Construction and Characterization of a High Performance Supercritical Fluid Chromatography System

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1. Introduction

The goal of this project is to design and build an SFC instrument capable of doing fast separations with short columns packed with small particles. In order to do that the system must be capable of withstanding high pressures, have limited extra column effects, and be able to acquire data at a sufficient rate.

Supercritical Fluid Chromatography (SFC) was first thought of as an extension of gas chromatography (GC) in the early 1960's [1]. It was not until later that Giddings brought up the possibility of using higher pressures (pressures up to 2000 atm) in gas chromatography to gain some of the chromatographic properties of liquid chromatography (LC) [1]. In the 1970's LC became more mainstream leaving little development in SFC. Early studies of SFC were done using packed columns. In the 1970's packed columns were abandoned for concern of the compressibility of carbon dioxide causing density changes along the column affecting the retention factors [1]. These beliefs led to the pursuit of using capillary columns similar to those used in GC. Unfortunately, the optimal flow rates that these columns produced were low and maintaining consistent modifier concentrations and backpressures proved difficult. In the 1980's it was shown that a conventional HPLC instrument could be modified to SFC separations [1]. It was not until after 1992 when packed column SFC was used widely for commercial purposes [2]. This has led to SFC being commonly used in the areas of preparative chromatography, pharmaceuticals, and chiral separations [1].

SFC has several advantages over ultra high performance liquid chromatography (UHPLC) and GC. SFC is not limited to volatile compounds that limit the analytes that can be separated in GC. SFC requires less organic solvent and modifier than are typically used in HPLC. UHPLC columns are typically shorter and narrower in order to limit the effects of frictional heating gradient that is generated. This limits the number of theoretical plates that an HPLC column can have. SFC separations can be done four times faster than HPLC methods and with twice the column efficiency [3]. The pressure drops in SFC can be as low as 20% of that seen in HPLC [2].

In SFC, there is enthalpic expansion of carbon dioxide that leads to an axial cooling gradient, similar to the heating gradient seen in UHPLC. At certain temperatures and pressures the heating and cooling terms offset, which creates a zero heat balance zone [4]. At these zero heat balance regions the negative impacts of the temperature gradients would be minimized. To reach these zero heat balance regions higher pressures are needed. Most common SFC is done at 100-400 bar and 20-40^{o}C [1]. This is only a small section in the range of possible conditions that SFC can be done. Also, at these pressures carbon dioxide behaves more like a liquid. To reach the zero heat balance zone at 40^{o}C the pressure needs to be at least 400 bar. This is pushing the limits of current systems. If higher pressures can be obtained, not only will faster separations be achievable but longer columns and/or smaller particles could be used. The range of pressures of particular interest is from 400-600 bar. Most commercial instruments can only handle pressures up to around 400 bar. The Agilent 1260 Infinity Analytical SFC

System is the only commercially available instrument that is able to reach pressures of 600 bar.

There are certain characteristics needed for a high pressure and high performance system. First, all tubing, connectors, and flow cell need to work under that pressure. Second, when operating at higher flow rates a fast detector is needed. Lastly, a pump of sufficient volume that can withstand the high pressures is needed. Once assembled, the instrument then needs to be optimized to minimize the variance contributions that are not from the column. This suggests that shorter tubing connections, smaller injection volumes, and small flow cell sizes would be optimal for creating a fast high-pressure SFC system.

2. Construction of SFC system

2.1 SFC components

The instrument consists of the following (Figure 1). Two pumps are required, one for carbon dioxide and another for modifier addition. The two pumps are connected with a mixer system used to deliver the modified mobile phase. Sample vessels containing various samples dissolved in neat CO_2 are a useful feature. Both pumps and sample vessels are connected to the injector. A column preheater is placed prior to the column heater. The column is mounted inside the column heater. The detector is placed at the end of the column. The backpressure regulators are placed downstream from the detector.

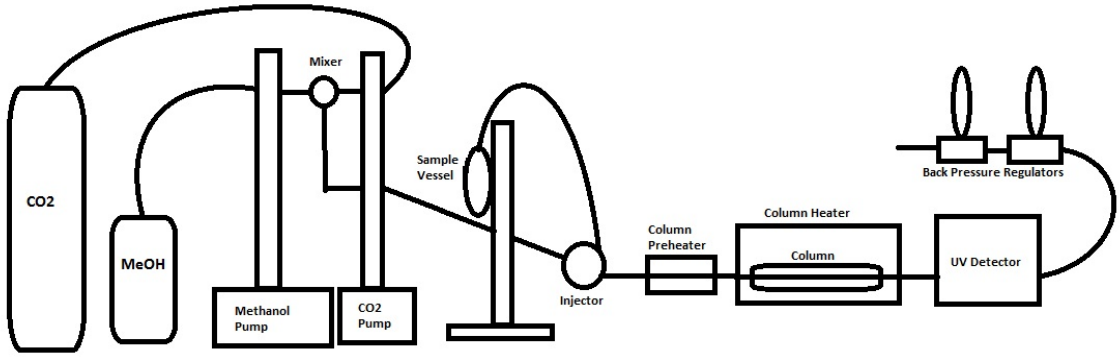

Figure 1. Diagram of the SFC system.

2.2. Description of the Individual Components

2.2.1 Pumping System

The pump used for the SFC system was an ISCO model 260D syringe pump. The pump has a volume of 266 mL and a pressure rating of 515 bar. An ISCO model 100DM syringe pump was later added to modify the carbon dioxide mobile phase. This modifier pump has a volume of 100 mL and a pressure rating of 695 bar. Both pumps were connected with an ISCO modifier addition kit for non-continuous pumping.

2.2.2. CO_2 Solvent-Based Sampling System

For injection samples dissolved in CO_2, sample solutions were made in a Double-Ended DOT-Compliant Sample Cylinders, model 304L-HDF4-150, with a working pressure of 124 bar from Swagelok. Sample vessels are set in parallel and samples are controlled with valves. Sample vessels are attached to a wooden frame to mount all vessels. The vessels were pressurized using the pumping system when pressures fell below 95 bar. The vessels were pressurized with carbon dioxide.

2.2.3 Injection System

The pumps were connected with stainless steel tubing to a six-port injector. The injector used was a VICI Cheminert model 11P-0154H six-port injector. The injector was operated by an actuator and was rotated using helium. The injector has a pressure rating of 690 bar. Sample loops available for the injector included a 1-µL and 5-µL loop.

2.2.4. Temperature System

The injector was connected to the column preheater. The preheater was constructed using a 30-mm x 42-mm x 12-mm aluminum block (Figure 2). Three holes were drilled into the block. One hole 30 mm deep and 4 mm in diameter was for the heating cartridge. A 10-cm length of 0.007" ID tubing was inserted into a 2-mm ID hole. The final hole drilled was 3-mm ID for the temperature sensor. Thermal paste was used to ensure contact and good heat transfer. The temperature for the preheater was controlled using an Omega model CSI32 RTD proportional integral derivative (PID) controller.

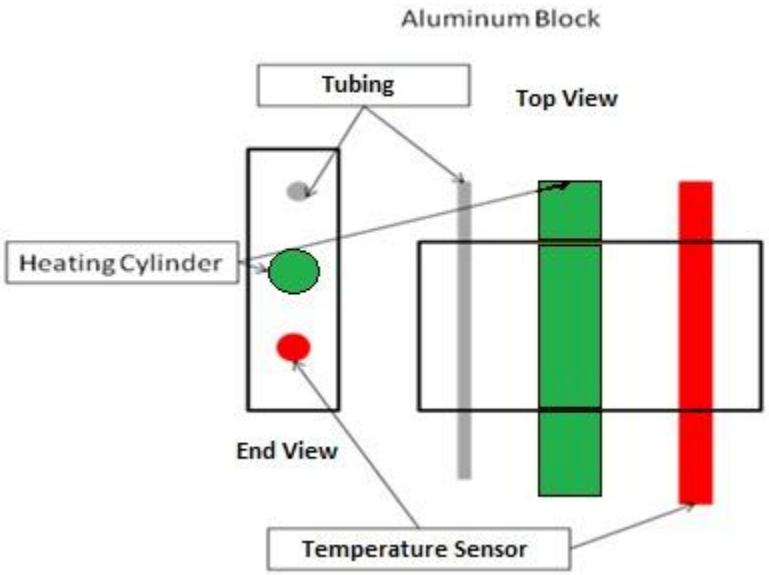

Figure 2. Image of column preheater construction.

A PID controller monitors three different parameters: the proportional, the integral, and the derivative terms. First, the temperature is set to 50 ^{o}C for both the BPR heater and the column preheater. The parameters for the controllers are as follows; self disable, %low 0, %high 99 for BPR heater and 11 for column preheater, controller type PID, Action type reverse, Auto PID enabled, Anti integral enabled, start auto tune PID disabled, cycle time 7 seconds, and damping factor 3. The column preheater was wrapped in fiberglass insulation to minimize heat loss to the surroundings.

The column was placed in a Brinkman model CH-30 column heater. The temperature was monitored using an Omega recording system prior to the column inlet and the column heater was set to give a reading of 50 ^{o}C.

2.2.5. Pressure System

A pressure transducer was placed before the injector to measure the inlet pressure. A second pressure transducer was placed downstream of the detector to

measure the outlet pressure. The outlet and inlet pressure transducers were Sensotec model TJE pressure transducers with a pressure rating of 515 bar. The pressure transducers were connected to a model GM Sensotec display. An Ashcroft model K1 pressure transducer was used to monitor sample vessel pressures. The Ashcroft transducer is rated to 690 bar. It was connected to a display built in-house.

It is important to maintain the stability of the backpressure because retention factors, diffusion coefficients, and mobile phase parameters such as viscosity and linear velocity depend on the density changes of the mobile phase, which are affected by changes in pressure. Two backpressure regulators (BPR) were connected downstream from the detector. A Tescom 10000 psi regulator was placed in series with a 4000 psi regulator. Two BPRs were used rather than using a single BPR to allow for better stability.

2.2.6. Connector and Detector System

The supply tubing used upstream from the injector was 0.020" ID stainless steel tubing. All tubing used to connect between the injector and detector was 0.007" ID tubing unless noted. The tubing used past the detector outlet was 0.020" ID or larger reduce the pressure drop between the detector and the outlet pressure transducer.

A Jasco Model X-LC 3075UV detector modified to reach an acquisition rate of 200Hz was used in all experiments. This detector had a flow cell volume of 4 μL.

3. Performance Characteristics

At different stages of development, the SFC system was tested for important performance characteristics. The principle characteristics tested include the system

holdup time and extra column variance, the repeatability of elution times, and the short-term and long-term stability of temperature and pressure conditions. In addition, representative data for elution of model solute systems on packed columns using both neat CO_2 and methanol-modified CO_2 are presented.

3.1. Temperature and Pressure Regulation

3.1.1. Temperature Control

The temperature stability of the preheater was measured over several different flow rates (Figures 3 & 4). The temperature sensors were not calibrated to specific temperatures but were used to examine the stability of the heater. Thus, it can be seen that the preheater does an adequate job of maintaining the desired temperature. At 1 mL/min, the temperature of the preheater does not vary by more than a tenth of a degree. At the lower flow rate of 0.2 mL/min the temperature changes more significantly but not by more than two tenths of a degree. The average drift over a two-hour period was measured at 0.15 ^{o}C.

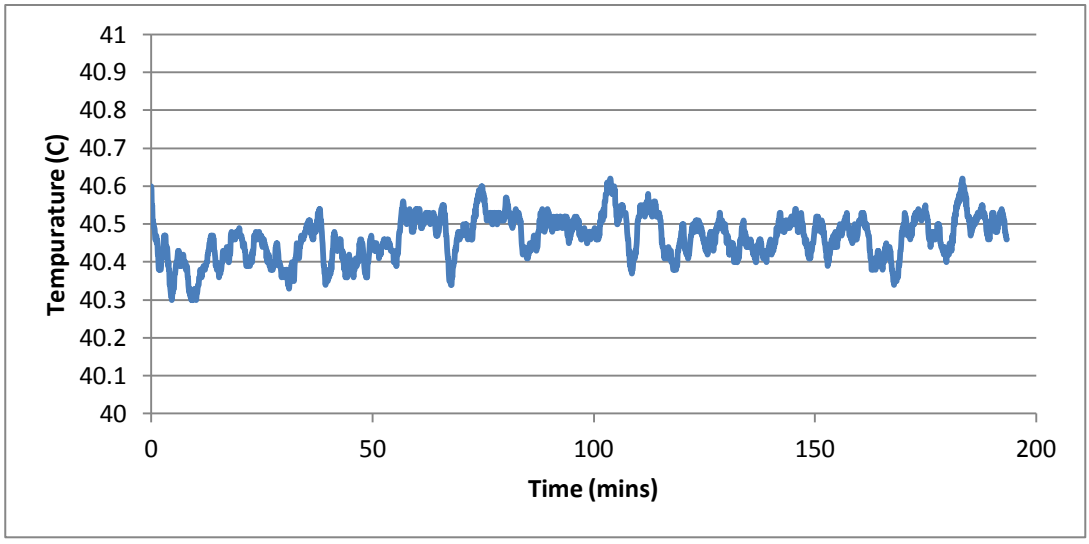

Figure 3. A plot of the pre-column temperature over time. The preheater was set to 40°C. The pump settings were neat carbon dioxide pumped at a flow rate of 1 mL/min and an outlet pressure of 150 bar.

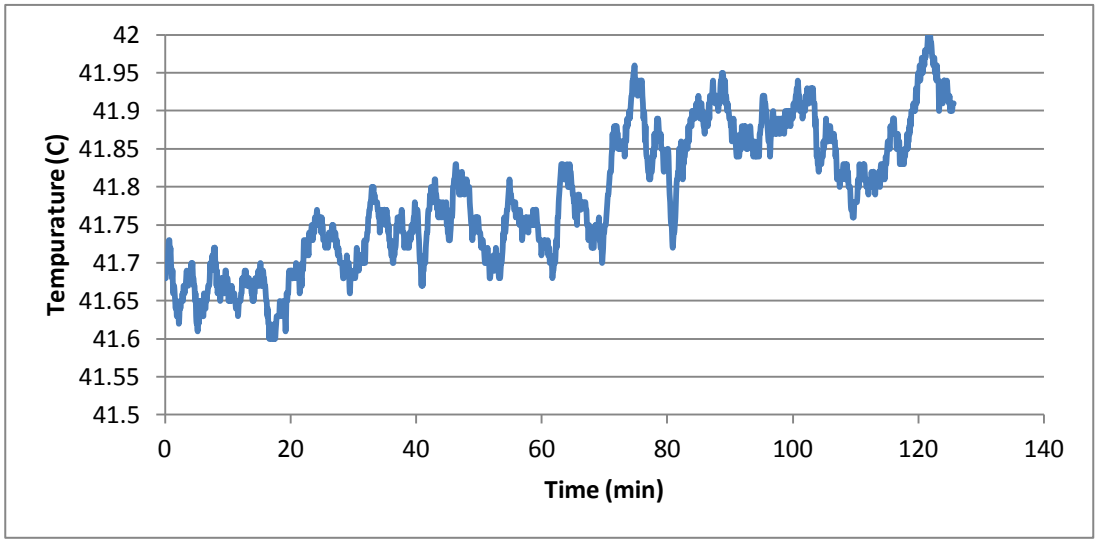

Figure 4. A plot of the pre-column temperature over time. The preheater was set to 40°C. The pump settings were neat carbon dioxide pumped at a flow rate of 0.2 mL/min and an outlet pressure of 150 bar.

3.1.2. Pressure Control

When examining a single BPR (Figure 5) the stability of the backpressure was poor. The average drift in the pressure was found to be 0.9 bar/hour over a two-hour period. The stability

with two BPRs was examined over a four-hour period (Figure 6). This shows that the backpressure remains within 0.15 bar after a warming up period of approximately 100 minutes. The BPRs are also placed on a heated aluminum plate. This has enhanced the stability of the back pressure to 0.1 bar for long-term drift. The heated aluminum plate is controlled with another Omega model CSI32 RTD PID controller.

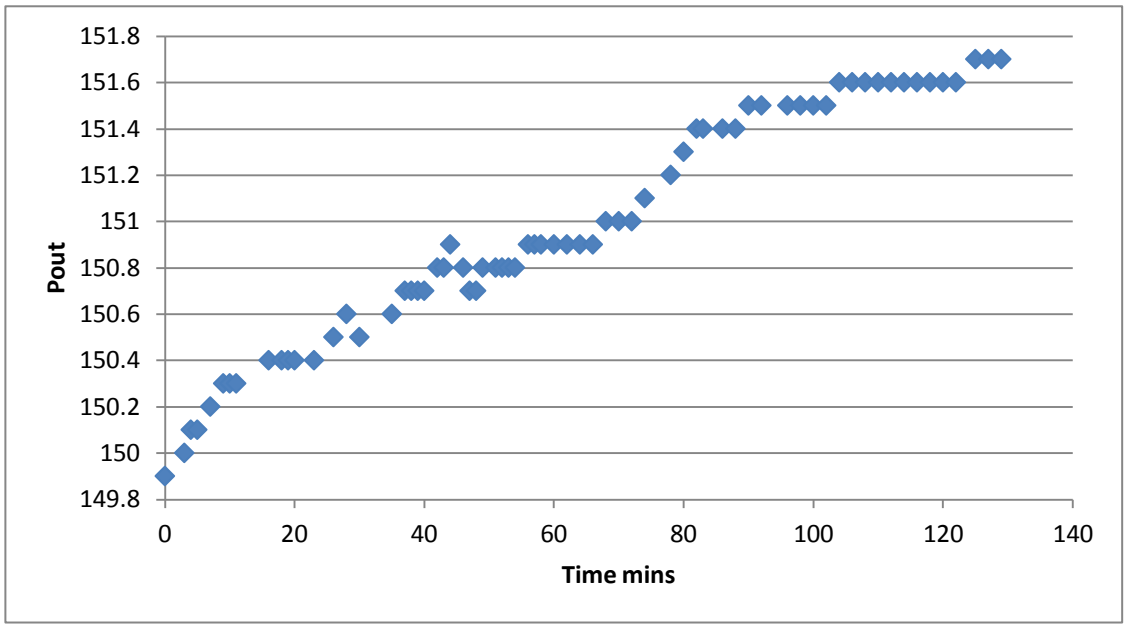

Figure 5. A plot of the backpressure over time for a single backpressure regulator. The outlet pressure was set to 150 bar.

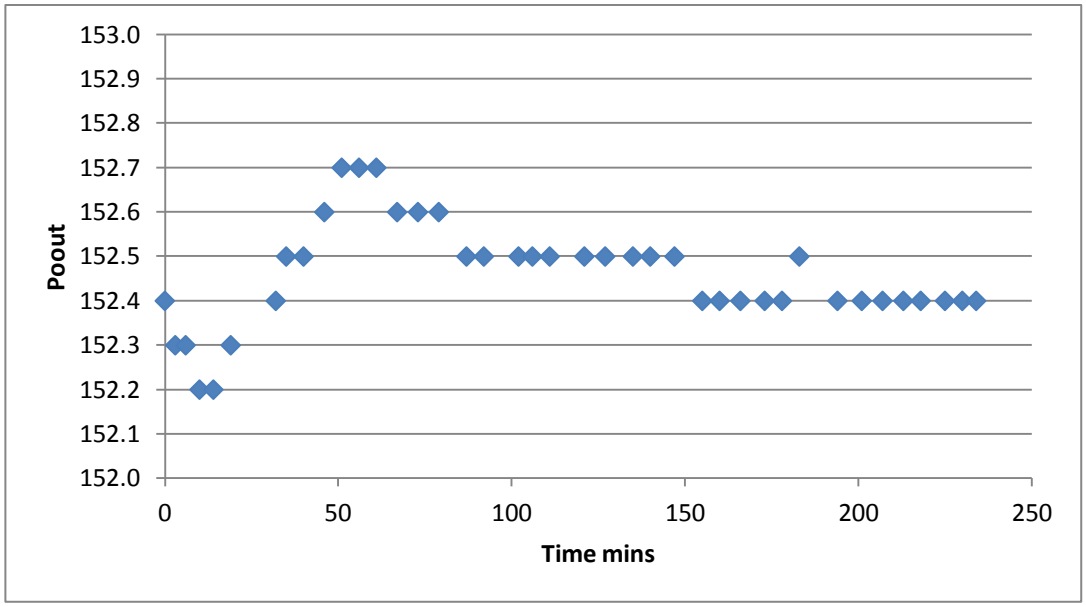

Figure 6. A plot of the backpressure over time with two backpressure regulators in series. The outlet pressure was set to 150 bar.

3.2. System Holdup Time and Variance

Once a SFC system is constructed, it needs to be characterized. By characterizing the system, the best operating conditions can be determined. These conditions include material conditions as well as setting conditions. Material conditions are any adjustments that can be made to the physical system to enhance performance, such as changing the sample loop size or changing columns. Setting conditions can be adjusted without dismantling the system, such as adjusting the acquisition rate of the detector. There are two ways to examine these conditions. First, analysis can be done by looking at the retention times and peak shape of eluted compounds with a column present. The second way is looking at extra column volume and variance. Extra column volume is the volume that is added by the connecting tubing, the injector and the flow cell of the detector, but does not include the column. Ideally, the extra column volume should be as close to zero as possible. A major objective is to minimize any broadening of peaks due to dispersion processes in these components. Variance is the measure of the degree of dispersion of

the solute bands in the system. To characterize the system fully, both methods need to be applied under various conditions.

3.2.1. System Characteristics. Theory

In order to characterize the system retention time, the extra column volume and the variance need to be defined. Retention time can be measured in two different ways. The first way is by taking the time at the peak maximum and using that as the retention time. The second technique is using the first statistical moment of the peak. Statistical moment data can be calculated using the following formula [5].

$$< \boldsymbol{y^n} > = \frac{\int_{-\infty}^{\infty} \boldsymbol{y^n} * \boldsymbol{c(y,t)} * \boldsymbol{dy}}{\int_{-\infty}^{\infty} \boldsymbol{c(y,t)} * \boldsymbol{dy}} \tag{3.1}$$

This calculates the *n*th moment for a set of data where *c(y, t)* denotes the function for the set of data that forms the peak. The first moment is taken with respect to the origin, where as greater moments are calculated with respect to the first moment [5]. The first moment can be thought of as the position of the center of mass for the peak. The first moment was used as the primary measure for retention time for these studies.

Extra column volume was calculated using the following equation when no column is present.

$$V_{ec} = \frac{t_{ec} * F_{pump} * \rho_{pump}}{\rho_{conn}} \tag{3.2}$$

where t_{ec} is the retention time without a column present, F_{pump} is the volumetric flow rate at the pump in μL/sec, ρ_{pump} is the density of carbon dioxide at the pump (at -2°C and the inlet pressure reading), and ρ_{conn} is the density of the carbon dioxide at the connectors (based on the temperature in the column heater and average of inlet pressure and outlet

pressure). Extra column volume can also be calculated by plotting the retention time verses 1/F. The results are then fit to a linear regression according to the following equation.

$$t_r = V_{ec} * \left(\frac{\rho_{conn}}{\rho_{pump}}\right) * \frac{1}{F} \quad (3.3)$$

Variance or the second moment can be interpreted in terms of total variance and the variance of the individual parts of the system. It can also be calculated for a set of data using Equation 3.1. The total variance (σ_v^2) is defined as the sum of the variances from the column $\sigma_v^2{}_{Col}$, the injector $\sigma_v^2{}_{Inj}$, the tubing $\sigma_v^2{}_{Conn}$, the flow cell $\sigma_v^2{}_{cell}$, and the detector electronics $\sigma_v^2{}_{Elect}$. [6]

$$\sigma_v^2 = \sigma_{v\,Col}^2 + \sigma_{v\,Inj}^2 + \sigma_{v\,Conn}^2 + \sigma_{v\,Cell}^2 + \sigma_{v\,Elect}^2 \quad (3.4)$$

Ideally, the largest contributor to the total variance should come from the column All formulas for the calculation of the variance for the various components were taken from one paper by Gritti and Guiochon [6].

3.2.1.1. The column

The variance for the column can be calculated using the following equation

$$\sigma_{v\,Col}^2 = \frac{V_0^2}{N} * (1+k)^2 \quad (3.5)$$

where V_0 is the void volume of the column, N is the number of theoretical plates generated by the column and k is the retention factor. The void volume (V_0) can be calculated using the following equation

$$V_0 = \xi_t * \pi * r_c^2 * L \quad (3.6)$$

where ξ_t is the total porosity, r_c is the inner diameter of the column, L is the length of the column, and k is the retention factor. Retention factor (k) is defined as

$$k = \frac{t_r - t_m}{t_m} \tag{3.7}$$

where t_r is the retention time of the analyte and t_m is the time required for the solvent to pass through the column. The porosity is typically estimated at 0.63 for a column packed with fully porous particles, and 0.55 for core shell particles.

3.2.1.2. The injector and detector

The variance for the injector is

$$\sigma^2_{v\,inj} = \frac{V_{inj}^2}{12} \tag{3.8}$$

where V_{inj} is the volume of the sample loop *in* the injector.

3.2.1.3. The connectors

The connector variance can be calculated by

$$\sigma^2_{v\,conn} = \frac{\pi * 2 * r_c^4 * L^2}{3 + (24 * L * {}^{D_m}/_{F})} \tag{3.9}$$

where D_m is diffusion coefficient of the analyte and F is the flow rate. The diffusion coefficient can be calculated from a modified version of the Wilke Chang equation [7].

$$D_m = (7.4 * 10^{-15}) * T * \frac{M_S^{1/2}}{\eta * V^{0.6}} \tag{3.10}$$

where *T* is the temperature in Kelvin, M_s is the molar mass of the solvent, η is the viscosity of the mobile phase, and V is molar volume of the analyte compound at ambient temperature. This is easily calculated using the molecular weight and the density (Table 1).

Table 1. Calculated diffusion coefficients for various alkylbenzenes used in these studies.

Compound	Density (g/mL)	Molecular Volume (mL/mol)	Diffusion Coefficient in neat CO_2 at 50 ^oC and 150 bar (m^2/s)
Decylbenzene	0.859	254	1.01E-08
Dodecylbenzene	0.858	287	9.40E-09
Tetradecylbenzene	0.857	320	8.81E-09
Octadecylbenzene	0.856	386	7.87E-09

3.2.1.4. The detector flow cell

Contributions from the detector include the flow cell and the detector electronics. The variance for the flow cell is

$$\sigma^2_{v\,Cell} = \frac{V_{Cell}^2}{K} \tag{3.11}$$

where V_{cell} is the volume of the flow cell and K is the mixing coefficient. K = 12 is no mixing in the flow cell and K = 1 is a perfect mixer. A value of between 5 and 6 is a typical expected value [6]. A comparison of flow cell variances for constructed system and commercially available system can be seen in table 2.

Table 2 : Calculations for detector variance for Jasco detector and another commercially available system (13 μL) at different mixing coefficients (*K*).

flow cell volume (μL)	K value	variance of flow cell μL^2
4	3	5.33
4	5	3.2
4	7	2.29
13	3	56.33
13	5	33.8
13	7	24.14

3.2.1.5. The detector electronics

The volume variance from the detector electronics can be calculated using the following equation assuming a uniform distribution:

$$\sigma^2_{v,rate} = \sigma^2_{t,rate} * F_v^2 \quad (3.12)$$

where F_v is the flow rate and $\sigma^2_{t,rate}$ is the time variance. $\sigma^2_{t,rate}$ is defined by Fountain et al (2011) is related to the sampling time by the following equation.

$$\sigma^2_{t,rate} = \frac{t_s^2}{12} \quad (3.13)$$

where t_s is the sampling time and $t_s=1/\nu$, where ν is the sampling frequency [8]. The volumetric variance due to the sampling rate is then

$$\sigma^2_{v,rate} = \frac{t_s^2}{12} * F_v^2 \quad (3.14)$$

where ν is the acquisition rate in hertz. This leads to τ in equation 3.13 being equal to the sampling time. Thus, equation 3.13 can be written in terms of acquisition rate.

$$\sigma^2_{v,rate} = \frac{1}{12*\nu^2} * F_v^2 \quad (3.15)$$

Values for detector electronics were calculated for acquisition rate of 200Hz. In table 3 acquisition rates of 200 and 60 are available to the detector and acquisition rates of 60 and 30 are available to the data system server.

Table 3. Calculation for detector rate variance for various acquisition rates.

Flow Rate mL/min	Flow Rate uL/sec	Detector variance 200 Hz μL^2	Detector variance 100 Hz μL^2	Detector variance 60 Hz μL^2	Detector variance 30 Hz μL^2
0.2	3.33	2.31E-05	9.24E-05	2.57E-04	1.03E-03
0.5	8.33	1.45E-04	5.78E-04	1.61E-03	6.42E-03
1	16.67	5.79E-04	2.32E-03	6.43E-03	2.57E-02
1.5	25	1.30E-03	5.21E-03	1.45E-02	5.79E-02
2	33.33	2.31E-03	9.26E-03	2.57E-02	1.03E-01
2.5	41.67	3.62E-03	1.45E-02	4.02E-02	1.61E-01
3	50	5.21E-03	2.08E-02	5.79E-02	2.31E-01

3.2.2. System Characteristics. Experimental Work

3.2.2.1. Sample Preparation

Data were obtained for four different alkylbenzenes (decylbenzene, dodecylbenzene, tetradecylbenzene, and octadecylbenzene). A solution of a mixture of decylbenzene, tetradecylbenzene, and octadecylbenzene (1.0 µL/mL or 1.0 µg/mL) was created by adding 150µL of decylbenzene and tetradecylbenzene along with 150 mg of octadecylbenzene in a 150-mL stainless steel sample vessel. The mixture was then subjected to vacuum briefly to remove air

from the vessel and then 4 bar of nitrous oxide was added to act as a t_0 marker. The sample vessel was then pressurized with carbon dioxide to a total pressure of 120 bar. The sample was then mixed with a magnetic stir bar overnight. The sample of dodecylbenzene was prepared earlier at a concentration of 1 μL/mL. The sample vessel was then connected to the system.

3.2.2.2. Experimental Procedures

All samples were tested under the same conditions. Samples were evaluated at flow rates of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL/min, unless otherwise noted. The columns consisted of a Luna 100 x 3 mm packed with 2.5-μm particles with a stationary phase being C18(2)-HST, a Kinetex 2.6 μm 150 x 2.1 mm column with a C18 100A stationary phase, and a Luna 5-μm 150 x 4.6mm column with a C18(2) 100A stationary phase. The injections were done with a 1-μL injection loop. The detector settings were run in X-LC mode in the fast response setting with an output range of 0.16 AU/10 mV at a wavelength of 205 nm. The column preheater and BPR heater were set to 50 ^{o}C.

3.2.2.3. System Configuration and Test Procedure

Several different configurations were examined. For all experiments in this section the column was replaced with a 10-cm length of 0.005" ID stainless steel tubing (column proxy) connected by a zero-dead-volume union. The sample injection loop was either 1 or 5 μL. Neat Laser Star grade CO_2 (Praxair, 99.995%, H_2O < 5 ppm, O_2 < 5 ppm, THC as CH_4 < 1 ppm) was used as the mobile phase. The detector settings were a wavelength of 208 nm for alkylbenzenes and 195 nm for nitrous oxide samples, response time of 0.03 s, and output of 0.16 AU/10 mV. A column preheater was used to heat the mobile phase prior to entering the column heater. The column heater when used was controlled to yield a column proxy temperature of 50 ^{o}C. Three unique tubing configurations were used to examine the contribution of the tubing variance to the total variance.

- Configuration A was done using 72 cm of 0.007" ID tubing and an additional 10 cm length of 0.007" ID tubing connecting the injector and detector
- Configuration B was done with a piece of 72 cm 0.007" ID tubing and a 10 cm 0.005" ID tubing (used as column proxy).
- Configuration C was done with a total of 154 cm of 0.007" ID tubing along with a 10 cm length of 0.005" ID tubing.

3.2.2.4. Injections using liquid solvents

To examine these properties several different sample injections were done. The first sample was done with octadecylbenzene in methanol. The octadecylbenzene was purchased from Tokyo Chemical Industry (TCI America) and was dissolved in Fisher Scientific HPLC grade methanol. A stock solution of 250 mL 1mg/mL was created. Other concentrations were created ranging down to 0.05 mg/mL from the stock solution were also prepared.

3.2.2.5. Injections using carbon dioxide as solvent.

A second sample of a mix of decylbenzene, tetradecylbenzene, and octadecylbenzene was made using carbon dioxide as the solvent. The sample was created in a stainless steel vessel at a concentration of 1 μL/mL. The sample was pressurized with carbon dioxide to approximately 125 bar. Once the vessel was pressurized, the sample was mixed overnight with a stir bar.

3.2.2.6. Injection method

A new analysis sequence was created for each new experiment. The pump was filled with CO_2 and allowed to cool to -2^{o}C and reach equilibrium before starting

injections (about 4 hours). Injections were done with syringes for the liquid solvent injections and with sample vessels and stainless steel tubing for the CO_2 solvent injections. The sample was passed through the sample loop to load the sample. Once filled the start button on the chromatography server was pressed and the injector was switched to inject. Once all injections at a flow rate were done, the flow rate was adjusted to the next desired value and allowed to equilibrate (15 – 30 minutes). Injections were done in triplicate for every flow rate examined.

3.2.3. System Characteristics, Results and Discussion.

3.2.3.1. Suitability of different solvent systems

The suitability of the different solvent systems for studies on the system variables with no column present are presented in this section.

3.2.3.1.1. Liquid solvent injection

A series of injections using the octadecylbenzene in methanol sample were done in triplicate. A sample chromatogram is shown in Figure 8. There is a very large tail seen in the peaks that lasts approximately 70 seconds. This distorts the analyte peak so that it cannot be used for determining retention time. Two peaks can also be seen in the chromatogram. The first peak on the chromatogram was assumed to be due to the methanol solvent, which tails into the second peak for the analyte. The extra volume was then calculated with the octadecylbenzene sample and found to be around 82.2 μL, where the expected V_{ec} is 8.27 μL. The expected extra column volume was based on the calculated volume of the injector, connecting tubing, and flow cell. This confirms that there is something adding to the peak broadening and causing a loss of efficiency.

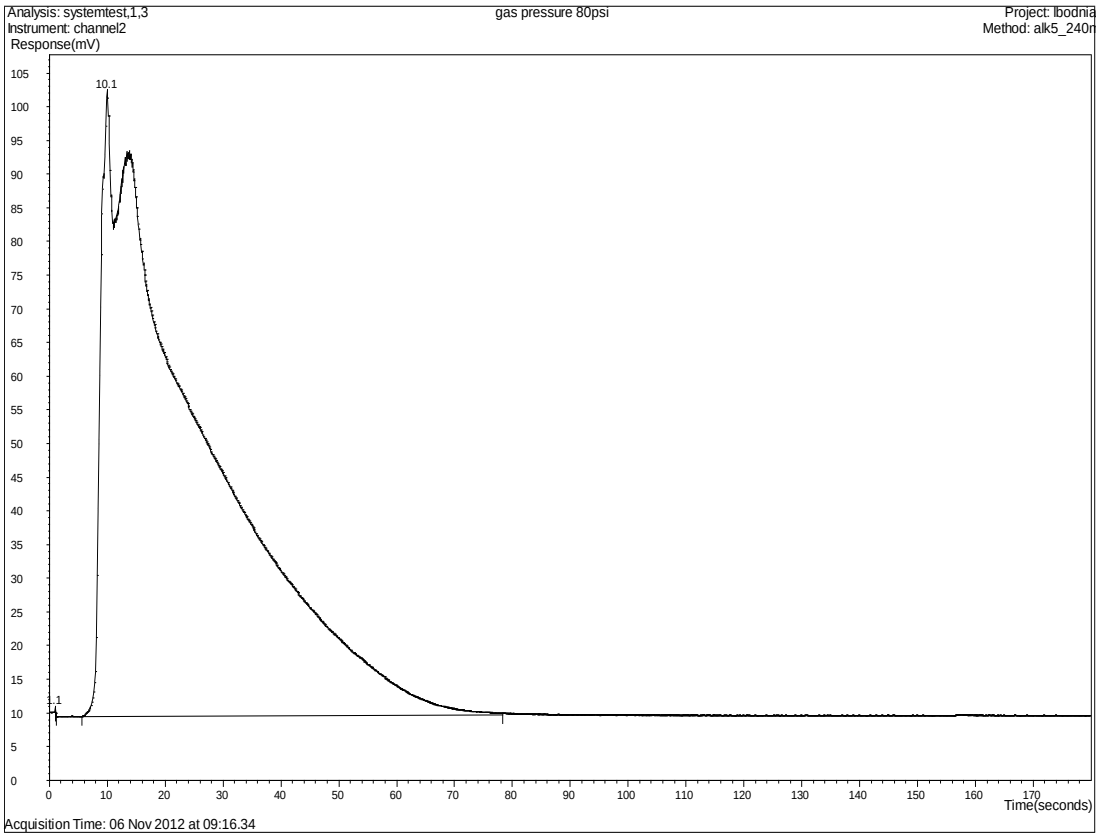

Figure 7. A chromatogram using 1 mg/mL octadecylbenzene in methanol. The conditions were no column present, done at room temperature, a flow rate of 1mL/min, and an outlet pressure of 150 bar. Injection volume was 5 μL and tubing used was configuration B.

3.2.3.2.2. Carbon dioxide solvent system

The sample of dodecylbenzene in carbon dioxide solvent was also tested with similar conditions. When comparing the chromatogram done with neat CO_2 (figure 8) and the chromatogram with methanol based solvent (figure 7) a significant difference is seen. The CO_2 based solvent injections had a much less tailing than in liquid solvent injections. The width of the base in the CO_2 based injections was around 8 seconds, were the methanol based injections had a peak width of almost 70 seconds. The peak width of the methanol based injections is almost 10 times that seen in the CO_2 based injections.

Since the only change was from the sample injected, there is no reason why the change in analytes would lead to this excessive broadening. The broadening must be due to the change of solvent used. CO_2 is inert and should not be retained on the tubing. Methanol and water however has been seen to be retained on the inside of stainless steel tubes [9,10].

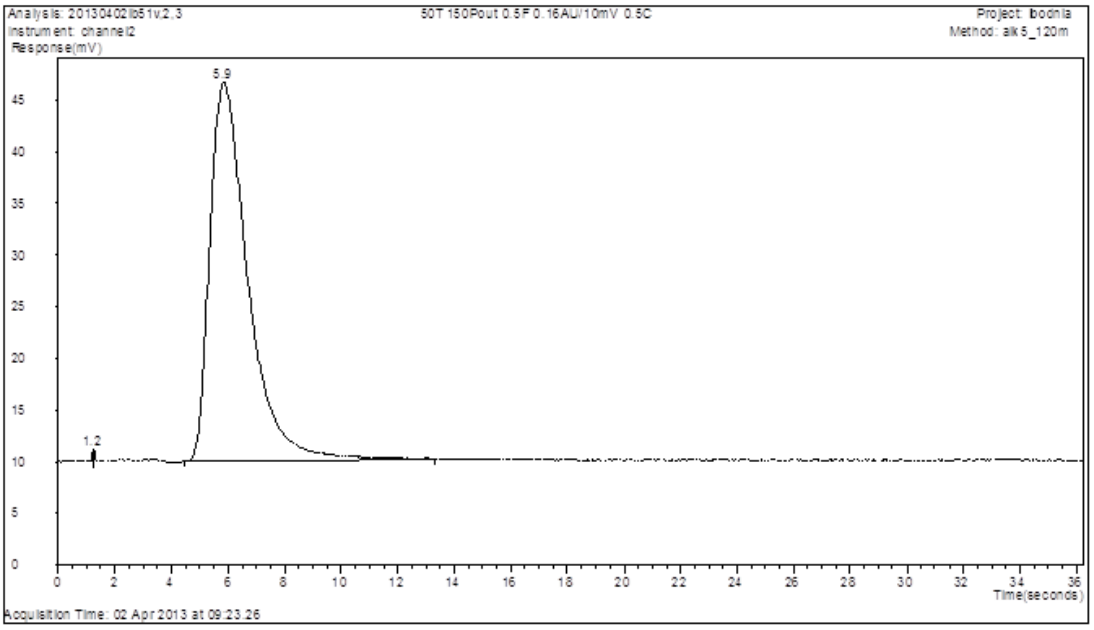

Figure 8. A chromatogram using 1 μL/mL dodecylbenzene in neat CO_2. The conditions were no column present, done at 50 ^oC, a flow rate of 0.5 mL/min, and an outlet pressure of 150 bar.

3.2.3.2. Results for the System Holdup Time

The data for injections with carbon dioxide solvent were compared to those with the liquid solvents. The calculated extra column volume for dodecylbenzene in carbon dioxide was found to be 14.83μL once the density ratio is considered (Figure 9). The expected value was 8.27μL. The volume using neat carbon dioxide is as the injection solvent 18% of the volume calculated using methanol and much closer to the expected value. This means that the choice of solvent could be the only possible source for such a

dramatic increase in the extra column volume and unusual peaks. The methanol solvent creates broader peaks and leads to higher variance. Similar phenomena have been seen with water in open tubular columns [9]. Water was found to form a layer on the inside of the stainless steel tubing. Since methanol has some properties similar to water, the same phenomena may be occurring. A more recent study has found that methanol does act in a similar fashion to water and forms a layer around the silica particles of a packed column [10]. This phenomenon is not seen when the mobile phase is modified with methanol. This is likely due to the methanol in the mobile phase being sufficient to cover the inside of the tubing and being replenished by the new methanol coming in, so a constant layer is formed.

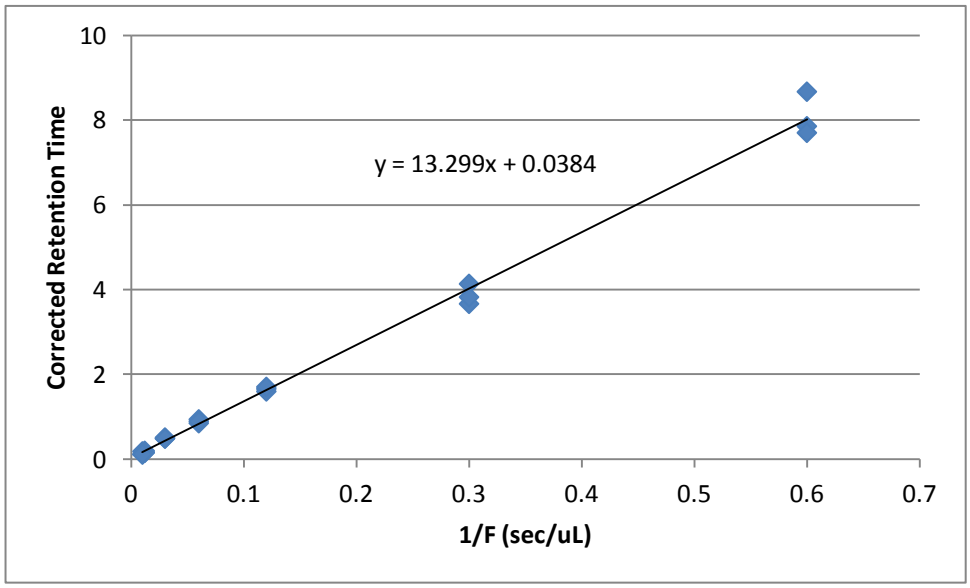

Figure 9. A graph of the corrected retention time against the inverse flow rate. The slope of the line is proportional to the extra column volume.

3.2.3.3. Results for the System Variance

Each contribution to the total variance was examined. The equations 3.4, 3.5, 3.8, 3.9, 3.11, and 3.12 were employed to calculate the individual contributions.

- The calculated variance for the injector was 0.08 μL^2 for a sample loop of 1 μL and 2.08 μL^2 for a sample loop of 5 μL.
- The calculated variance for the 4-uL detector flow cell (using mixing constant K = 5) was 3.2 μL^2 (Table 2). For comparison, for a common commercial SFC instrument (Agilent 1260 Infinity SFC system has a flow cell of 13 μL) the calculated detector variance is 33.8 μL^2.
- Three different tubing configurations were used during these experiments (Table 4). Tubing variances were calculated using equation 3.9 for a diffusion coefficient equal to 0.00836 mm^2/s (representing tetradecylbenzene and assuming neat CO_2 mobile phase, temperature 50 ^oC, 150 bar) [7]. The calculated variance for each flow rate listed in Table 4 is the sum of the injector, flow cell, and tubing variances.
- The detector electronic variance was calculated for several flow rates used for this study (Table 3). Most injections were done in X-LC Fast mode with the fastest acquisition rate (200 Hz). Comparing the detector electronic variance to the variance on table 4, it is clear that the detector electronic variance is negligible. Since the detector electronic variance is so small it does not contribute significantly to the overall system variance.

The total extra column variance was compared for both the calculated and experimental values (Table 4). Several trends can be seen from these data. Perhaps the most striking is that the experimental variance decreases as the flow rate increases, whereas the calculated variance increases as the flow rate increases. A possible

explanation for this apparent contradiction is that equation (3.9), which assumes laminar flow, does not apply under these experimental conditions. The decrease in the variance with increasing flow rate could be the result of increased turbulence, which would improve mixing and result in a decrease in the variance. The large experimental variance at low flow rates suggests the presence of an unidentified source of dispersion.

For most cases in Table 4 the variance increases as the tubing length and volume increase, with configuration C giving the largest variance. The largest differences between the calculated and experimental variances occur at the low and high flow rates, with the largest difference being around 60 μL^2 for configuration C at 0.2 mL/min. The calculated and experimental variances are similar at moderate flow rates.

Table 4. Experimental and calculated extra column variances for three different configurations with 1.0-μL injection volume.

	Configuration A		Configuration B		Configuration C	
	Length of 0.007" ID Tubing (mm)	Length of 0.005" ID Tubing (mm)	Length of 0.007" ID Tubing (mm)	Length of 0.005" ID Tubing (mm)	Length of 0.007" ID Tubing (mm)	Length of 0.005" ID Tubing (mm)
	820	0	720	100	1540	100
Flow rate (mL/min)	Calculated Extra Column Variance μL^2	Experimental Extra Column Variance μL^2	Calculated Extra Column Variance μL^2	Experimental Extra Column Variance μL^2	Calculated Extra Column Variance μL^2	Experimental Extra Column Variance μL^2
0.2	5.91	38.52	5.65	40.00	8.33	67.18
0.5	9.67	34.75	9.00	44.98	15.69	64.39
1.0	15.49	27.21	14.11	29.64	27.43	41.61
1.5	20.82	19.70	18.70	25.24	38.59	34.33
2.0	25.72	18.94	22.87	22.46	49.21	31.65
2.5	30.23	20.97	26.68	19.13	59.33	26.75
3.0	34.41	21.31	30.16	17.46	69.00	25.79

3.2.3.4. Impact of System Variance on Total Variance for Model Column

The effect of the extra-column contributions to the system variance on the total variance with different columns is examined in this section. Variance was calculated for

three test columns (Table 5) with different void volumes and plate numbers for a retained solute with $k = 2$ when operated under optimum conditions (reduced theoretical plate heigh of 2). The 150 x 2.1-mm column was a Phenomenex Kinetex C18 column packed with 2.6-μm core-shell particles, and the 100 x 3-mm column and the 150 x 4.6-mm column were Phenomenex Luna C18 columns packed with totally porous particles. The column variance for the Kinetex column is much smaller than the column variance for the two Luna columns. Ideally, the extra column variance should be <10% of the total variance. In order to obtain this in configuration A using a 1-μL sample loop the column variance has to be greater than 200 to 400 μL^2, depending on the maximum flow rate, in order to make the extra column variance be less than 10%. This suggests that the Kinetex column cannot work for the system. Under the current conditions the 150 x 4.6 mm ID Luna column meets the requirement of the extra column variance to be less than 10% of the column variance for both calculated and experimental variances. The 100 x 3.0 mm ID column only meets variance level for the calculated values but the experimental variance exceeds 10% of the column variance. The 150 x 2.1 mm ID Kinetex column does not meet the requirement for either the calculated or the experimental variances. This means that in order to use the smaller volume columns changes in the system need to be made in order to reduce the extra column variance contributions.

Table 5. Specifications for columns used in this study. The 250 mm Luna column was replaced with a 100x3.0 mm Luna column packed with 2.5-µm C18 particles. The number of theoretical plates was calculated assuming a reduced plate height of 2. The variance calculations are assuming a $k = 2$.

Column Name	Length (mm)	Inner Diameter (mm)	Type of Stationary Phase	Particle Size (µm)	Pore Diameter (nm)	Number of Theoretical Plates	Column Volume (µL)	Column Variance (μL^2)	Maximum EC Variance (μL^2)
Kinetex	150	2.1	Core Shell C18	2.6	9.2	30000	286	24.5	2.45
Luna	100	3	Fully Porous C18(2)-HST	2.5	10	20000	445	89.2	8.92
Luna	150	4.6	Fully Porous C18 100A	5	10	25000	1570	887.9	88.79

3.3. Chromatographic Separations

3.3.1. Modified CO_2 Preparation

A series of injections was done to test the system's ability to separate a series of alkylbenzenes using 5% methanol modified carbon dioxide as the mobile phase. The methanol pump was filled with HPLC grade methanol and then purged for about 10 minutes to remove any air bubbles in the pump. The pumps were operated independently and both were pressurized to 150 bar. The pumps were then allowed to stabilize for 20 minutes, then were switched to constant flow mode. Net flow rates used in this study were the same as in previous studies. Since both pumps were operated independently the methanol was pumped at 5% of the net flow rate and the carbon dioxide was pumped at 95% of the net flow rate (Table 6). The system was allowed to equilibrate for another 20 minutes and allowed to reach a stable backpressure. Injections were done with the mixed alkyl benzene sample. The column used in this study was the Luna 100 x 3 mm column with 2.5-µm particles.

Table 6. Table of flow rates used for methanol modified injections

Net Flow Rate mL/min	Flow Rate CO2 mL/min	Flow Rate MeOH mL/min
0.2	0.19	0.01
0.5	0.475	0.025
1	0.95	0.05
1.5	1.425	0.075
2	1.9	0.1
2.5	2.375	0.125
3	2.85	0.15

3.3.2. Results and Discussion

3.3.2.1. Separations Using Neat CO_2.

Chromatograms were generated in triplicate for each flow rate. Figures 10-12 show chromatograms obtained at 1.5mL/min for each of the columns. Plate height curves were then generated for each compound on each column (Figures 13-21). The theoretical plate height was calculated in two different ways. The first being the uncorrected plate height which was calculated normally. The second being the corrected plate height where the extra column variance was subtracted from the total variance and was calculated using the following equation [11]

$$k = \frac{L}{d_p} * \frac{\mu_{2\,total} - \mu_{2\,ec}}{(\mu_{1\,total} - \mu_{1\,ec})^2} \quad (3.16)$$

where L is the length of the column in mm, d_p is the particle size in mm, μ_1 and μ_2 are the first and second moments for with the column (total) and without the column (ec). The predicted moment data for the extra column moments was taken using the same 72 cm of 0.007" ID tubing along with the 10cm length of 0.005" tubing used as a column proxy. Though this is not ideal, it should give a reasonable estimate of the extra column

variance. This allows the extra column variance to be taken out so an improved measure of the theoretical plate height can be obtained. The inlet pressure for the Kinetex column became too great at a flow rates higher than of 2mL/min and high flow rates could not be reached. The data for the 150 x 4.6 mm Luna column was only gathered at the higher flow rates as the chromatograms for the low flow rates would take up to an hour to finish. From these plate height curves, it can be seen that the corrected data yields a lower reduced plate height compared to the traditional uncorrected data. It can also be determined that at greater retention factors (k), the contribution of the extra column variance becomes minimal. This is due to the retention factor affecting the column variance and the larger the column variance is the smaller the contribution from the extra column variance. The optimum flow rate for most of the plate height curves seems to be around 1-2 mL/min. An assumed minimum reduced plate height is typically around 2. If this is taken into account, only the Luna columns come close to that range and the minimum reduced plate height for the Kinetex column is around 4-5. This is about twice as high as would be expected to see. The Kinetex column also had the greatest difference when the extra column variance was subtracted out. This would likely be because the Kinetex column has the smallest volume as well as the particles being core shelled which also reduces the column volume. The smaller the volume of the column, the smaller the column variance, this leads to larger contributions from the extra column variance. This

is most likely the reason the reduced plate height did not reach expected values.

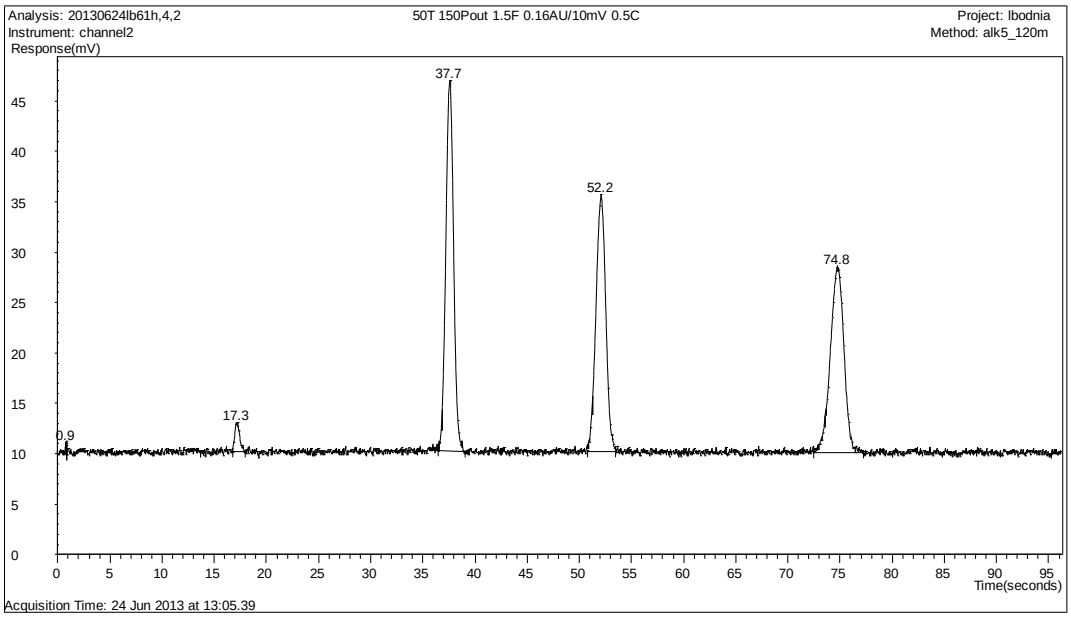

Figure 10. Chromatogram of mixture of decylbenzene, tetradecylbenzene, and octadecylbenzene on a 100 x 3 mm Luna column packed with 2.5 μm particles. Conditions were flow rate of 1.5 mL/min, inlet pressure of 216.7 bar, outlet pressure 150.0 bar, and column temperature of 50 ^{o}C.

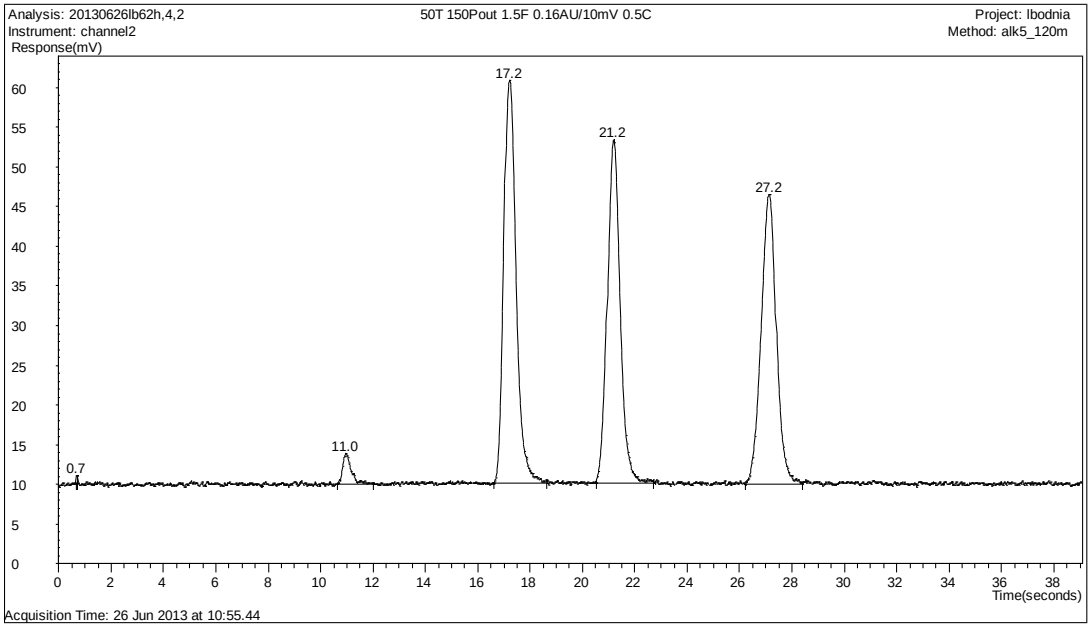

Figure 11. Chromatogram of mixture of decylbenzene, tetradecylbenzene, and octadecylbenzene on a 150 x 2.1 mm Kinetex column packed with 2.6 µm particles. Conditions were flow rate of 1.5 mL/min, inlet pressure of 342.2 bar, outlet pressure 150.1 bar, and column temperature of 50 ^{o}C.

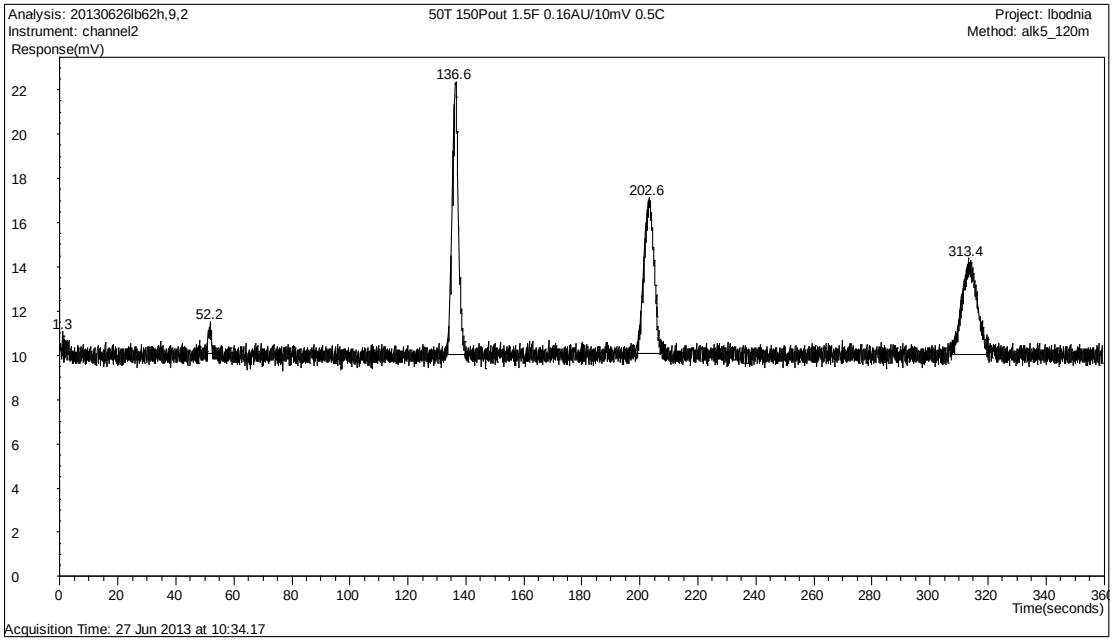

Figure 12. Chromatogram of mixture of decylbenzene, tetradecylbenzene, and octadecylbenzene on a 150 x 4.6 mm Luna column packed with 5 µm particles. Conditions were flow rate of 1.5 mL/min, inlet pressure of 159.7 bar, outlet pressure 150 bar, and column temperature of 50 ^{o}C.

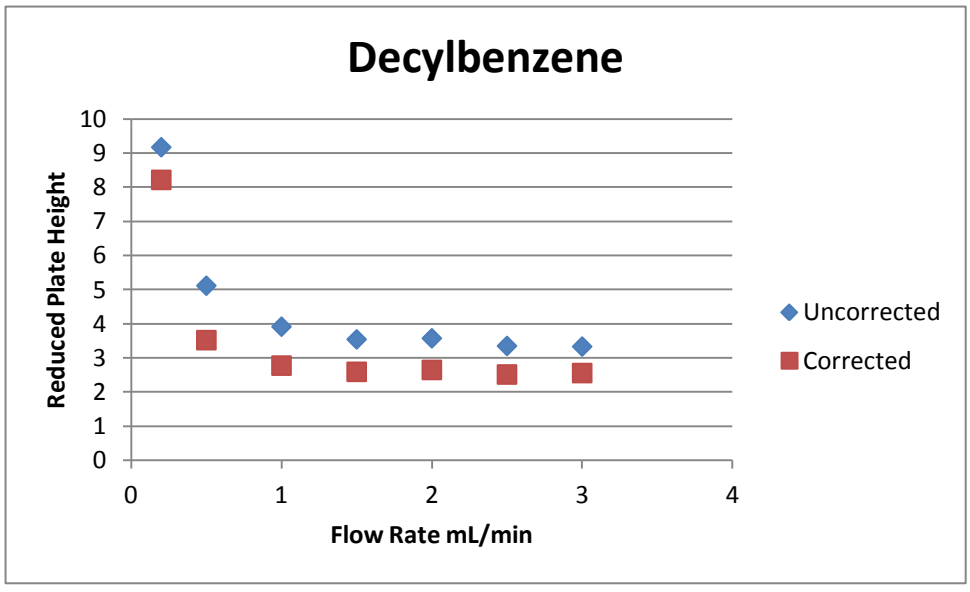

Figure 13. Decylbenzene data for Luna 100 x 3 mm 2.5 µm column.

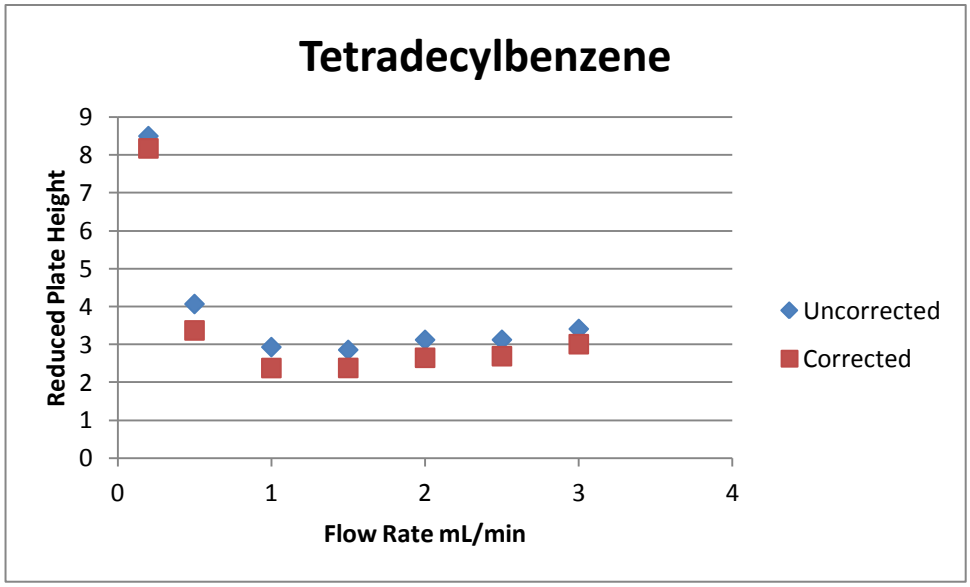

Figure 14. Tetradecylbenzene data for Luna 100 x 3 mm 2.5 µm column.

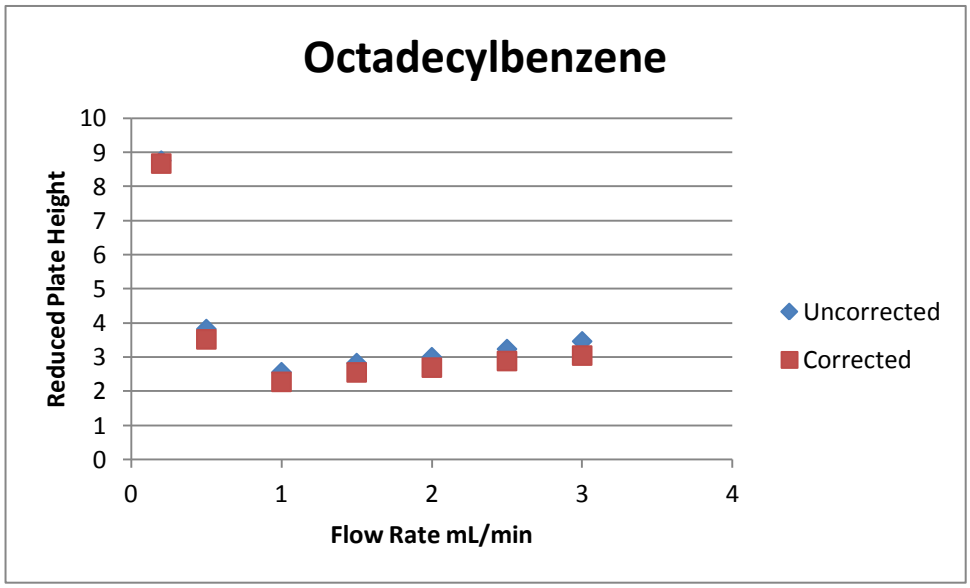

Figure 15. Octadecylbenzene data for Luna 100 x 3 mm 2.5 µm column.

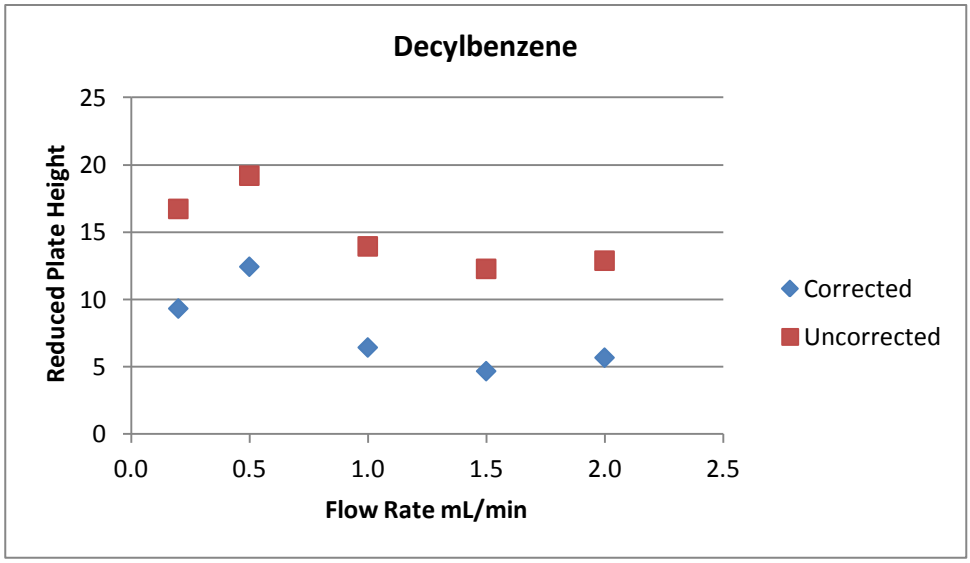

Figure 16. Decylbenzene data for Kinetex 150 x 2.1 mm 2.6 µm column.

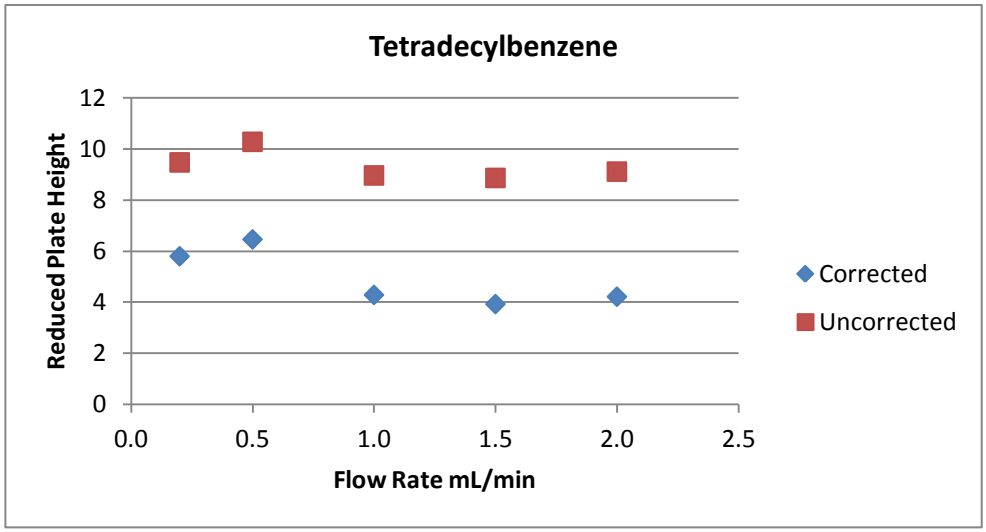

Figure 17. Tetradecylbenzene data for Kinetex 150 x 2.1 mm 2.6 µm column.

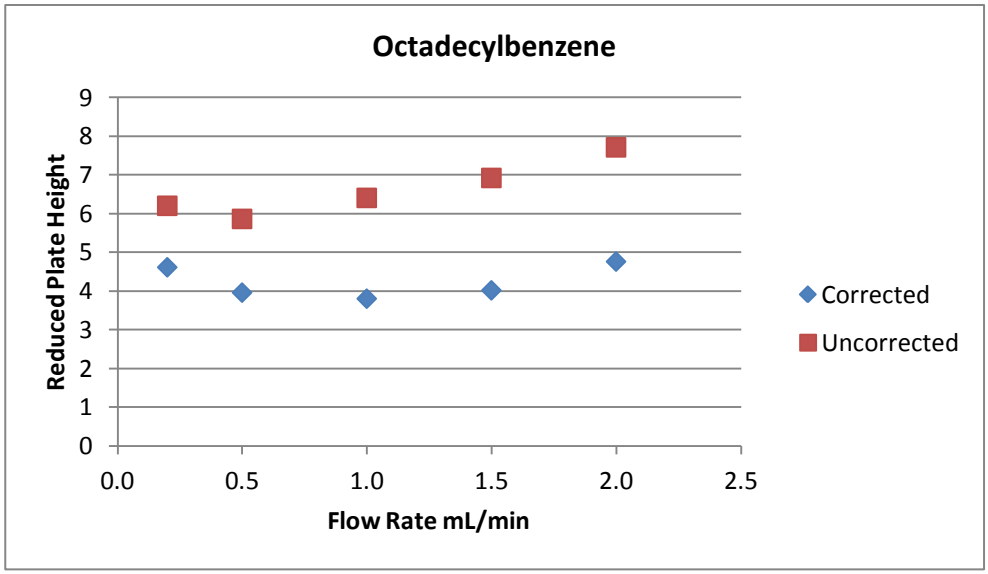

Figure 18. Octadecylbenzene data for Kinetex 150 x 2.1 mm 2.6 µm column.

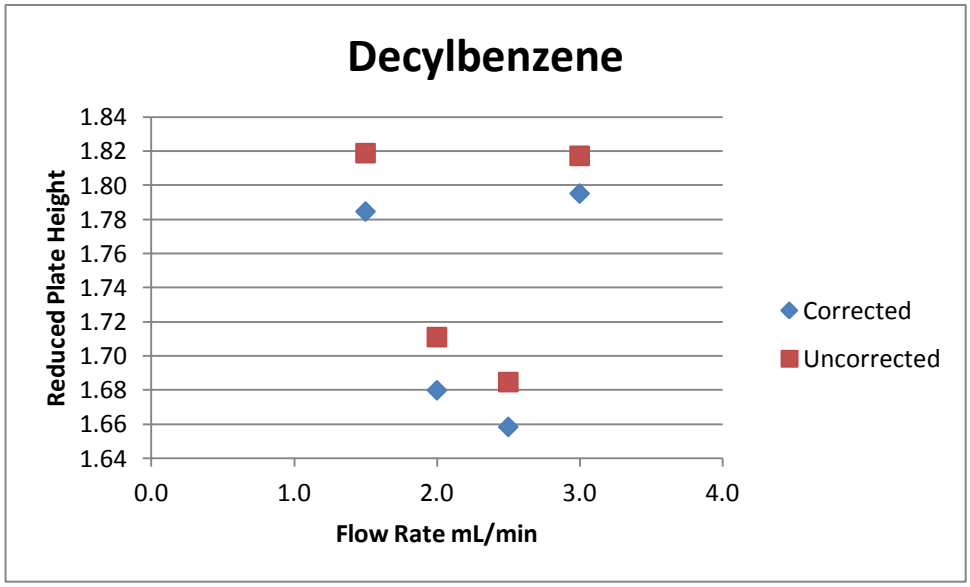

Figure 19. Decylbenzene data for Luna 150 x 4.61 mm 5.18 µm column.

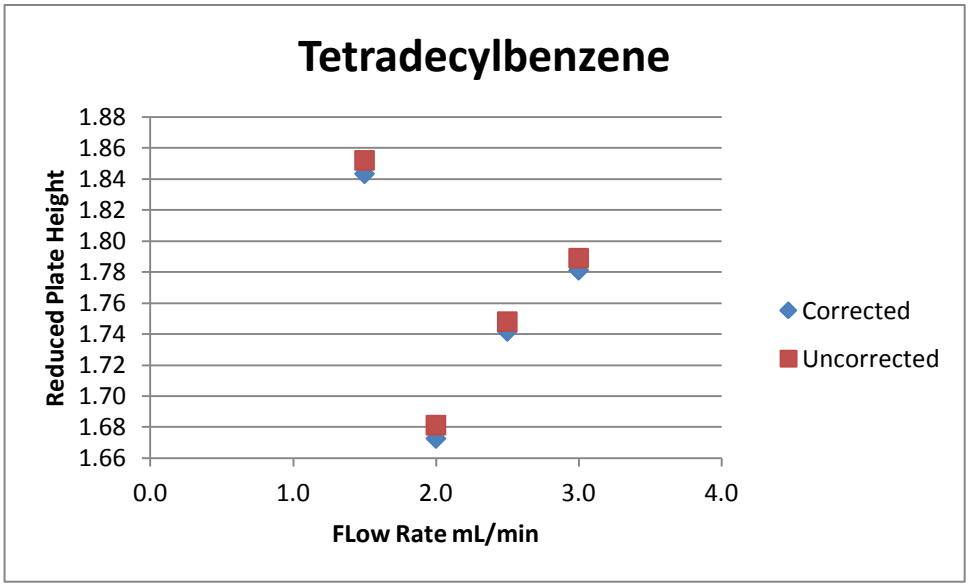

Figure 20. Tetradecylbenzene data for Luna 150 x 4.61 mm 5.18 µm column.

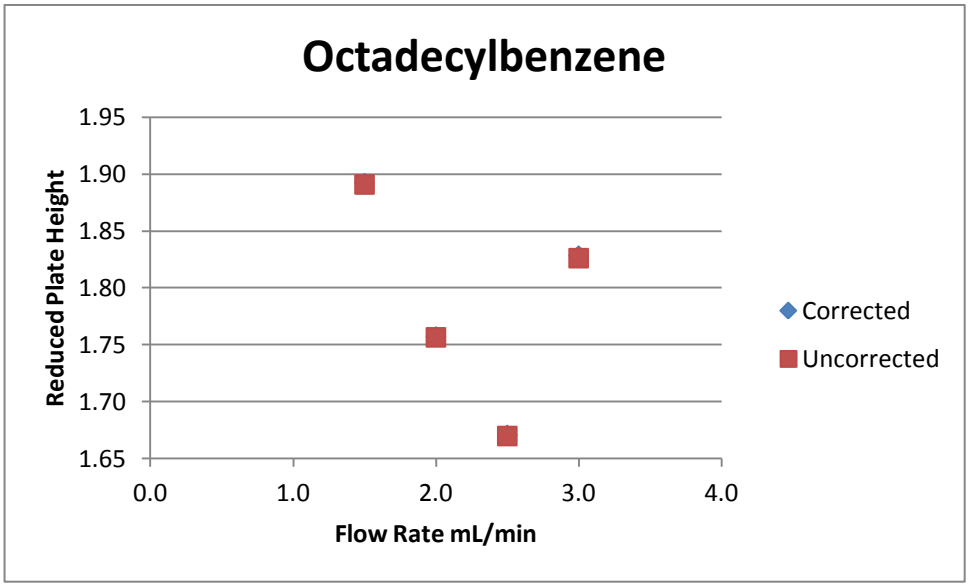

Figure 21. Octadecylbenzene data for Luna 150 x 4.61 mm 5.18 µm column.

3.2.3.2. Separations Using Modified CO_2

Plate height curves for the separations using modified CO_2 are shown in Figures 22-24. Except for the plate height curves for the low flow rates the modified mobile phase agrees with

the neat CO_2 mobile phase. At the optimal flow rate (based on figures 13-21) between 1.0-1.5 mL/min the reduced plate height differed by no more than 0.2 for all of the injections. This means that the system is capable of getting good separations with the modified mobile phase. The first moment of the last peak (octadecylbenzene) was examined to determine how fast separations were done using modified mobile phase. It was found that the retention factors of all of the analytes examined were significantly shorter in the mixed mobile phase as opposed to neat CO_2. This is a common behavior in SFC and methanol is typically used to shorten retention factors of strongly retained compounds. From all this data it can be determined that with a 5% methanol modified mobile phase there is no significant loss of column performance, separations are done faster, and there is less variance seen than when using neat CO_2 as the mobile phase.

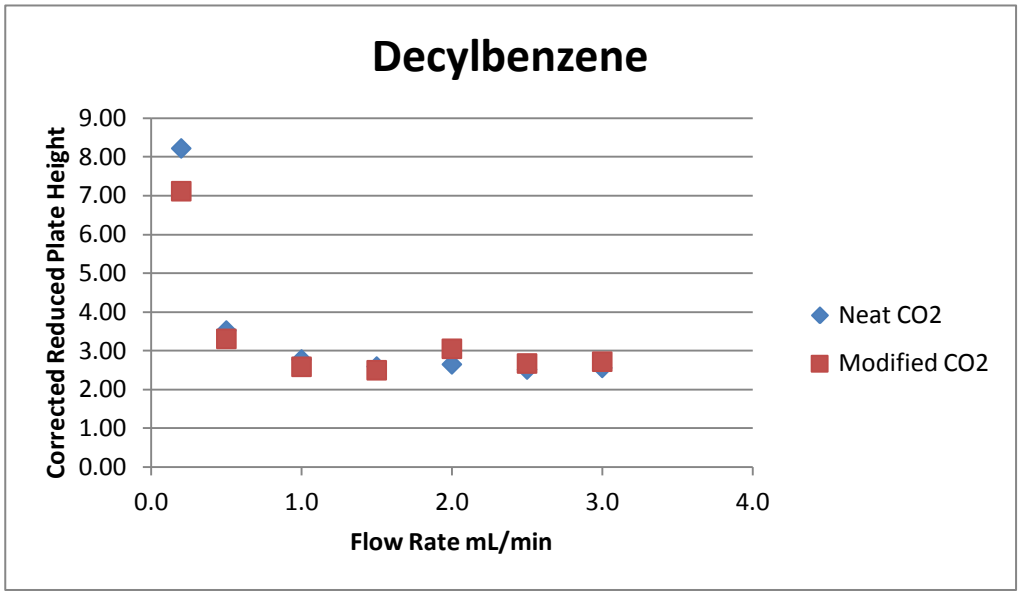

Figure 22. Plate height curve for decylbenzene using a 5% methanol modified CO_2 mobile phase. A Luna 100 x 3mm column with 2.5 µm particles was used.

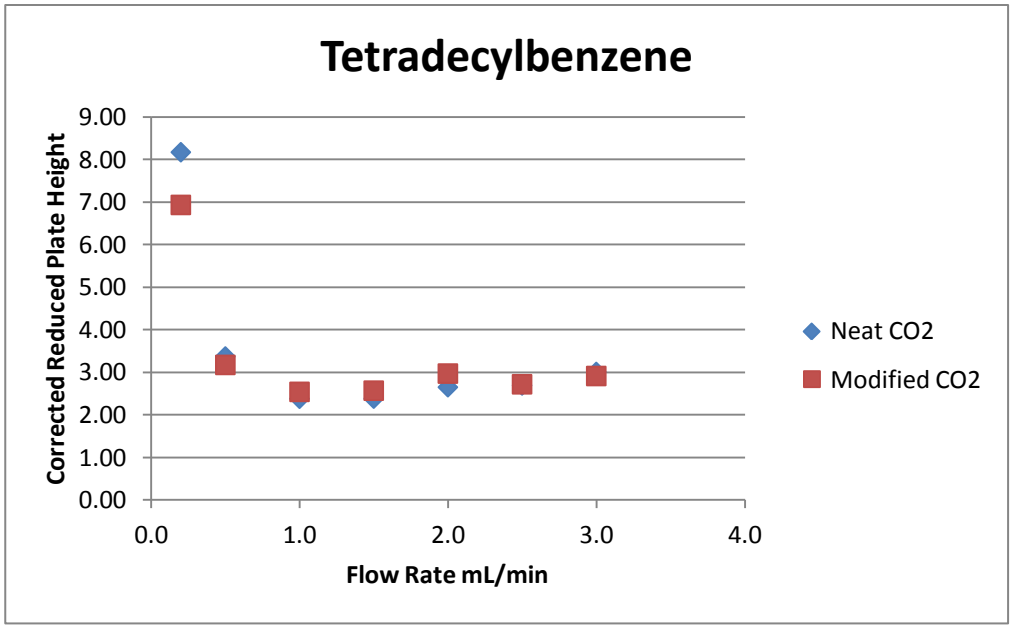

Figure 23. Plate height curve for tetradecylbenzene using a 5% methanol modified CO_2 mobile phase. A Luna 100 x 3mm column with 2.5 µm particles was used.

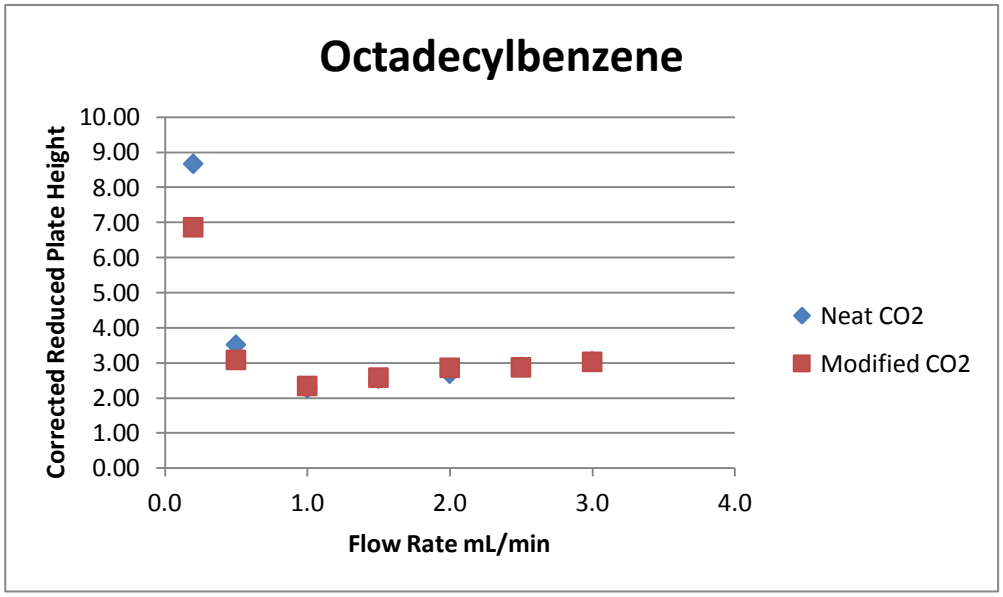

Figure 24. Plate height curve for octadecylbenzene using a 5% methanol modified CO_2 mobile phase. A Luna 100 x 3mm column with 2.5 µm particles was used.

3.2.3.3. Comparison of Experimental and Calculated Variances

For the separations using neat CO_2 as mobile phase, the variances of the injections used to generate the reduced plate height curves were also examined. The variance was taken from the second moment obtained from the peak data. The percent extra column variance was calculated for both experimental data as well as theoretical data (Table 7). Direct comparison can be done by multiplying the time based variance by the squared flow rate. Using a percentage of the contribution of the extra column variance to the total variance, the data indicate for almost all the sets a larger contribution of extra column variance is seen in the experimental data than in the calculated. Three trends can be seen in the data sets. First, the percentages get closer the faster the flow rate becomes. Second, the higher the retention factor the lower percentage of extra column variance is seen. This makes sense as the column variance increases as the retention factor increases, which would contribute to larger variance coming from the column. Lastly, the smaller the volume in the column the greater the percentage extra column variance is. This also makes sense as for small columns the smaller variance for the column is making the extra column effects more prominent. If a criterion for the percentage of variance was no more the 10% it would not be possible to use the Kinetex column at the optimal flow rate. To be around the 10% range a compound with a retention factor of 7 would be needed. This would lead to doing longer trials and consume a lot of carbon dioxide in our pumps. The 150 x 4.6 mm Luna column provide good preformance under any of the conditions that were tested as the largest percentage seen was 3.39%. While this column more than met the desired separation parameters, it has a large volume that would require a larger sample loop increasing the extra column variance. The noise seen when using the 150x4.6mm Luna column is also rather large and could partially be corrected by decreasing the acquisition rate of the detector and by increasing the sample size. The peak widths using this column are on the order of tens of seconds and a response time of 0.03 seconds adds more noise and is not needed to be that fast. This column had 15000-16000 plates, which was the

best of the columns that were tested. The 100 x 3 mm Luna column could be used with octadecylbenzene at most flow rates.

Table 7. Percent of extra column variance as part of the total variance for both experimental and calculated values.

Column	Analyte	Flow Rate mL/min	Retention Factor *k*	%EC experimental	%EC calculated
Luna 100x3mm 2.5µm	Decylbenzene	0.2	1.88	14.75	3.18
		0.5	1.63	34.78	5.88
		1	1.37	32.92	10.72
		1.5	1.23	30.94	15.21
		2	1.15	30.03	19.23
		2.5	1.1	29.02	22.51
		3	1.04	27.53	25.8
	Tetradecylbenzene	0.2	3.43	6.75	1.37
		0.5	2.9	19.95	2.76
		1	2.38	21.76	5.59
		1.5	2.11	19.96	8.47
		2	1.94	18.52	11.25
		2.5	1.85	17.3	13.65
		3	1.74	15.39	16.22
	Octadecylbenzene	0.2	6.1	2.56	0.54
		0.5	4.98	9.18	1.19
		1	3.98	11.78	2.65
		1.5	3.49	10.1	4.26
		2	3.17	10.04	5.93
		2.5	2.99	9.11	7.44
		3	2.79	8.63	9.15

Column	Analyte	Flow Rate mL/min	Retention Factor *k*	%EC experimental	%EC calculated
Kinetex 150x2.1mm 2.6µm	Decylbenzene	0.2	1.15	49.82	17.59
		0.5	0.95	41.18	29.12
		1	0.7	58.69	45.85
		1.5	0.6	66.45	55.97
		2	0.55	61.1	62.34
	Tetradecylbenzene	0.2	2.07	43.07	9.48
		0.5	1.66	41.26	18.15
		1	1.17	56.32	34.25
		1.5	0.98	60.26	45.34
		2	0.89	58.6	52.74
	Octadecylbenzene	0.2	3.62	29.03	4.42
		0.5	2.79	35.51	9.83
		1	1.89	44.42	22.73
		1.5	1.55	46.43	33.35
		2	1.39	43.12	41.14
Luna 150x4.6mm 5.18µm	Decylbenzene	1.5	1.67	3.39	1.49
		2	1.66	3.26	1.83
		2.5	1.6	2.99	2.23
		3	1.54	2.67	2.63
	Tetradecylbenzene	1.5	2.99	1.5	0.67
		2	2.97	1.49	0.83
		2.5	2.84	1.36	1.04
		3	2.71	1.41	1.25
	Octadecylbenzene	1.5	5.17	0.61	0.28
		2	5.13	0.6	0.35
		2.5	4.88	0.59	0.44
		3	4.63	0.54	0.55

3.2.3.4. Repeatability of Chromatographic Properties

A series of injections was done to determine the repeatability of chromatographic data. Injections were done using the 100 x 3 mm Luna column, at a flow rate of 1 mL/min, backpressure 150 bar, and column temperature of 50 ^{o}C. Thirty injections were done under these conditions. The repeatability of the peak area and retention times were examined (Table 8). The standard deviations of the retention times for all analytes were found to be less than 1.25% of the average retention time. This means that there was no significant change in the retention times over the series of injections. The standard deviations for the peak areas were less than 0.25% of the average area for each analyte. This shows that not only are the retention times reproducible but the peak areas are as well. This verifies that the instrument has good repeatability.

Table 8. The areas and retention times for a set of 30 injections using the 100 x 3 mm Luna column at 50 ^{o}C and backpressure of 150 bar.

Compound	Average Area	Standard Deviation Area	%Stdv of Ave Area	Average Retention Time (s)	Standard Deviation Retention Time (s)	%Stdv of Ave Retention Time
Decylbenzene	43.898	0.354	0.81%	55.728	0.134	0.24%
Tetradecylbenzene	38.186	0.342	0.90%	80.027	0.19	0.24%
Octadecylbenzene	37.279	0.455	1.22%	119.096	0.269	0.23%

3.4. Effect of Detector Settings

An experiment was done to determine the effect the detector acquisition rate had on the chromatograms. There are six different acquisition settings. The detector can be in either LC or XLC mode and each mode can be set to fast, standard, or slow responses. For the fastest acquisition rate (XLC – fast) there was a considerable amount of noise to the point where it was becoming difficult to distinguish the t_0 marker (N_2O). A series of 3 injections were done at each of the different detector acquisition settings. The column used for this experiment was the Luna 4.6 x 150 mm column with 5.18-µm particles. This column showed the most noise of the three columns examined. Injections were done with the mixed alkylbenzenes at the optimal flow rate (2 mL/min). The temperature was set to 50 ^{o}C and the outlet pressure was set to 150 bar. A sample of the noise level seen in X-LC fast mode can be seen in figure 25. The noise level varies by about 0.5 mV. This gives a signal to noise ratio of about 8 (for octadecylbenzene). At this ratio (S/N = 16) it becomes harder to distinguish features from the peak and from the noise. The N_2O peak has a signal to noise ratio of 8 and cannot be fully distinguished from the noise. This chromatogram was taken at 1 mL/min, an outlet pressure of 150 bar, and a column temperature of 50 ^{o}C. Further discussion on the detector is detailed in Appendix A.

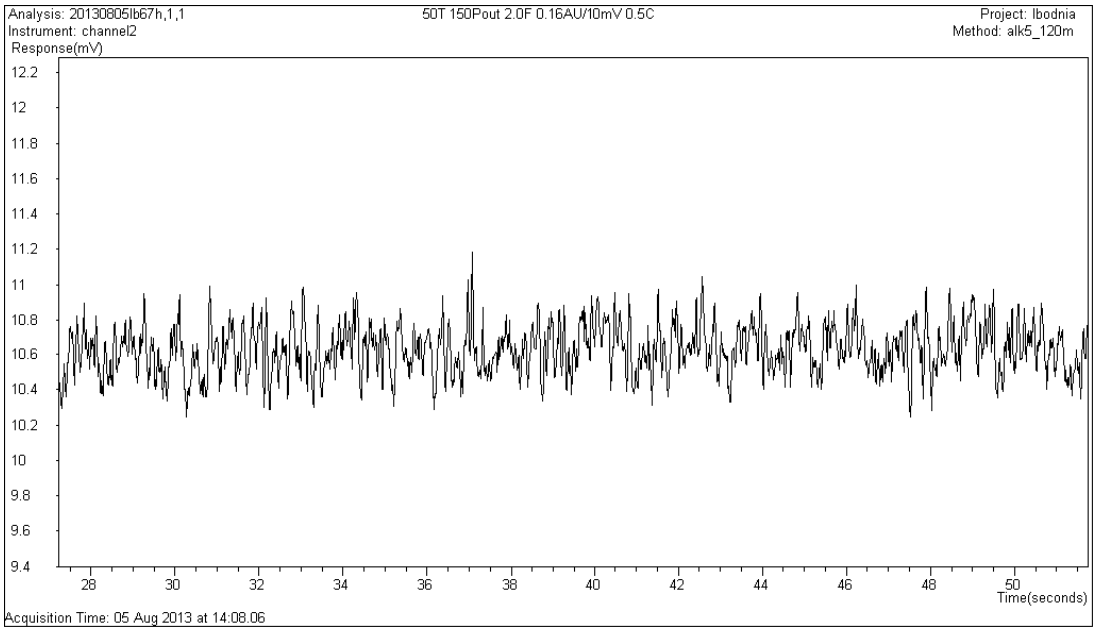

Figure 25. – A section of a chromatogram taken at 1 mL/min, an outlet pressure of 150 bar, and a column temperature of 50 ^{o}C.

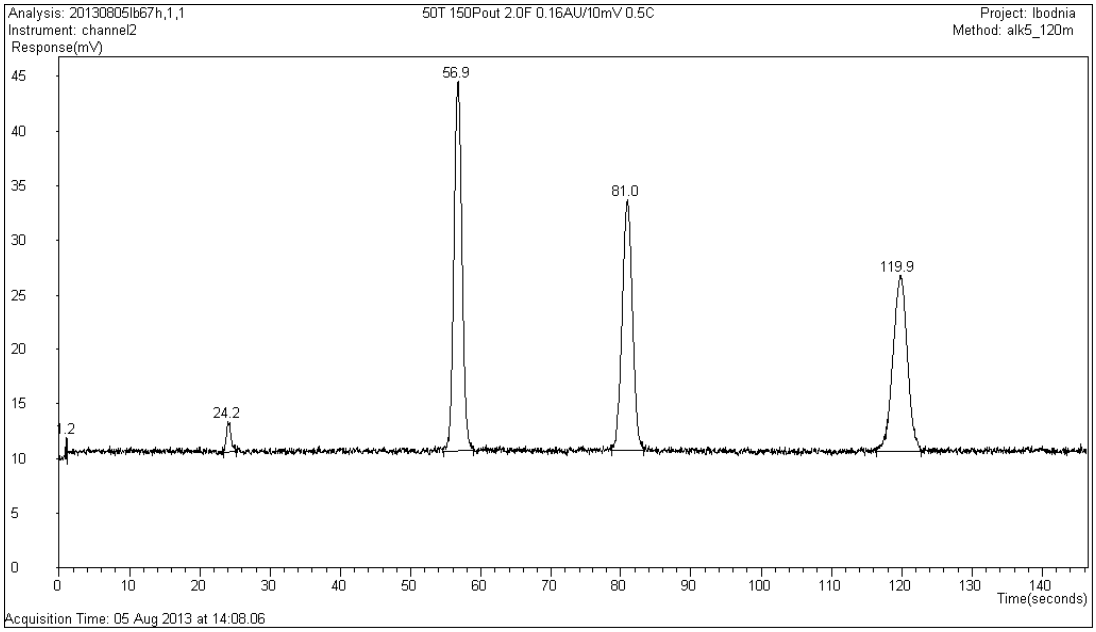

Figure 26. Full chromatogram from figure 25. 1 mL/min 150 bar using 100 x 3.0 mm ID Luna column with 2.5-µm particles.

4. Conclusion

The constructed system meets some of the desired criteria. The system can handle higher pressures than most normal SFC systems. Inlet pressures of ≈420 bar have been used and led to good chromatograms. There is a good relative stability for the backpressure regulators, where typical variations are less than 0.2 bar. The temperature stability is also rather consistent (within 0.3 ^{o}C), once adjusted after switching flow rates. The system can handle carbon dioxide based solvents with either a neat CO_2 mobile phase or with methanol modified mobile phase. The use of methanol modified CO_2 allows for faster separations without the loss of column efficiency. With a modified mobile phase, compounds with high retention factors would come out earlier, saving time as well as conserving the mobile phase. The current system is capable of providing good performance with columns with an inner diameter of 4.6 mm easily with little variance and volume contributions from extra column sources. The best column performance was from a Luna 150 x 4.61 mm with 5.18-µm particles, having a reduced plate height of around 1.6 at the optimal flow rate.

The contributions from the extra column sources were examined. In the current configuration it becomes difficult to work with small diameter columns. To limit the variance contribution from the tubing, the tubing can be changed to 0.005" ID. Then columns with 3mm ID can be used with little contribution to the total variance. A secondary option to examine would be the possibility of using PEEKsil tubing that could decrease the ID down to 25µm. This would decrease the volume down to <4% of the 0.005" ID setup and would decrease the variance down to 0.15% of the 0.005" setup (at 1

mL/min with octadecylbenzene). Assuming the PEEKsil tubing behaves as the stainless steel tubing, this would allow for 2.1 mm ID columns packed with 2-µm particles, which is not capable of in the setup of current system. This would make the largest variance contribution come from the detector flow cell, which cannot readily be changed. The only issue with this is that the small ID of the tubing might make pumping the mobile phase difficult due to an increased pressure drop. The change in changing the tubing it would allow for more efficient separations. This would also allow for the use of small core shell packed columns. As currently configured the system is giving a reduced plate height of up to 20 with the Kinetex 2.6-µm particles in a 150 x 2.1 mm ID column. With the variance from the connecting tubing, better reduced plate heights should be achievable.

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Appendix A.

Detector Settings and Band Variance[1]

The selection of an appropriate data acquisition rate is based on two primary considerations. First, the rate must be fast enough to avoid distortion of the recorded peak signal due to slow detector response. Second, increasing the acquisition rate results in increased noise in the signal. Increasing the data rate beyond the minimum setting required to obtain an accurate peak profile does little to improve the estimation of the peak variance [Gritti and Guiochon, JCA 1218 (2011) 4452], the increased noise at high data rates suggests that the acquisition rate should not be increased unnecessarily.

Detector electronics have an intrinsic time constant that affects the response time that determines the fast practical data acquisition rate. Slower acquisition rates are achieved to applying one or more types of data filters. The Jasco XLC 3075 UV detector employs different filtering methods depending on the selected acquisition mode, shown in Table 1.

Table 1. Jasco 3075 Detector Filtering Methods

Method	**Description**	**XLC mode**	**LC mode**
Time Accumulation	Moving average	X	X
CR Filter	Simulated low-pass filter (RC)		X
Digital Filter	FIR (finite impulse filter)		X

[1] The material in this appendix was contributed by Professor Donald Poe, University of Minnesota Duluth

For this study, most of the data were acquired using the XLC mode, which uses only the Time Accumulation method. Only this mode is discussed here. Table 2 gives the response times associated with the different detector settings provided by Jasco when using the XLC mode. The response time corresponds to the time required for the detector output to reach 95% of its maximum value, or input signal, and is equal to three times the time constant.

Table 2. Time Constants and Response Time

Response Setting	**Response Time**	**Time Constant**
Fast	0.015 s	0.005 s
Std	0.050 s	0.017 s
Slow	0.15 s	0.050 s

The detector was modified at the factory to increase the fundamental data acquisition rate from 100 Hz to 200 Hz and decreasing the time constant and response time by a factor to two. The resulting response times listed in Table 2 are one-half of the values printed in the user manual. According to Fountain et al [JCA 1216 (2009) 5979], the sampling time can be treated as the time constant of the detector electronics. The time constant τ of the detector corresponds to the sampling time, t_s, which is the reciprocal of the sampling rate,

$$\tau = t_s = \frac{1}{\nu} \tag{A.1}$$

where ν is the data acquisition rate.

A common approach used to determine the optimum acquisition rate is based on the peak width. A recorded peak profile should include at least 15 points per standard deviation σ [Gritti and Guiochon, JCA 1218 (2011) 4452], either 60 data points over a range of 4 standard deviations, or 75 data points over 5 sigma.

The effect of acquisition rate on the peak variance in volume units can be related to the volumetric flow rate. The relationship between the detector time constant and its contribution to the band variance is

$$\sigma^2_{v,Rate} = \frac{1}{12}\tau^2 F_v{}^2 \qquad \text{(A.2)}$$

where τ is the time constant and F_v is the volumetric flow rate in the flow cell [Gritti and Guiochon, JCA 1218 (2011) 4632]. Table 3 provides example data for different detector settings and flow rates.

Table 3. Detector Settings, Flow Rate and Band Variance for XLC Mode

Detector Setting	Time Constant, s	Effective Rate (Hz)	Variance Contribution (uL^2) at F_v (µL/s)		
			1.0	10	50
Fast	0.005	200	2.08E-06	2.08E-04	5.21E-03
Std	0.017	60	2.31E-05	2.31E-03	5.78E-02
Slow	0.050	20	2.08E-04	2.08E-02	5.21E-01

The data in Table 3 show that at the Fast setting for the highest indicated flow rate of 50 µL/s (3.0 mL/min), the contribution of the detector electronics is less than 0.01 µL.

For the smallest column used in this study, 2.1-µm Kinetex 150 x 2.1 mm, the column void volume is 286 µL and the estimated peak variance for an unretained solute

with reduced plate height of 2 is 2.7 μL^2 (Table 5, section 3.2). Using one percent as the maximum relative contribution from the detector electronics to the column variance, any setting in Table 3 that yields a variance contribution less than 0.027 µL would be satisfactory as long as the data rate provides at least 15 points per sigma, which depends on the temporal standard deviation. The minimum required temporal standard deviation is

$$\sigma_t = \frac{15}{\nu} \tag{A.3}$$

yielding a minimum σ_t of 0.075 s at 200 Hz. The corresponding flow rate is

$$F_v = \frac{\sqrt{\sigma_v^2}}{\sigma_t} \tag{A.4}$$

which yields a maximum flow rate of 22 µL/s for a column with σ_v^2 = 2.7 μL^2.

In our system, the output of the Jasco detector is supplied to the VG Chromatography Server, which has a fundamental acquisition frequency of 960 Hz. The accessible rates are obtained by dividing this rate by 2^n, yielding frequencies such as 240, 120, 60, 30 and 15 Hz. The acquisition rates of both units, the detector and the server, should be equal to or greater than the requirements set by the peak variance. Higher rates would serve no purpose except to introduce additional noise.

Appendix B. Images of the constructed system.

Figure B1 – Photograph of the mobile phase pumps. The left of the pump is used for modifier and the right pump is for CO_2. A mixer system is added in-between the two pumps.

Figure B2 – A photograph of the sample vessels: A is the sample vessels, B is a knob that allows to choose which sample to deliver, and C is the sample shut off valve for sample vessel.

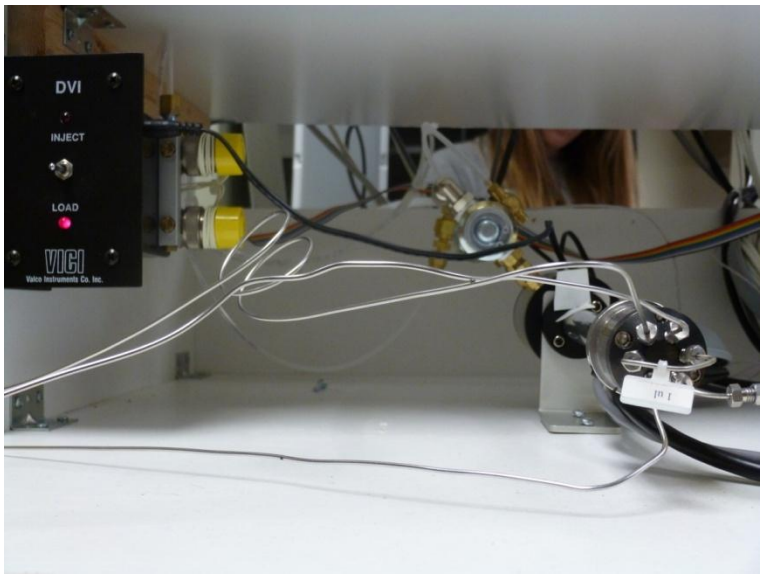

Figure B3 – A photograph of the injector. The black rectangle is the injection switch and the actuator is the grey metallic cylinder at the back of the injector.

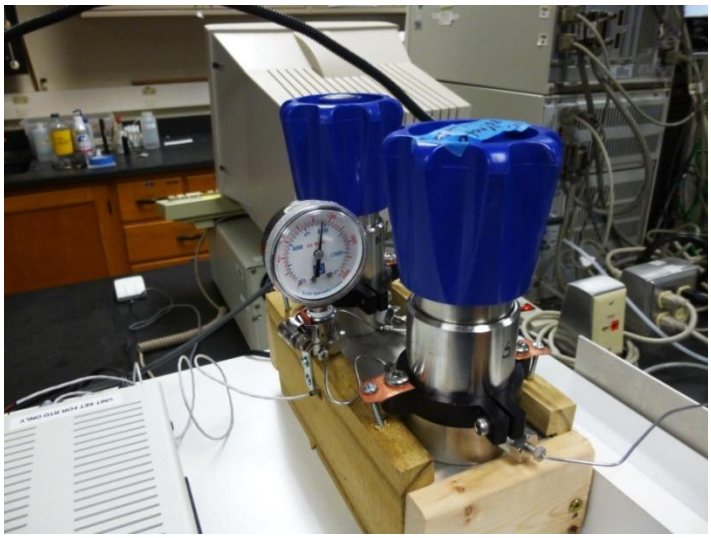

Figure B4 – A photograph of the back pressure regulators.

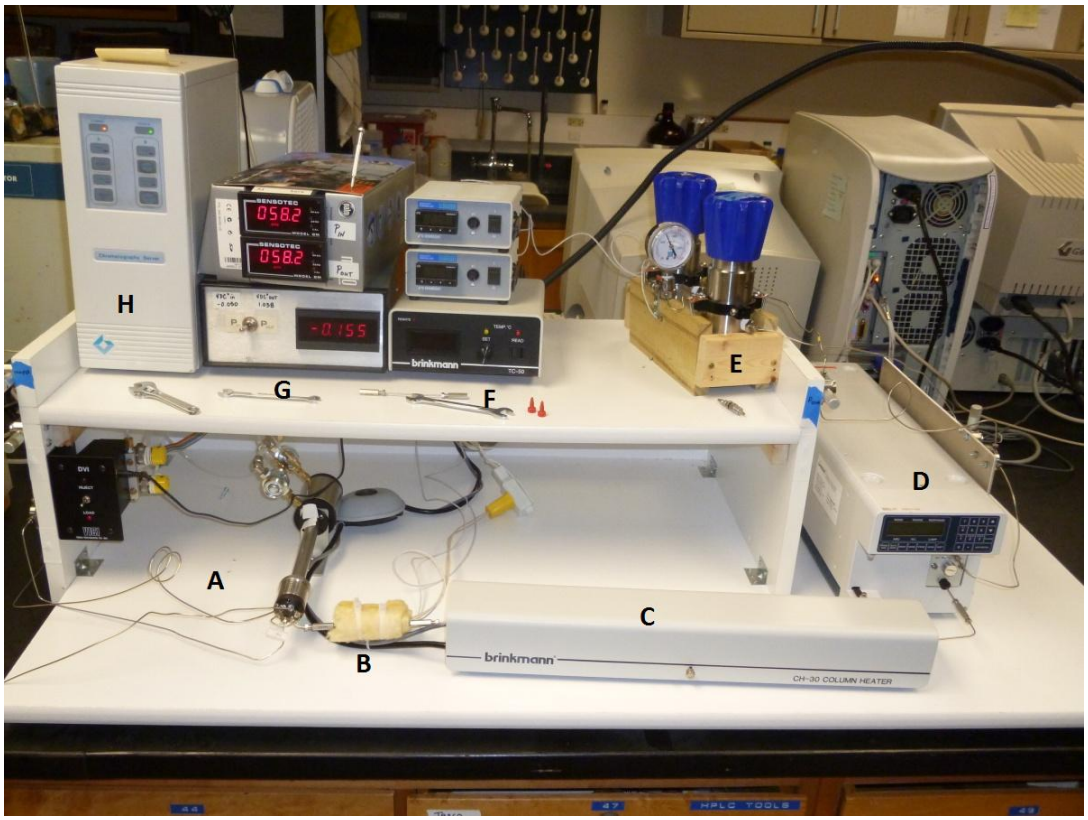

Figure B5 – A photograph of the overall system: A is the injector. B is the column preheater, C is the column heater, D is the detector, E is the backpressure regulators, F is the 2 PID controllers on top and the column heater controller beneath them, G is the pressure desplays (top to bottom, inlet, outlet, and sample vessel pressures), and H is the data acquisition server.