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CHROMATOGRAPHIC SILANOL ACTIVITY TESTS: THE DEVELOPMENT OF A
COMPREHENSIVE TEST PROCEDURE

By

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I would like to dedicate this dissertation to my parents, Kenneth and Mary Rogers. All that I am and all that I will ever be are due to their constant love and support. In my high school yearbook they gave me a challenge: “Do not go where the path may lead, go instead where there is no path and leave a trail.” I hope that I have blazed that trail for future generations.

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ABSTRACT

There is great interest in the analysis of stationary phases used in reversed-phase liquid chromatography, with a strong emphasis on the evaluation of residual silanols. A number of techniques have been developed in order to assess the level of silanol activity in reversed-phased columns; however, to date there is no universally accepted test procedure. The overall goal of this work was to develop such a test. The invention of a universally accepted test would allow for a classification system for reversed-phase columns that would enable researchers to select columns more appropriately.

The project consisted of two phases. In phase I of this study, several different silanol activity tests were compared. Test procedures were performed on a large collection of columns, including some of the most popular C₁₈ phases used for the analysis of basic solutes. Column rankings, ranging from a low silanol activity to a high silanol activity, were determined according to the specifications of each test. Column rankings varied significantly among tests, suggesting that the current chromatographic silanol activity tests are not all testing for the same property. A number of unresolved issues from the literature were also addressed in this study. It was revealed that the older test procedures were inept at distinguishing between newer generation phases. Newer test procedures were shown to be more in agreement with each other than older test procedures. Test procedures were examined as candidates for a comprehensive procedure; however, none of the current tests were found to include all of the necessary components. Results indicated the need for a new approach in the development of a universal test procedure.

In Phase II of this study, an attempt has been made to develop a universally accepted chromatographic silanol activity test procedure. This procedure analyzed all of the major interactions which comprise the term 'silanol activity', namely hydrogen bonding and ionic interactions. Aromatic compounds with nitrogen groups were selected as test solutes. These compounds had pK_a values between 4 and 11. The new procedure took into account the pK_a shifts that occur upon the addition of organic modifier. This test analyzed the basic compounds at mobile phase pH values ranging from 2.5 to 7.5 by utilizing a single mobile phase system which can be used in a pH gradient. This approach allowed for the analysis of the columns performance at all possible pH ranges for nondestructive use of the column. Results from the isocratic portion of this procedure illustrated the ability of the procedure to predict silanol activity at different pH levels when compared to results from everyday analyses. This work further demonstrated the need for testing columns over their entire useful pH range. An attempt was made to shorten the procedure by using the mobile phase system to create a pH gradient. Although analysis times were decreased greatly, the ability of the test to predict a column's performance according to silanol activity was also diminished. Overall, gradient experiments provided solid foundational investigations into a radically different approach to silanol activity assessment.

CHAPTER 1

INTRODUCTION

Reversed-Phase Chromatography

Chromatography is an analytical method in which chemical components in complex mixtures are separated and identified. Russian botanist Mikhail Tswett invented this technique shortly after the turn of the century [1]. Although this term can be applied to a number of diverse techniques, all chromatographic methods use both a stationary phase and a mobile phase. The mobile phase carries chemical compounds through the stationary phase using the flow of a gaseous or liquid fluid. In chromatography, the separation is based on differences in migration rates between sample components as they repetitively interact with the stationary phase on their way through the column.

Early liquid chromatography used glass columns with inner diameters of 10 to 50 mm that were packed with 50 to 500 μm solid particles. During the late 1960's, the technology for producing columns with particle diameters of 5 to 10 μm was developed in conjunction with the sophisticated instrumentation required to use these columns. It is this technology that distinguishes high-performance liquid chromatography (HPLC) from liquid chromatography. HPLC uses a liquid mobile phase and stainless steel columns (5 to 30 cm long with inner diameters of 2 to 10 mm) packed with 3 to 5 μm particles as the stationary phase. Today, the vast majority of separations for biomedical, pharmaceutical, and environmental analyses are performed using HPLC. HPLC gained its position as the most important chromatographic technique after the introduction of reversed-phase

columns by Kirkland in 1971 [2]. These materials became available commercially in 1974.

The most widely used method of HPLC is reversed-phase liquid chromatography (RPLC). In RPLC, the separation mechanism is dependent upon interactions between analyte components, the mobile phase, and the surface of the stationary phase. Stationary phases utilized for RPLC consist of nonpolar, hydrophobic alkyl chains attached to the surface of the silica support by siloxane bonds. The synthesis of these chemically bonded silica supports can be performed by either surface modification, where there is a reaction between porous silica and a reactive organosilane, or by forming a bond during the formation of the porous support [3]. There are two main types of silica particles most often used in reversed-phase packing materials. SilGel particles are made by gelling soluble silicates. They are characterized by higher surface areas, higher porosities, and irregular pore shapes with variable wall thickness. SolGel silicas are made by aggregating silica and they have lower surface areas, lower porosities, and thicker walls [4]. Both types of particles are readily used in reversed-phase columns; however, it has been shown that SolGel silicas produce columns which are more durable [5]. The silica particles used in packings today are generally spherical, with particle sizes between 1.5 and 10 μm and pore sizes between 60 and 400 \AA .

In spite of recent advances in the investigation of alternative packings such as zirconia, alumina, titania, and polymer-based packings, microparticulate silica continues to be the most commonly used packing material for reversed-phase columns [6]. Polymer-based packings are applicable in the pH range from 1 to 13 and they have strong hydrophobic retention. Alumina and zirconia phases also have an extended pH range. However the disadvantages of these packings far outweighs the advantages. Polymer-based columns have lower column efficiencies and the support often swells differently in the presence of different organic modifiers. Alumina-based columns are not commercially available with a large range of functionalities on the surface of the stationary phase. In addition to that, the columns cannot be used with carboxylic acids due to irreversible binding. Some columns have been acknowledged to be very active

towards basic compounds. Zirconia-based columns have been known to bind with hard Lewis acids, carboxylic acids, and sulfonic acids.

The silica surface remains the most dominant packing due to its versatility, high column efficiency, mechanical stability, and easily controlled particle size and porosity. Porous particles can be prepared with a narrow pore-size distribution and with a wide choice of pore sizes and particle sizes. This allows for the availability of packings for both small and large molecules and for both analytical and preparative applications [7]. The mechanical strength of silica allows for the use of the material at high pressures, which gives faster mass transfer conditions. The silica surface contains both silanols (Si-OH) and siloxanes (Si-O-Si). Siloxanes are hydrophobic and have very little to do with solute retention [8]. Silanol groups are considered to be the strong adsorption sites and are hydrophilic in nature. These groups allow for the easy modification of silica surface.

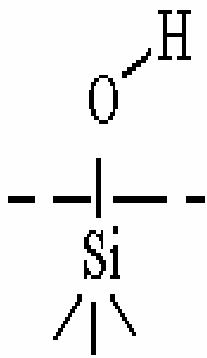
Although superior to the other available packings, silica is by no means an “ideal” support for reversed-phase LC. Silica-based stationary phases are limited to operating within certain pH limits. Below a pH of 2 siloxane bonds begin to hydrolyze and above a pH of 8 silica particles begin to dissolve. This phenomenon is intensified in highly aqueous mobile phases, at elevated temperatures, and when aggressive mobile phase buffers are used. In addition, porous silica particles are synthesized by a variety of methods which leads to differences in surface area, purity, and pore-size distribution, hence causing differences in chromatographic results. Another problem hampering the performance of reversed-phase columns is the presence of unreacted silanol groups. Hydrated silica surfaces have a layer of silanol groups which can be removed by full hydroxylation to give a maximum surface concentration of about $8 \mu\text{mol}/\text{m}^2$ [6]. However, due to steric hindrance, a problem arises when the maximum concentration of C_8 or C_{18} ligands is less than the density of the silanols found on chromatographic grade silica [9]. This results in residual silanols within the bonded phase. The unreacted silanol groups are weakly acidic, and their presence causes difficulty when analyzing basic compounds such as those found in biomedical, pharmaceutical, and environmental compounds.

Similarly, the presence of metals in contaminated silicas also causes silanols to become highly acidic due to the activation of surface silanol groups. Silica purity can be hindered by the contamination of the silica with certain metals such as iron, zinc, nickel, and aluminum. Metal impurities are found to be present at 0.1 to 0.3% for chromatographic grade silica [10]. These compounds can also complex with chelating solutes.

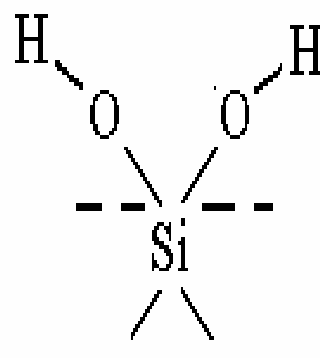
Surface Silanols

There are three types of silanols found on the surface of amorphous silica with porous structures: geminal, vicinal, and isolated (Figure 1-1). The identification and concentration of the different types of silanols can be determined by a number of spectroscopic techniques such as ^{29}Si cross-polarization magic-angle-spinning NMR (^{29}Si -CP-MAS NMR), proton-spin-counting solid-state NMR, and diffuse-reflectance infrared Fourier-transform spectroscopy (DRIFT) [6]. The three types of silanols have different adsorption activities and their relative distribution greatly affects the characteristics of reversed-phase columns. A number of studies have been performed in order to determine which silanol group dominates as the primary reaction and adsorption site, yet no definitive answer has been provided [11-16]. Some argue that the vicinal silanols are stronger due to an increased acidity of the proton not engaged in hydrogen bonding. However, this is disputed by the fact that vicinal silanols would rather form linear or two-dimensional structures than pairs [6]. Others point to NMR or IR data which indicate that isolated silanols are the main culprit of problems associated with unreacted silanols.

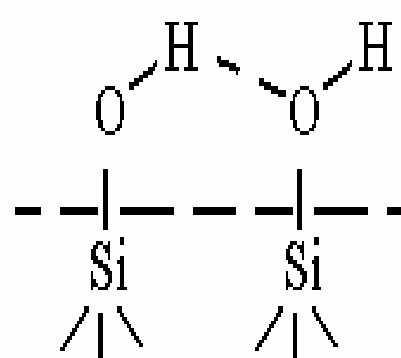
A debate also rages concerning the pK_a value of silanol groups. The average pK_a value is generally accepted to be 7.1 ± 0.5 , however values have been reported in the literature from 1.5 to 10 [10]. This range can be attributed to different manufacturing methods which lead to various impurity levels. A recent investigation by Neue and co-workers examine the acidity of silanols in C_{18} bonded and underivatized silica columns [17]. They found that various commercial columns were found to have very different pK_a values for their silanols. For example, the Resolve C_{18} column was found



Isolated



Geminal

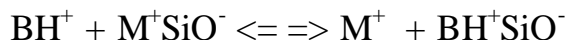


Vicinal

Figure 1-1. Various Types of Silanols

to have two types of silanols, one that is very acidic, with a pK_a between 3.4 and 4.6, and one that is not as acidic, with a pK_a between 6.2 and 6.8. However, residual silanols were not detected for the Symmetry C₁₈ and Xterra C₁₈ packings within the 3 to 7 pH range. This study further proved that the pK_a of residual silanols is heavily dependent on differences in the manufacturing steps and varies greatly from one column to the next.

Despite the fact that a fully hydroxylated silica surface generally has a silanol concentration of approximately 8 $\mu\text{mols}/\text{m}^2$, the maximum coverage of bonded ligand is never more than 4 – 4.5 $\mu\text{mols}/\text{m}^2$ [18]. This results in a number of unreacted silanols on the surface of the column with various activities and pK_a values. Basic compounds interact with residual silanols by ionic interactions together with hydrophobic interactions. This is demonstrated by the equation below, where BH^+ is a protonated base and M^+ is the mobile phase buffer cation.



A number of different interactions take place between residual silanols and solutes such as ion-ion, ion-dipole, dipole-dipole, dipole-induced dipole, and induced dipole-induced dipole [19]. The two most important interactions are the ion-ion (ion-exchange) interactions and the dipole-dipole (hydrogen bonding) interactions [20]. Hydrogen bonding interactions will occur when the silanols are still protonated and no ion-exchange is available. Once the silanols are deprotonated, ion-exchange interactions dominate. It has been proved that these secondary interactions are the cause of many of the problems encountered by chromatographers when analyzing basic compounds [6,21]. When analyzing basic substances using RPLC, chromatographers may observe asymmetric peaks, low column efficiency, poor reproducibility and retention times which are larger than expected. This can be a vast problem for chromatographers in the pharmaceutical industry where asymmetry factors of less than 1.5 are needed in order to perform quantitative analysis [22].

Stationary Phase Improvements

The problem of residual silanols has been attacked by both manufacturers and chromatographers on a number of different fronts. Various approaches have been used to minimize silanol activity, with varying degrees of success. Manufacturers have worked to improve the underlying silica by reducing metal impurities and thermal pretreatment procedures. They have also employed a number of different bonding technologies. Chromatographers have endeavored to manipulate experimental conditions in order to avoid problems associated with residual silanols. They have also developed a number of characterization tests to analyze the various reversed-phase columns. These tests are designed to determine the “silanol activity” of the various packings. Silanol activity can be defined as the ability of the most reactive silanols to affect the quality of a separation. This term is not merely a reflection of the number of silanols on the surface of the column because not all silanols adversely affect the outcome of a separation.

As previously stated, metal impurities contribute greatly to peak tailing by chelating with various solutes and by increasing the acidity, and thus activity, of residual silanols on the surface of the column. Different metal species make different contributions to the acidity of the different silanol sites on the surface of the packing. While some metals, such as aluminum and titanium, generate high levels of acidity, other metals, such as sodium, appear to have the opposite effect. Metal impurities can also influence the chemical modification process, therefore altering the chromatographic performance of the column, when metal impurities serve as adsorption sites themselves. Column manufacturers have greatly improved the purity of the silica used in today's columns by removing many of these metal impurities via acid wash techniques. This step is usually performed before the preparation of the alkyl modified stationary phases. Acids commonly used for this procedure include sulfuric acid, nitric acid, and hydrochloric acid. Acid treatment will generally result in the removal of two thirds of the metals from the silica [4]. Silica with this reduced metal content is commercially referred to as “high purity silica”.

Acid washing does not totally solve the problem of residual silanols. In fact, it is highly unlikely that all impurities will be removed from the silica because many impurities are buried within the matrix of the particles making them inaccessible. In addition to this, the efficiency of the removal of the metal impurities depends on the surface area and the wall thickness of the silica support. It was found that the efficiency increased as the surface area increased and as the wall thickness decreased [10]. Some acids are more effective at removing certain metals but they do not effectively remove others. For example, both hydrochloric acid and sulfuric acid remove 90% of iron contaminants while removing very little aluminum or titanium. Despite the drawbacks, almost all of the reversed-phase columns manufactured today use purified silica in their stationary phases.

Chromatographic stationary phase manufacturers have also employed thermal pretreatment steps. Due to the fact that the relative distribution of the different types of silanols greatly affects the characteristics of the stationary phase more than the absolute number of silanols, it is helpful to try to control which silanols are abundantly present on the surface of the column. Methods used to achieve this goal are varied depending on which types of silanols are thought to contribute most to derivitization and adsorption. Silanols present on the silica surface can be thermally removed by forming siloxane bonds and releasing water molecules. Above 200°C all physically adsorbed water is removed. The water on the surface plays an important role in bridging any gaps between adjacent silanols on the silica surface. Further heating (200 – 400°C), results in dehydroxylation of the bonded silanols. Above a temperature of 400°C the number of isolated silanols decreases. The process of rehydroxylation can then be used to increase the number of bonded silanols on the silica surface. If the goal is to produce an abundance of free silanols, the surface should be partially dehydroxylated to remove the associated silanols. Because of the reduction in steric hindrance, the remaining free silanols can then be nearly completely derivatized by high-temperature silylation. Despite the advantage of having a more homogenous surface through the hydroxylation/rehydroxylation process, bonding densities higher than about 4 – 4.5 $\mu\text{mols}/\text{m}^2$ have not been reported.

In addition to the various pretreatment procedures, manufacturers have also designed a number of different bonding technologies over the past 10 years. The overall goal is to minimize the interaction between basic analytes and residual silanols. One of the first procedures which attempted to reduce the effects of residual silanols was endcapping (Figure 1-2). In this procedure relatively small reagents are used to react with the residual silanols found on the surface of the column. Usually short alkylsilanes, such as trimethylchlorosilane (TCMS) and hexamethyldisilazane (HMDS), are used as end-capping reagents. It was found that HMDS was a more suitable endcapping reagent for the analysis of bases than TCMS [4]. End-capping was found to be fairly proficient at improving chromatographic results for columns when analyzing basic substances. In fact, many deactivated supports available today employ a double end-capping treatment [3].

Polymer encapsulated columns use a thin layer of organic silicone polymer to coat the silica surface. The coating process solves the problem of residual silanols by providing an inert surface free from residual silanols and metal impurities. This type of column also provides a pH stability range up to a pH of 10. Positive results were demonstrated by giving good peak profiles for pyridine; however, after NMR studies were conducted, it was found that residual silanols were still present on the surface of the column [4].

Horizontal Polymerization involves the mixing of long and short chain trifunctional silanes to produce a high-density stationary phase (Figure 1-3). These columns can be used within a pH range of about 2-10. This type of column relies on the notion that there is significant Si-O-Si bridging parallel to the silica substrate, thus creating a dense, two-dimensional cross-linked network over the chromatographic silica surface. This effectively hinders the residual silanols. This same concept has been extended to the use of C₁ and C₁₈ mixed stationary phases [3]. These surface modified stationary phases have CH₃ groups in place of OH groups in the silica backbone. For ordinary phases, the ratio between OH and alkyl chains is 1:1. However, for these phases the ratio is now 1/3 OH, 1/3 alkyl, and 1/3 CH₃. An additional advantage of these phases is that they have an extended pH range of 1-12.

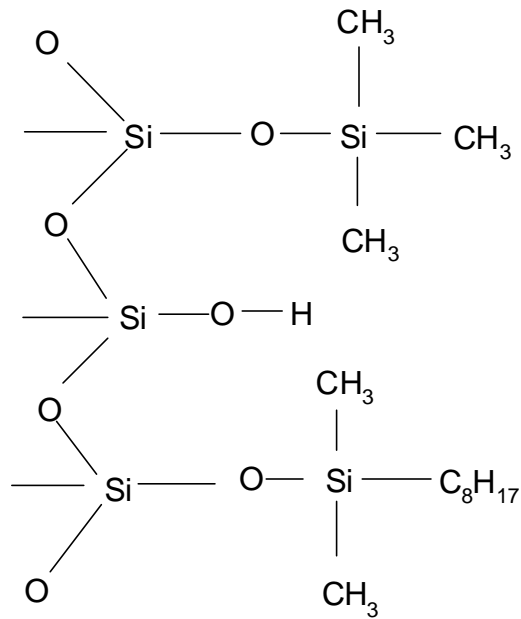


Figure 1-2. Schematic of Endcapped C₈ Stationary Phase.

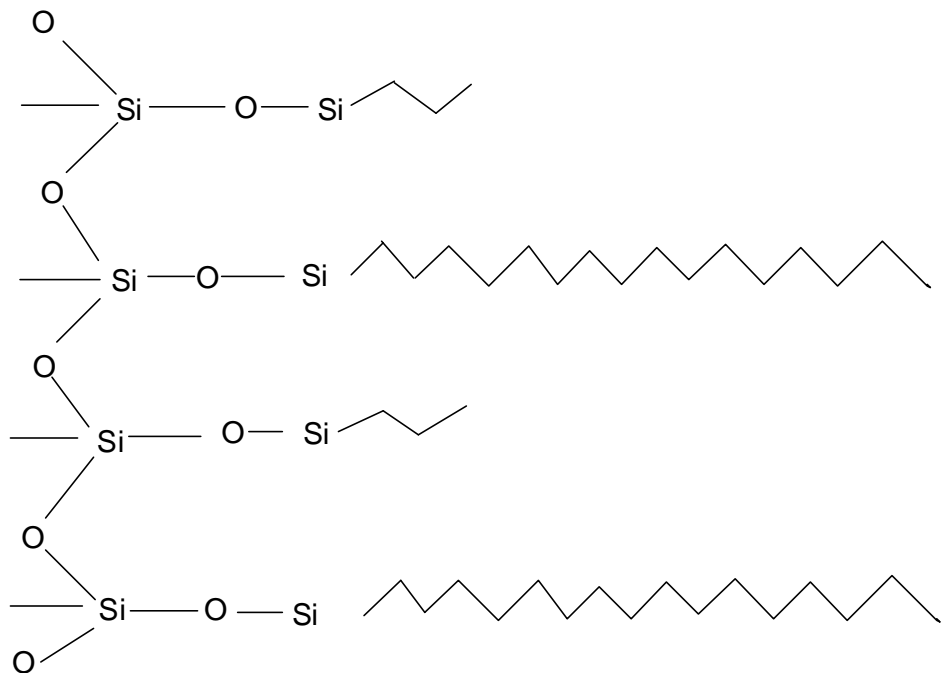


Figure 1-3. Schematic of Mixed Stationary Phase Technology.

Reversed-phase columns have been designed to prevent access to residual silanols both electrostatically and sterically. Sterically protected silica is formed by reaction of a sterically encumbered monochlorosilane (Figure 1-4). These groups are used to stabilize both long and short chain monofunctional ligands. Bulky diisobutyl or diisopropyl side chains also provide protection from hydrolysis and loss at low pHs. For electrostatic shielding, polar groups, amides, or esters are embedded into the backbone of the column (Figure 1-5). These groups act as a shield for the silanols from highly polar analytes by direct interaction with solutes through hydrogen bonding, by competing with the analyte for interaction with the silanols, and by stabilizing the water in the surface layer of the embedded polar group. The columns have an added advantage; they can be used with eluents containing large amounts of water. Bidentate stationary phases also restrict access to residual silanols, while providing stability at high and low mobile phase pHs. These columns incorporate a bidentate alkyl chain on the surface of the packing.

Recently, a number of hybrid supports have been introduced [3]. This technology incorporates a mixture of organic and inorganic groups in the optimal balance. Columns with high inorganic material, like silica, will provide residual silanol groups and inadequate stability with aggressive buffers. Columns which have a high organic content will exhibit low mechanical strength and efficiency. The correct mixture of the two provides increased surface coverage and an increased pH stability range of 1-12.

Despite all of the advances in column technology, there is not an ideal stationary phase. In addition to this, chromatographers now have the daunting task of selecting the best column for a particular separation from the more than 600 available phases that are now available on the market [23].

Optional Experimental Conditions

Chromatographers have tried to eliminate the problems associated with residual silanols by adjusting different experimental conditions. One of the options that are available is to work at an optimum mobile phase pH. This would occur at a point where

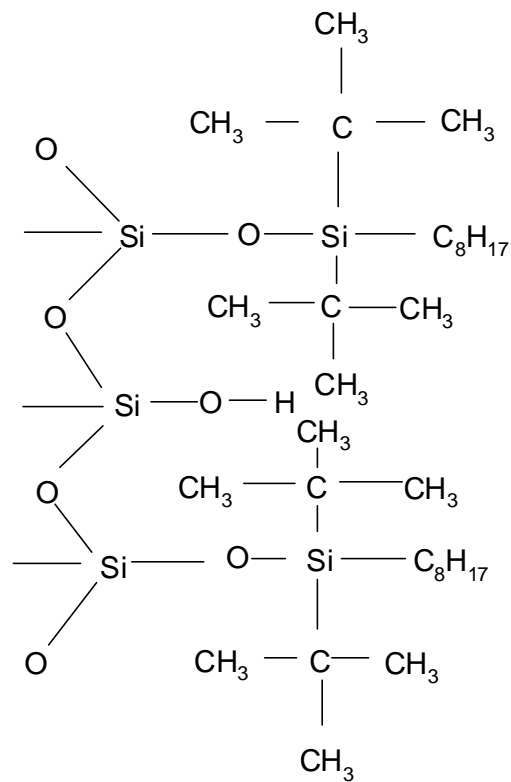


Figure 1-4. Schematic of Sterically Protected Column Technology.

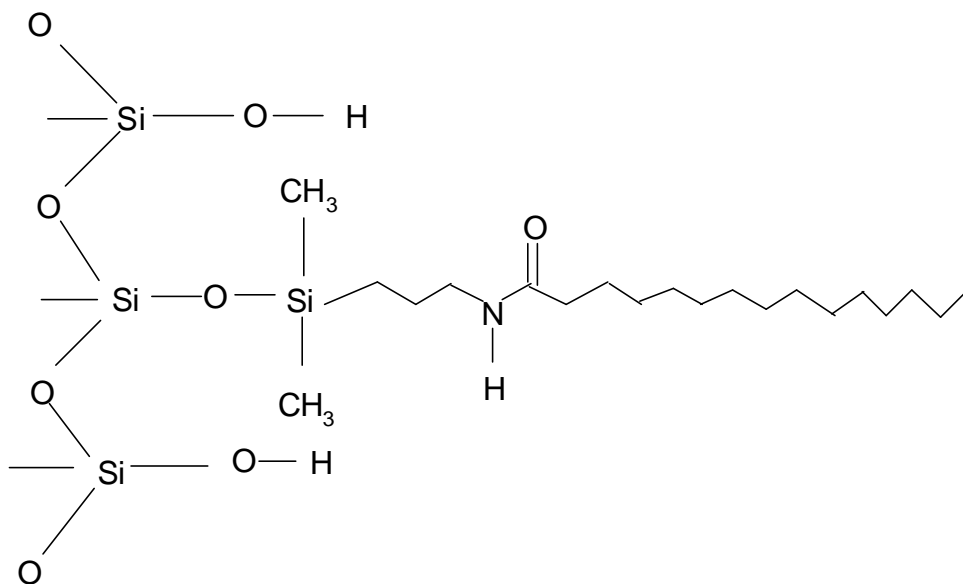


Figure 1-5. Schematic of Polar Embedded Stationary Phases.

ion-exchange interactions between silanols and analytes are minimized. Either the silanols must be uncharged or the basic analytes must be uncharged. Due to the pH limitations of silica, this can be a difficult task. At a low pH, basic analytes are still protonated but residual silanols should still be uncharged. Most silanols are believed to be protonated at or below a pH of 3. Working at a pH well above the pK_a value of the compound you are analyzing is theoretically also a good idea. However, many of the problematic basic compounds have high pK_a 's (above 8). Silica-based columns do not provide stability at pHs high enough to allow operation above the pK_a value of these compounds.

Another option includes the use of amine modifiers to block residual silanols. The more basic compounds, such as triethylamine, will interact more strongly with the silanol groups, thus allowing the less basic solutes to interact solely with the alkyl ligands in the stationary phase. Again, while this approach theoretically shows some promise, it does not hold up experimentally. It has been found that amine modifiers fail to show improved peak shapes for some compounds [18]. In addition, column equilibration is slow and many modifiers are difficult to remove from the stationary phases when they are able to be removed at all. Some modifiers are aggressive towards the stationary phase and in some cases the organic modifiers may interact with the solutes to be analyzed.

Another idea which is comparable to the addition of organic modifiers is to increase the ionic strength of the mobile phase. This provides a competing ion for the residual silanols to bind. This particular method is limited by instrumentation. Buffer concentrations higher than 0.1M may cause problems with pump maintenance and buffer precipitation. Instead of increasing the ionic strength, the buffer cation can be exchanged with one that exchanges more strongly with residual silanols. Sodium ions could be replaced with potassium or rubidium ions. Sample concentrations may also be altered to alleviate saturation of acidic silanol sites.

Stationary Phase Characterization

The introduction of the vast number of stationary phases available for reversed-phase analysis led to the development of characterization methods by many chromatographers. There have been a number of publications dedicated to the research done on evaluation methods [20,24-28]. In fact, the understanding of the properties of commercial columns is of great interest to both practitioners of HPLC and theoreticians interested in retention mechanisms. Theoreticians are interested in knowing the separation mechanism, which is dependent upon the properties of the packings and the analytes. Chromatographers are interested in knowing the similarities and differences between different packings. This aids in the column selection process during method development and reproduction of previous methods. Some scientists do not recognize the fact that nominally identical materials may show very different chromatographic properties. Technical information provided by manufacturers proves to be insufficient for the column selection process and many manufacturers test their columns using protocols that are designed to be beneficial to their product, thus preventing chromatographers from comparing the various product claims. This leaves users to rely on previous experience and intuition when choosing an appropriate column.

The presently available evaluation methods for RPLC columns can be divided into the following subdivisions:

- Determination of physico-chemical properties of the stationary phase
- Spectroscopic techniques
- Thermodynamic measurements
- Evaluation of chromatographic data using statistical methods
- Chromatographic test procedures

Physico-chemical properties includes items such as carbon content, particle size and shape, specific surface area, pore size and porosity, and particle strength. While all of these properties provide very useful information about the column, they do not provide enough information to predict how columns will behave towards compounds under various mobile phase conditions. In addition, these procedures are not easy to perform and cannot be carried out without destroying the column.

Spectroscopic techniques provide invaluable information about the different types of silanols on the surface of the silica. Infrared (IR) spectroscopy data can provide specific information about the occurrence of free and geminal or vicinal silanols in the silica substrate and on the bonded phase. This method is also very useful for the study of reactions and kinetic studies of RPLC columns. ^{29}Si and ^{13}C NMR techniques also give detailed information on the various groups present on the surface of the column. These techniques are superior to IR because they can readily distinguish between free and geminal silanols. In IR these groups absorb at almost the same wavenumber [6]. NMR techniques can also provide information about the alkyl chain and the nature of bonding to the surface.

Thermodynamic measurements involve the construction of Van't Hoff plots. From these plots, enthalpy and entropy contributions to chromatographic retention and/or selectivity can be derived. As these measurements are carried over a wide temperature range, it can be deduced whether or not the retention mechanism changes over the temperature range analyzed.

The evaluation of chromatographic data using statistical analysis involves the application of test methods on a large number of potential columns. This type of evaluation is most useful for determining a suitable column for a specific application. After gathering all of the data from the various columns, chemometric techniques such as principal component analysis or factor analysis is used to determine clusters of similar or dissimilar columns. This process can help to reduce the number of potential column candidates for a particular analysis.

Despite the potential of the previous test methods, chromatographic test procedures (without additional statistical applications) remain the most effective way to analyze reversed-phase columns. Subtle yet decisive differences in the chromatographic properties of RPLC columns can only be detected using these methods. What may seem to be minor difference in the properties of two different columns can mean success or failure in a separation method. These methods are nondestructive and they allow for columns to be tested in environments similar to those in which they will normally operate.

Most chromatographic test procedures focus on one particular property of the column. Properties such as hydrophobicity, silanol activity, shape selectivity, and metal impurity level can all be characterized using this type of method. Several papers have been published proposing different chromatographic tests for the characterization of commercial columns [19,20,28-33]. To this date, not a single procedure has been widely accepted [34]. For the purposes of the work conducted here, we will focus solely on chromatographic silanol activity test procedures.

Silanol Activity Test Procedures

Silanol activity tests are designed to give chromatographers a means by which they can quickly and easily characterize columns according to their silanol activity. Silanol activity can be defined as the ability of the most reactive silanols to affect the quality of a separation. The various procedures in the literature vary in terms of their specific design; however, they all include the same essential components. Compounds used in test mixtures usually include at least one basic substance with a pK_a value between 5 and 10. In some cases neutral and acidic compounds are included in the test mixture as well. The performance of a column can be strongly affected by the choice of test solutes; therefore, great care is taken in the selection of these items. While weakly retained compounds will sometimes show unrealistically favorable results, high pK_a compounds with steric hindrance around the nitrogen atom may show the worst interpretation of a column's performance. A variety of different mobile phases with different organic modifiers have been used as the test eluent. Some of the test procedures

utilize a buffered mobile phase, yet some procedures do not. Test procedures which do include the use of a buffered mobile phase will choose a buffer at pHs at or below 3 and/or at or above 7. This allows for the assessment of contributions from hydrogen bonding and the maximum contribution from ionic interactions. One of the major differences between the current tests lies in the choice for the silanol activity indicator. This is the property which is used to classify columns as having a high or low silanol activity. Important parameters for this assessment include asymmetry factors and tailing factors, efficiency, and capacity factors.

Currently there are a number of different tests which use a variety of the aforementioned options in their test procedure; however, all of these tests claim to be testing for the same property [28]. A number of tests have been proposed, but only a few particular studies have risen to prominence [19,29,35]. In the literature there are three studies which are mentioned most often; the Engelhardt test, the Tanaka test, and the McCalley studies [20,32,36].

The Engelhardt test was developed in an attempt to provide a universal test method which could be used to compare the various selectivities of reversed-phase stationary phases. The isocratic elution system consisted of either methanol/water (49:51,v/v) or methanol/1 mM phosphate buffer (pH 7); nevertheless, the unbuffered eluent was used most often. Engelhardt suggests that the influence on retention when using a buffer should not be noticeable; however, he found this to be untrue in his study. It should be noted that the use of mobile phase without the buffer restricts the range of compounds that can be used based on their pK_a values. In most cases, buffered eluents are used in routine analysis in industry, and the use of unbuffered mobile phases would produce an environment which is dissimilar to that in which the column would be used for analysis.

The Engelhardt test mixture contains toluene and ethylbenzene, which are used to monitor the hydrophobic properties; aniline, toluidine isomers, and N, N-dimethylaniline, which were chosen to monitor silanophilic interactions; and phenol and benzoic acid

ethyl ester to check for polar interactions. The toluidine isomers are interchangeable with phenylaniline isomers. The compounds within each set of isomers are dissimilar only by their pK_a values; this was presumed to show that separation was based on silanophilic interactions only, with no contribution from hydrophobic interaction.

Silanophilic interactions are determined by the asymmetry value and elution order of the basic solutes, aniline and phenol. It was found that silanophilic interactions can be deemed as negligible when aniline elutes before phenol. Interactions are also considered negligible when the isomers are not separated and elute with symmetric peaks. It was also proposed that the asymmetry value at 10% of the p-ethyl aniline can be used to characterize silanophilic properties. Three classes of columns were established according to this classification: columns which had asymmetry values less than two, those with asymmetry values between two and four, and those which had values greater than four.

When evaluating the columns for residual silanol effects the peak asymmetry of the basic samples was found to reveal the largest differences. It was suggested that the relative retention values of basic samples should be consistent regardless of whether or not the eluent contained buffer. It was noted that the application of buffers and neutral salts disguised retention contributions and resulted in symmetrical peak shapes and reduced retention. Numerous studies were carried out evaluating the effects of mobile phase composition and temperature on retention. Optimum conditions were selected at 40 °C and mobile phase compounds were prepared by weight. According to Engelhardt et al. columns were classified as “good” if aniline eluted before phenol, the ratio of peak asymmetry values for aniline and phenol was less than 1.3, the isomers were hardly separated and the ratio of the k' values was less than 1.3, and N-N, dimethylaniline (DMA) eluted before toluene [37].

Kimata and co-workers developed a test procedure for the characterization of silica C_{18} packing material [20]. Solutes used in the test mixture were easily obtained if not already present in a well-equipped analytical lab. The isocratic gradient contained

either methanol/water (20:80) or methanol/buffer (40:60, pH 7.6 or 2.4). Hydrophobic properties were examined by alkyl benzenes with various alkyl groups. The shape selectivity was examined by triphenylene and ortho-terphenyl, while caffeine and theophylline were used to test the hydrogen bonding ability of the stationary phase. Alkyl amines with pK_a values greater than 9 were used to examine the contribution to the silica surface from exchange sites. The retention of the polar amines was normalized by the use of phenol and benzyl alcohol to conceal any differences in the hydrophobic properties of the columns. It was found that the retention of caffeine was a good measurement of the number of residual silanols. Separation factors were used to attempt to cancel the effect of hydrophobic interaction as much as possible. It was concluded that the procedure was sufficient for the estimation of the extent of trimethylsilylation for stationary phases. The method was deemed as suitable for the characterization of surface coverings, types of silanes, amounts of silanol present, and the amount of ion exchange sites.

McCalley performed a number of experiments designed to address many of the problems and possible solutions for evaluating reversed-phase packings [18,31,33,36,38-43] From his studies, he not only came up with a number of suggestions for the analysis of basic substances, but he also developed a silanol activity test in the process. From McCalley's studies it was concluded that:

- Sample amounts of 0.5 μg or less should be used at low pH
- Acetonitrile is the best organic modifier at a pH of 3.0.
- Methanol is the best organic modifier at a pH of 7.0.
- Potassium salts will give better results than sodium salts
- Buffer concentrations of 0.025 M or less should be used
- Acidic pH values should be used in the mobile phase for strong bases
- A pH of 7 should be used in the mobile phase for weak bases
- High pK_a bases will yield the worst asymmetry.
- Steric hindrance around the basic center reduces asymmetry.

The test procedure that McCalley developed used basic compounds which were generally available such as: pyridine, benzylamine, codeine, diphenhydramine, amphetamine, nicotine, quinine, and nortriptyline. Amphetamine and codeine are controlled drugs and special licensing must be used to obtain these compounds. These compounds were used to evaluate columns at both a pH of 7 and a pH of 3 using both methanol and acetonitrile as the organic modifier. Silanol activity was assessed using both asymmetry factors and theoretical plates.

Research Goals and Significance

Although several chromatographic tests have been proposed and several studies on the analysis of reversed-phase columns have been conducted, ultimately there is no universally accepted method of measurement available to determine the residual silanol activity toward solute molecules in reversed-phase LC [39]. In order for a single test to be accepted, a number of discrepancies have to be addressed. For example, whether or not to test in buffered or unbuffered mobile phases, at which pH to run, and to what degree do the organic modifiers affect the pK_a 's of the analyte and the silanols. Once these matters have been settled a more accurate method can be produced.

A test of this nature would be highly beneficial for chromatographers as well as for the biomedical and pharmaceutical industries. It would also aid column manufacturers in testing batch and column reproducibility. This type of test would allow for the classification of reversed-phase columns into similar groups. Currently a sufficient classification system is nonexistent. The United States Pharmacopoeia's column classification system groups all of the C_{18} packings commonly used in RPLC (3-10 μ m) into one category [44]. The development of such a test procedure would also enable researchers to select columns more appropriately and with greater confidence. The goal of this research is to perform a comprehensive comparison study of the current silanol activity tests and to develop a new approach to testing columns for silanol activity.

There has been very little investigation into the conformity between results from the various silanol activity tests. A study performed by Claessens and coworkers compared test procedures with respect to their ionic and hydrophobic properties [35]. In

this study, it was found that while the hydrophobic test procedures were in agreement, the ionic procedures were not. Vervoort and colleagues published a study that looked at three of the same tests used in the Claessens study, as well as the procedure that was developed by McCalley [19]. In that study, only the ionic properties were evaluated and results were compared to chromatographic data obtained from a pharmaceutical sample. Both of the previous studies showed a lack of agreement in results from silanol activity test procedures; however, a number of key issues were left unanswered. In this study the objective is to provide a more comprehensive comparison of the literature tests and address the unrequited issues. In addition, this study includes an examination into the feasibility of one of the current tests as a universally used chromatographic silanol activity test procedure.

An attempt to develop a universally accepted, comprehensive silanol activity test procedure has also been made. In this attempt, the gaps left by previous tests have been filled in order to design a test procedure that can accurately predict silanol activity at all pH ranges for which reversed-phase columns can be used. The changes that occur to pH and pK_a values upon the addition of organic modifier to the mobile phase have also been examined. In addition, the foundation for a totally different approach to reversed-phase column analysis has been set.

Chapter 2

Experimental

This chapter describes the general experimental information for the entire body of work. Brief experimental sections will be included for each subsequent chapter to describe detailed material which pertains solely to that particular study.

Apparatus

Chromatographic measurements were performed on a Shimadzu LC-10ADVP liquid chromatograph system (Kyoto, Japan) and a Spectra Physics SP8800 (Mountain View, CA) liquid chromatograph system. A Waters 490 Programmable Multiwavelength Detector (Milford, MA) and an Applied Biosystems 757 Absorbance Detector (Foster City, CA) were used for detection. Sample injections were performed using a Valco (Houston, TX) injection valve fitted with a 10 μ L sample loop. Column temperatures were controlled using a Fisher Scientific (Fair Lawn, NJ) Isotemp Refrigerated Circulator Model 901. Chromatograms were collected using Turbochrom Chromatographic Workstation Version 4.0. Figure 2-1 shows a typical LC set-up. The pH was monitored constantly for experiments performed in Chapters 4 and 5 using a Sensorex (Garden Grove, CA) 200 μ L internal volume flow cell. The cell, made of acrylic, was supplied complete with 1/16" inlet and outlet fittings. The cell used a Sensorex 450C Flat Surface Combination pH/Reference Electrode (Figure 2-2). The entire set-up was attached to the LC system after the detector.

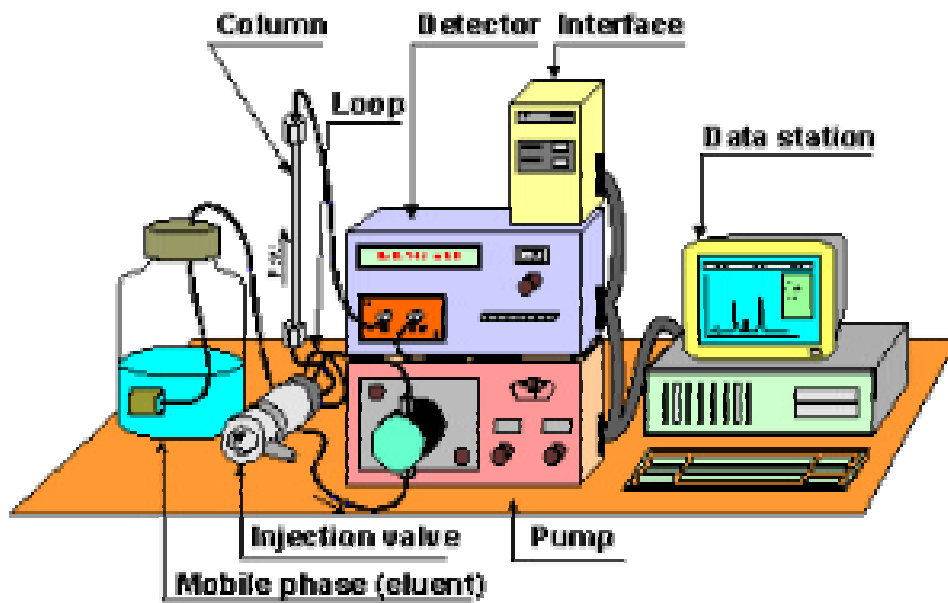


Figure 2-1. Complete LC System.

Adapted from http://hplc.chem.shu.edu/NEW/HPLC_Book/index.html



Figure 2-2. Sensorex HPLC Flow Cell and pH Electrode.

Chemicals and columns

Test solutes purchased from Fisher Scientific Co. (Fair Lawn, NJ) include: thiourea, toluene, aniline, phenol, benzylamine, ethylbenzoate, 4-bromoaniline, dibasic anhydrous potassium phosphate, anhydrous citric acid, phosphoric acid, sodium acetate, sodium hydroxide, and potassium hydroxide. HPLC-grade methanol and acetonitrile were also purchased from Fisher Scientific Co. Ethylbenzene, N,N-dimethylaniline, 2-ethylaniline, 3-ethylaniline, 4-ethylaniline, naphthalene, nitronaphthalene, acetylacetone, dimethyl phthalate, diethyl phthalate, uracil, quinine, sorbic acid, diphenhydramine, 2-picoline, 3-picoline, 4-picoline, 2,5-lutidine, 2,4-lutidine, 3,5-lutidine, 4-hydroxyquinoline, 8-hydroxyquinoline, 2-aminophenol, 3-aminophenol, 4-aminophenol, 2-nitrophenol, 3-nitrophenol, lidocaine, amitriptyline, and piroxicam were all purchased from Sigma-Aldrich (St. Louis, MO). Procaine, monobasic potassium phosphate, and dibasic potassium phosphate were purchased from Spectrum Chemicals (Gardena, CA). Caffeine was obtained from ICN Biomedicals (Aurora, OH). Nicotine was purchased from Chemical Service (West Chester, PA) and 4-nitrophenol was purchased from Eastman Kodak (Rochester, NY). Standard Reference Material (SRM) 870 was donated from the National Institute of Standards and Technologies (NIST) in Gaithersburg, Maryland. Helium used to purge the mobile phase was purchased from Air Products (Allentown, PA).

The columns used for this body of work are listed in Table 2-1. The columns used in this study were all C₁₈ phases and had inner diameters of 4.6 mm and column lengths of 15 cm. All columns had particle sizes of 5 μm with the exception of the Novapak and μBondapak columns, which had particle sizes of 4 μm and 10 μm respectively. Columns were selected based on their popularity of use by chromatographers for the analysis of basic substances. Abbreviations for the columns will be utilized for data analysis in the ensuing chapters.

Table 2-1. Reversed-phase Columns Used.

Column Name	Manufacturer	Abbreviation	Bonding Characteristics
Discovery C ₁₈	Supelco	DISC	Endcapped
Supelcosil-LC-18-DB	Supelco	SUPL	Deactivated Silica
Symmetry 300C ₁₈	Waters	SYM3	ODS w/ 300 Å pores
Symmetry C ₁₈	Waters	SYMM	Deactivated Silica
Symmetry Shield RP18	Waters	SYMS	Polar Embedded Ligand
YMC Pro 18	Waters	YMCP	Endcapped
YMC Basic S5	Waters	YMCB	Base Deactivated
Spherisorb ODS-2	Waters	SPH2	Endcapped
Spherisorb ODS-1	Waters	SPH1	ODS
μBondapack C ₁₈	Waters	UBON	Endcapped
Nova-Pak C ₁₈	Waters	NOVP	Endcapped
Zorbax Rx-C ₁₈	Agilent	ZXRX	ODS
Eclipse XDB-C ₁₈	Agilent	ECLP	Double Endcap
Zorbax SB-C ₁₈	Agilent	ZXSB	Sterically Protected Group
Zorbax ODS	Agilent	ZXOD	Endcapped
Xterra RP ₁₈	Waters	XTRA	Hybrid Bonding

Calculations

The Foley-Dorsey equation was used to calculate efficiency[45]:

$$N = \frac{41.7 \times (t_r / W_{0.1})^2}{(B/A + 1.25)}$$

where t_r is the retention time, $W_{0.1}$ is the peak width at 10% of the peak height, and B/A is the asymmetry factor of the peak at 10% of the peak height. All peak shape calculations were also calculated by determining the asymmetry factor (A_s) at 10 % of the peak height.

Capacity factor calculations were calculated using the following equation:

$$k' = \frac{t_r - t_o}{t_o}$$

where t_r is the retention time of the solute and t_o is the time it takes for an unretained solute to elute.

Selectivity calculations were performed by dividing the capacity factor of one compound by another.

$$\alpha = \frac{k'_2}{k'_1}$$

Chapter 3

Comparison of Silanol Activity Test Procedures

Introduction

Reversed-phase liquid chromatography (RPLC) is one of the most popular separation techniques used today. Despite its limitations, silica remains the most dominant packing material. Although far superior to other materials, silica is encumbered by secondary interactions between residual silanols and analytes [6]. Residual silanol groups found on the surface of the packing interact with the analyte's ionizable functional groups to cause tailing peaks, increased retention, and loss of resolution [21].

There have been numerous attempts to improve the deficiencies of silica [3,46]. The column industry has employed a number of technologies to improve the performance of silica-based columns. Currently there are over 600 different reversed-phase packings available and this has elevated the level of difficulty in column selection for chromatographers [47]. In response, chromatographers have presented evaluation methods in the literature which can be used as tools to classify columns based on their silanol activity [28,29]. The term silanol activity refers to the assessment of the ability of the most reactive silanols to affect a separation. These chromatographic evaluations vary greatly in terms of their specific experimental parameters and silanol activity indicators. However, the numerous attempts have yet to produce a universally accepted test procedure [48].

There has been very little investigation into the conformity between results from the various silanol activity tests. In fact, to date only two studies have been executed. A

study performed by Claessens and co-workers compared test procedures with respect to their ionic and hydrophobic properties [35]. In this study, Claessens compared results from the Engelhardt, Tanaka, Galushko, and Walters Test. Five different tests were compared in the silanol activity evaluations, each of the tests previously mentioned and a “modified” Engelhardt test. The modification to the test was the addition of a buffer to the mobile phase. It was found that while the hydrophobicity tests seemed to produce interchangeable data, the results of the silanol activity tests varied significantly. Columns which would be ranked as having low silanol activity with one testing procedure would be ranked as having a high silanol activity by another method. It was also found that the modified Engelhardt test and the unbuffered Engelhardt test were poorly correlated. In addition, it was determined that column classifications which are based on silanol activity values are dependent upon which test procedure is employed.

Vervoort and colleagues published a study that looked at three of the same tests used in the Claessens study [19]. The Engelhardt, Tanaka, and Galushko tests were compared again, as well as a procedure that was developed by McCalley. In this study, only the ionic properties were evaluated in order to evaluate the applicability of the stationary phases in the analysis of basic compounds. Results were compared to chromatographic data obtained from analyzing a selection of pharmaceutical compounds. Again, it was concluded that the results from the various test procedures resulted in different rankings for columns depending on which test procedure was used for characterization. In general, it was found that the McCalley test showed the greatest correlation to the results from the analysis of the pharmaceutical compounds.

While both of the previous studies showed a lack of agreement in results from silanol activity test procedures, a number of key issues were left unanswered. The Vervoort study questioned the ability of some of the older procedures at distinguishing between new generation columns based on the results obtained from a set group of columns. However, this study failed to actually compare results from newer test procedures on that same group of columns to see if the newer tests would fair better than the older procedures. In fact, only one procedure in that study had been recently

developed. This not only left the effectiveness of the older procedures in doubt, but it also leads one to examine the possibility of agreement between the results from newer silanol activity procedures. The more recent tests were all designed on the newer generation stationary phases. There is also a need to consider the possibility that perhaps a comprehensive silanol activity test has already been developed.

A thorough comparison of the current silanol activity tests must include a fundamental evaluation of the test procedures for theoretical consistency and applicability in a laboratory setting. A comprehensive silanol activity test would be the one test procedure that will be universally used to classify columns based on their silanol activity. This test would aid chromatographers in column selection and reproducibility of written procedures.

In this study, the aim was to address the key issues which were left unanswered in the literature. A wide variety of silanol activity test procedures are compared using a number of commonly used reversed-phase columns. The objectives were:

- To further display the inconsistencies between the current tests;
- To demonstrate the ineffectiveness of older procedures in distinguishing between newer phases;
- To determine if rankings from newer procedures will be in agreement with each other;
- To examine the test procedures as candidates for a comprehensive test.

It is important to note that this study is not an investigation into which phases have the lowest silanol activity, nor does it indicate which columns are the “best” columns. This study demonstrates that the actual test procedures used are not testing for the same properties; therefore, it is difficult to justify using them to determine which columns are better than others.

Experimental

Test Procedures. All procedures were performed according to the conditions specified by the authors in the literature. However, amphetamine was not used in the McCalley procedure and procaine was substituted for procainamide in the Manufacturer procedures. The “modified” Engelhardt procedure, which uses a buffer, was used instead of the standard Engelhardt procedure. All test mixtures were dissolved in the mobile phase solution, even if the author specified differently.

1a. McCalley Test – Hydrogen Bonding (MCLH) [36]

Test Compounds: uracil, quinine, nicotine, benzylamine, diphenhydramine, pyridine, codeine, nortriptyline

Mobile Phase Conditions: Methanol/ 25mM Phosphate Buffer pH 3 55:45 (v/v), 30:70 (v/v)

Silanol Activity Indicator: A_s values of all compounds, theoretical plates for all compounds

1b. McCalley Test – Ionic Interactions (MCLI) [36]

Test Compounds: uracil, quinine, nicotine, benzylamine, diphenhydramine, pyridine, codeine, nortriptyline

Mobile Phase Conditions: Methanol/ 25mM Phosphate Buffer pH 7 55:45 (v/v), 30:70 (v/v)

Silanol Activity Indicator: A_s values of all compounds, theoretical plates for all compounds

2a. Tanaka Test – Hydrogen Bonding (TNKH) [20]

Test Compounds: thiourea, uracil, caffeine, phenol, amylbenzene, butylbenzene, triphenylene, o-terphenyl, benzylamine

Mobile Phase Conditions: Methanol/Water 30:70 (v/v)

Silanol Activity Indicator: $k'_{\text{caffeine}}/k'_{\text{phenol}}$

2b. Tanaka Test – Ionic Interactions at pH 3 (TKI3) [20]

Test Compounds: thiourea, uracil, caffeine, phenol, amylbenzene, butylbenzene, triphenylene, o-terphenyl, benzylamine

Mobile Phase Conditions: Methanol/ 0.02 M Phosphate Buffer pH 2.7 30:70 (v/v)

Silanol Activity Indicator: $k'_{\text{benzylamine}}/k'_{\text{phenol}}$

2c. Tanaka Test - Ionic Interactions at pH 7 (TKI7) [20]

Test Compounds: thiourea, uracil, caffeine, phenol, amylbenzene, butylbenzene, triphenylene, o-terphenyl, benzylamine

Mobile Phase Conditions: Methanol/ 0.02 M Phosphate Buffer pH 7.6 30:70 (v/v)

Silanol Activity Indicator: $k'_{\text{benzylamine}}/k'_{\text{phenol}}$

3a. Manufacturer Test – Hydrogen Bonding (MANH) [49]

Test Compounds: uracil, procaine, N, N-dimethylaniline, caffeine, phenol, Sorbic acid

Mobile Phase Conditions: Methanol/ 25mM Phosphate Buffer pH 2.5 17:83 (v/v)

Silanol Activity Indicator: A_s ratio for caffeine and phenol peaks

3b. Manufacturer Test – Ionic Interactions (MANI) [49]

Test Compounds: uracil, procaine, N,N-dimethylaniline, caffeine, phenol, sorbic acid

Mobile Phase Conditions: Methanol/ 25mM Phosphate Buffer pH 7.5 17:83 (v/v)

Silanol Activity Indicator: A_s ratio for caffeine and phenol peaks

4. Engelhardt Test (ENGL) [32]

Test Compounds: thiourea, toluene, ethylbenzene, aniline, phenol, N, N-dimethylaniline, ethylbenzoate, p-ethylaniline, m-ethylaniline, o-ethylaniline

Mobile Phase Conditions: Methanol/1 mM Phosphate Buffer pH 7 55:45 (v/v)

Silanol Activity Indicator: elution order of aniline and phenol peaks and A_s p-ethylaniline peak

5. Verzele and Dewaele Test (VERD) [50]

Test Compounds: acetylacetone, naphthalene, nitronaphthalene

Mobile Phase Conditions: Methanol/Water w/ 0.5% Sodium Acetate 60:40 (v/v)

Silanol Activity Indicator: $k'_{\text{naphthalene}}/k'_{\text{nitronaphthalene}}$

6a. Goldberg Polar (GOLP) [51]

Test Compounds: dimethyl phthalate, diethyl phthalate, thiourea,

Mobile Phase Conditions: Methanol/ Water 65:35 (v/v)

Silanol Activity Indicator: $k'_{\text{dimethyl phthalate}}/k'_{\text{diethyl phthalate}}$

6b. Goldberg - Basic (GOLB) [51]

Test Compounds: thiourea, caffeine, theophylline

Mobile Phase Conditions: Acetonitrile/10mM Sodium Acetate pH 4.5 20:80 (v/v)

Silanol Activity Indicator: $k'_{\text{caffeine}}/k'_{\text{theophylline}}$

7. Standard Reference Material 870 (S870) [52]

Test Compounds: uracil, toluene, ethylbenzene, quinizarin, amitriptyline

Mobile Phase Conditions: Methanol/ 5mM Phosphate Buffer pH 7 80:20 (v/v)

Silanol Activity Indicator: A_s for amitriptyline peak

Standard Reference Material (SRM) 870 served a dual role in this study. It was mainly used as a tool to detect any column performance degradation over the course of the study, but it also can be used as a tool for the classification of columns based on their silanol activity [52]. Therefore, it was also used as one of the test procedures in the comparison study. Results from SRM 870 performed at the onset of the study and after completion of the study did not indicate any decay of column performance over the course of the study. Results from the initial SRM 870 analysis were used for comparative purposes.

Columns. Columns used in this study include all of the columns in Table 2-1 except the Xterra RP₁₈ column. Only one column of each type was studied. However, all columns were new and were provided by the manufacturer, so they are assumed to be representative samples.

Calculations. Columns were characterized according to the parameters specified by the various authors. The Foley-Dorsey equation was used for all plate calculations [45]. Asymmetry calculations for the SRM 870 test were taken at the 10% level as opposed to the 5 % level as the authors of the test indicated.

Results and Discussion

Assessment of Current Test Procedures. Test procedures were performed according to the specifications of each test. Triplicate runs were completed for each column for each test procedure to produce average values. The results from all fifteen columns for each of the procedures are included in Appendix Tables 1-12. Test procedures were qualitatively compared using the ranking system adopted from Vervoort and co-workers according to the specifications of each individual test [19]. Rankings range from a low silanol activity (1) to a high silanol activity (6). Lower rankings indicate a better aptness of the columns to be used in the analysis of basic compounds. Rankings are based on the range of values given from the 15 columns to give a normalized assessment of silanol activity. Ranking criteria were designed according to the specifications of each individual test procedure, the ranking criteria for each test procedure are displayed in the following tables.

Table 3-1. McCalley (pH 3) Silanol Activity Test Column Ranking Criteria

Rankings	Average A_s Value
1	1.27-1.57
2	1.58-1.88
3	1.89-2.19
4	2.20-2.50
5	2.51-2.81
6	2.82 and above

Table 3-2. McCalley (pH 7) Silanol Activity Test Column Ranking Criteria

Rankings	Average A_s Value
1	1.05-1.71
2	1.72-2.38
3	2.39-3.05
4	3.06-3.72
5	3.73-4.39
6	4.40 and above

Table 3-3. Tanaka Hydrogen Bonding Silanol Activity Test Column Ranking Criteria

Rankings	$k'_{\text{benzylamine}} / k'_{\text{phenol}}$
1	1.00-1.26
2	1.27-1.53
3	1.54-1.80
4	1.81-2.07
5	2.07-2.33
6	2.34-2.60

Table 3-4. Tanaka Ionic Exchange (pH 7) Silanol Activity Test Column Ranking Criteria

Rankings	$k'_{\text{benzylamine}} / k'_{\text{phenol}}$
1	0.230-0.366
2	0.337-0.443
3	0.444-0.550
4	0.551-0.657
5	0.658-0.764
6	0.765-0.871

Table 3-5. Tanaka Ionic Exchange (pH 3) Silanol Activity Test Column Ranking Criteria

Rankings	$k'_{\text{benzylamine}} / k'_{\text{phenol}}$
1	0.030-0.063
2	0.064-0.097
3	0.098-0.131
4	0.132-0.165
5	0.166-0.199
6	0.200-0.233

Table 3-6. Goldberg Basic Silanol Activity Test Column Ranking Criteria

Rankings	$k'_{\text{caffeine}} / k'_{\text{theophylline}}$
1	1.18-1.95
2	1.96-2.75
3	2.76-.3.53
4	3.54-4.32
5	4.33-5.10
6	5.11 and above

Table 3-7. Goldberg Polar Silanol Activity Test Column Ranking Criteria

Rankings	k' dimethyl phthalate/ k' diethyl phthalate
1	1.48-1.70
2	1.71-1.93
3	1.94-2.16
4	2.17-2.39
5	2.40-2.62
6	2.63-2.85

Table 3-8. Manufacturer Hydrogen Bonding Silanol Activity Test Column Ranking Criteria

Ranking	Elution of Peaks	A_s Caffeine/ A_s Phenol
1	All peaks separate	0.90-1.20
2	All peaks separate	0.59-0.89, 1.21-1.51
3	All peaks separate	0.28-0.58, 1.51 and above
4	Coelution of N,N-Dimethylaniline and Procaine	Any
5	Coelution of Caffeine and Phenol	N/A
6	Coelution of N,N-Dimethylaniline and Procaine Coelution of Caffeine and Phenol	N/A

Table 3-9. Manufacturer Ionic Interactions Silanol Activity Test Column Ranking Criteria

Rankings	Elution of Peaks	A_s Caffeine/ A_s Phenol
1	All peaks separate	0.90-1.20
2	All peaks separate	0.68-0.89, 1.21-2.08
3	All peaks separate	0.67 and below, 2.09 and above
4	Coelution of N,N-Dimethylaniline and Procaine	Any
5	Coelution of Caffeine and Phenol	N/A
6	Coelution of N,N-Dimethylaniline and Procaine Coelution of Caffeine and Phenol	N/A

Table 3-10. Verzele and Dewaele Silanol Activity Test Column Ranking Criteria

Ranking	$k'_{\text{Naphthalene}}/k'_{\text{Nitronaphthalene}}$
1	2.26 and above
2	2.01-2.25
3	1.76-2.0
4	1.50-1.75
5	1.26-1.50
6	1.0-1.25

Table 3-11. SRM 870 Silanol Activity Test Column Ranking Criteria

Ranking	A_s for Amitriptyline peak
1	1.31-2.11
2	2.12-2.91
3	2.92-3.71
4	3.72-4.51
5	4.52-5.31
6	5.31 and above

Table 3-12. Engelhardt Silanol Activity Test Column Ranking Criteria

Ranking	A_s of p-ethylaniline
1	1.10-1.33
2	1.34-1.57
3	1.58-1.81
4	1.82-2.05
5	2.06-2.29
6	2.30 and above

As seen in previous studies, the various test procedures give varying appraisals of the same column. For any given column, rankings of low silanol activity and high silanol activity can be found, depending on which procedure is used (Figure 3-1). When average values are rounded to the nearest whole number, 13 of the columns have an average ranking of either 3 or 4 (Table 3-13). This would indicate that all 13 of these columns would give the same type of results in the analysis of basic substances. In reality, rarely did more than two or three of the columns demonstrate similar results. These results further prove that the current procedures, although designed specifically to determine silanol activity, are not all testing for the same property of the column.

Old Test Procedures versus New Column Technologies. A number of the test procedures were designed before column manufacturers started using the high purity supports and other column technologies invented to improve the problem of residual silanols. Therefore, these tests were suitable for the determination of vast differences between the older phases, however it was unclear whether or not these tests would be effective at distinguishing between the subtle differences in the newer phases [48].

In this study, four older procedures were examined: the Engelhardt test [32]; the Verzele and Dewaele test [50]; the Goldberg test [51]; and the Tanaka test [20], with the most recent procedure first being published in 1991. In general, it was found that the older tests were not as effective at characterizing columns as the newer procedures. For example, the results from the Verzele and Dewaele and the Tanaka Ionic-7 test procedures both show only 4 and 5 columns with rankings poorer than a 1 or 2 (Table 3-14), respectively. This suggests that at least 10 out of the 15 columns would be highly effective in the separation and analysis of basic solutes.

In some cases, this fact is not as evident when looking at the ranking chart. Any one of the tests has rankings ranging from 1 to 6; however, these rankings are based on the range of values given from the results of the 15 columns. The actual numerical

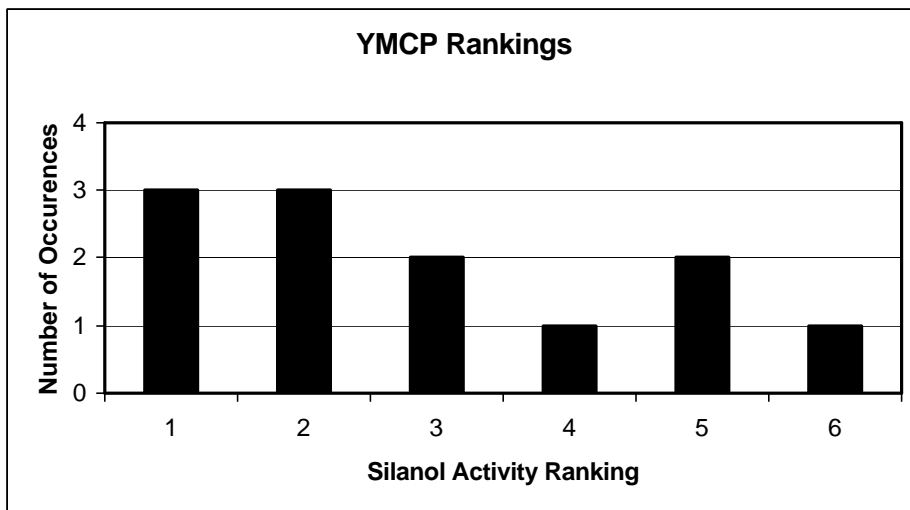
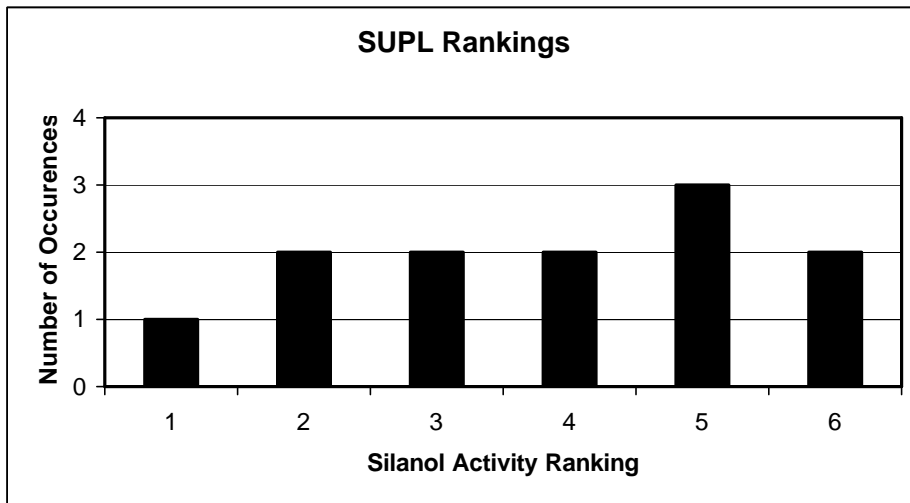
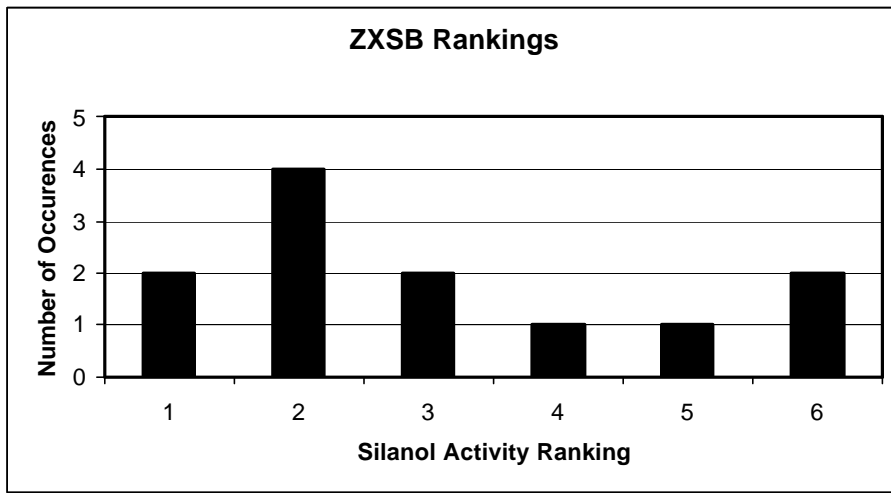


Figure 3-1. Silanol Activity Rankings from All of the Test Procedures for Three of the Columns Used in the Study.

Table 3-13. Silanol Activity Rankings for Columns According to Each Test Procedure and Average Ranking Rounded to the Nearest Whole Number.

Column	S870	ENGL	TNKH	TKI7	TKI3	VERD	MANH	MANI	GOLP	GOLB	MCLI	MCLH	Total Avg
DISC	1	1	4	1	2	1	2	4	5	2	1	1	2
SUPL	6	2	5	2	3	2	4	1	5	3	5	4	4
SYM3	1	6	6	1	2	1	2	1	5	3	2	1	3
SYMM	4	1	5	2	1	1	3	2	5	3	5	1	3
SYMS	2	1	5	1	1	5	2	4	2	1	2	1	2
YMCP	3	1	4	1	2	2	5	6	5	3	2	1	3
YMCB	2	1	4	2	2	5	3	4	3	2	3	1	3
SPH2	6	2	1	1	5	2	5	6	2	5	1	6	4
SPH1	6	2	6	2	6	6	6	6	1	6	2	6	4
UBON	3	1	2	3	4	3	2	4	1	3	2	1	3
NOVP	5	6	3	5	3	1	3	6	4	4	3	3	4
ZRRX	6	1	2	5	2	1	3	2	4	6	1	2	3
ECLP	1	2	4	3	3	1	5	1	4	4	3	1	3
ZXSB	2	2	2	6	3	2	3	4	6	5	1	1	3
ZXOD	6	1	2	1	5	1	6	4	6	6	1	3	3

Table 3-14. Rankings from the Verzele and Dewaele and Tanaka Ionic Exchange (pH 7) Test Procedures.

Columns	VERD Ranking	TKI7 Ranking
DISC	1	1
SUPL	2	2
SYM3	1	1
SYMM	1	2
SYMS	5	1
YMCP	2	1
YMCB	5	2
SPH2	2	1
SPH1	6	2
UBON	3	3
NOVP	1	5
ZXRX	1	5
ECLP	1	3
ZXSB	2	6
ZXOD	1	1

values for the silanol activity indicator may not vary very much or in some cases the lowest values may still be considered acceptable according to the author's guidelines. For example, the Engelhardt paper states that all columns with asymmetry values less than 2 are suitable for the analysis of bases [37]. In this study, there were columns which displayed values higher than 2. However, in our study only two columns displayed asymmetry values greater than two (Table 3-15). The remaining thirteen columns displayed asymmetry values that were less than or equal to 1.56. According to the Engelhardt study, this makes all of these columns suitable for the analysis of bases.

In all three of these cases it can be assumed that all of the columns which are deemed suitable by these procedures are not going to give equal or even similar performance when analyzing an actual pharmaceutical sample. It appears that the procedures are just not sensitive enough to distinguish between the subtle differences in today's modern phases.

3.3 Comparison of Rankings from the Newer Procedures. Three newer test procedures were examined: SRM 870 [52], the McCalley test [36], and the Manufacturer test [49]. These tests were published in 2003, 1997, and 2000 respectively. It was found that the newer test procedures were more in agreement than the older tests. This is especially evident when looking at the results from procedures which were performed at the same pH. It is generally believed that in order to assess any contribution to silanol activity from hydrogen bonding, the pH of the mobile phase must be at or below 3 where silanols should be protonated. The maximum contribution to silanol activity can be assessed at or above a pH of 7. The McCalley and Manufacturer tests, which both include a test at pH 3 and a test at pH 7, show agreement in their assessment of several columns. For 9 out of the 15 columns, a number of rankings are exactly the same or differ in ranking by only one number (Table 3-16). Although SRM 870 is not performed at a pH of 3, it does give similar results to the McCalley test procedure which is also performed at a pH of 7 (Table 3-17). Both tests use the same type of silanol activity indicator, asymmetry values of high pK_a basic substances. The similarity in rankings for 9 out of the 15 columns, when analyzed by the modern procedures, suggests that

Table 3-15. Ranking and Asymmetry values from Engelhardt Test Results.

Columns	Rankings	Asymmetry Values
DISC	1	1.25
SUPL	2	1.46
SYM3	6	3.20
SYMM	1	1.30
SYMS	1	1.16
YMCP	1	1.20
YMCB	1	1.31
SPH2	2	1.50
SPH1	2	1.56
UBON	1	1.10
NOVP	6	2.47
ZXRX	1	1.23
ECLP	2	1.44
ZXSB	2	1.54
ZXOD	1	1.25

Table 3-16. Rankings from the McCalley (pH 7) and Manufacturer Ionic Interactions Test Procedures.

Columns	MCLH Results	MANH Results
DISC	1	2
SUPL	4	4
SYM3	1	2
SYMS	1	2
SPH2	6	5
SPH1	6	6
UBON	1	2
NOVP	3	3
ZXRX	2	3

Table 3-17. Rankings from McCalley (pH 7) test and Standard Reference Mixture 870 Test Procedures.

Columns	S870 Results	MCLI Results
DISC	2	1
SUPL	6	5
SYMM	5	5
SYMS	2	2
YMCP	2	2
YMCB	3	3
UBON	1	2
NOVP	2	3
ECLP	2	3

examining the columns for specific interactions using specific silanol activity indicators may aid in bringing some uniformity to these test procedures.

Characteristics of a Comprehensive Test Procedures. When dealing with silanol activity there are a number of criteria which must be met in order to design a comprehensive test. This test would be used in laboratories world wide for the assessment of a column's silanol activity. In order to produce such results the test must include some key factors.

- The test must be fast and efficient in order to be accepted by industrial laboratories.
- The test must contain compounds that are readily available.
- The test mixture must contain some of the most problematic compounds.
- The test must be robust.
- The test must account for the two most important components of silanol activity.

After reviewing the current tests, it was found that none of the procedures met the criteria for comprehensive procedure. One of the main problems in the analysis of the stationary phases lies in the applicability of the full definition of silanol activity to the tests. Silanol activity includes a number of different interactions between the solute and the stationary phase. The two most important of these are hydrogen bonding and ionic interactions. A number of tests fail to examine both of these types of interactions, thus giving an inaccurate assessment of the column's silanol activity [28,29]. Out of all of the procedures reviewed here, only three tests took into account the two most important types of interactions with regard to silanol activity; the Tanaka, Manufacturer, and McCalley test [20,36,49]. Of those three tests, only the McCalley test actually uses several of the problem causing, high pK_a basic compounds in the test mixture [36]. However, this test requires single injections of each test solute, one at a time, for each pH; therefore making the procedure too lengthy to be used in an industrial setting for column classification.

Overall, we found that each of these tests failed in two very critical areas. There is currently not a single test that examines columns over their entire useful pH range. Tests are usually performed at the extremes of this range, at or below a pH of 3 and at or

above a pH of 7, with the absolute minimum and maximum of pH 2 and 8, respectively. A study conducted by Mendez and co-workers showed that different columns exhibit different minimum pK_a levels for their most acidic silanols; therefore, examination of the column's performance over the entire useful pH range for a reversed-phase column should be explored [17]. The information gathered is especially important for chromatographers who may want to perform an analysis at a pH of 5, for example. According to the Mendez study, there are some columns that have silanols that are still protonated at pH's as high as 7.0.

We also found that none of the tests took into account the changes in the pH of the mobile phase and the pK_a of the solutes that occurs upon the addition of organic modifier. A detailed review by Roses and Bosch discusses the need for chromatographers to take the effects of adding organic modifier to the mobile phase into account, especially when dealing with pH dependent analyses [53]. This implies that any test which assumes a particular compound is ionized under specific experimental conditions, but does not take the pK_a shifts into consideration, is theoretically defunct.

Conclusion

In this study the aim was to comprehensively examine the unanswered questions as they pertained to the current silanol activity test procedures. As expected, the classifications of the different columns based on the measurement of their silanol activities by different tests are vastly inconsistent. Columns are shown to have different levels of active silanol sites depending on which test procedure is used for analysis. Results from this study are in agreement with results from previous studies. We also showed that older test procedures are less effective at distinguishing between the subtle differences in newer generation phases. Results from older procedures indicate that a majority of the vastly different columns will give similar results when analyzing basic substances, when in fact, they do not. Results from new test procedures showed more agreement in rankings, especially when test procedures at similar pH levels were examined. Test procedures were considered as candidates for a comprehensive procedure and it was determined that none of the current tests were sufficient according to the

criteria laid forth in this analysis. Close scrutiny of the current tests show that the test procedures do not always use the full definition of silanol activity. Those few tests which do take all interactions into account fail to use pertinent basic substances in their evaluations. It is very important to test the effectiveness of the columns at separating basic and polar compounds by using probes that are very similar to the types of compounds for which the columns will eventually be used. It was noted that there is not a single test which examines the columns over their entire useful pH range. While it is believed that the maximum ionic contribution to silanol activity occurs at a pH of 7, there can be contributions to silanol activity from silanols at pH levels between 3 and 7. Chromatographers who perform analyses in this range could benefit from any knowledge gained at these intervals. Most disturbing was the fact that none of these tests took into account the changes that occur to pH and pK_a values upon the addition of organic modifiers. To date there is no universally accepted chromatographic silanol activity test procedure and until these gaps have been filled, no one test will be deemed as a universal procedure.

Chapter 4

Development of a Silanol Activity Test Procedure

Introduction

Stationary phases are now available that have been specially designed for the analysis of basic compounds [54]. However, asymmetric peaks are still obtained for basic compounds using these newly designed stationary phases. Testing of these phases before they are utilized in development or in quality control applications is essential. The need to objectively distinguish between the chromatographic properties of RPLC phases was recognized early and this led to a plethora of characterization tests in the literature. Despite numerous attempts, there is not a universally accepted chromatographic silanol activity test procedure [28].

Comparison studies performed indicate that the various silanol activity test procedures are not all testing for the same property of the stationary phase [19,35]. These evaluations, along with the study conducted in this body of work, have provided some insight into which qualities future tests would need to include in order to be universally used and accepted. There have also been a number of studies conducted which have explored the effects of different experimental parameters on the analysis of basic substances by RPLC that can contribute to the guidelines for the development of an improved silanol activity test procedure [18,31,33,36,38-43,48].

In this work, the aim is to combine the knowledge gained from all of these studies in order to develop a silanol activity test that provides the information that has been deficient in previous tests. Focus was placed on using basic compounds with wide ranges of pK_a 's, using a mobile phase system that covered the entire useful pH range for typical

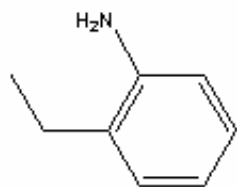
RPLC columns, and monitoring the pH of the mobile phase after the addition of organic modifier. The test procedure developed was used to analyze a select group of columns and the accuracy of this test procedure was tested as well.

Experimental

Mobile Phase System. The mobile phase consists of a citrate/phosphate buffer system with HPLC grade acetonitrile added as the organic modifier in varying amounts. Solution A is a 0.01 M citrate solution with a pH of 2.57 before the addition of organic modifier. Solution B consists of a 0.02M phosphate solution with a pH of 8.05 before the addition of organic modifier. Changes to the mobile phase pH were monitored at all times using a Sensorex 200 μ L flow cell apparatus (Figure 1-1).

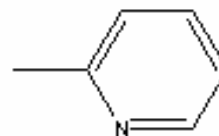
Compounds. Sets of configurational isomers were used in this study. In most cases the ortho, meta, and para-substituents were used for each isomer set. The only exception is the hydroxyquinoline isomers, in this case 4-hydroxyquinoline and 8-hydroxyquinoline were used. Each set of isomers possesses pK_a values within a certain range. The structure of one isomer from each group is shown in Figure 4-1. All of the compounds are aromatic and contain a nitrogen group in the structure. Table 4-1 shows the literature pK_a value of all of the compounds used in this study. 0.05 mg/mL solutions of each single compound were used with a 10 μ l injection loop for both chromatographic pK_a and silanol activity test evaluations.

Columns and Detection. All experiments were carried out at 25°C. UV/VIS detection was used at 210 nm. All preliminary data were collected on an Xterra RP₁₈ (XTRA) column. The Xterra column uses a hybrid packing that has both organic and inorganic



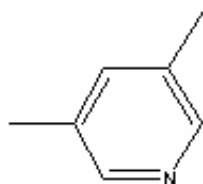
Ethylaniline Isomers

pK_a 4-5



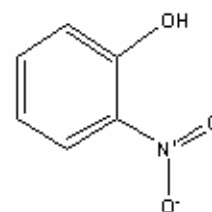
Picoline Isomers

pK_a 5-6



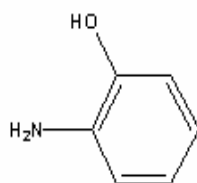
Lutidine Isomers

pK_a 6-7



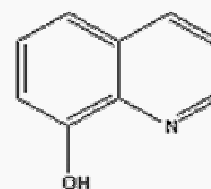
Nitrophenol Isomers

pK_a 7-8



Aminophenol Isomers

pK_a 8-10



Hydroxyquinoline Isomers

pK_a 10-11

Figure 4-1. Structures of One Isomer From Each Isomer Set

Table 4-1. Literature pK_a Values of All Compounds

Compound	pK _a
2-Ethylaniline	4.42
3-Ethylaniline	4.70
4-Ethylaniline	5.00
2-Picoline	5.96
3-Picoline	5.68
4-Picoline	6.00
2,4-Lutidine	6.74
2,5-Lutidine	6.43
3,5-Lutidine	6.09
2-Nitrophenol	7.22
3-Nitrophenol	8.36
4-Nitrophenol	7.15
2-Aminophenol	9.28, 9.72
3-Aminophenol	9.83, 9.87
4-Aminophenol	8.50, 11.30
4-Hydroxyquinoline	2.27, 11.25
8-Hydroxyquinoline	4.91, 9.81

* All pK_a values were determined in water at 25°C with the exception of the hydroxyquinoline isomers. Vales for these isomers were determined at 20°C. Literature values were obtained from Lange's Handbook of Chemistry [55].

precursors. This technology allows for the safe use of the column over a pH range of 1–12. This column was used for all chromatographic pK_a determinations as well as in the development of the silanol activity test procedure. In combination with the Xterra RP₁₈ column, the efficacy of the test procedure was analyzed using a SymmetryShield RP 18 (SYMS) column, a Discovery C₁₈ (DISC) column, a Symmetry C₁₈ (SYMM) column, and an Eclipse XDB-C₁₈ (ECLP) column. These columns were selected based on the various column technology employed for each column. The SymmetryShield column has a polar embedded group in the stationary phase, while the Discovery column uses endcapping technology. The Eclipse column utilizes extra dense bonding in conjunction with double-endcapping and the Symmetry column was produced with high purity silica.

Results and Discussion

Selection of the Mobile Phase. Because the relative performance of columns is significantly different at high and low pH, meaningful evaluation of columns must be performed using at least two pH values. Also testing at these levels ensures that both hydrogen bonding and ionic interactions are evaluated. In addition, there is an indication that column rankings will change depending on the pH at which a test is carried out. Current procedures do not provide any information that could be used to assess a column's performance at pH levels between 3 and 7.

One of the major objectives of the study was to evaluate reversed-phase stationary phases over the entire useful pH range for these columns. Comprehensive characterization of the properties of packings requires chromatographic testing at multiple mobile phase compositions [52]. Typical reversed-phase columns may be used safely between a pH of 2-8. Around a pH of 2 siloxane bonds are hydrolyzed and above a pH of 8 silica particles tend to dissolve. To ensure that this testing procedure would not cause harm to columns, a pH range of 2.5-7.5 was selected for operation. This would allow for testing below a pH of 3, where hydrogen- bonding contributions could be assessed. It also would allow for testing above a pH of 7, where the maximum contribution to silanol activity from ionic interactions could be evaluated.

In order to ensure that this procedure could be considered for use in industrial settings, a single mobile phase system which could provide this pH range had to be utilized. Preparation of multiple mobile phase buffers would be too time consuming for chromatographers in an industrial setting to find a test of this nature useful and applicable on an ordinary basis. The selected mobile phase design was adopted from the system used in a retention prediction study of Buck and Tomellini [56]. For this system, a 0.01M citrate solution in reservoir A was used in conjunction with a 0.02M phosphate solution in reservoir B. Before the addition of organic modifier, solution A had a mobile phase pH of 2.57 and solution B had a mobile phase pH of 8.05. Variation of the solvent composition resulted in an isocratic mobile phase system with a pH range available from 2.57 to 8.05.

Other precautions concerning the mobile phase pH include the examination of the influence of the addition of organic modifier on the pH of the mobile phase and the pK_a values of the test solutes. The accuracy of these values is extremely important when characterizing columns based on their silanol activity. Differences in the actual pH or pK_a values versus the perceived pH and pK_a values result in inaccurate testing theories and column rankings. In order to promote the elution of test solutes in a suitable time frame, organic modifier must be added to the buffer solutions. To assure that there are not any shifts in mobile phase strength, equal amounts of organic modifier are added to both buffer solutions. Generally, mobile phase pH is determined before the addition of organic modifier, however, the addition of organic modifier to a buffer solution has been proved to change the overall pH of the solution [29]. An additional goal for this project was to account for the changes in mobile phase pH after the addition of organic modifier.

The International Union of Pure and Applied Chemistry (IUPAC) has recently clarified the notational and practical definitions of pH and a more rigorous procedure for measuring the pH of chromatographic mobile phase systems has been suggested [57]. The procedure involves measuring the pH of the mobile phase after the addition of organic modifier using an electrode system which can be calibrated with aqueous buffers or with buffers prepared in the same solvent composition as the mobile phase [58].

pH is defined in terms of activity, which is dependent upon the standard state of activity. When buffer pH values are determined before the addition of organic modifier, the standard state for the activity is infinite dilution of the hydrogen ion in water. When the pH of the mobile phase is determined after the addition of organic modifier, there are two different standard states which are possible. Infinite dilution of the hydrogen ion in a solution which has the same solvent composition as the mobile phase or infinite dilution of the hydrogen ion in water [53]. This leads to the ^spH and the ^wpH scales respectively. In this study, the ^wpH scale was used for all ensuing analyses.

To determine the effect of adding organic modifier to the mobile phase on the final pH of the system, titrations were performed with both acetonitrile and methanol as the choice for organic modifier. In this experiment, equal amounts of organic modifier were added to each buffer solution. The initial ^wpH of the citrate solution was determined and then increasing amounts of the phosphate solution were added. The ^wpH was monitored during the addition of the phosphate solution. This procedure was performed with 20%, 40%, 60%, and 80% organic modifier. When using methanol as the organic modifier, the addition of 20 % produced a ^wpH range of 2.69 – 7.90 (Figure 4-2). The addition of 40% and 60 % resulted in a ^wpH range of 2.87-8.43 and 3.19-8.09 respectively. Adding 80% methanol to both solutions gave a ^wpH range of 3.59 to 8.99. The addition of 20% acetonitrile to both buffer solutions resulted in a ^wpH range of 2.78 - 8.10, whereas the addition of 40% acetonitrile produced a ^wpH range of 3.13 – 8.53 (Figure 4-3). The addition of 60 and 80 % acetonitrile to both solutions presents ^wpH ranges of 3.33 - 8.81 and 3.63 - 9.20 respectively. In order to remain within the initial pH range (2.5 to 7.5), acetonitrile could be added to the buffer solutions in amounts of 20% or less. Methanol could be used as the organic modifier if less than 40% was added to the buffer solutions in order to still permit analysis of the columns below a pH of 3.

Decisions on which organic modifier to use were made using recommendations from previous studies conducted on the influence of organic modifiers on the separation of organic bases [41,43,59]. It has been proved that the mobile phase is an important factor influencing the peak shape and efficiency of the column. The authors of these studies agreed that acetonitrile gave more asymmetrical peaks when compared to methanol and tetrahydrofuran. This asymmetry is attributed to the inability of acetonitrile to form hydrogen bonds with residual silanols [43]. For the purposes of this study, any additional contributions to silanol activity or interaction with residual silanols were kept to a minimum whenever possible. To ensure that the silanol activity assessments would solely be based on the performance of the stationary phase and not any other additional interference, acetonitrile was used as the organic modifier. Based on chromatographic data, 15 % acetonitrile was added to both the citrate solution and the phosphate solution. This resulted in a pH_w gradient range of 2.65 to 8.00.

Test Compounds. The performance of a column is greatly dependent on the test solute, therefore tremendous consideration is used when choosing the compounds. For example, a substance with a low capacity factor may cause deactivation of the stationary phase by a loading effect, which would give idealistic results [29]. Also, basic compounds which exhibit a great challenge at one pH, are not necessarily the most challenging at another pH. In order to ensure that columns are being evaluated by compounds similar to those for which the column will ultimately be used, it is important to include a wide range of basic compounds. Although several authors have recommended that column evaluation be performed with a set of compounds covering a wide range of pK_a values and stereochemistries, many of the previous test procedures only utilized compounds with low to moderate pK_a values.

In order to guarantee that columns were exposed to compounds with a wide range of pK_a 's, groups of configurational isomers were selected to cover a pK_a range of 4-11.

Figure 4-2. Titration of Citrate and Phosphate Solutions with Varying Amounts of Methanol Added to Each Solution

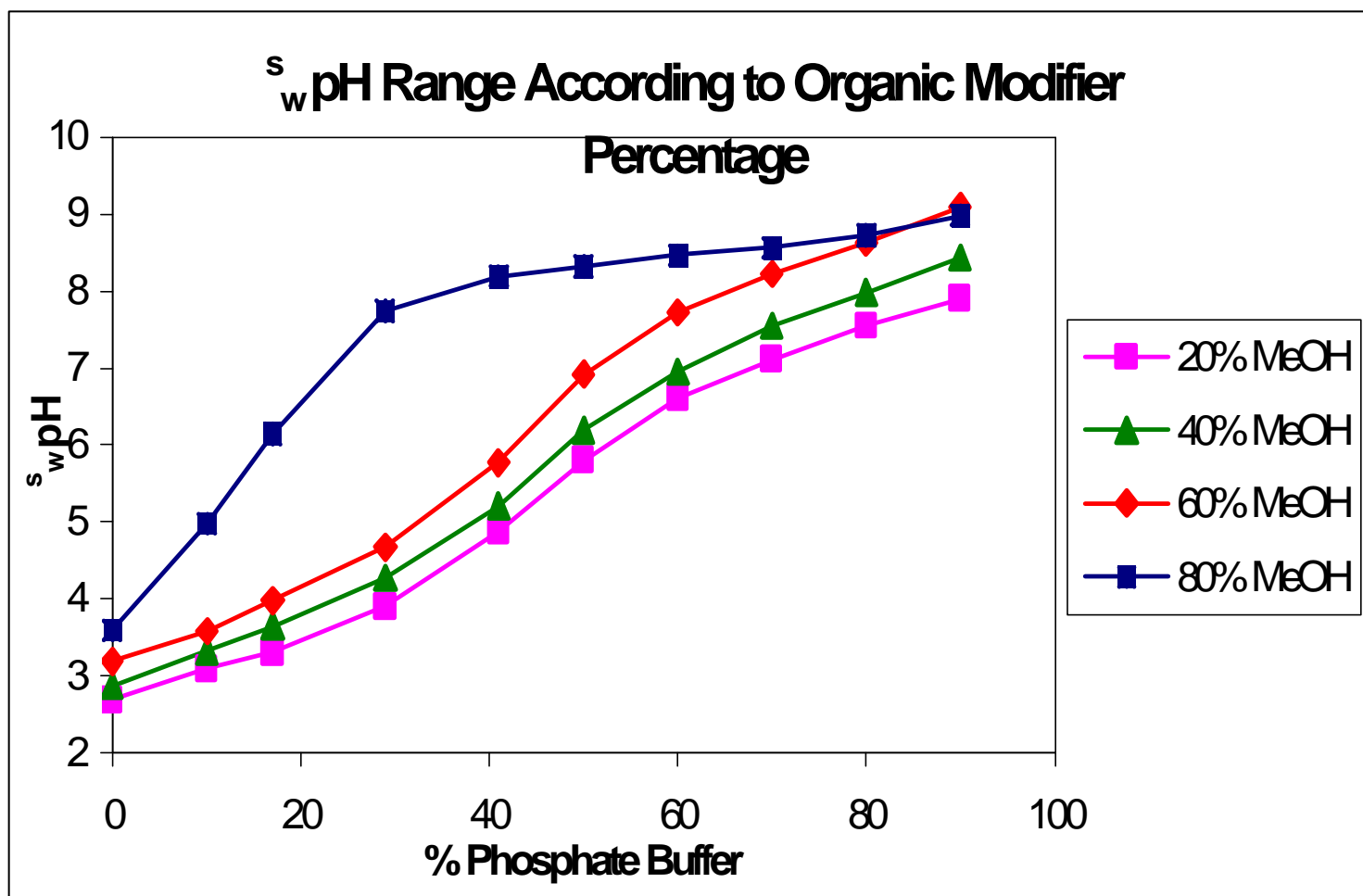
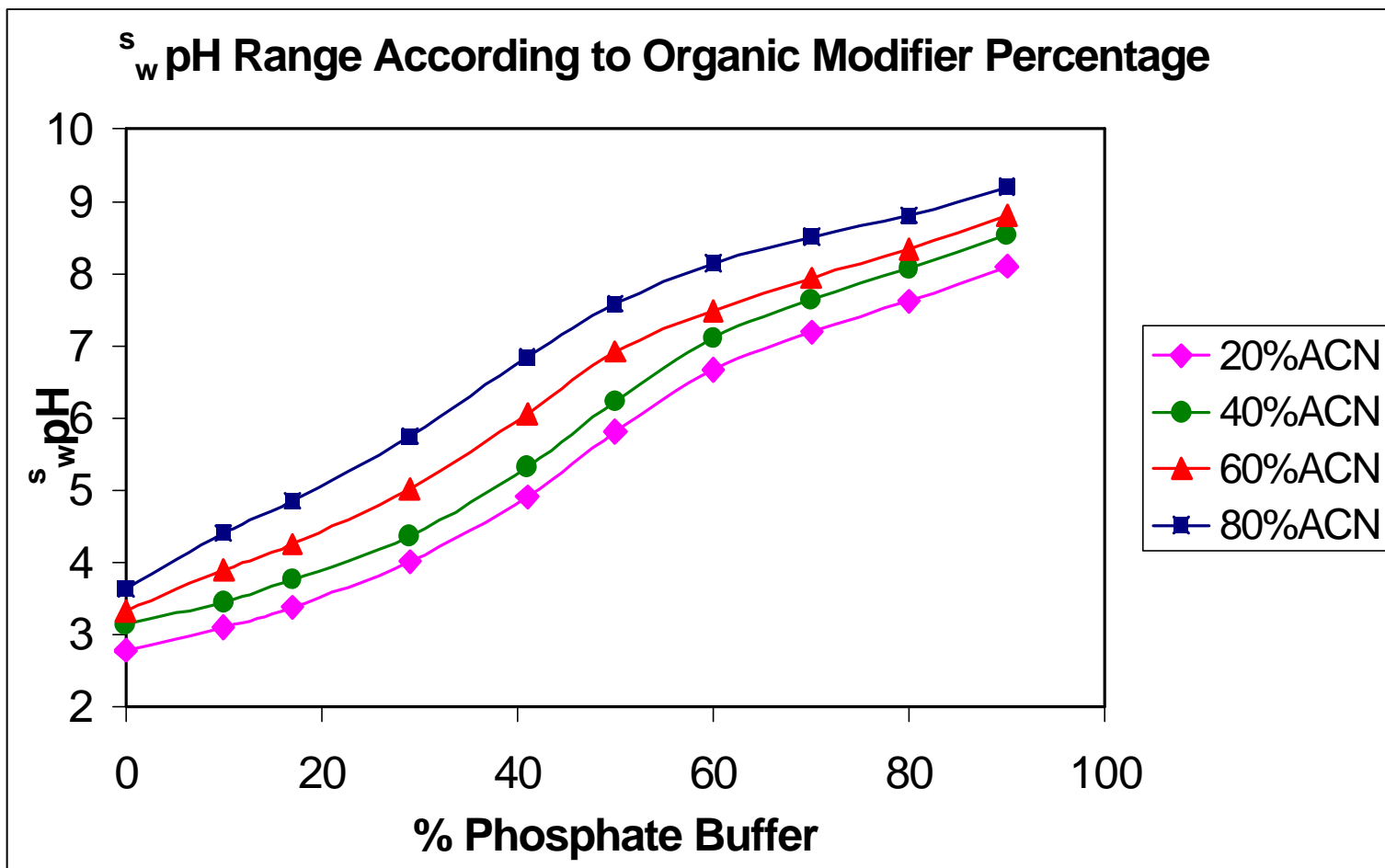


Figure 4-3. Titration of Citrate and Phosphate Solutions with Varying Amounts of Acetonitrile Added to Each Solution



Compounds selected were aromatic and contained at least one nitrogen group in the structure. Previous procedures did not expose columns to a wide range of basic compounds. Test procedures which only used low or moderately basic compounds in the test mixture fail to evaluate the column's reaction to highly basic compounds. Many of the previous silanol activity test procedures used only one basic compound as a test probe, although it has been suggested that at least five or six compounds should be used when analyzing silanol activity [31].

An additional aspiration of this project was to account for the shift in solute pK_a that occurs upon the addition of organic modifier. Most literature values reported for organic compounds have been determined in water. The ionization of bases in an organic-aqueous mixture differs from the ionization of these same compounds in water. Therefore, the chromatographic pK_a values cannot be directly correlated to the potentiometrically determined pK_a values. In principle, the variation of pK_a values in mixed solvents is different for each compound because preferential solvation of the compound by the components of the solvent mixture affects the pK_a variation [53]. Before determining how to appraise a column's level of silanol activity with the selected compounds, the chromatographic pK_a values of the compounds had to be determined. This guarantees that, unlike previous test procedures, any theoretical basis for silanol activity determination would be based on accurate values.

Horvath and co-workers first derived the equation that is used to describe the retention effect of the ionization of basic solutes (Figure 4-5) [60]. In this equation, k_0 and k_i are the retention factors of the neutral and ionized solutes respectively, BH^+ is the protonated basic solute, $K_a(BH^+)$ is the acid dissociation constant for the base and $[H^+]$ is the proton concentration. The retention of basic compounds as a function of pH shows a sigmoidal dependence as seen in Figure 4-4.

$$k = \frac{k_0 + k_i \times \frac{[H^+]}{K_a(BH^+) + [H^+]}}{1 + \frac{[H^+]}{K_a(BH^+)}}$$

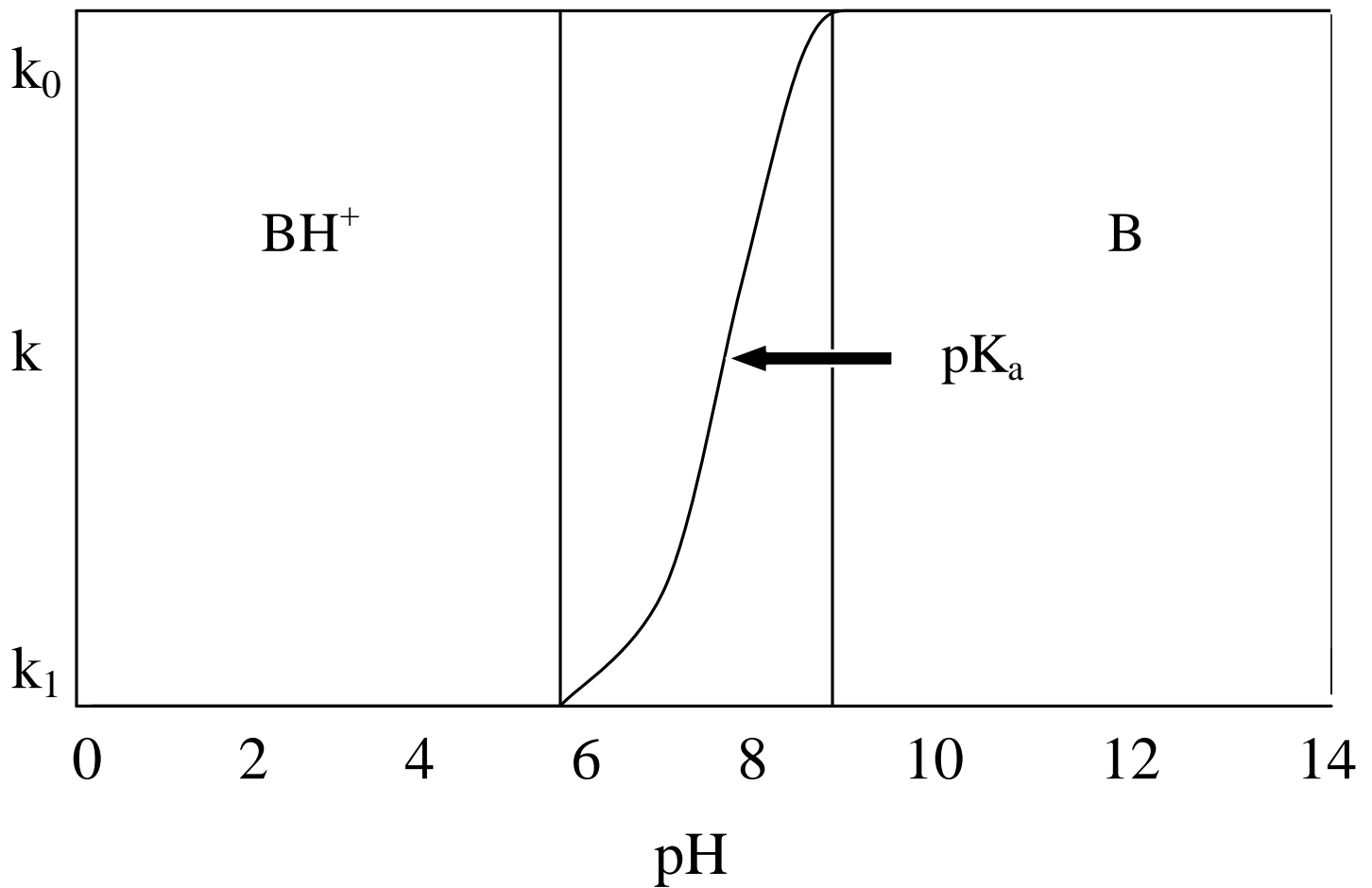


Figure 4-4. Equation and Theoretical Curve Showing Basic Analyte Retention as a Function of pH.

The pK_a of the analyte corresponds to the pH at the inflection point of the curve. The greatest retention is seen at pH values above the analyte pK_a , where B represents a deprotonated base. At this high pH, basic solutes show greater hydrophobic interaction with the C_{18} stationary phase. This curve demonstrates the significant changes in retention which occur within ± 2 units of the pK_a . In this range, analytes show both fronting and tailing peaks shapes [61].

The procedure for determining the chromatographic pK_a 's consists of measuring the retention time or capacity factor of the solute for several pH values [62]. In this study, the chromatographic pK_a values were determined using an Xterra RP_{18} column at 25°C . This temperature was selected so that chromatographic values would be comparable to literature values. Capacity factor values were determined for each compound at a ^spH of 2.65, 3, 4, 5, 6, and 7.5 using the citrate/phosphate mobile phase system with 15% acetonitrile added to both buffer solutions. Plots of capacity factor versus ^spH were constructed for each compound (Figure 4-6). From these plots and experimental data, chromatographic pK_a values were determined.

In order to determine pK_a values from the plots and experimental data, a full plot must be constructed. Due to the ^spH limitations of the mobile phase system (2.65-8.00), results could not be obtained for compounds with higher pK_a values. These compounds would have required evaluation at ^spH values up to about 12 in order to construct a useful plot. Despite the limitations, useful information about the retention of these compounds for the purposes of the silanol activity test procedure was collected.

Table 4-2 shows the results for the compounds for which full plots were constructed. Results found in this study were consistent with results from previous studies, which show a significant shift down in pK_a value for basic compounds in mixed mobile phases [53,61,62]. Acidic compounds usually show an increase in pK_a value in the presence of mixed mobile phases.

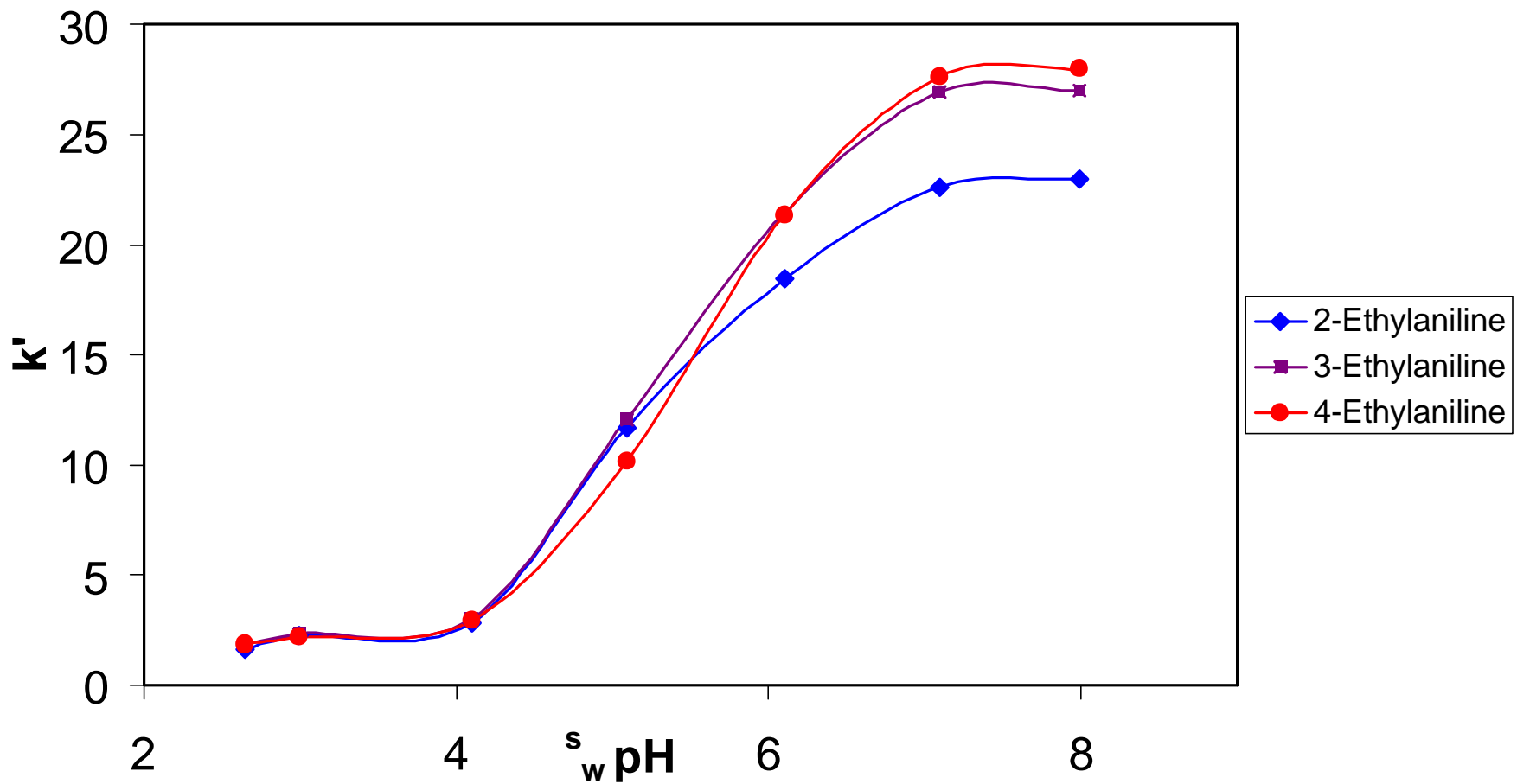


Figure 4-5. Plot of Capacity Factor versus pH for Ethylaniline Isomers

Table 4-2. Chromatographic pK_a Values versus Literature pK_a Values.

Compound	Chromatographic pK_a 25°C in Citrate/Phosphate System	Literature pK_a 25°C in Water
2-Ethylaniline	4.14	4.42
3-Ethylaniline	4.26	4.70
4-Ethylaniline	4.42	5.00
2-Picoline	5.79	5.96
3-Picoline	5.08	5.68
4-Picoline	5.41	6.00
2,5-Lutidine	6.18	6.43
3,5-Lutidine	5.52	6.09
2,4-Lutidine	6.29	6.74

Literature values were obtained from Lange's Handbook of Chemistry [55].

Sets of configurational isomers were originally selected in order to pattern the test procedure after a portion of the Engelhardt silanol activity test procedure [37]. In this test, Engelhardt evaluates ortho-, meta-, and para- ethylaniline to determine if there are any silanophilic contributions to retention. The isomers are structurally the same, therefore it is assumed that hydrophobic contributions should be constant and the only other contribution to retention comes from the fact that the isomers differ by their basicity. When the isomers are injected, if they elute in one peak, Engelhardt states that the contribution from silanophilic interactions can be deemed as negligible. However, if the isomers elute with multiple peaks this is due to their different interactions with residual silanols. For the new procedure, it was eventually decided that peak asymmetry would be a better indicator of silanol activity in order for the test to be universally accepted and used. Peak tailing is the most frequent characteristic associated with silanol activity and peak asymmetry is commonly used as a figure of merit for this property [52].

In order to simplify the procedure, it was decided to only use one compound from each isomer set. In order to maximize the exposure of the compound to the residual silanols, the para substituted isomer was selected from each set. According to studies conducted by McCalley, steric hindrance around the basic nitrogen atom of the solute reduces the interactions with silanols [42,43,48]. The final test solution included 4-ethylaniline, 4-picoline, 2,4-lutidine, 4-hydroxyquinoline, 4-nitrophenol, and 4-aminophenol.

Comprehensive Silanol Activity Test Procedure. Preliminary steps, molded by adherence to the goals and objectives set forth, led to a citrate/phosphate mobile phase system for which an accurate s_w pH value had been determined after the addition of organic modifier. In addition, compounds covering a wide range of pK_a values were selected and the pK_a values for these compounds were determined for the mobile phase in which the compounds would be analyzed. From this large set of compounds, a smaller sub-set was selected to use for analysis purposes. In order to get an accurate depiction of a column's response to a range of basic compounds at various s_w pH levels, it was decided

to determine the asymmetry factor of each compound at a s_w pH level of 2.65, 3.5, 4.5, 5.5, 6.5, and 7.40. The average A_s at each s_w pH level was then determined and used as the silanol activity indicator. This method provided an assessment of the column's silanol activity at every pH for which a typical reversed-phase column can be used. Because compounds with pK_a values from 4-11 are used as test probes, the column is exposed to fully protonated, fully deprotonated and partially protonated/deprotonated basic compounds at each pH level. This gives the most comprehensive assessment of silanol activity to date.


This procedure was tested on a group of four reversed-phase columns in addition to the Xterra RP₁₈ column. Average A_s values for each column at each s_w pH are shown in Table 4-3. These results were used to rank the columns in order of lowest to highest silanol activity at each s_w pH. Columns with the lowest average A_s value were judged to have the lowest silanol activity at a given pH. Columns with higher average A_s values were judged to have higher silanol activities. Column rankings can be seen in Table 4-4. Differences in average A_s values at a given s_w pH between columns with the lowest silanol activity and columns with the highest were shown to differ as much as 58%.

Table 4-4 clearly demonstrates the need to evaluate columns over the entire pH range versus the extremities. If this test was performed only at a s_w pH of 2.65 and 7.4, the SymmetryShield column would be considered the best column to use for the analysis of basic substances. This is an appropriate choice at those particular s_w pH levels and at a s_w pH of 3.50, however it is not the best choice at s_w pH levels 4.5, 5.5, and 6.5. The various columns demonstrate varying degrees of silanol activity at different s_w pH levels. This is probably due to the differences in pK_a of the silanols on the surface of the different packings. A study by Mendez and co-workers compared the acidity of residual silanol groups in several liquid chromatography columns [17]. This study found that while some columns exhibited two types of silanols, one which is very acidic and

Table 4-3. Average Theoretical Plates and Asymmetry Factor Values for Para-Substituted Isomers at Each pH

pH	ECLP	XTRA	SYMS	DISC	SYMM	Best Column
2.65						SYMS
A _s	1.80	2.08	1.15	2.54	1.54	
Plates	3800	3600	3100	1100	2500	
3.50						SYMS
A _s	2.09	1.96	1.39	2.14	1.74	
Plates	3500	3300	4000	1500	3000	
4.50						SYMM
A _s	1.79	1.36	1.38	1.51	1.31	
Plates	5100	6100	5300	1800	2200	
5.50						XTRA
A _s	1.58	1.14	2	1.3	1.26	
Plates	2400	5100	2800	2100	2100	
6.50						XTRA
A _s	1.46	1.24	1.76	1.71	1.86	
Plates	2200	9300	5900	2800	3000	
7.40						SYMS
A _s	1.65	1.56	1.52	1.93	2.06	
Plates	2100	4900	5000	2000	4400	
Avg. A_s	1.71	1.45	1.63	1.71	1.65	XTRA
Avg. Plates	3124	5746	4582	2074	2917	

Table 4-4. Column Rankings from Low to High Silanol Activity

pH	2.65	3.5	4.5	5.5	6.5	7.5
Lowest Silanol Activity  Highest Silanol Activity	SYMS	SYMS	SYMM	XTRA	XTRA	SYMS
	SYMM	SYMM	XTRA	SYMM	ECLP	XTRA
	ECLP	XTRA	SYMS	DISC	DISC	ECLP
	XTRA	ECLP	DISC	ECLP	SYMS	DISC
	DISC	DISC	ECLP	SYMS	SYMM	SYMM

one which is less acidic, some columns did not reveal the presence of any residual silanols between a pH of 3-7, thus proving the importance of testing over the entire useful pH range.

In order to test the accuracy of this procedure at predicting column performance based on silanol activity, an arbitrary analysis was used for comparison. All of the previous columns were used to analyze a mixture of piroxicam, phenol, and nortriptyline. These basic compounds have literature pK_a values of 5.10, 7.17, and 9.70 respectively. The mobile phase used for this analysis consisted of a 0.02M citrate buffer prepared to a pH of 4.0 before the addition of organic modifier. The organic modifier used was 70 % acetonitrile. The s_w pH of the final mobile phase was 4.65. This analysis was carried out at 25°C and 210 nm UV detection. The compounds were evaluated and asymmetry factor values for each compound were determined (Figures 4-6 and 4-7). The average of these values was used to determine column rankings. Columns were ranked from a low to high silanol activity and these rankings were compared to rankings from the silanol activity test procedure at a pH of 4.5. This particular set of rankings was compared at a s_w pH closest to that used in the test analysis. Comparison of the results from the silanol activity test at s_w pH 4.5 and the random test analysis showed identical rankings for all of the columns used in this study.

Conclusions

In this work, a silanol activity test that provides the components that have been lacking in previous tests has been developed. This test was designed with the goal of using a group of bases with a wide range of pK_a 's, using a mobile phase system that covered the entire useful pH range for RPLC columns, and monitoring the pH of the mobile phase after the addition of organic modifier. All goals were accomplished.

Results from this test procedure further indicate the need for testing over the entire pH range. Column rankings from a set of selected columns varied from one pH level to another, suggesting varying pK_a values for residual silanols on the surface of the packing. Columns labeled with the lowest silanol activity at one pH may be deemed as the column with the highest silanol activity at another pH. The accuracy of the test procedure was evaluated using a randomly selected group of basic compounds as test probes. The results from the test analysis matched with results from the silanol activity test procedure, thus suggesting that the silanol activity test was a good predictor of column performance at any given pH.

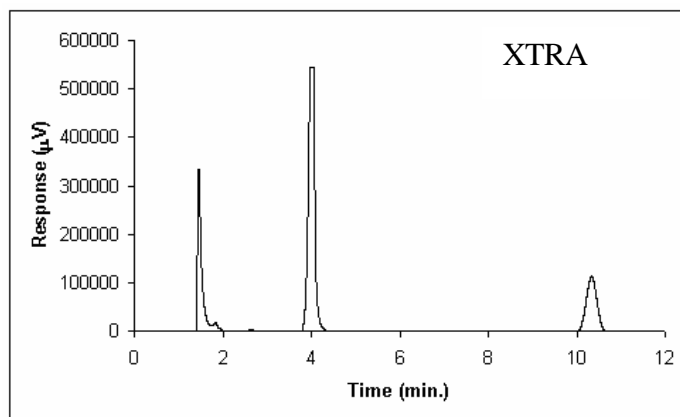
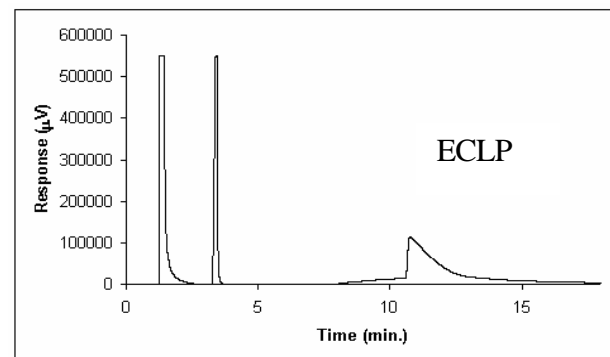
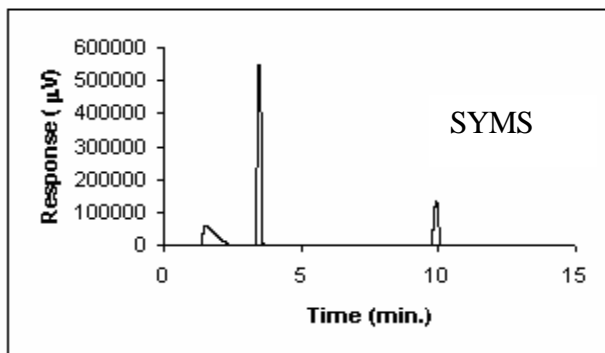
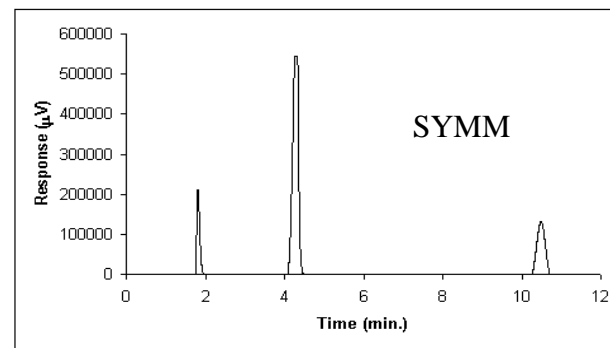
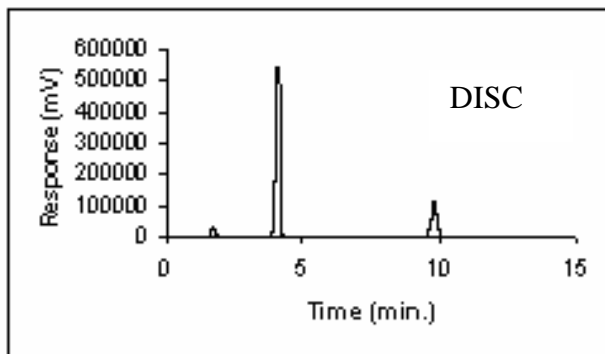


Figure 4-7. Results from Prediction Accuracy Test Method.

Piroxicam, Phenol, Nortriptyline in Acetonitrile/0.02M Citrate Buffer pH 4.0, 25°C, 254nm.

Chapter 5

Silanol Activity Test Procedure Utilizing a pH Gradient

Introduction

A more comprehensive silanol activity test procedure has been proposed in this body of work. The new procedure uses a single mobile phase system which has the ability to analyze reversed-phased columns over a $\frac{s}{w}$ pH range from 2.65 to 8.00. The new procedure has been shown to produce accurate predictions of column performance based on silanol activity at different pH levels. Although the test procedure is theoretically sound and analysis times are reduced because multiple buffers do not need to be prepared, the test procedure may be perceived as being too lengthy to perform on a routine basis.

One of the advantages of using a single mobile phase system is the ability to program the LC system, if an autosampler is available, to analyze all compounds at all pH levels. Although this advantage decreases the quantity of manpower required, it does not decrease the total time that the LC system is being occupied. In order to shorten this analysis time an investigation into the possibility of analyzing columns in one run, using the same fundamental tools from the previous test procedure, has been explored. In this study, the citrate/phosphate mobile phase system is used to produce an isocratic strength mobile phase with a pH gradient. This system is then applied to the analysis of basic compounds for the purposes of determining levels of silanol activity in reversed-phase columns.

Experimental

Mobile Phase. The mobile phase consists of a citrate/phosphate buffer system with 15% HPLC grade acetonitrile added to each solution. Solution A is a 0.01 M citrate solution with a s_w pH of 2.65. Solution B consists of a 0.02M phosphate solution with a s_w pH of 8.00. The forward linear gradient program provided a change in s_w pH from 2.80 to 8.00 and was set as follows:

<u>Citrate Solution</u>	<u>Phosphate Solution</u>	<u>Time (min.)</u>
90%	10%	0.0
0%	100%	15.0

The reverse linear gradient program provided a change in s_w pH from 7.90 to 2.65 and the program was set as follows:

<u>Citrate Solution</u>	<u>Phosphate Solution</u>	<u>Time(min.)</u>
10%	90%	0.0
100%	0%	15.0

Changes to the mobile phase pH were monitored at all times using a Sensorex 200 μ L flow cell apparatus (Figure 1-1).

Compounds and Columns. Compounds used in the final test mixture included uracil, 4-aminophenol, 4-hydroxyquinoline, 4-picoline, 2,4-lutidine, lidocaine, and 4-bromoaniline. All compounds except 4-aminophenol were mixed in a single solution with concentrations of 0.01mg/mL for thiourea, 0.08mg/mL for lidocaine and 4-bromoaniline, and 0.05mg/mL for all others. 4-aminophenol was added to 10 mL of the test mixture on a daily basis for a final concentration of 0.05 mg/mL. This was done to manage the photooxidizing effects of this particular compound.

The literature pK_a values for lidocaine and 4-bromoaniline are 8.01 and 3.88 respectively, values for all other compounds are shown in Table 4-2. The chromatographic pK_a for 4-bromoaniline was determined using procedures described in chapter 4. 4-Bromoaniline was found to have a chromatographic pK_a of 3.32. As with other high pK_a compounds, the chromatographic pK_a for lidocaine could not be determined due to the limited pH range of the mobile phase system.

An Xterra RP₁₈ (XTRA) column was used in the development of this particular procedure. After final test conditions had been determined, the procedure was performed on three other columns for comparative purposes. Columns selected were the same as those used in the analysis of the performance of the previous silanol activity test procedure, these include a SymmetryShield RP18 (SYMS) column, a Symmetry C₁₈ (SYMM) column, and a Eclipse XDB-C₁₈ (ECLP) column. The Discovery C₁₈ column was not used in this study because it started to show signs of deterioration.

Other Experimental Parameters. UV/VIS detection was used for this analysis at several wavelengths between 210 and 254 nm. Experiments were performed at 25°C. In order to compensate for the dwell volume, injections were made 3.6 minutes into the pH gradient program. This time coincided with the moment at which a change in pH would reach the top of the LC column. This was determined by connecting the flow cell to the system where the column would normally be positioned to detect changes in pH. On average the reequilibration time was about 10 minutes, however injections were not made until the pH returned to its initial value as indicated by the flow cell system.

Results and Discussion

Initial experiments were conducted using the same compounds from the previous silanol activity test procedure. These experiments were conducted using the forward linear gradient program. As seen in Figure 5-1, this was highly unsuccessful.

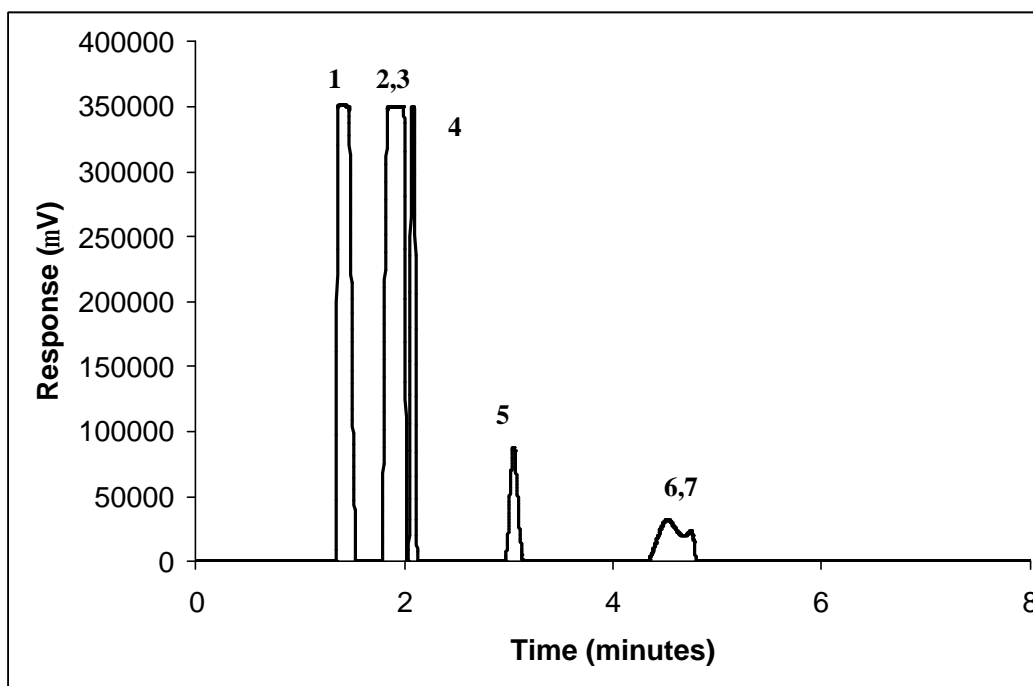


Figure 5-1. Forward Gradient Test Procedure Results
15 minute Linear Gradient 10% to 100% B
Peaks 1-7 corresponds to the following compounds: Sodium Nitrate,
4-aminophenol, 4-hydroxyquinoline, 4-picoline, 2,4-lutidine,
4-ethylaniline, 4-nitrophenol

This could be attributed to the early elution of many of the compounds at low pH levels. By the time the s_pH_w gradient had reached a s_pH_w of 4 or above, most compounds had already eluted. Data gathered from chromatographic pK_a studies indicated that most of the compounds would coelute until s_pH_w values of 4 or above were reached. Below this pH, the compounds are all fully charged and are not retained by the stationary phase. Above a s_pH_w of 4 the pK_a values of the various compounds are reached and they begin to become deprotonated. Once compounds are deprotonated they can interact with both the alkyl chains and the residual silanols.

This dilemma was resolved by using a reverse gradient method. The reverse linear gradient program allowed for the elution of all of the compounds with good resolution and peak shapes in less than 25 minutes, thus reducing the analysis time as compared to the previous test procedure. Initially, problems with baseline drift were encountered using this reverse system at 210 nm. This was due to the increasing amounts of citrate buffer in the mobile phase as the program progressed. Citrate solutions can be detected using UV/VIS detection at wavelengths up to 230 nm [7]. A number of wavelengths were evaluated until a flat base line was achieved (Figure 5-2). It was noted that as the wavelength was increased the intensity of the t_0 marker decreased and it could no longer be detected, therefore the t_0 marker was changed to uracil. In order to ensure that the selected compounds covered the range of elution pH values, substitutions were made to the initial compounds used in the previous silanol activity test. 4-ethylaniline was replaced by 4-bromoaniline and 4-nitrophenol was replaced by lidocaine. This produced peaks which did not overlap and it more evenly distributed compounds across the elution pH range, the final detection wavelength was set at 254nm (Figure 5-3).

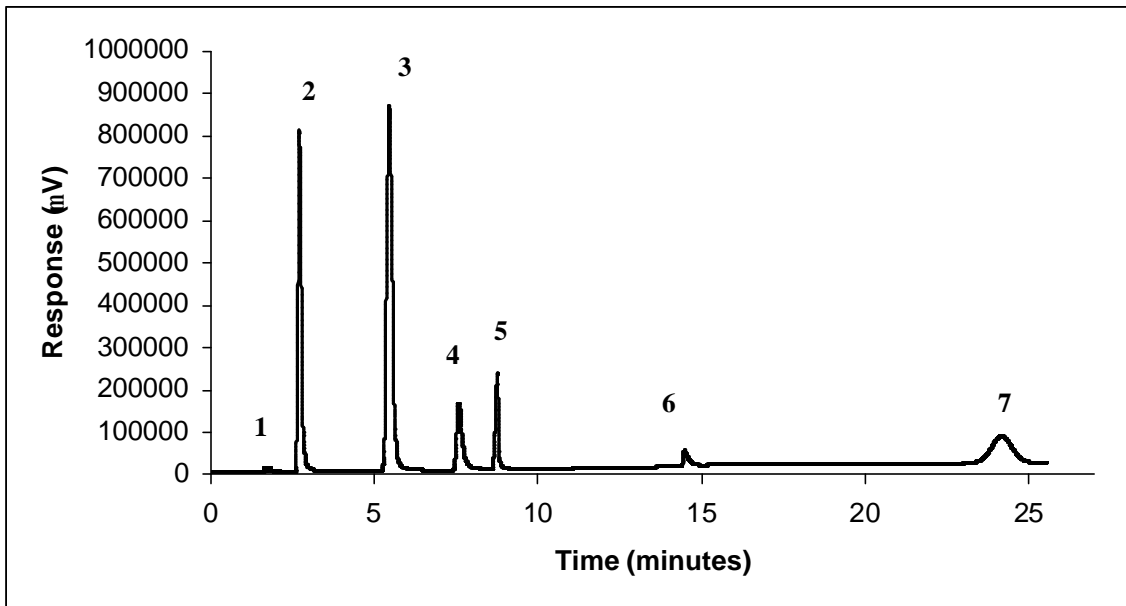
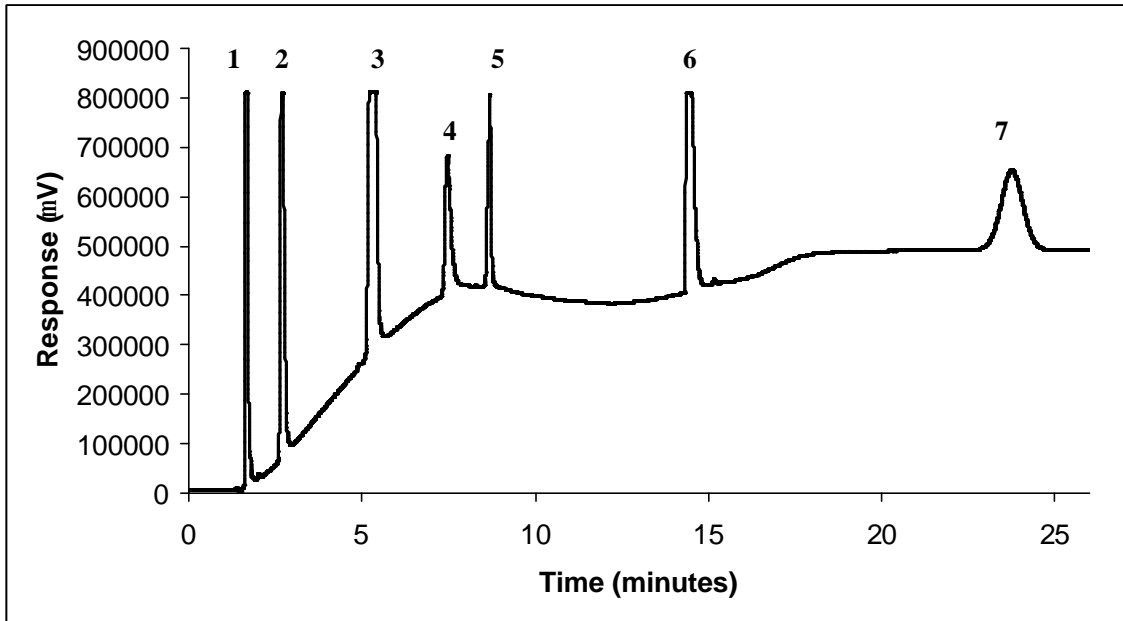


Figure 5-2. Reverse Gradient Test Procedure Results at 210nm and 245nm.

15 minute Linear Gradient 90% to 0% B

Peaks 1-7 corresponds to the following compounds: Sodium Nitrate,
 4-aminophenol, 4-hydroxyquinoline, 4-picoline, 2,4-lutidine,
 4-ethylaniline, 4-nitrophenol

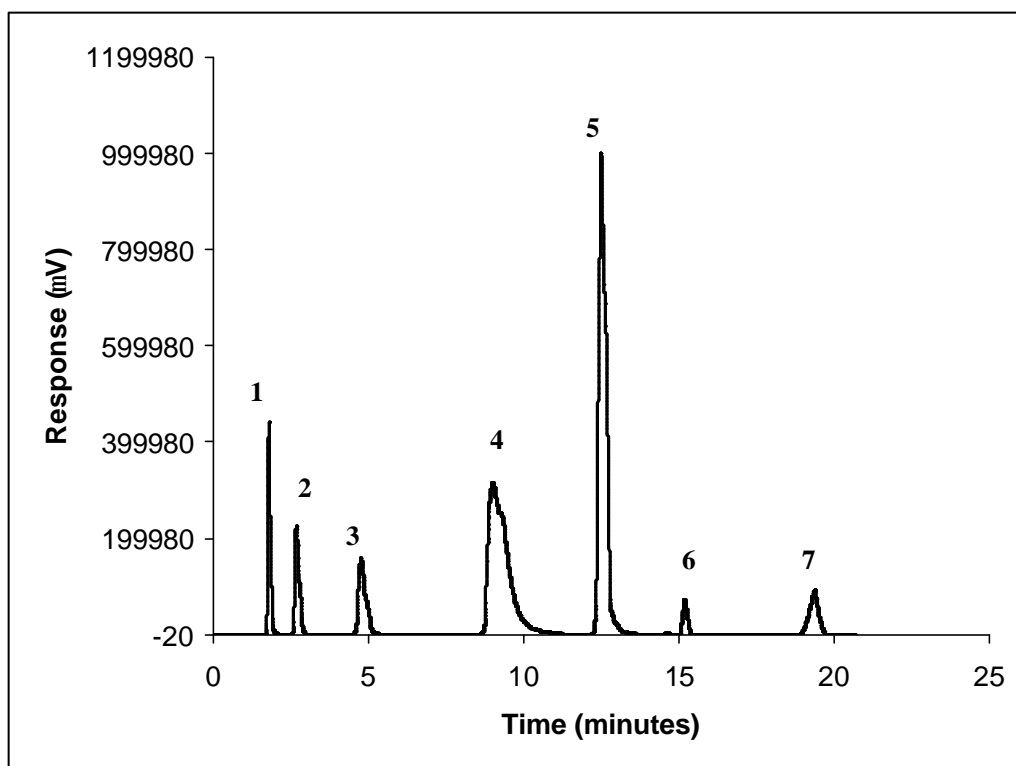


Figure 5-3. Reversed Gradient Test Procedure Results with Final Experimental Conditions.

15 minute Linear Gradient 90% to 0% B
Peaks 1-7 corresponds to the following compounds: Uracil,
4-aminophenol, 4-hydroxyquinoline, 4-picoline, 2,4-lutidine,
lidocaine, 4-bromoaniline

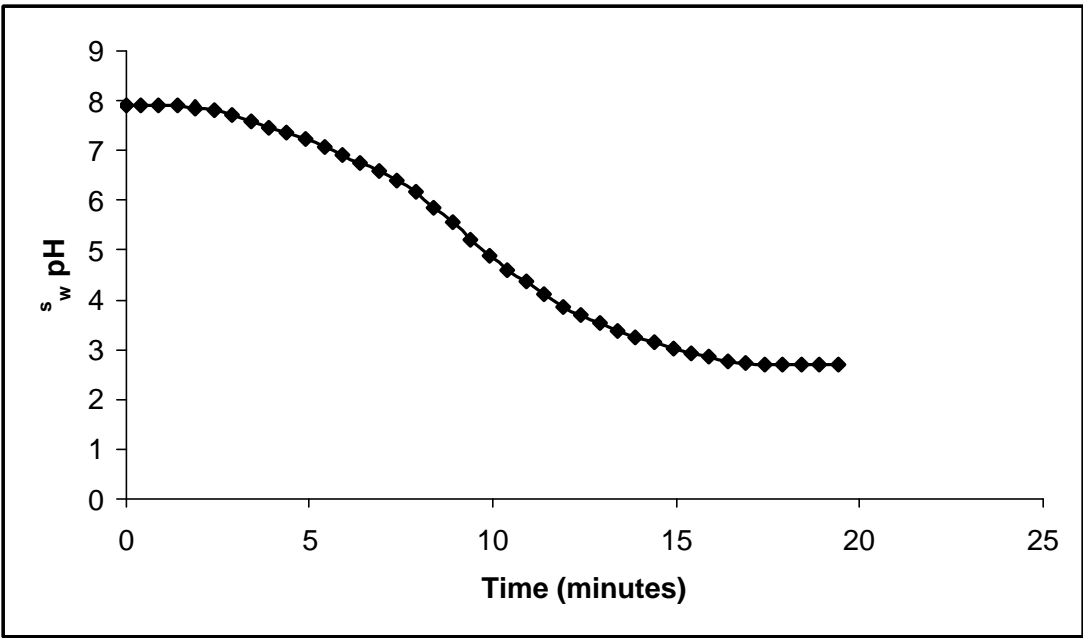


Figure 5-4. Plot of s_w pH versus Time for Reversed Gradient Test Procedure

Table 5-1. Elution Order and Elution s_w pH Values for Test Compounds

Compounds	Literature pK_a	Chromatographic pK_a	Elution s_w pH
Uracil	*	*	8.0
4-aminophenol	8.50	*	7.8
4-hydroxyquinoline	11.25	*	7.4
4-picoline	6.0	5.41	6.8
4-lutidine	6.74	6.29	6.1
Lidocaine	8.01	*	4.3
4-bromoaniline	3.88	3.32	2.7

Literature values were obtained from Lange's Handbook of Chemistry [55].

*Values not measured.

Once initial experimental considerations were designed, elution order and elution ^spH needed to be determined. A graph of the mobile phase ^spH versus time was constructed using the flow cell apparatus to determine the ^spH of the mobile phase at any time during the course of the analysis (Figure 5-4). Compounds in Table 5-1 are listed in order of elution seen in the chromatogram. Literature pK_a values and elution pH 's are listed as well. The chromatographic pK_a values, where applicable, are also listed. This procedure was performed on three other columns in order to see if the procedure transferred well from one column to another. The results from these columns are presented in Table 5-2 along with results from the Xterra RP_{18} column.

Unlike the previous test procedure, determinations about column performance at each pH were not as easy to ascertain. While some pH levels were represented by the elution of two compounds, none of the selected compounds eluted at a ^spH of 3 or 5 for the Xterra RP_{18} column. Examination of column rankings, according to the A_s values of peaks that eluted at certain pH levels, did not show any correlation between results from this procedure and results at the same pH level using the test procedure described in Chapter 4. This can be attributed to the fact that A_s values in the current study are dependent on only one or two compounds per pH level, while results from the previous procedure consisted of average A_s values for six different basic compounds. Collecting average figures from six basic compounds with pK_a values ranging from 4-11 allowed for the assessment of the column's reaction to all possible forms of basic compounds. At any given pH , the column was exposed to compounds that were in various stages of the protonation/deprotonation process. The current procedure exposes the column to compounds at different stages of this process depending on which pH is selected. For example with the Xterra RP_{18} column, lidocaine ($\text{pK}_a=8.0$) elutes at a ^spH of 4.3. At this point the compound is still fully protonated and thus available to react with deprotonated silanols available on the surface of the column. 4-picoline ($\text{pK}_a=5.41$) elutes at a ^spH of 6.8, where the compound is already present in its deprotonated form

Table 5-2. Reversed Gradient Results for Various Columns

	4-Aminophenol	4-Hydroxyquinoline	4-Picoline	2,4-Lutidine	Lidocaine	4-Bromophenol
XTRA						
Plates	1700	1000	4100	2300	6200	8400
A _s	3.27	3.59	4.29	1.84	3.6	1.6
Elution _w ^s pH	7.8	7.4	6.8	6.2	4.3	2.7
SYMS						
Plates	1100	1100	3500	5600	6100	9700
A _s	2	2.16	4.09	1.62	2.73	0.8902
Elution _w ^s pH	7.8	7.2	5.6	3.7	3	2.6
ECLP						
Plates	2200	1300	Coelution w/ 2,4-lutidine	Coelution w/ Picoline	8200	7800
A _s	1.21	2.09	Coelution w/ 2,4-lutidine	Coelution w/ Picoline	3.75	0.6366
Elution _w ^s pH	7.8	7.6	Coelution w/ 2,4-lutidine	Coelution w/ Picoline	3.2	2.6
SYMM						
Plates	1700	3000	2900	7400	8800	5000
A _s	0.9286	0.9675	2.83	0.664	5.46	0.5478
Elution _w ^s pH	7.8	7.7	5.8	5.3	3.3	2.6

and unavailable for ionic interactions. Meanwhile, 4-lutidine ($pK_a=6.29$) elutes at a $^s pH_w$ of 6.1. Here, the compound is most likely available in nearly equal amounts of the protonated and deprotonated form. The present conditions not only make it difficult to compare results to the previous test procedure, but it also makes comparisons to itself impossible. Comparing a column's performance with a fully deprotonated compound at one pH to the column's performance with a fully protonated compound at another pH does not produce any useful information in the realm of silanol activity assessment. In addition, elution $^s pH_w$ values for a particular compound varied from column to column. Thus, one column may not have any compounds to elute at a $^s pH_w$ of 5, while another column may not have any compounds to elute at a $^s pH_w$ of 6.

Conclusion

In this study, a new approach to examining silanol activity was explored using the foundation from a proven procedure in conjunction with a novel idea for the mobile phase system. This approach was examined in order to reduce the analysis time and introduce a more sufficient test for industrial use. The mobile phase system from the previous procedure was used to create an isocratic strength mobile phase gradient. The mobile phase $^s pH_w$ was set to decrease from 7.90 to 2.65 over the course of 15 minutes. All compounds in the final test mixture eluted in less than 25 minutes for all columns used in this study. While analysis times were significantly decreased, the ability of the test procedure to predict a column's silanol activity at any given pH was also diminished. Overall, this study introduces a fresh approach for silanol activity testing at numerous pH levels without making new buffer solutions and with greatly reduced analysis times. Improvements to the current procedure should include the selection of new test compounds which will allow for results from one pH to be compared to results from an additional pH.

Chapter 6

Summary and Recommendations

Although an ideal support has not been developed for reversed-phase stationary phases, the vast majority of stationary phases in RPLC have been and are still being prepared using microparticulate silica. The silica surface has a number of properties which make it attractive for derivatization, including easily controlled particle size and porosity and mechanical stability. However, there are a number of characteristics of silica which are undesirable. For example, the operation of silica-based stationary phases is limited to certain pH levels. Below a pH of 2 siloxane bonds begin to hydrolyze and above a pH of 8 silica particles begin to dissolve. Another problem impeding the performance of reversed-phase columns is the presence of unreacted silanol groups. A fully hydroxylated silica surface has a maximum surface concentration of silanol sites of about $8 \mu\text{mol}/\text{m}^2$. However, due to steric hindrance, only about half of these sites are ever used for bonding. This results in residual silanols within the bonded phase. There are several types of surface silanols, which have their own unique properties that affect both chemical derivatization reactions and adsorptive interactions with solutes. Unfortunately, there is still no universally agreed upon method to measure the accessibility or interaction of these silanols with solute molecules.

The overall goal of this project was to develop a universally accepted chromatographic silanol activity test. The invention of such a test would allow for a column classification system for reversed-phase columns. This classification system would enable researchers to select columns more appropriately and with greater

confidence. The project consisted of two distinct phases. In phase one, the current silanol activity tests were examined in an intensive comparison study. This study not only answered unrequited questions about the current test procedures, but it also revealed gaps in the development of these procedures. In phase two an attempt to devise a universally accepted test was made. In order to make the test unique, it was designed specifically to encompass areas which had been left uncovered by previous test procedures.

In phase one, an investigation into the conformity between results from various silanol activity tests was made. There had been very little investigation into this matter in the literature. In fact, before this study was conducted only two other studies had been executed. Previous studies found that the results of the various silanol activity tests varied significantly. Columns which would be ranked as having low silanol activity with one testing procedure would be ranked as having a high silanol activity by another method. In addition, it was determined that column classifications which are based on silanol activity values are dependent upon which test procedure is employed. While both of the previous studies showed a lack of agreement in results from silanol activity test procedures, a number of key concerns were left unanswered.

In this body of work, the goal was to address the vital issues which remained unanswered in the literature. A wide variety of silanol activity test procedures were compared using a number of commonly used reversed-phase columns. The objectives were to further display inconsistencies between the current tests, to demonstrate the ineptness of older procedures at distinguishing between newer generation phases, and to determine if rankings from newer procedures were in agreement with each other. In addition, a thorough examination of the current silanol activity tests was included. Tests were evaluated for theoretical consistency and applicability in a laboratory setting.

As expected, the classifications of the different columns based on the measurement of their silanol activities by diverse tests were vastly inconsistent. Columns were shown to have different levels of active silanol sites depending on which test procedure is used for analysis. This study also demonstrated that older test procedures

were less effective at distinguishing between the subtle differences in newer generation phases. Results from older procedures indicated that the vastly different columns would produce similar results when analyzing basic substances, although this is not accurate. Results from new test procedures showed more agreement in results, especially when test procedures at similar pH levels were examined.

It was also determined that none of the current tests could serve sufficiently as the one test used for silanol activity across the board. Examination of the tests showed that the test procedures do not always use the full definition of silanol activity. Those few tests which do take all interactions into account, fail to use relevant basic substances in their assessments. It was noted that there is not a single test which examines the columns over their entire useful pH range, although there can be contributions to silanol activity from silanols at pH levels between 3 and 7. One of the biggest problems with the current tests was that they failed to account for the changes that occur to pH and pK_a values upon the addition of organic modifier.

There have been a number of studies conducted which have examined the effects of different experimental parameters on the analysis of basic substances by RPLC. These evaluations, along with the study conducted in this body of work, provided some insight into which qualities future tests would need to include in order to be universally used and accepted. In phase two, an attempt was made to develop such a test. The goal was to develop a silanol activity test that provides the measurements that have been deficient in previous tests. Focus was placed on using basic compounds with wide ranges of pK_a 's, using a mobile phase system that covered the entire useful pH range for typical RPLC columns, and monitoring the pH of the mobile phase after the addition of organic modifier.

The newly designed test procedure used a single mobile phase system to examine columns. This citrate/phosphate buffer system, with 15% acetonitrile added to both solutions, resulted in a pH_w range of 2.65-8.00. This more rigorous pH scale accounts for the addition of organic modifier to the mobile phase. Test solutes included aromatic

compounds with nitrogen groups in the structure. These compounds had pK_a values which ranged from 4-11. In order to account for changes in the pK_a values as a result of the presence of the acetonitrile in the mobile phase, chromatographic pK_a values were determined in the citrate/phosphate mobile phase system. The silanol activity indicator selected ensured the assessment of the column's reaction to a diverse group of compounds. The average A_s value of all of the compounds at each pH was used to provide predictions about column performance when analyzing basic substances at any given pH within the useful pH range for reversed-phase columns. Results from this test procedure also further indicated the need for testing over the entire pH range. Column rankings from a set of selected columns varied from one pH level to another. Columns labeled with the lowest silanol activity at one pH would be deemed as the column with the highest silanol activity at another pH, thus suggesting that there are varying pK_a values for residual silanols on the surface of the packing. The accuracy of the test procedure was evaluated using a randomly selected group of basic compounds as test probes. The results from the test analysis matched with results from the silanol activity test procedure, thus suggesting that the silanol activity test was a good predictor of column performance at any given pH.

Despite the theoretical aptitude of the test and the reduced analysis times due to the avoidable multiple buffer preparations, the newly designed procedure could be perceived as being too lengthy to perform on a repetitive basis. In order to shorten this analysis time an investigation into the possibility of analyzing columns in one run, using the same fundamental tools from the previous test procedure, was explored. The citrate/phosphate mobile phase system was used to produce an isocratic strength mobile phase with a pH gradient. The mobile phase pH_w decreased from 7.90 to 2.65 using a 15-minute linear gradient. All compounds in the final test mixture eluted in less than 25 minutes for all columns used in this study. Unfortunately, the decrease in analysis time also resulted in a decline in the ability of the procedure to effectively analyze columns based on silanol activity. The procedure exposed the column to compounds at different stages of the protonation/deprotonation process at different pH levels making accurate silanol activity assessments impossible. Comparing a column's performance with a fully

deprotonated compound at one pH to the column's performance with a fully protonated compound at another pH does not produce any useful information in the realm of silanol activity assessment. In addition, elution s_w pH values for a particular compound varied from column to column. Therefore, there were some columns which did not have compounds eluting at certain s_w pH values. Overall, this particular study produced a novel approach for silanol activity testing at numerous pH levels without making new buffer solutions and with greatly reduced analysis times.

The work presented here demonstrates promising results in the area of silanol activity characterization methods. The proposed isocratic procedure is fundamentally sound and has been proven to predict column performance according to silanol activity. The acceptance of this procedure as a universally accepted test would need to involve a study on the robustness of the test procedure. In addition the column performance prediction capabilities should be tested at various pH levels. An examination into the differences between results when using methanol as the organic modifier for the test procedure would also be an interesting investigation. Ultimately the test procedure should also be performed using a larger number of different reversed-phased columns.

Although the gradient procedure did not produce a test which could accurately be used to examine silanol activity, further investigation may lead to the development of a useful procedure. The use of different test solutes should be extensively examined. The ideal situation is to have test solutes which will elute during the same stage of the protonation/deprotonation process. Test solutes should be selected that will elute when they are still fully protonated in order to assess any contributions from secondary interactions. Although aromatic compounds must be used to ensure detection at the current wavelength, the test solute selection should not be limited to isomeric compounds. Final test solutes should then be examined by the same processes suggested for the isocratic test procedure to ensure reliability and robustness.

APPENDIX

Initial results from the comparison of silanol activity tests are presented. Results represent the average value of three consecutive experiments for each column.

Table A-1. Engelhardt Silanol Activity Test Results

Methanol/1 mM Phosphate Buffer pH 7 55:45 (v/v), 40°C, 1mL/min, 254nm

Column	A_s p-Ethylaniline
Discovery C ₁₈	1.25
Supelcosil-LC-18-DB	1.46
Symmetry 300C ₁₈	3.20
Symmetry C ₁₈	1.30
Symmetry Shield RP18	1.16
YMC Pro 18	1.20
YMC Basic S5	1.31
Spherisorb ODS-2	1.50
Spherisorb ODS-1	1.56
μBondapack C ₁₈	1.10
Nova-Pak C ₁₈	2.47
Zorbax Rx-C ₁₈	1.23
Eclipse XDB-C ₁₈	1.44
Zorbax SB-C ₁₈	1.54
Zorbax ODS	1.25

Table A-2. Goldberg-Basic Silanol Activity Test Results

Acetonitrile/ 10mM Sodium Acetate pH 4.5 20:80(v/v), 30°C, 1mL/min, 254nm

Column	k' caffeine/ k' theophylline
Discovery C ₁₈	2.11
Supelcosil-LC-18-DB	2.21
Symmetry 300C ₁₈	2.17
Symmetry C ₁₈	2.27
Symmetry Shield RP18	1.84
YMC Pro 18	2.23
YMC Basic S5	2.09
Spherisorb ODS-2	2.44
Spherisorb ODS-1	2.69
μBondapack C ₁₈	2.14
Nova-Pak C ₁₈	2.40
Zorbax Rx-C ₁₈	2.70
Eclipse XDB-C ₁₈	2.36
Zorbax SB-C ₁₈	2.51
Zorbax ODS	2.59

Table A-3. Goldberg-Polar Silanol Activity Test Results

Methanol/Water 65:35(v/v), 30°C, 1mL/min, 254nm

Column	k' dimethyl phthalate/ k' diethyl phthalate
Discovery C ₁₈	2.53
Supelcosil-LC-18-DB	2.60
Symmetry 300C ₁₈	2.54
Symmetry C ₁₈	2.43
Symmetry Shield RP18	1.93
YMC Pro 18	2.56
YMC Basic S5	2.07
Spherisorb ODS-2	1.92
Spherisorb ODS-1	1.48
μBondapack C ₁₈	1.53
Nova-Pak C ₁₈	2.34
Zorbax Rx-C ₁₈	2.27
Eclipse XDB-C ₁₈	2.34
Zorbax SB-C ₁₈	2.77
Zorbax ODS	2.81

Table A-4. Tanaka Hydrogen Bonding Silanol Activity Results

Methanol/Water 30:70(v/v), 30°C, 1mL/min, 254nm

Column	$k'_{\text{caffeine}} / k'_{\text{phenol}}$
Discovery C ₁₈	1.92
Supelcosil-LC-18-DB	2.18
Symmetry 300C ₁₈	2.47
Symmetry C ₁₈	2.11
Symmetry Shield RP18	2.13
YMC Pro 18	1.91
YMC Basic S5	1.88
Spherisorb ODS-2	1.03
Spherisorb ODS-1	2.57
μBondapack C ₁₈	1.27
Nova-Pak C ₁₈	1.67
Zorbax Rx-C ₁₈	1.41
Eclipse XDB-C ₁₈	1.88
Zorbax SB-C ₁₈	1.45
Zorbax ODS	1.52

A-5. Tanaka Ionic Exchange (pH 7) Silanol Activity Test Results

Methanol/ 0.02 M Phosphate Buffer pH 7.6 30:70(v/v), 30°C, 1mL/min, 254nm

Column	$k'_{\text{benzylamine}} / k'_{\text{phenol}}$
Discovery C ₁₈	0.30
Supelcosil-LC-18-DB	0.35
Symmetry 300C ₁₈	0.30
Symmetry C ₁₈	0.37
Symmetry Shield RP18	0.27
YMC Pro 18	0.27
YMC Basic S5	0.39
Spherisorb ODS-2	0.23
Spherisorb ODS-1	0.41
μBondapack C ₁₈	0.49
Nova-Pak C ₁₈	0.73
Zorbax Rx-C ₁₈	0.71
Eclipse XDB-C ₁₈	0.49
Zorbax SB-C ₁₈	0.87
Zorbax ODS	0.35

Table A-6. Tanaka Ionic Exchange (pH 3) Silanol Activity Results

Methanol/ 0.02 M Phosphate Buffer pH 2.7 30:70(v/v), 30°C, 1mL/min, 254nm

Column	$k'_{\text{benzylamine}} / k'_{\text{phenol}}$
Discovery C ₁₈	0.09
Supelcosil-LC-18-DB	0.12
Symmetry 300C ₁₈	0.09
Symmetry C ₁₈	0.06
Symmetry Shield RP18	0.03
YMC Pro 18	0.07
YMC Basic S5	0.09
Spherisorb ODS-2	0.17
Spherisorb ODS-1	0.23
μBondapack C ₁₈	0.15
Nova-Pak C ₁₈	0.11
Zorbax Rx-C ₁₈	0.07
Eclipse XDB-C ₁₈	0.08
Zorbax SB-C ₁₈	0.09
Zorbax ODS	0.14

Table A-7. Verzele and Dewaele Silanol Activity Test Results

Methanol/ Water w/ 0.5% Sodium Acetate 60:40(v/v), 25°C, 1mL/min, 254nm

Column	$k'_{\text{naphthalene}} / k'_{\text{nitronaphthalene}}$
Discovery C ₁₈	2.56
Supelcosil-LC-18-DB	1.64
Symmetry 300C ₁₈	1.68
Symmetry C ₁₈	1.81
Symmetry Shield RP18	1.25
YMC Pro 18	1.65
YMC Basic S5	1.20
Spherisorb ODS-2	1.60
Spherisorb ODS-1	1.11
μBondapack C ₁₈	1.45
Nova-Pak C ₁₈	1.74
Zorbax Rx-C ₁₈	1.92
Eclipse XDB-C ₁₈	1.74
Zorbax SB-C ₁₈	1.62
Zorbax ODS	1.75

Table A-8. Manufacturer Hydrogen Bonding Silanol Activity Test

Methanol/ 25mM Phosphate Buffer pH 2.5 17:83 (v/v), 30°C, 2 mL/min, 260nm

Column	A_s of Caffeine/ A_s of Phenol
Discovery C ₁₈	0.740
Supelcosil-LC-18-DB	0.668
Symmetry 300C ₁₈	0.664
Symmetry C ₁₈	0.451
Symmetry Shield RP18	0.772
YMC Pro 18	Coelution of Caffeine and Phenol peaks
YMC Basic S5	0.408
Spherisorb ODS-2	Coelution of Caffeine and Phenol peaks
Spherisorb ODS-1	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks
μBondapack C ₁₈	1.43
Nova-Pak C ₁₈	1.55
Zorbax Rx-C ₁₈	2.40
Eclipse XDB-C ₁₈	Coelution of Caffeine and Phenol peaks
Zorbax SB-C ₁₈	2.21
Zorbax ODS	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks

Table A-9. Manufacturer Ionic Interactions Silanol Activity Results

Methanol/ 25mM Phosphate Buffer pH 7.5 17:83 (v/v), 30°C, 2 mL/min, 260nm

Column	A_s of Caffeine/ A_s of Phenol
Discovery C ₁₈	0.82
Supelcosil-LC-18-DB	1.17
Symmetry 300C ₁₈	1.20
Symmetry C ₁₈	1.21
Symmetry Shield RP18	0.82
YMC Pro 18	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks
YMC Basic S5	0.65
Spherisorb ODS-2	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks
Spherisorb ODS-1	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks
μBondapak C ₁₈	1.91
Nova-Pak C ₁₈	Coelution of Caffeine and Phenol peaks Coelution of Procaine and N,N-Dimethylaniline peaks
Zorbax Rx-C ₁₈	0.76
Eclipse XDB-C ₁₈	1.16
Zorbax SB-C ₁₈	1.91
Zorbax ODS	5.91

Table A-10. McCalley (pH 3) Silanol Activity Test

Methanol/ 25mM Phosphate Buffer pH 3.0 30:70 (v/v), 30°C, 1 mL/min, 254 or 210 nm

Column	Average A_s
Discovery C ₁₈	1.28
Supelcosil-LC-18-DB	2.51
Symmetry 300C ₁₈	1.37
Symmetry C ₁₈	1.27
Symmetry Shield RP18	1.33
YMC Pro 18	1.45
YMC Basic S5	1.38
Spherisorb ODS-2	3.14
Spherisorb ODS-1	2.91
μBondapack C ₁₈	1.47
Nova-Pak C ₁₈	2.00
Zorbax Rx-C ₁₈	1.65
Eclipse XDB-C ₁₈	1.54
Zorbax SB-C ₁₈	1.45
Zorbax ODS	1.98

Table A-11. McCalley (pH 7) Silanol Activity Test Results

Methanol/ 25mM Phosphate Buffer pH 7.0 30:70 (v/v), 30°C, 1 mL/min, 254 or 210 nm

Column	Average A_s
Discovery C ₁₈	1.39
Supelcosil-LC-18-DB	4.17
Symmetry 300C ₁₈	2.05
Symmetry C ₁₈	4.07
Symmetry Shield RP18	1.78
YMC Pro 18	2.09
YMC Basic S5	2.48
Spherisorb ODS-2	1.68
Spherisorb ODS-1	2.11
μBondapack C ₁₈	2.07
Nova-Pak C ₁₈	2.64
Zorbax Rx-C ₁₈	1.63
Eclipse XDB-C ₁₈	2.48
Zorbax SB-C ₁₈	1.53
Zorbax ODS	1.10

Table A-12. SRM 870 Silanol Activity Test Results

Methanol/ 5mM Phosphate Buffer pH 7.0 80:20 (v/v), 25°C, 2 mL/min, 254 nm

Column	A_s Amitriptyline
Discovery C ₁₈	1.89
Supelcosil-LC-18-DB	8.90
Symmetry 300C ₁₈	1.31
Symmetry C ₁₈	4.21
Symmetry Shield RP18	2.47
YMC Pro 18	3.18
YMC Basic S5	2.51
Spherisorb ODS-2	8.50
Spherisorb ODS-1	6.30
μBondapack C ₁₈	3.63
Nova-Pak C ₁₈	5.06
Zorbax Rx-C ₁₈	8.34
Eclipse XDB-C ₁₈	1.55
Zorbax SB-C ₁₈	2.27
Zorbax ODS	6.22

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BIOGRAPHICAL SKETCH

Sydana D. Rogers was born in Ft. Dix, New Jersey on July 2, 1977 to Kenneth and Mary Rogers. Her father's military career resulted in her upbringing in a number of different locations on the east coast, in England, and in Germany, where she graduated from Ramstein American High School. Upon graduation, Sydana was awarded a full scholarship through the Dozoretz National Institute for Minorities in Applied Sciences (DNIMAS) Scholarship Program at Norfolk State University in Norfolk, VA. Sydana graduated magna cum laude with an ACS certified degree in chemistry from Norfolk State University in the Spring of 1998. In the fall of 1998, Sydana began the graduate program at Florida State University and she joined the Dorsey research group in the Spring of 1999. Sydana earned her doctorate in August of 2003, making her the first African-american to earn a Ph.D. in Chemistry at Florida State University. After graduation she took a position with Philip Morris USA in Richmond, Virginia.