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Gradient Elution in Reversed-Phase HPLC

Commonly Used Terms in Chromatography

A, B, C	Knox equation coefficients (Equation 5)
d_c	column diameter
D_m	solute diffusion coefficient
d_p	particle diameter
F	flow rate of mobile phase
h	reduced plate height
H	plate height (=L/N)
k'	solute capacity factor
$\bar{k}$	average k' value in gradient elution
k_0	value of k' at beginning of separation
L	column length
N	column plate number
PC	peak capacity—maximum number of peaks that can be fit into the chromatogram with unit resolution
R_s	resolution function
S	change in log k' for unit change in ϕ (isocratic elution)
t_g	retention time, gradient elution
t_G	time from start to end of gradient
t_G^0	value of t_G for full-range gradient (ϕ changes from 0 to 1)
t_R	retention time, isocratic elution
Δt_R, Δt_G	difference in retention time (t_R or t_g) for adjacent bands
t_0	column dead time
u	mobile-phase velocity
V_G	gradient volume (=t_GF)
V_m	column dead volume (=t_0F)
α	separation factor
ν	reduced mobile-phase velocity
ϕ	volume fraction of stronger (organic) solvent
$\Delta\phi$	change in ϕ during gradient ($\Delta\phi$ = 1 for 0–100% gradient)
σ	isocratic bandwidth (1 SD)
σ_g	gradient bandwidth (1 SD)

The chromatographic separation of large molecules (10^3–10^7 daltons) began in the early 1950s, with the first separations of proteins by ion exchange (*1*). Then, as now, it was necessary to use gradient elution for the resolution of these macromolecules. More recently gradient elution has been extended to the separation of other large molecules such as nucleic acids, DNA and RNA fragments, synthetic polymers, and oligomeric carbohydrates (*2*, *3*). These developments have coincided with the commercial introduction of much more efficient high-performance liquid chromatography (HPLC) columns for such separations, and it is now possible to resolve macromolecular samples in less than an hour.

Chemists are more familiar with the behavior of small molecules than that of macromolecules, while chromatographers are accustomed to working with isocratic elution rather than with gradient elution. It is not surprising then that there is some confusion when it comes to the principles of the gradient separation of macromolecules. Practitioners often are puzzled by the outcome of some change in separation conditions. The apparent absence of predictability and of guidelines for method development also prevents the full potential of these gradient separations from being achieved.

Such apparent anomalies in the gradient separation of macromolecules

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Report

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Elution Gradient Elution Gradient Elution Gradient Elution Gradient Elution Gradient Elution

Separation of Macromolecules

have led workers to make new proposals for the basis of such separations. Many chromatographers assume that macromolecules are retained at the column inlet, until at some point in the gradient they are desorbed completely and then move through the column without further interaction with the stationary phase. Other workers have suggested that chromatographic migration in these systems occurs by a precipitation–redissolution process rather than by interaction of solute molecules with the stationary phase; that is, solute solubility determines separation. Another view is that the multipoint attachment of macromolecules to the surface of the stationary phase leads to a retention process that differs fundamentally from that for small molecules. On the other hand, we believe that the application of existing "small molecule" chromatographic theory to these separations can explain many of the anomalies reported so far. That is, the gradient separation of macromolecules differs from the isocratic separation of small molecules in degree, not in kind.

Isocratic Elution

The separation of a three-component mixture by *isocratic elution* is represented in Figures 1a and 1b. The fractional migration of solutes *a*, *b*, and *c* along the column as a function of time after sample injection is shown in Figure 1a, and the resulting chromatogram is pictured in Figure 1b. The final separation can be described by the well-known equations summarized in Table I for retention (Equation 1), bandwidth (Equation 2), and resolution (Equation 3). A good theoretical understanding of column efficiency or plate number N as a function of experimental conditions now exists, and can be summarized by the so-called Knox equation (*4*):

$$h = A\nu^{1/3} + B/\nu + C\nu \qquad (5)$$

Here the reduced plate height h is equal to H/d_p, where H is the usual plate height value (column length L divided by plate number N) and d_p is the diameter of particles used to pack the column; ν is the reduced mobile-phase velocity, equal to ud_p/D_m (u is the mobile phase velocity and D_m is the solute diffusion coefficient). In principle, Equation 5 allows the calculation of N as a function of such experimental variables as column length, column diameter, mobile-phase flow rate, temperature, sample molecular

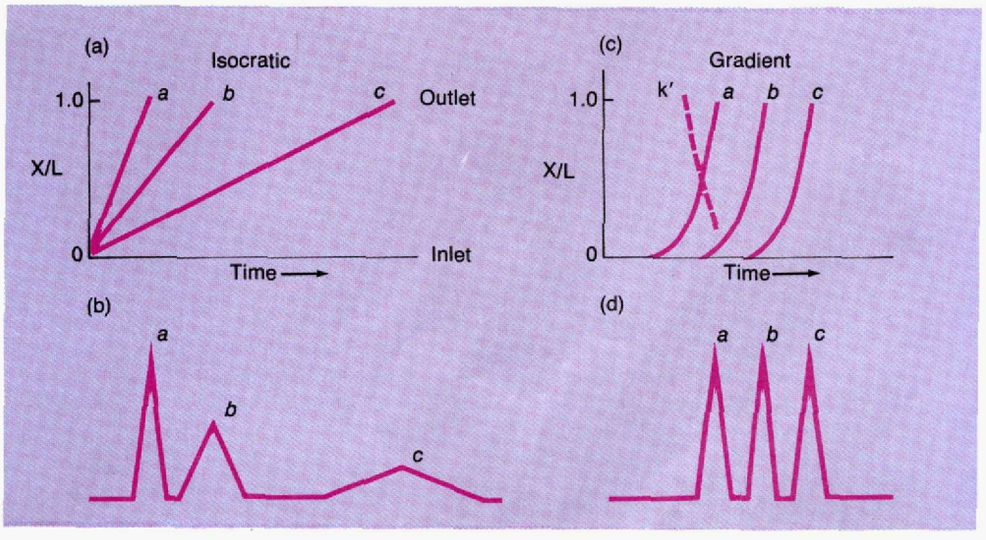


Figure 1. Fractional migration (X/L) of solute bands along the column and resulting chromatograms for isocratic vs. gradient elution

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Table I. Basic Equations for Isocratic and Gradient Elution

Relationship	Isocratic elution	Gradient elution [a]
Retention time (s)	(1) $t_R = t_0k' + t_0$	(1a) $t_g = t_0\bar{k} \log(2.3k_0/\bar{k}) + t_0$
Bandwidth (mL)	(2) $\sigma = V_m(1 + k')N^{-1/2}$	(2a) $\sigma_g = V_m(1 + \bar{k})N^{-1/2}/2$
Resolution ($\Delta t_R/4\sigma$ or $\Delta t_g/4\sigma_g$)	(3) $R_s = [(1/4)(\alpha - 1)N^{1/2}][k'/(1 + k')]$	(3a) $R_s = [(1/4)(\alpha - 1)N^{1/2}][\bar{k}/(1 + \bar{k})]$
Capacity factor	(4) $k' = (t_R - t_0)/t_0$	(4a) $\bar{k} = t_G/\Delta\phi St_0 = t_GF/\Delta\phi S\ V_m$

[a] Approximate equations for so-called gradient conditions (*8*)

weight, etc. For application to a broad range of experimental systems, particularly involving both large and small molecules, it is also necessary to recognize that B and C of Equation 5 depend on the solute capacity factor k′ and molecular weight (*5*, *6*). A further discussion of all aspects of isocratic HPLC is given in Reference 7.

Gradient Elution

The corresponding separation by *gradient elution* of the same three-component sample is shown in Figures 1c and 1d. The migration of each compound along the column is pictured in Figure 1c, where a different pattern is seen compared to the isocratic separation shown in Figure 1a: All three compounds are initially retained at the column inlet after the gradient is begun. Eventually the k′ value for compound *a* (dashed curve in Figure 1c) becomes small enough to allow migration of *a* along the column; *a* then moves increasingly faster along the column until it appears in the chromatogram (Figure 1d). The same pattern is repeated at a later time in the separation for compound *b*, followed eventually by similar migration of *c*. Compared to isocratic elution (Figure 1b), gradient elution (Figure 1d) provides more nearly equal bandwidths for compounds *a*–*c*, and faster overall separation.

Equations in gradient elution for retention, bandwidth, and resolution also are given in Table I (Equations 1a, 2a, 3a) (*8*). These are expressed in terms of the same parameters as for isocratic elution (Equations 1, 2, 3), except that k′ is replaced by the average k′ value during gradient elution, $\bar{k}$. Bandwidth and resolution are of special interest; it can be seen that Equations 2 and 2a and Equations 3 and 3a are virtually the same, except that k′ is replaced by $\bar{k}$ in gradient elution. That is, separation and peak heights or detection sensitivity are controlled by the same factors in both isocratic and gradient elution. Method development therefore proceeds in similar fashion for both HPLC procedures (*7*, *8*).

Of special interest in gradient elution are so-called linear-solvent-strength (LSS) gradients. These can be defined as gradients that provide roughly equal values of $\bar{k}$ for compounds eluting at different times during the separation. LSS gradients thus yield constant bandwidths for the various compounds separated (Equation 2a) and equal resolution for band pairs with similar values of the separation factor α (Equation 3a). As in isocratic elution for k′, there is a roughly optimum value of $\bar{k}$: $1 < \bar{k} < 10$. Equation 4a describes $\bar{k}$ as a function of gradient time t_G, flow rate F, and column dead volume V_m. This is in contrast to isocratic elution, where k′ is independent of separation time, flow rate, and column dimensions (other conditions being constant). This difference can lead to confusion when gradient and isocratic separations are compared.

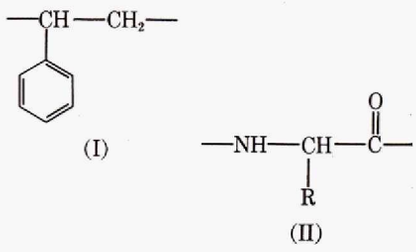
Retention time t_g, resolution R_s, and bandwidth σ_g in gradient elution can be predicted from data for corresponding isocratic systems (Equations 1a–4a). Thus it is necessary to know values of the isocratic parameters k_0, t_0, N, and S. For many isocratic systems (e.g., reversed-phase HPLC) retention is given by:

$$\log k' = \log k_0 - S\,\phi \qquad (6)$$

As illustrated in Figure 2, the important parameter S is the slope of these plots of log k′ vs. volume fraction of organic. When plots of log k′ vs. ϕ are nonlinear, the tangent to the curve at $k' = \bar{k}$ serves as an adequate approximation to linear log k′–ϕ plots for purposes of relating gradient and isocratic data. However, in the remainder of this REPORT we will assume that linear log k′–ϕ plots as in Figure 2 describe isocratic data and that linear gradients (ϕ varying linearly with time) are employed. For examples of the prediction of gradient data from isocratic experiments see References 8 and 9.

Macromolecular Solutes and Chromatographic Separation

Macromolecular solutes can be regarded as polymers Q_n of some repeating unit Q. For polystyrene solutes as in Figure 2a, Q is a phenethyl group (I). For peptides as in Figure 2b, Q is an amino acid residue (II):



As the number of repeating units, n, increases and the solute molecule becomes larger, the molecule assumes a three-dimensional (3D) structure. For peptides and proteins, there will exist a preferred or "native" configuration, while most synthetic polymers assume a "random-coil" structure. This 3D configuration of macromolecules is critical to some chromatographic pro-

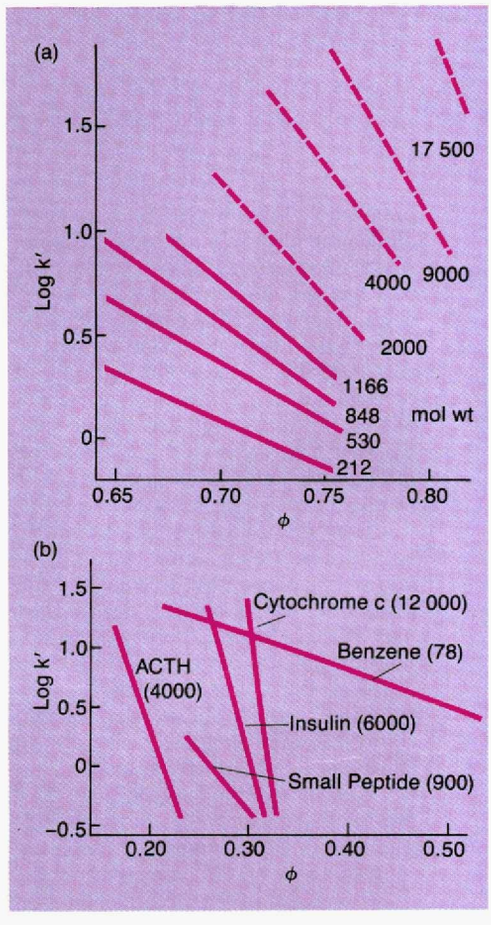
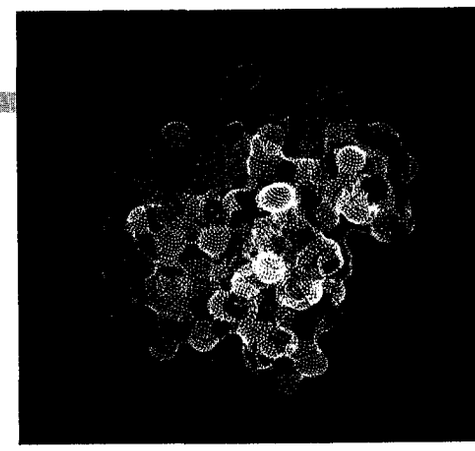
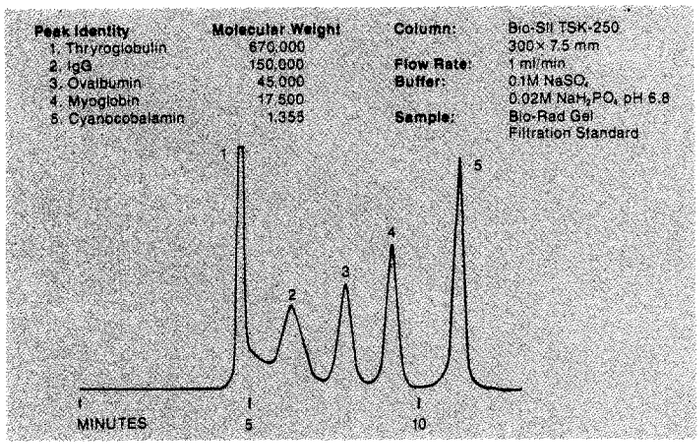


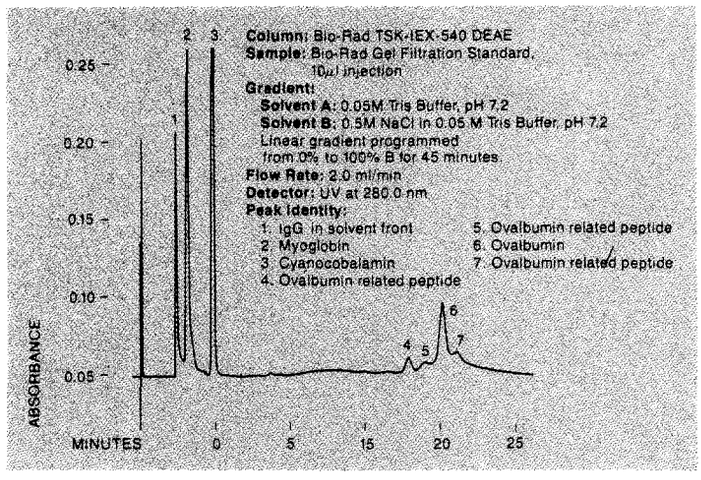
Figure 2. Variation of sample retention k′ with the volume fraction ϕ of the strong solvent B in a solvent mixture A–B

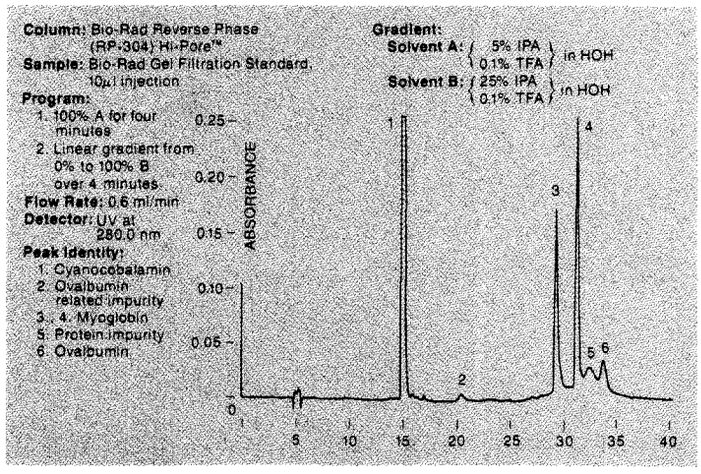
(a) Polystyrene samples of varying molecular weight eluted from C_{18}-silica by mixtures of water (A) and tetrahydrofuran (B). Solid line derived from isocratic data; dashed line derived from gradient data. Adapted from Reference 9. (b) Peptide samples and benzene eluted from C_{18}-silica by mixtures of buffer (A) and acetonitrile (B); solute molecular weights shown in parentheses. Adapted from Reference 10

Applications Report: New Column Technology









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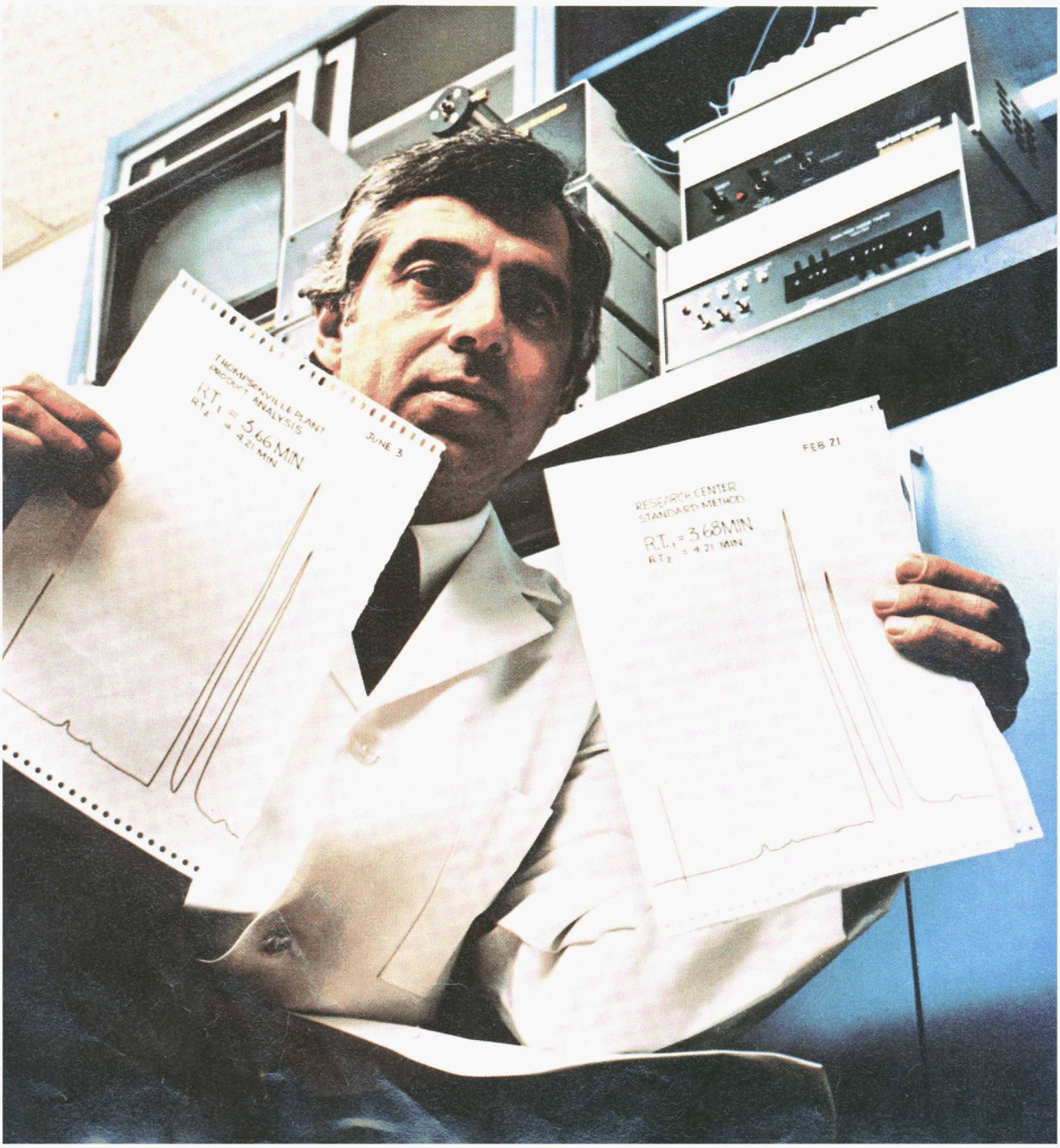
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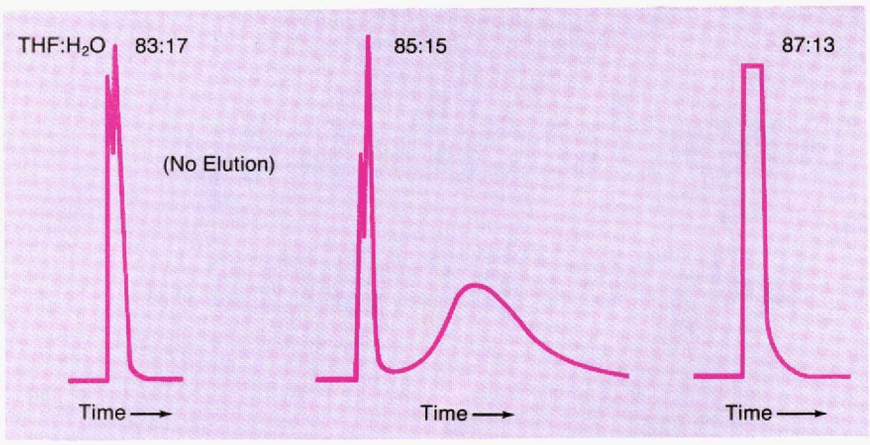


Figure 3. Isocratic reversed-phase separation of 50 000-dalton polystyrene sample with tetrahydrofuran–water mixtures
Adapted from Reference 9

cesses, but less important in others.

Chromatographic Retention. Retention in HPLC can effectively be regarded as an equilibrium process:

$$Q_n(\text{mobile phase}) \rightleftharpoons Q_n(\text{stationary phase})$$

In many HPLC systems this equilibrium appears to involve the competitive displacement of mobile-phase molecules from the stationary phase (see various discussions of liquid–solid [*11*], ion exchange [*8*], and reversed-phase chromatography [*12*]). If so, the above equilibrium could then be written:

$$Q_{n(m)} + rM_{(s)} \rightleftharpoons Q_{n(s)} + rM_{(m)} \quad (7)$$

where M refers to a molecule of mobile phase or solvent, and (m) and (s) refer to molecules in the mobile phase or stationary phase, respectively. Whether displacement is the most important factor in every macromolecular system cannot be stated at the present time. Other phenomena (solute–solvent interactions) may also be important.

It can be seen that one molecule of solute Q_n displaces some number r of solvent molecules M upon sorption of the solute. The value of r in Equation 7 will be related to the size of the molecule Q_n because a larger solute molecule will cover a larger part of the stationary phase surface, and therefore displace a larger number of solvent molecules upon sorption of Q_n. This means that plots of log k′ vs. ϕ will be steeper for larger molecules Q_n (mass action effect—Equation 7), or the S value (Equation 6) for larger solute molecules will be larger. This is clearly seen in Figures 2a and 2b, where the slope (value of S) for these plots increases with increasing solute molecular size; see also the protein–ion-exchange examples of Reference 13 and the reversed-phase separations in Reference 14. For very large solute molecules (>10^5 daltons) values of S can be 30- to 100-fold greater than for small molecules, meaning that retention is then extremely sensitive to change in ϕ. This is illustrated for the isocratic separation of a 50 000-dalton polystyrene fraction in the reversed-phase system of Figure 2a, shown in Figure 3. For 87% tetrahydrofuran–water (ϕ = 0.87) this sample elutes at t_0; for ϕ = 0.85, the compound exhibits a normal band at about k′ = 2. For ϕ = 0.83, the sample appears not to elute at all. This behavior of large solute molecules may appear strange to chromatographers, who are more familiar with the separation of smaller molecules (lower values of S). Because of the dependence of $\bar{k}$ on S in gradient elution (Equation 4a), the gradient separation of macromolecular samples requires different experimental conditions to achieve optimum values of k′ during the separation.

Pore Size Effects. Solutes separated by the usual porous HPLC packings will be retained almost exclusively on the internal surface of the particle, because only a small fraction of the stationary phase is found on the outside of the particle. If the large size of a macromolecular solute prevents its access to the small pores of a particle, retention of the solute will be correspondingly decreased. A similar effect forms the basis of size exclusion chromatography (SEC) (*7*), illustrated in Figure 4 for a column packing having uniform-size pores. In this example, macromolecules A and B are shown in the preferred coiled configuration as opposed to small molecule C. In SEC (see Reference 8 and calibration plot of Figure 4), large molecule A is almost totally excluded from the pore. Compound B of intermediate size is partially excluded, whereas small molecule C is not excluded. Exclusion in SEC is seen to be related to the ease with which the coiled molecule "fits" within the pore.

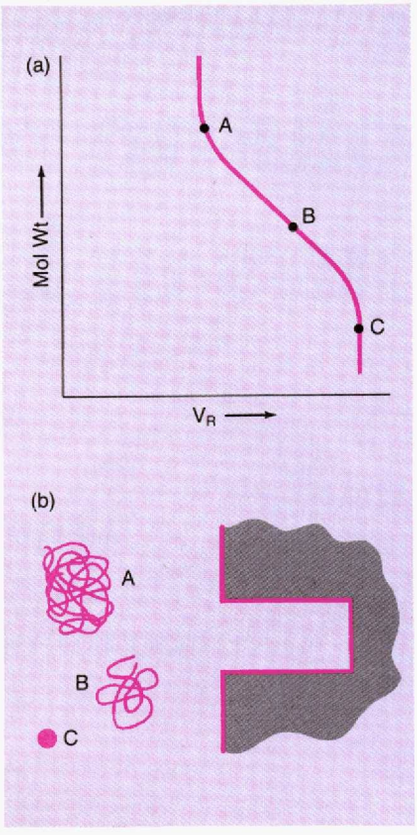


Figure 4. Size exclusion of large molecules from the pores of the column packing
(a) Plot of solute molecular weight vs. solute retention volume. (b) Size of solute molecules (A, B, C) compared to diameter of particle pore

For other HPLC methods, however, the accommodation of the coiled molecule within the pore is less important. Thus the coiled structure can be disrupted as a result of attachment of the solute molecule to the stationary phase, or the solute molecule can unravel partially, thereby decreasing its molecular cross section and permitting its access to small pores. At the same time, the amount of stationary phase within the particle tends to be *inversely* proportional to pore diameter (proportional to surface area). This means that in the absence of exclusion effects, retention for non-SEC methods in HPLC should decrease with increasing pore size of the particle—the opposite of the case for SEC as in Figure 4. This is illustrated by the data in Table II for the reversed-phase gradient elution of various polystyrene samples. Here retention is measured by the ϕ value at the column outlet at the time of elution; a larger value of ϕ means later elution and stronger retention. For each polystyrene sample, retention is stronger (larger ϕ) for the small-pore column. That is, the larger surface area of the small-pore column more than outweighs any exclusion of large molecules from the pores of this column. In Table II the extent of SEC

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Table II. Reversed-phase HPLC Separation of Polystyrenes Using Columns of Different Pore Size [a]

Sample mol wt	Volume fraction organic solvent (ϕ) at elution		SEC % exclusion [b]	
	6-nm pore column	30-nm pore column	6-nm pore	30-nm pore
2000	0.804	0.750	66	14
4000	0.830	0.788	78	19
9000	0.853	0.826	90	26
17 500	0.863	0.853	100	40
50 000	0.873	0.870	100	60

[a] Tetrahydrofuran/water gradient (linear), 40-min gradient time and 2 mL/min flow rate, C_{18} columns
[b] Decrease in size exclusion retention vs. totally permeating molecule; equals about 100% for A in Figure 4a and 0% for C
Data taken from Reference 9.

exclusion (coiled molecule) is also shown for each column and the various polystyrene samples. The greater exclusion of these samples from the small-pore column (66–100%) vs. the large-pore column (14–60%) contrasts with the stronger retention of these same samples on the small-pore, high-surface column under reversed-phase conditions.

For larger molecules (>50 000 daltons) or more compact (e.g., cross-linked) molecules than the polystyrenes of Table II, pore exclusion effects can be more important than surface area effects. In these cases it is possible to see greater retention on large-pore vs. small-pore columns (see discussion of Reference 15).

Column Efficiency. Chromatographers who have compared the resolving power of different columns for both large and small molecules have often drawn two conclusions: first, that the plate numbers measured for large-solute molecules are generally much smaller than those measured for small molecules (same column) and second, that "good" columns for small-molecule separations (large N values) are often "poor" when applied to macromolecular samples and vice versa. This again has been interpreted as evidence for a nonchromatographic process in the separation of macromolecules by HPLC. On the other hand, chromatographic theory holds that the Knox equation (Equation 5) applies to *all* samples for a given column (*16*). Let us see if we can reconcile these apparently contradictory observations.

The reduced velocity ν of Equation 5 is proportional to $(1/D_m)$. The solute diffusion coefficient D_m is roughly proportional to (solute mol wt)$^{-0.4}$ (*7*), which means that ν will be proportional to (mol wt)$^{0.4}$, other factors being equal. Thus, present separations of small molecules with 3–5-μm particles usually involve ν values in the range $3 < \nu < 15$, whereas macromolecule separations typically involve ν values greater than 50. This means that Equation 5 is approximately given as:

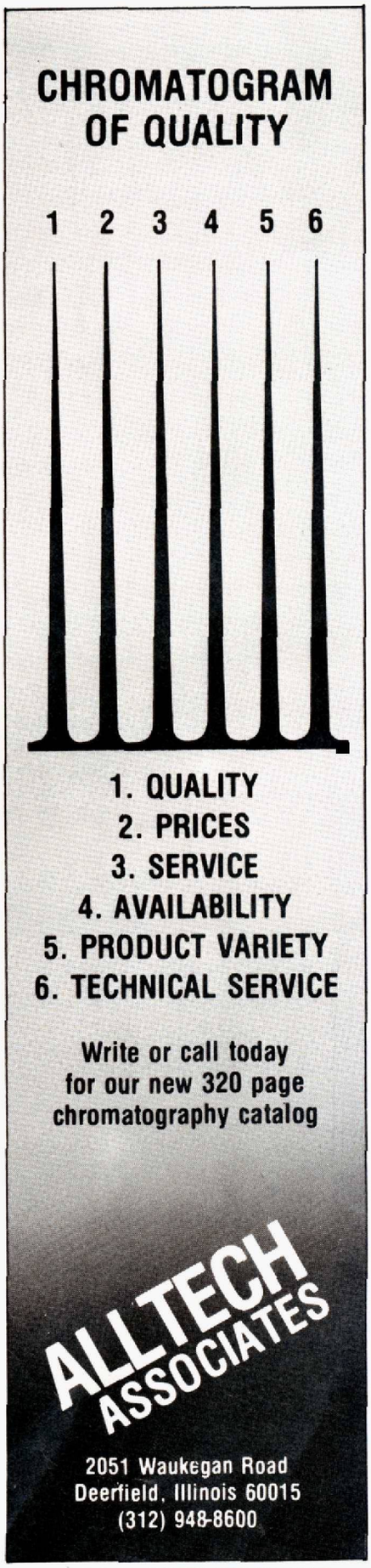
$$h = A\nu^{1/3} \qquad (3 < \nu < 15) \qquad (8)$$

for small molecules, and as

$$h \approx C\nu \qquad (\nu > 50) \qquad (8a)$$

for macromolecules, because only the above terms from Equation 5 are significant in the indicated range of ν values.

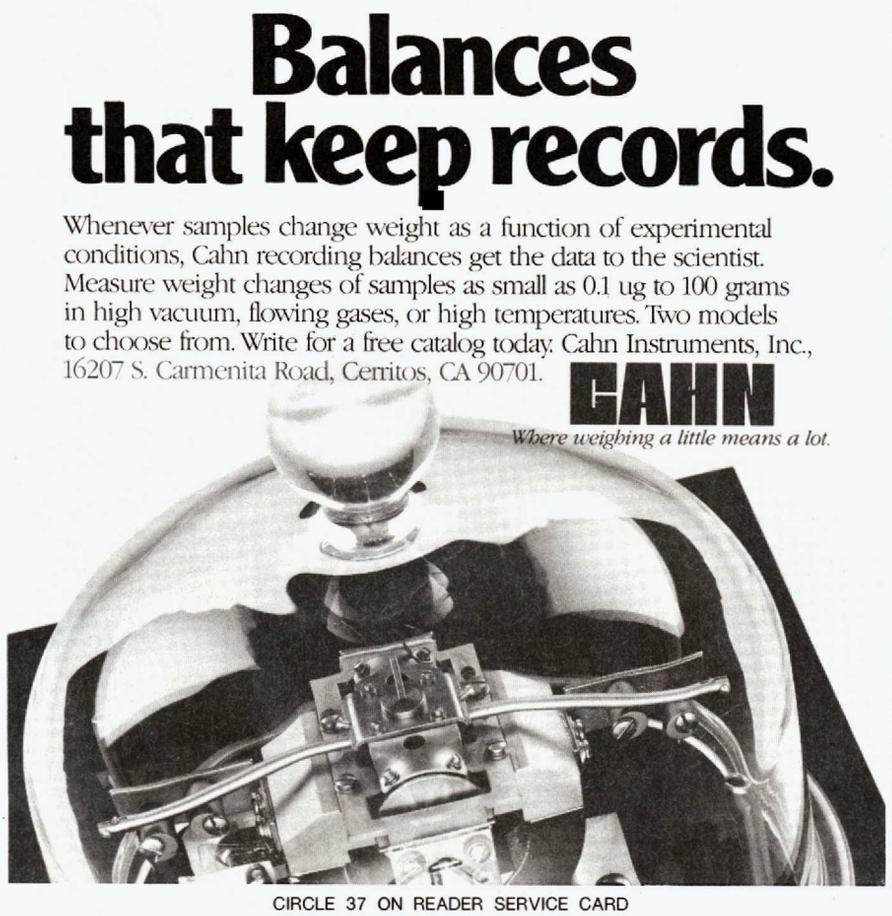
For typical values of A, B, and C in Equation 5, h has a minimum value (largest N value) at about $\nu = 3$, and then increases with increase in ν. Thus, macromolecule separations with their large values of ν will necessarily involve larger values of h (and smaller N values) compared to small-molecule separations on the same column. This is illustrated in Figure 5. Also, other conditions being equal (d_p, column dimensions, etc.), a "good" column for small-molecule separations means a column with a small A value (Equation 8), i.e., a well-packed column. A "good" column for macromolecule separations means a column with a small C value (Equation 8a), implying particles with favorable mass-transfer properties (e.g., large pores, fast diffusion in the stationary phase, no retention sites that are much stronger than average, etc.) (*5*, *6*). Thus, the factors that make a "good" column for separating small molecules are indeed different from the factors that yield large N values in the separation of large molecules, yet the Knox equation is fully able to explain the observations of previous workers trying to separate macromolecules by HPLC. It should be noted also that the value of C is a function of both the column and the sample. Thus, columns with 10–20-nm pores can have "good" values of C for small solute molecules, yet much larger ("poor") values of C for macromole-



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cules, because of slower diffusion of large molecules within small pores (*5*). Finally, columns with small values of both A and C for solutes of all sizes will be "good" columns for all samples.

Application to Studies from the Literature

We can now apply the preceding discussion to several otherwise puzzling observations on the separation of macromolecular samples by gradient elution. First, however, it is useful to express peak capacity (or average R_s value) and peak height (or $1/\sigma_g$) in terms of the primary variables: column length L and diameter d_c, flow rate F, gradient time t_G, and particle diameter d_p. Table III summarizes the resulting relationships for both isocratic elution (small molecules) and gradient elution for macromolecules (*17*).

The starting point in Table III is the relationships for reduced plate height h: Equation 8 for small molecules and Equation 8a for large molcules (Knox equation). These equations plus the definitions of $N(=L/H)$, $h(=H/d_p)$ and $\nu(=ud_p/D_m)$ lead to expressions for the plate number N for each case. The proportionality of N to certain separation variables is then given by Equations 9 and 9a in Table III. For gradient elution, it is convenient to express N in terms of the mean capacity factor $\bar{k}$, proportional to $t_G^0\ F/L\ d_c^2$ (t_G^0 is defined equal to $t_G/\Delta\phi$, the time for a 0–100% gradient). For the final derivation of resolution or peak capacity from Equations 3 or 3a, and peak height or $(1/\sigma_v)$ from Equations 2 or 2a, the value of N from Equations 9 or 9a is used. In the case of gradient elution, advantage is taken of the approximate, empirical relationships:

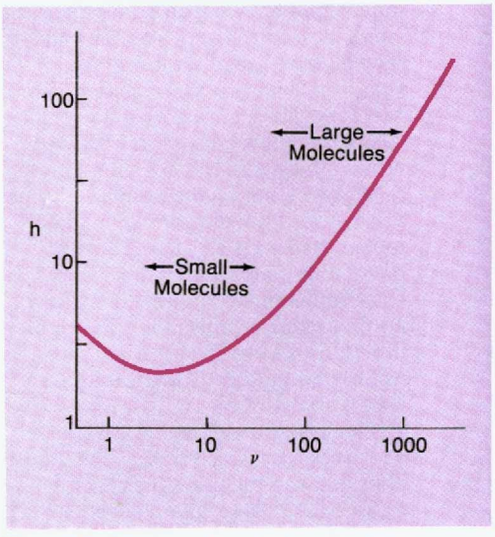
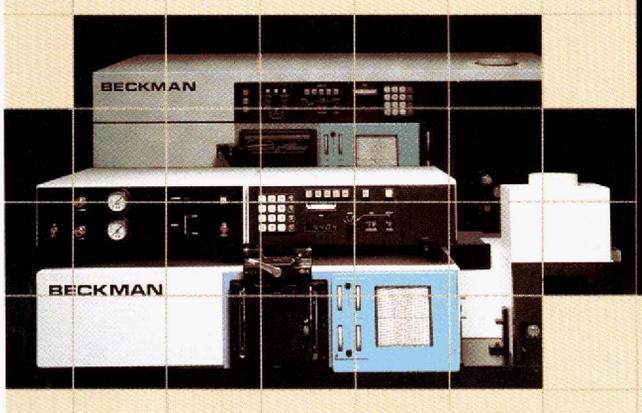
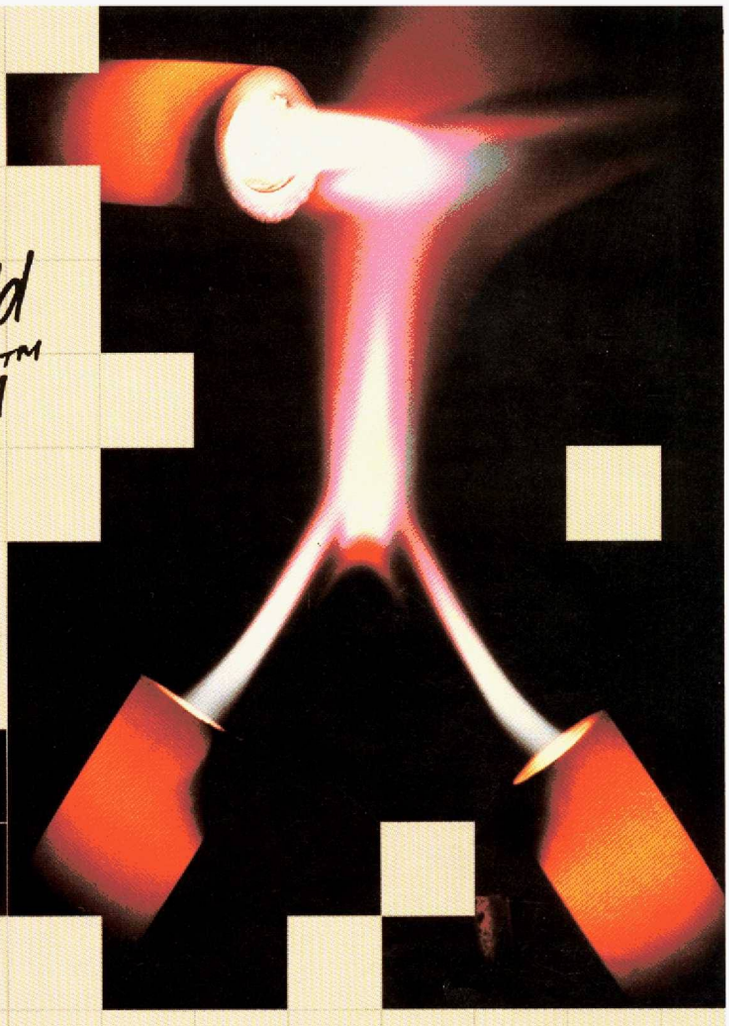


Figure 5. Plot of reduced plate height h vs. reduced velocity (Knox Equation 5)

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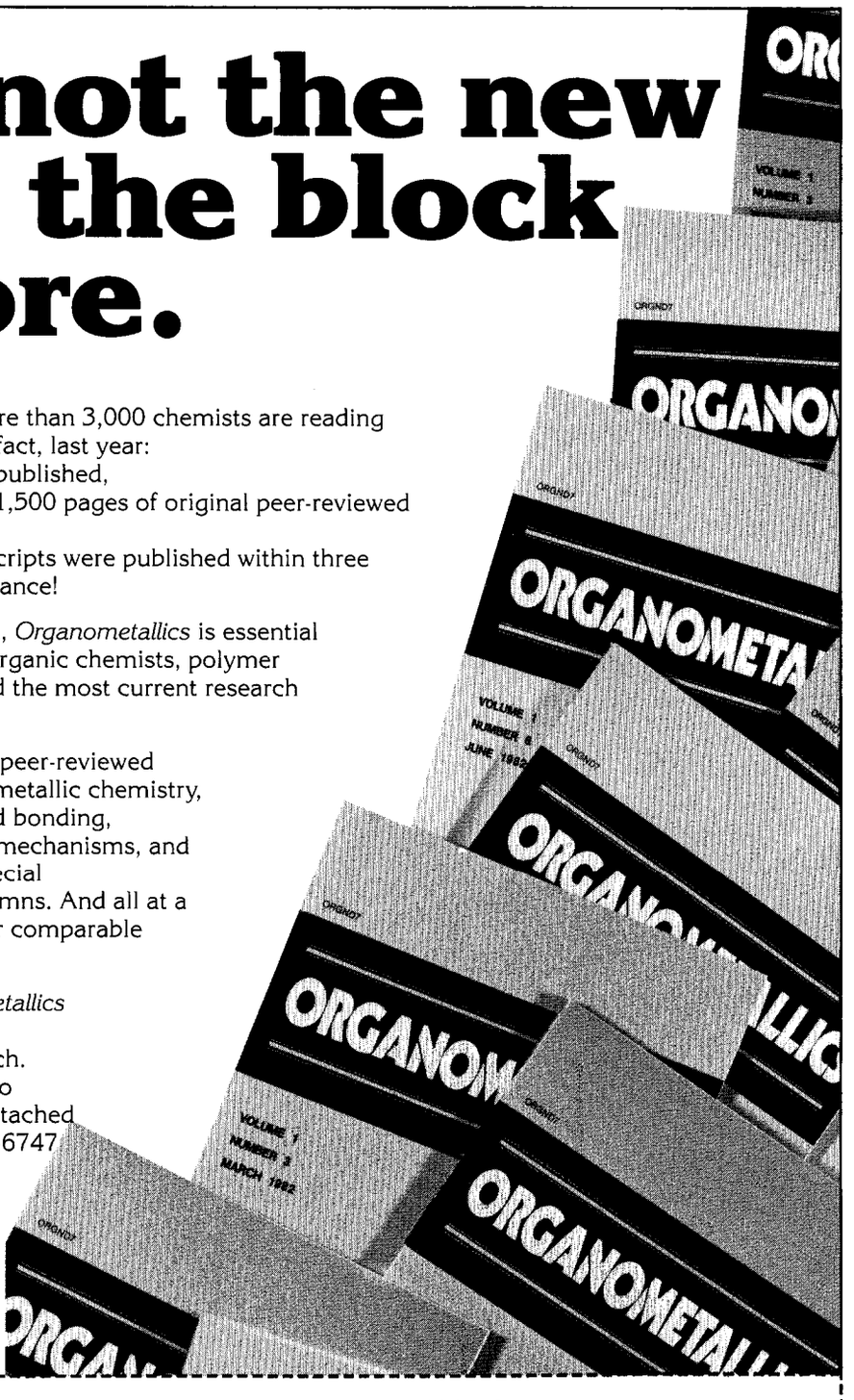
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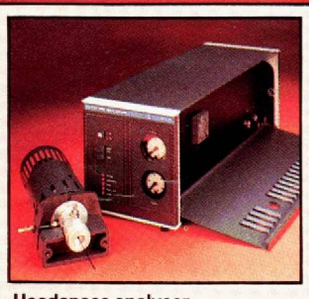
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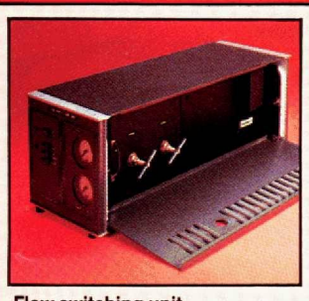
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Table III. Isocratic Separation of Small Molecules vs. Gradient Elution of Large Molecules—Dependence on Experimental Conditions

Relationship	Small molecules, isocratic elution	Large molecules, gradient elution
Plate height h	(8) $A\nu^{1/3}$	(8a) $C\nu$
Plate number N	(9) $D_m^{1/3}Ld_c^{2/3}F^{-1/3}d_p^{-4/3}$	(9a) $D_m t_G^0(\bar{k}Cd_p^2)^{-1}$
Resolution R_s, peak capacity PC	(10) $[D_m^{1/6}L^{1/2}d_c^{1/3}F^{-1/6}d_p^{-2/3}]$ $[k'/(1+k')]$	(10a) $D_m^{1/2}(t_G^0)^{1/2}(\bar{k})^{-(0.5+n)/2}d_p^{-1}$
Peak height or $(1/\sigma)$	(11) $[D_m^{1/6}L^{-1/2}d_c^{-5/3}F^{-1/6}d_p^{-2/3}]$ $[1/(1+k')]$	(11a) $D_m^{1/2}(t_G^0)^{-1/2}F^{-1}(\bar{k})^{-(0.5+n)/2}d_p^{-1}$

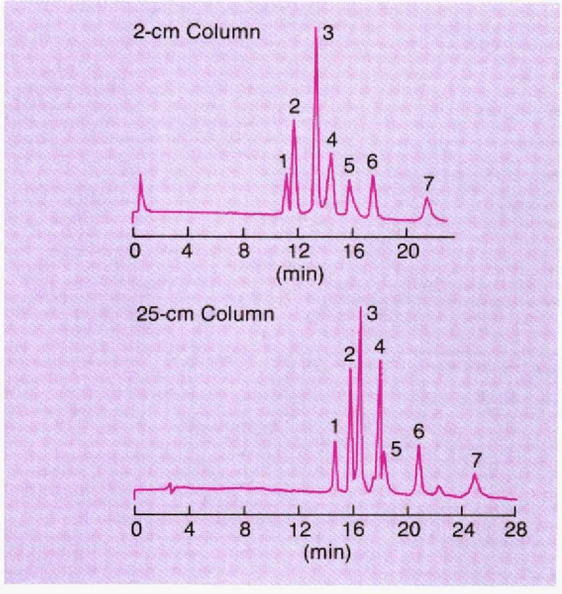


Figure 6. Reversed-phase gradient elution separation of proteins as a function of column length

Diphenyl-silica column, 10–90% isopropanol–water gradient, 0.46-cm column diameter, 1 mL/min flow rate. 1, ribonuclease A (13.7 kilodaltons); 2, insulin (6.0); 3, cytochrome c (12.2); 4, lysozyme (14.0); 5, bovine serum albumin (66.3); 6, β-lactoglobulin (35.0); and 7, ovalbumin (44.0). Adapted from Reference 18

$\bar{k}/(1+\bar{k}) \propto (\bar{k})^{1/4}$, and
$C \propto (\bar{k})^n$
where $-0.5 \leq n \leq 0.3$ (*6*).

Equations 10a and 11a apply to the separation of large molecules by gradient elution. We have observed that the column parameter n is close to −0.5 for reversed-phase systems (either large or small molecules), but should be larger if stationary-phase diffusion is markedly retarded (probably the case in ion exchange and adsorption chromatography). For reversed-phase systems, with n taken as −0.5, Equations 10a and 11a then yield simplified equations for gradient elution of macromolecules: Peak capacity or average R_s is proportional to

$$D_m^{1/2}\, d_p^{-1}\, t_G^{0.5} \qquad (12)$$

and peak height is proportional to

$$D_m^{1/2}\, F^{-1}\, d_p^{-1}\, t_G^{-1/2} \qquad (13)$$

Equations 12 and 13 can be contrasted with corresponding relationships (Equations 10 and 11) for resolution and peak heights in isocratic elution. Unlike isocratic elution, gradient elution separations are somewhat insensitive to the column dimensions chosen. This is confirmed in Figure 6 for the separation of a mixture of proteins by the same gradient from two columns of different length. Peak capacities (or average resolution) and average peak heights are similar for the two separations.

Average resolution and peak capacity also are predicted (Equation 12) to be independent of flow rate, and this is shown experimentally in Figure 7a for peak capacity vs. flow rate and gradient time for the reversed-phase separation of a mixture of peptides. These data also show that peak capacity increases regularly with gradient time t_G, as predicted by Equation 12. In Figure 7b the effects of flow rate and gradient time on peak height values are shown for the same system. Here the decrease in peak height predicted by Equation 13 vs. increase in t_G or F is again confirmed. This same study (*19*) also showed that a 5-μm-particle column gave roughly double the peak capacity found for a 10-μm column, again in agreement with Equation 12. Other examples of the applicability of Equations 12 and 13 for the reversed-phase separation of peptides and proteins using gradient elution are presented in Reference 17, where quantitative agreement has been observed between experimental data and a model based on small-molecule HPLC.

The failure of average resolution or peak capacity to change with column length L or flow rate F (Figures 6 and 7) deserves comment. The plate number N increases with L and decreases with F for macromolecular gradient separation. However, this is counterbalanced by a change in $\bar{k}$, since $\bar{k}$ is proportional to (F/L), and resolution increases with $\bar{k}/(1+\bar{k})$. Equations 12 and 13 thus arise from the fortuitous cancellation of two effects.

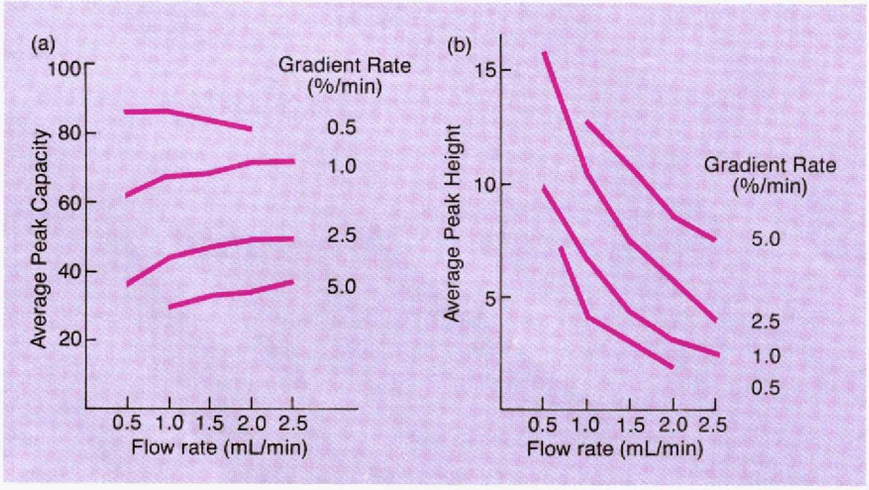


Figure 7. Peak capacity (a) and peak height (b) as a function of flow rate and gradient time t_G^0 for separation of various peptides (300–6000 daltons) on a C_{18}-silica column

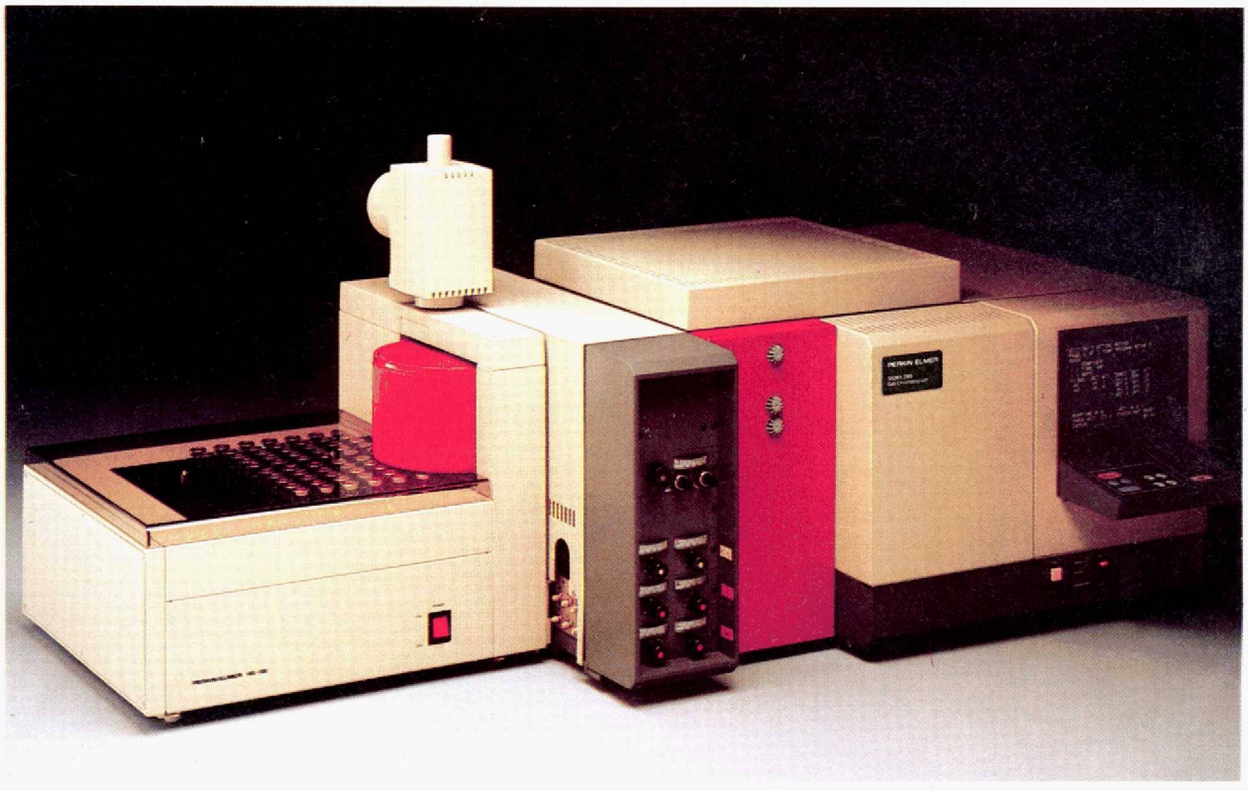
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Other Predictions of the Model

The model described here also provides insight about the question of how macromolecules move through the column during gradient elution: unretained vs. retained (the normal case for small molecules). Figure 8 illustrates how band migration in reversed-phase liquid chromatography changes with change in the sample molecular weight, *other conditions held constant*. In Figure 8a the elution of a 100-dalton (small molecule) solute is shown under conditions (Equation 4a) that yield a value of $\bar{k}$ equal to 3. This separation can be compared with that of Figure 1c. It is seen that the k′ value of the band varies from about 10 to 1.5 during most of the migration process. In Figure 8b the similar separation of a 10 000-dalton solute is shown. Because

(*Continued on p. 1429 A*)

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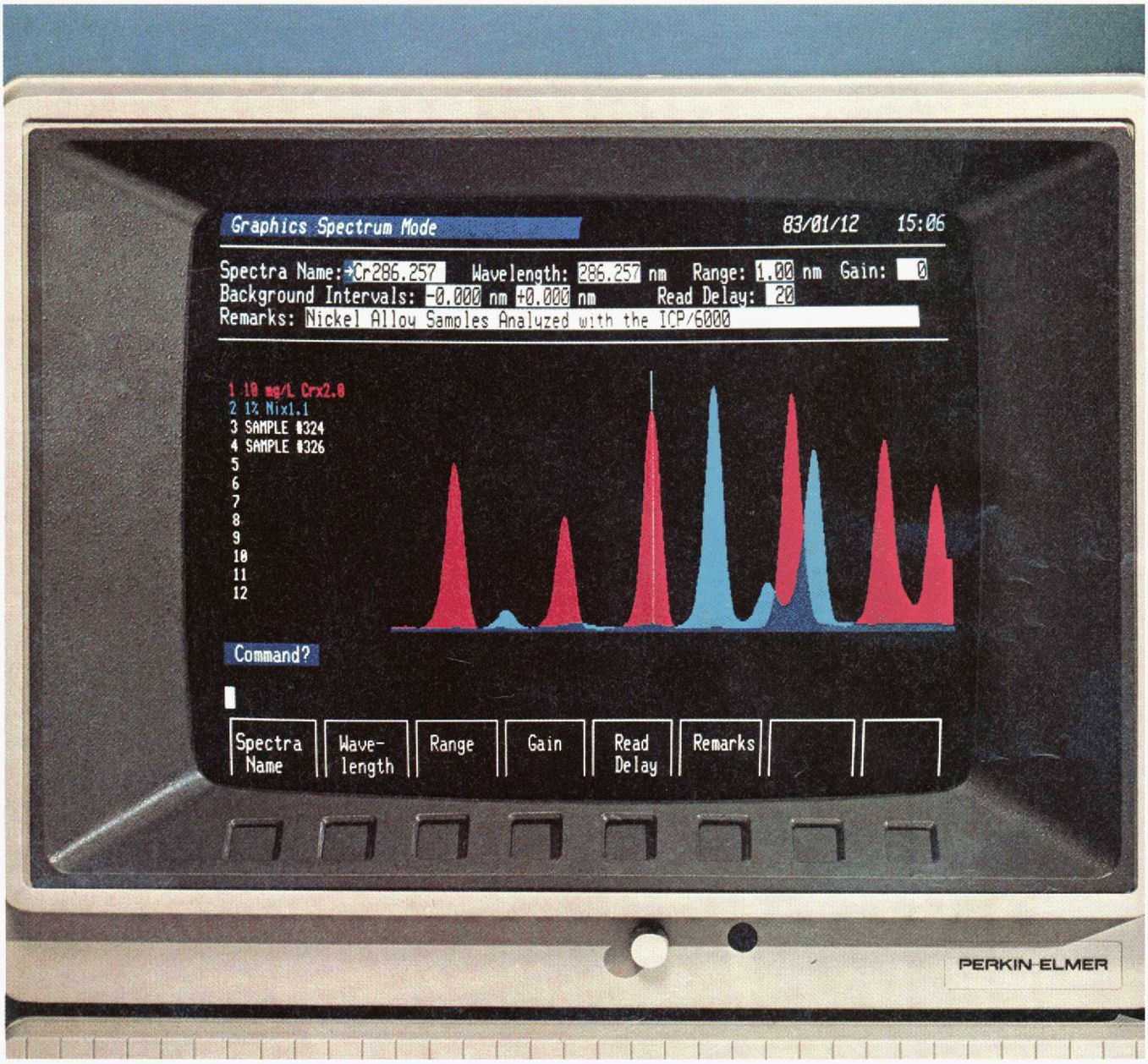
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Brings the world's best background correction to light.

Name: Smith-Hieftje* (S-H) Background Correction System.

Availability: Only in the new IL *Video 22*™ Atomic Absorption Spectrophotometer.

Principle: The hollow cathode lamp serves as its own background corrector (Fig. 1). At the normal lamp current, light is absorbed by the element and also by the background. Then a very brief, high-current pulse broadens the emission, which is then absorbed primarily by the background. The difference of the two signals produces the corrected absorbance. An IL patent is pending.

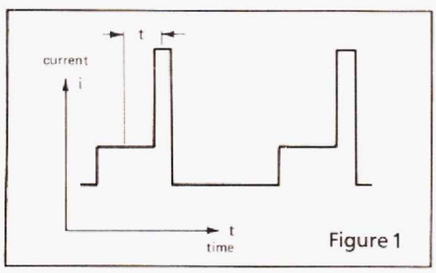


Figure 1

*Stanley B. Smith, Jr., Director of R&D, IL Analytical Instrument Division. Gary M. Hieftje, (Heef'yeh), Professor of Chemistry, Indiana University.

Advantages:
S-H System compared to conventional deuterium-arc correction:

- Corrects "structured" background.
- Eliminates some spectral interferences.
- Also corrects in visible range.
- Cannot be misaligned.
- Provides better light throughput.
- Corrects higher absorbances (Fig. 2).
- Costs less.

S-H System compared to Zeeman system:

- No rollover of working curves.
- No "anomalous" elements.
- No obstruction of sample area.
- No splitters or polarizers to impede light throughput.
- Costs much less.

A winning combination:
IL has introduced a new video AA, complete with graphics, methods storage and storage of analytical curves. The *dual*-channel, *double*-beam Video *22* AA can determine almost any two elements simultaneously, can utilize the internal standard technique, can also be used as a first-rate UV spectrophotometer, and is *equipped with the Smith-Hieftje system as standard.*

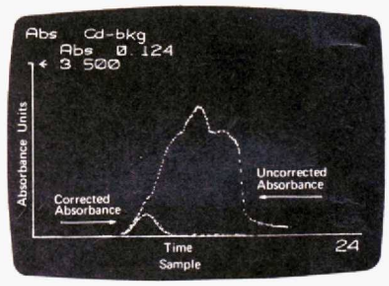


Figure 2 IL Video 22 AA screen during the determination of Cd in 2% NaCl, with furnace atomizer. Background absorption is 2.8 absorbance units (99.8% absorption).

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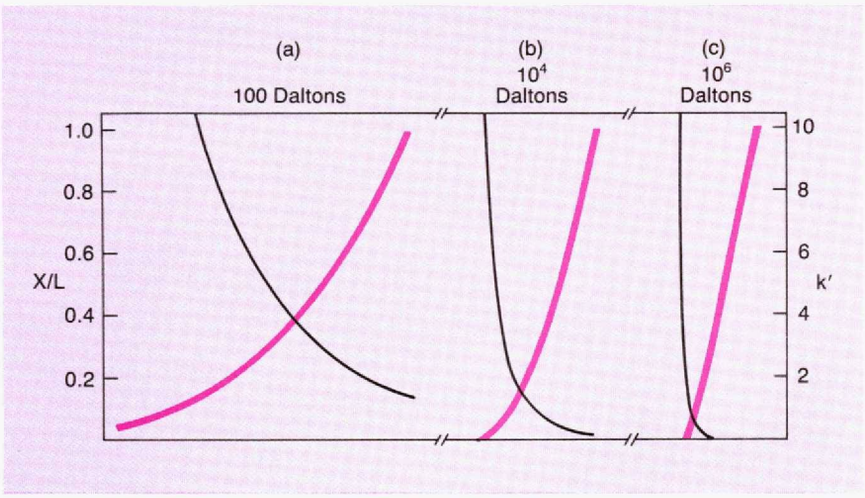


Figure 8. Calculated band migration in the gradient elution separation of compounds of varying molecular weight

(a) 100-dalton solute, conditions chosen to give $\bar{k} = 3$; (b) 10 000-dalton solute, other conditions the same; (c) 1-million-dalton solute, other conditions the same. X/L is fractional migration distance; colored line, X/L; black line, k′

of the larger value of S for this compound (*17*), the value of $\bar{k}$ is reduced to 0.5 (Equation 4a), and k′ varies from about 2 to 0.2 during migration. However, significant retention of the band exists over the entire course of its elution from the column. In Figure 8c, for the gradient separation of a 1-million-dalton sample, the value of $\bar{k}$ is only 0.07, and the sample is essentially unretained during its elution ($0.05 < k' < 0.5$). The separation conditions of Figure 8c are suboptimal for the separation of such macromolecular samples, and a change in column dimensions or flow rate (so as to increase $\bar{k}$) would be expected to improve separation markedly—without any change in t_G. It is likely that many gradient separations of macromolecular samples are carried out as in Figure 8c—without significant retention of the sample during elution. This is particularly true of step-gradient procedures as shown in Figure 9 for the separation of egg white proteins by ion-exchange chromatography.

Table II illustrates another characteristic feature of the gradient separation of macromolecules. Here it is seen that as solute molecular weight becomes large, the band is eluted in almost the same mobile phase composition ϕ for the two different columns: For the 50 000-dalton solute, ϕ = 0.873 for the 6-nm-pore column vs. ϕ = 0.870 for the 30-nm-pore column (corresponding ϕ values for the 2000-dalton solute are 0.804 and 0.750). That is, it *appears* that the column plays no role in determining separation for large molecules.

It is also found that elution of large molecules at constant ϕ occurs when other conditions (column length or diameter, flow rate, and gradient time) are changed—unlike the case for small molecules. Actually, the present model predicts just what has been observed: elution of large molecules at approximately constant ϕ. Thus Equations 1a and 4a can be combined with the dependence of k_0 on column surface area (*9*) to yield (for a 0–100% gradient):

$$\phi \text{ (at elution)} = (t_g - t_0)/t_G^0$$
$$= (\log 2.3\, K_0)/S + (\log \psi)/S + (\log \bar{k})/S \quad (14)$$
$$\text{(i)} \qquad \text{(ii)} \qquad \text{(iii)}$$

Here $k_0 = K_0\psi$, where ψ is the stationary-phase volume for a given column vs. a standard column with $k_0 = K_0$ by definition (*9*). Differences in solute retention between two C_{18} columns of differing pore diameter (as in Table II) are described by term ii of Equation 14. Likewise, differences in t_G, F, or column dimensions affect $\bar{k}$ and term iii of Equation 14. For a given solute, its value of S will become large as its molecular weight increases, thereby making terms ii and iii small. Therefore in the limit, for very large sample molecules, terms ii and iii can become negligible, so that ϕ at elution becomes constant for columns of different surface area and for different experimental conditions (t_G, F, V_m, etc). Note that differences in the solute also affect term i of Equation 14. Because K_0 increases with solute molecular weight, however, term i does not become small for large-solute molecules, as do terms ii and iii.

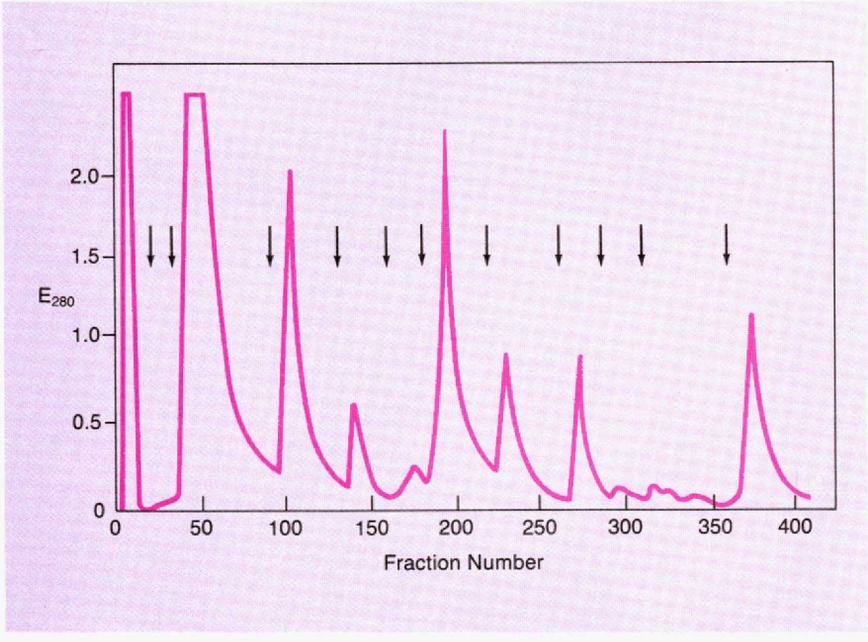


Figure 9. Separation of egg white proteins by step-gradient elution. Mobile-phase composition changed (↓) for elution of each band

Adapted from Reference 20

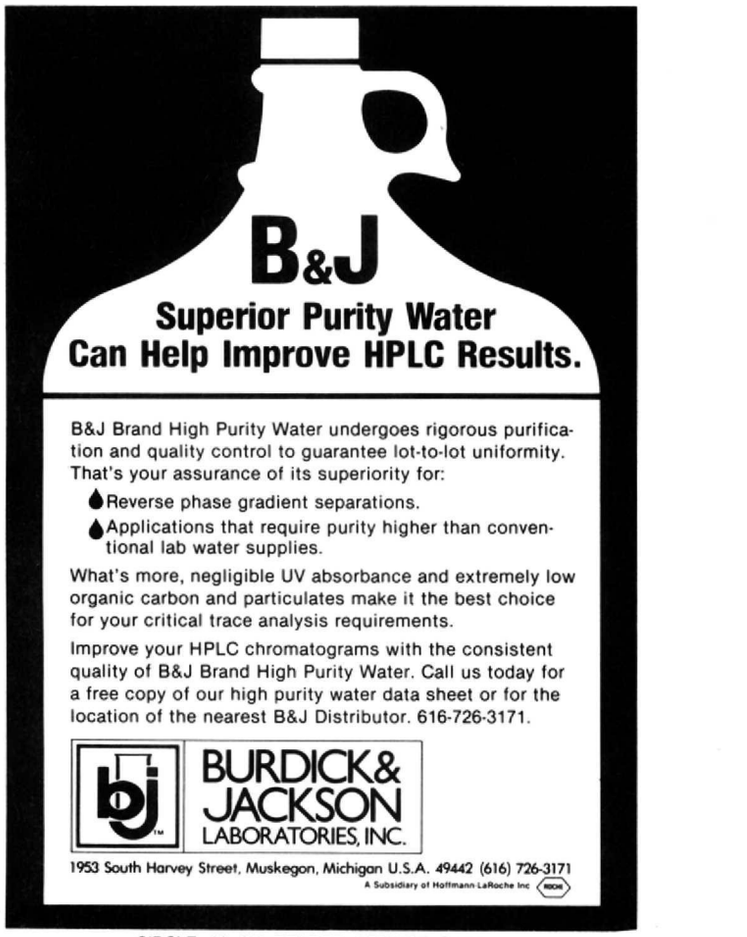
Conclusion

The separation of many macromolecular samples by gradient elution can be described by the same model (*8*) that has been verified for the gradient separation of small molecules. A simplified form of this model (Equations 12–14) predicts how separation varies with experimental conditions, and this in turn explains some of the anomalies reported by previous workers. A key feature of these macromolecular solutes is the potentially strong dependence of retention on mobile phase composition ϕ, and this possibility should be considered in the development of optimized separation procedures (to avoid elution as in Figure 8c). A strong dependence of solute retention on ϕ also leads to some interesting consequences, such as relative independence of gradient reten-

tion time t_g on certain experimental conditions. It should be noted that we have simplified many features of these macromolecular separations, and there is still much to learn about the "fine structure" of this area of chromatography. Most experimental work relating to this picture has been collected for the reversed-phase separation of molecules with molecular weights of 100 000 and under. The characteristics of these systems may not be typical of macromolecular separation is general. However, at this time, there is no reason to believe that the gradient separation of macromolecules cannot be understood in terms of the same principles that have served to explain the chromatographic separation of small molecules.

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Lloyd R. Snyder is currently a consultant to Du Pont and other companies that use HPLC. He graduated with BS and PhD degrees in chemistry from the University of California at Berkeley, and prior to 1982 worked for Union Oil and Technicon. His current research interests include several areas of HPLC: retention optimization strategies, separations of large biomolecules, the characterization and design of new columns, and gradient elution theory.



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