity, i.e. selectivity of each CH_2 group. Corresponding data is summarized in **Table 3.2** to **Table 3.5**. Correlation of ΔG to alkyl benzene and alkane homologues at different organic / water ratios are also plotted in **Figure 3.4** and **Figure 3.5**. The graphs showed that ΔG of both homologue series were increasing with increasing analyte alkyl chain length and decreasing with increasing mobile phase organic ratio. These results match the general concept of alkyl compound with longer chain length exhibiting higher affinity to hydrophobic surface and

mobile phase with higher organic ratio. The test results also showed that the determined ΔG for alkyl benzene compounds were lower than the alkane compounds with same total number of carbon atom. For instance, the chromatographic Gibbs free energy for ethyl benzene with a 60% acetonitrile mobile phase was 6837 Joul/mole where the Gibb's free energy for octane was 11917 Joul/mole. This indicates that alkyl benzene is more polar than alkane and showed a lower affinity to the alkyl surface.

The methylene (CH_2) selectivity of both set of compounds is comparable among each pair of analytes within the same homologue. Alkanes generally show higher methylene selectivity than alkyl benzenes. The difference become more significant at mobile phases with a higher acetonitrile percentage. The methylene selectivity also decreases with increasing acetonitrile percentage. Reflecting that the hydrophobic interaction exerted per methylene group of each compound to the reversed-phase surface within its own homolog is similar but is different from different homologues. As a fact, alkyl column will provide longer retention and better separation for alkane compounds than alkyl benzene, particularly at mobile phase with higher acetonitrile percentage.

3.5. Conclusion

Traditionally, the Gibbs free energy (ΔG) of an analyte in a chromatographic process are often estimated by the linear Van't Hoff plot [125]:

$$lnk' = \frac{-\Delta G}{RT} + ln\emptyset$$

ΔG may be obtained from the slope of the linear plot of lnk' versus 1/T where k' is the capacity factor of the analyte, T is the absolute temperature and ∅ is the phase ratio of the column. However nonlinear Van't Hoff plots have been observed for temperature studies of reversed-phase stationary phases due to phase transition. Typically, temperature ranges of 45° C or more have been evaluated in the studies showing nonlinear Van't Hoff plots. These phase transitions have been found to be much more pronounced on high bonding density alkyl stationary phases [126] [127].

Using the equilibrium constant calculated by the analyte concentration in bulk mobile phase and the surface absorbed solvent layer is a more versatile method to estimate the associated Gibbs free energy change for reversed-phase liquid chromatography. Connecting RP-HPLC to the excess adsorption model can directly calculate the Gibbs free energy of a chromatographic process from its analyte retention volume and the volume of the adsorbed liquid layer, thus avoid anticipating in the ambiguity of bonded ligand stationary phase volume determination and the problem of nonlinear temperature plot. The result trends of both alkyl benzene and alkane homologues match the general concept of alkyl compounds with longer alkyl chain length exhibiting higher affinity to hydrophobic surface and relatively non-polar mobile phase. As compared to alkanes, alkyl benzenes release lower Gibbs free energy when transferring from mobile phase to stationary phase. However, the methylene (CH_2) selectivity of both set of compounds shows that the hydrophobic interaction from each methylene group of these compounds to the alkyl surface is comparable within its own homologue but different from different homologues. This difference becomes more significant with mobile phases at high acetonitrile percent.

Appendix

Table *A-1.* *Acetonitrile / water minor disturbance peak retention volume and excess adsorption for custom made alkyl bonded columns*

Acetonitrile %(v/v)	Column							
	Alkyl C_4		Alkyl C_8		Alkyl C_{12}		Alkyl C_{18}	
	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**
0	2.865	0.0000	3.600	0.0000	4.106	0.0000	4.037	0.0000
1	2.525	0.4221	2.918	0.7287	2.937	0.9087	3.003	0.8965
5	2.435	1.6705	2.535	2.5534	2.407	2.8044	2.502	2.9114
10	2.384	3.0505	2.408	4.1818	2.240	4.2823	2.358	4.6049
20	2.270	5.3884	2.250	6.7093	2.125	6.5165	2.212	7.2496
30	2.104	7.0098	2.034	8.2798	1.962	8.0393	1.986	8.9425
40	1.815	7.4669	1.725	8.5069	1.658	8.3672	1.655	9.2102
50	1.573	6.5653	1.494	7.3522	1.450	7.3849	1.423	8.0372
60	1.411	4.6300	1.348	5.2328	1.289	5.4585	1.288	5.9251
70	1.409	2.2749	1.346	2.7348	1.291	3.1251	1.279	3.4445
80	1.524	0.2091	1.459	0.5207	1.390	1.0502	1.388	1.2198
90	1.731	-1.0329	1.652	-0.9103	1.543	-0.3798	1.572	-0.2551
95	1.906	-1.1651	1.827	-1.1550	1.682	-0.7212	1.747	-0.5332
99	2.308	-0.6803	2.254	-0.7346	1.990	-0.5369	2.160	-0.1539
100	4.091	0.0000	4.287	0.0000	3.600	0.0000	1.978	0.0000

*: V_R = Retention volume in mL
**: Γ = Excess adsorption in μmole/m^2

TableA-2. *Acetonitrile / water minor disturbance peak retention volume and excess adsorption for commercially available columns*

Acetonitrile %(v/v)	Column							
	Zorbax SB-C_{18}		Curosil PFP		Luna Phen C_6		Colgent UDC	
	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**
0	2.754	0.0000	3.291	0.0000	3.277	0.0000	3.133	0.0000
1	2.353	1.1836	2.799	0.9535	2.704	0.9945	2.502	0.5799
5	1.947	4.2017	2.458	3.5516	2.329	3.5890	2.227	1.9086
10	1.821	6.5602	2.312	5.9108	2.149	5.8196	2.144	3.0800
20	1.716	10.0489	2.162	9.5492	1.989	9.0402	2.044	4.9225
30	1.573	12.2190	1.988	12.0055	1.821	11.0641	1.895	6.0841
40	1.358	12.4858	1.688	12.7323	1.555	11.5044	1.652	6.1739
50	1.220	10.8758	1.410	11.3502	1.330	10.1533	1.428	4.9868
60	1.142	8.1174	1.283	8.4904	1.225	7.5981	1.306	2.8536
70	1.144	4.9549	1.279	5.1526	1.229	4.6744	1.375	0.5755
80	1.204	2.1221	1.389	2.2016	1.311	2.0645	1.520	-1.1175
90	1.295	0.0921	1.562	0.2831	1.455	0.2792	1.809	-1.6237
95	1.373	-0.4737	1.660	-0.1817	1.540	-0.1957	2.061	-1.1373
99	1.531	-0.4244	1.874	-0.0982	1.771	-0.1145	2.224	-0.2942
100	2.148	0.0000	1.872	0.0000	1.798	0.0000	2.366	0.0000

*: V_R = Retention volume in mL
**: Γ = Excess adsorption in μmole/m^2

Table*A-3.* *Methanol / water minor disturbance peak retention volume and excess adsorption for custom made alkyl bonded columns*

Methanol %(v/v)	Column							
	Alkyl C_4		Alkyl C_8		Alkyl C_{12}		Alkyl C_{18}	
	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**
0	2.188	0.0000	2.352	0.0000	2.414	0.0000	2.405	0.0000
1	2.141	0.1923	2.257	0.3142	2.291	0.3843	2.266	0.3902
5	2.066	0.8005	2.105	1.2448	2.085	1.4868	2.043	1.4729
10	2.022	1.3642	2.009	1.9986	1.959	2.3169	1.915	2.2466
20	1.955	2.1252	1.904	2.8424	1.841	3.1712	1.802	2.9984
30	1.894	2.4634	1.832	3.1017	1.767	3.3915	1.737	3.1623
40	1.840	2.4219	1.776	2.9384	1.714	3.1924	1.690	2.9565
50	1.798	2.0634	1.742	2.4778	1.677	2.6961	1.657	2.4864
60	1.782	1.5133	1.724	1.8455	1.658	2.0149	1.641	1.8545
70	1.776	0.8906	1.722	1.1472	1.659	1.2742	1.642	1.1731
80	1.798	0.3208	1.738	0.4951	1.676	0.5930	1.664	0.5676
90	1.837	-0.0477	1.773	0.0115	1.707	0.0703	1.686	0.1075
95	1.853	-0.1411	1.803	-0.1231	1.737	-0.0903	1.703	-0.0582
99	1.920	-0.1061	1.870	-0.1025	1.803	-0.0920	1.762	-0.0904
100	2.148	0.0000	2.098	0.0000	2.017	0.0000	2.001	0.0000

*: V_R = Retention volume in mL
**: Γ = Excess adsorption in µmole/m^2

TableA-4. *Methanol / water minor disturbance peak retention volume and excess adsorption for commercially available columns*

Methanol %(v/v)	Column							
	Zorbax SB-C_{18}		Curosil PFP		Luna Phen C_6		Colgent UDC	
	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**	V_R*	Γ**
0	1.810	0.0000	2.179	0.0000	2.086	0.0000	2.151	0.0000
1	1.711	0.4763	2.126	0.3952	2.010	0.3989	2.065	0.2545
5	1.551	1.6705	2.011	1.6598	1.875	1.5977	1.960	1.0030
10	1.467	2.3262	1.916	2.7471	1.778	2.5509	1.886	1.6229
20	1.409	2.6634	1.808	3.9672	1.679	3.5359	1.814	2.3474
30	1.405	2.5752	1.738	4.3505	1.616	3.7593	1.761	2.6308
40	1.396	2.3977	1.690	4.1790	1.576	3.4984	1.709	2.5437
50	1.375	2.0145	1.656	3.6221	1.551	2.9319	1.675	2.1532
60	1.372	1.4665	1.634	2.8019	1.532	2.1586	1.657	1.5791
70	1.371	0.8911	1.637	1.8923	1.535	1.3101	1.664	0.9663
80	1.389	0.4324	1.652	1.0674	1.551	0.5509	1.665	0.3817
90	1.397	0.1520	1.672	0.4070	1.579	-0.0015	1.720	-0.0054
95	1.403	0.0599	1.682	0.1473	1.599	-0.1648	1.738	-0.0701
99	1.403	0.0026	1.701	-0.0059	1.649	-0.1638	1.774	-0.0456
100	1.420	0.0000	1.776	0.0000	1.947	0.0000	1.850	0.0000

*: V_R = Retention volume in mL
**: Γ = Excess adsorption in µmole/m^2

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