

IMPROVED PURIFICATION OUTCOMES FOR PROBLEMATIC COMPOUNDS USING AN INERT COLUMN WITH POLAR SORBENT

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INTRODUCTION

Whether the goal is to obtain enough intermediate for the next step in a synthetic scheme, to polish a final product, or to create a suitable standard for compound identification, preparative liquid chromatography remains a primary technique for isolating compounds from complex mixtures. Recovering the target compound at the desired purity and yield in the least amount of time is of utmost importance.

Polar targets that do not retain, or “sticky” compounds that seemingly disappear on the column are two groups that many consider to be ‘problematic’ in purification. “Sticky” compounds, also known as non-specific adsorbers (NSAs), are metal-sensitive targets what can adsorb to positively charged metal surfaces in the LC system flow path and column, making them difficult to detect and collect effectively. Although other methods, such as column pre-conditioning, can be implemented for isolating compounds known to be interactive, this option can be time-consuming and lead to sample loss and excessive solvent waste.

Although lab scale purifications in which tens to hundreds of milligrams of target compound are isolated from crude mixtures are still performed routinely, the trend toward smaller scale purification is becoming more prevalent. Laboratories are increasingly opting to isolate only a few milligrams of target compound for preliminary studies. Successful target candidates are then isolated in greater amounts to satisfy ensuing experiments. Whether the objective is to isolate a small amount of target compound for exploratory studies, or to purify greater amounts for their use as reference standards, a column which can resolve eluting impurities efficiently is required.

Echinacea is an herbaceous flowering plant in the daisy family^{1,2} and is one of the most important medical herbs widely used to treat the common cold and other infectious diseases.³ One of the most abundant compounds in echinacea is chicoric acid (Figure 1), a phenylpropanoid and caffeic acid derivative.^{4,5} In this scale-up study, chicoric acid, a suspected “sticky” target polar compound was isolated from a complex echinacea tea extract using two inert XSelect™ HSS T3 MaxPeak Premier OBD Columns with different particle sizes and dimensions (3.5 µm 10 x 100 mm and 5 µm 10 x 150 mm). The MaxPeak Premier OBD preparative HPLC columns are considered ‘inert’ because they are enabled with Waters High Performance Surface (HPS) Technology. The chicoric acid isolated using the 3.5 µm column was of equivalent UV purity to the product isolated using the 5 µm column, with a 34% savings in run time. Smaller particle preparative isolations using columns with inert hardware are a practical option for those laboratories where reduced amounts of target are needed for projects conducted on accelerated timelines.

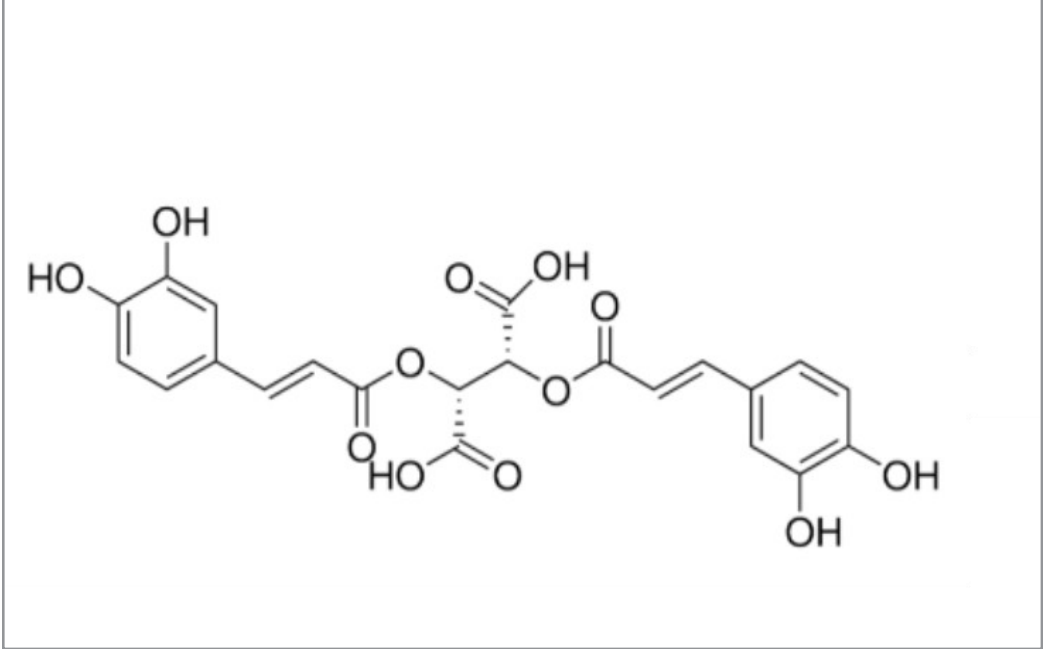


Figure 1. Chemical structure of chicoric acid

METHODS

Chicoric Acid Isolation Conditions:

Analytical Columns and flow rate: ACQUITY™ Premier HSS T3 and ACQUITY UPLC™ HSS T3 Columns; 1.8 µm, 2.1x50 mm; 0.35 mL/min

Prep Columns and flow rate: XSelect HSS T3 OBD Prep Column and XSelect Premier HSS T3 OBD Prep (custom) Columns, 5 µm, 10x150 mm; XSelect Premier HSS T3 OBD Prep Column, 3.5 µm, 10x100 mm; 5.4 mL/min

Mobile phase A: Water with 0.1% Formic Acid

Mobile phase B: Methanol with 0.1% Formic Acid

Wavelengths: 254 and 330 nm

Injection volumes: Analytical 5 µL; Preparative 227 µL (10x100 mm column); 340 µL (10x150 mm column)

Sample: Four lemon and echinacea herbal tea bags in 16 ounces of hot water were stirred at room temperature overnight in a glass bottle. The tea extract was filtered with a Thermo Scientific Nalgene® Rapid Flow 90 mm Filter Unit. The glass bottle was rinsed two times with ~10 mL Milli-Q® Water and the rinses were added to the filtered tea extract. The total volume of tea extract was ~300 mL.

Instrumentation

Analytical: ACQUITY UPLC H-Class System; Quaternary Solvent Manager, Sample Manager FTN-H, Column Manager, TUV Detector, ACQUITY QDa™ Detector

Prep: Waters AutoPurification™ System; 2545 Binary Gradient Module, 2767 Sample Manager, System Fluidics Organizer, 2998 Photodiode Array Detector

RESULTS AND DISCUSSION

High mechanical strength, extensive compatibility with chemicals, and ease of fabrication make stainless steel an attractive material for the manufacture of high-performance liquid chromatography columns and instruments. Despite these favorable characteristics, stainless steel can negatively impact the peak shape and recovery of some compounds. Negatively charged molecules can interact ionically with the positively charged metal oxide surfaces of the column and LC flow path in neutral and acidic pH environments and lead to non-specific adsorption.⁶ This non-specific adsorption (NSA) occurs with stainless steel and titanium, as well as other metal alloys. Metal sensitive compounds with strong acidic moieties like sulfates, phosphates, or carboxylates are prone to adsorption (Figure 2).

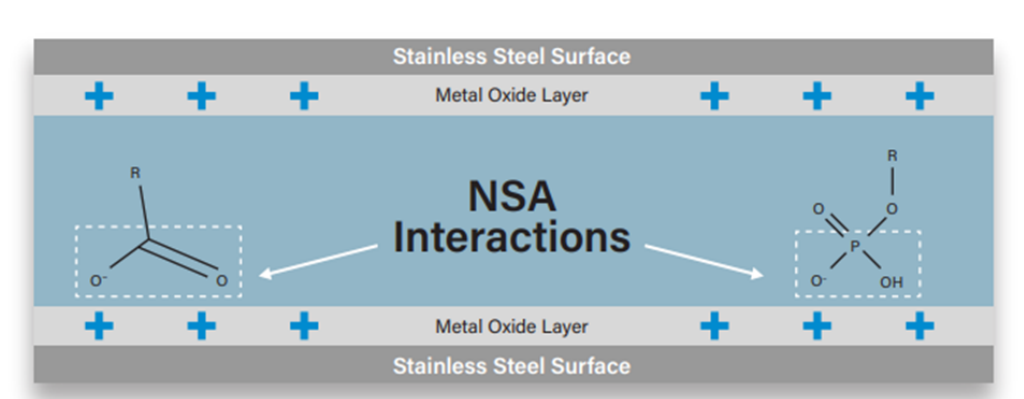


Figure 2. “Sticky” Non-Specific Adsorbers result in bad peak shapes and poor or inadequate fraction triggering, and can lead to compromised target purity and recovery.

Non-specific adsorption can cause practical problems in the purification lab, impeding efficiency and compromising both target purity and recovery. NSA may cause peak shape problems and inconsistent retention times and/or peak areas, missing peaks or low sensitivity, or increasing peak areas over time. Approaches that reduce or eliminate non-specific adsorption must be implemented for

successful isolations. Preconditioning the column before use with sample or matrix, or passivating the column with acid or other metal-chelating additives in the mobile phase are time-consuming and unstable and may need to be repeated. These strategies increase sample and waste handling and slow the purification process. The easiest way to prevent non-specific adsorption on metal surfaces is to use a column with an inert surface.

An ACQUITY Premier HSS T3 Column was used to evaluate the crude echinacea extract. The lambda max for chicoric acid is about 330 nm, which simplifies the analysis by reducing the absorbance of the compounds which elute later. Impurities eluting very closely to chicoric acid would prevent the isolation of pure target, so the gradient was focused⁷ to increase the resolution between the chicoric acid and the other extract components (Figures 3, 4) and analyzed on a stainless steel column and on an inert column. The inert column showed a 7% improvement in peak area and a 2% improvement in peak height for chicoric acid. Chicoric acid was positively identified in the crude echinacea extract by injecting a standard (not shown).

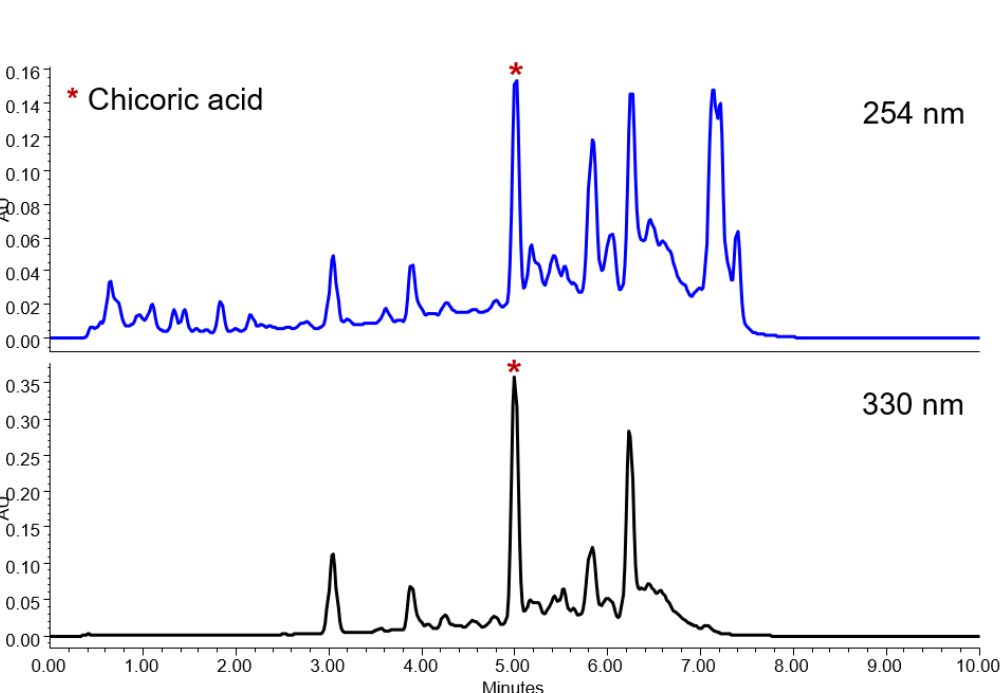


Figure 3. Analytical separation for the echinacea tea extract using the 5-50% B scouting gradient: ACQUITY Premier HSS T3 Column; 2.1x50 mm, 1.8 µm; Inj. Vol. 5 µL.

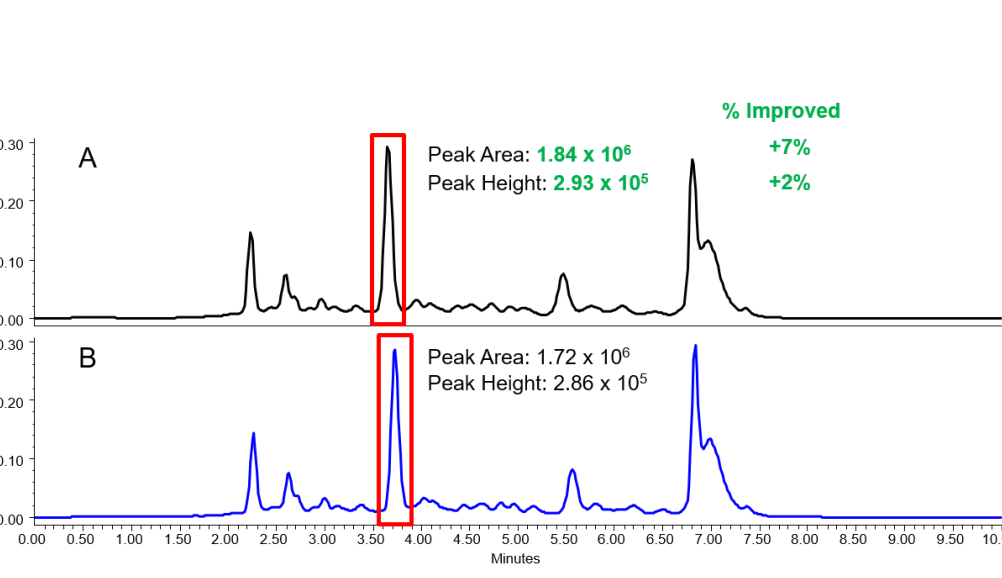


Figure 4. Analytical separation for the echinacea tea extract using the 32-40% B focused gradient on the 2.1x50 mm, 1.8 µm columns. A) ACQUITY Premier HSS T3 Column; B) ACQUITY HSS T3 Column; Inj. Vol. 5 µL; 330 nm

Figures 5 and 6 show the direct scalability from UPLC to prep when OBD preparative columns are employed and the rules of scaleup are applied.⁸ As laboratories work to develop more sustainable processes, strategies that improve overall productivity and efficiency are highly desired. The isolation performed on the 3.5 µm prep column saved >16 minutes of run time and ~88 mL of solvent per run.

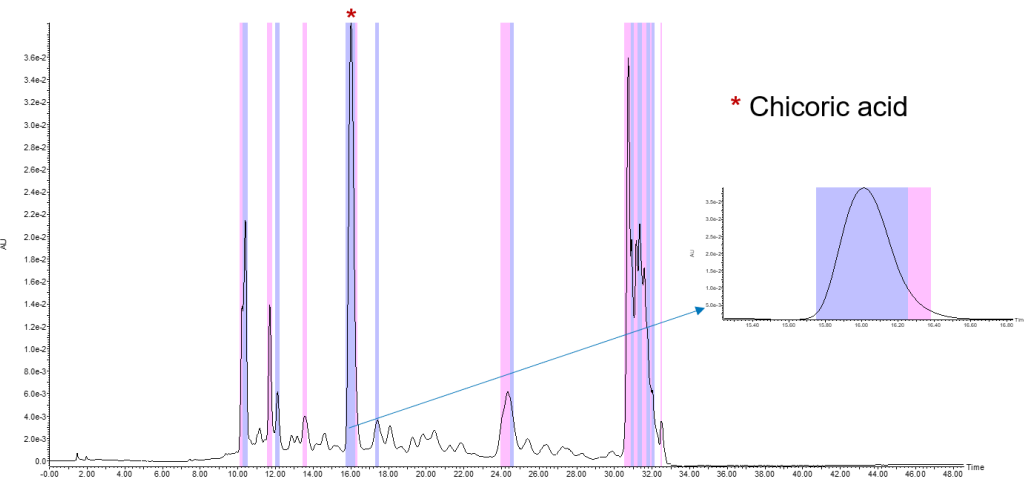


Figure 5. Chicoric acid isolation; 32-40% B focused gradient; 5 µm 10x150 mm XSelect Premier HSS T3 OBD Prep Column; Inj. Vol. 340 µL

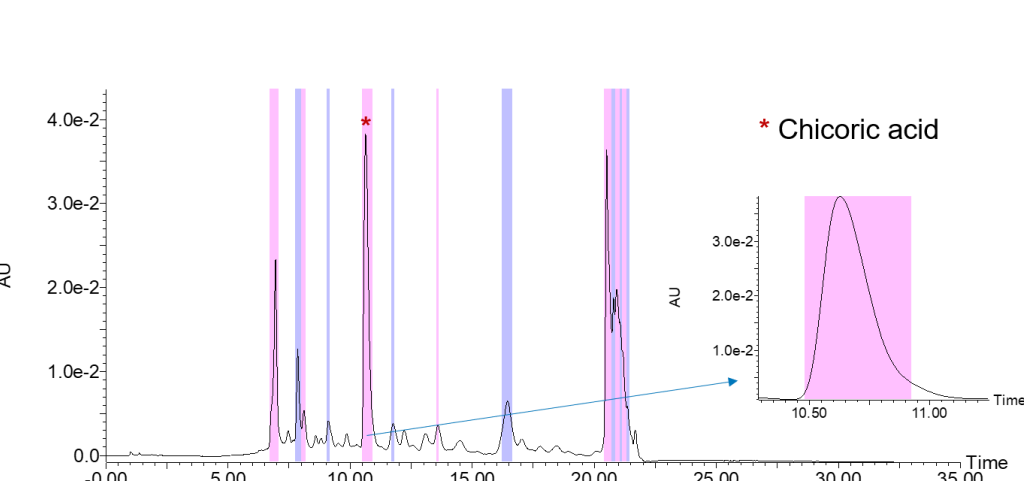


Figure 6. Chicoric acid isolation; 32-40% B focused gradient; 3.5 µm 10x100 mm XSelect Premier HSS T3 OBD Prep Column; Inj. Vol. 227 µL

The chicoric acid isolated from the 3.5 µm column was of equivalent purity to the target recovered from the 5 µm column. These results demonstrate the value of using 3.5 µm columns with inert hardware for isolating reduced target amounts for drug discovery and research.

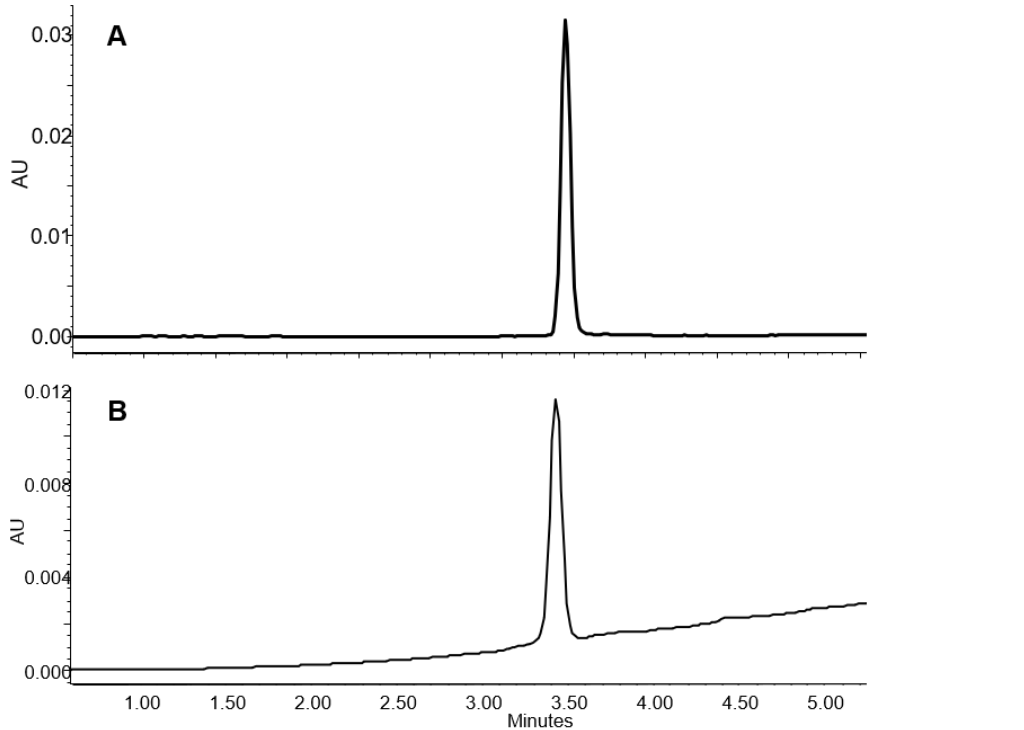


Figure 7. Fraction pool analysis using ACQUITY Premier HSS T3 Column, 2.1x50 mm, 1.8 µm, 5-95% B; Inj. Vol. 5 µL; 330 nm for the preparative isolations on A) 5 µm 10x150 mm XSelect Premier HSS T3 Column; B) 3.5 µm 10x100 mm XSelect Premier HSS T3 Column.

Stationary phase particle size also affects the target peak fraction volume. Narrow, well resolved peaks improve peak detection and fraction triggering. As shown in Figure 8, the average chicoric acid fraction volume from the 3.5 µm column was about 10% less than the average fraction volume collected when using the 5 µm column. Smaller fraction volumes lead to shorter lyophilization times during the final steps of the isolation process.

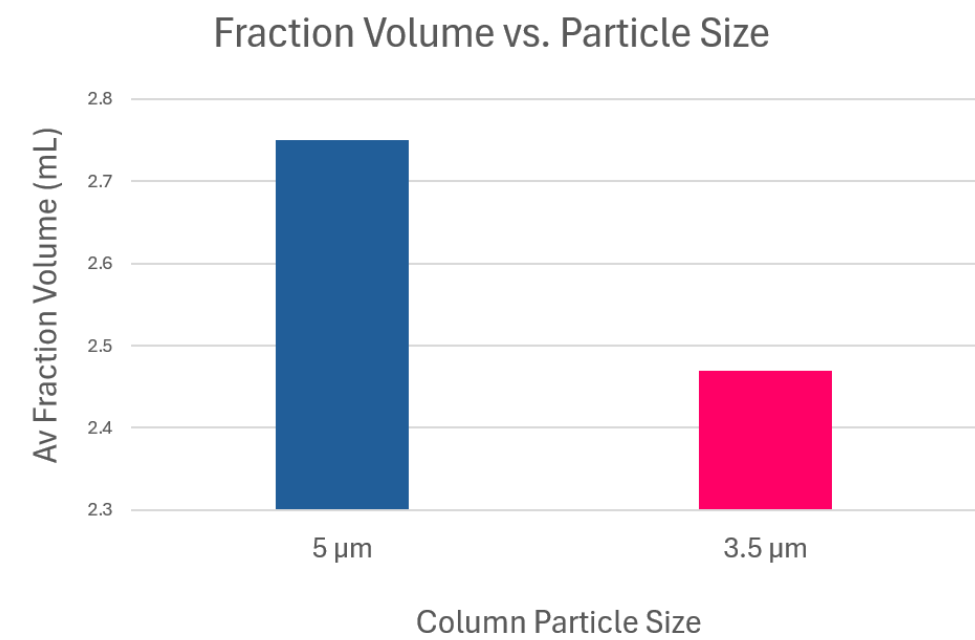


Figure 8. Average chicoric acid fraction volume comparison for three isolations performed on the 5 µm and 3.5 µm XSelect Premier HSS T3 Columns.

Sometimes the purification scientist must isolate target compounds that are present in the crude sample mixture at very low levels. In other cases, the sample has unknown compounds that might not be targeted as moieties of interest, that is, until they are identified as peaks in the chromatogram. The chromatographic separation must be sensitive enough to determine whether these low level unknowns are worthy of further investigation. MaxPeak Premier Columns are suitable for these types of purification applications. Figure 9 shows the separation of the echinacea tea extract injected on the 5 µm 10x150 mm XSelect Premier HSS T3 OBD Prep Column and the XSelect HSS T3 OBD Prep Column at one-tenth the fully scaled injection volume, i.e., 34 µL instead of 340 µL.

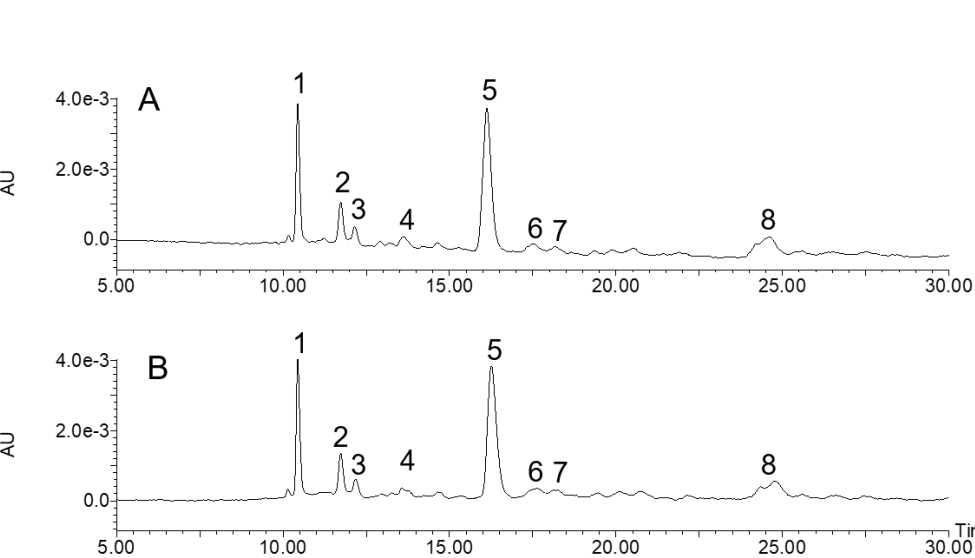


Figure 9. Preparative chromatography at low loading on the 5 µm, 10x150 mm A) XSelect Premier HSS T3 and B) XSelect HSS T3 OBD Preparative Columns; 32-40% B; Inj. Vol. 34 µL.

All but one of the numbered peaks in the chromatograms showed increased area and height on the MaxPeak Premier Column. The percent improvement in peak area and height at this low level of sample loading is illustrated in Figure 10. Since multiple injections were not performed at this low loading level, the statistical significance of these differences was not calculated. Although the area and height differences are small, the MaxPeak Premier Column plays an important role in the quest to identify, detect, or isolate low level targets in a crude sample mixture. This is especially important when limited amounts of sample are available, when only small amounts are needed for testing, or when an unknown peak appears during analysis and isolation. Scientists charged with isolating very small amounts of target from 96 and 384 well sample plates execute their purification workflow expeditiously with single injections and small improvements in peak detection are often crucial to their success.

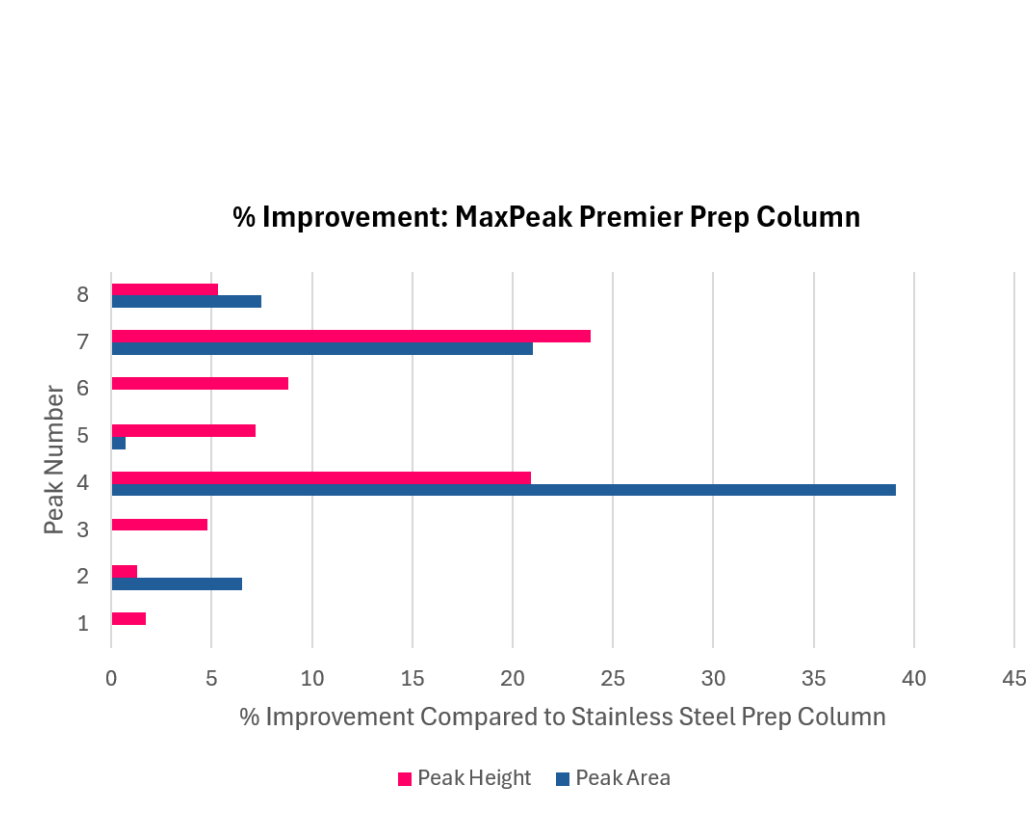


Figure 10. Percent improvement in peak areas and heights for the XSelect Premier HSS T3 OBD Prep Column compared to the stainless steel XSelect HSS T3 OBD Prep Column at low loading; 5 µm, 10x150 mm; 32-40% B; Inj. Vol. 34 µL.

CONCLUSIONS

- Chicoric acid was analyzed and isolated using analytical and preparative MaxPeak Premier HSS T3 Columns with inert surfaces, which increased the peak area and height for improved detection and collection.
- Chicoric acid was successfully isolated using 3.5 and 5 µm XSelect Premier HSS T3 OBD Prep Columns and fraction pool analysis showed equivalent UV purity, which suggests the viability of incorporating small particle preparative chromatography into the purification workflow.
- 3.5 µm preparative columns improve purification process efficiency and promote sustainability by reducing method run time, solvent usage, and fraction lyophilization time.
- Inert MaxPeak Premier column hardware, OBD Technology, and small particle preparative columns are well-suited for the demands of the purification laboratory when results must be generated efficiently to satisfy accelerated production timelines.

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