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Factors affecting the separation and loading capacity of proteins in preparative gradient elution high-performance liquid chromatography

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ABSTRACT

The optimum conditions for the purification of proteins by gradient elution in reversed-phase liquid chromatography were studied, with emphasis on the column length. Because of the strong dependence of the retention of proteins on the mobile phase composition, very short columns can be used successfully to perform analytical separations. A similar conclusion is extended to preparative separations. Columns with different lengths and diameters were used. The dependence of the loading capacity for touching band separation on the column length, diameter and volume was studied, in addition to the regeneration time between successive runs, the starting mobile phase composition and the necessary column efficiency.

INTRODUCTION

The preparative purification of proteins by gradient elution raises problems which are different from those found in the separation of low-molecular-weight compounds in gradient elution or in isocratic purifications. Under isocratic conditions, the mechanism of band broadening in preparative chromatography is fairly well understood [1] and it is experimentally easy, if tedious, to determine the equilibrium isotherms necessary for the calculation of band profiles [2]. Then, agreement between calculated and experimental band profiles is excellent [3,4]. Modeling gradient elution separations is more difficult [5,6]. This is possible, however, if one can measure the equilibrium isotherms over the whole useful range of mobile phase compositions [6]. This has been done for small molecules, for which this range is wide, and excellent agreement has been reported between experimental and calculated profiles for single-component band profiles for 3-phenylpropanol [7].

The modeling of protein separations in gradient elution is much more complex. Under isocratic conditions, the protein isotherms in reversed-phase chromatography tend to be rectangular [8]. The useful range of concentrations is very narrow and the reproducibility of the measurements is questionable [9]. Serious experimental problems are also found. Until these problems have been solved, this method cannot provide much help and an empirical approach becomes necessary.

The conclusions of theoretical studies of isocratic separations is that concentration overload is necessary to achieve high production rates and to reduce the cost of recovery of the purified product from the mobile phase, and that the most important factor limiting the production rate is related to the minimum column efficiency necessary to achieve a sufficient degree of separation between the bands of the

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main component and of the closest impurities [10–14]. However, these conclusions do not extend simply to the separation of proteins in gradient elution. In this instance, proteins seem to be released from the column very rapidly, as if a trap door were open when the mobile phase reaches a certain composition [15].

Hence the column length has little effect on the separation between proteins under analytical conditions [15–18]. The classical equations relating the number of theoretical plates of the column, the resolution between two components, their separation and retention factors do not apply in this instance. It is difficult to derive an alternative relationship involving these parameters and the gradient rate for the separation of small molecules [19]. This does not seem to be either possible or useful with proteins.

The major factors that control the separation of proteins in gradient elution seem to be the gradient rate and the dependence of the retention factor of the two components on the mobile phase composition [15–20]. This dependence is usually logarithmic, but the slope of dlog k'/dC, where k' is the retention factor of the component of interest and C the concentration of the strong solvent in the mobile phase, depends considerably on the molecular weight of the solute considered. It is gradual for small molecules, intermediate for peptides and very steep for proteins [15].

It has been reported that, in gradient elution, the dependence of the resolution between two compounds of similar molecular weight on the column efficiency decreases steadily with increasing molecular weight [15–20]. It is considered that, for most proteins, only a thin layer at the top of the column contributes effectively to the separation. This is so because a very small change in the mobile phase composition is sufficient to shift the adsorption behavior of the protein molecule from totally adsorbed to almost not adsorbed. Hence, once a molecule has been released from its original adsorption site, it undergoes almost no adsorption again. The remainder of the column contributes to band broadening but not to retention. Therefore, the extent of the influence of the column efficiency on the resolution depends on the fractional length of the column at which the retention factor becomes lower than about 0.5. Beyond that position, the molecule is essentially unretained. The influence of the column efficiency is stronger for shallow gradients, but it is impractical to set the gradient rate below a certain limit [19]. In the literature, there are examples of gradient separations performed on 1- and 25-cm columns [17,18] and showing the same resolution. In general, however, the poorer separation is achieved on the shorter column for small molecules (*e.g.*, small peptides) [20].

Although there have been applications of this phenomenon for the rapid analytical separations of proteins with very short columns [15–18], its consequences in the preparation of purified proteins by preparative chromatography have not yet been investigated in detail. A separation strategy based on the use of very short columns would be of great value, however. The industrial separation of proteins is an important application of preparative chromatography. It is very costly, requiring large volumes of solvents which are more difficult to recycle in gradient elution than under isocratic conditions. The use of a column longer than necessary results in excessive expense, the need for too high an inlet pressure and the processing of undue solvent volumes, all contributing to increase production costs.

At this stage, however, there is little theoretical background available for the optimization of the experimental conditions of a separation based on gradient elution. The models available in analytical chromatography for the optimization of the gradient slope are too limited in their range of applicability [19,21,22]. They either borrow or adapt equations and concepts from isocratic chromatography. Their validity is questionable. Their extension to overloaded elution chromatography is impractical. Therefore, experiments are necessary to develop an understanding of the separation process and assist in the development of the proper models.

This work was undertaken to elucidate the influence of the column length and efficiency on the separation of proteins in gradient elution. As a first step, we investigated the experimental conditions resulting in touching band separation, as it is a situation where the competitive interactions of the two proteins with the stationary phase still contribute moderately to the separation. The displacement effect remains moderate and the influence of column efficiency is relatively more important than under overlapping band conditions. We also studied

the dependence of the column saturation capacity on the cross-sectional area and the influence of the initial solvent composition. The time needed to regenerate the column between two successive runs was also determined.

EXPERIMENTAL

Apparatus

The separations were studied using two liquid chromatographs. The first was assembled from two ConstaMetrics-III metering pump (LDC/Milton Roy, Riviera Beach, FL, USA), a dynamic on-line solvent mixer of volume 2.4 ml (Beckman, Fullerton, CA, USA), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA), a SpectroMonitor D variable-wavelength UV detector (LDC/Milton Roy), an IBM-compatible PC computer (Equus/Systems, Hesperia, CA, USA) with an OkiData printer (Oki America, Mount Laurel, NJ, USA) and a strip-chart recorder (Kipp & Zonen, Veendam, Netherlands). The system was operated with a program developed in-house.

The second system was assembled from a Model 2360 gradient programmer (Isco, Lincoln, NE, USA), an IsoChrom LC pump (Spectra-Physics, San Jose, CA, USA), a Model 7126 injector with different-sized loops (Rheodyne), an L-3000 photodiode-array detector (Hitachi, Tokyo, Japan), an IBM-compatible PC (Equus/Systems), an IBM PC graphic printer (IBM, Valhalla, NY, USA) and a 900 Series intelligent interface (Nelson Analytical, Cupertino, CA, USA), which connects the detector to the computer integrator with dual channels converting analog to digital data.

Products and chemicals

Two lots of Vydac 218TP silica C_{18} (The Separations Group, Hesperia, CA, USA) packing materials with different particle sizes were used: the 5-μm particle size lot is typical of the materials used in the analysis of proteins and the 10-μm particle size lot is typical of those used in preparative applications. The influence of the particle size in the separation of proteins in reversed-phase chromatography has been discussed in detail in ref. 18.

Lysozyme (molecular weight, MW = 14 307, grade I from chicken egg white) and cytochrome *c* (MW 12 300, type III from horse heart) were purchased from Sigma (St. Louis, MO, USA), trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA) and HPLC-grade acetonitrile (ACN) from J. T. Baker (Phillipsburg, NJ, USA). All chemicals were used as received.

Chromatographic conditions and procedures

The 5-and 10-μm Vydac 218TP silica C_{18} packing materials were slurry packed into stainless-steel columns of different lengths and diameters, as needed.

The mobile phases were prepared from 0.1% (v/v) TFA in water as solvent A and 0.1% TFA in pure ACN as solvent B. Prior to use, all solvents were degassed by sonication under vacuum. The gradients were run at a flow-rate of 1 ml/min and at a rate of 2%B/min, unless indicated otherwise. The initial gradient composition was variable.

Lysozyme and cytochrome *c* were dissolved in solvent A at concentrations of 10 and 5 mg/ml, respectively. Different loading amounts were achieved by adjusting the sample volume, except for one series of experiments in which the concentration was adjusted.

For most of the single-component experiments, the elution profiles were monitored with the SpectroMonitor D variable-wavelength UV detector at 300 nm, where the response is lower than at 254 or 280 nm. Experiments with binary mixtures were monitored at 300 and 320 nm with the L-3000 photodiode-array detector. Both proteins were detected at the former wavelength, but only cytochrome *c* at the latter. The detector sensitivity was adjusted depending on the sample size.

Retention volumes and band widths were measured from the recorded chromatogram. The band width is the width of the recorded profile at half-height. The detector gave a linear response in the sample size range used. The column efficiency was measured with biphenyl, using ACN–water (60:40) as the mobile phase, at a flow-rate of 1 ml/min, unless indicated otherwise. The retention factor is 2.0.

RESULTS AND DISCUSSION

Band width in gradient elution and column efficiency

Although the column length has little influence on the band width of proteins in gradient elution, this does not necessarily mean that the column effi-

TABLE I

INFLUENCE OF COLUMN PLATE NUMBER ON PEAK WIDTH OF PROTEIN

Column No.	Column length (cm)[a]	V_R (ml)	$W_{1/2}$[b] (ml)	N[c]	h
1	5	16.05	0.18	2459	4.0
2	15	18.17	0.20	9157	3.3
3	25	19.81	0.22	14198	3.5

[a] All columns 0.46 cm I.D.

[b] Peak width at half-height. Lysozyme injected, 50 μg/ml column volume. Other conditions as in Fig. 2.

[c] Column plate number was determined using ACN–water (60:40) as the mobile phase (1.0 ml/min; $k' = 2$) and biphenyl as the solute.

ciency is without effect. The column efficiency depends also on the quality of the packing and on the mobile phase velocity. We performed two series of experiments to clarify some issues related to the influence of the column efficiency on the band width.

The influence of the total column efficiency was studied by making three columns of different lengths (Table I) with Vydac 218TP C_{18} silica (5 μm, 300 Å), using the same packing method. The efficiencies of the three columns are proportional to the column length, with an average reduced plate height of 3.5. Sample sizes proportional to the column volume (50 μl of the lysozyme solution per ml of column volume) were injected. The band widths measured are reported in Table I. The band width varies in the same order as the column efficiency, the more efficient column, which is also the longer column, giving the wider band. The same relationship is observed under isocratic conditions, as the HETP of the three columns is the same. However, whereas under isocratic conditions the distance between two bands increases in proportion to the column length, we see in Table I that this is no longer true under the conditions of gradient elution. The retention volumes, and hence the distance between two bands, increase only slightly with increasing column length, less than 25% for a fivefold increase in the column length. The ratio $w_{1/2}/t_R$ is constant within the precision of the measurements (the values are 0.0112, 0.0110 and 0.0111 for the 5-, 15- and 25-cm columns, respectively). Hence we cannot expect an improvement in performance with increasing column length.

Fig. 1 compares the chromatograms obtained with two columns of the same length, but packed differently with the same material and having different reduced plate heights (3.0 and 5.6) for the same mobile phase velocity. The band width of the two proteins are 0.21 and 0.23 ml on the first column and 0.23 and 0.26 ml on the second column, *i.e.*, 10 and 13% larger on the less efficient column, respectively. The distances between the two bands are the same on the two chromatograms. Hence the resolution between the two proteins is better on the more efficient column. This is also demonstrated by the band of the lysozyme impurity (Fig. 1), which is well resolved on the first chromatogram but appears as a shoulder on the second. Although the

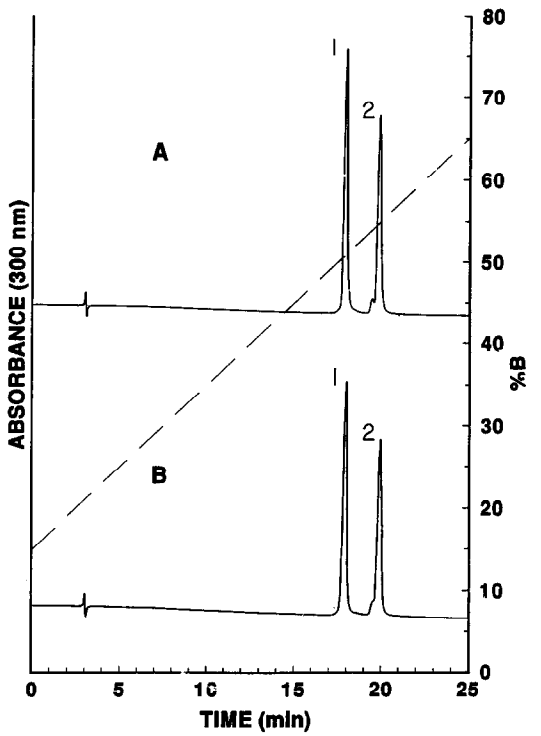

Fig. 1. Comparison of protein separations on columns of different efficiencies. Experimental conditions: 25 × 0.46 cm I.D. columns packed with Vydac 218TP, 5 μm, 300 Å (lot number 900201-25RE); flow-rate, 1.0 ml/min; gradient from 15 to 65% B in 25 min; UV detection at 300 nM; sample size, 20 μl of a mixture of cytochrome *c* (peak 1) and lysozyme (peak 2); column efficiency: (A) 65 208 plates/m (or $h = 3$) and (B) 35 900 plates/m (or $h = 5.6$).

gain in resolution is certain, however, it is not proportional to the square root of the plate number (*i.e.*, 1.37), as would happen under isocratic conditions.

In summary, the column HETP has an influence on the resolution achieved in gradient elution and the column should certainly be as well packed as possible, but the effect is not important. It seems probable that this effect would decrease to the point of being negligible under overlapping band conditions, when the band broadening due to the axial dispersion and mass-transfer resistance becomes negligible compared with the thermodynamic band broadening due to the non-linear behavior of the isotherm. The effect of the column length at a constant value of the plate height is also small. We now consider this effect in more detail.

Loading amount and column length

The elution band width under gradient conditions for the three columns in Table I is plotted in Fig. 2 *versus* the amount of lysozyme injected (gradient from 15 to 65% B in 25 min) for large sample sizes and in Fig. 3 for very small loadings. For a very small sample size (less than *ca.* 20 μg), the bands have nearly the same widths on all three columns (see also Table I). When the sample size increases, all three curves rise and tend to become linear at high loadings. For a given sample size, the longer column gives the smaller band width, but the effect is due to the larger loading factor of the shorter column at constant sample amount, obviously causing more band broadening of thermodynamic origin.

This effect is apparent on comparing Fig. 2 and Fig. 4, in which the band width is now plotted *versus* the loading ratio (in μg/ml column volume). For a given loading ratio, the band width is larger for the longer column. The ratio of the band widths for the 25- and 5-cm columns also increases with increasing loading factor. This suggests that the separation performance should be better with the shorter column.

Fig. 5 shows the plot of the resolution between lysozyme and cytochrome *c* as a function of the loading ratio. There is not much difference between the results obtained with the three columns. However, for a given loading ratio and a given column

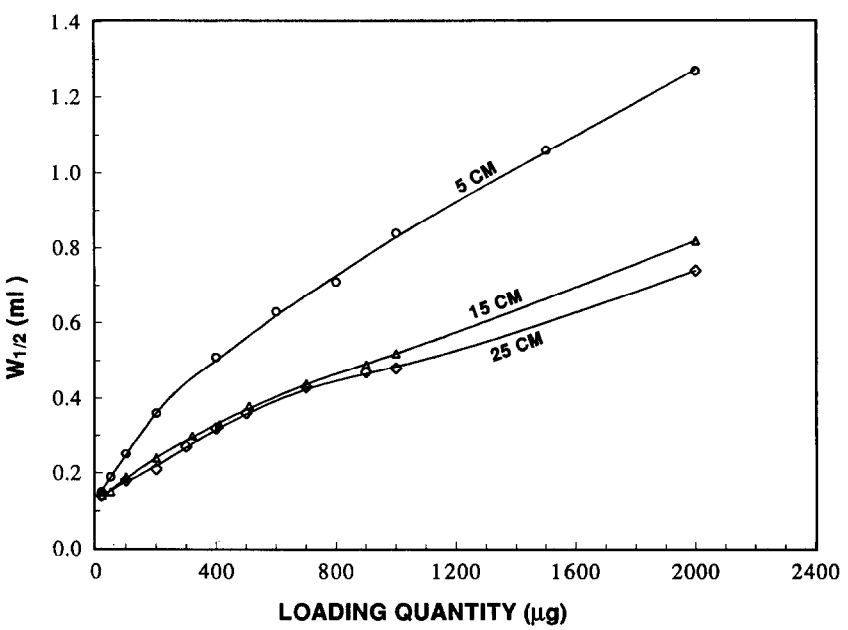

Fig. 2. Plot of peak width *versus* sample size on columns of different lengths. Experimental conditions: three columns, 5, 15 and 25 cm × 0.46 cm I.D., were packed with Vydac 218TP, 5 μm, 300 Å (lot number 890706-14); flow-rate, 1.0 ml/min; gradient, 15 to 65% B in 25 min; detection, UV at 300 nm; sample, lysozyme, 1% in 0.1% TFA–water, injected by varying the sample volume.

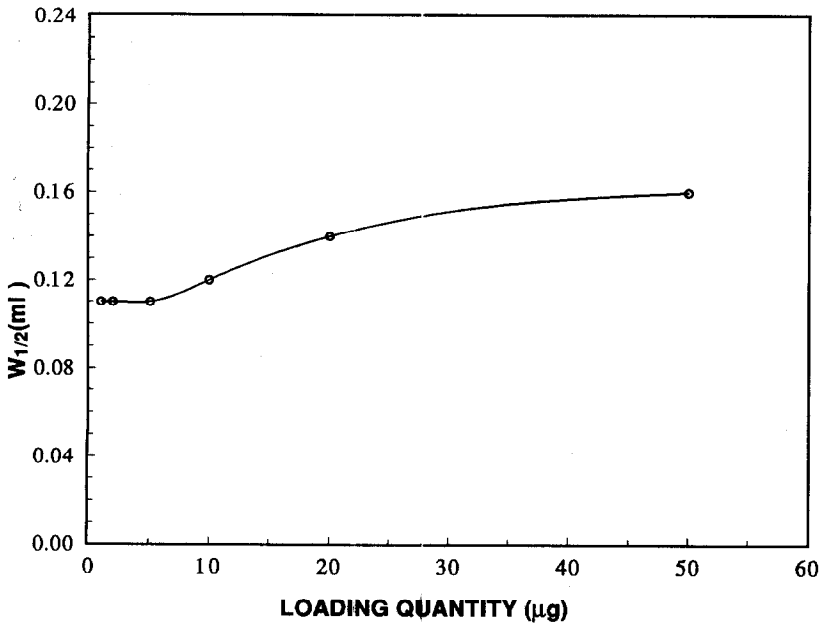

Fig. 3. Plot of peak width *versus* sample size at low loadings on the 15-cm column. Experimental conditions as in Fig. 2.

diameter, the resolution obtained with the shorter column is slightly better. The performance seems to be best with the 12.5-cm column. All the results tend towards the same limit at large loading ratios.

Column regeneration time

It is particularly critical in the gradient elution chromatography of proteins, whether analytical or preparative, to achieve reproducible results. One

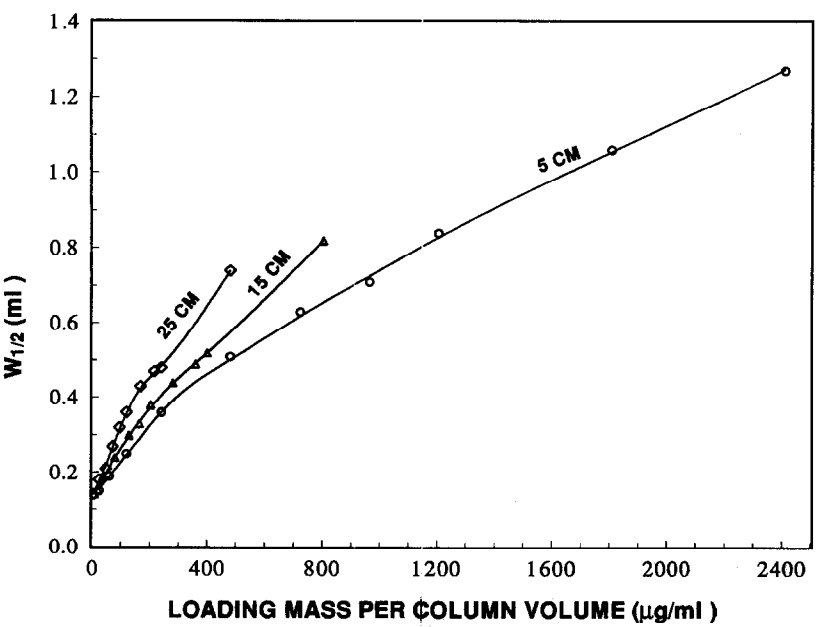

Fig. 4. Plot of peak width *versus* specific sample size on columns of different lengths. Experimental conditions as in Fig. 2.

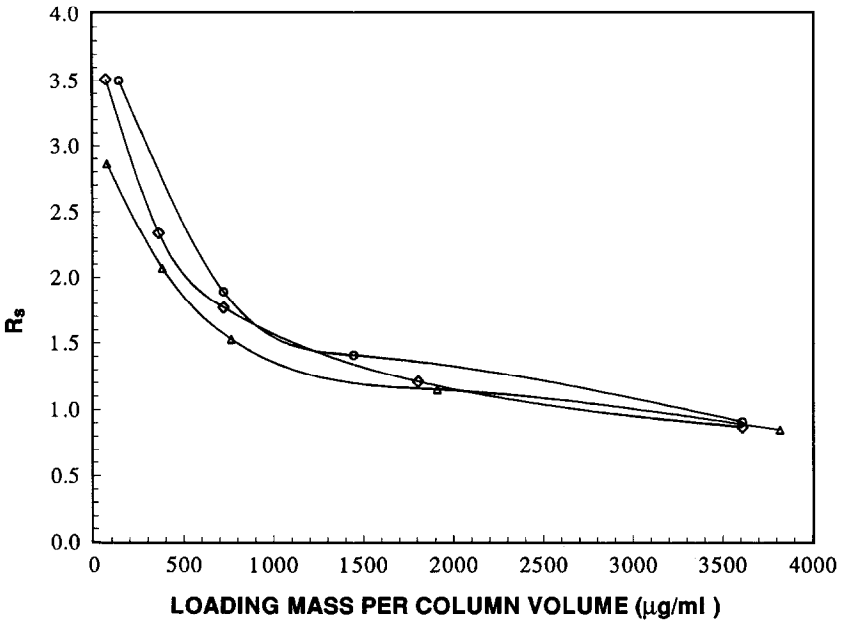

Fig. 5. Plot of resolution between cytochrome *c* and lysozyme *versus* sample size. Experimental conditions: (◇) 25 × 0.46 cm, (○) 12.5 × 0.46 cm and (△)5 × 1.0 cm I.D. columns packed with Vydac 218TP, 10 µm, 300 Å (lot number 891208-21); flow-rate, 1.0 ml/min; gradient, 15 to 65% B in 25 min; detection, UV at 300 nm; sample, cytochrome *c* (0.5%) and lysozyme (1%) in solvent A.

important factor in this respect is the re-equilibration of the column between successive runs. All the components of the feed sample must be eluted from the column. This may require that the column be washed with a suitable solution, and then that the initial solvent be pumped through the column for a period. Conventionally, analysts pump about 15–20 column volumes of the initial eluent before making a new injection [21]. This is a very costly proposition in preparative chromatography as much time is wasted waiting for the column to be operational again, and the solvent cannot be recycled, otherwise the impurities extracted from the column would be returned to it. It is useful to determine in various instances what amount of solvent must be pumped through the column to achieve reproducible results.

In Fig. 6, the band width and the retention volume of lysozyme on the three columns are plotted *versus* the time during which the initial solvent is pumped into the column before a second run is started again. The volume needed to return to a constant chromatogram is only 5 min, a value which is surprisingly independent of the column volume. This time is twice that needed to flush the mixer, which may explain the result. Then, the regeneration time needed could be made still shorter by emptying the mixer and filling it between each run.

This result is independent of the composition of the initial solvent, up to 25% B. When the initial solvent composition is 35% B, the results are no longer reproducible, although we have never observed any split peak. This is related to the fact that the isocratic elution of lysozyme with a finite, reasonable retention time can be achieved in the concentration range of 34–40% B. We have also observed that when the column is flushed with pure solvent B for a long time, reconditioning takes longer.

Band width and initial solvent composition

The higher the initial solvent composition, the shorter is the cycle and the higher is the production rate, as long as the feed components are eluted under gradient conditions. With proteins, it is easier to define an optimum upper concentration of the

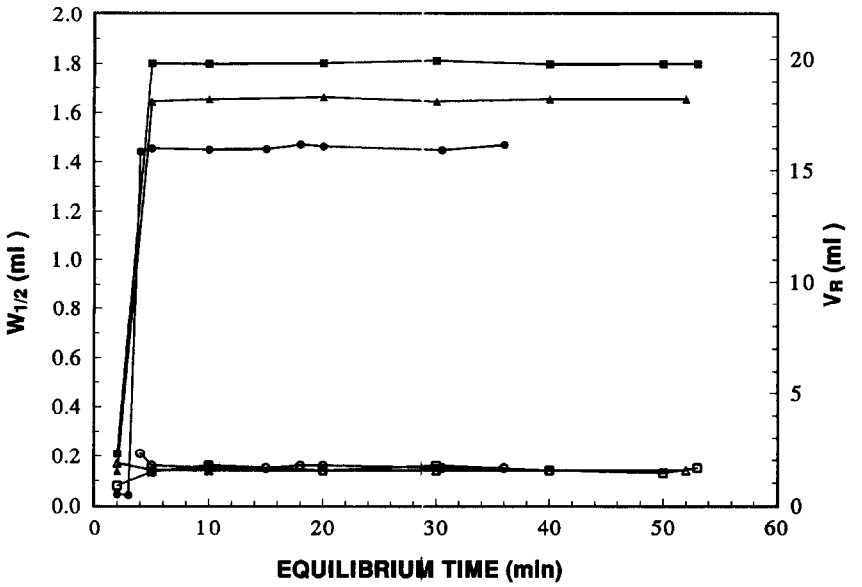

Fig. 6. Influence of regeneration time on the retention time (V_R) and peak width ($W_{1/2}$) of lysozyme. Experimental conditions as in Fig. 2, except sample volume (2 μl). V_R: ● = 5; ▲ = 15; ■ = 25 cm. $W_{1/2}$: ○ = 5; △ = 15; □ = 25 cm.

strong solvent in the initial mobile phase, as the retention of proteins is very high until it falls abruptly to low values.

Fig. 7 shows a plot of the band width *versus* the loading factor for the 25-cm column for different initial compositions. There is little difference between the results obtained with initial concentrations between 0 and 25% B. The best results seem to

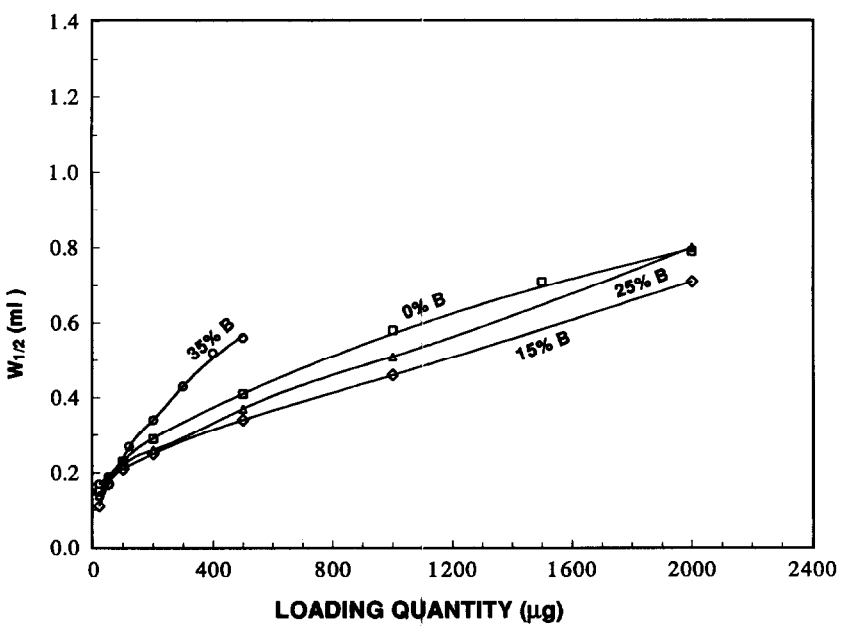

Fig. 7. Plot of peak width *versus* sample size for different initial gradient solvent compositions. Experimental conditions as in Fig. 2, except 25 × 0.46 cm I.D. column, packed with Vydac 218TP, 5 μm, 300 Å (lot number 890706-17A).

have been obtained with 15% B, but this optimum is barely significant. Given the reduction in cycle time achieved by starting with 25% B, this is probably the most economical condition.

The result becomes very different, however, if the initial concentration is 35%. Although the elution of a single-mode band is always observed, this elution is carried out through a mixed process, which is no longer pure gradient elution but involves a significant migration rate as soon as the sample is injected. The characteristics of the chromatogram are close to those observed under isocratic conditions.

Volume and concentration loading

The amount of feed injected into the column is given by the product of the sample volume and its concentration. It is still not entirely clear whether there is any good reason to prefer the injection of large volumes of a dilute solution or of small volumes of a concentrated solution, and also it seems that an intermediate combination can be optimum [23]. However, the result does not need to be the same in gradient elution and under isocratic conditions.

Fig. 8 compares the dependence of the band width and the retention volume of lysozyme on the loading amount for two sets of different, fairly extreme conditions. In the first instance volumes increasing from 2.2 to 200 μl of a 1% solution were injected into the column, and in the second, a constant volume (200 μl) of solutions with concentrations increasing from 0.001 to 1% was injected.

There is very little difference between the two sets of results obtained. This conclusion is in agreement with the assumptions of our model of gradient elution chromatography. The sample is strongly adsorbed in a thin slice at the top of the column. This slice is nearly saturated, in agreement with a rectangular isotherm. When the sample size increases, the thickness of the slice also increases, but the stationary phase concentration and the thickness of the saturated slice do not depend on the concentration of the sample used, only on its total amount.

As a consequence, gradient elution could be used to concentrate dilute solutions. A 0.001% solution can be introduced in the column. With a very steep gradient a strong concentration of the band takes place and the eluate solution may reach a concentration of *ca.* 10%. In fact, the solubility limit and the increase in the band viscosity beyond 2–3 cP

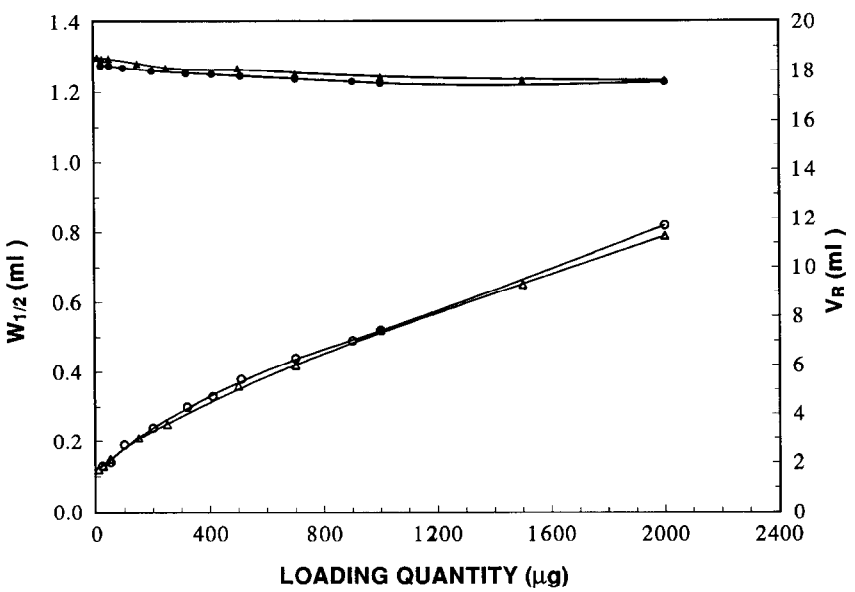

Fig. 8. Plots of retention volume and peak width *versus* sample size when the sample amount is adjusted at constant volume or constant concentration. Experimental conditions as in Fig. 2, except 15 × 0.46 cm I.D. column; sample, lysozyme; volume change by injecting 1% solution, concentration change by injecting 200 μl of different concentrations. V_R: ▲ = constant volume (200 μl); ● = constant concentration (1%). $W_{1/2}$: △ = constant volume (200 μl); ○ = constant concentration (1%).

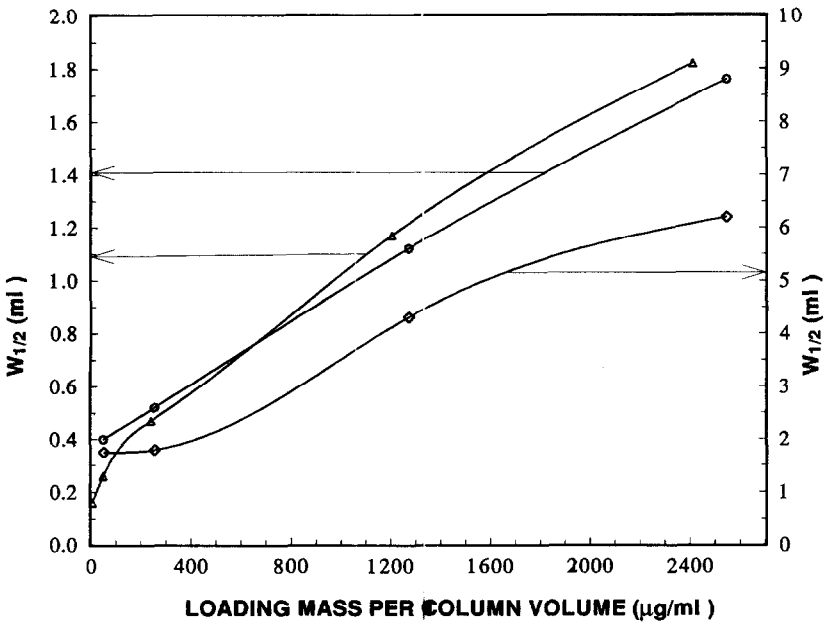

Fig. 9. Plot of peak width *versus* sample size for columns of similar volume and different length and diameter. Columns: (△) 25 × 0.46 cm I.D. (volume 4.15 ml, N = 8200 plates, h = 3.05 at 1 ml/min, with biphenyl) and (○, ◇) 5 × 1.0 cm I.D. [volume 3.93 ml, N = 1600 plates, h = 3.1 at (○) 1 ml/min and N = 1400 plates, h = 3.6 at (◇) 4.73 ml/min, with biphenyl] packed with Vydac 10 μm, 300 Å (lot number 891208-21); flow-rate, 1.0 and 4.73 ml/min; gradient, 15 to 65% B in 25 min; detection, UV at 300 nm; chart speed, 1.0 cm/min; sample, lysozyme injected by varying the volume.

and the fingering effects which could arise as a consequence of this viscosity difference [24] constitute the only practical limits to the sample concentration.

Influence of column diameter and volume on the band width

Two columns having nearly the same volume (4 ml) were packed with 10-μm Vydac 218TP silica C_{18}, one 25 cm × 0.46 cm I.D. and the other 5 cm × 1.0 cm I.D. with volumes of 4.15 and 3.93 ml, respectively. The former column was operated at 1 ml/min and the latter successively at 1 ml/min (constant flow-rate) and 4.73 ml/min (constant flow velocity). When the volume flow-rate was increased, the gradient slope (in % ACN/min) was kept constant. The band width of lysozyme on these two columns were measured for increasing sample amounts. The results are shown in Fig. 9.

Similar results were obtained with the two columns at constant volume flow-rate (1.0 ml/min). This is consistent with the fact that the reduced plate heights (h) of the two columns are about the same (3.1) at 1.0 ml/min. The short, wide column, however, is operated at a much lower velocity than the longer column. The wide column shows an increased performance with increasing loading amount. At sample loadings above 0.66 mg/ml, narrower peaks are obtained on the 1 cm than on the 0.46 cm I.D. column. When the sample loading drops below 0.66 mg/ml, broader peaks are obtained, particularly at very low sample sizes. This indicates that the small diameter column is more advantageous than the large diameter column in the analytical range (small sample sizes), and *vice versa* in high loading applications.

At constant flow velocity, much broader volumetric peak widths (see the right-hand ordinate in Fig. 9) are obtained on the wide column, which is operated at a higher volume flow-rate. However, the comparison must take into account the change in retention volume. In gradient elution, a higher flow-rate results in an earlier elution of the band, and hence in its elution at a lower solvent strength. This means that the elution volume will be higher and so will be the volume band width. For example,

when comparing the chromatograms on the two columns at the same mobile phase velocity, with a 1270 mg/ml loading, there is a 2.9-fold increase in the retention volume and a 3.5-fold increase in the volumetric band width for the wider column. This performance decrease seems to be a consequence of the larger reduced plate height (3.6 instead of 3.1 at 1 ml/min). In addition, when the flow-rate is increased, the gradient delay due to the system dead volume changes, although the gradient slope (in % ACN/min) was kept constant [19,21,22].

Assuming that the separation peformances of the two columns of equal volume are comparable, the advantages of the short, wide column can be summarized as follows. First, even if we keep the mobile phase velocity constant, the inlet pressure is considerably decreased, in proportion to the ratio of the column lengths. Second, the cycle time can be dramatically reduced, as it is possible to use a very high flow-rate during the column regeneration (see Fig. 6), the flow-rate resistance of a short column being so low. The production rate increases in proportion to the reverse of the cycle time. Third, the band width is slightly narrower with the shorter, wider column at high sample loadings, which may permit the use of a still shorter column, thus decreasing the expense and the amount of solvent consumed and increasing further the production rate.

It was not possible to detect the bands obtained on the wider column with a sample of less than 20 μg, showing a significantly higher degree of dilution in the mobile phase. The phenomenon was observed at both flow-rates, 1.0 and 4.73 ml/min. If we operate the columns at constant velocity, the dilution of the product and the volume of solvent needed increase. If we operate the column at constant flow-rate, the production rate, the amount of solvent needed and the degree of dilution remain nearly constant. These results suggest that the flow velocity may not necessarily be kept constant when a separation procedure using gradient elution is scaled-up by increasing the column diameter.

Elution profiles

Fig. 10 compares the elution profiles obtained on the two columns of constant volume, with the same sample amounts. These profiles are nearly identical at high loadings. At low loadings, the profile obtained on the wider column is much worse, which

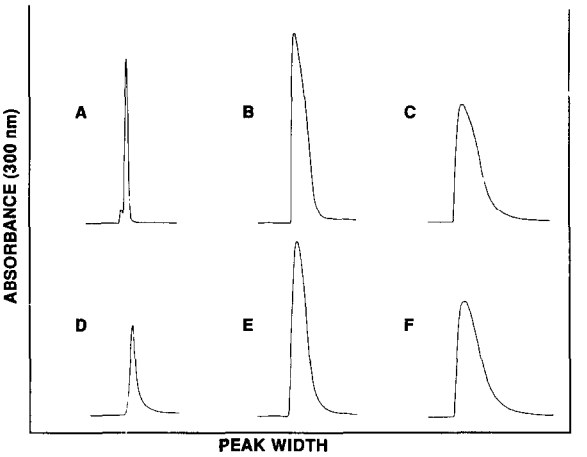

Fig. 10. Comparison of chromatograms obtained with lysozyme on columns of the same volume and different diameter. Columns and experimental conditions as in Fig. 9, except all chromatograms with a flow-rate of 1 ml/min. Peaks A, B and C (20-, 500- and 1000-μl injection, respectively) were obtained with the 25 × 0.46 cm I.D. column and D, E and F (20-, 500- and 1000-μl injection, respectively) with the 5 × 1.0 cm I.D. column.

explains the justified favor that narrow columns enjoy among analysts. There is no reason for prejudices justified in the analytical applications of chromatography to be extended to its preparative applications, where they are unwarranted.

Whereas analytical columns for the fast analysis of proteins by gradient elution should be narrow, preparative columns should be wide and shorter than analytical columns.

Touching band separations

Band profiles and band widths are useful characteristics of separations and their study has permitted a detailed analysis of the band broadening mechanism observed when a column is overloaded with proteins under gradient elution conditions. Another approach for this study is the comparison of chromatograms obtained under touching band conditions, a simple and moderate degree of column overloading.

Fig. 11 compares the chromatograms obtained on three columns, the two columns studied in the previous section (25 × 0.46 cm I.D. and 5 × 1 cm I.D.) and a 12.5 x 0.46 cm I.D. column. The same chromatogram, corresponding to touching band conditions, is eventually obtained with the three

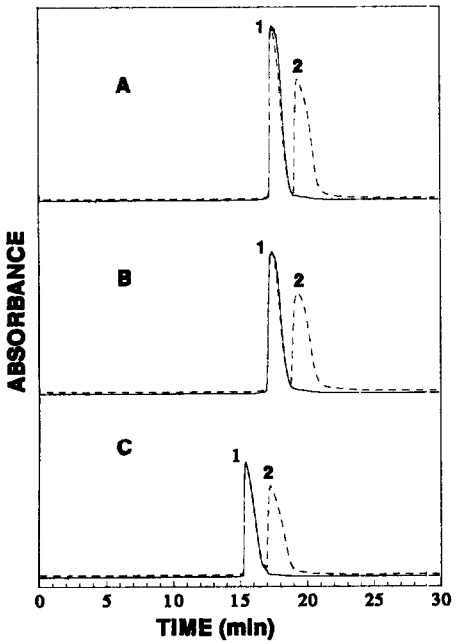

Fig. 11. Comparison of the touching band chromatograms obtained on columns of different dimensions. Columns and experimental conditions as in Fig. 5. Chromatograms A, B and C were obtained with the 25 × 0.46 cm, 5 × 1.0 cm and 12.5 × 0.46 cm I.D. columns, respectively. Injection volumes: (A and B) 500 μl; (C) 250 μl. Dashed lines, detection at 300 nm; solid lines, detection at 320 nm. 1, cytochrome *c*; 2, lysozyme.

columns when the sample amount injected is progressively increased. The sample amount corresponding to touching band conditions is identical for the two columns which have the same volume (5 mg of lysozyme and 2.5 mg of cytochrome *c*), and it is twice as small for the column which has half the volume of the other two columns.

CONCLUSIONS

We have shown that the loading capacity of a chromatographic column in gradient elution is proportional to its volume. Under isocratic conditions, there is a plate number threshold below which the separation is impossible. As the column length has little influence on the resolution, there is no such threshold in gradient elution. Within a wide range of combinations, short, wide columns give nearly the same production rate as long, narrow columns of the same volume.

The packing quality of the column, *i.e.*, its reduced plate height under analytical conditions, remains important and efforts should be made to pack preparative columns at least as carefully as analytical columns and to select high-quality packing materials.

It remains unclear, at this stage, how thin a column operated under gradient elution can be. There is a lower limit set by the homogeneity of the hydrodynamic performance. The flow velocity should be the same all over the column cross-section. This can probably be achieved with fairly thin columns. Specifications regarding column homogeneity and also the semantic problem of deciding when a thin column becomes a membrane may decide. A more practical limitation results from the extra-column sources of band broadening.

Finally, preparative chromatography does not deal with single-component profiles or with the resolution between independent bands. It deals with the production of pure components and their extraction from complex feeds. At high concentrations displacement effects take place in gradient elution as under isocratic conditions. These effects increase considerably the production rate above that which could be suggested by chromatograms. The investigation of their influence on the production rate under gradient elution conditions is in progress and the results will be reported later [25].

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