

# Ultra Performance Liquid Chromatography (UPLC): An Introduction



ACQUITY Ultra Performance LC™ Systems take advantage of technological strides made in particle chemistry performance, system optimization, detector design, and data processing and control. When taken together, these achievements have created a step-function improvement in chromatographic performance. Defined as UPLC™, this new category of analytical separation science retains the practicality and principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity, and resolution.

**H**igh performance liquid chromatography (HPLC) is a proven technique that has been used in laboratories worldwide over the past 30-plus years. One of the primary drivers for the growth of this technique has been the evolution of packing materials used to effect the separation. The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). Since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance.

According to the van Deemter equation, as the particle size decreases to less than 2.5 μm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separations) can be extended to new limits, termed Ultra Performance Liquid Chromatography, or UPLC. The technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity.

Figure 1 shows a stability indicating assay of five related substances accomplished in under one minute, proving that the resolving power of UPLC is not compromised even at high speed. The current USP lists

multiple HPLC methods for the analysis of these same compounds with run times approaching 20 min, with broad, tailed peaks.

## Chemistry of Small Particles

As shown in Figure 1, smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as HPLC. In the fundamental resolution ( $R_s$ ) equation:

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

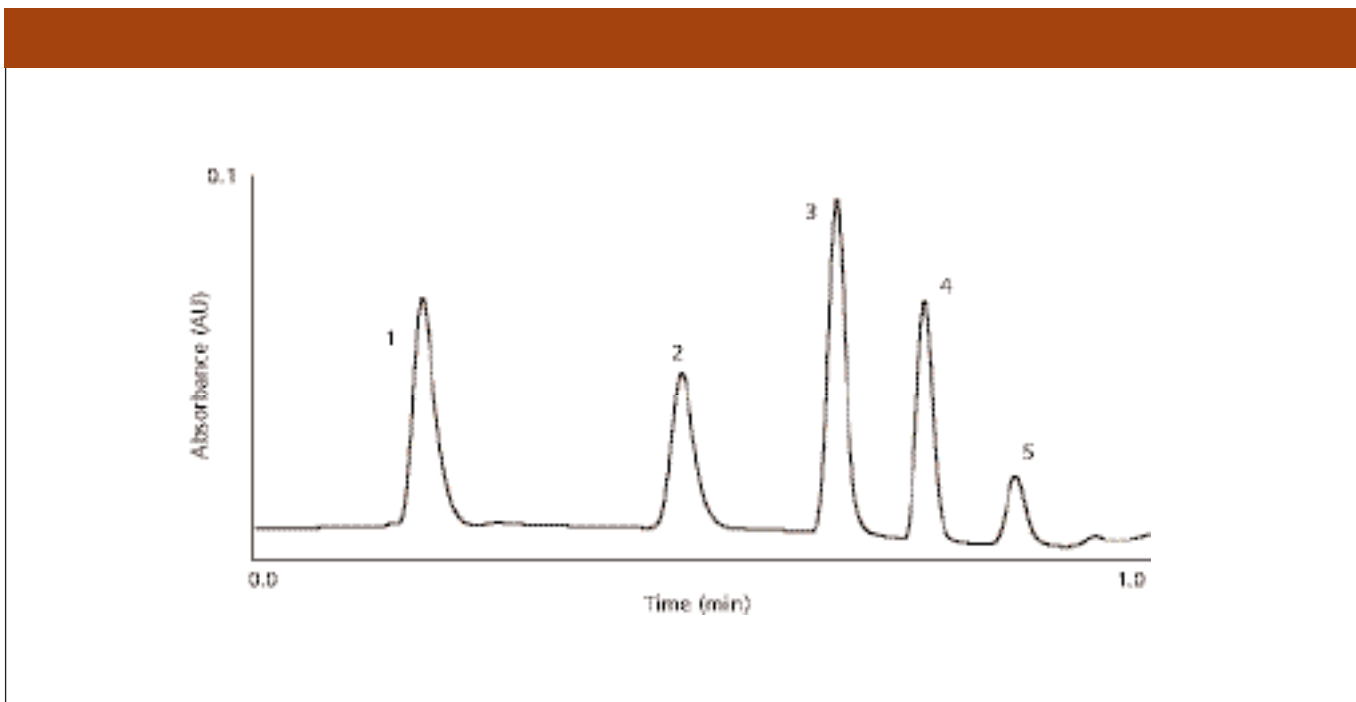
System
Selectivity
Retentivity  
Efficiency

resolution is proportional to the square root of  $N$ . But since  $N$  is inversely proportional to particle size ( $dp$ ):

$$N \propto \frac{1}{dp}$$

as the particle size is lowered by a factor of three, from, for example, 5 μm (HPLC-scale) to 1.7 μm (UPLC-scale),  $N$  is increased by three and resolution by the square root of three or 1.7.  $N$  is also inversely proportional to the square of the peak width:

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**Figure 1:** UPLC stability indicating assay. UPLC conditions: Column:  $2.1 \times 30$  mm  $1.7\mu\text{m}$  ACQUITY BEH C18 at  $30^\circ\text{C}$ . A 45 s, 5–85%B linear gradient, at a flow rate of 0.8 mL/min was used. Mobile phase A was 10 mM ammonium formate, pH 4.0, B was acetonitrile. UV detection at 273 nm and 40 pts/s. Peaks are in order: 5-nitroso-2,4,6-triaminopyrimidine, 4-amino-6-chloro-1,3-benzenesulfanamide, hydrochlorothiazide, triamterine, and methylbenzenesulfanamide; 5  $\mu\text{L}$  injection, 0.1 mg/mL each.

$$N \propto \frac{l}{w^2}$$

$$H \propto \frac{l}{w}$$

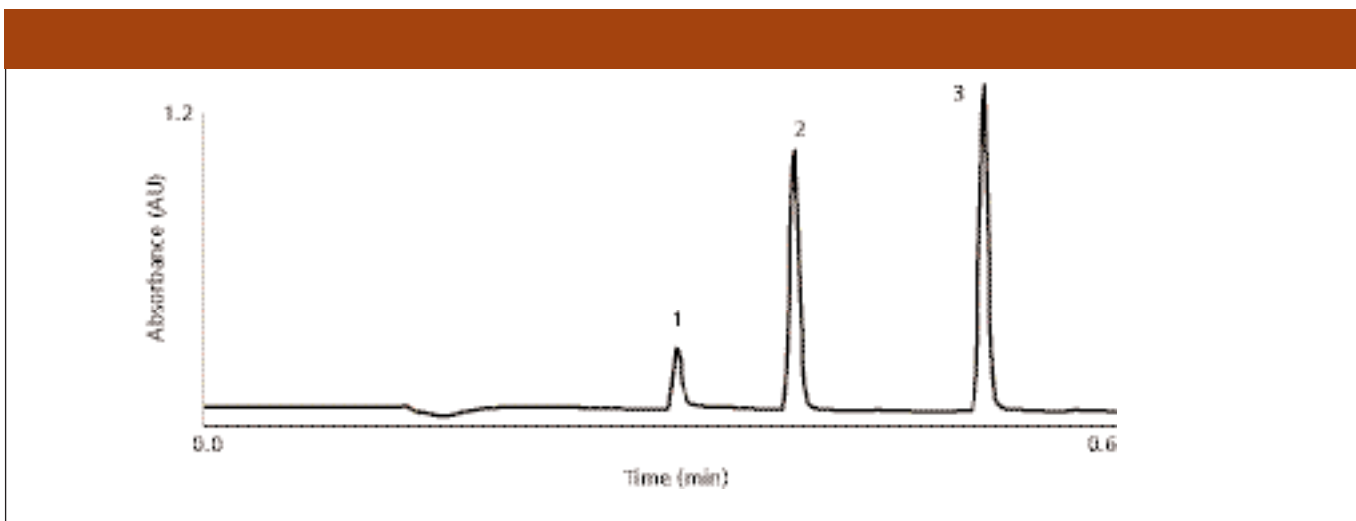
This illustrates that the narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

So as the particle size decreases to increase  $N$  and subsequently  $R_s$ , an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient sep-

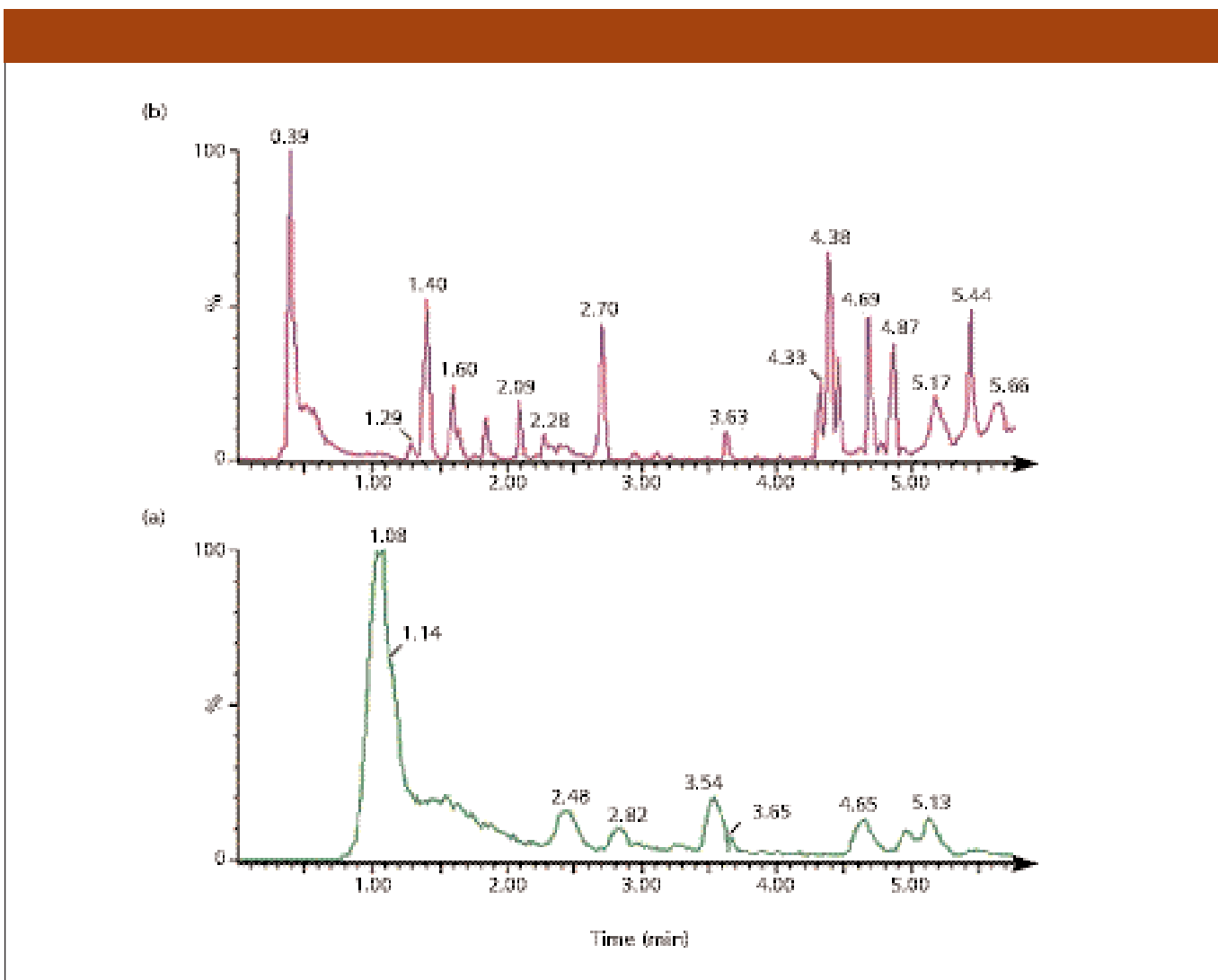
arations, desirable for many applications, e.g., peptide maps.

Still another equation comes into play when migrating toward smaller particles:

$$F_{opt} \propto \frac{1}{dp}$$



**Figure 2:** Conversion of an HPLC method to an UPLC method. Column:  $2.1 \times 50$  mm,  $1.7\mu\text{m}$  ACQUITY BEH C18 at  $40^\circ\text{C}$ . A 10 mM ammonium phosphate (pH 9.2) acetonitrile linear gradient from 10–90% acetonitrile at a flow rate of 1.0 mL/min was used, with UV detection at 210 nm. Sample was a 3  $\mu\text{L}$  injection of a 5 mg/mL mixture of each of the compounds. Peaks are in order: pseudoephedrine, ibuprofen, and butylparaben internal standard.



**Figure 3:** Full scan ToF MS for HPLC (Figure 3a) and UPLC analyses (Figure 3b) of the metabolites of dextromethorphan. (Reproduced with permission from reference 12, copyright John Wiley and Sons Limited 2005.)

This relationship also is revealed from the van Deemter plot. As particle size decreases, the optimum flow  $F_{\text{opt}}$  to reach maximum  $N$  increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressures, and a system properly designed to capitalize on the efficiency gains; A system that can both reliably deliver the requisite pressures and that can maintain the separation efficiency of the small particles with tightly managed volumes.

Higher resolution and efficiency can be leveraged even further, however, when analysis speed is the primary objective. Efficiency is proportional to column length and inversely proportional to the particle size:

$$N \propto \frac{L}{dp}$$

Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to the smaller particles and shortening the column by one third (again due to the smaller particles), the separation is completed in 1/9 the time while maintaining resolution. So if speed, throughput, or sample capacity is a concern, theory can be further leveraged to get much higher throughput.

But the design and development of sub- $2 \mu\text{m}$  particles is a significant challenge, and researchers have been active in this area for some time, trying to capitalize on their advantages (2–4). Although high efficiency,

nonporous  $1.5\text{-}\mu\text{m}$  particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. Silica-based particles have good mechanical strength but can suffer from a number of disadvantages, which include a limited pH range and tailing of basic analytes. Polymeric columns can overcome pH limitations, but they have their own issues including low efficiencies, limited loading capacities, and poor mechanical strength.

In 2000, Waters introduced XTerra®, a first generation hybrid chemistry that took advantage of the best of both the silica and polymeric column worlds. XTerra columns are mechanically strong, with high efficiency, and operate over an extended pH range. They are produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups. But in order to

provide the necessary mechanical stability for UPLC, a second generation bridged ethyl hybrid (BEH) technology was developed. Called ACQUITY BEH, these 1.7- $\mu\text{m}$  particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix.

Packing 1.7- $\mu\text{m}$  particles into reproducible and rugged columns was also a challenge that needed to be overcome. Requirements include a smoother interior surface of the column hardware and re-designing the end frits to retain the small particles and resist clogging. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations. All ACQUITY UPLC BEH columns also include eCord™ microchip technology that captures the manufacturing information for each col-

umn, including the quality control tests and certificates of analysis. When used in the Waters® ACQUITY UPLC™ System, the eCord database is also updated with real time method information, such as the number of injections, or pressure and temperature information, to maintain a complete continuous column history.

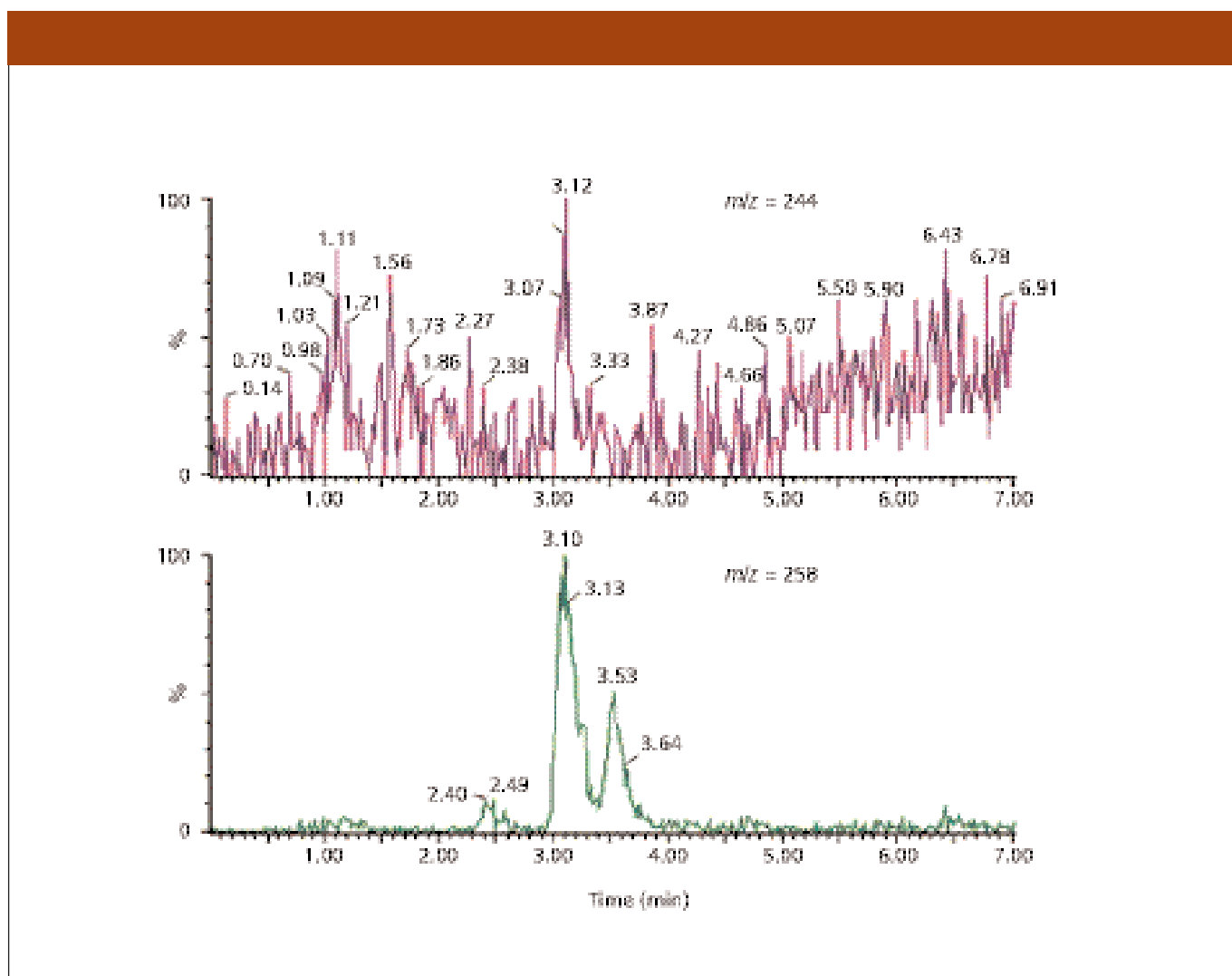
#### Capitalizing on Smaller Particles

Instrument technology also had to keep pace to truly take advantage of the increased speed, superior resolution and sensitivity afforded by smaller particles. Standard HPLC technology simply doesn't have the capability to take full advantage of sub-2  $\mu\text{m}$  particles. A completely new system design with advanced technology in the solvent and sample manager, auto sampler, detector, data system, and service diagnostics is required.

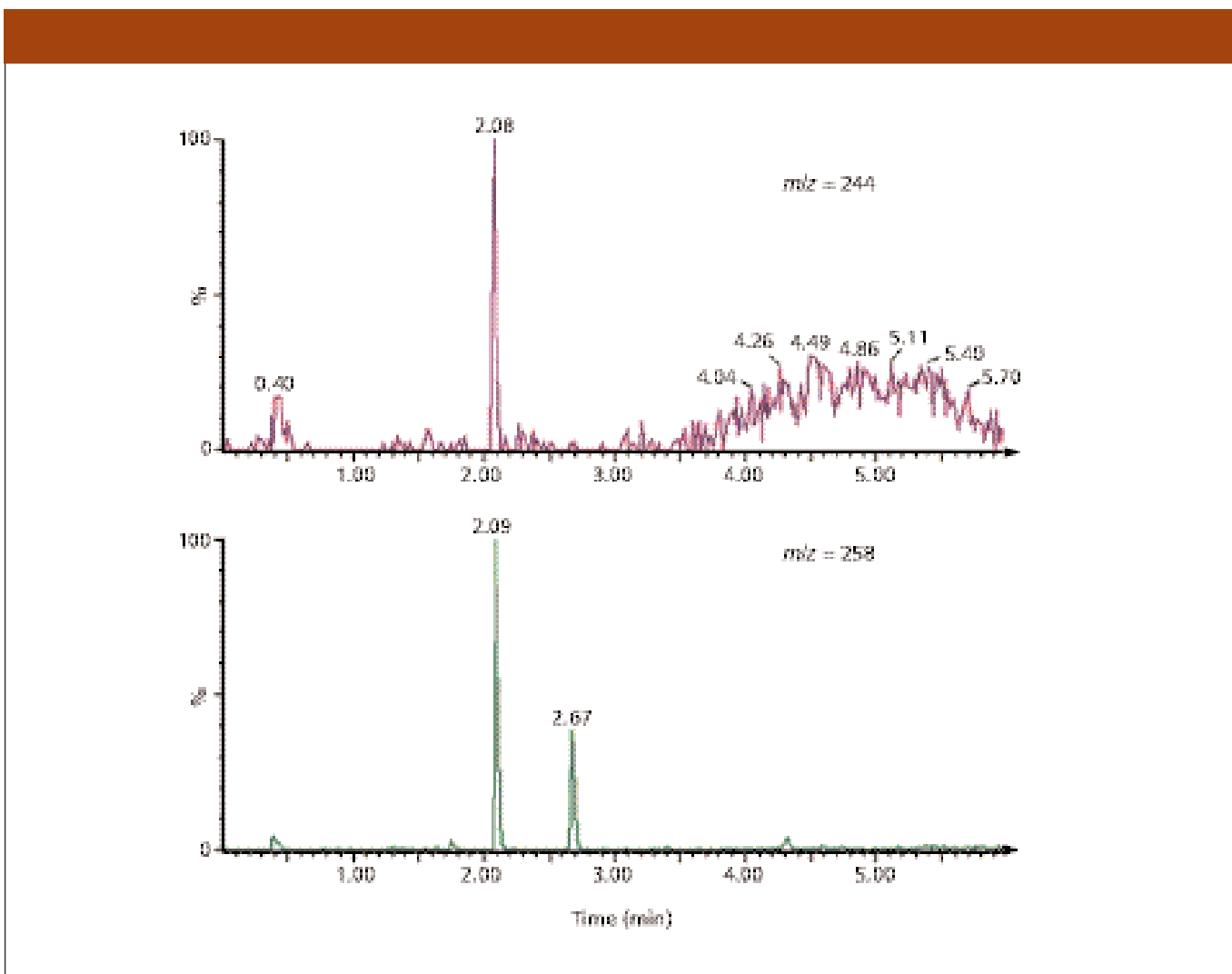
The ACQUITY UPLC System has been holistically designed for low system and dwell volume to minimize dispersion and take full advantage of small particle technology.

As alluded to previously, achieving small particle, high peak capacity separations requires a greater pressure range than that achievable by today's HPLC instrumentation. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15-cm long column packed with 1.7- $\mu\text{m}$  particles is approximately 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures and that can compensate for solvent compressibility, while operating in both the gradient and isocratic separation modes is required.

With 1.7- $\mu\text{m}$  particles, half-height peak



**Figure 4:** Extracted ion chromatograms for major N and O dealkylated and double de-alkylation metabolites of dextromethorphan by HPLC/Tof MS. (Reproduced with permission from reference 12, copyright John Wiley and Sons Limited 2005.)



**Figure 5:** Extracted ion chromatograms for major N and O dealkylated and double de-alkylation metabolites of Dextromethorphan by UPLC/Tof MS. (Reproduced with permission from reference 12, copyright John Wiley and Sons Limited 2005.)

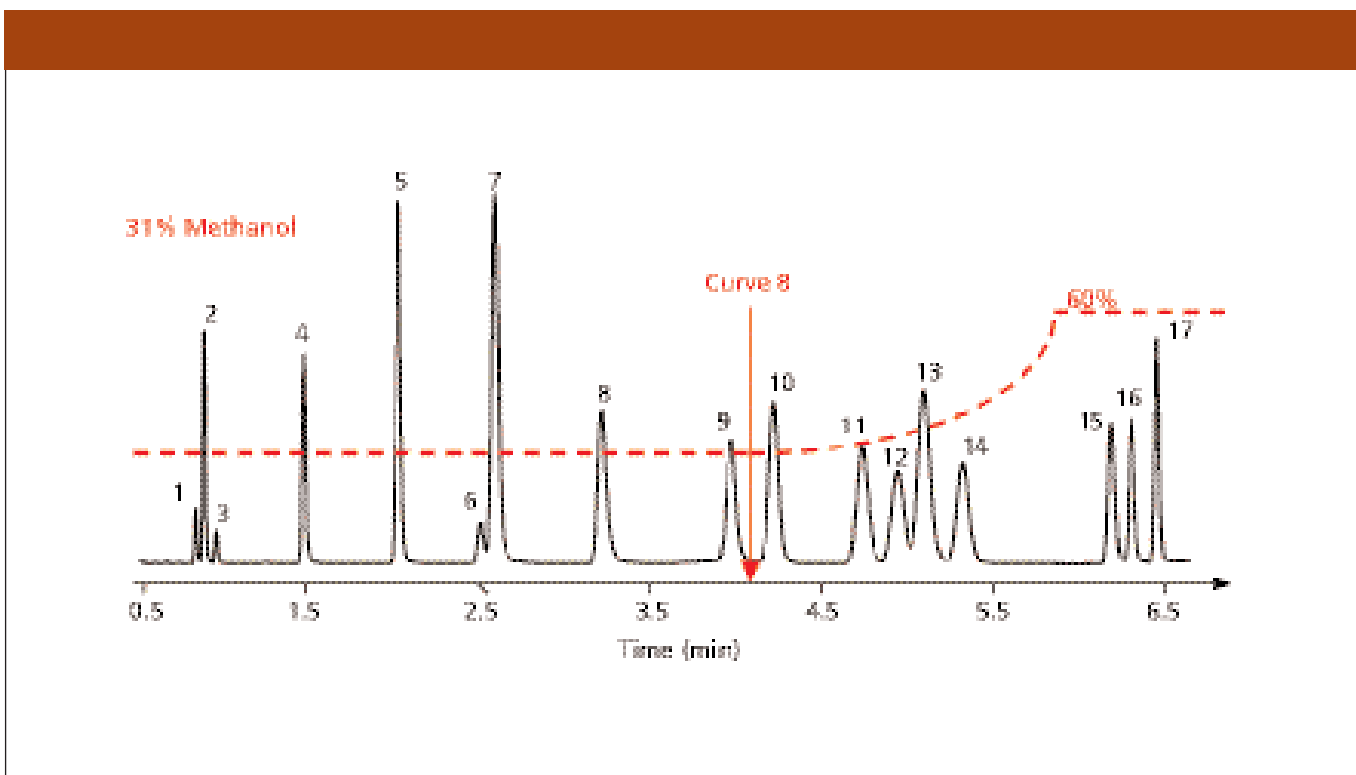
widths of less than one second can be obtained, posing significant challenges for the detector. In order to accurately and reproducibly integrate an analyte peak, the detector sampling rate must be high enough to capture enough data points across the peak. In addition, the detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2–3 times higher than HPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates (no flow splitting) promotes increased source ionization efficiencies (reduced ion suppression) for improved sensitivity.

Sample introduction is also critical. Con-

ventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from experiencing extreme pressure fluctuations, the injection process must be relatively pulse-free. The swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to realize the increased sensitivity benefits.

The ACQUITY UPLC System consists of a binary solvent manager, sample manager (including the column heater), detector, and optional sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient

mixed under high pressure. There are built-in solvent degassing as well as solvent select valves to choose from up to four solvents. There is a 15,000 psi pressure limit (about 1000 bar) to take full advantage of the sub-2- $\mu\text{m}$  particles. The sample manager also incorporates several technology advancements. Low dispersion is maintained through the injection process using pressure assist sample introduction, and a series of pressure transducers facilitate self monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and a needle calibration sensor increases accuracy. Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry over. A variety of microtiter plate formats (deep-well, mid-height, or vials) can also be accommodated in a thermostatically controlled environment. Using



**Figure 6:** Explosives analysis using ACQUITY UPLC. Column:  $2.1 \times 100$  mm  $1.7 \mu\text{m}$  ACQUITY UPLC BEH C18. A water methanol gradient from 31–60% methanol was used as shown, at a flow rate of 0.5 mL/min, with UV detection at 254 nm. Sample was a 10  $\mu\text{L}$  injection of a 10 ppm mixture of each of the compounds. Peaks are in order: 2,6 diamino- 4 nitrotoluene, HMX, 2,4 diamino- 6 nitrotoluene, RDX, 1,3,5-trinitrobenzene, 1,2-dinitrobenzene, 1,3-dinitrobenzene, nitrobenzene, tetryl, 2,4,6-Trinitrotoluene, 2-Amino-4,6-dinitrotoluene, 4-Amino-2,6-dinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene, 2-nitrotoluene, 4-nitrotoluene, 3-nitrotoluene.

the optional sample organizer, the sample manager can inject from samples from up to 22 microtiter plates. The sample manager also controls the column heater. Column temperatures up to 65 °C can be attained. A “pivot out” design provides versatility to allow the column outlet to be placed in closer proximity to the source inlet of an MS detector to minimize excess tubing and sample dispersion.

The tunable UV–vis and PDA detector s include new electronics and firmware to support Ethernet communications at the high data rates necessary for UPLC detection. Conventional absorbance-based optical detectors are concentration-sensitive detectors, and for UPLC, the flow cell volume would have to be reduced in standard UV–vis detectors to maintain concentration and signal. However, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends; and worse: a reduction in cross-section means the light path is reduced, and transmission drops, increasing noise. Therefore, if a conventional HPLC flow cell is used, UPLC sensitivity would be compromised. The ACQUITY UPLC System

detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10-mm flow cell path length with a volume of only 500 nL. Tubing and connec-

**Higher sample throughput with more information per sample may decrease the time to market, an important driving force in today’s pharmaceutical industry.**

tions in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems.

## Applications

Scientists are used to making compromises; and one of the most common scenarios involves sacrificing resolution for speed. With UPLC increased resolution in shorter run times can generate more information faster without sacrifices.

Higher sample throughput with more information per sample may decrease the time to market, an important driving force in today’s pharmaceutical industry.

Figure 2 illustrates a method converted from HPLC to UPLC that takes advantage of the speed of UPLC. The corresponding HPLC separation takes in excess of 12 min; UPLC accomplishes the same separation in under 30 s.

UPLC can also be used to significantly improve the success of the drug discovery process. Drug discovery is heavily dependant upon the early prediction of metabolic fate and interactions of drug candidate molecules. To prevent “poor” candidates from progressing through the discovery process, factors such as metabolic stability, toxic metabolite production, p450 inhibition, and induction are all routinely monitored. By the mid-1990s, high performance liquid

chromatography (HPLC) directly coupled to mass spectrometry (MS) was in routine use in drug metabolism laboratories for these types of studies (5–12). Enhanced selectivity and sensitivity, and rapid, generic gradients made LC–MS the predominate technology for both quantitative and qualitative analyses. However, with the ever-increasing numbers and diversity of compounds entering development, and the complex nature of the biological matrices being analyzed, new analytical procedures and technology were required to keep pace with the testing demands. Unexpected, reactive, or toxic metabolites must be identified as early as possible to reduce the very costly attrition rate. This quest for more accurate data meant improving the chromatographic resolution to obtain higher peak capacity, reducing the co-elution of metabolites, while enhancing the sensitivity and decreasing ion suppression in the MS.

The power of the ACQUITY UPLC System when used in drug discovery can be illustrated by the analysis of the in-vitro metabolism of dextromethorphan. Dextromethorphan undergoes O-dealkylation in two positions leading to three major phase I metabolites. These products can be further metabolized via conjugation with glucuronic acid to form metabolites of masses  $MH^+ = 434$  and  $420$ . The data in Figure 3a and 3b shows the HPLC–MS and UPLC–MS separations, respectively, of the in-vitro incubation of dextromethorphan with rat liver microsomes. As shown, the chromatographic performance of the ACQUITY UPLC BEH 1.7- $\mu\text{m}$  particles is significantly better than that produced by the 3.5  $\mu\text{m}$  material. The 1.7- $\mu\text{m}$  material gives peaks of width 4 s at the base, resulting in a peak capacity of over 100, whereas with HPLC the average peak width was 20 s at the base giving a total peak capacity of just 20, resulting in a 5-fold increase in the performance of the UPLC system.

The extracted ion chromatogram  $m/z = 258$  and  $m/z = 244$  for the HPLC/MS analysis is shown in Figure 4.

In Figure 4, we can clearly see the two O-dealkylated metabolites of dextromethorphan  $m/z = 258$ , these two metabolites are resolved to about 80%, while the 244 metabolite is barely visible. These results can be compared to those obtained by UPLC, here we can see that the two 258 ions are clearly resolved and that the 244 ion is now easily detected, as illustrated in Figure 5.

This data clearly illustrates the improved

resolution and sensitivity of the UPLC system. This extra resolution is particularly important when analyzing isobaric compounds such as these dealkylated metabolites. By incorporating a more efficient UPLC separation into the MS there is less ion suppression from competing compounds in the source and therefore more discreet ionization of the metabolites. Without the resolution generated by UPLC it would be possible to falsely assign the structure of a metabolite or miss a potential toxic moiety. The extra sensitivity produced by the UPLC system ensures more low concentration metabolites will be detected, helping to prevent potentially toxic compounds from progressing further into the drug discovery process. This added sensitivity is extremely important when performing MS–MS experiments as it can make the difference between obtaining an interpretable spectrum or not.

## **At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography.**

Sensitivity, selectivity, and analysis time (sample throughput) are also some of the challenges analysts face when analyzing environmental samples such as soil and water.

Explosives residues in soil or environmental waters are of both forensic and environmental interest. These types of assays prove challenging because of the selectivity needed to resolve positional isomers. Typical HPLC analyses require viscous, buffered mobile phases operated at high temperatures, and analysis times exceeding 30 min.

Figure 6 shows the separation of a complex mixture of explosive compounds in less than seven minutes, with a much simpler, more robust mobile phase than that commonly used in HPLC assays. The simpler nonbuffered mobile phase also is ideal for MS detection if desired.

## **Conclusion**

ACQUITY UPLC using 1.7- $\mu\text{m}$  particles and a properly holistically designed system provide significantly more resolution (information) while reducing run times, and improve sensitivity for the analyses of many compound types. At a time when many scientists have reached separation barriers with conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography. New ACQUITY technology in both chemistry and instrumentation boosts productivity by providing more information per unit of work as UPLC fulfills the promise of increased resolution, speed, and sensitivity predicted for liquid chromatography.

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