HUNGARIAN JOURNAL
OF INDUSTRY AND CHEMISTRY
VESZPRÉM
Vol. 42(1) pp. 25-29 (2014)

IMPACT OF SIZE HETEROGENEITY OF CORE-SHELL PACKING MATERIALS ON CHROMATOGRAPHIC SEPARATION OF LARGE BIOMOLECULES

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The effect of particle size heterogeneity of core-shell stationary phases on the efficiency of chromatographic separation of large biomolecules was studied. It was shown that the column efficiency was affected significantly by the breadth of particle size distribution. The chromatographic efficiency decreased as the heterogeneity of particle sizes increased. Although the absolute decrease of separation efficiency was affected by the linear velocity, u, of the eluent, the relative change of HETP was independent of u in the practical range of eluent velocities. The results showed that the affect of particle size distribution was the highest in case of fully porous phases, and it decreased as the diameter of the inner core decreased. It was shown that, in the usual range of particle size heterogeneity of core-shell phase, the peak capacities did not change significantly even at high eluent velocities.

Keywords: high performance liquid chromatography, particle size heterogeneity, separation efficiency, core-shell phases

Introduction

Modern analytical applications of liquid chromatography require efficient stationary phases. Before the introduction of core-shell particles – particles with a porous layer surrounding a solid core (*Fig. 1*) – the use of monolithic silica rods [1, 2], or of sub-2 μm particles offered today the most satisfactory results. Due to their excessive radial heterogeneity, however, the efficiency of monolithic columns currently is lower than that of sub-2 μm particles. Columns packed with fine particles, however, have a low permeability [5–7]. Thus, efficiency of columns packed with core-shell particles similar to the columns packed with sub-2 μm particles, but can be operated with the same instruments as those used for conventional columns. The use of core-shell phases is particularly advantageous in the separation of large biomolecules [8]. Recently, core-shell phases were optimized for the separation of large biomolecules [9, 10]. Besides their unique structure, the high efficiency of core-shell phases is in part due to the very narrow particle size distribution, with a relative standard deviation around 5–10% vs. 20–40% for most totally porous particles [7].

In literature, fairly contradictory information can be found about the effect of particle size distribution, PSD, on the separation efficiency. Some results suggest that the large particle size variance has no influence on the column efficiency, while according to other data the wide PSD decreases the efficiency of chromatographic separation. Halász and Naefe [11] were the first ones who examined the effect of particle size distribution

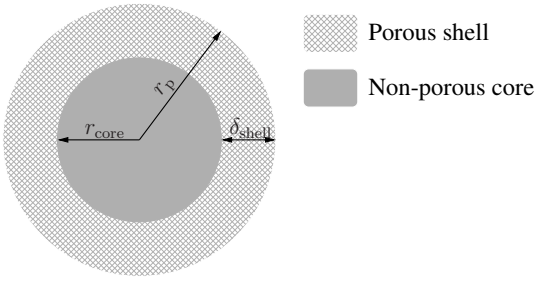

Figure 1: Structure of core-shell particles

on the column efficiency in case of particles that were larger than 50 μm. In their work, no experimental result was found for the change of chromatographic efficiency even in case of wide PSDs ($\sim$ 40%). Two years after Halász's work, significantly smaller, 1-10 μm, particles were tested by Endele *et al.* [12]. No change in the permeability and in the chromatographic efficiency were observed as long as the average particle size remained the same. Dewaele and Verzele prepared columns packed with mixtures of different compositions of reversed phase particles of 3 and 8 μm and investigated the effect of the PSD on the column efficiency [13] with similar results.

Billen *et al.* found that the breadth of the distribution of the stationary phase particles has no effect on the chromatographic efficiency as long as no fines are present. In the presence of fines, the efficiency decreases significantly [14]. They emphasized that

different conclusions can be drawn about the goodness of a chromatographic column depending on the definition used for the particle size distribution.

The effect of the PSD on the chromatographic efficiency of sub-2 μm particles was studied by Cabooter *et al.* by analyzing kinetic plots of these phases [15]. In their work, they showed that not just the PSD is important but also the separation efficiency one wants to achieve. It was concluded that the greater the achievable number of theoretical plates, the more the PSD affects the separation efficiency. In their experiments, the authors obtained better results by using columns with a narrower PSD than by using columns of the same dimensions but packed with particles with broader PSD.

Recently, Gritti *et al.* found that the chromatographic efficiency of the columns can be optimized if a small amount of greater particles is added to the sub-3 μm particles which leads to better bead homogeneity [16]. Manufacturers practice matches these, because on their confession they add a small amount of larger particles to the bathes to optimize the pressure drop along the column.

Experimental study of the effect of PSD on chromatographic efficiency is rather complex, because the column packing procedure is hardly reproducible. It is particularly true when we work with stationary phases which have different PSDs. The quality of column packing also affects the column efficiency significantly. Theoretical models can provide more reliable data on this field because it does not affected by the variability of column packing. The goal of this work is to study the effect of particle size heterogeneity on the chromatographic efficiency of separation of large biomolecules on core-shell stationary phases on a theoretical basis.

Theory

In HPLC, one of the measure of separation efficiency is the height equivalent to a theoretical plate, HETP [17]. The higher the plate height, the less efficient the separation is. The general rate model of chromatography [18] permits the calculation of HETP of columns packed by core-shell phases [8]:

$$H(d_\mathrm{p}) = \frac{2D_\mathrm{L}}{u} + \frac{k_1^2}{1+k_1^2}\left(\frac{\Omega\, u\, d_\mathrm{p}^2}{30FD_\mathrm{p}} + \frac{u\, d_\mathrm{p}}{3Fk_\mathrm{f}}\right), \quad (1)$$

where d_p is the diameter of particles of packing material, u the linear velocity of the eluent in the interstitial volume, k_1 the interstitial retention factor, F the phase ratio, D_L and D_p the axial and pores diffusion coefficients, respectively, and k_f the external mass transfer coefficient. Ω is given by the relationship

$$\Omega = (1-\rho)\,\frac{1+3\rho+6\rho^2+5\rho^3}{\left(1+\rho+\rho^2\right)^2}, \quad (2)$$

where ρ represents the ratio of the radius of the inner solid core, r_core, to the radius of the particle, r_p (see *Fig. 1*):

$$\rho = \frac{r_\mathrm{core}}{r_\mathrm{p}}. \quad (3)$$

Accordingly, ρ is zero for fully porous particle and one for non-porous particle.

For a core-shell particle, the interstitial retention factor is

$$k_1 = \frac{(1-\varepsilon_\mathrm{e})\,(1-\rho^3)}{\varepsilon_\mathrm{e}}\left[K_\mathrm{a}(1-\varepsilon_\mathrm{p})+\varepsilon_\mathrm{p}\right], \quad (4)$$

where ε_e is the external bed porosity, ε_p is the porosity of the porous shell, and K_a is the adsorption equilibrium constant (Henry constant).

The particle size distribution is usually described by log-normal distribution [19]. The PSD of a packing material with mean μ and variance σ^2 is

$$f_{d_\mathrm{p}} = \frac{1}{d_\mathrm{p}\sqrt{2\pi\theta}}\exp\left(-\frac{\left(\ln\frac{d_\mathrm{p}}{\mu}+\frac{1}{2}\theta\right)^2}{2\theta}\right), \quad (5)$$

where

$$\theta = \ln\left(\frac{\sigma^2}{\mu^2}+1\right). \quad (6)$$

On the basis of the equations above, the probability density function of local HETPs, f_H, can be derived by applying the change-of-variables rule [20]:

$$f_H = \left|\frac{\mathrm{d}}{\mathrm{d}H}d_\mathrm{p}(H)\right| f_{r_\mathrm{p}}\left(d_\mathrm{p}(H)\right), \quad (7)$$

where $d_\mathrm{p}(H)$ is the inverse function of $H(d_\mathrm{p})$ (see *Eq. (1)*).

The observable HETP of the column is the first moment of *Eq. (7)*:

$$H_\mathrm{col} = \int_0^\infty H\, f_H\, \mathrm{d}H. \quad (8)$$

Methods of Calculations

For the calculation of H_col (*Eq. (8)*) a software written in C++ language, using the adaptive quadrature routine provided by GNU Scientific Library (GSL, v. 1.15) [21]. The integration region was divided into subintervals, and on each iteration the subinterval with the largest estimated error was bisected. As a result, the overall error reduced rapidly, as the subintervals became concentrated around local difficulties in the integrand. These subintervals were managed by the GSL library, which handled the memory for the subinterval ranges, results and error estimates too. The relative error of integration was set to 10^{-10}. The source code of the program was compiled by g++ shipped by GNU

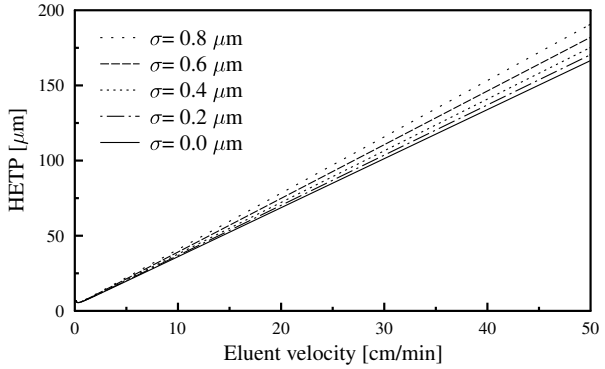

Figure 2: Van Deemter plots of large biomolecules in case of different standard deviations of particle size distributions of 2.6 μm core-shell stationary phases

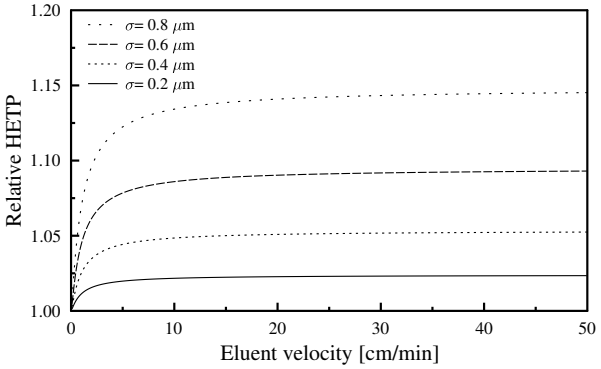

Figure 3: Relative van Deemter plots of large biomolecules in case of different standard deviations of particle size distributions of 2.6 μm core-shell stationary phases

Compiler Collection ver. 4.7.2 using O1 optimization. The calculations were performed on a Pentium IV computer (2.80 GHz) running GNU Linux operating system (Debian Wheezy).

The function f_H (*Eq. (7)*) for the numerical integration was derived symbolically by Mathematica 8.0 (Wolfram Research, Inc.). H (*Eq. (1)*) was calculated as it is described in Ref. [8]. The internal (ε_p) and external column porosities (ε_e), were 0.05 and 0.4, respectively. The distribution coefficient of the compounds were set to 3. The ratio of the molecule size and the average pore diameter were assumed to be 0.68. The molecular diffusivity was 2.5×10^{-5} cm^2/min. The value of "eddy" diffusion term, the internal and external obstruction factors were 1.3, 0.31 and 0.6, respectively. The linear velocity of the eluent was varied between 0.1 and 50 cm/min. The average pore size was 80 Å. It was assumed that the quality of the column packing remained identical in all cases.

Results and Discussion

In *Fig. 2* the calculated van-Deemter curves of large biomolecules separated on 2.6 μm core-shell phases can be seen. The ratio of the radius of the inner solid core to the radius of the particle, ρ, is 0.7 representing the typical core size of commercial core-shell phases. The standard deviation of particle size distribution is varied between 0.0–0.8 μm that corresponds to 0–30% relative standard deviation. Close examination of *Fig. 2* highlights that the HETP values of large biomolecules are approximately directly proportional to the eluent velocity.

Fig. 2 shows that the heterogeneity of particle sizes has a significant effect on the separation efficiency of large biomolecules. The separation efficiency is the highest when the variance of particle size distribution is zero (solid black line). Increasing breadth of particle size distribution decreases the efficiency of separation. The decrease in the efficiency is more significant at higher eluent velocities. At 10 cm/min, the difference between the two extremes ($\sigma = 0$ and $\sigma = 0.8$) is less than 5 μm that is approximately 15% of the total plate height at this eluent velocity in ideal case ($\sigma = 0$). In case of 30, 40, and 50 cm/min the differences are significantly larger: $\sim$15, $\sim$20, and $\sim$25 μm, respectively. Note, however, that the relative increase is $\sim$15% in each case suggesting that the relative change of HETP is independent from the eluent velocity.

In *Fig. 3* the relative HETP, H_rel, values of large biomolecules can be seen as a function of eluent velocity. H_rel were calculated as

$$H_\mathrm{rel} = \frac{H_\sigma}{H_0}, \tag{9}$$

where H_σ and H_0 are HETP values at σ^2 and 0 variance of PSD.

In *Fig. 3*, it can be seen that raising eluent velocity increases the relative HETP. Above $\sim$8 cm/min, however, a plateau is reached and the change of relative HETP becomes negligible. It suggests that, at low eluent velocities, the interstitial dispersion is the governing effect in band dispersion. As the velocity of eluent increases, the pore diffusion becomes more and more significant. Accordingly, in practice ($u > 10$ cm/min), it is the dominant effect in band dispersion.

Fig. 4 shows the effect of core size on the chromatographic efficiency at different breadth of core-shell particle distribution. The linear velocity of the eluent was 15 cm/min that is a typical value in chromatographic applications. It can be seen that, as expected from *Eq. (1)*, the efficiency of stationary phase increases as the thickness of porous layer decreases. The effect of width of particle size distribution is less significant at larger ρ values. While in case of fully porous particles, the difference is almost 17 μm between the HETP values of the two extremes ($\sigma = 0$ and $\sigma = 0.8$), it is slightly more than 4 μm at $\rho = 0.8$.

The relative HETPs calculated as in case of *Fig. 3* do not change significantly with the diameter of non-porous core until $\rho = 0.8$ (*Fig. 5*). Above that value,

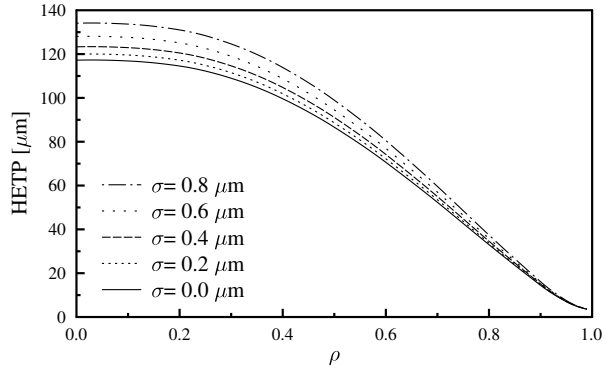

Figure 4: HETP of columns packed with 2.6 μm core-shell particles

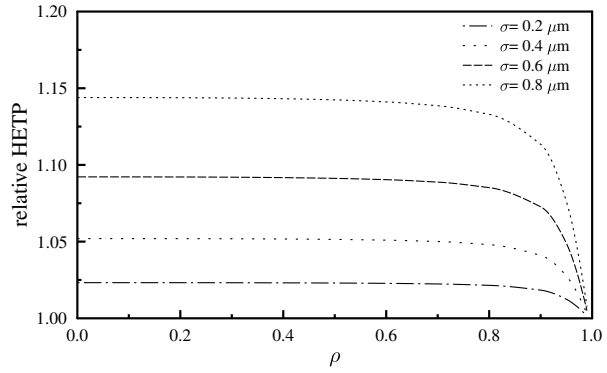

Figure 5: Relative HETPs of columns packed with 2.6 μm core-shell particles

H_{rel}s decrease abruptly, at $\rho = 1$ there is no difference between the cases. It can be explained by considering that the separation efficiency of large biomolecules is affected mainly by the pore diffusion (second term of *Eq. (1)*). As the thickness of porous layer of core-shell particles decrease, the diffusion paths reduce as well. As a result, the impact of pore diffusion on band broadening is less and less significant. The difference between the distinct particles vanishes. Finally, at $\rho = 1$, only the interstitial band spreading (first term of *Eq. (1)*) influences the efficiency of separation. Since D_{L} is not affected by the particle size distribution, H_{rel}s become unity.

Besides the plate heights, the change of peak capacities were also studied. Peak capacity, n_{c}, is a practical measure of separation potential in HPLC. n_{c} is defined as the maximum number of components that can be resolved completely between the peaks of the least and most retained solutes [22]. Several expressions exist for the calculation of peak capacity [23] depending on the mode of chromatography. In case of isocratic mode of separation, n_c can be calculated as

$$n_c = \frac{1}{4}\sqrt{N}\,(t_{\max} - t_{\min}) \ln \frac{t_{\max}}{t_{\min}}, \qquad (10)$$

where $t_{\max}$ and $t_{\min}$ are the retention times of the first and last eluting peaks, and N the number of theoretical plates ($N = L/H$).

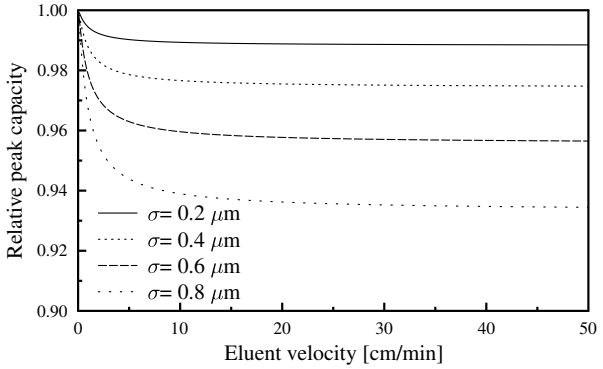

Figure 6: Relative peak capacities of columns packed by 2.6 μm core-shell particles; $L = 10\,\mathrm{cm}$, $t_0 = 10/9$, $t_{\max} = 100/9$

In *Fig. 6* the peak capacities of 2.6 μm core-shell phases can be seen as a function of eluent velocity in case of separation of large biomolecules. The calculated peak capacities were normalized for value that belongs to zero variance of PSD. The figure shows that the achievable peak capacity of a column packed with core-shell particles is affected by the heterogeneity of particle sizes. The decrease of peak capacities, however, is significantly smaller than the decrease of column efficiencies (*Fig. 2*). That is because n_{c} is proportional to the reciprocal square root of H. Accordingly, 15% increase in the plate height results in ∼7% loss of peak capacity that is not significant in practice.

Conclusions

Since their introduction, core-shell stationary phases became very popular for the analysis of large biomolecules. The morphology of these phases results in less band broadening compared to fully porous particles and thus delivers extremely high efficiencies. In this work, the effect of particle size heterogeneity of core-shell stationary phases on the efficiency of chromatographic separation of large biomolecules was studied. The results showed that slopes of van Deemter curves were affected by the breadth of particle size distribution. As a result of widening PSD the column efficiencies decreased significantly. The analysis of peak capacities showed that the maximum number of large biomolecules that can be resolved by core-shell phases was not affected significantly, even if the PSD was wide. Considering that the relative standard deviations of PDSs of core-shell phases are between 0.2–0.4 μm for 2.6 μm particles, it can be concluded that the efficiency of these phases is affected significantly by the size heterogeneity of the particles. Further efforts from column manufacturers in order to improve the PSD do not give any more advantages and are not profitable.

Acknowledgements

This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/2-11/1-2012-0001 'National Excellence Program'. The research infrastructure was supported by the Hungarian Scientific Research Fund (OTKA PD104819) and the Hungarian State and the European Union under the TÁMOP-4.2.2.A-11/1/KONV-2012-0071 project.

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