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Possibilities of new generation columns packed with 1.3 μm core–shell particles in gradient elution mode

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ABSTRACT

The aim of this work was to evaluate the practical possibilities in gradient elution mode of a column packed with 1.3 μm core–shell particles recently released on the market. For this purpose, two types of analytes possessing different diffusion coefficients were selected (small molecule and peptide). It appears that the new 1.3 μm material was particularly well suited for fast separations, compared to other existing core–shell particle dimensions in gradient mode. The new material systematically outperforms the other existing ones for peak capacity up to 300 for small molecules and 700 (corresponding to $t_0 = 15$ min) for peptides. Based on these cut-off values, the advantage of column packed with 1.3 μm was much more obvious for peptides vs. small molecules analysis. Further improvements in terms of column mechanical stability and system upper pressure capability could expand the limits of separation speed and efficiency to a different level. Again, because of the current pressure limitation and low permeability, a column length of more than 5–8 cm is never desired for small molecules analysis in gradient elution. On the contrary, longer columns were useful for peptide analysis. As example, a column of 28 cm packed with 1.3 μm particles provides a peak capacity of 1000 in the case of peptides analysis. All the predicted values were experimentally confirmed using a standardized extract of *Ginkgo biloba* and a tryptic digest of a monoclonal antibody (Panitumumab). For the plant extract, the better performance was always achieved with a 5 cm long column ($P = 267$ and 268 for the 5 and 15 cm, respectively, using a gradient time of 10 and 40 min, respectively). Finally, in the case of peptide mapping, a 15 cm long column packed with 1.3 μm particles was the best choice ($P = 176$ and 311 for the 5 and 15 cm, respectively, using a gradient time of 10 and 40 min, respectively).

1. Introduction

Since their introduction on the market in 2007, interest for chromatographic columns packed with sub-3 μm core–shell (superficially porous, fused-core) particles has constantly increased [1]. Today, most industry and academic analysts used and studied these new columns, with the goal to improve current separation methods that were developed on columns packed with classical fully porous particles [2–10].

Numerous vendors proposed core–shell particles, presenting a size between 2.6 and 2.7 μm (the so-called sub-3 μm core–shell particles). Sub-2 μm core–shell particles (1.7 μm) were first commercialized in 2009 [11,12]. Several wide-pore 2.7 or 3.6 μm particles were also proposed for the analysis of large biomolecules [8,9,13–15]. Currently columns packed with 5 μm core–shell particles were also released to meet the requirements of pharmaceutical industry and pharmacopeias [1,16,17].

The kinetic efficiency of columns packed with core–shell particles is improved when the porous shell thickness is decreased, especially for compounds having low diffusion (high molecular weight compounds). However, the optimum shell thickness is likely to be a compromise between efficiency, sample loading capacity and analyte retention. Now, it seems that the structure of the last generation of shell-particles is very close to its optimum. Indeed, using a relatively thick shell (e.g. between 0.20 and 0.50 μm), the low loading capacity which hindered the success of earlier generation particles produced by Horváth and Kirkland in the late 1960s [18–20], was largely overcome [21]. The so called "core–shell advantage" lies mostly in the favorable eddy dispersion and longitudinal diffusion, when separating low molecular weight compounds.

According to several experimental studies, the eddy dispersion contribution (A-term of van Deemter equation) of core–shell particles is significantly reduced (∼30–40%), compared to fully porous particles [2,10,22–26]. Core–shell particles have a higher density and are rougher than fully porous particles [2,10]. This might

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have an influence on the packing quality and could explain the improvement of A-term [22–26]. On the other hand, the particle size distribution is also narrower for core–shell particles than for fully porous ones. The presence of a solid core inside the particles also has a direct consequence on the longitudinal diffusion (B-term of van Deemter equation), since its contribution to the plate height is decreased by about 20%, when the ratio of the core to the particle diameter is equal to $\rho = 0.63$ (Halo, Ascentis Express, Poroshell) and about 30% when $\rho = 0.73$ (Kinetex) [2,10]. To conclude on the efficiency of core–shell particles, the success of these materials in the separation of small molecules is not primarily a result of the decrease in the mass transfer resistance (C-term). Most importantly, the exceptional performance of these phases is caused by the significant reduction of the eddy dispersion term [26].

Recently columns packed with 1.3 μm core–shell particles were released to further improve the kinetic efficiency [27,28]. Theoretically, the fastest separations could be obtained with the smallest particle size [29]. Indeed, particle size affects both the eddy dispersion and mass transfer resistance. As demonstrated in early works, small particle diameters induce an increase in efficiency, optimal velocity and mass transfer [30–34]. This is probably the reason why very fine (1.3 μm) core–shell material was recently launched, allowing very high plate counts and fast separations.

The aim of this work was to perform a critical evaluation of the practical possibilities and limitations of this new 1.3 μm core–shell material in gradient elution mode. It was recently showed that when separating small analytes (MW ~200 g/mol), it is difficult to reach the optimal flow rate, due to the (1) low permeability of 1.3 μm packing and (2) high molecular diffusivity of small solutes. In several cases, these 1.3 μm columns can be operated only in the B-term dominated region [28]. Therefore, in this study we focused on both small solutes and peptides (~1.3 kDa). For peptides, probably this column would be more advantageous due to their lower diffusivity. Gradient kinetic plots were predicted and the possible analysis time and optimal column length for small analytes and peptides are discussed for 1.3, 1.7, 2.6 and 5 μm core–shell packings. Practical real-life separations were also performed to verify the predictions. Metabolites of *Ginkgo biloba* extracts and peptides coming from recombinant monoclonal antibody tryptic digest were separated under several gradient conditions, using 5, 10 and 15 cm long columns.

2. Experimental

2.1. Chemicals, columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Acetonitrile, was purchased from Sigma–Aldrich (Buchs, Switzerland). Test analytes such as uracil, methylparaben, ethylparaben, propylparaben and butylparaben, were purchased from Sigma–Aldrich. Model decapeptide such as CH-870 (1295.5 g/mol) was purchased from ChinaPeptides Co. Ltd. (Shanghai, China). This model peptide is an analog of the commercial therapeutic peptide, triptorelin in which only one amino acid was altered.

Standardized *G. biloba* extract to meet pharmacopeia requirements from Indena (Milan, Italy) was a generous gift from Prof. Jean-Luc Wolfender, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Switzerland.

Commercially available IgG1 and IgG2 monoclonal antibodies (Rituximab and Panitumumab) were purchased from Roche and Amgen (Switzerland). Dithiothreitol (DTT), trypsin (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated, from bovine pancreas), trifluoroacetic acid (TFA), formic acid (FA) and ammonium hydrogen-carbonate were all obtained from Sigma–Aldrich (Switzerland).

Kinetex 5 μm XB-C18 (150 mm × 4.6 mm), Kinetex 2.6 μm and 1.7 μm C18 (50 mm × 2.1 mm) columns and the new Kinetex 1.3 μm columns (50 mm × 2.1 mm) were generous gifts from Phenomenex (Torrance, CA, USA).

2.2. Equipment, software

The measurements on 50 mm × 2.1 mm columns were performed using a Waters Acquity UPLC^{TM} I-Class system equipped with a binary solvent delivery pump (maximum flow rate of 2 mL/min and upper pressure limit of 1200 bar), an autosampler and UV detector. The system includes a flow through needle (FTN) injection system with 15 μL needle and a 0.5 μL UV flow-cell. The connection tube between injector and column inlet was 0.003″ I.D. and 200 mm long (active preheater included), while the capillary located between the column and detector was 0.004″ I.D. and 200 mm long. The overall extra-column volume (V_{ext}) was about 8.5 μL as measured from the injection seat of the auto-sampler to the detector cell. The average extra-column peak variance of our system was found to be around σ^2_{ec}~0.5–2.5 μL^2 (depending on the flow rate, injected volume, mobile phase composition and solute). The extra-column peak variance was not negligible, when using these very efficient narrow-bore columns, therefore plate counts were corrected from extra-column variance to estimate the “true” column efficiency. Data acquisition and instrument control was performed by Empower Pro 2 Software (Waters).

For the 150 mm × 4.6 mm column, experiments were performed on a Waters Breeze^{TM} HPLC system equipped with a binary solvent delivery pump (module 1525), an autosampler (module 2707) and UV detector (module 2489). Standard bore columns require flow rate higher than 2 mL/min for efficiency estimation, therefore a conventional system was employed for these measurements. This system enables mobile phase flow rate up to 10 mL/min. It includes an injection system with 100 μL loop and a 10 μL standard UV flow-cell. All the parts were connected with relatively long 0.005″ I.D. tubing (because external column oven was applied). Despite the fact that the extra-column variance of the system was significantly larger (σ^2_{ec}~50–100 μL^2) than for the UHPLC system, it was found to be negligible with the 150 mm × 4.6 mm, 5 μm Kinetex column (for retained analytes, e.g. $k > 3$), due to the large column volume.

Calculation and data transferring were achieved by using a home made MS Excel template. Curve fitting and chromatogram re-plotting were performed using OriginPro 8 SRO (OriginLab Corporation).

2.3. Apparatus and methodology

2.3.1. Peak variances, extra-column effects

Band profiles were recorded in isocratic elution mode for a series of flow rates. Achievable plate heights were calculated for low molecular weight analyte (butylparaben, 194.2 g/mol) and also for a decapeptide (CH-870, 1295.5 g/mol).

The extra-column variances were calculated according to the moment method. The first (μ_1) and the second (μ_2) central moments of the band profiles were determined by numerical integration of the full band profiles [35,36]:

$$\mu_1 = \frac{\sum_{i=1}^{n-1}(C_i + C_{i+1})(t_i + t_{i+1})}{2\sum_{i=1}^{n-1}(C_i + C_{i+1})} \quad (1)$$

$$\mu_2 = \frac{\sum_{i=1}^{n-1}(C_i + C_{i+1})(((t_i + t_{i+1})/2) - \mu_1)^2}{\sum_{i=1}^{n-1}(C_i + C_{i+1})} = \sigma^2_{ec} \quad (2)$$