

The Theory of HPLC

Chromatographic Parameters



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.

Aims and Objectives

Aims

- To introduce and explain the concept of Chromatographic Resolution (R_s)
- To define the Resolution equation and illustrate its dependence on the chromatographic parameters – Retention Factor (k), Selectivity (α), and Efficiency (N)
- To define Retention Factor (k), Selectivity (α), and Efficiency (N) in chromatography and show how each can be determined and optimized
- To interactively demonstrate how each parameter may be manipulated to optimize chromatographic resolution

Objectives

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

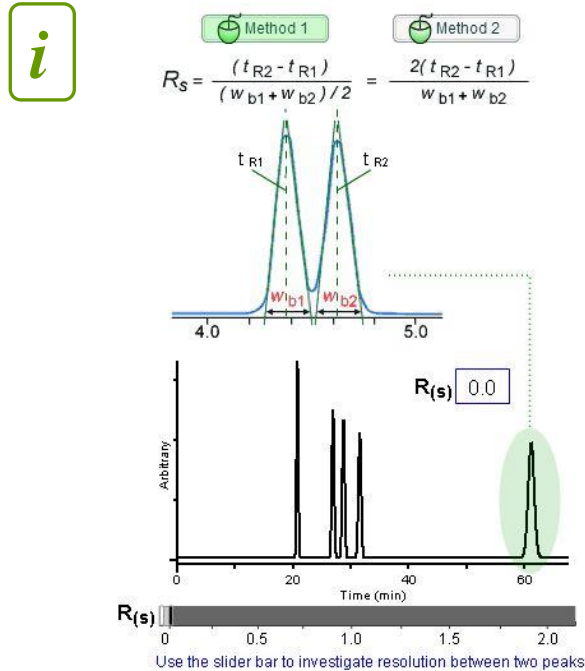
- Understand and optimize the main chromatographic parameters; retention factor (k), selectivity (α), and efficiency (N)
- Explain and demonstrate the concept of peak Asymmetry (A_s)
- How to practically improve chromatographic separations

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Resolution R_s

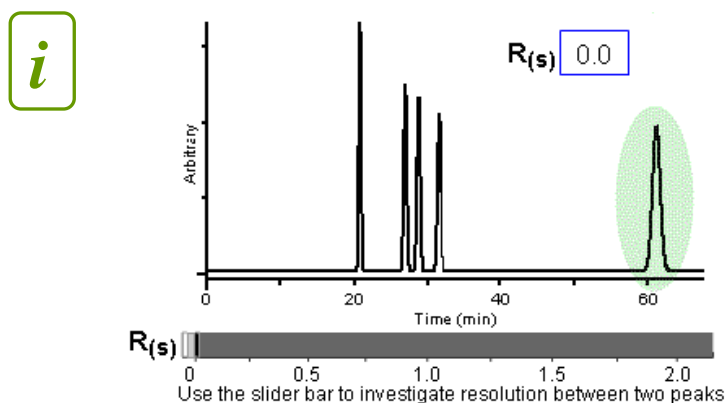
The most important thing in HPLC is to obtain the **optimum resolution in the minimum time**. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well (baseline) separated to a degree at which the area or height of each peak may be accurately measured.



Calculation of Chromatographic Resolution (R_s)

Resolution is calculated using the separation of two peaks in terms of their average peak width at the base ($t_{R2} > t_{R1}$). In the case of two adjacent peaks, it may be assumed that the peak width at the base $w_{b1} \approx w_{b2}$, and thus, the width of the second peak may be substituted for the average value.

The width at the base of each peak is the segment of the peak base intercepted by the tangents drawn to the inflection points on either side of the peak as shown. It should be noted that as resolution increases so does the time required for the separation. As a chromatographer, you will have to balance the desire for rugged separations with that of time and materials.



The On-line course contains an interactive Resolution experiment

The Resolution Equation

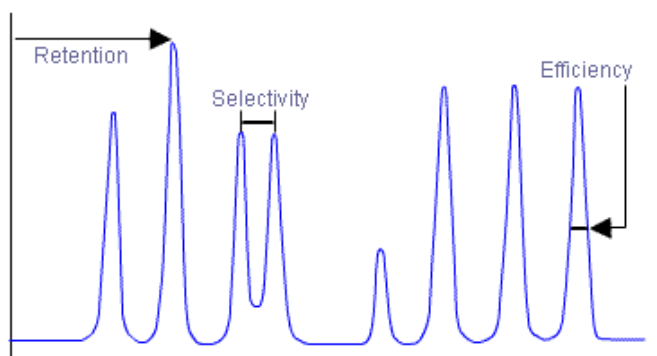
How are you able to control the resolution obtained from a chromatographic separation? The Fundamental Resolution Equation (shown) indicates that resolution is affected by three important parameters:

- Selectivity (Separation factor)
- Efficiency
- Retention (Capacity factor)

Therefore, how do each of these factors contribute to the resolution of the separation and are there target values to aim for, or limits beyond which a change in the parameter will have no meaningful effect on resolution? The answer to these questions is yes, and it is important to understand the way in which each parameter affects resolution. The following pages will describe each of the parameters, how to change them, and what affect they have on the chromatographic resolution.

$$R_s = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

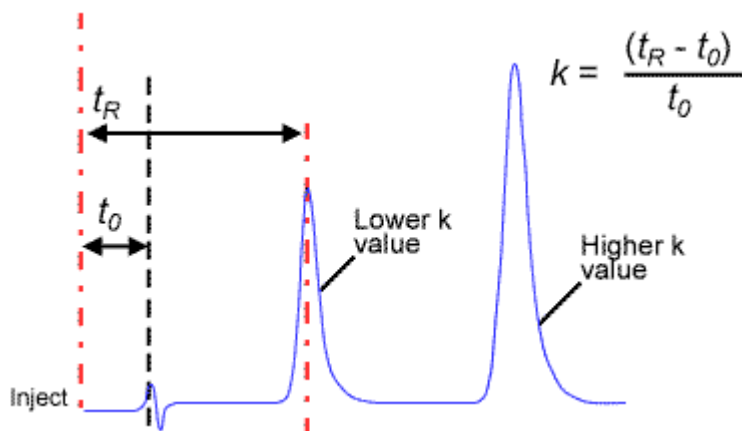
The Fundamental Resolution Equation



The Fundamental Resolution Equation and contributing parameters

Retention (Capacity) Factor (k)

The retention (or capacity) factor (k) is a means of measuring the retention of an analyte on the chromatographic column.



A high k value indicates that the sample is highly retained and has spent a significant amount of time interacting with the stationary phase.

Determination of Retention Factor (k)

The retention factor is equal to the ratio of retention time of the analyte on the column to the retention time of a non-retained compound. The non-retained compound has no affinity for the stationary phase and elutes with the solvent front at a time t_0 , which is also known as the 'hold-up time' or 'dead time'.

There are several ways to determine t_0 including:

- The time at the baseline disturbance seen due to differences in absorbance or refractive index as the injection solvent passes through the detector
- Retention time of uracil (reversed phase HPLC)
- Retention time of hexane (normal phase HPLC)

Retention factor is independent of some key variable factors including small flow rate variations and column dimensions. Therefore, it is a useful parameter when comparing retention of chromatographic peaks obtained using different HPLC systems.

Calculation of Typical Retention Factor (k) values

Chromatographers like to keep k values between 1 and 10 for good separations. If the t_0 time of the system was 1.0 minute, this would equate to a retention time range of:

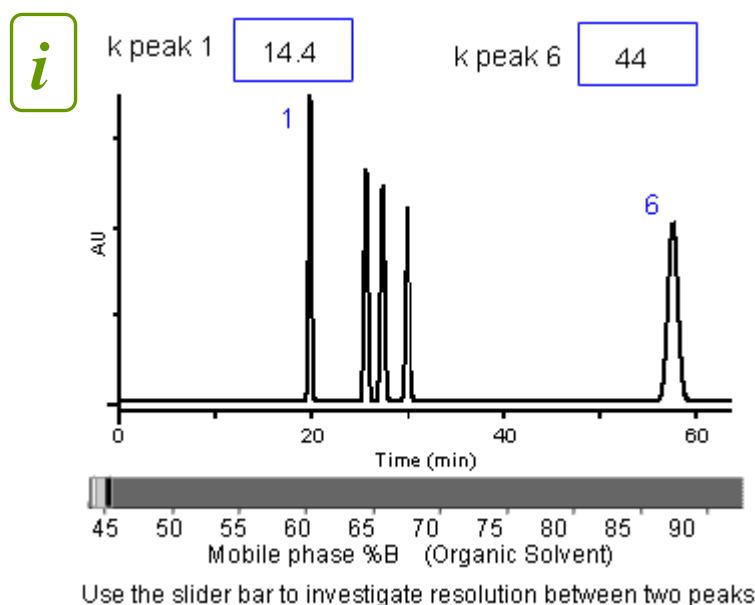
$$t_R = (k \times t_0) + t_0 = \begin{matrix} 2.0 \text{ min} \\ (k = 1) \end{matrix} \quad \text{to} \quad \begin{matrix} 11.0 \text{ min} \\ (k = 10) \end{matrix}$$

How to change Retention (Capacity) Factor

The most effective and convenient way to alter the retention factor of a peak is to adjust the 'solvent strength' of the chromatographic mobile phase. This is usually achieved in reversed phase chromatography by changing the amount of organic solvent (modifier) in the mobile phase mixture.

Characteristically reversed phase HPLC has a non-polar stationary phase, therefore, increasing the polarity of the mobile phase will increasingly repel the hydrophobic (non-polar) sections of the analyte molecules into the stationary phase and the analyte will be retained for longer on the column. The converse is also true as can be seen in the interactive example.

An increase in the organic content of the mobile phase of 10% decreases k for each component by a factor of 2 to 3. Altering the mobile phase composition is a very powerful separation tool to use when optimizing HPLC separations.



Effect on Retention Factor (k) values of changing mobile phase organic composition

Analytical conditions:

Column: C18, 15 cm \times 0.46 mm \times 5 μ m

Flow: 2.0 mL/min

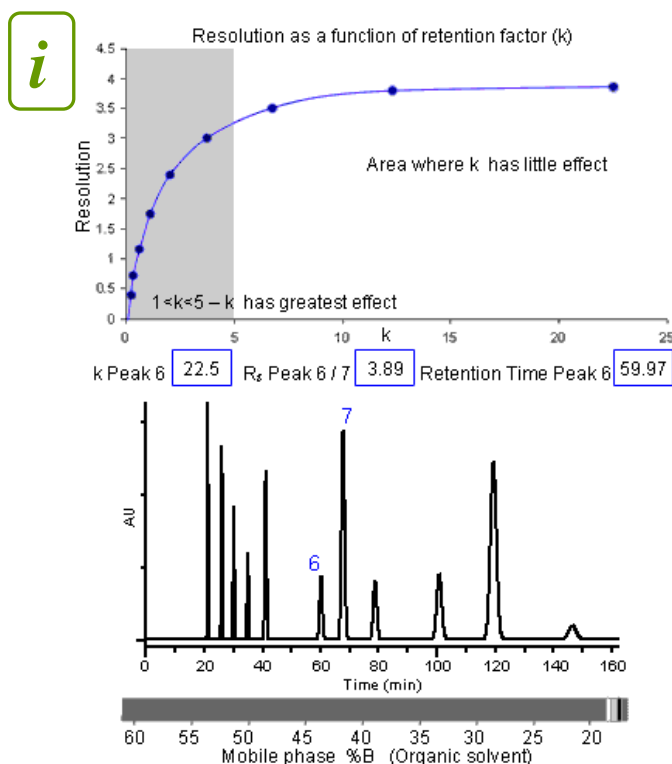
t_0 : 1.28 min

At low mobile phase organic solvent composition, the retention factor is high – the analytes are interacting strongly with the stationary phase

Increasing the organic composition by 10%B brings about a 2-3 fold reduction in retention factor

When retention factors are very high or very low, the quality of the separation is reduced. Retention factors below 1, for any of the analytes, generally indicate that the separation will be poor

Effects of Retention Factor on Resolution



Effect of Altering Retention Factor (K) (by altering mobile phase composition) on Resolution

The On-line course contains an interactive Resolution experiment (comprising the two peaks)

$$R_s = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

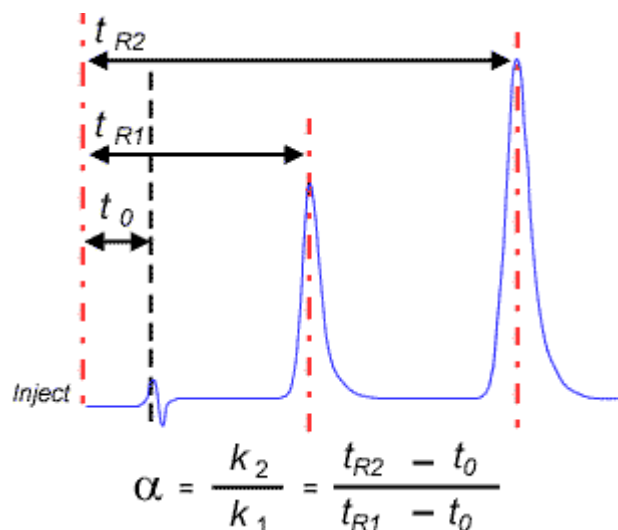
The largest gain in resolution is achieved when the k value is between 1 and 5. As k is very simple to change it is often worthwhile adjusting the k range of peaks within the chromatogram to obtain an optimum resolution

k values less than 1 are unreliable as analytes may be eluting with other sample components or solvent.

Above a k value of approximately 5, increasing retention only provides minimal increases in resolution. Too much retention wastes valuable analysis time and the chromatographic peak height will decrease as the bandwidth of the peaks increases. For more complex sample mixtures, the useful k range may be extended to $2 < k < 10$. If you have not achieved the desired resolution and the k values of your sample components are above 10, you will find that increasing the selectivity or efficiency of your separation will be more useful.

Selectivity (Separation) Factor (α)

The selectivity (or separation) factor (α) is the ability of the chromatographic system to 'chemically' distinguish between sample components. It is usually measured as a ratio of the retention (capacity) factors (k) of the two peaks in question and can be visualized as the distance between the apices of the two peaks.



Determination of Selectivity (α)

High α values indicate good separating power and a good separation between the APEX of each peak. However, the alpha value is NOT directly indicative of the resolution.

By definition, the selectivity is always greater than one – as when α is equal to one, the two peaks are co-eluting (i.e. their retention factor values are identical). The greater the selectivity value, the further apart the apices of the two peaks become.

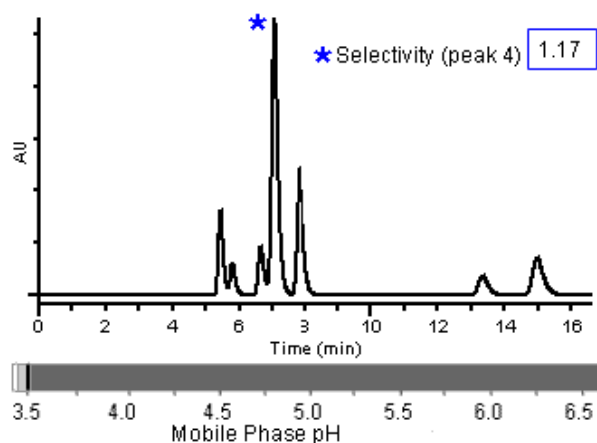
As the selectivity of a separation is dependent upon the chemistry of the analyte, mobile, and stationary phases all of these factors may be altered in order to change or optimize the selectivity of an HPLC separation.

How to change Selectivity (Separation) Factor (α)

Some of the many factors that can be used to manipulate the selectivity of HPLC separations are shown in Table 1.

Table 1. Parameters affecting selectivity in reversed phase HPLC

Parameter	Usage
Sub 2: Organic solvent	Changing to a different solvent (e.g. methanol to acetonitrile in reversed phase HPLC) will alter the selectivity
Sub 3: Mobile phase pH	Can alter the degree of ionization of some analytes – affecting their hydrophobicity
Sub 4: Solvent strength and additives	Can be adjusted to affect selectivity as well as retention (capacity) factor
Sub 5: Stationary phase	One of the most popular ways to alter the selectivity of a separation
Sub 6: Temperature	Can have an effect with certain analytes in reversed phase and Chiral HPLC



- The mobile phase pH can have a drastic effect on the selectivity of a separation, especially when acidic or basic analyte molecules are involved
- For acidic analytes the pH range 2.5 to 6.5 is particularly important
- Small changes in pH can bring about large changes in retention behavior of certain peaks within the chromatogram
- For this particular separation, it would be difficult to identify a single pH value at which all peaks are satisfactorily resolved

Effect on Separation Factor (α) values of changing mobile phase pH

The On-line course contains an interactive Selectivity experiment (in which selectivity is affected by changing the pH)

Analytical conditions:

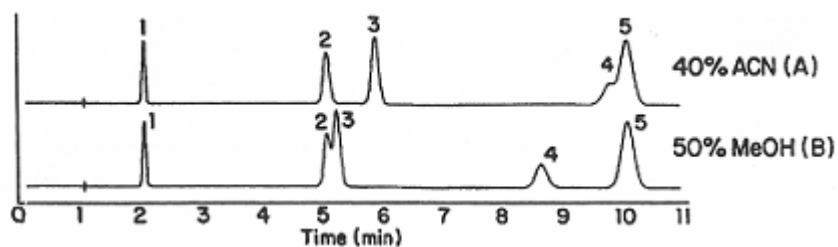
Column: C18, 15 cm × 0.46 mm × 5 μ m

Flow: 1.0 mL/min

t_0 : 1.68 min

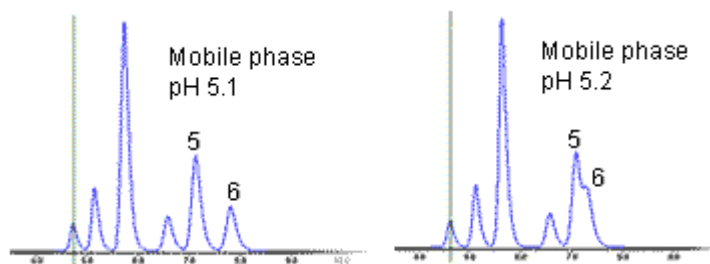
Organic Solvent Type

In this example, the organic solvent has been changed, and the solvent strength altered to make the eluent systems **isoeluotropic** (i.e. the k value of the last peak is the same in both cases). It can be clearly seen that although the overall analysis time is the same, the selectivity of the separation between peaks 2 and 3 and peaks 4 and 5 is different when methanol is substituted for acetonitrile.



Effect of changing organic modifier type on chromatographic selectivity in Reversed Phase HPLC

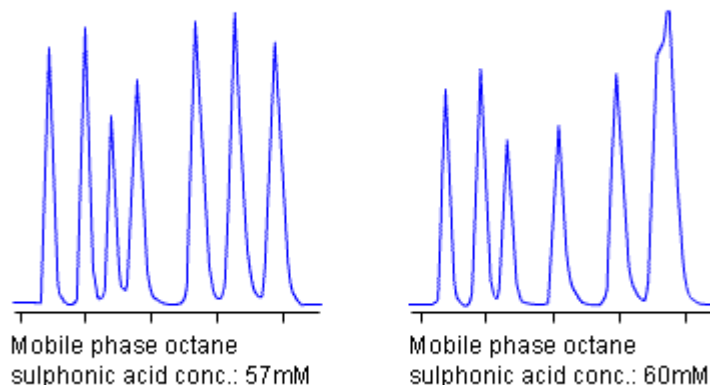
Mobile Phase pH



Effect of changing mobile phase pH on chromatographic selectivity in Reversed Phase HPLC

In this reversed phase separation of acidic analytes on a C8 column it is clear that a change in pH of just 0.1 units can bring about a substantial change in selectivity between peaks 5 and 6.

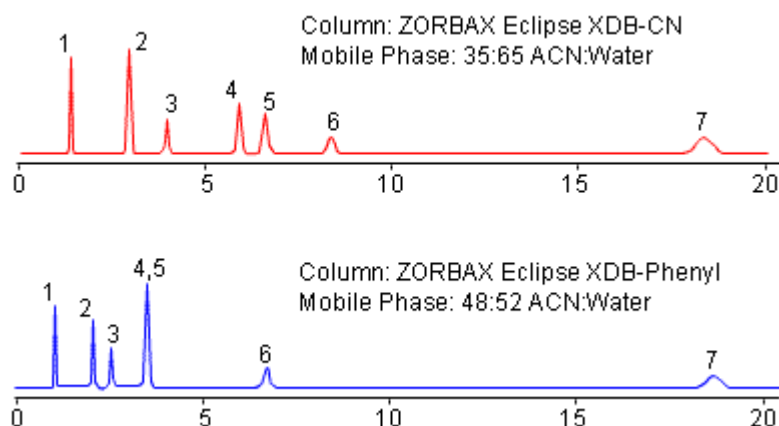
The pH of the mobile phase is usually a key parameter for selectivity optimization when dealing with analyte molecules that have **ionizable groups**. Changes in mobile phase pH should be undertaken carefully as not all silica based HPLC columns are resistant to extremes of pH.



Changes in separation selectivity caused by small alterations to mobile phase ion-pair concentration in reversed phase HPLC

In this example of changing selectivity, **octanesulfonic acid** (an **ion-pairing reagent**), has been added to the mobile phase in differing concentrations. As can be seen, even small adjustments in ion-pair concentration can produce large differences in the chemical separating power of the chromatographic system. The analytes in this case are basic pharmaceutical compounds, separated on a C18 column.

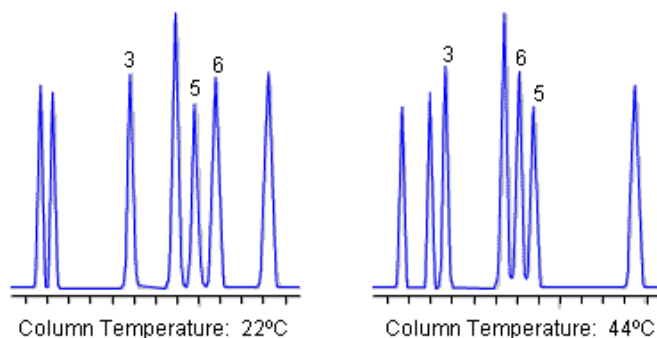
Stationary Phase Type



Changes in separation selectivity caused by altering the chemical nature of the stationary phase in reversed phase HPLC

Here we can see how changing the column stationary phase can affect the selectivity of a separation. The hydrophobicity, polarity, and nature of the base silica all play a critical role in the physicochemical interaction with the analyte. As a chromatographer one of the most powerful options to change a separation will come from changing the stationary phase. Picking the correct stationary phase is amongst the most important choices to be made when developing methods.

Column Temperature



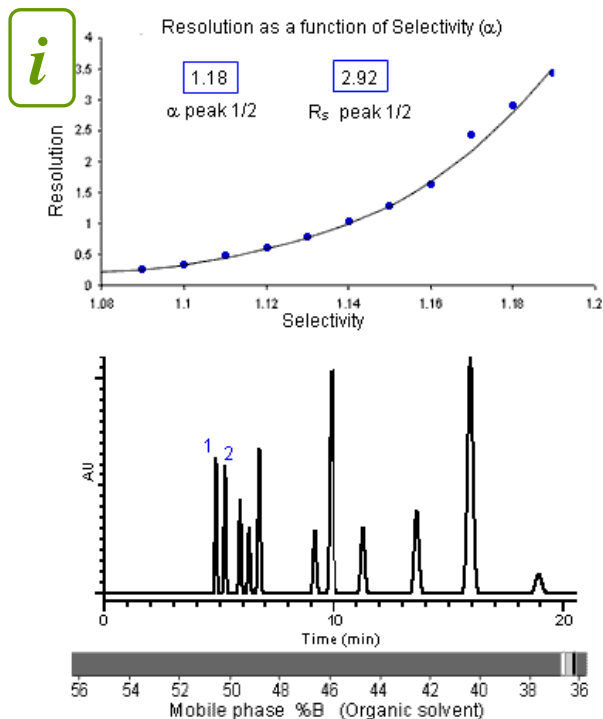
Changes in separation selectivity caused by altering the column temperature in reversed phase HPLC

Temperature of the column (and hence the mobile phase) is used in this example to alter the selectivity of the separation. It is interesting to note that peaks 5 and 6 have swapped as the temperature is adjusted.

Temperature produces the smallest changes in selectivity; however, it can be usefully used to optimize separations, especially when the analytes involved are ionizable.

Effects of Selectivity (α) on Resolution

The graph shown illustrates the effect of changing the selectivity (separation factor) on the resolution between two early eluting peaks in the chromatogram.



- Changing selectivity can have a dramatic effect on the chromatographic resolution
- Selectivity is relatively simple to alter, with mobile phase constituents (solvent type, ion pair reagents etc.) and pH being the most frequently used methods of adjustment
- If suitable resolution cannot be achieved by altering the mobile phase constituents, an alternative column chemistry should be investigated as a means of altering the selectivity of the separation

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

Efficiency Selectivity Retention

Effect of Altering Selectivity (α) (by altering mobile phase composition) on Resolution (R_S)

In this example the selectivity has been altered using the solvent strength of the mobile phase. It can be seen that above α values of around 1.1, the resolution begins to increase exponentially.

Altering the system selectivity provides an excellent means of optimizing the chromatographic resolution as small changes in selectivity can lead to large changes in resolution.

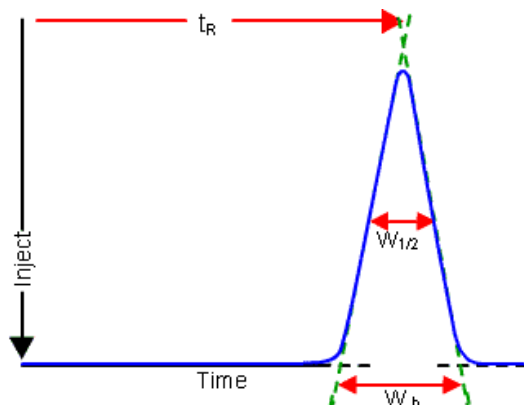
Efficiency

The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travels 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.

The plate number (N) is a measure of the peak dispersion on the HPLC column, which reflects the column performance. Efficiency is derived from an analogy of Martyn and Syngé 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 are possible, and the better quality the separation.

$$N = 16 \left(\frac{t_R}{w_b} \right)^2 = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2$$



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 eluting peaks may have a higher plate count.

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

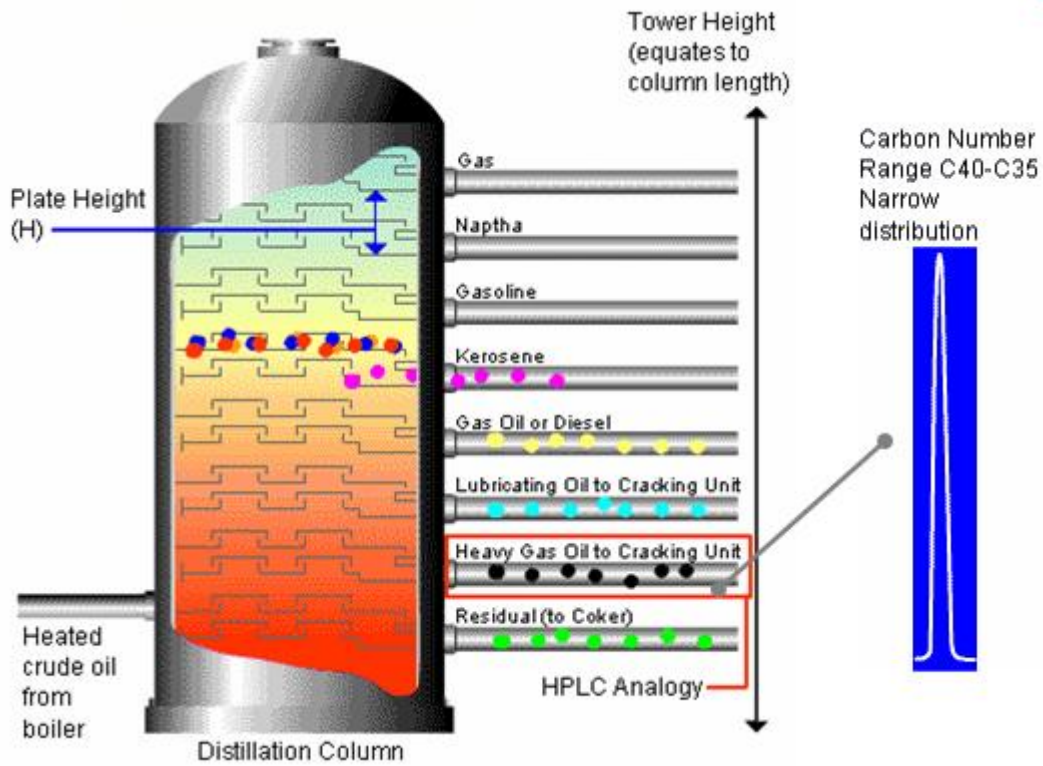
Determination of Efficiency (N)

The method for calculating column efficiency is shown. A typical plate number for a 4.6 × 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. This links directly to the concept of 'Plate Height', which is explained in the following sections.

Factional Distillation Model of Efficiency Theory

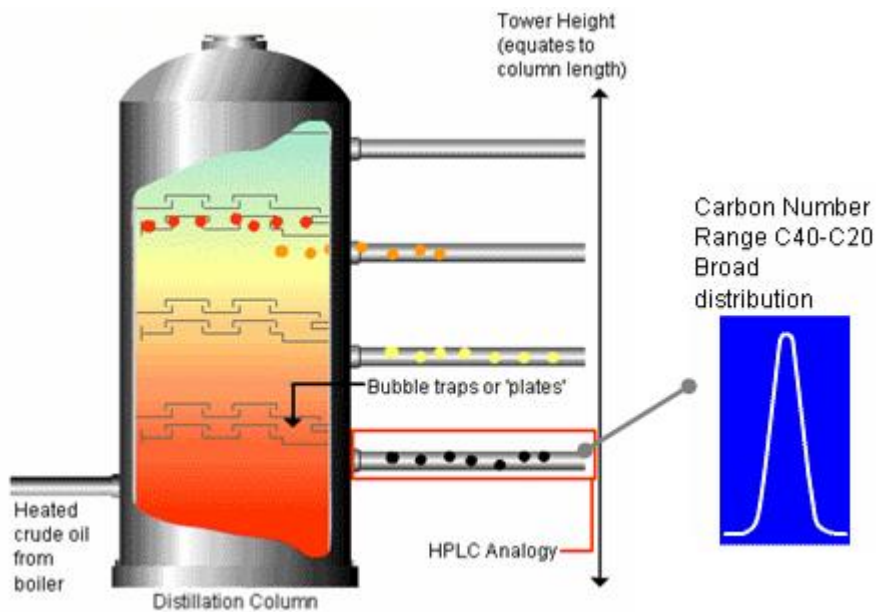
The more plates (N) there are the narrower the distribution of carbon numbers from each trap (or plate). Therefore, the higher the number of plates (N) the narrower the 'peak' obtained from that trap – this can be directly related to the peak 'efficiency' in HPLC where a column with a high number of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

i



High Efficiency Distillation Process

i



Low Efficiency Distillation Process

Similarly, for a fractionating tower of a given length (L), the higher the number of plates, the lower 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:

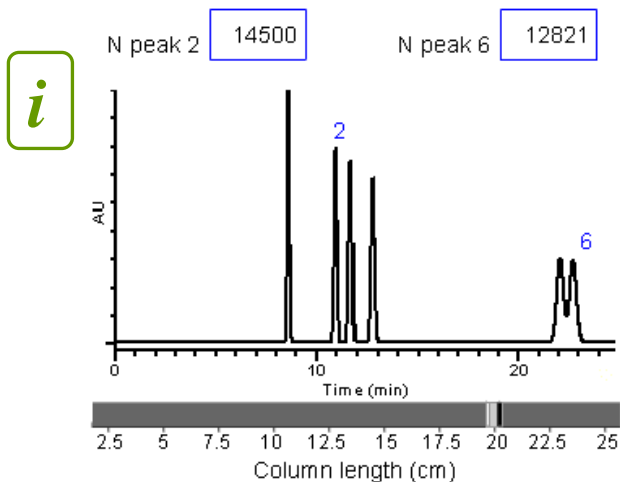
$$H = \frac{L}{N}$$

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 peak broadening– 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, the column dimensions, and any voids that may have formed within the column all play a part in determining the column efficiency.



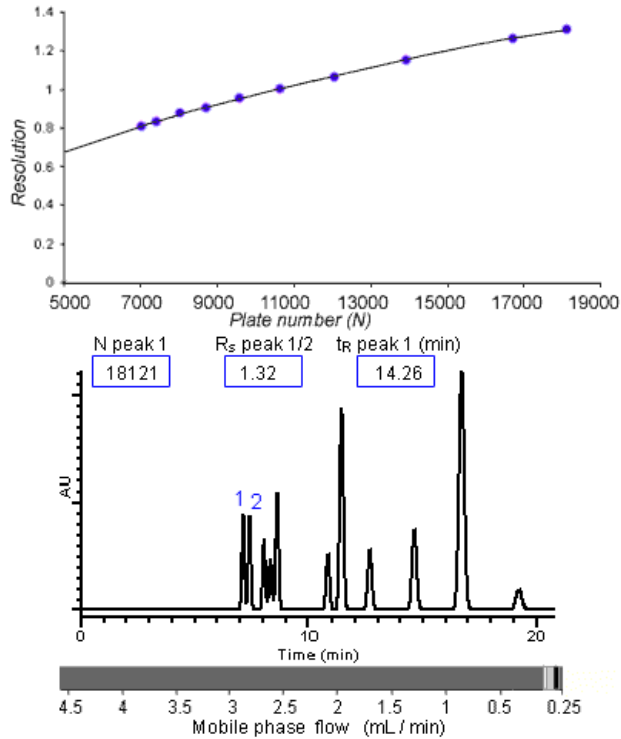
Effect on Separation Factor (α) values by changing the column length

- 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 25 cm) 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 x 0.46 cm column with 5 μ 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

Effect of Efficiency on Resolution



Effect of Altering Efficiency (N) (by altering mobile phase flow rate) on Resolution (R_s)

- In this example, efficiency increases as flow rate is reduced – it should be noted that this cannot be assumed as will be explained later
- 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:

Doubling column length, doubles efficiency, doubles analysis time but only increases resolution by a factor of 1.42 times

$$R = \underbrace{1/4 \sqrt{N}}_{\text{Efficiency}} \times \frac{\alpha - 1}{\alpha} \times \frac{k'}{1 + k'}$$

Selectivity
Retention

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

The chromatograms show the separation of various real life test compounds (A and B) and steroids (C). In each case chromatograms are shown for the 'before' and 'after' situations – the aim was to improve resolution using the parameters we have studied in this section.

Select which of three parameters k , N and α , has been altered to achieve the change in resolution.

The aim of the exercise is to highlight the possibilities in achieving increased Resolution and how this would be achieved in a practical situation. You should choose the 'easiest'

way to achieve the changes in a practical situation. You need to achieve a tick in each box in order to proceed.

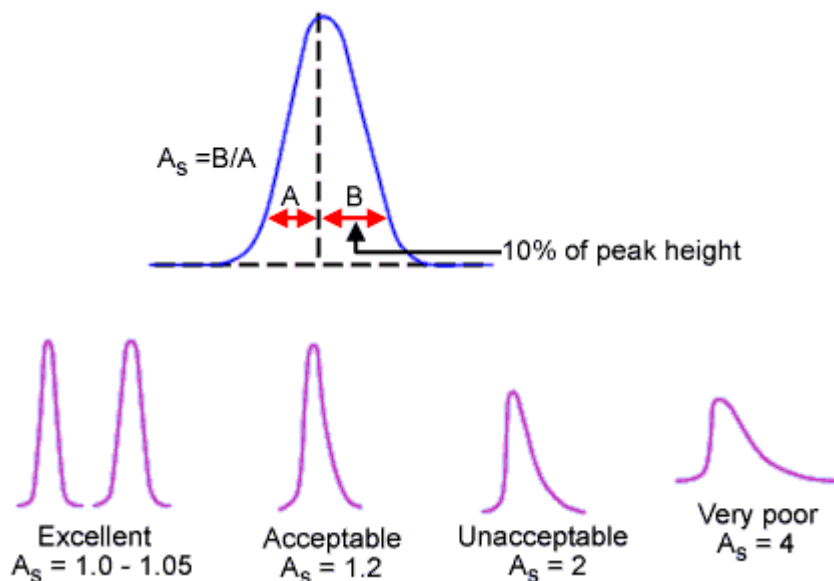


Interactive tutorial exercise – see on-line course for full details

Peak Asymmetry

In the ideal world all chromatographic peaks would be symmetrical (or Gaussian).

However, due to the effects of instrument dead-volume, adsorptive effects of the stationary phase and the quality of the column packing, peaks may often show a tailing behavior. Tailing describes a peak whose tail portion (distance 'B' in the diagram) is wider than the front portion (distance 'A' in the diagram). Also, if the sample concentration is too high or if the column is damaged and contains 'channels' then a fronting peak shape may occur.



Determination of Peak Asymmetry (A_s) and examples of good and poor peak shape

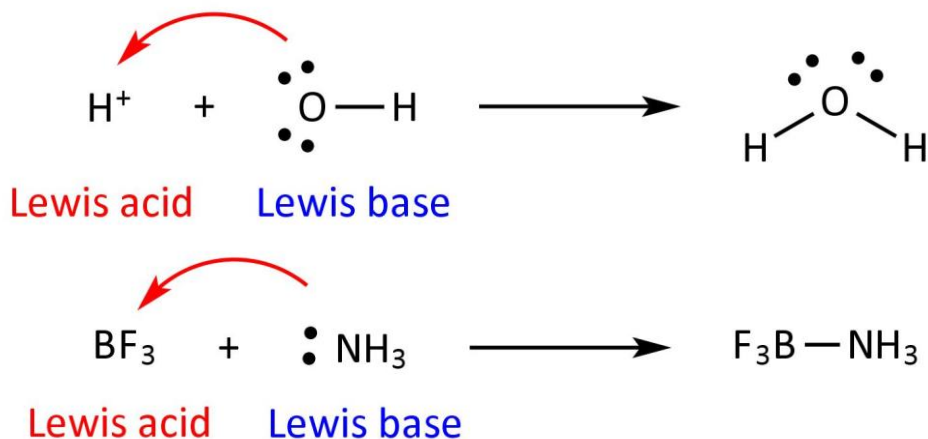
Asymmetric peaks often present problems with resolution and quantitation of the peaks within the chromatogram. Asymmetrical peaks are more difficult to resolve, therefore, integration of the peak to provide a peak area for quantitation will also be much less reproducible.

Often, chromatographers will set limits for peak asymmetry beyond which chromatography will be deemed unsuitable. Some examples are shown for reference.

Glossary

Isoeluotropic – mobile phase compositions that produce a separation within the same time are known as isoeluotropic. In HPLC, an eluotropic series is a list of solvents or mixtures listed in increasing order of being able to displace and analyte from the stationary phase, and hence, elute it from the column.

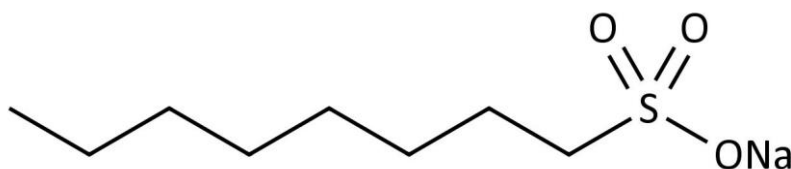
Ionizable groups – any group within an analyte molecule that is capable of acting with acidic or basic properties over a changing pH range. Brief definitions of acidic and basic behavior are:



Lewis acid: A molecule that can act as an electron-pair acceptor, such as the H^+ ion

Lewis base: A molecule that can act as an electron-pair donor, such as ammonia NH_3

Octanesulfonic acid – a popular ion-pair reagent for the analysis of basic analytes.



Octane sulfonic acid sodium salt

Ion-pair reagent – an ion pair reagent is usually an ionic compound containing a long hydrophobic chain which imparts some hydrophobic character on the ion-pair complex allowing it to be retained, and therefore, separated on a reversed phase HPLC column. The formation of ion pairs is promoted by adjusting the pH to 7.5 for acids and 3.5 for bases.

