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Modeling and optimization for separation of ionic solutes in pressurized flow capillary electrochromatography

By manipulation of applied pressure or voltage, pressurized flow capillary electrochromatography (P-CEC) permits unique control of selectivity for ionic solutes. A simple mathematical model has been developed to describe the quantitative relationship between the electrochromatographic retention factor (k^*) of charged solutes and the applied voltage and pressure. The validity of the model was verified experimentally with hydrophilic interaction mode CEC (HI-CEC). On the basis of the model developed, it was found that the value of k^* could be predicted accurately using only a limited number of data points from the initial experiments at different voltages or pressures. Correlation between the experimentally measured and calculated k^* was excellent, with a correlation coefficient greater than 0.999. Optimization for the separation of peptides by P-CEC was also performed successfully on the basis of the proposed model.

Key Words: Pressurized flow; Capillary electrochromatography; Theoretical model

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1 Introduction

Capillary electrochromatography (CEC) is a hybrid technique which combines the advantages of capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) [1–3]. The chief benefits of this new technique are its high separation efficiency, which is often superior to that of HPLC because of the flat profile of electroosmotic flow (EOF) in CEC, and the possibility of combining electrophoretic and chromatographic mechanisms. However, some limitations are apparent when only EOF is used as the driving force. First, the magnitude of EOF is determined by the charge density on the surface of packing material; low charge density will result in low EOF and long analysis time. Secondly, charged solutes migrating against the EOF may have too low a velocity to elute in a reasonable time. Accordingly, it has been recommended that acidic compounds be analyzed in the ion suppressed mode in reversed-phase CEC at a low pH value in order to eliminate the counter migration of anions with EOF [4]. Finally, CEC experiments may often be interrupted by the formation of bubbles in the capillary.

Many of the problems encountered in CEC with EOF as the only driving force can be minimized or eliminated if

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pressure is applied to one side of the capillary column during the separation [5]. For example, bubble formation could be easily suppressed in P-CEC [6, 7]. This mode of CEC is often called pressurized flow CEC (P-CEC) [8–10]. However, in P-CEC the parabolic flow profile of pressurized flow is superimposed on the plug-like flow profile of EOF, reducing the column efficiency in P-CEC compared with pure CEC [8].

The retention and separation selectivity of ionic solutes can be manipulated by changing the applied voltage or pressure in P-CEC [9–13]. Theory has been developed to explain selectivity tuning in P-CEC; however, the effect of the applied voltage or pressure on the retention of solutes has not been investigated in detail [9, 10, 11, 14]. The retention time of a cationic analyte in P-CEC was calculated from the chromatographic capacity factor and the electrophoretic mobility; however, the EOF effect was not taken into account in this model [9]. Due to the difficulty of quantitatively describing the migration behavior of analytes in P-CEC, an artificial neural network was applied to optimize the separation of ionic solutes in P-CEC [8]. In this paper, a mathematical model is developed to relate the electrochromatographic retention factor (k^*) of ionic solutes to the applied voltage and the applied pressure in P-CEC. The validity of the model was verified experimentally. The developed model was also applied to optimize the separation of peptides in P-CEC.

2 Theory

The linear velocity of a charged solute in CEC (u_{mig}) can reasonably be described as a weighted average velocity in the mobile phase (u_{m}) and stationary phase (u_{s}) [3, 14], and can be given as

$$u_{\text{mig}} = F_{\text{s}}u_{\text{s}} + F_{\text{m}}u_{\text{m}} \quad (1)$$

where F_{s} and F_{m} are the fractions of the charged solute in the stationary phase and mobile phase, respectively. It is assumed that the charged solute migration in the stationary phase due to the electrical field is much slower than that in the mobile phase; therefore u_{s} could be neglected and Eq. (1) simplified to:

$$u_{\text{mig}} = F_{\text{m}}u_{\text{m}} \quad (2)$$

According to the definition of capacity factor (k') in HPLC, the fraction of solute in the mobile phase can be expressed as:

$$F_{\text{m}} = 1/(1 + k') \quad (3)$$

In P-CEC, the linear velocity of a charged solute in the mobile phase (u_{m}) is the sum of the velocities contributed from the electrophoretic mobility (u_{ep}), pressurized flow (u_{p}), and electroosmotic flow (u_{eo}), respectively, and thus is given by

$$u_{\text{m}} = u_{\text{eo}} + u_{\text{ep}} + u_{\text{p}} = \mu_{\text{eo}}E + \mu_{\text{ep}}E + u_{\text{p}} \quad (4)$$

where μ_{eo} and μ_{ep} are the electroosmotic mobility and electrophoretic mobility, and E is the applied electrical field strength. Substituting Eqs. (3) and (4) into Eq. (2), the following equation is obtained:

$$u_{\text{mig}} = \frac{\mu_{\text{eo}}E + \mu_{\text{ep}}E + u_{\text{p}}}{1 + k'} \quad (5)$$

The electrochromatographic retention factor (k^*) is defined as [11, 14–16]:

$$k^* = (t_{\text{r}} - t_0)/t_0 \quad (6)$$

where t_{r} and t_0 are the migration times of the charged solute and unretained neutral solute, respectively, and they can be expressed by

$$t_{\text{r}} = L_{\text{id}}/u_{\text{mig}} \quad (7)$$

$$t_0 = L_{\text{id}}/(u_{\text{eo}} + u_{\text{p}}) \quad (8)$$

where L_{id} is the length of capillary from injection end to detection window. Combining Eqs. (6) – (8), we have

$$k^* = (u_{\text{eo}} + u_{\text{p}})/u_{\text{mig}} - 1 \quad (9)$$

Substituting Eq. (5) into Eq. (9) yields:

$$k^* = \frac{k'(\mu_{\text{eo}} + u_{\text{p}}/E) - \mu_{\text{ep}}}{\mu_{\text{eo}} + u_{\text{p}}/E + \mu_{\text{ep}}} \quad (10)$$

This equation is identical to that reported by Wu et al. [11] but in a simpler form. In the case of CEC without pressurized flow, u_{p} equals zero, and Eq. (10) reduces to

$$k^* = \frac{k' - \mu_{\text{ep}}/\mu_{\text{eo}}}{1 + \mu_{\text{ep}}/\mu_{\text{eo}}} \quad (11)$$

The electrical field E term does not appear in Eq. (11). If it is assumed that the electric field has no influence on k' , μ_{eo} , and μ_{ep} , then the k^* value does not depend on the electric field in CEC without pressurized flow. Accordingly, it is impossible to tune the retention of solutes by the applied electrical field only. In practice, slight changes in retention of solutes at different electrical fields could possibly occur due to the column temperature changes caused by Joule heating. However, in a P-CEC system, as can be seen from Eq. (10), the k^* value is no longer independent of the applied electrical field. Furthermore, the applied pressure is also available to adjust the k^* value. Therefore, the tuning of the elution can be achieved by properly adjusting the applied electrical field and the pressure.

Influence of E and u_{p} on the k^* values of a hypothetical solute at different parameters in P-CEC are calculated according to Eq. (10). It is assumed that the EOF is to the cathode in this system, and the results obtained are shown in **Figure 1** and **Figure 2**, respectively. For neutral solutes, $\mu_{\text{ep}} = 0$, therefore $k^* = k'$ according to Eq. (10). As shown in Figure 1 and Figure 2, E and u_{p} have no influence on the migration behavior of neutral solutes. However, the situation for charged solutes is different. As shown in Figure 1, the k^* value of anionic solutes (solute 1, 2, and 3) increases, but that of cationic solutes (solute 5, 6, and 7) decreases with increasing E . The elution of an anionic solute from the column will be decelerated because its electrophoretic mobility is opposite that of the solvent flow. At a given applied pressure, the contribution of the electrophoretic mechanism to the migration of anionic solutes increases and accordingly the k^* values in P-CEC increase with increasing strength of the applied electrical field. The effect of the electrical field on cationic solutes is different from that on anionic solutes because electrophoretic mobility will accelerate their elution from the column, which will result in a decreasing k^* value. As shown in Figure 1, solutes with the same k' value (solute 1, 3, 5, and 7) cannot be separated in liquid chromatography (LC); however, they can be separated in P-CEC

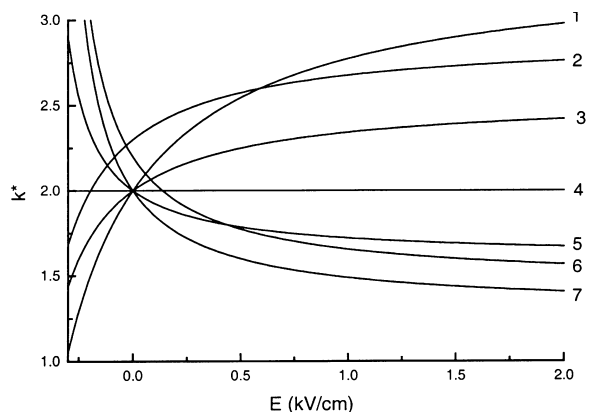


Figure 1. Numerical simulation by Eq. (10) of the effect of electrical field (E) on the k^* values of ionic solutes in P-CEC. Parameters: $u_p = 6.5$ cm/min, $\mu_{eo} = 13$. (1) $k' = 2$, $\mu_{ep} = -2$; (2) $k' = 2.3$, $\mu_{ep} = -2$; (3) $k' = 2$, $\mu_{ep} = -2$; (4) $k' = 2$, $\mu_{ep} = 0$; (5) $k' = 2$, $\mu_{ep} = 2$; (6) $k' = 2.2$, $\mu_{ep} = 4$; (7) $k' = 2$, $\mu_{ep} = 4$. μ_{eo} and μ_{ep} are in units of $\text{cm}^2/\text{min kV}$.

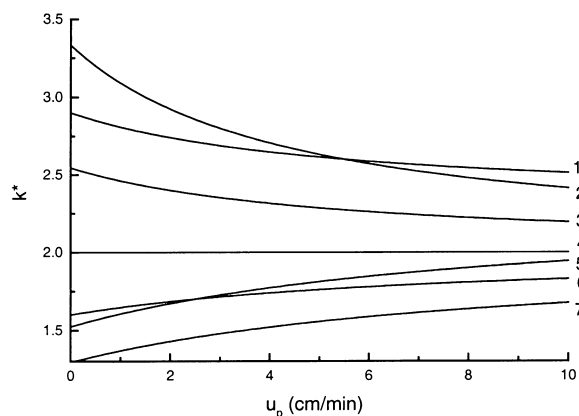


Figure 2. Numerical simulation by Eq. (10) of the effect of the pressurized flow velocity (u_p) on the k^* values of ionic solutes in P-CEC. Parameters: $E = 0.5$ kV/cm, $\mu_{eo} = 13$. (1) $k' = 2.3$, $\mu_{ep} = -2$; (2) $k' = 2$, $\mu_{ep} = -4$; (3) $k' = 2$, $\mu_{ep} = -2$; (4) $k' = 2$, $\mu_{ep} = 0$; (5) $k' = 2.3$, $\mu_{ep} = 4$; (6) $k' = 2$, $\mu_{ep} = 2$; (7) $k' = 2$, $\mu_{ep} = 4$. μ_{eo} and μ_{ep} are in units of $\text{cm}^2/\text{min kV}$.

because of their different electrophoretic mobilities. Similarly, solutes with the same electrophoretic mobility (solute 2 and 3, and solutes 6 and 7) cannot be separated in capillary zone electrophoresis (CZE), but can be separated in P-CEC. Therefore, P-CEC is a complementary technique to LC and CZE. The extent of the influence of the electric field on the k^* value of charged solutes may be different due to their different k' and/or μ_{ep} , and therefore the separation selectivity can be adjusted by changing the applied voltage.

It can be seen from Figure 2 that the k^* value of anionic solutes decreases and that of cationic solutes increases with increasing u_p . This can be explained by a decreasing contribution of electrophoretic mechanisms to the migra-

tion with an increase in applied pressure. This also results in decreasing retention of anionic solutes and increasing retention of cationic solutes. Figure 2 also demonstrates that the separation selectivity can be tuned by changing u_p .

Unlike in capillary zone electrophoresis and microscale high-performance liquid chromatography, the CEC columns are unique as they consist of a packed and an open capillary segment having different conductances and consequently different electric field strength [17, 18]. Due to conservation of volumetric flow rate, in most case an intersegmental pressure develops at the interface of the packed and the open segments because EOF has different velocities in these two sections [17]. However, the packed segment is much less permeable than the open segment; the flow velocity in the packed segment is not significantly influenced by the pressure gradient [17]. In other words, the effect of EOF in the open section on the overall flow velocity could be ignored. Since, in columns most commonly used in CEC at present, the detection window is located right after the packing, the chromatograms will not be affected by the open section. Therefore, the effect of the open section on separation was not considered in this study. The electrical field E in Eq. (10) is that applied across the packed section. Since the open section and the packed section are coupled in series, E should be proportional to the voltage applied to the total column (V_{applied}), and can be expressed as

$$E = K_V V_{\text{applied}} \quad (12)$$

where K_V is a constant. Substituting Eq. (12) into Eq. (10), after rearrangement we have

$$k^* = \frac{k' + \frac{(k'\mu_{eo} - \mu_{ep})K_V}{u_p} V_{\text{applied}}}{1 + \frac{(\mu_{eo} + \mu_{ep})K_V}{u_p} V_{\text{applied}}} = \frac{B_V + C_V V_{\text{applied}}}{1 + A_V V_{\text{applied}}} \quad (13)$$

It is assumed that Joule heating is not significant, therefore μ_{eo} , μ_{ep} , and k' should be constant with a given column and mobile phase. The u_p value is kept constant at a given applied pressure. Then the parameters in Eq. (13), A_V , B_V , and C_V , are constant. The k^* value at different voltages can be predicted according to Eq. (13). Since Eq. (13) contains only three parameters, these parameters can be determined by a minimum of three experimental data points.

Similarly, the pressurized flow velocity u_p is proportional to the applied pressure P_{applied} , and we have

$$u_p = K_P P_{\text{applied}} \quad (14)$$

where K_p is a constant. Substituting Eq. (14) into Eq. (10) gives

$$k^* = \frac{\frac{(k'\mu_{eo} - \mu_{ep})}{(\mu_{eo} + \mu_{ep})} + \frac{k'}{(\mu_{eo} + \mu_{ep})E} K_p P_{\text{applied}}}{1 + \frac{1}{(\mu_{eo} + \mu_{ep})E} K_p P_{\text{applied}}} = \frac{B_p + C_p P_{\text{applied}}}{1 + A_p P_{\text{applied}}} \quad (15)$$

Analogous to Eq. (13), A_p , B_p , and C_p also should be constant at a given applied voltage if Joule heating is not significant. The k^* value at a specific pressure can be predicted according to Eq. (15).

The resolution for two peaks is given by

$$R_s = \frac{t_{r(2)} - t_{r(1)}}{w_{1/2}} \quad (16)$$

where $t_{r(2)}$, $t_{r(1)}$ are the migration times of the two peaks and $w_{1/2}$ is the average peak width at half peak height. According to the definition of k^* and column efficiency, Eq. (16) can be expressed as the following:

$$R_s = \left(\frac{t_{r(2)}}{t_{r(1)}} - 1 \right) \frac{t_{r(1)}}{w_{1/2}} = \frac{k_2^* - k_1^*}{1 + k_2^*} \sqrt{\frac{N_1}{5.54}} \quad (17)$$

where k_1^* and k_2^* are the electrochromatographic retention factors of the solutes and N_1 is the column efficiency for the first peak.

3 Experimental section

3.1 Materials

Peptides used in this study were purchased from Serva Feinbiochemica Company (Heidelberg, Germany). Acetonitrile was of chromatographic grade. Other reagents were analytical reagent grade. Ultra-pure water used for preparing solutions was produced by a Milli-Q water system (Millipore Corp., Bedford, MA, USA). Stock solutions of triethylamine phosphate (TEAP), 500 mM in phosphate, was prepared by addition of triethylamine to a solution of phosphoric acid till pH 3.0 or 2.8 was obtained. The TEAP stock solutions were filtered through a 0.45 μm membrane. Mobile phases were prepared by addition of the required volume of acetonitrile and TEAP buffer to a volumetric flask, followed by addition of water to 1 mL below the scale marker. Then the flask was placed in a sonicator bath for 5 min. Water was then added to the scale marker after the solution reached room tempera-

ture. Before a run, the mobile phase was degassed in an ultrasonic bath for 20 minutes, and returned to room temperature.

3.2 Column preparation

Fused silica capillary (75 μm ID, 365 μm OD) was obtained from Yongnian Optic Fiber Plant (Hebei, China). PolyHYDROXYETHYL A (5 μm , 300-Å) was a gift from PolyLC (Columbia, MD, USA). CEC columns were packed in-house by a slurry packing technique as reported in the literature [19]. All columns were 31 cm long with a packed length of 10 cm. Before a run, the column was first flushed with mobile phase for 10 min with a syringe, then the column was conditioned on the instrument with the mobile phase by applying 620 kPa pressure at the inlet end of the CEC column.

3.3 P-CEC Apparatus

Both P-CEC and LC experiments were performed on a capillary electrophoresis P/ACE system MDQ (Beckman; Fullerton, CA, USA). This instrument can apply gas pressure as high as 690 kPa to one or both ends of the capillary. Supplementary pressure was applied at the inlet end of the CEC column during the separation in this work. Data collection and instrument control were performed with P/ACE System MDQ version 1.5 software.

3.4 Separation conditions

Pressure applied at the inlet end of CEC column varied from 138 kPa to 690 kPa. The voltage applied across the CEC column varied from -2 kV to 5 kV. Positive voltage indicates that the anode was placed at the inlet side and the cathode at the outlet side of the column, while negative voltage indicates the polarity of the electrodes is opposite that with the positive voltage arrangement. Injection was performed by applying 690 kPa pressure at the inlet end of the column for 20 s. The detection wavelength was set at 200 nm. The column temperature was set at 20°C.

If not otherwise stated, the mobile phases contained 60% acetonitrile and 10 mM TEAP buffer (pH 3.0). As with HPLC, toluene was selected to mark the void time [20].

4 Results and discussion

4.1 Hydrophilic interaction CEC

In this work, charged peptides were selected as the test solutes. Peptides have typically been separated by reversed-phase or ion-exchange CEC and P-CEC [11,

16, 21]. Hydrophilic interaction chromatography in essence is a kind of normal-phase liquid chromatography (NPLC) where polar sorbents and apolar mobile phases are used, but is unique regarding the presence of water in the mobile phase [20]. As a variant of NPLC, separation in hydrophilic interaction chromatography depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase, and solutes are eluted in the order of increasing hydrophilicity. Recently, hydrophilic interaction CEC with a strong cation-exchange material, PolySULFOETHYL A, has successfully been applied for separation of highly polar compounds [22]. In order to avoid the strong electrostatic interaction of peptides with the ion-exchange stationary phase, a neutral, polar stationary phase, PolyHYDROXYETHYL A, which was used for separation of peptides in HPLC [20], was adopted in this work. Hydrophilic interaction can be promoted by increasing the organic modifier concentration in the mobile phase, and the typical acetonitrile concentration for separation of peptides is about 80%. However, liquid chromatography (LC) and P-CEC were performed here on the electrophoresis instrument, which has a maximum applied pressure limit of 690 kPa. Therefore, in order to shorten analysis time, relatively weak retention of peptides was preferable. The acetonitrile level should not be so low, however, that solutes are unretained in a pure chromatography mode. Thus, an acetonitrile concentration of 60% was adopted in most separations of this study. **Figure 3** shows a typical chromatogram for separation of six peptides in the LC mode under these conditions. The retention of peptides by the stationary phase was moderate, and the separation was achieved in 14.7 min. The mobile phase of 60% acetonitrile and 10 mM TEAP (pH 3.0) was adopted for most subsequent LC and P-CEC experiments for the sake of analysis time.

One advantage of P-CEC is that bubble formation could be suppressed by applying pressure at the inlet end, thereby providing stable flow of the mobile phase. It was found in this work that the current was not stable when applying voltage of 5 kV across the column without applied pressure, but was stabilized by applying a pressure of 620 kPa. Although PolyHYDROXYETHYL A is a neutral stationary phase, a CEC column packed with this material still could generate electroosmotic flow. **Figure 4** shows the dependency of linear velocity of the mobile phase on the applied voltage and the pressure. It can be seen from Figure 4.a that linear velocity increases with increasing voltage. This means that the generated EOF has the same direction as that of pressurized flow when positive voltage is applied. Therefore, the direction for EOF is from anode to cathode. Accordingly, the packing surface is negatively charged. Presumably this reflects the charge on residual silanols. Peptides were positively charged and their elution accelerated from the column at

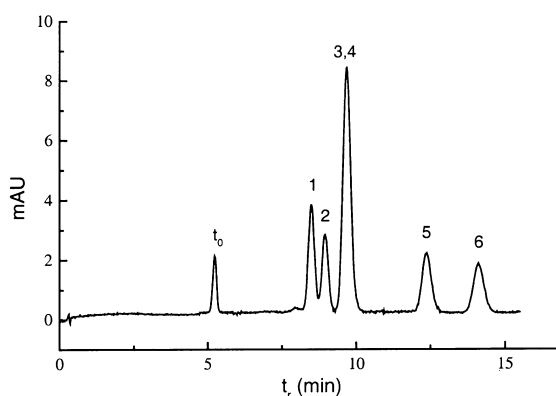


Figure 3. Separation of peptides in hydrophilic interaction chromatography. Conditions: column, 31 cm (with packed length of 10 cm) \times 75 μ m ID capillary packed with PolyHYDROXYETHYL A (5 μ m, 300 Å); mobile phase, 60% acetonitrile in 10 mM TEAP buffer (pH 3.0); hydrodynamic injection, 69 kPa \times 20 s; applied pressure, 620 kPa; applied voltage, 0 kV. Solutes: 1 Gly-PheM; 2 Gly-Ile; 3 Gly-Val; 4 Gly-Tyr; 5 Gly-Thr; 6 Gly-Asp.

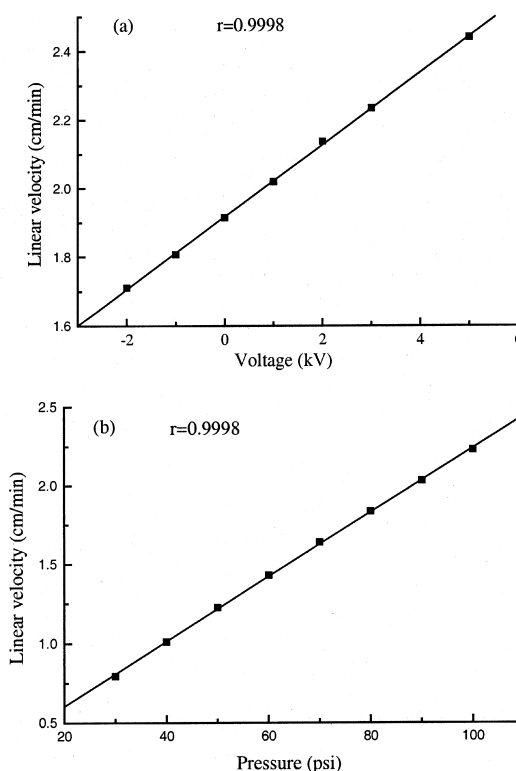


Figure 4. Dependence of linear velocity on the (a) applied voltage and (b) applied pressure. Condition: (a) applied pressure, 620 kPa; applied voltage varied from -2 kV to 5 kV. (b) Applied voltage, 1 kV; applied pressure varied from 207 to 690 kPa. Other conditions as in Figure 3.

the mobile phase pH of 3.0 because their electrophoretic mobility is in the same direction as that of EOF.

A good linear relationship between the linear velocity of the mobile phase and the applied voltage was obtained

Table 1. Comparison of the predicted and experimentally observed k^* values for peptides at different voltages. Conditions: applied pressure, 620 kPa; (a)–(d), mobile phase, 60% acetonitrile in 10 mM TEAP buffer (pH 3.0); Parameters A_v , B_v , and C_v , in Eq. (13) were determined by the k^* values at -2, 2, and 5 kV; (e) mobile phase, 75% acetonitrile in 10 mM TEAP buffer (pH 2.8); Parameters A_v , B_v , and C_v , in Eq. (13) were determined by the k^* values at 0, 1, and 3.5 kV. Other conditions as in Figure 3.

| a) -1 kV | | | | b) 0kV | | | |
|----------|------------|------------|-------|----------|------------|------------|-------|
| Peptides | $k^*(exp)$ | $k^*(pre)$ | RD% | Peptides | $k^*(exp)$ | $k^*(pre)$ | RD% |
| Gly-Phe | 0.840 | 0.835 | -0.64 | Gly-Phe | 0.623 | 0.620 | -0.58 |
| Gly-Ile | 1.084 | 1.087 | 0.26 | Gly-Ile | 0.710 | 0.729 | 2.64 |
| Gly-Val | 1.243 | 1.252 | 0.73 | Gly-Val | 0.851 | 0.879 | 3.25 |
| Gly-Tyr | 1.084 | 1.087 | 0.27 | Gly-Tyr | 0.851 | 0.851 | -0.03 |
| Gly-Thr | 1.643 | 1.636 | -0.45 | Gly-Thr | 1.364 | 1.365 | 0.07 |
| Gly-Asp | 1.935 | 1.934 | -0.06 | Gly-Asp | 1.697 | 1.700 | 0.16 |
| Gly-Leu | 1.002 | 0.995 | -0.69 | Gly-Leu | 0.652 | 0.696 | 6.58 |
| Gly-Trp | 0.901 | 0.870 | -3.55 | Gly-Trp | 0.652 | 0.628 | -3.72 |
| Gly-Glu | 1.797 | 1.797 | 0.02 | Gly-Glu | 1.568 | 1.596 | 1.80 |
| Gly-Ser | 1.879 | 1.831 | -2.59 | Gly-Ser | 1.643 | 1.610 | -2.00 |
| c) 1 kV | | | | d) 3 kV | | | |
| Peptides | $k^*(exp)$ | $k^*(pre)$ | RD% | Peptides | $k^*(exp)$ | $k^*(pre)$ | RD% |
| Gly-Phe | 0.468 | 0.465 | -0.65 | Gly-Phe | 0.265 | 0.258 | -2.78 |
| Gly-Ile | 0.468 | 0.488 | 4.21 | Gly-Ile | 0.199 | 0.184 | -7.84 |
| Gly-Val | 0.619 | 0.629 | 1.67 | Gly-Val | 0.327 | 0.315 | -3.65 |
| Gly-Tyr | 0.703 | 0.679 | -3.47 | Gly-Tyr | 0.445 | 0.446 | 0.04 |
| Gly-Thr | 1.167 | 1.174 | 0.52 | Gly-Thr | 0.922 | 0.921 | -0.16 |
| Gly-Asp | 1.514 | 1.523 | 0.55 | Gly-Asp | 1.273 | 1.271 | -0.10 |
| Gly-Leu | 0.463 | 0.490 | 5.51 | Gly-Leu | 0.232 | 0.224 | -3.83 |
| Gly-Trp | 0.463 | 0.461 | -0.55 | Gly-Trp | 0.249 | 0.246 | -1.30 |
| Gly-Glu | 1.384 | 1.447 | 4.48 | Gly-Glu | 1.237 | 1.240 | 0.21 |
| Gly-Ser | 1.460 | 1.451 | -0.58 | Gly-Ser | 1.237 | 1.238 | 0.11 |
| e) 2 kV | | | | | | | |
| Peptides | $k^*(exp)$ | $k^*(pre)$ | RD% | | | | |
| Gly-Trp | 1.033 | 1.025 | -0.81 | | | | |
| Gly-Ile | 1.078 | 1.054 | -2.31 | | | | |
| Gly-Val | 1.583 | 1.563 | -1.26 | | | | |
| Gly-Tyr | 1.892 | 1.845 | -2.53 | | | | |
| Gly-Ala | 3.020 | 3.041 | 0.71 | | | | |
| Gly-Glu | 4.201 | 4.135 | -1.57 | | | | |
| Gly-Thr | 4.201 | 4.238 | 0.88 | | | | |
| Gly-Ser | 5.749 | 5.775 | 0.44 | | | | |
| Gly-Asp | 6.671 | 6.707 | 0.55 | | | | |

with a correlation coefficient (r) of 0.9998 as shown in Figure 4.a. The EOF velocity increases linearly with increasing applied voltage, which supports the assumption that the Joule heating was negligible. The velocity of the mobile phase in P-CEC was also found to increase

linearly ($r = 0.998$) with increasing applied pressure when a voltage of 1 kV was applied. The result is shown in Figure 4.b. This means that the velocity derived from pressure increases linearly with increasing applied pressure.

4.2 Modeling and prediction

According to Eq. (13), the retention of charged solutes in P-CEC is dependent on the applied voltage at a given applied pressure. The experimentally obtained k^* values for peptides were calculated from Eq. (6) in this study. The k^* values of peptides at -2 , -1 , 0 , 1 , 2 , 3 , and 5 kV were measured experimentally, respectively, while maintaining the applied pressure at 620 kPa. Since Eq. (13) contains only three parameters, these parameters can be determined by a minimum of three experimental data points. In this work, the three parameters, A_V , B_V , and C_V , in Eq. (13) were calculated according to the three k^* values of peptides at -2 , 2 , and 5 kV. Subsequently the k^* values at other voltages could be predicted with Eq. (13). The k^* values experimentally observed and predicted by Eq. (13) at -1 , 0 , 1 , and 3 kV for ten peptides are listed in **Table 1.a–d**. Their relative differences (RD) are also listed in the same table. The RD for Gly-Ile at 3 kV was -7.8% , which was the largest; the RDs for Gly-Leu at 0 and 1 kV were also greater than 5%, but the RDs for all other points were less than 5%. In order to illustrate the prediction capability of the proposed model, the predicted k^* of ten peptides at 4 different voltages are plotted against the observed values as shown in **Figure 5** ($n = 40$). A correlation coefficient of 0.9992 was obtained, indicating that the k^* values of peptides in P-CEC can be predicted well on the basis of three experimental data points. The retention of peptides in HI-CEC at 60% acetonitrile was weak, their k^* values at different voltages were all less than 2.3. The prediction accuracy with relatively high k^* value need to be investigated. As we know, the hydrophilic interaction could be promoted by increasing the acetonitrile fraction [20]. It was found that the retention of peptides increased quickly when the acetonitrile concentration increased from 60% to 75%. The k^* values experimentally observed and predicted under this condition at 2 kV for nine peptides are listed in **Table 1.e**. The three parameters in Eq. (13) were determined by the k^* values at 0, 1, and 3.5 kV. As can be seen from Table 1.e, the RDs for all the 9 peptides were less than 2.53%, which means the prediction is also accurate with relatively high k^* values.

The predicted effects of the applied voltage on the k^* values of 6 peptides are plotted in **Figure 6.a**. It is apparent that the observed k^* values at the four voltages lie close to the predicted line, which also indicates the accuracy of the prediction. As addressed above, the peptides are positively charged under the experimental conditions, and the influence of the applied voltage on the retention of peptides was very similar to the pattern of the hypothetical cationic solutes shown in Figure 1. Because electrophoretic migration of positively charged peptides has the same direction as that of EOF, the k^* values decrease with increasing applied voltage. Figure 6.a clearly shows that

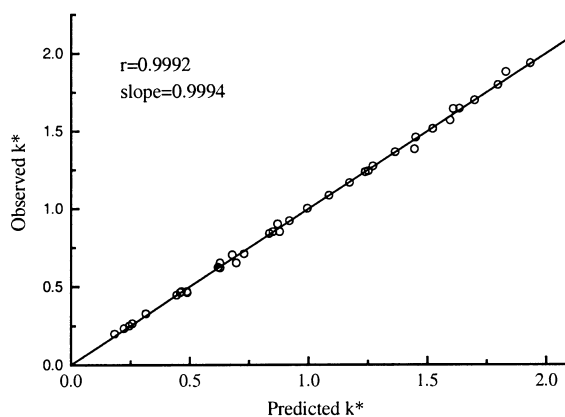


Figure 5. Correlation plot of the experimentally observed and calculated k^* values for ten peptides at different voltages. Conditions as in Table 1.a–d.

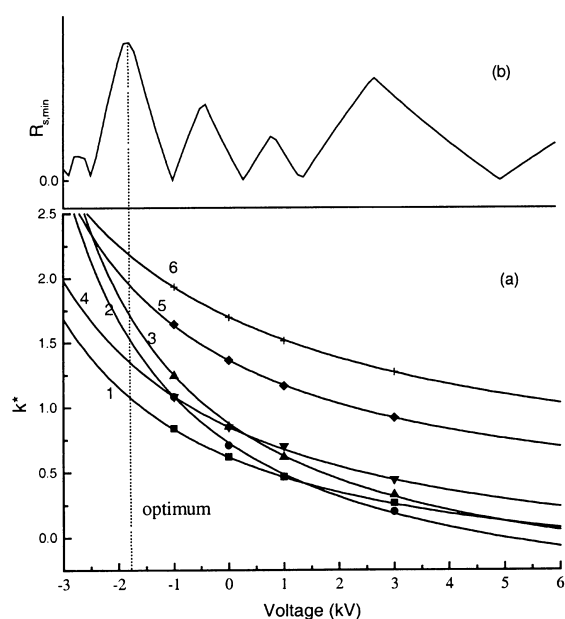


Figure 6. Predicted effect of the applied voltage on the k^* values for six peptides according to Eq. (13) and their minimum resolution pattern according to Eq. (17). Conditions: applied pressure, 620 kPa; other conditions and solutes as in Figure 3. Parameters A_V , B_V , and C_V , in Eq. (13) were determined by the k^* values at -2 , 2 , and 5 kV.

the separation selectivity of peptides in P-CEC can be adjusted by changing the applied voltage.

The k^* values of peptides were also measured with the applied pressure varied from 207 kPa to 690 kPa while maintaining the applied voltage at 1 kV. Similarly, the parameters A_P , B_P , and C_P in Eq. (15) were determined by the k^* values at 207, 345, and 690 kPa pressure. Accordingly, the k^* values of ten peptides could be predicted by Eq. (15) at other applied pressures, and the results obtained are listed in **Table 2**. The RDs for all 40 experimental points are less than 5%, which means that

Table 2. Comparison of the predicted and experimentally observed k^* values for peptides at different applied pressures. Conditions: applied voltage, 1 kV; other conditions as in Figure 3. Parameters A_P , B_P , and C_P in Eq. (15) were determined by the k^* values at 207, 345, and 690 kPa pressure.

| a) 80 psi | | | | b) 70 psi | | | |
|-----------|-------------------|-------------------|-------|-----------|-------------------|-------------------|-------|
| Peptides | $k^*(\text{exp})$ | $k^*(\text{pre})$ | RD% | Peptides | $k^*(\text{exp})$ | $k^*(\text{pre})$ | RD% |
| Gly-Phe | 0.489 | 0.484 | -0.93 | Gly-Phe | 0.477 | 0.465 | -2.61 |
| Gly-Ile | 0.489 | 0.489 | -0.07 | Gly-Ile | 0.477 | 0.470 | -1.47 |
| Gly-Val | 0.715 | 0.681 | -4.91 | Gly-Val | 0.631 | 0.645 | 2.20 |
| Gly-Tyr | 0.715 | 0.708 | -0.92 | Gly-Tyr | 0.719 | 0.690 | -4.20 |
| Gly-Thr | 1.206 | 1.203 | -0.33 | Gly-Thr | 1.191 | 1.182 | -0.76 |
| Gly-Asp | 1.564 | 1.552 | -0.77 | Gly-Asp | 1.546 | 1.535 | -0.72 |
| Gly-Leu | 0.471 | 0.475 | 0.86 | Gly-Leu | 0.447 | 0.453 | 1.30 |
| Gly-Trp | 0.471 | 0.472 | 0.18 | Gly-Trp | 0.447 | 0.449 | 0.40 |
| Gly-Glu | 1.400 | 1.458 | 4.08 | Gly-Glu | 1.417 | 1.441 | 1.71 |
| Gly-Ser | 1.464 | 1.458 | -0.42 | Gly-Ser | 1.447 | 1.441 | -0.42 |

| c) 60 psi | | | | d) 40 psi | | | |
|-----------|-------------------|-------------------|-------|-----------|-------------------|-------------------|-------|
| Peptides | $k^*(\text{exp})$ | $k^*(\text{pre})$ | RD% | Peptides | $k^*(\text{exp})$ | $k^*(\text{pre})$ | RD% |
| Gly-Phe | 0.445 | 0.441 | -0.89 | Gly-Phe | 0.371 | 0.371 | -0.08 |
| Gly-Ile | 0.445 | 0.446 | 0.17 | Gly-Ile | 0.347 | 0.356 | 2.68 |
| Gly-Val | 0.597 | 0.603 | 1.04 | Gly-Val | 0.486 | 0.484 | -0.24 |
| Gly-Tyr | 0.670 | 0.665 | -0.70 | Gly-Tyr | 0.587 | 0.585 | -0.33 |
| Gly-Thr | 1.162 | 1.155 | -0.56 | Gly-Thr | 1.060 | 1.067 | 0.65 |
| Gly-Asp | 1.514 | 1.512 | -0.17 | Gly-Asp | 1.422 | 1.427 | 0.36 |
| Gly-Leu | 0.417 | 0.424 | 1.55 | Gly-Leu | 0.337 | 0.326 | -3.34 |
| Gly-Trp | 0.417 | 0.420 | 0.72 | Gly-Trp | 0.337 | 0.336 | -0.32 |
| Gly-Glu | 1.423 | 1.418 | -0.31 | Gly-Glu | 1.341 | 1.343 | 0.18 |
| Gly-Ser | 1.423 | 1.418 | -0.31 | Gly-Ser | 1.341 | 1.343 | 0.18 |

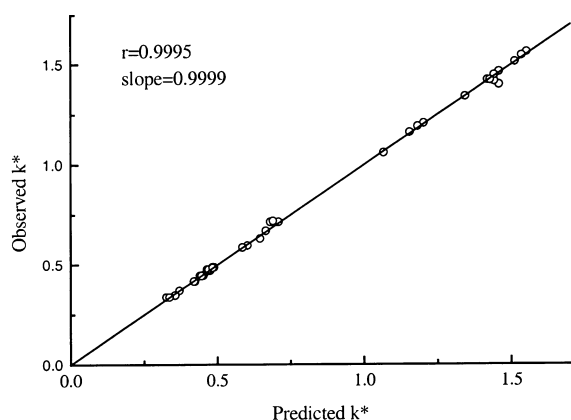


Figure 7. Correlation plot of experimentally observed and calculated k^* values for ten peptides at different pressures. Conditions as in Table 2.

Eq. (15) can be used to predict retention quite accurately on the basis of three experimental data points. In order to demonstrate the prediction capacity of the proposed

model, the predicted k^* values of ten peptides at 4 different pressures are plotted against the observed values in **Figure 7** ($n = 40$). A correlation coefficient of 0.9995 was obtained.

The predicted effect of applied pressure on the k^* values of 6 peptides is shown in **Figure 8.a**. It can be seen from **Figure 8.a** that the observed k^* values virtually coincide with the line of predicted values. Compared with that in **Figure 2**, the influence of the applied pressure on the k^* values of peptides is similar to that of cationic solutes. The retention of peptides increases with increasing applied pressure when the applied voltage is held constant.

4.3 Optimization

According to Eq. (17), the resolution of ionic solutes is dependent on the retention and column efficiency. The retention of ionic solutes in P-CEC at different applied voltages and pressures could be modeled by Eq. (13) and Eq. (15), respectively. However, the effects of applied

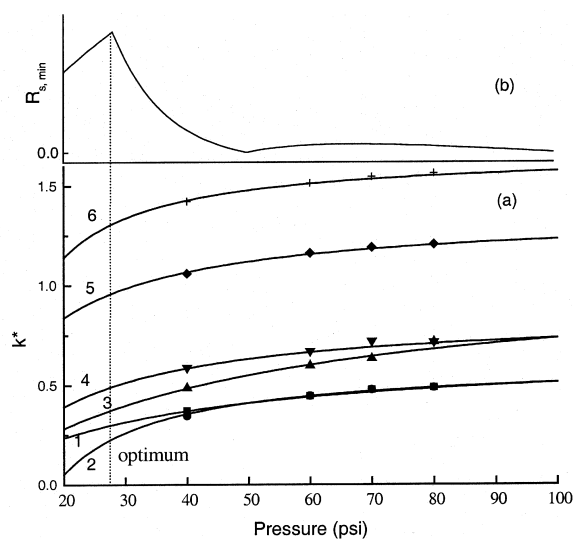


Figure 8. Predicted effect of the applied pressure on the k^* values for six peptides according to Eq. (15) and their minimum resolution pattern according to Eq. (17). Conditions: applied voltage, 1 kV; other conditions and solutes as in Figure 3. Parameters A_P , B_P , and C_P in Eq. (15) were determined by the k^* values at 207, 345, and 690 kPa pressure.

voltage or pressure on the column efficiency are complex. **Figure 9.a** shows the dependence of column efficiency on the applied voltage in the separation of two peptides. It is generally believed that efficiency in P-CEC is lower than that in pure CEC, but higher than that in LC because the parabolic flow profile of the pressurized flow is superimposed on the plug-like flow profile of EOF [10]. However, it can be seen from Figure 9.a that the column efficiency seems to be related to the polarity of the applied voltage. The efficiency was poorer when negative voltages were applied, while the efficiency was improved when positive voltages were applied. The reason is still not clear. Maybe the counter direction of EOF and electrophoretic migration of positively charged peptides with the pressurized flow when negative voltage was applied may result in the increase of eddy diffusion, thereby decreasing the efficiency. The dependence of column efficiency on the applied pressure is also shown in **Figure 9.b**. At constant voltage, the column efficiency tended to decrease with increasing applied pressure. A possible reason is that the relatively flat flow profile approaches that of the one with pure pressure at high applied pressure, which results in the increase of eddy diffusion. The relative standard deviations of the column efficiencies at different voltages and pressures are both less than 12%. The effect of efficiency on resolution was not considered in this work in order to simplify the optimization procedure.

Figure 6.b is a theoretical pattern of the minimum resolution versus applied voltages for the separation of a mixture of 6 peptides calculated from Eq. (17). The N_1 for all

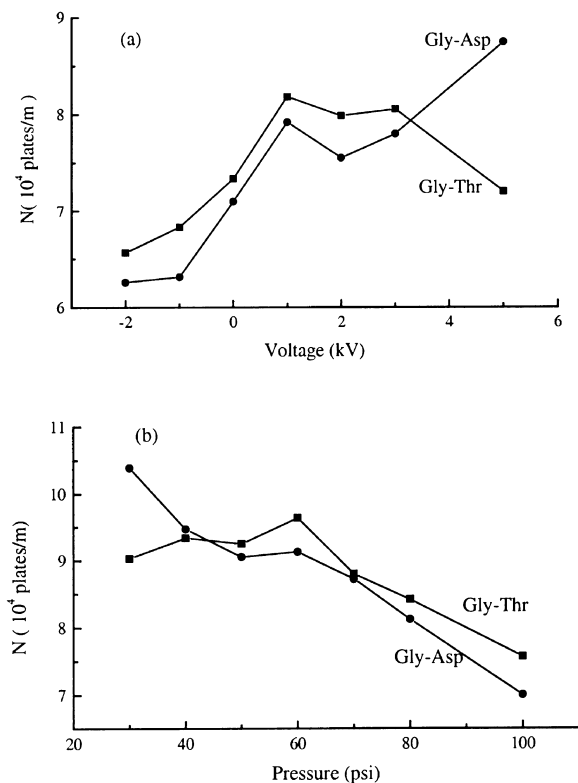


Figure 9. Dependence of column efficiency on (a) the applied voltage and (b) the applied pressure. Conditions as in Figure 4.

solutes was the average efficiency of Gly-Asp at different voltages. It is apparent that the optimum voltage is about -1.8 kV. Separation of the actual mixture at -1.8 kV is shown in **Figure 10.a**; all six peptides were baseline separated. As can be seen from Figure 6.b, the second optimum voltage is about 2.7 kV. The six peptides may be separated under these conditions because higher efficiency can be obtained by applying positive voltage. **Figure 10.b** shows the chromatogram of the separation of the six peptides at 3 kV. The first three peaks were not baseline resolved. According to Figure 6.a, resolution for Gly-Phe and Gly-Ile will worsen with decreasing voltage. Therefore, it is also impossible to accomplish baseline separation of the six peptides at 2.7 kV. Accordingly, -1.8 kV seems to be the optimal voltage for separation of this mixture.

The minimum resolution pattern predicted by Eq. (17) as a function of the applied pressure is shown in **Figure 8.b**. It indicates that the optimum pressure should be about 186 kPa. Separation of the mixture at this pressure is shown in **Figure 11**. The first three peaks were not in fact baseline separated. Baseline separation cannot be obtained across the entire range of the applied pressure, because the resolution for any pair of the three peptides decreases whenever the applied pressure increases or

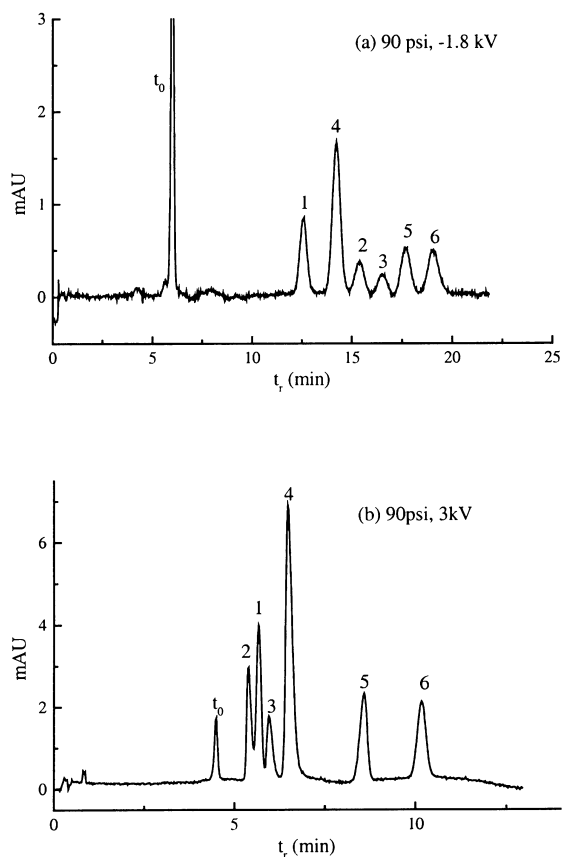


Figure 10. Chromatograms for separation of six peptides at different voltages. Conditions: applied voltage, 620 kPa; applied voltage, (a) -1.8 kV and (b) 3 kV; other conditions and solutes as in Figure 3.

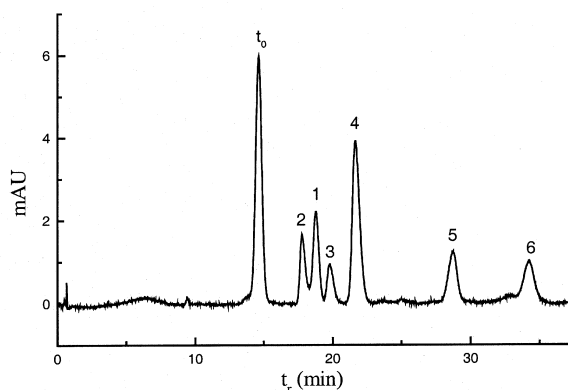


Figure 11. Chromatogram for separation of six peptides at the optimal applied pressure. Conditions: applied voltage, 1 kV; applied pressure, 186 kPa. Other conditions and solutes as in Figure 3.

decreases according to the effect of pressure on the k^* value as shown in Figure 8.a.

5 Conclusion

A theoretical model was developed to quantitatively describe the migration of ionic solutes in P-CEC. The predicted effect of the applied voltage or pressure on the retention is reasonably consistent with that observed in experiment. The electrochromatographic retention factor (k^*) of ionic solutes could be accurately predicted by the proposed model on the basis of the experimental data. The applied voltage and pressure are two tunable parameters for adjustment of selectivity in P-CEC. Optimization was successfully achieved by maintaining one parameter constant and varying the other parameter. In the future, a model should be established to address the optimization of the separation by changing the applied voltage and the pressure simultaneously.

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