Post Separation Pause II: Determination of Maximum Sample Load

Abstract

The Post Separation Pause function implemented in the ACCQ*Prep* and EZ Prep automation allows fine-tuning of preparative chromatography. The Post Separation Pause is designed to be used during multiple injections of the same sample.

The Post Separation Pause can be used to determine the maximum loading for a particular sample, column, and gradient after optimizing the gradient. The loading of a sample is based on the resolution from its nearest impu-rity. As the loading is increased, the resolution is decreased due to column loading and solvent effects. This application note demonstrates how resolution is measured and how to translate resolution into a max-imum injection volume.

Overview

It is important to minimize solvent usage and purifi-cation time when running preparative chromatography. One way to minimize time is to optimize the gradient so that the desired compound elutes quickly while still eluting away from the impurities. A focused gradient allows maximum resolution while minimizing run time and solvent usage. A focused gradient also allows for some variation in solvent delivery between an analytical system and a preparative system. The Post Separation Pause allows fine-tuning of the gradient.

Another way to reduce total purification time is to maximize the loading on a column thereby reducing the number of runs required to purify a given amount of sample. In preparative chromatography, efficiency is determined by maximizing the load with less concern for peak shape. Maximum efficiency occurs when the peak for the desired compound barely touches the peak for the nearest impurity.
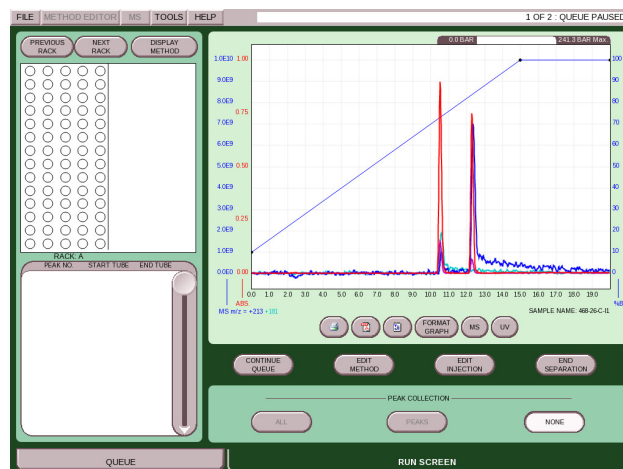

Figure 1: Display of Post Separation Pause after injected purification is complete.

If the Post Separation Pause is enabled, a screen similar to that in Figure 1 is displayed. The CONTINUE QUEUE button runs the next injection with no changes. EDIT METHOD allows any method parameter to be changed prior to the next injection. This includes detection parameters as well as gradient and solvent parameters, but not injec-tion parameters. The EDIT INJECTION button allows one to change the volume of sample injected. The button on the far right, END SEPARATION, ends all injections for this sample. The sample loading is adjusted using the EDIT METHOD control. The procedure below allows to adjust the loading by volume, rather than mass, so the data entry is easier.

Theoretical Estimation of Maximum Sample Load

The gradient should be adjusted prior to optimizing the sample load because the loading is dependent on the peak resolution. The sample load can't be deter-mined if the resolution is being changed during gradient adjustments. The peak resolution is determined, in part, by the gradient used and may be optimized as described in Technical Note 37 *Why Use a Post Separation Pause? I-Optimizing Gradient*.

After the gradient is adjusted, the sample load on a column can be maximize. The amount loaded on a column depends on the resolution (distance) between two peaks and the column efficiency[1].

1. Dolan, J.W. How Much Can I Inject? Part 1: Injecting in Mobile Phase. *LCGC North Am.* 32(10).

If the sample is injected in mobile phase under isocratic conditions, without overloading, resolution is determined by *Equation 2*:

$$R_s = (t_2 - t_1)/(0.5[w1 + w2])$$

Where t_2 and t_1 are the retention times of the second and first eluting peak and w_2 and w_1 are their peak widths.

When injecting a larger sample, dissolved in the mobile phase, into the column at the same concentration as a smaller injection, the first part of the injection behaves the same as a smaller injection. For example, if doubling the injection volume, the first half of the injection will elute down the column the same as the original injection. The rest of the injection will increase the width of the peak(s) as shown in Figure 2.
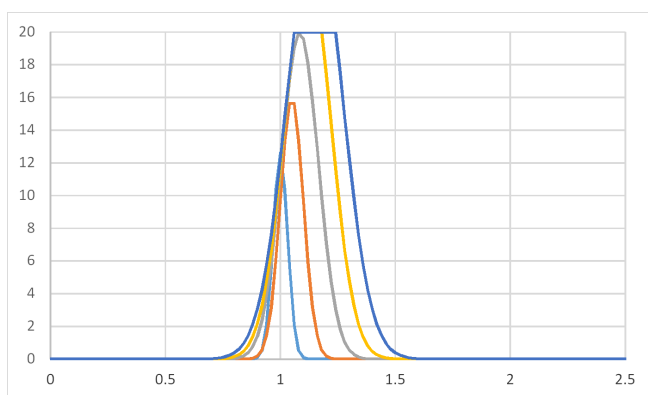

Figure 2: Increasing injection volume under conditions where the column is not overloaded. Yellow and blue traces shows detector overloading.

In the model in Figure 2, doubling the injection volume does not double the peak width, in practice, since the peak also becomes taller. The increase in peak width can be estimated by the "15% rule"[1] where increasing the sample load by 15% causes the peak width to increase by 1%.

Factors Affecting Actual Sample Load

In real life, peak width increases due to column overloading and the effects of the solvent used to dissolve the sample. In Figure 3, the sample was dissolved in a solvent weaker than the mobile phase, allowing for concentration of the sample at the head of the column. The "shark fin" peak shape suggests column overloading. As the injection volume is increased, the front of the peaks appear earlier in the column elution.
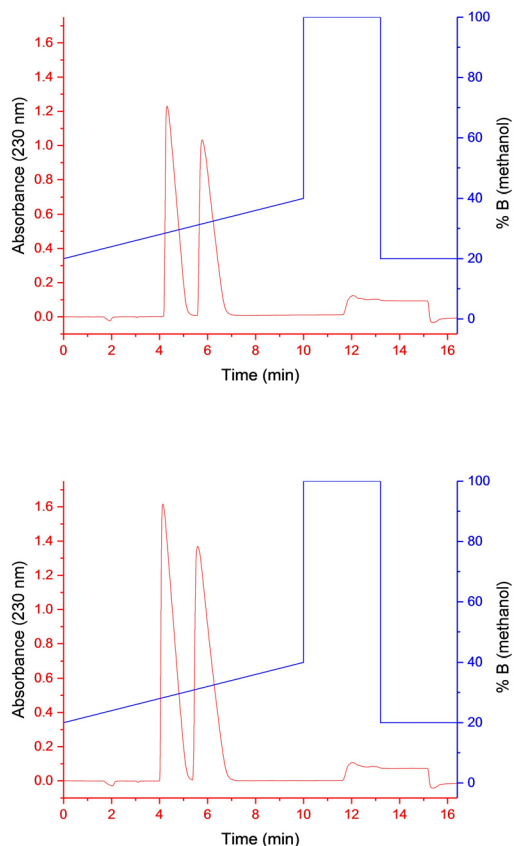

Figure 3: Front of peaks show reduced retention as loading increased from 2.5 to 4 mL

Another deviation from the ideal is caused by gradients. Shallow gradients allow for error in determining the solvent system which elutes the compound. Gradients tend to focus the sample on the column. The tailing part of the peak is in a stronger solvent system then the leading portion of the peak. This tends to cause the compound contained in that portion of the peak to run faster through the column. The front of the peak is in a weaker solvent and runs slower compared to the tail. This causes the peak to become focused. Since the molecules at the tail of the peak catch up to the front of the peak we again may reach the overloaded condition described above.

It is common to use solvents such as DMSO and DMF to dissolve samples. However, these solvents often reduce the sample loading because they "drag" the sample down the column, smearing peaks together (Figure 4). This may then reduce resolution, and loading. The DMSO, DMF, or other "strong" dissolution solvent acts locally as a gradient. Although the solvent is diluted by the mobile phase, it carries the sample down the column until diluted so that the solvent strength is weaker than the elution solvent.
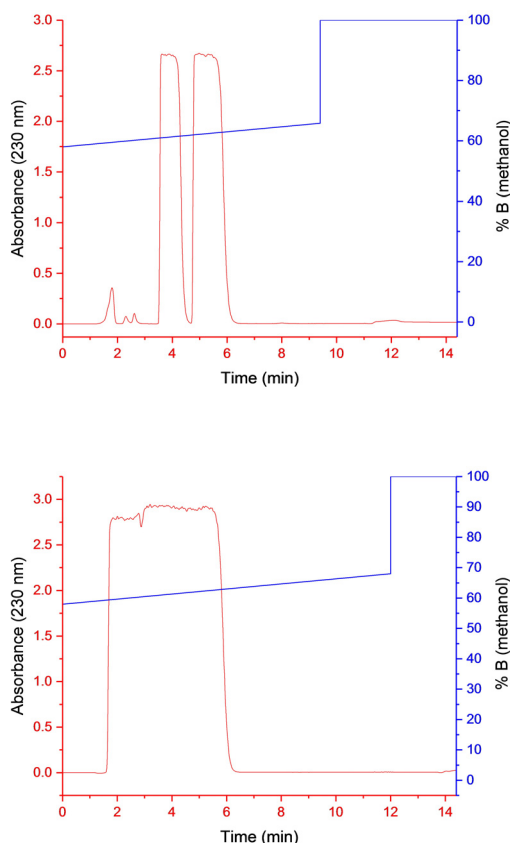
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Figure 4: Sample dissolved in mobile phase (3.90 mL), and same sample at same concentration dissolved in DMSO

Calculation of Sample Load from Chromatographic Information

After optimizing the chromatographic conditions, as discussed in Technical Note 37 *Why Use a Post Separation Pause? I-Optimizing Gradient* the next step is to estimate the maximum loading. Take the optimized run and use the touch screen to find the start and end of the peak containing the desired compound. Find the nearest impurity (eluting either prior to, or after, the desired compound) and note the starting and ending times for this peak. As the flow rate is not being changed, time can be used instead of volume in the calculations. Subtract the start time from the end time to find the peak widths for each peak (W_p). Subtract the time of the end of the first eluting peak from the end of the second eluting peak to get the maximum peak width (W_{max}) which is the maximum peak width that "uses" all the available resolution. This corresponds to the maximum width for the second eluting peak of interest. Peaks tend to creep forward rather than elute later, especially when

gradients are involved (Figure 3), so the maximum peak width is defined from the second peak. Calculate the allowable increase in the peak 2 width (W_{max}- W_{p2}) and multiply this value by 15 (the 15% rule) to make a multiplier which is then multiplied by the injection volume. Finally, multiply this volume by (1-0.25) to create the maximum injection volume. The 0.25 value allows for column overloading and consequent "peak creep".
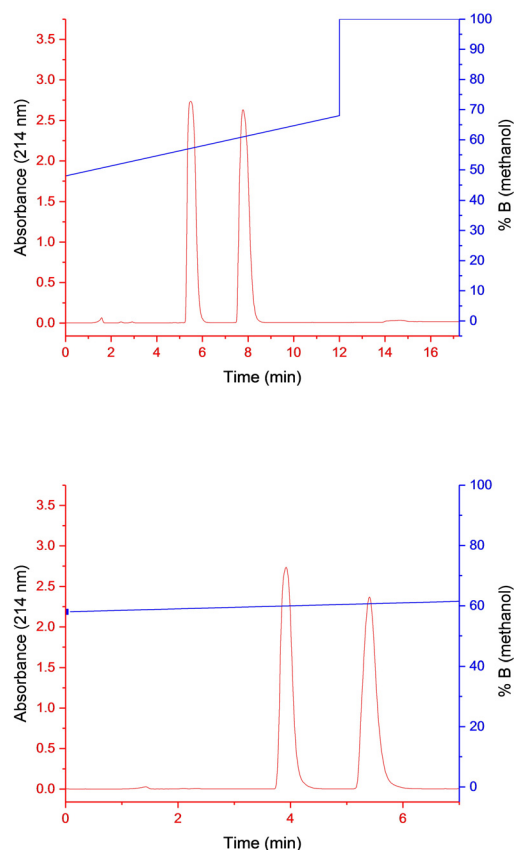

Figure 5: Original chromatogram

If the sample is dissolved in a solvent such as DMSO of DMF, a value of 0.5 may be more appropriate to account for the injection solvent smearing the peaks as shown in Figure 4. Due to a combination of solvent smearing, peak shape, and column loading effects, it is suggested that the initial injection scale-up be 50% of the value suggested by the algorithm to avoid wasting a run.

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As an example, the run in Figure 5 can be scaled up in the following table:

Injection Volume	Peak 1		Peak 2		Peak 1 Width	Peak 2 Width	Max Width	Width Increase	Rule	Ideal Load Scale-Up	Ideal Injection Volume	Peak Creep Correction	Injection Volume
	Start	End	Start	End			W_{max}						
	$P1_{Start}$	$P1_{End}$	$P2_{Start}$	$P2_{End}$	$P1_{End}$ - $P1_{Start}$	$P2_{End}$ - $P2_{Start}$	$P2_{End}$- $P1_{End}$	W_{Max}- $Width_{P2}$	Rule 15% used here	(Rule)* (Width Increase)	(Ideal Load Scale-up) * (Injection volume)	Empirically determined	(Ideal Injection Volume) * (1- (Peak Creep Correction))
					(4.40-3.72)	(5.81-5.10)	(5.80-4.40)	(1.40-0.70)	15	(0.70*15)	(10.5*0.5)	0.25	(5.25*(1-0.25)
0.5	3.72	4.40	5.10	5.80	0.68	0.70	1.40	0.70		10.5	5.25		3.94

From the table, an injection of just under 4 mL is deemed ideal. However, the loop volume needs to be considered; using the AutoSampler or AutoInjector, one can inject up to the (loop volume -1 mL). The 5 mL loop is used to inject up to 4 mL; a 9 mL injection may be used on a 10 mL loop. Pressing the EDIT INJECTION button in the Post Separation Pause screen (Figure 1) will show a screen similar to that in Figure 6.
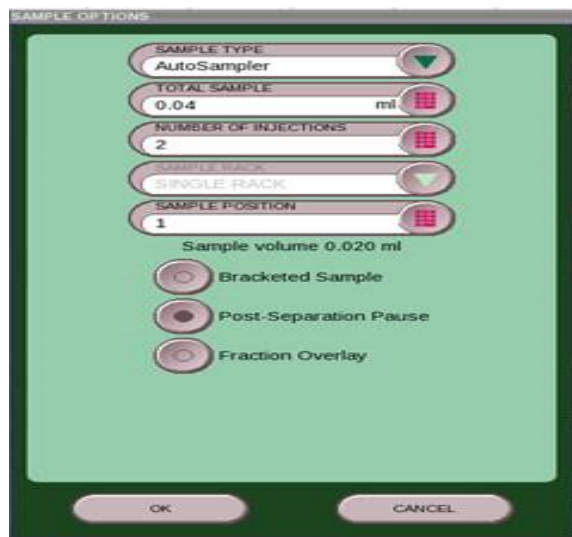

Figure 6: EDIT INJECTION dialog on system equipped with an AutoSampler

Change the volume for the following runs by altering the number of injections. Assuming the calculations above are used, and there is only 10 mL of sample, the number of injection is (10 mL/3.94 mL) to give 2.56 injections. Since we obviously cannot make 0.56 injections, we round up to 3 injections.

It may be wise to limit the initial scale-up to a smaller injection volume than calculated. For example, the purification of ionizable compounds in a solvent modifier, such as an amine purified using an acidic solvent modifier may not scale up according to the calculations. If the loading of the compound is sufficiently large, it could "use up" all the modifier leading to a mixture of free base and conjugate acid causing a broader peak. Another factor may be the requirement to dissolve the sample in a strong solvent, as mentioned earlier.

Conclusion

The Post Separation Pause is useful not only for adjusting gradients to improve resolution and reduce run times, but can also be used to determine the maximum loading for a particular sample. The maximum load should be approached in one or two steps due to changes in peak shape and solvent effects. For example, peak shape could be altered with higher loading by an ionizable compound which interacts with a solvent modifier. If the loading of the compound is sufficiently large, it could "use up" all the modifier leading to a mixture of ionized and unionized compound and a very large increase in peak width. The calculation is still useful to reduce the number of trials required to optimize sample loading.