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High-performance liquid chromatography of amino acids, peptides and proteins

CXXXII☆. Optimisation of operating parameters for protein purification with chromatographic columns

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ABSTRACT

In large-scale chromatography, process optimisation is one of the key elements for success. This paper presents a method for determining the optimum operating parameters for affinity and ion-exchange chromatographic columns when used for protein purification. Based on a mathematical model developed as part of our association investigations, computer programs have been developed to describe the dynamic relationships acting within the chromatographic system. Two basic operating parameters, the flow-rate and the effluent concentration at which the adsorption stage is terminated, can be optimised to give a maximum production rate. The sample loading volume and the processing time then can be determined. The effect of washing conditions on the production rate and the yield is also discussed. Examples are given for a specific system where the optimisation is based on the yield and the percentage utilisation of the column capacity. Contour plots are generated to aid the determination of the range of controlling parameters, and to guide further system design.

INTRODUCTION

The importance of using chromatographic methods for both small- and large-scale purification of high-value protein and other polypeptide products has been well documented [1,2]. Competitive commercial pressures have meant that process scale-up and optimisation are now being subjected to increasing attention [3–7]. Although it is generally accepted that optimisation work carried out through simulation with mathematical models should be cheaper, more comprehensive and more versatile than solely through repetitive laboratory experiments, the majority of large-scale processes are still the result of considerable trial and error requiring a long period of time. Whilst a large number of mathematical models has been developed for individual components of the process (*e.g.* adsorption, washing, elution and regeneration), when addressing the scaling-up of the total chromatographic procedure, there is no one accepted method to follow. Most published works in the scientific literature on the validation of the physical relevance of the different models have been limited to studies with breakthrough curves. Recently, some effort has been devoted to the application of mathematical modelling to the optimisation of actual processes [6–14]. Dantigny *et al.* [6] for example have studied the influence of the adsorption kinetic constants, maximum capacity of the column and axial dispersion on the extent of product loss, or the use of column capacity and productivity for a

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column loaded to an effluent concentration of 5% of the influent value, followed by washing. Mao *et al.* [7] have also studied the effect of flow-rate and the terminating effluent concentration on the production rate, the yield and the column capacity utilisation for the adsorption of proteins with non-porous particle systems.

The present paper is aimed at providing a practical procedure based on the application of a mathematical model for the estimation of optimum operating parameters. This procedure has been developed for both porous and non-porous particles used as affinity and ion-exchange sorbents in chromatographic columns as part of protein purification processes. Such processes will normally involve a number of stages, such as adsorption, washing and elution. The primary objective was to achieve a maximum production rate for each stage, while ensuring the basic requirements of product purity and yield were met. To achieve these criteria the operating parameters, such as the fluid flow-rate, the volume of the loading solution, and the time to terminate a particular stage, must be optimised. These investigations demonstrate that the exact values of these parameters (flow-rate, volume and time) will be dependent upon the economics of the specific procedures.

MATERIALS AND METHODS

System parameters required for the simulation, such as adsorption isotherm constants and various kinetic constants, were obtained from experimental data generated with a laboratory-scale system described previously [15,16]. In this case, the adsorbate was lysozyme and the adsorbent was Cibacron Blue F3GA immobilised to Fractosil Diol gel (E. Merck, Darmstadt, Germany) [17]. The particle size was 63–100 μm. The biomimetic dye adsorbent was packed into columns 4.0 cm × 1.6 cm I.D. The flow-rates used were between 2 and 9.5 ml/min, corresponding to a fluid velocity range of 0.17 to 0.79 mm/s. The influent concentration of lysozyme was 1.0 mg/ml. The values of equilibrium constant and maximum adsorption capacity for this lysozyme–Cibacron Blue F3GA system were obtained using the procedure established in this laboratory [15]. Concentration–time profiles for the adsorption stage (breakthrough curve) and the washing stage were obtained using frontal analysis [16,18]. The liquid film mass transfer coefficients were calculated from a literature correlation [19]. A mathematical model developed in this Centre was then used to fit the experimental breakthrough curves to extract the surface interaction rate constants. Detailed derivation and discussions of the model, and its solution can be found in separate publications [20–22]. The product yield, column capacity utilisation, and the production rate then were calculated. The computer programs (BEDSTP and BEDSTS) using the model were written in FORTRAN and the computation was carried out using a personal computer and a VAX 8700 computer.

RESULTS AND DISCUSSION

The sectional model of a chromatographic column developed in this Centre represented the basis of the optimisation study reported here. In this model the column is divided into small sections. Each section is then treated as a finite bath with the equations used in the batch adsorption model developed earlier [21] forming the key algorithm of the model. The model is similar to the discrete cell model or the discrete stage model available in the literature [23,24]. The main difference between this type model and the Tanks in Series Model [25], for example, is that the flow is not continuous. At the end of each time increment, the content of the liquid phase in each section is transferred to the next section. It was found that when the number of the sections, n, was larger than 16 in the test case, the effect of "numerical dispersion" was negligible and the breakthrough curves produced by the sectional model and the analytical solution of a packed bed [22] were synonymous and overlapped. The advantage of this mode is its versatility. The adsorption stage may be terminated at any time without causing any difficulty in the calculation of the time–concentration curves in the washing stage. This model is capable of independently addressing both the external mass transfer and surface interaction, yet retains the

simplicity of analytical solution. Non-linear Langmuir adsorption isotherms were assumed.

For a large number of protein–ligand systems, the adsorption of protein molecules to the adsorbent is a reversible process. During the washing stage, some protein which has already been adsorbed onto the solid phase will re-enter the liquid phase and may be carried out of the column. Hence, at the end of the washing stage the amount of the protein retained in the column may not be the same as at the end of the adsorption stage, with the binding efficiency (=yield in the present paper) normally less than 100% [5]. For this reason, the adsorption and washing stages were considered as one operating unit in the present investigations.

The effect of washing on the breakthrough curves and the amount of protein retained in the column is shown in Fig. 1. In Fig. 1a the time–concentration curves are shown for loading the column to an effluent concentration of 2, 10 and 99% of the influent concentration respectively, followed by washing. The numbers of the 10% curve indicate the different stages of the operation. The stages are as follows: the column at the end of loading (No. 1), the column washed with one column volume of buffer (No. 2), the column washed with 10 column volumes (No. 3) and with 30 column volumes (No. 4). The dimensionless concentration profiles in the solid-phase for the 10% curve at these stages of the process are shown in Fig. 1b with the corresponding curves marked with the same numbering as in Fig. 1a. It can be seen from Fig. 1a that although the protein solution was no longer applied to the column, the effluent concentration continued to rise even after the column was washed with ten column volumes of buffer solution (No. 3). Examination of the concentration profile at this stage in Fig. 1b shows that the highest concentration section is still in the column, although it has moved closer to the outlet of the column. Only when the concentration peak moves out of the column will the effluent concentration start to decline. By then, however, the amount of protein still remained in the column has reduced, represented by the area under each concentration profile. In all three cases of loading the column to an effluent concentration of 2, 10 and 99% of the influent concentration, the concentrations in the effluent continued to rise after the loading has been terminated and the washing with buffer solution started. Reducing the terminating effluent concentration from 10 to 2% of the influent concentration only decreased the maximum effluent concentration from *ca.* 80 to *ca.* 70%.

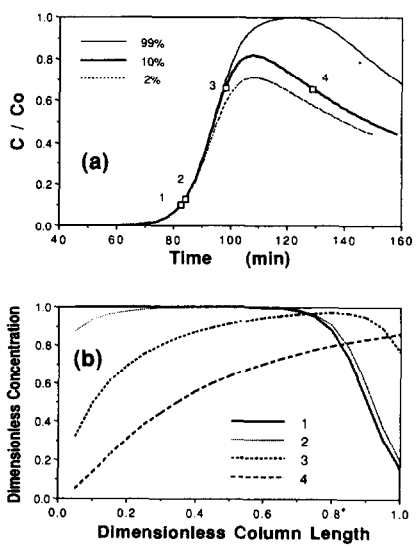

Fig. 1. (a) Time–concentration curves for loading to effluent concentration of 2, 10 and 99% of influent respectively, followed by washing. (b) Concentration profiles in the solid-phase corresponding to the numbered times on the 10% effluent curve in (a). The terms C and C_0 are the protein concentrations in the effluent and influent, respectively.

The production rate of the process can be defined as the mass of protein retained after washing per unit volume of the adsorbent per unit total processing time. The total time for a chromatographic process includes not only the actual time used for adsorption and washing, but also the time associated with preparation and reconditioning the column. In the present simulation, these latter times were fixed at 10 min. The yield was defined as the mass of protein retained by the column after washing as a percentage of total mass of protein applied. The column capacity utilisation was calculated form dividing the mass of protein retained by the column after washing, by the attainable column capacity, and was also expressed as a percentage. The attainable column capacity is a fraction of

the maximum capacity as a function of the protein concentration in the inlet solution.

The potential applications of the methodology are demonstrated by consideration of a specific biomimetic affinity system similar to that used to extract system parameters. Hence the protein used was again lysozyme, and the ligand was Cibacron Blue F3GA immobilised onto porous silica of average particle size 83 μm. The column size used was 10 cm × 1.6 cm I.D. and the fluid velocity was varied between 0.2 and 8.0 mm/s. The protein concentration in the inlet solution was 0.2 mg/ml. The system parameters required for the simulation were obtained from experimental data as described in the Materials and Methods section. The values of equilibrium constant and maximum adsorption capacity used were 0.107 and 15.4 mg/ml particle, respectively. Correlations for estimating the values of liquid film mass transfer coefficient [19] have been incorporated into the BEDSTP program as it is a function of the fluid velocity. The value of the surface interaction rate constant was extracted from experimental data and a value of 0.0207 ml/mg s was used. A value of 20 was chosen to be the number of the sections used in the programs BEDSTP and BEDSTS for simulation.

In an actual process, the operating conditions of the washing stage such as the amount of washing solution used and the washing flow-rate, should be determined according to the nature of the contaminants (*e.g.* in terms of their molecular masses and concentrations) and the purity requirement of the target protein. Hence these operating conditions are fixed for any particular system and will be essentially independent of those of the adsorption stage when the contaminants are not involved in direct competition with the binding sites for the target protein. In these cases, the operating conditions of the washing stage can be addressed separately as an independent problem. IN the present simulation, 10 column volumes of buffer solution were used at a fluid velocity of 0.5 mm/s for the washing stage. The adsorption time, the percentage utilisation of the attainable column capacity, and the protein yield at different fluid velocities for each of the terminating effluent concentrations were then calculated using the computer program BEDSTP.

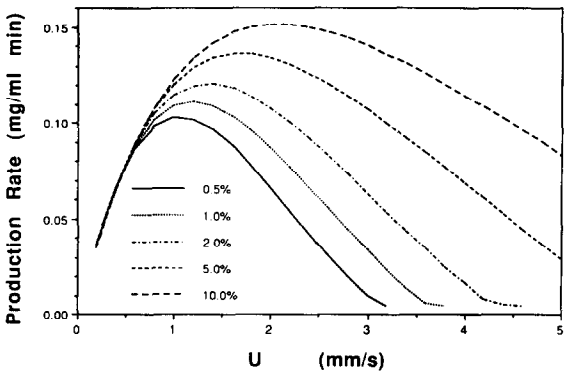

Fig. 2. Production rate *versus* superficial liquid velocity for different effluent concentrations (0.5, 1.0, 2.0, 5.0 and 10% of inlet concentration) for the lysozyme–Cibacron Blue F3GA silica system.

Fig. 2 shows the variation in production rate for the preparative lysozyme–Cibacron Blue F3GA silica system described above at different fluid velocities for the different terminating effluent concentrations. It can be seen that there is a fluid velocity at which a maximum production rate for this biomimetic affinity system is achieved. This fluid velocity increases with increase in the allowable effluent concentration. The maximum production rate also increases with the increase of the terminating effluent concentration. However, at fluid velocities lower than the optimum velocities, the effect of the terminating effluent concentration becomes less important. Fig. 2 also shows that by using a flow-rate at the maximum capacity of the pump (or to the pressure limit of the system) as often is practised, the most desirable production rate could not be achieved. This conclusion has been also supported by other experimental data on large-scale chromatographic purification of proteins with ion-exchange sorbents [26].

The product yield also has a maximum value at a specific fluid velocity for a given terminating effluent concentration, as shown in Fig. 3. The velocity at which maximum product yield occurs increases with the increase of terminating effluent concentration. The value of the maximum yield decreases with the increase of the terminating effluent concentration. Moreover, the

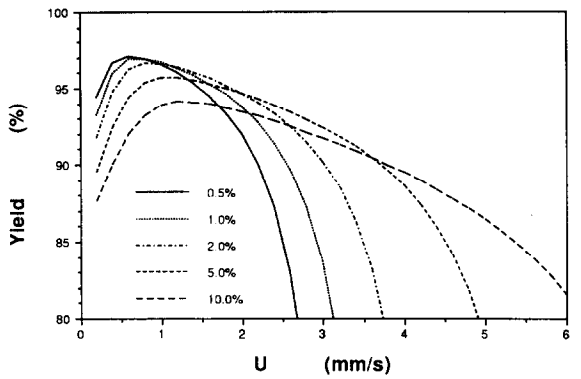

Fig. 3. Product yield *versus* superficial liquid velocity for different effluent concentrations (as percentages of inlet concentration) for the lysozyme–Cibacron Blue F3GA silica system.

results shown in Fig. 3 indicate that a proper choice of the fluid velocity is even more important if a high yield was required, as the yield drops rapidly when the fluid velocity exceeds a certain value, *e.g.* above 1 mm/s in the case of a 0.5% terminating effluent concentration for this particular chromatographic system.

The most appropriate fluid velocity and the terminating effluent concentration for a required production rate or yield value cannot be directly determined from Figs. 2 and 3. Therefore, contour plots were generated to aid the identification of the appropriate ranges of these operating parameters for the lysozyme–Cibacron Blue F3GA silica system. Fig. 4 shows the variation of yield with fluid velocity and effluent concentration. It can be seen that for any required yield of lysozyme within a defined parameter range, there is a particular fluid velocity which will result in the maximum allowable terminating effluent concentration. It should be noted from these investigations that the highest yield can be achieved only with low effluent concentrations and low fluid velocity. For any required yield value, there is a maximum fluid velocity, beyond which the required value will not be achieved.

The column capacity utilisation reduces when the fluid velocity increases, but increases with the increase of effluent concentration, as shown in Fig. 5. Thus, for a column capacity utilisation of 60% with a fluid velocity of 2 mm/s, a $C_{(out)}/C_{(in)}$ value of *ca.* 10% was required with the system. Although an effluent breakthrough of 10% may be acceptable with low value commodity proteins such as lysozyme, lower values of the $C_{(out)}/C_{(in)}$ would be essential for proteins of much higher commercial value thus requiring lower fluid velocities if the same system configuration had to be employed.

The results given in Fig. 6 show the production rate as a function of both the fluid velocity and the effluent concentration. It can be seen from Fig. 6 that for any required production rate in a designated range, there is a particular fluid velocity which will result in the minimum effluent concentration. Similar to the data shown in Fig. 4, the plots shown in Figs. 5 and 6 also

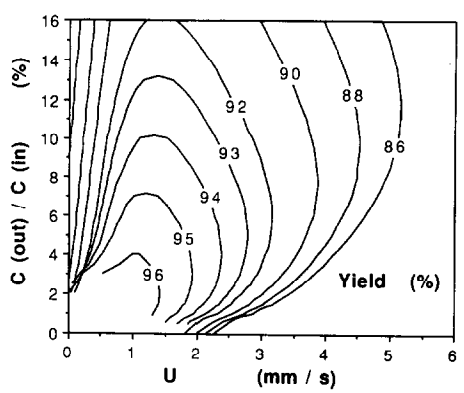

Fig. 4. Effects of liquid velocity and effluent concentration on the yield of a lysozyme–Cibacron Blue F3GA silica affinity chromatographic column.

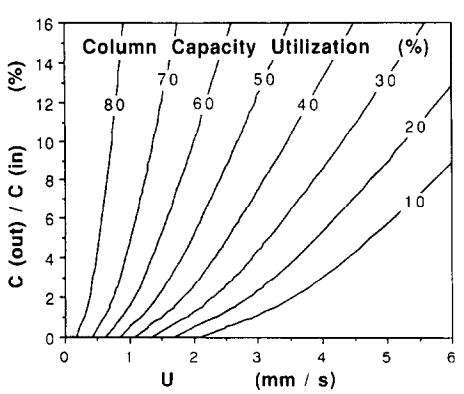

Fig. 5. Effects of liquid velocity and effluent concentration on the capacity utilisation of a lysozyme–Cibacron Blue F3GA silica affinity chromatographic column.

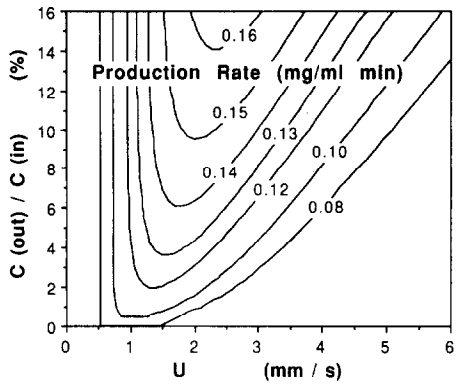

Fig. 6. Effects of liquid velocity and effluent concentration on the production rate (mg/ml min) of a lysozyme–Cibacron Blue F3GA silica affinity chromatographic column.

indicate that there are limitations on the values of the operating parameters which can be used for any required production rate or capacity utilisation.

The application of these contour plots can be given for the following two examples. One example is based on the requirement of yield, the other example is based on the requirement of maximum utilisation of the column capacity, since in some cases the cost of the packing is significant [27]. The first example is shown in Fig. 7, in which the optimisation is based on a primary requirement of 94% yield. If a 60% column capacity utilisation is preferred, the operation must be carried out in the region enclosed by the 94% yield curve and to the left of the 60% capacity curve. It can be seen that the highest production rate in the region occurs at the intersection of the two curves. Similar operational boundaries can be determined for the cases where 70 or 50% are the preferred column capacities. The exact values of fluid velocity and effluent concentration at these points can also be calculated by the BEDSTP program.

Example 2 is based on 70% utilisation of column capacity as illustrated in Fig. 8. Three yield curves are shown. Again it can be seen that the highest production rates which satisfy production requirement lie at the operational parameter intersections. However, if the requirement for capacity utilisation is not critical, the process may be operated at the highest production rates for each yield requirement. For the present simulation, the utilisation of the attainable column capacity is about 60% at the maximum production rate. Calculations have shown that when the non-adsorption time increases, the capacity utilisation at the maximum production rate also increases.

The simulations presented so far are all for finite terminating effluent concentration. Hence the yield value will always be less than 100% as part of the target protein has been lost in the effluent. For certain cases, a yield value approaching 100% is preferred. The operating conditions which need to be optimised then should be the loading volume and the flow-rate. Through computer simulation, a proper loading

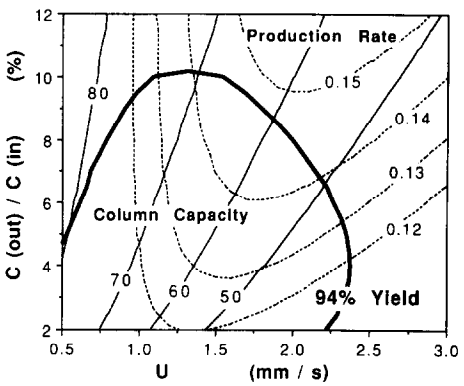

Fig. 7. Operating regions at 94% yield for four (50, 60, 70 and 80%) column capacity utilisation levels for the lysozyme–Cibacron Blue F3GA silica system.

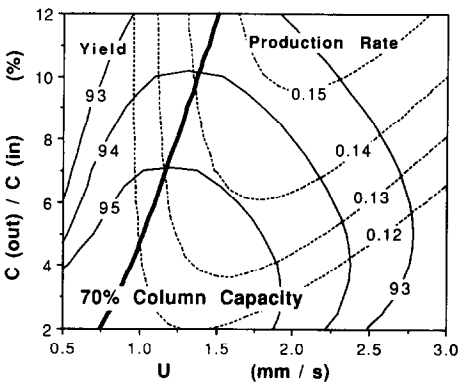

Fig. 8. Operating conditions for three (93, 94 and 95%) yield levels at 70% column capacity for the lysozyme–Cibacron Blue F3GA silica system.

volume can be found to ensure that the effluent concentration of the target protein stays just below the detectable limit (*e.g.* 0.1% of influent) at the end of washing. There will be no breakthrough curve. However, the solid-phase concentrations as shown in Fig. 9 can be used to demonstrate this case (calculated by program BEDSTS). In Fig. 9 the dashed line corresponds to the concentration profile at the end of ten column volumes of washing, whilst the solid line corresponds to the concentration profile at the end of loading. From these two profiles the loading volume can be readily calculated. It can be seen that although the profile has shifted, the area under these two curves are nearly the same, indicating that there is almost no protein loss during the washing.

These computer programs can also be used for design purpose. The choice of optimum fluid velocity determined from Figs. 4 to 8 is also subject to the practical pressure-drop limitation [1,2]. The relationships between packed bed pressure drop, superficial fluid velocity, and particle size are illustrated in Fig. 10. The calculation is based on the Blake–Kozeny equation (see ref. 28), assuming rigid particles and with a bed porosity of 0.4. It can be seen that for a fluid flow-rate above 0.1 mm/s, 10-μm particles based on silica or other mechanically rigid support materials would be the practical lower particle diameter limit for process scale applications. Therefore, if the predicted optimum fluid velocity exceeded the limit imposed by the system pressure drop, a shorter column with a larger diameter must be used. Fig. 11 shows the superficial liquid velocity at which the maximum production rate occurs *versus* column length for different terminating effluent concentrations. As the relationship between the column length and the optimum fluid velocity is almost linear, the proper design of a suitable column should be easily achieved.

In the development of a new protein purification process, not only will the choice of particle size affect the performance of the chromatographic outcome, but the selection of particle pore size will also influence the overall pro-

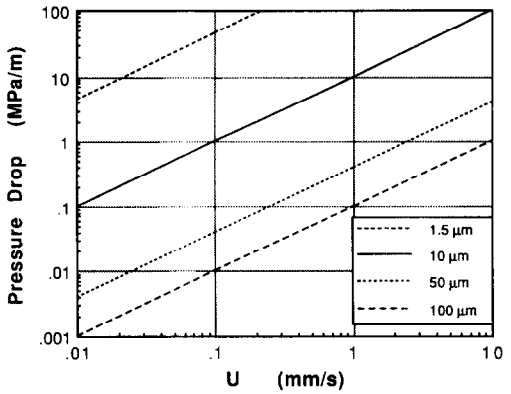

Fig. 10. pressure drop in a packed bed as a function of superficial liquid (water) velocity (U) for 1.5-, 10-, 50- and 100-μm particles.

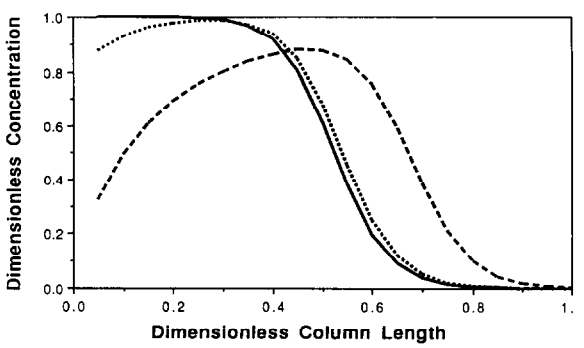

Fig. 9. Concentration profiles in the solid phase at the end of loading (solid line), the end of one column volume of washing (dotted line), and the end of 10 column volumes of washing (dashed line), in a non-breakthrough situation.

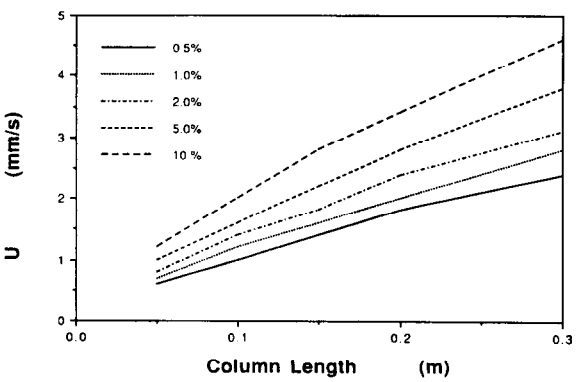

Fig. 11. Superficial liquid velocity at which the maximum production rate occurs, *versus* column length for different terminating effluent concentrations for the lysozyme–Cibacron Blue F3GA silica system.

duction rate. Currently, the pore size of most commercial packing materials is in the range of 6 to 400 nm. As a general rule, the ratio of the protein molecular diameter to the mean pore diameter should be less than 0.1 to eliminate restricted pore diffusion effects [29]. For biomacromolecules, a large pore size is required. However, the trade-off here is that a sorbent with a large pore size will exhibit a small surface are, as the total surface area of a porous particle is approximately proportional to the inverse of the average pore diameter, if the pore volume remains constant. As a result, the adsorption capacity of sorbents will reduce as the pore size increases and this ultimately will affect the overall production rate. On the other hand, use of sorbents with small pore sizes will result in reduced mass transfer of the protein molecules to the internal surface of the particle. This reduced mass transfer rate will also result in a reduction to the overall production rate. Furthermore, when the protein to pore size ratio becomes equivalent, most protein molecules will no longer be able to access the binding sites inside the pores and this effect will result in a great reduction in the effective adsorption capacity. Therefore, a compromise in terms of particle size and pore size must be reached in order to obtain the maximum overall production rate. A set of detailed selection criteria can only be established when specific data become available on each type of sorbent and the application usage. For the present, an initial selection can be made based on a preferred protein to pore size ratio of 0.1, with the sorbent choice limiting the adsorption capacity. Under these boundary conditions the computer programs described in this paper can then be used to evaluate which packing will offer the higher production rate.

CONCLUSIONS

A method has been developed to enable optimization of the cost-effective operating parameters in purification of proteins using chromatographic columns. The method employs computer simulations using a mathematical model based on non-linear adsorption isotherms and a limited set of data obtained from laboratory-scale experiments. The contour plots generated aid the selection of the appropriate fluid velocity and sample loading volume for a desired yield and production rate. For an existing process, the illustrated approach can be used to evaluate the performance, and identify individual components in the process where improvements can be made. For an existing equipment system, this method can also help to determine the operating parameters by permitting a key set of laboratory-scale and pilot-scale experimental studies to be identified from previous computer simulation rather than the more traditional trial-and-error approach. For a new process system this method can assist in the design of the column and the selection of the specifications of the associated equipment, such as pumps, tubing, etc.

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