Application Note

Pharma

Agilent InfinityLab

Developing Fast Purification Methods for Natural Products Using an Agilent InfinityLab Poroshell 120 SB-C18 Preparative LC Column

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Abstract

A fast and simple purification method for withaferin A in ashwagandha extract was successfully developed using an Agilent InfinityLab Poroshell 120 SB-C18 preparative LC column. The InfinityLab Poroshell 120 column provided better resolution and a 45% decrease in run time over a traditional preparative column. The performance benefits of InfinityLab Poroshell preparative columns, especially at high flow rates, align well with the needs of pharmaceutical discovery laboratories and other high-throughput environments.

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Introduction

Superficially porous particle (SPP) chromatography columns are a popular choice for analytical method development. Columns packed with SPPs have higher efficiency and lower backpressure than their totally porous particle (TPP) counterparts.[1] Figure 1 compares the two particle types.[1] A superficially porous particle consists of a solid silica core surrounded by a porous outer layer. The solid core prevents the analyte from traveling too far into the particle, shortening its diffusion path, resulting in a narrower chromatographic peak.

Van Deemter curves provide a simple way to compare the efficiency of SPP and TPP columns. An isocratic separation of a void volume marker and analyte mixture is run across a wide range of flow rates. The efficiency, or plate number (N), of the analyte is calculated using Equation 1, where tr is the analyte retention time and $W_{1/2}$ is the peak width at half height.

$$N = 5.54 (t_r/W_{1/2})^2$$

Equation 1. USP plate number calculation.

From N, the plate height (H) can be calculated using Equation 2, where L is the length of the column.

$$H = L/N$$

Equation 2. Plate height calculation.

H is plotted against linear velocity (u), which is calculated from the retention time of the void volume marker (t_0) using Equation 3.

$$u = L/t_0$$

Equation 3. Linear velocity calculation.

The Van Deemter curve for a 4 µm SPP column and a 5 µm TPP column of the same dimension are shown in Figure 2.

Keeping in mind that H is inversely proportional to N, the curve with the smaller values of H has better performance. Looking at the curves, it is obvious that the 4 µm column performs better than its 5 µm counterpart. The linear velocity at the minimum of each curve (u_{opt}) corresponds to the flow rate at which the column will have the highest efficiency. The SPP column's u_{opt} is 1.5 times faster than the TPP column's u_{opt}, which means that the SPP column can provide a better separation in less time than the TPP column.

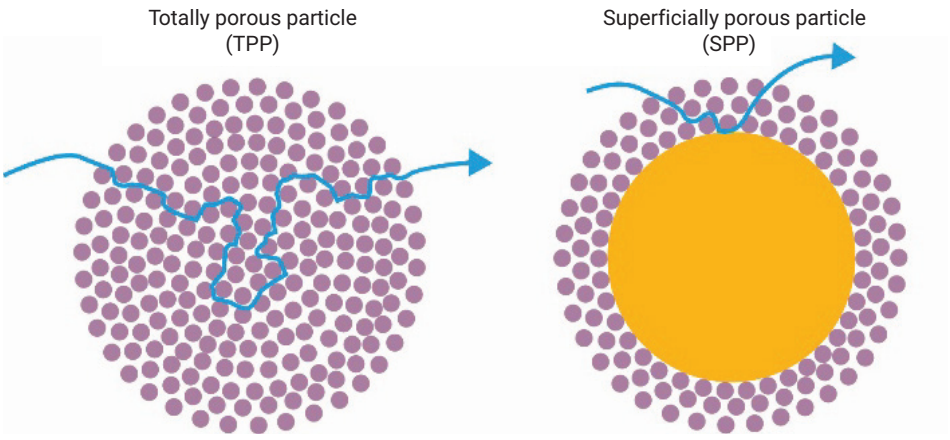

Figure 1. Analyte diffusion path for a totally porous particle versus a superficially porous particle.

The data can then be fit to the Van Deemter equation to calculate the A, B, and C terms (Equation 4).

$$H = A + B/u + Cu$$

Equation 4. Van Deemter equation.

These constants represent the main contributions to band broadening. The impacts of the A term (eddy diffusion) and the B term (longitudinal diffusion) are significant at low linear velocities (<0.5 mm/s) and are discussed elsewhere.[2] At normal or above normal chromatographic linear velocities (>1.5 mm/s), the C term (resistance to mass transfer) is the main contributor to band broadening. For a TPP column, the analyte's long diffusion path within the particle causes band broadening. The resistance to mass transfer only gets worse as the velocity increases. In contrast, the porous layer in an SPP column is much shorter. As a result, H is considerably lower for the SPP column, and the slope of the Van Deemter curve at high flow rates (>3 mm/s) is shallower than it is for the TPP column. A lower H value allows the user to run SPP columns at higher flow rates without experiencing a significant decrease in performance.

Pharmaceutical discovery laboratories and other high-throughput environments regularly utilize preparative chromatography to purify large batches of samples. They require small amounts (10 to 100 mg) of high purity fractions for downstream workup and characterization. Given the fast-paced nature of these environments, not much time can be spent on method development. Therefore, these customers could benefit from SPP columns that provide high resolution and throughput.

SPP columns have not been widely adopted in preparative LC for several reasons. Most analytical instrumentation has been designed to minimize system volume and exceed pressures of

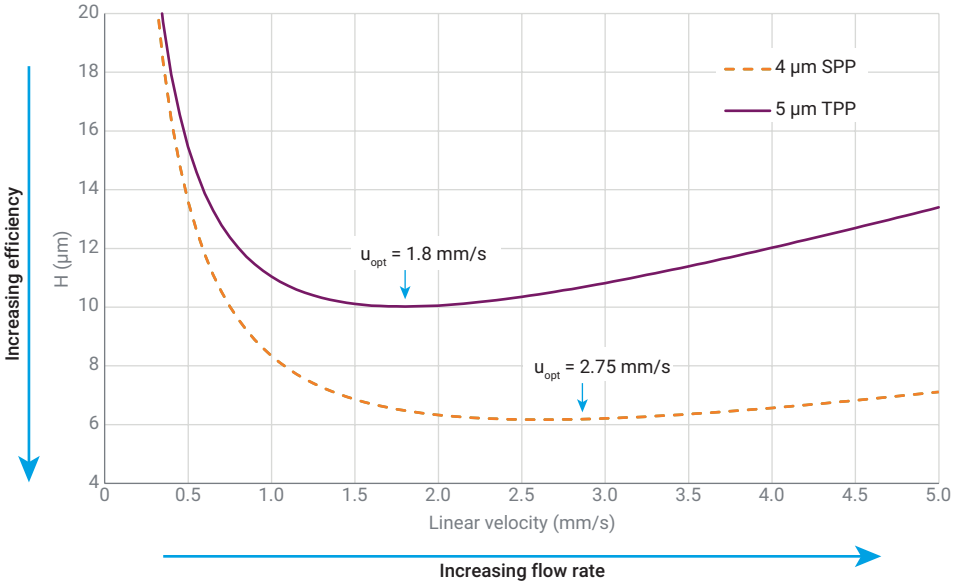

Figure 2. Van Deemter plots of 4 µm superficially porous particle (SPP) and 5 µm total porous particle (TPP) columns.

400 bar, whereas most preparative instrumentation has large system volume to prevent overpressuring the pump. Using smaller-sized SPPs in a preparative column could cause excessive pressure, and a large system volume could negate the chromatographic benefits.

The new line of Agilent InfinityLab Poroshell 120 preparative LC columns, packed with 4 µm superficially porous particles, mitigates these concerns. This particle size is large enough to operate well within the pressure range of traditional preparative instruments without significant band broadening, while still performing better than traditional TPP preparative columns, especially at higher flow rates.

Purification of bioactive components in natural products represents a similar challenge to that of drug candidates in crude mixtures – both have complicated matrices. *Withania somnifera* (L.) Dunal, well known as ashwagandha, is a plant that contains many withanolides, which are natural steroids. Withaferin A (WFA) is the most bioactive withanolide in ashwagandha. This study focuses on the efficacy of a generic purification method for WFA on both a 4 µm SPP and traditional 5 µm TPP column at standard and elevated flow rates.

Experimental

Instrumentation

All work was performed on an Agilent 1290 Infinity II autoscale preparative LC system.

Columns and supplies

Purification methods were developed on two columns. The first column was an InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm preparative LC column (part number 670150-902). The second was a traditional C18, 19 × 150 mm, 5 µm preparative LC column. Ashwagandha extract in 2:1 ethanol:water (100 mg/mL) was purchased from Banyan Botanicals (Albuquerque, NM). Withaferin A standard was purchased from Sigma-Aldrich (St. Louis, MO). LC grade solvents were acquired from Burdick and Jackson. The extract was filtered using an Agilent Captiva premium syringe filter, 0.2 µm (part number 5190-5116).

Gradient separation at optimal flow rate

The optimal flow rate for each column was determined by its respective Van Deemter curve (not shown). A previously published analytical method was used to select the mobile phase.[3] A generic gradient was run on both columns, adjusting gradient time to keep the number of column volumes in the gradient consistent. A summary of run conditions is shown in Table 1.

Table 1. Summary of data acquisition parameters at optimal flow rate.

Parameter	Agilent	Competitor
Column	Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm	Competitor C18, 19 × 150 mm, 5 µm
Flow Rate	25 mL/min	17 mL/min
Mobile Phase	A: Acetonitrile + 0.1% formic acid B: Water + 0.1% formic acid	
Gradient	5 to 95% B in 15 min	5 to 95% B in 18 min
Injection Volume	1,000 µL	
Wavelength	220 nm	

Gradient separation at elevated flow rate

Each column was run at 50% above its optimal flow rate. The gradient time was adjusted to keep the number of column volumes in the gradient consistent. A summary of run conditions is shown in Table 2.

Table 2. Summary of data acquisition parameters at elevated flow rate.

Parameter	Agilent	Competitor
Column	Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm	Competitor C18, 19 × 150 mm, 5 µm
Flow Rate	37.5 mL/min	25.5 mL/min
Mobile Phase	A: Acetonitrile + 0.1% formic acid B: Water + 0.1% formic acid	
Gradient	5 to 95% B in 10 min	5 to 95% B in 12 min
Injection Volume	1,000 µL	
Wavelength	220 nm	

Results and discussion

Figure 3 is a comparison of both columns run at their respective optimal flows. The total mass on each column is 100 mg. The WFA peak elutes at 8.59 minutes on the Poroshell column and at 9.66 minutes on the competitor column. Both columns provide separation between the WFA peak and the adjacent impurities. The impurities have baseline separation with WFA on the Poroshell column. However, on the competitor column, one of the impurities appears as a shoulder of the WFA peak.

When the flow was increased by 50% (Figure 4), the Poroshell column maintained separation between WFA (retention time = 5.77 minutes) and its impurities. On the competitor column, the leading impurity partially coelutes with WFA (retention time = 6.47 minutes), and the trailing impurity completely coelutes with the target compound.

The competitor column was only able to separate impurities at its optimal flow rate, which was an 18-minute gradient. Meanwhile, the faster gradient on the Poroshell column was able to separate all peaks in 10 minutes, reducing the run time by 45%.

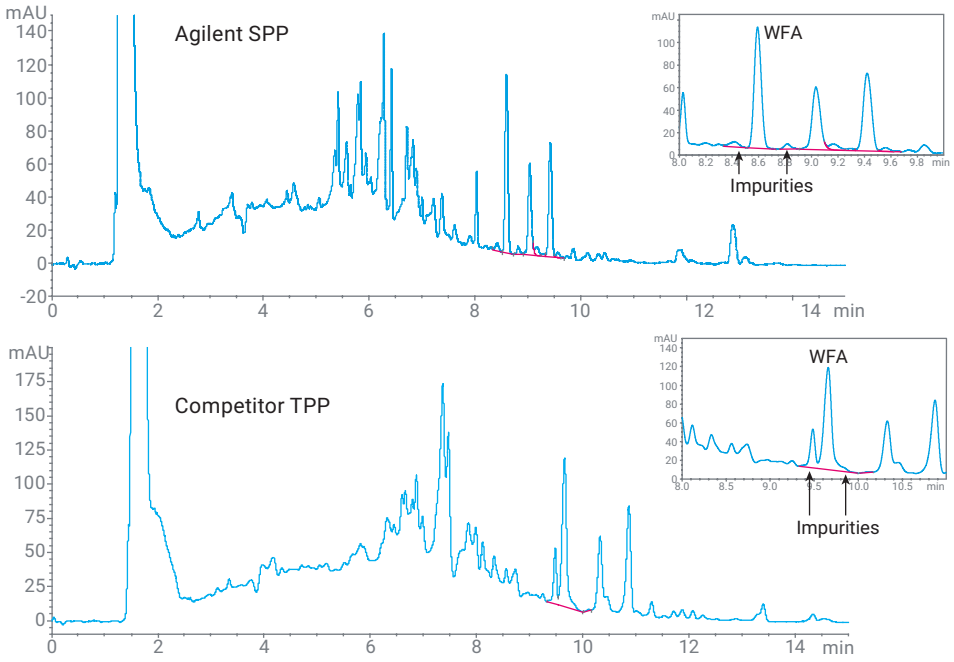

Figure 3. Withaferin A separation on preparative LC columns. Gradient time: Agilent – 15 minutes; competitor – 18 minutes.

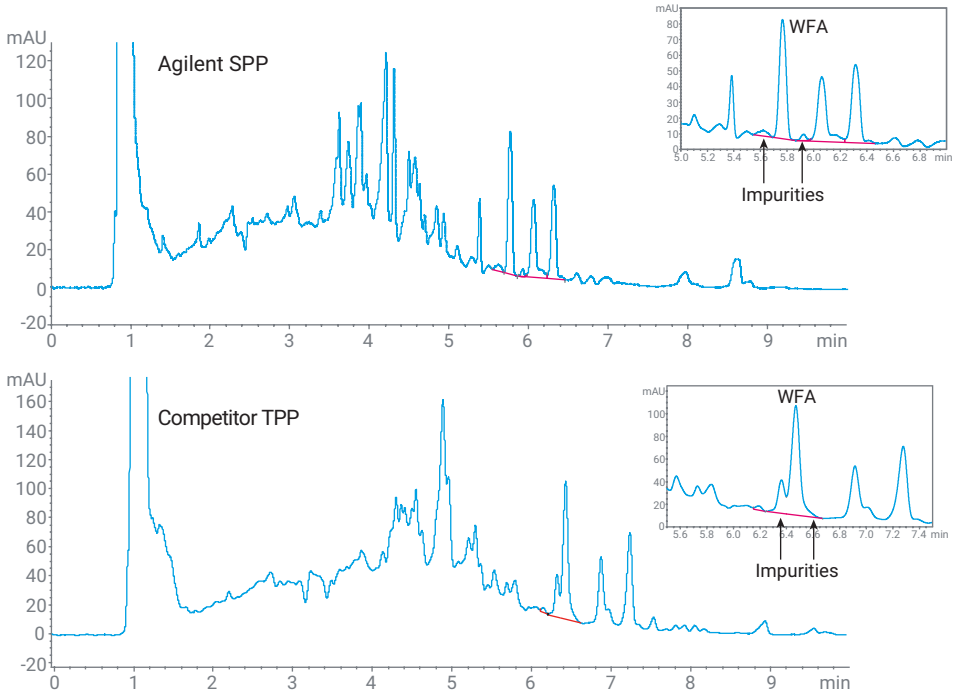

Figure 4. Withaferin A separation on preparative LC columns. Gradient time: Agilent – 10 minutes; competitor – 12 minutes.

Conclusion

The Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm preparative LC column successfully separated withaferin A from adjacent impurities with minimal method development. When run at 1.5× the optimal flow, the InfinityLab Poroshell 120 column maintained the separation whereas the competitor column had significant coelution. The faster Poroshell method results in a 45% decrease in run time over the competitor method at standard flow. The speed and loadability of the InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm preparative LC column are well suited to the needs of high-throughput discovery laboratories.

References

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