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Effect of stationary phase solvation on shape selectivity in reversed-phase high-performance liquid chromatography*

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ABSTRACT

The effect of stationary phase solvation on reversed-phase chromatographic shape selectivity has been investigated using n-hexanol as an additive to methanol-water mobile phases. A wide range of mobile phase compositions was evaluated to normalize for solvent strength selectivity differences. Monomeric C_{18} stationary phases of both high and low bonding density were synthesized and used to correlate selectivity changes caused by stationary phase ordering with those seen by the addition of n-hexanol. The temperature dependence of retention and selectivity was also investigated using Van 't Hoff plots, which provided insight into the nature of selectivity behavior for estrogens and polyaromatic hydrocarbons. The results showed that using n-hexanol as a mobile phase additive did not provide higher shape selectivity, suggesting that changes in the solvation of the stationary phase did not impart a significant change in the level of surface ordering or morphology. However, n-hexanol did impart solvent selectivity changes in the separation of estrogen diastereomers that could prove useful in future methods development schemes.

INTRODUCTION

Chromatographic selectivity, α, is generally defined as the interaction difference that two solutes experience between the mobile and stationary phases, such that

$$\ln \alpha = -\Delta(\Delta G/RT) \quad \text{(I)}$$

where AG is the Gibbs free energy of transfer between the mobile and stationary phase, R is the gas constant, and T is the absolute temperature. Because small increases in selectivity can lead to substantial increases in resolution, with concomitant decreases in analysis time, considerable effort has been expended to better understand the relevant interactions that govern selectivity in reversed-phase high-performance liquid chromatography (RP-HPLC) and the chromatographic conditions that can maximize selectivity.

In what proved to be great foresight, Bakalyar wrote in 1977 [1]: "It may well be that the standard column (for RP-HPLC) becomes a hydrocarbon bonded phase (analogous to the nitrogen gas mobile phase of gas chromatography) and that selectivity is adjusted by changing the mobile phase only (analogous to the column in GC)". Indeed, while changing the polarity of the stationary phase to optimize selectivity has been explored, selectivity is most often adjusted by changing either the mobile phase composition (solvent strength optimization) or the mobile phase organic modifier (solvent selectivity optimization). Extensive litera-

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ture has been published for each of these three strategies [2–4].

The success of mobile phase or stationary phase optimization generally relies on the polar interactions of the solutes with the two phases. One class of compounds that does not show significant selectivity enhancement with changes in mobile phase or stationary phase polarity is that of shape isomers. Because they have identical structural composition, and differ only in geometric shape, shape isomers often have similar solubilities and thus similar retention properties, resulting in coelution. Sander and Wise [5–7] have studied shape isomer separations extensively using C_{18} stationary phases. Their results suggest that the separation of shape isomers is not enhanced by mobile phase optimization as compared to stationary phase effects. However, the greatest enhancement in shape selectivity occurs not from stationary phase polarity changes, but from the degree of stationary phase surface ordering [6,7]. The trends of this work show that shape selectivity is highest for polymerically bound (i.e., trichlorosilanes with water in the reaction mixture) C_{18} stationary phases at low temperatures. By increasing the networking of the stationary phase by using polymeric bonding chemistry, and by increasing the stationary phase chain rigidity by decreasing the temperature, Sander and Wise propose that linear, planar solutes can partition more readily into the ordered surface than solutes that are bent, or non-planar, thus allowing a means of separation [6,8]. Similar results were reported by Sentell and Dorsey [9], who demonstrated that monomerically derivatized stationary phases of high bonding density provided higher shape selectivity than low bonding density columns. Again this increase in selectivity with bonding density was attributed to greater ordering of the stationary phase chains at high chain densities.

Martire and Boehm [10] proposed a stationary phase model that incorporated the idea of a "breathing" surface that could change its three-dimensional structure from a collapsed state in poor wetting solvents, to a more extended bristle-like structure in mobile phases of good wetting ability. The implications of this model are that the chromatographic properties of the system change not only as a function of mobile phase polarity, but that the mobile phase can change the nature of the stationary phase. A large number of scientists have reported evidence of mobile phase modifiers partitioning into stationary phases [11–13]. Although most non-polar modifiers are relatively strong eluents, it is not uncommon for solute retention to increase as a result of adding the modifier. This increase can be attributed to an increase in stationary phase volumes as the modifier becomes part of the stationary phase.

MacCrehan and Schonberger [14,15] showed that the addition of 10% n-butanol to methanol–water mobile phases reduced the retention of the shape isomers *cis*/*trans*-retinol and *cis*/*trans*-P-carotene dramatically, while maintaining separation selectivity. One proposed mechanism for this selectivity phenomenon was stationary phase solvation, in which n-butanol partitioned into the stationary phase to provide a more extended, ordered surface, thus improving the shape selectivity. However, this hypothesis was not investigated.

In an effort to improve shape selectivity for the wide range of stationary phases currently available, and to gain insight into the relationship between stationary phase solvation and shape selectivity, we have studied the systematic addition of n-alcohols to mobile phases to determine their effect on shape selectivity. The alcohols evaluated included n-propanol through n-octanol to determine which would provide the greatest solvation while maintaining system compatibility. Because of the great effect of solvent strength on selectivity, a wide range of mobile phase compositions was evaluated. Methanol was selected as the organic modifier to minimize the effect of solvent selectivity, as methanol is in the same solvent family as the n-alcohols and thus should provide more similar solution properties than other modifiers such as acetonitrile or tetrahydrofuran [16]. In order to evaluate the role of stationary phase ordering on selectivity, both high and low bonding density stationary phases were used such that selectivity changes caused by an increase in bonding density could be compared to those seen by adding the *n*-alcohol.

EXPERIMENTAL

Apparatus

All chromatographic data was collected on one of two HPLC systems comprised of a SP8800 ternary HPLC pump (Spectra-Physics, San Jose, CA, USA), a Rheodyne 7125 injector with a 20-μl loop (Rheodyne, Cotati, CA, USA) and either an Applied Biosystems 757 absorbance detector or an Applied Biosystems 1000S diode array detector (Applied Biosystems, Foster City, CA, USA). Constant temperature ($\pm$0.3°C) was maintained using a water or water-ethylene glycol bath pumped through both a pre-column and column glass jacket using a Model 9000 Isotemp Refrigerated Circulator Bath (Fisher Scientific, Springfield, NJ, USA). The detector output was recorded on an HP3394A integratoi (Hewlett-Packard, Avondale, PA, USA). All experiments were conducted at 30°C unless otherwise specified, at controlled flow-rates of 1.0, 1.5 or 2.0 ml/min, which were calibrated regularly. The detection wavelength was adjusted to the absorbance maximum of each class of solutes studied.

Replicate injections of all solutes were made until the retention times were reproducible to within $\pm$1% R.S.D. Solutes were dissolved in methanol and diluted with methanol-water with the exception of SRM 869 which was provided as an acetonitrile solution that was diluted with acetonitrile-water. Capacity factors were calculated using a t_0 value obtained from either the solvent disturbance at the beginning of the chromatogram, or by injecting water.

Columns

Stationary phase derivatization materials included dimethyloctadecylmonochlorosilane (Hüls America, Bristol, PA, USA), Novapak spherical silica, 5 μm diameter, 60-Å pores, and 120 m^2/g (a gift from Waters Chromatography Division, Millipore, Milford, MA, USA), N,N-dimethylaminopyridine (Nepera, Harriman, NY, USA), and dichloromethane (Fisher Scientific). Corroborative silica surface area analysis was provided gratis by Union Carbide, the results of which compared within 2% of the nominal values. The silica was derivatized according to the procedure outlined by Sentell et *al.* [17]. The only exception to this procedure was that the reaction was refluxed with stirring for 24 h rather than reacted under ultrasound. Two batches of silica were derivatized, one with a two-fold excess of silane (based on a 5 $\mu mol/m^2$ estimated surface silanol density) and one with an 80% charge of silane to produce phases of higher and lower bonding density. Samples were submitted for C, H and N analysis, and the bonding densities were calculated to be 3.3 and 2.5 $\mu mol/m^2$, respectively. The derivatized silicas were slurry packed into stainless-steel columns (25 cm and 15 cm length, respectively x 4.6 mm I.D.).

Reagents

Methanol and acetonitrile were of HPLC grade from Fisher Scientific, and were used without further purification. Water was distilled, followed by purification with a Bamstead Nanopure system to produce 17.8-MΩ or higher resistivity. The n-alkanols, n-propanol, *n*-butanol, n-pentanol, n-heptanol and n-octanol were of reagent grade and were obtained from Fisher Scientific. The n-hexanol (99%), estradiol-17α, estradiol-17β and equilin were obtained from Sigma Chemical (St. Louis, MO, USA). Napthacene was obtained from Eastman Kodak (Rochester, NY, USA), and benz[*a*]anthracene from K & K Laboratories (Plainview, NY, USA). Benzo[c]phenanthrene was purchased through the Alfred Bader Library of rare chemicals, Aldrich (Milwaukee, WI, USA). Benzo[a]pyrene (BaP), phenanthro[3,4-*c*]phenanthrene (PhPh), and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) were obtained as test mixture SRM 869 as a gift from Dr. Lane Sander, National Institute of Standards and Technology (Gaithersburg, MD, USA).

RESULTS AND DISCUSSION

The solutes in SRM 869 were separated on both the 2.5 and 3.3 $\mu mol/m^2$ columns using the recommended acetonitrile-water (85: 15) mobile phase with UV detection at 254 nm [7]. The structures are shown in Fig. 1. The selectivity between TBN and BaP can be used as a measure

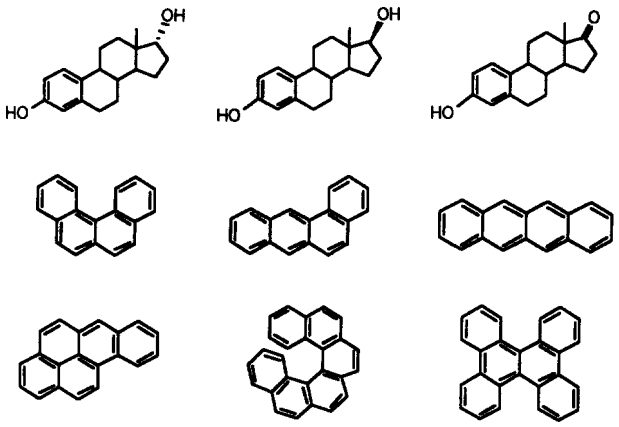

Fig. 1. Structures of the solutes used in these studies: top row: estradiol-17α, estradiol-17β, equilin; middle row: benzo(c)-phenanthrene (BcP), benz(a)anthracene (BaA), naphthacene (NAP); bottom row: benzo(a)pyrene (BaP), phenanthrophenanthrene (PhPh), tetrabenzonaphthalene (TBN).

of shape selectivity, with lower values of $\alpha_{TBN/BaP}$ indicating higher surface ordering and shape recognition. The $\alpha_{TBN/BaP}$ values for the 2.5 and 3.3 $\mu mol/m^2$ stationary phases were 1.74 and 1.65, respectively. These values are both near the border of "monomeric" ($\alpha_{TBN/BaP} >$ 1.7) and "intermediate" ($\alpha_{TBN/BaP} <$ 1.7 and >1.0) stationary phase behavior, with the higher bonding density showing more ordered behavior as predicted [7].

Initial screenings of n-alcohols ranging from *n*-propanol to n-octanol were conducted by adding 3% (v/v) of the alcohol to methanol-water mobile phases such that a final composition of methanol-n-alcohol-water (62:3:35) was achieved. A variety of solutes was screened using mobile phases with and without n-alcohol present. The result of these studies showed that a maximum in selectivity was achieved when using n-hexanol as the additive. Based on these studies, n-hexanol was selected for further study. It should be noted that all of the other n-alcohols, such as the *n*-butanol used by MacCrehan and Schonberger [14] showed significant chromatographic changes at the 3% (v/v) level. The optimum level of n-hexanol was somewhat governed by the solubility and viscosity of *n*-hexanol. At a level of 3% n-hexanol, the system back pressure was increased by approximately 10% and mobile phases of lower than about 45% methanol developed significant cloudiness due to the insolubility of n-hexanol. Given these properties, a maximum level of 5% n-hexanol in the methanol fraction was used. When the level of n-hexanol was decreased to 1% of the mobile phase volume, significantly lower selectivity changes were observed. Thus only higher percentages of n-hexanol were investigated.

In an effort to more closely compare chain ordering to the addition of n-hexanol, two classes of solutes were investigated. These included the estrogens equilin, estradiol-17α, and estradiol-17β, and the polyaromatic hydrocarbons (PAHs) benzo[c]phenanthrene, benz[a]-anthracene and naphthacene. The structures of these compounds are shown in Fig. 1. The three estrogens were previously studied by Olsson et *al.* [18], who showed that methanol-water mobile phases provided the best resolution of the three compounds. Equilin was used as a retention reference, and the compounds were separated on the 2.5 and 3.3 $\mu mol/m^2$ columns with detection at 280 nm. Baseline resolution of the α- and β-estradiols could only be achieved on the 3.3 $\mu mol/m^2$ column. Likewise the PAHs benzo[c]phenanthrene, benz[a]anthracene and naphthacene showed an increase in selectivity with an increase in stationary phase bonding density (the linear naphthacene showed an increase in separation from the angular benzo[c]-phenanthrene) .

Mobile phases containing n-hexanol were prepared for the estrogen and PAH studies by dissolving 5% n-hexanol in methanol, and then using the n-hexanol-methanol mixture as the organic portion of the hydro-organic mobile phase. This made mobile phase preparation easy and accurate, and produced mobile phases that had a constant ratio of methanol to n-hexanol. Thus at a volume fraction of organic modifier, φ, of 0.5 (i.e., 50% organic) the actual mobile phase composition was methanol–*n*-hexanol–water (47.5:2.5:50).

Under all conditions the selectivity of the α/β-estradiol pair was higher on the 3.3 $\mu mol/m^2$ column than on the 2.5 $\mu mol/m^2$ column. For a methanol-water (50:50) mobile phase at 30°C, the selectivity values for α/β were 1.22 and 1.02

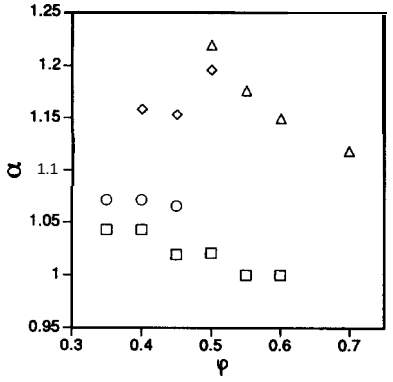

Fig. 2. Plot of the selectivity of estradiol-17α/estradiol-17β versus the volume fraction of methanol in the mobile phase for the 3.3 μmol/m² column (A) **without and (0) with, and the *2.5* μmol/m² column (Cl) without and (0) with 5% n-hexanol in the methanol fraction of the mobile phase.**

for the 3.3 and 2.5 **μmol/m²** columns, respectively. Fig. 2 shows the effect of the volume fraction of methanol, **φ,** on the selectivity of the **α/β** pair for both the 3.3 and 2.5 **μmol/m²** columns with and without 5% n-hexanol in the mobile phase. It is apparent that the high bonding density column yields higher selectivity, and that as the mobile phase strength is increased the selectivity between the pair is decreased. When 5% n-hexanol is added to the methanol fraction of the mobile phase, retention is decreased dramatically [with ***k'* (estradiol-17α)** values of 15.3 and 2.77 on the 3.3 **μmol/m²** column at **φ** = 0.5 without and with n-hexanol, respectively]. Despite this difference in mobile phase strength, the selectivity for the **α/β** pair remains fairly constant with values of 1.22 and 1.20 without and with n-hexanol, respectively. However, the relationship between a and ₀is entirely different for methanol-water systems and methanol-n-hexanol-water systems. While methanol-water mobile phases show a steady increase in selectivity as ₀is decreased, the system that contains 5% n-hexanol in the methanol does not show a predictable pattern of selectivity. The most plausible reason for this incongruity in the selectivity ***versus*** ₀plot is that while the strength of the mobile phase increases with increasing ₀ values, and thus should result in lower a values, the volume fraction of n-hexanol also increases. It is apparent that the n-hexanol is contributing to the selectivity of the system in a manner that is not found when just using methanol. Thus the increase in mobile phase strength is somewhat counterbalanced by the concomitant increase in n-hexanol concentration.

Because the estrogens contain polar hydroxyl functionalities, and because the shape difference between the estrogens was based on the position of the 17-hydroxyl moiety, further study was warranted. One concern was the effect of residual silanols on the separation selectivity. To ensure that the separation differences between the high and low bonding density columns were not caused by differences in the level of surface silanols, methanol-15 **m*M*** phosphate buffer **(50:50)** mobile phases were prepared with and without 0.2% triethylamine (TEA) at **pH** 3.0. The use of TEA to reduce the effects of residual silanols is well documented, and its effect should be most pronounced on the low bonding density column. Comparison of the selectivity of the **α/β** estradiol pair on the 2.5 **μmol/m²** column showed virtually no difference in selectivity upon the addition of 0.2% TEA. This provides clear evidence that the surface silanols are not a significant contributor to the separation.

While the estrogen separation improved by adding n-hexanol, it was not confirmed that the selectivity differences were caused by solvation or ordering of the stationary phase. This study was expanded to the **PAHs benzo[*c*]phenanthrene (BcP),** benz[a]anthracene **(BaA)** and naphthacene (NAP). The selectivity between **BcP,** the most angular solute of the three, and NAP, the most planar solute of the three, provided a measure of shape selectivity for different stationary phases. Using a **methanol–**water **(80:20)** mobile phase with the 2.5 and 3.3 **μmol/m²** columns yielded $\alpha_{NAP/BcP}$ values of 1.39 and 1.69, respectively. This increase in selectivity with bonding density is in agreement with previous work **[9].** Fig. 3 shows a plot of the selectivity of the BaA/BcP and **NAP/BcP** pairs as a function of ₀on the 2.5 **μmol/m²** column using mobile phases with and without 5% ***n*-hexanol** in the methanol fraction. The data for the methanol-water mobile phase show that the selectivity of the system is decreased in a regular manner with increasing methanol concentration.

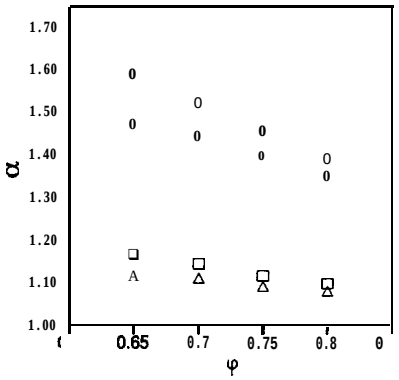

Fig. 3. Plot of the selectivity versus the volume fraction of methanol on the 2.5 μmol/m^2 column for the **NAP/BcP** pair (0) without and (0) with, and the **BaA/BcP** pair (0) without and (A) with 5% n-hexanol in the methanol fraction of the mobile phase.

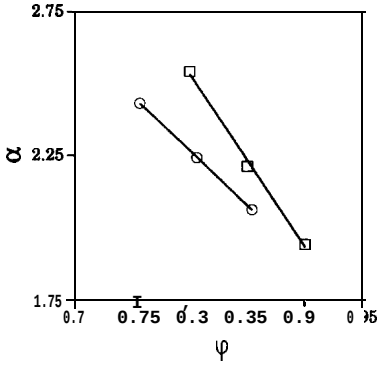

Fig. 4. Plot of the selectivity of the **TBN/BaP** pair *versus* the volume fraction of (□) methanol and (0) methanol with 5% n-hexanol on the 2.5 μmol/m^2 column.

The negative slope of the **NAP/BcP** pair is larger than that of the BaA/BcP pair, indicating that the mobile phase polarity is a significant contributor to the system selectivity. Similar trends are seen for the n-hexanol containing system in terms of the decrease in selectivity *versus* φ, and the steeper slope for the **NAP/BcP** pair relative to the BaA/BcP pair. The only substantial difference between the data is the magnitude of the selectivity values, which are consistently smaller for the n-hexanol containing mobile phase. This decrease was thought to occur because of the greater solvent strength of the n-hexanol containing mobile phase. In an effort to normalize for this solvent strength difference, the selectivity values obtained with both systems were compared at equivalent retention *(i.e.,* eluent strength). However, even at equivalent retention, no selectivity enhancement was observed by adding n-hexanol.

The test mix SRM 869 was also evaluated on both the 2.5 and 3.3 μmol/m^2 columns. Mobile phases with n-hexanol were prepared by adding 3% (v/v) n-hexanol directly to methanol-water mobile phases of fixed composition *(i.e.,* 80:3:20 methanol-n-hexanol-water). Fig. 4 is a plot of $\alpha_{TBN/BaP}$, the numerical indicator of shape selectivity, *versus* the volume fraction of organic, φ on the 2.5 μmol/m^2 column. As the volume fraction of organic is increased, the value of $\alpha_{TBN/BaP}$ is decreased. While a decrease in this term is typically associated with increased shape selectivity for a fixed mobile phase composition, these data indicate that the mobile phase strength can also produce changes in $\alpha_{TBN/BaP}$ that are not associated with shape selectivity. A thorough study of this phenomenon must include a normalization for this mobile phase strength effect. Examination of the $\alpha_{TBN/BaP}$ values for the mobile phase with 3% n-hexanol added show that the $\alpha_{TBN/BaP}$ values are consistently lower than the values obtained using methanol/water mobile phases at any composition. However, the 3% n-hexanol containing mobile phases are also stronger eluents than the corresponding methanol-water mobile phases of similar composition. Fig. 5 is a plot of $\alpha_{TBN/BaP}$ *versus* analysis

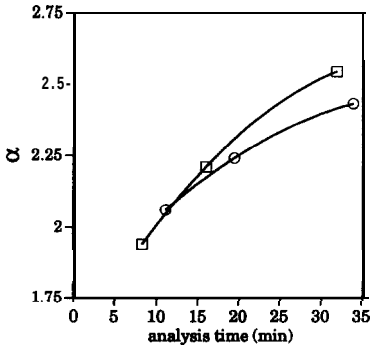

Fig. 5. Plot of the selectivity of the **TBN/BaP** pair *versus* analysis time for mobile phases using (Cl) methanol, and (0) methanol with 5% n-hexanol as the organic modifier on the 2.5 μmol/m^2 column.

time on the 2.5 $\mu mol/m^2$ column for mobile

magnitude of this change is small
pared to the overall selectivity range
and thus does not indicate that

PAHs le greater selectivity for the n d
SRM 869, improved separation of the estrogens was accomplished. To better understand the nature of these selectivity differences, a study of the temperature dependence of retention was conducted. Numerous scientists have examined the effect of temperature on chromatographic separations. The general findings are that as the temperature increases, retention decreases and
19].

The relationship often invoked to describe the temperature dependence of retention is known as the Van 't Hoff relationship and is expressed as

$$\ln k' = -\frac{\Delta H^0}{RT} \quad \frac{\Delta S^0}{} + \ln \Phi \qquad (2)$$

ΔH^0 a n d ΔS^0

Phase, the gas constant, T is the absolute temperature in K, and Φ is the volume phase ratio of the stationary and mobile phase respectively [20]. Experimentally, retention data is collected over a wide temperature range and the data are plotted as $\ln k'$ *vs.* $1/T$. Eqn. 2 predicts a linear relationship between these two variables, with the slope of the line equal to $-\Delta H^0/R$, and the intercept equal to $\Delta S^0/R + \ln \Phi$. Thus the thermodynamic constants ΔH^0 and ΔS^0 can be determined if the value of Φ is known.

In addition to the thermodynamic information [20–26]. These non-linear plots typically exhibit either a steady curve away from linearity, or distinct breaks from linearity at a particular temperature. The nature of these deviations has been debated. One theory suggests that as the temperature is varied high bonding density stationary phases undergo a phase transition from a more solid, ordered, state at low temperature to a more fluid, liquid- [8,21]. Thus the stationary phase retention properties are not homogeneous throughout the temperature range, and deviations from linearity would be predicted. It has also been postulated that the mobile phase properties, such as heat capacity and hydrogen bonding, may not remain constant throughout the temperature range which could also lead to deviations from predictable retention behavior [25,26].

Whether linear or non-linear Van 't Hoff behavior is observed, and regardless of the cause of any deviation from linearity, Van 't Hoff analysis can provide a qualitative assessment of retention mechanism changes that occur throughout the temperature range investigated. A series of chromatograms was collected on both the 2.5 and 3.3 $\mu mol/m^2$ column over a temperature range of 0 to 70°C. This study included the solutes estradiol-17α and -β, which had previously shown improved separation when n-hexanol was added to the mobile phase, and the PAHs BcP, BaA and NAP which showed no shape selectivity enhancement upon the addition of n-hexanol. Mobile phases were prepared by mixing methanol or 5% n-hexanol in methanol with water to appropriate compositions. Because of the polarity differences between the solutes, 50% methanol or methanol with n-hexanol mobile phases were used for the estrogens, while 80% methanol or methanol with n-hexanol mobile phases were used for the PAHs. The chromatographic experiments were performed as above except at temperatures of 20°C or lower, which required flow-rates of 1.0, 0.75 or 0.5 ml/min to compensate for increased system backpressure.

The results of these studies were not defini-

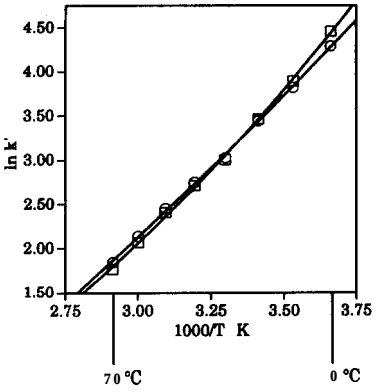

Fig. 6. Plot of In k' of (0) estradiol-17α and (Cl) estradiol-17β versus 1/T for the 2.5 μmol/m^2 column using a methanol-water (50:50) mobile phase.

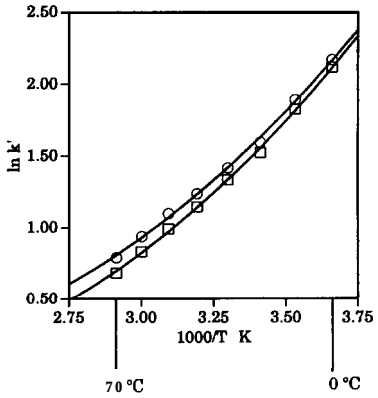

Fig. 7. Plot of In k' of (0) estradiol-17α and (□) estradiol-17β *versus* l/T for the 2.5 μmol/m^2 column using a methanol-n-hexanol-water (47.5:2.5:50) mobile phase.

tive, but gave insight into the nature of the selectivity effects seen. Figs. 6 and 7 show the Van 't Hoff plots of the estradiol isomers on the 2.5 μmol/m^2 column using methanol-water (50:50) and methanol-n-hexanol-water (47.5:2.5:50) mobile phases, respectively. The system that contained no n-hexanol shows a retention inversion of the α and β isomers at 30°C. This provided a clear explanation as to why the isomers were not separated at 30°C even under a wide range of mobile phase compositions, as illustrated by the selectivity values in Fig. 2. The addition of 2.5% n-hexanol to the system caused a shift in this retention inversion point to lower temperatures, allowing faster separation of the isomers in the room temperature regime.

The Van 't Hoff plot for the 3.3 μmol/m^2 column with methanol-water (50:50) mobile phase showed a retention inversion at 10°C, much lower than that of the 2.5 μmol/m^2 column. This inversion point is also shifted to lower temperature with the addition of *n*-hexanol, such that coelution does not occur over the entire temperature range investigated. The shift to lower temperature of the retention inversion point is observed both when n-hexanol is added, and when the bonding density is increased. If the n-hexanol is inducing greater ordering of the C_{18} chains to change the retention process, then this behavior should also be seen for the PAH solutes.

Figs. 8 and 9 show Van 't Hoff plots of the PAH data obtained on the 3.3 μmol/m^2 column using methanol-water (80:20) or methanol–n-hexanol-water (76:4:20) mobile phases. There are several trends worth noting. First, as the temperature is decreased, not only is retention increased, but the changes in the slope of the plot are solute dependent. The curves for NAP and BcP show distinct curvature toward higher and lower retention, respectively, as the temperature is decreased. The net result of this retention trend is that higher shape selectivity is seen at low temperatures, which is in agreement with previous work [8,24]. Similar results were observed for the 2.5 μmol/m^2 column, though at

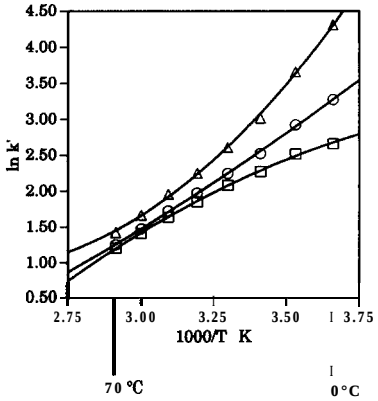

Fig. 8. Plot of In k' of (A) **NAP, (0) BaA, and (Cl) BcP for the 3.3 μmol/m^2 column using a methanol-water (80:20) mobile phase.**

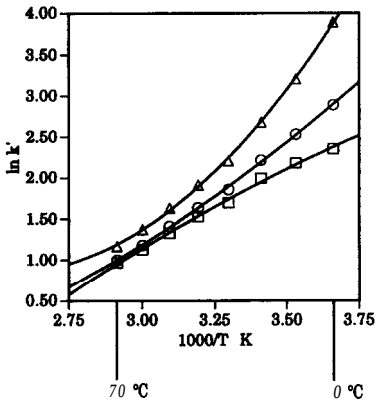

Fig. 9. Plot of ln k' of (A) NAP, (0) BaA, and (0) BcP for the 3.3 μmol/m^2 column using a methanol-n-hexanol-water (76:4:20) mobile phase.

any given temperature the selectivity was always highest for the 3.3 μmol/m^2 column. The temperature induced selectivity increase is consistent with greater chain ordering at low temperatures in that the linear NAP was progressively more retained while the angular BcP was progressively less retained as the temperature was decreased. If surface ordering were occurring at lower temperatures, as has been shown by Sander and Wise, one would expect that the linear, planar solutes such as NAP would partition more readily than bent or torqued solutes such as BcP [8].

When n-hexanol was added to the mobile phase, there was a decrease in retention as expected, but the basic shape of the plot remained the same as in the methanol-water mobile phase case. If increased ordering were induced by the addition of n-hexanol, the magnitude of the "breaks" from linearity in the Van 't Hoff plot for NAP and BcP should be more pronounced, and higher shape selectivity should be seen for the n-hexanol containing mobile phase.

Clearly if surface ordering is occurring from stationary phase solvation by n-hexanol, the effect is insignificant when compared to that of stationary phase bonding density and system temperature. A recent study by Montgomery et al. [27] examined the effect of water, methanol–water (80:20), and n-propanol-water (5:95) on the contact angle measurements and frequency-domain fluorescence anisotropy of a probe molecule on a C_{18} silica surface. The results showed that while the addition of alcohol to the system provided better interfacial wetting of the surface, "The results do not support the idea that a small amount of alcohol causes the C_{18} chains to become extended toward the surface normal" [27].

The enhanced separation of the estrogen pair is most likely a result of a solvent selectivity difference between methanol and the higher n-alcohols. While methanol is in the same solvent family as the higher alcohols, Snyder noted that for solvents that undergo strong self-hydrogen bonding, such as alcohols and amides, changes in selectivity between lower and higher homologues can be significant [16]. This type of interaction could well explain the selectivity differences seen in these experiments.

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