Technical Overview

A Tale of Two Samples: Understanding the Purification Workflow from Different Perspectives

Part 1: Bulk purification

Introduction

Reversed-phase liquid chromatography (RPLC) is often associated with quantitative- or analytical-scale analysis, but it is also a useful preparative-scale tool for purification. For any purification project, there are three possible objectives: purity, yield, and throughput. These objectives are related and are commonly represented as a triangle, as shown in Figure 1. Two of these targets can be achieved at the expense of the third. Prioritizing the objectives of the purification project before developing the method can help clarify the path forward. Most projects center around either bulk purification (the purification of one sample over multiple injections) or high-throughput purification (the purification of small amounts of many different samples). Bulk purifications prioritize purity and yield at the expense of throughput, whereas high-throughput purifications prioritize purity and throughput at the expense of yield.

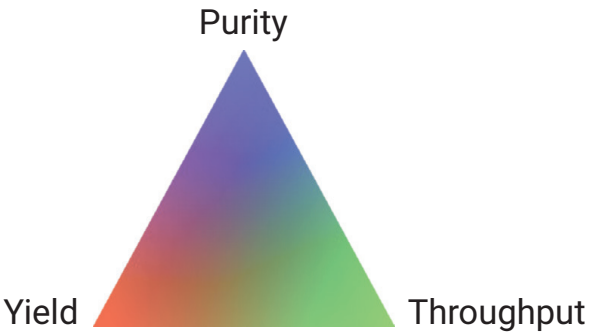

Figure 1. Purification triangle.

This technical overview is a two-part series that examines the workflow for each project type and how prioritization influences the preparative workflow. Part 1 focuses on the bulk purification workflow and part 2[1] gives an overview of the high-throughput purification workflow.

Instrumentation and supplies

All analytical-scale work was performed on an Agilent 1260 Infinity II LC system, and all preparative-scale work was performed on an Agilent 1290 Infinity II preparative LC system. The following columns were used:

- Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 150 mm, 4 µm (part number 683970-302)
- Agilent InfinityLab Pursuit XRs C18, 4.6 × 150 mm, 5 µm (part number A6000150X046)
- Agilent InfinityLab Pursuit XRs C18, 30.0 × 150 mm, 5 µm (part number INF6000150X300)

A proprietary small-molecule crude mix was provided courtesy of a collaborator. All samples were filtered with 0.45 µm Agilent Captiva Premium syringe filters (part number 5190-5093). HPLC-grade acetonitrile and water were acquired from Honeywell Burdick & Jackson. Formic acid (part number G2453-85060) was added to all mobile-phase solvents.

Purification workflow

The general purification workflow (Figure 2) is as follows:

1. Check the sample solubility.
2. Screen the stationary phases and mobile-phase solvents.
3. Optimize the critical pair separation.
4. Determine the maximum sample load.
5. Scale the method to the preparative instrument.
6. Purify the target compound from the sample.

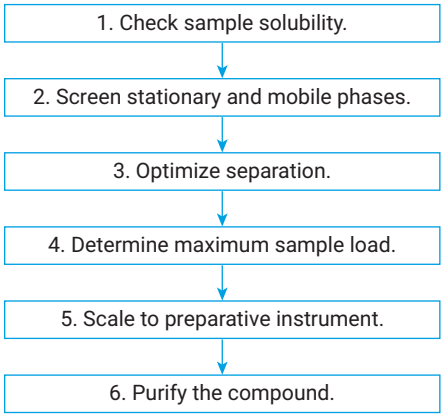

Figure 2. Purification workflow.

Purification of a crude mix while prioritizing purity and yield

A collaborator has synthesized a proprietary small molecule. They would like to have 200 to 300 mg of their target compound purified from the crude mix for additional characterization. The collaborator has developed an analytical method on a C18 column that is not available in a preparative dimension.

The objective of this purification was to collect a high-purity fraction of the synthesized compound while maximizing the yield.

Step 1: Check sample solubility

While low-concentration levels make solubility issues less of a concern in analytical LC, a sample that precipitates or crashes out of solution can clog both the column and the preparative LC instrument. Sample solutions that are submitted for purification are commonly left in the solvent that is used for synthesis. These solvents (such as dimethylsulfoxide or dimethylformamide) are stronger than the usual aqueous-based mobile phase and can provide higher sample solubility. When the sample is injected onto the column, the sample diluent may elute before the rest of the sample, leaving the solute in the mobile phase. If more sample is present than what is soluble in the mobile phase, the remaining sample will precipitate in the LC flow path. Therefore, it is important to confirm that the preparative solution has at least some solubility in the mobile phase.

In this case, the sample was previously dried and provided as a solid. The crude mix was found to be soluble in a mixture of 25:75 ethanol:water, up to 20 mg/mL. If the sample is already in solution, the solubility can be tested by adding a small volume of sample to a beaker with mobile-phase components at initial gradient conditions. If the sample does not precipitate, the sample will most likely be compatible with the mobile phase. The crude mix solution was found to be soluble in 95:5 of both water:acetonitrile and water/methanol. If the sample was found to be only partially soluble, then the preparative injection volume or solution concentration could be lowered to reduce the mass of sample on the column.

Step 2: Screen stationary phases and mobile-phase solvents

Similar to analytical RPLC method development, screening columns and mobile phase solvents are the foundation for developing a solid purification method. The stationary and mobile phases have the biggest impact on the separation between the target compound and adjacent impurities (the critical pair). A good column tool kit contains several different stationary phases with complementary selectivity, which can separate a wide range of compounds. Gradients using water, paired with either acetonitrile or methanol, are typical choices for initial screening work. The pH of the mobile phase should also be considered. If the pH is too close to the compound's pK (dissociation constant), the compound will have two peaks: one for the compound target and the other for its conjugate species. This issue can be avoided by operating at a pH that is one to two units away from the pK.

For this sample, the collaborator's initial screening work suggests that a C18 phase paired with acetonitrile and formic acid mobile phase provides the best separation. A slightly modified screening experiment was completed using two C18 phases that are also available in preparative dimensions: an Agilent InfinityLab Poroshell 120 SB-C18 column, 3.0 ×150 mm, 5 µm and Agilent InfinityLab Pursuit XRs C18 column, 4.6 × 150 mm, 5 µm. A nominal concentration (5 mg/mL) of the crude mix was screened on both columns with generic gradients. The results are shown in Figure 3. Note that the noise seen at the apex of some of the peaks is due to detector saturation and can be avoided by using a less-sensitive flow cell.

If developing an analytical method, the InfinityLab Poroshell 120 SB-C18 column would appear to be the preferred choice since it resolves many of the peaks in the mixture. However, for a purification method, the primary focus is on the separation of the critical pair. From this perspective, both columns provide very similar separations and would satisfy purity requirements. The next objective is to maximize yield. The sample load of a given injection can be increased by injecting a higher concentration sample, injecting a larger volume, or a combination of both. Since the sample concentration is capped by the crude mix's relatively low solubility, the sample load can be increased through injection volume, which can be maximized by using a column with the largest internal diameter (id) available. The InfinityLab Pursuit XRs C18 column, optimized for yield and available in larger ids, was chosen in a 30.0 × 150 mm dimension for additional work.

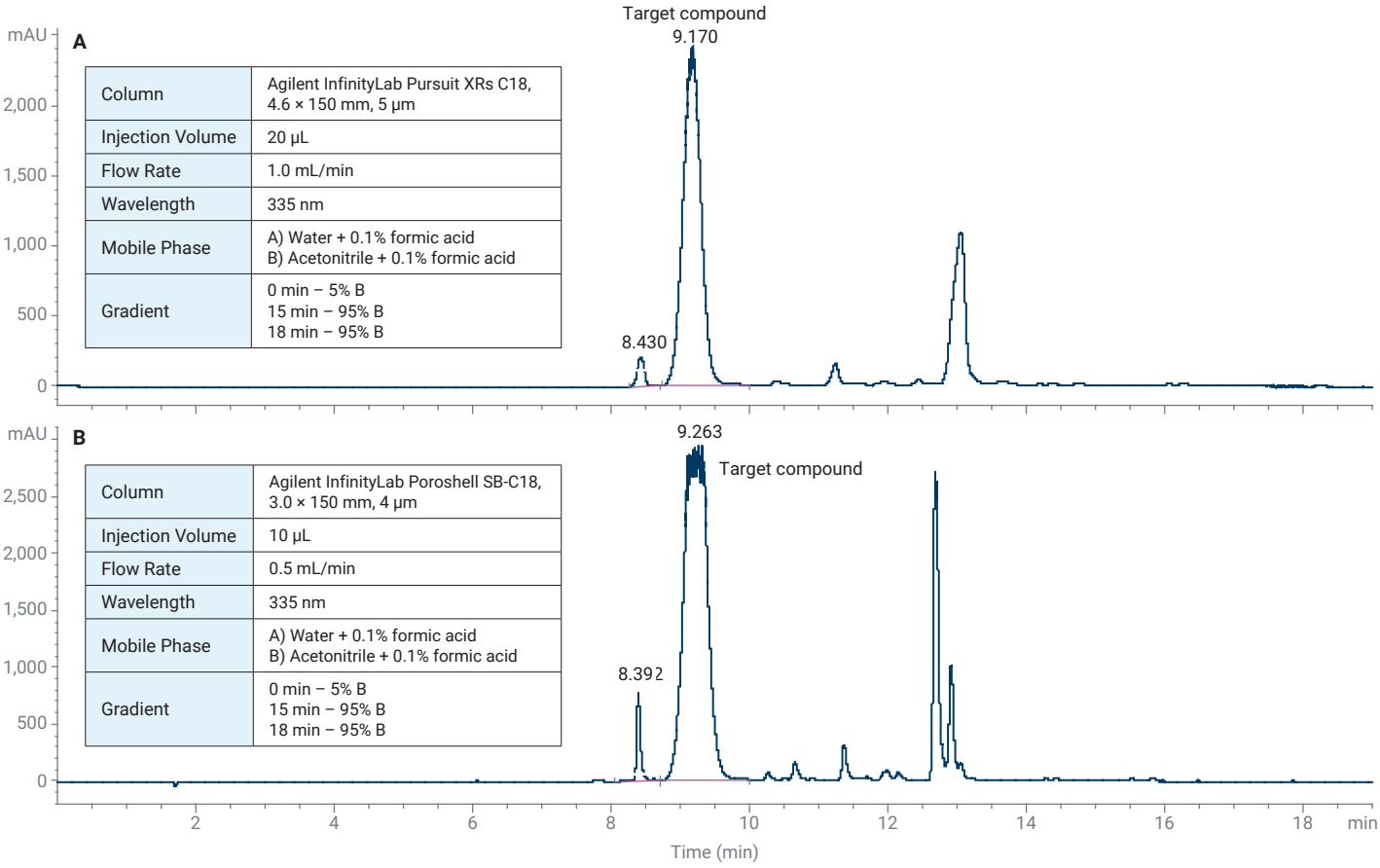

Figure 3. Generic screen of crude mixture (5 mg/mL). (A) Agilent InfinityLab Pursuit XRs C18, 4.6 × 150 mm, 5 µm; (B) Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 150 mm, 4 µm.

Step 3: Optimize the separation

When sample is scarce, optimizing chromatographic conditions using an analytical instrument is an excellent option. Many combinations of various chromatographic parameters can be run without significant depletion of sample or mobile phase. Using the slope of the gradient and correcting for system delay time, the target compound was determined to elute at approximately 44% B.[2] This elution point was used as the basis for additional method development. Significant time was spent maximizing the separation between the target compound and the adjacent impurity to allow for the greatest sample load on the preparative column. A comparison of the chromatograms from the generic gradient and the optimized method is shown in Figure 4.

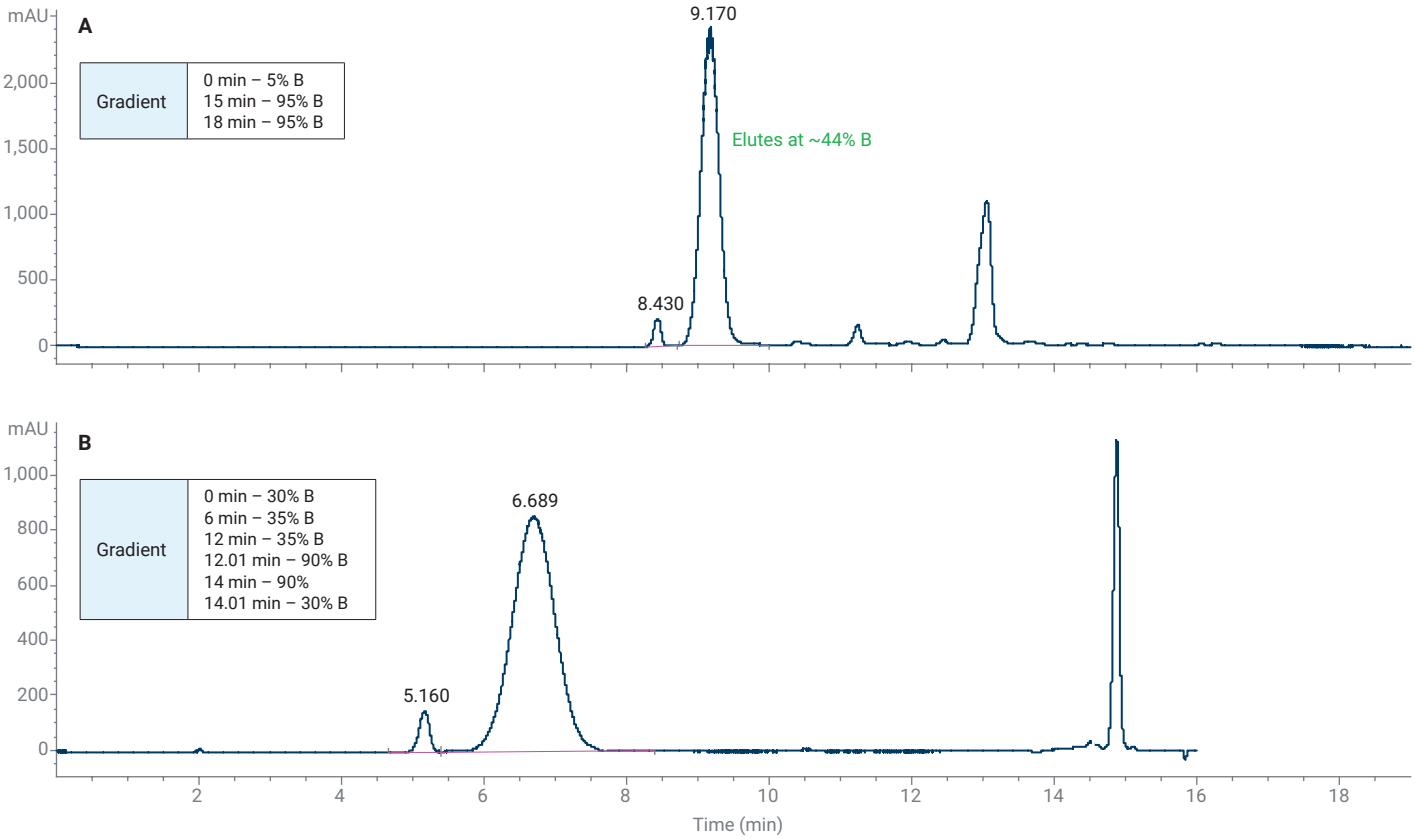

Figure 4. Optimized separation of crude mixture (5 mg/mL) on an Agilent InfinityLab Pursuit XRs C18, 4.5 × 150 mm, 5 µm column. (A) Generic gradient; (B) optimized method.

Step 4: Determine the maximum sample load

The maximum sample load on the column was determined by using the highest concentration solution (20 mg/mL) to make several injections of increasing volume (10, 40, and 70 µL). The largest injection volume (70 µL) was chosen to correspond with the capacity of the sample loop configured on the preparative instrument (3,000 µL). More details on scaling are provided in the next step. The results from the loading study are shown in Figure 5. Even at the highest injection volume (70 µL), the peaks still maintain separation. This injection volume was scaled up and used for the purification method.

Step 5: Scale method to preparative instrument

The InfinityLab Pursuit XRs C18, 30.0 × 150 mm preparative column is the largest dimension available and therefore allows the largest injection and sample load. Assuming that the analytical and preparative column are of the same length and packed with the same particle size material, the injection volume and flow rate are geometrically scaled from the analytical method using Equations 1 and 2.

Equation 1. Preparative column flow rate (f_p) calculation.

$$f_p = f_a \left(\frac{id_p}{id_a} \right)^2$$

Where f_a is the flow rate of the analytical column, id_p is the internal diameter of the preparative column, and id_a is the internal diameter of the analytical column.

Equation 2. Preparative column injection volume (V_p) calculation.

$$V_p = V_a \left(\frac{id_p}{id_a} \right)^2$$

Where V_a is the injection volume on the analytical column, id_p is the internal diameter of the preparative column and id_a is the internal diameter of the analytical column.

The calculated flow rate for the 30.0 × 150 mm column is 42 mL/min, with an injection volume of 3,000 µL (rounded from 2,977 µL for convenience). The sample load per injection (sample concentration × injection volume) is 60 mg.

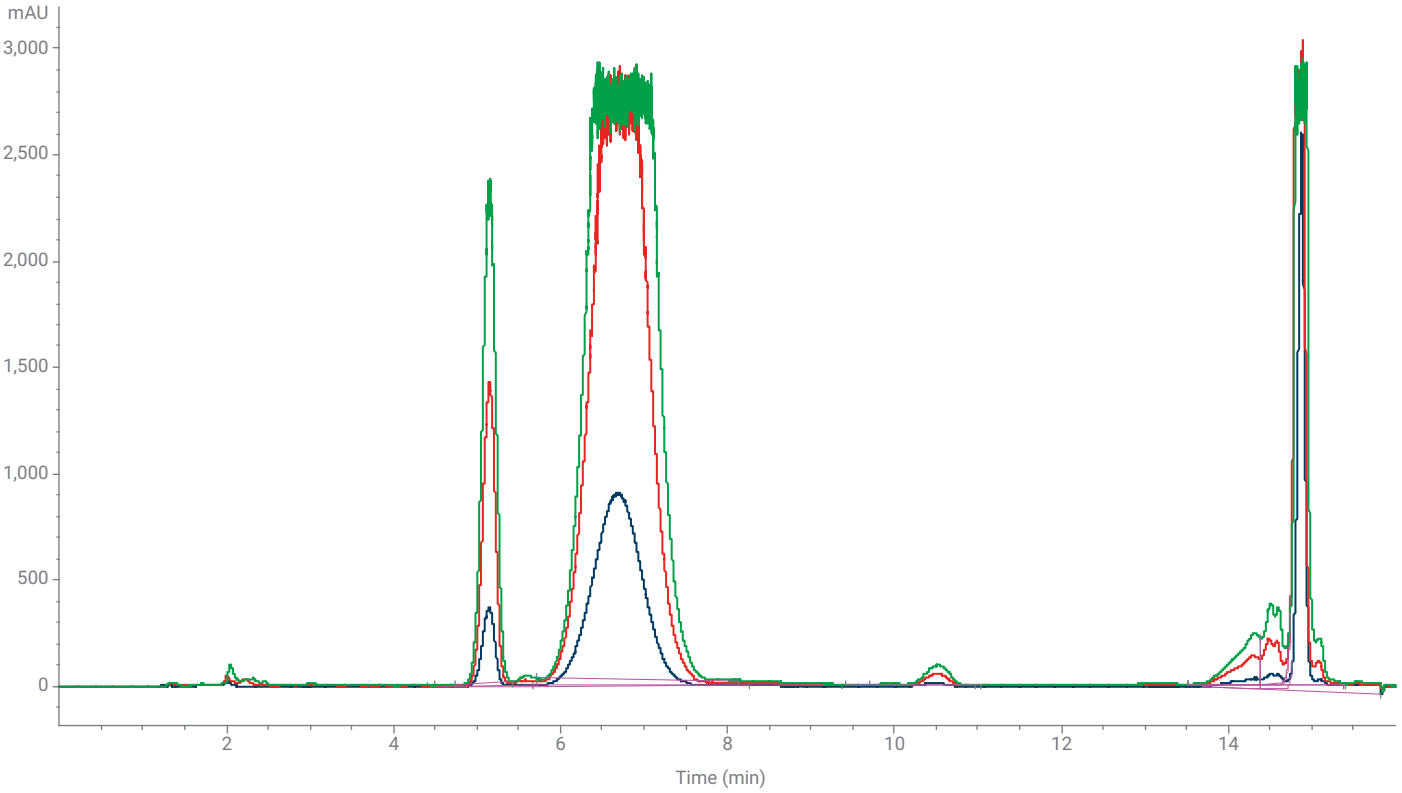

Figure 5. Results from 20 mg/mL sample loading study. Blue: 10 µL injection; red: 40 µL; green: 70 µL. All injection volumes provide adequate resolution between target and impurity peaks.

One factor that can impact scaling gradient methods is the difference in instrument dwell times. The dwell time is the amount of time it takes for the mobile phase to travel from the mixing point in the pump to the head of the column. The dwell time acts like an isocratic hold at the beginning of the method. The type of solvent mixing, autosampler, tubing length, and id can all impact the dwell time. When scaling an analytical gradient method to a preparative instrument, it is important to factor dwell time into the gradient method. At a minimum, not factoring additional hold time at the beginning of the method can cause differences between analytical and preparative retention times; the hold time can even change chromatographic separations.

By using the methods outlined in the Agilent primer *Principles and Practical Aspects of Preparative Liquid Chromatography* (publication number 5994-1016EN)[3], the difference in dwell time was calculated to be 0.8 minutes. This time was added as an isocratic hold to the beginning of the preparative method.

Step 6: Purify the compound

The crude mix was injected onto the preparative column using the parameters in Table 1. The collection of the target compound was based on the 335 nm UV chromatogram using a combination of threshold and time window. This combination prevents accidentally collecting another peak while minimizing the collection volume. The chromatograms for the 70 µL analytical injection and the preparative injection are shown in Figure 6.

Table 1. Acquisition and collection parameters for crude mix.

Parameter	Value
Column	Agilent InfinityLab Pursuit XRs C18, 30.0 × 150 mm, 5 µm
Injection Volume	3,000 µL
Flow Rate	42 mL/min
Wavelength	335 nm
Mobile Phase	A) Water and 0.1% formic acid B) Acetonitrile and 0.1% formic acid
Gradient	0 min: 30% B 0.8 min: 30% B 6.8 min: 35% B 12.8 min: 35% B 12.81 min: 90% B 14.8 min: 90% B 14.81 min: 30% B
Collection	Between 6 and 8 min and above 500 mAU

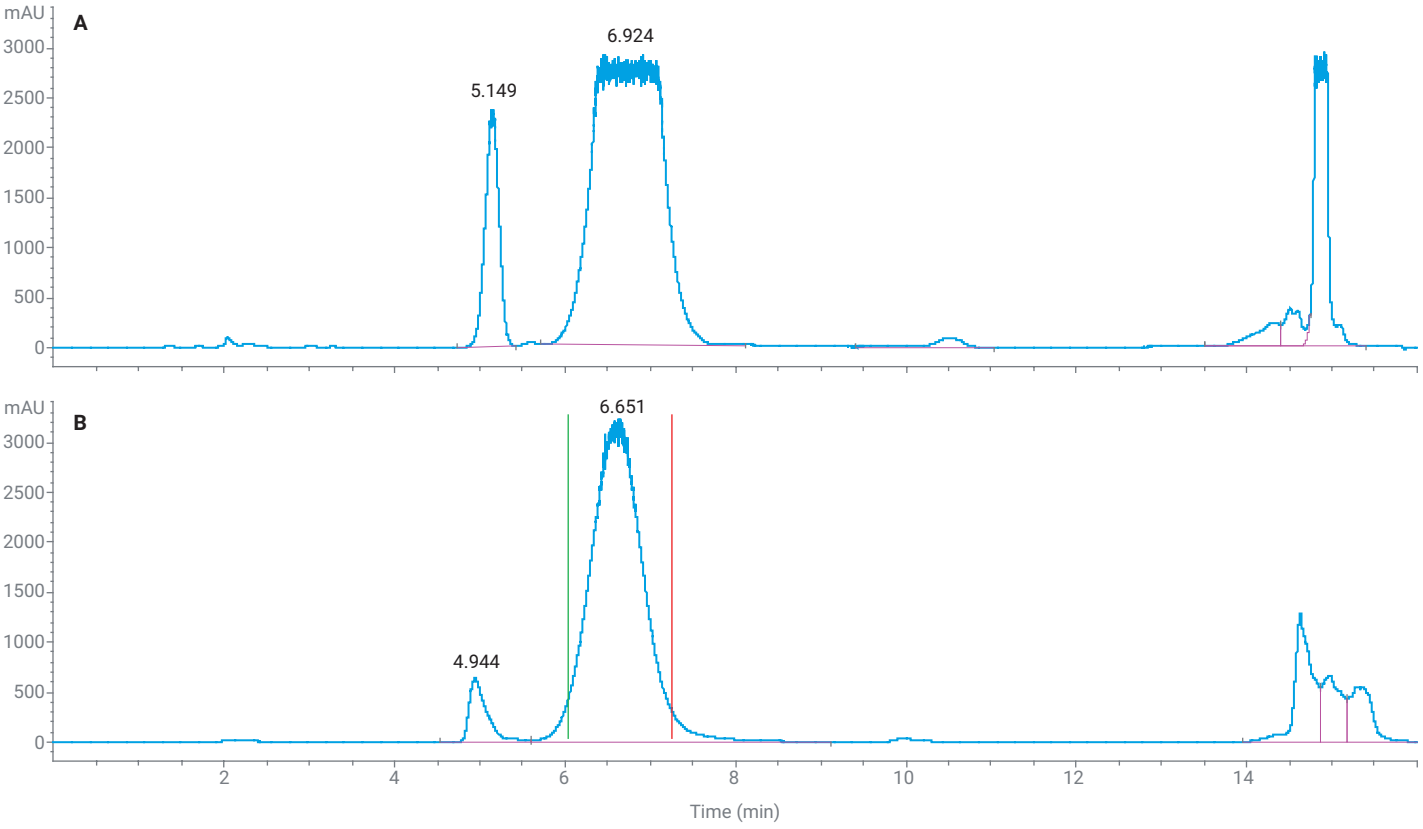

Figure 6. (A) Analytical (70 µL), and (B) preparative (3000 µL) chromatograms on the Agilent InfinityLab Pursuit XRs C18, 30 × 150 mm, 5 µm column.

The column was allowed to re-equilibrate to initial conditions during a postrun. When making multiple injections, it is important to allow the column to flush 8 to 10 column volumes before the next injection is made. Otherwise, retention times may shift in the next injection. Several injections were made to collect the desired amount of product. The fractions were combined and then reinjected on the analytical system to confirm purity (Figure 7). The injection confirms the presence of the target compound. No detectable impurity peaks were found, indicating >99% purity.

Key points for bulk purification

- The purification goals prioritize yield and purity at the expense of throughput.
- The best column just needs to resolve the target compound from impurities. All the other peaks can coelute.
- Verify that the sample is soluble in mobile-phase solvents.
- For bulk purification, determine the method and loading conditions on the analytical system before scaling them to the preparative system.
- Remember to incorporate an isocratic hold (where necessary) to account for dwell volume differences.

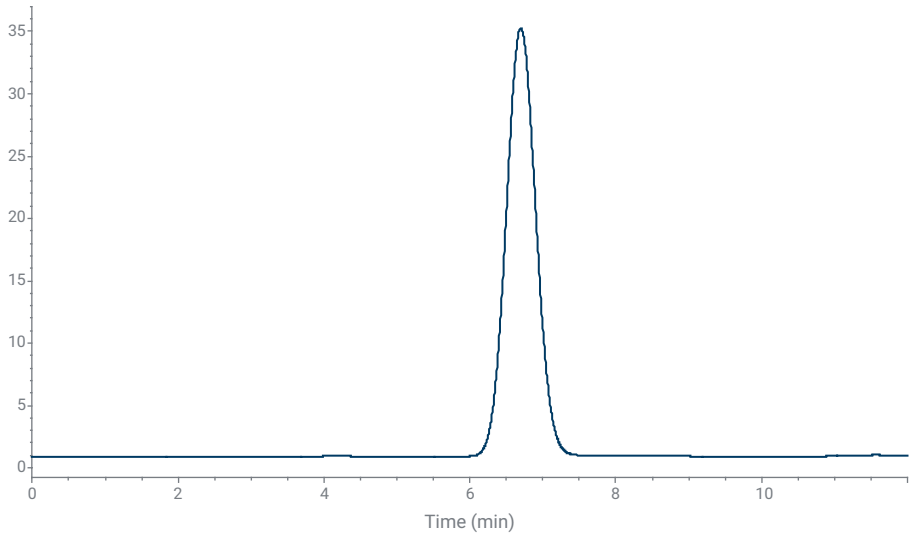

Figure 7. Reinjection of collected crude mix, confirming purity.

Conclusion

In the crude mix example, yield and purity were prioritized at the expense of throughput. The critical pair proved to be a challenging separation that required significant method development. However, not all separations require extensive method development. Many discovery and other high-throughput laboratories are inundated with requests for the purification of batches of samples where there is little time for method development. These environments, which prioritize throughput and purity over yield, could benefit from faster screening and purification methods, and will be the focus of the second part of this series.

References

1. A Tale of Two Samples: Understanding the Purification Workflow from Different Perspectives Part 2: High-Throughput Purification, *Agilent Technologies technical overview*, publication number 5994-4708EN, **2022**.
2. The LC Handbook, Guide to LC Columns and Method Development, *Agilent Technologies*, publication number 5990-7595EN, **2016**, p. 84. https://www.agilent.com/cs/library/primers/public/LC-Handbook-Complete-2.pdf.
3. Principles and Practical Aspects of Preparative Liquid Chromatography, *Agilent Technologies Primer*, publication number 5994-1016EN, **2019**. https://www.agilent.com/cs/library/primers/public/primer-preparative-liquid-chromatography-5994-1016EN-agilent.pdf.

www.agilent.com

DE60718789

Printed in the USA, March 11, 2022
5994-4707EN

Agilent
Trusted Answers