The Use of Micron-Sized Particles in Ultrahigh-Pressure Liquid Chromatography

Anton D. Jerkovich, J. Scott Mellors and James W. Jorgenson,
University of North Carolina at Chapel Hill, North Carolina, USA.

Over the past 20 years several approaches to improve upon the separation power of standard (20 000 theoretical plate) chromatography columns have been investigated. Packing conventional 3 and 5 µm packing materials into long fused-silica capillary columns is one means to generate theoretical plate numbers in the hundreds of thousands.1 A drawback of this approach is that run times may range from several hours to the better part of a day. Another approach is the preparation of monolithic columns, in which a porous packing structure is synthesized in situ in the column by sol-gel or polymerization chemistry.2 It is hoped that, through optimization of this strategy, highly efficient columns with modest flow resistances can be prepared. To date, however, separation efficiencies demonstrated for columns prepared in this manner are not extraordinary.

Reducing the particle diameter of the packing material in an LC column is the time-proven method to achieve both increased separation power and faster analysis times. Consequently, the use of packings in the 1–2 µm size range is a logical approach to improve resolution. As we shall see, the pressures required for pumping mobile phase through a relatively long column packed with such small particles can be prohibitive for standard HPLC hardware. There are two strategies that have been developed to address this problem. One approach is to drive the flow of mobile phase using electroosmosis, in which a high voltage, typically tens of thousands of volts, is used to pump mobile phase through the column.3–4 This technique has come to be known as capillary electrochromatography (CEC). The principle drawback of this technique is that the chromatographic packing material must serve two roles. It must act as the stationary phase for chromatography, as well as the material that generates the electroosmotic flow (i.e., the “pump”). In order to serve as an efficient electroosmotic pump, the packing material must have considerable surface charge when in contact with the mobile phase. This requires that the mobile phase be relatively polar, containing water and/or polar organic solvents such as acetonitrile or methanol. This precludes the use of CEC with much of normal-phase chromatography, although reversed phase is feasible. Also, most modern highly deactivated reversed-phase packing materials were developed specifically to reduce the presence of surface charge, and thus serve as poor CEC “pumps.” Finally, knowing how a particular packing will interact with a particular mobile phase to produce electroosmotic flow, and thus knowing what voltage to apply to achieve a particular flow, is largely an empirical matter, not a desirable characteristic of a pump.

A more straightforward way to achieve the required flow in long columns packed with micron-sized particles is to develop pumps, valves and columns that are capable of operating at much higher pressures than conventional HPLC systems.5–7 While conventional HPLC technology is limited to pressures below 400 bar (6000 psi), this new technology will be required to operate at pressures at least ten times higher. This technology has been termed ultrahigh-pressure liquid chromatography (UHPLC).

The efficiency of a chromatography column may be described by the height equivalent of a theoretical plate (H), where the lower the plate height, the more efficient a column. Plate height is significantly reduced by the use of small particles. The relationship of H with mobile-phase flow velocity (u) is described by the van Deemter equation:

$$H = A + \frac{B}{u} + Cu$$  [1]

where A, B and C are the coefficients for eddy diffusion, longitudinal diffusion and resistance to mass transfer, respectively. Decreasing the contributions to plate height made by these terms is desirable. The A-term is generally found to be proportional to the stationary phase particle diameter (dp), while the C-term is proportional to dp2. In addition, the optimum mobile phase velocity increases with decreasing particle diameter, allowing for faster separations. This effect is illustrated by the van Deemter plots in Figure 1. Shown are the theoretical performances of columns packed with 1, 3 and 5 µm particles. Not only is the minimum plate height (Hmin) reduced significantly using smaller particles, but the slope of the high-velocity side of the curve (dominated by the C-term) also drops dramatically, allowing for operation at higher flow-rates without sacrificing much in efficiency.

The use of micron-sized particles, however, requires increased pumping pressure. The pressure drop (∆P) over a column is inversely proportional to the square of the particle diameter, and is given by:
The optimum flow velocity is also inversely proportional to column length and a fivefold decrease in analysis time; however, the pressure drop required to achieve significantly faster analysis times. However, this yields no improvement in overall column efficiency. Table 1 compares the expected performance and pressure requirements of 25 cm long columns, packed with particles ranging from 0.75 to 5 µm in size, operated at the optimum flow velocity for a small organic analyte. Reducing the particle diameter from 5 µm to 1 µm yields a fivefold increase in theoretical plates and a fivefold decrease in analysis time; however, the pressure requirements increase 125-fold. To take full advantage of the performance of small particles, these pressure limitations must be overcome. A few research laboratories have developed pumping systems capable of pressures up to 3500 bar (50 000 psi) or greater. Columns greater than 50 cm in length have been prepared with 1 µm packing materials, yielding in excess of 350 000 theoretical plates.

Commercial packing materials smaller than 2 µm in size are fairly scarce. Some commercial sources include Eichrom Scientific (Darien, Illinois, USA), Alltech (Deerfield, Illinois, USA), Zirchrom Separations (Anoka, Minnesota, USA) and Chemie Uetikon (Uetikon, Switzerland). Some research laboratories have synthesized and bonded their own material. Cimtron and Colmán have described a one-step process to synthesize uniform organo-silica spherical particles for use with UHPLC.

Several concerns arise when using such extreme mobile-phase pressures. The engineering challenges involved with sealing and operating an LC system at ultra-high pressures have required in-house construction of column fittings and injectors. Operating an LC system at ultra-high pressures have required in-house construction of column fittings and injectors. However, with proper consideration of the pressure limitations of materials, tubing and other instrument components, and with careful design and normal laboratory safety precautions, UHPLC can be safe to use.

Studies on the effects of conventional HPLC–type pressures on analyte retention in reversed-phase HPLC found retention factors (k') increase with pressure. By extrapolation of these studies to much higher pressures, one could speculate that the increase in retention would be so great as to place an upper limit on the pressure feasible for liquid chromatography. Experimental results in UHPLC have so far shown only a modest linear increase in k' with pressure.

The power (heat/time) dissipation caused by flow through a packed bed is equal to the product of the flow-rate (F) and the pressure drop:

\[ \text{power} = F \times P \]  

This frictional heating of the mobile phase will occur and, under UHPLC conditions, could cause the temperature inside the column to rise significantly. This will result in uneven temperature profiles, both across the column’s diameter and along its length. An uneven radial temperature profile, in particular, could lead to additional peak broadening as a result of temperature’s effect on mobile-phase viscosity and solute retention. Reducing the column diameter to capillary dimensions largely eliminates this concern. Table 2 illustrates the power dissipated by flowing mobile phase through 25 cm long columns of different inner diameters at 2000 bar (30 000 psi), near the optimum flow velocity. The nearly three watts of power dissipated in the 4.6 mm diameter column would be problematic. Four orders of magnitude less heat, however, is produced in the 50 µm i.d. column as compared with the 4.6 mm column. Based upon experience with heat dissipation in electrophoresis capillaries, we know such low power dissipation will cause negligible temperature gradients, and thus will not be a problem. The low flow-rates, excellent

### Table 1: Pressure requirements and performance expected for differing stationary-phase particle diameters in a 25 cm long column. Values calculated for an analyte with k' = 2, \(D_m = 6.0 \times 10^{-6} \text{cm}^2/\text{s}\) and a mobile-phase viscosity (\(\eta\)) = 1.0 cP.

<table>
<thead>
<tr>
<th>(d_p) (µm)</th>
<th>∆P (psi)</th>
<th>Theoretical plates</th>
<th>Ret. time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>210</td>
<td>25 000</td>
<td>35</td>
</tr>
<tr>
<td>3.0</td>
<td>1000</td>
<td>42 000</td>
<td>21</td>
</tr>
<tr>
<td>1.5</td>
<td>8000</td>
<td>83 000</td>
<td>10.5</td>
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<tr>
<td>1.0</td>
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<td>7</td>
</tr>
<tr>
<td>0.75</td>
<td>62 000</td>
<td>166 000</td>
<td>5</td>
</tr>
</tbody>
</table>

\[ \Delta P = \frac{\Phi \eta L u}{d_p^2} \]  

where \(\Phi\) is the flow resistance factor, \(\eta\) is viscosity, \(L\) is column length and \(u\) is mobile-phase linear velocity. Because the optimum flow velocity is also inversely proportional to \(d_p\), the pressure required to run at the optimum velocity (the minimum of the \(H vs u\) curve) is inversely related to the cube of the particle diameter. Most conventional HPLC pumps have pressure limits of approximately 400 bar (6000 psi), which has restricted the use of small particles to short columns. A current trend in conventional HPLC separations is to use smaller particles (<4 µm) packed into shorter columns (<10 cm) to achieve significantly faster analysis times. However, this yields no improvement in overall column efficiency. Table 1 compares the expected performance and pressure requirements of 25 cm long columns, packed with particles ranging from 0.75 to 5 µm in size, operated at the optimum flow velocity for a small organic analyte. Reducing the particle diameter from 5 µm to 1 µm yields a fivefold increase in theoretical plates and a fivefold decrease in analysis time; however, the pressure

![Figure 1: Theoretical performance of columns packed with (a) 5 µm, (b) 3 µm and (c) 1 µm particles.](image-url)
heat dissipation and high tensile strength of small-diameter fused-silica capillary columns make them particularly suitable for use in UHPLC.

Figure 2(a) is a chromatogram obtained under isocratic conditions on a 43 cm long capillary column packed with 1.0 µm non-porous C18 particles (Eichrom Scientific). Five compounds: ascorbic acid (dead-time marker), hydroquinone, resorcinol, catechol and 4-methyl catechol, were eluted with a 10/90 (v/v) acetonitrile/water mobile phase containing 0.1% trifluoroacetic acid as an electrolyte, and detected with amperometric detection (+1.0 V vs Ag/AgCl). The chromatogram was obtained near the optimum linear velocity at a run pressure of 3000 bar (45 000 psi). All compounds eluted in less than 8 min, with efficiencies ranging from a low of 244 000 plates for 4-methyl catechol to as high as 330 000 plates for hydroquinone. The peaks are Gaussian and approximately 5 s wide. In Figure 2(b), at about 7000 bar (103 000 psi), the same separation occurred in less than 4 min, at only a small loss of efficiency (196 000 plates for 4-methyl catechol and 310 000 plates for hydroquinone).

Plate height vs flow velocity data for hydroquinone and catechol are plotted and fit (non-linear least squares fit) to the van Deemter equation in Figure 3. Runs were performed on a 30 µm i.d. × 37 cm long capillary packed with 1.0 µm non-porous C18 particles. The minimum plate height is approximately 2 µm for catechol and 1.6 µm for hydroquinone, which correspond to 500 000 and 625 000 plates/m, respectively. These values compare favourably to what is considered to be good performance for well-packed particles ($H_{min} \approx 2d_p$). As can be seen from the high-velocity side (C-term dominated side) of the curves, fast separations can occur while low plate heights (high efficiencies) are still maintained.

While isocratic analyses are useful for demonstrating the separation power of UHPLC using micron-sized particles, gradient elution must be used if this technique is to be applicable to samples containing analytes of wide-ranging polarity. For samples containing hundreds of detectable compounds, separations with large peak capacities are required.6–7,16 Gradient elution of a tryptic digest of the protein ovalbumin is shown in Figure 4. The sample was run using constant-flow pumps at 52 000 psi on a 38 cm long capillary packed with 1.0 µm C18 particles. The peptides from the digest were tagged with the fluorophore tetramethylrhodamine isothiocyanate (TRITC) and detected by laser-induced fluorescence. Because of the high flow-rates of the pumps, it was necessary to split the flow through a splitter capillary. The run is approximately 170 min long with a 0.28%/min gradient between acetonitrile and a 15/85 (v/v) acetonitrile/water mixture. As it is not valid to calculate theoretical plates under mobile-phase gradient conditions, peak capacity is used as an alternative measure of the separating power of the column.
power of a system. Peak capacity is defined as the total number of peaks separable with unit resolution in a given separation space. In the chromatogram shown, the peak capacity between 48 and 168 min is approximately 500, with an average peak width of 14.5 s. This is significantly higher than the peak capacities of conventional HPLC columns packed with 5 µm particles, which tend to be below 200 for similar samples.

Low flow-rates and narrow peak widths make capillary UHPLC particularly suitable for coupling with mass spectrometry via nanoelectrospray ionization. Wu and co-workers\(^9,11\) have conducted fast separations on short capillary columns packed with 1.5 µm particles using time-of-flight (TOF) mass spectrometric detection. Selected herbicides and combinatorial chemistry samples were separated in less than 100 s. Tolley and co-workers\(^7\) have used more moderate pressures of around 1000 bar (15 000 psi) to separate bovine serum albumin digests on columns packed with 1.5 µm reversed-phase particles by gradient elution, with quadrupole/time-of-flight (Q-TOF) tandem mass spectrometry for detection. A 20-fold enhancement in sensitivity over nanoelectrospray MS/MS was observed. Shen et al.\(^16\) developed a two-dimensional LC system with FTICR mass spectrometric detection for high-throughput proteomics studies. Commercial LC pumps operating at 10 000 psi were used with capillary columns packed with porous 1.5 µm C18 particles to analyse whole cell proteolytic digests.

Despite the engineering challenges associated with using ultra-high pressures, micron-sized particles have been shown to be a viable route towards increasing speed and resolution in reversed-phase liquid chromatography. Additional modes of liquid chromatography, such as ion exchange, normal phase and size exclusion, should be investigated under ultra-high pressure conditions. Development of micron-sized porous particles with a narrow size distribution is another important goal that needs to be realized. Most critical to the development of this field, however, is the introduction of reliable commercial equipment capable of operation under ultra-high pressure conditions. While 7000 bar (100 000 psi) is perhaps too ambitious a goal for commercial equipment in the immediate future, a commercial system capable of pressures of 2000 bar (30 000 psi) should be a reasonable target for the near term.

Such a system would permit the use of fairly long and efficient capillary columns packed with 1–2 µm particles, and would provide a very significant and overdue improvement in the separation power of LC columns.

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Anton D. Jerkovich earned his BA in Chemistry at Illinois Wesleyan University, and is in the fifth year of his PhD thesis research at the University of North Carolina.

J. Scott Mellors earned his BS in Chemistry at the University of Richmond and is currently in the third year of his PhD thesis research at the University of North Carolina.

James W. Jorgenson has been on the faculty of the Chemistry Department of the University of North Carolina for 24 years, and is currently William Rand Kenan Jr Distinguished Professor of