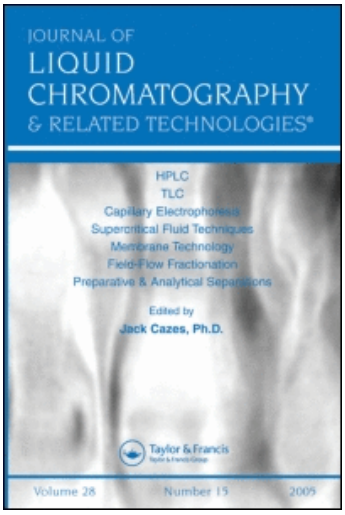


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Ion Interaction Chromatographic Separation of Amino Acids Using a Basic-Tetraalkylammonium Salt Mobile Phase

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**To cite this Article** Rigas, Pantelis G. , Arvanitis, Steve J. and Pietrzyk, Donald J.(1987) 'Ion Interaction Chromatographic Separation of Amino Acids Using a Basic-Tetraalkylammonium Salt Mobile Phase', Journal of Liquid Chromatography & Related Technologies, 10: 13, 2891 — 2910
**To link to this Article: DOI:** 10.1080/01483918708066834
**URL:** http://dx.doi.org/10.1080/01483918708066834

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(13), 2891-2910 (1987)

# ION INTERACTION CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS USING A BASIC-TETRAALKYLAMMONIUM SALT MOBILE PHASE

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## ABSTRACT

A basic mobile phase containing a tetraalkylammonium ($R_4N^+$) salt was used to enhance the retention of free amino acids (AA) in their anion form on a polystyrene divinylbenzene copolymeric (Hamilton PRP-1) nonpolar stationary phase adsorbent. Major variables, which can be readily manipulated to alter this retention and resolve complex AA mixtures, are: structure and concentration of $R_4N^+$ salt, type and amount of organic modifier in the mobile phase solvent, concentration and selectivity of the counteranion present, and mobile phase pH and ionic strength. Mobile phase gradients based on a pH change, or an ionic strength change and their combination, while all other variables are constant, were evaluated for the separation of complex AA mixtures. Detection was accomplished by absorbance or fluorescence after a post-column ortho-phthalaldehyde reaction.

## INTRODUCTION

Most high performance liquid chromatographic (HPLC) strategies for the separation of amino acids (AA) utilize either

ion exchangers or reverse stationary phases. In the former and usually with cation exchangers, AA are separated as cations using an acidic mobile phase while in the latter the AA are first derivatized and these AA derivatives are subsequently separated. Several recent reviews have surveyed these kinds of HPLC methodologies for AA separations and have focused on the chromatographic parameters, resolution, efficiency, detection, sensitivity, accuracy, and scope of application (1-4).

Recently, we reported on the use of alkylsulfonate salts as mobile phase additives (ion interaction reagents) for the ion interaction chromatographic (IIC) separation of AA (5,6) and small chain peptides (7). An acidic mobile phase was used to convert the AA and peptides into cations. The enhanced AA and peptide retention is the result of two major equilibria. One describes the retention of the II reagent onto the stationary phase and the other an ion exchange-like selectivity between the AA and peptide cation and the II reagent countercation or other countercations in the mobile phase. Manipulation of the parameters which influence these equilibria, subsequently leads to resolution of the AA or peptides. Evidence supporting IIC (5-9) and applications of IIC reagents (ion pairing reagents) in AA and peptide separations (5-7,10,11) are discussed elsewhere.

This report focuses on the use of tetraalkylammonium ($R_4N^+$) salts as mobile phase II reagents to enhance the retention of underivatized AA and subsequently to resolve complex AA mixtures. Since the AA are separated as anions, AA elution order differs

from cation exchange, reverse phase, and $RSO_3^-$ II reagent separation strategies.

## EXPERIMENTAL

Reagents and Instrumentation. Amino acids, 2-mercaptoethanol, and ortho-phthalaladehyde (OPA) were obtained from Sigma Chem. Co. Tetraalkylammonium ($R_4N^+$) salts were purchased from Eastman Kodak and Aldrich Chemical Co. Conversion to a specific counteranion form was achieved by passing an aqueous solution of the $R_4N^+Br^-$ salt through a strong base, anion exchanger (Amberlite IRA-400) charged in the appropriate anion form. All organic solvents were LC Quality and LC water was prepared by passing distilled water through a Sybron/Barnstead water purification unit. A DuPont 8800 gradient controller and 870 pump, a Rheodyne 7125 injector and a Beckman 160 fixed wave-length detector (340 nm) were used. Post column derivatization was achieved with an OPA solution at 1.1 mL/min using a Beckman 110A pump, a Lee Visco Jet Micromixer mixing T(10 µL internal volume), and 0.01 inch id SS connecting tubing. PRP-1 columns were obtained from Hamilton Co. as 5 µm spherical, polystyrene divinylbenzene copolymeric beads prepacked in 4.1 mm x 150 mm SS column tubing and end fittings.

Procedures. Aqueous AA samples of about 1 mg/5mL were used. Typical conditions were at least 1 hr column equilibration with the $R_4N^+$ salt mobile phase following the $R_4N^+$ column breakthrough, 2 to 10 µL sample aliquots, 1.0 mL/min flow rate, inlet pressure

of 500 to 1200 psi depending on the mobile phase, and a column temperature of 25°C. Mixed solvents in isocratic and gradient elution are percent volume. Mobile phase pH was maintained by adding $Na_2CO_3$ and inert salts were added to establish ionic strength and/or introduce specific counteranions. Column breakthrough volumes were determined (5) and these data were used to calculate apparent anion exchange capacities.

The OPA solution for post column derivatization (3,12) was made by mixing 0.5 g OPA, KOH solution to yield pH = 10.0, and 10 mL of 1.43M 2-mercaptoethanol with an aqueous solution containing 0.10 mole of $K_3BO_3$ and dilution of the mixture to 1 L. For gradients and isocratic mobile phases, where organic modifier amount was appreciable, organic solvent was also included in the OPA solution. The OPA reagent was refrigerated when not in use (freshly made after 2 days).



## RESULTS AND DISCUSSION

When using a $R_4N^+$ salt as a mobile phase ion interaction reagent two major equilibria contribute to enhanced AA retention. These are: 1) retention of the $R_4N^+$ salt on the PRP-1 stationary phase, A, (see eq. 1), and 2) an ion exchange like selectivity between the counteranions, $C^-$, provided by the $R_4N^+$ salt, the ionic strength salt, and/or buffer salts in the mobile phase and the AA analyte anion, $X^-$, (see eq. 2). By using a basic

$$A + R_4N^+ + C^- \rightleftharpoons A\cdots R_4N^+C^- \qquad (1)$$

$$A\cdots R_4N^+C^- + X^- \rightleftharpoons A\cdots R_4N^+X^- + C^- \qquad (2)$$

mobile phase, the AA is converted to and maintained in its anionic form. If the AA side chains also contain an acidic group, these will also be in their anionic form because of the mobile phase pH used.

If an ion interaction enhanced retention (see 5-9 for supporting experimental evidence) as shown in eqs. 1 and 2 describes the major equilbria, the controllable experimental parameters are those that influence these equilibria. These are: 1) the type of reverse stationary phase (the PRP-1 was used throughout these studies because of its stability in a basic environment), 2) the hydrophobic character of the $R_4N^+$ salt, 3) the $R_4N^+$ salt mobile phase concentration, 4) mobile phase pH, 5) the presence of an organic modifier, its type, and concentration, 6) mobile phase ionic strength and 7) the mobile phase counteranions provided by the $R_4N^+$ salt, the buffer, and the ionic strength salt.



$R_4N^+$ Salt. Figure 1 correlates the enhancement in AA retention to $R_4N^+$ salt structure. As alkyl chain length (hydrophobic property) increases, which is expressed as total carbon number in Fig. 1 (only symmetrical $R_4N^+$ salts were studied), AA retention and selectivity increases sharply initially and then decreases, depending on the AA structure, at higher $R_4N^+$ salt carbon numbers. The latter result is due to the retention of the AA itself onto the PRP-1 stationary phase. As the AA side chain hydrophobicity increases, AA retention on PRP-1 increases and this reverse phase interaction competes with the $R_4N^+$ salt retention (see eq. 1) resulting in a reduced apparent anion exchange capacity. The net effect reduces AA retention.

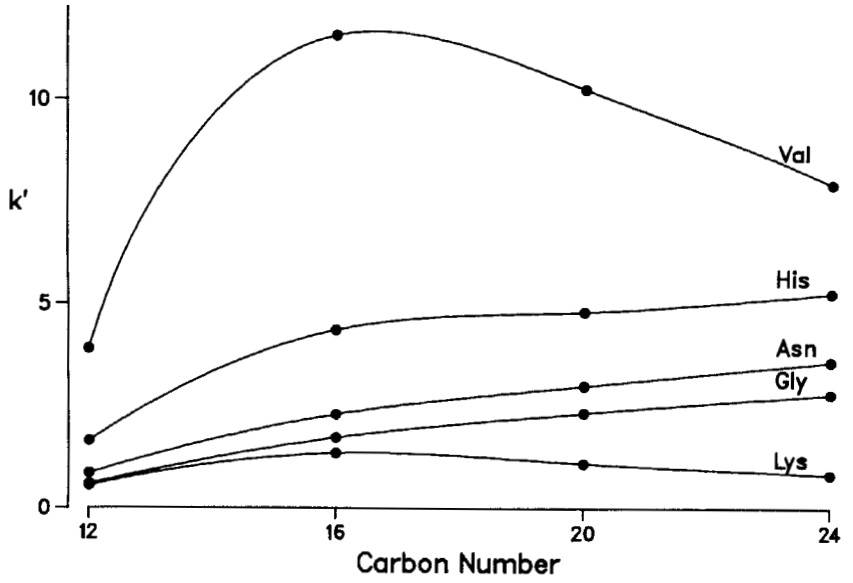


FIGURE 1

Effect of $R_4N^+$ Carbon Number on AA Retention

A 4.1 mm x 150 mm, 5 μm PRP-1 column and 99.5: 0.5 $H_2O$:$CH_3CN$, 1.0 mM $R_4NBr$, 0.50 mM $Na_2CO_3$, pH = 10.0 mobile phase at 1.0 mL/min.



If $R_4N^+$ salt mobile phase concentration increases, the equilibrium amount of $R_4N^+$ retained by the PRP-1 increases (see eq. 1). From $R_4N^+$ salt column breakthrough measurements (13) apparent anion exchange capacities due to the retained $R_4N^+$ salt can be calculated. For example, for a 10 μm, 4.1 mm x 150 mm PRP-1 column and a 1.0mM (pentyl)$_4$NF (TPeAF), 1:4 $CH_3CN$:$H_2O$ mobile phase the equilibrium amount of TPeAF retained, which is also a measure of the equilibrium generated column anion exchange capacity, is about 17 μmoles/column. Increasing the mobile phase organic modi-

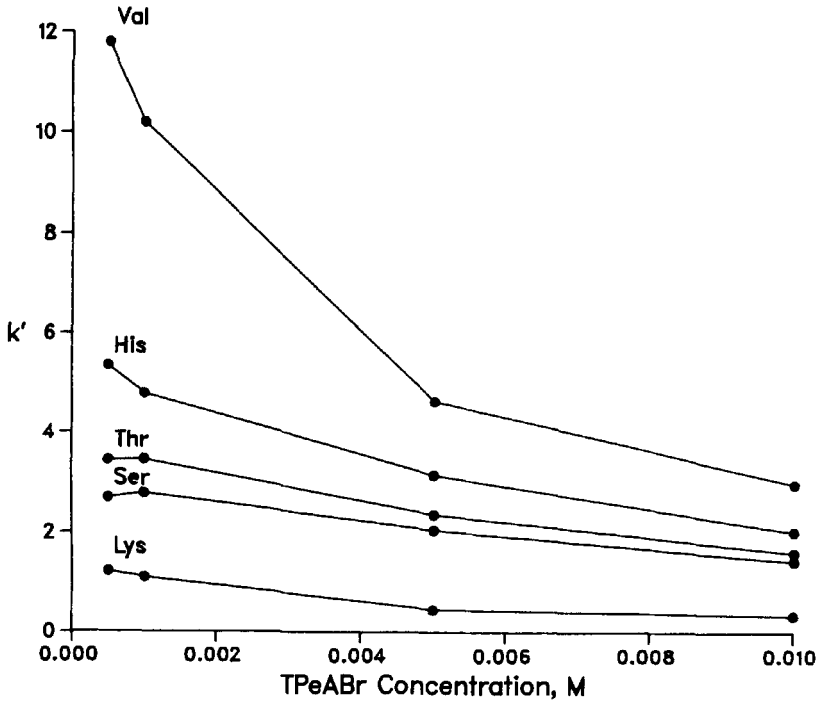


FIGURE 2

Effect of TPeABr Concentration on Amino Acid Retention

Same as Figure 1 except varying TPeABr concentration.



fier decreases the equilibrium retention (and the apparent anion exchange capacity) due to the retained TPeAF (13).

The enhanced retention of the AA analyte anion is affected by the $R_4N^+$ salt mobile phase concentration. In the absence of $R_4N^+$ salt AA retention is low (13) and increases sharply as $R_4N^+$ salt concentration approaches about 0.5 mM. This effect is illustrated in Fig. 2. Above 0.5mM, AA retention drops even though the retention of the $R_4N^+$ salt and the resulting apparent anion exchange

capacity due to $R_4N^+$ salt retention continues to increase. A maximum in retention results because of the presence and increased concentration of counteranion that takes place as it is introduced with the $R_4N^+$ salt. As the counteranion concentration increases, it competes more favorably with the AA analyte for the apparent anion exchange site as shown in eq. 2. The position of the maximum and the retention of the AA at the maximum are both dependent on the type of counteranion and the AA analyte structure. Counteranion selectivity influences the former (the greater the anion selectivity is the lower the AA retention becomes) while the latter effect depends on the AA side chain hydrophobicity since the more hydrophobic the side chain is the more readily the AA competes with the $R_4N^+$ salt by a reverse phase retention process onto the PRP-1 surface.



Mobile Phase pH. The major effect of mobile phase pH is that it determines the charge of the AA analyte. In a basic direction the AA anionic form is favored and enhanced retention of the AA in the presence of the $R_4N^+$ salt is the greatest. As the zwitterion pH is approached AA retention decreases. A carbonate salt rather than NaOH was used to achieve a basic mobile phase pH even though $CO_3^{-2}$ is a stronger eluent anion than $OH^-$. When using NaOH, AA retention times were found to decrease in reproducibility studies. This was shown to occur because the eluents containing NaOH were slowly absorbing atmospheric $CO_2$. Using $CO_3^{-2}$ salts for pH control eliminates this problem. The increased eluting strength of $CO_3^{-2}$ was compensated for by a low mobile phase $CO_3^{-2}$ concentration.

Organic Modifier. Increasing the organic modifier in the mobile phase decreases $R_4N^+$ salt retention on PRP-1. This results in a lower apparent anion exchange capacity (see eq. 1) and consequently a lower AA retention. The extent of this change in retention is illustrated in Table I where AA retention is listed as a function of $CH_3CN:H_2O$ ratio. $EtOH:H_2O$ produces the same effect except that the decrease in AA retention is more gradual as EtOH concentration increases.

Mobile Phase Ionic Strength-Counteranion Effects. Figure 3 demonstrates that increasing the counteranion concentration decreases AA analyte anion retention. In these experiments the mobile phase TPeABr concentration is constant and counteranion concentration is increased by adding NaBr to the mobile phase. When different counteranions are compared the effect can be correlated to counteranion ion exchange selectivity. The more favorable the counteranion selectivity (or its eluent power) the more rapid the decrease in AA analyte anion retention (see eq. 2). Thus, for common halide eluent anions eluting power follows the order $I^- > Br^- > Cl^- > F^-$.

AA Separations. The data in Fig. 1 to 3, Table I, and other preliminary experiments establish the major mobile phase parameters that enhance AA retention and how these can be manipulated in order to achieve optimum resolution in AA separations. The key parameters and their effects are summarized in the following. 1) Increasing the hydrophobicity within limits or the concentration of the $R_4N^+$ salt, increases $R_4N^+$ salt retention and the equilibrium

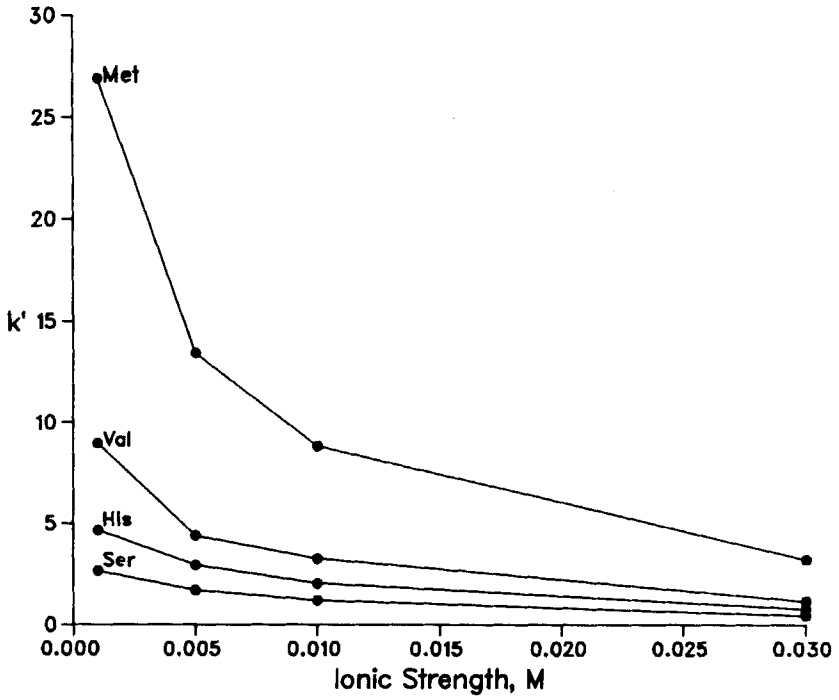


FIGURE 3

Effect of NaBr Concentration on Amino Acid Retention

Same as Figure 1 except 100% $H_2O$ and varying NaBr concentration.

apparent anion exchange capacity on the PRP-1 surface (see eq. 1); this property is also counteranion dependent. AA retention increases up to the point where the counteranion competes favorably with the AA anion for the apparent exchange site (see eq. 2). The more hydrophobic the AA side chain, the more it competes with $R_4N^+$ salt retention and mixed reverse phase-ion interaction retention of the AA occurs. 2) Increasing the organic modifier reduces the retention of the $R_4N^+$ salt and the equilibrium apparent anion

TABLE I

Effect of Organic Modifier on Amino Acid Retention

Capacity Factor, k'

| Amino Acid | Percent $CH_3CN$[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0.5 | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 |
| L-Arg[b] | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| L-Lys | 1.54 | 1.21 | 1.03 | 0.83 | 0.76 | 0.64 |
| Gly | 2.83 | 2.61 | 2.35 | 1.96 | 1.75 | 1.36 |
| L-Ala | 3.26 | 2.83 | 2.41 | 1.97 | 1.72 | 1.35 |
| L-Ser | 3.28 | 2.93 | 2.58 | 2.11 | 1.90 | 1.44 |
| L-Asn | 3.37 | 2.96 | 2.59 | 2.14 | 1.96 | 1.49 |
| L-Gln | 3.55 | 2.98 | 2.54 | 2.08 | 1.87 | 1.45 |
| L-Thr | 4.60 | 3.69 | 2.90 | 2.30 | 2.02 | 1.53 |
| L-His | 6.19 | 4.46 | 3.25 | 2.40 | 2.06 | 1.55 |
| L-Val | | 11.2 | 6.11 | 3.75 | 2.80 | 1.88 |
| L-Met | | | 11.6 | 6.00 | 4.08 | 2.51 |
| L-Ile | | | | 6.31 | 4.04 | 2.43 |
| L-Leu | | | 15.8 | 7.15 | 4.54 | 2.59 |
| L-Nle | | | | 7.96 | 4.91 | 2.73 |
| L-Phe | | | | 20.5 | 10.0 | 4.49 |
| L-Tyr | | | | | 13.0 | 5.64 |
| L-Trp | | | | | 16.9 | 6.19 |
| L-Asp | | | | | 20.8 | 11.9 |
| L-Glu | | | | | 22.6 | 12.4 |
| L-Cys | | | | | | 16.2 |

a. A $H_2O$-$CH_3CN$ mobile phase containing 1.0 mM TPeAF and 0.50 mM $Na_2CO_3$ at 1.0 mL/min flow rate on PRP-1 column.

b. $V_o$ = 1.42 to 1.22 mL for L-Arg.

exchange capacity on the PRP-1 surface which results in lower AA retention. 3) The more favorable the anion exchange selectivity for the counteranion, the stronger its eluent strength and the more it reduces AA retention. 4) An increase in counteranion (ionic strength) concentration leads to two competing effects. One causes AA retention to decrease by mass action (see eq. 2) while the other causes AA retention to increase because the retention of the $R_4N^+$ salt and the equilibrium apparent anion exchange capacity increases with ionic strength increase. Over a modest ionic strength change, in general, the first effect, particularly for these counteranions that have favorable anion exchange selectivities, is more significant. 5) Mobile phase pH enhances AA retention if the pH is adjusted to favor AA dissociation into its anionic form. The counteranion and its concentration provided by the buffer salt also influence AA retention.



Figure 4 illustrates isocratic separations of a series of AA. The elution order is polar and basic AA elute first followed by nonpolar and acidic AA. The pH effect is particularly significant with the acidic AA since the basic mobile phase pH causes dissociation of acidic side chains and formation of dianionic charged AA which leads to high retention. In cation exchange (1-4) and $RSO_3^-$ mobile phase modified IIC (5,6) acidic amino acids are in the polar group and basic AA are in the nonpolar group. In Fig. 4A AA retention is reduced because the organic modifier concentration is high. When the organic modifier is reduced AA retention increases. For example, in Fig. 4B, where retention of

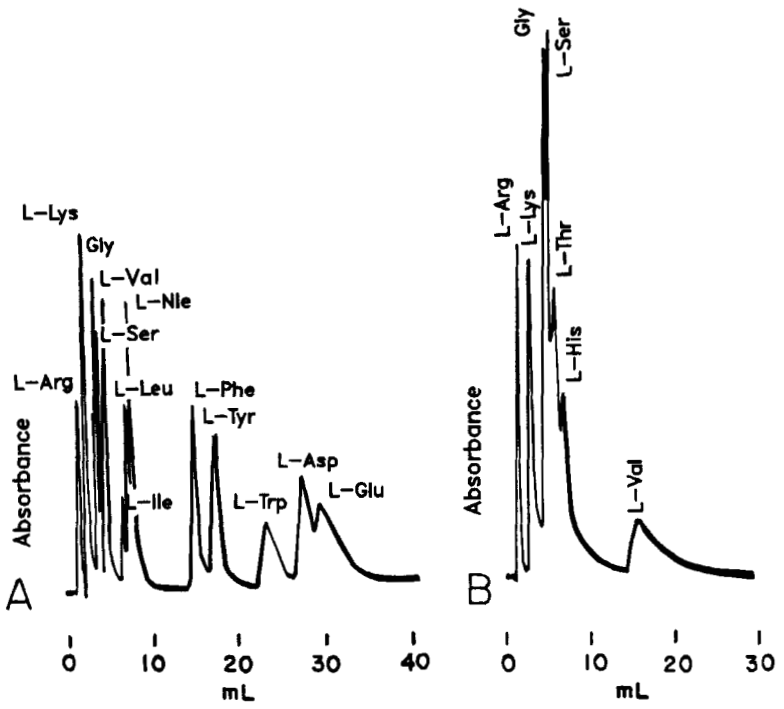


FIGURE 4

Effect of Organic Modifier on the Isocratic Separation of Amino Acids

Same as Figure 1 except 4:1 $H_2O$:$CH_3CN$ in (A) and 95:5 $H_2O$:$CH_3CN$ in (B).

only the polar AA are shown, $CH_3CN$ concentration is reduced to 5%. Although not shown, changing $TPeA^+$ counteranion to a stronger eluent counteranion decreases AA retention. For example, using the conditions in Fig. 4B and TPeAF, TPeABr, and TPeAI capacity factor for the retention of L-Val changes in the order 11.2, 6.92, and 3.76, respectively.

While isocratic elution in the presence of $R_4N^+$ salts is applicable to the separation of simpler AA mixtures on PRP-1, resolution of more complex AA mixtures is more readily obtained by gradient elution. Furthermore, a change in solvent composition (see Table I), ionic strength (see Figure 3), or counteranion and their combination in a step or linear gradient is possible. While a pH gradient is also feasible, the former strategies provide the better gradient conditions.

Figure 5A shows the isocratic separation of nonpolar AA. Because of the contribution of the hydrophobic side chain to retention, retention is high and this is compensated for by using a high organic modifier concentration. If an organic modifier, linear gradient is used as in Fig. 5B, resolution of the early eluting nonpolar AA is improved. Figure 6 illustrates the separation and improved resolution (compare to Fig. 4) of polar AA using a step gradient where $CH_3CN$:$H_2O$ solvent composition changes (Fig. 6A) and where both solvent composition and $Br^-$ counteranion concentration changes (Fig. 6B). Tetrabutylammonium ($TBuA^+$) salt was used in Fig. 6A and B in combination with $Br^-$ a modestly strong eluent counteranion. The difference between $TBuA^+$ and $TPeA^+$ salts as ion interaction reagents are small when complex mixtures of AA are compared, however, for simple AA mixtures resolution tends to be more favorable with the $TPeA^+$ salt when all other factors are equal.

Figure 7A and B illustrate the separation of a complex mixture of AA using step gradients. In Fig. 7A a changing $CH_3CN$:$H_2O$ ratio

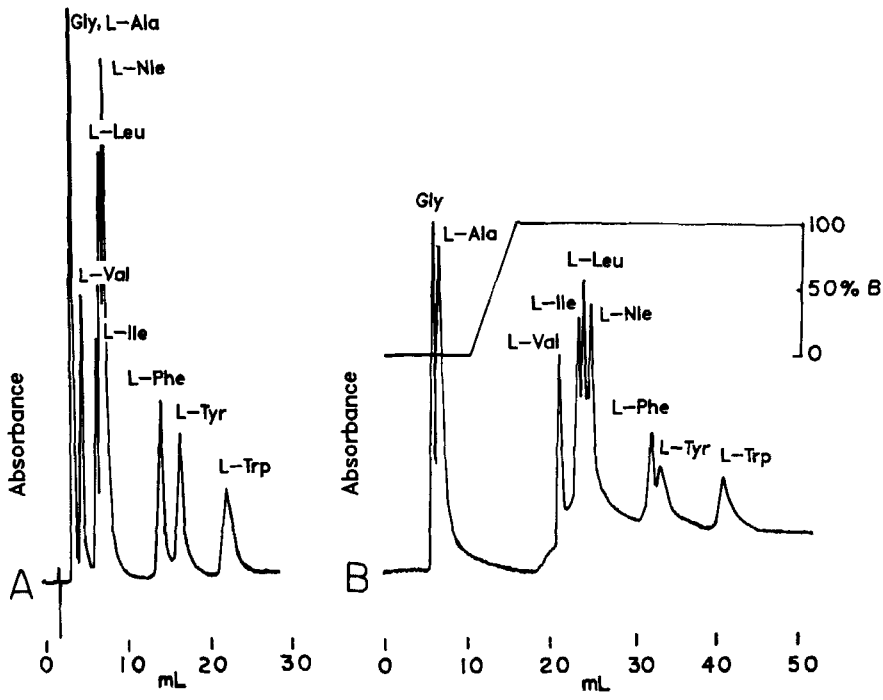


FIGURE 5

Comparison of Isocratic and Organic Modifier Linear Gradient for the Separation of Nonpolar Amino Acids

Same as Figure 1 except 4:1 $H_2O$:$CH_3CN$ in (A) and a linear gradient of A solvent of 1.0 mM TPeAF, 0.50 mM $Na_2CO_3$, 99.5:0.5 $H_2O$:$CH_3CN$ and B solvent of the same except 4:1 $H_2O$:$CH_3CN$ in (B).

is used in combination with $F^-$ a weak counteranion at constant concentration. Since a 1:5 $CH_3CN$:$H_2O$ ratio is achieved at the third step, retention times, particularly at the latter stages of the separation are significantly reduced. In Fig. 7B the solvent is 100% water and $Br^-$ a stronger eluent counteranion than $F^-$ is increased in concentration in three steps. The final elution

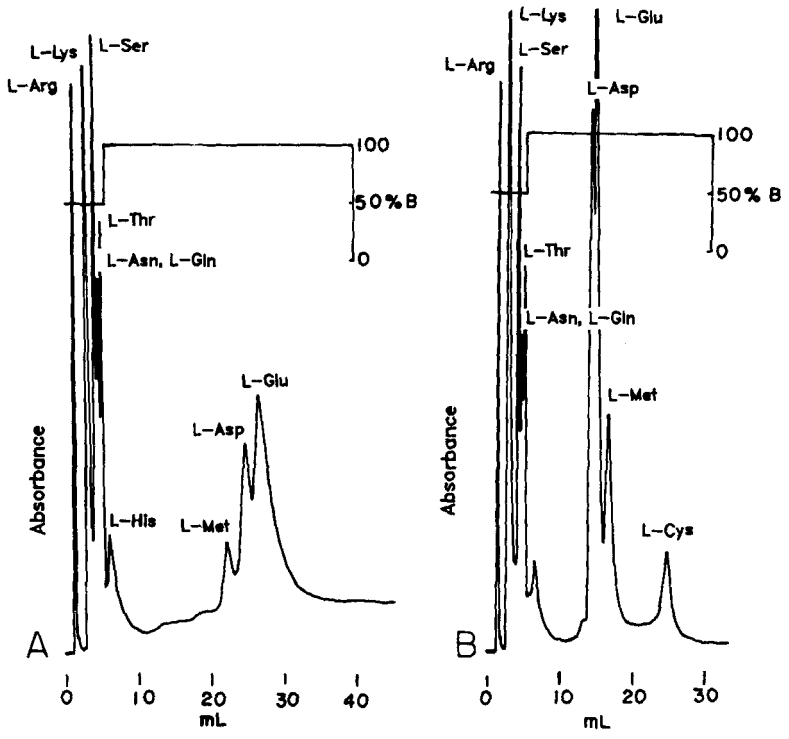


FIGURE 6

Separation of Polar Amino Acids with an Organic Modifier Step Gradient (A) and an Organic Modifier–Ionic Strength Step Gradient

A 4.1 mm x 150 mm, 5 μm PRP-1 column and a step gradient of A solvent of 1.0 mM TBuABr, 0.50mM $Na_2CO_3$, 99.5:0.5 $H_2O$:$CH_3CN$ and B solvent of 1.0 mM TBuABr, 0.50 mM $Na_2CO_3$, 95:5.0 $H_2O$:$CH_3CN$ in (A) and the same in (B) except B solvent which also contains 10 mM NaBr at 1.0 mL/min.

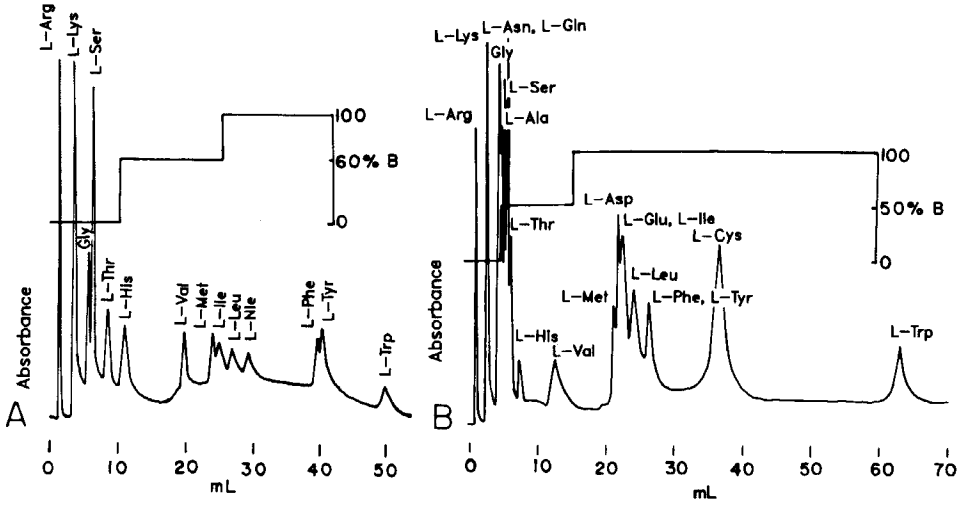


FIGURE 7

Comparison of Amino Acid Separation by Organic Modifier Step Gradient and Ionic Strength Step Gradient

A 4.1 mm x 150 mm, 5 μm PRP-1 column and a step gradient of A solvent of 1.0 mM TPeAF, 0.50 mM $Na_2CO_3$, 99.5:0.5 $H_2O$:$CH_3CN$ and a B solvent of the same except 4:1 $H_2O$:$CH_3CN$ in (A) and a two step gradient of an aqueous A solvent of 1.0 mM TPeABr, 0.50 mM $Na_2CO_3$ and a B solvent of the same with 20 mM NaBr at 1.0 mL/min.

mixture in this case is a weaker eluent than in the latter stages of the organic modifier gradient (Fig. 7A) and consequently AA retention times are higher.

Figure 8 shows a separation where both the $CH_3CN$:$H_2O$ ratio and counteranion are changed in a linear gradient. The counteranion changes from $Br^-$ (TPeABr) to the stronger $I^-$ counteranion (TPeAI). If the weaker eluent counteranion $F^-$ is maintained, retention times are longer. Similarly, if the solvent composition is changed more gradually or if the percent organic modifier is

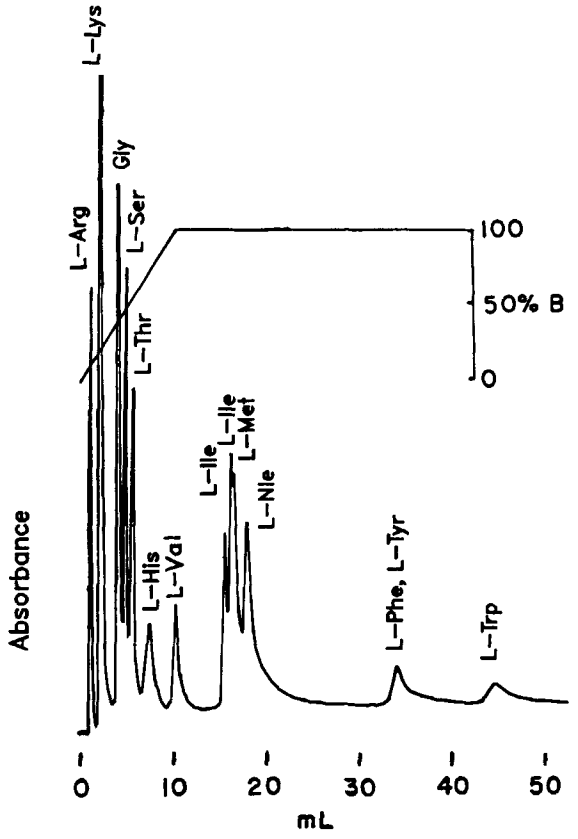


FIGURE 8

Separation of Amino Acids Using an Organic Modifier-Ionic Strength Linear Gradient

Same as Figure 7 except the B solvent is 1.0 mM TPeAI, 0.50 mM $Na_2CO_3$, 9:1 $H_2O$:$CH_3CN$.

less than that achieved in the Fig. 8 gradient, retention times are longer. This is particularly true for nonpolar AA because of a mixed AA retention due to ion interaction (see eq. 1 and 2) and reverse phase retention due to ion interaction (see eq. 1 and 2) and $R_4N^+$ salt in retention onto the PRP-1 surface.

Figures 4 to 8 demonstrate the scope of the major elution parameters and their influence in isocratic and gradient elution and in AA resolution. AA elution order can also be altered and this option can be used advantageously in AA separations. First, since AA are being separated as anions, basic AA elute early and acidic AA elute late. This is opposite to most other AA separation strategies involving their separation as cations. Furthermore, altering mobile phase ionic strength (compare Figs. 6A to 6B and 7A to 7B) alters elution order for acidic AA acids. Other AA reversals are possible and depend on the mobile phase variable that is altered. In all examples studied AA chromatographic peaks were well defined and reproducible. For complex AA mixtures an organic modifier gradient, an ionic strength gradient in the absence of or at low organic modifier, or their combination appear to be the most versatile. For simple AA mixtures several options are available and the optimum one depends on the AA mixture.



## ACKNOWLEDGEMENTS

Part of this work was supported by AM28077 awarded by the National Institutes of Health. Part of this work was presented at the Twenty-first Midwest Regional ACS Meeting, Kansas City, MO, November, 1986, Paper 222.

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