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PERFORMANCE EVALUATION OF NON-POROUS *VERSUS* POROUS ION-EXCHANGE PACKINGS IN THE SEPARATION OF PROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The performance of a non-porous, anion-exchange packing was evaluated and compared with a number of similar porous high-performance liquid chromatography packings. The non-porous columns were found to be equally efficient for proteins spanning a wide range of molecular weights, while the porous columns exhibited decreasing efficiency as the proteins became larger. The porous materials also exhibited size exclusion effects that were not seen with the non-porous materials, which partially accounts for the loss of efficiency with large proteins. When increasingly steep gradients were employed, the loss of resolution was less with the non-porous materials.

INTRODUCTION

Recently, there has been increased interest in the use of non-porous packing materials for both high-performance liquid chromatography (HPLC)[1–4] and affinity chromatography[5]. The major advantage of non-porous materials is that there is no diffusion of solute into and out of pores which, in porous materials, leads to band-broadening and, consequently, a loss of efficiency and resolution. Separations on non-porous column materials are characterized by narrow peak-widths and very short analysis times, generally on the order of 1–10 min[1–3]. The absence of pores also obviates entrapment of solute and leads to high recoveries[1,2].

An apparent drawback to the use of non-porous beads as chromatographic column materials is the reduced surface area and, hence, lower capacity. One way to overcome this problem is to use very small beads. Beads as small as 1.5 μm in diameter have been employed succesfully in reversed-phase chromatography[4]. Another approach that has been used for anion exchangers is to take non-porous 7 μm beads and to couple polyethyleneimine (PEI) covalently to their surface[1].

According to Snyder and Kirkland[6], the efficiency (H) of a well packed column is related to the following:

$$H = Au^{0.33} + B/u + Cu \quad (1)$$

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TABLE I

COLUMNS TESTED

The surface area was determined by mercury porosimetry. Pore volumes in ml of pores per ml of beads were determined by comparing elution volumes of an excluded molecule (DNA) to that of an included molecule (glycyl-tyrosine) under conditions where each was unretained. NA = information not available.

Column	*Name*	*Source*	*Material*	*Dimensions*	*Pore size (nm)*	*Surface area (m^2/g)*	*Pore volume (ml/ml)*
A Porous	MONO Q	Pharmacia	Polymer	5 cm × 5.0 mm	100	NA	0.38
Non-porous	MA7P(10 μm)	Bio-Rad	Polymer	5 cm × 4.6 mm	0	1.3	0
B Porous	DEAE-5-PW	Bio-Rad	Polymer	7.5 cm × 7.5 mm	100	58	0.43
Non-porous	MA7P(10 μm)	Bio-Rad	Polymer	7.5 cm × 7.5 mm	0	1.3	0
C Porous	AX-1000	Rainin	Silica	250 cm × 4.6 mm	100	NA	0.40
Non-porous	MA7P(10 μm)	Bio-Rad	Polymer	250 cm × 4.0 mm	0	1.3	0

where u is the mobile phase velocity, the A term is due to eddy diffusion and interparticle mobile phase mass transfer, B is the longitudinal diffusion coefficient and C is the intraparticulate mass transfer effect. The use of non-porous materials largely eliminates the C term of this equation for unretained peaks, and the use of the same size spherical particles packed in the same size columns should keep the A and B terms relatively constant. If intraparticulate mass transfer is a significant contributor to band broadening, then it is expected that non-porous columns will be more efficient than porous ones. As protein molecules become larger this mass transfer effect will become more pronounced because once the larger molecules enter the pores the slower diffusion will make it more difficult for them to diffuse back out again.

The objective of this study was to compare non-porous PEI packings with other commercially available porous anion exchangers in terms of efficiency, resolution, and speed of separation. Of particular interest is the way each of these column materials perform with increasingly large proteins at different flow-rates and gradient times. A major difficulty in comparing columns from many different commercial sources is that the material comes in a variety of particle size ranges and is often packed in a variety of column formats. In order to compare the columns fairly it was necessary to match the columns packed with non-porous beads as closely as possible to the porous bead columns. Fortunately, one thing that a majority of anion-exchange columns have in common is an average particle size of approximately 10 μm. We therefore obtained some 10-μm non-porous polymethacrylate beads and covalently coupled PEI to the surface according to the method of Burke *et al.*[1]. This material was then packed into columns of the same length and similar inner diameter as the other columns. Slight differences in the column I.D. were compensated for by adjusting the linear flow-rates.

MATERIALS AND METHODS

Columns

Three sets of columns were tested, all packed with 10-μm spherical material with the porous columns having the following column length and diameter: (A) 5 cm × 5.0 mm Mono Q (Pharmacia, Piscataway, NJ, U.S.A.), (B) 7.5 cm × 7.5 mm Bio-Gel TSK DEAE-5-PW (Bio-Rad Labs., Richmond, CA, U.S.A.), and (C) 25 cm × 4.6 mm Synchropak AX-1000 (Rainin Instrument, Emeryville, CA, U.S.A.). The corresponding non-porous Microanalyzer MA7P columns (Bio-Rad Labs.) measured 5 cm × 4.6 mm, 7.5 cm × 7.5 mm, and 25 cm × 4.0 mm (Table I).

HPLC system

The HPLC system used was a Bio-Rad Protein Microanalyzer System, operated by an Apple IIe computer with dual-disk drive, ProFile hard-disk option, and Bio-Rad gradient processor system (version 3.8) software. Data from the Bio-Rad Model 1305A detector were integrated with a Bio-Rad Model 3392A integrator interfaced with the computer.

Samples were injected with a Bio-Rad Model AS-48 autosampler, and gradients were formed by two Bio-Rad Model 1330 pumps and a 250-μl Lee Visco Jet® micro-mixer (Lee Company, Westbrook, CT, U.S.A.). Dead-volumes between the mixer and the injector, between the injector and the column, and between the column

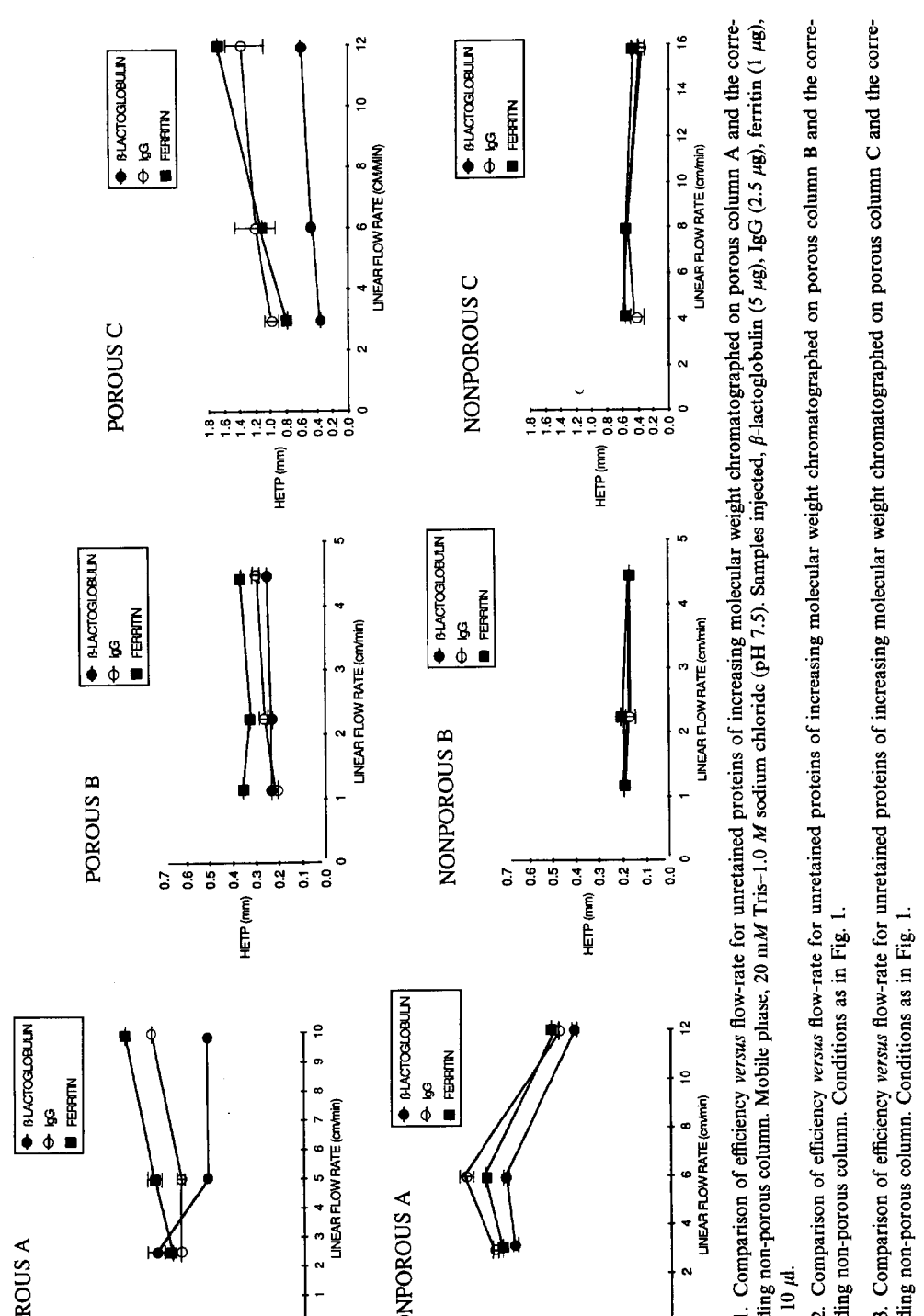

Fig. 1. Comparison of efficiency *versus* flow-rate for unretained proteins of increasing molecular weight chromatographed on porous column A and the corresponding non-porous column. Mobile phase, 20 m*M* Tris–1.0 *M* sodium chloride (pH 7.5). Samples injected, β-lactoglobulin (5 μg), IgG (2.5 μg), ferritin (1 μg), all in 10 μl.

Fig. 2. Comparison of efficiency *versus* flow-rate for unretained protcins of increasing molecular weight chromatographed on porous column B and the corresponding non-porous column. Conditions as in Fig. 1.

Fig. 3. Comparison of efficiency *versus* flow-rate for unretained proteins of increasing molecular weight chromatographed on porous column C and the corresponding non-porous column. Conditions as in Fig. 1.

and the detector were kept to a minimum by using short, 0.01-in. I.D. tubing. The tubing between the injector and the detector was 10 cm long. Since the detector cell had a volume of 8 μl, the extra-column volume (injector through detector) was *ca.* 20 μl.

Materials

All proteins used were from Sigma (St. Louis MO, U.S.A.). Ascites fluids from Sigma were from myeloma lines MOPC 21 (IgG_1), UPC 10 (IgG_{2a}), and FLOPC 21 (IgG_{2b}). DNA was from Bio-Rad. All buffers were made with distilled, deionized water and reagent grade solutes. Buffer solutions were passed through a 0.45-μm filter and degassed prior to use.

RESULTS

Efficiency as a function of molecular weight

The efficiency of each column was determined by using proteins spanning a wide range of molecular weights. Each substance was injected onto the column under conditions where it would be unretained, at flow-rates ranging from 0.2 to 2.0 ml/min. The number of theoretical plates was calculated according to the following equation, averaged over three runs

$$N = 5.54(t/w_h)^2 \tag{2}$$

where t is the retention time and w_h is the peak width at half height. The resulting efficiency, expressed as H, was calculated as

$$H = L/N \tag{3}$$

where L is the column length in millimeters.

The efficiency *versus* flow-rate was plotted for the non-porous and the porous column materials for three different proteins (Figs. 1–3). These proteins can be classified as small (β-lactoglobulin, MW 35 000), medium (IgG, MW 150 000) and large (ferritin, MW 470 000). In each case the efficiency of the non-porous column remains relatively constant, regardless of the size of the protein. In contrast, each of the porous columns loses efficiency with increasingly larger proteins. This is especially noticeable at higher flow-rates where the intraparticulate mass transfer term is predominant.

Size-exclusion effects

In porous materials there is usually a broad pore size distribution. The reported pore size represents a mean value, with many pores both larger and smaller than this mean. A significant number of these pores are small enough that average sized proteins are excluded, leading to gel permeation effects. This broad range of pore sizes can contribute to increased intraparticulate mass transfer resulting in band broadening and a loss of efficiency. This does not occur with non-porous materials.

The interparticle volume for a column, V_0, was determined by passing a very large molecule over the column under conditions where it would not be retained (1.0

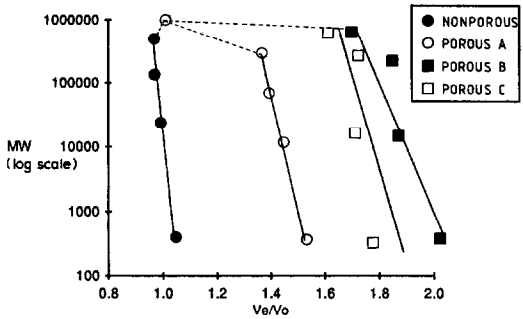

Fig. 4. Size exclusion effects on porous and non-porous columns. Flow-rate, 1 ml/min. Mobile phase, 20 m*M* Tris–1.0 *M* sodium chloride (pH 7.5). Samples injected: glycyl-tyrosine (M_r = 350), β-lactoglobulin (M_r = 35 000), IgG (M_r = 150 000), ferritin (M_r = 470 000). Void volume marker, DNA ($M_r > 10^6$).

M sodium chloride) and would not fully penetrate the available pore volume. The large void volume marker used in this case was a large DNA restriction fragment from *Escherichia coli*. It was excluded from all the pores of the 1000-Å materials. Other molecules spanning a wide range of molecular weights were similarly chromatographed and their elution volumes (V_e) determined. A plot of V_e/V_0 *versus* molecular weight is shown in Fig. 4. Each porous column exhibited a substantial pore volume that excluded proteins of higher molecular weight, while non-porous beads exhibited only a slight effect, probably due to hydronamic chromatography effects at the bead to bead contact points.

Effect of gradient time on resolution

The effect of gradient steepness on resolution was shown by chromatographing β-lactoglobulins A and B with a 0.0 to 1.0 *M* salt gradient over 30, 20, 10, and 5 min and plotting the loss of resolution (%) as a function of gradient time (Figs. 5–7). The resolution of the protein pair with a 30-min gradient was taken as being 100%. Buffer conditions were established so that the separation selectivity was nearly identical for each pair of columns. Consequently, differences in resolution were a function of band-broadening.

Fig. 5 shows that from a 30-min to a 10-min gradient both the porous column A and the corresponding non-porous column lost 18% of their resolution. However when the gradient time was shortened to 5 min the loss resolution on the porous column dropped sharply (53%) compared with the non-porous (29%). Resolution on the porous column B decreased much more than on the corresponding non-porous column until with a 5-min gradient it could no longer resolve the proteins at all (Fig. 6). A similar pattern was seen with the porous column C in Fig. 7.

CONCLUSIONS

Several factors contribute to the efficiency of HPLC columns, the most significant of these being eddy diffusion, interparticle mobile phase mass transfer effects and intraparticulate mass transfer effects, as expressed in eqn. 1. The use of non-

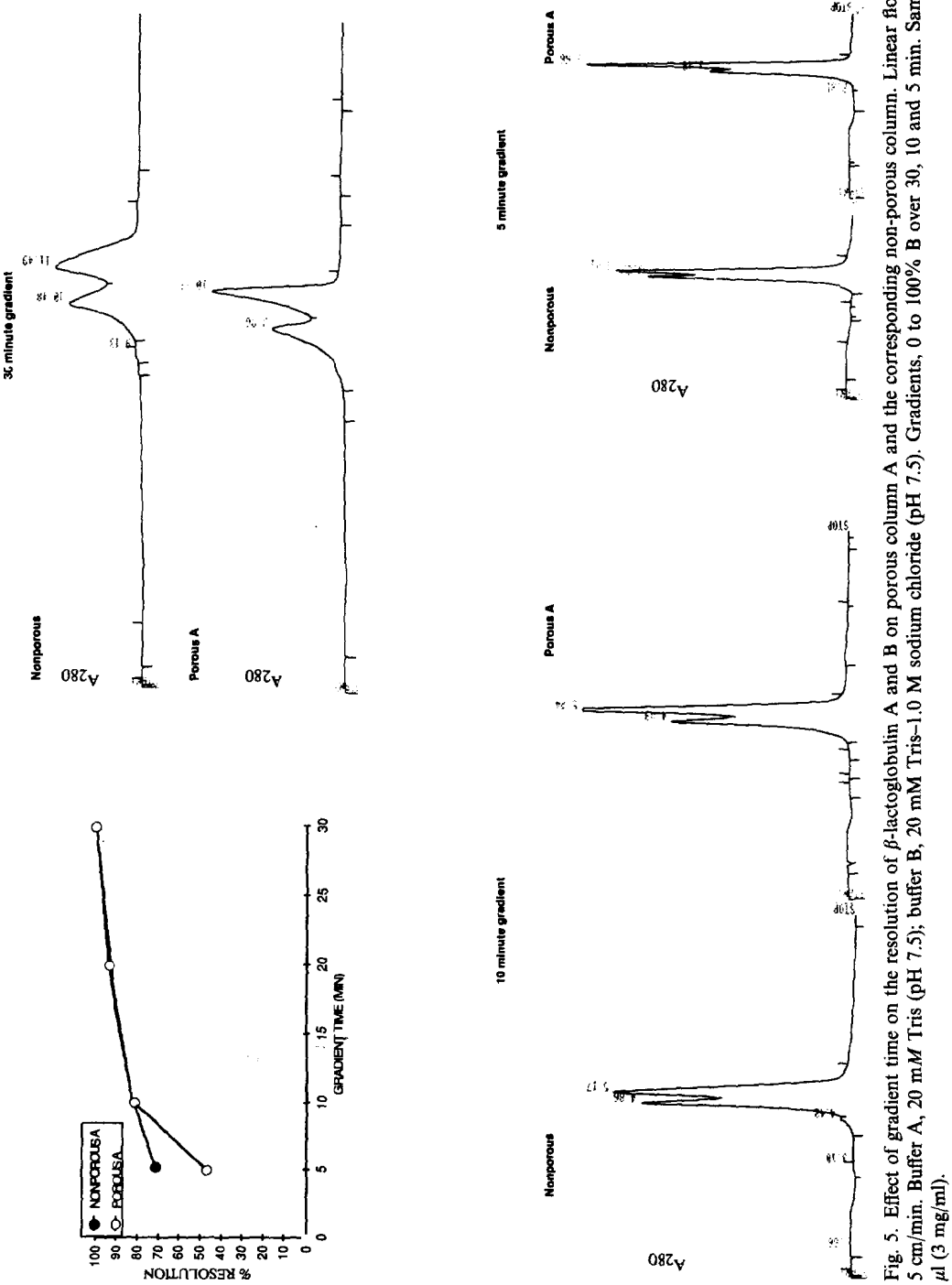

Fig. 5. Effect of gradient time on the resolution of β-lactoglobulin A and B on porous column A and the corresponding non-porous column. Linear flow-rate, 5 cm/min. Buffer A, 20 m*M* Tris (pH 7.5); buffer B, 20 mM Tris–1.0 M sodium chloride (pH 7.5). Gradients, 0 to 100% B over 30, 10 and 5 min. Sample, 20 μl (3 mg/ml).

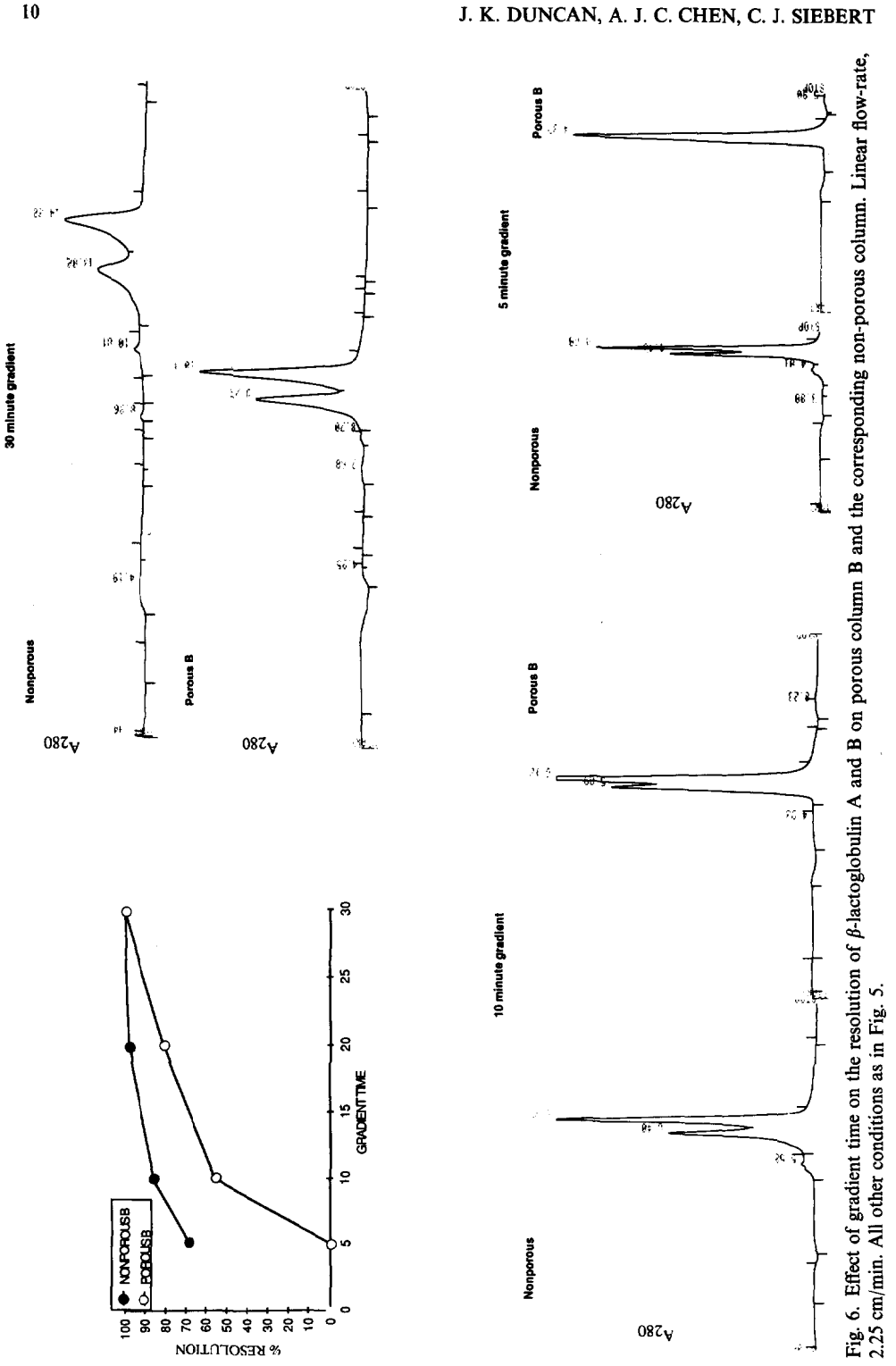

Fig. 6. Effect of gradient time on the resolution of β-lactoglobulin A and B on porous column B and the corresponding non-porous column. Linear flow-rate, 2.25 cm/min. All other conditions as in Fig. 5.

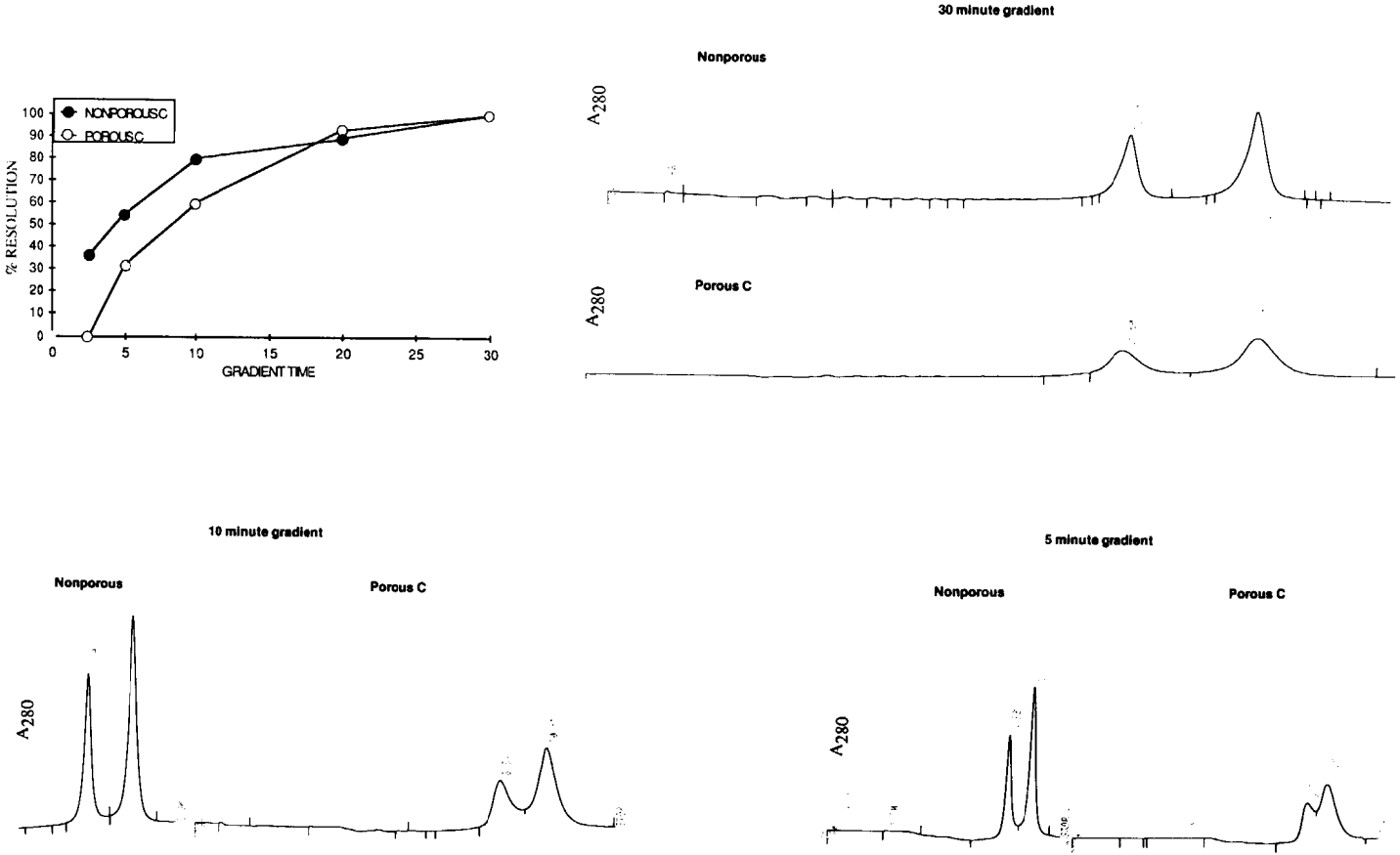

Fig. 7. Effect of gradient time on the resolution of β-lactoglobulin A and B on porous column C and the corresponding non-porous column. Linear flow-rate, 6 cm/min. Buffer A, 20 m*M* Tris, (pH 6.0); buffer B, 20 m*M* Tris–1.0 *M* sodium acetate (pH 6.0). All other conditions as in Fig. 5.

porous materials essentially eliminates intraparticulate mass transfer leading to more efficient columns. This is especially true with large proteins, as the intraparticulate diffusion with these molecules is much slower. All of the porous columns tested showed a progressive loss of efficiency with increasingly large proteins over the range of flow-rates measured. In contrast, the non-porous columns were equally efficient with proteins ranging from 35 to 470 kilodaltons over the same flow-rate ranges.

The size exclusion effects on porous columns contribute to loss of efficiency, especially for larger proteins. As the solute moves through the column it diffuses freely into and out of the larger pores and is excluded from the smaller pores. The pores that include the protein range from those providing a "tight fit" for the molecule to those allowing relatively free diffusion. This results in a differential rate of intraparticulate mass transfer and increases band broadening.

In ion-exchange separations of proteins, as gradient steepness increases the bands broaden progressively more on porous columns that on non-porous columns. The ability of non-porous columns to retain efficiency with increased gradient steepness appears to be inversely related to the apparent pore volumes indicated in Fig. 4. This phenomenon can be explained by the slow mass transfer of the solutes inside the pores. With the non-porous materials, gradient solvents are carried by the flow at or near the surface of the particles. The solutes are therefore desorbed and carried away by the flow as the gradient is being changed. With porous materials, the pores serve as mixing chambers for the solutes. As the gradient is being changed rapidly outside the pore, the desorbed solutes inside the pores cannot immediately be carried away by the flow, due to the slow intraparticulate mass transfer. As a result, the desorbed solutes mix with the solutes that desorb at a higher gradient concentration, leading to decreased resolution.

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