



General Interest

## USLC™ Columns Put the Right Tools in Your LC Method Development Toolbox

### Understanding Selectivity in Reversed Phase Separations – A Simplified Approach to HPLC and UHPLC Column Selection

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*The most significant influence on chromatographic peak separation, or resolution, is column selectivity. Unfortunately, column selectivity is also the least understood and most underutilized parameter. To improve selectivity, method developers often concentrate on manually altering mobile phases, operational parameters, and instrumentation. But because stationary phases offer more significant selectivity differences, you can drastically speed up HPLC and UHPLC method development by instead focusing on column choice. In this article, we discuss column selection for reversed phase separations and, using the hydrophobic subtraction model (H-S model), identify a set of just 4 stationary phases—Restek's USLC™ column set—that encompasses the widest selectivity range available on the market.*

#### The Role of Selectivity in Liquid Separations

When performing a liquid separation, we generally focus on choosing the right instrumentation—especially since the recent advent of UHPLC—and end up choosing columns rather hastily, either by proximity (using the column that is already on the instrument or in the closest drawer) or by habit (using a column that has offered problem-free service in the past). While never optimal, this practice should be particularly concerning for a method developer because improper column choice can lead to needlessly labor- and time-intensive method development. If we consider the impact of column selectivity on peak separation, or resolution, we can see why choosing the right column can be so advantageous.

Resolution is the result of 3 cumulative terms: efficiency (N), retention capacity (k), and selectivity (α). So, how well we resolve our analytes, and how quickly we do so, depends upon our ability to control these 3 factors. Of the 3, the selectivity term mathematically affects resolution to the greatest degree (Equation 1). Put another way, resolution is largely a function of selectivity.

**Equation 1** Selectivity is the driving parameter of resolution, as it affects peak separation to the greatest degree.

$$R = \frac{1}{4} \sqrt{N} \times \left( \frac{k}{k+1} \right) \times (\alpha - 1)$$

*Efficiency   Retention Factor   Selectivity*



## Changing Columns to Create Significant Changes in Selectivity

Since resolution is largely a function of selectivity, any discussion about improving resolution should focus primarily on altering selectivity. It has often been taught in HPLC method development that one can effectively alter selectivity by adjusting mobile phases to reach a desired separation. This, of course, is true. However, mobile phase adjustment can be laborious—often involving many preparation adjustments and column equilibration times—and typically creates only marginal selectivity differences. In addition, some elution profiles are not practical with certain mobile phases and detection modes, including mass spectrometry (MS) and refractive index (RI).

On the other hand, changing stationary phases (i.e., columns) can be much easier and can also result in more significant selectivity differences because stationary phases can offer alternate and even orthogonal separations. These alternate separations can also be scouted very quickly using precise scouting gradients.

With the number of columns commercially available today, choosing the right one can be difficult, even overwhelming. By quantifying stationary phase selectivity, we can create new guidelines for effectively and easily choosing columns to help reduce method development time and increase method ruggedness.

## Quantifying Column Selectivity Using the Hydrophobic Subtraction Model (H-S Model)

Many models exist for choosing solvents and mobile phase additives, but not until recently has stationary phase characterization received much attention. Column selectivity has been largely overlooked due, in part, to its complexity, particularly for liquid separations. But now that Snyder et al. have proposed their popular hydrophobic subtraction model (H-S model) [1], we can begin to compare and quantify stationary phase selectivity in reversed phase separations and determine (often through orthogonal separations) which stationary phases produce the greatest degree and range of selectivity differences. Only then can we identify a small set of columns that will form the contents of an efficient and effective method development toolbox.

The H-S model is a novel treatment that empirically defines reversed phase selectivity by analyzing a varied collection of solute test probes and then utilizing 5 established selectivity parameters—hydrophobicity (H), steric hindrance ( $S^*$ ), hydrogen bond acidity (A), hydrogen bond basicity (B), and cation exchange activity (C)—to identify the contributions of silica sorbents and stationary phases on selectivity. This model has been used by many organizations, including United States Pharmacopeia (USP), to find column equivalency.

The selectivity value ( $F_s$ ) of the H-S model is normally used to find the similarity between columns, but it can conversely be used to find column *dissimilarity*, even orthogonality, to highlight selectivity differences and simplify column selection. Table I compares a variety of stationary phases and reveals which phases offer increased selectivity. (Because the H-S model evaluates the contributions of both stationary phase and silica support on selectivity, we intentionally kept the silica support constant throughout our experiments to isolate the effect of stationary phases on selectivity.) Each value was calculated relative to a C18 benchmark. The columns showing high  $F_s$  values—like the 4 Restek USLC™ phases shown in blue—exhibit the greatest dissimilarity in selectivity relative to the C18, so they are excellent choices when a C18 does not provide the selectivity needed.

**Table I** The  $F_s$  term of the hydrophobic subtraction model (H-S model) can numerically determine which stationary phases are most dissimilar to a C18, illustrating the phases needed to extend the selectivity range in reversed phase chromatography. The 4 Restek USLC™ phases are shown in blue.

Terms Calculated from the Hydrophobic Subtraction Model (H-S Model)							
Stationary Phase Type	Hydrophobicity	Steric Hindrance	Hydrogen Bond Acidity	Hydrogen Bond Basicity	Cation Exchange Activity	Selectivity Function	Rank Dissimilarity
	H	$S^*$	A	B	C	$F_s$	
Ultra C18 (control)	1.051	0.033	-0.032	-0.023	0.057	0.0	—
Ultra C8	0.0871	0.013	-0.0199	0.019	-0.032	11.2	8
Ultra C4	0.0738	-0.010	-0.276	0.019	0.032	11.3	7
Ultra C1	0.613	-0.054	-0.408	0.016	-0.032	17.9	6
Ultra Aqueous C18	0.808	-0.128	0.378	0.013	0.0229	25.4	5
Ultra Biphenyl	0.661	-0.189	-0.283	0.042	0.204	28.4	4
Ultra Cyano <sup>†</sup>	0.409	-0.041	-0.801	-0.011	-0.110	29.1	3
Ultra PFP Propyl	0.671	-0.092	-0.213	-0.007	0.658	52.0	2
Ultra IBD	0.672	-0.035	-0.052	0.233	-0.564	63.7	1

All columns were tested using the same silica support.

<sup>†</sup> NOTE: The cyano phase also ranks high in terms of dissimilarity, but the more rugged PFP propyl phase was ultimately chosen for the USLC™ column set because it better withstands the low pH levels required for mass spectrometry while offering equally heightened retention of basic compounds.

## Characterizing Selectivity at the Molecular Level

Often during method development, after we have made our initial column choice, we still find ourselves struggling to resolve compounds as we try to find a “better” column. This difficulty is often due to an inability to find a column with *alternate* selectivity. Quantifying stationary phase selectivity (Table I) is a very important step in identifying a small and effective column set for method development, but we must further define selectivity at a molecular level to ensure that the columns in our method development toolbox exhibit not just *high* selectivity, but also *alternate* selectivity based on potential analyte types.

Selectivity ( $\alpha$ ) is practically determined from the difference in retention factors ( $k$ ) of 2 peaks. Therefore, to produce alternate selectivity, we must alter the retention of one peak relative to the other. (Increasing the retention of both peaks equally results in higher retention capacity, but no change in selectivity because the difference between the 2 peaks does not change.) If we focus column selection on intermolecular interactions, we can see how specific phases create selectivity by altering the retention profile of specific solutes in relation to others—true selectivity.

So before we can confirm alternate selectivity, we first need to characterize the types of intermolecular interactions commonly encountered in reversed phase chromatography (RPC). In our experiments, we measured 4 major types of interactions—dispersion, polarizability, hydrogen bond donor, and cation exchange. To further simplify things and more easily define a guideline, we can relate these measured interactions to chemical properties as noted below:

- **Dispersion** is the term for the van der Waals interactions that exist to some extent in all organic molecules, including polar molecules. It is the major driver for RPC and is a major retention mechanism for alkyl phases (i.e., C1 through C18). Since the retention is proportionate to the hydrophobicity of the molecule, we can call these interactions *hydrophobic retention*.
- **Polarizability** is the ability of a stationary phase to change its electron distribution in the presence of an analyte and induce a dipole interaction. It is commonly seen in phenyl-based columns and is the main reason we often switch from a C18 to a phenyl to find alternate selectivity. The Restek Biphenyl column has 2 phenyl rings to enhance polarizability. These interactions are most commonly seen in dipolar, unsaturated, or conjugated compounds and fused-ring compounds with electron withdrawing groups (like nitro groups). For our purposes, we will define these interactions simply as *dipolar retention*.
- **Hydrogen bond donor** is often used in RPC when a solute acts as a proton donor with a proton accepting functionality embedded in the stationary phase. Because this interaction is most commonly observed as an increased retention for acidic compounds, we will call it *acidic retention*.
- **Cation exchange** is an electrostatic interaction between a cationic solute and an anion within the stationary phase. Cation exchange, or electrostatic interaction, is most commonly employed in RPC for the retention of protonated bases. Therefore, for simplicity, we will call it *basic retention*.

Table II outlines the common solute retention profiles for the specific interactions we measured in our experimentation. With these intermolecular interactions defined, we can now use their retention profiles to determine which highly selective columns produce alternate selectivity for specific compound types, thereby radically simplifying column selection.

**Table II** Common retention profiles measured for modern reversed phase columns as they relate to molecular interactions.

Solute Interaction	Type of Solute Retained	Common Phase Category	H-S Model Term	Probes Measured
Dispersion	Hydrophobic	C18	H	Toluene, Ethylbenzene
Polarizability	Dipolar	Biphenyl	n/a*	Anisole, Benzonitrile
Hydrogen Bond Donor	Acidic	Polar Embedded	B	4-Butylbenzoic Acid, Mefenamic Acid
Cation Exchange	Basic	Fluorinated Phenyl	C	Berberine, Amitriptyline, Nortriptyline

\* Because polarizability is not measured by the H-S model, Restek used anisole and benzonitrile probes to mathematically determine the degree of polarizability of each stationary phase.

## Extending the H-S Model to Simplify Column Choice

To determine a simplified guideline for column selection, Restek has extended the H-S model by analyzing empirical selectivity data of our stationary phases (Table I) against the RPC molecular interactions described in Table II. Through matching stationary phases to specific solute types based on these measured intermolecular attractions, we can aid method development in 2 significant ways: First, we can find a small set of columns with a wide range of alternate selectivity for use in method development. Second, we can define a process for selecting columns based on the chemical properties of our analytes when scouting column selectivity.

Extrapolating the retention data for the solute probes in the H-S model allows us to correlate the retention characteristics of specific solutes to stationary phase types. Ultimately, this correlation has enabled us to match column type to the selective retention of our analytes' chemical properties, making column selection truly definable by the chemical composition of our analytes.

Figure 1 illustrates the retention profile of a C18 compared with the profiles of the 4 Restek Ultra Selective Liquid Chromatography™ (USLC™) columns. We can see changes in selectivity across these columns as illustrated by the circled areas showing heightened retention for particular solute types. (Selectivity is the retention of one solute relative to another.) The 4 USLC™ columns exhibit varied retention profiles based upon solute type and, therefore, will exhibit alternate selectivity relative to one another. Because we have a small, quantified column set—4 Restek USLC™ phases—that is highly selective *and* exhibits significantly different retention profiles based on specific solute chemical properties, we can now match columns to specific analytes and, thus, simplify method development.

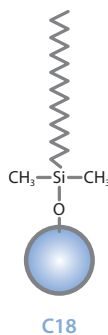
**Figure 1** Stationary phase selectivity can be determined by looking for column types with varying retention profiles. When compared to a C18, the 4 Restek USLC™ phases offer diverse retention profiles—that is, a true range in selectivity.

Restek Phase:  
**C18**

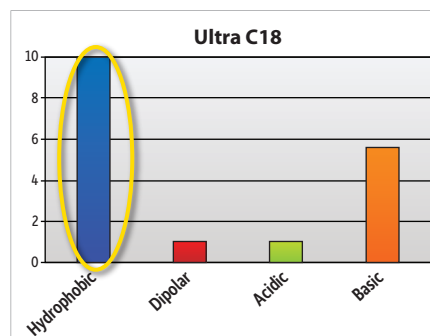
Stationary Phase Category:  
C18 (L1)

Ligand Type:  
Densely bonded and fully end-capped octadecyl silane

- Properties:
- General purpose.
  - Strong hydrophobic retention.



C18 Benchmark

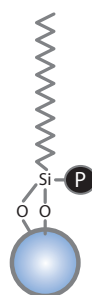


Restek USLC™ Phase:  
**Aqueous C18**

Stationary Phase Category:  
Modified C18 (L1)

Ligand Type:  
Proprietary polar modified and functionally bonded C18

- Properties:
- General purpose with a well-balanced retention profile.
  - Increased retention for acids and bases.
  - Resistant to dewetting—compatible with 100% aqueous mobile phases.



Well-balanced retention profile.

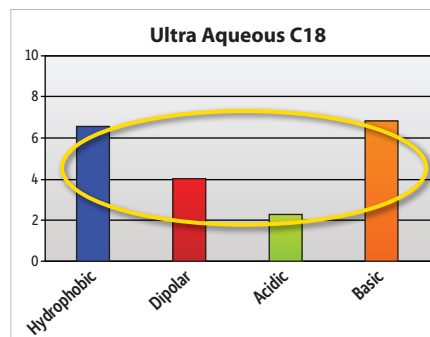


Figure 1, continued

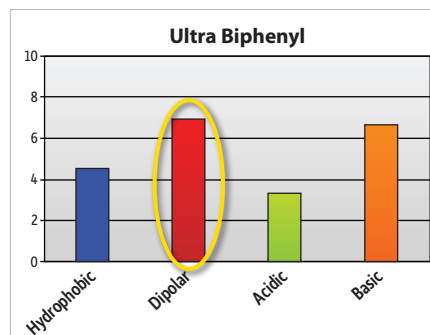
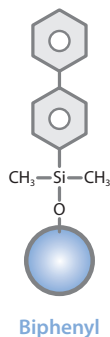
Restek USLC™ Phase:  
**Biphenyl**

Stationary Phase Category:  
Phenyl (L11)

Ligand Type:  
Unique Biphenyl

Properties:

- Increased retention for dipolar, unsaturated, or conjugated solutes.
- Increased retention for fused-ring solutes containing electron withdrawing ring substituents.
- Enhanced selectivity when used with methanolic mobile phase.



Heightened retention for dipolar compounds.

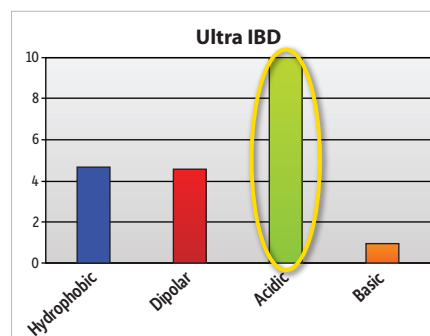
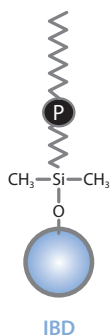
Restek USLC™ Phase:  
**IBD**

Stationary Phase Category:  
Polar Embedded Alkyl (L68)

Ligand Type:  
Proprietary polar functional embedded alkyl

Properties:

- Increased retention for acids.
- Moderate retention for hydrophobic and dipolar solutes.
- Resistant to dewetting—compatible with 100% aqueous mobile phases.
- Capable of multi-mode mechanisms.



Heightened retention for acidic compounds.

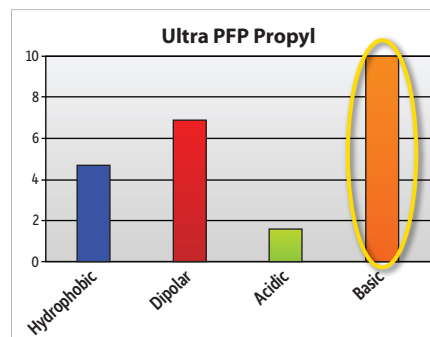
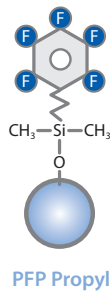
Restek USLC™ Phase:  
**PFP Propyl**

Stationary Phase Category:  
Proprietary end-capped pentafluorophenyl propyl (L43)

Ligand Type:  
Fluorophenyl

Properties:

- Increased retention for protonated bases.
- Increased retention for solutes containing dipolar moieties.
- Capable of multi-mode mechanisms.



Heightened retention for basic compounds.

All columns were tested using the same silica support.

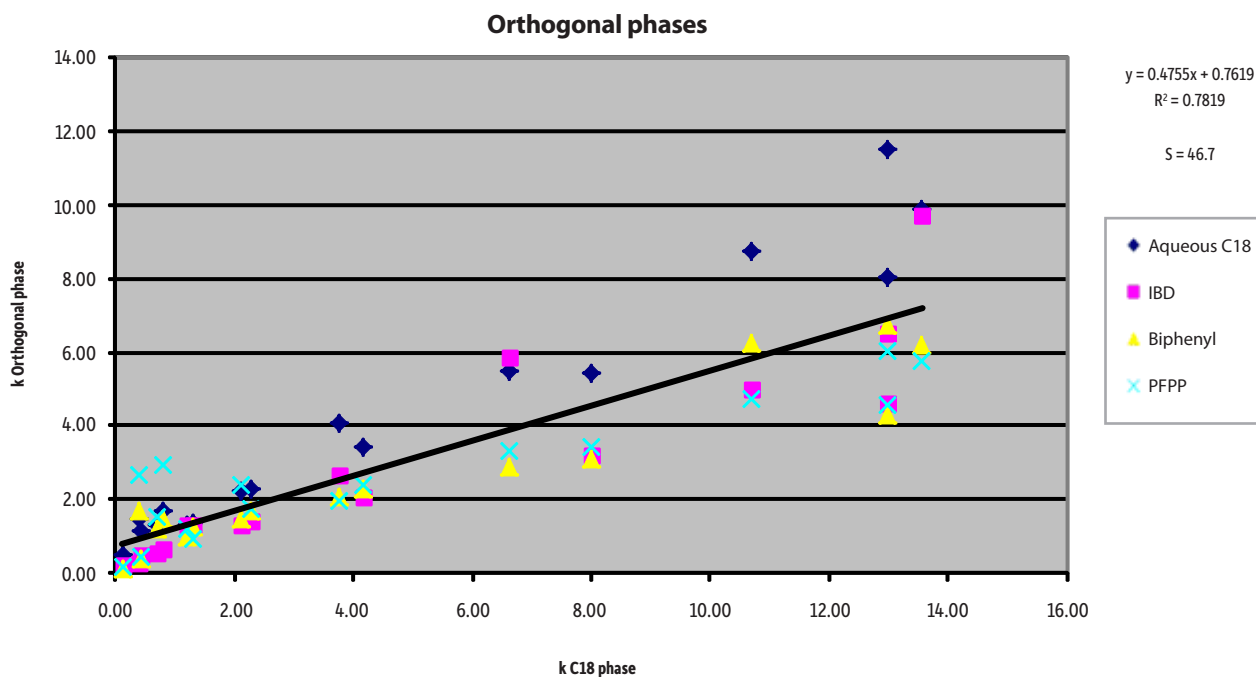
## Confirming the Alternate Selectivity of the USLC™ Column Set

To further confirm that each USLC™ column provides alternate selectivity—not only when compared to the C18 benchmark, but also when compared to the other columns in the set—we quantified the column set's range of selectivity (S) as described by Neue et al. [2]. Looking at the retention characteristics of the H-S model solute probes, we can define selectivity as the degree of scatter along the regression line when comparing stationary phases to the conventional C18 benchmark (Figure 2).

Two very similar stationary phases will produce similar retention for the solute probes and, when graphed, will show high linearity and high correlation. Two very *dissimilar*, or alternately selective, stationary phases that differ in the retention of the solute probes will show a high degree of scatter around the regression line. More scatter reveals that columns are more different, or orthogonal, from one another because it shows larger differences in selectivity. To measure this difference and use it as a means of comparing stationary phases, we can calculate a selectivity (S) value for the columns in the USLC™ column set. Note that because silica and mobile phase contributions could also alter the retention of the test probes, it is important to use identical silica supports and mobile phase compositions as to not bias the results and to allow focus only on the stationary phase contributions to selectivity.

With a selectivity (S) value of 46.7, Restek USLC™ phases produce the highest range of alternate selectivity available on the market today—using only 4 columns.

**Figure 2** Restek has extended the selectivity range for commercially available columns and defined a column set—the 4 USLC™ phases—that is ideal for simplified column selection and ease of method development.



All columns were tested using the same silica support.

## Conclusion: The Right Tools for Maximum Selectivity

The H-S model offers the chromatographic method developer a practical approach to column selection. With a simplified model described here, we can now easily create predictable and alternate selectivity, effectively influencing the most significant factor contributing to resolution. Now that we have identified the small USLC™ column set with a wide range of quantified selectivity, we can quickly determine the best column for almost any reversed phase situation by referencing predefined retention profiles. This column set can also be used to get the most out of column switching by providing a functional column set.

The Restek USLC™ column set, consisting of a balanced aqueous C18, a Biphenyl, a fluorinated PFP propyl, and a polar embedded IBD, has a profile that encompasses the widest range of reversed phase selectivity available today. Putting the right tools—like the USLC™ column set—in your method development toolbox means maximum alternate selectivity and peak separation with minimal effort.

## Acknowledgements

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## References

- [1] L.R. Snyder, J.W. Dolan, P.W. Carr, The Hydrophobic-Subtraction Model of Reversed-Phase Column Selectivity, J. Chromatogr. A 1060 (2004) 77.
- [2] U.D. Neue, J.E. O’Gara, A. Mendez, Selectivity in Reversed-Phase Separations Influence of the Stationary Phase, J. Chromatogr. A 1127 (2006) 161.

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