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Theoretical study of the ion-exchange preparative chromatography of a two-protein mixture

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ABSTRACT

Preparative ion-exchange chromatography of a two-protein mixture is theoretically considered by the use of numerical simulations. The mathematical model is a combination of the semi-ideal model for the chromatograpbic process, with the stoichiometric displacement model for the basic interactions between proteins and the stationary phase. A study of the selectivity of the adsorption, which appears to be dependent on the loading of the column, makes possible a better understanding of the chromatograms obtained with either isocratic or gradient elution. Special stress is been laid on the effect of overloading the column by pointing out displacement effects between proteins. The influence of important adsorption parameters, such as the characteristic charge or maximum loading capacity, was investigated by considering criteria of production rate, recovery yield and enrichment of products.

INTRODUCTION

The preparative purification of biomolecules from complex mixtures is now emerging as an important challenge for the pharmaceutical and biotechnology industries and, among all the techniques at their disposal, ion-exchange chromatography is one of the most effective. This technique is nowadays widely used for the analysis and purification of peptides, proteins or polynucleotides [1]. However, despite its numerous applications, this useful technique really suffers from a lack of the theoretical bases necessary for a good understanding of the retention of polymeric ions, such as proteins.

A great amount of work has already been carried out in order to understand the phenomena encountered with small organic or inorganic ions [2–4]. Considering amino acids, Saunders et al. [5] and Dye et al. [6] have contributed to the understanding of their retention behaviour on ion-exchange resins. All these models were satisfactory but their extension to larger molecules such as proteins, usually characterized by a complex three-dimensional structure, is not straightforward. In addition, the number and spatial distribution of the molecule charges interacting with the support are not easy to evaluate.

Nevertheless, a simple model has been developed during the last decade to describe the retention of proteins in ion-exchange chromatography. This model, the stoichiometric displacement model (S.D.M.), was sucessfully used to predict and simulate the chromatographic behaviour of proteins under analytical conditions. Nevertheless, numerical problems arising from the non-linearity of adsorption isotherms in the high concentration range, combined with the mathematical complexity of their expression, have limited the use of the S.D.M. to analytical chromatography.

Recently, the development of a numerical method using the S.D.M., and simulating the

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isocratic or gradient elution of a single protein in ion-exchange chromatography for mass overload conditions, was reported by Cysewski et al. [7]. This original work, applied to the study of the chromatographic behaviour of concentrated proteins, is a milestone in the research on the optimum operating conditions in the preparative ion-exchange chromatography of proteins. Their paper also contributes to a better understanding of the chromatographic peak shapes.

In this work, we extended the study of Cysewski *et* al. [7] to the theoretical study of the separation of a two-protein mixture. Our aim was not to describe accurately all the physical phenomena that occur in ion-exchange chromatography, which is not possible when considering the simplifying hypotheses of the S.D.M. combined with the shortcomings of the numerical modelling, but to study and compare as a whole the respective behaviour of isocratic and gradient preparative elution in order to draw some general conclusions. This was done first through a study of the evolution of the selectivity with the loading factor, and then through the calculation of parameters specific to preparative separations such as the recovery yield, the enrichment factor and the production rate.

MODELLING THE ADSORPTION OF PROTEINS

Introduction

The ion-exchange chromatography of proteins is often operated with pH or counter-ion concentration variations, especially when gradient elution is used. These variations may affect the tertiary structure of the protein and therefore the charge distribution which notably influences, but in a little known and unpredictable way, the affinity of the protein for the support [8]. In addition, the characteristics of the stationary phase, such as its charge density and its physical structure, take on real importance. A model taking into account all these effects and parameters would be of extreme complexity and would need too much information about the proteins and the stationary phase concerned. It has been found more appropriate to develop a model requiring less information and which, even though restricted by its initial hypotheses, could give correct predictions for a limited range of operating conditions. This has been the basic idea for the S.D.M.

Historical background

The basic principles of ion-exchange chromatography, *i.e.,* electrostatic interaction chromatography, were established by Walton [9]. However, they were restricted to small molecules, either organic or otherwise. In order to describe adsorption of polymeric ions with mono-, di- or trivalent counter ions, a simple model derived from the work of Boardman and Partridge [10] was developed by Regnier and co-workers [1,11]. Its major features were restated precisely by Velayudhan and Horváth [12] who, 2 years later, presented a detailed analysis of the formalism of the model [13]. An interesting study was also reported by Whitley et *al.* [8].

Basic hypotheses

This is a non-mechanistic model based on the mass action law, whose main principle is that the displacement of an adsorbed solute is followed by the stoichiometric adsorption of the displacing agent, in such a way as to maintain the electroneutrality of the stationary phase [10,14]. Therefore, the adsorbed salt concentration must be considered to account for the competition for the adsorption sites between this salt and the mobile phase solutes.

In the equations of the model presented below, the proteins in solution will be assumed to behave ideally, *i.e.,* their liquid phase concentration will be considered to be a correct measurement of their activity. Their activity coefficient will be then taken as equal to 1 over the whole concentration range. This hypothesis, unrealistic even in the preparative mode characterized by mass overload conditions, is nevertheless commonly accepted by most workers for the sake of simplicity [13].

For well defined experimental conditions, the S.D.M. considers two distinct co-ion categories: co-ions of the first type, which are liberated from the protein when it binds to the support, and co-ions of the second type, which remain attached during this adsorption process. From this point of view, the protein may therefore be

considered as a neutral salt which is totally dissociated in solution into a poly-ion **, *i.e.,*** the protein is bound to co-ions of the second type, and **first-type** co-ions are free in solution **[13].** These latter therefore remain thermodynamically unchanged during the adsorption process, and simply maintain the solution electroneutrality. In fact, they are not considered in the final equations of the model.

The properties of a biopolymer, e.g., its charge, valency, tertiary structure and size, are assumed not to vary during the separation process operated at constant **pH.**

The stationary phase must be homogeneous and must have pores of constant shape and size with a sufficiently large diameter to prevent any parallel separation by exclusion.

This model does not take into account hydrophobic interactions **[7,15].**

Description of the model

The adsorption-desorption equilibrium of a protein in ion exchange may be represented by the following equation:

$$m_A P_i + m_i A^s = m_A P_i^s + m_i A \qquad (1)$$

where P_i and P_i^s represent the protein in the flowing mobile phase and adsorbed on the stationary phase, respectively. A is the counter ion (or displacing ion) in the mobile phase and A^s is the counter ion adsorbed; the valency of the counter ion is m_A and the protein is m_i-valent with respect to the chromatographic support. Let us specify that when a mixture of proteins is considered, an equilibrium equation such as that in eqn. 1 is written for each protein.

In a first approach, m_i, called the characteristic charge **[16],** may be defined as the number of monovalent counter ions displaced when the protein adsorbs or, in other words, the number of charged residues of the protein in direct contact with the fixed charges of the support **[13].** Also, m_i has another significance which seems more complex to handle. In fact, it accounts for several possible associations between the protein and the support. Indeed, for given operating conditions, such as **pH** and salt concentration, several preferential orientations may co-exist, as shown Whitley ***et al.*** **[8],** who pointed out at least two kinds of different binding forms for each protein they studied. These preferential orientations may depend on the adsorbed protein concentration **[13].** In conclusion, we can say that m_i represents an average for competing binding forms and therefore its experimental value may not be an integer. Further, if all the previous hypotheses oversimplify a specific system, it will lead to an experimental value of m_i accounting for all the deviations from the ideal situation, and then m_i, initially a simple value derived from a stoichiometric equation, will be transformed into an empirical parameter. However, this does not affect the formalism of the model, as emphasized by Velayudhan and **Horváth [13].**

K_i, the equilibrium constant of eqn. 1, is as follows:

$$K_i = \frac{q_i^{m_A} C_A^{m_i}}{C_i^{m_A} q_A^{m_i}} \qquad (2)$$

where q_i and q_A are the adsorbed protein and counter-ion concentration, respectively, calculated per unit volume of particle skeleton. The other concentrations (C_i and C_A) refer to the product in the mobile phase.

The maximum loading capacity of the exchanger, Q_x, may be defined as the sum of the sites occupied by all the proteins of the mixture and the counter ion:

$$Q_x = \sum_{i=1}^{N_c} m_i q_i + m_A q_A \qquad (3)$$

where N_c is the total number of proteins. We can see that this last equation represents the stationary phase electroneutrality condition.

Substituting eqn. 3 in eqn. 2, we obtain the following isotherm equation:

$$C_i = \frac{q_i}{K_i^{1/m_A}} \left(\frac{m_A C_A}{Q_x - \sum_{i=1}^{N_c} m_i q_i} \right)^z \qquad (4)$$

where Z ($= m_i/m_A$) is the stoichiometric charge ratio of the protein and the counter ion.

As already stated in a previous paper **[17],** in reversed-phase chromatography it is not straightforward to extrapolate from individual adsorption isotherms to competitive isotherms. It is

surprising that, when considering the ion-exchange process, this derivation does not need any other parameters and requires only a modification of the mass balance for the stationary phase, as it already appears in eqn. 3 through the summation term. We can also emphasize the ability of the S.D.M. to take into account the counter-ion concentration, which offers among other advantages the possibility of accounting for gradient elution chromatography.

It must be stressed that eqn. 4 has the disadvantage of being an implicit form of the adsorbed product concentration as a function of the liquid phase product concentration. This unusual feature is of great importance and may introduce some complications during the numerical handling of the equations of the model. Indeed, when several proteins are considered it is necessary to use a numerical method (the Newton-Raphson algorithm) for solving nonlinear systems of eqns. 4 (one equation for each protein).

For very dilute solutions, the total adsorbed protein concentration becomes negligible compared with Q,, and then eqn. 4 becomes a very easy to handle explicit equation:

$$q_i = C_i\left(\frac{Q_x}{m_A C_A}\right)^z K_i^{1/m_A} \qquad (5)$$

NUMERICAL MODEL

Hypotheses and preliminary remarks

In order to have a good description of the various physical phenomena encountered in the separation process of biomolecules in preparative liquid chromatography, several models have been proposed. They may be classified into three main categories: the theory of interferences, the plate theory and the continuity equations. The last category offers the greatest number of developments for both analytical and numerical descriptions [18]. This kind of modelling was therefore chosen here and, in particular, we have used the semi-ideal model, whose numerical resolution has been undertaken using the R.G.S. method (from Rouchon and Golshan-Shirazi, its authors [29]), which is a particular algorithm based on finite differences. The simulation program was performed in FORTRAN.

The simulations will be based on an isothermal porous fixed bed, uniformly packed with one-sized spherical particles, where concentration profiles are assumed to be one-dimensional. Diffusion coefficients of solutes in the mobile and stationary phases are considered to be independent of concentration. Moreover, adsorption equilibrium is assumed to be instantaneous.

The semi-ideal model

In the general case, the continuity equation in the flowing mobile phase for each component is written as

$$\frac{\partial}{\partial t}(Fq_i + C_i) + u\cdot\frac{\partial C_i}{\partial z} = D_{i,ax}\cdot\frac{\partial^2 C_i}{\partial z^2} \qquad (6)$$

z and t being spatial and time coordinates, respectively, F the phase ratio, u the interstitial fluid velocity and $D_{i,ax}$ the axial dispersion coefficient for component i. This description of the phenomena occurring during the separation should be completed by the continuity equation for the stationary phase, accounting for internal mass transfer resistances, and an expression accounting for the mass transfer rate from the mobile phase towards the stationary phase (external mass transfer resistance).

The semi-ideal model assumes that all mass transfer phenomena are very fast. The axial dispersion coefficient in eqn. 6, which accounts for molecular diffusion and dispersion due to the irregular flow through the porous medium, is therefore replaced with an apparent dispersion coefficient $D_{i,ap}$ that accounts also for external and internal mass transfer kinetics that are always considered fast but not infinitely fast [19]. Guiochon *et al.* [20] specified once again that the molecular diffusion coefficient remains constant during the separation, which is true for the range of concentrations used in preparative chromatography. Therefore, the apparent dispersion coefficient, related to this molecular coefficient, is constant.

It must be noted that, for proteins, mass transfer kinetics are usually slow [21,22]. However, as the efficiency of columns is commonly over several hundred theoretical plates, we shall assume that these limitations are correctly taken

into account by the lumped coefficient used by Guiochon *et al.* [20], *i.e.,* the apparent dispersion coefficient. Considering this coefficient, Giddings [23] has established the following expression:

$$D_{i,ap} = \frac{H\,L}{2t_0} = \frac{Hu}{2} \qquad (7)$$

where t_0 is the retention time of an unretained molecule, L the length of the column and H the height equivalent to a theoretical plate.

The accurate numerical resolution of the semi-ideal model is not straightforward, owing to intrinsic errors of the numerical method. One solution, proposed by Guiochon et *al.* [20], is to consider an infinite efficiency of the column, *i.e.,* an infinite number of theoretical plates, and therefore a zero axial dispersion coefficient. This leads to the consideration of the ideal model, written as follows:

$$\frac{\partial}{\partial t}(Fq_i + C_i) + u \cdot \frac{\partial C_i}{\partial z} = 0 \qquad (8)$$

The finite difference method has proved to be a suitable way of solving the ideal model equation, but it results in chromatographic profiles affected by a so-called "numerical diffusion", due to the finite value of the time and space increments of the discretization. Nevertheless, it does correlate well with some experimental curves. This artificial diffusion may be known, mastered and tuned to stick to the physical phenomena and the band broadening well accounted for by the semi-ideal model. Therefore, while trying to solve numerically the ideal model, a systematic error is made, resulting in the solution of the semi-ideal model. This method proved to be very effective in the work of Guiochon *et al.* [20].

NUMERICAL METHOD

Finite difference method

In this method, the movement of products, from their injection to their exit from the column, is represented by a discrete distribution of mass on a grid of spatial and temporal coordinates. Time and spatial derivatives may be transformed into finite differences using various algorithms [24]. In this work, we chose a simple numerical scheme developed by Rouchon *et al.* [25] and successfully used in numerous studies [20,26–28].

Presentation of the numerical scheme

To evaluate spatial and time derivatives we have used the following expressions:

$$\frac{\partial C_i}{\partial z} = \frac{C^j_{i,n} - C^j_{i,n-1}}{\sigma} \qquad (9a)$$

$$\frac{\partial C_i}{\partial t} = \frac{C^j_{i,n-1} - C^{j-1}_{i,n-1}}{\tau} \qquad (9b)$$

and finally for the stationary phase:

$$\frac{\partial q_i}{\partial t} = \frac{q^j_{i,n-1} - q^{j-1}_{i,n-1}}{\tau} \qquad (9c)$$

where σ and z are the space and time increments, respectively, n refers to the space location and j to time. By defining T_i as the ratio of stationary and mobile phase concentrations $(= q_i/C_i)$ and by inserting eqns. 9a–c in eqn. 8, the following scheme is obtained [24]:

$$C^j_{i,n} = C^j_{i,n-1} - \frac{\sigma}{u\tau}\left[(1 + FT^j_{i,n-1})C^j_{i,n-1} - (1 + FT^{j-1}_{i,n-1})C^{j-1}_{i,n-1}\right] = 0 \qquad (10)$$

Eqn. 10 is easy to solve as it expresses explicitly the concentration of component i in the mobile phase for the cell $\binom{j}{n}$ as a function of previously calculated concentrations.

A numerical analysis of this scheme from Guiochon and co-workers [24,29,30] has shown that this algorithm leads to a numerical dispersion, and therefore to an artificial H given by

$$H = \sigma\left[\frac{u\tau}{(1 + Fk_0)\sigma} - 1\right]_{\mathrm{I}} = \sigma(P - 1) \qquad (11)$$

where k_0 is the average value of all the initial slopes of the components isotherm and P the Courant number, written as follows [24,30]:

$$P = \frac{u\tau}{(1 + Fk_0)\sigma} \qquad (12)$$

As we have already stated, the replacement of actual derivatives of the ideal model by finite differences generates a numerical error at each calculation step. If time and space increments

are not carefully chosen, it will be very difficult to control their evolution, and oscillations or divergence phenomena may appear. A stability condition is therefore necessary, and the most commonly used method to check this stability is the Von Neuman analysis [30]. In our case, the deduced condition is [24]:

$$P \geqslant 1 \tag{13}$$

Eqn. 13 imposes a Courant number greater than 1. Czok and Guiochon [24] proposed a value of 2 for P. This choice, satisfying the stability condition, also has the advantage of fixing the space increment value equal to the H leading to (from eqn. 11)

$$\sigma = H \tag{14}$$

The time increment is then written as

$$\tau = \frac{2H(1 + Fk_0)}{U} \tag{15}$$

Using eqns. 14 and 15, this finite difference method is termed R.G.S., from its authors' names, Rouchon and Golshan-Shirazi [29].

From these considerations, it is now possible, from the actual value of the height equivalent to a theoretical plate of the column, to simulate, with the ideal model, actual separations affected by an apparent physical dispersion, by generating the corresponding numerical diffusion in the numerical resolution.

DESCRIPTION OF THE PROBLEM

Protein characterization

We chose two proteins, P_1 and P_2, with relatively close molecular masses ($M \approx 20\,000$ g mol^{-1}) and with a molecular diffusion coefficient equal to 10^{-6} cm^2 s^{-1} [31].

Column characteristics

The ion-exchange column chosen for the computations is 5 cm x 4.6 mm I.D. It is filled with 13-μm spherical porous particles. The total porosity (E) is 0.85. The pore size is assumed to be large enough (1000 A) to avoid any diffusional limitations. The phase ratio [$F = (1 - \varepsilon)/\varepsilon$] is therefore equal to 0.176. The maximum loading capacity of the exchanger (Q_c) is 11 mM, which means that 11 mM of a monovalent ion are necessary to saturate all the adsorption sites (Q_c = 11 mequiv. l^{-1}). The column efficiency is calculated using the Knox equation [32]:

$$h = \frac{2}{\nu} + \nu^{1/3} + \frac{\nu}{10} \tag{16}$$

where h is the- reduced plate height ($h = H/d_p$), ν the reduced mobile phase velocity ($\nu = ud_p/D_m$), d_p the average particle diameter, u the interstitial fluid velocity and D_m the molecular diffusion coefficient of the protein; u will be taken as equal to 0.01 cm/s for the whole study.

The reduced mobile phase velocity is then 13, and is nearly five times larger than the mathematical value of the optimum reduced velocity ($\nu_{opt} = 2.71$, from eqn. 16) that gives the smallest H value for our operating conditions. For all the production rate calculations done below, we specify that no optimization of the mobile phase velocity has been achieved. Therefore, this velocity remains low, but we must stress that it is not very important, as our aim is to illustrate the value of the S.D.M. on a few examples. A strategy for optimization of the experimental conditions is not our purpose.

When using these numerical values, we may calculate an H value of 50 μm for both proteins, which means a number of theoretical plates of 1000 for the whole column and which corresponds to 20 000 theoretical plates per metre. The efficiency of this column is therefore excellent owing to the small particle diameter chosen and the large porosity of the column that is operated with an adequate mobile phase velocity. Further, the molecular diffusion coefficient is relatively high compared with the usual values encountered for most proteins, as this coefficient characterizes rather small proteins.

The gradient

For our study, the gradient profile we used is always linear. This linear variation of the counter-ion concentration is started directly after the feed injection. The counter ion is a small molecule that is assumed not to be affected by the axial dispersion [33]. Further, we shall assume that this displacing ion will migrate at the same velocity as the mobile phase, and therefore that

the gradient profile does not undergo any deformation [7]. No deformation of the gradient profile assumes that the protein solid-phase concentration does not affect the velocity of the counter-ion concentration to a large extent.

Definitions

Loading factor [34]. This is the ratio of the amount of injected component to the saturation capacity of the column for this considered component. It is therefore directly related to the concentration of the injected component:

$$Lf_i = \frac{C_{i,0} V_{inj} m_i}{Q_x (1-\varepsilon) SL} \quad (17)$$

where $C_{i,0}$ is the initial concentration of the injected component, V_{inj} the injection volume and S the column cross-sectional area of the column. The other parameters (m_i, Q,, ε and L) have been defined previously.

Recovery yield. This is the ratio of the recovered amount of the desired component to the amount of this component injected at the column entrance:

$$Y_i = \frac{\int_{t_1}^{t_2} C_i \, dt}{C_{i,0} t_{inj}} \quad (18)$$

where t_{inj} is the injection time and t_1 and t_2 the time limits of the recovery of the considered component for a given degree of purity that has been fixed, in the whole study, at a value of 0.98.

Production rate. This is the amount of recovered component, at a given purity, per unit time and per unit column cross-sectional area:

$$Pr_i = \frac{\varepsilon u \int_{t_1}^{t_2} C_i \, dt}{t_c} = \frac{\varepsilon u C_{i,0} t_{inj} Y_i}{t_c} \quad (19)$$

where t_c is the cycle time. The total cycle time usually takes into account the time of feed introduction, the duration of elution and the duration of a regeneration or washing step if necessary. To simplify, we shall consider here that this regeneration duration is small enough to be neglected. The cycle time will therefore be defined as the time between the injection of the products and the time when traces of the most retained component have left the column. This simplified definition will provide us with a very convenient tool.

Enrichment factor. This is the ratio between the average of the recovered concentrations of the desired component and the initial concentration of this component:

$$E_i = \frac{\int_{t_1}^{t_2} C_i \, dt}{(t_2 - t_1) C_{i,0}} \quad (20)$$

Selectivity. The selectivity characterizes, for a binary mixture of known composition, the ability of the stationary phase to induce different migration velocities for each component, and therefore its ability to separate this mixture:

$$\alpha = (q_{P_2}/C_{P_2})/(q_{P_1}/C_{P_1}) \quad (21)$$

RESULTS AND DISCUSSION

Selectivity

We shall concentrate here on a very interesting feature of the S.D.M. that departs from the well known competitive **Langmuir** model generally used in reversed-phase chromatography and very occasionally in ion-exchange adsorption of proteins.

The competitive **Langmuir** model predicts a constant selectivity for two components, and does not account for their concentration influence or the influence of other components. This is not the case for the S.D.M., which makes it possible to obtain a much more realistic value of the selectivity in the case of ion-exchange chromatography. Regnier and **Mazsaroff [35]** stressed the opportunity of applying to a certain extent the S.D.M. in reversed-phase chromatography, an opportunity less rigorously examined, but which perhaps in the future will lead to an actual improvement of the modelling of liquid chromatography equilibria.

From the basic equation of the model (see eqn. **4),** the selectivity is formulated as follows:

$$\alpha = \frac{(q_{P_2}/C_{P_2})}{(q_{P_1}/C_{P_1})} = \left(\frac{K_{P_2}}{K_{P_1}}\right)^{1/m_A} X \left(\frac{Q_x - m_{P_1} q_{P_1} - m_{P_2} q_{P_2}}{m_A C_A}\right)^{(m_{P_2} - m_{P_1})/m_A} \quad (22)$$

Eqn. 22 shows that selectivity depends on, among other things, the concentration of the adsorbed components P_1 and P_2 and on the counter-ion concentration in the mobile phase. The theoretical study of such a selectivity, which includes also the influence of the respective **valency** of all the components in the system, will be very useful in the understanding of the performances of isocratic or gradient ion-exchange chromatography. It should be noted that Golshan-Shirazi and Guiochon [36] emphasized that the **LeVan–Vermeulen** model [37] can also lead to a very effective selectivity, but this work was restricted to reversed-phase chromatography. First, we shall investigate the influence of the counter-ion concentration through a particular situation.

We shall consider two proteins, P_1 and P_2, having $m_{P_1} = 6$ and $m_{P_2} = 4$ as characteristic charges, and $K_{P_1} = 3200$ and $K_{P_2} = 640$ as equilibrium constant values. These values were chosen with regard to the experimental work presented by Whitley et *al.* [8], where parameters characterizing the S.D.M. were determined. Fig. la shows the competitive adsorption isotherms of the two proteins, with constant relative concentrations chosen as 1:1, and also the evolution of the selectivity a. These curves are plotted as a function of the loading factor of the protein P_1. The maximum loading capacity of the stationary phase (Q,) is 11 mM (11 mequiv. l^{-1}), the counter ion is monovalent and its concentration is 20 mM.

It can be seen in Fig. la that protein P_1 saturates the adsorption sites of the support ($Q_{x_{P_1}} = 11/6 = 1.83$ mM) faster than does protein P_2 ($Q_{x_P} = 2.75$ mM), this latter being, in concentrated' solution, more retained than P,. Nevertheless, in very dilute solution, the initial slope of the P_1 isotherm (88.57, from eqn. 5) is greater than that of P_2 (58.56). Therefore, in linear ion-exchange chromatography, protein P_1 is more strongly retained than P_2, but this latter, owing to its smaller size, will have a higher saturation limit. The selectivity, below 1 for dilute solutions, exceeds this value as it increases with increasing protein concentration. This evolution therefore gives evidence of a selectivity

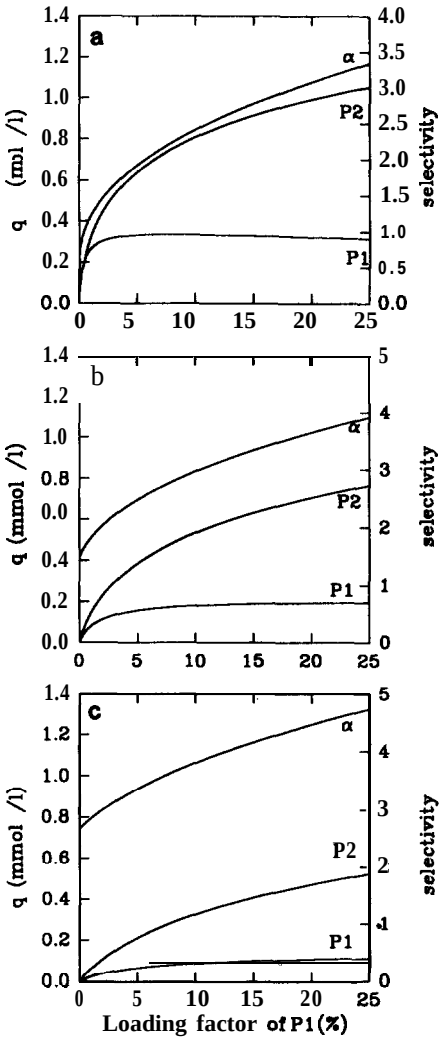

Fig. 1. Selectivity and competitive adsorption isotherms for a 1:1 binary mixture of proteins. $Q_x = 11$ mM; $m_{P_1} = 6$; $m_{P_2} = 4$; $m_A = 1$; $K_{P_1} = 3200$; $K_{P_2} = 640$. (a) $C_A = 20$ mM; (b) $C_A = 30$ mM; (c) $C_A = 40$ mM.

inversion as a function of concentrations, when the parameters are carefully chosen.

Still considering Fig. la, it is seen that the non-linearity of the isotherms is strongly marked, especially for protein P_1 which quickly reaches its maximum concentration on the stationary phase. However, this concentration is not the saturation limit for this protein ($Q_{x_{P_1}} = 1.83$ mM), which cannot be reached **because of** strong competition for the adsorption sites. The adsorbed P_1 concentration reaches its maximum

value of 0.4 m*M* for a 10% value of its loading factor, and then decreases slowly, owing to the displacement of P_1 by more strongly linked P_2 molecules. The latter will reach their saturation limit only for very high concentrations, because they will first have to displace all P_1 and counter-ion molecules. Consequently, the selectivity can only increase, and this fact is of great interest in revealing that two very close-lying products under analytical conditions may, under certain conditions, be separated more easily in the preparative mode. We shall present further a specific example of this situation.

Fig. lb and c show the same curves as in Fig. la, but with different counter-ion concentrations (30 and 40 m*M*, respectively). These figures show a similar evolution of the selectivity as in Fig. la, but the selectivity inversion phenomenon previously mentioned is not present here. In addition, it is worth noting that, at the same loading factor, selectivity is increased with increasing counter-ion concentration.

Concerning the adsorption isotherms, it is noticeable that, for a given P_1 loading factor, the adsorbed concentration diminishes with increasing counter-ion concentration, which is directly related to eqn. 1, and gives evidence of the displacement effects of the counter ion, which is a basic feature of the S.D.M. In Fig. lb and c, protein P_2 will saturate the stationary phase less and less easily, and therefore will displace P_1 at much higher concentrations than in Fig. la.

Fig. 2 presents the same parameters as in Fig. la, but using a divalent counter ion at 20 m*M* concentration. We note that the competitive isotherms have lost their strong non-linear character previously observed for a monovalent counter ion. These isotherms are much more oblique, illustrating the increased displacing effect of the counter ion and the difficulty, for the protein, to saturate the stationary phase. Considering isocratic elution, Cysewski *et al.* [7] demonstrated the benefit of using displacing ions with a high valency in order to limit the tails of peaks in mass overload conditions.

Fig. 3a and b show the influence of the equilibrium constants on the selectivity and the effect, on this parameter, of a large difference between the saturation capacities of the two

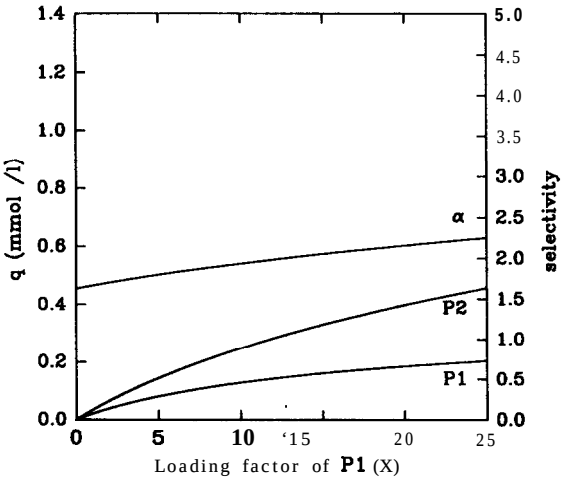

Fig. 2. Selectivity and competitive adsorption isotherms for a 1:1 binary mixture of proteins. Conditions as in Fig. 1, except the valency of the counter ion is 2.

proteins. The monovalent counter-ion concentration is 30 m*M* and the maximum loading capacity of the stationary phase is still 11 m*M* (11 mequiv. l^{-1}). Proteins P_1 and P_2 considered in Fig. 3a now have $m_{P_1} = 6$ and $m_{P_2} = 2$ as characteristic charge. The maximum adsorption capacity for P_1 is therefore 1.83 m*M* and for P_2 5.5 m*M*. In this instance the isotherms for the two proteins have similar initial slopes. Here, selectivity is increasing with the loading factor, but this evolution is markedly faster than that observed in Fig. la. These two proteins, whose selectivity is around 1 in dilute solution, *i.e.*, with a very close affinity for the stationary phase, exhibit a selectivity of around 7 for a 10% value of the loading factor of P_1. Above 5% for this loading factor, protein P_2, which adsorbs only on two sites ($m_{P_2} = 2$), displaces readily the over-large protein P_1, and this occurs despite the fact that the adsorption constant of P_1 ($K_{P_1} = 8200$) is much greater than that of P_2 ($K_{P_2} = 148$). In Fig. 3b, for the same previously defined characteristics, except that the P_2 valency is now taken as 3, we observe very different results. Indeed, one can see here the major influence of the equilibrium constants. These, in this instance, predominate over the differences in saturation values of the stationary phase for each protein, so that displacement of P_1 by P_2, observed in Fig. 3a, is not seen here and will appear only for very high

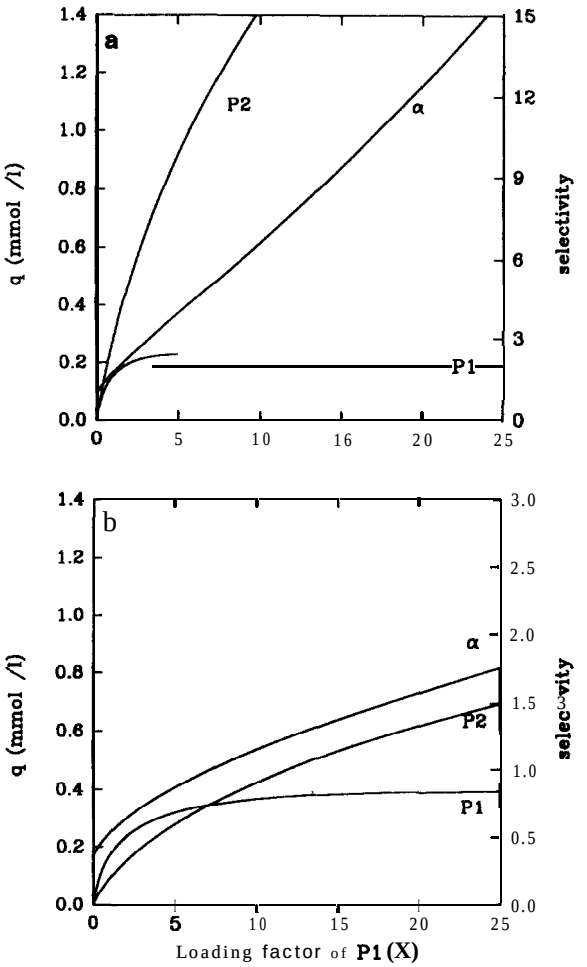

Fig. 3. Selectivity and competitive adsorption isotherms for a 1:1 binary mixture of proteins. $Q_x = 11$ mM; $K_{P_1} = 8200$; $K_{P_2} = 148$; $m_A = 1$; $C_A = 30$ mM. **(a)** $m_{P_1} = 6$; $m_{P_2} = 2$; **(b)** $m_{P_1} = 6$; $m_{P_2} = 3$.

loading factors. In other words, the great affinity of P_1 for the stationary phase compensates in a better way for the influence of its high characteristic charge.

In conclusion, we would stress the value of an accurate study of competitive isotherms in order to determine the evolution of the selectivity as a function of the protein load. This will make possible a better understanding of the results of a binary separation and may possibly provide an effective framework to the choice of good operating conditions.

As these few comments about the presented curves have demonstrated numerous parameters are linked to influence the selectivity and this is one of the main advantages of the S.D.M. in accounting, even imperfectly, for their interactions. This also emphasizes the difficulty in establishing general rules about their respective influence.

Isocratic elution and gradient elution

For ion-exchange chromatography, elution is termed isocratic when product separation occurs at a constant counter-ion concentration. When this counter-ion concentration increases during the separation, linearly or not, it is termed gradient elution. Isocratic elution (IE) and gradient elution (GE) are widely used in analytical chromatography, where numerous theoretical studies have contributed to a closer understanding of their characteristics. On the other hand, concerning the preparative mode, very few

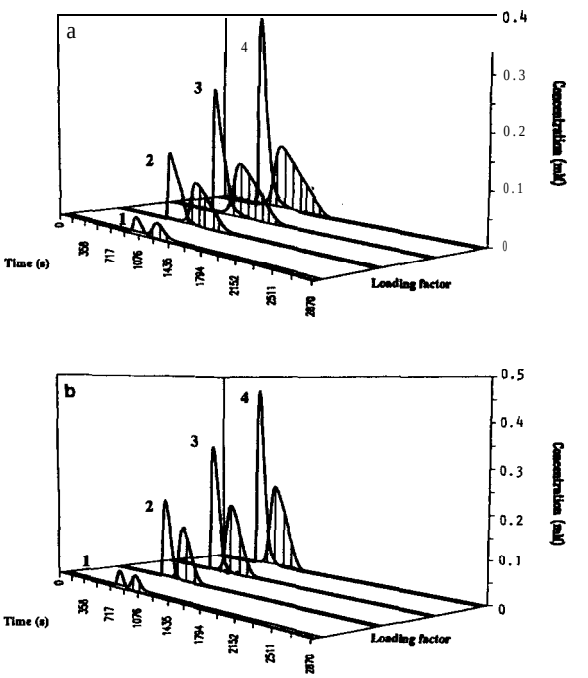

Fig. 4. Gradient elution of a 1:1 binary mixture of proteins in ion-exchange chromatography. Influence of the loading factor and the gradient steepness. (a) $\beta = 0.025$ mM s^{-1}; **(b)** $\beta = 0.055$ mM s^{-1}. $Q_x = 11$ mM; $L = 5$ cm; u = 0.01 cm s^{-1}; $\varepsilon = 0.85$; HETP = 50 μm; $m_{P_1} = 6$; $m_{P_2} = 4$; $m_A = 1$; $C_{A,i} = 20$ mM; $K_{P_1} = 3200$; $K_{P_2} = 640$. (1) $Lf_{P_1} = 2.61$; $Lf_{P_2} = 1.44$; (2) $Lf_{P_1} = 12.96$; $Lf_{P_2} = 8.64$; (3) $Lf_{P_1} = 21.6$; $Lf_{P_2} = 14.4$; (4) $Lf_{P_1} = 30.24$; $Lf_{P_2} = 20.16$.

studies have been devoted to the comparison of these two techniques. For instance, we may quote the work of **Antia** and Horvhh **[33],** but restricted to reversed-phase chromatography. Our work, in ion-exchange chromatography, was inspired by their study, and some conclusions will prove identical, but specific features of the S.D.M. will be pointed out.

In preparative chromatography, the last steps of the purification usually concern a small number of components. We therefore chose here to study the separation of a mixture of two proteins, P_1 and P_2, with constant relative concentrations (1:1), with $m_{P_1} = 6$ and $m_{P_2} = 4$ as characteristic charges, and with equilibrium constants $K_{P_1} = 3200$ and $K_{P_2} = 640$. The counter ion is monovalent and the ion-exchange column has been described in a previous section. In every case considered, protein P_2 will be the most retained component, and therefore the last eluted.

Fig. 4 shows, for two different values of the gradient steepness, the evolution of **chromatograms** with increasing values of the loading factor. On a qualitative basis, Fig. 4 is very interesting, as one can observe the global evolution of mixing zones, and these curves will serve as an illustration for our conclusions concerning the comparison of IE and GE.

Fig. 5 presents for each protein P_1 and P_2 the evolution for 98% purity, in GE, of the recovery yield, the enrichment factor and the production rate, as a function of the loading factor of the protein under consideration. As a first general remark, we observe, whatever the gradient steepness, values of the recovery yield, the enrichment factor and the production rate that are always greater for protein P_1 than for P_2. The competition for adsorption sites and the greater affinity of P_2 for the stationary phase explain these results, which are, in fact, the consequence of the displacement of P_1 by P_2. This will be of great interest when the product to be purified is the one that is "pushed", like P_1, and therefore concentrated.

The study of the yield *versus* the loading factor provides very useful information concerning the mixing zones between products. For a fixed gradient steepness, we notice in Fig. **5a** and b,

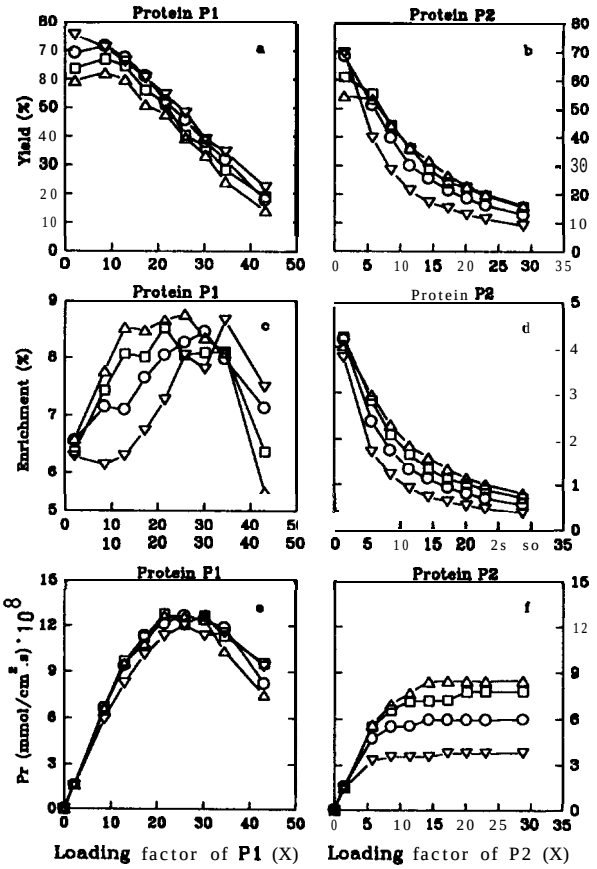

Fig. 5. Gradient elution of a 1:1 binary mixture of proteins in ion-exchange chromatography. Yield, enrichment and production rate as a function of the loading factor, for 98% purity. $L = 5$ cm; HETP = 50 μm; $\varepsilon = 0.85$; $Q_x = 11$ mM; $C_{A,i} = 20$ mM; $u = 0.01$ cm s^{-1}; $m_{P_1} = 6$; $m_{P_2} = 4$; $m_A = 1$; $K_{P_1} = 3200$; $K_{P_2} = 640$. V = $\beta = 0.025$ mM s^{-1}; 0 = $\beta = 0.035$ mM s^{-1}; ❑ = $\beta = 0.045$ mM s^{-1}; A = $\beta = 0.055$ mM s^{-1}.

for both proteins, a slow decay in the yield when the loading factor increases, leading to a degradation of the separation that could have been qualitatively seen in Fig. 4. For a **fixed** loading factor, the recovery yield for the P_1 is inversely proportional to the gradient steepness, and this tendency is reversed for P_2, except for low loading factors. As we have seen previously (see Fig. 1), although the selectivity increases with increasing counter-ion concentration, the isotherms become simultaneously more and more oblique, leading to a weaker affinity of the protein for the stationary phase. Therefore, the more the gradient steepness increases, the less

the products are retained and have time to organize themselves in separated bands. This explains the evolution of the recovery yield for P_1 but not for P_2. For the latter, we must consider the tails of P_1 peaks that degrade the P_2 yield. Indeed, these tailings-off shorten as the gradient steepness increases, showing by the way higher displacing effects of the counter ion (Fig. 4).

Finally, there is an increase in selectivity with increasing loading factor (Fig. 1), which explains why the yield of P_1 shows a maximum for a 10% loading factor, except for the lowest gradient steepness (Fig. 5a). Above this value, the selectivity does not increase fast enough to compensate for mixing effects.

The enrichment factor is an important parameter for evaluating the performance of a separation technique and indicates the evolution of the average recovered concentration of a component with respect to the input concentration. The curves in Fig. 5c and d illustrate the displacing power of the counter ion, as one can observe, for both proteins (except for P_1 at loading factors greater than 45%), an enrichment factor in direct proportion to the gradient steepness. In addition, enrichment of P_1 increases with the loading factor, exhibits a maximum at about a 30% loading factor and then drops sharply, because of the predominance of mixing zones. We can say, therefore, that the more the column is loaded, the larger is the added displacing effect of P_2 towards P,, so much so that the mixing zones remain negligible. P_2 enrichment decreases continuously with increasing loading factor because the tails of P_1, although displaced by P_2 and the counter ion, degrade the P_2 zone in proportion to the load, affecting zones where P_2 is the most concentrated. Respecting a fixed high degree of purity for P_2 leads to the recovery of more and more dilute fractions of P_2, explaining the small values of the enrichment.

The production rate is a useful parameter for an economic evaluation of the process. In Fig. 5e and f, the production rate of P_1 increases to a maximum for a 25–30% value of the loading factor. For P_2, it increases and then stabilizes. Hence, to a certain extent, the injection of concentrated products increases the production rate and compensates, for a while, the appearance of growing mixing zones. A weak effect, for P_1 production rate, of the gradient steepness is also seen in Fig. 5e, up to the attainment of a maximum value. In contrast, the P_2 production rate is greatly dependent on the gradient steepness and increases with increasing linear rate of change of counter-ion concentration at the inlet.

Fig. 6 gives a qualitative illustration of the influence of the loading factor and the counter-ion concentration on chromatograms in IE, and is as relevant as Fig. 4. Fig. 7 shows the same study as Fig. 5, with the same proteins, but undergoing IE. As a first overall comment, one can observe that all the parameters' values in this instance are, whatever the protein, clearly below those obtained in GE. This is an effect of the displacing power of the counter-ion gradient,

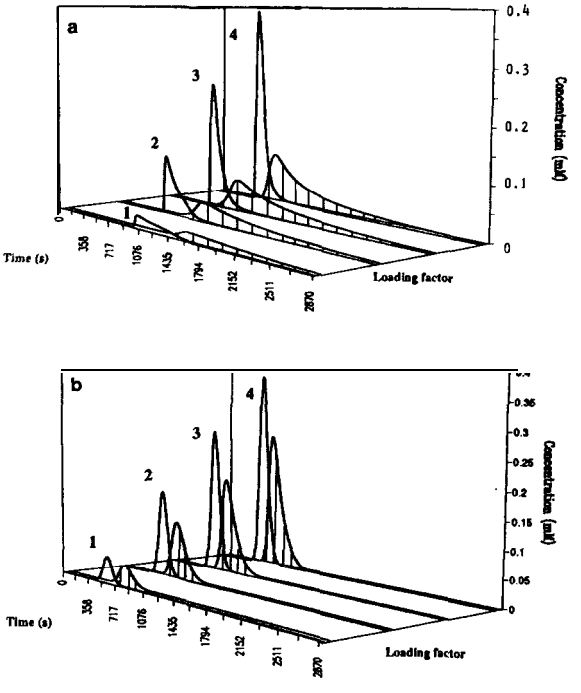

Fig. 6. Isocratic elution of a 1:1 binary mixture of proteins in ion-exchange chromatography. Influence of the loading factor and the counter-ion concentration. (a) $C_A = 25$ mM; (b) $C_A = 40$ mM. $Q_x = 11$ mM; $L = 5$ cm; $u = 0.01$ cm s^{-1}; $\varepsilon = 0.85$; HETP = 50 μm; $m_{P_1} = 6$; $m_{P_2} = 4$; $m_A = 1$; $K_{P_1} = 3200$; $K_{P_2} = 640$. (1) $Lf_{P_1} = 2.16$; $Lf_{P_2} = 1.44$; (2) $Lf_{P_1} = 12.96$; $Lf_{P_2} = 8.64$; (3) $Lf_{P_1} = 21.6$; $Lf_{P_2} = 14.4$; (4) $Lf_{P_1} = 30.24$; $Lf_{P_2} = 20.16$.

which "squeezes" the peaks, concentrates the products and favours the competition for adsorption sites. In addition, we can say that GE reduces the cycle time by limiting appreciably the tail of the last peak [33].

The recovery yield of the two proteins as a function of the loading factor (Fig. 7a and b) shows the same evolution as in GE, *i.e.*, a decrease with increasing loading factor. For a 25 m*M* counter-ion concentration, one can notice (Fig. 7a) that the P_1 recovery yield shows a maximum, accounting for the fact that the selectivity increases with the loading factor and that this augmentation predominates first over the mixing zone increase, whereas for higher counter-ion concentrations substantial mixing occurs rapidly. For a loading factor of P_1 larger than 10%, and as already observed in GE, large counter-ion concentrations lead to too fast an elution for competition phenomena to organize the components in separated zones, and therefore to improve the yield of the separation (see Fig. 7a). Lastly, very weak counter-ion concentration variations result in noticeable differences in yields (they were smaller in the GE case).

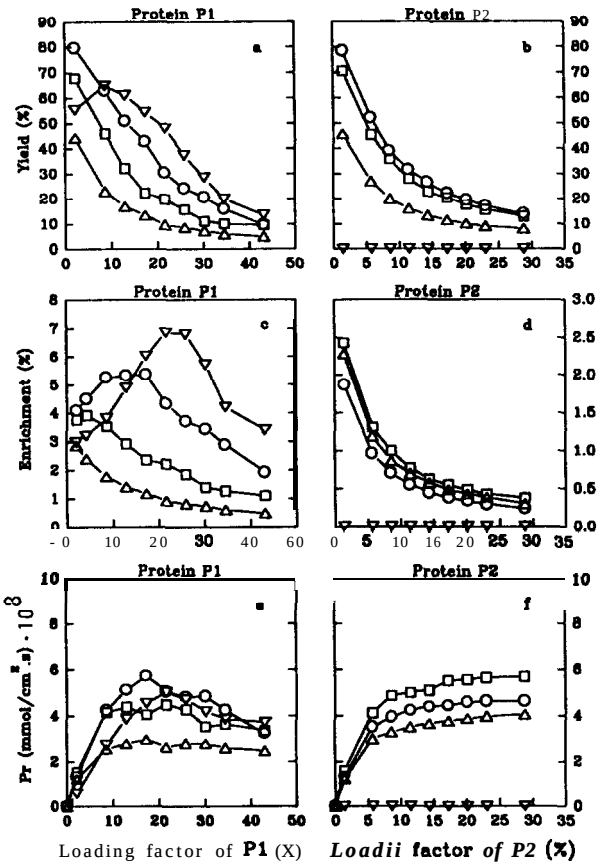

Fig. 7. Isocratic elution of a 1:1 binary mixture of proteins in ion-exchange chromatography. Yield, enrichment and production rate as a function of the loading factor, for 98% purity. L = 5 cm; HETP = 50 μm; $\varepsilon = 0.85$; Q_x = 11 mM; u = 0.01 cm s^{-1}; m_{P_1} = 6; m_{P_2} = 4; m_A = 1; K_{P_1} = 3200; K_{P_2} = 640. ▽ = C_A = 25 mM; ○ = C_A = 30 mM; ❑ =c,=35 mM; △ = C_A = 40 mM.

Concerning the P_2 yield (see Fig. 7b), the same tendencies as for protein P_1 are observed, except for a 25 m*M* counter-ion concentration, where the yield tends to a zero value, whatever the loading factor. These very small values mainly derive from the long tails of the P_1 peaks that "contaminate" P_2 peaks, and because the required purity is high this phenomenon artificially decreases the recovery yield of P_2. Other calculations (not shown here) have revealed that, for a 0.97 purity, the P_2 yield increases sharply, thus confirming this explanation.

It can also be seen that, for a given loading factor and a counter-ion concentration different from 25 m*M*, the influence of the displacing ion concentration on the P_2 yield is not the same as was observed for P_2 in GE. To explain this fact, we must consider that the tails of protein P_2, a direct consequence of a marked non-linearity of the isotherm, are much more spread in IE than in GE, and therefore the yield loss, induced by the tails of protein P_1, affects the results much less as the overlapping of the two peaks is smaller.

The evolution in IE of the enrichment for protein P_2 as a function of the loading factor (see Fig. 7d) is similar to that observed in GE, except for the particular case of a 25 m*M* counter-ion concentration. The enrichment factor for the isocratic case is anyway inferior to the gradient case, demonstrating once again the reduced displacing power of a constant counter-ion concentration.

Concerning protein P_1, we note that enrichment curves (see Fig. 7c) present a maximum. The lower the counter-ion concentration, the higher is this maximum value, the latter being obtained for higher loading factor values. For a

high counter-ion concentration (40 m*M*), the enrichment factor decays slowly without exhibiting a maximum. These results may be explained if we consider again the influence of the selectivity that depends on protein and counter-ion concentrations. For a low counter-ion concentration and dilute concentrations of injected products, the retention characteristics of the two proteins are more or less similar (see Fig. la for a 20 m*M* counter-ion concentration and analytical conditions), therefore leading to a poor separation. As the loading factor increases, the selectivity increases and protein P_1 is more and more displaced by protein P_2, which results in much more concentrated recovered fractions with respect to P_1. The enrichment reaches a maximum, then drops when mixing zones become too large. The more the counter-ion concentration increases, the more rapidly is the effect of the mixing zones noticed, explaining the movement of the maxima.

The evolution of the production rate of protein P_2 as a function of the loading factor (see Fig. 7f) is similar to that observed in GE, but with lower values. However, there is an optimum 35 m*M* counter-ion concentration, beyond which the production rate drops sharply. Concerning protein P_1 (Fig. 7e), we would also point out that a counter-ion concentration greater than 30-35 m*M* results in a noticeable drop in the production rate. Therefore, it is not worthwhile in IE to use high counter-ion concentrations, either for the production rate or for enrichment.

In conclusion in this section, we would stress once again the value of the S.D.M. in investigating optimum operating conditions for a separation, but we must add, as did Ghodbane and Guiochon [38] in their study on reversed-phase chromatography, that it is very difficult, at a quantitative level, to extrapolate these results to other protein mixtures. On the other hand, it seems that the overall evolution of the parameters under study may be observed in other situations, provided that the latter remain conventional. However, considering non-standard configurations, very surprising results may arise, as will be seen in the next section.

Particular case of a separation markedly favoured by a selectivity inversion

In this section, we shall show that a rapidly varying selectivity (with respect to the loading factor) and corresponding to the case in Fig. 3a, may result in increasing yields and production rates when operating at a preparative level. Antia and Horváth [33] have also shown, in reversed-phase chromatography, that the more

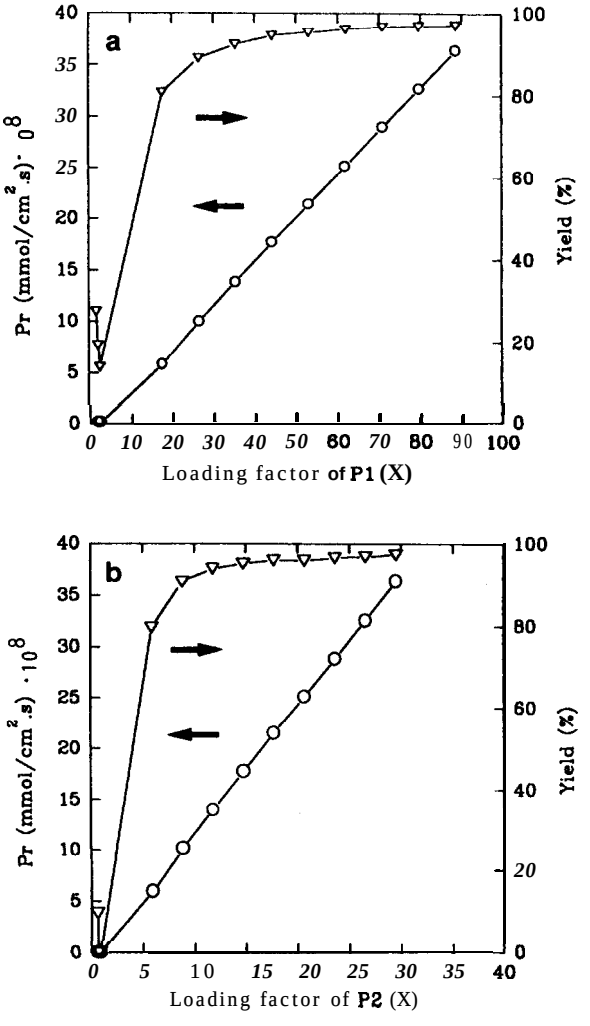

Fig. 8. Gradient elution of a 1:1 binary mixture of proteins in preparative ion-exchange chromatography. Yield and production rate for 98% purity, as a function of load. L = 5 cm; HETP = 50 μm; ε = 0.85; β = 0.005 mM s^{-1}; Q_x = 11 mM; u = 0.01 cm s^{-1}; m_{P_1} = 6; m_{P_2} = 2; m_A = 1; K_{P_1} = 8200; K_{P_2} = 148. (a) Protein P_1; (b) protein P_2. 0 = Production rate; V = yield.

saturated the stationary phase is in the products they considered, the greater is the tendency for them to separate.

The two proteins P_1 and P_2 considered here were the same as above (see Fig. 3). Their characteristic charges are $m_{P_1} = 6$ and $m_{P_2} = 2$ and their equilibrium constants $K_{P_1} = 8200$ and $K_{P_2} = 148$. As we already specified in the comments on Fig. 3, these two proteins have very dissimilar maxima adsorption capacities, which explains the rapid evolution of the selectivity with respect to the loading factor. Proteins are injected in equimolar concentration on to the column previously equilibrated at a 20 m*M* counter-ion concentration, the latter being monovalent. The separation is performed in GE with a very low gradient steepness ($\beta = 0.005$ m*M* s^{-1}) in order to eluate the products slowly enough. Fig. 8a and b show the evolution of the yield and the production rate versus the loading factor of each protein and Fig. 9 shows, for different loading factors, the shape of the chromatograms for this separation.

It is noted first that the loading factor of protein P_1, whose characteristic charge is the larger, reaches high values, greater than 80%. Protein P_2, with a lower characteristic charge, although injected at the same concentration as P_1, saturates the stationary phase very slowly and does not pass beyond a 30% loading factor

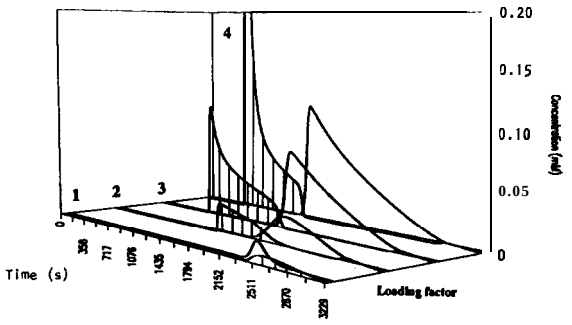

Fig. 9. Gradient elution of a 1:1 binary mixture of proteins in preparative ion-exchange chromatography. Influence of the load. $\beta = 0.005$ m*M* s^{-1}; $Q_x = 11$ m*M*; $L = 5$ cm; $u = 0.01$ cm s^{-1}; $\varepsilon = 0.85$; HETP = 50 μm; $m_{P_1} = 6$; $m_{P_2} = 2$; $m_A = 1$; $C_{A,i} = 20$ m*M*; $K_{P_1} = 8200$; $K_{P_2} = 148$. (1) $Lf_{P_1} = 1.72$; $Lf_{P_2} = 0.576$; (2) $Lf_{P_1} = 8.6$; $Lf_{P_2} = 2.88$; (3) $Lf_{P_1} = 25.8$; $Lf_{P_2} = 8.64$; (4) $Lf_{P_1} = 43$; $Lf_{P_2} = 14.4$.

value, leading to different scales for the two graphs in Fig. 8a and b.

In highly dilute solutions, at a 20 m*M* counter-ion concentration, proteins P_1 and P_2 can easily be separated in IE ($\alpha = k'_2/k'_1 = 0.19$, where k'_1 and k'_2 are the respective capacity factors). Let us specify that, under these conditions, protein P_1 is the most retained component (see the definition of the selectivity, eqn. 21). Indeed, its equilibrium constant ($K_{P_1} = 8200$) is much higher than that of P_2, and at the analytical level its poor saturation capacity (Qx, = 1.83) does not exert a great influence. Using GE, under the same analytical conditions as above, the separation is also good and protein P_1 is still eluted last.

At low loading factor values, the quality of the separation diminishes with increasing loading factor, as the selectivity quickly reaches a value of 1, indicating a similar affinity for both proteins (see Fig. 3a, for a 30 m*M* counter-ion concentration). The observed yield therefore drops and the production rate remains negligible. Under these conditions, we may note, on curve 1 in Fig. 9, the poor quality of the separation. As the selectivity increases continuously with increasing the loading factor, one can see an improvement in the separation through a rapid increase in the yield, up to nearly 100%, and through the linear increase of the production rate in proportion to the loading factor. Protein P_2 is now the most retained component.

-Finally, two, proteins, separable at an analytical level, may surprisingly show, after an inversion of the elution order, high yields and considerable production rates at a preparative level, provided that their selectivity increases very sharply with the loading factor. This result is noteworthy, but we must keep in mind that the S.D.M., which has made such calculations possible, has its limitations and its validity decreases at too high protein concentrations [7]. It is still a very useful tool that has brought to light such unconventional situations that appear probable but still deserve experimental validation.

As a conclusion to this section, it has been shown that an advantage of the S.D.M. lies in its multi-parameter character. Indeed, when experimenting in the zone where the selectivity was

near to 1, and considering that the retention time was the essential parameter, one is tempted to believe that proteins showing similar chromatographic behaviour also have similar ion-exchange interactions with the stationary phase. One might therefore have directed efforts toward modifying this interaction. In fact, as has been demonstrated here, this situation resulted from a coincidence as regards the retention time governed by two separate parameters, i.e., the characteristic charge of the protein and its equilibrium constant, and this does not invalidate the ion-exchange process, merely the operating conditions chosen. Although this situation has been artificially constructed here, it serves as a clear example of the new insight given by this approach.

CONCLUSION

The stoichiometric displacement model is a first step in the understanding of protein adsorption mechanisms occurring during an ion-exchange process, and it provides a simple tool for investigating the behaviour of these polymeric ions. Although it is still restricted by oversimplified hypotheses, it remains an interesting research topic that in the future will undergo, more or less important modifications of its theoretical bases.

The work presented here has illustrated the competitive adsorption of two proteins and has considered the influence of various relevant parameters such as the counter-ion concentration, the loading factor and the saturation value of the stationary phase concentration for the protein. The preliminary study of the selectivity from competitive isotherms has proved to be a good data source in understanding isocratic or gradient elution and we have stressed the value of using gradient elution in the preparative mode. All these computations were done with a computer program, written in FORTRAN, and there is no major problem in extending its use to mixtures with more than two proteins.

Work is in progress to check experimentally the validity of the S.D.M., and to provide actual values of the parameters in our computations.

SYMBOLS

$C_{A,i}$	Initial counter-ion concentration (kmol m^{-3})
$C_{i,0}$	Concentration of component i at the column entrance (kmol m^{-3})
C_i	Concentration of component i in the flowing mobile phase (kmol m^{-3})
$D_{i,ap}$	Apparent diffusion coefficient of component i ($m^2 s^{-1}$)
$D_{i,ax}$	Axial dispersion coefficient of component i ($m^2 s^{-1}$)
$D_{i,m}$	Molecular diffusion coefficient of component i in the flowing mobile phase ($m^2 s^{-1}$)
d_p	Particle diameter (m)
E_i	Enrichment factor of component i
F	Phase ratio of the column packing [= (1 − &)I&]
h	Reduced plate height (= H/d_p)
H	Height equivalent to a theoretical plate (HETP) (m)
K_i	Equilibrium constant of component i
k_0	Average value of all the initial slopes of the components' individual isotherms
k'_i	Capacity factor for component i
L	Column length (m)
Lf_i	Load factor of component i
m_A	Valency of the counter ion
m_i	Characteristic charge of the protein i
M	Molecular mass (g mol^{-1})
N	Number of theoretical plates (= L/HETP)
N_c	Number of components
P	Courant number
Pr_i	Production rate of component i (kmol $m^{-2} s^{-1}$)
Q_x	Maximum loading capacity of the exchanger (km01 m^{-3})
Q_{x_i}	Saturation value of stationary phase concentration for component i (= Q_x/m_i)
q_i	Concentration of component i adsorbed on the stationary phase (calculated per unit volume of particle skeleton) (kmol m^{-3})
S	Column cross-sectional area (m^2)
t	Time coordinate (s)
t_1, t_2	Integration limits for eqns. 18-20 (s)
t_c	Cycle time (s)

t_{inj} Time of feed introduction (s)
t_0 Residence time of an unretained compound (s)
T_i Mass distribution coefficient; ratio between the amount of component i in the stationary and the mobile phases ($= q_i/C_i$)
u Interstitial fluid velocity (m s^{-1})
V_{inj} Volume of feed injection (m^3)
Z Stoichiometric charge ratio of the protein and the counter ion
z Axial coordinate (m)
Y_i Recovery yield of component i

Greek letters

α Selectivity [$= (q_i/C_i)/(q_j/C_j)$]
β Linear rate of change of counter-ion concentration at the inlet (km01 $m^{-3} s^{-1}$)
ε Total porosity of the column packing
ν Reduced mobile phase velocity ($= ud_p/D_m$)
ν_{opt} Optimum reduced mobile phase velocity
σ Space increment of the grid for the finite difference method (m)
τ Time increment of the grid for the finite difference method (s)

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