Ultra HPLC System Configuration

TELEDYNE ISCO
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Syringe Pump Application Note AN4

Introduction

Ultra-HPLC uses the same separation methodology as conventional HPLC, but typically uses columns packed with particles smaller than 2 µm. These smaller particles dramatically increase column efficiency, which in turn increases mass sensitivity, analytical resolution, and speed. These attributes are important for such applications as proteomics and pharmaceutical samples.[1]

While instrumentation for Ultra-HPLC requires components similar to conventional HPLC, smaller column particle size results in higher system pressures and requires components, such as pumps, with high pressure ratings. In addition, the ideal pump should provide flow rates that are pulseless and stable. Teledyne ISCO pumps are excellent Ultra-HPLC pumps.

Theory & Practice

The key to understanding the importance of using smaller column packing materials follows from the van Deemter equation, which is fundamental to chromatographic theory. According to the van Deemter equation:

$$H = A + B/\mu + C\mu$$

where: H is the column's plate height, and µ is the mobile phase linear flow rate. A, B, and C are constants. A smaller plate height H indicates higher separation resolution.[2]

The constant A is the eddy diffusion term. Eddy diffusion results from multiple flow paths in the column and is independent of mobile phase flow rate. Due to the packing particles, analyte molecules can follow multiple pathways of differing path lengths. These multiple pathways of differing length spread the analyte molecules apart and cause peak broadening. Voids in the stationary phase can further contribute to peak broadening due to channeling. In contrast, smaller packing particles offer smaller differences in path length, thus reducing peak broadening. The A term depends on the compactness of the stationary phase.

The constant B is the longitudinal diffusion coefficient. It is related to the diffusion coefficient of the analyte molecules in the mobile phase. Shorter resident time of the analyte molecules in the column reduces peak broadening due to longitudinal diffusion. Faster mobile phase flow rates reduce resident time. This reduction contributes to better separation efficiencies, since the analyte molecules have less opportunity to diffuse, thus explaining the 1/µ factor in this term.

The constant C is the analyte mass transfer coefficient. It is related to the time needed for the analyte molecules to equilibrate between the mobile and stationary phases. If this equilibration is too slow, then some of the analyte molecules, which did not have enough time to bond to the stationary phase, will flow down the column with the mobile phase; whereas, the other molecules, which did not have enough time to detach from the stationary phase, are left behind. Higher mobile phase flow rates will contribute to the spreading of the analyte molecules, thus explaining the µ factor in this term. Smaller stationary phase particles are expected to reduce this equilibration time.

It follows that smaller stationary phase particles should contribute to smaller A and C values. The A term will contribute less to H and allow for higher resolution. As the C term becomes less significant to the value of H, an increase in mobile phase flow rates will not sacrifice separation performance as much. This would allow for faster separations with the same resolution. Even though smaller column particles may not directly affect the B value, the higher flow rates reduce this contribution to H. Smaller column packing material would allow for faster separations with higher resolution.[3]

Smaller beads will pack with smaller interstitial spaces and offer higher resistance to solvent flow. In turn, this requires higher driving pressures. For optimal separation flow rates, column back pressure increases as an inverse cubic function of the stationary phase particle size, i.e. P a 1/d.[3] Typically, pressures of 20,000 psi and above are required, hence the name Ultra-HPLC, or UHPLC for short.[1,4]

These smaller stationary phase particles can be packed in either capillary columns or in stainless steel columns. Capillary columns offer less solvent consumption and can assay tiny amounts of sample. Due to very low mobile phase consumption, capillary columns interface more naturally with mass spectrometer detectors. With the higher flow rates and pressure drops across the columns, Joule heating can occur in the column during an UHPLC analysis. The power of the generated heat is the product of the pressure drop and volumetric flow rate. Capillary columns are better for dissipating such heat due to their greater surface area to volume aspect ratio and lower volumetric flow rate (µL/min) for a given linear flow rate (mm/sec). As a result of heating, stainless steel column types tend to be limited to microbore (< 2mm diameter bore).[4] Stainless steel columns offer the advantage of assaying larger sample sizes due to higher carbon load, which in turn aids in detecting and quantifying minor components in a mixture. This can be a major advantage in analyzing pharmaceutical samples.[4] The choice of capillary or stainless steel columns gives UHPLC added flexibility.

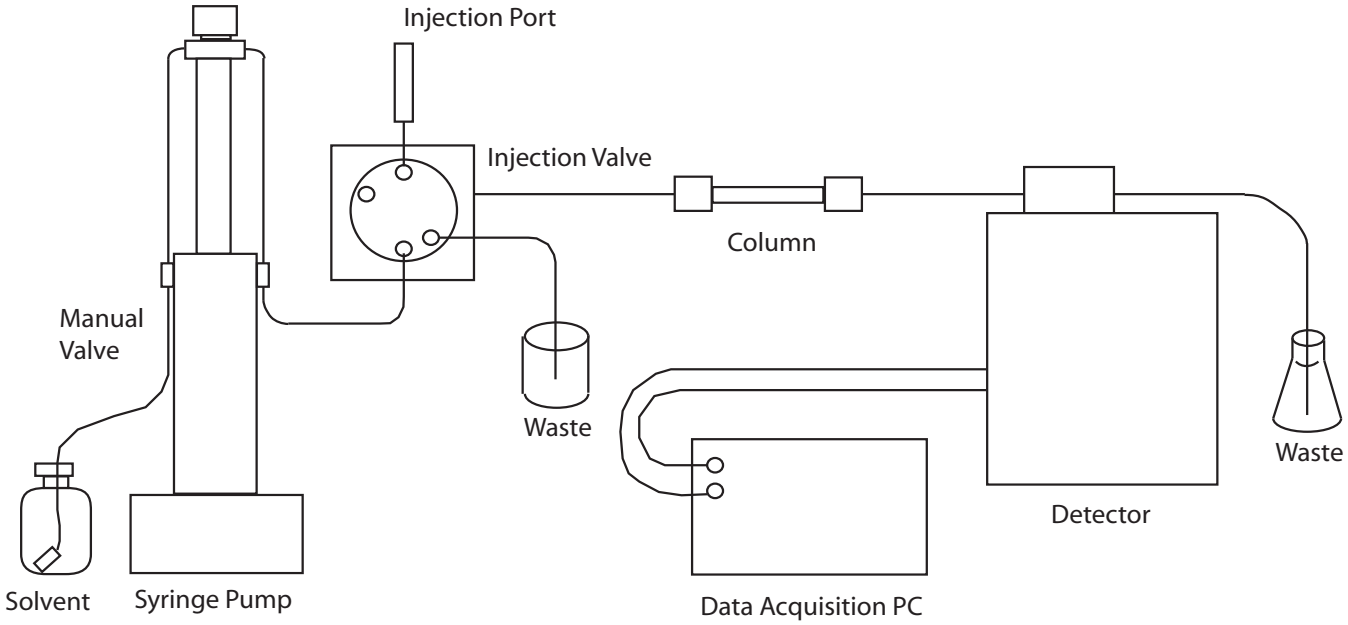

Figure 1: System Configuration

Table 1: System Components

Item	Component	Details	Recommended Vendor	Part Number
1	65x Pump/Controller [see note]	Rated at 1,360 Bar (20K psi)	Teledyne ISCO	68-1240-850
2	Refill Valve Package	Rated at 1,360 Bar (20K psi)	Teledyne ISCO	60-1267-023
3	Check Valve	Rated at 1,360 Bar (20K psi)	HIP	20-41LF4
4	1/4" F250 to 1/8" Reducer	Rated at 751 Bar (10.9K psi)	Swageloc	SS-44M -A-200
5	1/8" Teflon Tubing	Specify length - low pressure	Teledyne ISCO	023-0504-02
6	10 micron filter		Teledyne ISCO	209-9012-10
7	Outlet Valve Package	Rated at 1,360 Bar (20K psi)	Teledyne ISCO	60-1267-023
8	1/4" - F250 to 1/32" Reducer	Rated at 1,360 Bar (20K psi)	Valco	ZA.5UHP
9	1/4" - F250 Nut	Combine with reducer	Autoclave Engineers	AGL40
10	1/4" - F250 Ferrule (not shown)	Combine with reducer	Autoclave Engineers	ACL40
11	1/32" SST Tubing	Rated at 1,360 Bar (20K psi)	Valco	T100N5D
12	1/32" Male Nut (not shown)	Use as replacement or extras	Valco	ZN.5H
13	1/32" Ferrule (not shown)	Use as replacement or extras	Valco	RF.5 -.83S6
14	Injection Valve - 10 nanoliter loop	Rated at 1,360 Bar (20K psi)	Valco	C4NX-4904-01EH
15	Column - 1.5 micron		TBD	
16	Absorbance Detector		TBD	
17	Data Acquisition PC		TBD	

Experimental Method & Results

To demonstrate the advantage of smaller stationary phase material, the van Deemter equations for three columns packed with different particle sizes are determined. The columns have the dimensions of 1x150mm and are packed with RP-derivatized silica beads. Two columns are purchased from Alltech Associates (Deerfield, IL). One column contains 5µm Alltima C18-silica beads; whereas, the other contains 3µm Alltima C18-silica beads. The third column is packed with 1.5µm Kovasil C14-silica beads, purchased from Zeochem AG (Uetikon, Switzerland). This column is packed under 30,000 psi pressure. For the mobile phase, a 58:42% Acetonitrile in water solvent mix is prepared and degassed. A standard mix of Uracil, Benzyl Alcohol, Benzene, Toluene, and Ethyl Benzene is prepared in Acetonitrile.

Using the system described above, the standard mix is separated for each column isocratically for different volumetric flow rates. The Teledyne ISCO (Lincoln, NE) 65D syringe pump [see note] delivers the isocratic 58:42 Acetonitrile in water mobile phase with volumetric precision. The standard mix is injected onto the column using a Valco (Houston, TX) Ultrahigh pressure injector with a 10nL internal sample loop. After the column, the elution is delivered to a Teledyne ISCO CV4 UV-Visible capillary absorbance detector by a 375µm OD x 200µm ID fused-silica capillary from Polymicro (Phoenix, AZ). The detector is tuned to 215nm for maximum sensitivity. The analog signal is digitized using a Digital Multimeter with RS-232 interface. A personal computer is used to store and analyze the data.

The Uracil is used as a void marker to measure the linear flow rate µ of the mobile phase and the retention void of the column. The effective plate number of each column for the other analytes is calculated using the formula:

$$N = 5.546 \, (t_r / w_{1/2})^2$$

where: N is the effective plate number, t_r is the retention time subtracted by the retention void (as determined by the Uracil peak), and $w_{1/2}$ is the full-width half-maximum of each of the other analyte peaks.[2,5,6] The plate height H is calculated as L / N, where L is the length of the column (in this case, L=150mm). A program is written in C++ to find each peak and perform the above calculations. Using SigmaPlot, the van Deemter data points H vs. µ are plotted. Also using SigmaPlot, the van Deemter coefficients A, B, and C are determined from least squares regression by fitting a parabola to a plot of (µH) vs. µ since: $\mu H = \mu(A + B/\mu + C\mu) = A\mu v + B + C\mu^2$.

The van Deemter equations for each of the columns are plotted in Figures 2 and 3. Figure 2 includes the fitted data points; Figure 3 shows a closer view of the curves. The van Deemter curve for the Alltech Alltima C18 5µm column has a minimum (i.e. highest chromatographic resolution) near a mobile phase linear flow rate of 1.4 mm/sec. The minimum for the Alltech C18 3µm column occurs near 1.6 mm/sec. During each run, the syringe pump pressure is recorded in order to estimate the column back pressure for the different flow rates. These pressure data are plotted in Figure 4. A sample chromatogram is shown in Figure 5.

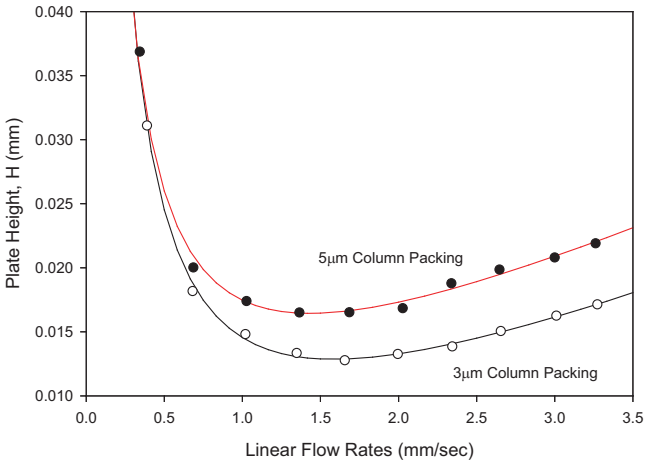

Figure 2: van Deemter Plots (for columns containing 5, 3, and 1.5 µm C18 packing material)

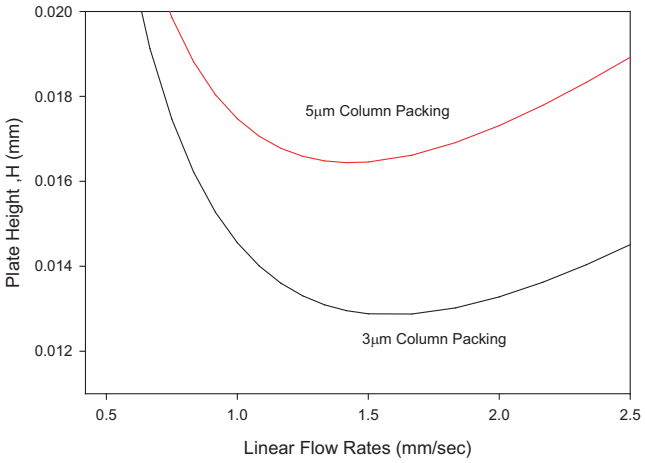

Figure 3: Close-up View of van Deemter Plots (minus data points)

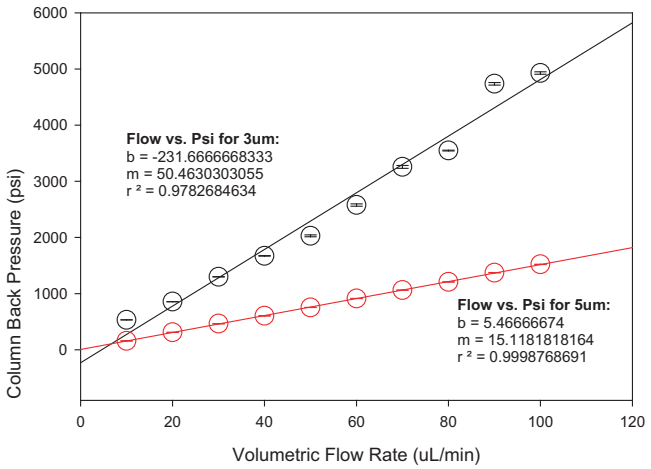

Figure 4: Column Back Pressures

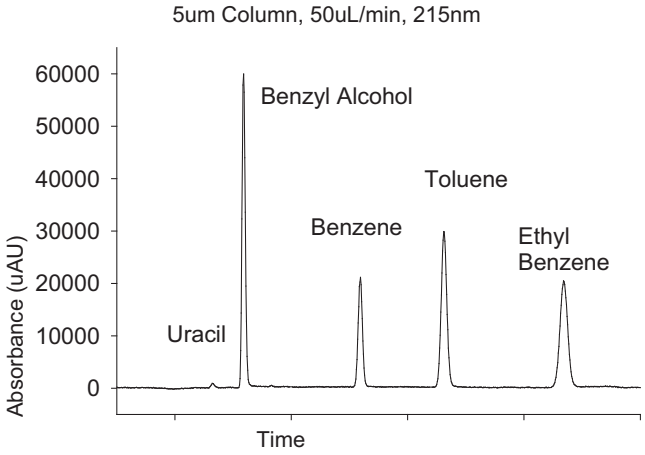

Figure 5: Chromatogram

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Note:

The 65D model pump, which were used during the original experiment, is discontinued. Current model 65x is the recommended replacement for the older 65D model.

September 28, 2012; revised November 6, 2023
Product model names have been updated in this document to reflect current pump offerings.

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