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Wide injection zone compression in gradient reversed-phase liquid chromatography

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ABSTRACT

Chromatographic zone broadening is a common issue in microfluidic chromatography, where the sample volume introduced on column often exceeds the column void volume. To better understand the propagation of wide chromatographic zones on a separation device, a series of MS Excel spreadsheets were developed to simulate the process. To computationally simplify these simulations, we investigated the effects of injection related zone broadening and its gradient related zone compression by tracking only the movements of zone boundaries on column. The effects of sample volume, sample solvent, gradient slope, and column length on zone broadening were evaluated and compared to experiments performed on 0.32 mm I.D. microfluidic columns. The repetitive injection method (RIM) was implemented to generate experimental chromatograms where large sample volume scenarios can be emulated by injecting two discrete small injection plugs spaced in time. A good match between predicted and experimental RIM chromatograms was observed. We discuss the performance of selected retention models on the accuracy of predictions and use the developed spreadsheets for illustration of gradient zone focusing for both small molecules and peptides.

1. Introduction

When a large sample volume is loaded onto a chromatographic column, zone broadening invariably occurs [1,2], an effect that is compounded when the sample is loaded under conditions where it is poorly retained [3–5]. This effect is often observed in preparative chromatography, two-dimensional (2D) liquid chromatography (LC) [1,6], microfluidic liquid chromatography (μLC) [7], or any other separation technique where the sample volume might exceed the column void volume V_0.

The extent of initial zone broadening (or compression) upon the injection of a large sample volume was investigated by Snyder and Saunders [8] and others [1,5,6]. Sanchez et al. [2] derived the equations necessary for calculation of the zone width formed by a given injection.

μLC is becoming a popular tool in analytical laboratories [9–13], especially in proteomics research [14,15]. For applications such as this, microliter(s) injection volumes (multiple column volumes) are common to derive high-sensitivity analyses. Relatively large sample volumes may lead to injection related zone broadening or even to sample breakthrough. Users typically expect that gradient elution will compress the injected zones into Gaussian peaks, but this may not be the case for all analytes. Some analytes may elute as wide rectangular zones, reducing the achievable analytical sensitivity.

In recent publication [7], we have studied injection induced zone broadening in μLC, for which an Excel spreadsheet was developed to model the movement of analytes on a chromatographic column. In these simulations, the propagation of the zone front and zone rear boundaries through the column can be tracked independently; the zone broadening and focusing during gradient elution is then calculated either in spatial units (width on column) or in time units (difference between front and rear boundary elution time). The visualization of zones on column is useful and can be employed as an educational tool to understand zone movements during chromatography.

In this study, we will further demonstrate the utility of the calculations performed in this Excel spreadsheet with respect to zone broadening, expanding to more thorough examinations of the effects of sample volume, sample solvent strength, gradient slope,

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type of analytes, and column length. Computational simulations are compared with chromatographic experiments throughout.

2. Experimental

2.1. Materials and reagents

Formic acid (FA), >99%, was purchased from Sigma (St. Louis, MO, USA). Analytes in this study: uracil (U), acetophenone (AP), propiophenone (PP), butyrophenone (BP), valerophenone (VP), hexanophenone (HP), reserpine (RES), and bombesin (BOM) were obtained from Sigma. HPLC grade acetonitrile (MeCN) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for preparation of HPLC mobile phases. MassPREP Digestion Standard Mix 1 was obtained from Waters (Milford, MA, USA).

2.2. LC instrumentation, columns, and conditions

Chromatographic experiments were carried out as described previously. Briefly, a microfluidic LC system consisting of a 20 nL Cheminert UHPLC internal loop sample injector (Valco Instruments, Houston, TX, USA) or 6-port valve with 6 μL external loop (Valco) were used for sample introduction. Mobile phases were delivered by a nanoACQUITY binary solvent manager (BSM) pump. Analytes were detected using an ACQUITY TUV detector equipped with a 10 nL bubble cell (Waters, Milford, MA, USA). A stand-alone column heater module and a Temperature Control Module II (Waters) were used for maintaining the column temperature. The system was controlled by Empower software (Waters). Prototype titanium microfluidic tile-based columns were machined and packed in house using 1.8 μm High Strength Silica (HSS) T3 sorbent (C18-bonded silica, Waters). Each channel was 100 mm long with a rectangular cross section equivalent to a tubular column with a 0.32 mm internal diameter. Mobile phase A was 0.1% (v/v) FA in water, mobile phase B 0.1% FA in acetonitrile. Column flow rate was 6 μL per minute, with the temperature held at 40 °C. Detector wavelength was set to 243 or 260 nm. Gradient conditions are given in the figure captions.

Retention factors in a wide range of acetonitrile concentrations were measured with an ACQUITY UPLC I-Class system, a flow-through needle sample manager and a TUV detector fitted with a 250 nL cell (Waters). Data were obtained using a 50 × 2.1 mm, HSS T3 column packed with 1.8 μm sorbent (Waters). Mobile phase and chromatographic conditions were as above; flow rate was set to 294 μL per minute.

LC–MS analysis of tryptic peptides was performed on an ionKey/MS system in which a NanoACQUITY UPLC (Waters) was interfaced to a Xevo TQ MS triple quadrupole mass spectrometer (Waters). Emitter was set to 3.5 kV, cone to 30 V, collision energy was optimized for each MRM transitions. MassPREP Digestion Standard Mix 1 was dissolved in an aqueous solution of 0.1% FA containing 3% MeCN. Final concentration was 50 fmol/μL. 20 μL of sample was directly injected on an iKey separation device with a built in column heater, integrated electrospray emitter, and μLC column of 0.15 × 50 mm dimensions packed with 1.7 μm BEH C18 sorbent. Mobile phase A was 0.1% FA in water, B was 0.1% FA in acetonitrile. Gradient was 5 to 45% B in 10 min, flow rate 3 μL/min, and column temperature was set to 60 °C. Selected eluting peptides (IGDYAGIK, AEFVEVTK, VNQIGTLSESIK, SIVPSGASTGVHEALEMR, LVNELTEFAK, and VVGLSTLPEIYEK) were detected by MRM MS.

2.3. Repetitive injection method

The μLC system used in this study was capable of injecting either one large volume plug of sample using an injector with an external sample loop, or repetitive small volume injections at pre-programmed time intervals using an injector with a 20 nL internal sample loop. This technique (as described previously [7]) is termed the repetitive injection method (RIM) and emulates large sample volume injections by using two temporally spaced small volume injections. The method is performed as follows: two separate injections (spaced by a defined period of time) are performed and the gradient elution is performed, with gradient initiation beginning at the completion of the second injection. The spacing between these two discrete peaks (corresponding to the front and rear boundaries of a wide chromatographic zone) is changing during gradient elution and can be used to study gradient compression. RIM is useful for investigation of gradient zone focusing without requiring a change of the sample loop. Rather, the injection timing can be easily changed instead.

2.4. Excel spreadsheet retention prediction and zone visualization

Excel Supplementary spreadsheet S1 was used to predict zone movement through a column as described previously [7]. Two zone positions (as introduced by a RIM experiment) were calculated independently, providing the positions of the front and rear boundaries of a hypothetical wide zone. It is important to understand that the spreadsheet does not calculate the band compression of a single peak (or peak compression factor) as described in literature [16–18]. It is designed to visualize the compression of wide rectangular zones that significantly exceed the width of a typical chromatographic Gaussian peak. After the injection of a wide zone we neglect kinetic factors and mass transfer effect, and assume no further band broadening. This is acceptable simplification when the column dispersion is insignificant in comparison to initial zone width.

Calculation of the movement of each zone boundary is performed in step-wise fashion. (i) The mobile phase strength is calculated at a given column position; (ii) the retention factor of an analyte at that position is obtained; (iii) analyte speed is calculated; (iv) the distance migrated in a given time step is obtained; and (v) new on column position of the zone boundary is determined. The computation is then iterated, calculating the zone's on-column position until the boundary "elutes" from the column outlet. The time difference between zone front and rear arrival represents elution zone width. Simulated RIM chromatograms were generated with Supplemental spreadsheet S2.

This calculation is dependent on the assumption that no mass overload takes place (generally the case in trace LC–MS analysis), and that the gradient moves through the column with the speed of the mobile phase (no delay or distortion due to the solvent adsorption on the stationary phase is considered [19]).

3. Results and discussion

3.1. Validation of RIM and retention model accuracy

Fig. 1 shows an overlay of three chromatograms. Panel A represents an injection of 6 μL of sample; this volume exceeds the μLC column void volume (4.8 μL) and generates relatively wide initial zones. This is due, in part, to the sample solvent (30% MeCN in this case) temporarily acts as the mobile phase during sample injection, until the sample solvent is replaced by the actual mobile phase gradient. The zone widths calculated by Supplementary spreadsheet S1 for the experiment presented in Fig. 1 were 21.8, 10.6, 5.3, 2.5, and 1.1 mm for AP, PP, BP, VP, and HP, respectively. It is important to note that the zone width for each analyte depends primarily on its retention factor k in the sample solvent [2,8]. The zone widths are further compressed in gradient elution, however, Fig. 1 shows