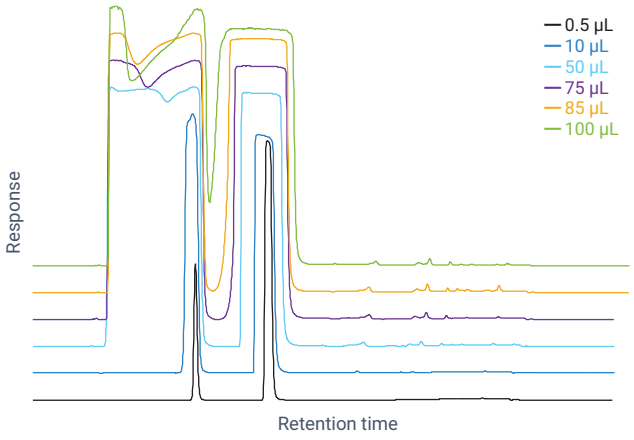
Technical Overview

Analytical-Scale LC Purification with High Load and Low Dispersion

The Agilent 1260 Infinity II Analytical-Scale LC Purification Systems



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Abstract

Analytical high-performance liquid chromatography (HPLC) systems can be turned into purification systems by adding a fraction collector to the stack. The analytical column can be used for purification as well, as long as the method is properly optimized with respect to selectivity and sample load. This technical overview presents the purification of a small molecule using an Agilent 1260 Infinity II LC, combined with an Agilent 1260 Infinity II Analytical-Scale Fraction Collector and the OpenLab CDS software suite. Three different analytical columns are compared with respect to their sample capacity. While all columns deliver excellent purity and recovery, the highest throughput is achieved using an Agilent Pursuit XRs column. These results demonstrate that the same system, column, and software can be used to switch from analytical to preparative HPLC, just by investing in a fraction collector.

Introduction

HPLC is the method of choice in many different industries for analyses of nonvolatile small molecule mixtures. Whenever these mixtures are not only to be separated but to have their constituent compounds individually isolated, chromatographers turn to preparative HPLC. In many cases, preparative HPLC implies large-scale instrumentation with high flow rates and large separation columns. However, purifying single compounds of a small-scale synthesis or low-concentrated natural product extract is possible using an analytical HPLC system that has been extended by a fraction collector.

Agilent InfinityLab LC systems have a modular design that enables the simple switch from an analytical to a purification system. The 1260 Infinity II Analytical-Scale Fraction Collector (G1364F) can be added to any analytical LC system and enables fraction collection at flow rates up to 10 mL/min. Its design allows its inclusion in an existing LC stack without increasing the footprint of the system. The flow path is optimized to ensure lowest dispersion on an analytical scale, yielding highest purity and recovery of the collected compounds. Purification features have been added to revision 2.5 of Agilent OpenLab CDS, which enables control of the entire LC system by a single software and does not require users to get familiar with another software if they want to switch from analytical to preparative work. This not only facilitates operation but also saves time and cost of additional training.

To purify a certain amount of a compound in as little time as possible, it is necessary to load as much sample onto the column as possible. This technical overview describes the process of method development and column loading experiments of an analytical-scale purification workflow. Different columns are compared with respect to maximum sample load as well as purity and recovery of the purified compound.

Experimental

Instrumentation

An analytical Agilent 1260 Infinity II LC was extended by a fraction collector. The system consisted of the following modules:

- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm analytical flow cell (option #018)
- Agilent 1260 Infinity II Analytical-Scale Fraction Collector (G1364F)

Columns

- Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (part number 959993-902)
- Agilent InfinityLab Poroshell 120 EC-C18, 4.6 × 150 mm, 4 µm (part number 693970-902)
- Agilent Pursuit XRs C18, 4.6 × 150 mm, 5 µm (part number A6000150X046)

Software

Agilent OpenLab CDS 2.5

Solvents and sample

All solvents used were LC grade and purchased from VWR (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). 3,5-Dihydroxybenzoic acid (DHBA) and 4-hydroxybenzoic (HBA) acid were purchased from Sigma (Taufkirchen, Germany).

For loading studies and column comparison, a sample of DHBA and HBA at concentrations of 8 and 40 mg/mL, respectively, was prepared in acetonitrile/water 20/80 (v/v). This sample was diluted with acetonitrile/water 10:90 (v/v) to concentrations of 1,000, 500, 250, 125, and 62.5 µg/mL for calibration purposes. A second sample at concentrations of 32 mg/mL DHBA and 160 mg/mL HBA was prepared in acetonitrile/water 1:1 (v/v) for overloading the best performing column.

Calibration

On each column, a calibration curve was constructed by six points including a solvent blank. Points were measured in triplicate and in increasing order of concentration.

Results and discussion

Gradient optimization and calibration

A mixture of 3,5-dihydroxybenzoic acid and 4-hydroxybenzoic acid was separated using a linear gradient from 2 to 98% acetonitrile in 9.6 minutes, with a total run time of 13 minutes. Based on the retention time of the target compounds, a focused gradient with a shallower slope from 10 to 30% in 3.5 minutes and 8 minutes run time was created. This gradient provided optimum separation of the compounds on each column used. Figure 1 shows a chromatogram overlay of a calibration standard separated by a focused gradient on different columns. The Pursuit XRs column produced the highest retention of the two compounds, followed by the ZORBAX and the InfinityLab Poroshell 120. Selectivity of the target compound, however, was lowest on the Pursuit XRs column (1.60), and about the same on the ZORBAX and InfinityLab Poroshell 120 (1.71 and 1.73, respectively). Resolution of 4-hydroxybenzoic acid was comparable on the two fully porous columns, whereas the InfinityLab Poroshell 120 produced an even higher resolution. This is because resolution is calculated based on peak width, which is smaller on a superficially porous particle (InfinityLab Poroshell 120) because of the shorter diffusion path.

Calibration curves were constructed measuring six points in triplicate on each column. For the target compound 4-hydroxybenzoic acid, excellent linearity was achieved on all three columns in a range of 62.5 to 1,000 µg/mL (r >0.999).

Method settings

Table 1. Chromatographic conditions.

Parameter	Value
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	1.5 mL/min
Optimized Gradient	Time (min) %B 0.0 10 3.5 30 4.0 98 5.0 98 5.5 10
Stop Time	8.0 min
Injection Volume	0.5 µL for quantitation, 75 to 100 µL for purification
Needle Wash	10 s with acetonitrile/water 1:1 (v:v)
Temperature	Ambient
UV Detection	254 nm, no reference Peak width >0.05 min (1 s response time, 5 Hz)
Fraction Collection	Peak-based from 2.35 to 4.5 min, triggered on threshold and slope Threshold: 10 mAU Upslope: 5 mAU/s Downslope: 5 mAU/s Upper threshold: 1,000 mAU

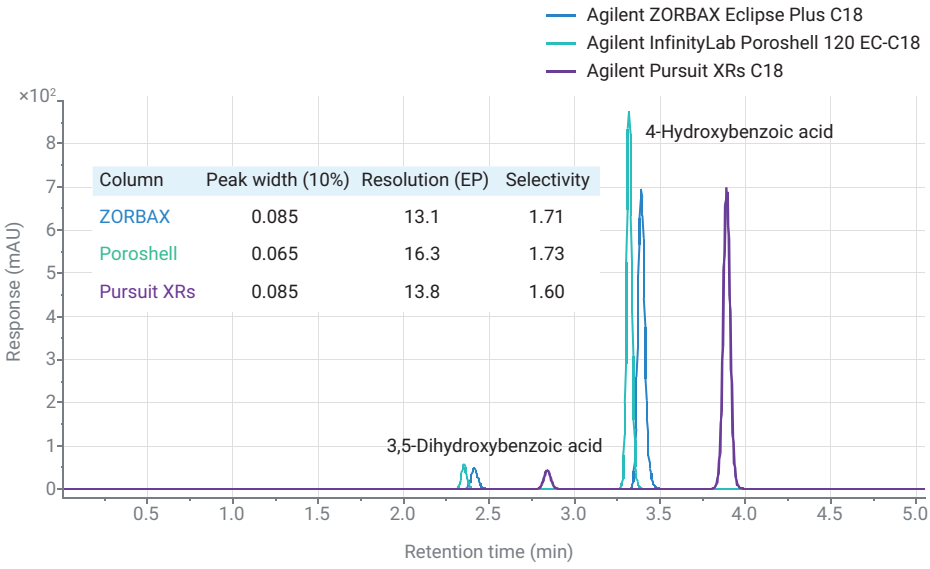

Figure 1. Chromatogram overlay of the 1,000 µg/mL calibration standard, separated on different columns with a focused gradient.

Sample loading and purification

The sample containing 40 mg/mL of the target compound was injected several times onto the ZORBAX column with increasing injection volume. Figure 2 shows an overlay of the separation of different sample amounts using the optimized gradient. Peak width of both compounds increased with increasing injection volume, reducing the resolution. However, baseline separation was maintained, up to an injection volume of 75 µL. Only at 85 µL did the peaks begin to overlap slightly. Therefore, further purification runs were carried out using 80 µL injection volume, which is equivalent to 3.2 mg target compound per run.

A similar loading experiment was conducted using an InfinityLab Poroshell 120 of the same length and diameter (data not shown). Due to the smaller peak width that is achieved with superficially porous particles, baseline resolution could be maintained with slightly higher load than on the ZORBAX column (until about 85 µL injection volume). For purification runs, 90 µL injection volume was chosen, equal to 3.6 mg target compound per run.

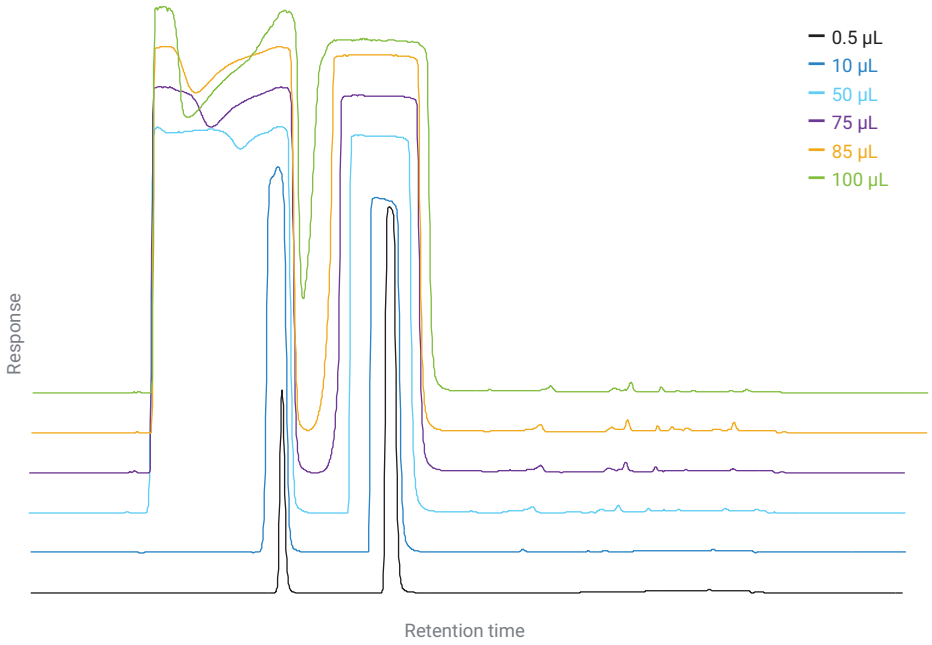

Figure 2. Chromatogram overlay of different sample volumes injected on an Agilent ZORBAX Eclipse Plus C18 column.

For further comparison, a third column was loaded with the sample. The Pursuit XRs stationary phase has a larger surface area and hence is specifically suited for high sample load. Despite identical length and diameter, the Pursuit XRs column was able to maintain baseline separation even at 100 µL injection volume (4 mg target compound); the other two columns showed coeluting peaks at this sample load (Figure 3). As this volume was the maximum possible with the autosampler used, purification on this column was carried out using this setting.

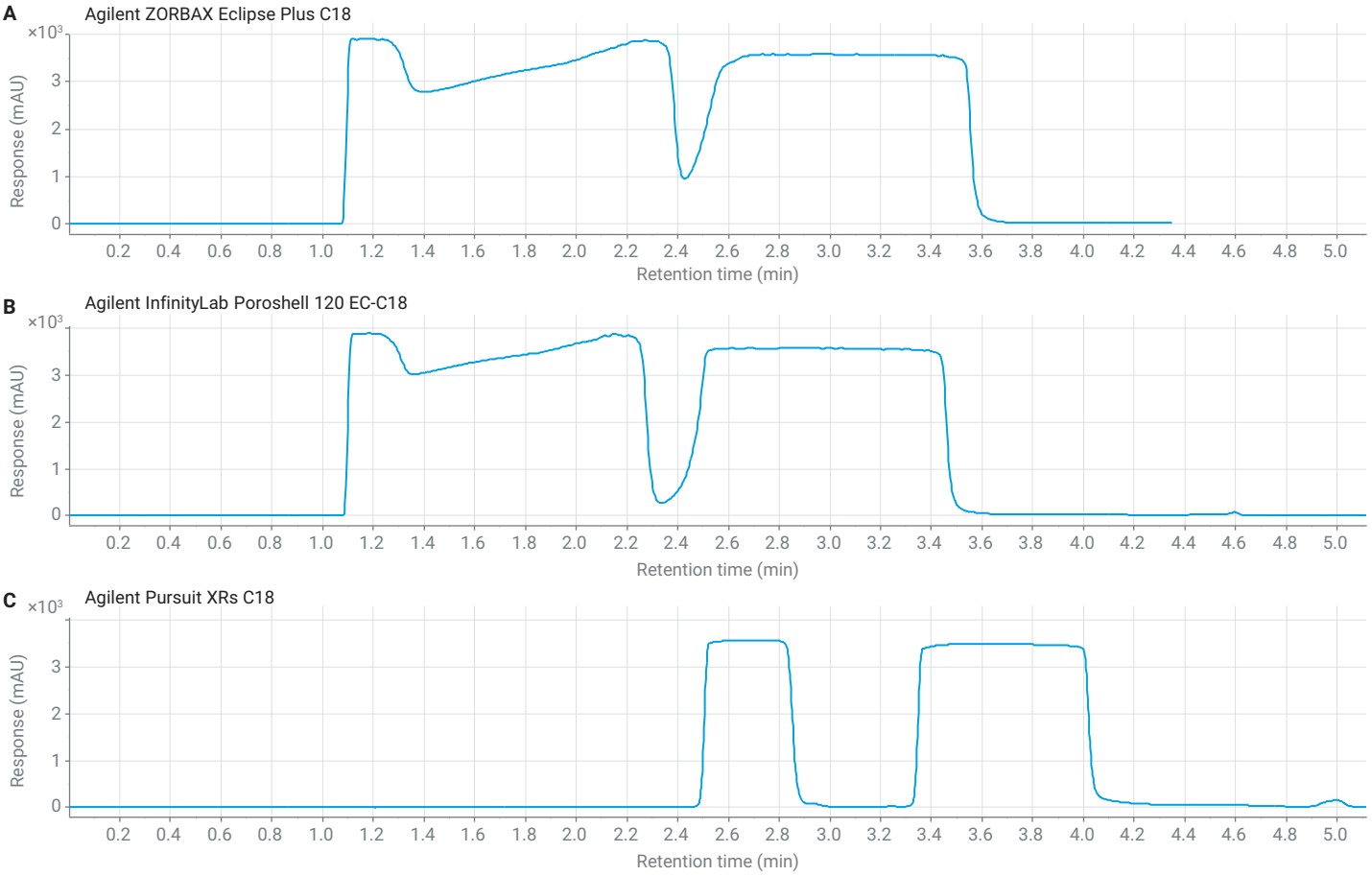

Figure 3. Comparison of the separation of 100 µL of sample on three different columns with same dimensions. Agilent Pursuit XRs stationary phase (C) maintains baseline separation, whereas the peaks on Agilent ZORBAX Eclipse Plus C18 (A) and Agilent InfinityLab Poroshell 120 EC-C18 (B) start to coelute.

Figure 4 shows chromatograms that compare the separation and fraction collection on the three columns tested. The first compound is clearly separated from the target compound on all columns. Fractions of six consecutive runs were collected and reanalyzed with respect to purity and recovery. Table 2 lists the reanalysis results. Highly pure fractions (>99%) were collected using any of the three columns tested. Recovery was 96 to 97% on average, and thus at a high level, too.

As the impurity was still well separated from the target compound using the Pursuit XRs column at 100 µL injection volume, another sample solution was prepared, which had the highest concentration possible using 50% aqueous acetonitrile as a solvent.

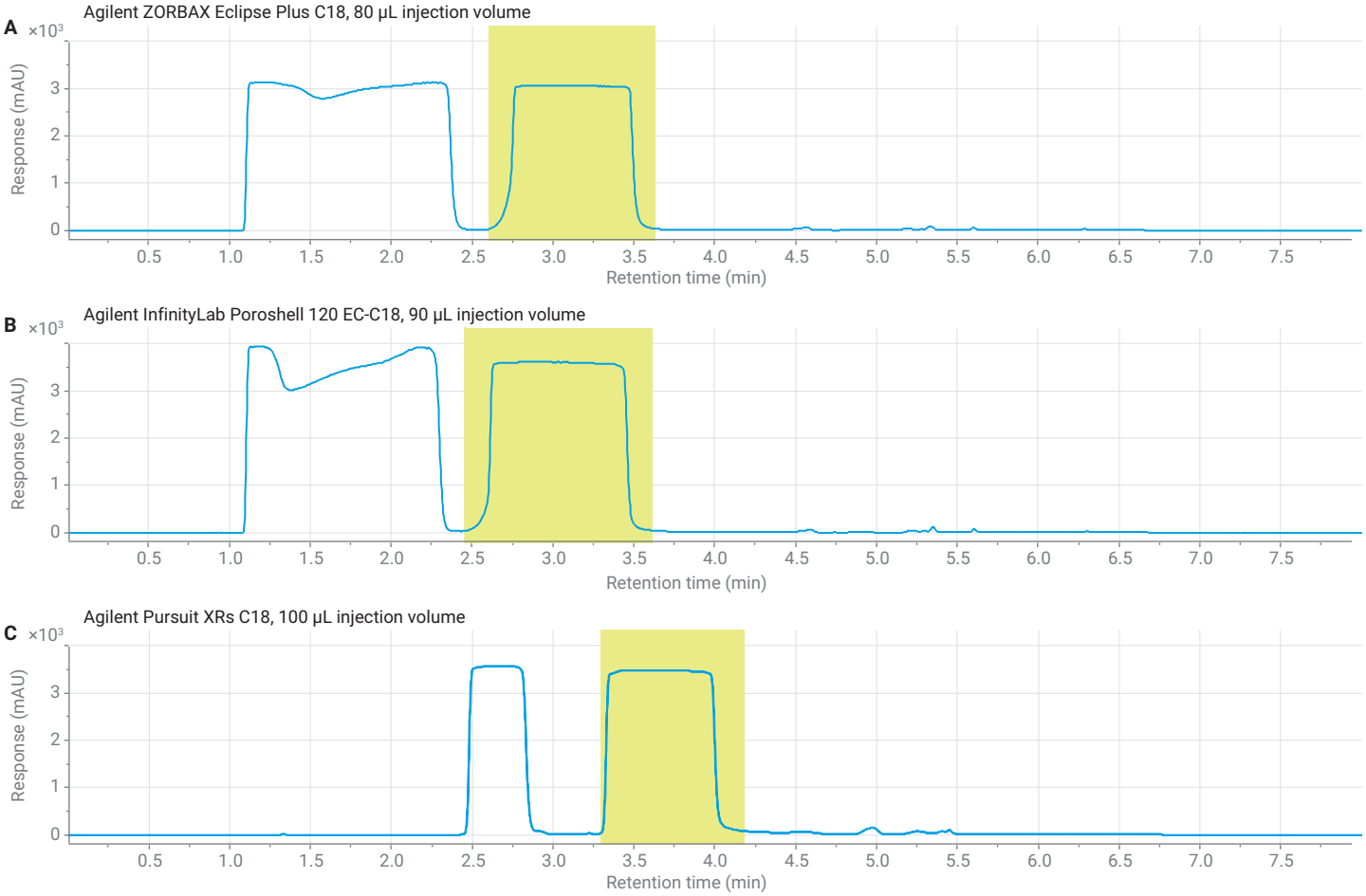

Figure 4. Purification runs separated on an Agilent ZORBAX Eclipse-Plus C18 (A), Agilent InfinityLab Poroshell 120 EC-C18 (B), and Agilent Pursuit XRs (C) column, each with 4.6 × 150 mm dimension. Injection volumes were 80, 90, and 100 µL, respectively. Yellow bands indicate fraction collection.

Table 2. Purification results (n = 6) of the 40 mg/mL sample separated on three different columns.

Column	Injection Volume (µL)	Purity	Recovery
ZORBAX Eclipse Plus C18	80	>99%	97%
InfinityLab Poroshell 120 EC-C18	90	>99%	97%
Pursuit XRs	100	>99%	96%

Loading experiments were repeated on the Pursuit XRs column using this sample. The maximum possible injection volume without significant coelution of the two peaks was 80 µL (see Figure 5). Fractions of multiple runs were collected and reanalyzed, showing that the high purity of >99% could be maintained, while recovery was 97% on average. These data show that the Pursuit XRs stationary phase can be loaded with 3.5 to 4 times the sample load of InfinityLab Poroshell 120 or ZORBAX stationary phase, while purity and recovery of the compounds are maintained at the same high level. This makes Pursuit XRs columns ideal for analytical-scale purification: in four runs, more than 50 mg of target compound could be purified. This amount can be sufficient for purposes such as activity testing or structure elucidation by NMR.

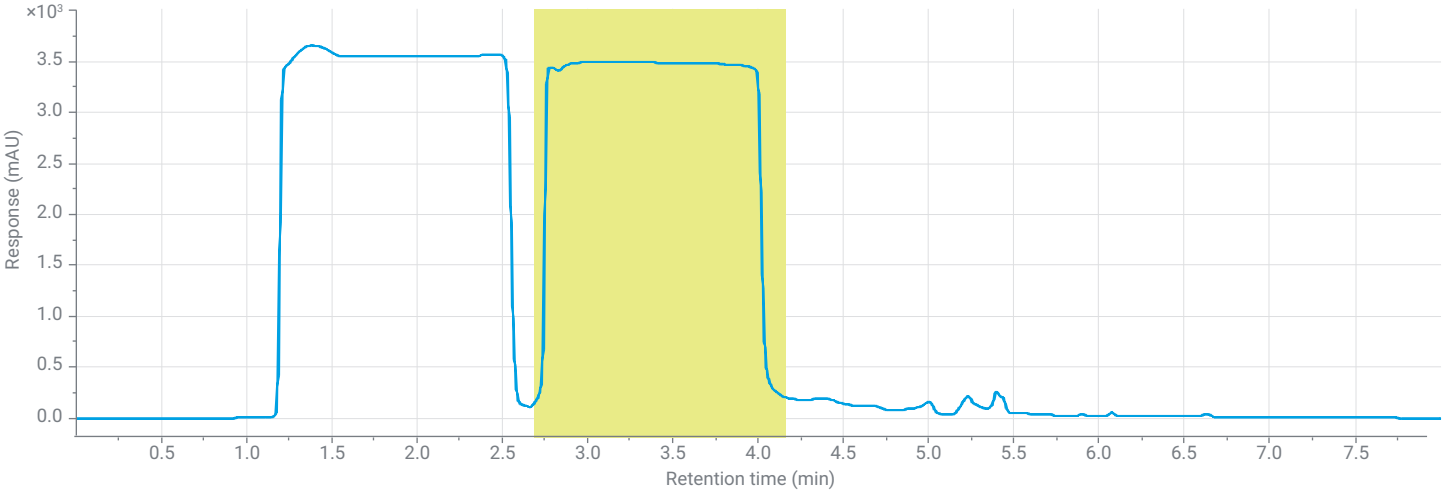

Figure 5. Chromatogram (254 nm) of the separation of the 160 mg/mL sample on an Agilent Pursuit XRs column, 80 µL injection volume (12.8 mg target compound). The yellow band indicates fraction collection.

Conclusion

Analytical-scale purification was carried out using an Agilent 1260 Infinity II LC, extended by an Agilent 1260 Infinity II Analytical-Scale Fraction Collector. Three different columns were evaluated with respect to maximum capacity of a small molecule sample. Purification of the target compound was successful on all columns, yielding a purity of >99% and recoveries between 96 and 97%. The Agilent Pursuit XRs column showed the highest sample capacity, which was 3.5 to 4 times higher than on Agilent ZORBAX Eclipse Plus C18 and Agilent InfinityLab Poroshell 120 EC-C18 columns. These data show that, for successful analytical-scale purification, an analytical Agilent HPLC system can simply be extended by a fraction collector, with no need for further investment into hardware or software.

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