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Capillary electrochromatographic separation of ionizable compounds with a molecular imprinted monolithic cationic exchange column

A polymer-based monolithic capillary column imprinted with 4-aminopyridine (4-AP) was prepared by a thermally-initiated polymerization process; and its performance as a capillary electrochromatographic medium was evaluated in separating 4-AP and 2-AP isomers. The effects of experimental parameters, such as pH value and ionic strength of the buffer, the acetonitrile content in the mobile phase, and the applied voltage, on the resolution of these isomers had been carefully investigated. It was found that in the retention process there were interplays of multiple mechanisms of ion-exchange, molecular imprinting, and electrophoresis. These mechanisms allowed more sophisticated control of experimental parameters in the separation of ionizable compounds.

Key Words: Molecular imprinted polymer; Monolithic column; Cationic exchange column; Capillary electrochromatography; Ion exchange chromatography; Structural isomers; Ionizable compounds; 4-Aminopyridine; 2-Aminopyridine

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1 Introduction

Molecular imprinting is a technology in which specific recognition sites are formed in a polymer matrix by synthesis in the presence of a template molecule. The imprinted polymer is capable of selectively rebinding the template molecule. Molecular imprinted polymers (MIP) can be used as attractive alternatives or complements to native antibodies and receptors [1–10]. They have also been used for chromatographic separation [11–12], solid-phase extraction [13–14], and membrane separation [5, 15]. The unique features of MIP, in particular, high selectivity and physicochemical stability, have led to their fast development. Molecular imprinting is now maturing from a subject of academic interest to a practical technique of analytical chemists [16].

Capillary electrochromatography (CEC) is a hybrid separation technique combining the conventional stationary phase of liquid chromatography with the electroosmotically driven mobile phase characteristic of electrophoresis. CEC provides high separation efficiency, short separation times, and minimal consumption of packing materials, solvents, and samples. This leads to a cost-effective and environmentally friendly separation technique with high efficiency, high selectivity, and the ability to analyze samples available only in limited amount. The

utility of the MIP-based technology in CEC is still limited [16–18]. There are several methods of preparing MIP capillaries: (1) conventional MIP particles packed into a capillary with a retaining frit [19–22] or immobilized by a “trapping” medium to hold the particles in the column [23, 24]; (2) *in situ* preparation by a dispersive polymerization method [25]; (3) MIP is synthesized as a thin film *in situ* and attached to the capillary wall by covalent bonding [26, 27]; and (4) capillaries filled with a monolithic, imprinted polymer obtained by an *in situ* photo-initiated polymerization process [28–31]. Also, MIP particles have been used as a mobile phase additive for enantiomeric separations in the CEC mode [32]. Most of the research articles published to date have been focused on the preparation of MIP stationary phases within fused-silica capillaries. Little work has been done on the optimization of separation parameters, particularly for ionizable compounds.

In this paper, we report the preparation and evaluation of a 4-aminopyridine (4-AP) imprinted, polymer-based monolithic capillary column. Our goal is to investigate the effectiveness of the capillary column in CEC as well as the effects of experimental parameters on the separation, such as pH value, salt concentration of the buffer, the content of the organic modifier in the mobile phase, and applied voltage. The MIP-CEC results demonstrated highly specific recognition of the imprinted molecule. The migration and separation of the imprinted molecule and its structural isomer are found to be greatly affected by the experimental parameters. These results indicated that in the separation process there were triple retention/migra-

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tion mechanisms of molecular imprinting, ion exchange, and electrophoresis. These interplaying retention/migration mechanisms allow a high degree of control of electrochromatographic resolution.

2 Experimental

2.1 Chemicals and materials

Fused-silica capillaries of 100 μm ID and 375 μm OD were purchased from Hebei Yongnian Optical Fiber Factory (Yongnian, Hebei, China). The chemical reagents were purchased from the sources indicated in parentheses: 3-(trimethoxysilyl) propyl methacrylate (Acros, Geel, Belgium); 4-aminopyridine (4-AP) and 2-aminopyridine (2-AP) (Sigma-Aldrich, St. Louis, MO, USA); methacrylic acid (MAA) (Beijing Donghuan Chemical Reagent Factory, Beijing, China); ethylene glycol dimethacrylate (EGDMA) (Suzhou Anli Chemical & Engineering Co. Ltd., Suzhou, China); 2,2'-azobis (2-isobutyronitrile) (AIBN), (Special Chemical Reagent Factory, Nankai University, Tianjin, China). Other analytical reagents such as thiourea were purchased from Tianjin Chemical Reagent Co. Lt. (Tianjin, China). Acetic acid (HOAc), sodium acetate (NaOAc) and acetonitrile were HPLC grade.

2.2 Column preparation

A fused-silica capillary was silanized with 3-(trimethoxysilyl)propyl methacrylate, according to the procedure published by H \acute{e} rt \acute{e} n [33]. A mixture of 4-AP (0.25 mmol), MAA (1 mmol), EGDMA (5 mmol) and initiator AIBN were dissolved in proper volume of acetonitrile. The mixture was degassed for 10 min by ultrasonication. A 40-cm length capillary was attached to a syringe and filled with the degassed polymerization mixture to a length of 13 cm. After each end was plugged with a piece of rubber, the capillary was submerged in a 60°C bath for 12 h. Subsequently, the column was moved out of the water bath and immediately washed with 5% acetic acid in acetonitrile and acetonitrile, respectively. A detection window was created near the end of the continuous polymer bed by burning off a 2 mm segment of the polyimide outer coating. Both ends of the capillary were cut off to fit a Beckman P/ACE system MDQ cartridge. The final capillary column had a total length of 31.2 cm and the effective length with MIP-based stationary phase was 10 cm.

2.3 Capillary electrochromatography: apparatus and procedure

CEC separation was performed on a P/ACE system MDQ capillary electrophoresis apparatus (Beckman-Coulter, Fullerton, CA, USA) equipped with a diode array detector.

The retention or the migration time was measured in triplicate. The relative standard deviation was less than 5%.

The capillary column was installed, in a reverse mode, in a Beckman P/ACE system MDQ cartridge and then inserted into the P/ACE system MDQ instrument. The section of the capillary (10 cm) filled with the polymer stationary phase is on the "outlet" side in order to minimize the dead volume (the detection window is located at 10 cm from the outlet end). The polarity of the applied voltage of the instrument was also reversed (positive electrode in the "outlet" vial). The column was first rinsed with mobile phase under 20 psi (ca. 138 kPa) pressure in the "outlet" vial, and then equilibrated with a mobile phase at a voltage of 15 kV under 20 psi in both inlet and outlet vials until a constant baseline was obtained. The column temperature was set at 25°C. The sample was injected by applying 0.5 psi (3.45 kPa) pressure for 3 seconds in the "outlet" vial. Separations were carried out by applying certain voltages with 20 psi pressure at both ends. The electrolyte consisted of acetonitrile with varied amounts of acetate buffer at various pH values. The samples were prepared at 10 mM concentration in water and then diluted with water to the desired concentrations. All the buffer and sample solution were made using doubly distilled water, and filtered before use through a 0.2 μm porosity membrane.

2.4 Capillary electrochromatography: equations

The EOF velocity depends on the density of charges on the polymer matrix as well as the properties of the electrolyte. The relation between the electroosmotic velocity, v_{eof} , and zeta potential, ζ , is given by [34]:

$$v_{\text{eof}} = \frac{\epsilon \epsilon_{\text{eo}} \zeta}{\eta}$$

where E is the electric field strength, ϵ_{eo} is the dielectric constant of free space, ϵ the dielectric constant of the medium, and η the viscosity of the medium.

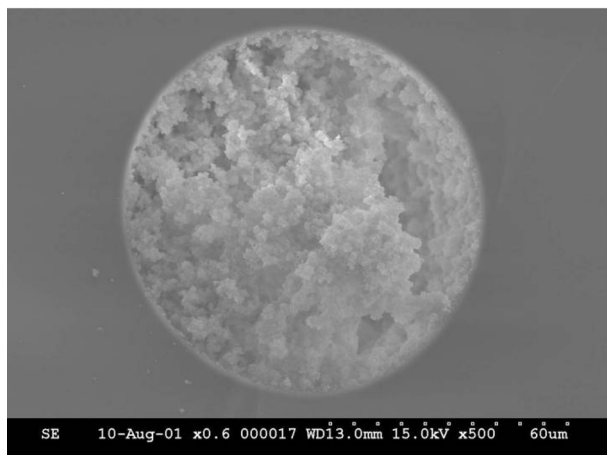
The resolution factor, R_s , was calculated by a slightly modified method: a straight line was drawn from the peak maximum to the baseline, and the baseline bandwidth divided into two halves [35]. Since the peaks were asymmetric, only the baseline half bandwidth adjacent to the next peak was selected, referred to as $w_1^{1/2}$ or $w_2^{1/2}$ and the resolution was defined as $R_s = (t_2 - t_1)/(w_1^{1/2} + w_2^{1/2})$.

3 Results and discussion

3.1 Column preparation and characterization

In this study, molecular imprinting of 4-AP in methacrylic-type polymers was performed in situ by a thermally initiated polymerization process in a 100 μm silanized fused-silica capillary. The morphology of the resultant

A)



B)

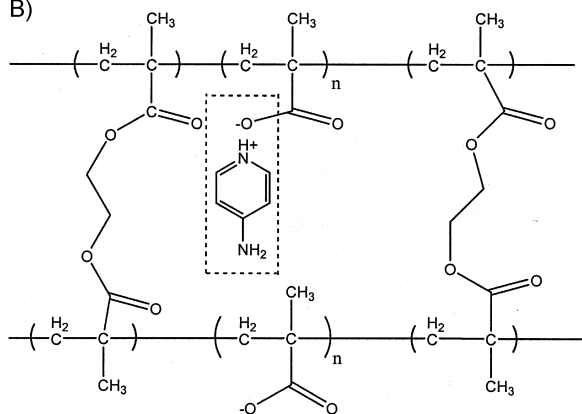


Figure 1. Scanning electron micrograph of the 4-AP MIP based capillary (A) and structure of polymer matrix (B).

polymer was a porous monolith. **Figure 1.A** shows the electron micrograph, which indicates that there were aggregates of micrometer size globular particles throughout the capillary. The aggregates were surrounded by 0.1–5 μm interconnected pores that permitted bulk flow through the capillary. The polymer was attached covalently to the inner wall by a bifunctional reagent, 3-(trimethoxysilyl)propyl methacrylate. The inner wall of the capillary was thus also coated with the MIP and the silanol groups of the capillary were either sterically shielded or chemically modified. The column had good permeability since it could be easily rinsed under 20 psi pressure.

The stationary phase in the capillary column in CEC has two main functions: (1) to generate electroosmotic flow (EOF) of the mobile phase upon applying electric field to the column and (2) to selectively interact with the analytes.

The EOF was generated by the carboxylic acid ($-\text{COOH}$) functional groups on the 4-AP MIP matrix that can be

deprotonated at an appropriate pH. This will be discussed in detail in the following sections. Because of the carboxylic acid functionality, the polymeric matrix is essentially a weak cationic exchanger, as demonstrated in **Figure 1.B** and in the following sections.

The selectivity originated from the “imprinting” recognition. In MIP, the shape, size, and chemical function of the cavities are complementary to the imprinting molecule. Thus, these cavities give rise to a specific affinity for the imprinting molecule (the “template molecule”) during the CEC separation process and it is always more strongly retained than its structural analogues (the “non-template molecule”) when non-specific interactions between all of these structurally similar molecules and the MIP stationary phase are the same. In this study, the MIP cavities were created by the specific ionic interaction and hydrogen bonding between the carboxylic acid groups on the polymeric matrix and the 1-N atom in the pyridine ring and the amino group of the imprinted 4-AP molecules. The mixture of 4-AP and its structural isomer, 2-AP, was used in the demonstration of the selectivity.

3.2 Mobile phase

Acetonitrile was chosen as an organic modifier in the mobile phase. Methanol was also tested. However, the separation was not as good as with acetonitrile (data not shown). As in the HPLC mode [36, 37], it was advantageous to use the same electrolyte solvent for CEC as for the polymerization process. In addition, acetonitrile is an aprotic solvent. It does not adversely interfere with the ionic and hydrogen bonds between the imprinted molecule and the carboxylic acid group on the MIP.

3.3 EOF

The transport of mobile phase in CEC is achieved by EOF. The origin of this flow is the electrical double layer that is formed at the solid-liquid interface of a charged surface in contact with an electrolyte solution. In the 4-AP MIP-based capillary, the carboxylic acid groups on the polymer matrix are negatively charged at certain pHs, typically higher than 4.5, the estimated $\text{p}K_{\text{a}}$ of the carboxylic acid group (based on MAA). Consequently, the solution at the interface bears a net positive charge. When an electrical field is applied to the column the ions migrate towards the cathode, moving the bulk solution by viscous drag.

3.4 Effect of applied voltage

CEC separations were performed at 15, 13, 12, 10, and 7 kV, corresponding to the field strength of 480, 420, 390, 320, and 225 V/cm, respectively. The EOF mobility was

determined from the elution time of thiourea, a neutral marker. The EOF increased approximately linearly with increasing voltage from 7 to 15 kV with the mobile phase of HOAc-NaOAc (0.01 M, pH 6.0)/acetonitrile (10/90) (data not shown). This indicates that the Joule heating is not significant in this system under the experimental conditions, which may be due to the large ratio of acetonitrile (90%) in the mobile phase and the low salt concentration (10% of the 10 mM buffer). The current was only 1.85 mA at 15 kV. The limited Joule heat was sufficiently removed by the liquid cooling system.

The separation of 4-AP and 2-AP on the 4-AP MIP-based column was achieved at different voltages: 15, 13, 12, 10, 7 kV. As expected the migration time decreased with increasing applied voltage.

3.5 Effect of salt concentration of the buffer

The effect of salt concentration was studied using different ionic strength of electrolyte, 0.01, 0.02, 0.04, 0.08, 0.1 M HOAc-NaOAc (pH 6.0)/acetonitrile (10/90 v/v). The EOF velocity was decreased by increasing the salt concentration at a constant content of acetonitrile (90%). This trend can be explained by the relatively smaller thickness of the electrical double layer at higher ionic strength. The EOF velocity is plotted against the reciprocal of the square root of salt concentration (**Figure 2**). The EOF velocity increases linearly with the reciprocal of the square root of the salt concentration in the 0.01–0.1 M range ($v_{\text{eof}} = 0.1089/I^{1/2} + 1.1414$, $R^2 = 0.9912$), demonstrating the normal electrophoretic mechanism in the migration process.

The experimental results showed that the effect of ionic strength on the migration time and retention factor of the 4-AP and 2-AP was profound, demonstrated by the resolution factor R_s in **Figure 3**. The migration time of non-imprinting molecule 2-AP was hardly affected, while imprinting molecule (4-AP) was greatly affected by the salt concentration of the buffer, especially in the low concentration range. After correction of the EOF dependence on the salt concentration, the migration and retention factor of 4-AP increased sharply with the decrease of the ionic strength when the buffer concentration was below 0.02 M (**Figure 3**). This trend is similar to that observed in HPLC [38]. It indicates that chromatographic retention based on cationic exchange plays a vital role in the separation.

The pK_a of 4-AP and 2-AP are 9.2 and 6.8, respectively (Beilstein). When the acetonitrile content was as high as 90% in the mobile phase, the 4-AP remained fully protonated in the mobile phase (HOAc/NaOAc, pH 6.0: acetonitrile 10/90). Thus, the retention of 4-AP followed the traditional cationic-exchange chromatographic pattern, that

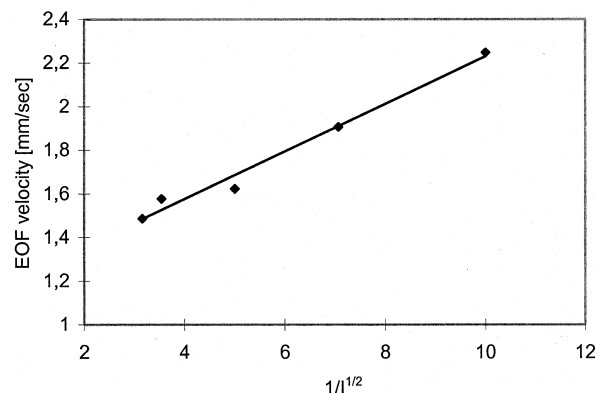


Figure 2. Effect of salt concentration on EOF: the EOF velocity versus the reciprocal of the square root of salt concentration. 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μm ID]; electrolyte: HOAc-NaOAc (pH 6.0)-acetonitrile (10/90, v/v) mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s (reverse); sample concentration: 2 mg/mL.

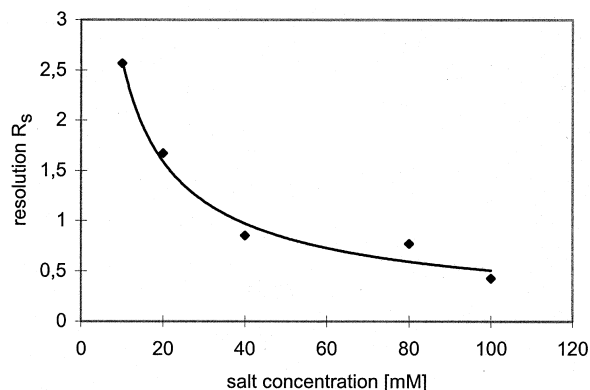


Figure 3. Effect of salt concentration on resolution. 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μm ID]; electrolyte: HOAc-NaOAc (pH 6.0)-acetonitrile (10/90, v/v) mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s (reverse); sample concentration: 2 mg/mL.

is, the higher the salt concentration, the higher the elution strength of the mobile phase, and hence, the shorter the retention or faster the migration of the cationic analytes. On the other hand, 2-AP's pK_a (6.8) is significantly lower than that of 4-AP (9.2). Under similar conditions, 2-AP remained unprotonated or neutral in 90% acetonitrile. Thus, the 2-AP did not follow the cationic exchange retention mechanism and the salt concentration had little effect on its migration or retention. Although the relationship between the retention based on the contribution of the ionic interaction in the molecular imprinting mechanism and the salt concentration is very similar to that of the cationic exchange, it is unclear that the fingerprint type of recognition such as "shape recognition" did play a role in the retention based on this set of data. The proof of selec-

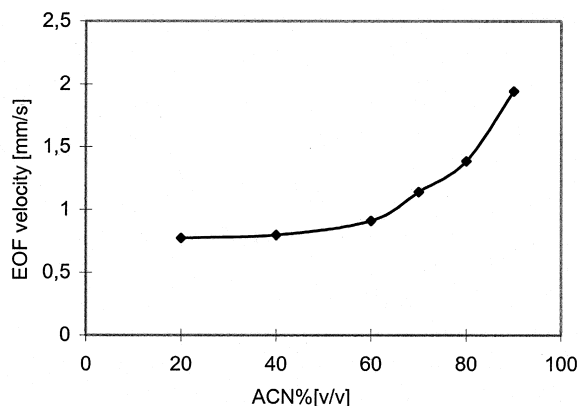


Figure 4. Effect of acetonitrile content (ACN%) on EOF: the EOF velocity versus the percentage of ACN. 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μ m ID]; electrolyte: HOAc-NaOAc (pH 6.0)-acetonitrile mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s (reverse); sample concentration: 2 mg/mL.

tivity based on true molecular imprinting is demonstrated in the following sections.

3.6 Effect of organic modifier

The EOF was influenced by the percentage of organic modifier in the mobile phase. **Figure 4** shows the plot of the EOF velocity versus the acetonitrile content. In this set of experiments, the acetonitrile content (v/v) was varied from 20% to 90%. The EOF increased from 0.8 mm/s to 1.9 mm/s when the acetonitrile content increased from 20% to 90%. Such an EOF dependence on the acetonitrile content can be explained from two sides: 1) The increase of acetonitrile decreases the viscosity of the mobile phase; 2) the increase of acetonitrile decreases the buffer concentration or the ionic strength. This effect is very similar to that of the salt concentration in the buffer on the EOF described previously.

The migration times of 4-AP and 2-AP were very different at different contents of acetonitrile, as shown in **Figure 5**. The migration time of 4-AP decreased rapidly as the acetonitrile content decreased. This can be explained by the cationic exchange mechanism discussed previously. Over the full range of the acetonitrile content studied in **Figure 5**, 4-AP remained protonated in the presence of the acetate buffer of pH 6.0. It should be noted that the real pH of the acetonitrile/buffer mixture was unknown. The decrease of acetonitrile increased the buffer concentration or the elution strength. Thus, the migration time or the retention time decreased with the acetonitrile content. It should also be noted that the dissociation of the carboxylic acid groups on the stationary phase is also influ-

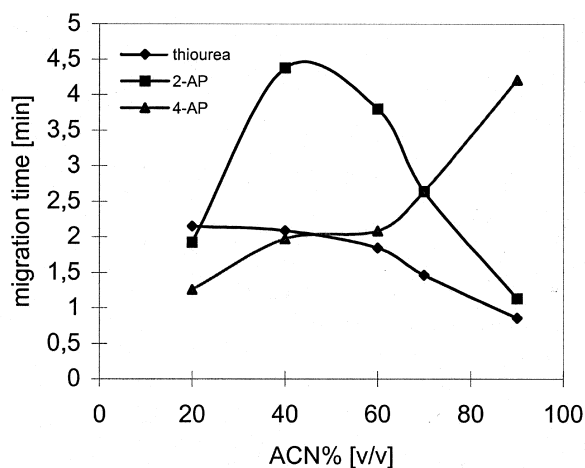


Figure 5. Effect of acetonitrile content on the migration time, 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μ m ID]; electrolyte: HOAc-NaOAc (0.01 M, pH 6.0)-acetonitrile mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s; sample concentration: 2 mg/mL.

enced by the content of the organic modifier, which can affect the EOF.

The retention or migration of 2-AP was influenced by two counter-acting mechanisms: 1). The varied degree of ionization in the mobile phase mixture of a varied amount of acetonitrile or the buffer: the increase of the buffer concentration increases the ionization of 2-AP and hence increases the ionic interaction with the carboxylic acid groups on the stationary phase. 2). The varied elution strength of mobile phase as a result of the varied amount of acetonitrile or the buffer. As acetonitrile content was decreased from 90% to 20%, 2-AP became more ionized (partially protonated) and interacted more strongly with the stationary phase via the cationic-carboxylic group interaction mechanism. The retention time or migration time would be longer. However, on the other hand, the elution strength of the mobile phase was increased and the retention/migration time was reduced. The counter-balance of these two factors resulted a parabolic profile of the retention/migration time dependence on the content of acetonitrile.

3.7 Effect of pH value and retention by molecular imprinting

The effect of pH of the buffer on the separation was studied in HOAc-NaOAc (0.01 M)/acetonitrile (10/90) mobile phase. **Figure 6** shows the migration time of 4-AP, 2-AP, and thiourea at various pH values of the buffer.

The pH-migration time profile of 4-AP showed a classical sigmoid shape (**Figure 6**). The apparent pK_a of 4-AP could be estimated from this curve to be approximately 5.8,

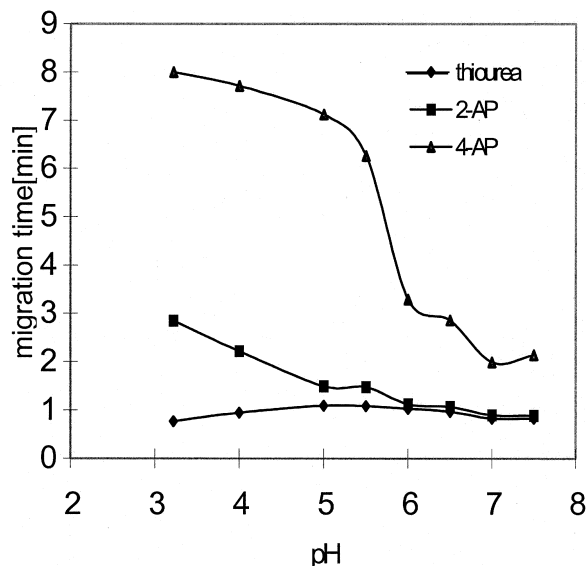


Figure 6. Effect of pH of the buffer on the migration time, 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μ m ID]; electrolyte: HOAc-NaOAc (0.01 M)-acetonitrile (10/90, v/v) mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s (reverse); sample concentration: 2 mg/mL.

downshifted by 3.4 units from 9.2 in a purely aqueous solution. At lower pH values (< 5), 4-AP was fully protonated and the retention process followed a cationic exchange mechanism. It was noted with interest that the EOF was largely independent of the pH (from pH 3 to pH 7.5) based on the migration time of thiourea, suggesting that the pK_a of the carboxylic groups on the stationary phase could be lower than the theoretical value of 4.5 in the mobile phase of 90% acetonitrile. In other words, the apparent pK_a of the carboxylic acid groups in 90% acetonitrile would be downshifted by at least 1.5 units to below 3. The alternative explanation is that the pH of the ACN/buffer mixture is higher than the apparent pH (3–7.5), at which the carboxylic groups (pK_a 4.5) fully deprotonate.

At higher pH values (> 7), 4-AP was largely unprotonated. Thus the retention of 4-AP followed the molecular imprinting mechanism. The molecular recognition was significant when compared with the retention/migration profiles of thiourea and 2-AP at the same pH of 7 and 7.5. At these high pHs, all molecules were largely neutral. Hence, the forces that differentiate 2-AP and 4-AP arose from molecular imprinting. The resolution of 4-AP from 2-AP via the imprinting mechanism is demonstrated in **Figure 7** (pH 7.5).

The pH-migration time profile of 2-AP showed that the retention of 2-AP was largely driven by ionization. Assuming the same solvent effect on the pK_a downshift (3.5) as in the case of 4-AP, the apparent pK_a of 2-AP in 90% acetonitrile would be $6.8 - 3.5 = 3.3$. Thus, 2-AP would

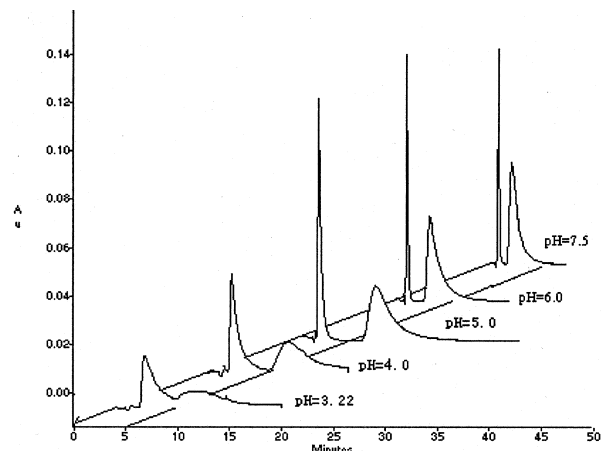


Figure 7. Electrochromatogram of 2-AP (first peak, less retained) and 4-AP (second peak, more retained) at various pH on a 4-AP molecular imprinted polymer-based capillary column [31.2 cm (10 cm packed) 100 μ m ID]; electrolyte: HOAc-NaOAc (0.01 M)-acetonitrile (10/90, v/v) mixture; temperature: 25°C; voltage: 15 kV; UV detection: 246 nm; injection: 0.5 psi, 3 s (reverse); sample concentration: 2 mg/mL.

remained unprotonated across the pH range of (5–7.5) because, unlike 4-AP, 2-AP lacked the specific “imprint” and it did not follow the retention mechanism of the molecular imprinting, it had a migration or retention pattern nearly identical to the neutral marker thiourea in the pH range of 5–7.5. In the low pH range (3–5), 2-AP became partially ionized and the retention time became longer than thiourea.

4 Conclusion

The molecular imprinted polymer-based monolithic column was prepared by in situ thermally initiated polymerization for 4-aminopyridine, an ionizable basic compound. The capillary column was evaluated under various conditions in the capillary electrochromatography mode. The mechanism of the separation is based on the interplay among molecular imprinting recognition, ion-exchange chromatographic retention, and electrophoretic migration. Studies on other classes of compounds and further investigation of the molecular imprinting mechanism are in progress.

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