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Peak capacity of columns for size-exclusion chromatography*

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ABSTRACT

The practical peak capacity of columns for size-exclusion chromatography (SEC) is related to the pore fraction (V_p/V_t), plate number (N) and resolution (R_s) and may be estimated from $n_{SEC} \approx 1 + V_p/V_t) N^{1/2}/(4R_s)$. This equation yields a value of 80–90% of the maximum peak capacity, in contrast to earlier proposed equations which yield a large overestimate of the peak capacity. The practical peak capacities of columns packed with microparticulate materials is *ca.* 13 for completely resolved peaks, which is roughly equal to the peak capacity of columns traditionally used for SEC, the reason being the small pore volume of rigid materials and the short column lengths used. However, the maximum peak capacity is obtained with a considerably shorter separation time.

INTRODUCTION

In size-exclusion chromatography (SEC) (also known as gel filtration for aqueous system and gel permeation chromatography for non-aqueous systems), solutes are, ideally, eluted strictly according to decreasing size. This is due to the increasing proportion of the pore volume that becomes sterically available to successively smaller molecules with an increased retention time as a result. The maximum effective separation volume is thus restricted by the total pore volume of the packing material used. This limits the theoretical peak capacity of columns for SEC to a fraction of that for adsorptive chromatographic techniques for which solutes may be selectively retained and eluted after several column volumes of effluent [1]. As the peak-to-peak distance in SEC is limited, minimizing the peak widths becomes an important factor for achieving high peak capacities. Peak widths can be kept small through the use of columns of high efficiency and the following simple equation has been proposed for the calculation of the peak capacity (n) from the column plate number (N) (see, *e.g.*, refs. 2–4):

$$n \approx 1 + 0.2N^{1/2} \quad (1)$$

This relationship implies that peak capacities of 23–33 should be common for columns packed with microparticulate materials with $N = 12\,500$–$25\,000$. However, such peak capacities have so far not been realized in practice. This is due to the fact that eqn. 1 is an oversimplification and, further, it does not accurately account for variations in the pore volume. Chang *et al.* [5] illustrated the effect of pore volume on resolution in SEC and concluded that 2.6 times as many plates were required for Glycophase G/CPG for obtaining a resolution factor comparable to that for Sephadex G-200, owing to the small pore volume of the former material.

The complete equation for peak capacity (the maximum number of peaks separated on a given column) in gel filtration as described by Giddings [1] is

$$n = 1 + \ln(V_t/V_0)N^{1/2}/m \quad (2)$$

where V_t is the total liquid volume, V_0 is the void

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volume of the column and m is the spacing between peaks in terms of the standard deviation of solutes within a peak. The equation was derived under the assumption that the plate number is constant over the separation range. However, this assumption seems not to be valid as variations of N have been found to be considerable whereas variations in peak widths are small for various solutes [6–9]. From a study of the broadening effect in the SEC of polyethylene by Tung *et al.* [6], it may be calculated that the peak widths varied by 2.6 times and the plate numbers by 28.7 times over the separation range. Hagel and Andersson [7] presented data on proteins from which variations in peak widths of 2 times and in plate counts of 13 times may be inferred. Basedow and co-workers [8,9] found that the variation in peak widths of narrow dextran fractions was almost negligible when the solutes were chromatographed at low flow-rates.

Horváth and Lipsky [10] derived an equation for peak capacity in gradient elution, where all peaks have the same peak width. The equation was generalized by Grushka [11] to

$$n = 1 + N^{1/2}/4[(V_p/V_t) - 1] \quad (3)$$

where N is the plate number of the first peak and V_p is the pore volume of the material. This equation should also be applicable to the calculation of peak capacities of columns for SEC provided that the plate number of the first peak, *i.e.*, the void peak, is readily attainable. Unfortunately, in practice, this is seldom the case.

In a recent study of the separation characteristics of gel filtration by simulations it was found that the equations suggested (*i.e.*, eqns. 1 and 2) were not in accordance with the expected peak capacities [12]. Further, published chromatograms indicates peak capacities of *ca.* 7–8 also for columns packed with microparticulate materials [3,13–17], which shows that the simple dependence of merely plate number as expressed by eqn. 1, is erroneous. Thus, for estimations of the inherent performance of different media–column combinations, it is necessary to find a more accurate equation for expressing the peak capacity in SEC.

THEORETICAL

The zone broadening of solutes in SEC can be calculated from the well known Van Deemter equation as adapted to gel filtration by Giddings and Mallik [18], and Knox and Scott [19]:

$$H = 2\lambda d_p + 2[0.6D_m + \gamma_s D_m(1/R - 1)]/u + R(1 - R)d_p^2 u/30\gamma_s D_m \quad (4)$$

assuming that coupling effects between column dispersion and axial diffusion can be neglected (this is normally the case in SEC, which is carried out at reduced velocities exceeding 1) [20]. The plate height, H, is dependent on a geometric factor λ, the particle size d_p, the diffusion coefficient of the solute D_m, the obstruction factor to diffusion in the porous material γ_s, the relative zone velocity R and the interstitial velocity of the mobile phase u. A characteristic of the Van Deemter equation is the minimum plate height, which for SEC of macromolecules is given by [12]

$$H_{min} = d_p(2\lambda + 2\{2/30[0.6R(1 - R)/\gamma_s + (1 - R)]^2\}^{1/2}) \approx \text{constant} \cdot d_p \quad (5)$$

The proportionality constant, called the minimum reduced plate height, h_{min}, is dependent on, among other factors, how well the column is packed. Minimum reduced plate heights around 2 are regarded as excellent and values around 4 acceptable for high-performance columns [21,22].

The plate number and the peak width can be calculated from the plate height by

$$N = L/H \quad (6)$$

and

$$\sigma = V_R/N^{1/2} = V_R(H/L)^{1/2} \quad (7)$$

where L is the column length, V_R the retention volume of the solute and σ the peak width expressed in standard deviation, assuming a Gaussian distribution of solutes within a peak.

The maximum number of resolved peaks may be estimated from simulations of chromatograms using eqns. 4 and 7 as illustrated in Fig. 1. Identical separation patterns for different columns were obtained by selecting parameters in accordance with published data for commercial columns, as stated in the caption. For traditional media, a long column is necessary for providing high peak capacity. Note that the peak widths are roughly constant over the separation range at these flow velocities (as found

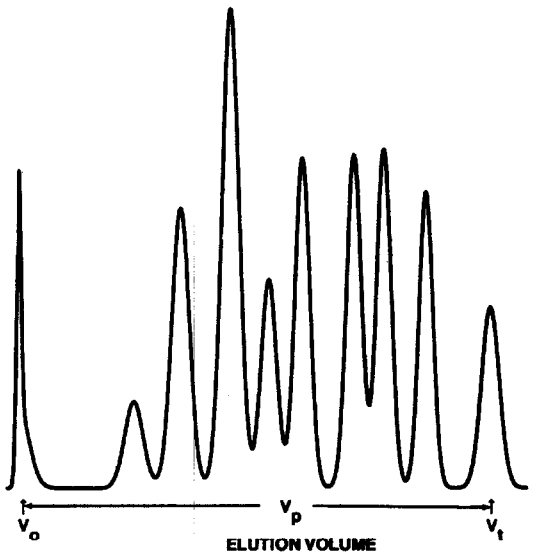

Fig. 1. Theoretical peak capacities of SEC columns as obtained by computer simulations. Peak widths are calculated from eqn. 7 for the solutes used under Experimental (see caption to Fig. 6). Conditions were selected according to published data [7,23,24], except for the column length of the 100-μm material, and identical separation patterns were obtained by minor adjustments of the pore fraction of the various materials:

Particle size (μm)	5	10	30	100
Bead pore fraction (%)	52	75	92	96
Nominal velocity (cm/h)	54	27	9	3
Column length (cm)	25	30	60	214

The resolutions between adjacent peaks are, from left to right, 2.93, 1.29, 1.45, 1.17, 1.10, 1.75, 1.02, 1.49 and 2.04. Peaks are, in ideal SEC, only eluted in the pore volume region, V_p, between the void volume, V_0, and the total liquid volume, V_t.

experimentally by Basedow *et al.* [8]). The separation systems seem to be able to resolve approximately eleven peaks with baseline separation. As illustrated in Figs. 2 and 3, the variations in peak width are considerably smaller than variations in plate number over the separation range normally encountered in SEC. It is also seen that the peak widths are virtually constant over a large portion of the separation range at the flow velocities commonly recommended for protein separations, *i.e.*, 27 cm/h for a 50-kilodalton protein chromatographed on a 10-μm material [23].

These results, together with experimental observations, encourage the use of eqn. 3 for the calculation of column peak capacity in SEC. However, determination of the plate number for the void peak is difficult. Instead, an equation that is related to the

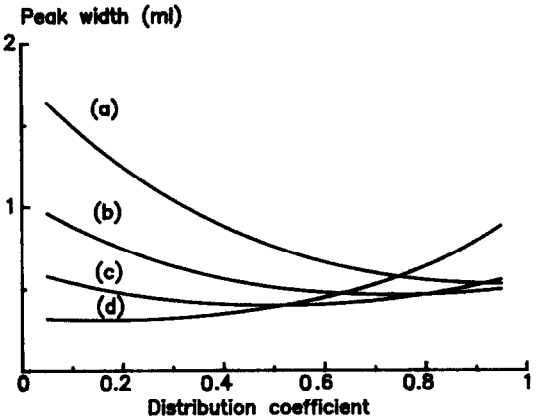

Fig. 2. Variations in peak widths over the separation range of SEC at various eluent velocities. Peak widths in ml calculated from eqn. 7 for solutes with molecular weights from 100 to 10 000 000 chromatographed on a 30 × 1 cm I.D. column packed with a 10-μm material. Nominal velocity: (a) 90; (b) 30; (c) 10; (d) 2 cm/h.

maximum plate number should be very useful for purposes of comparison between different materials packed in different columns. This may be obtained by a slight modification of the discussion made by Grushka [11] (see Fig. 1). The volume between the void peak, V_0, and the totally permeating peak, V_t, is equal to the pore volume, V_p, of the chromatographic material. This space may be filled with V_p/W peaks of width W. The width of a peak can be

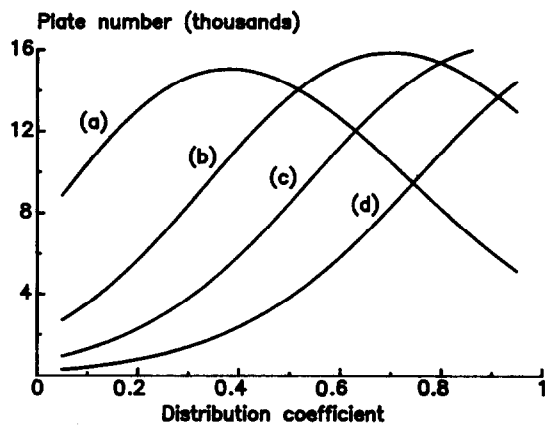

Fig. 3. Variations in plate numbers over the separation range of SEC at various eluent velocities. Plate numbers calculated from eqn. 6 with conditions given in the caption to Fig. 2. Nominal velocity: (a) 2; (b) 10; (c) 30; (d) 90 cm/h.

expressed in multiples of the standard deviation, σ, assuming a Gaussian distribution of solutes within the peak. The multiple factor is related to the resolution, R_s, of adjacent peaks. A spacing of the centre of two peaks (of equal widths) of 6σ, *i.e.*, $W = 6\sigma$, will yield $R_s = 1.5$, whereas $W = 4\sigma$ will yield $R_s = 1.0$. The influence of the resolution may be incorporated into the equation for peak capacity by expressing the width as $W = 4\sigma R_s$.

The width of the peaks will vary with both eluent velocity and retention volume (Fig. 2). At very low velocities, excessive broadening of fast-diffusing solutes will take place owing to the dominating influence of axial diffusion, and at very high velocities non-equilibrium effects will cause broadening of slow-diffusing species. A guideline for high-resolution gel filtration of proteins is to use a flow velocity yielding a reduced plate height of four for the solute of interest [23]. Assuming that this solute is eluted in the middle of the separation range (*i.e.*, a situation between curves b and c in Fig. 2), the peak width may, with the aid of eqn. 7, be calculated from

$$\sigma = (V_0 + K_D V_p)(4h)^{1/2}\sigma_{t,min}/[V_t(2h)^{1/2}] = (1.0 \pm 0.1)\sigma_{t,min} \quad (8)$$

where the distribution coefficient, K_D, is set to 0.5, the permeability, V_p/V_0, is between 0.8 and 2.2 and $\sigma_{t,min}$ is the minimum peak width of totally permeating solutes in standard deviations. Thus, at the eluent velocities commonly recommended for protein separations, the peak widths are approximately constant (Fig. 2) and roughly equal to the minimum peak width of a totally permeating peak (eqn. 8). From these assumptions and with the use of eqn. 7, and realizing that owing to column dispersion (and the definitions of V_0 and V_t), half a peak will appear before V_0 and half a peak will appear after V_t, the total number of peaks that, in practice, can be separated by SEC may be estimated from

$$n_{SEC} = V_p/[4(V_R/N^{1/2})R_s] + 0.5 + 0.5 = 1 + (V_p/V_t)(N_{max})^{1/2}/(4R_s) \quad (9)$$

where the plate count is calculated for the totally permeating peak (hence V_R becomes V_t). The practical peak capacity as calculated from eqn. 9 will be fairly close to the maximum theoretical peak capacity of the column (see below).

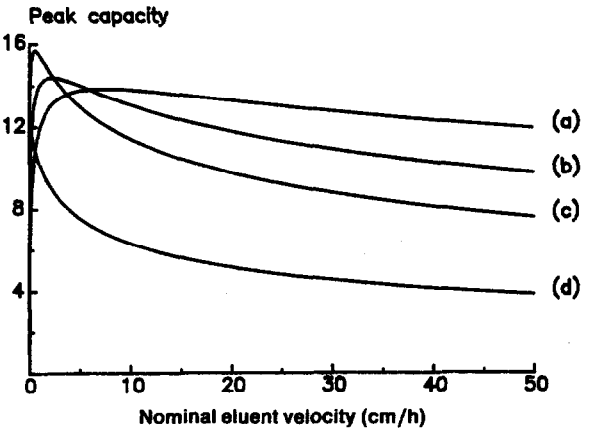

Fig. 4. Theoretical peak capacities of SEC columns. Number of peaks resolved by $R_s = 1.5$ obtained by successive calculations of peak widths with aid of eqn. 4, starting with the void peak. Separation range same as in Fig. 2. Column parameters:

	d_p (μm)	V_p/V_0	h_{min}	L (cm)
(a)	4	0.78	2.5	25
(b)	10	1.30	2.0	30
(c)	30	2.20	2.0	60
(d)	100	2.25	2.0	100

The plate number may be expressed in terms of the minimum reduced plate height from eqns. 5 and 6, which yields

$$n_{SEC} = 1 + (V_p/V_t)[L/(h_{min}d_p)]^{1/2}/(4R_s) \quad (10)$$

Hence the practical peak capacity is roughly inversely proportional to the resolution and to the square root of the particle size and proportional to the pore fraction and to the square root of the column length. It is also evident that optimum peak capacity is only obtained with columnss that are well packed. The influence of some of these parameters is illustrated in Fig. 4, showing the peak capacities of optimally packed columns at various eluent velocities. The peak capacity at each velocity was obtained by successive calculations of peak widths [14], starting from the void peak, with the aid of eqns. 4 and 7. The total peak capacity will decrease at very low eluent velocities owing to the excessive broadening of low-molecular-weight solute peaks and at very high velocities owing to broadening of high-molecular-weight solute peaks. The latter effect is reduced by using small-particle-size materials, possessing small diffusion distances. The maximum peak capacity is obtained, in most instances, at an unrealistically low

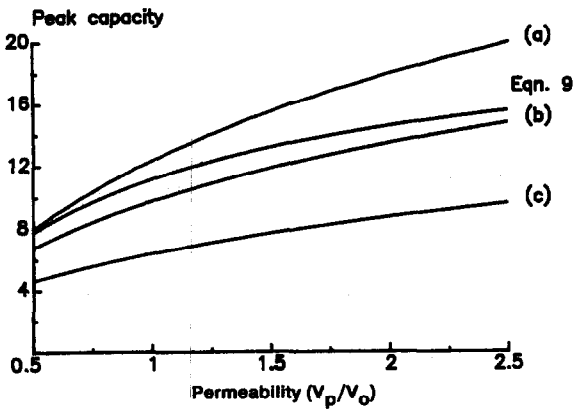

Fig. 5. Comparison of theoretical peak capacities and that calculated from eqn. 9 at various permeabilities, V_p/V_o, of the chromatographic material. Maximum peak capacity derived as for Fig. 4b with the following nominal eluent velocities: (a) 2; (b) 27; (c) 150 cm/h.

eluent velocity. In the calculation of the practical peak capacity, the eluent velocity exceeds the optimum velocity (as often is the case in experimental SEC) and therefore the peak capacity calculated with the aid of eqn. 9 will be 15–20% lower than the true peak capacity of the column (Fig. 5a). However, as illustrated in Fig. 5, the peak capacity as calculated from eqn. 9 will be close to the true peak capacity obtained at recommended eluent velocities (Fig. 5b), which was the assumption in eqn. 9.

The eluent velocity yielding the minimum plate height, and thus minimum zone broadening of a particular solute, is given by [23]

$$u_{opt} = (D_m/d_p)\gamma_s(60\{2/[3\gamma_s R(1-R)] + 1/R^2\})^{1/2} \quad (11)$$

and is thus inversely proportional to the particle size of the chromatographic material employed. It can be shown that the flow velocity will be proportional to D_m/d_p also when $H = 2H_{min}$. This means that the inherent peak capacity of small-particle-size media is obtained at a higher eluent velocity and consequently with a shorter time of analysis (Fig. 4). By combining eqns. 10 and 11, it is seen that the practical peak capacity is held constant by keeping the column length proportional to the particle size and the flow velocity inversely proportional to the particle size, which results in an analysis time proportional to $[d_p/(V_p/V_t)]^2$. However, this is only true for media of similar pore fractions, as the pore fraction will influence R and thus u_{opt}. Low pore fractions will result in relatively longer separation times, as is evident from the data in the caption to Fig. 1. The analysis time on the 5-μm material is twice as long as expected on the basis of differences in particle sizes of the materials.

EXPERIMENTAL

For an experimental study of the peak capacity as a function of the particle size, it is necessary to use a set of materials possessing very similar selectivity properties. For this purpose, we selected a family of gels based on 6% agarose of various particle sizes. The peak capacities of Superose 6 ($d \approx 13$ μm), Superose 6 Prep Grade ($d \approx 33$ μm) and Sepharose CL 6B ($d_p \approx 95$ μm), packed into columns of various lengths (Table I), were examined by size exclusion of a protein mixture using phosphate buffer as mobile phase (Fig. 6). The instrumentation used was a Model P-500 high-precision pump, a Model V-7 injector, equipped with a 200- or 500-μl loop, a Model UV-M UV detector with a 10-μl flow cell and 280-nm filter and a Model REC-2 recorder (Pharmacia LKB Biotechnology, Uppsala, Sweden).

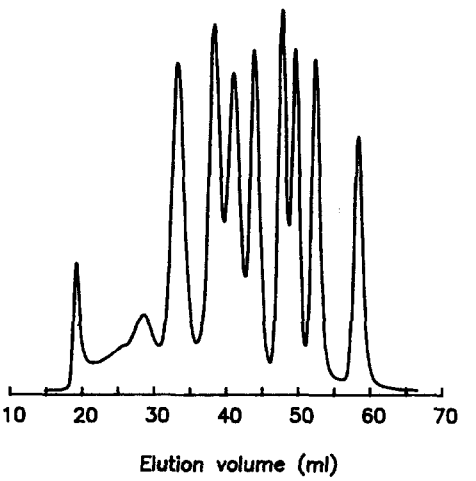

Fig. 6. Separation of a protein mixture by fast SEC. Column: 31.6 × 1.6 cm I.D. packed with Superose 6. Sample: 200 μl of a mixture of thyroglobulin (2 mg), ferritin (0.6 mg), IgG (1 mg), bovine serum albumin (1.6 mg), carbonic anhydrase (0.6 mg), cytochrome *c* (0.4 mg), aprotinin (1.4 mg) and glycyltyrosine (0.2 mg). Eluent: 0.05 M phosphate in 0.15 *M* NaCl (pH 7.0). Flow-rate: 50 ml/h.

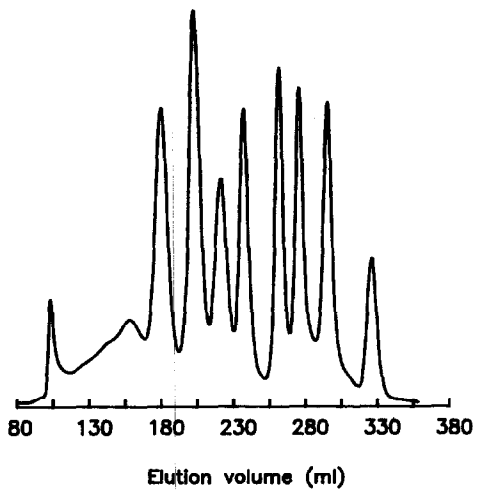

Fig. 7. Separation of a protein mixture by standard SEC. Column: 64.0 × 2.6 cm I.D. packed with Superose 6 Prep Grade. Solutes and eluent as in Fig. 6. Sample volume: 200 μl. Flow-rate: 43 ml/h.

Simulations were performed on a Hewlett-Packard Series 9000/200 computer.

RESULTS AND DISCUSSION

The chromatograms illustrated in Figs. 6–8 confirm that the predicted peak capacities of the different columns, as calculated from eqn. 9 (see Table I, are in good accordance with those found experimentally and that similar separation patterns and peak capacities are obtained under optimum conditions for traditional, standard and fast SEC. Further, the chromatograms are very similar to the separation patterns expected from the simulations shown in Fig. 1, although a lower resolution between peaks is obtained experimentally. The peak widths are fairly constant over the entire separation range. It is evident from the chromatograms that the selectivities of the materials differ slightly, especially at the extremes of the separation range. The separation in the high-molecular-weight region is overestimated by the computer simulation, which is due to the problem of assigning a correct obstruction factor for solutes eluted at $K_D < 0.2$ [23]. The separation on Superose 6 Prep Grade is better than that on Superose 6, which can be attributed to the too high eluent velocity used with the latter column (*i.e.*, 20 cm/h should give a more realistic picture of the separation ability of this material [23]). For Sepharose CL 6B, a change in the experimental conditions to L = 156 cm and u_{nom} = 2.5 cm/h can be expected to yield a similar separation to that with Superose 6 Prep Grade.

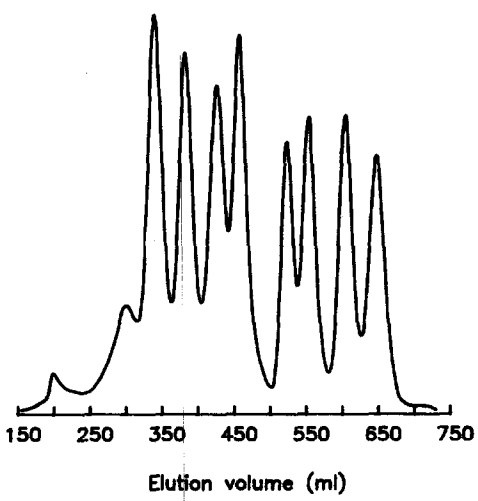

Fig. 8. Separation of a protein mixture by traditional SEC. Column: 137.9 × 2.5 cm I.D. packed with Sepharose CL 6B. Solutes and eluent as in Fig. 6. Sample volume: 500 μl. Flow-rate: 9.6 ml/h.

TABLE I

EXPERIMENTAL PARAMETERS

Parameter	Superose 6	Superose 6 Prep Grade	Sepharose CL 6B
$d_{p,50,v}$ (μm)	13.6	29.2	95.0
V_o (ml)[a]	19.7	105.7	204.8
V_t (ml)[b]	58.8	328.2	652.8
V_p/V_t	0.664	0.678	0.686
L (cm)	31.6	64.0	137.9
N_{max}[c]	10 000	12 500	5140
u_{nom} (cm/h)	24.9	8.1	1.96
$L/\{u_{nom}[d_p/(V_p/V_t)]^2\}$	$3.0 \cdot 10^5$	$4.2 \cdot 10^5$	$3.7 \cdot 10^5$
$n_{1.5}$ from eqn. 9	12.0	13.6	9.2
$n_{1.5}$ experimental[d]	11.6	13.6	8.7

[a] Calculated from the peak apex of the void peak.
[b] Calculated from the peak apex of glycyltyrosine.
[c] Determined from the peak width at half-height of acetone.
[d] Calculated from the mean peak widths of bovine serum albumin and carbonic anhydrase in Figs. 6–8 and the distance between the void volume and the total volume.

TABLE II

THEORETICAL PEAK CAPACITIES OF SOME COMMERCIAL COLUMNS FOR AQUEOUS SEC

Column	L (cm)	d_p (μm)	V_p/V_t	N	$n_{1.0}$	$n_{1.5}$
Prepacked columns[a]						
Zorbax GF-250	25.0	4	0.44	25 000[b]	18	13
Superose 12	30.0	10	0.65	12 000	18	13
TSK 3000 SW	30.5	10	0.57	9220	15	10
Synchropak GPC 300	24.5	10	0.59	4200	11	7
Waters I-125	25.0	10	0.48	4950	10	7
Synchropak GPC 100	25.0	10	0.55	2080	6	4
LiChrosorb Diol	30.0	10	0.39	1760	5	4
Laboratory-packed columns[a]						
Superose 6 Prep Grade	30.0	33	0.69	3640	11	8
Superose 6 Prep Grade	60.0	33	0.69	7200	16	11
Sephacryl S 300 HR	60.0	54	0.62	6900	14	10
Sepharose CL 6B	100.0	110	0.70	4170	12	9

[a] Data for the columns collected from refs. 23 and 24.
[b] Calculated assuming $h_{min} = 2.5$.

The peak capcities of some different commercially availble columns for high-performance SEC and those of traditional media packed in laboratory columns, as calculated from eqn. 9, are given in Table II. It is seen tht the inherent high peak capacity of small-particle-size media is in some instances ruined by an extremely low pore volume of the material or sacrificed for a decrease in column length as compared with traditional media. As noted elsewhere [25], the plate numbers determined for some of the columns are surprisingly low, which might yield an unfair picture of the expected peak capacity of these silica-based materials. Assuming that the columns are well packed, *e.g.*, $h_{min} = 2.5$, the peak capacity for completely resolved peaks, *i.e.*, $R_s = 1.5$, varies between 8 and 13 owing to the different pore volumes of the different materials. This shows that the peak capacity of columns for fast SEC are of the same order as laboratory-packed columns of traditional media, provided that the columns are operated close to the optimum velocity. This is also illustrated by Fig. 4, showing the peak capacities of optimally packed columns at various flow velocities. However, the peak capacities of microparticulate materials are substantially less sensitive to high flow velocities, owing to the small diffusion distances of these materials. Hence the optimum performance is not dramatically [higher] but [faster] with microparticulate materials with the column lengths normally used. This is also evident from Fig. 1, where the difference between the materials is not peak capacity but separation time, *i.e.*, the number of peaks separated per unit time. Grushka [26] discussed the influence of particle size, column length and eluent velocity on the "rate of peak capacity production".

The detrimental impact of large matrix volumes on the peak capacities of microparticulate materials was noted by Engelhardt and Ahr [14]. They found that the theoretical peak capacities of 10- and 3-μm silica materials packed in 25-cm columns were 15 and 27, respectively. Corresponding data from eqn. 9 using their data for pore fractions, plate height and resolution factor, *e.g.* $R_s = 1.0$, are 13 and 23, respectively, which is in fair agreement considering that eqn. 9 will yield a slight underestimate of the maximum peak capacity, as outlined above.

The example given is valid for macromolecules. However, the discussion is equally applicable to small solutes, the maximum peak capacity will appear at higher flow velocities (proportional to the diffusivity) and as the slope of the C term of the Van Deemter plot will be smaller than for solutes of large D_m, the operational range of high peak capacities

will be large. This is also the case for smaller-particle-size materials. It may be noted that in general it seems to be more difficult to obtain optimally packed beds of smaller particles, which also partially explains the modest peak capacities displayed by some novel media. The small zone broadening of microparticulate media will, of course, be very important when low sample dilution is critical, *e.g.*, in micropreparative work or high-sensitivity analysis.

As the optimum eluent velocity is directly related to the diffusivity of the solute, a continuous increase in velocity as the solutes are being eluted should not only provide more optimum resolution but also a decrease in separation time, sometimes by a factor of two [23]. In the ideal case, it may be expected that a flow gradient may be constructed such as to allow each solute to elute with $H = H_{min}$. However, this situation can never be realized as the low eluent velocity necessary to yield small peak widths of early eluting substances will have to be constant for 30–40% of the run, and this will have a detrimental effect on the peak width of fast-diffusing solutes.

A resolution of 1 is assumed in the calculations of peak capacity by eqns. 1 and 2. However, as can be seen from Fig. 1, $R_s = 1$ will result in overlapping peaks (*e.g.*, peaks 7 and 8 from the left), whereas $R_s = 1.5$ yields almost completely resolved peaks (*e.g.*, peaks 8 and 9 from the left). It may therefore be recommended that the peak capacity be calculated from $R_s = 1.5$.

CONCLUSIONS

The practical peak capacities of columns for SEC can be calculated from

$$n = 1 + (V_p/V_t)[L/(h_{min}d_p)]^{1/2}/(4R_s)$$

Owing to the reduction in the relative pore volume and column length with decreasing particle size, the practical peak capacity of columns for SEC is around 10–15. This value will be lower for non-optimally packed columns.

The maximum peak capacity is obtained at a flow velocity inversely proportional to the particle size and, thus, the gain achieved with using microparticulate media is in separation speed and low sample dilution, not peak capacity.

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