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TANDEM COLUMNS AND MIXED-BED COLUMNS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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SUMMARY

By using a cation- and an anion-exchange column in series, mixtures of acidic and basic proteins were separated in a single chromatographic run with increasing salt gradient at pH 7.0. The serial order of the columns was found to affect the chromatographic results, and the effect was attributed to alteration of the salt gradient profile upon traversing the first ion-exchange column. Single columns, packed with a binary mixture of a cation and an anion exchanger gave similar chromatographic results as the tandem columns and thus offered an alternative approach to the separation of both acidic and basic proteins in a single chromatographic run. A ternary mixed phase was obtained by adding a mildly hydrophobic stationary phase to the mixture of the two ion exchangers. This column could be used with increasing salt gradient as a cation exchanger for the separation of basic proteins, or as an anion exchanger for the separation of acidic proteins. Furthermore, it could be used as a "bipolar" electrostatic-interaction column with increasing salt gradient and as a hydrophobic-interaction column with decreasing salt gradient for the separation of both types of proteins in a single chromatographic run. The constituent stationary phases used in the mixed-bed columns were prepared from the same silica support, *i.e.*, they had the same particle and pore dimensions, density, and pore volume. Besides their obvious advantages in analytical applications, appropriate mixed stationary phases, all having retentive properties for the components to be separated, are expected to be useful also in preparative chromatography to "tailor" column selectivity for a given separation problem without loss of separating capacity.

INTRODUCTION

The selectivity of a given stationary phase toward the components of a mixture to be separated can be too low and/or too high under the chromatographic conditions employed. In the case of low selectivity, the relative retention of certain eluite pair(s) may be insufficient to effect their resolution. When the selectivity is too high, certain sample components may be retained beyond practical limits or, in turn, some may be eluted in the column void volume. The latter situation arises in electrostatic-interaction chromatography of proteins, *e.g.*, when the negatively and positively charged sample components may not be retained by a cation-exchange and anion-exchange column, respectively.

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We have investigated the tandem use of a cation- and anion-exchange column with gradient elution in order to facilitate the analytical resolution of protein mixtures having components with a wide range of isoelectric points by electrostatic-interaction chromatography. Furthermore, we have found that the separation of such samples could also be carried out with a single column, packed with a mixture of a suitable cation-exchange and anion-exchange material. Such mixed-bed columns not only allow the separation of acidic and basic proteins in a single chromatographic run, but also can have a higher selectivity toward the components of interest than columns packed with the appropriate single stationary phase. The significance of mixed bed columns may transcend analytical applications, and the approach may be useful to tailor the properties of the stationary phase for preparative separations. The need for column engineering, *i.e.*, design of the column and the stationary phase for a particular application, arises from the growing employment of chromatographic methods for isolation and purification of proteins on a production scale in biotechnology.

Tandem columns in their present application represent a special case of coupled columns, which have been used to enlarge the scope of chromatographic separations when the components of the mixture exhibit a wide range of retention behavior[1]. A variety of schemes have been suggested[2,3], most of them involving the use of not only more than one column but also elaborate valving and pumping systems for the different eluents required[4,5]. Our approach described here is much more modest, as it employs two columns in series, which are operated with the same mobile phase. Such a technique has been used in biochemical purifications[6,7], and our goal is to demonstrate the advantages and certain peculiarities of tandem columns in analytical separations by high-performance liquid chromatography (HPLC) with gradient elution.

Columns with mixed beds were described for use in liquid chromatography as early as forty years ago[8]. The subject was extensively investigated in gas chromatography[9–11], since this technique facilitated rapid and precise measurements and, consequently, the physico-chemical investigation of retention behavior in mixed-bed columns. Nevertheless, the volatility differences among the stationary phases employed in gas–liquid chromatography have greatly limited the acceptance of this approach.

Remarkably enough, mixed-bed columns have received little attention in HPLC, instead, modulation of column selectivity was attempted by tedious surface modification of bonded stationary phases by chemical means[12] but tailoring the stationary phase surface in a desirable and reproducible fashion for various separation problems is beset by difficulties. Fortunately, a wide array of mobile-phase additives, *e.g.* hetaerons[13], are available for modulating the selectivity in reversed-phase chromatography, so far the most popular branch of HPLC and, therefore, a limited number of stationary phases suffices to carry out most separations by this technique.

However, under conditions of reversed-phase chromatography, denaturation of biopolymers usually occurs. Therefore, HPLC of biopolymers without compromising the integrity of their native molecular structure represents a challenge. Various types of microparticulate, macroporous bonded stationary phases with a hydrophilic surface layer, containing weak binding sites, have become available recently[14–17]. The most common techniques for carrying out protein separations by interactive chromatography are electrostatic-interaction chromatography with such "soft" ion exchangers and hydrophobic-interaction chromatography with mildly hydrophobic sta-

tionary phases, which may include ion exchangers[18]. Recently, metal chelate-interaction chromatography[19] has also shown promise in protein HPLC. The latter technique employs a chelating bonded phase. Column selectivity can be varied by changing the metal, complexed to the fixed chelating functions of the stationary phase. Such a flexibility is not available with the stationary phases employed in the other branches of interactive protein chromatography, where columns in tandem or packed with mixed stationary phases are expected to be of major significance.

The stationary phases used here are silica-based sorbents having a "soft" hydrophilic surface and thereby allowing the proteins to interact with the appropriately spaced fixed charges and/or hydrophobic functions. Due to their rigidity and dimensional stability, siliceous stationary phases are much more suitable for the preparation of mixed-bed columns than the cellulosic and other polysaccharide-based column materials traditionally employed for protein chromatography. The components of the mixed stationary phases used in this study were made from the same parent silica support by using similar chemistry as far as the "soft" surface layer is concerned. Since each of them featured the same particle size and pore-size distribution, neither the uniformity of the packed bed was compromised nor the size-exclusion properties of the mixed stationary phases were different from those of the individual packing materials.

EXPERIMENTAL

Instruments

The liquid chromatograph consisted of a Micromeritics (Norcross, GA, U.S.A.) Model 750 pump with a Model 753 ternary solvent mixer and a Model 740 control module. Samples were injected by a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve with 100-μl sample loop, and a Kratos Analytical (Ramsey, NJ, U.S.A.) Model 770R variable-wavelength UV detector was used to monitor the column effluent at 280 nm. Chromatograms were recorded with a Model C-R3A integrator (Shimadzu, Columbia, MD, U.S.A.).

Columns

Four ion-exchange columns measuring 80 × 6.2 mm were packed with Zorbax Bio Series WCX-300, SCX-300, WAX-300 and SAX-300, stationary phases obtained from DuPont de Nemours (Wilmington, DE, U.S.A.). According to the manufacturer, the fixed ionogenic functions of the stationary phases are: $-COOH$, $-SO_3H$, $-NH_2$ and $-N(CH_3)_3$ for WCX, SCX, WAX and SAX, respectively. The support of these stationary phases is zirconia-treated Zorbax PSM 300, a spherical, porous silica[20], having a mean particle and pore diameter of 7.5 μm and 300 Å, respectively. Tandem columns were connected in series via 50 × 0.25 mm capillary tubing, and No. 316 stainless-steel frits, having 2-μm pores, were used at the inlet and outlet of all columns. Phenyl Zorbax-300 stationary phase was prepared from Zorbax Bio Series GF-450 diol by a procedure similar to that described previously[17]. Mixed-bed columns were packed by using a methanolic slurry of appropriate mixtures of the above-mentioned ion exchangers into stainless-steel tubes (Handy and Harman, Norristown, PA, U.S.A.) at 8000 p.s.i.[21]. Prior to packing, the dry stationary phases were mixed in a beaker by shaking, then 10 ml methanol was added per gram of powder, and after vigorously shaking it again, the slurry was sonicated for 3 min.

TABLE I

PROTEINS USED IN THIS STUDY

Protein	*Symbol*	*MW*	*pI*	*Source*
Cytochrome *c*	CYT	12 200	10.6	Horse heart
α-Chymotrypsinogen A	CHY	25 500	9.5	Bovine pancreas
Ribonuclease A	RNase	13 700	9.4	Bovine pancreas
Transferrin	TR	77 000	5.0	Bovine
Lysozyme	LYS	14 000	11.0	Chicken egg white
Conalbumin	CON	93 000	6.8	Chicken egg white
β-Lactoglobulin A	LAC A	35 000	5.1	Bovine milk
β-Lactoglobulin B	LAC B	35 000	5.1	Bovine milk
Ovalbumin	OVA	44 000	4.7	Chicken egg
Hemoglobin	Hb	68 000	6.8	Bovine blood

Materials

The proteins listed in Table I were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade disodium hydrogen phosphate, ammonium sulphate, sodium chloride, Tris base, phosphoric acid, as well as HPLC-grade methanol and acetonitrile were obtained from Fischer (Pittsburgh, PA, U.S.A.). Distilled water was prepared with a Barnstead unit.

RESULTS AND DISCUSSION

Tandem columns

A mixture of seven proteins having a wide range of isoelectric points was chromatographed by using a linear gradient of sodium chloride from 0 to 0.8 *M* in 25 m*M* phosphate buffer (pH 7.0) with a gradient time of 40 min. The two chromatograms, obtained with the single weak anion- or cation-exchange column, are illustrated in Fig. 1. The basic proteins, ribonuclease A, lysozyme, the oxidized and reduced forms of cytochrome *c* were eluted in the void volume of the weak anion-

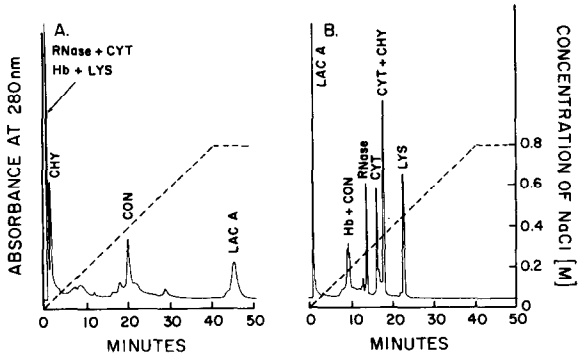

Fig. 1. Chromatograms of proteins on weak anion (A) and weak cation (B) exchangers. Columns, 80 × 6.2 mm, Zorbax WAX-300 (A) and Zorbax WCX-300 (B); mean particle diameter, 7.5 μm; flow-rate, 1.5 ml/min; temperature, 25°C. Linear gradient in 40 min from 0 to 0.8 *M* sodium chloride in 20 m*M* Tris–HCl (pH 7.0).

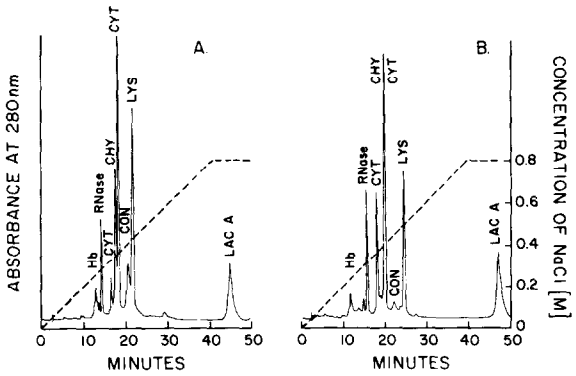

Fig. 2. Separation of proteins on weak anion- and cation-exchange columns in series. (A) The anion-exchange column is first, followed by the cation exchanger. (B) The cation-exchange column is first, followed by the anion exchanger. Columns and conditions as in Fig. 1.

exchange column, while α-chymotrypsinogen A was slightly retained. The acidic protein, β-lactoglobulin A, was retained significantly, together with conalbumin, although hemoglobin, which according to the literature has the same p*I* as conalbumin, was eluted in the column void volume. On the weak cation-exchange column, all proteins were retained, except β-lactoglobulin A, but neither the eluite pair hemoglobin and conalbumin nor the pair cytochrome *c* and α-chymotrypsinogen A were resolved.

However, by using the two columns in series the separation of all proteins could be accomplished. Chromatograms obtained with the tandem column system under the same elution conditions as those used with the individual columns are illustrated in Fig. 2. An unexpected result of these experiments, as seen in Fig. 2, is that upon reversing the order of the two columns in the series there is a change in the elution profiles. Generally, better resolution was obtained and cytochrome *c* was separated from α-chymotrypsinogen A when the cation exchanger followed the anion exchanger.

The same protein mixture was also chromatographed on both, the strong cat-

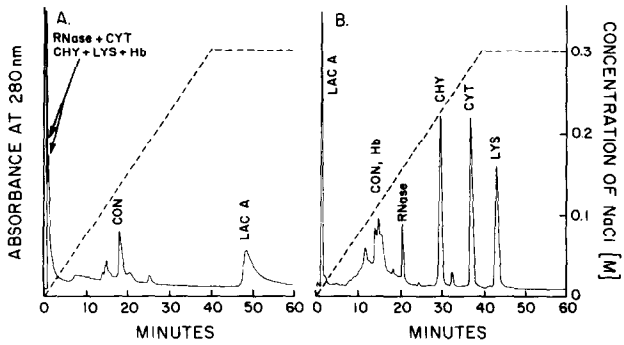

Fig. 3. Chromatograms of proteins on strong anion (A) and strong cation (B) exchangers. Columns, 80 × 6.2 mm, Zorbax SAX-300 (A) and Zorbax SCX-300 (B); mean particle diameter, 7.5 μm. Conditions as in Fig. 1, except the final concentration of sodium chloride was 0.3 *M*.

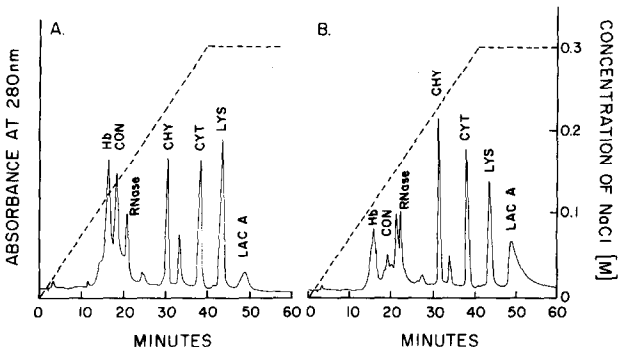

Fig. 4. Separation of proteins on strong ion-exchange columns in series. (A) The cation-exchange column is first, followed by the anion exchanger. (B) The anion-exchange column is first, followed by the cation exchanger. Columns and conditions as in Fig. 3.

ion- and anion-exchange columns. The chromatograms (Fig. 3) show that the strong anion exchanger retained and separated only conalbumin and β-lactoglobulin A, while the other proteins were eluted in the void volume. On the other hand, only β-lactoglobulin A was eluted in the void volume of the strong cation-exchange column, while the other proteins were retained and separated, except for the eluite pair conalbumin and hemoglobin. However, when the two columns were used in tandem, all the proteins were separated, although the selectivity for the early peaks was dependent on the serial order of the columns, as seen in Fig. 4.

In liquid chromatography under isocratic elution conditions one does not expect the serial order of equally sized tandem columns to have an effect on the final elution pattern, as the system is linear and, therefore, the contributions of the columns to the retention of any given eluite are additive. The differences observed in the chromatograms depicted in Figs. 2 and 4 must therefore be attributed to the use of gradient elution. The movement of the eluites through the column under conditions where the eluent strength changes with axial position and time, such as those which prevail in gradient elution, is most appropriately described by their trajectories. These are conveniently illustrated by plotting the axial position of the peaks as a

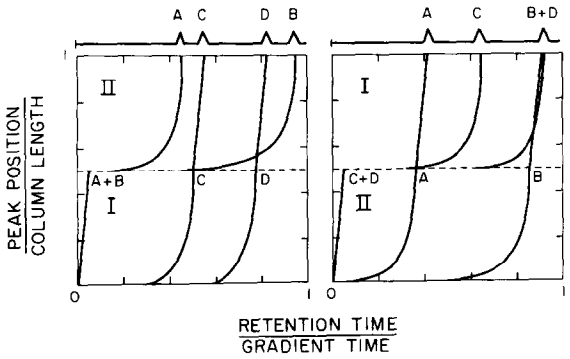

Fig. 5. Normalized time *versus* distance diagrams for schematic illustration of eluite trajectories with gradient elution in dual tandem columns. On the left, column I is first and column II is second in the series, whereas on the right the order is reversed.

function of time, *i.e.* by a time *versus* distance diagram[1]. A schematic illustration of such trajectories in a dual tandem-column system is illustrated in Fig. 5 for two cases in which the serial order of the columns is reversed. When column I and column II in the series are packed with an anion exchanger and a cation exchanger, respectively, the basic proteins A and B pass through column I without retention, whereas the acidic proteins C and D interact with the anion exchanger and follow trajectories typical for gradient elution. As the eluites enter column II, the situation is reversed so that A and B follow a quasi-exponential trajectory, whereas C and D pass through the column unretained. The chromatogram produced when the four components emerge at the outlet of column II is illustrated on the left at the top of Fig. 5.

When the order of the tandem columns is reversed, one would expect that the individual trajectories will be the same as before in each column, and their addition in a fashion shown before gives rises to the same chromatogram, irrespective of the serial order of the two columns. The different pictures shown in Fig. 5 are believed to arise from the modification of the salt gradient upon passing through the first ion-exchange column in the series so that the gradient shape will not be the same in column II as it was when that column was the first in the series. As a result, the trajectories of retained eluites in a given column depend on the position of the column in the series and, thus, the elution patterns obtained from the tandem column systems depend on the serial order of the columns. As seen in Figs. 2 and 4, the effect is small and the chromatograms exhibit only slightly different elution patterns. As expected, the differences are most pronounced for early peaks, the retention of which is most affected by the column-mediated alteration of the gradient shape. In gas chromatography with tandem columns a similar dependence of retention on the serial order of the columns was observed and attributed to the compressibility of the carrier gas[10].

Mixed-bed columns

Binary stationary phase. The same stationary phases as those used in the tan-

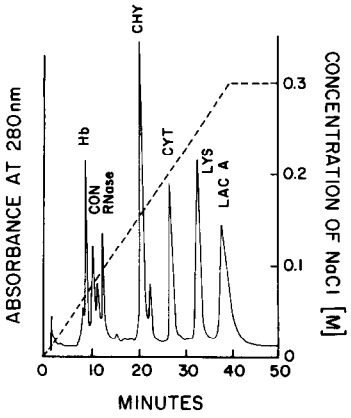

Fig. 6. Separation of proteins on a binary mixed-bed column, containing a 1:1 mixture of strong anion and cation exchanger. Column, 100 × 4.6 mm; mean particle diameter, 7.5 μm; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient in 40 min from 0 to 0.3 *M* sodium chloride in 20 m*M* Tris–HCl (pH 7.0).

dem columns were mixed and packed into a single column in order to investigate the potential of such mixed-bed columns for protein separations. Fig. 6 shows a chromatogram obtained with a mixed-bed column that contained a 1:1 mixture of the strong cation and anion exchangers. Linear gradient elution with increasing sodium chloride concentration was used, as in the previous experiments. The elution pattern is similar to that obtained by using tandem columns when each of them is packed with one of these stationary phases (*cf.* Fig. 4).

Ternary stationary phase. In another set of experiments we investigated the properties of columns packed with a mixture of three stationary phases: a weak anion and cation exchanger and a mildly hydrophobic stationary phase in a 1:1:1 ratio. The column packed with the ternary stationary phase was used in both electrostatic- and hydrophobic-interaction chromatography of proteins. The separation of a protein mixture with increasing salt gradient is illustrated in Fig. 7, which shows the use of the ternary stationary phase as a "biopolar" ion exchanger for the separation of proteins having a wide range of isoelectric points in a single run. In addition, the column can be used either as an anion exchanger for the separation of proteins of low p*I* values, or as a cation exchanger for the separation of proteins of high p*I* values as shown in Fig. 8. Furthermore, the same column can also be used for the separation of proteins with decreasing salt gradient by hydrophobic-interaction chromatography. This is illustrated in Fig. 9, which also shows for comparison a chromatogram obtained under conditions identical with those for a column packed with only the "neutral" stationary phase for hydrophobic-interaction chromatography. It is seen that the chromatograms are very similar and that replacement of 67% of the hydrophobic stationary phase by ion exchangers resulted in only a modest decrease in retention.

An earlier report from our laboratory[18] deals with the interplay of electrostatic and hydrophobic interactions in protein chromatography and shows that soft ion

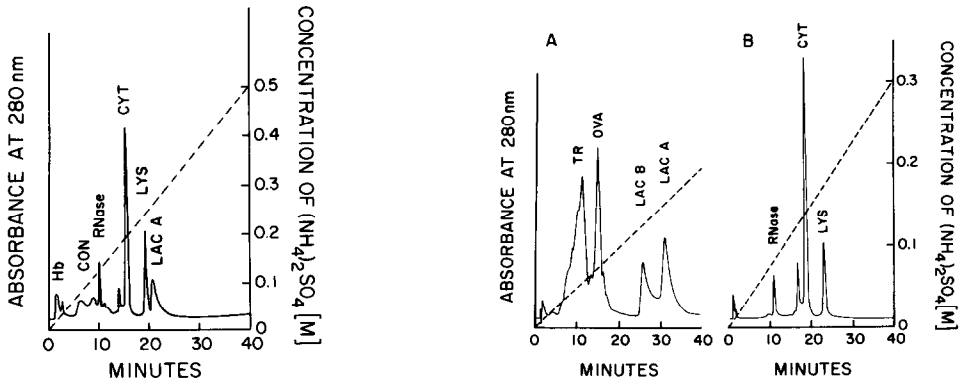

Fig. 7. Separation of proteins on a ternary mixed-bed column, containing equal parts of a weak anion exchanger, a weak cation exchanger, and a weakly hydrophobic stationary phase. Column, 100 × 4.6 mm; mean particle diameter, 7.5 μm; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient: in 40 min from 0 to 0.5 *M* ammonium sulphate in 20 m*M* Tris–HCl (pH 7.0).

Fig. 8. Separation of proteins of low (A) and high (B) p*I* values on a ternary mixed-bed column. Column and conditions as in Fig. 7, except the final concentation of ammonium sulphate was 0.2 *M* in A and 0.3 *M* in B.

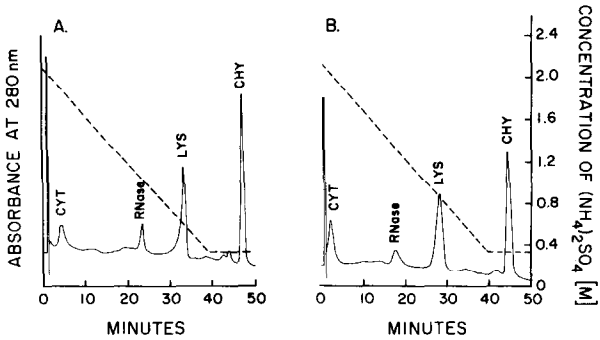

Fig. 9. Separation of proteins on a hydrophobic-interaction column (A) and on the ternary mixed-bed column used in Fig. 8 (B). Columns, 100 × 4.6 mm; flow-rate, 1.0 ml/min; temperature, 25°C. Linear gradient: in 40 min from 2.2 *M* to 0.35 *M* ammonium sulphate in 25 m*M* phosphate buffer (pH 6.0).

exchangers can be used for protein separation not only by electrostatic-interaction chromatography with increasing salt gradient, but also by hydrophobic-interaction chromatography with decreasing salt gradient. Therefore, the binary mixed ion-exchange column can also be used in hydrophobic-interaction chromatography at sufficiently high salt concentrations in the eluent. However, addition of the mild hydrophobic stationary phase, renders the column more hydrophobic, so that the ternary mixed-bed column can be used in hydrophobic interaction chromatography at significantly lower salt concentrations. Yet, the presence of the weakly hydrophobic stationary phase does not preclude the use of the column in electrostatic-interaction chromatography at sufficiently low salt concentrations. Thus, the ternary mixed-bed column can be employed as an anion exchanger, a cation exchanger, a hybrid "bipolar" ion exchanger, or as a hydrophobic-interaction column.

CONCLUSIONS

It is shown that, by using columns in series, analytical chromatography of samples having components of wide-ranging retention properties can be facilitated. Even though the tandem columns described here were of equal dimensions, further flexibility in modulating the selectivity of the system may arise from using columns of different dimensions, *i.e.*, inner diameter and/or length, in series. In this way, the relative amounts of the stationary phases in contact with the mobile phase and the relative values of the linear flow velocities in the individual columns can be varied to bring about the resolution of the sample components of interest.

Another modification of column selectivity is shown by the use of mixed-bed columns. This approach involves mixing of various types of stationary phases made by surface modification of the same support material, although this is not a requirement, as long as the mixed bed has the desired chromatographic behavior and operational stability. Mixed-bed columns exhibit the properties of each of the constituent stationary phases and, accordingly, they can be used in the corresponding separating modes. By varying the mixing ratio, column selectivity can be modulated.

Both, tandem columns and mixed-bed columns, offer approaches to expanding the scope of biopolymer chromatography in analytical work. When a maximal utilization of the stationary phase capacity is required, *i.e.*, in preparative or process-

scale chromatography, the employment of tandem or mixed-bed columns, containing stationary phases of widely different retention properties, appears to be less attractive. Nonetheless, for such applications mixing of stationary phases having similar retentive properties but different selectivities can be an excellent expedient for "tuning" column selectivity without loss of separating capacity.

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