Reverse Phase Flash Method Development Using Analytical LC Systems

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Chromatography Technical Note TN62

Abstract

The Focused Gradient Generator allows users to quickly create efficient preparative gradient methods using compatible reverse phase columns on their analytical HPLC systems. The resulting preparative flash gradients are 12 column volumes long, are focused for increased resolution around the peak of interest, and have a wash step at the end. A simple calibration step using Universal Test Mix allows calculation of focused gradients from analytical systems.

Being able to quickly determine a preparative method with increased resolution around the target peak enables methods that offer better separation between the target compound and other impurities. Such methods offer increased purity from preparative flash runs or the ability to increase sample loading for preparative runs. Performing more efficient flash chromatography helps decrease overall solvent usage and waste solvent generated.

The first step to calculate a focused gradient is to run a scouting gradient using a small amount of sample. The scouting run typically requires the same amount of time to run as a single thin layer chromatography (TLC) plate. In addition to providing a retention time to calculate a focused gradient, the scouting gradient answers the following questions:

- Will the compound elute with a particular column or solvent system?
- Does the method need a modifier such as trifluoroacetic acid or triethylamine to force the compound to a protonated or unprotonated state, prevent tailing, or improve peak shape?
- Can it be purified by flash? Is there enough resolution? Changing the column or solvent system may allow more resolution.

It also provides additional information:

- Estimated sample loading for a column and solvent system. Peaks separated by <~3% B solvent in a focused gradient or less will be subject to a light loading.

It is very common for chemists to develop flash methods on analytical HPLC for reverse phase because they are faster than using reverse phase TLC plates. Teledyne ISCO has Redi*Sep*® analytical HPLC columns containing media that match the flash column chemistry available in C18, C18AQ, and C8. These columns are 4.6x150 mm and 2x50 mm.

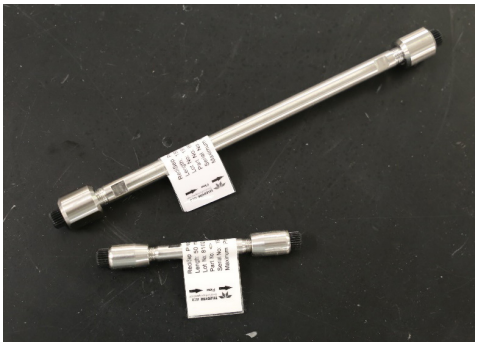


Figure 1. Teledyne ISCO Redi*Sep* analytical columns match Teledyne ISCO Redi*Sep* flash columns.

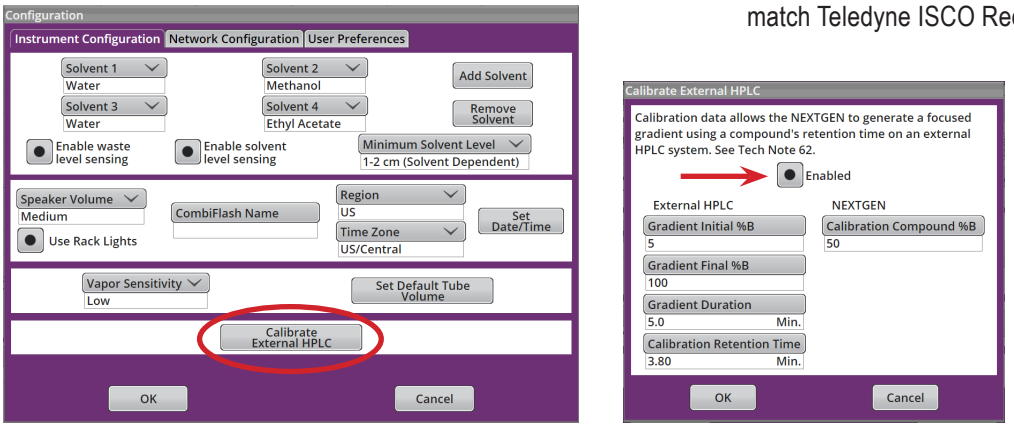


Figure 2. Instrument configuration and external HPLC calibration screen.

Analytical system calibration

A common scouting gradient for a C18 2x50 mm columns is from 5 to 100% B over 5 minutes with a 2-minute isocratic hold at 100% B at 0.5 mL/min, while 4.5x150 mm columns work well with a gradient from 5 to 100% B over 6 minutes with a 6-minute isocratic hold for 6 minutes at 100% B at 1.0 mL/min. Run Universal Test mix in your focused gradient. For analytical systems, one drop of test mix per 2 mL runs well with 1 to 10 µL injection.

To enable this feature, open the Configuration window under the Tools menu. Under the Instrument Configuration tab, select Calibrate External HPLC as shown in Figure 2. Enter the scouting gradient parameters into the Calibrate External HPLC window. Enter the retention for a Universal Test Mix peak as per Table 1. If same scouting gradient is used for other Redi*Sep* Prep columns, no recalibration is needed. Press the Enabled button; "HPLC Focus" will then be an additional method option when a column is selected on the Main screen. Scouting gradients do not require an isocratic hold at the start of the gradient.

	Solvent	
Column Type	**Methanol**–use first eluting peak retention time	**Acetonitrile**–use second eluting peak retention time
C18	50%	50%
C18AQ	50%	50%
C8	40 %	40%

Table 1. Values to enter for NextGen Calibration Compound %B for different columns and reverse phase solvents

As an aid for entering the values into the Combi*Flash* NextGen system, use enter your HPLC parameters into Table 2 below.

HPLC Parameter	**Value to be entered to NextGen**
Gradient starting %B:	
Gradient Ending %B:	
Gradient Duration:	
Retention time of calibration compound:	
NextGen Parameter	**Value to be entered to NextGen**
%B for the compound, solvent, and column listed in Table 1:	

Table 2. Worksheet for HPLC data for the "Calibrate External HPLC" window in PeakTrak.

Running analytical scouting gradients and calculating focused gradients

After calibration is complete, run the scouting run on the analytical system and note the retention time for the peak that needs to be purified.

Load a column on the Combi*Flash* NextGen system. From the Main screen, select the column and choose HPLC Focus. This opens the HPLC Focus window (Figure 3). Enter the retention time into the Retention Time control.

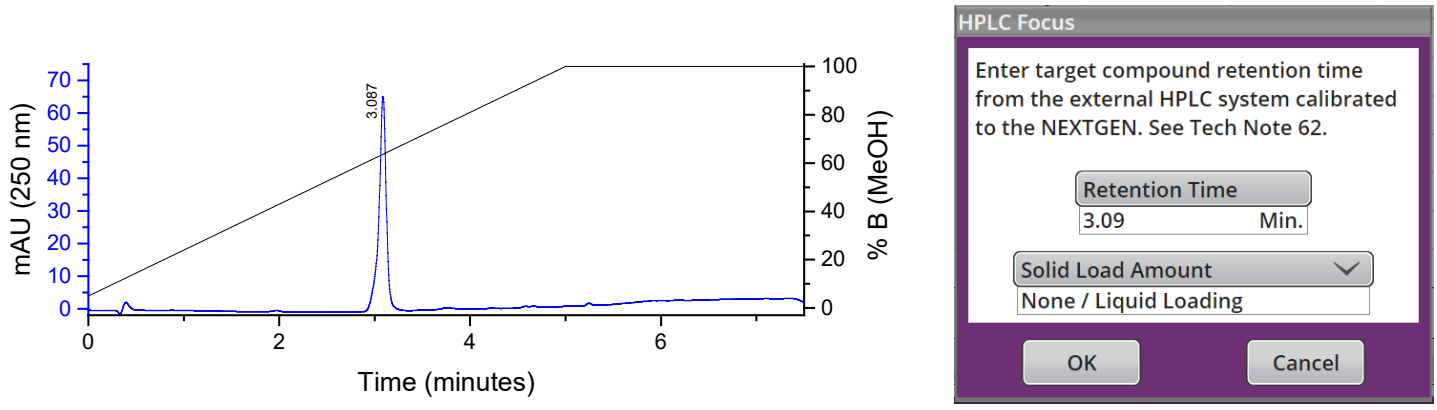


Figure 3. Analytical run retention time entered into the HPLC focus window.

Next, enter the loading type into the window—either liquid load or the amount of material in the solid load cartridge. Pressing "OK" generates a preparative gradient that you can run.

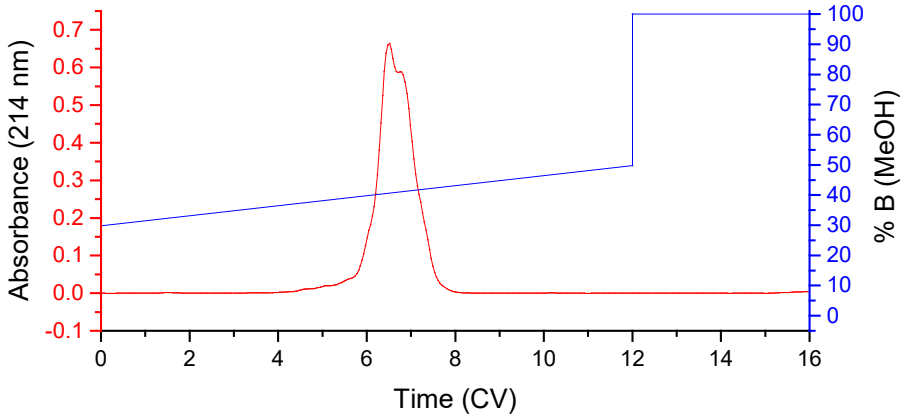


Figure 4. Focused gradient elution using the data in Figure 3. The calibration values are those used in Figure 2.

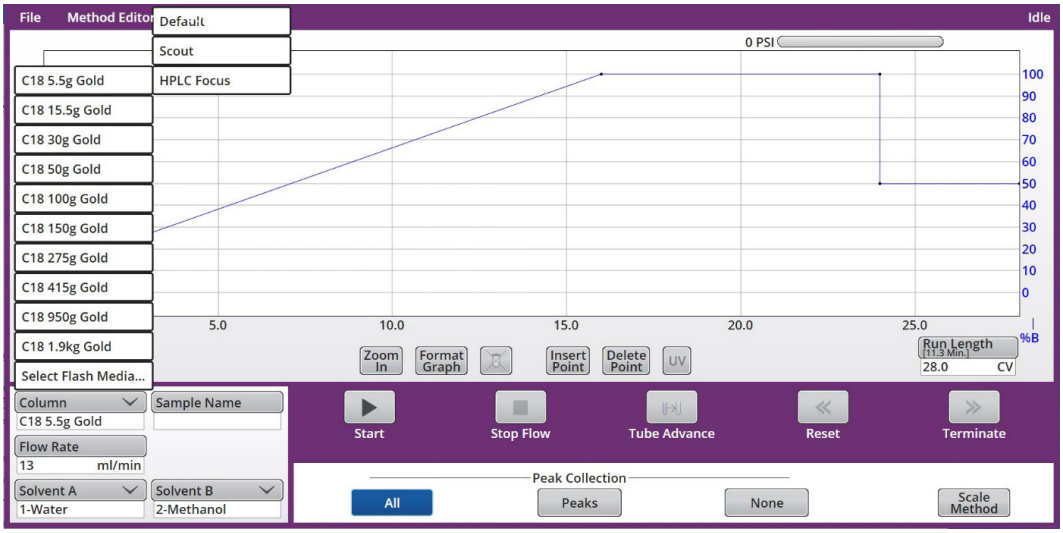


Figure 5. Column and method selection.

Always use the same mobile phases for scouting and flash gradients to ensure the correct retention time in the focused gradient run! A successful focused gradient elutes between ~2 and ~10 column volumes on the flash system.

Retention time and sample loading

Sample loading is primarily affected by resolution—greater resolution between peaks allows higher sample loading. We can see a difference in resolution on the scouting run when peaks are resolved from one another. Empirical data indicates that peaks eluting with a ~3% difference in B solvent in the scouting gradient have ~1 column volume difference in retention in a focused preparative gradient. HPLC equipment uses time as a measure of retention rather than solvent composition. To determine the difference in elution solvent composition for two peaks, use the following two equations:

Equation 1: $m = \frac{(B_e - B_s)}{T}$, where B_e is the ending solvent composition (usually 100%) and B_s is the scouting gradient initial composition (usually 5 or 10%). So long as the scouting run is unchanged, the calculated slope can be used as a constant in equation 2 below.

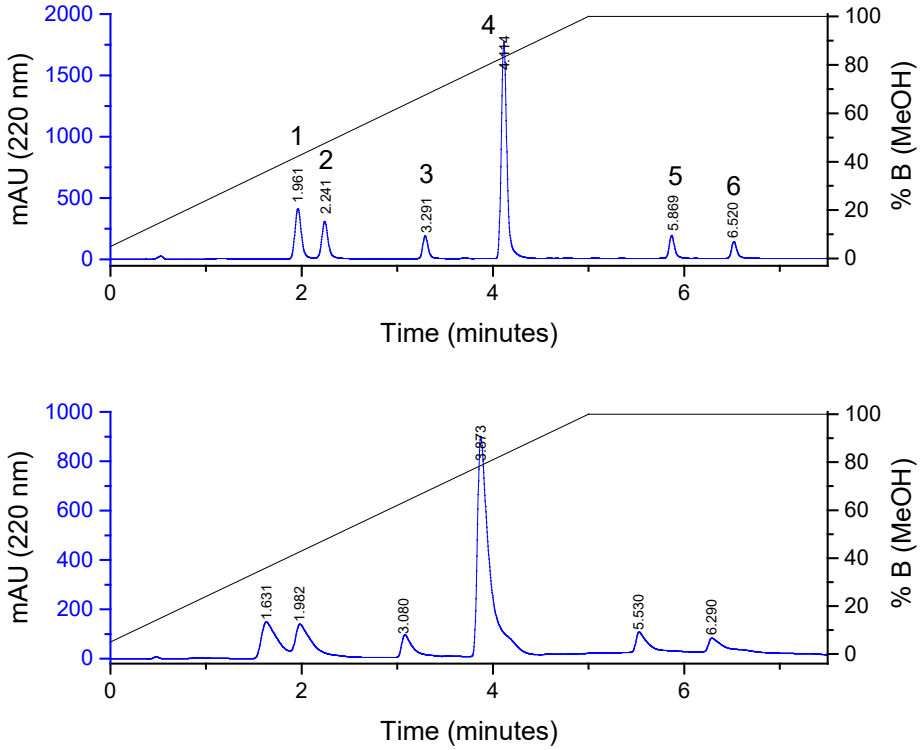
Equation 2: $\Delta\%B = (T_{peak\,2} - T_{peak\,1}) * m$, where T_{peak2} is the elution time for the second eluting peak, and T_{peak1} is the elution time for the first peak.

Δ%B	Sample Loading (% column mass, Reverse phase)
4	0.1
6	1
10	1.5

There are many factors that affect the amount of sample that can be loaded on a column. Loading conditions play a role; DMSO and DMF often work well in small volumes but cause poor peak shape in larger injection volumes. Larger sample masses often need buffers, as the concentration of the eluting compound may exceed that of 1% TFA.

Why use Teledyne ISCO matching analytical columns?

Teledyne ISCO Redi*Sep* Prep HPLC columns are made with packing that has the same chemistry as Redi*Sep* flash columns. This means they have the same selectivity, so different compounds elute the same way and retention time data from scouting gradients will accurately calculate focused gradients. In Figure 6, Teledyne ISCO Redi*Sep* Prep analytical HPLC columns (2x50 mm) were compared to other columns of the same size using the same gradient method and solvents. A mixture of six compounds were run. The calculated gradients for each compound and column are listed in Table 3. Although all columns gave similar results near the center of the gradient, the results diverged at the ends of the gradient. The results from columns B and C would suggest that compounds 1 and 2 couldn't be run in a focused gradient, although the Teledyne ISCO analytical column allows calculation of a gradient for these compounds that do run on Teledyne ISCO column flash columns.



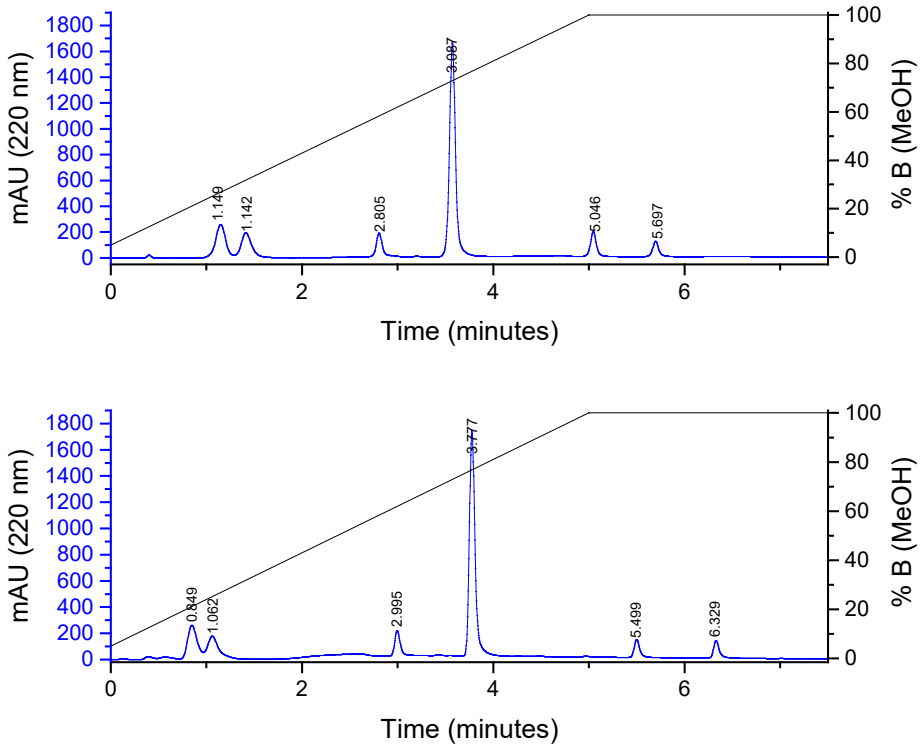


Figure 6. Scouting runs from 2x50 mm UHPLC columns from different manufacturers (in water/acetonitrile).

	1	2	3	4	5	6
Teledyne ISCO	1.3-21.3	6.6-26.6	26.5-46.5	42.1-62.1	75.5-95.5	87.9-100
Column A	N/A*	6.2-26.2	27.1-47.1	42.1-62.1	73.6-93.6	88.1-100
Column B	N/A*	1.1-21.1	27.7-47.7	42.1-62.1	70.2-90.2	82.6-100
Column C	N/A*	N/A*	27.3-47.3	42.1-62.1	74.8-94.8	90.6-100

* "N/A" means the compound eluted too early or too late to calculate a gradient

Table 3. Calculated gradients for six compounds.

Conclusion

The Flash Focused Gradient Generator allows rapid reverse phase development from analytical columns. Method development and transfer to the analytical system are done at the same time. The method development uses the same time as a silica TLC plate.

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