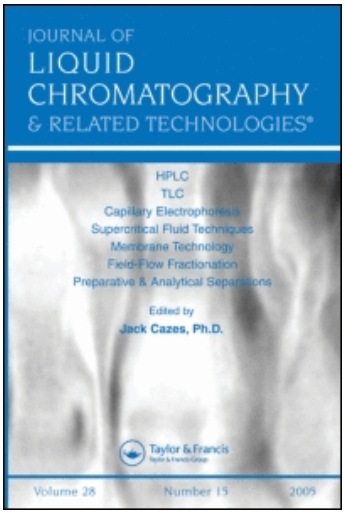

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MIXED REVERSED PHASE/BETA CYCLODEXTRIN PACKINGS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: SINGLE MIXED SUPPORT COLUMN VERSUS TWO COLUMNS IN SERIES*

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ABSTRACT

A study of mixed C_{18}/β packings in high performance liquid chromatography was undertaken to evaluate the utility of such phases. The results show that non additive retention times are obtained when a mixed phase column is used while the retention times are additive when two columns, one packed with C_{18} and the other with β are coupled in a series. A plot of corrected retention times vs % β in C_{18} gave a non-linear result. This means that the prediction of retention times on a mixed phase column may not be easily done. As a result, in this case, it will be easier to couple two different columns of different selectivities to achieve a difficult separation.

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INTRODUCTION

High Performance Liquid Chromatography (HPLC) has gained great acceptance as an analytical tool and is found in almost every laboratory. This is due to its relative ease of operation, the wealth of research effort into the optimization of the mobile phase, the wide range of commercially available column supports of different modes, its applicability to a wide range of compounds (normal, ionic, polar, polymeric and biomolecules of all sizes) and the relatively economical instrumentation (pumps, detectors...etc). Today's columns come in different sizes packed with different supports - silica base or polymeric, normal, reversed phase (bonded akyl chains of various lengths), ionic (anion and cation), gel for size exclusion, or alkyl chains bonded to the silica and to another modifier such as cyclodextrins (α, β or γ), a phenyl-, cyano-, amino- or others. All the above mentioned supports are available commercially in a single mode, that is a column packed with one support. Unlike gas chromatography, where mixed support columns are used almost routinely, in HPLC there has been no commercially available mixed support column. In fact, a search of the scientific literature reveals little activity into this area. In a recent article Rassi and Horvath (1) wrote "remarkably enough mixed-bed columns have received little attention in HPLC". This is disappointing in light of the facts that (a) mixed phases are used in GC; (b) coupled columns of different selectivities are used in GC and HPLC; and (c) one can safely assume that a single peak obtained with a single mode column may be resolved into several peaks on a mixed-mode support column. This means that a column can be made to achieve the resolution of a mixture which is not separated on the individual support columns. In addition the column can be used in different chromatographic modes as will be discussed later. Wise et al (2) has shown that selectivity in HPLC can be modified by using a mixed supports column. In their study, better separation of a polycyclic aromatic hydrocarbon (PAH) mixture was

achieved on reversed phase HPLC using a column made of a mixture of 5 µm polymeric C_{18} material from two different lots with high and low C_{18} surface concentrations, than by using the individual columns. They also reported that selectivity factors for these mixed phase columns were found to be similar to those predicted by the linear addition of the selectivities of the two individual phases. Rassi et al (3) found that the resolution obtained by using columns packed with mixed anion and cation exchangers, does not depend linearly on the fraction of the retentive ion exchanger particles under isocratic mobile phase conditions. The advantages of using a mixed phase anion/cation column is that such a column can be used as an anion, cation or hydrophobic interaction column. Ogan and Katz (4) used columns made of a mixture of Lichrosorb RP-2 and RP-18, and claimed that the relationship between retention times and % composition of the stationary phase was linear.

The present research was undertaken in order to evaluate the use of mixed support columns in HPLC. The supports selected are C_{18} reversed phase (C_{18}) and β-cyclodextrin bonded to silica gel (β). The aim of the study is to evaluate (a) feasibility and useability of such mixed phase, mixed mode columns; (b) what effect, if any, each support in the column has on the others selectivity; (c) are the retention times obtained on the mixed phase column linear and predictable; (d) can columns be easily tailored to achieve a specific separation and (e) is it easier to use a mixed phase column or two coupled columns in series. For this study five columns packed with C_{18}, β, and a mixture of both by volume (75%, 50% and 25% C_{18} in β) were selected. The mobile phase used is methanol/water. Three groups of compounds, naphthalene and biphenyl; 2-phenylphenol and 4-phenylphenol; and an isomeric mixture of 2-, 3-, and 4-hydroxybenzyl alcohols (HBA) were used. The C_{18} and β were bonded to a 5 µm spherical silica and packed into steel columns.

EXPERIMENTAL

Materials

The compounds used in this study (biphenyl, 4-phenylphenol, 2-phenylphenol, 2-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, triethylamine) were purchased from Aldrich Chemical Company, (Milwaukee, WI) except naphthalene which was purchased from Eastman Organic Chemicals, (Rochester, NY) and glacial acetic acid from Baker Analytical, (Phillipsburg, NJ). The methanol used was glass distilled UV grade, Burdick & Jackson, (Muskegon, MI). Water was glass distilled deionized. All of the columns were supplied by Advanced Separations Technology, Inc., (Whippany, NJ) and contained C_{18} and/or β-cyclodextrin (cyclobond I) bonded to 5 µm spherical silica particles. All columns had the same dimensions, 25 cm x 4.6 mm.

Apparatus

A Perkin-Elmer Series 4 Liquid Chromatograph equipped with a Kratos Spectroflow 783 programmable absorbance detector, Waters WISP automatic injector, strip chart recorder, and a Hewlett-Packard 3357 integration system was used. 10 µl of solution was injected and monitored for UV absorbance at 254 nm. 0.01 M triethylaminoacetate (TEAA) buffer was made by adding 1.4 ml triethylamine to 1 liter of water and titrating to pH 4.5 with glacial acetic acid. All mobile phases were filtered and degassed before use and maintained under helium throughout the experiments. The mobile phase flow rate was 1 ml/min.

RESULTS AND DISCUSSION

One of the objectives of this study was to compare the retention times obtained on two columns of different selectivities coupled in series, and

individually. A C_{18} reversed phase and β-cyclodextrin bonded columns having the same base silica from the same batch were used. These two types of columns were selected because they have different mechanisms of separation, one (C_{18}) by partition the other (β) by inclusion mechanism. The mobile phase, methanol/water, is compatible with both column packings, which is a requirement when mixed stationary phases are used.

Table 1 lists the retention times obtained using C_{18} column, β-cyclodextrin column and C_{18}-β and β-C_{18} columns connected in series. The table shows no appreciable difference in the sum of retention times obtained using the single columns or the columns in series. This means that the order by which the columns are coupled has no relevence on the final retention time. These results which agree with those published (1) show that when columns of different selectivities are connected in series the retention times are additive and linear. This gives the chromatographer the unique capability of tailoring coupled columns to specific separations. For example, difficult separations of a mixture which cannot be resolved on a single column may be resolved on a series of coupled columns of different selectivities. In such a case the mobile phase should be compatible with both the columns used. To illustrate the above let us look at the retention times in Table 1 for naphthalene, biphenyl, 2-phenylphenol and 4-phenylphenol. It is clear that 2-phenylphenol was not resolved from biphenyl on C_{18} but was resolved on β column. Again naphthalene and 4-phenylphenol were resolved by 0.5 min on β and 2.4 min on C_{18} under the same experimental conditions. By coupling both columns in series, the four compounds are separated from each other by at least 1.1 min. It was of interest in light of the above linear and additive results to find out if one can predict the retention times using a column made of mixed supports (C_{18} and β) rather than coupled columns in series. The rationale for doing so was (a) only one column would be used which means lower back pressure; (b) one can tailor a support mixture to achieve difficult separations, using the window diagram (5), a procedure

TABLE 1
COMPARISON OF RETENTION TIMES OBTAINED FOR THE TEST SOLUTES ON C_{18}, β, C_{18}–β AND β–C_{18}

		Columns				
		t_R(min.)				
Compound	Mobile Phase MeOH/H_2O	C_{18}	β	Sum	C_{18}–β	β–C_{18}
Naphtalene	70:30	8.3	4.3	12.6	12.8	12.6
Biphenyl	70:30	11.6	5.5	17.1	17.6	17.2
2–Phenylphenol	70:30	5.9	3.7	9.6	9.7	9.6
4–Phenylphenol	70:30	5.9	4.8	10.7	10.9	10.7
2–HBA	10:90	16.0	7.3	23.3	23.1	22.5
3–HBA	10:90	13.7	6.4	21.1	20.3	19.6
4–HBA	10:90	11.0	7.3	18.3	18.4	17.7

which was previously used to predict a binary mobile phase for optimum resolution of a mixture.

In this study, unlike that of Wise et al (2) who used a mixture of polymeric reversed phase supports with high and low C_{18} surface concentration and unlike Katz and Ogan (4) who used a mixture of Lichrosorb C_2 and C_{18}, a mixture of two supports having different selectivities and mechanisms of separation (partition and inclusion) were selected for this study. Five columns were used which have the same dimensions and silica from the same batch. The columns were packed with: 100% C_{18}, 75%, 50% and 25% C_{18} and 100% β. Three groups of compounds were used, naphthalene and biphenyl, 2- and 4-phenylphenol and 2-, 3-, and 4-hydroxbenzyl alcohols. Figure 1 is a plot of corrected retention times (t'_R) of naphthalene, biphenyl, 2-phenylphenol and 4-phenylphenol using the five columns in this study. The results clearly show that a curve was obtained for all the compounds except 4-phenylphenol. This was surprising in light of the linear results obtained for the coupled columns, Table 1. The experiment was repeated but the results were the same. The results also showed, Figure 1, that the higher the retention time, the steeper the curve.

It was suggested (6) that since the column is made of a mixture of two different supports, each is being diluted by the other and as a result the amount of solute injected into the mixed support column may be too large compared with that injected onto a single phase column. The concentration of the test solution was diluted by 100 fold from 10 µg/injection to 0.1 µg/injection. The results, Figure 2, show no appreciable difference in the shape of the curve or the retention times. This means that the solute concentration is not a factor in this case.

It is a fact that due to steric hindrances some silanol groups remain underivatized on the silica surface. It was thought that these silanol groups

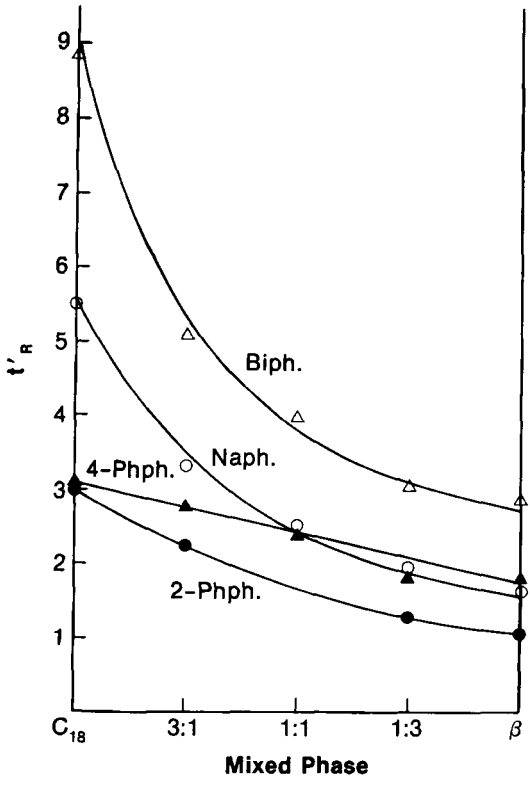

Figure 1. A plot of corrected retention times vs. mixed phase C_{18}/β composition using a mobile phase of 70% methanol/H_2O at a flow rate of 1 ml/min. A solution containing 10 µg of each test compound was injected and analyzed.

may contribute somehow to the non-linearity of the results. Therefore, in order to block these groups, 0.01 M triethylammonium acetate (TEAA) was added to the mobile phase. Using 0.1 µg/l injection and methanol: 0.01 M TEAA (70:30) there was again no appreciable difference in the results, Figure 3. A plot of lnk' vs % β in the phase mixture gave a linear relationship, Figure 4. If the curve in Figures 1, 2, 3 were linear, the plot of lnk' vs % β should not be linear as Figure 4 indicates.

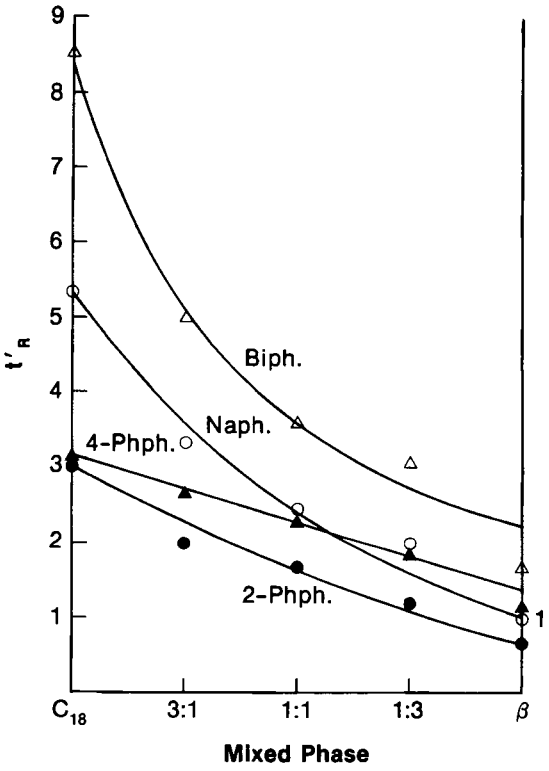

Figure 2. Same as Figure 1 except a solution containing 0.1 µg of each test compound was injected and analyzed.

Since methanol is a strong solvent for ß phases, it was decided to use a series of compounds which can be eluted at lower percentage of methanol in the mobile phase. A group of three hydroxybenzyl alcohol isomers were selected. A mobile phase of 10% methanol/water was used. The results, Figure 5, show clearly a non-linear relationship when t'_R is plotted against % ß in the mixed phases.

In a recent discussion (7) Scott suggested that it is possible that 10% MeOH may not cover the support surface completely and this may contribute to non-

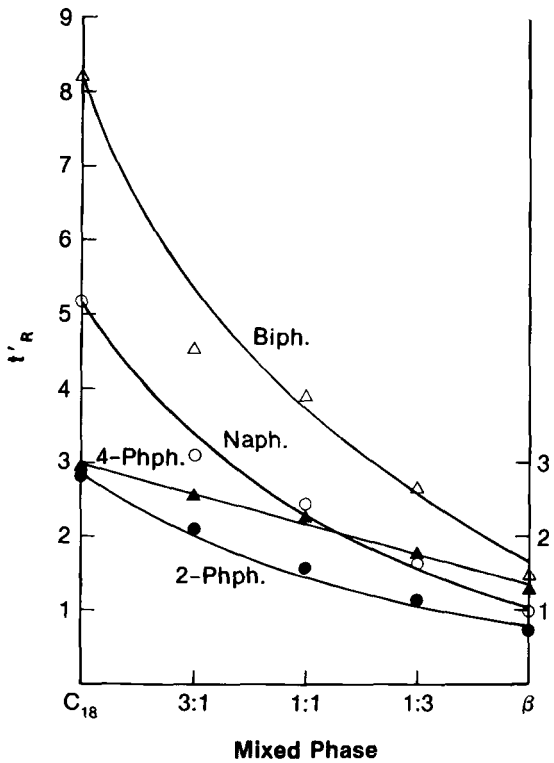

Figure 3. A plot of corrected retention times vs. mixed phase C_{18}/β composition using a mobile phase of 70% methanol/30% triethylammonium acetate (0.01 M) pH 4.5. A solution containing 0.1 µg of the test solution was injected and analyzed. Flow rate 1 ml/min.

linearity of the results. A new experiment was designed where three different concentrations of methanol in the mobile phase were used: 10% MeOH; 17.5% MeOH and 25% MeOH. The results are given in Figure 6, which shows that non-linear curves were obtained in each case. The only observed effect of the increase of the volume of methanol is the faster elution of the test compound, which is expected.

At this point we have no reasonable acceptable explanation as to why when two different selectivity columns are connected in series, the results are

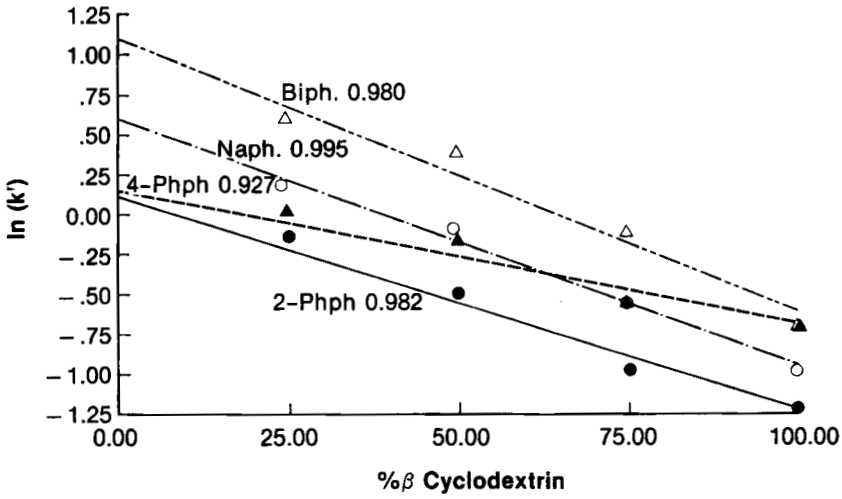

Figure 4. A plot of lnk' vs. mixed phase C_{18}/β composition. Experimental conditions as in Figure 3.

linear and when the phases are mixed then packed into the column the results are not linear. Rassi el al (3) in explaining the non-linearity of the mixed anion/cation exchange column by assuming that the accessibility of the retentive particles for the eluite and thus the effective phase ratio is reduced by the presence of oppositely charged ion exchanger particles in the surroundings. As a result, they speculated, the retention factor is smaller than would be expected in the absence of such coupling phenomena which are caused by electrostatic shielding.

The present study shows that there is a decrease of retention when a column made of a mixture of the phases is used. One can talk of shielding of the C_{18} chains on the silica surface by adjacent β-cyclodextrin molecules, or vice versa, however, most of the separation occurs in the pores of the silica where the majority of the C_{18} and β groups are located. Also, the size of the silica spheres, 5 μm diameter is too large compared to the C_{18} chains and the β molecules for shielding to be a major factor. The bulk

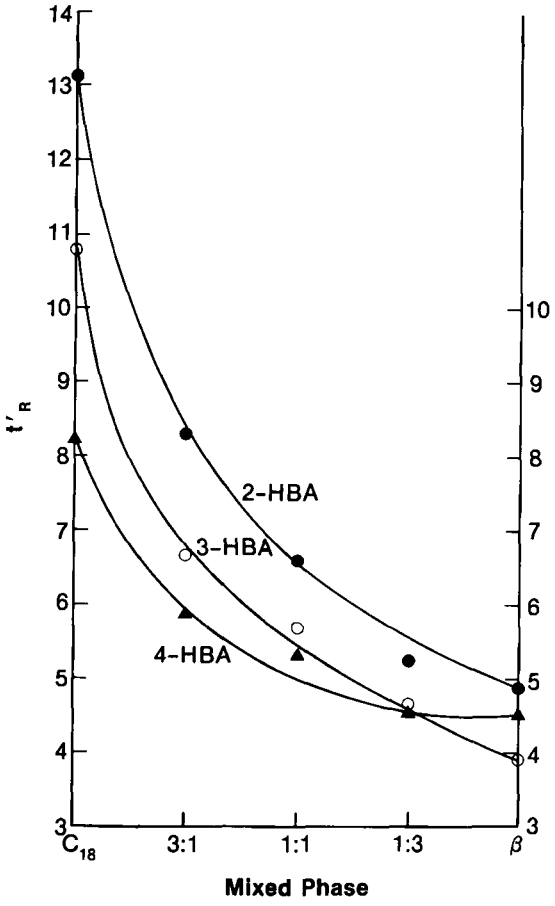

Figure 5. A plot of corrected retention times of 2-, 3-, and 4-hydroxybenzyl alcohols using 10% methanol/water at a flow rate of 1 ml/min. A solution containing 0.1 µg of each compound was injected and analyzed.

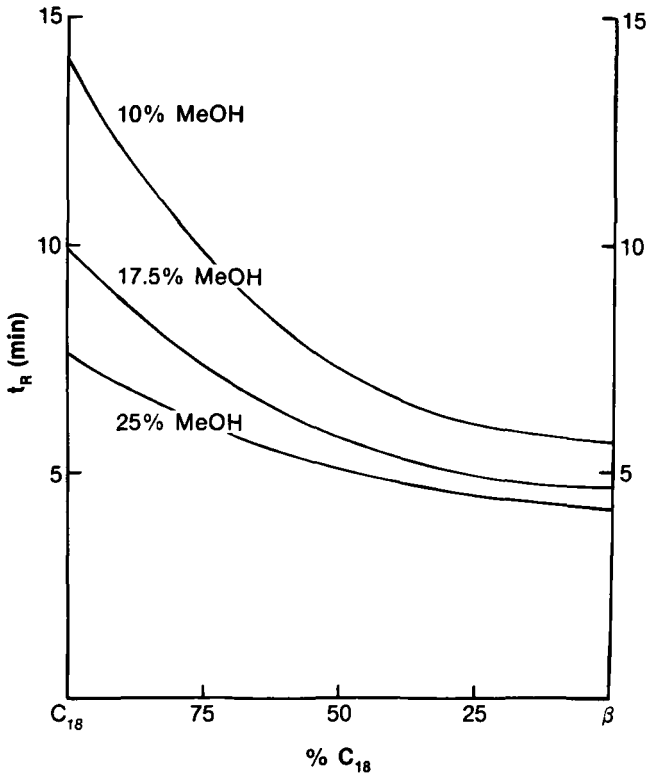

Figure 6. A plot of retention times of 2-hydroxylbenzyl alcohol on the five test columns (see text for details). Experimental conditions as in Figure 5.

density differences of C_{18} and β cannot account for the differences observed.

Another possible explanation is that water molecules from the mobile phase are trapped in the β cavity and as a result there is more methanol in the mobile phase which would decrease the retention of the solute molecules. This would result in faster elution times. One can also argue that this is of such small effect, because the mobile phase is in equilibrium at all times. It is also possible that the water molecules in the β-cavity render

it ineffective in operating as an inclusion complex, which would result in less retention of the solute molecules. Another explanation which seems realistic is that there are two different mechanisms which, when taken individually, are linear. Therefore, it is possible that the addition of two linear plots having different slopes would result in a non-linear relationship (8).

Wise et al (2) found in their study that the linear relationships hold only when the C_{18} phases were prepared on silica of the same surface area. In our study silica from the same batch was used, therefore, the surface area effect could not contribute to the non-linearity of the results. Ogan and Katz (4) evaluated the retention and selectivity of 12 PAHs on columns packed with 100% Lichrosorb RP-18, and three columns packed with 25%, 50% and 75% Lichrosorb RP-18 in Lichrosorb RP-2. Their data indicated that the contribution of the individual components were simply additive. Upon examination of their results (Figure 8, ref. 4) it is clear that the plot of K' vs % RP-18 is non-linear for the majority (11 out of 12) of the tested PAHs. In another part of the study Ogan and Katz (4) found that when columns packed with C_{18} bonded silicas having different pore diameters, the results were inconclusive. This finding agrees with that observed later by Wise et al (2).

Pietrzyk and Brown (9) used a mixed anion and cation exchangers column for the separation of inorganic ions. Their results show that anions retention (Cl^- and NO_3^-) decreased by 16-21% and cation retention increased by only 5% although cation exchanger weight in the column was increased by 33% (from 1:1 to 2:1 cation:anion ratios). This indicates a non-linear relationship between retention and the ratio of cation:anion in the mixed bed column, which agrees with the results of Rassi, et al (3) for the separation of proteins on a mixed-bed anion/cation exchange column. Crowther and Hartwick (10) synthesized chemically bonded multi-functional stationary

phases which are largely reversed phase in nature, but also contain significant ion exchange properties. By bonding ionic and hydrophobic groups to the same silica they were able to achieve unique selectivities for nucleotides and nucleosides separation. A near linear increase in R' was observed upon going from 50 to 100% quaternized groups.

An HPLC phase was synthesized in which a mixture of two silanes, an alkylorganosilane and a nitrilorganosilane, were bonded to the same silica gel (11). The results show that improvent in selectivity of PAH was achieved. However, when the ratios of ODS to cyanopropyl on the silica were changed from 100% ODS to a mixture of both, non linear K' values were observed in most cases (figures 2, 3, 4, and 6 in ref. 11).

CONCLUSION

It is clear from published and present results that, to date, most of the results obtained using mixed-bed or mixed ligands on the same particle, showed a non-linear relationship between t'_R or K' and the ratios of the mixed-beds or ligands. This means that % change in retention does not faithfully reproduce the % change in the mixed-bed or ligands.

The results in this study indicate that the retention times obtained by coupling two different selectivity and separation mechanisms columns in series gave linear, predictable and additive results when the individual columns are used. Therefore, one can achieve the resolution of a mixture of solutes by selecting a series of different length columns of different selectivities. On the other hand the results show that the retention times obtained using a column made of a mixture of two different selectivity or different separation mechanism particles is not linear and therefore may not be easily predicted in advance. Therefore, it is concluded that it will be easier to predict the elution times of a mixture by coupling 2 columns in a

series rather than by mixing 2 different phases in the column. Also, as observed earlier (2, 4) the surface of the silica particles for both phases should be the same. The advantage of having one mixed phase column rather than 2 coupled columns is that less back pressure results from using one column. It is not clear at this time why mixed-phase columns give non-linear relationship, but our efforts are continuing into understanding this phenomenon.

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