

An Introduction to Capillary HPLC

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Decreasing the size of packed HPLC columns into the capillary range, where the column ID is between 100–500 μm and flow rates are typically 0.4–100 $\mu\text{L}/\text{min}$, brings advantages to the separation scientist by improving the speed and mass sensitivity of a separation.

Capillary HPLC has been used to separate biomolecules with mass spectrometer detection for years, primarily because the low flow rates inherent in the technique offer improved sensitivity with nanospray sources. Because these sources must vaporize the solvent, their ionization efficiency goes down as the flow rate becomes too high. With capillary columns a higher percentage of the molecules are successfully ionized.

Historically HPLC instrumentation limited capillary column performance and prevented wider adoption of the technique. Instruments which did not minimize the delay volumes before the injector and the extra column volumes after it, gave poor resolution and long separations. Recent improvements however, have allowed the advantages inherent in capillary HPLC to be tapped for a wider range of separations. This report will discuss some of these advantages and the instrumentation required to realize them.

Greater Mass Sensitivity

The improvement in mass sensitivity one achieves with capillary HPLC is perhaps the most intuitive advantage of the technique, and historically one of the most important in the field of proteomics research. A researcher faces the challenge of extracting the maximum amount of information from a protein sample but might have only a few 100 attomoles available. Using a 100 μm ID column this can still be readily characterized.

In situations where sample mass is limiting this advantage is key. Concentration sensitive detection, such as UV absorption, produces signals linear in the concentration of the eluted analyte. The best sensitivity for a fixed mass is achieved when that mass is diluted in the minimum volume. Since in general this volume scales as the square of the inner diameter of the column, a 300 μm capillary format offers 235 times more sensitivity than a column with an ID of 4.6 mm, for the same size injection.

Decreased Analysis Time

Three aspects of capillary LC combine to offer the separation scientist a route to faster analyses: faster column equilibration, smaller solvent volumes involved, and lower backpressures.

Helpful Hints and Practical Advice for HPLC

To get good retention time reproducibility with a gradient method on a conventional 4.6 mm system, a separation scientist often uses more than 10 column volumes of the initial mobile phase to reequilibrate the system after the gradient run. Many times this reequilibration time is on the order of the separation time itself. These large reequilibration times become much smaller in capillary HPLC as many methods need fewer than 2 column volumes to satisfactorily reequilibrate. The advantage here is the much smaller delay volumes seen in modern capillary HPLC instrumentation.

As can be seen in Table 1, the delay volume in a conventional system often exceeds the volume of the column itself. This is not the case for a capillary system with passive flow mixing like the Eksigent Technologies ExpressLC 100 where pre-column components are kept to a minimum — leading to much faster system reequilibration with gradient chromatography.

Table 1. System delay volumes

| Vendor | diameter, mm | delay, μL | $V_{\text{delay}}/V_{\text{column}}$ |
|------------------------|--------------|----------------------|--------------------------------------|
| Eksigent ExpressLC 100 | 0.3 | 0.35 | 0.1 |
| Conventional System A | 2.1 | 560 | 3.2 |
| High Pressure System B | 2.1 | 150 | 0.9 |
| High Pressure System B | 1 | 150 | 3.8 |

Using a 0.3×50 mm capillary column for a small molecule separation might take 5 minutes at 10 $\mu\text{L}/\text{min}$ (equivalent to 2.35 mL/min with a 4.6 mm column) and use a total of 60 μL of solvent with a pre and post wash. At these volumes instrument vendors can design more efficient mixing schemes than those used in conventional systems. To achieve very fast gradients in a conventional system that uses reciprocating pumps and pulse dampers, active mixers are employed. Again these components all add delay volume to the instrument which acts to slow the separation.

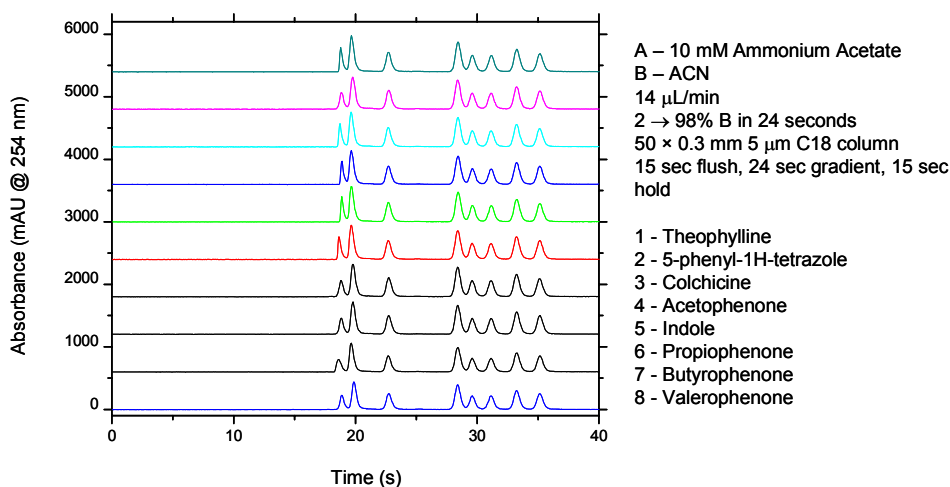


Figure 1. 10 separations in 10 minutes with the Eksigent ExpressLC 100

The ExpressLC 100 has 9–38 times less relative delay than do popular conventional instruments. With this system gradients which change solvent composition from 2%→100% in less than 30 seconds are not unusual — a sharpness which can be tuned for a particular mixture to decrease its sep-

aration time. As shown in Figure 1, such a scheme offers excellent separation for an eight component mixture. Here 10 separations are completed within 10 minutes using a 24 second gradient.

The back pressure a column develops depends on the interstitial porosity of the packed bed, i.e. the space between the particles. When the ratio of the column ID to the particle size decreases the interstitial porosity of the column goes up, and per the Kozeny-Carman equation, the back pressure goes down sharply (for a description of the Kozeny-Carman equation see [HPLC Columns](#) by Uwe Neue). This can be seen in Figure 2 where the expected back pressure (Δp with a 0.3×50 mm column, 3 μm particles, 9 $\mu\text{L}/\text{min}$, 30/70 water/ACN at 30°) is plotted against interstitial porosity (ϵ_i).

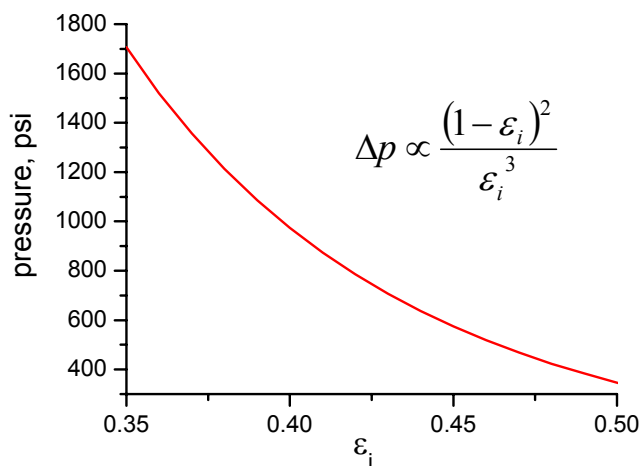


Figure 2. Pressure as a function of porosity

Experimental results for C18 columns (Table 2), obtained by analyzing the pressures on four vendors' data sheets, have shown that for 300 μm ID columns with 3 μm particles run near 40/60 water/ACN (v/v), one sees a porosity average between 0.43 and 0.44. This compares with a typical porosity in a 4.6 mm column with 3 μm particles of 0.40. This difference corresponds to up to 50% less back pressure for comparable linear velocities. That is for a given maximum pressure on an HPLC system, separations can be 50% faster with a capillary column.

Table 2. Interstitial porosities for capillary columns from different vendors

| Vendor | ID, μm | particle size, μm | ϵ_i |
|--------|-------------------|------------------------------|--------------|
| A | 300 | 3 | 0.44 |
| B | 300 | 3 | 0.42 |
| C | 300 | 3 | 0.45 |
| D | 300 | 3 | 0.43 |

Instrument Requirements

The advantages from capillary HPLC however come at a price. A typical HPLC cannot use capillary columns as is, since care must be taken to accurately deliver solvents at these low flow rates and every effort made to minimize extra column volume in the entire sample path.

Extra column volume in any system causes a 'plug' injected into the instrument to broaden due to diffusion. This broadening shows up at the detector as wider peaks. Generally the total dispersion introduced by the several elements of an HPLC system is represented by a variance (σ^2). The total variance of a system then is the sum of the variances from the several stages: column, injector, tubing, fittings, and detector.

$$\sigma^2 = \sigma_{\text{col}}^2 + \sigma_{\text{inj}}^2 + \sigma_{\text{tube}}^2 + \sigma_{\text{fittings}}^2 + \sigma_{\text{det}}^2$$

The limiting component to the total variance in any well designed system should be the column. In fact the sum of all extra-column variances, as a general design rule, should be less than 10% of the column's variance. When this rule is followed the efficiency of a peak is not a function of the peak's retention factor (typically k or k'). Figure 3 shows this for the Eksigent ExpressLC 100 where the number of theoretical plates changes very little (127,000 plates/m \rightarrow 130,000 plates/m) while the retention factor varies from 1.1 (acetophenone) \rightarrow 5.0 (heptanophenone).

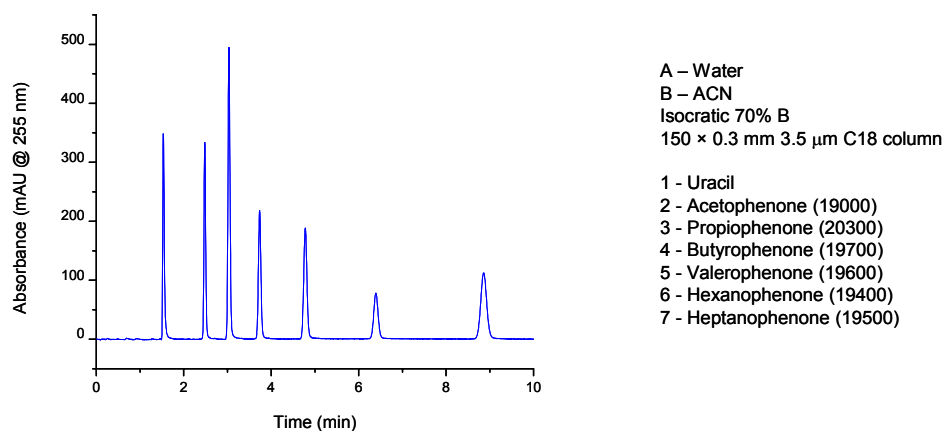


Figure 3. Efficiency as a function of retention factor

The ExpressLC 100 achieves this by judicious choice of tubing and injector together with a custom 45 nL, micro-fabricated detection cell having a 4 mm absorption pathlength. As can be seen from Figure 4, this system gives excellent performance using a 0.3 × 150 mm column with 3.5 μm particles. Here the van Deemter minimum corresponds to a reduced plate height of nearly 2.

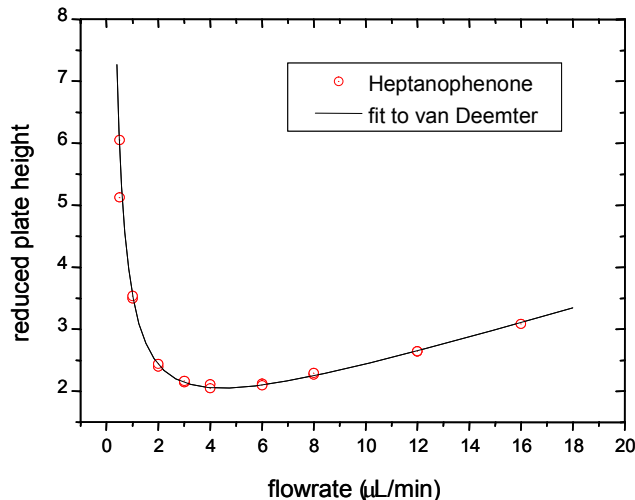


Figure 4. Eksigent ExpressLC-100 with a 300 μm capillary column

With the instrumentation available today, capillary HPLC now offers resolution equal to or better than that of a conventional 4.6 mm system. Several groups have reported particular improvements to the van Deemter C-term when using capillary HPLC, which becomes very important with fast separations. These advantages combine to offer the opportunity for faster separations with greater resolution and mass sensitivity.



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