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Surface Characterization of Mixed-mode Adsorbents using BET C-Constant Energy Scale

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Dinah C. Lee has successfully defended and made the required modifications to the text of the doctoral dissertation for the Ph.D.
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Dedicated to

My Mother-In-Law Sandra Lee
My Husband Adam & My Son Brady
My Mother Gloria Sandig

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Mixed-mode chromatography is becoming increasingly popular in pharmaceutical, biopharmaceutical, environmental, food and other industrial applications due to its unique selectivity and retention of a variety of compounds. Mixed-mode chromatography is a chromatographic method in which solutes interact with stationary phase through more than one interaction mode or mechanism. It is because of these multiple complex interactions, which makes it difficult to predict chromatographic behavior of analytes on Mixed-mode columns. In order to fully understand the retention mechanisms on Mixed-mode columns, the packing material properties must be well characterized since each characteristic may contribute to the overall chromatographic performance of the column. Many non-chromatographic and chromatographic techniques are available to characterize and compare Mixed-mode columns in terms of their interaction abilities, retentivity, surface chemistry, chemical properties and geometry. Characterization of different Mixed-mode columns on the basis of interaction energy characteristics has not been explored.

In this study, different ratios of porous silica (Axia Luna Silica) and C_{18} (Axia Luna C_{18}), which are commonly used single mode adsorbents were blended in various ratios to simulate Mixed-mode materials and analyzed by Low Temperature Nitrogen Adsorption (LTNA). Adsorption isotherms, surface area, and BET C-Constants were obtained for all the blended materials and a linear relationship between the BET C-Constant and the blend ratio of silica has been observed. A new BET C-Constant energy scale was created from a plot of non-specified average interaction energy vs. percent silica surface.

A variety of commercially available Mixed-mode columns were unpacked and the materials were analyzed by LTNA in order to explore the use of the BET C-Constant energy scale

as a universal indexation system to characterize Mixed-mode materials. Adsorption isotherms, surface area, and BET C-Constants were obtained for all the commercial Mixed-mode materials. The non-specified average interaction energy and the equivalent percent silica surface was calculated from the BET C-Constant and plotted on the BET C-Constant energy scale to enable Mixed-mode column comparison based on average interaction energy. The data in this research project suggests that the BET C-Constant may be a viable parameter that can be used to characterize commercial Mixed-mode columns on the basis of average interaction energy.

Utilizing this BET C-Constant energy scale in conjunction with the study of retention behaviors on Mixed-mode material could lead to improvements in the understanding of method development on Mixed-mode columns, allow the comparison and selection of commercialized Mixed-mode columns, and even predict retention behavior of analytes in Mixed-mode columns.

CHAPTER 1 – AN INTRODUCTION TO MIXED-MODE CHROMATOGRAPHY (MMC)

1.1 The Basic Theory of High Performance Liquid Chromatographic (HPLC) Separation Technique

High-performance liquid chromatography (HPLC) is a common analytical technique that is used to separate, identify, and quantitate an analyte present in a liquid mixture. HPLC utilizes pumps to pass a highly pressurized solvent or "mobile phase" and a liquid sample mixture through a column filled with a solid adsorbent material or "stationary phase". The adsorbent, which plays a major role in the separation of the target analyte from other components within the mixture, is typically comprised of a granular porous material made of solid particles (i.e.- silica gels, porous polymers, etc.).

Typical mobile phases for HPLC are comprised of mixtures of organic solvents (i.e. – methanol, acetonitrile) and water or aqueous buffers. Although the separation of the analytes is primarily based on the differences in the analyte interactions with the adsorbent surface, the mobile phase composition and properties, such as pH and ionic strength, also affects the separation process by influencing the interactions that occur between the analyte and the stationary phase. Once adequate separation of the components in the mixture is achieved, the target analyte can then be identified and quantitated using a variety of detectors (i.e.- UV-VIS, PDA, etc.), and the chromatographic signal is processed using an appropriate computer-based data acquisition software. A typical HPLC system consists of a solvent reservoir, pump, sample injector, column, detector and data acquisition software. A common set up for a HPLC system is illustrated in Figure 1-1. HPLC separations can be classified according to the physical molecular interactions (i.e. -

hydrophobic (dispersive), dipole–dipole, polar, ionic, etc.) of the analyte with the stationary phase and the approach to method development will depend on the type of HPLC separation employed.

There are many factors to consider when developing an HPLC method. First and foremost, the goal of the HPLC separation must clearly be defined, specifically the analyst must identify the target analyte that must be separated. The next step is to collect important information about the target analyte's physicochemical properties (chemical structure, molecular weight, p*Ka*, log *P*, and solubility). Sample preparation must be considered, especially if the composition of the sample matrix is complex. Selecting the appropriate mobile phase composition and gradient conditions will depend on the physicochemical properties of the target analyte. Stationary phase selection is a critical step in the method development process since characteristics of the adsorbent material will have the most significant impact on the change in analyte selectivity. Finally, a suitable, highly sensitive detector should be selected based on the properties of the analyte and the minimum detection needs of the analysis. Various types of detection systems are available such as photodiode array (PDA), ultraviolet (UV), fluorescence, refractive index (RI), electron light scattering (ELS), and mass spectrometry (MS).

The ideal HPLC method should be simple, robust, reproducible, precise and accurate in its performance under typical daily laboratory conditions. To complete the method development process, a thorough understanding of all the method variables (i.e.- mobile phase composition, temperature, pH, stability) is required to be able to successfully troubleshoot problems that may arise in the future.

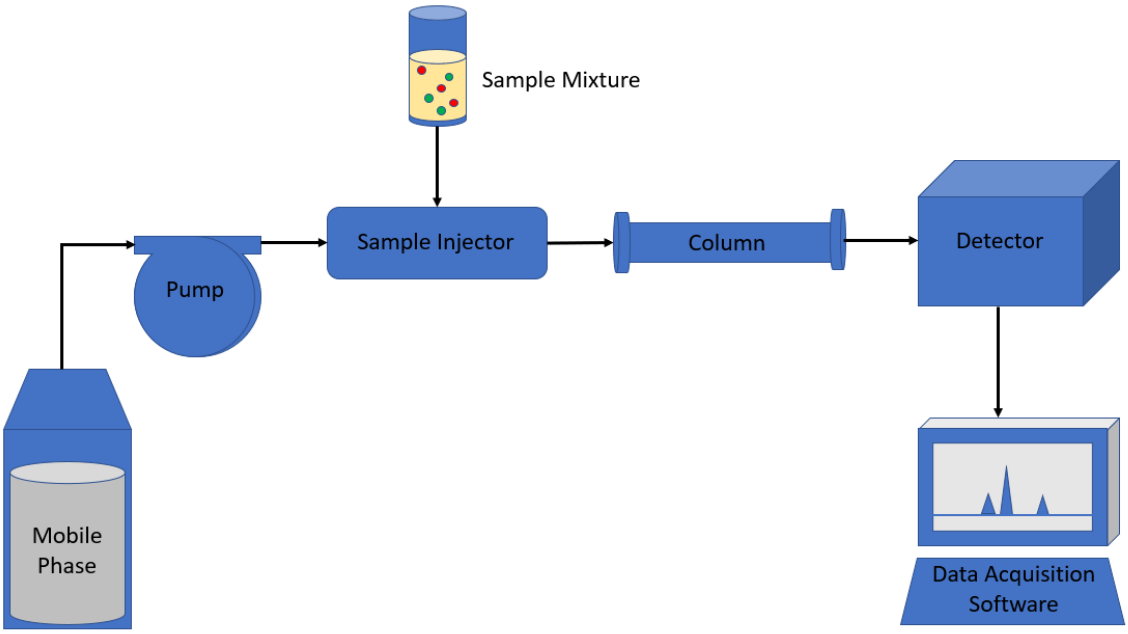

Figure 1-1 A Schematic Diagram of a Typical Liquid Chromatographic System

1.2 Classical Modes of Chromatography

Historically, classical HPLC consisted of four main modes: Reversed-Phase (RPLC), Normal-Phase (NPLC), Ion-Exchange (IEX), and Size-Exclusion (SEC). The main characteristic that defines each HPLC mode is based on the dominant type of molecular interactions that is used. A description of the basic principles for each HPLC mode is shown in Table 1-1.

1.2.1 Reversed-Phase (RPLC)

Reversed-phase HPLC (RPLC) is considered the most popular and well-known mode of liquid chromatography. The principles of RPLC are routinely applied during analytical analysis in a vast majority of fields from pharmaceutical, food, medical, agriculture, cosmetics and industrial chemistry. RPLC is the most heavily leveraged technique in HPLC and is often used as the default chromatographic mode for separation of mixtures. RPLC is highly preferred because this mode is able to separate very closely related compounds with high efficiency. The method development process is also well understood. RPLC offers some practical advantages, such as faster mobile phase equilibration, chromatographic reproducibility, and compatibility with a variety of detectors including highly sensitive MS detection techniques.

The molecular interactions between the analyte and the stationary phase are based on hydrophobic interactions. The stationary phase consists of porous silica supports chemically modified with hydrophobic ligands (i.e. – C_{18}, C_8, Phenyl, Cyano, etc.) while the mobile phase is composed mainly of polar solvents such as water, acetonitrile and methanol. Dispersive forces that are employed in this separation mode are the weakest intermolecular forces, thereby making the

Mode	Separation Based On	Stationary Phase	Mobile Phase	Analytes & Retention Behavior
Reversed Phase (RPLC)	Hydrophobic Interactions	Non-Polar Hydrophobic Ligands (C_{18}, C_8, C_4, Phenyl, Cyano, Amino etc.)	Polar, Aqueous solvents and Buffer solutions	• Non-polar, hydrophobic, Neutral, Weak Acids, Weak Bases • Non-Polar analytes are retained longer
Normal Phase (NPLC)	Polar Interactions	Polar Hydrophilic Ligands (Silica, Amino, Cyano, Diol)	Non-Polar Organic solvents	• Polar, hydrophilic compounds • Polar analytes are retained longer
Ion Exchange (IEX)	Ionic Interactions	Charged ligands (Anionic, Cationic)	Polar, Aqueous solvents and Buffer solutions	• Ionic, Inorganic Ions • Charged analytes are retained longer on oppositely charged stationary phase
Size Exclusion (SEC)	Molecular Size of Analyte No interactions	• Polymeric (GPC), • Polysaccharides, Silica (GFC)	• Non-Polar Organic (GPC) • Polar, Aqueous solvents and Buffer solutions (GPC)	• High molecular weight analytes, Polymers • Larger molecules elute faster than smaller molecules which elute slower due to penetration into the pores

Table 1-1 The Basic Principles for Each HPLC Mode

overall background interaction energy in the chromatographic system very low compared to other separation techniques.[1] This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes.[1]

A wide variety of commercially available RPLC columns are available on the market, some with large differences in selectivity as well as manufacturing standards. Most of them differ from each other only subtly, but the difference is often sufficient to achieve a required separation, otherwise not attainable with another column.[2] Because the subtle differences in the available RPLC columns can have a big impact on the resolution of critical peaks, column selection in RPLC is a critical step in the development of RPLC methods.

1.2.2 Normal-Phase (NPLC)

In Normal-phase liquid chromatography (NPLC), the polarities of the stationary phase and mobile phase is inverted compared to RPLC. The molecular interactions between the analyte and the stationary phase are based on polar interactions such as hydrogen-bonding or dipole-dipole interactions. The stationary phase consists of inorganic adsorbents or polar bonded phases (i.e. – silica, amino, cyano, diols, etc.) while the mobile phase is composed mainly of organic solvents. NPLC is not as commonly used as RPLC because the presence of water or the formation of a protic organic solvent layer on the silica surface may change the analyte interactions with the stationary phase and cause drifting retention times, resulting in poor reproducibility. Also since organic solvents that have low boiling points are used, the analysis is more prone to evaporation than RPLC, resulting shifting retention times due to the presence of bubbles, thus leading to more frequent troubleshooting and longer equilibration times.

Despite the disadvantages, NPLC is still a useful technique for samples that may not be soluble or stable in aqueous solutions. Also, samples that may be too hydrophilic or too hydrophobic to be retained in RPLC can be easily retained using NPLC. In NPLC, separation is a competitive process. Analytes compete with the mobile phase molecules for the adsorption sites on the stationary phase surface. The stronger the mobile phase interactions with the stationary phase, the lower the difference between the stationary phase interactions with the analyte, thus the lower the analyte retention.

1.2.3 Ion-Exchange Chromatography (IEX)

Ion-Exchange Chromatography (IEX) is also a widely used mode of chromatography. IEX is typically used for separating small biomolecules and proteins because they have the ability to exist as charged molecules. The molecular interactions between the charged analyte and the stationary phase is based on reversible electrostatic interactions of the opposite charge and can be classified as either anion-exchange or a cation-exchange. Anion-exchange columns have positively charged basic functional groups (i.e. – secondary, tertiary, quaternary amines) covalently bonded to the adsorbent surface and are used to retain negatively charged analytes. Cation-exchange columns have negatively charged acidic functional groups (i.e. - sulfonic acids or carboxylic acids) covalently bonded to the adsorbent surface and are used to retain positively charged analytes.

Cross-linked polystyrenes, cellulose, agarose, and polyacrylamides are all typical base materials for ion-exchange adsorbents. For this chromatographic mode, the surface charge of the functional groups will depend on the mobile phase pH and ionic strength, thus directly affecting the separation process by influencing the interactions that occur between the analyte and the

stationary phase. The ion exchange capacity of an IEX column measures the concentration of the ion exchange groups on the surface of the adsorbent and ultimately influences the ionic strength needed for retention or elution of an analyte from the column.

1.2.4 Size-Exclusion Chromatography (SEC)

Size-Exclusion Chromatography is typically used for the separation and characterization of polymers and large biomolecules such as proteins. SEC separates analytes on the basis of molecular size or hydrodynamic volume in solution. In a mixture of large and small molecules, the smaller molecules will diffuse into the pores of the adsorbent material and elute later than larger molecules which cannot enter the pores, and thereby will elute from the column faster.

A calibration curve can be established using standards of known molecular weights in order to determine the molecular weight of unknown molecules. A calibration curve is created by plotting the log molecular weight of the analyte vs. the elution volume to create the distribution coefficient. The retention time of the molecule can then be correlated to the molecular size of the molecule but will be dependent on pore size of the adsorbent. Because separation is based on the molecular size, interactions between the molecule and the adsorbent material should be avoided. Method development is focused on selecting the appropriate column with pore sizes that will provide the required resolution of the analytes and selecting a mobile phase that is compatible with the sample to minimize interactions between the adsorbent surface.

SEC can be classified into two types: Gel Permeation Chromatography (GPC) and Gel Filtration Chromatography (GFC). GPC is typically used for synthetic polymers and uses primarily organic solvents and cross-linked polystyrene stationary phases. GFC is typically used for

biopolymers and proteins and uses primarily aqueous solvents and polysaccharide, or silica based stationary phases.

1.3 Alternative Modes of Chromatography

The demand to analyze more complex mixtures over the last few decades has led to the development of alternative modes of chromatography with more complex types of surface interactions. These alternative modes of chromatography involve complex adsorbents with different types of active surface adsorption sites and more fundamentally complicated retention mechanisms. Two such alternative modes are Hydrophilic Interaction Liquid Chromatography (HILIC) and Hydrophobic Interaction Chromatography (HIC).

1.3.1 Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic Interaction Liquid Chromatography (HILIC) is used to separate highly polar compounds under highly aqueous conditions. Like Normal Phase Liquid Chromatography, HILIC stationary phases are typically highly polar material such as silica or functionalized ligands (i.e.- Amides, Diols, Cyano, etc.) on silica supports. While NPLC uses completely organic mobile phases for the analysis, HILIC mobile phases are typically aqueous mixtures or buffers, yet the retention behavior of both NPLC and HILIC are similar in that highly polar analytes are retained on the polar column. Although the HILIC retention behavior is similar to NPLC, the retention mechanism is more complex and is still being debated. The original theory devised by Alpert in 1990 is that HILIC is a multi-modal partition technique involving hydrogen bonding or dipole-

dipole interactions. The explanation is that polar analyte partitions between the bulk organic mobile phase and a water-rich layer that forms on the surface of the polar stationary phase. As the aqueous portion of the mobile phase increases, the polar analyte partitions out of the water-rich layer back to the bulk mobile phase and elutes from the column.[3] A schematic of the HILIC retention mechanism is illustrated in Figure 1-2.

According to a literary review by Buszewski[4] outlining fundamental developments in HILIC, there may be three possible ways to model the separation mechanism. The first is analyte partitioning between the mobile and stationary phases; the second is the adsorption of the analyte onto the surface of the adsorbent; the third assumes the preferential adsorption of the organic mobile phase modifier onto the adsorbent surface, followed by the partitioning of this analyte into the adsorbed layer.

Another theory exploring the retention mechanism of HILIC suggests that a complex combination of the analyte properties and experimental parameters will ultimately determine whether partitioning or adsorption will dominate.[5] Significant work was also achieved in understanding selectivity and multi-modality in HILIC columns. In their work involving separation of flavonoids, Jandera et al.[6] had observed both Reversed Phase and HILIC mechanisms on the same polar column. Just by varying the composition of acetonitrile in the mobile phase, they confirmed that both hydrophobic and hydrophilic separations can be achieved on the same HILIC stationary phase.[6]

Regardless of which retention mechanism is the more fundamentally correct, the HILIC mechanism ultimately depends on a combination of factors, such as the physicochemical properties of the analyte and stationary phase and the mobile phase composition simultaneously working together.[4,5] The HILIC retention mechanism, is considered a multi-modal mechanism, which may

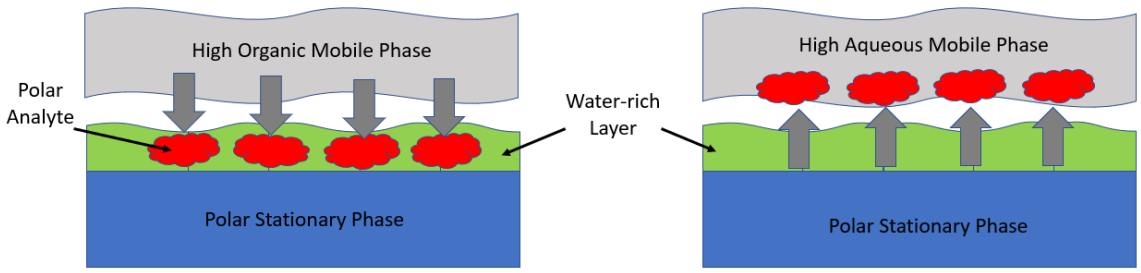

Figure 1-2 A Schematic Illustration of the HILIC Retention Mechanism

include one or more of the following mechanisms: partitioning between stagnant water layer on the surface of the stationary phase and aqueous organic-rich mobile phase, adsorptive interactions such as hydrogen bonding, electrostatic interactions and dipolar interactions.[7]

HILIC stationary phases have also evolved to be multi-modal as well. HILIC column manufacturing has progressed into a wide range of different stationary phases, many involving mixed or multiple-interaction solid phases.[4,7] HILIC column selection and subsequent method development must be carefully considered because of these complexities. Successful method development and validation using HILIC columns has been demonstrated across literature, such as in the quantification of metformin hydrochloride and its impurities in bulk pharmaceutical and finished dosage forms[8] and also in the analysis of therapeutic proteins and monoclonal antibodies.[9]

1.3.2 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic Interaction Chromatography (HIC) is a technique used to separate biomolecules using a mildly hydrophobic stationary phase. HIC stationary phases are less hydrophobic than RPLC and the aqueous buffered mobile phases contain a high concentration of a non-denaturing salt. The use of HIC is desirable if the goal of separation is to avoid denaturing the biomolecules and preserve its native structure to avoid loss of bioactivity. HIC therefore is commonly used in the purification of proteins and other biologically active molecules since the molecules elute in an unchanged stable conformation.[10]

The retention mechanism of HIC can be compared to Ion-Exchange Chromatography and Size Exclusion Chromatography where the mobile phase pH and ionic strength directly affects analyte retention and selectivity. The retention mechanism of HIC was described as a displacement

process where both water and salt are displaced when the biomolecule adsorbs onto the stationary phase.[11] When a biomolecule is subjected to high salt concentration of the mobile phase, the high salt concentration will increase the molecular interaction with hydrophobic stationary phase resulting in retention and decreasing the salt gradient will result in decreased interactions with the stationary phase, resulting in elution from the column in order of increasing hydrophobicity.[12] A schematic of the HIC retention mechanism is illustrated in Figure 1-3.

HIC stationary phases typically consist of silica supports chemically modified with short alkyl chains. Historically, techniques for HIC separations were expanded to include the use of multiple columns in series and different combinations of HIC, IEX and SEC columns were successfully used to purify proteins.[10] This encouraged more interest in creating newer types of HIC resins to support the growing interest in overcoming existing HIC limitations: low binding capacity and high salt concentration. Recent efforts to improve hydrophobic interaction chromatography (HIC) for use in monoclonal antibody (mAb) purification have focused on two approaches: optimization of resin pore size to facilitate monoclonal antibody mass transport, and use of novel hydrophobic charge induction (HCIC) Mixed-mode ligands that allow binding of monoclonal antibodies under low salt conditions.[13] Chen et. al.[13] evaluated a new generation of HIC-related chromatography resins that allowed higher binding capacity under decreased salt concentration thus improving the efficiency of the monoclonal antibody purification process. Just like HILIC however, new developments in multiple-interaction HIC column resins, complicates the HIC column selection, and therefore subsequent method development must be carefully considered.

There have been numerous efforts over the years to understand the retention mechanisms and provide recommendations for method development on HIC columns. One of the earlier works by Melander in 1989, studied the effect of salt on the retention behavior of proteins. According to

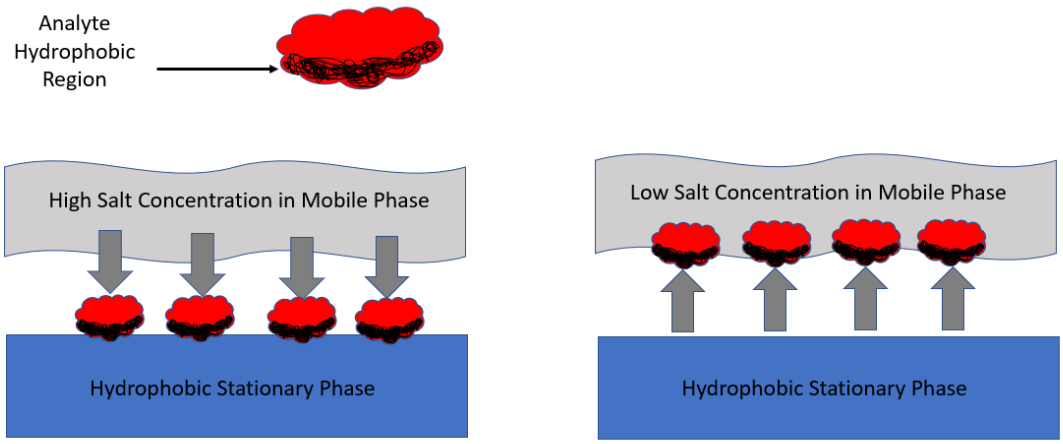

Figure 1-3 A Schematic Illustration of the HIC Retention Mechanism

Melander's devised theory, the magnitude of the hydrophobic interactions is determined by the hydrophobic contact area upon protein binding at the stationary phase surface and the properties of the salt as measured by its molal surface tension increment. The theory also states that the electrostatic interactions depends on the charge of the protein and the salt counterion which governs the change of retention with the salt concentration.[14] The theory was successful in explaining the observed effect of the nature and concentration of salt in the eluent, the pH and the effect of the density of fixed charges at the surface of the stationary phase in the absences of specific salt effects. More recent efforts by Srikoti[15] in 2020, has evaluated the influence of elution conditions and the impact of mobile phase composition such as salt composition and organic modifier on the separation of monoclonal antibodies and biomolecules. Efforts in the study of retention mechanisms and method development are expected to continue with increasing interest in biomolecule characterization in the pharmaceutical industry.

1.4 Mixed-mode Chromatography (MMC)

Combined with the demand to separate more complex sample mixtures and the emergence of alternative modes of HPLC such as HILIC and HIC, the interest in developing novel HPLC stationary phases has increased significantly. The definition of Mixed-mode chromatography (MMC) is clear, although the understanding of Mixed-mode materials and retention mechanisms are still evolving. Mixed-mode Chromatography (MMC) or Multi-Modal Chromatography uses more than one mode of interaction between the stationary phase and analytes in order to achieve the desired separation. These multiple modes of interactions exist simultaneously on one stationary phase and are controlled by varying the eluent composition, pH, salt type and concentration, which

allows for a much wider range of applications as compared to a single mode of interaction.[16] The schematic illustration of a Mixed-mode separation is illustrated in Figure 1-4. Figure 1-4 shows a mixture of analytes 1-5 with different physico-chemical properties and retention behavior on Single Mode Columns 1 and 2, and a Mixed-mode Column. Figure 1-4(a) shows that analytes 3,4,5 are well retained, while analytes 1,2 are less retained using Single Mode 1 column. Figure 1-4(b) shows analytes 1,2 are well retained, while analytes 3,4,5 are less retained using Single Mode 2 column. Figure 1-4(c) shows all analytes retained with acceptable resolution on a Mixed-mode column.

Mixed-mode chromatography (MMC) has become increasingly popular across different industries, such as environmental, food, and biopharmaceutical applications due to its unique selectivity and retention of a variety of compounds. MMC has been successfully applied in pharmaceutics for counter ion analysis, polar and charged API, impurities, formulation excipients, environmental and biological samples.[17] Long before the development of modern Mixed-mode Chromatography, many early experiments had already been exploring the possibility of rapidly separating different classes of nucleic acids in one chromatographic run by connecting a Reversed-Phase column and Ion-Exchange column through an automatic switching valve.[18] The success of this experiment demonstrated that Mixed-mode chromatography could provide better separation for a complex mixture of analytes than Single-Mode chromatography.

1.4.1 Advantages of Mixed-mode Chromatography

Mixed-mode columns have the advantage of being operated under a single mode or combination mode. A Mixed-mode column operating under a single mode of interaction takes advantage of one

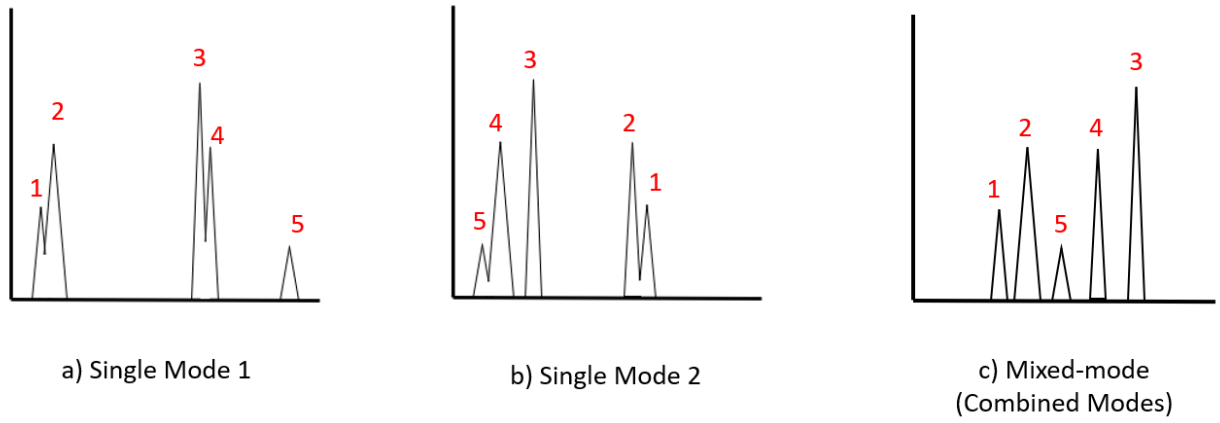

Figure 1-4 A Schematic Illustration of Mixed-mode Chromatography showing a mixture of analytes 1-5 with different physico-chemical properties and retention behavior on Single Mode Columns and a Mixed-mode Column.

interaction mode a time, depending on the mobile phase conditions (i.e. - the ratio of organic/aqueous portion, pH, or buffer concentration). An example of this was demonstrated by Lämmerhofer in 2010, when a Mixed-mode column with reversed phase/weak anion-exchange stationary phase material was created to separate peptide molecules by lipophilicity and charge differences under hydroorganic reversed-phase type elution conditions.[19] Lämmerhofer claimed that the column that was created was able to exploit the attractive or repulsive electrostatic interactions or hydrophobic or hydrophilic interactions, therefore the column can be operated under a multitude of modes (RPLC, IEX, HILIC or HIC) and allowed flexible adjustment of retention and selectivity by tuning experimental conditions.[19] In all the above distinct chromatographic modes that were established, the experiment used the same three mobile phase solvents (water, buffer, acetonitrile), that were adjusted depending on the analyte properties and elution conditions. Mixed-mode columns that can operate under different modes, have the advantage of versatility and can effectively reduce the cost of method development by eliminating the need to purchase individual columns that can only operate in a single mode. The Mixed-mode column can essentially become a "one column fits all" as elution conditions can be modified to meet the desired separation. An example of a Mixed-mode column operated under single mode conditions is illustrated in Figure 1-5.

A Mixed-mode column operating under combined modes of interaction takes advantage of all the available interaction modes in order to separate all the analytes of interest. An example of this was demonstrated by Zhang[20] in 2010, when a commercially available Mixed-mode column with reversed phase/ion-exchange stationary phase material was used to simultaneously separate 25 commonly used pharmaceutical counterions, including both inorganic cations and anions, within 20 minutes using a single method. The method was used successfully for screening

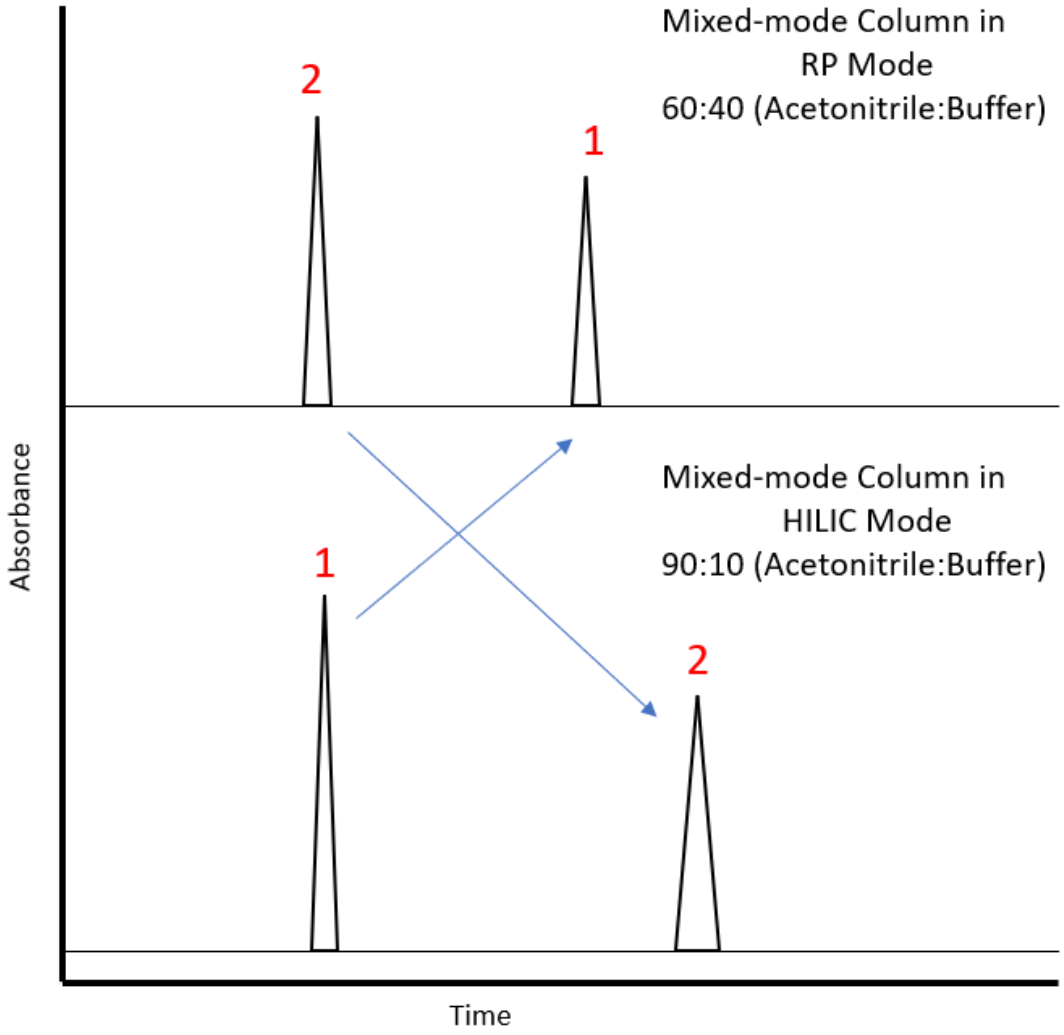

Figure 1-5 A Mixed-mode column operated under single mode conditions.

quantitative analysis of counterions, unknown ionic impurities and salts in active pharmaceutical ingredients and in-process control samples with excellent accuracy, precision and sensitivity. Mixed-mode columns that can simultaneously operate under a combination of different modes also have the advantage of versatility in that they effectively improve the efforts of method development by eliminating the need to develop multiple methods needed for complex mixtures containing analytes with different physico-chemical properties. Where once existed the need for two different methods to separate cation and anions or hydrophilic and hydrophobic analytes, multiple methods can now be combined into one method using one single Mixed-mode column. There have been numerous successful examples published over the years that demonstrate the ability to apply Mixed-mode chromatography to solve complex separation challenges, and undoubtedly, the examples will continue to grow as the use of Mixed-mode chromatography continues to rise.

1.4.2 Types of Mixed-mode Materials

There are two types of Mixed-mode materials. The first type of Mixed-mode material is created when the two or more functional groups are present in the material, however each functional group remains physically discrete from each other. The second type of Mixed-mode material is created when two or more functionalities are chemically modified onto one ligand to create a single Mixed-mode stationary phase. Types of Mixed-mode materials are illustrated in Figure 1-6.

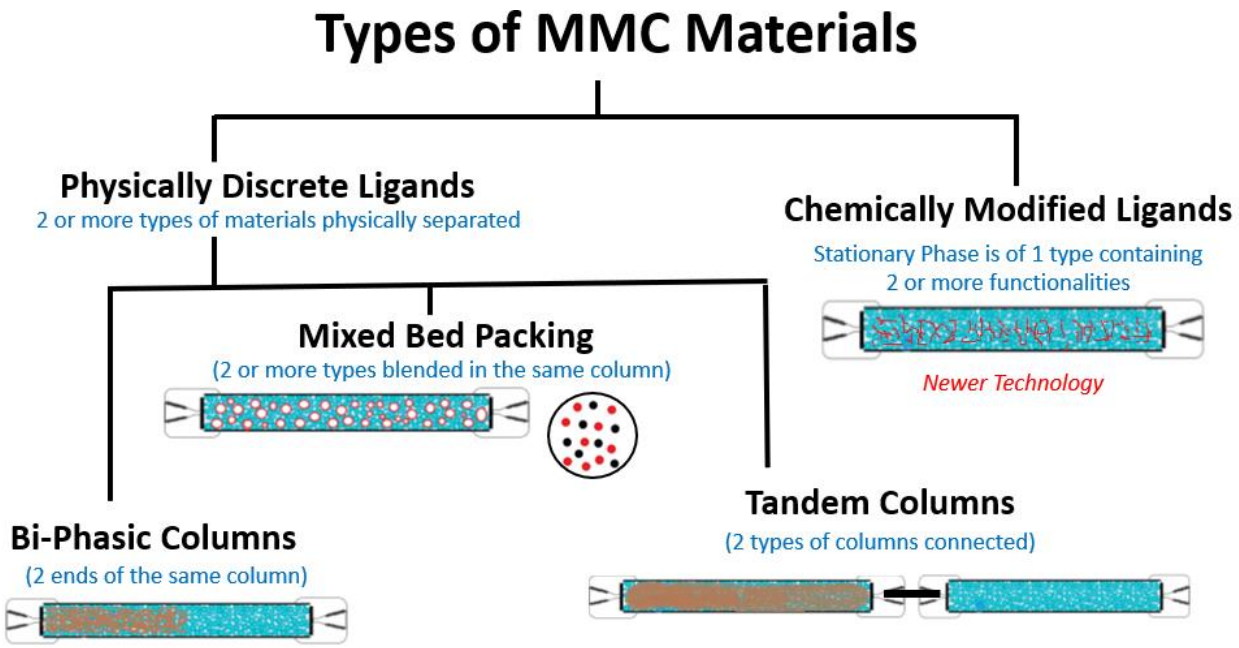

Figure 1-6 Types of Mixed-mode Materials

1.4.2.1 Physically Discrete Mixed-mode Materials

1.4.2.1.1 Mixed-Bed

Mixed-mode materials that are made of physically discrete ligands can be further categorized into three types: Mixed-Bed, Tandem, and Bi-Phasic materials.[21] The Mixed-Bed approach is the physical blending of two different stationary phases packed into a single column in order to create the "Mixed-mode" effect.[16,17] In a study conducted by Wise et al.[22], reversed-phase separations selectivity was modified relative to the mixed ratio of monomeric and polymeric C_{18} materials present, and a linear relationship between solute retention and support composition was observed. Selectivity factors for these columns were found to be similar to those predicted by the linear addition of the selectivities of the two individual phases. This study indicates that columns of specific selectivity can be prepared by mixing two different C_{18} materials.

Another study by Rassi and Horvath[23] (1986) explored the use of a single column packed with a binary mixture of cation and anion exchange column materials to successfully separate both acidic and basic proteins in a single chromatographic run. The experiment demonstrated that the mixed-bed columns which exhibited both cationic exchange and anionic exchange properties, can be used in either separation mode, and that by varying the mixing ratio of the materials, the column selectivity can be adjusted.

In a later study by Walshe, a mixed-bed column was prepared by filling a column with blend of equal amounts of C_{18} and sulphonate-modified silica stationary phase to mimic an RPLC and Strong Cation-Exchange (SCX) Mixed-mode column.[24] The retention behavior and capacity factor of 25 charged and un-charged compounds were studied in terms of the effects of pH, ionic

strength, type and percent of organic modifier. The study concluded that the dual nature of the retention mechanism allowed the retention of ionizable molecules to be adjusted by altering the composition of the aqueous component of the mobile phase, while compounds uncharged over the pH range investigated remained unaffected. Although the column was found to exhibit chromatographic properties characteristic of both phases, the data obtained in the study indicated that ion exchange was a significant force in determining the retention behavior of the more strongly basic analytes on the Mixed-mode column.

1.4.2.1.2 Tandem

A tandem column is when two columns of different stationary phase modes are connected in series, and is the simplest approach to achieve MMC, as long as the two mobile phases are compatible.[21] The same study by Rassi and Horvath[23] (1986) explored the tandem use of a cation and anion exchange column with gradient elution in order to successfully separate a mixture of proteins with a wide range of isoelectric points in a single chromatographic run. Their experiment employed the use of two columns that were connected in series, which utilized the same mobile phase. Regardless of the serial order of the columns, the chromatographic behavior of all the proteins were the same. Finally, when they compared the chromatographic behavior of the tandem columns with the mixed-bed single column, the results showed similar chromatographic results.[23]

The process of connecting columns in tandem is also known as Two-Dimensional Liquid Chromatography (2D-LC). The essential concept in 2D-LC is that at least one fraction of the effluent from the first column (1D) is transferred to a second column (2D), and the task of the 2D column is to separate all the unresolved analytes present in each fraction of the 1D effluent based

on the differences in selectivity of the two dimensions.[25] A typical set up of the hardware commonly used for online comprehensive 2D-LC separations is illustrated in Figure 1-7. The modulator valve, which interfaces the 1D and 2D columns, is integral to a 2D-LC set up.

A major challenge associated with 2D-LC is transferring the sample from the first-dimension column to the second-dimension column. When the sample is transferred from the first column to the second column, one must ensure that the transfer solute is compatible with the mobile phase in the second column. Furthermore, the collection of sample fractions from the first mode and re-injection of these fractions into the second mode require careful consideration of quantitative calculations in order to avoid errors in both accuracy and precision.

A single Mixed-mode column containing both separation modes has the advantage of replacing the dual columns of 2D-LC. This would cut down the cost and complexity of a 2D-LC set up and simplify quantitative calculations. This was demonstrated in a series of experiments by Geng[26] in 2009, in which the entire on-line 2D-LC operations was performed using a single Mixed-mode column to separate native proteins. This method of using on-line 2D-LC using only a single column was termed 2D-LC-1C. The resolution of this method was comparable to that obtained when two individual conventional chromatography columns were used for the separation of native proteins.

1.4.2.1.3 Bi-Phasic

A Bi-Phasic column is defined as two different stationary phases packed sequentially into different parts of the same column. Bi-phasic columns are more commonly used in capillary columns for separation or identification of proteins. A bi-phasic capillary column of Strong Cation Exchange

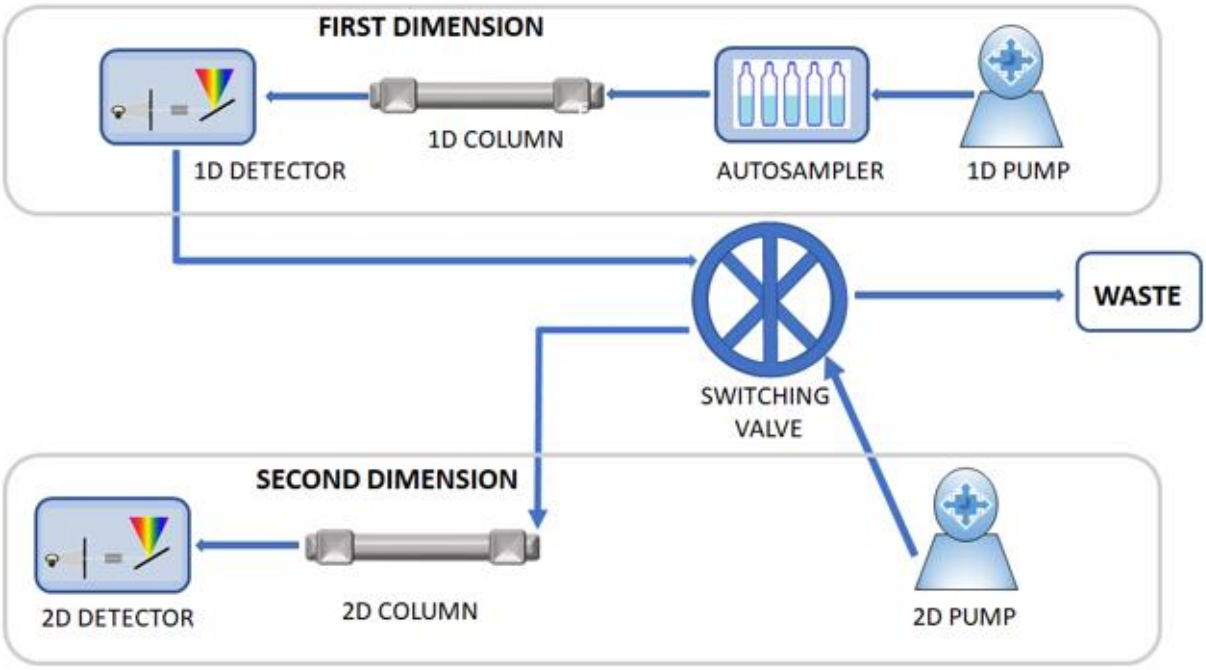

Figure 1-7 A Schematic Illustration of a 2D-LC setup.

The switching valve is either 8-port or 10-port valve containing 2 sample loops. Loop volume defines the velocity ratio between 1st and 2nd dimension pumps.

(SCX) and RPLC with a ratio of 1:1 was first used for peptide separation in proteomics by Yates in 2000.[27,28] Later, the same group prepared a Tri-Phasic column by adding 3.0 cm of RPLC material onto one end of a Bi-Phasic column.[29] The use of these bi-phasic and tri-phasic columns coupled with MS/MS demonstrated that this type of Mixed-mode column is a highly efficient tool in proteomics to separate peptides.[29]

1.4.2.2 Chemically Modified Mixed-mode Materials

Modern Mixed-mode stationary phases are designed with the purpose of creating controllable multiple-interactions, and to provide reproducibility, high efficiency and loadability.[16] These modern Mixed-mode stationary phases are often created through covalent chemical modification of ligands with the desired functional groups. Through chemical modification, many different geometric and spatial arrangements of the functional groups are possible. A visual representation of these different types of geometric and spatial arrangements is shown in Figure 1-8. Because of the combination of different modes, compounds with very different physical-chemical properties can be retained and separated on the same column, and column selectivity can be optimized by adjusting mobile phase ionic strength, pH or organic solvent.[16,17]

In the mixed-ligand approach, two chemically different ligands are both chemically bonded to the solid silica support.[17] The Mixed-mode stationary phases introduced in recent years uses a single ligand chemically modified with two or more functional groups. These functional groups may be located at the tip of the ligand or embedded within the ligand. Furthermore, tri-modal Mixed-mode stationary phases are possible when 2 different functional groups are chemically modified onto one single ligand or 2 different ligands are chemically bonded onto one silica

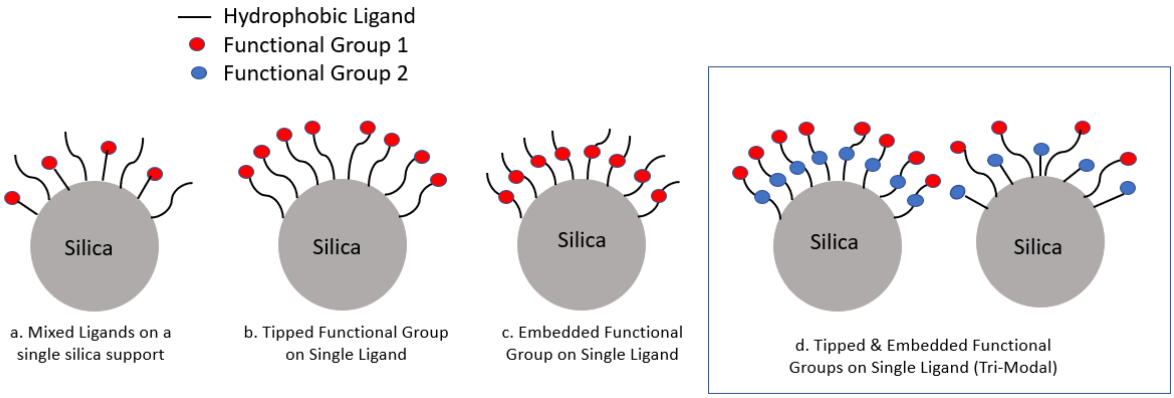

Figure 1-8 Chemically Modified Mixed-mode Materials with different combinations of interaction modes and different geometrical and spatial arrangements of the functional groups.

support. As Mixed-mode chromatography gains popularity, exploration in the creation of more novel Mixed-mode stationary phases will increase. A majority of the published research over the past few years has focused on the development of novel stationary phases to create different combinations of Mixed-mode columns.

Qiao et al.[7] published research on the development of a Mixed-mode stationary phase using ionic liquids. In their research, a glucaminium-based ionic liquid was synthesized and chemically bonded to the surface of 3-mercaptopropyl modified silica materials. The resulting stationary phase was shown to exhibit a hydrophilic interaction and anion-exchange Mixed-mode retention mechanism. Mixtures of nucleotides and flavonoids were successfully separated on this novel Mixed-mode column showing that ionic liquid Mixed-mode columns could offer flexible selectivity toward polar and hydrophilic compounds.

More recently in 2020, Gao[30] and others published a paper describing the preparation of a novel Mixed-mode stationary phase based on modified dialdehyde cellulose. The authors indicate that with this new stationary phase, a combination of HILIC and IEX mode was used to separate strongly polar compounds, phenylamines, and chiral compounds. In the same year, Shields published the development of a Mixed-mode, reversed-phase cation-exchange stationary phase using a thiol-yne reaction, a type of "click chemistry" to create a Mixed-mode column with low pH stability. The successful separation of monoamine neurotransmitters to demonstrated that the new stationary phase showed characteristics of both the ion-exchange and HILIC partitioning mechanisms.[31]

A list of other examples of different types of Mixed-mode combinations, the type of ligands created, and the types of analytes that were separated are shown in Table 1-2. As more and more

Modes	Ligand Composition	Separated Analytes	Information from Reference
RPLC/IEX	C18 + quaternary ammonium groups	Alkylbenzenes	32
	C18 + sulfonylphenyl	Phosphopeptides	33
	C18 + 3-carboxypropyl	Peptides	34
	C21 + quaternary ammonium group	Peptides	35
RPLC/HILIC	sulfonic-azobenzene	Alkylbenzenes, steroids, bases, nucleosides	36
	Alkyl carbon + glycol terminus	Alklyphenol derivatives, ethoxylated alcohols	37
	Humic acid	Nucleosides, nucleobases	38
	Poly-L-lysine	Anilines, phenols	39
HILIC/IEX	Glutathione	Saccharides, Peptides	40
	Polysulfoethylaspartamide	Peptides	41
	Propargylamine	Inorganic anions, nucleosides	42
	Glucaminium-based ionic liquids	Nucleosides, flavanoids	43
RPLC/HILIC/IEX	Poly(1-vinyl-3-octadecylimidazolium bromide	Alkylbenzenes, steroids, terphenyl isomers	44
	Dendritic polymer	Alkylbenzenes, charged solutes, nucleobases	45
RPLC/HILIC/IEX/π-π	Hybrid carbon, silica monolithic	Alkylbenzenes, nucleosides, aromatic acids	46
IEX/π-π/H-binding	Inylbenzyltrimethyl-ammonium chloride and tris	PAHs, phenols, estrogens, aromatic amines	47
RPLC/HILIC/IEX/π-π/H-binding	C18 + amide	Alkylbenzenes, nucleosides, isomers	48

Table 1-2 Novel Mixed-mode Stationary Phases, their ligand composition, and analytes separated.

(Information taken from the reference listed in the last column)

research into Mixed-mode stationary phases, the number of examples and published research is expected to grow.

1.4.3 Challenges of Mixed-mode Chromatography

Mixed-mode chromatography is powerful, but complex. The use of Mixed-mode chromatography is sometimes avoided, because of the complexity of the method development process. When multiple retention mechanisms and complex interactions are involved, many variables must be controlled to produce a robust and repeatable method. It is due to these complex interactions, however, that it is difficult to predict chromatographic behavior of analytes on Mixed-mode columns. A diversity of ligands are now available for use in Mixed-mode chromatography. Although the influence of their primary mechanisms can be demonstrated fairly easily, for example, electrostatic and hydrophobic interactions, the practical contributions and control of secondary functionalities, such as metal coordination, pi-pi bonding, hydrogen bonding, and van der Waals forces, are poorly understood.[49] These uncertainties increase the unpredictability of Mixed-mode columns that is fueled by variations in ligand density and physical configurations among ligands of similar chemical character.[49] Furthermore, there is usually very little information that is publicly disclosed by column manufacturers about the column properties such as the surface chemistry, bonded ligand geometry types, and bonding density; thus making chromatographic behavior that much harder to predict. Even similarly classified Mixed-mode columns manufactured across different manufacturers may show different chromatographic behavior for the same compound, for a variety of reasons, making column selection for method development complicated[50] and successful method validation difficult to meet.

1.5 Conclusion

The demand to separate more complex sample mixtures combined with the emergence of alternative modes of chromatography such as HILIC and HIC have paved the way for evolution of Mixed-mode chromatography. More and more novel Mixed-mode stationary phases are expected to grow and become commercially available, therefore a better understanding of the underlying retention mechanism behind Mixed-mode materials is needed. Although significant steps have been achieved toward the understanding of interactions contributing to retention and selectivity in Mixed-mode columns there are still many opportunities to optimize the selectivity in Mixed-mode columns. As the understanding of Mixed-mode chromatography continues to improve, along with its robust capabilities and wide applications, the use of this technique will increase.

Mixed-mode chromatography has been applied to areas such as oligonucleotides, peptides, proteins, pharmaceutical and environmental analysis and continues to meet the on-going need for separating complex mixtures. Published literature has demonstrated the efficiency of Mixed-mode chromatography for the combined analysis of not just small, non-polar, polar, and charged compounds, but also for larger molecules such as peptides or proteins. As a result of the increased efficiency, Mixed-mode chromatography is primarily used in pharmaceutical analysis for impurity profiling of active pharmaceutical ingredients, counterions, drugs and biopharmaceuticals. In a typical 2D-LC set up, one Mixed-mode column can effectively replace the dual columns typically used.

As the understanding of Mixed-mode retention mechanisms continues to increase, so will the requirement of a deeper understanding of the process of method development on Mixed-mode

materials. Because method development using Mixed-mode columns is challenging, fundamental studies that provide the chromatographer with information to help guide method development and understand both the potential and limitations of Mixed-mode stationary phases is critical for success. Finally, additional research into the understanding and characterization of novel stationary phases and commercially available Mixed-mode columns is expected to improve the understanding of retention behavior of analytes on Mixed-mode stationary phases.

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CHAPTER 2 – CHROMATOGRAPHIC ADSORBENTS AND THE USE OF C-CONSTANT OF THE BET EQUATION TO CHARACTERIZE MIXED-MODE ADSORBENTS

2.1 Introduction to Chromatographic Adsorbents

Chromatography is widely used around the world in many industries such as pharmaceutical, environmental, industrial, forensic, agricultural and cosmetics, as an effective analytical technique that separates mixtures into their individual components. In general, there are four main types of chromatography: Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), Thin-Layer Chromatography (TLC), and Paper Chromatography. Although each type is very different from each other and each type has its own advantages and specific applications, the one commonality across all chromatographic techniques is the use of adsorbents for separating mixtures.

GC technique is used to separate analytes in volatile mixtures and uses an inert gas or "carrier gas" as the mobile phase. The stationary phase is contained in packed columns with porous adsorbents, or capillary columns coated with either a thin film of viscous liquid-like polymer or a thin, porous layer on the walls.[1] HPLC is used to separate analytes in non-volatile mixtures and uses a liquid solvent as the mobile phase that carries the mixture through an instrument using a high pressure pump. The stationary phase is an adsorbent packed into a glass or stainless steel column and the mixture is separated into its constituents within the column. TLC is best suited for small scale experiments and uses a liquid solvent that travels by capillary action up a silica or alumina stationary phase that is coated onto a thin glass or plastic plate. Although a wide variety

of adsorbents are available for use as the stationary phase of a chromatographic separations process, this research will focus on HPLC adsorbents and their characterization.

2.2 Chromatographic Parameters

There are four basic parameters to describe characteristics of a chromatographic separation: Retention factor (*k*), Efficiency (*N*), Selectivity (α), and Resolution (*R*).

2.2.1 Retention Factor *(k)*

Retention factor (*k*) measures the retention of an analyte on a particular chromatographic system and is calculated according to Equation 2-1:

$$k = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} \qquad \text{Equation 2-1}$$

Where V_R is the analyte retention volume, V_0 is the volume of the liquid phase in the chromatographic system, t_R is the analyte retention time, and t_0 is the retention time of a non-retained analyte.[1] The retention factor is sometimes referred to as the "capacity factor" and does not depend on the mobile phase flow rate and column dimensions.

2.2.2 Efficiency

When an analyte is injected into the column, it moves through the theoretical plates of the column and the resulting peak experiences band-broadening or an increase in peak width. The degree of band-broadening is a measure of the column's efficiency and is expressed in number of theoretical plates (*N*) according to Equation 2-2:

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad \text{Equation 2-2}$$

Where t_R is the analyte retention time and *w* is the peak width at the baseline.[1] The greater the number of theoretical plates, the better the separation outcome, therefore efficiency a property of the column and is dependent on the quality of the column packing, particle size, flow rate and instrumental optimization.

The van Deemter equation shown in Equation 2-3 is a useful expression which describes the theory of band broadening and column efficiency. HETP is the height equivalent to a theoretical plate.

$$HETP = A + \frac{B}{v} + Cv \qquad \text{Equation 2-3}$$

The terms A, B, and C represent the three different processes that contribute to the overall chromatographic band-broadening, while *v* is the mobile phase linear velocity.[1] The term A represents eddy diffusion or the different paths that a molecule takes to travel through the column and is independent of the flow rate. The term B represents molecular diffusion and is inversely

proportional to the flow rate, meaning that at slower flow rates, the longer the molecule stays in the column, causing the peak to widen.[1] Term C is proportional to the flow rate and represents mass transfer, or the molecules migrating into the stationary phase surface pores and staying in the column, thus causing band-broadening.

The van Deemter curve shown in Figure 2-1 illustrates the contribution of all three terms of the van Deemter equation to efficiency and plots HETP as a function of flow velocity. The curve shows that maximum column efficiency can be achieved at a specific flow rate at the minimum of the curve, therefore it is important to select a suitable flow rate to minimize molecular diffusion and mass transfer. In order to minimize the effect of eddy diffusion, well packed columns should be used. Efficiency will decrease as particle size increases and faster flow rates are required for longer columns to achieve the desired theoretical plates.[1]

2.2.3 Selectivity

Selectivity is defined as the ability of the chromatographic system to discriminate different analytes and is ideally dependent on the difference in the analytes interaction with the stationary phase.[1] Selectivity is defined by Equation 2-3 as the ratio of the retention factors of the two analytes, or the ratio of the reduced retention times,

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \qquad \text{Equation 2-3}$$

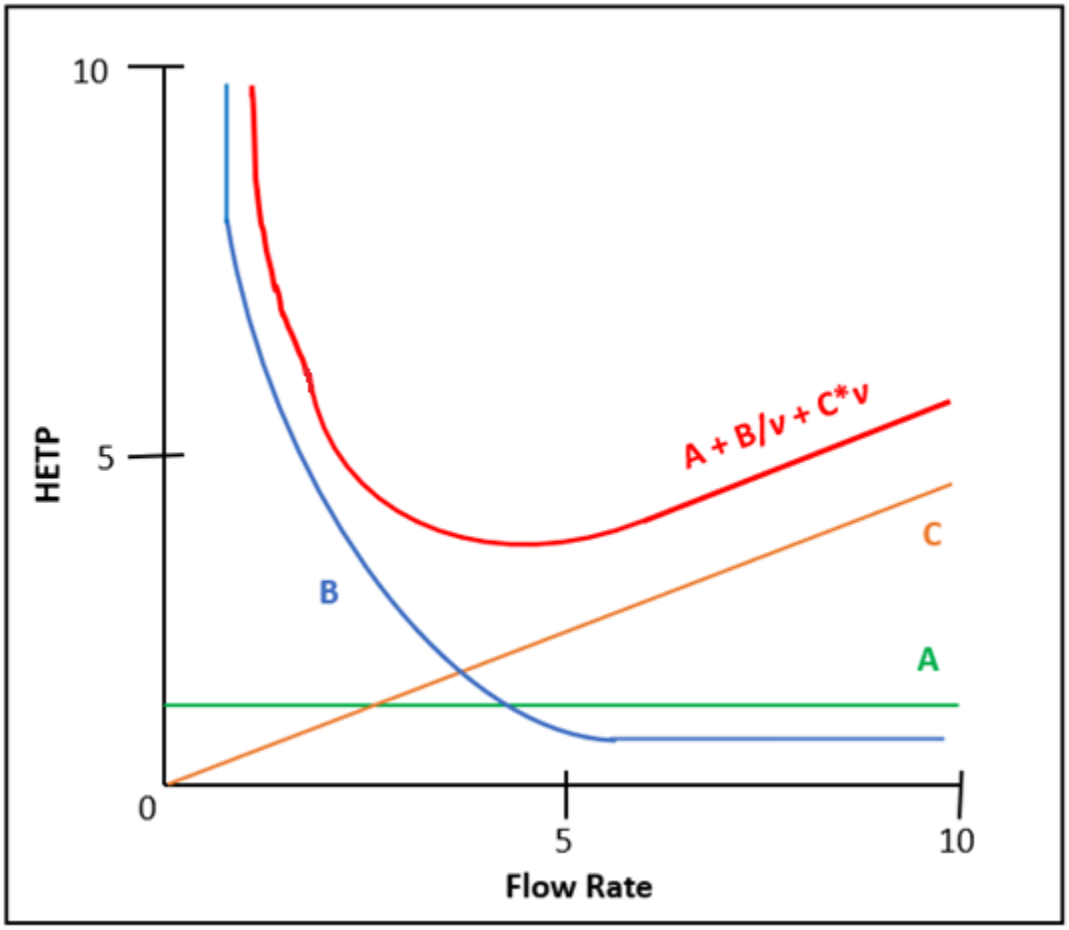

Figure 2-1 The van Deemter curve

Where *k* is the capacity factor, t_R is the retention time of the analyte, and t_0 is the retention time of the un-retained analyte.[1] Selectivity is primarily dependent on the stationary phase properties and the nature of the analytes, although the mobile phase type and composition will also have an effect on the analyte's interaction with the stationary phase. Chapter 1 discusses the different HPLC modes (Reversed-Phase Liquid Chromatography, Normal Phase Liquid Chromatography, Ion-Exchange Chromatography, Size Exclusion Chromatography, and Mixed-mode Chromatography) in terms of the retention mechanism and analyte interactions on the different stationary phases. Later sections in this chapter will discuss the various characteristics of adsorbents that have an effect on selectivity.

2.2.4 Resolution

Resolution is the ability of the column to resolve analytes in separate peaks or chromatographic zones.[1] In terms of peak width, resolution can be defined as the ratio of the distance between two peaks to the average width of these peaks at the baseline and is defined according to Equation 2-4:

$$R = 2\,\frac{t_{R2} - t_{R1}}{(w_2 + w_1)} \qquad \text{Equation 2-4}$$

Where t_R is the analyte retention time and *w* is the peak width at the baseline.[1] All the factors together: efficiency, selectivity, and retention all contribute to the overall approximate resolution and is expressed in the master resolution equation, Equation 2-5:

$$R = \left[\frac{\alpha - 1}{\alpha}\right]\left[\frac{k_2}{1 + k_2}\right]\left[\frac{\sqrt{N}}{4}\right] \qquad \text{Equation 2-5}$$

where *N* is the number of theoretical plates or the efficiency of the column, α is the selectivity of the analytes for the stationary phase, and *k* is the retention factor of two closely eluting analytes.[1,2] This equation is only approximately applicable for closely eluted components (critical pairs) and has no direct mathematical relationship with the definition of Resolution (Equation 2-4).

2.3 HPLC Columns

The HPLC column is the main component in an HPLC system and is largely responsible for the success of an HPLC separation. An HPLC column is comprised two main parts: the column housing, typically a stainless steel tube, and the stationary phase material. An illustration of the structure of a typical HPLC column is shown in Figure 2-2. There are thousands of HPLC columns across all categories of HPLC modes (i.e. - Reverse-Phase Liquid Chromatography, Ion-Exchange Chromatography, Normal Phase Chromatography, Size Exclusion Chromatography, and Mixed-mode Chromatography) that are commercially available on the market and have distinct differences in selectivity as well as manufacturing processes. Within each HPLC mode type, most of the columns may seem to differ from each other only slightly, however the difference is often sufficient to achieve a required separation that is otherwise not achievable with a similar column.

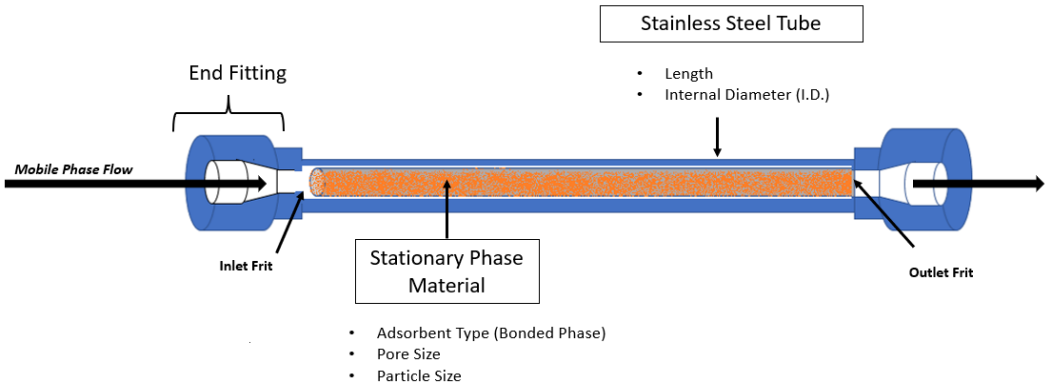

Figure 2-2 Structure of an HPLC Column

When developing an HPLC method, it is important to understand the different attributes of the column and how they will affect the chromatographic separation, therefore column selection is the most critical segment in method development and is becoming especially important due to rising sample complexity.

2.3.1 Column Dimensions

The column dimensions of a typical analytical HPLC column are between 30 mm to 300 mm in length, with inner diameters between 1.7 mm to 4.6 mm. The effect of column dimensions on chromatography was examined by Simpson[3] in 1985. In this study, several C_{18} columns, varying in column length as well as packing diameter, were used to separate mixtures of biochemical compounds, and the column performances were compared qualitatively and quantitatively in terms of efficiency, peak resolution, sensitivity, cost per analysis, and analysis times. Shorter columns provided the fastest separations, lowest cost per analysis, highest sensitivity, and lowest solvent consumption, however there was a lower number of theoretical plates and limited resolution between peaks. Longer columns with smaller inner diameters had the highest number of theoretical plates, highest overall resolution, and highest sensitivity, however the analysis was subjected to long analysis times, high solvent consumption, and high cost per analysis. Careful selection of column dimensions is needed during method development, in order to achieve the right balance between analysis cost and speed, sensitivity, efficiency and resolution.

Another column characteristic that affects the overall column properties and separation performance is its particle size distribution (PSD). Particle size distribution (PSD) is the measure of the size distribution of the particles in a packed LC column. It has long been known that a

packed column bed is not radially homogeneous due to the variations in the complexity of the column packing process.[4] This non-homogeneity may lead to decreased column efficiency, column-to-column variability and poor reproducibility. Generally, in HPLC, a narrow particle size distribution is desirable. Although PSD itself does not affect chemical behavior of the adsorbent, it is known to influence the efficiency of packed columns, since big size differences between particles in the column decreases overall column efficiency.[1] This relationship was confirmed by Horvath[5] who showed that the width of PSD affects the height equivalent to a theoretical plate, as the width of PSD increases, the height equivalent for a theoretical plate decreases.

2.3.2 Column Stationary Phases

The term “stationary phase” when applied to HPLC packing material is a bit ambiguous. Common chromatography theory treats analyte retention as the result of its partitioning between mobile and stationary phases. Thus, the stationary phase is understood to be permeable for analyte molecules. In reality, HPLC packing materials are mainly porous silica with various chemically bonded ligands on the solid non-permeable surface. The main process governing HPLC retention is adsorption of analyte molecules on the surface of solid packing material, which is also commonly named “Stationary phase”, but should not be treated as permeable phase where analyte could be distributed.

While the column dimensions will have an effect on the efficiency, speed, and sensitivity of the chromatographic analysis, it is the stationary phase characteristics that will have the biggest impact on selectivity and retention factor of the target analytes. Column stationary phases can be classified according to characteristics such as the type of packing material, geometry, surface

chemistry, and base material, with each characteristic contributing to the overall chromatographic performance of the column.[1]

2.3.2.1 Type of Packing Material

The type of packing material can be categorized as either internally porous or non-porous. A standalone type is monolithic where the whole column is a porous monolithic silica rod placed in plastic housing. Most packing materials being used in HPLC columns are made of porous particles between 3 µm to 10 µm in diameter. Porous particles increase the necessary surface area, usually between 100 m^2/g to 400 m^2/g, which provides adequate analyte retention.[1] Porous particles resembles a sponge, with analytes diffusing in and out of the pores, interacts with the stationary phase and achieving separation.

Stationary phases are made of non-porous particles, either physically or covalently attached with a layer of functional groups on its surface.[6] Although the reduction in surface area for non-porous materials results in a decrease of column loading capacity, non-porous materials have some advantages in analyzing biomolecules. Non-porous packing materials are used for studying the fundamental retention mechanism of proteins and low-molecular mass solutes, since both the kinetics and thermodynamic properties of the interactions between the solute in the mobile phase and the ligand in the stationary phase can be studied separate from the effects of the porous structure.[7] Non-porous silica materials were successfully used to study the conformational change of proteins since the protein is adsorbed exclusively at the external surface of the beads and only a small amount of protein was necessary to saturate the column.[8]

Monolithic columns are made from one single rod of either porous silica or an organic polymer instead of small silica particles. The single rod contains mesopores and macropores that form a series of channels that increase surface area, which contributes to the permeability of the column. Monolithic columns can be made of silica-based or polymer-based materials. Silica-based materials are typically used for separating small molecules while, polymer-based are used for separating large protein molecules.

2.3.2.2 Geometry

Characteristics such as the particle shape and size, particle size distribution, surface area, pore volume and pore diameter, all make up the geometry of the adsorbent material. The particle shape can either be classified as spherical or irregular in shape. Most materials used in HPLC are spherical due to higher efficiency and reproducibility.

Particle size is the average particle size of the adsorbent material and for HPLC is usually 1.7 µm to 5 µm. The use of lower particle size however, results in an increase of column backpressure since the plate number doubles, thus lower particle size values are utilized more often in Ultra High Pressure Liquid Chromatography (UHPLC) applications. Particle size distribution (PSD) is the measure of the size distribution of the particles in a packed LC column and the effects on column efficiency are discussed in section 2.3.1. In general, a narrow particle size distribution is desirable.

Pore size, typically expressed in angstroms (Å), is the average size of a pore in the porous packing material. The pore size determines whether a molecule can diffuse into and out of the packing material. Smaller pore size packings (80Å to 120Å) are typically used for the analysis of

small molecules with molecular weights up to 2000 g/mol. For molecules larger than 2000 g/mol, wider pore size materials are required.

Surface area is the most important factor in analyte retention. Surface area is directly proportional to retention.[1] For adsorbent materials, the higher the surface area, the greater the analyte retention, according to Equation 2-6:

$$V_R = V_0 + SK_H \qquad \text{Equation 2-6}$$

where V_R is the retention volume, V_0 is the Void volume, S is the surface area, and K_H is known as the Henry constant.[1]

2.3.2.3 Surface Chemistry

It is important to understand the surface chemistry of the packing material since this will define the type of retention mechanism of the analyte's interactions on the surface. The surface of the base material may be chemically modified in order to introduce specific functional groups that have specific interactions with different analytes, however chemical modifications will decrease the surface area of the porous material.[1]

Silica-based adsorbents are the most widely used base materials for packings of HPLC columns due to their physical stability and well-known and controllable pore structure and morphology, which results in high reproducibility, rapid mass transfer.[9] Furthermore the high mechanical strength can resist a large back pressure when operated at a high flow-rates.[10] Silica consists of silicon, oxygen, and hydrogen atoms. Each silicon atom is mobile and linked to oxygen

and hydrogen atoms. Porous silica provides the highest surface area necessary for successful separation and is easily chemically modified to accommodate different functional groups.[1]

The microproperties of silica that include the specific surface area, pore shape and diameter, specific pore volume, and pore size distribution play a significant role both in silica derivatization and in the chromatographic features of silica based columns.[9] Silica has disadvantages, however, in that it readily solubilizes in water at high pH and also has a very polar surface. Alternative base materials include zirconia, polymeric materials, and porous graphitized carbon, both of which are stable in high pH and temperatures, but also have drawbacks including decreased retention, selectivity, and efficiency.[1]

Bonding density is defined as the surface concentration of the bonded ligands, typically expressed in number of moles per square meter (μmole/m^2). The higher the bonding density, the better the shielding of residual silanols, and the higher the hydrolytic stability and is measured indirectly through elemental analysis (CHN) to determine carbon content.[1] When silica is chemically modified, there always exists a significant amount of unreacted silanols on the silica surface called residual silanols that may contribute to unwanted interactions. However, if the bonding density is high and the silica surface is uniform, the bonded ligands may form a shielding effect, and the residual silanols will not be accessible for surface interactions.

As discussed in Chapter 1, Section 1.4.2.2, modern Mixed-mode stationary phases emerged with the purpose of creating multiple controllable interactions through covalent chemical modification of ligands with the desired functional groups. Through chemical modification of silica and other base particles, many different geometric and spatial arrangements of the functional groups are possible and are illustrated in Figure 1-8. Ultimately, the surface chemistry will define the type of molecular interactions available.

2.4 Column Classification and Characterization

Column selection has traditionally been based on trial-and-error and using the collected knowledge and experience of the analyst, which can be labor-intensive, cost-prohibitive and can lead to conflicting results. In an attempt to facilitate the selection of appropriate HPLC columns by the analyst, the United States Pharmacopeia (USP) created a classification system for liquid chromatography columns that is based on the properties of the packing material. The USP also implemented a set of standard reference materials from the National Institute of Standards and Technology (NIST) to be used for column performance characterization on the basis of theoretical plate count, peak symmetry, tailing factor and shape selectivity.[11]

When the USP 20 was published in 1980, only seven columns were classified and given a "L" designation along with a general description.[11] The list has since grown to over a hundred "L" designated columns. A partial list of columns classified by the USP is presented in Table 2-1 along with their assigned description.[12] Although the USP Column Classification system may aid the analyst in selecting columns of similar adsorbents, the accuracy of this classification system is heavily disputed, as it often does not necessarily correlate to similar retention behavior of analytes. A good column classification system based on objective criteria and a system for ranking of the columns, is currently needed.

In order to build a good classification system for HPLC columns, proper characterization of the stationary phase materials and a clear description of the bonded group is required. The challenges of HPLC column characterization have long been the subject of intensive research and the emergence of Mixed-mode columns has further complicated existing classification systems.

USP Number	Description of Stationary Phase Material
L1	Octadecyl silane chemically bonded to porous or non-porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod.
L2	Octadecyl silane chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30 to 50 µm in diameter
L3	Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L4	Silica gel of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter
L5	Alumina of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter
L6	Strong cation-exchange packing-sulfonated fluorocarbon polymer coated on a solid spherical core, 30 to 50 µm in diameter
L7	Octylsilane chemically bonded to totally or superficially porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L8	An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L9	Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating, 3 to 10 µm in diameter.
L10	Nitrile groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L11	Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L12	A strong anion-exchange packing made by chemically bonding a quaternary amine to a solid silica spherical core, 30 to 50 µm in diameter
L13	Trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter
L14	Silica gel having a chemically bonded strongly basic quaternary ammonium anion-exchange coating, 5 to 10 µm in diameter.
L15	Hexylsilane chemically bonded to totally porous silica particles, 3 to 10 µm in diameter
L16	Dimethylsilane chemically bonded to porous silica particles, 5 to 10 µm in diameter
L17	Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 6 to 12 µm in diameter
L18	Amino and cyano groups chemically bonded to porous silica particles, 3 to 10 µ in diameter
L20	Dihydroxypropane groups chemically bonded to porous silica or hybrid particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.
L21	A rigid, spherical styrene-divinylbenzene copolymer, 3 to 30 µm in diameter
L22	A cation-exchange resin made of porous polystyrene gel with sulfonic acid groups, 5 – 15 µm in diameter.

Table 2-1 A partial list of “L” columns classified by the USP

(Information taken from reference 12)

The many types of chromatographic column classification systems and characterization techniques that were developed for Reversed-Phase HPLC systems over the last 40 years are discussed and reviewed by Zuvela[9] in order to highlight the many attempts to develop a universal column selection system for Reversed-Phase materials. Column classification systems are continuing to evolve and more complex approaches to column selection, using advanced chemometric data processing, have been proposed.

With the emergence of Mixed-mode stationary phases, many researchers have been using the chromatographic and non-chromatographic techniques that were originally developed for Reversed-Phase materials to also evaluate the differences in the characteristics and separation mechanisms of Mixed-mode stationary phases. Analysts are now faced with not only selecting an appropriate Mixed-mode column and also selecting a suitable column classification system.

2.4.1 Column Characterization by Chromatographic Techniques

The structure of new Mixed-mode stationary phases should be defined and understood prior to using them for HPLC analysis. Characterization of commercially available Mixed-mode columns is often difficult since complete information on the chemistry and structure of the stationary phase is often proprietary and not shared with end-users. Column characterization tests based on chromatographic techniques are important for end-users who may not have the knowledge or access to non-chromatographic instruments that may be used as characterization tools for the stationary phases.

Many recent attempts have been made to characterize and compare Mixed-mode columns to traditional Reversed-Phase columns using chromatographic parameters in terms of their

interaction abilities, retentivity, peak symmetry, and applicability for separation of molecules.[13] Kadlekova[14] used basic and acidic analytes to investigate the ionic interaction participation in retention behavior of selected Reversed-Phase and polar columns. A chromatographic method was developed and used to study the ionic interactions of stationary phases in the pH range between 2.5 and 9.0. Kadlekova concluded that the method can provide only a relative comparison of ionic and hydrophobic interactions for the tested adsorbents, since the overall retention depends not only on the immobilized ligands, but also on the adsorbents phase ratio that is closely related to sorbent pore size or morphology.

Later, Kadlekova[13] investigated the chromatographic attributes of commercially available Mixed-mode columns that combined C_{18} and anion-exchange ligands. The simple Walters test was used to obtain general information about the differences in silanophilic interactions and hydrophobic interactions of the tested columns. The effect of buffer pH and concentration on retention and peak symmetry of peptides demonstrated the similarity and differences of two different Mixed-mode columns at specific buffer pHs.

The Walters Test was devised by Walters[15] in 1987 for classifying C_{18} columns on the basis of hydrophobic and silanophilic interactions, which are dependent on the hydrocarbon coverage and the unreacted silanol sites, respectively. Hydrophobic interactions are represented by the retention factor ratio of anthracene-benzene in an acetonitrile-water mobile phase, while the unreacted silanols are represented by the *N, N*-diethyltoluamide-anthracene retention factor ratio with acetonitrile as the mobile phase. The Walters Test was intended as an aid in the selection of similar or equivalent columns and in defining the type of column suitable for an analytical method.

An example of a column classification system using chemometric data processing is Quantitative Structure-Retention Relationships (QSRR) for chromatographic column selection.

QSRRs appeared in the early 1970s as an innovative approach for the prediction of chromatographic retention from the molecular structure.[6,16-19] One of the earliest QSRR models, Linear Free Energy Relationships (LFER), used solvophobic parameters derived from equilibrium measurements to predict retention factors and the regression coefficients determined by multiple regression analyses are assumed to characterize the stationary phase/mobile phase system being investigated.[9] Although LFER models have been used repeatedly to study Reversed-Phase columns,[20-24] recent applications of QSRR models were successfully applied to Mixed-mode columns in order to improve the understanding of the retention mechanisms and interaction capabilities of Mixed-mode Reversed-Phase/Ion-Exchange columns.[25]

2.4.2 Column Characterization by Non-Chromatographic Techniques

Non-Chromatographic techniques provide valuable information on the chemistry and structure of stationary phases that may be able to provide more insight in evaluating retention behavior of analytes on Mixed-mode materials. A list of non-chromatographic techniques that are commonly applied in the characterization of HPLC columns is provided in Table 2-2. Low Temperature Nitrogen Adsorption (LTNA) measures the surface area, pore characteristics, and pore size distribution of stationary phase materials based on nitrogen gas adsorption on solid surfaces and is routinely used to characterize a wide variety of stationary phases.[26,46-48] This technique will be discussed in detail in Section 2.5.

Microscopy techniques such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Atomic Force Microscopy (ATM) provide high resolution visual

Method Type	Technique	Characterization Information Obtained from Technique
Adsorption	Low Temperature Nitrogen Adsorption (LTNA)	Surface Area, Pore Structure, Pore Size, Pore Volume, Pore Size Distribution [26,46-48]
Microscopy	• Scanning Electron Microscopy (SEM) • Transmission Electron Microscopy (TEM) • Atomic Force Microscopy (ATM)	Visual imaging of particle structure and surfaces[27]
Thermal Analysis	Elemental Analysis (CHN)	Elemental content (Carbon, Hydrogen, Nitrogen) of materials[28,29]
Thermal Analysis	• Thermogravimetric Analysis (TGA) • Differential Scanning Calorimetry (DSC)	Surface coverage of chemically modified silica calculated by weight loss following thermal decomposition of organic groups. Heat capacity[30-33]
Thermal Analysis	Micro-calorimetry	Heat capacity changes due to interactions with solvent molecules may indicate presence of residual silanols and surface accessibility for interactions[34-38]
Spectroscopic	• Nuclear Magnetic Resonance (NMR) • Fourier-Transform Infrared Spectroscopy (FTIR) • Raman Spectroscopy	Ligand structure & conformation, Distribution of silanols/siloxane groups, surface coverage[39-41,49]
Spectroscopic	• X-Ray Photoelectron Spectroscopy (XPS) • Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)	Elemental analysis of surface, Structural information related to bound functional groups[9,30,42,43]
Electron Migration	Zeta Potential	Charge distribution on the surface of materials based on the potential of the electric field formed on solid-liquid interface.[44,45]

Table 2-2 Commonly used Non-Chromatographic Techniques for Column Characterization

imaging of particle structure and surfaces and can also provide additional information regarding elemental composition.[27,49] SEM can be used to capture the changes in particle shape and size of the spherical silica gel due to a result of chemical modification. By comparing the SEM spectra of blank silica gel to that of the synthesized Mixed-mode material, particle size distribution can be determined in order to evaluate the geometry and reproducibility of the synthesis process.[49]

Elemental Analysis (CHN) provides information about the elemental content (Carbon, Hydrogen, Nitrogen) of stationary materials and the data obtained can be used to calculate the ligand coverage density.[28-30,47] Elemental analysis, however, has been inferior with regard to determining both the total coverage on the stationary phase and the ratio of these ligands, thus improved methods of analysis are needed.

Thermogravimetric Analysis (TGA) is a technique that provides information about the surface coverage of chemically modified silica by exposing the materials to very high temperatures in the range of 200°C to 600°C, which thermally degrades the material, resulting in a weight loss, and is usually performed with Differential Scanning Calorimetry (DSC) to determine the heat capacity of the material.[30-33]

Microcalorimetry can be used to determine the ligand conformation and their interactions with solvent molecules.[9] Interactions such as hydrogen bonding, van der Waals, and London forces, generate heat that can be directly measured. Overall, the changes in the surface accessibility for interactions with solvent molecules cause changes in the heat of immersion.[9,34-36] Microcalorimetry has also been used to determine the presence of residual silanols on the stationary phase surfaces.[37,38]

Some commonly used spectroscopic techniques for stationary phase evaluation are Nuclear Magnetic Resonance (NMR), Fourier-Transform Infrared Spectroscopy (FTIR), and Raman

Spectroscopy and can be used to determine the chemical composition, structure, and conformation of the materials and the manner in which the ligands are functionalized onto the silica surface.[39-41,49] Stationary phase materials can also be investigated using X-Ray Photoelectron spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) to obtain elemental analysis of a silica surface and provide structural information related to the bound functional groups.[9,30,42,43]

Zeta potential is a technique that can be used to study the charge distribution on the surface of stationary phase materials. Zeta potential is the electric field formed on the solid-liquid interface. If charges are present on the surface, this would allow for the characterization of surface properties of Ion-Exchange stationary phases, thus provide insight into the retention mechanism.[44,45] Recently, zeta potential measurement was used to study the surface properties of Mixed-mode materials. In a study conducted by Krzeminska et al.[51] zeta potential values of hydrophobic and polar Mixed-mode stationary phases suspended in water, organic solvent and their mixtures were measured, and selectivity coefficients were calculated based on retention factor. Although the study confirms that the zeta potential investigation is a useful tool in the characterization of the stationary phase surface, knowing the properties of the polar embedded stationary phase is critical in understanding column selectivity and ultimately, retention behavior.[51]

2.5 Introduction to Low Temperature Nitrogen Adsorption (LTNA)

Low Temperature Nitrogen Adsorption (LTNA), a technique that was mentioned in section 2.4.2, is routinely used to characterize a wide variety of stationary phase materials. LTNA is a type of gas adsorption technique commonly used to measure the specific surface area and pore size

distribution of various materials. The principle is based on the adsorption behavior of nitrogen gas molecules on solid surfaces at the temperature of liquid nitrogen.[26] The specific area, total pore volume and average pore size of Mixed-mode stationary phase materials can be measured by the nitrogen adsorption method using BET analysis.[26] There are a number of other well-known methods for the characterization of surfaces, particularly the estimation of surface area, such as those based on adsorption from solution, heat of immersion, chemisorption, and on the application of the Gibbs adsorption equation.[52] In the field of gas adsorption, however, calculations using the BET theory remains the most widely used procedure for the determination of surface area.

The main steps in the process of an LTNA analysis on a typical surface area and porosity analyzer instrument are illustrated in Figure 2-3. The first step to an LTNA analysis is to weigh and prepare a dry sample for analysis. The amount of sample to be analyzed depends on the expected surface area of the material. Generally, a smaller sample sizes are recommended for larger surface areas. Prior to the determination of an adsorption isotherm the sample must be degassed by under vacuum or by flushing the sample with a gas such as N_2 often under elevated temperatures. The appropriate temperature for heating the sample should be determined in order to avoid irreversible changes to the material surface.

Prior characterization of the material using thermal analysis techniques such as TGA or DSC discussed in Section 2.4.2 may be performed in order to determine the thermal stability limits of the sample. After degassing, the cooled sample must be re-weighed in order to account for any mass loss during the degassing step. During the adsorption step, the adsorbate gas (N_2) is introduced to the sample tubes either in doses or as a slow continuous flow. The incoming nitrogen gas is cooled to the boiling point of nitrogen at 77K by a vessel of liquid nitrogen surrounding the sample tube. At this temperature, the nitrogen gas is below the critical temperature and so it

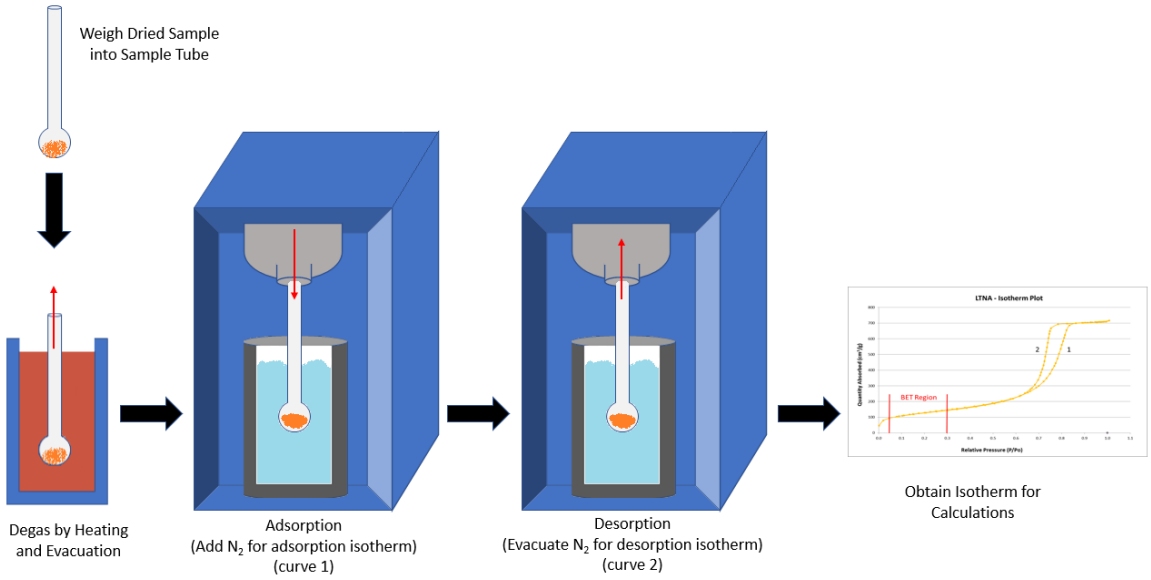

Figure 2-3 The main steps in the process of an LTNA analysis on a typical surface area and porosity analyzer instrument

condenses on the surface in a monolayer. As the nitrogen gas begins to adsorb on the sample surface, the pressure in the sample tube continues to fall until the adsorbate and the adsorptive are in equilibrium. The amount of adsorbate at the equilibrium pressure is the difference between the amount of gas that is introduced and the amount of adsorptive remaining in the gas phase.[52] At the end of the adsorption step, an adsorption isotherm is generated over a selected range of relative pressures (P/P_0), where P is the partial pressure of nitrogen and P_0 is the saturated vapour pressure of nitrogen under the temperature of liquid nitrogen.[26,52]

A typical example of an isotherm and the adsorption process is shown in Figure 2-4. When P/P_0 is in the range of 0.05–0.35, the adsorption and relative pressure P/P_0 can be used to measure the specific surface area using the Brunauer, Emmett and Teller (BET) equations.[26,52] At relative pressures P/P_0 greater than 0.35, nitrogen begins to condense in the micropores due to capillary condensation and form multi-layers.[26,52] The molecules in an adsorbed layer not only interact with the solid surface, but also with neighboring molecules within the layer, and ultimately a densely occupied monolayer will act as an extension of the solid, attracting further molecules from the gas phase, resulting in a multi-layer at higher relative pressures.[52]

The pore volume, pore size, and pore distribution can then be calculated from the full adsorption isotherm. If a desorption isotherm must be generated, a vacuum is then applied to the sample tube to remove the adsorbate from the sample surface. Since the technique is non-destructive, samples can be re-used and re-analyzed if necessary. For a standard analysis for BET surface area, the analysis may take around 45 minutes, however a full isotherm may take up to 16 hours to generate.

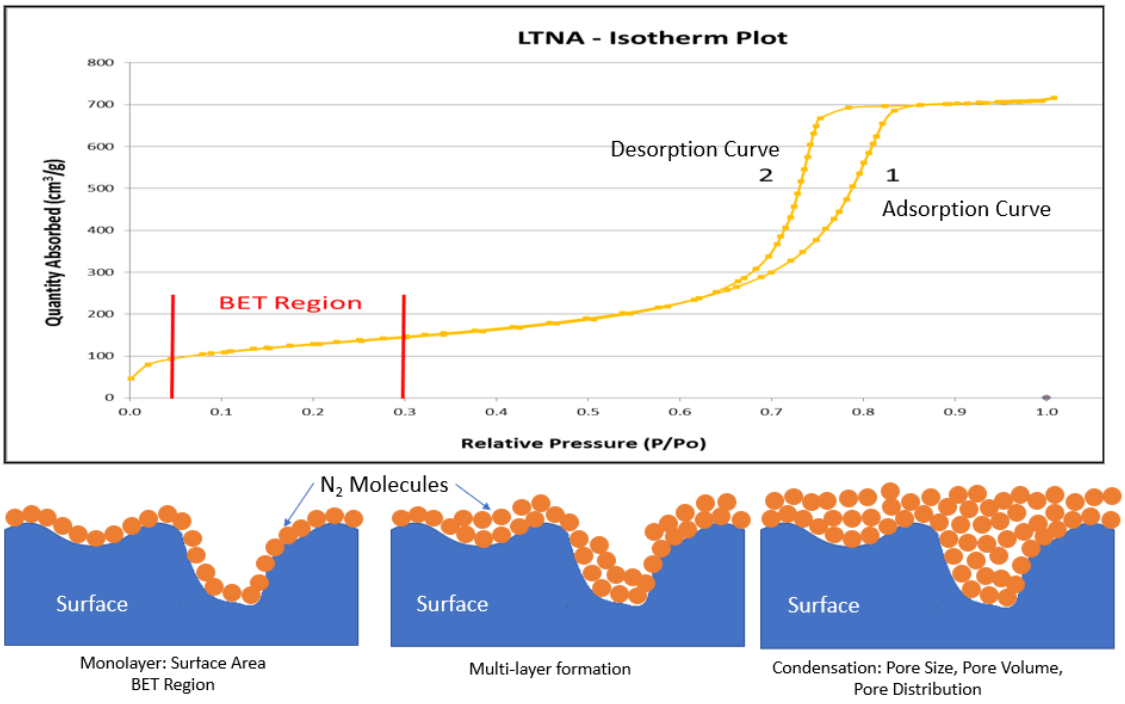

Figure 2-4 Illustration of a typical isotherm and the adsorption process

According to Brunauer[26,53,55] there are five types of adsorption isotherms. These isotherms are illustrated in Figure 2-5. The BET method is applicable only to adsorption isotherms of Type II (disperse, nonporous or macroporous solids) and type IV (mesoporous solids, pore diameter between 2 nm and 50 nm).[26,52-55] Type 1 is obtained when P/P_o (partial pressure) < 1 and C (BET C-Constant) > 1 in the BET equation, which is related to the adsorption energy of the first monolayer and varies from solid to solid.[55,56] The characterization of microporous materials creates this type of isotherm. Type 2 represents the formation of a monolayer and is obtained when c > 1 in the BET equation.[55,56] At very low pressures, the micropores fill with nitrogen gas, followed by monolayer formation, then multilayer formation at medium pressure. Finally, at higher pressures, capillary condensation occurs.[55,56] Type 3 isotherms are created when c < 1 and shows the formation of a multilayer, however there is no asymptote in the curve, therefore no monolayer is formed and BET is not applicable.[55,56] Type 4 isotherms occur when capillary condensation occurs in the capillary pores of the solid at pressures below the saturation pressure of the gas. At the lower pressure regions, a monolayer is formed followed by the formation of multilayers.[55,56] BET surface area characterization of mesoporous materials (pore diameters between 2 - 50 nm) characterizes this type of isotherm. The last type of isotherm, Type 5 isotherms are very similar to Type 4 isotherms and are not applicable to BET.

2.6 Discussion of the BET Equation and Evaluation of C-Constant

As discussed in the previous section, The BET method can be used to determine the surface area and pore size distribution of disperse or porous solids by measuring the amount of physically adsorbed gas. BET represents the initials of the three people (Brunauer, Emmett, and Teller) who

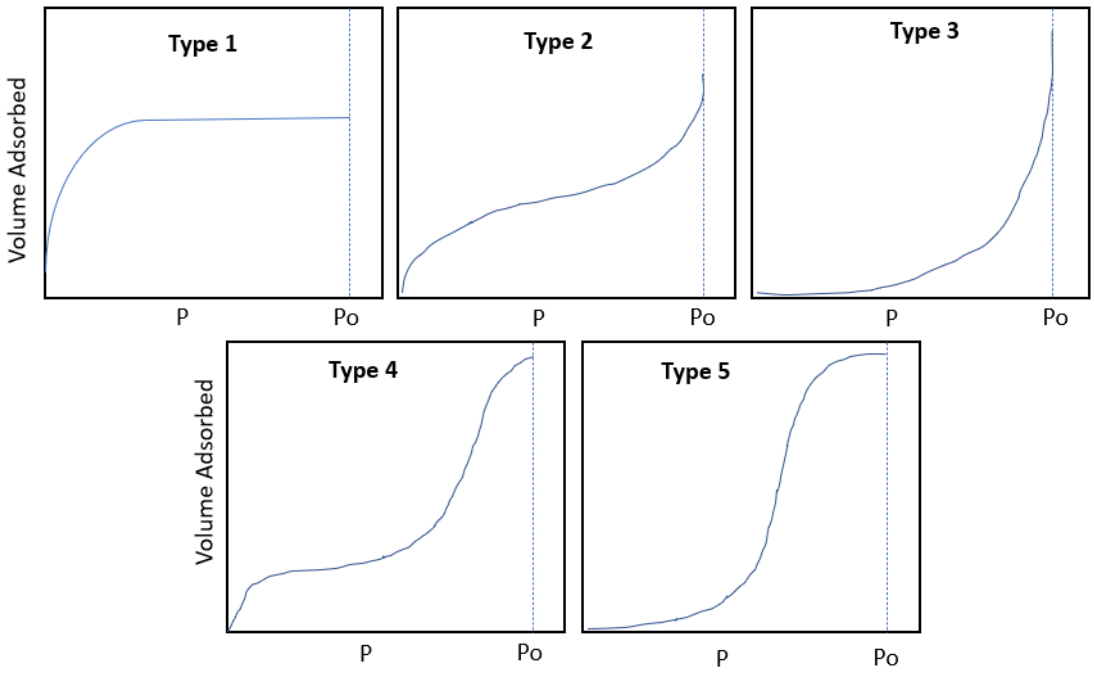

Figure 2-5 Brunauer's Five Types of Adsorption Isotherms

developed the mathematical equation for the measurements. Although nitrogen gas is typically used, others such as argon gas may be used, provided it is physically adsorbed by weak bonds (i.e.-van der Waals) at the surface of the solid and can be desorbed by a decrease in pressure at the same temperature.[26]

The BET equation used to calculate the surface area of a sample is shown below in Equation 2-7:

$$\frac{{}^{p}/_{p_0}}{n(1-{}^{p}/_{p_0})} = \frac{1}{n_m C} + \frac{C-1}{n_m C} * \frac{p}{p_0} \qquad \text{Equation 2-7}$$

Where p/p_0 is the relative pressure, n is the measured amount of adsorbed nitrogen, n_m is the number of moles of nitrogen in the first monomolecular layer (monolayer capacity), and C is the BET Constant.[1,26,52] To calculate these variables, the BET equation is plotted linearly typically at relative pressures p/p_0 between 0.05-0.35. An example of a BET plot is shown in Figure 2-6. The monolayer capacity n_m can then be calculated from the slope, and the surface area can be calculated using the molecular cross-sectional area of nitrogen.[56]

The amount of adsorbed nitrogen gas can be calculated using the size of the nitrogen gas molecule or cross sectional area. It is generally assumed that a nitrogen molecule occupies a 16.4 $Å^2$ on the polar silica surface, therefore the adsorbent surface area is then calculated as a product of the total amount of nitrogen in the monolayer (n_m) and the nitrogen molecular area (16.4 $Å^2$).[1,26,52,57] The assessment of the surface area of modified adsorbents maybe complicated due to the uncertainty of the molecular cross sectional area of nitrogen. Nitrogen occupies a larger area on hydrophobic surfaces than on polar surfaces and is estimated to be between 19 and 22 $Å^2$.[1,26,52,57]

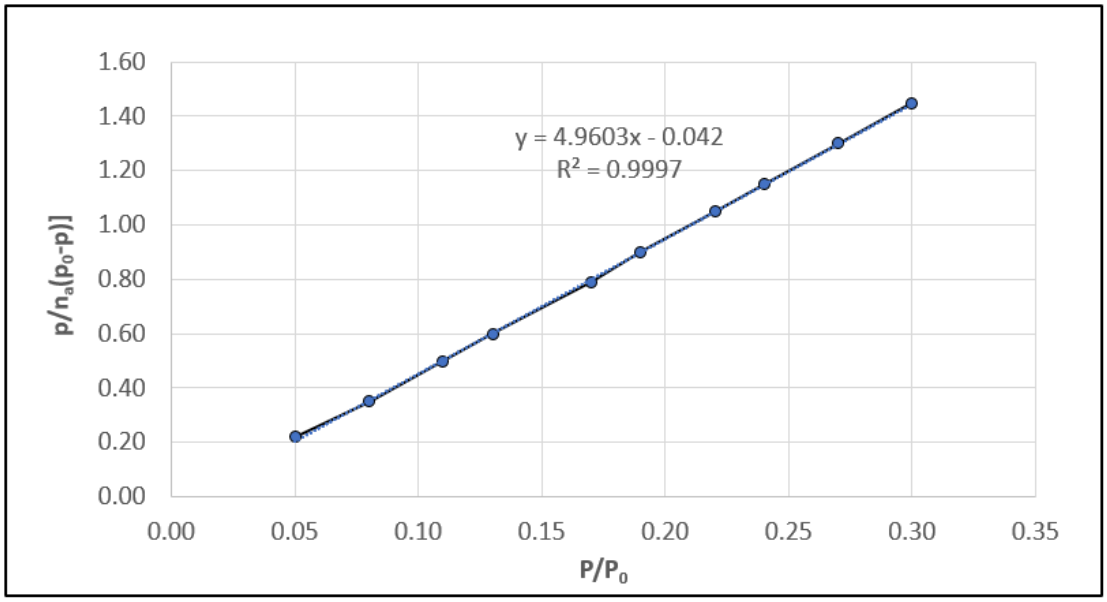

Figure 2-6 Example of a BET Plot

The total surface area can then be calculated according to Equation 2-8:

$$s\,(total) = \frac{n_m N s}{V}$$ Equation 2-8

Where n_m is the number of moles of nitrogen in the first monomolecular layer (monolayer capacity), *N* is Avogadro's number, *s* the adsorption cross section of nitrogen, and *V* is the molar volume of the nitrogen gas.

C-Constant can be represented by Equation 2-9:

$$C = \frac{a_1 v_2}{a_2 v_1} \exp\left(\frac{Q_1 - Q_L}{RT}\right)$$ Equation 2-9

where Q_1 is the heat of adsorption in first monolayer and Q_L is the heat of condensation, therefore $(Q_1 - Q_L)$ is the net heat of nitrogen adsorption on the surface. *R* is the gas constant, and *T* is absolute temperature. According to Gregg and Sing[7], the ratio of term (a_1v_2/a_2v_1), in which the constants are associated with condensation and evaporation of the first and higher layers of an adsorbate can be assumed as 1, therefore C-Constant can be simply calculated by Equation 2-10:

$$C = \exp\left(\frac{Q_1 - Q_L}{RT}\right)$$ Equation 2-10

The BET constant (C-Constant) is calculated from the intercept and is related to the energy of adsorption in the first adsorbed monolayer. The value of C is a dimensionless, energy related constant that reflects the interaction (adsorption) energy between nitrogen and the adsorbent surface.[1,52] At the low temperature of 77K, excess energy is dampened down and only significant

energy is distinguished. C-Constant then represents the average interaction energy present in the material and may indicate the magnitude of the adsorbent and adsorbate interactions. Typical C-Constant values for non-modified and modified silica surfaces previously studied and reported in literature fall within the range of polar and non-polar C-Constant values (10 – 200).[54,58] Low values of C-Constants indicate low energy of surface interactions, while high values of C-Constants indicate higher energy surface interactions. Polar materials, such as hydroxylated silica, has a surface that allows hydrogen bonding to occur on the charged surface thus is hydrophilic. Non-polar materials, such as paraffin, have a long chain of carbons that do not interact with water molecules therefore is hydrophobic. C-Constant values for nitrogen adsorption on hydroxylated silica typically range from 180 – 200, while on the paraffin surface C-Constant values are approximately 20.[52]

An interesting observation of the electrical conductivity on dry silica surface was made by Muroya[59,60] from various experiments which concluded that conductivity was clearly due to the protons on the SiOH groups, therefore the silanol surface was acting as the zone of conductivity. In water suspension free from soluble electrolytes, the surface of silica acts as a conductor to the degree that the surface is charged and counterions are available to provide charge transfer. Due to this nature of the silica surface, it can be deduced that interaction energy increases with increasing surface polarity. This further supports the possibility that Mixed-mode adsorbents can be characterized according to the degree of surface polarity using a type of interaction energy scale.

We may be able to use C-Constant to characterize chromatographic stationary phases on the basis of interaction energy. Assuming that materials such as silica lies on the high end of the interaction energy materials as most hydrophilic and materials such as paraffin lies on the low end of the interaction energy materials as most hydrophobic, one can create a type of universal

interaction energy scale as a way to evaluate an adsorbent's degree of hydrophobicity or hydrophilicity.

2.7 Conclusion

As new applications for Mixed-mode chromatography emerge, new columns with novel stationary phases are constantly being manufactured in order to meet the demand of separating complex sample mixtures. While the column dimensions will influence the efficiency, speed, and sensitivity of the chromatographic analysis, the stationary phase characteristics that will have the biggest impact on selectivity and retention factor of the target analytes. Many column classification systems are available in order to aid the end user in selecting the appropriate columns for their chromatographic separation goals, however the application of these classification systems to Mixed-mode materials may not be fully understood or appropriate due to the complex nature of multiple functional groups that make up the stationary phase and different types of interaction mechanisms that are available.

Although Mixed-mode chromatography has been successfully used for the separation of complex mixtures of analytes, understanding of the chromatographic behavior of commercially available Mixed-mode columns is limited.[14] In order to fully understand the retention mechanisms on Mixed-mode columns, the characteristics of the stationary phase must be well characterized since each characteristic may contribute to the overall chromatographic performance of the column.

Different chromatographic and non-chromatographic characterization techniques that have been applied to single mode adsorbents have also been applied to Mixed-mode adsorbents. Although many of the characterization techniques may be appropriately applied to single mode

adsorbents to study retention behavior, the same characterization techniques may not help predict the retention behavior on Mixed-mode adsorbents, since factors such as the mobile phase will have a significant influence on retention behavior an analyte. Presently, the non-chromatographic techniques for column characterization are not suitable for the general purpose of qualitative and quantitative analysis of Mixed-mode supports. A qualitative or quantitative method for analyzing of Mixed-mode supports may be able to provide more insight in evaluating retention behavior of analytes on Mixed-mode materials. Currently, none of the aforementioned non-chromatographic characterization techniques have been used to correlate retention behavior of analytes on Mixed-mode materials.

Low Temperature Nitrogen Adsorption (LTNA), a dry technique based on gas adsorption on solid surfaces, is able to provide surface energy characteristics of an adsorbent material. The specific area, total pore volume and average pore size of Mixed-mode stationary phase materials can be measured by the nitrogen adsorption method using BET analysis. Furthermore, interaction energy of the Mixed-mode adsorbent material can be evaluated with the C-Constant. By evaluating C-Constant, we can use the values to characterize Mixed-mode materials according to the degree of surface polarity and create a type of interaction energy scale as way to evaluate an adsorbent's degree of hydrophobicity or hydrophilicity.

Characterization of Mixed-mode adsorbents on the basis of average interaction energy has not been evaluated. This study aims to determine whether the BET C-Constant is a viable parameter that can be used to characterize commercial Mixed-mode columns on the basis of average interaction energy, and whether this characterization technique can be used to further evaluate retention behavior of analytes on Mixed-mode materials.

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CHAPTER 3 – CREATION OF BET C-CONSTANT ENERGY SCALE TO BE UTILIZED AS A UNIVERSAL INDEXATION SYSTEM FOR MIXED-MODE MATERIALS

3.1 Introduction

As new applications for Mixed-mode chromatography emerge, new columns with novel stationary phases are constantly being manufactured in order to meet the demand of separating complex sample mixtures. Although Mixed-mode chromatography has been successfully used for the separation of complex mixtures of analytes, understanding of the chromatographic behavior of commercially available Mixed-mode columns remains limited.[1]

Mixed-mode materials are not fully understood due to the complex nature of multiple functional groups that make up the stationary phase. Since the stationary phase will have the biggest impact on selectivity and retention factor of the target analytes, the characteristics of the stationary phase must be well characterized since each characteristic may contribute to the overall chromatographic performance of the column.

Qualitative or quantitative methods for analyzing Mixed-mode packing material are needed in order to provide more insight in evaluating retention behavior of analytes on Mixed-mode materials. Low Temperature Nitrogen Adsorption (LTNA), a dry technique based on gas adsorption on solid surfaces, is able to provide the specific surface area, total pore volume and average pore size of stationary phase materials using BET analysis. In addition to surface area and pore characterization, LTNA can also provide insight on surface energy characteristics of an adsorbent material. Surface energy of the adsorbent material can be evaluated with the C-Constant. C-Constant values can be used to characterize Mixed-mode materials according to the degree of

surface polarity and create a type of interaction energy scale as way to evaluate an adsorbent's degree of hydrophobicity or hydrophilicity.

Characterization of Mixed-mode adsorbents on the basis of interaction energy has not been previously evaluated. The primary focus of this study is to determine whether the BET C-Constant is a viable parameter that can be used to characterize commercial Mixed-mode columns on the basis of interaction energy, and whether this characterization technique can be used to further evaluate retention behavior of analytes on Mixed-mode materials.

3.2 Experimental

3.2.1 Materials

Two preparative scale columns from Phenomenex (Torrance, CA, USA): Axia Luna Silica (2), 5 µm, 100Å, 250 mm x 21.2 mm I.D. and Axia Luna C_{18} (2), 5 µm, 100Å, 250 mm x 21.2 mm I.D., were purchased as starting materials to construct the interaction energy scale. To prepare the materials for analysis, both columns were washed in order to remove any residual impurities that may have been left behind from the column manufacturing process. The Axia Luna Silica Prep Column was washed with approximately 10 column volumes of deionized water followed by 10 column volumes of methanol. The Axia Luna Silica column packing material was then unpacked from the column housing and the slurry was dried to constant weight in a furnace oven at 50°C under vacuum. It is important to dehydrate sample material as much as possible prior to degassing on the LTNA instrument, therefore the Axia Luna Silica column material was then further dried in an oven at 400°C under vacuum for 6 hours to drive out any residual water, then removed and

stored in a desiccator. The Axia Luna C_{18} column was washed with approximately 10 column volumes of acetonitrile, then 10 column volumes of deionized water, followed by 10 column volumes of acetonitrile. The Axia Luna C_{18} column was then unpacked from the column housing and the slurry was dried to constant weight in an oven at 50°C under vacuum. The Axia Luna C_{18} material was then removed from the oven and stored in a desiccator.

Acetonitrile (MeCN) and methanol (MeOH), used to wash the columns prior to unpacking were Optima Grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

3.2.2 Sample Preparation

Axia Luna C_{18} and Axia Luna Silica adsorbent materials were selected to create Mixed-Mode blended materials and construct a BET C-Constant interaction energy scale based on polarity. The properties of C_{18} and Silica material are well characterized adsorbents and theories of retention mechanisms on these materials are widely accepted. The silica material will represent the high end of the energy scale since silica is polar hydrophilic material. On the opposite end of the scale will be the C_{18} material representing low energy, since C_{18} adsorbents are non-polar hydrophobic material. To simulate Mixed-mode material, a simple Mixed-Bed approach, discussed in Chapter 1.4.2.1.1, was selected in order to avoid the complexity of chemically modifying ligands to create Mixed-mode material.

To create a simple indexation scale, the average adsorbent interaction energy of various blend ratios of Axia Luna Silica and Axia Luna C_{18}-modified materials must be evaluated. Axia Luna Silica-C_{18} blends were prepared starting with 100% Axia Luna Silica surface and progressively increasing the percentage of Axia Luna C_{18} surface in increments of 5-10% up to

100% Axia Luna C_{18} surface. Since the Axia Luna Silica and Axia C_{18} materials have different surface areas, the difference must be accounted for to create the correct surface area blend ratio. In order to prepare the blend ratios according to % Silica Polar surface, first the surface area of Axia Luna Silica material and Axia Luna C_{18} material was each determined by LTNA analysis.

Details of the instrumental analysis method and parameters are discussed in section 3.2.3. The results of LTNA analysis using the BET method for the Axia Luna Silica material and Axia Luna C_{18} material were found to be 405 m^2/g and 253 m^2/g respectively and are presented in Table 3-1. The standard deviation (STDEV) and percent relative standard deviation (%RSD) results were calculated from the preparation and analysis of three sample replicates, analyzed on the same instrument at the same time in order to demonstrate the precision or variability of the measurements. According to Gregg and Sing[7], the accuracy of the specific surface area obtained from BET analysis can vary by at least 10% from the actual area of the solid, due to factors not limited to laboratory conditions, variations in temperature during sample analysis, and nitrogen purity. Although the variability of the sample measurements were low, the accuracy of the experimentally determined BET specific surface area may vary by at least 10%. The error may be propagated to the calculation of C-Constant where high variability for low C-Constant values are more significant than high variability for high C-Constant values.

As discussed in Chapter 2.6, the amount of adsorbed nitrogen gas can be calculated using the size of the nitrogen gas molecule or cross-sectional area. It is generally assumed that a nitrogen molecule occupies a 16.2 $Å^2$ on the polar silica surface, and an average[2-5] of 20.1 $Å^2$ on modified and hydrophobic surfaces,[6-9] therefore a value of 21 $Å^2$ will be used to calculate the BET surface area of the Axia Luna C_{18} material. For the blend samples, intermediate values of nitrogen cross sectional area can be used to calculate the BET surface area, based on the percent of silica polar

Table 3-1 (a) BET Analysis – Surface Area								
Material	Sample Weight (mg)	N_2 Molecular Cross Sectional Area (nm^2)	Experimental BET Surface Area (m^2/g)					
			Rep 1	Rep 2	Rep 3	AVG	STDEV	RSD (%)
Axia Luna C_{18} Material (Non-polar)	100	0.210	195	197	200	253*	2.2	1.1
Axia Luna Silica Material (Polar)	100	0.162	404	404	409	405	2.6	0.7

**Corrected using Nitrogen cross-sectional area of 21Å.*

Table 3-1 (b) BET Analysis – BET C-Constant						
Material	Experimental BET C-Constant					
	Rep 1	Rep 2	Rep 3	AVG	STDEV	RSD (%)
Axia Luna C_{18} Material (Non-polar)	21	21	21	21	0.1	0.5
Axia Luna Silica Material (Polar)	203	207	199	203	4.3	2.1

Table 3-1 Results of LTNA using BET analysis (a) Surface Area and (b) BET C Constant for the Axia Luna Silica material and Axia Luna C_{18} material.

surface area. For each blend sample, the intermediate nitrogen cross-sectional area value A (nm^2) can be calculated according to Equation 3-1:

$$A_{b\%} = \left(\frac{21 - 16.2}{100}\right) * S_{b\%} + 16.2 \qquad \text{Equation 3-1}$$

Where $A_{b\%}$ is the nitrogen cross-sectional area (nm^2) for % Silica surface blend sample, 21 $Å^2$ is the nitrogen cross-sectional area for C_{18} material, 16.2 $Å^2$ is the nitrogen cross-sectional area for Silica material, and $S_{b\%}$ is the % Silica polar surface in the blend sample. Intermediate values of nitrogen cross-sectional area for each blend sample are shown in Table 3-2.

An accurate value of the nitrogen cross-sectional area for Mixed-mode materials is difficult to determine because the nitrogen molecule cross-sectional area may be different on neighboring surfaces. In this experiment, the average nitrogen cross-sectional area of the blended materials was estimated to range from the accepted value of 16.2 $Å^2$ for polar surfaces and 21 $Å^2$ for non-polar surfaces. Space requirements for the adsorption of nitrogen on self-assembled monolayers (SAMs) was previously explored and surface areas used the corrected values of nitrogen cross-sectional areas in the range 14.8 - 18.2 $Å^2$.[10] The uncertainty of the values may be as high as 20%. An estimated value of the nitrogen cross-sectional area of each of the Mixed-mode sample blends may be determined, however this analysis is beyond the scope of this work.

% Silica (Target % Polar Surface Area)	Axia Luna Silica Material Weight (mg)	Axia Luna C_{18} Material Weight (mg)	Total Blend Sample Weight (mg)	N_2 Molecular Cross Sectional Area $A_{b\%}$ (nm^2)	Axia Luna Silica Absolute Total Surface Area in Blend (m^2/g)	Axia Luna C_{18} Absolute Total Surface Area in Blend (m^2/g)	% Silica (Actual % Polar Surface Area)
0	0	100	100	16.2	0	25	0
10	12	178	190	16.7	5	45	10
20	25	158	183	17.2	10	40	20
30	38	112	150	17.9	15	28	35
35	38	99	137	18.0	15	25	38
40	38	87	125	18.2	15	22	41
45	38	74	112	18.4	15	19	45
50	38	62	100	18.6	15	16	50
60	38	50	88	18.8	15	13	55
65	38	37	75	19.2	15	9	62
75	38	25	63	19.6	15	6	71
85	38	12	50	20.2	15	3	83
90	111	20	131	20.5	45	5	90
100	100	0	100	21.0	41	0	100

Table 3-2 Blend Sample Weights and Ratios

The Axia Luna Silica and Axia Luna C_{18} material were each weighed and physically blended in the proportions where their surface area was incremented by 5-10% for each indexation point chosen for the indexation scale. The blend ratios were X/(100-X) with X being a percent of silica surface with 5-10% increments from 0 to 100.

The amount of each adsorbent by weight in the blend were calculated using the following expressions:

To get *n* grams of blend, the necessary weight of Axia Luna silica material, w_{Si}, is calculated according to Equation 3-2:

$$w_{Si} = \frac{n}{1+\frac{S_{Si}}{S_{C18}}\frac{100-X}{X}} \qquad \text{Equation 3-2}$$

Where S_{Si} is the specific surface area of silica adsorbent and S_{C18} is the specific surface area of Axia Luna C18 adsorbent.

The weight of the Axia Luna C_{18} part of the same blend, w_{Si}, is calculated according to Equation 3-3:

$$w_{C18} = w_{Si}\frac{S_{Si}}{S_{C18}}\frac{100-X}{X} \qquad \text{Equation 3-3}$$

The absolute total surface area for the Axia Luna Silica material in the blend sample is calculated according to Equation 3-4:

$$\text{Absolute Surface Area of Silica in Blend} = \frac{(w_{Si} * S_{Si})}{1000} \qquad \text{Equation 3-4}$$

Where w_{Si} is the weight of silica adsorbent in the blend sample and S_{Si} is the specific surface area of Axia Luna Silica adsorbent (405 m^2/g) determined experimentally.

Likewise, the absolute total surface area for the Axia Luna C_{18} material in the blend sample is calculated according to Equation 3-5:

$$\text{Absolute Surface Area of } C_{18} \text{ in Blend} = \frac{(w_{C18} * S_{C18})}{1000} \qquad \text{Equation 3-5}$$

Where w_{C18} is the weight of silica adsorbent in the blend sample and S_{C18} is the specific surface area of Axia Luna C_{18} adsorbent (253 m^2/g) determined experimentally.

The % Silica (Actual Polar Surface Area) that is in each blend sample can be calculated according to Equation 3-6, using the values obtained from Equations 3-4 and 3-5:

$$\%\, Silica\ (Polar\ Surface) = \left(\frac{\text{Absolute SA}_{Si}}{Absolute\ SA_{Si} + \text{Absolute SA}_{C18}}\right) * 100\% \qquad \text{Equation 3-6}$$

The blend sample weights and ratios are shown in Table 3-2.

3.2.3 Instrumental Parameters and Methods

All dried and dehydrated blend samples prepared in Section 3.2.1, were weighed into ½ inch diameter round bottom sample tubes with a filler rod and an isothermal jacket and prepared in triplicate to assess sample analysis variability. The sample weights (~0.1g) corresponded to approximately 30 m^2 – 50 m^2 total surface area as recommended by LTNA instrument

manufacturer for best results.[11] The filler rod, inserted into the sample tube, is used to ensure accuracy in low total surface area samples (less than 100 m^2) by reducing free-volume space and preventing adsorption of the gas to the internal glass surface of the sample tube.[11] The isothermal jacket, wrapped around the neck of the sample tube, controls the cold zone around the sample tube throughout the analysis, preventing errors and fluctuations in P_0.[10]

The LTNA degassing step is required to evacuate any moisture of other physically adsorbed molecules in the sample that will negatively impact the surface interaction with the nitrogen gas.[11,12] Different temperatures and times for degassing various materials can be found in literature, however the general recommendation is to select appropriate temperatures to avoid decomposition or drastic changes to surface modifications of the material. For typical C_{18} and other chemically modified silica, various literature[13-16] has reported mass loss between 200 and 600°C obtained from TGA experiments, however some literature[17] reports mass loss starting at 150°C. Silica material is highly hydrophilic, therefore it requires higher temperatures in order to drive out residual water that may adsorbed within the pores. Physically adsorbed water is removed from the silica surface at 115°C, and between 115°C and 600°C was stable with minimum loss of surface area.[18] The blend samples and Axia Luna C_{18} material were degassed with ultra-pure nitrogen at 90°C for approximately 1 hour. The Axia Luna Silica material was degassed with ultra-pure nitrogen at 130°C for 2 hours. After heating and degassing, samples were cooled, weighed, and placed into the instrument for analysis. The main steps in the process of an LTNA analysis are discussed in detail in Chapter 2.5.

Overall, the sample preparation and instrumental analysis followed the same general practice applied by Rustamov[19] for the determination of geometry of chemically modified silica using LTNA. Instrument calibration was performed using silica alumina reference material (Surface

Area = 215 m^2/g) to confirm accurate analysis of samples. All samples (Axia Luna Silica, Axia Luna C_{18}, and Blend Samples, Silica Alumina) were then analyzed by LTNA using a BET Surface Area and Porosity Analyzer instrument (Micromeritics, Norcross, GA, USA) Tri-Star II Plus model. Instrumental method and parameters for the LTNA analysis are shown in Table 3-3.

3.3 Results and Discussion

Adsorption isotherms for the Axia Luna C_{18} and Axia Luna Silica are shown in Figure 3-1 and is similar to that of a Type 4 isotherm, therefore BET method can be applied for surface area and C-Constant calculations.[7,8,20-22] The adsorption isotherm and BET Plot for the 50% Silica Blend sample shown in Figure 3-2 also resembles a Type 4 isotherm. All other blend samples were also observed to generate Type 4 isotherms.

As discussed in Chapter 2.5 and 2.6, a linear relationship is obtained when the BET Equation (Equation 2-7) is applied in plotting the amount of adsorbed nitrogen at relative pressures p/p_0 between 0.05-0.35. The BET plot for the Axia Luna C_{18} and Axia Luna Silica materials are shown in Figure 3-3. The BET surface area, and the BET C-Constants for the Axia Luna C_{18}, Axia Luna Silica, and blend samples can then be calculated from the slope and y-intercept using Equations 2-8 (Total Surface Area) and Equation 2-10 (BET C-Constant). BET Surface Areas and BET C-Constants for all the analyzed materials are summarized in Table 3-4.

Results from the analysis show that the Axia Luna C_{18} material had the lowest BET surface area (253 m^2/g) and Axia Luna Silica material had the highest BET surface area (405 m^2/g). Overall, a clear trend was observed of increasing BET surface area with increasing % Silica polar

LTNA Instrument Method and Parameters for Blend Samples	
Instrument Model	Micromeritics BET Surface Area & Porosity Analyzer Tristar II Plus Model
Sample Description	• ½ inch round bottom glass sample tube with filler rod & isothermal jacket • Enter sample weight (~0.1g) following degas heating phase
Degas Conditions	• Evacuation Gas (back fill) = Helium • Evacuation Rate = 5.0 mmHg/s • Evacuation Time = 10 min • Evacuation Target Temperature = 30°C • Temperature Ramp Rate = 10.0°C/min • Heating Phase (Axia Luna C_{18}/Blend Samples) = 90°C for 1 hr. under N_2 Heating Phase (Axia Luna Silica) = 130°C for 1 hr. under N_2 Heating Phase (Silica Alumina Ref. Std) = 350°C for 240 min. under N_2
Adsorptive Properties	• Adsorptive = Nitrogen • Temperature = 77.35K • Molecular Cross-Sectional Area = 0.162 nm^2 (Silica); 0.21 mm^2 (C_{18}) • Hard Sphere Diameter = 3.860 Å • Adsorbate molecular weight = 28.01
Analysis	• Full Adsorption Isotherm Collection & BET Surface Area Analysis • Target Relative Pressures (P/P_o) = 0.05 to 0.998 • Equilibration Interval = 10s • Evacuation Time = 0.20h
Reporting	• Full Adsorption Isotherm Collection & BET Surface Area Analysis • Relative Pressures (P/P_o) = 0.05 to 0.998

Table 3-3 LTNA Instrumental Method and Parameters for Blend Samples

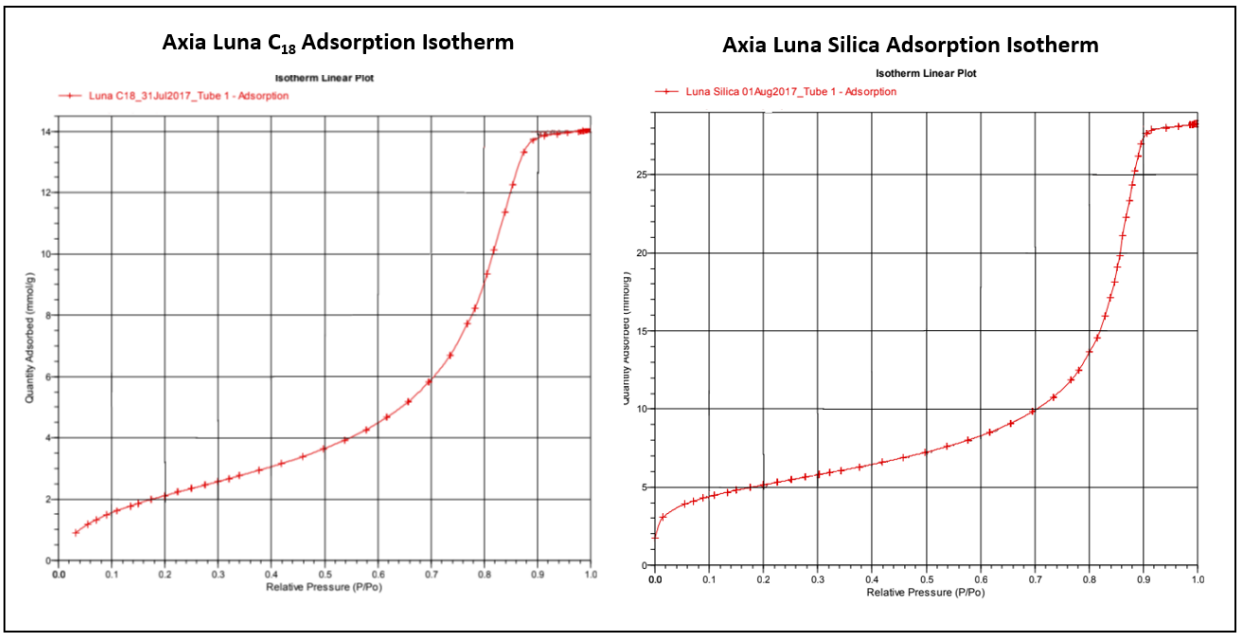

Figure 3-1 Adsorption isotherms for Axia Luna C_{18} and Axia Luna Silica Material

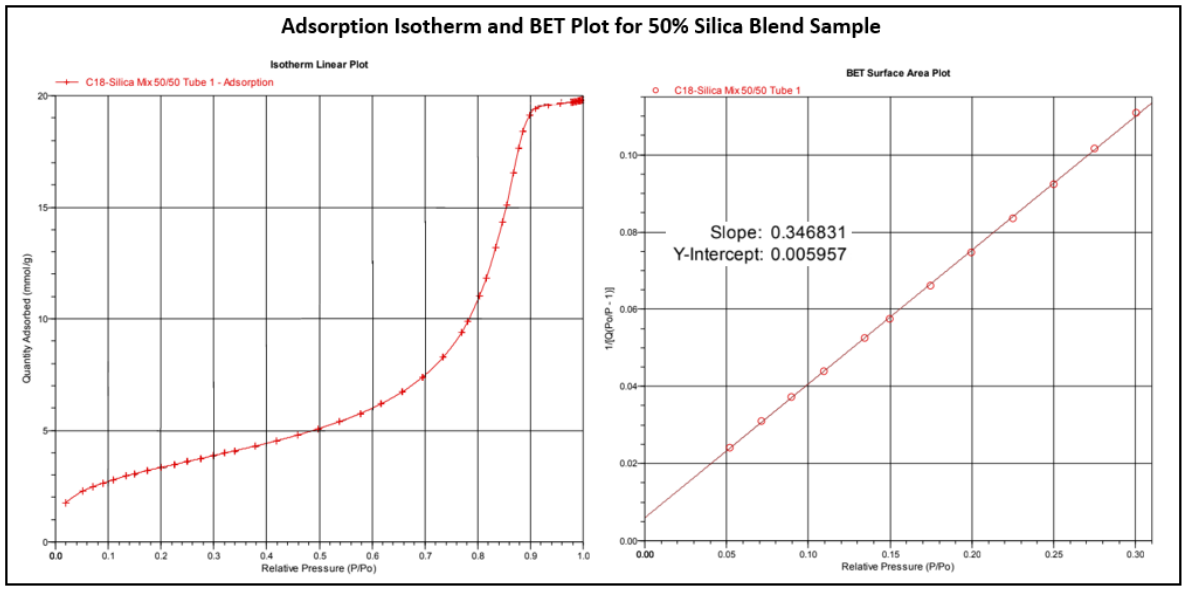

Figure 3-2 The adsorption isotherm and BET Plot for the 50% Silica Blend sample

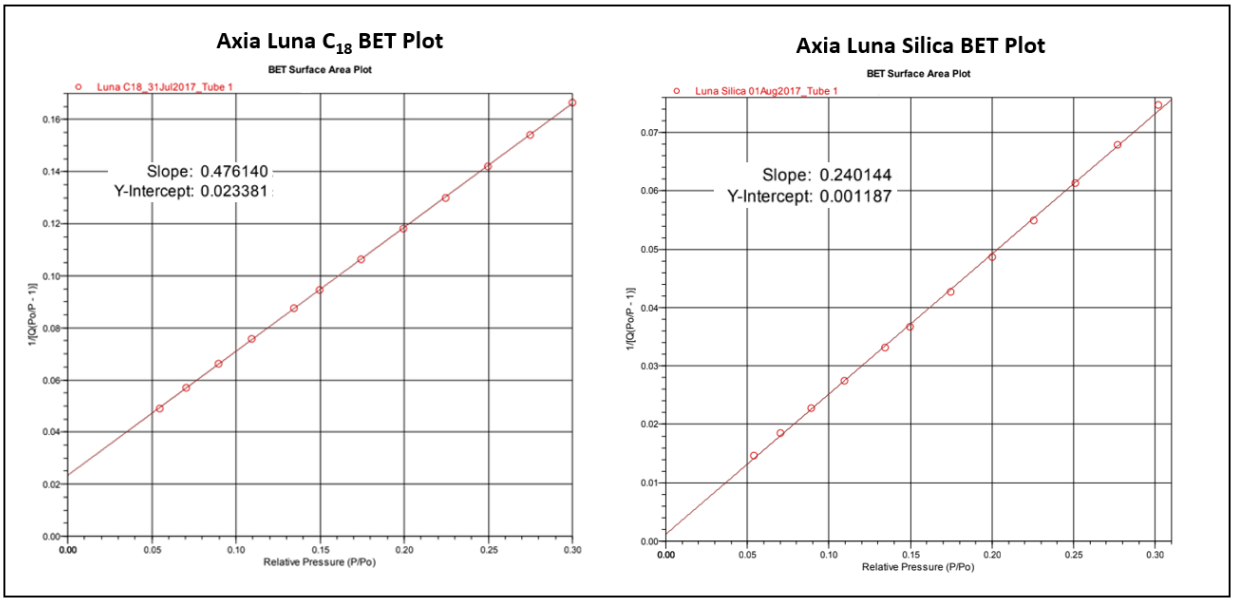

Figure 3-3 The BET plot for the Axia Luna C_{18} and Axia Luna Silica materials

% Silica (Target % Polar Surface Area)	% Silica (Actual % Polar Surface Area)	Experimental AVG BET Surface Area (m^2/g)	RSD (%) n=3 BET Surface Area	Experimental AVG C-Constant	RSD (%) C-Constant	ln C
0*	0*	253	1.1	21	0.5	3.06
10	10	197	5.2	26	2.4	3.27
20	20	245	0.8	32	0.8	3.45
30	35	245	1.5	42	2.8	3.73
35	38	254	1.2	44	1.4	3.78
40	41	266	1.1	46	1.5	3.83
45	45	270	1.1	49	1.4	3.90
50	50	281	3.1	57	1.1	4.04
60	55	290	0.7	61	1.0	4.11
65	62	294	1.3	76	1.9	4.33
75	71	329	2.3	87	5.4	4.46
85	83	350	0.3	118	2.5	4.77
90	90	378	2.1	148	1.2	5.00
100	100	405	0.7	203	2.1	5.31

**0% Silica Polar Surface is equivalent to 100% Axia Luna C_{18} non-polar surface area*

Table 3-4 BET analysis of nitrogen adsorption results for all studied materials (Axia Luna Silica, Axia Luna C_{18}, and Blend Samples)

surface area. BET surface area values of different types of C_{18} material were reported to vary in the range of 91 – 291 m^2/g using a nitrogen cross-sectional area of 20.5 $Å^2$.[5] BET surface area values of different types of unmodified bare silica material are reported to be much higher and ranged from 263 – 459 m^2/g using a nitrogen cross-sectional area of 16.2 $Å^2$.[4]

BET surface area values obtained in this study were within the ranges observed for various types of C_{18} and silica materials. In a study conducted by Zouari[23] to graft octadecyldimethyl(dimethylamino)silane to silica surface, results showed that specific surface area dropped drastically from 580 m^2/g down to 125 – 192 m^2/g after grafting the C_{18} material, confirming that chemical modification of silica will decrease surface area and surface energy of adsorption interactions.[3,6,24]

The same study by Zouari[23] also showed that the bare silica demonstrated high values of C-Constants (80 – 97) obtained from the BET method, and after surface modification, the grafted C_{18} materials showed C-Constants of 27-28. These C-Constant values agreed with those reported by Giaquinto[5], for various C_{18} material ranging from 20–22. Other studies have reported C-Constant values of other modified silica containing other types of functional groups to be in the range of 15-60.[4,25,26] C-Constant values for silica have been reported in various literature to range from 70-200.[4,24,26] The BET C-Constant, is used to assess adsorption energy and surface polarity. Low C-Constant values indicate non-polar, low average interaction energy, while high C-Constant values indicate high average interaction energy of the polar silanol groups.[7,24-26]

The blend samples experimental BET surface area (left y-axis) vs. % Silica Surface is plotted in Figure 3-4. Total surface area increases with increasing % Silica surface in the blend sample, and total surface area shows a linear dependence with a regression coefficient of 0.98. The

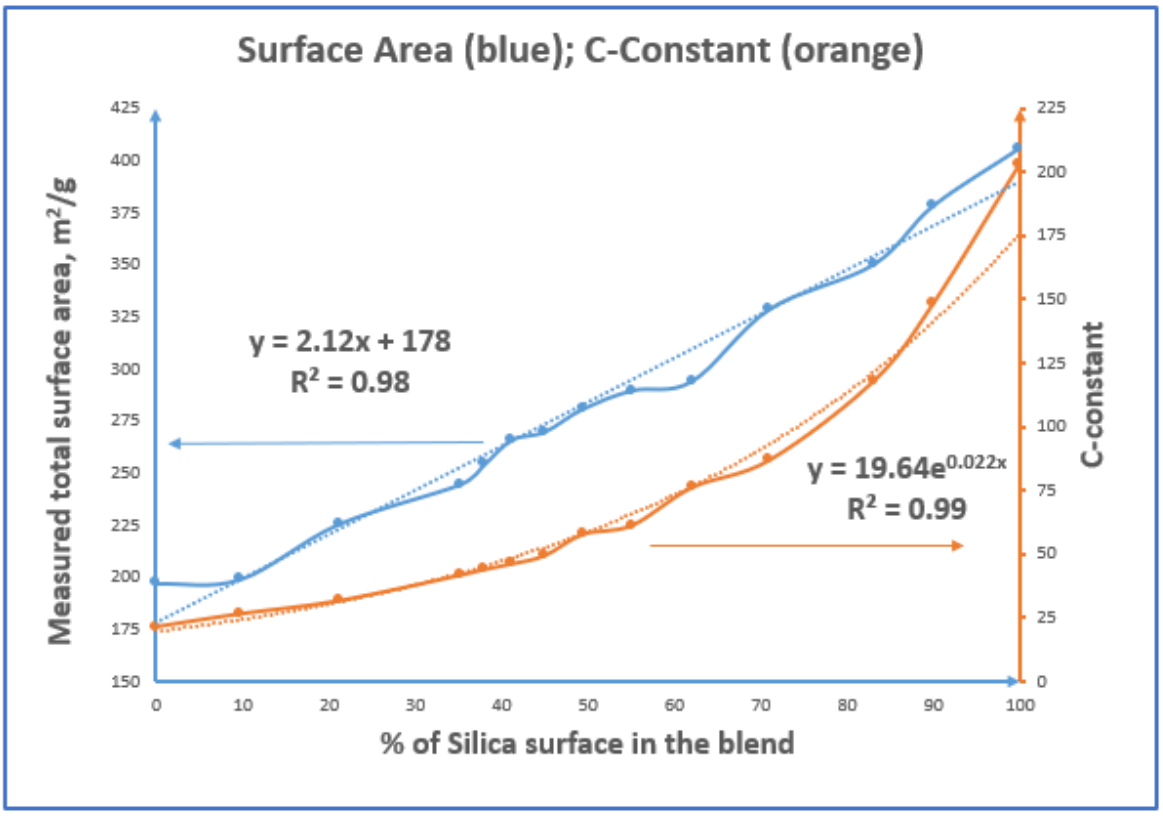

Figure 3-4 Plot of BET surface area (left y-axis) vs. % Silica Surface and BET C-Constant (right y-axis) vs. % Silica Surface

BET C-Constant (right y-axis) vs. % Silica Surface is also plotted on the same graph in Figure 3-4. Results demonstrate that C-Constant increases with increasing % Silica surface in the blend sample, and that the C-Constant dependence has an exponential fit with a regression coefficient of 0.99. When the ln (C) is calculated, a plot of ln (C) vs. % Silica Surface (Figure 3-5) shows that a linear relationship is observed between the C-Constant and the % Silica Blend ratio.

Ln (C) represents the excess of the nitrogen adsorption energy over its condensation energy. The average interaction energy can be calculated using Equation 2-10 (detailed in Chapter 2). Average interaction energy is then calculated for each blend sample and presented in Table 3-5. An interaction energy scale can now be created (shown in Figure 3-6) by plotting the calculated interaction energy vs. percent polar silica surface in the blend sample and the linear dependence is confirmed with a regression coefficient of 0.99. This interaction energy scale represents the adsorbent excessive non-specific interaction energy at the boiling point temperature of liquid nitrogen (77K). This low temperature suppresses all specific interactions, thus what remains is the average interaction energy without any specificity that this surface may demonstrate at normal HPLC conditions toward specific analytes.

Although at 77K, the energetic effects are relatively small, ranging from 2-3.5 kJ/mol, the full range of non-polar to polar materials are covered in the newly created interaction energy scale. This linear dependence of BET C-Constant and nitrogen adsorption energy on the fractions of Axia Luna Silica and Axia Luna C_{18} surfaces in the blended sample material allows for a non-chromatographic technique for the energetical indexation of Mixed-mode adsorbents using their BET C-Constant. These results confirm that it is possible to create an index of any other Mixed-Mode adsorbent using its C-Constant relative to the percentage of silica polar surface scale.

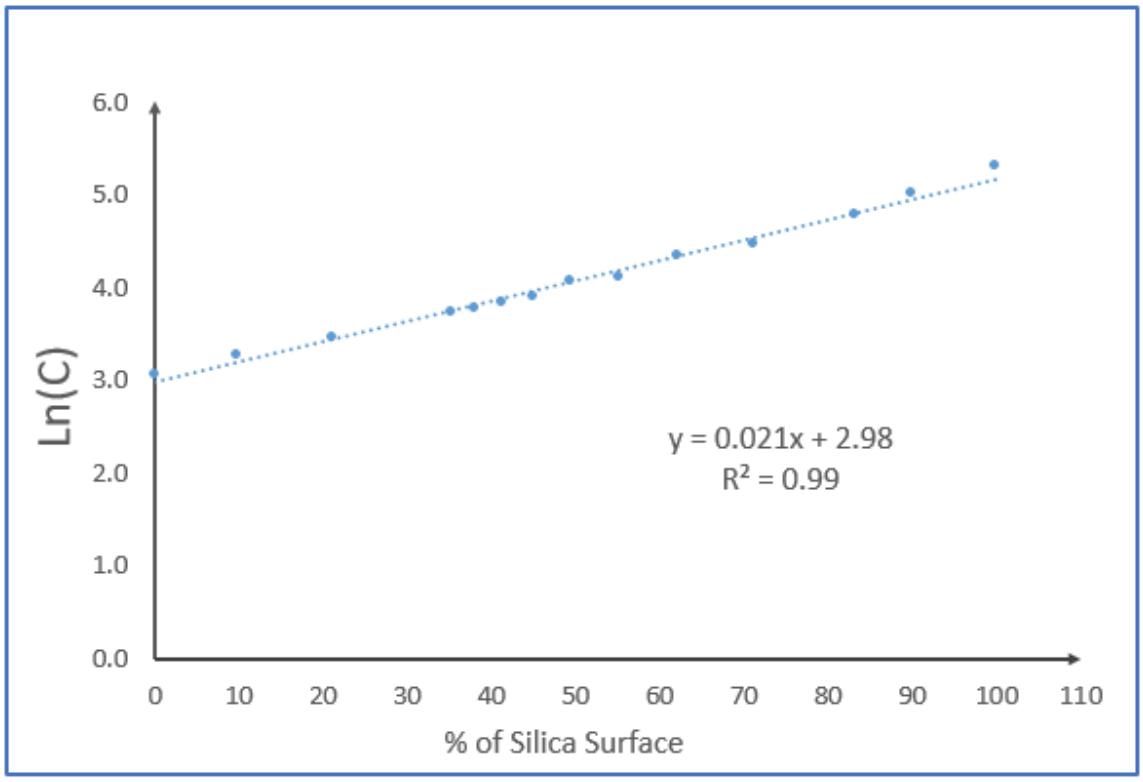

Figure 3-5 Plot of BET C-Constant vs. % Silica Surface

% Silica (Target % Polar Surface Area)	% Silica (Actual % Polar Surface Area)	Experimental AVG C-Constant	Ln C	RTLnC	Average Interaction Energy (kJ/mol)
0	0	21	3.06	1958.94	1.96
10	10	26	3.27	2093.38	2.09
20	20	32	3.45	2208.61	2.21
30	35	42	3.73	2387.86	2.39
35	38	44	3.78	2419.87	2.42
40	41	46	3.83	2451.88	2.45
45	45	49	3.90	2496.69	2.50
50	50	57	4.04	2592.72	2.59
60	55	61	4.11	2631.13	2.63
65	62	76	4.33	2771.97	2.77
75	71	87	4.46	2855.19	2.86
85	83	118	4.77	3053.65	3.05
90	90	148	5.00	3200.89	3.20
100	100	203	5.31	3399.35	3.40

Table 3-5 Calculation of Average Interaction Energy from BET C-Constant values

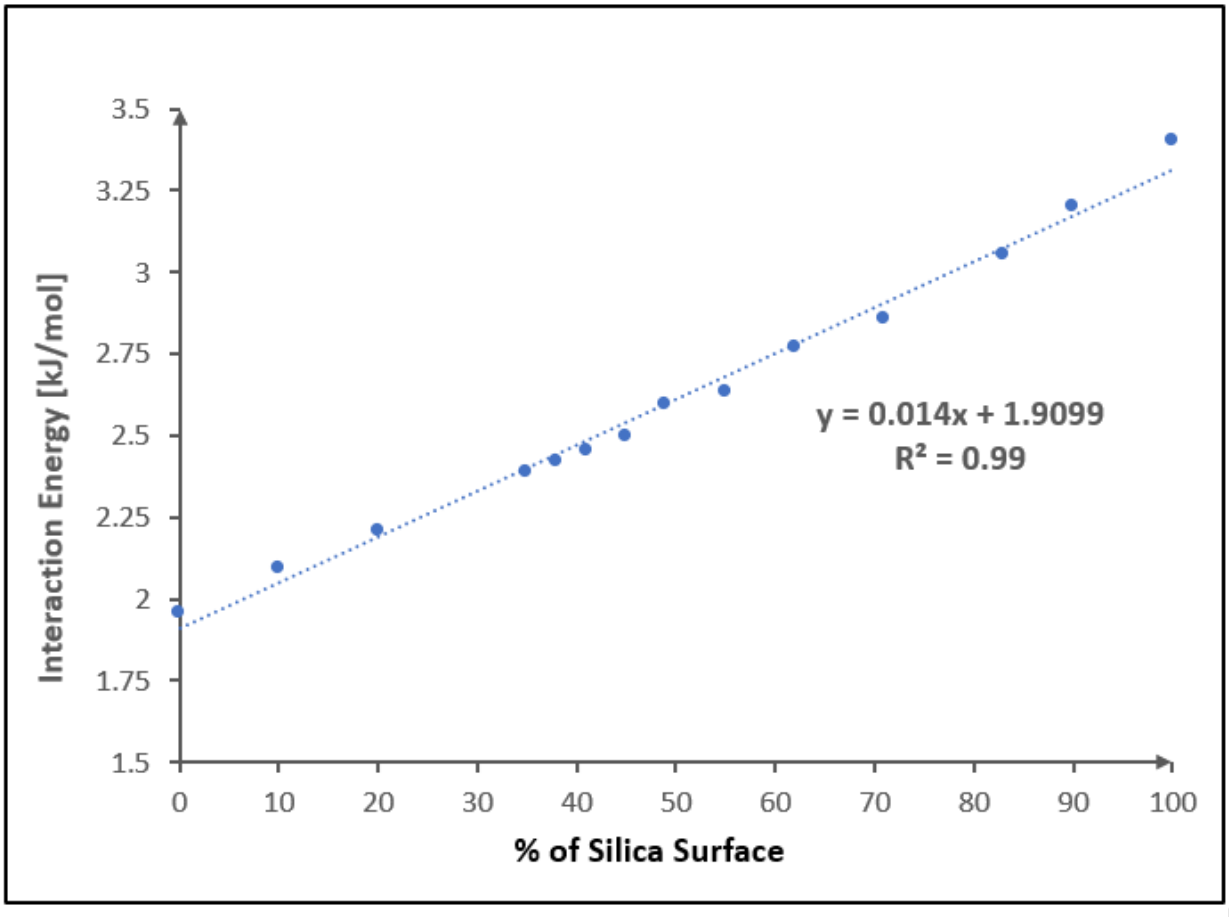

Figure 3-6 Plot of Average Interaction Energy vs % Silica Surface

3.4 Conclusion

In this study, a mixed-bed approach was used to simulate Mixed-mode materials of various blend ratios using well characterized Silica and C_{18} materials. The blended materials were analyzed by a commonly used non-chromatographic LTNA technique to generate adsorption isotherms. The BET method of analysis was applied to obtain surface areas and the BET C-Constants of the Silica, C_{18}, and blended samples. The BET C-Constants were evaluated and a linear correlation was established between the BET C-Constant and the % Silica Surface in the blended samples. Results suggested that the BET C-Constant may be a viable parameter that can be used to characterize Mixed-mode materials based on average interaction energy. This linear correlation that was established serves as the basis for the newly created BET C-Constant interaction energy scale.

This BET C-Constant energy scale may be used as a universal indexing system to characterize a wide array of adsorbents, including Mixed-mode materials on the basis of average interaction energy. This indexing system may assist the end-user in Mixed-mode column selection and improve the understanding of method development on Mixed-mode columns. Finally, this non-chromatographic characterization technique may be used to further explore possible correlations between analyte retention behavior and interaction energy of Mixed-mode materials.

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CHAPTER 4 – CHARACTERIZATION OF COMMERCIAL MIXED-MODE STATIONARY PHASES USING THE BET C-CONSTANT ENERGY SCALE

4.1 Introduction

New applications for Mixed-mode chromatography continue to emerge, and new columns with novel stationary phases and different types of interaction mechanisms are constantly being manufactured. To fully understand the interaction mechanisms on Mixed-mode columns, the characteristics of the stationary phase should be studied in order to evaluate the contribution to the overall chromatographic performance of the column. Characterization of commercially available Mixed-mode columns is often difficult since complete information on the chemistry and structure of the stationary phase is often proprietary and not shared with end-users.

In Chapter 3, a non-chromatographic technique (LTNA) was used to create an interaction energy scale that maybe used as a universal indexation system to characterize a wide array of adsorbents, such as Mixed-mode materials on the basis of interaction energy. A mixed-bed approach was used to simulate Mixed-mode materials of various blend ratios using well characterized Silica and C_{18} materials. The BET C-Constants were evaluated and a linear correlation was established between the BET C-Constant and the % Silica Polar Surface in the blended samples. Results from these studies suggested that the BET C-Constant may be a viable parameter that can be used to characterize Mixed-mode materials based on interaction energy.

The studies conducted and presented in this chapter will evaluate a variety of commercially available Mixed-mode column materials by characterizing them on the basis of average interaction energy using the BET C-Constant energy scale that was created in Chapter 3.

4.2 Experimental

4.2.1 Materials

Eight different analytical scale columns, each used for different modes of chromatography (Reversed Phase, Ion-Exchange, Normal Phase, HILIC and Mixed-mode) were purchased from various column manufacturers: Restek Corp. (Bellefonte, PA, USA), Waters Corp. (Milford, MA, USA), SIELC Technologies (Wheeling, IL, USA), (Phenomenex (Torrance, CA, USA) and Thermo Scientific (Waltham, MA, USA). The descriptions and details of the commercial column materials are provided in Table 4-1. Acetonitrile (MeCN) used to wash the columns prior to unpacking were Optima Grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.2.2 Sample Preparation

Prior to unpacking the stationary phase materials from the stainless steel column housing, all columns were washed in order to remove any residual impurities that may have been left behind from the column manufacturing process. All columns were washed with approximately 10 column volumes of deionized water followed by 10 column volumes of Acetonitrile. All column materials were then unpacked from the column housing and the slurry was dried to constant weight in a furnace oven at 50°C under vacuum. All materials were then removed from the oven and stored in a desiccator.

Material #	Commercial Column	Manufacturer	Column Dimensions (mm)	Particle Size (µm)	Pore Size (Å)	Manufacturer's Description of Stationary Phase
1	Allure Silica	Restek	4.6 x 150	5	60	Silica for Normal Phase LC Separations[1]
2	BEH HILIC Silica	Waters	2.1 x 150	1.7	130	Unbonded BEH Silica for HILIC separations[2]
3	Acquity CSH Phenyl-Hexyl	Waters	2.1 x 150	1.7	130	C_6 phenyl ligand[2]
4	Acquity Peptide CSH C_{18}	Waters	2.1 x 150	1.7	130	C_{18} Charged Surface[2]
5	Promix MP	SIELC	4.6 x 250	5	300	Reversed Phase + IEX[3]
6	Luna Omega Polar C_{18}	Phenomenex	4.6 x 100	5	100	C_{18} + Polar Modified Particle Surface[4]
7	Acclaim Mixed-mode HILIC-1	Thermo Scientific	4.6 x 150	5	120	RP + HILIC (hydrophobic alkyl chain with diol group)[5]
8	Luna Omega PS C_{18}	Phenomenex	4.6 x 100	5	100	C_{18} + Positively Charged Surface[4]

Table 4-1 The description and details of the commercial column materials analyzed.

4.2.3 Instrumental Parameters and Methods

To prepare the materials for analysis, all dried and dehydrated samples prepared in Section 4.2.2, were weighed into ½ inch diameter round bottom sample tubes with a filler rod and an isothermal jacket and prepared in triplicate to assess sample analysis variability. The sample weights (~0.1g) corresponded to approximately 30 m^2 – 50 m^2 total surface area as recommended by LTNA instrument manufacturer for best results.[6] The filler rod, inserted into the sample tube, was used to ensure accuracy in low total surface area samples (less than 100 m^2) by reducing free-volume space and prevent adsorption of the gas to the internal glass surface of the sample tube.[6] The isothermal jacket, wrapped around the neck of the sample tube to control the cold zone around the sample tube throughout the analysis, was used in order to prevent errors and fluctuations in P_0.[6]

The LTNA degassing step was performed in order to evacuate any moisture of other physically adsorbed molecules in the commercial material samples that may negatively impact the surface interaction with the nitrogen gas.[6,7] As discussed in Chapter 3.2.3, different temperatures and times for degassing various materials were found in literature, with an overall recommendation to select appropriate temperatures that would avoid decomposition and drastic changes to surface modifications of the material. Since previous literature[8-12] has reported mass loss between 150 and 600°C for C_{18} and other chemically modified silica, this experiment used the same temperature conditions used to analyze the Axia Luna C_{18} material in Chapter 3.2.3, therefore all commercial materials from Table 4-1 were degassed with ultra-pure nitrogen at 90°C for approximately 1 hour. After heating and degassing, all samples were cooled, weighed, and placed into the instrument for analysis. The main steps in the process of an LTNA analysis are discussed in detail in Chapter 2.5.

Instrument calibration was performed using silica alumina reference material (Surface Area = 215 m^2/g) to confirm accurate analysis of samples. All commercial material samples from Table 4-1 were then analyzed by LTNA using a BET Surface Area and Porosity Analyzer instrument (Micromeritics, Norcross, GA, USA) Tri-Star II Plus model. Instrumental method and parameters for the LTNA analysis are shown in Table 4-2.

4.3 Results and Discussion

Adsorption isotherms for all the commercial material samples tested in Table 4-1 are shown in Figure 4-1 and all adsorption isotherms resemble that of a Type 4 isotherm, confirming that BET analysis can be applied for surface area and C-Constant calculations.[13-17] As discussed in Chapter 2.5 and 2.6, a linear relationship is obtained when the BET Equation (Equation 2-7) is applied in plotting the amount of adsorbed nitrogen at relative pressures p/p_0 between 0.05-0.35. The BET Plots for all the commercial material samples tested are shown in Figure 4-2. The BET surface area, and the BET C-Constants for the commercial material samples can then be calculated from the slope and y-intercept using Equations 2-8 (Total Surface Area) and Equation 2-10 (BET C-Constant). BET Surface Areas and BET C-Constants for all the analyzed materials are summarized in Table 4-3. As discussed in Chapter 2.6, the amount of adsorbed nitrogen gas is calculated using the nitrogen molecular cross-sectional area. Since it is generally assumed that a nitrogen molecule occupies a 16.2 $Å^2$ on the polar silica surfaces, 16.2 $Å^2$ was used for commercial silica materials #1 and #2. Since an average nitrogen cross-sectional area value of 20.1 $Å^2$ is reported[18-21] for modified and hydrophobic surfaces,[13,17,22,23] a value of 21 $Å^2$ was used to calculate the BET surface area of the commercial materials #3 through #8.

LTNA Instrument Method and Parameters for Commercial Materials	
Instrument Model	Micromeritics BET Surface Area & Porosity Analyzer Tristar II Plus Model
Sample Description	• ½ inch round bottom glass sample tube with filler rod & isothermal jacket • Enter sample weight (~0.1g) following degas heating phase
Degas Conditions	• Evacuation Gas (back fill) = Helium • Evacuation Rate = 5.0 mmHg/s • Evacuation Time = 10 min • Evacuation Target Temperature = 30°C • Temperature Ramp Rate = 10.0°C/min • Heating Phase (All Commercial Materials) = 90°C for 1 hr. under N_2
Adsorptive Properties	• Adsorptive = Nitrogen • Temperature = 77.35K • Molecular Cross-Sectional Area = 0.16 mm^2 (Material #1-2) • Molecular Cross-Sectional Area = 0.21 mm^2 (Material #3-8) • Hard Sphere Diameter = 3.860 Å • Adsorbate molecular weight = 28.01
Analysis	• Full Adsorption Isotherm Collection & BET Surface Area Analysis • Target Relative Pressures (P/P_o) = 0.05 to 0.998 • Equilibration Interval = 10s • Evacuation Time = 0.20h
Reporting	• Full Adsorption Isotherm Collection & BET Surface Area Analysis • Relative Pressures (P/P_o) = 0.05 to 0.998

Table 4-2 LTNA Instrumental Method and Parameters for Commercial Materials

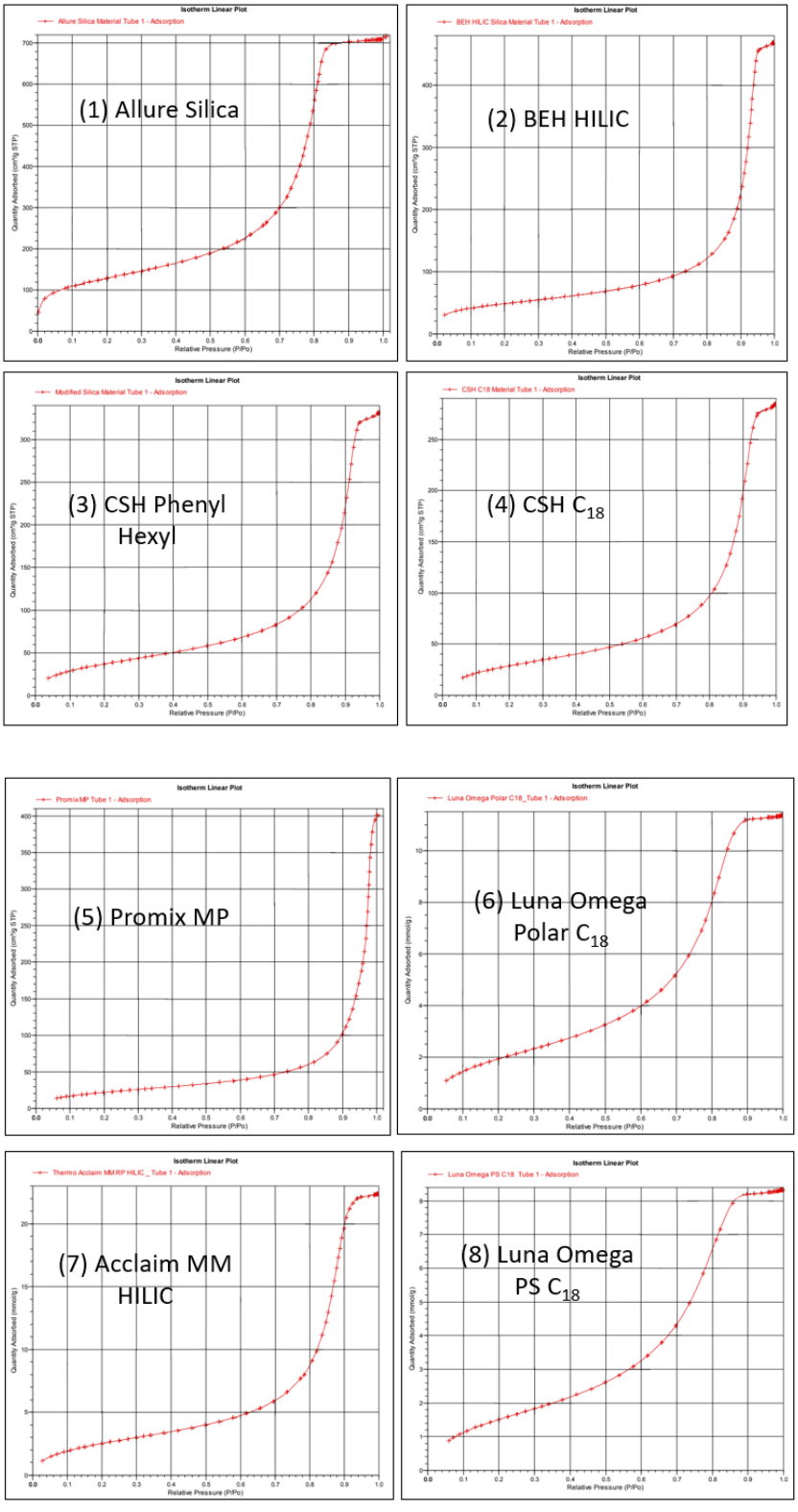

Figure 4-1 Adsorption isotherms for commercial materials

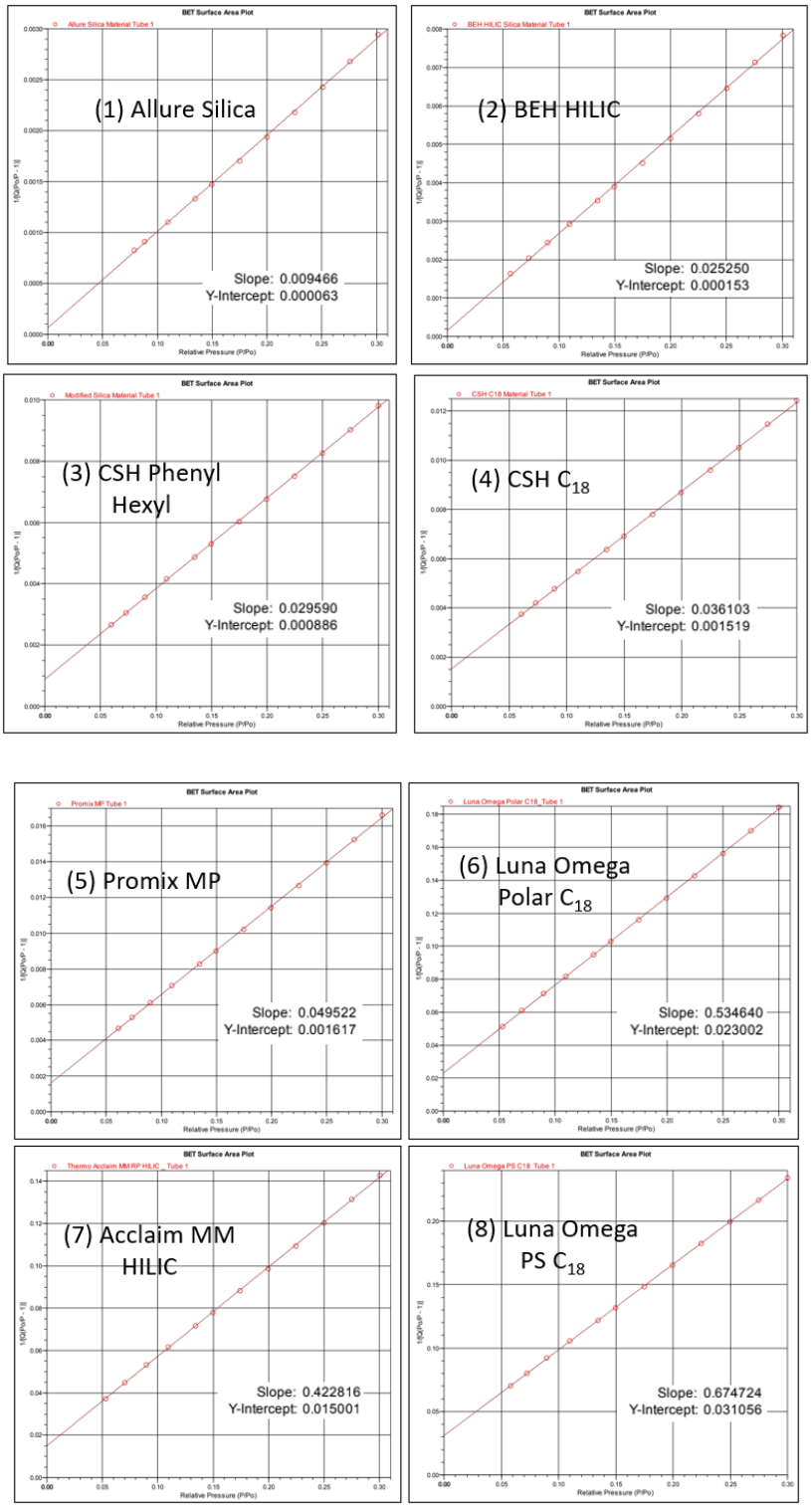

Figure 4-2 BET plots for commercial materials

Material #	Commercial Material	Experimental AVG BET Surface Area (m^2/g)	RSD (%) n=3 BET Surface Area	N_2 Molecular Cross Sectional Area (nm^2)	Corrected BET Surface Area (m^2/g)	Experimental AVG C-Constant
1	Allure Silica	451	1.9	16.2	451	149
2	BEH HILIC Silica	176	4.7	16.2	176	172
3	Acquity CSH Phenyl Hexyl	143	0.3	21.0	185	35
4	Acquity Peptide CSH C_{18}	110	7.2	21.0	143	25
5	Promix MP	83	2.6	21.0	107	33
6	Luna Omega Polar C_{18}	173	1.8	21.0	224	24
7	Acclaim Mixed-mode HILIC-1	221	1.0	21.0	287	29
8	Luna Omega PS C_{18}	139	0.7	21.0	180	23

Table 4-3 BET Surface Areas and BET C-Constants for Commercial Materials

$RTln(C)$ represents the excess of the nitrogen adsorption energy over its condensation energy and can be used in the calculation of the average interaction energy. Average interaction energy can be calculated using Equation 2-10 (detailed in Chapter 2). In order to use the Axia Luna BET C-Constant energy scale that was created in Chapter 3 (Figure 3-6) as a indexation system for commercial Mixed-mode materials, the corresponding percent silica polar surface must be determined for each commercial material. The equivalent percent silica polar surface for each commercial material can be calculated from the average interaction energy according to Equation 4-1, which is the linear equation from the average interaction energy scale shown in Figure 3-5:

$$y = 0.014x + 1.91 \qquad \text{Equation 4-1}$$

Equation 4-1 can be re-arranged to Equation 4-2 in order to obtain the commercial material's equivalent % silica polar surface:

$$Equivalent\ \%\ silica\ polar\ surface\ (x) = \frac{y - 1.91}{0.014} \qquad \text{Equation 4-2}$$

Where 0.014 is the slope, 1.91 is the y-intercept, and y is the calculated average interaction energy of the commercial material in kJ/mol. The average interaction energy and equivalent percent silica polar surface for each commercial material is presented in Table 4-4.

Material #	Commercial Material	Experimental AVG C-Constant	Ln C	RTLnC	Average Interaction Energy (kJ/mol)	Calculated Equivalent % Silica Polar Surface
1	Allure Silica	149	5.00	3201.26	3.20	92
2	BEH HILIC Silica	172	5.14	3293.45	3.29	99
3	Acquity CSH Phenyl Hexyl	35	3.54	2266.84	2.27	26
4	Acquity Peptide CSH C_{18}	25	3.22	2060.65	2.06	11
5	Promix MP	33	3.48	2230.58	2.23	24
6	Luna Omega Polar C_{18}	24	3.19	2039.83	2.04	9
7	Acclaim Mixed-mode HILIC-1	29	3.37	2155.67	2.16	18
8	Luna Omega PS C_{18}	23	3.11	1993.20	1.99	7

Table 4-4 Average Interaction Energy and the Calculated Equivalent % Silica Polar Surface for All Analyzed Commercial Materials

4.3.1 Evaluation of Commercial Materials

The results shown in Table 4-4 for all the analyzed commercial materials revealed that C-Constant values for all chemically modified commercial Mixed-mode materials (Materials #3-8) were between 22 and 35, which corresponds to a range of 6% to 26% of polar surface. For the silica materials (Materials #1-2), the C-Constant values came close to 100% of polar surface as would be expected. Each commercial material sample that was analyzed was plotted on the BET C-Constant energy scale according to their calculated average interaction energy and equivalent percent silica polar surface. The position of each of the studied commercial Mixed-mode adsorbents are shown on the BET C-Constant energy scale in Figure 4-3. According to the results, the commercial materials that are listed in Table 4-1 could be characterized into three groups: Highly Hydrophilic Adsorbents, Intermediate Adsorbents, Highly Hydrophobic Adsorbents.

4.3.1.1 Highly Hydrophilic Adsorbents (Materials #1 and 2)

The interaction energy characteristics of two highly hydrophilic column materials were studied in this experiment. Allure Silica column (Material #1) contains unbonded silica designed for normal phase separations of highly polar compounds.[1] BEH HILIC Silica (Material #2) contains unbonded Bridged Ethylene Hybrid (BEH) particles and is designed to retain and separate very polar basic compounds under HILIC conditions.[2] As was expected, the results showed that both Materials 1 and 2 had high C-Constant values, which are indicative of the higher interaction energy characteristics of polar surfaces.[15,24]

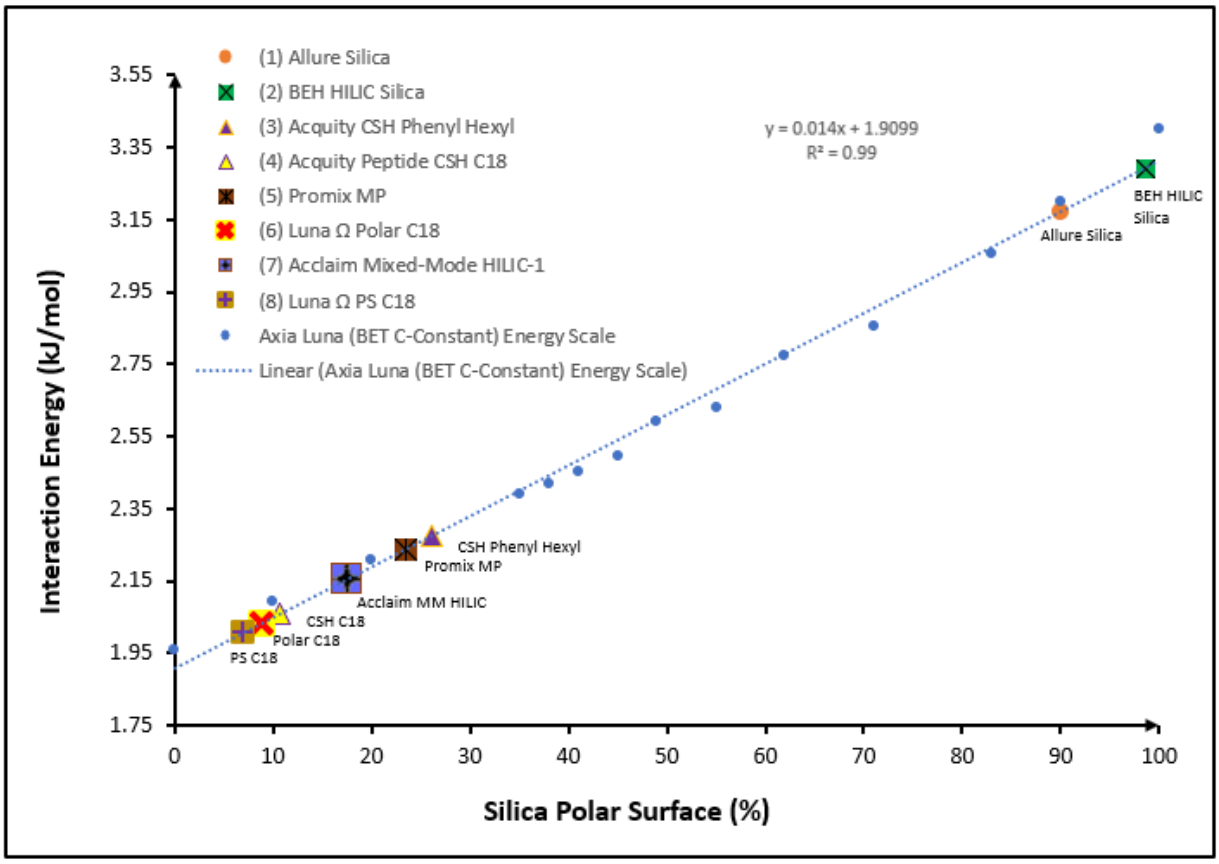

Figure 4-3 Position of Each of the Analyzed Commercial Adsorbent Materials on the BET C-Constant Energy Scale.

Allure Silica (Material #1) and BEH HILIC Silica (Material #2) are positioned highest on the interaction energy scale. Allure Silica (Material #1) demonstrates a slightly lower interaction energy possibly due to a smaller pore diameter (60 Å) and partial dehydroxylation of the surface, while BEH HILIC Silica which has a larger pore diameter (130 Å) demonstrates a high degree of hydroxylation. As the temperature is raised, hydroxyl groups condense to form siloxane bonds and water is evolved.[25] The effect of particle size and pore diameter was studied by Brunauer[15,25] who determined that smaller silica particles with smaller pore diameters were dehydrated at lower temperatures than larger silica particles with larger pore diameters. These results support the claim that the change in the surface nature of the adsorbents is due to the change in the degree of hydroxylation, and that nitrogen adsorption on a dehydroxylated silica gel is 20-25% less than on a hydroxylated one.[26] The results indicate that dehydroxylation of the silica surface can have an effect on the interaction energy characteristics.

4.3.1.2 Highly Hydrophobic Adsorbents (Materials # 4, 6, 8).

The interaction energy characteristics of three highly hydrophobic column materials were studied in this experiment. Acquity Peptide CSH C_{18} column (Material #4) is advertised as a Trifunctional C_{18}, fully end-capped, and bonded to Charged Surface Hybrid Bridged Ethylene Hybrid (BEH) substrate for the separation of non-polar basic analytes.[2] The Luna Omega PS C_{18} column (Material #8) is marketed as a Mixed-mode stationary phase for the separation of polar and non-polar molecules.[4] According to the manufacturer, the surface of the Luna Omega PS C_{18} adsorbent contains a positive charge which aids in the retention of acidic compounds through ionic interactions (IEX), while the C_{18} ligand promotes general reversed phase retention.[4] The Acquity

Peptide CSH C_{18} adsorbent (Material #4) and the Luna Omega PS C_{18} (Material #8) are modified C_{18} hydrophobic materials and are expected to have low adsorption energy. The results show that the C-Constant values for Materials #4 and #8 were 23 and 25 respectively. These C-Constant values are within the range (20-25) of typical values reported[20,21,24] for C_{18} materials. Since the Acquity Peptide CSH C_{18} column (Material #4) and the Luna Omega PS C_{18} column (Material #8) have ionizable functional groups that are dependent on the normal liquid chromatography environment such as the mobile phase, the ion-exchange properties of the adsorbent cannot be evaluated in the dried environment of an LTNA analysis. Only the reversed phase properties can be evaluated in this experiment.

Luna Omega Polar C_{18} column (Material #6) contains a C_{18} ligand with a proprietary polar modified particle surface that is designed for separation of polar and non-polar molecules, with enhanced retention of polar compounds.[4] The Luna Omega Polar C_{18} adsorbent material (Material #6), given its name, one may expect it to have a significant mix of both hydrophobic and hydrophilic characteristics, since according to the manufacture[4] its surface is modified with octadecyl ligands and proprietary polar groups. The results however show that the C-Constant value is low (24) which is indicative of the lower interaction energy characteristics of non-polar surfaces.[15,24] Reversed phase adsorbents have a high surface concentration (bonding density) of hydrophobic ligands. Depending on the homogeneity and bonding density, the hydrophobic ligand may exhibit shielding effects of residual silanols or other polar groups.[22] The adsorption results for the Luna Omega Polar C_{18} adsorbent material (Material #6), suggests that the C_{18} ligands may be effectively shielding the polar groups, resulting in the lower C-Constant values.

Overall, the results for the highly hydrophobic commercial materials all show C-Constant values in the range of 20 to 25, an average interaction energy of approximately 2.1 kJ/mol, and polar surface in the range of 6% to 11%.

4.3.1.3 Adsorbents with Intermediate Hydrophobicity (Materials # 3, 5, 7)

The interaction energy characteristics of three intermediate hydrophobicity column materials were studied in this experiment. The CSH Phenyl Hexyl column (Material #3) is a Trifunctional C_6 Phenyl, non-end-capped, and bonded to Charged Surface Hybrid BEH substrate designed for retention of non-polar analytes. The phenyl-hexyl phase offers a Mixed-mode separation mechanism in which the C_6 chain is responsible for hydrophobic interactions and the phenyl ring is responsible for π-π interactions.[2,27] The results show that the C-Constant value obtained (35) is within the range (31-40) of typical values reported[20] for Phenyl-type adsorbent materials. The average interaction energy of 2.3 kJ/mol corresponded to approximately 26% silica polar surface.

The Promix MP column (Material #5) is classified as a Type III Embedded Mixed-mode material which utilizes reversed phase (C_{12}) and ion-exclusion mechanism of interaction.[3,28] This type of Mixed-mode column has been used to explore the principles of both hydrophobic and hydrophilic interactions with additional electrostatic interactions and have also been studied in the development of HILIC and ion exchange modes for the separation of peptides and phosphopeptides.[29,30] Since this adsorbent material also contains ionizable functional groups that are dependent on the mobile phase composition, only the reversed phase properties can be evaluated. The C-Constant value obtained (33) is significantly higher than the typical C-Constant

values reported for reversed phase materials. No other information regarding the adsorbent properties and functional groups is provided by the manufacturer, therefore one can only speculate that embedded functional group adds some degree of polar surface characteristics to the Mixed-mode column. The average interaction energy of 2.23 kJ/mol corresponded to approximately 23% silica polar surface.

The Acclaim Mixed-mode HILIC-1 (Material #7) is marketed as a Mixed-mode column which utilizes HILIC and Reversed Phase mechanisms of interaction for the separation of polar and non-polar molecules. According to the manufacturer, the material consists of a hydrophobic alkyl chain with a diol group at the terminus. The hydrophobic moiety supplies reversed-phase retention and the terminal diol group facilitates hydrophilic interactions.[5] Given the HILIC and Reversed phase functional groups present, one may expect the material to have a distinct mix of both hydrophobic and hydrophilic characteristics. The results however show that the C-Constant value (29) for this material is closer to the C-Constant values reported for non-polar materials. The average interaction energy of 2.16 kJ/mol corresponded to approximately 18% silica polar surface. Overall, the results for the intermediate hydrophobicity commercial Mixed-mode materials all show C-Constant values in the range of 29 to 35, an average interaction energy of approximately 2.2 - 2.3 kJ/mol, and polar surface in the range of 18% to 26%.

4.4 Conclusions

In this study, a variety of commercial Mixed-mode columns were characterized on the basis of average interaction energy. The commercial Mixed-mode materials were analyzed by a commonly used non-chromatographic LTNA technique to generate adsorption isotherms. The BET method

of analysis was applied to obtain surface areas and the BET C-Constants of the commercial Mixed-mode materials. The average interaction energy was calculated for each commercial Mixed-mode material and the equivalent % silica polar surface was calculated according to the linear correlation established for the BET C-Constant energy scale that was created in Chapter 3. The equivalent percent silica polar surface for all of the commercial Mixed-mode materials that were analyzed fell in the range below 30% of silica surface on the interaction energy scale.

Results suggested that the BET C-Constant may be a viable parameter that can be used to characterize Mixed-mode materials based on interaction energy. The results from this chapter demonstrated that the interaction energy scale may be used as a universal indexing system to characterize a wide array of adsorbents, including Mixed-mode materials on the basis of interaction energy. This new universal indexing system may assist the end-user in Mixed-mode column selection and classification. The interaction energy scale will allow for the evaluation of the relative behavior of hydrophobic non-ionic analytes, and offer a basis for selection of starting chromatographic conditions for method development.

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OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Overall Conclusions

This research project has shown that the newly created BET C-Constant energy scale can be used to characterize commercial Mixed-mode materials based on average interaction energy.

A. A BET C-Constant energy scale was created by mixing various ratios of well characterized C_{18} and Silica materials in order to simulate Mixed-mode materials of varying surface polarity. The non-chromatographic LTNA technique was used to generate adsorption isotherms and BET analysis was applied to obtain surface areas and the BET C-Constants of the Silica, C_{18}, and blended samples.

B. The BET C-Constants were evaluated and a linear correlation was established between the BET C-Constant and the % silica surface in the blended samples. The average interaction energy was then calculated from the C-Constant and the new interaction energy scale was created by plotting the average interaction energy vs % silica surface. Results confirmed that the BET C-Constant may be a viable parameter that can be used to characterize Mixed-mode materials based on interaction energy.

C. A BET C-Constant energy scale was created using Axia Luna C_{18}/Silica mixtures and was used as a universal indexation system to characterize commercially available Mixed-mode column materials based on interaction energy. The same LTNA technique was used to generate adsorption isotherms and BET analysis was then applied to obtain the BET C-Constants of the commercial Mixed-mode materials. The average interaction energy was calculated from the C-Constant and the equivalent % silica surface was determined.

D. We demonstrated that the interaction energy scale can be used to rank a variety of commercial Mixed-mode columns according to their degree of surface polarity and as way to evaluate an adsorbent's degree of hydrophobicity or hydrophilicity. Since the BET C-Constant represents the average interaction energy present in the material, its value may indicate the magnitude of the adsorbent and adsorbate interactions. This indexing system based on interaction energy may promote the understanding of retention mechanisms on Mixed-mode adsorbents and may be able to assist the end-user in column selection and classification during method development.

E. The evaluation of adsorbent surface properties using the interaction energy scale does not allow us to directly predict its possible specific interactions with any analyte, since the HPLC system, eluent composition, pH, salt type and concentration will specifically affect the analyte and its surface properties and thus analyte retention.

F. The evaluation of adsorbent surface properties using the interaction energy scale may however allow for the evaluation of the relative behavior of hydrophobic non-ionic analytes, offering a basis for the selection of starting chromatographic conditions for method development. More and more novel Mixed-mode stationary phases are expected to become commercially available, therefore a better understanding of the underlying retention mechanism behind Mixed-mode materials is needed.

G. Using this interaction energy scale in conjunction with the study of retention behaviors on Mixed-mode material could improve the understanding of method development on Mixed-mode columns and allow the comparison of commercial Mixed-mode columns.

5.2 Future Directions

As new applications of Mixed-mode chromatography continue to emerge, so will the creation of Mixed-mode columns with novel stationary phases. The interaction energy scale can be used as a universal indexing system in the characterization of a wide array of adsorbents. In this research project, only a small selection of Mixed-mode columns were evaluated. This research can be expanded to explore the interaction energy characteristics of a wider variety of Mixed-mode columns with novel stationary phases. This study will allow for a wider comparison of Mixed-mode adsorbents and aid in column characterization and selection.

Because retention behavior is specifically influcenced by HPLC parameters such as eluent composition, concentration, pH, and analyte properties, the interaction energy scale cannot be used to directly predict analyte retention behavior. This research can be further explored to see if a correlation can be made between interaction energy characteristics of Mixed-mode materials and chromatographic behavior of analytes on Mixed-mode columns. By using this interaction energy scale together with understanding chromatographic of retention behaviors on Mixed-mode material, perhaps we may be able to predict retention behavior of an analyte on Mixed-mode columns.