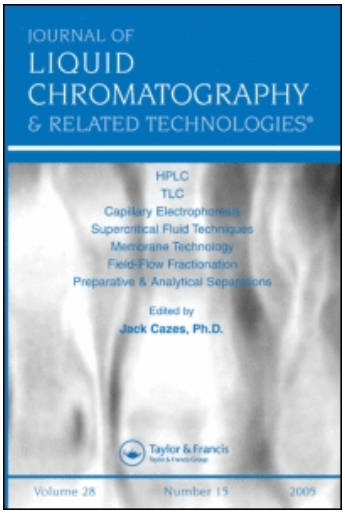

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Electrophoretic Selectivity as a Function of Operating Parameters in Free-Solution Capillary Electrophoretic Separation of Dipeptides

Nong Chen[a]; Lei Wang[a]; Yukui Zhang[a]
[a] National Chromatographic R. & A. Center Dalian Institute of Chemical Physics Chinese Academy of Sciences, Dalian, People's Republic of China

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ELECTROPHORETIC SELECTIVITY AS A FUNCTION OF OPERATING PARAMETERS IN FREE–SOLUTION CAPILLARY ELECTROPHORETIC SEPARATION OF DIPEPTIDES

NONG CHEN*, LEI WANG, AND YUKUI ZHANG
National Chromatographic R. & A. Center
Dalian Institute of Chemical Physics
Chinese Academy of Sciences
Dalian 116011, People's Republic of China

ABSTRACT

Free—solution capillary electrophoretic separation of some dipeptides were carried out under acidic and alkaline conditions. The reproducibility of two migration parameters (migration time and relative migration time) for some dipeptides was examined under various operating conditions. "The electrophoretic selectivity "or relative migration time which is defined as the ratio of the migration time of test component to that of the reference standard shows better reproducibility than migration time. In addition, better reproducibility in migration times was obtained from the capillary previously stored in acidic conditions for a certain period of time. Migration times of a neutral marker (t_o) and dipeptides (t_m) and relative migration times were examined as functions of operating column temperature and applied current (I). A quantitative linear correlation between logarithm of migration time (log

t_o or log t_m) and the reciprocal of column temperature (1/T) under constant applied voltage has been derived . Plots of the reciprocal of migration times ($1/t_m$) for dipeptides versus the applied current(I) yielded parallel lines. The parallel linear relationships between log t_m and 1/T, $1/t_m$ and I implied that the electrophoretic selectivity was independent of column temperature and operating current.

INTRODUCTION

High performance capillary electrophoresis (HPCE) has become an important separation tool as a result of its high resolving power and speed. Although several modes of operation in HPCE are possible, much of the work has used the mode of free—solution capillary electrophoresis (FSCE). FSCE exhibits simplicity and was expected to separate components based on the subtle changes in charge density and hydrophobicity[1—2]. Optimization of the electrophoretic behavior of dipeptides in FSCE requires further understanding of the relationship between the migration time and the operating parameters. In addition, the reproducibility of the results obtained by FSCE should be first settled. The reproducibility of the results obtained by HPCE has been investigated by several researchers[3—8]. They demonstrated that the fluctuations of the migration time result from the fluctuations in electroosmotic flow, the magnitude of which is dependent on the nature and concentration of the buffer. The effects of ionic strength, pre—run and post—run washing on the reproducibility have also been examined. It was also found that the number of runs required to obtain consistent migration times had a greater influence on migration reproducibility in FSCE[3].

Some researchers coated or bonded columns with hydrophilic polymers. These coatings were designed to bind onto the capillary surface in a uniform stable and reproducible manner[7—8].

In a study of the influence of operating parameters on the electrophoretic behavior of peptides in FSCE, most of the researchers focused on the effect of buffer pH and peptide composition on the selectivity of peptide separation by FSCE[9—16]. Applied voltage and buffer concentration have also been extensively investigated in the optimization of resolution of amino acids in FSCE[9—10]. There are factors other than pH in FSCE which affect the solute electrophoretic behaviors, such as column temperature and electrical field strength etc..

In this paper, free—solution capillary electrophoretic separations of dipeptides were carried out with alkaline and acidic buffers. The reproducibility of two migration parameters (migration time and relative migration time) of some dipeptides was examined under various operating conditions. Migration times and electrophoretic selectivities in FSCE separation of dipeptides were examined as a function of column temperature and applied current. A quantitative relationship between migration times and column temperature in FSCE was derived in this peper.

EXPERIMENTAL

A SPECTRA PHORESIS 500 capillary electrophoresis system (Spectra—Physics Analytical, Fremont, USA) with 50 μm and 75 μm fused silica capillary was used in the present study. On column ultraviolet (UV) detector was set at 210 nm. The samples were injected by hydrodynamically mode for 1 second.

Peptides were separated under acidic and alkaline conditions. Dipeptides were received from Serva Feinbiochemical (Meidelberg, Germany). Benzyl alcohol was used as a neutral marker to measure the electroosmotic flow.

Sodium phosphate buffer pH 2. 51, sodium glycinate buffer pH 10. 92, and borate buffer pH 9. 8 were used. Water was distilled twice.

RESULTS AND DISCUSSION

Separation in HPCE is achieved via the distinct migration velocities of the analytes under the influence of an electric field. An analyte is identified by its migration time(t_m) in an electropherogram, which can be expressed as follows:

$$t_m = \frac{\eta L}{\varepsilon E\left[\zeta_c + \frac{2\zeta_a}{3} f(\kappa\alpha)\right]} \qquad (1)$$

where η is the viscosity coefficient and ε the dielectric constant of the medium, ζ_a, ζ_c the zeta potential of the analyte and the inner wall of the capillary, respectively. κ is the reciprocal of the analyte double layer thickness, α is the "radius" of the analyte. f ($\kappa\alpha$) are functions dependent upon the shape and $\kappa\alpha$ of the analyte in the buffer. L is the length of the capillary, E(=V/L) is the local electric field, V is the applied potential. While, the electroosmotic migration time is given by:

$$t_o = \frac{\eta L}{\varepsilon E \zeta_c} \qquad (2)$$

The electrophoretic separations of dipeptides were carried out under acidic and alkaline conditions. It was found that the reproducibility of the migration time is better in acidic buffer than in alkaline buffer which indicated that fluctuations in the electroosmotic flow results in fluctuations in migration time of the dipeptide. Fig. 1 shows the capillary electrophoretic separations of four dipeptides in glycinate buffer.

Fig. 1

Free solution electrophoretic separation of dipeptides in glycinate buffer. FSCE conditions: 75 cm * 75 μm I. D. ,25 kV,25 ℃,1 second hydrodynamic injection,20 mM glycinate buffer pH 10. 92,1 =trp−leu,2=phe−gly,3=tyr−phe,4=tyr−gly,t_o=H2O.

The criteria of the electrophoretic selectivity (γ) or relative migration time is given by

$$\gamma=\frac{t_m}{t_m(r)}=\frac{\zeta_c+\frac{2\zeta_a(r)}{3}f(\kappa\alpha)(r)}{\zeta_c+\frac{2\zeta_a}{3}f(\kappa\alpha)} \qquad (3)$$

If the reference standard was neutral marker, γ can be written as

$$\gamma=t_m/t_o=\frac{\zeta_c}{\zeta_c+\frac{2\zeta_a}{3}f(\kappa\alpha)}$$

TABLE I
COMPARISON OF THE REPRODUCIBILITY OF MIGRATION TIME (MIN) AND RELATIVE MIGRATION TIME FOR TYR—GLY IN GLYCINATE BUFFER*

Run No.	1	2	3	4	5	6	7	8	RSD%
t_o	4.02	3.74	3.82	4.01	3.98	4.02	4.00	3.73	3.92
t_m	5.98	5.57	5.73	6.02	5.96	6.02	5.94	5.54	3.40
t_m/t_o	1.49	1.49	1.50	1.50	1.50	1.50	1.49	1.49	0.45

* 20 mM sodium glycinate buffer—NaOH pH 10.88, 25°C, 25kV. Between every run, capillary was washed with 0.1 N NaOH for 1 min..

Table I showed the comparison of the reproducibility of two migration parameters (migration time and relative migration time) for tyr—gly in glycinate buffer.

As shown in Table I, the fluctuations in migration time coincide with those in t_o, therefore the fluctuations in t_m is due to irregularities in electroosmotic flow rate, while t_m/t_o which compensates for electroosmotic fluctuations shows greater stability than migration time (see also Fig. 2). The criteria of t_m/t_o can be used as a parameter in FSCE for peak identifications in alkaline conditions. Separation of the dipeptides has also been carried out in acidic buffer. Figure 3 illustrated the electropherogram of some dipeptides in phosphate buffer.

Preconditions of capillary column were attempted in acidic buffer in order to obtain reproducible results. We have found, when the capillary was stored in 0.5 mM phosphate buffer for 16 hrs, it can produce reproducible results. Table II shows the comparison in the reproducibility of migration times for five dipeptides before

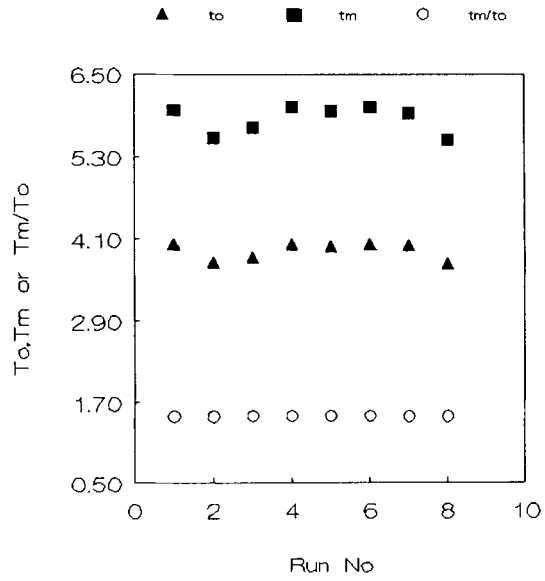

Fig. 2
Migration times of tyr—gly(t_m), benzyl alcohol(t_o) and t_m/t_o as a function of the injection number(n). For FSCE conditions, see Table I.

and after the capillary was stored overnight in 0.5 mM phosphate buffer.

Temperature can potentially be an important parameter in HPCE, good temperature control is important for migration reproducibility, fluctuations in temperature can lead to changes in migration velocities of the analytes by altering their mobilities[16—20].

In studying the influence of column temperature in FSCE, there is at least one aspect that need to be considered: the impact of temperature on the electrophoretic behaviour due to the changes in electroosmotic and electrophoretic mobility.

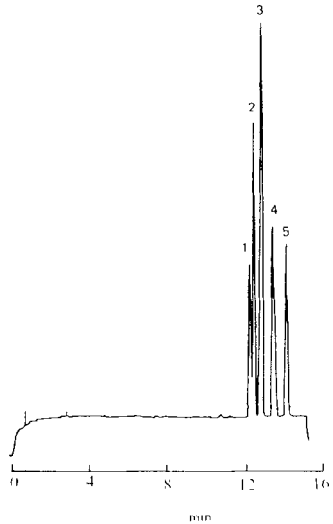

Fig. 3

Electropherogram of five dipeptides in phosphate buffer. FSCE conditions: 75 cm * 75 μm I. D. fused silica column, 25 kV, 20℃, 50 mM phosphate buffer pH = 2. 51, 1 second hydrodynamic injection. 1 = gly—phe, 2 = phe—gly, 3 = tyr—gly, 4 = trp—leu, 5 = tyr—phe.

In the fundamental migration equation, the term η is temperature—dependent, while all the other terms are all relatively temperature insensitive. The phosphate buffer and borate buffer have a relatively small temperature—coefficient. It appears temperature—induced viscosity changes predominantly account for the change in electroosmotic and electrophoretic migration time. The viscosity of the buffer has the following simplified relationship with temperature(18):

$$\eta = A * 10^{B/T}$$

TABLE II
REPRODUCIBILITY OF t_m FOR FIVE DIPEPTIDES BEFORE AND AFTER THE CAPILLARY WAS STORED OVERNIGHT IN 0.5 mM PHOSPHATE BUFFER

Dipeptides	Gly—phe	phe—gly	tyr—gly	trp—leu	tyr—phe
Average t_m*	12.04	12.22	12.59	13.20	13.90
RSD%	1.90	1.90	1.89	1.85	1.81
Avreage t_m**	11.52	11.65	12.06	12.72	13.29
RSD%	0.47	0.43	0.43	0.47	0.42

* Five consecutive injections were performed before the preconditioning of the capillary. For FSCE conditions, see Fig. 3.

* * Six consecutive injections were performed after the preonditioning of the column.

where A, B are constants characterizing the properties of the running buffer. B is mainly determined by the activation energy of diffusion ($\triangle E_a$); $B=\frac{\triangle E_a}{R}$.

Therefore, we obtained:

$$\log t_m=\log Q+\frac{B}{T} \qquad (6)$$

$$\log t_o=\log Q_1+\frac{B}{T}$$

where $Q=AL/(\varepsilon E[\zeta_c+\frac{2\zeta_a}{3}f(\kappa\alpha)])$, $Q_1=\frac{AL}{\varepsilon E\zeta_c}$, t_o is the migration time for neutral marker. In a linear portion of the plot of the operating current versus voltage, a quantitative linear correlation between log t_m or log t_o and the reciprocal of column temperature

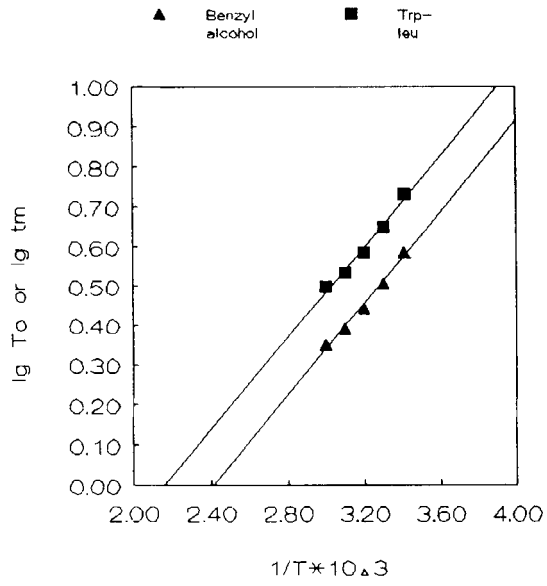

Fig. 4
Plots of log t_o and log t_m versus 1/T obtained with borate buffer. For FSCE conditions, see Table III.

was derived under constant field strength. In the present study, benzyl alcohol was used as a probe to measure the electroosmotic flow.

Fig. 4 illustrates the linear relationships between log t_m and log t_o and 1/T for dipeptide and benzyl alcohol. Under constant voltage, the plots are parallel and linear with correlation coefficients of 0.995, 0.995. The slope indicates the characteristic constant for the borate buffer and plays the role of the activation energy of diffusion in FSCE. The parallel electroosmotic and electrophoretic behaviors against column temperature illustrated that with increasing column temperature, the migration was faster, which will shorten the time of the analysis.

TABLE III

VARIATION OF MIGRATION TIMES(MIN)WITH THE OPERATING COLUMN TEMPERATURE. THE RELATIVE MIGRATION TIME BETWEEN BENZYL ALCOHOL AND DIPEPTIDE ARE ALSO GIVEN *

℃	t_o	$t_m(1)$	$t_m(2)$	$t_m(1)/t_o$	$t_m(2)/t_o$
20	3.86	5.39	5.70	1.40	1.48
30	3.20	4.47	4.47	1.40	1.48
40	2.77	3.86	4.09	1.39	1.48
50	2.46	3.42	3.63	1.39	1.48
60	2.24	3.15	3.35	1.41	1.49

* Fused silica capillary 70cm * 50 μm I. D., constant voltage: 30KV, borate buffer(8 mM, pH8.97), t_o is the migration time of benzyl alcohol, $t_m(1)$ and $t_m(2)$ refer to the migration time for trp—leu and phe—gly—gly, respectively.

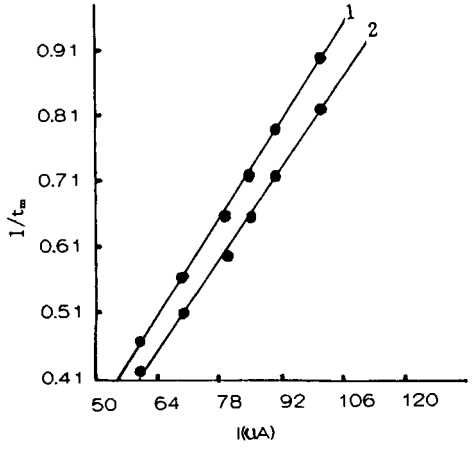

Fig. 5

Plots of $1/t_m$ versus I for two dipeptides. 1,2 denote gly—phe, trp—leu, respectively. For FSCE conditions, see Table IV.

TABLE IV
VARIATION OF MIGRATION TIME (MIN) OF FOUR DIPEPTIDES WITH THE APPLIED CURRENT. THE AND RELATIVE MIGRATION TIME BETWEEN EACH PAIR OF PEPTIDES ARE ALSO GIVEN *

I(μA)	$t_m(1)$	$t_m(2)$	$t_m(2)/t_m(1)$	$t_m(3)$	$t_m(3)/t_m(1)$	$t_m(4)$	$t_m(4)/t_m(1)$
100	11.01	11.44	1.04	12.09	1.10	12.64	1.15
90	12.57	13.07	1.04	13.86	1.10	14.49	1.15
85	13.89	14.46	1.04	15.19	1.09	16.06	1.16
80	15.17	15.82	1.04	16.85	1.11	17.60	1.16
70	17.62	18.36	1.04	19.61	1.11	20.45	1.16
60	21.14	22.06	1.04	23.61	1.12	24.71	1.17

* $t_m(1)$, $t_m(2)$, $t_m(3)$, $t_m(4)$ are the migration times for gly—phe, tyr—gly, trp—leu, tyr—phe, respectively, fused silica capillary, 50 mM phosphate buffer (pH 2.51), 20℃, prior to use, the column was stored in 0.5 M phosphate buffer for 16 hrs.

Therefore the electrophoretic selectivity remains the same despite a considerable variation in migration times between a pair of the dipeptide and benzyl alcohol(see Table III).

The effect of the applied voltage or operating current on solute migration times in FSCE was investigated by several authors[10 — 11]. In the present study, we demonstrated how changes in applied current(I) affected the values of the reciprocal of migration time ($1/t_m$) and the electrophoretic selectivity. According to eqn(1), in the linear portion of an Ohm's law plot, the reciprocal of solute migration time or the solute velocity is linearly proportional to the applied current. Fig. 5 shows the parallel plots of 1/

t_m versus I for two dipeptides, which implies that the operating current does not affect the values of relative migration times of the dipeptides (see Table IV). Therefore, the parallel linear relationships between lg t_m and 1/T, $1/t_m$ and I shows that the electrophoretic selectivities were independent of column temperature and operating current.

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