LAB NOTES
An Overview of Systematic RPLC Method Development Using Multiple Organic Modifiers and Different pH Buffers

An efficient and effective strategy for reversed-phase method development for HPLC and UHPLC usually utilizes several stationary phases with different selectivities (Table 1). Those stationary phases, ideally, should have different mechanisms for retaining and separating various types of analytes, such as hydrophobic interactions (common for all alkyl bonded phases), p–p interactions, dipole-dipole interactions, hydrogen bonding interactions, and steric interactions (shape selectivity). For each of the phase types we've listed some pertinent ACE column phases that fall into those categories. It's also wise to select columns from a manufacturer that has a proven history of producing and delivering high quality, reproducible (column-to-column and batch-to-batch) columns in a variety of geometries and particle sizes.

Table 1 Analyte-Phase Interactions for Common RPLC Stationary Phase Types

Phase Type/Description	Analyte-Phase Interactions	Most Selective for
C18, C8 **ACE SuperC18** **UltraCore SuperC18**	• Hydrophobic	• Diverse polar, moderately polar, and nonpolar acidic, basic, and neutral analytes
Phenyl **ACE C18-AR** **UltraCore SuperPhenylHexyl**	• Hydrophobic • p-p •Dipole-Dipole •Hydrogen-bonding	• Analytes with electron-withdrawing groups (e.g., halogens, nitro groups, ketones, esters, acids) •Analytes having aromatic rings, differing dipole moments, or protic groups
Polar-Embedded **ACE C18-Amide**	• Hydrophobic • Hydrogen-bonding	• Proton donors (e.g., alcohols, phenols, polar acids) •Proton acceptors (e.g., amines, anilines, ketones)
Cyano **ACE CN-ES**	• Hydrophobic •Dipole-Dipole	• Analytes with double or triple bonds, or with polarizable functional groups (acids, amines, alcohols, esters, carbonyl compounds, ethers, organic halides, aromatics, alkenes, alkynes) •Works well for normal-phase separations
PFP **ACE C18-PFP**	• Hydrophobic • p-p •Dipole-Dipole •Hydrogen-bonding •Steric (Shape selective)	• Analytes with electron-donating groups (e.g., phenols, alcohols, aromatic ethers, amines) •Analytes having aromatic rings, different dipole moments, or protic groups •Structural isomers

Example Using 2 Organic Modifiers and Aqueous Buffers Having 3 pHs

In this example (Scenario A of Figure 1), we show how a single, versatile phase with a broad useable pH range, ACE SuperC18, can be used in screening experiments for RPLC method development. In these experiments, we compared the separations of a 15-analyte mixture of acids, bases, and neutrals under various conditions. Notably, the ACE SuperC18 phase is not subject to memory effects (hysteresis) from previous conditions at low or high pH or with different modifiers, when cycling between various conditions. This benefit makes it ideal for method scouting and development.
In this LabNote we present an example in which one of these phases, the ACE Excel SuperC18, is used in a one-factor-at-a-time (OFAT) screening approach (with two different organic modifiers and several pHs) to identify conditions to use for a sample of fifteen acids, bases, and neutral analytes.

Method Development Strategies

Systematic method development can vary from simple to quite complex (Figure 1), depending on the nature of the sample. Some methods can be developed quickly by carrying out several gradient separations with different gradient slopes at only a single temperature, with a preferred organic modifier at a single pH (Scenario A). More complex projects such as related substances methods or multi-analyte environmental methods may require a comprehensive approach (Scenario B and scenarios in Figure 1). Other advantages of using gradient mode for method development are: (1) it allows you to quickly assess the complexity of your sample; (2) it ensures that you won't "miss" any analytes that may be present; and (3) it allows cleaning of the column with each run so that late-eluting components do not affect subsequent runs.

When evaluating different combinations of stationary phase, organic modifier, pH, and temperature, it is often important to be able to track peak identities among those various conditions from one run to another. This is especially important when you want to use those runs as inputs into a computer simulation and optimization program such as DryLab®. Peak tracking is most easily accomplished when you have access to LC-MS detection, but can also be accomplished using LC-UV spectra from diode-array detectors or using peak areas, if they're sufficiently dissimilar. However, you must be careful when changing pH dramatically as the UV spectra and peak areas can vary dramatically for many analytes, especially for large pH changes.

Figure 1 Examples of Different Strategies of Varying Complexity for RPLC Method Development

High Resolution and Peak Capacity

Conditions:

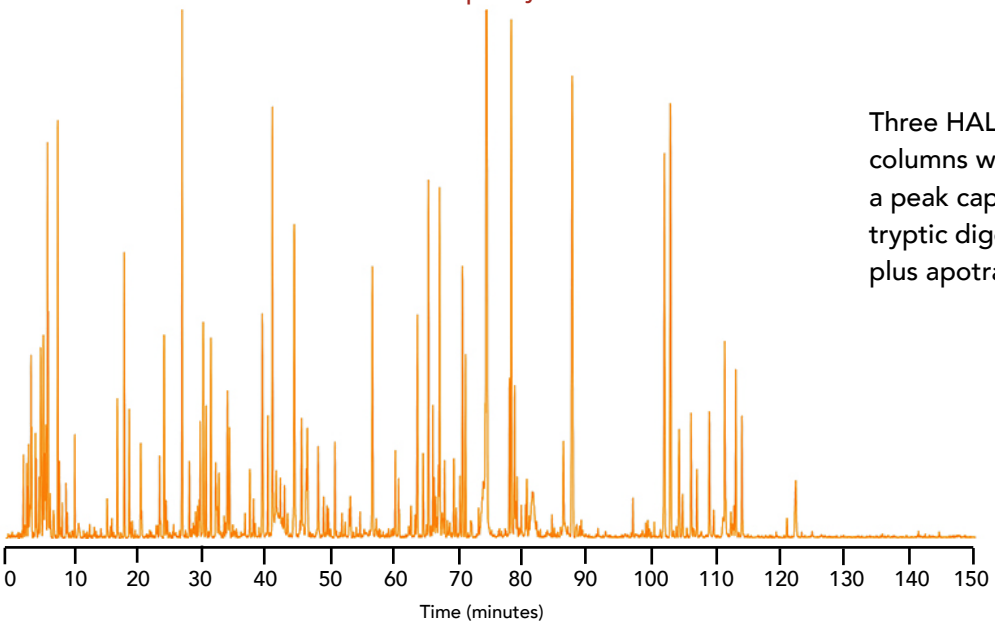
Column Dimensions: 2.1 x 450mm
(Three HALO Peptide ES-C18 columns connected in series)
Mobile Phase: A = Water with 0.1% formic acid/20 mM ammonium formate, pH 3.6
B = 20% A, 80% Acetonitrile

Gradient: 5 to 55% B, 150 min.
Flow Rate: 0.5 ml/min.
Temperature: 70 °C

Sample:

Tryptic digest of a-1 glycoprotein and apotransferrin

Peak capacity: 560



Three HALO Peptide ES-C18 2.1 x 150mm columns were connected in series to achieve a peak capacity of 560 for this mixture of tryptic digests of a-1-glycoprotein plus apotransferrin.

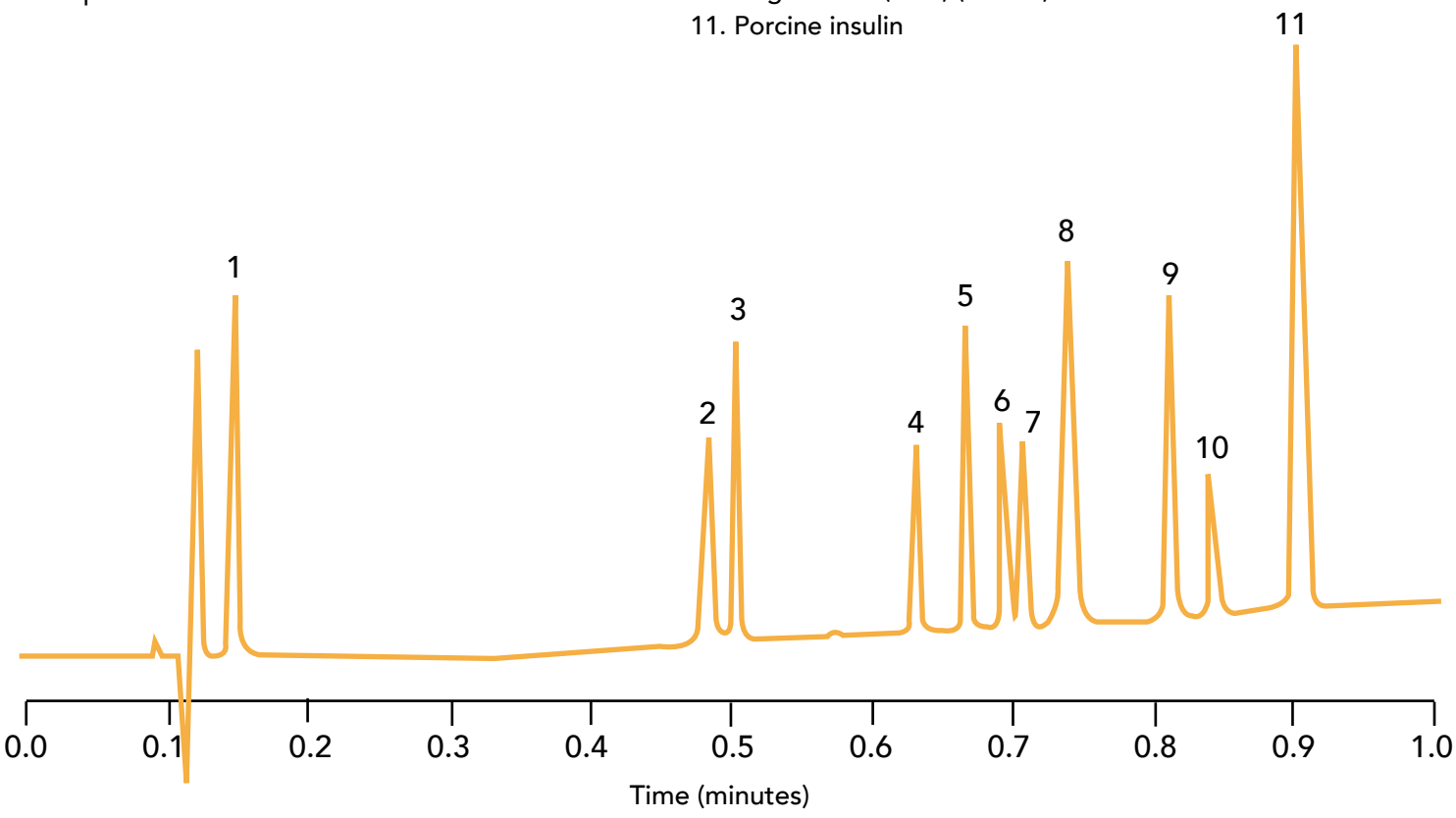
A short efficient column works well for this type of screening gradient or method development approach. For this example, a 2.1 x 50 mm ACE Excel SuperC18 2 mm UHPLC column was used at 0.5 mL/min at 30°C with short 8-minute gradient times from 5 to 80% organic using both acetonitrile and methanol as organic modifiers, with pH 2.75, 4.75 and 10.5 aqueous components (Figure 2).

Figure 2 Gradient Separations Using ACE SuperC18 with 2 Organic Modifiers and 3 pHs

Fast Separation of Peptides and Small Proteins

Conditions:
Column: HALO Peptide ES-C18, 4.6 x 50 mm
Mobile Phase: A = 90% Water with 0.1% TFA and 10% Acetonitrile, B = 70% Acetonitrile and 30% Water with 0.1% TFA
Gradient: 0 to 87.5% B, 1 minute
Flow Rate: 5.0 mL/min
Temperature: 60 °C

Sample:
1. Gly-Tyr
2. Val-Tyr-Val
3. Angiotensin 1 and 2 (1-7) amide
4. Met-enk
5. Angiotensin 1 and 2 (1-8) amide
6. Angiotensin II
7. Leu-enk
8. Ribonuclease A
9. Angiotensin (1-12) (human)
10. Angiotensin (1-12) (mouse)
11. Porcine insulin



Eleven peptides and small proteins are well separated in less than 60 seconds on a HALO Peptide ES-C18 column.

Fast Separation of Proteins

Conditions:

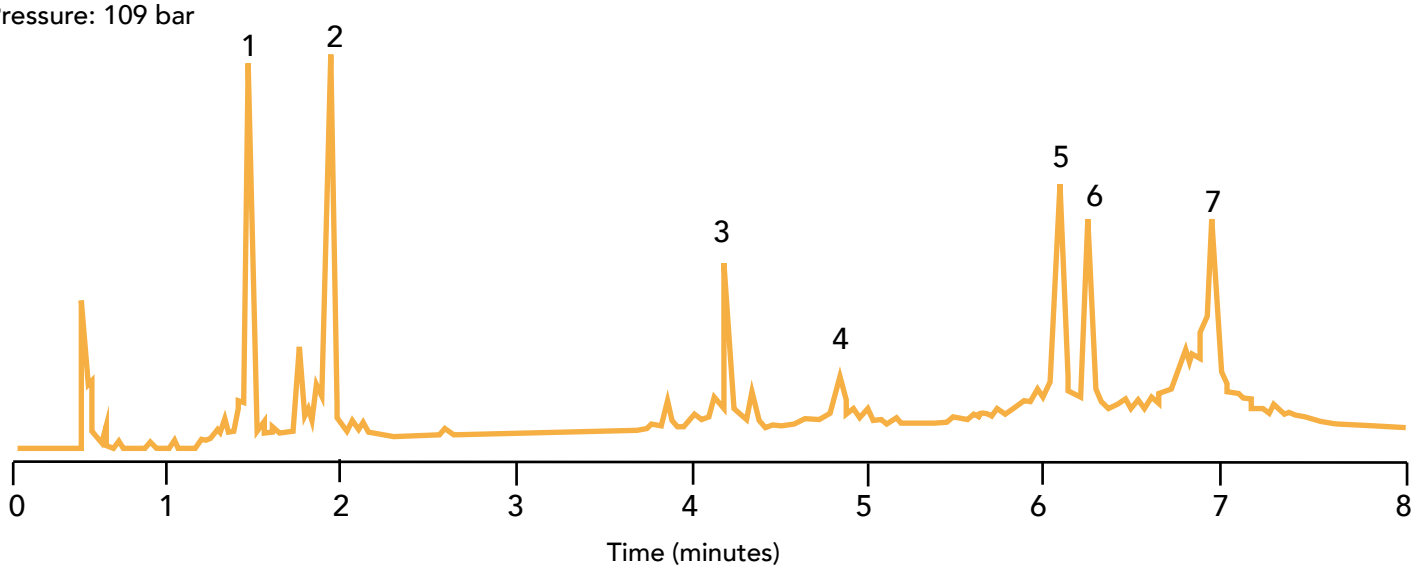
Column: HALO Protein C4, 2.1 x 100 mm
Mobile Phase: A = Water with 0.1% TFA,
B = 80% Acetonitrile and
20% Water with 0.1% TFA
Gradient:

Time	0 min.	2 min.	8 min.
%B	35	47.5	60

Flow Rate: 0.5 mL/min
Temperature: 60 °C
Pressure: 109 bar

Sample:

1. Cytochrome c (12.4 kDa)
2. Lysozome (14.3 kDa)
3. a-Chymotrypsin (25 kDa)
4. Catalase (250 kDa)
5. Carbonic anhydrase (29 kDa)
6. Enolase (46.7 kDa)
7. β-Amylase (200 kDa)



Seven proteins are separated in less than 8 minutes with high resolution on a HALO Protein C4 column. The backpressure is a low 109 bar.

Note: Unfortunately, chromatographic runs using methanol at pH 10.5 could not be carried out due to an instrument problem at the end of a limited method development window. It is expected that those results would also have been useful in selecting conditions for further development and optimization.

By comparing separations carried out using such an experimental design, one can quickly decide which combination(s) of organic modifier and pH are most promising for optimization and finalization of a method, based on the number of peaks detected and peak shapes of the various analytes. Based on the chromatograms shown in Figure 2, the separations obtained with methanol at both pH 2.75 and 4.75, with 14 out of 15 detected peaks, showed the most promise for additional work. The overall separation and peak shapes were also quite good for the pH 10.5 conditions with acetonitrile, although there were several co-elutions and little retention for one of the acidic analytes.

In summary, a systematic method development strategy can be a very productive approach for surveying various parameters that affect selectivity, resolution, and peak shape for RPLC. The ACE SuperC18 phase, with its superior inertness and stability over a broad pH range (1.5 to 11.5), is an excellent column choice, combined with the ability to study the effects of different organic modifiers, modifier blends, and pH.

Learn more about the **Avantor ACE Excel SuperC18** and **Avantor ACE UltraCore SuperC18**