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Effect of gradient steepness on the kinetic performance limits and peak compression for reversed-phase gradient separations of small molecules

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ABSTRACT

The effect of gradient steepness on the kinetic performance limits and peak compression effects has been assessed in gradient mode for the separation of phenol derivatives using columns packed with 2.6 μm core–shell particles. The effect of mobile-phase velocity on peak capacity was measured on a column with fixed length while maintaining the retention factor at the moment of elution and the peak-compression factor constant. Next, the performance limits were determined at the maximum system pressure of 100 MPa while varying the gradient steepness. For the separation of small molecules applying a linear gradient with a broad span, the best performance limits in terms of peak capacity and analysis time were obtained applying a gradient-time-to-column-dead-time (t_G/t_0) ratio of 12. The magnitude of the peak-compression factor was assessed by comparing the isocratic performance with that in gradient mode applying different gradient times. Therefore, the retention factors for different analytes were determined in gradient mode and the mobile-phase composition in isocratic mode was tuned such that the difference in retention factor was smaller than 2%. Peak-compression factors were quantitatively determined between 0.95 and 0.65 depending on gradient steepness and the gradient retention factor.

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1. Introduction

The development of robust baseline separations within the shortest possible analysis time is one of the key objectives in HPLC method development [1,2]. Different metrics have been introduced over the years to describe the chromatographic performance, such as the resolution (R_s), different plate-height (H) equations providing a description of band dispersion as a function of column parameters and mobile-phase velocity [3–5], the separation impedance (E) [6], *etc*. However, to assess and compare the chromatographic performance limits that can be achieved by using new support types, such as packed *versus* monolithic columns, or for example separation modes, *i.e.*, pressure- *versus* electro-driven separations, these approaches alone no longer suffice [7,8]. To visualize the performance limits that can be achieved in terms of efficiency and analysis time, Giddings proposed a representation that extrapolates the performance that can be achieved as a function of column length and particle size while operating at the maximum column (or system) pressure [9]. This approach has been refined by Poppe [10] and Desmet [11] and has now been widely adopted to optimize separations, since this approach, for example, allows practitioners to select the optimal column length-particle size combination for a given critical-pair separation [12].

Since the introduction of HPLC there has been a trend to decrease the particle size in order to increase the separation efficiency and to reduce the analysis time. However, the decrease in particle size is ultimately limited by the pressure drop across the column. A major break-through in liquid chromatography was the introduction of UHPLC instrumentation, allowing the user to operate at system pressures of 100 MPa, in combination with columns packed with porous sub-2-micron silica particles in 2004 [13]. Recently instrumentation allowing to operate at 150 MPa has been introduced [14]. In addition, stationary-phase design has evolved [15]. For example, monolithic stationary-phase with large through-pores have been developed to enhance separation efficiency [16], whereas monolithic materials featuring macropore and polymer microglobule

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sizes in the range of 50–200 nm have been designed allowing for sub-minute gradient separations [17]. One of the most promising stationary-phase materials is the new generation of core–shell particles that exhibit significantly lower separation impedance than their fully porous counterpart [18]. It has been stated that this performance improvement is due to the improved A-, B-, and C-term characteristics affecting the magnitude of the H [18]. It has also been reported that the presence of a solid core lowers the internal porosity which leads to an increases the column permeability ($K_{v,0}$), since $K_{v,0}$ is based on the time measurement of an unretained marker [19]. Since kinetic plots take both the effects of H and $K_{v,0}$ into account, the peak-production rate that can be reached with core–shell columns is significantly increased.

An additional gain in efficiency can be realized when applying the gradient-elution mode instead of isocratic mode. When a band migrates through the analytical column and a mobile phase is applied with increased solvent strength, the tail of the peak will move in a mobile phase that is slightly stronger compared to the front of the same band. As a result, the tail of the peak tends to move faster and a peak-focusing effect is observed, which is referred to as gradient peak compression (G) [20]. The theoretical values of G as a function of gradient conditions has been derived for linear gradients using a numerical procedure, assuming the validity of the linear-solvent-strength (LSS) model, by Snyder et al. [21]. Based on the mass-transport equation and dispersion modelling, Poppe [22] derived an analytical expression for the peak compression factor that can be applied for both linear and step gradients. In practice, the existence of significant peak-compression effects in reversed-phase gradient chromatography has been debated [23]. Neue et al. [23] identified different experimental phenomena, including extra-column band broadening, variation in separation efficiency with mobile-phase composition, viscous-fingering effects, deviation from the linear-solvent-strength model affecting the solvent strength parameter estimation, and stationary-phase diffusion, that contribute to band broadening and therefore (partially) mask the peak-compression effect.

In the present study, the effect of gradient steepness on kinetic performance limits has been assessed for small-molecule separations, using phenol derivatives as test compounds. In order to realize a better understanding of the effect of gradient steepness on the resulting peak width, separations were conducted in both the isocratic and the gradient mode to determine the retention factor at the point of elution, the gradient retention factors and corresponding peak widths, allowing to assess possible gradient peak-compression effects. Finally, the experimental values of peak compression are compared to the G factors predicted by Poppe [22].

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile (ACN, HPLC supra-gradient quality) was obtained from Biosolve B.V. (Valkenswaard, The Netherlands). Deionized HPLC-grade water (conductivity $\leq$0.055 μS cm^{-1}) was produced in-house using a Mili-Q water purification system (Millipore, Molsheim, France). Uracil, 5-nitro-2-aminophenol, 4-nitrophenol, 3-nitrophenol, o-cresol, 2-nitrophenol, 4-bromphenol, 3,4-dimethylphenol, 4-bromo-2-nitrophenol, 4-bromo-2,6-xylenol, acetanilide, acetophenone, propiophenone, butyrophenone, benzophenone, valerophenone, hexanophenone, heptanophenone were purchased from Sigma–Aldrich (Steinheim, Germany). For each compound, a stock solution was prepared by dissolving 500 ppm in 70:30% (v/v) ACN:water. Sample mixtures were prepared by diluting the phenol stock solutions to 20 ppm in the desired mobile-phase composition. Separations were performed on 2.1 mm i.d. × 100 mm Accucore columns packed with 2.6 μm silica C_{18} core–shell particles (Thermo Fisher Scientific, Runcorn, UK).

2.2. Instrumentation and gradient HPLC conditions

HPLC experiments were performed using an Ultimate 3000 RSLC system (Thermo Fisher Scientific, Germering, Germany) composed of a degasser, a binary high-pressure pump, an autosampler equipped with a six-port injection valve containing a 1.2 μL injection loop, a thermostated column compartment, and a diode array detector with a 2.5 μL flow cell. NanoViper tubing (Thermo Fisher Scientific) was used to connect the column with injector and detector (inlet tubing 0.075 mm i.d. × 350 mm, outlet tubing 0.065 mm i.d. × 250 mm). The inlet capillary of the UV flow cell was replaced by 65 μm i.d. tubing. In this way, the extra-column volume after the column was reduced from 1.58 μL to only 0.48 μL. During the separation the column was maintained at adiabatic conditions (30 °C), by wrapping the columns in insulation material. Phenols were separated by applying linear aqueous acetonitrile gradients with starting composition of 20:80% (v/v) ACN:water and final composition of 70:30% (v/v) ACN:water. UV detection was performed at 280 nm using 50 Hz data collection rate and 0.1 s response time.

The column dead time (t_0) was determined by injecting uracil and applying a mobile-phase composition of 70:30% (v/v) ACN:water at the optimal Van-Deemter flow rate F = 0.3 mL/min. The external time and pressure contributions induced by the connection tubing were determined by injecting uracil and replacing the column with a zero-dead-volume union. The external contributions to the band broadening were determined by measuring the retention volumes of eight phenones at the optimal Van-Deemter flow rate F = 0.3 mL/min. The dwell time was determined using a step gradient while spiking the mobile phase with 0.1% (v/v) acetone and applying UV detection at 265 nm.

3. Results and discussion

3.1. Effect of flow rate and gradient steepness on kinetic performance

Peak capacity (n_c) is a metric for the kinetic performance, that expresses the maximum number of components that can be separated in the gradient window with unit resolution (R_s = 1), according to [24]:

$$n_c = 1 + \frac{t_G}{t_0} \cdot \frac{1}{R_s} \cdot \frac{\sqrt{L}}{4 \cdot (1 + k_e) \cdot \sqrt{H} \cdot G} \quad (1)$$

where t_G is the gradient time and t_0 the column dead time, defining the gradient steepness, L is the column length, k_e the retention factor at the point of elution, H the height equivalent to a theoretical plate [23], and G the peak-compression factor. H in gradient mode is a function of the location (L) inside the column according to [23,25,26]:

$$H = \frac{d\sigma_L^2}{dL} \quad (2)$$

and the corresponding peak variance (σ_L^2) is defined in Eq. (3) [23]:

$$\sigma_L^2 = \int_0^L H(L)dL \quad (3)$$

Similar like in isocratic elution, the magnitude of H in gradient mode is affected by the A, B, and C term contributions, and hence H depends on the diffusion coefficient and the retention factor experienced during the solvent gradient.

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