

Properties of Multivariate Binding Isotherms in Capillary Electrophoresis

Michael T. Bowser, Andrea R. Kranack, and David D. Y. Chen*

Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Z1

When more than one complexation additive is used in capillary electrophoresis (CE), the migration behavior of an analyte can be described using contour plots and profile plots of the multivariate binding isotherms. At a certain concentration of one additive, the net electrophoretic mobility of the analyte is not affected by the concentration of the second additive, even though the second additive does alter the mobility of the analyte when used alone. The concentration of the first additive, in this situation, is defined as the dengsu concentration (dengsu means "same speed" in Chinese). The presence of a dengsu concentration for one additive is a strong indication that the second additive interacts with the analyte in a 1:1 (analyte–additive) stoichiometry. The binding isotherms in a profile plot can be used to unambiguously determine the binding stoichiometry of the analytes, as well as to determine the effect of interactions between the additives. The apparent complex mobilities obtained from the profile plots can also be used to determine whether there are interactions between the additives or whether the analyte can bind both additives at the same time.

The use of complexation additives to modify analyte migration behavior in capillary electrophoresis (CE) has increased dramatically in recent years. Terabe et al. were the first to suggest the use of additives (surfactants) in CE with their development of micellar electrokinetic capillary chromatography (MECC).¹ Since then, applications using additives such as cyclodextrins,^{2–4} polyethers,^{5–7} and macrocyclic antibiotics^{8–10} have been reported.

Recent developments in separation science, considering interactions at the molecular level, have extended CE theory to the

point where analyte migration behavior can be described quantitatively. A number of papers discussing analyte–additive interactions in protein binding,¹¹ protein–sugar interactions,^{12,13} chiral separations,^{4,14,15} cyclodextrin complexes,^{3,8,16–22} glycopeptide interactions,²³ antigen–antibody complexes,²⁴ and nonaqueous separations^{25,26} have been published. All of these papers describe analyte migration behavior based on a dynamic equilibrium process between the analyte and a single additive with a 1:1 binding stoichiometry.

More complicated interactions, such as multiple-additive or multiple-stoichiometry equilibria, pose a greater challenge in the quantitative prediction of analyte mobility, since the net analyte mobility is determined by the fundamental parameters associated with each interaction. The accurate measurement of these parameters is crucial in correctly predicting the analyte mobility. Through the introduction of a viscosity correction factor, which normalized the observed mobilities to an ideal state where all mobility changes were caused by shifts in equilibria, Peng et al.²¹ provided the first quantitative description of analyte migration behavior in CE with more than one buffer additive. Kranack et al.²⁷ expanded on multiple additive CE by demonstrating the usefulness of separation systems utilizing both charged and uncharged cyclodextrins. Bowser and Chen²⁸ have demonstrated the effects of higher order interactions to account for analyte–

* To whom correspondence should be addressed. Tel.: 604-822-0878. Fax: 604-822-2847. E-mail: chen@chem.ubc.ca.

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additive interactions that take place with binding stoichiometries other than 1:1. The theories developed to account for multiple-additive and higher order interactions have recently been combined and put into the context of a unified separation science.²⁹

The aim of this paper is to discuss some of the unique features of multivariate binding isotherms using applied mathematics. Insight into the properties of these isotherms furthers our understanding of how the separation environment (i.e., buffer system, counterions, pH, other interactions, etc.) affects the chemical equilibria in CE separations and provides the basis required to describe multiple-additive CE systems. Although this is a purely theoretical discussion, comparisons can be made to the experimental binding isotherms presented by Peng et al.²¹ and Kranack et al.²⁷

DISCUSSION

1:1 Binding in the Presence of Two Additives. When binding occurs at a 1:1 (analyte–additive) stoichiometry with two additives present in a CE system, the analyte migration behavior can be described by the following equation:²¹

$$\nu\mu_{\text{ep}}^{\text{A}} = \frac{\mu_{\text{ep,A}} + K_{\text{AC}}[\text{C}]\mu_{\text{ep,AC}} + K_{\text{AD}}[\text{D}]\mu_{\text{ep,AD}}}{1 + K_{\text{AC}}[\text{C}] + K_{\text{AD}}[\text{D}]} \quad (1)$$

where ν is a correction factor, $\mu_{\text{ep}}^{\text{A}}$ is the net electrophoretic mobility of the analyte, $\mu_{\text{ep,A}}$ is the mobility of the uncomplexed analyte (A), C and D are the additives, K_{AC} and K_{AD} are the formation constants for the complexes AC and AD, and $\mu_{\text{ep,AC}}$ and $\mu_{\text{ep,AD}}$ are the electrophoretic mobilities of the complexes AC and AD, respectively. This model (eq 1) assumes that there are no interactions between additive C and D and that the analyte can only bind one of the additives at a time. Another assumption is that all equilibria take place at a fast rate in relation to the overall separation time. The subscripts denoting the two additives are slightly different from those presented by Peng et al.²¹ in order to more clearly describe the complicated cases discussed later in this paper. To observe the phenomena discussed in this paper, $\mu_{\text{ep}}^{\text{A}}$ must be corrected to account for any changes in the mobility of the analyte caused by factors other than the equilibrium.²¹ The most important factor to consider is the viscosity of the buffer, which often varies with the additive concentration.

Equation 1 describes a binding isotherm surface (with two variables) from which the net mobility of the analyte can be determined for any concentration of the additives C and D. Three-dimensional plots of isotherms calculated according to eq 1 have been presented elsewhere.^{21,27} While this presentation style is useful for a quick assessment of the shape of the isotherm, other presentation methods emphasize certain properties of the isotherm more clearly. One alternative presentation method is the use of a contour plot. For any isotherm surface described by eq 1, it is possible to draw contour lines, along which $\nu\mu_{\text{ep}}^{\text{A}}$ remains constant. A contour plot is made by plotting these contour curves at constant increments of $\nu\mu_{\text{ep}}^{\text{A}}$. Contour plots describe the surface in a manner similar to contour maps. Areas where the contour curves are close together represent portions of the surface with a high slope; areas where the contour curves are farther apart

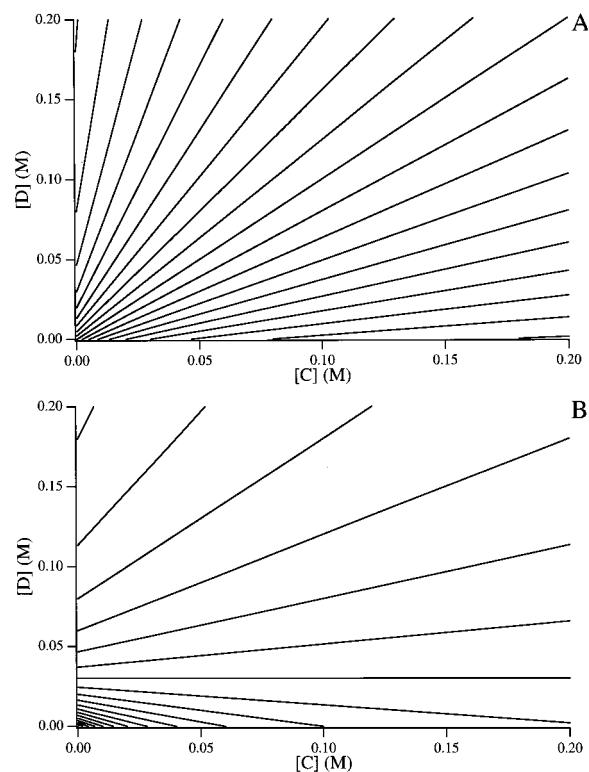


Figure 1. Contour plots of a binding isotherm surface where both additives interact with the analyte with 1:1 binding stoichiometries (eq 1). The additives shift $\nu\mu_{\text{ep}}^{\text{A}}$ in opposite directions in (A) and in the same direction in (B). The constants are $K_{\text{AC}} = 50 \text{ M}^{-1}$; $K_{\text{AD}} = 50 \text{ M}^{-1}$; $\mu_{\text{ep,A}} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{\text{ep,AD}} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; and $\mu_{\text{ep,AC}} =$ (A) -5×10^{-5} and (B) $3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. In (A), the contour curves range from $\nu\mu_{\text{ep}}^{\text{A}} = -4.5 \times 10^{-5}$ to $4.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, with increments of $0.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ between the contour lines. In (B), the contour curves range from $\nu\mu_{\text{ep}}^{\text{A}} = 0$ to $4.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, with increments of $0.25 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ between the contour lines.

represent flatter portions of the surface. Figure 1 shows an example of a pair of contour plots which describe binding isotherm surfaces made according to eq 1. The slope of the contour curve for any $\nu\mu_{\text{ep}}^{\text{A}}$ is equal to the partial derivative of [D] with respect to [C]:

$$\frac{\partial[\text{D}]}{\partial[\text{C}]} = -\frac{K_{\text{AC}}(\nu\mu_{\text{ep}}^{\text{A}} - \mu_{\text{ep,AC}})}{K_{\text{AD}}(\nu\mu_{\text{ep}}^{\text{A}} - \mu_{\text{ep,AD}})} \quad (2)$$

Because eq 2 is independent of either [C] or [D], the contour curves for any isotherm described by eq 1 (at any $\nu\mu_{\text{ep}}^{\text{A}}$) will always be linear, as shown in Figure 1.

There are two cases to consider when describing the binding isotherm surfaces for systems with two additives. One case is when the two additives have an opposite effect on the net analyte mobility (i.e., one additive increases the net analyte mobility and the other decreases the net analyte mobility), as shown in Figure 1A. The contour curve passing through the origin corresponds to $\nu\mu_{\text{ep}}^{\text{A}} = \mu_{\text{ep,A}}$. It is possible to adjust [C] and [D] along the contour curve without changing the net mobility of the analyte. Other contour lines in Figure 1 indicate the [C] and [D] coordinate pairs necessary to set $\nu\mu_{\text{ep}}^{\text{A}}$ to those mobility values.

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The intercepts of the contour lines are determined by the interaction between the analyte and the individual additives. The analyte mobility can range from $\mu_{ep,A}$ to $\mu_{ep,AC}$ using additive C alone and from $\mu_{ep,A}$ to $\mu_{ep,AD}$ using only additive D. If both additives are used, $\nu\mu_{ep}^A$ can range from $\mu_{ep,AD}$ to $\mu_{ep,AC}$. According to eq 2, when the additives shift the net mobility of the analyte in opposite directions, the slope of the contour curves will be positive over the entire range of $\nu\mu_{ep}^A$ values. This is expected because the ranges of mobilities obtainable by each additive do not overlap. The mobilities obtained using only additive C cannot be achieved using additive D alone; therefore, it is not possible for a contour line to intersect both the [C] and [D] axes (i.e., have a negative slope). According to eq 2, for the slope of a contour curve to be zero or infinity (i.e., perpendicular to one of the concentration axes), the net mobility of the analyte must be equal to $\mu_{ep,AD}$ or $\mu_{ep,AC}$. Although the net analyte mobility is limited by $\mu_{ep,AD}$ and $\mu_{ep,AC}$, $\nu\mu_{ep}^A = \mu_{ep,AD}$ or $\mu_{ep,AC}$ only at infinite concentrations of that additive. For practical purposes, the slope of the contour lines for binding isotherms described by eq 1 will not be equal to zero or infinity when the two additives shift the analyte mobility in opposite directions.

The other case to be considered is illustrated in Figure 1B, where both additives shift the net mobility of the analyte in the same direction. In this example, $|\mu_{ep,AD} - \mu_{ep,A}| > |\mu_{ep,AC} - \mu_{ep,A}|$. Because both additives shift the analyte mobility in the same direction, and $|\mu_{ep,AD} - \mu_{ep,A}| > |\mu_{ep,AC} - \mu_{ep,A}|$, $\nu\mu_{ep}^A$ can range from $\mu_{ep,A}$ to $\mu_{ep,AD}$. According to eq 2, the slope of the contour lines will be negative when $\nu\mu_{ep}^A < \mu_{ep,AC}$. Over this range, it is possible to reach a certain $\nu\mu_{ep}^A$ using either additive alone. Therefore, the contour lines will intersect both the [C] and the [D] axes. When $\nu\mu_{ep}^A > \mu_{ep,AC}$, it is impossible to reach $\nu\mu_{ep}^A$ using only additive C. The contour lines for this range will not cross the [C] axis. There is a special case when $\nu\mu_{ep}^A = \mu_{ep,AC}$. According to eq 2, the slope of the contour line corresponding to $\nu\mu_{ep}^A = \mu_{ep,AC}$ is equal to 0, indicating that, at a certain concentration of D, the net mobility of the analyte will not be affected by the concentration of additive C. This has been referred to previously as a dengsu point.²¹ Dengsu means "same speed" in Chinese. The additive concentration at which a dengsu point occurs is referred to as the dengsu concentration and is denoted by a subscript D (e.g., [D]_D). At the dengsu concentration, the slope of the binding surface perpendicular to the [D] axis is equal to 0. The dengsu concentration can, therefore, be determined by setting the partial derivative of $\nu\mu_{ep}^A$ with respect to [C] to 0 and solving for [D]:

$$\frac{\partial(\nu\mu_{ep}^A)}{\partial[C]} = \frac{K_{AC}(\mu_{ep,AC} - \mu_{ep,A}) + K_{AC}K_{AD}[D](\mu_{ep,AC} - \mu_{ep,AD})}{(1 + K_{AC}[C] + K_{AD}[D])^2} = 0 \quad (3)$$

$$[D]_D = -\frac{(\mu_{ep,AC} - \mu_{ep,A})}{K_{AD}(\mu_{ep,AC} - \mu_{ep,AD})} \quad (4)$$

Equation 4 is identical with that proposed by Peng et al.²¹ Equation 4 will give a positive concentration only when both additives shift the mobility of the analyte in the same direction and $|\mu_{ep,AD} -$

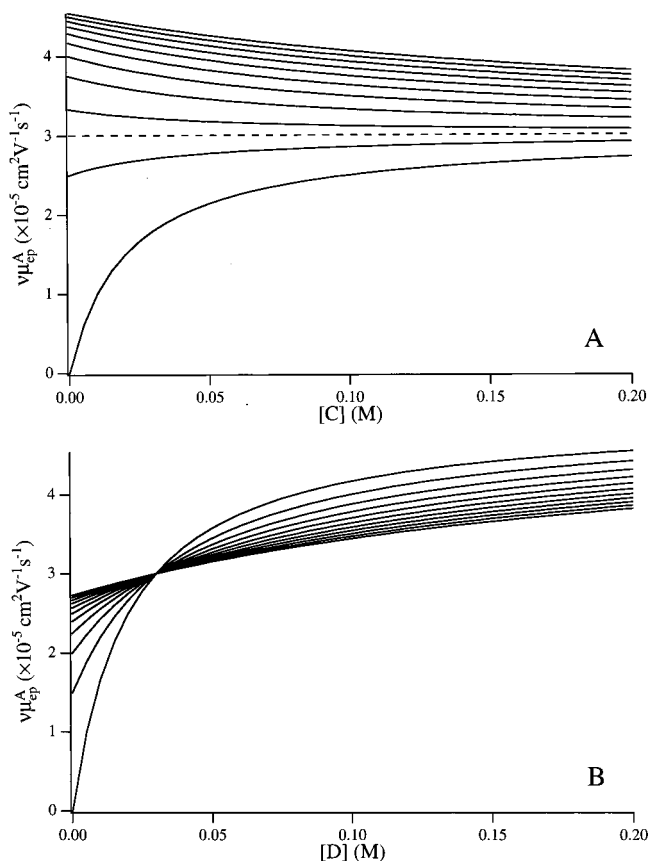


Figure 2. Profile plots of a binding isotherm surface where both additives interact with the analyte with 1:1 binding stoichiometries (eq 1). The constants are $K_{AC} = 50 \text{ M}^{-1}$; $K_{AD} = 50 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AD} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The second additive concentration ranges from 0 to 0.2 M in increments of 0.02 M. The dashed line in (A) is the binding isotherm for C when [D] is at the dengsu concentration.

$|\mu_{ep,A}| > |\mu_{ep,AC} - \mu_{ep,A}|$. Conversely, if $|\mu_{ep,AC} - \mu_{ep,A}| > |\mu_{ep,AD} - \mu_{ep,A}|$, there will be a dengsu concentration for C. When the two complex mobilities for the additives are equal, according to eq 4, the dengsu concentration for both additives will be infinity. This is the only case where there will not be an observable dengsu concentration for one of the additives when binding occurs with a 1:1 stoichiometry. When [D] is higher than the dengsu concentration, increasing [C] causes a decrease in the net mobility of the analyte. This may seem counterintuitive, since increasing [C] in the absence of D causes an increase in $\nu\mu_{ep}^A$. It is important to remember that, when [D] is above the dengsu concentration, $\nu\mu_{ep}^A$ is above $\mu_{ep,AC}$. Increasing the concentration of an additive always shifts the net mobility of the analyte toward the mobility of that analyte–additive complex. In the case where [D] is above the dengsu concentration, an increase in [C] shifts $\nu\mu_{ep}^A$ toward $\mu_{ep,AC}$, which decreases the net mobility of the analyte.

Profile plots are obtained by plotting binding isotherms for one additive at constant concentrations of the second additive. If the concentration of the second additive is varied at fixed increments, the result is analogous to viewing the profile of the binding isotherm surface perpendicular to one of the concentration axes. The dengsu point is easily identified on profile plots. Figure 2 shows the profile plots of an isotherm surface where both additives

increase the net mobility of the analyte (the case shown in Figure 1B). Figure 2A shows the profile of the surface when viewed perpendicular to the [C] axis. Since $|\mu_{ep,AC} - \mu_{ep,A}| < |\mu_{ep,AD} - \mu_{ep,A}|$, there is no dengsu concentration for additive C. The dengsu point for additive D is illustrated as a horizontal dashed line in Figure 2A, demonstrating that the net analyte mobility is not affected by [C] when [D] is at the dengsu concentration. Figure 2B shows the profile of the surface perpendicular to the [D] axis. The dengsu point is where all of the isotherms intersect. At $[D]_D$, the net mobility of the analyte is the same for any [C]. Therefore, any isotherm for D must intersect at the dengsu concentration, regardless of the concentration of C. The net analyte mobility at the dengsu concentration (i.e., the intersection on the profile plots) is equal to $\mu_{ep,AC}$. It should be noted that a dengsu point can be detected using profile plots, regardless of the concentrations of C at which the binding isotherms are measured. If several binding isotherms for D, measured at different [C], have a common intersection point, then a dengsu point is present.

When [D] is constant, as is the case in the individual isotherms in Figure 2A, eq 1 can be simplified to

$$v\mu_{ep}^A = \frac{\mu_{ep,A}^* + K_{AC}^*[C]\mu_{ep,AC}}{1 + K_{AC}^*[C]} \quad (5)$$

where the asterisks indicate the apparent parameters in the presence of constant [D]. Therefore,

$$\mu_{ep,A}^* = \frac{\mu_{ep,A} + K_{AD}[D]\mu_{ep,AD}}{1 + K_{AD}[D]} \quad (6)$$

$$K_{AC}^* = \frac{K_{AC}}{1 + K_{AD}[D]} \quad (7)$$

Equation 5 has the same form as the equation for a one-additive system. It is, therefore, impossible to determine if a second equilibrium is taking place from the shape of the isotherm when the concentration of the second additive remains constant. The apparent free mobility ($\mu_{ep,A}^*$) is determined by the interaction between the analyte and D, as shown in Figure 2A, where $\mu_{ep,A}^*$ increases with the concentration of D. It is this shift in the apparent free mobility that gives rise to the dengsu point. When [D] is at the dengsu concentration, $\mu_{ep,A}^* = \mu_{ep,AC}$. Substituting $\mu_{ep,AC}$ for $\mu_{ep,A}^*$ in eq 5 gives $v\mu_{ep}^A = \mu_{ep,AC}$. Since the apparent free mobility is equal to the complex mobility at the dengsu point, formation of the complex AC does not affect the net analyte mobility.

As binding with D increases, the apparent equilibrium constant for the binding of A and C (K_{AC}^*) decreases. This can be seen clearly in Figure 2A, where the curvature of the isotherms decreases as the concentration of D increases. Changing [D] is analogous to changing the environment in which the analyte interacts with C. It is important to remember that the constants measured depend heavily on the experimental conditions under which they are measured. It is impossible in CE, and most other methods, to study an equilibrium independent of other interactions. In CE, there are always going to be interactions between the analyte and the solvent, buffer, counterions, or other com-

pounds in solution. When the experimental conditions are changed, such as a change in the concentration of D or the addition of an organic modifier, a new set of apparent constants are often measured without considering the relationship between the two sets of conditions. Instead of empirically measuring apparent constants for different D concentrations, the interaction between the analyte and D can be considered as a second equilibrium, allowing a more systematic description of the system. The effect of solvent and buffer interactions on the apparent constants are often similar to changing the concentration of D. Generally speaking, if the interactions between an analyte and the solvent increase, the apparent equilibrium constant between the analyte and the additive will decrease.^{25,30} This solvation effect can be thought of as a secondary equilibrium which competes with the analyte–additive interaction. It should be possible to describe analyte–solvent and analyte–buffer interactions in terms of equilibria, providing a better understanding of how these parameters affect analyte mobility.

The presence of an unknown secondary interaction, such as an analyte–solvent or analyte–buffer interaction, can have important consequences in the application of single-additive CE experiments. Although the shape of the binding isotherm can still be described using eq 5, secondary equilibria may cause results that seem puzzling. This can be demonstrated using the profile plots in Figure 2. If the analyte is involved in a secondary interaction with D, the binding isotherm for C could take the shape of any of the curves shown in Figure 2A, depending on the experimental conditions. In some cases C would cause an increase in the net analyte mobility, and in others it would cause a decrease. If [D] is at the dengsu concentration, it will appear that K_{AC} is 0 even if binding does occur. Secondary equilibria need to be considered to systematically describe an analyte–additive interaction.

Higher Order Interactions. Not all analyte–additive interactions in CE take place with a 1:1 stoichiometry.²⁸ It is, therefore, important to consider the effect of higher order interactions on binding isotherms when more than one additive is used. The simplest case to consider is when one additive (D) interacts with the analyte with a 1:1 stoichiometry and another additive (C) interacts with both 1:1 and 1:2 stoichiometries. The equation that describes the net mobility of the analyte can be determined using the general equation presented by Bowser et al.:²⁹

$$v\mu_{ep}^A = \frac{\sum_{i=1}^m K_i \mu_{ep,i}}{\sum_{i=1}^m K_i} \quad (8)$$

where m is the number of species of A, and K_i and $\mu_{ep,i}$ are the capacity factor and the electrophoretic mobility of species i , respectively. The capacity factor for the second-order interaction is²⁸

$$k_{AC_2}' = K_{AC}K_{AC_2}[C]^2 \quad (9)$$

Therefore, the net analyte mobility is

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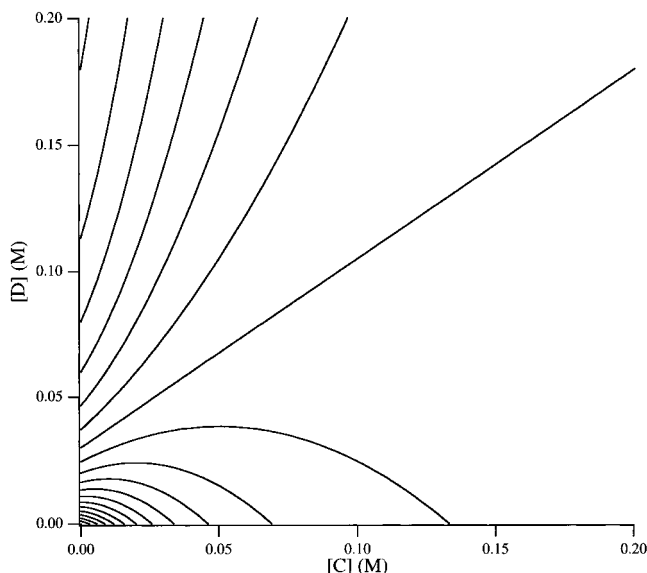


Figure 3. Contour plot of a binding isotherm surface where additive C interacts with both 1:1 and 1:2 stoichiometries and additive D has a 1:1 binding stoichiometry (eq 10). The constants are $K_{AC} = 50 \text{ M}^{-1}$; $K_{AC_2} = 50 \text{ M}^{-1}$; $K_{AD} = 50 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC} = 1.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC_2} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AD} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The contour curves range from $\nu\mu_{ep}^A = 0$ to $4.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, with increments of $0.25 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ between the contour lines.

$$\nu\mu_{ep}^A = \frac{k_A'\mu_{ep,A} + k_{AC}'\mu_{ep,AC} + k_{AC_2}'\mu_{ep,AC_2} + k_{AD}'\mu_{ep,AD}}{k_A' + k_{AC}' + k_{AC_2}' + k_{AD}'} \quad (10)$$

$$\nu\mu_{ep}^A = \frac{\mu_{ep,A} + K_{AC}[C]\mu_{ep,AC} + K_{AC}K_{AC_2}[C]^2\mu_{ep,AC_2} + K_{AD}[D]\mu_{ep,AD}}{1 + K_{AC}[C] + K_{AC}K_{AC_2}[C]^2 + K_{AD}[D]} \quad (11)$$

Figure 3 shows a contour plot of an isotherm surface described by eq 11. The slopes of the contour curves are equal to the partial derivative of $[D]$ with respect to $[C]$:

$$\frac{\partial[D]}{\partial[C]} = \frac{K_{AC}(\nu\mu_{ep}^A - \mu_{ep,AC}) + 2K_{AC}K_{AC_2}[C](\nu\mu_{ep}^A - \mu_{ep,AC_2})}{K_{AD}(\nu\mu_{ep}^A - \mu_{ep,AD})} \quad (12)$$

The slope of the contour curves is now dependent on the concentration of C, indicating that the contour curves are generally not straight lines. The only case where the contour curve will be a straight line is when $\nu\mu_{ep}^A = \mu_{ep,AC_2}$ or $\mu_{ep,AD}$. For a dengsu point to exist, there must be a linear contour curve that is perpendicular to one of the concentration axes. If the slope of the contour curve is 0, there will be a dengsu concentration for D; if the slope is infinity, there will be a dengsu concentration for C.

There will be a dengsu concentration for C whenever $\nu\mu_{ep}^A = \mu_{ep,AD}$, which is possible if $|\mu_{ep,AC} - \mu_{ep,A}|$ or $|\mu_{ep,AC_2} - \mu_{ep,A}| > |\mu_{ep,AD} - \mu_{ep,A}|$ and both additives shift the net mobility of the analyte in

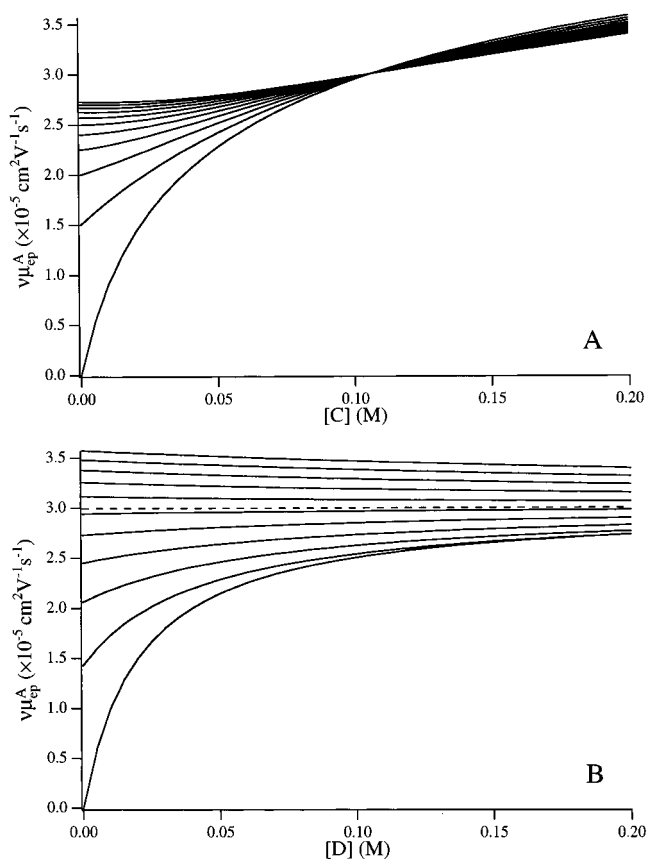


Figure 4. Profile plots of a binding isotherm surface where additive C interacts with both 1:1 and 1:2 stoichiometries and additive D has a 1:1 binding stoichiometry (eq 10). The constants are $K_{AC} = 50 \text{ M}^{-1}$; $K_{AC_2} = 50 \text{ M}^{-1}$; $K_{AD} = 50 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC} = 2.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC_2} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; and $\mu_{ep,AD} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The second additive concentration ranges from 0 to 0.2 M in increments of 0.02 M. The dashed line in (B) is the binding isotherm for D when $[C]$ is at the dengsu concentration.

the same direction. Figure 4 shows profile plots made using eq 11 when $|\mu_{ep,AC_2} - \mu_{ep,A}| > |\mu_{ep,AD} - \mu_{ep,A}|$. The binding isotherms in Figure 4A have a common intersection point, indicating that there is a dengsu concentration for C. The dengsu concentration can be determined by setting the partial derivative of $\nu\mu_{ep}^A$ with respect to $[D]$ to 0, and solving for $[C]$:

$$\frac{\partial\nu\mu_{ep}^A}{\partial[D]} = \{K_{AD}(\mu_{ep,AD} - \mu_{ep,A}) + K_{AC}K_{AD}[C](\mu_{ep,AD} - \mu_{ep,AC}) + K_{AC}K_{AC_2}K_{AD}[C]^2(\mu_{ep,AD} - \mu_{ep,AC_2})\} / (1 + K_{AC}[C] + K_{AC}K_{AC_2}[C]^2 + K_{AD}[D])^2 \quad (13)$$

$$0 = K_{AD}(\mu_{ep,AD} - \mu_{ep,A}) + K_{AC}K_{AD}[C]_D(\mu_{ep,AD} - \mu_{ep,AC}) + K_{AC}K_{AC_2}K_{AD}[C]_D^2(\mu_{ep,AD} - \mu_{ep,AC_2}) \quad (14)$$

The dengsu concentration for C (i.e., $[C]_D$) can be obtained by solving this quadratic equation (eq 14). The positive solutions correspond to the concentration of C, where the net mobility is equal to $\mu_{ep,AD}$. If $|\mu_{ep,AC} - \mu_{ep,A}| > |\mu_{ep,AD} - \mu_{ep,A}| > |\mu_{ep,AC_2} -$

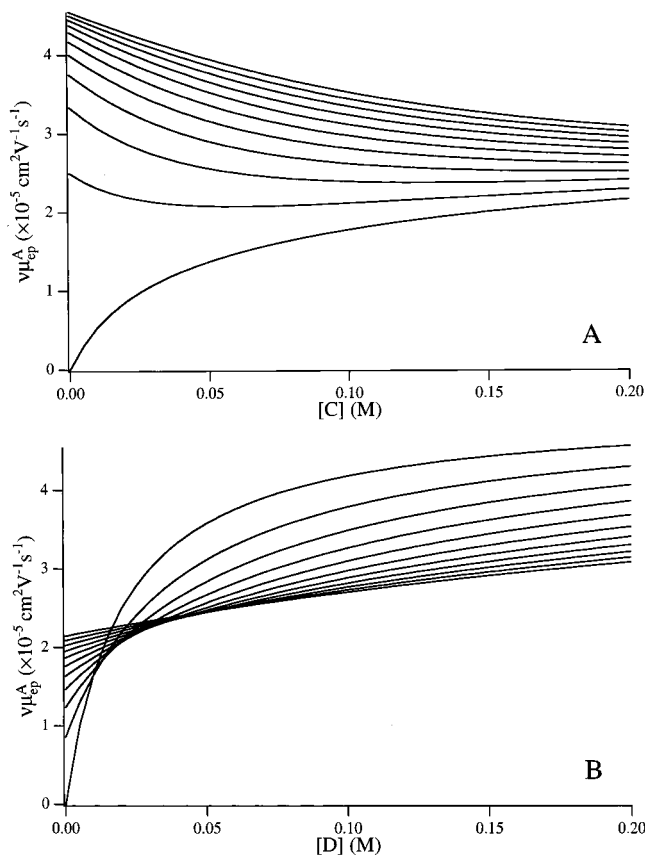


Figure 5. Profile plots of a binding isotherm surface where additive C interacts with both 1:1 and 1:2 stoichiometries and additive D has a 1:1 binding stoichiometry (eq 10). The constants are $K_{AC} = 50 \text{ M}^{-1}$; $K_{AC_2} = 50 \text{ M}^{-1}$; $K_{AD} = 50 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC} = 1.5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC_2} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AD} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The second additive concentration ranges from 0 to 0.2 M, and the increments between the profile lines are 0.02 M.

$\mu_{ep,A}$, there will be two positive solutions and, therefore, two dengsu points. This will be very uncommon in CE because $|\mu_{ep,AC_2} - \mu_{ep,A}|$ is generally larger than $|\mu_{ep,AC} - \mu_{ep,A}|$.

Figure 5 shows profile plots made using eq 11 when $|\mu_{ep,AD} - \mu_{ep,A}| > |\mu_{ep,AC_2} - \mu_{ep,A}|$. The isotherms intersect at different concentrations of D, as shown in Figure 5B, indicating that there is no dengsu point. In most situations where higher order interactions take place with one additive, there will be no dengsu concentration for the second additive. There are several exceptions when a dengsu concentration for D will exist, even when there are higher order interactions between the analyte and C.

The most obvious case is when $K_{AC_2} = 0$. No higher order interactions take place, and, therefore, the conditions for the presence of a dengsu point are identical with those for a system with only 1:1 binding.

The second case is when $K_{AC} = 0$, as demonstrated in Figure 6. This corresponds to strongly cooperative binding, where complexation of the second additive is more favorable than that of the first.^{28,30} Cooperative binding is relatively uncommon for small molecules because steric effects usually make the second binding less favorable than the first. However, it is more common in interactions involving larger biological molecules such as DNA or proteins. Cases where C does interact with the analyte cooperatively are easily identified using profile plots. The sigmoidal shape of the binding isotherm at $[D] = 0$ in Figure 6A

indicates that cooperative binding is taking place.^{28,30} Cases when cooperative binding causes a dengsu point for one additive, even though higher order interactions take place with the second additive, are easily identified by this distinctive sigmoidal-shaped isotherm in the profile plot.

The third case for the presence of a dengsu concentration for D, when C undergoes higher order interactions with the analyte, is when $\mu_{ep,AC} = \mu_{ep,AC_2} = \nu\mu_{ep}^A$. This case is extremely uncommon in CE. The purpose of using complexation additives is to modify the net mobility of an analyte. It is difficult to imagine any case where the binding of one additive changes the mobility of an analyte but the mobility does not change with further complexation.

In the exceptions, when there is a dengsu concentration for D when C undergoes higher order interactions with the analyte, $[D]_D$ can be determined by setting the partial derivative of $\nu\mu_{ep}^A$ with respect to $[C]$ to 0 and solving for $[D]$:

$$\frac{\partial \nu\mu_{ep}^A}{\partial [D]} = \{K_{AC}(\mu_{ep,AC} - \mu_{ep,A}) + K_{AC}^2 K_{AC_2} [C]^2 (\mu_{ep,AC_2} - \mu_{ep,AC}) + K_{AC} K_{AD} [D] (\mu_{ep,AC} - \mu_{ep,AD}) + 2K_{AC} K_{AC_2} [C] (\mu_{ep,AC_2} - \mu_{ep,A}) + 2K_{AC} K_{AC_2} K_{AD} [C] [D] (\mu_{ep,AC_2} - \mu_{ep,AD})\} / (1 + K_{AC} [C] + K_{AC} K_{AC_2} [C]^2 + K_{AD} [D])^2 \quad (15)$$

When $K_{AC} = 0$ (highly cooperative 1:2 interaction) or $\mu_{ep,AC} = \mu_{ep,AC_2}$, setting eq 15 to 0 gives

$$[D]_D = \frac{(\mu_{ep,A} - \mu_{ep,AC_2})}{K_{AD} (\mu_{ep,AC_2} - \mu_{ep,AD})} \quad (16)$$

which is identical with eq 4.

As stated earlier, it is uncommon for a dengsu concentration to exist when the second additive undergoes higher order interactions with the analyte. The exceptions to these cases either are easily identified or rarely occur. Therefore, the presence of a dengsu concentration for one additive is a strong indication that the second additive interacts with the analyte in a 1:1 stoichiometry over the range of additive concentrations tested. In practical applications, a dengsu point can be used as a diagnostic tool to determine if higher order interactions need to be considered to describe a particular analyte–additive interaction. Several binding isotherms for the interaction between the analyte and a second additive can be measured at different concentrations of the additive for which the binding stoichiometry is to be determined. If the isotherms share a common intersection point and the second additive does not undergo cooperative binding with the analyte, then the additive binds the analyte with a 1:1 stoichiometry. It should be emphasized that this does not exclude higher order interactions taking place at higher additive concentrations.²⁸ If the binding isotherms intersect but there is no dengsu point, it is almost certain that more complicated interactions are taking place.

Although only one additive can have a dengsu concentration, the profile plots of the two-additive system allow the unambiguous

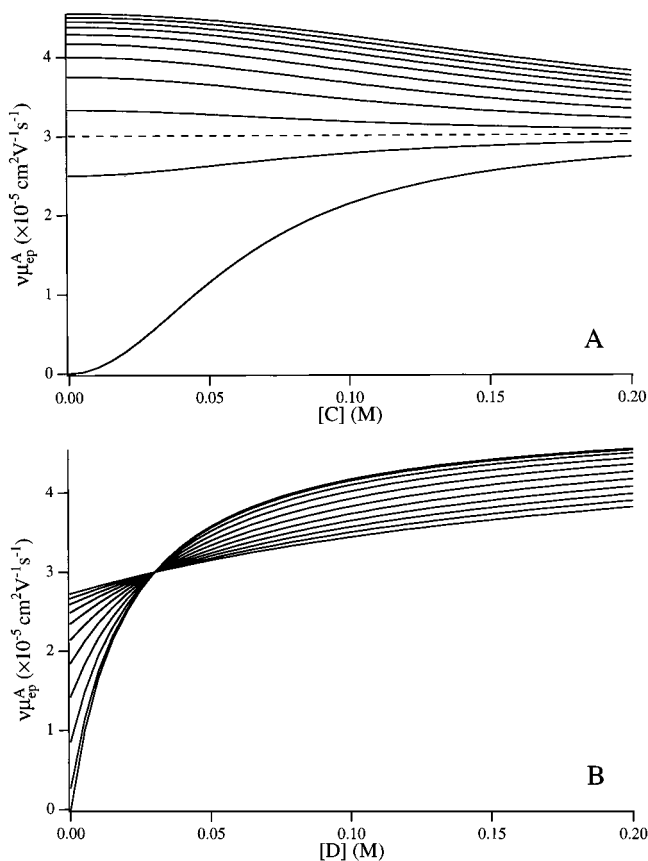


Figure 6. Profile plots of a binding isotherm surface where additive C interacts with a 1:2 stoichiometry and additive D has a 1:1 binding stoichiometry (eq 10). The constants are $K_{AC} = 0 \text{ M}^{-1}$; $K_{AC}K_{AC_2} = 250 \text{ M}^{-2}$; $K_{AD} = 50 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC_2} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AD} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The second additive concentration ranges from 0 to 0.2 M, with 0.02 M increments between the profile lines. The dashed line in (A) is where the concentration of D equals the dengsu concentration.

determination of the binding stoichiometry for both additives. As in the case where both additives interact with a 1:1 stoichiometry, the isotherms in the profile plot are drawn by keeping the concentration of the second additive constant. If [C] is constant, eq 13 can be simplified to

$$\nu\mu_{ep}^A = \frac{\mu_{ep,A}^* + K_{AD}^*[D]\mu_{ep,AD}}{1 + K_{AD}^*[D]} \quad (17)$$

where

$$\mu_{ep,A}^* = \frac{\mu_{ep,A} + K_{AC}[C]\mu_{ep,AC} + K_{AC}K_{AC_2}[C]^2\mu_{ep,AC_2}}{1 + K_{AC}[C] + K_{AC}K_{AC_2}[C]^2} \quad (18)$$

$$K_{AD}^* = \frac{K_{AD}}{1 + K_{AC}[C] + K_{AC}K_{AC_2}[C]^2} \quad (19)$$

Even though the analyte undergoes higher order interactions with C, when [C] is constant, the binding isotherms for D can be described using the equation for a first-order, one-additive system, with apparent constants given by eqs 18 and 19.

The isotherms for C keeping [D] constant are described by the equation

$$\nu\mu_{ep}^A = \frac{\mu_{ep,A}^* + K_{AC}^*[C]\mu_{ep,AC} + K_{AC}^*K_{AC_2}[C]^2\mu_{ep,AC_2}}{1 + K_{AC}^*[C] + K_{AC}^*K_{AC_2}[C]^2} \quad (20)$$

where

$$\mu_{ep,A}^* = \frac{\mu_{ep,A} + K_{AD}[D]\mu_{ep,AD}}{1 + K_{AD}[D]} \quad (21)$$

$$K_{AC}^* = \frac{K_{AC}}{1 + K_{AD}[D]} \quad (22)$$

The isotherms for C at constant [D] can be described using the equation for interactions with a single additive when binding occurs at stoichiometries of 1:1 and 1:2. It is generally true, when the concentration of the second additive is kept constant, that the shape of the isotherms for an additive is determined by the stoichiometry of the interactions between that additive and the analyte. The apparent constants in the equation describing the isotherms change with the concentration of the second additive.

Bowser et al.²⁸ have previously described a method for determining the effect of higher order interactions on the net mobility of the analyte. That method can also be used to fit binding isotherms in which higher order interactions take place and to obtain the constants that describe the analyte migration behavior. It was noted that, when the microscopic equilibrium constants and complex mobilities are equal, it is impossible to detect higher order interactions. For a system with multiple equilibria, it would be more accurate to say that, when the apparent microscopic constants are equal, it will be impossible to detect higher order interactions from a binding isotherm. As shown by eq 22, the apparent equilibrium constants are dependent on the concentration of the second additive. If binding isotherms for C are measured at different [D], the apparent microscopic constants cannot be equal in all cases. If higher order interactions are not detected in any of the isotherms, then binding occurs with a 1:1 stoichiometry. If higher order interactions take place, it will be noticeable in most, if not all, of the binding isotherms. This suggests that a second additive could be added to a one-additive system to unambiguously determine the binding stoichiometry of the first additive.

The conditions for a dengsu point to occur when higher order interactions take place (i.e., $K_{AC} = 0$) are very different from those when higher order interactions are not detectable in the binding isotherm (i.e., the microscopic constants are equal). If both methods are used in conjunction, higher order interactions can always be detected in at least one of the methods.

Interactions between Additives. Implicit in the above discussion was the absence of interaction between the additives C and D. When this assumption is valid, the constants measured for each additive can be used in eq 1 to predict the net mobility of the analyte for any combination of the two additives. The discussion in this section will demonstrate how the dengsu point and the binding isotherms in the profile plots can be used to determine if interactions between the additives need to be considered.

One of the common assumptions made in CE with additives is that the initial concentrations of the additives ($[C]_0$ and $[D]_0$) are not changed significantly during the dynamic complexation process. In systems where the additives only interact with the analyte, $[C]_0$ and $[D]_0$ can be substituted by $[C]$ and $[D]$ if the concentrations of the additives are much larger than those of the analyte ($[C]$ and $[D]$ are the concentrations of uncomplexed additives after the system reaches equilibrium). If there is significant interaction between C and D, then the above assumption is no longer valid. The concentration of C can be determined by solving the following quadratic equation:

$$0 = K_{CD}[C]^2 + (K_{CD}[C]_0 + K_{CD}[D]_0 + 1)[C] - [C]_0 \quad (23)$$

where K_{CD} is the formation constant for the complex CD. A similar expression can be written for $[D]$. To calculate $[C]$ and $[D]$, K_{CD} must be measured.

Substituting $[D]_0$ and $[C]_0$ with $[C]$ and $[D]$ when it is not valid to do so can have a major effect on the binding isotherms. This is true even if there is no interaction between the analyte and the complex formed by the two different additives (CD). If there is no interaction between the analyte and CD, then the net mobility of the analyte can still be predicted by eq 1. Therefore, if the binding isotherms are plotted using $[C]$ and $[D]$, the profile plots will be identical with those shown in Figure 2. Figure 7 shows the profile plots made with $[C]_0$ and $[D]_0$ when C and D interact. Clearly, there is no dengsu point. Also, the shapes of the isotherms cannot be described on the basis of equilibria with a 1:1 stoichiometry. Therefore, the presence of a dengsu concentration for either additive indicates that there is no significant interaction between C and D, and the assumptions that $[C] = [C]_0$ and $[D] = [D]_0$ are valid.

Even when the assumptions that $[C] = [C]_0$ and $[D] = [D]_0$ are valid, if the analyte can bind both additives at the same time, the constants measured for each additive individually may not accurately describe the net analyte mobility when both additives are used. If the analyte can bind both of the additives at the same time (forming the complex ACD), the net mobility of the analyte can be described by the equation

$$v\mu_{ep}^A = \frac{\mu_{ep,A} + K_{AC}[C]\mu_{ep,AC} + K_{AD}[D]\mu_{ep,AD} + K_{ACD}[C][D]\mu_{ep,ACD}}{1 + K_{AC}[C] + K_{AD}[D] + K_{ACD}[C][D]} \quad (24)$$

where K_{ACD} is the formation constant of the complex ACD, and $\mu_{ep,ACD}$ is the mobility of the complex ACD.

The formation of a complex ACD can be detected using the isotherms of a profile plot. If $[D]$ is kept constant, eq 24 can be simplified to

$$v\mu_{ep}^A = \frac{\mu_{ep,A}^* + K_{AC}^*[C]\mu_{ep,AC}^*}{1 + K_{AC}^*[C]} \quad (25)$$

where

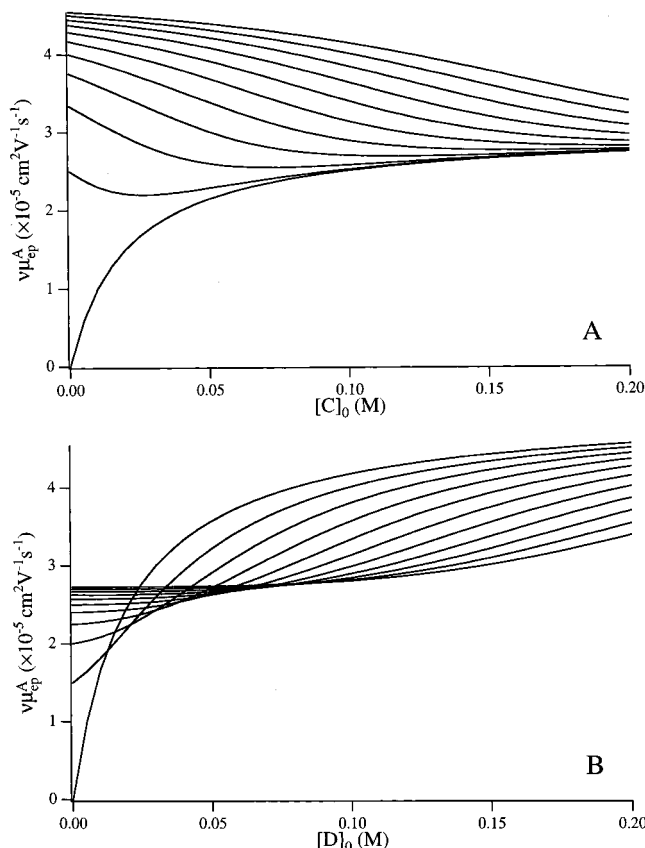


Figure 7. Profile plots of a binding isotherm surface where interactions between the additives invalidate the assumption $[C] = [C]_0$ and $[D] = [D]_0$ (eq 24). The constants are $K_{AC} = 50 \text{ M}^{-1}$; $K_{AD} = 50 \text{ M}^{-1}$; $K_{CD} = 50 \text{ M}^{-1}$; $K_{ACD} = 0 \text{ M}^{-1}$; $\mu_{ep,A} = 0 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AC} = 3 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$; $\mu_{ep,AD} = 5 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The second additive concentration ranges from 0 to 0.2 M, with 0.02 M increments between the profile lines.

$$\mu_{ep,A}^* = \frac{\mu_{ep,A} + K_{AD}[D]\mu_{ep,AD}}{1 + K_{AD}[D]} \quad (26)$$

$$K_{AC}^* = \frac{K_{AC} + K_{ACD}[D]}{1 + K_{AD}[D]} \quad (27)$$

$$\mu_{ep,AC}^* = \frac{K_{AC}\mu_{ep,AC} + K_{ACD}[D]\mu_{ep,ACD}}{K_{AC} + K_{ACD}[D]} \quad (28)$$

The major difference between eq 5 and eq 25 is the presence of an apparent complex mobility ($\mu_{ep,AC}^*$) in eq 25. The apparent complex mobility for a particular isotherm is now dependent on $[D]$. Binding isotherms for C at different $[D]$ will give different values for $\mu_{ep,AC}^*$, unless $\mu_{ep,AC} = \mu_{ep,ACD}$. It will be uncommon for $\mu_{ep,AC}$ to be equal to $\mu_{ep,ACD}$, since additives are chosen to modify the mobility of the analyte. Attaching a second additive to a singly bound analyte will almost always change the mobility of the analyte. The dependence of $\mu_{ep,AC}^*$ on $[D]$ can be used to detect cases where the analyte can interact with both additives at the same time. If the apparent complex mobility remains constant at different $[D]$, then the effect of complex ACD on the net mobility of the analyte does not need to be considered. If the apparent complex mobility changes at different $[D]$, then it is a strong indication that interactions between the additives need to be considered, and constants obtained using the individual additives

may not accurately describe the mobility of the analyte when the additives are used together.

CONCLUSION

This paper presented some of the unique properties of multivariate binding isotherms when two additives are used in CE. The dengsu concentration, when present, can be easily identified by a common intersection point in the profile plot. The presence of a dengsu point is a strong indication that the second additive interacts with the analyte in a 1:1 stoichiometry and there is no significant interaction between the additives. The shapes of the binding isotherms in the profile plots can be used to further confirm the binding stoichiometries of the two additives, as well as to determine whether the analyte can bind with both additives at the same time.

Although the discussion has been made in the context of a two-additive system, many of the points made in this paper are relevant to systems which employ a single additive and to systems with more than two additives. We have shown how a second additive can be used to unambiguously determine the stoichiometry of a particular analyte–additive interaction. It is necessary to understand the effect of a second additive on a binding isotherm, even if the concentration of the second additive remains constant. Buffer ions, solvents, or other compounds present in

the CE system may interact with the analyte. Although their concentrations may be constant if experimental conditions are kept the same, secondary equilibria may cause misleading results when studying an analyte–additive interaction if the possibility of more complicated equilibria is not considered. It is also important to remember that equilibrium constants are generally not measured independently of other interactions. Many secondary interactions are currently accounted for by reporting apparent constants for specific experimental conditions. In the future, it will be important to determine what phenomena are better described systematically using multiple equilibria, rather than relying on apparent constants measured for a specific set of experimental conditions.

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