Developing Columns for UPLC: Design Considerations and Recent Developments

The challenges in developing a new chromatographic particle for UPLC™ separations are described. Columns packed with this new UPLC particle must meet or exceed reproducibility and longevity expected for other modern HPLC columns under conditions that are more mechanically and chemically demanding. Additionally, several new bonded phases provide flexibility for methods development, enabling the introduction of new products to be brought to market faster.

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Chromatographers are faced with the challenge of developing separations that completely characterize the constituents of their sample. A new tool for meeting these challenges became generally available in 2004. This new class of separation science, Ultra Performance Liquid Chromatography or UPLC, provides improved resolution, speed, and sensitivity. This is achieved through the use of columns with very small particle packings and a matching family of instruments developed simultaneously to provide full compatibility between chemistry and instrumentation. In describing this new separation power, it is essential to consider the key parameters that influence peak resolution and ultimately lead to successful chromatographic methods.

Resolution between two chromatographic peaks is determined by the distance between two peaks relative to their respective peak widths. The resolution equation provides a quantitative model for the three parameters that control resolution: efficiency, selectivity and retentivity.

Retention (k or k') and selectivity (a) are

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

chemical factors describing the interaction among the analyte molecules, the mobile phase, and the stationary phase. In contrast, efficiency (N or plate count) describes the physical process of band-broadening during the separation. Developing a chromatographic method is based upon the systematic manipulation of these three parameters.

Most method development strategies focus on retention and selectivity because they are easy and economical to manipulate. Resolution is improved by increasing the retention (k) of all of the peaks. Increasing retention, however, increases peak width, resulting in lower sensitivity, and reduces sample throughput. Selectivity describes the elution sequence of the peaks relative to one another, that is, relative retention. It can be manipulated by several parameters including mobile phase pH, organic modifier, and bonded phase.

Efficiency is less often used to improve a separation because it is difficult to change experimentally and because any improvements only contribute to resolution as the square root. Efficiency, however, can be significantly improved by reducing the diameter of the particle. A column packed with 1.7 µm particles would offer a 1.7 fold improvement in resolution compared to a column packed with 5 µm material. This resolution increase is defined by narrower, lower volume peaks so sensitivity is also increased. This paper will focus on the challenges of improving resolution and efficiency by utilizing highly efficient 1.7 µm particle packed columns. The requirements include the design and development of the

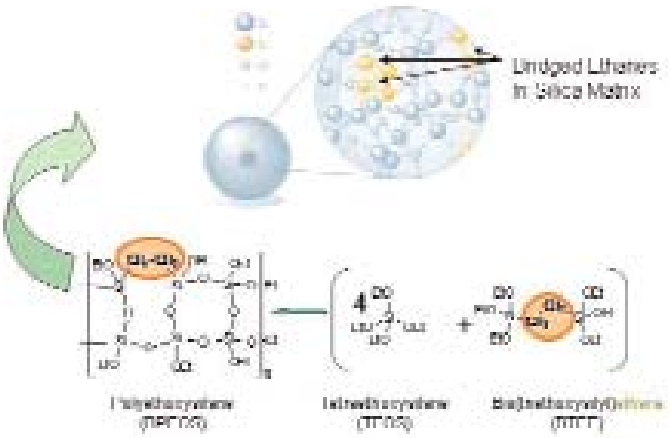

Figure 1: Bridged ethylsiloxane/silica hybrid particle provides improved mechanical and chemical stability for UPLC separations.

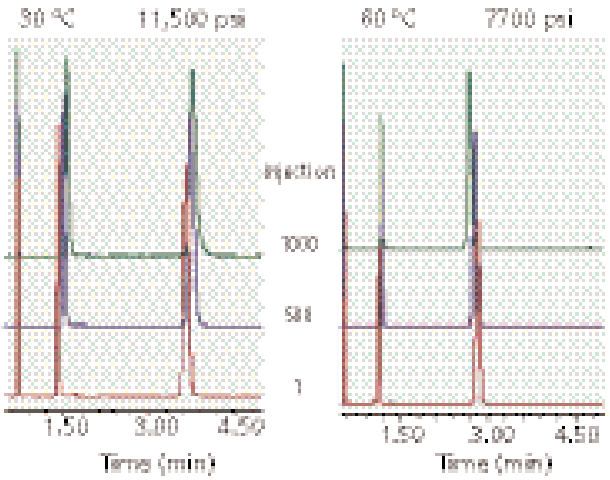

Figure 2: Mechanical and chemical aging study over 1000 injections at pH 11.3. Column: ACQUITY UPLC BEH C18 2.1 × 50 mm, 1.7 µm. Conditions: acetonitrile-methyl pyrrolidine buffer, 45:55 (v/v) at 0.9 mL/min. Test probes: butyrophenone, protriptyline and amitriptyline.

base chromatographic particle, the preparation of reproducible columns with maximized life using these particles, and the provision of modern reversed-phase selectivity with different stationary phase ligands.

Table I: BEH particle

Pore Diameter*	130 Å
Pore Volume*	0.7 mL/g
Surface Area*	185 m^2/g
90/10 Ratio*	1.5

*Expected or approximate values.

New Particle Technology

The use of smaller particle packing materials increases resistance to flow so that the columns operate at higher backpressure. In addition, the optimal linear velocity for maximum separation efficiency of a 1.7 µm particle necessitates operation at higher flow rates, generating even higher pressures, sometimes as high as 15,000 psi. Silica-based materials do not possess the mechanical strength or efficiency necessary to meet the demands of UPLC separations. The definition of a new particle to meet these requirements must, therefore, include improved physical stability. This strength must be achieved without compromising the mass loading capacity of the material that is related to the large surface area produced with fully porous packing material. The new material must also be stable to a wide range of chemical operating conditions while minimizing any secondary or mixed mode interactions with a wide range of analytes.

A new bridged ethylsiloxane/silica (BEH) hybrid particle was synthesized to meet these demands (Figure 1). It provides improved mechanical strength even when formed into fully porous particles. The narrow size distribution of the particles facilitates packing into high efficiency columns. The organic-

Table II

ACQUITY UPLC BEH Chemistry	C_{18}	C_8	Shield RP_{18}	Phenyl
Ligand Type	Trifunctional C_{18}	Trifunctional C_8	Monofunctional Embedded Polar Group	Trifunctional C_6 Phenyl
Ligand Density	3.1 µmol/m^2	3.2 µmol/m^2	3.3 µmol/m^2	3.0 µmol/m^2
Carbon Load	18%	13%	17%	15%
Endcap Style	Proprietary	Proprietary	TMS	Proprietary
pH Range	1–12	1–12	2–12	2–12

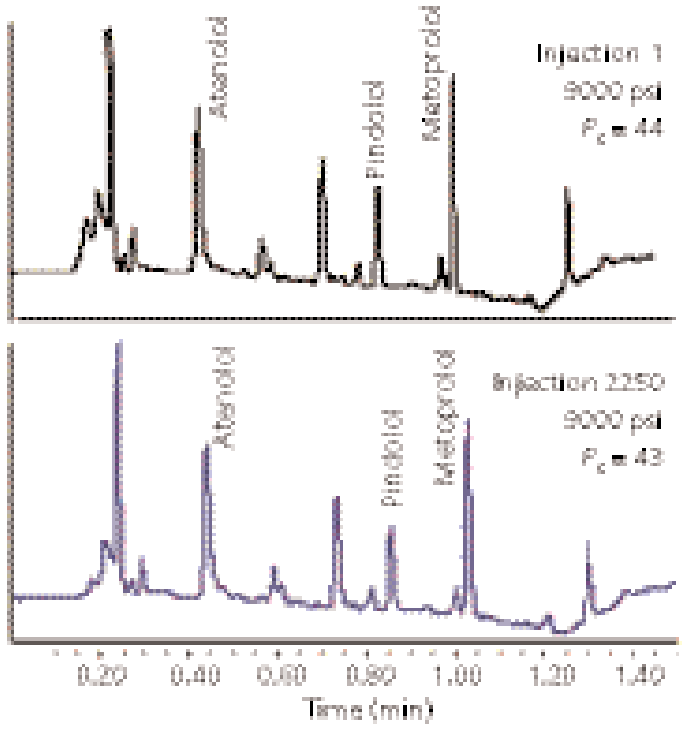

Figure 3: Column stability maintained over 2200 analyses at pH 2.0 with protein precipitated rat plasma samples. Column: ACQUITY UPLC BEH C18 2.1 × 50 mm, 1.7 µm. Mobile phase A: 0.1% triflouroacetic acid in water, mobile phase B: 0.08% triflouroacetic acid in acetonitrile. Gradient from 10–40% B over 1 minute curve 7, 40–90% B from 1.0 to 1.1 minutes, hold for 0.4 min, 95–10% B from 1.5–1.6 minutes, hold for 0.5 min. Flow rate 0.7 mL/min, 5.0 µL injection; temperature 30 °C; detection UV at 272 nm.

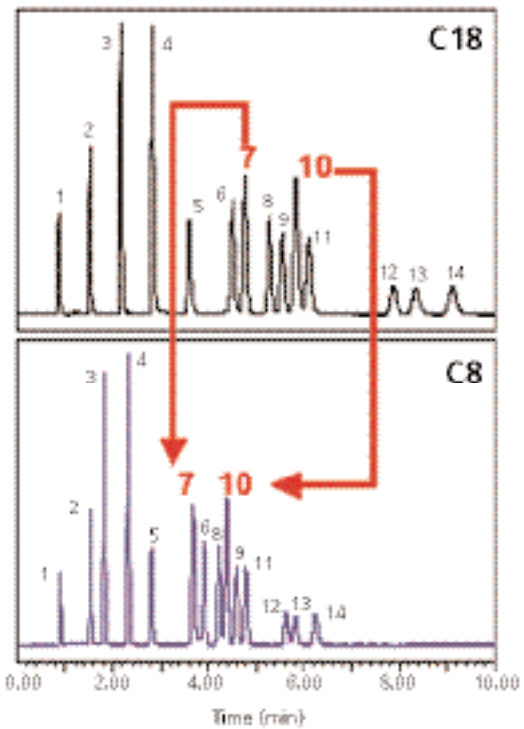

Figure 4: Selectivity difference between C18 and C8 alkyl chain columns. Column: ACQUITY UPLC BEH C18 and C8 2.1 × 100 mm, 1.7 µm. Isocratic 28% methanol; at flow rate 0.5 mL/min; temperature 50 °C; 5.0 µL injection; detection UV at 254 nm. Analytes: 1 HMX, 2 RDX, 3 1,3,5-TNB, 4 1,3-DNB, 5 NB, 6 Tetryl, 7 TNT, 8 2-Am-4,6-DNT, 9 4-Am-2,6-DNT, 10 2,4-DNT, 11 2,6-DNT, 12 2-NT, 13 4-NT, 14 3-NT.

inorganic hybrid, with ethylsiloxane bridges both on the surface and throughout the body of the material, provides a broader range of chemical stability, especially the pH operating range (pH 1–12), while minimizing interactions of the matrix with any analyte functionalities. The properties of this packing material are summarized in Table I. These characteristics are typical of modern reversed-phase HPLC packings. The slightly larger pore size improves the accessibility for larger analytes, while the surface area is about the same as first generation methyl hybrid packings. The particle size distribution is among the narrowest of modern packings.

Column Lifetime

Columns packed with this new UPLC particle must meet or exceed the reproducibility and longevity expected for other modern HPLC columns. Column lifetime is a broad term that reflects both physical and chemical changes to the packing as well as the adsorption of sample components. Chemical stability depends primarily on the effect of mobile phase pH and solvent selection. The higher operating pressures associated with sub-2 µm particle packed column could also

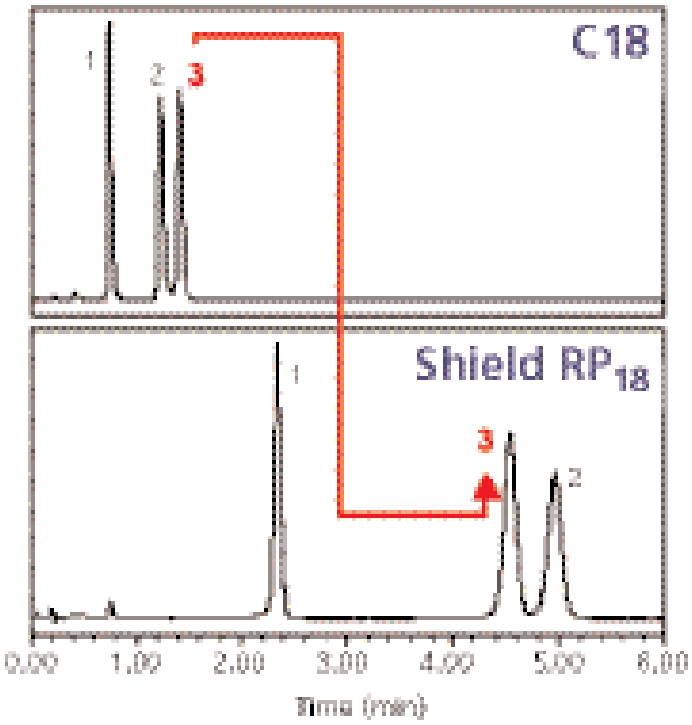

Figure 5: Selectivity difference between C18 and embedded polar group columns for phenolic compounds. Column: ACQUITY UPLC BEH C18 and Shield RP18 2.1 × 50 mm, 1.7 µm. Isocratic 45% methanol with 0.1% formic acid; at flow rate 0.6 mL/min; temperature 40 °C; 5.0 µL injection; detection UV at 270 nm. Analytes: 1 quercetin, 2 kaempferol, 3 isorhamnetin.

compromise bed stability. All of these phenomena are accelerated at elevated temperatures. The physical and chemical effects of mobile phase extremes were examined as shown in Figure 2. Both the mechanical (pressure tolerance) and chemical (pH and temperature) stability were measured as retention and efficiency, with no losses over 2000 injections. The same long term stability and performance is observed when using complex sample matrices shown in Figure 3. In this case, protein precipitated rat plasma was injected after evaporation and reconstitution in the initial mobile phase conditions. The column maintained initial peak capacity and selectivity for over 2200 injections. While it is never possible to predict column life absolutely for all combinations of sample and operating conditions, these experiments are consistent with this new UPLC particle meeting or exceeding the number of injections expected for traditional HPLC columns.

Column Selectivity

A 1.7 µm particle packed column provides significant improvements in resolution because efficiency is better. Separation of the

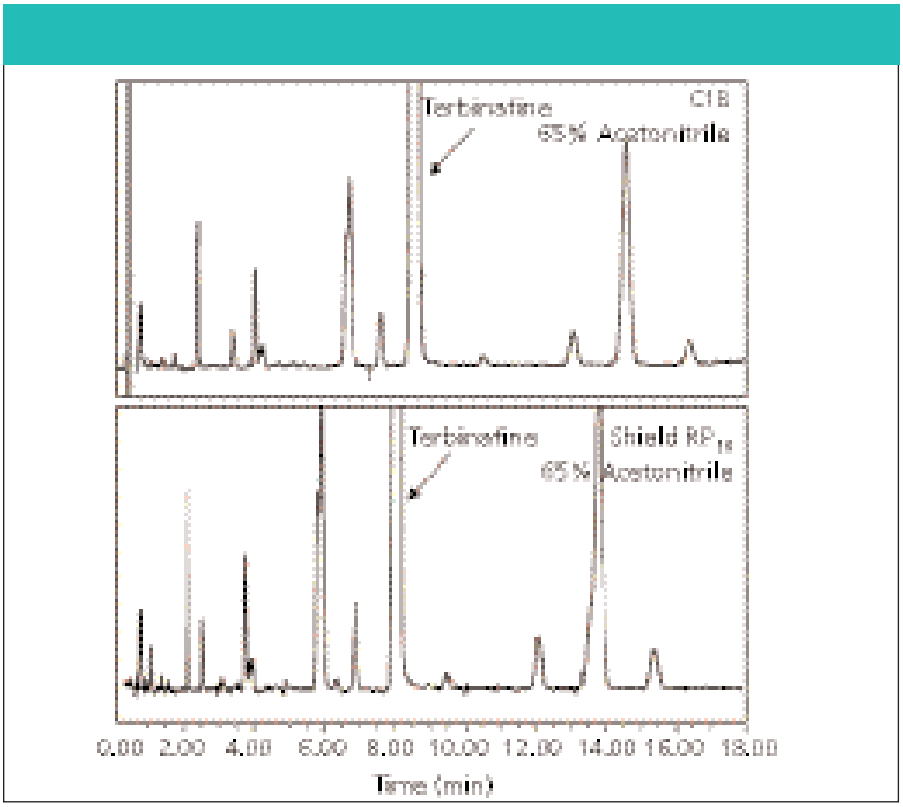

Figure 6: Similar selectivity is often observed between C18 and embedded polar group. Isoelutropic conditions are used to distinguish changes in retentivity and selectivity. Column: ACQUITY UPLC BEH C18 and Shield RP18 2.1 × 100 mm, 1.7 µm Mobile phase A: 20 mM ammonium bicarbonate pH 10.0, mobile phase B: acetonitrile; at flow rate 0.5 mL/min; temperature 30 °C; 10.0 µL injection; detection UV at 210 nm. Analytes: forced degradation of terbinafine HCl by 8.0 N hydrochloric acid.

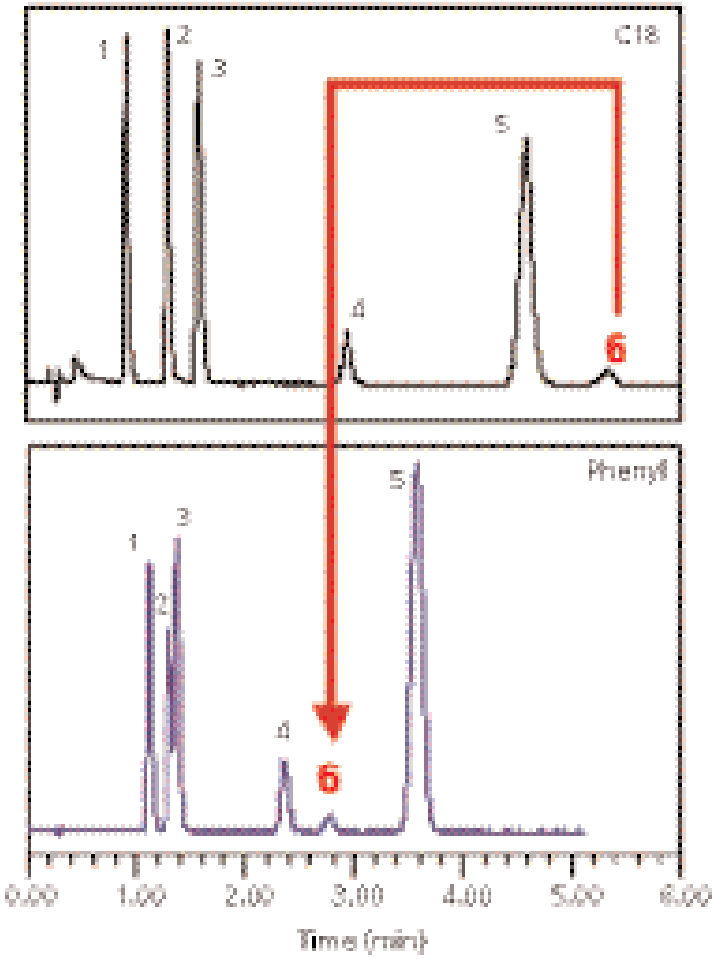

Figure 7: Selectivity difference between C18 and phenyl columns for aromatic compounds. Column: ACQUITY UPLC BEH C18 and Phenyl 2.1 × 50 mm, 1.7 µm. Isocratic 55% methanol with 0.1% formic acid; at flow rate 0.5 mL/min; temperature 30 °C; 5.0 µL injection; detection UV at 254 nm. Analytes: 1. suprofen, 2. tolmetin, 3. naproxen, 4. fenoprofen, 5. diclofenac, 6. ibuprofen.

components of a sample, however, still requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations: ACQUITY UPLC™ BEH C18 and C8 (straight chain alkyl columns), ACQUITY UPLC BEH Shield RP18 (embedded polar group column), and ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl). The characteristics of these stationary phases are summarized in Table II. Each provides a different combination of hydrophobicity, silanol activity, hydrolytic stability, and chemical interaction with the analytes. The effect of these properties on separations can be described briefly.

The C18 and C8 UPLC columns have alkyl chain bonded phases trifunctionally bonded to the particle surface to ensure the best hydrolytic stability. Compared to the C18 column, the shorter chain length C8 bonded phase is less hydrophobic, and, therefore, less retentive in general. Although selectivity differences seldom result from chain length differences, changes in peak elution order can occur. As shown in Figure 4, a set of 14 nitroaromatic compounds were analyzed on both C18 and C8 stationary phases. Baseline resolution was achieved on both stationary phases. However, less retentivity is observed on the C8 column. There is also a change in the elution order for the peaks.

An embedded polar group column can exhibit significantly different selectivity compared to linear alkanes (1,2). The ACQUITY UPLC BEH Shield RP18 column includes an embedded carbamate group that shows preferential retention of hydrogen-bond donors. Figure 5 demonstrates the selectivity differences between the straight chain alkyl C18 and the embedded polar group column for a set of flavanoids. For analytes that do not specifically interact with the embedded polar group, the column behaves as a shorter chain length alkyl column, as shown in Figure 6. The embedded polar functionality also suppresses surface silanol activity, reducing peak tailing, especially for basic analytes. Finally, the embedded polar group provides compatibility with highly aqueous mobile phases. The embedded carbamate group allows the stationary phase to resist pore dewetting by increasing the water concentration at the surface layer of the pores. The combination of the characteristics of ligands with embedded polar groups provides unique features, most importantly, an alternative selectivity to alkyl ligands.

Columns with phenyl ligands provide another alternate selectivity. Due to the π–π bonding orbital interactions, phenyl columns provide unique and specific selectivity with aromatic compounds and other analytes with similar π electrons. In Figure 7, the separation of nonsteroidal anti-inflammatory drugs on the phenyl column is compared to that on a C18 column. The selectivity differences can be magnified by changing the organic modifier from acetonitrile to methanol, increasing the retention of π-acids (2).

Conclusion

The performance barriers of traditional chromatographic packings have been removed with the development of a new, highly efficient, mechanically strong, 1.7 µm bridged ethylsiloxane/silica hybrid particles developed specifically to meet the challenge of routinely applying UPLC in the modern chromatographic laboratory. These particles can be packed in columns that meet or exceed the lifetimes expected for modern HPLC columns. Stability over a broad pH operating range combined with the several available bonded phases provide flexibility for methods development. This flexibility enables methods development to be more efficient, allowing products to be brought to market faster. The power of these ultra-efficient columns is combined with a low dispersion Ultra Performance LC™ system to successfully transfer existing HPLC methods or to develop new, fast chromatographic methods that offer substantial improvements in resolution, sensitivity and sample throughput.