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Repeatability of Gradient UHPLC-MS/MS Methods in Instrument-Controlled Thermal Environments

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Abstract

The impact of viscous friction on eluent temperature and column efficiency in liquid chromatography is of renewed interest as the need for pressures exceeding 1000 bar to use with columns packed with sub-2 μm particles has grown. One way the development of axial and radial temperature gradients that arise due to viscous friction can be affected is by the thermal environment the column is placed in. In this study, a new column oven integrated into an ultrahigh pressure liquid chromatograph that enables both still-air and forced-air operating modes is investigated to find the magnitude of the effect of the axial thermal gradient that forms in 2.1×100 mm columns packed with sub-2 μm particles in these modes. Temperature increases of nearly 30 K were observed when the generated power of the column exceeded 25 W/m. The impact of the heating due to viscous friction on the repeatability of peak capacity, elution time, and peak area ratio to an internal standard for a gradient UHPLC-MS/MS method to analyze neurotransmitters was found to be limited. This result indicates that high speed UHPLC-MS/MS methods under conditions of high viscous friction may be possible without the negative effects typically observed with isocratic separations under similar conditions.

Keywords

UHPLC; Gradient Liquid Chromatography; Mass Spectrometry; Viscous Friction; Neurochemistry; Column Oven

1. Introduction

One obstacle that continues to limit column performance at the high backpressures observed in ultrahigh pressure liquid chromatography (UHPLC) is viscous heating that leads to thermal broadening effects. [1–6] Because this heat generation is directly proportional to both the flow rate (F) and backpressure (P), [7]:

$$\text{Power} = F \Delta P \quad (1)$$

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early research in UHPLC focused on capillary columns ($<100\ \mu\text{m}$ i.d. (inner diameter)) where flow rates well below $1\ \mu\text{L}/\text{min}$ prevent any noticeable viscous heating. [7,8] For commercial UHPLC columns, although i.d.'s have decreased from $4.6\ \text{mm}$ to $2.1\ \text{mm}$ (and even $1.0\ \text{mm}$ in some cases [9,10]) to lower the necessary flow rates to reduce thermal broadening [11], viscous heating can still have a detrimental impact on chromatographic efficiency. [4,12–18]

Several strategies to overcome this problem have been implemented. The use of superficially porous particles (SPPs) in LC columns not only improves column performance [19–21], but also increases the thermal conductivity (λ) of the packed bed [22] which can reduce the magnitude of temperature gradients generated due to viscous friction. Radial thermal gradients are known to decrease column performance [5] and reducing their magnitude can improve column efficiency. [23–25] Thermal conductivity of the bed can be increased in monolithic structures by utilizing embedded metal scaffolds. [26] Another way to reduce the radial temperature gradient is by insulating the column [27], housing it in a vacuum chamber [28], or placing it in a still-air oven. [4,29–31] However, these quasi-adiabatic thermal environments increase the axial thermal gradient which can increase the overall column temperature and reduce retention time repeatability. [27] This is problematic as retention time can greatly fluctuate with temperature [32] and may cause problems in peak resolution during method development or method transfer (from a lower to higher pressure instrument) when trying to speed up methods by using higher flow rates. To achieve a more isothermal column environment and maintain steady retention times, a forced-air oven [30] or water jacket/bath [4,25,29,33,34] can be utilized, but the column efficiency suffers due to larger radial temperature gradients that occur with an isothermal column wall. Another strategy to counteract the heating effects that occur as pressure is dropped across the column bed is to segment a longer column into shorter, connected portions (i.e. a $15\ \text{cm}$ column segmented into $3 \times 5\ \text{cm}$ columns) with actively cooled tubing between each column. [35,36] With this technique, column segments can be insulated to reduce the radial thermal gradient; the actively cooled portions of the flow path between the columns reduce the magnitude of the axial thermal gradient that typically forms with such a quasi-adiabatic environment. This method is effective, but has increased costs due to the use of extra columns and hardware.

In addition to the isocratic studies described above, the impact of frictional heating can also lead to changes in both chromatographic selectivity and analyte retention for gradient methods. [37] Typically, water-acetonitrile gradients on reversed phase columns are run from low to high organic in a constant flow mode, with the backpressure dropping as the viscosity of the mobile phase decreases at later stages of the gradients. Based on Equation 1, if the flow rate is constant and the pressure drop across the column decreases over the gradient time, the amount of viscous heating should decrease. Alternatively, techniques to decrease separation times using constant pressure gradient methods [38] could actually lead to an increase in this heating because the flow rate goes up as the organic content increases at the end of the gradient.

To obtain a more stable mobile phase temperature in gradient LC, both constant frictional heat [39] and constant wall heat [40] gradient modes have been developed. To achieve constant frictional heat, the power generated by flow through the column (Equation 1) is

kept steady by modifying the instrument flow rate throughout the method to account for pressure changes that occur as the mobile phase composition changes (taking into account viscosity fluctuations due to this composition variation). [39] The constant wall heat mode expands the constant frictional heat calculations to include thermal expansion coefficients and heat capacities of various mobile phase mixtures that occur during the gradient as well as heat lost to radial dissipation and radiation through the column wall. [40] While these methods do help control mobile phase temperature fluctuations that occur due to viscous heating compared to constant flow gradients, the improvements in retention time repeatability are nominal (<0.1% RSD) [41] at the expense of complex instrument methods containing 30–60 gradient steps based on calculations from the constant friction and constant wall heat models [39,40,42] which may not be realistic for more standard gradient LC methods. [37]

Depending on the mobile phase viscosity and particle size, the maximum instrument pump pressure can hinder the achievable flow rate for a given column, which limits the viscous heating generated. [43,44] However, the newest UHPLC systems now push maximum pressures well beyond 1000 bar and have expanded the flow rate range that can be used at these high pressures. This increased capability can intensify temperature effects that occur due to higher flows and pressures. [45,46] To give users flexibility in controlling this heating, column ovens that enable both still-air and forced-air thermal environments are now integrated into some LC instruments. [46,47]

In this study, we explore the use of such an integrated oven in a UHPLC instrument with a 1500 bar limit and its impact on gradient UHPLC-MS/MS separations. The effect of oven thermal environment on the mobile phase temperature in fully porous particle (FPP) and SPP columns is investigated with the wider range of generated power enabled by the higher pressure limit. Then, the impact of thermal effects on retention time, peak capacity, and peak area ratio repeatability in different oven modes for a subset of compounds from a segmented gradient method used for quantitative neurotransmitter analysis is described. [48] Few reports on the impact of viscous heating in gradient UHPLC-MS for compounds with a wide range of chemical functionality [37], like the neurochemicals explored here, are available. The current work further explores how the column thermal environment impacts chromatographic repeatability when using tandem-MS detection with segmented gradient UHPLC. Here, we demonstrate how the control of thermal environments in an integrated column oven can decrease the magnitude of eluent temperature changes in conditions with high viscous friction, even during gradient runs with constantly shifting viscous friction values. We also show how the impact of these conditions on the repeatability of a standard UHPLC-MS/MS method is limited, which should enable the use of higher pressures and flow rates to increase the speed of other such analytical methods.

2. Materials and Methods

2.1 Chemicals and Reagents

Unless otherwise noted, all chemicals were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of 1 M choline (Ch), 25 mM acetylcholine (ACh), 20 mM glutamate (Glu), 20 mM 4-aminobutyric acid (GABA), 20 mM phenylalanine (Phe), 20 mM serotonin (5HT),

20 mM norepinephrine (NE), and 20 mM dopamine (DA) were prepared in HPLC grade water (Burdick & Jackson, VWR, Radnor, PA). Then a concentrated standard mixture, which included other neurotransmitters (see Table S1), was prepared using the listed stocks and diluted 100-fold in artificial cerebrospinal fluid (aCSF). aCSF was used because it is a similar matrix to that in which *in vivo* neurotransmitters are found. aCSF contained 145 mM NaCl, 2.68 mM KCl, 1.4 mM CaCl₂, 1.0 mM MgSO₄, 1.5 mM Na₂HPO₄, and 0.45 mM NaH₂PO₄ adjusted to pH 7.4 (with NaOH). 10 μ L aliquots of this concentrated mixture were stored at 193 K until the day of analysis.

2.2 Sample Preparation and Derivatization

Benzoyl chloride (BzCl) derivatization of neurotransmitter samples has been described previously [48,49] and is only summarized here. Prior to analysis, the thawed 10 μ L concentrated mixture described above was diluted 10-fold in HPLC grade water to a final volume of 100 μ L. Then, 50 μ L of each of the following solutions were added in sequential order (with vortexing in between): (1) 100 mM carbonate buffer (sodium carbonate monohydrate), (2) BzCl (2% v/v in HPLC grade acetonitrile (Burdick & Jackson, VWR, Radnor, PA)), and (3) an internal standard solution. The internal standard solution was prepared by first diluting 250 μ L of the standard mixture described in Section 2.1 with 249 μ L of HPLC grade water, 250 μ L of 100 mM carbonate buffer, 250 μ L of ¹³C₆-BzCl (2% v/v in HPLC grade acetonitrile), and 1 μ L of formic acid. Then, 10 μ L of this solution was diluted with 10 μ L of concentrated sulfuric acid, 489 μ L of HPLC grade water, 489 μ L of HPLC grade acetonitrile, and 2 μ L of a deuterated stock of non-¹³C-labeled internal standards (12.5 μ M d₄-ACh and 500 μ M d₄-Ch (C/D/N isotopes, Pointe-Claire, Canada) in HPLC grade water). Further information regarding the final concentrations of BzCl-labeled neurotransmitter standards and ¹³C₆-BzCl-labeled internal standards can be found in Table S1.

2.3 UHPLC-MS/MS Instrumentation and Analysis

Derivatized neurotransmitter samples were analyzed by UHPLC-MS/MS using a Vanquish UHPLC (Thermo Fisher Scientific, Gering, Germany) coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For each analysis, 5 μ L of the sample described in Section 2.2 was injected from the standard Vanquish 25 μ L injection loop. The active column preheater was set to 303 K for all experiments. The column compartment of the Vanquish UHPLC can operate in both still-air and forced-air heating modes and both were utilized here with the temperature always set to 303 K in this study. The forced-air mode allows further control of the oven fan speed (with levels ranging from 1–7); moderate (level 3) and high (level 7) speeds were tested.

To analyze peak capacity and retention time repeatability, the MS software (XCalibur 3.0) was set to detect peak transitions for the ¹³C₆-BzCl-labeled internal standards in selected reaction monitoring (SRM) mode. For these measurements, only internal standards were monitored to reduce the number of co-eluting peaks and ensure a high number of MS scans per peak (MS scan rate was set at 0.1 s). Raw data were exported into Igor Pro 6.0 (Wavemetrics, Inc., Lake Oswego, OR) to determine elution time and peak width (4 σ) data

using the Gaussian peak fitting algorithm included in the software. Peak capacities (n_c) were calculated using the following equation [50]:

$$n_c = 1 + \frac{t_g}{(4\sigma)_{\text{avg}}} \quad (2)$$

where t_g is the difference in elution time between the first (Ch) and last (DA) eluting peaks. To determine the repeatability in concentration calculations, the peak area ratio of the BzCl-labeled standard and $^{13}\text{C}_6$ -BzCl-labeled internal standard was calculated with 16 peak transitions used in these instances (see Table S2) by the QuanBrowser in the Xcalibur 3.0 software.

Two columns were characterized in this study: a 2.1×100 mm Hypersil GOLD C18 column (1.9 μm , fully porous) and a 2.1×100 mm Accucore Vanquish C18+ column (1.5 μm , superficially porous), both from Thermo Fisher Scientific (Bellefonte, PA). Mobile phase A was 10 mM ammonium formate (in HPLC grade water) with 0.15% formic acid and mobile phase B was HPLC grade acetonitrile. Flow rates were selected to match the elution time (and thus, linear velocity) for an unretained neurotransmitter analyte (Ch) on columns with different dead volumes; 120 and 720 $\mu\text{L}/\text{min}$ for the Hypersil column (referred to as the FPP column) and 100 and 600 $\mu\text{L}/\text{min}$ for the Accucore column (referred to as the SPP column), respectively. For repeatability studies, the columns were equilibrated at the base flow rate at 5% B (the gradient initial condition). Then, three consecutive gradient separations were performed at the higher flow rate to determine standard deviations of elution time, peak capacity, and peak area ratio for the three different oven modes. The gradient method was: initial condition, 5% B; 0.01 min, 5% B; 0.33 min, 19% B; 0.67 min, 26% B; 1.00 min, 75% B; 1.67 min, 100% B; 2.00 min, 100% B; 2.10 min, 5% B; and 2.50 min, 5% B (Table S3). During method development, it was observed that immediately going from the starting condition flow rate directly to the flow rate used for the method would lead to wide variations in the elution times early in the gradient. To remedy this, a 1 min “flow rate ramp up” step was added to the beginning of the method sequence to ensure that the pump was operating at the expected flow rate. Furthermore, while the total method time was set for 2.5 min, an instrument delay of approximately 45–50 s was needed for the injection process (including needle washes) in the autosampler. All calculations were performed in Microsoft Excel (Redmond, WA) and all data were plotted in Igor Pro 6.0 and Prism 7 (GraphPad, La Jolla, CA).

2.4 Mobile Phase Temperature Measurements

The measurement of the mobile phase temperature at the column outlet has been described previously [25,27] and is only briefly detailed here. A Type-T (Copper-Constantan) HYP-1 (.012” outer diameter) thermocouple probe (Omega Engineering, Stamford, CT) was inserted into a 1.4 cm segment of 0.015” i.d. PEEK tubing (Idex Health and Science, Oak Harbor, WA) adjacent to the outlet frit. The temperature was recorded using a OM-EL-USB-TC Data Logger with Easy-Log USB Software, both from Omega Engineering.

The impact of viscous heating on the mobile phase temperature in the three different thermal environments in the column oven described in Section 2.3 was studied for the two columns in two ways. First, after starting at an initial gradient condition (100 $\mu\text{L}/\text{min}$, 5% B) to determine a base temperature and power (Equation 1), the mobile phase was switched to its lowest viscosity value (100% B) and a series of flow rates were selected to observe the temperature change (following a 20 min thermal equilibration period). Five flow rates from 400 – 2,000 $\mu\text{L}/\text{min}$ for the FPP column and 400 – 1,300 $\mu\text{L}/\text{min}$ for the SPP column were tested and compared by the temperature and power change to the initial gradient condition. Second, the exact process of a low flow rate initial condition followed by three gradient runs (described in Table S3) to study chromatographic repeatability was replicated with the temperature probe in place. In these experiments, a 10-point moving average calculation was used to smooth the measured temperature due to the 0.5 K resolution of the USB data logger.

3. Results and Discussion

3.1 Mobile Phase Temperature Changes Due to Frictional Heating

With the different thermal environment modes integrated into the column heater used here (still air and forced air heating), conditions to enhance the development of either axial or radial thermal gradients can be selected. With still air, the oven chamber fan is turned off which limits the heat removed by convection from moving air. This mode of operation maximizes the axial thermal gradient along the column and leads to higher mobile phase temperatures at the outlet. In the forced air mode, the oven fan blows and increases the heat removed through the column walls as a way to maintain a more constant temperature at the column wall. This mode of operation increases the radial thermal gradient, reduces the axial thermal gradient, and limits the temperature increase of the mobile phase along the column length. These trends are demonstrated in Figure 1 for both FPP (Figure 1A) and SPP (Figure 1B) columns. In both columns the mobile phase temperature is noticeably higher in the still-air mode than in the forced-air mode as viscous heating occurs. As the speed of the fan in forced-air mode increases from ‘Level 3’ to ‘Level 7’, the magnitude of the axial thermal gradient is reduced even further. Temperature increases of nearly 30 K were observed in the still air mode when conditions led to thermal friction exceeding 25 – 35 W/m. This amount of power was achievable on 10 cm columns with the 1500 bar pressure limit enabled with this instrument and far exceeds the value (4 W/m) [29] where radial thermal gradients are expected to reduce column performance.

Based on previous results [25], it was anticipated that the use of the forced-air mode would lead to larger temperature drops with the SPPs than it would with the FPPs. However, in this study the impact of the forced air oven at its highest speed (Level 7) was similar for columns packed with both particle types. The most likely cause is that the use of flowing water to help maintain an isothermal environment is more effective than forced air because of differences in their thermal conductivity ($\lambda_{\text{water}} = 0.60 \text{ W/m/K}$ and $\lambda_{\text{air}} = 0.03 \text{ W/m/K}$). [28] While beds packed with SPPs are better at moving heat to the column wall (with a thermal conductivity about twice that of a column packed with FPPs [22]), having a lower capacity to remove the heat from the wall with air (relative to water) would reduce the decrease in

measured temperature. Additionally, as the columns tested were shorter than previous reports (15 and 10 cm, respectively) [25], they had less surface area to enable the removal of generated heat by the external environment (leading to longer columns having diminished axial temperature gradients at identical viscous friction levels). [12]

The temperature change at the mobile phase outlet was also monitored across three consecutive runs of the gradient used for neurotransmitter separation (see Figure 2). During the 1 min pump equilibration step at the initial mobile phase condition (5% B), the temperature increases quickly due to the increase in flow rate. As the gradient method is run, the temperature initially increases due to the higher viscous friction at the peak viscosity of the water/acetonitrile mixture (near 20% B [51,52]). Then, as the acetonitrile content of the mobile phase gradient increases, the column backpressure decreases and the thermal expansion coefficient of the mobile phase increases, which both lead to a decrease in the observed temperature at the outlet. [39] Finally, during the gradient re-equilibration step (which includes 24 s in the programmed timetable and 45–50 s in instrument delay time), the outlet mobile phase temperature increases again due to the higher backpressure at 5% B compared to 100% B. This general trend matches similar results observed for constant flow gradient methods in previous reports. [39,40]

Beyond the temperature change observed for each individual chromatographic run, an overall greater temperature increase occurs as the axial thermal gradient develops over time due to the flow rate increase 500 $\mu\text{L}/\text{min}$. While the maximum temperature change never reaches the level observed for an isocratic flow rate change at initial conditions (5% B, indicated by the horizontal dashed lines in Figure 2), the peak temperature for each run is progressively higher. Similar to the isocratic measurements shown in Figure 1, the magnitude of the axial thermal gradient is lower in the forced-air mode than it is in the still-air mode for both columns shown in Figure 2. The consequences of this constantly changing mobile phase temperature (due to the increase in flow rate and pressure as well as further changes in pressure related to the mobile phase composition) on chromatographic repeatability are explored in the next section.

3.2 Chromatographic Repeatability in Gradient UHPLC-MS/MS with Different Oven Modes

As described in the previous section, when the flow is increased from a low initial rate to a higher rate for consecutive gradient methods, the magnitude of the axial thermal gradient increases over time with smaller, more frequent fluctuations based on the changing mobile phase composition (Figure 2). These shifts in temperature throughout a set of analyses can affect chromatographic repeatability [53,54], but the impact of different integrated oven modes that can reduce such temperature changes on the repeatability in UHPLC-MS/MS analyses has yet to be explored.

During the development of radial thermal gradients in the column after the flow rate is increased, the peak capacity would be expected to drop due to increases in peak width (Equation 2) because of a loss of efficiency. [5] With the three consecutive runs investigated here (an example chromatogram is shown in Figure 3), a higher RSD value across runs would be expected as the peak capacity may decrease in each consecutive chromatogram, especially for the forced-air oven modes. However, as demonstrated in Figure 4, the

measured peak capacity did not drastically change over three consecutive runs with the percent RSD value being less than 6.9% for the SPP column and less than 5.4% for the FPP column. In a previous study that explored efficiency loss during gradient analyses where a radial temperature gradient had developed, peak capacity reduction was no greater than 10%, but this observation was only made for a still-air oven. [37] The reduction of peak capacity might be expected to change further when conditions where the magnitude of the radial temperature gradient would be larger due to the active removal of heat at the column wall, such as with the forced-air oven mode tested here. In this study, no statistically significant difference ($p > 0.05$) in the peak capacity for a given column between the three heating modes employed was found. This is likely due to the cooling period at the end of each gradient method, where viscosity is lower due to the higher organic content of the mobile phase (Figure 2); this serves to decrease the generated viscous heat and diminishes the magnitude of the radial temperature gradient across all runs in the same manner it does for the axial thermal gradient (Figure 2).

For all three oven modes, the SPP column gave higher peak capacity values than the FPP column, which would be expected for a column packed with smaller particles with higher column efficiency. [55] However, the only statistically significant difference ($p > 0.05$) in peak capacity between columns was found in the forced-air mode when the fan speed was set to 7, which could be an indication of the improved thermal conductivity of columns packed with SPPs. [22–24] As discussed in Section 3.1, this difference would likely be greater if the active cooling mode utilized flowing water rather than flowing air. [25]

For the quantitative UHPLC-MS/MS analyses used to monitor neurotransmitters regularly employed in our lab [48,49], the most important parameters for identifying and quantifying peaks are the elution time and peak area ratio between the analyte and its ^{13}C -labeled internal standard. Because the axial thermal gradient is higher in quasi-adiabatic environments like the still-air oven mode, larger shifts in the elution time between consecutive runs could be observed. In Figure 5, it is shown that even under conditions where the mobile phase temperature is changing between consecutive runs as well as within each given run (see Figure 2), the maximum percent RSD value in elution time for both columns is 1.42% with most values falling well below 1%. When the elution time of each compound is taken into account, the maximum elution shift for any of the eight compounds in any oven mode for either column is 1.1 s. Although the percent RSD values are an order of magnitude higher than those found with a linear gradient separation of a reversed-phase test mixture with repeated injections at constant flow [41], they are smaller than the retention shifts observed for some pharmaceutical compounds with segmented gradients having a similar temperature increase to that observed during these analyses [37]. Furthermore, through the use of UHPLC-MS/MS in this method, compound identification is enabled by retention time, m/z value, and the observed loss of the chemical tag (except for Ch and ACh, which are not derivatized by BzCl). Even with a maximum elution shift of 1.1 s, analytes of interest can still be detected by their known parent mass and mass shift. These results (Figures 4 and 5) are most applicable to gradient elution (where peak capacity and elution time repeatability can be aided by peak compression effects [55,56]) and thermal effects would likely have a greater impact on these parameters in isocratic separations under similar viscous heating conditions.

Between the different oven modes, six of the eight compounds had the highest elution time percent RSD value for the FPP column with the still-air mode, as would be expected for the thermal environment with the largest axial temperature gradient. These six compounds were the last to elute (Glu, GABA, Phe, 5HT, NE, and DA) which indicates a higher retention value that would likely have a higher shift in elution time at higher temperatures (compared to an unretained compound like Ch). For the SPP column, the oven mode with the highest percent RSD value for a given compound varied between the three modes and no obvious trend was observed.

The same comparison that was described above for elution times is shown in Figure 6 for the peak-area ratio between the analyte and its ^{13}C -labeled internal standard. Previously reported methods for measuring derivatized neurotransmitters and other metabolites have had maximum percent RSD values for a single compound range from 7% [49] to 13% [48], similar to the 8% found here. The percent RSD values in [48] and [49] were measured using an instrument with a maximum flow rate of 100 $\mu\text{L}/\text{min}$ and a maximum pressure of 690 bar with 1.0 mm diameter columns. With those constraints, the largest magnitudes of both the axial and radial thermal gradients would be far lower than those observed here; this indicates that the negative consequences of frictional heating on peak quantification are limited for this segmented gradient UHPLC-MS/MS method.

No general trends in the peak area ratio percent RSD value between columns or thermal environments were observed, likely due to the ratio values having less to do with elution time and peak capacity (parameters directly affected by frictional heating) and relating more to sample preparation and MS conditions. When this method is used to analyze dialysate samples [48,49], standards like those analyzed here are used to construct the necessary calibration curve. These percent RSD values were measured for three consecutive runs that would constitute a single point on such a calibration curve under conditions when the magnitude of the axial thermal gradient is trending upward toward an equilibrium value; simultaneously, this magnitude is constantly changing due to the fluctuating column backpressure related to the shifting mobile phase composition. Even in unstable thermal conditions like these, the impact on quantifying the analytes of interest is limited (although it may not be for larger biomolecules [57]) and suggests that fast UHPLC runs at pressures up to 1500 bar should be feasible (within the limits of MS scan rate to ensure sufficient ion counts). A follow-up study on how the UHPLC-MS/MS neurotransmitter analysis method might be affected by column dimensions [58] and extra-column volumes [59,60] at these high speeds is ongoing.

4. Conclusions

As demonstrated here, the integration of thermal environment options into UHPLC instrument ovens enables control over the magnitude of the thermal gradients that form inside the column. Temperature increases of nearly 30 K were observed in columns packed with sub-2 μm particles at viscous friction levels exceeding 25 W/m in the still-air oven mode; this increase diminished by nearly 10 K when using the forced-air oven mode. During gradient methods, the eluent temperature is constantly shifting due to changes in the mobile phase viscosity. However, shifts as large as 7 K within a single run did not greatly impact the

run-to-run repeatability of the peak capacity, analyte elution time, or peak area ratio to an internal standard. Anticipated decreases in peak capacity in forced-air mode and shifts in retention times in still-air mode were minimal. These observations were made under conditions where an axial thermal gradient is developed over time with periodic changes due to the mobile phase gradient that constitute parameters where thermal effects should have the most detrimental effects on the chromatographic method. Because the performance of this method was maintained throughout triplicate runs used to construct calibration curves for quantitative neurotransmitter measurements [48], increasing the method speed is feasible without significant performance losses due to viscous heating. The impact of these changes to the method and the column thermal environment on the detection limits and sensitivity for neurotransmitter measurements will be investigated in a future report.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Halász I, Ende R, Asshauer J. Ultimate limits in high-pressure liquid chromatography. *J. Chromatogr. A*. 1975; 112:37–60.
2. Lin H, Horváth S. Viscous dissipation in packed beds. *Chem. Eng. Sci.* 1981; 36:47–55.
3. Martin M, Guiochon G. Effects of high pressure in liquid chromatography. *J. Chromatogr. A*. 2005; 1090:16–38. [PubMed: 16196131]
4. de Villiers A, Lauer H, Szucs R, Goodall S, Sandra P. Influence of frictional heating on temperature gradients in ultra-high-pressure liquid chromatography on 2.1mm I.D. columns. *J. Chromatogr. A*. 2006; 1113:84–91. [PubMed: 16476437]
5. Gritti F, Guiochon G. Consequences of the radial heterogeneity of the column temperature at high mobile phase velocity. *J. Chromatogr. A*. 2007; 1166:47–60. [PubMed: 17720174]
6. Broeckhoven K, Desmet G. The future of UHPLC: Towards higher pressure and/or smaller particles? *TrAC Trends Anal. Chem.* 2014; 63:65–75.
7. Jorgenson JW. Capillary Liquid Chromatography at Ultrahigh Pressures. *Annu. Rev. Anal. Chem.* 2010; 3:129–150.
8. MacNair JE, Lewis KC, Jorgenson JW. Ultrahigh-Pressure Reversed-Phase Liquid Chromatography in Packed Capillary Columns. *Anal. Chem.* 1997; 69:983–989. [PubMed: 9075400]
9. Colón LA, Cintrón JM, Anspach JA, Fermier AM, Swinney KA. Very high pressure HPLC with 1 mm id columns. *Analyst*. 2004; 129:503–504. [PubMed: 15152325]
10. Anspach JA, Maloney TD, Colón LA. Ultrahigh-pressure liquid chromatography using a 1-mm id column packed with 1.5- μ m porous particles. *J. Sep. Sci.* 2007; 30:1207–1213. [PubMed: 17595956]
11. Mazzeo JR, Neue UD, Kele M, Plumb RS. Advancing LC Performance with Smaller Particles and Higher Pressure. *Anal. Chem.* 2005; 77:460 A–467 A.
12. Gritti F, Guiochon G. Complete Temperature Profiles in Ultra-High-Pressure Liquid Chromatography Columns. *Anal. Chem.* 2008; 80:5009–5020. [PubMed: 18529067]

13. Gritti F, Guiochon G. Heat Exchanges in Fast, High-Performance Liquid Chromatography. A Complete Thermodynamic Study. *Anal. Chem.* 2008; 80:6488–6499. [PubMed: 18690696]
14. Gritti F, Martin M, Guiochon G. Influence of Viscous Friction Heating on the Efficiency of Columns Operated under Very High Pressures. *Anal. Chem.* 2009; 81:3365–3384. [PubMed: 19361228]
15. McCalley DV. The impact of pressure and frictional heating on retention, selectivity and efficiency in ultra-high-pressure liquid chromatography. *TrAC Trends Anal. Chem.* 2014; 63:31–43.
16. Neue UD, Kele M. Performance of idealized column structures under high pressure. *J. Chromatogr. A.* 2007; 1149:236–244. [PubMed: 17382948]
17. Fekete S, Schappler J, Veuthey J-L. Current and future trends in UHPLC. *TrAC Trends Anal. Chem.* 2014; 63:2–13.
18. de Villiers A, Lestremiau F, Szucs R, Gélébart S, David F, Sandra P. Evaluation of ultra performance liquid chromatography: Part I. Possibilities and limitations. *J. Chromatogr. A.* 2006; 1127:60–69. [PubMed: 16797562]
19. Brice RW, Zhang X, Colón LA. Fused-core, sub-2 μm packings, and monolithic HPLC columns: a comparative evaluation. *J. Sep. Sci.* 2009; 32:2723–2731. [PubMed: 19603389]
20. Baker JS, Vinci JC, Moore AD, Colón LA. Physical characterization and evaluation of HPLC columns packed with superficially porous particles. *J. Sep. Sci.* 2010; 33:2547–2557. [PubMed: 20806238]
21. Guiochon G, Gritti F. Shell particles, trials, tribulations and triumphs. *J. Chromatogr. A.* 2011; 1218:1915–1938. [PubMed: 21353228]
22. Gritti F, Guiochon G. Comparison of heat friction effects in narrow-bore columns packed with core-shell and totally porous particles. *Chem. Eng. Sci.* 2010; 65:6310–6319.
23. Gritti F, Guiochon G. Mass transfer resistance in narrow-bore columns packed with 1.7 μm particles in very high pressure liquid chromatography. *J. Chromatogr. A.* 2010; 1217:5069–5083. [PubMed: 20579655]
24. McCalley DV. Some practical comparisons of the efficiency and overloading behaviour of sub-2 μm porous and sub-3 μm shell particles in reversed-phase liquid chromatography. *J. Chromatogr. A.* 2011; 1218:2887–2897. [PubMed: 21450300]
25. Grinias JP, Keil DS, Jorgenson JW. Observation of enhanced heat dissipation in columns packed with superficially porous particles. *J. Chromatogr. A.* 2014; 1371:261–264. [PubMed: 25456605]
26. Vonk RJ, Aalbers T, Eeltink S, Schoenmakers PJ. Temperature control in large-internal-diameter scaffolded monolithic columns operated at ultra-high pressures. *J. Chromatogr. A.* 2015; 1401:60–68. [PubMed: 26003621]
27. Gritti F, Guiochon G. Measurement of the axial and radial temperature profiles of a chromatographic column: Influence of thermal insulation on column efficiency. *J. Chromatogr. A.* 2007; 1138:141–157. [PubMed: 17141792]
28. Gritti F, Gilar M, Jarrell JA. Achieving quasi-adiabatic thermal environment to maximize resolution power in very high-pressure liquid chromatography: Theory, models, and experiments. *J. Chromatogr. A.* 2016; 1444:86–98. [PubMed: 27040511]
29. Gritti F, Guiochon G. Optimization of the thermal environment of columns packed with very fine particles. *J. Chromatogr. A.* 2009; 1216:1353–1362. [PubMed: 19150538]
30. Cabooter D, Lestremiau F, de Villiers A, Broeckhoven K, Lynen F, Sandra P, et al. Investigation of the validity of the kinetic plot method to predict the performance of coupled column systems operated at very high pressures under different thermal conditions. *J. Chromatogr. A.* 2009; 1216:3895–3903. [PubMed: 19285677]
31. de Villiers A, Lynen F, Sandra P. Effect of analyte properties on the kinetic performance of liquid chromatographic separations. *J. Chromatogr. A.* 2009; 1216:3431–3442. [PubMed: 19110259]
32. Lestremiau F, de Villiers A, Lynen F, Cooper A, Szucs R, Sandra P. High efficiency liquid chromatography on conventional columns and instrumentation by using temperature as a variable: Kinetic plots and experimental verification. *J. Chromatogr. A.* 2007; 1138:120–131. [PubMed: 17097097]

33. Gritti F, Guiochon G. Fundamental chromatographic equations designed for columns packed with very fine particles and operated at very high pressures: Applications to the prediction of elution times and the column efficiencies. *J. Chromatogr. A*. 2008; 1206:113–122. [PubMed: 18775540]
34. Fallas MM, Hadley MR, McCalley DV. Practical assessment of frictional heating effects and thermostat design on the performance of conventional (3 μ m and 5 μ m) columns in reversed-phase high-performance liquid chromatography. *J. Chromatogr. A*. 2009; 1216:3961–3969. [PubMed: 19339017]
35. Broeckhoven K, Billen J, Verstraeten M, Choikhet K, Dittmann M, Rozing G, et al. Towards a solution for viscous heating in ultra-high pressure liquid chromatography using intermediate cooling. *J. Chromatogr. A*. 2010; 1217:2022–2031. [PubMed: 20181348]
36. De Pauw R, Degreef B, Ritchie H, Eeltink S, Desmet G, Broeckhoven K. Extending the limits of operating pressure of narrow-bore column liquid chromatography instrumentation. *J. Chromatogr. A*. 2014; 1347:56–62. [PubMed: 24797393]
37. Nováková L, Veuthey JL, Guilleme D. Practical method transfer from high performance liquid chromatography to ultra-high performance liquid chromatography: The importance of frictional heating. *J. Chromatogr. A*. 2011; 1218:7971–7981. [PubMed: 21944847]
38. Verstraeten M, Broeckhoven K, Lynen F, Choikhet K, Dittmann M, Witt K, et al. Comparison of the quantitative performance of constant pressure versus constant flow rate gradient elution separations using concentration-sensitive detectors. *J. Chromatogr. A*. 2012; 1232:65–76. [PubMed: 22055519]
39. Gritti F, Guiochon G. Gradient chromatography under constant frictional heat: Realization and application. *J. Chromatogr. A*. 2013; 1289:1–12. [PubMed: 23566917]
40. Gritti F, Guiochon G. Realization and potential advantages of gradient separations performed under steady state temperature regime. *J. Chromatogr. A*. 2013; 1291:104–113. [PubMed: 23608405]
41. Stankovich JJ, Gritti F, Beaver LA, Stevenson PG, Guiochon G. Fast gradient separation by very high pressure liquid chromatography: Reproducibility of analytical data and influence of delay between successive runs. *J. Chromatogr. A*. 2013; 1318:122–133. [PubMed: 24209296]
42. Stankovich JJ, Gritti F, Stevenson PG, Vajda P, Beaver LA, Guiochon G. Volume based vs. time based chromatograms: Reproducibility of data for gradient separations under high and low pressure conditions. *J. Chromatogr. A*. 2014; 1343:79–90. [PubMed: 24717849]
43. Fekete S, Fekete J, Guilleme D. Estimation of the effects of longitudinal temperature gradients caused by frictional heating on the solute retention using fully porous and superficially porous sub-2 μ m materials. *J. Chromatogr. A*. 2014; 1359:124–130. [PubMed: 25069746]
44. Tanaka N, McCalley DV. Core–Shell, Ultrasmall Particles, Monoliths, and Other Support Materials in High-Performance Liquid Chromatography. *Anal. Chem.* 2016; 88:279–298. [PubMed: 26540635]
45. De Vos J, De Pra M, Desmet G, Swart R, Edge T, Steiner F, et al. High-speed isocratic and gradient liquid-chromatography separations at 1500bar. *J. Chromatogr. A*. 2015; 1409:138–145. [PubMed: 26199099]
46. De Vos J, Broeckhoven K, Eeltink S. Advances in Ultrahigh-Pressure Liquid Chromatography Technology and System Design. *Anal. Chem.* 2016; 88:262–278. [PubMed: 26599965]
47. Fekete S, Veuthey J-L, Guilleme D. Comparison of the most recent chromatographic approaches applied for fast and high resolution separations: Theory and practice. *J. Chromatogr. A*. 2015; 1408:1–14. [PubMed: 26187764]
48. Song P, Mabrouk OS, Hershey ND, Kennedy RT. In Vivo Neurochemical Monitoring Using Benzoyl Chloride Derivatization and Liquid Chromatography–Mass Spectrometry. *Anal. Chem.* 2012; 84:412–419. [PubMed: 22118158]
49. Wong J-MT, Malec PA, Mabrouk OS, Ro J, Dus M, Kennedy RT. Benzoyl chloride derivatization with liquid chromatography–mass spectrometry for targeted metabolomics of neurochemicals in biological samples. *J. Chromatogr. A*. 2016; 1446:78–90. [PubMed: 27083258]
50. Neue UD. Theory of peak capacity in gradient elution. *J. Chromatogr. A*. 2005; 1079:153–161. [PubMed: 16038301]
51. Chen H, Horváth C. High-speed high-performance liquid chromatography of peptides and proteins. *J. Chromatogr. A*. 1995; 705:3–20. [PubMed: 7620571]

52. Thompson JD, Carr PW. High-Speed Liquid Chromatography by Simultaneous Optimization of Temperature and Eluent Composition. *Anal. Chem.* 2002; 74:4150–4159. [PubMed: 12199587]
53. Stankovich JJ, Gritti F, Stevenson PG, Beaver LA, Guiochon G. Very high pressure liquid chromatography using fully porous particles: Quantitative analysis of fast gradient separations without post-run times. *J. Chromatogr. A.* 2014; 1324:155–163. [PubMed: 24296292]
54. Stankovich JJ, Gritti F, Stevenson PG, Beaver LA, Guiochon G. Very high pressure liquid chromatography using core-shell particles: Quantitative analysis of fast gradient separations without post-run times. *J. Chromatogr. A.* 2014; 1325:99–108. [PubMed: 24370000]
55. Jandera P. Can the theory of gradient liquid chromatography be useful in solving practical problems? *J. Chromatogr. A.* 2006; 1126:195–218. [PubMed: 16787650]
56. Neue UD, Marchand DH, Snyder LR. Peak compression in reversed-phase gradient elution. *J. Chromatogr. A.* 2006; 1111:32–39. [PubMed: 16473362]
57. Fekete S, Guilleme D. Estimation of pressure-, temperature- and frictional heating-related effects on proteins' retention under ultra-high-pressure liquid chromatographic conditions. *J. Chromatogr. A.* 2015; 1393:73–80. [PubMed: 25823888]
58. Van Schoors J, Maes K, Van Wansele Y, Broeckhoven K, Van Eeckhaut A. Miniaturized ultra-high performance liquid chromatography coupled to electrochemical detection: Investigation of system performance for neurochemical analysis. *J. Chromatogr. A.* 2016; 1427:69–78. [PubMed: 26687168]
59. Spaggiari D, Fekete S, Eugster PJ, Veuthey J-L, Geiser L, Rudaz S, et al. Contribution of various types of liquid chromatography–mass spectrometry instruments to band broadening in fast analysis. *J. Chromatogr. A.* 2013; 1310:45–55. [PubMed: 23993747]
60. Vanderlinden K, Broeckhoven K, Vanderheyden Y, Desmet G. Effect of pre- and post-column band broadening on the performance of high-speed chromatography columns under isocratic and gradient conditions. *J. Chromatogr. A.* 2016; 1442:73–82. [PubMed: 26987413]

Figure 1.

Temperature increases observed for fully porous (1A) and superficially porous (1B) sub-2 μm particle columns (both 2.1×100 mm). The base temperature was measured at an initial gradient condition (100 $\mu\text{L}/\text{min}$, 5% B) and then the mobile phase was changed to 100% B at increasing flow rates (and pressures). The x-axis was calculated using the column flow rate and pressure with Equation 1. Red circles indicate still-air mode, black squares indicate forced-air mode at fan speed 3, and blue triangles indicate forced-air mode at fan speed 7. Lines between points are included as a guide to the eye.

Figure 2. Temperature increases observed for fully porous (2A) and superficially porous (2B) sub-2 μm particle columns (both $2.1 \times 100 \text{ mm}$) during the gradient method described in Table S3. The base temperature was measured at an initial gradient condition ($120 \mu\text{L/min}$ in 2A and $100 \mu\text{L/min}$ in 2B, 5% B) and then monitored throughout three consecutive gradient runs at a higher flow rate ($720 \mu\text{L/min}$ in 2A and $600 \mu\text{L/min}$ in 2B, 5% B). Prior to the first

gradient, a 1 min run at 5% B was used to increase the flow rate from the initial to final flow rate. Red solid lines indicate still-air mode, black solid lines indicate forced-air mode at fan speed 3, and blue solid lines indicate forced-air mode at fan speed 7 (all smoothed by a 10-point moving average). Dashed horizontal lines of the same color indicate the temperature increase by just changing the flow rate at an isocratic 5% B. The black dotted vertical lines indicate the starts of individual runs.

Figure 3.

Extracted ion chromatograms for 24 neurotransmitters and metabolites using the gradient method described in Table S3 on a 2.1×100 mm sub-2 μ m superficially porous particle column at 600 μ L/min. Each extracted chromatogram is normalized to 100% abundance individually. Analyte information (including full compound names) can be found in Table S1. Compounds in red (with an asterisk next to their abbreviation) indicate key compounds focused on in the main article.

Figure 4.

Average peak capacities (calculated using Equation 2) for eight compounds separated on fully porous (solid bars) and superficially porous (patterned bars) sub-2 μm particle columns (both 2.1×100 mm) over three consecutive gradient runs (error bars indicate 1 standard deviation of the mean). Red bars indicate still-air mode, black bars indicate forced-air mode at fan speed 3, and blue bars indicate forced-air mode at fan speed 7. Data values can be found in Table S4.

Figure 5.

Average elution times for eight compounds separated on fully porous (5A) and superficially porous (5B) sub-2 μm particle columns (both 2.1×100 mm) over three consecutive gradient runs (error bars indicate 1 standard deviation of the mean). Red bars indicate still-air mode, black bars indicate forced-air mode at fan speed 3, and blue bars indicate forced-air mode at fan speed 7. Data values can be found in Table S5.

Figure 6. Relative standard deviations (as percentages) for eight compounds separated on fully porous (6A) and superficially porous (6B) sub-2 μm particle columns (both 2.1×100 mm) over three consecutive gradient runs. Red bars indicate still-air mode, black bars indicate forced-air mode at fan speed 3, and blue bars indicate forced-air mode at fan speed 7.