Reverse-Phase Liquid Chromatography Of Small Molecules Using Silica Colloidal Crystals

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For the degree of Master of Science

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ABSTRACT


Capillaries packed with a silica colloidal crystal (SCC) of 470 nm nonporous particles have recently been shown to offer significant improvements in the peak width, resolution, and speed of protein analysis due to the reduction of the A and C term broadening contributions of the Van Deemter equation. While protein separations using this technology have shown vast improvements, small molecules remain largely unexplored. As the SCC allows for an approximately diffusion limited system, extremely high plate numbers are expected for small molecule separations. The research presented herein studies the feasibility of pressure-driven small molecule separation using SCCs and commercial instrumentation. Studies are performed both in capillaries and stainless steel columns.

Separating small molecules presents a unique challenge due to their size and high diffusion coefficients. For capillary SCC separations, these properties are shown here to exacerbate problems in the nano-ultra high performance liquid chromatography (UHPLC) commercial instrumentation, such as extra-column band broadening and adherence to tubing walls. While this is less of a problem for gradient separations, isocratic separations are considerably affected. Thus, the full potential of these capillary
columns cannot currently be realized. Separations using UHPLC and stainless steel columns circumvent these problems. However, an unprecedented amount of broadening from longitudinal diffusion was found to limit the achievable plate heights in these columns to about 200,000 plates/m, which is on par with the latest commercially available columns. Significantly higher flow rates will need to be achieved to increase efficiency.
CHAPTER 1: INTRODUCTION

1.1 Current Role of Separation Science in Pharmaceutical Analysis

Pharmaceutical analysis laboratories are constantly striving to increase sample throughput and analysis speed to meet drug development timelines. These analyses are primarily performed using high performance liquid chromatography (HPLC) because the numerous combinations of mobile and stationary phase allow for the separation and analysis of a wide range of compounds\textsuperscript{1,2}. This technique is well-established and the methods are generally reproducible and accurate.

HPLC analysis is used in all stages of pharmaceutical drug development, from early drug discovery to final quality control laboratories. There is therefore considerable interest in reducing HPLC method run time both in terms of decreasing analysis time and solvent consumption. However, this separation time can be reduced only insofar as the resolution of the peaks of interest is not compromised. Peak resolution is dictated by three main factors: column efficiency, selectivity, and the retention factor.

When considering column efficiency, the greatest strides in improvement have come from the development of columns packed with sub-2 µm particles. Relatively short (5-10 cm) columns packed with porous sub-2 µm particles have been shown to exhibit both high speeds and extremely efficient separations compared to the more traditional larger particles\textsuperscript{1,3,4}. However, these small particles are also associated with significantly
higher backpressures, which restricts the analysis speeds when using conventional HPLC due to the instrument’s operational limits. Ultra-high performance liquid chromatography (UHPLC) instruments address this problem by extending the operational pressure range. UHPLC using sub-2µm particles is therefore the preferred instrumentation in this field.

Many analytical laboratories are still equipped with traditional HPLC instruments. Several strategies have therefore been developed to perform fast and efficient analyses using this technology. Specifically, monolith columns and core-shell particles meet this need. Both technologies can increase efficiency and analysis speed by reducing the diffusion path of the analyte inside the stationary phase while keeping column resistance low. Furthermore, high temperature liquid chromatography (HTLC) has also been shown to reduce mobile phase viscosity and backpressure. High flow rates can therefore be used even with long columns or smaller particle sizes. These techniques are discussed in more detail in section 1.3.

While many of these strategies have been very effective at increasing resolution and efficiency, there is still ample room for improvement. Pharmaceutical manufacturers continue to strive for better resolution, which means further improving column efficiency.
1.2 Review of Chromatographic Principles

1.2.1 Column Efficiency

The efficiency of a column can be measured both by its plate number (N) and its plate height (H). N is defined as the number of theoretical plates, where one theoretical plate represents one equilibration of the sample between the mobile phase and the stationary phase. The number of theoretical plates that a chromatographic column possesses can be calculated by Equations 1 and 2

\[
N = 16 \left( \frac{t_r}{w_b} \right)^2
\]  

(1)

\[
N = 5.454 \left( \frac{t_r - t_0}{w_{1/2}} \right)^2
\]  

(2)

where \( t_r \) is the retention time, \( t_0 \) is the retention time of an unretained peak, \( w_b \) is the base peak width in time, \( w_b = 4\sigma \), and \( w_{1/2} \) is the half peak width in time, \( w_{1/2} = (\ln 2)\sigma \). \( \sigma \) is the standard deviation of the peak. Equation 2 is easier to use for peaks which are not well resolved.

The plate number (N) can then be used to determine the plate height (H) of the column. This can be found by dividing the length of the column, L, by the plate number, as shown in Equation 3.

\[
H = \frac{L}{N}
\]  

(3)

Thus, for a given separation distance, an increase in the number of theoretical plates will lead to a lower plate height, signifying a more efficient column.
1.2.2 Peak Resolution

Peak resolution, $R_s$, is an invaluable tool in the evaluation of a chromatographic method. It is defined as the ability to separate two peaks. Resolution can be defined either by Equation 4 for base peak width or Equation 5 for peak width at half height

$$R_s = \frac{2(t_{r2}-t_{r1})}{(w_{b1}-w_{b2})}$$  \hspace{1cm} (4)

$$R_s = \frac{2(t_{r2}-t_{r1})}{1.7(w_{0.5,1}-w_{0.5,2})}$$  \hspace{1cm} (5)

Equation 5 is easier to use, especially for peaks that are not well resolved. Two peaks are considered baseline resolved if $R_s$ is 1.5. This is the point at which the valley between two adjacent peaks just touches the baseline. Minimum accepted resolution is normally considered to be closer to 1.75 or 2.

Resolution can also be expressed as shown in Equation 6

$$R = \frac{1}{4} \sqrt{\frac{L}{H}} \left( \frac{\alpha-1}{\alpha} \right) \left( \frac{k'}{1+k'} \right)$$  \hspace{1cm} (6)

where $\alpha$ is the selectivity factor and $k'$ is the retention factor. These two factors can be varied for a given column and analyte by regulating the mobile phase composition and column temperature. While this may increase resolution a little, either the length of the column or plate height need to be adjusted in order to make large gains in resolution. As column length and plate height are independent of analyte, the resolution of all peaks should increase. A reduction in plate height is especially desirable as increasing column length will also increase analysis time.
1.2.3 Plate Height

In chromatography, the plate height and efficiency of a column can be expressed via the Van Deemter equation, shown in Equation 7.

\[ H = A + \frac{B}{v} + Cv \]  

(7)

The Van Deemter equation is composed of three terms: eddy diffusion (A), longitudinal diffusion (B), and mass transfer between phases (C). This equation provides a way to relate \( H \) to the linear velocity of the mobile phase, \( v \). The A term describes the eddy diffusion that arises when the packing of the column is such that multiple paths exist throughout the column. Analytes which travel shorter paths will arrive before those traveling longer paths, thus broadening the peak. This is mainly caused by the fact that there is a radial distribution of particle densities in a cylindrical column\(^{12} \). This term scales with particle size, \( dp \), as shown in Equation 8.

\[ A = 2\lambda dp \]  

(8)

The packing factor, \( \lambda \), is a measure of the distribution of particle size and packing quality. It is therefore evident that uniformly packing a column with monodispersed particles would mitigate this term.

The B term describing longitudinal diffusion is caused by an analyte’s natural inclination to diffuse outward from the concentration center of the peak. This term is independent of particle size, though it inversely scales with linear velocity as shown in Equation 9.

\[ B = 2\gamma Dm \]  

(9)
Here, $\gamma$ is known as the obstruction factor and is also a measure of packing efficiency. It is generally accepted to have a value between 0.6 and 0.7. $D_m$ is the diffusion coefficient of the analyte in the mobile phase. This term will be especially important for smaller analytes with higher diffusion coefficients.

The $C$ term describing mass transfer arises from the slow transfer of analytes between the mobile phase and the stationary phase. This term can be further broken down into the three contributions, as demonstrated in Equation 10.

$$C = c_m \left( \frac{d_p^2}{2D_m} \right) v + c_p \left( \frac{d_p^2}{2D_m} \right) v + c_s v$$  \hspace{1cm} (10)

The $C_m$ term arises from the spread of analytes due to the Poiselle flow profile through the column. The $C_p$ term arises from the fact that, for a porous medium, an analyte must diffuse into and back out of the stationary phase before it can rejoin the analytes in the mobile phase. Finally, the $C_s$ contribution is due to the slow desorption of analytes from the stationary phase.

1.3 Recent Improvements in High Pressure Liquid Chromatography

1.3.1 Monolith Columns

Although monolith technology has been available since the 1970s, only in the last decade or so has it become truly viable and commercially available\textsuperscript{13}. Monolith columns differ from the traditionally packed particle columns in that they consist of a silica rod with a bimodal structure consisting of $\sim$2 $\mu$m macropores and $\sim$13 nm mesopores. The high porosity and short diffusion path length derived from these macropores both lower
the plate height and significantly decrease the hydraulic resistance of the mobile phase flow. Therefore, very fast and efficient separations with flow rates as high as 9 mL/min can be performed due to the incredibly low back pressures. These columns are therefore well suited for fast analyses with conventional HPLC systems. However, the overall efficiency is similar to a conventional column packed with ~3 µm porous particles\textsuperscript{2,14}. Thus the achievable plate heights do not rival what has been achieved with the technologies described in the sections below.

1.3.2 Core-Shell Particles

Similar to monolith columns, core-shell particles use the principle of reducing diffusion path length in order to lower plate height. A core-shell particle consists of a solid core that is surrounded by a relatively thin layer of porous material (Figure 1.1b). The advantage is that, when an analyte diffuses into the stationary phase, it will not waste time traveling through the particle because the solid core will block the path inwards. This lowers the $C_p$ contribution to the $C$ term. Furthermore, the solid core blocks analytes from diffusing outwards which effectively lowers the $B$ term contribution as well. Finally, these particles have been shown to have a very narrow size distribution, which leads to a very low $A$ term (plate height terms $A$, $B$, and $C$ previously discussed in Section 1.2.3.).

There are several different kinds of commercial available core-shell particles, including Halo from AMT, Kinetex from Phenomenex, and Poroshell from Agilent. These columns can maintain very low backpressures and are therefore compatible with traditional HPLC equipment. The latest generation of these columns feature reduced
particle sizes of sub-2 µm. Plate heights as low as 2 µm have been reported with Kinetex 1.3 µm particles, which is significantly lower than what traditional porous particles are currently able to achieve\textsuperscript{15}.

\subsection*{1.3.3 High Temperature Liquid Chromatography}

High temperature liquid chromatography (HTLC) is LC analysis performed at temperatures above ambient and below super critical temperatures\textsuperscript{16}. Temperatures as high as 200 ºC have been used in order to decrease analysis time\textsuperscript{17}. This technique is effective because mobile phase viscosity is significantly lowered at high temperatures which allows for the use of much higher flow rates. This is especially important for columns with high back pressures such as those with sub-2 µm particles as discussed in the next section.

High temperatures also increase the efficiency because the mass transfer term, normally a concern at high flow rates, is minimized due to the increased kinetics of the adsorption/desorption process. Furthermore, increasing the temperature decreases the dielectric constant of the water, thus giving it a more organic character. This allows one to tune the selectivity of a column\textsuperscript{2}.

HTLC at very elevated temperatures is not often used due to concerns about the stability of the stationary phase and the analyte. However, slightly elevated temperatures, up to 80 ºC, have been used with sub-2 µm particles to help decrease backpressure.
1.3.4 Sub-2 µm Particles

Over the last several decades, as particle preparation technology has advanced, particle sizes have continuously decreased. In the last decade, sub-2 µm particles have become readily available. The reason for the increasingly small particle sizes becomes evident when looking at the Van Deemter equation as described in Section 1.2.3.

As the A term (Equation 8) and C term (Equation 10) both decrease with decreasing particle size, there is a significant reduction in plate height. Furthermore, the reduction of the C term means that a column packed with sub-2 µm particles can be operated at higher flow rates without a significant decrease in efficiency. This increase in efficiency also allows for the use of shorter columns to obtain similar resolutions. It is for this reason that sub-2 µm particles are able to offer improved speed in pharmaceutical analysis.

However, the Kozeny-Carmen Equation\(^{12}\), shown in Equation 11, demonstrates that the use of such small particles leads to the generation of much higher backpressures.

\[
P = \frac{180\eta \nu (1 - \varepsilon)^2}{d_p^2 \varepsilon^2}
\]

(11)

Here, \(L\) is the column length, \(\eta\) is the solvent viscosity, and \(\varepsilon\) is the porosity of the column. A two-fold reduction in particle size is thus associated with a four-fold increase in backpressure. For this reason, conventional HPLC instrumentation cannot provide the backpressure necessary to operate such columns.

Jorgenson was the first to study and demonstrate the feasibility of creating an ultra-high pressure instrument\(^{18-19}\). In 2004, Waters Corporation introduced the first commercially available ultrahigh pressure liquid chromatography (UHPLC) instrument.
Similar instruments are now available from a variety of manufacturers and can exceed 1400 bar. This development has popularized the use of sub-2 µm particles to provide extremely fast separations without compromising efficiency.

1.3.5 Nonporous Particles

Nonporous silica particles (Figure 1.1c) extend upon the idea of reducing intraparticle diffusion. While core-shell particles reduce this effect, in this case it is eliminated altogether as the entire particle is a solid sphere. This leads to a significant reduction in the C term of the Van Deemter Equation (Equation 10) and thus higher flow rates can be used without a significant decrease in efficiency. Nonporous particles can also be manufactured to be very monodisperse, which leads to a reduced A term due to homogenous packing. They are also mechanically strong enough to withstand high temperatures, which is an advantage when attempting to reduce backpressure. However, nonporous particles suffer from low surface area, which lowers column capacity and resolution due to the decreased retention factor, k’.

Figure 1.1. Illustration of the structure of a (A) fully porous particle (B) core-shell particle and (C) non-porous particle.
1.4 Silica Colloidal Crystals as a Separation Medium

The introduction of submicron, nonporous silica particles builds upon the success of sub-2 µm particles. These particles self-assemble into face-centered cubic crystals and on the capillary scale have been shown to exhibit a blue color due to Bragg diffraction from the incredibly even spacing of the particles\(^{21}\). In nature, these particles form gem-quality opals (Figure 1.2). This extreme packing efficiency serves to lower the radial distribution of densities throughout the column and significantly decrease the A term. However, even randomly packed 1.5 µm nonporous particles have demonstrated plate heights as low as 3 µm\(^{20}\). This is due to the reduction of the A and C term as discussed above. Lowering the particle size further should offer even lower plate heights. The use of such crystals made with submicron particles as a separation medium for small molecules has heretofore not been well studied.

Figure 1.2. (A) Opal gem found in nature exhibiting opalescence (B) Packed capillary with an artificial silica colloidal crystal exhibiting opalescence.
1.5 Thesis Overview

The work presented in this thesis focuses on determining the feasibility of using silica colloidal crystals as a packing material to increase the efficiency and resolution of small molecule separations. These separations were studied both in fused-silica capillary and stainless steel columns. Commercial Nano-UHPLC and UHPLC instruments were used to study these columns, respectively. Fluorescence microscopy was used to observe the separation performance in capillary columns.

The first chapter reviews the current role of small molecule separation in the pharmaceutical industry. Improvement strategies and trends are discussed. Basic chromatographic principles are reviewed and then applied to recent improvements in HPLC. Advantages, disadvantages, and the current state of these technologies are evaluated. This is followed by a discussion on the properties of silica colloidal crystals.

Chapter 2 discusses the capillary packing method, particle preparation, and capillary modification process. The highly ordered packing of the silica colloidal crystal inside the capillary is characterized. Evaluation of a separation in isocratic mode using a pure dye (Rhodamine 6G) demonstrates that sub-µm plate heights can be achieved, but reveals limitations in the instrumentation that limit the efficiency of these capillary columns.
In Chapter 3, this separation process is tested in stainless steel columns. The column packing method and particle modification process is discussed. Packing quality and column efficiency is analyzed. The analysis of Rhodamine 6G using UHPLC instrumentation achieves a plate height similar to a commercial core-shell column. The vast majority of this plate height is found to be attributed to longitudinal diffusion.

Chapter 4 discusses the conclusions and possible future directions of this research.
1.6 References


CHAPTER 2: NANO-UHPLC SEPARATION OF SMALL MOLECULES WITH SILICA COLLOIDAL CRYSTAL PACKED CAPILLARY COLUMNS

2.1 Introduction

Two of the most popular ways to increase efficiency in capillary reverse-phase liquid chromatography (RPLC) are to use smaller particle sizes and to increase the capillary length. The drawback to both of these techniques is the large amount of backpressure generated. Jorgenson et. al. demonstrated this concept using 25 cm long capillaries packed with ever decreasing particle sizes. Using 1 μm particles, Jorgenson et al. observed a plate number of 125,000 for the separation of small organic molecules in under 10 minutes. This separation required pressures of 2,300 bar (34,000 psi) and required the construction of a custom modified Haskel pneumatic high-pressure pump\(^1\). This type of setup is not used in practice as it is not commercially available and is prone to technical difficulties. Achieving similar plate numbers with commercially available instruments (backpressures typically < 1400 bar) would require larger particles and much longer lengths. As such, these separations can take hours to complete\(^2\)\(^-\)\(^3\). The development and use of sub-μm silica colloidal crystal capillaries may be able to circumvent this problem.

As discussed in Sections 1.3.4 and 1.4, both the A and C terms of the Van Deemter equation with sub-μm silica colloidal crystal capillaries are almost negligible due to the small particle size. Therefore, the plate height would be dominated by the B
term (longitudinal diffusion). This is especially true for small molecules as they have high diffusion coefficients. If the plate height is, in fact, diffusion limited, then it can be demonstrated that the number of plates becomes independent of column length; instead, it is dictated by pressure. If this is the case, good column efficiencies can be obtained even at short column lengths. The theoretical proof for this theory is below.

In a diffusion limited setting, the plate height is dictated by the B term, which is a function of the diffusion coefficient, $D$, column porosity $\varepsilon$, and the fluid velocity, $v$, as demonstrated in Equation 1.

$$H = \frac{2\varepsilon D}{v} \quad (1)$$

The value of the fluid velocity, in turn, is proportional to the pressure, $P$, and flow enhancement from slip flow, $E$. It is inversely proportional to the length of the column, $L$, and the flow restriction under a no-slip condition, $\Omega$. This is demonstrated in Equation 2.

$$v = \frac{PE}{L \Omega} \quad (2)$$

By substituting Equation (2) into Equation (1), a value of plate height, $H$ directly proportional to column length, $L$ is obtained (Eq. 3).

$$H = \frac{2\varepsilon DL\Omega}{PE} \quad (3)$$

$$N = \frac{L}{H} \quad (4)$$

By substituting Equation (3) into Equation (4) and rearranging, it becomes evident that $N$ is now proportional to pressure and independent of column length.

$$N = \frac{PE}{2\varepsilon D\Omega} \quad (5)$$

Applying Equation 5 to various particles sizes for which there exists flow data (125-470 nm)$^4$, theoretical plate numbers of hundreds of thousands are feasible, assuming
a maximum pressure of 800 bar, and using the diffusion coefficient for a 500 Da analyte. This analyte size represents an average small molecule. As the length is arbitrary, columns as short as 1 cm can be used, assuming the diffusion limit is maintained. This should allow us to achieve these plate numbers in extremely short separation times. The caveat is that contributions from extra-column broadening increase for shorter columns.

In this work, small molecule separations are performed using a commercial nano-UHPLC and silica colloidal crystal packed capillaries in order to test the validity of this hypothesis. Isocratic elution of a pure dye (Rhodamine 6G) is used to measure column efficiency.

2.2 Experimental

2.2.1 Chemicals and Materials

The silica nanoparticles were purchased from Fiber Optic Center Inc. (New Bedford, MA). Silanization reagents were purchased from Gelest Inc. (Morrisville, PA). Toluene, acetonitrile, and nitric acid were purchased from Sigma Aldrich (St. Louis, MO). Fused silica capillaries were purchased from Polymicro (Phoenix, AZ) (TSP150375, 150 µm I.D.). Rhodamine 6G dye was purchased from Life Technologies (Carlsbad, CA).
2.2.2 SCC Capillary Column Preparation

2.2.2.1 Calcination and Annealing of Silica Nanoparticles

500 nm non-porous silica colloidal particles were calcined at 600°C for 8h in a furnace (Thermo Fisher Lindberg/Blue M), and then cooled and resuspended in ethanol. This process was repeated three times. The particles were then annealed at 1050°C for 3h. Annealing for three hours has been shown to notably reduce the amount of isolated silanols and peak tailing. Annealing times longer than three hours were found to not make a substantial difference in FTIR spectra. The annealed particles were sonicated in ultrapure water (Millipore, Milli-Q) for 1h to achieve a good suspension, and then rehydroxylated by refluxing in a 50:50 mixture of concentrated nitric acid and ultrapure water overnight. This was followed by rinsing with ultrapure water until the pH was neutral. The particles were subsequently filtered through a 2-um stainless steel mesh (TWP, Berkely, CA) in order to eliminate any potential aggregates. These particles were mixed with ultrapure water to form a 35% w/w slurry and sonicated overnight to ensure homogeneity.

2.2.2.2 SCC Capillary Column Packing and Modification

The inner wall of the 150µm capillary was cleaned and conditioned by pumping (Harvard Apparatus PhD 2000) 0.2M sodium hydroxide (Sigma Aldrich) through the capillary for 30 minutes. The capillary was then rinsed first with ultrapure water for 30 minutes and then with 100% ethanol for 30 minutes. The capillary was then cut into 12cm pieces and placed in a vacuum oven (VWR) to dry for 1 hour at 60 °C. The cleaned
and dried capillaries were then dipped into the slurry where capillary forces act to draw the slurry into the capillaries. The filled capillaries were then packed under pressure at 7500 PSI using a mechanical pump (Lab Alliance, State College, PA) with sonication for 15 minutes using a 0.5µm frit. This compacts the slurry into a silica colloidal crystal (typically 2.5cm in length). After packing, the frit was removed and the capillary bed was allowed to dry at room temperature for 48 hours prior to surface modification. Capillary opalescence was imaged using a Nikon Zoom microscope (Nikon Instruments Inc., Melville, NY). A schematic demonstrating the capillary packing procedure is shown in Figure 2.1.

Figure 2.1 Schematic of setup used for pressure packing silica colloidal crystals into fused silica capillaries.
The colloidal crystals were reacted with trichlorosilanes to achieve horizontal polymerization, as has been previously established by Wirth et al.\(^6\) Horizontal polymerization minimizes the amount of exposed silanol groups on the surface. These silanol groups act as strong binding sights and significantly increase mass transfer contributions to the plate height\(^7\).

Prior to the silanization process, part of the polyimide coating on the capillaries was burned off using matches to make fluorescence detection of the packed crystal possible. The capillaries were then placed into a humidity chamber with a controlled 50% humidity environment for 45 minutes. This has been shown to create the monolayer of water on the particles necessary for optimal surface coverage of alkyl chains on the particles\(^6\). A silane solution of 8% octadecyltrichlorosilane and 2% methyltrichlorosilane was prepared in dry toluene. The silane solution was then wicked into the capillaries and allowed to react at room temperature, under nitrogen, overnight. The capillaries were rinsed by flowing dry toluene through them for 15 minutes. Finally, the capillaries were placed into a 120 °C oven for 2 hours to dry and condense unreacted silanol groups.

2.2.3 RPLC with SCC Capillary Columns

The mobile phase was prepared by separately mixing HPLC-grade acetonitrile and ultrapure water with 0.1% (v/v) trifluoroacetic acid (TFA) (99%, Sigma Aldrich). Rhodamine 6G solutions were prepared in 100% ultrapure water to give final solution concentrations of 0.005 mg/mL.

Liquid chromatography experiments were performed using a commercial nano-UHPLC (Thermo-Fischer UltiMate 3000). Packed capillaries with 2.5cm packed beds
were used in these studies. Prior to use, each capillary was rinsed with 90:10 acetonitrile:water with 0.1% TFA for 15 minutes to remove any organic residue on the stationary phase. The column was then conditioned with the starting mobile phase composition of the separation for 15 minutes. The separation process was monitored with an inverted fluorescence microscope equipped with a mercury lamp (Eclipse Ti-S, Nikon Instruments, Inc., Melville, NY) and 2X objective (Nikon Eclipse TE2000U). Wavelengths were selected using Omega Optical filter cubes. The emission was collected using a CCD camera (ProEM 512, Princeton Instruments, Trenton, NJ) and recorded with a 0.2 s acquisition time using Winview software (Princeton Instruments, Trenton, NJ). Data was analyzed using Origin (Microcal, Northampton, MA).

2.3 Results and Discussion

2.3.1 Capillary Packing Quality

Prior to use, all capillaries were inspected for packing quality. Figure 1.1 demonstrates the blue-green color that a capillary with a crystalline structure should possess. This color is caused by Bragg diffraction due to the even spacing of the particles. The entire capillary may be examined by filling the capillary with ethanol, which increases the optical transmission due to index-matching. Any capillaries found to possess cracks or visible wall gaps were discarded.
Once crystallinity was visually confirmed, the porosity ($\varepsilon$) of the capillary was determined using the Kozeny-Carmen equation (Equation 6):

$$P = \frac{180\eta \nu (1-\varepsilon)^2}{d_p^2 \varepsilon^2} \quad (6)$$

Figure 2.2 shows a plot of the pressure versus flow for a 150 µm inner diameter capillary packed with a 2.5 cm colloidal crystal of 470 nm particles. The pressure was measured using toluene as a solvent. It has been found that the viscosity of toluene has a pressure dependence\(^8\). The flow rate for each data point was therefore multiplied by viscosity in order to normalize the data.

Using the best fit line, the porosity was calculated to be 0.288, which very closely matches previously determined values for this type of capillary\(^4\). This value is much lower than the porosity of randomly packed spheres (0.40), and approaches that of a perfect face-centered cubic crystal (0.26). It is therefore evident that the packing quality is very good and should not contribute significantly to peak broadening.
Figure 2.2. Pressure vs. flow for 2.5cm long capillaries packed with 470nm particles. Flow has been normalized for viscosity. Theoretical Hagen-Poiselle pressure vs. flow, as calculated by the Kozeny-Carmen equation, is shown with a dashed line.

2.3.2 Column Efficiency Characterization

Rhodamine 6G was chosen as the test analyte to characterize the efficiency of these columns with regard to small molecule separation using commercial instrumentation. This is a relatively pure dye with only one main peak, which makes isocratic separations easy to analyze. A chromatogram of an isocratic separation of Rhodamine 6G is shown in Figure 2.3a. The column was first equilibrated with 1:99 acetonitrile:water with 0.1% TFA for 15 minutes. The analyte was then injected under these retention conditions in order to stack the analyte as a narrow plug at the front of the column. This stacking serves to minimize the effects of the contribution of injected width
to extra-column band broadening. This condition was held for 2.5 minutes and the mobile phase was then switched to the elution condition of 70:30 acetonitrile:water. The full width at half maximum (FWHM) of the main peak is about 2 seconds, which is extremely narrow. The main peak elutes at 9.7 min, but it is unclear how much of the retention time is truly due to retention and how much is due to gradient delay. The instrument may take a significant amount of time to switch the mobile phase from the stacking condition to the elution condition and then deliver the new solvent composition to the column. Therefore, it is difficult to measure plate number using Equation 7 since $t_r$ must be known.

$$N = 5.454 \left( \frac{t_r}{w_{1/2}} \right)^2 \quad (7)$$

An alternative way to measure plate number is by using the variance of the peak in distance units. Equation 8 can be utilized to find the plate height and then Equation 4 can provide the plate number.

$$H = \frac{\sigma^2}{L} \quad (8)$$

Here, the peak width at half height is $(ln2)\sigma$ and $L$ is the separation distance. Figure 2.3b shows the fluorescence image of the Rhodamine 6G as it reaches a separation distance of 2.3 cm. A Gaussian fit reveals $\sigma$ to be 115.2 µm, and the plate height is calculated to be 0.577 µm. This is interesting because it is 4x lower than the best results that have been achieved using wide bore stainless steel columns and commercial UHPLC. Since the column length is also shorter, from Equation 4, the plate number ($N$) is calculated to be only about 40,000. This is an order of magnitude lower than what is theoretically possible according to what was calculated in Section 2.1. In the next section, possible causes of this unexpected amount of broadening are explored.
Figure 2.3. A. Chromatogram of Rhodamine 6G separated by an SCC capillary with 470nm C18/C1 modified particles. The separation distance was 2.3cm. A mobile phase of 70:30 acetonitrile:water with 0.1% TFA was flowed at 270nL/min after 2.5 minutes of stacking with 1:99 acetonitrile:water. The pressure was 540 bar. B. Fluorescence image of Rhodamine 6G as it reaches the separation distance and plot of the data points in the distance dimension. A fit of the Gaussian is also shown.

2.3.3 Instrument Limitations

Upon further investigation, it was found that the majority of the retention time for the peak shown in Figure 2.3 was not due to retention on the column, but rather due to a significant gradient delay that occurs when switching between the stacking condition and the eluting condition. At the typical flow rates used for these columns, the gradient delay can approach over 10 minutes depending on the steepness of the gradient. Furthermore, it was found that, under stacking conditions, the analyte is retained on the instrumentation
tubing rather than at the head of the column, mostly defeating the purpose of stacking. This is shown in Figure 2.4.

Figure 2.4 demonstrates a capillary packed with headspace so that the head of the column could be monitored. The first two panels demonstrate that, under stacking conditions, the analyte does not reach the head of the column even after 13 minutes. Once the conditions are switched to elution conditions (and the gradient has fully switched over 11 minutes later), the peak emerges. Stacking therefore has no appreciable benefit and significantly increases observed retention time. Thus, it is clear that the analyte will have to be injected without stacking as there is no other tubing currently available.

Injection without stacking has its own challenges. As the analyte is not first concentrated onto the head of the column, pre-column dispersion begins to become a problem. Figure 2.5 demonstrates Rhodamine 6G being injected under conditions without stacking. It can be seen that the peak begins to emerge around 10 seconds and continues to enter the column even at 90 seconds. It can be seen that the peak experiences significant broadening before it even reaches the head of the column.
Figure 2.4 Time-lapse fluorescent image of a Rhodamine 6G isocratic injection. 13 minutes of stacking under 1:99 acetonitrile:water with 0.1% TFA followed by elution using 70:30 acetonitrile:water. The headspace is monitored and the peak can be seen emerging from the tubing only 11 minutes after switching to elution conditions. The dashed line represents the interface between the liquid and the start of the packing material.
When considering a given plate height, the contribution from injection and detection must also be taken into account as demonstrated in Equation 9.

\[ H = H_{col} + H_{inj} + H_{det} \]  

(9)

It is more than likely that the contribution from injection is making up the majority of the plate height. The full potential of the column efficiency cannot be exploited without using a diffusion injection. This is not a practical solution since this is a time-consuming process that cannot be automated. A new design of the injector is needed.
2.4 Conclusion

These results show that capillaries packed with silica colloidal crystals of 470 nm particles can achieve plate heights as low as 0.577 μm and plate numbers of 40,000 using commercial instrumentation. However, this efficiency is much lower than what is theoretically calculated. It was found that the performance is significantly hindered by instrumental limitations. Analyte retention before the column under retention conditions prevents stacking a narrow injection zone onto the head of the column. Injection without stacking leads to a large amount of extra-column band broadening, presumably from the parabolic flow profile. As there is currently no viable means of injecting the analyte onto the head of the column, the efficiency of these columns cannot be exploited at this time.
2.5 References


CHAPTER 3: UHPLC SMALL MOLECULE SEPARATION WITH SILICA COLLOIDAL CRYSTAL PACKED STAINLESS STEEL COLUMNS

3.1 Introduction

Performing separations in stainless steel columns instead of capillaries offers several distinct advantages and disadvantages. Stainless steel columns are the industry preferred platform, as most labs are already set up with instruments to run them. They are also very robust and easy to handle. The main disadvantage, however, is their inherently wider inner diameter as compared to a capillary. A 2.1 mm i.d. is commonly used in stainless steel columns as opposed to the 150 µm i.d. found in capillaries. The one drawback is that less is known about packing of colloidal crystals in stainless steel columns. Nevertheless, excellent results should be possible due to the natural advantages of the small, nonporous particles (discussed previously in Section 1.4).

Industry-leading stainless steel columns are currently packed with a hybrid of nonporous and fully porous particles, known as core-shell particles. As discussed in Section 1.3.2, these particles contain a solid nonporous core surrounded by a relatively thin layer of porous material. In this way the C term contribution from analyte diffusion within the particles is reduced. The latest generation of core-shell particles have achieved plate heights as low as 2 µm.²
3.2 Experimental

3.2.1 Chemicals and Materials

Silica nanoparticles were purchased from Fiber Optic Center, Inc. (New Bedford, MA). Stainless steel columns and frits (0.5 µm) were purchased from IDEX Health and Science (Oak Harbor, WA). Stainless steel fittings and connection tubing was purchased from Vici Valco Instruments Co. Inc. (Houston, TX). Silanes were purchased from Gelest, Inc. (Morrisville, PA). Toluene, acetonitrile, hexanes, and biphenyl were purchased from Sigma Aldrich (St. Louis, MO). Rhodamine 6G dyes was purchased from Life Technologies (Carlsbad, CA.).

3.2.2 SCC Stainless Steel Column Preparation

3.2.2.1 Modification of Silica Nanoparticles

500 nm and 750 nm non-porous silica particles were calcined, annealed, and rehydroxylated as outlined in Section 2.2.2.1. After thermal treatment and rehydroxylation, the average particle diameter was determined to be 470 nm and 680 nm, respectively, as determined by scanning electron microscopy (SEM). The rehydroxylated particles were dried in a 60°C vacuum oven and then humidified using a humidity chamber with a controlled 50% humidity environment for 45 minutes.

The particles were modified with trichlorosilanes so as to achieve horizontal polymerization as previously discussed. The fused silica particles were poured into a 250 mL round-bottom flask and suspended in 150 mL dry toluene. A 4:1 mixture of
octadecyltrichlorosilane and methyltrichlorosilane was added to create a final 2% concentration. The solution was sonicated for 30 minutes to ensure suspension and finally stirred at room temperature, under nitrogen, overnight. The particles were then transferred to 50mL conical tubes and centrifuged. The solvent was poured off and the particles were rinsed four times with dry toluene and one time with hexanes. The particles were then transferred to a glass vial and dried at 120 °C for 2 hours to condense unreacted silanols.

3.2.2.2 Stainless Steel Column Packing

The prepared 470 nm particles were used to make a slurry using 0.2 g of particles and 2.4 mL of toluene. The excess of particles was used to account for any loss during transfer. The 680 nm particles were prepared into a slurry with 0.01 g of particles and 2.4 mL of toluene. The slurries were sonicated overnight to ensure solution homogeneity. Figure 3.1 shows the packing assembly used. A 30 mm x 2.1 mm stainless steel column was attached to a 150 mm x 4.6 mm reservoir column using a 50 mm x 1mm piece of connection tubing. One end of both the packing and reservoir column contained a 0.5µm stainless steel frit while the other contained the same frit assembly but with the frit bored out. This allows the slurry to be pushed down from the reservoir column into the packing column. A 100 mm x 0.12 mm piece of tubing connected the reservoir column to the high pressure packing pump.

The 680 nm silica slurry was transferred into the reservoir column and attached to the packing pump. The slurry was flowed into the packing column using toluene as the solvent at a volume flow rate of 1 mL/min for 15 minutes under sonication. This creates a
thin layer of 680 nm packed particles against the frit. This is done to ensure that the 480 nm particles, which are smaller than the porosity of the frit, do not clog the frit and cause unnecessarily high back pressures.

After the flow was turned off, the column was allowed to depressurize and the solvent was drained from the reservoir column and replaced with the 480 nm particle silica slurry. This slurry was then flowed into the packing column using a volume flow rate of 0.05 mL/min for 1 hour under sonication and a maximum pressure of 17,500 psi. The pump was then turned off and the column allowed to depressurize. The bored out frit was then replaced by a regular frit and both ends of the column were capped for storage.

Figure 3.1 Schematic of setup used for pressure packing silica nanoparticles into stainless steel columns.
3.2.3 RPLC with SCC Stainless Steel Columns

The mobile phase was prepared by separately mixing HPLC-grade acetonitrile and ultrapure water with 0.1% (v/v) trifluoroacetic acid (TFA) (99%, Sigma Aldrich). Rhodamine 6G solutions were prepared in 100% ultrapure water to give a final solution concentration of 0.05 mg/mL. Biphenyl solutions were prepared in 50/50 acetonitrile:water to give a final solution concentration of 0.05 mg/mL.

The liquid chromatography experiments were performed using a commercial UHPLC (Thermo Scientific Accela). In-house-packed 3cm stainless steel columns were used in these studies. A commercial core-shell column (Supelco Acentis Express C18, 2.7µm) was used for comparison studies. Prior to use each column was rinsed with 90:10 acetonitrile:water with 0.1%TFA for 15 minutes in order to clean out any organic residue on the stationary phase. The column was then conditioned with the starting mobile phase composition of the separation for 15 minutes. All separations were run under isocratic conditions. The separation process was monitored and recorded with Thermo Xcalibur software. Data was analyzed using Origin (Microcal, Northampton, MA).

3.3 Results and Discussion

3.3.1 Column Packing Quality

The porosity (ε) of the column bed was determined using the Kozeny-Carmen equation (Equation 1)

\[
P = \frac{180\eta
\mu (1-\varepsilon)^2}{d_p^2 \varepsilon^2}
\]

(1)
Figure 3.2 shows a plot of the pressure versus flow for a 30 mm x 2.1 mm i.d. packed column of 470 nm particles. The pressure was measured using toluene as the solvent. As it has been previously found that the viscosity of toluene has a pressure dependence\(^4\), the flow rate for each data point was multiplied by viscosity in order to normalize the data.

Using the best fit line, the porosity was calculated to be 0.382. This value is only slightly lower than the porosity of randomly packed spheres (0.40). This is likely due to the fact that, unlike in capillaries, the particles are modified prior to packing. This disrupts hydrogen bonding networks and electrostatic interactions which may help the particles self-assemble into a crystal. Nevertheless, even randomly packed 1.5 \(\mu\)m nonporous particles without sonication have demonstrated plate heights as low as 3 \(\mu\)m. This will therefore likely not be a major contributing factor to plate height.

![Figure 3.2. Pressure vs. flow for 3cm x 2.1mm stainless steel column packed with 470nm particles. Flow has been normalized for viscosity. Theoretical Hagen-Poiselle pressure vs. flow, as calculated by the Kozeny-Carmen equation, is shown with a dashed line.](image)
3.3.2 Column Efficiency Characterization

In order to fully evaluate the column efficiency, all comparisons were made using a 2.7 μm core-shell C18 column (Supelco Acentis Express C18, 10cm x 4.6mm). Core-shell columns currently provide the highest efficiencies for small molecule separations. In order to characterize efficiency, Rhodamine 6G was separated isocratically on both columns under conditions as to achieve similar retention times. Figure 3.3 shows the comparison between our 30 mm x 2.1mm stainless steel column packed with 470 nm C18 modified nonporous particles and the commercial column. Table 3.1 summarizes the efficiency parameters for each column. Plate number (N) was calculated according to Equation 2 using peak 4 and plate height (H) was calculated according to Equation 3. The quoted FWHM (full width at half maximum) in Table 3.1 is for peak 4.

\[
N = 5.454 \left( \frac{t_r - t_0}{w_{1/2}} \right)^2 \tag{2}
\]

Figure 3.3 Rhodamine 6G dye isocratic separation. A. 3cm x 2.1mm i.d., TC18/C1 modified 470 nm particle column. 35:65 acetonitrile:water with 0.1%TFA was flowed at 25 °C at 70 μL/min. B. Supelco Acentis Express C18 10 cm x 4.6 mm i.d.. 50:50 acetonitrile:water with 0.1%TFA was flowed at 25 °C at 500 μL/min.
Table 3.1. Efficiency parameters for separation in Figure 3.3. R1R2 refers to the resolution between peak 1 and 2, and so forth. FWHM, N, and H is quoted for peak 4.

<table>
<thead>
<tr>
<th></th>
<th>R1R2</th>
<th>R2R3</th>
<th>R3R4</th>
<th>FWHM</th>
<th>N</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house column</td>
<td>9.105</td>
<td>1.112</td>
<td>4.567</td>
<td>13.57s</td>
<td>6224</td>
<td>4.82µm</td>
</tr>
<tr>
<td>Commercial column</td>
<td>9.553</td>
<td>1.067</td>
<td>7.078</td>
<td>8.52s</td>
<td>20427</td>
<td>4.89µm</td>
</tr>
</tbody>
</table>

\[ H = \frac{L}{N} \] (3)

For the main peak (peak 4), the in-house custom column provided 6224 plates with a plate height of 4.82 µm. The commercial column provided 20427 plates with a plate height of 4.89 µm. Though the plate heights are similar, the in-house column resolution is still not quite as good as the commercial column. The reason becomes evident by looking at the equation for resolution (Eq. 4).

\[ R_s = \frac{2 (t_{r2} - t_{r1})}{1.7 (w_{0.5,1} - w_{0.5,2})} \] (4)

The difference in retention time for all the peaks using the in-house column are similar if not greater than those for the commercial column. However, peak widths, if looking at the FWHM, are significantly wider. Therefore, the resolution suffers considerably.

Relating H to the linear velocity of the mobile phase, v, via the Van Deemter equation for this separation offers some insight into the cause of the peak width. This plot is shown in Figure 3.4 and is fit to the Van Deemter equation, Equation 5.

\[ H = A + \frac{B}{v} + Cv \] (5)
As discussed in Section 1.2.3, this equation is composed of three terms: eddy diffusion (A term), longitudinal diffusion (B term), and mass transfer between phases (C term). Looking at the plot for this separation, the plate height has leveled off at the maximum pressure, and the flow rate can only go up to 90 μL/min before the system pressure limit is reached. This is 5-10x slower than what is typically seen for a commercial column, and is especially a problem since longitudinal diffusion is inversely related to the flow rate. As small molecules are already likely limited by longitudinal diffusion, this means that the B term contribution to the plate height must be very large.

Figure 3.4 Van Deemter plot for Rhodamine 6G dye separation shown in figure 3.3. Separation was run on a 3cm x 2.1mm i.d. column packed with 470nm TC18/C1 modified particles. 35:65 acetonitrile:water with 0.1%TFA was flowed at 25 ºC at various flow rates. A theoretical fit to the Van Deemter equation is shown in red and the minimum plate height is extrapolated to the y-axis.
3.3.3 Longitudinal Diffusion Studies

To confirm this theory, the B term contribution was measured using the arrested elution method as used by Knox et al. In this method, the analyte is allowed to enter and travel about halfway through the column. The flow is then stopped for various periods of time during which the analyte diffuses outwards. The flow is then restarted and the width of the resultant peak is measured. This peak width can then be used to find the B term contribution using Equation 6 and Equation 7.

\[ \sigma_t^2 = \sigma_0^2 + \frac{2D_{\text{eff}}}{u^2} \Delta t_{\text{off}} \]  

Here, \( \sigma_t^2 \) represents the peak variance after the flow has been stopped for a period of time, \( \Delta t_{\text{off}} \). \( \sigma_0^2 \) is the peak variance if the flow is not stopped. The chromatographic velocity, \( u \), is defined as \( u = \frac{L}{t_r} \). Finally, \( D_{\text{eff}} \) is the effective diffusion coefficient and can be found through the use of the slope when the above equation is plotted in Figure 3.5. The B term contribution is then calculated according to Equation 7.

\[ B = \frac{2D_{\text{eff}}}{u} \]  

Using this method, the contribution from longitudinal diffusion was measured at 70 \( \mu \text{L/min} \), and was found to be 3.5 \( \mu \text{m} \). This means that 73% of the plate height contribution is coming from longitudinal diffusion. The question then becomes whether this amount of diffusion is to be expected at this flow rate, or whether this is unreasonably large.

The expected contribution to longitudinal diffusion can be calculated using Equation 8, where \( D_m \) is the diffusion coefficient of the analyte in the mobile phase, \( u \) is
the linear velocity of the mobile phase, and $\gamma$ is the obstructive factor. This factor is known to be about 0.65 for a randomly packed bed of solid spheres\textsuperscript{5}.

$$B = \frac{2\gamma D_m}{u}$$  \hspace{1cm} (8)

Figure 3.5 Arrested elution curve for Rhodamine 6G dye separation shown in figure 3.3. Separation was run on a 3cm x 2.1mm i.d. column packed with 470nm TC18/C1 modified particles. 35:65 acetonitrile:water with 0.1%TFA was flowed at 25 °C at 70 µL/min. Flow was stopped when the analyte was halfway through the column for various amounts of time ($t_{off}$). A best fit line with equation $y = 0.0546 + 55.868$ is plotted.

A literature value for the diffusion of Rhodamine 6G in 100% water was found to be $3.89 \times 10^{-6}$ cm\textsuperscript{2}/s.\textsuperscript{6} As the longitudinal diffusion measurements were made in 35:65 acetonitrile:water, this value was adjusted for viscosity to be $5.326 \times 10^{-6}$ cm\textsuperscript{2}/s. This equates to a B term contribution of 1.39 µm at 70µL/min. This theoretical value is less than half of the diffusion that was actually observed.
This theoretical value only accounts for the longitudinal diffusion that occurs in the mobile phase. The higher value for the experiment is likely due to a contribution from diffusion on the stationary phase. In porous particles, this is probably less of an issue because the connections between particles are rare compared to the surface area of one particle. In nonporous particles, the connections are much more frequent, which would facilitate diffusion over longer distances. This may account for the larger than predicted B term observed in nonporous particles.

Elevated B term contributions for nonporous as opposed to porous particles of the same size have been observed before\(^7\). Here, the contribution to the B term from the nonporous particle was found to be almost 50% larger than that of the porous particle. This was attributed to the solid sphere structure and smooth surface of the particle. The increase we observe is apparently larger. This is consistent with the idea that the smaller surface area per particle, combined with the same number of connections between particles, facilitates transport by surface diffusion.

A Van Deemter curve for a smaller analyte, biphenyl, is shown in Figure 3.6. The minimum plate height is approximately 10 \(\mu m\). The B term contribution was measured to be 7.8 \(\mu m\). This larger B term contribution demonstrates that the diffusion effect increases as the analyte size is decreased and it hydrophobicity is increased.
Figure 3.6 Van Deemter plot for biphenyl. Separation was run on a 3cm x 2.1mm i.d. column packed with 470 nm TC18/C1 modified particles. 35:65 acetonitrile:water with 0.1%TFA was flowed at 25 ºC at various flow rates. A theoretical fit to the Van Deemter equation is shown in red.

3.4 Conclusion

These results show that columns packed with 470 nm silica nanoparticles can, for a given separation, achieve plate heights on par with a commercial core-shell column for small molecules. However, the plate number and corresponding resolution are much lower because of the shorter column length, lower flow rate, and higher contribution from surface diffusion. Reasons for higher surface diffusion include the small-diameter, solid-sphere structure, and smooth surface of the particle. Since the contribution from longitudinal diffusion to plate height is inversely related to flow rate, linear flow rates at
least 2.5x higher will be needed to achieve performance exceeding current commercial
technology. Such flow rates would correspond to pressures of over 2000 bar, and thus are
currently restricted by instrumental pressure limitations.
3.5 References


CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

4.1 Conclusion of Results

Small molecule separations using SCCs of 470nm nonporous particles have been studied both in capillaries and stainless steel columns. Using a commercial nano-UHPLC, it was found that these capillaries can achieve plate heights as low as 0.577 µm and plate numbers of 40,000. The efficiency is an order of magnitude lower than what is expected. Long gradient delays, retention on the tubing, and a large amount of extra-column band broadening are all instrumental limitations which were found to significantly hinder the performance of these columns. New, more compatible tubing will likely need to be introduced before these capillaries can meet their expected efficiencies.

Separations using UHPLC and stainless steel columns alleviate these problems. These results have shown that, for a given separation, these columns can achieve plate heights on par with a commercial core-shell column. However, the achievable plate height of 4.82 µm is much higher than expected due to a nearly three-fold larger amount of longitudinal diffusion. This problem is exacerbated by the high backpressures of these columns, and thus the maximum flow rates are inadequate due to instrumental pressure limitations.
4.2 Future Directions

As presented in this work, longitudinal diffusion, the B term of the Van Deemter equation, accounts for almost 75% of the peak width when performing small molecule separations using stainless steel columns packed with SCCs of 470nm nonporous particles. The B term is inversely proportional to linear flow rate because the more time an analyte spends in the column the more time it has to diffuse outwards. Small molecules in particular suffer from this problem due to their high diffusion coefficients. This means much higher flow rates must be achieved in order to reduce peak widths.

Currently, the pressure limit of the instrument prevents the use of sufficiently high flow rates to reduce peak widths. However, the phenomenon of slip flow may be the best solution. Slip flow in chromatography was discovered recently by Wirth et al.\textsuperscript{1} and arises as a result of weak interactions between the mobile phase and the walls of the stationary phase, as is the case in RPLC. This leads to a significant enhancement in flow rate that can be achieved as compared to traditional poiseuille flow\textsuperscript{1-2}. For 470 nm particles, this enhancement can be as great as 5x.\textsuperscript{3} This would lower the B term contribution to below 1 \( \mu \)m.
4.3 References


