

## A New Mixed-Mode Reversed-Phase/Anion-Exchange Stationary Phase Based on Hybrid Particles

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### Abstract

This application note highlights the characteristics of a new mixed-mode RP/AX stationary phase that offers very different selectivity compared to traditional RP stationary phases.

### Benefits

- Improved retention of polar acidic analytes in reversed-phase chromatography
  - Chromatographic selectivity complements that of traditional reversed-phase columns
  - Excellent stationary phase batch-to-batch reproducibility
  - Stable over a wide pH range (2–10) for method flexibility
  - Outstanding peak shapes for basic analytes using mobile phase containing formic acid
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### Introduction

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While there is a great diversity of reversed-phase (RP) columns currently available, there are relatively few that are designed to retain and separate polar acidic analytes. Widely used silica-based RP packing materials typically exhibit both reversed-phase and cation-exchange behavior, with the latter arising from ionized silanols.<sup>1</sup> The presence of ionized silanols creates a negative surface charge, which repels negatively charged analytes such as ionized acids. One strategy to retain ionized acids is to use ion-pairing reagents in the mobile phase.<sup>2</sup> This approach has several disadvantages including long equilibration times and high background levels. Another strategy involves derivatizing the analytes to form more hydrophobic neutral species.<sup>3</sup> The disadvantage of this approach is the greater time needed for sample preparation and the potential for increased variability in quantitative measurements. The limitations of these strategies led to the development of mixed-mode RP/anion-exchange (AX) columns, which contain stationary phases having both hydrophobic and AX functionalities.<sup>4</sup> However, many mixed-mode RP/AX columns suffer from poor batch-to-batch reproducibility and a limited pH range. To overcome these issues, we have developed a new stationary phase based on hybrid organic/inorganic particles that has excellent batch-to-batch reproducibility and is stable over a wide pH range. The chromatographic selectivity of the new Atlantis BEH C<sub>18</sub> AX stationary phase is described in this note. This material is packed into new MaxPeak High Performance Surfaces column hardware, which is designed to increase analyte recovery by minimizing interactions with stainless steel that can lead to losses. The packed columns are named Atlantis Premier BEH C<sub>18</sub> AX Columns.

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## Experimental

### Polar mixture separation

The sample contained 5 µg/mL thiourea, 12.5 µg/mL 5-fluorouracil, 25 µg/mL nicotinamide, 37.5 µg/mL adenosine- 5'-monophosphate, 37.5 µg/mL procainamide and 125 µg/mL resorcinol in 10 mM ammonium formate pH 3.00 (aq). Four consecutive separations were carried out, and the retention times from the last three injections were used to calculate the retention factors. Void time ( $t_0$ ) measurements were made using thiourea with an acetonitrile mobile phase.

Instrument: ACQUITY UPLC I-Class System  
with PDA Detector

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Data management: Empower 3 CDS

## LC method conditions

Column size: 2.1 × 50 mm

Column temp.: 30 °C

Injection volume: 1.5 µL

Flow rate: 0.2 mL/min

Mobile phase: 10 mM ammonium formate pH  
3.00 (aq)

UV detection: 254 nm

## Broad polarity range mixture separation

Samples containing 5 µg/mL thiourea, 50 µg/mL resorcinol, 10 µg/mL caffeine, 5 µg/mL 2-nitrobenzoic acid, 100 µg/mL metoprolol, 10 µg/mL 2-nitrobenzyl alcohol, 2 µg/mL papaverine, 125 µg/mL 2-chlorobenzoic acid, 20 µg/mL propranolol, 25 µg/mL 4-nitrophenol, 10 µg/mL berberine, 25 µg/mL 3-nitrophenol, 100 µg/mL 2-chlorophenol, 25 µg/mL 2-nitrophenol, 10 µg/mL amitriptyline, 500 µg/mL fluoxetine, 25 µg/mL diethylphthalate, 100 µg/mL fenoprofen, and 25 µg/mL dipropylphthalate were prepared in 15.4 mM ammonium formate pH 3.00 (aq). Three consecutive separations were carried out and the average retention times were used to calculate the gradient retention factors. Thiourea was used as the void time ( $t_0$ ) marker with a 100% acetonitrile mobile phase.

Instrument: ACQUITY UPLC System with  
PDA Detector

Data management: Empower 3 CDS

## LC method conditions

Column size:	2.1 × 50 mm
Column temp.:	30 °C
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Mobile phase A:	15.4 mM ammonium formate pH 3.00 (aq)
Mobile phase B:	Acetonitrile
Gradient:	5–95% B in 5.3 min (linear)
UV detection:	254 nm

## Retention loss after stopping and restarting flow

The sample contained 10 µg/mL thiourea and 12 µg/mL thymine in 10 mM ammonium formate pH 3.00 (aq). The instrument and separation conditions were the same as for the polar mixture separation. After separating the polar mixture four times, the flow was stopped for 10 min. The flow was then restarted at 0.2 mL/min. After 0.6 min, two injections of the thiourea/thymine sample were made. The retention factor of thymine was calculated using the average retention time of thymine and the  $t_0$  measurement previously obtained using thiourea with an acetonitrile mobile phase.

## pH 10 stability study

### Acid, base, and neutral mixture separation

The sample contained 10 µg/mL thiourea, 100 µg/mL guanosine-5'-monophosphate, 100 µg/mL resorcinol, and 100 µg/mL nicotinamide in water.

Instrument: ACQUITY UPLC H-Class  
System with PDA Detector

Data management: Empower 3 CDS

## LC method conditions

Column size: 2.1 × 50 mm

Column temp.: 30 °C

Injection volume: 2.0 µL

Flow rate: 0.2 mL/min

Mobile phase: (95/5 v/v) 10 mM ammonium  
bicarbonate pH 10.08  
(aq)/methanol

UV detection: 254 nm

## Basic analyte mixture separations

The sample contained 200 µg/mL metoprolol tartrate, 10 µg/mL papaverine, and 50 µg/mL amitriptyline in methanol/water (1/5 v/v). Three consecutive separations were carried out and the average peak widths at 13.4% peak height were determined for each analyte.

Instrument: ACQUITY UPLC System with  
TUV Detector

Data management: Empower 3 CDS

## LC method conditions

Column size:	2.1 × 50 mm
Column temp.:	30 °C
Injection volume:	2 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% formic acid (aq)
Mobile phase B:	Acetonitrile
Gradient:	15–95% B in 7.37 min (linear)
UV detection:	260 nm

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## Results and Discussion

### Chemical and Physical Properties of Atlantis BEH C<sub>18</sub> AX

The properties of the Atlantis BEH C<sub>18</sub> AX stationary phase are summarized in Table 1, and compared to those of BEH C<sub>18</sub>, CSH C<sub>18</sub>, and HSS T3. The Atlantis BEH C<sub>18</sub> AX stationary phase is based on a 95 Å ethylene-bridged hybrid (BEH) particle, which has a smaller pore size than the 130 Å particles used in BEH and CSH stationary phases. The smaller pore size particles have a 50% higher surface area, which leads to greater retention. The retention factors of neutrals for Atlantis Premier BEH C<sub>18</sub> AX columns are very similar to those for HSS T3 columns. The Atlantis BEH C<sub>18</sub> AX stationary phase contains not only C<sub>18</sub> groups, but also tertiary alkylamine moieties, creating a positive surface charge below approximately pH 8. Like Atlantis BEH C<sub>18</sub> AX, CSH C<sub>18</sub> also has both C<sub>18</sub> and anion-exchange groups. However, with anion-exchange groups containing pyridine moieties, CSH C<sub>18</sub> is positively charged only below approximately pH 5. Atlantis BEH C<sub>18</sub> AX is endcapped to reduce the concentration of residual silanols and to improve the base stability of the stationary phase.

Stationary phase	Particle properties				Bonded phase properties		
	Material	Pore size (Å)	Pore volume (cm <sup>3</sup> /g)	Surface area (m <sup>2</sup> /g)	C <sub>18</sub> surface concentration (μmol/m <sup>2</sup> )	Endcapping	Charge modifier
Atlantis BEH C <sub>18</sub> AX	BEH	95	0.7	270	1.6	Yes	Alkylamine
CSH C <sub>18</sub>	BEH	130	0.7	185	2.3	Yes	Pyridyl
BEH C <sub>18</sub>	BEH	130	0.7	185	3.1	Yes	None
HSS T3	Silica	100	0.7	230	1.6	Yes	None

Table 1. Comparison of the chemical and physical properties of four stationary phases.

## Compatibility with 100% Aqueous Mobile Phases

To obtain sufficient retention of highly polar analytes in RP separations, highly aqueous mobile phases are often needed. Not all RP columns are compatible with these mobile phases, due to retention losses that occur when the flow is stopped and restarted.<sup>5,6</sup> High-coverage C<sub>18</sub> and C<sub>8</sub> bonded phases based on particles with average pore diameters less than about 100 Å are particularly prone to this phenomenon, with retention losses approaching 100%. The use of an intermediate C<sub>18</sub> surface concentration (1.6 μmol/m<sup>2</sup>) together with the AX groups makes Atlantis BEH C<sub>18</sub> AX compatible with highly aqueous mobile phases. An experiment in which the flow of a 100% aqueous mobile phase (at 30 °C) was stopped and restarted for an Atlantis Premier BEH C<sub>18</sub> AX, 1.7 μm, 2.1 × 50 mm Column found only a 3.7% decrease in retention for thymine. This is lower than the approximately 10% retention loss observed for HSS T3.

## Retention and Selectivity

The Atlantis BEH C<sub>18</sub> AX stationary phase has a positive surface charge below approximately pH 8. Relative to conventional reversed-phase materials, the positive surface charge gives increased retention of anions, such as ionized acids, and decreased retention of cations, such as protonated bases. These differences are readily observed in the chromatograms shown in Figure 1. The sample used for this study contains a mixture of six polar analytes, one which is negatively charged in the pH 3 mobile phase (adenosine 5'-monophosphate, AMP), two which are positively charged (nicotinamide and procainamide) and three which are neutral (thiourea, 5-fluorouracil, and resorcinol). Relative to HSS T3 and CSH C<sub>18</sub> columns, the Atlantis Premier BEH C<sub>18</sub> AX Column gives the highest retention for AMP. The neutral compounds have similar retention on the Atlantis Premier BEH C<sub>18</sub> AX and HSS T3 columns, while the CSH C<sub>18</sub> column has the lowest retention of these analytes, due to the lower surface area of the 130 Å particles used for this material. The positively-charged analytes nicotinamide and

procainamide are most retained on the HSS T3 column, and least retained on the Atlantis Premier BEH C<sub>18</sub> AX Column.

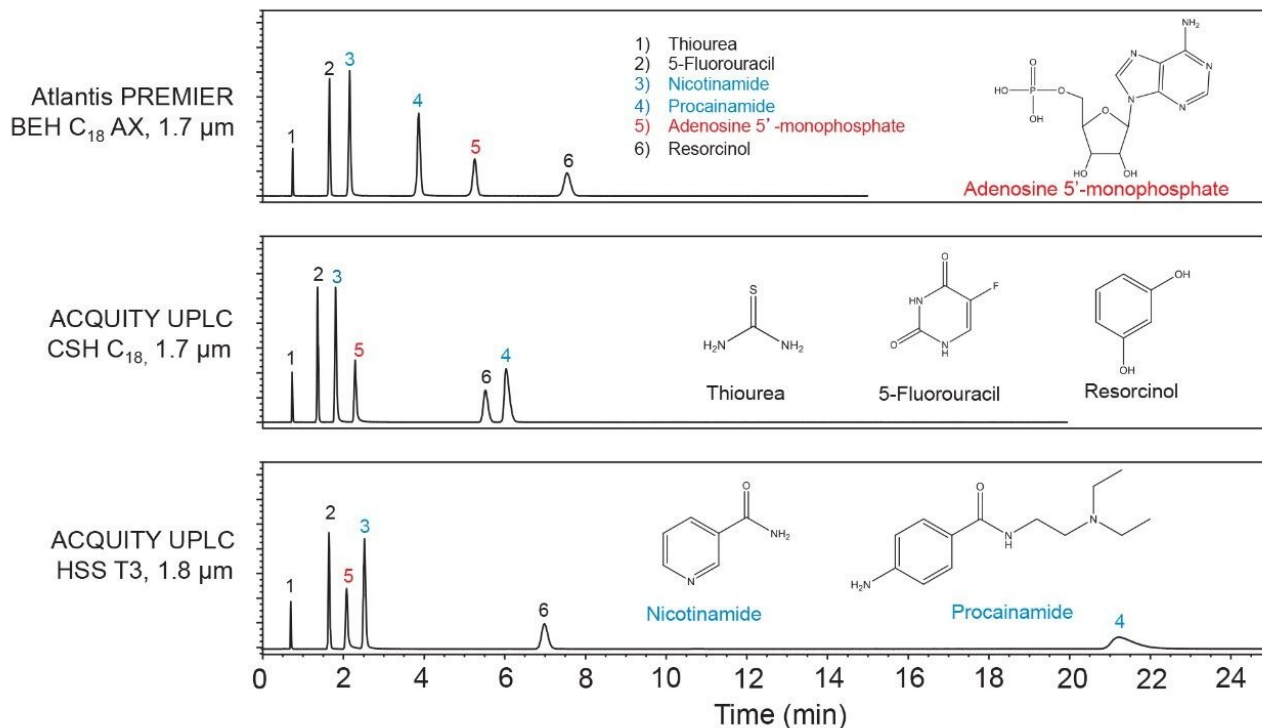


Figure 1. Isocratic separations of a mixture of polar analytes. Blue signifies compounds that are positively charged in the pH 3.00 mobile phase, red signifies the negatively charged compound, and black signifies the neutral compounds.

These trends have also been observed in studies with larger sets of analytes. For example, a set of 18 compounds covering a range of polarities and acid/base properties was separated using an acetonitrile gradient with an aqueous mobile phase containing a pH 3 ammonium formate buffer. The results are shown in Figure 2 as plots of gradient retention factors ( $k_g$ ) for Atlantis BEH C<sub>18</sub> AX vs.  $k_g$  for BEH C<sub>18</sub> and CSH C<sub>18</sub>. The data points for the analytes that are negatively charged at pH 3 are shown as red circles, those for the positively charged analytes as blue diamonds and those for the neutrals as black triangles. The Atlantis Premier BEH C<sub>18</sub> AX Column gives the highest  $k_g$  values for the negatively charged compounds, and the lowest  $k_g$  values for the positively charged analytes. To quantify the selectivity differences between these columns, we calculated the  $s^2$  values as



$$s^2 = 1 - r^2$$

where  $r$  is the correlation coefficient for the  $k_f$  vs  $k_r$  plots.<sup>7</sup> The lower the correlation coefficient, the greater is the  $s^2$  value, indicating a larger selectivity difference between the columns. The  $s^2$  values range between 0 and 1. The results show  $s^2$  values of 0.58 for Atlantis BEH C<sub>18</sub> AX relative to BEH C<sub>18</sub> and 0.47 for Atlantis BEH C<sub>18</sub> AX relative to CSH C<sub>18</sub>. These are large values, approximately four times greater than the largest value reported for a set of different RP bonded phases.<sup>7</sup> Atlantis BEH C<sub>18</sub> AX significantly expands the stationary phase selectivity range for reversed-phase columns when separating mixtures containing ionized analytes.

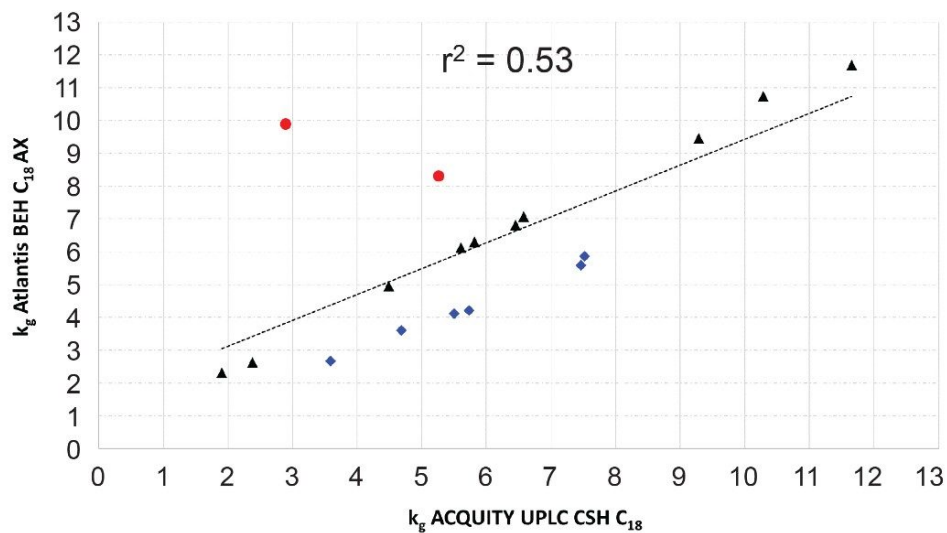
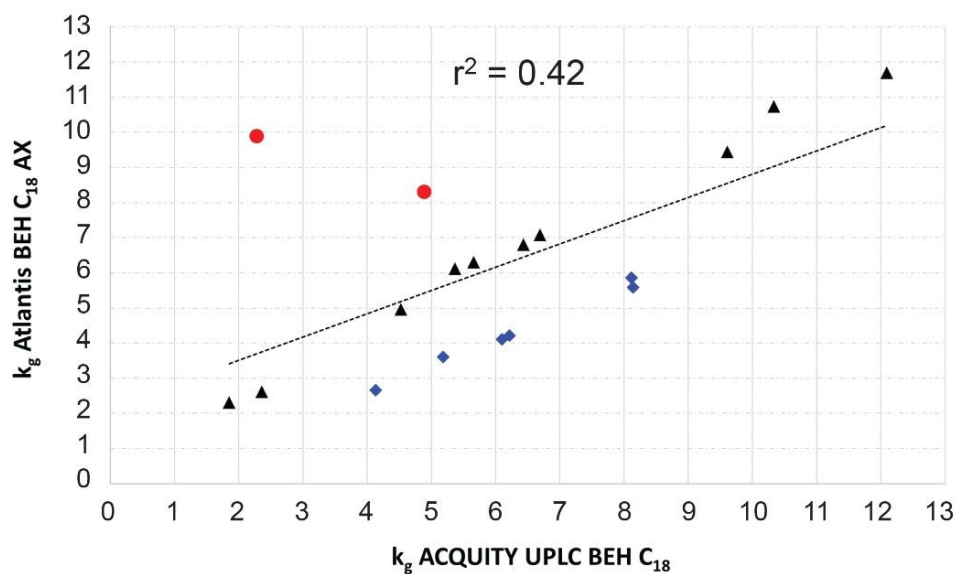


Figure 2. Correlation of gradient retention factors for Atlantis BEH C<sub>18</sub> AX vs. BEH C<sub>18</sub> (left) and vs. CSH C<sub>18</sub> (right). Color has the same meaning as in Figure 1. The line shows the least squares fit and the squares of the correlation coefficients ( $r^2$ ) are given.

## Batch-To-Batch Reproducibility and Particle Size Scalability

As for CSH C<sub>18</sub>,<sup>8</sup> the surface modification of Atlantis BEH C<sub>18</sub> AX involves separate steps to incorporate the charge modifier, the C<sub>18</sub> groups and the endcap. This approach gives excellent control of the surface chemistry,

resulting in high batch-to-batch reproducibility. The chromatographic reproducibility of Atlantis BEH C<sub>18</sub> AX was assessed by testing twenty-seven different batches of 1.7, 2.5, and 5 μm materials using the separation of the polar mixture shown in Figure 1. For the relative retentions, the results showed relative standard deviations (RSD) ranging from 1 to 3% (Figure 3), indicating excellent reproducibility, comparable to some of the most reproducible conventional C<sub>18</sub> bonded phases.<sup>9</sup> No statistically significant differences were found in the relative retentions of the different particle sizes, facilitating scalability across columns packed with different particle sizes.

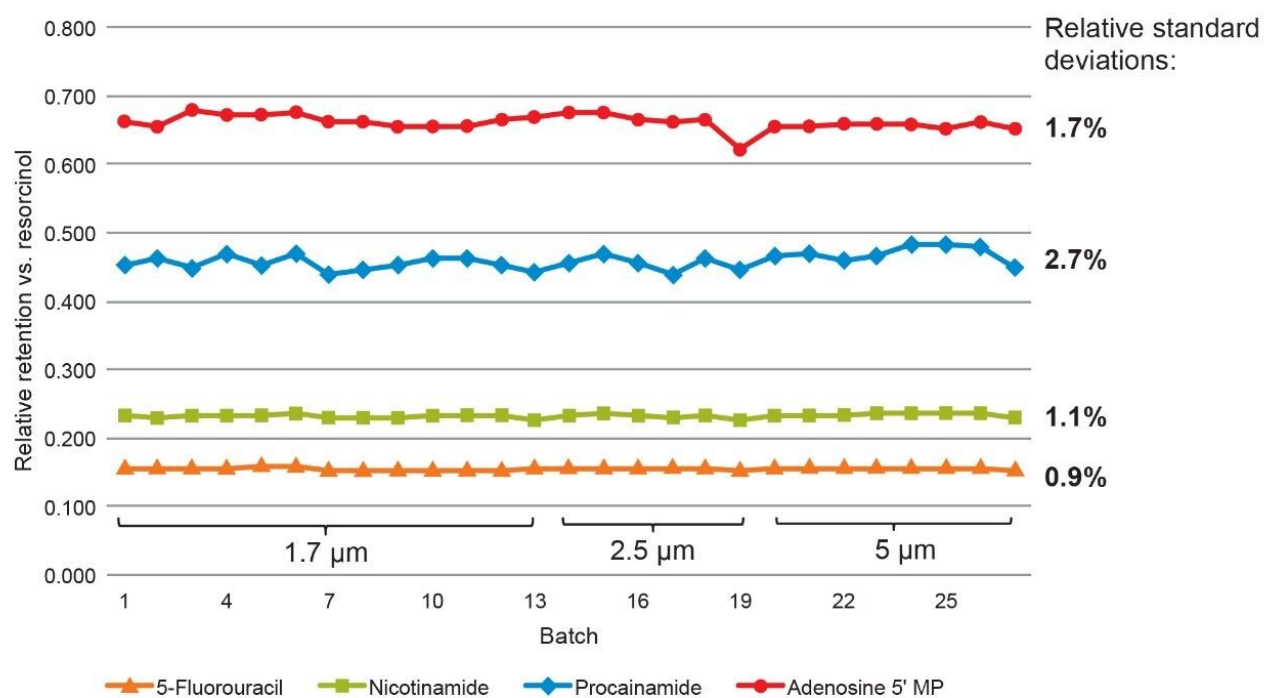


Figure 3. Relative retentions for 27 batches of Atlantis BEH C<sub>18</sub> AX, for the polar mixture separation shown in Figure 1.

## pH Stability

Combining the base-stable BEH particle<sup>10</sup> with stable bonding chemistries, the Atlantis BEH C<sub>18</sub> AX material shows exceptional resistance to both acidic and basic mobile phases for a mixed-mode stationary phase. The recommended pH range for Atlantis Premier BEH C<sub>18</sub> AX Columns is compared to those of two leading mixed-mode RP/AX columns in Table 2.

Stationary phase	Recommended pH range
Atlantis BEH C <sub>18</sub> AX	2 to 10
SiELC Primesep® B	1.5 to 4.5
Thermo Acclaim™ Mixed-Mode WAX-1	2.5 to 7.5

*Table 2. Recommended pH ranges for three mixed-mode RP/AX stationary phases.*

The most notable differences are in the upper limits, with Atlantis BEH C<sub>18</sub> AX recommended for use up to pH 10 vs. pH 4.5 or 7.5 for the other columns, which are based on silica particles. The pH range for Atlantis BEH C<sub>18</sub> AX was established based on the results of accelerated tests similar to those which have previously been described.<sup>10,11</sup> To verify the upper pH limit, we carried out a stability study using a pH 10 mobile phase and a temperature of 30 °C. A three-component sample mixture was injected 340 consecutive times, with a run time of one hour per injection.

The changes in retention time for resorcinol, guanosine-5'-monophosphate, and nicotinamide were found to be +0.1%, -14.5%, and -8.5%, respectively (Figure 4). The extended high pH stability of Atlantis Premier BEH C<sub>18</sub> AX Columns allows a wider range of mobile phase pH values to be used. For samples containing ionizable analytes, mobile phase pH has been shown to be the most powerful variable to adjust selectivity.<sup>12</sup>

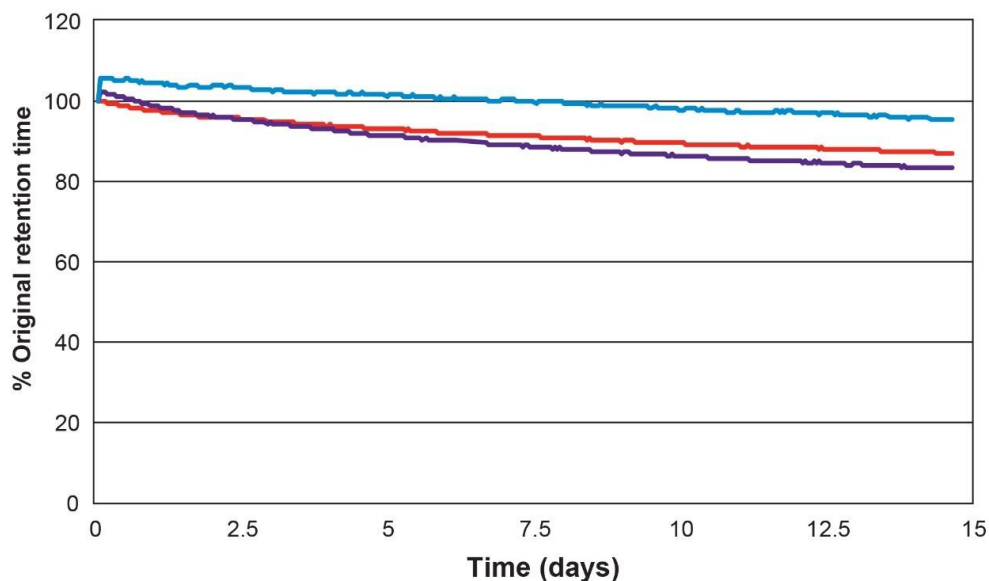


Figure 4. Plot of % original retention time throughout a pH 10 stability study for resorcinol (black), nicotinamide (green), and guanosine-5'-monophosphate (red).

## Effect of Mobile Phase pH and Buffer Concentration on Retention and Selectivity

To investigate the dependence of retention on pH for an Atlantis Premier BEH C<sub>18</sub> AX Column, the mixture of Figure 1 was separated using a series of mobile phases varying in pH, but with constant buffer concentration. The 100% aqueous mobile phases were prepared from ammonium formate with variable amounts of formic acid or ammonium hydroxide added to adjust the pH. The results are shown in Figure 5. The retention factors of three of the analytes (AMP, nicotinamide, and procainamide) show a pronounced dependence on pH, while the retention factors of the other two analytes (5-fluorouracil and resorcinol) vary only slightly.

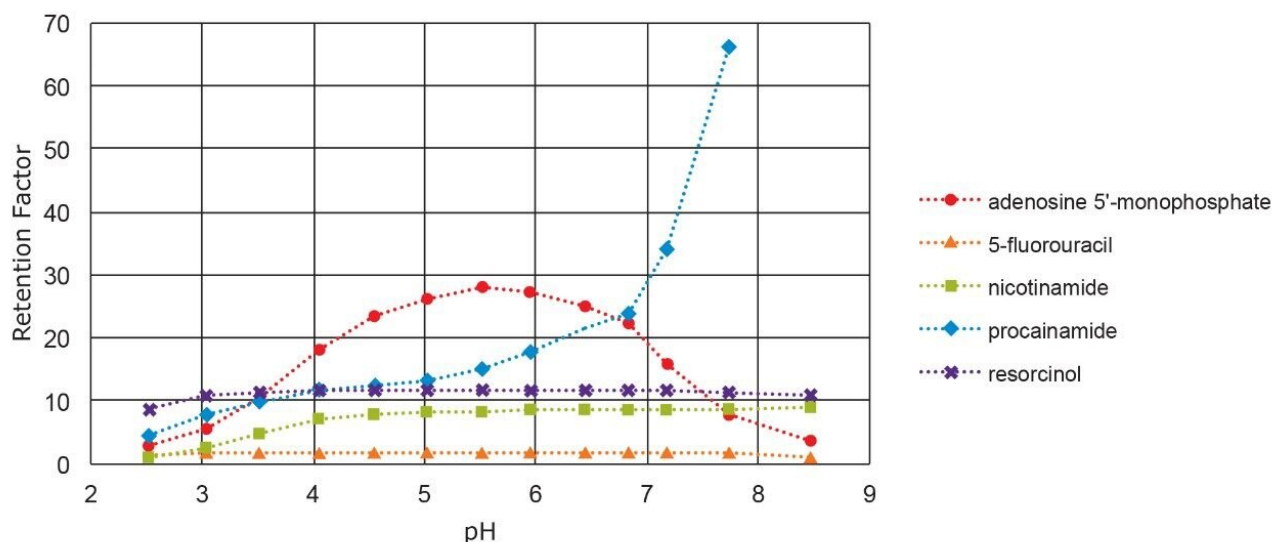


Figure 5. Retention factors vs. mobile phase pH for an Atlantis Premier BEH C<sub>18</sub> AX Column. All buffer concentrations were 20.0 mM.

The behavior of nicotinamide ( $pK_a = 3.4$ ) and procainamide ( $pK_a = 4.6, 9.3$ ) is similar to that of other bases in RP chromatography: the retention factors are lower below their  $pK_a$ , where they are protonated, then increase above their  $pK_a$ , where they are unprotonated.<sup>13</sup> The differences in retention factors between the protonated and unprotonated forms are large: a factor of 9 for nicotinamide and more than 14 for procainamide.

The surface charge of the stationary phase also transitions from positive to neutral over this pH range, which may accentuate the retention factor difference since the protonated forms of the analytes are partially excluded from the positively-charged surface due to ionic repulsion. The retention factor of AMP shows a different dependence on pH. AMP has three ionizable groups: the two acidic P-OH groups ( $pK_a = 0.9$  and  $6.2$ ) and the basic adenine moiety ( $pK_a = 3.8$ ).

The ionic charge of AMP varies from 0 at approximately pH 2.5 to -2 at pH 7. The increasing negative charge of AMP accounts for its increasing retention factor between pH 2.5 and 5.5. The decrease in its retention factor above pH 5.5 is due to the decreasing positive charge on the stationary phase.

A second important mobile phase variable is the concentration of the buffer, which strongly affects the ion-exchange retention. To investigate this variable, the same polar mixture was separated using a series of mobile

phases containing pH 3.00 ammonium formate at concentrations ranging from 5 to 50 mM. The results are shown in Figure 6. Two of the analytes (AMP and procainamide) show a strong dependence of retention on buffer concentration, with opposite trends. The retention factor of negatively charged AMP decreases with increasing buffer concentration, due to the formate anions displacing the AMP from the AX sites on the stationary phase. In contrast, the retention factor of positively charged procainamide increases with increasing buffer concentration. This is attributed to a weakening of the ionic repulsion between protonated procainamide and the positively-charged surface as the buffer concentration increases.

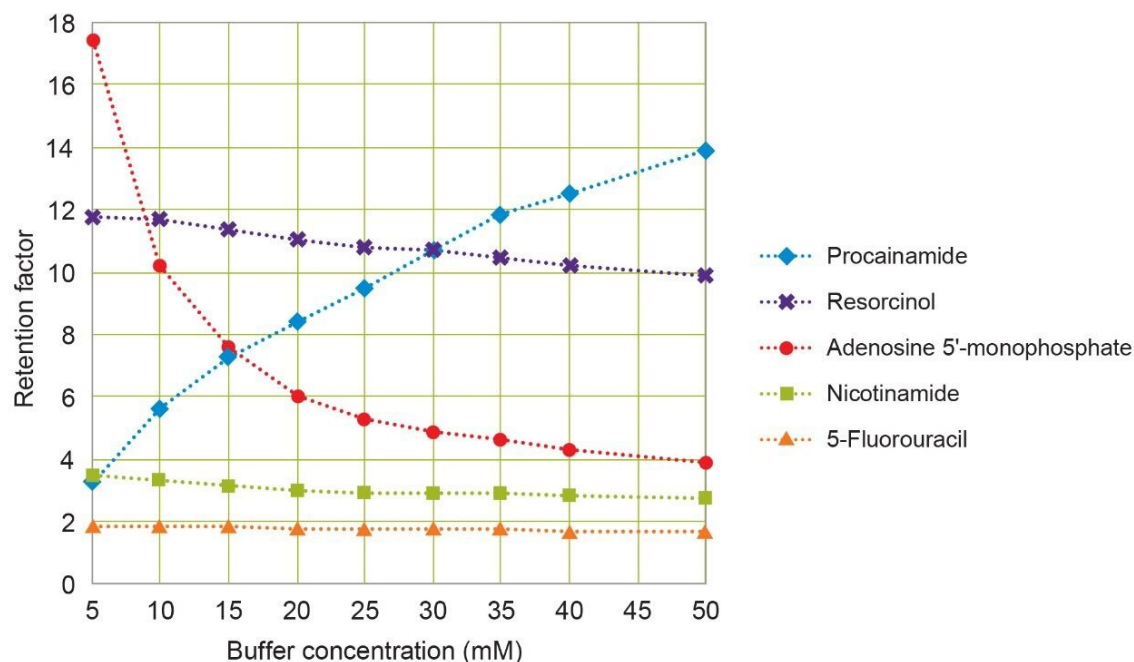


Figure 6. Retention factors vs. mobile phase buffer concentration for an Atlantis Premier BEH C<sub>18</sub> AX Column. All mobile phases had a pH of 3.00.

## Improved Peak Shapes for Basic Analytes using 0.1% Formic Acid

It has been demonstrated that CSH C<sub>18</sub> columns give a large improvement in the sharpness and symmetry of peaks from cationic analytes, such as protonated bases, when using low ionic-strength mobile phases, like those containing formic acid.<sup>8</sup> Atlantis Premier BEH C<sub>18</sub> AX Columns also show this effect, but to an even greater extent. Shown in Figure 7 are chromatograms for the separation of three basic analytes on three different

columns using an acetonitrile gradient and an aqueous mobile phase containing 0.1% formic acid. The results show that the metoprolol and amitriptyline peaks become narrower and more symmetric moving from BEH C<sub>18</sub> to CSH C<sub>18</sub> to Atlantis BEH C<sub>18</sub> AX.

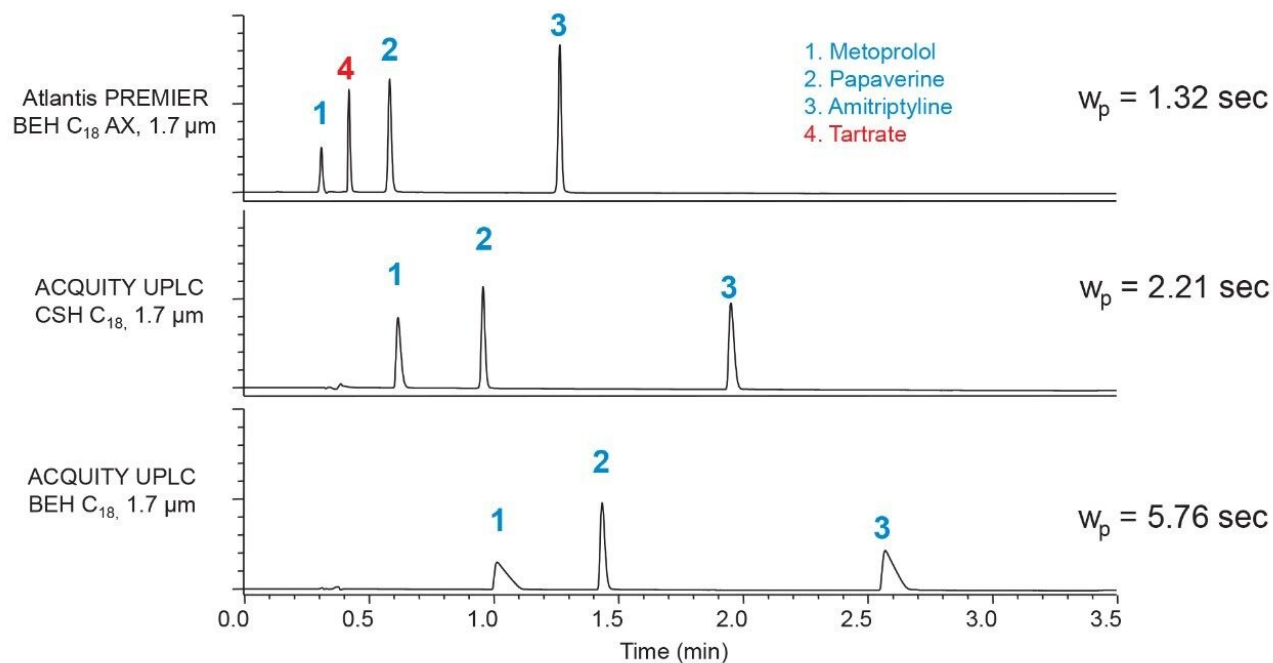


Figure 7. Gradient separations of three basic analytes using a mobile phase containing 0.1% formic acid. The average peak widths at 13.4% peak height ( $w_p$ ) were calculated from the metoprolol and amitriptyline peaks.

The retention times also decrease, as a result of ionic repulsion of the protonated analytes from the surfaces of the CSH C<sub>18</sub> and Atlantis BEH C<sub>18</sub> AX stationary phases. It's notable that the tartrate counterion of metoprolol is retained on the Atlantis Premier BEH C<sub>18</sub> AX Column, but not on the other two columns. To quantify the improved peak sharpness for the Atlantis Premier BEH C<sub>18</sub> AX Column, we determined the average peak widths for the metoprolol and amitriptyline peaks. The results show a 40% decrease moving from CSH C<sub>18</sub> to Atlantis BEH C<sub>18</sub> AX. This is a large improvement that offers the possibility of higher resolution separations for basic analytes when using low ionic-strength mobile phases. However, the peak shapes for ionized acids may not be optimal when using such mobile phases. Buffer concentrations of at least 10 mM are recommended for ionized acidic analytes with Atlantis Premier BEH C<sub>18</sub> AX Columns.



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## Conclusion

This application note highlights the characteristics of a new mixed-mode RP/AX stationary phase that offers very different selectivity compared to traditional RP stationary phases. Atlantis BEH C<sub>18</sub> AX has excellent batch-to-batch reproducibility and exceptional high-pH stability relative to existing mixed-mode RP/AX products. Compared to conventional RP stationary phases, when used with mobile phase pH values less than about 8, Atlantis BEH C<sub>18</sub> AX shows increased retention of anions, such as ionized acids, and decreased retention of cations, such as protonated bases. The selectivity for ionizable analytes may be varied over a wide range by adjusting the pH and buffer concentration of the mobile phase. When using low ionic strength mobile phases (e.g., containing 0.1% formic acid), Atlantis BEH C<sub>18</sub> AX shows extraordinarily sharp and symmetric peaks for protonated bases. This new stationary phase is complementary to existing RP stationary phases, making it useful for column screening during method development.

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