Continuous biospecific affinity purification of enzymes by simulated moving-bed chromatography
Theoretical description and experimental results

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Abstract

A system for continuous biospecific affinity purification of enzymes by simulated moving-bed chromatography was designed. The performance of steady-state operation was predicted by a model based on a linear driving force approximation. The system was tested for the purification of bovine α-chymotrypsin on immobilized soybean trypsin inhibitor. The theoretical results showed rather good agreement with the experimental results. The recovery was found to be dependent on the extract purity. For higher purities and recoveries the columns must be purged with non-desorbing buffer before shifting from the adsorption to the desorption zone.

Keywords: Simulated moving-bed chromatography; Affinity adsorbents; Adsorption isotherms; Enzymes; α-Chymotrypsin; Trypsin inhibitor, soybean

1. Introduction

Conventional liquid chromatography is widely used not only in the analytical field but also for preparative purposes. The two major disadvantages of chromatographic separations are the discontinuity of the process and the dilution of the product.

In continuous systems in which the solid-phase flows in the opposite direction to the fluid phase the mass transfer profile remains stationary and the adsorbent is used more efficiently [1]. In most cases this solid movement is achieved by periodical shifting of the dis-

crete columns (simulated moving bed). The required adsorbent volume in a countercurrent system is about one-quarter of that required for batch chromatography [2]. Since 1964 continuous chromatographic systems have been used at industrial scale mainly in the petro-chemical and sugar processing industries. The most widely known process is the SORBEX process developed by UOP (United Oil Products, Des Plaines, IL, USA) [3].

The operation of a simulated moving-bed (SMB) apparatus requires the optimization of more process parameters than in conventional chromatography. The following parameters can be varied: The fluid velocity in each section along with the solid velocity (in SMB devices

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given by the switching time), the total volume of adsorbent used, the number of columns in each section and the column dimensions. In SMB devices used at industrial scale the solid phase as well as the fluid phase is circulated.

Because of the multitude of variables, mathematical modelling is indispensable. Different models for the simulation of liquid chromatography which have been extended to continuous systems are available in the literature [4,5]. The models have been developed for low-molecular-mass compounds.

For the purification of biopolymers, especially proteins, from complex biological mixtures the use of biospecific affinity chromatography is advantageous. This chromatography technique requires non-isocratic conditions. In a first step the desired component is being adsorbed at the stationary phase. This step is followed by an elution step, where this component is being desorbed by changing the fluid-phase composition. This method is also applicable for continuous (SMB) processes.

A system for biospecific affinity purification of enzymes has, however, only been studied by Huang et al. [6]. In contrast to the system described in that work, here the adsorption and desorption zones are separated completely, which allowed better control of the operating conditions. Only the solid phase is circulated.

The purpose of this work is to investigate the relevant design parameters of an SMB device applied to biospecific affinity chromatography. The experimental verification of the theoretical description of the simulated moving-bed technique is restricted to low-molecular-mass compounds. Due to their slower diffusion rate, high-molecular-mass compounds such as enzymes might need a different approach to mathematical modelling. Furthermore, the effect of transportation of impurities in the void volume on the extract purity is investigated.

As a model system for biospecific affinity chromatography of enzymes the well-characterized interaction between α-chymotrypsin and immobilized soybean trypsin inhibitor [7] was used.

2. Theoretical description of the SMB device

Considering a chromatographic column packed with spherical particles of uniform size, a mass balance for the desired feed component in the fluid phase leads to the expression

$$D_{ax}\frac{\partial^2 c}{\partial z^2} - v\frac{\partial c}{\partial z} - \left(\frac{1-\epsilon_b}{\epsilon_b}\right)\frac{\partial q}{\partial t} = \frac{\partial c}{\partial t} \qquad (1)$$

Considering countercurrent flow of fluid and solid phase, Eq. 1 reduces at steady-state operation of an SMB device [8] to

$$D_{ax}\frac{d^2 c}{dz^2} - v\frac{dc}{dz} - \left(\frac{1-\epsilon_b}{\epsilon_b}\right)\frac{dq}{dt} = 0 \qquad (2)$$

A mass balance for the solid phase assuming plug flow leads to

$$u\frac{dq}{dz} + \frac{dq}{dt} = 0 \qquad (3)$$

here the exchange rate between fluid and solid phase is approximated by a linear driving force expression:

for the adsorption zone $\dfrac{dq}{dt} = k_{ad}(q^* - q)$ (4)

for the desorption zone $\dfrac{dq}{dt} = -k_{des}q$ (5)

In the adsorption section the driving force is given by the difference between the actual concentration in the solid phase and the equilibrium concentration at the present fluid-phase concentration. In most cases the adsorption equilibrium is of the Langmuir type with:

$$q^* = \frac{q_{max}c^*}{K_1 + c^*} \qquad (6)$$

In the desorption zone the fluid-phase composition is changed and no adsorption can occur. This means that the rate of desorption is only determined by the present solid-phase concentration. These equations imply that all adsorption sites are equally accessible and are of the same type, which has to be proved experimentally.

Eqs. 2 and 3 were transformed into a set of

ordinary partial equations of first order, which were solved numerically by the fourth-order Runge–Kutta method with the following boundary conditions:

Adsorption zone: $z = 0$: $c = c_0$ and $q = 0$,

$z = L$: $dc/dz = 0$.

Desorption zone: $z = 0$: $c = 0$ and $q = q_0$,

$z = L$: $dc/dz = 0$.

Once the governing parameters k_{ad} and k_{des} and the equilibrium data (q_{max} and K_1) are known, the performance of the SMB device can be predicted. At a desired liquid flow-rate different calculation runs have to be made to find the optimum solid velocity u (in SMB devices given by $u = H/T$).

For the evaluation of the theoretical description no purge step between adsorption and desorption is necessary. However, a buffer system with high buffering capacity has to be used to minimize the time required for the pH shift from a high value in the adsorption section to a low value in the desorption section.

3. Experimental

Bovine α-chymotrypsin (EC 3.4.21.1) and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO, USA). As immobilization matrix an oxirane–acryl polymer (Eupergit C250L) was used, which was kindly provided by Röhm (Darmstadt, Germany). The average particle diameter of Eupergit C250L is $dp_{50} = 163$ μm.

N-Benzoyl-L-tyrosin-ethylester (BTEE) and bovine hemoglobin were purchased from Serva (Heidelberg, Germany) and chymotrypsinogen A was purchased from Worthington (Feehold, NJ, USA)

All other chemicals were of analytical grade.

3.1. Preparation of affinity chromatography matrix

Five grammes of Eupergit C250L were incubated for 72 h with soybean trypsin inhibitor

(STI) solution (300 mg STI in 20 ml 1 M phosphate buffer, pH 7.5) at room temperature. After incubation the carrier was washed five times with 20 ml 0.1 M phosphate buffer, pH 7.5. The amount of bound STI was determined by measuring the absorbance at 280 nm of the supernatant and the washing buffer. The ligate density was 8.4 mg STI per ml wet support.

For removing residual oxirane groups of Eupergit C250L the carrier was incubated 16 h at room temperature with 20 ml ethanolamine solution (1 M) of pH 8.0

3.2. Determination of enzyme concentration and activity

In the experiments performed with pure α-chymotrypsin the enzyme concentration was determined by measuring the absorbance at 280 nm (A_{280}) with a spectrophotometer (Uvicon 930; Kontron, Zurich, Switzerland) using an ϵ_{280} value of $4.9 \cdot 10^4$ M^{-1} cm^{-1} [9].

The enzymatic activity was used as a concentration equivalent in the enzyme purification experiments. The rate of hydrolyzation of BTEE was determined at 25°C in phosphate buffer of pH 8.0 ($I = 0.2$). The change of absorption at 256 nm (ΔA_{256}) was measured as described in Ref. [10].

3.3. SMB apparatus

Fig. 1 depicts the simulated moving-bed device used in this work. The system consists of eight chromatographic columns (2 cm I.D.) packed with 1-cm height adsorbent. Each column is equipped with a movable piston, which allows an axial compression of the bed. Feed and desorbent streams are controlled by two HPLC pumps (Model 2150; LKB-Pharmacia, Uppsala, Sweden). Three-way solenoid valves (Model 368; Asco, Ratingen, Germany) and eight-port valves are controlled by a computer (PC 386). The software was written in Visual Basic (Microsoft, Redmond, WA, USA).

The eight-port rotating valves controlling the feed, desorbent, raffinate and extract streams are
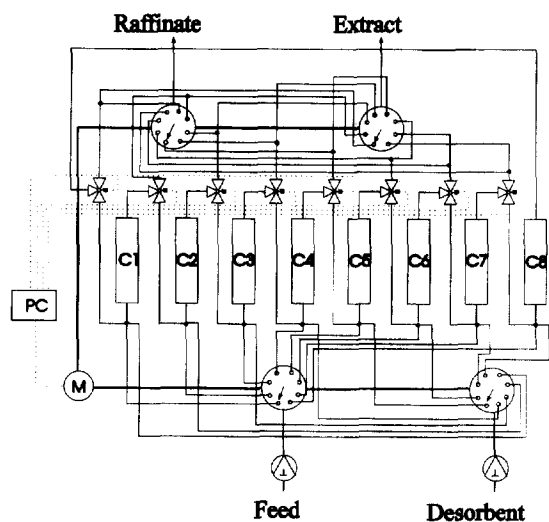
Raffinate   **Extract**

Fig. 1. Flow chart of the simulated moving-bed system consisting of eight columns (C), whose inlets can be connected to either the feed or desorbent stream by eight-port valves. The outlets can either be connected to the next column by a three-way valve or to either the raffinate or extract stream via eight-port valves. The eight-port valves are mounted on a single drive (M). All valves are controlled by a computer (PC).
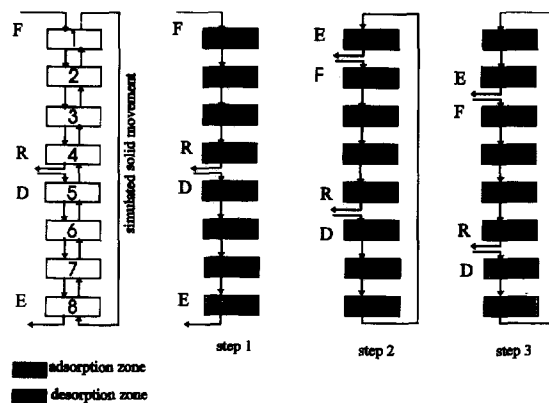
Feed   **Desorbent**

Fig. 2. Operating principle of the simulated moving-bed system. The first three switching steps achieving the solid movement are shown. F = feed stream, R = raffinate stream, D = desorbent stream, E = extract stream (containing the desired feed component).

mounted on one axle and are shifted simultaneously. The valves consists of two stationary heads with one and eight outlets, respectively, made of PTFE, interconnected by a stainless-steel rotating head.

Fig. 2 shows the principle of operation of the SMB device. The first three steps are shown.

During step 1 the feed containing the desired enzyme is fed through the eight-port valve to column 1. It then flows through columns 1 to 4. The interconnection to column 5 is closed by a three-way valve. In this step adsorption takes place in columns 1 to 3. In column 4 the pH is shifted from pH 3.0 to pH 7.5.

Desorbent buffer at pH 3.0 is fed to column 5. It then flows through columns 5 to 8. The interconnection to column 1 is closed by a three-way valve. The desired component is being desorbed in columns 5 to 7 while in column 8 the pH is shifted from pH 7.5 to pH 3.0.

In the next step all inlet and outlet streams are shifted one position, this is equivalent to a movement of the columns in the opposite direc-

tion. Column 1 is now situated in the desorption section while column 8 is shifted to the desorption section. In this step the interconnections between columns 1 and 2 and columns 5 and 6 are closed by a three-way valve. Hereby a movement of the solid-phase is achieved.

On-line measurements of the protein concentration in extract or raffinate stream are performed by measuring the absorbance at 280 nm with an UV detector (Model 2151, LKB-Pharmacia).

3.4. Determination of adsorption equilibrium

A suspension of the chromatographic support in 0.1 M phosphate buffer, pH 7.5, was incubated 2 h at 25°C with α-chymotrypsin solutions of different concentrations. Following incubation, the amount of remaining α-chymotrypsin was determined by measuring the absorbance at 280 nm.

3.5. Determination of mass transfer coefficients

Mixed vessel experiments were performed to determine the rate of adsorption and desorption. α-Chymotrypsin solution (8.8 ml) in phosphate buffer (pH 7.5; $I = 0.1$) was filled in a glass

vessel. Then 1.85 ml adsorbent was added and the change of absorbance at 280 nm was observed. Eqs. 4 and 5 respectively were solved numerically by the method of Euler. The values of k_{ad} and k_{des} were determined by fitting the calculated adsorption and desorption curves to the experimental results.

4. Results and discussion

4.1. Buffer system

To achieve steady-state operation it is necessary to shift the pH of each column to pH 7.5 while it is situated in the adsorption zone and to pH 3.0 while situated in the desorption zone. This has to be done within one shifting interval to ensure a stable pH gradient.

The time necessary for shifting the pH from a high value to a low value and vice versa has been tested in a single column. This time is not only determined by the buffering capacity of the feed and desorption fluids but also by the buffering capacity of the chromatographic support itself.

The best pH values for adsorption and desorption were pH 7.5 and pH 3.0, respectively, as shown previously [11]. Various buffers have been tested and optimum results were obtained with 0.3 M phosphate buffer (pH 7.5) and 0.2 M formiate buffer (pH 3.0). With this buffer system 8 min were required to shift the pH in a single column from a high value to a low value and vice versa at a flow-rate of 1 ml/min.

4.2. Adsorption equilibrium and mass transfer coefficients

Fig. 3 shows the result of the adsorption equilibrium measurement. The adsorption isotherm was of the Langmuir type. Using the sedimentation volume of a particle suspension as the adsorbent volume, the isotherm parameters were found to be $K_1 = 0.25$ mg/ml and $q_{max} = 6.5$ mg/ml$_{adsorbens}$.

Adsorption and desorption experiments were performed in a mixed vessel with different start concentrations c_0 and q_0, respectively. The mass
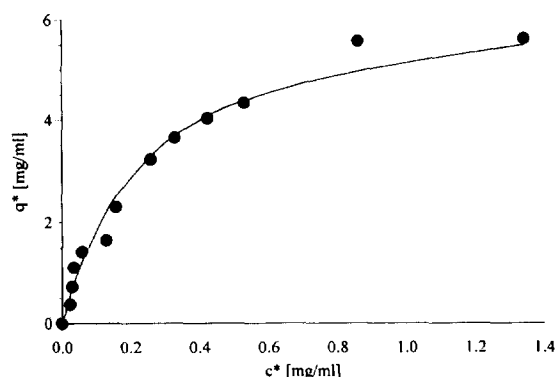

Fig. 3. Adsorption isotherm of α-chymotrypsin on STI–Eupergit C250L in phosphate buffer of pH 7.5 at 20°C. The ionic strength of the buffer was $I = 0.1$.

transfer constants k_{ad} and k_{des} were determined from the initial rate of adsorption and desorption. The adsorption rate was found to be concentration dependent, with $k_{ad}(c_0) = (0.004c_0 + 0.002)$ s^{-1} within the investigated concentration range (0.1–1.0 mg/ml). No concentration dependency was found for the desorption mass transfer coefficient within experimental error: $k_{des} = 0.03$ s^{-1}. (See also Fig. 4.)

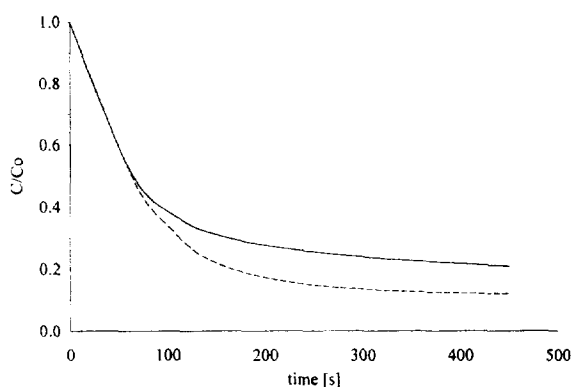These values are much lower than the film

Fig. 4. Concentration decay curve for the adsorption of α-chymotrypsin on STI–Eupergit C250L in phosphate buffer of pH 7.5 ($I = 0.1$) at 20°C. The concentration decay was determined in a batch experiment with a start concentration of $c_0 = 0.1$ mg/ml (solid line) and calculated with the linear driving force approximation of Eq. 4 (broken line).

mass transport parameter $k_1 a = 0.21$ s^{-1} calculated from $k_1 = D$ Sh$/(2R)$, with Sh $= 2 + 0.6$Re$^{1/2}$Sc$^{1/2}$ [12]. This indicates that pore diffusion rather than film diffusion is the rate limiting step.

The calculated batch concentration differs from the measured concentration at low values of c/c_0. This indicates an inhomogeneity of the adsorption sites. Within the first 1 to 2 min of each uptake experiment equilibration with the easily accessible binding sites was achieved. Then the remaining (less accessible) adsorption sites became rate limiting. For the modelling of the SMB device this was not taken into consideration.

4.3. Steady-state concentration profile

In these experiments 1 ml/min α-chymotrypsin solution (0.5 mg/ml) was fed into the SMB device. The protein concentration in the outlet streams was monitored. The outlet of one column was collected during one switching interval. After collecting the sample the apparatus was allowed to reach steady-state operation again, and the next sample was collected. The bed porosity was found to be 0.3 and was used to calculate the interstitial fluid velocity. The total volume of each column (including all interconnections) was 15.2 ml, filled with 8.5 ml of particles. This dead volume was taken into consideration by calculating a new overall porosity including the part of the SMB device not filled with adsorbent.

The calculated concentration profile was compared with the experimental results (Fig. 5). In the adsorption zone a good agreement of the experimental and theoretical results was observed. The mass transport in the SMB device was even faster than the calculated mass transport. However, the raffinate concentration, which is the important parameter for optimizing the operating conditions in this zone, was calculated correctly. In the desorption zone greater differences between the calculated and measured concentration were observed.
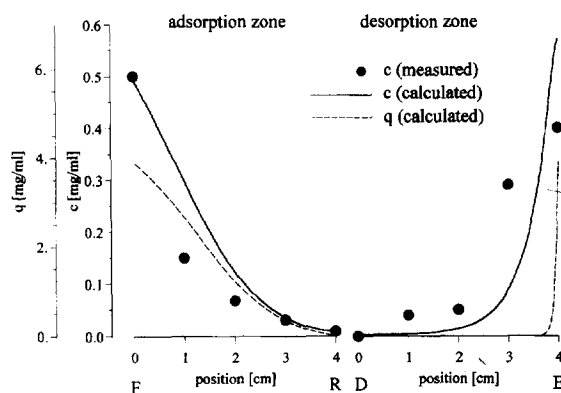

Fig. 5. Experimental and calculated enzyme concentration profile within the SMB system. Feed, 1 ml/min α-chymotrypsin (0.5 mg/ml in 0.3 M phosphate buffer of pH 7.5); desorbent, 1 ml/min (0.2 M formiate buffer of pH 3.0); switching interval, 9 min. The simulation parameter were: $q_{max} = 6.5$ mg/ml, $K_1 = 0.25$ mg/ml, $k_{ad} = 0.0036c + 0.002$ s^{-1}, $k_{des} = 0.03$ s^{-1}, $D_{ax} = 0.008$ cm^2/s, $\epsilon = 0.44$, $v = 0.018$ cm/s, $u = 0.00185$ cm/s.

4.4. Enzyme purification

A 0.5 mg/ml α-chymotrypsin solution mixed with different concentrations of impurities was applied to the SMB device. The operating conditions were the same as in the previous experiments. Raffinate and extract were collected and the specific activity (sa $= \Delta A_{256}/A_{280}$) was determined.

Since no purge step is employed one can expect that most of the impurities elute within the first minutes of each switching interval. A higher specific activity was achieved by discarding the extract eluted within the first minutes of each switching period. (See also Fig. 6.)

By discarding 7 ml of extract of each switching interval, almost pure α-chymotrypsin could be collected. To visualize this effect, experiments with 0.5 mg/ml α-chymotrypsin and 2.0 mg/ml hemoglobin were carried out. The spectra show only slight contamination of the remaining extract (Fig. 7). The desorption process started before all the impurities have left the column. Hence, the recovery decreased to 10%. That implies that each column should be purged with
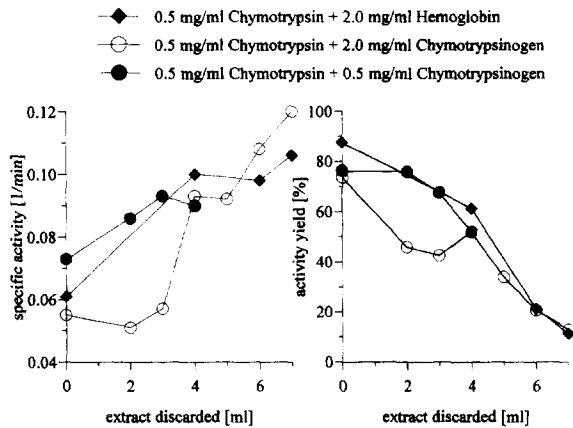
Fig. 6. Specific activity and activity yield as a function of discarded extract volume in each switching interval for the enzyme purification in the SMB system. Feed, 1 ml/min (0.3 M phosphate buffer of pH 7.5) of different compositions; desorbent, 1 ml/min (0.2 M formiate buffer of pH 3.0); switching interval, 9 min.
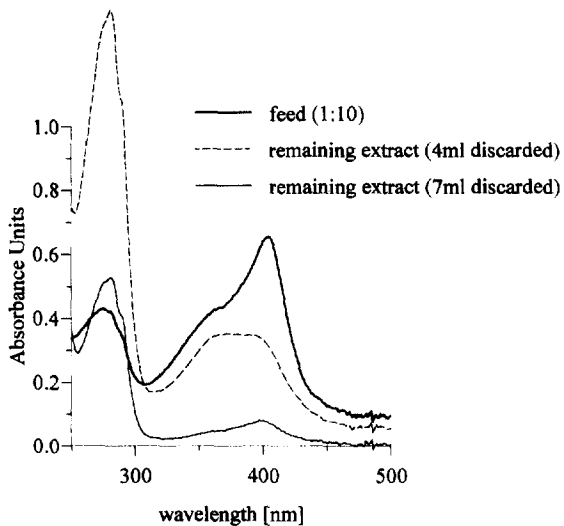

Fig. 7. Absorbance spectra of feed and extract of the enzyme purification experiment. The feed composition was 0.5 mg/ml α-chymotrypsin and 2.0 mg/ml hemoglobin in 0.3 M phosphate buffer of pH 7.5. The desorbent was 0.2 M formiate buffer of pH 3.0. The flow-rates of feed and extract were 1 ml/min; switching interval, 9 min.

high-pH buffer before entering the desorption section.

5. Conclusions

Based on a linear driving force approximation the predicted concentration profiles showed rather good agreement with the experimental results. With this model only steady-state concentration profiles can be calculated. The volume of the required tubing for an SMB device of this size was high compared to the volume of the solid phase. In this case the steady-state assumption of Eq. 2 is a rough approximation. The solution of Eq. 1 allows the calculation of the time necessary to achieve steady-state operation. A closer agreement with the experimental results can be expected since this model takes into account the stepwise movement of the columns.

The recovery was found to be dependent on the extract purity. For higher purities and recoveries the columns must be purged with high-pH buffer before shifting from the adsorption to desorption zone. This allows the problem of shifting the pH from high to low values to be overcome. Since the flow-rate of the purge stream can be chosen independently, it is possible to purge each column with several bed volumes of high-pH buffer before entering the desorption zone. This should allow the use of buffers with lower ionic strength.

Acknowledgement

List of symbols

c fluid-phase concentration of desired component (mg/ml)

c_0 start concentration in fluid phase (in SMB

experiments: concentration in feed stream) (mg/ml)

c^* fluid-phase concentration in equilibrium (mg/ml)

D diffusion coefficient (m^2/s)

D_{ax} dispersion coefficient (m^2/s)

H height of adsorbens bed in each column (m)

k_{ad} mass transfer coefficient for adsorption (s^{-1})

k_{des} mass transfer coefficient for desorption (s^{-1})

$k_l a$ film mass transfer coefficient × specific surface area (s^{-1})

K_l parameter in Langmuir isotherm (mg/ml)

L length of adsorption and desorption section (m)

q solid-phase concentration of desired component ($\text{mg/ml}_{adsorbens}$)

q_0 start concentration (in SMB experiments: maximum concentration achieved in adsorption section) ($\text{mg/ml}_{adsorbens}$)

q^* solid-phase concentration in equilibrium ($\text{mg/ml}_{adsorbens}$)

q_{max} parameter in Langmuir isotherm ($\text{mg/ml}_{adsorbens}$)

R particle radius (m)

Sc   Schmidt-number

Sh   Sherwood-number

t time (s)

T switching time (s)

u solid velocity (m/s)

v interstitial fluid velocity (m/s)

z axial coordinate in the adsorption and desorption section (m)

ϵ_b bed porosity

References

[1] G.J. Rossiter, in Subramanian (Editor), Preparative and Process-Scale Liquid Chromatography, Ellis Horwood, Chichester, 1991.

[2] D.M. Ruthven, Principles of Adsorption and Adsorption Processes, John Wiley, New York, 1984.

[3] G. Ganetsos and P.E. Barker (Editors), Preparative and Production Scale Chromatography, Chromatographic Science Series, Vol. 61, Marcel Dekker, New York, 1993.

[4] J.C. Bellot and J.S. Condoret, Process Biochemistry, 26 (1991) 363–376.

[5] D.M. Ruthven and C.B. Ching, Chem. Eng. Sci., 44 (1989) 1011–1038.

[6] S.Y. Huang, C.K. Lin, W.H. Chang and W.S. Lee, Chem. Eng. Commun., 45 (1986) 291–309.

[7] V. Kasche, Studia Biophysica, 35 (1973) 45–56.

[8] P. Deckert and W. Arlt, Chem. Ing. Technol., 66 (1994) 1334–1340.

[9] V. Kasche, H. Amnéus, D. Gabel and L. Näslund, Biochim. Biophys. Acta, 490 (1977) 1–18.

[10] B.C.W. Hummel, Can. J. Biochem. Physiol., 37 (1959).

[11] V. Kasche and B. Galunsky, in T. Gribneau and J. Visser (Editors), Affinity Chromatography IV, Elsevier, Amsterdam, 1982, pp. 93–110.

[12] T.K. Sherwood, L.R. Pigford and C.R. Wilke, Mass Transfer, McGraw-Hill, 1975, p. 221.