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MIXED-BED ION-EXCHANGE COLUMNS FOR PROTEIN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

YIH-FEN MAA, FIROZ D. ANTIA, ZIAD EL RASSI and CSABA HORVÁTH*
Department of Chemical Engineering, Yale University, New Haven, CT 06520 (U.S.A.)

SUMMARY

Protein retention is investigated on high-performance liquid chromatography columns packed with mixtures of ion exchangers. Retention factors are measured at both low and high salt concentrations in the eluent and their dependence on the bed composition is found to be linear in some cases, but non-linear in others. The physical basis for the observed non-linear retention behavior has not been established and an empirical mixing rule is employed to express the dependence of protein retention on bed composition. Protein separations are carried out on the mixed-bed columns by using gradient elution with increasing salt concentration and the process is modelled mathematically. The retention times predicted by computer calculations correspond closely to the experimental findings. Optimal selection of the mixed-bed composition and the gradient steepness for the separation of four proteins is illustrated by using the window diagram technique. Although the experimental results presented here deal with electrostatic interaction chromatography of proteins only the applicability of mixed sorbents is expected to extend to all branches of liquid chromatography. It is anticipated that mixed-sorbent columns will find extensive use in the large-scale purification of biological compounds and in routine analysis.

INTRODUCTION

In liquid chromatography (LC), retention and selectivity are most conveniently adjusted to the desired value by appropriately changing the composition of the mobile phase. Whereas this practice is widespread, in certain instances the freedom to select the mobile phase composition is curtailed or the available means are insufficient to tailor retention behavior in the required fashion. Such a situation may occur in the rapidly growing field of large-scale chromatography where process design may entail the specification of a stationary phase obtained by mixing two or more sorbents in order to attain retentive properties appropriate for solving the separation problem at hand. On the other hand in analytical high-performance liquid chromatography

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(HPLC) we may want to maximize the selectivity of the chromatographic system for the components of a given sample to be analyzed routinely.

Mixed stationary phases have been used in gas–liquid chromatography where it is more effective to manipulate the properties of the liquid stationary phase than those of the carrier gas[1–4]. Columns with multiple stationary phases have been obtained in three ways: (a) with a series of columns, each packed with a different stationary phase, (b) by coating a mixture of liquids onto the support and (c) by packing support particles coated with different liquids in a mixed bed. Each has its parallel in LC; tandem or mixed-bed columns have been shown to offer a convenient means to manipulate stationary phase selectivity[5] and stationary phases with mixed ligates in LC[6] can be likened to mixed-liquid phases in gas chromatography (GC) since in both cases mixing is on the molecular scale.

Our primary concern in this study is to examine the use of columns packed with mixed ion exchangers in the linear elution chromatography of proteins. Mixed-bed columns of anion and cation exchangers were first used for desalination in 1951[7], and are still employed for the deionization of water and other non-ionic substances. In chromatography, however, they have so far found very limited applications[5,8,9]. We shall therefore begin with an examination of the relationship between the retention factor and the composition of binary mixed-bed columns. The relationship will then be used to predict protein retention in gradient elution on such columns and to select an optimal bed composition for the separation of acidic and basic proteins on a column packed with mixed anion and cation exchangers.

Since the primary interaction responsible for retention in ion-exchange columns often changes with increasing ionic strength in the mobile phase from electrostatic to hydrophobic[10], the effect of bed composition on retention will be examined under a wide range of salt concentrations. For simplicity the studies on gradient elution and optimization will be restricted to electrostatic interaction chromatography (EIC).

EXPERIMENTAL

Instrumentation

The chromatograph was assembled from a Micromeritics (Norcross, GA, U.S.A.) Model 750 solvent delivery pump with a Model 753 ternary solvent mixer and a Model 740 control module. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 sampling valve with a 100-μl sample loop was used for sample injection. A Kratos (Ramsey, NJ, U.S.A.) Model 770R variable-wavelength detector was used to monitor the effluent at 280 nm. Chromatograms were recorded with a Shimadzu (Columbia, MD, U.S.A.) Model C-R3A integrator.

Columns

Zorbax BioSeries WCX-300 (B-WCX), SCX-300 (B-SCX), WAX-300 (B-WAX) and DEAE-300 (B-DEAE) were obtained from DuPont (Wilmington, DE, U.S.A.). According to the manufacturer, the fixed ionogenic functions are $-COOH$, $-SO_3H$, $-NH_2$ and $-N(C_2H_5)_2$ on the stationary phases B-WCX, B-SCX, B-WAX and B-DEAE, respectively. The support of these stationary phases is zirconia-treated Zorbax PSM 300[11], a spherical silica, having mean particle and pore diameters of

7.5 μm and 300 Å, respectively. Three other stationary phases, H-WCX, H-WAX and H-diol, were prepared in this laboratory by using established procedures[12,13] from Vydac silica gel (5 μm; 300 Å) supplied by The Separations Group (Hesperia, CA, U.S.A.) and have fixed –COOH, $-N(C_2H_5)_2$ and –OH functions at the surface, respectively. Under typical conditions, the stationary phase particles were mixed together in a given proportion and 1.5 g of the mixture was suspended in 25 ml methanol by sonication for 1 min. The resulting slurry was packed into a 100 × 4.6 mm I.D. No. 316 stainless-steel column with methanol as the packing solvent at 8000 p.s.i. for 20 min by using an air-driven pump (Haskel, Burbank, CA, U.S.A.).

As a test to determine whether column properties were influenced by electrostatically induced aggregation or non-uniform mixing of the ion-exchanger particles, another set of H-WCX–H-WAX mixed-bed columns was packed with a slurry of the stationary phase mixture in 20 m*M* aqueous phosphate buffer, pH 2.0, containing 200 m*M* sodium chloride. Results with these columns were found to match those obtained with columns packed with methanol slurry. The stability of the mixed-sorbent columns was tested by passing through the columns 4 l of 20 m*M* Tris–HCl, pH 7.0, containing 80 m*M* ammonium sulphate; the retention factors of proteins measured before and after this treatment differed only marginally.

Materials

Lysozyme (LYS), conalbumin (CON), and ovalbumin (OVA), all from chicken egg, α-chymotrypsinogen A (CHY) from bovine pancreas and hemoglobin (HEM) from bovine blood were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade ammonium sulfate, monobasic sodium phosphate, and sodium chloride were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade methanol was obtained from Fisher Scientific (Springfield, NJ, U.S.A.). Distilled water was prepared with a Barnstead unit (Barnstead, MA, U.S.A.).

Procedures

Mobile phases of appropriate salt concentrations were made by diluting a stock solution containing 0.4 *M* sodium chloride, 1.0 *M* or 2.0 *M* ammonium sulphate in 20 m*M* Tris–HCl buffer, pH 7.0, with the buffer solution. Columns were equilibrated with at least 100 ml of mobile phase before the first injection. Each protein was chromatographed at least twice, under isocratic elution conditions at a flow-rate of 1 ml/min at room temperature. Retention times, t_R, were measured at the intersection of the tangents drawn to the two inflection points of the peaks. For lack of uncharged homomorphs of the proteins, the retention time of fructose was taken as the mobile phase hold-up time, t_0. For convenience, the retention factors of proteins eluting earlier than fructose have been reported as negative values.

A mixture of lysozyme, ovalbumin, conalbumin and α-chymotrypsinogen A was chromatographed on each of the three mixed-bed columns composed of H-WCX–H-WAX (25:75), H-WCX–H-WAX (50:50) and H-WCX–H-WAX (75:25), respectively, by a linear gradient elution. The gradient elution was run from 0.05 *M* to 0.3 *M* sodium chloride in 20 m*M* Tris buffer, pH 7.0, at 25°C in 30 min.

Computations

All computations were performed on a MicroVAX computer at the Depart-

ment of Chemical Engineering at Yale University. Three dimensional plots were generated using the Td3 graphics routine accessed through the Yale Computer Center.

RESULTS AND DISCUSSION

Retention factors versus bed composition

In linear elution chromatography with a mixed-sorbent column, the dependence of the retention factor, k', on the bed composition is expected to follow the simple additivity relationship

$$k' = (1/V_M) \sum_{i=1}^{N} (V_{Si} K_i) \tag{1}$$

where V_{Si} is the volume of sorbent i in the column, V_M is the volume of mobile phase, K_i is the equilibrium constant for the partitioning of the eluite between stationary phase i and the mobile phase and N is the number of different sorbents in the mixed-bed. As discussed in the Appendix, when the different sorbent particles have the same density, diameter and porosity, the retention factor of a given eluite can be expressed as a function of the retention factors, k'_i, measured on single-sorbent columns packed in a manner identical with the mixed-sorbent column, and the individual sorbent weight fractions in the mixed-bed, w_i, according to

$$k' = \sum_{i=1}^{N} w_i k'_i \tag{2}$$

Since the sum of the weight fractions is unity, according to eqn. 2 the retention factor is a function of $N-1$ independent weight fractions.

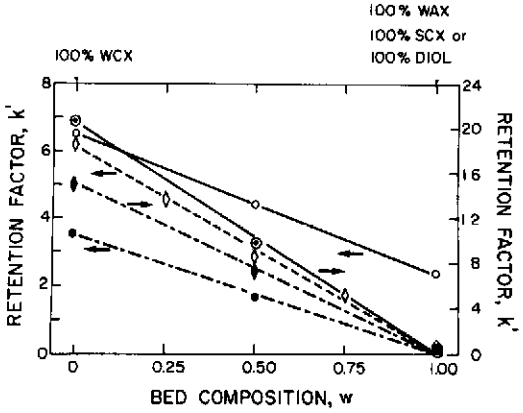

Fig. 1. Plots of retention factors against bed composition for lysozyme and α-chymotrypsinogen on columns packed with the sorbent mixtures H-WCX–H-diol (----), B-WCX–B-SCX (———) and B-WCX–B-WAX (- - - -) at different salt concentrations in 20 m*M* Tris–HCl, pH 7.0. Lysozyme: (◇) 0.22 *M* NaCl; (◆) 0.1 *M* $(NH_4)_2SO_4$. α-Chymotrypsinogen A: (○) 0.08 *M*; (●) 0.1 *M*; (⊙) 2.0 *M* $(NH_4)_2SO_4$. Column, 100 × 4.6 mm I.D.: flow-rate, 1.0 ml/min; temperature, 25°C.

In this study, we shall examine protein retention on binary mixed-sorbent columns packed by the standard procedure outlined previously with stationary phases prepared from the same support. Fig. 1 shows the retention factors of two proteins plotted against the bed composition of columns packed with mixtures of either H-WCX–H-diol, B-WCX–B-SCX, or B-WCX–B-WAX. Linear behavior is observed in each case. The proteins do not interact with the H-diol phase, which acts merely as a diluent for the H-WCX. Retention data on the mixed cation-exchangers, B-WCX and B-SCX, are shown at both low and high salt concentrations. The retention *versus* bed composition relationship is linear in both cases, despite change in the retention mechanism from electrostatic to hydrophobic interactions. In these experiments the linear nature of the relationship is conserved even when the mixed sorbents carry oppositely charged groups, as with both the B-WCX–B-WAX and the B-WCX–B-DEAE mixtures.

In other cases, however, the *k' versus w* relationship is found to be markedly non-linear, as shown in Fig. 2 by plots of the retention factor against the bed composition of mixed H-WCX and H-WAX sorbents at low salt concentration in the eluent. The curves for the four basic proteins exhibit greater departures from linearity than that for ovalbumin that is acidic at the eluent pH. For the same set of columns, the non-linearity persists at high salt concentrations, as can be seen in Fig. 3.

Non-linear dependence of the retention on bed composition was also observed in the LC of organic compounds on columns packed with mixed polar adsorbents[8]. N-Alkyl substituted aromatic amines were found to adsorb more strongly on a mixture of boron oxide and silicic acid than on either sorbent alone and the magnitude of the enhanced adsorption was dependent on the particular eluite. The effect was ascribed to "interactions" between the adsorbents but the precise nature of these

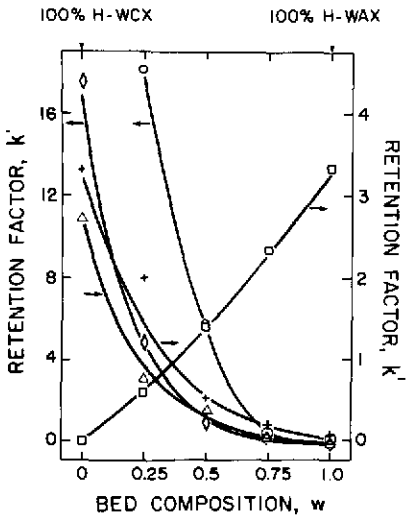

Fig. 2. Plots of retention factors against the bed composition of an H-WCX–H-WAX column at relatively low concentrations of $(NH_4)_2SO_4$ in 20 m*M* Tris–HCl, pH 7.0. (◇) Lysozyme, 80 m*M*; (○) α-chymotrypsinogen A, 30 m*M*; (□) ovalbumin, 30 m*M*; (△) hemoglobin, 10 m*M*; (+) conalbumin, 40 m*M*. Other conditions as in Fig. 1.

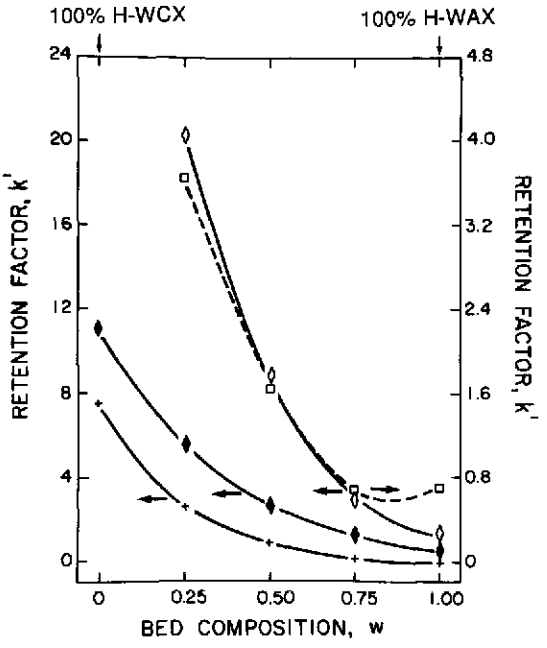

Fig. 3. Plots of retention factors against the bed composition of an H-WCX–H-WAX column at relatively high concentrations of $(NH_4)_2SO_4$ in the eluent. (◇) Lysozyme; (□) ovalbumin; (+) conalbumin, all with 1.8 *M* salt in the eluent; (◆) lysozyme, 1.6 *M* salt. Other conditions as in Fig. 1.

interactions could not be determined. Neither has the origin of the non-linearity observed in our case been discovered. The possibility of electrostatic interactions between ion-exchanger particles which carry oppositely charged functional groups is remote, since the Debye length at appreciable salt concentrations is far smaller than the particle diameter and drops sharply with increasing salt concentration, whereas the non-linearity is manifest even at high ionic strength. A similar argument precludes non-linearity due to Donnan exclusion of the proteins from the interior of like charged particles. It is also unlikely that possible small differences in porosity between the two sorbents could account for the non-linearity: they would have to differ by a factor greater than 3 to explain the data. Numerous other reasons for the observed behavior have been considered but no satisfactory explanation has been found.

An empirical mixing rule has been formulated that fits, without any adjustable parameters, the non-linear results obtained with the H-WCX–H-WAX mixed-beds. If k'_A and k'_B are the observed retention factors for the eluite on columns containing only the respective stationary phase of type A and B and w_A is the weight fraction of stationary phase A in the mixture then the retention factor, k', observed in the full range of stationary phase composition is given by

$$k' = \{w_A[k'_A/(1+k'_A)] + (1-w_A)[k'_B/(1+k'_B)]\}[1+w_A k'_A+(1-w_A)k'_B] \quad (3)$$

Retention data calculated by eqn. 3 are compared to experimental results in Fig. 4 and the agreement is remarkably accurate. Some of the data collected, however,

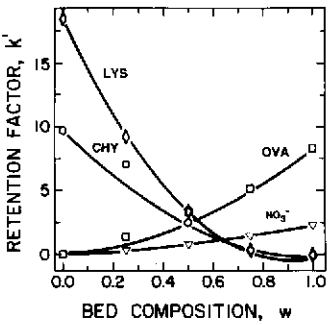

Fig. 4. Measured and predicted retention factors as a function of the bed composition in mixed H-WCX–H-WAX columns. Solid lines were calculated by using eqn. 3 and the data points were measured by using 20 m*M* Tris–HCl, pH 7.0, as the eluent. Lysozyme, 0.18 *M* NaCl; α-chymotrypsinogen A, 0.10 *M* NaCl; ovalbumin, 0.08 *M*; nitrate, 0.01 *M* $(NH_4)_2SO_4$.

displayed even stronger non-linearities than that predicted by this equation, and could not be reconciled with other generalized mixing rules (unpublished results), either.

Gradient elution with mixed-bed columns

The theory of retention in gradient elution has been discussed at length by several authors[14–18] and verified experimentally for reversed-phase chromatography[19] and EIC[20] of proteins. Here we shall put it to use for the prediction of protein retention in EIC on mixed-sorbent columns with gradient elution.

The migration of the center of an eluite band down the column packed with a single sorbent is described by

$$dz/dt = u_0/(1 + V_S K/V_M) \tag{4}$$

where z is the axial position of the eluite band, u_0 is the mobile phase velocity, K is the equilibrium constant for the retention and V_S and V_M are the volumes of stationary and mobile phases in the column, respectively. In gradient elution, K depends on the concentration of the mobile phase modulator —the salt in EIC— which in turn varies with time and distance along the column. The retention time, t_R, is determined by the integration of eqn. 4 with due regard for the time and position dependence of K. The term $V_S K/V_M$ may be considered the instantaneous value of the retention factor, k', at a given axial position.

In EIC retention depends on salt concentration according to

$$\log k' = \kappa_1 - Z \log m \tag{5}$$

where m is the salt concentration in the mobile phase, Z is a characteristic number for the interaction between the eluite and the stationary phase in the presence of the salt[21,22] and κ_1 is the logarithm of the retention factor when the salt concentration is unity. Table I shows κ_1 and Z values for proteins measured on H-WCX and H-

TABLE I

EXPERIMENTALLY DETERMINED κ_1 AND Z VALUES FOR PROTEINS ON SINGLE SORBENT COLUMNS PACKED WITH EITHER H-WCX OR H-WAX ION EXCHANGERS BY USING SODIUM CHLORIDE OR AMMONIUM SULPHATE IN THE ELUENT

Protein	*H-WCX*				*H-WAX*			
	NaCl		$(NH_4)_2SO_4$		*NaCl*		$(NH_4)_2SO_4$	
	$-\kappa_1$	Z	$-\kappa_1$	Z	$-\kappa_1$	Z	$-\kappa_1$	Z
α-Chymotrypsinogen A	2.90	3.88	3.61	3.32	N.R.	N.R.	N.R.	N.R.
Conalbumin	3.29	2.72	6.10	3.16	4.31	2.39	4.30	1.82
Lysozyme	1.81	4.09	3.12	3.98	N.R.	N.R.	N.R.	N.R.
Ovalbumin	N.R.	N.R.	N.R.	N.R.	6.39	6.66	5.70	4.09

N.R. = Not retained.

WAX stationary phases. Each pair of values was regressed from at least four data points measured at different salt concentrations.

If a linear salt gradient is transported unchanged through a chromatographic column, the salt concentration as a function of time and distance is given by

$$m(z,t) = \begin{cases} m_s & t-(z/u_0)-\tau < 0 \\ m_s + (m_f-m_s)[t-(z/u_0)-\tau]/t_G & 0 < t-(z/u_0)-\tau < t_G \\ m_f & t-(z/u_0)-\tau > t_G \end{cases} \quad (6)$$

where it is assumed that the gradient is both preceded and followed by isocratic plateaus with the respective starting and final salt concentrations m_s and m_f, t_G is the gradient time and τ the gradient delay due to the pertinent dead volume in the system.

Eqns. 4–6 yield a first order ordinary differential equation of the form

$$dz/dt = f(z,t) \quad (7)$$

The initial condition, which reflects the time and position at which the salt gradient, delayed by a time τ, catches up with the center of the eluting band, is

$$z = u_0\tau/k'(m_s) \text{ at } t = \tau[1 + 1/k'(m_s)] \quad (8)$$

where $k'(m_s)$ is the retention factor with the starting eluent. Integration of eqn. 7 with the condition given in eqn. 8 yields the retention time of the eluite when the value of z equals that of L, the column length.

In chromatography with mixed-sorbent columns, the retention factor observed at a given salt concentration can be expresed as a function of the pertinent k' values measured on the appropriate single-sorbent columns by a mixing rule such as eqn. 2 or eqn. 3. In EIC the single-sorbent k' values for the eluites depend on the salt

concentration according to eqn. 5. A combination of eqns. 4–6 and the appropriate form of the mixing rule yields a differential equation of the form

$$dz/dt = f(w; z,t) \quad (9)$$

that expresses the movement of peaks through the column also as a function of the bed composition, w. Integration of eqn. 9 with the condition in eqn. 8 yields the retention time.

A closed form solution to such a problem is difficult, if not impossible, so we used a numerical technique —a 4th order Runge–Kutta[23] algorithm— for the integration. The procedure was terminated when the value of z exceeded the length of the column and the retention time was determined by linear interpolation between the last two time and position values.

The chromatograms in Fig. 5 illustrate the retention of four proteins, three basic and one acidic, in mixed H-WCX–H-WAX columns of different sorbent compositions under fixed gradient conditions. The retention times of the four proteins were calculated by integrating eqn. 9 with the use of the mixing rule in eqn. 3 and the corresponding values from Table I. The results are plotted against the bed composition in Fig. 6 where the pertinent experimental data points from Fig. 5 are also illustrated. The match between the calculated and measured data is quite satisfactory, considering that the mixing rule holds only approximately in some of the cases considered. Indeed, it appears that the possible sources of error, including that incurred by changes in gradient shape due to salt adsorption on the column, have either minimal effect, or compensate each other in a fortuitous manner; thus, the predictive method can be used with some degree of confidence.

Optimum bed composition

A number of optimization techniques may be used to select the bed composition most advantageous for the separation problem at hand[24]. One is the "window diagram", sometimes known as an "overlapping resolution map". It is the result of a graphical procedure in which the resolution between the two least separated peaks is maximized with respect to one[25] or two[26,27] variables, all others being held constant. A plot of the resolution of the least resolved peaks *versus* the variables of

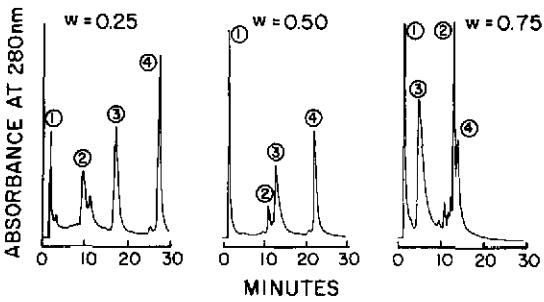

Fig. 5. Chromatograms of four proteins obtained on mixed H-WCX–H-WAX columns of different composition with gradient elution from 0.05 M to 0.3 M NaCl in 20 mM Tris HCl, pH 7.0, in 30 min with a 3-min delay. Proteins: 1, conalbumin; 2, ovalbumin; 3, α-chymotrypsinogen A; 4, lysozyme.

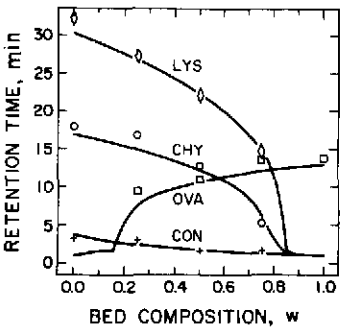

Fig. 6. Comparison of the predicted and observed dependence of protein retention on the stationary phase composition in mixed-bed columns. Solid lines were obtained by numerical integration of eqn. 9 with the use of eqn. 3 and the data from Table I. Conditions as in Fig. 5.

interest results in a surface containing a number of maxima, which, in a two-dimensional plot, may be likened to a series of "windows". The "optimum" conditions are considered to be those corresponding to the tallest window.

Whereas resolution can be readily determined in isocratic elution chromatograpy, it is not as easily predicted in gradient elution because there is no reliable theory to calculate band broadening. For simplicity, therefore, we have used the difference in retention times between the least separated peaks in a chromatogram, $\mathscr{S}$, as a measure of the separation. It is defined as

$$\mathscr{S} = \min_{j \neq i} | t_{R,j} - t_{R,i} | \tag{10}$$

where both i and j index all the peaks in the chromatogram, and the minimum is selected from all pairs of peaks. Since, in a given chromatogram, all bands have approximately the same width[17] the crude measure represented in eqn. 10 suffices for our purposes.

As an illustrative example we will examine the separation of the four proteins considered previously, *vide* Figs. 5 and 6, on mixed H-WCX–H-WAX columns at pH 7. At neutral pH three of the four proteins carry a net positive and one a net negative charge. A "catholic" ion-exchange column packed with mixed cation and anion exchangers offers a convenient means to retain each constituent of the sample, as is required in chromatographic analysis.

The search for optimum bed composition and gradient program was carried out by using the model outlined previously and the data in Table I. An additional variable was the gradient steepness G, defined as

$$G = (m_f - m_s)/t_G \tag{11}$$

For simplicity, m_s was held constant, and regions where the separation took longer than 30 min or any one of the peaks was unretained were not considered. The results are presented in Fig. 7 as a three-dimensional plot of $\mathscr{S}$ against w and G. There are two distinct regions in which $\mathscr{S}$ attains large values. Separation with a column con-

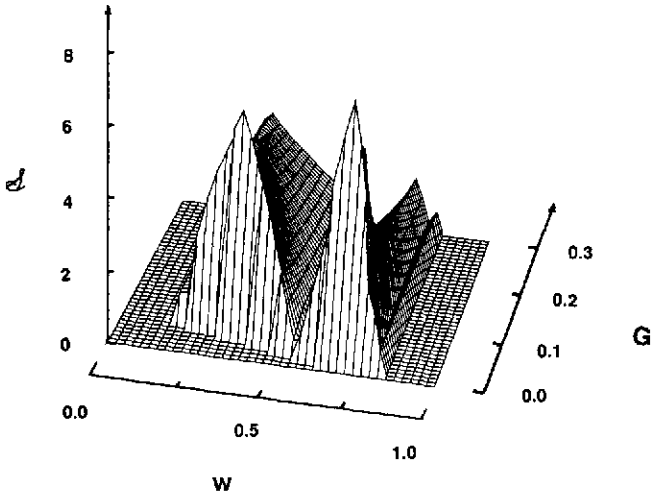

Fig. 7. Illustration of the separation of four proteins as measured by $\mathscr{S}$ with gradient elution in mixed ion-exchanger columns as a function of the bed composition, w, and the gradient steepness, G. Other conditions as in Fig. 5. Only domains with retention time $t_0 < t_R < 30$ min are shown.

taining 75% of H-WCX and 25% of H-WAX and at a G value of approximately 0.08, which lies in a region with a high $\mathscr{S}$ value, is illustrated in Fig. 5 ($w = 0.25$) and it is seen that the peaks are well resolved in agreement with the prediction.

Since $\mathscr{S}$ measures only the extent of separation, other criteria may also have to be applied in the optimization process. One method of locating the optimum in such circumstances is to procede in a stepwise manner, statisfying one criterion at a time[24-28]. For example, in the above problem, one may wish to achieve the fastest separation by confining the search to regions of sufficiently high $\mathscr{S}$ values in the window diagram such as in Fig. 7. The optimal value for G in our example would then be the maximum G that yields sufficiently high $\mathscr{S}$ values. However, other means also exist to satisfy multiple criteria simultaneously and these have been discussed in the literature[24,29].

The optimization scheme can be extended to include more than two independent variables, *e.g.* the column length could be a third variable in the above example. Although the visual advantage of the window diagram is then lost, computer algorithms can be readily devised to find the optimum conditions[30]. In the event that eqn. 1 holds, extension to any number of stationary phases is straightforward. When the dependence of the retention on bed composition is non-linear the task is more difficult. We have found, however, that by using a mixing rule of the form of eqn. 3 retention times in gradient elution can be predicted with resonable accuracy even if the fit for isocratic data is not entirely satisfactory.

A more general form of this empirical equation for multiple stationary phases is

$$k' = (1 + \sum_{i-1}^{N} w_i k_i') \sum_{i}^{N} [w_i k_i' / (1 + k_i')] \qquad (12)$$

where the symbols have the same meaning as in eqn. 2.

Although our discussion has so far been restricted to ion exchangers, the need for a mixing rule to facilitate the selection of the optimal bed composition is universal to any mixed-bed separation. It must be stressed that mixed beds appear to be of maximum utility in preparative applications or routine analysis. In many instances it may be more convenient to use several columns in series provided columns of appropriate dimensions and packing are readily available.

The retention behavior of proteins on mixed-bed and tandem columns was found to be similar, although with tandem columns selectivity in gradient elution was dependent on column sequence[5]. Whereas tandem columns have the advantage that they can be individually examined and replaced at will, for routine application the convenience of a single column with mixed sorbents might prove attractive.

The "general elution problem" put forward by Snyder[17], refers to the fundamental difficulties in the separation under isocratic conditions of a sample having components with widely disparate retention factors. They may be partially alleviated by using an appropriate blend of stationary phases so that the retention of the light and heavy ends of the sample is increased and decreased, respectively. This approach could eliminate the need for gradient elution that requires complex instrumentation and time consuming re-equilibration of the column with the starting eluent after each run. Columns with mixed sorbents, therefore, are likely to find applications in rapid HPLC for routine analysis and *a fortiori* in process monitoring and control by ultrahigh-speed HPLC.

Other important applications for mixed-sorbent columns are expected to be found in the burgeoning field of preparative chromatography. This approach not only permits the manipulation of selectivity for separation of the desired product but also may make the use of difficult-to-remove mobile phase additives superfluous. Furthermore, mixed stationary phases may offer certain advantages in displacement[31,32] and in other non-linear chromatographic techniques. In such cases the pertinent adsorption isotherms on multisorbents have to be evaluated by frontal chromatography[33] or other means.

Degradation of sorbents in mixed-bed columns may follow patterns different from those observed with columns packed with single sorbents as a result of intimate contact between different stationary phases. A disadvantage of a mixed-bed is that it may be difficult to determine the extent of degradation of any of the individual sorbents. Furthermore the properties of the column may change significantly upon moderate decomposition of a single stationary phase component. From this it follows that stable stationary phases are required to exploit the potential benefits of columns packed with mixed sorbents.

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APPENDIX

Consider a thin section within a uniform mixed-bed column that corresponds

to a theoretical plate. Let the total mobile phase volume and the volume of each individual sorbent in the plate be V_M and V_{Si} respectively, such that

$$\sum_{i=1}^{N} V_{Si} = V_S \tag{A1}$$

where V_S is the total volume of the stationary phase in the plate and N is the number of individual sorbents in the mixed bed.

Let an amount of eluite equal to $C_0 V_M$ where C_0 is some arbitrary concentration be charged into the plate and allowed to equilibrate between the mobile and stationary phases. At equilibrium, the mass balance for the eluite is

$$C_0 V_M = C_M V_M + \sum_{i=1}^{N} C_{Si} V_{Si} \tag{A2}$$

where C_M and C_{Si} are the equilibrium concentrations in the mobile phase and the sorbent i, respectively. If adsorption takes place at the sorbent surface, C_{Si} must be expressed per unit area and V_{Si} replaced by $a_{Si} V_{Si}$, a_{Si} being the surface area per unit volume of sorbent i. The results that follow, however, are not affected by this consideration.

Let it be assumed that there are no interactions between the sorbents and adsorption on each one is governed by a linear isotherm. Then, since each sorbent is individually at equilibrium with the homogenous mobile phase, the N equilibrium constants K_i are represented by

$$K_i = C_{Si}/C_M \tag{A3}$$

The observed retention factor, k', for such a mixed-bed column is given by the expression

$$k' = \left(\sum_{i=1}^{N} C_{Si} V_{Si} \right) / C_M V_M \tag{A4}$$

Substitution of eqn. A3 in eqn. A4 yields eqn. 1.

If we define

$$\varphi_i \equiv V_{Si}/V_{Mi} \tag{A5}$$

where V_{Mi} is some fraction of the mobile phase volume such that

$$\sum_{i=1}^{N} V_{Mi} = V_M \tag{A6}$$

we can rewrite eqn. 1 as

$$k' = \left(\sum_{i=1}^{N} K_i V_{Si}\right) \Big/ \left(\sum_{i=1}^{N} V_{Si}/\varphi_i\right) \tag{A7}$$

This equation can be recast in terms of the weight fractions of the individual sorbents, w_i. Since V_{Si} is proportional to w_i/ρ_i, where ρ_i is the density of sorbent i, eqn. A7 becomes

$$k' = \left(\sum_{i=1}^{N} K_i w_i/\rho_i\right) \Big/ \left(\sum_{i=1}^{N} w_i/\rho_i\varphi_i\right) \tag{A8}$$

where ρ_i is the density of sorbent i. When both φ_i and ρ_i are identical for each sorbent in the mixed bed, eqn. A8 reduces to

$$k' = \varphi \sum_{i=1}^{N} K_i w_i \tag{A9}$$

where φ is now the common value for φ_i. The terms φK_i correspond to retention factors measured on columns packed with the individual sorbents, k_i', provided that their packing structure is identical to that of the mixed-bed column. When the different sorbent particles have equal diameter and porosity, and the respective columns are packed by the same procedure, the conditions necessary for eqn. 1 to hold are satisfied and lead to the linear mixing rule embodied in eqn. 2.

On the other hand several non-linear mixing rules can be formulated if the mobile phase is considered to be non-homogeneous, *i.e.* the composition of the mobile phase in the vicinity of the different types of sorbent particles is different. Eqn. 3 is the result of an *ad hoc* combination of two such rules.

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