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A Theory of Gradient Hydroxyapatite Chromatography: Approximate Calculation of Chromatograms Taking into Account Both Mutual Molecular Interactions and Longitudinal Diffusion

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Abstract

A method for the approximate calculation of theoretical chromatograms with gradient chromatography is proposed for a mixutre of molecules with the same dimensions and the same shape. Account is taken of both the mutal interactions among molecules adsorbed on the crystal surfaces of hydroxyapatite and the longitudinal molecular diffusion in the column. The parameter that is related to geometrical configurations of a molecule on the crystal surface is specified.

INTRODUCTION

On the basis of experimental data, a model has been derived for the adsorption and desorption phenomena on the crystal surfaces of hydroxyapatite (HA) in a column (*1; 2,* Appendix I; *3*, Introduction Section). This model can be stated briefly as follows: Adsorbing sites are arranged in some manner on the surfaces of HA, and sample molecules with adsorption groups compete with particular ions from the buffer for these crystal sites. Both the distribution and stereochemical structures of the adsorbing sites on the surfaces of HA were explored (*4, 5*). Thus two types of sites referred to as C and P sites, exist on different crystal surfaces. Nucleoside phosphates and nucleic acids are adsorbed onto C sites by using phosphate groups. Acidic polypeptides, acidic proteins, and collagen (in the presence of sodium chloride) are adsorbed mainly onto C sites by using carboxyl groups. All these molecules compete with phosphate ions from the buffer that are also adsorbed onto C sites. Basic polypeptides and basic proteins are adsorbed mainly onto P sites by using basic groups, and they compete with cations

(sodium or potassium ions) from the buffer (*2*, Appendix I; *3*, Introduction Section; *6–8*; for collagen, see Ref. *9*).

The chromatographic process on a HA column is virtually a quasi-static process. Virtually no deformation of the chromatogram or change in elution position occurs when the flow rate is changed (*3*). Longitudinal diffusion in the column is essentially caused by the heterogeneity in the flow rate of the solution occurring within each vertical section of the column; it can be deduced that the heterogeneity in the flow rate is provoked by the heterogeneity in interspaces among HA crystals packed in the column (*3*). A new theory of HA chromatography with small sample loads was developed for the case when the elution of molecules is carried out by using a linear molarity gradient of competing ions, where account is taken of the longitudinal diffusions of both the sample molecules and the competing ions in the column (*3, 10*). With small sample loads the effect of the mutual interactions among sample molecules is negligible; the chromatographic behavior of any single component in the mixture is independent of the presence of the other components (*3*). The theory in Refs. *3* and *10* was confirmed experimentally (*11*).

On the other hand, we have a theory of gradient HA chromatography where account is taken of the mutual interactions among sample molecules adsorbed on the crystal surfaces of HA (*12–14*). The interactions occurring in the interstitial liquid in the column are negligible since the concentration of molecules in solution is usually low. In this theory, however, the effect of longitudinal diffusion in the column is not taken into consideration. Further only the case of macromolecules with rod like shapes is treated. From a thermodynamic consideration, it can be deduced (*12*, Appendix I) that, even in the case of molecules with elongated shapes with some flexibility, mutually superimposed adsorption does not occur on the crystal surface. When the molecular density on the crystal surface is high, the molecules should be adsorbed side-by-side in parallel with one another, avoiding the mutual superposition. If the adsorption energy per molecule in the state of no superposition is large enough for the strong adsorption necessary for chromatography to occur, then the loss of adsorption energy due to superposition should also be large. The loss of energy due to superposition should be much more important than the gain of entropy occurring, provided that molecules are orienting at random on the crystal surface and allowing the mutual superposition. Therefore, with rod like molecules, the energetic interactions should occur mainly through the sides of rods oriented in parallel with one another on the crystal surface (*12*). The theory (*12–14*) should be valid, however, for molecules with any shapes except the form of the funtion that represents the molecular interactions occurring on the crystal surface (cf. Eq. 8).

It can be deduced (*12, 13*) that repulsive interactions usually reside among

molecules existing on the crystal surface. Actually, beside sample molecules, the solvent (water, competing, and other ions) can be assumed to exist on the HA surface. The surface of a sample molecule that is not in contact with other sample molecules nor the crystal surface itself is in contact with the solvent. A molecule (or an ion) of the solvent is in contact with sample molecules and/or other solvent molecules (or ions). The precise meaning of the interaction energy between sample molecules, therefore, is the change in total (free) energy of the system occurring when the isolated molecules existing on the crystal surface come in contact with one another. In the theories (*1–5, 10–14*) it is assumed, however, that the total (free) energy of the system does not depend upon the ratios among the amounts of different components of the solvent that are in contact with a sample molecule (but see Discussion Section E in Ref. *9*). From a general thermodynamic consideration, when the density of molecules on the crystal surface is high enough, molecules with high total energies involved in the reaction with crystal sites should be adsorbed on the crystal surface preferentially to those with small energies (*13*). Because of repulsive interactions, however, the effective density of molecules on the crystal surface, which is necessary for preferential adsorption among different molecules (see above), should be reducted in comparison with the case of no interactions. Because of the reduction in the effective molecular density, the effect of preferential molecular adsorption should, in fact, appear in gradient chromatography as the displacement effect. Thus the elution molarity of the molecular component with small adsorption energy should be displaced to a smaller molarity in the presence of the component with large adsorption energy. As a result of the repulsive interactions or the displacement effect, the shape of the total chromatogram of the mixture should be considerably deformed (*13*).

Experimentally, the displacement effect is explicitly demonstrated in the case of DNA. For instance, with a mixture of yeast mitochondrial DNA that is considerably heterogeneous in both molecular dimensions and base composition, molecules with small dimensions and low AT content are generally eluted at lower phosphate molarities than molecules with large dimensions and high AT content (*15*). It can be assumed that the higher the AT content, the larger the adsorption energy per unit molecular length of DNA (*6, 15*). Nevertheless, the mean elution molarity of the mixture is almost independent of both the mean molecular dimensions and the mean AT content (*15*). This demonstrates that molecular fractionation on the column occurs due to the displacement effect and not due to differences in characteristic elution molarities among the respective components. Other good examples of the displacement effect can be seen for the mixture of lysozyme and cytochrome *c* (*16*), and for collagen (*9, 17*).

In Ref. *14*, neglecting the effect of longitudinal diffusion in the column but taking into account the repulsive molecular interactions occurring on the

crystal surfaces, a method for the general analytical calculation of the chromatogram was proposed for a mixture of molecules with the same dimensions and the same shape. In the present paper, by combining the older theory (*14*) and the recently developed theory (*3, 10*), a method for the approximate calculation of the chromatogram for a mixture of molecules with the same dimensions and the same shape is developed, where account is taken of both molecular interactions on the HA surfaces and longitudinal diffusion in the column. It is always assumed that adsorption occurs only on a single type of crystal site, which is a reasonable assumption for collagen (see above and Ref. *9*).

In the Appendix, the parameter $\tau_{(\rho')}$, which is related to the geometrical configurations of a molecule on the crystal surface, is specified.

In Ref. 9, on the basis of the theory developed in this paper, experimental chromatograms of collagen that have previously been obtained (*17*) will be analyzed.

THEORETICAL

With linear gradient chromatography a chromatogram can, in general, be represented as a function of molarity m of competing ions from the buffer used to make the gradient when the experimental parameter s (see below) is given. Under the assumption of no longitudinal diffusion in the column, the total chromatogram, $\hat{F}_s(m)$, for a given mixture of components 1, $2, \ldots, \rho', \ldots, \rho$ can be represented as a sum of contributions, $\hat{f}_{(\rho'),s}(m)$ (where $\rho' = 1, 2, \ldots, \rho$), from the respective components as

$$\hat{F}_s(m) = \sum_{\rho'=1}^{\rho} \hat{f}_{(\rho'),s}(m) \tag{1}$$

where the symbol $\hat{}$ indicates that the assumption of no longitudinal diffusion is involved. The subscript s is defined as

$$s = gL \tag{2}$$

where L and g represent the length of the column and the slope of the molarity gradient of competing ions, respectively. g is expressed as the increase in ion molarity per unit length of the column measured from the bottom to the top of the column in order for s to have a dimension of molarity. The function $f_{(\rho'),s}(m)$ (for the case when all molecules in the mixture have the same dimensions and the same shape) can be represented by both Eqs. (34) and (33′) in Ref *14*, which can be rewritten with slight modifications as

$$\hat{f}_{(\rho'),s}(m) = \frac{1}{\sum_{\rho''=1}^{\rho} \chi^*_{(\rho'')}} \frac{(\varphi' m + 1)^{x'}}{q_{(\rho')} s} \left[\frac{dY_{(\rho')}(\chi_{(\rho')})}{d\chi_{(\rho')}} \right]^2 \left[\frac{d^2 Y_{(\rho')}(\chi_{(\rho')})}{d\chi^2_{(\rho')}} \right]^{-1} \tag{3}$$

and

$$m = \frac{1}{\varphi'} \left[\left\{ (x' + 1)\varphi' q_{(\rho')} s \left[\frac{dY_{(\rho')}(\chi_{(\rho')})}{d\chi_{(\rho')}} \right]^{-1} + (\varphi' m_{\text{in}} + 1)^{x'+1} \right\}^{1/(x'+1)} - 1 \right] \tag{4}$$

$\chi_{(\rho')}$ being an intermediate parameter. [For the physical meaning of $\chi_{(\rho')}$, see below.] In both Eqs. (3) and (4), φ' is a positive constant representing the property of the competing ions. x' is the average number (in the equilibrium state) of sites of HA on which the adsorption of competing ions is impossible due to the presence of an adsorbed molecule; x' therefore represents the effective dimensions of the molecule assumed to be the same for any component of the mixture. $\chi^*_{(1)}, \ldots, \chi^*_{(\rho'')}, \ldots, \chi^*_{(\rho)}$ [which are also involved in the function $Y_{(\rho')}(\chi_{(\rho')})$ as parameters, see below], respectively, are the amounts of components $1, \ldots, \rho'', \ldots, \rho$ loaded initially on the column, expressed in unit such that $\chi^*_{(\rho'')} = 1$ provided the whole column is saturated only with componrnt ρ''. m_{in} is the initial molarity of competing ions at the beginning of the gradient (for some remark on the beginning of the gradient, see Ref. *10*). $q_{(\rho')}$ is defined as

$$q_{(\rho')} = \beta \tau_{(\rho')} e^{x_{(\rho')} \varepsilon / kT} \tag{5}$$

where $x_{(\rho')}$ is the average number (in the equilibrium state) of adsorption groups per molecule of component ρ' that react with sites of HA (cf. Eq. A2 in the Appendix), $-\varepsilon\,(\varepsilon > 0)$ is the adsorption energy of a functional group of the molecule on one of the sites of HA, T is the absolute temperature, k is the Boltzmann constant, and β and $\tau_{(\rho')}$ are positive constants related to the properties of the column and the molecule of component ρ', respectively. (For $\tau_{(\rho')}$, see Eq. A3 in the Appendix).

Finally, in Eqs. (3) and (4), $Y_{(\rho')}(\chi_{(\rho')})$ is the function concerning the mutual molecular interactions occurring on the crystal surface of HA, which is defined as

$$Y_{(\rho')}(\chi_{(\rho')}) = H(\chi)\chi_{(\rho')} \tag{6}$$

where

$$\chi\left(= \sum_{\rho''=1}^{\rho} \chi_{(\rho'')} \right) = \sum_{\rho''=1}^{\rho} \chi^*_{(\rho'')} \left[\frac{\chi_{(\rho')}}{\chi^*_{(\rho')}} \right]^{q(\rho')/q(\rho'')} \tag{7}$$

and

$$H(\chi) = \frac{e^{(\tilde{E}/kT)\sqrt{\chi}}}{1-\chi} \tag{8}$$

In Eqs. (6) and (7), the parameter $\chi_{(\rho')}$, which is the intermediate parameter between Eqs. (3) and (4) (see above), has a physical meaning of the molecular density of component ρ' on the surfaces of HA within the last infinitesimal section at the bottom of the column, or the proportion of the effective crystal surfaces occupied by molecules of component ρ' within that column section. $\chi_{(\rho')}$ is unity, provided the crystal surfaces are saturated only with component ρ' (*14*). Therefore, χ, which is defined as a sum of densities $\chi_{(\rho'')}$ for all ρ'' [the intermediate term (in parentheses) in eq. 7], represents the proportion of the effective surfaces of HA within the last section of the column that is occupied by molecules of all components 1, 2, . . . , ρ; χ is unity provided the crystal surfaces are saturated with these molecules. χ can be represented, however, as a function of only the density $\chi_{(\rho')}$ for a given component ρ' (the right-hand side of Eq. 7). The right-hand term in Eq. (7) shows that χ involves $\chi^*_{(\rho'')}$ as parameters for all ρ''. In fact, the value of $\chi_{(\rho'')}$ for any ρ'' can be determined when both the value of $\chi_{(\rho')}$ for the given ρ' and the values of $\chi^*_{(\rho'')}$ for all ρ'' are given. This is the reason why, in Eq. (7), the intermediate term (in parentheses) can be rewritten as the right-hand term. $H(\chi)$ (Eq. 8) represents the mutual interactions among sample molecules occurring on the crystal surfaces within the last section of the column. The numerator and denominator on the right-hand side of Eq. (8) are concerned with energetic and geometrical interactions, respectively. The expression in the numerator can be applied only to the case of rodlike molecules, however. Thus $\tilde{E}$ represents the interaction energy for one molecule with others on the crystal surface provided that one of the two sides of the rod is completely brought into contact with (or, more precisely, keeps the minimum distance from) other molecules that are adsorbed side-by-side in the same orientation (see Introduction Section). It is assumed (a) that the distribution of the molecules on the crystal surfaces follows a Bragg-Williams approximation, (b) that only the short-range interactions are of importance, and (c) that the probability that any part of a side of a rodlike molecule is brought into contact with other molecules is proportional to $\sqrt{\chi}$. With usual repulsive interactions (Introduction Section), $\tilde{E}$ is defined as positive. The formula of the numerator on the right-hand side of Eq. (8) can therefore be different if a

different approximation is used and/or the shape of the molecule differs from that of a rod. The denominator on the right-hand side of Eq. (8) is an approximate expression of the probability [denoted by $p_{(\rho')}(\chi_{(1)}, \ldots, \chi_{(\rho)})$ or $p(\chi)$ in Refs. *12* and *13*] that, when a new molecule is added at random to the crystal surface, a proportion χ of which is already occupied by molecules, it is not superimposed on the already adsorbed molecules. The final chromatogram depends only slighly upon the shape of the function $p_{(\rho')}$ or p (*12, 13*).

The term $1/\Sigma_{\rho''=1}^{\rho}\chi^*_{(\rho'')}$ on the right-hand side of Eq. (3) (which does not appear in Eq. 34 in Ref. *14*) normalizes the total chromatogram of the mixture such that

$$\int_{m_{in}}^{\infty} \hat{F}_s(m)\, dm = 1 \tag{9}$$

With small sample loads when χ tends to zero, mutual interactions among molecules are negligible. $H(\chi)$ (Eq. 8) tends to unity, and $Y_{(\rho')}(\chi_{(\rho')})$ and $dY_{(\rho')}(\chi_{(\rho')}))/d\chi_{(\rho')}$ (Eq. 6) tend to $\chi_{(\rho')}$ and unity, respectively. In this instance, Eq. (4) reduces to

$$m = \frac{1}{\varphi'}[\{(x' + 1)\varphi' q_{(\rho')} s + (\varphi' m_{in} + 1)^{x'+1}\}^{1/(x'+1)} - 1] \tag{10}$$

which no longer involves the parameter $\chi_{(\rho')}$ nor the parameters $\chi^*_{(1)}, \ldots, \chi^*_{(\rho)}$. This means (*3, 14*) that, in the absence of both molecular interactions and longitudinal diffusion in the column, a chromatographic peak has an infinitesimal width. The elution molarity m of the sharp peak of component ρ' can be represented by Eq. (10) independently of the presence of all the other components.

Actually, even with small sample loads, the chromatographic peak has a finite width due to longitudinal diffusion in the column (see Introduction Section). The theoretical chromatogram for for any component ρ' of the mixture obtained by taking into account longitudinal diffusion [denoted by $f^{\circ}_{(\rho'),s}(m)$; the symbol $^{\circ}$ indicates the infinitesimal sample load] can be represented by both Eqs. (36′) and (34) in Ref. *3* or both Eqs. (62) and (73) in Ref. *10*. These equations can be rewritten, with slight modifications, as

$$f^{\circ}_{(\rho'),s}(m) = \frac{\Phi_{(\rho')}}{\sqrt{4\pi\theta_0 gs}}\, e^{-[r_{(\rho')}(m_\lambda)-s]^2/4\theta_0 gs}\, \frac{dr_{(\rho')}(m_\lambda)/dm_\lambda}{1 + dr_{(\rho')}(m_\lambda)/dm_\lambda} \tag{11}$$

and

$$m = m_\lambda + r_{(\rho')}(m_\lambda) - s \tag{12}$$

where m_λ is an intermediate parameter. $r_{(\rho')}(m_\lambda)$ and $dr_{(\rho')}(m_\lambda)/dm_\lambda$ are defined as

$$r_{(\rho')}(m_\lambda) = \frac{1}{q_{(\rho')}\varphi'(x'+1)}[(\varphi' m_\lambda + 1)^{x'+1} - (\varphi' m_{in} + 1)^{x'+1}] \quad (13)$$

and

$$\frac{dr_{(\rho')}(m_\lambda)}{dm_\lambda} = \frac{1}{q_{(\rho')}}(\varphi' m_\lambda + 1)^{x'} \quad (14)$$

In Eq. (11), θ_0 is a positive constant (with a dimension of length) that measures the longitudinal diffusion of molecules in the column. θ_0 is independent of the type of molecules since the longitudinal diffusion is essentially due to the heterogeneity in the flow rate in the column (Introduction Section). $\phi_{(\rho')}$ (which does not appear in Eq. 36′ in Ref. *3* or Eq. 62 in Ref. *10*) represents the ratio of the amount of component ρ' to the total amount of molecules in the mixture. This can be written as

$$\phi_{(\rho')} = \frac{X^*_{(\rho')}}{\sum_{\rho''=1}^{\rho} X^*_{(\rho'')}} \quad (15)$$

so that

$$\sum_{\rho'=1}^{\rho} \phi_{(\rho')} = 1 \quad (16)$$

With the factor $\phi_{(\rho')}$, the total chromatogram,

$$F^\circ{}_s(m) = \sum_{\rho'=1}^{\rho} f^\circ{}_{(\rho'),s}(m) \quad (17)$$

is normalized such that

$$\int_{m_{in}}^{\infty} F^\circ_s(m)\, dm = 1 \quad (18)$$

In Ref. *11* it was shown that the shape of the function $f^\circ_{(\rho'),s}(m)$ is almost Gaussian. Both the center of gravity and the position at the maximum height

of a theoretical chromatogram given by this function can be represented with good approximations by using Eq. (10) (see Figs. 5 and 11 in Ref. *11*; see also Ref. *18*). Moreover, the shape of the theoretical peak depends only slightly upon the value of $q_{(\rho')}$ (Eq. 5) if x' is constant. As a result, when the parameter s (Eq. 2) is given, or under a given experimental condition (see above), the shape of the chromatogram is almost independent of the mean position of the chromatogram as long as x' is constant. The mean position of the chromatogram is determined by $q_{(\rho')}$ (see Figs. 5 and 11 in Ref. *11*). This means that the function $f^{\circ}_{(\rho'),s}(m)$ can be represented approximately as

$$f^{\circ}_{(\rho'),s}(m) = \phi_{(\rho')} G(m;\ \mu_{(\rho'),s},\ \bar{\sigma}_s) \tag{19}$$

where

$$G(m;\ \mu_{(\rho'),s},\ \bar{\sigma}_s) = \frac{1}{\sqrt{2\pi}\bar{\sigma}_s} e^{-(m-\mu_{(\rho'),s})^2/2\bar{\sigma}_s^2} \tag{20}$$

represents a Gaussian function for m. The mean value, $\mu_{(\rho'),s}$, of m is given by Eq. (10). The standard deviation $\bar{\sigma}_s$ depends only slightly upon the mean position, $\mu_{(\rho'),s}$, of the chromatographic peak if x' is constant (see above). Therefore, the value of $\bar{\sigma}_s$ for any value of s and for any component of the given mixture can approximately be estimated if the value of θ_0 (see Eq. 11) is given. θ_0 is a constant that is independent of the type of molecule (see above). In Ref. *11* the value of θ_0 was estimated from the experiment of lysozyme. Practically, it is convenient to estimate first the value of $\bar{\sigma}_s$ for the component that appears near the center of the total chromatogram of the mixture. It can be assumed that $\bar{\sigma}_s$ values for any other components are identical with the former value (see above and Ref. *9*).

Now, the total chromatogram, $F^{\circ}_s(m)$ (Eq. 17), can be represented approximately as

$$F^{\circ}_s(m) = \sum_{\rho'=1}^{\rho} \phi_{(\rho')} G(m;\ \mu_{(\rho'),s},\ \bar{\sigma}_s) \tag{21}$$

which can be rewritten as

$$F^{\circ}_s(m) = \int_{m_{in}}^{\infty} \phi_s(\mu) G(m;\ \mu,\ \bar{\sigma}_s)\, d\mu \tag{22}$$

where $\phi_s(\mu)\, d\mu$ represents the probability that any molecule appears between molarity μ and $\mu + d\mu$ of competing ions (under the experimental condition where s is given), provided that there is no longitudinal diffusion in the column. If the components $1, 2, \ldots, \rho$ are distributed discretely in the mixture, $\phi_s(\mu)$ can be expressed as

$$\phi_s(\mu) = \sum_{\rho'=1}^{\rho} \phi_{(\rho')}\delta(\mu - \mu_{(\rho'),s}) \tag{23}$$

where δ is a delta function. Equation (22) is generally valid for the case of a continuous distribution of the components in the mixture. The continuous chromatogram that is obtained in this situation in the absence of longitidinal diffusion in the column can be represented by the function $\phi_s(\mu)$.

In the presence of repulsive molecular interactions on the crystal surfaces and in the absence of longitudinal diffusion in the column, a continuous chromatogram is obtained even when the distribution of the components in the mixture is discrete. Thus, due to molecular interactions, the width of the chromatographic peak for any component of the mixture becomes finite; mathematically, any peak in the total chromatogram extends over an infinite range with a finite standard deviation (cf. Eqs. 3 and 4). Therefore, the total chromatogram represents a continuous distribtuion of molecules. This type of chromatogram can also be represented by the function $\phi_s(\mu)$ in Eq. (22). In fact, since the longitudinal molecular diffusion is essentially due to the heterogeneity in the flow rate in the column (Introduction Section), $\phi_s(\mu)$ can represent any chromatogram that can be obtained provided that the flow rate is homogeneous and that there is no longitudinal diffusion in the column. This statement is true independent of how the continuous chromatogram $\phi_s(\mu)$ is produced. This means that the function $\phi_s(\mu)$ in Eq. (22) can be replaced by the function $\hat{F}_s(\mu)$ defined by Eq. (1), giving $F_s^\circ(m)$ a physical meaning of the total chromatogram for any mixture that can be obtained in the presence of both the molecular interactions and the longitudinal diffusion in the column. Hence, writing $F_s(m)$ instead of $F_s^\circ(m)$, we finally have

$$F_s(m) = \int_{m_{in}}^{\infty} \hat{F}_s(\mu) G(m; \mu, \bar{\sigma}_s)\, d\mu \tag{24}$$

$F_s(m)$ is normalized such that

$$\int_{m_{in}}^{\infty} F_s(m)\, dm = 1 \tag{25}$$

DISCUSSION

Earlier (*10*) it was shown that, with gradient chromatography, it is impossible in principle for a chromatographic process to be described on the basis of a continuity equation for the actual molecular flux occurring on the column. It is an abstract molecular flux occurring on the molarity gradient of the competing ions that is fundamental. The mathematical structures of both actual and abstract fluxes are mutually derivable from the structures of one

another. In the process of these derivations, an intermediate abstract flux appears. This flux, with density $\chi_{(\rho')}$ (see Eqs. 3 and 4), occurs on the gradient made by the quantity s (Eq. 2). As far as this flux is concerned, molecular diffusion cannot occur since the flux concerns only molecules existing on the crystal surfaces [with density $\chi_{(\rho')}$]. In contrast with the actual chromatographic process in gradient chromatography that can be described only by using the continuity equation for the abstract flux (see above), the idealized chromatographic process in the absence of longitudinal diffusion in the column can be described on the basis of both abstract and intermediate abstract fluxes (see Eqs. 18 and 20 in Ref *10*). In Ref. *10*, however, the arguments above were made only for the case of small sample loads when there are no mutual molecular interactions. We confirm below the fact that the intermediate abstract flux is conceivable even in the presence of molecular interactions, and that it can be applied to the flow of molecules of any component in the mixture. This is necessary because Eqs. (3) and (4) (which give an idealized chromatogram occurring both in the presence of mutual molecular interactions and in the absence of longitudinal diffusion in the column) are derived from the solution of the simultaneous continuity equations for the intermediate abstract flux (Eq. 21 in Ref. *14*). In the presence of molecular interactions, the simultaneous continuity equations for the intermediate abstract flux would have much simpler structures than the equations for the abstract flux, because it is on the crystal surfaces that the molecular interactions occur (Introduction Section) while the intermediate abstract flux concerns only molecules existing on the crystal surfaces (see above). The method of the approximate calculation of the chromatogram applied in the present paper would greatly reduce the mathematical difficulty appearing in the method consisting of solving the simultaneous continuity equations for the abstract flux occurring in the presence of both molecular interactions and diffusion.

Now, in the case of small sample loads when there are no molecular interactions, the authenticities of the mathematical structures of both the intermediate abstract flux (see Eq. 20 in Ref. *10*) and the abstract flux (see Eq. 17 or Eqs. 2 and 14 in Ref. *10*) can be confirmed from the fact that these can be derived from the mathematical structure of the actual molecular flux occurring on the column itself (see Eq. 29 or Eqs. 1 and 33 in Ref. *10*). The mathematical structure of the actual flux *a priori* is true (*10*). The fact that the intermediate abstract flux concerns only molecules on the crystal surfaces (see above) is an indispensable condition for the derivation processes of the two abstract fluxes (from the actual flux) to be justified (*10*; *3*, Appendix II). Even in the presence of the molecular interactions, the mathematical expression of the actual flux (Eq. 30 or 33 in Ref. *10*) can be considered to be *a* true *priori*. The continuity equation for the intermediate abstract flux (Eq. 20 in Ref. *10*) can simply be replaced with simultaneous continuity equations in the presence of molecular interactions (Eq. 21 in Ref.

14), because the latter equations are also concerned only with molecules on the crystal surfaces (see above). This means that the intermediate abstract flux, in fact, is conceivable even in the presence of molecular interactions, thus justifying the method for the calculation of the chromatogram applied in the present paper.

APPENDIX

Here, we specify the parameter $x_{(\rho')}$ and the entropy factor $\tau_{(\rho')}$, both appearing in Eq. (5). Equation (5) can be rewritten as

$$\begin{aligned} Q_{(\rho')} &\equiv -kT(\ln q_{(\rho')} - \ln \beta) \\ &= -x_{(\rho')}\varepsilon - kT \ln \tau_{(\rho')} \end{aligned} \tag{A1}$$

$Q_{(\rho')}$ represents a type of free energy per molecule of component ρ' on the crystal surface from which the contribution of the interaction energies with other molecules is subtracted. This latter energy is assumed to be the same for any type of molecule in the mixture provided both the dimensions and the shape of any molecules are the same. The first and the second terms on the right-hand side of Eq. (A1) represent the energy and entropy terms of the free energy, respectively; $-x_{(\rho')}\varepsilon$ represents the average adsorption energy per molecule of component ρ' in equilibrium state, and $\tau_{(\rho')}$ is the number of effective geometrical configuration(s) of the molecule on the crystal surface in the equilibrium state. $\tau_{(\rho')}$, in general, is related to both the distribution of functional groups on the molecular surface and the flexibility (or the rigidity) of the molecular structure. In the special case where the flexibility (or the rigidity) of the molecular structure is the same in both solution and the adsorbed state, which should be true at least with rigid or native molecules, $\tau_{(\rho')}$ should be related only to the distribution of the adsorption groups on the molecular surface (neglecting a solvent effect).

It can be considered that a molecule on the crystal surface is a canonical system. This means that, at least with a rigid or native molecule, $x_{(\rho')}$ and $\ln \tau_{(\rho')}$ in the energy and entropy terms in Eq. (A1) can be written as

$$x_{(\rho')} = \sum_{j=1}^{\nu} x_j g_{(\rho')}(x_j) \tag{A2}$$

and

$$\ln \tau_{(\rho')} = -\sum_{j=1}^{\nu} g_{(\rho')}(x_j) \ln g_{(\rho')}(x_j) \tag{A3}$$

respectively, where

$$g_{(\rho')}(x_j) = \left[\frac{e^{x_j \varepsilon / kT}}{\sum_{j=1}^{\nu} e^{x_j \varepsilon / kT}} \right]_{(\rho')} \tag{A4}$$

In Eqs. (A2)–(A4), j and ν represent a configuration of the molecule on the crystal surface and the total number of possible configurations, respectively. x_j is the number of adsorption groups that react with sites of HA when the jth type of configuration is realized. Therefore, provided ε is large, $x_{(\rho')}$ should be equal to the maximum value of x_j (i.e., $\max_{j=1}^{\nu} x_j$), and $\tau_{(\rho')}$ should represent the number of possible configuration(s) of the molecule that can be realized by using the maximum possible number of functional groups.

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