

Nota applicativa

## Leveraging Mobile Phase pH to Optimize Separations for Improved Prep Performance Using Columns Packed With Waters™ Hybrid Particles

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Jo-Ann M. Jablonski, Kim Haynes, Kathy Lawrence

Waters Corporation

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### Abstract

This application note illustrates the value of leveraging mobile phase pH to improve peak shape, retentivity, and mass loading for preparative separations. The Waters columns, manufactured with Hybrid Particle<sup>1,2</sup> and Optimum Bed Density [(OBD™)]<sup>3</sup> Technologies, enable the purification scientist to exploit the advantages of changing pH to improve chromatographic separations while maintaining consistent column-to-column performance, long column lifetimes, and direct scalability from analytical to prep. The OBD preparative columns resulted in better purification outcomes when compared to Vendor Y's Column using the conditions specified in this study.

### Benefits

- Changing the mobile phase pH can lead to enhanced performance with changes in retentivity and selectivity, improved peak shape, and increased mass loading, which save purification processing time.
- XBridge™ BEH™ C<sub>18</sub> and XSelect™ CSH™ C<sub>18</sub> Columns are stable over a wide pH range (1–12 and 1–11,

respectively), effectively eliminating concerns about damaging the stationary phase due to low/high pH conditions within these ranges.

- Different C<sub>18</sub> stationary phases provide additional selectivity choices (*i.e.*, impacting the resolution and order of peak elution) for separating target compounds.
- The highly controlled OBD column packing process ensures preparative columns are of similar bed density to analytical columns of the same chemistry, thus making preparative chromatographic profiles analogous to analytical runs. Similar chromatography at the analytical and preparative scales simplifies scaleup and compound isolation.

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## Introduction

Purification scientists are constantly tasked with isolating synthetic intermediates and purified products in the shortest amount of time at the highest yield and purity. Sometimes generic separation methods successfully resolve sample components easily without having to develop customized gradients. At other times, compounds in the mixture require a different approach to effectively separate them from their closely eluting impurities. Ideally, suggestions for separation improvement should be easy to implement. For mixtures containing ionizable compounds, one of the most straightforward approaches to changing the separation lies with changing the pH of the mobile phase. With columns that are compatible with both high and low pH conditions, such as XBridge BEH C<sub>18</sub> and XSelect CSH C<sub>18</sub> Columns (both stationary phases manufactured with hybrid particle technology), changing the pH to optimize a separation becomes routine. Indeed, in some high throughput laboratories, pH switching is standard protocol for finding the best conditions before scaling to prep. Leveraging the pH to improve the separation will often result in improved peak shape and increased sample mass loading, both of which contribute to enhancements in yield, purity, and time savings.

This study illustrates how leveraging pH is a useful approach for quickly optimizing a separation and improving mass loading.

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## Experimental

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## Sample Description

A sample mixture, which included two acids (benzoic acid, diclofenac), three bases (benzamide, clomipramine, diphenhydramine) and three neutrals (hydrocortisone, estradiol, flavone) was prepared by combining different masses of each of the components in a 20 mL scintillation vial and dissolving in 20 mL of dimethyl sulfoxide (DMSO). The final sample mixture concentration was 78.5 mg/mL.

## LC Conditions

LC system:	AutoPurification™ System
UV detection:	2998 Photodiode Array Detector; 254 nm channel extracted
Mass detection:	ACQUITY QDa™ Mass range: 100-900 amu Run time: 15.25 minutes Sampling frequency: 2 Hz Detector gain: 1 Probe temperature: 500 °C Ionization mode: Electrospray positive Capillary voltage: 1.5, positive Cone voltage: 15 V Data mode: Centroid Makeup solvents: 50 water/50 acetonitrile with 0.01% formic acid or 0.01% ammonium hydroxide
Columns:	XBridge BEH C <sub>18</sub> , 5 µm Column, 4.6 x 50 mm, p/n: 186003113 XBridge BEH C <sub>18</sub> OBD Prep Column, 5 µm, 30 x 50

mm, p/n: 186002980

XSelect CSH C<sub>18</sub> Column, 5 µm, 4.6 x 50 mm, p/n:  
186005287

XSelect CSH C<sub>18</sub> Column, 5 µm, 30 x 50 mm, p/n:  
186005423

Vendor Y C<sub>18</sub> Column, 4.6 x 50 mm, 5 µm

Vendor Y C<sub>18</sub> Column, 30 x 50 mm, 5 µm

Column temperature:

Ambient

Sample temperature:

Ambient

Injection volumes:

As noted in figures

Flow rates:

Analytical 0.7 mL/min; Prep 29.8 mL/min

Mobile phase A:

Water with 0.1% formic acid (HCOOH) or 0.1%  
ammonium hydroxide (NH<sub>4</sub>OH)

Mobile phase B:

Acetonitrile with 0.1% formic acid or 0.1%  
ammonium hydroxide

## Gradient Table: Analytical Method

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.00	0.7	95	5	6
7.14	0.7	5	95	6
8.57	0.7	5	95	6
9.29	0.7	95	5	6
14.29	0.7	95	5	6

## Gradient Table: Preparative Method

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.00	29.8	95	5	6
0.96	29.8	95	5	6
8.10	29.8	5	95	6
9.53	29.8	5	95	6
10.25	29.8	95	5	6
15.25	29.8	95	5	6

## Data Management

Chromatography software:

MassLynx™ version 4.2

Application manager:

FractionLynx

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## Results and Discussion

The chromatography for a compound mixture containing acidic, basic, and neutral compounds was compared on three C<sub>18</sub> columns run under both low and high pH conditions. Selectivity differences between the C<sub>18</sub> stationary phases run under both acidic and basic conditions influenced the separations by changing elution order, peak shape, and retentivity. Although such changes are noteworthy for analytical separations, they are especially useful for preparative separations. A change in pH may also increase the resolution between the target compound and other closely eluting impurities, effectively driving them further apart chromatographically, which then promotes increased mass loading. With improved mass loading, the number of preparative runs needed to produce the required amount of target is reduced, effectively leading to savings in time and solvent usage.

Silica-based reversed phase columns exhibit two major shortcomings:

- Many basic compounds chromatograph as broad, tailing peaks at acidic and neutral pH.

- They have short lifetimes when the mobile phase pH is outside the 2–8 pH range.

When the pH is below 2, the bonded phase may be hydrolyzed, and the hydrolysis products eluted from the stationary phase. At pH values above 8, the silica support begins to hydrolyze, ultimately leading to column voiding. The Waters' Hybrid Particle Technology eliminates these obstacles, allowing the chemist to manipulate separations over a wide pH range (1–12 for XBridge BEH C<sub>18</sub> and 1–11 for XSelect CSH C<sub>18</sub>). This flexibility for compound screening, method development and for preparative separations simplifies the target molecule isolation and purification workflow. Leveraging pH, instead of changing the column, may be the easiest route to obtaining pure compound in adequate yield for the next step in the process.

Mobile phase pH is a powerful tool for improving peak shape, compound retention, and mass loading for ionizable compounds.<sup>4</sup> Working at the optimum pH for the target compounds in a complex mixture is important in preparative chromatography because along with the improved peak shape comes reduced fraction volume and evaporation time, which saves processing and handling time. The retention map shown in Figure 1 illustrates the behavior of acidic, basic, and neutral compounds with changes in pH. The inflection points of the acid and base curves represent the pK, the pH at which 50% of the ionized and un-ionized forms of the molecule exist. To maximize compound retention and peak shape improvement, the compound needs to be in its un-ionized form. The best peak shape and retention occurs at low pH for acids and at high pH for bases.

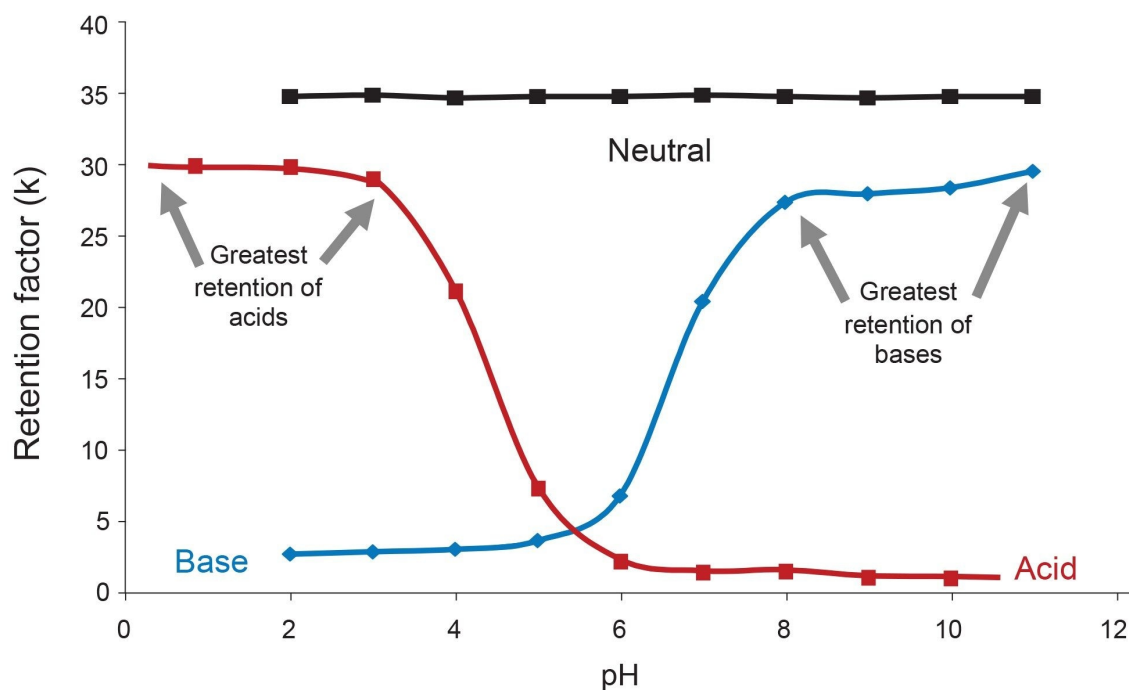


Figure 1. Retention factors for a representative acid, base, and neutral at different pH values for an XBridge BEH C<sub>18</sub> Column.

The differences in the separation of the sample mixture between the three analytical C<sub>18</sub> columns with 0.1% formic acid in the mobile phase is illustrated in Figure 2. On the XBridge BEH C<sub>18</sub> Column, the basic compound clomipramine tails significantly while the neutral compound, flavone, and the acidic compound, diclofenac, are well-resolved. The separation observed using the XSelect CSH C<sub>18</sub> Column shows clomipramine eluting earlier in the chromatogram as compared to the separation obtained using an XBridge BEH C<sub>18</sub> Column due to the positively charged surface on the CSH particle.<sup>5,6</sup> For the same reason the acid, diclofenac, elutes later in the chromatogram. Flavone and diclofenac are better resolved than on the XBridge Column, even in the slightly overloaded condition (which is acceptable for discerning maximum sample load on the analytical column), prior to scale up. The CSH stationary phase was created to facilitate desirable peak shapes for bases run with low ionic strength acidic mobile phases.<sup>7</sup> Vendor Y's C<sub>18</sub> Column adequately resolves clomipramine from the other targets in the sample mixture with acceptable peak shape (but inferior to CSH). This C<sub>18</sub> column, however, is not suitable for separating flavone and diclofenac under these conditions, as shown by the low resolution for these compounds.

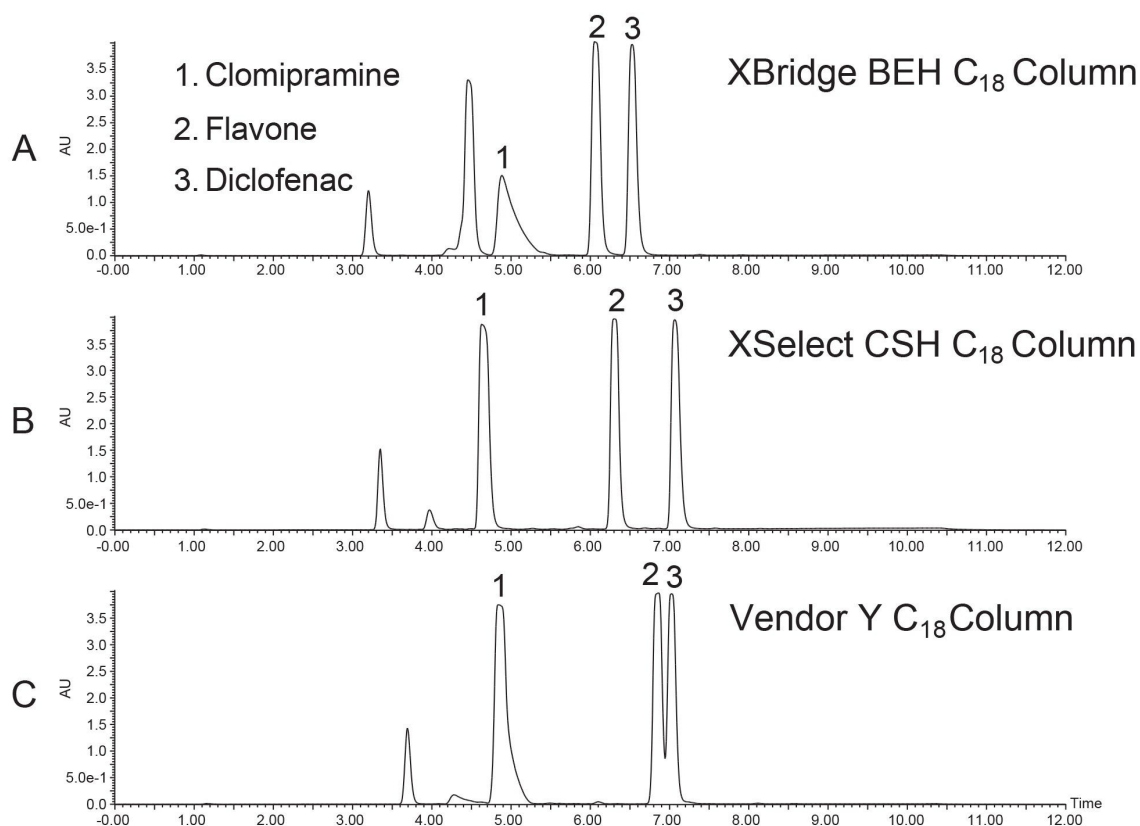


Figure 2. Comparison of the separations obtained on the 4.6 x 50 mm analytical columns using 0.1% HCOOH in the mobile phases.

For the compound mixture used in this study, migrating from low pH to high pH had a profound effect on the separations on each of the three C<sub>18</sub> columns. Figure 3 illustrates the changes in compound retentivity and peak shape when the mobile phase additive was changed from 0.1% formic acid to 0.1% ammonium hydroxide. All three columns show diclofenac (acid) moving from the most retained compound in 0.1% formic acid to the least retained of the three target compounds in 0.1% ammonium hydroxide. Conversely, clomipramine (base) moves from the least retained (with poor peak shape on BEH C<sub>18</sub>) of the three target compounds in acidic mobile phase to the most retained in basic mobile phase. The XBridge BEH C<sub>18</sub> Column has the best overall peak shape for the three target compounds when using the basic mobile phase. Of note is the peak shape of the base, clomipramine. In the acidic mobile phase, clomipramine elutes earlier and tails significantly. In the basic mobile phase, clomipramine elutes later, in higher % organic solvent, and with nearly symmetrical peak shape. Scaling to prep (Figure 4) shows that in the acidic mobile phase, the clomipramine required three fraction collection



tubes, whereas in the basic mobile phase with clomipramine's improved peak shape, only one fraction collection tube was needed. Therefore, better peak shape with higher percent organic elution and fewer fraction tubes leads to faster fraction evaporation, handling, and processing time.

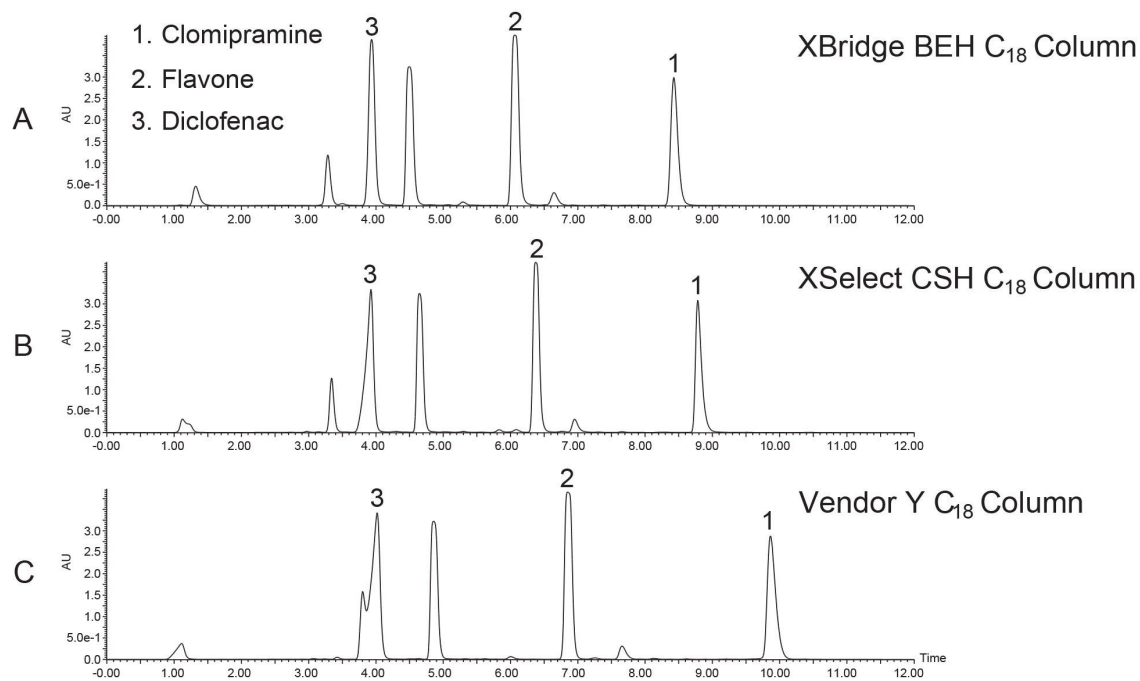


Figure 3. Comparison of the separations obtained on the 4.6 x 50 mm analytical columns using 0.1% NH<sub>4</sub>OH in the mobile phases.

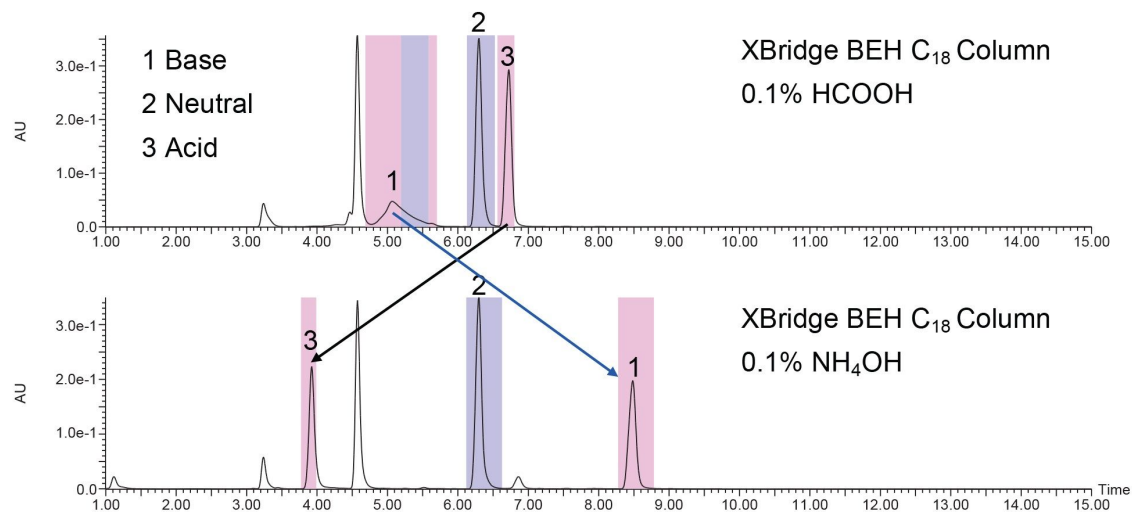


Figure 4. Comparison of the separations obtained on a 30 x 50 mm 5  $\mu$ m XBridge BEH C<sub>18</sub> OBD prep column using 0.1% HCOOH or 0.1% NH<sub>4</sub>OH in the mobile phases. The injection volume was 85.1  $\mu$ L, loading 6.7 mg of loading 6.7 mg of the sample mixture.

Each of the three target compounds on the XSelect CSH C<sub>18</sub> Column also have good peak shape, even though the diclofenac shows slight fronting. The Vendor Y C<sub>18</sub> Column would be an acceptable choice for the isolation of the neutral and basic compounds, but upon the pH change, diclofenac coeluted with another compound in the sample mixture.

Not only did the acidic and basic compounds change elution order, but the peaks were much better resolved from the other compounds in the sample mixture, which impacted sample loading. When compounds are further apart, the amount of sample that is introduced to the column in a single injection may be increased. Improved loading reduces the total number of injections required to produce the quantity of target needed for the next process step.

An important procedural recommendation for moving from low pH to high pH includes the necessity for flushing the column with water before each change to prevent the formation of salts, which may precipitate and plug the column or cause a high-pressure shutdown on the LC system. Therefore, if chromatography is performed in low pH mobile phases, flush the column and the LC system with water before moving to the high pH mobile phases, and vice versa.

Many purification scientists forego performing a loading study prior to scaling up to prep because they may be familiar with compound characteristics, or the purification process strategy. A loading study is helpful, however, for easily determining the amount of sample that can be introduced to the analytical column before adequate resolution is lost between the compounds in the mixture. Once the maximum loading mass for the analytical column is known, that mass can be scaled for the isolation on the preparative column. Figures 5 and 6 show loading studies performed on the XBridge BEH C<sub>18</sub> and XSelect CSH C<sub>18</sub> Columns at low pH. The maximum load on the 4.6 x 50 mm XBridge Column was 2  $\mu$ L (0.16 mg) of the 78.5 mg/mL sample mixture. Doubling the injection volume resulted in overlap between the peak eluting at about 4.1 min and clomipramine. The maximum load on the XSelect CSH Column was 8  $\mu$ L (0.63mg) of the 78.5 mg/mL sample, a four-fold increase. Loading studies were not performed on the Vendor Y Column because the flavone and diclofenac coeluted in low pH conditions.

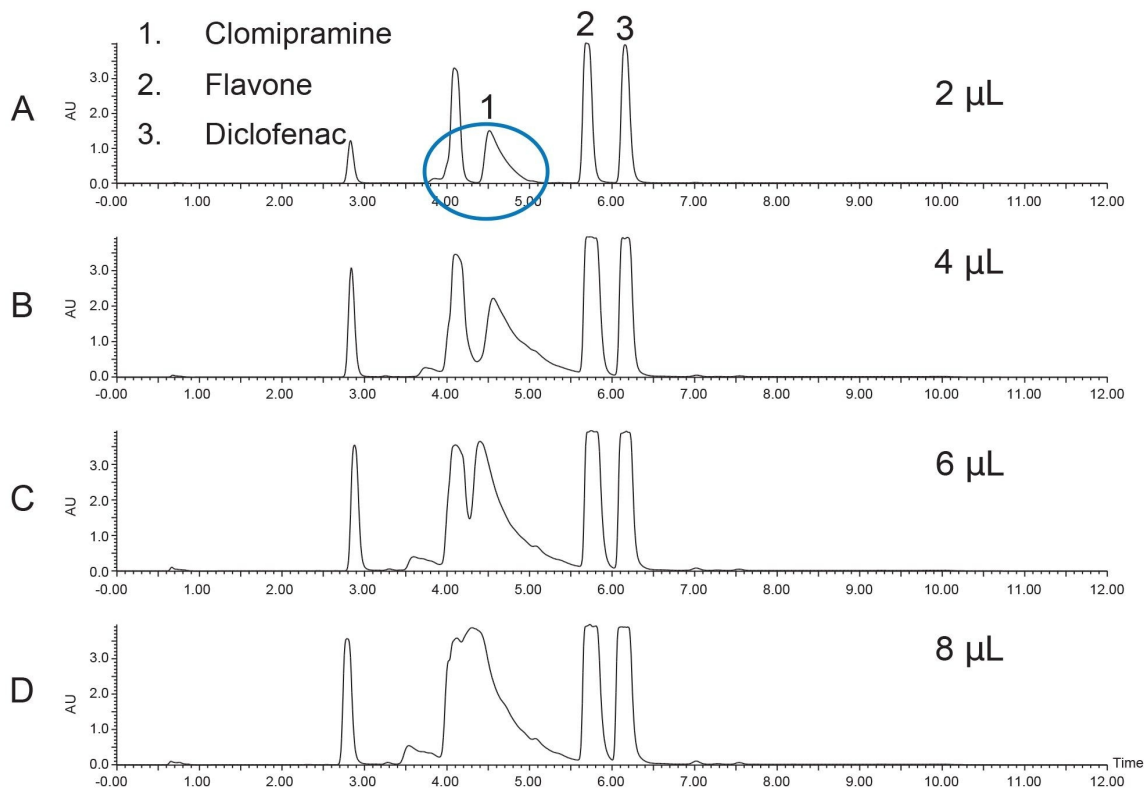


Figure 5. Loading Study on the 4.6 x 50 mm XBridge BEH C<sub>18</sub> Column using 0.1% HCOOH in the mobile phases.

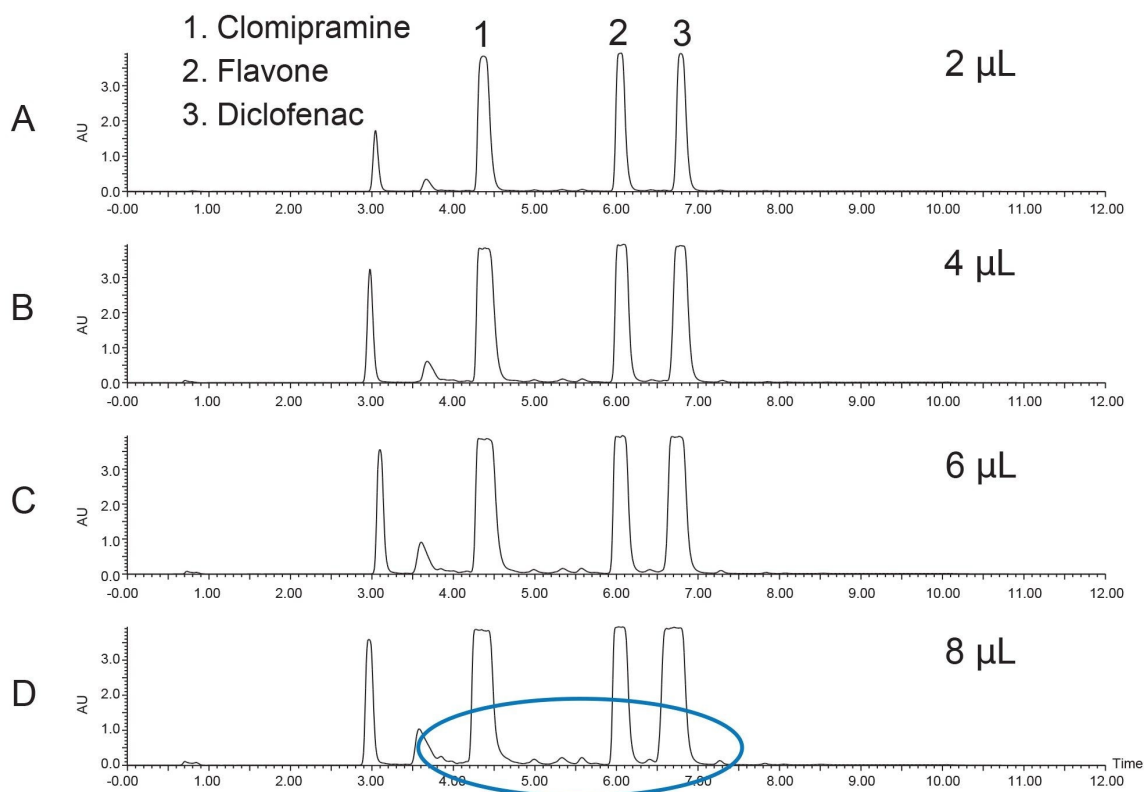


Figure 6. Loading Study on the 4.6 x 50 mm XSelect CSH C<sub>18</sub> Column using 0.1% HCOOH in the mobile phases.

Loading studies were also performed on all three columns at high pH. The results are shown in Figures 7, 8, and 9. The XBridge BEH C<sub>18</sub> Column (Figure 7) shows a maximum load of 8 μL (0.63 mg) on the 4.6 x 50 mm column and all three target compounds are baseline-resolved. The XSelect CSH C<sub>18</sub> Column (Figure 8) also shows a maximum load of 8 μL, although the diclofenac peak fronts slightly. Although Vendor Y's C<sub>18</sub> Column could also have a maximum load of 8 μL in the basic mobile phase and adequately separate flavone and clomipramine, the diclofenac now coelutes with another peak in the chromatogram (Figure 9).

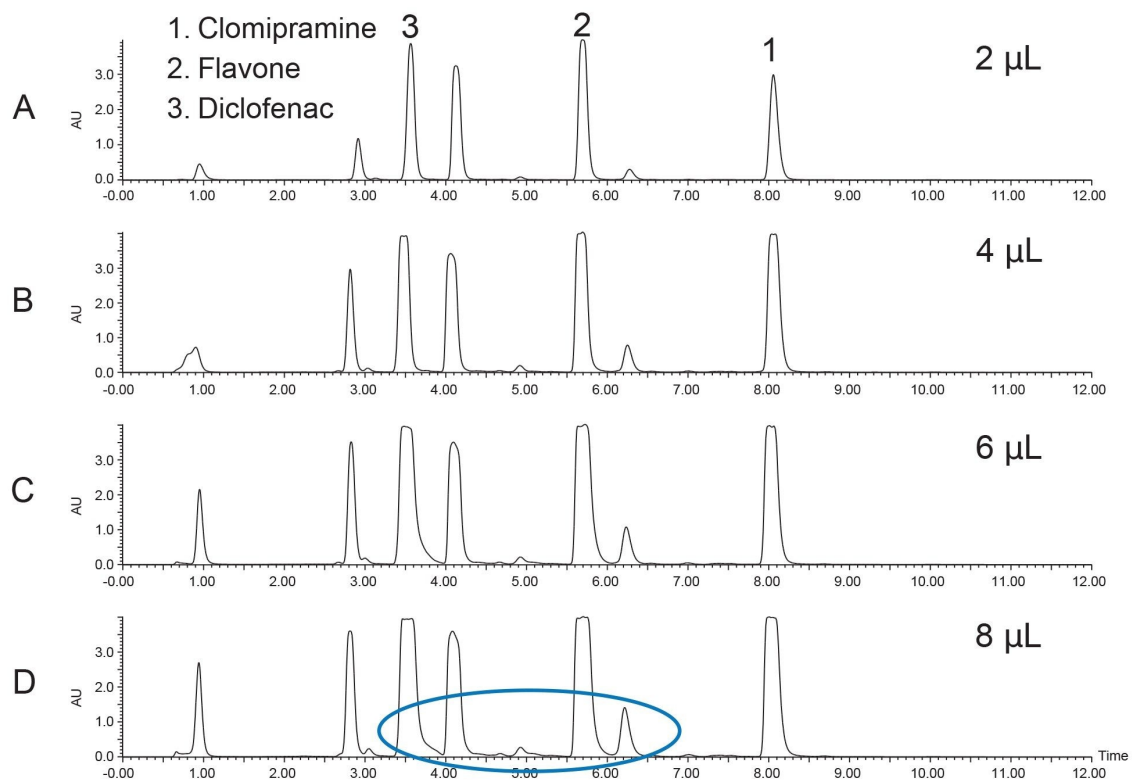


Figure 7. Loading Study on the 4.6 x 50 mm XBridge BEH C<sub>18</sub> Column using 0.1% NH<sub>4</sub>OH in the mobile phase.

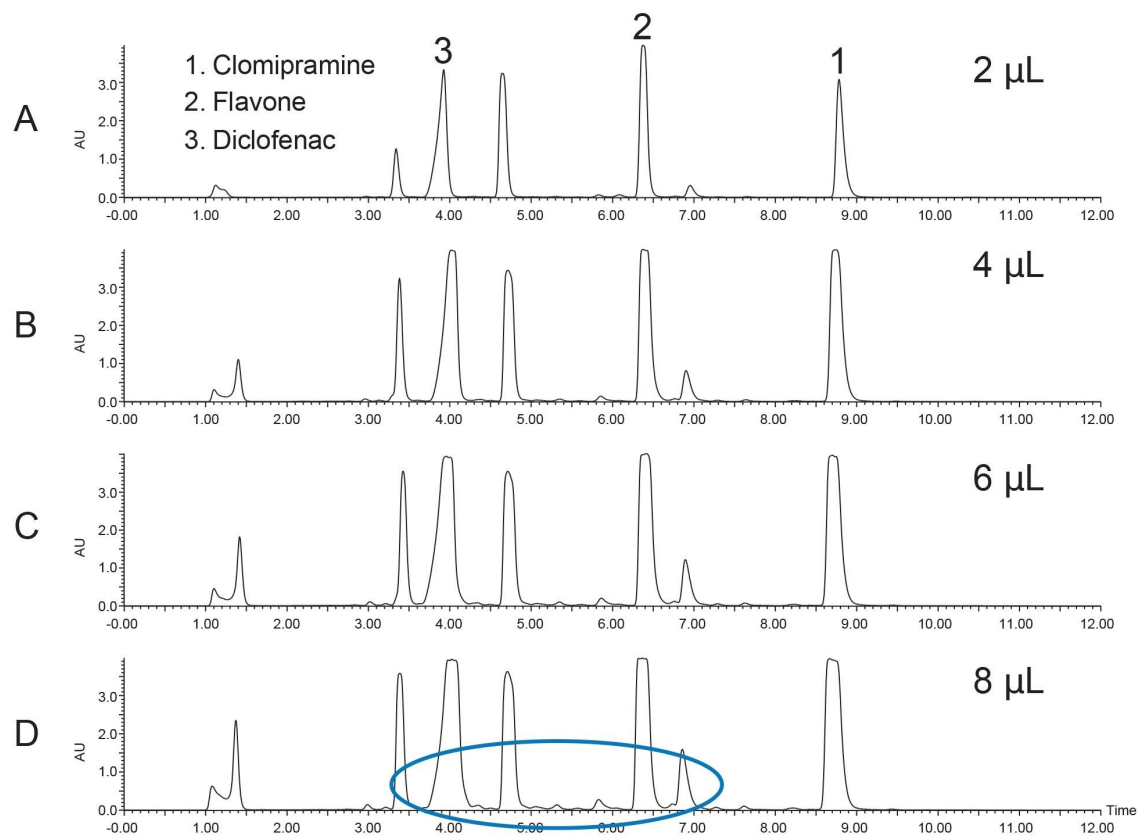


Figure 8. Loading Study on the 4.6 x 50 mm XSelect CSH C<sub>18</sub> Column using 0.1% NH<sub>4</sub>OH in the mobile phase.

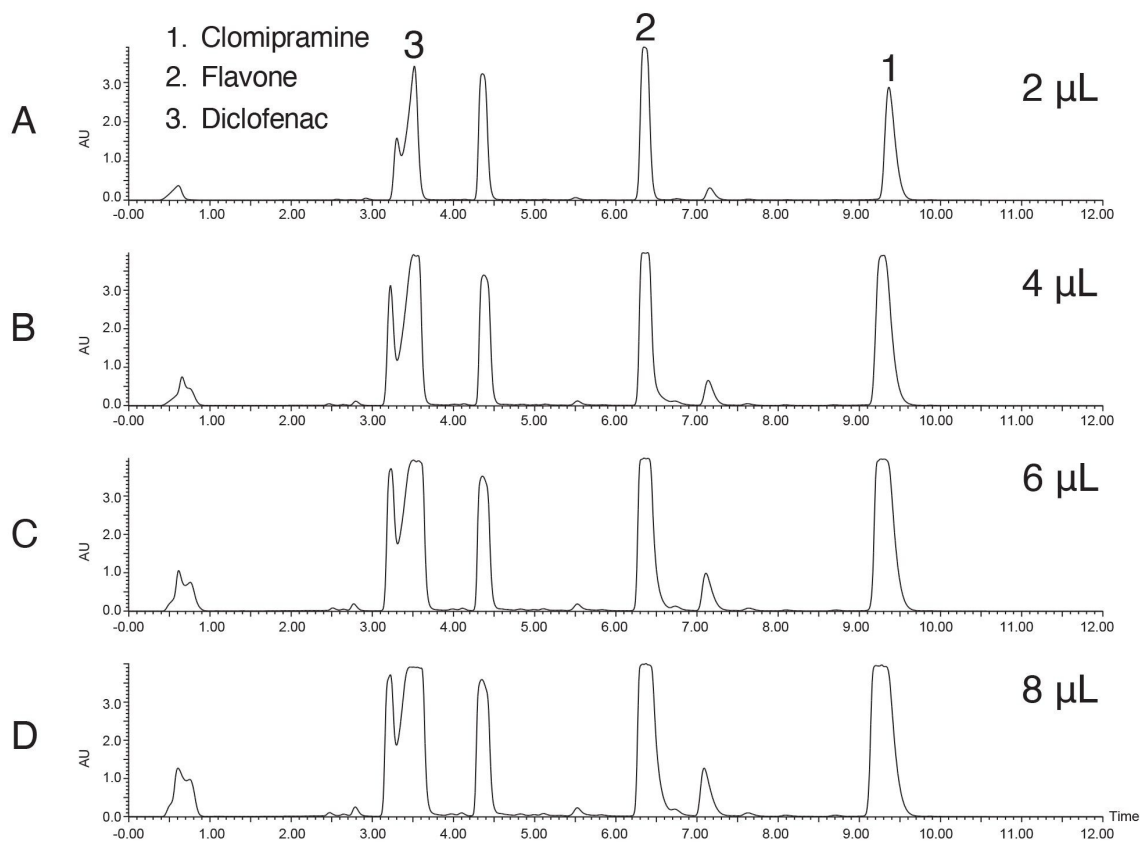


Figure 9. Loading Study on the 4.6 x 50 mm Vendor Y C<sub>18</sub> Column using 0.1% NH<sub>4</sub>OH in the mobile phase.

Once the loading study was completed, the next logical step in the purification workflow was scaling to prep. Geometric scaling from the 4.6 x 50 mm analytical columns to the 30 x 50 mm preparative columns was performed in both low and high pH mobile phases for the XBridge and XSelect Column chemistries.<sup>7</sup> Only scaling in high pH mobile phase was completed on the Vendor Y C<sub>18</sub> Column since two of the target compounds could be isolated. Only one of the three target compounds could be separated in low pH mobile phase, so scaleup under these conditions was not performed on the Vendor Y Column.

Because purification scientists want preparative chromatograms to look like their analytical profiles, preparative columns packed with the same bed density as their analytical counterparts help to ensure predictable scaleup. The Optimum Bed Density (OBD) columns are produced with a highly controlled process that ensures the column bed is uniform throughout the entire length and width of the column. These strict controls in column

packing and manufacturing lead to predictable scaleup, longer column lifetimes, and to improvements in the purification process. The XBridge BEH C<sub>18</sub> and XSelect CSH C<sub>18</sub> preparative columns used in this study were manufactured using the OBD process.

Figure 10 shows the separation of the sample mixture scaled from the 4.6 x 50 mm XBridge BEH C<sub>18</sub> Column to the 30 x 50 mm preparative column in 0.1% formic acid. All three target compounds were collected, with the clomipramine peak requiring three fraction collection tubes due to the tailing peak shape of this base in acidic conditions. The limit on loading at the analytical scale was 2 µL (0.2 mg), while about 85 µL (6.7 mg) was injected on the 30 x 50 mm column. The resolution between each of the peaks with their closely eluting contaminants was maintained, thus giving predictable scaleup.

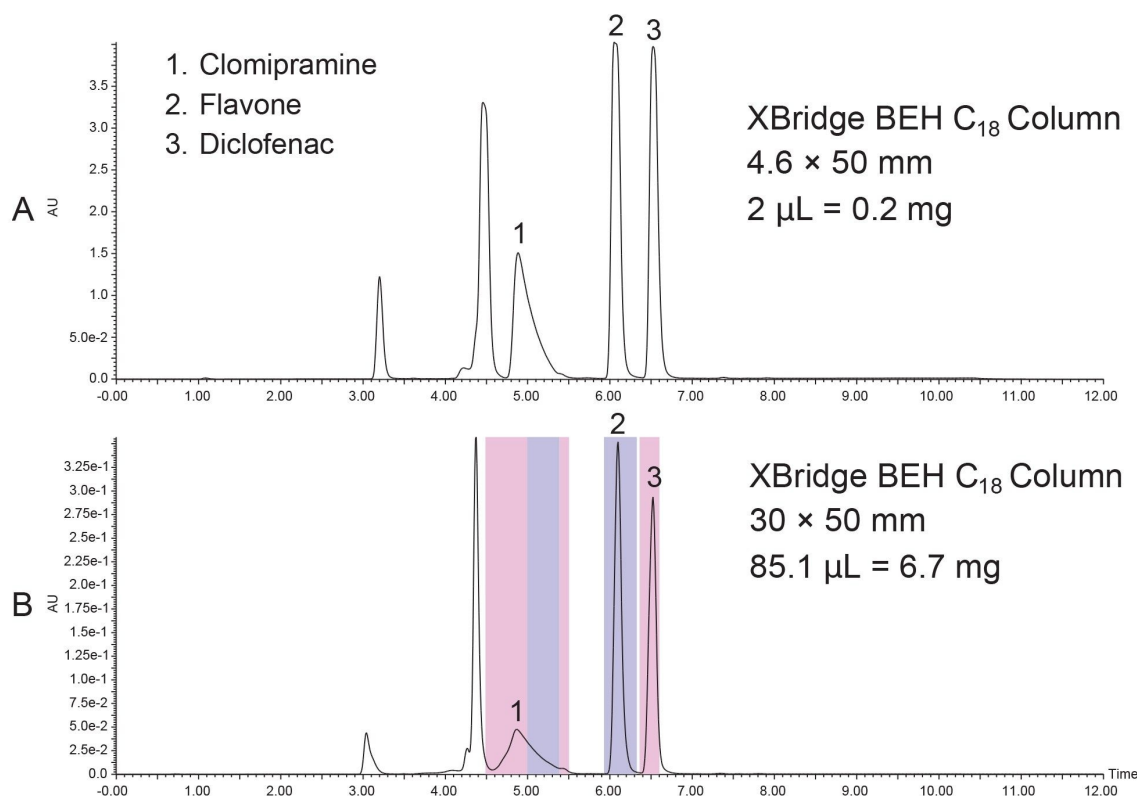


Figure 10. Scaling the separation using XBridge BEH C<sub>18</sub> Columns with 0.1% HCOOH in the mobile phases. The shaded areas show the fractions that were collected.

Scaling from analytical to prep on the XSelect CSH C<sub>18</sub> Column with mobile phases containing 0.1% formic acid



shows improved separation between the three target compounds, even at increased load (Figure 11). The scaled 26.7 mg preparative separation shows a small shoulder on the front of the clomipramine peak. Since the preparative system was configured with a mass detector, analysis of the clomipramine peak indicated the presence of another compound with a mass-to-charge ratio in positive ion mode ( $m/z$ ) of 363. Systems configured with mass detectors make compound analysis quick and easy for determining the presence or identity of possible target compound contaminants. Fraction collection using mass triggering is also less ambiguous than using UV detection alone.<sup>8,9</sup>

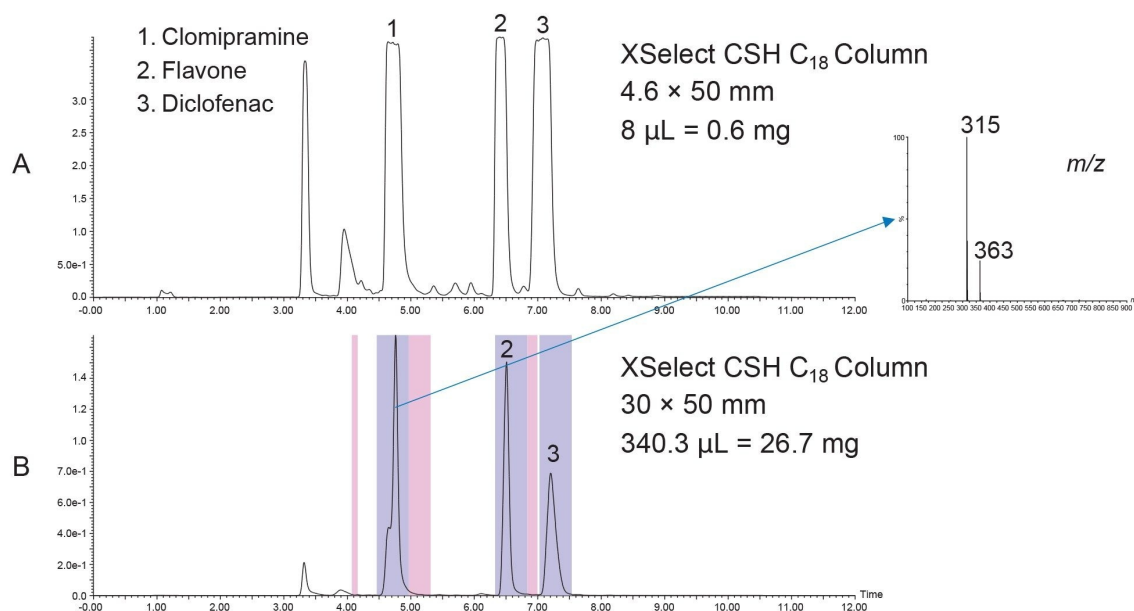


Figure 11. Scaling the separation using XSelect CSH C<sub>18</sub> Columns with 0.1% HCOOH in the mobile phases. The inset shows the mass spectrum of peak 1. The shaded areas show the fractions that were collected.

Figures 12, 13, and 14 show the scaleup of the separation on the three preparative columns with 0.1% ammonium hydroxide in the mobile phase. As mentioned above, the maximum loading on the analytical column for each of the three chemistries was 8 μL, or 0.6 mg. Geometrically scaling this injection volume to 340.3 μL, or 26.7 mg, resulted in the best separation on the XBridge BEH C<sub>18</sub> Column, with well-resolved peaks with good peak shape collected in one fraction tube. The XSelect CSH C<sub>18</sub> Column also had well-resolved peaks in one fraction tube, but the diclofenac peak exhibited a bit of fronting. Vendor Y's C<sub>18</sub> Column showed good peak shape for flavone

and clomipramine and one-tube fraction collection, but incomplete resolution of diclofenac with another contaminant in the sample mixture, as noted earlier.

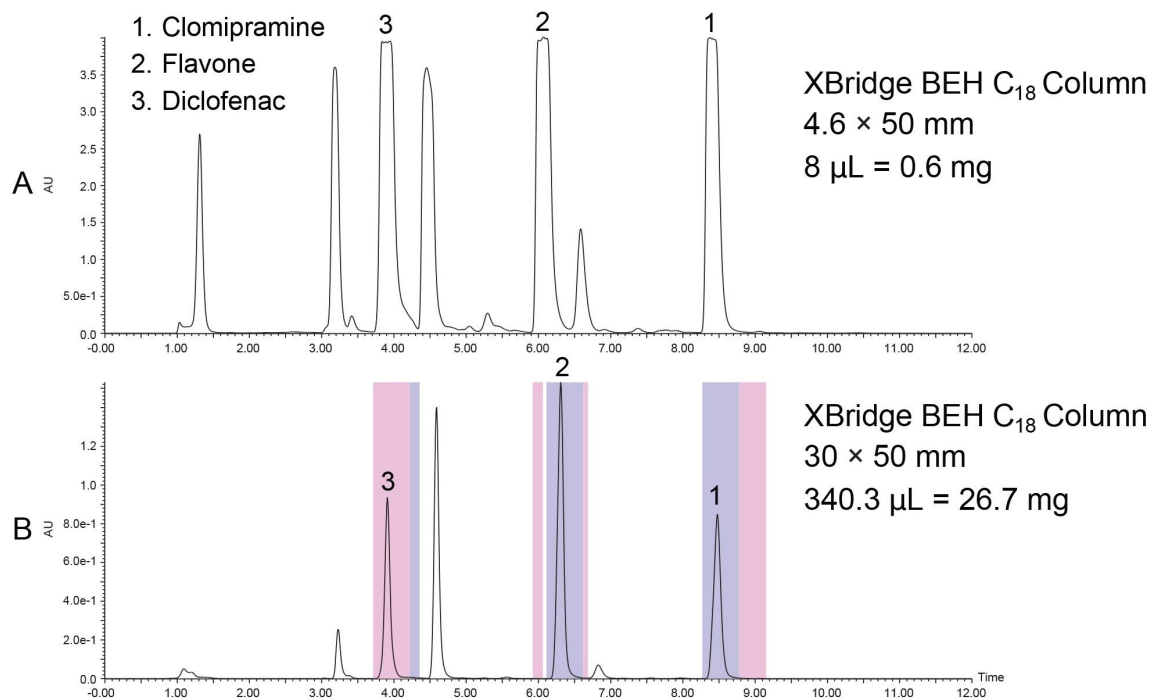


Figure 12. Scaling the separation using XBridge BEH C<sub>18</sub> Columns with 0.1% NH<sub>4</sub>OH in the mobile phase. The shaded areas show the fractions that were collected.

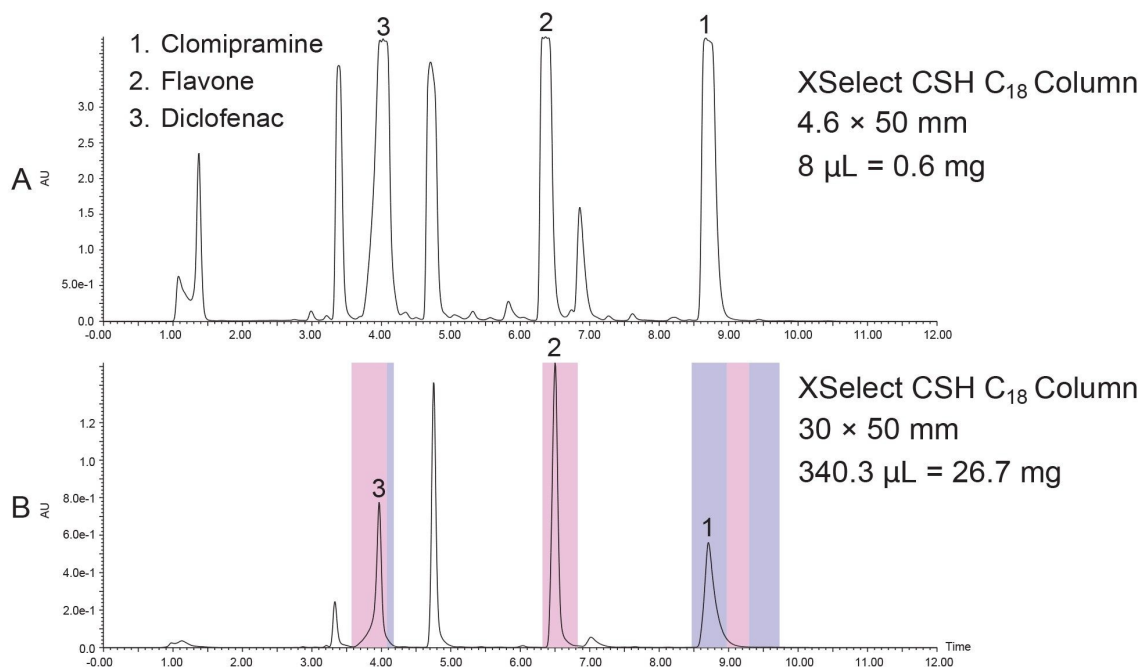


Figure 13. Scaling the separation using XSelect CSH C<sub>18</sub> Columns with 0.1% NH<sub>4</sub>OH in the mobile phase. The shaded areas show the fractions that were collected.

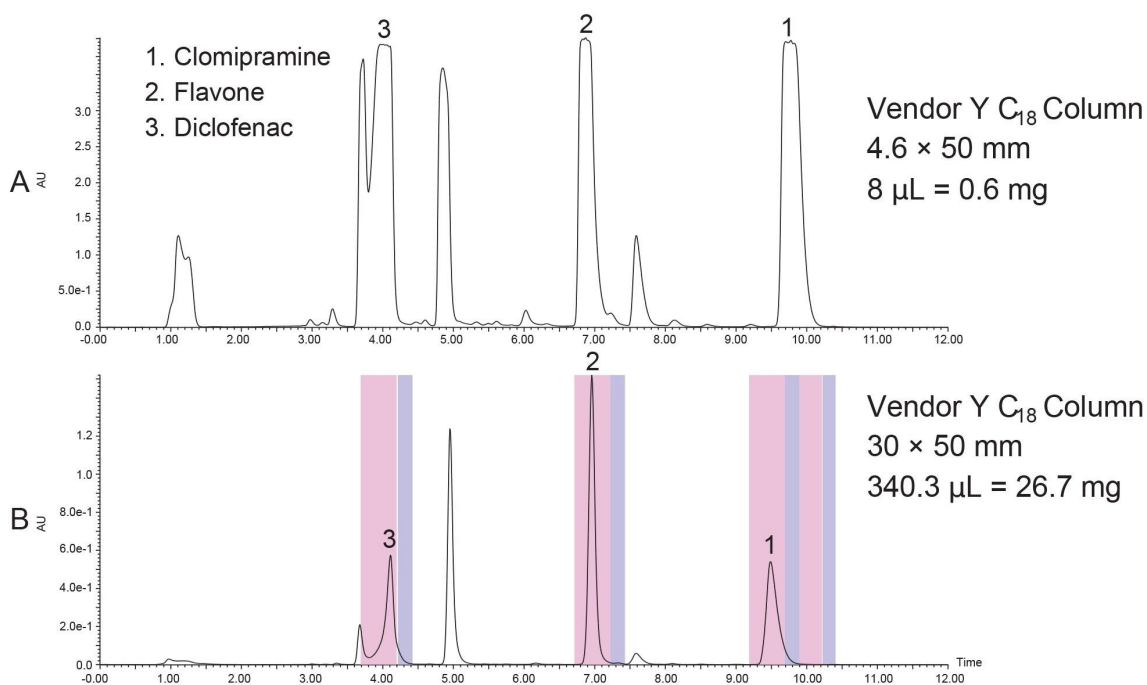


Figure 14. Scaling the separation using Vendor Y C<sub>18</sub> Columns with 0.1% NH<sub>4</sub>OH in the mobile phase. The shaded areas show the fractions that were collected.

As mentioned above, preparative LC systems configured with mass detectors are useful for determining the presence of contaminants or for finding the target compound in complex sample mixtures. Figure 15 also demonstrates the advantage of having a mass detector in the system. Sometimes compounds in the sample mixture do not have good UV absorbance but ionize very well. Take, for example, the contaminant which elutes after flavone in Figure 15. The UV absorbance of the contaminant peak is very low, yet the magnitude of the same peak in the total ion mass chromatogram (TIC) is large. The size of the peak in the mass chromatogram might, in fact, be the limiting factor for compound loading for some separations. Therefore, dual detection is a system option that is often advantageous for the purification scientist who isolates large numbers of diverse compounds.

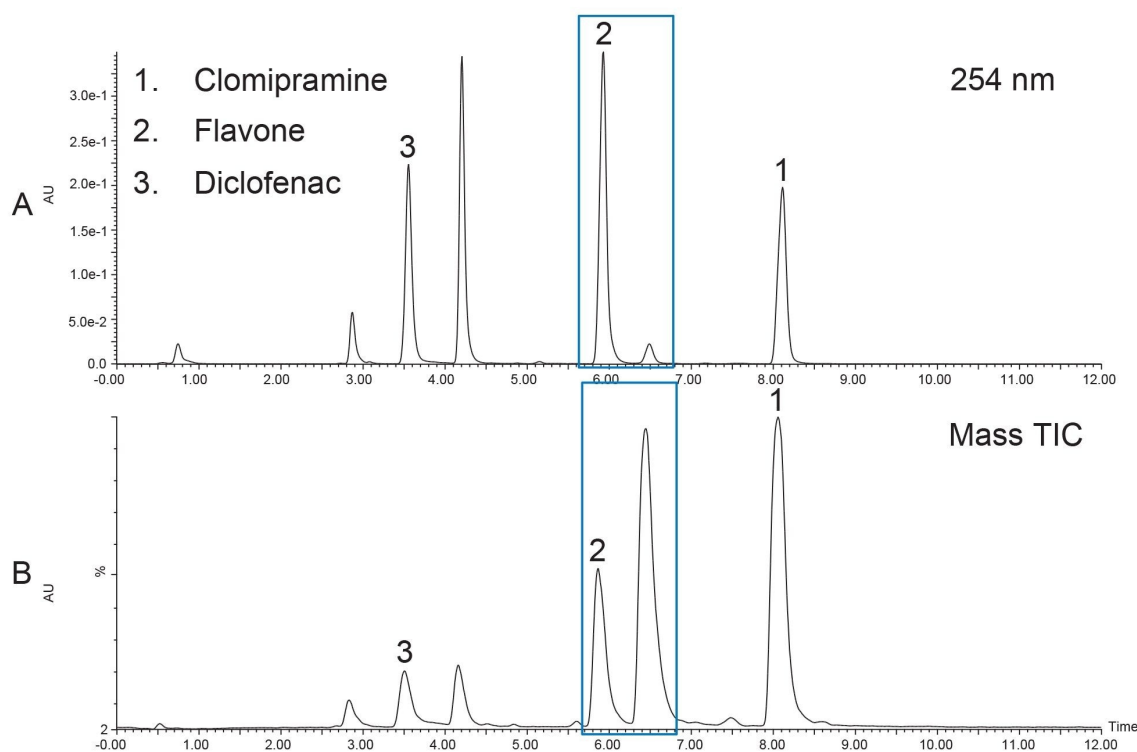


Figure 15. Sample mixture on XBridge BEH C<sub>18</sub> Column (30 x 50 mm) with 0.1% NH<sub>4</sub>OH in the mobile phase; A = UV detection (254 nm); B = MS detection (TIC).

Tables 1 and 2 report the peak widths and USP Tailing values for each of the target compounds on each of the three preparative columns using both low pH and high pH mobile phases. The peak widths were calculated at 5% of the peak height. The reported values reinforce the observations regarding the chromatography previously discussed. Clomipramine on the BEH C<sub>18</sub> column shows a wide, tailing peak at low pH. In high pH mobile phase, the clomipramine peak width is narrow with no evidence of tailing. The peak width and tailing factors are much improved on the CSH C<sub>18</sub> column for clomipramine at low pH with 0.1% formic acid. Slightly wider peaks and slightly higher tailing factors are also evident with high pH on the CSH C<sub>18</sub> Column.

Table 1. Peak Width Comparison in Mobile Phases Containing 0.1% Formic Acid and 0.1% Ammonium Hydroxide

Peak width (min)						
	BEH C <sub>18</sub>		CSH C <sub>18</sub>		Vendor Y C <sub>18</sub>	
	HCOOH	NH <sub>4</sub> OH	HCOOH	NH <sub>4</sub> OH	HCOOH	NH <sub>4</sub> OH
Clomipramine	0.58	0.25	0.24	0.51	0.35	0.28
Flavone	0.23	0.23	0.22	0.29	NA	0.26
Diclofenac	0.19	0.22	0.24	0.37	NA	0.24

Table 2. USP Tailing Factor Comparison in Mobile Phases Containing 0.1% Formic Acid and 0.1% Ammonium Hydroxide

USP tailing factor						
	BEH C <sub>18</sub>		CSH C <sub>18</sub>		Vendor Y C <sub>18</sub>	
	HCOOH	NH <sub>4</sub> OH	HCOOH	NH <sub>4</sub> OH	HCOOH	NH <sub>4</sub> OH
Clomipramine	1.46	1.03	1.21	1.15	1.03	1.09
Flavone	1.18	1.20	1.06	1.28	NA	1.17
Diclofenac	0.97	1.18	0.99	1.67	NA	0.92

## Conclusion

One of the most straightforward ways to modify a separation is to change the pH of the mobile phase. Columns packed with robust chromatographic particles designed for purification that are compatible with both low and high pH conditions, such as those manufactured with Hybrid Particle Technology like XBridge BEH and XSelect CSH, make separation method development for isolation and purification fast and efficient. Changing the pH of a separation often results in improved peak shape, reduced tailing, and increased mass loading, all of which contribute to increased yield, purity, and time savings. XBridge and XSelect CSH stationary phases, combined with the highly controlled OBD column packing process, ensure preparative columns are of similar bed density to the analytical column of the same chemistry, thus making preparative chromatographic profiles analogous to the

analytical run. Similar chromatography at the analytical and preparative scales simplifies scale-up and compound isolation. The OBD preparative columns used in this study produced better purification outcomes when compared (using identical experimental conditions) to the Vendor Y Column at both low and high pH.

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[MassLynx MS Software](#) <<https://www.waters.com/nextgen/global/products/informatics-and-software/mass-spectrometry-software/masslynx-mass-spectrometry-software.html>>

720008431, August 2024



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