Journal of Chromatography, 326 (1985) 3–32
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 572

THEORETICAL INVESTIGATION OF THE OPTIMUM PARTICLE SIZE FOR THE RESOLUTION OF PROTEINS BY SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

A theoretical discussion is presented of the possibilities of the dramatic acceleration of the size-exclusion chromatographic separation of proteins as a result of a marked decrease in the diameter of the gel particles used to pack the column. The use of very small particles, in the range 1–2 μm, is made compatible with the use of moderate inlet pressures by the requirement of a low mobile phase velocity, due to the small diffusion coefficient of high-molecular-weight compounds. Thus, conditions regarding (i) the suppression of eluent flow in the intra-particle pores, (ii) the avoidance of shear degradation of proteins because of excessive viscous stress and (iii) the avoidance of size exclusion of protein molecules from some interstitial channels can be met for proteins with molecular weights up to several hundred thousand.

The optimum particle diameter is discussed, and it is shown that efficiencies of a few tens of thousands plates, permitting peak capacities of up to 30–40, could be generated by using 20–30 cm long columns packed with 1- or 2-μm particles. The analysis time depends on the molecular weight range but, with these columns, it would be of the order of 1 h.

The results of this discussion are supported by experimental data, illustrating the practical possibilities of this approach.

INTRODUCTION

For a long time, following the pioneering work by Porath and Flodin[1], size-exclusion chromatography (SEC) has been used in the separation of proteins[1–5]. Until recently, the main drawbacks of this method were the relatively low resolution achieved and the extremely long time necessary to carry out the separation of a complex sample.

It is well known in chromatography that both the resolution power and the speed of analysis are related to some parameters of the column design and operation, namely the column length, L, the average diameter of the particles used to pack this

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0021-9673/85/$03.30

column, d_P, and the mobile phase flow velocity, u. The column efficiency also depends on the diffusion coefficient of the sample component in the solvent, which determines the rate at which the molecules of a given species spread from their initial position (axial diffusion) and diffuse into, inside and out of the packing particles (resistances to mass transfer)[6-8].

Most of these concepts extend easily to SEC[9], although they were originally developed for high-performance liquid chromatography (HPLC). Their validity has been extensively demonstrated by the wealth of data collected during studies on the influence on column performance of the design and operation parameters of HPLC columns[10].

A detailed theoretical study, dealing with the fundamental obstacles to the use of SEC for the analysis of macromolecules of very high molecular weight, has recently been published by Giddings[11]. Although this work was mainly related to the exploration of the problems to be solved for the analysis of molecules with molecular weights in the multi-million dalton range and above, the same basic approach can be used to discuss possible improvements in the SEC analysis of proteins with molecular weight between 10 000 and 1 000 000 daltons, which is the topic of the present work. The problems will just be simpler to solve and as will be seen, the potential for improvement is more important.

In this paper, we show that it is possible to apply these concepts in order to improve markedly both the resolving power of the SEC columns used for the separation of proteins and their analysis times by using smaller particles to pack the columns and by operating them at a higher velocity. It is shown that the increase in the inlet pressure remains acceptable. We also discuss the extent to which it is possible to proceed further along these lines and achieve additional improvements in the resolving power and the speed of analysis of SEC when it is applied to the separation of proteins and at the same time, to find conditions under which shear degradation, the polarization effect and other potential disturbances are avoided.

A paper on this subject was published recently by Engelhardt and Ahr[12], but referring to the SEC of synthetic polymers.

THEORETICAL

Flow velocity

In conventional liquid chromatography, the flow velocity is traditionally defined as the ratio of the column length to the breakthrough time of a non-retained solute. This gives a linear velocity of the mobile phase that is averaged over the entire fraction of the cross-section of the column available to the solvent[6]. This velocity is usually designated u.

In SEC it is conventional, and more appropriate, to define the flow velocity as the ratio of the column length to the retention time of a totally excluded compound. This defines the flow velocity as the average over the fraction of the column cross-section that is outside the particles of packing, available for the actual flow of the mobile solvent[9]. This velocity is usually designated v_0 and called the interstitial velocity.

Obviously, the two velocities are proportional for a given column, and therefore the relationships involving the velocity of the mobile phase are formally the

same, whatever the definition of the velocity used. The numerical values of the parameters, however, are different. The interstitial porosity of an SEC column is usually of the order of half the total porosity, so that the interstitial velocity, v_0, will be about double the average velocity, u.

Column efficiency

It has been shown by Knox and Saleem[13] that the height equivalent to a theoretical plate (HETP) of a chromatographic column, H, is related to the flow velocity of the mobile phase by the following equation:

$$H = \frac{B}{v_0} + Av_0^{1/3} + Cv_0 \quad (1)$$

where A, B and C represent the different phenomena that are responsible for band broadening in chromatography[6,13]. They all depend on the average size of the particles used to pack the column and/or on the diffusion coefficient of the sample in the solvent. In HPLC, eqn. 1 is usually written by using the linear velocity, u. This equation is also valid in SEC when the interstitial velocity, v_0, is used, the only change being in the numerical values of A, B and C.

The first coefficient, B, is proportional to the diffusion coefficient and accounts for the band broadening by molecular diffusion in the axial direction. In the system of coordinates of the mass centre of the zone, molecules diffuse away from this mass centre, following Fick's law, which in this simple instance can be expressed by the integrated form of the Einstein relationship[14]:

$$s^2 = 2\gamma D_m t \quad (2)$$

The coefficient B is thus equal to $2\gamma D_m$, while γ, close to but smaller than 1 in HPLC, results from the complexity of the structure of the packing, which acts to slow down molecular diffusion. In SEC, because of the different definition of the flow velocity and of the sluggish diffusion of large molecules in the pores[7], the value of γ is around 0.7. There is nothing that can be done to decrease the importance of this contribution to band broadening, except to reduce the time spent by the zone inside the column by increasing the flow velocity. The diffusion coefficient (D_m) is essentially a function of the nature of the sample and is only marginally modified by a change in the solvent used (see *Diffusion coefficients*).

The second coefficient, A, accounts for the irregularity of the packing. As a packing of more or less irregular particles, having a more or less broad size distribution, cannot be homogeneous, regions appear in the packing where the density of the particles is larger than average. In these regions the average distance between particles is smaller than in other places, the local permeability is smaller than average and, accordingly, the local velocity of the mobile phase is lower. Conversely, in other parts of the packing the local solvent velocity will be larger. The solutes are carried downstream by the mobile phase at a velocity that is proportional to the solvent velocity. Such a distribution of the solvent velocity over the column cross-section results in a broadening of the solute band, the extent of which is limited only by radial diffusion. The importance of this contribution to band broadening is a function

of the degree of heterogeneity of the packing, which is expressed by the coefficient A in eqn. 1. This coefficient depends on both the particle size and the solute diffusion coefficient[9,13].

A good packing method permits the achievement of a significant reduction in the value of A, which explains the major importance of the quality of the column packing. As will be seen later, a decrease in A results not only in a smaller plate height, but also in a higher optimum flow velocity. Hence the speed of analysis is increased, both because a shorter column is required and because it is operated at a larger velocity. As explained above, because of the difference in the definition of the solvent velocity, similar columns would have A values about 1.25 times smaller in SEC than in HPLC.

The third term in eqn. 1 accounts for the resistance to mass transfer inside the particles: in SEC, retained molecules have to diffuse into and out of the particles, a process that takes time. The order of magnitude of this time is given by the condition that the Fick number, Fi,

$$Fi = \frac{d_p^2}{2D_m t} \tag{3}$$

be close to unity. This condition is related to the integrated Einstein eqn. 2 if applied to the time it takes a molecule to diffuse over a length d_p. As in axial diffusion, the complex structure of the inner porosity of the particles slows down diffusion and results in the Fick number being smaller than unity. This is also accounted for by the coefficient C. Finally, this coefficient depends on the fraction of the retention time spent by the compound studied inside the particles of the packing.

It has been shown by Giddings *et al.*[8,11] that, in SEC, the coefficient C is given by the equation

$$C = \frac{R\,(1 - R)\,d_p^2}{30\,\gamma_S D_m} \tag{4}$$

where γ_S is the obstructive factor for the inner particle structure, D_m is the coefficient of molecular diffusion and R is the retention ratio, or ratio of the volume of the interstitial mobile phase, V_0, to the retention volume of the corresponding compound, V_R, with:

$$V_R = V_0 + K_{SEC} V_i \tag{5}$$

where K_{SEC} is the equilibrium constant and V_i the intra-particular solvent volume. K_{SEC} is between 0 (totally excluded compounds) and 1 (compounds that have access to all the pores). R is thus

$$R = \frac{V_0}{V_R} \tag{6}$$

R varies between $V_0/(V_0 + V_i)$ and 1. Because V_0 and V_i are comparable for most SEC packings, it turns out that R usually varies between 0.5 and 1. In some instances, values as low as 0.38 have been reported[14].

Theoretical studies on the dependence of the column efficiency on the average size of the particles used to pack the column have stressed the importance of reduced parameters[6,10,13]; the reduced plate height,

$$h = H/d_p \tag{7}$$

and the reduced velocity,

$$v = v_0 d_p/D_m \tag{8}$$

The reduced plate height and velocity are dimensionless numbers and, accordingly, as long as similar packings having the same degree of homogeneity but only a different geometrical scale are achieved, these numbers should suffice to describe the column performance, independently of the particle size and diffusion coefficient.

A large number of systematic experimental studies over the last 10 years have shown the validity of this approach. Solutes having markedly different diffusion coefficients, analysed on columns differing considerably in length and particle size, give plots of reduced plate height *vs.* reduced velocity that are almost impossible to distinguish[13]. This applies to columns having lengths between a few centimetres and 1 m, packed with particles ranging in average diameter from 2 to over 50 μm, and to combinations of solvents and solutes with diffusion coefficients between $1 \cdot 10^{-6}$ and $3 \cdot 10^{-5}$ cm^2/sec.

These curves can be represented by the following equation:

$$h = \frac{b}{v} + av^{1/3} + cv \tag{9}$$

This equation is derived from the Knox plate-height equation (eqn. 1). In eqn. 7, when it is applied to SEC, b is close to 1.4, a is usually between 0.8 (excellent columns) and 3.2 (fair columns) and c is given by the following relationship, derived from eqns. 4–6:

$$c = \frac{R(1 - R)}{30\,\gamma_s} \tag{10}$$

c is zero for $R = 1$ and is maximal for $R = 0.5$. This maximum value of c is between 0.01 and 0.02, but usually close to 0.01.

Although among the many authors who have discussed this question there is no agreement regarding the description of a good packing technique for LC columns, there is, on the other hand, an amazingly narrow distribution of the best results obtained so far in HPLC. In most instances, the values of a, b and c are between 1 and 2 for a, 1.2 and 1.5 for b and 0.02 and 0.1 for c[13–17]. Accordingly, we may expect to find, in SEC, values of b also between 1.2 and 1.5, values of a between 0.8 and 1.6 for silica-based stationary phases, but probably larger values between 2.5 and 3.5 for organic gels, which are usually softer and more difficult to pack than silica particles, and values of c between 0.01 and 0.02. Some values of reduced HETP derived from these data are reported in Table I.

TABLE I

REDUCED HETP OF SEC COLUMNS

a	*c*	*Reduced velocity*	*Reduced* plate height*	*Optimal velocity (cm/sec)*	*Minimal reduced plate height*
0.80	0.01	2.00	1.73	3.27	1.65
0.80	0.01	4.00	1.66		
0.80	0.01	8.00	1.86		
0.80	0.01	16.00	2.26		
0.80	0.01	32.00	2.90		
1.00	0.01	2.00	1.98	2.81	1.94
1.00	0.01	4.00	1.98		
1.00	0.01	8.00	2.26		
1.00	0.01	16.00	2.77		
1.00	0.01	32.00	3.54		
2.00	0.01	2.00	3.24	1.72	3.23
2.00	0.01	4.00	3.56		
2.00	0.01	8.00	4.26		
2.00	0.01	16.00	5.29		
2.00	0.01	32.00	6.71		
3.00	0.01	2.00	4.50	1.28	4.36
3.00	0.01	4.00	5.15		
3.00	0.01	8.00	6.26		
3.00	0.01	16.00	7.81		
3.00	0.02	2.00	4.52	1.26	4.38
3.00	0.02	4.00	5.19		
3.00	0.02	8.00	6.34		
3.00	0.02	16.00	7.97		

* After eqn. 9, with $b = 1.4$.

As a consequence, the minimum value of h is most often around 2 for silica-based particles, which are easy to pack, around 3–4 for organic gels and rarely larger than 4. The corresponding value of the reduced velocity is close to 3 for silica-based particles and around 1.5 for organic gels. When maximum efficiency is not critical, a small part of the column efficiency can be traded for a significant reduction in the analysis time, especially when a is small, and the column can be operated at a reduced velocity of up to 15–20, while the plate height usually does not exceed about 4. Sometimes it can even be as low as 2.5 if a column packing of excellent quality can be achieved and a is close to 1, which is not unusual in HPLC.

Combination of eqns. 7–10 shows that decreasing the average particle size permits both the achievement of a smaller plate height and operation of the column at a larger flow velocity. Hence, if a certain column efficiency is required for performing a given separation, a shorter column packed with finer particles will achieve it, but this column will be operated at a higher solvent velocity. Both changes result in a reduction in the analysis time.

Analysis time

It has been shown by Giddings[11] that, for a constant column efficiency, the

analysis time is proportional to the square of the particle diameter, to the retention volume and to the ratio of the reduced plate height to the reduced velocity, and inversely proportional to the diffusion coefficient. It also depends on the efficiency deemed necessary to achieve the requested separation, *i.e*, on the column selectivity and on the ratio $\Delta M/M$ of the difference in the molecular weights of the polymers to be resolved to their average molecular weight.

If we define the analysis time, t_a, as the time necessary to elute the molecules that have free access to all pores, we obtain

$$t_a = \frac{L}{u} = \frac{Nhd_p^2}{vD_m\varphi} \tag{11}$$

Eqn. 11 is obtained by combining the definition of the analysis time with eqns. 7 and 8. N is the plate number necessary to achieve the desired separation and φ is the ratio $V_0/(V_0 + V_i)$ of the interstitial to the total porosity.

The dependence of the analysis time on the particle diameter is very strong. For example, replacing 50- by 10-μm particles would permit a decrease in the analysis time by a factor of 25, a five-times shorter column being used at a five-times higher velocity. Alternately, part of this gain can be traded for an improvement in the column efficiency (*cf.*, Table II). The resolving power of the column is proportional to the square root of its length and the analysis time is proportional to the column length (*cf.*, Table II).

TABLE II

PERFORMANCE OF HPLC COLUMNS UNDER PRACTICAL OPTIMUM CONDITIONS

Viscosity, 1 cP; diffusion coefficient, $1 \cdot 10^{-5}$ cm^2/sec; reduced velocity, 10; reduced plate height, 3.0.

L (cm)	*dp (μm)*	*u (cm/sec)*	t_m *(sec)*	*F (ml/min)**	*P (bar)*	*N (plates)*
3.0	10.0	0.10	30	0.80	3.0	1000
3.0	5.0	0.20	15	1.60	24.0	2000
3.0	3.0	0.33	9	2.66	111.1	3300
5.0	20.0	0.05	100	0.40	0.6	830
5.0	10.0	0.10	50	0.80	5.0	1700
5.0	5.0	0.20	25	1.60	40.0	3300
5.0	3.0	0.33	15	2.66	185.2	5600
10.0	50.0	0.02	500	0.16	0.1	700
10.0	20.0	0.05	200	0.40	1.3	1700
10.0	10.0	0.10	100	0.80	10.0	3300
10.0	5.0	0.20	50	1.60	80.0	6700
10.0	3.0	0.33	30	2.66	370.4	11 100
20.0	20.0	0.05	400	0.40	2.5	3300
20.0	10.0	0.10	200	0.80	20.0	6700
20.0	5.0	0.20	100	1.60	160.0	13 300
50.0	10.0	0.10	500	0.80	50.0	16 700
50.0	5.0	0.20	250	1.60	400.0	33 300

* Column inner diameter, 4.6 mm. Total porosity, 0.80.

For example, under the same conditions, we can replace 50- by 10-μm particles and keep the same column length. Then, the efficiency is five times greater and the analysis time still five times shorter. Other compromises between efficiency and analysis time are possible.

Inlet pressure

However, there is a price to be paid in all instances when one decreases the particle size, namely that the column permeability also decreases in proportion to the square of the particle diameter, so that the inlet pressure, which is proportional to the solvent velocity, increases in proportion to the inverse of the square of the particle diameter at constant column efficiency (the decrease in column length compensating for the increase in solvent velocity), and in proportion to the cube of the particle diameter at constant column length. For example, in HPLC, with a constant column length of 10 cm, a typical inlet pressure would be 10 atm with 10-μm silica particles, 80 atm with 5-μm particles and 370 atm with 3-μm particles (*cf.*, Table II). These values assume the use of a conventional reversed-phase solvent (viscosity 1 cP) at a typical reduced flow velocity of 10 (flow-rate 1 ml/min with 10-μm particles for a 4.6 mm I.D. column) and a low-molecular-weight solute (diffusion coefficient *ca.* $1 \cdot 10^{-5}$ cm^2/sec). The pressure rapidly becomes prohibitive for packing materials that are neither as hard nor as mechanically strong as silica.

It should be emphasized, however, that if these values are conventional in HPLC, this is only because the solutes typically analysed using this method have a relatively low molecular weight, usually a few hundreds to a thousand daltons. Accordingly, their diffusion coefficients are in the range between $5 \cdot 10^{-6}$ and $1.5 \cdot 10^{-5}$ cm^2/sec. As shown by eqn. 8, the actual solvent velocity corresponding to a given value of the reduced velocity is proportional to the diffusion coefficient. Accordingly, the necessary inlet pressure, which is related to the parameters of the column that must be used to achieve a given separation, is supplied by the following relationship, derived from the Darcy equation[16]:

$$P = \frac{u\eta L}{k_0 d_p^2} = N \cdot \frac{h v \eta D_m \varphi}{k_0 d_p^2} \tag{12}$$

where η is the solvent viscosity and k_0 the specific permeability of the column, usually between $1 \cdot 10^{-3}$ and $1.3 \cdot 10^{-3}$.

For high-molecular-weight compounds, such as proteins, the actual solvent velocity corresponding to a given value of the reduced velocity is much smaller than for conventional HPLC solutes, in the ratio of the diffusion coefficients, and so is the inlet pressure. Hence it is important to have a correct estimate of the diffusion coefficients of molecules with high molecular weights.

Diffusion coefficients

The order of magnitude of the diffusion coefficient of a solute is classically obtained in HPLC by using the conventional empirical equation derived by Wilke and Chang[18]:

$$D_m = 7.4 \cdot 10^{-10} \cdot \frac{(\psi M)^{0.5}\, T}{\eta V^{0.6}} \tag{13}$$

where M is the molecular weight of the solvent, V the molecular volume of the solute, η the viscosity of the solvent, T the absolute temperature of the column and ψ an association constant, which is equal to 1 for solvents with no hydrogen bond association and can be as high as 2.7 for water[18]. The value of 41 assumed for ψM in the numerical calculations (*cf.*, Table III) is exact for acetonitrile and close to the values corresponding to water and methanol.

TABLE III

DIFFUSION COEFFICIENTS OF HIGH-MOLECULAR-WEIGHT COMPOUNDS

Solvent viscosity, 1 cP; solvent molecular weight, 41 daltons; temperature, 293°K.

M (daltons)	D_m *(cm²/sec)* × 1.0^{-6}*				
	A	*B*	*C*	*D*	*E*
100	8.7601	21.7646	12.8411	4.2900	5.2646
300	4.5314	11.8550	6.9945	2.4768	3.6503
1000	2.2004	6.0919	3.5942	1.3566	2.4436
3000	1.1382	3.3182	1.9577	0.7832	1.6943
10 000	0.5527	1.7051	1.0060	0.4290	1.1342
30 000	0.2859	0.9288	0.5480	0.2477	0.7864
100 000	0.1388	0.4773	0.2816	0.1357	0.5265
300 000	0.0718	0.2600	0.1534	0.0783	0.3650
1 000 000	0.0349	0.1336	0.0788	0.0429	0.2444

* A, After eqn. 13 (Wilke and Chang[18]). Solute density: 1. B, After eqn. 14 (Giddings *et al.*[19]). Solvent: toluene, η = 0.59 cP. C, Values from column 2, corrected for a viscosity of 1 cP. D, After eqn. 15 (Stokes–Einstein equation for random coils). E, After eqn. 16 (Stokes–Einstein equation for globular molecules).

Eqn. 13 shows that the diffusion coefficient decreases as the power 0.6, *i.e.*, slightly faster than the square root, of the molecular volume of the compounds studied. Accordingly, the optimum actual flow velocity of the solvent, which is proportional to the diffusion coefficient (*cf.*, eqn. 8) as the optimum reduced velocity is constant, also decreases as the power 0.6 of the molecular volume of the solute. The specific volume of proteins is almost constant, around 0.73 (ref. 20), so we can consider, as a first approximation, that their molecular volume is directly proportional to their molecular weight. Hence for heavy polypeptides and proteins, the diffusion coefficient is bound to be small, often below $1 \cdot 10^{-6}$ cm²/sec (*cf.*, Table III).

It must be noted, however, that eqn. 13 is an approximation that does not differentiate between globular and statistically coiled molecules and, further, has been derived empirically from data obtained in the molecular weight range 100–500[18]. Accordingly, eqn. 13 cannot be expected to provide a good estimate of the diffusion coefficients of proteins, and the error may well exceed 50%.

It has been determined by Giddings *et al.*[19] that the diffusion coefficient of polystyrene in toluene is given by

$$D_m = M^{-0.553} \exp\left(-3.8029 - \frac{1285}{T}\right) \quad (14)$$

This is in agreement with a molecular size proportional to the square root of the mean end-to-end distance. This polystyrene-equivalent diffusion coefficient is also given in Table III. As shown by the comparison between eqns. 13 and 14, the dependence on the molecular weight is very similar (exponents -0.6 and -0.553, respectively). The values derived from eqn. 14, however, are about three times larger than those derived from eqn. 13 (*cf.*, Table III). Part of this difference is explained by the lower viscosity of toluene, 0.59 cP, compared with 1 cP for water (for acetonitrile it is 0.35 cP), but after correction a substantial disagreement remains (*cf.*, Table III, column 3). While there is no doubt that the order of magnitude of the diffusion coefficient derived from eqn. 14 is correct, a better estimate would be highly desirable. In this paper we use eqn. 14 as an approximation for the calculation of the diffusion coefficient of random coil proteins. Most proteins are not random coils, however, but have a more compact globular structure, resulting in a smaller molecular diameter and a larger diffusion coefficient.

For globular proteins, a group that accounts for the larger proportion of proteins and the most important known ones, an approximation of the diffusion coefficient can be derived from the Stokes–Einstein equation:

$$D_m = \frac{kT}{3\,\pi\eta d_S} \tag{15}$$

where d_S is the Stokes diameter of the molecule and k the Boltzman constant[11,20]. This equation does not give good results for random coils, but affords an excellent estimate for solutes with molecular diameters that are large compared with the solvent molecular diameter and that are roughly spherical in shape. Globular proteins meet these conditions. A recent study[20] on the correlation between molecular weight and diffusion coefficient for 301 proteins was based on eqn. 15. It was shown that for globular proteins the following relationship is valid:

$$D_m = 8.34 \cdot 10^{-10} \cdot \frac{T}{\eta M^{1/3}} \tag{16}$$

(the use of the exponent -8 instead of the correct -10 in ref. 20 seems to originate from the incorrect use of cP as the unit of viscosity).

If in eqn. 15 the Stokes diameter is calculated as the diameter of a sphere of density 0.73 cm^3/g, which is the measured average partial specific volume of proteins[20], the numerical coefficient in eqn. 16 should be 8% higher than the reported value. This suggests that the Stokes diameter is, on average, 8% larger than the molecular diameter calculated from the molecular weight and density, because a hydration shell is attached to the diffusing protein. This is in agreement with the data reported in Table IV.

Over 75% of the globular proteins have diffusion coefficients within 20% of the value predicted by eqn. 16[20], which provides probably the best estimate for globular proteins.

In all further calculations the values provided by eqn. 16 have been used for estimating the diffusion coefficients of globular proteins.

It must be noted in passing that there is no practical way of changing markedly

TABLE IV

MOLECULAR DIAMETERS OF SOME PROTEINS

Protein	*M (daltons)*	*V (g/ml)*	*d (1)**	*d (2)**	*Difference, d(2) − d(1) (%)*
Cytochrome C, bovine heart	13 370	0.728	38.8	41.7	8
α-Chymotrypsin, bovine pancreas	21 600	0.736	45.3	48.9	8
α-Amylase, *B. subtilis*	96 920	0.717	75.4	80.7	7
Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	136 800	0.725	84.3	90.5	7
Cytochrome C_1, bovine heart	370 500	0.762	115.5	126.1	9

* d (1), Molecular diameter derived from the molar volume (Å); d (2), molecular diameter derived from the diffusion coefficient (Å). The molecules are assumed to be rigid spheres.

the diffusion coefficient of a given compound. Heating the column and replacing the solvent with a less viscous one (of lower molecular weight) are the only possibilities suggested by eqns. 13–16. By nature, samples of polypeptides and proteins can be very susceptible to temperatures above 310–330°K and many lose their biological activity in certain solvents[21]. Hence the choice of solvent is essentially determined by the requirement that the biological activity of the sample be conserved, and it is very difficult to take into account the influence of the solvent on the diffusion coefficient when selecting the mobile phase. Accordingly, an increase in the diffusion coefficient by more than 20–50% cannot be realistically expected. Diffusion coefficients tend, however, to increase faster with increasing temperature than predicted by eqns. 13, 15 and 16[22]. If diffusion is really an activated process, as these results show, and if eqn. 14 provides a correct estimate of the activation energy, an increase in the analysis temperature from 293 to 310°K would afford an increase in D_m of 27% and permit a comparable reduction in the analysis time.

It would be tempting to conclude that proteins should be analysed at the highest temperature compatible with their stability, but other phenomena may tend to make the situation more complex.

Restrictive conditions for SEC optimization

Before we apply the classical results of the optimization theory of LC analytical conditions to the SEC analysis of proteins, we must make sure that we restrict the field of column design and operating parameters to the range of values where SEC can be carried out without undue interference with its proper mechanism.

This problem has been reviewed recently in detail by Giddings[11], who raised several issues. Fundamentally, SEC must be carried out under conditions where (i) the flow contrast between the outside of the packing particles and their inside is maintained, *i.e.*, the fluid in the pores must be stagnant, (ii) the macromolecules in the interstitial space must be unhindered, as in a free space, (iii) the flow pattern must be mild enough to avoid significant shear degradation and (iv) chromatography must be linear.

The first condition imposes a limit on the ratio between intra-particle pore size and particle diameter. The second and third conditions impose limitations on the

particle diameter. It will be assumed here that SEC analyses are carried out with a packing material that is inert towards the proteins and does not retain them by a partition or adsorption processes, and that the sample is small enough that none of the "overloading phenomena" are experienced, in order to satisfy the fourth requirement.

The consequences of these prerequisites are now discussed in further detail. In this discussion M_c represents the maximum molecular weight that must not to be exceeded if the phenomenon discussed is to be avoided or at least if its influence is to be kept reasonably small, and d_{pc} represents the corresponding minimum particle size.

Condition for flow suppression in pores

We have just seen that if we want to increase the performance of SEC analysis, we have to use smaller particles at a higher flow velocity. There are limits in this direction, however. First, it becomes increasingly difficult to manufacture the packing material, and especially to achieve the narrow size distribution that is necessary to take advantage of the possibilities thus offered by the theory.

Assuming that this synthesis is possible, the minimum size of the packing material that can be used is a function of the size of the molecules to be separated. There is a relationship between the size of the pores and the molecular weight of the compounds to be analysed. The molecules of these compounds must neither be completely excluded from these pores nor have free access to their entire volume. On the other hand, if we are to prevent significant flow through the particles across the pores (which should not be swept unless the SEC mechanism is spoiled), we must use particles having diameters that are markedly larger than their pores.

Giddings[11] has shown that, in order to experience a decrease in resolution smaller than 10%, the particle size should be larger than about 30 molecular mean diameters. This ensures that the largest pores, which must be larger than the largest molecules of the samples to be analysed, are small enough compared with the openings around the particles and that the solvent flow velocity inside these pores is negligible compared with the flow velocity in the channels around the particles.

As we have seen in the discussion of the diffusion coefficient, the molecular diameter increases roughly as the square root of the molecular weight for random coil polymers and as the cube root of the molecular weight for globular polymers, like most proteins.

Accordingly, to analyse globular proteins of molecular weight M, without losing resolution because of significant solvent flow through the pores, we must use particles with an average size of at least 32 times the molecular diameter, *i.e.*,

$$d_{pc} = 5.62 \cdot 10^{-7} M^{1/3} \tag{17a}$$

with d_{pc} in cm and M in daltons. The molecular diameter is obtained by combining eqns. 15 and 16. For our purpose, eqn. 17a gives a proper estimate of the minimum particle diameter. For random coil proteins, the molecular diameter can be derived from the data presented by Giddings (ref. 11, Fig. 2), and the minimum particle size becomes

$$d_{pc} = 2.10 \cdot 10^{-7} M^{1/2} \tag{17b}$$

This condition can also be stated in the opposite way: with a packing material of average particle size d_p, it will not be possible to analyse samples containing globular compounds of maximum molecular weight larger than

$$M_c = 5.63 \cdot 10^{18} d_p^3 \tag{18}$$

Although necessary, these conditions are not sufficient. SEC analysis will be possible only if the material has the correct pore size distribution, including pores as large as 2–3 times the maximum allowed molecular diameter (*cf.*, ref. 11, eqn. 19).

Eqns. 17a and 17b show that if we want to analyse proteins with molecular weights up to 1 000 000 daltons, we have to use particles larger than 2.1 μm (random coil proteins) or 0.6 μm (globular proteins). This is markedly smaller than any packing material used previously in this field, and leaves much room for performance improvement.

Avoidance of shear degradation

Degradation may occur during the elution of polymers, owing to shear processes, especially those related to the velocity gradients associated with the rapid opening and closing of the flow paths around packing particles. A significant velocity gradient may occur in these regions, large enough for viscous forces resulting from the interaction between the macromolecule and the solvent molecules flowing around it to generate a shear force sufficient to break the molecule backbone and tear this molecule apart.

Giddings[11] derived a semi-empirical expression for the critical molecular weight below which only insignificant shear degradation occurs. This expression contains an empirical factor, θ, which takes into account the fact that types of shear other than tangential shear take place in packed columns. Using experimental correlations between the critical molecular weight and tangential shear stress obtained in a Couette viscosimeter for polyacrylamide[23] and data on the shear degradation of this polymer, measured by observing the reduction in the intrinsic viscosity of the polymer solution after passage through the column, he found[11] that θ was equal to 1000. This factor may well be higher, however, as Giddings assumed a viscosity of 10 cP for a 0.05–0.1% (w/w) aqueous solution of polyamide in 0.1 *M* $Na_2S_2O_3$. For dilute polymer solutions as typically encountered in SEC, when significant band broadening further dilutes the sample solution, the viscosity approaches that of the pure solvent. In this instance, θ would be close to 10 000.

These asumptions may be more drastic than necessary for proteins, at least for those which have a globular structure. Nevertheless, the condition derived by Giddings states that compounds analysed on an SEC column should have a molecular weight smaller than

$$M'_c = 3.6 \cdot 10^8 \left(\frac{d_p^2}{600\,000\, \eta v D_m} \right)^{0.41} \tag{19}$$

or

$$M'_c = 1.53 \cdot 10^6 \left(\frac{d_p^2}{\eta v D_m} \right)^{0.41} \tag{19a}$$

where M'_c is the critical maximum molecular weight of the solute above which shear degradation may take place. The numerical coefficient in eqn. 19 differs from the coefficient in the corresponding equation in ref. 11, because it has been corrected for a viscosity of the polymer solution of 1 cP.

This equation can be applied to random coil proteins but not to globular ones. The latter are less sensitive to shear degradation than random coils for a given velocity gradient, as they are smaller, more compact and usually held together by several strong bonds, which require larger shear forces to break the molecules and make repair more probable.

As the molecular diffusion coefficient in eqn. 19 is a function of the molecular weight (*cf.*, eqns. 15 and 16) and assuming that this equation applies equally well to random coil proteins and to polystyrene in toluene, it is possible to obtain a simpler relationship for this condition. We use eqn. 14, which seems the most appropriate for this discussion, but correct it for a solvent viscosity of 1 cP. Combining it with eqn. 19 and solving for M'_c gives

$$M'_c = 1.02 \cdot 10^{10} \left(\frac{d_p^2}{\eta v}\right)^{0.53} \tag{20}$$

In the derivation of eqn. 20, the diffusion coefficient has been corrected for the solvent viscosity by using the conventional relationship (*cf.*, eqn. 15 and Table III). The condition expressed by eqn. 20 is not very drastic: with an average particle diameter of 0.5 μm, which is very small by present standards, it would still be possible to analyse random coil polymers with molecular weights up to $0.95 \cdot 10^6$ daltons a reduced velocity of 10. The maximum molecular weight would be $1.8 \cdot 10^6$ daltons at a reduced velocity of 3. It will certainly be possible to analyse much higher molecular weight globular proteins before shear degradation is observed, because globular proteins are more compact, experience a smaller stress along their backbone in a given velocity gradient and because, in most instances, their degradation requires the breaking of several bonds. The entropy of such a process is known to be highly unfavourable.

Accordingly, in most instances, condition 18 will limit column performance more than condition 20.

Exclusion from some interstitial channels

When the pore diameter approaches the diameter of interstitial channels, *i.e.*, the diameter of the narrower openings between particles, the difference between the solvent contained inside the pores of the particles, which constitutes the stationary phase and the solvent around these particles, which constitutes the mobile phase, begins to vanish. The narrowest channel (diameter of the opening between three tangent spheres) in a bed of equal spheres is 6.5 times smaller than the particle diameter. In a random packing of particles with a finite width of size distribution, this ratio is probably smaller. Unger and Roumeliotis[24] showed that in this instance it is probably smaller than 2.

In addition to the obvious problems that can lead to rapid and total column obstruction when part of the sample material has a molecular diameter of the same

order as these interstitial channels, retention occurs only by reference to the behaviour of the molecules in the mobile phase.

Giddings[11] suggested that the diameter of the particles should be at least 16 times larger than that of their larger pores, so the minimum allowable ratio of pore size to molecular diameter would be 2. In such a case, however, the ratio of the diameters of the largest internal pores to the narrowest channels would be *ca.* 0.41, which is too large. From the discussion of the effect of interstitial channel diameter[11], it appears that this ratio should not exceed 0.25.

Accordingly, the particle diameter should be at least about 50 times larger than the molecular diameter of the excluded molecules.

Table V gives the minimum particle size as a function of molecular weight. Obviously, smaller particles could be used, but then a decrease in retention and resolution for the heavier fraction of the sample will be experienced.

TABLE V

MINIMUM PARTICLE SIZE

M (daltons)	*Random coil proteins**		*Globular proteins***	
	d (Å)	d_p *(μm)*	*d (Å)*	d_p *(μm)*
1000	26	0.13	16	0.08
3000	45	0.22	24	0.12
10 000	82	0.41	35	0.18
30 000	141	0.71	51	0.25
100 000	258	1.29	76	0.38
300 000	447	2.23	109	0.55
1 000 000	816	4.08	163	0.82

* d, Molecular diameter ($0.816\sqrt{M}$) (Å). d_p, Minimum particle size ($50d$; *cf.*, section *Exclusion from some interstitial channels*).

** d, Molecular diameter (from specific volume and molecular weight). d_p, Minimum particle size ($50d$).

It should be noted that separations of macromolecules can still be performed when the pore diameter approaches the size of the interstitial channels, although in this instance the porous space loses the nature of a stationary phase. Steric exclusion from the low-velocity regions near the surface of the particles leads to the earlier elution of larger molecules, a process known as hydrodynamic chromatography[25,26]. Although the elution order is the same than in SEC, this mechanism gives a markedly lower separation power. Accordingly, for optimum resolution, the minimum particle sizes indicated in Table V should be respected.

Optimization of particle size and flow-rate

Optimization in liquid chromatography is ambiguous. It can be carried out to minimize retention time at a constant resolution or separation power, or to maximize resolution or column efficiency at a constant analysis time[16]. There are a number of possible intermediate trade-offs between resolution and efficiency that are difficult to formalize but important to consider, as experimentalists are understandably reluctant

to make frequent column changes. Further, some other constraints, which are difficult to quantify, come into play. The value of the inlet pressure is a good case in point. The pressure is of no or little importance when it is low, but it cannot exceed some threshold value, depending on the pump used or on the mechanical stability of the packing material.

Rather than trying to derive theoretically optimum conditions, the validity of which would be contingent upon the exact sample being analysed, we have calculated the performance of a number of hypothetical columns operated under different conditions. This serves our aim, which is to illustrate the potential advantages to be derived from the preparation and use of smaller particles.

In the following calculations we have restricted the range of variation of the experimental parameters so that only realistic values are used for column dimensions (column diameter 1 cm, length between 3 and 60 cm) and reasonable results are obtained for analysis time and inlet pressure. Average particle sizes between 0.5 and 10 μm have been considered. In all instances, the molecular weight was kept below 1 000 000 daltons, as there are few proteins with larger molecular weights. The minimum particle size considered is that derived in Table V, although this limit is not well established. Calculations have been restricted to cases where the analysis time is less than a few hours and the inlet pressure smaller than about 500 atm. Such a large pressure would certainly not be realistic with most polymer gels, but attractive silica-based materials do exist now for the SEC analysis of proteins.

In the calculations made in order to obtain the data in Tables VI–XI, a value of 10 was chosen in almost all instances for the reduced velocity of the solvent, in agreement with the current practice of trading a reasonable decrease in column efficiency for a marked reduction in analysis time. The corresponding reduced plate height has been assumed to be 3. These numerical values correspond to $a = 1.25$, $b = 1.4$ and $c = 0.01$–0.02 in eqn. 9, which are typical of the current performance of columns packed with good silica-based material. A value of 1 cP has been selected for the solvent viscosity, that of water. The effect of a lower viscosity was also studied. The diffusion coefficients were derived from eqn. 16 (globular proteins, Tables VI–IX and XI) or eqn. 14 (random coil proteins, Table X).

Results of the optimization calculations

The results for globular proteins are summarized in Tables VI–IX and those dealing with random coil proteins, which are given much less importance, in Table X. In each table, calculations have been made for proteins with molecular weights between 1000 and 1 000 000 daltons whenever possible, or with a narrower range, if some of the exclusion rules detailed in the previous section apply.

Table VI relates to the large or moderate sized particles that are currently available for SEC. They are a basis for assessing the value of our theoretical approach, and illustrate the considerable improvement in performance (combined large reduction in analysis time and marked increase in separation power) made possible by the 10-μm particles that appeared a few years ago. Table VI explains, for example, how reducing the particle size from 40 to 10 μm has permitted both a four-fold decrease in analysis time and a four-fold increase in column efficiency. With 10-μm particles, however, analysis requiring a good column efficiency still take several hours (*cf.*, Table VI).

TABLE VI

CALCULATED PERFORMANCE OF SEC SEPARATIONS OF GLOBULAR PROTEINS USING CONVENTIONAL PARTICLES

Column diameter, 1.0 cm. Total porosity, 0.8. Interstitial porosity, 0.4. Reduced velocity, 10. Reduced plate height, 3. Solvent viscosity, 1 cP. Specific permeability, 0.001. Temperatures, 293°K. D_m calculated after eqn. 16.

M (daltons)	*L (cm)*	d_p *(μm)*	v_0 *(cm/sec)*	*F (μl/min)*	t_m *(h)*	*P (bar)*
1000	60	10.0	0.0244	460.6	1.4	7.3
10 000	60	10.0	0.0113	213.8	2.9	3.4
100 000	60	10.0	0.0053	99.2	6.3	1.6
1 000 000	60	10.0	0.0024	46.1	13.6	0.7
N = 20 000						
1000	60	20.0	0.0122	230.3	2.7	0.9
10 000	60	20.0	0.0057	106.9	5.9	0.4
100 000	60	20.0	0.0026	49.3	12.7	0.2
1 000 000	60	20.0	0.0012	23.0	27.3	0.1
N = 10 000						
1000	60	40.0	0.0061	115.2	5.5	0.1
10 000	60	40.0	0.0028	53.4	11.8	0.1
100 000	60	40.0	0.0013	24.8	25.3	0
1 000 000	60	40.0	0.0006	11.5	54.6	0
N = 5000						
1000	60	60.0	0.0041	76.8	8.2	0
10 000	60	60.0	0.0019	35.6	17.6	0
100 000	60	60.0	0.0009	16.5	38.0	0
1 000 000	60	60.0	0.0004	7.7	81.8	0
N = 3300						

Table VII indicates the performance that could be expected from small particle size packing material, having dimensions comparable to those of the stationary phases now in use in HPLC, *i.e.*, 3–10 μm. The technology to prepare these products with a reasonably narrow size distribution and to pack efficient columns with them is available. The preparation of materials in this size range, which would be suitable for SEC analysis of proteins, is currently under investigation.

The use of 10-μm particles in the SEC separation of large peptides and of proteins does not require that the packing material used to prepare the column exhibit unusual mechanical characteristics, as the pressure needed is modest, even for the lightest compounds. Particles of size 5 and 3 μm offer a very significant decrease in analysis time at constant column efficiency (*cf.*, Table VII). The efficiency available is large, at least 20 000 plates, which is probably sufficient for the solution of most practical problems. For globular proteins, the analysis of 100 000 dalton compounds takes more than 6 h with 10-μm particles, about 1.5 h with 5-μm particles and less than 40 min with 3-μm particles (*cf.*, Table VII). Samples containing random coil proteins with a molecular weight larger than 300 000 daltons could prove difficult to analyse with 3-μm particles (*cf.*, Table V and the corresponding discussion). At the other end of the molecular weight range, the analysis of peptides lighter than 3000 daltons becomes difficult because of the requirement of too high a pressure.

TABLE VII

PREDICTED PERFORMANCE OF SEC SEPARATIONS OF GLOBULAR PROTEINS ON COLUMNS OF SMALL SIZE PARTICLES

Retention time $t_m = L/u$ of molecules which have free access to all pores. Column diameter, 1.0 cm. Total porosity, 0.80. Interstitial porosity, 0.4. Reduced velocity, 10. Reduced plate height, 3. Solvent viscosity, 1 cP. Specific permeability, 0.001. Temperature, 293°K. Constant efficiency, *ca.* 20 000 plates. D_m calculated after eqn. 16.

M (daltons)	*L (cm)*	d_p *(μm)*	v_0 *(cm/sec)*	*F (μl/min)*	t_m	*P (bar)*
1000	60	10.0	0.0244	460.6	1.4 h	7.33
3000	60	10.0	0.0169	319.4	2.0 h	5.08
10 000	60	10.0	0.0113	213.8	2.9 h	3.40
30 000	60	10.0	0.0079	148.2	4.2 h	2.36
100 000	60	10.0	0.0053	99.2	6.3 h	1.58
300 000	60	10.0	0.0037	68.8	9.1 h	1.10
1 000 000	60	10.0	0.0024	46.1	13.6 h	0.73
1000	30	5.0	0.0489	921.2	20.5 min	29.32
3000	30	5.0	0.0339	638.7	29.5 min	20.33
10 000	30	5.0	0.0227	427.6	44.1 min	13.61
30 000	30	5.0	0.0157	296.5	1.1 h	9.44
100 000	30	5.0	0.0105	198.5	1.6 h	6.32
300 000	30	5.0	0.0073	137.6	2.3 h	4.38
1 000 000	30	5.0	0.0049	92.1	3.4 h	2.93
1000	20	3.0	0.0815	1535.4	8.2 min	90.50
3000	20	3.0	0.0565	1064.6	11.8 min	62.75
10 000	20	3.0	0.0378	712.7	17.6 min	42.01
30 000	20	3.0	0.0262	494.1	25.4 min	29.13
100 000	20	3.0	0.0175	330.8	38.0 min	19.50
300 000	20	3.0	0.0122	229.4	54.8 min	13.52
1 000 000	20	3.0	0.0081	153.5	1.4 h	9.05

Tables VIII–X deal with very fine (micro) particles, with dimensions between 3 and 0.5 μm. Nothing in this range has been systematically used in HPLC yet, in spite of some impressive pioneering work carried out by Dewaele and Verzele[27] and Unger *et al.*[28]. Two situations have been investigated for globular proteins, depending on whether a high efficiency is sought or a moderate efficiency is sufficient. In the last case, the analysis time would be five times shorter and (which may be more important for the feasability of the work) the pressure required would be also five times smaller at a constant mobile phase velocity. To illustrate more clearly the tremendous improvement in the performance of SEC analysis that could be brought about by the use of very small particles, a further reduction in pressure is achieved by using a reduced velocity of 3. In this way, part of the gain in analysis time is sacrificed for a reduction in pressure and a moderate increase (about 20–25%) in column efficiency. Nevertheless, all the analyses mentioned in Table IX are achieved in less than 1 h. It takes less than 15 min to analyse (with a 10 000 plate column) globular proteins with a molecular weight of 300 000 daltons when 2-μm particles are used.

The results in Tables VIII and IX also illustrate how the range of molecular weights of peptides and proteins that can be analysed efficiently becomes narrower

TABLE VIII

CALCULATED PERFORMANCE OF SEC SEPARATIONS OF GLOBULAR PROTEINS ON COLUMNS USING MICRO PARTICLES

Retention time $t_m = L/u$ of molecules which have free access to all pores. Column diameter, 1.0 cm. Total porosity, 0.80. Interstitial porosity, 0.40. Reduced velocity, 10. Reduced plate height, 3. Solvent viscosity, 1 cP. Specific permeability, 0.001. Temperature, 293°K. Constant efficiency, 50 000 plates. D_m calculated after eqn. 16.

M (daltons)	*L (cm)*	d_p *(cm)*	v_0 *(cm/sec)*	*F (μl/min)*	t_m *(min)*	*P (bar)*
1000	45	3.0	0.0815	1535.4	18.4	203.6
3000	45	3.0	0.0565	1064.6	26.6	141.2
10 000	45	3.0	0.0378	712.7	39.7	94.5
30 000	45	3.0	0.0262	494.1	57.2	65.5
100 000	45	3.0	0.0175	330.8	85.5	43.9
300 000	45	3.0	0.0122	229.4	123.3	30.4
1 000 000	45	3.0	0.0081	153.5	184.2	20.4
1000	30	2.0	0.1222	2303.0	8.2	458.2
3000	30	2.0	0.0847	1596.8	11.8	317.7
10 000	30	2.0	0.0567	1069.0	17.6	212.7
30 000	30	2.0	0.0393	741.2	25.4	147.5
100 000	30	2.0	0.0263	496.2	38.0	98.7
300 000	30	2.0	0.0183	344.0	54.8	68.4
1 000 000	30	2.0	0.0122	230.3	81.8	45.8
30 000	15	1.0	0.0786	1482.4	6.4	589.8
100 000	15	1.0	0.0526	992.3	9.5	394.8
300 000	15	1.0	0.0365	688.1	13.7	273.8
1 000 000	15	1.0	0.0244	460.6	20.5	183.3

and narrower when one attempts to use smaller and smaller particles. On the one hand, the actual velocity at which the solvent must flow through the column in order to achieve a reduced velocity in the optimum range of 3–10 requires the use of inlet pressures that are too high when peptides are analysed, and on the other hand the restriction on the dimensions of the particles used, whose diameter must be at least about 50 times the molecular diameter of the analyte, restricts the application to the larger proteins.

Hence it can be seen from Table IX and eqn. 12 that it would be almost impossible to use a high-efficiency column packed with 0.5-μm particles. The pressure would be too high, even at a reduced velocity of 3, for analysing proteins with a molecular weight lower than $2 \cdot 10^4$–$3 \cdot 10^4$ daltons, and difficulties due to exclusion of the globular proteins from some interstitial channels could begin at molecular weights above about $3 \cdot 10^5$ daltons (*cf.*, Table V).

Similar conclusions can be derived from the results of the calculations concerning the random coil proteins (*cf.*, Table X), the main difference being that the requirement that no significant exclusion from interstitial channels occurs prevents the use of particles that are as small as those used for the separation of globular proteins (*cf.*, Table V). No problem of this kind would be expected, however, with the most common proteins, in the 10 000–100 000 dalton range, for the analysis of which 1-μm particles could be used.

It is striking that only moderate pressures are required in most of the analyses

TABLE IX

CALCULATED PERFORMANCE OF SEC SEPARATIONS OF GLOBULAR PROTEINS ON COLUMNS USING MICRO PARTICLES

Retention time $t_m = L/u$ of molecules which have free access to all pores. Column diameter 1.0 cm. Total porosity, 0.80. Interstitial porosity, 0.40. Reduced velocity, 10. Reduced plate height, 3. Solvent viscosity, 1 cP. Specific permeability, 0.001. Temperature, 293°K. Constant efficiency, 10 000 plates. D_m calculated after eqn. 16.

M (daltons)	*L (cm)*	d_p *(μm)*	v_0 *(cm/sec)*	*F (μl/min)*	t_m *(min)*	*P (bar)*
1000	9	3.0	0.0815	1535.4	3.7	40.73
3000	9	3.0	0.0565	1064.6	5.3	28.24
10 000	9	3.0	0.0378	712.7	7.9	18.90
30 000	9	3.0	0.0262	494.1	11.4	13.11
100 000	9	3.0	0.0175	330.8	17.1	8.77
300 000	9	3.0	0.0122	229.4	24.7	6.08
1 000 000	9	3.0	0.0081	153.5	36.8	4.07
1000	6	2.0	0.1222	2303.0	1.6	91.64
3000	6	2.0	0.0847	1596.8	2.4	63.54
10 000	6	2.0	0.0567	1069.0	3.5	42.53
30 000	6	2.0	0.0393	741.2	5.1	29.49
100 000	6	2.0	0.0263	496.2	7.6	19.74
300 000	6	2.0	0.0183	344.0	11.0	13.69
1 000 000	6	2.0	0.0122	230.3	16.4	9.16
1000	3	1.0	0.2444	4606.1	0.4	366.54
3000	3	1.0	0.1694	3193.7	0.6	254.14
10 000	3	1.0	0.1134	2138.0	0.9	170.13
30 000	3	1.0	0.0786	1482.4	1.3	117.96
100 000	3	1.0	0.0526	992.3	1.9	78.97
300 000	3	1.0	0.0365	688.1	2.7	54.75
1 000 000	3	1.0	0.0244	460.6	4.1	36.65
10 000	1.5	0.5	0.2268	4275.9	0.2	680.53
30 000	1.5	0.5	0.1573	2964.7	0.3	471.85
100 000	1.5	0.5	0.1053	1984.7	0.5	315.87
300 000	1.5	0.5	0.0730	1376.1	0.7	219.02
1 000 000	1.5	0.5	0.0489	921.2	1.0	146.62

described in Table IX. It is only when (i) the use of extremely small particles is contemplated, (ii) very high efficiencies are required for the achievement of difficult separations or (iii) low-molecular-weight peptides must be analysed that the pressures become larger than 50 atm.

Influence of solvent viscosity and temperature on column performance

Table XI gives the results of a simulation made on the column performance for the separation of globular proteins, using values of the solvent viscosity between 0.4 and 1 cP. The analysis time decreases markedly with decreasing solvent viscosity, but the pressure remains unchanged. This result derives from the relationship between diffusion coefficient and viscosity. When the viscosity decreases, the diffusion coefficient increases in proportion to the inverse of the viscosity (*cf.*, eqns. 13–16). The product of the viscosity and the diffusion coefficient remains constant. The inlet pressure is proportional to this product, as shown by eqn. 12.

TABLE X

CALCULATED PERFORMANCE OF SEC SEPARATIONS OF RANDOM COIL PROTEINS ON COLUMNS USING VARIOUS KINDS OF PARTICLES

Column diameter, 1 cm. Total porosity, 0.80. Interstitial porosity, 0.40. Reduced velocity, 10. Reduced plate height, 3. Solvent viscosity, 1 cP. Specific permeability, 0.001. Temperature, 293°K. D_m calculated after eqn. 14.

M (daltons)	*L (cm)*	d_p *(μm)*	v_0 *(cm/sec)*	*F (μl/min)*	t_m	*P (bar)*
1000	60	50.0	0.0122	202.5	2.7 h	0.1
10 000	60	50.0	0.0034	56.7	9.8 h	0
100 000	60	50.0	0.0010	15.9	34.9 h	0
1 000 000	60	50.0	0.0003	4.4	124.8 h	0
N = 4000						
1000	60	10.0	0.0609	1012.4	32.8 min	18.3
10 000	60	10.0	0.0171	283.4	2.0 h	5.1
100 000	60	10.0	0.0048	79.3	7.0 h	1.4
1 000 000	60	10.0	0.0013	22.2	25.0 h	0.4
N = 20 000						
1000	30	5.0	0.1218	2024.8	8.2 min	73.1
10 000	30	5.0	0.0341	566.7	29.3 min	20.5
100 000	30	5.0	0.0095	158.6	1.7 h	5.7
1 000 000	30	5.0	0.0027	44.4	6.2 h	1.6
N = 20 000						
1000	20	3.0	0.2031	3374.6	3.3 min	225.6
10 000	20	3.0	0.0568	944.5	11.7 min	63.1
100 000	20	3.0	0.0159	264.4	41.9 min	17.7
1 000 000	20	3.0	0.0045	74.0	2.5 h	4.9
N = 22 000						
3000	20	2.0	0.1659	2757.2	4.0 min	414.8
10 000	20	2.0	0.0853	1416.8	7.8 min	213.1
30 000	20	2.0	0.0464	771.7	14.4 min	116.1
100 000	20	2.0	0.0239	396.6	27.9 min	59.7
300 000	20	2.0	0.0130	216.0	51.3 min	32.5
N = 33 000						

The influence of temperature is much less significant. Assuming a linear dependence of the diffusion coefficient on the absolute temperature, we observe a reduction in the analysis time of about 20% with an increase in temperature from 0 to 60°C (*cf.*, Table XI). If, however, diffusion is an activation process and eqn. 14 gives a correct estimate of the activation energy, the decrease in analysis time with increase in temperature could be much more significant. Probably the temperature dependence of the diffusion coefficient is the result of a similar dependence of the viscosity of the solvent. Then, again, the inlet pressure remains constant and the analysis time decreases in the same proportion as the viscosity[22].

Programmed flow-rate SEC analysis of proteins

Finally, it should be observed that, because the optimum column performance is achieved at a reduced flow velocity between 3 and 10, the value of the optimum

TABLE XI

INFLUENCE OF VISCOSITY AND TEMPERATURE ON THE PERFORMANCE OF SEC SEPARATIONS OF GLOBULAR PROTEINS

Same conditions as for Table VII.

Parameter	*M (daltons)*	*η (cP)*	*T (°K)*	*L (cm)*	d_p *(μm)*	v_0 *(cm/sec)*	t_m *(min)*	*P (bar)*
Solvent viscosity	1000	1.0	293	30	5	0.0489	20.5	29.3
	10 000	1.0	293	30	5	0.0227	44.1	13.6
	100 000	1.0	293	30	5	0.0105	95.0	6.3
	1 000 000	1.0	293	30	5	0.0049	204.6	2.9
	1000	0.8	293	30	5	0.0611	16.4	29.3
	10 000	0.8	293	30	5	0.0284	35.3	13.6
	100 000	0.8	293	30	5	0.0132	76.0	6.3
	1 000 000	0.8	293	30	5	0.0061	163.7	2.9
	1000	0.6	293	30	5	0.0815	12.3	29.3
	10 000	0.6	293	30	5	0.0378	26.4	13.6
	100 000	0.6	293	30	5	0.0175	57.0	6.3
	1 000 000	0.6	293	30	5	0.0081	122.8	2.9
	1000	0.4	293	30	5	0.1222	8.2	29.3
	10 000	0.4	293	30	5	0.0567	17.6	13.6
	100 000	0.4	293	30	5	0.0263	38.0	6.3
	1 000 000	0.4	293	30	5	0.0122	81.8	2.9
Temperature	1000	1.0	273	30	5	0.0455	22.0	27.3
	10 000	1.0	273	30	5	0.0211	47.3	12.7
	100 000	1.0	273	30	5	0.0098	101.9	5.9
	1 000 000	1.0	273	30	5	0.0046	219.6	2.7
	1000	1.0	313	30	5	0.0522	19.2	31.3
	10 000	1.0	313	30	5	0.0242	41.3	14.5
	100 000	1.0	313	30	5	0.0112	88.9	6.7
	1 000 000	1.0	313	30	5	0.0052	191.5	3.1
	1000	1.0	333	30	5	0.0555	18.0	33.3
	10 000	1.0	333	30	5	0.0258	38.8	15.5
	100 000	1.0	333	30	5	0.0120	83.6	7.2
	1 000 000	1.0	333	30	5	0.0056	180.0	3.3

actual flow velocity depends on the diffusion coefficient and hence on the molecular weight. The optimum flow velocity, or flow-rate of the column, decreases steadily with increasing molecular weight.

Accordingly, if the column is operated at constant flow-rate, a compromise must be chosen. Depending on the circumstances, this will favour the lowest or the highest molecular weight components of the mixture. If the ratio of the highest to the lowest molecular weight of the components in the sample is 100, the ratio of the corresponding optimum velocities is 4.6 for globular proteins and 12.8 for random coil proteins. In the first instance, the effect on the resolution will be small, provided that the compromise is properly chosen, the velocity being nearly optimum for the lightest components of the mixture (*cf.*, eqn. 8). It will be too large for the heaviest components, but still acceptable, as at first the column HETP increases only slowly with increasing reduced flow velocity (*cf.*, Table I). With random coil proteins, it is difficult to find a satisfactory compromise for a molecular weight ratio greater than 25.

An alternative approach is to programme the flow-rate. As in SEC high-molecular-weight compounds are eluted first, followed by compounds of smaller and smaller molecular weight, the analysis should be started at a flow velocity corresponding to the optimum for the compounds eluted first and then be progressively increased to a maximum value close to the optimum flow velocity for the last eluted compounds. In this way, as in constant flow-rate analyses, no compound experiences the optimum velocity that corresponds to its molecular weight during its entire elution, but the compromise is closer to the optimum during most of the analysis. Owing to the long analysis times that one has to face when analysing mixtures containing components with really high molecular weights, the reduction in analysis time can be significant, especially when long, efficient columns packed with particles of intermediate or large average size are used.

Peak capacity

The peak capacity is the most general description of column performance in chromatography[29]. The peak capacity, n, of a column is given by the equation derived by Grushka[30]:

$$n = 1 + \frac{N^{1/2}}{4} \cdot \ln (V_{R2}/V_{R1}) \tag{21}$$

where N is the average plate number of the column and V_{R1} and V_{R2} are the retention volumes of the first and the last peak considered, respectively.

The peak capacity is the number of peaks with a resolution of unity that can be placed on the chromatogram between the first and the last peaks. It is the largest number of components that in principal can be resolved by the column. As in SEC there is a systematic variation of the retention time with increasing molecular weight, the statistical limitations derived by Davis and Giddings[31] on the number of compounds that one can hope to separate in actual practice with a column of given peak capacity do not apply.

The concept of peak capacity has not yet been used much in the SEC analysis of proteins, because this method is mainly used for the analysis of polymers that are

more or less dispersed (ref. 9, p. 102). As proteins are well defined compounds, it becomes a valuable tool, as it is in HPLC.

As explained above (*cf.* first section, *Flow velocity*; and eqn. 6) the maximum ratio V_{R2}/V_{R1} is equal to $(V_0 + V_i)/V_0$ in SEC in practice of the order of 2–2.5. Consequently, the peak capacity is closely approximated by $0.17\sqrt{N}$. Accordingly, whereas conventional SEC permits the achievement of peak capacities that rarely exceed about 10, it seems that the use of very small particles, in the 1- to 2-μm range, will permit the achievement of values between 30 and 40 within about 1 h.

EXPERIMENTAL AND RESULTS

So far, no experiments have been directly performed to check the validity of the theoretical results detailed above. However, a number of reports from various sources support our general conclusions, together with the well known historical trends in HPLC. When this chromatographic technique started in the late 1960s, 60–80-μm particles were most often used, whereas most applications are now carried out mainly with 3–10-μm particles. The same fundamental phenomena call for the use of small particles in SEC, the only present hindrance being the lack of availability of suitable stationary phases. Synthesis of good stationary phases is difficult, as is their packing, but significant efforts are now being made in this field.

There are few data in the literature that would permit one to ascertain that the concepts of reduced plate height and velocity may be applied in SEC and that a similar *h versus u* plot is obtained in SEC and in HPLC. Table XII shows data derived from a previous study, in which plots of actual plate height *versus* actual velocity, obtained for a 10-μm particle column with three compounds of different molecular weight were reported[32]. The data are imprecise, but the comparison shows that the assumptions that we made above are reasonable.

TABLE XII

INFLUENCE OF MOLECULAR WEIGHT ON COLUMN PERFORMANCE

Data from Fig. 1, ref. 32.

Sample	*M (daltons)*	D_m *(cm²/sec)*	v_{opt} *(cm/sec)*	v_0
Tyrosine	180	5.600	0.01380	2.46
Myoglobin	17 000	0.950	0.00194	2.04
Albumin	68 000	0.599	0.00075	1.25

Some preliminary tests of our results can be made by comparing results obtained with the classical gel particles, typically 50–60 μm in average diameter, and with the second generation of packing material, which appeared a few years ago. Typical results are described in Figs. 1–4 and summarized in Table XIII. Several important conclusions can be drawn.

First, although relatively low flow-rates of mobile phase were used, it is only in Fig. 1 that a satisfactory efficiency and separation power could be achieved[32]. The reduced flow velocity is still ten times higher than the value corresponding to maxi-

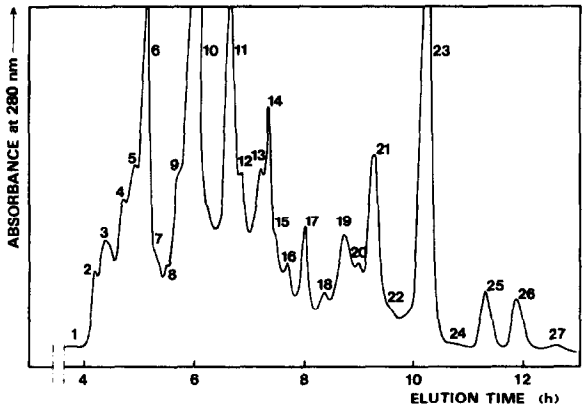

Fig. 1. Very high-resolution SEC analysis of a degraded mixture of ceruloplasmin and L-amino acid oxidase. Sample, 200 μl of a solution containing 1 mg of degraded mixture in 1 m*M* Na_2HPO_4–0.1 *M* KCl–0.05% NaN_3 (pH 7.4). Column: packing, LKB 2135 Ultropac TSK SW 3000; dimensions, 60 × 0.75 cm I.D.; flow velocity, 40 μl/min; eluent, same as sample solvent. (Reproduced with permission from ref. 32.)

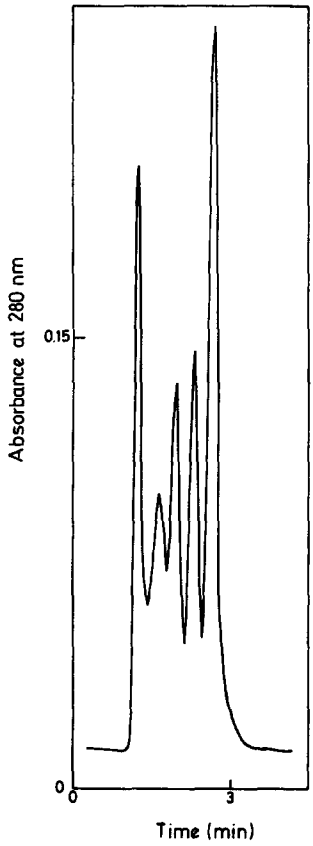

Fig. 2. Very high-speed SEC analysis of a protein mixture. Sample: thyroglobulin, γ-globulin, ovalbumin, myoglobulin and vitamin B_{12}. Column: packing, LKB 2135 Ultropac TSK; dimensions, 6.0 × 0.75 cm I.D.; flow velocity, 1.0 ml/min; eluent, 0.1 *M* $NaPO_4$ buffer (pH 6.8). (Reproduced with permission from ref. 33.)

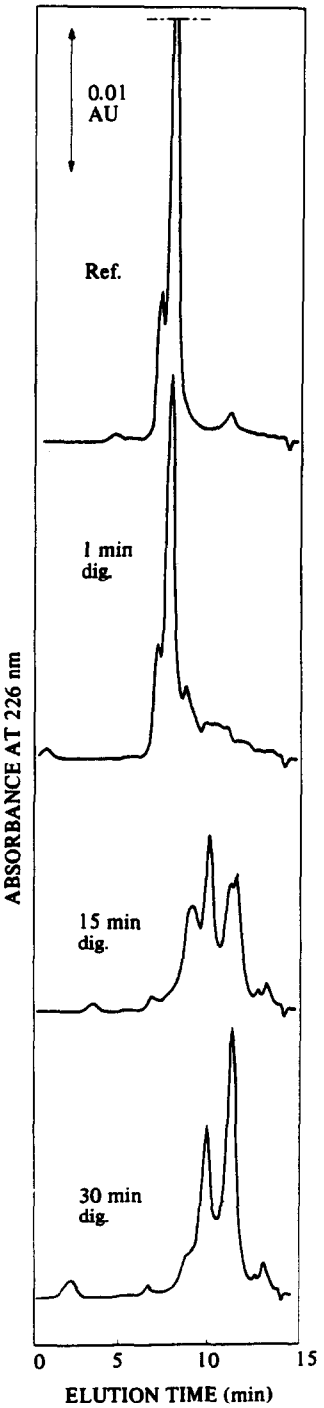

Fig. 3. High-speed SEC analysis. Sample: 50 μl from an enzymic digestion of staphylococcal protein A, after various digestion (dig.) times. Column: packing, LKB Ultropac TSK G 2000 SW; dimensions, 30 × 0.75 cm I.D.; flow velocity, 1.0 ml/min; eluent, 0.1 *M* Tris–HCl (pH 7.5). (Reproduced with permission from ref. 34.)

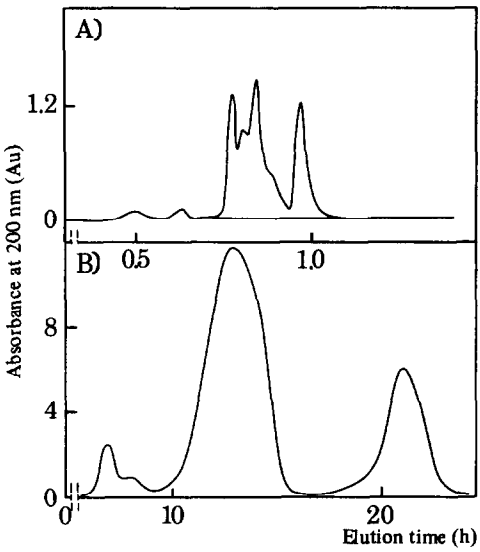

Fig. 4. Comparison between high-performance SEC and soft matrix gel filtration. Sample: freeze-dried whole venom from black manta. Eluent: 0.1 *M* NH_4OAc (pH 6.5). Column A: packing, LKB Ultropac TSK G 2000 SWG; dimensions, 60 × 2 cm I.D.; flow velocity, 3.2 ml/min. Column B: packing, Dextran beads; dimensions, 145 × 2 cm I.D.; flow velocity, 0.4 ml/min. (Reproduced with permission from ref. 35.)

mum efficiency, but in view of the analysis time and the reduced efficiency achieved, the compromise can be considered satisfactory. In the other instances, the reduced velocity far exceeds what would be considered reasonable in HPLC. Comparison of Fig. 1 with Figs. 3 and 4a shows that the decrease in resolution, although severe, is accompanied by a considerable reduction in analysis time.

In Fig. 1, the retention volume of the last eluted compound (No. 27) is 30 ml, while the geometrical column volume is 26.5 ml. The liquid phase hold-up volume is around 21–22 ml[14]. It is possible that some adsorption takes place, contributing to the retention. It seems more probable, however, that the flow velocity determination (one significant figure in ref. 33) is imprecise. If this is so, the actual reduced flow velocity would be lower than that indicated in Table XIII, in agreement with the excellent efficiency observed.

The chromatogram in Fig. 2 was obtained under extreme conditions, but it illustrates very clearly that fast SEC analysis is a real possibility[34]. In this instance, a very short column (6 cm) is operated at an extremely high velocity of 0.038 cm/sec. This corresponds to a reduced velocity of 54 for ovalbumin ($M = 4.3 \cdot 10^4$ daltons) and a reduced plate height of more than 5.2. The efficiency achieved is small but sufficient to achieve the separation of four proteins and an additional non-retained compound, well spread over the molecular weight range separated by the packing material used.

The chromatogram shown in Fig. 3 demonstrates the analytical possibilities of SEC in following relatively rapid reactions[35]. Although the conditions under which the column is operated here are less extreme, the very high reduced flow velocity

TABLE XIII

COMPARISON BETWEEN THEORETICAL PREDICTIONS AND EXPERIMENTAL RESULTS

Flow-rate, particle size and column length are from the references cited. The reduced velocity is derived from the flow velocity and estimated molecular weight. The reduced plate height is calculated (eqn. 9). Theoretical values of retention time and efficiency are derived from these parameters.

Fig. No.	*F (ml/min)*	*u (cm/sec)*	d_0 *(μm)*	*M (daltons)*	*v*	*h*	*L (cm)*	*Theoretical*			*Experimental*		*P* (atm)*	*Ref.*
								N	*n*	t_R *(min)*	*N*	t_R *(min)*		
1	0.040	0.0015	10	200 000	36.1	3.75	60	16 002	22.9	662.7	14 500	780	0.91	32
2	1.000	0.0377	10	100 000	716.6	16.12	7.5	465	4.7	3.3	810	3	2.83	33
3	1.000	0.0377	10	7000	295.3	9.62	30	3118	10.7	13.3	2600	14	11.32	34
4	3.200	0.0147	10	50 000	221.5	8.28	60	7248	15.7	68.1	5000 (?)	60	8.81	35
4	0.400	0.0018	50	50 000	138.4	6.58	145	4408	12.5	1316.1	na	1260	0.11	35

* Pressure is calculated (*cf.*, eqn. 12) assuming a solvent viscosity of 1 cP.

results in a poor column efficiency. A three times larger efficiency and a 1.7 times better resolution would be achieved at a ten times smaller flow velocity. The analysis would then take 2.5 h.

Comparison between Fig. 4a and b clearly shows the outcome of a five-fold reduction in particle diameter[33]. The column used with the finer particles is less than half the length and the flow velocity is almost ten times faster. Although, compared with the other column (*cf.*, Fig. 4b), the reduced velocity at which the column packed with the smaller particles (*cf.*, Fig. 4a) is operated is greater (1.6 times), this small particle column still offers a larger efficiency (1.65 times) and the end result is a markedly better separation achieved in a much shorter time.

The analysis times calculated from the equations discussed above are in excellent agreement with those observed in the chromatograms in Figs. 1–4. The values of the efficiency derived from some of the peaks compare favourably with those predicted, especially if one takes into account the very large error that results from the combination of the facts that the plate numbers were measured from printed chromatograms, which are usually very small, and that most of the prominent peaks, which can best be used for this task, are probably composite. This is the reason why no efficiency could be measured for the chromatogram in Fig. 4b.

Finally, we observe in Table XIII that the pressure required to operate the columns is still very low, only a few atmospheres, except in Fig. 3 where it is 11 atm. The pressure will remain acceptable with a marked reduction in particle diameter if shorter columns are operated at comparable or lower velocities. In this instance, the efficiency will increase markedly (much lower reduced velocity and lower particle diameter), while the analysis time is slightly reduced (shorter column). If the columns can be operated at high pressure, then a considerable improvement in performance can be expected, as shown in Tables VIII and IX.

These results demonstrate the great advantages achieved by reducing the particle diameter. One further gain, illustrated by comparison between Fig. 4a and b, is that the use of smaller particles, resulting in the preparation of more efficient columns, permits greater flexibility. Some of the additional efficiency can be traded for a further reduction in analysis time. The same column could be used at a lower flow velocity to achieve a much larger peak capacity, if needed.

The use of small or very small particles in SEC would permit much greater flexibility than is presently available. As in HPLC, the analyst would have a choice between short columns, operated at medium or high pressures, for the rapid analysis of relatively simple mixtures, long columns, operated at high pressures, for the analysis of complex mixtures and columns of medium length and performance, working at moderate pressure, for the safe reproducible achievement of routine analyses.

ACKNOWLEDGEMENT

We are grateful to Dr. Jorgen Sjodhal (Bromma, Sweden) for very informative discussions and suggestions.

REFERENCES

1 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657.
2 T. Kremmer and L. Boross, *Gel Chromatography, Theory, Methodology, Applications*, Wiley, New York, 1979.
3 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 185 (1980) 305.
4 P. Roumeliotis and K. K. Unger, *J. Chromatogr.*, 218 (1981) 535.
5 F. E. Regnier, *Science*, 222 (1983) 245.
6 J. C. Giddings, *Dynamics of Chromatography*, Marcel Dekker, New York, 1965.
7 J. H. Knox and F. McLennan, *J. Chromatogr.*, 185 (1979) 289.
8 J. C. Giddings, L. M. Bowman, Jr. and M. N. Myers, *Macromolecules*, 10 (1977) 443.
9 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size Exclusion Liquid Chromatography*, Wiley, New York, 1979.
10 J. H. Knox, in C. F. Simpson (Editor), *Practical High Performance Liquid Chromatography*, Heyden, London, 1976.
11 J. C. Giddings, *Advan. Chromatogr.*, 20 (1982) 217.
12 H. Engelhardt and G. Ahr, *J. Chromatogr.*, 282 (1983) 385.
13 J. H. Knox and M. Saleem, *J. Chromatogr. Sci.*, 7 (1969) 614.
14 S. M. E. Himmel and Ph. G. Squire, *Int. J. Peptide Res.*, 17 (1981) 365.
15 J. C. Giddings, in E. Heftmann (Editor), *Chromatography*, Van Nostrand, New York, 3rd. ed., 1976, Ch. 3, p. 27.
16 G. Guiochon, in Cs. Horváth (Editor), *HPLC–Advances and Perspectives*, Wiley, New York, 1982, p. 1.
17 J. H. Knox, G. R. Laird and P. A. Raven, *J. Chromatogr.*, 122 (1976) 129.
18 C. R. Wilke and P. Chang, *AIChE J.*, 1 (1955) 264.
19 J. C. Giddings, K. D. Caldwell and M. N. Myers, *Macromolecules*, 9 (1976) 106.
20 M. E. Young, P. A. Carroad and R. L. Bell, *Biotechnol. Bioeng.*, 22 (1980) 947.
21 S. A. Cohen, K. P. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
22 H. Colin, J. C. Diez-Masa, G. Guiochon, T. Czajkowska and I. Miedziak, *J. Chromatogr.*, 167 (1978) 41.
23 A. H. Abdel-Alim and A. E. Hamielec, *J. Appl. Polym. Sci.*, 17 (1973) 3769.
24 K. K. Unger, J. N. Kinkel, B. Anspach and H. Giesche, *J. Chromatogr.*, 296 (1984) 3.
25 C. M. Guttman and E. A. Di Marzio, *Macromolecules*, 3 (1970) 681.
26 H. Small, *J. Colloid Interface Sci.*, 48 (1974) 147.
27 C. Dewaele and M. Verzele, *J. Chromatogr.*, 282 (1983) 341.
28 K. K. Unger, W. Messer and K. F. Krebs, *J. Chromatogr.*, 149 (1978) 1.
29 J. C. Giddings, *Anal. Chem.*, 39 (1967) 1027.
30 E. Grushka, *Anal. Chem.*, 42 (1970) 1142.
31 J. M. Davis and J. C. Giddings, *Anal. Chem.*, 55 (1983) 418.
32 J. Sjodahl, *Sci. Tools*, 27 (1981) 54.
33 *LKB HPLC Systems Revolutionize Biology, Biochemistry and Biotechnology, HPLC-E01 6/83.50M*, LKB, Bromma, 1983.
34 J. Sjodahl and A. Winter, *Protides Biol. Fluids, Proc. Colloq.*, (1982) 693.
35 *High Performance Liquid Chromatography in Protein Research, Liquid Chromatography Application Note 354*, LKB, Bromma, 1983.