Back to Basics

Explaining Resolution

2020

What is Resolution?

Chromatography is the act of separating components in a mixture. There are different measurements to determine the success of a separation, one of which is resolution (R_s). Resolution is determined between two peaks of interest, which are usually adjacent. The least resolved peak pairs are known as the critical pair and the aim is to achieve baseline resolution between all peaks. Baseline resolution is required in order to have accurate quantitation of peaks, which is where the detector trace returns to baseline between the peaks. This value must be greater than 1.5 to be classed as baseline resolved, however, during method development, the required value is often greater (>2.0).

In practical terms, resolution is regularly measured using Equation 1 or 2, although there are many different variations available. The two displayed are the approved methods determined by the (Eq. 1) United States Pharmacopeia (USP) and (Eq. 2) European Pharmacopeia (EP). The two differ by the position the width measurement is taken (Figure 1) and the adjustment factor associated with that width. These values can often be calculated using LC software, however, it is important to know which calculation is being used. Within LabSolutions, Equation 1 is set as standard, however, it also allows the user to define which method they would like to use for their analysis. As resolution is related to the retention time and width, the value of resolution can be increased by increasing the peak spacing between the two peaks, or by decreasing peak widths to have narrower peaks.

$$R_s = \frac{2 \times (t_{R2} - t_{R1})}{W_{b1} + Wb_2} \qquad \textit{Eq. 1}$$

$$R_s = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{W_{\frac{1}{2}1} + W_{\frac{1}{2}2}} \right) \qquad \textit{Eq. 2}$$

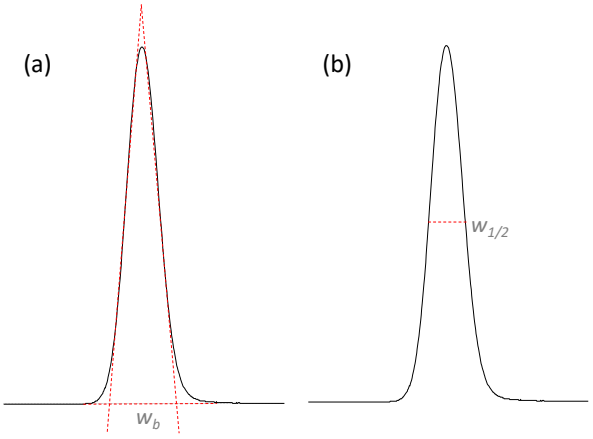

Figure 1 Position of W_b with inflection lines (used in Eq. 1) and $W_{1/2}$ used to measure resolution (used in Eq. 2).

Although it is not necessary to remember every resolution calculation, it is worth knowing or understanding the fundamental resolution equation. This equation, otherwise known as the Purnell equation, describes the underlying parameters responsible for a separation and can assist in improving resolution. Optimisation of these parameters is essential to obtain suitable resolution between critical pairs.

What is the Fundamental Resolution Equation?

The fundamental resolution equation, which is applicable for isocratic separations, is comprised of three components; 1. Efficiency, 2. Retentivity and 3. Selectivity (Equation 3), where *N* is efficiency or column plate count, *k* is retention factor and α is selectivity value. For gradient separations, there is a marginally different equation applied, to take into account the changing mobile phase composition. This will not be discussed in this article.

$$R_s = \underbrace{\frac{\sqrt{N}}{4}}_{1} \times \underbrace{\left(\frac{k}{k+1}\right)}_{2} \times \underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{3} \qquad \textit{Eq. 3}$$

Each component of the equation can be separately plotted against resolution to elucidate the impact (Figure 2). Selectivity (α) gives a linear response to resolution, whilst both efficiency (*N*) and retentivity (*k*) both provide curved responses.

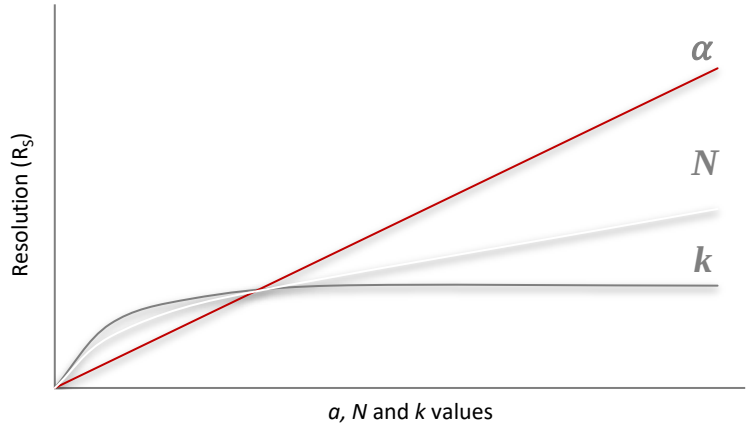

Figure 2 *Effect of selectivity (α), efficiency (N) and retentivity (k) on resolution (R_s).*

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk

1. Efficiency Term

Efficiency (*N*), or column plate count, describes the ability of a column to produce narrow peaks. The more efficient the column, the narrower the peak widths which can be achieved. The narrow peak widths are crucial for obtaining acceptable resolution, as illustrated in Figure 3. The two peaks have the same retention time, however, in Figure 3(a), the peaks are considerably narrower than the peaks in Figure 3(b). Thus, the critical pair are baseline resolved in (a) due to the more efficient separation, whereas the peaks are not baseline resolved in (b).

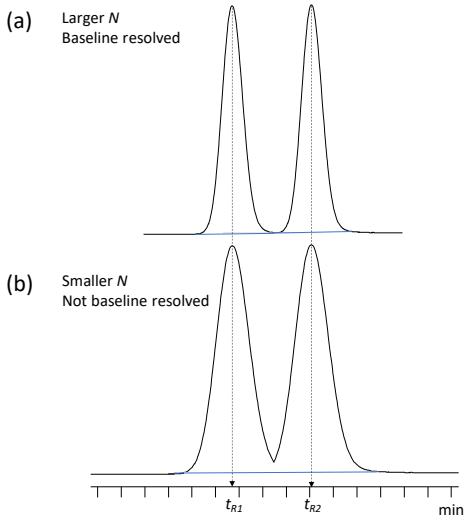

Figure 3 Comparison of two peaks separated on a column with (a) larger N, and (b) smaller N (i.e. (a) utilises a smaller particle size than (b)). With the lower efficiency in (b), the two peaks no longer have baseline resolution as the peak widths are greater than those in (a), despite having the same retention time.

There are various means of improving the efficiency, such as:

- Increase the column length.
    - N is proportional to column length, therefore as column length is increased, the efficiency increases (Equation 4). If the column length is doubled, the resolution should increase by a factor of ~1.4 (Equation 5). However, an increase in column length also increases the run time and pressure. Thus, there is a compromise which needs to be made between resolution, run time and pressure constraints of the column and LC instrument.

$$N = \left(\frac{1}{H}\right) L \qquad Eq.\ 4$$

Where H is the column plate height (a measure of column efficiency per unit length of the column).

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk

$$R_s \propto \sqrt{N} \qquad Eq.\ 5$$

- Decrease the particle size (d_p).
    - Efficiency is inversely proportional to the particle size (Equation 6). As the particle size decreases, the efficiency increases. However, as with the increased column length, the pressure can increase. HPLC analyses often employ 3, 5 and 10 µm totally porous particle (TPP) sizes on lower pressure systems (such as Shimadzu's Prominence series <440 bar). However, to utilise smaller particles, such as 1.8 and 2 µm TPP and 2.7 µm superficially porous particles (SPP), specialist instruments known as uHPLC (such as Nexera-I or XR) and UHPLC (such as Nexera XS or X3) are required which have greater pressure limits (<660 and <1300 bar systems, respectively)

$$N \propto \frac{1}{d_p} \qquad Eq.\ 6$$

- Use an appropriate flow rate.
    - Each column has an optimum flow rate to obtain the most efficiency. This is related to the mobile phase mass transfer of the analyte band. This information is often displayed in a van Deemter plot.

Remember
Improving the dispersion of an LC system can assist in minimising peak broadening which can help to reduce the peak width. For assistance in understanding and measuring dispersion, see our useful "Back to Basics: Dispersion" protocol.

2. Retentivity Term

Analytes must interact sufficiently with the stationary phase to obtain adequate retention. In reversed-phase isocratic separations, this is often achieved by altering the organic component in the mobile phase (i.e. decrease organic, increase retention).

Retention times can be normalised to compare between different column formats and operating conditions by using retention factor (k) which removes the column volume contribution (Equation 7), where t_0 is the retention time of an unretained peak (normally water).

$$k = \frac{t_R - t_0}{t_0} \qquad Eq.\ 7$$

It is regularly stated in literature that k values of analytes should be between 2-10. The graph in Figure 2 explains why this is the case. If the k value is below 2, there is insufficient retention due to poor interactions with the stationary phase surface and limited resolution. There is also the potential that peaks of interest are affected by matrix effects which elute near the void of the column.

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk

However, *k* should be less than 10, as there is little gain in resolution above 10 (approximately where the three traces cross in Figure 2). A *k* of greater than 10 will mean excessive elution times, thus prolonged run time and greater solvent consumption, as well as increased band spreading and decreased peak height.

3. Selectivity Term

The greatest potential for increasing resolution is to increase selectivity (Equation 8). Although with the advent of UHPLC which offers improved efficiencies, the *N* term only provides diminishing returns in resolution, therefore it is important to also have good selectivity to optimise resolution. Selectivity is a measure of the degree of separation between two peaks of interest. A value of 1 equates to coelution, thus unresolved peaks.

$$\alpha = \frac{k_2}{k_1} \qquad Eq.\ 8$$

Selectivity can be changed by varying:

- Column type
  - Selecting a different stationary phase is one of the most effective methods to alter selectivity in reversed-phase chromatography. There is a plethora of stationary phases available which offer different modes of interaction which can alter selectivity.
- Organic modifier
  - Altering the type of organic modifier is also considered one of the most effective means of changing selectivity. The most common solvents employed in reversed-phase separations are methanol and acetonitrile.
- Organic composition (%B)
  - The organic composition not only changes the retentivity of compounds but can also alter elution orders, hence selectivity changes. The selectivity differences aren't as dramatic as utilising different stationary phases or organic modifier but is still an effective tool.
- Temperature
  - Temperature can be very effective to fine tune a separation, but it is often considered less important than other factors assessed for neutral species.
- Mobile phase pH (for ionisable species)
  - The pH of a mobile phase should be 2 pH units above or below the pK_a of the ionisable species analysed, which is often a compromise for a mixture. In general, most methods utilise a pH which places the analyte in the unionised form, however, switching to the ionised form will alter the retention mechanisms and therefore possibly the selectivity. However, it is important to be aware of any pH restrictions for the stationary phase.

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk

- Buffer concentration
    - The buffer concentration can offer some differences in selectivity for ionisable species. Basic and acidic analytes may behave differently with a change in buffer concentration due to the different retention mechanisms.

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk

Shimadzu UK Ltd.
Mill Court
Featherstone Road
☎ : +44 1908 552209
Web : www.shimadzu.co.uk