Solvent modulation in liquid chromatography: General concept and theory

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vantage of the dual column approach is that when problems or questions are encountered relative to the analytical system, they are more easily diagnosed. It is seldom that both columns fail or experience the same problems at the same time.

ACKNOWLEDGMENT

We thank D. W. Krogmann for the gift of the *Cyanobacterium anabaena* PCC 7120 extract and helpful discussions regarding the composition of the sample. We also thank D. Culler and R. W. Stringham for their assistance in the preparative chromatography of the sample.

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Solvent Modulation in Liquid Chromatography: Experimental Verification and Comparison with Conventional Premixed Mobile Phases

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Experimental verification of the theory of solute retention is performed under the condition of solvent modulation. These results are compared to those obtained by using conventional premixed mobile-phase systems. Seven test solutes are used to examine a range of chemical interactions for strength and selectivity changes of the mobile phase: phenol, diphenylamine, nitrobenzene, toluene, naphthalene, anthracene, and pyrene. The mobile phases used are 100% methanol and 75% methanol–25% water for the solvent strength studies and 75% methanol–25% water and 58% acetonitrile–42% water for the solvent selectivity studies. Experimental results utilizing premixed mobile phases show a systematic deviation as great as −16% for the solvent strength studies and +60% for the solvent selectivity studies from the theoretically predicted solute retention. However, a random error of approximately ±10% occurs when utilizing solvent modulation for either solvent strength or selectivity. From the results and observations presented, it is concluded that solvent modulation offers a simple, versatile, and accurately modeled means to control and predict solute retention in liquid chromatography.

INTRODUCTION

In liquid chromatography, the retention characteristics of a solute are altered by modifying the composition of the mobile phase. Because of nonideal solute–solvent and solvent–solvent interactions, however, solute retention is not always a simple predictable function of the mobile-phase composition. Recently, solvent modulation was introduced as a practical and versatile alternative to premixed mobile phases (*1*). In solvent modulation, the individual components that comprise the mobile phase are never physically mixed. Instead, solvent zones of predetermined composition are introduced in either a repeating or random pattern along the chromatographic column. Because the solvent zones are spatially and temporally separated from one another, solute retention should be controlled independently within each solvent zone. Consequently, solute retention should be more accurately and precisely predicted by using solvent modulation (*1*). The goal of this work is to verify the theoretical model of solute retention and to demonstrate the versatility of solvent modulation compared to conventional premixed mobile phases in liquid chromatography.

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THEORY OF SOLUTE RETENTION

Premixed Mobile-Phase Model. Many models, including theoretical as well as empirical and semiempirical models, have been developed to describe the effect of mobile-phase composition on solute retention (*2–19*). In general, these models rely on an accurate description or understanding of the chemical environment of the solvent system, which is particularly difficult when a mixed solvent system is used. The chemical interactions in mixed solvents can be qualitatively predicted if the system behaves in a ideal manner, where the average interaction between two differing species (A–B) is similar to the interactions between the individual components (A–A and B–B). Alternatively, ideal behavior is expected if one component of the solvent system is at infinite dilution. However, neither of these conditions is met regarding the mobile-phase chemical environment, especially in reversed-phase liquid chromatography where polar solvent systems are used. Recently, more rigorous treatments of molecular interactions in liquid chromatography have been developed that are based on a lattice-type model. These models include the solvophobic theory of Horvath and co-workers (*3*), the statistical thermodynamic theories of Martire and Boehm (*4*) and of Dill et al. (*5*, *6*), and the solubility parameter theory (*7–9*) adapted by Tijssen and co-workers (*10–12*). Each of these

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Table I. Solubility Parameters of Common Solvents for Reversed-Phase Liquid Chromatography ($MPa^{1/2}$) (8)

solvent	δ	δ_d	δ_p	δ_h
acetonitrile	24.4	15.3	18.0	6.1
methanol	29.6	15.1	12.3	22.3
water	47.8	15.5	16.0	42.3

theories allows specific chemical interactions to be considered and yields qualitatively similar conclusions. However, because of the abundance of relevant physical constants and supporting data, the solubility parameter theory was chosen for this study. Therefore, a brief discussion of this theory and its application to liquid chromatography follows.

The solubility parameter theory, which is a thermodynamic model based on regular solution theory, defines the solubility parameter, δ, of a pure liquid as the square root of the cohesive energy density (7, 8). This theory has been extended to polar solvents by including the contributions of dispersion (δ_d), permanent dipole orientation (δ_o), and dipole induction (δ_{ind}) as well as hydrogen-bonding interactions of acidic (δ_a) and basic (δ_b) nature. In general, these individual components are assumed to be independent, and the overall solubility parameter is represented by the following summation (12):

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad (1)$$

where the polar component is defined as $\delta_p^2 = \delta_o^2 + 2\delta_{ind}\delta_d$, and the hydrogen bonding component is $\delta_h^2 = 2\delta_a\delta_b$.

In Table I, the total solubility parameter and the individual component values are listed for three common solvents used in reversed-phase liquid chromatography. The total solubility parameter (δ) increases as the polarity of the solvent increases, but the values of the individual components differ notably. The dispersive component (δ_d) is comparable among the three solvents; however, acetonitrile has the greater polar component (δ_p) due to a strong permanent dipole. Hydrogen-bonding interactions (δ_h) are dominant in water, moderate in methanol, and minor in acetonitrile. Thus, it is possible to use the solubility parameter as a direct measure of the overall polarity or strength of the solvent. Further, because the solubility parameter is a function of the general types of intermolecular interactions, the magnitude of the individual contributions defines the selective nature of the solvent.

The solubility parameter theory may be extended to mixed solvent systems, where the total solubility parameter is equal to the arithmetic sum of the individual solubility parameters (δ_j) weighted according to their volume fractions (ϕ_j) in the mixture.

$$\delta = \sum \phi_j \delta_j \quad (2)$$

Thus, it is possible to create two mixed solvent systems having the same solvent strength while their predominant chemical interactions differ drastically. In this paper, the term solvent strength will be used to refer to the magnitude of the total solubility parameter and the term solvent selectivity will refer to the magnitude of the individual components contributing to the total solubility parameter.

The solubility parameter theory can be used to predict solute retention in liquid chromatography for either a pure or a mixed solvent system. In this development, the activity coefficient (γ) for solute (i) dissolved in solvent (j) is related to the individual solubility parameters by the following equation (9):

$$RT \ln \gamma_i = V_i(\delta_i - \delta_j)^2 \quad (3)$$

where V_i is the molar volume of the solute and is presumed to be identical with the solvent at infinite dilution, R is the gas constant, and T is the absolute temperature. In turn, the distribution constant of the solute (K_i) is mathematically related to the ratio of its activity coefficient in the mobile and stationary phases (9, 12). Thus, the solute capacity factor (k_i) is expressed by the following equation:

$$\ln k_i = \ln\left(\frac{K_i}{\beta}\right) = \frac{V_i}{RT}[(\delta_i - \delta_M)^2 - (\delta_i - \delta_S)^2] - \ln \beta \quad (4)$$

As a result, solute retention is a function of the solubility parameters of the solute (δ_i), the mobile phase (δ_M), and the stationary phase (δ_S), as well as the volume ratio of the mobile and stationary phases (β).

If the solubility parameter model correctly accounts for all solute–solvent and solvent–solvent interactions, then a quadratic relationship should exist between the logarithm of the capacity factor and the volume fraction of one component in the mobile phase. This quadratic relationship is obtained by combining eqs 2 and 4 for a simple binary mobile-phase mixture of components A and B and expressing the result as a function of the volume fraction (ϕ_B):

$$\ln k_i = a\phi_B^2 + b\phi_B + c \quad (5)$$

where the coefficients are equivalent to

$$a = \frac{V_i}{RT}(\delta_B - \delta_A)^2$$

$$b = \frac{V_i}{RT}[(\delta_i - \delta_B)^2 - (\delta_i - \delta_A)^2 - (\delta_B - \delta_A)^2]$$

$$c = \frac{V_i}{RT}[(\delta_i - \delta_A)^2 - (\delta_i - \delta_S)^2] - \ln \beta$$

Experimental results have verified that such a quadratic relationship in solute retention exists when using binary or ternary solvent mixtures (5, 6, 12, 15, 16).

Because the rigorous theoretical prediction of solute retention requires an accurate estimation of five variables (δ_i, δ_M, δ_S, V_i, and β), eq 5 is commonly used in a semiempirical manner. As a result, at least three preliminary measurements are required to estimate the coefficients in eq 5. However, this equation can be expressed in a more convenient linear form by subtracting the solute capacity factor (k_i°) when the volume fraction of component B is zero ($\phi_B = 0$). Upon rearrangement, the linear form is shown in the following expression:

$$\frac{1}{\phi_B} \ln\left(\frac{k_i}{k_i^\circ}\right) = a\phi_B + b \quad (6)$$

where $\ln k_i^\circ$ is equal to coefficient c in eq 5. Consequently, a linear relationship should be obtained from eq 6 by plotting $(1/\phi_B) \ln (k_i/k_i^\circ)$ as a function of the volume fraction of the organic modifier (ϕ_B) in the mobile phase. It is noteworthy that this equation is mathematically equivalent to the more rigorous form developed by Dill et al. (5, 6), wherein binary interaction parameters are substituted for the unitary solubility parameters.

Several shortcomings of the solubility parameter treatment of solute retention may lead to deviations in any of the above expressions. First, eq 2 assumes that solvent–solvent interactions are independent; thus, each solute is assumed to interact independently with each mobile-phase component. Second, the solute and solvents are assumed to have equal molar volumes, and no change in volume occurs upon mixing. Third, eq 3 presumes that binary interaction parameters may be approximated by using the geometric mean of the individual interaction parameters. As a result of the geometric mean approximation, the activity coefficient for a solute is always positive. This implies that the enthalpy of mixing

(ΔH^M) is also positive so that it is always unfavorable for mixing to occur between solute–solvent or solvent–solvent. In addition, it is assumed that the solute and the mobile-phase components are randomly mixed. This neglects any preferential orientation or clustering due to strong intermolecular interactions, such as proton donor–acceptor interactions. Similarly, solute interactions with the stationary phase are presumed to be isotropic and independent of the mobile-phase composition. Finally, the volume ratio of the mobile and stationary phases (β) is assumed constant for an individual solute in the compositional ranges examined. As a result of these assumptions, the solubility parameter model may not always adequately represent solute–solvent and solvent–solvent interactions in solvent mixtures.

Solvent Modulation Model. Solvent modulation has been shown to offer a practical alternative to conventional premixed mobile phases in liquid chromatography (*1*). In solvent modulation, solvent zones of differing composition and time duration are sequentially introduced to the chromatographic column in a known random or repeating pattern. Because the individual solvents are never physically mixed, any nonideal intermolecular interactions that may exist within a premixed solvent system are avoided. Thus, solute retention may be controlled independently within each solvent zone and is a function of several experimental variables. The number and composition of the individual solvents are selected to provide differential retention for the solutes of interest. The length of each solvent zone in the modulation sequence is defined in terms of the solvent proportion and repetition rate (*1*). The proportion represents the fractional length of each solvent zone within one cycle of the modulation sequence, which is analogous to the compositional ratio in a premixed solvent system. The repetition rate represents the real or integer number of modulation cycles that may reside simultaneously on the chromatographic column. The selected solvent sequence may be repeated without alteration, which is analogous to isocratic elution with premixed mobile phases. Alternatively, the proportion or repetition rate may be changed continuously or discontinuously during the analysis, comparable to linear or stepwise gradient elution. Thus, solvent modulation offers an extremely versatile means to control both solvent strength and selectivity in liquid chromatography.

The time (t_j) that the solute spends in a solvent zone of length x_j is determined by

$$t_j = \frac{x_j}{u}\left(\frac{1 + k_{ij}}{k_{ij}}\right) \tag{7}$$

where k_{ij} is the individual capacity factor of solute i in solvent zone j and u is the average linear velocity of the modulated mobile phase in a column of length L and void time t_0. If the solute undergoes independent interactions within each solvent zone, then the overall retention time is a sum of the individual retention times. As a result, the overall capacity factor (k_i) under the conditions of solvent modulation is given by

$$k_i = \sum_{j=0}^{n}\frac{t_j}{t_0} - 1 = \sum_{j=0}^{n}\frac{x_j\left(\frac{1 + k_{ij}}{k_{ij}}\right)}{L} - 1 \tag{8}$$

The limit of the summing index (n) is equal to the number of solvent zones encountered by the solute during the separation. If this limit has a noninteger value, the summation in eq 8 is performed in the normal manner for solvent segments 0 to n, and the remainder is treated as a fractional multiplier for the last $n + 1$ solvent segment (*1*).

The fundamental assumption underlying the theory of solvent modulation is that solute retention is controlled independently within each solvent zone. It is, therefore, necessary that the solvent segments be of known sequence and length and not be intermixed at the boundary. Another assumption implicit in this model is that rapid mass transfer exists at the interface of the mobile and stationary phases. Therefore, the column is assumed to achieve steady-state conditions and behavior very quickly after each change in solvent composition. This assumption is reasonable when solvents of similar composition are modulated but may not be valid for solvents of significantly different strength. Finally, it is assumed that the phase ratio and the separation mechanism, such as partition or adsorption, do not change with the solvent composition.

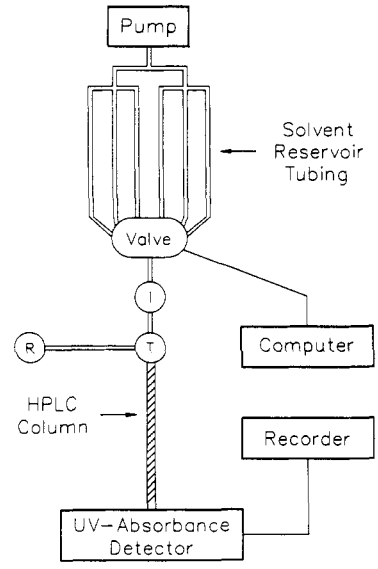

Figure 1. Schematic diagram of experimental system. V = solvent selection valve; I = sample injection valve; T = effluent splitter tee; R = restrictor or metering valve.

In this paper, we present the results of a systematic study designed to examine the validity of the theoretical model developed for the solvent modulation technique. Solute retention is measured under various modulation sequences for changes in both solvent strength and selectivity. The retention measured from each of these experiments is compared with that predicted by eq 8. Further, solute retention is measured using analogous premixed mobile phases and comparisons are made between the accuracy of the two methodologies.

EXPERIMENTAL SECTION

The chromatographic system used for this research is shown schematically in Figure 1 and is described in detail below.

Chromatographic System. A single-piston reciprocating pump (Model 114M, Beckman Instruments) is used in the constant-pressure mode for solvent delivery. Six sections of passivated stainless steel tubing (3.18 mm o.d., 2.16 mm i.d., 2.00 m length) contain the individual solvents and are connected to a six-port solvent selection valve (Model ECSD6PXN6, Valco Instruments Co.). The solvent selection valve is controlled through software written in the BASIC programming language that is executed on an IBM XT computer.

Sample introduction is achieved by using a 1.0-μL injection valve (Model ECI4W1., Valco Instruments Co.), after which the effluent stream is split (S.R. 30:1) and applied to the chromatographic column, producing a nominal volumetric flow rate of 1 μL/min. The microcolumn utilized in this study is prepared from fused-silica capillary tubing (200 μm i.d., 25 cm length, Hewlett-Packard), which is packed with an octadecylsilica material (Spheri-5 RP-18, 5 μm, Applied Biosystems), as described previously (*20*).

Solute detection is accomplished by using a variable-wavelength UV–vis absorbance detector (λ_{abs} = 250 nm, 0.010 AUFS, Model Uvidec 100-V, Japan Spectroscopic Co.) with a modified 25-nL capillary flow cell (80 μm i.d., 0.5 cm length). The signal from

the detector is shown on a chart recorder (Model 585, Linear Instruments).

Materials and Methods. Reagent- or technical-grade phenol, diphenylamine, nitrobenzene, toluene, naphthalene, anthracene, and pyrene are obtained from MCB Manufacturing Chemists and are used without further purification. Organic solvents are high-purity, distilled-in-glass grade (Baxter Healthcare, Burdick & Jackson Division); water is deionized and double distilled in glass (Model MP-3A, Corning Glass Works).

RESULTS AND DISCUSSION

Seven test solutes were selected for their differing chemical properties. A series of polynuclear aromatic compounds was chosen to represent dispersion and induced dipole interactions: toluene, naphthalene, anthracene, and pyrene. Permanent dipole interactions were examined with nitrobenzene as the test solute. Proton donor and acceptor interactions were probed by using phenol and diphenylamine, respectively. Two chromatographic studies were performed: the first study investigated changes in the mobile-phase strength, and the second investigated changes in the mobile-phase selectivity. In each study, two solvent systems were chosen to examine solute–solvent and solvent–solvent interactions in both premixed and solvent-modulated mobile phases. Under these conditions, the retention of the seven solutes was measured and compared with the theoretically predicted values.

Solvent Strength Study. The strength of the mobile phase determines the rate of solute migration along the chromatographic column. In reversed-phase liquid chromatography, the strength of the mobile phase is controlled by adjusting the amount of organic modifier present in the solvent system; the stronger or less polar the solvent, the less the solute is retained. In this study, the individual solvent systems utilized were 75% methanol–25% water (A) and 100% methanol (B), having solubility parameters equal to 34.2 and 29.6 $MPa^{1/2}$, respectively. Shown in Figure 2 are the chromatograms obtained by using these two mobile phases for the separation of the test solutes. On comparison of these separations, the difference in solvent strength is apparent as the analysis times differ by approximately an order of magnitude. However, the general elution order of the solutes does not change because the selective nature of both mobile phases is similar.

Premixed Mobile Phases. In the formulation of eq 2, intermolecular interactions are assumed to be independent in a mixed solvent system. If this assumption is correct, then a quadratic relationship is expected between the logarithm of the solute capacity factor and the volume fraction of each component in the mobile phase (eq 5). If the assumption is incorrect, the direction and magnitude of deviation from the quadratic relationship are an indication of the chemical environment that exists during the separation. To determine if this premise is correct for significant changes in solvent strength, the two methanol–water solvent systems (A and B) are mixed in various ratios and the capacity factors of the seven solutes are measured as a function of the mobile-phase composition. These experimental results are summarized in the top diagram of Figure 3, where the solute capacity factor is shown on a logarithmic scale as a function of the volume percent methanol in the mobile phase. Experimentally, all seven solutes show slightly nonlinear retention with maximum deviation at a mobile-phase composition of approximately 87.5% methanol. In addition, the general slope for the basic solute, diphenylamine, is substantially different than for the other test solutes. This difference in slope may arise because of a mixed retention mechanism involving both partition in the octadecyl stationary phase and adsorption on residual acidic silanol groups. This conclusion is consistent with the low bonding density of octadecylsilane on this packing material (*21*).

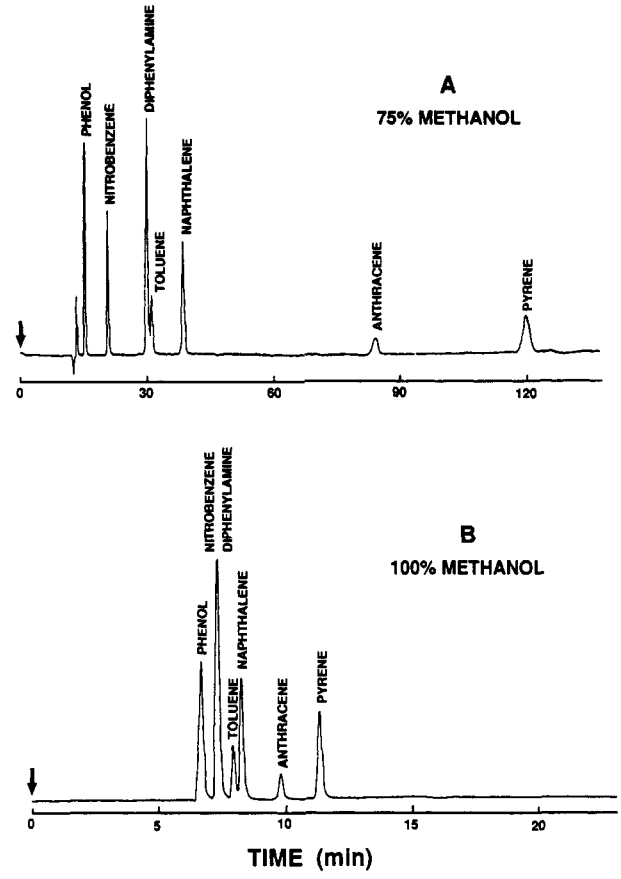

Figure 2. Chromatograms of the seven test solutes in individual mobile phases chosen for the solvent strength studies. Column: 200 μm i.d., 25 cm length, packed with Spheri-5 RP-18. Mobile phase: 75% methanol–25% water (A), 100% methanol (B), 1 μL/min. Detector: UV–vis absorbance, 250 nm, 0.010 AUFS.

These experimental capacity factors are next compared with those predicted by theory. To predict the solute capacity factor as a function of the mobile-phase composition, which is based on measurements in the two solvent systems (A and B), a linear expression is required. Equation 6 predicts that a linear relationship is obtained between the solute capacity factor and the volume fraction of methanol in the mobile phase if $(1/\phi_{MeOH})\ \ln\ (k_i/k_i^o)$ is plotted as a function of ϕ_{MeOH}. Accordingly, an accurate estimate of the solute capacity factor at 0% methanol (k_i^o) is required. Because a nearly linear dependence of $\ln k_i$ on the volume fraction of methanol has been shown (Figure 3, top) and discussed previously (*5*, *6*), k_i^o was estimated by linear extrapolation of the logarithm of the solute capacity factors using the two mobile-phase compositions 75% methanol–25% water (A) and 100% methanol (B). The theoretical results predicted by using eq 6 are summarized in the center diagram of Figure 3. As expected theoretically, a constant value of $(1/\phi_{MeOH})\ \ln\ (k_i/k_i^o)$ is obtained for each solute because of the manner in which k_i^o was determined. In the bottom diagram of Figure 3, the relative error between the measured capacity factor and that determined by using eq 6, which is calculated as $(k_{Exp} - k_{Theory})/k_{Theory}$, is shown as a function of the volume percent of methanol. All solutes except phenol and diphenylamine display a systematic negative deviation in retention with decreasing solvent strength. This negative deviation indicates that the actual strength of the premixed mobile phase is greater (δ_M is smaller) than predicted by simple linear additivity using eq 2. This conclusion is in qualitative agreement with Katz and co-workers (*17*, *18*), who noted that aggregation in methanol–water mixtures may lead to a substantial increase in solvent strength for reversed-phase separations. The solutes

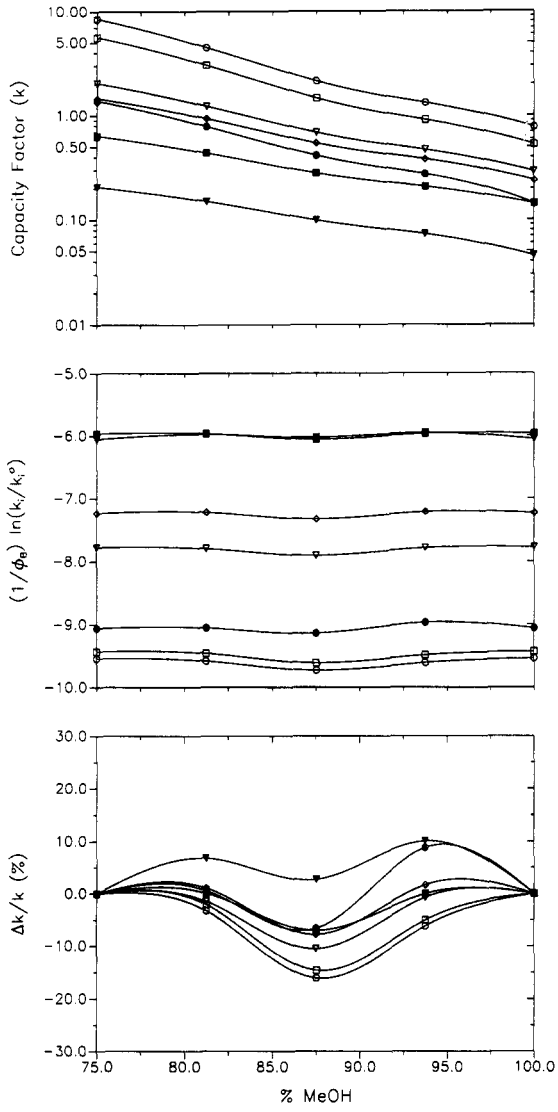

Figure 3. Experimentally measured solute retention for premixed mobile phases varying in solvent strength. The capacity factor is presented as a logarithmic function of composition (top) and according to eq 6 (center). The relative error between the experimental and theoretical solute retention based on the solubility parameter model is calculated as $(k_{Exp} - k_{Theory})/k_{Theory}$ (bottom). Chromatographic conditions as described in Figure 2. Solutes: phenol (▼), nitrobenzene (■), diphenylamine (●), toluene (◇), naphthalene (▽), anthracene (□), pyrene (○).

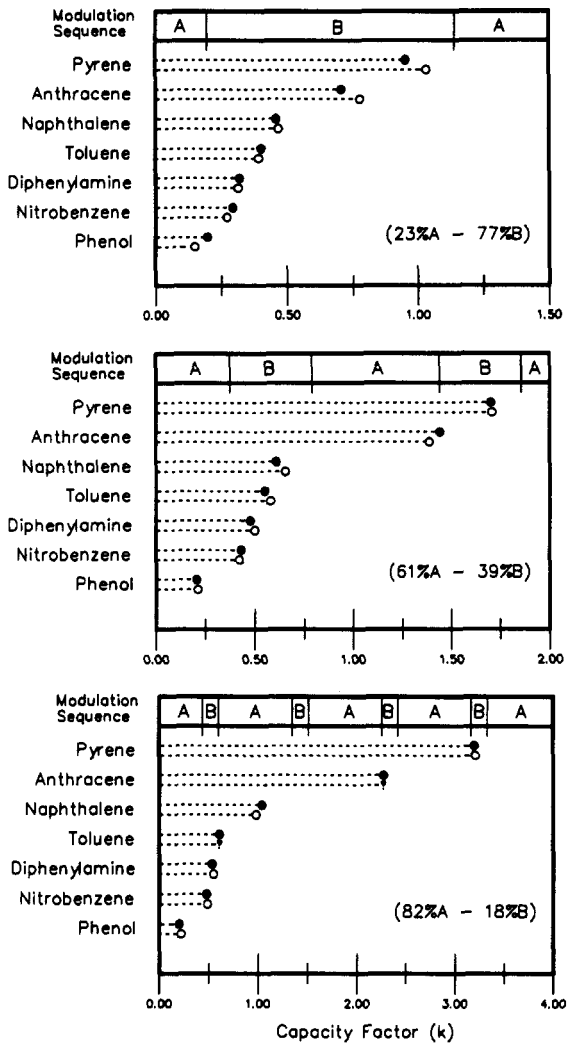

Figure 4. Solute retention in solvent-modulated mobile phases for varying solvent proportion and constant repetition rate (RR = 1). At the top of each figure, the order and relative length of the solvent zones used in the separation are depicted, and at the bottom of each figure the range and scale of the overall capacity factors are listed. The open circles (○) represent the experimentally measured capacity factors and the filled circles (●) are theoretically predicted from eq 8. Chromatographic conditions as described in Figure 2.

phenol and diphenylamine, which may not be separated by a simple partition mechanism, show positive deviation in retention. This disparity may result from proton donor–acceptor interactions with residual silanol groups present in the stationary phase. The calculations of Katz and co-workers (*17*, *18*) suggest that the concentration of free water, which may displace the adsorbed solutes from the silanol groups, is less than expected due to methanol–water aggregation. In addition to the assumption of linear additivity given by eq 2, other assumptions may contribute to the observed deviation in retention for all solutes. For example, the value of k_i° is estimated linearly from a rather limited range of mobile-phase compositions. Consequently, the estimated value of k_i° is larger for phenol and smaller for the remaining solutes than that obtained by nonlinear extrapolation of a more extensive data set.

In summary, solutes that are retained primarily by a reversed-phase partition process show a systematic negative deviation in capacity factor for premixed mobile phases differing in solvent strength. Such solutes exhibit maximum deviation in retention at 87.5% methanol–12.5% water, where errors are as greater as −16%. For solutes that are retained by a mixed partition–adsorption mechanism, the situation is more complex: phenol shows a systematic positive deviation in capacity factor, whereas diphenylamine shows both positive and negative deviation.

Solvent Modulation. The solvent modulation technique is evaluated by comparing the experimentally determined solute capacity factors with those predicted by eq 8 using the two individual solvent systems 75% methanol–25% water (A) and 100% methanol (B). The effects of varying the solvent proportion and the repetition rate within a solvent modulation sequence are examined separately. These results are summarized in Figures 4 and 5, where the capacity factor is shown as a linear function of the modulation sequence.

Figure 4 illustrates the results obtained by varying the solvent proportion while maintaining a constant repetition rate of approximately one. For a solvent proportion of 23% A–77% B (Figure 4, top), all solutes elute from the column within the first two solvent zones with the most retained solute, pyrene, having a capacity factor just greater than one. As the proportion of the weaker mobile phase is increased to 61% A–39% B (Figure 4, center), longer overall solute re-

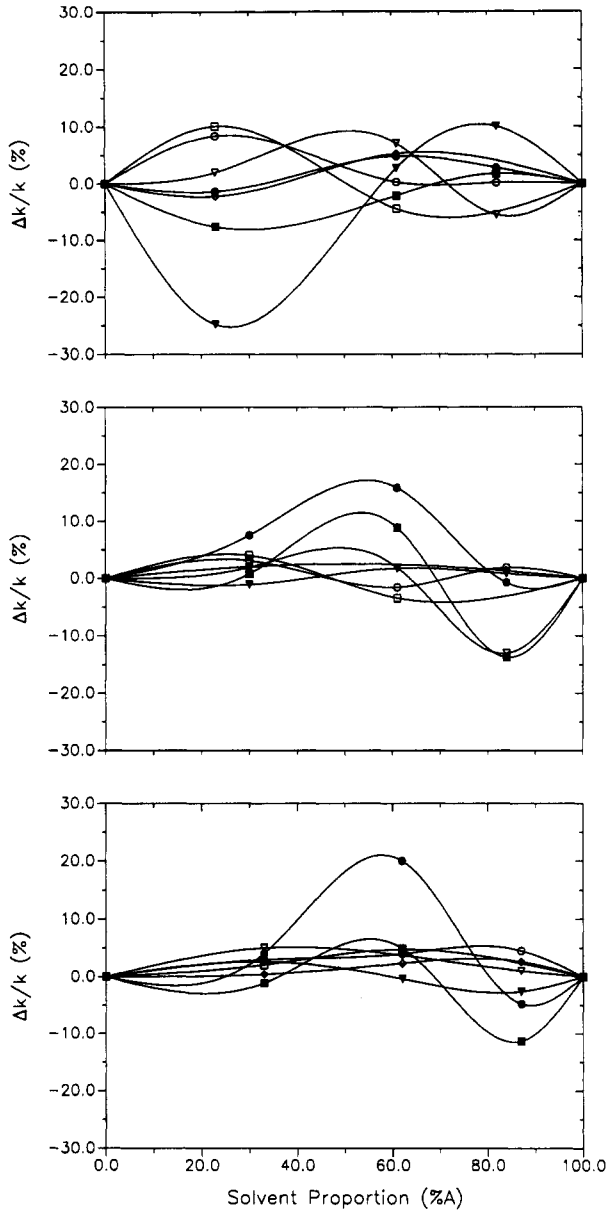

Figure 5. Relative error between the measured capacity factors and those predicted by eq 8 for repetition rates nominally equal to one (top), two (center), and three (bottom). Chromatographic conditions as described in Figure 2. Solutes: phenol (▼), nitrobenzene (■), diphenylamine (●), toluene (◇), naphthalene (▽), anthracene (□), pyrene (○).

tention is observed. This increase in solute retention is especially noticeable for anthracene and pyrene, which show a dramatic increase in capacity factor relative to the other five solutes because of the increase in the number of solvent zones encountered. Likewise, with the final increase in the fractional length of the weak solvent to 82% A–18% B (Figure 4, bottom), a corresponding increase occurs in the overall capacity factor range where pyrene, still the most retained solute, has a capacity factor just greater than three. To facilitate direct comparison of the solvent modulation technique with premixed mobile phases, the relative error between the measured capacity factor and that determined by using eq 8 is calculated as $(k_{Exp} - k_{Theory})/k_{Theory}$. As shown in the top diagram of Figure 5, all seven solutes display a statistically random deviation in retention with solvent strength, compared to the systematic deviations observed using premixed mobile phases (Figure 3, bottom). The deviation between the measured and the predicted capacity factors has a mean value of +0.5% with a range of ±10% for all solutes. Consequently, good agreement is observed between the measured and predicted capacity factors for all solutes, regardless of the nature of chemical interactions.

The results obtained by increasing the repetition rate of the solvent modulation sequence are summarized in Figure 5, where the relative error between the measured capacity factor and that determined by eq 8 is examined as a function of solvent proportion. In the center and bottom diagrams of Figure 5, the repetition rate is increased to values of approximately two and three, respectively. In all cases, the relative error still fluctuates randomly about a mean value of approximately zero, but the fluctuations appear to decrease as the repetition rate increases.

These observations give interesting insight into the nature of mass-transport processes in liquid chromatography using premixed mobile phases and solvent modulation. The octadecylsilica stationary phase is commonly believed to consist of a nonpolar surface layer that equilibrates rapidly with the mobile-phase components and other strongly interacting (silanols and Lewis acids) or less accessible sites that equilibrate more slowly. In addition, the physical structure or orientation of the nonpolar octadecyl groups may be influenced by the mobile-phase composition (*22, 23*). Because all of these environments must be completely equilibrated when premixed mobile phases are used, many column volumes of solvent are necessary after a change in composition or upon gradient elution (*2, 22–26*). In solvent modulation, however, the volume of each solvent zone is typically less than the column volume and decreases with increasing repetition rate. Consequently, it is unlikely that the mobile-phase components are exchanged to any significant extent at the slowly equilibrating sites in the stationary phase. Moreover, it is unlikely that the physical structure of the stationary phase changes to an appreciable extent. If partial exchange or rearrangement is achieved within the solvent zone, then solute retention would be expected to deviate from theoretical predictions as repetition rate is increased. Because the deviation is observed experimentally to decrease with repetition rate (Figure 5), it may be inferred that the stationary phase attains a steady-state (nonequilibrium) composition and structure during solvent modulation. This conclusion is signficant because it implies that the proportion and repetition rate, which are used to control retention in solvent modulation, may be altered rapidly between successive analyses, whereas premixed mobile phases require much longer equilibration times. An exception to the general behavior described above is observed for the solute diphenylamine, which shows a relatively constant deviation in capacity factor with increasing repetition rate. This difference may result from strong proton donor–acceptor interactions between diphenylamine and residual silanol groups in the stationary phase. Because an adsorption process often has slower mass-transport dynamics than a partition process, the mass transport of diphenylamine is expected to be slower than that of the other six solutes. Nevertheless, good agreement between the theoretically predicted and experimentally measured capacity factors for all solutes is observed under the conditions of solvent modulation. In general, these results are very encouraging because the strength of the solvent systems being modulated in these experiments alters solute retention by approximately an order of magnitude.

Solvent Selectivity Study. The selectivity of the mobile phase generally determines the ability to discriminate between solutes based on the type of intermolecular interactions, for example, dispersion, dipole induction or orientation, or proton donor–acceptor interactions. In reversed-phase liquid chromatography, the selectivity is controlled by altering the type of organic modifier present in the mobile phase. In this study, the individual solvent systems are 75% methanol–25% water

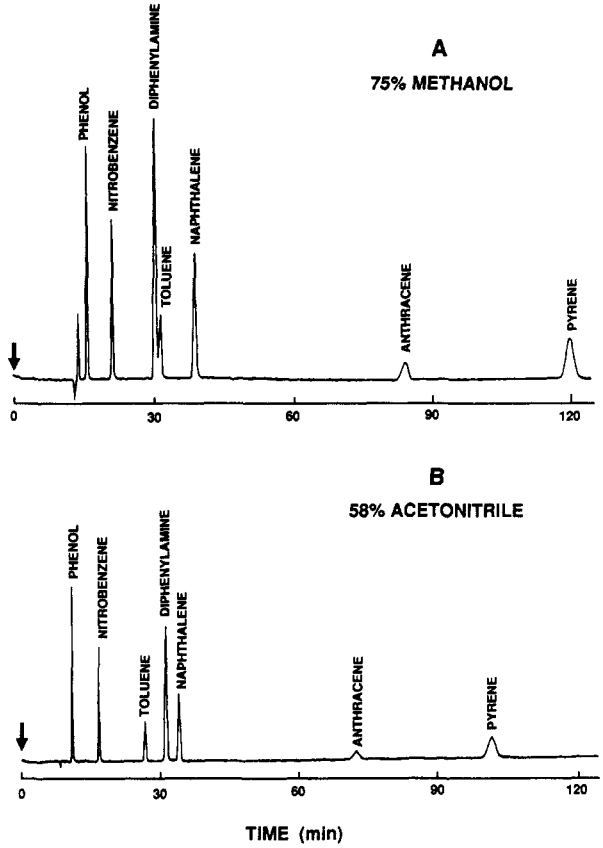

Figure 6. Chromatograms of the seven test solutes in individual mobile phases chosen for the solvent selectivity studies. Column: 200 μm i.d., 25 cm length, packed with Spheri-5 RP-18. Mobile phase: 75% methanol–25% water (A), 58% acetonitrile–42% water (B), 1 μL/min. Detector: UV–vis absorbance, 250 nm, 0.010 AUFS.

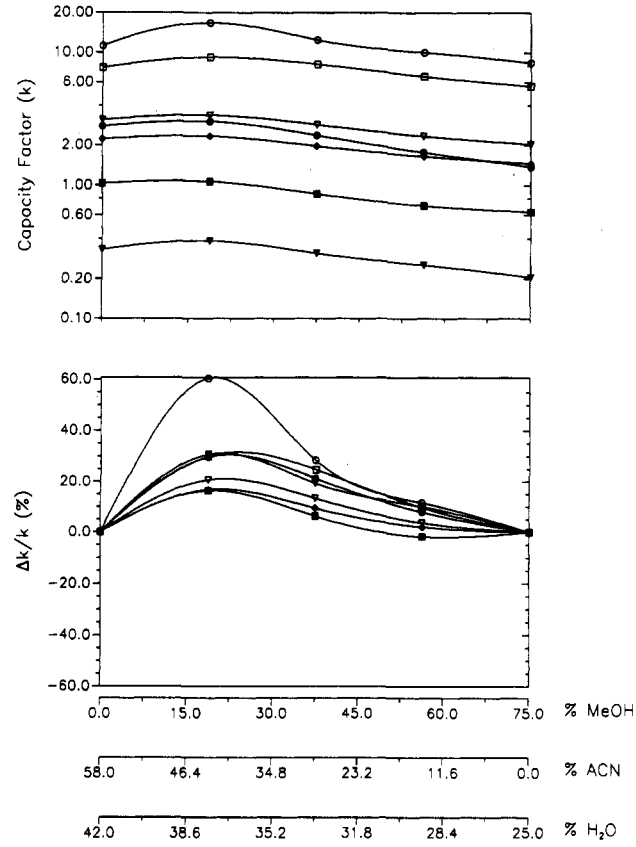

Figure 7. Experimentally measured solute retention for premixed mobile phases varying in solvent selectivity (top). The relative error in solute retention (bottom) is calculated as $(k_{Exp} - k_{Theory})/k_{Theory}$, based on the solubility parameter model in eq 6. Chromatographic conditions as described in Figure 6. Solutes: phenol (▼), nitrobenzene (■), diphenylamine (●), toluene (◇), naphthalene (▽), anthracene (□), pyrene (○).

(A), representative of proton donor–acceptor interactions, and 58% acetonitrile–42% water (B), representative of dipole-(induced) dipole interactions. At these compositions, the solvent systems have approximately equal solvent strength according to eq 2, with a total solubility parameter of 34.2 $MPa^{1/2}$. Shown in Figure 6 are the chromatograms for the seven solutes obtained with these mobile phases. Because the solvent systems have equal solvent strength, all solutes are eluted with capacity factors ranging from 0 to 10 despite the mobile phase used. However, the order of elution for diphenylamine and toluene is reversed, because the solvent systems have different selectivity. This reversal in elution order arises because the base diphenylamine has greater proton donor–acceptor interactions with the methanol–water system (A) and is less retained than toluene. Conversely, because the proton donor–acceptor interaction is reduced in the acetonitrile–water system (B), diphenylamine has greater interaction with the stationary phase and is thus more retained.

Premixed Mobile Phases. The assumption of independent intermolecular interaction in a mixed solvent system is evaluated by measuring the retention time of the seven solutes in a manner similar to the solvent strength study. However, in this case, a linear relationship is expected between the logarithm of capacity factor and the mobile-phase composition because the two solvent systems have identical solvent strength according to eq 2. These data are summarized in the top diagram of Figure 7, where the measured solute capacity factors are shown as a logarithmic function of mobile-phase composition. In fact, retention reaches a maximum for all solutes at a mobile-phase composition of approximately 18% methanol–44% acetonitrile–38% water, where capacity factors are +16% to +60% greater than predicted by simple linear interpolation (Figure 7, bottom). This positive deviation in solute retention indicates that the actual strength of the mobile phase is less (δ_M is greater) than that predicted by eq 2 for ternary mixtures of methanol, acetonitrile, and water. This conclusion is in qualitative agreement with the results of Lochmuller and co-workers (*19*), who determined that the formation constants for acetonitrile–water and acetonitrile–methanol aggregates are substantially less than for methanol–water aggregates. Consequently, the replacement of methanol (associating) by acetonitrile (nonassociating) reduces the extent of aggregation in the ternary mixtures, thereby increasing the effective polarity. It is noteworthy that all solutes, regardless of the nature of chemical interactions, display the same direction of error and that the magnitude of the deviation increases with capacity factor. These results clearly illustrate the difficulty of predicting solute retention accurately in premixed mobile phases of aqueous methanol and acetonitrile.

Solvent Modulation. Solute retention is examined next under the condition of solvent modulation using the individual 75% methanol–25% water (A) and 58% acetonitrile–42% water (B) solvent systems. The experimentally measured capacity factors for the seven solutes are compared to theoretically predicted values by using eq 8. These results are summarized in Figure 8, where the capacity factor is shown as a linear function for modulation sequences with a repetition rate of approximately one. For a solvent proportion of 45% A–55% B (Figure 8, top), the capacity factors of all solutes are less than 10 and show excellent agreement with the theoretically predicted values, regardless of the nature of chemical interactions. Also, solute retention remains relatively constant

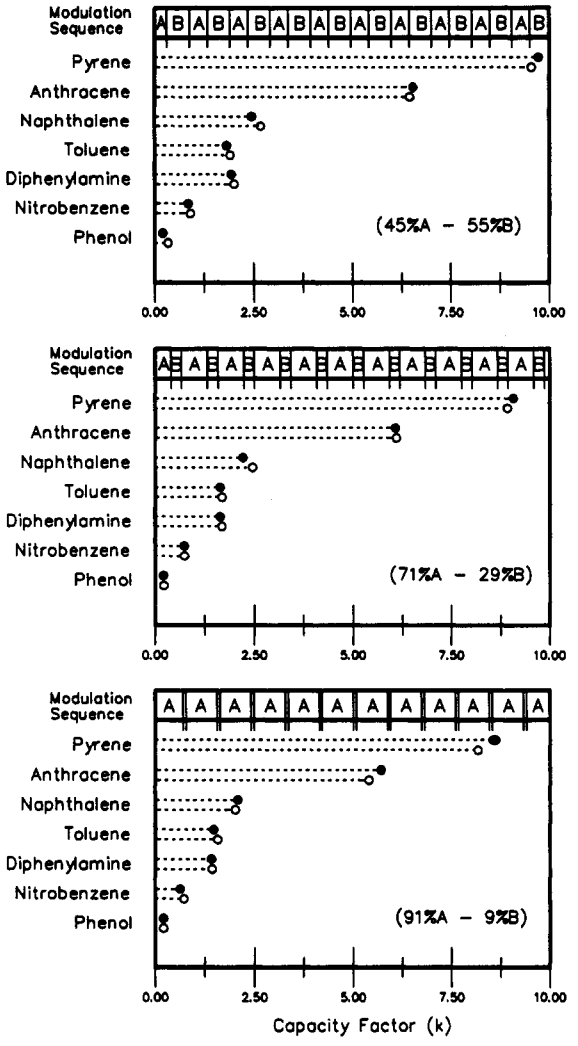

Figure 8. Solute retention in solvent-modulated mobile phases for varying solvent proportion and constant repetition rate (RR = 1). The open circles (O) represent the experimentally measured capacity factors and the filled circles (●) are theoretically predicted from eq 8. Chromatographic conditions as described in Figure 6.

with increasing solvent proportion because the strength of the individual mobile phases is the same. However, the selectivity varies with solvent proportion, as shown by the changing elution order of the solutes toluene and diphenylamine. In the top diagram of Figure 8, diphenylamine elutes after toluene. As the proportion of the stronger proton donor–acceptor mobile phase (75% methanol–25% water) is increased to 71% A–29% B (Figure 8, center), diphenylamine coelutes with toluene. Yet, with the final increase in the solvent proportion to 91% A–9% B (Figure 8, bottom), diphenylamine elutes prior to toluene. This reversal in elution order illustrates how the simple control of solvent proportion can be used to influence specific solute–solvent interactions and produce predictable changes in solute retention.

The relative error between the measured capacity factors and those determined by eq 8 is examined as a function of solvent proportion in Figure 9. All seven solutes display a statistically random error in retention with solvent selectivity, compared to the systematic error observed with premixed mobile phases (Figure 7, bottom). The agreement between the measured and predicted capacity factors for all solutes is +2.5% with a range of ±11.5%, which is within the confidence limits of the experimental measurements (*1*). This agreement is particularly impressive considering that the most retained solute (pyrene) has encountered approximately 20 solvent zones. This excellent agreement further supports the

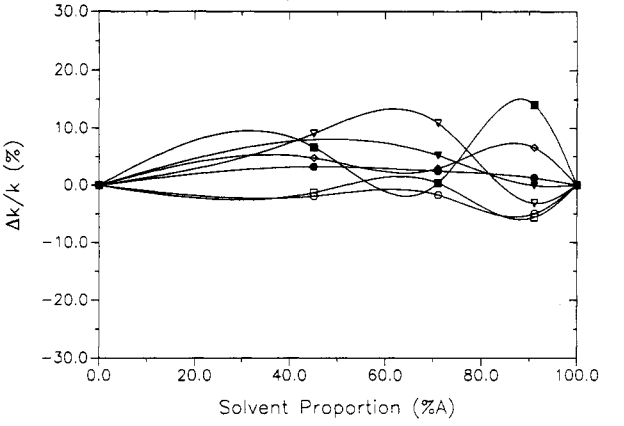

Figure 9. Relative error between the measured capacity factors and those predicted by eq 8 for repetition rate of one. Chromatographic conditions as described in Figure 6. Solutes: phenol (▼), nitrobenzene (■), diphenylamine (●), toluene (◇), naphthalene (▽), anthracene (□), pyrene (○).

fundamental assumption that solute–solvent and solvent–solvent interactions are independent under the condition of solvent modulation. Moreover, the assumption of steady-state mass-transport processes between the mobile and stationary phases, which was proposed in the strength study, also appears to be valid when solvent selectivity is modulated.

CONCLUSIONS

Solvent modulation appears to offer a powerful and versatile strategy to control solute retention in liquid chromatography. The benefits of solvent modulation arise because the individual solvent components are separated spatially and temporally along the chromatographic column rather than mixed physically. Solute retention may be more accurately predicted by using the time-averaged behavior in independent solvent zones than by estimating the likely behavior in premixed solvent systems. Furthermore, solvent modulation allows independent control of solvent strength and selectivity to emulate either isocratic or gradient elution. Thus, it is concluded that solvent modulation offers a simple, versatile, and accurately modeled means to control and predict solute retention in liquid chromatography.

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Capillary Electrophoretic Separations of Proteins Using Nonionic Surfactant Coatings

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Capillary zone electrophoretic separations of proteins have been achieved by using nonionic surfactant coated capillaries. Capillaries were prepared by derivatization of the silica surface with octadecylsilane followed by the deposition of a layer of nonionic surfactant from an aqueous solution above the critical micelle concentration. This coating is of sufficient thickness and hydrophilicity to reduce both protein adsorption and electroosmotic pumping. This hydrophilic coating reduces electroosmotic pumping 5–8-fold while resolving proteins quickly and efficiently with good recovery. The coating provides a stable and reproducible means of deactivation, while the rate of electroosmotic pumping stays relatively constant throughout the pH range 4–11. This allows the pH to be varied to enhance selectivity without adversely affecting the flow rate.

INTRODUCTION

High-resolution capillary electrophoresis is proving to be of great utility in the separation of small molecules such as inorganic ions (*1*, *2*), amino acids (*3–6*), small organic ions (*7–10*), peptides (*11–13*), and oligonucleotides (*14*, *15*). Unfortunately, the enormous resolving power of capillary electrophoresis has been of minimal value in the separation of proteins. The difficulty in applying this technique to proteins arises from silanol groups on the surface of fused-silica capillaries. Silanols ionize above pH 4 and greatly increase band spreading and peak tailing through adsorption or denaturation of many proteins on the capillary walls.

Although acidic pH may be used to repress the ionization of silanols (*16*), or basic pH to produce a net negative charge on the protein, which is then repelled by the negatively charged capillary wall (*17*, *18*), these approaches introduce several new problems. Many proteins are denatured by extremes in pH, and the full pH range is necessary to discriminate between proteins on the basis of charge. Attempts to deactivate capillary walls by either silane derivatization (*19*, *20*) or physically coating the silica surface (*21*, *22*) have been reported. These approaches, however, have been of limited success due to a lack of consistency in intercolumn performance, rapid deterioration of column efficiency, and limited utility at neutral pH. An alternative strategy to reduce protein adsorption has been to reverse the charge on the capillary wall from negative to positive through coating (*23*) or the addition of an amine polymer to the buffer (*24*). These two approaches are well suited for the analysis of positively charged solutes where they are repelled from the surface, but severe wall interactions result when anionic proteins are analyzed. Stable, reproducible deactivation of the capillary is needed before capillary zone electrophoresis (CZE) will be of general utility in protein separation.

The goal of this study was to develop a stable, reproducible coating that would reduce protein adsorption on capillaries and provide good recovery while some electroosmotic flow was maintained. The use of ionic surfactants has been used to control electroosmotic flow in both CZE (*25*, *26*) and open tubular chromatography (*27*). Nonionic surfactants have been used for quite a different reason. It has been determined, in the case of chromatography, that nonionic surfactants can be hydrophobically adsorbed onto an alkylsilane-derivatized surface to create a hydrophilic layer that will exclude proteins from the surface (*28–35*). Borgerding and Hinze (*29*) examined the chromatographic effects of polyoxyethylene[23]–dodecanol (BRIJ 35) on an octadecylsilane (C_{18}) column and found that unlike ionic surfactants, BRIJ 35 adsorbs in substantial amounts onto the reversed-phase surface. Other papers demonstrated that after the modification of reversed-phase columns by nonionic surfactant adsorption, proteins could be eluted with aqueous mobile phase either when the surfactant was in the mobile phase (*32*) or when it was deleted (*33*). Deschamps (*32*) found that surfactants in the mobile phase improved efficiency by reducing the denaturation of proteins. Chang (*33*) recognized that the hydrophilic layer established by the surfactants over a wide-pore reversed-phase sorbent creates a nearly permanent and ideal surface for the size-exclusion separation of proteins. The most definitive work on the effects of nonionic surfactants in exclusion media for large molecules was contributed by Desilets et al. (*28*). This study examined the chromatographic effects of polyoxyethylene surfactant size and structure on protein exclusion and small analyte separations. Adsorption of polyoxyethylene-based surfactants apparently creates a semipermeable, hydrophilic layer of adsorbed surfactant on an alkylsilane-derivatized surface that prevents the adsorption of proteins.

A hydrophilic network is apparently created by the polyoxyethylene portion of the surfactant that may include polyoxyethylene loops, trains, and tails. It is suggested that this hydrophilic "forest" would keep proteins at a sufficient distance from either the reversed-phase surface or the residual silanol groups on the capillary wall to prevent adsorption and denaturation (*28*). The TWEEN and BRIJ series surfactants

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