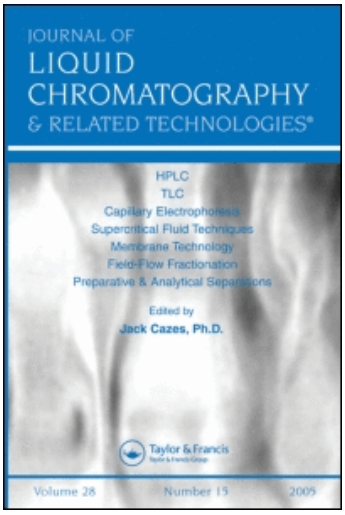

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UPLC™: An Introduction and Review

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UPLC™: An Introduction and Review

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Abstract: Ultra performance liquid chromatography™ (UPLC) takes advantage of technological strides made in particle chemistry performance, system optimization, detector design, and data processing and control. Using sub-2 μm particles and mobile phases at high linear velocities, and instrumentation that operates at higher pressures than those used in HPLC, dramatic increases in resolution, sensitivity, and speed of analysis can be obtained. This new category of analytical separation science retains the practicality and principles of HPLC while creating a step-function improvement in chromatographic performance.

This review introduces the theory of UPLC, and summarizes some of the most recent work in the field.

Keywords: UPLC, Ultra performance liquid chromatography

INTRODUCTION

High performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide during 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 separations. The underlying principles of this evolution are governed by the van Deemter equation, with which any student of chromatography is intimately familiar.[1] The van Deemter equation is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP, or column efficiency). And, since particle size is one of the variables, a van Deemter curve can be used to investigate chromatographic performance.

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For many years, researchers have looked at "fast LC" as a way to speed up analyses.[2,3] The "need for speed" has been driven by the sheer numbers of samples in some laboratories (particularly in drug discovery) and the availability of affordable, easy to use mass spectrometers. Smaller columns and faster flow rates (amongst other parameters) have been used. Elevated temperature, having the dual advantages of lowering viscosity, and increasing mass transfer by increasing the diffusivity of the analytes, has also been investigated.[4] However, using conventional particle sizes and pressures, limitations are soon reached and compromises must be made, sacrificing resolution for time.

However, as illustrated in Figure 1, as the particle size decreases to less than 2.5 μm, not only is there a significant gain in efficiency; but the efficiency doesn't diminish at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits, termed Ultra Performance Liquid Chromatography, or UPLC™. This introduction and review traces some of the developments and technological advancements made in producing the first commercially available UPLC instrument.

SMALL PARTICLE CHEMISTRY

The promises of the van Deemter equation cannot be fulfilled without smaller particles than those traditionally used in HPLC. The design and development

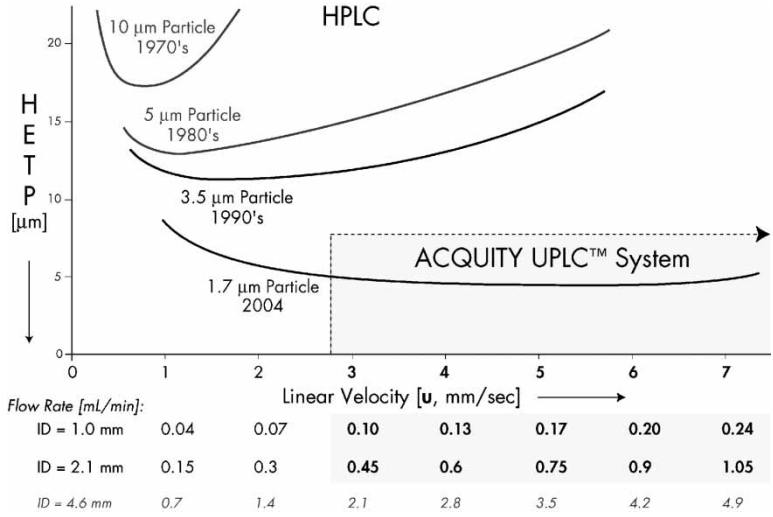

Figure 1. van Deemter plot, illustrating the evolution of particle sizes over the last three decades.

of sub-2 μm particles is a significant challenge, and researchers have been active in this area for some time to capitalize on their advantages.[5–7] Although high efficiency, non-porous 1.5 μm particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. To maintain retention and capacity similar to HPLC, UPLC must use novel porous particles that can withstand high pressures. 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 and limited capacities.

In 2000, a first generation hybrid chemistry that took advantage of the best of both the silica and polymeric column worlds was introduced.[8,9] Produced using a classical sol-gel synthesis that incorporates carbon in the form of methyl groups, these columns are mechanically strong, with high efficiency, and operate over an extended pH range. But, in order to provide the kind of enhanced mechanical stability required for UPLC, a second generation bridged ethane hybrid (BEH) technology was developed.[10] These 1.7 μm particles derive their enhanced mechanical stability by bridging the methyl groups in the silica matrix.

Packing 1.7 μm 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.

In addition, at high pressures, frictional heating of the mobile phase can be quite significant and must be considered.[11] With column diameters typically used in HPLC (3.0 to 4.6 mm), a consequence of frictional heating is the loss of performance due to temperature induced non uniform flow. To minimize the effects of frictional heating, smaller diameter columns (1–2.1 mm) are typically used for UPLC.[12,13]

CAPITALIZING ON SMALLER PARTICLES

Small particles alone do not make it possible to fulfill the promises of the van Deemter equation (Figure 1). 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 (pumps, injectors, and detectors) simply doesn't have the horsepower to take full advantage of sub-2 μm particles.

One-of-a-kind systems, capable of delivering the pressures required to realize the potential of UPLC have been reported in the literature and elsewhere.[14–16]

Lee et al. described the design of injection valves and separation reproducibility,[14] and the use of a carbon dioxide enhanced slurry packing method on the capillary scale for the separation of some benzodiazepines, herbicides, and various pharmaceutical compounds.[17] Jorgenson et al. modified a commercially available HPLC system to operate at 17,500 psi and used 22 cm long capillaries packed with 1.5 μm C_{18}-modified particles for the analysis of proteins.[15]

These reports illustrated that, to take full advantage of low dispersion and small particle technology to achieve high peak capacity UPLC separations, a greater pressure range than that achievable by today's HPLC instrumentation was required. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15 cm long column packed with 1.7 μm particles is about 15,000 psi. Therefore, a pump capable of delivering solvent smoothly and reproducibly at these pressures, that can compensate for solvent compressibility, and can operate in both the gradient and isocratic separation modes, was required.

Sample introduction is also critical. Conventional 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.

With 1.7 μm particles, half-height peak widths of less than one second are 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 with HPLC separations, depending on the detection technique that is used. Conventional absorbance-based optical detectors are concentration sensitive detectors and, for UPLC use, the flow cell volume would have to be reduced in standard UV/Visible detectors to maintain concentration and signal, while avoiding Beers' Law limitations.

In early 2004, the first commercially available UPLC system that embodied these requirements was described for the separation of various pharmaceutical related small organic molecules, proteins, and peptides; it is called the ACQUITY UPLC™ System.[18–20]

Using UPLC, it is now possible to take full advantage of chromatographic principles to run separations using shorter columns, and/or higher flow rates for increased speed, with superior resolution and sensitivity. Figures 2 and 3

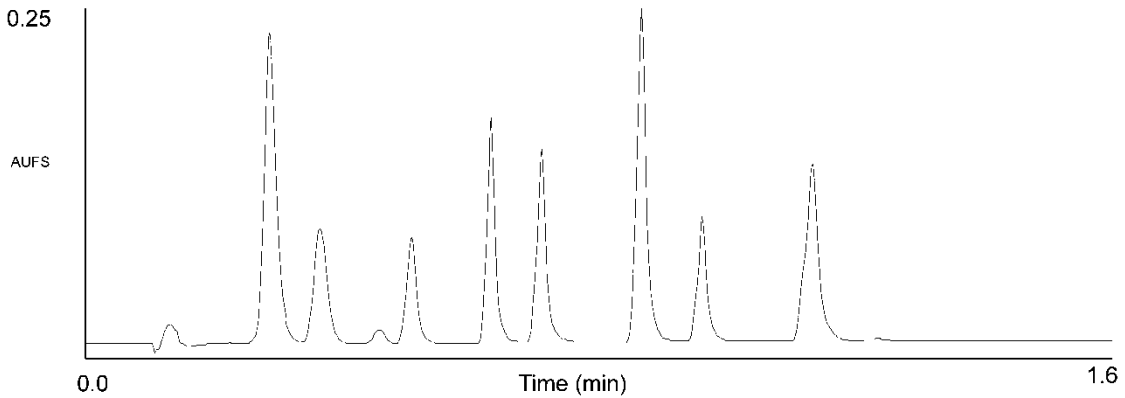

Figure 2. UPLC separation of eight diuretics. Column: 2.1 by 30 mm 1.7 μm ACQUITY UPLC BEH C_{18} @ 35°C. A 9–45%B linear gradient over 0.8 minutes, at a flow rate of 0.86 mL/min was used. Mobile phase A was 0.1% formic acid, B was acetonitrile. UV detection @ 273 nm. Peaks are in order: Acetazolamide, Hydrochlorothiazide, Impurity, Hydroflumethiazide, Clopamide, Trichlormethiazide, Indapamide, Bendroflumethiazide, and Spironolactone, 0.1 mg/mL each in water.

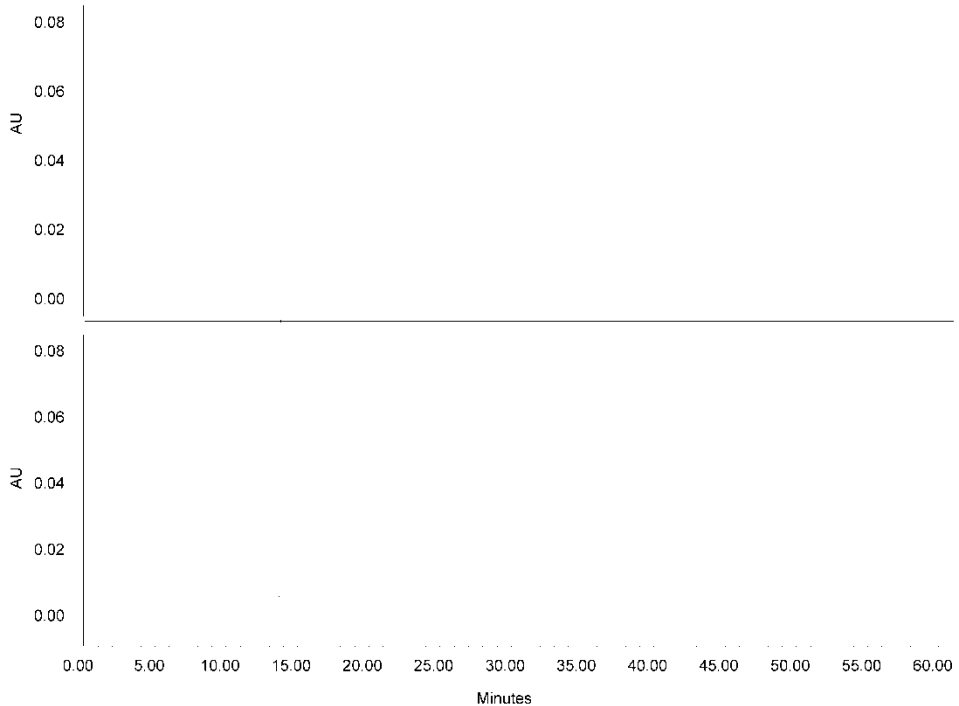

Figure 3. HPLC vs. UPLC peak capacity. In this gradient peptide map separation, the HPLC (top) separation (on a 5 μm C18 column) yields 70 peaks, or a peak capacity of 143, while the UPLC separation (bottom) run under identical conditions yields 168 peaks, or a peak capacity of 360, a 2.5X increase.

illustrate UPLC in action. In Figure 2 a separation of eight diuretics is accomplished in under 1.6 minutes. The same separation on a 2.1 by 100 mm 5 μm C_{18} HPLC column yields comparable resolution, but takes over ten minutes. For some analyses, however, speed is of secondary importance; peak capacity and resolution take center stage. Figure 3 shows a peptide map where the desired goal is to maximize the number of peaks. In this application, the increased peak capacity (number of peaks resolved per unit time) of UPLC dramatically improves the quality of the data resulting in a more definitive map

APPLICATIONS

Chromatographers are accustomed to making compromises; one of the most common scenarios involves sacrificing resolution for speed. In addition, for complex samples like natural product extracts, added resolution can provide more information in the form of additional peaks. Figure 4 shows an HPLC

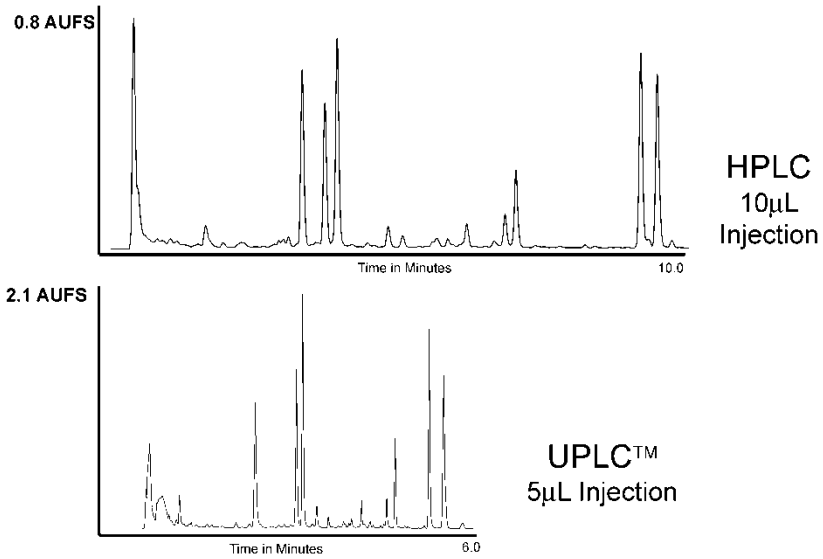

Figure 4. Comparison HPLC and. UPLC for the separation of a ginger root extract. HPLC conditions: Column: 2.1 by 100 mm 5.0 μm prototype BEH C18 at 28°C. A 25–96%B linear gradient over 10 minutes, at a flow rate of 1.0 mL/min was used. Mobile phase A was water, B was acetonitrile. UV detection @ 230 nm, 10 μL injection. UPLC conditions: Column: 2.1 by 100 mm 1.7 μm ACQUITY BEH C18 at 28°C. A 50–100%B linear gradient from 1.4 to 3.7 minutes, followed by a hold until 6.0 minutes, at a flow rate of 0.3 mL/min was used. Mobile phase A was water, B was acetonitrile. UV detection @ 230 nm, 5 μL injection.

versus UPLC separation comparison of a ginger root extract sample where both speed and resolution are improved, as well as an increase in sensitivity. DryLab software was used to model and redevelop the separation and transfer it to the ACQUITY UPLC System and BEH chemistry.

Faster separations can lead to higher throughput and time savings when running multiple samples. But, a significant amount of time can also be consumed in developing the method in the first place. Faster, higher resolution UPLC separations can cut method development time from days, to hours, or even minutes. Figure 5 is an example of an UPLC separation of several closely related coumarins and a metabolite that was developed in under an hour, including UPLC scouting runs for gradient optimization, and individual runs for elution order identification. These runs were performed in a fraction of the time that would be necessary by conventional HPLC, resulting in significant time savings in the method development laboratory.

As alluded to previously, mass spectrometry has gained widespread acceptance as an analytical tool for the qualitative and quantitative analysis

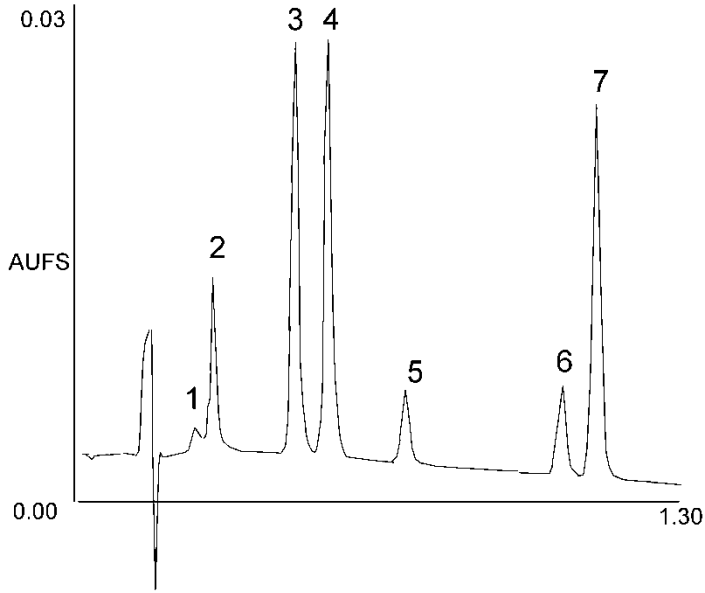

Figure 5. UPLC Separation of Seven Coumarins illustrating fast method development. Column: 2.1 by 30 mm 1.7 μm ACQUITY UPLC BEH C_{18} @ 35°C. A 20–40%B linear gradient over 1.0 minute, at a flow rate of 0.86 mL/min was used. Mobile phase A was 0.1% formic acid, B was acetonitrile. UV detection @ 254 nm and 40 pts/sec. Peaks are in order: 1: 7-hydroxycoumarin-glucuronide, 7-hydroxycoumarin, 4-hydroxycoumarin, coumarin, 7-methoxycoumarin, 7-ethoxycoumarin, and 4-ethoxycoumarin.

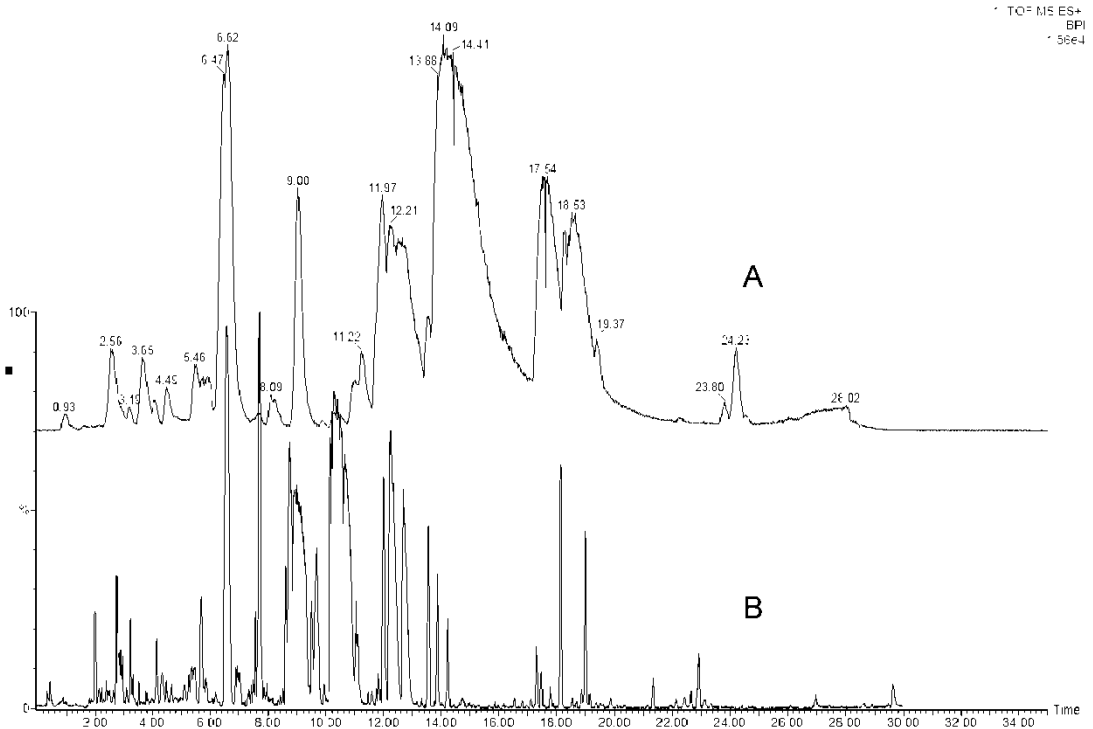

Figure 6. Separation of rat bile following the administration of midolazam at 5 mg/kg: A) 30 minute separation on a 2.1 by 100 mm 3.5 μm C18 HPLC Column and B) 30 minute separation on a 2.1 by 100 mm 1.7 μm C18 UPLC column. Reprinted by permission from reference 21.

of many types of compounds. 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. Jorgenson et al. have shown that higher chromatographic efficiency, resulting from the use of UPLC, translates into better resolution and higher peak capacity, which is particularly important for the analysis of peptides and proteins.[15] The increased resolving power made the resulting data easier to interpret, since more of the MS peaks consisted of a single compound, and up to a 20-fold improvement in the quality of the spectral information (vs. nanoelectrospray) was obtained. Lee et al. also used MS detection for the analysis of low molecular weight compounds similar to those that might comprise a combinatorial library.[14,17] It was demonstrated that, in order to address the very narrow peaks produced by UPLC, it is necessary to use a very high data capture rate MS such as a TOF or quadrapole with fast scan rates. Lee et al. also pointed out that, in some instances, related compounds of the same molecular weight and similar structures could not be differentiated by MS, necessitating chromatographic resolution on the UPLC time scale.[17]

Plumb et al. have investigated the use of UPLC/MS for the analysis of metabolites,[21,22] and as a tool for differential metabolic pathway profiling in functional genomic studies.[23] Their data illustrate the benefit obtained from the extra resolution of UPLC, both in terms of specificity and spectral quality, revealing new information and reducing the risk of not detecting potentially important metabolites. Figure 6 shows the benefits of UPLC versus HPLC for monitoring the *in-vivo* metabolism of midazolam (an anticonvulsant) in rat liver bile. This is a challenging separation due to the high concentration of bile salts that can interfere, and the presence of bilirubin that can cause ion pairing. The resolution is dramatically improved, and the number of discreet peaks has more than doubled.

CONCLUSION

UPLC presents the ability to extend and expand the utility of separation science at a time when many scientists have reached separation barriers, pushing the limits of conventional HPLC. New chemistry and instrumentation technology can provide more information per unit of work as UPLC begins to fulfill the promise of increased speed, resolution, and sensitivity predicted for liquid chromatography.

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