Selectivity Optimization in Tandem Column Liquid Chromatography Using the Eluent Composition as the Tuning Variable

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DEDICATION

I would like to dedicate this thesis to both my father, Henry John Bigert, Jr., and my wife, Jaime Lynn Kupillas.

ABSTRACT

Clearly, in typical separations in HPLC, selectivity is the most important variable for improving resolution and the optimization of selectivity is the primary focus of most method development effort in reversed-phase liquid chromatography (RPLC). We have developed a family of novel techniques in which two columns with *markedly different chromatographic selectivities* are combined in tandem. One then uses another chromatographic variable (e.g. temperature, flow rate, eluent composition) which has different values for the two columns to continuously "tune" the selectivity. We call this family of techniques XT^2C, meaning that the variable X (temperature, etc.) is used to continuously tune the combined selectivity of the tandem *c*olumns. By serially coupling the columns, our approach eliminates the common "selectivity discontinuity" problem encountered by analysts when the type of column used is changed. Previously, we utilized the thermally tuned tandem column (T^3C) concept in which selectivity was adjusted by independently tuning the two column temperatures. The primary difference between the experimental setup for T^3C and XT^2C is that a second pump and static micro-mixer are placed between the two columns. In this study, we describe the *eluent* tuned tandem column concept in which selectivity is continuously tuned by independently controlling the eluent composition of each column. When the percent organic modifier in the eluent (%B) is adjusted, the concept is denoted "solvent tuned tandem column (ST^2C)". Likewise, when the ionic strength (buffer concentration) is tuned, the concept is referred to as ionic strength tuned tandem column (IT^2C). The objective of the present study is to evaluate the applicability of IT^2C and ST^2C to

mixtures of ionic and non-ionic analytes, respectively. We also describe the use of a simple computer assisted optimization strategy based on the window diagram method. This strategy allows for XT^2C optimization based on only four to six initial data acquisition runs. ST^2C and IT^2C are comparable in terms of chromatographic performance to T^3C and provide flexibility in optimizing selectivity but do not require thermally stable columns.

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INTRODUCTION

The primary goal of any chromatographic method optimization is the complete separation of the analytes in the sample. Selectivity ($\alpha \equiv k_2'/k_1'$) has the most impressive impact on resolution.[1, 2] Moreover, trial-and-error methods involved in searching for the appropriate stationary phase for a particular separation are time-consuming and unsystematic. In many cases, if a separation cannot be performed using one column type, a new stationary phase possibly providing a quite different selectivity would be evaluated. The analyst must then re-optimize the eluent conditions, which may require a variety of approaches such as switching between several eluent types, changing the pH of the eluent, or adding ion-pairing agents to the eluent. This process is common; however, it affords no *continuous* change in selectivity upon changing the column type. The discontinuous process of altering selectivity via a change in column selectivity is obviously quite inefficient. The method described here avoids the discontinuity problem by pairing two pre-selected columns with decidedly different chromatographic selectivities and varying the fraction of the total retention time spent on each column by tuning the two column temperatures, flow rates or compositions.

Our technique was inspired by conceptual papers from the 1960s, pertaining to mixed stationary phases and coupled columns in series for gas chromatography.[3, 4] Additional experimental GC work was performed over the next several decades to support the use of relative retention times and retention factors for the prediction of optimized separations (window diagrams) and to drive selectivity optimization with

variation in key variables such as flow rate, pressure, and temperature.[5, 6, 7, 8, 9, 10, 11, 12]

In a series of recent publications, T^3C was shown to be useful for the separation of complex mixtures of environmental, biological, and pharmaceutical samples.[1, 13, 14, 15, 16, 17] Temperature was used to change the *relative* residence time of the solutes on the individual columns, thereby altering the relative contribution each stationary phase makes to the net selectivity of the T^3C system. Until the past decade, temperature has received rather little attention as an operating variable in LC even though it can significantly impact selectivity.[2, 18, 19, 20, 21, 22, 23] Unfortunately, there is a limited effect of temperature upon retention (e.g. in RPLC a 50 °C change in temperature typically changes *k'* by less than 3-fold), which only allows for about one third of the selectivity difference between two columns in T^3C to be exploited.[24] There are 3 basic problems with T^3C. First, T^3C requires the use of at least one thermally stable column. Although a few RPLC phases are thermally stable up to 200 °C, use of high temperatures is still not widely accepted as a method to improve separations. The resistance to using higher temperatures stems from the fact that majority of conventional silica-bonded phases are thermally unstable above 50 – 60 °C, which restricts the temperature dimension that can be used during method optimization. Furthermore, many workers are fearful of analyte instability, an issue which is often unwarranted.[20, 25, 26] Second, an extra heating apparatus is generally needed to heat the second column; and furthermore thermal mismatch induced broadening must be scrupulously avoided.[27, 28, 29, 30, 31] Third, the critical pair of analytes on each column must be different. This third

restriction is crucial, because if each column has the same critical pair, T^3C offers little benefit to the overall separation. Because the fundamental basis for the utility of the T^3C concept is the big difference in selectivity achievable by varying the column type, any approach that allows the amount of retention delivered by each column in a tandem pair could allow profound variations in selectivity provided by T^3C.

By far the most popular way to adjust band spacing is by changing the eluent type and eluent composition.[2] Snyder has shown that a change in the stationary phase type or the eluent type will produce greater changes in selectivity than will a change in volume fraction of mixed eluents .[2] DeStefano and co-workers have stated that in many cases it is more desirable to change the stationary phase rather than the eluent conditions.[32] However, practical issues such as slow equilibration upon change in type of eluent or stationary phase and unpredictable changes in selectivity and discontinuities in selectivity upon change in column type has proven to be time consuming and inconvenient.[33, 34] Optimization of selectivity through manipulation of %B (and also temperature especially in gradient elution) remains the preferred means for adjusting retention time and selectivity.[2] The primary advantage of the ST^2C and IT^2C approaches is that selectivity can be continuously adjusted.

An approach to the eluent tuned tandem column concept is shown schematically in Figure 1. Inspired by T^3C, the "eluent variable (X) tuned tandem column (XT^2C)" optimization concept uses two serially coupled columns with markedly different chromatographic selectivities. A second pump and static micro-mixer are added between the two columns, and the temperatures of the respective columns are fixed and not used

to adjust selectivity. Instead, selectivity is tuned by adjusting the eluent composition (e.g. %B or buffer concentration) in the two columns by using different eluent compositions at pumps 1 and 2. Typically the flow rate of each pump is 1.0 mL/min which yields a flow rate of 1.0 and 2.0 mL/min on the first and second columns, respectively.

THEORY

If ϕ_1 and ϕ_2 are the volume fractions of organic modifier from pump 1 and 2 respectively, the eluent composition on column 1 and column 2 are, assuming no volume change upon mixing, as follows:

$$\phi_{1,1} = \phi_1 \tag{1}$$

$$\phi_{2,2} = \frac{F_1}{F_1 + F_2}\phi_1 + \frac{F_2}{F_1 + F_2}\phi_2 \tag{2}$$

where F_1 (mL/min) is the flow rate from pump 1, F_2 (mL/min) is the flow rate from pump 2, $\phi_{1,1}$ is the eluent composition on column 1 from pump 1, and $\phi_{2,2}$ is the eluent composition on column 2 and is a function of both ϕ_1 and ϕ_2.

ST^2C and IT^2C offer some potential advantages over T^3C. For instance, because we are dealing with changes in eluent composition rather than temperature, thermally stable columns and analytes are not needed. Retention times can be changed over a much wider range since changes in ϕ can often affect a much bigger relative change in *k'* than can changing column temperature. Solvent strength or ionic strength can change the selectivity of XT^2C in a similar manner as does temperature in T^3C. Moreover, the method optimization process of T^3C, which allowed for optimal temperatures on each column to be rapidly located with the assistance of a computer program, can be easily modified and applied to either ST^2C or IT^2C.

Similar to T^3C, ST^2C and IT^2C require that one does four initial measurements of *k'* on each analyte to obtain a window diagram wherein resolution of the worst separated pair is plotted versus the operating variable on each column. In T^3C, the first step is to

determine the effect of temperature on retention on each separate column. A linear van't Hoff relation ($\log k' = A + B/T$) allows for estimation of retention factors as a function of temperature (T). However, in ST^2C and IT^2C, retention is modeled as a function of %B and ionic strength, respectively. The effect of volume fraction of organic in the eluent composition (ϕ) on the retention factor is usually described by the equation:

$$\log k' = A + B\phi \tag{3}$$

where *k'* is the retention factor and A and B are constants for a given solute. Assuming linearity in ϕ, B is the free energy of retention from pure organic (%B = 100) to pure water. Generally, B is negative, meaning that an increase in %B leads to a decrease in the retention factor.[2] Prediction of isocratic retention data involves the estimation of the retention factor for each compound on each column from plots of 1og *k'* vs. ϕ. Equation 3 provides a good linear relationship between log *k'* and ϕ only over a relatively narrow range.[35, 36, 37, 38] The retention factors at other %B compositions within a certain *k'* window can be calculated based on the intercept (A) and slope (B) for each solute.

Similarly, the effect of buffer (displacer) concentration (C) on the retention factor is usually described by the equation:

$$\log k' = A + B\log C \tag{4}$$

where A and B are constants for a given solute and displacer. Generally, an increase in buffer concentration leads to a decrease in the retention factor. For cation-exchange chromatography, the retention factor for a cationic analyte as a function of the salt concentration in the eluent is usually estimated using Equation 4 which predicts that a

plot of log *k'* versus the logarithm of buffer concentration yields a straight line.[39] When both the analyte and displacer have the same charge type (e.g. for example both are univalent) other linearizing relationships are possible.[40, 41, 42, 43, 44, 45, 46, 47] Equations 3 and 4 can be used to estimate retention times for each solute on both columns based on only two initial measurements per column.

Retention times can then be estimated for each solute on both columns over a range in conditions ($t_R = t_o (1+k')$). The total retention time for the XT^2C set is calculated for each solute at all possible combinations of conditions of the two columns. To a first approximation, the net retention time is the sum of the retention times from each individual column and is given by the following equation:

$$t_{R,net} = t_{R,1} + t_{R,2} - t_{ex} \quad (5)$$

where $t_{R,1}$ and $t_{R,2}$ are the retention times on columns 1 and 2, respectively, and t_{ex} is the time that a solute spends outside the column (in the injector loop, connection tubing and detector). The resolution is conveniently estimated based on equation 6:

$$R_s = \frac{\sqrt{N}}{4}\frac{(t_{R,2} - t_{R,1})}{t_{R,average}} \quad (6)$$

in which R_s is resolution, $t_{R,average}$ is the average of the retention times and N is the theoretical plate number.[48] N was assumed to be about 5,000 for a single 5 cm column packed with 5 μm particles (100,000 plates/m), and 10,000 for the two columns in the XT^2C set.

The goal of the present study is to investigate, compare and contrast the advantages and disadvantages of pairing of the same stationary phase types used by Mao and Carr in their

T^3C work but using adjustments in eluent strength (ϕ) (ST^2C) and buffer concentration (IT^2C) instead of temperature.[1, 15]

EXPERIMENTAL

Equipment

All chromatographic experiments were conducted with a Hewlett-Packard 1090 chromatograph, equipped with a binary pump, an autosampler, a thermostatted-column compartment, and a variable wavelength UV detector (Hewlett Packard S.A., Wilmington, DE). All data was processed with Chemstation software. A second Hewlett-Packard 1090 chromatograph was used as the auxiliary pump for the second column. A PEEK static mixer (P/N 413-0250B, Analytical Scientific Instruments, Inc., Richmond, CA) with a 50 μL mixer cartridge (P/N 410-0050B) was used for mixing the effluent from the upstream column with the eluent from pump 2 to form the eluent entering the downstream column. In the work of Mao, it was shown that the order of connection of the two columns in the T^3C set had no effect on the total retention.[1] For experiments involving T^3C, the hotter of the two columns is placed second. The order of placement of the two columns in ST^2C and IT^2C is important due to differences in flow rates for the first and second column. This is discussed below. Window diagrams were constructed using Microsoft Excel software. Drylab 2000 software (LC Resources, Walnut Creek, CA) was used to predict the maximum resolution separation conditions on each individual column.

Analytical Columns

A 50 X 4.6 mm i.d. Zorbax Extend-C18 (octadecylsilane) column was obtained from Agilent Technologies (Wilmington, DE). The particle size is 5 μm. The carbon content of the Extend-C18 particle is 12.5% (w/w). A 50 X 4.6 mm i.d. carbon-coated

zirconia (C-ZrO_2) column and 50 X 4.6 mm i.d. polybutadiene-coated zirconia (PBD-ZrO_2) column were provided by ZirChrom (ZirChrom Separations, Inc., Anoka, MN). The C-ZrO_2 particle has 1.6% (w/w) carbon content and the particle size is 3 μm. The PBD-ZrO_2 particle has 3% carbon content (w/w) and the particle size is 3 μm.

Reagents

All chemicals were reagent grade or better. HPLC grade acetonitrile (ACN) was purchased from Burdick & Jackson (Allied Signal, Muskegon, MI). HPLC water was obtained from a Barnstead Nanopure deionizing system (Dubuque, IA) passed through an "organic-free" cartridge followed by a 0.2 μm final filter. When zirconia-based columns were used the water was boiled to remove carbon dioxide.[49] All water containing eluents were filtered through a 0.20 μm filter (Lida Manufacturing Corp., Kenosha, WI) before use. Antihistamine drugs were purchased from the Theta Corporation (Newtown Square, PA). Other solutes used in this study were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI). Analyte (concentrations of 1 – 2 mg/mL) were dissolved in 100% acetonitrile.

Samples

Four alkylbenzenes (toluene, ethylbenzene, propylbenzene and butylbenzene) were used during ST^2C retention prediction work on ODS and PBD-ZrO_2 columns. Another set of non-ionic aromatic solutes were used during ST^2C separations on C-ZrO2 and PBD-ZrO_2 columns: benzonitrile, anisole, methyl benzoate, ethylbenzene, p-xylene and n-propylbenzene. The antihistamine solutes (and their pK_a values) used during IT^2C separations on ODS and PBD-ZrO_2 columns are as follows: alkylamines (pheniramine

(9.3, 4.2), chlorpheniramine (9.0) and brompheniramine (9.8, 3.6)), ethylenediamines (thenyldiamine (8.9, 3.9) and thonzylamine (9.0, 2.2)), and piperazines (cyclizine (8.0, 2.5), meclizine (6.2, 3.1) and promethazine (9.1)). The structures and pK_a values of the ionic compounds are given in Appendix 1.

3.5 Experimental Conditions

All chromatograms were obtained at a flow rate of 1–2 mL/min and detected at a wavelength of 254 nm. The injection volumes were 1–2 μL. The dead time was determined by injecting uracil and acetone for the silica and zirconia columns, respectively. The extra-column time (t_{ex}) was determined by injecting uracil without any column under otherwise the same chromatographic conditions. The aqueous buffer was prepared by dissolving the proper amount (mM) of monobasic ammonium phosphate ($NH_4H_2PO_4$) in water; the pH was adjusted to 7.0 with ammonium hydroxide (NH_4OH) using a pH meter. The mobile phases were then prepared by weighing the appropriate amount of aqueous buffer and pure organic solvent and pre-mixing them before use; consequently, all pH values refer to that of the 100% aqueous buffer.

RESULTS AND DISCUSSION

Predicting Retention on the XT^{2}C System

We assumed that the total retention on the tandem column system is the sum of the retentions on each column corrected for the additional time spent outside of the column (in the injector loop, connection tubing, mixer, and detector, see equations 5 and 7). We used gravimetrically metered pre-mixed mobile phases to avoid both mixing and retention time errors caused by backpressure fluctuations in the system.[1, 50, 51] In preliminary work, we showed that the endothermic mixing of the various ACN/water mobile phases in the mixer could cause peak distortion due the thermal mismatch broadening in the downstream column. This was ameliorated by the simple expedient of inserting a short length of stainless steel tubing (~5 cm length, 0.005" i.d.) coiled around a solid copper rod between the mixer and the second column to ensure adequate heat dissipation.[27] This additional tubing and copper rod hardware was housed inside the temperature controlled second column compartment.

In order to calculate the total retention time as the sum of retention times on individual columns, we assume negligible compressibility of the mobile phases, no volume change upon mixing and that the retention time is independent of the total applied pressure.[52, 53] We tested the accuracy of equation 2 for the mixing of various eluent compositions by comparing both experimental and estimated ST^{2}C and IT^{2}C retention times. The individual experimental ST^{2}C retention times of four alkylbenzenes on both ODS (column 1) and PBD-ZrO_2 (column 2) are shown in Table 1. ST^{2}C retention times were then predicted using a modification of the retention equation by Mao:

$$t_{R,net} = t_{R,1} + t_{R,2} - t_{ex} + t_{mixer} \quad (7)$$

where t_{mixer} is the time spent in the mixer ($t_{(mixer)} = t_{ex(with\ mixer)} - t_{ex(no\ mixer)}$).[1] The value of t_{ex} (0.027 min) was determined by injecting uracil without any columns under the same chromatographic conditions as the actual tandem column runs. The value of t_{mixer} (0.064 min at a flow rate of 2.0 mL/min) was determined by injecting uracil without any column with the mixer present in the chromatographic setup. Equation 7 was used because the measured retention time ($t_{R,2}$ and $t_{R,1}$) on each individual column consists of the "true" retention time and the extra-column time. The calculated ST^{2}C retention times are also listed in Table 1 and compared to the average experimental ST^{2}C retention times. Table 1 shows retention time data for two mixing compositions: (1) 70/30 ACN/water on ODS at 1.0 mL/min mixed with 70/30 ACN/water at 1.0 mL/min from the second pump for an equivalent of 70/30 ACN/water at 2.0 mL/min on PBD-ZrO_2 and (2) 40/60 ACN/water on ODS at 1.0 mL/min mixed with 100% ACN at 1.0 mL/min from the second pump for an equivalent of 70/30 ACN/water at 2.0 mL/min on PBD-ZrO_2. The agreement between the retention times is very good with an error less than 0.3%, however it should be noted that there is rather little retention on column 2.

For IT^{2}C, we tested the accuracy of Equation 7 by comparing experimental and predicted retention times for a set of nine antihistamines in which the buffer concentration in the aqueous portion of the eluent (at constant ϕ) was altered at the mixer. Table 2 shows the individual experimental retention times of nine antihistamines on both the ODS (column 1) and the PBD-ZrO_2 (column 2). The calculated IT^{2}C retention times are also listed in Table 2 and compared to the average of the experimental IT^{2}C retention

time. Table 2 shows retention time data for 45/55 ACN/10 mM ammonium phosphate buffer (pH 7) on ODS at 1.0 mL/min mixed with 45/55 ACN/40 mM ammonium phosphate buffer (pH 7) at 1.0 mL/min from the second pump for an equivalent eluent composition of 45/55 ACN/25 mM ammonium phosphate buffer (pH 7) at 2.0 mL/min on PBD-ZrO_2. The agreement between the measured and calculated retention times is good to within 0.4 %.

Separation of Non-Ionic Aromatic Solutes using ST^2C

Initial Separations of Non-Ionic Aromatic Solutes on C-ZrO_2 and PBD-ZrO_2 Columns

For direct comparison to T^3C, we used the same set of six non-ionic aromatic solutes for ST^2C experiments under nearly identical chromatographic conditions.[1] According to our expectations, under 40/60 acetonitrile/water eluent conditions at 40 °C, neither the PBD-ZrO_2 column nor the C-ZrO_2 column can baseline separate the six-component mixture (see Experimental section). Figure 2 shows the individual chromatograms for the six solutes on PBD-ZrO_2 and C-ZrO_2 at 40/60 ACN/water mobile phase conditions (other parameters: temperature, 40°C; flow rate, 1.0 mL/min; detection, 254nm). These findings agree with data of Mao using the same type of stationary phases under numerous eluent and temperature conditions.[1] Compound 4 (ethylbenzene) and compound 5 (*p*-xylene) co-elute on PBD-ZrO_2 because they are functional group isomers and partition-like RP materials are less sensitive to structural isomerization whereas phases that are adsorption-like, such as the carbon clad ZrO_2 are more sensitive to structural isomerization. However, the C-ZrO_2 column, which is well-known for its ability to discriminate between structural isomers is easily able to separate compounds 4

and 5 with an α of 1.60.[54, 55, 56] Unfortunately, the C-ZrO_2 failed to completely resolve the mixture due to the co-elution of compounds 1 and 2. Obviously, neither of these two columns has the required selectivity to baseline resolve this mixture.

Under the same chromatographic conditions, the retention factors of the six solutes on C-ZrO_2 are two to five times larger than those on PBD-ZrO_2, meaning that the overall retention (thus selectivity) in ST^2C will be nearly completely dominated by the C-ZrO_2 column if both columns were run at the same flow rate and percent organic composition. Therefore, we decided to place the C-ZrO_2 column second in the tandem setup because the flow rate on this column is 2.0 mL/min. After the two initial trial runs, four additional runs were needed to fit the data to Equation 3 on each column within an acceptable *k'* range ($1 < k' < 20$). Two additional data sets at 1.0 mL/min on PBD-ZrO_2 (25/75 and 35/65 ACN/water) and two additional data sets at 2.0 mL/min on C-ZrO_2 (40/60 and 50/50 ACN/water) were acquired. Using our simple, computer-based program no additional runs were needed.

Selectivity Comparison of Non-Ionic Aromatic Solutes using ST^2C

A κ–κ plot was made to quantitatively compare the selectivity difference between PBD-ZrO_2 and C-ZrO_2 for this specific mixture (see Appendix 2A).[57] The low correlation coefficient and relatively high standard error ($r^2 = 0.366$, s.d. = 0.16) indicate that the two stationary phases have rather different selectivities. These values are in accordance with the prior results of Mao using 10 cm PBD-ZrO_2 and C-ZrO_2 columns ($r^2 = 0.332$, s.d. = 0.18) in comparison to the 5 cm columns used in this study.[1] In agreement with the prior data, we observe that the critical pair on the PBD phase (solutes

4/5) are well separated on the carbon phase;[1] in contradistinction, the critical pair on the carbon phase (solutes 1/2) co-elute but are well separated on the PBD column. For additional reference, Appendix 2B shows a plot in which the retentions on the PBD-ZrO_2 and C-ZrO_2 columns are plotted against their log k' values.

For any XT^2C technique to achieve baseline separation, *every pair of solutes must be well separated on at least one of the two phases*. Additionally, new critical pairs that are more poorly resolved that the critical pairs on the individual columns must not be formed when the two columns are combined in the tandem column system. This is a fundamental difference between tandem column chromatography and two-dimensional chromatography as propounded by Giddings.[58, 59] There are many conditions where numerous solute pairs are well resolved and the likelihood of total resolution can be achieved provided that the eluent conditions are judiciously chosen.[60, 61] In this case, ST^2C will be useful for improving the separation.

Window Diagram Optimization of the Non-Aromatic Solutes using ST^2C

In ST^2C, selectivity can be adjusted by simultaneous variation of the %B applied to each column. The optimization of ST^2C involves locating the optimum %Bs on both columns that give the best overall resolution. Equation 3 was used to estimate the retention data within various *k'* windows on C-ZrO_2 and PBD-ZrO_2. We further calculated the overall ST^2C retention times at all combinations of conditions on the two columns using Eq 7. Finally, the optimization is based on calculating the resolution of the critical pair at each combination of %B on each column. Figure 2 shows a three-dimensional (3-D) window diagram which is a plot of resolution versus %B on each

respective column. Given the fact that resolutions larger than 1.5 produce near baseline separation for equal height peaks, Figure 2 shows that many combinations of %B in ST^2C will give quite adequate separation. The highest point in the window diagram (condition A) corresponds to 25/75 ACN/water on PBD-ZrO_2 and 45/55 ACN/water on C-ZrO_2 where the resolution is approximately 3.5, and all solutes are resolved in less than 13.5 min (other parameters: temperature, 40°C; flow rate, 1.0 mL/min on C-ZrO_2 and 2.0 mL/min on PBD-ZrO_2; detection, 254nm).

A 3-D window diagram plot such as Figure 2 is useful for rapidly visualizing the conditions that give the best overall separation. For instance, condition B corresponds to 35/65 ACN/water on PBD-ZrO_2 and 50/50 ACN/water on C-ZrO_2 where the resolution is approximately 3.0. At point B, there is only a small loss in resolution, but a significant improvement in analysis time and all solutes are resolved in less than 5.5 min. The combination of the superior selectivity of the ST^2C set of columns and manipulation of %B allows for the baseline separation of the six solutes in less time than using either single column. The individual chromatograms at points A and B of the window diagram are given in Appendix 3.

Separation of Ionic Antihistamines using IT^2C

Initial Separation of Ionic Antihistamines on ODS and PBD-ZrO_2 Columns

In this study, we attempted to separate nine antihistamines using the IT^2C approach. Other than meclizine, all the solutes have pK_a values greater than 8.0 and should be nearly fully protonated at pH 7.0.[45] The antihistamine mixture used in this study is the same as the prior T^3C mixture except promethazine replaced chlorcyclizine

(solutes: x, impurity; 1, pheniramine; 2, thenyldiamine; 3, chlorpheniramine; 4, brompheniramine; 5, cyclizine; 6, thonzylamine; 7, meclizine; 8, promethazine; 9, pyrrobutamine).[15]

Figure 3 shows the separation of the nine antihistamines on both ODS and PBD-ZrO_2 under 40/60 ACN/25 mM ammonium phosphate buffer (pH 7.0) mobile phase conditions (other parameters: temperature, 40 ^oC; flow rate, 1.0 mL/min; detection, 254 nm). This particular mobile phase composition and phosphate buffer were initially chosen so that we could compare them to previous T^3C separation of these solutes.[15] However, ammonium phosphate was used instead of potassium phosphate in this study due to its greater solubility in organic/aqueous buffer systems.[62] Use of phosphate buffers at intermediate pH are known to result in increased retention and stronger cation-exchange interactions on PBD-ZrO_2 phases as well as improved selectivity and pH stability in contrast to ODS phases.[2, 15, 63, 64, 65, 66]

Figure 3 shows that thenyldiamine, chlorpheniramine, and brompheniramine (solutes 2/3/4) overlapped on the ODS column and promethazine and pyrrobutamine (solutes 8/9) were not baseline separated on the ODS column. The overall separation is better on PBD-ZrO_2 except for the incomplete separation of thonzylamine and pheniramine (solutes 6/1) and co-elution of chlorpheniramine and cyclizine (solutes 3/5). In fact, the peak shapes on the ODS column are more tailed than on the PBD-ZrO_2 column due to the detrimental cation-exchange interactions between the basic solutes and the surface silanol groups.[40, 41, 42, 67, 68, 63] The PBD-ZrO_2 column also operates under a mixed-mode mechanism and retention is the result of cation-exchange

interactions and reversed-phase interactions with the polybutadiene coating.[40, 41, 42] These findings are in accordance with similar results by Mao using the same types of stationary phases.[15]

Selectivity Comparison of Antihistamines using IT²C

Under the similar chromatographic conditions employed by Mao (40/60 ACN/25 mM ammonium phosphate buffer at pH 7.0), the *k'* values of the antihistamines on the ODS phase range from 0.93 to 27.5.[15] The *k'* values on the PBD-ZrO_2 phase range from 3.47 to 28. To work within an acceptable *k'* range, the mobile phase was changed to 45/55 acetonitrile/25 mM ammonium phosphate buffer at pH 7.0. Clearly, the PBD-ZrO_2 column has more retention, higher efficiency, and more numerous and likely stronger cation-exchange interactions than did the ODS column. Because the PBD-ZrO_2 seemed to offer more selectivity than the ODS column, we decided to position the PBD-ZrO_2 column second in the tandem column set. Retention on the PBD-ZrO_2 column can be varied much more than on ODS by changes in buffer concentration despite operating at 2.0 mL/min.[40] Two additional runs at 45/55 acetonitrile/25 mM ammonium phosphate buffer at pH 7.0 were performed on the ODS and PBD-ZrO_2 columns at 40 °C. To quantitatively compare the selectivity differences between the ODS and PBD-ZrO_2 phases, we constructed a κ–κ plot, in which the log *k'* of the antihistamines on the ODS column was plotted against the log *k'* on the PBD-ZrO_2 column. The weak correlation coefficient (r^2 = 0.159, s.d. = 0.44, see Appendix 4A) indicates that the two stationary phases have quite different selectivities and that there is minimal correlation between the retention on the ODS and PBD-ZrO_2 columns. These values confirm prior results of Mao

(r^2 = 0.271, s.d. = 0.66).[15] Clearly the reversed phase and Coulombic retention mechanism operate to quite different extents on the ODS and PBD-ZrO_2 columns as is also supported by the differences in retention of neutral and ionic compounds in this mixture. For example, the neutral compound meclizine (solute 7) eluted last on the ODS column but eluted first on the PBD-ZrO_2 column. Meclizine can only be retained by reversed-phase interactions which are significantly higher on the ODS phase compared to PBD-ZrO_2. Additionally, pheniramine (solute 1) is positively charged and four times more retained on the PBD-ZrO_2 column due to the stronger cation-exchange interactions on this phase. Most important to this work are the quite different retention orders and critical pairs on the two stationary phases.

It is vital to understand that better separations can only be obtained when the critical pairs on the two columns are different.[1, 13, 14, 15] Appendix 4B shows a plot in which the logarithmic *k'* values on the ODS and PBD-ZrO_2 columns are plotted against one another and the elution sequences and selectivities of the antihistamine solute set are shown for both phases. Fifteen crossovers of the solid lines connecting the same solutes on each phase can be seen, indicating dramatic changes in the elution order from one phase to another. Newly formed critical pairs (solutes 3/2/4 and solutes 9/8 on ODS, and solutes 3/5 and solutes 6/1 on PBD-ZrO_2) are evident at the intersection points of the crossover lines connecting the solutes on each phase. These same solute crossovers occurred during T^3C optimization.[1]

The critical pairs (solutes 3/2 and solutes 9/8) which co-eluted on the ODS column are well resolved on the PBD-ZrO_2 column. Additionally, the critical pairs

(solutes 6/1 and solutes 5/3) on the PBD-ZrO_2 column are better separated on the ODS column. The existence of the crossovers means that there are 15 conditions in which a new critical pair has been formed. Finding the best set of separation conditions for the IT^2C set of columns using a trial-and-error method would be extremely difficult. Our 3-D window diagram (see below) easily shows whether the pairing of the two columns will prove useful for the separation of the antihistamines. In this case, IT^2C is useful for improving the separation.

Window Diagram Optimization of the Ionic Antihistamines using IT^2C

In addition to 45/55 ACN/25 mM ammonium phosphate (pH 7.0), additional runs using 10 mM ammonium phosphate and 40 mM ammonium phosphate buffer (pH 7.0) were performed on ODS and PBD-ZrO_2 at 1.0 and 2.0 mL/min, respectively. These four additional runs were needed to fit the data to Eq 3 on each column within an acceptable *k'* range. In IT^2C, selectivity can be adjusted by simultaneously varying the buffer concentration on each column. The optimization of IT^2C involves locating the buffer concentration on both columns that gives the best overall resolution. Equation 3 was used to estimate retention within the *k'* windows on ODS and PBD-ZrO_2. We further calculated the overall IT^2C retention times at all combinations of conditions on the two columns via Eq 7. Finally, the optimization is performed by calculating the resolution of the critical pair at each combination of conditions on each column.

Figure 3 shows a 3-D window diagram which is a plot of the critical pair resolution versus phosphate buffer concentration on ODS and PBD-ZrO_2. As described above, 3-D window diagrams such as Figure 3 allow for easy visualization of the

conditions that give baseline separation. The optimum set of buffer concentrations is indicated as point A in Figure 3, which corresponds to 45/55 ACN/12 mM ammonium phosphate buffer on ODS and 45/55 ACN/15 mM ammonium phosphate buffer on PBD-ZrO_2 (other parameters: temperature, 40°C; flow rate, 1.0 mL/min on ODS and 2.0 mL/min on PBD-ZrO_2; detection, 254nm). Under the chromatographic conditions of point A, the best critical pair resolution is approximately 2.5 and all solutes are eluted in less than 14 min. Similarly, point B is another local optima but some resolution of the critical pair is sacrificed (1.9 at B vs. 2.5 at A) to gain a faster separation (9 minutes at B vs. 12 minutes at A). Point B corresponds to 45/55 ACN/10 mM ammonium phosphate buffer on ODS and 45/55 ACN/25 mM ammonium phosphate buffer on PBD-ZrO_2.. Example separation chromatograms corresponding to points A and B from the 3-D window diagram are shown in Appendix 5. The combination of the superior selectivity of the IT^2C set of columns and manipulation of buffer concentration allow for baseline separation of the nine solutes.

General Comparison of the XT^2C Family of Techniques

The advantage of all XT^2C techniques is that they use the selectivity differences between two quite different columns to allow the *continuous* tuning of selectivity. Once all four necessary initial runs are performed and the data, entered to generate the relevant window diagram we readily learn if acceptable resolution can be achieved by XT^2C in a reasonable time with no further experimentation. In the end, this saves time and significantly simplifies method development in comparison to single column optimization in which the stationary phase might have to be changed, and the mobile

phase optimized. In general, most researchers still find it easier to optimize conditions on one column at a time. In many if not most cases, using a single column is satisfactory. Based on a general knowledge of compound structures and chemistry, picking different phases and mobile phase conditions to perform the desired separation on a single column is the best approach especially since the evolution of the hydrophobic subtraction method (HSM) for classifying RPC stationary phases in terms of their selectivity.[69, 70, 71, 72, 73, 74, 75, 76, 77, 78] However, if the separation is difficult, pairing two columns with radically different selectivities and *continuously* tuning selectivity by varying temperature, %B, or buffer concentration (ionic strength) can prove advantageous.

The eluent variable tuned tandem column concepts (ST^2C and IT^2C) are useful, general approaches to HPLC method development. In terms of accurate predictability of *k'* during method development, IT^2C is better able to model results compared to T^3C and ST^2C since the range in salt concentration is limited and the k' is not a very strong function of buffer concentration. Deviations from linearity are commonly observed when a wide range of ϕ is used.[79] However, regarding the ability to change *k'* and thus selectivity in tandem column chromatography we believe that the order is as follows: $ST^2C > T^3C > IT^2C$. ST^2C has the best ability to change *k'* (3 – 4 orders of magnitude for $0 < \phi < 1$). The effect that temperature can *realistically* have on relative *k'* in T^3C is somewhat limited because of the narrow ranges of temperatures commonly utilized on silica-based stationary phases (maximum increase in temperature of 30 – 40 °C above ambient).[26] Even with the use of thermally stable stationary phases (up to 200 °C), *k'* can only be changed by perhaps only a factor of 10.[27] In addition, there are problems

that arise with the implementation of HPLC at higher temperatures.[27, 28, 29, 80] Although IT^{2}C is limited to a small range in salt concentration (typically 5–50 mM, possibly only up to a maximum of 100–200 mM) and affords smaller changes in *k'*, IT^{2}C can be used to fine-tune separations when mixed-mode retention mechanisms take place. Flow tuning (FT^{2}C) is also possible using the ST^{2}C and IT^{2}C system setup, but cannot be used to dramatically change *k'* before exceeding the pressure capabilities of typical HPLC systems. Unfortunately, ST^{2}C and IT^{2}C are more complicated instrumentally in comparison to T^{3}C and require the use of a mixer and an additional pump in contrast to T^{3}C which requires only an oven for each column.

CONCLUSIONS

Analogous to T^3C, ST^2C and IT^2C can be used to continuously and systematically vary the overall chromatographic selectivity of two columns placed in tandem. The columns must have different (that is weakly correlated retentions), the critical analyte pairs must be different on both columns and no new poorly resolved pairs must be formed. Only two or three initial runs are needed on each column to locate the optimum set of conditions from a 3-D window diagram. We have demonstrated that eluent tuning of selectivity is analogous to column temperature tuning of selectivity and additionally that the combination of an aliphatic phase and carbon-coated phase is an extremely powerful tool for the separation of non-electrolyte solutes. Moreover, the combination of a silica-based and a zirconia-based column, when operated in the presence of a Lewis base buffer, is useful for the separation of basic (cationic) analytes. If co-eluted or overlapped peaks on one column type can be resolved on the other column type, XT^2C will more than likely provide a better separation than on either individual column.

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Table 1. Comparison of Measured and Predicted Retention Times (t_R, min) for Tandem Columns on the ST^{2}C System

Solutes	Measured t_R(min)[a]			Predicted t_R (min)[b]	% error[c]
	Column 1	Column 2	Col 1 + Col 2		
	70/30	70/30	*Total 70/30*		
Toluene	1.05	0.34	1.388	1.390	0.14
Ethylbenzene	1.31	0.35	1.660	1.657	-0.18
Propylbenzene	1.75	0.37	2.120	2.123	0.14
Butylbenzene	2.38	0.41	2.789	2.792	0.11
	40/60	*70/30*			
Toluene	4.48	0.34	4.821	4.811	-0.21
Ethylbenzene	7.72	0.35	8.068	8.056	-0.15
Propylbenzene	14.43	0.37	14.796	14.837	0.28
Butylbenzene	27.43	0.41	27.842	27.751	0.33

[a] Retention times measured in various pre-mixed acetonitrile/water eluents at 40 °C. The flow rate is 1.0 mL/min on ODS (column 1) and 2.0 mL/min on PBD-ZrO_2 (column 2).

[b] Calculated using equation 7.

[c] % error = 100 * (predicted ST^{2}C t_R - average measured ST^{2}C t_R)/ average measured ST^{2}C t_R.

Table 2. Comparison of Measured and Predicted Retention Times (t_R, min) for Tandem Columns on the IT^{2}C System

Solutes	Measured t_R(min)[a]			Predicted t_R (min)[b]	% error[c]
	Column 1	Column 2	Col 1 + Col 2		
	10 mM	*25 mM*			
Pheniramine	0.72	0.93		1.644	-0.30
Thenyldiamine	1.18	0.99	2.165	2.170	0.23
Chlorpheniramine	1.12	1.47	2.596	2.604	0.31
Brompheniramine	1.25	1.73	2.985	2.993	0.27
Cyclizine	3.20	1.30	4.509	4.495	-0.31
Thonzylamine	5.19	1.01	6.211	6.198	-0.21
Meclizine	6.73	0.95	7.677	7.704	0.36
Promethazine	5.01	3.00	8.010	7.982	-0.35
Pyrrobutamine	5.34	4.01	9.353	9.372	0.20

[a] Retention times measured in various pre-mixed 45/55 acetonitrile/ammonium phosphate buffer eluents (pH 7.0) at 40 °C. The flow rate is 1.0 mL/min on ODS (column 1) and 2.0 mL/min on PBD-ZrO_2 (column 2).

[b] Calculated using equation 7.

[c] % error = 100 * (predicted IT^{2}C t_R - average measured IT^{2}C t_R)/ average measured IT^{2}C t_R.

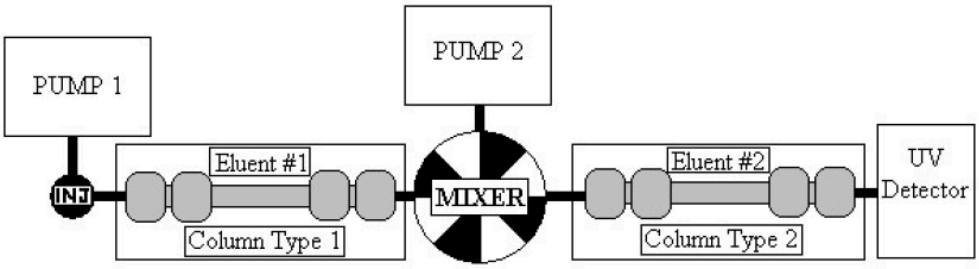


Figure 1. Block diagram of the eluent variable tuned tandem column system.

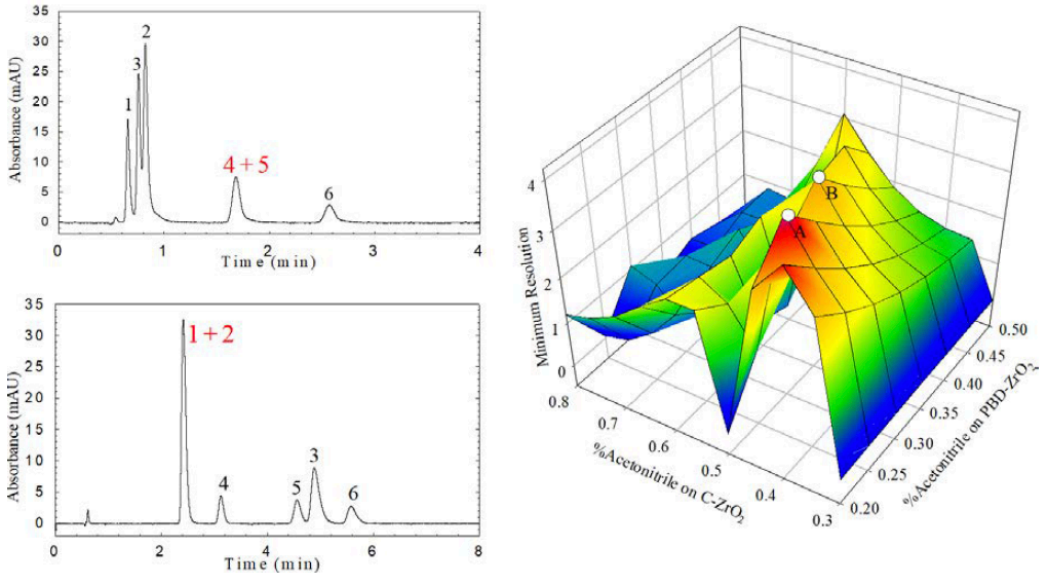


Figure 2. Chromatograms showing the individual separation of aromatic compounds on PBD-ZrO_2 (top left) and C-ZrO_2 (bottom left) HPLC columns, and a 3-D window diagram showing the resolution of the critical pair for the six solutes versus the percent acetonitrile in the eluent on the PBD-ZrO_2 and C-ZrO_2 columns. Experimental conditions on both PBD-ZrO_2 and C-ZrO_2 columns: temperature, 40 °C; mobile phase, 40/60 ACN/water; flow rate, 1.0mL/min; detection, 254nm.

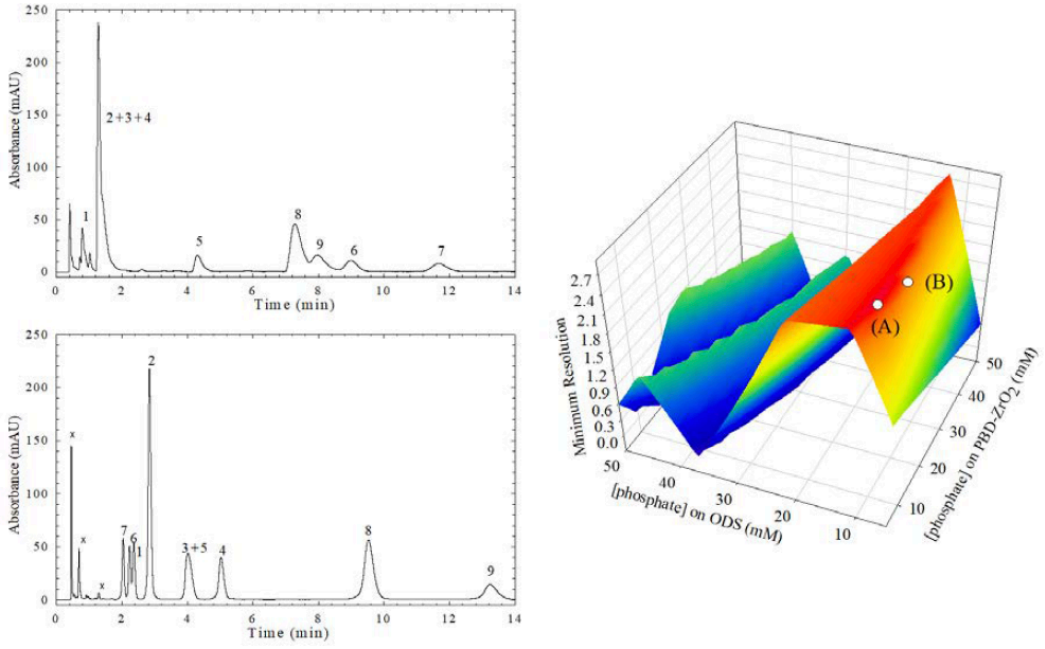
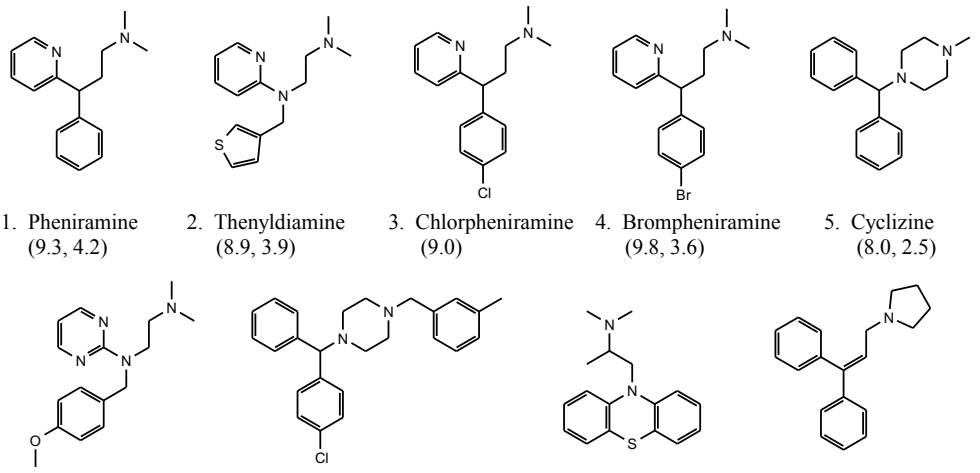


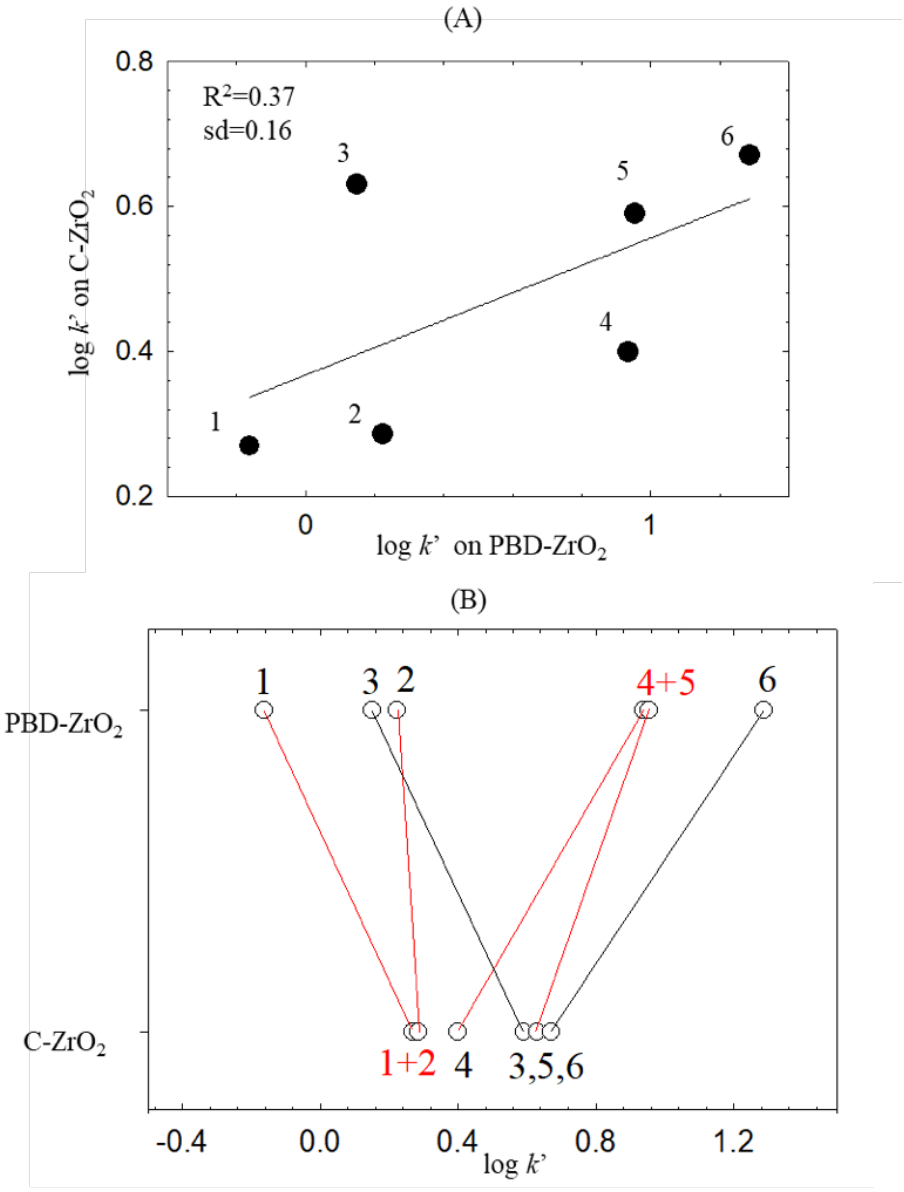
Figure 3. Chromatograms showing the individual separation of the antihistamine mixture on ODS column (top left) and PBD-ZrO_2 column (bottom left), and a 3-D window diagram showing the resolution of the critical pair for antihistamines versus the phosphate buffer concentration on ODS and PBD-ZrO2 columns. Experimental conditions on both ODS and PBD-ZrO_2 columns: temperature, 40 ^{o}C; mobile phase, 40/60 acetonitrile/25 mM ammonium phosphate buffer at pH 7.0; flow rate, 1.0 mL/min; detection, 254 nm. Solutes: x, impurity; 1, pheniramine; 2, thenyldiamine; 3, chlorpheniramine; 4, brompheniramine; 5, cyclizine; 6, thonzylamine; 7, meclizine; 8, promethazine; 9, pyrrobutamine.

APPENDIX 1



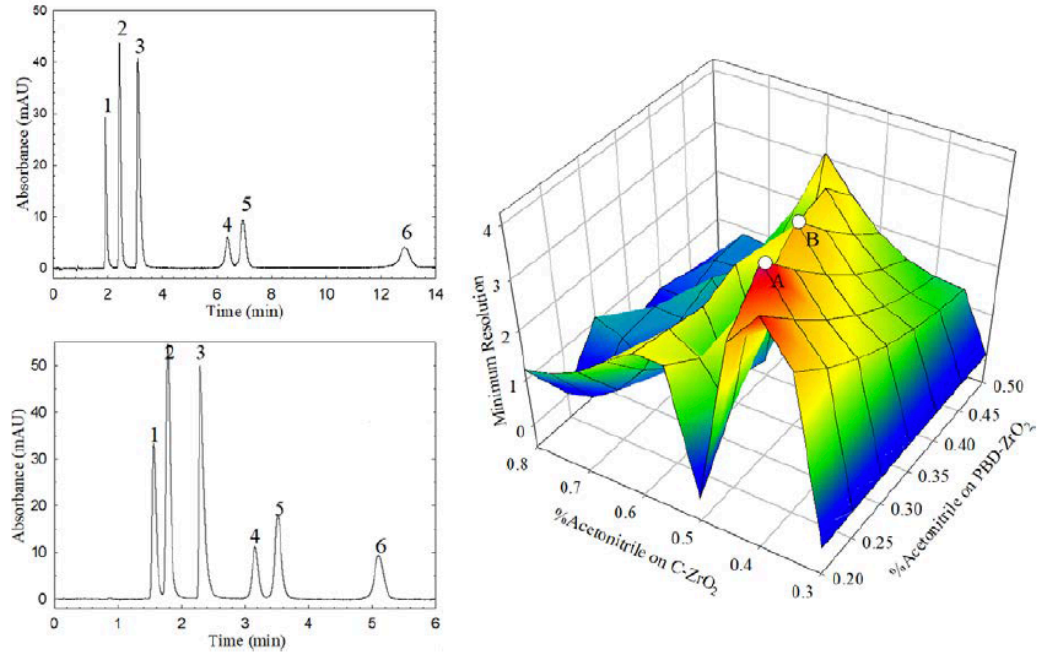
Appendix 1. Structures and pK_a values of antihistamines used during IT^{2}C separations on ODS and PBD-ZrO_2 columns includes alkylamines (pheniramine (9.3, 4.2), chlorpheniramine (9.0) and brompheniramine (9.8, 3.6)), ethylenediamines (thenyldiamine (8.9, 3.9) and thonzylamine (9.0, 2.2)), and piperazines (cyclizine (8.0, 2.5), meclizine (6.2, 3.1) and promethazine (9.1)).

APPENDIX 2



Appendix 2. (A) Plot of log k' on C-ZrO_2 vs. log k' on PBD-ZrO_2 for benzonitrile, anisole, methylbenzoate, ethylbenzene, p-xylene and n-propylbenzene. (B) Comparison of elution order (log k') of the six solutes on C-ZrO_2 and PBD-ZrO_2. Experimental conditions: mobile phase, 40/60 ACN/water; flow rate, 1.0mL/min; detection, 254nm.

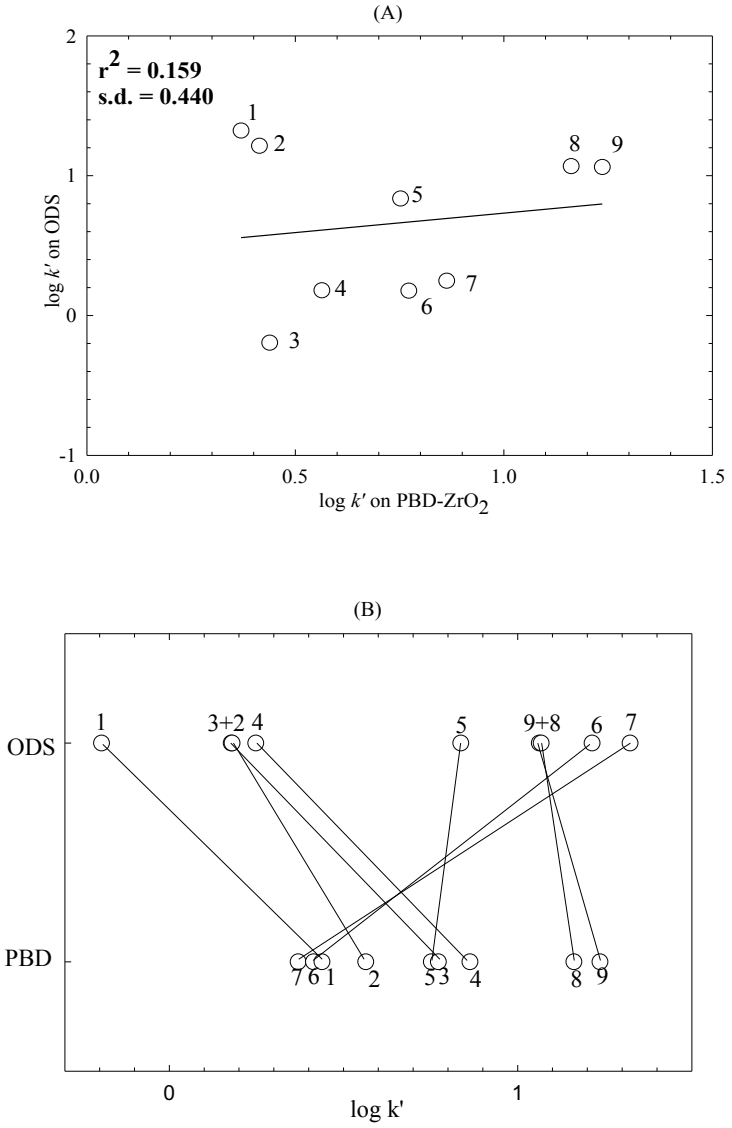
APPENDIX 3



Appendix 3. Chromatograms showing the separation of aromatic compounds on PBD-ZrO_2 and C-ZrO_2 tandem columns at condition A (top left) and condition B (bottom left) from the 3-D window diagram of minimum resolution of the critical pair (right). The window diagram shows the minimum resolution of the critical pair for the six solutes versus the percent acetonitrile in the eluent on the PBD-ZrO_2 and C-ZrO_2 columns.

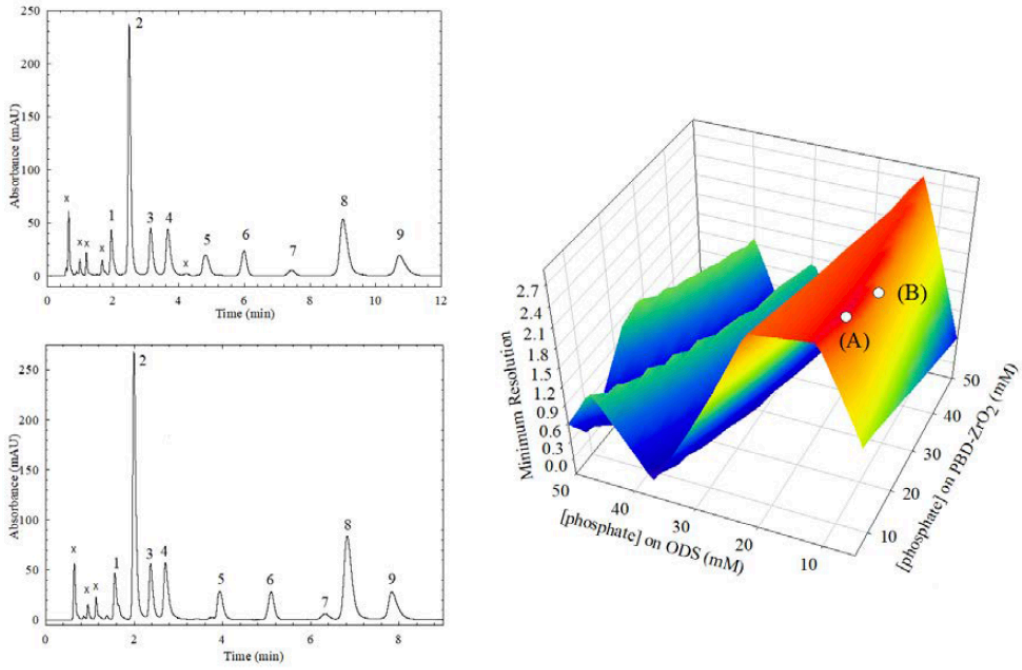
Condition A predicts that the greatest resolution will occur at 25/75 ACN/water on PBD-ZrO_2 and 45/55 ACN/water on C-ZrO_2. Condition B corresponds to another set of optimal conditions (35/65 ACN/water on PBD-ZrO_2 and 50/50 ACN/water on C-ZrO_2) where resolution is still adequate for a separation. Other experimental conditions: temperature, 40°C; flow rate, 1.0 mL/min on C-ZrO_2 and 2.0 mL/min on PBD-ZrO_2; detection, 254nm.

APPENDIX 4



Appendix 4. (A) Plot of log k' on PBD-ZrO_2 vs. log k' on ODS for the antihistamines. (B) Comparison of elution order (log k') of the antihistamine mixture on PBD-ZrO_2 and ODS. Experimental conditions: 45/55 acetonitrile/25 mM ammonium phosphate buffer at pH 7.0; flow rate, 1.0 mL/min on ODS and 2.0 mL/min on PBD-ZrO_2; detection, 254 nm; temperature, 40 °C. Solutes are listed in Appendix 1.

APPENDIX 5



Appendix 5. Chromatograms showing the separation of the antihistamine mixture on ODS and PBD-ZrO_2 tandem columns at condition A (top left) and condition B (bottom left) from the 3-D window diagram of minimum resolution of the critical pair (right). The window diagram shows the minimum resolution of the critical pair for antihistamines versus the phosphate buffer concentration on ODS and PBD-ZrO_2 columns. Experimental conditions: temperature, 40 °C; mobile phase, 45/55 ACN/ammonium phosphate buffer at pH 7.0; flow rate, 1.0 mL/min on ODS and 2 mL/min on PBD-ZrO_2; detection, 254 nm. The buffer concentration for condition A is 12 mM (ODS) and 15 mM (PBD-ZrO_2) ammonium phosphate (pH 7.0). The buffer concentration for condition B is 10 mM (ODS) and 25 mM (PBD-ZrO_2) ammonium phosphate (pH 7.0).