Supplementary Material, Appendices A-D

Appendix A. Retention Fitting for Model Metabolites on BEH-C18 and Hypercarb

Retention data for a series of model metabolites was measured over a range of water/MeOH compositions for both BEH-C18 and Hypercarb columns. In order to fit the data and allow projection of retention across the range of mobile phase compositions, these experimental retention data was fit with an empirical model (Equation 2). This model, which was suggested by Neue, allows for curvature of the retention data across wide ranges in mobile phase composition. Figure A.1 shows the experimental retention data that was measured on the BEH-C18 column for the model metabolites. Table A.1 and Table A.2 display the fit parameters that were produced when the Hypercarb (Figure 3) and BEH-C18 (Figure A.1) were fit with Equation 2, respectively.

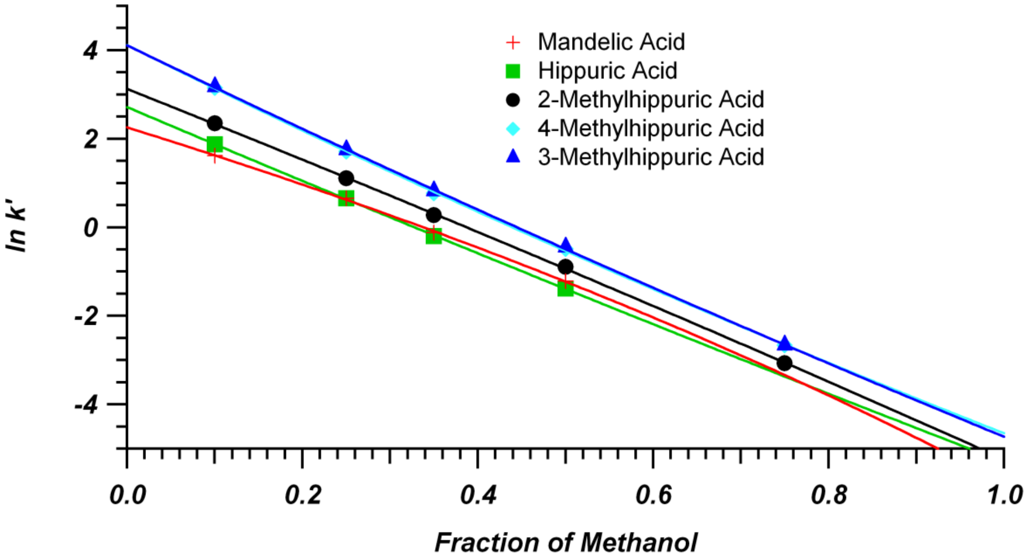

Figure A.1. Plot of natural log of retention factors of the model metabolites as a function of methanol volume fraction in the mobile phase on the BEH-C18 column (2.1 mm x 50 mm, 1.7 µm). Measurements made in water/MeOH + 0.1% FA mobile phases at 30 °C. Experimental data fit with Equation 2.

Table A.1. $\ln k'_{00}$, a, and B_{sol} values from the fit of model metabolite retention data on the Hypercarb (4.6 mm x 100 mm, 3 μm) using Equation 2. Retention measurements were taken under a variety of mobile phase compositions (water/MeOH + 0.1% FA) at 30 °C.

Analyte	$\ln k'_{00}$	a	B_{sol}
MA	3.86	0.63	11.1
2MHA	8.09	1.03	20.5
HA	8.66	1.17	21.6
3MHA	9.5	0.77	17.0
4MHA	9.3	0.66	15.0

Table A.2. $\ln k'_{00}$, a, and B_{sol} values from the fit of model metabolite retention data on the BEH-C18 (2.1 mm x 50 mm, 1.7 μm) column using Equation 2. Retention measurements were taken under a variety of mobile phase compositions (water/MeOH + 0.1% FA) at 30 °C.

Analyte	$\ln k'_{00}$	a	B_{sol}
MA	2.26	-0.23	6.2
2MHA	3.13	-0.06	7.9
HA	2.72	0.05	8.4
3MHA	4.12	0.09	9.6
4MHA	4.12	0.13	9.9

Appendix B. Setup and Measurement of D_m

D_m was measured using a capillary, dual-UV system. This system is used to monitor the broadening of an analyte front as it flows between a series of detection regions. A diagram for the dual-UV setup can be seen below in Figure B.1. Polyimide was removed from two regions of the 50 µm capillary, roughly 2.5 m apart. These windows were then centered in the UV/Vis Spectrophotometers to allow detection of the analyte front as it flows by. These analyte fronts are then differentiated to create Gaussian peaks which were fit in order to determine their variance and ultimately for the calculation of D_m using Equations 3, 4, and 5. An example of the detected analyte fronts and differentiated signal can be seen in Figure B.2. At least triplicate measurements were made for each solute/mobile phase combination.

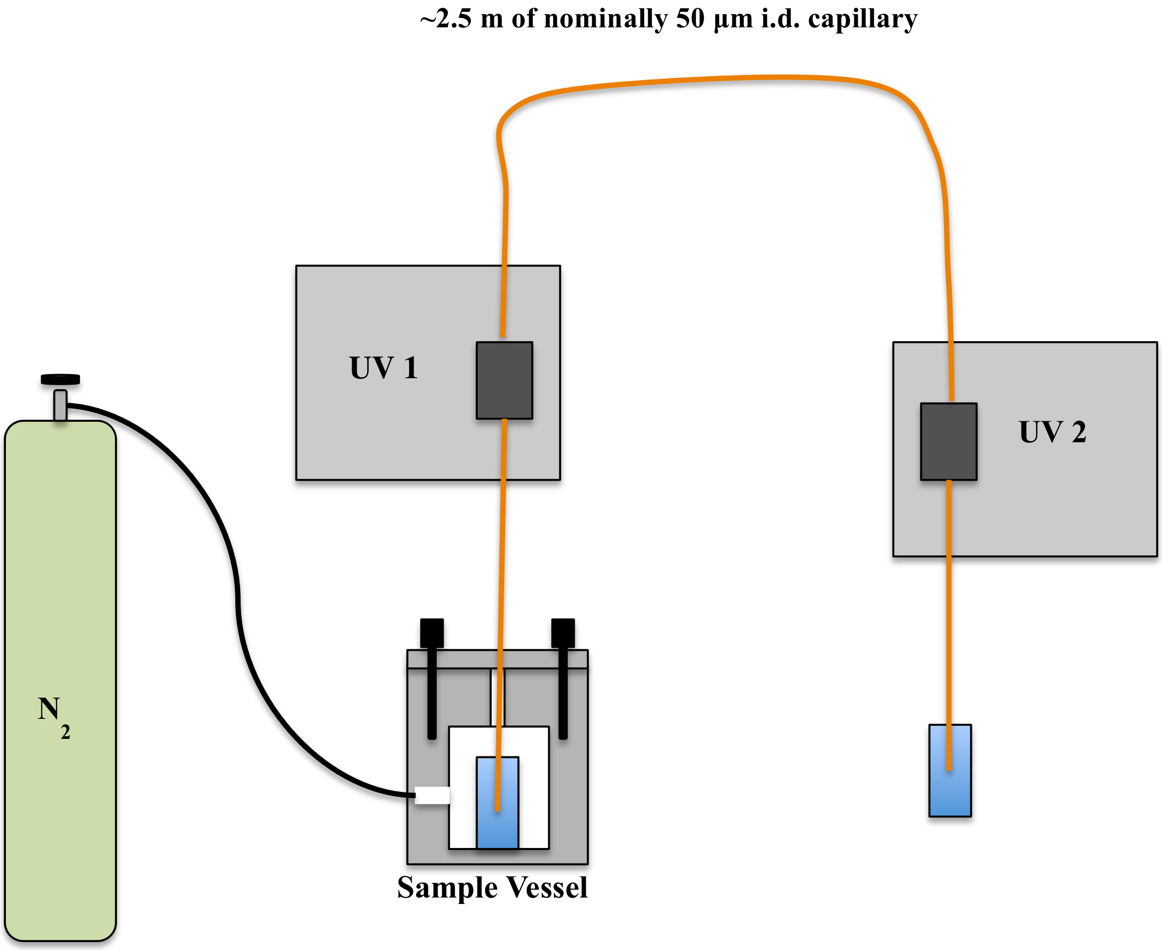

Figure B.1. Dual UV setup for D_m determination studies.

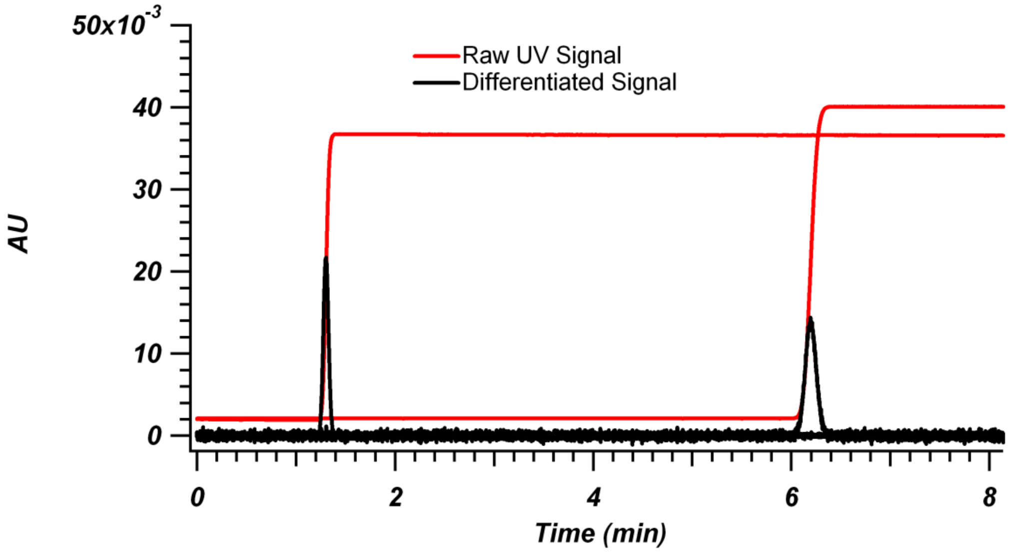

Figure B.2. Mobile phase diffusion coefficient measurement for 4-methylhippuric acid in 5/95 (v/v) water/methanol + 0.1% FA performed using a capillary, dual-UV setup. Raw signal (red) is sigmoidal due to analyte fronts passing by the two detectors. These sigmoidal curves are differentiated into Gaussian peaks (black) in order to calculate D_m from the change in variance between the peaks using Equations 3, 4, and 5. UV settings: 240 nm, 0.1 s rise time.

Appendix C. Peak-parking experiments for BEH-C18 and Hypercarb

In order to determine D_{eff} for all analyte and mobile phase combinations, peak-parking experiments were performed. By plotting the spatial variance of peaks that experienced a range of stop times, it is possible to determine D_{eff} from the slope of a linear trend line as given by Equation 9. Here, Figure C.1 shows the data corresponding to the peak-parking experiments for BEH-C18 at 30 °C (A) and Hypercarb at 55 °C (B). All trend lines have $R^2 > 0.96$. D_{eff} is equal to $\frac{slope}{2}$.

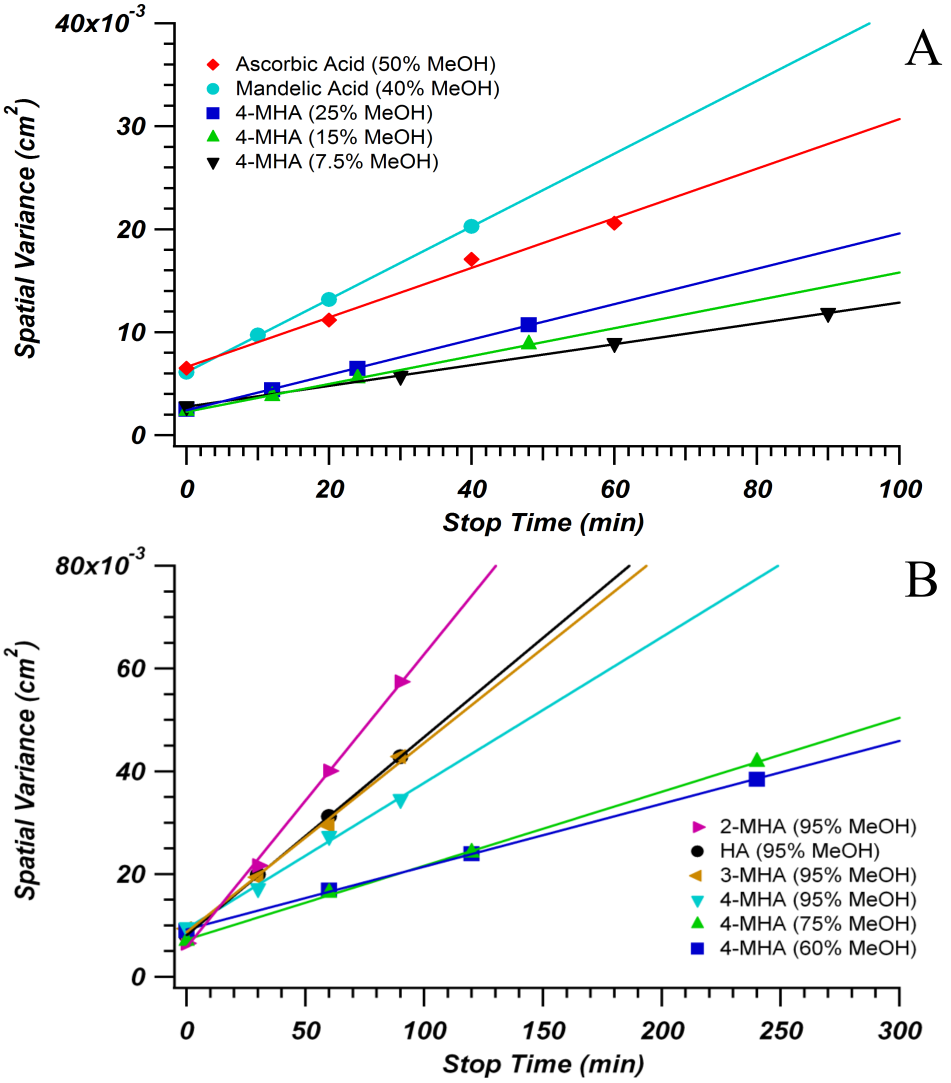

Figure C.1. Change in spatial variance of peaks as a function of stop time for BEH-C18 at 30 °C (A) and Hypercarb at 55 °C (B) peak-parking experiments. In order to cover a range of retention factors, different analytes and mobile phase conditions were used.

Tables C.1, C.2, and C.3 show the results for linear trend lines being fit to the peak-parking data for Hypercarb at 30 °C, BEH-C18 at 30 °C, and Hypercarb at 55 °C, respectively.

Table C.1. Values for slope, intercept and R^2 for the linear fit of $\sigma_{ax,L}^2$ vs t_{stop} for the peak-parking experiments using the Hypercarb column (4.6 mm x 100 mm, 3 μm) at 30 °C. Analyte and mobile phase conditions are included.

Analyte	**Mobile phase**	**R^2**	**Slope (cm^2/s)**	**Intercept (cm^2)**
Acetone	5/95 Water/MeOH	0.99	2.5×10^{-5}	1.1×10^{-2}
2-MHA	5/95 Water/MeOH	0.99	5.8×10^{-6}	5.7×10^{-3}
Hippuric acid	5/95 Water/MeOH	0.99	4.0×10^{-6}	7.1×10^{-3}
3-MHA	5/95 Water/MeOH	0.99	3.0×10^{-6}	7.1×10^{-3}
4-MHA	5/95 Water/MeOH	0.99	2.7×10^{-6}	7.3×10^{-3}
4-MHA	25/75 Water/MeOH	0.99	1.4×10^{-6}	7.7×10^{-3}
4-MHA	40/60 Water/MeOH	0.98	9.4×10^{-7}	8.5×10^{-3}

Table C.2. Values for slope, intercept and R^2 for the linear fit of $\sigma_{ax,L}^2$ vs t_{stop} for the peak-parking experiments using the BEH-C18 column (2.1 mm x 50 mm, 1.7 μm) at 30 °C. Analyte and mobile phase conditions are included.

Analyte	**Mobile phase**	**R^2**	**Slope (cm^2/s)**	**Intercept (cm^2)**
Ascorbic Acid	50/50 water/MeOH	0.98	4.0×10^{-6}	6.6×10^{-3}
Mandelic Acid	60/40 water/MeOH	0.99	5.9×10^{-6}	6.1×10^{-3}
4-MHA	75/25 water/MeOH	0.99	2.85×10^{-6}	2.5×10^{-3}
4-MHA	85/15 water/MeOH	0.99	2.25×10^{-6}	2.3×10^{-3}
4-MHA	92.5/7.5 water/MeOH	0.99	1.68×10^{-6}	2.7×10^{-3}

Table C.3. Values for slope, intercept and R^2 for the linear fit of $\sigma_{ax,L}^2$ vs t_{stop} for the peak-parking experiments using the Hypercarb column (4.6 mm x 100 mm, 3 µm) at 55 °C. Analyte and mobile phase conditions are included.

Analyte	**Mobile phase**	**R^2**	**Slope (cm^2/s)**	**Intercept (cm^2)**
2-MHA	5/95 Water/MeOH	0.99	9.5×10^{-6}	6.2×10^{-3}
Hippuric acid	5/95 Water/MeOH	0.99	6.5×10^{-6}	8.1×10^{-3}
3-MHA	5/95 Water/MeOH	0.96	5.4×10^{-6}	1×10^{-2}
4-MHA	5/95 Water/MeOH	0.98	4.6×10^{-6}	1×10^{-2}
4-MHA	25/75 Water/MeOH	0.99	2.4×10^{-6}	7.2×10^{-3}
4-MHA	40/60 Water/MeOH	0.99	2.0×10^{-7}	9.2×10^{-3}

Appendix D. Calculation of Error in $\frac{D_{eff}}{D_m}$

Due to the number of measurements and calculations needed to study effective diffusion, an in depth analysis of error was needed. This section will detail the calculation and propagation of error through the measurement of the effective diffusion properties of BEH-C18 and Hypercarb materials. The standard deviation of any value will be written as σ_x with the select term as the subscript. For example, the standard deviation in the retention factor (k') will be written as $\sigma_{k'}$.

The measurement of analyte diffusion coefficient in the mobile phase (D_m) was performed using frontal analysis with a capillary scale, dual-UV setup. The measured sigmoids were differentiated to form Gaussian peaks and calculate D_m using:

$$D_m = \frac{d_c^2\ \Delta t}{96\ \Delta\sigma_{t\_net}^2} \tag{D-1}$$

The standard deviation in d_c^2 can be calculated by:

$$\frac{\sigma_{d_c^2}}{d_c^2} = 2(\frac{\sigma_{d_c}}{d_c}) \tag{D-2}$$

σ_{D_m} can then be calculated by accounting for the error in the measurements of the terms in Equation D-1. As measurements were taken using a variety of pressures to assure no variability with pressure, Δt and $\Delta\sigma_{t\_net}^2$ were assumed to be constants within any measurement.

$$\frac{\sigma_{D_m}}{D_m} = \sqrt{(\frac{\sigma_{d_c^2}}{d_c^2})^2 + (\frac{\sigma_{\Delta t}}{\Delta t})^2 + (\frac{\sigma_{\Delta\sigma_{t\_net}^2}}{\Delta\sigma_{t\_net}^2})^2} = \frac{\sigma_{d_c^2}}{d_c^2} \tag{D-3}$$

The peak-parking experiments were then used to calculate D_{eff} of an analyte using Equation D-4:

$$\sigma_{ax,L}^2 = 2D_{eff}t_{stop} \qquad \text{D-4}$$

By plotting all of the measured $\sigma_{ax,L}^2$ and t_{stop} pairs, D_{eff} is calculated using the slope of a linear trend line and is equal to $\frac{slope}{2}$. $\sigma_{D_{eff}}$ can then be calculated using the following equation.

$$\frac{\sigma_{D_{eff}}}{D_{eff}} = \sqrt{(\frac{\sigma_{slope}}{slope})^2} = \frac{\sigma_{slope}}{slope} \qquad \text{D-5}$$

$$\sigma_{D_{eff}} = \frac{\sigma_{slope}}{2} \qquad \text{D-6}$$

Now that D_{eff} and D_m are both known, it is possible to calculate $\frac{D_{eff}}{D_m}$ and $\sigma_{\frac{D_{eff}}{D_m}}$.

$$\frac{\sigma_{\frac{D_{eff}}{D_m}}}{\frac{D_{eff}}{D_m}} = \sqrt{(\frac{\sigma_{D_{eff}}}{D_{eff}})^2 + (\frac{\sigma_{D_m}}{D_m})^2} \qquad \text{D-7}$$

For the purpose of building the plot seen in Figure 5, it is also necessary to calculate the error in the measurement of k'.

$$k' = \frac{t_R - t_m}{t_m} \qquad \text{D-8}$$

$$\sigma_{t_R - t_m} = \sqrt{\sigma_{t_R}^2 + \sigma_{t_m}^2} = \sigma_{t_R} \qquad \text{D-9}$$

$$\frac{\sigma_{k'}}{k'} = \sqrt{(\frac{\sigma_{t_R - t_m}}{t_R - t_m})^2 + (\frac{\sigma_{t_m}}{t_m})^2} = \frac{\sigma_{t_R - t_m}}{t_R - t_m} \qquad \text{D-10}$$

In the following section an example calculation will be shown for the Hypercarb measurements using 2-methylhippuric acid in 5/95 water/MeOH + 0.1% FA at 30 °C. D_m measurements for 2-MHA were taken in 5/95 water/MeOH + 0.1% FA using the capillary, dual-UV system. In order to find σ_{D_m}, it is first necessary to know $\sigma_{d_c^2}$.

$$\sigma_{d_c^2} = 2d_c^2\left(\frac{\sigma_{d_c}}{d_c}\right) = 2(2.15\ x\ 10^3\ \mu m^2)\left(\frac{0.41\ \mu m}{46.39\ \mu m}\right) = 38.4\ \mu m^2 \qquad \text{D-11}$$

This is then used to find σ_{D_m} for 2-MHA based on measured values of D_m.

$$\sigma_{D_{m,2MHA}} = D_{m,2MHA}\left(\frac{\sigma_{d_c^2}}{d_c^2}\right) = \left(9.6\ x\ 10^{-6}\ \frac{cm^2}{s}\right)\left(\frac{38.4\ \mu m^2}{2.15\ x\ 10^3\ \mu m^2}\right) \qquad \text{D-12}$$

$$\sigma_{D_{m,2MHA}} = 1.7\ x\ 10^{-7}\,\frac{cm^2}{s} \qquad \text{D-13}$$

It is now necessary to calculate $\sigma_{D_{eff}}$ for 2-MHA based on the plot of σ_L^2 and t_{stop} created using stopped-flow experiments.

$$\sigma_{D_{eff,2MHA}} = \frac{\sigma_{slope,2MHA}}{2} = \frac{1.58\ x\ 10^{-7}\ \frac{cm^2}{s}}{2} = 7.9\ x\ 10^{-8}\ \frac{cm^2}{s} \qquad \text{D-14}$$

Now that D_{eff} for 2-MHA has been calculated it is possible to determine $\frac{D_{eff,2MHA}}{D_m,2MHA}$ and $\sigma_{\frac{D_{eff,2MHA}}{D_m,2MHA}}$ for 2-MHA on the Hypercarb column.

$$\frac{D_{eff,2MHA}}{D_{m,2MHA}} = \left(\frac{2.92\ x\ 10^{-6}\ \frac{cm^2}{s}}{9.6\ x\ 10^{-6}\ \frac{cm^2}{s}}\right) = 0.30 \qquad \text{D-15}$$

$$\frac{\sigma_{\frac{D_{eff,2MHA}}{D_m,2MHA}}}{\frac{D_{eff,2MHA}}{D_{m,2MHA}}} = \sqrt{\left(\frac{\sigma_{D_{eff,2MHA}}}{D_{eff,2MHA}}\right)^2 + \left(\frac{\sigma_{D_m,2MHA}}{D_{m,2MHA}}\right)^2} \qquad \text{D-16}$$

$$\sigma_{\frac{D_{eff,2MHA}}{D_m,2MHA}} = 0.30\sqrt{\left(\frac{7.9\ x\ 10^{-8}\ \frac{cm^2}{s}}{2.92\ x\ 10^{-6}\ \frac{cm^2}{s}}\right)^2 + \left(\frac{1.7\ x\ 10^{-7}\ \frac{cm^2}{s}}{9.6\ x\ 10^{-6}\ \frac{cm^2}{s}}\right)^2} \qquad \text{D-17}$$

$$\sigma_{\frac{D_{eff,2MHA}}{D_m,2MHA}} = 9.8\ x\ 10^{-3} \qquad \text{D-18}$$

Finally, it is necessary to calculate $\sigma_{k'}$.

$$\sigma_{k'} = k'\left(\frac{\sigma_{t_R - t_m}}{t_R - t_m}\right) = 0.87\left(\frac{2.31\ x\ 10^{-3}\ min}{2.08\ min}\right) \qquad \text{D-19}$$

$$\sigma_{k'} = 9.6\ x\ 10^{-4} \qquad \text{D-20}$$

These calculations were performed for each mobile phase and analyte in order to determine the error associated with the plotted data in Figure 5.