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Selectivity of organic solvents in micellar liquid chromatography of amino acids and peptides

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ABSTRACT

The influence of the type of organic modifiers on retention behavior in micellar liquid chromatography is studied. A group of amino acids and small peptides was used as the test mixture. It is shown that the chromatographic selectivities of 2-propanol, acetonitrile and tetrahydrofuran which belong to three different groups in Snyder's classification, are considerably similar in the presence of micelles for the test mixture. On the other hand, the selectivities of 2-propanol and butanol which belong to the same solvent selectivity group are different for these solutes in the micellar mobile phases.

INTRODUCTION

At the present time reversed-phase liquid chromatography (RPLC) using *n*-alkyl bonded phases is the most frequently used technique for separation of non-volatile compounds [1]. One of the advantages of this technique is the feasibility of manipulating retention by a careful selection of mobile phase parameters [2,3]. The most important among these parameters are type and concentration of organic solvents, pH, and addition of surfactant. Organic modifiers are used in the RPLC mobile phases to control the solvent strength as well as to improve selectivity [4].

A widely accepted technique for characterizing of LC solvents is the Snyder's selectivity triangle [3,5]. This technique classifies various organic solvents on the basis of their relative ability to engage in proton accepting, proton donating and strong dipolar interactions. When the resulting values are plotted on three axes in the form of a triangle, solvents having similar functionalities tend to fall within the same area of the triangular plot. In principle, the solvents grouped in the same area of the triangle should have similar chromatographic selectivity, while solvents from other groups should exhibit different selectivity for a given separation [5]. This theory has been widely accepted and has often formed the rationale for solvent selection for optimizing a given HPLC separation [6–10].

The use of secondary chemical equilibria in RPLC has greatly extended its separation capability [11]. A good example of these secondary equilibria is micellar liquid chromatography (MLC). The retention of a solute in MLC is determined by three competing equilibria among micelle, bulk aqueous solvent and stationary phase. It has been demonstrated that the use of organic modifiers in MLC has a great influence on chromatographic behavior [12–18]. In previous papers, we reported a rather unique phenomenon of simultaneous enhancement of solvent strength and selectivity in MLC through optimizing the concentrations of an organic modifier and micelles [13–18]. However, adequate attention has not been paid to the chromatographic selectivity of different organic solvents in the presence of micelles. In this paper, the results of an explora-

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tory study on the chromatographic selectivity of organic solvents in micellar media are described.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a pump (Model 400; Applied Biosystems, Foster City, CA, USA) and a variable-wavelength absorbance detector (Model 783 A, Applied Biosystems) set at 210 nm, controlled by Chemresearch chromatographic data management system controller software (ISCO, Lincoln, NE, USA) running on a PC-88 Turbo personal computer (IDS, Paramount).

The retention behavior of individual solutes was studied using a 5-μm particle size Ultremex C_{18} column (Phenomenex, Torrance, CA, USA), 100 × 4.6 mm I.D. The column dead volume (0.6 ml) was measured by making multiple injections of water samples. The test mixture was separated using a longer (250 × 4.6 mm I.D.) Ultremex column (dead volume: 2.1 ml) in order to generate larger number of theoretical plates. The colums were thermostated at 40°C by a water circulator bath (Lauda Model MT-6; Brinkmann Instruments, Westbury, NY, USA). A silica precolumn was used to saturate the mobile phase with silicates and to protect the analytical column.

The software used to evaluate the separation at different mobile phase compositions was based on an extended version of the iterative regression optimization strategy [17–20]. The simulated chromatograms are based on a Gaussian peak shape, using the plate-count and dead volume observed in chromatographic experiments.

Reagents

The stock solution of sodium dodecyl sulfate (SDS) was prepared by dissolving the required amount of surfactant in doubly distilled deionized water and filtering through a 0.45-μm nylon membrane filter (Gelman, Ann Arbor, MI, USA). The test solutes were: (1) tyrosine (Y), (2) methionine (M), (3) alanyl-tyrosine (AY), (4) tryptophan (W), (5) aspartyl-phenylalanine (DF), (6) leucyl-tyrosine (LY), (7) glycyl-leucyl-tyrosine (GLY), (8) leucyl-tryptophan (LW) and (9) phenylalanyl-phenylalanine (FF). The sample solutions were prepared by diluting stock solutions (10 mg/ml in water or tetrahydrofuran) in the mobile phase. The ionic strength was adjusted by adding phosphate buffer such that the total buffer concentration in the final concentration was 0.02 *M*. After adding the required amount of 2-propanol the pH was adjusted to 2.5.

RESULTS AND DISCUSSION

In the following sections, the influence of four organic modifiers on the chromatographic behavior of a group of amino acids and small peptides in MLC will be discussed. The organic solvents are 2-propanol (PROH) and butanol (BUOH) (which both belong to group II of Snyder's triangle), acetonitrile (ACN) (group VIb) and tetrahydrofuran (THF) (group III).

2-Propanol, acetonitrile and tetrahydrofuran

In RPLC using hydro–organic mobile phases the relationship between retention factor and volume fraction of organic modifier (φ) is often a quadratic equation as eqn. 1 [4].

$$\ln k' = -A\varphi^2 + B\varphi + \ln k'_w \quad (1)$$

where a coefficient A is expected to be positive, B is a negative, and $\ln k'_w$ is the natural logarithm of the retention factor in pure water.

Over a limited range of φ the relationship between retention factor and φ can be written as eqn. 2:

$$\ln k' = -S\varphi + \ln k'_w \quad (2)$$

where S is solvent strength parameter. The linearity of eqn. 2 deteriorates in the low (less than 10%) and high (more than 90%) concentration ranges of organic solvents. In conventional RPLC with water-rich eluents ($\varphi < 10\%$), even a quadratic fit of $\ln k'$ *vs.* φ would be inadequate.

Likewise, in MLC with hybrid eluents of micelles–organic modifier, the relationship between retention and volume fraction of organic modifiers is also linear:

$$\ln k' = -S_{hyb}\varphi + \ln k'_0 \quad (3)$$

where S_{hyb} is solvent strength parameter in hybrid system and $\ln k'_0$ is the retention in purely aqueous micellar eluent [13–16].

Eqn. 3 adequately described ($r^2 > 0.986$) the retention behavior of the test mixture of amino acids

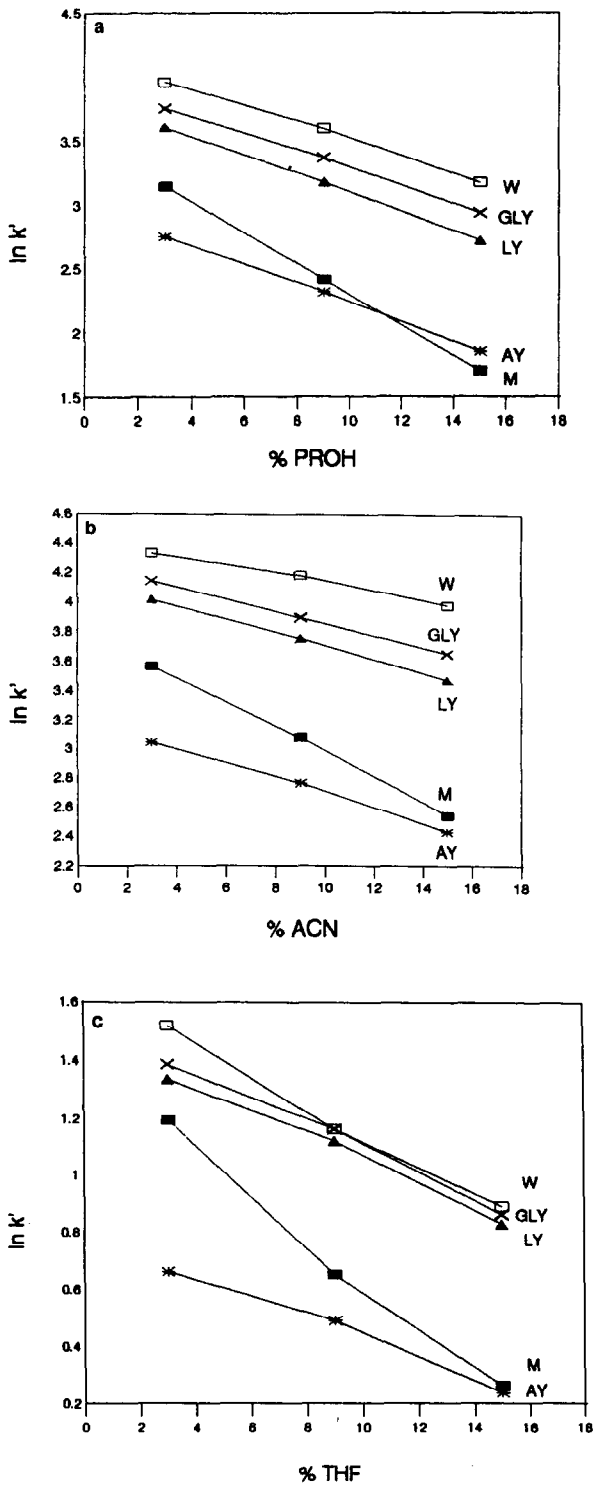

Fig. 1. Plots of ln k' *vs.* φ for (a) PROH, (b) ACN and (c) THF.

and small peptides in MLC with hybrid mobile phases as a function of volume fraction of three organic modifiers, 2-propanol acetonitrile, and tetrahydrofuran, over a range of 3–15% (Fig. 1).

The solvent strength parameters (*i.e.* S and S_{hyb}, the slopes of eqns. 2 and 3), represent the sensitivity of solutes retention with volume fraction of organic modifiers in hydroorganic and hybrid systems respectively. The relation between the slope and the intercept of these equations has a significant effect on chromatographic selectivity. The selectivity between those solutes whose slopes and intercepts are directly related to one another, would decrease with an increase in organic solvent concentration [13,14]. In contrast, for cases where there is no direct relationship between the slope and the intercept, the selectivity would increase with volume fraction of organic modifiers [14]. In RPLC with methanol–water mobile phases, linear correlations have been reported for the slope *vs.* intercept of eqn. 2 for a large group of compounds [4]. For hybrid eluents in MLC, the results of slope *vs.* intercept of eqn. 3 are illustrated in Fig. 2. As shown, unlike conventional hydro–organic eluents in the presence of micelles no correlation was observed between S_{hyb} and ln k'_0 for PROH-, ACN- and THF-modified micellar eluents. One can then anticipate a different selectivity behavior for PROH, ACN and THF in the presence of micelles.

Fig. 3 illustrates the chromatographic selectivities of PROH, ACN and THF in the presence of a fixed concentration of micelle (0.02 M SDS) for a sample mixture of seven amino acids and peptides. The volume fraction of the organic solvents are adjusted so that the solvent strength (analysis time) of all three mobile phases remain approximately the same. Interestingly, the elution order and selectivity of all solutes are similar for THF, PROH and ACN except for the different elution order of peaks 1 and 3 for PROH as compared to those for THF and ACN, and poor resolution of peaks 5 and 6 for PROH and ACN (Fig. 3).

As was discussed previously, both micelle concentration and volume fraction of an organic modifier influence the elution strength and selectivity [13–18]. Likewise, in order to achieve an understanding of the influence of the type of organic modifier, one should also simultaneously consider the role of micelles. In other words, one should

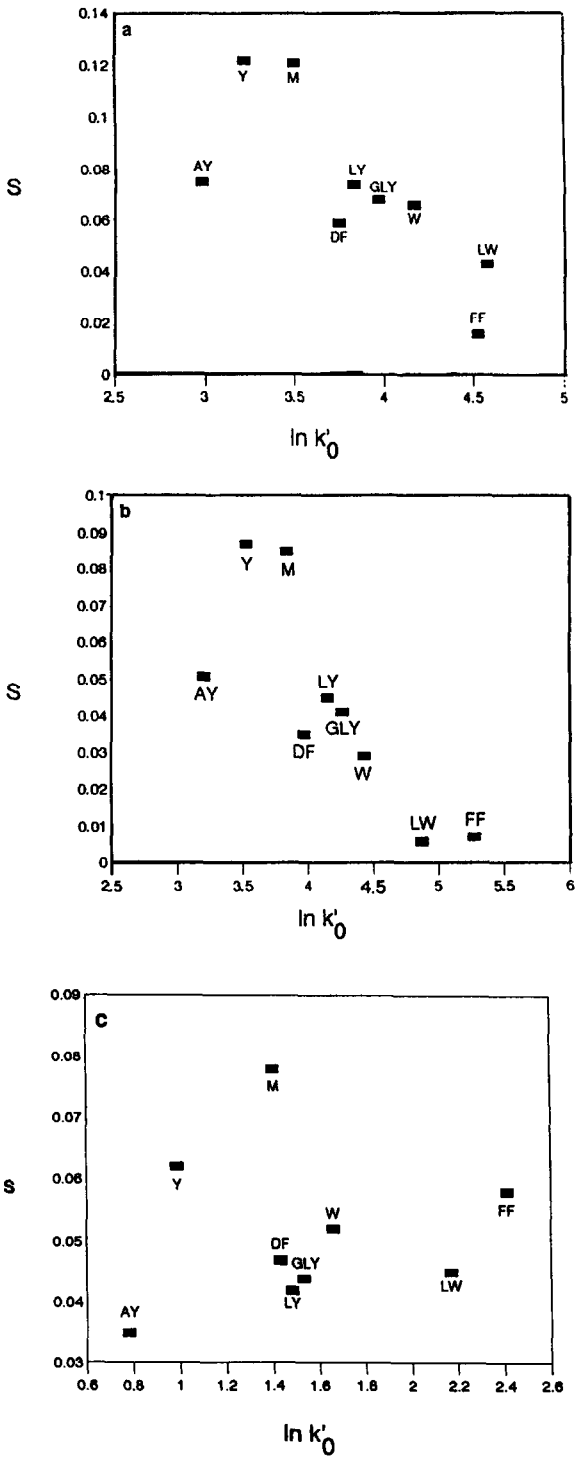

Fig. 2. The relation between S value and ln k'_0 for (a) PROH, (b) ACN and (c) THF.

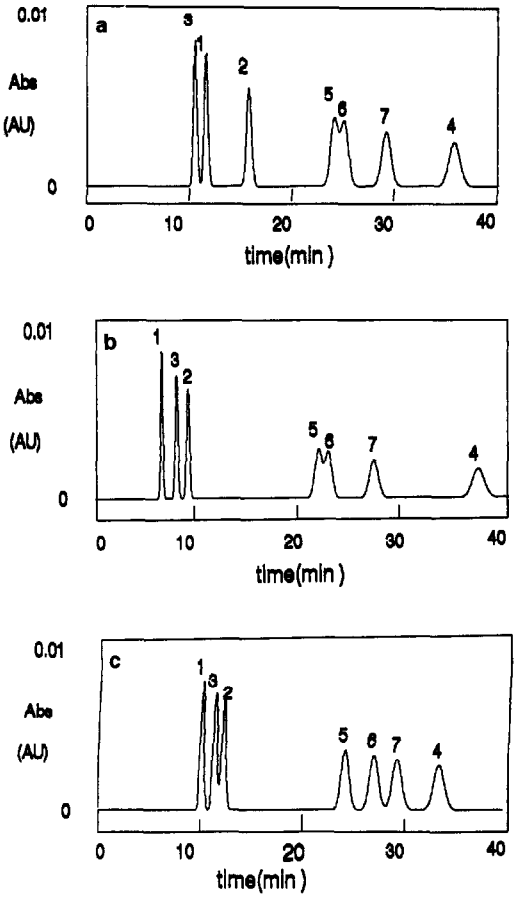

Fig. 3. The reconstructed chromatograms of a mixture of amino acids and peptides based on experimental retention data at (a) 0.02 M SDS and 3% PROH, (b) 0.02 SDS and 12.5% ACN and (c) 0.02 M SDS and 3% THF. The solutes are identified in the Experimental section.

compare the three organic modifiers under optimized elution strength and selectivity.

For this purpose, we used iterative regression (IR) procedure to predict the optimum mobile phase compositions (*i.e.* micelle concentration and organic modifier volume fraction). This procedure was originally described by Drouen *et al.* [19] and extended by Van Renesse *et al.* [20]. We have recently reported the successful application of this technique for the two- and three-parameter optimization of micelle concentration, PROH% and pH [17,18].

A two-dimensional parameter space (surfactant and organic solvent concentration) was used as is

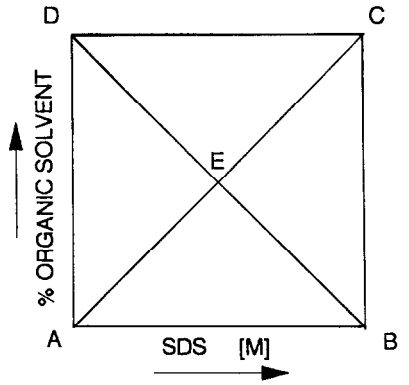

Fig. 4. The parameter space for iterative regression procedure. The five initial measurements were performed using the following mobile phases: (A) 0.02 *M* SDS + 3% organic solvent, (B) 0.20 *M* SDS + 3% organic solvent, (C) 0.20 *M* SDS + 15% organic solvent, (D) 0.02 *M* SDS + 15% organic solvent and (E) 0.11 *M* SDS + 9% organic solvent. This parameter space was used for the three organic solvents: PROH, ACN and THF.

shown in Fig. 4. This optimization procedure is based on linear modelling of the solutes retention (ln k') in the mixture as a function of mobile phase parameters using a limited number of initial experiments [16–10]. The retention of the solutes in a mixture will then be predicted within the parameter space through interpolation of the assumed linear model of ln k' *vs.* parameters. Based on the predicted retention behavior of all compounds in a mixture, the quality of separation (*e.g.* minimum resolution) at all mobile phase compositions within the parameter space will be calculated and an optimum is predicted. If the observed quality of separation at the predicted optimum mobile phase compositions is not satisfactory, more experiments will have to be performed (*i.e.* through an iterative process) in order to locate the global optimum in the parameter space. The success or failure of finding the optimum parameter mobile phase composition would largely depend on the correctness of the linearity assumption of the model [16–20].

The retention of seven amino acids and peptides were measured at five mobile phase compositions. Four measurements at the corners of the selected two dimensional parameter space, and one measurement at the center. The parameter space consists of four triangle subspaces defined by three of the five measurements, *i.e.* two corner points and the central point. The boundaries of the parameter space are determined by practical limitations of the chromatographic system. The lower surfactant con-

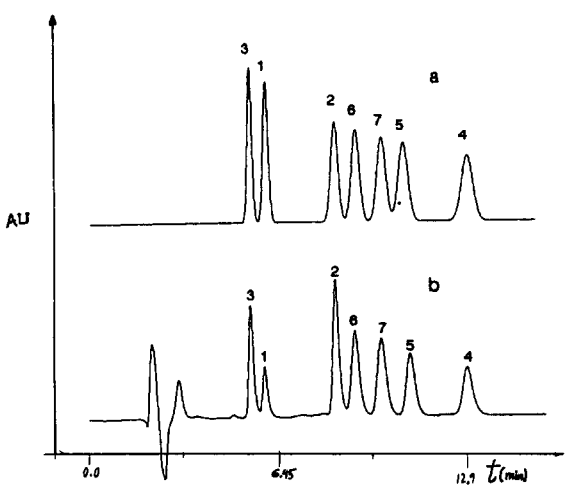

Fig. 5. The predicted (a) and measured (b) chromatograms for amino acids and peptides at 0.17 *M* SDS + 12.6% PROH. The identities of solutes are listed in the Experimental section of the text.

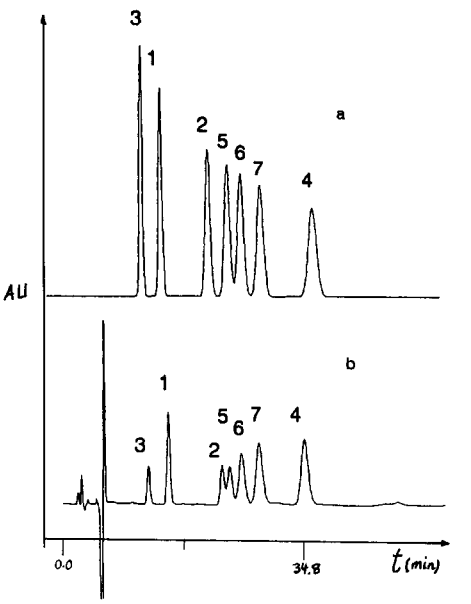

Fig. 6. The predicted (a) and measured (b) chromatograms for amino acids and peptides at 0.12 *M* SDS + 7.5% ACN. The identities of solutes are listed in the Experimental section of the text.

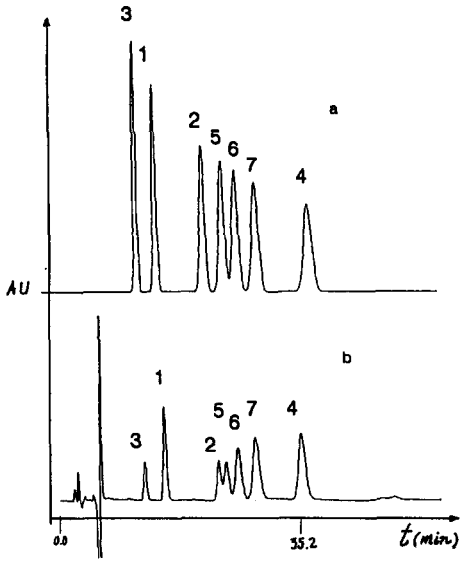

Fig. 7. The predicted (a) and measured (b) chromatograms for amino acids and peptides at 0.07 *M* SDS + 4.2% THF. The identities of solutes are listed in the Experimental section of the text.

centration must be well above the critical micelle concentration (8 m*M* at room temperature and in pure water) and must be strong enough to cause elution of solutes. The upper surfactant concentration is determined by a combination of solubility of the surfactant, the viscosity of the resulting mobile phase, and degradation of efficiency at higher concentration [17]. The volume fraction of organic solvent was limited to a maximum of 15% to protect the integrity of micelles.

The applicability of the IR procedure for these three solvents was examined. The agreements between predicted and measured chromatograms for PROH–SDS-, ACN–SDS- and THF–SDS-modified eluents are excellent, good and fair, respectively, as shown in Figs. 5–7. It is shown that there is a considerable similarity between the chromatograms for THF, ACN and PROH.

2-Propanol vs. butanol

According to Snyder's solvent classification the chromatographic selectivity for 2-propanol and butanol should be the same at equal solvent strengths in hydro–organic mobile phases because they belong to the same selectivity group.

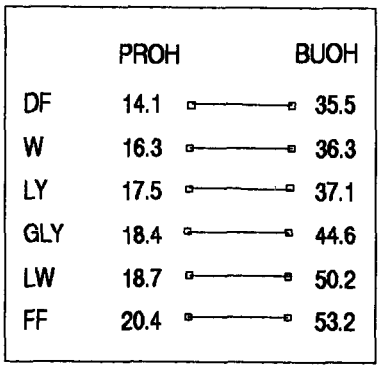

Fig. 8. The *S* values of some amino acids and peptides for PROH–water and BUOH–water mobile phases.

The *S* values of most of solutes used in this study for hydro-organic eluents (*i.e.* PROH–water and BUOH–water) are ranked in Fig. 8. An attempt for measuring the *S* value of the rest of solutes for these mobile phases (*i.e.* AY, Y and M) was unsuccessful due to the lack of adequate retention. As expected, the *S* values of solutes in BUOH containing eluents are larger than those for PROH, however, the ranks of *S* values of different solutes for both BUOH and PROH are the same. In other words, selectivity in retention of solutes in BUOH–water and PROH–water mobile phases should be similar at equal solvent strength. Since in MLC organic modifiers associate with micelles and on the other hand compete with micelles to interact with solute, the chromatographic selectivity of organic solvents may no longer be the same. The S_{hyb} values for the test solutes in the hybrid systems of PROH–SDS and BUOH–SDS at a micelle concentration are shown in Fig. 9.

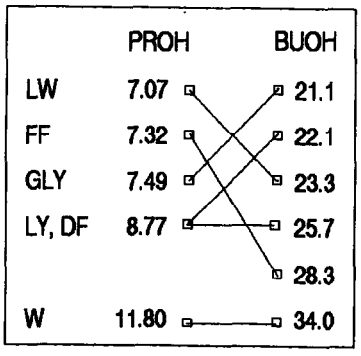

Fig. 9. The *S* values of some amino acids and peptides for PROH and BUOH at 0.02 *M* SDS.

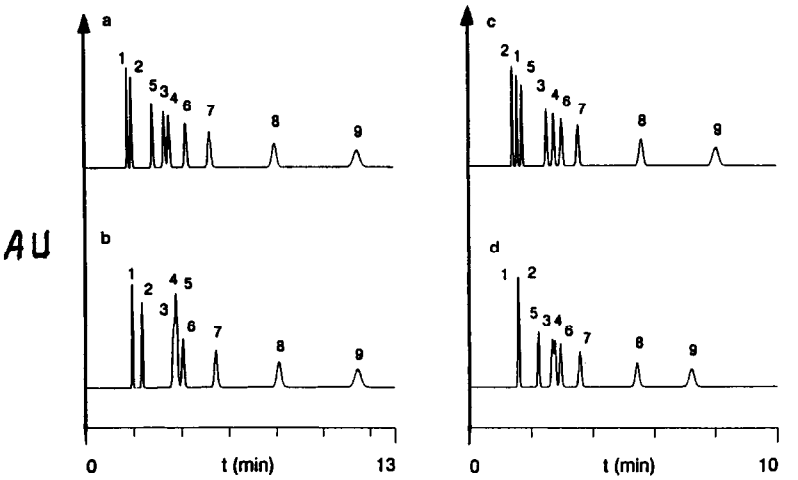

Fig. 10. The reconstructed chromatograms on the basis of measured retentions for nine amino acids and peptides at 0.08 *M* SDS and (a) 8% PROH, (b) 1.9% BUOH, (c) 14% PROH, and (d) 3.6% BUOH.

The rank of S_{hyb} values of some solutes for BUOH is different from that for PROH. For example, at 0.02 *M* SDS the S_{hyb} value of FF for PROH is the second highest and for BUOH is the second lowest, or the S_{hyb} value of LY for PROH is the second lowest while for BUOH is the second highest (Fig. 9). A comparison of the Figs. 8 and 9 shows that the *S* values of all solutes in both PROH and BUOH decrease due to the inclusion of micelles in the aqueous–organic media [*i.e.* $S_{(hyb)} < S$(hydro–organic)] [13,14]. The magnitude of the reduction in *S* values depend upon the degree of interactions of solutes and organic solvents with micelle [13–15]. Micelles control the solvation ability of organic solvent and as a result their chromatographic selectivity. The ranks and the magnitudes of the S_{hyb} values for PROH and BUOH changed with the micelle concentration. Consquently, one can expect that the organic solvent selectivity in MLC be a function of micelle concentration. The reasons behind these observations have been reported elsewhere [14,15].

The chromatograms of amino acids and peptides for different concentrations of BUOH and PROH at a fixed micelle concentration (0.08 *M* SDS) in the hybrid systems are illustrated in Fig. 10. In this figure the strengths of the PROH and BUOH hybrid mobile phases are adjusted so that the retention of the last peak remains the same. One can observe that although all peaks are well separated for PROH, there exist strong overlaps and coelution of peaks 3,'4 and 5 in BUOH. The elution strength for chromatograms c (PROH) and d (BUOH) are also the same. A close look at chromatograms c and d shows that, again all peaks for PROH are well resolved, while for BUOH peaks 1 and 2 are coeluted and peaks 3 and 4 have very poor resolution. As a result of these observations one can conclude that the chromatographic selectivity of BUOH is different as compared to that of PROH.

CONCLUSIONS

The presence of micelles in the mobile phases of RPLC has a great influence on the chromatographic selectivity of organic solvents. As a result the classification of organic solvents established by Snyder may not be fully valid in MLC. Further investigations should be made on the effect of micelles on chromatographic selectivity of organic solvents.

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