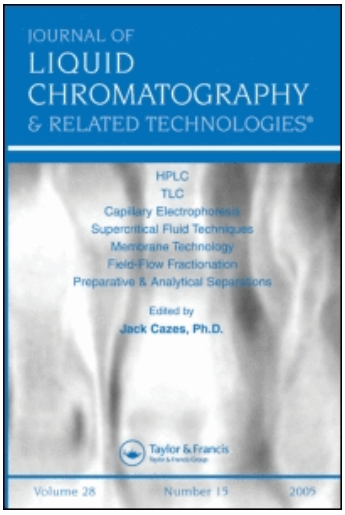

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Effect of Column Dimensions on HPLC Separations Using Constant Volume Columns

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EFFECT OF COLUMN DIMENSIONS ON HPLC SEPARATIONS USING CONSTANT VOLUME COLUMNS*

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ABSTRACT

The effect of column dimension on resolution, sample capacity, retention time, efficiency and mobile phase composition were studied, using both constant flow rate and constant linear velocity. The four columns selected (A = 238 x 3.2 mm, B = 153 x 4.0 mm, C = 116 x 4.6 mm and D = 50 x 7 mm) had the same volume. K' values were found to be constant, within experimental error, for all columns. At constant linear velocity, the retention time was found to be a linear function of column length, while at constant flow rate retention time was constant for all columns. The longest column (A) generated the largest N values while columns B and C gave the lowest H values, for dilute solutions, while they decreased with decreasing column length. On the other hand, it was observed that as the sample size increased, N generated by column A decreased more rapidly and eventually fell below the values generated by columns B and C. These two columns (B & C) can tolerate a larger sample size with less reduction in N value than the longest column. It is important to note that although there were minor differences in performance between columns B and C, there were significant differences between them (B and C) and the other two columns (A and D). Column A offered the highest sensitivity (narrower peaks) for dilute solutions, while columns B and C offered higher loadability. The volume of organic modifier in the mobile phase affected

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the retention equally in the four columns. It was also found that equal separation (α) was obtained for each column at constant flow rate and constant linear velocity, except with the latter the retention times were longer.

INTRODUCTION

High performance liquid chromatography (HPLC) is becoming one of the most widely used analytical techniques, due to its applicability to small as well as large polymeric and biomolecules, organic as well as inorganic compounds. Recent research into HPLC methods development and application have been mostly concerned with mobile phase optimization, type of column support materials, particle and pore size effects and applications to different fields. Although there have been studies into the effect of column size, it is not an easy task to order a column for a specific need. Today's analytical HPLC columns have different dimensions ranging from approximately 1 to 7 mm in diameter and 30 to 300 mm in length. So, it becomes difficult to decide which column to use in order to achieve optimum resolution of a mixture, and/or maximum sample loadability.

The design of the column is a very important aspect in analytical as well as preparative scale liquid chromatography. The processes involved in peak broadening are basically isotherm nonlinearity and column dispersion. The mathematical treatment is complex and there are no analytical solutions to the set of differential equations describing the combined effect. Except at infinite dilution (linear chromatography) and perhaps moderate overload, no theory is yet available to describe peak profile (1-6). In preparative liquid chromatography, theories stress the complex interdependence of optimum performance on a number of parameters including column length and diameter in addition to linear flow velocity, particle size, sample size and concentration and number of theoretical plates (7). In an attempt at simplifying the mathematical complexity, Hupe and co-workers (8,9) presented a simple relationship which allows a semi-quantitative determination of some of the parameters that optimize the amount of sample that can be separated per unit time (the production rate). Among the many parameters involved, column length and internal diameter received more attention. It is

concluded that the production rate increases with increasing both column length and cross-section. However, while the increase with column cross-section is linear, that with column length asymptotically approaches a maximum value.

In this study we present experimental results pertaining to the relation between column dimensions and column loadability or sample capacity, column efficiency and resolution of solute probes, as well as effect of organic modifier concentration using four columns with different dimensions (length and internal diameter), where all other parameters, including column volume were kept

EXPERIMENTAL

Materials:

The columns selected for this study had the following dimensions: Column A: 238 x 3.2 mm; Column B: 153 x 4 mm; Column C: 116 x 4.6 mm and Column D: 50 x 7 mm. All columns have the same volume of 1.923 ± 0.007 mm^3. Each column was packed with about 1.25 grams of 5 µm spherical, reversed phase C_{18} bonded silica (Advanced Separations Technologies, Inc., Whippany, NJ). The test solutes were nitrobenzene from Chem Services (Westchester, PA), biphenyl (BP) and 2-phenylphenol (2PP) from Aldrich Chemical Co. (Milwaukee, WI), diphenylamine from Fisher Scientific (Fairlawn, NJ) and toluene from Burdick and Jackson (Muskegon, MI). The mobile phase was acetonitrile/water (75:25). Its viscocity at 25°C was 0.59 centipoise ($mNsm^{-2}$). The diffusion coefficients of BP and 2PP in this solvent system were estimated to be 1.53 x 10^{-5} cm^2/s and 1.39 x 10^{-5} cm^2/s, respectively (10).

Apparatus:

A Hewlett-Packard liquid chromatograph (Model 1090M) equipped with a photodiode array detector, an integrator and an auto injector was used. All mobile phases were filtered and degassed before use and maintained under helium throughout the experiments. Effluents were monitored at 254 nm. Flow rates, solution concentrations and injection volumes are as specified in the figure legends.

RESULTS AND DISCUSSION

Column Selection:

The four columns used in this study were selected based on availability and reasonable column dimensions, diameter and length, which are in the median of commercially available analytical HPLC columns. It was decided that the medium size column should be approximately 15 cm in length and the shortest 5 cm. Longer columns with smaller diameters would be too long (a 2 mm diameter column having the same volume as those selected would have to be 61.2 cm long), while shorter columns (than 5 cm) would have too wide an internal diameter, and would prove to be impractical.

Column Testing:

For each column, a chromatogram of a 5 µl injection of the two solutes BP and 2PP (.014 µg/µl of each) was obtained at several linear velocities between 0.5 and 5 mm/s; and characteristic reduced plate height versus reduced velocity curves were determined (results not shown). The sample concentration was sufficiently low to establish the necessary condition of linear chromatography. Based on these results, the flow rates for this study were chosen to have linear velocities at the minimum of the reduced plate height versus reduced velocity plots. Void volumes were determined using sodium nitrite as a non-retained solute.

In the first set of experiments, the columns were evaluated at a flow rate of 0.7 mL/min each. In this case, the residence time of each solute is the same for each column. The linear velocities were as follows: Column A: 3.16 mm/s; Column B: 2.05 mm/s; Column C: 1.54 mm/s; and Column D: 0.66 mm/s.

In the second set of experiments, the flow rates for each column were varied in order to have solute data at the same linear velocity of 0.67 mm/s for each column. The flow rates were as follows: Column A: 0.15 ml/min; Column B: 0.23 ml/min; Column C: 0.3 ml/min; and Column D: 0.7 ml/min.

Furthermore, the column performance parameters K°, ϕ and E were determined. These parameters are defined in the following. In HPLC the velocity of the mobile phase (μ) which is constant along the column is related to the column parameters by the equation

$$\mu = K^\circ \, dp^2 \, \Delta P/\eta L \qquad (1)$$

where K° (a dimensionless quantity) is the specific column permeability; dp is the average diameter of the packing material; η is the viscocity of the mobile phase; ΔP is the column pressure drop which is effectively the reading of the inlet pressure gauge; and L is the column length.

The parameter $\phi = 1/K^\circ$ is the flow resistance factor, the importance of which is explained by Bristow and Knox (11).

The parameter E (the separation impedance) was also introduced by Bristow and Knox (11) and is meant to measure the performance of a given column in consideration of the number of theoretical plates generated by the column (N), void time (t_o), pressure drop (ΔP) and mobile phase viscocity (η). It is obtained via the equation

$$E = h^2\phi$$

where h is the reduced plate height (H/dp) and H is the height equivalent to a theoretical plate ($H = L/N$).

The value of K° can be evaluated from the Kozeny-Carman equation

$$K^\circ = \varepsilon^3/180(1-\varepsilon)^2$$

where ε the column external porosity, that is the fraction of the column volume not occupied by the particles and is therefore available to the solvent flowing around the particles.

ε can be calculated from experimental variables as follows:

$$\varepsilon = Ft_o/\pi r^2 L$$

where F is the flow rate and r is the column radius. For a well packed conventional column ε typically lies between 0.4 and 0.5 (12).

The column performance parameters described above were calculated for all columns and are presented in Table 1.

Several important observations about column performance are revealed from the results presented in Table 1. It is noted that h is lowest for columns B and C, even though column A has the highest value of N. Column D showed poor performance as indicated by the value of h = 9.4 and the values of the other parameters. The values of K° and the related parameter ϕ falls within the range of well packed conventional columns. Typically K° values are around 1×10^{-3} and ϕ values are therefore about 1×10^{3} (13,14).

The large E value for column A is a result of somewhat high value of h, while the very large value for column D is a combination of both large ϕ value and large h value. The results presented in Table 1 indicate that the dimensions chosen for columns B and C yield the best values for the column performance parameters out of the set of columns chosen for this study. Column D (50 x 7 mm) was repacked more than once by different manufacturers, but gave relatively poor results in each case.

Effect of Sample Size on N, H and R:

In the limit of linear chromatography (very small sample size), the two solute probes (BP and 2PP) and sodium nitrite were chromatographed first at

TABLE 1. Column performance parameters

Column (mm)[a]	h	$K^{\circ}10^{3}$	ϕ	E	ε
238 x 3.2	3.6	1.23	813	10537	0.459
153 x 4.0	2.4	1.28	781	4499	0.452
116 x 4.6	2.4	1.10	909	5236	0.457
50 x 7.0	9.4	0.27	3704	327285	0.556

[a]The values were calculated for the solute biphenyl (k'=1.61±0.02 at a flow rate of 0.7 mL/min.

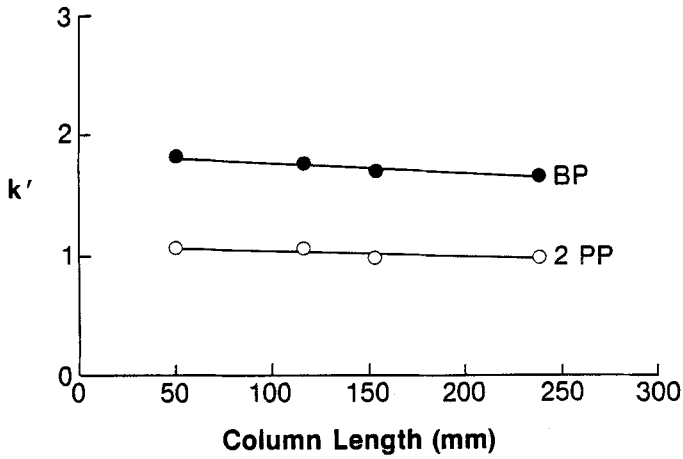

Figure 1 Capacity factor versus column length for columns of equal volumes packed with 5 µm spherical reversed phase C_{18} material and a mobile phase of acetonitrile/water (75:25) at a flow rate of 0.7 ml/min. 5 µl of a solution of 0.024 µg/µl biphenyl and 2-phenylphenol were injected and the effluent was monitored at 254 nm.

constant linear velocity of 0.67±.02 mm/s (variable flow rate) and then at constant flow rate of 0.7 ml/min. (variable linear velocity) using acetonitrile/-water (75:25 v/v).

Figure 1 shows a plot of k' versus column length. As expected k' values are constant, within experimental error, for all columns, at the two flow rates used for each column.

On the other hand, when a constant linear velocity is used, the retention time is a linear function of column length irrespective of column cross-section. This result is clearly illustrated in Figure 2. Furthermore, when the solutes are chromatographed at constant flow rate (0.7 ml/min) the retention time for each solute is essentially constant, on all the columns, within experimental error for all columns (see Table 2).

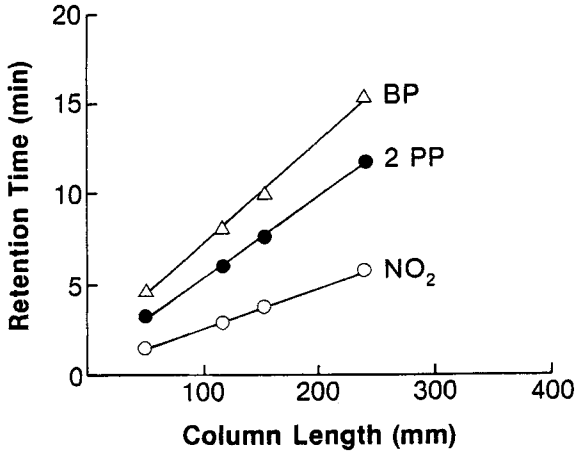

Figure 2 Retention time versus column length. All experimental condition are the same as figure 1 except a linear velocity of 0.67 mm/s (i.e. variable flow rate, see text for details).

TABLE 2

Retention times for BP and 2PP on column A, B, C and D using a mobile phase of acetonitrile/water (75:25) and a flow rate of 0.7 ml/min. Sample size: 5 ml of 0.024 µg/µl of each BP and 2PP.

Column	A	B	C	D
BP	2.77	2.72	2.75	2.84
PP	3.59	3.54	3.59	3.68

The above results are the average of three readings, having a relative standard deviation of 0.02.

The decrease in column efficiency with increasing injected quantity is a matter of concern in analytical liquid chromatography. It is even more critical in preparative liquid chromatography because a certain minimum number of plates is required to obtain a substance with a given degree of purity. A large sample size could be delivered either as a small volume of a concentrated solution or a large volume of a dilute solution. It has been established that an adverse overload effect appears more rapidly on a more efficient column compared to a less efficient column. Furthermore it was shown (15) that the linear capacity of the column is higher for diluted than for concentrated sample solutions for the same amount of solute. In this work, we attempted to compare the four columns with respect to efficiency and sample capacity (loadability) as a function of injected sample size by both sample overload (fixed volume of solutions with different concentration) and volume overload (different volumes of a dilute solution). Since the columns studied in this work showed a slightly better efficiency (lower H values and higher resolution for the solutes BP and 2PP) at the fixed flow rate of 0.7 ml/min. we will show in what follows the results obtained at constant flow rate. This should be acceptable because the trends observed in both modes of operation were nearly identical.

Figure 3 shows a plot of N (theoretical plate height) versus sample size in the mass overload mode at a constant flow rate of 0.7 ml/min. Figure 4 shows a corresponding plot of H versus sample size. Examination of these two figures revealed that the longer column (A) generated a large N value in the linear range and that N decreases with decreasing column length. On the other hand, H which measure column efficiency per unit length had lower values for the wider and shorter columns B and C compared to the longer and narrower column A. Furthermore it was observed that as the sample size increased, N generated by column A decreases more rapidly and eventually approaches and falls below the values generated by columns B and C at a sample size of about 100 μg (80 μg

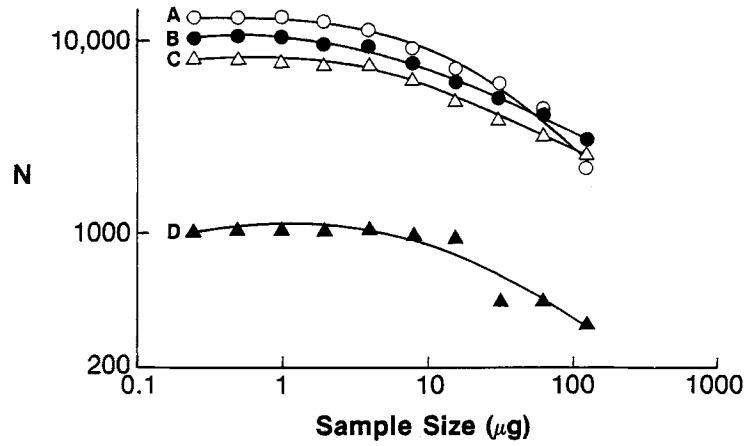

Figure 3 Effect of sample size (biphenyl) on N for columns of equal volume. Experimental conditions are the same as for figure 1.

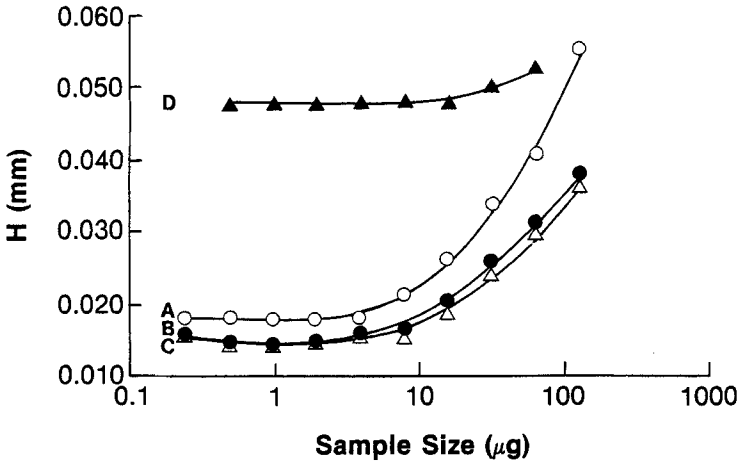

Figure 4 Effect of sample size on H. All experimental conditions are the same as for figure 3.

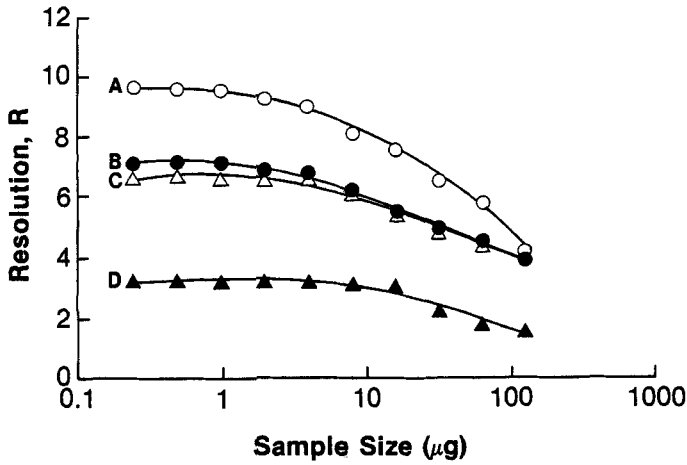

Figure 5 Effect of sample size on resolution. All conditions are the same as for figure 3.

sample/g packing). It was also observed that the linear range of columns B and C is wider, i.e. they can tolerate a larger sample size with relatively less reduction in N. It was evident that column A offers the advantage of greater sensitivity (narrower peaks) in its linear range (small sample) while columns B and C offer the advantage of higher loadability. Note that although the solvent consumption and solute residence time is the same for all columns under the chosen experimental conditions, columns B and C were operated at lower inlet pressures compared to column A because of their wider diameter. Column D which gave poorer N and H values has a wider linear range than the other three columns. Figure 5 shows the effect of sample size on the resolution of BP and 2PP. All the conclusions drawn from figures 3 and 4 are more clearly illustrated in this figure. It is significant to note that there are minor differences in the performance of columns B and C and both are widely different from columns A and D. It is also important to note that while peak width is significantly affected by increasing sample size, k' values in this sample size range (0.12 μg - 125

µg) are only slightly affected. At a sample size of 125 µg k' was only 2% less than that at the linear range of 1-5 µg. Figure 5 also shows that although column A gave much better resolution of BP and 2PP than that of the other columns at 0.12 µg, the resolution was equal to that obtained on columns B and C when 125 µg of sample was chromatographed. Note also that column D had a wider linear range while column A had the narrowest and the highest drop in resolution with increasing sample size.

Sample Size vs Sample Volume:

The differences between the two modes of injection namely sample overload and volume overload were evaluated. Sample load can be studied in two different ways; (a) increased injection volume of the same dilute solution i.e. increased volume, increased concentration; or (b) injection of different volumes having the same concentration, where only the volume injected affects the resolution and not the concentration of the solute. For figures 6 and 7 the increased volume, increased concentration mode was used while for figure 8 increased volume, constant concentration mode was used. Figure 6 gives a plot of resolution versus sample size for both modes of sample introduction using column A, and figure 7 gives a corresponding comparison on column B. It was observed that the sample overload mode gave higher resolution compared to volume overload at large sample size; however, the magnitude of the difference diminished when columns B or C were substituted for column A. Figure 8 shows the effect of volume injected on resolution using the four columns. The results for columns A, B, and C show an almost threefold drop in resolution between a 2 µl and 60 µl injection. The results also show that Columns A, B, and C gave equivalent resolution (within experimental error).

Effect of Acetonitrile on Retention:

In a previous study (16), it was observed that for a set of columns having the same diameters but different lengths (i.e. different volumes) the sample

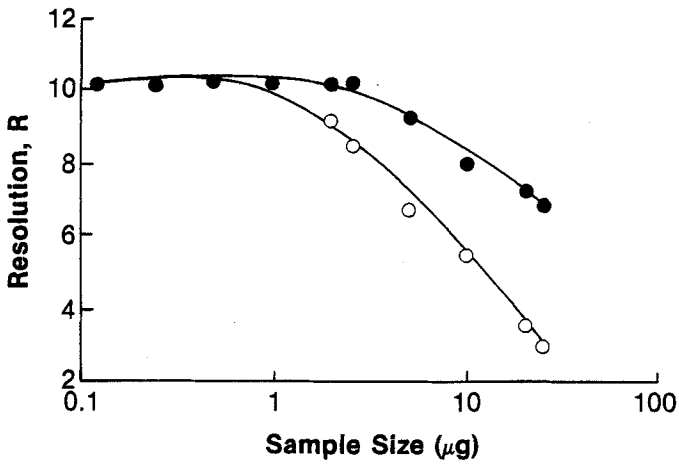

Figure 6 Comparison of the plots of resolution versus sample size on the 238 x 3.2 mm column. O = volume overload (sample concentration 1 μg/μl), ● = sample overload (5 μl sample injected). Solutes BP and 2PP. Other conditions are the same as for figure 5.

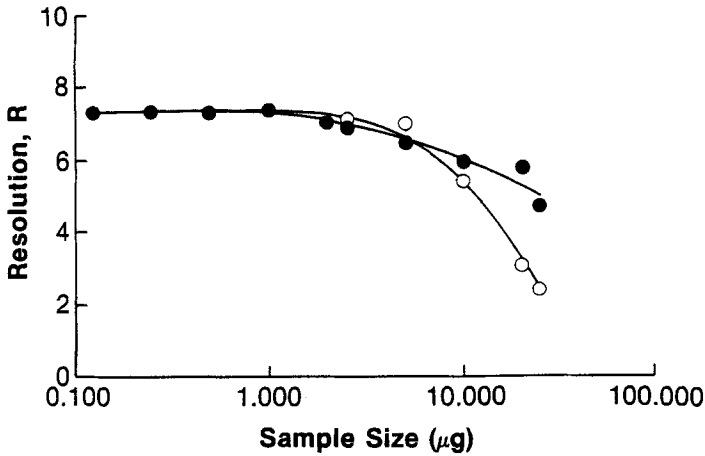

Figure 7 Comparison of the plots of resolution versus sample size on the 153 x 4.0 mm column. Experimental conditions are the same as for figure 6.

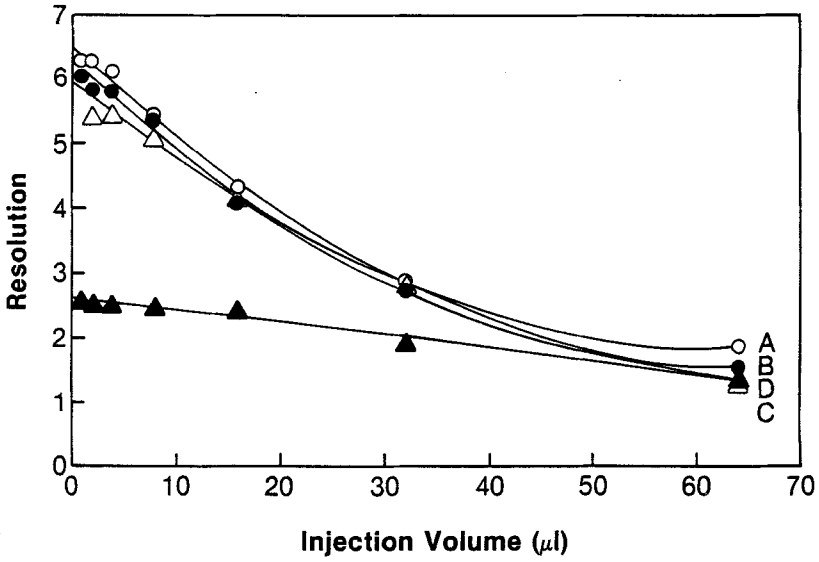

Figure 8 Comparison of the plots of resolution versus volume injected on Columns A, B, C, and D. All experimental conditions are the same as for figure 3.

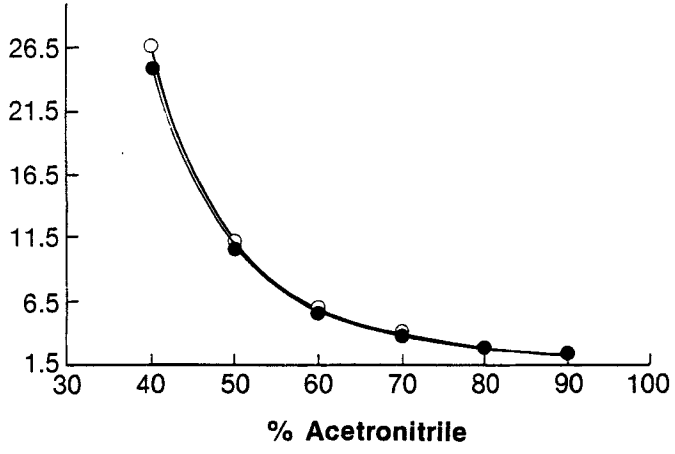

Figure 9 The influence of acetonitrile concentration in the acetonitrile/water mobile phase for BP on the 4 columns having the same volume. All experimental conditions are the same as for figure 1.

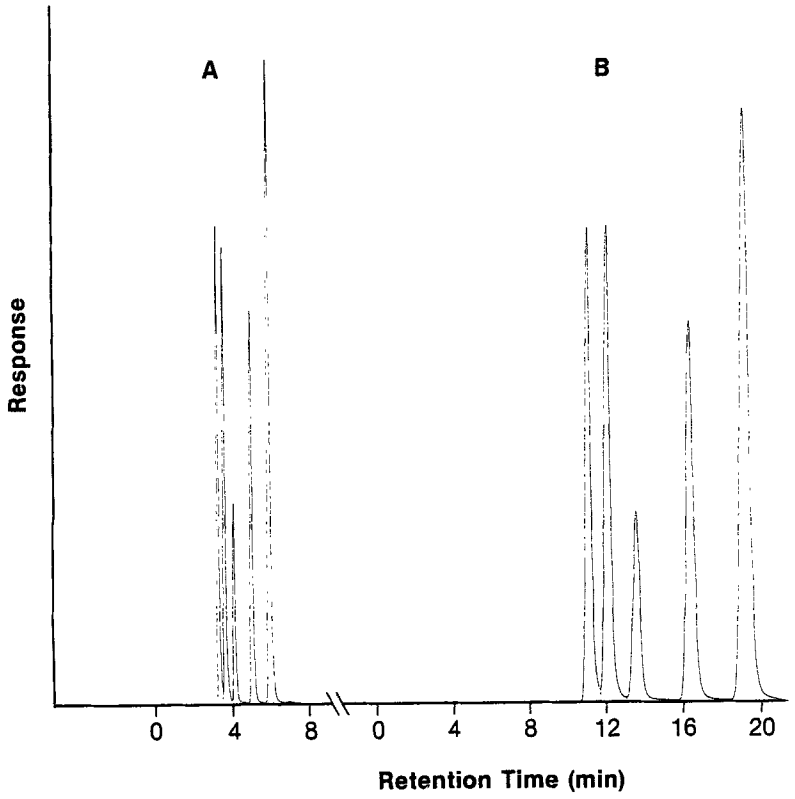

Figure 10 Chromatograms of the separation of a mixture of nitrobenzene, 2PP, toluene, diphenylamine and BP using column B (153 x 4.0 mm) and a mobile phase of 60% acetonitrile/water at (a) constant flow rate (0.7 ml/min) and (b) constant linear velocity of 0.67 mm/s (a flow rate of 0.23 ml/min). Other conditions are the same as for figure 1.

residence time in the column is important and can affect resolution. This meant that if a mixture of A and B is resolved on a 25 cm RP C-18 column using 80% acetonitrile/water, the same mixture can be resolved on a shorter column, giving equivalent separation, if the percent acetonitrile in the mobile phase is decreased. In this study, it was of interest to see if the lengths of the same volumn columns have such an effect. The results show that the retention times obtained for BP on the four columns were not affected by the column dimensions, and gave equivalent retention times under the same experimental conditions. This is due to the efficient distribution of the sample solution at the head of the column. Figure 9 shows the values for columns A and B. Those for columns C and D overlap with those of A and B.

Constant Flow Rate vs. Constant Linear Velocity

Since the columns used in this study had the same volume, but different length and diameter it was of interest to see which mode (constant flow rate or constant linear velocity) would give a better separation of a mixture. Figure 10 shows the separation of a mixture of nitrobenzene, 2PP, toluene, diphenylamine and BP on column B at a constant flow rate (0.7 ml/min) and constant linear velocity 0.67 mm/s (0.23 ml/min). No appreciable difference in the separation (α) of the mixture is observed, except that the retention times are longer at constant linear velocity. The results (not shown) were similar for columns A, C, and D.

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