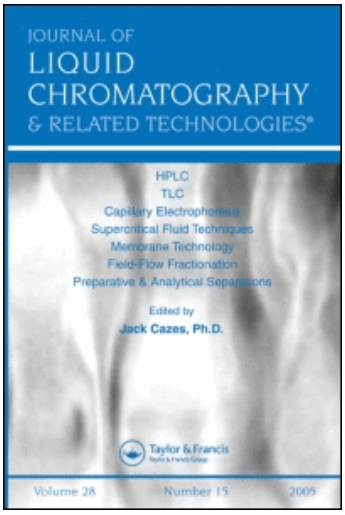

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Hydrophilic Interaction Liquid Chromatographic (HILIC)/Ion Exchange Separation of Picolinic and Nicotinic Acids

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Abstract: A hydrophilic interaction liquid chromatography (HILIC)/ion-exchange method for the analysis of nicotinic and picolinic acid is presented. Separation of nicotinic and picolinic acid was achieved at relatively high acetonitrile concentration (70%). Various aspects of the separation were examined, including buffer concentration, column temperature, buffer pH, and column configuration. Throughout the study, the method demonstrated characteristics of both ion-exchange and HILIC retention. Studies performed indicate an increased influence of HILIC on the separation at aqueous concentrations less than 20%. Finally, a decrease in solvent consumption was noted using a small bore column without negative impact on the separation.

Keywords: Hydrophilic interaction, HILIC, Ion exchange, Picolinic acid, Nicotinic acid

INTRODUCTION

The retention and separation of small polar molecules presents unique challenges to the chromatographer. Typically, separations occur using highly aqueous mobile phases using specialty columns with polar embedded groups and/or endcapping to avoid phase collapse. Additionally, ion-pair reagents have been employed to increase the retention and selectivity. These sets of conditions present challenges to the chromatographer when

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developing a stability indicating test method to be compatible with mass spectroscopy.

Hydrophillic interaction liquid chromatography (HILIC) is an alternate approach to the retention and separation of small polar molecules. Similar to normal phase chromatography, HILIC employs the use of polar stationary phases (e.g., silica, amine) with a hydrophobic mobile phase.[1] However, unlike normal phase chromatography, reversed phase solvents (THF, methanol, acetonitrile) are combined with an aqueous phase, where the aqueous phase is the strong solvent. This lends the separation technique to be more amenable to mass spectroscopy, but also alleviates instrumental, safety, and environmental concerns of the use of traditional normal phase solvents.

To evaluate the potential of this separation technique, a test method was developed for the analysis of picolinic and nicotinic acid. Picolinic and nicotinic acid are pyridine carboxylic acids. They are structural isomers of each other, differing only in the position of the carboxylic acid group in relation to the nitrogen in the pyridine ring. Both nicotinic and picolinic acid are zwitterions and have two pKa's of interest. For nicotinic acid the pKa's are pKa_1: 2.2 and pKa_2: 4.8; while picolinic acid's are pKa_1: 1.0 and pKa_2: 5.4. Both molecules are highly soluble in water. The chemical structures of picolinic and nicotinic acid are presented in Figure 1.

Several methods were identified for the analysis of niacin by HPLC utilizing either highly aqueous mobile phases[2–4] or ion-pair reagents.[5–8] One HILIC method was identified for the analysis of nicotinic acid.[9] However, this method shows poor retention of nicotinic acid (<1 min). One method was identified for the analysis of picolinic acid using ion-pair chromatography.[10]

EXPERIMENTAL

Chemicals and Reagents

Picolinic acid and nicotinic acid (niacin) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ACS grade ammonium hydroxide and glacial acetic acid were obtained from Fisher (Hanover Park, IL, USA). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). HPLC grade water was obtained using a Millipore Milli-Q Gradient water purification system (Bedford, MA, USA).

Apparatus

The chromatographic systems consisted of an Agilent 1100 HPLC System (Palo Alto, CA, USA) equipped with a variable wavelength detector (Model G1314A) at 264 nm, thermostatted column compartment (Model G1316A),

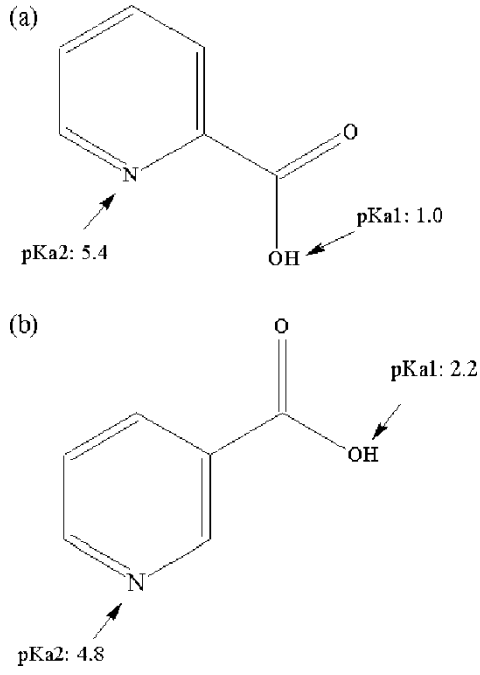

Figure 1. Structures of (a) picolinic acid and (b) nicotinic acid with associated pKa's.

well plate autosampler (Model G1367A), and quaternary pump (Model G1311A) equipped with a degasser (Model G1379A). All data was acquired and stored using Waters Millennium Data Acquisition Software v. 4.0 (Waters Corporation, Milford, MA, USA).

The HPLC column was a Phenomenex Luna NH_2, 5 μm, 150 × 4.6 mm and (Phenomenex, Torrance, CA, USA).

Preparation of Solutions

Mobile phase buffers were prepared by mixing the appropriate amount of ammonium hydroxide and glacial acetic acid in water to obtain the desired concentrations, as dictated by the experimental conditions. Mobile phase buffers were mixed with the appropriate amount of acetonitrile as dictated by the experimental conditions.

Samples

Samples of picolinic acid and nicotinic acid were prepared at 200 μg/mL in acetonitrile. Due to the solubility of the materials, sonication was employed to

aid in dissolving the sample. All samples were filtered through a 0.45 μm PTFE syringe filter (Pall Corporation, East Hills, NY, USA), discarding the first 3 mL of filtrate.

RESULTS AND DISCUSSION

Initial Development

The use of propylamine columns for HILIC separations was previously reported.[11,12] For acidic compounds, propylamine columns add additional separation functionality through ion-exchange. At acidic and neutral conditions, the propylamine bonded phase (pKa $\sim$ 10.9) will be protonated. This will allow for ion-exchange interactions for acidic compounds. Since both compounds of interest are acids, a Phenomenex Luna NH_2 was chosen. The column is a silica based propylamine column without endcapping. The column has a claimed pH range of 1.5 to 11.0. The propylamine functionality will allow for additional separation power due to its ion-exchange capability.

Both picolinic and nicotinic acid are ionizable molecules. Therefore, control of the mobile phase pH is required to ensure a robust separation. Between pKa_1 and pKa_2, picolinic and nicotinic acid exist as zwitterions with the amine functionality protonated and the carboxylic acid functionality deprotonated. However, at a pH above pKa_2, the amine functionality of both molecules is not protonated. Therefore, in order to take full advantage of the ion-exchange capabilities of the column, the mobile phase pH will be controlled greater than 5.4.

To accomplish this, a buffering system of 200 mM ammonium hydroxide and 100 mM glacial acetic acid was chosen. This would provide adequate buffering capacity over a pH range of about 8 to 10. Furthermore, since the buffer components are volatile, this system could potentially be transferable to ELSD and/or mass spectrometry detection. The inherent pH of the buffer is about 9.2. Since this is within the pH range of the column and well above the pKa's of both picolinic and nicotinic acid, no pH adjustments of the buffer was made. It should be noted that at first glance the buffer concentrations appear relatively high. However, when mixed at mobile phase conditions, this equates to a much lower effective buffer concentration on column.

To investigate the elution conditions, a scouting gradient analysis was performed using acetonitrile and the aforementioned buffer preparation. Since the aqueous phase is the strong solvent in HILIC, initial conditions were 95% acetonitrile and 5% buffer. The acetonitrile content was decreased over 20 minutes to a final value of 50%. Results demonstrate that this separation can be accomplished in a reasonable time using isocratic conditions. From the results of the scouting gradient, a mobile phase of 70% acetonitrile/30% buffer at a flow rate of 1 mL/min was proposed. Since nicotinic and picolinic acid share similar UV chromophores, a wavelength

of 264 nm was used for analysis. A representative chromatogram is presented in Figure 2.

Buffer Concentration

The effect of the buffer concentration on the retention was evaluated. Buffer solutions at various concentrations were prepared and mixed with acetonitrile at a ratio of 70:30 acetonitrile:buffer. Results demonstrate an increase in retention for both picolinic and nicotinic acid with lower buffer concentrations. This observation is evidence of the ion-exchange capacity of the propylamine stationary phase. A decrease in the buffer concentration would lead to a decrease in the counter-ion concentration in the buffer and, hence, an increase in the retention time of the analytes. This observation is typical of ion-exchange separations.[13] Results are presented in Figure 3.

Column Temperature

The effect of column temperature on the separation was investigated over the range of 15°C to 55°C for various mobile phase conditions listed in Table 1. For each experiment, the buffer concentration was adjusted accordingly to maintain a constant on-column buffer concentration of 60 mM ammonium hydroxide/30 mM acetic acid. Results from the analysis were used to create van't Hoff curves. A van't Hoff curve describes the relationship between temperature and capacity factor. The equation can be expressed as follows:

$$\ln k' = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi^{[14]}$$

where k' is the capacity factor, ΔH is the molar enthalpy of transfer of a solute from the mobile phase to the stationary phase, R is the ideal gas constant, T is

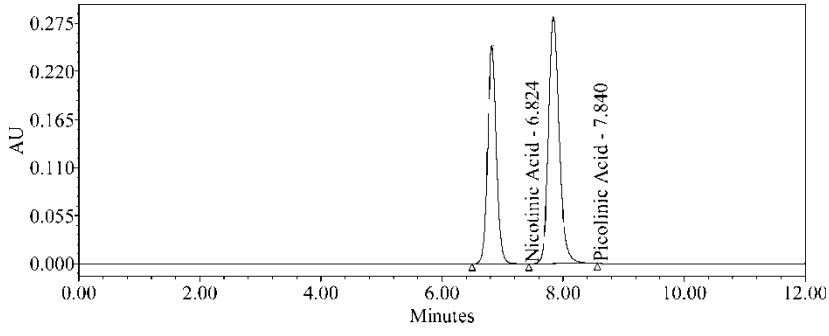

Figure 2. Representative chromatogram of nicotinic and picolinic acid at 70:30 acetonitrile:buffer.

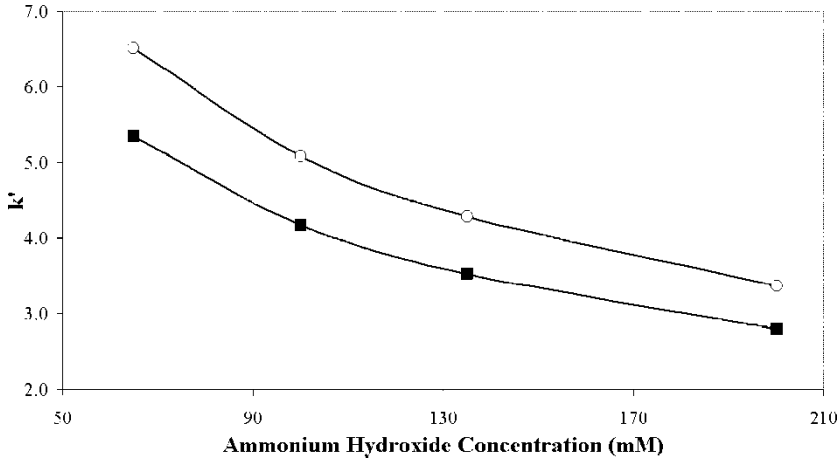

Figure 3. Effect of buffer concentration on the retention of nicotinic acid (■) and picolinic acid (○).

the temperature (in Kelvin), ΔS is the molar entropy of transfer of a solute from the mobile phase to the stationary phase, and Φ is the phase ratio. For any given mobile phase, the ln k' of the solute is plotted against $1/T$ and linear regression performed. Assuming $-\Delta H/R$ is equal to the slope, and that the phase ratio is constant, ΔH can be calculated.[15]

Results from the experiments are presented in Table 1 and graphically in Figures 4–7. A linear relationship between ln k' and $1/T$ was demonstrated in experiments 1 and 2 for both peaks of interest. The ΔH calculated for nicotinic acid and picolinic acid were 7.6 KJ/mole and 7.4 KJ/mole, respectively, for experiment 1 and 6.3 KJ/mole and 6.2 KJ/mole, respectively, for experiment 2. In both cases, a positive ΔH was observed, indicating that the transfer of the solute from the mobile phase to the stationary phase is endothermic. For both picolinic and nicotinic acid, the ΔH dropped as the acetonitrile concentration was decreased. This phenomenon has previously been observed in HILIC retention of acids on propylamine phases and is attributed to the ion-exchange effect of the propylamine phase.[12] The ΔH for both picolinic and nicotinic were relatively constant in any given mobile phase condition,

Table 1. Enthalpy of transfer in various mobile phase conditions

Experiment	ACN (%)	Buffer (%)	ΔH Nicotinic acid (KJ/mole)	ΔH Picolinic acid (KJ/mole)
1	70	30	7.6	7.4
2	60	40	6.3	6.2
3	80	20	3.3	4.6

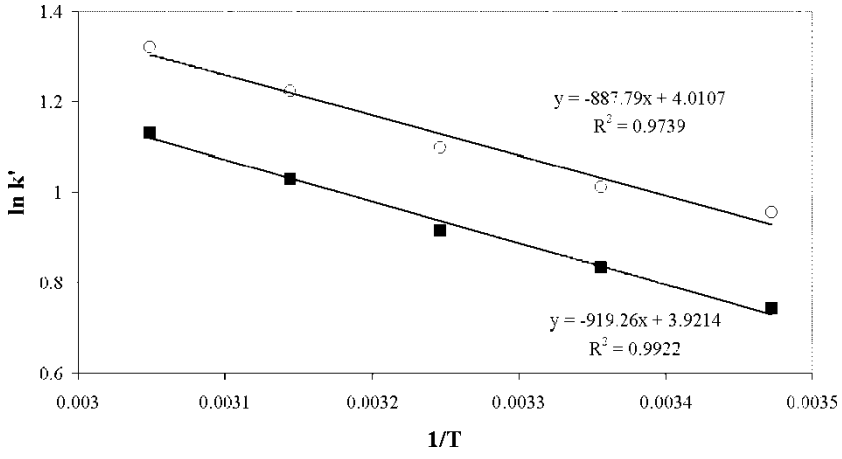

Figure 4. The van't Hoff curves for nicotinic acid (■) and picolinic acid (○) at 70:30 acetonitrile:buffer.

indicating the separation mechanism is comparable between the two molecules. This is not surprising given the similarities between the two molecules. Additional evaluation of the data from the three experiments demonstrates the increased retention of both nicotinic and picolinic acid with increased concentrations of acetonitirile. This is most likely due to the increased contribution of the HILIC retention mechanism on the separation at higher organic concentrations.

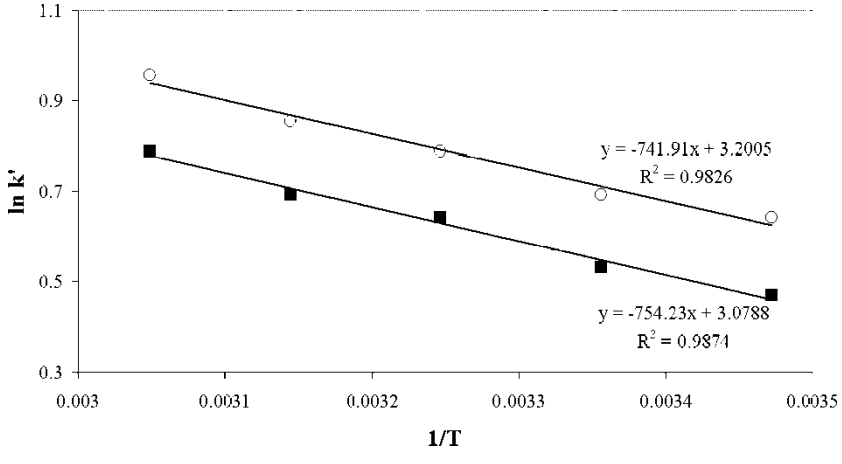

Figure 5. The van't Hoff curves for nicotinic acid (■) and picolinic acid (○) at 60:40 acetonitrile:buffer.

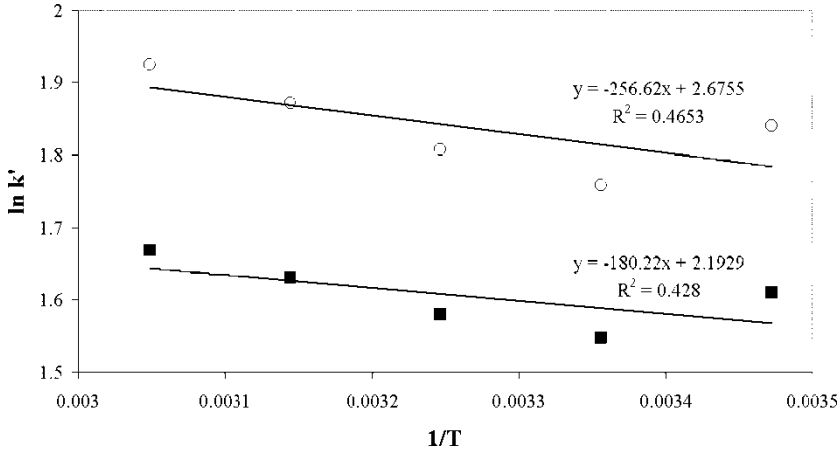

Figure 6. The van't Hoff curves for nicotinic acid (■) and picolinic acid (○) at 80:20 acetonitrile:buffer.

Experiment 3 demonstrated a non-linear relationship between ln k′ and 1/T. From the plot, it is apparent that the non-linear relationship is due to the deviation of the 15°C data point from the trend line.It is possible that the increased retention observed at the 15°C time point is due to lack of miscibility of the buffer and organic at this temperature. Elimination of this point demonstrates a linear van't Hoff curve with ΔH calculated to be 3.3 KJ/mole and 4.6 KJ/mole for nicotinic and picolinic acid, respectively. Interestingly, we see a divergence in the ΔH calculated between nicotinic and picolinic

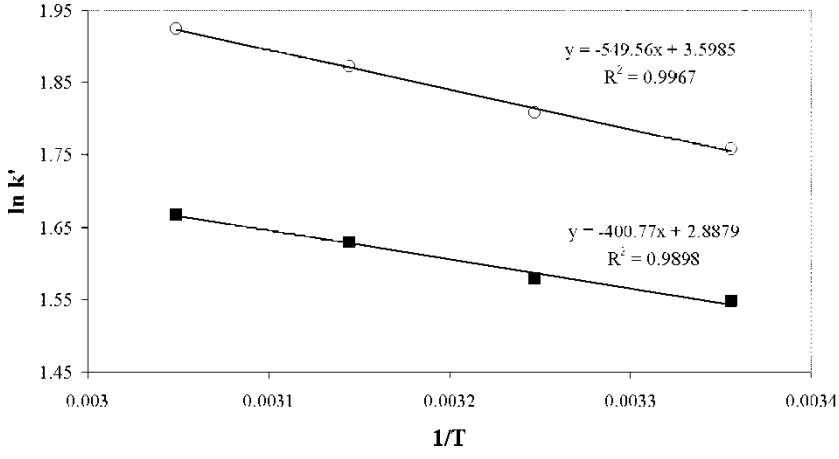

Figure 7. The van't Hoff curves for nicotinic acid (■) and picolinic acid (○) at 80:20 acetonitrile:buffer from 25° to 55°C.

acid. It is postulated that this difference is due to the increased influence of the HILIC retention mechanism on the separation at higher organic percentages in the mobile phase.

As previously mentioned, the retention of both nicotinic and picolinic acid is governed by both ion-exchange and HILIC retention mechanism. As the percent organic is increased in the mobile phase, it is intuitive that we would see an increased contribution of the HILIC retention mechanism on the separation. Based on the experiments, this increased contribution is having a greater affect on the retention of the nicotinic acid compared to the picolinic acid, possibly due to the proximity of the nitrogen in the pyridine ring to the carboxylic acid functional group, and the resulting carboxylate anion formed through resonance. This is demonstrated in Figure 8. For niacin, through resonance a carbocation is formed at the γ position (relative to nitrogen). This carbocation has a net neutralizing effect on the carboxylate anion. However, for picolinic acid the carbocation is formed at the α position. Therefore, the neutralizing effect for the carboxylate ion is not as prominent due to presence of the nitrogen anion. This results in a higher effective charge for the carboxylate anion in the picolinic acid molecule compared to the nicotinic acid. This higher effective charge for the picolinic acid molecule results in the continued influence of ion-exchange on the separation.

Based on experiments 1 and 2, it was anticipated that the calculated ΔH's would be higher for experiment 3 than those reported in experiment 1.

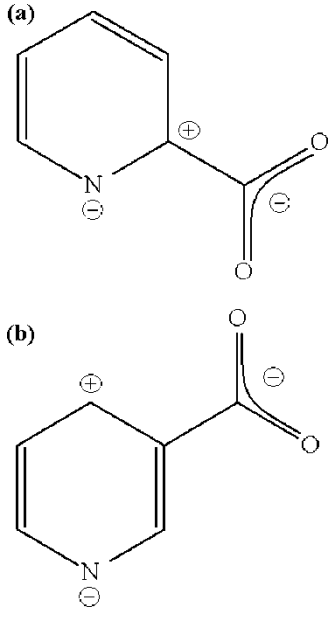

Figure 8. Resonant carboxylate anion forms for (a) picolinic and (b) nicotinic acid.

However, as demonstrated in Table 1, this is not the case. Although ΔH's for this experiment still demonstrate the transfer process as being endothermic, the magnitude is significantly less (57% for nicotinic acid and 38% for picolinic acid) than that from experiment 1. Assuming that ion-exchange is responsible for the endothermic ΔH's calculated,[12] this data also suggests the increasing influence of the HILIC retention mechanism on the separation with increasing acetonitrile concentrations. This is also supported by the increased retention times observed with these mobile phase conditions, as well as the diverging ΔH's discussed previously.

Effect of Aqueous Content

The effect of the aqueous content of the mobile phase was examined over a range of 20% to 60%. To examine only the aqueous contribution, the buffer concentration was adjusted accordingly to maintain an on-column buffer concentration of 60 mM ammonium hydroxide, 30 mM acetic acid. Results are presented in Figure 9. Results demonstrate a decrease in the retention of both nicotinic and picolinic acid with increasing proportions of the aqueous content of the mobile phase. This observation is typical of HILIC separations since water is considered the strong solvent for the separation. Interestingly, as demonstrated in Figure 9, the nicotinic acid was retained less on the column at higher organic concentrations compared to picolinic acid. As previously discussed, this may demonstrate the increased influence of HILIC on the retention of both molecules, as well as a larger ion-exchange effect on the picolinic acid (compared to the nicotinic acid).

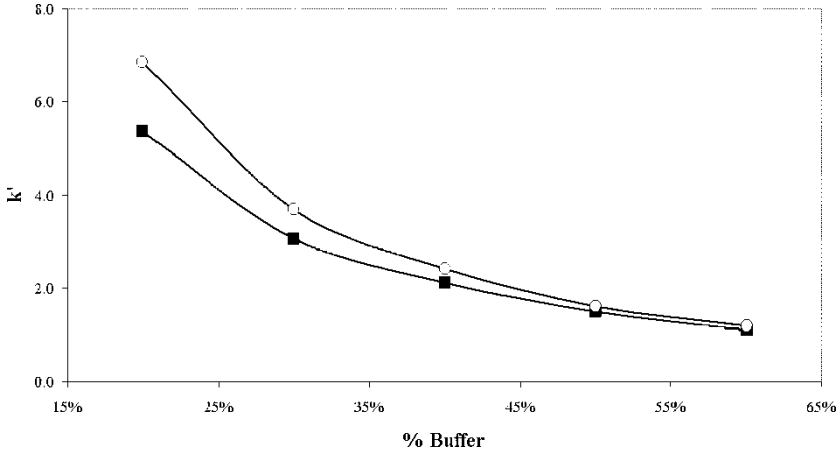

Figure 9. Effect of aqueous content on the retention of nicotinic acid (■) and picolinic acid (○).

Buffer pH

The effect of the pH of the buffer was examined over the range of the buffering system (8.2 to 10.0). Separate aliquots of the mobile phase buffer were adjusted to a pH of 8.2 and 10.0 using either glacial acetic acid or ammonium hydroxide. Separate mobile phases were prepared with these buffers at 70:30 acetonitrile:buffer and analysis was performed. Results from this analysis were compared against a non-pH adjusted mobile phase (pH = 9.2). As demonstrated in Figure 10, decreasing the pH caused an increase in the retention of both nicotinic and picolinic acid. Conversely, increasing the pH caused a decrease in the retention of both nicotinic and picolinic acid. These retention changes are most likely due to changes in the bonded phase polarity. Decreasing the pH increases the protonation of the bonded phase, leading to an increase in polarity. Increasing the pH has an opposite effect, leading to less retention. Figure 10 also demonstrates that the retention of picolinic acid was more susceptible to changes in pH, which is most likely due to a more predominate ion-exchange interaction of the picolinic acid.

Column Configuration

As an alternate to the Phenomenex Luna NH_2, 5 μm, 150 × 4.6 mm examined above, a 150 × 2.0 mm column, packed with the same bonded phase was examined. Due to the narrower column, the flow rate was adjusted accordingly

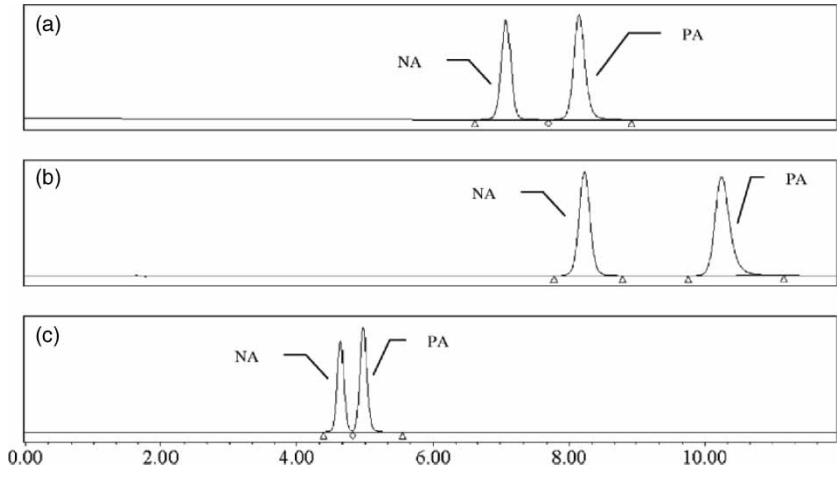

Figure 10. Effect of the buffer pH on the separation of nicotinic (NA) and picolinic (PA) acid at (a) control conditions, (b) pH = 8.2, and (c) pH = 10.0.

to account for the difference in column volume. Using the following, the column volume was calculated for both configurations:

$$\text{Column Volume} = \pi \cdot r^2 \cdot L \cdot 0.6$$

where r is the radius of the column, L is the column length, and 0.6 is the assumed packing porosity (60%). Following calculation of the column volume, the flow rate for the narrow bore configuration was determined using the following calculation:

$$\frac{V_1}{F_1} = \frac{V_2}{F_2}$$

where V_1 and F_1 are the volume and flow rate for the 150 × 4.6 mm configuration, respectively, and V_2 and F_2 are the volume and flow rate for the 150 × 2.0 mm configuration, respectively. Based on these calculations, a flow rate of 0.2 mL/min was required to maintain the same linear velocity. In addition, to avoid column overload, the injection volume was reduced from 10 μL to 5 μL. As shown in Figure 11, analysis performed at this flow rate and column configuration demonstrated chromatography relatively equivalent to that from the analysis at 1 mL/min with the 150 × 4.6 mm column configuration.Assuming a 12 minute run time, this equates to a 500% decrease in solvent consumption (~9.6 mL/injection).

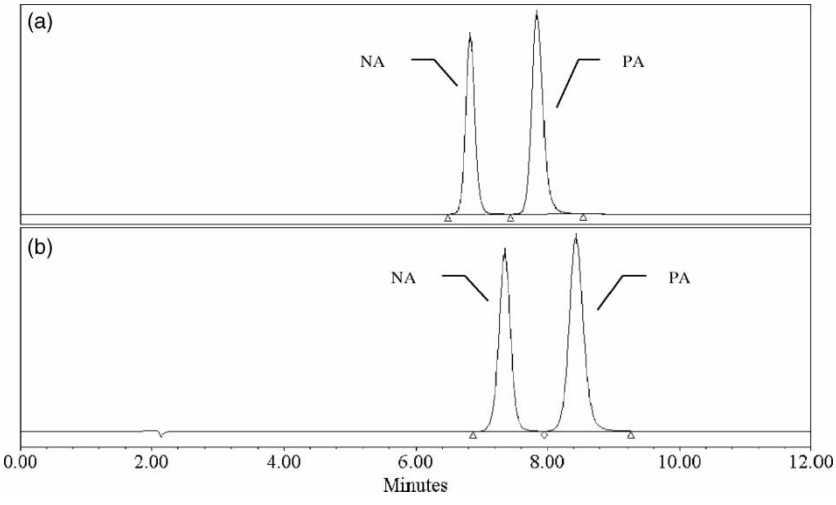

Figure 11. Separation of nicotinic (NA) and picolinic (PA) acid using an (a) 150 × 4.6 mm and (b) 150 × 2.0 mm column.

CONCLUSION

A hydrophilic interaction liquid chromatography (HILIC)/ion-exchange method for the analysis of nicotinic and picolinic acid is presented. Various aspects of the separation were examined, including buffer concentration, column temperature, and column configuration.Variation of the buffer concentration demonstrated retention changes typical of ion-exchange separations. Through the analysis of van't Hoff curves, changes in column temperature demonstrate that the process of transferring the solute from the mobile phase to the stationary phase is endothermic with mobile phase containing 80% of acetonitrile or less, which is attributed to ion-exchange retention. However, with mobile phases containing greater than 80% acetonitrile, the separation appears to demonstrate an increase in the influence of the HILIC retention mechanism on the separation. Changes in the pH of the buffer over the buffering range demonstrated significant changes in the retention likely due to changes in the polarity of the bonded phase. Finally, the method was demonstrated to be scalable to a smaller bore column, which resulted in a 500% decrease in solvent consumption.

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