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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 gradient methods under conditions of high viscous friction may be possible without the negative effects typically observed with isocratic separations under similar conditions.

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]:

$$Power = F\Delta P \quad (1)$$

early research in UHPLC focused on capillary columns (<100 μm i.d. (inner diameter)) where flow rates well below 1 μL/min prevent any noticeable viscous heating [7,8]. For commercial UHPLC columns, although i.d.'s have decreased from 4.6 mm to 2.1 mm (and even 1.0 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

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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 cm column segmented into 3 × 5 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 Eq. (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 (Eq. (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 norephinephrine (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 is 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 $CaCl_2$, 1.0 mM $MgSO_4$, 1.5 mM Na_2HPO_4, and 0.45 mM NaH_2PO_4 adjusted to pH 7.4 (with NaOH). 10 μL 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 μL concentrated mixture described above was diluted 10-fold in HPLC grade water to a final volume of 100 μL. Then, 50 μL 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 μL of the standard mixture described in Section 2.1 with 249 μL of HPLC grade water, 250 μL of 100 mM carbonate buffer, 250 μL of $^{13}C_6$-BzCl (2% v/v in HPLC grade acetonitrile), and 1 μL of formic acid. Then, 10 μL of this solution was diluted with 10 μL of concentrated sulfuric acid, 489 μL of HPLC grade water, 489 μL of HPLC grade acetonitrile, and 2 μL of a deuterated stock of non-^{13}C-labeled internal standards (12.5 μM d_4-ACh and 500 μM d_4-Ch (C/D/N isotopes, Pointe-Claire, Canada) in HPLC grade water). Further information regarding the final concentrations of BzCl-labeled neurotransmitter standards and $^{13}C_6$-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, Gemering, Germany) coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). For each analysis, 5 μL of the sample described in Section 2.2 was injected from the standard Vanquish 25 μL 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

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