Separation of Underivatized Amino Acids and Dipeptides Using 1-Butylimidazolium Based Stationary Phase and UV Detection

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Amanda Blackburn
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Dr. Kyle Strode, Director — Date

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Dr. Ron Wilde, Reader — Date

Debra Bernardi — 04.05.04

Dr. Debra Bernardi, Reader — Date

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Abstract

Isocratic elution of aromatic amino acids was achieved using a butyl-imidazolium based stationary phase and UV detection. For this study butyl-imidazolium bromide was bonded to a silica substrate and packed into an HPLC column. This study focused on the effects of ionic strength and organic modifier on retention. Retention of aromatic dipeptides was also explored to gain further insight into the retention mechanisms exhibited by this new stationary phase. The stationary phase was found to exhibit both reverse-phase and ion-exchange retention mechanisms.

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Introduction

Amino acids, the building blocks of proteins, are biologically active molecules. They serve many vital roles in biological functions. They serve as matrices for bone structures, act as enzymes and serve as carriers for nutrient transport.[1] As interest in proteomics increases with the completion of the Human Genome Project, there will be a demand for better techniques to separate proteins and peptides from cell mixtures.[2]

Much attention has already been paid to improve chromatographic separations in the area of amino acids, peptides and proteins. Traditionally, high-performance liquid chromatography (HPLC) has been a widely utilized method for separations. Reverse phase liquid chromatography (RPLC) is often used in tandem with other methods including ion-pairing, size-exclusion chromatography and ion-exchange chromatography.[3,4,5,6] While RPLC shows good separations for hydrophobic peptides, polar amino acids and peptides are often eluted near the void volume.[7]

Other areas of LC have been explored in an effort to improve the universality of amino acid and peptide separations from more complex mixtures. Ion-pairing agents have been explored as additives in the mobile phase. It has been reported that the

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[1] T. M. Devlin, **Textbook of Biochemistry: With Clinical Correlations**, T.M. Devlin, 4th Ed, New York John Wiley & Sons, Inc, New York, 1997, 24.
[2] H.J. Issaq, "The role of separation science in proteomics research", *Electrophoresis*, 22, 2001, 3629.
[3] G.J. Opiteck; J.W. Jorgenson, "Two-Dimensional SEC/RPLC Coupled to Mass Spectrometry for the Analysis of Peptides", *Anal. Chem.*, 69,1997, 2283.
[4] M.T. Davis;J. Bierele; E.T. Bures; M.D. McGinley; J. Mort; J.H. Robinson; C.S. Spahr; W. Yu; R. Leuthy; S.D. Patterson, "Automated LC-LC-MS-MS platform using binary ion-exchange and gradient reversed-phase chromatography for improved proteomic analyses", *J. Chromatogr. B*, 752, 2001, 281.
[5] G. J. Opiteck; K.C. Lewis; J.W. Jorgenson, "Comprehensive On-Line LC/LC/MS of Proteins", *Anal. Chem.*, 69, 1997, 1518.
[6] A. J. Link; J. Eng; D.M. Schieltz; E. Carmack; G.J. Mize, D.R. Morris; B.M. Garvik; J.R. Yates III, "Direct analysis of protein complexes using mass spectrometry", *Nature*, 17, 1999, 676.
[7] H.J. Chaves-das-Neves; Z.B. Morais, "HPLC Assay of Underivatized Free Amino Acids with Column Switching and Evaporative Light-Scattering Detection", *J. High Resolut Chromatogr.*, 20, 1997, 115.

addition of cyclohexylamine diminished the effects of surface silanols, thus improving peak shape and separation efficiency.[8] The use of perfluorinated carboxylic acids as additive using gradient elution provided successful separations of all 20 underivatized amino acids.[9] Perfluorinated carboxylic acids have also been used as an ion-pairing agents for separations of polar amino acids.[10] Ion-exchange mechanisms have also been explored in peptide separations.[11,12]

Multidimensional Separations

Recent investigations on improving the separation of complex mixtures have focused on multidimensional separations. These multidimensional techniques show improved separation abilities and improve the peak capacity. Peak capacity of a two dimensional (2-D) system is the product of the peak capacity of each individual system. For these 2-D systems to realize their full potential for these separations, the individual systems must exhibit orthogonality, in which each system utilizes a different separation technique with a different mechanism for separation. The orthogonality between the two systems has the ability to confirm peak purity or to separate samples that co-elute on a different dimension.[13] It should be noted that the true potential of 2-D separation relies on the probability that, while molecules may exhibit similarities in one dimension (e.g.,

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[8] S.R. Cole; J.G. Dorsey, "Cyclohexylamine Additives for Enhanced Peptide Separations in Reversed Phase Liquid Chromatography", *Biomed. Chromatogr.*, 11, 1997, 167.

[9] P. Chaimbault; K. Petritis; C. Elfakir; M. Dreux, "Ion-Pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids", *J. Chromatogr. A.*, 870, 2000, 245.

[10] K.N. Petritis; P. Chaimbault; C. Elfakir; M. Dreux, "Ion-Pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent", *J. Chromatogr. A*, 833, 1999, 147.

[11] L.E. Welch; W.R. LaCourse; D.A. Mead; D. C. Johnson; T. Hu, "Comparison of Pulsed Coulometric Detection and Potential-Sweep Pulsed Coulometric Detection for Underivatized Amino Acids in Liquid Chromatography", *Anal. Chem.*, 61, 1989, 555.

[12] P. Luo; F. Zhang; R.P. Baldwin, "Constant Potential Amperometric Detection of Underizatized Amino Acids and Peptides at a Copper Electrode", *Anal. Chem.*, 63, 1991, 1702.

[13] P.J. Slonecker; X. Li; T.H. Ridgway; J.G. Dorsey, "Informational Orthogonality of Two-Dimensional Chromatographic Separations", *Anal. Chem.*, 68, 1996, 682.

hydrophobicities), they will not be similar in all their properties (e.g., charges, sizes, or affinities.)[14]

Many of the reported multidimensional chromatographic systems have involved linking at least two columns in series. One reported system linked a strong cation exchange (SCX) column in series with an RPLC column. This system reported an increase in the resolution power over conventional 1-D systems.[4] Other systems utilizing SCX and RPLC used in separations of proteins reported a theoretical peak capacity of 512 as compared to a peak capacity of 16 for SCX and 32 for RPLC.[5] Another study reported the use of size exclusion chromatography (SEC) paired with RPLC in the analysis of peptides. Using this system, it was possible to attain separations of complex peptide mixtures with improved resolution over previously used systems. Furthermore, this system was able to reduce remixing of the samples between columns through elimination of storage loops and creating a system that switched between two RPLC columns.[3] Another system further improved the problem of samples remixing between columns by constructing a biphasic microcapillary column by first packing 8 cm of RP particles, then 4 cm of strong-cation exchange particles in a microcapillary column.[6]

Certain issues exist with the current protocols for 2-D systems. One consideration is the choice of mobile phases. For many SCX columns and SEC columns, mobile phases commonly used in RPLC, such as those containing organic modifiers, are incompatible.[3] Also, the systems containing multiple columns connected in series often have longer dead times and increased elution times. Furthermore large volumes of mobile phase are necessary. Synchronization between the two platforms must occur in correlating the optimum flow rates and the capacity load for the second column. The

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[14] A. Link, "Multidimensional peptide separations in proteomics", *Trends in Biotechnology*, 20, 2002, S8.

instrumentation described by M.T. Davis et al. discussed one system which involved trapping the analyte after it was eluted from a SCX column, then switching the column valve, backflushing the column, then introducing the analytes to the RP column using an organic gradient.[4] Another system involved a series of six SEC columns which then connected to an injector pump. After equilibration with the mobile phase for the RP column, the SEC eluent was then flushed for four minutes onto an alpha RP column. After four minutes, the injector valve was then switched and flushed the eluent onto a beta RP column. Creating this two column system allowed the large flow rate without filtering the analytes out of the eluent from the SEC column. For these systems to be functional, without the dual columns in the second dimension, the second dimension must have a high flow rate.[3]

Detection Methods

In order to ensure the separation has occurred, much attention has been paid to the detection methods following separation. In recent years, there has been a focus on separations of underivatized amino acids and peptides. Ultraviolet (UV) detection has been a common protocol; however, it is not the method of choice since only a limited number of amino acids contain an aromatic group. Fluorometric detection is also not a viable method for underivatized detection. Mass spectrometry (MS) has been highly utilized as a detection method for identifying amino acids after separation. MS has also been considered by some as a third dimension for multidimensional separations because of the power of MS to identify co-eluting peaks.[3] Evaporative light scattering detection (ELSD) is considered a universal detection method and has shown good results in detecting amino acids.[7] Other methods include various forms of coulometric detection.

Pulsed coulometric detection using gold, platinum and copper electrodes have proven to be "direct, sensitive and simple" detection methods.[11,12]

Ionic Liquids

Ionic liquids have been used in a large variety of recent scientific investigations in the area of chromatography. They have been used as electrolytes in capillary electroforesis (CE),[15,16] additives in HPLC,[17] and as stationary phases in gas chromatography.[18] When used as electrolytes in CE, as the concentration of ionic liquids increased, the electroosmotic flow (EOF) decreased. In the same study, it was shown that the addition of ionic liquids to the solvent enabled electrophoretic separations for uncharged molecules.[15] Improved CE separations of polyphenols were reported with the addition of 1-alkyl-3-methylimidazolium to water.[16] When used as an additive in RPLC, improved peak symmetry and shortened retention times were reported for the separation of catecholamines. Furthermore, it was reported that certain ionic liquids change the effects of the C18 column. It was suggested that at low concentrations, (i.e. 10mM) ionic liquids played no specific role in interacting with the analyte, but coat the surface of the stationary phase thereby changing the analyte/stationary phase interactions. However, at high concentrations (i.e. 25mM), ionic liquids change the retention behavior of the analytes, thus shortening the retention times.[17] When used as a stationary phase for GC it was reported that ionic liquids appear to have a dual nature. Non-polar compounds have

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[15] M. Vaher; M. Koel; M. Kaljurand, "Ionic liquids as electrolytes for nonaqueous capillary electrophoresis", *Electrophoresis*, 23, 2002, 426.
[16] E.G. Yanes; S.R. Gratz; M.J. Baldwin; S.E. Robinson; A.M. Stalcup, "Capillary Electrophoretic Application of 1-Alkyl-3-methylimidazolium-Based Ionic Liquids", *Anal. Chem.*, 73, 2001, 3838.
[17] W. Zhang; J. He; Y. Gu; X. Liu; S. Jiang, "Effect of Ionic Liquids as Mobile Phase Additives on Retention of Catecholamines in Reversed-Phase High-Performance Liquid Chromatography", *Anal. Letters, 36(4), 2003, 827.*
[18] D.W. Armstrong; L. He; Y.S. Liu, "Examination of Ionic Liquids and Their Interaction with Molecules, When Used as Stationary Phases in Gas Chromatography", *Anal. Chem.*, 71, 1999, 3873.

the same separation behavior as on a non-polar stationary phase, but highly polar molecules act very differently; they are strongly retained in the ionic liquid stationary phase. These stationary phases exhibit strong proton donor and dipolar interactions, followed by proton acceptor abilities.[18]

Recently, a new stationary phase for HPLC incorporating ionic liquids was developed.[19] Butyl-imidizolium bromide was bound to a silica substrate and packed into a HPLC column. Initial studies using this new stationary phase have shown separations of various phenols and suggest promising applications for other molecules. Other preliminary studies have shown the stationary phase exhibits both ion exchange and hydrophobic separation mechanisms. In this work, separations of amino acids and dipeptides are explored using a butyl-imidazolium based stationary phase with a primary focus on the effect of increasing the ionic strength and the organic content of the mobile phase.

[19] Personal communication. Dr. Apryll Stalcup, 7 July 2004.

Experimental:

Chemicals

All amino acids and dipeptides were analytical grade reagents. The amino acids were obtained from Aldrich Chemical Co, Inc. (Milwaukee, WI). The dipeptides were purchased from Sigma Chemical Co (St. Louis, MO). HPLC grade water, ammonium acetate and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC grade methanol was obtained from Pharmco Products, Inc. (Brookfield, CT).

Apparatus

The HPLC system was composed of a Shimadzu LC-10AT Liquid Chromatograph, a Shimadzu SPD-6A UV Spectophotometric Detector and a Shimadzu CR501 Chromatopac (Tokyo, Japan). The HPLC column stationary phase was synthesized in the Stalcup lab group and consisted of butyl-imidazolium bromide bound to a silica substrate (Nucleosil, 100 Å, 5 μm particle size).

The imidazole based-stationary phase was synthesized by adding 50 mmoles of 8-bromo-1-octene and dimethylchlorosilane, with approximately 70 mL toluene and a grain of hexachloroplatinate. This solution was refluxed at 90° C. After 2 hours, silica and 3 mL pyridine were added and the mixture was refluxed for 4 hours at 70° C. Chlorotrimethylsilane (40 mmoles) was added to the reaction flask for endcapping with 3 mL pyridine and refluxed for 4 hours. The reaction mixture was then filtered. The sorbent was rinsed with toluene, methanol, methanol/water, water and methanol; then it was air dried overnight. Sufficient 1-butylimidazole was then added to cover the sorbent and the mixture was gently stirred for 24 hours. The reaction mixture was then filtered, and the sorbent was rinsed with acetone, methanol, methanol/water, water and methanol, then air dried. The sorbent was then packed into an HPLC column (4.6mm x 25 cm, Advanced Separation Technologies, Inc. Whippany, NJ).

Chromatographic Conditions

The mobile phases used were ammonium acetate buffers with ionic strengths varying from 5mM to 50mM. Also, mobile phases with varying organic concentrations of 10mM ammonium acetate/methanol (0-80% organic modifier) were examined with the concentration of the ammonium acetate given for the hydro-organic mixture. The pH of the mobile phases was adjusted to approximately 5.9 using a 1 M acetic acid solution.

The separations were performed by isocratic elution with a flow rate of 1 mL/min. The injection volume was 20 μL. All experiments were performed at room temperature. The detection wavelength was set at 220 nm.

Amino acids and dipeptides all contain aromatic side chains making UV detection possible. All sample concentrations were approximately 1mg/mL. The amino acids were dissolved in 5 mM acetic acid. The dipeptides were all dissolved in 10mM ammonium acetate/methanol solution (90/10).

Results and Discussion

Isocratic elution of aromatic amino acids was achieved using butyl-imidazolium based-stationary phase. In general, the elution order of the aromatic amino acids was DOPA, tyrosine, phenylalanine, then tryptophan. Typical chromatograms for these amino acids are illustrated in Figure 1 (DOPA), Figure 2 (tyrosine), Figure 3 (phenylalanine) and Figure 4 (tryptophan).

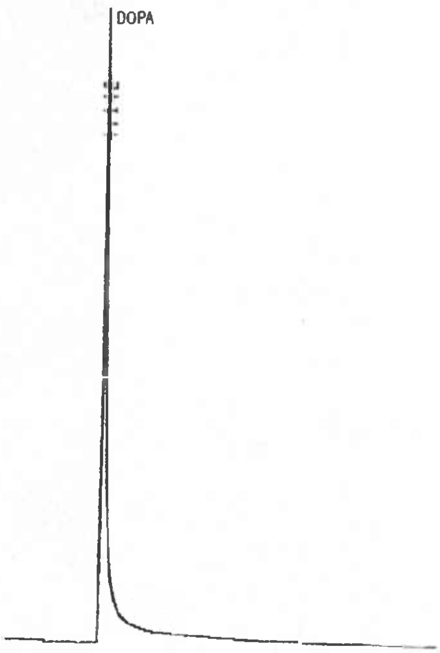

Figure 1: Chromatogram of DOPA. Mobile Phase: 10mM Ammonium Acetate/MeOH 80/20). pH-5.9

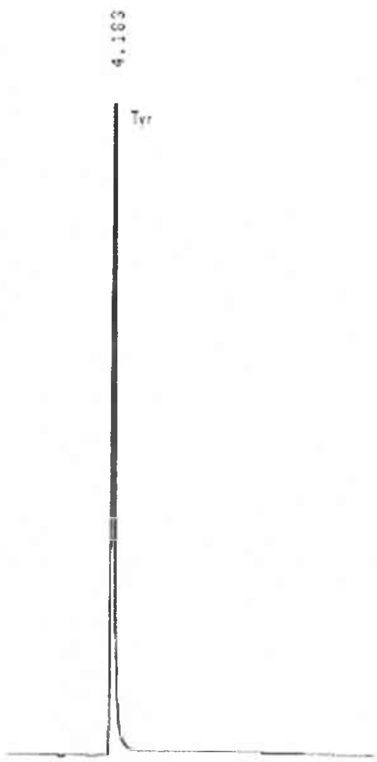

Figure 2: Chromatogram of tyrosine. Mobile Phase: 10mM Ammonium Acetate/MeOH 80/20) pH-5.9

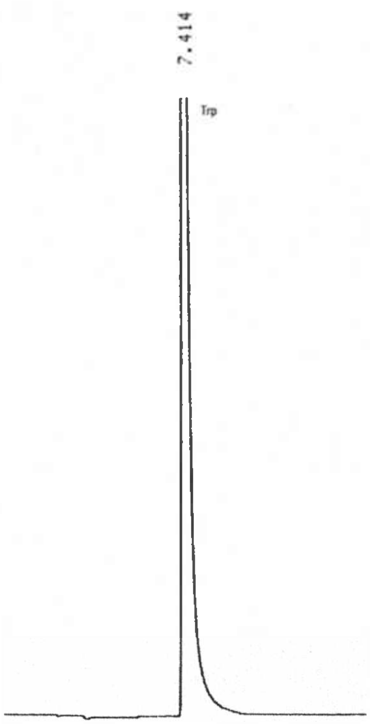

Figure 4: Chromatogram of tryptophan. Mobile Phase: 10mM Ammonium Acetate /MeOH 80/20) pH-5.9

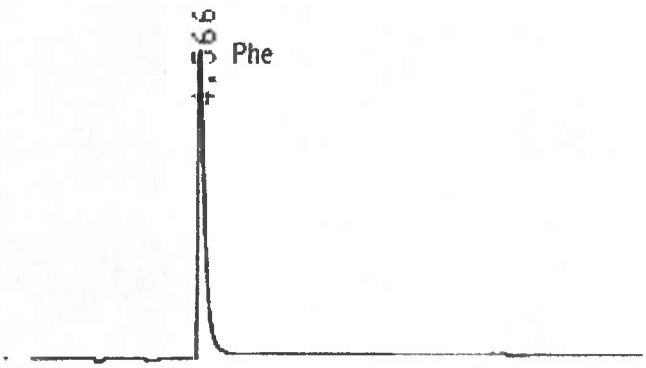

Figure 3: Chromatogram ofphenylalanine. Mobile Phase: 10mM Ammonium Acetate/MeOH 80/20) pH-5.9

The similar retention times of DOPA, phenylalanine and tyrosine is not surprising, since they vary only through the addition of a hydroxyl group. It appears the more substituted the phenyl group on these three amino acids, the lower the retention time. Because these amino acids exist as zwitterions at a pH of 5.9, they would exhibit similar sensitivities to changes in charge; thus the retention times decreased similarly.

The major difference in overall retention times suggests a large role in retention due to the hydrophobicities of the amino acids. Hydrophobicity scales for a pH of approximately 6.0 predict that tyrosine should elute first, followed by phenylalanine then tryptophan for a reverse phase column.[20] DOPA was not ranked in their scale. However, since DOPA contains an additional hydroxyl group, it is reasonable to expect that it should elute before tyrosine. Based on the relative hydrophobicities the elution order correlates to the degree of the analyte's hydrophobicity which may account for the longer retention of tryptophan.

Other studies have reported using gradient elution for their buffer systems with the buffer varying from 50mM sodium formate to 1000mM sodium formate.[5] Another system, showing similar elution order used a buffer system consisting of 1% citric acid in water, and 1% sodium citrate in water.[21] With my system, high selectivity and lower capacity factors were observed with 25 mM ammonium acetate, lessening the need for high salt concentrations.

Effect of Ionic Strength

The effect of varying ionic strength was studied using four amino acids: phenylalanine, 3,4-dihydroxyl-L-phenylalanine (DOPA), tyrosine and tryptophan. The chromatographic data are summarized in Table 1.

Amino Acids	k'	α
DOPA	0.68	
		1.10
Tyrosine	0.75	
		1.22
Phenylalanine	0.90	
		2.69
Tryptophan	3.30	

Table 1: Chromatographic results of isocratic elution of aromatic amino acids.
Mobile phase: 10mM ammonium acetate.

[20] J.L. Meek; Z.L. Rossetti, "Factors Affecting Retention and Resolution of Peptides in High-Performance Liquid Chromatography", *J. Chromatogr*, 211, 1981, 15.
[21] G. W. Fong; E. Grushka, "High-Pressure Liquid Chromatography of Amino Acids and Dipeptides on a Tripeptide Bonded Stationary Phase", *J. Chromatogr*, 142, 1977, 299.

As indicated in the table, the capacity factors for DOPA, tyrosine and phenylalanine are relatively close. The capacity factor (k') is the ratio of the reduced retention volume to the dead volume. Between each of the amino acids, there is not baseline separation; however, the individual peaks are distinguishable. Baseline separations are achieved between phenyalanine and tryptophan, and even between DOPA and phenylalanine. By increasing the ionic strength to 50 mM ammonium acetate, elution of the four amino acids occurs in less than 13 minutes with little effect on the selectivities. The selectivity (α) is the ratio between the capacity factors for two compounds.

The results for all ionic strengths are illustrated in Figure 5. As the figure shows, an increase in the ionic strength causes a decrease in the capacity factor. A change in ionic strength between 5 and 10mM ammonium acetate has a larger effect on capacity factors than increasing the ionic strength >10mM.

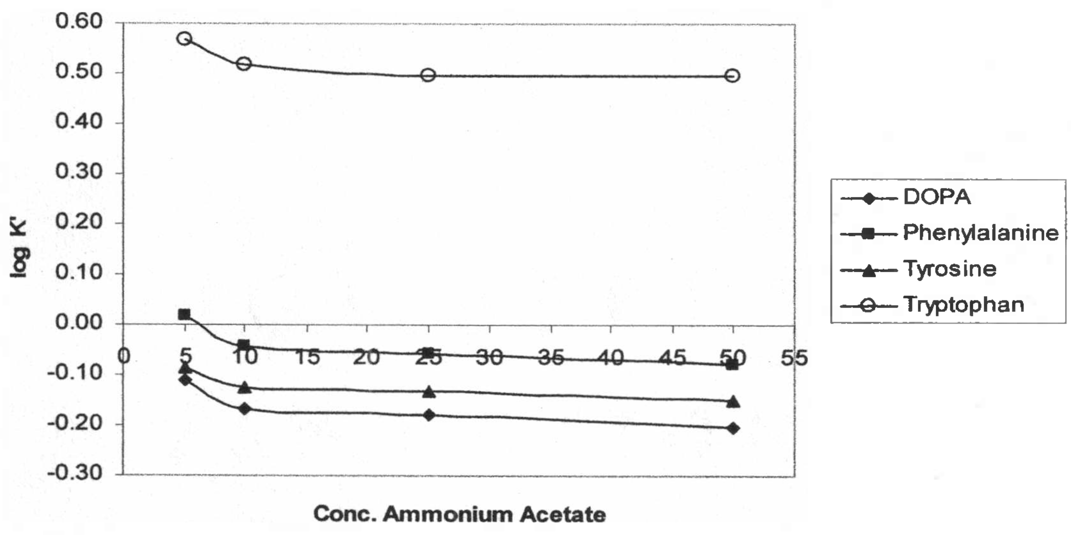

Figure 5:Effect of ionic strength on capacity factor of amino acids. Mobile phase: Ammonium acetate

The decrease in the capacity factor suggests that an electrostatic component is involved in the retention mechanism. Because these amino acids exist as zwitterions at a pH of 5.9, they show an affinity to interact with the positively charged imidazolium cation on the stationary phase. By increasing the salt concentration, the acetate ion dominates interactions with the positively charged imidazolium ion in the stationary phase thus inhibiting interaction between the analytes and the stationary phase. Also, increasing the salt concentration in the mobile phase can create a more favorable environment for the charged analytes, thereby decreasing their affinity for the stationary phase. Consequently, the decrease in interaction between the analyte and stationary phase leads to the decrease in the retention time.

The selectivities exhibited by the amino acids remain relatively similar as the ionic strength increases. One exception is the selectivity of phenylalanine to tryptophan. With increasing ionic strength, the selectivity increases approximately by 2 percent.

Effect of Organic Modifier

Methanol was selected as the organic modifier in this study. Previous trials using the imidazolium based-stationary phase with acetonitrile as the organic modifier resulted in high backpressures. The results for the amino acids are summarized in Table 2.

Amino Acids	k'	α
DOPA	0.40	
		1.10
Tyrosine	0.44	
		1.22
Phenylalanine	0.54	
		2.69
Tryptophan	1.45	

Table 2:Chromatographic results of isocratic elution of aromatic amino acids. Mobile phase: 10mM ammonium acetate/MeOH 80/20 (pH-5.9).

When comparing the results from Table 2 with Table 1, a decrease in capacity factors and selectivities shows a significant impact due to the addition of an organic modifier.

The effect of methanol concentration on capacity factors is shown in Figure 6. As Figure 6 illustrates, increasing the concentration of methanol decreases the capacity factor. One interesting exception is DOPA. Its capacity factor as a function of increasing organic modifier appears to have a minimum (at 60 percent). At lower concentrations (<60 percent) increasing the organic modifier decreases k'_{DOPA}. However, in the presence of high methanol concentrations (>60 percent) k'_{DOPA} increases.

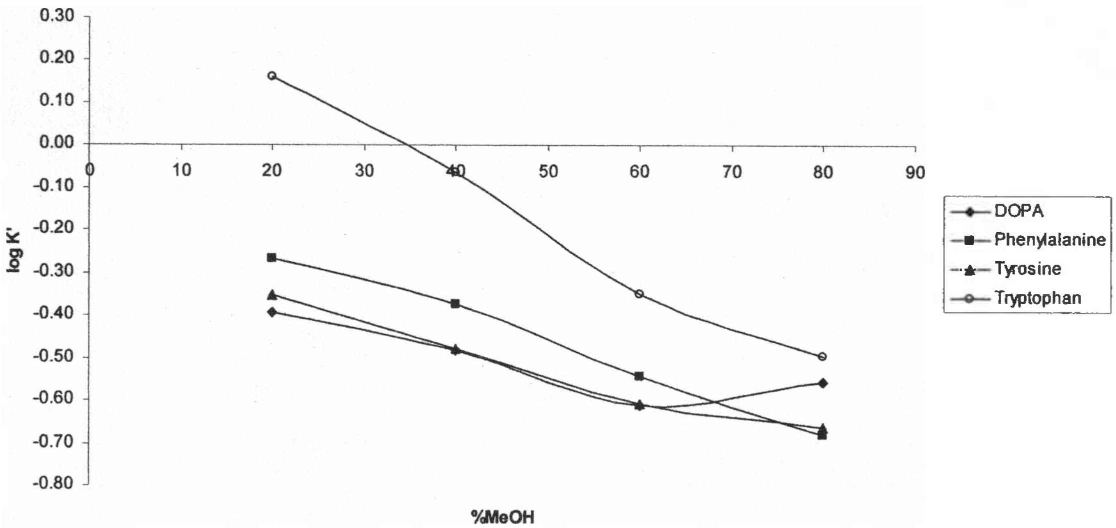

Figure 6: Effect of organic modifier on log k' for amino acids.
Mobile phase: 10mM ammonium acetate/MeOH (pH-5.9)

The presence of a minimum in k' with increasing the percent organic modifier, as illustrated in Figure 6, suggests multiple retention mechanisms. One such consideration is electrostatic interactions described previously. At higher levels of organic content, the role of hydrophobic interactions in the retention mechanism decreases and other mechanisms become more pronounced.

In typical RPLC, when the mobile phase has high water content, the more hydrophobic molecules (i.e. tryptophan) interact strongly with the stationary phase. The stationary phase contains 1-butylimidazolium and residual alkyl chains creating a relatively hydrophobic environment despite the charge on the imidazolium. As the analytes are flushed through the column they interact with the stationary phase. Because hydrophobic molecules tend to associate with other less polar molecules, they exhibit an affinity to interact with the stationary phase, resulting in longer retention times. They resist interactions with the more polar mobile phase. As the volume of organic modifier (i.e. methanol) increases, the mobile phase decreases in polarity, thus increasing the affinity of hydrophobic molecules for the mobile phase. Therefore, they are eluted earlier.

Another factor leading to the longer retention times in the mobile phases containing a low volume of organic modifier is $\pi-\pi$ interactions. Ring stacking ($\pi-\pi$ stacking) between phenols and 1-ethyl-3-methylimidizolium tetrafluoroborate which was enhanced through hydrophobic interactions.[22] The $\pi-\pi$ interactions may therefore play a large role in the retention of the more hydrophobic amino acids, resulting in longer elution times.

The effect of increasing the organic modifier on the selectivity was also explored. Figure 7 summarizes the change in selectivities as a function of increasing percent organic modifier. As Figure 7 illustrates, there appears to be little effect on the selectivity between DOPA and tyrosine, and between tyrosine and phenylalanine. The lack of a significant change in selectivity due to an increase in organic modifier for these

[22] B. Cabovska; G.P. Krieshman; D.F. Wassell; A.M. Stalcup, "Capillary electrophoretic and nuclear magnetic resonance studies of interactions between halophenols and ionic liquid or tetraalkylammonium cations", *J. Chromatogr. A,* 1007, 2003, 179.

three analytes is consistent with their similar structures. Since these three amino acids only vary through the addition of hydroxyl groups (phenylalanine-none, tyrosine-1, DOPA-2) changes in the organic phase should affect these molecules similarly.

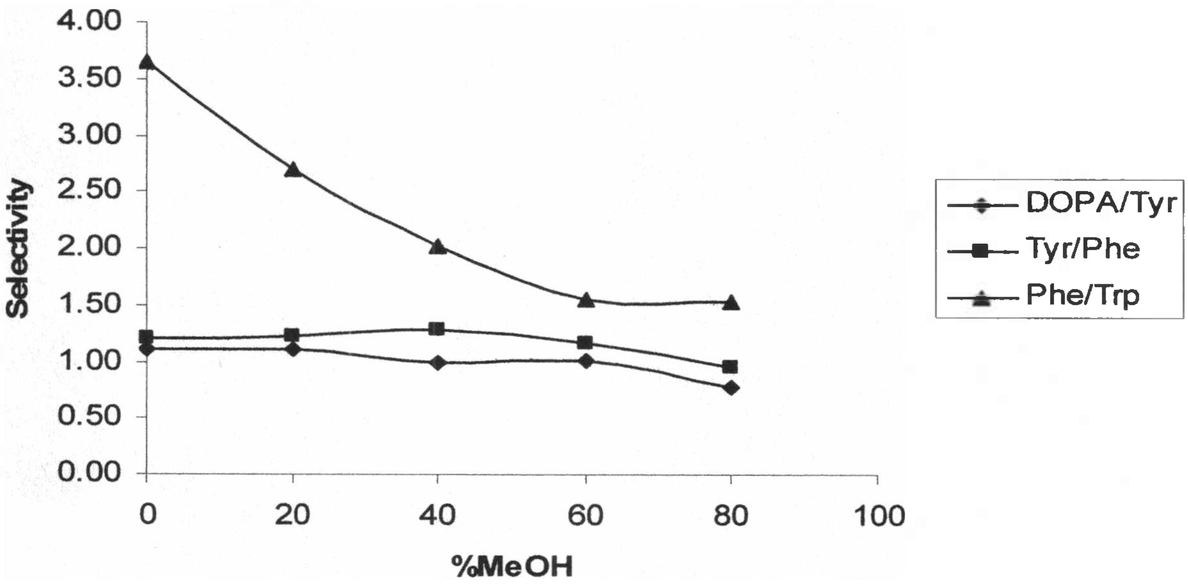

Figure 7: Effect of increasing organic modifier on selectivity.
Mobile phase: 10mM ammonium acetate/MeOH (pH-5.9).

Figure 7 shows the large impact on the selectivity between phenylalanine and tryptophan, however. The selectivity decreases drastically, from 3.65 at zero percent methanol to 1.52 in 80 percent methanol. The change in selectivity between tryptophan and phenylalanine reflects the large decrease in capacity factor of tryptophan as the organic modifier is increased as seen in Figure 6. As the role of hydrophobic interactions decreases with increasing organic modifier volume, the degree of $\pi - \pi$ stacking also decreases. Since both of these mechanisms play a large role in the retention of tryptophan, the decrease in hydrophobicity would have a significant impact on the elution time.

Separation of Dipeptides

To gain more insight into the retention mechanism separations of dipeptides were performed. All dipeptides were also selected on the basis of containing an aromatic group allowing UV detection. Data were obtained using mobile phases containing zero to 20 percent organic modifier. A summary of the capacity factors and selectivities can be found in Table 3.

Dipeptides	k'	A
Tyr-Lys	0.06	1.01
Lys-Tyr	0.06	4.46
Trp-Lys	0.28	1.81
Lys-Trp	0.51	1.03
Gly-Tyr	0.52	1.33
His-Gly	0.69	1.74
His-Leu	1.20	1.13
Gly-Trp	1.35	1.11
Trp-Gly	1.50	1.02
Leu-Tyr	1.53	1.09
Phe-Pro	1.67	1.31
His-Phe	2.18	1.34
Phe-Val	2.92	1.50
Trp-Leu	4.38	1.12
Leu-Trp	4.91	1.17
Glu-Tyr	5.74	1.34
Trp-Tyr	7.72	1.94
Glu-Trp	14.93	

Table 3: Chromatographic results of isocratic elution of dipeptides.
Mobile phase: 10mM ammonium acetate/MeOH 80/20 (pH-5.9).

As with the amino acids, an increase in the organic modifier resulted in a decrease in capacity factor. Figure 8 illustrates the decrease in the capacity factors for substituted tyrosine dipeptides. With the exception of tyrosine-lysine (Tyr-Lys) all capacity factors decreased as the percent organic modifier increased. The presence of lysine, a polar, positively charged amino acid at pH 5.9, resulted in elution at or near the void volume in the tyrosine dipeptides, and the tryptophan-lysine dipeptides eluted at approximately 4 minutes. The polarity would promote association with the polar mobile phase, while the less polar tryptophan would have an affinity for the stationary phase. Furthermore, the positively charged lysine would be inhibited from interacting with the stationary phase because of the electrostatic repulsion from the positively charged imidazolium ion.

Further evidence on the role of the electrostatic interactions is the high retention of glutamic acid-tryptophan and glutamate-tyrosine amino acids. The addition of glutamic acid to tyrosine increased the capacity factor from 0.44 to 5.74 for tyrosine and 1.45 to 14.93 for tryptophan dipeptides in a mobile phase composed of 10mM ammonium acetate/20 percent MeOH. Glutamic acid is a negatively charged, polar amino acid. Because of the polarity, glutamic acid would preferentially associate with the polar mobile phase decreasing the capacity factor. However, due to its negative charge, it associates with the imidazolium ion resulting in longer retention time. In the case of the glutamic acid-tryptophan dipeptides, the hydrophobic association of tryptophan and the negatively charged glutamic acid coincide to produce much longer retention times.

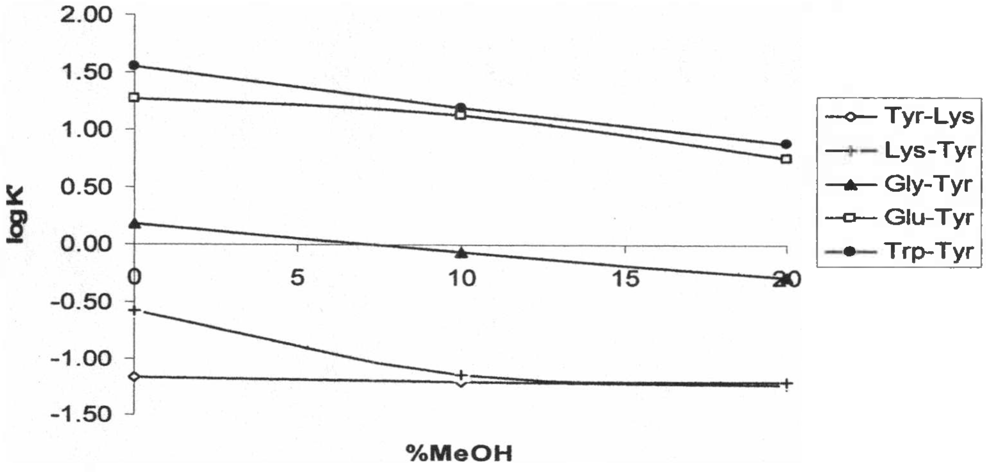

Figure 8:Effect of organic modifier on log K' for tyrosine dipeptides.

Mobile phase: 10mM ammonium acetate/MeOH 80/20 (pH-5.9).

The substitution of the second amino acid in the tryptophan dipeptides and the order in which the amino acids were substituted also played a role in the elution order. Figure 9 illustrates a comparison of the selectivities of the dipeptides to tryptophan alone. Both charge and relative hydrophobicities play a role in changing the selectivity. The dipeptides that elute before tryptophan are less hydrophobic and carry a positive charge. Not surprisingly, glycine, the smallest amino acid appears to have little impact on changing the retention relative to tryptophan. Leucine, a nonpolar molecule, when substituted as the second amino acid increased the retention of tryptophan. Addition of glutamic acid, a negative charged amino acid, increased the selectivity and retention time of tryptophan.

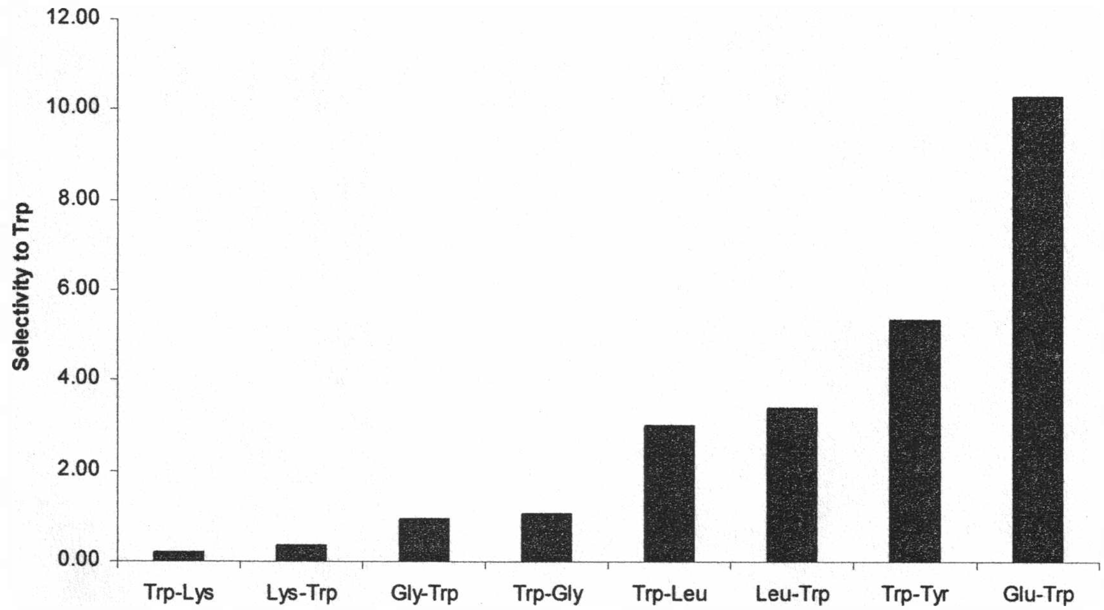

Figure 9: Change in selectivity of substituted Trp dipeptides.
Mobile phase: 10mM ammonium acetate/MeOH 80/20 (pH-5.9).

It is interesting to note the order of substitution appears to play a role in the retention of dipeptides and changes in the mobile phase appear to affect these dipeptides differently as Table 4 shows. F. Zou et al. reported similar results in a study on the retention behavior of dipeptide isomers in RPLC. In that study, it was found that increasing the size of the molecule resulted in an increase in log k'. The same study also found a pH between 5.7 and 7 resulted in lower elution times.[23] Another study on dipeptide isomers found that changes in pH resulted in reversal of elution order. It was proposed that the mechanisms for this reversal were due to protonation of the terminal amine group. If this protonated group was near an aromatic ring, it hindered the interaction between the ring and the bonded phase.[24]

Dipeptide	k' in 10% MeOH	k' in 20% MeOH
Lys-Trp	0.64	0.51
Trp-Lys	0.42	0.28
Trp-Gly	2.20	1.50
Gly-Trp	2.21	1.35
Leu-Trp	10.77	4.91
Trp-Leu	12.29	4.38

Table 4: Effect of increasing organic modifier on k' of Trp dipeptides. Mobile phase: 10mM ammonium acetate/MeOH 90/10 and 80/20, respectively (pH-5.9).

In comparing the capacity factors for Lys-Trp and Trp-Lys, Trp-Lys elutes first in both mobile phase conditions. For the Trp-Lys dipeptide, Trp contains a free α-amino group at the N-terminus and Lys contains an amino group on its side chain. At a pH 5.9, both the Lys's side chain amino group and the N-terminal amino group of Trp would be protonated.[21] This protonated amine group would sterically hinder the interaction of the

[23] F. Zou; Y.K. Zhang; L.F. Dong, P.C Lu, "Retention Behavior of Dipeptide Isomersi n Reversed-Phase High-Performance Liquid Chromatography", *Chromatographia*, 31 (1/2), 1991, 27.
[24] G.W. Fong; E. Grushka, "High-Pressure Liquid Chromatography of Amino Acids and Dipeptides on a Tripeptide Bonded Stationary Phase", *J. Chromatogr*, 142, 1977, 299.

Trp aromatic group with the stationary phase, resulting in a more rapid elution. In comparison, Lys-Trp dipeptides would have two free amine groups on Lys and a carboxyl group on the Trp. Under acidic conditions, the carboxyl group on Trp would not be protonated, thus it would not hinder interaction with the stationary phase.

The order of substitution for Leu-Trp and Trp-Leu provide more insight into the retention mechansims. Both of these amino acids are hydrophobic with Trp showing more hydrophobic tendencies. At an organic concentration of 10 percent, Leu-Trp is eluted first. However, at an organic concentration of 20 percent, Trp-Leu is eluted first. The mechanism behind this reversal in elution order is unclear at present, but a combination of electrostatic forces and hydrophobicities may play a large role. This reversal may be due the interplay of the mechanisms, and which one plays a larger role in retention based on the organic phase (i.e. in higher organic concentrations, electrostatic forces become more important).

The elution of Phe-Pro dipeptide produces intriguing results which are illustrated in Figure 10. The elution of this dipeptide produced "batman peaks." Batman peaks can be considered two chromatographic peaks without baseline separation occurring as a result of one analyte. This may be due to the presence of D/L diasteromers. In previous studies E.P. Kroeff reported that retention times for alanine diasteromers was different. In the same study it was also shown that decreasing the pH of the mobile phase would increase the separation of the diasteromers.[25] It may be possible to determine if the presence of diasteromers is the cause of the "batman" peaks through a series of separations at varying pH values.

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[25] E.P. Kroeff; D.J. Pletrzyk, "High Performance Liquid Chromatographic Study of the Retention and Separation of Short Chain Peptide Diastereomers on a C_8 Bonded Phase", *Anal. Chem.*, 50 (9), 1978, 1353.

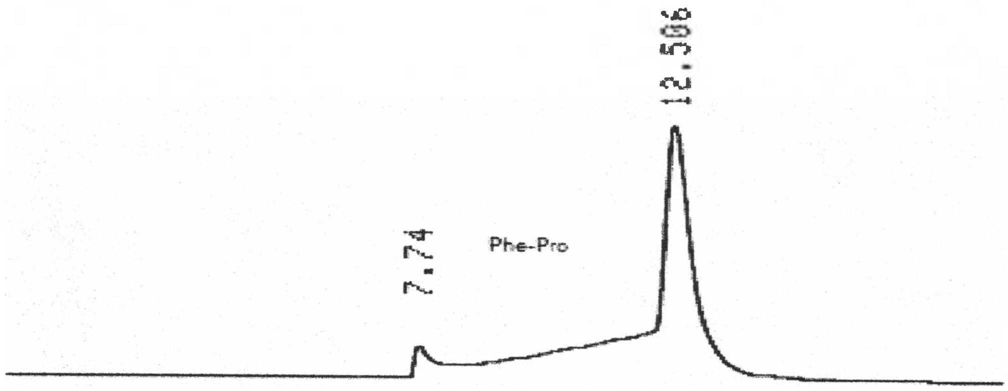

Figure 10: Chromatogram of Phe-Pro dipeptide. Mobile phase: 10 mM Ammonium acetate/ MeOH 90/10 (pH-5.9)

Conclusion

Based on the amino acids, the optimum conditions for amino acid separations appear to be 10mM ammonium acetate/MeOH (80/20). At this concentration, the greatest selectivity between amino acids occurred. This also gives the most selectivity for the dipeptides with the shortest retention times. However, higher volumes of both organic modifier and ionic strength were not explored.

Separations of the amino acids and dipeptides provided insight into the retention mechanisms of the butyl-imidazolium stationary phase. It was shown that the butyl-imidazolium stationary phase acts in a dual nature, exhibiting both ion-exchange and reversed-phase retention mechanisms. Furthermore, it was shown that separations of aromatic amino acids and the selected dipeptides can be accomplished using this stationary phase with short retention times. This system maximizes on the benefits of many 2-D systems, without many of their current limitations

Future Work

Future work on optimizing the conditions and varying the conditions may lead to better separations of amino acids and dipeptides. Since this system has shown good separations with low salt concentrations, with further optimization, this system may be coupled to a mass spectrometer, allowing detection of all 20 underivatized amino acids and further examining the applications of this column. Also, work on larger peptides may further illuminate the retention mechanisms and the resolving capabilities of ionic-liquid based stationary phases. It may be possible, through variations of pH, to increase the separation abilities of this system for dipeptide isomers and diasteromers.