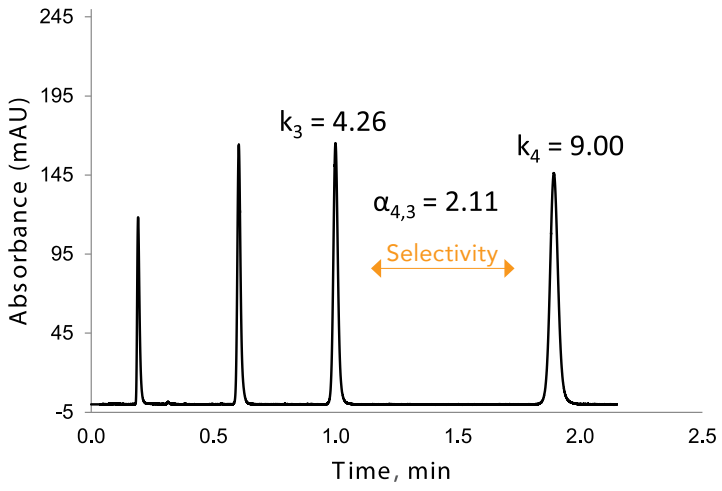
Using Selectivity of Fused-Core® Particles to Optimize Resolution for Improved LC and LCMS Method Development

Today's chromatographers face the same challenges that chromatographers faced twenty-five years ago – to develop methods that maximize resolution between their analytes of interest. The difference is that today there are more bonded phases and particle morphologies from which to choose. With increased options, method development should be easy. However, the analytes that are being separated are more complex and often have more closely related substances. Furthermore, in many work environments, the method development must be completed in a compressed time frame. This is where the skilled chromatographer must rely on their knowledge of both advanced HPLC column technology and selectivity to aid them in their method screening and development process. By using fast, efficient Fused-Core® technology columns like HALO® with a variety of bonded phases, chromatographers are able to quickly develop methods for analyzing the most challenging samples.

Selectivity (α) (also known as separation factor) is the space between peaks in an HPLC separation. Mathematically, it is defined as the ratio of retention factors (k) between neighboring peaks and is always 1.0 or greater: $\alpha = k_2 / k_1$



PEAK IDENTITIES (IN ORDER):
uracil, pyrene, decanophenone, dodecanophenone

TEST CONDITIONS:
Column: HALO 90 Å C18, 2 µm 2.1 x 50 mm
Mobile Phase: 85/15 ACN/Water
Flow rate: 0.5 mL/min
Temperature: Ambient (~25 °C)
Detector: UV 254 nm
Sample Volume: 0.2 µL

Figure 1. Example of selectivity between two peaks.

How does Selectivity Influence Resolution?

The master resolution equation is comprised of an *efficiency* term, a *selectivity* term, and a *retention* term.

$$R_S = \left(\frac{\sqrt{N}}{4}\right) x \left[\frac{(\alpha - 1)}{\alpha}\right] x \left[\frac{k_2}{(1+k_2)}\right]$$

Efficiency Selectivity Retention

N = plates
α = selectivity
k = retention factor

Of these three, selectivity is the most effective parameter to change to increase resolution as shown in Figure 2. Resolution vs. selectivity is nearly linear in the range of selectivity from 1.00 to 2.00.

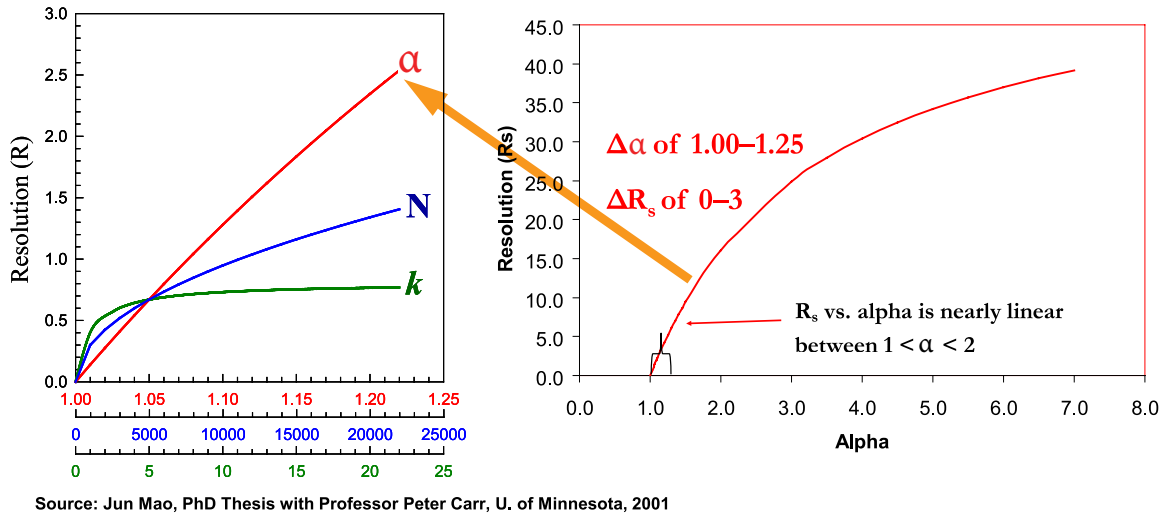
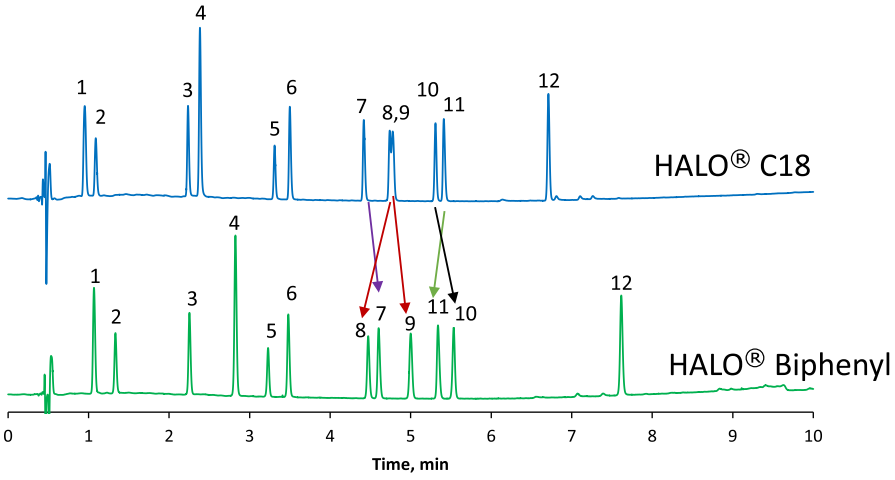


Figure 2. Plot of resolution vs. selectivity (red), efficiency (blue), and retention factor (green).

An example of the differences in selectivity from two bonded phases (C18 and Biphenyl) is shown in Figure 3. The Biphenyl phase shows increased retention for most of the β-blockers in this sample since it is able to interact with the aromatic moieties of these compounds. Additionally, elution order has changed on the Biphenyl column relative to the C18 column.



PEAK IDENTITIES:

1. Atenolol
2. Sotalol
3. Nadolol
4. Pindolol
5. Acebutolol
6. Metoprolol
7. Oxprenolol
8. Bisoprolol
9. Labetalol
10. Propranolol
11. Alprenolol
12. Carvedilol

TEST CONDITIONS:

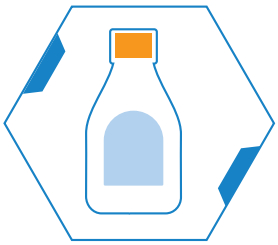
Column: 2.7 µm HALO, phases as indicated 2.1 x 100 mm SPP columns
Gradient: 10-50% in 10 min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN
Injection Volume: 1 µL
Flow Rate: 0.50 mL/min
Temperature: 30 °C
Detection: 220 nm

Figure 3. Comparison of β-blocker separations on HALO® C18 and HALO® Biphenyl.

If not for running standards or using mass identification, one might think that the elution order is the same when using an LC detector. Data systems can create confusion because peaks are usually numbered in elution order which frequently changes for complex samples. Retention order on a C18 column is often chosen as a reference to interpret subsequent change in selectivity. While both columns detect the same number of peaks, the Biphenyl column has better peak spacing and greater overall resolution for this sample.

Parameters for Optimizing Selectivity

There are several parameters that can be modified in order to optimize selectivity; this paper will discuss mobile phase, column type, pH, and temperature. Other parameters, which provide influence to selectivity, but will not be discussed, include ion pair concentration, % B (organic) solvent/gradient slope, and buffer concentration [1].



1. MOBILE PHASE

The organic component of the mobile phase has a significant impact on the selectivity of an HPLC separation. Although changing the column stationary phase can be equally important in altering k, the mobile phase is often evaluated first because it is more convenient instrumentally. In addition, users often prefer to develop methods on their favorite column so they first make certain that adequate resolution cannot be accomplished with a convenient mobile phase.

A recent article in Chromatography Today [2] describes the characteristics of acetonitrile and methanol, the two most widely used solvents in HPLC. Advantages of acetonitrile (polar-aprotic solvent) include its lower UV cutoff and viscosity compared to methanol. On the other hand, methanol (polar-protic solvent) is less toxic and less expensive than acetonitrile. Ultimately, the article concludes that screening both solvents is necessary for effective method development. A separation that shows coelutions with 100% acetonitrile as the B solvent may benefit from a switch to methanol or a combination of acetonitrile and methanol as shown in Figure 4.

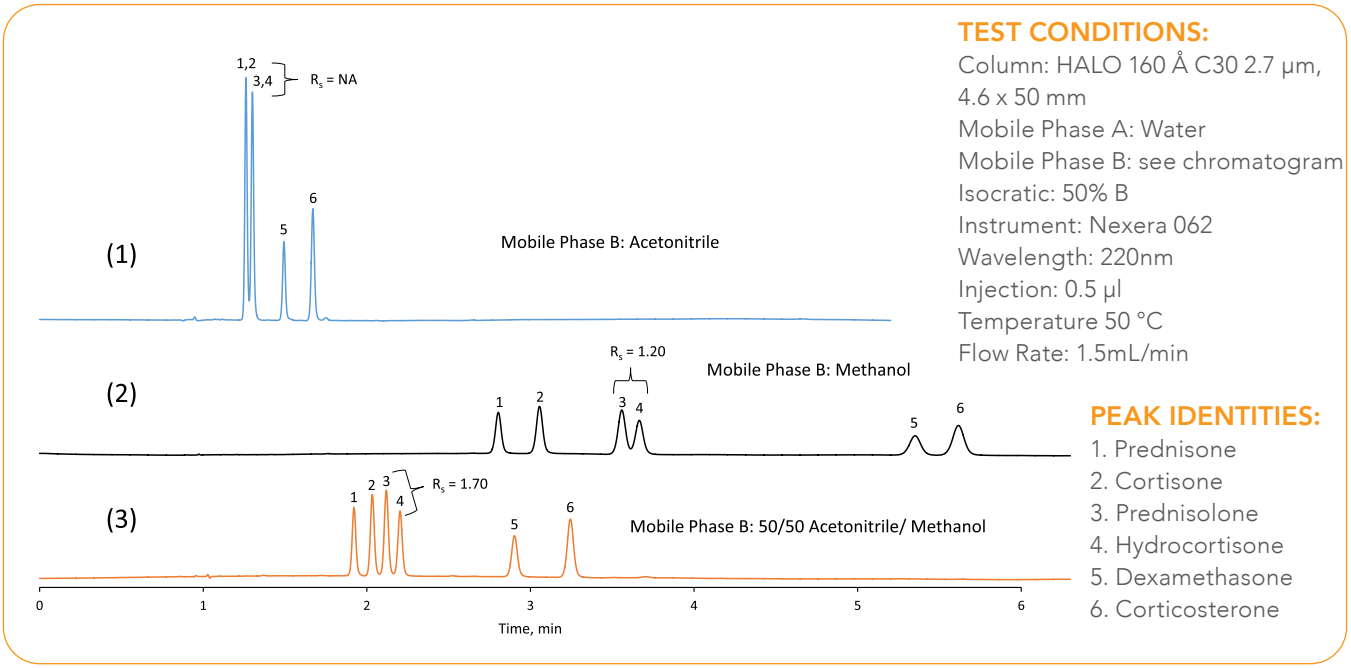
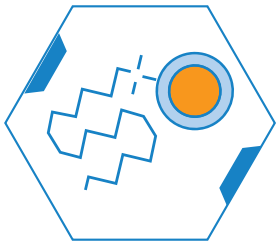


Figure 4. Effect of different mobile phase solvents on the separation of steroids.

The top chromatogram (1) uses 100% acetonitrile and critical pairs 1,2 and 3,4 are not resolved. The middle chromatogram (2) uses 100% methanol and critical pair 3,4 is not well resolved (resolution is 1.20). Neither of these separations provides adequate resolution for all six components. However, a 50/50 mixture of acetonitrile and methanol (3) enables baseline resolution (1.70 for critical pair 3,4) for all of the critical pairs in the sample along with a compromise in terms of the total time of the separation.



2. COLUMN TYPE

The chemistry of the stationary phase determines the selectivity of the column. When columns show different elution order and selectivity, they are said to be *orthogonal*. When columns show the same elution order and selectivity, they are called *equivalent*. A table of the available reversed-phase HALO® bonded phases is shown in Table 1.

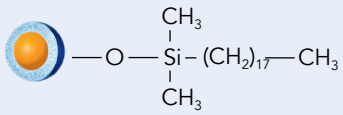
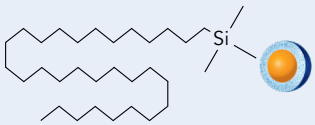
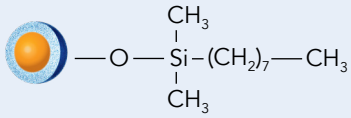
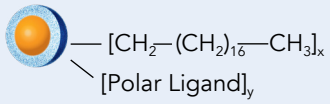
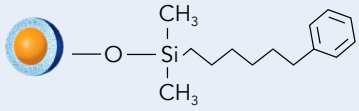
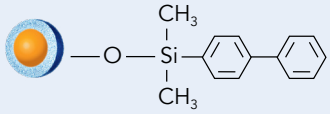
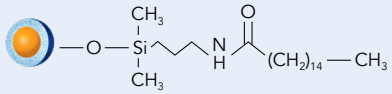
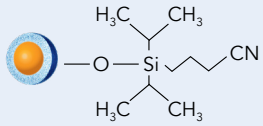
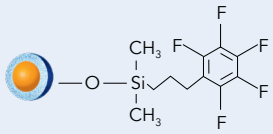
HALO® BONDED PHASE	STRUCTURE	CHEMISTRY	TYPES OF INTERACTIONS
C18		Alkyl	• Hydrophobic
C30		Alkyl	• Hydrophobic
C8		Alkyl	• Hydrophobic
AQ-C18		Alkyl / Polar	• Mainly hydrophobic • Some dipole-dipole
Phenyl-Hexyl		Aromatic / Alkyl	• Hydrophobic • π - π
Biphenyl		Aromatic	• π - π • Hydrophobic
RP-Amide		Polar / Alkyl	• Hydrogen Bonding • Hydrophobic
ES-CN		Polar	• Dipole-dipole • Hydrophobic
PFP		Aromatic / Polar	• Hydrophobic • π - π • Dipole-dipole • Hydrogen bonding

Table 1. Available HALO® phases, structures, chemistry, and types of interactions for use in reversed-phase HPLC.

Chromatographers are most familiar with C18 bonded phase and gravitate to beginning method development on this particular phase since it works well for a broad range of analyte polarities. However, it is not ideal for very polar compounds and this is when other phases, such as RP-Amide and PFP are needed. The HALO® phases may be classified by three main chemistry designations: alkyl, aromatic, and polar. See Venn diagram in Figure 5. Non-aromatic alkyl chemistry has the general formula of C_nH2_n+1. Aromatic chemistry contains one or more 6-carbon rings with the general formula C_6H_6. Polar chemistry contains any of the following functional groups or element: amide, cyano, or fluorine. C18, C8, and C30 are all in the alkyl designation. Phenyl-Hexyl, while aromatic, demonstrates alkyl characteristics. Solely aromatic is Biphenyl. PFP shares characteristics of both aromatic and polar chemistries. In the polar section is ES-CN. Finally, in the overlap section between polar and alkyl are AQ-C18 and RP-Amide. They are mostly polar, but often exhibit some hydrophobic selectivity. When selecting screening phases for method development, it is good practice to choose phases from each main chemistry designation or from overlap sections if necessary to achieve your separation.

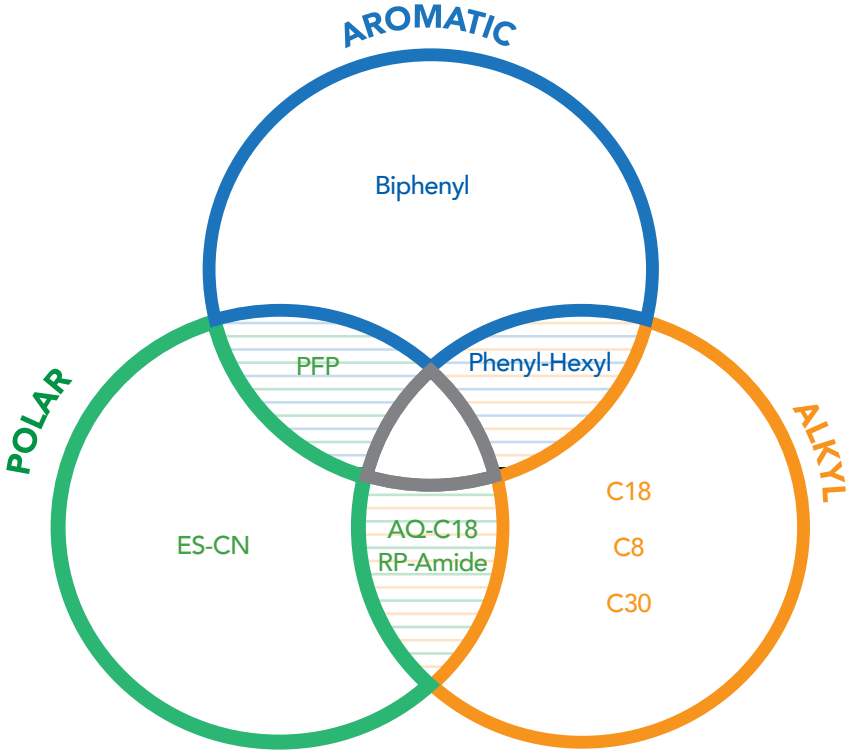


Figure 5. Chemical classification of HALO® reversed-phase stationary phases.

The *critical separation factor* is the smallest value for all peaks identified in a separation. An example chromatogram indicating the retention factors of two adjacent peaks and the selectivity between them is shown in Figure 1. Numerous methods for characterizing stationary phases have been developed, including the Hydrophobic-Subtraction Model (HSM) [3]. For the HSM, five different parameters (H, S*, A, B, and C) are used to measure the physicochemical properties of a chromatographic bonded phase.

H = hydrophobicity of the phase.
S* = resistance of the stationary phase to penetration by a solute molecule.
A = hydrogen-bond acidity of the phase.
B = hydrogen-bond basicity of the phase.
C = interaction of the phase with ionized solute molecules and is measured at pH 2.8 and 7.0.

The combination of these parameters along with parameters that characterize a solute (η, σ, β, α, κ) is then related to the solute's retention (k_x) relative to the retention of ethylbenzene (k_{EB}) by the equation below:

$$log\left(\frac{k_x}{k_{EB}}\right) = \eta H - \sigma S^* + \beta A + \alpha B + \kappa C$$

where:
η = solute hydrophobicity
σ = bulkiness of the solute molecule
β = hydrogen-bond basicity of the solute
α = hydrogen-bond acidity of the solute
κ = ionization state of the solute molecule

HALO® phases have been evaluated and compared to over 700 commercial HPLC columns using HSM and the data is available via **hplccolumns.org** and via the USP website **apps.usp.org/app/USPNF/columnsDB.html**. Table 2 lists HALO® bonded phases, USP designations, and HSM coefficients. HSM phase parameters may be compared for similarity or difference (orthogonal) using an F_s value calculated using the following equation:

$$F_S = \sqrt{\left(w_H(H_1 - H_2)\right)^2 + \left(w_S(S^*_1 - S^*_2)\right)^2 + \left(w_A(A_1 - A_2)\right)^2 + \left(w_B(B_1 - B_2)\right)^2 + \left(w_{C_{2.8}}\left(C_{2.8_1} - C_{2.8_2}\right)\right)^2}$$

where:

$H_1 - H_2$	is the difference between the hydrophobicity parameters of columns 1 and 2
$S^*_1 - S^*_2$	is the difference between the steric parameters of columns 1 and 2
$A_1 - A_2$	is the difference between the hydrogen bond acidity parameters of columns 1 and 2
$B_1 - B_2$	is the difference between the hydrogen bond basicity parameters of columns 1 and 2
$C_{2.8_1} - C_{2.8_2}$	is the difference between the charge interaction parameters (at pH 2.8) of columns 1 and 2
w_H	is the weighting factor for the difference in hydrophobicity
w_{S^*}	is the weighting factor for the difference in steric interactions
w_A	is the weighting factor for the difference in hydrogen bond acidity
w_B	is the weighting factor for the difference in hydrogen bond basicity
$w_{C2.8}$	is the weighting factor for the difference in charge interactions at pH 2.8

Two phases are considered equivalent when F_s value is < 12 and orthogonal when F_s value is > 12 with larger F_s values being more orthogonal. F_s values relative to HALO® C18 are listed in Table 2. Note in the Table that HALO® C18, C8 and AQ-C18 are almost equivalent in selectivity while HALO® RP-Amide and PFP are very different in selectivity (orthogonal). After initial column screening, choose columns with small F_s changes as needed, or choose columns with orthogonal behavior (large F_s values) when poor resolution is observed and greater changes in selectivity are needed. Ideally, a set of columns that span a range of F_s values should be used for method screening. 50 or 100 mm length columns in 2.1 mm ID are suggested for LC-MS screening while 3.0 mm ID columns are suggested for UV detection. Both UV and MS detection are recommended when screening mixes of unknowns.

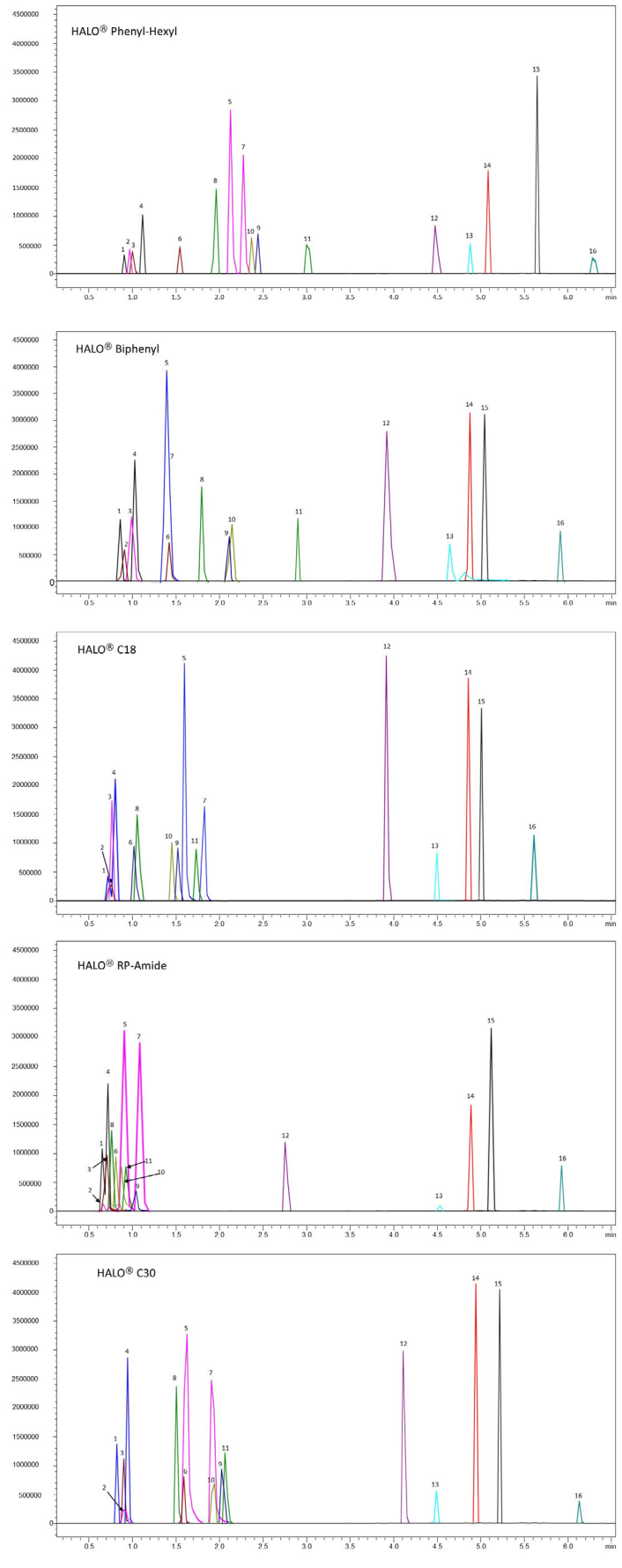
Decreased Similarity to C18

Fs	Phase	USP type	H	S*	A	B	C (pH 2.8)	C (pH 7.0)
0	HALO C18	L1	1.100	0.040	0.000	-0.050	0.050	0.040
10.04	HALO C8	L7	0.910	0.020	-0.130	0.000	-0.010	0.180
12.07	HALO AQ-C18	L1	1.000	-0.036	0.099	-0.048	0.156	0.864
17.35	HALO Phenyl-Hexyl	L11	0.780	-0.090	-0.230	0.000	0.100	0.450
17.43	HALO C30	L62	0.938	-0.046	-0.140	0.023	0.170	0.350
22.78	HALO ES-CN	L10	0.566	-0.110	-0.344	0.021	0.126	1.150
26.76	HALO Biphenyl	L11	0.708	-0.183	-0.279	0.028	0.047	0.990
52.83	HALO RP-Amide	L60	0.850	0.080	-0.380	0.190	-0.410	0.310
94.45	HALO PFP	L43	0.702	-0.117	-0.073	-0.062	1.170	0.972

*Note HSM values listed are for 2.7 µm particle size.

Table 2. HALO® Reversed-Phase with USP type, HSM coefficients, and F_s values relative to HALO® C18.

Screening different bonded phases while keeping the mobile phase constant is a common way to compare and optimize selectivity. A column switching valve can be added to most HPLC instrumentation for automated screening increasing the productivity of developing methods. A mix of 16 different drugs of abuse and metabolites were screened using LC-MS on 5 different HALO® phases. The most promising phase in screening was HALO® Phenyl-Hexyl as shown in Figure 6 because it had the least number of coelutions.



PEAK IDENTITIES

Peak Number	Compound	m/z
1	Hydromorphone	286
2	Oxymorphone	302
3	Noroxycodone	302
4	Morphine	286
5	Methamphetamine	150
6	Naloxone	328
7	Phentermine	150
8	Codeine	300
9	Naltrexone	342
10	Oxycodone	316
11	Hydrocodone	300
12	Meperidine	248
13	Fentanyl	337
14	Buprenorphine	468
15	Methadone	310
16	THC	304

TEST CONDITIONS:

Columns: HALO 90 Å or 160 Å phase as indicated, 2.7 µm, 2.1 x 100 mm
Mobile Phase A: water/0.1% formic acid
B: methanol/0.1% formic acid

Gradient:

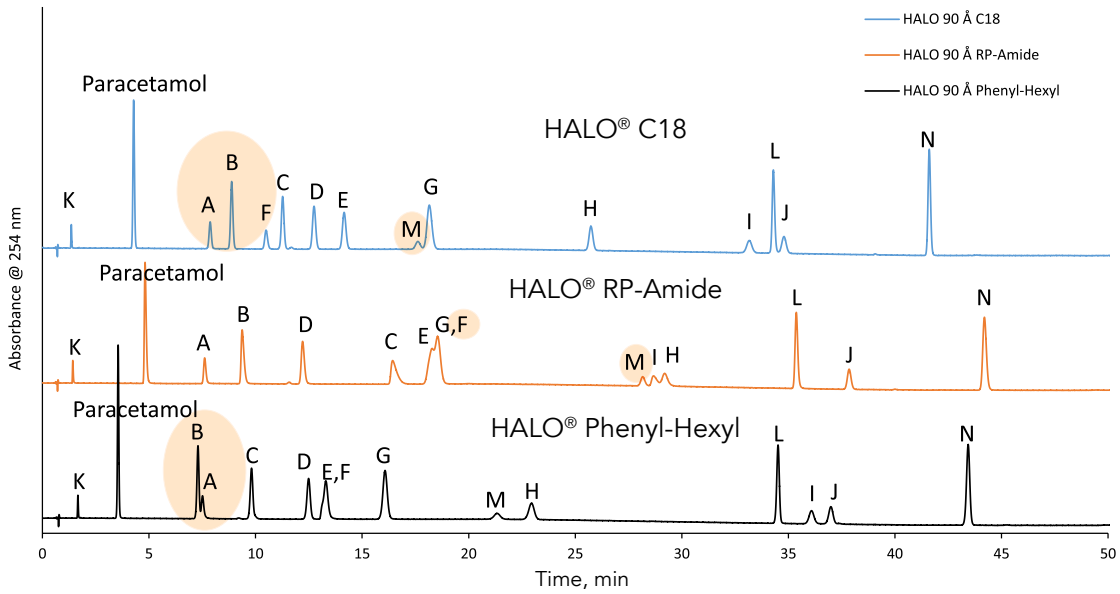
Time	% B
0.00	10
3.00	20
6.00	80
7.00	80
7.01	10
9.00	10

Flow Rate: 0.4 mL/min
Temperature: 30 °C
Detection: MS
Injection Volume: 0.2 µL of 1 ppb drugs of abuse and metabolites

Figure 6. LCMS screening of five HALO® phases for drugs of abuse and metabolites.

Selecting a few columns which are orthogonal to each other is a common screening approach because using a variety of different stationary phases provides a higher probability of succcess. Figure 7 shows a separation of paracetamol (acetaminophen) and its 14 impurities, following EP 9.4, using three orthogonal phases (HALO® C18, HALO® RP-Amide, and HALO® Phenyl-Hexyl) to demonstrate selectivity differences between phases. Interesting elution order changes were observed between HALO® C18 and HALO® RP-Amide (F_s value 52 from Table 2). Several impurity components (in particular F and M) were retained longer on the HALO® RP-Amide column compared to HALO® C18 since RP-Amide has more retention for phenol-containing compounds. This behavior is often observed and can be attributed to very strong hydrogen bonding between solute phenol and carbonyl of the amide phase on top of general hydrophobic attraction between sample and phase.

Additionally, there was an elution order switch between impurity A and B on HALO® C18 compared to HALO® Phenyl-Hexyl. These compounds differ by only a methyl group and the position of the hydroxyl group on the phenyl ring so it is reasonable that C18 would retain impurity B more than Phenyl-Hexyl. Although there were two coelutions on the HALO® RP-Amide and HALO® Phenyl-Hexyl phases, this should not be considered discouraging since other samples may benefit from the selectivity of these phases. Also, the unresolved compounds may become separated in another mobile phase. Screening multiple bonded phases from trusted suppliers is highly recommended because resolution order cannot be predicted for compounds of similar chemical structure.



TEST CONDITIONS:

Column: HALO 90 Å C18, 2.7 µm, 2.1 x 100 mm
Guard Column: HALO 90 Å C18, 2.7 µm, 2.1 x 5 mm
Mobile Phase A: pH 7 Phosphate Buffer (1.7 g potassium dihydrogen phosphate and 1.8 g dipotassium hydrogen in 1000 mL)
Mobile Phase B: Methanol

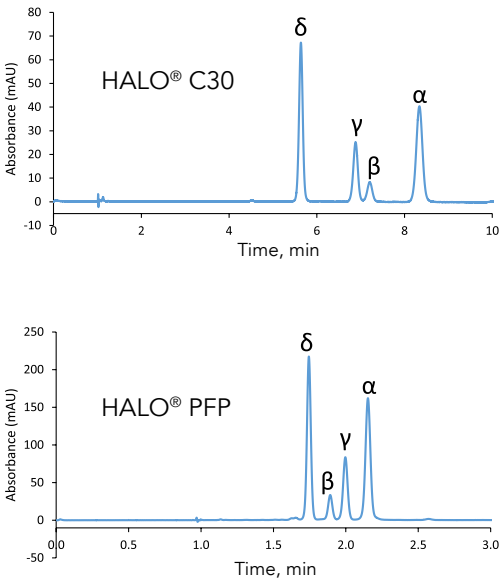
Gradient:

Time	% B
0.0	5
1.0	5
10.0	10
20.0	10
40.0	34
50.0	34

Flow Rate: 0.3 mL/min
Initial Pressure: 246 bar
Temperature: 30 °C
Detection: 254 nm, PDA
Injection Volume: 1 µL

Figure 7. Paracetamol and associated impurities screened on HALO® C18, HALO® RP-Amide, and HALO® Phenyl-Hexyl.

Another example of the benefit in screening different bonded phases is shown in Figure 8, where HALO® C18, HALO® C30, and HALO® PFP were used to screen the same sample of four tocopherols. HALO® C30 and HALO® PFP exhibit shape selectivity properties, which is important for isomer separations as in this separation below. While retention in the same mobile phase was highest with HALO® C18, there was no resolution between the beta and gamma-tocopherols under these mobile phase conditions.



PEAK IDENTITIES (IN ORDER):

δ-tocopherol, γ-tocopherol,
β-tocopherol, α-tocopherol
(4 different congeners of vitamin E)

TEST CONDITIONS:

Columns: HALO 160 Å C30, 2.7 µm
HALO 90 Å C18, 2.7 µm
HALO 90 Å PFP, 2.7 µm
All 4.6 x 150 mm
Mobile Phase A: Water
Mobile Phase B: Methanol
Isocratic: 95% B
Wavelength: 290 nm
Injection Volume: 1.5 µl
Temperature: 10 °C
Flow Rate: 1.5 mL/min

Figure 8. Tocopherol separation screened on HALO® C30, HALO® C18, and HALO® PFP.

For this particular sample and mobile phase, shape selectivity that probably is related to the aromatic structure had more impact than hydrophobicity. The highest resolution and least retention was observed with the HALO® PFP phase followed by the HALO® C30 phase. An elution order reversal was also observed between the HALO® PFP and the HALO® C30 phases.

3. pH

pH manipulation is another parameter to vary to evaluate the effects on the separation. Weak acids (HA) and bases (B) are partially dissociated in water. They exist in an equilibrium between their neutral and charged/ionized states as shown in the following equations:

$HA \leftrightarrow H^+ + A^-$

$B + H^+ \leftrightarrow BH^+$

More retained Less retained

With increased pH, retention for an acid decreases under reversed-phase conditions while retention for a base increases. Depending upon the nature of the analytes, changing the pH of the mobile phase can have a large impact on the selectivity of the separation. The pK_a of 3-nitrobenzoic acid is 3.46. At pH 7 (Figure 9, top chromatogram), 3-nitrobenzoic acid is in its anionic/charged form which explains its lower retention compared to when the compound is neutral at pH 2 (Figure 9, bottom chromatogram). The retention of fenuron is unaffected whether the pH is 2 or 7. One must be careful to use a pH that is 2 pH units away from the pK_a of the analytes in the separation. Peaks might become asymmetrical at pH too near pK_a if the acid or base form tails. In addition, pH must be controlled tightly to prevent peaks from too much retention time variation. In general, starting with low pH conditions is preferred because low pH suppresses ionization of surface silanols so the stationary phase selectivity can be fully utilized.

PEAK IDENTITIES (IN ORDER):
uracil, 3-nitrobenzoic acid, fenuron (top chromatogram)

TEST CONDITIONS:
Column: HALO 90 Å C8, 2 µm 2.1 x 50 mm
Mobile Phase A: 20mM Potassium Phosphate buffer, pH 2 and 7
Mobile Phase B: Acetonitrile
Gradient: 0-50% B in 5.5 min
Wavelength: 254 nm
Injection Volume: 1 µL
Temperature: 30 °C
Flow Rate: 0.3 mL/min

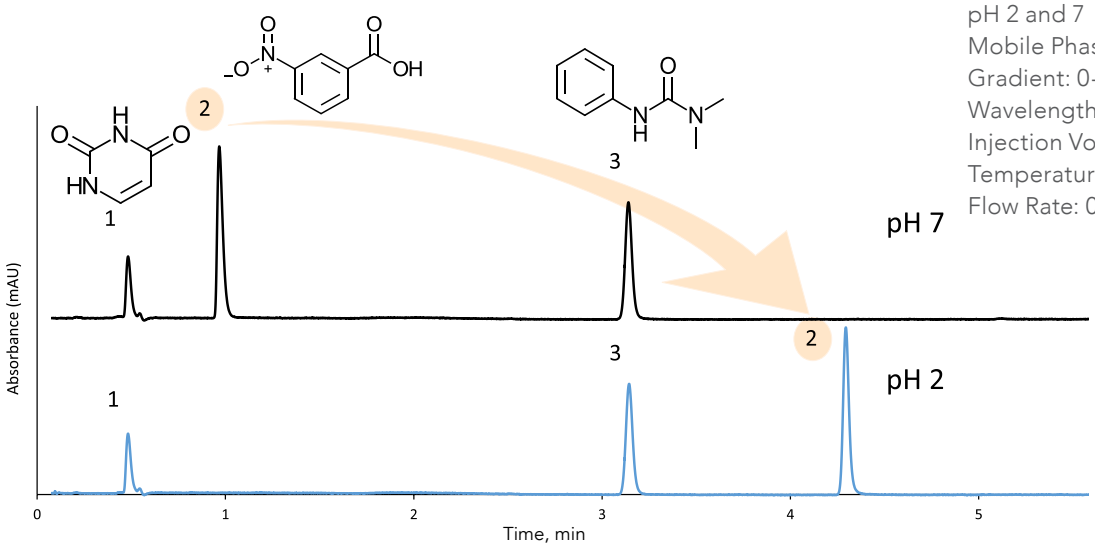
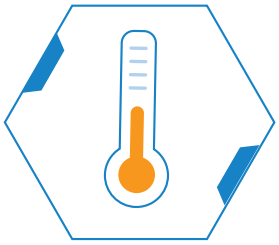


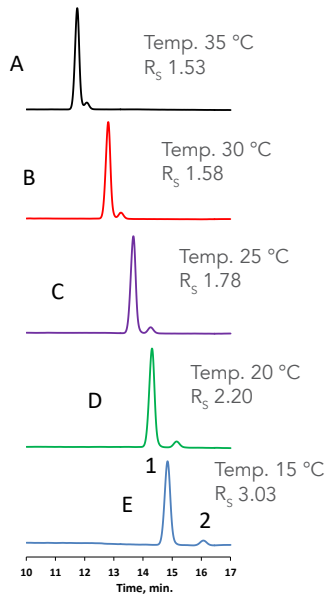
Figure 9. Effect of pH on retention time.



4. TEMPERATURE

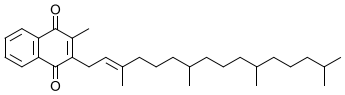
As sample matrix and component properties are impacted with temperature, it is another way to optimize selectivity, but it has less impact on selectivity compared to mobile phase, column type, and pH. Temperature is generally optimized with the mobile phase percent or gradient steepness using commercially available method optimization software [4]. Macromolecular samples (both biological and industrial polymers) often show better results at elevated temperatures (60 °C and higher) as long as compounds are thermally stable. This is due to the fact that zone broadening is reduced with higher temperature.

In contrast, isomer separations of vitamins can benefit from lower temperatures as shown in Figure 10. This improved selectivity may be caused by samples and stationary phases becoming more rigid at lower temperature, allowing an increase in shape selective interactions.



PEAK IDENTITIES:

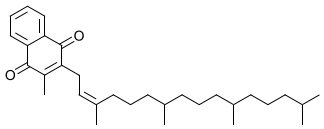
1. 2,3-trans-phylloquinone (K1)
2. cis-phylloquinone (K1)



Vitamin K1: 2,3-*trans*-phylloquinone

TEST CONDITIONS:

Column: HALO 160 Å C30, 2.7 µm, 4.6 x 150 mm
Mobile Phase A: Water
Mobile Phase B: Methanol
Isocratic: 95% B
Flow Rate: 1.5 mL/min
Pressure: 341 bar
Detection: 280 nm, PDA
Injection Volume: 1.0 µL
Sample Solvent: Methanol



Vitamin K1: *cis*-phylloquinone

Figure 10. Effect of temperature on resolution using HALO® C30.

Particularly with HALO® C30, running sub-ambient conditions has been shown to increase resolution and retention. The lower the temperature, the better the resolution as is shown in this vitamin separation. The trans and cis isomers of vitamin K1 are resolved the best at 15 °C on the HALO® C30 column compared to the resolution of the separation at 35 °C.

Conclusion

As has been described, there are several parameters, which can be investigated individually or in combination, that can be used to maximize selectivity and optimize resolution. Beginning by screening both acetonitrile and methanol mobile phases with one column or a set of orthogonal columns is a good option for method development. Selection of an appropriate pH for the analytes of interest is critical for ionizable compounds. Once the stationary phase, organic solvent, and pH, are selected, vary temperature and gradient time. Then use of a commercially available method optimization software package is recommended. While C18 is a good all-purpose phase for initial method development, the other phases offer alternate selectivities. It is good practice to screen multiple phases to ensure the best possible separation.

References

[1] Introduction to Modern Liquid Chromatography, 3rd Edition, L. R. Snyder, J. J. Kirkland, J. W. Dolan; p. 29, 2010, John Wiley & Sons, inc.

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[3] The hydrophobic-subtraction model of reversed-phase column selectivity, L.R. Snyder, J.W. Dolan,P.W. Carr, Journal of Chromatography A, 1060 (2004) 77–116.

[4] Temperature selectivity in reversed-phase high performance liquid chromatography, J.W. Dolan, Journal of Chromatography A, 965 (2002) 195–205.