Journal of Chromatography, 609 (1992) 61–68
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 423

Short fibrous supports for preparative chromatographic separations of biomolecules

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(First received February 20th, 1991; revised manuscript received June 1st, 1992)

ABSTRACT

The characteristics of short, fibrous supports for chromatographic separations of biomolecules have been studied. A non-porous, silica-based fiber with an anion-exchange functionality was developed, and the performance of this support for protein separations was determined. It was established that the short-fiber column exhibits a low pressure drop, allowing separations to be performed at elevated flow-rates. The dynamic adsorption capacity of the column for three proteins, myoglobin, ovalbumin and bovine serum albumin, have been determined as a function of solution pH and salt concentration. It has been shown that the column can be used to separate mixtures of these proteins at elevated flow-rates.

INTRODUCTION

The significant growth in the market for bioproducts has led to the need to adapt chromatography to the process scale [1]. The criteria for evaluating the performance of a process-scale purification have been shown to be product purity, throughput, and cost [2]. For this reason, systems developed for analytical separations are not always suitable for process-scale separations. In recognition of this, substantial efforts are now being devoted to the development of systems that involve new operating procedures [3] and novel equipment configurations [4–9].

The use of chromatographic supports with a fibrous geometry is one novel approach to developing chromatographic systems for scaled-up separations. Two types of fiber columns have recently been developed. In one type, fibers, either solid or hollow, are packed into a column such that the longest dimension of the fibers is aligned with the direction of flow of the mobile phase [10–13]. The second type uses relatively short fibers, and these are packed with random orientations [14]. The short-fiber column and columns packed with aligned hollow fibers have been shown to exhibit very low pressure drops [11,14], making them potentially suitable for high-throughput separations.

Though both types of columns have potential applications in high-throughput separations, the randomly packed short-fiber columns are of immediate interest, since the procedures for packing and operating these columns are essentially the same as those used for conventional columns packed with spherical supports. Thus, the developmental task involves only the synthesis of appropriate supports, and not the development of packing and operating procedures that is required for systems that are radically different.

At the present time, only one type of short-fiber support [14], a silica-based affinity support, has been developed. In this paper, the synthesis of a short-fiber anion-exchange support is described; an ion-exchange support was developed because a great number of downstream processing protocols use an ion-exchange step. The characteristics of this

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0021-9673/92/$05.00

support for protein separations have been evaluated.

EXPERIMENTAL

Materials

Bulk Q-106 quartz fiber was a gift from Manville Sales (Toledo, OH, USA). This non-porous fiber consists of 98.5% (w/w) silica (SiO_2) and has a nominal fiber diameter of 0.7 μm. PAE-300, a porous silica-based chromatographic packing, was purchased from Amicon (Danvers, MA, USA).

Reagents

3-Glycidoxypropyltrimethoxysilane (Z-6040) was a gift from Dow Corning (Midland, MI, USA). Polyethyleneimine (PEI-6, molecular mass 600) was purchased from Polysciences (Warrington, PA, USA). Solvents (acetone, methanol, etc.) and reagents (sulfuric acid, nitric acid, etc.) were purchased from Fisher Scientific (Cincinnati, OH, USA).

Proteins

The proteins, bovine serum albumin (BSA), ovalbumin (OVA) and myoglobin (MYO) from horse heart, were purchased from Sigma (St. Louis, MO, USA).

Instrumentation

A SP8800 ternary HPLC pump system (Spectra-Physics, San Jose, CA, USA) was used for the chromatographic evaluations. Absorbance was monitored with a Model 2550 UV detector (Varian, Walnut Creek, CA, USA).

Preparation of fiber substrate

Bulk quartz fiber Q-106 was cut in water with a homogenizer (Biospec Products, Bartlesville, OK, USA). The nominal length of the cut fiber, characterized with an optical microscope, is 115 μm. The fiber was cleaned by washing with acetone and deionized water. It was then hydroxylated, to increase the number of reactive silanol groups on the surface. This was achieved by refluxing in a 50:50 (v/v) mixture of concentrated sulfuric acid and nitric acid for 8 h [15,16]. Subsequently, the fiber was thoroughly washed with deionized water and dried in a vacuum oven at 110°C for 2 h.

Surface modification of hydroxylated fiber

A 2-g amount of the hydroxylated fiber was refluxed in 10% (v/v) Z-6040 solution in dry toluene (dried over sodium metal) for 24 h [14,15,17,18]. After filtering off the silane solution, the fiber was dried in a vacuum oven at 110°C for 8 h. It was then washed with 60 ml acetone and dried in a vacuum oven at room temperature for 8 h. At the end of this step, the Z-6040 is covalently attached to the fiber through the silanol group.

Immobilization of polyethyleneimine on the fiber

The Z-6040 bonded fiber was treated with a solution of 20% (w/v) PEI-6 in methanol for 12 h [17,19,20]. After filtering off the PEI-6 solution, the fiber was dried in a vacuum oven at 80°C for 12 h. Subsequently, it was washed with 60 ml methanol and 60 ml acetone, and dried in vacuum at room temperature for 8 h. This process immobilizes the PEI-6 on the fiber by covalent attachment to the Z-6040. The procedure used here to attach the PEI-6 to the silica fiber is similar to that developed by Regnier and co-workers [17,19–24] for spherical silica supports, indicating that already developed activation methods can be adapted to making silica supports with a fibrous geometry.

Column packing

A packing procedure involving sedimentation followed by pressurization was used to pack the anion-exchange fiber into a standard high-performance liquid chromatography (HPLC) (150 mm × 4.6 mm I.D.). The column was vertically oriented, and the fiber suspended in methanol was poured into the top end. A weak vacuum was applied to the bottom to remove the solvent. The column was then attached to a HPLC pump and a pressure drop of 2400 p.s.i. was applied for about 10 min. This compressed the packing in the column. The entire process was repeated a number of times until the column was full and tightly packed.

In order to allow a comparison of pressure drop characteristics with conventional columns, PAE-300 (10 μm), a commercial, porous, silica support, was packed in a column of the same dimensions. In this case, conventional slurry packing at a pressure of 6000 p.s.i. was used. A Haskel air-driven liquid pump purchased from Alltech (Deerfield, IL, USA) was utilized for this purpose.

Measurement of column pressure drop

The column pressure drop was determined by subtracting the system pressure drop in the absence of the column from that when the column was part of the flow circuit. These measurements were made for both the Q-106 fiber column and the PAE-300 column. A 0.05 mg/ml BSA in 0.01 *M* Tris–HCl (pH 8.0) solution was used in all cases. The pressure drop behavior was studied for flow-rates between 1 and 10 ml/min.

Measurement of dynamic capacity

The dynamic capacity of the fiber column was determined with frontal chromatography. The capacity was determined for three proteins, BSA, OVA and MYO, at pH 6.0 and 8.0, and at two salt concentrations. The buffers used were 0.01 *M* histidine at pH 6.0 and 0.01 *M* Tris at pH 8.0, and NaCl was used as the salt.

The frontal experiments involved washing the column with 0.5 *M* NaCl and equilibrating it with the required carrier solution. The column was then disconnected, and the upstream tubing was filled with the protein solution. The column was reconnected, and the frontal injection was initiated. Column response was monitored with the UV detector at 280 nm. All the experiments were performed at room temperature.

Chromatographic evaluation

Chromatographic evaluations of the column were made in the gradient elution mode. A sample size of 10 μl was used in all cases. The gradient elutions involved a 20-min linear gradient of NaCl from 0.0 to 0.5 *M*. Three buffers, Tris (pH 8.0), imidizole (pH 7.0) and histidine (pH 6.0), were used. The column response was monitored at 280 nm.

RESULTS AND DISCUSSION

Column pressure drop

The experimentally obtrained pressure drop characteristics of the fiber column and reference PAE-300 column are shown in Fig. 1. It is immediately evident that the fiber column exhibits a substantially lower pressure drop, with pressure drops of no more than about 20% of those in the PAE-300 column at the same flow rate.

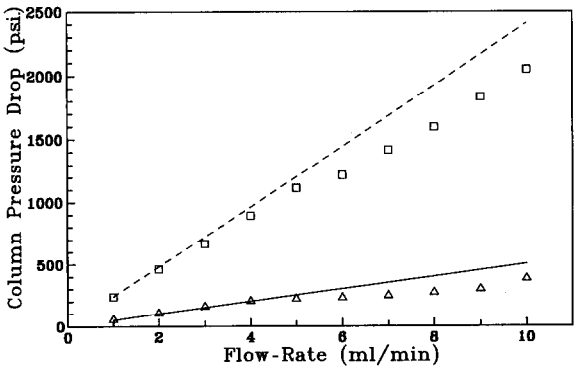

Fig. 1. Column pressure drop as a function of flow-rate for the fiber column (△) and PAE-300 column (□). Column length, 15 cm; ---- and ——, theoretical predictions [25].

Due to equipment limitations on the maximum volumetric throughput, the pressure drop experiments could not be extended to flow-rates above 10 ml/min. However, an attempt was made to theoretically estimate the pressure drop at higher volumetric flow rates. A correlation developed by Leva [25] for packed beds was used for this purpose:

$$\Delta P = \frac{2f_m G^2 L(1-\varepsilon)^{3-n}}{dg_c \rho \Phi_s^{3-n} \varepsilon^3} \quad (1)$$

The predictions of this correlation for the two columns studied experimentally are shown in Fig. 1; the bed porosities, 0.4 for the PAE-300 column and 0.92 for the fiber column, were calculated independently from unretained component data. It is seen that Leva's correlation predicts the pressure drop reasonably well; the slight over prediction at higher flow-rates is suspected to be due to pump characteristics at 6 ml/min and higher, rather than a failure of the equation. Thus, for the purposes of a rough theoretical estimate at flow-rates beyond the experimental range the correlation should be adequate. Shown in Table I are the predictions at higher flow rates. These data show that it will be possible to operate the 15-cm fiber column at 40–50 ml/min while maintaining acceptable pressure drops. This implies linear velocities of 240–300 cm/min at pressure drops of about 140 p.s.i./cm.

Dynamic adsorption capacity

Frontal chromatography was used to evaluate the dynamic adsorption capacity of the fiber col-

TABLE I

PREDICTIONS OF PRESSURE DROP AT HIGHER FLOW-RATES

Flow-rate (ml/min)	Linear velocity (cm/min)	Column	ΔP (p.s.i.)	$\Delta P/L$ (p.s.i./cm)
20	120	Q-106	1037	69
		PAE-300	4717	315
30	181	Q-106	1459	97
		PAE-300	7308	487
40	241	Q-106	2021	135
		PASE-300	9666	644
50	301	Q-106	2600	173
		PAE-300	12 144	810

umn. Three proteins, BSA, OVA and MYO, were used. The adsorption isotherms constructed from the breakthrough curves for each of these proteins and for a number of mobile phase conditions are shown in Figs. 2–5. These capacities are calculated on the basis of the breakthrough of 50% of the feed concentration. Figs. 2 and 3 show the effect of pH on capacity. For both cases, the adsorption isotherm is roughly Langmuirian in shape. At pH 8.0 the fiber has a capacity of about 1.2 mg/g for BSA, 0.6 mg/g for OVA, and virtually no capacity for MYO. At the lower pH of 6.0, the capacity for BSA drops to about 0.8 mg/g, while for OVA it increase slightly to about 0.7 mg/g. Once again there is virtually no capacity for MYO. The observed effects of pH can be qualitatively explained on the basis of the p*I* values of the proteins (Table II) and the weakly basic nature of the ion-exchange fiber. The low p*I* values of BSA and OVA, 4.9 and 4.7, respectively, ensure that these proteins have a high charge density at pH 8.0. When the pH is lowered to 6.0, the ion-exchange capacity of the weakly basic support is increased, but the charge density of both proteins is lowered. In the case of BSA these oppositely acting effects lead to a net decrease in capacity, while OVA shows a slight increase. For MYO, a p*I* of 7.3 should give, as observed, no capacity at pH 6.0. At pH 8.0, the protein will have a low charge density, but the ion-exchange capacity of the support is lowered, once again giving virtually no adsorption. Though the adsorption behavior can be explained qualitatively on the basis of charge density, other factors such as charge asymmetry, hydrophobic interactions, conformational changes, and hydrogen

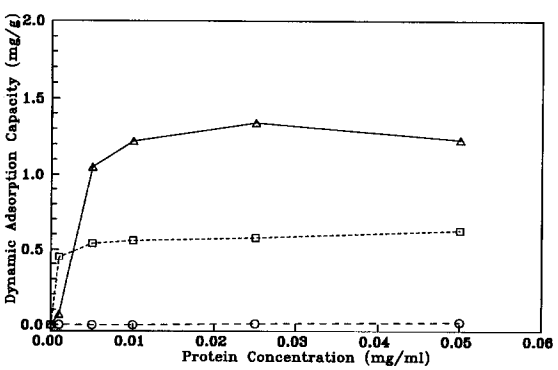

Fig. 2. Adsorption isotherms of BSA (△), OVA (□) and MYO (○) at pH 8.0.

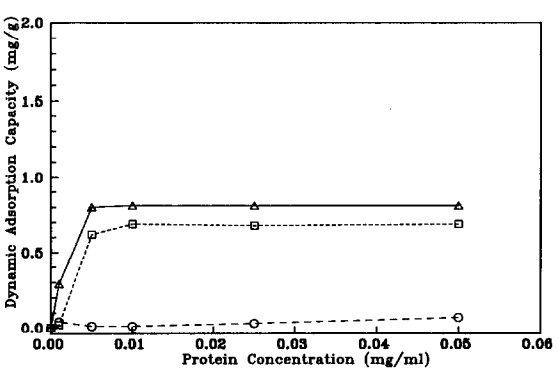

Fig. 3. Adsorption isotherms of BSA (△), OVA (□) and MYO (○) at pH 6.0.

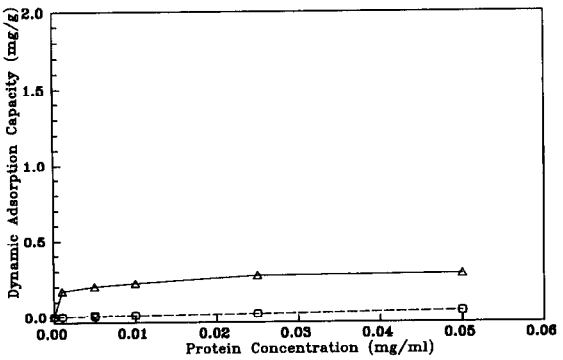

Fig. 4. Adsorption isotherms of BSA (△), OVA (□) and MYO (○) at pH 8.0 with 0.05 *M* NaCl.

bonding can also be expected to influence the adsorption capacity [24,26,27].

The effects of salt concentration on adsorption are shown in Figs. 4 and 5. Two salt concentrations were used: 0.05 *M* NaCl (Fig. 4) and 0.1 *M* NaCl (Fig. 5). Once again, the adsorption isotherms are roughly Langmuirian. However, it is clear that the presence of salt leads to a rapid decrease in protein capacity, with virtually no capacity remaining at 0.1 *M* NaCl.

An important factor for columns operating at elevated flow rates is the sensitivity of the column capacity to the mobile phase velocity. Shown in Fig. 6 is the typical dependence of column capacity on flow-rate. These data were obtained with frontal experiments with BSA at pH 8.0. Once again, 50% of the feed concentration was used to determine capacity. It can be seen that the dynamic capacity is maintained at higher flow-rates. The reason for this is that the fiber is non-porous, restricting the mass transfer resistance to the liquid phase. In this case, since the fluid velocities are in the laminar flow region, the liquid mass transfer coefficient is essentially independent of flow-rate. Thus, the shape of the concentration fronts is not affected significantly by mass transfer, leading to a dynamic capacity that is essentially independent of flow-rate.

TABLE II

CHARACTERISTICS OF PROTEINS

Protein	Isoelectric point	Molecular mass
BSA	4.9	69 000
OVA	4.7	44 000
MYO	7.3 (major)	17 500
	6.9 (minor)	

Gradient elution chromatography

The separation of BSA, OVA and MYO was attempted on the fiber column using gradient elution. A mixture of 5 mg/ml BSA, 5 mg/ml OVA and 0.5 mg/ml MYO was used for all the separations. Shown in Fig. 7 are typical chromatograms obtained at 1 ml/min for carrier solutions at pH 6.0, 7.0 and 8.0. While the MYO is separated cleanly from the other two proteins, since it is essentially unretained, the separation of BSA from OVA is strongly dependent on the solution pH. A good sep-

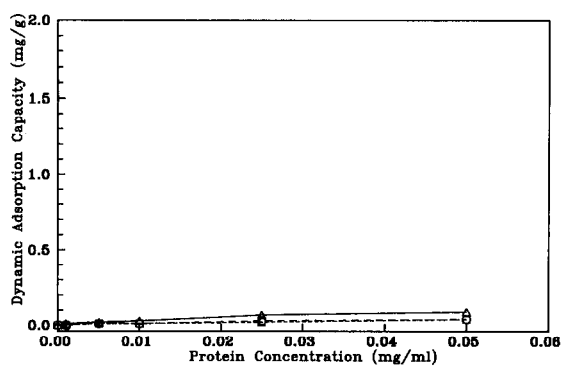

Fig. 5. Adsorption isotherms of BSA (△), OVA (□) and MYO (○) at pH 8.0 with 0.10 *M* NaCl.

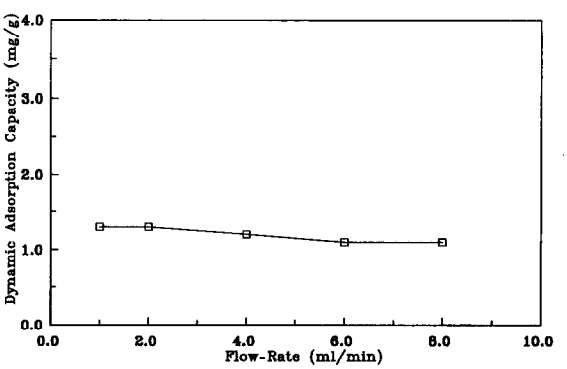

Fig. 6. Dynamic adsorption capacity of fiber column for BSA as a function of flow-rate.

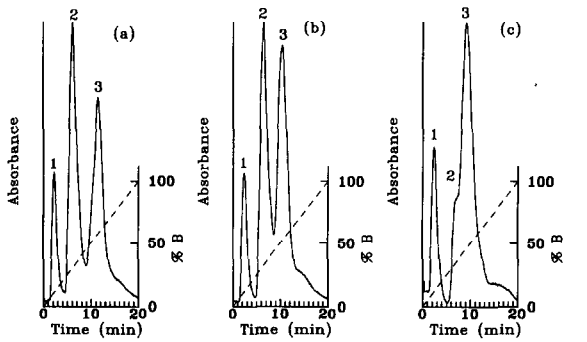

Fig. 7. Gradient elution separation of BSA, OVA and MYO as a function of pH. Flow-rate, 1 ml/min. Gradient, 20 min, linear, 0.0 to 0.5 *M* NaCl. (a) pH 8; (b) pH 7; (c) pH 6.

aration is obtained at pH 8.0, a poor separation at pH 6.0, and a separation intermediate between these at pH 7.0. With regard to the resolution between OVA and BSA, a decrease is observed with decreasing pH. This can be explained on the basis of the pH dependence of the retention times. Retention times for BSA and OVA, with each obtained separately using the same gradient protocol as was used for the mixtures, changed in opposite directions with an increase in pH; an increase in the retention time for BSA and a decrease for OVA were observed. This behavior is consistent with the frontal capacity data, and, as was stated earlier, is related to the charge density of the proteins and the ion-exchange capacity of the support.

A significant characteristic of the fiber column is the low pressure drop it exhibits at high flow-rates.

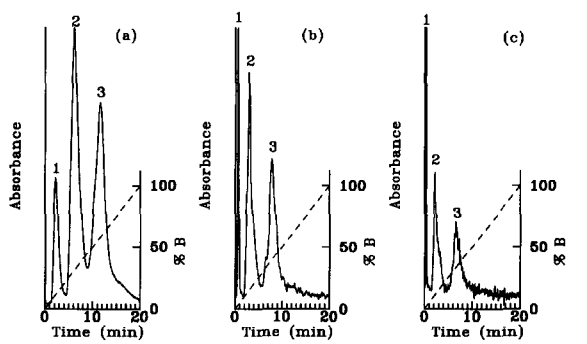

Fig. 8. Gradient elution separation of BSA, OVA and MYO as a function of flow-rate. pH, 8.0. Gradient, 20 min, linear, 0.0 to 0.5 *M* NaCl. (a) 1 ml/min; (b) 4 ml/min; (c) 8 ml/min.

Thus, it is important to determine if elution separations can be achieved at higher flow-rates. Fig. 8 shows the effect of flow-rate on the separation of MYO, OVA and BSA at pH 8.0. Three chromatograms at 1, 4 and 8 ml/min are shown. Identical 20-min salt gradients were used for all three cases. It should be noted that the noisy signal obtained at the highest flow-rate is due to slight flow fluctuations produced by the pump, which is operating close to its upper limit; these fluctuations are independent of the column. From Fig. 8 it is clear that the proteins can be separated on the fiber column at higher flow rates. In fact the resolution between the OVA and BSA peaks actually improves with increasing flow-rate. However, this improved resolution at higher flow-rates is due to the salt gradient applied, rather than any particular characteristic of the column. If the salt gradients are compared on the basis of volume of eluent rather than time, it is immediately evident that the steepest gradient was applied at the lowest flow-rate. Thus, with increasing flow-rate the retention time difference between OVA and BSA increases. This results in improved resolution between peaks. The smaller gradient at higher flow-rates also results in lowered peak concentrations as is clearly evident in Fig. 8.

Implications of experimental results

The attainment of high flow-rates in chromatographic columns does not by itself guarantee high column throughputs. Equilibrium and kinetic characteristics are essential factors that must also be considered. The non-porous fiber support used in this work shows good pressure-drop characteristics, but has a low adsorption capacity. Thus, the throughput attainable is very limited, even at high fluid velocities. An obvious way of enhancing capacity, without losing the favorable pressure drop characteristics, is to use a porous fiber in place of the non-porous support. Based on the surface area reported for equivalent porous carbon fibers, it is expected that the available surface area in the porous silica fiber will be approximately two orders of magnitude greater than the non-porous fiber. This will give the fiber a capacity comparable to that of conventional, porous, spherical supports.

Porous supports provide the high capacities required for preparative separations, but their kinetic characteristics are generally inferior to non-porous

supports, due to the additional mass transfer resistance in the stationary phase. Thus, poor chroamtographic resolution can result, especially in the separation of large biomolecules. In order to overcome this problem, small-diameter spherical supports ($\leqslant 20$ μm) are used. However, since small diameters lead to high pressure drops, 2 μm is generally recognized [28] as the lower limit for the particle diameter; at smaller diameters, excessive pressure drops lead to detrimental effects associated with localized viscous heating, even at low linear velocities. The pressure drop behavior observed with the fiber column indicates that with a short-fiber support the lower limit on the diameter can be reduced to the sub-micron range. In fact, a sub-micron diameter of 0.7 μm was used in this study. The implication is that with a porous, sub-micron fiber good intra-particle mass transfer characteristics will be obtained in combination with low pressure drops and high capacities. These are highly desirable characteristics for preparative separations.

One disadvantage of fiber columns is that flow dispersion can be expected to be significantly higher than in comparable columns packed with spherical supports, and, depending on characteristics of the separation and on operating conditions, this effect may partially or completely counter the advantage gained with respect to intra-particle mass transfer. Consequently, the low pressure drop and intra-particle mass transfer characteristics of fiber columns will not necessarily translate into higher throughputs than sphere packed columns. A complete comparison requires the optimization of both systems with respect to throughput, in order to compare the maximum throughput attainable in each.

CONCLUSIONS

The potential of using chromatographic packings with a short, fibrous geometry for preparative separations of proteins has been investigated. Specifically, a non-prous, anion-exchange silica fiber was synthesized. It has been established that short-fiber supports give very porous beds, and, consequently, high linear velocities can be achieved at acceptable column pressure drops; it is estimated that linear velocities of 240–300 cm/min can be attained at pressure drops of about 140 p.s.i./cm. Also, because of the non-porous nature of the support, the dynamic column capacity is maintained at high flow-rates, indicating good mass transfer characteristics. A sample protein separation was performed on the column using gradient elution. It has been shown that the peaks can be resolved even at elevated flow-rates. One disadvantage of the non-porous support was found to be its low capacity. However, it is anticipated that replacing the sub-micron non-porous fiber with an equivalent sub-micron porous fiber will result in an adequate enhancement in capacity, while retaining good mass transfer characteristics.

SYMBOLS

d	diameter of particle
f_m	friction factor
g_c	gravitational constant
G	mass flow-rate
L	column length
n	state of flow factor
ΔP	pressure drop of column

Greek symbols

ε	bed porosity
ρ	fluid density
Φ_s	shape factor

ACKNOWLEDGEMENTS

This work was supported by Grant No. CTS-8909742 from the National Science Foundation, and by The Center for Excellence in Membrane Technology at The University of Cincinnati. This support is gratefully acknowledged.

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