Supporting Information

Improving the Sensitivity, Resolution, and Peak Capacity of Gradient Elution in Capillary Liquid Chromatography With Large-Volume Injections by Using Temperature-Assisted On-column Solute Focusing

Rachael E. Wilson, Stephen R. Groskreutz, Stephen G. Weber*

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260

*corresponding author: sweber@pitt.edu Fax: 412-624-1668

Flow Rate Calibration

To verify the flow of solvent delivered by the system, varying injection volumes of 1 mM uracil were delivered into a 55 cm x 25 µm ID open capillary spanning the injection valve and the UV detector flow cell. The mobile phase was 50:50 0.1% TFA ACN/ 0.1% TFA H_2O and the flow rate was set to 1.00 µL/min. Uracil absorbance was measured at 254 nm. Timed injections were used to deliver injection volumes of 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 µL in random order. Each sample was repeated at least three times. Under these conditions, all peaks had flat tops so the half-width, $w_{1/2}$, of the peak should be equivalent to the width in time units of the injection, t_{inj}, as seen in Equation S1.

$$t_{inj} = w_{1/2} = \frac{1}{F} V_{inj} \qquad \text{(S-1)}$$

Thus, a plot of width vs. injection time should yield a line with a slope of one, as seen in Figure S1. The equation of this plot was y=0.9989 (±0.0012)x + 0.00252 (±0.00054) with 95% confidence. This resulted in a calculated flow rate of 1.001 ±0.002 µL/min, which is tolerable in the scope of this work.

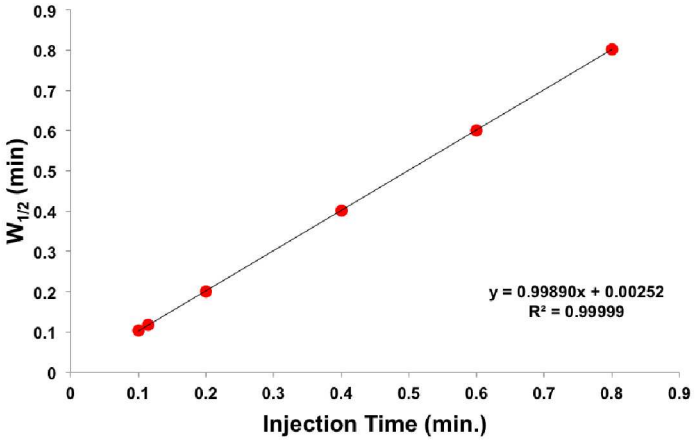

Figure S-1: Peak half-widths for varying injection volumes into an open tube for the flow rate validation. Each point represents n = 3 replicates.

Dwell Time Determination

The dwell time of the system was measured using a method described elsewhere.[1] The mobile phase consisted of pure water in channel A and 0.1% acetone in water in channel B. The injection valve was connected to the flow cell by the same 55 cm x 25 µm ID open capillary used throughout the work reported here. A gradient of 10-90% B was delivered over ten minutes using flow rates of 1.00 µL/min and 1.20 µL/min. Runs were repeated at each flow rate in triplicate. One such chromatogram can be found in Figure S-2.

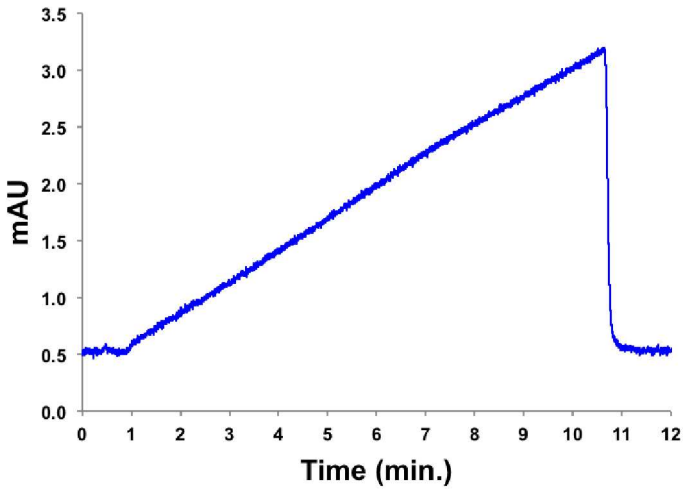

Figure S-2: Chromatogram of 10-90% 0.1% acetone/ H_2O gradient over 10 minutes for the determination of the dwell volume. Chromatogram is representative of n = 3 replicates.

To determine the dwell time, a baseline was drawn at the beginning and end of the gradient and the time at which the detector response is at the half point of the gradient, $t_{1/2}$, was determined. The dwell time, t_d, was then calculated using Equation S2 where t_g is the gradient time. This was calculated to be 0.907 ±0.025 minutes.

$$t_d = t_{1/2} - \frac{1}{2} t_g \qquad \text{(S-2)}$$

TASF Hardware

The TEC temperature was controlled via a feedback loop in which the voltage at the desired temperature was 'stored' allowing set temperatures to be achieved quickly and reproducibly following several training runs prior to the start of the experiments. The feedback loop operated by slowly increasing or decreasing the current depending on the overall difference between the actual temperature and desired temperature. For example, following a cooling cycle, the voltage was raised rapidly to 95% of the maximum voltage then the increment by which the voltage was raised further slowed until the desired temperature was reached. This is demonstrated in the temperature trace shown in Figure S-3A. This trace was reproducible for all experiments and once the desired temperature was reached, temperatures did not fluctuate more than ±0.1 °C from the set point (Figure S-4).

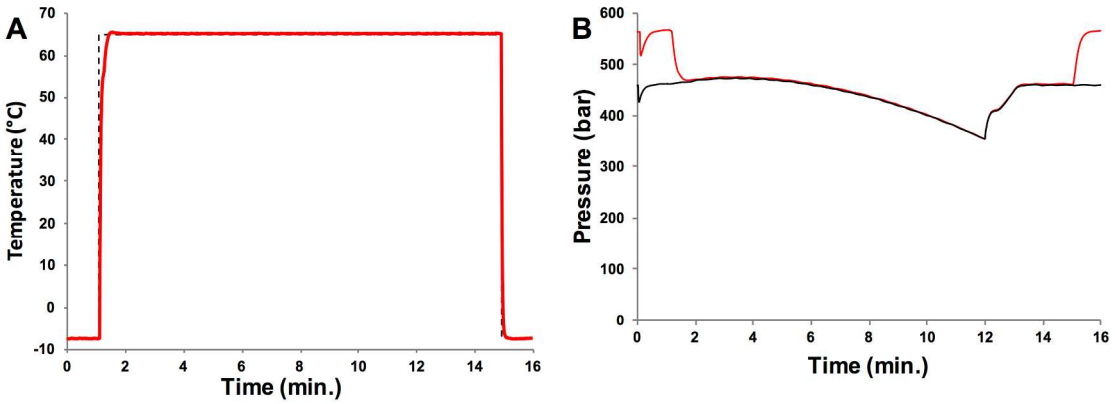

Figure S-3: Panel A is the TEC temperature profile of the small molecule mixture separation (Fig. 2). Panel B is the corresponding pressure trace under isothermal (black line) and TASF (red line) conditions.

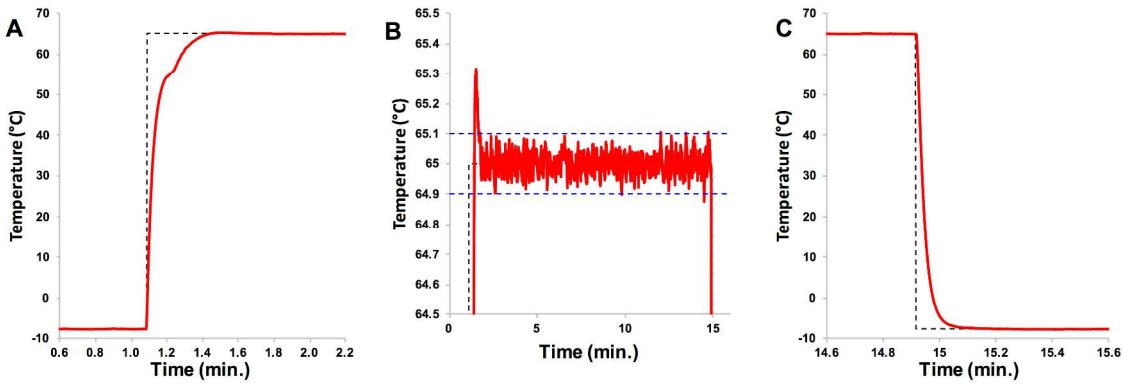

Figure S-4: Demonstration of the programmed control of the TEC. Panel A shows the TEC transition from focusing to separation. Panel B shows the temperature trace during the separation phase.

As expected, changing temperatures are also reflected in changing pressures due to the change in viscosity of the mobile phase. Figure S-3B demonstrates the pressure cost of TASF (red line) over isothermal separation of the small molecule mixture (black line). It is seen for both traces that valve actuation causes a pressure drop during injection and then pressure decreases accordingly with the increase of organic modifier in the mobile phase. Pressure then increases again during mobile phase re-equilibration. This is consistent with what has been described previously.[2] The focusing temperature used resulted in approximately 100 bar greater pressures during injection and is well within the capabilities of most HPLC pumps and a reasonable compromise for improved peak shapes and separation efficiencies. The pressure increase at the end of the run corresponds to cooling the TEC prior to the start of the next run. These pressure traces were consistent over all runs and sample types (Figure S-5).

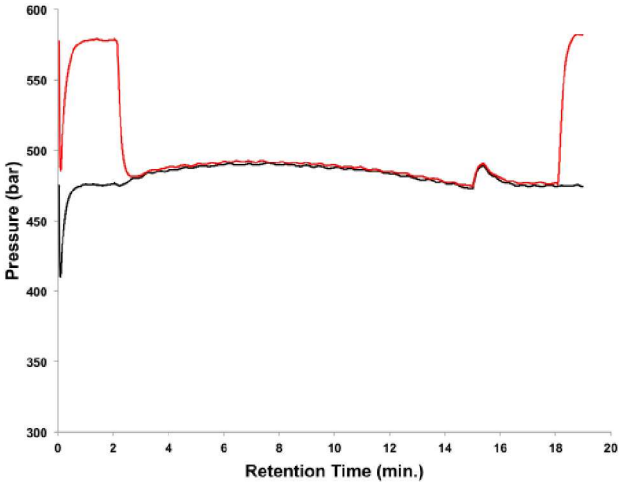

Figure S-5: The pressure trace of the separation of the BSA tryptic digest (Figure 7) under isothermal (black line) and TASF (red line) conditions.

Determination of Retention Factors

Table S-1: Mobile Phase Compositions[a]

	% ACN										
Solute	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**
Acetanilide	X	X	X	X							
Methylparaben	X	X	X	X	X						
Ethylparaben		X	X	X	X	X					
Acetophenone	X	X	X	X	X						
Propylphenone	X	X	X	X	X						
Butylphenone			X	X	X	X	X				
Benzophenone					X	X	X	X	X		
Valerophenone						X	X	X	X	X	
Hexanophenone						X	X	X	X	X	X
Heptanophenone								X	X	X	X
Octanophenone								X	X	X	X

[a] Retention times were measured on a 100 μm ID x 5.5 cm. column packed as described in the experimental section with a flow rate of 1 µL/ min. Parabens and phenones were analyzed separately to avoid cross-over.

Table S-2: Calculated Retention Factors at Φ=0.05[b]

	k'	
Solute	**-7.5 °C**	**65 °C**
Acetanilide	21.3	5.46
Methylparaben	107	13.5
Acetophenone	59.8	16.4
Ethylparaben	410	42.4
Propiophenone	210	49.3
Butylphenone	851	147
Benzophenone	2251	565
Valerophenone	3134	761
Hexanophenone	11800	1640
Heptanophenone	44600	3710
Octanophenone	168000	16200

[b] Retention factors calculated based on the experimental data from Table S-1 and Equation 6.

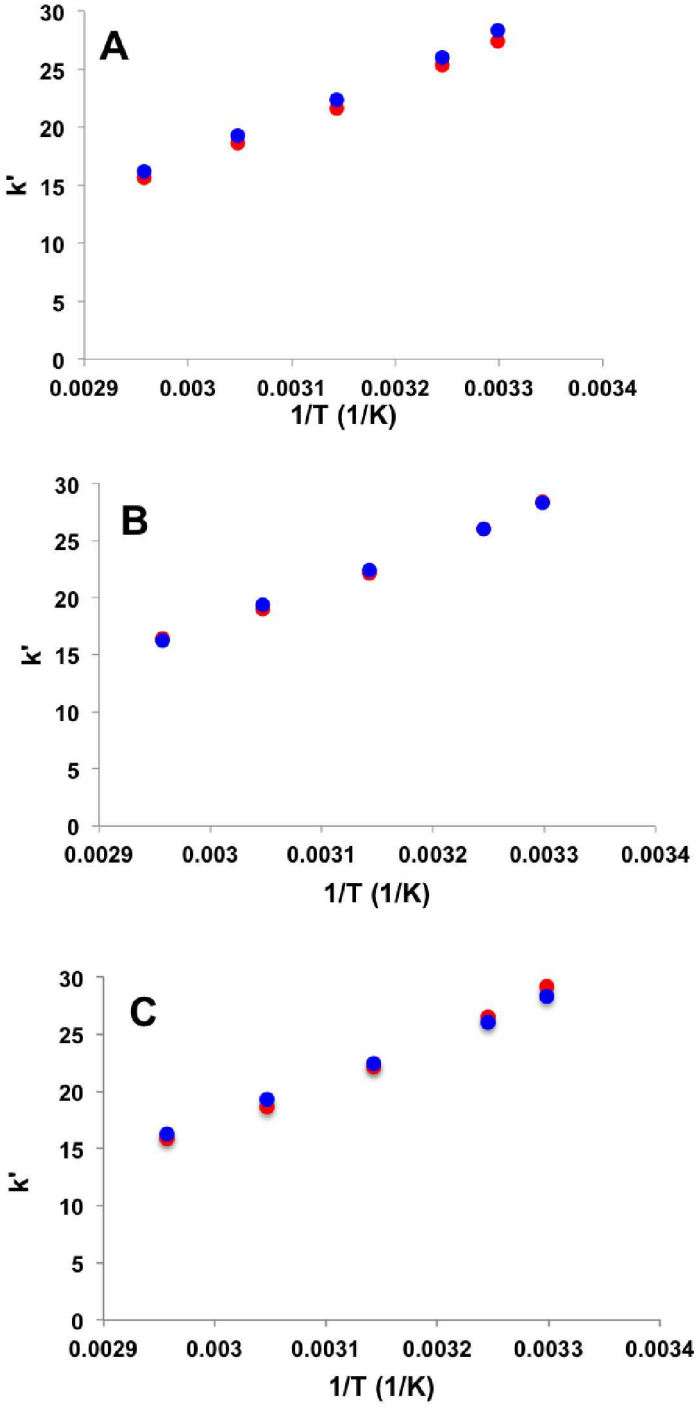

Figure S-6: Comparison of van't Hoff plots using extrapolated retention factors (red dots) and experimental data (blue dots) for acetophenone at 30 °C and Φ = 0.05. The extrapolated retention factors were calculated using Equation 5 for Panel A, Equation 6 for Panel B, and Equation 7 for Panel C.

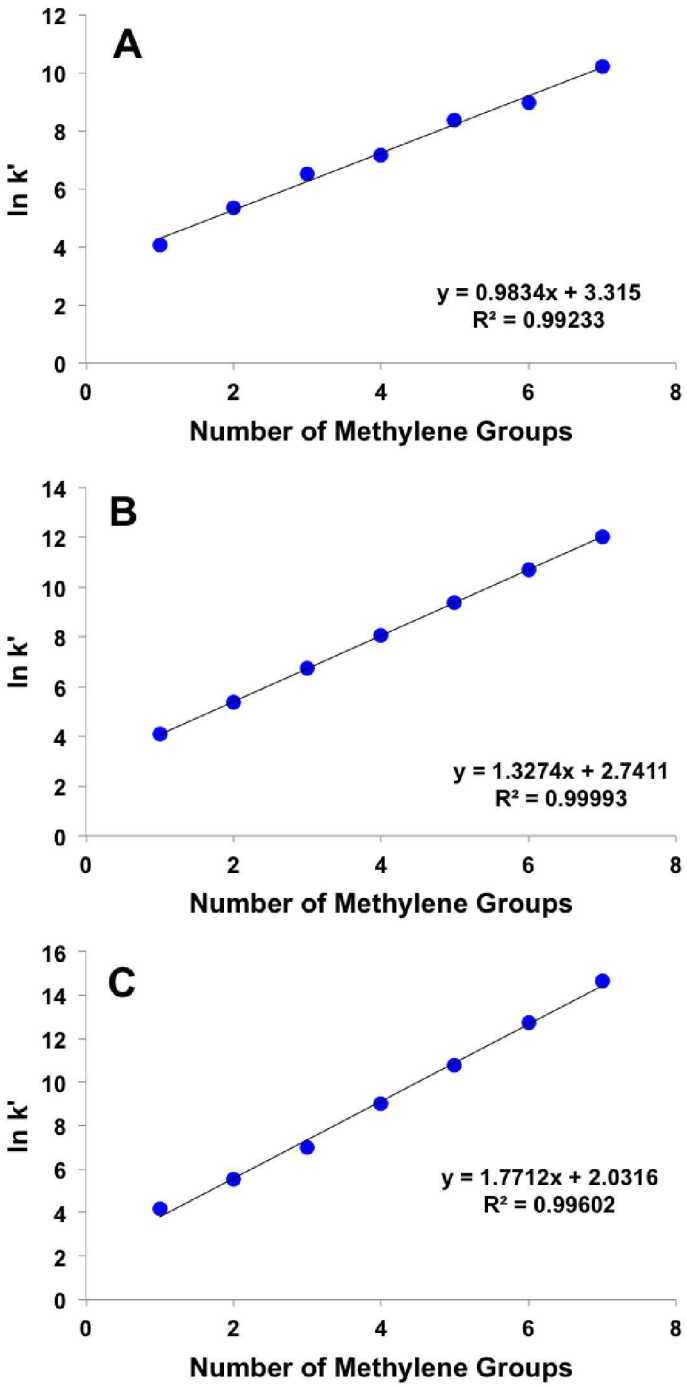

Figure S-7: Determination of ln (α_{CH2}) using extrapolated retention factors at -7.5°C and Φ = 0.05. The values in Panel A were calculated using Equation 5, Equation 6 was used for Panel B, and Equation 7 was used for Panel C.

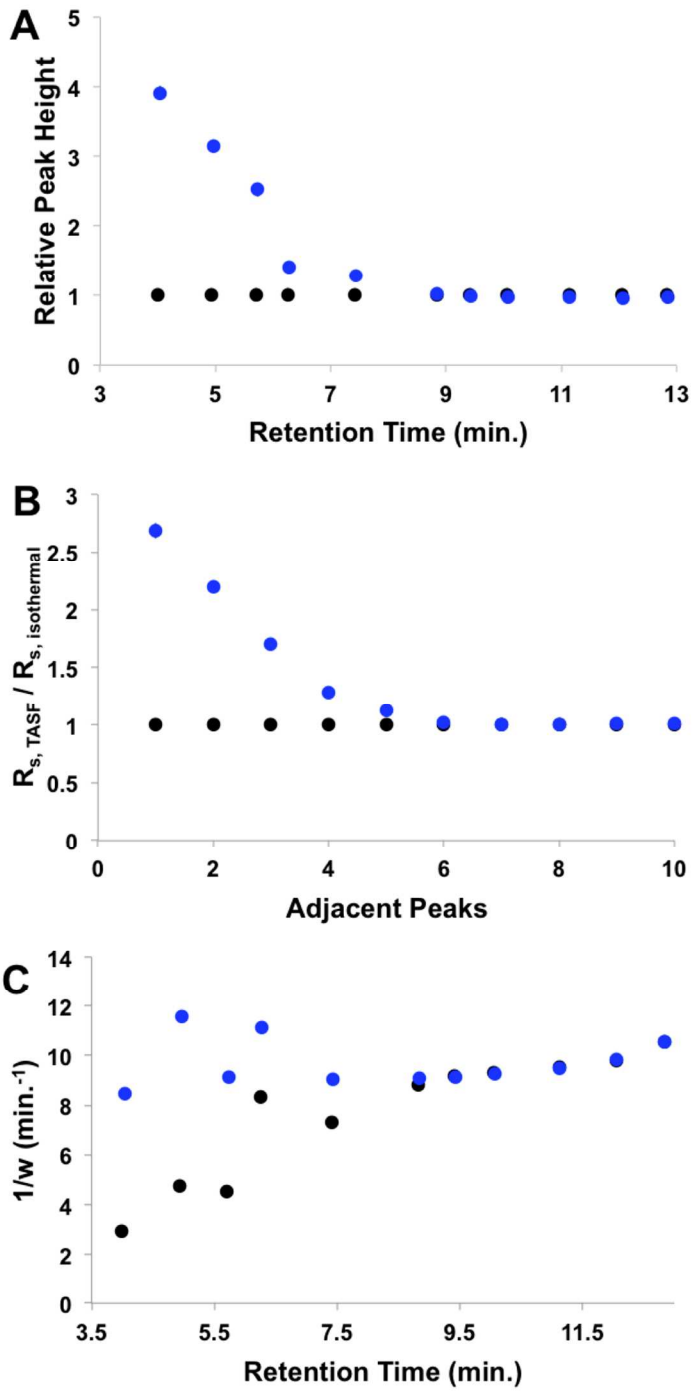

Figure S-8: Comparison of peak height (Panel A), resolution (Panel B), and peak capacity (Panel C) of isothermal (black dots) and TASF (blue dots) separations of the small molecule mixture depicted in Figure 2.

References

(1) Snyder, L. R.; Dolan, J. W. *High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model*; John Wiley and Sons, Inc., 2007.
(2) Eghbali, H.; Sandra, K.; Tienpont, B.; Eeltink, S.; Sandra, P.; Desmet, G. *Anal. Chem.* **2012**, *84*, 2031-2037.