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Multiple-injection technique for isolating a target protein from multicomponent mixtures☆

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ABSTRACT

An integrated chromatographic process comprising ion exchange (IEC) and hydrophobic interaction chromatography (HIC) for isolating a target protein form multicomponent mixtures has been analyzed. The model mixture contained immunoglobulin G that was the key product of the separation process, cytochrome C and ovalbumin. The adsorption characteristics and the mass transport kinetics of the model proteins have been determined along with their dependencies on the operating variables such as pH, temperature and the salt concentration for IEC as well as HIC media. Limitations of the process efficiency resulting from kinetic effects, solubility constraints and the necessity of the mobile phase exchange between chromatographic steps have been discussed. To improve the performance of the integrated process the multiple-injection technique has been suggested. This technique consisted in loading feed mixtures dissolved in a good solvent onto the column by several small-volume injections under conditions of strong protein adsorption. It allowed diminishing interactions between the sample-solvent and protein and elimination of undesired effects such as band splitting and band broadening. For the process design and optimization a dynamic model has been used accounting for thermodynamics and kinetics of the process. The optimization results indicated superiority of the multiple-injection technique over standard isocratic injections in terms of the process yield and productivity.

1. Introduction

Nowadays large demand for proteins is recognized, particularly by pharmaceutical industry and biotechnology. The increasing regulatory requirements enforce the necessity of manufacturing high-purity proteins; therefore, downstream processing is a critical step of the overall production process.

Purification of macromolecules may comprise several high-resolution separation stages (two, three, four or more) and in many cases final polishing when product is used for therapeutical applications. Chromatography as a highly selective separation method is often a key operation in the downstream processing. To isolate a target protein from complex multicomponent mixtures a combination of different chromatographic techniques have to be employed. Usually few chromatographic stages differing in the separation mechanism are necessary to achieve desired selectivity of the separation [1–3].

Integration of different chromatographic techniques requires determining and synchronizing a number of operating parameters such as the mobile phase composition, pH and temperature to ensure high efficiency of the whole operation. Each of chromatographic techniques utilizes different type and composition of buffered mobile phases; therefore, additional step of buffer exchange between the stages is necessary. Moreover, chromatographic elution of proteins is accompanied by strong dilution of feed mixtures originating from kinetic effects. Thus, transferring mixtures to be separated between subsequent chromatographic stages requires additional operation of protein concentration. Another performance limitation is the solubility constraint. Solubility of proteins strongly depends on pH, type and concentration of salt in solutions used as the mobile phase [1].

To overcome those limits different solvents can be used to inject protein mixtures and to elute them. The choice of the solvent for the feed stream depends on the sequence of chromatographic stages, solubility constraints and separation selectivity. However, such an injection methodology may cause undesired effects, such as band splitting and band deformations [4–16]. In our previous study these phenomena were analyzed in hydrophobic interaction chromatography [16]. It was shown that proper selection of the operating conditions for the sample injection allowed increase of the mass loadings while undesirable effects of band deformations could be avoided. The method was indicated to be suitable for increasing the loading concentration; however, it forced a restriction on the loading volume.

In this study that idea was extended to increase both the concentration and volume loading. The multiple-injection technique

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Nomenclature

c	concentration in the mobile phase
D_L	axial dispersion coefficient
F	phase ratio, $(1-\varepsilon_t)/\varepsilon_t$
k	kinetic coefficient
K	equilibrium constant
L	column length
N	number of theoretical plates
Pr	productivity
Pu	purity
q	concentration in the adsorbed phase
x	space coordinate
t	time coordinate
t,T	temperature, in proper scale
u	superficial velocity
$\dot{V}$	flowrate
w	interstitial velocity
Y	yield

Greek symbols

ε_t, ε_e	total and external bed porosity, respectively

Subscripts and superscripts

a	denotes adsorption
d	denotes desorption
f	denotes folding
F	denotes feed
in	denotes inlet
inj	denotes injection
n	denotes native form
p	denotes protein
salt	denotes salt
sol	denotes solubility
unf	denotes unfolding
wash	denotes washing
0	denotes initial conditions

was exploited to overcome performance limitations of chromatographic elution and to integrate different chromatographic techniques. The integrated process comprised two chromatographic stages typically used in an industrial scale: ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC).

The mechanism of IEC separation is based on electrostatic interactions between the protein and charged column matrix. The proteins are separated according to the nature and degree of their ionic charge. Elution is effected by increasing the ionic strength and, thus, concentration of salt containing counter ions that compete with the protein molecules for the charged active centers on the matrix [1,2]. Because of the nature of the separating mechanism, pH plays important roles in controlling the separation.

HIC is often employed as an alternative or supplementary technique to IEC [17,18]. It exploits the interaction between hydrophobic surface sites of both native proteins and column matrix, requiring consequently strong salting-out solvents for binding [19]. Protein adsorption is induced by high concentration of structure-enhancing salts (cosmotrope) such those used in precipitation (e.g. ammonium sulphate) and followed by elution with a descending salt concentration. The retention properties of proteins in HIC are strongly altered by temperature. In general, increasing temperature enhances hydrophobic interactions and the protein retention whereas lowering temperature promotes the protein elution [17,20–22].

It is evident that both these techniques utilize different operating conditions; hence, their proper integration is of major importance for the purification performance.

In this study HIC and IEC were integrated to isolate a target protein from a ternary mixture. For the process design a lumped dynamic model was used accounting for underlying kinetics, i.e., rates of adsorption–desorption and conformational changes. Relevant kinetic dependencies indispensable to the process design were experimentally quantified and incorporated into the model.

2. Theory

2.1. Mathematical modeling

To simulate band profiles the kinetic-dispersive model was employed. It consists of the mass balance equation of the component i in the mobile phase:

$$\varepsilon_t \frac{\partial c_i}{\partial t} + u\frac{\partial c_i}{\partial x} + (1-\varepsilon_t)\frac{\partial q_i}{\partial t} = \varepsilon_e D_{L,i}\frac{\partial^2 c_i}{\partial x^2} \tag{1}$$

where c_i is the concentration of species in the mobile phase; $i = p$ (*protein*) or *salt*; u is the superficial velocity, t, x are time and axial coordinates, D_L is the axial dispersion coefficient, q_i is the adsorbed phase concentration, ε_t, ε_e are the total and external bed porosity, respectively.

For the Langmuir type of adsorption behavior Eq. (1) can be combined with the following kinetic equation:

$$\frac{\partial q_i}{\partial t} = k_{d,i}(K_{e,i}c_i(q^\infty - q_i) - q_i) \tag{2}$$

where q^∞ is the saturation capacity, $K_{e,i} = k_{a,i}/k_{d,i}$ is the equilibrium constant, $k_{a,i}$, $k_{d,i}$ are the rate coefficients of adsorption and desorption.

Due to kinetic limitations chromatographic elution of protein is usually accompanied by strong band dilution. In this case $q^\infty >> q_i$ and Eq. (2) simplifies to:

$$\frac{\partial q_i}{\partial t} = k_{d,i}(K_i c_i - q_i) \tag{3}$$

where $K_i = K_{e,i}q^\infty$ is the distribution coefficient, termed also as the Henry constant.

For non-retained salts the value of the distribution coefficient can be set equal to zero $K_{salt} = 0$.

The protein unfolding was assumed to occur in the adsorbed phase according to the reversible reaction mechanism:

$$P \underset{k_d}{\overset{k_a}{\rightleftharpoons}} P_n \underset{k_f}{\overset{k_{unf}}{\rightleftharpoons}} P_{unf} \tag{4}$$

represented by the following kinetic equations [16,23,24]:

$$\frac{\partial q_n}{\partial t} = k_{d,p}(K_p c_p - q_n) - k_f(K_{unf}q_n - q_{unf}) \tag{5}$$

$$\frac{\partial q_{unf}}{\partial t} = k_f(K_{unf}q_n - q_{unf}) \tag{6}$$

where $K_{unf} = k_{unf}/k_f$ is the unfolding equilibrium constant, k_f and k_{unf} are the folding and unfolding rate coefficients, respectively, q_n, q_{unf} is the concentration of the protein (P) adsorbed on the surface in the native (P_n) and unfolded form (P_{unf}), respectively.

Then, the accumulation term in Eq. (1) can be expressed as:

$$\frac{\partial q_p}{\partial t} = \frac{\partial q_n}{\partial t} + \frac{\partial q_{unf}}{\partial t} \tag{7}$$