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Buffer effects in the desionoselective/ionoselective/ duoselective separation selectivity model-assisted optimization of the capillary electrophoretic separation of enantiomers I. Low-pH phosphate buffers

Robert L. Williams, Gyula Vigh*

Department of Chemistry, Texas A&M University, College Station, TX 77843-3255, USA

Abstract

The complexation of aromatic permanently charged anions by β-cyclodextrin in capillary electrophoresis has been studied using phosphate background electrolytes in which the pH was varied from 2.0 to 3.6. The complex formation constants and ionic mobilities proved independent of the pH, indicating that the electrophoretically determined parameters of the multiple equilibria-based separation selectivity model (DID model) are meaningful and should be suitable for the optimization of enantiomer separations. Therefore, the effective mobilities of the enantiomers of α-methoxy-α-trifluoromethyl phenylacetic acid, suspected to belong to the families of ionoselective or duoselective separations, were measured as a function of pH and β-cyclodextrin concentration in the $2 < pH < 4$ and $0 < c_{CD} < 15$ mM ranges in phosphoric acid-based background electrolytes. The effective mobilities were fitted to the DID model to extract the respective acid dissociation and complex formation constants and ionic mobilities. Peak resolution for the duoselective separation was calculated with these parameters as a function of pH and the β-cyclodextrin concentration of the background electrolyte, as well as the dimensionless electroosmotic flow coefficient. The shapes of the predicted and observed peak resolution curves agreed well, demonstrating that the DID model can be used for the optimization of the CE separations of enantiomers.

1. Introduction

Over the last few years the tools available for the quantitative description of peak resolution in the cyclodextrin-mediated capillary electrophoretic (CE) separations of enantiomers [1–10] have improved significantly. First, a resolution equation has been proposed which describes peak resolution, R_s, as a function of the effective part of the applied potential, separation selectivity, and the effective charge of the analyte [1], as well as the dimensionless electroosmotic flow [2]. Second, equilibrium models have been presented to describe the mobility of the enantiomers as a function of the cyclodextrin (CD) concentration [3–5] of the background electrolyte (BGE). Subsequently, it was shown that not only the CD concentration, but also the hydronium ion concentration of the BGE plays a crucial role in the

* Corresponding author.

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success of the separation, and the desionoselective/ionoselective/duoselective separation selectivity model (DID model) was introduced [6–10] to aid in the rational selection of the optimum separation conditions.

It was demonstrated that the acid dissociation and complex formation constants, as well as the ionic mobilities of the free and cyclodextrin-complexed enantiomers can be determined from three simple sets of CE experiments [2,6–10]. The first set has to be carried out in the absence of CD by varying the pH of the BGE and evaluation of the measured mobilities yields the pK_a and μ^0 values. In the second set of experiments, the CD concentration is varied while pH is kept constant at a value where the analyte is fully ionized. This set of experiments yields the complexation constants for the dissociated forms of the analyte. In the third set, the CD concentration is varied again while the pH is kept constant at a value where the analyte is about one third ionized. This set of experiments, together with the previously determined constants, yields the complexation constants for the non-dissociated forms of the analyte.

Some concerns were expressed [11] about the possible effects that the competitively binding BGE components might have on the complexation constants one can obtain from CE mobility measurements as the pH of the background electrolyte is changed from a value where the analyte is fully dissociated to a value where the analyte is only one third dissociated. The present paper addresses this concern in the case of one of the most frequently used CE buffering systems, the phosphate buffer, and shows the utility of the DID model and the experimentally determined model parameters in the optimization of the separation of the enantiomers of a weak acid analyte that displays duoselective separation behavior.

2. Experimental

A diode-array detector equipped P/ACE 5500 system (Beckman Instruments, Fullerton, CA, USA) was used for the mobility measurements, with the detector electrode kept at high positive potential. The thermostatting cartridge temperature was 37°C. Untreated, 25 μm and 50 μm I.D., 180 μm O.D. (40.7 cm from injector to detector, 47.4 cm total length) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) and 50 μm I.D., 375 μm O.D. coated capillaries with a neutral coating (eCAP Neutral Capillary, P/N 477441, Beckman Instruments) and an amine coating (eCAP Amine Capillary, P/N 477430, Beckman Instruments) were used for the separations. The field strength was varied between 100 and 256 V/cm to maintain power dissipation levels below 500 mW/m.

BGEs of 100 m*M* balanced cation-concentration [2,8–10] were prepared by weighing the required amount of LiOH (Aldrich, Milwaukee, WI, USA) into the volumetric flask and adjusting the pH of the solution with phosphoric acid (Aldrich) in such a way that the total cation concentration (hydronium ion concentration plus lithium ion concentration) remained constant. β-Cyclodextrin (American Maize Products, Hammond, IN, USA) was used as chiral resolving agent. To minimize the risk of cyclodextrin hydrolysis at very low pH values (hydrolysis half-life at pH = −0.133 and 40°C: 48 days [14]), the BGEs were freshly prepared before use. The test solutes included *p*-toluenesulfonic acid (PTSA) and 4-ethylbenzene sulfonic acid (EBSA) (Aldrich) as permanently charged anionic markers, α-methoxy-α-trifluoromethyl phenylacetic acid (MTPA) (a generous gift from H. Billiet, Technical University of Delft, Netherlands) as chiral probe, and benzyl alcohol as the electroosmotic flow marker. The initial 0.3-min long potential rise time of the P/ACE 5500 was taken into account in the calculation of the correct mobility values [12].

The parameters of the mobility equation were evaluated with the TableCurve 2D software package (Jandel Scientific, San Rafael, CA, USA). They were then used to calculate the mobility, effective charge, selectivity and resolution surfaces with the Origin Ver. 3.5 software package (MicroCal, Northampton, MA, USA) running on a 486DX4 100 MHz 20 MB RAM

personal computer (ARM Computers, San Jose, CA, USA).

3. Results and discussions

3.1. Formation constants for the cyclodextrin complexes of permanently charged anions in $2.0 < pH < 3.6$ phosphate BGEs

According to Ref. [1], the effective mobility of an analyte can be described as a linear combination of the ionic mobilities and the mole fractions of all possible forms of the species present. For a permanently charged anion, P^-, which complexes with CD, the effective mobility, μ_P^{eff}, becomes:

$$\mu_P^{eff} = \frac{\mu_{P^-}^0 + \mu_{PCD^-}^0 K_{PCD^-}[CD]}{1 + K_{PCD^-}[CD]} \quad (1)$$

where $\mu_{P^-}^0$ and $\mu_{PCD^-}^0$ are the ionic mobilities of the free and the complexed forms of P^-, K_{PCD^-} is the formation constant for the cyclodextrin complex of P^-, and [CD] is the cyclodextrin concentration, respectively. When the analytical concentration of P^- is much smaller than the analytical concentration of CD, and the BGE components do not complex strongly with CD, or when the analytical concentration of the BGE components is high [6], the analytical concentration of CD, c_{CD} can be used instead of [CD]. If it can be shown that the $\mu_{PCD^-}^0$ and K_{PCD^-} values for a series of weakly binding permanently charged anions remain constant in cation concentration-balanced [2] phosphate BGEs of varying pH, the K and μ^0 values of the more strongly binding analyte enantiomers will also remain independent of the pH of the phosphate BGE and be suitable for the optimization of the resolution of the enantiomers.

Thus, a series of phosphate BGEs were prepared with pH values varying between 2.0 and 3.6. β-Cyclodextrin was dissolved in these BGEs to obtain twelve different CD concentrations in the 0 to 15 mM range. The μ_P^{eff} effective mobilities of the two aromatic sulfonate test solutes, PTSA and EBSA, were determined from μ_P^{obs}, their observed mobilities, and μ_{eo}, the coefficient of the electroosmotic flow, as:

$$\mu_P^{obs} = \mu_P^{eff} + \mu_{eo} \quad (2)$$

The μ_P^{eff} values (at least five replicate measurements at each cyclodextrin concentration level) were then fitted to Eq. 1 to calculate parameters $\mu_{PCD^-}^0$ and K_{PCD^-} as listed in Table 1. The K_{PCD^-} values are small (about 100) for PTSA, moderate for EBSA (about 330). There is no clearly discernible trend in either the $\mu_{PCD^-}^0$ or the K_{PCD^-} values as pH is increased from 2.0 to 3.6. This indicates that the components of the phosphate buffer have a negligible effect on the ionic mobilities and complex formation constants of even the weakly binding PTSA. Consequently, the DID model and its parameters should be suitable for the optimization of the separation of enantiomers whenever the K values are at least 100 or larger.

3.2. Formation constants for the cyclodextrin complexes of the MTPA enantiomers

A previous CE study on the separation of the enantiomers of nine mandelic acid analogs [13] found that only the enantiomers of MTPA and 2-phenylpropionic acid could be separated with β-cyclodextrin as resolving agent. Since these separations occurred at high pH, well above the expected pK_a values of the acids, according to the DID separation selectivity model the separations must behave as either *ionoselective* or *duoselective* separations [2,6–10]. (In an *ionoselective* separation, only the dissociated forms of the enantiomers complex selectively. In a *duoselective* separation, both the dissociated and the non-dissociated forms of the enantiomers complex selectively [2,6–10].) Therefore, it was of interest to see if this suspected separation type assignment, based on a limiting pK_a value (less than 6) and a single resolution value, was correct because, if so, the separation can be optimized very simply.

According to Refs. [2,6–10], the effective

Table 1
Complex formation constants and ionic mobilities for permanently charged anions in phosphate BGEs at different pH values

Solute	pH	μ_P^0 ($\cdot 10^{-5}$ cm^2/Vs)	$\mu_{PCD^-}^0$ ($\cdot 10^{-5}$ cm^2/Vs)	K_{PCD^-}
PTSA	2.0	32.6 ± 0.1	7.3 ± 0.4	90 ± 6
	2.5	32.1 ± 0.1	7.8 ± 0.3	102 ± 5
	2.9	32.1 ± 0.1	7.8 ± 0.6	106 ± 6
	3.6	32.1 ± 0.1	7.6 ± 0.7	95 ± 6
EBSA	2.0	30.6 ± 0.1	8.7 ± 0.2	323 ± 6
	2.5	29.9 ± 0.1	8.1 ± 0.1	316 ± 9
	2.9	30.0 ± 0.1	8.2 ± 0.2	337 ± 10
	3.6	30.1 ± 0.1	8.4 ± 0.2	341 ± 16

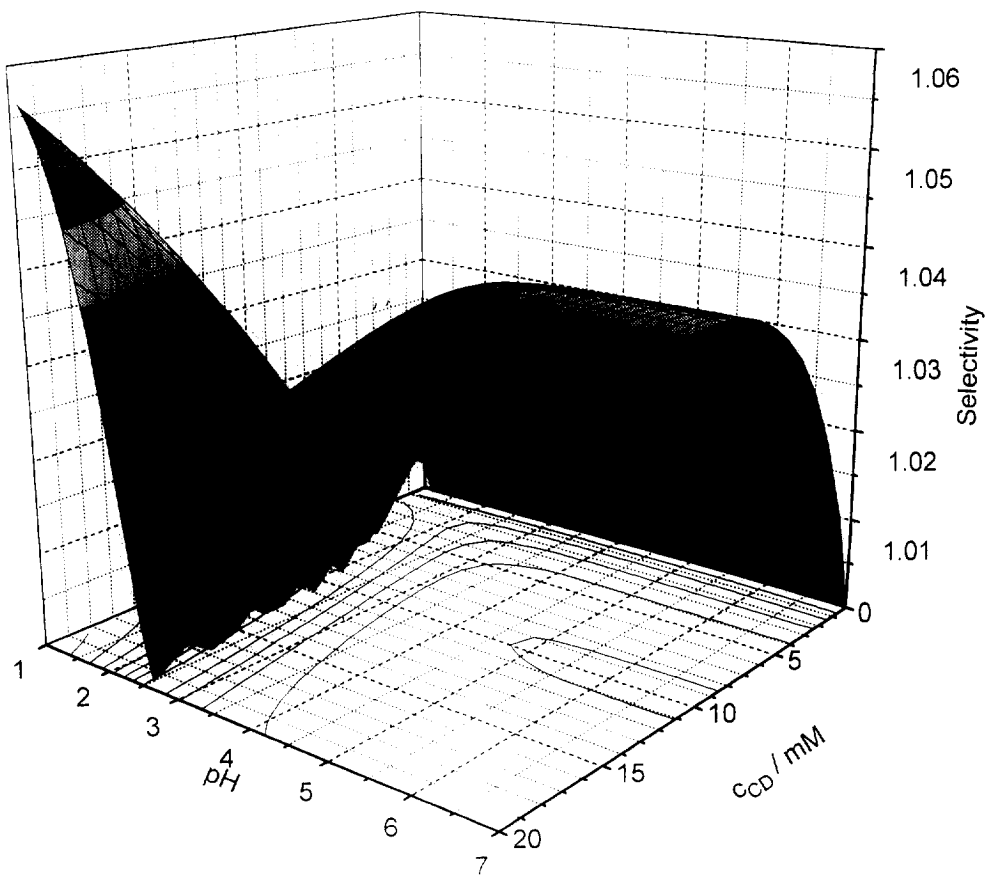

Fig. 1. Selectivity surface for the separation of the enantiomers of MTPA as a function of the β-cyclodextrin concentration and the pH in 100 m*M* cation concentration-balanced phosphate BGEs. The surface was calculated with Eq. 5 and the constants in Table 2.

Table 2
Estimated model parameter values for MTPA

μ_-^0 (10^{-5} cm^2/Vs)	25.0 ± 0.2
$10^2 K_a$	3.1 ± 0.1
pK_a	1.5
$\mu_{RCD^-}^0$ (10^{-5} cm^2/Vs)	7.1 ± 0.2
$\mu_{SCD^-}^0$ (10^{-5} cm^2/Vs)	7.4 ± 0.2
$K_{RCD^-}^0$	127 ± 4
$K_{SCD^-}^0$	149 ± 4
K_{HRCD}	1257 ± 19
K_{HSCD}	1292 ± 15

mobility of a weak acid *R*-enantiomer, μ_R^{eff}, is:

$$\mu_R^{eff} = \frac{\mu_-^0 + \mu_{RCD^-}^0 K_{RCD^-}[CD]}{1 + K_{RCD^-}[CD] + \frac{[H_3O^+]}{K_a}(1 + K_{HRCD}[CD])} \quad (3)$$

where μ_-^0 and $\mu_{RCD^-}^0$ are the ionic mobilities of the fully dissociated free and CD-complexed

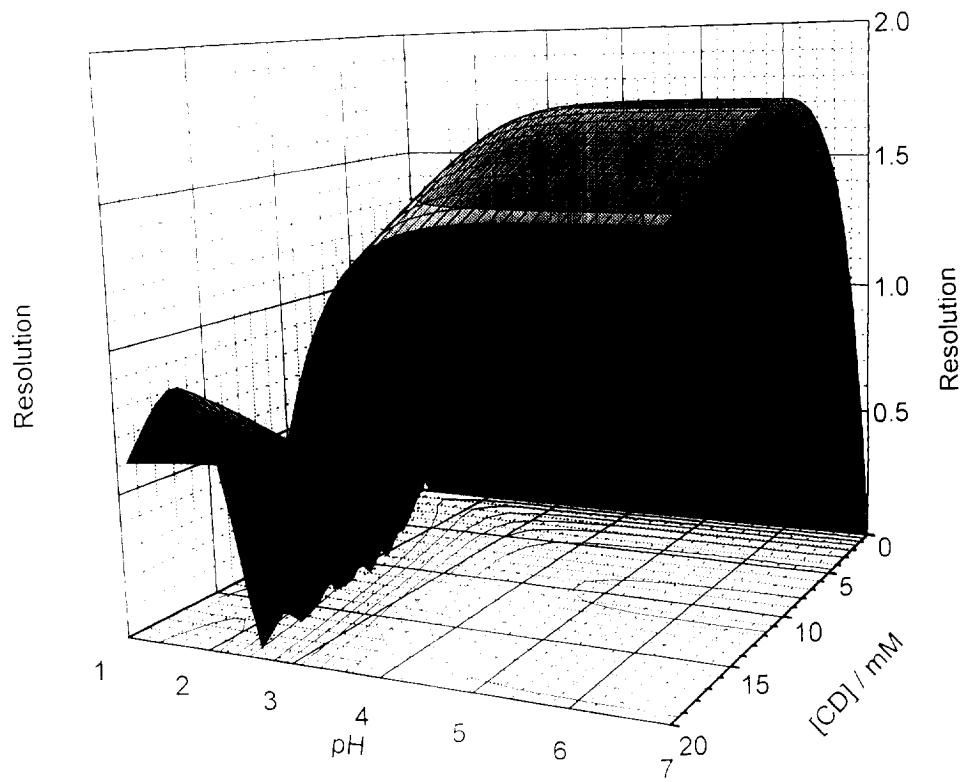

Fig. 2. Peak resolution surface for the separation of the enantiomers of MTPA as a function of the β-cyclodextrin concentration and the pH in 100 m*M* cation concentration-balanced phosphate BGEs. The surface was calculated with Eq. 6 and the constants in Table 2 for $\beta = 0$ and $E = 265$ V/cm, $T = 310$ K, and $l = 40.7$ cm.

enantiomers, K_a is the acid dissociation constant of the enantiomer, K_{RCD^-} and K_{HRCD} are the formation constants for the cyclodextrin complexes of the dissociated and non-dissociated forms of the enantiomer, and $[H_3O^+]$ and [CD] are the hydronium ion and cyclodextrin concentrations. The parameters in Eq. 3 have been determined as outlined in the Introduction, and described in detail in [2,6–10]. The results, shown in Table 2, indicate that the separation of the enantiomers of MTPA belongs to the family of duoselective separations ($K_{RCD^-} \neq K_{SCD^-}$, $K_{HRCD} \neq K_{HSCD}$, and $\mu^0_{RCD^-} \neq \mu^0_{SCD^-}$), as expected on the basis of the single-pH resolution data in [13].

3.3. Separation selectivity and peak resolution surfaces

Once the K values are known, the absolute value of effective charge of the enantiomer, Z^{eff}, can be calculated as [2]:

$$Z_R^{eff} = \frac{Z^0_- + Z^0_{RCD^-} K_{RCD^-}[CD]}{1 + K_{RCD^-}[CD] + \frac{[H_3O^+]}{K_a}(1 + K_{HRCD}[CD])} \quad (4)$$

where Z^0_- and $Z^0_{RCD^-}$ are the absolute values of

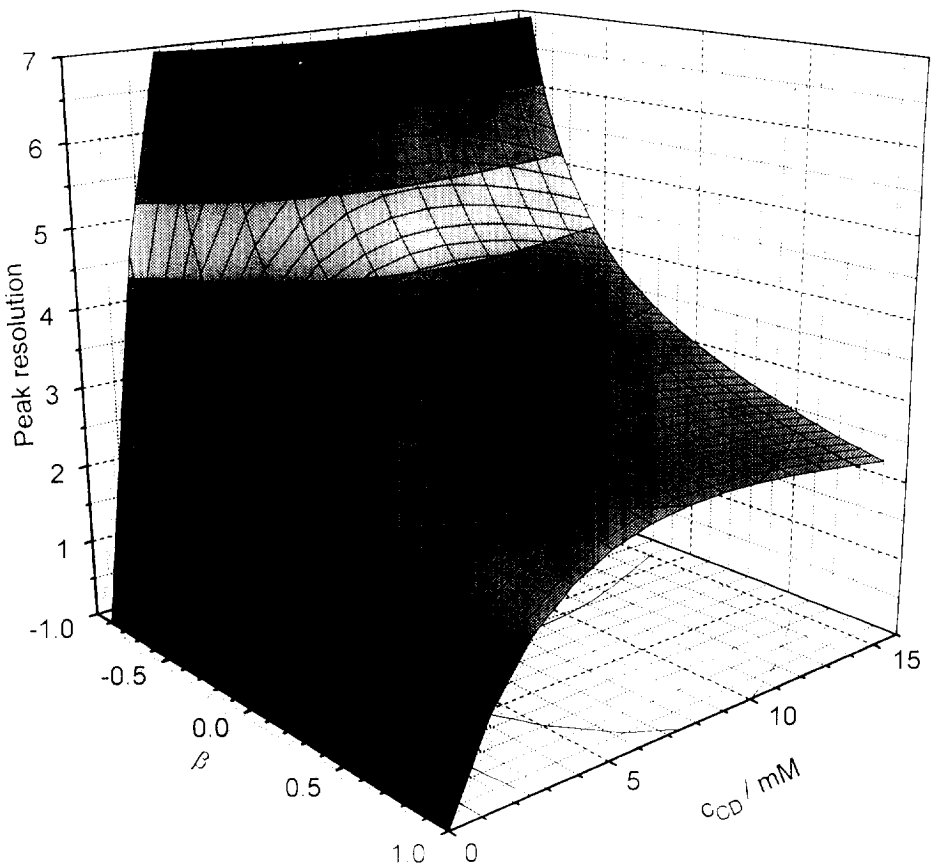

Fig. 3. Peak resolution surface for the separation of the enantiomers of MTPA as a function of pH and β in 100 mM cation concentration-balanced phosphate BGEs. The surface was calculated with Eq. 6 and the constants in Table 2 for $c_{CD} = 15$ mM, $E = 265$ V/cm, $T = 310$ K, and $l = 40.7$ cm.

the ionic charges of the fully dissociated free and CD-complexed enantiomers, respectively. Separation selectivity, α, the ratio of the effective mobilities of the two enantiomers, can be calculated as [6]:

$$\alpha = \frac{\mu_-^0 + \mu_{RCD}^0 \cdot K_{RCD} \cdot [CD]}{\mu_-^0 + \mu_{SCD}^0 \cdot K_{SCD} \cdot [CD]} \frac{1 + K_{SCD} \cdot [CD] + \frac{[H_3O^+]}{K_a}(1 + K_{HSCD}[CD])}{1 + K_{RCD} \cdot [CD] + \frac{[H_3O^+]}{K_a}(1 + K_{HRCD}[CD])} \quad (5)$$

assuming, for the sake of discussion, that the *S*-enantiomer is the less mobile enantiomer. If α and Z^{eff} are known, peak resolution can be obtained [2,8–10] as:

$$R_s = \sqrt{\frac{Ele_0}{8kT}} \frac{\text{abs}(\alpha - 1)\sqrt{\text{abs}(\alpha + \beta)}\sqrt{\text{abs}(1 + \beta)}\sqrt{Z_R^{eff}}\sqrt{Z_S^{eff}}}{\sqrt{\text{abs}[(\alpha + \beta)^3]Z_R^{eff}} + \sqrt{\alpha\ \text{abs}[(1 + \beta)^3]Z_S^{eff}}}, \quad (6)$$

where E is the field strength, l the effective length of the capillary, e_0 the electric charge, k the Boltzman constant, T the absolute temperature, and β the dimensionless electroosmotic flow coefficient, defined as:

$$\beta = \frac{\mu_{eo}}{\mu_S^{eff}} \quad (7)$$

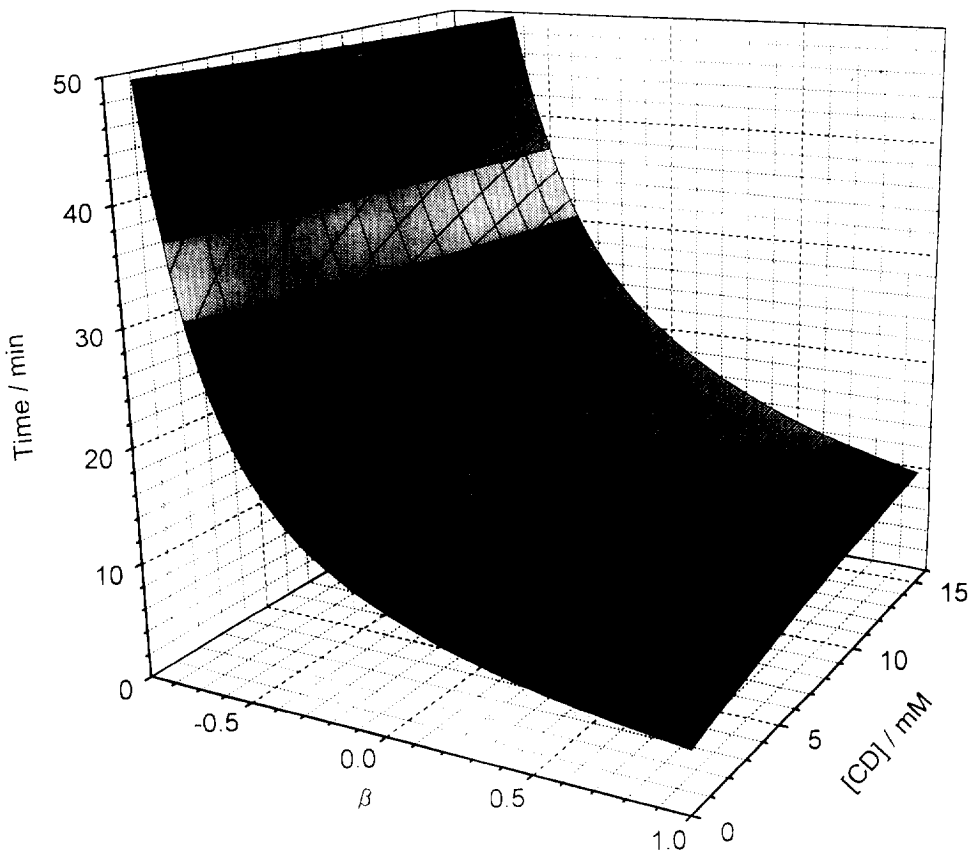

Fig. 4. Separation time surface for the resolution of the enantiomers of MTPA as a function of pH and β in 100 m*M* cation concentration-balanced phosphate BGEs. The surface was calculated with Eq. 6 and the constants in Table 2 for $c_{CD} = 15$ m*M*, $E = 265$ V/cm, $T = 310$ K, and $l = 40.7$ cm.

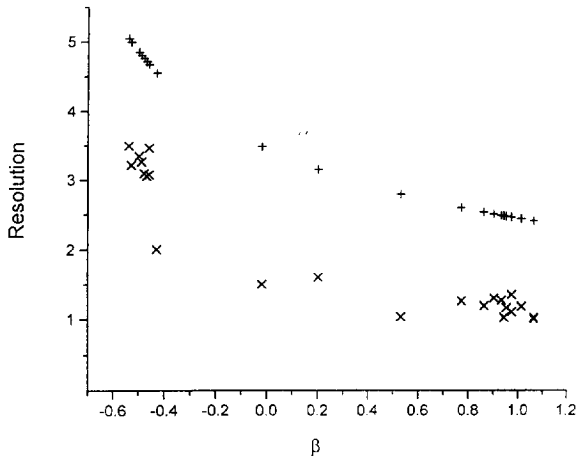

Fig. 5. Comparison of the predicted (symbol +) and measured (symbol ×) R_s values for MTPA as a function of β in 100 mM cation concentration-balanced phosphate, 15 mM β-cyclodextrin BGEs. The measured values were obtained using $E = 265$ V/cm, with β values varying in the -0.6 to 1.06 range.

The separation selectivity surface for MTPA is shown in Fig. 1 as a function of c_{CD} and pH. When the pH is at least 1 unit above the pK_a, selectivity passes a maximum as a function of c_{CD}. If the CD concentration is kept constant close to the solubility limit, $c_{CD} = 15$ mM, and pH is decreased from 3.6, selectivity first remains constant, then decreases, becomes unity, then the migration order reverses and α increases again. The features of this selectivity surface agree with the theoretically predicted features of a *duoselective* enantiomer separation [6].

The peak resolution surface calculated for $\beta = 0$ (no electroosmotic flow) is shown in Fig. 2. The two lobes of the resolution surface are separated by an $R_s = 0$ valley in the vicinity of the pK_a value. On the primary lobe, above the pK_a, there is an R_s maximum at $c_{CD} = 10$ mM. The separation is rugged in terms of the pH variable. On the secondary lobe, R_s increases as c_{CD} is increased toward the solubility limit. However, as pH is decreased further, R_s passes through a local maximum at pH = 1.75 because the effective charge is lost. The migration order is opposite on the two lobes. The R_s surface in Fig. 2 agrees with the theoretically predicted resolution surface of a *duoselective* separation and indicates that the enantiomers can be readily separated as long as the pH is kept at least one pH unit above the pK_a.

Since the separation is superior on the primary lobe, it is instructive to see the effects of varying β on both R_s and the separation time. For this calculation $E = 256$ V/cm and $c_{CD} = 15$ mM were chosen, and β was varied in the -1 to 1 range. As it can be seen in Fig. 3, R_s improves dramatically as one approaches the $\beta = -1$ value. However, the separation time also increases

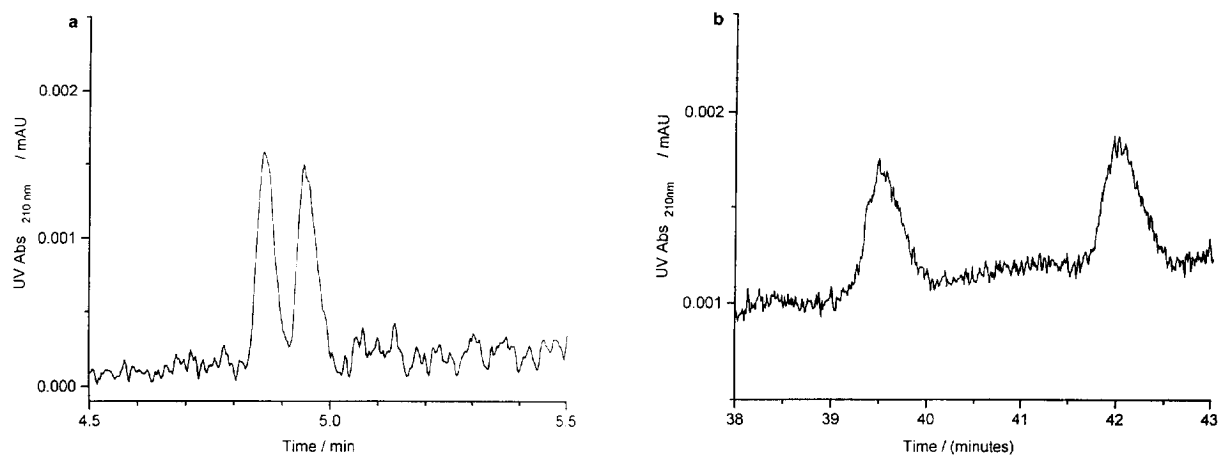

Fig. 6. Electropherograms of an MTPA sample, obtained in 100 mM cation concentration-balanced phosphate, pH 3.6, 15 mM β-cyclodextrin BGEs, with $\beta = 1.06$ (a) and $\beta = -0.6$ (b). $E = 256$ V/cm.

dramatically (Fig. 4). A good compromise could be obtained by selecting a $\beta = -0.6$ value: compared to $\beta = 1$, R_s can be more than doubled for a four- to five-fold increase in the separation time.

In Fig. 5 the theoretically predicted (symbol +) and actually measured (symbol ×) R_s values are compared for different dimensionless electroosmotic flows (β). Both curves follow the same trend, but the measured R_s values are about 20 to 30% lower, because the shape of the analyte peak is distorted by electromigration dispersion. The electropherograms of MTPA at $\beta = -0.6$ and 1.06 are compared in Fig. 6.

4. Conclusions

The complexation equilibria-based DID model was used to describe the mobilities of permanently charged anions and MTPA as a function of the cyclodextrin concentration and pH of phosphate BGEs. The complex formation constants and ionic mobilities of the permanently charged anions remain constant while the pH of the phosphate BGE is varied in the 2.0 to 3.6 range. This indicates that the formation constants obtained for the moderately or strongly complexing analyte enantiomers are also independent of the pH of the phosphate buffer and can be used for optimization of the separation. The separation of MTPA represents a duoselective enantiomer separation, because all four complexation constants are different. Rugged separations can be achieved on the primary lobe of the resolution surface (at pH values at least one unit above the pK_a of MTPA). By selecting negative β values around the -0.6 level, R_s can be easily doubled at the cost of quadrupled separation time. The measured peak resolution values are 20–30% lower than the theoretically predicted ones, because the separation is plagued by electromigration dispersion. Nevertheless, the DID model can be used both for the rationalization of the migration behavior of the MTPA enantiomers and the selection of the optimum separation conditions.

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