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Effects of isotherm non-linearity on the determination of the binding constant in affinity chromatography

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SUMMARY

A systematic study was made of non-linear effects on the determination of the binding constant in affinity chromatography. The criteria for the applicable range of linear theory are derived for both frontal and zonal analysis. The difference in the criteria between zonal and frontal analysis indicates that the latter may not be preferred in some instances. Investigation of experimental data from the literature showed that the effect of isotherm non-linearity on elution volume is not small in affinity chromatography, where the binding constant is very large and the experimental design does not permit work in the linear region. A universal function $\Phi(a)$ for zonal analysis has been derived. The strong correlation of experimental and calculated $\Phi(a)$ demonstrates that the equations derived in this work are valuable. In applying these equations, one can determine both the binding constant and the maximum binding capacity from experiments by evaluating the capacity factor at various sample concentrations with or without an inhibitor in the mobile phase.

INTRODUCTION

Affinity chromatography is widely used in the investigation of specific interactions between biomolecules. Both zonal and frontal elution approaches have been developed in order to determine quantitatively the interaction constant[1]. Differences have been found when two methods are applied to evaluate the inhibitory constant of the complex of an enzyme and its soluble or immobilized inhibitor[2]. Whichever method is used, a working equation for the elution volume has to be used to interpret the experimental data. The observation of elution volume depending on the sample concentration or inhibitor concentration is essentially a result of a non-linear equilibrium isotherm, which is conventionally of the Langmuir type. Hence affinity chromatography in the case of a Langmuir isotherm is non-linear chromatography.

Arnold *et al.*[3] pointed out some inconsistencies in the theoretical treatments of

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analytical affinity chromatography and identified these as the sources of erroneous results. Recently, Golshan-Shirazi and Guiochon[4–6] derived analytical solutions of elution time as a function of sample volume and sample concentration for the ideal (equilibrium) model of non-linear chromatography. However, the elution time they obtained was the retention time of the shock front. A thorough examination of the non-linear problem in affinity chromatography was made by Muller and Carr[7]. No theory was available in their work to interpret the effects of isotherm non-linearity on the capacity factor and desorption rate constant. Recent work on non-linear affinity chromatography includes that of Anderson and co-workers[8–10].

In this paper, a systematic approach is presented by first introducing a more realistic model to describe the chromatographic behavior; the equations relating elution volume and capacity factor to concentrations of immobilized and soluble ligand, inhibitor, etc., are derived by following the clarified definitions and a correlation is given to demonstrate the effects of isotherm non-linearity. By using the equations derived in this work, a proper determination of the binding constant, the interaction constant between the soluble solute and its immobilized ligand, can be achieved in affinity chromatography.

MATHEMATICAL MODEL AND DEFINITIONS OF THE FIRST MOMENT

The governing equation of affinity chromatography can be derived from a material balance. For a simple system contaning one single solute, it is given as

$$\varepsilon \frac{\partial C}{\partial t} = - u_0 \frac{\partial C}{\partial z} + \varepsilon D \frac{\partial^2 C}{\partial z^2} + r'' \tag{1}$$

where r'' denotes the net mass transfer rate from the bulk fluid to the adsorbed phase and can be expanded as shown in Table I. The model, including eqn. 1 and Table I, may be the most sophisticated and realistic one for the description of affinity chromatography. In this model, a uniform diameter d_p is assumed for monodisperse porous media, an imaginary external film separating the bulk fluid and solid phase is present and an effective diffusion coefficient D_i based on the entire particle volume is used to describe the diffusion of solute into the pores. The concentrations, in pores and in the bulk liquid surrounding the particle, c and C, are coupled by the mass-transfer rate through the film, which is accounted by k_f. The parameter k_a accounts for the desorption rate; q is the concentration on the inner surface of the solid. The equilibrium isotherm describes the relationship between concentrations c and q. It represents a plot of amount adsorbed in equilibrium with the concentration of free solute in the pores. To account for the possibility that all sites can be filled with the adsorbent, many workers considered the equilibrium relation to be of the Langmuir type:

$$q^* = \frac{q_s K_L c}{1 + K_L c} \tag{2}$$

The model presented above has been reported elsewhere by Arnold *et al.*[11] and

TABLE I

MODEL OF FLUID-TO-PARTICLE DIFFUSION AND BIO-SPECIFIC ADSORPTION

Pore diffusion with external film resistance (spherical particles).

$$-r'' = \frac{6\,(1-\varepsilon)}{d_p} \cdot D_i \frac{\partial c}{\partial r}\bigg|_{r=d_p/2}$$

$$D_i\left(\frac{\partial^2 c}{\partial r^2} + \frac{2}{r}\cdot\frac{\partial c}{\partial r}\right) - \varepsilon_p \cdot \frac{\partial c}{\partial t} - \rho_p \cdot \frac{\partial q}{\partial t} = 0$$

$$k_f\,(C - c\,|_{r=d_p/2}) = D_i \cdot \frac{\partial c}{\partial r}\bigg|_{r=d_p/2}$$

$$\frac{\partial c}{\partial r}\bigg|_{r=0} = 0$$

$$\frac{\partial q}{\partial t} = k_a\,(q^* - q)$$

$$q^* = \frac{q_s\,K_L\,c}{1 + K_L\,c}$$

McCoy[12]. It could also be reduced to the model of Horváth and Lin[13] by defining the film mass transfer coefficient as a linear function of $u_0^{1/3}$.

The use of the statistical moments to characterize chromatographic processes is well known, but some inconsistencies may be frequently found in the literature. Mathematically, the moments for each distribution function can be evaluated as soon as the function is well defined. In zonal elution chromatography, the effluent concentration profile can be regarded as a distribution function, by which the expressions of the moments are derived. For frontal elution chromatography, the effluent concentration profile, which is called a breakthrough curve, is no longer a distribution function. In general, the breakthrough curve can be treated as the integral of the distribution function of the quantity t. Therefore, the function $\partial c/\partial t$ can be treated as the distribution function of this quantity. The definition of the first moment for frontal elution is then given by

$$\mu_1^{\mathrm{F}} = \frac{\int_0^\infty t\frac{\partial C^{\mathrm{F}}}{\partial t}\,\mathrm{d}t}{\int_0^\infty \frac{\partial C^{\mathrm{F}}}{\partial t}\,\mathrm{d}t} \tag{3}$$

and that for zonal elution by

$$\mu_1^{\mathrm{Z}} = \frac{\int_0^\infty t\,C^{\mathrm{Z}}\,\mathrm{d}t}{\int_0^\infty C^{\mathrm{Z}}\,\mathrm{d}t} \tag{4}$$

TABLE II

RESULTS OF THE FIRST MOMENT

Isotherm	$\mu_1^F = \dfrac{\int_0^\infty t \cdot \dfrac{\partial C^F}{\partial t} \cdot dt}{\int_0^\infty \dfrac{\partial C^F}{\partial t} \cdot dt}$	$\mu_1^Z = \dfrac{\int_0^\infty t\ C^Z\ dt}{\int_0^\infty C^Z\ dt}$
Linear isotherm	$\dfrac{L}{u_0}[\varepsilon + (1-\varepsilon)\varepsilon_p + (1-\varepsilon)\rho_p q_s K_L]$	$\dfrac{L}{u_0}[\varepsilon + (1-\varepsilon)\varepsilon_p + (1-\varepsilon)\rho_p q_s K_L]$
Rectangular isotherm	$\dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s}{C_0}\right]$	$\dfrac{L}{u_0}\left\{\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{\varepsilon + (1-\varepsilon)\varepsilon_p}{\mu_0} \cdot \left(\dfrac{L}{u_0}\right) \cdot \dfrac{(1-\varepsilon)\rho_p q_s}{C_0}\left[\dfrac{1}{2} - \dfrac{\varepsilon D}{L u_0}\right] - \dfrac{(1-\varepsilon)\rho_p q_s}{\mu_0 C_0}\left[\dfrac{R\ \varepsilon_p}{3k_f} + \dfrac{1}{k_a} + \dfrac{1}{15} \cdot \dfrac{R^2 \varepsilon_p}{D_i}\right]\right\}$ where $\mu_0 = \int_0^\infty C^Z\, dt = 1 - \dfrac{L}{u_0} \cdot \dfrac{(1-\varepsilon)\rho_p q_s}{C_0}$
Langmuir isotherm:		
Equilibrium	$\dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s K_L}{1 + K_L C_0}\right]$	$\dfrac{L}{u_0}[\varepsilon + (1-\varepsilon)\varepsilon_p + (1-\varepsilon)\rho_p q_s K_L \Phi(a)]$
Finite mass-transfer rate	$\dfrac{\varepsilon L}{u_0}(1 + k'')$ with $k'' = \mathrm{f}\,(v, v_i, Bi, K_a, K_L^+, q_s^+, \varepsilon_p^+)$ (numerical value)	$\dfrac{\varepsilon L}{u_0}(1 + k'')$ with $k'' = \mathrm{f}\,(t_0^+, v, v_i, Bi, K_a, K_L^+, q_s^+, \varepsilon_p^+)$ (numerical value)

where $t_0^+ = \dfrac{u_0 t_0}{\varepsilon L}$, $v = \dfrac{u_0 L}{2\ \varepsilon D}$, $v_i = \dfrac{\varepsilon_p R^2 u_0}{\varepsilon L D_i}$, $Bi = \dfrac{R k_f}{D_i}$, $K_a = \dfrac{\varepsilon k_a L}{u_0}$, $K_L^+ = K_L C_0$, $\varepsilon_p^+ = \left(\dfrac{1-\varepsilon}{\varepsilon}\right)\varepsilon_p$, $q_s^+ = \dfrac{\rho_p q_s}{\varepsilon_p C_0}$

In eqns. 3 and 4, the superscripts F and Z represent frontal and zonal elutions (analyses), respectively. In frontal analysis, the concentration C^F is the effluent concentration normalized by dividing by sample concentration, *i.e.*, $C^F = C/C_0$.

Solving eqn. 1, along with proper initial/boundary conditions gives the concentration profiles in either zonal or frontal analysis. After substituting the profiles into eqns. 3 and 4, we can obtain the predicted values of the first moments. The results are summarized in Table II for various isotherm cases. It is noted that with the Langmuir isotherm, analytical solutions are not available when the mass transfer effects are considered. In the following sections, the results in Table II will be discussed in detail.

It has been proved that eqn. 3 is equivalent to the following equation:

$$\mu_1^F = \int_0^\infty (1 - C^F)dt \tag{5}$$

Eqn. 5 has been used in the literature to evaluate the first moment from experimental data[14]. The quantity in eqn. 5 is the area behind the breakthrough curve and represents the dynamic capacity of the affinity column in frontal elution. The equality between eqns. 3 and 5 indicates that eqn. 3 is a proper definition of the retention time. The resulting expressions of the first moments derived from eqns. 3 and 4 and are not identical except for the case of a linear isotherm. We have proved that the equality between eqns. 3 and 4 exists only when the mathematical model is linear. In this instance, the equilibrium isotherm will be linear.

ELUTION VOLUME AND BINDING CONSTANT DETERMINATION

Basically, all the equations of elution volume used in affinity chromatography are related to the expressions of the first moment. We usually call the first moment μ_1 the average retention time. The elution volume, V_t, also called retention volume, is simply the product of μ_1 and volumetric flow-rate, *i.e.*,

$$V_t = \mu_1 Q \tag{6}$$

Typical elution volume equations can be found in ref. 15 for frontal elution and in ref. 16 for zonal elution. Both analyses were used by Malanikova and Turkova[2] for the determination of binding (or association) constants, K_L, of complexes of trypsin and its low-molecular-weight inhibitors.

The elution volume is a measure of the extent to which the soluble component is retained in the packing bed of a chromatographic system or, in other words, the degree of retardation of the component in a matrix. The retardation of a solute in a matrix certainly depends on the intensity of interaction between the solute and its immobilized ligand. In a physical sense, an increase in the association constant between the solute and the ligand (or binding site) increases the capacity of the bio-adsorption, and hence increases the elution volume.

The elution volume is widely used to determine the binding constants in affinity chromatography because it is assumed to be independent of the mass-transfer rate and

the column dispersity. Actually, this common belief is not completely correct. Many factors may affect the value of the first moment or elution volume. For example, different initial/boundary conditions that come from different methods of sample injection and system considerations may result in different expressions for the first moment. Table III lists four sets of commonly used initial/boundary conditions for zonal analysis in the literature: (1) Kucera[17], (2) Aust[18], (3) Horváth and Lin[19] and (4) Chung and Hsu[20]. A comparison of the solutions of μ_1 in Table III shows that all four sets will lead to the same solution when the sample size t_0 and column dispersion $\varepsilon D/\mu_0 L$ are sufficiently small. Horváth and Lin's initial/boundary conditions can be regarded as an ideal case of Chung and Hsu's. In this work, the last set of initial/boundary conditions with $t_0 \to 0$ is used. A dependence of elution time on flow-rate was also reported by Heathcote and DeLisi[21]. It is believed that the dispersive effect of the boundary conditions can be made trivial by a reasonable design of the experiment.

It should be noted that the solutions in Table III are true only for linear isotherms, *i.e.*, in the range $K_L c \ll 1$ in eqn. 2. Under this assumption, μ_1 value is directly related to the total void fraction, $\varepsilon + (1-\varepsilon)\varepsilon_p$, and a product of the two quantities, $(1-\varepsilon)\rho_p q_s$ and K_L. The quantity $(1-\varepsilon)\rho_p q_s$ represents the maximum

TABLE III

EFFECTS OF INITIAL/BOUNDARY CONDITIONS ON THE FIRST MOMENT IN ZONAL ANALYSIS

Initial/boundary conditions[a]	μ_1^Z
(1) Kucera[17]: $C = q = c = 0;\ t < 0$ and $z = \pm\infty$; $t = 0;\ q = c = 0;\ -\infty < z < \infty;\ 0 < r < \frac{d_p}{2}$; $C = M\delta(z);\ 0 < z < L$	$\left(1 + \frac{2\varepsilon D}{u_0 L}\right)\left[1 + \frac{(1-\varepsilon)}{\varepsilon}\cdot\varepsilon_p + \left(\frac{1-\varepsilon}{\varepsilon}\right)\rho_p q_s K_L\right]\varepsilon\cdot\frac{L}{u_0}$
(2) Aust[18]: $C = q = c = 0;\ t = 0$; $\varepsilon D\left.\frac{\partial C}{\partial z}\right\|_{z=0^+} = u_0\,[C(z=0^+) - C(z=0^-)]$; $\left.\frac{\partial C}{\partial z}\right\|_{z=L} = 1;\ C(z=0^-) = \delta(t)M$	$\left[1 + \frac{(1-\varepsilon)}{\varepsilon}\cdot\varepsilon_p + \left(\frac{1-\varepsilon}{\varepsilon}\right)\rho_p q_s K_L\right]\varepsilon\cdot\frac{L}{u_0}$
(3) Horvath and Lin[19]: $C = q = c = 0;\ t = 0$; $C = 0;\ z = \infty$; $C = \delta(t)M;\ z = 0$	$\left[1 + \frac{(1-\varepsilon)}{\varepsilon}\cdot\varepsilon_p + \left(\frac{1-\varepsilon}{\varepsilon}\right)\rho_p q_s K_L\right]\varepsilon\cdot\frac{L}{u_0}$
(4) Chung and Hsu[20]: $C = q = c = 0;\ t = 0$; $C = C_0\,[u(t) - u(t-t_0)];\ z = 0$; $C = 0;\ z = \infty$	$\left[1 + \frac{(1-\varepsilon)}{\varepsilon}\cdot\varepsilon_p + \left(\frac{1-\varepsilon}{\varepsilon}\right)\rho_p q_s K_L\right]\varepsilon\cdot\frac{L}{u_0} + \frac{t_0}{2}$

[a] $\int_a^b f(z)\,\delta(z-z')\,dz = \begin{cases} f(z') & \text{if } a < z' < b \\ 0 & \text{otherwise} \end{cases}$

number of moles of solute that can be adsorbed per unit volume of packing bed. In other words, it is the concentration of accessible binding sites. In an affinity chromatographic system, the K_L value could be as large as 10^6 l mol^{-1}. By the linear theory, K_L is determined from the plot of elution volume *vs.* $(1-\varepsilon)\rho_p q_s$.

Elution volume can also be expressed in terms of the total penetrable volume of the colum, $V_0\{= [\varepsilon + (1-\varepsilon)\varepsilon_p]V_B\}$, which is measured by the elution of an unretained component having the same degree of penetration as a desired component but no specific affinity for the immobilized ligand. The ratio, which is referred to as the capacity factor (retention factor), k', is given by the following expression:

$$k' = \frac{V_t - V_0}{V_0} \tag{7}$$

The quantity k' is related to the thermodynamics of distribution between the mobile phase and stationary phase in chromatography. As one of the most important chromatographic parameters, its value is characteristic of individual solutes and the selection of the chromatographic system. For example, the ratio of the capacity factors is directly related to the resolution, which is the selectivity of two components in chromatography.

EFFECT OF ISOTHERM NON-LINEARITY

As shown in Table II, $\mu_1^F = \mu_1^Z$ when the isotherm is linear. Another idealized model was proposed[22] for which the absorbate is bound irreversibly, and other species are totally unadsorbed. The equilibrium isotherm is considered to be irreversible and is of the rectangular type, *i.e.*,

$$\begin{aligned} q^* &= q_s;\ c > 0 \\ q^* &= 0;\ c = 0 \end{aligned} \tag{8}$$

For the first two types of isotherm, Laplace transformation has been used to solve the governing equation and hence to derive the expressions of μ_1^F and μ_1^Z. The effects of column dispersion and fluid-to-particle mass transfer are accounted for in these cases. As expected, these expressions are independent of dispersion and mass transfer except the rectangular isotherm in zonal analysis, where the parameters accounting for dispersion and mass transfer appear. The effect of dispersion and mass transfer will be small if we take the values $R/k_f \to 0$, $R^2/D_i \to 0$ and $\mu_0 L/D \to \infty$. When the parameters values are in the above range, the system is in local equilibrium. Even for the equilibrium case, $\mu_1^F \neq \mu_1^Z$.

Both a linear isotherm and a rectangular isotherm can be regarded as special cases of the Langmuir isotherm. When all mass-transfer resistances are negligible, the method of the particle-volume average can reduce the governing equations to being analytically solvable[12]. Therefore, the method of characteristics serves to provide solutions of eqn. 1[23]. The expressions of the first moment were derived by substituting the resulting concentration profiles into eqns. 4 and 5. As shown in Table II, the deviation of the Langmuir from the linear isotherm is due to the effect of sample inlet concentration, C_0. In frontal analysis, the factor is $1/(1+K_L C_0)$. In zonal analysis, we

derived a universal function $\Phi(a)$. This function results from the method of characteristics and can be represented as

$$\Phi = \frac{1}{4} + \frac{\frac{1-a}{2}(2a-a^2)^{3/2}}{\frac{\pi}{2} - \sin^{-1}|1-a| - (1-a)(2a-a^2)^{1/2}}; \qquad a \leqslant 1 \tag{9}$$

$$\Phi = \frac{\frac{\pi}{8}}{a^2 - 1 + \frac{\pi}{2}}; \qquad a \geqslant 1 \tag{10}$$

where

$$a = \sqrt{\left(\frac{u_0 t_0}{L}\right) \frac{C_0}{(1-\varepsilon)\rho_p q_s}} \tag{11}$$

The function can also be represented as in Fig. 1. It is tedious to obtain the results, *i.e.*, eqns. 9–11; we ignore the mathematical details here. Fig. 1 shows that the effect of isotherm non-linearity is relatively small (1% deviation), provided that the value of a is less than 0.01. The a value reflects the amounts of relative feed concentration and sample size. The smaller the feed concentration, the smaller is a and hence the smaller is the effect of non-linearity. The contribution of sample size to elution time was also examined by Golshan-Shirazi and Guiochon[4–6], but the retention time they derived is the retention time of the shock front, not the true one as defined in eqn. 4.

If we take a 10% deviation from linear theory as an acceptable figure, then the criteria for the applicable range of linear theory can be derived as $a \ll 0.09$ for zonal analysis and $K_L C_0 \ll 0.11$ for frontal analysis. In addition to the sample concentration C_0, the a value also depends on the sample size and the quantity $(1-\varepsilon)\rho_p q_s$. For an affinity chromatographic system with large K_L and $(1-\varepsilon)\rho_p q_s$, zonal elution could be preferable, *i.e.*, it could be operated with a linear behavior over a wide range of sample concentrations, as shown in Fig. 2. The system in the linear region may take advantage

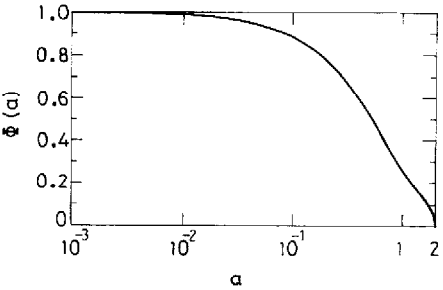

Fig. 1. Universal function of isotherm non-linearity effects in zonal analysis.

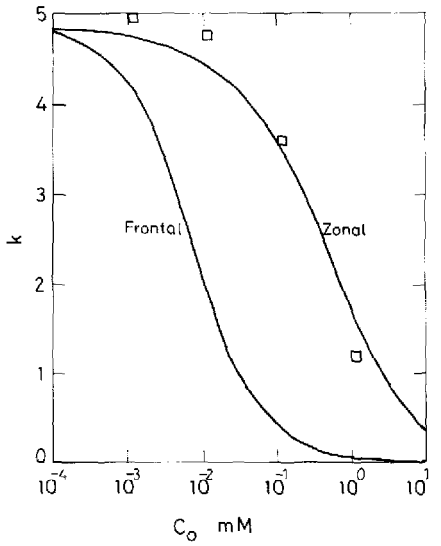

Fig. 2. Effects of sample concentration on the capacity factor. Data after Anderson and Walters[9] in their Fig. 1; binding of 4-methylumbelliferyl α-D-mannopyranoside on concanavalin A in a column using Hypersil 300 as support. $t_0 = 0.01$ min, $V_B = 0.66$ ml, $\varepsilon = 0.35$, $\varepsilon_p = 0.44$, $L = 5$ cm, flow-rate = 1 ml min^{-1}; $(1-\varepsilon)\rho_p q_s K_L = 3.1$ and $K_L = 1.17 \cdot 10^5$ l mol^{-1} are estimated from inhibition experiments (Fig. 2 in Anderson and Walters'[9] paper).

of using the theories in linear chromatography, for example, the height equivalent to a theoretical plate (HETP). The difference in the criteria between zonal and frontal analysis indicates that frontal analysis may not be preferable in some instances. Also, these criteria are different from that in the work of Arnold *et al.*[3].

For the Langmuir isotherm, the expression of μ_1^F in Table II is the same as that given by Arnold *et al.*[3], which was obtained from a local equilibrium approach. This elution volume equation with different nomenclature was also derived by Kasei and Oda[15]. Arnold *et al.* pointed out that the elution volume equation derived by Dunn[24] and Chaiken[16] is similar to this expression of μ_1^F. However, Dunn and Chaiken[25,26] used the equation to estimate the K_L value with zonal elution data.

The effects of concentration on the capacity factor in affinity chromatography are show in Fig. 2. Both predicted values and experimental data from the literature[9] are shown. The predicted values were calculated with the equations in Table II for a Langmuir isotherm at equilibrium, *i.e.*,

$$k' \text{ (zonal)} = \frac{(1-\varepsilon)\rho_p q_s K_L}{\varepsilon + (1-\varepsilon)\varepsilon_p} \cdot \Phi(a) \tag{12}$$

$$k' \text{ (frontal)} = \frac{(1-\varepsilon)\rho_p q_s K_L}{[\varepsilon + (1-\varepsilon)\varepsilon_p](1 + K_L C_0)} \tag{13}$$

TABLE IV

EXPRESSIONS OF THE FIRST MOMENT AND CAPACITY FACTOR IN INHIBITION EXPERIMENTS

Inhibitor	*Frontal analysis, μ_1^F and k'*		*Zonal analysis, μ_1^Z and k'*	
Competing free (soluble) inhibitor:				
$P + L = PL$: $K_L = \dfrac{[PL]}{[L]C_p}$	$\mu_1^F = \dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s K_L}{(1+K_I C_I)(1+K_L C_0)}\right]^a$	(F1)	$\mu_1^Z = \dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s K_L}{(1+K_I C_I)} \cdot \Phi(a)\right]$	(Z1)
$P + I = PI$: $K_I = \dfrac{C_{PI}}{C_I C_p}$	$k' = \dfrac{(1-\varepsilon)\rho_p q_s K_L}{[\varepsilon+(1-\varepsilon)\varepsilon_p](1+K_I C_I)(1+K_L C_0)}$	(F2)	$k' = \dfrac{(1-\varepsilon)\rho_p q_s K_L}{[\varepsilon+(1-\varepsilon)\varepsilon_p](1+K_I C_I)} \cdot \Phi(a)$	(Z2)
			$a = \sqrt{\left(\dfrac{u_0\ t_0}{L}\right)\dfrac{C_0\ (1+K_I C_I)}{(1-\varepsilon)\rho_p q_s}}$	(Z3)
Competing binding inhibitor:				
$P + L = PL$: $K_L = \dfrac{[PL]}{[L]C_p}$	$\mu_1^F = \dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s K_L}{(1+K_I C_I+K_L C_0)}\right]$	(F3)	$\mu_1^Z = \dfrac{L}{u_0}\left[\varepsilon + (1-\varepsilon)\varepsilon_p + \dfrac{(1-\varepsilon)\rho_p q_s K_L}{(1+K_I C_I)} \cdot \Phi(a)\right]$	(Z1)
$I + L = IL$: $K_I = \dfrac{[IL]}{[L]C_I}$	$k' = \dfrac{(1-\varepsilon)\rho_p q_s K_L}{[\varepsilon+(1-\varepsilon)\varepsilon_p](1+K_I C_I+K_L C_0)}$	(F4)	$k' = \dfrac{(1-\varepsilon)\rho_p q_s K_L}{[\varepsilon+(1-\varepsilon)\varepsilon_p](1+K_I C_I)} \cdot \Phi(a)$	(Z2)
			$a = \sqrt{\left(\dfrac{u_0\ t_0}{L}\right)\dfrac{C_0}{(1-\varepsilon)\rho_p q_s}}$	(Z4)

[a] Where C_0 is the concentration C_p in the influent solution.

It is obvious that the theoretical results fit very well the data for zonal elution experiments with high-performance affinity chromatography. The linearity of the capacity factor no longer exists in either zonal or frontal analysis.

Eqns. 12 and 13 provide a means of estimating the values of K_L and $(1-\varepsilon)\rho_p q_s$ from the experimental data for concentration C_0 *vs.* capacity factor. Note that the dependence of the capacity factor on the binding site concentration, $(1-\varepsilon)\rho_p q_s$, is different from that of linear theory. As shown in Table II, K_L cannot be distinguished from $(1-\varepsilon)\rho_p q_s$ in the expression of the first moment for a linear isotherm. On the contrary, K_L and $(1-\varepsilon)\rho_p q_s$ can be determined simulaneously from a reciprocal plot or from a non-linear regression of C_0 *vs.* capacity factor for a Langmuir isotherm.

Sometimes the K_L and $(1-\varepsilon)\rho_p q_s$ values were obtained from other independent experiments, *e.g.*, batch isotherm measurement or inhibition experiments. In this case, a correlation can be drawn between the calculated and experimental values of Φ. The calculated values of Φ are obtained directly from eqns. 9–11, and the experimental values are from the capacity factors obtained by experiments with various C_0. Fig. 3 shows the correlation with data from the literature[7,9]. The strong correlation demonstrates that the equations in Table II are valuable.

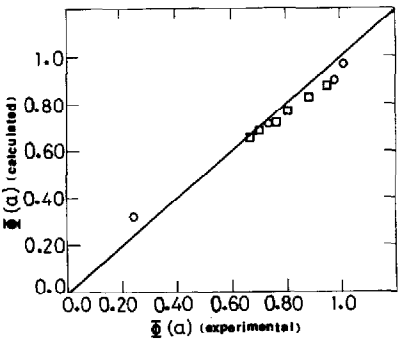

Fig. 3. Correlation of the universal function $\Phi(a)$ in zonal analysis. (○) Data after Anderson and Walters[9], same source as in Fig. 2; (□) data after Muller and Carr[7] in their Fig. 4; binding of *p*-nitrophenyl α-D-mannopyranoside on concanavalin A immobilized in 50-μm silica particle. $t_0 = 0.025$ min, $V_B = 1.06$ ml, $V_0 = 0.85$ ml, $\varepsilon = 0.5$, $\varepsilon_p = 0.6$, $Q = 1$ ml min^{-1}, $L = 15$ cm, $K_L = 1.5 \cdot 10^4$ l mol^{-1} and $(1-\varepsilon)\rho_p q_s = 6.39 \cdot 10^{-4}$ M are estimated with eqns. Z2 and Z4 using the data in Muller and Carr's Fig. 3.

INHIBITION EXPERIMENTS AND FINITE MASS-TRANSFER EFFECTS

As the K_L value in a common affinity chromatographic system is very large, the volume needed to saturate the packing bed (in the frontal mode) or to elute isocratically the desired component (in the zonal mode) is large. The use of a competing inhibitor will reduce the elution volume to a manageable level. Two types of inhibitor for inhibition experiments have been used, namely competing free (soluble) inhibitor and competing binding inhibitor, as in this work. In the former instance, a soluble inhibitor (I), which competes with an immobilized ligand (L) for the desired macromolecular compound (P), is present in the sample solution and pre-equilibrated buffer[16]. In the latter a macromolecular compound (L) is im-

mobilized and is saturated with sample containing a solute (P) and a competing inhibitor (I), or a solute (P) is isocratically eluted using a competing inhibitor (I) in the mobile phase. The latter case may also be called reversed-role affinity chromatography[9]. As shown in Table IV, expressions of the first moment and capacity factor for both zonal and frontal analyses have been derived. It should be noted that there is a small mistake in the paper by Arnold *et al.*[3]. They used the equation for the second type of inhibition, *i.e.*, eqn. F3 in Table IV, to calculate the association constant from the first type of inhibition experiment. A correction should replace eqn. 11 in their paper by eqn. F1 in Table IV.

As an example of using inhibition experiments for determining binding constants, we consider the work of Dunn and Chaiken[26] in which nuclease was adsorbed on pdTpAp-Sepharose with soluble pdTp as competing soluble inhibitor. Fig. 1 in that paper gives a plot of elution volume *vs.* concentration of immobilized ligand, $(1-\varepsilon)\rho_p q_s$, from a zonal elution experiment. They used an equation similar to eqn. F2 in Table IV (for frontal analysis) to estimate the constant K_L to be $3.85 \cdot 10^5$ M. If we use eqns. Z2 and Z3 to calculate the binding constant, it will be $K_L = 2.5 \cdot 10^5$ M by non-linear regression. They overestimated the K_L value by 54%.

In applying the equations in this work to determine the binding constant, one may test first whether the experiment can be performed in the linear region by running it at various sample concentrations C_0 with or without inhibitors. If the resulting capacity factor k' is independent of C_0, then linear theory is required to calculate K_L. In contrast, if a dependence of k' on C_0 is obvious, eqn. 12 or 13 or the equation in Table IV is required to determine K_L and also $(1-\varepsilon)\rho_p q_s$ from the experimental data for k' *vs.* C_0.

Previous investigators have reported that the major effect of column dispersion and mass-transfer resistances is zone spreading. Their effect on elution volume is believed to be relatively small. Numerical calculations were made for frontal analysis and it was observed that the μ_1 values are nearly independent of parameters that characterize the dispersion and mass-transfer resistances. If we consider the column for μ_1^F in Table II, it will be no surprise that the effect of column dispersion and mass transfer on frontal elution is negligible. Although no results are reported at this stage for zonal analysis, it is believed that the effect of dispersion and mass transfer on elution volume is relatively small compared with that of concentration, *i.e.*, the isotherm non-linearity.

SYMBOLS

c concentration of solute in pores, M
C concentration of solute in bulk fluid phase, M
C_0 sample concentration, M
C_p concentration of component P, M
C_I concentration of inhibitor I, M
d_p diameter of particle, cm
D dispersion coefficient in column, $cm^2\ s^{-1}$
D_i pore diffusivity of solute, $cm^2\ s^{-1}$
k' capacity factor
k_a desorption rate constant, s^{-1}

k_f fluid film mass transfer coefficient of solute, cm s^{-1}
K_I inhibition constant, l mol^{-1}
K_L binding constant, l mol^{-1}
L length of the column, cm
q sorbate concentration, mmol (g particle)$^{-1}$
q^* equilibrium value of q
q_s maximum number of available binding sites, mmol (g particle)$^{-1}$
Q volumetric flow-rate, cm^3 s^{-1}
R radius of particle, cm
r radial distance in particle, cm
t time, s
t_0 time interval of sample injection, s
u_0 liquid superficial velocity, cm s^{-1}
V_0 unretarded void volume, cm^3
V_B total bed volume, *i.e.*, the empty column volume, cm^3
V_t retention (elution) volume, cm^3
z linear coordinate along the packed bed, cm

ε void fraction of the packed bed, equal to the volume outside the particles divided by the empty column volume
ε_p porosity of particle
ρ_p particle density, the density of packed stationary phase in column, g (cm^3 particle)$^{-1}$
μ_1 first moment, s

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