LC Troubleshooting Series
Gradients Overview

Introduction

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This overview offers a look at gradients and how they are developed. We will also discuss common pitfalls and how to address them.

Why Use Gradients?

Gradients are useful for:

- Separating a number of unknown compounds
- Combinatorial chemistry
- Impurity analyses
- Separating compounds that differ widely in polarity, are of high molecular weight, such as peptides and proteins.

The Gradient Equation

The gradient equation is important to understand. See below.

Gradient Retention (k*)

$$k^* = \frac{t_G F}{S \Delta\Phi V_m}$$

$\Delta\Phi$ = change in volume fraction of B solvent
S = constant
F = flow rate (mL/min.)
t_G = gradient time (min.)
V_m = column voice volume (mL)

S ≈ 4 – 6 for small molecules
10 < S < 1000 for peptides and proteins

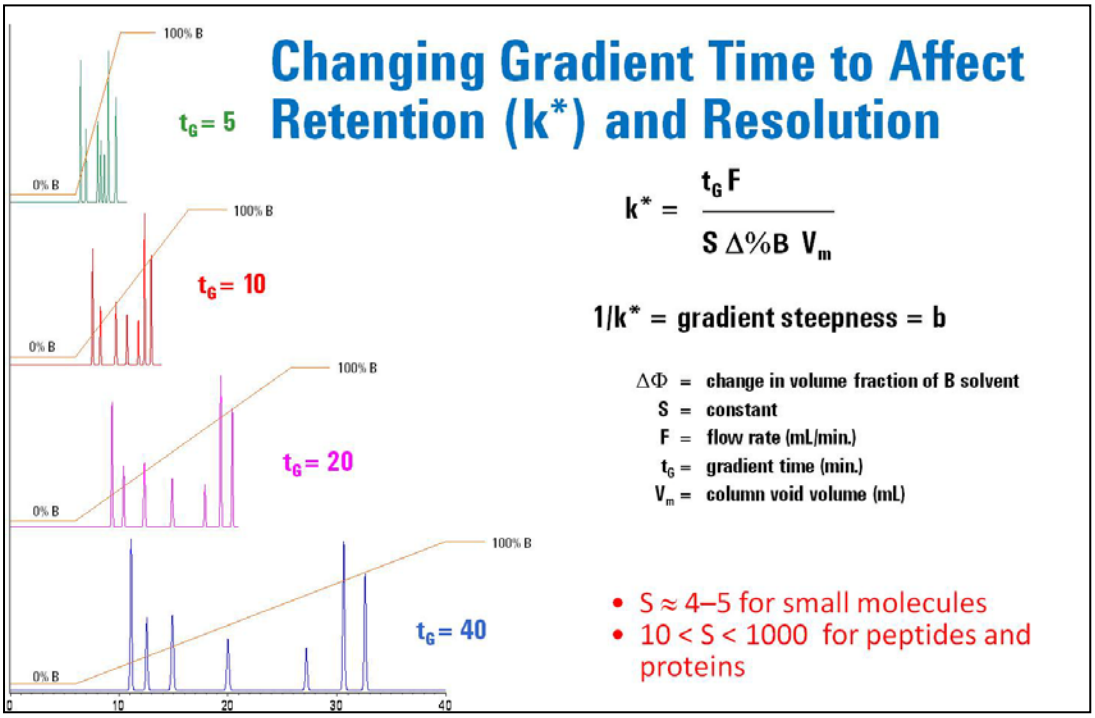
Changes in the numerator (gradient time, flow rate) need to be offset by changes in the denominator (gradient range, column volume, etc.), and vice versa.

Look for the LC Method Translator at Agilent.com. It is a good tool to help you adjust your method.

Available:
www.agilent.com/chem/rrlc_mt

Gradient Steepness

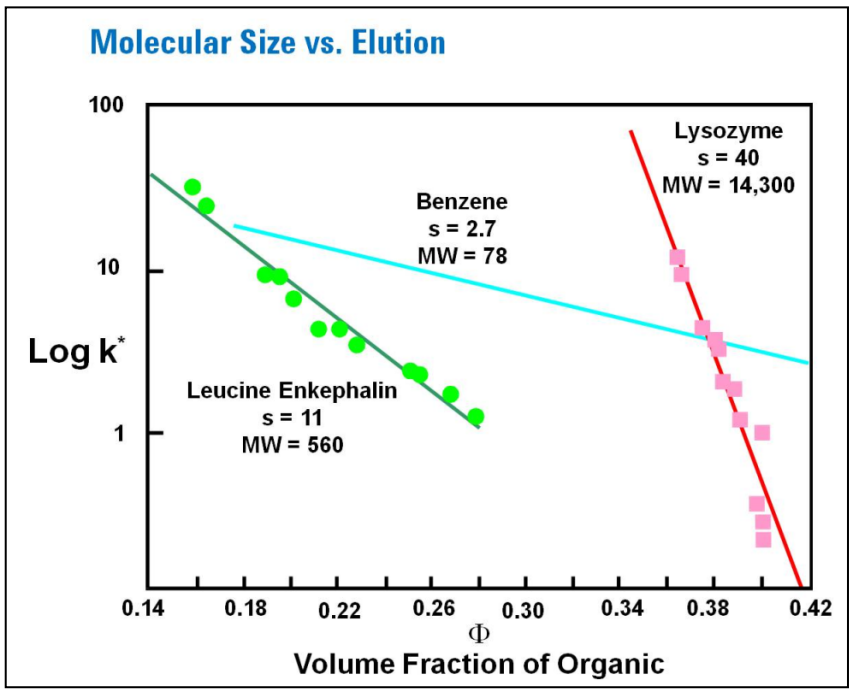
Gradient steepness is analogous to percent organic in isocratic separations. See the example below.



When gradient steepness is decreased, retention increases; when gradient steepness is increased, retention decreases.

Tips to remember:

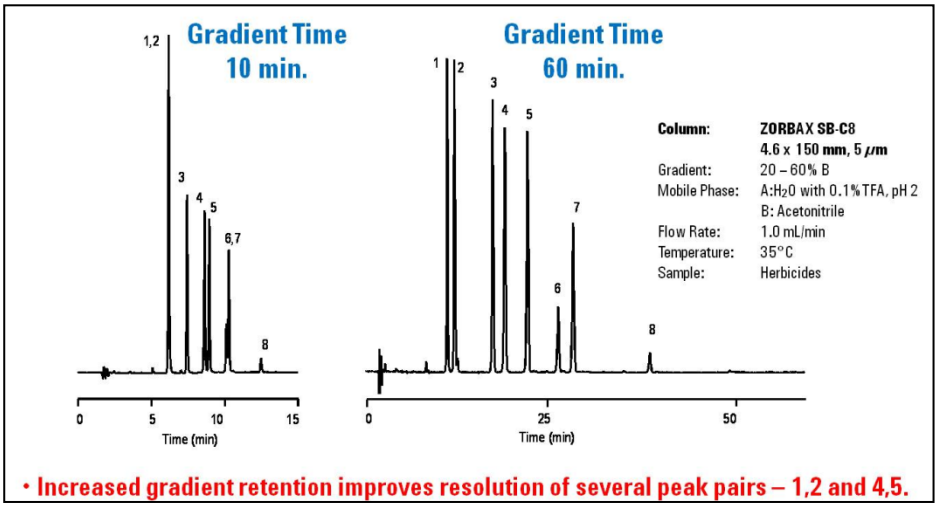
- As long as b is kept constant, your peaks will elute with the same relative pattern.
- Decreasing gradient steepness does not necessarily mean better retention or resolution. Results will vary depending on your analytes.



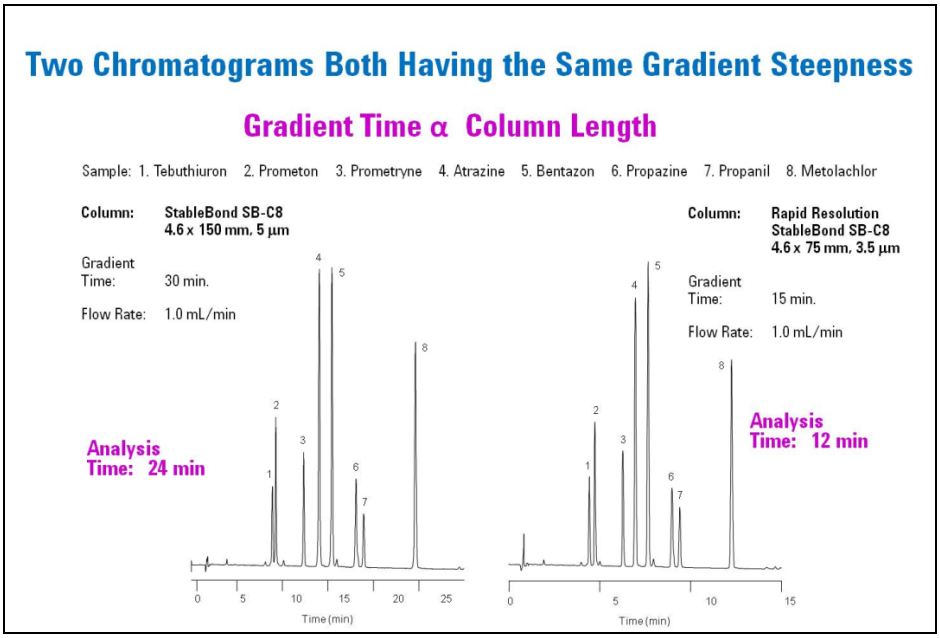
Different analytes have characteristic S values which reflect a molecule's sensitivity to the % organic in the mobile phase. See the example at the left.

For most small molecules (<a few hundred Da), S values are about 4-5, and will not have much impact. However, S values for peptides and proteins are much larger and can vary from each other over 2-3 orders of magnitude.

To illustrate how this equation's concepts manifest themselves in your chromatography, let's look at a few examples.



The longer gradient time results in a shallower gradient. By decreasing the gradient steepness, we're able to separate peaks 1 and 2, and 6 and 7.



See the example above. In the chromatogram on the left, our sample is well separated on a 4.6 x 150 mm, 5um column using a gradient time of 30 minutes at 1.0 mL/min. We achieved a significant reduction in analysis time by reducing the column length to 75 mm. The gradient steepness was unchanged but the analysis time *decreased by 50%.* A reduction in solvent consumption and re-equilibration time was also achieved.

Mobile Phase Modifiers and Drifting Baselines

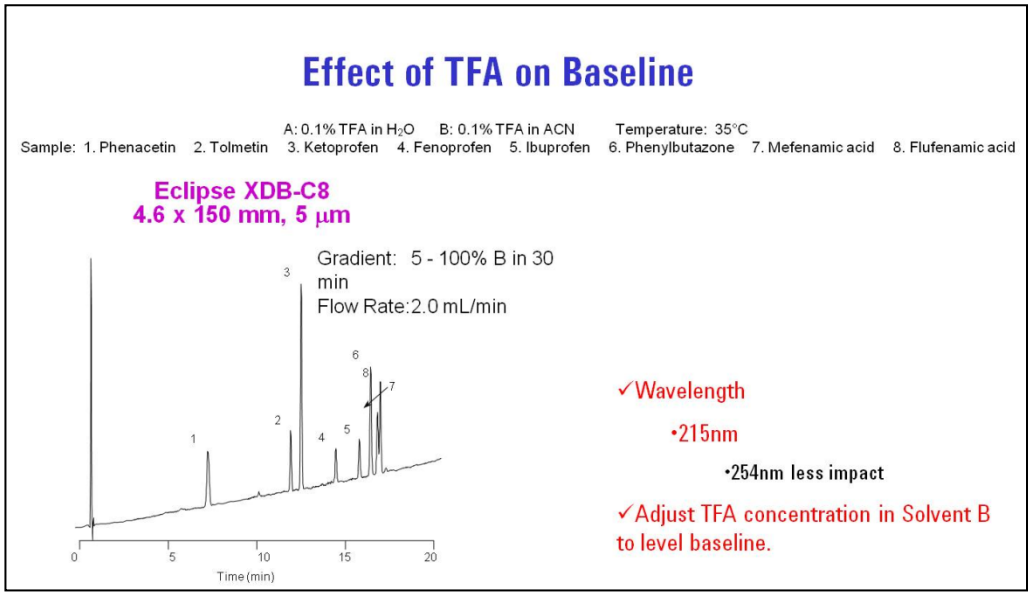
Baseline drift is a common issue with gradients. It is usually related to the mobile phase, and its relationship to the UV absorbance.

UV Cutoff for Common Mobile Phase Components

Buffer	pK_a	pH Range	UV Cutoff (A > 0.5)
Trifluoroacetic acid	<< 2 (0.5)	1.5-2.5	210 nm (0.1%)
KH_2PO_4/phosphoric acid	2.12	1.1-3.1	<200 nm (0.1%)
Ammonium hydroxide./ammonia	9.2	8.2-10.2	200 nm (10mM)
Acetonitrile	na	na	190 nm
Methanol	na	na	205nm
Tetrahydrofuran, unstabilized	na	na	212 nm, when fresh

For many applications, it is ideal to have the same level of buffer or modifier in both mobile phase A and B.

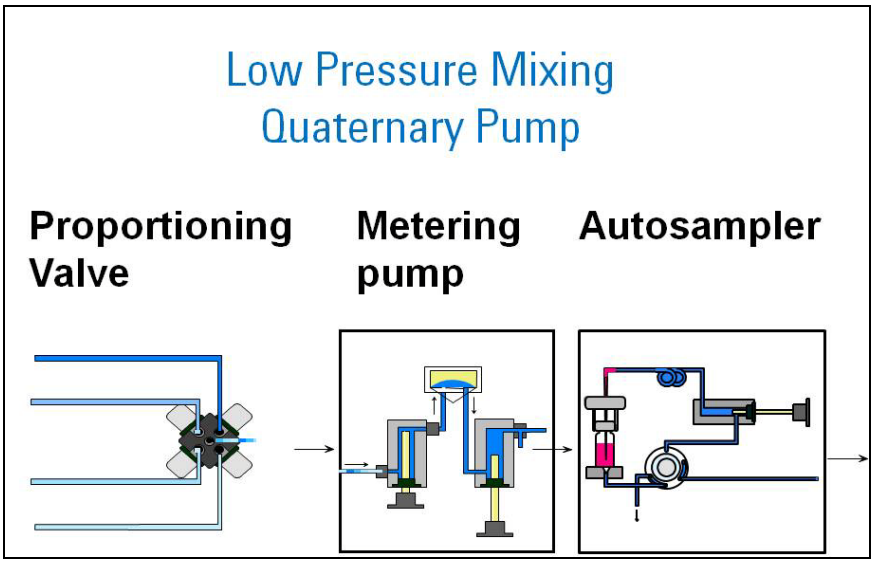
When using TFA, try using 0.1% TFA in solvent A and about 0.09% in solvent B.



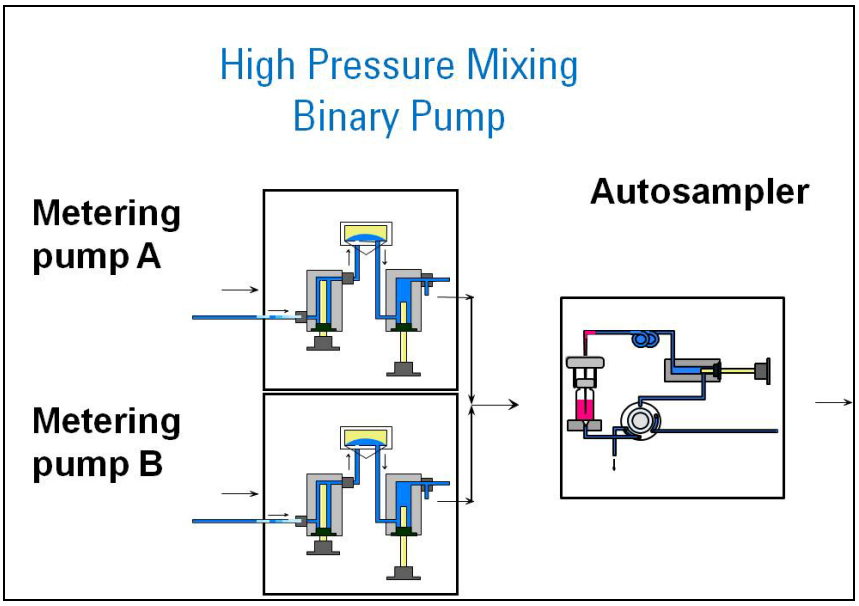
Dwell Volumes

Dwell volume equals all of the volume from where the solvents first meet, after the two metering pumps, to the head of the column.

For low pressure mixing systems, dwell volume equals all of the volume from the proportioning valve through the pump and on to the head of the column. See below.

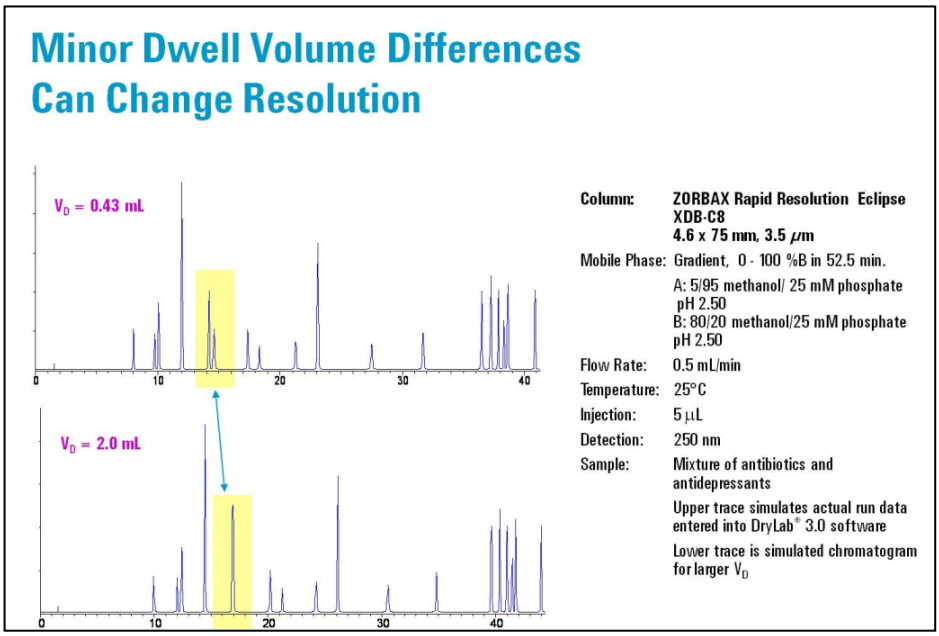


For high pressure mixing systems, dwell volume equals all of the volume from where the solvents first meet, after the two metering pumps, to the head of the column.



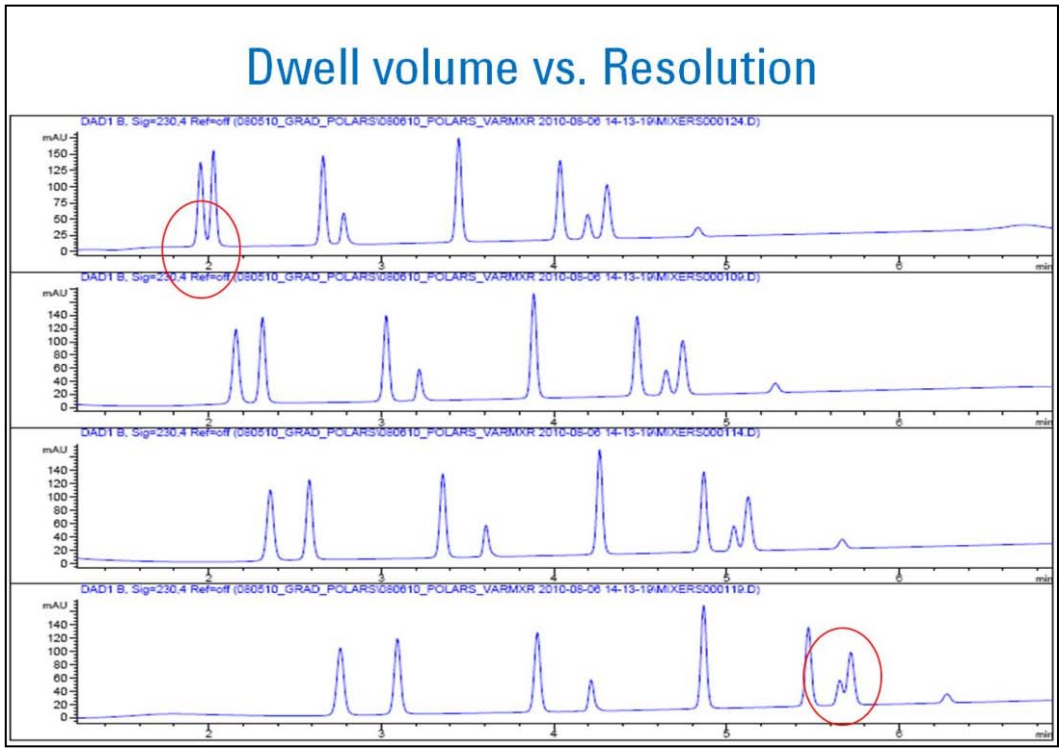
Dwell volume imposes a de facto isocratic hold time at the beginning of the gradient equal to the dwell volume divided by the flow rate.

Dwell volume differences can change both peak widths and relative retention in gradient separations.



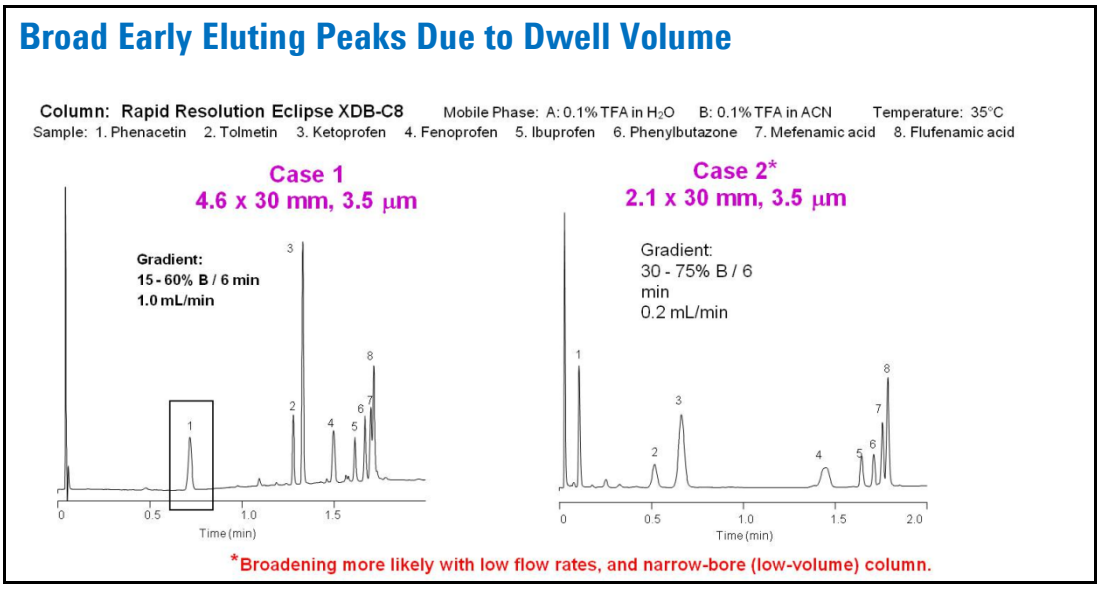
The example on the left represents simulated gradient chromatograms for separations of a complex mixture. The upper trace is a simulation of the actual input data. The lower trace is a simulated chromatogram of the same separation carried out on an instrument having a dwell volume of 2.0 mL instead of 0.43 mL. Notice the peak pair that separated on one instrument completely co-eluted on the instrument with the larger dwell volume.

Below, you can see four different gradient runs of the same sample on the same system. The gradient time program is the same but the instrument was reconfigured to represent a wide range of delay volumes. What we see is that, for this particular set of gradient conditions, the smallest and largest delay volumes have resolution problems



Effect of Dwell Volume on Ruggedness—Gradient Separations

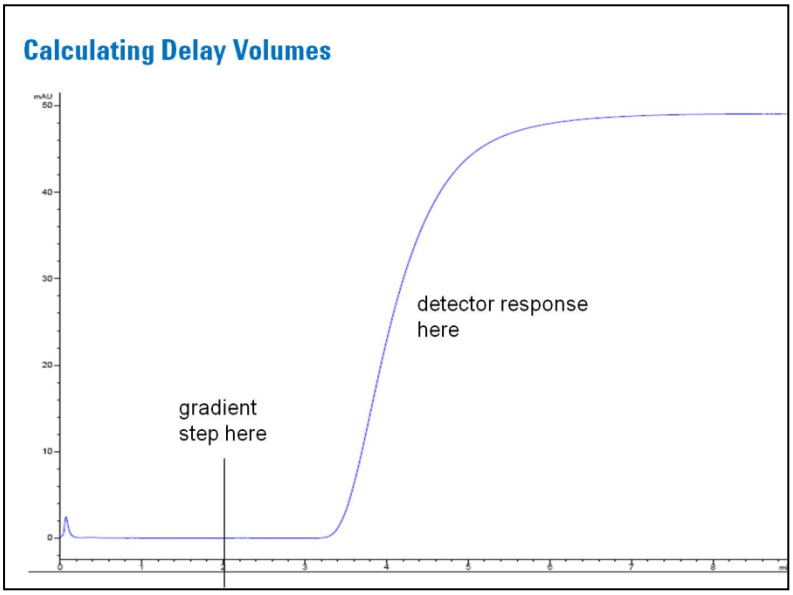
Below is an LC troubleshooting example that deals with dwell volume. Note that early eluting peaks are broader—they are eluting late due to the dwell volume.



Knowing the dwell volume is important when transferring gradient methods to other instruments and laboratories.

For direct measurement of instrument dwell volume:

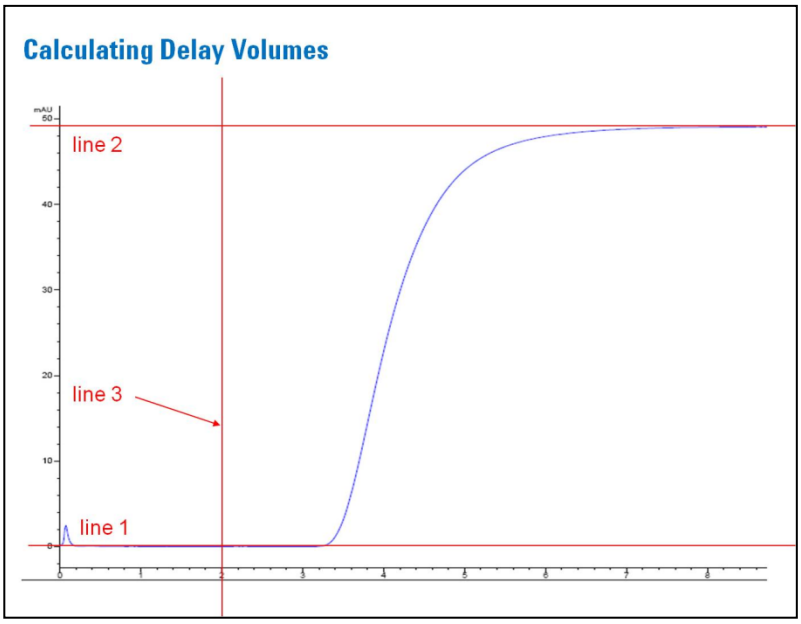
First, prepare a full page landscape printout of the step gradient trace, or alternately copy and paste the image to Microsoft Office PowerPoint as we have done here:



Using a ruler on paper or by inserting lines in PowerPoint, draw lines parallel to the x or y axis at the following points:

1. at the zero signal defined by the region of 0% B
2. at the maximum stable signal defined by the region 100%B
3. a vertical line at 2.0 minutes

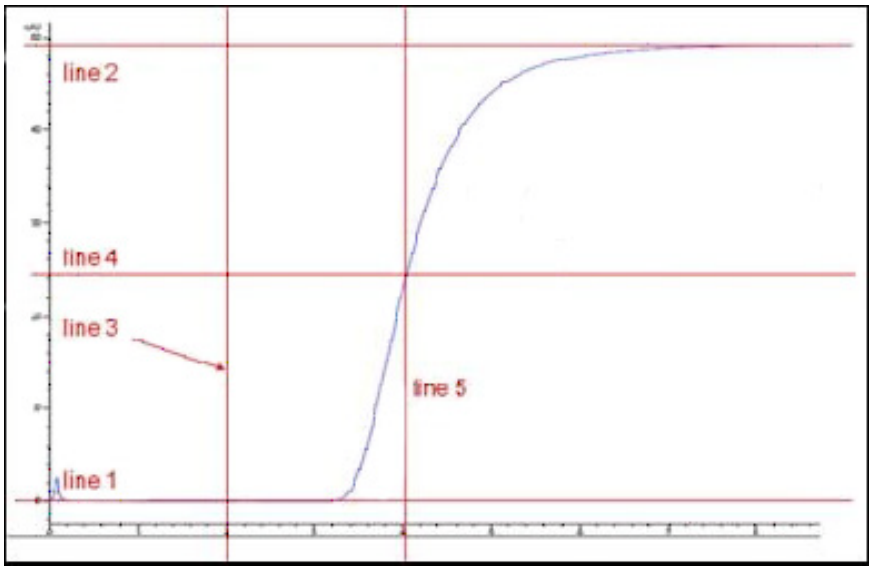
Your image should resemble the figure below:



After the three lines have been added, add these two additional lines:

4. Calculate the 50% response of the step, in this case 24.5 mAU, and draw a horizontal line across the image.
5. Draw a vertical line that intersects the junction of the 50% response line and the observed detector signal.

Your new image should look like the slide below:

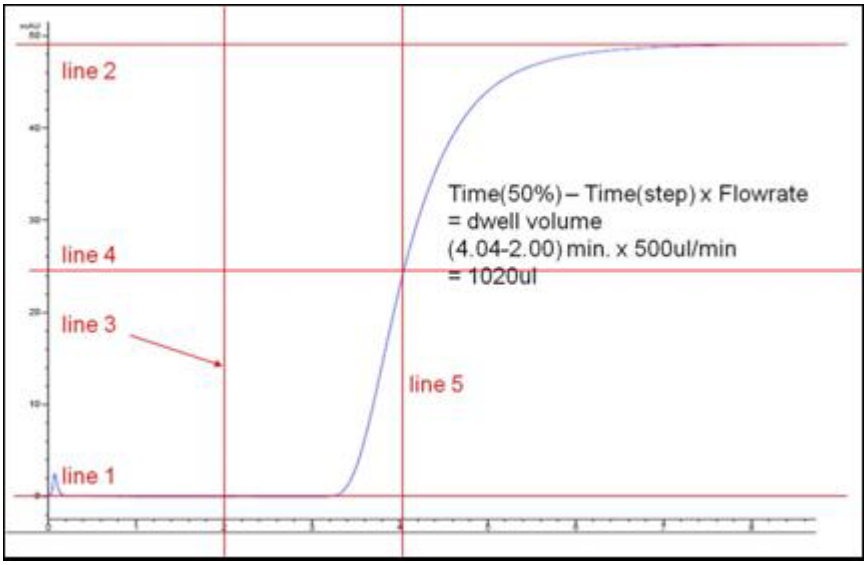


Follow the x axis to the 50% B vertical line and determine the time, as closely as possible, at which the 50% response is observed.

An error of 0.1 minute will be a 50µl error in your delay volume estimate.

In this example, we estimated the 50% time at 4.04 minutes.

To calculate the dwell volume, the simple formula is Time (50%) – Time(step) x Flow rate = dwell volume.



In this example, then, (4.04-2.00) minutes x 500µl/min = 1020µl.

Autosampler Programming to Assist with Gradient Optimization

Dwell volume also includes injector volume, so it is important to minimize the internal volume and connecting tubing around the injector.

You can reduce analysis time in rapid gradient separations by overlapping injections.

Don't forget that it is also important to minimize extra column volume and inject in an appropriate sample diluent if you have early eluting peaks in your gradient run.

It is necessary to make sure the detector response time is set correctly to catch enough points on the quickly eluting peaks. If it is not, then distorted, broad peaks may appear.

Summary

Tips for working with gradients:

- Familiarize yourself with the gradient equation, and remember to adjust your gradient to account for changing method variables as needed.
- Adjust modifiers to ensure UV backgrounds are consistent, to avoid baseline drift
- Understand your system dwell volume, and adjust for it in method development.
- Always run a gradient blank before a sample/standard.
- Consider removing the mixer to reduce dwell volume.

If you require additional assistance, you can always contact Agilent technical support by logging on to www.agilent.com/chem/contactus.