The Evolution of Ultra High-Performance Liquid Chromatography: Expanding the Future of Separation Technologies

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The adoption of UHPLC (ultra high-performance liquid chromatography), with the advancements made in both UHPLC instrumentation and UHPLC column technology, has changed scientists' expectations of chromatographic performance. This article will discuss the challenges of liquid chromatography (LC), in terms of increasing separation efficiency, which led to the innovations of UHPLC technology. We take a closer look at UHPLC and how the benefits of speed, sensitivity, and resolution of UHPLC separations have expanded outside routine small molecule analysis. We will also discuss the evolution of UHPLC and its expansion into other chromatographic application areas such as size-exclusion chromatography (SEC) and supercritical fluid chromatography (SFC).

Introduction

UHPLC (Ultra High-Performance Liquid Chromatography) is a separation technology that increases chromatographic speed, sensitivity and resolution, over traditional HPLC separations by providing higher chromatographic efficiency per unit time [1]. The greatest gains are obtained when using columns packed with sub-2-µm particles in narrow bore (< 2.1 mm internal diameter) columns and run at or above their optimal linear velocity using low dispersion instrumentation. However, using columns packed with small particles, and realising the expected gains in efficiency, require improvements to the instrumentation, column hardware and particle technology [1].

Columns packed with smaller particles produce greater chromatographic efficiency than columns packed with larger particles by reducing both the eddy dispersion and the mass transfer contributions to peak broadening [1]. Efficiency is directly proportional to the length of the column and inversely proportional to the particles size; resolution of the separation can be maintained by keeping the column length to particle size ratio (L/dp) equivalent. Using shorter columns packed with smaller particle decreases the overall run time while increasing sensitivity. Figure 1 shows the chromatographic improvements in speed and sensitivity when utilising smaller higher efficiency columns.

Figure 2 shows the measured gains in efficiency as the particle size is reduced when operated at their optimal flow rates. Operating columns packed with smaller particles and run at their optimal flow rates generates higher column backpressure compared to columns that are packed with

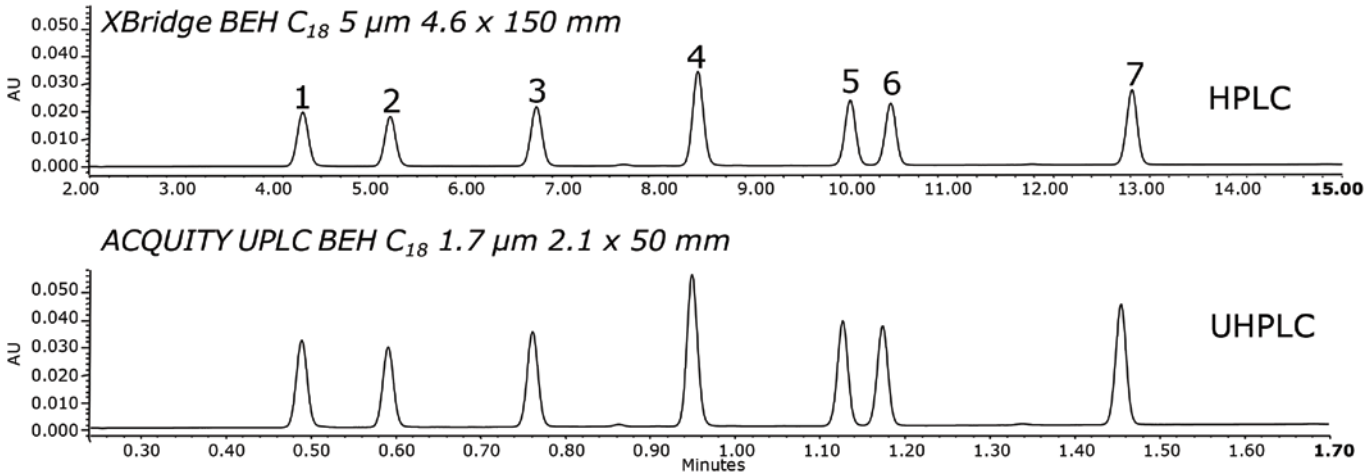

Figure 1. Scaled separation of 7 steroid standards (Prednisone, Hydrocortisone, Dexamethazone, Estradiol' 17α – Hydroxyprogesterone, Levonorgestrel, Progesterone) on an XBridge BEH C_{18} 5 µm 4.6 x 150 mm column (top trace) and a ACQUITY UPLC BEH C_{18} 1.7 µm 2.1 x 50 mm column. Highlighted are the improvements in speed and sensitivity when using the shorter column packed with smaller particles.

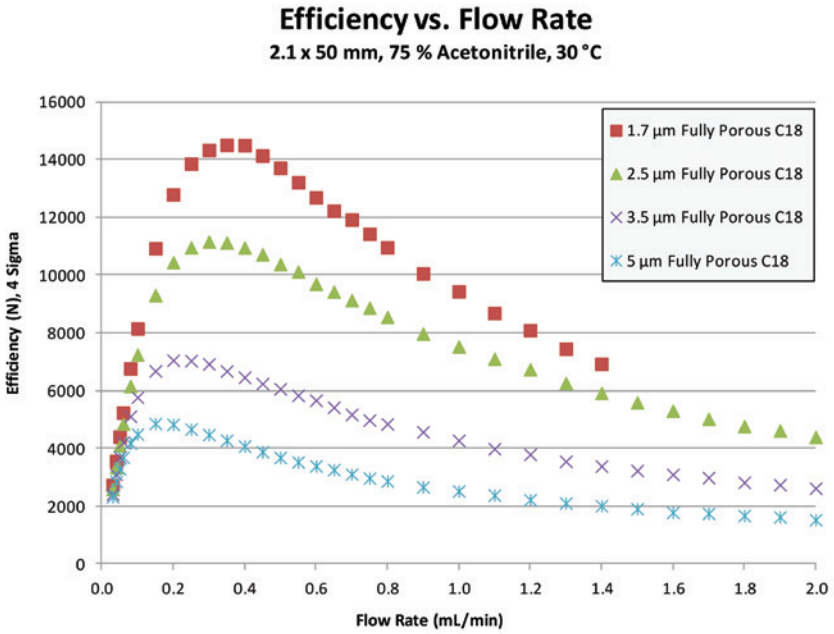

Figure 2. Curves showing the increases in column efficiency as the particle size is decreased across multiple flow rates. Efficiency for all columns is measured at 4 sigma peak width using a neutral compound. All 2.1 x 50 mm columns are packed with fully porous BEH C_{18} particles of various particle sizes.

larger particles. The observed pressure at the optimal flow rate increases as the inverse cube of the particle size ($\Delta P \propto 1/dp^3$). Improvements to the UHPLC instrument pump, valves, and fluidic pathway need to be compatible with the increased pressure that these columns produce.

The efficiency gains of a narrow bore column (< 2.1 mm internal diameter) packed with small particles can only be obtained by minimising instrument band spreading. Traditional HPLC systems have large fluidic pathways that are inherent to the design of the injector, tubing, and flow cell. This increased extra-column dispersion dilutes the chromatographic band, which negates the efficiency gains of using high efficiency columns [2]. This effect is more pronounced for early eluting peaks where the peak volume is small and is more impacted by the system band spreading contribution [2]. The impact of this effect increases as the column internal diameter is further reduced. Large bore columns compensate for this effect by increasing the eluting peak volume; the relative ratio of system dispersion to peak volume is reduced as the column volume increases. An often overlooked beneficiary of this is in the application of nano- and micro-flow liquid chromatography. The advantages of low dispersion systems developed for sub-2-µm LC separations are also utilised for nano-and micro-flow LC separations. This development significantly enhances the advantages of the smaller scale chromatographic separation and further increases sensitivity [3]. Figure 3 shows the measured system dispersion values for two systems and contribution of the system dispersion on the chromatography.

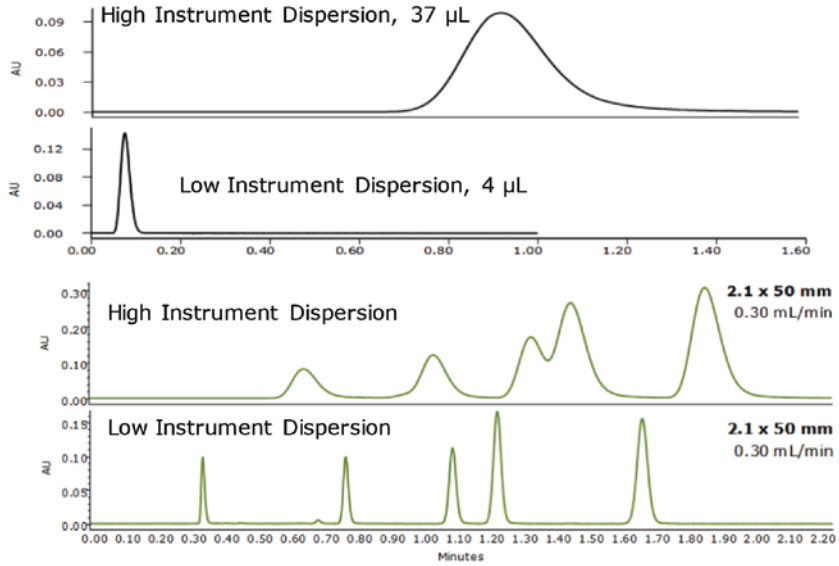

Figure 3. Calculated dispersion values of two LC instruments, high dispersion system [first] and a low dispersion system [second]. The impact of the instrument dispersion on the chromatography for the isocratic separation of five neutral compounds using the same 2.1 mm internal diameter column packed with 2.7 µm particles is shown for comparison [third & fourth].

Using smaller particle packed columns on systems that are not optimally configured for UHPLC can introduce problems that are not commonly encountered when using columns of a larger particle size. For example, as the mobile phase flows through the packed bed, the column temperature increases due to frictional heating. This causes the temperature to vary across the column length and radius. The radial temperature gradients can increase band broadening [4]. Narrow bore columns help to reduce radial temperature gradients. The relatively larger surface area-to-volume ratio of smaller diameter columns compared to larger bore columns help to dissipate frictional heating. Using narrow bore columns with a well designed column oven with optimised thermal control can dramatically improve separation efficiency and resolving power [4].

UHPLC Particle Technologies

Along with the instrument, there have been significant innovations in particle technology used to drive separation performance. Silica-based particles have been widely used over the years due to their wide applicability for a broad range of separation conditions. Many traditional silica-based columns that are used for HPLC have particles with open pore structure and high pore volume, which makes them mechanically unstable when operated at UHPLC pressures. Ethylene-bridged hybrid particles, which combine attributes of both silica particles and polymeric particles, were the first to provide increased mechanical stability, while maintaining separation efficiency [5]. Hybrid particles also have increased chemical stability over silica based particles, giving them the ability to be used over a broader mobile phase pH range [5]. Innovations in silica-based particle synthesis allowed manufacturers to produce higher strength silica particles that can tolerate UHPLC operating pressures. With the development of high strength silica, chromatographers are able to transfer methods developed on HPLC silica particles to UHPLC. Superficially porous silica particles were the next innovation to increase separation efficiency.

Columns packed with these particles further reduce the eddy dispersion and also reduce longitudinal diffusion [6]. Understanding these particle technology developments has allowed manufacturers to provide innovative columns applicable for other areas outside of routine small molecule analysis, such as size exclusion chromatography and supercritical fluid chromatography.

Evolution of UHPLC for Size - Exclusion Chromatography

For the purposes of this discussion, size-exclusion chromatography (SEC) and gel-permeation chromatography (GPC) differ only in application area. They are both size-based separation modes with SEC commonly associated with biomolecule separations and GPC referring to separations for synthetic or natural polymers. Significant improvements in UHPLC technology using smaller particle columns coupled with low dispersion fluidic paths increased resolving power with a subsequent reduction in analysis time for both techniques [7,8]. As innovation in biomolecular research continues, scientists are required to fully characterise and identify biomolecular species present in the sample that may impact the efficacy or safety of the drug product. In the polymer industry, the physicochemical properties of the polymer, such as chemical resistance or tensile strength, are correlated to molecular data to tailor the material characteristics required for the end use product. For these examples size-exclusion chromatography is the preferred technique.

Similar to small molecule separations, any extra-column band spreading resulting from a poorly optimised UHPLC system can undermine the gains achieved from the highly efficient separation. For SEC, the separation takes place within the pore volume contained within the column; it is even more important to minimise the contribution of extra column band broadening. For this reason isocratic systems with extremely low system dispersion are preferred. Figure 4 shows the impact of two systems: one with high system dispersion and one with low system dispersion. The UHPLC based low dispersion system maintains the resolution of the separation especially for the low molecular weight species. Even though both systems use the same column series the high dispersion system cannot take advantage of using smaller particle columns due to the increased system dispersion.

It is imperative that the peak width remains at a minimum to maintain resolution; however, speed is the main benefit of UHPLC. Short columns that are packed with 1.7 µm particles can produce equivalent resolution, in approximately 1/9 the time, compared to traditionally longer columns that are packed with 5 µm particles [2]. In cases where more resolving power is required, longer columns can now be used more effectively without requiring long analysis times. Also, the difference in solvent consumption is significant. The original separation shown in Figure 4 used 30 mL of solvent compared to the 5 mL of solvent used for the low dispersion UHPLC GPC system. The cost per analysis is significantly impacted, both during the analysis and for the final disposal of the waste stream. In this situation, using reducing the amount of toxic solvent used also improves the analysts work environment.

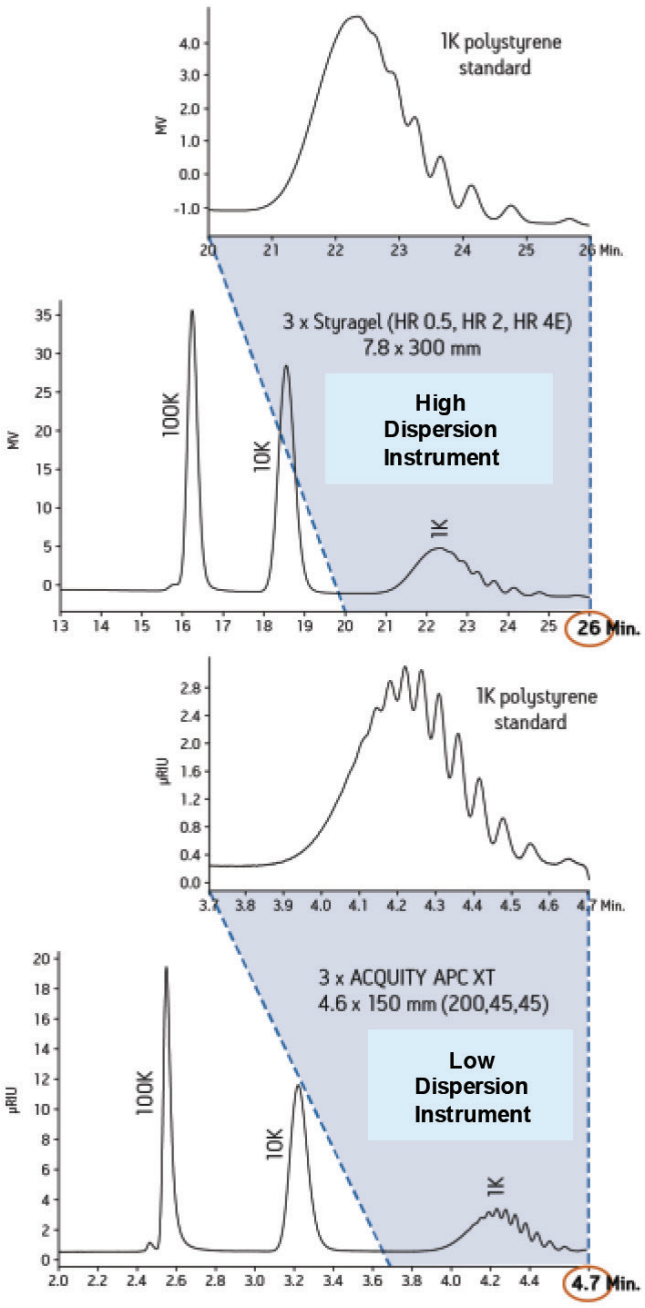

Figure 4. The effects of extra-column band broadening on chromatographic resolution. Compared to the low dispersion system [bottom], the extra-column band broadening introduced by the high dispersion GPC system negates efficiency gains of the 2.5 µm particle column set-up used for the separation. The three-column set-up used for this polystyrene separation (two ACQUITY APC XT 45 columns (4.6 x 150 mm) and one ACQUITY APC XT 200 column (4.6mm x 150 mm)); THF mobile phase, 1.0 mL/minute was the same for both systems. Image reproduced by permission, Waters Corporation.

The lack of modern column chemistries optimised for high performance GPC have limited the potential for developing new, more efficient UHPLC GPC separations. To fully realise the efficiency benefits of smaller particle columns, the mobile phase needs to be pumped through the chromatographic bed at a higher linear velocity. Using this approach for traditional 7.8 mm i.d. SEC columns will waste a considerable amount of mobile phase for very little chromatographic benefit. Additionally, achieving maximum resolution for SEC separations requires stationary phases with large pore volumes. This is challenging for small particles because increasing pore volume significantly decreases the structural integrity of the particle. Poor particle strength is the main disadvantage of polymer-based resins, where the relatively non-rigid structure can compress or collapse under high pressure operating conditions. The most recent developments for GPC stationary phases use fully porous hybrid particles [5], which maintain the required mechanical rigidity while providing the improvements in separation efficiency.

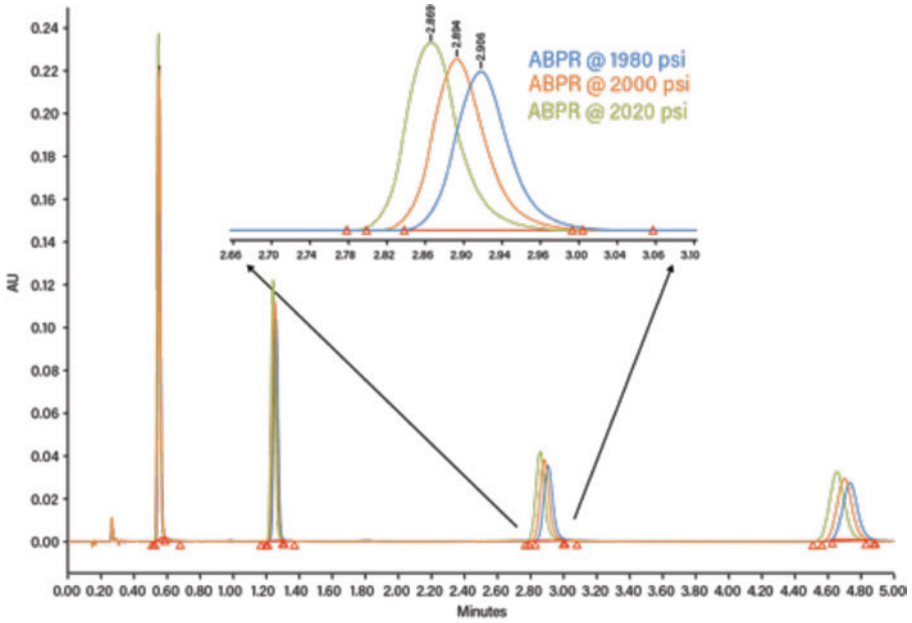

Figure 5. The effect of system pressure on SFC retention. Changes in system pressure ABPR (active backpressure regulator) result in variable retention due to changes in mobile phase density and solvent strength. Image reproduced by permission, Waters Corporation.

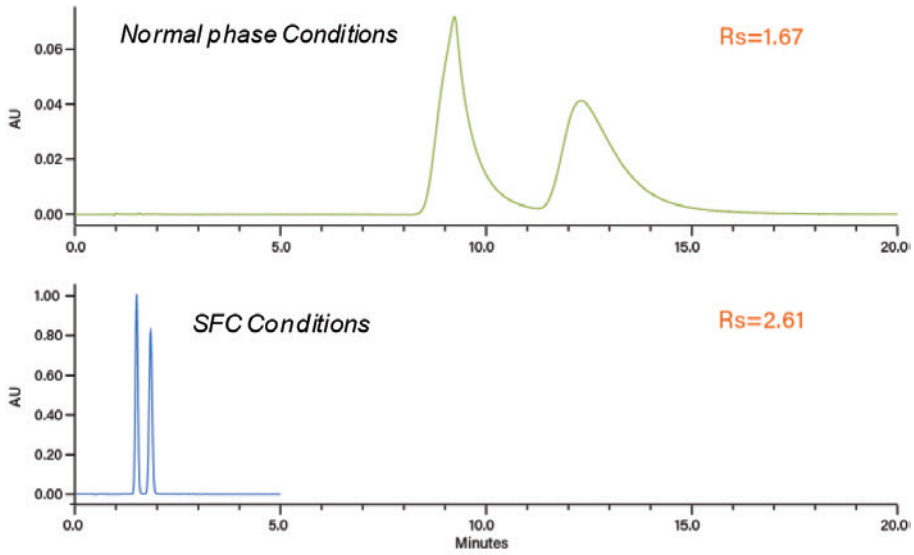

Figure 6. Efficiency and separation speed of SFC compared to conventional normal phase HPLC for the separation of enantiomers. Image reproduced by permission, Waters Corporation.

Evolutions in SFC

Another recent advancement for UHPLC separations is the exploration and use of alternative solvents and mobile phases to achieve analyte selectivity and retention. Supercritical fluid chromatography (SFC) is one such area that uses alternative solvents, such as liquid carbon dioxide along with co-solvents like methanol, as the mobile phase. The pressure required to maintain carbon dioxide in the critical fluid region is typically between 100 and 400 bar. Carbon dioxide is a highly compressible fluid, which places greater demand on the fluidics and pumping algorithms compared to systems designed for pumping non-compressible fluids commonly used for LC. Any small deviation in system pressure will change the solvent density and subsequent solvent strength, thus impacting analyte retention reproducibility [9]. Historically, older HPLC instrument technology could not consistently deliver compressed carbon dioxide so method reliability and repeatability suffered.

Most LC instrumentation is designed to transfer incompressible fluids and therefore struggle to maintain compositional accuracy and flow precision under SFC operational conditions [9]. Improvements in UHPLC system engineering changed the understanding of pump design and pump control algorithms to compensate for compressible fluids to allow for reproducible retention times and extremely low baseline noise. Additionally, gradient SFC separations are now commonplace compared to methods that were primarily limited by older technology and isocratic conditions.

Backpressure regulation within the system is the most important aspect to achieve reliable SFC separations. Poor pressure regulation greatly affects mobile phase density and the resulting chromatographic reproducibility. Traditional SFC instrumentation was plagued by poor pressure monitoring capability and slow to react feedback loops. By using both active and static pressure control, the user can accurately control both pressure and flow rate to achieve highly repeatable results under all separation conditions. In this case, the static control maintains a set minimum pressure while the enhanced active control fine tunes the set point required by the user. Figure 5 shows the effect of small variations in system pressure over the observed retention time.

The higher diffusion rates of SFC separations combined with smaller particles results in faster mass transfer of the analyte between the stationary phase and mobile phase. This results in highly efficient separations using high mobile phase linear velocities. For example, chiral separations that were developed for normal-phase conditions can now take advantage of SFC to significantly reduce sample run times and solvent cost. Figure 6 shows the improvement in separation speed while maintaining peak resolution compared to traditional HPLC based normal-phase approaches.

Conclusion

Combining columns packed with small particles with a low dispersion UHPLC system is the key to UHPLC technology. Small particle technology has been around for many years; however, the particle alone cannot provide the expected increases in chromatographic performance. It's the holistic approach to system design that considers the instrument, column hardware and particle development that provides true UHPLC performance.

The evolution of UHPLC technology over the past fourteen years has enabled scientists from different application areas to advance their research. Adapting UHPLC concepts that originated with small molecule theory are now being applied to additional chromatographic techniques such as SEC and SFC. SEC and SFC are not designed to replace conventional UHPLC techniques but rather complement it by providing

different separation modes. Independent of the application, UHPLC has produced significant advancements to reduce analysis time, produce sharper peaks for increased sensitivity, and to enhance resolution for better characterisation and quantification of sample constituents.

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