Performance PerSPECtives

Gradient Chromatography

Column Reequilibration

In gradient chromatography the composition of the mobile phase is changed with time to increase the selectivity of the separation, permitting better separation of the chemically different analytes. At the end of the separation, reequilibration must be performed to restore the system and column to the initial mobile phase condition prior to the next injection. This is a time consuming part of each analysis. The amount of time depends on the HPLC system or gradient delay volume, the column size and volume, and the flow rate.

Traditionally HPLC has been done on colums that are 3.9 x 150 mm and 4.6 x 250 mm in size. Today, there are many different dimension columns to choose from. The various column dimensions offer benefits, such as saving solvent (narrower or shorter columns), faster analyses (shorter columns) or higher sensitivity (narrower columns). Determining the reequilibration time for a new method is typically based on past experience (e.g. ten column volumes). When a different HPLC system, column size, or flow rate is used, reequilibration time may not be sufficient, causing retention times to vary. Can one calculate the reequilibration time from known parameters?

A method of estimating reequilibration time has been determined experimentally. Reequilibration was divided into two parts, flushing out the HPLC system volume and reequilibrating the column to initial conditions. The HPLC system volume, from the point where the solvents (e.g. A and B) are first mixed to the head of the column, can vary from a low 600 µL in the Waters Alliance™ HPLC System to several mL in more traditional HPLC systems. Experiments showed that three system volumes of initial mobile phase are required to return the system to initial conditions. Then approximately five column volumes ($\pi R^2 * L$) are required to fully reequilibrate the column.

Figure 1

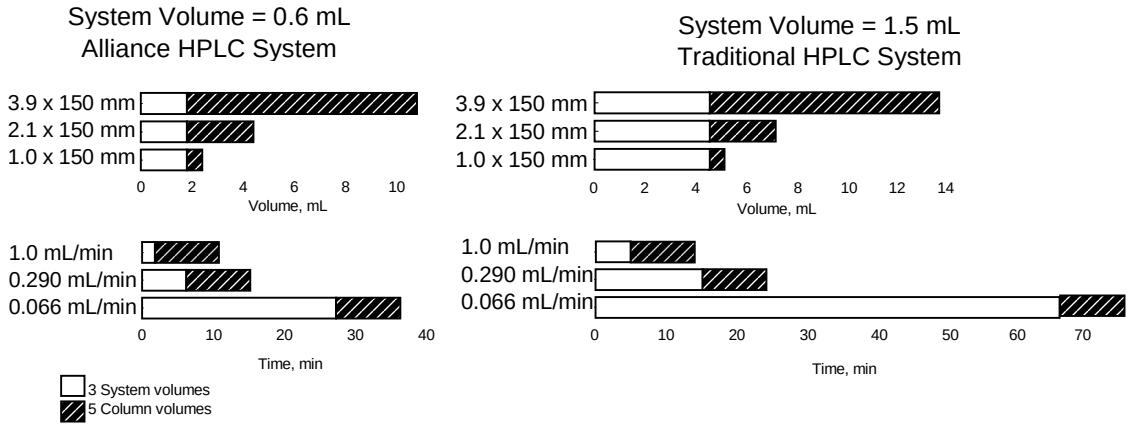

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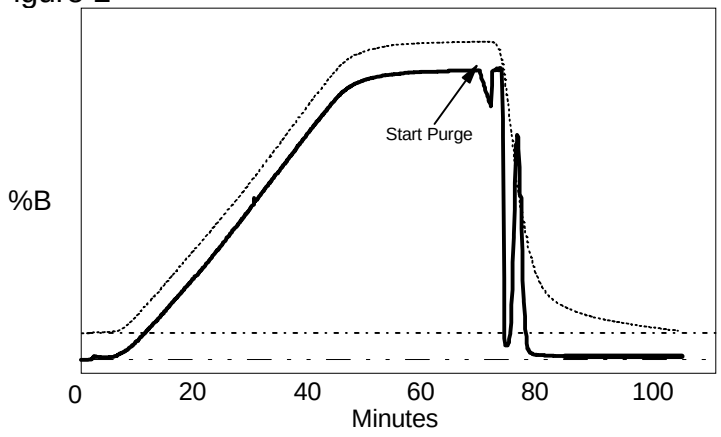
Figure 1 diagrams the mobile phase volumes (upper panels) and the times (lower panels) required for reequilibration for analytical (3.9 mm i.d. at 1.0 mL/min), narrowbore (2.1 mm i.d. at 0.29 mL/min) and microbore (1.0 mm i.d. at 0.066 mL/min) columns. The results for two different HPLC systems are shown. On the left is the Waters Alliance™ HPLC System with 0.6 mL system volume and on the right is a system with 1.5 mL system volume.

The advantage of a low system volume for gradient chromatography is shown in Figure 1. In the top two panels with a constant flow, it will take longer to equilibrate the larger volume system (right side). With a constant flow, column reequilibration volume would decrease with column diameter. In the lower panels the equilibration issue is shown as a function of time. In practice, when the column diameter decreases, the flow rate is also decreased to give the same mobile phase linear velocity for each column. Therefore the time to reequilibrate the column (5 volumes) remains the same regardless of column diameter. On the other hand, the HPLC system reequilibration time becomes longer with narrower columns because the flow rates are lower and the system volume remains constant. Therefore, the smaller system volume (lower left) requires a shorter reequilibration time (3 volumes) at a given flow rate. Thus, a smaller system volume results in faster injection to injection cycle time in gradient chromatography especially at low flow rates.

Although it is best to reequilibrate a column at the flow rate used for the analysis, the system reequilibration time can be decreased by purging the HPLC system volume at a higher flow rate. On the Waters Alliance™ system this can be accomplished automatically by programming a "Wet Prime" at 5 mL/min for 0.5 minutes after each gradient run. This is illustrated in Figure 2. In this figure the microbore gradient (66 µL/min) was generated with methanol as the A solvent and methanol containing propylparaben as the B solvent, and monitored at 254 nm. This was a linear gradient of 0 to 40%B in 40 minutes, a 30 minute hold at 40% with a step back to initial conditions of 0%B at 70 minutes. The upper trace (dashed line) is the standard reequilibration that requires 36 minutes to return the column to initial conditions (>105 minutes total time). In the lower trace (solid line), at 70 minutes the column is by-passed to permit a rapid equilibration of the system volume at 5 mL/min for 0.5 min. The flow is then returned through the column at analytical flow rates (0.066 mL/min) and within 10 minutes the column is also fully reequilibrated and the system ready for the next injection (80 minutes total time). This is a savings of more than 25 minutes in the total run time. This advanced capability is the results of coordination hardware and software features of the Waters 2690 Separations Module in the Alliance™ System and provides the ability to shorten gradient cycle times even with narrow columns.

Figure 2



Gradient Trace
Waters Alliance™ HPLC System
Symmetry® C18 1.0x150mm
A = methanol
B = methanol + propylparaben
Linear gradient 0-60%B in 75 min
Reequilibration = step to 0%B
without purge
with purge
Flow rate = 0.066 mL/min