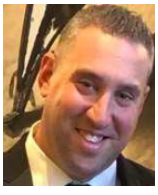
An Executive Summary

# UHPLC/HPLC Method Development for Pharmaceutical-Related Substance



**Geoff Faden**
*National Sales Manager*
MAC-MOD Analytical, Inc.

## How to translate methods, maximize resolution, and comply with *USP* <621>

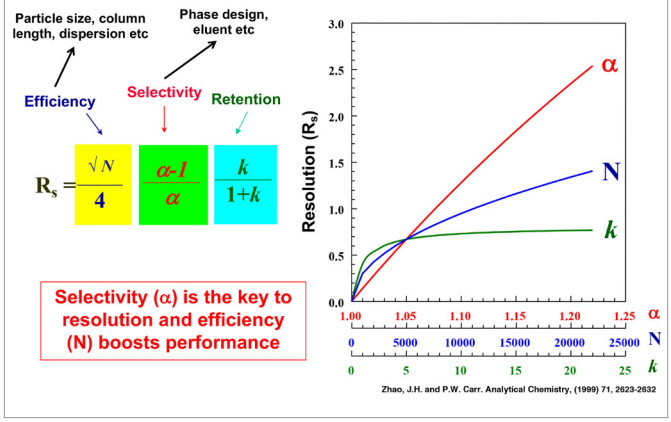
### Introduction

The *United States Pharmacopeia* general chapter on chromatography (*USP* <621>) has incorporated a significant degree of flexibility for the conversion of older, traditional isocratic pharmacopeia methods to newer technologies. Systematic method development workflows using a stationary phase-based screening approach can be used to maximize resolution and speed for isocratic and gradient methods. Solid-core particles can be used to achieve ultra-fast methods even on standard high performance liquid chromatography (HPLC) systems. In addition, concatenating solid-core columns yield greater peak capacity and resolution for complex samples, such as various natural products, without exceeding pressure limits.

### Selectivity Is the Key to Resolution

Selectivity is the ability of the chromatographic system to retain and separate sample components of interest. It determines the order of elution of sample components as well as the spacing between the two adjacent peaks. The selectivity factor (α) is calculated as the ratio of retention factors (*k*) between two adjacent peaks, $\alpha = k1/k2$, and is the key factor in optimizing resolution (*R*), as shown in **Figure 1**.

**Figure 1:** Resolution, selectivity, efficiency and retention.




The powerful parameter that affects selectivity in isocratic separations is the column stationary phase type. Other key factors include pH; organic modifier type and percentage; buffer type and concentration; and temperature. For gradient separations, the additional parameters of gradient slope, dwell volume, and column dimensions must

SPONSORED BY

be considered. The more factors that are explored, the more complex the method development becomes. Fortunately, the top three factors during method development are stationary phase, pH, and organic modifier.

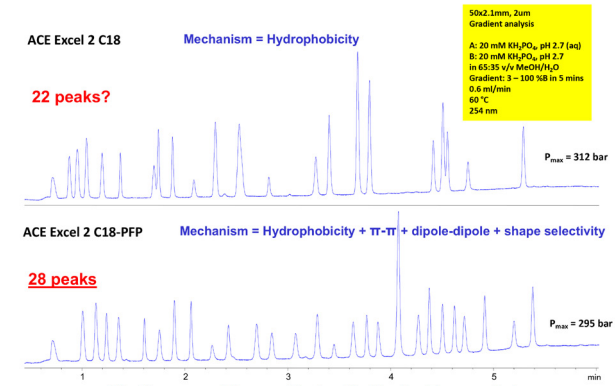
> The more factors that are explored, the more complex the method development becomes.

The specific choice of which stationary phase is selected is a powerful parameter for selectivity. For example, the selectivity of the bonded phase types, which are based on alkyl chains, such as SuperC18, C18 and C8 phases, is dominated by hydrophobic interactions between the analytes and the stationary phase. Aromatic stationary phases, which contain an aromatic delocalized ring system, such as a phenyl, alkyl phenyls, and even pentafluorophenyl types, are dominated by π-π interactions. The pentafluorophenyl group will yield some dipole-dipole interactions as well as shape selectivity interactions. Polar-embedded phases, such as C18-Amide or Cyano, yield yet another slightly different modes of interaction. These types of phases are all useful in method development because they provide differing mechanisms of interaction that affect selectivity.

To illustrate how stationary phase selection can help maximize resolution via selectivity, a group of analytical scientists used a general scouting gradient with a standard C18 column to analyze a degraded sample with an unknown number of peaks. In general, the method had good peak capacity, but some peaks were not well resolved. Keeping all other conditions the same, the team switched columns from the primarily hydrophobic interaction of the C18 to a column that delivers multiple modes of interaction like the C18-PFP column. The C18-PFP is composed of a delocalized ring system that also contains hydrophobic interactions, a moderate dipole moment interaction due to the fluorines that are on the aromatic ring, and it also provides a degree of ape selectivity. Thus, the analysts could use a column that delivers multiple modes of interaction with this column compared to the standard C18. The change in selectivity yielded more peaks and better resolution (see **Figure 2**).

One can chart the retention times of the peaks between the two columns and track peak movement to gain

**Figure 2:** Degraded sample with unknown number of peaks.




information on the functional groups and composition of the analytes. Furthermore, the linear regression of that chart can be plugged into the Neue Selectivity equation: $S$=100x√(1–$R$^2). Selectivity values from approximately eight and greater indicate suitable differences between the two columns for a class of analytes.

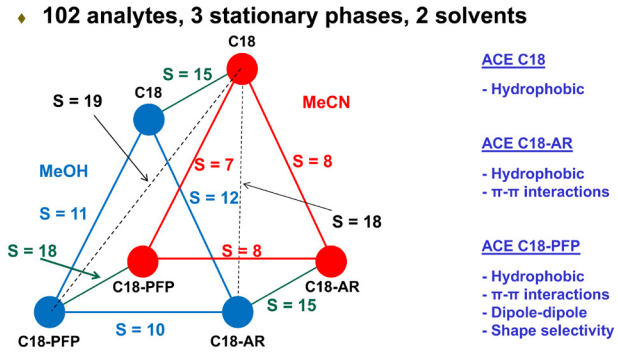
## Optimizing HPLC/UHPLC Method Development Workflows

In addition to stationary phase, organic solvent selection can have a strong effect on elution order and selectivity. The two most common organic modifiers are acetonitrile and methanol. Acetonitrile, which is an aprotic solvent, has advantages such as lower viscosity, which keeps pressure low. Methanol, which is protic solvent, is known as a useful solvent for mass spectrometry because it can help with ionization of analytes, as well as provide additional benefits that impact selectivity.

> In addition to stationary phase, organic solvent selection can have a strong effect on elution order and selectivity.

Using a pragmatic method development platform, one can explore selectivity using three stationary phases and two solvents, chart the retention times, and determine the selectivity differences. In this example, analysts selected ACE C18, ACE C18-AR, and ACE C18-PFP because

**Figure 3:** Three column–two solvent workflow.




they have different mechanisms of interaction that yield different selectivity values. The selectivity values from the three column–two solvent workflow for a complex sample are summarized in **Figure 3**.

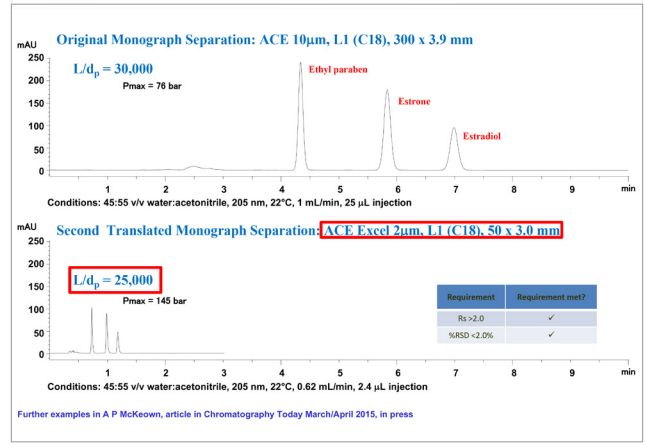
The starting conditions for the method development workflow are based on the pH required for reversed-phase retention of the specific analyte(s), the column dimensions, particle size, and pressure limits of the system. Once the column and organic solvent are selected, one can use modeling software to rapidly optimize certain parameters such as temperature and gradient slope for method robustness. Last, once multiple lots of the column selected are evaluated, the method is ready to be validated and transferred for routine analysis.

## *USP* <621> Broadens the Criteria for Isocratic Method Translation

When teams are working with an older compendial method or are transferring a method to a laboratory with different instrumentation, they may need to refer to the guidance for compendial methods.

Fortunately, *USP* <6.21> (updated in August 2014) has broadened the criteria for isocratic method translation to provide more flexibility. Isocratic LC methods can be converted using the column length to particle size ratio ($L/d_p$). And in fact, one can even use -25% to +50% of the original $L/d_p$ ratio specified in the monograph. It is important to know and understand the capabilities and configuration of your instrument. Note, when moving to more modern columns, the analyst should be aware that extra column band broadening due to the extra column volume of the analysts system can cause dispersion effects on the separation, which may impact the maximum resolution that can be achieved. In some cases, using a larger inner diameter column, which has a larger column volume, can overcome those challenges. In addition,

**Figure 4:** Method translations: isocratic estradiol porous → porous (III)



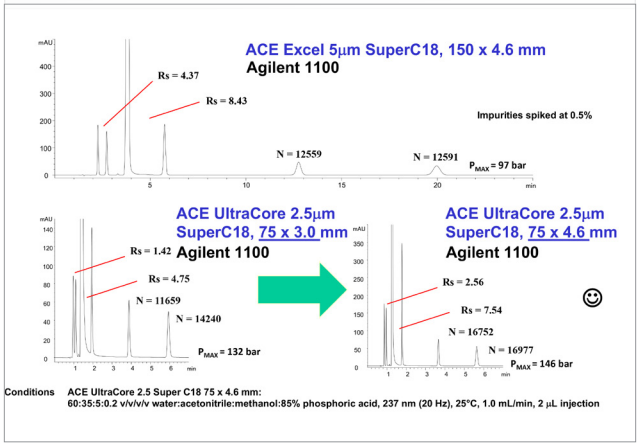

similar columns from different vendors may perform very differently due to variations in platform silicas and other variables caused by differing manufacturing protocols. One must use scalable bonded phases that maintain the same selectivity characteristics across the range of column formats. Similar selectivity and resolution should be achieved by keeping the same $L/d_p$ ratio when methods are translated.

Once a new column or set of columns has been determined, one can simply geometrically scale the flow rate and injection volume to create the improved method. With the new guidance, one can take that a step further and use a smaller $L/d_p$ ratio to choose a new column to even further improve the method, as shown in **Figure 4**.

> One must use scalable bonded phases that maintain the same selectivity characteristics across the range of column formats.

Figure 4 shows that a resolution value of greater than 2, between estrone and estradiol and a %RSD of less than 2, was achieved with the new column, in compliance with the *USP* system suitability requirements. Run time was reduced to less than three minutes.

**Figure 5:** Isocratic method translations: aspirin porous → solid core (IV).



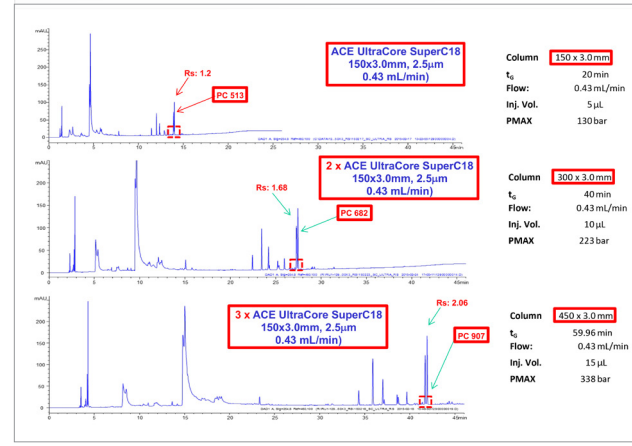

## Translate Isocratic and Gradient Methods Effectively Between Totally Porous and Solid-Core Particle Columns

Laboratories can also take advantage of the reduced hydrophobicity of solid-core particles to get a faster separation. The use of solid-core particles can enable ultra-fast separations on standard HPLC systems. An example of this, is an isocratic method for aspirin, where analysts adjusted for the extra column band broadening of the system (see **Figure 5**). In addition, the method can be shortened even further when using solid-core particles by increasing the flow rate without excessively increasing the back pressure. This is a clear advantage of using solid-core particles over porous particles.

> Laboratories can also take advantage of the reduced hydrophobicity of solid-core particles to get a faster separation.

With gradient methods, one must calculate column volumes and translate gradient times in addition to translating the flow rates and injection volumes. The time of injection relative to the time of the start of the gradient ramp may also need adjustment for the most accurate translations because of changes to the system dwell volume to column volume ratio.

**Figure 5:** High resolution: echinacea, constant flow/longer column.




## Natural Product Samples: When More Resolution and Peak Capacity is Needed

In addition to shorter faster methods, one may need methods with greater resolution. This is especially relevant for natural product samples, which can be very complex mixtures. Analysts can increase the column length or add another column to the separation to increase the overall bed length for greater resolution and peak capacity, without changing selectivity. Again, one can calculate column volume, translate the gradient time, scale the injection volume, and calculate whether the start of the injection time should be adjusted relative to the start of the gradient. In this case, the flow rate did not need adjustment. While adding extra column length will also increase the overall runtime, this can be an important tool for achieving extra resolution when it is necessary (see **Figure 6**).

## Conclusion

Selectivity is a principal concept in chromatography. Screening columns with differing retention mechanisms is a useful first step for method development. The chromatographer should focus on key variables to optimize workflows. One can maintain retention information while simultaneously reducing development time, using a three phase–two solvent optimized method development platform, based on selectivity.

To summarize, column coupling can provide improved sample detail and high peak capacities for complex samples. Relatively accurate method translations are achievable for isocratic method translations using the $L/d_p$ ratio and scalable bonded phases. Gradient translations are more complex, but the calculations are readily available.