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응용 자료

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Abstract

This application note illustrated a systematic preparative HPLC method development to isolate a minor component from peppermint extract using an AutoPurification System. The overall workflow included screening different column chemistries, applying focused gradients, scaling up, and employing an ACD injection scheme. With proper scale-up from an optimized analytical chromatographic condition, employing ACD increased the sample loading by five-fold while maintaining the resolution on the preparative scale. The techniques demonstrated in this case study have general applicability for laboratories routinely performing natural product isolation using preparative HPLC.

Benefits

- Focused gradients improve the resolution of closely eluting components, thereby increasing the column loading for more efficient target compound purification.
- At-column dilution alleviates the peak distortion and loss of resolution attributed to the injection of large volumes of strong solvent, leading to improved resolution, column loading, and overall productivity in natural product isolation.

Introduction

Natural products are widely used in the pharmaceutical, food supplement, nutraceutical, and alternative

medicine industries.[1-4] Chromatography has long been an integral part of natural product research, including chemical fingerprinting, structural elucidation, and isolation of bioactive compounds on the preparative scale. Since natural product extracts are usually complex mixtures comprised of many different compound classes with a variety of functional groups, acid-base properties, and molecular sizes, reversed-phase liquid chromatography (RPLC) often lends itself as the technique of choice for the analysis and purification of natural products, largely due to its general applicability.

The use of preparative high performance liquid chromatography (prep HPLC) has become a mainstay in the isolation of most classes of natural products over the last ten years.[4] In target compound purification, adequate resolution between target analytes and their adjacent interference peaks is a prerequisite for successful preparative chromatography. Typical approaches for improving resolution include the following: evaluating different stationary phases, mobile phases, and modifiers; changing the temperature of the separation; and varying the gradient slope. However, the ultimate objective for prep chromatography is to efficiently collect target compounds of desired purity. Consequently, experimental parameters such as sample diluents and injection techniques and their impact on solvent consumption and productivity should also be considered in the overall method development strategy.[5] This is particularly important for natural product isolation, since the desired compounds often exist at low concentrations within very complex matrices. To that end, at-column dilution (ACD) has proven to be a viable alternative to conventional injection techniques. ACD allows for injections of large volumes of sample in strong solvents while preserving chromatographic integrity and resolution, thereby improving overall purification productivity.[6]

This application note uses peppermint extract[7] to demonstrate a typical prep HPLC method development workflow, systematically improving resolution and column loading for the isolation of a minor component in a natural product.

Experimental

Sample description

A total of 3.3 g dried peppermint was extracted with a 20 mL 80:20 methanol/water mixture for six hours at room temperature.[7] The supernatant was filtered with an Acrodisc Syringe Filter with GHP Membrane, 25 mm, 0.45 µm

LC conditions

System:	AutoPurification
Columns:	XSelect CSH C_{18} 4.6 x 100 mm, 5 µm; XSelect CSH Phenyl-Hexyl 4.6 x 100 mm, 5 µm; XSelect CSH Fluoro-Phenyl 4.6 x 100 mm, 5 µm; XSelect C_{18} Prep OBD 19 x 100 mm, 5 µm
Mobile phase A:	0.1% trifluoroacetic acid (TFA) in water
Mobile phase B:	0.1% TFA in acetonitrile
UV wavelength:	220 nm
Flow rate:	1.46 mL/min for analytical and 25.0 mL/min for preparative experiments

The analytical and preparative gradients used in this study are summarized in Table 1. For the ACD injections, a separate ACD pump delivered a constant 1.3 mL/min acetonitrile (5% of the total flow rate) directly to the injection valve while the gradient pump delivered the gradient at a flow of 23.7 mL/min. The two flow streams were combined at the head of the column. The number of column volumes (CV) per gradient segment was constant for all three methods, ensuring that the chromatography at the prep scale was identical to the chromatography at the analytical scale. Other key experimental parameters are listed in the respective figure captions.

Analytical		Prep *conventional injection		Prep **ACD	
Time (min)	%B	Time (min)	%B	Time (min)	%B
0.0	5.0	0.0	5.0	0.0	0.0
1.0	17.4	0.4	5.0	4.3	0.0
11.7	25.4	1.4	17.4	5.3	12.4
12.2	95.0	12.2	25.4	16.1	20.4
17.2	95.0	12.6	95.0	16.5	90.0
17.4	5.0	17.6	95.0	21.5	90.0
25.4	5.0	17.8	5.0	21.7	0.0
		25.8	5.0	29.7	0.0

** 2-mL loop, system volume = 6.3 mL*

*** 5-mL loop, system volume = 9.3 mL; ACD pump flows at 1.3 mL/min, total flow rate was 25.0 mL/min*

Table 1. Gradients used in the study. The analytical flow rate was 1.46 mL/min and the preparative flow rate was 25.0 mL/min.

Results and Discussion

Column screening and focused gradients

Prep chromatography shares many basic principles with its analytical counterpart. As a result, preparative HPLC method development often starts with an analytical LC followed by geometric scale-up to prep. LC/UV chromatograms of the peppermint extract using a generic gradient on three different columns is shown in Figure 1. The target compound, as well as other minor components in the crude extract, was best resolved on the XSelect CSH C_{18} Column, as shown in Figure 1A. The XSelect CSH C_{18} Column chemistry was, therefore, chosen for all ensuing experiments.

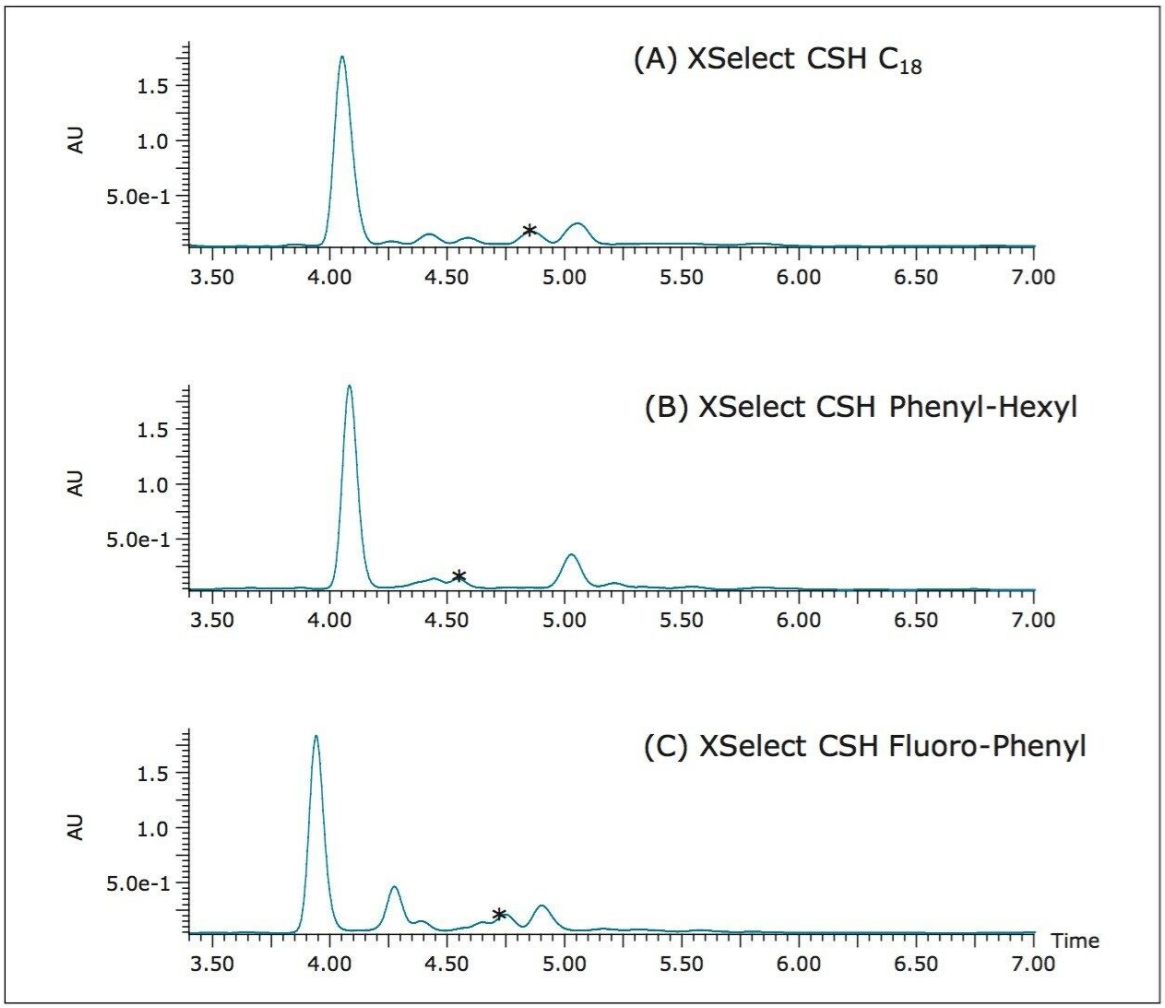

Figure 1. LC/UV chromatograms of the peppermint extract obtained on three different columns: (A) XSelect CSH C_{18} ; (B) XSelect CSH Phenyl-Hexyl; and (C) XSelect CSH Fluoro-Phenyl. The asterisk denotes the target compound peak. All column dimensions were 4.6 x 100 mm with 5-µm particles. The injection volume was 10 µL. A generic gradient from 5% to 95%B in 12 minutes was used, and the total run time was 20 minutes.

Since the analyte of interest eluted at ~22% B in the initial generic gradient, shown in Figure 2A, focused gradients ranging from 17% to 25% B were employed to further improve the resolution, as shown in Figures 2B and 2C. Focused gradients increase the residence time of closely eluting compounds on the column for better partition, improving the selectivity (α) between compounds with minute polarity differences.[8] However, decreased gradient slope also increases retentivity (k′), which in turn leads to broader peaks, reduced peak

heights, prolonged run time, and greater solvent cost for prep chromatography. Therefore, caution should be exercised when using focused gradients to ensure the balance between resolution and run time. In the current study, at 0.72 %B/CV, shown in Figure 2C, the target peak was clearly baseline resolved from all adjacent peaks with a total run time of 25 minutes. For a shorter run time, the method could be terminated immediately after target peak collection with column washing steps to follow.

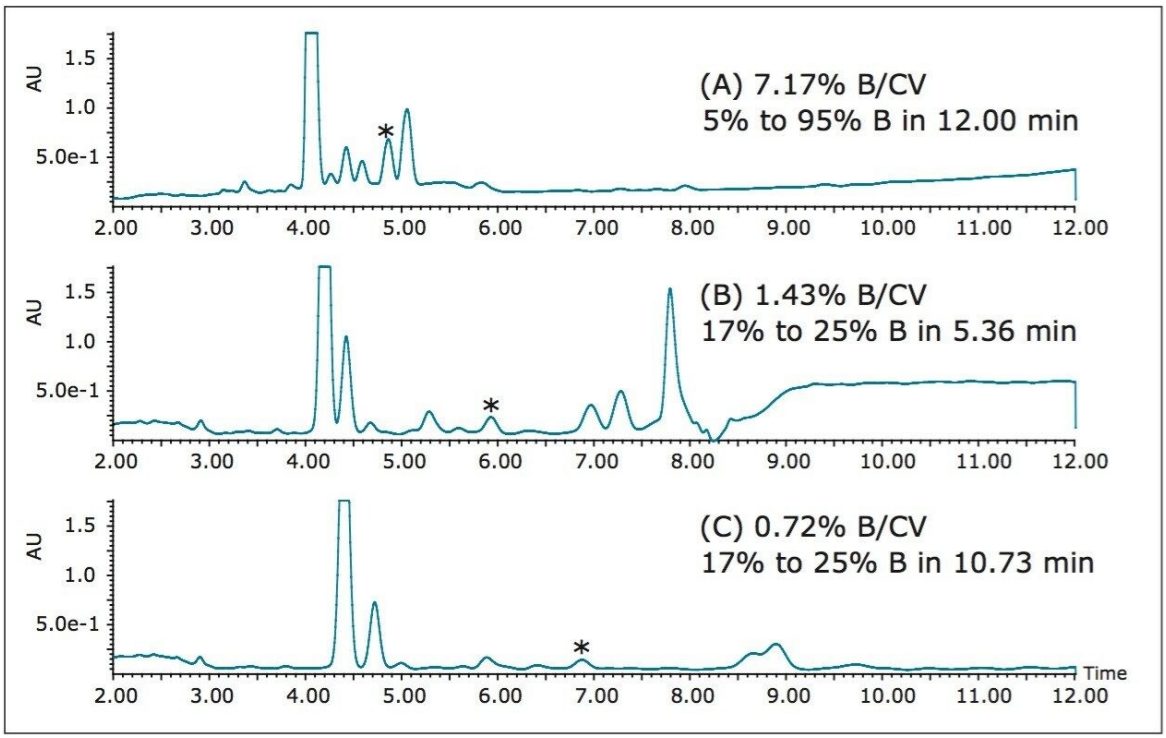

Figure 2. LC/UV chromatograms of the peppermint extract on an XSelect CSH C_{18} 4.6 x 100 mm, 5 µm Column using three different gradients.

Scale-up

Proper scaling from analytical to prep requires the gradient slope (change in %B/CV) to be maintained for each step of the chromatography.[9] The flow rate and the injection volume are scaled geometrically. Table 2 summarizes the flow rate and injection volumes used when the chromatography was scaled from the analytical column to the preparative column. The properly scaled focused gradient methods are shown in Table 1.

	Analytical	Prep
Column dimension	4.6 x 100 mm	19 x 100 mm
Column volume	1.4 mL	23.8 mL
Flow rate	1.46 mL/min	25.0 mL/min
Injection volume	30 µL	512 µL
Injection volume	40 µL	682 µL

Table 2. Summary of scale-up parameters.

A loading study (not shown) performed on the XSelect CSH C_{18} 4.6 x 100 mm Column showed that 30 µL was the maximum volume that could be injected without losing resolution between the target compound and the impurity. Geometrically, an injection volume of 30 µL on the analytical column scales to 512 µL on a 19 x 100 mm prep column. In the conventional injection technique, the target peak is resolved from its closely eluting neighbors, as shown in Figure 3A. Further increasing the injection volume, such as the 682-µL injection in Table 2, shown in Figure 3B, resulted in decreased resolution between the peak of interest and its closely eluting neighbors. For the peppermint extract in this study, a sensible injection volume for a 19 x 100 mm column was, therefore, limited to 512 µL using the conventional injection technique. The observed resolution loss was partially due to the strong solvent used as the sample diluent. Natural products sometimes require the use of strong organic solvents, such as methanol, ethanol, and acetone, to extract them from the sample matrix. However, large injection volumes of strong solvents often distort chromatography and result in a loss of chromatographic resolution. Sample molecules entering the column in a strong solvent do not retain. Instead, they move through the column until the strong solvent is diluted sufficiently by the initial-strength mobile phase to promote retention. As a result, the samples retain on the column as wide bands. The samples elute from the column as broad, poorly resolved peaks, thereby limiting the chromatographic efficiency and overall productivity.

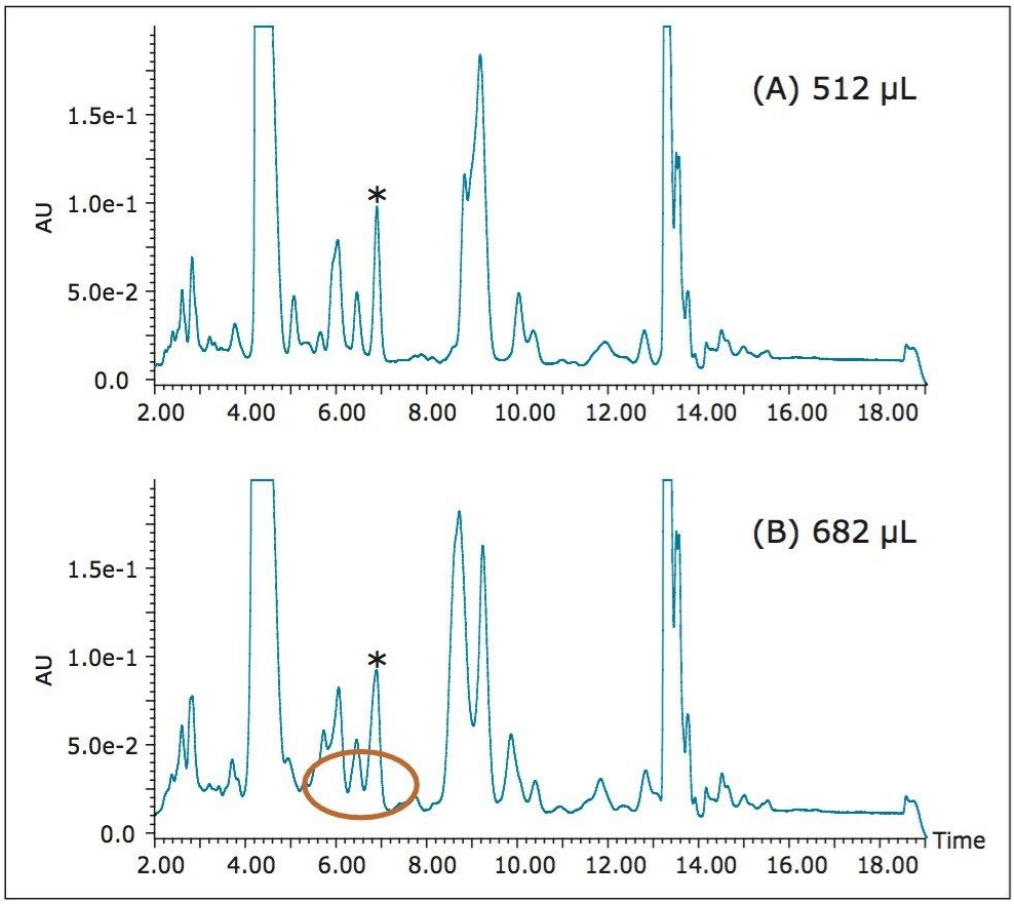

Figure 3. 512-µL and 682-µL injections of peppermint extract on an XSelect C_{18} Prep OBD 19 x 100, 5 µm Column with the Waters AutoPurification System plumbed in the conventional mode.

At-column dilution

ACD, an alternative injection technique, permits the injection of large volumes of strong solvents and concurrently improves sample solubility, column loading, and resolution.[6] With ACD, the chromatographic system is plumbed so that the sample in strong solvent is diluted at the head of the column with aqueous mobile phase. The sample is deposited on the column and the strong solvent flushes from the column before sample elution begins. Once the gradient is initiated, the sample components elute as narrow, sharply resolved bands, as shown in Figure 4. The strong solvent effect is effectively alleviated and the resolution is preserved. Furthermore, because the sample is continually surrounded by organic solvent until the point of dilution at the head of the

column, no sample precipitation occurs.

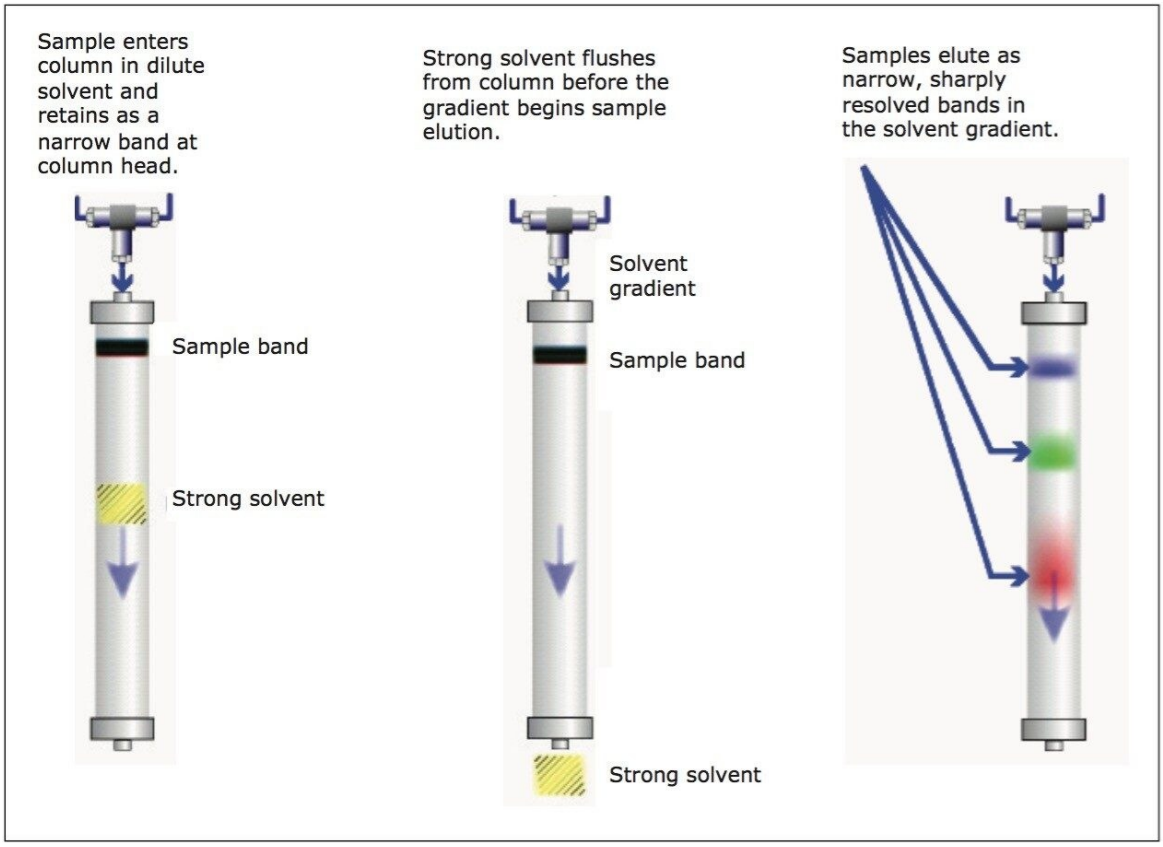

Figure 4. Schematic of at-column dilution.

Figures 5A and 5B show the chromatograms using the conventional injection technique and ACD with the same 682-µL injection volume. Clearly, the one with the ACD, as shown in Figure 5B, provides improved resolution of the target compound from the closely eluting neighboring peaks. With ACD, a maximum injection volume of 2.7 mL was possible without the loss of resolution, as shown in Figure 5C. This represents a five-fold increase in column loading compared to the 512-µL injection volume using the conventional injection technique.

It is important to note that the initial hold at the beginning of the ACD method ensures a complete sweeping of the sample loop. For example, a 5-mL loop was used for the 2.7-mL sample injection in Figure 5C, so an extra four minutes was added to the initial hold at 1.3 mL/min, as shown in Table 1.

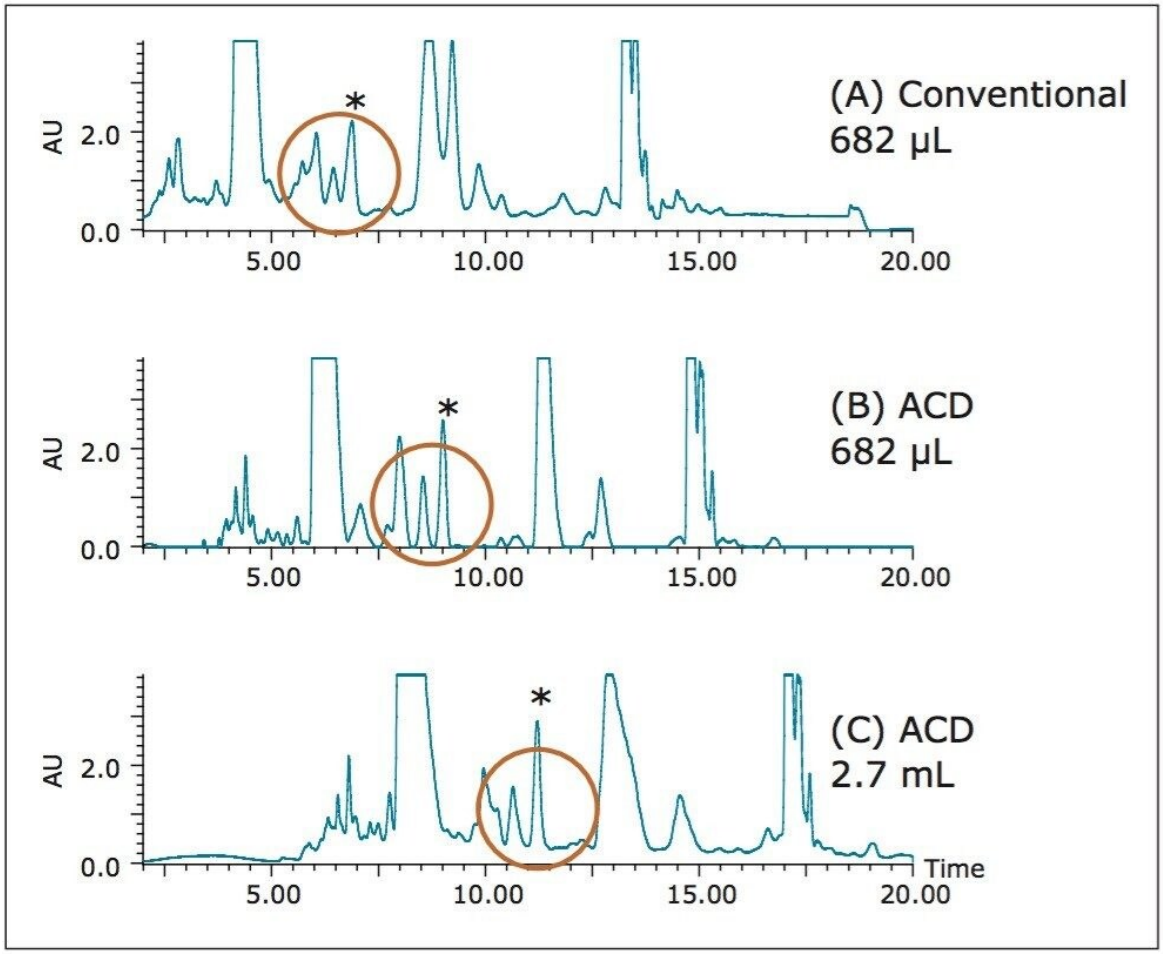

Figure 5. Comparison of prep LC/UV chromatograms with 682-µL and 2.7-mL injections of peppermint extract on an, XSelect C_{18} Prep OBD 19 x 100, 5 µm Column with the AutoPurification System plumbed in conventional and at-column dilution modes.

The target minor component was successfully isolated from the 2.7-mL sample load in a fraction with a purity of 94% by UV analysis, as shown in Figure 6.

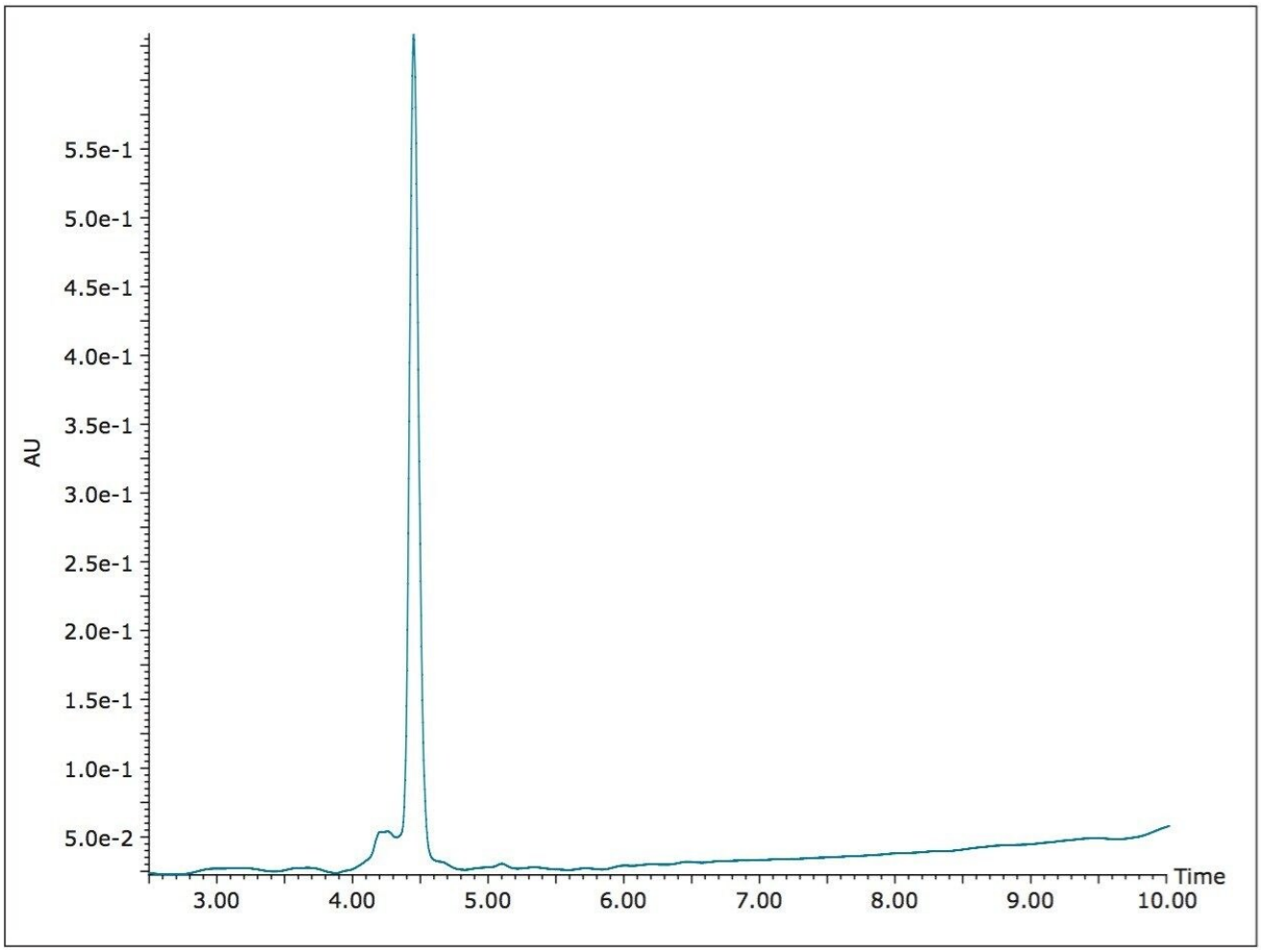

Figure 6. Purity analysis by UV of the fraction from a 2.7-mL injection with ACD.

Conclusion

This application note illustrated a systematic preparative HPLC method development to isolate a minor component from peppermint extract using an AutoPurification System. The overall workflow included screening different column chemistries, applying focused gradients, scaling up, and employing an ACD injection scheme. With proper scale-up from an optimized analytical chromatographic condition, employing ACD increased the sample loading by five-fold while maintaining the resolution on the preparative scale. The techniques

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References

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