still required for the synthesis of such amino acids due to their important biological activities and medicinal interests.[45] Furthermore, it is suggested that a hydroxyl group placed on the backbone of an amino acid is an active site when bound to a receptor protein or a biomembrane. When such amino acids are incorporated into peptides, the hydroxyl group plays an essential role in constraining the peptide structure into a specific conformation through intramolecular or external hydrogen bonding.[1a,b,25] Recent interest in the family of unusual amino acids is focused not only on chemistry but also on the impacts on interdisciplinary scientific fields. These amino acids are expected to function as useful probes to investigate molecular mechanisms of a variety of biological functions.[1,46]

It is a pleasure to acknowledge the contribution of my colleagues: their names are recorded in the references. I am grateful to Professor Koji Nakanishi for his continuous encouragement. The financial support of a grant-in-aid from the Ministry of Education, Sciences, and Culture, Japan, is appreciated.

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Prediction of High Concentration Band Profiles in Liquid Chromatography

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Introduction

When samples of increasingly large sizes of a pure component are injected in a chromatographic column, the elution profiles change progressively from a nearly Gaussian shape at very low sizes to an increasingly wide, highly unsymmetrical outline, usually characterized by an extremely steep front and a continuous rear boundary (Figure 1). More complicated profiles are also possible (Figure 2a). For mixtures, increasing the sample size leads to higher degrees of interference between the component bands. The decrease in band resolution is accompanied by increasing band interactions, and the profile of a band is modified by the presence of other components. Thus, the elution profile of a component in a mixture becomes different from the profile obtained for the same amount of the same compound injected alone (Figure 3). It is the essential purpose of this Account to show how these phenomena can be accounted for, both qualitatively and quantitatively.

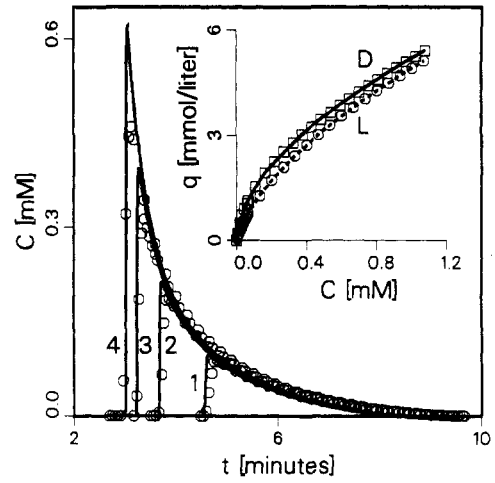

Figure 1. Comparison of experimental and calculated single-component overloaded elution profiles of *N*-benzoyl-L-alanine, using a bilangmuir isotherm model. Effect of sample size:[11] (1) 0.145, (2) 0.290, (3) 0.434, and (4) 0.580 μmol. Experimental conditions: L = 15 cm, i.d. = 0.4 cm; immobilized BSA on silica; mobile phase, 10 mM phosphate buffer at pH 6.8 with 3% 1-propanol, 1 mL/min. Insert: Experimental adsorption isotherm data (symbols) and best fit with the bilangmuir model (lines) for the D isomer (□, —) and the L isomer (○, - - -).

Their most common interpretation is that, because the band width increases, the column loses its efficiency when the sample size is increased. Under various forms,

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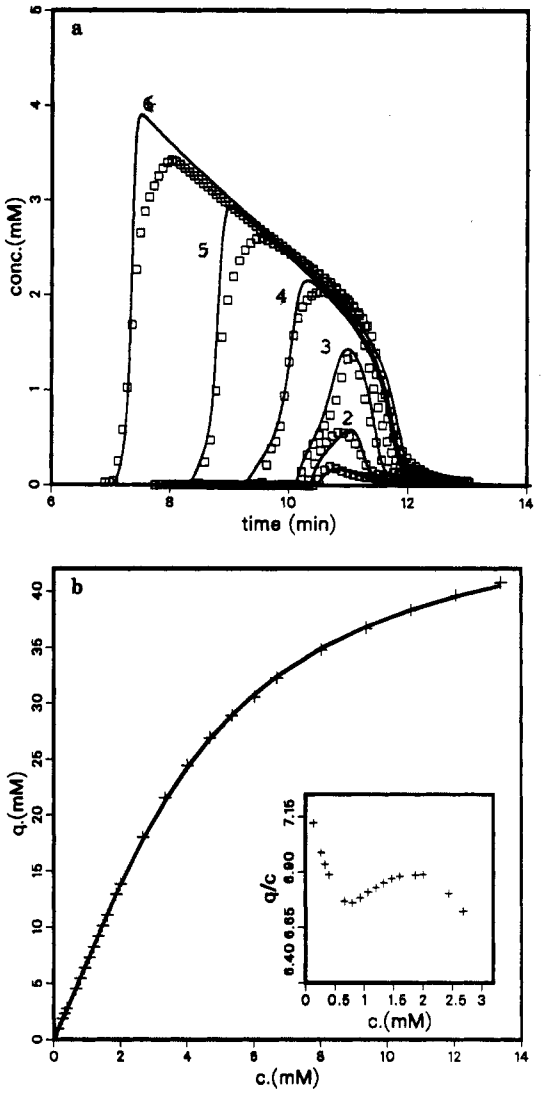

Figure 2. Comparison of experimental and calculated single-component overloaded elution profiles of phenyldodecane using a composite isotherm model: (a) comparison of experimental and calculated overloaded elution profiles of phenyldodecane; mobile phase, acetonitrile, 1 mL/min; sample, 67 mM solution, (1) 5, (2) 10, (3) 30, (4) 50, (5) 100, and (6) 180 μL; column length, 30 cm, i.d. 0.46 cm, T = 50 °C;[13] (b) adsorption isotherm of phenyldodecane on graphitized carbon black. Insert: Plot of q/C versus C, demonstrating the two inflection points.[13]

this erroneous statement is often found in the scientific literature. We show below that the correct explanation is entirely thermodynamic in nature. It stems from the competition of solute molecules for the finite number of adsorption sites on the stationary phase and, thus, from the nonlinear behavior of phase equilibria at high concentrations. Most importantly, the shape of these isotherms controls the band profiles. At high concentrations the equilibrium isotherm of any component depends on the concentration of all of the other mixture components. Therefore, competitive isotherms are complex. They are not yet fully understood.

The theory of nonlinear chromatography permits the accurate prediction of band profiles if the equilibrium isotherms of the mixture components and the column operating conditions are known. Thus, this theory provides the background needed for the optimization of the experimental conditions of a separation. This explains its importance, since interest in industrial-scale

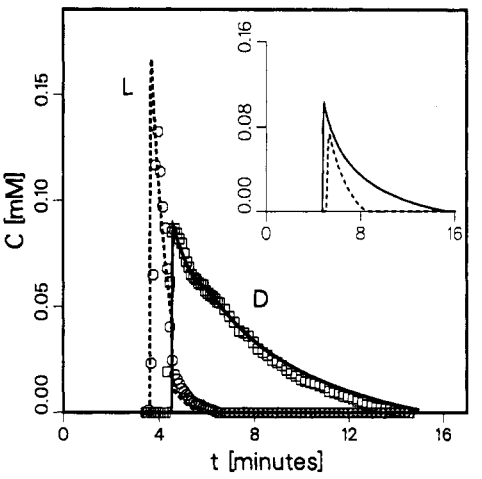

Figure 3. Comparison of experimental and calculated profiles for the separation of *N*-benzoyl-D- and -L-alanine on immobilized BSA (ref 11, Figure 6): 1/3 mixture, 0.105 μmol of L isomer, 0.392 μmol of D isomer. Same experimental conditions as for Figure 1. Insert: Calculated profiles assuming no interaction at the same sample size as for the main figure.

preparative chromatography is rapidly growing in the pharmaceutical industry. Applications such as isolating pure enantiomers from racemic mixtures, peptides from a Merrifield synthesis, or proteins from fermentation broths are now common. The application of the theory of nonlinear chromatography to preparative chromatography is discussed elsewhere.[1]

Theory

The profiles of chromatographic bands result from the interaction of various thermodynamic and kinetic phenomena.[1] In liquid chromatography, a solvent mixture called the mobile phase percolates through a tightly packed particle bed, the stationary phase. This stationary phase can be an adsorbent, an ion-exchange resin, or a liquid dispersed on a solid support. We consider here only liquid–solid chromatography, a mode of chromatography using an adsorbent as the stationary phase. Most of our conclusions could be extended to the other modes.

A relatively narrow pulse of a mixture is injected into the column. Our goal is to predict the individual elution profiles of each component of a mixture. These profiles depend on the competitive equilibrium isotherms of each component, the rate of axial dispersion of the solute bands, and the kinetics of mass transfer of the components between the two phases. The link is provided by the equation of conservation of mass in a chromatographic system, derived by Wilson.[2]

I. The Mass Balance Equation. This equation states that the rate of increase of the amount of a component in an infinitely narrow slice of column (first two terms of eq 1 below) is given by the balance of what enters and leaves the slice (third term of eq 1) and the balance of what diffuses in and out of the slice (last term of eq 1). For the two components of a binary mixture, the differential mass balances are as follows:

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$$\frac{\partial C_1}{\partial t} + F\frac{\partial q_1}{\partial t} + u\frac{\partial C_1}{\partial z} = D_{ap}\frac{\partial^2 C_1}{\partial z^2}$$
$$\frac{\partial C_2}{\partial t} + F\frac{\partial q_2}{\partial t} + u\frac{\partial C_2}{\partial z} = D_{ap}\frac{\partial^2 C_2}{\partial z^2} \quad (1)$$

where q_i and C_i are the concentrations of the component i in the stationary and mobile phases, respectively, at time t and distance z along the column, F is the ratio of the volumes occupied by the two phases contained in the column ($F = (1 - \epsilon)/\epsilon$, ϵ is the total column porosity or fraction of the column volume occupied by the mobile phase), u is the linear mobile phase velocity (ratio of the mobile-phase flow rate and the cross section of the column available to the mobile phase), and D_{ap} is the apparent axial dispersion coefficient, a lumped kinetic coefficient (eq 3) which accounts for the net effect of the axial dispersion and the resistance to mass transfer.[1,3] Equation 1 is completed by the equilibrium isotherms,

$$q_1 = f_1(C_1,C_2)$$
$$q_2 = f_2(C_1,C_2) \quad (2)$$

and by a relationship between the apparent dispersion coefficient and the column height equivalent to a theoretical plate, H, a parameter which is easily measured.

$$D_{ap} = \frac{HL}{2t_0} \quad (3)$$

The column length, L, the dead time, t_0, and the initial and boundary conditions are known experimental quantities. The initial conditions describe the concentration of the adsorbing species in the column at the beginning of the experiment. Usually the column is filled with pure mobile phase, and $C_i(z,t=0) = 0$. The boundary conditions give the composition of the adsorbing species at the column inlet during the time period of the experiment, $C_i(z=0,t) = f_i(t)$. This includes the sample injection and, possibly, progressive changes of the mobile-phase composition, such as during gradient elution. The mathematical properties of this system of equations have been studied in detail.[4] Its solution is particularly difficult for multicomponent mixtures because the isotherms in eqs 2 relate or couple the mass balance eqs 1. There are no analytical solutions, but various algorithms[1,3,5] permit the calculations of numerical solutions.

The critical step in predicting individual band profiles (necessary for the optimization of the conditions of a separation) is the accurate experimental determination of the equilibrium isotherms and the fitting of these data to a functional form for use in eq 2.

II. Equilibrium Isotherms. Physical chemists have paid considerable attention to the representation of isotherm data. The most useful equations are listed in Table I (the a's and b's are numerical coefficients, and q_s is the saturation capacity of a monolayer, i.e., the amount of component needed to form a dense monolayer on the adsorbent surface).

Equation 4 corresponds to the Langmuir model (curve 2, Figure 4a) and assumes that the surface is homogeneous, there is monolayer adsorption, there are no adsorbate–adsorbate interactions, and both the mobile and stationary phases are ideal. The isotherm depends on two parameters: the saturation capacity (q tends toward q_s at high concentrations) and the adsorption energy at infinite dilution, i.e., under linear conditions. The retention factor of analytical chromatography is proportional to bq_s. With Langmuir isotherms, b is positive, and the isotherm is convex upward.

Equation 5 is the bilangmuir model, the sum of two Langmuir isotherms (curve 3 in Figure 4a). It corresponds to a model that assumes the adsorbent surface is heterogeneous, a quilt of two different surfaces, noted 1 and 2. For example, in reversed-phase chromatography, a part of the surface is covered with C18 chemically bonded groups and the other part with unreacted silanols; in enantiomeric separations, one term accounts for the chiral selective interactions and the other for the nonchiral ones. These two parts are independent, and there is no cooperative adsorption. Each part has a separate Langmuir contribution to the adsorption isotherm. Obviously, under analytical conditions, there are no possibilities to distinguish the two contributions, and the retention factor is proportional to $(b_1q_{s,1} + b_2q_{s,2})$.

Equation 6 is a three-parameter isotherm, suggested by statistical thermodynamics (curve 4, Figure 4a). It permits a satisfactory representation of isotherms having an inflection point (also called S-shaped isotherms), which is not possible with the Langmuir model. S-shaped isotherms are usually convex downward at concentrations below the inflection point.

Finally, eqs 7 and 8 are the competitive or "binary" isotherms corresponding to the Langmuir and the bilangmuir isotherms, respectively. They express the facts that the surface area is finite and that an adsorption site can be occupied by only a single molecule. These simple competition models are valid only if the saturation capacities for the two components are equal. Otherwise, a more complex treatment is needed.[6]

Many other models have been suggested.[7] Their number indicates the complexity of the problem and the lack of a strong theoretical basis. Experimental methods of isotherm measurements were reviewed.[1] Accurate data should be acquired in a wide concentration range in order to permit the precise determination of the isotherm parameters. Calculations[3,5] and experiments[1] show that band profiles are sensitive to small changes in the isotherms. In Figure 4a, we show four isotherms with the same initial slope, but corresponding to different isotherm models. The same Gaussian peak will be obtained at very low sample sizes, but quite different profiles result at large sample sizes (Figure 4b).

III. Qualitative Relationships between Isotherms and Band Profiles. While the equations displayed above permit the calculation of band profiles, it is useful to understand on a qualitative basis how band profiles depend on component concentrations. The theory shows that, if we neglect the dispersive effect of a finite efficiency, each concentration of the

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Table I
Isotherm Models

Langmuir isotherm	$q = \frac{q_s b C}{1 + bC}$	(4)
bilangmuir isotherm	$q = \frac{q_{s,1} b_1 C}{1 + b_1 C} + \frac{q_{s,2} b_2 C}{1 + b_2 C}$	(5)
quadratic isotherm	$q = \frac{q_s C(b_1 + 2b_2 C)}{1 + b_1 C + b_2 C^2}$	(6)
competitive Langmuir isotherm	$q_i = \frac{q_s b_i C_i}{1 + b_1 C_1 + b_2 C_2}$	(7)
competitive bilangmuir isotherm	$q_i = \frac{q_{s,1} b_{i,1} C_i}{1 + b_{1,1} C_1 + b_{2,1} C_2} + \frac{q_{s,2} b_{i,2} C_i}{1 + b_{1,2} C_1 + b_{2,2} C_2}$	(8)

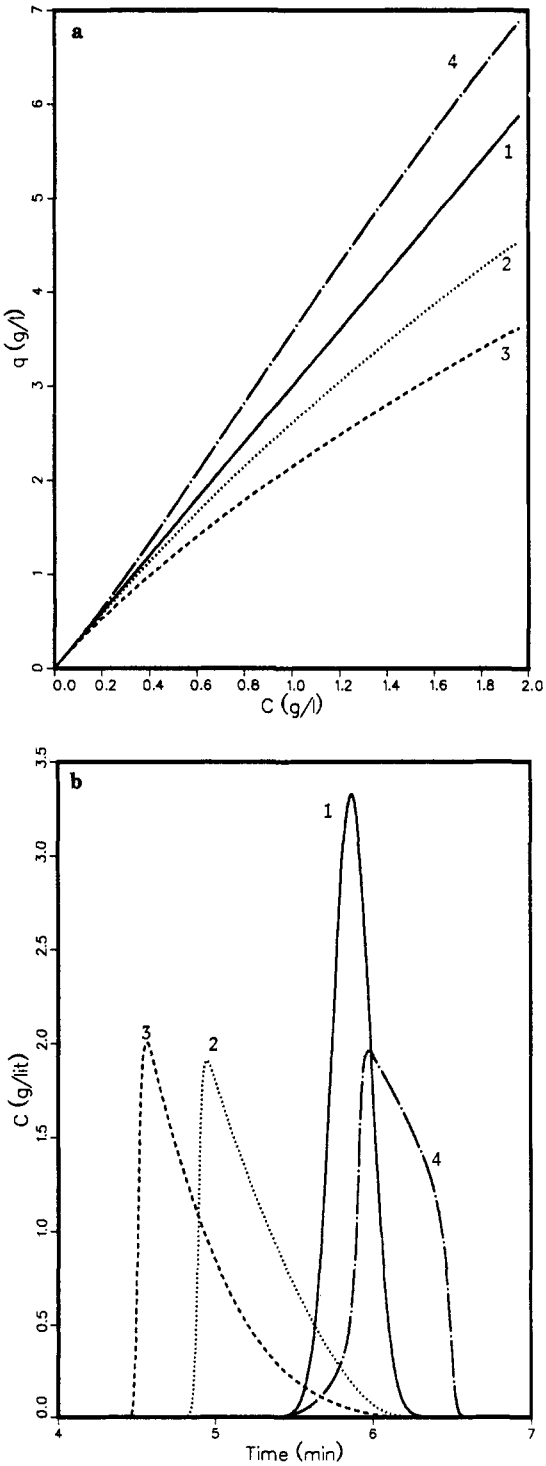

Figure 4. Equilibrium isotherms and band profiles at high concentrations: (a) all four isotherms have the same initial slope, isotherm models 1 linear, 2 Langmuir, 3 bilangmuir, 4 quadratic (see Table I); (b) band profiles corresponding to a 1-mg sample, with the four isotherms.

injected profile moves at its own velocity. The velocity associated with a concentration increases with decreasing slope of the isotherm. With a convex upward isotherm (e.g., Langmuir, curve 2 in Figure 4a), the concentration in the stationary phase at equilibrium increases less rapidly than the mobile-phase concentration; hence, high concentrations are less retained than low ones, which explains the profiles in Figure 1. The rear boundary is directly related to the derivative of the isotherm. The high concentrations cannot pass the low ones, however, and they all pile up into a discontinuity. This discontinuity moves more slowly than the highest concentration on the rear boundary; hence, the band top erodes.

For real columns, the concentration discontinuity is unstable, the infinite concentration gradient results in an infinite diffusive flux, and the discontinuity is replaced by a steep front. This explains the profiles seen in Figure 1. For a convex downward isotherm, retention increases with increasing concentrations, and the converse holds true. This explains the profiles in Figure 2.

Single-Component Elution Bands

The elution of small organic molecules (e.g., phenol, acetophenone, 2-phenylethanol) on silica or chemically bonded silica is the simplest case study. In almost all cases, the Langmuir isotherm (Table I, eq 4) accounts well for equilibrium data.[8,9] When the experimental isotherm is accurately known, there is excellent agreement between calculated and experimental band profiles.[1,8,9]

We consider now the case of L and D amino acid derivatives, using bovine serum albumin (BSA) immobilized on silica as the stationary phase. The bilangmuir isotherm (Table I, eq 5) models the experimental data (Figure 1) very well, because the surface is heterogeneous and contains two types of adsorption sites.[8,9] A hydrophobic pouch of BSA constitutes the chiral selective sites. The other sites include the residual silica, the peptide chains, and the side groups of the amino acids, all of which are nonselective sites. Since the free energy of adsorption on the nonselective sites is low, they can be considered together as a homogeneous surface.

The insert to Figure 1 presents the experimental isotherm data fitted to the bilangmuir isotherm model. Although this model requires four parameters for each enantiomer, only five are needed for each pair of enantiomers, in agreement with the retention mecha-

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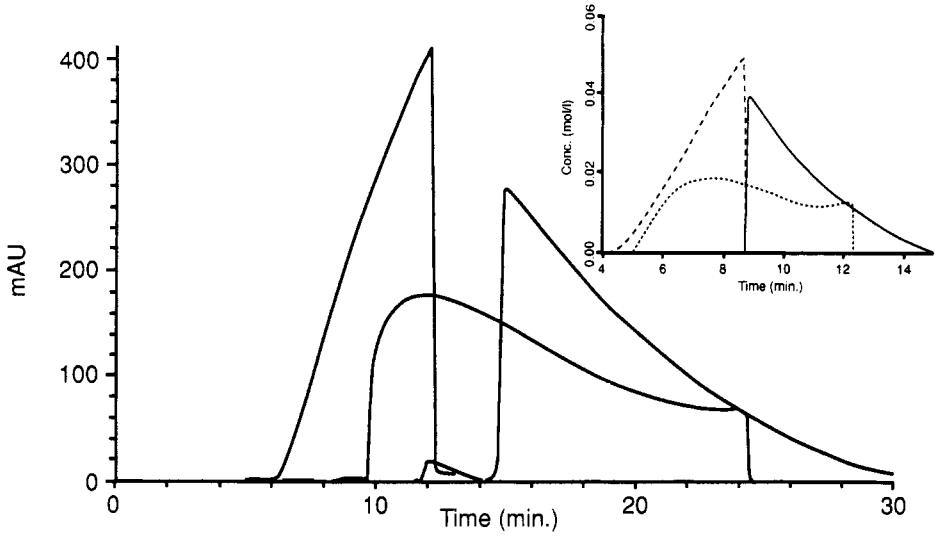

Figure 5. Qualitative comparison of experimental and calculated single-component overloaded elution profiles with a binary mobile phase. Effect of the concentration of the mobile-phase additive on the experimental profile of 10 μL of 3-phenyl-1-propanol. Mobile phase: (1) 0.02%, (2) 0.1%, and (3) 0.4% 2-propanol in dichloromethane. L = 25 cm, i.d. = 0.46 cm, 2 mL/min, on silica (ref 15, Figure 17). Inset: Calculated profiles of 3-phenyl-1-propanol (ref 14, Figure 6). Reprinted with permission from refs 14 and 15. Copyright 1989 Elsevier.

nism.[10,11] The Langmuir term, which accounts for nonselective interactions between the molecule and the adsorbent, is the same for both enantiomers. The active site is the hydrophobic cavity of the albumin, and the column saturation capacity is the same for both enantiomers. Figure 1 presents the elution profile of *N*-benzoyl-L-alanine at four different sample sizes. The agreement between experimental and calculated band profiles is excellent.

The adsorption of compounds with long alkyl chains on the surface of graphitized carbons has long been recognized as characterized by strong adsorbate–adsorbate interactions.[12] Due to the density of carbon atoms and its high degree of homogeneity, graphite has a high surface energy. When adsorbed on its surface, molecules lie with the largest possible number of "heavy" atoms (i.e., C, N, O) in contact with the surface.[12] An alkyl chain will lie in a zig-zag conformation, maximizing the contact area.[12] While small polar groups or phenyl groups give small adsorbate–adsorbate interactions, long, straight, parallel alkyl chains interact strongly together. Thus, the adsorption of a second alkyl molecule will generate a higher energy than that of the first one. In this case, the amount adsorbed at equilibrium will increase faster than the mobile-phase concentration, and the isotherm will be concave upward. Because of the finite saturation capacity, an inflection point will appear at some intermediate concentration. Equation 6 of Table I can fit this type of data.

The surface of graphitized carbon adsorbents available for liquid chromatography[13] is heterogeneous, however, and a small fraction is made of defects on which the adsorption energy is high. Figure 2b presents the equilibrium isotherm of phenyldodecane in acetonitrile on graphitized carbon. A composite isotherm model, the sum of a Langmuir term and a quadratic term is used to fit the experimental data. The former term is for adsorption on the surface defects, and the latter term represents adsorption on the bulk surface. Although at first glance the equilibrium isotherm may seem linear, it is not. It is S-shaped and has two inflection points, as illustrated in the insert, in the form of a q/C vs C plot.

Figure 2a presents the elution of phenyldodecane at several sample sizes. The appearance of a sharp rear boundary and a steep front boundary is typical of S-shaped isotherms. The agreement between experimental and theoretical profiles is excellent and is attributed primarily to the excellent fit of the isotherm to the adsorption data. This result also illustrates how isotherms which may appear nearly linear lead to elution profiles which differ considerably from a Gaussian distribution. Finally, from the experimental conditions, we derive that for the largest sample the maximum concentration in the elution band is approximately 15 times more dilute than the feed. This illustrates one of the fundamental problems of preparative chromatography. Isocratic elution is a dilution process, as required by the second principle of thermodynamics. The optimization of experimental conditions can reduce the extent of dilution but cannot prevent it. Gradient elution, by contrast, can significantly concentrate the bands.

In reversed-phase (RPLC) and normal-phase (NPLC) liquid chromatography, adjustment of the retention behavior is achieved with the addition of a mobile-phase modifier. In RPLC, an *n*-alkyl group is chemically bonded to silica and used as the adsorbent, the mobile phase is water, and the modifier is generally methanol or acetonitrile. These modifiers are much less retained than the sample components, but increasing the modifier concentration increases the sample solubility in the mobile phase, thereby decreasing the component retention. In contrast, in NPLC, straight silica is used as the adsorbent, the modifier is retained, sometimes

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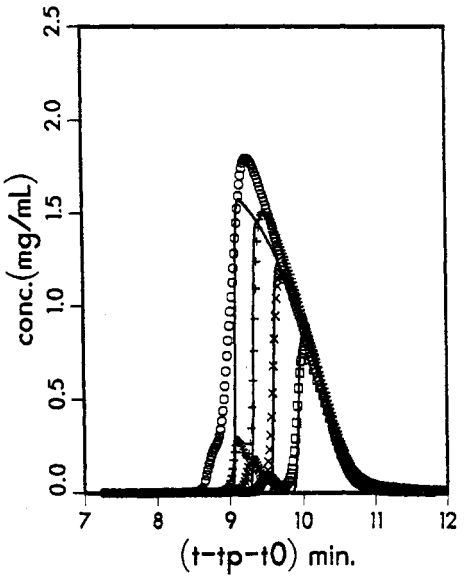

Figure 6. Comparison of experimental and calculated single-component gradient elution profiles of lysozyme.[16] Sample sizes: 1, 2, 3, and 4 mL of a 0.405 mg/mL solution of lysozyme. Loading factor: 2.9, 5.8, 8.7, and 11.6%. Gradient: 1% ACN/min. Reprinted with permission from ref 16. Copyright 1992 Wiley.

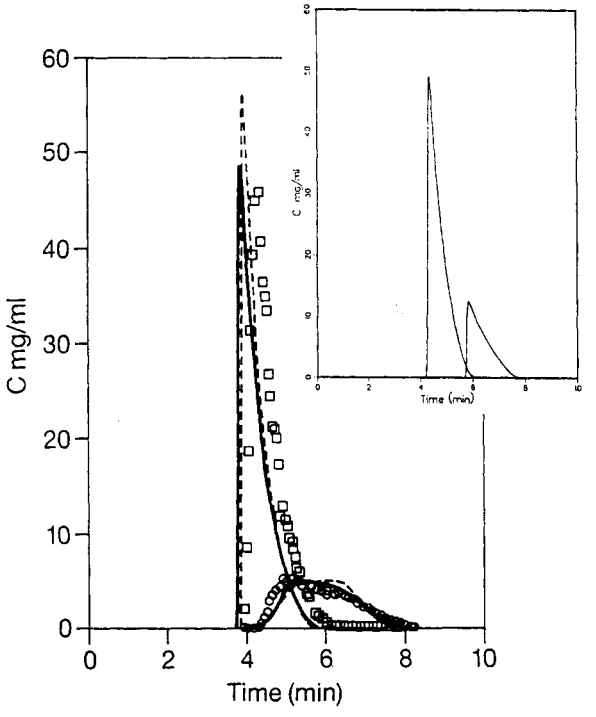

Figure 7. Comparison of experimental and calculated profiles for the separation of 2-phenylethanol and 3-phenylpropanol. The coefficients of the competitive Langmuir isotherms are measured according to two methods.[9] Sample: 2-phenylethanol 7 mg, 1-phenylpropanol 21 mg. Mobile phase: 1/1 methanol/water at 1 mL/min. Column: L = 25 cm, i.d. = 0.46 cm, ODS silica. Insert: Calculated profiles assuming no interaction at the same sample size as the main figure. Reprinted with permission from ref 9 and Katti, A. M.; Czok, M.; Guiochon, G. *J. Chromatogr.* **1991**, *556*, 205. Copyright 1990 and 1991 Elsevier.

more than the sample components, and their retentions decrease because of increasing competition with the modifier.[14]

Figure 5 shows the experimental profiles of 3-phenyl-1-propanol with increasing concentration of the

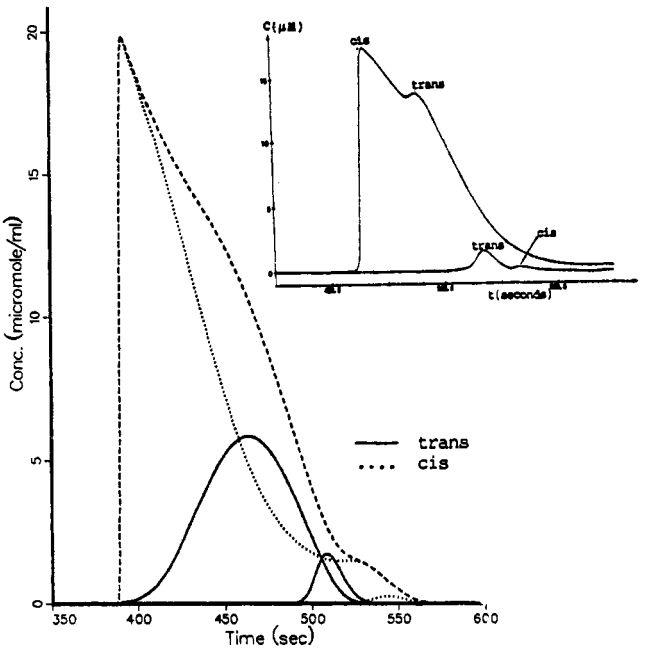

Figure 8. Qualitative prediction of the elution order reversal of *cis*- and *trans*-androsterone.[6] Analytical and preparative sample size. Insert: Experimental data. Stationary phase: silica modified with a pH 6.8 phosphate buffer. Mobile phase: 9/1 acetonitrile/dichloromethane, 0.98 mL/min. Samples: *cis*-androsterone 0.026 and 5.2 mg, *trans*-androsterone 0.15 and 1.8 mg.

modifier 2-propanol in NPLC.[15] Qualitative comparisons with theoretical data are presented in the inset of Figure 5. The theoretical predictions are made from assumed Langmuir isotherms representing the adsorption behavior of the modifier and the sample. A competitive Langmuir isotherm model (Table I, eq 7) enables the calculation of theoretical profiles.[14] The competition between sample and modifier explains the dramatic changes in band profile with increasing modifier concentration observed in these figures.

The elution behavior of proteins in RPLC is highly sensitive to the concentration of organic modifier in the mobile phase. While the logarithm of the retention factor decreases linearly with increasing modifier concentration in the mobile phase, the decrease is steep for proteins. For example, a decrease in acetonitrile concentration by 0.6% doubles the retention time of lysozyme.[16]

It is possible to quantitatively model the adsorption behavior of proteins in RPLC with a bilangmuir isotherm model.[16,17] However, the chromatography of proteins is normally conducted under gradient elution, a process where the mobile-phase concentration of the modifier at the column inlet increases over the separation time. The elution of lysozyme on C-18 modified silica using an acetonitrile/water gradient is presented in Figure 6 at four different sample sizes. The equilibrium adsorption isotherm was determined at several acetonitrile/water concentrations differing by only a few tenths of one percent. The dependence of the bilangmuir coefficients on the acetonitrile concentration was determined and used to calculate the band profiles.

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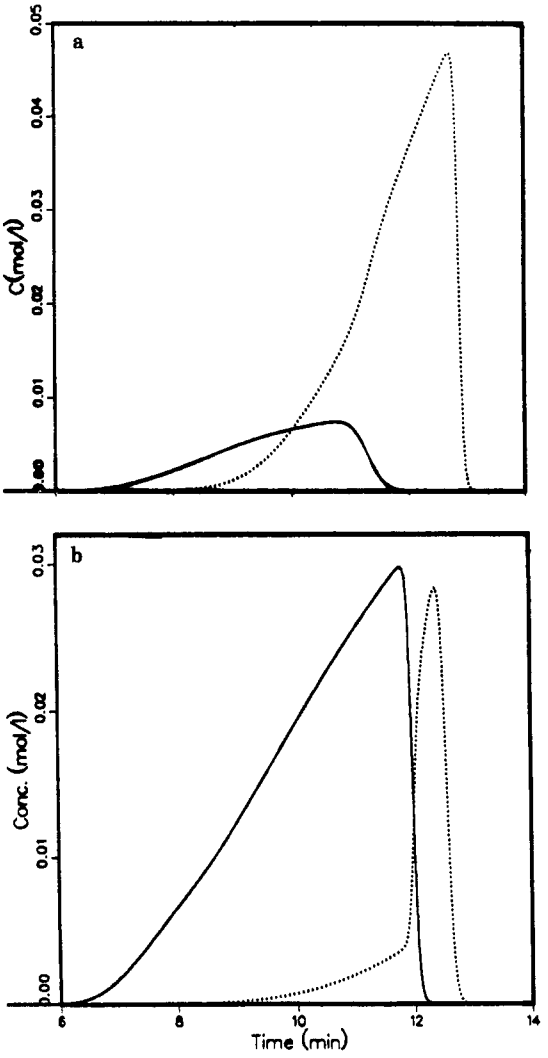

Figure 9. Illustration of the inversion of the band asymmetry in the case of a strongly adsorbed additive (ref 20, Figure 6): (a) calculated profiles for a 1/4 mixture, first component (···) 16 mmol, second component (—) 65 mmol, L = 25 cm, flow velocity 0.122 cm/s; (b) calculated profiles for a 4/1 mixture. Same conditions as for Figure 5a.

The agreement between theory and experiment is again excellent.

Two-Component Elution Bands

Individual band profiles of the components of a mixture depend on the mixture composition and the sample size because of the coupling of the mass balance equations (eqs 1) due to the competitive isotherms (eqs 2, 7, and 8). Competitive isotherms are more difficult to measure accurately than single-component isotherms because of their greater complexity (they are surfaces instead of curves) and the larger errors introduced by the intricacy of the experimental measurements. It is also more difficult to model and fit the adsorption data to a surface.

From a theoretical point of view, one of the simplest separation problems is that of a racemic mixture. This simplicity arises from the facts that the mixture has only two components and that all of the parameters characterizing the behavior of the two enantiomers are equal, except the chiral selective mechanism. This problem also has high practical value. An example is the separation of mixtures of *N*-benzoyl-D- and -L-alanine on immobilized BSA (Figure 3). Because the chiral selective retention mechanism involves adsorption of the enantiomers in the hydrophobic cavity of BSA, the column saturation capacity is the same for the two enantiomers. Thus, a competitive bilangmuir isotherm (Table I, eq 8) was employed, using the parameters obtained from single-component isotherm measurements.

While Figure 3 shows the individual band profiles for a 1/3 mixture, the insert shows an overlay of the chromatogram for each pure enantiomer. The significance of the competitive isotherms can be seen by comparing this insert and the main figure. The interaction of the two components results in the forward displacement, concentration, and narrowing of the first component band by the second. Since the second component is more strongly adsorbed to the stationary phase, its presence behind the first band forces an

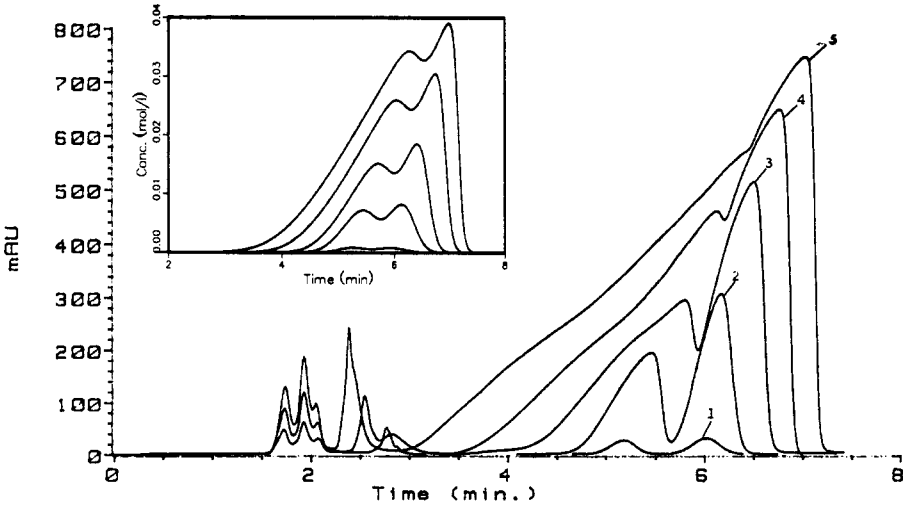

Figure 10. Qualitative agreement between experimental and calculated two-component overloaded profiles with a mobile-phase additive. Effect of sample size for a 1/1 mixture (ref 21, Figure 13). Sample: 1/3.6 solution of 2-phenylethanol and 3-phenylpropanol. Sample size: (1) 2, (2) 20, (3) 50, (4) 100, and (5) 150 μL. L = 25 cm, i.d. 0.46 cm, silica 2 mL/min. Concentration of propanol in the mobile phase: 0.133 M. Inset: Calculated profiles.

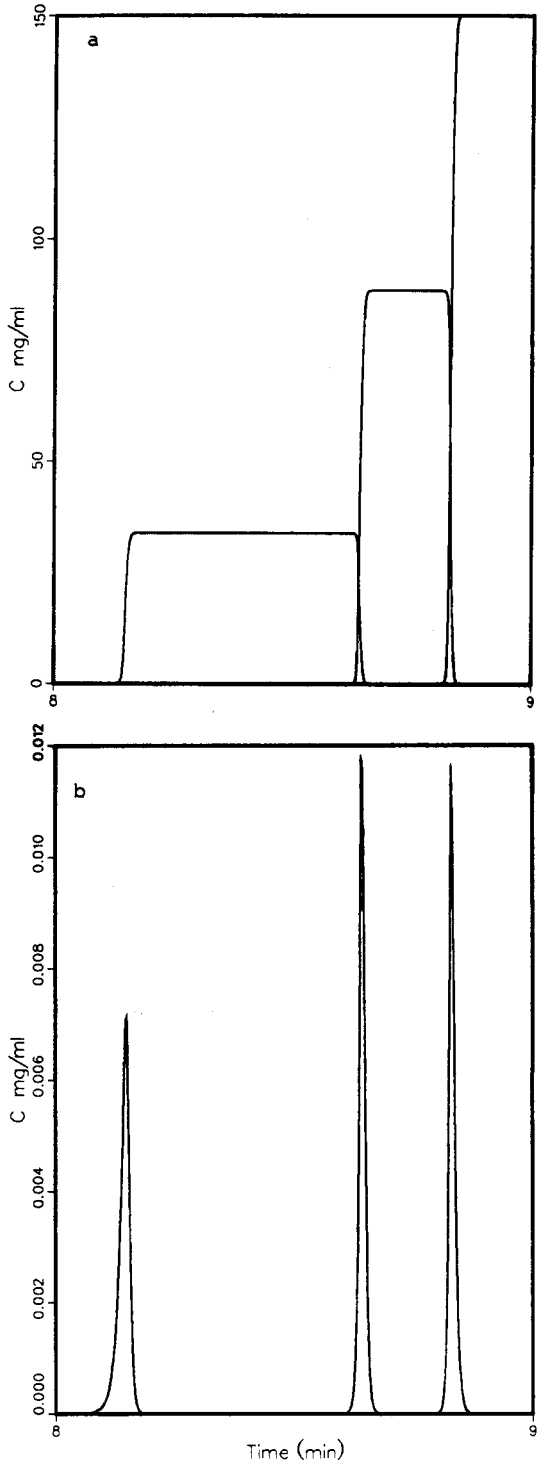

Figure 11. Calculated profile illustrating trace enrichment in displacement chromatography[24]: (a) displacement train, 100 mg of first and second components, displacer concentration 150 mg/mL, column efficiency 16700 theoretical plates; (b) profiles of impurities eluted in a displacement train. Concentration of each impurity: 5 ppm.

earlier desorption of the less strongly adsorbed first component and causes it to elute in the mobile phase as a more concentrated band. The maximum concentration of the first band is 0.17 mM in the 1/3 mixture containing 0.105 μmol, while it is 0.08 mM under noncompetitive conditions[11] (feed concentration, 10 mM). Because the retention mechanism is well accounted for by the bilangmuir model, the comparison of calculated and experimental results has been excellent for all pairs of enantiomers studied on BSA.[10,11] These results cannot be generalized to the separation of enantiomers on other chiral selective adsorbents.[18]

A second comparison between theory and experiment for a binary 3/1 mixture is the separation of 2-phenylethanol and 3-phenylpropanol shown in Figure 7.[9,19] Comparison of the insert showing profiles calculated with a noncompetitive isotherm and the experimental data in the main figure illustrates how competitive interactions between the mixture components affect the elution profiles. The later eluting band is dragged forward into the first component band. At the time when the tail of the first component band ends, a concentration plateau appears in the second component profile. This phenomenon is called the tag-along effect. It is strong when the first component is in greater amounts. However, a slight tag-along effect for a 1/3 mixture can be seen in Figure 3 where an inflection appears in the profile at 5 min. Physically, this effect can be explained by the reduction in the fraction of molecules of the second component adsorbed at equilibrium in the presence of an increasingly large excess of the first component. Even though the second component is more strongly adsorbed, because of the competition only a reduced fraction of its molecules are adsorbed, and thus it elutes faster than alone. This results in a poor separation at high concentrations.

As a last example, we qualitatively compare the separation of *cis*- and *trans*-androsterone.[6] As in the previous examples, the pure component adsorption data of both isomers were fitted to a Langmuir isotherm. Qualitative agreement was obtained between experimental and calculated single-component elution profiles. However, the column saturation capacities differed by 30%, which is explained by the very different geometrical structures of these two isomers. When the column saturation capacities are different, the Langmuir isotherm is no longer thermodynamically consistent. A more complex competitive isotherm model should be used. The experimental adsorption data fit well with this latter model.[6]

Figure 8 insert illustrates the experimental elution profiles of *cis*- and *trans*-androsterone at two different sample sizes. The small analytical size shows the trans isomer eluting first, before the cis isomer. At the large sample size, the peak maximum of the cis isomer elutes first and the trans second. Band profiles calculated using the proper competitive isotherm model under the same conditions as the experiment are shown in Figure 8. At the small sample size the peak maximum of the cis isomer elutes at 550 s and the trans isomer at 510 s. At the large sample size the peak maximum of the cis isomer elutes at 400 s and the trans isomer at 460 s. This complex phenomenon of elution order reversal at high concentrations cannot be accounted for by simple models (eq 7 or 8).

More Complex Phenomena

The separation of binary mixtures in normal-phase chromatography using a solvent with a strongly retained

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additive as the mobile phase is a three-component problem, because competition for adsorption takes place between the mobile-phase additive and the two components of the mixture. We have shown in Figure 5 how complex band profiles arise for the single-component sample. For binary mixtures, competition results in most complex chromatograms, as illustrated in Figures 9a,b which correspond to 4/1 and 1/4 mixtures, respectively. Effects which are the converse of the displacement and tag-along effects appear on the rear and the front boundary, respectively. It is often difficult to realize that such complex overlapping band profiles can be observed when the isotherms for both solutes and for the additive follow the simple competitive Langmuir model.[20] Because of their complexity, these profiles are often mistaken for profiles due to S-shaped isotherms (cf. Figure 2a).

Figure 10 compares qualitatively calculated and experimental band profiles at five different sample sizes for a 1/1 mixture. The experimental profiles (Figure 10) show stronger competition than the theoretical ones (Figure 10, inset), but otherwise the qualitative comparison is good. The interaction between the two bands is complex. As the sample size increases, both the front and rear boundaries move apart.[21]

There are few analytical applications of nonlinear chromatography. The most important is the concentration of trace components by displacement chromatography. In this process, after completion of a large sample injection, a stream of a relatively concentrated solution of a displacer is pumped into the column. The displacer is a component which is more strongly adsorbed than any component of the mixture. The displacer front pushes the sample band in front of it, causing separation of the component bands. It is possible to show[22,23] that, after a certain time, a train of rectangularly shaped bands is formed, each component being separated from its neighbors (Figure 11a). These bands are eluted in increasing order of the initial slope of the isotherm. The height of a band depends only on the adsorption isotherms of the component and the displacer and on the displacer concentration. The width of each band is proportional to the amount of each component in the sample. Axial dispersion and mass-transfer resistances cause the boundaries between the bands to be slightly curved.

Thus, it follows that, when trace components elute between these rectangular bands, they are considerably enriched as they are squeezed between the sharp boundaries of the adjacent main components (Figure 11b). The enrichment factor depends on the column efficiency. This phenomenon can be used for trace analysis to increase the detection limit of certain species.[24] It has been used in displacement LC/MS, where trace impurities have been detected at the boundaries between major component bands, permitting their identification in peptide mapping of recombinant proteins.[25]

Conclusion

Our results demonstrate that chromatographic band profiles at high concentrations are essentially controlled by thermodynamics, that is, by the competitive equilibrium isotherms of the mixture components. In almost all cases, the contributions of the finite rate of mass transfer in the column and the axial dispersion are limited to the smoothing of these profiles. This effect can be accurately accounted for by a dispersion term simply related to the column height equivalent to a theoretical plate (see eqs 1 and 3).

Given the competitive isotherms, it is possible to accurately predict the individual component band profiles. Accordingly, using a Simplex procedure, we can determine the optimum parameters of a chromatographic separation for maximum production rate or minimum cost, with or without constraints, e.g., a minimum recovery yield or a maximum inlet pressure.[26,27] Thus, the complete design of preparative chromatography units becomes possible, provided competitive isotherms are measured.

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