Watch

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

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Capillary columns packed with 1-µm particles can provide separations far superior to those of conventional high performance liquid chromatography (HPLC) columns and maintain normal HPLC analysis times.

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Column Watch Editor

During the past 20 years, researchers have investigated several approaches for improving the separation power of standard chromatography columns, which provide 20,000 theoretical plates. Packing conventional 3- and 5-µm particle diameter (d_p) packing materials into long fused-silica capillary columns is one means to generate theoretical plate numbers in the 100,000–300,000 plate range (1). A drawback of this approach is that run times can 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). Chromatographers hope that the optimization of this strategy will enable preparation of highly efficient columns with modest flow resistances. To date, however, separation efficiencies demonstrated for columns prepared in this manner are not extraordinary.

Reducing the particle diameter of the packing material in a liquid chromatography (LC) column is the time-proven method to achieve both increased separation power and faster analysis times. Consequently, using packings in the 1–2 µm size range is a logical approach to improving resolution. As we will demonstrate, the pressures required for pumping mobile phase through a relatively long column packed with such small particles can be prohibitive for standard high performance liquid chromatography (HPLC) hardware. Two strategies 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 10,000–50,000 V, is used to pump mobile phase through a column (3,4). This technique has come to be known as capillary electrochromatography (CEC). The principal drawback of this technique is that the chromatographic packing material must serve two roles. It must act as the stationary phase for chromatography and serve as the material that generates the electroosmotic flow (in other words, the pump). To serve as an efficient electroosmotic pump, the packing material must have considerable surface charge when it is in contact with the mobile phase. Because of this requirement the mobile phase must be relatively polar and contain water or polar organic solvents such as acetonitrile or methanol. This limitation precludes the use of CEC with most normal-phase chromatography applications, although reversed-phase chromatography is feasible. Most modern highly deactivated reversed-phase packing materials were developed specifically to reduce the presence of surface charge, so they 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, largely is an empirical matter, not a desirable characteristic of a pump.

A more straightforward way to achieve the required flow in long columns packed with micrometer-sized particles is to develop pumps, valves, and columns that can operate at much higher pressures than conventional HPLC systems (5–7). Although conventional HPLC technology is limited to pressures less than 400 bar (6000 psi), this new technology will be necessary to operate at pressures at least ten times higher. This technology is called *ultrahigh pressure liquid chromatography* (ultra-HPLC).

The efficiency of a chromatography column can be described by the height equivalent of a theoretical plate (H) in which the lower the plate height, the more efficient a

column. Plate height is significantly reduced by the use of small particles. The van Deemter equation describes the relationship of H and mobile-phase flow velocity (u)

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

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 generally is found to be proportional to the stationary-phase particle diameter (d_p), and the C term is proportional to d_p^2. In addition, the optimum mobile-phase velocity increases with decreasing particle diameter, which enables faster separations.

Figure 1 illustrates this effect using van Deemter plots. The figure shows the theoretical performances of columns packed with 1-, 3-, and 5-µm d_p media. Not only is the minimum plate height (H_{min}) reduced significantly using smaller particles, but the slope of the high-velocity side of the curve (dominated by the C term) also decreases dramatically, which enables operation at higher flow rates without sacrificing efficiency.

The use of micrometer-sized particles, however, requires increased pumping pressure. The pressure drop (ΔP) in a column is inversely proportional to the square of the particle diameter and is given by

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

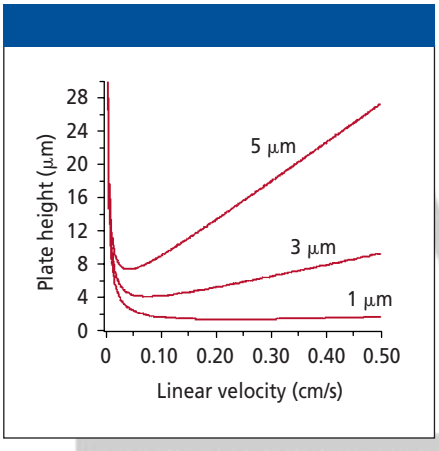

Figure 1: Theoretical performance of columns packed with 5-µm, 3-µm, and 1-µm particles.

where Φ is the flow resistance factor, η is mobile-phase viscosity, L is column length, and u is mobile-phase linear velocity. Because the optimum flow velocity also is inversely proportional to d_p, the pressure required to operate at the optimum velocity (the minimum of the H versus 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 restricts the use of small particles to short columns. A current trend in conventional HPLC separations is to use smaller particles ($d_p < 4$ µm) packed into shorter columns ($L < 10$ cm) to achieve significantly faster analysis times (8). However, this approach yields no improvement in overall column efficiency.

Table I compares the expected performance and pressure requirements of 25-cm-long columns packed with particles ranging in diameter from 0.75 µm to 5 µm and operated at the optimum flow velocity for a small organic analyte. Reducing the particle diameter from 5 µm to 1 µm yielded a fivefold increase in theoretical plates and a fivefold decrease in analysis time; however, the pressure requirements increased 125-fold.

To take full advantage of the performance of small particles, analysts must overcome these pressure limitations. A few research laboratories have developed pumping systems that can generate pressures as great as 3500 bar (50,000 psi) or more (5–7,9–16). We have prepared columns longer than 50 cm in our laboratory with 1-µm d_p packing materials to yield more than 350,000 theoretical plates.

Commercial packing materials smaller than 2-µm d_p are fairly scarce. Some commercial sources include Alltech Associates, Inc. (Deerfield, Illinois, USA); Chemie Uetikon AG (Uetikon, Switzerland); Eichrom Technologies, Inc. (Darien, Illinois, USA); and ZirChrom Separations, Inc. (Anoka, Minnesota, USA). Some research laboratories have synthesized and bonded their own materials. Cintrón and Colón (15) described a one-step process to synthesize uniform organosilica spherical particles for use in ultra-HPLC.

Several concerns arise when using such extreme mobile-phase pressures. The engineering challenges involved with sealing and operating an LC system at ultrahigh pressures have required in-house construction of column fittings and injectors (6,7,9,10). The columns also are individually packed in-house by a slurry-packing method. The safety of using this type of system is a concern. Seals, valves, and other parts must be able to withstand the extreme pressures. If the column fitting fails, it could cause the rapid ejection of the column. With careful design and normal laboratory safety precautions, however, ultra-HPLC can be safe to use. Analysts must carefully consider the pressure limitations of materials, tubing, and other instrument components (17).

Studies of the effects of conventional HPLC–type pressures upon analyte retention in reversed-phase HPLC found that the retention factor (k) increases with pressure (18,19). By extrapolating these studies to much higher pressures, we can speculate that the increase in retention would be so great as to place an upper limit on the pressure feasible for LC. Experimental results in ultra-HPLC so far have 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 (ΔP)

$$\text{power} = F\Delta P \qquad [3]$$

This frictional heating of the mobile phase will occur and, under ultra-HPLC conditions, could cause the temperature inside the column to rise significantly. This

Table I: Pressure requirements and performance expected for differing stationary-phase particle diameters in a 25-cm-long column*

d_p (µm)	ΔP (psi)	Theoretical Plates	Retention Time (min)
5.0	210	25,000	35
3.0	1000	42,000	21
1.5	8000	83,000	10.5
1.0	26,000	125,000	7
0.75	62,000	166,000	5

*Values calculated for an analyte with $k = 2$, $D_m = 6.0 \times 10^{-6}$ cm^2/s, and $\eta = 1.0$ cP.

temperature's effect on mobile-phase viscosity (η) and solute retention. Reducing the column diameter to capillary dimensions largely eliminates this concern.

Table II illustrates the power dissipated by flowing mobile phase through 25-cm-long columns of different inner diameters at 2000 bar (30,000 psi), which is near the optimum flow velocity. The nearly 3 W of power dissipated in the 4.6-mm column would be problematic. Four orders of magnitude less heat, however, is produced in the 50-µm column, as compared with the 4.6-mm column. Based upon experience with heat dissipation in electrophoresis capillaries, we know that this low power dissipation will cause negligible temperature gradients and thus will be no problem. The low flow rates, excellent heat dissipation, and high tensile strength of small-diameter fused-silica capillary columns make them particularly well suited for use in ultra-HPLC.

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

Figure 3 shows plate height versus flow velocity data for hydroquinone and catechol that have been plotted and fit (nonlinear least squares fit) to the van Deemter equation. Runs were performed on a 37 cm × 30 µm capillary packed with 1.0-µm nonporous C18 particles. The minimum plate height was 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 favorably 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, chromatographers can obtain fast separations with low plate heights (high efficiencies).

Although isocratic analyses are useful for demonstrating the separation power of ultra-HPLC using micrometer-sized particles, gradient elution must be used if this technique is to be applicable to samples that contain analytes of wide-ranging polarity. For samples with hundreds of detectable compounds, separations with large peak capacities are necessary (6,7,16).

Figure 4 shows a gradient elution of a tryptic digest of the protein ovalbumin. The sample was analyzed using constant-flow pumps at 3600 bar (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, we needed to split the flow through a splitter capillary. The run was approximately 170 min with a 0.28%/min gradient between acetonitrile and a 15:85 (v/v) acetonitrile–water mixture. Because it is invalid to calculate theoretical plates under mobile-phase gradient conditions, we used peak capacity as an alternative measure of the separating power of a system. Peak capacity is defined as the total number of peaks separable with unit resolution in a given separation space. In Figure 4, the peak capacity between 48 and 168 min is approximately 500, with an average peak width of 14.5 s. This peak capacity is significantly higher than the peak capacities of conventional HPLC columns packed with 5-µm particles, which tend to be less than 200 for similar samples.

Low flow rates and narrow peak widths make capillary ultra-HPLC particularly suitable for coupling with mass spectrometry (MS) via nanoelectrospray ionization. Wu and co-workers (9,11) conducted fast separations on short capillary columns packed with 1.5-µm particles using time-of-flight (TOF) MS detection. The selected herbicides and combinatorial chemistry

Table II: Flow rates and power generated for various column inner diameters*

Column i.d.	Flow Rate	Power Generated
4.6 mm	820 µL/min	2.8 W
1.0 mm	38 µL/min	0.13 W
300 µm	3.5 µL/min	12 mW
100 µm	380 nL/min	1.3 mW
50 µm	96 nL/min	0.33 mW

*Values calculated for 25-cm-long columns packed with 1.0-µm particles, operated at 2000 bar (30,000 psi), near the optimum linear velocity for small molecules.

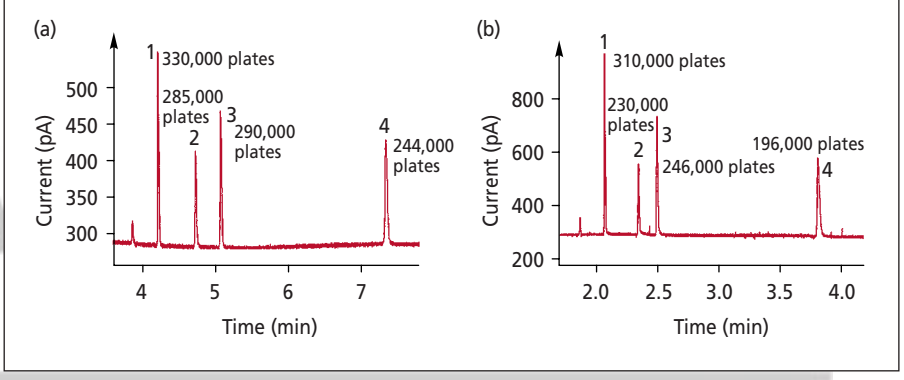

Figure 2: Chromatograms obtained on a column packed with 1.0-µm particles at run pressures of (a) 45,000 psi and (b) 103,000 psi. See text for separation conditions. Peaks: 1 = hydroquinone, 2 = resorcinol, 3 = catechol, 4 = 4-methylcatechol.

samples were separated in less than 100 s using pressures as great as 2700 bar (40,000 psi). Tolley and colleagues (7) used more-moderate pressures of approximately 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 TOF MS–MS for detection. They observed a 20-fold enhancement in sensitivity compared with nanoelectrospray MS–MS. Shen and co-workers (16) developed a two-dimensional LC system with Fourier transform ion cyclotron resonance MS detection for high-throughput proteomics studies. They used commercial LC pumps operating at 10,000 psi with capillary columns packed with porous 1.5-µm C18 particles to analyze whole-cell proteolytic digests.

Despite the engineering challenges associated with using ultrahigh pressures, micrometer-sized particles have proved to be a viable route toward increasing speed and resolution in reversed-phase LC. Researchers should investigate additional modes of LC — such as ion-exchange, normal-phase, and size-exclusion chromatography — under ultrahigh pressure conditions. The development of micrometer-sized porous particles with a narrow size distribution is another important goal that needs to be realized. Most important to the development of this field, however, is the introduction of reliable commercial equipment that can operate under ultrahigh pressure conditions. Although 7000 bar (100,000 psi) is perhaps too ambitious a goal for commercial equipment in the immediate future, a commercial system capable of withstanding pressures of 2000 bar (30,000 psi) should be a reasonable target in the near future. This type of 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.

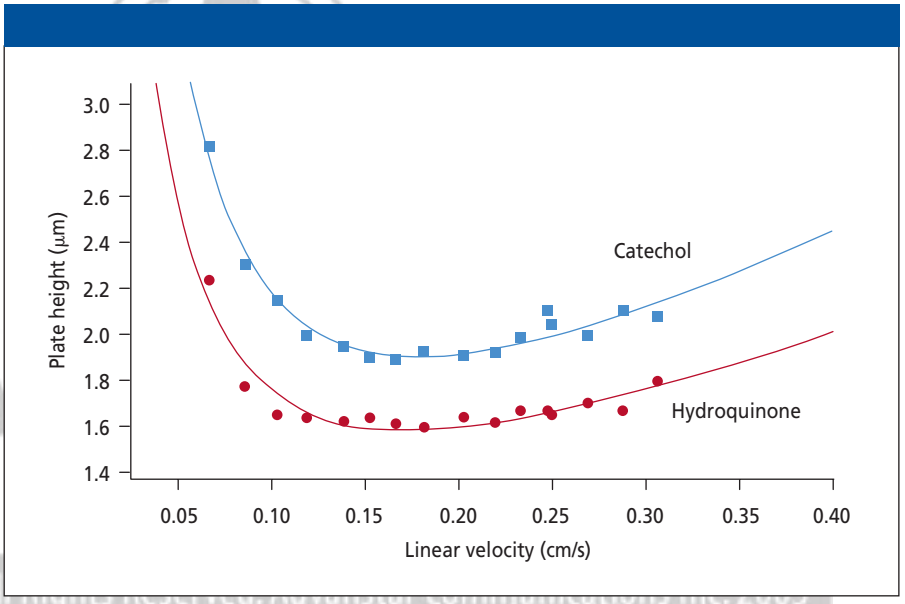

Figure 3: Van Deemter curves for hydroquinone and catechol on a 37 cm × 30 µm, 1.0-µm d_p column.

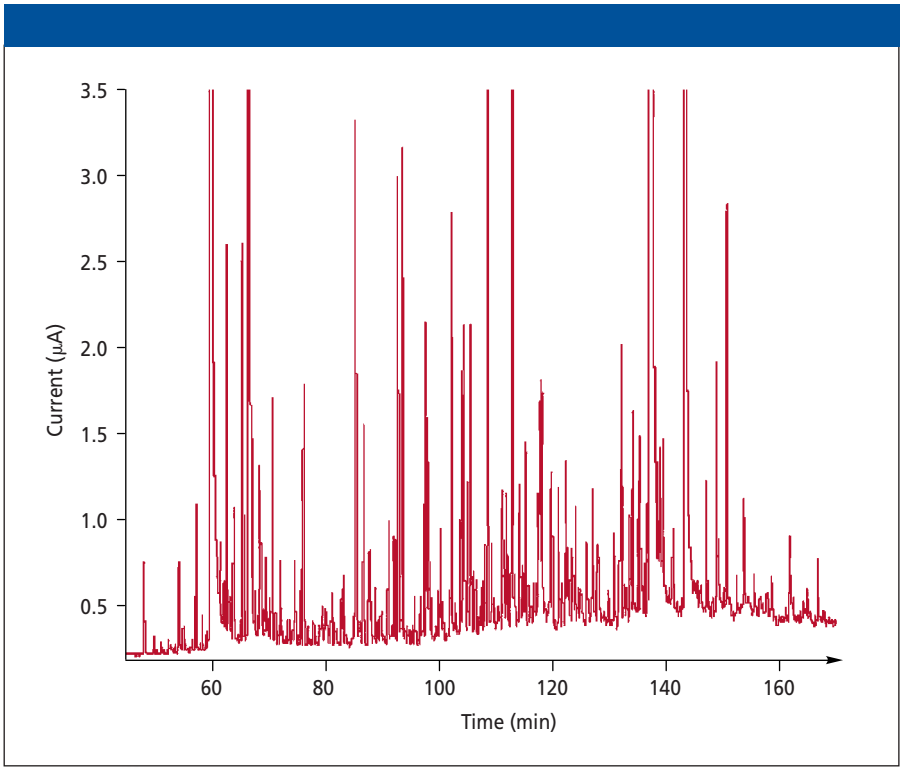

Figure 4: Ultra-HPLC gradient separation of a tryptic digest of bovine serum albumin. A peak capacity of 500 was obtained between 48 and 168 min.

Acknowledgments

The authors gratefully wish to acknowledge the Waters Corp. (Milford, Massachusetts, USA) and grant number GM-39515 from the National Institutes of Health (Bethesda, Maryland, USA) for their generous financial support for this work. We also thank Bruce Compton, Ed Bouvier, Geoff Gerhardt, and Keith Fadgen of Waters for invaluable conversations, help, and input throughout the years. Finally, we wish to acknowledge the work of John MacNair, Kamlesh Patel, and Ken Lewis, former members of our research group, whose dedicated work made this report possible.

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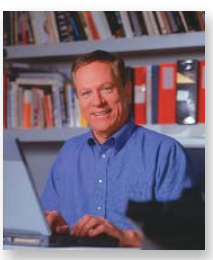

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