At Last: Easy Preparative Supercritical Fluid Chromatography Method Development

TELEDYNE ISCO
Everywhereyoulook™
teledyneisco.com

Abstract

A facile technique for developing preparative Supercritical Fluid Chromatography (SFC) methods using focused gradients is described. The method correlates the retention of a model compound in an analytical scouting gradient to the retention of the same compound in a preparative SFC run to determine an apparent gradient delay. Using the same scouting gradient, this delay is applied to calculate focused gradients for compounds to be purified. The method is based on the "Time-on-Target" algorithm that has been successfully applied to preparative liquid chromatography. The algorithm saves time and sample because a single scouting run allows calculation of a focused gradient with the desired retention time for the targeted compound. Once the initial calibration is complete, that same calibration can be used for other columns and solvent modifiers. This technique, when combined with analytical column and modifier screening, allows rapid method development.

Background

Preparative SFC is increasingly used to purify compounds in high-throughput synthesis labs. Efficient preparative SFC is fast, allowing many compounds to be purified within a short period of time. Short purification times also reduce solvent usage and waste generation. It is also of importance to purify compounds as thoroughly as possible as impurities may lead to false positive or negative results in biological assays. Preparative SFC only requires that the compound of interest be resolved, in contrast to analytical techniques that require resolution of all compounds for quantitation.

SFC uses a wide variety of columns and systems. Generally, a preparative SFC elution time of 3 minutes for the compound of interest is desirable within a 6 minute run. The gradient should be calculated from the analytical scouting run that was used to determine the quality of the synthesis. Given the wide variety of compounds and systems, it is desirable to find an optimization method that applies to as many compounds as possible and requires little further optimization.

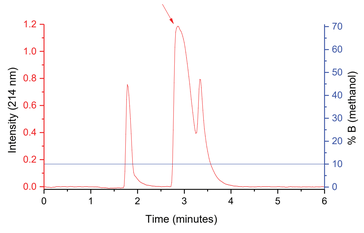
There are very few methods to create a purification method for SFC. One method, Compound Specific Method Optimization[1,2], assigns a focused gradient to various time ranges of a fast scouting gradient on an analytical system. The gradient from these "zones" is usable, but not optimized for any particular compound. It also takes time to create and validate the focused gradients for a particular preparative system such that the results of the analytical system properly transfer to the preparative unit. However, this technique was found to work for open access SFC systems[3]. Another approach is to develop the method on an analytical SFC system, and then scale the method geometrically so as to maintain the same mobile phase linear velocity[4] as is commonly done with preparative HPLC.

Another method is the Time-on-Target algorithm[5]. A model compound is eluted in an isocratic run with the desired retention time to purify compounds. Once the solvent composition for the desired elution time is determined, the model compound is run on a column with matching chemistry on the analytical system with a scouting gradient. The retention time for the model compound on the analytical system is used to set the solvent composition to that used on the preparative system analytical run. The apparent delay in the gradient is determined for the analytical system and is applied to the retention times for compounds to be purified to determine the actual solvent composition which will elute the compounds on the preparative system with the desired retention time as set in the isocratic run. A focused gradient was chosen over isocratic conditions because it allows for some error in measuring the retention time, and for some daily variation between the analytical and preparative systems that can affect retention time, such as pressure variation. The gradient range used brackets the target solvent modifier concentration as calculated by the algorithm, typically starting 5% below the target and ending 5% after. The solvent concentration range in the focused gradient is still a reasonable approximation of an isocratic method. The focused gradient is adjusted for the dwell and column volumes for the preparative system. This technique transfers well to SFC. In fact, no modifications to the algorithm were required for acceptable SFC results. Once the focused gradient is tested, the focused gradient is easily "flattened" to isocratic conditions, if desired.

Calibration of Preparative SFC

This requires three steps, the same as for preparative HPLC

1. Determine the Dwell Volume. As the experiment used a modified Teledyne ISCO ACCQ*Prep*®, the dwell volume was known to be 7 mL, including the injection loop. The ACCQ*Prep* was modified with Teledyne SSI pumps with chilled pump heads and a back-pressure regulator set to ~100 Bar.
2. Determine the column volume. As Teledyne ISCO Silica RediSep® Prep columns (25x150 mm, PN 69-2203-826) were used, the column volume was known to be 25 mL. The column volume is very close to the void volume.
3. Set the elution time for the model compound using an isocratic run at the column flow rate. Use the same solvent and modifiers as typically run on the analytical system. Adjust the mobile phase composition to elute the compound at the desired time for the preparative runs, using the flow rate appropriate for the prep column. Teledyne Universal Test Mix (N-benzylbenzamide and phenacetin) was used for calibration compounds.



Iscocratic calibration for elution time of ~3 minutes. N-benzylbenzamide (2.8 minute elution) used for calibration with a methanol modifier at 60 mL/min.

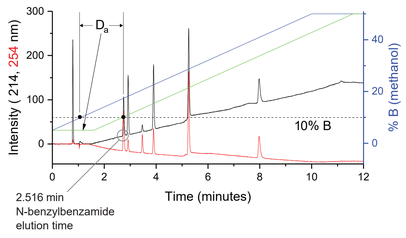
Calibration of Analytical System

Calibration only requires running the model compound with the scouting gradient used to evaluate synthesized compounds. This gradient is typically 5% to 50% modifier with no isocratic hold at the start. The same solvent system used to calibrate the preparative system, including any additives, such as trifluoroacetic acid, is used for this step. Columns with matching chemistries should be used.

$$M_a = \frac{(\%B_{Ea} - \%B_{Sa})}{L_a}$$

$$P = \frac{(\%B_I - \%B_{Sa})}{M_a}$$

After calibration, the programmed gradient time for the calibration compound may be calculated using the equations to the left. M_a= Analytical gradient slope. P = Programmed gradient %B; $\%B_I$ = %B eluting compound in prep isocratic run; $\%B_{Sa}$= Starting %B for analytical gradient; $\%B_{Ea}$ = End %B for analytical gradient; L_a = Gradient length for the analytical run. The apparent gradient delay (D_a) is the difference between the time the calibration compound elutes, and the time on the programmed gradient corresponding to the solvent composition used to elute the compound isocratically. The concentration of strong solvent used to elute the compound is %B.



Samples and calibration run on an Agilent 1260 SFC using a RediSep Prep 4.6 x 150 mm Silica column (Teledyne ISCO PN 69-2203-802) with a methanol gradient at 2.5 mL/min. The peak eluting at 2.516 minutes (N-benzylbenzamide) is deemed to elute at 10% methanol.

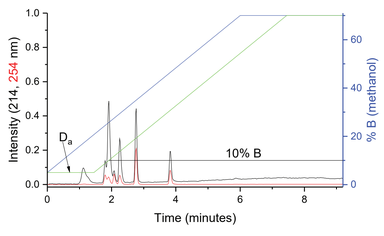
For this example:
M_a = (50–5)/10 = 4.5
P = (10–5)/4.5 = 0.889 minutes

The Apparent Gradient Delay (D_a) = 2.516–0.889 = 1.63 minutes

The other peaks are compounds run so that gradients may be calculated and tested.

Calibration of Preparative SFC Scouting Gradient

The modified ACCQ*Prep* was found to have linear gradients to 70% organic modifier, allowing a greater range of compounds to be analyzed than on the analytical SFC which is limited to a maximum of 50% modifier, while still giving good results. In addition, the ability to use the preparative column for scouting runs allows a gradient to be determined if there is no matching analytical column available. Although the ACCQ*Prep* PeakTrak® software can calculate focused gradients, the parameters in the software are appropriate for the flow rates and retention for preparative HPLC, so a calibration was performed for SFC conditions.



Samples and calibration run on an ACCQ*Prep* SFC using a RediSep Prep 20x150 mm Silica column (Teledyne ISCO PN 69-2203-826) with a methanol gradient at 60 mL/min. The peak eluting at 1.90 minutes (N-benzylbenzamide) is deemed to elute at 10% methanol.

For this example:
M_a = (70–5)/6 = 10.83
P = (10–5)/10.83= 0.462 minutes

The Apparent Gradient Delay (Da) = 1.90–0.462 = 1.45 minutes

The other peaks are compounds run so that gradients may be calculated and tested.

Calculation of Focused Gradient

Calculation requires four steps:

1. Run the compound to be purified on the analytical system using the same gradient as the initial calibration.
2. Using the calculated value D_a, determine the actual %B that elutes the compound with the desired retention time.
3. Set a focused gradient encompassing the calculated %B
4. Correct the gradient for the dwell and column volumes of the preparative system. The dwell and column volumes were found to change the gradient only slightly, by 1% B or less in the system used for the preparative runs.

$$\%B_{Corr} = (T_{Ea} - D_a) * M_a + \%B_{Sa}$$

The corrected solvent composition for the desired compound is $\%B_{Corr}$; T_{Ea} = the elution time for the desired compound in the analytical run. The other terms are the same as the earlier equations.

$$D_p = \frac{(V_{Dp} + V_{Cp})}{F_p}$$

The final steps to calculating the gradient involve correcting the focused gradient for the preparative system dwell and column volume by increasing the strong solvent concentration. For the equations to the left, D_p, the preparative system delay, is determined by adding the prep system dwell volume (V_{Dp}) to the prep column volume (V_{Cp}) and dividing by the prep flow rate. The amount to increase the strong solvent concentration (Δ%B) is calculated by dividing the range (R) by the prep gradient length (L_p), then multiplying by D_p. For the modified ACCQ*Prep*, V_{Dp} = 7.0 mL (with 5 mL loop); V_{Cp} = 24.81 mL (silica 20x150 mm RediSep Prep column), run at 60 mL/min. The chosen range (R) is 10%, with a length (L_p) of 6 minutes.

D_p = (7.0+24.81)/60 = 0.530 min

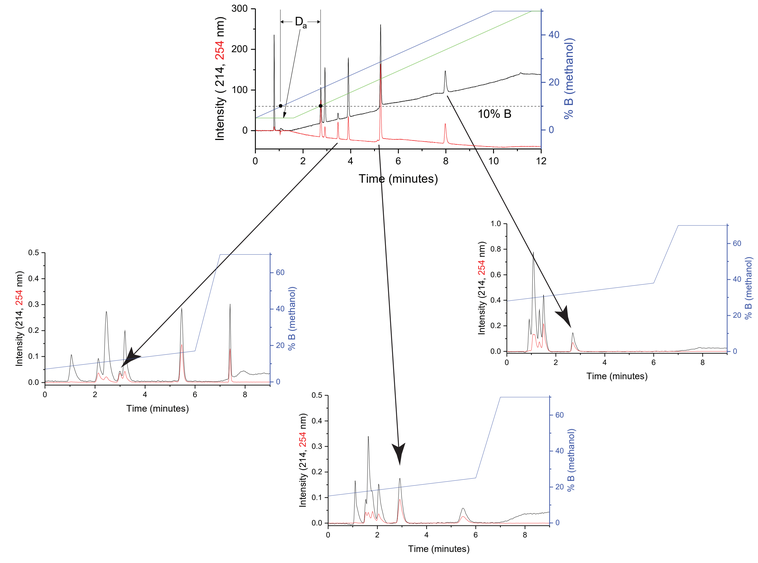
Δ%B = 10/6*0.530 = 0.88%

$$\Delta\%B = \frac{R}{L_p * D_p}$$

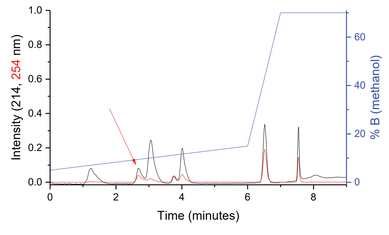
These last two calculations are done regardless of whether the scouting run is done on the analytical or preparative SFC system.

Calculated Preparative Gradients from Analytical SFC

The apparent gradient delay, D_a, calculated for the analytical SFC was used for the runs in this section. The dwell and column volume compensation calculated earlier in this poster was also used. The scouting run used for the calibration contained toluene, ketoprofen, N-benzylbenzamide, phenacetin, theoplylline, sulfanilamide, and adenine. Focused gradients could be calculated for all compounds other than toluene.



Calculated Preparative Gradients from Preparative SFC



The apparent gradient delay, D_a, calculated for the preparative SFC was used for the runs in this section. The dwell and column volume compensation calculated earlier in this poster was also used. The scouting run used for the calibration contained toluene, ketoprofen, N-benzylbenzamide, phenacetin, theoplylline, sulfanilamide, and adenine. Only the ketoprofen run is shown here, but the gradient calculations produced identical results to those for the analytical SFC runs. In all cases, the retention time for the desired compound was the same as that used for the isocratic calibration run.

Conclusion

A simple method iss presented to calibrate an analytical SFC to match the performance of a preparative system so that efficient focused gradients can be used to purify samples. Calibrations performed with methanol have been found to apply to other modifiers such as ethanol and ethyl acetate. Earlier work with preparative HPLC demonstrates that the same calibration can be used for different column chemistries.

Some important notes:

1. The analytical and preparative columns should have the same chemistry, from the same manufacturer, to avoid errors due to selectivity differences.
2. The calibration solvent system modifiers and additives must be the same for the preparative and analytical systems
3. After calibration, the analytical and prep columns may be run with different modifiers than the calibration runs, but the analytical and prep runs for a sample must use the same solvent system.

This technique can be used for other chromatography including, but not limited to, supercritical fluid chromatography, ion exchange, and other packing materials than those listed here. The technique is very amenable for use in high-throughput purification labs since, after calibration is complete, one only needs to run compounds and note their retention times to generate an efficient gradients. If the injected mixture has several compounds of interest, one can use the average retention on the analytical run if they elute closely together. If they elute further apart, create a focused gradient for both the first and last eluting compounds of interest. The calculated gradient causes the compound to elute at a predictable time, such that time windows can be used for fraction collection to reduce the number of fractions collected. The predictable elution time also allows the possibility of ending the run after elution of the target compound, saving time and solvent.

[1] Blom, K. F.; Sparks, R.; Doughty, J.; Everlof, J. G.; Haque, T.; Combs, A. P. Optimizing Preparative LC/MS Configurations and Methods for Parallel Synthesis Purification. *J. Comb. Chem.* **2003**, *5*, 670–683

[2] Blom, K. F.; Glass, B.; Sparks, R.; Combs, A. P. Preparative LC–MS Purification: Improved Compound-Specific Method Optimization. *J. Comb. Chem.* **2004**, *6* (6), 874–883

[3] Michaels, P.; Neef, J.; Galyan, K.; Ginsburg-Moraff, C.; Zhou, X.; Dunstan, D.; Poirier, J.; Reilly, J. Enabling Chiral Sepa ions in Discovery Chemistry with Open-Access Chiral Supercritical Fluid Chromatography. *Chirality* **2019**, *31* (8), 575–582.

[4] Enmark, M.; Åsberg, D.; Leek, H.; Öhlén, K.; Klarqvist, M.; Samuelsson, J.; Fornstedt, T. Evaluation of Scale-up from Anlytical to Preparative Supercritical Fluid Chromatography. *Journal of Chromatography A* **2015**, *1425*, 280–286.

[5] Silver, J. Overview of Analytical-to-Preparative Liquid Chromatography Method Development. *ACS Combinatorial Science* **2019**, *21* (9), 609–613.