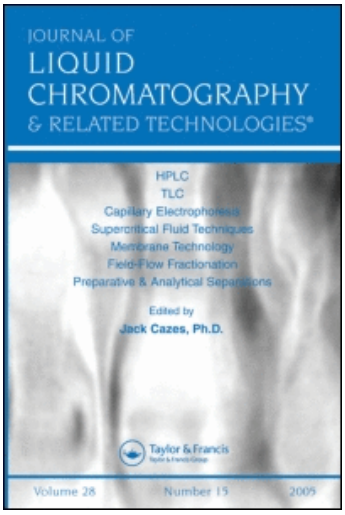

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Donald J. Pietrzyk[a]; Ziad Iskandarani[b]; Gary L. Schmitt[c]
[a] Department of Chemistry Iowa City, The University of Iowa, Iowa [b] Dow Chemical Company Midland, Michigan [c] PPG Industries Barberton, Ohio

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ANION EXCHANGE-ADSORPTION ON LOW CAPACITY ANION EXCHANGERS: SEPARATION OF ORGANIC ACIDS, AMINO ACIDS, SMALL CHAIN PEPTIDES

Donald J. Pietrzyk[1]*, Ziad Iskandarani[2], and Gary L. Schmitt[3]

[1]*The University of Iowa*
Department of Chemistry
Iowa City, Iowa 52242
[2]*Dow Chemical Company*
Midland, Michigan 48640
[3]*PPG Industries*
Barberton, Ohio 44203

ABSTRACT

The variables that influence the retention of organic analyte anions on a macroporous, high surface area polystyrenedivinylbenzene copolymer that is chemically modified by attaching tetraalkylammonium groups to the copolymer surface are identified and studied as a function of anion exchange capacity. A combined adsorption-anion exchange retention of the organic analyte anion is possible providing the analyte has both a hydrophophic center and an anionic charge site. As the column anion exchange capacity (0 to 173 μeq of anion exchange sites/column was studied) increases, analyte retention due to adsorption decreases and retention due to anion exchange increases. The factors influencing organic analyte anion retention by adsorption are low anion exchange capacity and mobile phase solvent composition, type of organic modifier, and pH for analytes that are weak organic acids. For anion exchange the major factors are a high anion exchange

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capacity, mobile phase ionic strength and pH, and the anion selectivity for the mobile phase counteranion. Separations of mixtures of benzoic acid derivatives, amino acids, and small chain peptides illustrate the effects of combined adsorption-anion exchange on selectivity and resolution.

INTRODUCTION

Low capacity ion exchangers are excellent stationary phases for the ion exchange separation of inorganic and simple organic ions (1). A polystyrene divinylbenzene (PSDB) co-polymer, a typical backbone for ion exchangers, is a favorable reverse phase adsorbent for the retention and separation of organic analytes particularly when it has a high surface area and macroporous type structure (2-4). Recently, Cantwell and coworkers prepared low capacity anion (5,6) and cation exchangers (5,7) where tetraalkylammonium and sulfonate sites were chemically attached to a macroporous PSDB backbone, respectively. They proposed that organic ion sorption under appropriate conditions is due to: 1) ion exchange of the analyte ion in the diffuse part of the electrical double layer resulting from the exchange site and its counterion, and 2) adsorption of the organic analyte ion providing it also has a hydrophobic center onto the PSDB surface. The significance and contribution of double layer effects in chromatographic retention is supported by other investigations which have focused on the retention of analyte ions from mobile phases containing alkylsulfonate or tetraalkylammonium salts as mobile phase additives (6,8-16 and references within).

In the present study we have modified Hamilton PRP-1 (a 10 μm, spherical, high surface area PSDB), which provides excellent

reverse stationary phase properties, by chemically bonding tetra-alkylammonium groups to its surface. Since only a small portion of the surface is quaternized, the stationary phase still provides most of its adsorbent surface area. Thus, the quaternized PRP-1 has the property of providing both adsorption and anion exchange sites and it should be a useful stationary phase for the chromatographic separation of organic analytes that can participate in either or both of these processes. This study focuses on identifying and optimizing the mobile phase conditions affecting a combined retention or favoring one over the other. While many kinds of organic analytes are suited to this type of mixed retention, we have focused in this report primarily on benzoic acid derivatives, amino acids, and small chain peptides as analytes.

EXPERIMENTAL SECTION

Reagents. Amino acids, peptides, and benzoic acid derivatives were obtained from Eastman Kodak, Sigma Chemical, Chemalog, and Aldrich Chemical. Acetonitrile and water were LC quality. Inorganic salts were analytical reagent grade.

PRP-1, a macroporous, 10 μm, spherical PSDB copolymer was obtained from Hamilton Co. and quaternized (6,15). A 1.5 g sample of PRP-1 was swollen in a mixture of 10 mL of chloromethyl ether (CARCINOGEN), 40 mL CH_3Cl, and 3 mL CH_3NO_2 under N_2 for 1 hr. The reaction was initiated by the careful addition of 1.2 g dried $ZnCl_2$, allowed to proceed for a controlled time period (about 5 min for 10 μeq/g exchange capacity), and was stopped by the addition of cold water. The quality of the chloromethyl ether, the

reaction time, and temperature will determine the degree of quaternization; increasing the time was used to increase the quaternization. The chloromethylated resin was filtered with a sintered glass size F funnel, washed with MeOH and H_2O (excess carcinogenic chloromethyl ether should be disposed of carefully), and air-dried overnight. The particles were suspended in 50 mL of MeOH and trimethylamine gas (generated by the slow addition of aqueous 7M NaOH to an aqueous $(CH_3)_3N \cdot HCl$ solution) was slowly bubbled through the suspension overnight. The quaternized PRP-1 was filtered and washed with 1M HCl, 2-propanol, MeOH, and LC water. Quaternized PRP-1 in the Cl-form was slurry packed into 4.1 mm i.d. x 150 mm ss tubes with 2 μm fittings.

Procedures. Ion exchange capacities were determined for bulk-form and column-packed quaternized PRP-1. Bulk Cl-form particles were dried under vacuum at 60°C for 1 hr. A 1 g weighed portion was suspended in 50 mL of 0.1 M $NaNO_3$ for 2 hrs, filtered, and the supernatant was titrated with standard 10^{-3} M $AgNO_3$ using a Ag-SCE potentiometric endpoint. Column anion exchange capacity was determined by passing about 60 mL of 0.1 M $NaNO_3$ through the Cl-form column (all flow rates were 1 mL/min) and the collected effluent was titrated potentiometrically with standard 10^{-3} M $AgNO_3$. Bulk exchange capacities were 11×10^{-3}, 31×10^{-3}, 33×10^{-3}, 210×10^{-3} μeq/g and corresponding column capacities were 7.3×10^{-3}, 12×10^{-3}, 15×10^{-3}, and 173×10^{-3} μeq/column. A column of 110 μeq/column was also obtained from Hamilton Co.

Analyte solutions (1 mg/mL) were prepared in water, MeOH, or their mixture. Sample aliquots were introduced by syringe (1 to

10 μL). Mixed solvents are percent by volume. Sodium salts were used to introduce specific counteranions and to adjust ionic strength. Capacity factors, k', were calculated in the usual way; V_o was determined with analytes that were not retained for the mobile phase conditions under study.

RESULTS AND DISCUSSION

Cantwell and coworkers (5-7) suggest that analytes that possess both a nonpolar property and an anionic charge site are retained by a combined ion exchange and adsorption process providing the stationary phase is nonpolar, of high surface area, and provides relatively few anion exchange sites. The ion exchange, IE, and adsorption, Ads, processes can be viewed as

$$A\text{-}\overset{+}{N}Me_3C^- + R\text{-}X^- + M^+ \underset{}{\overset{IE}{\rightleftharpoons}} A\text{-}\overset{+}{N}Me_3{}^-X\text{-}R + C^- + M^+ \quad (1)$$

$$A\text{-}\overset{+}{N}Me_3C^- + R\text{-}X^- + M^+ \underset{}{\overset{Ads}{\rightleftharpoons}} M^{+-}X\text{-}R\cdots A\text{-}\overset{+}{N}Me_3C^- \quad (2)$$

where A is the PRP-1 PSDB copolymeric matrix, C^- is a counteranion, $R\text{-}X^-$ is an analyte with an anionic site X^- and a hydrophobic center R, and M^+ is the mobile phase countercation. If the two processes are treated separately the major mobile-stationary phase parameters influencing each of them can be identified since both anion exchange and adsorption are well understood.

Anion exchange will be affected by ionic strength, type and concentration of counteranion, and mobile phase pH and stationary phase anion exchange capacity. Increasing ionic strength reduces analyte retention by reversing the exchange equilibrium shown in eq. 1. Similarly, changing the counteranion will alter the mobile

phase elution strength according to the anion exchange selectivity defined by eq. 1. Mobile phase pH will influence analyte dissociation, if it is a weak acid, while buffer components will determine the ionic strength and type of mobile phase counteranions. Increasing the anion exchange capacity provides more exchange sites; for a given column dimension this leads to greater analyte anion retention. Furthermore, more exchange sites will consume more PSDB surface area reducing its participation in adsorption. Anion exchange selectivities will be affected by mobile phase solvent composition. However, if the solvent change is modest the effect on anion exchange will often be small.

Adsorption will be influenced by mobile phase solvent composition, ionic strength and pH, if the analyte is a weak acid, stationary phase nonpolarity, and analyte hydrophobicity. As the analyte hydrophobic center increases in hydrophobicity, retention increases. Addition of organic modifier will decrease retention and the extent of the effect is dependent on organic modifier hydrophobicity. The stationary phase hydrophobicity will be determined by the number of anion exchange sites on the stationary phase; as these increase in number hydrophobic activity of the surface decreases. Adjustment of mobile phase pH determines the charge of a weak acid analyte and consequently its hydrophobicity while ionic strength adjustment causes a modest change in adsorption when dealing with retention of a charged analyte.

Stationary Phase-Analytes. The exchange capacity for the series of columns studied was from 7 to 173 μeq/15 cm column. A

PRP-1 column (no exchange sites) was used to determine reverse phase retention as a function of the mobile phase conditions. A halide, NO_3^-, NO_2^- mixture and an aqueous 2×10^{-3} M Na benzoate mobile phase was used as the test sample and eluent, respectively, to monitor column efficiency and peak shape. When the capacity or efficiency measurements showed significant changes, which indicate column deterioration, a new column was prepared.

Since low capacity anion exchangers have the potential to participate in a combined adsorption-anion exchange, analytes suited to this stationary phase are those that have acidic sites, hydrophobic centers, and their combination. Initially, our goal was to explore how this mixed retention could be used advantageously for amino acid and peptide separations. At an acidic mobile phase pH they are cations, at intermediate pH they are zwitterions, and at a basic pH they are anions. The latter two conditions would thus favor anion exchange. Adsorption should be possible through interactions between amino acid and peptide side chain groups and the adsorbent surface. Studies of amino acid and peptide retention on a PSDB stationary phase are reported elsewhere (3). As side chain hydrophobicity increases adsorption also increases. Amino acids and peptides, however, are not ideal analytes for studying the variables affecting this mixed mode of interaction because these analytes are always charged. Even in an acidic mobile phase they are cations and this charge will reduce adsorption effects. Weak acid analytes on the other hand do not suffer from this problem. Depending on their K_a value a mobile

phase pH can be adjusted to one where the analyte is either undissociated or is anionic; the former favors adsorption and the latter anion exchange. Several mono- and di-substituted benzoic acid (BA) analytes were chosen as model analytes. These test analytes varied widely in their K_a values; also, retention data on PSDB adsorbents were available from previous studies (2,16).

Table I shows how retention changes for several BA analytes as a function of column anion exchange capacity at a mobile phase pH of 1.3 and 11.0, respectively. The former pH favors undissociation and the fraction of ionized analyte present depends on its K_a value. The latter pH ensures that all BA analytes are in an ionized anion form. Although not shown retention was also determined at intermediate pH values. When these data were plotted versus pH the graph shape changes as the anion exchange capacity increases. In the absence of exchange sites an S-shape curve of high retention at low pH (BA analyte is undissociated) and low retention at high pH (BA analyte is dissociated) is obtained which is consistent with an equation that correlates analyte retention to pH (17), with the break occurring at pH = pK_a. When the number of anion exchange sites is low an S-shaped curve that fits the equation is still obtained, however, the graph is shifted since the retention is greater at both low and high pH. As the exchange capacity increases retention at low pH (BA analytes are not appreciably dissociated) rises gradually and approaches a maximum at about 100 μeq of sites/column and then decreases. This is consistent with retention that initially is mainly due to adsorption

TABLE I

Retention of Benzoic Acid Derivatives as a Function of Anion Exchange Sites/Column from an Acidic and Basic Mobile Phase

Benzoic Acid Derivative	k' at pH = 1.3[a]				k' at pH = 11.0[b]			
	μeq Exchange Sites/Column				μeq Exchange Sites/Column			
	0	8.7	108	173	0	8.7	108	173
4-OH	1.24	1.24	1.93	2.04	0	0.03	1.40	1.49
3-OH	1.49	1.53	2.48	2.61	0	0.09	2.52	3.12
H	4.66	4.86	7.02	5.66	0.12	0.28	3.00	3.62
4-F	5.94	6.17	9.00	7.38	0.17	0.43	4.60	5.54
3-NO_2	6.92	7.34	11.6	9.02	0.22	0.76	8.84	11.4
4-NO_2	7.70	8.20	13.1	10.2	0.24	0.78	9.02	11.2
4-CH_3	8.54	8.89	13.3	10.2	0.18	0.44	4.83	5.69
3-CH_3	9.11	9.55	14.3	11.1	0.18	0.46	5.00	6.02
4-Cl	13.5	14.6	24.2	19.4	0.28	0.96	11.0	14.5
4-Br	18.6	19.8	35.0	27.6	0.34	1.28	14.8	20.3
3,5-NO_2	12.5	12.5	21.7	16.2	0.47	2.48	29.4	39.2
4-Cl-3-NO_2	16.9	17.6	31.2	24.3	0.48	2.32	28.1	37.1

a. A 3:7 $CH_3CN:H_2O$, 5×10^{-2} M HCl, NaCl to give μ = 0.10 M mobile phase at 1.0 mL/min.

b. A 3:7 $CH_3CN:H_2O$, 1×10^{-3} M NaOH, NaCl to give μ = 0.10 M mobile phase at 1.0 mL/min.

and only partially to anion exchange. The adsorption eventually decreases as the adsorbent surface is converted into a charged surface. At a high pH (BA analytes are predominately anions) retention increases rapidly as the exchange capacity increases indicating a major dependence on anion exchange. These general trends, indicated by comparing the data in Table I for the two pH extremes for any given number of anion exchange sites, were observed for all 12 BA analytes studied and are consistent with a contribution of ion exchange and adsorption to the observed retention. As the exchange sites increase anion exchange is greater while adsorption decreases. If the mobile phase CH_3CN concentration is reduced the trends are the same except that retention is at a higher level.

BA analyte retention was determined as a function of anion exchange capacity and CH_3CN:H_2O mobile phase solvent ratio at an acidic pH where the BA analytes are undissociated and a basic pH where they are dissociated. Results for two BA derivatives are listed in Table II; the combined anion exchange-adsorption trends indicated in Table II were observed for the other ten BA analytes studied except that retention was shifted depending upon the substituent (compare to Table I for an estimate of the substituent effect). At both acidic and basic mobile phase conditions and at all levels of anion exchange capacity studied BA analyte retention decreases as CH_3CN mobile phase concentration increases. The rate of change, however, differs for the two conditions. In the acidic mobile phase, where the BA analyte is undissociated and

TABLE II

Retention of Two Benzoic Acid Derivatives as a Function of Anion Exchange Sites/Column and $CH_3CN:H_2O$ Ratio from an Acidic and Basic Mobile Phase

μeq of Sites per column	k', Acidic Mobile Phase[a]					
	Benzoic Acid			4-Methyl-Benzoic Acid		
	%CH_3CN			%CH_3CN		
	25%	30%	35%	25%	30%	35%
0	6.84	4.40	2.91	13.8	8.02	4.89
8.7	6.73	4.44	2.82	13.5	8.10	4.58
108	9.88	6.00	4.26	20.4	11.1	7.38
173	8.28	5.56	3.37	16.0	9.44	5.35
	k', Basic Mobile Phase[b]					
	20%	30%	40%	20%	30%	40%
0	0.10	0.04	0.02	0.23	0.10	0.04
8.7	0.68	0.39	0.02	1.36	0.64	0.41
108	10.1	6.04	4.20	20.3	9.80	5.99
173	12.1	7.66	5.24	23.2	12.6	7.40

a. A $CH_3CN:H_2O$, 5.5×10^{-3} M HCl, NaCl to give μ = 0.050 M mobile phase at 1.0 mL/min.

b. A $CH_3CN:H_2O$, 1.0×10^{-3} M NaOH. NaCl to give μ = 0.050 M mobile phase at 1.0 mL/min.

adsorption should be favored, the decrease in retention is greater than in the basic mobile phase, where the BA analyte is dissociated and anion exchange is favored. Also, retention in the acidic mobile phase increases modestly as the exchange capacity increases and passes through a retention maximum at about 100 μeq of anion sites/column. The maximum is present because as the number of sites increase adsorption decreases due to the increased charge on the stationary phase surface while retention due to anion exchange increases. Since the BA analyte is not dissociated retention by anion exchange is only a modest contribution. In contrast in the basic mobile phase, retention of the BA analyte increases sharply due to anion exchange as column anion exchange capacity increases.

Altering the mobile phase ionic strength affects BA analyte retention and depends on the pH (determines analyte dissociation) and the number of column anion exchange sites. Figure 1 illustrates the trends using benzoic acid as the analyte. Similar effects were observed for all the BA analytes accept that the level of retention differed according to the type and position of the substituent. At low pH, Fig. 1A, where BA is unionized favoring adsorption, retention increases slightly as ionic strength increases even though anion exchange favors the opposite. The retention increase is consistent with previous studies on ionic strength effects on uncharged analyte retention on PSDB absorbents (2,16). At a basic pH, Fig. 1B, where BA is ionized favoring anion exchange, the reverse occurs or retention decreases as ionic

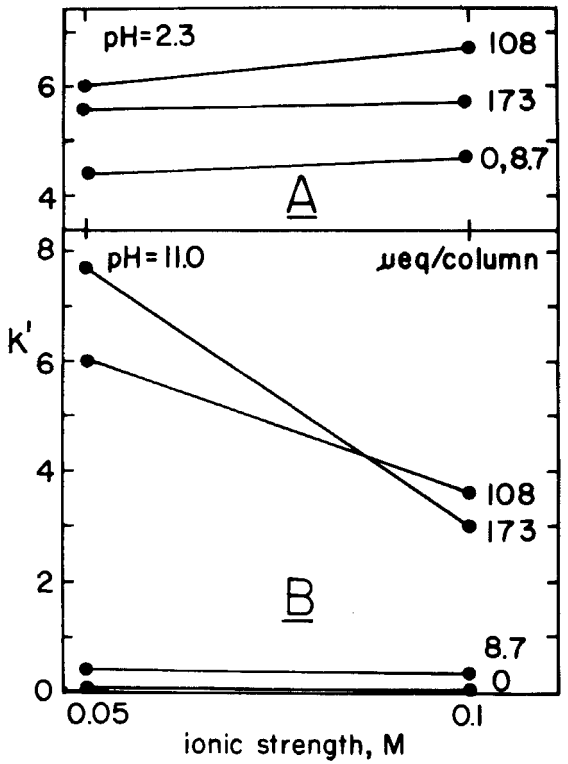

FIGURE 1

Retention of Benzoic Acid from an Acidic (A) and a Basic (B) Mobile Phase as a Function of Column Anion Exchange Capacity and Mobile Phase Ionic Strength

A. A 3:7 $CH_3CN:H_2O$, 5.5×10^{-2} M HCl, pH = 2.3, varying ionic strength (NaCl) mobile phase and 0, 8.7, 108, and 173 µeq of anion exchange sites/column.

B. Same as A except 5.5×10^{-3} M NaOH, pH = 11.0.

strength increases and the rate of change is greater as exchange capacity increases. If the counteranion in the basic mobile phase is changed while ionic strength is maintained the effect on BA analyte retention is consistent with the counteranion exchange selectivity typical of anion exchange (18). Thus, for example, SO_4^{-2} is a stronger eluent counteranion than Cl^-.

When Na alkylsulfonates, which are strong electrolytes, were used as analytes the anion exchange sites significantly enhance their retention. This is shown in Fig. 2 where retention of the alkylsulfonate anions increases as the alkyl chain hydrophobicity increases. In curves a to d alkylsulfonate retention sharply drops as the ionic strength increases for a column containing 8.7 μeq of anion exchange sites. If the capacity increases (compare curves e to d in Fig. 2) analyte retention is also sharply increased. Reducing the CH_3CN concentration increases retention particularly for the lower number of anion exchange sites. If anion exchange sites are absent alkylsulfonate retention using the conditions in Fig. 2 is not observed until the alkyl chain is appreciably hydrophobic; for example, pentylsulfonate has a $k' < 1$ in the absence of anion exchange sites at the conditions used in Fig. 2.

Because of a dual retention and a low number of column anion exchange sites, it is necessary to verify that the analyte concentration used is within the linear portion of the retention isotherm. The upper limit should increase as exchange capacity increases since anion exchange becomes a major contribution par-

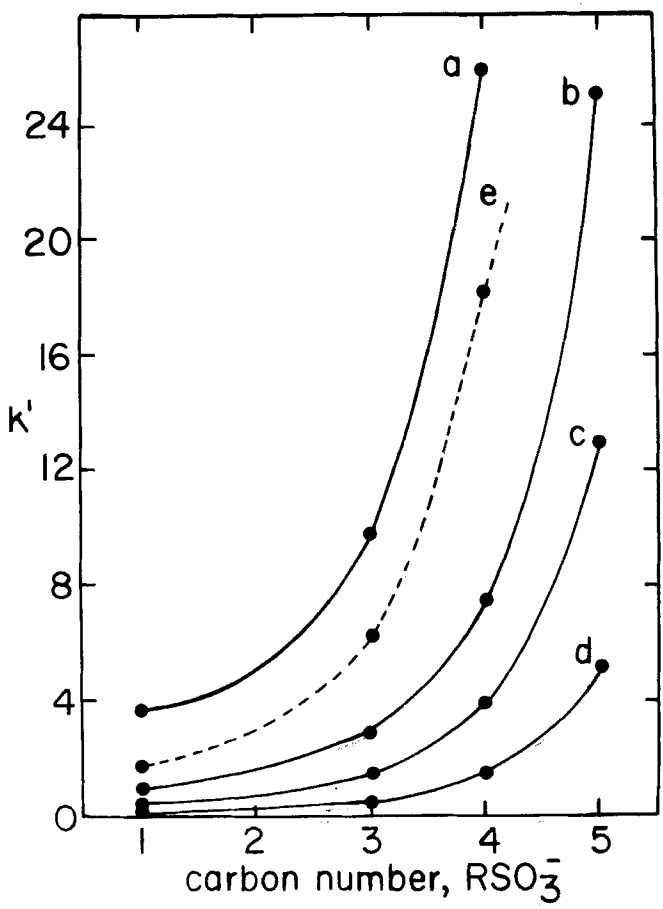

FIGURE 2

Retention of Alkylsulfonates as a Function of Mobile Phase Ionic Strength and Column Anion Exchange Capacity

a-d. A 1:9 $CH_3CN:H_2O$, 1.0×10^{-3} M (a), 5.0×10^{-3} M (b) 1.0×10^{-2} M (c), 5.0×10^{-2} M (d) ionic strength (NaCl), mobile phase and 8.7 μeq of anion exchange sites/column.

e. Same as d except the column contains 173 μeq of anion exchange sites.

ticularly when the analyte is in an anion form. In these studies analyte loading was always below 5×10^{-4} μmole of analyte. When the retention of $(L\text{-}Ala)_2$ as an anion was determined on columns with capacities of 7 and 12 μeq/column using an aqueous, 0.010 M NaF, pH = 11.0 mobile phase retention for both columns was independent of concentration below this loading. At higher loadings (up to 5×10^{-3} μmole of analyte loading was studied) retention changed by about 10% when concentration was doubled. Previous studies had shown that higher analyte loadings were permissible when retention was due only to adsorption (3,8,9,16).

As alkylsulfonate hydrophobicity increases, retention is greater. Also, increasing the column exchange capacity increases retention (see Fig. 2). A similar result, Fig. 3, was found when using a series of $(L\text{-}Ala)_n$ peptides as the analytes and a basic mobile phase to ensure their anion form. Retention data for $(L\text{-}Ala)_n$ in the absence of anion exchange sites are also included in Fig. 3. The enhanced retention due to the contribution of anion exchange and adsorption through the side chain groups provides a better selectivity which becomes even more favorable as the chain length of the peptide increases.

Retention of peptide anions like the BA analytes is counter-anion dependent which is typical of anion exchange. This is shown in Fig. 4 where salts that differ in the anion are used for adjustment of ionic strength. The effect on retention, which is typical of strong base anion exchange selectivity (18), is $NO_3^- > Cl^- > F^-$. The data in Fig. 4 also indicate that retention is

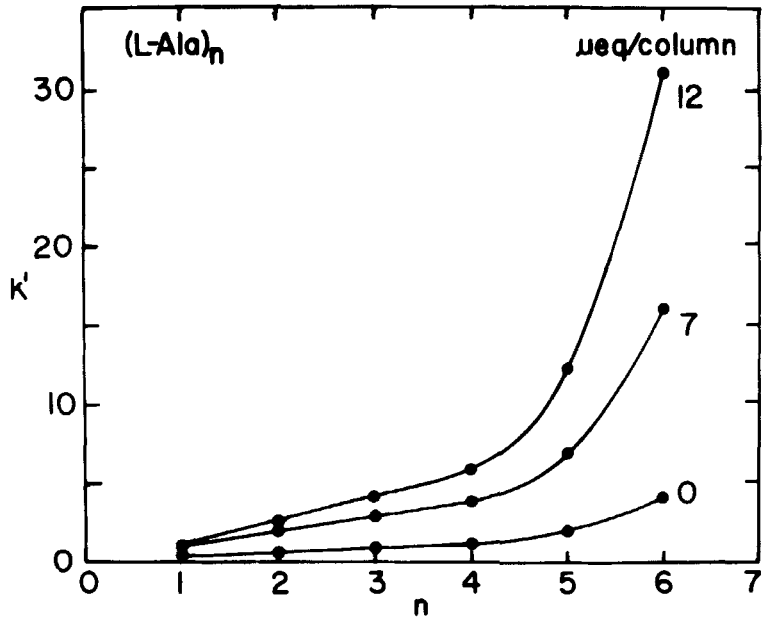

FIGURE 3

The Effect of Side Chain Hydrophobicity for $(L\text{-}Ala)_n$ Peptides on Retention as a Function of Chain Length and Column Anion Exchange Capacity

A 100% H_2O, 1.0×10^{-2} M NaF, 1.0×10^{-3} M NaOH, pH = 11.0 mobile phase and 0, 7, and 12 μeq of anion exchange sites/column.

consistent with side chain structural effects on anion exchange and adsorption. The Phe dipeptides are highly retained due to the hydrophobic Phe side chain. The presence of anion exchange sites causes their retention to be 2 to 3 times higher compared to a column that has no exchange sites. As observed when only adsorption is possible (3), retention on the low capacity anion exchanger is higher when the hydrophobic side chain is located

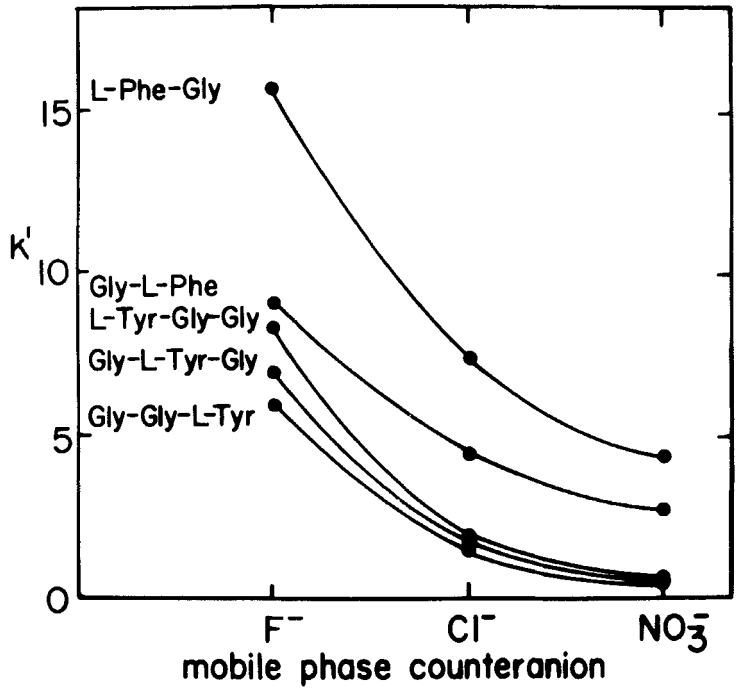

FIGURE 4

The Effect of Mobile Phase Counteranion on the Retention of a Series of Di- and Tripeptides

A 5:95 $CH_3CN:H_2O$, 1.0×10^{-3} M NaOH, pH = 11.0, 1.0×10^{-2} M salt mobile phase and 12 μeq of anion exchange sites/column.

away from the anionic charge center, thus, L-Phe-Gly is more retained than Gly-L-Phe. The L-Tyr tripeptides in Fig. 4 contain two anionic sites since at pH = 11.0 the L-Tyr side chain is also appreciably dissociated. In the absence of anion exchange sites these tripeptides are not retained by adsorption at pH = 11 even when the solvent is 100% H_2O. In the presence of anion exchange sites retention is favorable due to anion exchange. The larger

retention is favored by the dianion that has the largest charge separation. That is, retention changes in the order L-Tyr-Gly-Gly > Gly-L-Tyr-Gly > Gly-Gly-L-Tyr which corresponds to a change in anion charge location from 1,3, to 2,3, to a 3,3 position.

Figure 5 shows that manipulation of the mobile phase solvent mixture, which has a large effect on adsorption and a small effect on ion exchange, can result in selectivity reversal. At low CH_3CN L-Phe (Fig. 5A) and L-Phe dipeptides (Fig. 5B) are highly retained because of the hydrophobic side chain and their retention drops sharply as CH_3CN is increased. In contrast retention of L-Tyr, L-Asp, L-Glu (Fig. 5A) and L-Tyr dipeptides (Fig. 5B), which have acidic side chains and are predominately dianions at this pH, is almost independent of solvent composition. Since their retention remains nearly constant (in the absence of anion exchange sites these analytes are not retained by adsorption at these conditions), they end up being more retained than L-Phe or its dipeptides at the higher CH_3CN concentration.

When the studies in Figs. 3-5 were done using an anion exchange capacity of 173 μeq/column, retention sharply increased. Similar trends were observed except that stronger eluents were required to reduce retention times into a more favorable elution time range. This was accomplished by either increasing counter-anion concentration, organic modifier, or both.

Separations. Examination of Tables I-II and Figs. 1-5 indicate that changes in analyte selectivity including reversals, and subsequently resolution are affected by adjusting the mobile-

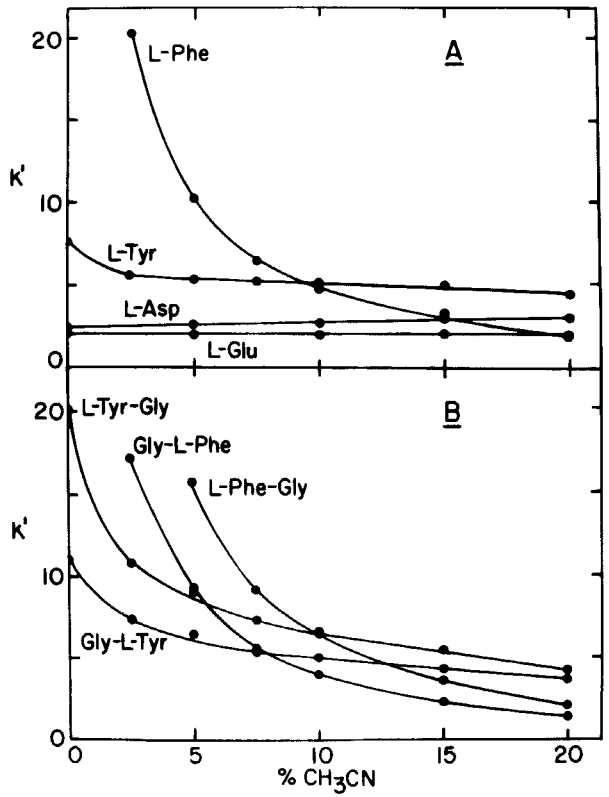

FIGURE 5

The Effect of $CH_3CN:H_2O$ Ratio on the Retention of Several Amino Acid and Dipeptide Analyte Anions

A $CH_3CN:H_2O$, 1.0×10^{-2} M NaF, 1.0×10^{-3} M NaOH, pH = 11.0 mobile phase and 12 μeq of anion exchange sites/column.

stationary phase parameters that favor analyte adsorption, anion exchange or their combination. Anion exchange at the expense of adsorption is favored by increasing the number of anion exchange sites. Mobile phase ionic strength, pH, and counteranion selectivity are the major elution variables affecting anion exchange while mobile phase solvent composition, pH, and type of organic modifier are the major ones affecting adsorption. At an intermediate number of anion exchange sites, which appears to be about 125 μeq/g (about 100 μeq/column), a combined adsorption-anion exchange is possible depending on the analyte anion structure. Thus, at these conditions selectivity and resolution changes are possible by focusing on parameters that influence either adsorption or anion exchange. Figures 6-8 illustrate this strategy.

Figure 6A shows a separation of 12 BA analytes on a column containing 8.7 μeq of anion exchange sites. Since an acidic mobile phase is used the BA analytes are undissociated and the major source of retention is adsorption. If the capacity is increased a modest increase in retention is produced while decreasing the CH_3CN sharply increases the retention. For example, when CH_3CN in Fig. 6A was 25% CH_3CN, separation time increased by about 13 min. In Fig. 6B BA analytes are separated as anions on a column containing 108 μeq of anion exchange sites. Increasing the capacity increases retention. For example, using the conditions in Fig. 6B and a column containing 173 μeq of exchange sites BA analyte retention times increase to 11.5, 19.8, 24.5, 33.6, 36.9, 75, and 78 min, respectively. If anion exchange

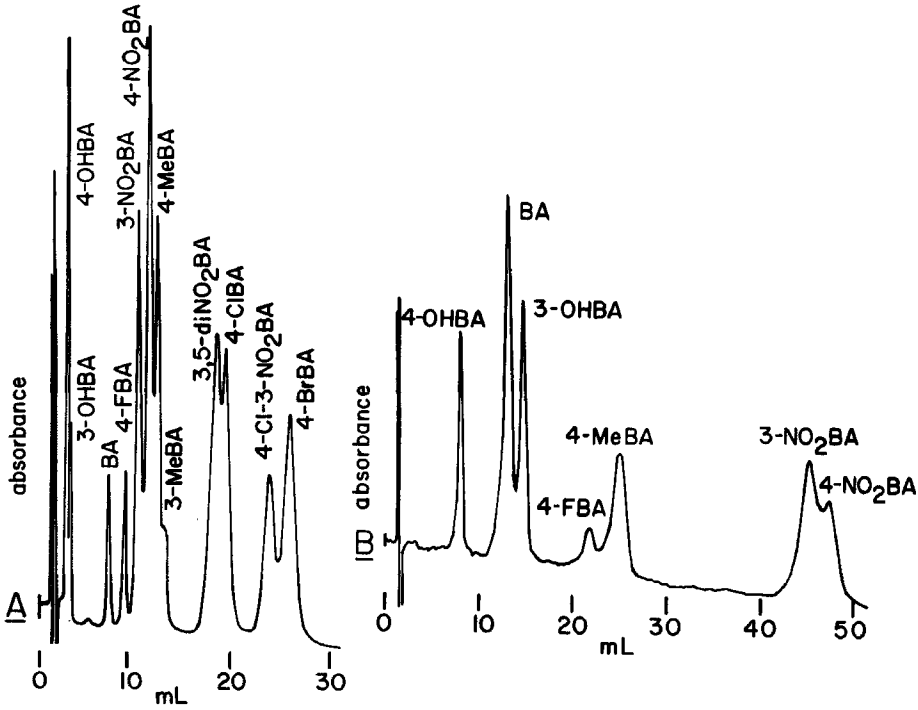

FIGURE 6

Separation of Substituted Benzoic Acid (BA) Derivatives from an Acidic (A) and Basic (B) Mobile Phase

A. A 3:7 $CH_3CN:H_2O$, 5.0×10^{-2} M NaCl, 5.5×10^{-3} M HCl, pH = 2.3 mobile phase and 8.7 μeq of anion exchange sites/column.

B. A 1:5 $CH_3CN:H_2O$, 5.0×10^{-2} M NaCl, 1.0×10^{-3} M NaOH, pH = 11.0 mobile phase and 108 μeq of anion exchange sites/column.

sites are absent BA analyte elution is less than 2 min. Although anion exchange is the major interaction, increasing the CH_3CN concentration reduces BA retention. For example, if $CH_3CN:H_2O$ in Fig. 6B is changed from 1:4 to 2:3 $CH_3CN:H_2O$ the time for the separation is about 14 min. Resolution is also affected and under

these latter conditions 3-OH, 4-F, and 4-Me BA derivatives are poorly resolved while the 3-NO_2 and 4-NO_2 BA derivatives are not resolved. Increasing the counteranion concentration or switching to a stronger eluent counteranion decreases BA analyte retention.

Figure 2 indicates that only a small number of anion exchange sites are needed in combination with adsorption to significantly increase the selectivity, retention, and resolution of alkylsulfonate salts. For example, for the conditions used in Fig. 2B retention times for methyl, propyl, butyl, and pentyl sulfonic acids are 95, 165, 287, 621, and 1830 sec, respectively. Increasing the counteranion concentration, switching to a stronger eluent counteranion, or increasing organic modifier concentration will decrease alkylsulfonate retention while increasing exchange capacity sharply increases retention. A similar combined anion exchange-adsorption leads to better resolution for the separation of $(L\text{-}Ala)_n$ for n = 1 to 6. In the absence of anion exchange sites retention is less than 5 min. and resolution is poor using the conditions in Fig. 3. In the presence of 12 μeq of anion sites/column retention, selectivity, and resolution, particularly for the longer $(L\text{-}Ala)_n$ chains, is significantly increased. Adding organic modifier, increasing counteranion concentration or using a strong eluent counteranion decreases retention.

Figure 7 shows the separation of basic and acidic side chain amino acids and peptides. In Fig. 7A a column containing 108 μeq anion sites is used. The effects of counteranion, its concentration, number of anion exchange sites, and organic modifier on

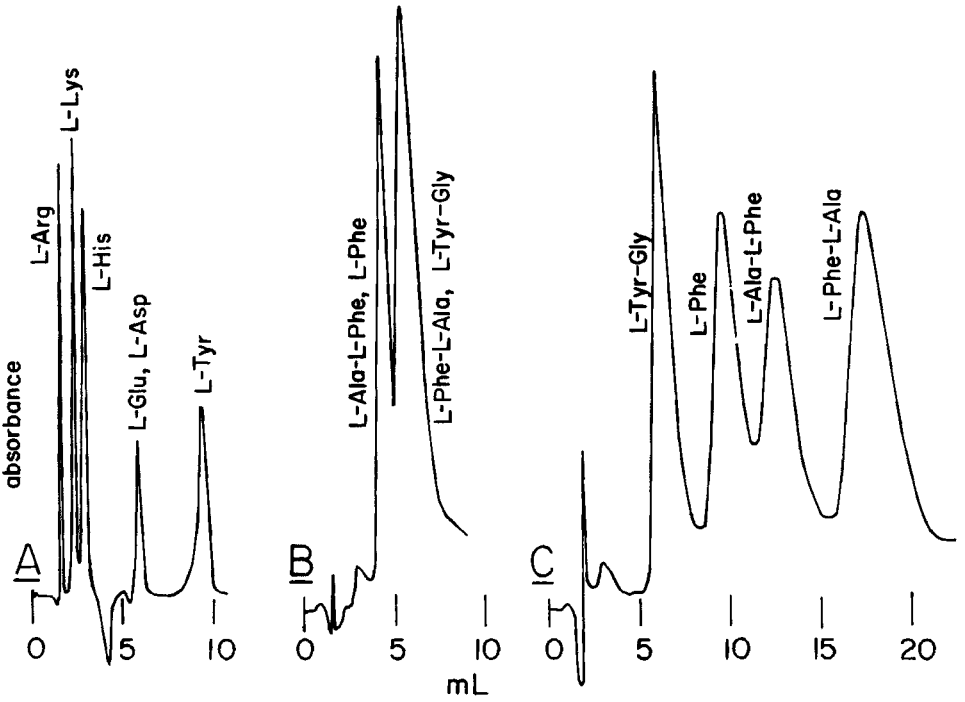

FIGURE 7

Separation of Basic and Acidic Side Chain Amino Acids and Peptides from a Basic Mobile Phase

A. Conditions are same as Fig. 6B except 5:95 $CH_3CN:H_2O$.

B. A 1:4 $CH_3CN:H_2O$, 1.0×10^{-2} M NaF, 1.0×10^{-3} M NaOH, pH = 11.0 mobile phase and 7 μeq of anion exchange sites/column.

C. Same as B except 1:9 $CH_3CN:H_2O$.

amino acid retention are similar to the trends observed when studying other analytes.

Figures 7B and C illustrates selectivity reversal and resolution improvement by manipulating adsorption rather than anion exchange as in previous examples. In Fig. 7B all analytes are anions due to the pH = 11.0 mobile phase. Thus, their retention is largely due to anion exchange since the mobile phase organic modifier is at a level that reduces adsorption due to side chain hydrophobic groups. Reducing the organic modifier from 20% to 10% CH_3CN increases retention for analytes that have a stronger participation in adsorption compared to anion exchange; this modest change has only a minor effect on anion exchange. Thus, as shown in Fig. 7C, L-Tyr-Gly retention is relatively constant because it is a dianion while the other three analyte's retention compared to Fig. 7B sharply increase because of their side chain hydrophobicity. The location of the hydrophobic center relative to the charge center has a significant effect on retention (3). Thus, in Fig. 7C L-Phe-L-Ala is more retained than L-Ala-L-Phe because the more hydrophobic side chain is one subunit away from the charge center in the former dipeptide while in the latter it is in the subunit containing the anion charge center.

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