Successful Selection of an "Orthogonal" Column During HPLC Method Development

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ABSTRACT

The selection of reversed-phase conditions for orthogonal separation is of interest in two situations: "missing" peaks or 2-D separation. For either application, the goal is to achieve a maximum difference in selectivity between two separations, so that any two overlapping peaks in one separation are likely to be resolved in the other separation. Changes in selectivity are most readily achieved by changes in the column, B-solvent (e.g., MeOH vs. ACN), or mobile-phase pH. The effects of the B-solvent or pH on selectivity can be determined with a few experiments, but the selection of a column of differing selectivity can be more challenging. One approach to predicting differences in column selectivity is based on the so-called *hydrophobic subtraction model* [1]. The use of this approach, combined with a change in the B-solvent, has been tested and found promising[2]. However, a closer look at the latter procedure [3], suggests that this approach may be less effective for some columns. The present poster explores an alternative procedure for developing orthogonal separations, based on a change of column.

1. "A New Look at the Selectivity of Reversed-phase HPLC Columns", L. R. Snyder, J. W. Dolan and P. W. Car, *Anal. Chem.*, 79 (2007) 3255.

2. "Orthogonal Separations for Reversed-phase Liquid Chromatography", J. Pellett et al, *J. Chromatogr. A*, 1101 (2006) 122.

3. "Characterization and Applications of Reversed-phase Column Selectivity", D. M. Marchand, L. R. Snyder, and J. W. Dolan, *J. Chromatogr. A*, (2008) (in press).
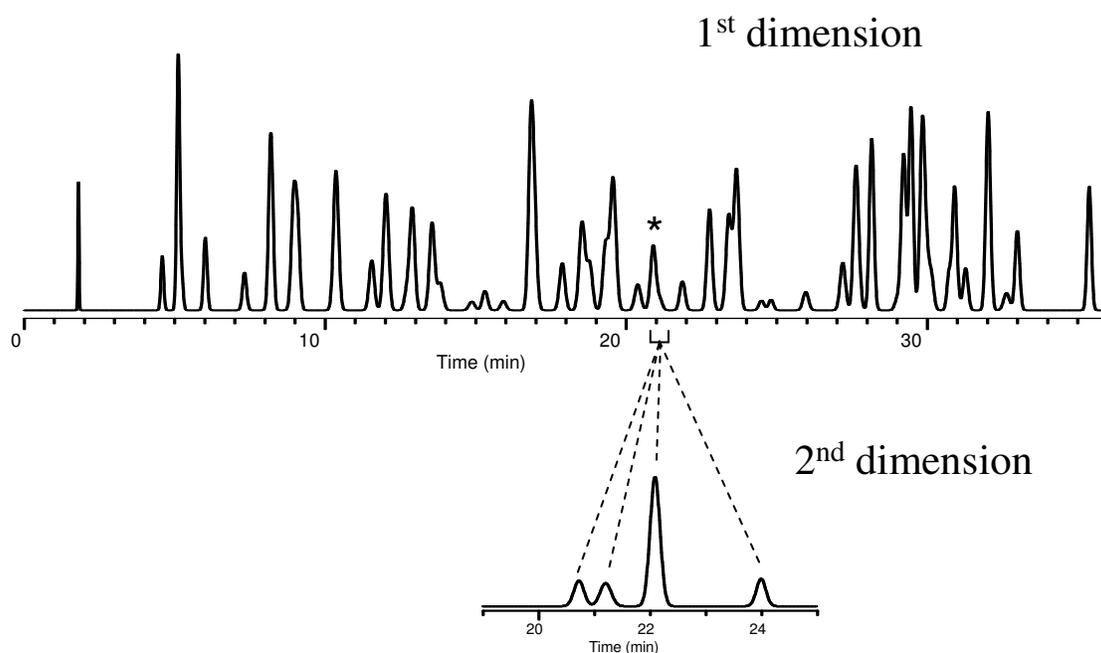
Why Orthogonal Separation?

- Selectivity change during method development

- Find "hidden" peaks

- 2-D Separation

In this poster we will emphasize the detection of hidden peaks, but our results should also be of interest for both improving resolution and the development of a 2-D separation.
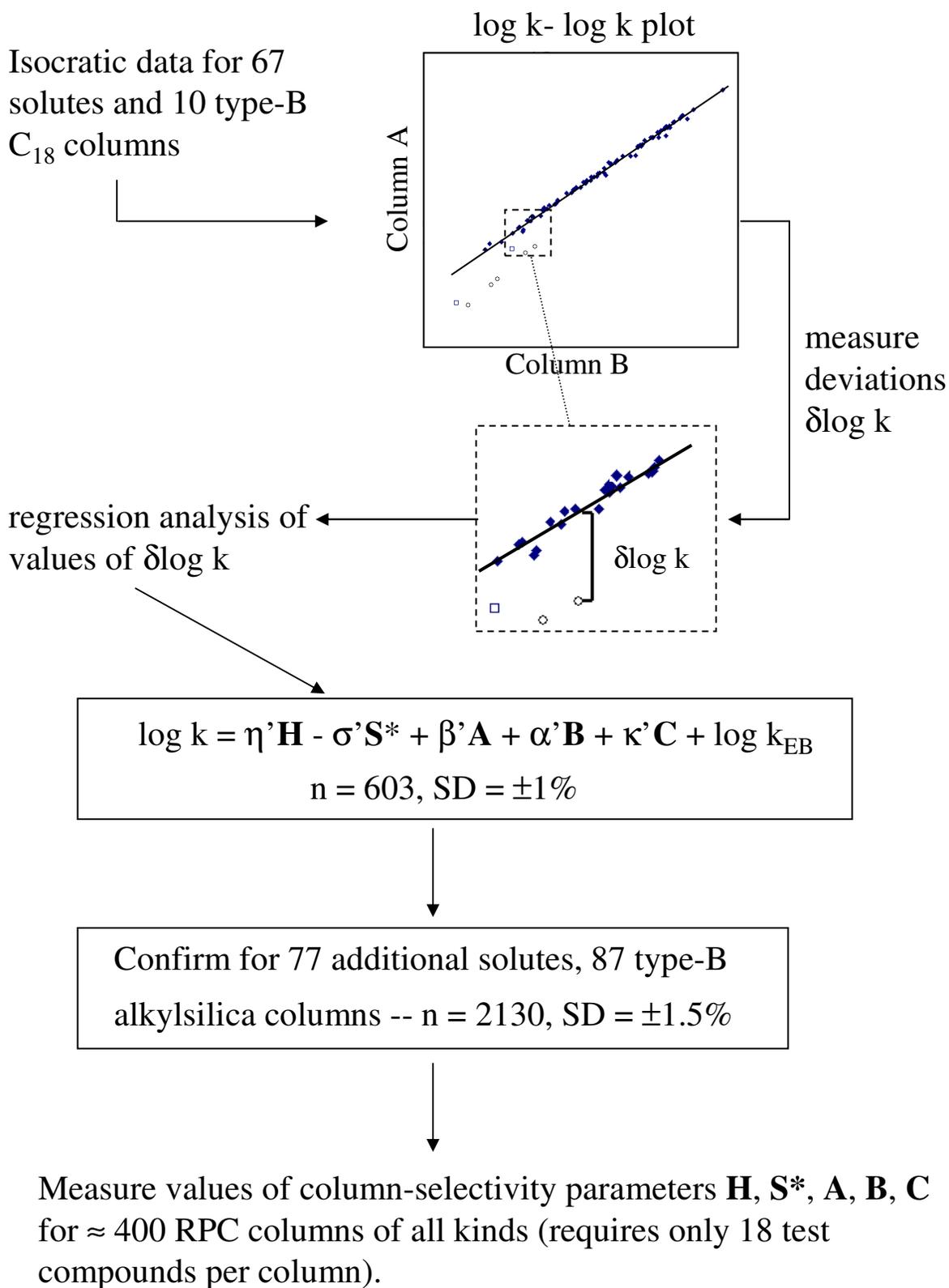
Achieving Orthogonal Separation
(maximum change in selectivity)

For ionizable solutes such as amines and carboxylic acids, a large change in selectivity can be achieved by a change of mobile phase pH from low to high (e.g., from pH-3 to pH-10). A change in the B-solvent from acetonitrile to methanol can also change selectivity for both neutral and ionizable compounds. The selection of a suitable change in pH or B-solvent for orthogonal separation is easily achieved. Large changes in selectivity (for all solute types) can also be achieved by a change in the column, but an optimum choice of column is less obvious. *When conditions are selected for orthogonal separation, a combined change in pH, B-solvent, and column is recommended.* The present poster will deal primarily with the selection of an orthogonal column, assuming simultaneous changes (as above) in pH and B-solvent.
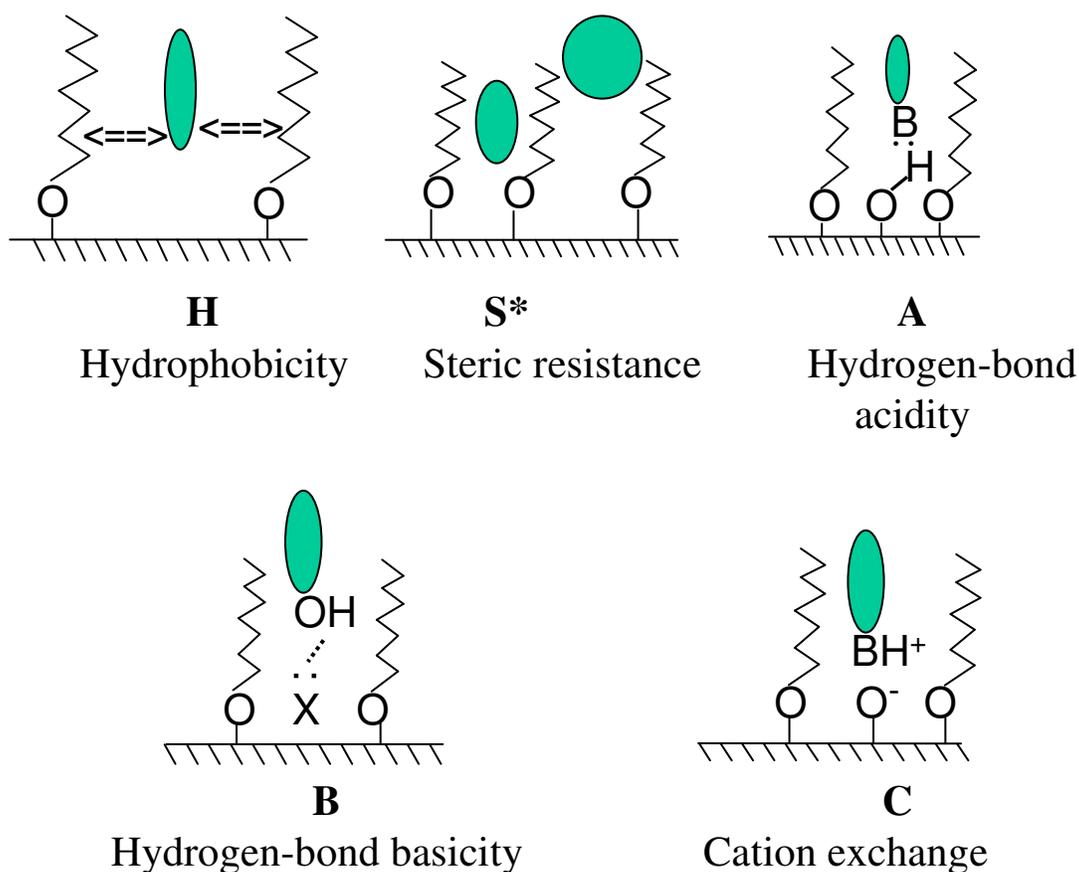
Measuring Separation Orthogonality

For gradient separations (assumed in this poster), the usual measure of separation orthogonality is the correlation (r^2) of retention data for the 1st vs. 2nd separations. For samples that contain both neutral and ionizable compounds, a value of r^2 can be a poor measure of overall separation orthogonality. This is because a change in pH will lead to large changes in the relative retention of a mixture of acids and bases, but will have little effect on the relative retention of neutral compounds. Similarly, a change in the B-solvent or column will usually have a larger effect on the retention of ionizable compounds than for neutral compounds. Consequently, when pH, B-solvent and column are changed, a large value of r^2 provides information mainly about changes in relative retention for *ionizable* species with differing values of pK_a.

The Hydrophobic-Subtraction Model of Reversed-Phase Column Selectivity (1)

log k- log k plot

Isocratic data for 67 solutes and 10 type-B C_{18} columns

Column A

Column B

measure deviations $\delta\log k$

regression analysis of values of $\delta\log k$

$\delta\log k$

$$\log k = \eta'\mathbf{H} - \sigma'\mathbf{S^*} + \beta'\mathbf{A} + \alpha'\mathbf{B} + \kappa'\mathbf{C} + \log k_{EB}$$

$$n = 603, SD = \pm 1\%$$

Confirm for 77 additional solutes, 87 type-B alkylsilica columns -- n = 2130, SD = ±1.5%

Measure values of column-selectivity parameters **H**, **S***, **A**, **B**, **C** for ≈ 400 RPC columns of all kinds (requires only 18 test compounds per column).

The Hydrophobic-Subtraction Model of Reversed-Phase Column Selectivity (2)

The preceding procedure allows the quantitative measurement of 5 column-selectivity parameters for any reversed-phase column. These parameters have been shown to measure the following sample-column interactions:

H
Hydrophobicity

S*
Steric resistance

A
Hydrogen-bond acidity

B
Hydrogen-bond basicity

C
Cation exchange

Two columns 1 and 2 can be compared in terms of selectivity by means of the column comparison function F_s:

$$F_s = \{(H_2-H_1)^2 + (S^*_2-S^*_1)^2 + (A_2-A_1)^2 + (B_2-B_1)^2 + (C_2-C_1)^2\}^{1/2}$$

(plus weighting of each column parameter).

Comparing Columns

When *similar columns* are required (as for column replacement), the value of F_s should be small; preferably $F_s \leq 3$.

When *orthogonal columns* are required, the value of F_s should be large. **However**, the value of F_s can be misleading in this connection The problem is that the cation exchange parameter **C** has a much larger effect on F_s than values of **H**, **S***, **A**, or **B**. Thus, ionized sample components largely determine values of F_s – particularly for large values of F_s, as when selecting an orthogonal column.

As an example, consider two columns: Capcell C18 UG120 and YMC PackPro, for which F_s is fairly large ($F_s = 60$). When retention (values of log k) are compared for these two columns, however, only the 3 protonated bases in the sample show a significant change in relative retention:

log *k* (Capcell)
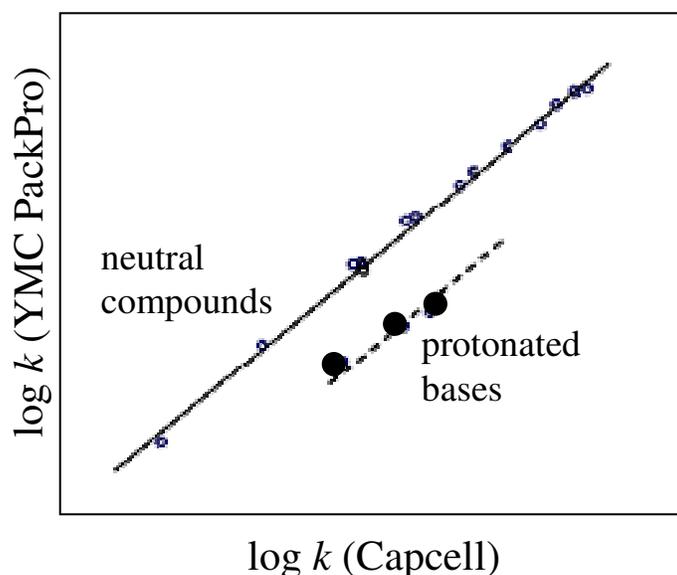
Consequently, these two columns might be considered as somewhat "orthogonal" for the entire sample, but not for the neutral compounds in the sample.

An Alternative Approach for Achieving Column "Orthogonality"

If an orthogonal separation is developed by changing mobile phase pH, as well as the B-solvent and column, there is less need for the column to provide changes in relative retention for ionizable compounds (changes in retention for ionizable compounds will automatically occur as a result of the change in pH). For this reason, it is more important that the column provide changes in relative retention for *neutral* compounds in the sample. This suggests dropping the C-term of the F_s equation:

$$F_s(-C) = \{(\mathbf{H}_2-\mathbf{H}_1)^2 + (\mathbf{S}^*_2-\mathbf{S}^*_1)^2 + (\mathbf{A}_2-\mathbf{A}_1)^2 + (\mathbf{B}_2-\mathbf{B}_1)^2\}^{1/2}$$

By seeking a maximum value of $F_s(-C)$, we can expect a greater change in relative retention for neutral compounds – the part of the sample that presents the greatest challenge. A related consideration is the fact that ionizable compounds with similar pK_a values will be less likely to be separated by a change in pH – in this sense behaving more like neutral molecules when pH is changed.

Evaluating Column Orthogonality

We have seen that retention correlations (values of r^2) may be of limited value as a measure of of column orthogonality. This will be especially true for recognizing "missing" peaks, when two neutral compounds overlap in a routine assay procedure. The ultimate test of separation orthogonality is the demonstration for a broad range of samples that peaks which overlap in one separation are resolved in the corresponding orthogonal separation. However, such a test of column orthogonality is impractical for "real" separations, if the goal is the demonstration that any "hidden" peaks are resolved (we have no way of knowing whether any "hidden" peaks were not resolved).

Testing Column Orthogonality

In the absence of a large number of "real" separations for comparison, an alternative is presented by data acquired during the development of the hydrophobic-subtraction model. Values of the sample parameters η', σ', β', α', and κ' were obtained for 64 neutral compounds of widely varying molecular structure. In addition, the effects of %B on retention were also obtained (values of $d[\log k]/d\phi = S$), so that retention can be predicted for either isocratic or gradient elution as a function of the column. This then allows gradient separations to be compared for any two columns, in turn allowing various comparisons of column orthogonality. These test compounds are summarized below.

|			
1. benzene	17. anisole	33. cis-chalcone	71. Biphenyl
2. toluene	18. benzyl alcohol	34. trans-chalcone	72. 2-Nitrobiphenyl
3. ethylbenzene	19. 3-phenyl propanol	35. cis-4-nitrochalcone	73. 3-Nitrobiphenyl
4. p-xylene	20. 5- phenyl pentanol	36. trans-4-nitrochalcone	74. 2-BiphenylCH$_2$OH
5. propylbenzene	21. phenol	37. cis-4-methoxychalcone	75, 2,2'-Biphenol
6. butylbenzene	22. p-chlorophenol	38. trans-4methoxychalcone	76. 4,4'-Biphenol
7. napthalene	23. 2,3 dihydroxy-napthalene	39. prednisone	77. Diphenyl-butyrolactone
8. p-chlorotoluene	24. 1,3 dihydroxy-napthalene	40. hydrocortisone	78. Fluorescamine
9. dichlorobenzene	25. eugenol	41. mephenytoin	79. Camphorquinone
10. benzotrichloride	26. danthron	42. oxazepam	80. Ferrocene
11. bromobenzene	27. n-propyl formate	43. flunitrazepam	81.N,N-diethyl-acetamide
12. 1-nitropropane	28. methylbenzoate	44. 5,5 diphenyl-hydantoin	82. 3-Nitrophenol
13. nitrobenzene	29. benzonitrile	45. N,N-dimethyl acetamide	83. 4-Nitrophenol
14. p-nitrotoluene	30. coumarin	68. 1,2-dinitrobenzene	85. 2,5-Nitrophenol
15. p-nitrobenzyl Cl	31. acetophenone	69. 1,3-dinitrobenzene	87. Fisetin Hydrate
16. N-benzyl-formamide	32. benzophenone	70. Nitrocyclohexane	88. Biochanin A

The data and numbering for these compounds are taken from Wilson et al, *J. Chromatogr. A*. 961 (2002) 171, 217.

The Primary Column

The "primary" column corresponds to our initial column or the 1st-dimension column for a 2-D separation. We can then compare separations for the primary and above orthogonal columns, using the same gradient conditions (0-100% acetonitrile in 30 min), same column conditions (100 x 4.6-mm, 3 µm column with a flow rate of 1.0 mL/min), pH-2.8, and a temperature of 35°C. As primary column we have selected Capcell
C18 UG120, because it represents an average type-B C_{18} column (that is, its coefficients . **H**, **S***, etc. are nearly identical to the average values of all type-B C_{18} columns). Resulting chromatograms for different columns can be predicted from previously determined values of parameters for both the column (**H**, **S***, etc.) and solute (η', σ', etc.).

Separation of 64 neutral compounds on the Capcell C18 UG120 column:
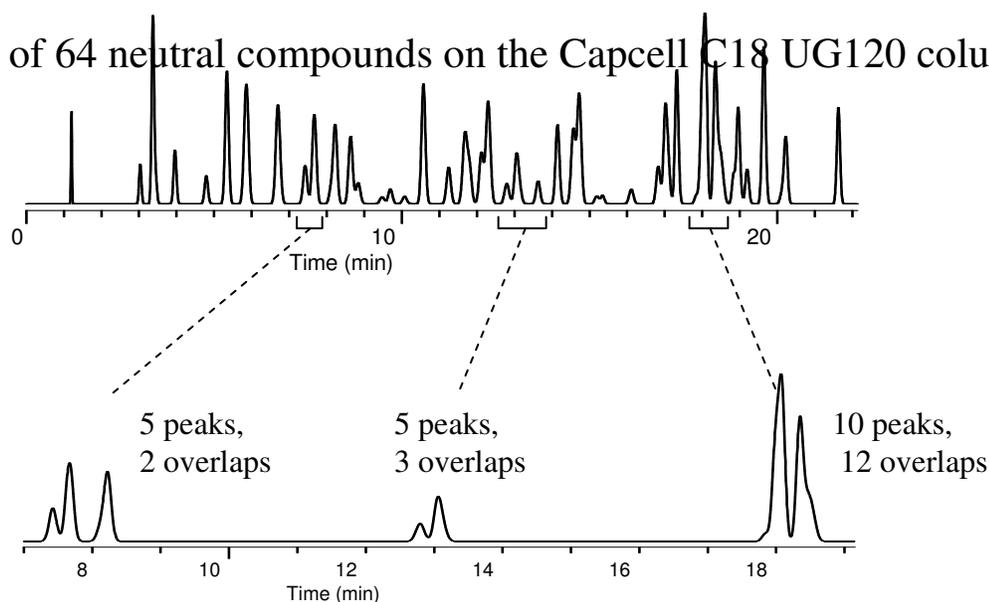
For the entire sample, there are 37 peak-pairs that are overlapped with $R_s < 1.0$. An orthogonal separation should (ideally) resolve all or most of these overlapped peak-pairs with $R_s > 1.0$. We will next compare column "orthogonality" for columns selected either for maximum F_s or maximum F_s(-C).

Top 20 columns with highest values of F_s (type-B only)*

Column	F_s	Type	Column	F_s	Type
EC Nucleosil Protect 1	268	EP	Ascentis RP-Amide	73	EP
Zorbax Bonus RP	253	EP	Inertsil ODS-EP	71	C_{18}
Hypersil Prism C18 RP	236	EP	Fluophase PFP	69	FP
Inertsil CN-3	216	CN	Acclaim Polar Advantage II	69	EP
BetaMax Acid	181	EP	Cogent UDC Cholesterol	66	C
Hypurity Advance	118	EP	Symmetry Shield C18	64	EP
Purospher RP-18	95	C_{18}	Discovery HS F5	61	C_5
Prevail Select C18	91	C_{18}	UltraSep ES AMID H	61	EP
BAS MF-8954	88	C_{18}	Allure PFP propyl	60	FP
Fluophase RP	88	FP			

Top 20 columns with highest values of F_s(-C) (type-B only)*

| Column	F(-C)	Type	Column	F(-C)	Type
Zorbax Bonus RP	68	EP	Hypurity Advance	42	EP
BetaMax Acid	63	EP	ProntoSIL C18 ace-EPS	41	EP
Purospher RP-18	52	C_{18}	Discovery Amide C16	41	EP
EC Nucleosil Protect 1	51	EP	Ultra IBD	38	EP
Hypersil Prism C18 RP	51	EP	Hypersil Prism C18 RPN	38	EP
BioBasic Phenyl	50	Phenyl	Ascentis RP-Amide	34	EP
Inertsil ODS-EP	48	C_{18}	Acclaim Polar Advantage II	34	EP
Nautilus C18	46	EP	Discovery HS PEG	32	EP
Prontosil C8 ace-EPS	44	EP	Inertsil CN	30	CN
UltraSep ES AMID H	42	EP	ACE CN	28	CN

EP, polar-embedded; FP, fluorophase, CN, cyano
* all values relative to the "average" C18 column represented by Capcell C18 UG120

Embedded-polar-phase columns are seen to be favored generally.

Comparison of Reference and "Orthogonal" Columns

With chromatograms for the various columns listed above, we can compare column orthogonality for the 64 neutral compounds in the sample. For example, we can compare the number of overlaps in the reference column that are separated with $R_s > 1$ on the orthogonal column. For one such comparison,

Capcell C18 UG120 (reference column) top,
Zorbax Bonus RP (orthogonal column) bottom
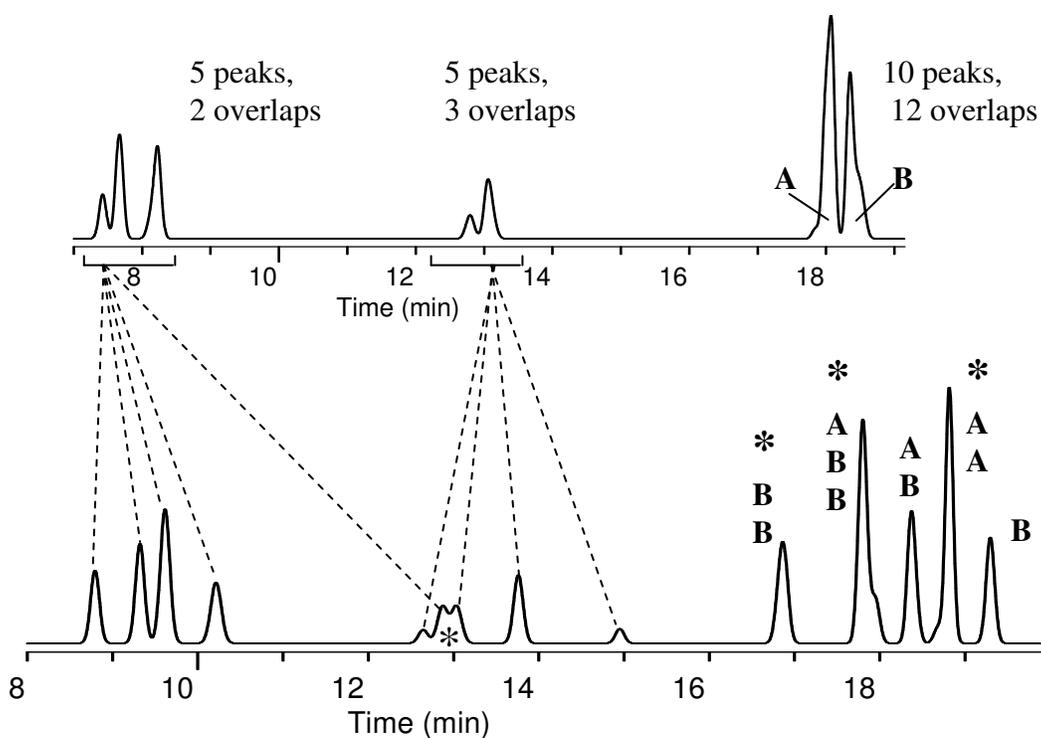
For the first and second group of peaks, every overlapped band-pair from the reference column is separated with $R_s > 1$ by the orthogonal column. Two peaks (*) separated with the reference column are overlapped on the orthogonal column with $R_s = 0.6$. However, these peaks were resolved in the first separation, so would not contribute to the "missing peak" problem.

For the third group of peaks, there are three overlaps marked by * (A-A or B-B). For the entire chromatogram, there were 5 peak-pairs (out of 37) at were unresolved on both the reference and Zorbax Bonus RP columns.

Two Different Measures of "Othogonality"

Once we have simulated chromatograms for the reference and orthogonal columns in this way, there are two possible measures of "orthogonality" with respect to neutral compounds: the number of overlaps on the reference column that are resolved with $R_s > 1$ on the orthogonal column, and the correlation coefficient r^2 for the two columns. Each of these measures of orthogonality can be compared for columns selected on the basis of $F_s(-C)$ or F_s.

Unresolved Overlaps

The number of overlaps *not* separated on the orthogonal column decrease for columns with higher values of $F_s(-C)$ – as expected.


$$y = 2.4 + 113/F_s(-C)$$
$$r^2 = 0.75, SD = 2.4$$

20 most orthogonal columns (●)
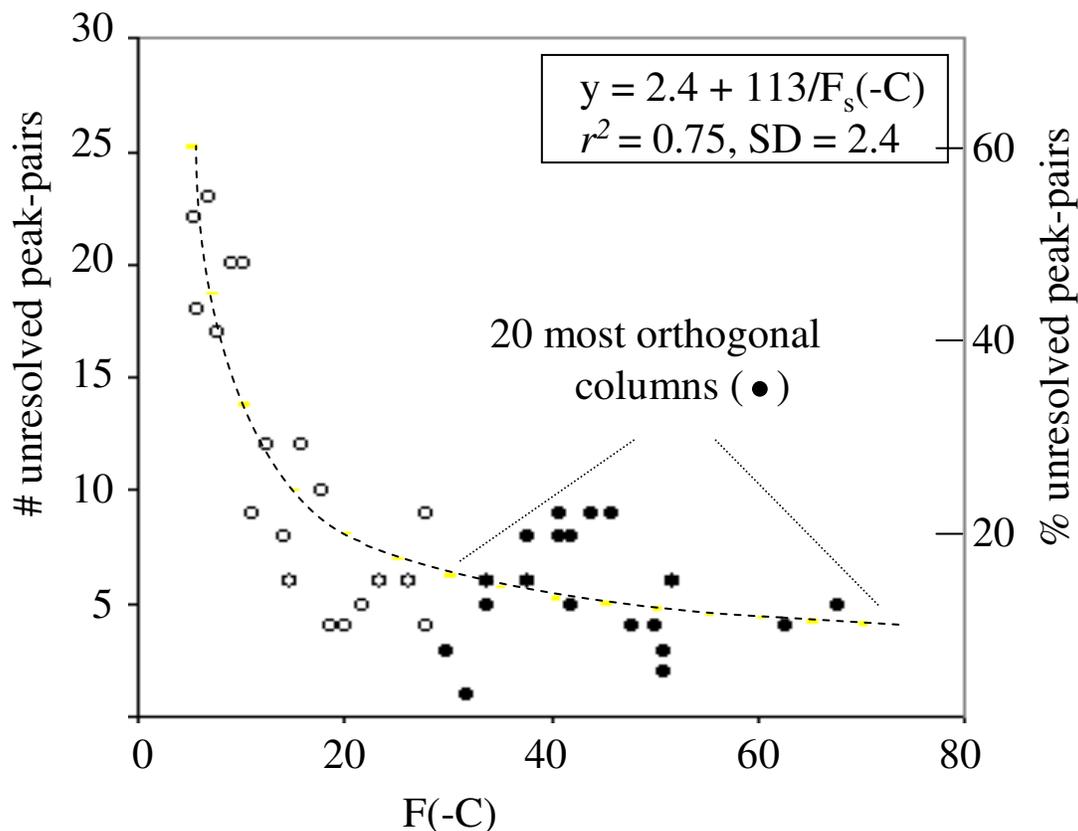
The *%* of unresolved peaks is shown at the right.

A rough correlation with Fs(-C) is also noted for the number of peak-pairs that remain unseparated on the orthogonal column. The scatter in the above plot reflects the range in molecular structures of the compounds examined. On average, however, a value of $F_s(-C) > 30$ means an *average* chance of separating a previously overlapped peak-pair of about 5 in 6. When a change in B-solvent is combined with the use of a reasonably orthogonal column, there is then a reasonable likelihood that an unresolved neutral peak-pair from the primary separation will be resolved in the orthogonal separation.

For a similar plot as above vs. the function F_s, (# of overlaps) = 5.9 + 87/Fs, with $r^2 = 0.50$ and SD = 5.9 (a poorer correlation, as expected), so removal of the C-term adds discrimination to the model for neutral analytes.

Values of r^2

The scatter (or lack of correlation) of retention-time plots is another measure of column orthogonality; e.g., for the Zorbax Bonus RP column vs. the reference column.:

$$y = 2.6 + .86 \, x$$
$$r^2 = 0.93$$
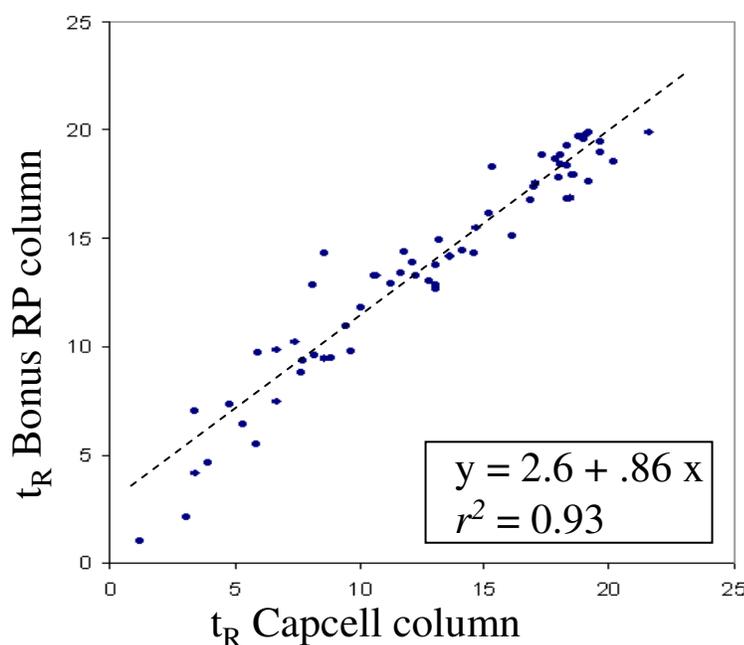
The scatter r^2 for plots, such as this, correlates reasonably well with values of F_s(-C); e.g., for F_s(-C) equal a maximum value of 68, $r^2 = 0.9$. Orthogonality measured in this way may seem modest in comparison with the usual goal of $r^2 \approx 0.00$ (poor correlation is desired for orthogonality). It should be recalled that orthogonal separation will always be much more difficult for neutral compounds (the present case). A change in the B-solvent can further increase separation orthogonality.. **However**, we feel that the number of overlaps is a better general measure of column orthogonality than r^2.

Orthogonality and 2-D Separation

When developing a separation for the purpose of ruling out "missing" peaks, the primary or initial column will often be a "conventional" column, similar to the reference column assumed here (Capcell C18 UG120). However, the selection of columns for orthogonal separation allows any column we wish for both the 1st and 2nd dimension. This suggests starting with one of the previously-selected orthogonal columns for the 1st dimension, followed by selecting a maximally orthogonal second column for the 2nd dimension. We investigated this possibility for the 6 maximally-orthogonal columns (values of $50 \leq F_s[-C] \leq 68$, relative to the Capcell column).

Zorbax Bonus RP	BetaMax Acid	Purospher RP-18
EC Nucleosil Protect 1	Hypersil Prism C18 RP	BioBasic Phenyl

The most orthogonal columns found, relative to the above 6 columns, had F_s(-C) values vs. these columns that were only marginally higher: $64 \leq F_s$(-C)≤ 79. The fraction of unresolved peak-pairs for the second column (e.g., Prontosil 120-3-C30) vs. the first column (e.g., Zorbax Bonus RP) was quite similar to values reported above ($\approx 10\%$) for values of F_s(-C) > 50. That is, the latter procedure provides columns that are only slightly more orthogonal than starting with the "average" (Capcell) column.

Effect of Solute Structure

Certain peak-pairs were found more likely to be unresolved on two columns with large values of F_s(-C). It is useful to examine the structural similarities that can result in a high probability that peak overlap will be maintained for two columns with large values of F_s(-C). Only three peak-pairs were found to be unresolved on different combinations of two orthogonal columns with a frequency > 50%:

cis-4-nitrochalcone / *cis*-4-methoxychalcone   (89% unresolved)

ethylbenzene / *p*-xylene   (63% unresolved)

prednisone / hydrocortisone   (53% unresolved)

The solute parameters (η', σ', etc.) are quite similar for each of these solute pairs, which accounts for their difficult separation. The poor separation of *cis*-4-nitrochalcone from *cis*-4-methoxychalcone suggests that other compounds differing only in the substitution of nitro for methoxy might also prove difficult to separate, but this was not the case for nitrobenzene and anisole. Rather, other features in the chalcone molecule appear to combine with differences in nitro vs. methoxy to create the difficulty seen for nitro vs. methoxy chalcone.

The difficult separation of ethylbenzene and *p*-xylene is understandable both in terms of the close similarity of these two isomers, as well as the absence of functional groups within the molecule that are capable of hydrogen bonding. However, this situation is highly atypical.

Finally, the two steroids, prednisone and hydrocortisone, are structurally similar and are also relative large molecules. This peak-pair is likely more indicative of difficultly separable compounds likely to be encountered in "real" samples.

Summary and Conclusions

The present study provides support for an alternative procedure for selecting orthogonal columns. Column selectivity parameters based on the hydrophobic-subtraction model are now available for about 400 different reversed-phase columns. The use of these data for comparing column selectivity has been validated in previous studies. However, when used to select orthogonal columns, this past procedure fails to properly weight differences in selectivity for neutral compounds within the sample. A modified procedure based on values of F_s(-C) is described in this poster. The choice of column for either the starting separation or the first dimension in 2-D chromatography is up to the user. A second (orthogonal) column can be selected based on a maximum value of F_s(-C). The USP computer program available at **http://www.usp. org/USPNF/columnsDB. html** can be used for this purpose (leave the "bases present" checkbox empty). Alternatively, contact one of the authors for further assistance.

The use of orthogonal columns selected in this way can reduce the likelihood of peak overlap for a given pair of compounds by a factor of about 6. Further changes in experimental conditions (pH, B-solvent) for the two separations should minimize the possibility of two peaks being overlapped in both separations – but no orthogonal separation can completely eliminate peak overlap and "missing" peaks.

The present procedure can be extended for the selection of a third column that is orthogonal to the second column. However, the added orthogonality that can be achieved in this way appears to be marginal.