A Practical Investigation of Extracolumn Dead Volume and its Effects in HPLC

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Abstract

The theoretical effects of extracolumn dead volume on band broadening and the resulting chromatography have been well studied and documented. These effects, however, have primarily been studied in isocratic systems. In this investigation we have taken a practical look at dead volume and studied the effects on gradient separations. In addition to evaluating band broadening due to dead volume in the sample flow path, we have investigated the effects of gradient delay/dwell volume, and the interaction between band broadening and gradient delay. The specific amounts and locations of dead volume in the mobile phase flow path were correlated to effects on theoretical plates, resolution, and gradient delay time. These factors, along with the variables of column dimension and analyte retention, were studied to give a more complete understanding of the effects of total extracolumn volume under both isocratic and gradient conditions.

Equipment

Hewlett-Packard series 1100 liquid chromatograph consisting of binary gradient pump, auto sampler, column compartment with switching valve, and photo diode array detector.

Columns

- Pinnacle II™ C18 5µm 150x4.6mm
- Pinnacle II™ C18 5µm 50x4.6mm

Mobile Phase

- Isocratic conditions: 80% methanol 20% water
- Gradient conditions:
  - 80-100% methanol 20-0% water in 10 minutes for the 150mm column
  - 80-100% methanol 20-0% water in 3.3 minutes for the 50mm column

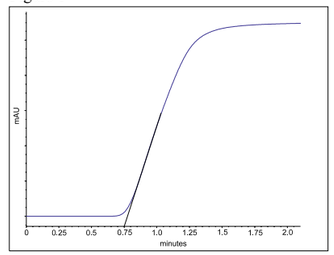
Test Sample

- uracil, toluene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene
- Concentration: uracil 0.05mg/mL, all others 10mg/mL

Measurement of Gradient Delay Volume

To determine the gradient delay volume, the system was set up with with 100% methanol in channel A, 99% methanol 1% acetone in channel B, and a zero dead volume union in place of the column. The system was equilibrated by pumping 100% channel A at 1 mL/min. A step gradient was then run that changed from 100% A to 100% B at time zero. As shown in **Figure 1**, the detector response was recorded and a tangent line drawn to determine the volume. The total gradient delay volume was determined to be 750µL.

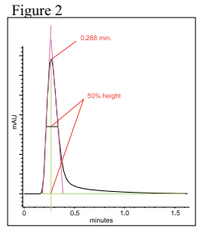
Figure 1



Measurement of Sample Flow Path Volume

To determine the sample flow path volume, the system was set up in the same configuration with a mobile phase of 80:20 methanol:water and a flow rate of 200µL/min (**Figure 2**). A 1µL sample of uracil was injected, producing a peak with a retention time of 0.268 min. and a peak width at half height of 0.115 min. The total sample path volume was determined to be 53µL.

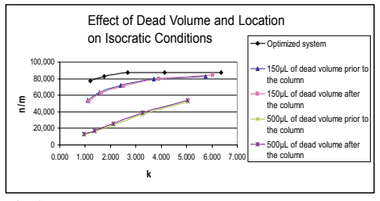
Figure 2



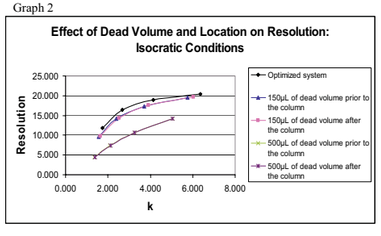
Effect of Dead Volume and Location on Efficiency: Isocratic Conditions

Graph 1 and **Graph 2** show the effect on efficiency and resolution (plates/meter, n/m) caused by adding 150µL or 500µL of dead volume to the system when using a 150x4.6mm C18 column under isocratic conditions. As expected, regardless of the dead volume location in the sample flow path, when it is increased both efficiency and resolution decrease. It is also important to note in **Graph 1** that beyond a k of 2, efficiency in the original separation reaches the optimum plateau, whereas with extra dead volume, full column efficiency is not attained even at a k of 6.

Graph 1



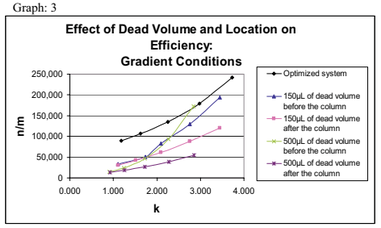
Graph 2



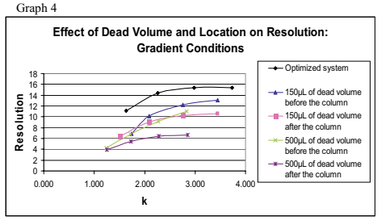
Effect of Dead Volume and Location: Gradient Conditions

Graph 3 and **Graph 4** show the effect on efficiency and resolution caused by increased dead volume when using a 150 x 4.6mm C18 column under gradient conditions. Chromatographic performance deteriorates, as expected. Unlike observations from isocratic separations, however, extracolumn volume in the portion of the sample path between the column and the detector has a more significant effect than extracolumn volume in the tubing, connections, guard column, etc. located before the column inlet. In fact, for the later-eluting compounds in the test mix, 150µL of extra volume after the column had the same effect as 500µL of extra volume before the column.

Graph: 3



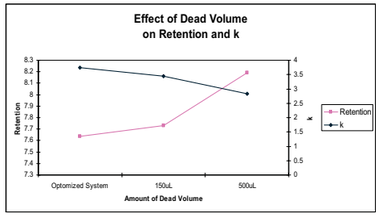
Graph 4



Effect of Dead Volume on Retention and k

Graph 5 shows the effect of dead volume on analyte retention and k. The retention time/volume increased proportionately to the amount of dead volume added to the flow path, while k decreased proportionately to the increase in void time/volume. The location of the dead volume had no significant effect on retention or k in any analysis performed in this study.

Graph: 5



Conclusions

In isocratic analysis, the amount of extracolumn volume in the sample flow path affects chromatographic performance.

In gradient analysis, the location of extracolumn volume in the sample flow path, as well as the amount, affects chromatographic performance: extracolumn volume in the system after the column has greater effect than extracolumn volume prior to the column.

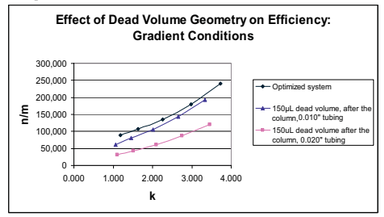
The geometry of extracolumn volume in the sample flow path, as well as the amount affects chromatographic performance.

Extracolumn volume in the sample flow path has greater effect on shorter columns.

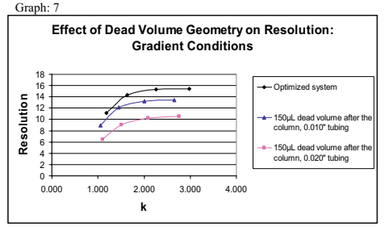
Effect of Dead Volume Geometry: Gradient Conditions

A variable related to dead volume is the geometry of the void. For this experiment 150µL of dead volume, after the column, in two alternative configurations, was added to the system. In one run, 296 cm of 0.010" ID tubing was used, in a second run 74 cm of 0.020" was used. As shown by **Graph 6** and **Graph 7**, the shorter tubing with larger ID had the greater effect on efficiency and resolution. This experiment was repeated in isocratic mode, and produced similar results.

Graph: 6



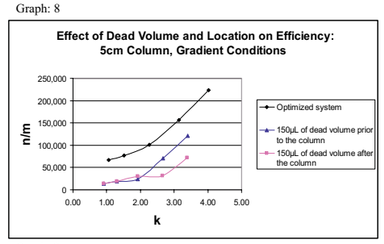
Graph: 7



Effect of Dead Volume and Location: 5cm Column, Gradient Conditions

Graph 8 and **Graph 9** show the effect on efficiency and resolution caused by increased dead volume when using a 50 x 4.6mm C18 column. Since the peak volume is much smaller for this column, a given amount of extracolumn volume has greater effect than on a 150mm column. In this system, 150µL of extra volume before the column reduced efficiency by 46%, whereas with the 150mm column the efficiency loss was only 20%.

Graph: 8



Graph: 9