

# Leveraging Bonded Phase Selectivity to Achieve Better Reversed Phase HPLC Separations



## INTRODUCTION

There has been a great deal of emphasis in the past several years on column efficiency (theoretical plates, N) and using ultra-efficient columns, so called “UHPLC” columns, as a means of achieving separation goals, especially high speed separations. UHPLC columns deliver higher numbers of theoretical plates per column length than conventional HPLC columns and can benefit from the use of higher mobile phase flow velocities. Therefore, shorter column lengths and high mobile phase velocities can be used to achieve remarkably fast separations with little or no compromise in resolution. In addition, longer versions of UHPLC columns can be employed to achieve separation of complex mixtures by virtue of their high plate counts.

However, it should be recognized that there is a considerable price to pay for relying on ultra-high column efficiency to achieve separation goals. UHPLC columns are more expensive to buy, require the use of more expensive and complex ultra-high pressure equipment, require more care in use, and are subject to frictional heating problems. In addition, if UHPLC is used for fast method development, considerable time and effort may have to be spent in altering these methods so that they can be transferred to labs that may only have conventional HPLC equipment.

Nonetheless, UHPLC has proved its value for faster method development and UHPLC’s contribution to improvements in laboratory productivity is well documented. However, chromatographic selectivity is often overlooked and its importance overshadowed by the recent emphasis on column efficiency. One reason for this focus on efficiency is that the relationship between column efficiency and resolution ( $R_s$ ) is fairly simple to understand. If you want to increase  $R_s$ , you simply increase N by increasing column length and/or reducing the column packing particle size. It is also true that increasing selectivity ( $\alpha$ ) increases  $R_s$ , but because of the numerous variables that affect selectivity, it is not so simple to understand how to actually increase selectivity. It is even more difficult to understand how to optimize selectivity for all peak pairs of interest in a sample. We view increasing N as a means to increase  $R_s$  as a “sledge hammer” approach to developing a separation, whereas optimizing  $\alpha$  is a more nuanced approach that requires a higher level of knowledge to implement effectively. Figure 1 compares an ultra-high speed separation on a UHPLC column to a similarly fast separation achieved on an HPLC column that has better selectivity for the two peak pairs. Although the successful adjustment of selectivity gives a better “ultra-fast” separation of this sample than does the UHPLC approach, it does require a higher level of chromatographic skill.

**TABLE 1: Definition of Terms**

### Theoretical Plates, N

Theoretical plates, expressed with the symbol N, is a measure of the efficiency of a column.

$$N = 16(t_r/w_b)^2$$

Where:  $t_r$  is the retention time of an analyte  
 $w_b$  is the peak width at baseline

### Retention, k

Retention, expressed with the symbol k, is a measure of the number of column volumes of mobile phase required to elute an analyte from the column.

$$k = (t_r - t_0)/t_0$$

Where:  $t_r$  is the retention time of the analyte  
 $t_0$  is the void volume of the column

### Selectivity, $\alpha$

Selectivity, expressed with the symbol  $\alpha$ , is a measure of the relative retention of two analytes. It is also referred to as separation factor.

$$\alpha = k_2/k_1$$

Where:  $k_2$  is the k value for peak 2  
 $k_1$  is the k value for peak 1

### Resolution, $R_s$

Resolution, expressed with the symbol  $R_s$ , is a measure of the degree of separation between two chromatographic peaks.

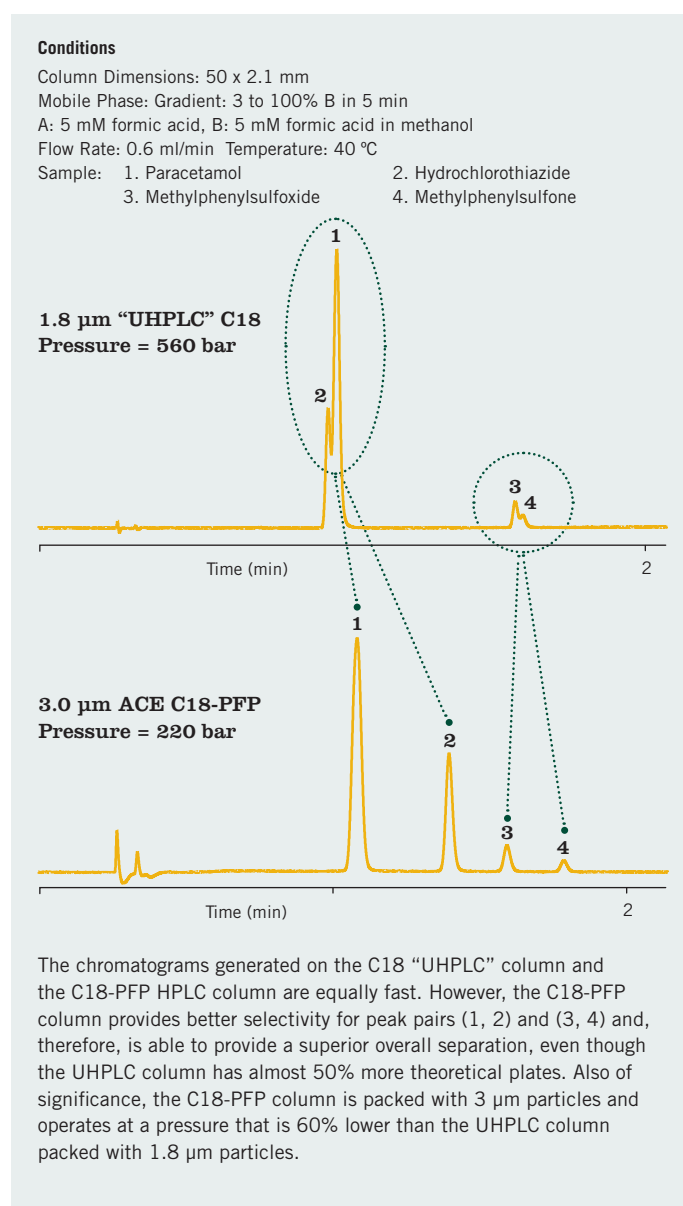
$$R_s = 2(t_{r2} - t_{r1})/(w_2 + w_1)$$

Where:  $t_{r2}$  is the retention time of analyte 2  
 $t_{r1}$  is the retention time of analyte 1  
 $w_2$  is the peak width of peak 2  
 $w_1$  is the peak width of peak 1

Resolution can also be defined by the chromatographic parameters that affect it.

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

**FIGURE 1: Comparison of ultra-fast separations using a UHPLC column with sub-optimal selectivity and an HPLC column with better selectivity**



It is important to note here that  $N$  and  $\alpha$  are independent variables and you do not have to choose to use one rather than the other when developing a separation. You should optimize both, as well as  $k$ , if you want to achieve the best overall results.

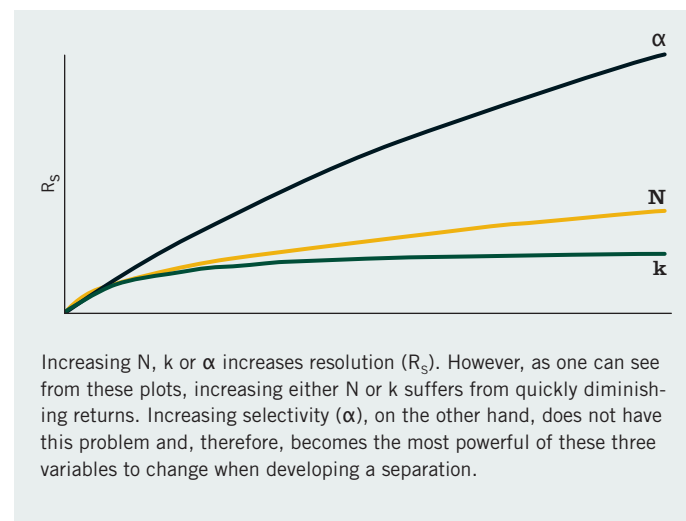
#### THE RESOLUTION EQUATION

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{k + 1}$$

The resolution equation identifies the parameters that contribute to resolution,  $N$ ,  $k$  and  $\alpha$ . The power and limitation of each of these parameters is illustrated in Figure 2. Resolution increases as  $N$  increases, but since resolution increases only with the square root of the increase in  $N$ , it is a case of diminishing returns with an ac-

companying “cost” of increased pressure and/or increased separation time. (Note: Pressure is directly proportional to column length and inversely proportional to the square of particle size.) Similarly, increasing retention ( $k$ ) increases resolution slightly, but also with rapidly diminishing returns and at a cost of longer separation times. Increasing selectivity ( $\alpha$ ) increases resolution, but without having to pay the price of either higher pressure or longer separation times observed when increasing  $N$  or  $k$ . Clearly, selectivity is the most powerful of these three parameters to increase. In addition, manipulating selectivity provides us the opportunity to optimize the separation of all peak pairs of interest to achieve a better overall separation.

**FIGURE 2: Effects of  $k$ ,  $N$  and  $\alpha$  on Resolution ( $R_s$ )**



It would seem that the decision to optimize selectivity is obvious when developing a separation. However, there are complex interactions among the analytes, stationary phase and mobile phase which can make optimizing selectivity difficult. Consequently, many practicing chromatographers just choose a C18 bonded phase and then attempt to manipulate  $N$  and  $k$  to achieve their separation goals.

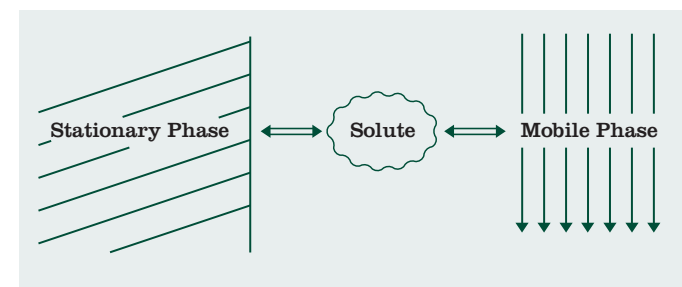
This technical report will attempt to explain stationary phase selectivity in a way that will make optimizing selectivity less daunting and, hopefully, encourage more people to include this powerful variable in their separation development. In the interest of brevity, this technical report will focus on reversed phase chromatography and the more commonly used bonded phases.

#### MECHANISMS OF SEPARATION

We will begin this discussion of bonded phase selectivity by exploring the different types of interactions that can occur between analytes and a variety of bonded phase types. We have chosen to ignore interactions between polar analytes and exposed silanols on the surface of silica stationary phase supports in this report. These ion exchange interactions can affect retention, but they are seldom useful to exploit when developing a separation. In fact, they are viewed as undesirable interactions because they can cause band broadening and poor lot-to-lot reproducibility. It is generally advisable to try to minimize these interactions by choosing to use highly inert stationary phases and/or by modifying the mobile phase.

#### HYDROPHOBIC INTERACTIONS

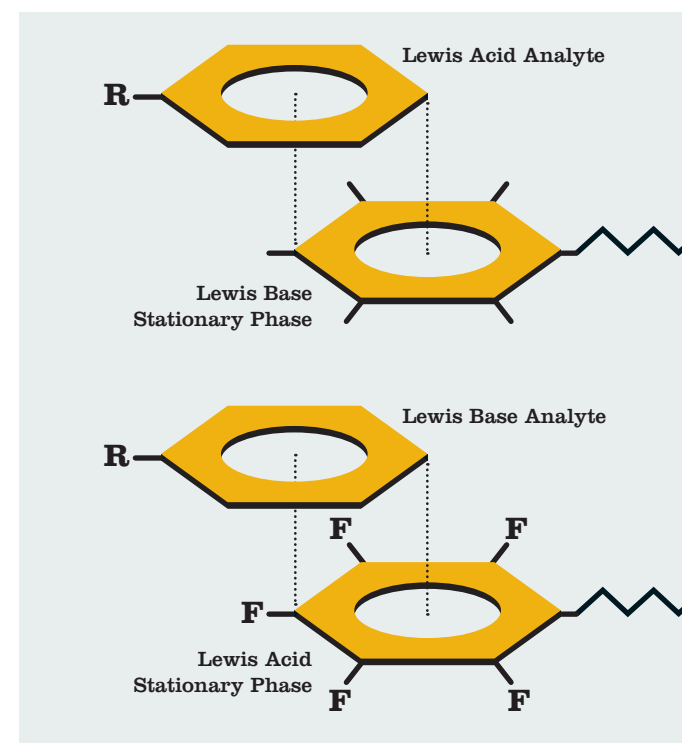
**FIGURE 3: Schematic of Hydrophobic Interactions**



The dominant separation mechanism in reversed phase chromatography is hydrophobic binding interaction between solute molecules in the mobile phase and the stationary phase, i.e. the bonded phase, such as C18, C8, etc. Although the actual retention mechanism is not well understood, it has been found useful to describe the process as solute partitioning from the mobile phase into the bonded phase.

#### $\pi$ - $\pi$ INTERACTIONS

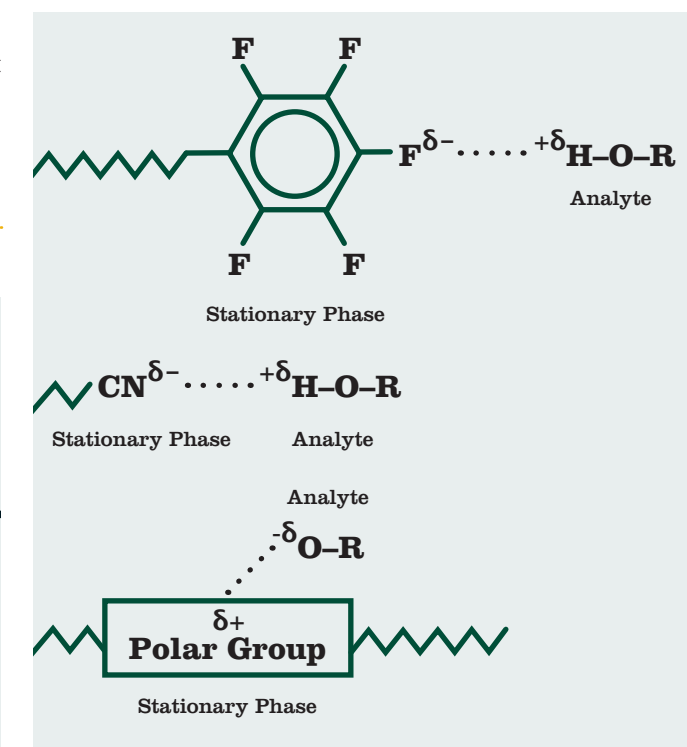
**FIGURE 4: Schematic of  $\pi$ - $\pi$  interactions**



$\pi$ - $\pi$  interactions occur due to intermolecular overlapping of p-orbitals in p-conjugated systems. Figure 4 provides a cartoon representation of the  $\pi$ - $\pi$  interactions between an aromatic analyte and the aromatic group of the stationary phase. The phenyl group of the stationary phase is a Lewis base (electron rich) and will interact most strongly with Lewis acid (electron poor) analytes (Figure 4). The pentafluorophenyl group is a Lewis acid and will interact most strongly with Lewis base analytes (Figure 4). Such  $\pi$ - $\pi$  interactions can be significant contributors to the separation of a mixture containing aromatic compounds.

#### HYDROGEN BONDING INTERACTION

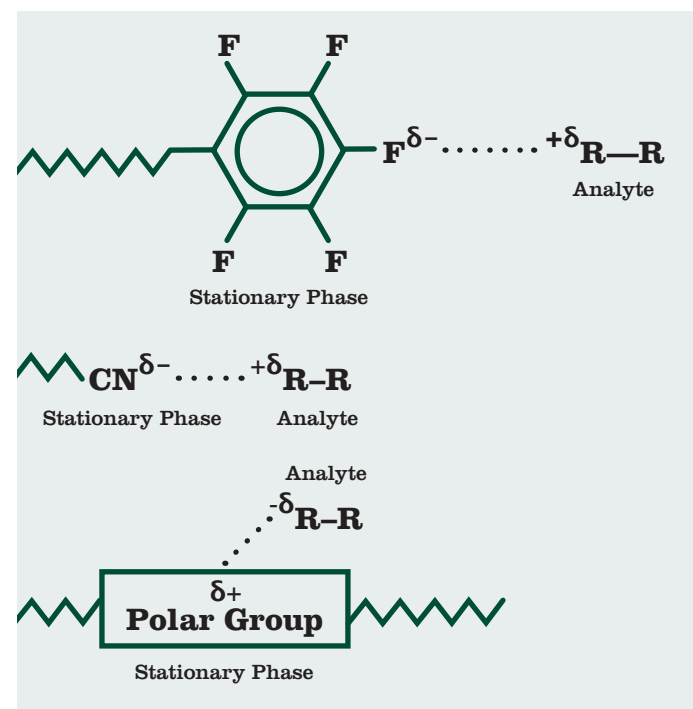
**FIGURE 5: Schematic of Hydrogen Bonding Interaction**



Hydrogen bonding interaction is a type of dipole-dipole interaction that occurs when the interaction involves a hydrogen atom bonded to a heteroatom, such as oxygen, nitrogen or sulfur. Hydrogen bonding interactions occur between hydrogen atoms attached to heteroatoms and the electronegative fluorine atoms in pentafluorophenyl (PFP) bonded phases, and they occur between heteroatom hydrogens and electronegative groups, such as -CN groups in cyanopropyl bonded phases and amide (-NHC=O) groups in polar embedded phases.

## DIPOLE-DIPOLE INTERACTION

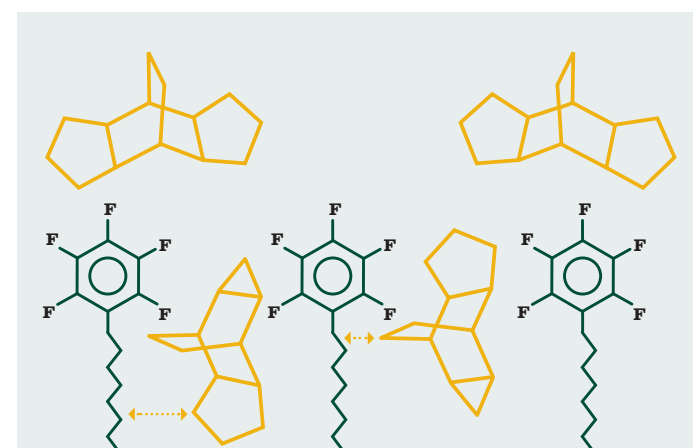
**FIGURE 6:** Schematic of Dipole-Dipole Interaction



Polar molecules have electric dipole moments because of an uneven distribution of electron charges on the molecules due to differences in electronegativities of the atoms. Dipole-dipole interactions occur when electric moments (dipoles) of molecules are attracted to oppositely charged dipoles of the bonded phase. The interaction energy depends on the strength and relative orientation of the two dipoles. Although this type of interaction is weaker than hydrogen bonding, it can still promote differences in relative retention when you are trying to separate analytes that are not adequately resolved on a typical alkyl phase, such as C18, C8, C4, etc.

## SHAPE SELECTIVITY

**FIGURE 7:** Schematic of Shape Selectivity



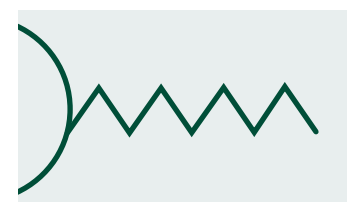
A rigid bonded phase can sterically hinder the way analytes interact with the bonded phase. The amount of hindrance will vary according to the shape of the molecule in solution, permitting stronger interaction and greater retention for some molecules than others. In this way more rigid bonded phases will have more shape selectivity and, thus, may offer a better separation of some sample mixtures. Bonded phases based on long alkyl chains, such as C30, are rigid and offer shape selectivity. Fluorine adds significant structural rigidity to a bonded phase, making bonded phases with PFP groups especially useful when shape selectivity is desirable.

## DESCRIPTION OF BONDED PHASES FOR REVERSED PHASE HPLC/UHPLC

This section will describe a number of bonded phases that are used for reversed phase HPLC. This is not a complete list of all bonded phases, but it does contain descriptions of the bonded phases that are used most often. It should be assumed that these phases are bonded to silica stationary phase supports or “hybrid” silica stationary phase supports.

### Alkylbonded Phases (C18, C8, and C4)

**FIGURE 8:** Schematic of C8

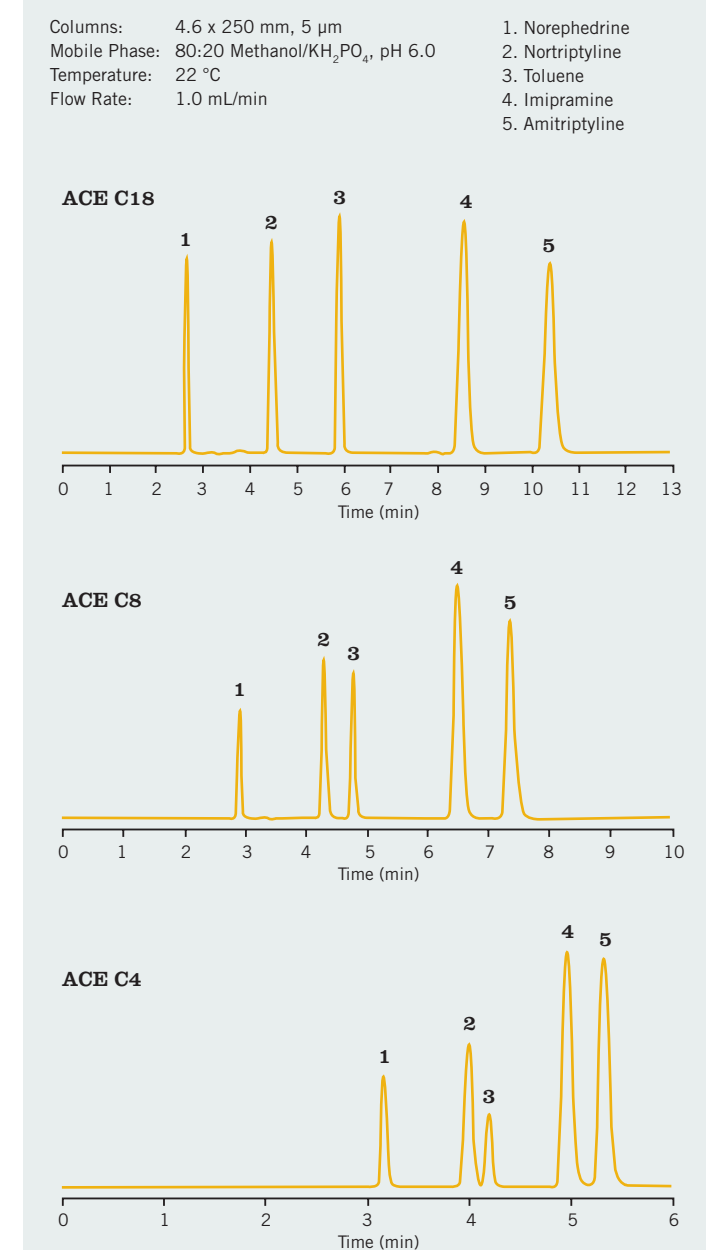


Alkyl bonded phases (C18, C8, and C4) are the most commonly used bonded phases for reversed phase HPLC. The mechanism for retention is primarily via hydrophobic interaction, which makes these bonded phases useful for separating a variety of analytes of widely differing polarities. One reason that alkyl bonded phases are often preferred is that they are among the oldest phase types and many methods have been developed using these phases. Another reason for their popularity is the excellent stability of the bonded phases that gives acceptable column lifetimes and provides robust and rugged separations.

In general, the longer the hydrocarbon chain, the more hydrophobic the phase, and the greater the retention of analytes. Figure 9 compares chromatograms of a mixture of four antidepressants and toluene generated on a C18, a C8, and a C4 phase. All three phases use the identical ultra-inert silica stationary phase support (ACE®) to eliminate the effects of silanols on retention. The retention times become shorter as the chain length is decreased, and there are even some minor changes in relative retention (selectivity).

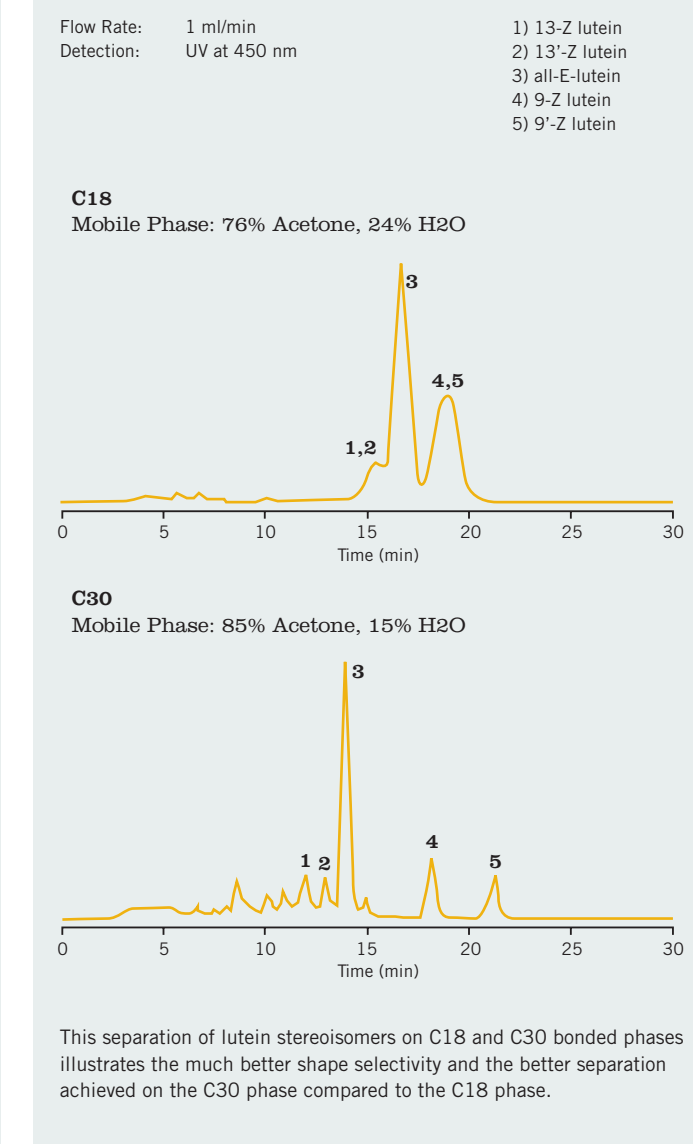
Steric interactions can also diminish or accentuate the degree of hydrophobic binding interaction that analytes have with the stationary phase. It's been demonstrated that monomerically bonded phases have less shape selectivity than polymeric bonded phases that are made with di- or trifunctional silanes. Very long alkyl bonded phases, such as C30, offer significantly better shape selectivity than shorter, e.g., C18, C8, alkyl bonded phases. (See Figure 10.)

**FIGURE 9:** The effect of alkyl chain length on Retention and Selectivity



A C18 phase, because of its greater hydrophobicity, is more retentive. An exception is the highly polar norephedrine, which is retained slightly longer on the C4. This is likely due to the better solvent penetration into the shorter C4 phase under highly aqueous mobile phase conditions which permits better interaction between norephedrine and the bonded phase. Note that there are also some minor differences in selectivity among these three alkyl phases, especially for peak pairs (2,3).

**FIGURE 10:** A C30 Phase Provides Better Shape Selectivity than even a C18



## PHENYL PHASES

**FIGURE 11:** Schematic of Phenyl Phase



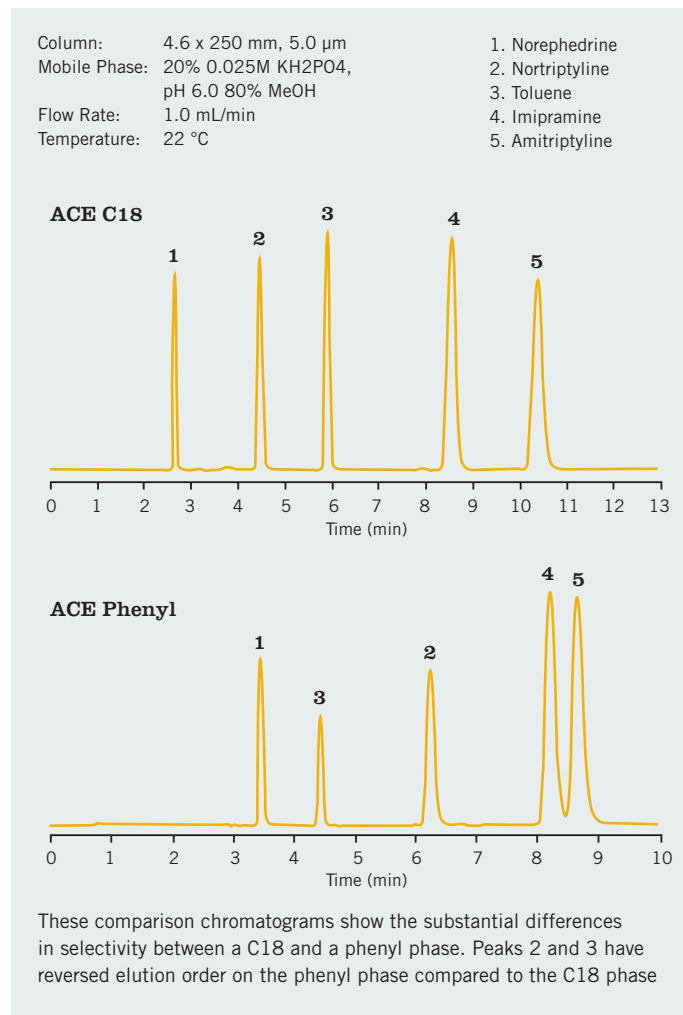
Phenyl bonded phases are usually manufactured with a short chain alkyl silane that attaches the phenyl ring to the silanols on the silica surface (Figure 11), although a new breed of phenyl phase has recently been introduced, the C18-AR, that uses a much longer alkyl chain. Phenyl phases are more polar than alkylbonded phases (C18, C8) and will generally provide less retention for hydrophobic analytes, with the exception of C18-AR, which provides the same retention as other C18

phases. Phenyl phases interact with analytes via  $\pi$ - $\pi$  interactions (see Figure 4) from the overlap of the delocalized electrons on the bonded phase phenyl group and those of analytes having aromatic character and unsaturated groups. Polar compounds that have aromatic or unsaturated functional groups will often be retained longer on phenyl phases than alkyl phases. Polar aromatic compounds with electron-withdrawing groups like nitro, chloro, fluoro, etc. interact very strongly with phenyl groups in the stationary phase due to Lewis acid – Lewis base and dipole-dipole interactions.

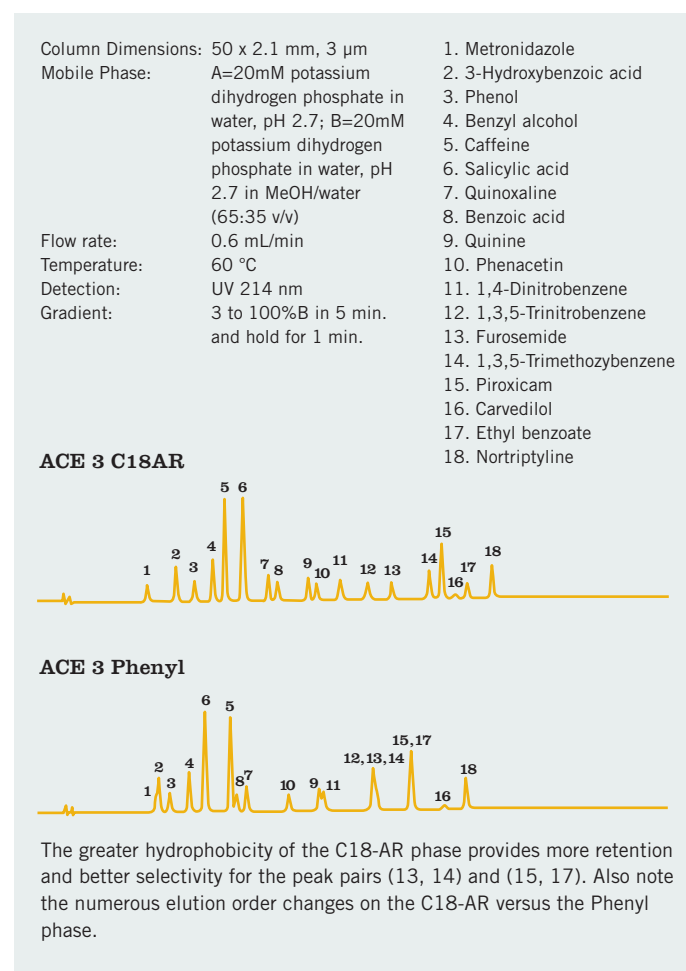
When a phenyl group is attached at the end of a long alkyl chain, as is the case with the C18-AR, the bonded phase will have a moderate amount of rigidity that can exhibit shape selectivity. This type of bonded phase will offer multiple mechanisms of separation, including  $\pi$ - $\pi$  interactions, dipole-dipole interactions, hydrophobic interactions and some slight shape selectivity.

Figure 12 illustrates the differences in retention and selectivity of a C18 and phenyl phase. Figure 13 shows the effect on retention and selectivity of bonding a phenyl group at the end of a long alkyl chain (C18-AR) compared to a more typical phenylpropyl phase.

**FIGURE 12: Comparison of Selectivity differences between C18 and Phenyl phases**



**FIGURE 13: Comparison of Selectivity Differences Between Phenyl and C18-AR Phases**



## CYANO PHASES (CN)

**FIGURE 14: Schematic of Cyanopropyl Phase**

The most common CN phase is the cyanopropyl bonded phase. This type of bonded phases has a three carbon alkyl chain with the CN group at the end of the alkyl chain. Cyanopropyl phases

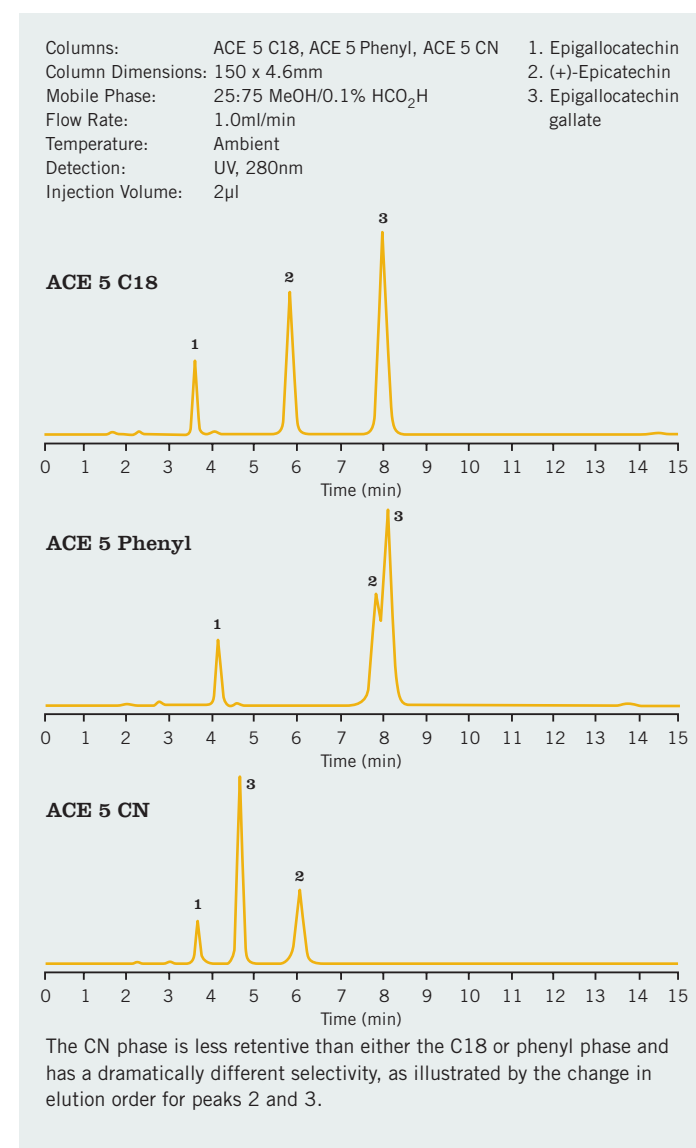
are much less retentive than C18, C8 or phenyl phases and are unusual in that they can be used in reversed-phase mode and also in normal-phase mode. Because it's much less retentive, you must use a much lower percentage of organic modifier in the mobile phase to obtain adequate retention.

The difference in selectivity of a CN phase compared to a C18, C8, C4/C3 or Phenyl phase is due to its very low hydrophobic interaction and its strong dipole-dipole interaction. The distinctly different mechanisms of separation offered by a CN phase may be useful when a C18 or phenyl phase fails to deliver adequate selectivity for all peak pairs. This difference in selectivity is illustrated in Figure 15

where the retention order for peaks 2 and 3 is different on a CN phase compared to a phenyl and a C18 phase. The lower retention of hydrophobic analytes on CN phases is sometimes useful when trying to achieve an isocratic separation of a sample that contain analytes with a wide range of polarities.

As stated before, CN phases can be used in both reversed-phase mode and normal-phase mode. However, for best results we recommend dedicating a CN column for either reversed-phase or normal-phase and avoid using a column previously used for reversed-phase for normal-phase, and vice versa.

**FIGURE 15: Comparison of Selectivities of ACE C18, Phenyl and CN Columns**



## POLAR EMBEDDED PHASE

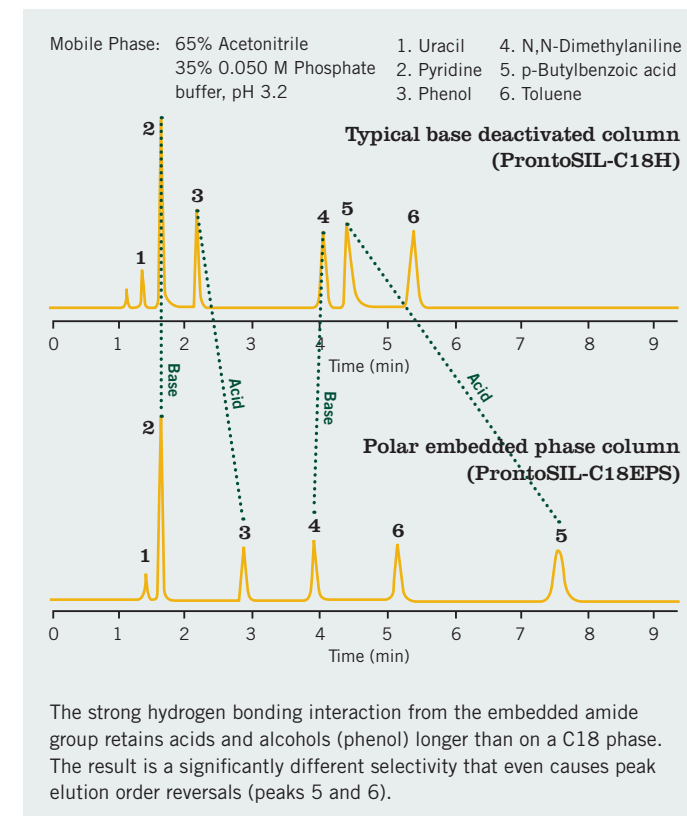
**FIGURE 16: Schematic of a Polar Embedded Phase**



A polar embedded phase commonly has an amide functional group (-NHC=O) located near the silica surface, usually separated by a short alkyl chain, with a longer alkyl chain beyond the polar group (see Figure 16). Other types of polar embedded phases have ether, aryl, carbamate, or other polar groups in the ligand. Due to the presence of the polar group, polar embedded phases can be used with highly aqueous mobile phases without concern for pore dewetting effects that cause retention loss and poor reproducibility with many C18 and C8 phases.

Polar embedded phases may offer several different mechanisms of separation, depending on the particular polar group that is used, but strong hydrogen bonding interactions combined with moderately strong hydrophobic binding interactions are what are typically expected. In the case of amide polar embedded phases, analytes whose functional groups can form hydrogen bonds with the -N-H

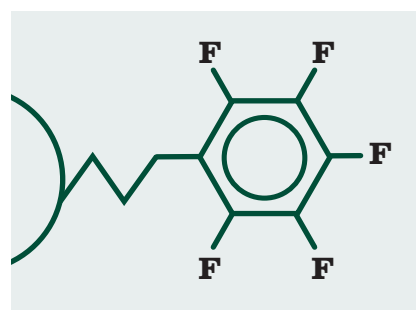
**FIGURE 17: Comparison of a separation on a Polar Embedded Phase and a C18 Phase**



or C=O bond of the amide group are retained longer than on a C18 phase column. Therefore, alcohols, phenols, and acids will generally show increased retention on an amide polar embedded phase compared to a C18 phase. Conversely, analytes with amine groups tend to be retained somewhat less on a polar embedded phase. (See Figure 17.)

### PENTAFLUOROPHENYL PHASE (PFP)

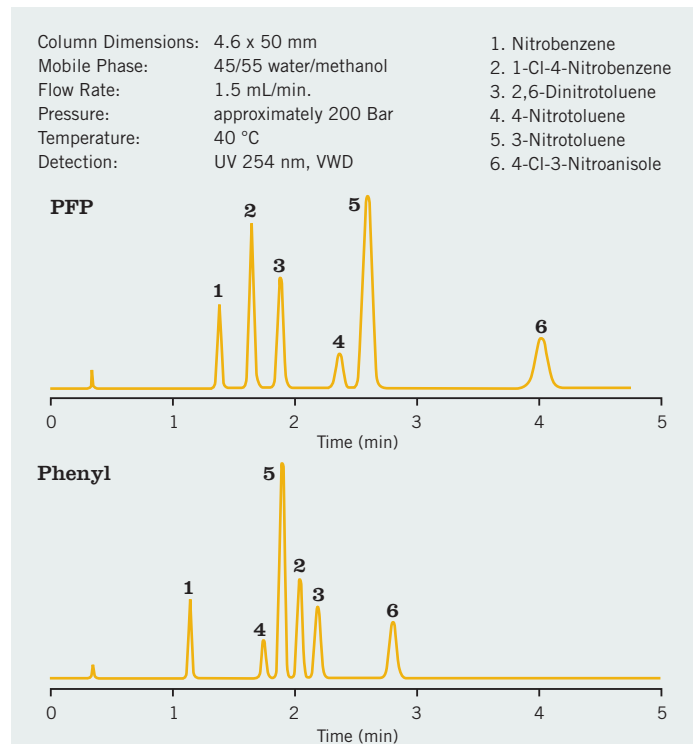
**FIGURE 18:** Schematic of typical PFP phase



PFP phases achieve retention primarily through  $\pi$ - $\pi$  interaction, hydrogen bonding interactions and, to a lesser extent, dipole-dipole interaction. As discussed in the section on mechanisms of separation, the pentafluorophenyl group is

Lewis acid (electron poor) and will interact strongly with Lewis base analytes (electron rich). This  $\pi$ - $\pi$  interaction is typically the strongest contributor to the retention of aromatic compounds and is what gives the PFP phases their unique selectivity compared to typical alkyl phases (C18, C8, etc.) and phenyl phases.

**FIGURE 19:** Comparison of Retention and Selectivity of a Phenyl and a PFP Phase

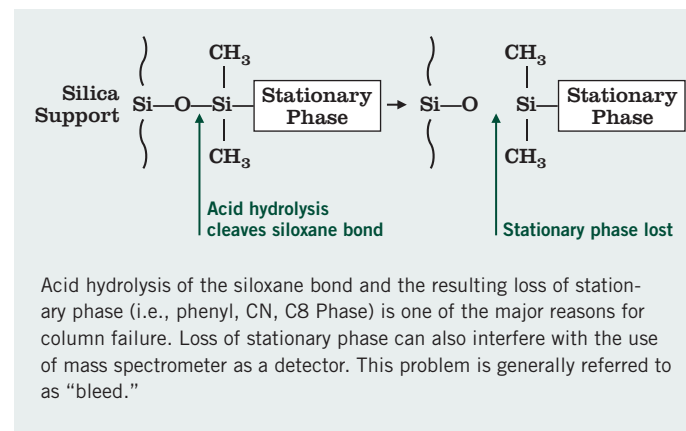


The difference in selectivity between the phenyl and the PFP phase is likely due to differences in the contributions of hydrogen bonding and  $\pi$ - $\pi$  interactions to retention.

### A NEW GENERATION OF BONDED PHASES

The bonded phases discussed thus far have armed chromatographers with a wide range of stationary phase selectivities to use in developing separations. However, some of these bonded phases have failed to deliver the ruggedness and robustness desired. In particular, CN, phenyl, and to a lesser extent PFP, have been prone to loss of stationary phases due to hydrolysis of the siloxane bond that attaches the stationary phase to the silica stationary phase support. (See Figure 20.) This loss of stationary phase shortens column life and can cause stationary phase “bleed,” which can interfere with mass spec detection. This isn’t a problem with all chromatographic conditions, but those that require acidic mobile phases are particularly vulnerable to this problem. Also, the ever growing challenge to develop separations of “difficult” sample mixtures, plus the demand to improve productivity in the analytical laboratory has driven the development of a new generation of bonded phases. These new bonded phases have taken the advantages of a C18 bonded phase (stability, reproducibility, and general applicability to separating a wide range of sample mixtures) and “added” functional groups that provide additional mechanisms of separation. There are currently two of these new types of bonded phases commercially available: the C18-AR and the C18-PFP. (See Figures 21, 22 and 23.)

**FIGURE 20:** Loss of Bonded Phase Due to Hydrolysis of the Siloxane Bond

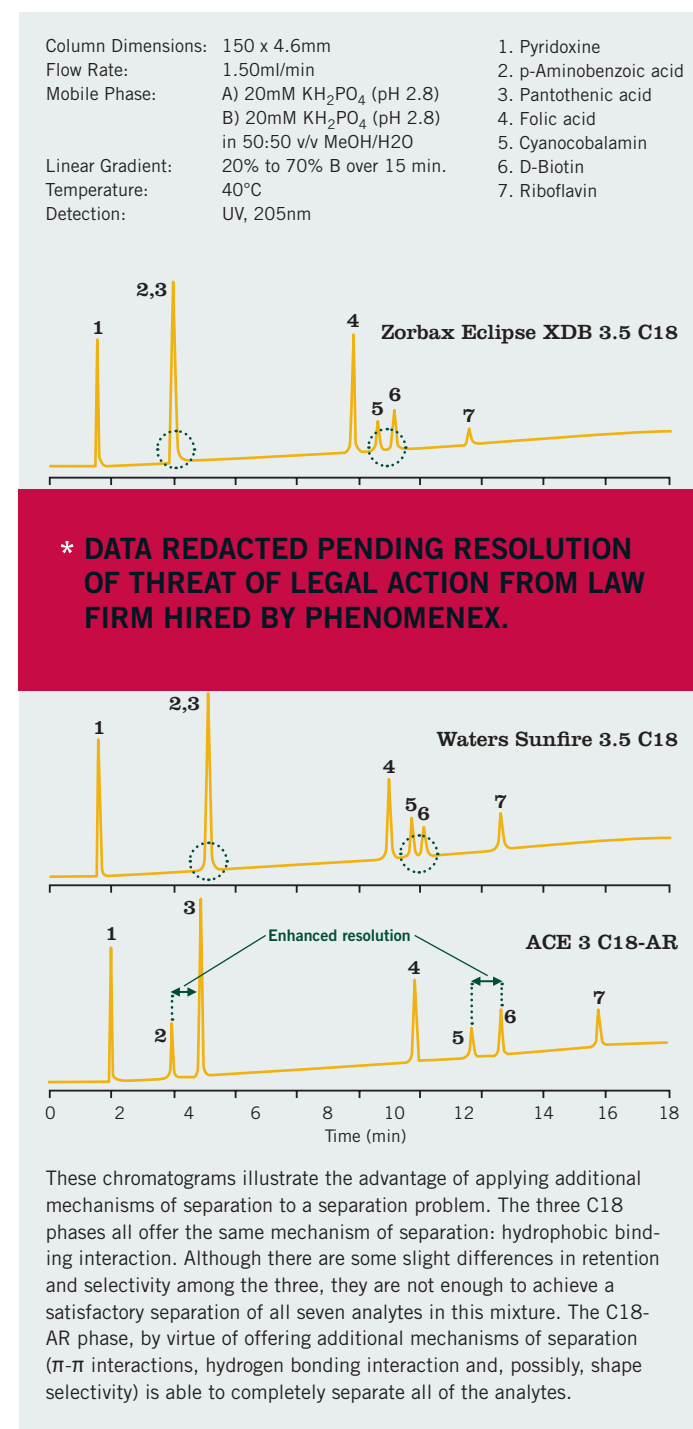


Acid hydrolysis of the siloxane bond and the resulting loss of stationary phase (i.e., phenyl, CN, C8 Phase) is one of the major reasons for column failure. Loss of stationary phase can also interfere with the use of mass spectrometer as a detector. This problem is generally referred to as “bleed.”

### C18-AR

The C18-AR phase contains a phenyl group on the end of a long alkyl chain. Unlike typical phenyl phases, C18-AR, as its name implies, has a long alkyl chain to provide strong hydrophobic binding interactions with analytes, just like any other C18 phase. The phenyl group of the C18-AR phase is placed at the end of the alkyl chain where it can interact more strongly with analytes.

**FIGURE 21:** Comparison of Retention and Selectivity of several C18 phases and a C18-AR phase



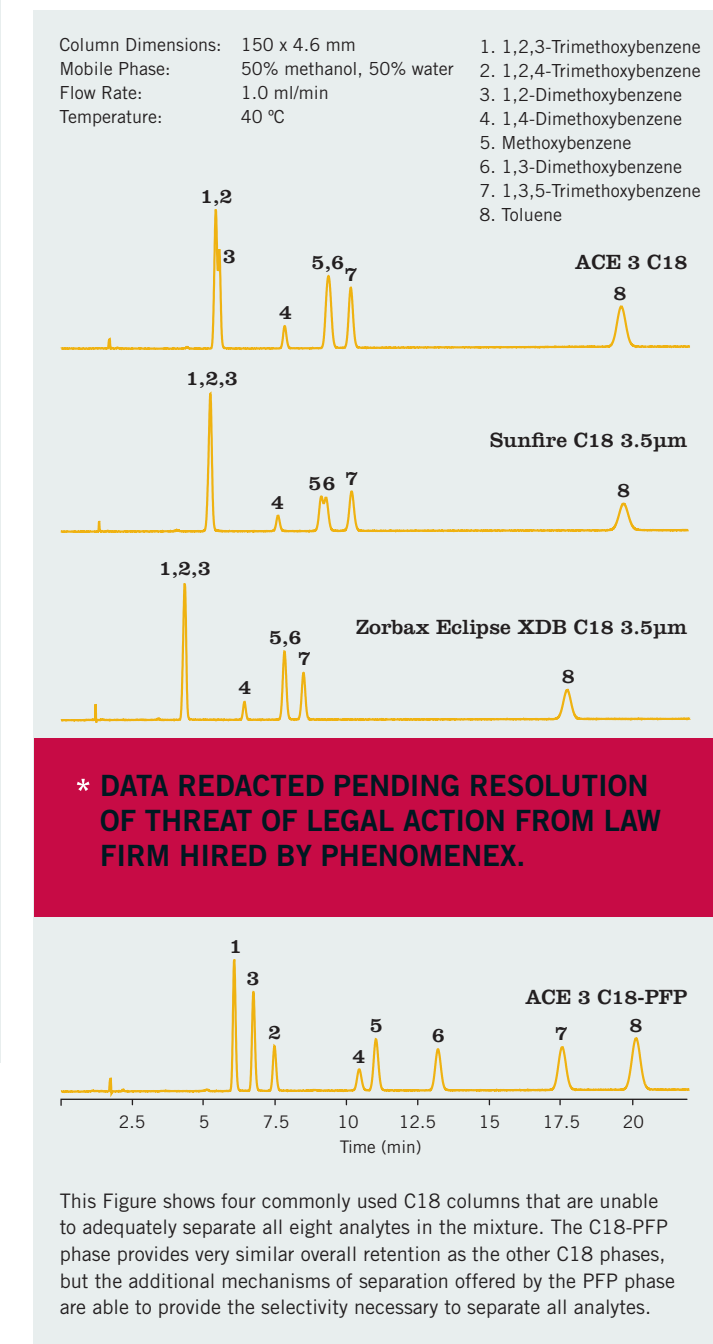
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These chromatograms illustrate the advantage of applying additional mechanisms of separation to a separation problem. The three C18 phases all offer the same mechanism of separation: hydrophobic binding interaction. Although there are some slight differences in retention and selectivity among the three, they are not enough to achieve a satisfactory separation of all seven analytes in this mixture. The C18-AR phase, by virtue of offering additional mechanisms of separation ( $\pi$ - $\pi$  interactions, hydrogen bonding interaction and, possibly, shape selectivity) is able to completely separate all of the analytes.

### C18-PFP

Similar to the C18-AR, the C18-PFP phase has a pentafluorophenyl group attached to the end of a long alkyl chain. It combines the stability and hydrophobic binding interaction characteristics of a C18 bonded phase with the additional mechanisms of separation offered by a PFP group. It differs from other PFP bonded phases by offering greater retention and selectivity for many analytes, due to its much stronger hydrophobic interaction. The C18-PFP phase is also much more hydrolytically stable than typical PFP phases due to the attachment of the PFP group to the silica support via a long, stable alkyl chain.

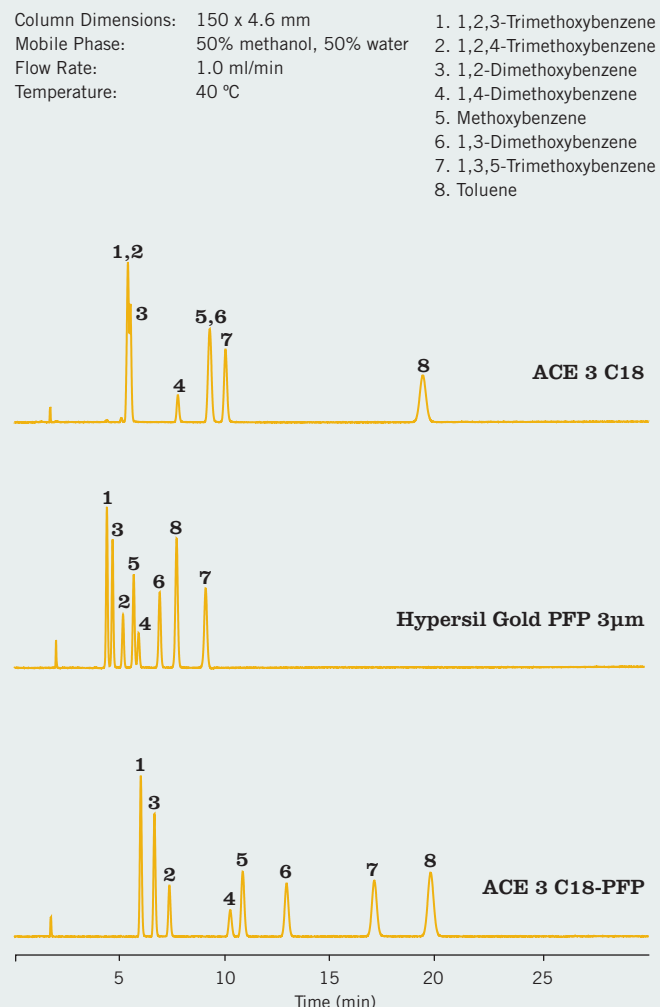
**FIGURE 22:** Comparison of Retention and Selectivity of Several C18 Phases and a C18-PFP Phase



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This Figure shows four commonly used C18 columns that are unable to adequately separate all eight analytes in the mixture. The C18-PFP phase provides very similar overall retention as the other C18 phases, but the additional mechanisms of separation offered by the PFP phase are able to provide the selectivity necessary to separate all analytes.

**FIGURE 23:** ACE C18-PFP columns offer greater retention and dramatic differences in selectivity compared to typical PFP columns.



The typical PFP phase (Hypersil Gold PFP) shows better overall selectivity than the C18 phase. However, it still does not yield a satisfactory separation. The C18-PFP phase delivers the beneficial selectivity provided by a PFP group, but also provides the strong hydrophobic binding interaction of a C18 phase to yield baseline separation of all analytes. Note that the retention order for peak pairs (2, 3) and (5, 6) are characteristic of the additional mechanisms of separation offered by a PFP group and not present in a typical C18 phase. In addition, the C18-PFP phase has the retention characteristics of a C18 phase (retention of toluene, peak 8) and selectivity for some peak pairs (4, 5 and 7, 8) that is more like a C18 column than a typical PFP column.

## LEVERAGING BONDED PHASE SELECTIVITY TO ACHIEVE A SEPARATION

Table 2 provides some guidelines for choosing a bonded phase for a sample mixture. With this guidance it is assumed that you know something about the types of compounds in your sample, which may not always be the case. However, these guidelines only give you a starting point. In the search to find the best bonded phase for a sample mixture, it is always necessary to run experiments. Although we may think we know what will provide the best separation, the interactions that take place among analyte, mobile phase and stationary phase are just too complex to predict relative retention for two analytes, much less for a mixture containing multiple analytes.

The approach we recommend for finding the best bonded phase for a sample is to use the guidelines offered in Table 2 to select the bonded phase to screen first for selectivity and peak shape. If this phase choice does not provide adequate selectivity for all peak pairs of interest, then choose a bonded phase that is “different” from the first bonded phase you tried. The rationale in this approach is that if one type of bonded phase does not provide adequate selectivity, a similar bonded phase that offers the same mechanism of separation is also unlikely to provide adequate selectivity. A better approach is to choose a bonded phase that offers a different mechanism of separation, or perhaps one that offers additional or fewer mechanisms of separation. This will increase the odds of achieving an adequate separation of all components. Exploring different bonded phases may also identify unknown compounds that co-elute with other compounds on some bonded phases.

**TABLE 2:** Guidelines for Choosing a Bonded Phase for Developing a Separation

Bonded Phase	Mechanism(s) of Separation	Strength of Interaction	Target Analytes	Recommended Applications
C18	Hydrophobic binding interaction	Very strong	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity</li> <li>Polar, moderately polar, and nonpolar analytes</li> <li>Uncharged acids and bases</li> <li>Ionized acids or bases using ion-pairing</li> </ul>	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity</li> <li>Homologous compounds differing by –CH<sub>2</sub></li> </ul>
	Shape selectivity	Weak (polymeric C18 bonded phases have moderate shape selectivity)		
C8	Hydrophobic binding interaction	Strong	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity</li> <li>Polar, moderately polar, and nonpolar analytes</li> <li>Uncharged acids and bases</li> <li>Ionized acids or bases using ion-pairing</li> </ul>	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity,</li> <li>Homologous compounds differing by –CH<sub>2</sub></li> <li>Mixtures containing polar and very hydrophobic analytes</li> <li>Good starting phase for developing separation of proteins and large polypeptides. (300Å recommended)</li> </ul>
C4	Hydrophobic binding interaction	Moderate/Weak	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity</li> <li>Polar, moderately polar, and nonpolar analytes</li> <li>Uncharged acids and bases</li> <li>Ionized acids or bases using ion-pairing</li> </ul>	<ul style="list-style-type: none"> <li>Analytes differing in hydrophobicity</li> <li>Homologous compounds differing by –CH<sub>2</sub></li> <li>Larger molecules, such as polypeptides and proteins</li> </ul>
C30	Hydrophobic binding interaction	Very strong	<ul style="list-style-type: none"> <li>Hydrophobic, long-chain, structural isomers</li> <li>Analytes differing in hydrophobicity</li> </ul>	<ul style="list-style-type: none"> <li>Separation of isomers, such as plant extracts, food, and biological tissues that contain structurally different carotenoids and mixtures of geometric isomers</li> <li>Stereoisomers of vitamin E and vitamin A</li> <li>Polynuclear aromatic hydrocarbons (PAHs)</li> <li>Carotenoids and tocopherol derivatives</li> </ul>
	Shape selectivity	Strong		
Phenyl	$\pi$ - $\pi$ interactions	Strong	<ul style="list-style-type: none"> <li>Analytes with <math>\pi</math> bonding</li> <li>Analytes with electron delocalization and electron-withdrawing groups, such as halogens, nitro groups, ketones, esters, and acids</li> <li>Analytes with proton donor groups</li> <li>Analytes with different dipole moments</li> </ul>	<ul style="list-style-type: none"> <li>Stereoisomers</li> <li>Steroids</li> <li>Taxanes</li> <li>Substituted aromatics</li> <li>Highly aqueous conditions</li> </ul>
	Dipole-dipole interactions	Strong		
	Hydrophobic binding interaction	Moderate		

## CN

### Mechanism(s) of Separation

Dipole-dipole interactions ..... Strong  
Hydrophobic binding interaction ..... Weak

### Target Analytes

- Polar analytes
- Analytes with double and triple bonds
- Nonpolar analytes having too much retention on alkyl phases

### Strength of Interaction

### Recommended Applications

- Mixtures of very polar and polar analytes
- Antihistamines
- Anesthetics
- As an orthogonal phase in RPLC method development

## Polar Embedded Phase

### Mechanism(s) of Separation

Hydrogen bonding interaction ..... Strong  
Hydrophobic binding interaction depending on the particular phase ..... Moderate to very strong

### Target Analytes

- Analytes containing groups that can form hydrogen bonds, such as NH and CO
- Analytes differing in hydrophobicity
- Polar, moderately polar, and nonpolar analytes

### Strength of Interaction

### Recommended Applications

- Alcohols, phenols, and acids will generally show increased retention on an amide polar embedded phase compared to a C18 phase. Conversely, analytes with amine groups tend to be retained somewhat less on a polar embedded phase.
- Highly aqueous mobile phase conditions

## PFP

(pentafluorophenyl)

### Mechanism(s) of Separation

$\pi$ - $\pi$  interactions ..... Strong  
Dipole-dipole interactions ..... Strong  
Hydrophobic binding interaction ..... Moderate  
Shape selectivity ..... Moderate

### Target Analytes

- Analytes with  $\pi$  bonds
- Analytes with electron donating groups, such as phenols, aromatic ethers and amines
- Analytes with proton donor groups
- Analytes with different dipole moments

### Strength of Interaction

### Recommended Applications

- Stereoisomers
- Steroids
- Taxanes
- Substituted aromatics
- Highly aqueous mobile phase conditions

## C18-AR

(C18 with Phenyl group)

### Mechanism(s) of Separation

$\pi$ - $\pi$  interactions ..... Strong  
Dipole-dipole interactions ..... Strong  
Hydrophobic binding interaction ..... Strong  
Shape selectivity ..... Moderate

### Target Analytes

- Analytes with  $\pi$  bonding
- Analytes with electron delocalization and electron-withdrawing groups, such as halogens, nitro groups, ketones, esters, and acids
- Analytes with different dipole moments
- Analytes differing in hydrophobicity
- Polar, moderately polar, and nonpolar analytes
- uncharged acids and bases

### Strength of Interaction

### Recommended Applications

- Analytes differing in hydrophobicity, homologous compounds differing by  $-\text{CH}_2$
- Stereoisomers
- Steroids
- Taxanes
- Substituted aromatics
- Highly aqueous conditions
- Particularly recommended for applications where a typical C18 does not provide an adequate separation

## C18-PFP

(C18 with pentafluorophenyl group)

### Mechanism(s) of Separation

$\pi$ - $\pi$  interactions ..... Strong  
Dipole-dipole interactions ..... Strong  
Hydrophobic binding interaction ..... Strong  
Shape selectivity ..... Strong

### Target Analytes

- Analytes with  $\pi$  bonds
- Analytes with electron donating groups, such as phenols, aromatic ethers and amines
- Analytes with proton donor groups
- Analytes with different dipole moments
- Analytes differing in hydrophobicity
- Polar, moderately polar, and nonpolar analytes
- Uncharged acids and bases

### Strength of Interaction

### Recommended Applications

- Stereoisomers
- Steroids
- Taxanes
- Substituted aromatics
- Analytes differing in hydrophobicity, homologous compounds differing by  $-\text{CH}_2$
- Highly aqueous mobile phase conditions
- Particularly recommended for applications where a typical C18 does not provide an adequate separation

## MOBILE PHASE CONSIDERATION

A discussion of mobile phase selectivity is beyond the scope of this technical report. However, there is one issue concerning the choice of mobile phase that should be considered. Using acetonitrile or THF as mobile phase modifiers will typically decrease  $\pi$ - $\pi$  interactions between aromatic and polarizable analytes and phenyl groups in bonded phases. Methanol generally accentuates those same interactions, producing increased retention and greater changes in selectivity. Therefore, methanol is the preferred organic modifier to use for enhancing selectivity when using phenyl or PFP bonded phases, including C18-AR and C18-PFP. This does not mean that the use of acetonitrile should be avoided. There may be cases where better overall separations can be achieved when  $\pi$ - $\pi$  interactions contribute modestly to retention of some analytes. In these cases, acetonitrile will be the preferred mobile phase modifier.

## CONCLUSION

Bonded phase selectivity is often ignored when developing a reversed phase separation. Part of the reason for this is the marketing hype that comes from manufacturers of so-called “UPLC” and “UHPLC” equipment who emphasize pressure and column efficiency. Another reason is the propensity to always choose a C18 phase, either out of habit or because it is familiar, since the majority of HPLC separations have been carried out on C18 bonded phases. The main reason for ignoring bonded phase selectivity, however, is a lack of understanding of the mechanisms of separations offered by different bonded phases and how they contribute to retention and separation. It is hoped that this technical report has contributed to this understanding and provided a basis for leveraging selectivity to achieve better overall separations.



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