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PROPERTIES, IN THEORY AND PRACTICE, OF NOVEL GEL FILTRATION MEDIA FOR STANDARD LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of experimental parameters on the separation result in gel filtration may readily be predicted from a few basic equations, as demonstrated with the aid of experimental observations on Sephacryl® HR. Excellent agreement between the predicted resolution and that determined experimentally was found for parameters such as temperature, particle size, column length, flow velocity, sample volume and gel porosity. The theoretical prediction of an optimum sample volume for a constant processing rate was also experimentally verified. An exhaustive investigation of the physical, chemical and functional properties of Sephacryl HR was undertaken to facilitate the interpretation of experimental observations.

INTRODUCTION

During the last decade several new media designed for aqueous size-exclusion chromatography (gel filtration) have been introduced[1–10]. The majority of products has been developed to meet demands for high resolution at short separation times, *e.g.*, pre-packed columns of micro-particulate gels at the expense of cost and system flexibility.

Recently, a new family of chromatography packings, Sephacryl® High Resolution, claimed to offer a rational compromise between chromatographic performance, system flexibility and economy for standard liquid chromatography, was introduced by Pharmacia[11]. Information on the physico-chemical and functional properties of new matrices facilitates interpretation of experimental observations and is thus of vital importance to the researcher. We therefore wish to communicate an exhaustive examination of the characteristics of the new packing materials.

Predictability of separation results is important in order to facilitate the development of chromatographic separation schemes. This know-how is sometimes commercialized into expert or knowledge-based systems[12,13]. This study illustrates the application of some fundamental theoretical relationships of size-exclusion parameters to produce simple guidelines for work with standard gel filtration chromatography.

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THEORETICAL

Size-exclusion chromatography (SEC) is, in the ideal mode, based on a relatively uncomplicated separation principle, *i.e.*, elution is solely governed by the differences between the solute and pore dimensions[14–16]. Thus, estimates of the separation result are readily inferred from some basic equations[17–20]. The resolution, R_s, between two solutes of molecular weights M_1 and M_2 may conceptually be described by

$$R_s = \frac{1}{4} \log M_1/M_2 \left(\frac{b}{V_0/V_p + \bar{K}_D} \right) \sqrt{\frac{L}{\bar{H}}} \tag{1}$$

where b is the slope of the selectivity curve, *i.e.*, dlog $M/\mathrm{d}K_D$, $\bar{K}_D$ is the mean value of the distribution coefficients, *i.e.*, $\frac{1}{2}(K_{D1} + K_{D2})$, V_0 is the void volume of the column, V_p the pore volume of the packing material, L the length of the column and $\bar{H}$ the packing efficiency of the column, expressed as the mean value of the plate heights for the two solutes[20]. For very precise calculations the expression $(V_0/V_p + \bar{K}_D)\sqrt{\bar{H}}$ in eqn. 1 must be replaced by:

$$\frac{1}{2}(V_0/V_p + K_{D1})\sqrt{H_1} + \frac{1}{2}(V_0/V_p + K_{D2})\sqrt{H_2}$$

The equation for the plate height may, for macromolecules, be reduced to

$$H = 2\lambda d_p + R(1 - R)d_p^2 u/30\gamma_s D_m \tag{2}$$

i.e., the B term is in most cases negligible compared to the C term of the Van Deemter equation[20]. In eqn. 2, λ is a geometrical factor close to 1 for many gel filtration columns[20], R is the ratio of the zone velocity to the mobile phase velocity, *i.e.*, V_0/V_R, d_p is the particle size, u is the interstitial linear flow velocity, D_m is the diffusion coefficient in the mobile phase and γ_s is the obstruction factor to diffusion in the pores[20].

The diffusion coefficient (cm^2/s) of globular proteins may be estimated from[20,21]:

$$D_{25,H_2O} \approx 2.6 \cdot 10^{-5}\ M^{-1/3} \tag{3}$$

The diffusivity is affected by the temperature according to[22]

$$D_{t1}/D_{t2} = T_1/T_2 \cdot \eta_{t2}/\eta_{t1} \tag{4}$$

where T is the absolute temperature (K) and η the viscosity of the solution at t°C. The viscosity of water (in cP) is fairly accurately given by[23]

$$\eta_t = \exp\{1301/[998.333 + 8.1855\ (t - 20) + 0.00585\ (t - 20)^2]\} - 1.30233 \tag{5}$$

where t is the temperature in °C between 0 and 30°C.

The extra-column peak broadening is, with well designed systems, mainly a function of the sample volume and the total zone broadening may then be calculated from[24,25]

$$\sigma^2_{\mathrm{tot}} = \sigma^2_{\mathrm{inj}} + \sigma^2_{\mathrm{column}} \approx V^2_{\mathrm{inj}}K^{-1}_{\mathrm{inj}} + V^2_{\mathrm{R}}N^{-1} \qquad (6)$$

where V_{inj} is the sample injection volume, K_{inj} an injector-dependent constant (empirically close to 5 for our injection device[25]) and N the plate number of the column. The plate number was calculated, assuming a Gaussian distribution of solute molecules, from

$$N = 5.54\left(\frac{V_{\mathrm{R}}}{W_{\mathrm{h}}}\right)^2 \qquad (7)$$

where W_{h} is the peak width at half peak-height.

For maximum process economy it is of interest to find an optimum combination of cycle time, *i.e.*, flow velocity and cycle number, *i.e.*, processed sample volume per cycle. This may be inferred by combining eqns. 1, 2 and 6. The result is illustrated in Fig. 1 indicating that an optimum combination exists for each feed. The relative feed, F_{rel}, expresses the sample volume, in terms of column bed volume, V_{c}, that is processed per hour.

Approximations of the optimum sample volume and flow-rate for processing totally F ml sample per hour may be found by combining eqns. 2 and 6 and differentiation of H_{tot} with respect to V_{inj} which yields:

$$V_{\mathrm{inj,opt}} \approx (FK_{\mathrm{inj}}V_{\mathrm{c}}V_{\mathrm{p}}d^2_{\mathrm{p}}/15D_{\mathrm{m}})^{1/3} \qquad (8)$$

and

$$F_{\mathrm{opt}} = FL/V_{\mathrm{inj,opt}} \qquad (9)$$

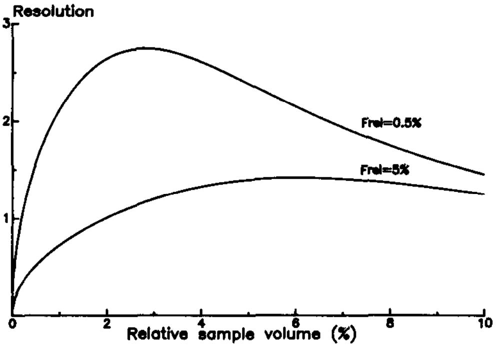


Fig. 1. Resolution as a function of sample volume at constant processing rate. Relative sample volume, $V_{\mathrm{rel}} = V_{\mathrm{inj}}/V_{\mathrm{c}}$, and relative feed, $F_{\mathrm{rel}} = V_{\mathrm{rel}}$ per hour. Calculated from eqns. 1, 2 and 6 for bovine serum albumin and myoglobin on Sephacryl S-200 HR.

EXPERIMENTAL

Pore and surface structure

The surface topology of Sephacryl HR was studied by scanning-electron microscopy (SEM), using a Philips (Eindhoven, Holland) 400, FEG at 60 kV, with a spot size of 2 nm, providing a magnification factor of 100 000 times. Before SEM, the beads were freeze-dried by first immersing the fixed beads in liquid nitrogen and then allowing the eutectic ice thus formed to sublime for 48 h at −78°C and 10^{-5} mmHg. The beads were then vapour phase impregnated with osmium to yield a very small extra surface layer and still a sufficiently large emission of scattered electrons[26].

Bead size distribution

The particle-size distribution of Sephacryl HR was routinely analysed on a Coulter Counter. The instrument was calibrated with the aid of absolute data, obtained by microscopy and image analysis with the aid of an IBAS 2000 (Kontron, Eching/Munich, F.R.G.)[27]. The particle size, d_p, in this context is the mean of the volume-size distribution. This estimate is, for this size distribution of beads, useful for the calculation of the reduced plate height, *i.e.*, $h = H/d_p$ and the pressure drops over gel beds[3].

Matrix rigidity

The effect on bead rigidity of the improved cross-linking procedure was tested by running a flow gradient through a packed column and monitoring the pressure drop generated over the column. The data were compared with the results expected on the basis of the pressure-drop equation. The procedure has been described in detail elsewhere[3].

Packing procedure

A new packing procedure was developed in order to pack this new media efficiently. The principle is to form an homogeneous gel bed by first packing the bed at a medium flow velocity to avoid a compressed zone of beads at the end piece and then stabilizing the bed at an higher flow velocity.

We found that typical flow velocities for the steps were 30 cm/h for 2 h and 60 cm/h for 1 h, respectively, when packing a 70-cm column. Optimum flow velocities are due to the column length (increasing flow with decreasing length) as well as the rigidity of the gel. Detailed packing instructions for various column dimensions and gel types are now available from the manufacturer[28]. The columns were packed according to the two-step procedure in the reverse order of elution, *i.e.*, towards the inlet adaptor, thus providing a undisturbed, dense and homogeneous bed at the sample application zone.

The efficiency of the packing procedure was evaluated by passing 200 μl of acetone (5 μl/ml water) through the column at 30 cm/h. A leading peak indicates channeling and the column had to be repacked at a reduced flow velocity, *e.g.*, 10% less in step 2. A tailing peak may be caused by too loose a packing, but this can be adjusted by increasing the flow velocity in step 2 by 5–20%. This packing procedure should yield columns with high efficiencies, expressed by a reduced plate height of approximately 2 (corresponding to more than 10 000 plates per metre), and a peak symmetry of 1.0 ± 0.2.

Chromatographic performance

All equipment and gels were obtained from Pharmacia (Uppsala, Sweden). The efficiency of the columns was checked by evaluating the plate number and symmetry for acetone, as outlined above. The selectivity and the separation range were evaluated by chromatographing mixtures of model proteins, listed in Table I. The chromatographic system typically was comprised of an high precision pump (P-500), injection valve (MV-7), UV monitor (UV-M, 280 nm), recorder (REC-482) and a controller unit (LCC-500 Plus).

TABLE I

PROTEINS USED FOR CHARACTERIZATION OF SEPHACRYL® HR

Sources: A = Pharmacia LKB Biotechnology; B = Sigma; C = Boehringer Mannheim.

Substance	*Source*	*Lot. No.*	*Molecular weight (kilodaltons)*	*Yield determined on Sephacryl S-100 HR (%)*
Blue Dextran 2000	A	C 619	2 000	97
Ferritin	A	C 620	440	99
Catalase	A	C 621	232	96
Aldolase	A	C 627	158	100
Bovine serum albumin	A	C 623	67	100
Ovalbumin	A	C 626	43	99
β-Lactoglobulin A+B	B	106F-8120	35	101
Chymotrypsinogen A	A	C 624	25	99
Myoglobin	B	34F-7180	17.6	99
Lysozyme	C	10321922-61	14.4	96
Ribonuclease A	A	C 625	13.7	104
Cytochrome *c*	B	56F-7020	12.4	99
Immunoglobulin G	B	54F-9390	160	
Human serum albumin	A		67	
Transferrin	B	14F-9425	81	

The columns XK 16/70 (70 cm × 1.6 cm I.D.), XK 26/70 and XK 50/100 were packed with Sephacryl S-100 HR, S-200 HR, S-300 HR, S-400 HR and S-500 HR according to the new packing procedure. The eluent was 0.05 *M* phosphate buffer in 0.15 *M* sodium chloride, adjusted to pH 7.0 and containing 0.04% sodium azide as a preservative.

Adsorption of model proteins on Sephacryl under recommended chromatographic conditions was determined by applying 1 mg of protein to 24 ml of fresh gel, packed into an HR 10/30 column, and calculating the decrease in absorption of the collected peak as compared to a reference sample. Sephacryl S-100 HR, being the packing material of largest matrix volume, was chosen for this test.

The influence of temperature was studied in a cold room (3°C) and at room temperature (22°C). The impact of particle size on the resolution was elucidated by comparing the performance of columns packed with Sephacryl S-200 HR (47 μm) and S-200 SF (70 μm). The sample volume was varied with the aid of a Superloop. The effects of the column length and dead-volume were studied by connecting columns in series. The influence of all these parameters as well as that of the flow velocity and solute molecular weight was compared to results predicted by the theoretical relationships.

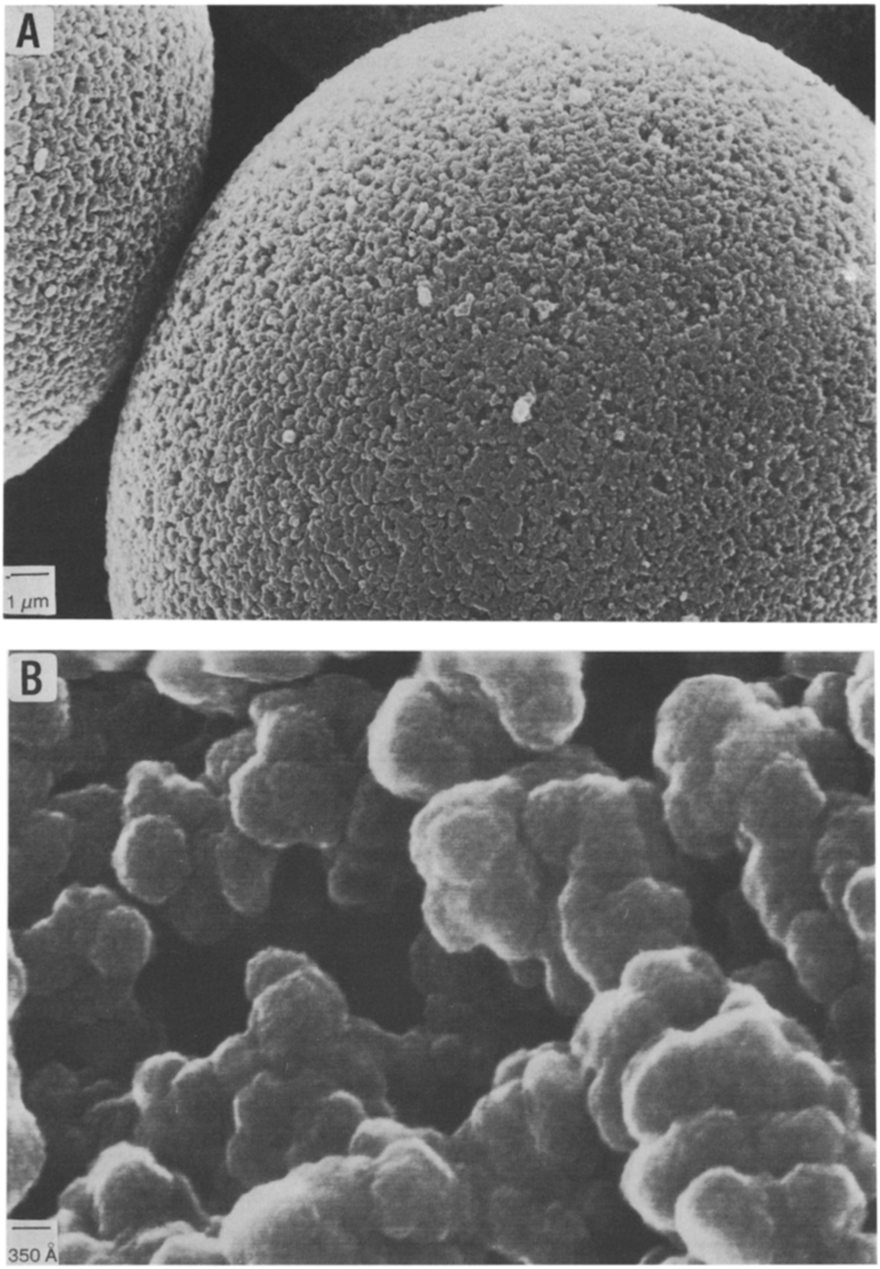


Fig. 2. Scanning electron micrographs of Sephacryl S-500 HR at different magnification factors (courtesy of B. Medin, Institute of Biochemistry, Uppsala University).

RESULTS AND DISCUSSION

Pore and surface structure

The structure of Sephacryl HR, as observed by SEM, is shown in Fig. 2. The topology of the surface is not identical to that shown for agarose-based media[29].

From the micrograph in Fig. 2B it can be concluded that Sephacryl S-500 HR contains cavities of widths in the range of 750–2000 Å. The granular structures of polymerized bis(acrylamide), assumed to form the macroreticular structure, are clearly visualized in Fig. 2B. Allyldextran is believed to be co-polymerized with or in some cases trapped in the polymerized granular structures[30]. We have noticed affinity of lentil lectin to Sephacryl which indicates that sugar structures, *i.e.*, dextran are exposed to solutes[31]. Other workers have found that treatment of Sephacryl with dextranase yields an increase in porosity but no substantial decrease in rigidity[32]. These observations provide support for the tentative structure of Sephacryl given here.

Bead size distribution

The particle-size distribution of Sephacryl is fairly narrow, *i.e.*, $s = 20\%$ of the mean as shown in Table II. The optimum particle size of a chromatographic material is a compromise between considerations such as the allowable column zone broadening and tolerable column pressure drop at the flow velocities required and the extra column contribution, *e.g.*, introduced by large injection volumes[25]. Whereas efficient column packing techniques for standard media, *e.g.*, $20 \leqslant d_p \leqslant 200$ μm are well established, the packing of micro-particulate materials, *e.g.*, $d_p \leqslant 5$ μm is still considered to be very difficult[33]. The particle size range of 30–50 μm is often claimed to be optimal for preparative liquid chromatography[34,35]. It may be noted that overload conditions, *i.e.*, an experimental situation where the sample volume and/or concentration is the major cause of the total peak width, is for gel filtration achieved at smaller sample volumes for smaller bead sizes[25].

TABLE II

PROPERTIES OF SEPHACRYL® HR USED IN THIS STUDY

Parameter	*Gel type*				
	S-100	*S-200*	*S-300*	*S-400*	*S-500*
Particle size (μm)					
mean of volume distribution	48	48	43	44	46
width (5–95%)	31–68	31–67	29–63	31–63	32–66
Separation range, $K_D = 0.1$–0.8					
proteins	$(1–75) \cdot 10^3$	$(3–160) \cdot 10^3$	$(5–1000) \cdot 10^3$	$(2–2000) \cdot 10^4$	$2 \cdot 10^4–3 \cdot 10^9$
dextrans[a]				$3 \cdot 10^3–1.6 \cdot 10^6$	$10^4–2 \cdot 10^7$
Matrix volume (%)	15	12	10	10	8
Permeability, V_p/V_0	1.5	1.7	1.6	1.5	1.6
Void fraction, V_0/V_c	0.36	0.34	0.36	0.37	0.37
Packing efficiency, h	1.9	2.1	2.1	2.1	2.1

[a] In-house preparations and substances from Pharmacosmos, Denmark.

Matrix rigidity

The enhanced flow properties due to an increase in matrix rigidity of the different types of Sephacryl is illustrated in Fig. 3. As can be noted, the flow pressure characteristics are in accordance with the pressure-drop equation, and this indicates that the column bed is very homogenous, *i.e.*, the void fraction is constant over the entire column length. An abnormal drop in the pressure curve at very high flow velocities may be noted with Sephacryl. This is probably caused by cracking of the bed, leading to void channels. Maximal flow resistance of semi-rigid media is due not only to the bead strength but also to the column internal diameter and length. The flow velocities obtained with Sephacryl HR are sufficient for most standard gel filtration chromatography separations.

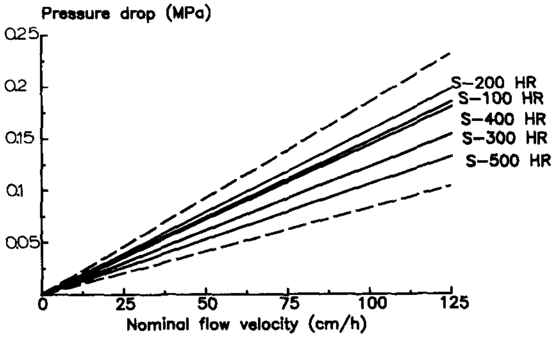


Fig. 3. Pressure–flow relationships for Sephacryl HR. Bed dimensions, void fractions as listed in Table II. Dotted curves illustrate theoretical limits for homogeneous beds, calculated for the actual particle size and void fractions[3].

Column packing

The optimum packing procedure of a certain material is related to a large number of parameters, and one may therefore assume that each type of material must be treated on an individual basis[33]. With the proposed method we routinely achieved columns with a reduced plate height of better than 2.1, *i.e.*, 10 000 theoretical plates per metre and a symmetry in the range 0.9–1.1 for acetone.

Chromatographic performance

The adsorption of model proteins on Sephacryl is very low, when a buffer of intermediate ionic strength is used, as indicated by the data in Table I. The yield is very similar to that reported for agarose gels[3,5]. It should be noted that elution of lysozyme is delayed, indicating a non-ideal behaviour of this protein. The behaviour is probably not due to the shape of the protein, *i.e.*, other more asymmetric proteins are eluted as expected, but is caused by a "salting-out" effect. Reduction of the ionic strength of the buffer was accompanied by a reduction in the elution volume of lysozyme. Lysozyme is claimed to undergo strong hydrophobic interactions[36].

The chemical stability of Sephacryl HR allows the continuous use of eluents of pH between 2 and 11 (ref. 37). Anionic interactions at low ionic strengths have been

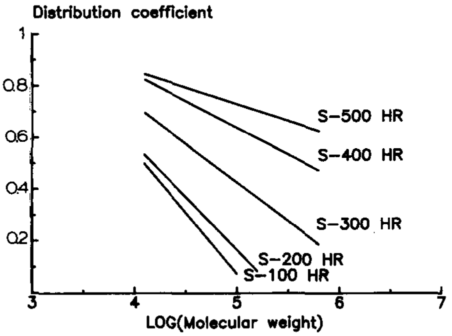


Fig. 4. Selectivity curves of Sephacryl HR for globular proteins.

noted, and the effect was eliminated by the use of neutral buffers at intermediate ionic strength, *e.g.*, the buffer used in this study[37].

The selectivity of Sephacryl HR for globular proteins is illustrated in Fig. 4. By comparison with published data for Sephacryl SF it can be concluded that the separation ranges for the two types of supports are very similar (see also Fig. 11)[38]. The separation range given by the molecular weight of globular proteins at $K_D = 0.1$ and 0.8 is given in Table II.

The influence of the porosity on the resolution, *e.g.*, eqns. 1 and 2 indicates that optimum resolution is achieved for a medium from which the substances are eluted at *ca.* $K_D = 0.2$, provided the selectivity is constant[20]. This is illustrated in Fig. 5, showing the elution pattern of protein mixtures for Sephacryl HR of different porosities. Immunoglobulin G–human serum albumin is best resolved on S-200 HR and ferritin–aldolase on S-300 HR. Even though the effect is not dramatic, *i.e.*, a maximum gain in resolution of 20% may be expected[20], selection of a gel from which the substances are eluted at $K_D = 0.2$ is favourable for the resolution. This is illustrated in Figs. 6–8, for which different porosities of Sephacryl HR have been selected for the final purification of important proteins.

The effect of the flow velocity on the resolution may be estimated with the aid of eqns. 1 and 2. The effect on the peak zone broadening for various types of Sephacryl HR is shown Fig. 9, which also illustrates the effect of porosity on the obstruction factor.

A change in temperature will influence the diffusion coefficient, predominantly through the change in solvent viscosity. The largest effect is obtained in situations where the C term dominates over the A term and H becomes proportional to $1/D_m$. Changing the temperature from 22 to 3°C will, in this situation, reduce the resolution by 26%, *i.e.*, $R_{s,3}/R_{s,22} \approx \sqrt{D_3/D_{22}} = 0.74$.

The result in Fig. 10 shows that the reduction due to temperature was *ca.* 20% (the A term was not negligible compared to the C term). The increase in peak width due to decreased temperature may be compensated for by a decrease in flow velocity (see eqn. 2). This is also illustrated in Fig. 10. However, it should be noted that an increase in viscosity is accompanied by an equally large increase in column pressure drop. Transfer of columns between extreme temperatures must therefore be made with great care, *e.g.*, at a reduced flow-rate.

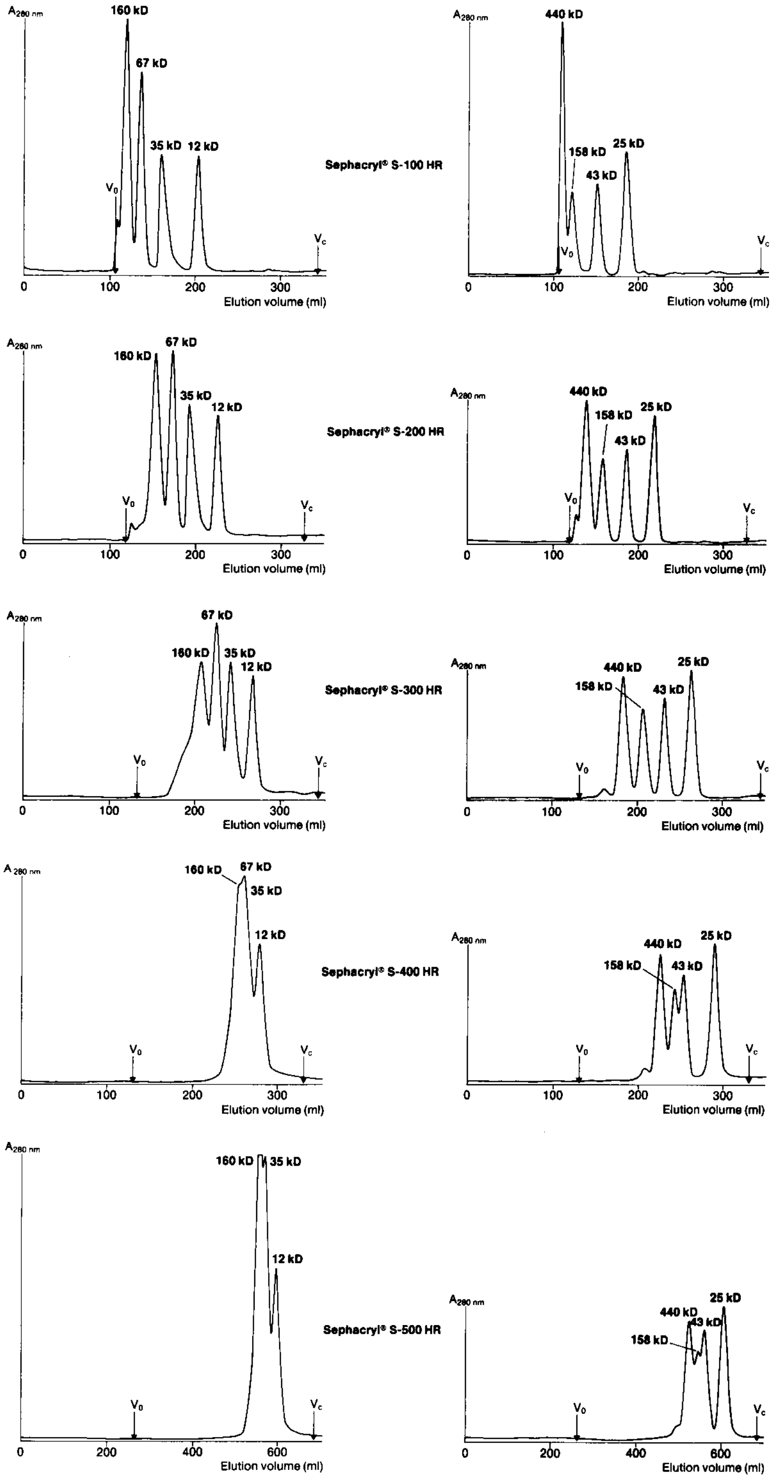


Fig. 5. Separation characteristics of Sephacryl HR of different pore sizes. Solutes: (left) immunoglobulin G, human serum albumin, β-lactoglobulin and cytochrome *c*; (right) ferritin, aldolase, ovalbumin and chymotrypsinogen A. Sample volume: 500 μl. Bed dimensions: 62–65 cm × 2.6 cm (column XK 26/70). Eluent: 0.05 *M* phosphate in 0.15 *M* NaCl (pH 7.0).

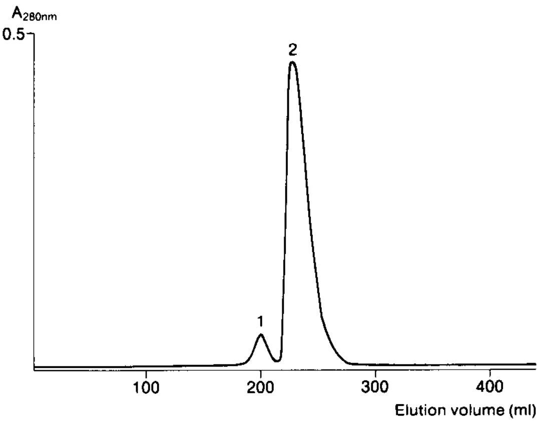
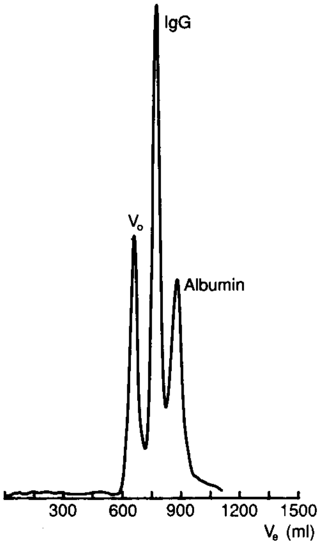


Fig. 6. Separation of human growth hormone (hGH) on Sephacryl S-100 HR. Sample: 14 ml hGH solution. Bed dimensions: 90 cm × 2.6 cm (K 26/100). Eluent: glycine phosphate (pH 7.0); flow-rate 21 ml/h (courtesy of KabiVitrum, Peptide Hormones, R&D). Peaks: 1 = hGH dimers; 2 = hGH monomers.

The influence of particle size on resolution is readily predicted from eqns. 1 and 2. A reduction of the particle size from 65.5 to 46.5 μm would theoretically increase the resolution by 20–30%. This was experimentally confirmed for a protein mixture eluted from Sephacryl S-200 SF and S-200 HR (see Fig. 11). The gain in peak sharpness from



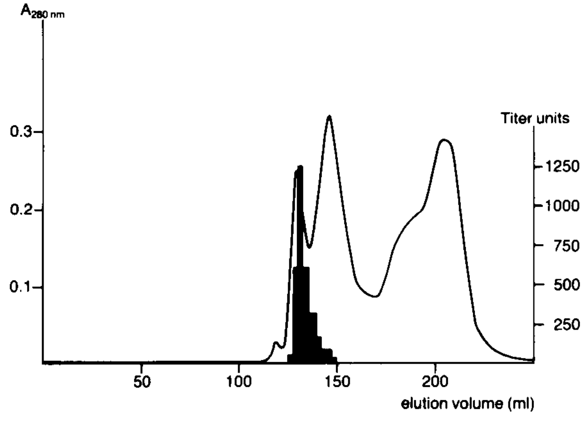


Fig. 7. Final purification of monoclonal immunoglobulin G on Sephacryl S-200 HR. Sample: 30 ml pre-purified solution from cell culture; bed dimensions 90 cm × 5 cm (K 50/100). Eluent: 0.05 *M* phosphate in 0.15 *M* NaCl (pH 7.4); flow-rate, 126 ml/h (courtesy of G. Vestin, Pharmacia Diagnostics).

Fig. 8. Purification of immunoglobulin M monoclonal antibodies on Sephacryl S-300 HR. Sample: 2 ml pre-concentrated solution from hybridoma cell culture. Bed dimensions: 63 cm × 2.6 cm (K 26/70). Eluent: 0.02 *M* phosphate in 0.5 *M* NaCl (pH 6.5); flow-rate 60 ml/h. Antibody titre determined by haemolytic test (courtesy of A. Domicelj, Pharmacia LKB Biotechnology).

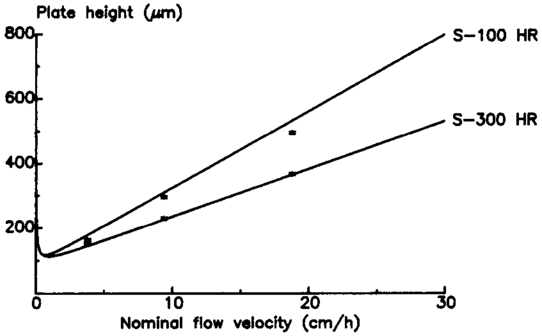


Fig. 9. Zone broadening of macromolecules as a function of flow velocity. Solid lines are theoretical curves, calculated for bovine serum albumin. Dots represent experimentally found peak widths of bovine serum albumin on Sephacryl HR.

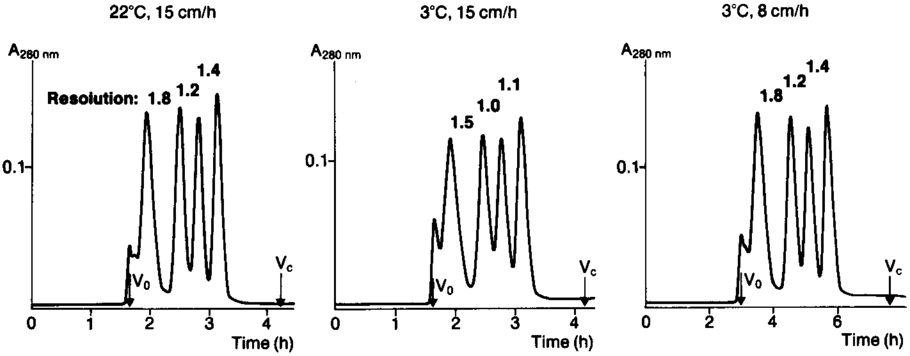


Fig. 10. Influence of temperature and flow-rate on the resolution on Sephacryl S-300 HR. Sample: 3.3 ml of a protein mixture, consisting of thyroglobulin, aldolase, ovalbumin and myoglobin. Bed dimensions: 63.2 cm × 2.6 cm (XK 26/70). Eluent: 0.05 *M* phosphate in 0.15 *M* NaCl (pH 7.0).

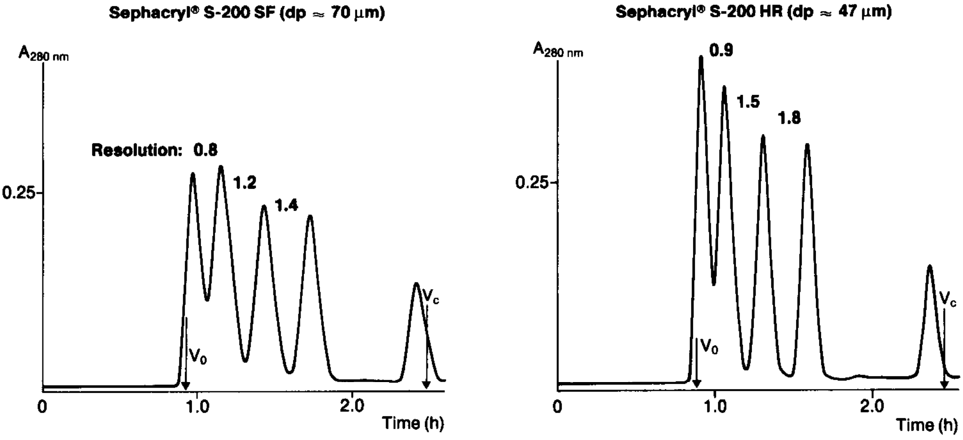


Fig. 11. Influence of particle size on the peak width and resolution of Sephacryl S-200. Sample: 3.3 ml of a mixture consisting of ferritin, aldolase, ovalbumin, myoglobin and glycyltyrosine. Bed dimensions: Sephacryl S-200 SF, 62.1 cm × 2.6 cm; Sephacryl S-200 HR, 61.3 cm × 2.6 cm. Eluent as in Fig. 10.

the use of a smaller particle size may be utilized either to improve the separation and/or to decrease the separation time (in this case by a factor of 2) with retained resolution.

Another way to increase the resolution is to use long columns (see eqn. 1). Since it is generally more difficult to produce optimum packing of very long columns, the desired length may be achieved by connecting several medium-length columns in series, provided that the proper design of connectors is used to minimize extra-column zone broadening. However, the use of very long columns is only meaningful for very difficult separations. For many separations of macromolecules, approximately equal

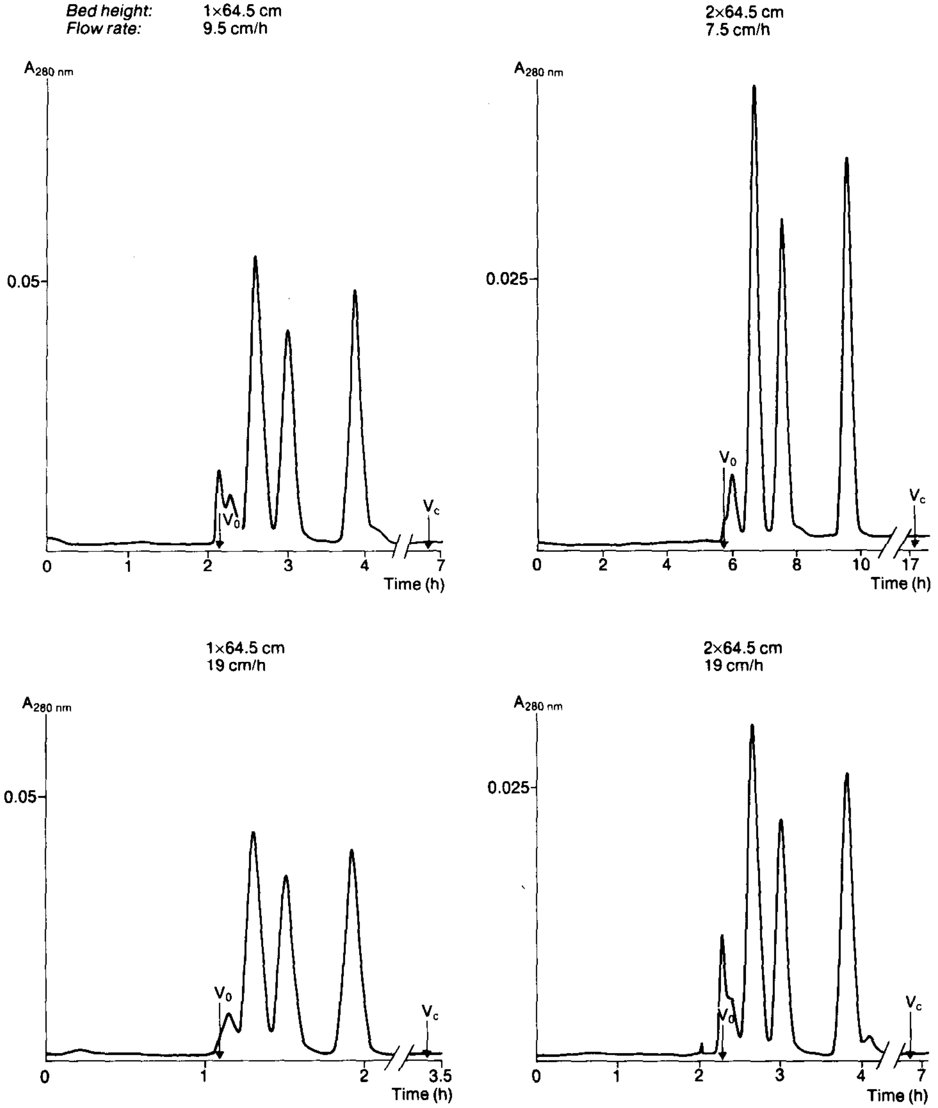


Fig. 12. Resolution at various column lengths and flow velocities on Sephacryl S-100 HR. Sample: 500 μl of transferrin, ovalbumin and myoglobin. Eluent as in Fig. 10.

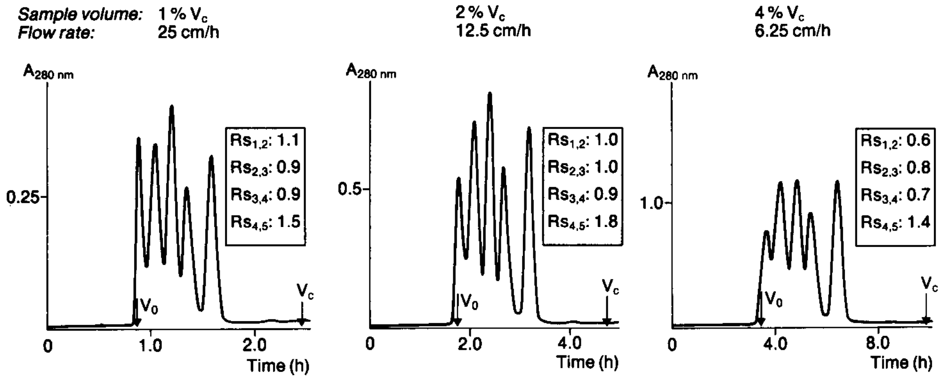


Fig. 13. Resolution for various sample volumes at a constant processing rate (ml sample/hour). Peaks in order of elution: aggregates; immunoglobulin G; bovine serum albumin; β-lactoglobulin; myoglobin. Processing rate: 1.33 ml/h (0.004 V_c/h). Bed dimensions: 61.3 cm × 2.6 cm (XK 26/70, V_c = 325 ml). Eluent as in Fig. 10.

resolution per unit time will be achieved through rapid elution of a long column or slow elution of a short column, *i.e.*, keeping L/H constant. These theoretically based rules-of-thumb are illustrated experimentally with a protein mixture in Fig. 12.

The optimum sample volume is directly related to the application of the chromatography step. For analytical purposes, the extra-column zone broadening is to be mininized, and the sample volume is therefore kept very small, typically less than 0.2% of the column bed volume[25]. The approach is drastically different for processing larger sample volumes, and an optimum of column zone broadening, *i.e.*, flow velocity and extra-column zone broadening, *i.e.*, sample volume is sought. Theory, as illustrated by Fig. 1, predicts an optimum sample volume of 2–3% of the column bed volume when 0.5% of the column volume is processed per hour. This is confirmed by the chromatograms shown in Fig. 13.

For purification of very large sample volumes it is of interest to prepare columns of larger diameters. We found that the packing method proposed for Sephacryl HR was applicable to columns even as large as 50 mm I.D. As illustrated in Fig. 14, the separation efficiencies of these columns were as good as those of the analytical columns.

TABLE III

INFLUENCE OF EXPERIMENTAL PARAMETERS ON RESOLUTION

Parameter	*Variation*	*Change in resolution (%)*	
		Predicted	*Experimental (Figure; peaks)*
Particle size (μm)	65.5–46.5	35	33 (11; 3, 4)
Flow velocity (cm/h)	19–9.5	25	27 (12; 2, 3)
Column length (cm)	64.5–129	40	33 (12; 2, 3)
Temperature (°C)	3–22	26	25 (10; 1, 2, 3)
Sample volume (%) at constant feed	2–1	23	13 (13; 3, 5)

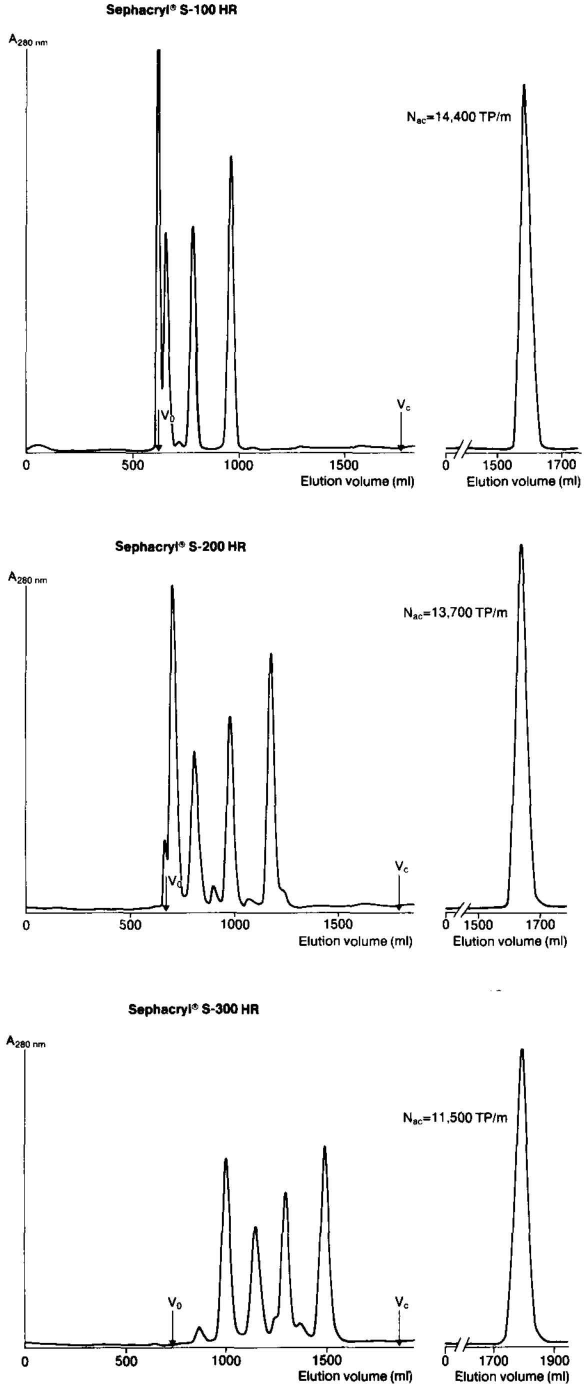


Fig. 14. Separation efficiencies (TP = theoretical plates) of Sephacryl on a small production scale. Sample: 10 ml of ferritin (4 mg), aldolase (30 mg), ovalbumin (25 mg) and chymotrypsinogen A (15 mg). Bed dimensions: 91–94 cm × 5 cm (XK 50/100). Eluent as in Fig. 10.

In conclusion, with the aid of some basic equations, the resolution obtainable in SEC of proteins may readily be predicted, and furthermore, the detrimental impact of one parameter, *e.g.*, a decrease in temperature, may be compensated for by proper adjustment of another parameter, as illustrated in Table III.

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