Fast HPLC
Theory and Practice of Fast HPLC

Aims and Objectives

Aims

• Introduce a variety of methods to speed up HPLC separations
• Explain fundamental approaches of speeding up HPLC
• Investigate the balance between speed and resolution in HPLC separations
• Introduce a variety of methods to achieve faster HPLC separations
• Highlight advantages and limitations of the various approaches to speeding up separations

Objectives

At the end of this Section you should be able to:

• Understand the benefits and limitations of the various approaches to speeding up HPLC separations and chose between them as appropriate
• Recognise some of the most important parameters which can be manipulated to reduce HPLC analysis time and the manner in which these might be used
• Demonstrate an awareness of the limitations of each approach and appreciate the relative advantages and disadvantages

Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.
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Introduction

‘Fast HPLC’ has come to refer to the technique of decreasing analysis time using a variety of novel column and instrument technologies. Speed increases are usually measured using ‘Peak Capacity’ - the number of compounds separated with satisfactory resolution per unit time. For example a separation of ten compounds in five minutes is more efficient (‘faster’) than a separation of five compounds in the same timescale.

In many modern analytical laboratories, there is a growing demand for faster results without compromising the quality of the separation. Similarly, the technology used to produced fast HPLC separations can also be used to significantly increase chromatographic resolution within the original timeframe – an additional benefit which is of interest to every laboratory!

Several approaches have commonly been used for fast chromatography. Short and/or reduced internal diameter columns, increased column temperature and high flow rates can all be used to decrease analysis time. However, the current advances have been lead by various improvements in column packing materials, including the advent of reliable and stable sub 2µm packings, superficially porous materials as well as monolithic columns.

HPLC versus fast HPLC analysis of ceftizoxime (most intense peak) sodium

**Mobile Phase:** A:10%citric acid/ sodium phosphate buffer (pH=3.6). B: acetonitrile.

**Flow rate:** 2.0 mL/min

**Fast HPLC Column:** C18, 50mm×4.6 mm, 1.8µm.

**HPLC Column:** C18, 250mm×4.6 mm, 5.0µm.
The instrumentation used in fast HPLC typically has to cope with higher pressures and some fast HPLC systems are designed to work at pressures exceeding 1000bar, which is significantly higher than traditional HPLC systems which typically operate below 400bar[1,2,3,4].

This module covers the most important factors to consider when speeding up HPLC separations.

**Separation Efficiency and Particle Size**

The efficiency of HPLC separations is heavily influenced by the particle size of the packing material and by the column length[2,3,4].

Performing HPLC separations in minimum time whilst maintaining high separation efficiency and good resolution are the main goals for the analytical scientist and this topic is dedicated to outlining how this is achieved in the modern HPLC laboratory.


In the application, baseline resolution can be maintained while analysis time is reduced by 80%. As particle size is reduced, the column length can also be reduced without compromising the plate numbers (efficiency) generated by the column. The analysis time can be shortened considerably even using the same flow rate for applications.
Fully porous particles in the 3-10 µm range form the basis for most of the HPLC columns currently available. However, when the packing material particle size is reduced (usually to 2.0 µm or below) then the column may show an appreciable increase in efficiency such that faster analyses become possible without compromising the resolution within the separation.

These so called ‘Sub 2µm’ and related ‘Fast HPLC’ particle types are also capable of delivering improved resolution over traditional particles when used under the same flow rate conditions as the initial separation due to their increased inherent efficiency. This can be highly advantageous when separating multiple components or when very high peak capacities are required as exemplified in the separation of protein digests.
**Back Pressure and Particle Size**

The efficiency of the separation is heavily influenced by the particle size of the packing material and by the column length.\(^{[2,3,4]}\)

Partially porous packing materials allow analyte molecules to diffuse through small areas (which depends upon the thickness of the porous layer).

In the example presented below, the same flow rate is used for each separation, however as the particle size reduces, the column length may be similarly reduced to reduce the analysis time. It should be noted that the selectivity and resolution of the separation remain approximately the same.

The back pressure generated by the system rises from 90 bar, to 180 bar and finally 260 bar, which can be achieved by most standard HPLC systems.

HPLC analysis of molecules typically used in sun block products by using columns (of the same dimensions) packed with three different sizes of the same partially porous packing material. Mobile Phase: 1.0 mL/min 85% Methanol in water. Sample: 1. 2-hydroxy-4-metoxy-benzophenone; 2. Padimate; 3. Octyl Methoxycinnamate; 4. 2-ethylhexylsalicylate
Superficially Porous Materials

Superficially porous silica particles are capable of delivering very high plate numbers whilst not suffering from the same magnitude in back pressure increase. These particles are partially porous, with a typical particle size between 2.5 and 3.00 μm. As can be seen above, this short, wide bore column can achieve a 10 component separation in under 1 minute with an eluent flow rate of 4 mL/min. at 60°C. This back pressure can be easily maintained by conventional pumping equipment – rendering the transition to faster techniques relatively inexpensive.

Peptide map of BSA (Bovine Serum Albumin) on three C18 columns with different particle sizes. The flow rates were adjusted to give the same retention time for the peaks.

**Increasing Resolution via Improved Efficiency**

It’s important to bear in mind that Sub 2µm and related ‘Fast HPLC’ particle types deliver improved resolution over traditional particles when used at ‘traditional’ HPLC flow rates. At flow rates <= 1mL/min. the high inherent efficiencies of new generation materials helps to deliver much higher resolution than would be possible with, say, 5um particles.

This approach to using new generation materials can be highly advantageous when separating multiple components or when very high peak capacities are required as exemplified in the separation of protein digests.
Improving resolution via increased efficiency at “traditional” HPLC flow rates.

**Speeding up HPLC Separations**

Simply shortening the HPLC column reduces separation time; however, resolution will also be reduced due to a decrease in the plate number (available stationary phase surface). In general column length is directly proportional to retention time, column efficiency and backpressure. Reduction of column length is acceptable as long as column efficiency remains sufficient for separation.

Working at high flow rates is another way to reduce analysis time (flow rate is inversely proportional to analysis time). Flow rates should be selected according to the Van Deemter equation to make sure high chromatographic efficiency during the analysis (see following pages). Unfortunately, flow rate is also proportional to the pressure drop across the column.

Increasing temperature can be used to speed up the HPLC analysis for several reasons. First, an increase in column temperature reduces the viscosity of the mobile phase and therefore the column backpressure is reduced, permitting faster flow rates. An increase in column temperature enhances analyte mass transfer (increasing efficiency). The use of high temperatures is limited by the boiling point of the mobile phase and the thermal stability of the analyte.

The use of small particles with shorter columns may compensate for reduced efficiency but will be limited again by the system backpressure (small particles produce high backpressure). As can be seen, the limiting factor in speeding up HPLC separations is the column back-pressure; therefore, traditional approaches to fast HPLC are inherently restricted by this parameter.
Effect of column length and particle size of packing material on column’s efficiency

<table>
<thead>
<tr>
<th>Length (mm)</th>
<th>Efficiency (in theoretical plates) for columns packed with particles of</th>
<th>Analysis time</th>
<th>Solvent Saving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0 µm</td>
<td>3.5 µm</td>
<td>1.8 µm</td>
</tr>
<tr>
<td>150</td>
<td>12,500</td>
<td>21,000</td>
<td>35,000</td>
</tr>
<tr>
<td>100</td>
<td>8,500</td>
<td>14,000</td>
<td>23,250</td>
</tr>
<tr>
<td>75</td>
<td>6,000</td>
<td>10,500</td>
<td>17,500</td>
</tr>
<tr>
<td>50</td>
<td>4,200</td>
<td>7,000</td>
<td>12,000</td>
</tr>
<tr>
<td>30</td>
<td>N.A.</td>
<td>4,200</td>
<td>6,500</td>
</tr>
<tr>
<td>15</td>
<td>N.A.</td>
<td>2,100</td>
<td>2,500</td>
</tr>
</tbody>
</table>

For a given column length, chromatographic efficiency increases with reducing stationary phase particle size, making high efficiencies possible even with short columns.

Note how a column of 150mm in length and packed with particles of 5.0µm presents almost the same efficiency than a similar column of 50mm packed with particles of 1.8 µm. Note also that the shorter column will result in considerable analysis time and solvent volume savings.

Reverse phase HPLC separation of doping agents at 30°C. Partially Porous Column: C18 (2.1mm I.D. x 50 mm, 1.7µm); mobile phase: 0.1% formic acid in water. In the present application flow rates between 1000 and 1500µL/min present highest efficiency (lowest HETP) values and should be aimed for optimum analysis results. Sample: a. Acetazolamide; b. Chlortalidone; c. Clopamide; d. Dexamethasone.
HPLC Method Conversion

There are a number of simple equations which can assist with translating HPLC methods to faster equivalents and some of these are explained in the example presented below.

An HPLC separation was performed on a C8, 150mm×4.6 mm, 5µm column, experimental conditions: injection volume 10 µL; flow rate 1.0mL/min; pressure 54 bar; the analysis was done under gradient conditions (see the experimental conditions table given below). Find the experimental conditions for the same analysis performed on a C8, 50mm×4.6 mm, 1.9µm column.

**Experimental conditions.**

<table>
<thead>
<tr>
<th>Conditions Column 1</th>
<th>Conditions Column 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column length (mm):</td>
<td>Column length (mm):</td>
</tr>
<tr>
<td>$L_1 = 150$</td>
<td>$L_2 = 50$</td>
</tr>
<tr>
<td>Column internal diameter (mm):</td>
<td>Column internal diameter (mm):</td>
</tr>
<tr>
<td>$D_{C1} = 4.6$</td>
<td>$D_{C2} = 4.6$</td>
</tr>
<tr>
<td>Column particle size (µm):</td>
<td>Column particle size (µm):</td>
</tr>
<tr>
<td>$d_{p1} = 5.0$</td>
<td>$d_{p2} = 1.9$</td>
</tr>
<tr>
<td>Flow rate (mL/min):</td>
<td>Flow rate (mL/min):</td>
</tr>
<tr>
<td>$F_1 = 1.0$</td>
<td>$F_2 = ?$</td>
</tr>
<tr>
<td>Injection volume (µL):</td>
<td>Injection volume (µL):</td>
</tr>
<tr>
<td>$V_{i1} = 10$</td>
<td>$V_{i2} = ?$</td>
</tr>
<tr>
<td>Pressure (bar):</td>
<td>Pressure (bar):</td>
</tr>
<tr>
<td>$P_1 = 54$</td>
<td>$P_2 = ?$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gradient conditions</th>
<th>Time</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Conditions</td>
<td>$t_{g1} = 0$</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Step 2 (initial hold)</td>
<td>$t_{g1} = 10$</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Step 3</td>
<td>$t_{g1} = 11$</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gradient conditions</th>
<th>Time</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Conditions</td>
<td>$t_{g2} = 0$</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Step 2 (initial hold)</td>
<td>$t_{g2} = ?$</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Step 3</td>
<td>$t_{g2} = ?$</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

\[
F_2 = F_1 \times \left( \frac{D_{C2}}{D_{C1}} \right)^2 \times \left( \frac{d_{p1}}{d_{p2}} \right)
\]

\[
F_2 = 1.0\text{mL/min} \times \left( \frac{4.6\text{mm}}{4.6\text{mm}} \right)^2 \times \left( \frac{5.0\mu\text{m}}{1.9\mu\text{m}} \right) = 2.63\text{mL/min}
\]

\[
V_{i2} = V_{i1} \times \left( \frac{D_{C2}}{D_{C1}} \right)^2 \times \left( \frac{L_2}{L_1} \right)
\]

\[
V_{i2} = 10\mu\text{L} \times \left( \frac{4.6\text{mm}}{4.6\text{mm}} \right)^2 \times \left( \frac{50\text{mm}}{150\text{mm}} \right) = 3.33\mu\text{L}
\]

\[
P_2 = P_1 \times \left( \frac{d_{p1} \times D_{C1}}{d_{p2} \times D_{C2}} \right)^2 \times \left( \frac{L_2 \times F_2}{L_1 \times F_1} \right)
\]

\[
P_2 = 54\text{bar} \times \left( \frac{5.0\mu\text{m} \times 4.6\text{mm}}{1.9\mu\text{m} \times 4.6\text{mm}} \right)^2 \times \left( \frac{50\text{mm} \times 2.63\text{mL/min}}{150\text{mm} \times 1.0\text{mL/min}} \right) = 327.84\text{bar}
\]
\[ V_{01} = \pi \times \left( \frac{D_{C1}}{2} \right)^2 \times L_1 \times 6.8 \times 10^{-4} \]

\[ V_{01} = \pi \times \left( \frac{4.6mm}{2} \right)^2 \times 150mm \times 6.8 \times 10^{-4} = 1.695mm^3 \]

\[ V_{02} = \pi \times \left( \frac{D_{C2}}{2} \right)^2 \times L_2 \times 6.8 \times 10^{-4} \]

\[ V_{02} = \pi \times \left( \frac{4.6mm}{2} \right)^2 \times 50mm \times 6.8 \times 10^{-4} = 0.565mm^3 \]

<table>
<thead>
<tr>
<th>Gradient conditions</th>
<th>Time</th>
<th>Time</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Conditions</td>
<td>( t_{g1} = 0 )</td>
<td>( t_{g2} = 0 )</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>Step 2 (initial hold)</td>
<td>( t_{g1} = 10 )</td>
<td>( t_{g2} = ? )</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Step 3</td>
<td>( t_{g1} = 11 )</td>
<td>( t_{g2} = ? )</td>
<td>5</td>
<td>95</td>
</tr>
</tbody>
</table>

\[ t_{g2} = t_{g1} \times \left( \frac{V_{02}}{V_{01}} \right)^2 \times \left( \frac{F_1}{F_2} \right) \]

\[ t_{g2} = 0\min \times \left( \frac{0.565mm^3}{1.695mm^3} \right) \times \left( \frac{1.0mL/min}{2.63mL/min} \right) = 0\min \]

\[ t_{g2} = 10\min \times \left( \frac{0.565mm^3}{1.695mm^3} \right) \times \left( \frac{1.0mL/min}{2.63mL/min} \right) = 1.27\min \]

\[ t_{g2} = 11\min \times \left( \frac{0.565mm^3}{1.695mm^3} \right) \times \left( \frac{1.0mL/min}{2.63mL/min} \right) = 1.39\min \]
Transferring Methods - Gradient Elution Considerations

Fast HPLC can be performed in either isocratic or gradient mode.

When translating methods from traditional to ‘Fast’ HPLC, due to changing column lengths and bed volumes, it is possible for the separation selectivity to change, leading to possible peak inversions (swapping of peak retention order), loss of resolution or peak-co-elution. A typical example of this problem is shown below.

This is obviously problematic when translating methods and is a barrier to the simple adoption of the enabling technologies.

It is necessary when transferring to ‘Fast’ HPLC to take into account certain gradient criteria and to translate the gradient profile in order to ensure the selectivity of the separation remains the same.

Column: C18, 2.1mm x 150 mm, 5µm.
Mobile Phase: 80%Water (0.1% TFA) : 20% ACN
Gradient: (20-60% B), 30 min.
Inj. Vol.: 5µL
Flow: 0.2 mL/min
Temperature: 35°C
Detection: UV 254 nm
**Column**: C18, 2.1mm x 50 mm, 3.5µm.  
**Mobile Phase**: 80%Water (0.1% TFA) : 20% ACN  
**Gradient**: (20-60% B), 30 min.  
**Inj. Vol.**: 2µL  
**Flow**: 0.2 mL/min  
**Temperature**: 35°C  
**Detection**: UV 254 nm  

**Transferring Methods – Calculating Gradient Conditions**

One of the most important parameters in gradient HPLC is the gradient ‘steepness’ or rate of change over time. Gradient steepness is given by the mobile phase starting and ending composition and the gradient time. The steepness of the mobile phase gradient can have a significant effect on the separation and is given by:

\[ b = \frac{S \times \Delta \Phi \times V_m}{tg \times F} \]

Where

- \( \Delta \Phi \): change in volume fraction of organic (\( \Delta \Phi = %B_{\text{final}} - %B_{\text{initial}} \))  
- \( S \): is a constant determined by the solvent (usually around 4)  
- \( F \): flow rate  
- \( tg \): gradient time  
- \( V_m \): column void volume (\( V_m \approx \pi r^2 L \times W \))  
- \( W \): experimental column void fraction, it depends upon the column packing.  
- \( r \): internal radius of the column  
- \( L \): column length
If the parameter “b” is kept constant from run-to-run, peaks will elute in the same relative pattern, or in other words, if gradient steepness is kept constant from run to run then peaks will elute in the same relative pattern.

**Column:** C8, 150mm x 4.6 mm, 5µm.  
**Mobile Phase:** 0.2mL/min (water (A) + CH₃CN (B))  
**Gradient:** (20-60% B), 30 min.

**Column:** C8, 75mm x 4.6 mm, 3.5µm.  
**Mobile Phase:** 0.2mL/min (water (A) + CH₃CN (B))  
**Gradient:** (20-60% B), 15 min.  

The gradient steepness is the same for both analyses (note how the elution order is the same in both chromatograms) but due to the reduction in column geometry and particle size combined with an adjusted gradient time, analysis time is reduced from 24 minutes to 12 minutes without loss of efficiency. See the ‘Calculation’ tab for more details.
Let’s consider the experimental results for the 150 column (column 1). We are interested in calculating the gradient time \( t_{g1} \) for the same analysis performed on a similar column of 75 mm length (column 2). We need to know this in order to keep the gradient steepness ‘b’ constant as we will not change the starting and ending mobile phase composition.

If we keep the gradient steepness constant (which will maintain elution order), then we can apply the equation of gradient steepness for the two columns to calculate \( t_{g2} \):

\[
b = \frac{S_1 \times \Delta \Phi_1 \times V_{m1}}{t_{g1} \times F_1} = \frac{S_2 \times \Delta \Phi_2 \times V_{m2}}{t_{g2} \times F_2}
\]

If we are dealing with the same eluent and analytes under the same flow rate conditions (note this may not always be the case when transferring methods), then \( S, \Delta \Phi, \) and \( F \) remain constant and we can rewrite the last equation:

\[
\frac{V_{m1}}{t_{g1}} = \frac{V_{m2}}{t_{g2}} \Rightarrow \frac{\pi \times r_1^2 \times L_1 \times W_1}{t_{g1}} = \frac{\pi \times r_2^2 \times L_2 \times W_2}{t_{g2}}
\]

Both columns have the same radius \( (r_1 = r_2) \) and the same packing material \( (W_1=W_2=68\% \) in this case):

\[
\frac{L_1}{t_{g1}} = \frac{L_2}{t_{g2}} \Rightarrow t_{g2} = \frac{t_{g1} \times L_2}{L_1}
\]

Gradient Time \( t_{g1} \) is 30mins, the column lengths are 75 and 150 mm, then:

\[
t_{g2} = \frac{t_{g1} \times L_2}{L_1} = \frac{30 \text{ min} \times 75 \text{ mm}}{150 \text{ mm}} \approx 15 \text{ min}
\]

The adjusted gradient time should be 15 minutes

**Transferring Methods – Rules of Thumb**

**Rule 1:** Equal linear velocity ensures that each column is operated at the same point in the van Deemter curve yielding comparable efficiency. The next relationship can be used:

\[
F_2 = F_1 \times \left( \frac{\text{Diam}_{Col1}}{\text{Diam}_{Col2}} \right)^2
\]

Example: Calculate the flow rate on a column of 4.6mm i.d. that yields equivalent linear velocities to the same eluent system flowing at 1.0mL/min on a 2.1mm i.d. Hint: neglect temperature effects.

\[
F_2 = 1.0\text{mL/min} \times \left( \frac{2.1\text{mm}}{4.6\text{mm}} \right)^2 = 0.21\text{mL/min}
\]

**Rule 2:** A reduction in the particle size of the column packing permits a similar reduction in column length without losing much resolution.

Example: A 5µm packed column of length \( L \) can be replace by a 1.8µm packed column (a third of the initial packing size) of length \( L/3 \) (or even \( L/5 \)).
Rule 3: The injection volume will depend upon the void volume of the column (which in turn depends upon the quality of the packing material). The next relationship can be used:

\[ V_{i2} = V_i \times \left( \frac{\text{Void} - \text{Vol}_{\text{Col1}}}{\text{Void} - \text{Vol}_{\text{Col2}}} \right) = V_i \times \left( \frac{\pi \times r_1^2 \times L_1 \times W_1}{\pi \times r_2^2 \times L_2 \times W_2} \right) \]

Note that the column void fraction \((W_1 \text{ and } W_2)\) depend upon the quality of the packing, if both columns present the same packing then they have the same value \((W_1=W_2)\).

Example: Calculate the injection volume for a C8, 75mm×4.6 mm, 5µm column if the same analysis will require 20µL on a C8, 150mm×4.6 mm, 5µm. Consider \(W_1=W_2=0.68\)

\[ V_{i2} = 20\mu L \times \left( \frac{\pi \times \left( \frac{4.6}{2} \right)^2 \times (75) \times 0.68}{\pi \times \left( \frac{4.6}{2} \right)^2 \times (150) \times 0.68} \right) = 10\mu L \]

Rule 4: The run time or gradient segment time can be estimated based upon experimental results for a similar column under the same experimental conditions (gradient steepness, packing material, etc). The next relationship can be used:

\[ t_2 = t_1 \times \left( \frac{L_2}{L_1} \right) \]

Example: The elution time for a separation performed on a C18, 250mm×2.1 mm, 3.0µm column is 25 mins, estimate the elution time for the same separation on a C18, 100mm×2.1 mm, 3µm under the same conditions.

\[ t_2 = 25 \text{ min} \times \left( \frac{100 \text{ mm}}{250 \text{ mm}} \right) = 10 \text{ min} \]

Rule 5: When working at high eluent flow rates, increasing the eluent flow rate linear velocity will proportionally decrease analysis time.

Example: If working at 1.0mL/min, a separation is achieved in 30 mins, then if working at 2.0mL/min the same separation will require around 15 mins.

Rule 6: The rules of thumb can be used in conjunction to speed up the HPLC separation.

Example: Reducing column length by a factor of 3 and increasing velocity by a factor of 4 then the method is 12 times faster

---

**When to stop speeding up your separation**

- When you achieve your target time
- When resolution is no longer satisfactory
- When flow limit of pump is reached
- When pressure is approaching maximum (e.g. 550 bar)
Efficiency

In order to fully appreciate the advances in column and hardware technology we need to gain a good grounding in the theory and practice of ‘Efficiency’ in HPLC. To this end the following pages serve as a general introduction to the topic prior to our more in-depth discussions on new generation high efficiency technologies.

The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travelled through the HPLC system and column. In an ideal world, chromatographic peaks would be pencil thin lines – however, due to dispersion effects the peaks take on their familiar ‘Gaussian’ shape.

![Diagram of Efficiency](image)

\[
N = 16 \left( \frac{t_r}{W_s} \right)^2 = 5.54 \left( \frac{t_r}{W_s} \right)^2
\]

Higher values for the Plate Number (N) are expected for subsequent peaks within a chromatogram. Later eluting peaks that look broad in comparison to early eluters may have a higher plate count.

If this is not the case then your system contains a large extra-column dead volume!

The plate number (N) is a measure of the peak dispersion on the HPLC column, reflecting the column performance. N is derived from an analogy of Martyn and Synge who likened column efficiency to fractional distillation, where the column is divided into Theoretical Plates.

Each plate is the distance over which the sample components achieve one equilibration between the stationary and mobile phase in the column. Therefore, the more (‘theoretical’) plates available within a column, the more equilibrations possible and the better quality the separation.

The method of calculating column efficiency is shown. A typical plate number for a 4.6 X 100 mm column with 5 µm particles is between 5000 and 8000. Of course, for a given column length, more plates means less dispersion of chromatographic bands, narrower peaks and a better quality separation.
**Factional Distillation Model of Efficiency Theory**

The more ‘Plates’ there are—the narrower the distribution of carbon numbers from each trap (or plate). Therefore – the higher the number of plates (N) the narrower the ‘peak’ obtained from that trap – this can be directly related to the peak ‘efficiency’ in HLC where a column with a high number of plates gives narrower (more efficient) peaks.

Similarly – for a fractionating tower of a given length (L), the higher the number of plates, the lower will be the distance between each plate, shown as plate height in the diagram. Therefore, for high efficiency separations, the plate number (N) will be high and the plate height (H) low. Note that plate height is often called – ‘Height Equivalent to a Theoretical Plate (HETP)’
These two terms are related through the expression: \( H = \frac{L}{N} \)

The number of theoretical plates is often used to establish the efficacy of a column for a given method. The method developer may decide that a given method is no longer valid when the plate number falls below a predetermined value. At that time, the column would be replaced with a new one.

**How to Change Efficiency**

There are many factors that contribute to the broadening of the peak – or actually the injected ‘band’ of analyte as it travels through the chromatographic system.

The biggest contributor to band broadening (and hence low efficiency) is usually the column itself. The quality of the column packing, the particle size and column dimensions, as well as any voids that may have formed within the column, all play a part in determining the column efficiency.

- As column length increases the peaks become narrower (more efficient)
- As the peak efficiency increases the separation quality increases
- As the column length is increased, the analysis time increases significantly (this should be intuitive)
- Increasing the column length by an order of magnitude (2.5 to 25cm) the efficiency of the peaks also increases by about one order of magnitude
- 10,000 -20,000 plates can be generated by a well-packed 15 × 0.46cm column with 5\(\mu\)m packing material

Several other factors also need to be taken into account including:

- Injection volume
- Dead volume (such as detector cell, tubing and connections etc.)
- Flow rate

Other pages within CHROMacademy are dedicated to investigating column efficiency and so we will leave the detail of this topic for now.
Effects of Efficiency (N) on Resolution

Use the slider to investigate the effect of changing the column efficiency (plate number) on the resolution between two peaks in the chromatogram.

- In this example, efficiency increases as flow rate is reduced – it should be noted that this cannot be assumed as will be explained later on.
- Although peak shape improves markedly as flow rate is decreased, baseline resolution between the peak pair of interest is not achieved ($R_s < 1.5$ in all cases).
- Whilst resolution is generally improved - the transition from a flow rate of 2.5 mL/min. to 0.25 mL/min. increases analysis time by over 200 times!
- An important general rule of thumb states:

$$R = \frac{1}{4\sqrt{N}} \times \frac{\alpha - 1}{\alpha} \times \frac{k'}{1+k'}$$

In this example the efficiency has been altered using the flow rate of the mobile phase. It can be seen that the increase in resolution follows an approximately straight line with shallow slope.

The slope of this line is important. It should be noted that doubling the column efficiency, which may mean doubling analysis time, will only increase the resolution by a factor of the square root of 2 (1.42). It is doubtful if this would be considered 'good value for money!'

As you investigate the relationship between efficiency and resolution, it's important to look at the retention time scale and notice the overall time for the separation.
Van Deemter Plots

Reducing the analysis time for an HPLC application makes sense only if the column efficiency remains sufficiently high to achieve the required resolution. Put another way – we have to use columns which can develop very high plate numbers in order to reduce analysis time without compromising the resolution between critical peak pairs.

Band broadening is a term which describes the processes which cause analyte peak widths in the chromatogram to increase and thus chromatographic efficiency to decrease. The degree of band broadening naturally increases with the age of the column.

The Van Deemter curve,\textsuperscript{[4,5,7,8]} plots height equivalent of a theoretical plate (HETP) (y-axis) against the eluent linear velocity (u) which is a function of eluent flow rate, column internal diameter and particle size. This curve is a composite of curves made up from three individual effects which contribute to band broadening –Eddy Diffusion (A-Term), Longitudinal Molecular Diffusion (B-Term) and Mass Transfer Effects (C-Term).

As HETP decreases, plate number (N) increases and chromatographic resolution will also increase. Band broadening is considerable at very low or very high eluent flow rates, with an optimum minimum value lying somewhere in-between. The challenge is to find a set of HPLC conditions and hardware whose optimum HETP values occur at higher flow rates with flat minima in order to speed up the HPLC process. It is worthy of once again noting however the fundamental technological compromise of Fast HPLC, the higher the eluent flow rate the higher the pressure drop across the column (system back pressure).

![Van Deemter Equation](image)

It is worth considering once again, the fundamental technological compromise in Fast HPLC, the higher the eluent flow rate the higher the pressure drop across the column (system back pressure).

Van Deemter plot:

- **The A term** is related to the eddy diffusion and is a constant over a range of linear velocities
- **The B term** is related to longitudinal diffusion and decreases approximately exponentially with increasing eluent linear velocity
- **The C term** is related to the mass transfer process and increases linearly with increasing eluent flow rate
Flow rate and linear velocity are related terms, the next relation has been proposed:

\[
V \approx \frac{F}{10 \times d^2}
\]

Where:
- \(V\) = linear velocity (cm/sec)
- \(F\) = Flow rate (mL/hr)
- \(d\) = Column internal diameter (mm)

Thus even though the flow rate for two different columns is the same, the linear velocities are different for columns of different internal diameters.

Small HPLC column diameters provide higher sensitivity than larger column diameters for the same injected mass because the concentration of the analyte in the mobile phase is greater. Smaller diameter columns also use less mobile phase per analysis because a slower flow rate is required to achieve the same linear velocity through the column. For columns of different internal diameter the next expression can be useful.

\[
F_2 = F_1 \times \left( \frac{d_2}{d_1} \right)^2
\]

Eluent flow rate for HPLC columns of 250mm length and selected internal diameters.

<table>
<thead>
<tr>
<th>Column Diameter (mm.)</th>
<th>Typical Flow Rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.5</td>
<td>24.0</td>
</tr>
<tr>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>4.6</td>
<td>1.0</td>
</tr>
<tr>
<td>3.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Eddy Diffusion (The A term)**

The A term in the Van Deemter equation is often used to describe variations in mobile phase flow or analyte path within the chromatographic column.\(^{[5,7]}\)

Eddy diffusion itself relates to the fact that an analyte molecule can take one of many possible paths through the column. These multiple paths arise due to inhomogeneities in column packing and variations in the particle size / shape of the packing material. In fact, the Eddy diffusion in the Van Deemter equation is often called the “packing” term as it reflects the quality of column packing.

Eddy diffusion can be minimized by:

- Selecting well packed columns
- Using reduced diameter packing materials
- Choosing packing material with a narrow particle size distribution
Important considerations for fast HPLC:

- Analyte molecules move through different paths within the column packing material – leading to reduction in efficiency (band broadening).
- Larger differences in path-length occur with larger particles.

**Longitudinal Diffusion (The B Term)**

A band of analytes contained in the injection solvent will tend to disperse in every direction due to the concentration gradient at the outer edges of the analyte band. The B term in the Van Deemter equation is related to the dispersion experienced by analyte molecules due to these concentration gradients. This phenomenon is known as ‘Longitudinal Molecular Diffusion’ because inside the column, the greatest scope for broadening is along the axis of flow. Longitudinal diffusion will occur within all system tubing but will be most pronounced in the column.\(^\text{[5,7,8]}\)

Longitudinal diffusion occurs whenever the HPLC system contains internal volumes that are larger than necessary:

- Tubing length too long and/or too high internal diameter
- Incorrectly connected zero dead volume fittings
- Using the wrong column nuts and ferrules
- Having a detector flow cell which is too large for the required analytical sensitivity

Longitudinal diffusion has a much larger effect at low mobile phase velocity (flow); therefore, longitudinal diffusion is reduced when using high linear velocities. However, these effects still need to be borne in mind when designing and using Fast HPLC equipment as gains in efficiency can be easily mitigated by small, but additive, extra column system volumes.
Mass Transfer (The C Term)

The C term in the Van Deemter equation is related to the analyte mass transfer. C accounts not only for the dispersive convection in the mobile phase between and within the packing material pores but also for sorption and desorption of the analyte from the stationary phase.\[5,7,8\]

Rather than presenting a unique residence time in the stationary phase, analyte molecules present a spectrum of retention times. As analyte molecules move through the stagnant mobile phase within the pores of the stationary phase support material they do so by diffusion only (i.e the mobile phase is not moving with the eluent flow but is ‘trapped’ within the pore). Analyte molecules entering the pore will be sorbed onto the stationary phase surface at different depths within the pore, as the diffusion process towards the surface is a fixed rate process, this will cause the analytes to elute from the pore at different times – broadening the band of analytes as they travel through the column.

Mass transfer effects can be minimized by:

- using smaller diameter stationary phase particles
- using low mobile phase flow rates (low linear velocities)
- increasing column temperature (at high temperatures the diffusion processes speed up and the differences in elution time from the particle are reduced)

The mass transfer process increases with the eluent flow rate, increasing the band broadening.
Diffusion of the analyte molecules into and out of the stagnant mobile phase in the silica pore. Different depths of the analyte penetration will cause the analyte band to broaden.

**Packing Material - Particle Size**

The efficiency of the separation (and the hence the speed of an analysis) is heavily influenced by the packing material (particle shape, size and size distribution).\(^2,3,4\)

‘Fast HPLC’ techniques require the very highest quality of packing materials available and are generally spherical, small in diameter, with very narrow particle size distribution.

HPLC separations should be ideally performed at the eluent linear velocity corresponding to the minimum plate height (HETP). In practice the optimum practical linear velocity is within a range of values (highlighted in yellow below). The challenge in Fast HPLC is to extend this optimum working range to higher linear velocities in order to obtain high eluent flow rates without significantly compromising resolution due to the band broadening associated with the increase in plate height (loss of efficiency). It should be noted that high linear velocity can also be obtained by working with reduced internal diameter (2.1mm) columns at flows rates up to 1ml/min. Controlling the particle size and particle size distribution are techniques often employed by column manufacturers to enhance the optimum linear velocity working range.\(^9,10\)

Let’s consider an HPLC analysis performed under the same conditions on three columns with the same specifications (length, internal diameter, column chemistry) but packed with particles of different size (top example below). Note how, when using smaller particles the plate height is at or near the minima at higher linear velocity and remains near the optimum at much higher linear velocity. This is a significant advantage when attempting
Fast HPLC separations and is primarily due to the reduction in pore depths associated with the smaller particle producing less band broadening, especially at higher flow rates. It is therefore a reduction in Mass Transfer within the van Demeter equation which drives Fast HPLC using Sub 2µm particles.

Efficiency versus resolution.

\[ R_S = \frac{1}{4} \times \sqrt{N} \times \frac{\alpha+1}{\alpha} \times \frac{k}{1+k} \]

Efficiency (N) is proportional to the inverse of particle size. Therefore reducing particle size by a factor of 3 (5µm – <2µm), then N increases by a factor of 3 and the resolution, as defined by the Fundamental Resolution Equation above, increases by a factor of \( \sqrt{3} \) (1.7).

Plate Height \[ H = \frac{L}{N} \]

(\text{where L is the column length and N the number of theoretical plates}). Therefore lower plate heights give higher Plate (N) numbers and higher efficiency!

\[ N \propto \frac{1}{W^2} \quad \text{and} \quad H \propto \frac{1}{W} \]
(where \( W \) is the width at the base of the chromatographic peaks)

Therefore higher plate numbers, and hence better resolution can be derived with narrower chromatographic peaks!

Packing Material - Particle Size Distribution

HPLC columns are usually packed under optimised conditions to be as homogeneous as possible, thus producing a chromatographic bed which results in the highest efficiency peaks due to the reduction in Eddy Diffusion. One factor which seriously limits the quality (homogeneity) of the packed stationary phase bed is the distribution of packing material sizes around the ‘nominal’ particle diameter value – known as the ‘particle size distribution’.

The particle size distribution in an HPLC column usually approximates a normal distribution as can be seen below. The narrower the distribution the higher the efficiency of the resulting column due to the resulting packing density / bed homogeneity.

A Sub 2\( \mu \)m Fast HPLC column may be packed with particles of nominally 1.8 \( \mu \)m which results in a very high surface area and hence increased efficiency. However the particle size distribution will play a significant role in dictating the column packing efficiency, and hence the number of theoretical plates that the column can generate. However, as usual there is a trade-off; very narrow particle size distributions result in higher column back pressures due to the resulting density of the packed bed.\([11]\)

Therefore both the particle size and the particle size distribution play a vital role in determining system back pressure. Some manufacturers may intentionally skew the particle size distribution so that it becomes bi-modal with a significant proportion of larger particle sizes (~2.2\( \mu \)m). This significantly reduces the back pressure of the system but only slightly reduces the column efficiency.
Column Internal Diameter and Linear Velocity

Increased linear velocity can be achieved in several ways, however the combination of eluent flow rate and column internal diameter ultimately define the achievable linear velocity with any particular column.

There have been two distinct approaches made to developing column technologies for Fast HPLC, especially when using Sub 2\(\mu\)m column packing materials.

Some manufacturers opt to achieve very high linear velocities using longer, relatively narrow HPLC columns at modest flow rates – some examples of typical column geometry combinations are shown below.

Other manufacturers have opted to achieve increased linear velocity using shorter, wider columns but with higher than conventional eluent flow rates. It should be noted that both approaches lead to increased back pressure, some examples of which are shown below.

From a practical standpoint, wider bore columns can sometimes be successfully used on conventional HPLC systems at modest flow rates before pressure limits are reached. Further, the narrower bore column approach does require that all extra column volume in the system is strictly minimized otherwise longitudinal molecular diffusion effects may negate any increase in efficiency derived from the Sub 2\(\mu\)m packing materials. On the flip side, narrow columns can achieve very high linear velocities which can result in ultra-fast HPLC separations.

Typical data is shown below to contrast pressure v's linear velocity for wide and narrow bore Fast HPLC columns.
<table>
<thead>
<tr>
<th>Particle Size</th>
<th>Flow rate</th>
<th>Back pressure (5% MeCN) (50 x 4.6 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>1</td>
<td>190 (14)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>380 (27)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>571 (41)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>761 (54)</td>
</tr>
<tr>
<td>3.5</td>
<td>1</td>
<td>388 (28)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>776 (55)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1165 (83)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1553 (111)</td>
</tr>
<tr>
<td>1.7</td>
<td>1</td>
<td>1645 (117)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3291 (235)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4936 (353)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1553 (470)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particle Size</th>
<th>Flow rate</th>
<th>Back pressure (30% MeCN) (100 x 2.1 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>0.45</td>
<td>8000 (571)</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>10700 (764)</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>13400 (957)</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>16100 (1150)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Particle Size</th>
<th>Flow rate</th>
<th>Linear Velocity mm/sec (100 x 2.1 mm)</th>
<th>Linear Velocity mm/sec (50 x 4.6 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>0.45</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1.8</td>
<td>2.19</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2.75</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4.25</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>
Packing Material - Morphology

Achieving optimal HPLC separations (usually defined by the minimum resolution between peaks within the chromatogram), in minimal time is the main concern of many analytical scientists. The reasons for this are quite understandable as rapid and reliable HPLC analyses not only save analysis time and cost per analysis but also decrease the amount of solvents, buffers and additives required for the separation to take place, making the analysis more environmentally friendly and economic. With this in mind, manufacturers have introduced new stationary phases, new column geometries and new instruments; all designed to reduce analysis time whilst maintaining (or even improving) the quality of the HPLC separation.\(^{[1]}\)

The HPLC column’s separation performance is highly influenced by the morphological properties of its packing material (porosity, particle size, shape and distribution). Different packing materials have been developed to enable operation at high flow and high efficiency, including:\(^{[2,3,4,12,13]}\)

- small (often sub 2µm) fully porous particles
- superficially porous spherical particles
- non-porous spherical particles
- monolithic stationary phases

**Monolithic cylindrical fiber.**
Silica monolithic porous rod is regarded as one of the representative stationary phases for fast HPLC. The silica skeleton is cylindrical and pores are homogeneous distributed.

Monolithic based systems are designed to deal with pressures not exceeding 400bar and are in general compatible with conventional HPLC systems (maximum allowable pressure 400bar).
Partially porous (also termed superficially porous) spherical particle.
The non-porous and inert core is located in the centre of the spherical particle (chromatographically inert), the porous layer is homogeneous and surrounds it. Sample molecules three-dimensionally diffuse in the radial direction of the superficial thin porous layer.

Partly porous systems are designed to deal with pressures not exceeding 600 bar and are in general compatible with conventional HPLC systems (maximum allowable pressure 400 bar).

Totally Porous:
A popular fast HPLC system uses the column packed with super-fine full-porous spherical particles; however, the instrumentation required must be especially designed to deal with pressures that are unusually high in traditional HPCL applications. Full porous systems have to deal with high pressures (which can exceed 1000 bar) and in general are not compatible with conventional HPLC systems (maximum allowable pressure 400 bar). The easiest way to overcome this problem consists in performing the separation at high temperatures.

Non Porous:
Chromatographic processes take place only on the external surface of the non-porous spherical particle. The inner non-porous particle is chromatographically inert.

Non-porous based systems are designed to deal with pressures not exceeding 400 bar and are in general compatible with conventional HPLC systems (maximum allowable pressure 400 bar).

Reduced Diameter / Sub 2µm Particles

The ability to produce rugged reproducible column packing materials below 2.0 µm in diameter has been an important and relatively recent breakthrough.

Efficiency increases markedly when using particles less than 2.5 µm in diameter due to the reduction in the Mass Transfer contribution to band broadening. Smaller particles have shallower pores (including through pores) which ultimately results in a smaller distribution of analyte ‘retention’ times within each pore during the ‘diffuse in / adsorb / desorb / diffuse out’ process.
Further, smaller particles may be more densely packed which can result in better bed homogeneity further reducing the eddy diffusion contribution to band broadening.

Several manufacturing challenges need to be overcome when producing Sub 2µm columns. The internal surfaces of the column wall need to be very highly polished to reduce the effects of non-laminar flow within the tube and the forces of frictional heating. Further, end frits used to retain packing material must be of low enough porosity to retain the packing material without becoming susceptible to clogging. The packing technology used must be capable of consistently producing highly homogenous and reproducible bed densities. Lastly, the particles must be high enough in strength to resist the sheer forces applied when the eluent is flowing. All of these challenges have now been overcome and several manufacturers produce Sub 2µm particles of very high quality and ruggedness.

Sub 2µm fully porous columns can be used with traditional HPLC equipment, however to achieve high eluent linear velocities HPLC equipment which is rated to higher pressures is often used. The eluents used with these columns must be ultra-filtered to ensure no particulate material can block the column and sample preparation must be carefully considered.

![HPLC v's UPLC peak capacity.](image)

In this gradient peptide map separation, the HPLC (top) separation (on a 5µm C18 column) yields 70 peaks, or a peak capacity of 143, while the UPLC separation (bottom) run under identical conditions yields 168 peaks, or a peak capacity of 360, a 2.5 x increase.
UPLC (Ultra High Performance Liquid Chromatography) separation of eight diuretics in <1 min with a <3 min cycle time.

**Column:** 2.1 by 30 mm 1.7 µm UPLC C18 at 35°C.

**Eluent:** A - 0.1% formic acid, B – acetonitrile.

**Gradient:** 9-45% B linear gradient over 0.8 minutes

**Flow Rate:** 0.86 mL/min

**Detection:** UV detection at 273nm

**Sample:** peaks are in order, acetazolamide, hydrochlorothiazide, impurity, hydroflumethiazide, clopamide, trichlormethiazide, indapamide, bendroflumethiazide, and spironolactone, 0.1mg/mL of each in water

**Superficially Porous Particles**

Superficially porous silica technology has been available since the mid-70's and consists of a solid silica core surrounded by a relatively shallow layer of porous silica material. The inner core is chromatographically inert whilst the porous outer layer is used for the separation of analytes.
These materials were originally designed for the separation of macromolecules and consisted of silica with a wide pore diameter (typically 300Å). Modern advances in superficially porous materials allow the materials to be produced with 90 - 120Å pores which is much more amenable to the separation of small molecules. The core of these materials is typically 1.5 – 2.0µm in diameter with a 0.5µm outer layer making the whole particle 2.5 - 3.0 mm diameter. The main advantage of these materials is that they typically generate one third of the back pressure seen with sub 2µm materials, whilst achieving comparable efficiencies.

Van Deemter curve for various particle sizes/types

Like their reduced diameter counterparts, superficially porous particles gain efficiency through the reduction in band broadening due to Mass Transfer. However, very recently it has been concluded that this process is insignificant for analyte molecules below about 600Da. Current thinking explains the increase in efficiency as being due to the very homogenous packed beds which result from superficially porous materials. Very narrow particle size distributions can be achieved when engineering these particles and as such the quality of the resulting packed beds increases and a significant reduction in eddy diffusion results in increased efficiency.
There were some initial concerns regarding the capacity of these materials as they theoretically have limited silica surface area. This issue is explored further in this module.

**Separation of aromatic acids**

**Column**: 4.6 x 50 mm, 2.7-µm C8.
**Mobile phase**: 55% methanol/45% 25 mM sodium phosphate buffer, pH2.5.
**Flow rate**: 2.20 mL/min.
**Temperature**: 24 °C.
**Column pressure**: 360 bar.
**Solutes**: 1) uracil, 2) phthalic acid, 3) 2-fl urobenzoic acid, 4) 3 nitrobenzoic acid, 5) 3-fl urobenzoic acid, 6) m-toluic acid.
Reduced Diameter Non-Porous Particles

Small non-porous particles have been available for chromatography for several years. These particles are typically 1.5µm non-porous polymeric materials with stationary phase applied to the outer surface of the particle.

The efficiency difference between porous and nonporous particles results mainly from the elimination of mass transfer resistance from the stagnant mobile phase in the pores. However it has been demonstrated that for small molecules the efficiency differences between non-porous 1.5µm and porous 1.7µm particles is minor. For larger molecules and proteins the non-porous materials show significantly increased efficiency.

The experimental results (presented below) of plate height as a function of mobile phase linear velocity for 1.5 and 3.0 µm nonporous and porous particles when nitromethane was used as an unretained solute and 100% acetonitrile as the mobile phase. In order to more accurately estimate the contribution of mass transfer resistance from the stagnant mobile phase, any contribution to mass transfer resistance from the stationary phase must be eliminated; therefore, an unretained solute (Nitromethane) with low molecular weight was chosen. It can be seen that at high linear velocities, columns packed with nonporous particles produced smaller plate heights for both 1.5 and 3.0µm particles. The difference in plate height between porous and nonporous particles, however, was reduced significantly from 3.0 to 1.5µm.
Separation of alkylbenzenes using porous and non-porous sub 2\(\mu\)m materials

**Conditions:**
- Detection: 254 nm UV detection
- Sample: 25 \(^\circ\)C; 1\(\mu\)L injection; 0.5 mgmL\(^{-1}\) each of alkylbenzenes (C\(_n\)H\(_{2n+1}\)C\(_6\)H\(_5\), \(n = 0–9\))
- Columns: 30 cm×75\(\mu\)m i.d.
- Temperature: Ambient
- Mobile Phase: 50:50 H\(_2\)O/ACN isocratic (porous) 68:32 H\(_2\)O/ACN isocratic (non-porous).

The mobile phase composition was adjusted so that the average retention factors were similar for both columns.

Peak Identifications: (1) benzene; (2) toluene; (3) ethylbenzene; (4) \(n\)-propylbenzene; (5) \(n\)-butylbenzene; (6) amylbenzene; (7) \(n\)-hexylbenzene; (8) \(n\)-heptylbenzene; (9) \(n\)-octylbenzene.

Separations of 10 alkylbenzenes using porous and nonporous particles are shown in the figure. It can be seen that porous particles provided better separation than nonporous particles for the early eluting components; while nonporous particles provided better separation than porous particles for the later eluting components.
Non-Porous Particles Considerations

Due to the reduced amount of stationary phase available when using non-porous materials, retention factors are usually much lower and this can cause problems with resolution of early eluting compounds.

Retention factors for amylbenzene on C18 columns packed with different packing materials.

Mob. Phase: water/acetonitrile  
Temperature: 25 °C  
Flow Rate: 0.3mLmin^{-1}  
Detection: 254 nm UV detection  
Columns: 50mm×2.1mm i.d.  
Sample: 0.5 mgmL^{-1} amyl benzene as solute

Pressure drops for nonporous particles are higher than porous particles in practice, since high aqueous mobile phases are required to obtain similar retention factors for the non-porous materials.

The sample loading capacity for porous particles is significantly higher than that for nonporous particles.

In general terms, the retention factor for porous materials is higher than the one for non-porous materials. Non-porous materials have much lower surface area, therefore lower carbon loading and decreased hydrophobic interaction and hence retention.
Sample loading capacity for porous and nonporous particles

**Mob. Phase:** 60:40 H₂O/ACN for porous particles; 95:5 H₂O/ACN for nonporous particles  
**Temperature:** 25 °C  
**Flow Rate:** 0.3mLmin⁻¹  
**Detection:** 254 nm UV detection  
**Columns:** 50mm×2.1mm i.d.  
**Solute:** Benzyl alcohol

The retention factors were 0.62 and 0.60 for porous and nonporous particles, respectively.

The figure above shows the relationship of width at half peak height (W₁/₂) with approximate sample amount injected for porous and nonporous particles. Little increase (~10%) in W₁/₂ was observed up to 15µg for a 5.0mm×2.1mm i.d. column packed with porous particles and 1.0µg for nonporous particles using benzyl alcohol as solute.

The sample loading capacity was approximately 15 times higher than that for nonporous particles, which agrees well with theoretical values.

**Monolithic Columns**

Monolithic HPLC columns are made of a single piece of porous cross-linked polymer or porous silica. The unusual feature of silica monolithic columns is their bi-modal pore structure. The macroporous structure is around 1.5µm pore diameter and forms large ‘through pores’ which offer reduced resistance to mobile phase flow. For Fast HPLC applications the through pores allow reduced flow resistance alongside fast mass transfer kinetics and as such high efficiency can be obtained at increased eluent flow rates without compromising the instrument pressure tolerances of conventional HPLC equipment (400 bar rated).[17,18]

Monolithic columns demonstrate increased mechanical stability provided by the integrated network structure, which allows elution at mobile phase linear velocities greater than 10 mm/s. The amount of silica within a monolithic column is less than in a particle-packed column, resulting in a smaller surface area, which in turn results in shorter retention times. k values found with monolithic silica-C18 columns are smaller by a factor of
2 – 5 typically, depending on the total porosity. The sample loading capacity, however, has been reported to be not as small as expected from the phase ratio.

At present monolithic columns commonly provide a plate height, $H$, of 8 – 20 $\mu$m at a linear velocity of 1 – 10 mm/s - similar to a column packed with 3 $\mu$m particles, but lower than the most sub 2 $\mu$m particles columns, especially at high linear velocity.
(BLUE) silica monolith: mobile phase acetonitrile/water (50:50, v:v)
(RED) 3 µm ODS porous particles: mobile phase acetonitrile/water (57.5: 42.5; v:v)
0.05 mg/mL naphthalene yielding the same retention factor on both columns

Silica monoliths provide plate height values similar to 3µm porous particles. It is worthy of note that the monolith can achieve much higher linear velocities (and therefore shorter separations) within the operating pressure range of traditional HPLC.

HPLC vs fast HPLC analysis of some notable xanthenes.
**Column:** C18, 50mm × 1.0 mm, 3.5µm.

**Analysis Conditions:** 140 Bar and 30°C.

**Mobile Phase:** 0.46 mL/min 70% acetonitrile in water (pH=2.6)

**Sample:** a. Xanthine; b. Theobromine; c. Theophyline; d. Caffeine

**Monolithic Columns Selected Applications**

**Food additives** are substances added to food to preserve flavour or improve its taste and appearance. The determination of selected food additives (in the concentration range of 13 - 100µg/mL) is presented.

![Image](image-url)

Fast separation of food additives on a monolithic silica column

**Column:** Chromolith ® SpeedROD RP-18e, 50-4.6mm

**Mobile phase:** A: Acetonitrile + B: 0.01M Phosphate Buffer pH 5.0

**Flow rate:** 4 mL/min

**Pressure:** 48-50Bar

**Detection:** 227nm

**Temp:** Ambient

**Injection Volume:** 10µL

**Sample:** 1. Acetasulfame-K (23µg/mL); 2. Saccarine (29µg/mL); 3. Benzoic acid (13µg/mL); 4. Sorbic acid (14µg/mL); 5. Caffeine (47µg/mL); 6. Aspartame (100µg/mL)

As the column is relatively short (50mm) the moderately high surface area and fast eluent flow rate (4mL/min.) combine to produce a satisfactory separation in around 2.5 mins.
Blood plasma is the yellow liquid component of blood. It is mostly water and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide. The determination of tocopherol from a plant extract was done by using a monolithic is presented.

Rapid Analysis of Tocopherols in plasma

**Column:** C18 monolithic column, 50mm×4.6mm  
**Mobile phase:** Water-Methanol (2:98, v/v)  
**Flow rate:** 7 mL/min  
**Pressure:** 48-50Bar  
**Detection:** Fluorescence  
**Injection Volume:** 1µL  
**Column temp:** 40°C  
**Sample:** Plasma precipitated with 3 volumes of 2-propanol

Here the monolithic column is used at very high flow rates (7 mL/min.) to obtain sufficient resolution to detect and quantify two key tocopherols by Fluorescence detection. Note that this separation would be more than adequate to resolve the materials from endogenous compounds prior to MS/MS detection.
**Plant extracts:** as a result of the potential for new chemical discovery (pesticides, drugs, cosmetics, etc), plants have attracted scientists from different disciplines, such as organic chemistry, bioorganic chemistry, pharmacology, biology and ecology. The determination of iridoid glycosides harpagoside from a plant extract was done by using a monolithic is presented.

![Fast HPLC vs HPLC separation](image)

**Fast HPLC Conditions**
- **Column:** Monolithic silica column 100mm × 4.6mm
- **Mob. Phase:** 1% MeCN – 50% MeCN in 2 mins
- **Flow rate:** 5.0 mL/min
- **Temperature:** 30 °C.

**HPLC Conditions**
- **Column:** ODS, 5µm particle size 125mm × 4mm
- **Mob. Phase:** 1% MeCN - 50% MeCN in 16 mins; (Solvent A – Water pH2)
- **Flow rate:** 0.8 mL/min
- **Temperature:** 30 °C.

A total monolithic column length of 200mm was used to give a high enough plate count for the fast separation of several medicinal plant extracts and internal standards. Note that even at an eluent flow rate of 5 mL/min. with a 200mm column the back pressure did not exceed system limits.

The determination of iridoid glycosides harpagoside from a plant extract was done by using a monolithic and a full porous packed columns columns.

As can be seen from the applications below, monolithic columns can produce a satisfactory separation in a reasonable time frame. The use of monolithic columns for fast HPLC is continuously growing.
Temperature

Temperature plays an important role in HPLC; this is because both the kinetics and thermodynamics of the chromatographic process are temperature dependent.

In nearly all reversed phase separations, an increase in temperature will reduce analyte retention. Additionally solvent viscosity is reduced at elevated temperature which in turn means lower backpressure.

At elevated temperature the solute transfer from the mobile phase to the stationary phase is more efficient. This leads to a flatter van Deemter curve (extended working range) at higher linear velocity or higher flow rate.

Effect of temperature on the HPLC separation. Column: 50 cm × 4.6 mm 1.8µm; solute: α-naphthol; mobile phase: 60% acetonitrile–40% water.

When working temperature is increased, then the optimum HPLC working range (where HETP finds minimum values, yellow area) flattens out at higher flow rate values.

By increasing the temperature, the amount of organic solvent in the mobile phase can be reduced to maintain retention. In some cases a small increase in temperature (of only a few degrees Celsius) produces a similar effect on retention as changing mobile phase composition.[9,14]

Increasing temperature will speed up the HPLC analysis for several reasons. First, an increase in column temperature reduces the viscosity of the mobile phase and therefore the column backpressure is reduced, permitting faster flow rates.[5] An increase in column temperature enhances analyte mass transfer (increasing efficiency). The use of high temperatures is limited by the boiling point of the mobile phase, and by thermal stability of the analyte and stationary phase.
NOTE: When transferring methods from traditional HPLC, it should be noted that altering the column temperature may also affect the selectivity of the separation, especially when there are ionisable compounds within the sample.

In the application below, a mixture of parabens was separated by using a sub-two micron column. The separation was performed at three different temperatures (the optimum flow rate for each temperature was selected), all the remaining parameters were kept constant.

![Fast HPLC analysis of a mixture of parabens (200 Bar). Column: C18 (2.1mm I.D.×50 mm, 1.7µm); mobile phase: water + 30%acetonitrile. Sample: a. Methylparaben; b. Ethylparaben; c. Propylparaben; d. Butylparaben. Kinetic Plots

A kinetic plot allows comparing column supports with different morphologies and/or sizes, as well as systems using different mobile phases.

Poppe plots are a kind of kinetic plot that provide a way to discuss the performance limits of HPLC separation systems. Particle size and pressure drop are factors that can be explored from such plots. Poppe plots can also be used to compare the performance of different chromatographic systems.[15,16]

The two factors that feature on the axes of a Poppe plot are the number of theoretical plates (N) and the time required to realize one theoretical plate (the "plate time", \( t_p \)). The parameter \( t_p \) describes the speed of the separation, fast separations present lower to values. The "plate time" (\( t_p \)) can be related to the plate height (H) and the interstitial mobile-phase velocity (\( u_0 \)), or to the un-retained time (\( t_0 \)) and the plate count:

\[
\frac{1}{t_p} = \frac{H}{u_0} = \frac{L/N}{t_0} = \frac{t_0}{N}
\]

Fast separations have a short "plate time" (small \( t_p \) values) and are located towards the bottom of the figure. High-resolution separations (high numbers of theoretical plates) are located towards the right in the Poppe plot.
Comparison of three columns of different particle size by using a kinetic Poppe Plot at a maximum pressure P.

The dashed lines correspond to zones of constant time (here the time duration $t_1$ is smaller than $t_2$). Note that for fast analysis (lasting $t_1$ minutes total time), the smaller the particle size of the packing material the more efficient the analysis ($N_1 < N_2 < N_3$). For long analysis times the situation can change note how for a longer analysis (lasting for $t_2$ minutes) the $3.5 \, \mu m$ packed column will present lower separation efficiency than the one packed with particles of $7.0 \, \mu m$ in diameter.

Comparison of three columns of different particle size by using a kinetic Poppe Plot.
The dashed lines correspond to zones of constant time (here the time duration $t_1$ is smaller than $t_2$). Note that for fast analysis (lasting $t_1$ minutes total time), the smaller the particle size of the packing material the more efficient the analysis ($N_1 < N_2 < N_3$). For long analysis times the situation can change note how for a longer analysis (lasting for $t_2$ minutes) the 3.5 µm packed column will present lower separation efficiency than the one packed with particles of 7.0 µm in diameter.

**Poppe Plots –Use and Description**

Poppe plots are used as a comprehensive column-characterization method that includes information on the plate number, the A and C terms (from the Van Deemter or knox equation), the analysis time, and the pressure that can be applied. All these parameters are reflected in a single graph.

In practice, these plots can directly be constructed from experimental data, i.e. from measured values of $N$, $t_0$, $\Delta P$, and $L$. The transformation corresponds to calculating the efficiency of an imaginary, longer, but otherwise identical column, operated at the same mobile-phase velocity, for which the pressure drop would be equal to the maximum permissible value.

As can be seen from the diagram below, in a Poppe plot the separation efficiency is plotted in the X-axis against plate time ($t_0/N$). The use of logarithmic coordinates is a common practice, because it facilitates the visualization and analysis of data comprised over a large range values in one single diagram.

The diagonal lines represent zones of constant analysis time; just consider an analysis lasting for 100 seconds total time, a column packed with 6.0µm particles will perform better (higher efficiency, $1.5 \times 10^4$ theoretical plates) than a second column packed with 10µm (lower efficiency, $7.1 \times 10^3$ theoretical plates).
Note that the situation changes at longer analysis times (see for example at 10000 seconds total analysis time).

**Impedance Plots – Use and Description**

Impedance plots are another type of kinetic plot widely used to compare column supports with different morphologies and/or sizes, as well as systems using different mobile phases.\[^{12}\]

The separation impedance ($E=t_0/N^2$), is a performance parameter that combines the reduced plate height and the flow resistance parameter of a column. The time required to obtain a certain resolution for a separation, with a specific pressure drop, is directly proportional to the separation impedance of the column. The lower the separation impedance, the better is the performance of a column. Although highly informative, this parameter fails in relating the performance of columns to variations in the A and C contributions.

In order to keep resemblance with the Van Deemter plot, it is a common practice to present kinetic plots with their X-axis scale decreasing from left to right; by doing this, the left hand side of the curve will be dominated by B term (low flow rates), while the right hand side of every curve will be dominated by the C term (high flow rates).\[^{20}\]

As with Poppe plots, the use of logarithmic coordinates is a common practice when dealing with impedance plots.
Thermal Considerations

While flowing through the column, the eluent system experience frictional forces that rise its temperature (frictional heating). These frictional forces are related to the speed of the eluent system, its chemical nature and the contact area with the packing material (which in turn depends upon the particle size).

Frictional heating of the mobile phase can cause a non-uniform increase in temperature inside the column that can adversely affect the separation. A typical frictional heating profile within a column is presented.\[17,18,19]\n
In order to avoid frictional heating associated problems, fast HPLC utilizes temperature control devices.

Under well-thermostatted conditions, a radial temperature profile will develop, with the centre of the column at higher temperature than the wall. In certain occasions altering the temperature of the eluent system before entering the column can be used to compensate for radial gradient, allowing more efficient analysis.
Power Plots

The use of very high pressures to achieve the required flow rates to produce chromatographic separations, as the particle diameter decreases, induces frictional heating of the mobile phase.\cite{17,19}

Frictional heating can cause a non-uniform increase in temperature inside the column that can adversely affect the separation. The power to be dissipated is dictated by the pressure applied and the flow rate through the packed column, and is given by:

$$Power = \Delta P \times F$$

Where:

- $F$ is the flow rate across the column (which depends upon the column cross-sectional area for a given linear velocity)
- $\Delta P$ is the pressure drop across the column and is dependent upon the eluent system, the column and the analysis conditions.

As an example, let’s consider a typical HPLC application with no power dissipation problems. First consider a standard HPLC column with dimensions of 150 mm length and 4.6 mm id, packed with 3 $\mu$m particles and an eluent system of 1 cP flowing at 1.0 mL/min; the power generation in such a column is about 280 mW. See below.

![Power generation on columns of 250 mm x 1.8 mm ID and packing material of different size (1.0, 1.5, 3.0 and 5.0$\mu$m).](image-url)
Consider a 250 mm column length with an internal diameter of 1.8 mm (vertical black line). The generated power decreases from 340mW (1.0 µm packing size) to 40mW (5.0 µm packing size).

For a given column, the power generated during analysis decreases while increasing packing size.

Fast HPLC columns should be selected in such a way that the power generated during analysis do not exceed 280 mW otherwise power dissipation problems should appear.

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