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Optimization of resolution in capillary zone electrophoresis: combined effect of applied voltage and buffer concentration

Ibrahim Z. Atamna, Haleem J. Issaq, Gary M. Muschik and George M. Janini*

Program Resources, Inc./Dyncorp, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702-1201 (USA)

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ABSTRACT

Expressions are formulated for the prediction of solute migration time and resolution as a function applied voltage and buffer concentration in capillary zone electrophoresis. The resolution equation assumes that solute diffusion is the only operative zone-broadening mechanism. A resolution surface in applied voltage and buffer concentration space is presented featuring isochrones that are used to predict the behavior of resolution under constant analysis time. In the resolution–voltage planes the resolution increases continuously with increasing voltage. At the high-voltage border, the resolution decreases continuously with increasing concentration, however, at the low-voltage border the resolution passes through a shallow maximum as the buffer concentration is increased. At constant analysis time, resolution is optimized by simultaneously increasing the voltage and the buffer concentration. In comparison, this theoretical approach, which predicts resolution from solute migration times only, gives values that are consistently about 40–50% higher than experimentally determined resolution.

INTRODUCTION

Since its introduction in 1937 [1] electrophoresis has witnessed tremendous advances and a variety of methods and modes of operation were developed [2]. In particular, capillary zone electrophoresis (CZE) is now recognized as a highly efficient and sensitive microanalytical technique. It has rapidly developed since Jorgenson and Lukacs [3,4] realized the advantage of using small-diameter (< 100 μm) fused-silica columns. Although CZE is compared to liquid chromatography yet it has a completely different separation mechanism. Charged substances are resolved according to their differential migration in semiconducting buffers under the influence of an electric field gradient.

Resolution in CZE, as is the case in chromatography, is a function of three parameters: selectivity, column efficiency and migration time. Each of these parameters is influenced by many factors including applied voltage, buffer pH, type, concentration and ionic strength, modifiers, and inner capillary wall treatment. The pH is a dominant factor that could be manipulated to control resolution [5–7]. Surface modification of capillary walls by either masking or deactivating the surface silanol groups is another factor that greatly influences migration time and peak shape [8–11]. In previous studies from this laboratory we studied the role of applied voltage, buffer type and concentration in CZE [12–15]. The objective of this work is to investigate the effect of applied voltage and buffer concentration on resolution under ideal experimental conditions where the only operative solute zone-broadening mechanism is solute diffusion. An optimization procedure for the attainment of the best performance for a system by adjusting these two variables either independently or under isochronal (time normalization) conditions is presented. Isochronal methods have been developed and successfully used for the optimization of resolu-

tion in gas–liquid chromatography [16] and high-performance liquid chromatography [17,18]. This work represents an effort on our part to extend the utility of this procedure to CZE.

THEORETICAL

The resolution (R_s) of two close-lying zones in an electropherogram is given by [19]:

$$R_s = \frac{1}{4}(N)^{\frac{1}{2}}\frac{\Delta v}{\bar{v}} \tag{1}$$

where N is the number of theoretical plates and $\Delta v/\bar{v}$ is the relative migration velocity difference of the two zones. The migration time (t_m), which is directly measured from the electropherogram, is related to the migration velocity (v) by the following equation:

$$v = \frac{l}{t_m} \tag{2}$$

where l is the column length from injector to detector. The number of theoretical plates is given by [19]

$$N = \frac{L^2}{\sigma^2} \tag{3}$$

where L is the column length and σ^2 is the zone variance. If longitudinal diffusion is the only source of zone broadening, which seems to be the case under conditions where Joule's heating effects are minimized, then zone variance is given by the Einstein equation [19]:

$$\sigma^2 = 2Dt_m \tag{4}$$

where D is solute diffusion coefficient. Substituting eqn. 4 in eqn. 3 yields an expression for N in terms of experimental electrophoretic parameters and solute intrinsic diffusion.

$$N = \frac{L^2}{2Dt_m} \tag{5}$$

It is to be noted that L^2 should be substituted by $L \cdot l$ if the total column length and the injector-to-detector length are not identical. If the relation expressed in eqn. 5 as well as the fact that $\Delta v/\bar{v} \simeq \Delta t/t_2$ for two close-lying zones are taken into consideration then the resolution equation could be rewritten as:

$$R = \left(\frac{L^2}{32D}\right)^{\frac{1}{2}}\frac{1}{\sqrt{t_{m,2}}}\frac{\Delta t}{t_{m,2}} \tag{6}$$

where Δt is the migration time difference of the two zones and $t_{m,2}$ is the migration time of the slower-migrating solute. Note that this resolution equation neglects some experimental parameters which, if present, can adversely affect column performance such as sample injection artifacts, Joule's heating effects and possible solute adsorption onto the capillary wall.

In a previous communication from this laboratory [15] we have demonstrated that, under ideal electrophoretic conditions (*i.e.* the Joule's heat generated inside the column is efficiently dissipated), both the electrophoretic and electroosmotic mobilities are directly proportional to the applied voltage and inversely proportional to the square root of concentration. Accordingly the following relations are obeyed.

(i) At constant applied voltage (V)

$$t_m = a + b\sqrt{C} \tag{7}$$

where a and b are constants.

(ii) At constant buffer concentration (C)

$$\frac{l}{t_m V} = m + nV \tag{8}$$

where m and n are constants. The excellent linearity of the plots presented in Figs. 2–4 testify to the validity of these relationships.

In this work we attempt to formulate an expression for migration time as a function of applied voltage and buffer concentration, $t_m(C,V)$, as follows: a and b of eqn. 7 are each fitted to a second-degree polynomial in V:

$$a = a_0 + a_1V + a_2V^2 \tag{9a}$$

$$b = b_0 + b_1V + b_2V^2 \tag{9b}$$

Similarly m and n of eqn. 8 are fitted to a polynomial in C:

$$m = m_0 + m_1C + m_2C^2 \tag{10a}$$

$$n = n_0 + n_1C + n_2C^2 \tag{10b}$$

By substituting for a and b in eqn. 7 and for m and

n in eqn. 8 and solving for t_m the following expression is obtained:

$$t_m(C,V) = \left[\frac{a_0 + a_1V + a_2V^2 + b_0\sqrt{C} + b_1V\sqrt{C} + b_2V^2\sqrt{C}}{m_0V + m_1CV + m_2C^2V + n_0V^2 + n_1CV^2 + n_2C^2V^2}\right]^{\frac{1}{2}} \quad (11)$$

Finally, utilizing eqn. 11, the expression for resolution (eqn. 6) could be rewritten as:

$$R_s(C,V) = \left(\frac{L^2}{32D}\right)^{\frac{1}{2}} \frac{\Delta t_m(C,V)}{[t_{m,2}(C,V)]^{\frac{3}{2}}} \quad (12)$$

where $\Delta t_m(C,V) = t_{m,2}(C,V) - t_{m,1}(C,V)$.

EXPERIMENTAL

A Beckman CZE System 2000 (Model P/ACE) equipped with a UV detector, an automatic injector, a column cartridge (50 cm × 75 μm I.D., surrounded by coolant), an autosampler and a printer was used in this study. All experiments were carried out at 25°C and were run at least in triplicates to insure reproducibility. Injections were made using the pressure mode for 1 s each. The acetate buffer solutions were degassed and filtered through 0.2-μm Nylon 66 filters. All experiments were conducted at pH 5.0. Solute standards were prepared to be about 10 μg/ml and were monitored at 254 nm with the highest instrument sensitivity setting. Water was distilled and deionized. Dansyl leucine and dansyl methionine were purchased from Sigma (St. Louis, MO, USA), sodium acetate and acetic acid were purchased from Johson Matthey Alpha Products (Danvers, MA, USA) and mesityl oxide was obtained from Aldrich (Milwaukee, WI, USA). All solutes were dissolved in the eluent buffer.

The migration times for the solutes dansyl leucine and dansyl methionine and for the marker mesityl oxide were measured at four different applied voltages (10–25 kV) using water and five different buffer concentrations (10–50 m*M*).

RESULTS AND DISCUSSION

Table I lists the migration times for the probe solutes used in this study at different applied voltages in water and acetate buffers. Each data point is an average of at least three readings with a standard deviation of ±1%. Fig. 1 shows plots of the data collected at 20 kV according to eqn. 7. All plots were linear with correlation coefficients in excess of 0.995. It is important to note that if the data are plotted as t_m *vs.* C or t_m *vs.* log C the plots will be linear only if the data point for pure water is excluded. Even then the correlation coefficients are not as good as reported for Fig. 1. It is more important to note that these plots will not extrapolate to a single point at infinite buffer dilution as is the case in Fig. 1. The data for dansyl methionine were plotted according to eqn. 7 (Fig. 2) and eqn. 8 (Fig. 3). The values of a and b obtained from plots of t_m *vs.* $C^{1/2}$ at different voltages were fitted to a polynomial in V according

TABLE I

SOLUTE MIGRATION TIMES AT DIFFERENT APPLIED VOLTAGES IN WATER AND ACETATE BUFFERS

All measurements except pure water were conducted at pH = 5.0.

Applied voltage (kV)	Buffer concentration (m*M*)	Migration time[a] (min)		
		Mesityl oxide	Dansyl leucine	Dansyl methionine
10	0	3.21	3.21	3.21
15	0	1.99	1.99	1.99
20	0	1.49	1.49	1.49
25	0	1.28	1.28	1.28
10	10	6.52	9.75	9.90
15	10	4.20	6.22	6.32
20	10	3.21	4.27	4.34
25	10	2.55	3.36	3.42
10	20	8.54	11.89	12.08
15	20	5.43	7.46	7.58
20	20	3.88	5.29	5.36
25	20	3.01	4.09	4.15
10	30	9.65	13.99	14.29
15	30	6.02	8.77	8.95
20	30	4.08	5.89	6.01
25	30	3.17	4.51	4.60
10	40	10.71	15.83	16.19
15	40	6.69	9.77	9.98
20	40	4.67	6.74	6.86
25	40	3.45	4.93	5.01
10	50	11.47	17.36	17.80
15	50	7.05	10.53	10.76
20	50	4.92	7.26	7.41
25	50	3.60	5.24	5.34

[a] Migration times ± 1.0%.

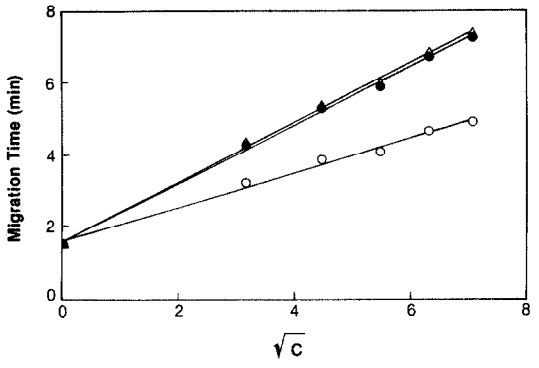

Fig. 1. Migration time as a function of the square root of buffer concentration. Buffer: acetate at pH 5.0; field strength: 400 V/cm; column: 50 cm × 75 μm I.D. fused silica; instrument: Beckman Model P/ACE System 2000. ○ = Mesityl oxide; ● = dansyl leucine; △ = dansyl methionine.

to eqns. 9a and 9b. Similarly the values of m and n obtained from plots of $1000/tV$ *vs.* V at different concentrations were fitted to a polynomial in C according to eqns. 10a and 10b. The coefficients thus obtained are reported in Table II. By substituting the values of the parameters for the appropriate solutes from Table II in eqns. 11 and 12 one could calculate the migration times and the resolution at any applied voltage and buffer concentration. In order to validate the fitting procedure described above the migration times for leucine and methionine were calculated according to eqn. 11. The results agreed with the experimental data reported in Table I to within ±2%.

It is important to emphasize that this treatment is based on the assumption that solute molecular diffusion is the only band-broadening mechanism in CZE. In practice many experimental parameters such as sample introduction [20], distortion of the flat flow profile by capillary walls [21], solute concentration, column length and diameter [5] and Joule's heating [22,23] can adversely affect column efficiency. Nevertheless, it is instructive to look at column efficiency and resolution based on this assumption since it is believed that all of these disruptive factors could be experimentally minimized or perhaps eliminated.

The resolution as a function of applied voltage and buffer concentration $R_s(C,V)$ is given by eqn. 12. Unlike chromatographic techniques where better resolution is achieved at the expense of longer analysis time, eqn. 12 suggests that resolution in CZE is not necessarily compromised if the analysis time is shortened. This appears to be in apparent contradiction with the Jorgenson and Lukacs [3] conclusion that resolution improves with increase in analysis time; however, it is to be noted that in their analysis they compared different columns with different electroosmotic properties while our analysis is based on using the same column and keeping all experimental parameters except applied voltage and buffer concentration constant.

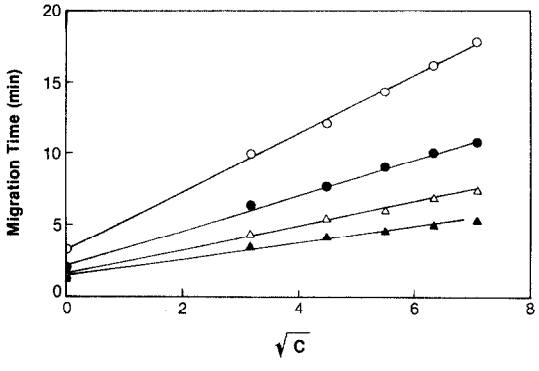

Fig. 2. Migration time for dansyl methionine *versus* square root of buffer concentration at different constant applied voltages. Experimental conditions as in Fig. 1. ○ = 10 kV; ● = 15 kV; △ = 20 kV; ▲ = 25 kV.

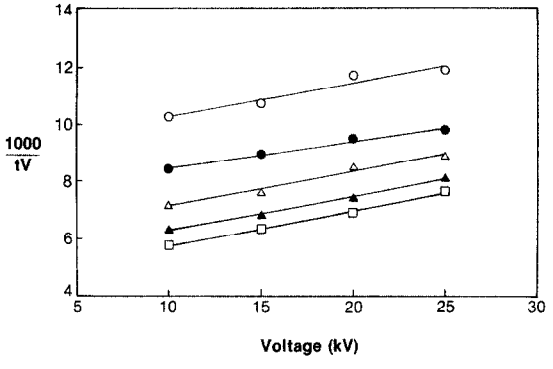

Fig. 3. Migration time for dansyl methionine as a function of applied voltage at different buffer concentrations. Experimental conditions as in Fig. 1. ○ = 10 m*M*; ● = 20 m*M*; △ = 30 m*M*; ▲ = 40 m*M*; □ = 50 m*M*.

TABLE II

VALUES OF THE COEFFICIENTS OF EQNS. 9 AND 10

Solute	a_0	a_1	a_2 ($\times 10^3$)	b_0	b_1	b_2 ($\times 10^3$)	m_0 ($\times 10^3$)	m_1 ($\times 10^3$)	m_2 ($\times 10^4$)	n_0 ($\times 10^4$)	n_1 ($\times 10^5$)	n_2 ($\times 10^5$)
Leucine	6.716	−0.456	9.680	4.246	−0.279	5.280	10.998	−0.217	0.017	0.744	0.156	−0.001
Methionine	6.662	−0.452	9.590	4.387	−0.289	5.480	10.917	−0.220	0.018	0.705	0.183	−0.001

The effect of applied voltage on resolution was investigated by several groups [5,6,24–27]. By all accounts experimentally determined resolution improves with increasing voltage, up to a certain point beyond which the voltage is so high as to contribute to zone broadening by Joule's heating effect. The effect of buffer concentration is not as dramatic as that of applied voltage [15,25,27]; however, it has been reported that resolution slightly increases with increasing buffer concentration [15,27].

In this work R_s values for dansyl methionine/dansyl alanine were calculated according to eqn. 12 given that the length of column used is 50 cm and assuming a value of $1 \cdot 10^{-5}$ cm^2 s^{-1} for the solute diffusion coefficient. The resolution was, then, plotted as a function of C and V resulting in the resolution surface shown in Fig. 4. Isochrons (*i.e.* lines of constant analysis time) were also calculated and plotted in Fig. 4 (dark lines). These lines are used for the prediction of the behavior of the resolution under constant analysis time. The R_s–V plane in this figure is featureless since R_s continuously increase with increase in V at all concentrations. With regards to buffer concentration effects the following observations are noted. At the high-voltage border, R_s decreases continuously with increasing concentration; however, at the low-voltage border R_s passes through a shallow maximum as the concentration is increased. For example, at 10 kV, R_s is maximum at C = 35 mM, while at 15 kV the maximum R_s value is at C = 20 mM. This behavior is mainly due to the fact that $\Delta T(C,V)$ is small at high voltages and only marginally increases with increasing concentration, while at low voltages ΔT is large and shows a relatively larger increase with increasing concentration. The isochronal lines show that at constant analysis time resolution is optimized by simultaneously increasing the voltage and the buffer concentration. A slight increase in voltage necessitates a large increase in buffer concentration in order to achieve higher resolution under isochronal conditions.

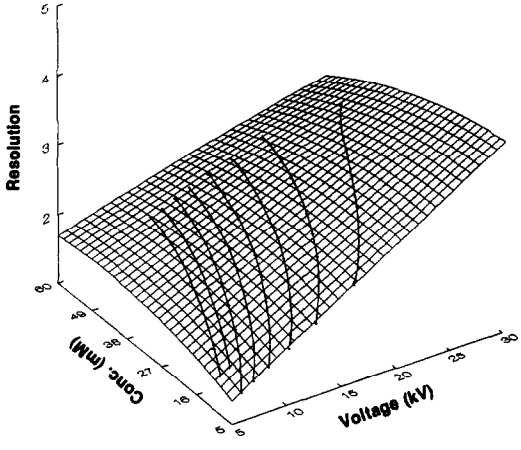

Fig. 4. Calculated resolution surface for dansyl methionine/dansyl leucine in applied voltage and buffer concentration space. The dark non-grid lines on the surface are isochrons, from 300 s (lower border) to 1000 s (higher border) in increments of 100 s.

Finally, in order to compare this theoretical prediction of R_s with experimental determination we measured R_s from migration times and peak widths at half-height, and examined the trends in R_s as V and C are changed. It is observed that: (a) the experimental R_s values are consistently about 40–50% lower than the theoretical calculation, indicating that zone-broadening mechanisms other than solute diffusion are also operative in the experimental set-up used in this study; (b) R_s increases with increasing V in agreement with theory, however, R_s levels off at the high-voltage border; and (c) R_s increases with increasing buffer concentration in agreement with the theoretical prediction at the low-voltage border.

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