

TOPICS IN LIQUID CHROMATOGRAPHY

Part 1. Designing a Reversed-Phase Column for Polar Compound Retention

Latest refinements reflect the culmination of more than three decades of proprietary experience in improving retention, selectivity, peak shape, and stability via substrate synthesis, particle morphology manipulation and surface modification.

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Affinity Underlies Retention, Separation, Selectivity

Liquid chromatography is a dynamic process involving distribution of sample molecules, in solution, between two intimate phases, one mobile and one stationary. Any analyte must, to some degree, be soluble in, or have an affinity for, both of these phases. To increase retention, the relative affinity of an analyte for the stationary phase must be strengthened.

To achieve a separation, two analytes must differ in their relative affinities for the two phases in a chromatographic system. On the thermodynamic level, a slight affinity difference, multiplied by the multitude of times each molecule moves between the two phases on its random path through the column, results in a separation—a measurable gap in time between the elution—of the respective bands for each type of molecule. The magnitude of the difference between the relative affinities of each analyte for the two phases in a chromatographic system is a measure of that system's selectivity.

The strength of this affinity is a measure of an analyte's chromatographic polarity. The higher its polarity, the stronger is the analyte's affinity for the more polar phase—the stationary phase in normal-phase LC—the mobile phase in reversed-phase LC. Conversely, the lower its polarity, the stronger is its affinity for the more non-polar phase—the mobile phase in normal-phase LC—the stationary phase in reversed-phase LC. Polar phases are typically aqueous or hydrophilic, non-polar phases non-aqueous or hydrophobic, in nature.

Polar Analytes Pose a Chromatographic Challenge

The overwhelming majority of LC separations today are done by using the reversed-phase mode. However, organic molecules at the extremes of chromatographic polarity pose particular problems for this separation mechanism. Non-polar, strongly hydrophobic molecules are highly retained, and poor selectivity is achieved in non-aqueous mobile phases sufficiently strong to elute them. Polar, hydrophilic molecules are poorly retained and may elute in the void volume, or may be excluded from pores and elute even earlier. Highly aqueous mobile phases, even water alone, may not be sufficiently weak to induce retention.

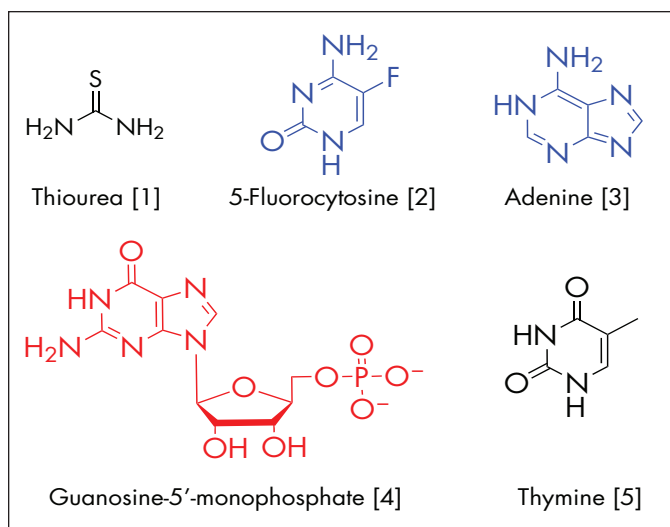


Figure 1. A selection of typical chromatographically polar analytes [neutral (1), basic (2,3), and acidic (4)] used as a test mixture to challenge reversed-phase columns designed for better retention of such small organic compounds.

Difficulties with polar compound separations are further compounded by several issues. Most modern high-performance silica-based reversed-phase LC packings are designed with a high surface density of long-chain alkyl moieties and exhaustive end-capping of accessible silanol groups so as to improve the separation efficiency and peak symmetry for basic compounds. [A preponderance of pharmaceutical analytes fall into this category.] Such packings may dewet when used with highly aqueous mobile phases; then all retention is lost [see discussion below].

Newer hybrid-particle-technology phases have lower surface silanol concentrations and more hydrophobicity within even the smallest, inaccessible pores via the methyl or ethylene groups embedded throughout the particle backbone.¹ But, as with the modern silica phases, minimizing access to silanols or the silica siloxane surface masks the very functionality that may impart desirable retention and selectivity to the separation of polar molecules.

Minimizing Pore Dewetting

In 1997, Walter, Iraneta, and Capparella² definitively debunked the myth of hydrophobic collapse. They found that bonded-phase pore size and column pressure played a key role in the dramatic loss of retention following stopping, then restarting, flow of highly aqueous [$> 90\%$] mobile phases through C_{18} and C_8 columns. They suggested the cause of this phenomenon to be extrusion of the mobile phase from the pores. Recently, they developed a procedure to quantify the retention loss. They also recommended ways to design reversed-phase sorbents to minimize pore dewetting and maximize the retention of polar analytes.²

Three stationary phase characteristics determined to reduce the degree of dewetting after flow stoppage were:

- larger pore size
- embedded-polar group in bonded alkyl chain
- lower surface coverage: e.g., no end-capping and/or lower primary ligand density.

Why Aren't Embedded-Polar Phases an Option?

Incorporation of a polar moiety [e.g., carbamate, amide] into a long-chain-alkylsilyl group nearest to the end attached via a siloxane bond to a silica surface successfully mitigates undesirable interaction of basic analytes with silanols.³ When combined with the advantages of a hybrid particle substrate, such “shield” phases, as predicted by some new notions on the mechanism of reversed-phase separations, deliver the most symmetrical peak shape for basic analytes.⁴ They are fully compatible with highly aqueous mobile phases and do not dewet. Compared with typical C_8 or C_{18} packings, they also exhibit unique selectivity and far stronger retention for hydrogen-bond-donor analytes such as phenols.⁵

So, with all these advantages, why aren't embedded-polar phases also good for polar analyte separations? To achieve peak symmetry for bases, syntheses sought to maximize primary-ligand population on accessible surface sites. As can be seen in Figure 2, however, consistent with findings by O'Gara et al.³, such embedded-polar phases do not retain polar analytes well.

Lower Ligand Density Is Better

Whether an adsorption or a partition process rules reversed-phase chromatography⁴, it seems logical that the retention of polar analytes is due to their affinity for, and the nature of, the polar siloxane and silanol surface functionalities. It is also likely that the hydrophobic alkyl ligands influence the composition of the layer of solvent closely associ-

ated with the stationary phase, and may, in fact, guide formation of conduits for longer-range interaction of analytes with nominally inaccessible surface silanols in smaller pores.⁴ Neue, Carr, and colleagues recently found strong evidence for a combined effect of silanols and hydrophobic ligands for charged basic analytes.⁶

Maximum interaction with accessible silanols may be achieved by omission of the end-capping step in the bonded-phase synthesis. However, this is not the most desirable way to increase polar analyte retention. Such phases exhibit poor peak shape for bases [Figures 4, 6].

Neue et al.⁵ have shown that, in general, retention also increases with an intermediate bonding level. While this may seem counter intuitive, they speculate such a condition likely improves accessibility to, or the effect of, the hydrophobic ligand. This coincides with the similar conclusions proposed by O'Gara et al.³ and by Walter et al.² based upon their recommendations for designing reversed-phase packings to minimize pore dewetting while maximizing polar-analyte retention in highly aqueous mobile phases.

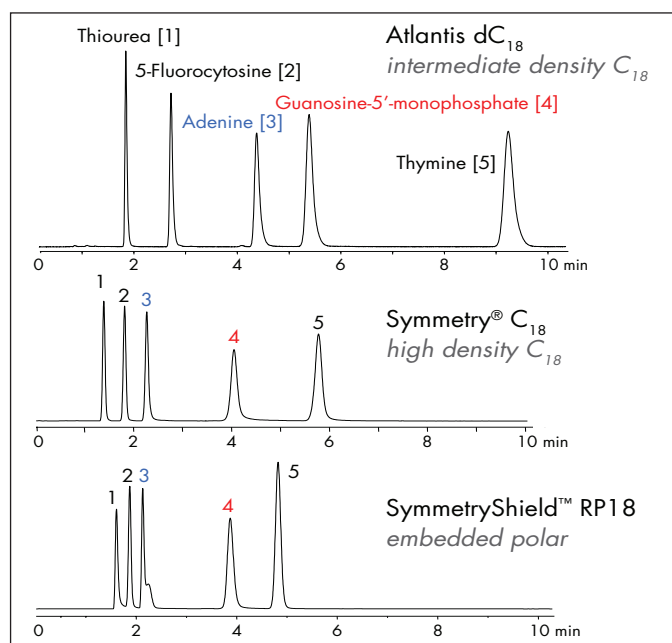


Figure 2. Validating the intermediate-primary-ligand-density approach to stationary phase design for the separation of polar compounds, Atlantis® dC₁₈ shows 35 – 90% more retention for the polar-analyte test mixture than does either a modern, conventional, fully-bonded and end-capped, high-density- C_{18} -ligand column or a high-density embedded-polar column.⁸

A New Generation: Atlantis dC₁₈

In 1973, a revolution in reversed-phase packing design and synthesis occurred when Vivilecchia and co-workers made μ Bondapak[®] C₁₈.⁷ They sought to optimize retention and selectivity reproducibly by balancing the nature and population of, and interaction with, inaccessible silanols and accessible surface alkyl ligands. In 2002, guided by these same principles and the knowledge gained subsequently in three decades of proprietary research and development, materials scientists at Waters created Atlantis dC₁₈. When compared to traditional, fully bonded and end-capped C₁₈ packings, this reversed-phase sorbent, bonded with a difunctional C₁₈-silane to an intermediate ligand density [1.6 μ moles/m²] and fully end-capped, did indeed show improved retention and selectivity for polar analytes [Figure 2].

Desirable Refinements

As with the design of any new LC sorbent, some compromise is always necessary. Atlantis dC₁₈ scored well against a target list of desirable attributes:

- compatible with on-line mass spectrometry [MS] [low bleed]
- good peak shape and chromatographic efficiency
- good retention for both polar and non-polar analytes
- excellent batch-to-batch reproducibility.

Though user acceptance of, and success with, Atlantis dC₁₈ has been gratifying, our scientists pursued further refinements in the silica-substrate-morphology modification, bonding, and end-capping procedures to deliver an even better column [compared to Atlantis dC₁₈] designed specifically to address requests for:

- superior column lifetime at low pH [0.1% TFA]
- increased retention for polar analytes
- similar selectivity [see Figure 3]
- better peak shape for basic compounds at pH 7
- easy methods transfer to UPLC[®] separations.

Figure 4. [at right] demonstrates how Atlantis T3 retention and selectivity compares to that of other commercially available columns designed for polar analyte separations. Unless otherwise indicated, all use an intermediate-primary-ligand-density approach.⁹ All are fully compatible with aqueous mobile phases.¹⁰ However, Atlantis T3 shows higher retention, esp. for thymine, and the best combination of retention, selectivity, and peak shape.

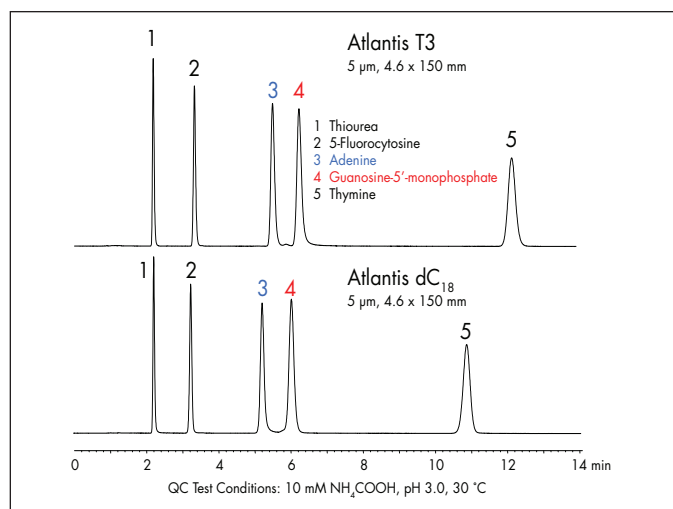
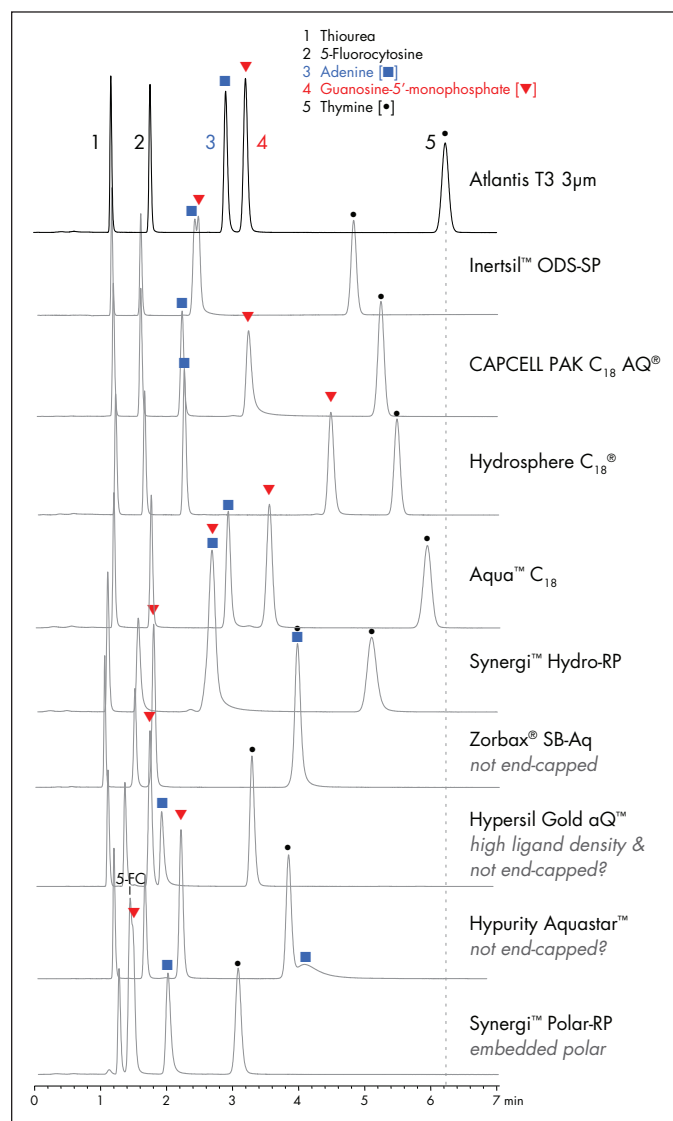


Figure 3. Atlantis T3 shows higher retention, especially for thymine, while overall selectivity between the test mixture analytes is very similar to that of Atlantis dC₁₈.



Proprietary T3-Bonding Process Maintains Selectivity

By controlling the reaction of a trifunctional C₁₈ silane with specially pre-treated silica particles followed by novel end-capping technology, they produced a new sorbent, Atlantis T3, with an intermediate primary-ligand density [1.6 $\mu\text{moles}/\text{m}^2$], approximately half that of most modern high-ligand-density C₁₈ phases [typically > 3.0 $\mu\text{moles}/\text{m}^2$]. This unique T3-bonding technology enhances the performance characteristics of Atlantis T3, enabling it to meet all the requests listed above.

A test to measure the chromatographic performance and reproducibility of every batch of both Atlantis dC₁₈ and T3 sorbents was designed to be the most stringent in the industry. Five acidic, basic, or neutral compounds [structures in Figure 1] are separated in an entirely aqueous mobile phase containing 10 mM ammonium formate at pH 3. Figure 3 shows a comparison of the results of this test on the two types of Atlantis column packings.

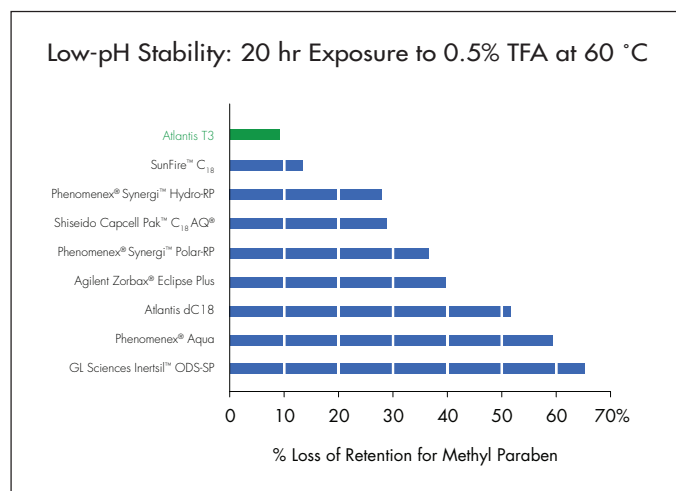


Figure 5. These accelerated low-pH-exposure test results verify that the new, proprietary, T3-bonding process enables the excellent stability of Atlantis T3.

Atlantis T3: Superior Stability at Low pH

Reversed-phase packings are typically prepared by binding primary and end-capping ligands to silica via siloxane bonds, the same type of link that forms the silica particle structure. Siloxane [Si–O–Si] bonds are prone to hydrolysis catalyzed by either base or acid. At higher pH [> 8], base-catalyzed cleavage of the silica backbone is favored. At low pH [< 2], the rate of acid-catalyzed cleavage of bonded ligands increases significantly.

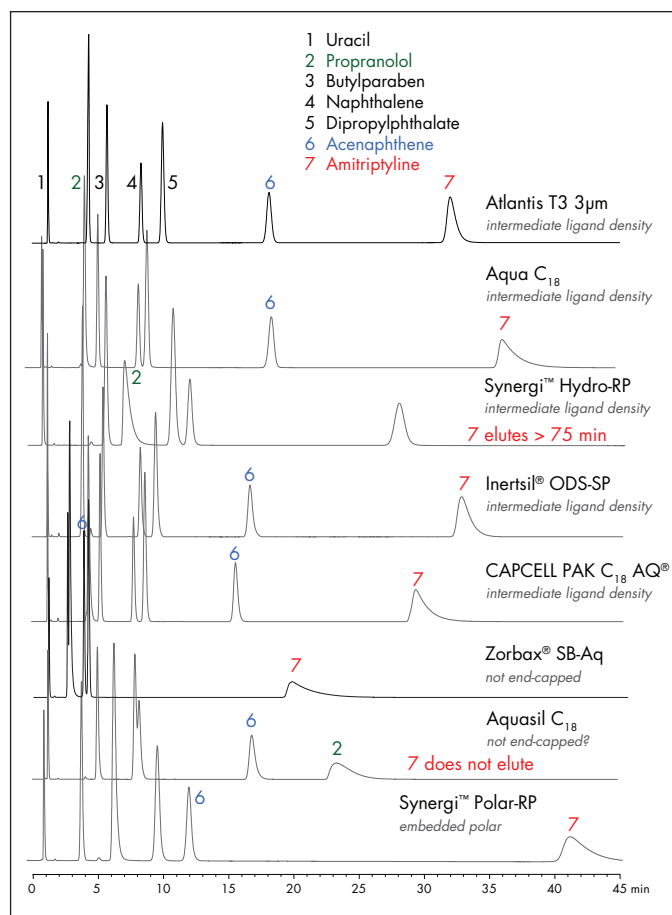


Figure 6. Atlantis T3 is unique in its ability to combine the polar compound retention and aqueous compatibility of intermediate-C₁₈-ligand-density columns with the superior pH 7 peak shape and stability of high-C₁₈-ligand-density columns.¹¹

In an aqueous environment, cleaved long-chain-alkyl ligands may still cling to the stationary phase; the column may appear to function normally—until it is washed with an amount of organic solvent sufficient to dissolve and carry away the freed ligands. Depending upon pH, temperature, mobile phase composition and history, the consequence of ligand cleavage during a column's lifetime is either a gradual or a catastrophic loss of efficiency.

When the population density of bulky ligands is lowered, it becomes easier for hydrolytic agents to access the critical siloxane bonds. A countermeasure is to use a di- or trifunctional ligand that forms more than one bond to the silica surface. This retards the rate of cleavage since to remove a single ligand, multiple bonds must be broken. As can be seen in Figure 5, the T3-bonding process yields a packing with superior stability at low pH.

Atlantis T3: Improving Peak Shape at pH 7

Basic compounds exhibit peak tailing at neutral pH if they interact with acidic silanols in the inaccessible pores⁴ or with non-end-capped silanols on the accessible surface. Traditional end-capping may not be sufficient to preclude undesirable silanol interaction with bases on intermediate-primary-ligand-density packings. As explained above, this may also shorten column lifetime due to hydrolysis of the silica's siloxane backbone. Our new T3-bonding process end-caps more silanols than do traditional methods. This results in excellent peak shape and stability, comparable to that of modern high-ligand-density packings [Figure 6]. Unlike these materials, however, Atlantis T3 performs well in highly aqueous [$> 90\%$] mobile phases and exhibits better retention, selectivity, and peak shape for polar analytes.

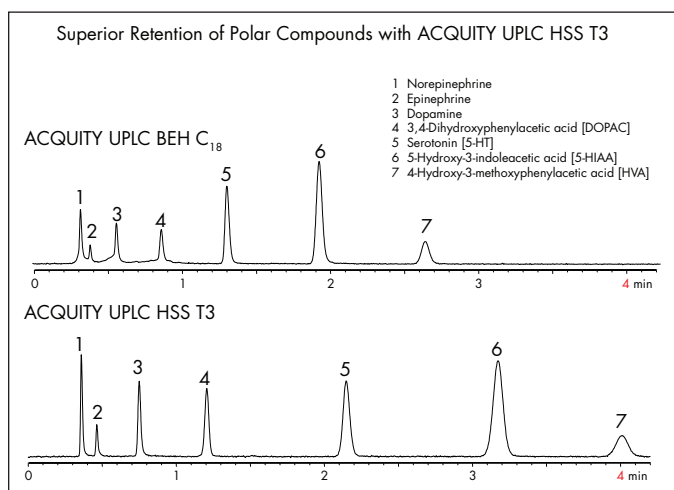


Figure 7. A mixture of phenol and catechol derivatives are more strongly retained on ACQUITY UPLC® HSS T3 than on the C_{18} -bonded hybrid-particle phase.¹²

Column Harmonization

With hundreds of reversed-phase LC columns available from a multitude of manufacturers, it is no wonder that large organizations seek to narrow the number of column choices in their preferred “toolkits”. This worldwide trend toward “harmonization” is driven by the desire to control consumable costs predictably, simplify methods transfer between laboratory locations, and prescreen LC columns for performance parameters. Atlantis T3 columns were designed with these goals in mind. With good retention of, and selectivity for, neutral, hydrophobic compounds as well as polar analytes and superior performance over a wider pH range, Atlantis T3 merits serious consideration as a more universal alternative to traditional C_{18} -reversed-phase LC columns.

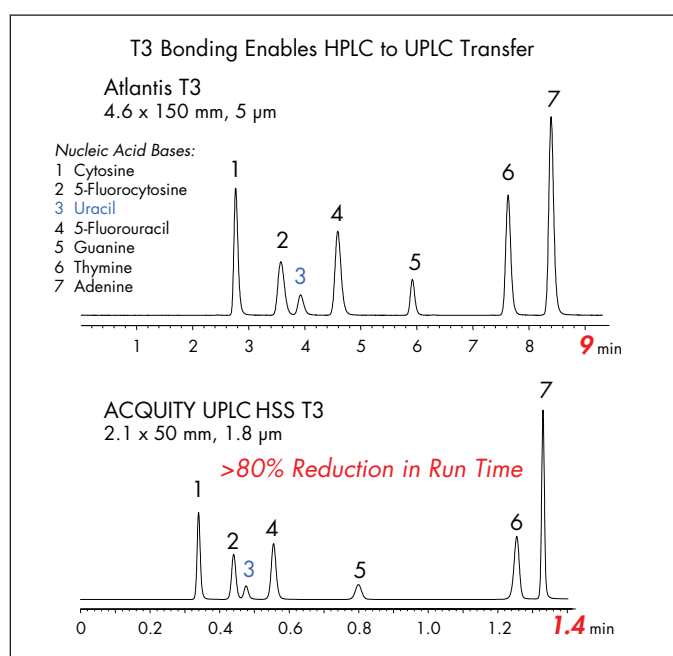


Figure 8. Transferring methods for polar analyte separation from HPLC to a UPLC system is straightforward using new T3 columns designed for each technique. Note retention of uracil, a traditional VO marker.¹³

	ACQUITY UPLC BEH Columns	ACQUITY UPLC HSS Columns	Atlantis HPLC Columns
Intended Use	UPLC	UPLC	HPLC
Particle Type	Ethylene Bridged Hybrid [BEH]	High Strength Silica [HSS]	Atlantis Silica
Available Chemistries	C_{18} , C_8 , Shield RP18, Phenyl, HILIC	T3 [C_{18}]	T3 [C_{18}]; d C_{18} ; Unbonded [HILIC]
pH Range	1–12; [RP18: 2–11]; [HILIC: 1–8]	2–8	2–8; 3–7; 1–5
Maximum Rated Pressure	15,000 psi [~1000 bar]	15,000 psi [~1000 bar]	6,000 psi [~400 bar]
Particle Size	1.7 µm	1.8 µm	3, 5, 10 µm
Pore Diameter/Volume	130 Å / 0.7 mL/g	100 Å / 0.7 mL/g	100 Å / 1.0 mL/g
Surface Area	185 m ² /g	230 m ² /g	330 m ² /g

Table 1. ACQUITY UPLC and Atlantis HPLC column families. Compare especially ACQUITY UPLC HSS T3 with Atlantis T3.

A UPLC Packing with the Advantages of T3-bonding

With the rapid acceptance of UPLC separation technology, and its extraordinary combination of speed, resolution, and efficiency, it was only natural to ask our materials scientists to find a way to transfer the unique selectivity and retention properties of Atlantis T3 to a packing engineered to withstand the rigors of ultra-high pressure. To do this, they first had to develop a new high-strength silica [HSS] particle with higher mechanical stability, as well as appropriate morphology. Then, they applied the proprietary T3-bonding process to this silica's surface to create ACQUITY UPLC HSS T3 columns. As shown in Figure 7, HSS T3 retains the superior polar-compound retention of Atlantis T3. Table 1 compares the characteristics of both T3 packings.

Method Transfer from HPLC to UPLC Separations

A key characteristic of a chromatographic packing substrate is its morphology [surface area, pore size distribution, pore volume, etc.]. This sterically directs the bonding process, and, ultimately, affects the retention and selectivity properties that govern the success of a separation. Using the same T3-bonding on two different silica substrates means that, even if their morphology is matched as closely as possible, not all separations may be transferred from one to the other without some degree of modification or optimization.

A compelling benefit of UPLC technology is the ease and speed of method development and optimization. Moving from XBridge™ HPLC to ACQUITY UPLC BEH columns is nearly seamless, as the hybrid-particle substrates, with corresponding surface chemistry, differ only in particle size. However, as can be seen in Figure 8, it should be straightforward to make simple adjustments to transfer a successful separation from an Atlantis T3 HPLC to an ACQUITY UPLC HSS T3 column.

Conclusions:

Building on over 30 years of proprietary experience, fully aqueous-compatible packings have been made by combining properly tailored silica substrates with a novel, new, T3-bonding process, designed to create bonded phases with enhanced stability in acidic solutions and excellent peak shape. These columns set a new performance standard for polar compound retention and selectivity in HPLC and UPLC separations. As we continue to gain more understanding of how reversed-phase separations work, we hope that our next generation of columns will be even better.

— Patrick D. McDonald, Douglas McCabe, Bonnie A. Alden,
Nicole Lawrence, Daniel P. Walsh, Pamela C. Iraneta,
Eric Grumbach, Fang Xia and Paula Hong

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NOTE: Where indicated below, use bold search term following * to access PDF document in Waters Library at <<http://www.waters.com>>.

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8. Conditions for separations in Figure 2: All columns: 4.6 x 150 mm, 5 µm; Mobile Phase: 10 mM NH₄COOH, pH 3.0; Flow Rate: 1.2 mL/min; Injection Volume: 7 µL; Detection: UV @ 254 nm
9. Conditions for separations in Figure 4: All columns contained 3 µm packings in either 4.6 x 100 mm or 4.6 x 75 mm standard sizes. Chromatograms were normalized on time axis based upon column volume. The same test was run on all columns: Eluent: 10 mM NH₄COOH, pH 3.00 ± 0.02; Temperature: 30 °C ± 1 °C; Flow Rate: 1.3 mL/min; Sample concentrations [µg/mL in eluent]: Thiourea [20], 5-Fluorocytosine [20], Adenine [7], Guanosine-5'-monophosphate [40], Thymine [24]. Detector: UV at 254 nm.
10. Conditions for dewetting test: Test is run as in Note 8. Then flow is stopped for 10 minutes, releasing column pressure. Flow is resumed for one minute. Then, another injection is made, and the capacity factor, k, for thymine is calculated. This value is compared to k for thymine in the initial run. The percent change in k between these two runs indicates the magnitude of dewetting [see Reference 2]. All columns here showed <10% dewetting.

11. Conditions for separations in Figure 6: All columns contained 3 μm packings in either 4.6 x 100 mm or 4.6 x 75 mm standard sizes. Chromatograms were normalized on time axis based upon column volume. The same Symmetry QC Test was run on all columns: Eluent: MeOH:20 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, [65:35 v/v], pH 7.00 \pm 0.02; Temperature: 23.4 $^\circ\text{C}$ \pm 0.1 $^\circ\text{C}$; Flow Rate: 1.0 mL/min; Sample concentrations [$\mu\text{g/mL}$ in eluent]: Uracil [8], Propranolol [400], Butylparaben [20], Naphthalene [60], Dipropylphthalate [340], Acenaphthene [200], Amitriptyline [100]; Detector: UV at 254 nm.
12. Conditions for separations in Figure 7: Both columns: 2.1 x 50 mm. Eluent: Isocratic: Mobile Phase A - 10 mM $\text{CH}_3\text{COONH}_4$, pH 5.0; Mobile Phase B - acetonitrile [98:2]; Temperature: 30 $^\circ\text{C}$; Flow Rate: 0.438 mL/min; Injection volume: 0.7 μL ; Detector: UV at 280 nm; Instrument: ACQUITY UPLC System with ACQUITY UPLC PDA.
13. Conditions for separations in Figure 8: Mobile Phase A: 10 mM $\text{CH}_3\text{COONH}_4$, pH 5.0; Mobile Phase B: acetonitrile; Gradient Profile: HPLC: %A: %B [time in min]: 100:0 [0.0], 94:6 [10], 94:6 [15], 100:0 [16.0], 100:0 [30.0]; UPLC: 99.9:0.1 [0.0], 94:6 [1.13], 94:6 [1.70], 99.9:0.1 [1.81], 99.9:0.1 [3.40]; Flow Rate: HPLC: 1.0 mL/min; UPLC: 0.613 mL/min; Temperature: 30 $^\circ\text{C}$; Injection Volume: HPLC [5 μL]; UPLC [0.3 μL]; Sample concentrations [$\mu\text{g/mL}$ in A]: Cytosine [80], 5-Fluorocytosine [80], Uracil [10], 5-Fluorouracil [40], Guanine [30], Thymine [80], Adenine [80]; Detection: UV @ 254 nm; Instrument: HPLC: Alliance[®] 2695 with 2996 PDA; UPLC: ACQUITY UPLC with ACQUITY UPLC PDA.

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