

Reversed-Phase Flash Method Development Using Analytical LC Systems

Abstract

The Focus Gradient Generator allows users to quickly create efficient preparative gradient methods using compatible reversed-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 tri-fluoroacetic 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 $< \sim 3\%$ 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 reversed-phase because they are faster than using reversed-phase TLC plates. Teledyne ISCO has RediSep® 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 RediSep analytical columns match Teledyne ISCO RediSep flash columns.

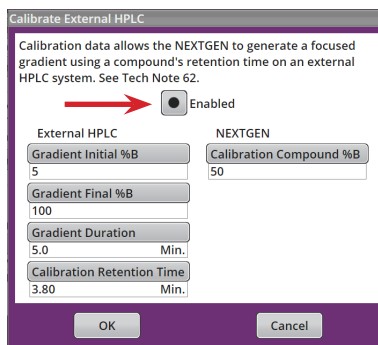
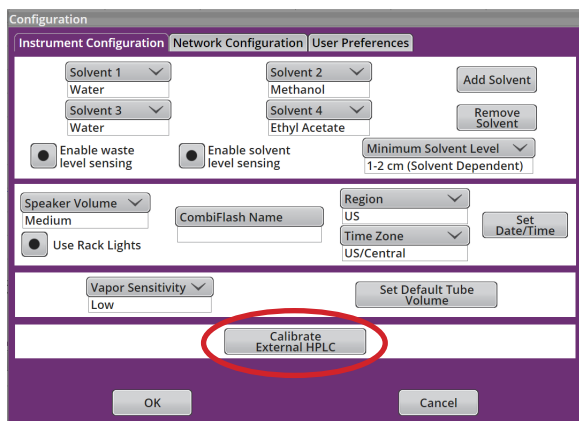


Figure 2. Instrument configuration and external HPLC calibration screen.

Analytical system calibration

A common scouting gradient for 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 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 μ L 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 the same scouting gradient is used for other RediSep 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 reversed-phase solvents

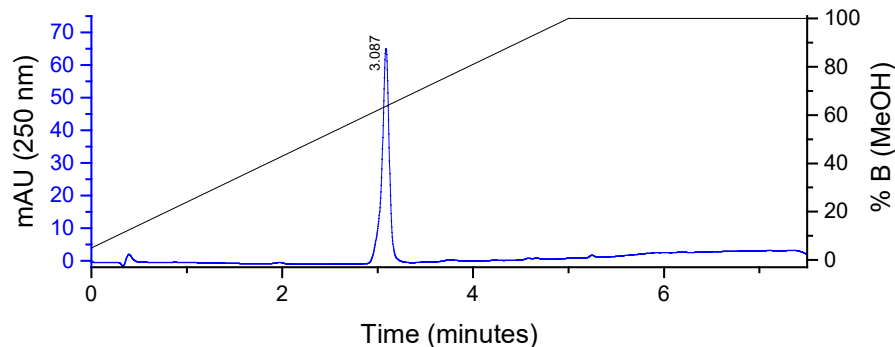


Figure 3. Analytical run retention time entered into the HPLC Focus window.

As an aid for entering the values into the CombiFlash NextGen system, enter your values for HPLC parameters into Table 2 below.

| HPLC Parameter | Value to be entered to NextGen |
|--|--------------------------------|
| GRADIENT INITIAL %B (starting): | |
| GRADIENT FINAL %B (ending): | |
| GRADIENT DURATION: | |
| CALIBRATION RETENTION TIME of compound: | |
| NextGen Parameter: CALIBRATION COMPOUND %B | 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 CombiFlash 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.

HPLC Focus

Enter target compound retention time from the external HPLC system calibrated to the NEXTGEN. See Tech Note 62.

Retention Time
3.09 Min.

Solid Load Amount ▼
None / Liquid Loading

OK Cancel

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.

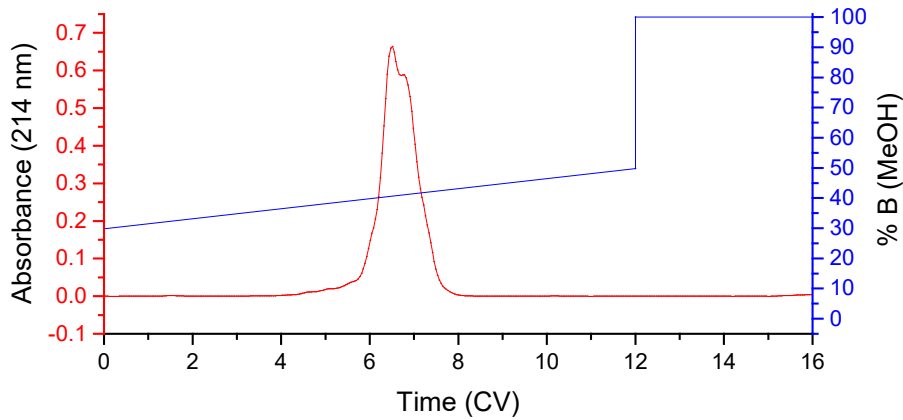


Figure 4. Focused gradient elution using the data in Figure 3. The calibration values are those used in Figure 2.

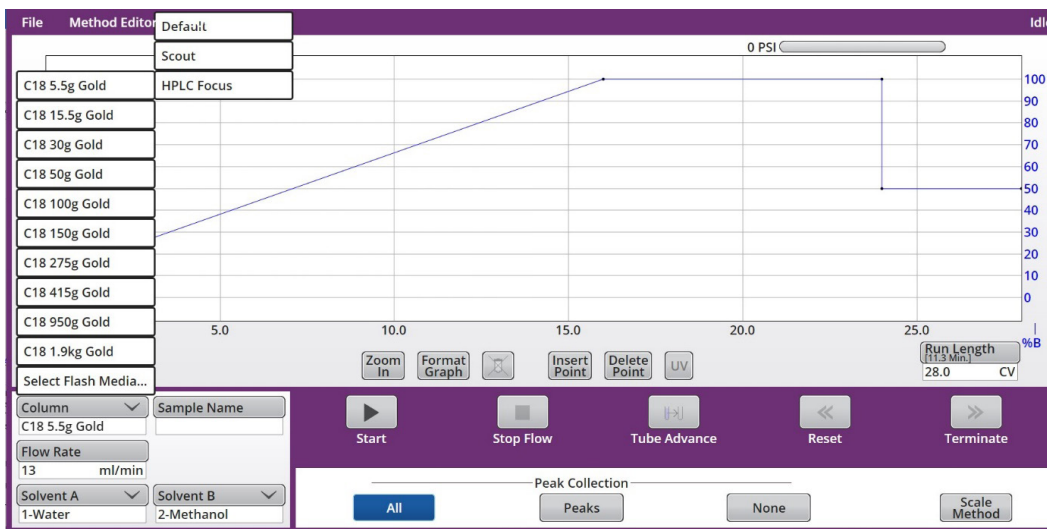


Figure 5. Column and method selection.

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

| $\Delta\%B$ | 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 RediSep Prep HPLC columns are made with packing that has the same chemistry as RediSep 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 RediSep 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 flash columns.

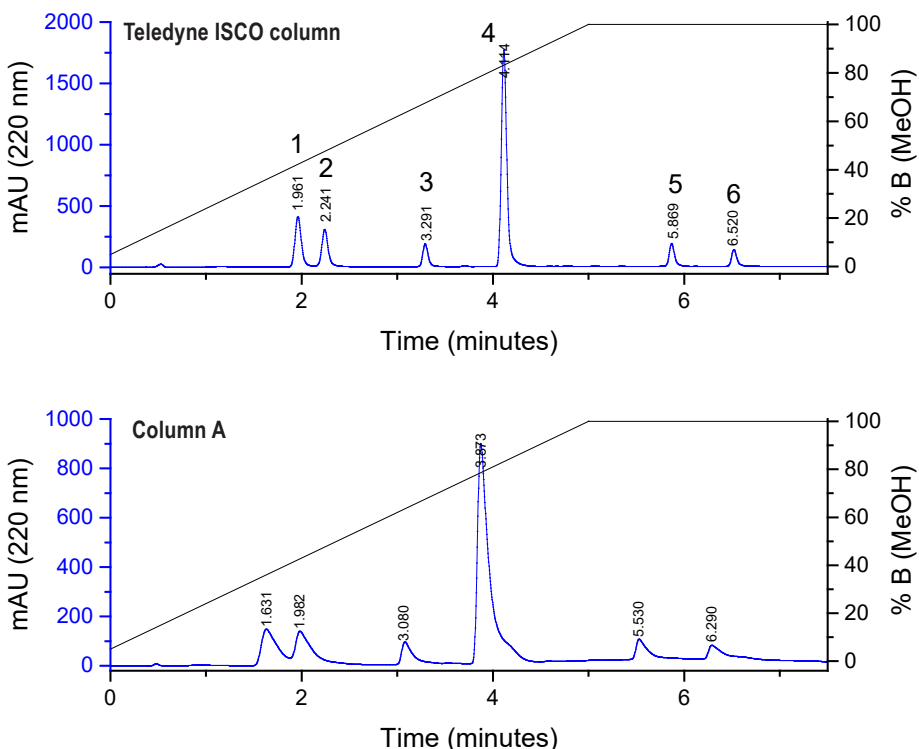


Figure 6. Scouting runs from 2x50 mm UHPLC columns from different manufacturers (in water/methanol).

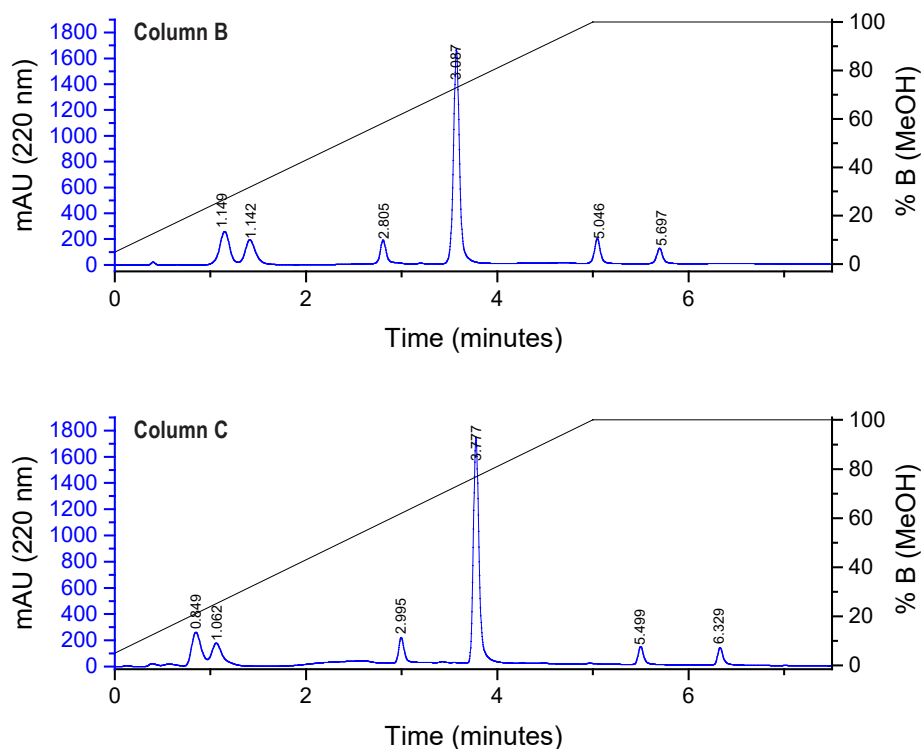


Figure 6 (continued). Scouting runs from 2x50 mm UHPLC columns from different manufacturers (in water/methanol).

| | 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 Focus Gradient Generator allows rapid reversed-phase development from analytical columns. Method development and transfer to the preparative system are done at the same time. The method development takes the same time as a silica TLC plate.

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