Technical Overview

Agilent
Trusted Answers

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

Part 2: High-throughput 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.

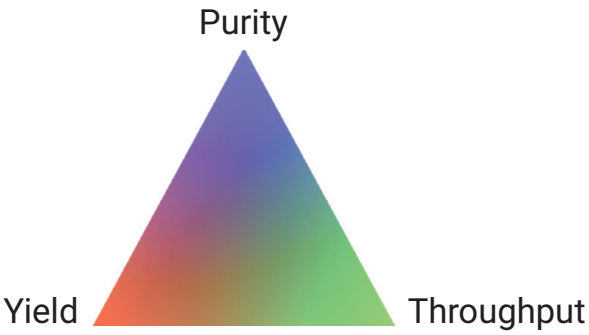


Figure 1. Purification triangle.

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[1] focuses on the bulk purification workflow and part 2 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 HPH-C18, 2.1 × 50 mm, 1.9 µm (part number 699675-702)
- Agilent InfinityLab Poroshell 120 HPH-C18, 21.2 × 150 mm, 4 µm (part number 670150-702)

Ginger extract was acquired from Herb Pharm (Williams, Oregon). 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.

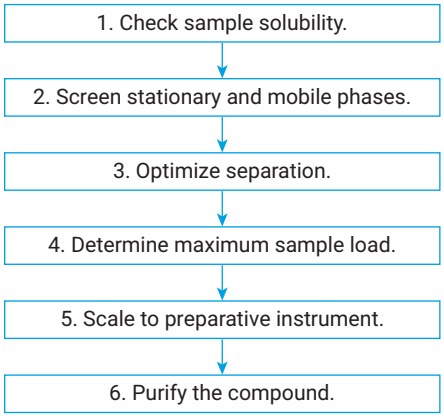


Figure 2. Purification workflow.

Purification of ginger extract while prioritizing purity and throughput

Purifying bioactive components from ginger extract and other natural products represent the type of challenges that discovery and other high-throughput environments face with separating their target compounds from complex matrices. In this example, the target compound was 6-gingerol.

The objective of this project was to quickly develop a purification method for 6-gingerol, prioritizing purity and throughput over yield.

To improve screening throughput, narrow-bore columns (2.1 × 50 mm or smaller) packed with smaller particles are used to quickly determine the best column/mobile phase combination, from which a full-fledged method can be developed. This is done by leveraging the resolving power of a column. The resolving power is calculated using Equation 1.

Equation 1. Resolving power calculation.

$$\text{Resolving power} = L/d_p$$

Where L is the length of the column, and d_p is the particle size of the stationary phase material.

Two columns with L/d_p values within –25 to 50% of each other are said to have similar resolving power.[2] For example, a 100 mm column packed with 1.9 µm particles would have a resolving power of 52.6, similar to the resolving power of a 250 mm column packed with 5 µm particles (L/d_p = 50).

Step 1: Check sample solubility

Samples purified using high-throughput workflows are often submitted in 1 to 2 mL of strong solvent (such as DMSO or DMF).[3] While sample precipitation is a common purification concern[1], the high-throughput yield requirements keep the sample load on the column small, reducing the chances of precipitation. The addition of an in-line filter in the flow path can collect any sample that does precipitate, preventing column clogging. The ginger extract was previously prepared in an ethanol:water solution, with a concentration of 40 mg/mL.

Step 2: Screen stationary phases and mobile-phase solvents

While it is generally considered good practice start developing a purification method by screening samples on several different types of columns and mobile-phase solvents, many discovery laboratories screen samples on either a low-pH system using an acid-stable column (such as the Agilent InfinityLab Poroshell 120 SB-C18 column) or a high-pH system with an end-capped column that can withstand basic conditions (such as the InfinityLab Poroshell HPH-C18 column). The volume of historical data indicates that many samples will separate on C18 phases. The screening process in high-throughput environments is a pass/fail test of sorts. The samples that can be separated by either C18 will move to the preparative instrument, whereas the samples that do not will be addressed elsewhere. The ginger extract was screened using a four-minute generic gradient on an InfinityLab Poroshell

HPH-C18 column, 2.1 × 50 mm, 1.9 µm. The resolving power (L/d_p) is 26.3, which is similar to that of a 150 mm column packed with 5 µm particles (L/d_p = 30), a dimension commonly used for traditional purification screening work, such as in part 1.[1] Despite them having similar L/d_p, the 1.9 µm HPH-C18 column is one-third of the length of its 5 µm counterpart, and could reduce the analytical screening time by up to 67%. The results from the screen are shown in Figure 3. The 6-gingerol peak elutes in the middle of the gradient as a sharp peak, with some closely eluting impurities, which could contaminate a collection and should be addressed when optimizing the method.

Step 3: Optimize the method

An effective method to maximize the resolution between the product and any impurity peaks is to run a focused gradient. This involves running a gradient that has a shallower slope than in the screening method, with a stop percentage specific to the peak(s) of interest. The chromatogram in Figure 3 is overlaid with both the programmed as well as the dwell time-adjusted gradients. After taking the dwell time (2 minutes) into account, the adjusted retention time of 6-gingerol is 2.2 minutes, which corresponds to 49.5% B in the gradient. Instead of making additional analytical injections to optimize the method, a focused gradient is created directly for the preparative column in step 5.

Sample	Ginger extract (40 mg/mL) in ethanol:water
Column	Agilent InfinityLab HPH-18, 2.1 × 50 mm, 1.9 µm
Injection Volume	0.5 µL
Flow Rate	0.5 mL/min
Wavelength	282 nm
Mobile Phase	A) water and 0.1% formic acid B) acetonitrile and 0.1% formic acid
Gradient	0 min: 5% B 4 min: 95% B 7.5 min: 95% B 7.6 min: 5% B

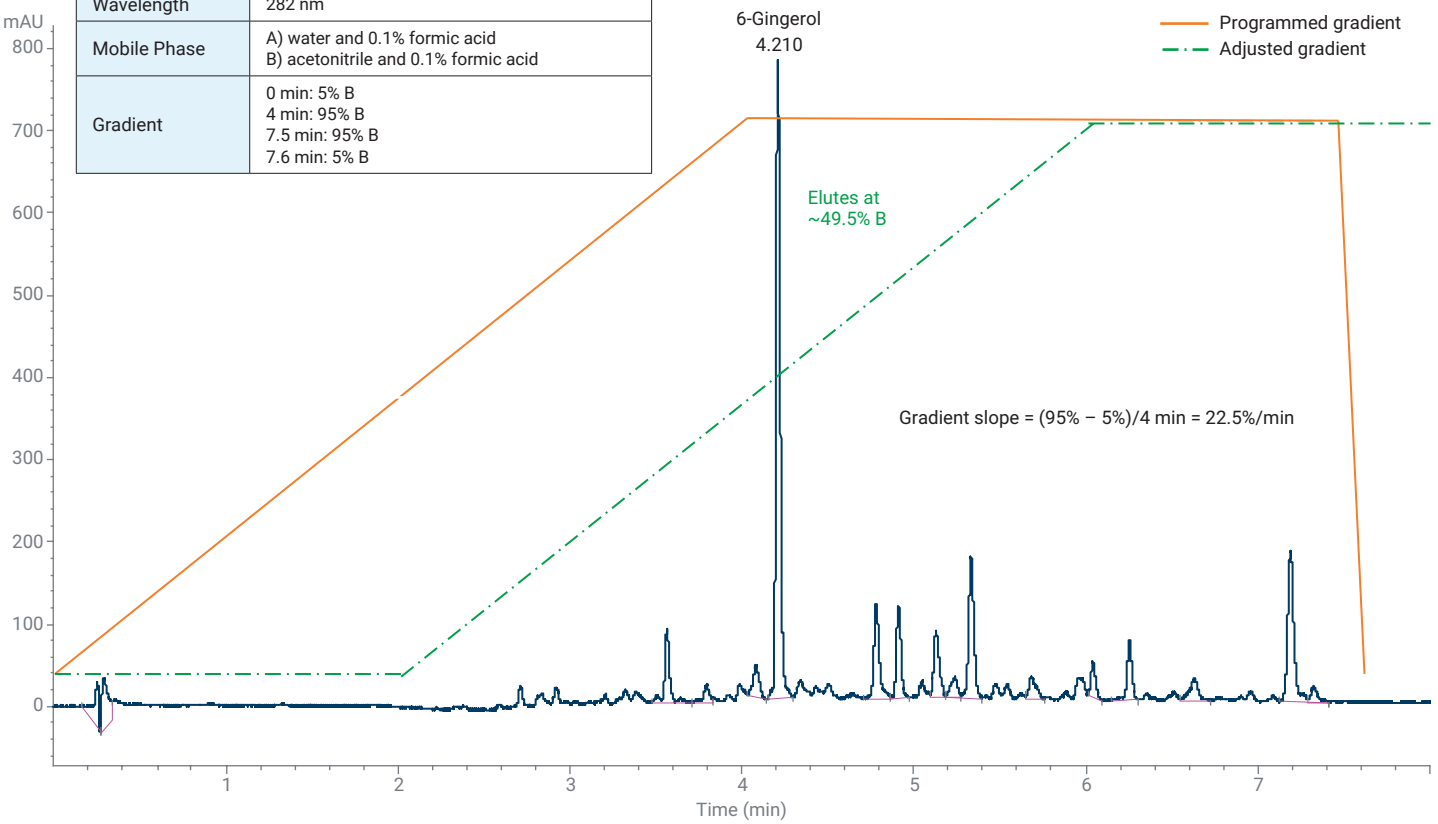


Figure 3. Chromatogram for ginger extract on an Agilent InfinityLab Poroshell HPH-C18, 2.1 x 50 mm, 1.9 µm column, with programmed and adjusted gradient traces overlaid.

Step 4: Determine maximum sample load

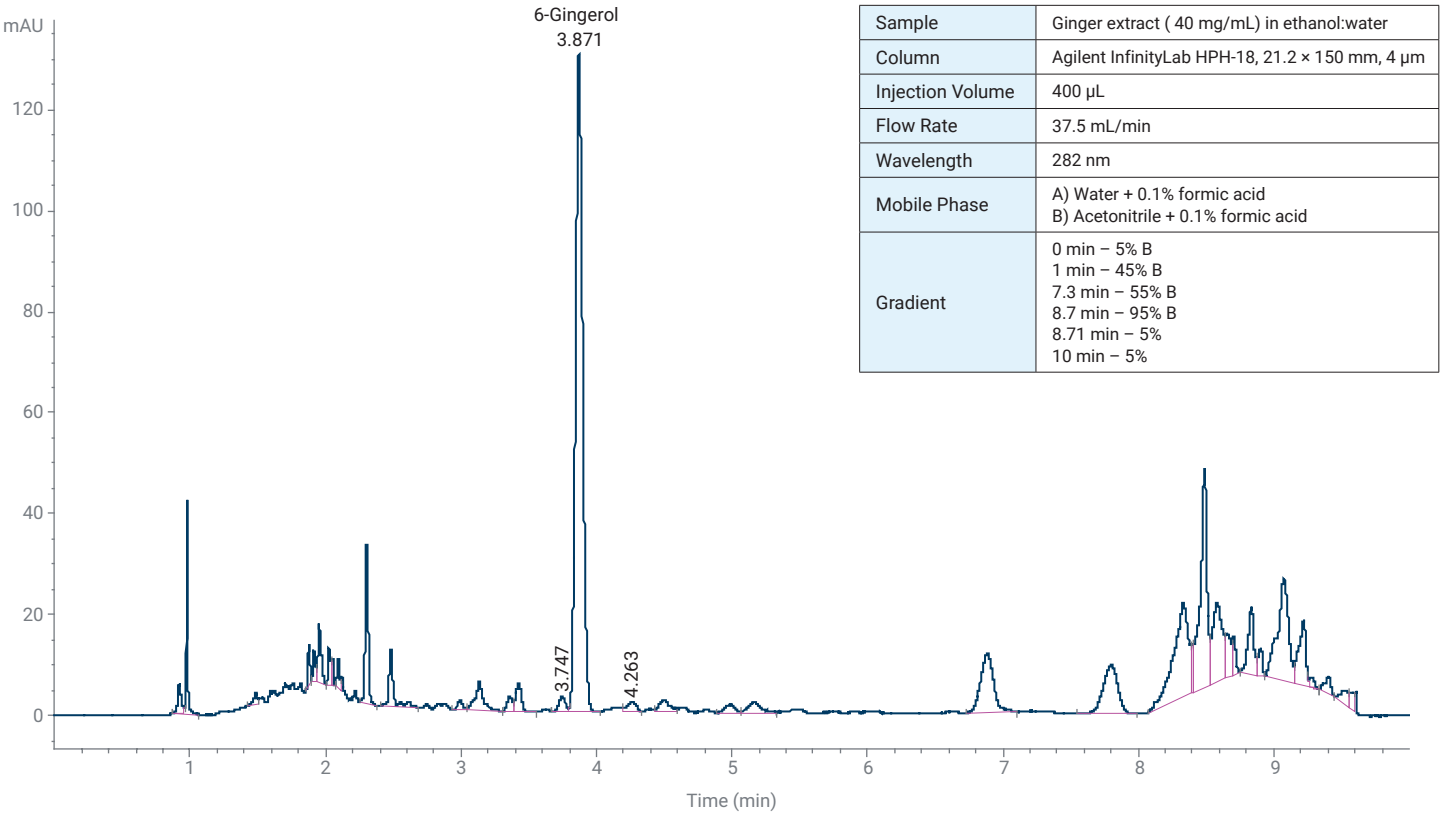
The objectives of this work prioritize purity and throughput over yield, so no loading experiments are necessary. If there are any partially coeluting compounds, a smaller slice of the peak can be collected, omitting the impurity from the fraction.

Steps 5 and 6: Scale to the preparative instrument and purify target compound

In traditional or bulk purification workflows, chromatographic parameters are geometrically scaled from the analytical method to ensure that the target compound has a similar retention time on the preparative column for accurate collection. In high-throughput workflows, scaling to the preparative column from a fast gradient can be problematic as it may scale to a flow rate at or above the operating limit for the preparative instrument or column. Instead, the preparative column is run at its optimal flow rate and the sample collection is triggered by slope, threshold, or other means, independent of time. For a conventional column packed with 5 µm, 21.2 mm internal diameter (id) totally porous particles (TPPs), the optimal flow rate is 21 mL/min.[4] As with any TPP column, if it is run significantly above its optimal flow, there is usually a loss of performance, which can cause peaks to coelute.

Rather than using a TPP column, a 4 µm, 21.2 × 150 mm InfinityLab Poroshell 120 HPH-C18 column (L/d_p = 37.5) was chosen for the purification work. The preparative column has slightly more resolving power than its analytical counterpart (L/d_p = 26.3), which will help separate the small impurities around the target compound. Both the analytical and preparative columns were packed with superficially porous particles (SPPs). An SPP has a solid silica core covered by a porous shell. SPPs create a shorter diffusion path for an analyte, providing chromatographic speed, efficiency, and throughput advantages over TPPs.[4] The optimal flow rate for a Poroshell preparative column is 25 mL/min; the column was run at 37.5 mL/min since Poroshell columns are designed to be run above their optimal flow rate without appreciable loss of performance.

From the calculations in step 5, the peak elutes ~50% B, so the focused gradient will run from 45 to 55% B in 6.3 minutes, with a slope of 1.6% per minute. The slope of this focused gradient is much lower than the original screening



Sample	Ginger extract (40 mg/mL) in ethanol:water
Column	Agilent InfinityLab HPH-18, 21.2 × 150 mm, 4 µm
Injection Volume	400 µL
Flow Rate	37.5 mL/min
Wavelength	282 nm
Mobile Phase	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0 min – 5% B 1 min – 45% B 7.3 min – 55% B 8.7 min – 95% B 8.71 min – 5% 10 min – 5%

Figure 4. Focused gradient for 6-gingerol; 45 to 55% in 6.3 minutes, or 1.6%/min slope.

gradient, to try to pull the adjacent peaks away from the 6-gingerol, while still realizing the sharper peak shape that comes from gradient separations. The method starts at 5%, then ramps to 45% in 1 minute to quickly elute any early impurities. After running the focused portion of the gradient, the method ramps to 95% to flush any remaining impurities.

The resulting preparative chromatogram is shown in Figure 4. Overall, there is increased separation between 6-gingerol and the adjacent peaks, with a closely eluting minor impurity present at 3.75 minutes. No collection was made for this sample, but this peak could be omitted by using higher-threshold values to trigger the collection, so that only the center of the 6-gingerol peak is collected. This will reduce the yield, but preserve the purity and throughput, which is consistent with the purification objectives. The run time for the preparative method was 10 minutes. Scaling this method to a 5 µm totally porous column of the same size at 21 mL/min would have resulted in an 18-minute method. The Poroshell preparative column almost doubles the throughput.

Instrument considerations for high-throughput work

- **Dwell volume:** The fastest screening methods (1 to 2 minutes) are run on sub-2 µm columns, 2.1 × 30 mm, such as the InfinityLab Poroshell HPH-C18, 1.9 µm column (part number 695775-502). For method development, the analytical instrument must be optimized and suitable for running short columns with small particle sizes. A system dwell time equal to the length of the gradient will double the run time and band broadening will negate the efficiency gains.
- **Detection:** Most screening instruments in high-throughput labs are equipped with mass spectrometers (MS) to identify the target compound without having a standard, and to detect impurities that lack chromophores. MS functionality allows more specificity during collection and prevents the collection of impurities that are not visible in UV.

Key points for high-throughput purification

- The purification goals prioritize purity and throughput over yield.
- Using narrow-bore columns with fast gradients (1 to 2 minutes) is an effective way to increase screening throughput, provided that the instrumentation dwell time has been minimized.
- Focused gradients are effective tools to improve separations with minimal intervention.
- SPP preparative columns, such as the InfinityLab Poroshell 120 HPH-C18 column, can increase throughput by providing superior performance at high flow rates.
- MS detection improves collection specificity, which minimizes the number of false positive fractions.

Conclusion

Method development for the purposes of purification follows the same principles as it would for analytical separation, but with a slightly different focus. Rather than separate all peaks, the goal of a purification method is to isolate the peak of interest from the others. The priority of purity, yield, and throughput will clarify method development and workflow decisions. Bulk purifications, such as the example from part 1 of the series, will require methods that provide the best separation and preparative columns that maximize load. High-throughput purifications will benefit from screening instruments and columns that can support ultrafast methods, and preparative columns with equally impressive performance.

References

1. A Tale of Two Samples: Understanding the Purification Workflow from Different Perspectives Part 1: Bulk purification, *Agilent Technologies technical overview*, publication number 5994-4707EN, **2022**.
2. General Chapter <621> "Chromatography" in United States Pharmacopeia 40 National Formulary 35 (USP 40–NF 35, United States Pharmacopeial Convention, Rockville, Maryland, 2017), pp. 508–520.
3. High-Throughput Purification Platform in Support of Drug Discovery. *ACS Comb. Sci*. **2012**, *14*, 51–59. dx.doi.org/10.1021/co200138h
4. Subbarao, L. Developing Fast Purification Methods for Natural Products Using an Agilent InfinityLab Poroshell 120 SB-C18 Preparative LC Column. *Agilent Technologies application note*, publication number 5994-3518EN, **2021**.

www.agilent.com

DE23371589

Printed in the USA, March 9, 2022
5994-4708EN

Agilent
Trusted Answers