Strategies for the Systematic Development of Reversed-Phase HPLC Separations

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August, 2005

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We will not be talking about method optimization. Optimization — defined in chromatographic terms as finding the best possible set of separation conditions — is a luxury afforded to very few of us in today’s environment. The pressures of limited time and resources combined with seemingly unlimited need for analytical methods mean that, at best, we have the opportunity to “adequatize” methods.
In practice, “adequate” resolution usually refers to $R_s > 1.7$ or so (for major peaks; greater resolution may be required for trace compounds eluting near major peaks). Note that this does not imply super-narrow peaks. Both of the upper chromatograms in this example represent “adequate” separation. In general, the more bands in the sample, the harder it is to achieve adequacy.

By the same token, ‘inadequate’ separation in LC simply means that the resolution between bands is insufficient to allow for precise or accurate quantitation. In practice, this means $R_s < \approx 1.5$, depending on the relative sizes of the peaks, will be unsatisfactory.

Note that it is possible to be “too good” (although this type of separation rarely occurs on a first try!). Resolution in excess of that needed for good quantitation (greater than 2 or so) adds nothing to the quantitation and wastes time. In almost all cases, this “excess” resolution can be traded for a shorter analysis time (by using a shorter column or a higher flow rate, for example).

Of the chromatograms shown here, the upper right would be considered adequate, while the lower right is obviously inadequate. The upper left chromatogram is “overkill”, while the lower left chromatogram is marginal, at best.

Paradoxically, band width measurements are easy to make when peaks are well resolved — and we really don’t need to measure resolution carefully so long as it is more than adequate. On the other hand, the resolution of a marginal separation is important to calculate (so we can try to improve it), but can be difficult to measure because the peaks overlap. Here is where resolution based on peak widths at half-height can be handy. Data systems can often be set up to calculate resolution. Visual comparison with standard resolution curves can also provide approximate values.
Critical resolution is the resolution between the worst-resolved pair of peaks of interest in the chromatogram.

A search through the literature of chromatographic optimization turns up a wide variety of criteria ("figures of merit") for judging a separation.

Resolution, $R_s$, is simply the ratio of the center-to-center separation between adjacent peaks to their average baseline width ($w_b$).

Our recommendation is the use of critical resolution (the resolution between the worst-resolved pair of peaks in the separation), plus a healthy dose of judgement in evaluating tradeoffs among resolution, run time, and pressure. We will define terminology and discuss the rationale for this selection in the next section.
Fundamental Resolution Equation

Physics (kinetics)  Chemistry (thermodynamics)

\[ R_S \approx \frac{1}{4} N^{1/2} (\alpha - 1) \left[ \frac{k'}{(k'+1)} \right] \]

efficiency  selectivity  retention

Another way to look at resolution is to express \( R_s \) in terms of \( N, \alpha, \) and \( k \). \( N \) is a “physics” (kinetics) parameter rather than “chemistry” (thermodynamics) and changes resolution only in relation to its square root. \( \alpha \) is most strongly affected by chemical changes in the column and mobile phase. \( k' \) measures retention, and is influenced mainly by solvent strength (part of system “chemistry”), but also influences \( \alpha \).
Let's look at these parameters one at a time. We can start with k' because it is the most powerful, as well as the easiest to control in a predictable manner. As k' approaches zero, resolution also drops to zero. Mathematically, this simply reflects the fact that 0/(1+0) is, indeed, equal to 0. Chromatographically, it reflects that fact that at k'=0, peaks are all "squashed" together at t_0. Chemically, it reflects the fact that at k'=0, the distribution of sample molecules is such that sample molecules spend all of their time in the mobile phase. As a result, there is essentially no interaction between the sample and the column packing (hence, no possibility of separation).

As k' is increased, resolution also increases. The increase is rapid at first, but the rate falls off as the factor [k'/(1+k')] asymptotically approaches one. There is a significant difference in resolution between k'=1 and k'=2, but a negligible difference between k'=10 and k'=11. At the same time that resolution increases, peak height decreases, reflecting the fact that more strongly retained peaks are broader and thus, for a given area, shorter. This decrease limits sensitivity at high k'-values. Finally, run time increases as a linear function of k'. The combination of these effects suggest that k' should not be allowed to range much below 2, and certainly not below 1 (much lower and resolution suffers significantly, not to mention the fact that quantitation becomes suspect because of early baseline upsets) nor above approximately 20 (any higher only increases run time for insignificant changes in R_s). The "ideal" range for a given pair of peaks is from approximately 2 to 5.
Both resolution and peak height increase with the square root of the plate number. 10,000 plates is a typical number for a "good" HPLC column. As a matter of practice, doubling plate count is usually an attainable goal. Further increases become expensive in terms of run time and operating pressure. Doubling the plate number only increases resolution by 40%.
Most of our method development effort is therefore concentrated on selectivity. Reversed-phase selectivity can only be controlled in a limited number of ways.
Continuous vs. Discontinuous Variables

**Continuous variables:**
- solvent strength
- temperature
- solvent type
- Additives

**Discontinuous variable:**
- column type

**“Quasi-discontinuous” variable:**
- pH

All selectivity control variables are not created equal. A distinction can be made between “continuous” variables, which can take any value and which therefore allow interpolation between selectivity extremes, and “discontinuous” variables which can only take on certain discrete values. Solvent strength, temperature, solvent type, and additive concentration are generally considered to be continuous variables. Column type is a discontinuous variable; there is no convenient way to interpolate between selectivity extremes. pH requires special discussion. While it is technically a continuous variable, the potential for robustness problems leads many chromatographers to treat it discontinuously by using only certain favored values of pH, with continuous variation left as a last resort.
A commercially available database can be searched for “most different” columns. The search can be refined to limit selectivity parameters, column chemistry, or vendor.

For this database, when $F_s$ is < 3, columns are expected to be nominally interchangeable. $F_s$ values of <5 should be close enough for many applications. At the bottom, we see columns that are dramatically different in selectivity; these might be chosen when different column selectivity is desired.

This program, “Column Match,” can be obtained from Rheodyne’s website (www.rheodyne.com) in a free 60-day trial version.
When a separation is carried out at a pH near the pKₐ of the analytes, small changes in pH can have a major impact on resolution, as shown here.
These data show the effect of changing pH on the spacing of ionic compounds in the pH region near the pKₐ of these materials. Since very large changes in band spacings occur with small changes in pH, the ruggedness of a separation in this pH range would be suspect. A more rugged and reproducible separation would probably result by working at about pH 3, and increasing the organic modifier content to 50% ACN to adjust the mixture in the desired k’ = 2 - 10 range.
When developing a strategy, we can distinguish between two general approaches. Incremental method development, in which variables are (usually) explored one at a time with decisions as to subsequent direction based on the outcome of each experiment, has the highest level of user involvement, but can be carried out in such a way as to minimize the amount of experimental data required. Structured method development, which is usually required if the interactions of multiple variables are to be exploited, usually requires more (or more complex) experiments carried out in a predetermined sequence.
Varying Selectivity: Factors to Consider

- maximum change in $\alpha$
- universal applicability
- maximum experimental convenience
- low-UV detection
- method robustness
- fast column equilibration

The specific order in which variables are explored is subjective. These are some of the considerations that will affect the selection.
Generally Useful Variable Pairs

- **Temperature and Solvent Strength (gradient steepness)**
  - easy to manipulate
  - effects relatively independent
  - provide robust methods
  - provide control equivalent to other variable pairs

- **Solvent Type and Solvent Strength (gradient steepness)**
  - classic “solvent triangle”

If two variables can be changed at a time, additional selectivity power is available. One could choose any two variables, such as %B and pH or gradient steepness and the blend of ACN and THF. Although many such combinations are possible, the use of temperature and gradient elution together provides a very convenient and powerful combination of variables. Most equipment can perform the experiments automatically, the methods are robust, and the effect on selectivity of temperature and gradient steepness are, for the most part, independent. The use of solvent type changes was popularized a generation ago as the “solvent triangle” approach to method development.
Other variable pairs can be useful in specific situations, primarily where analytes are weak acids or bases or are zwitterionic.
Gradient or Isocratic?

An initial gradient run can be used to quickly explore the best approach in method development. We recommend that a 15-cm column be used at a flow rate of 1.5-2 mL/min. A 5-95% B gradient should then be run in 20 min ($t_G = 20$ min.). With these standardized conditions, it is possible to determine (a) whether gradient elution is most suitable for the sample, and (b) reasonable initial conditions for an isocratic run or an appropriate range for a gradient run.

First measure the quantity $\Delta t_g$ as indicated in the figure. If $\Delta t_g / t_G > 0.40$, then gradient elution should be used. If $\Delta t_g / t_G < 0.25$, an isocratic run should be possible.

This same run can also be used to determine reasonable initial and final %B values for a gradient using the nomogram in the next figure.
The nomograms shown in the previous figures are specific for a particular column size, flow rate, and gradient time. A free spreadsheet allows you to estimate isocratic conditions from a gradient scouting run for any size column, flow rate, and/or gradient time. It also allows estimation of initial and final %B values for gradient separations. This program may be downloaded from:

Isocratic / Gradient Comparison

Isocratic Resolution:

\[ R_s = \frac{1}{4} \left( \frac{N^{1/2}}{2} \right) (\alpha - 1) \left[ \frac{k'}{1 + k'} \right] \]

Gradient Resolution:

\[ R_s = \frac{1}{4} \left( \frac{N^{1/2}}{2} \right) (\alpha - 1) \left[ \frac{k^*}{1 + k^*} \right] \]

The same method development strategy can be applied to both isocratic and can be extended beyond a mere qualitative similarity. It allows a quantitative description of gradient resolution in terms analogous to those we used earlier for isocratic LC. The difference is substitution of \( k^* \) for \( k' \) (necessitated by the fact that \( k' \) changes during the course of the gradient). \( k^* \) is the instantaneous isocratic \( k' \)-value of a peak when it reaches the midpoint of the column.
When the sample is injected onto the column at the beginning of the gradient, the mobile phase strength is low, so the sample molecules move only very slowly (in fact, they move approximately not at all!). As the solvent strength increases gradually, the sample molecules move down the column with increasing speed. Because solvent strength increases with time, the peak accelerates as it moves down the column. The speed with which it is moving when it is at the midpoint of the column determines $k^*$. Each peak in the sample moves down the column in turn, but each peak is moving at about the same speed when it hits the middle of the column, hence $k^*$ values are approximately the same for all peaks in a gradient separation.
Gradient Selectivity Varies with Flow and Column Dimensions

isocratic: \[ \log(k') = \log(k'_{0}) - S \Phi \]

gradient: \[ k^* \approx \frac{t_{G}}{\Delta \Phi} \frac{F}{V_{m}} \frac{1}{S} \]

In isocratic reversed-phase LC, retention is related only to the percent strong solvent in the mobile phase ($\Phi$ is the volume fraction strong solvent, $S$ is a slope factor, and $\log(k'_{0})$ is simply an intercept in a linear relationship). In gradient LC, $k^*$ depends not only on the steepness (time and range) but also on the flow and the internal volume of the column! $t_{G}$ is the gradient time (min), $F$ is the flow rate (mL/min), $\Delta \Phi$ is the gradient range (e.g., 5 – 95% B is 0.90), $V_{m}$ is the column volume (mL), and $S$ is the same as for isocratic separation.

Note that the gradient equation is only an approximation. Also note that the $S$-value is the same in both the isocratic and gradient equations; a value of $S = 5$ can be used as an approximation for samples of molecular weight <1000.
Selecting Gradient Time

\[ k^* \approx \frac{t_G F}{\Delta \Phi V_m S} \quad t_G \approx \frac{S k^* \Delta \Phi V_m}{F} \]

\[ t_G \approx 25 \Delta \Phi \frac{V_m}{F} \]

**Example:** what is a reasonable gradient time for a 5 - 100% gradient on a 15 x 0.46-cm column at a flow rate of 2 mL/min?

- \( V_m = 1.5 \)
- \( F = 2 \)
- \( \Delta \Phi = 0.95 \)

\[ t_G = (25) (0.95) (1.5) / (2) \approx 20 \text{ minutes} \]

We can use this relationship to advantage, because it lets us identify a gradient steepness that generates a favorable \( k^* \) value (in the range of 2-5) without having to go through the experiments required in the isocratic case. This figure assumes a desired value for \( k^* \) of 5 and a value for \( S \) of 5 (this is typical for small molecules). Thus we can see that a suitable scouting gradient can be designed for most samples before making the first injection – a full-range gradient with a 150 x 4.6 mm column run at 2 mL/min in 20 min will do a pretty good job.
The existence of dwell volume means that the gradient that we program does not match that delivered to the column: there is an offset.
Any gradient HPLC system is characterized by a “dwell volume” or “delay volume”, which is the volume between the point where the gradient is formed and the inlet of the column.

High-pressure mixing systems typically have a dwell volume on the order of 0.5-3.0 mL.
Low-pressure mixing systems have the added volume of the pump included in the dwell volume. Typical low-pressure mixing system dwell volumes range from 2 to 5 mL or more.

The presence or absence of an autosampler and the autosampler design can affect the dwell volume of both high- and low-pressure mixing systems.

Older LC systems typically have larger dwell volumes than newer systems. It is possible to modify many systems so that dwell volumes of < 0.5 mL can be obtained for use with LC-MS applications in which short, small particle columns are used.
Effect of Dwell Volume Differences

The impact of dwell volume differences is seen in retention time and resolution changes in gradient chromatograms. In this case, the top separation was the original method in which a resolution of about 1.7 was observed for the highlighted peaks. When moved to a system with 3 mL of dwell volume, resolution dropped to less than 1.5.

Overall retention increased for the 3 mL system, as well, because the (stronger) gradient reached the column later than for the 1 mL system.

Dwell volume changes shift the retention of all peaks. Dwell volume changes tend to impact selectivity of early-eluting peaks more than later ones, although changes throughout the chromatogram are not uncommon.

A specific dwell volume is not “better” than another one. One might think that smaller is better, and it is true that a smaller dwell volume will shorten the gradient delay, and in this case, the separation was degraded when the dwell volume was increased, but the effect on selectivity can go either way.

Because of the impact of dwell volume on the resulting separation, it is important to include the dwell volume in any written description of a method and, if possible, to “normalize” the time delay between sample injection and arrival at the column of the gradient.
Measuring Dwell Volume

- no column
- A = water
- B = 0.1% acetone / H₂O
- 265 nm
- 0-100% / 20 min
- 2 mL/min

It is important to know the dwell volume of each system, and although systems of the same brand, model, and configuration tend to have very similar dwell volumes, it is a good idea to measure dwell volume on each system.

A simple test shown here allows the gradient to be visualized by adding a UV-absorbing compound to the B reservoir and replacing the column with a piece of connecting tubing.

Some workers prefer using methanol instead of water for this test. The important thing is to use the same solvent in both reservoirs so that you aren’t mislead by changes in background absorbance when organic and water are blended during the gradient.
Compensating for Dwell Volume Differences

- Normalize gradient delay time:
- Identify “worst case” instrument among user population
- Establish an “adjustable” isocratic hold
- Hold time = $t_{D(\text{worst})} - t_D$

For the best transferability, the gradient delay time (the time difference between injection and when the gradient arrives at the head of the column) should be constant from instrument to instrument. An adjustable isocratic hold allows this delay to be normalized from instrument to instrument. It requires that the dwell volume on each system be known.
Incremental Method Development

- Start with reversed-phase conditions
- Initial gradient run
- Select isocratic or gradient elution
- Adjust %B for $2 < k' < 10 \ (1-20)$
  or $t_G$ for $2 < k^* < 8$
- Adjust selectivity
  - non-ionics
    - Solvent strength, temperature, solvent type, column type
  - Gradient
    - Gradient steepness, temperature, solvent type, column type
  - Ionics
    - Solvent strength, temperature, additives, pH, solvent type, column type
- Adjust column conditions

The general approach to HPLC method development we have used so far works well for most samples. To use this approach, not much information about the sample is required. Mainly we need to know if the sample is neutral, acidic or basic, and the molecular weight should be <5000 Da. The only two procedures used in this approach are reversed-phase and ion-pair HPLC.

Some samples, however, require a different strategy for best results. We will refer to these as “special” samples.
Structured Method Development

- Start with reversed-phase conditions; select pH based on sample type (acid/base/neutral/mix)
- Initial experimental matrix: two gradients X two temperatures
- Select isocratic or gradient elution; “optimum” combination of solvent strength & temperature
- Adjust selectivity if necessary
  - Change solvent
  - Change pH
  - Change column
- Adjust column conditions

The general approach to HPLC method development we have used so far works well for most samples. To use this approach, not much information about the sample is required. Mainly we need to know if the sample is neutral, acidic or basic, and the molecular weight should be <5000 Da. The only two procedures used in this approach are reversed-phase and ion-pair HPLC.

Some samples, however, require a different strategy for best results. We will refer to these as “special” samples.
Here is a “universal” strategy to allow development of gradient methods. Of course the resulting gradient method often can be converted to a simpler isocratic method, just like standard binary gradients.

First, a set of 4 runs is made, one each at two temperatures and two gradient times. It is a good idea with all gradient method development to make blank runs under each set of conditions so that extraneous background peaks aren’t confused with important sample peaks.
Here are the two low temperature runs for a sample of steroids related to testosterone. This doesn’t look promising for either run -- the resolution is less than 0.5 in each case. However, notice that the critical peak pair -- marked with an * -- is different in the two runs. In the 20 min run, the second two peaks overlap completely (*), whereas in the 40 min run, there is a little separation. Similarly, the critical pair in the 40 min run shows separation in the 20 min run (*). When the critical peak pair changes from one run condition to the next, an intermediate condition usually will give a better overall separation.
The high temperature runs look a lot more promising. In fact, the 40 min run doesn’t look too bad without any further optimization. The peaks are nearly baseline resolved. Notice once again, that the critical pair of peak changes between the run conditions, as indicated by the marked peaks (*). This tells us that we may get even better separation between the two run conditions.

However, we don’t need to do all this speculation and interpretation about how the peaks move with changes in conditions -- we can just input the data and let the computer give us a resolution map.
Here’s the resolution map for the testosterone samples. Our input runs were 20 and 40 min at 30 and 50°C. They bracket the maximum resolution region, which agrees with our earlier speculation that intermediate conditions were likely to be better for both the low and high temperature runs.

The optimum for this separation is with a 26 min gradient run at 44°C and should have a resolution of about 1.5.
“Optimized” Testosterone Separation (26-min, 44°)

(R_s = 1.5)

Here’s the chromatogram for the optimum from the resolution map. Notice that there some wasted time at the beginning -- the first peak doesn’t come out for 12 min. And there is room to shorten the run at the end -- the gradient runs to 26 min even though the last peak elutes at about 19 min. We could use the auto-optimization feature of DryLab to trim the gradient for us if we like.

It is possible to use this same data set to model the separation under isocratic conditions, but for this sample, the retention range is too broad to get a decent isocratic method in under about 40 min.
This is the optimum linear gradient. If we wanted to get fancy, we could cut the run time further by using a 2-segment gradient, with an initial segment designed to optimize the resolution of the early peaks followed by a steeper segment to decrease the run time.
"Tweaked" Testosterone Separation
(30% isocratic 5 min; 30-75% in 5 min, 44°)

(Rs = 1.65)

An isocratic hold at 30% followed by a fairly steep gradient segment cuts the run time and improves resolution somewhat.
Acknowledgements

John Dolan    Lloyd Snyder

For information on selecting variables, see:


For information on column selectivity, see:


For a review of linear solvent strength gradients, see:


Suggestions for further reading.

For information on selecting variables, see:


For information on column selectivity, see:


For a review of linear solvent strength gradients, see:

“Just relax, Greg, and get in touch with your inner chromatographer.”
Appendix on Column Selectivity

Optional material: more background on column selectivity.
What determines retention and column selectivity?

- the silica particle
- the bonded phase
- mobile phase pH

Column selectivity, or the contribution of the column to separation selectivity, depends on three things: the silica particles used to make the column packing, the bonded phase attached to the silica particles, and mobile phase pH. We'll say a little about each of these three contributions to column selectivity.
The silica particle that is used to make the column packing is mainly characterized by two properties: acidity and pore diameter.

Older (type A) columns were made with less pure silica that contained metal impurities such as iron or aluminum. These metal impurities increase the acidity of silanol groups in the final HPLC packing, which affects column selectivity and may increase band tailing. Columns introduced after 1990 are mainly type B, made from highly purified silica with much less metal contamination. These columns tend to be much less acidic and give better peak shapes.

Most small-molecule samples are separated on narrow-pore silicas, typically with pore diameters of 6-12 nanometers (or 60-120 Angstroms). Pore diameter affects column selectivity by its affect on the spacing of bonding groups attached to the silica. As illustrated above, the bonding groups or ligands become more compressed in narrow-pore silicas, with effects on column selectivity we will examine shortly. Although narrow-pore packings are used more commonly for samples with molecular weights < 1000, wide pore packings can offer different selectivity for smaller molecules.

There is an inverse relationship between pore diameter and surface area. Small-pore columns have larger surface areas and resulting larger values of $k'$. 

The Silica Particle

Type A (acidic)  
OH OH OH  
| | | | 
-- Si – O – Si – O – Si -- 

Type B (basic)  
OH OH OH  
| | | | 
-- Si – O – Si – O – Si -- 

Narrow pore  
10-nm-pore

Wide pore  
30-nm-pore
The Bonded Phase

- **functionality**
  - $C_3$, $C_4$, $C_8$, $C_{18}$, $C_{30}$
  - polar group (embedded, end-capped)
  - other ligand (phenyl, cyano, fluoro)

- **coverage (micromoles/m²)**

- **end-capping (yes or no)**

The bonding that is attached to the silica to make a reversed-phase column is important in determining the final selectivity of the column. Most reversed-phase columns are $C_8$ or $C_{18}$, but shorter or longer alkyl groups are also used. Ligand length contributes significantly to column selectivity.

A recent, fairly popular column bonding is based on the use of polar groups that are either inserted into an alkyl chain or used to end-cap unreacted silanols. Polar group columns are preferred when mobile phases of high water content are used (<10%B), because many $C_8$ and $C_{18}$ columns are not well wetted by low-organic mobile phase. The result may be poor separation, and changes in separation with time. Polar group columns are also very different in terms of selectivity. There are many other kinds of bonded phase, but we will not spend much time on these less popular column types.

Aside from the kind of bonded phase, the concentration of bonded groups also affects column selectivity. Today, most columns are highly bonded, with ligand concentrations of 2.5–4 micromoles/meter². Some columns, however, are deliberately underbonded (<2.5 micromoles/meter²) in order to achieve either different selectivity or better wetting by high-water-content mobile phases.

End-capping refers to the further reaction of unbonded silanols with a small silane (usually trimethylsilyl). End-capping reduces silanol activity and helps to stabilize the column at high pH.
Mobile phase pH affects column selectivity by varying the ionization of unreacted silanols. The chart shows how the negative charge on the column from silanol ionization varies with pH. At low pH, some silanols in type-A columns are ionized, but fewer type-B silanols are ionized. As pH increases, there is a continuing further ionization of silanols, especially for type-A columns. The relative charge on the column is extremely important in controlling the separation of ionized sample molecules (acids, and especially bases).
An Experimental Study of Column Selectivity

- 150 different sample compounds of all kinds
- 300 different alkyl-silica columns (C1 – C30, but mainly C8, C18)
- same mobile phase and temperature

\[
\log k' = \log k'_{EB} + \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C
\]

hydrophobic  steric  H-bonding  cation exchange

The rest of this section is mainly the result of a 6-year study of column. The beginning of the study consisted of an intense experimental program which resulted in the measurement of about 10,000 retention time values. A wide range of molecular structures were used as test compounds, so that resulting conclusions could be assumed to apply for any kind of sample. Similarly, alkyl-silica columns were used with widely varying ligand lengths (C1-C30), bonding density (1-5 µmoles/m²), and pore diameter (6-30 nanometers), as well as columns with and without end-capping. Resulting values of k could be correlated within ±2%, which means that all sample-column interactions that affect selectivity have been identified for alkyl-silica columns.

Values of k’ are seen to be related to k’ for a reference compound (ethylbenzene) and to five terms which represent different sample-column interactions. The small Greek letters refer to a property of the sample molecule, while the capital letters represent a corresponding property of the column. For example, \(\eta'\) is the hydrophobicity of the sample molecule, and H is the hydrophobicity of the column.

This equation with suitable experimental data allows the measurement of the five column selectivity parameters or properties: H, S*, A, B and C.
Comparing Column Selectivity

- measure H, S*, A, B, and C for each column
- how to compare?
- for neutral samples, only H, S*, A important

\[
\text{difference in selectivity} = F_s = \sqrt{(H_2-H_1)^2 + (S^*_2-S^*_1)^2 + (A_2-A_1)^2}
\]

so: small \( F_s \), similar columns
large \( F_s \), different columns

To compare selectivity between two columns, first we have to measure H, S*, A, B, and C under standard conditions. Once we have these values, we can use the Pythagorean theorem to compare the quantitative difference between columns. This is difficult to visualize in five dimensions, so we have shown a simpler example here.

As an example, suppose we have a sample that consists of neutral compounds only. In this case, the column parameters B and C will not be important, which simplifies our illustration. That is, column selectivity now depends only on hydrophobicity, H, steric interaction, S*, and column H-bond acidity, A.

In the 3-dimensional diagram on the left we show the positions of two columns in a plot of H, S*, and A. That is, values of H, S*, and A for each column determine the position of each data point. Now, it is logical to measure column similarity by the distance between two columns in this plot. Let's call that distance, measured in column parameter units, as \( F_s \). By analogy with the Pythagorean theorem, \( F_s \) is given by the expression on the right as a function of the values of H, S*, and A for the two columns.

When \( F_s \) is small, the difference between the columns is small; large \( F_s \) values mean the columns are different.
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For this database, when $F_s$ is < 3, columns are expected to be nominally interchangeable. $F_s$ values of <5 should be close enough for many applications. At the bottom, we see columns that are dramatically different in selectivity; these might be chosen when different column selectivity is desired.

This program, “Column Match,” can be obtained from Rheodyne’s website (www.rheodyne.com) in a free 60-day trial version.
In general, the greatest selectivity changes are seen when changing from an alkyl bonded phase column (C18 or C8) to an EPG column. This figure is taken from a presentation given at the 2005 Pittsburgh Conference. The low correlation between retention on these two columns indicates a large difference in selectivity. The largest possible difference in selectivity (perfectly “orthogonal” separations) would result in an $R^2$ value of 0.
While the general order of elution is similar, note that peak pair 2 / 3 has switched as have 3 / 4 and 5 / 6.