Chromatography Today Help Desk

Can Chromatography Theory Really Help Improve My Separation?

Introduction

Chromatography is a science and having a thorough understanding of the theory behind the science can have substantial benefits for optimising the separation, and for understanding why improvements in the separation process are occurring. This article will go through the method development process for a particular separation, investigating the theoretical interpretation of the practical observations. The separation under investigation is the separation of a polystyrene standard, PS580, using gel Permeation Chromatography (GPC), which is a form of size exclusion chromatography, where organic solvents are predominantly used to allow for the separation of polymers.

Size exclusion chromatography is not based on chemical interactions between the compound of interest and the stationary phase, but rather a molecular size based separation based on the hydrodynamic volume of the analytes that are being investigated. For true SEC/GPC, it is important that there are no chemical interactions occurring, and so the stationary phase is typically designed to reduce any form of chemical interactions, and this is combined with the use of a solvent that will reduce any potential interactions. The types of interactions that can occur will be very dependent on the analytes under investigation and also the nature of the stationary phase. The interactions can, however, be broadly based into three categories, non-polar, polar interactions and electrostatic interactions. The column being used can exhibit polar interactions, however the sample is a polystyrene sample and so would only retain on the surface by a non-polar, hydrophobic mode of interaction. The mobile phase is typically chosen to also reduce the interactions between the stationary phase and the analyte, thus for protein separations, where polar interactions can be prominent the use of high buffer concentrations is typical to reduce the amount of chemical interactions. It is also evident from this statement that gradients are not performed in SEC, since there is a potential to increase the interactions between the stationary phase and the analyte. One advantage of this approach is that detectors which require the use of isocratic mobile phases to generate a consistent baseline can be used. Examples of detectors which benefit from the use of isocratic mobile phases are Evaporative Light Scattering Detectors (ELSD), Charged Aerosol Detectors (CAD), and Refractive Index (RI) detectors. It should be noted that there are other approaches which can be employed that allow the use of these detectors in a gradient mode, such as the use of a secondary pump that runs a reverse gradient into the column eluent, ensuring that the final mobile phase composition reaching the detector is consistent.

The resolution between two peaks in a chromatogram can be defined as;

$$R_s = \frac{2(t_1 - t_2)}{(w_1 - w_2)}$$

Where;

t – is the retention time of the compound

w – is the width of the peak at the baseline

In both cases the subscripts relate to the first or second peak that is eluting.

This equation can be modified, using a few reasonable assumptions, into the Purnell equation. When optimising a separation, a thorough understanding the Purnell equation will give a better insight into why this can be important for resolution;

The Purnell equation is given by;

$$R_s = \left(\frac{1}{4}\right) N_2^{0.5} \left(\frac{k_2}{1+k_2}\right)\left(\frac{\alpha - 1}{\alpha}\right)$$

Where;

R_s – Resolution between two peaks.

N – Chromatographic efficiency of second peak, peak width relative to the retention time, subscript refers to the second peak.

k – Retention factor, dimensionless retention time, of second peak.

α - Separation factor between the two peaks, defined by the ratio of the retention factors of the two peaks.

The Purnell equation states that the resolution can be improved by increasing the chromatographic efficiency (N) of the HPLC system, so assuming that there is a difference in the hydrodynamic radius of the components that make up the polystyrene standard, increasing the efficiency of the chromatographic system will eventually result in separate peaks, with further improvements of the efficiency improving the resolution of those peaks.

It is interesting to note that this equation is often misquoted and that the subscripts are often missed, which in most cases does not have a significant effect on the determination of the resolution, since in most cases the peaks being discussed are quite close in terms of elution. A nice overview of the various forms of the resolution equation is given by Rozing [1]. It should also be noted that it is the efficiency is of the whole chromatographic system and not just of the column, this is important when using very high-performance columns, since any separation could be nullified by extra-column variances, such as inappropriate tubing, wrongly fitted connectors, large detector cell volumes, etc. Another consideration is that in an isocratic system, where there are no peak focussing effects, the minimum peak width will be determined by the injection volume, thus the initial injection volume will determine the optimal efficiency of the separation. Thus, the theory can give guidance into how to drive the separation, by minimising the dispersion processes within the chromatographic system.

Injection Volume

The effect of the injection volume can be seen in Table 1, where two injection volumes are compared. The initial injection volume was 5 µL, but by reducing the injection volume to 1 µL and increase in the efficiency of nearly 10% was seen, Table 1.

Table 1. The effect of varying injection volume on the chromatographic efficiency. Reducing the injection volume to 1 µL, resulted in nearly a 10% improvement in efficiency.

Injection Volume	Efficiency, plates/m
5 µL	83600
1 µL	90033

Investigation of Tubing and Data Sampling Rate

The efficiency is a measure of the dispersion processes within the whole chromatographic system, and optimising the extra-column variances will reduce the peak widths. It is quite common to standardise the connecting tubing between the various modules on a HPLC system, and there are a variety of different i.d. tubing that can be used. Figure 1 shows the effect of varying the tubing from the original experimental configuration which used red tubing (i.d. 0.12 mm), compared to black tubing (i.d. 0.075 mm). The tubing was replaced throughout the system replacing the tubing will affect the pressure across the chromatography system, and in gradient mode the location of the tubing which is replaced can have an effect. In a gradient mode, the smallest dead volume tubing should be placed after the column to minimise dispersion effects, although dwell volumes also need to be considered in this scenario as well. The theory would dictate that reducing dispersion (increasing the N term) will result in an improvement in the resolution, assuming the compounds are not co-eluting.

The number of data points associated with a peak is also an important consideration. A peak that is poorly defined due to a low sampling rates will cause a reduction in the performance of the separation of the analytes after they have left the column. Increasing the sampling rate results in an improved peak definition and so the point at which the peak starts and finishes can be better defined. Low data sampling rates will over-estimate the peak width and will result in the observed peak width not matching what is happening within the column. The limitation of increasing the data sampling rate can be associated with the detector, and some detectors do not have a very fast data sampling rate, some types of mass spectrometers are a good example here. Also as the data sampling rate is increased the amount of storage space for the chromatogram will increase, in general this may not be a limiting issue, unless spectral data is also being collected, so the use of diode array detectors or mass spectrometers can result in the generation of very large files if very high data sampling rates are used.

The combination of these results is quite dramatic as seen in Figure 1.

Effect of Column Length

In SEC, the partitioning is ideally driven by entropic processes and not by enthalpic/adsorption processes as was noted earlier. The Gibbs free energy can be used to determine the relative partitioning of the analyte between the mobile and the stationary phase, this equates to the retention time, with the generalised equation given by the Gibbs-Helmholtz equation;

$$\Delta G = \Delta H - T\Delta S$$

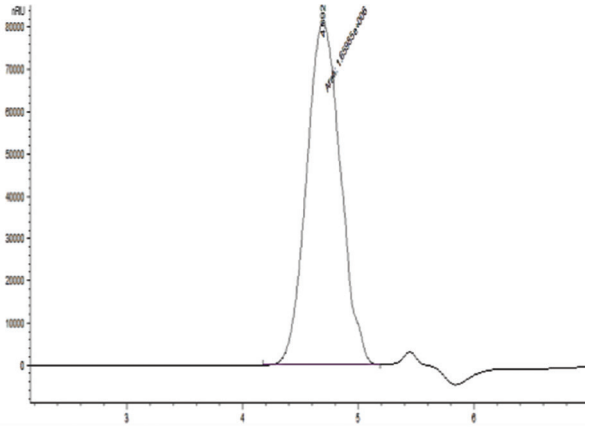

Figure 1a

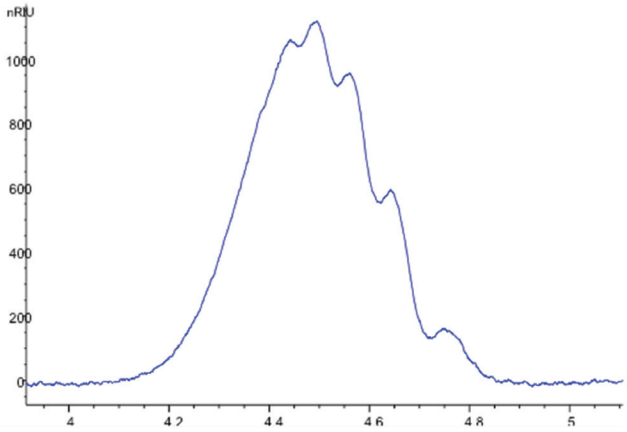

Figure 1b

Figure 1. Effect of lowering the injection volume, reducing the tubing length and diameter, and also altering the data sampling rate. The conditions for the left figure are; 100% THF, 0.35 ml/min flow rate, 5µl (PS-580) injection volume, column temperature 23°C, mRID 4 s response time, 10 minutes running time, with the experimental parameters for the right figure; 100% THF, 0.35 ml/min flow rate, 0.1 µl (PS-580) injection volume, Column temperature 23°C, mRID 0.13 s response time.

Where G – Gibbs free energy

$\boldsymbol{H}$ – heat of enthalpy which is assumed to be zero for SEC

$\boldsymbol{T}$ – thermodynamic temperature in K

$\boldsymbol{S}$ - Entropy

And since it is possible to relate the Gibbs free energy to the retention time, the following equation can be derived;

$$LnK_D = \left(\frac{\Delta S}{R}\right)$$

Where;

$\boldsymbol{R}$ - Universal gas constant

$\boldsymbol{K_D}$ – thermodynamic retention factor

It is important to note that the thermodynamic retention factor differs for different sized molecules, since they will have different levels of access to the available pore structure of the stationary phase.

From this it is possible to determine an equation that relates to the separation factor, which is given by;

$$Ln\alpha = \frac{\Delta S_1}{\Delta S_2}$$

These equations can be used to drive the resolution, since the resolution comprises both a retention factor term and also a separation factor term. So, for a SEC based separation increasing the entropy term, which is achieved by increasing the difference in pore volume will improve the separation.

Another analogous retention factor has also been described previously and this relates the available volume for an analyte to the interstitial volume;

$$k^* = \frac{V_{Elu} - V_z}{V_z}$$

Where $\boldsymbol{V_{Elu}}$ - the volume available to the analyte

$\boldsymbol{V_z}$ – total interstitial volume

This retention factor has also been related to the dispersion that occurs in the column through [2];

$$N = \frac{L}{3.5d_p + 1.3\frac{(1+k^*)D_m}{u} + 0.6\frac{k^*}{(1+k^*)^2}}$$

Where;

$\boldsymbol{D_m}$ – Diffusion coefficient for the analyte molecule

$\boldsymbol{L}$ – length of the column

$\boldsymbol{U}$ – the linear velocity of the mobile phase through the column

Thus, it is possible to see that in SEC, increasing the column length, which increases the total pore volume that all 3 parameters are improved within the Purnell equation.

The experimental data to support this statement is given in Figure 2, which demonstrates an increase in retention factor, an increase in efficiency and also an increase in resolution.

L – Length of column

H – Height equivalent theoretical plates, which is a measure of the chromatographic efficiency per unit length of column.

Conclusion

The development of a chromatographic separation can be aided by a greater understanding of the chromatographic processes that are occurring to drive or inhibit the separation. Dispersion is a key parameter that will reduce the chromatographic separation, ensuring that this is minimised will aid the chromatographic separation. This affects the efficiency of the chromatographic system, and since it is not just the performance of the column that is critical in obtaining a separation, it is also important to be aware of the effect that extra-column dispersion processes have on the separation of the sample. The column efficiency can be improved by increasing the length of the column and in this example, it helped to substantially improve the separation.

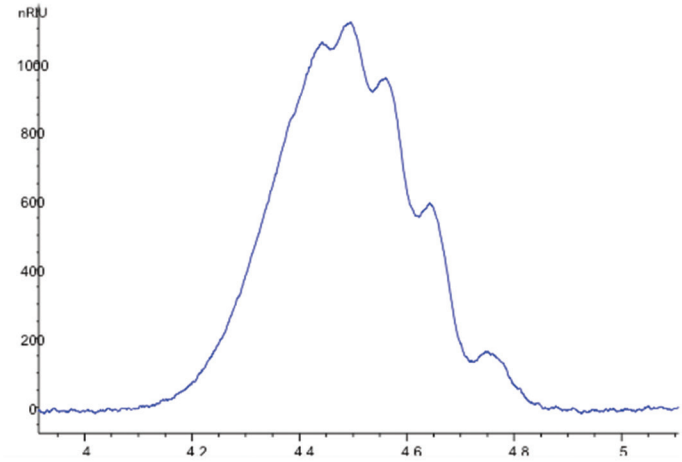

Figure 2a

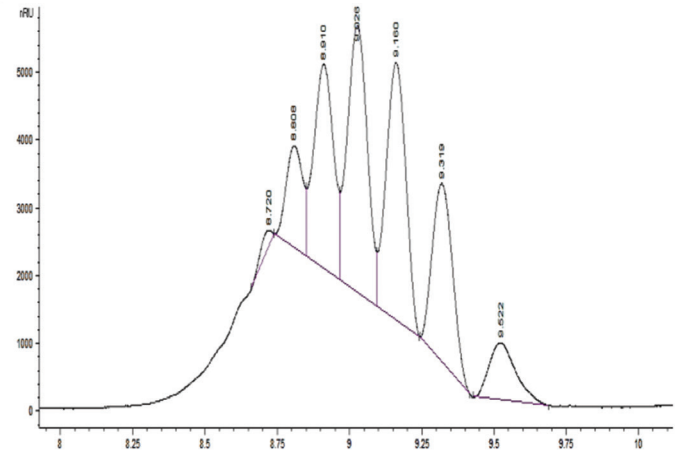

Figure 2b

Figure 2. The effect of altering the column length on the separation, the left-hand figure uses a 150 mm column, compared to the 300 mm column used on the right.

In SEC the total pore volume is also a critical parameter for obtaining resolution and using defined equations it was possible to demonstrate that theory could predict how to get a better separation, by improving all three components of the Purnell equation.

References

1. Rozing G. https://www.rozing.com/resolution-equations.html

2. http://www.chem.purdue.edu/courses/chm321/Lectures/Lecture%2038%20(12-5-2008).pdf