Comparison of Selectivity Differences Among Different Agilent ZORBAX Phenyl Columns using Acetonitrile or Methanol

Application Note

Pharmaceutical

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Abstract

Alkyl, most commonly C18, columns are used in reversed-phase HPLC method development. Occasionally additional bonded phases are tested in attempt to optimize the separation. Phenyl bonded phases are an excellent choice to consider when a C18 does not achieve separation. In this work, the unique selectivity of three Agilent ZORBAX phenyl phases is shown and compared against a ZORBAX C18. The phenyl columns are run in a methanol mobile phase, as well as acetonitrile. Scatter plots comparing the organic solvents, show increased slopes and stronger correlation with acetonitrile due to the suppression of π-π interactions that are present with methanol. While these π-π interactions are not solely responsible for retention on phenyl columns in methanol, they could provide a slight enhancement to complicated separations of closely related compounds.

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Introduction

Alkyl phases, such as C18 stationary phases, are the most widely used for reversed-phase high performance liquid chromatography (HPLC). However, analysts occasionally encounter difficult separations for which selectivity, ruggedness or reproducibility is not easily obtained. These separations might require the use of more selective chromatography. Differences in selectivity are determined by various interactions between column stationary phase and solute. The mechanisms of these interactions, such as hydrophobicity, hydrogen bonding, dipole-dipole and ion exchange, all play an important part in column selectivity. Another contributor to selectivity is π-π interactions. The π-π interactions that occur between molecules and stationary phases involve double bonds. In this case they are the phenyl phases exemplified by Agilent ZORBAX StableBond SB-Phenyl, Eclipse XDB-Phenyl and Eclipse Plus Phenyl-Hexyl columns with molecules such as substituted aromatic or polycyclic compounds. In many cases the presence of one double bond is sufficient to yield a π-π interaction.

The π-π interaction can be defined as the interaction between π-electrons of the phenyl phase and those of the solute species. An interaction between π-electron containing compounds is favored when the stationary phase (usually phenyl) is electron-rich and the analyte is electron-poor. In these systems the stationary phase can act as an electron donor (soft Lewis base), while the analyte can act as recipient (soft Lewis acid) of electrons.[1] These interactions do not contribute to the retention behavior as much as hydrophobic interactions or electrostatic interactions do. However, π-π interactions can successfully be utilized in the separation of closely related compounds like metabolites or degradation products (finely tuned separation).[2]

Previous work has shown that π-π interactions contribute to the retention of aromatic and other unsaturated solutes on phenyl columns. It was noted that retention on phenyl columns as compared to C18 columns tends to increase in the order aliphatics < substituted benzenes < PAH's ≈ nitro-substituted aromatics, which is the order of increasing π-activity of the solute. The preferential retention of PAH's and nitro-substituted aromatics on phenyl columns is also significantly greater when methanol (MeOH) is used as B-solvent, compared to the use of acetonitrile (ACN). It is plausible to attribute the reduced retention of aromatics with ACN to π-π interactions of ACN with either solutes in the mobile phase or phenyl groups in the stationary phase.[3]

Phenyl bonded phases are available in a variety of different types (phenyl-ethyl, phenyl-hexyl etc.), and each of these may yield different selectivity for a variety of compounds.[4-9] The silica base or other base material of the column will also affect selectivity with a phenyl bonded phase. This work compares three Agilent ZORBAX Phenyl columns to an Agilent ZORBAX C18 column made using the same silica, to probe the differences without the complication of different base silicas and to show how phenyl columns can be a powerful alternate selectivity choice for optimizing resolution.

Experimental

An Agilent 1100 Series HPLC with a well plate autosampler and a diode array detector was used throughout this work. The mobile phase consisted of methanol/water (60:40) or acetonitrile:water (40:60), which have similar solvent elution strengths, and was mixed by the instrument's binary pump. The flow rate was set at 2.0 mL/min with 5-µL injections used throughout this study. Detection was accomplished at 205 nm. The column temperature was controlled at 25 °C. A series of over 40 aliphatic, nitro-substituted aromatic, substituted benzene and polyaromatic compounds (≈1 mg/mL each in acetonitrile:water, 50:50) were injected onto four different Agilent ZORBAX 4.6 mm × 100 mm 5-µm columns; log k' of each compound was determined and plotted.

Results

Table 1 lists the compounds, their category and the retention index (k') of the compound determined in 60% methanol on four different columns. Table 2 lists the same information in 40% acetonitrile. Many of these compounds were chosen and used in Croes [3]. In some cases, some of the materials listed in Croes were not available and substitutions were made. In the Croes paper, the compounds were grouped into the three categories listed in column 2 of Tables 1 and 2. In this work, a case may be made that a fourth category should be used because nitro-substituted aromatics and PAHs appear to have two distinct retentive patterns according to the slope and correlation data found in Table 3. The slopes and R^2 values were determined from linear regression lines fit to the log k' of data found in Tables 1 and 2, where compounds on respective phenyl columns were plotted against the same compound on the C18 column.

Table 1. Summary of Compound k′ in Methanol:Water (60:40) on Three Agilent ZORBAX Phenyl Columns and one Agilent ZORBAX C18 Column

Compound	Compound Classifiation	Eclipse Plus Phenyl-Hexyl	Eclipse XDB-Phenyl	StableBond SB-Phenyl	Eclipse XDB-C18
Thiourea	Void marker	0.00	0.00	0.01	0.00
Dimethoxybenzene	Substituted Benzene	1.48	1.31	1.20	1.16
Ethylbenzene	Substituted Benzene	6.37	4.01	3.11	11.55
Anisole	Substituted Benzene	2.33	1.75	1.43	2.62
Benzonitrile	Substituted Benzene	1.14	1.10	1.05	0.79
Ethyl benzoate	Substituted Benzene	3.66	2.88	2.71	4.39
Toluene	Substituted Benzene	3.71	2.48	1.97	6.18
p-Xylene	Substituted Benzene	6.45	3.93	3.19	12.87
Acetophenone	Substituted Benzene	1.25	1.18	1.22	1.02
Butylphenylether	Substituted Benzene	13.44	8.34	6.24	23.13
Benzene	Substituted Benzene	2.09	1.51	1.22	2.96
Acetanilide	Substituted Benzene	0.54	0.57	0.62	0.43
Methyl benzoate	Substituted Benzene	2.17	1.81	1.80	2.30
Chlorobenzene	Substituted Benzene	4.17	2.74	2.08	5.80
Benzaldehyde	Substituted Benzene	1.06	1.00	0.99	0.83
Fluorobenzene	Substituted Benzene	2.22	1.64	1.32	2.92
Bromobenzene	Substituted Benzene	5.16	3.27	2.44	7.05
Phenyl acetate	Substituted Benzene	1.37	1.29	1.15	1.19
Iodobenzene	Substituted Benzene	7.16	4.39	3.14	9.66
Nitrobenzene	Nitro-substituted Aromatic	2.08	1.67	1.36	1.50
p-Nitrotoluene	Nitro-substituted Aromatic	3.53	2.65	2.20	2.98
1-Chloro-4-nitrobenzene	Nitro-substituted Aromatic	4.31	3.26	2.35	3.31
p-Dinitrobenzene	Nitro-substituted Aromatic	2.88	2.44	1.57	1.17
m-Dinitrobenzene	Nitro-substituted Aromatic	3.35	2.71	1.69	1.31
1,3,5-Trinitrobenzene	Nitro-substituted Aromatic	4.69	4.10	2.01	1.04
1-Nitronaphthalene	Nitro-substituted Aromatic	7.68	5.31	4.12	5.82
1,3-Dinitronaphthalene	Nitro-substituted Aromatic	15.50	11.42	6.83	6.22
1,5-Dinitronaphthalene	Nitro-substituted Aromatic	11.36	9.03	5.62	4.63
9-Nitroanthracene	Nitro-substituted Aromatic	29.46	18.81	13.53	25.40
Naphthalene	PAH	7.46	4.44	3.44	10.69
2-Methylnaphthalene	PAH	12.92	7.22	5.63	20.98
1-Methoxynaphthalene	PAH	10.73	6.24	4.76	13.48
Biphenyl	PAH	15.18	8.96	6.57	22.59
Phenanthrene	PAH	23.93	12.43	9.61	37.00
Anthracene	PAH	27.13	14.32	10.81	43.53
p-Terphenyl	PAH	109.55	64.06	43.66	198.00
Fluorene	PAH	20.30	11.08	8.16	31.43
Acenaphthene	PAH	18.64	10.02	7.23	28.30
Fluoranthene	PAH	38.97	19.06	14.96	65.05
Ethyl acetate	Aliphatic	0.46	0.50	0.53	0.44
n-Propyl acetate	Aliphatic	0.94	0.89	0.91	1.04
n-Butyl acetate	Aliphatic	1.82	1.58	1.55	2.28
n-Pentyl acetate	Aliphatic	3.44	2.72	2.64	4.80
1-Nitropropane	Aliphatic	0.69	0.67	0.55	0.43
1-Nitrobutane	Aliphatic	1.37	1.23	0.95	1.02
1-Bromopropane	Aliphatic	2.70	1.94	1.45	3.61
1-Bromobutane	Aliphatic	5.11	3.36	2.46	7.74
1-Bromopentane	Aliphatic	9.42	5.80	4.18	16.06
1-Bromohexane	Aliphatic	17.07	10.09	7.10	33.23

Table 2. Summary of Compound k′ in Acetonitrile:Water (40:60) on Three Agilent ZORBAX Phenyl Columns and one Agilent ZORBAX C18 Column

Compound	Compound Classifiation	Eclipse Plus Phenyl-Hexyl	Eclipse XDB-Phenyl	StableBond SB-Phenyl	Eclipse XDB-C18
Thiourea	Void marker	0.00	0.00	0.00	0.00
Dimethoxybenzene	Substituted Benzene	1.74	1.79	1.84	2.16
Ethylbenzene	Substituted Benzene	9.52	7.71	7.20	20.68
Anisole	Substituted Benzene	3.40	3.14	3.07	4.88
Benzonitrile	Substituted Benzene	2.00	2.05	2.15	2.38
Ethyl Benzoate	Substituted Benzene	4.62	4.21	4.34	8.04
Toluene	Substituted Benzene	5.72	4.80	4.58	10.64
p-Xylene	Substituted Benzene	9.22	7.34	6.97	21.48
Acetophenone	Substituted Benzene	1.62	1.68	1.84	2.05
Butylphenylether	Substituted Benzene	18.28	14.12	13.02	43.76
Benzene	Substituted Benzene	3.47	3.08	2.97	5.45
Acetanilide	Substituted Benzene	0.46	0.58	0.71	0.46
Methyl benzoate	Substituted Benzene	2.73	2.62	2.78	4.11
Chlorobenzene	Substituted Benzene	6.29	5.14	4.89	10.68
Benzaldehyde	Substituted Benzene	1.55	1.58	1.71	1.88
Fluorobenzene	Substituted Benzene	3.74	3.41	3.30	5.72
Bromobenzene	Substituted Benzene	7.53	6.02	5.59	12.76
Phenyl Acetate	Substituted Benzene	2.27	2.36	2.39	2.91
Iodobenzene	Substituted Benzene	10.14	7.73	7.12	17.89
Nitrobenzene	Nitro-Substituted Aromatic	3.00	2.94	2.93	3.66
p-Nitrotoluene	Nitro-Substituted Aromatic	4.89	4.46	4.51	7.16
1-Chloro-4-nitrobenzene	Nitro-Substituted Aromatic	5.86	5.23	4.95	7.69
p-Dinitrobenzene	Nitro-Substituted Aromatic	3.99	3.96	3.70	3.84
m-Dinitrobenzene	Nitro-Substituted Aromatic	3.86	3.72	3.55	3.69
1,3,5-Trinitrobenzene	Nitro-Substituted Aromatic	6.00	5.83	4.83	3.99
1-Nitronaphthalene	Nitro-Substituted Aromatic	8.93	7.70	7.72	12.72
1,3-Dinitronaphthalene	Nitro-Substituted Aromatic	13.06	11.52	10.87	14.17
1,5-Dinitronaphthalene	Nitro-Substituted Aromatic	10.79	9.62	9.20	11.86
9-Nitroanthracene	Nitro-Substituted Aromatic	28.67	21.37	21.27	50.82
Naphthalene	PAH	10.36	8.11	7.76	18.26
2-Methylnaphthalene	PAH	16.61	12.23	11.71	35.54
1-Methoxynaphthalene	PAH	12.95	10.12	9.67	23.30
Biphenyl	PAH	19.10	14.78	13.78	36.57
Phenanthrene	PAH	26.97	18.67	17.91	55.68
Anthracene	PAH	30.14	20.31	19.59	64.91
p-Terphenyl	PAH	93.87	63.26	59.97	248.68
Fluorene	PAH	21.85	15.95	14.85	44.46
Acenaphthene	PAH	20.00	14.11	13.03	41.00
Fluoranthene	PAH	40.09	26.06	25.77	92.99
Ethyl acetate	Aliphatic	0.56	0.67	0.71	0.72
n-Propyl acetate	Aliphatic	1.16	1.22	1.31	1.75
n-Butyl acetate	Aliphatic	2.20	2.16	2.28	3.74
n-Pentyl acetate	Aliphatic	3.99	3.67	3.83	7.92
1-Nitropropane	Aliphatic	1.37	1.45	1.46	1.57
1-Nitrobutane	Aliphatic	2.57	2.61	2.54	3.21
1-Bromopropane	Aliphatic	4.16	3.57	3.33	6.82
1-Bromobutane	Aliphatic	7.55	6.12	5.59	14.38
1-Bromopentane	Aliphatic	13.39	10.19	9.19	29.75
1-Bromohexane	Aliphatic	23.24	16.50	14.88	61.67

By plotting the k' of a compound on one column against the k' of another the relative strength of interaction and similarity can be determined. The slope of the line indicates the relative strength of the interaction as compared to the base column (in this case an Eclipse XDB-C18). When the value of the slope is 1, this indicates an equal strength of retention behavior. As an example, a C8 column will interact less with a group of analytes than a C18 column and so will have a slope of less than 1 when this convention is applied. As interactions with the target column become weaker relative to the control column, the value becomes lower.

The correlation coefficient (R^2) is an indication of orthogonality. In an orthogonal method, the objective is to take advantage of varied chemical interactions between analytes, solvents and column stationary phases in an attempt to maximize differences between two methods. The more orthogonal the methods are to each other the lower the correlation coefficient, producing visually more scattered data.

As an example a group of compounds is taken from Tables 1 and 2, and injected as a mixture onto Eclipse XDB-Phenyl and Eclipse XDB-C18 in both acetonitrile:water and methanol:water. The sample set is reduced to simplify the examples. Figure 1 shows closely correlating conditions, which show similar elution order with acetonitrile, and Figure 2 which shows more dissimilar correlation (with several peak order changes) with methanol. Most notable is the change in elution order in the methanol:water pairing. Not all peaks change order, but column solvent selectivity can be a useful tool in accomplishing difficult separations.

Hydrophobic interactions between the solute and the stationary phase generally dominate reverse phase HPLC. A scatter plot comparing retention on one C18 column to another made on the same silica with similar bonding chemistry would likely produce a linear (slope (m = 1), $R^2 \approx 1$) plot. Deviations from unity correlation coefficient ($R^2 < 1$) can be attributed to other non-hydrophobic interactions such as π-π, dipole-dipole, etc. The scatter plot data (Figures 3–5) infer that all phenyl bonded phases have more non-polar interactions when methanol mobile phase is used compared to acetonitrile. The π-π interactions are suppressed when acetonitrile is used, resulting in weaker retention of the nitrogen substituted aromatics and a more linear R^2 value with a slope closer to 1. Scatter plots comparing phenyl columns to C18 columns have lower correlation (R^2) and more deviation from 1 in slope values, indicating different selectivity.

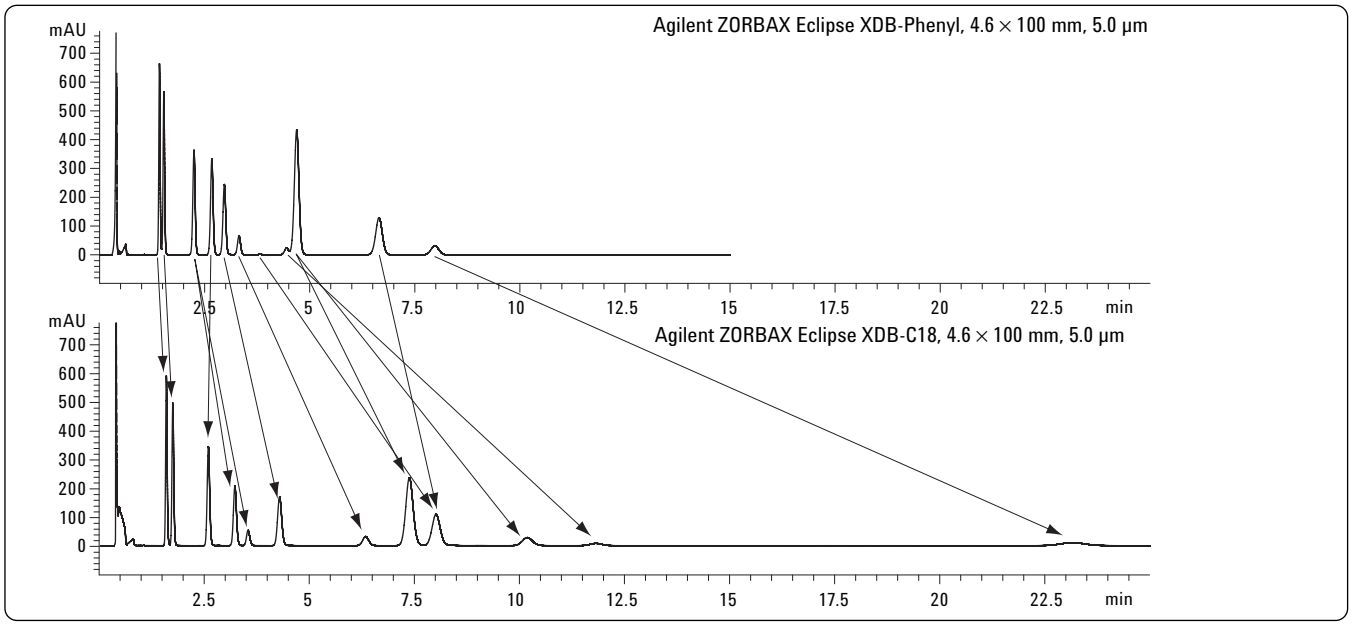

Figure 1. Closely correlating chromatograms in acetonitrile/water on Agilent ZORBAX Eclipse XDB-Phenyl and Agilent ZORBAX Eclipse XDB-C18.

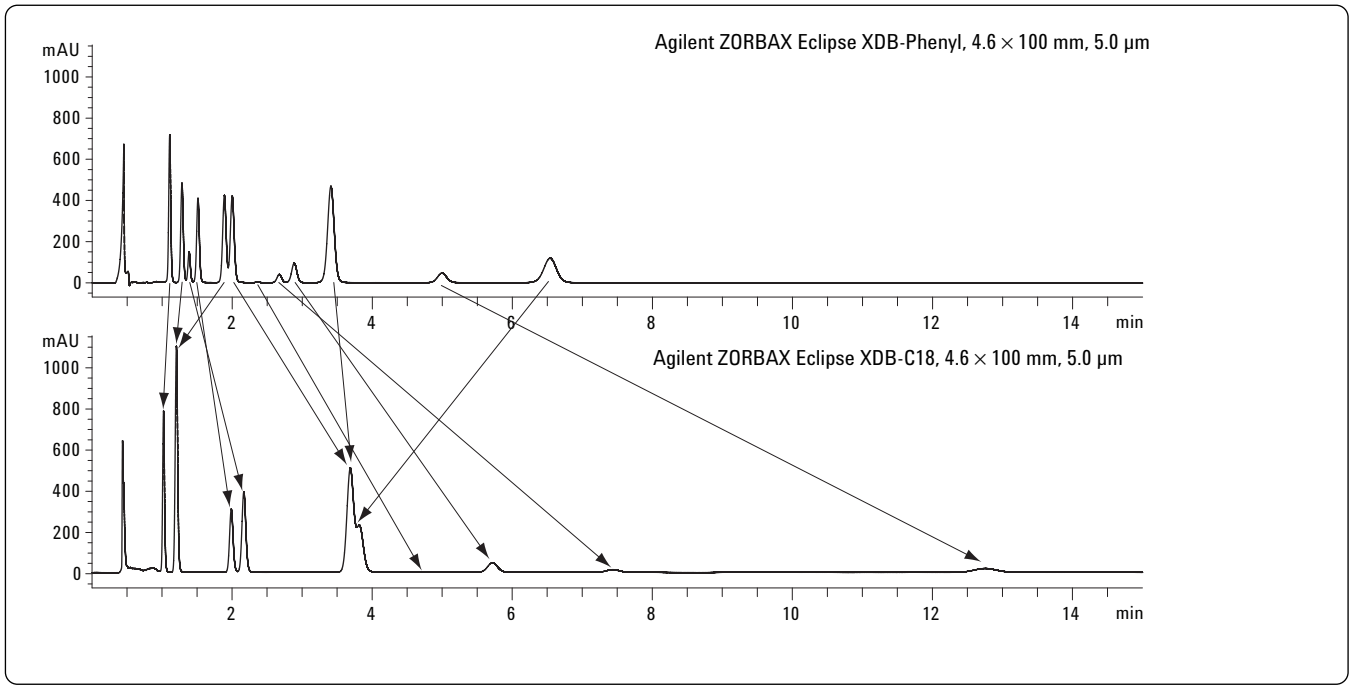

Figure 2. Chromatograms in methanol/water on Agilent ZORBAX Eclipse XDB-Phenyl and Agilent ZORBAX Eclipse XDB-C18 showing more varied selectivity. (elution order changes for several peaks).

Agilent ZORBAX phenyl phases start with Rx-Sil as a base material. As mentioned earlier, three phases are available: StableBond SB-Phenyl (an ethyl phenyl column, with two isobutyl protecting groups, on a nonend-capped type B silica), Eclipse XDB-Phenyl (an ethyl phenyl column, with two methyl side chains, on a double end-capped type B silica) and Eclipse Plus Phenyl-Hexyl (a hexyl phenyl column with two methyl side chains on a double end-capped type B silica). Of these three, the Eclipse Plus Phenyl-Hexyl is generally the most retentive, due to its longer alkyl linkage.

The scatter plot indicates that nitro-substituted aromatics have the greatest difference in retention on the phenyl versus C18, but the aliphatics and substituted benzenes also varied. This is shown by the correlation coefficient of the respective plots. It can also be seen that a methanol organic modifier changes selectivity between the two bonded phases more than acetonitrile.

Good correlation between the phenyl phases and the C18 phase occur when the interactions are controlled by a common mechanism. The slope of the respective lines is indicative of the relative strengths of these interactions. As can be seen in Table 3, the slopes are greater for the Eclipse Plus Phenyl-Hexyl versus Eclipse XDB-C18 plots than for either of the similarly plotted data for either Eclipse XDB-Phenyl or StableBond SB-Phenyl. The reason for this trend is that the phenyl-hexyl column with its six-carbon linkage and six-carbon phenyl ring yields a more highly lipohilic interaction (lower percentage of π-π interaction) than the Eclipse XDB-Phenyl or the StableBond SB-Phenyl with their two carbon ethyl phenyl linkages. These trends are especially evident in acetonitrile containing mobile phase.

The correlation coefficients (R^2) for all columns using either methanol or acetonitrile are between 0.98 and 0.99 for the aliphatic compounds indicating a high degree of similarity between the interactions involved in the separation on phenyl and C18 columns. Surprisingly, the PAH compounds yield similar relationships in both acetonitrile and methanol.

Table 3. Summary of Slope and Correlation Data for Agilent ZORBAX Phenyl and C18 Columns; Values are Derived from Linear Regression Data for Each Compound Class on the Respective Phenyl Column Versus C18 Column

	Substituted Benzenes		Nitro-substituted Aromatics		PAH's		Aliphatics	
Column (mobile phase)	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**
Eclipse Plus Phenyl-Hexyl (60% methanol)	0.9786	0.7271	0.7898	0.7517	0.9951	0.9020	0.9841	0.7770
Eclipse XDB-Phenyl (60% methanol)	0.9494	0.5686	0.7198	0.6635	0.9859	0.8768	0.9824	0.6403
StableBond SB-Phenyl (60% methanol)	0.8993	0.4734	0.8782	0.7117	0.9908	0.8463	0.9788	0.5724
Eclipse Plus Phenyl-Hexyl (40% acetonitrile)	0.9949	0.7905	0.9131	0.7761	0.9972	0.8444	0.9909	0.8228
Eclipse XDB-Phenyl (40% acetonitrile)	0.9942	0.6733	0.8810	0.6770	0.9899	0.7697	0.9885	0.7053
StableBond SB-Phenyl (40% acetontrile)	0.9935	0.6120	0.9292	0.7036	0.9915	0.7750	0.9917	0.6635

While the nitro-substituted aromatics exhibit the greatest differentiation when comparing methanol and acetonitrile based mobile phases, PAH compounds also show interesting differences. Comparing the graphs in Figures 3, 4, and 5, it is observed that in acetonitrile, the PAH trendline is virtually a continuation of the substituted benzene and aliphatic compound trendlines. However in methanol-based mobile phases, the trendline slope becomes more vertical indicating an affinity for the phenyl phases. This slope change is more dramatic on the Eclipse XDB-Phenyl and StableBond SB-Phenyl (ethyl phenyl phases) than on the Eclipse Plus Phenyl-Hexyl column.

The four classes of compounds in the study are substituted benzenes, nitro-substituted aromatics, polyaromatic hydrocarbons (PAH's) and aliphatics. Three of these compound groups (substituted benzenes, nitro-substituted aromatics and PAHs) can also be considered in the context of electron donating or electron withdrawing effects.

Examples of electron donating groups (EDG) are alkyl, alcohol and amino groups (–OMe, $–NH_2$). Electron donating groups can be recognized by the presence of lone pairs on the atom adjacent to the bond. Alkyl, aryl or vinyl groups can also achieve this effect through hyperconjugation. Examples of electron withdrawing groups are halogens, nitriles, carbonyls and nitro groups (–C=O, $–NO_2$). Electron withdrawing groups are recognized by the atom adjacent to the bond having several bonds to more electronegative atoms. Not all groups have equivalent effects; however the nitro groups have the greatest effect.

In the final graph of log k's for alkanes and substituted benzenes for Eclipse XDB-C18 and Eclipse XDB-Phenyl are plotted using methanol. As can be seen in other graphs and tables throughout this work, methanol allows the most differentiation in retention. Using the line formed by the plot of the non π active alkanes, the substituted benzenes (including nitrobenzene) are plotted (Figure 6). Interestingly, the compounds with electron withdrawing groups show greater retention than the alkane compounds and fall above the plotted line. In addition, the electron donating groups show less retention than the alkane reference.

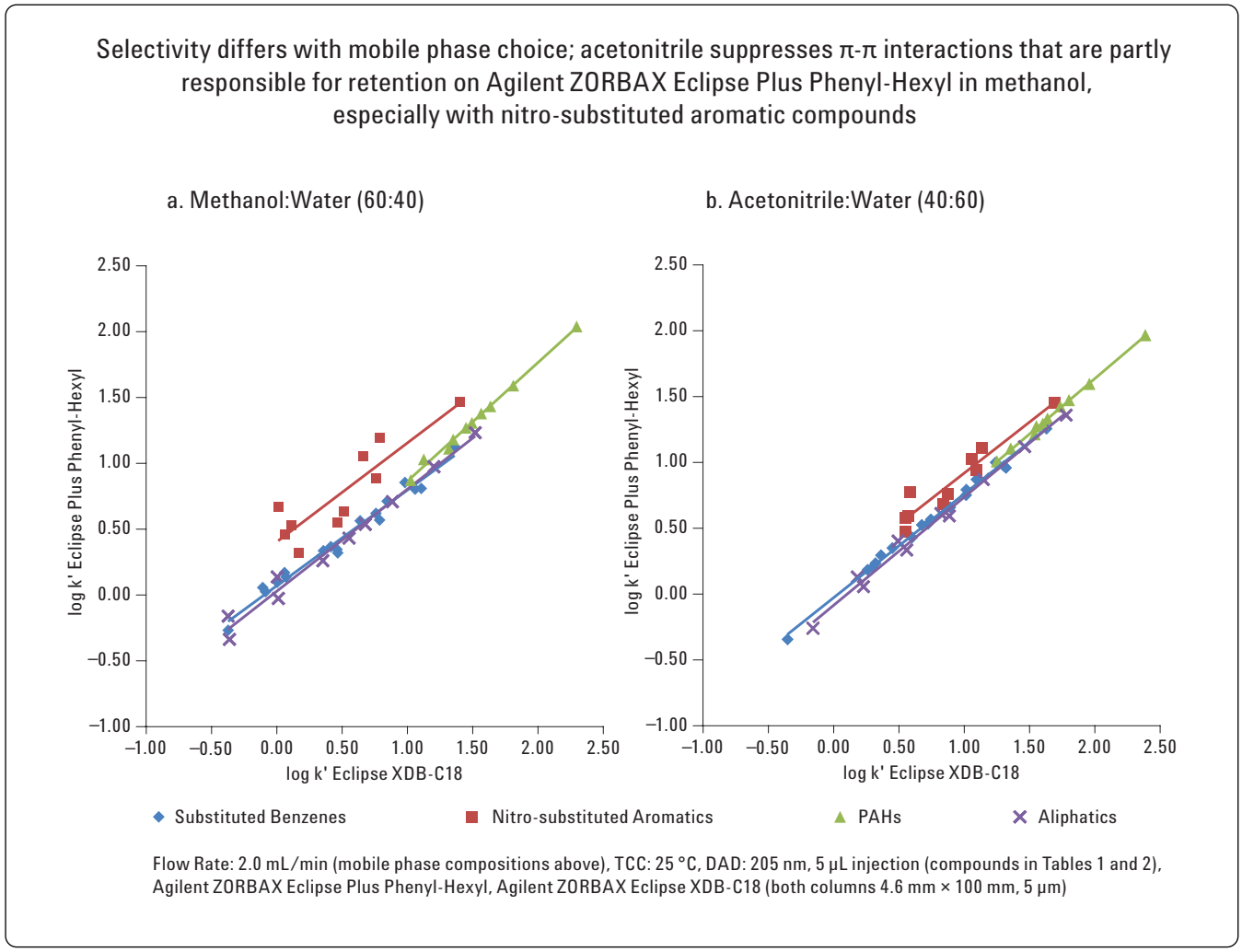

Figure 3. Scatter plot showing the correlation of retention of 40+ compounds by category on Agilent ZORBAX Eclipse Plus Phenyl-Hexyl and Eclipse XDB-C18 in methanol (a) or acetonitrile (b).

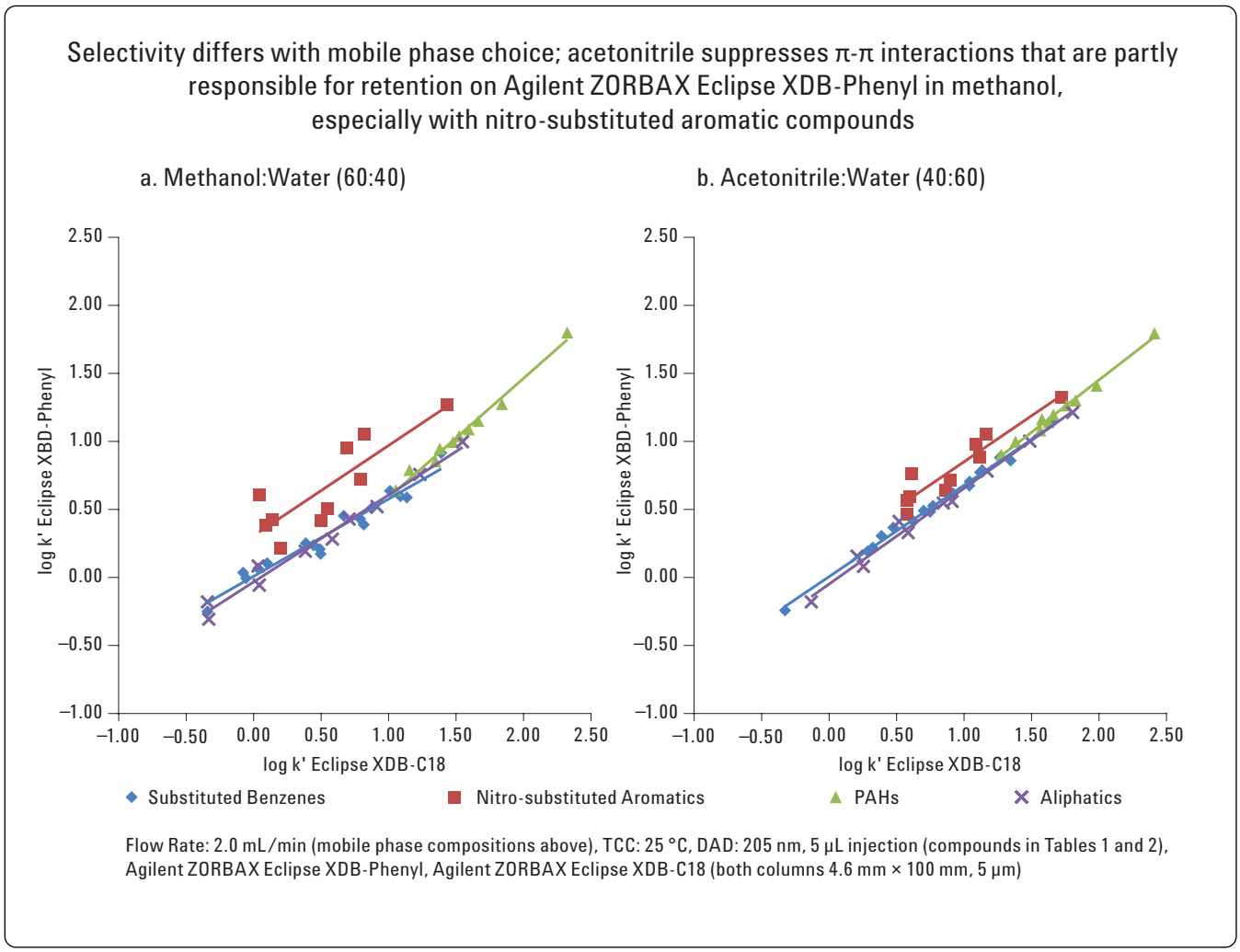

Figure 4. Scatter plot showing the correlation of retention of 40+ compounds by category on Agilent ZORBAX Eclipse XDB-Phenyl and Eclipse XDB-C18 in methanol (a) or acetonitrile (b).

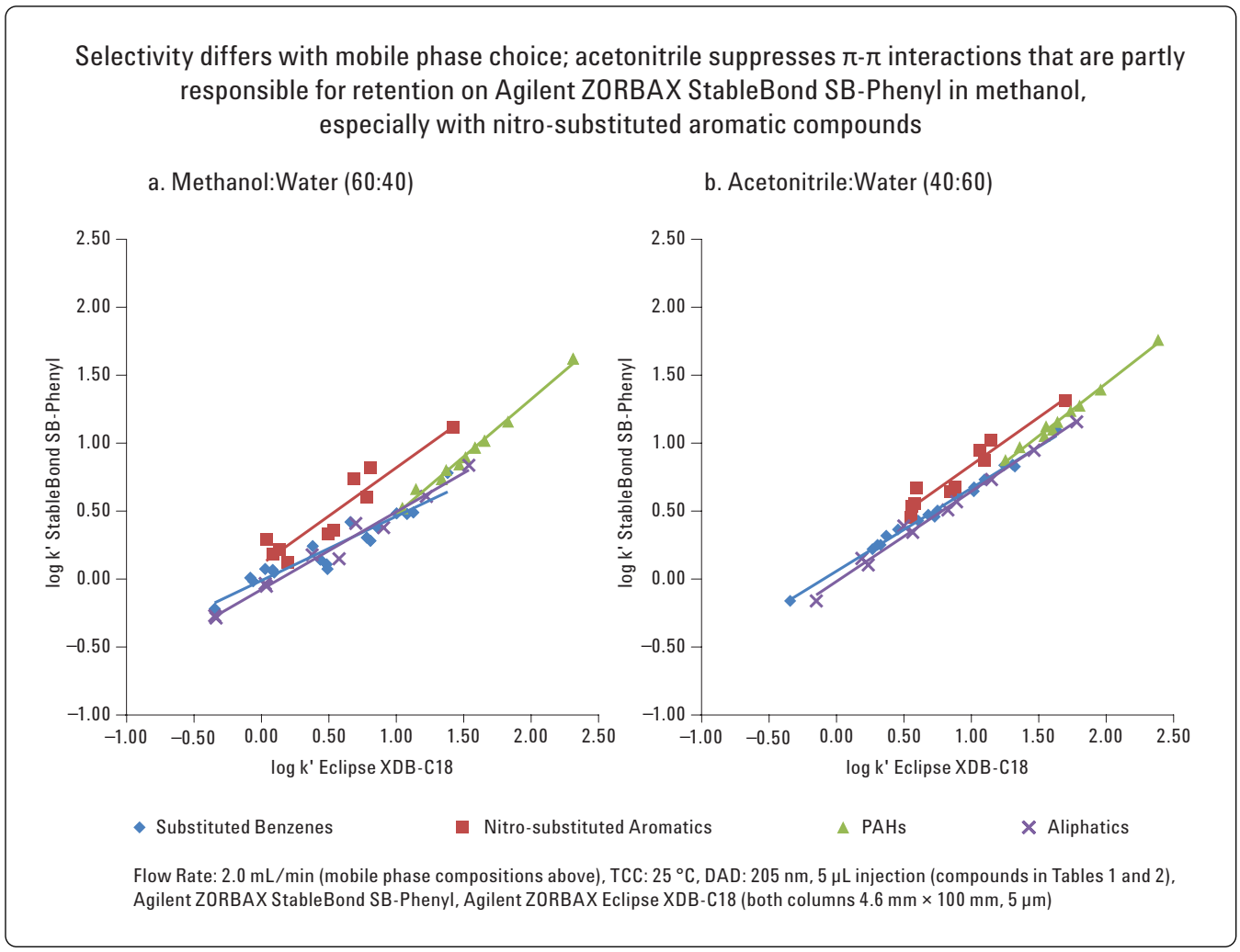

Figure 5. Scatter plot showing the correlation of retention of 40+ compounds by category on Agilent ZORBAX StableBond SB-Phenyl and Eclipse XDB-C18 in methanol (a) or acetonitrile (b).

While the aliphatic compounds show good correlation between both Agilent ZORBAX Eclipse XDB-Phenyl and C18 columns, substituted benzenes do not, further the compounds subtituted with electron withdrawing groups fall above the aliphatic trendline and those with electron donating groups fall below the trendline.

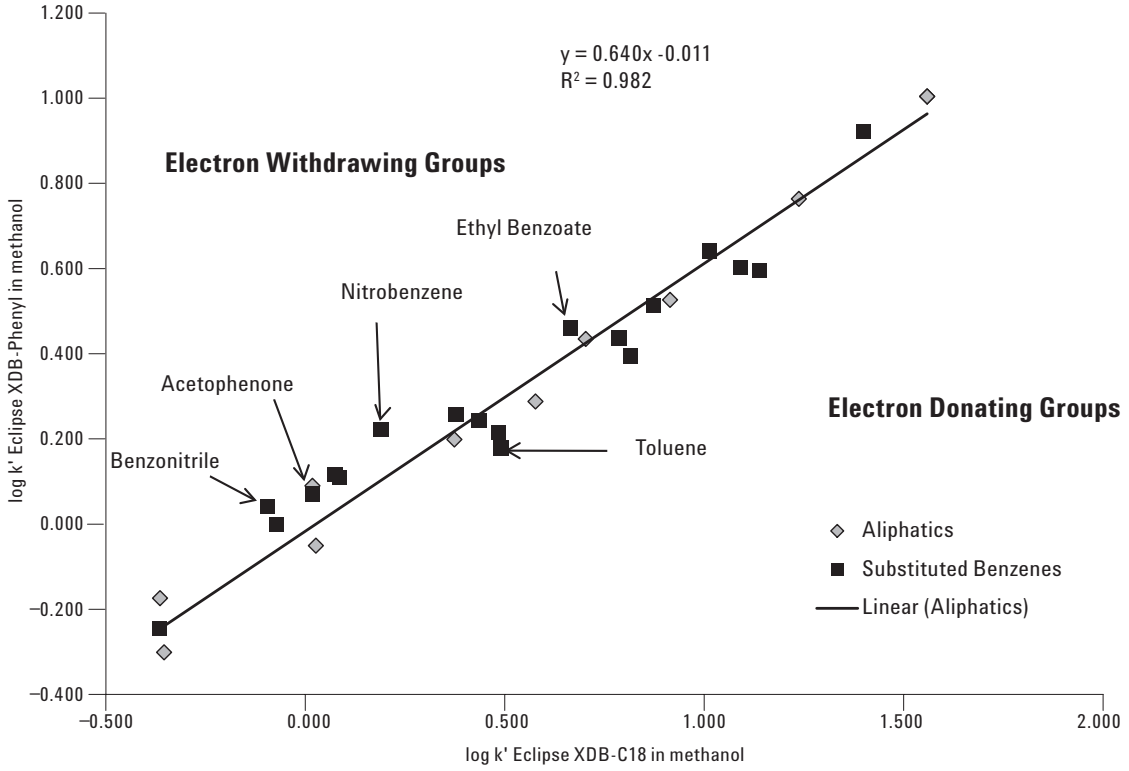

Figure 6. Aliphatic and substituted benzenes on Agilent ZORBAX Eclipse XDB-Phenyl and C18 in Methanol.

Conclusions

In this work, it has been shown that selectivity for substituted benzene compounds, nitro substituted aromatics and PAH compounds can be altered by choice of solvents and columns. Methanol is a good choice especially when paired with Phenyl columns.

Phenyl columns offer selectivity derived, at least in part, from π-π interactions in addition to hydrophobic interactions which dominate reverse phase high performance liquid chromatography.

These interactions can be reduced by using acetonitrile, which appears to interfere with π-π interactions due to competition from the acetonitrile triple bond (nitrile group). However, these interactions are only attenuated, not destroyed as many useful applications take place in acetonitrile. The different phenyl phases offered on highly consistent ZORBAX Rx-Sil allow a balanced mixture of retention mechanisms including hydrophobic and π-π interactions, as well as ionic interactions that can be exploited with StableBond.

While most separations are developed starting with C18 columns, these are not the only choices available for optimum resolution. Multiple bonded phases are frequently considered when desired resolution is not easily attained on a C18 column. Due to the presence of π-π interactions with phenyl bonded phases, phenyl columns provide an excellent alternative to C18 columns during method development, especially when fine tuning a difficult separation of closely related compounds.

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