Understanding Orthogonality in Reversed-Phase Liquid Chromatography for Easier Column Selection and Method Development

Application Note

Authors

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Introduction

Orthogonality in chromatography refers to alternative selectivity between separations. Orthogonal, or 2D separations are needed to address one of the major concerns in method development, insufficient resolution, which can mask an impurity or a sample degradation peak. Such separations can be achieved by modifying method parameters and/or by a choice of stationary phases with different selectivities[1]. Relative column selectivity does not change with conditions other than the change of organic modifier and of mobile phase pH[2]; hence, it is orthogonal columns that make 2D separations successful[1, 3]. Identification of orthogonal stationary phases has lately received special attention in the literature[1, 3, 4] and from column manufacturers[5]. In this work, we compared different Agilent Polaris and Agilent Pursuit stationary phases with respect to their selectivity by screening a broad range of analytes under identical conditions. We were able to identify pairs of columns, that exhibited orthogonal retention patterns. The degree of scatter observed for the retention factors of analytes chromatographed on any two columns served as the measure of orthogonality[1]. Stationary phases in this analysis represented different chemistries and base silicas that varied in both surface area and pore size. The goal of this research was to help end-users save time and effort on method development by facilitating efficient column selection.

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Instrumentation

All columns were screened using a Agilent ProStar HPLC System with CVM (model 500), 2 LC Pumps (model 210), Autosampler (model 410) and UV Detector (model 325).

Materials and Reagents

Ten premier Polaris and Pursuit columns with 5 µm particle size, 150 x 4.6 mm dimensions were selected for this project (Table 1).

Table 1. Columns and their physical properties

Polaris / Pursuit Column	Ligand	Pore Size (Å)	Surface Area (m^2/g)	Carbon Load (%)
Polaris C18 Ether	Octadecyl, polar end-capped	200	200	12
Polaris Amide C18	Octadecyl, polar- embedded	200	200	15
Polaris C18-A	Octadecyl, polar end-capped	200	200	14
Pursuit DP	Diphenyl, end-capped	200	200	7
Pursuit C8	Octyl, end-capped	200	200	7
Pursuit PFP	Pentafluorophenylpropyl, end-capped	200	200	6
Pursuit C18	Octadecyl, end-capped	200	200	13
Pursuit XRs DP	Diphenyl, end-capped	100	440	15
Pursuit XRs C8	Octyl, end-capped	100	440	15
Pursuit XRs C18	Octadecyl, end-capped	100	440	23

To investigate columns for orthogonality, commonly used basic and acidic pharmaceuticals, along with some homologous series, were selected and analyzed[5]. In order to study effects of stationary phase on selectivity, we attempted to keep all other separation parameters constant. Each analyte was injected individually in duplicates, and mean RSD values for retention times on duplicate runs were calculated. Analytes underwent screening under fixed testing conditions at 50% organic concentration (Table 2). Analytes that were not adequately retained at this mobile phase composition were later screened at 10% organic (Table 3), with all other variables kept constant. The two mobile phases (50% organic and 10% organic) were selected to represent realistic, but disparate, reversed-phase chromatography conditions for the purpose of screening a broader range of analytes under identical settings. The specified conditions should not be confused with the optimum conditions for resolution of individual analytes.

Table 2. List of compounds investigated under 50% organic conditions

Analytes	Log P	pKa*
Series 1 - Acids		
o-aminobenzoic acid	1.21	2.09
sorbic acid	1.33	4.80
o-nitrobenzoic acid	1.46	2.47
m-nitrobenzoic acid	1.83	3.46
p-nitrobenzoic acid	1.89	3.44
benzoic acid	1.87	4.19
o-toluic acid	2.46	3.98
furosemide	2.03	4.70
phenacetin	1.58	2.20
ibuprofen	3.97	4.91
salicylic acid	2.26	2.97
Series 2 - Phenyl Derivatives		
benzaldehyde	1.46	N/A
o-cresol	1.99	10.30
anisole	2.11	N/A
phenol	1.46	9.99
methylbenzoate	2.12	N/A
4-nitrophenol	1.91	7.15
Series 3 - Bases		
diphenhydramine	3.27	8.98
papaverine	2.95	6.40
fluoxetine	4.05	10.10
norfluoxetine	3.50	9.10
Series 4 - Bases (Tricyclic Antidepressants - Polycyclic Amines)		
nordoxepin	3.80	N/A
doxepin	4.29	8.00
nortriptyline	4.51	10.10
imipramine	4.80	9.40
protriptyline	4.89	10.00
desipramine	4.90	10.40
amitriptyline	4.92	9.40
trimipramine	5.43	8.00
Series 5 - Alkyl Parabens		
methylparaben	1.96	8.40
propylparaben	3.04	7.91
butylparaben	3.57	8.47
ethylparaben	2.47	8.34
Series 6 - Benzene Derivatives - Nitro Derivatives		
nitrobenzene	1.85	N/A
1,3-dinitrobenzene	1.50	N/A
nitrosobenzene	2.01	N/A
benzene	2.13	N/A
Series 7 - Benzene Derivatives - Alkyl Derivatives		
toluene	2.73	N/A
ethylbenzene	3.15	N/A
propylbenzene	3.69	N/A
butylbenzene	4.4	N/A

* denotes pKa values of BH+ species (protonated cations) for bases, N/A: Not Available.

Table 3. List of compounds investigated under 10% organic conditions

Analytes	Log P	pKa*
Bases		
nizatidine	-0.43	N/A
phentermine	1.90	10.10
benzylamine	1.09	9.33
procainamide	0.88	9.32
quinidine	2.60	4.00
codeine	1.19	8.21
lidocaine	2.10	8.01
hydrochlorothiazide	-0.07	7.90
pyridine	0.65	5.17
aniline	0.90	4.60
benzylalcohol	1.10	15.40
Acids		
phthalic acid	0.73	2.76
benzamide	0.64	N/A

* denotes pKa values of BH+ species (protonated cations) for bases, N/A: Not Available. Sources of Log P and pKa values: SRC (Interactive Physprop Database), Handbook of Organic Chemistry by John A. Dean, Drug Bank, A Practical Guide to Contemporary Pharmacy Practice by Judith E. Thompson, Appendix H, etc

Sample Preparation

All samples were diluted in methanol or methanol-water (50:50) to between 30 µg/mL and 1 mg/mL concentration, depending upon their UV absorbance.

HPLC Conditions and Detection

Compounds screened at 50% organic conditions (41 analytes listed in Table 2) were analyzed in duplicates under isocratic conditions using CH_3CN:H_2O + 0.1% TFA, pH 2.0 - 50:50 as the mobile phase at 1.0 mL/min flow rate at ambient temperatures. Those investigated under 10% organic conditions (13 analytes listed in Table 3) were analyzed in duplicates under isocratic conditions using CH_3CN:H_2O + 0.1% TFA, pH 2.0 - 10:90 as the mobile phase at 1.0 mL/min flow rate at ambient temperatures. UV Detection used for most analytes was 254 nm, while some were also examined at 220 nm.

Results and Discussion

To obtain numerical values for orthogonality between any two columns, plots of retention factors (k′) for analytes obtained on one column versus retention factors of the same analytes on another column were made. All retention factors were calculated as an average of two runs using uracil as a void volume marker. An r^2 value of a linear correlation between the two data sets was used as a measure of orthogonality. The lower the r^2 value, the higher the degree of orthogonality between any pair of columns[1]. Retention times observed at both organic compositions were within experimental error*.

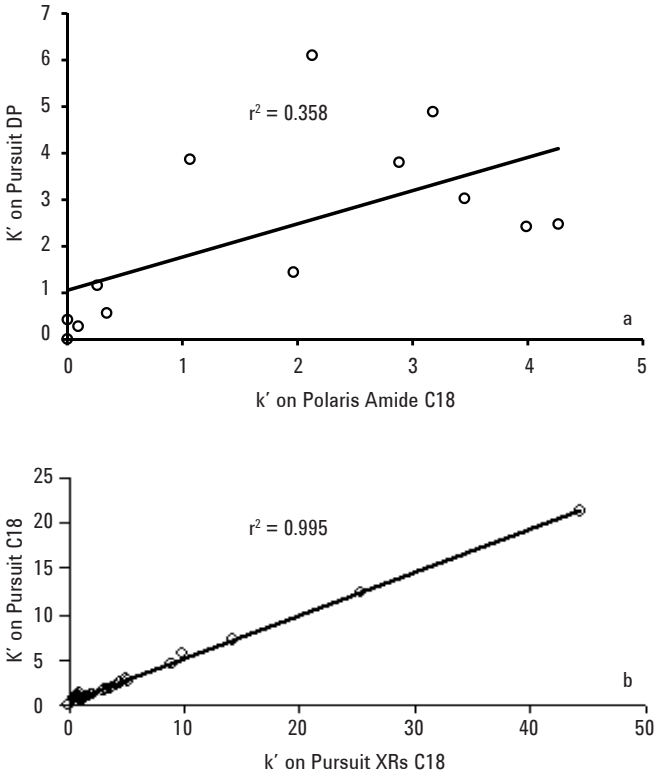

Figure 1. a) Retention factors of 13 compounds on Pursuit DP vs Polaris Amide C18 at 10% organic, pH 2
b) Retention factors of 41 compounds on Pursuit C18 vs Pursuit XRs C18 at 50% organic, pH 2

An example of an orthogonal and non-orthogonal separation is shown in Figure 1. A plot of retention factors obtained on Pursuit Diphenyl (DP) versus those obtained on Polaris Amide C18 (Figure 1a) showed a high degree of scatter (r^2 = 0.358). This is an example of an orthogonal pair of columns. The opposite is true for the retention plot of Pursuit C18 vs Pursuit XRs C18 (Figure 1b) where the two data sets were very well correlated (r^2 = 0.995). An r^2 value close to 1 indicates the highest degree of similarity.

* In order to negate the variance in experimental error and its possible contribution to the observed differences in phase chemistries, mean RSD values for retention times on duplicate runs were calculated at both organic compositions. They were < 0.6% at 50% organic and < 0.1% at 10% organic concentrations indicating that the orthogonality differences observed were not substantiated by differences in experimental error.

A chromatographic illustration of orthogonal selectivity for certain groups of analytes is given in Figure 2. It was evident that Polaris Amide C18 and Pursuit C18 displayed opposite retention patterns for acids and bases: bases co-eluted on Polaris Amide C18 (a) and were well separated on Pursuit C18 (b), whereas acid peaks were well resolved on Polaris Amide C18 (c) and partially overlapped on Pursuit C18 (d).

The r^2 values of the linear correlation of retention factors were derived for all combinations of ten investigated columns at 50% organic and 10% organic, and are listed in Tables 4 and 5, respectively.

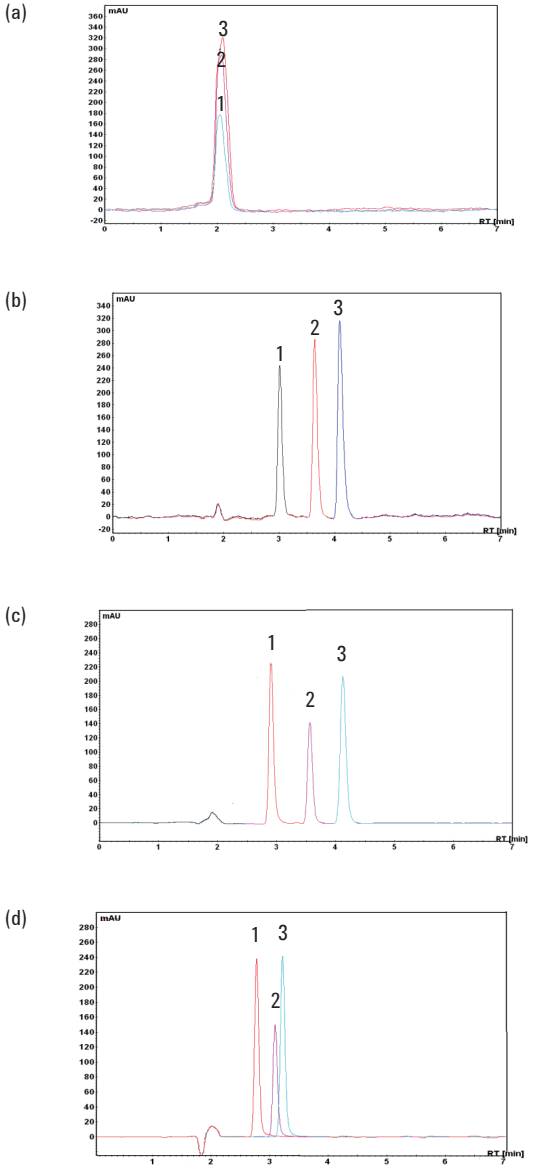

Figure 2. Bases on Polaris Amide C18 (a) and on Pursuit C18 (b).
Mobile phase: $CH_3CN:H_2O$ + 0.1% TFA, pH 2.0 - 50:50 (separate lines)
Samples: 1. Doxepin, 2. Nortriptyline, 3. Trimipramine

Acids on Polaris Amide C18 (c) and Pursuit C18 (d).
Mobile phase: $CH_3CN:H_2O$ + 0.1% TFA, pH 2.0 - 50:50 (separate lines)
Samples: 1. o-Nitrobenzoic acid, 2. p-Nitrobenzoic acid, 3. Salicylic acid

Table 4. r^2 values based on retention plots of 41 compounds at 50% organic

	Pursuit XRs DP	Pursuit XRs C8	Pursuit XRs C18	Polaris C18-A	Polaris C18 Ether	Polaris Amide C18	Pursuit DP	Pursuit PFP	Pursuit C8	Pursuit C18
Pursuit XRs DP	1	0.972	0.939	0.948	0.965	0.948	0.961	0.968	0.960	0.940
Pursuit XRs C8	0.972	1	0.993	0.993	0.998	0.971	0.952	0.967	0.991	0.989
Pursuit XRs C18	0.939	0.993	1	0.995	0.995	0.949	0.923	0.945	0.985	0.995
Polaris C18-A	0.948	0.993	0.995	1	0.997	0.960	0.954	0.965	0.996	0.998
Polaris C18 Ether	0.965	0.998	0.995	0.997	1	0.965	0.958	0.971	0.995	0.995
Polaris Amide C18	0.948	0.971	0.949	0.960	0.965	1	0.890	0.906	0.954	0.943
Pursuit DP	0.961	0.952	0.923	0.954	0.958	0.890	1	0.989	0.975	0.956
Pursuit PFP	0.968	0.967	0.945	0.965	0.971	0.906	0.989	1	0.981	0.966
Pursuit C8	0.960	0.991	0.985	0.996	0.995	0.954	0.975	0.981	1	0.995
Pursuit C18	0.940	0.989	0.995	0.998	0.995	0.943	0.956	0.966	0.995	1

Key:
blue – columns with a high degree of orthogonality ($r^2 < 0.95$), purple – columns with a high degree of similarity ($r^2 > 0.99$)

Table 5. r^2 values based on retention plots of 13 compounds at 10% organic

	Pursuit XRs DP	Pursuit XRs C8	Pursuit XRs C18	Polaris C18-A	Polaris C18 Ether	Polaris Amide C18	Pursuit DP	Pursuit PFP	Pursuit C8	Pursuit C18
Pursuit XRs DP	1	0.820	0.827	0.838	0.883	0.454	0.987	0.944	0.806	0.826
Pursuit XRs C8	0.820	1	0.999	0.993	0.984	0.542	0.807	0.829	0.988	0.986
Pursuit XRs C18	0.827	0.999	1	0.993	0.984	0.537	0.815	0.834	0.988	0.988
Polaris C18-A	0.838	0.993	0.993	1	0.991	0.487	0.837	0.865	0.996	0.997
Polaris C18 Ether	0.883	0.984	0.984	0.991	1	0.515	0.877	0.897	0.980	0.983
Polaris Amide C18	0.454	0.542	0.537	0.487	0.515	1	0.358	0.397	0.445	0.439
Pursuit DP	0.987	0.807	0.815	0.837	0.877	0.358	1	0.938	0.812	0.834
Pursuit PFP	0.944	0.829	0.834	0.865	0.897	0.397	0.938	1	0.837	0.854
Pursuit C8	0.806	0.988	0.988	0.996	0.980	0.445	0.812	0.837	1	0.999
Pursuit C18	0.826	0.986	0.988	0.997	0.983	0.439	0.834	0.854	0.999	1

Key:
blue – columns with a high degree of orthogonality ($r^2 < 0.83$), purple – columns with a high degree of similarity ($r^2 > 0.99$)

The r^2 values in Table 4 were significantly higher for most pairs of columns than in Table 5, which means that correlations were higher for the set of 41 analytes than for the set of 13 analytes. This was due to the presence of an homologous series of alkylparabens and alkylbenzenes in the larger set of compounds. Spacing of these homologs on all retention plots was due to a linear increase in retention with each additional methylene group in a side chain, the so-called "methylene increment"[6, 9 - 11]. A high degree of correlation between homologous series on each plot (Figure 4) resulted in overall higher correlation values for the set of 41 analytes.

Tables 4 and 5 clearly demonstrate that the highest degree of orthogonality was found between the columns with different bonded phase chemistries. The availability of complementary interaction sites and different contributions of hydrophobic, pi-pi, hydrogen-bonding, induced-dipole interaction forces and steric resistance had a significant effect on selectivity[6 - 13]. Pairs of phenyl-based and polar-embedded phases (Pursuit DP (PFP) and Polaris Amide C18) exhibited the lowest correlation coefficients: r^2 = 0.890 - (0.906) in Table 4; r^2 = 0.358 - 0.397 in Table 5. Any pair of columns involving Polaris Amide C18 displayed a very high degree of orthogonality due to the presence of a polar functionality in this stationary phase. Other examples of orthogonal selectivities were pairs of phenyl-based and pure alkyl-bonded columns with different pore sizes, e.g. Pursuit XRs C18 versus Pursuit DP (PFP) and Pursuit C18 vs Pursuit XRs DP. The r^2 values for these pairs varied from 0.923 to 0.945 for the set of analytes investigated at 50% organic, and from 0.815 to 0.834 for the set of analytes screened at 10% organic. In this case, both chemistry and surface area differences contributed to orthogonality.

In contrast to differences in column chemistry, differences in pore size/surface area and, therefore, in carbon load, alone did not lead to a high degree of orthogonality. For most analytes, a change in retention factors from one column to another was proportional to a change in a carbon load, which resulted in a strong correlation between selectivities. Higher retention was almost always observed on columns with higher carbon load, as in the case with Pursuit C18 vs Pursuit XRs C18 (Figure1b). Examples of strong correlation are Pursuit C18 and Pursuit XRs C18 (r^2 = 0.995 and 0.988 in Tables 4 and 5, respectively), Pursuit C8 and Pursuit XRs C8 (r^2 = 0.991 and 0.988), and Pursuit DP and Pursuit XRs DP (r^2 = 0.961 and 0.987).

An even higher degree of similarity was found between columns where the only difference was the length of an alkyl ligand. Thus, retention factors on Pursuit C18 and Pursuit C8 correlate with r^2 = 0.995 and 0.999, in Tables 4 and 5, respectively, and on Pursuit XRs C18 and Pursuit XRs C8, with r^2 = 0.993 and 0.999.

Since the retention mechanism on any given column was a sum of several modes of interaction between analytes and stationary phase[3, 9 - 12], retention of different analyte classes can be driven by different combination of forces. Thus, a pair of columns with a high degree of overall similarity can display an orthogonal pattern of retention for one or two analyte classes.

Percentile plots of retention factors (Figures 3, 7, 9) were used to demonstrate a change in retention on column A (in numerator) relative to retention on column B (in denominator) in percentile for different analytes from Table 4 (those compounds investigated at 50% organic mobile phase composition). A formula used to calculate values for individual compounds is shown on the plot. Each bar represents a percent of increased/decreased retention factor of a certain compound from a color-coded class in the legend. A value of zero on the y-axis indicated equal retention between the two columns. Positive values of y refer to a longer retention on column A relative to retention on column B. Consequently, negative values refer to a longer retention on column B relative to retention on column A.

A percentile plot in Figure 3 illustrates a consistent pattern of retention on Pursuit XRs C18 compared to Pursuit C18 at 50% organic with respect to all but two classes of compounds. The correlation coefficient for this pair of columns was high (r^2 = 0.995). Consistency in retention was due to similar chemistry, and higher surface area/carbon load accounts for 50-100% longer retention of most analytes on Pursuit XRs. However, non-polar ionized bases (including tricyclic antidepressants) fell out of this pattern – they were retained 40% less on Pursuit XRs C18 than on Pursuit C18. Interestingly, this effect was much smaller in C8 phases with different pore sizes and was absent in diphenyl phases.

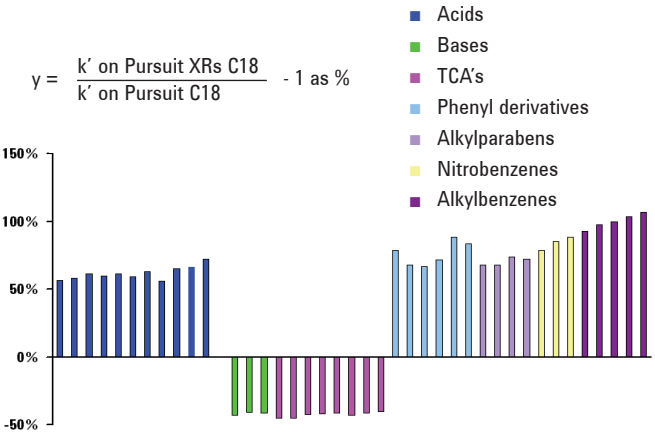

Figure 3. Percentile plot of retention factors on Pursuit XRs C18 relative to Pursuit C18

This example illustrates that although the r^2 values provided useful guidelines for understanding orthogonality, the next step in this kind of research should deal with isolating orthogonal behavior of stationary phases with respect to certain classes of compounds.

Retention of alkylbenzenes

As expected, the strongest retention of neutral analytes was provided by the most hydrophobic columns. In this study, strongest retention was exhibited by Pursuit XRs C18, an octadecyl column with 100Å pore size and the highest carbon (23%) of all columns tested (Table 1).

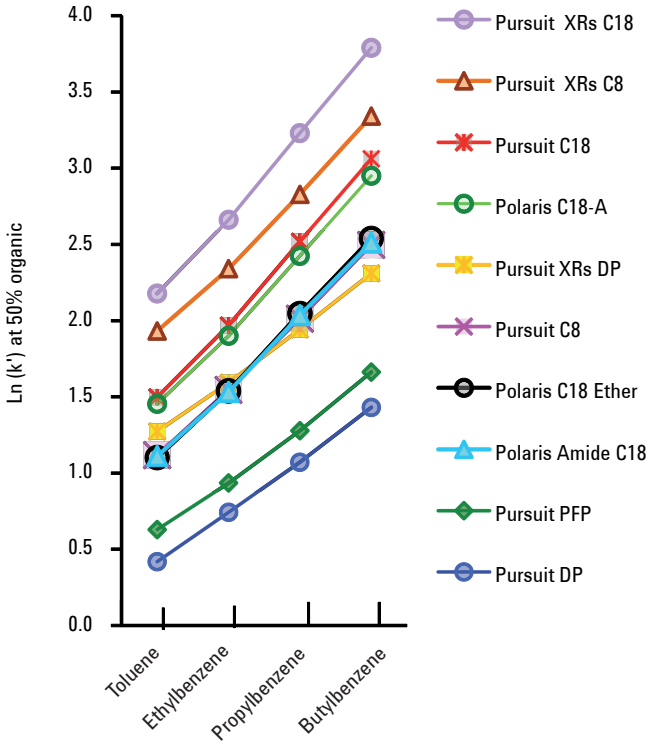

Figure 4. Retention of alkylbenzenes on alkyl-based columns

Figure 4 is a plot of retention factors of four alkylbenzenes. This plot illustrates a linear increase in retention with each additional methylene group in a side chain. Since retention of alkylbezenes was mostly due to hydrophobic interaction, this plot also represents a general order of hydrophobicity for the given columns with the most hydrophobic columns at the top of the plot.

A common way to rank columns by hydrophobicity is either by the NIST test which uses a retention ratio of ethylbenzene and toluene, or by the Tanaka test which uses a retention ratio of amylbenzene and butylbenzene[6]. To complement Figure 4, ratios were calculated for ethylbenzene and toluene, as well as butylbenzene and propylbenzene (Table 6).

According to the data shown in Figure 4 and Table 6, the most hydrophobic columns were Pursuit XRs C18 and Pursuit XRs C8, and the least hydrophobic were phenyl-based columns. Among pure alkyl phases (C8 and C18), the order of hydrophobicity correlated with the carbon load, which was a function of the surface area and the length of an alkyl ligand (Table 1). The 100Å columns with higher surface area showed enhanced retention compared to their 200Å analogs, and the C18 columns showed stronger retention than the C8 columns in both pore sizes (Figure 4). Among alkyl-based phases, 200Å polar-modified columns (Polaris C18 Ether, Polaris Amide C18, Polaris C18-A) clearly displayed lower hydrophobicity compared to their pure, non-functionalized counterparts (Pursuit C18). Their hydrophobicity ratios are very close to those of Pursuit C8 (Table 6), even though their carbon loads were almost twice as large (Table 1). This was in agreement with the observation stating that a presence of a polar-modified group decreased hydrophobic character of an alkyl-based phase[8, 12].

Table 6. Ranking Polaris and Pursuit phases by hydrophobicity

	Retention factor ratios	
	Ethylbenzene to toluene	**Butylbenzene to propylbenzene**
Pursuit XRs C18	1.62	1.75
Pursuit XRs C8	1.60	1.72
Pursuit C18	1.56	1.69
Pursuit C8	1.55	1.63
Polaris Amide C18	1.53	1.62
Polaris C18-A	1.51	1.67
Polaris C18 Ether	1.52	1.61
Pursuit DP	1.38	1.45
Pursuit XRs DP	1.38	1.43
Pursuit PFP	1.36	1.47

Lower hydrophobicity of phenyl-based columns such as Pursuit DP, Pursuit PFP, and Pursuit XRs DP compared to pure alkyl phases was well known[13]. This was due to the lower hydrophobicity of ring structures compared to alkanes (e.g., log P of benzene is 2.2, log P of hexane is 3.9, and log P of octane is 5.2). Phenyl columns appeared to be even less hydrophobic than polar-modified Polaris C18 columns (Figure 4 and Table 6). By the calculated ratios (Table 6), Pursuit XRs DP was equal or less hydrophobic than Pursuit DP, despite the higher surface area of the 100Å XRs phase. It followed that, in contrast to pure alkyl phases, a larger surface area and a higher carbon load in a diphenyl phase did not lead to increased hydrophobicity. Consequently, the degree of hydrophobicity was dictated by carbon load only in pure

alkyl-based columns, whereas in polar-embedded and phenyl-based packings, the chemistry of a bonded phase was more important.

Retention of acids

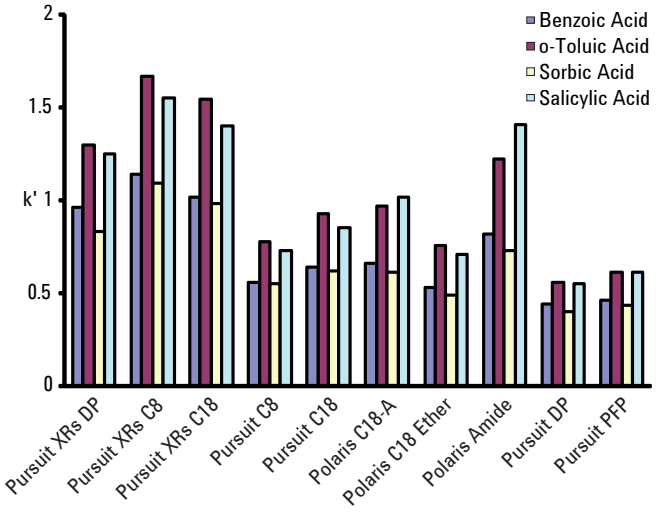

Figure 5. Retention of acids at 50% organic

Within the 200Å family of columns, the strongest retention of acids was found on Polaris Amide C18 – a polar-embedded alkyl-based stationary phase. Acids used in this screening had pKa values in the range of 2.1- 4.9 and were neutral or partly deprotonated at the pH of analysis*. Enhanced retention on Polaris Amide C18 may be attributed to hydrogen bonding with embedded polar groups. Neutral acids are H-bonding donors and at low pH can form hydrogen bonds with protonated amide groups[8] of Polaris Amide C18. Among 200Å columns, Polaris C18-A exhibited only slightly more retention than Pursuit C18. Phenyl phases showed lower acid retentivity, and the most orthogonal phases with respect to acid retention were Polaris Amide C18 and Pursuit DP (Figure 6a).

* Although the aqueous modifier in this experiment was at pH 2.0, the apparent pH of the mobile phase was higher due to the presence of CH_3CN; addition of 10% of CH_3CN increases the pH between 0.1-0.3 units. Thus, we assumed that with 50% CH_3CN, the apparent pH of the mobile phase was approximately 3.0, and with 10% CH_3CN – approximately 2.2.

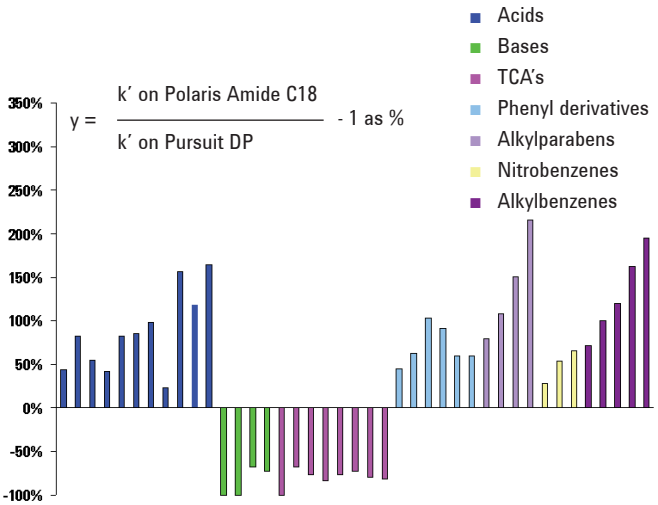

Figure 6a. Percentile plot of retention factors of 41 analytes on Polaris Amide C18 relative to Pursuit DP

Comparison of acid retention between 200Å and 100Å columns revealed that retention of all but salicylic acid was stronger on alkyl-bonded XRs packings, than on Polaris Amide C18 (Figures 5 and 6b). This implied that hydrophobic retentions on alkyl-bonded XRs packings were stronger for most acids than hydrogen bonding to the 200Å polar-embedded Polaris Amide C18.

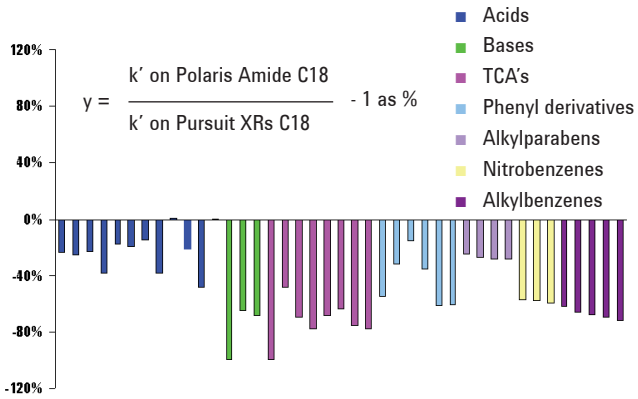

Figure 6b. Percentile plot of retention factors of 41 analytes on Polaris Amide C18 relative to Pursuit XRs C18

Retention of bases, including tricyclic antidepressants

In the screening experiments conducted, retention of bases, both polar and non-polar, was clearly enhanced on pure alkyl-bonded phases (Figures 7, 9). Interestingly, the non-polar bases and the TCA's analyzed at 50% organic were best retained on 200Å Pursuit C18 (Figures 3 and 7), whereas the polar bases analyzed at 10% organic were best retained on 100Å Pursuit XRs C18 (Figure 9).

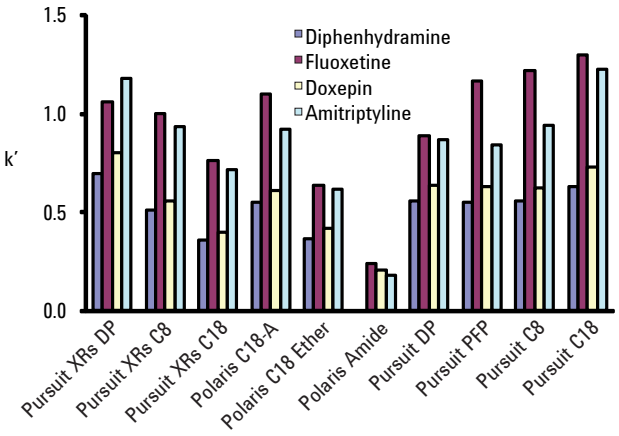

Figure 7. Retention of non-polar bases at 50% organic

Pursuit XRs C18 provided less retention of non-polar bases than other members of the XRs family (Pursuit XRs C8 and especially Pursuit XRs DP). In other words, retention of non-polar bases at 50% organic decreased with the increase in phase hydrophobicity (Figure 7). These results implied a mixed-mode retention where hydrophobic interactions were not the sole contributor to the retention mechanism. One factor that could interfere with the retention of bases (TCA's) is steric selectivity. For bulky solutes such as polycyclic aromatics, resistance to penetration into the stationary phase can decrease for larger pore diameters[3, 11], thus providing longer retention on 200Å Pursuit C18 vs 100Å Pursuit XRs C18.

Phenyl based phases provided good (Pursuit XRs DP) to intermediate (Pursuit DP/PFP) retention of bases, which was significantly enhanced with methanol as an organic modifier (Figure 8). The same set of compounds tested at 50% acetonitrile was re-tested on Pursuit DP at isoeluotropic concentrations of 60% methanol. Figure 8 demonstrates 40 - 100% increase in retention of non-polar bases with 60% methanol as organic modifier and 10 - 40% decrease in retention of most acids, phenolics, and benzenes. Increased retention with methanol vs acetonitrile was described for aromatic compounds[14, 15] and explained by the fact that π-π interactions between analytes and the stationary phase were enhanced by methanol. Acetonitrile, having the strongest π character of all polar organic solvents, is capable of forming π- π bonds (nitrile group), and is likely to impede such interactions. In this study, all compounds except sorbic acid possessed aromatic rings, and enhanced π- π interactions in the absence of acetonitrile could be expected not only for bases but for all other classes of compounds. However, under the given conditions, only bases showed increased retention. This could be due to two factors:

1. Unlike other analytes, all bases in this experiment possessed multiple aromatic rings.
2. Under low pH testing conditions, all bases were protonated. Protonated amine groups could participate in noncovalent molecular interactions with electron-rich systems (phenyl rings of the stationary phase); cation - π interactions are known to play an important role in stabilizing the three dimensional structures of proteins[16]. It appears that these interactions were promoted in the presence of methanol, and acetonitrile weakend them, as it interfered with the selective π- π interactions between analytes and phenyl bonded chemistries.

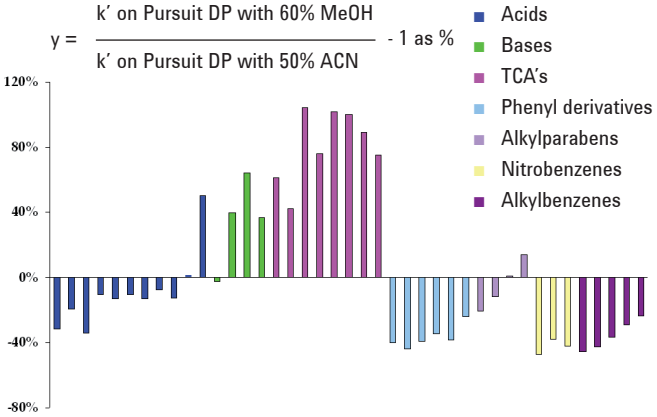

Figure 8. Effect of isoeluotropic concentrations of acetonitrile and methanol on Pursuit DP. Percentile plot of retention factors of 41 analytes with 60% CH_3OH relative to 50% CH_3CN

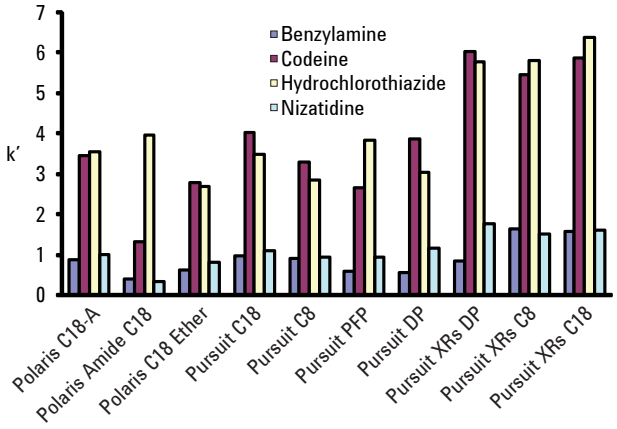

Figure 9. Retention of polar bases at 10% organic

Figure 9 indicates that more hydrophobic columns retained polar bases longer than less hydrophobic columns. Indeed, the order of alkyl-based columns for retention of polar bases at 10% organic followed the order of hydrophobicity given in Figure 5. This implied that a major retention mechanism under given conditions was a weak hydrophobic interaction between analytes and the stationary phase.

In both 50% organic and 10% organic experiments, Polaris Amide C18 appeared to retain almost all bases less than other columns (Figures 6a, 6b, 7, 9). A plausible explanation is that at pH 2-3, a protonated amide group might repel ionized bases[7, 12]. On Polaris Amide C18, most of the basic analytes coeluted, e.g. fluoxetine and TCAs at 50% organic (Figure 2a and 7); and, benzylamine and nizatidine at 10% organic (Figure 9). Thus, under low pH conditions, Polaris Amide can provide alternative selectivity options to straight chain alkyl-based columns for the retention of basic analytes. Phenyl-based phases and Polaris C18-A provided intermediate retention of bases both at 50% organic and 10% organic.

As mentioned earlier, the specified conditions were not necessarily optimal for a given class of compounds. For bases, adjusting the amount of organic modifier in the mobile phase could significantly enhance separation efficiency as compared to the results shown in Figures 7 and 9.

Retention of phenolic compounds

Among 200Å columns, phenolic compounds, including phenol, 4-nitrophenol, o-cresol and alkyl parabens, were best retained on Polaris Amide C18 and Polaris C18-A. Specific interactions of stationary phases with phenolic analytes that are not due to hydrophobic retention have been termed phenolic selectivity[6, 8]. To evaluate relative contribution of phenolic selectivity to column retention, various phenolic selectivity indices have been proposed, such as a retention factor ratio (or its logarithm) of butylparaben to dipropylphthalate[6], phenol and benzylalcohol, and phenol and toluene[8]. All three pairs comprised a non-phenolic compound and a phenol with a similar molecular structure. However, presence/absence of a phenolic -OH was not the only difference within each pair, and a change in retention cannot be attributed solely to phenolic selectivity. The difference in retention of phenol and benzylalcohol is not free from a "methylene increment" effect, described earlier. It seems that retention ratios of two compounds with the only difference in a presence/absence of phenolic -OH could provide better indices of phenolic selectivity. In this study, examples of such pairs of analytes were phenol and benzene, and methylparaben and methylbenzoate, all screened at 50% organic, pH 2. Figure 10 illustrates phenolic selectivity of ten Polaris and Pursuit columns as measured by phenol/benzene and methylparaben/methylbenzoate retention ratios. Despite a few small differences between the relative order in selectivity based on two ratios, all columns split into two groups: 1) a group with enhanced phenolic selectivity (Polaris Amide C18, Pursuit DP, Pursuit PFP and Pursuit XRs DP), and 2) a group with lower phenolic selectivity which covered the rest of the columns.

High phenolic selectivity of phenyl-based columns could be due to an increased π - π retention mechanism. Enhanced phenolic selectivity of polar-embedded amide phases has been described[8, 12]. It was attributed to hydrogen bonding of phenolics to polar groups in the stationary phase, namely, to the interaction of the phenolic proton and the highly polarized carbonyl oxygen[8]. In alkyl-based columns without a polar-embedded group, a hydrogen-bond acceptor in the stationary phase was assumed to be sorbed water[3, 12]. If this assumption is correct, the most hydrophobic phases, containing a smaller amount of sorbed water should possess the lowest phenolic selectivity. Indeed, the lowest phenolic selectivity in group 2 was found in Pursuit XRs C18, the most hydrophobic of all columns investigated. It was followed by Pursuit C18 and Pursuit XRs C8 (Figure 10). It can be seen from a comparison of Figures 4 and 10 that the more hydrophobic the stationary phase, the lower its phenolic selectivity.

Although the lowest calculated phenolic selectivity was found in Pursuit XRs C18, actual measured retention of phenolic compounds on this column appeared to be 20 - 50% longer than on Polaris Amide C18, which had the highest calculated phenolic selectivity (Figure 6b). Apparently, hydrophobicity-induced retention in Pursuit XRs C18 outweighed hydrogen-bonding-induced retention in Polaris Amide under the given mobile phase conditions.

The order of phenolic retention was similar to the order of acid retention, which was expected given that:

1. phenolic compounds were essentially very weak acids and were retained by the same combination of mechanisms (hydrophobic interaction and hydrogen bonding to a H-bond acceptor in a stationary phase),
2. all acids in this screening except sorbic acid possessed aromatic structure and therefore experience π - π interaction with phenyl phases.

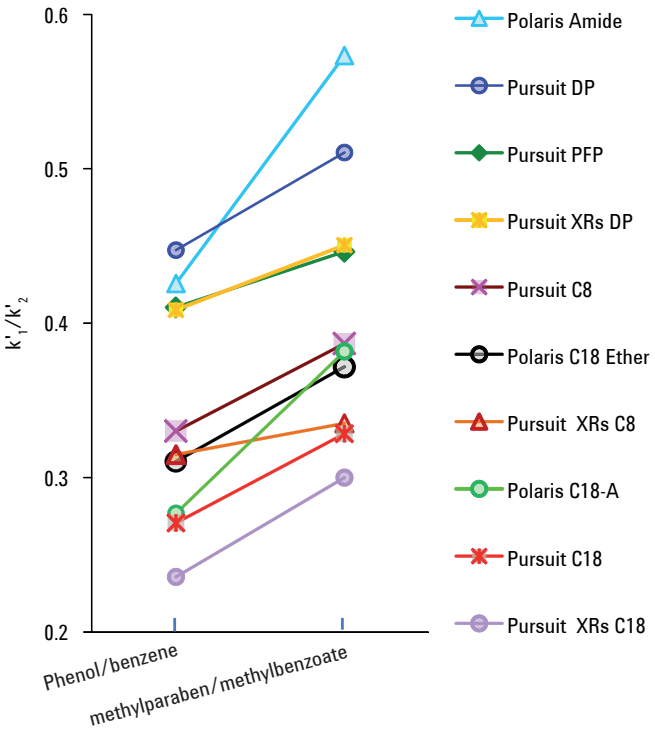

Figure 10. Phenolic selectivity in Polaris and Pursuit columns; 50% organic, pH 2

Conclusions

Screening of a broad range of analytes under identical low pH conditions revealed specific selectivities of Polaris and Pursuit columns to different classes of compounds and allowed identification of the most orthogonal pairs of columns:

- Combinations of columns involving Polaris Amide C18 and phenyl-based bonded phases (PFP and DP) were most complementary and provided the highest degree of orthogonality in this study due to the contribution of different chemistries involving diverse retention mechanisms. Selecting two members with different pore sizes can further enhance the degree of orthogonality.
- Within the 200Å suite of columns, enhanced acid and phenolic selectivity was found in polar-embedded Polaris Amide C18. It provided good retention of acids and phenolics but showed poor separation power for ionized bases under the low pH testing conditions.
- Within 200Å suite of columns, Pursuit C18 provided the strongest retention of alkylbenzenes and ionized bases.
- Compared to 200Å columns, all 100Å columns demonstrated stronger retention of neutrals, acids, and phenolics due to enhanced hydrophobic interaction. For these classes of compounds, alkyl-based XRs columns possessed the highest retentivity of all screened columns.
- Pure alkyl columns were good for retention of ionized bases, with Pursuit C18 providing strong retention for non-polar ionized bases at 50% organic, and Pursuit XRs C18 for polar ionized bases at 10% organic conditions.
- With acetonitrile as an organic modifier, phenyl-based columns provided intermediate retention of bases and shorter retention of acids, neutrals, and phenolics than alkyl-based columns. Increased retention of ionized bases can be achieved with methanol as an organic modifier. Pursuit XRs DP provided intermediate retention of all given classes of compounds between 100Å and 200Å columns.
- The order of phenolic selectivity of the columns appeared to be generally a reverse of their hydrophobicity order.

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Published in UK, October 6, 2010
SI-02425

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