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Impact of the column hardware volume on resolution in very high pressure liquid chromatography non-invasive investigations

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ABSTRACT

The impact of the column hardware volume (≃1.7 μL) on the optimum reduced plate heights of a series of short 2.1 mm × 50 mm columns (hold-up volume ≃80–90 μL) packed with 1.8 μm HSS-T_3, 1.7 μm BEH-C_{18}, 1.7 μm CSH-C_{18}, 1.6 μm CORTECS-C_{18}+, and 1.7 μm BEH-C_4 particles was investigated. A rapid and non-invasive method based on the reduction of the system dispersion (to only 0.15 μL^2) of an I-class Acquity system and on the corrected plate heights (for system dispersion) of five weakly retained *n*-alkanophenones in RPLC was proposed. Evidence for sample dispersion through the column hardware volume was also revealed from the experimental plot of the peak capacities for smooth linear gradients *versus* the corrected efficiency of a weakly retained alkanophenone (isocratic runs). The plot is built for a constant gradient steepness irrespective of the applied flow rates (0.01–0.30 mL/min) and column lengths (2, 3, 5, and 10 cm). The volume variance caused by column endfittings and frits was estimated in between 0.1 and 0.7 μL^2 depending on the applied flow rate. After correction for system and hardware dispersion, the minimum reduced plate heights of short (5 cm) and narrow-bore (2.1 mm i.d.) beds packed with sub-2 μm fully and superficially porous particles were found close to 1.5 and 0.7, respectively, instead of the classical *h* values of 2.0 and 1.4 for the whole column assembly.

1. Introduction

Pharmaceutical, biological, and food industries have constantly pushed column and instrument manufacturers towards the design of faster separation systems while maintaining their resolution performance. These systems should satisfy the demand of a higher analysis throughput. Early theories of chromatography predicted that faster separations would require shorter columns packed with finer particles [1,2]. Concomitantly, chromatographic instruments would have to deliver flow rates at pressures three times higher than the conventional 400 bar limit. In practice, very high-pressure liquid chromatography (vHPLC) emerged in 2004 with systems operating up to 1 kbar at 1 mL/min [3] able to operate 5–10 cm long narrow-bore columns packed with sub-2 μm particles at their optimum velocity. Inevitably, the elution peak volumes along these columns became so small that the experimenter could not operate them at their full resolution power. This was due to the significant dispersion of the sample zone along the vHPLC instrument itself [4–10]. System dispersion had then to be minimized by redesigning the different parts of standard vHPLC systems: they include the injection device, the connecting tubes, the detection cell, any connection between these three parts [11–18].

A decade later, the most advanced vHPLC system cannot deliver volume variances smaller than 1.0 μL^2 at flow rates larger than 0.5 mL/min for small molecules [12]. This is still representing a significant amount of extra-column dispersion because the volume variance of a non-retained compound eluted through a 2.1 mm × 50 mm column (hold-up volume around 90 μL) packed with 1.6 μm core–shell particles is no larger than 0.4 μL^2 at optimum speed (assuming $h = 1.4$). In addition, both ends of the columns are equipped with endfittings (flow distributor volume of 0.15 μL) and porous frits (porosity 0.2 μm, 20% void, dead volume of 0.69 μL) which both contribute to increase the sample dispersion along the column. Assuming that these dead volumes behave as ideal mixers, the increment of the volume variance at each end of the column would be at most $0.15^2 + 0.69^2 = 0.5\ \mu L^2$, which would be comparable or even larger than the volume variance due to the sole column. So far, it is commonly accepted that the minimum reduced plate heights of columns packed with fully porous and core–shell particles are around 2.0 and 1.4, respectively [19]. These values are based on the measurements performed either directly on large volume columns (4.6 mm i.d.) or on small volume columns (2.1 mm i.d.) after correction for system dispersion. They are necessarily overestimating the true minimum reduced

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plate heights of the sole packed bed present in between the two frits. Strategies are then needed to determine quantitatively the amount of dispersion caused by the endfitting/frit assembly in order to potentially optimize its current design for minimization of the total sample dispersion. In contrast to the radial heterogeneity of the packed bed [20–26] and the practical solutions (such as curtain and active flow technologies [27–33]) used to minimize the impact of the flow unevenness across the column diameter on the column efficiency, to the best of our knowledge, no detailed investigations have yet been reported on the impact of the dead volume of endfitting/frit assemblies on the observed sample dispersion along a chromatographic column. This potential source of sample dispersion has systematically been neglected in the field, yet, it could be highly relevant in ultrafast vHPLC, in comprehensive two-dimensional HPLC requiring a fast second dimension, and in any applications using short trap columns to increase sample sensitivity.

The goal of this work is to propose a simple experimental approach that will assess quantitatively the volume dispersion caused by the dead volumes of distributors and frits in modern chromatographic columns. Short (5 and 10 cm) and narrow-bore (2.1 mm i.d.) columns packed with sub-2 μm particles (1.8 μm HSS-T_3, 1.7 μm BEH-C_{18}, 1.7 μm CSH-C_{18}, 1.6 μm CORTECS-C_{18}+, and 1.7 μm BEH-C_4) designed for fast HPLC analyses are used in this study. These columns are intrinsically very efficient, so, it is most likely that the endfittings and porous frits should affect to some unknown extent their performance at optimum linear velocity. The corresponding loss of efficiency is also investigated from the analysis of the plot of peak capacities *versus* the observed efficiency using a series of 2, 3, 5, and 10 cm long columns packed with 1.8 μm HSS-T_3 particles. Finally, this work enables to assess the actual minimum reduced plate heights of beds packed in 2.1 mm × 50 mm columns with sub-2 μm fully porous and core–shell particles.

2. Theory

In this work, three different column efficiencies (or plate heights) are defined: (1) the observed column efficiency, N_{obs}, which is directly measured by the experimenter, (2) the apparent column efficiency, N_{app}, measured after correction for the dispersion caused by the vHPLC instrument, and (3) the intrinsic column efficiency, N_{int}, measured after correction for the contributions of both the instrument and the column distributors and frits to the total band spreading.

2.1. Observed, apparent, and intrinsic plate heights

The total volume variance, $\sigma^2_{v,total}$, of a sample zone percolating through the injection device, the packed column, and the detection cell is the sum of three distinct variances: (1) the system dispersion, $\sigma^2_{v,syst.}$, along the system volume V_{syst} (sample loop, injection valve, connecting tubes, and detection cell), (2) the sample dispersion, $\sigma^2_{v,ends}$, across the dead volume, V_{ends}, of the porous frits and endfittings present at both ends of the column, and (3) the sample dispersion along the packed bed of hold-up volume V_0.

By definition, the observed and apparent plate heights, H_{obs} and H_{app}, are written:

$$H_{obs} = L\frac{\sigma^2_{v,total}}{V_R^2} \tag{1}$$

$$= \frac{V_0^2 H_{\rm int}(1+k')^2}{(V_0[1+k']+V_{syst}+V_{ends})^2} + L\frac{\sigma^2_{v,syst.}+\sigma^2_{v,ends}}{(V_0[1+k']+V_{syst}+V_{ends})^2} \tag{2}$$

and

$$H_{app} = L\frac{\sigma^2_{v,total}-\sigma^2_{v,syst.}}{V_R^2} \tag{3}$$

$$= \frac{V_0^2 H_{\rm int}(1+k')^2}{(V_0[1+k']+V_{syst}+V_{ends})^2} + \frac{L\sigma^2_{v,ends}}{(V_0[1+k']+V_{syst}+V_{ends})^2} \tag{4}$$

where H_{int} is the intrinsic plate height of the packed bed and k' is the retention factor of the analyte.

Two extreme case scenarios can be distinguished:

(1) The column hold-up volume is much larger than the sum of the system and endfittings volumes ($V_0 \gg V_{syst}+V_{ends}$) and the column is packed with large particles. Then, $V_0^2 H_{\rm int} \gg L\sigma^2_{v,ends}$ and the contributions of the HPLC system and column endfittings to the observed efficiency are negligible. Therefore, the observed efficiency is close to the intrinsic one and

$$H_{obs} \simeq H_{\rm int} \tag{5}$$

(2) The column dimensions are small, the bed is packed with fine particles, and modern very-high pressure liquid chromatographs are used. Then, the term $V_0^2 H_{\rm int}$ becomes comparable or even smaller than $L\sigma^2_{v,ends}$ while, still, $V_0 \gg V_{syst}+V_{ends}$. Since $V_0 = \epsilon_t \pi r_c^2 L$, where r_c is the inner diameter of the column and ϵ_t is the total porosity of the column, Eq. (3) can be rewritten in a simpler form and the apparent efficiency is given by:

$$H_{app} \simeq H_{\rm int} + \frac{\sigma^2_{v,ends}}{L\epsilon_t^2\pi^2 r_c^4[1+k']^2} \tag{6}$$

Note that Eq. (5) would rarely holds for a non-retained compound ($k'=0$), a standard 4.6 mm × 250 mm bed ($V_0 \simeq 2.5$ mL, total porosity $\epsilon_t = 0.6$) packed with 5 μm particles ($H_{int} = 10$ μm) in between two 4.6 mm × 1 mm frits (porosity 40%, $V_{ends} \simeq 13$ μL), 2.0 μL cone distributor volume, and for a standard liquid chromatograph ($V_{syst} \simeq 50$ μL). Assuming that the distributor and frit assembly behave as ideal mixers, then $L\sigma^2_{v,ends} \simeq 0.0024\,\text{cm}^7$, which is only three times smaller than $V_0^2 H_{\rm int} \simeq 0.0063\,\text{cm}^7$.

Eq. (6) describes quantitatively the impact of dispersion along distributors and porous frits on the apparent efficiency measured after correction for the system dispersion. Consider a 2.1 mm × 50 mm column ($V_0 \simeq 90$ μL, total porosity $\epsilon_t = 0.52$) packed with 1.7 μm fully porous particles ($H_{int} = 3.4$ μm) in between two 2.1 mm × 1 mm frits (porosity 20%, $V_{ends} \simeq 2 \times 0.7 = 1.4$ μL), a total distributor volume of 0.3 μL, and a standard very high pressure liquid chromatograph ($V_{syst} \simeq 5$ μL). Again, assuming that the distributor and the frit behave as ideal mixers, $L\sigma^2_{v,ends} \simeq 5.8 \times 10^{-6}\,\text{cm}^7$, while $V_0^2 H_{\rm int} \simeq 2.8 \times 10^{-6}\,\text{cm}^7$. In other words, the efficiency of the column becomes seriously affected by the sample dispersion through the volume of the column hardware.

More generally, the volume dispersion through flow distributors and frits become larger than the volume dispersion through the packed bed for a non-retained compound when the product of the column length, L, by the particle diameter, d_p, is smaller than a critical value:

$$Ld_p < \frac{\sigma^2_{v,ends}}{\epsilon_t^2\pi^2 r_c^4 h_{\rm int}} \tag{7}$$

Eq. (7) insists on the importance of minimizing $\sigma^2_{v,ends}$ for short columns packed with fine particles.