Chromatography Solutions

Knowledge note #0007

Gradient Chromatography and *k**

INTRODUCTION

Gradient chromatography, where the % organic is changed from an initial value to a higher value over a set time (gradient time), is a versatile technique which can be used to separate analytes with a wide range of logP values and is well suited to the analysis of complex samples. This Knowledge Note introduces the key concepts of gradient chromatography and explains how to determine appropriate gradient conditions for a separation.

ISOCRATIC VS GRADIENT

In isocratic chromatography, the % organic is maintained at a set value. Whilst this is convenient and fairly robust, isocratic chromatography is inherently unsuitable for the analysis of compounds with widely varying hydrophobicities (logP / logD). This often results in poor resolution of early eluting (hydrophilic) components and broad peaks with excessively long retention times for very hydrophobic components.

In such cases, gradient chromatography is required to provide adequate retention for both hydrophobic and hydrophilic sample components within the same analytical run. Figure 1 shows the profile features of a typical linear gradient. In this example, the initial organic proportion is set to 5% and increased to 95% over 15 minutes (t_G). The dwell time is the time taken for the

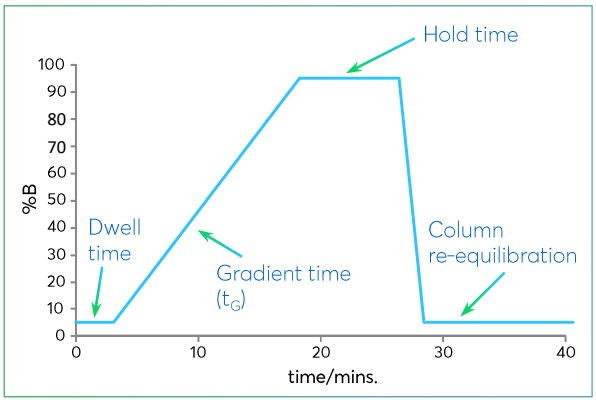

Figure 1: Features of a gradient.

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mobile phase to travel from the point of mixing to the head of the column and is dependant on the LC system dwell volume and flow rate (see AKN0001). At the top of the gradient, the %B is held at 95% to allow elution of all sample components, before being returned to the starting conditions to re-equilibrate the column.

Gradients also provide a means of cleaning the column of retained impurities, as the gradient sweeps from low to high organic solvent. It also produces narrow, consistent peak widths as the tail of the analyte is accelerated under the influence of the increasing strength of mobile phase.

GRADIENT RETENTION FACTOR (*k**)

In isocratic chromatography, retention is defined by the retention factor, *k*. In gradient chromatography, under the initial gradient conditions, sample analytes are immobilised on the stationary phase and begin to accelerate through the column as the % organic is increased. Therefore, *k* constantly changes for the analyte as the mobile phase varies through the gradient. In gradients we consider the average retention factor, *k**, which is the average retention factor as the analyte passes the midpoint of the column.

It is useful to consider the relationship between *k** and the various gradient parameters as defined by equation 1, where t_G is the gradient time in minutes, *F* the flow rate in mL/min, $\Delta\Phi$ the change in %B expressed as a decimal, V_M the column volume in mL, and *S* is a constant dependent on the molecular weight of the compounds to be analysed. For small molecules, the *S* value can be assumed to be 5, whilst the value for large molecules is significantly higher.

$$k^* = \frac{t_G \times F}{\Delta\phi \times VM \times S} \qquad \text{Equation 1}$$

This equation is important for chromatographers as it allows a suitable gradient method to be established. For gradient chromatography, an ideal *k** value is $2 < k^* < 5$. For example, on a 150 x 4.6 mm column at a flow rate of 1 mL/min, a 30 minute gradient time from 5-95% organic would be a suitable starting point for method development:

$$k^* = \frac{t_G \times F}{\Delta\phi \times VM \times S} = \frac{30 \times 1}{0.90 \times 1.571 \times 5} = 4.24$$

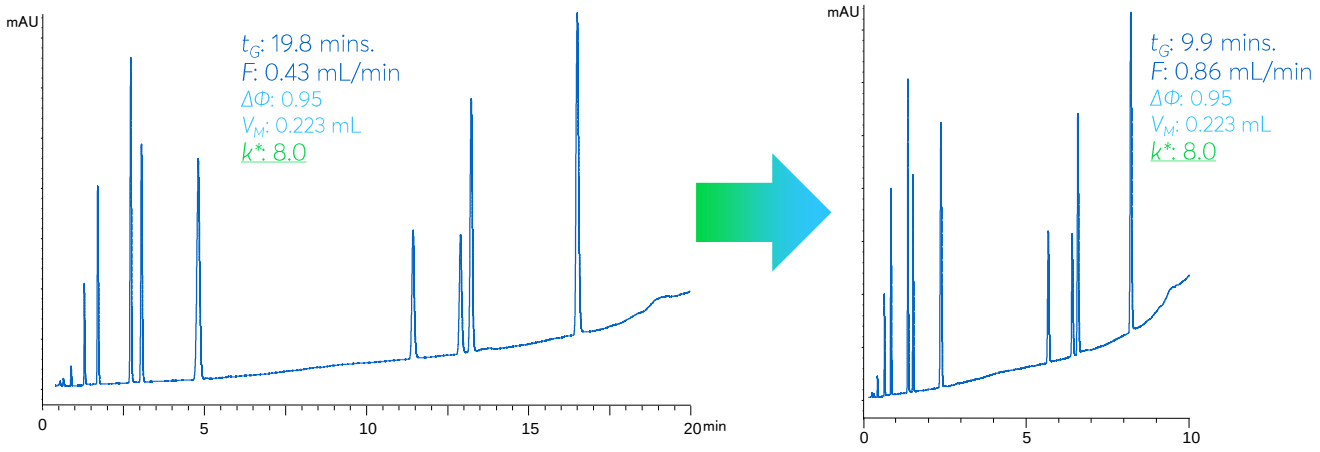

Figure 2: Decreasing run time of a gradient separation of bases (gradient range 5 – 95% B) by increasing flow rate and decreasing gradient time. Column: ACE Excel 2 C18-PFP, 50 x 3.0 mm.

OPTIMISING GRADIENT SEPARATIONS

Once a suitable gradient method has been identified, we can conveniently use the k^* relationship to make changes to flow rate or column length in order to decrease run time or increase resolution. The important point to consider is that the product of $(t_G F)/(\Delta\Phi V_M)$ must be kept constant in order to preserve the peak spacing. Therefore, an increase in flow rate should be accompanied by a proportional decrease in the gradient time. An increase in column length (and therefore column volume, see AKN0015 for further details) requires a corresponding increase in gradient time.

Figure 2 shows a separation of bases on an ACE 2 Excel C18-PFP. The flow rate was doubled in order to provide a faster separation. To maintain the selectivity of the separation, the gradient time was halved, thereby keeping k^* constant. As can be seen, the same separation was obtained in half the run time.

In Figure 3, a separation of NSAIDs on an ACE Excel 2 SuperC18 was moved to a longer column to increase resolution. By increasing both the column length (volume) and gradient time proportionally, increased resolution is achieved whilst the peak spacing remains identical to the original separation.

CONCLUSION

This Knowledge Note has shown the basic concepts of a gradient separation and the importance of the gradient retention factor, k^*. The relationship between k^* and the key gradient parameters is valuable for the manipulation of gradient methods to achieve increased throughput and resolution.

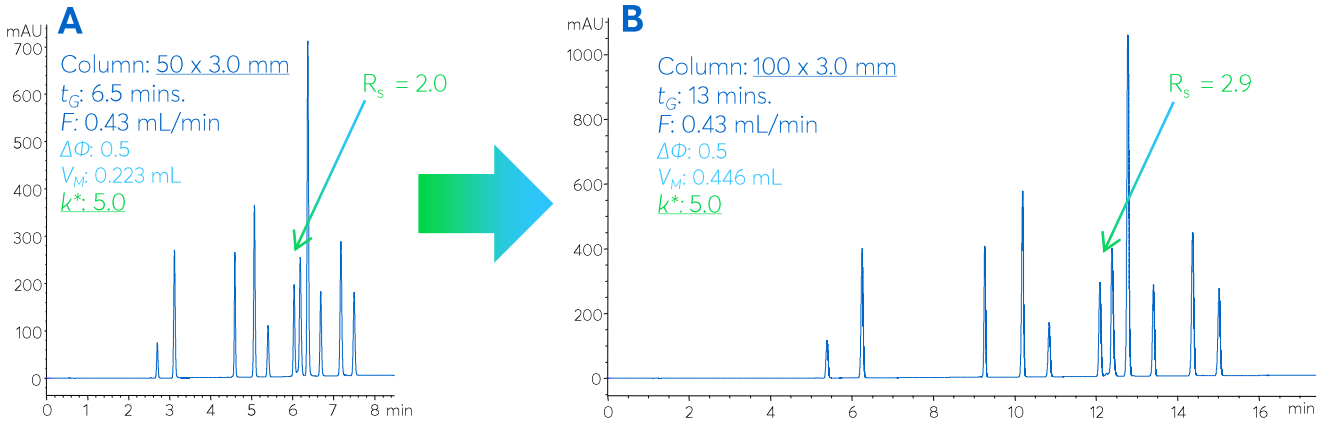

Figure 3: Increasing resolution of a gradient separation of NSAIDs (gradient range 20 – 50%B) by increasing column length and gradient time. Column: ACE Excel 2 SuperC18. Dimensions: A: 50 x 3.0 mm, B: 100 x 3.0 mm.