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Adjusting Selectivity in Liquid Chromatography by Use of the Thermally Tuned Tandem Column Concept

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In this study, we propose the novel "thermally tuned tandem column (T^3C)" concept for the optimization of selectivity in LC by continuous adjustment of the stationary phase. Two columns with distinctly different chromatographic selectivities (e.g., polybutadiene- and carbon-coated zirconia) are serially coupled and *independently* temperature-controlled. Selectivity is "tuned" by adjusting the individual temperatures of the two columns. The effect of changing column temperature is quite analogous to changing the relative column lengths, thereby altering the relative and absolute contribution each column makes to the overall retention time in T^3C. The distinct selectivity differences between polybutadiene- and carbon-coated zirconia as well as the extraordinary thermal stability of zirconia-based phases (thermally stable to 200 °C) allow us to tune the overall chromatographic selectivity over a very substantial range. We have developed a simplified useful model, which characterizes retention and selectivity for the T^3C system as a function of the two column temperatures. The model is in good agreement with the experimental results. We also describe a simple computer-assisted optimization strategy based on the window diagram method, which facilitates the optimization of the T^3C system with only four or five initial runs.

Reversed-phase liquid chromatography (RPLC) is the analytical method of choice for the separation of the preponderance of nonvolatile compounds ranging from small polar molecules to large biological macromolecules. To develop a RPLC separation method, major emphasis is usually placed on optimizing the chromatographic selectivity because it has the most dramatic impact on resolution.[1−3] A useful approach to optimizing selectivity is to choose an appropriate stationary phase and adjust the mobile phase (both the type and volume composition) until an acceptable separation is achieved. If this does not work, other conditions such as temperature and pH are then adjusted. Only when the above fails does one finally search for a new stationary phase which affords a different selectivity. This trial-and-error method is extremely time-consuming, and its success depends strongly on the experience of the chromatographer.

Snyder has critically analyzed and carefully compared the ability of all the common experimental variables (mobile phase type, composition, pH, temperature, stationary phase type) for their ability to adjust selectivity in RPLC.[4] He found that varying the mobile phase type (from acetonitrile to tetrahydrofuran or from methanol to tetrahydrofuran) usually has the greatest effect on selectivity, followed by changing the stationary phase type (e.g., among C8, phenyl, and cyano columns) as well as changing mobile phase volumetric composition (%B). Varying temperature has the least effect but quite often a useful impact on band spacing especially when used in combination with mobile phase type or %B. However, in a study in progress in this laboratory using a much more diverse set of reversed-type stationary phases with a fixed set of twenty-two judiciously selected but highly variegated nonelectrolyte probe solutes, we find that the largest changes in selectivity are brought about by varying the stationary phase type, particularly when an aliphatic bonded phase or coated-polymer phase is compared with a carbon phase or with an aromatic polymer-coated phase.

In addition to the ability to change selectivity, practical convenience is another important factor when choosing the best experimental variable to improve separation.[4] Temperature and %B are the most convenient variables to manipulate. However, varying the mobile phase type and the stationary phase type, which in our opinion have a greater effect on selectivity, both suffer from practical problems such as slow column equilibration. More importantly, in contrast with changing the mobile phase type which can be done gradually by using a ternary mixture, changing the stationary phase type is a totally discontinuous operation and cannot be used to systematically and continuously adjust selectivity. Thus it is very desirable to have an approach in which selectivity can be changed significantly, systematically, and continuously in such a way as to allow the optimum conditions to be easily located by a computer.

In this study, we propose the *"thermally tuned tandem column (T^3C)"* optimization concept in response to this need. The basic concept is shown schematically in Figure 1. It shows that two columns which *must have decidedly different chromatographic selectivities* are serially coupled and held in two *independently*

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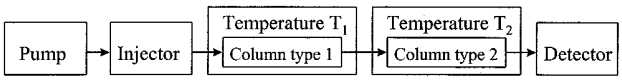

Figure 1. Block diagram of the T^3C system.

controllable temperature zones. Selectivity can be "tuned" by adjusting the individual temperatures of the two columns. The effect of changing temperature is quite analogous to varying the relative column lengths (see below); thus, changing the difference in column temperatures changes the contribution that each column makes to the overall retention time in T^3C. Temperature can change the selectivity of T^3C dramatically, continuously, and conveniently only if two radically different stationary phases are coupled. Fortunately, such phases are available.

T^3C can be considered to be an optimal combination of adjusting selectivity by varying stationary phase type and temperature. When temperature is varied on a single column, the resulting changes in selectivity for nonelectrolytes are small because differences in enthalpies of retention for closely related *nonionic* compounds are not large in RPLC. In this new T^3C approach, the change in selectivity is brought about by effectively and continuously changing from one type of column to a second type, while the practical problems of automatically switching columns are circumvented by connecting the columns in series.

The T^3C concept is *not* a form of multidimensional chromatography. Rather, the T^3C concept is closely related to the seminal concept of Laub and Purnell in their use of mixed stationary phases in gas chromatography (GC).[5,6] They studied the selectivity of the mixed stationary phase and introduced the "window diagram" concept in which selectivity or resolution of the worst separated pair of peaks was plotted versus some operating variables. Later, Purnell and co-workers described the coupling of two capillary columns and selectivity tuning by varying the length of the columns.[7,8] A more elegant approach is to use fixed-length columns and then tune selectivity by adjusting the carrier gas flow,[9−12] the temperature of the individual columns,[13−16] or both.[17] This technique has been used successfully and extensively to optimize separations of complex samples in GC.[9−21]

There have been numerous reports concerning the use of tandem columns in liquid chromatography, but most are used to address specific problems in column switching arrays[22−25] or in multidimensional chromatographic systems.[26−28] Only a few attempts have been made to adjust selectivity with mixed stationary phase or tandem columns in LC.[29−31] We believe that one of the key reasons for this situation is the long-held belief that retention and selectivity in RPLC are dominated by the mobile phase (i.e., the solvophobic theory).[32] Thus, the effect of the type of stationary phase on selectivity in HPLC has been underestimated. However, with the further elucidation of retention mechanisms in RPLC, more and more reports indicate that the stationary phase also plays a vital role.[33−36] As a result, many new RPLC phases that offer unique and useful selectivities have been synthesized.[37−46]

Another reason that tunable selectivity is difficult to achieve is that in LC, unlike GC, there has been no practical variable available for adjusting the tandem column selectivity. Glajch and co-workers coupled a cyano column and a benzyl column to separate phenylthiohydantoin amino acid derivatives, and the selectivity was adjusted by varying the size of the two columns.[29] Issaq and co-workers used a mixed-phase column for the separation of antidepressants and anticonvulsants; and they found that the retention factor on the mixed-phase column is not linearly related to the stationary-phase composition.[30] However, it is not practical to prepare columns with a specific mixture of stationary phases or cut very expensive LC columns to a certain length for a single specific application. Temperature, as an operating variable for tuning selectivity, has until recently been almost neglected in LC. This is because temperature has a smaller effect on retention in LC than in GC and has only a small, albeit sometimes very important, effect on selectivity.[1,47−49] More importantly, most

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conventional bonded phases which dominate the practice of RPLC are thermally unstable above 50−60 °C which limits the use of temperature as a dimension for varying selectivity. Novel zirconia-based RPLC phases are thermally stable to at least 200 °C.[50−54] This stability provides precisely the tool needed to make the T^{3}C concept useful.

In the present study, we will first describe the theory of the T^{3}C concept as to how temperature can influence the chromatographic selectivity and then present an example to demonstrate the separating power of T^{3}C.

THEORY SECTION

Similar to the fundamental relationships developed in GC for tandem columns,[20,21] the basic retention characteristic for columns connected in series is additive. We can write the relationships between the net dead time ($t_{n,0}$) and the net retention time ($t_{n,i}$) of the T^{3}C system and the individual columns as follows

$$t_{n,o} = t_{1,o} + t_{2,o} \tag{1}$$

$$t_{n,i} = t_{1,i} + t_{2,i} = t_{1,o}(1 + k'_{1,i}) + t_{2,o}(1 + k'_{2,i}) \tag{2}$$

where the first subscript denotes the column system and the second subscript denotes the solute; the subscript n refers to the net system and 1 and 2 refer to the first and second column, respectively. The above equations are strictly correct if the fluid is incompressible and the retention factor is independent of pressure. These are not bad first approximations for HPLC. The net retention factor of species i can then be expressed as

$$k'_{n,i} = \theta_1 k'_{1,i} + \theta_2 k'_{2,i} \tag{3}$$

where θ_1 and θ_2 are the dead time fractions of the first column ($t_{1,o}/t_{n,o}$) and second column ($t_{2,o}/t_{n,o}$), respectively.

The net selectivity for compounds i and j is:

$$\alpha_n = \frac{k'_{n,j}}{k'_{n,i}} = \frac{\theta_1 k_{1,j} + \theta_2 k_{2,j}}{\theta_1 k_{1,i} + \theta_2 k_{2,i}} \tag{4}$$

For T^{3}C, temperature affects the net selectivity by changing the retention factors of compounds i and j on each column. The effect of temperature on retention factor is usually described by the empirical form

$$\ln k' = A + B/T \tag{5}$$

where A and B are constants for a given solute and T is the absolute temperature. B is related to the enthalpy change for the solute transferring from the mobile to the stationary phase. For most RPLC systems, B is positive, meaning that an increase in temperature leads to a decrease in the retention factor, which is analogous to shortening the column length.

It is interesting to consider some limiting cases. In the limit that the retention factors on the second column ($k_{2,i}$ and $k_{2,j}$) are negligible compared with the retention factors on the first column ($k_{1,i}$ and $k_{1,j}$) due to the high temperature of column 2, the net selectivity of the system is reduced to the selectivity factor on column 1. Similarly, if the temperature on column 1 is much higher than that on column 2, so that the retention factors on the first column are negligible, the overall selectivity is the same as that on the second column. At a more equal intermediate temperature, the net selectivity can be adjusted to any value between α_1 and α_2. We call this thermal selectivity "tuning".

For a single column, the effect of temperature variation on selectivity depends on the difference in the two enthalpy changes for solutes i and j. The effect is usually not large, because for similar molecules which are hard to separate, the enthalpy difference between solutes i and j is usually small. *However, temperature affects the selectivity for the T^{3}C system in a totally different manner*. Temperature plays a role in tuning selectivity by changing the relative retention times of the solutes on the individual columns, thus changing the relative contribution of the selectivity of each stationary phase to the T^{3}C system. As long as column 1 and column 2 exhibit different selectivities, a small change in T_1 or T_2 can have a substantial effect on the overall selectivity.

To show more clearly how the selectivity of the T^{3}C (α_n) is related to the selectivity of the individual column ($\alpha_1 = k'_{1,j}/k'_{1,i}$, $\alpha_2 = k'_{2,j}/k'_{2,i}$), eq 4 can be rewritten as

$$\alpha_n = \frac{\theta_1 k'_{1,i}}{k'_{n,i}}\alpha_1 + \frac{\theta_2 k'_{2,i}}{k'_{n,i}}\alpha_2 \tag{6}$$

We can define

$$f_{1,i} \equiv \frac{\theta_1 k'_{1,i}}{k'_{n,i}}$$

and

$$f_{2,i} \equiv \frac{\theta_2 k'_{2,i}}{k'_{n,i}}$$

where $f_{1,i}$ and $f_{2,i}$ are the fractions of retention time of compound i on the first and second column, respectively. The values of $f_{1,i}$ and $f_{2,i}$ can be *continuously* adjusted by varying the isothermal temperature of each column. By definition, $f_{1,i} + f_{2,1} = 1$, eq 6 can be rewritten as follows:

$$\alpha_n = f_{1,i}(\alpha_1 - \alpha_2) + \alpha_2 \tag{7}$$

If α_1 and α_2 are assumed to be independent of temperature (because the effect of temperature on the selectivity of two similar species is usually small), for two α_n at two different temperatures, the change of the selectivity $\Delta\alpha_n$ with respect to temperature can

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be expressed as

$$\Delta\alpha_n = \alpha_n(T_2) - \alpha_n(T_1) = (f_{1,i}(T_2) - f_{1,i}(T_1))(\alpha_1 - \alpha_2)$$
$$= \Delta f_{1,i}\Delta\alpha \quad (8)$$

where $\Delta f_{1,i}$ is the difference between $f_{1,i}$ at two temperatures. Equation 8 shows that the ability to change selectivity by varying temperature for a T^3C system depends on the tunable range of $f_{1,i}$ as well as the selectivity difference between the two tandem columns. Notice that $\Delta\alpha$ is actually related to the stationary phase type controlled selectivity. This clearly shows that the T^3C system changes the selectivity in the same manner as does the stationary-phase type, except that instead of changing selectivity discontinuously, we can continuously "tune" the selectivity of the T^3C.

EXPERIMENTAL SECTION

Instruments. All chromatographic experiments were conducted with a Hewlett-Packard 1100 chromatography system, equipped with a quaternary pump, a vacuum degasser, an autosampler, a thermostated-column compartment, a variable-wavelength UV detector, and a computer-based Chemstation (Hewlett-Packard S. A., Wilmington, DE). An additional experimental heating apparatus from Systec (Systec, Inc., Minneapolis, MN) was used as a second heating zone when carrying out the T^3C experiments. The heating apparatus consists of a mobile phase preheater and a column heating jacket which can heat the column up to 200 °C. A pressure regulator (about 30 bar) was installed at the outlet of the detector to prevent boiling of the mobile phase.

Analytical Columns. A 10 cm × 4.6 mm i.d. carbon-coated zirconia (C-ZrO_2) column and polybutadiene-coated zirconia (PBD-ZrO_2) particles were provided by ZirChrom (ZirChrom Separations, Inc., Anoka, MN 55303). The PBD-ZrO_2 particle has 3% carbon content and the particle size is about 3 μm. A 10 cm × 4.6 mm i.d. PBD-ZrO_2 column was packed using the upward stirred slurry method.[55]

Reagents. All reagents used here are reagent-grade or better. ChromAR HPLC grade acetonitrile (Mallinckrodt Chemical Co., Paris, KY) was used as the organic modifier for LC. HPLC water was obtained from Barnsted Nanopure deionizing system (Dubuque, IA) with an "organic-free" cartridge followed by a 0.2-μm particle filter. The water was boiled to remove carbon dioxide. All solvents were filtered through a 0.45-μm filter (Lida Manufacturing Corp., Kenosha, WI) before use. Solutes used in this study were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI).

Chromatographic Conditions. All measurements were made at a flow rate of 1 mL/min and detected at a wavelength of 254 nm with a 1-μL injection of sample. The dead time was determined by injecting D_2O. The flow rate used in this work was not corrected for thermal expansion of the mobile phase inside the hot columns as required if our objective were to obtain enthalpies of transfer. It can be shown that thermal expansion of the mobile phase introduces a multiplicative error in both the apparent (uncorrected) retention times and dead times, which cancels out when the retention factors are computed. As shown below, the *apparent* column dead time does vary with column temperature. We therefore calculated the retention factor on the basis of the apparent dead time measured at that temperature. The apparent retention times at other temperatures are calculated using dead times estimated by interpolation from the measured apparent dead time with an interpolation scheme based on an equation of the same form as the one we use to predict retention factors. The resolution is conveniently estimated on the basis of eq 9:

$$R_s = \frac{\sqrt{N}}{4}\frac{t_{r2} - t_{r1}}{t_{r\ average}} \quad (9)$$

in which R_S is resolution, $t_{r\ average}$ is the average of the retention times, and N is the theoretical plate number. N is assumed to be 5000 for a single 10-cm column and 10 000 for the two column T^3C set.

Predicting Retention on the T^3C System. In the theory section, we assume that the total retention time on the tandem columns is the simple sum of the retention on each column. However, when two columns of equal dimensions are serially coupled, the inlet pressure at the first column is about twice as high as it is when it is not coupled. To calculate the total retention time as the sum of the retention times on individual columns, we must neglect the compressibility of the mobile phase, as well as assume that retention is independent of the total applied pressure.

Neglecting the mobile phase compressibility is not a bad approximation because pressure usually has minor effects on condensed phases such as liquids. The effect of pressure on retention is usually minor, but a few studies raise some concern that pressure might significantly influence retention.[56,57] We therefore tested the accuracy of eq 2 by comparing the experimental and estimated T^3C retention times. Table 1 shows the experimental retention times of four alkylbenzenes on PBD-ZrO_2 and C-ZrO_2 and on the T^3C system with different column orders under the same chromatographic conditions. Theoretical T^3C retention times were then calculated using a modification of eq 2

$$t_{n,i} = t_{1,i} + t_{2,i} - t_{ex} \quad (10)$$

where t_{ex} is the time that a solute spends outside the column (in

Table 1. Comparison of Experimental and Calculated Retention Times (t_R) on the T^3C System

	expertl t_R (min)[a]				calcd t_R (min)[b]	
solutes	PBD-ZrO_2	C-ZrO_2	PBD-ZrO_2 + C-ZrO_2	C-ZrO_2 + PBD-ZrO_2	T^3C	% error[c]
toluene	2.02	3.37	5.31	5.32	5.32	0.21
ethylbenzene	2.51	4.02	6.43	6.45	6.46	0.26
propylbenzene	3.37	6.25	9.52	9.57	9.56	0.15
butylbenzene	4.83	10.22	14.92	15.04	14.98	0.01

[a] Measured in 50/50 acetonitrile/water mixture at 30 °C and flow rate of 1 mL/min. [b] Calculated using eq 10. [c] % error = 100 (calculated T^3C t_R − average experimental T^3C t_R)/average experimental T^3C t_R.

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the injector, connection tubing, and detector). The value of t_{ex} was determined to be 0.067 min by injecting uracil without any column under chromatographic conditions which otherwise were the same. Equation 10 was used because the measured retention time ($t_{1,i}$ and $t_{2,i}$) on each individual column consists of the "true" retention time and the extra-column time. Therefore, t_{ex} is counted twice upon using eq 2. The calculated T^{3}C retention times via eq 10 are also listed in Table 1 and compared with the average of the experimental T^{3}C retention time. They agree with each other very well with an error of less than 0.3%. Thus, eq 10 is deemed accurate enough for our estimation purposes, and pressure effects seem negligible. We want to point out that, in this study, the mobile phase was prepared by weighing the proper amount of water and acetonitrile which were premixed before use. We avoided the machine-mixed mobile phase because we suspect that machine-made mobile-phase compositions might vary somewhat with a change in column back pressure.

RESULTS AND DISCUSSIONS

Dramatically Different Selectivity of C-ZrO$_2$. In order for the net selectivity in T^{3}C to be substantially and usefully tunable, there must be a significant difference in band spacing on the pair of coupled columns (see eq 8). It is therefore critical to locate two stationary phases with maximally different selectivities. The so-called $\kappa-\kappa$ plot, in which log k' for one stationary phase is plotted versus log k' for a second stationary phase as solute is varied, was used to evaluate selectivity differences between stationary phases. Horvath first used $\kappa-\kappa$ plots to compare the energetics of retention on alkyl-silica-bonded phases.[58] According to Horvath, a linear correlation means the same or a similar retention mechanism is operating on both columns, while a weak correlation indicates different retention mechanisms and different selectivities. Snyder further used the average standard deviation (s.d.) of the linear regression of $\kappa-\kappa$ plots as a quantitative criterion to compare selectivity differences between different stationary phases.[4] The thermal stability of zirconia-based HPLC materials makes them highly suitable for use in the T^{3}C concept. We therefore compared the selectivity difference between two zirconia-based phases (PBD-ZrO$_2$ versus C-ZrO$_2$) in Figure 2. The twenty-two nonionic solutes used in this study (for a list, see ref 59) were carefully chosen to cover a wide range of physicochemical properties (size, dipolarity, etc.), and all experiments were done in a 50/50 acetonitrile/water mobile-phase mixture.

The $\kappa-\kappa$ plot for C-ZrO$_2$ and PBD-ZrO$_2$ shows very considerable scatter of the retention data with only a minimal correlation ($r^2 = 0.401$). This indicates the dramatically different selectivities between these two phases. It is much more likely that two compounds will be well-resolved on PBD-ZrO$_2$ if they cannot be separated on C-ZrO$_2$ and vice versa. For instance, solutes a and b (see Figure 2) fall on almost the same vertical line, meaning they almost coelute on PBD-ZrO$_2$, but obviously they are well-resolved on C-ZrO$_2$. Similarly, solute c and d separate very well on PBD-ZrO$_2$, but coelute on C-ZrO$_2$. Therefore, a change from a PBD-ZrO$_2$ column to a C-ZrO$_2$ column will have a profound effect on selectivity. Carbon adsorbents as HPLC materials have recently drawn increasing attention.[37,40,60–66] C-ZrO$_2$ phase shows superior

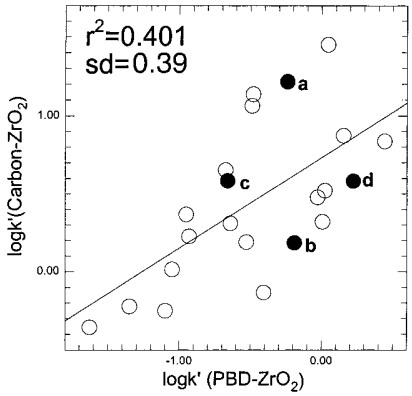

Figure 2. Plot of log k' on C-ZrO$_2$ versus log k' on PBD-ZrO$_2$ for twenty-two selected solutes in a 50/50 acetonitrile/water mixture at 30 °C. Solid line denotes the least-squares line, and a, b, c, d denote four different solutes (●).

chemical stability and unique selectivities toward isomers and diasteromers.[61,64] A LSER study qualitatively shows that C-ZrO$_2$ differs from conventional alkyl-bonded phases in that C-ZrO$_2$ possesses higher polarizability and higher hydrophobicity.[65]

Zhao and Carr used $\kappa-\kappa$ plots to compare retention characteristics of six reversed phases including both aromatic and aliphatic phases.[59] The lowest correlation was observed with polystyrene-coated zirconia and octadecylsilane (ODS) phases ($r^2 = 0.92$). Comparing their results with the selectivity difference between C-ZrO$_2$ and PBD-ZrO$_2$ ($r^2 = 0.401$, s.d. = 0.39), we conclude that C-ZrO$_2$ is one of the most different reversed-phase materials from conventional alkyl-bonded phases. By putting PBD-ZrO$_2$ and C-ZrO$_2$ in series, we can tune the selectivity over a wide range by manipulating the two column temperatures. Data not given here show that PBD-ZrO$_2$ and ODS have very much the same selectivity for the twenty-two probe solutes studied here, and thus, the ODS could be used in the T^{3}C system provided that its temperature was not set too high.

Effect of Temperature on Selectivity for a Single Column. In a similar manner, the $\kappa-\kappa$ plot can be used to characterize the effect of temperature on selectivity for a single column. In Figure 3, we plot the log k' of the same set of solutes at one temperature versus log k' at another temperature on C-ZrO$_2$ columns. For a temperature change from 30 to 130 °C on C-ZrO$_2$ at 40/60 acetonitrile/water mixture, the correlation coefficient is 0.974. Comparing the correlation coefficients in Figures 2 and 3 indicates that changing stationary-phase type (from PBD-ZrO$_2$ to C-ZrO$_2$) is a much more effective approach to varying chromatographic selectivity than changing temperature. It is important to note that temperature changes the T^{3}C selectivity in the same manner as does stationary-phase type; except that, in T^{3}C, selectivity can be tuned continuously and conveniently by adjusting the temperature. We point out again that the twenty-two solutes selected for this

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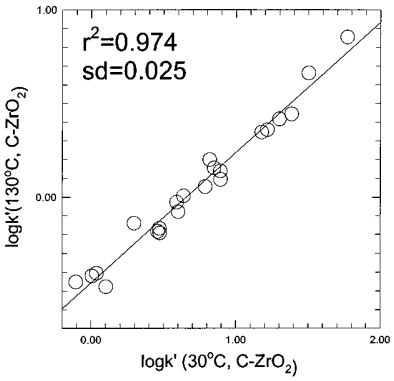

Figure 3. Plot of log k' at 30 °C versus log k' at 130 °C for the twenty-two selected solutes on C-ZrO_2 columns in a 40/60 acetonitrile/water mixture. Solid line denotes the least-squares line.

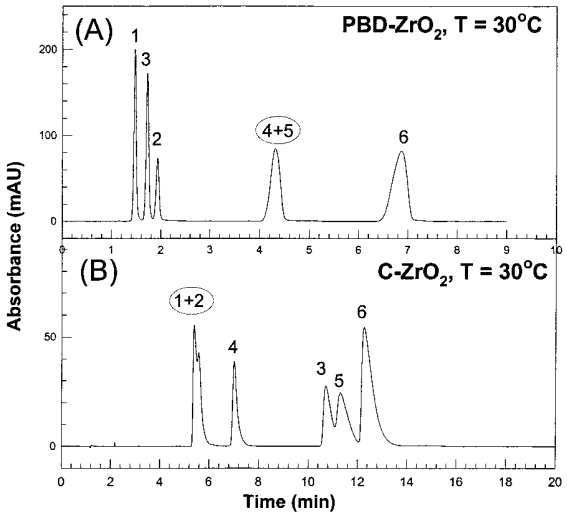

Figure 4. Chromatograms showing the separation of the mixture on PBD-ZrO_2 (A) and C-ZrO_2 (B) at 30 °C. Experimental conditions: mobile phase, 40/60 acetonitrile/water; flow rate, 1 mL/min. Solutes: 1, benzonitrile; 2, anisole; 3, methylbenzoate; 4, ethylbenzene; 5, *p*-xylene; 6, *n*-propylbenzene.

study are all nonelectrolytes, and it is usually the case that, for ionic compounds, temperature can have a much larger effect on selectivity. But our point is that, in general, for nonelectrolytes, temperature has far less effect on changing the selectivity on a single column than on a T^3C system.

Selectivity Tuning for T^3C. Figure 4 shows the separation of a six-component mixture on PBD-ZrO_2 (plot A) and on C-ZrO_2 (plot B) at 30 °C in a 40/60 acetonitrile/water mixture. The solutes and their retention factors are listed in Table 2. As can be seen from plot A, compound 4 (ethylbenzene) and compound 5 (*p*-xylene) coelute on PBD-ZrO_2. This is expected because the two compounds are structural isomers with the same molecular volume and hydrophobicity. An aliphatic phase like PBD-ZrO_2 or ODS, which interacts with solutes mainly by a partition mechanism, usually cannot discriminate between such similar structural isomers. In contrast, carbon-based supports are well-known for their superior selectivity toward structural isomers due to the rigid and highly polarizable carbon surface.[40,61,64] Plot B does show that compound 4 and 5 are well separated with an α of 1.56. However, C-ZrO_2 could not totally resolve the mixture because compounds 1 and 2 almost coelute; also, compounds 3 and 5 were not baseline-resolved. This clearly shows the disadvantage of varying stationary phase type since selectivity can only be changed discontinuously.

Table 2. Experimental Retention Factors of the Solutes on PBD-ZrO_2 and C-ZrO_2

solutes	k' (PBD-ZrO_2)[a] 30 °C	k' (C-ZrO_2)[a] 30 °C	70 °C	130 °C
(1) benzonitrile	0.30	3.68	1.98	0.83
(2) anisole	0.71	3.86	2.08	0.89
(3) methylbenzoate	0.52	8.87	4.58	1.79
(4) ethylbenzene	2.81	5.09	2.71	1.09
(5) *p*-xylene	2.81	8.37	4.32	1.63
(6) propylbenzene	5.04	9.89	4.99	1.78

[a] Measured in a 40/60 acetonitrile/water mixture.

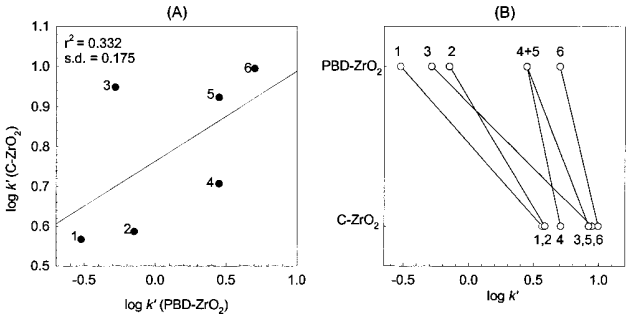

Figure 5. (A) Plot of log k' on C-ZrO_2 at 30 °C versus log k' on PBD-ZrO_2 at 30 °C for the mixture in a 40/60 acetonitrile/water mixture. (B) Comparison of elution order (log k') of the mixture on PBD-ZrO_2 and C-ZrO_2. Circles on the top row represent the retention data on the PBD-ZrO_2 column, and the circles on the bottom row denote the retention data on the C-ZrO_2 column. Solutes: 1, benzonitrile; 2, anisole; 3, methylbenzoate; 4, ethylbenzene; 5, *p*-xylene; 6, *n*-propylbenzene.

A κ–κ plot is shown in Figure 5 (plot A) to quantitatively compare the selectivity difference between PBD-ZrO_2 and C-ZrO_2 toward this specific mixture. The small correlation coefficient (r^2 = 0.332, s.d. = 0.175) indicates that the two stationary phases do show drastically different selectivities. However, the κ–κ plot only provides the general or average selectivity change for the compounds in the mixture; *the much more important factor is the change in selectivity for the critical pair, the pair having the worst resolution*. In plot B, we plot the retention data on the two phases against their respective log k' values. The circles on the top row are the retention data on PBD-ZrO_2; while the circles on the bottom row represent the retention data on C-ZrO_2. The solid lines simply connect the same compounds on the two phases. The difference in elution order on PBD-ZrO_2 and C-ZrO_2 phases is clearly shown in plot B by the crossover of the solid lines. But more importantly, it shows that the critical pairs on one phase can be resolved on the other phase, which means that T^3C will be useful for improving the separation.

Figure 5B also shows that, under the same chromatographic conditions, the retention factors of the six solutes on C-ZrO_2 are two to fifteen times larger than those on PBD-ZrO_2, meaning that the overall selectivity in T^3C will be dominated by the C-ZrO_2 column if both columns are held at 30 °C. We therefore decided to increase the temperature on the C-ZrO_2 column and decrease the relative contribution it makes to the T^3C system, thus gradually

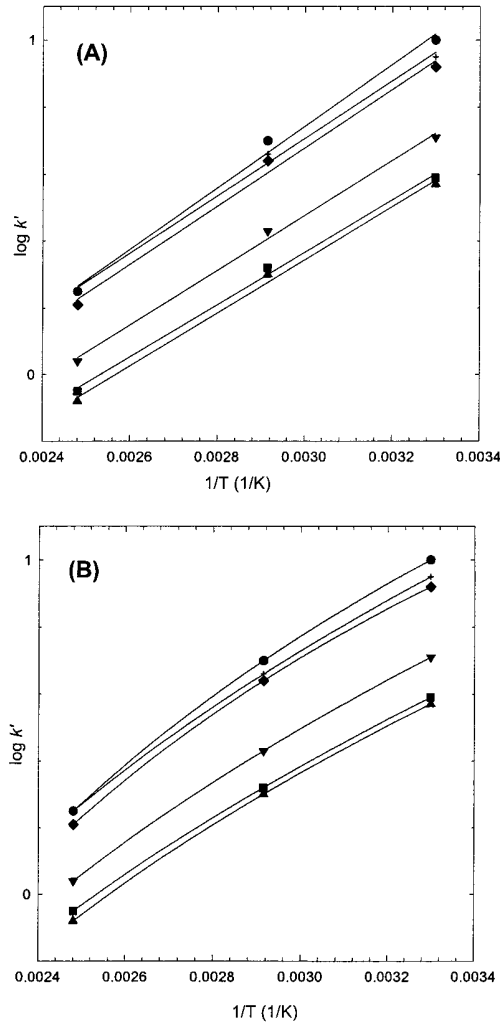

Figure 6. Plot of log k' versus inverse temperature on C-ZrO_2 in a 40/60 acetonitrile/water mixture. The points are experimental retention factors at 30, 70, and 130 °C. In plot A, the solid lines are linear regression lines, and in plot B, the solid lines are the regression lines according to eq 11. Solutes: (▲), benzonitrile; (■), anisole; (▼), ethylbenzene; (◆), *p*-xylene; (+), methylbenzoate; (●) *n*-propylbenzene.

adjusting the overall selectivity toward that of the PBD-ZrO_2 system.

Two additional data sets on C-ZrO_2 at 70 and 130 °C were acquired, and the retention factors are listed in Table 2. Comparison of the retention factors at 30 °C and those at 130 °C on C-ZrO_2 shows that for a 100 °C increase in temperature, the retention factors decrease an average of 5-fold. This is analogous to shortening the C-ZrO_2 column 5-fold and thus significantly decreasing the carbon column's contribution to the net retention in the T^3C system. We plot log k' on C-ZrO_2 versus the reciprocal of the temperature in Figure 6. In plot A, eq 5 ($\log k' = A + B/T$) was used to fit the data, and the solid line denotes the linear regression line with correlation coefficients ranging from 0.993−0.996. Downward curvature was observed for all solutes, suggesting that eq 5 might not be accurate enough to predict the retention factors at other temperatures on the carbon column. To fully use the three initial measurements and achieve more accurate predictions, a three-parameter equation was used to fit the data (as

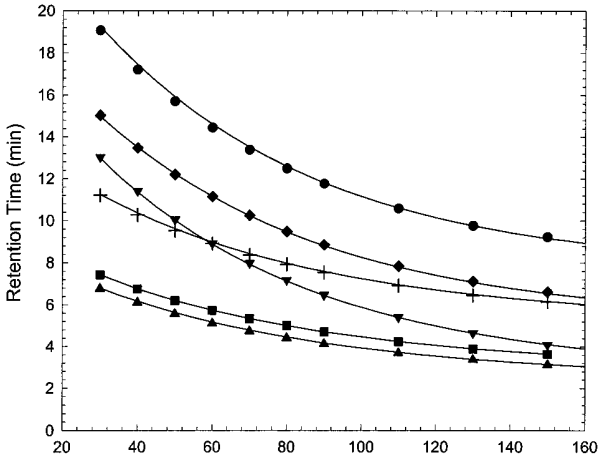

Figure 7. Plot of retention times in T^3C as a function of the temperature on the C-ZrO_2 column. The solid lines are the calculated retention times, and the points are experimental retention times. The symbols are the same as in Figure 6.

shown in Figure 6B)

$$\ln k' = A + B/T + C \ln T \qquad (11)$$

where A, B, and C are constants. We want to point out that it makes no practical difference which of many three-parameter equations we use to estimate retention time. In fact, excellent predictions of the retention times can be obtained by using other three-parameter (e.g., $\ln k' = A + B/T + C/T^2$) or even some two-parameter (e.g., $\ln k' = A + BT$) equations. As long as the equation can account for the deviation from linearity in the van't Hoff plot, it will allow interpolation of the retention times accurately enough for our optimization purposes; and the optimum condition predicted (see below) is essentially independent of which equation we choose. In Figure 6B we fit log k' of each solute to eq 11 as shown by the regression line. The resulting equation was then used to estimate k' on the carbon column at other temperatures.

Window Diagram Optimization for the T^3C System. We further calculated the T^3C retention time via eq 10. Figure 7 shows a plot of predicted T^3C retention times (lines) versus the temperature on the carbon column while keeping the PBD column at 30 °C. Note that when two lines in Figure 7 cross over, coelution of two solutes will occur at the corresponding temperature, while some degree of separation will occur at the other temperatures. To locate the optimum temperature more easily and precisely, a window diagram was constructed on the basis of the plots in Figure 7. The resulting window diagram is shown in Figure 8, in which the resolution of the most poorly separated pair (specified by the numbers above the curve) is plotted against the temperature of the carbon column. The window diagram indicates that adequate separation ($R_s > 1.5$) is achieved at many different carbon-column temperatures even though the mixture did not separate on either column at 30 °C. We choose a carbon column temperature of 90 °C as the optimum temperature since it is in the middle of the highest and rather flat "window". The circles in the window diagram at temperatures 30, 60, and 90 °C correspond to the chromatograms c, b, and a in Figure 9, respectively. We see good agreement between the experimental chromatograms

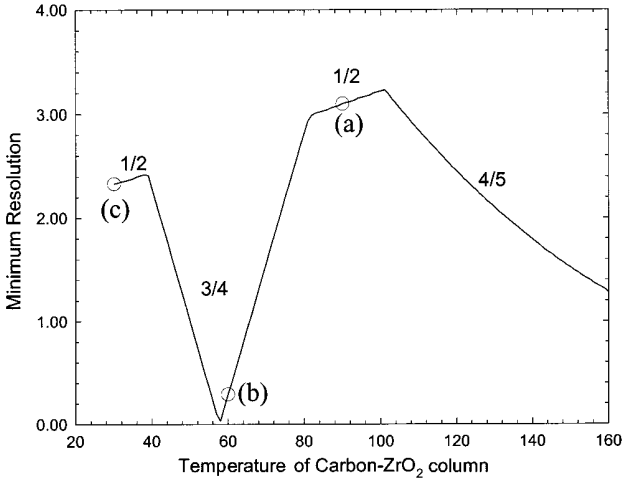

Figure 8. Window diagram showing the resolution of the critical pair versus the temperature of the C-ZrO_2 column. The corresponding critical analyte pairs are indicated above the line. The circles labeled a, b, and c correspond to the chromatograms in Figure 9.

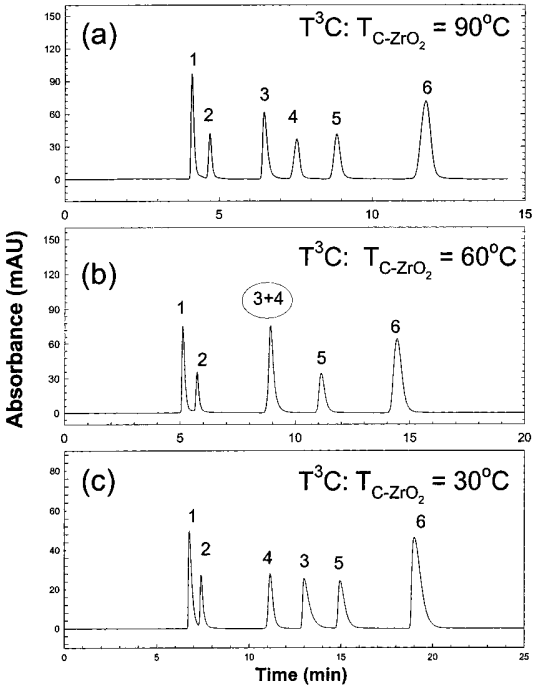

Figure 9. Chromatograms showing the separation of the mixture on T^3C with the PBD-ZrO_2 column at 30 °C and the C-ZrO_2 column at 90 °C (a), 60 °C (b), and 30 °C (c). Experimental conditions: mobile phase, 40/60 acetonitrile/water; flow rate, 1 mL/min. Solutes: 1, benzonitrile; 2, anisole; 3, methylbenzoate; 4, ethylbenzene; 5, *p*-xylene; 6, *n*-propylbenzene.

and the results predicted by the window diagram. Excellent separation was achieved with T^3C when the carbon column is set to either 90 °C (Figure 9a) or 30 °C (Figure 9c) and the PBD column temperature is fixed at 30 °C; whereas, no separation was obtained with either pure phase at 30 °C. Furthermore, chromatogram b did show the coelution of compounds 3 and 4 as predicted by the window diagram. In contrast to what is normally observed when a separation is optimized by varying the mobile phase composition, inspection of the chromatograms in Figure 9 indicates that there is no large change in k' of the last peak to

Table 3. Comparison of the Effect of C-ZrO_2 Column Temperature on the Separation of the Critical Pair for a Single C-ZrO_2 Column and T^3C System

	temperature of C-ZrO_2 (°C)	critical pair[c]	selectivity (α)	resolution (R_s)
single C-ZrO_2 column[a]	30	2/1	1.05	0.65
	70	2/1	1.05	0.57
	130	4/6	1.01	0.06
T^3C system (PBD-ZrO_2 + C-ZrO_2)[b]	30	2/1	1.15	2.17
	70	4/3	1.07	1.12
	130	5/4	1.16	2.24

[a] Separations are with a 40/60 acetonitrile/water mixture and a 1 mL/min flow rate. [b] Separations are with a 40/60 acetonitrile/water mixture and a 1 mL/min; the PBD-ZrO_2 column was held at 30 °C. [c] Solutes: 1, benzonitrile; 2, anisole; 3, methylbenzoate; 4, ethylbenzene; 5, *p*-xylene; 6, *n*-propylbenzene.

elute. However, in method development and resolution optimization, when one weakens the mobile phase, one usually must contend with large increases in retention, increased analysis time, and decreased sensitivity.

To validate the model quantitatively, as shown in Figure 7, we further compared the experimental and predicted retention times on T^3C at the C-ZrO_2 column temperatures of 40, 50, 70, 80, 110, 130, and 150 °C at a fixed PBD-ZrO_2 column temperature of 30 °C. The solid lines are the predicted retention times for six solutes while the points are the experimental retention times. Note that the solid lines are based only on the data taken at four temperatures (see Table 2). Excellent agreement is achieved with errors of less than 1.5%. Therefore, the model developed here is adequate to predict resolution on the basis of retention data taken in only four initial experiments.

Comparing with the Optimization on a Single Column. We further compared the effect of varying temperature on a single carbon column to that on the T^3C system (see Table 3). With just the carbon column, when the temperature is changed from 30 to 70 °C, the critical pair remains the same and the resolution decreases slightly (0.65 to 0.57). Although the critical pair changes at 130 °C, the resolution of the critical pair becomes even worse. Obviously, adjusting only the temperature on the carbon column cannot bring about the desired separation. On the other hand, we see a large change in resolution as well as change in the critical pair on the T^3C system as the temperature is varied. At 30 °C, the critical pair is the same on both the T^3C system and the carbon column because the carbon column dominates retention and the selectivity of the T^3C system. When the carbon column temperature is increased to 130 °C, the critical pair of the T^3C system changes to the same as that on the PBD-ZrO_2 column because, at that temperature, the PBD-ZrO_2 column dominates the retention of the T^3C system. The important point is that an adequate separation is easily achieved on the T^3C system at many different carbon column temperatures.

Since varying the mobile-phase composition is the most commonly used approach to optimizing RPLC separations, it would be very interesting to compare the separation using the T^3C approach with that achieved by optimizing mobile-phase composition. Table 4 summarizes the selectivity and resolution for the most poorly resolved pairs on both columns at various percentages of acetonitrile (from a 30/70 acetonitrile/water mixture to a 70/

Table 4. Effect of Mobile Phase Composition on the Separation of the Critical Pair on PBD-ZrO_2 and C-ZrO_2

mobile phase composition (% acetonitrile/% water)	PBD-ZrO_2[a]		C-ZrO_2[a]	
	selectivity (α)[b]	resolution (R_s)	selectivity (α)	resolution (R_s)
30/70	1.03	0.63	1.04[c]	0.79
35/65	1.03	0.62	1.01[c]	0.27
40/60	1.03	0.58	1.01[c]	0.19
45/65	1.04	0.63	1.04[c]	0.62
50/50	1.03	0.42	1.05[c]	0.74
55/45	1.04	0.44	1.02[d]	0.25
60/40	1.04	0.37	1.04[d]	0.51
70/30	1.05	0.29	1.08[e]	0.84

[a] Separation at 30 °C and 1 mL/min. [b] The critical pair is 4/5 (ethylbenzene/*p*-xylene). [c] The critical pair is 1/2 (anisole/benzonitrile). [d] The critical pair is 1/4 (benzonitrile/ethylbenzene). [e] The critical pair is 2/4 (ethylbenzene/anisole).

30 acetonitrile/water mixture). The highest resolution achieved was 0.84, meaning that this mixture cannot be baseline-resolved by simply varying the mobile-phase composition. It is possible that other types of mobile phases or stationary phases could separate the mixture, but our point is that optimization by T^3C is a reasonable approach to method development in HPLC which has not yet been investigated or applied to chemically interesting separations.

In summary, this simple example of a six-component sample shows that the T^3C approach can offer unique selectivities for the separation of mixtures that are difficult to resolve on single-column systems. The optimization strategy for T^3C in RPLC can be summarized in the following six steps: (1) Choose a carbon phase and an aliphatic hydrocarbon phase (ODS, PBD-ZrO_2, etc.) as the two stationary phases because they generally exhibit dramatically different selectivities. (2) Choose acetonitrile as the organic modifier for the mobile phase because of its low viscosity and favorable UV transmittance as well as the excellent efficiency observed in an acetonitrile/water mixture on most stationary phases. (3) On the basis of the retention times at low temperature (30−40 °C), select an appropriate mobile-phase composition (%B) such that the maximum retention factors on the two phases are both less than about 20. The mobile phase must be chosen such that, on at least one column, the smallest retention factor is one or greater. This will give enough retention to separate the least well retained compound and simultaneously avoid exceedingly long analysis time. (4) Use the elution order plot as shown in Figure 5 (plot B) to see if T^3C is potentially useful and which column needs to be the "hot" column in T^3C optimization. (5) Run one or two additional experiments at a higher temperature on either one or both columns. This depends on the relative retention times and the temperature ranges studied. (6) Estimate the T^3C retention times according to the model, and construct a window diagram to locate the best temperatures. For this specific sample, optimum chromatographic conditions are easily arrived at on the basis of only four initial experiments.

CONCLUSIONS

The thermally tuned tandem column (T^3C) concept is a useful, new, general approach to the practice of HPLC. It affords a mechanism for continuously tuning chromatographic selectivity by systematically adjusting the contribution to retention from the stationary phase. The two columns used in T^3C system must exhibit quite different selectivities and, more specifically, have different critical pairs to make T^3C useful. Temperature affects the T^3C selectivity through the continuous variation of the contribution each column makes to the overall selectivity; this is totally different from its effect on a single column. Selectivity in the T^3C system can often be changed over a wide range without a large change in retention time as is often observed in mobile-phase optimization. Only four initial runs were needed to locate the optimum temperature, and the predicted retention times agree well with the experimental data (error less than 1.5%). Changing the stationary phase often has a greater effect on selectivity than changing temperature and/or mobile-phase volume fraction. Until now, the key difficulty in changing the stationary phase in HPLC is that it is a discontinuous "hit or miss" proposition. Thus, it is very possible, if not probable, that separations that are otherwise impossible or extremely difficult will be "doable" with T^3C. Work is currently in progress in looking at other combinations of phase types including RPLC and ion exchange and other mechanisms of adjusting selectivity with coupled columns.

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