EVALUATION OF NEW MS-COMPATIBLE MIXED-MODE RP/AX HPLC COLUMNS

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INTRODUCTION

Reversed-phase (RP) liquid chromatography is widely used for the analysis of both neutral and ionizable analytes. However, the separation of polar acids using reversed-phase (RP) HPLC poses significant challenges for currently available RP columns, with many analytes of interest being poorly retained. Solutions to this challenge include the use of ion-pairing reagents or mixed-mode RP/anion exchange (AX) columns. However, most existing approaches are not compatible with mass spectrometry (MS) detection. In addition, binding of acidic analytes to metal surfaces in columns is a common problem. To overcome these challenges and to explore further increasing the retention of polar acidic analytes, we developed a new family of columns employing several novel technologies. The columns are named Atlantis™ PREMIER BEH C_{18} AX. The stationary phase is based on a new high phase-ratio BEH particle, bonded with C_{18} groups and a controlled low concentration of anion-exchange groups. The properties of the Atlantis BEH C_{18} AX stationary phase are summarized in Table 1, and compared to those of BEH C_{18}, CSH™ C_{18}, and HSS T3.

Table 1. Chemical and Physical Properties of Atlantis™ BEH C_{18} AX

	Particle Properties			Bonded phase properties	
Stationary Phase	Pore Size (Å)	Pore volume (cm^3/g)	Surface area (m2/g)	C_{18} Surface concentration (µmol/m2)	Charge Modifier
Atlantis BEH C_{18} AX	95	0.7	270	1.6	Alkylamine
CSH C_{18}	130	0.7	185	2.3	Pyridyl
BEH C_{18}	130	0.7	185	3.1	None
HSS T3	100	0.7	230	1.6	None

The use of bridged-ethyl hybrid particles allows the columns to be used with a broad range of mobile phase pH values. The material does not exhibit retention losses when used with 100% aqueous mobile phases. The anion-exchange groups have a pK_a of approximately 7.5. Consequently, the columns exhibit anion-exchange retention at mobile phase pH values up to 8. The columns are compatible with mass spectrometry detection, exhibiting minimal ion suppression or enhancement from column bleed. Novel column hardware was used to provide improved recovery of analytes that bind to metal surfaces in conventional column hardware. The chromatographic properties of the new columns have been evaluated and compared to existing RP columns designed for separating polar analytes.

METHODS

Polar Mixture Separations (Figures 1, 2)

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.

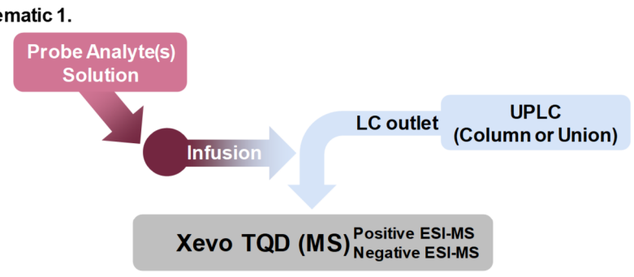
Instrument: ACQUITY UPLC I-Class System with PDA Detector
Data management: Empower 3 CDS
LC method conditions
Column size: 2.1 × 50 mm Column temp.: 30 °C
Injection volume: 1.5 µL Flow rate: 0.2 mL/min
Mobile phase: 10 mM ammonium formate pH 3.00 (aq)
UV detection: 254 nm

% Matrix Effects (Figure 3)

A sample containing 10 µg/mL thiourea, 20 µg/mL thymine, 10 µg/mL adenine, 2 µg/mL 5-fluorocytosine, 5 µg/mL thiamine, 15 µg/mL tryptophan and 1.5 µg/mL Niflumic acid in 1:1 (v/v) (Acetonitrile: 0.1% (v/v) formic acid in water) was used for positive ESI-MS. A sample containing 10 µg/mL citric acid, 10 µg/mL malic acid, 10 µg/mL guanosine-5′-monophosphate (G5MP), 10 µg/mL thymidine-5′-monophosphate (T5MP) and 1.5 µg/mL niflumic acid in 1:1 (v/v) (Acetonitrile: 0.1% (v/v) formic acid in water) was used for negative ESI-MS. The sample was infused post LC at 10 % and 90% of the organic/aqueous mobile phase and their individual peak areas measured for their corresponding multiple reaction monitoring (MRM) in the presence and absence of a column (**Schematic 1**). The matrix effect was calculated for each of the individual analytes.

Instrument: ACQUITY UPLC H-Class System with Xevo TQD-MS.
Data management: MassLynx V4.2
LC-ESI-MS method Conditions:
Column size: 2.1 x 150 mm Column temp.: 50 °C
Mobile phase flow rate: 0.2 mL/min
MS infusion flow rate: 20 µL/min
Mobile phase positive ESI-MS: Acetonitrile (organic), 0.1 % (v/v) formic acid in water (pH: 2.7)

Schematic 1.



Peak area of analyte(s) are measured in the presence of column bleed (Column) and absence of column bleed (Union)

$$\text{Matrix Factor (MF)} = \frac{\text{Peak area of Analyte from post-}\textbf{\textit{Column}}\text{ infusions}}{\text{Peak area of Analyte from post-}\textbf{\textit{Union}}\text{ infusions}}$$

$$\text{\% Matrix Effect (ME):} \quad \begin{cases} (MF-1)\,x\,100, & MF > 1 \\ (1-MF)\,x\,100, & MF < 1 \end{cases}$$

RESULTS

As shown in Table 1, the Atlantis BEH C_{18} 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 analyte retention. The Atlantis BEH C_{18} AX stationary phase contains not only C_{18} groups, but also tertiary alkylamine moieties, creating a positive surface charge below approximately pH 8. The separation of a mixture of six polar analytes was compared on an Atlantis PREMIER BEH C_{18} AX Column, CSH C_{18} Column, and an HSS T3 Column (Figure 1). The chromatographic reproducibility of the Atlantis BEH C_{18} AX stationary phase was assessed using relative standard deviations of the ionizable analytes to the neutral analyte resorcinol (Figure 2).

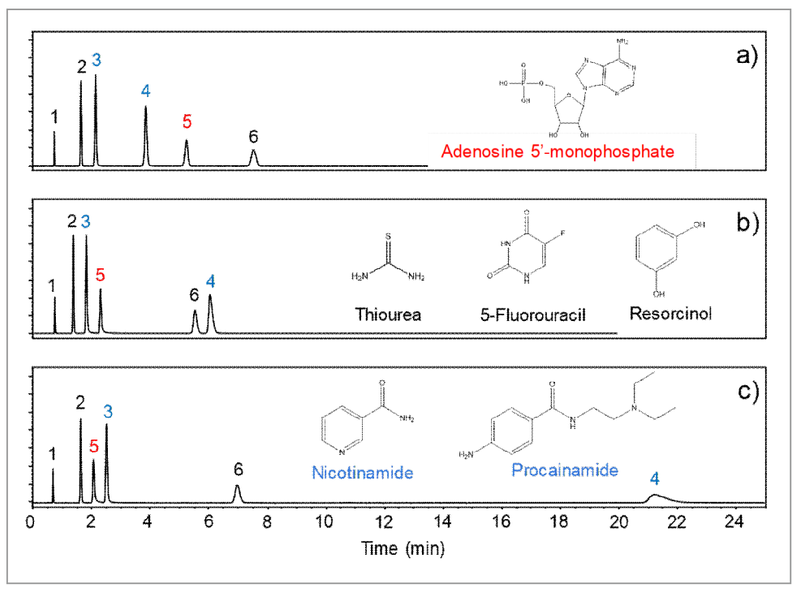


Figure 1. Isocratic separations of a mixture of polar analytes. Blue signifies compounds that are positively charged in the pH 3.00 mobile phase, red signifies the negatively charged compound and black signifies the neutral compounds. a) Atlantis PREMIER BEH C_{18} AX, b) ACQUITY UPLC CSH C_{18} 1.7 µm, and c) ACQUITY UPLC HSS T3 1.8 µm Sample ID: 1) Thiourea, 2) 5-Fluorocytosine, 3) Nicotinamide, 4) Procainamide, 5) Adenosine 5'-monophosphate, 6) Resorcinol

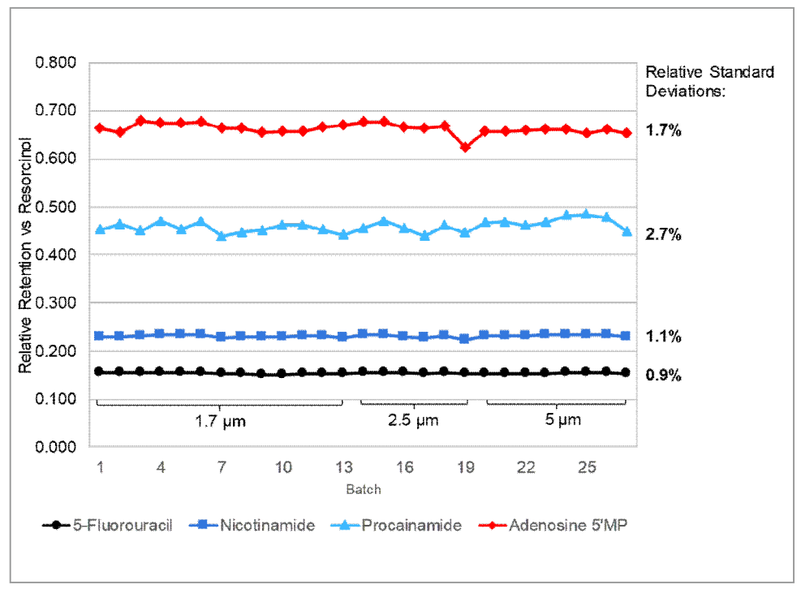


Figure 2. Relative retentions for 27 batches of Atlantis BEH C_{18} AX, for the polar mixture separation shown in Figure 1.

The % Matrix Effect (ME) was determined for several probe analytes using the Atlantis BEH C_{18} AX stationary phase. The results were compared to two leading mixed-mode columns. In negative ESI-MS, the % Matrix Effect for the analytes using the Atlantis PREMIER BEH C_{18} AX column were lower in all comparisons but one versus the other columns (Figure 3). In positive-ESI-MS, the same trend was observed: The % ME caused by bonded phase interferences were lower for the Atlantis PREMIER BEH C_{18} AX column.

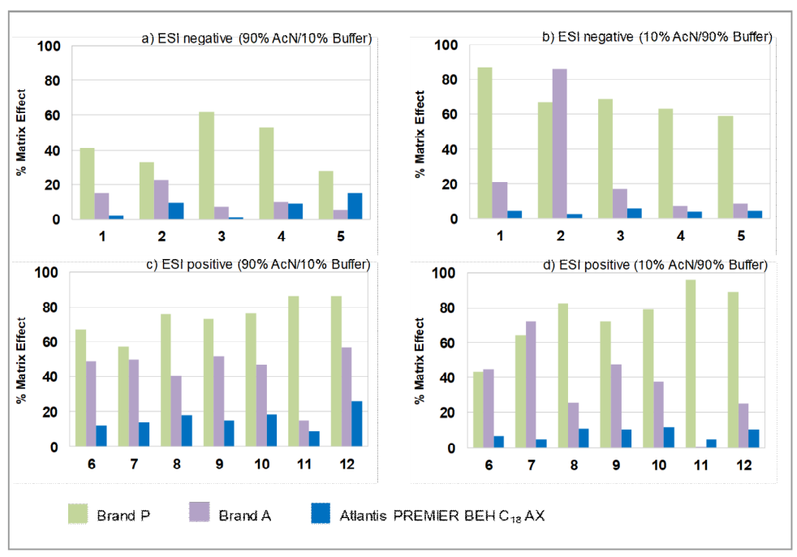


Figure 3. % Matrix Effect under negative ESI mode and positive ESI mode
Negative ESI mode: (a) 90% Acetonitrile/10% 10 mM ammonium formate (pH~6.4); (b) 10% Acetonitrile/90% 10 mM ammonium formate (pH~6.4). Sample ID: 1) Malic acid, 2) Citric acid, 3) Niflumic acid, 4) Thymidine 5'-monophosphate, 5) Guanosine 5'-monophosphate
Positive ESI mode: (c) 90% Acetonitrile with 0.1% Formic Acid (pH ~ 2.7) ; (d) 10% Acetonitrile with 0.1% Formic Acid (pH~ 2.7). Sample ID: 6) Niflumic acid, 7) Thiamine, 8) Tryptophan, 9) Adenine, 10) 5-Fluorocytosine, 11) Thymine, 12) Thiourea

DISCUSSION

Relative to conventional reversed-phase materials, the positive surface charge present on Atlantis BEH C_{18} AX stationary phase 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 was 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_{18} columns, the Atlantis PREMIER BEH C_{18} AX Column gives the highest retention for AMP. The neutral compounds have similar retention on the Atlantis PREMIER BEH C_{18} AX and HSS T3 columns, while the CSH C_{18} column has the lowest retention of these analytes, due to the lower surface area of the 130 Å particles. The positively-charged analytes nicotinamide and procainamide are most retained on the HSS T3 column, and least retained on the Atlantis PREMIER BEH C_{18} AX Column.

As for CSH C_{18}, the surface modification of Atlantis BEH C_{18} AX involves separate steps to incorporate the charge modifier, the C_{18} groups and the endcap. This approach gives excellent control of the surface chemistry, resulting in high batch-to-batch reproducibility. For the relative retentions, the results showed relative standard deviations (RSD) ranging from 1 to 3% (Figure 2), indicating excellent reproducibility, comparable to some of the most reproducible conventional C_{18} bonded phases.

Combining the base-stable BEH particle with stable bonding chemistries, the Atlantis BEH C_{18} 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_{18} AX Columns is compared to those of two leading mixed-mode RP/AX columns in Table 2. The most notable differences are in the upper limits, with Atlantis BEH C_{18} 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 extended high pH stability of Atlantis PREMIER BEH C_{18} 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.

Table 2. Recommended pH Ranges for Three Mixed-Mode RP/AX Stationary Phases

Stationary Phase	**Recommended pH Range**
Atlantis BEH C_{18} AX	2 to 10
Brand P	1.5 to 4.5
Brand A	2.5 to 7.5

Adsorption on steel surfaces in columns causes difficulties for a number of analytes containing carboxylate or phosphate groups. MaxPeak HPS hardware addresses these problems. Increased MS signal intensity is achieved for phosphorylated analytes when using HPS hardware. The improvement for AMP is 1.9X higher and for ATP, the intensity is 19X higher (Figure 4).

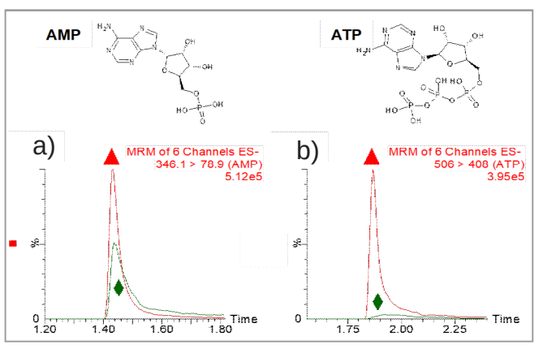


Figure 4. Separation of AMP (a) and ATP (b) on the Atlantis BEH C_{18} AX material with MaxPeak HPS column hardware (RED) and standard column hardware (GREEN)

Atlantis PREMIER BEH C_{18} AX Columns achieve a fast analysis of organic acids in juices. Atlantis PREMIER BEH C_{18} AX columns have good retention and separation efficiency for organic acids (Figure 5).

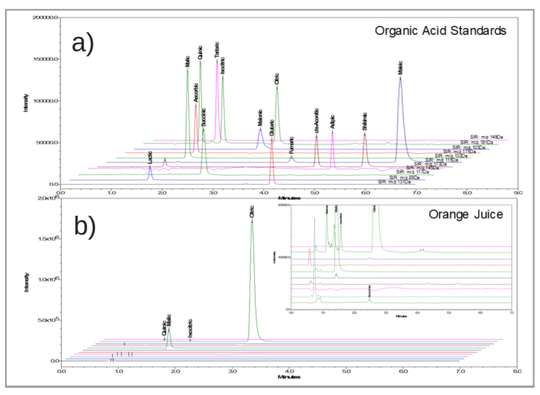


Figure 5. 20 ppm Organic acid standards (a) in 11 SIR channels using Atlantis PREMIER BEH C_{18} AX 1.7 um, 2.1 x 100 mm. SIR chromatogram overlay of a 100 % orange juice (b) with an insert of the enlarged baselines of these SIR channels.

CONCLUSION

- **Compared to conventional RP stationary phases, when used with mobile phase pH values < 8, Atlantis BEH C_{18} AX shows increased retention of anions, such as ionized acids, and decreased retention of cations, such as protonated bases.**
- **MS signal suppression or enhancement caused by bonded -phase hydrolysis products observed for analytes in positive ESI and negative ESI modes were significantly lower than two leading mixed-mode RP/AX columns.**
- **MaxPeak HPS hardware provides improved recovery, better peak shape and increased signal intensity and analysis reproducibility for metal-sensitive analytes.**
- **Atlantis PREMIER BEH C_{18} AX Columns have outstanding batch-to-batch reproducibility, and are stable over a wide pH range (2-10).**

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