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Characterization of peak capacity of microbore liquid chromatography columns using gradient kinetic plots☆

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ABSTRACT

The performance of micro-liquid chromatography columns with an inner diameter of 0.3 mm was investigated on a dedicated micro-LC system for gradient elution. Core-shell as well as fully porous particle packed columns were compared on the basis of peak capacity and gradient kinetic plot limits. The results for peak capacity showed the superior performance of columns packed with sub-2 μm fully porous particles compared to 3.0 μm fully porous and 2.7 μm core-shell particles within a range of different gradient time to column void time ratios. For ultra-fast chromatography a maximum peak capacity of 16 can be obtained using a 30 s gradient for the sub-2 μm fully porous particle packed column. A maximum peak capacity of 121 can be achieved using a 5 min gradient. In addition, the influence of an alternative detector cell on the basis of optical waveguide technology and contributing less to system variance was investigated showing an increased peak capacity for all applied gradient time/column void time ratios. Finally, the influence of pressure was evaluated indicating increased peak capacity for maximum performance whereas a limited benefit for ultra-fast chromatography with gradient times below 30 s was observed.

1. Introduction

Gradient elution in high performance liquid chromatography is nowadays the predominant separation mode [1]. The possibility to separate compounds with different physico-chemical properties in a single chromatographic run within a reasonable time frame favors its application in reversed liquid chromatography. Especially, the advantage of peak focusing related to peak compression in gradient elution methods is considered advantageous compared to isocratic separations [2]. During the last decade, the development of both instrument and column technology offered the opportunity to achieve fast and highly efficient separations due to increasing pressure capabilities of the chromatographic system including the stationary phase up to 1500 bar [3]. To achieve even faster separations at lower flow rates, which is a prerequisite for coupling with mass spectrometry (*MS*), the column inner diameter (i.d.) was subsequently decreased from 4.6 mm to 2.0 mm. Currently, this trend continues to even smaller column i.d.'s of 1.0 mm to 0.3 mm [4]. At the same time stationary phase materials, such as sub-2 μm fully porous particles with a minimum particle diameter (d_p) of 1.5 μm as well as core-shell particle packed columns with a d_p of 1.3 μm, were introduced to accomplish highly efficient separations and attain increased peak capacities in shorter analysis times when using decreased column i.d. and length [5–7]. To take full advantage of such small i.d. columns and subsequently small peak volumes, the system contribution to extra-column band broadening needs to be further minimized [8–12]. Especially, for miniaturized columns with an i.d. of 0.3 mm dedicated micro-LC systems have been developed. In particular, micro-LC systems are characterized by low gradient delay volumes allowing the use of fast cycle times of 30 s and smaller because of the immediate effectiveness of the solvent gradient. Due to the high linear velocities and decreased gradient delay volumes, fast separations and column re-equilibration can be achieved leading to reduced analysis cycle times, which is of utmost importance for routine analysis [7,13]. These properties are also favourable for the applicability of micro-LC as a second dimension of an online comprehensive two-dimensional chromatography (LCxLC) system as has been recently shown by Haun and Leonhardt [14,15]. In general, these benefits have been a major driver for implementing capillary columns – especially in combination

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with mass spectrometry – in different application areas [13,16–18]. To circumvent possible column overloading, several investigations have explored means to overcome the limited loadability of small i.d. columns by temperature focusing, online solid phase extraction or direct large volume injection, illustrating the effort of optimizing this separation dimension [17,19,20].

However, only little information about systematic efficiency characterization in gradient elution is available for miniaturized columns packed with commercially available state-of-the-art particles [21]. Therefore, this contribution focuses on the question, which peak capacity can be obtained using 300 μm i.d. micro-LC columns packed with various chromatographic supports on a dedicated micro-LC system. Furthermore, the influence of different chromatographic supports varying in d_p is investigated with respect to applications requiring high peak capacities and therefore decreased gradient slopes. In addition, the range of ultra-fast chromatography with a maximum gradient time of 30 s for the applicability of online LCxLC and high throughput analysis is evaluated. Moreover, an alternative miniaturized detector cell based on optical waveguide technology was evaluated with respect to its influence on peak capacity to demonstrate the impact of reduced extra-column volume. For data evaluation, the peak capacity was determined and subsequently transformed into gradient kinetic plot limits.

2. Materials and methods

All experiments were performed on an Eksigent ExpressLC Ultra system (Sciex, Dublin, CA) with a micro-LC flow module (flow rate range: 5–50 μL min^{-1}). The pneumatic pump was capable of 690 bar maximum pressure. Flow calibration was carried out at a flow rate of 25 μL min^{-1}. The sample was loaded by an HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). The installed fused-silica, surrounded by a polyetheretherketone (PEEKSil) sample loop, had an inner diameter of 75 μm and a length of 10 cm. The sample was injected through a built-in six-port valve using the full loop injection mode of the software injecting 442 nL. An integrated static air column oven was used for temperature control of the stationary phase. For data acquisition, a built-in diode array detector (DAD) was employed with a cell volume of 100 nL as well as an alternative detector cell on the basis of optical waveguide technology with a cell volume of 6 nL (KNAUER, Berlin, Germany). Chromatograms were recorded at 272 nm with a data acquisition rate of 40 Hz and 50 Hz depending on the applied detector.

Fused-silica capillaries with an outer diameter (*o.d.*) of 360 μm and an inner diameter (*i.d.*) of 50 μm were used for the connection prior to and after the column with a length of 10 cm and 13 cm. Using sleeves, the o.d. was expanded to 1/32" for the connection to the columns and detectors. For data acquisition and analysis the Eksigent control software (Version 4.2 Patch for ekspert nanoLC 400 and batch acquisition control) and ClarityChrom (V. 6.1.0) were used. Further data processing was performed using Origin Lab V. 9.3 and Microsoft Office Excel 2013. Table 1 presents an overview of the investigated columns.

Water and acetonitrile (ACN) were used as mobile phase constituents. In addition, acetone was used for the determination of the gradient delay volume. All solvents were purchased from Th. Geyer-Chemsolute (Renningen, Germany) with purity for LC–MS. Formic acid (FA), purchased from Sigma-Aldrich (Seelze, Germany), was used at 0.1% (v/v) as solvent additive to adjust the pH of the mobile phase and analyte mixture.

Table 2 contains the selected compounds including important physico-chemical properties, while the structural formulas are compiled in Fig. S1. All compounds except uracil are pharmaceuticals and have different log P values and thus different polarity to obtain a uniform distribution over the entire gradient window, which is of utmost importance for an appropriate determination of peak capacity [21].

The stock solutions for all analytes were prepared using a mixture of 50/50 H_2O/ACN (v/v) at a concentration of 1 mg mL^{-1}. For the analysis the single standards were merged before dilution with acidified water. The final concentrations are given in Table 2 with a composition of 96/4 H_2O/ACN + 0.1% FA (v/v).

For the determination of peak capacity, the flow rate was varied between 10 and 50 μL min^{-1} in 5-μL steps using a linear gradient from 5% to 95% B. The influence of the gradient slope was analyzed using four different gradient times (t_G). At a flow rate of 10 μL min^{-1}, t_G was set to 0.5, 1.0, 3.0 and 5.0 min. The first two gradient times were chosen to mimic ultra-fast chromatography whereas the latter two represent applications requiring high peak capacity. In order to ensure the same mobile phase history, the ratio between t_G and the column void time (t_0) needed to be kept constant. Therefore, the t_0 values were determined using uracil as column void marker at a mobile phase composition of 10/90 H_2O/ACN + 0.1% FA (v/v). Afterwards, the obtained t_0 times were corrected for the system void time ($t_{0,sys}$) by replacing the column by a zero-dead volume (*ZDV*) union. The gradient time was adjusted to ensure constant elution volumes resulting in gradient times between 5 s and 5 min depending on the column t_0 and flow rate (see Table S1–Table S4). In addition, the ratio between the delay time (t_d) and t_0 had to be kept constant to obtain comparable results. Consequently, the gradient delay volume had to be determined. Therefore, the column was replaced by a *ZDV* union using water as mobile phase for both solvent channels. In order to illustrate the gradient profile, 0.1% acetone was added to the water of solvent channel B. The use of acetone was not considered critical because no on-line degasser was used. The gradient delay volume was determined at three different flow rates (10, 20 and 40 μL min^{-1}) using a plateau of 0.5 min followed by a 1.0 min linear gradient from 5% to 95% B. Afterwards, the gradient delay volume was corrected for the volumes of the connection capillary after the column (V_{cap} = 255 nL) as well as for the detector cell volume (V_{det} = 100 nL). Since t_0 differed for all columns, an additional isocratic plateau (t_p) was added at the beginning of the analysis to obtain a constant ratio of t_d to t_0 of 2. In the following, t_d will be used as the sum of the gradient delay time (t_{dwell}) and t_p. All measurements were performed in triplicate at a column temperature of 30 °C.

3. Results and discussion

3.1. Determination of the gradient delay volume and retention factor

Since the ratio of t_d/t_0 had to be kept constant in order to provide comparable results, the gradient delay volume needed to be determined. This was achieved according to the procedure described in section 2. Fig. S2 shows the resulting gradient profile including the determination of the gradient delay volume for a flow rate of 40 μL min^{-1}. In order to characterize t_{dwell}, several determination strategies can be applied [23]. As can be identified in Fig. S2, the point of intersection between the linear regressions of the isocratic plateau and linear gradient was used. Thereby, t_{dwell} is estimated to 0.06 min. After multiplication by the flow rate, the experimentally determined gradient delay volume ($V_{d,exp}$) needs to be corrected for the corresponding volumes of the capillary after the column (V_{cap}) as well as the detector cell volume (V_{det}) according to Eq. (1) to obtain the corrected gradient delay volume ($V_{d,cor}$). Compared