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Characterization of a novel stationary phase derived from a hydrophilic polystyrene-based resin for protein cation-exchange high-performance liquid chromatography☆

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Abstract

A new strong cation-exchange (SCX) HPLC stationary phase derivatized from a hydrophilic polystyrene–divinylbenzene (PS–DVB)-based resin was characterized. We have evaluated the new stationary phase for its column efficiency, chemical and mechanical stability, protein retention behavior, loading capacity and non-specific adsorption features. A reduced plate height of 2.2 was obtained. The high column efficiency enabled high-resolution separations of proteins in a short time with relatively low salt concentration in the elution buffer. Prolonged exposure to large volume of strong acid and strong base did not change the chromatographic properties of the column. The dynamic protein loading capacity of the stationary phase was around 50 mg per ml column volume. Some noticeable differences were observed between this SCX column and other commercially available columns.

Keywords: Stationary phases, LC; Polystyrene–divinylbenzene resins

1. Introduction

Ion-exchange chromatography (IEC) is of increasing interest to separation scientists for protein chromatography. Because of the nature of the mechanism by which separations are carried out [1], ion-exchange chromatography provides a more gentle environment for solutes than reversed-phase chromatography. This can be particularly important for biologically active compounds which need to maintain their native forms. Since IEC can be carried out near physiological conditions, it causes less damage to sensitive biological macromolecules, such as proteins. In addition, ion-exchange chromatography provides greater flexibility for optimizing the separation during method development [2,3]. Furthermore, aqueous eluents (or mobile phases) used in ion-exchange chromatography can be disposed of more easily than most of the organic solvents used in reversed-phase chromatography. This can be important in reducing purification costs and environmental hazards.

As in all modes of chromatography, ion-exchange columns packed with good packing ma-

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terials are essential for the separation. In general, ideal ion-exchange packing materials for HPLC are mechanically stable, chemically inert, hydrophilic with no irreversible adsorption, and highly efficient.

Silica-based packing materials are most widely used in high-performance liquid chromatography (HPLC), because of their mechanical stability and a wide variety of derivatizations, as well as their relatively higher column efficiency. Unfortunately, silica-based supports also possess a series of drawbacks. One is their inherent low chemical stability at a pH above 8 and below 2 [4–6]. This drawback can cause dissolution of the silica support and loss of the bonded phase. It was reported [7] that the chemical stability at low pH was improved with proprietarily manufactured silica reversed-phase columns which can be rinsed with 0.1 *M* nitric acid (pH 1) overnight without damaging the column. In another report [8], introduction of a sterically-protected bonded phase increased the chemical stability of silica to some extent at low pH for reversed-phase chromatography. In reversed-phase chromatography, the silica surface is bonded with a layer of a hydrophobic ligand, which shields the surface from the hydrolysis to a certain extent. This reduces the degree of dissolution of the silica, particularly in the pH range 2–8. However, this chemical stability problem is more pronounced in ion-exchange chromatography, even at a pH between 2 and 8. In ion-exchange chromatography, a hydrophilic surface is required for protein elution. The hydrophilic surface makes it easier for water molecules to attack the silica surface. Silica dissolves in water at room temperature to the extent of 100 to 150 ppm between pH 2 and 9 [9]. The salts added in the mobile phases of ion-exchange chromatography can also accelerate the dissolution of silica.

To overcome the stability problem of silica, researchers have turned their attention to polymeric supports [10–15]. Polystyrene–divinylbenzene (PS–DVB)-based supports have been studied and utilized the most, due to their chemical stability in both strong base and acid [16–18]. However, their high hydrophobicity and poor efficiency have inhibited their use for protein separations in IEC. Recently, HPLC ion-exchange stationary phases have been developed [2,19,20] from the PS–DVB matrix by applying a hydrophilic coating on the surface. However, the column efficiency could not match that of most silica-based columns.

A new technique [21] has been developed to treat the surface of PS–DVB beads. With the new technique, we can derive an HPLC matrix from PS–DVB beads, which can then be derivatized into different ion-exchange materials. Columns packed with these materials can achieve column efficiencies comparable to those of silica-based columns, while retaining their chemical stability. This paper presents some results from the characterization and application of a strong cation-exchange (SCX) material derived from the matrix.

2. Experimental

2.1. Apparatus

Chromatography was performed with an assembled high-performance liquid chromatographic (HPLC) system, unless otherwise mentioned. The system consists of two ConstaMetric-III metering pumps (LDC/Milton Roy, Riviera Beach, FL, USA), a dynamic on-line solvent mixer with a volume of 2.4 ml (Beckman Instruments, Fullerton, CA, USA), a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA), a SpectoMonitor D variable-wavelength UV detector (LDC/Milton Roy), an IBM-compatible PC computer (Equus/Systems, Hesperia, CA, USA) with an OkiData printer (Oki America, Mount Laurel, NJ, USA) and a strip chart recorder (Kipp and Zonen, Veendam, Netherlands). The system is operated using a computer program developed in-house. Pore parameters of the packing materials were measured using an Autoscan 60 porosimeter (Quantachrome Corp., Syosset, NY, USA). An Elzone 280pc particle size analyzer (Particle Data, Elmhurst, IL, USA) was used for particle size analysis.

2.2. Materials

All proteins were purchased from Sigma (St. Louis, MO, USA), except myoglobin which was from Calbiochem (La Jolla, CA, USA). Cytosine was obtained from Calbiochem. The reagent grade tris(hydroxymethyl)aminomethane (Tris) was purchased from Sigma under the trade name of TRIZMA BASE. Sodium chloride (99 + %) was from Aldrich (Milwaukee, WI, USA). Hydrochloric acid (HCl) was from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained in-house by treating the tap water with a carbon filter, reversed-osmosis, a mixed bed of ion exchangers, and a 0.45-μm filter. All proteins and chemicals were used as received.

2.3. Columns

Highly crosslinked polystyrene–divinylbenzene (PS–DVB) beads were obtained by suspension co-polymerization. By adjusting the polymerization conditions, we can control the mean pore size of the PS–DVB bead from 80 to 4000 Å at a mean particle size of 2 μm and larger. A hydrophilic layer was then linked [21] to the hydrophobic surface of the PS–DVB bead through chemical modification. As a result, a new matrix with PS–DVB backbone and a neutral hydrophilic surface was developed. This hydrophilic PS–DVB matrix was further derivatized into a strong cation exchanger (SCX) by introducing sulphonic acid functional groups. The functional groups were chemically attached to the PS–DVB matrix with stable covalent bonds. No labile chemical bonds were employed in the entire bead. Columns were packed with distilled water using a slurry packing technique. The strong cation-exchange columns (Vydac 400VHP5 and 400VHP8 for particles of 5 μm and 8 μm, respectively, and mean pore size of 900 Å) were from The Separations Group (Hesperia, CA, USA). Other commercially available cation-exchange columns (Mono-S HR 5/5, particle size 10 μm; TSKgel SP-5PW, particle size 10 μm, and PL-SCX, particle size 8 μm) were purchased from Pharmacia Biotech (Uppsala, Sweden), TosoHaas (Montgomeryville, PA, USA) and Polymer Laboratories (Amherst, MA, USA), respectively.

2.4. Mass recovery test

The mass recovery was measured by comparing the peak area (A_p) of proteins with and without the testing column, a 5.0 × 0.46 cm I.D. (column 2). The protein peaks were monitored using a L-3000 photodiode array detector at 254 nm (Hitachi, Tokyo, Japan). The detection data was collected and integrated using a 900 Series data interface and PC integrator (Nelson Analytical, Cupertino, CA, USA). Without the testing column connected in the system, it was difficult to precisely integrate the peak area because of the fast, narrow, out-of-scale and asymmetrically eluted peak. In order to overcome this difficulty, a small 2.0 × 0.46 cm I.D. column (column 1) packed with the same material was used. The small column remained on the system when the testing column (column 2) was connected and disconnected. The protein peak area (A_p) from the small column (column 1) was compared with that obtained using both columns (column 1 and column 2), as shown below:

$$\text{Mass recovery} = \frac{A_{\text{p(col.1+col.2)}}}{A_{\text{p(col.1)}}} \cdot 100$$

The proteins were eluted with a gradient of 0 to 0.5 *M* NaCl in 10 m*M* Tris-HCl buffer, pH 7.3, at 1 ml/min flow-rate.

3. Results and discussion

3.1. Column efficiency

As discussed above, column efficiency is an important criterion for HPLC columns, particularly for columns packed with polymer-based packing materials. It has been traditionally understood [22] that polymer-based HPLC packing materials inherently have lower column efficiency. This is mostly attributed to the higher degree of shrinking and swelling, and to the existence of micropores and possibly closed

pores. In contrast to the low efficiency observed with polymer-based packing materials, we have obtained highly efficient columns with the new SCX materials (400VHP) derived from the hydrophilic PS–DVB based beads. Fig. 1 shows a plot of the plate height vs. flow-rate (H–u curve) obtained for a column of 5.0 × 0.75 cm I.D. packed with the SCX packing material (mean pore size of 900 Å, range 300–3000 Å, particle size of 5 μm). Cytosine ($k' = 3.2$) was used as the probe solute. The column efficiency was measured using the peak width at half peak height ($W_{1/2}$) method. The column plate number (N) was calculated from the equation: $N = 5.54(t_R / W_{1/2})^2$, where t_R is the retention time. The peak asymmetry factor at 10% of the peak height was 1.21. It can be seen that the optimum column efficiency is found at flow-rate of 0.8 ml/min. The minimum plate height is 11 μm, which is equivalent to a reduced plate height of 2.2 [23], and a column efficiency of more than 90 000 plates per meter. Very often, we can pack columns up to 100 000 plates per meter. This result is comparable to that found for "good" porous silica-based columns [24] in which a reduced plate height of 2.25 can be obtained at the optimum reduced velocity. This is also true when comparing silica-based reversed-phase packing materials [25].

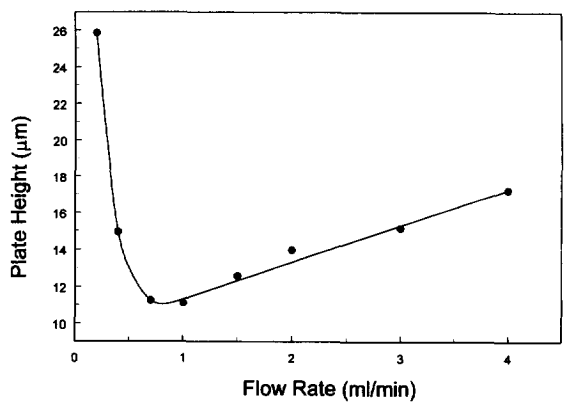

Fig. 1. Plate height vs. flow-rate (H–u) curve from the new SCX column. Column: 400VHP575 (5.0 × 0.75 cm I.D., 5 μm); mobile phase: 0.01 M $(NH_3)_2HPO_4$, pH 3 adjusted with H_3PO_4 (85%); test probe: 0.5 μl cytosine (0.1%); Temperature: ambient; detection: UV 254 nm.

To our knowledge, such a high column efficiency has not been reported previously on polymer-based porous packing materials designed for protein separations. The high column efficiency is also reflected in the chromatogram of standard proteins, shown in Fig. 2. These results demonstrate that the limitations on the column efficiency of polymer-based packing materials can be minimized.

3.2. Column compressibility

Column compressibility is another important feature for HPLC packing materials. This was tested using a column of 5.0 × 0.46 cm I.D. packed with the strong cation-exchange packing material of 7 μm particle size and 2000 Å pore size. The column back pressure was monitored with an IsoChrom pump (Spectra-Physics, San Jose, CA, USA). As shown in Fig. 3, a good linear relationship was obtained for pressures up to 24.13 MPa (3500 p.s.i.) and flow-rates up to 5 ml/min. This indicates that the packing material is rigid and stable at pressures up to at least 24.13 MPa (3500 p.s.i.), which is well above the operat-

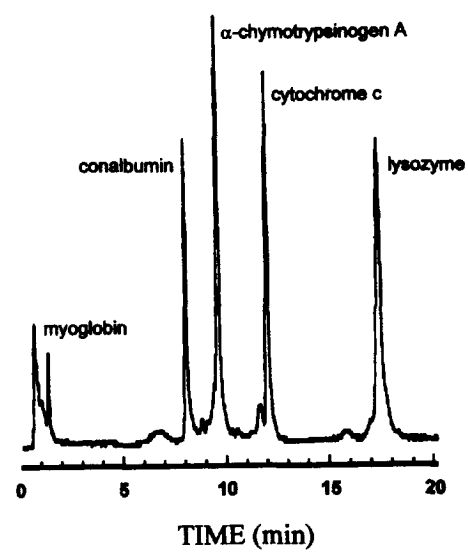

Fig. 2. An example of high-efficiency separation of five proteins. Column: 400VHP575 (5.0 × 0.75 cm I.D., 5 μm); mobile phase: A, 0.01 M tris-HCl pH 7.34; B, 0.5 M NaCl in "A"; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; detection: UV 254 nm.

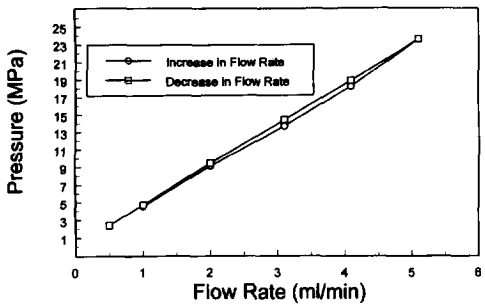

Fig. 3. Relationship between the column back pressure and flow-rate. Column: 5.0 × 0.46 cm I.D. packed with 400VHP (particle: 7 μm, pore: 2000 Å); mobile phase: distilled water. The column back pressure was monitored with an IsoChrom pump (Spectra-Physics).

ing range for almost all HPLC applications. However, the column back pressure is still higher than that of columns having the same dimensions packed with most silica-based packing materials with the same particle size.

3.3. Column stability

pH stability

It has long been realized that chemical stability is the major drawback of silica-based packing materials [4]. Bare PS–DVB beads show excellent chemical stability. However, does the modified PS–DVB still possess the same chemical stability? We sought to answer this question by exposing the column to extreme conditions. The separation of standard proteins was used to monitor possible changes before, during and after the treatments. The column was sequentially washed with 1000 ml of 1 *M* NaOH overnight at 1 ml/min and 1100 ml of 0.5 *M* H_2SO_4 over 24 h. Fig. 4 shows the chromatograms of the standard proteins separated on a freshly packed column, after washing the column with the base solution and after further washing the column with the acid solution. It can be seen that the three chromatograms are almost identical. This indicates that the column was not affected by the strong acid and strong base treatments. The demonstrated chemical stability allows the column to be used from pH 0 to pH 14. Thus, the column can be cleaned using very harsh conditions for column sterilization and for removing column contamination from dirty samples or other sources.

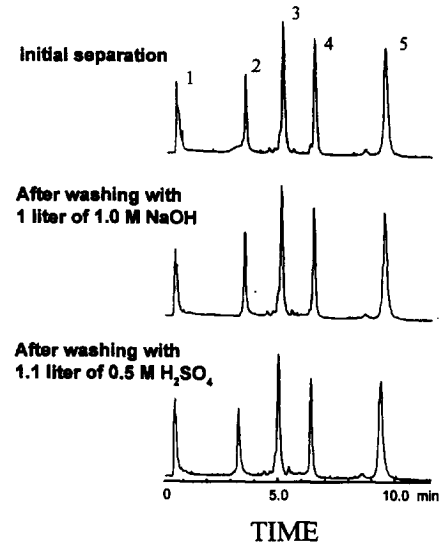

Fig. 4. Chemical stability test before and after treating the column with strong base and acid. Column: 5.0 × 0.46 cm I.D. packed with 400VHP5; mobile phase: A, 0.01 *M* tris-HCl, pH 7.34; B, 0.5 *M* NaCl in "A"; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; peaks: 1 = myoglobin, 2 = conalbumin, 3 = α-chymotrypsinogen A, 4 = cytochrome c, and 5 = lysozyme.

Frontal loading effect

In the column chromatography of proteins, one often encounters irreversible adsorption problems, particularly when a column has been overloaded with proteins [26]. The irreversibly adsorbed proteins cause loss of sample, change the chromatographic properties of the column and create ghost peaks. We examined the irreversible adsorption on the new cation-exchange column using a mixture of five protein standards. The chromatogram (see the upper chromatogram in Fig. 5) of the five protein mixture was first obtained on a fresh column. The column was then fully loaded (frontal loading) with lysozyme (1% solution in the "A" buffer as described in the caption of Fig. 5), followed by a gradient elution. This frontal loading and elution was repeated twice. The protein mixture was chromatographed (see the

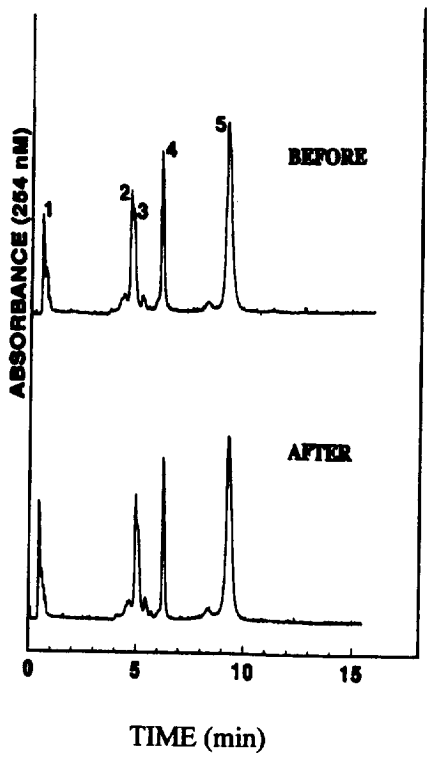

Fig. 5. Influence of protein frontal loading on column performance. The chromatograms were obtained before and after the column was used for lysozyme frontal loading twice. Column: 5.0 × 0.46 cm I.D. packed with 400VHP (particle: 7 μm, pore: 2000 Å); mobile phase: A, 0.01 *M* tris-HCl, pH 7.34; B, 0.5 *M* NaCl in "A"; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; peaks: 1 = myoglobin, 2 = ribonuclease A, 3 = α-chymotrypsinogen A, 4 = cytochrome c, and 5 = lysozyme.

lower chromatogram in Fig. 5) again on the column after the frontal loading. When comparing the two chromatograms in Fig. 5, only small differences can be seen. This implies that there is no significant irreversible adsorption of proteins on the new polystyrene–divinylbenzene-based cation-exchange column. Moreover, no ghost peaks were observed in a blank gradient run, performed after the frontal loading and the consecutive protein mixture chromatography.

3.4. Retention vs pH mapping

The high chemical stability of the packing material enables us to examine the retention

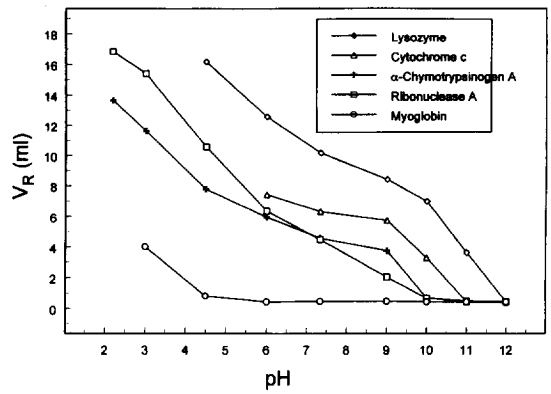

Fig. 6. pH vs. retention map of proteins on the 400VHP SCX column. Column: 5.0 × 0.46 cm I.D. (7 μm, 2000 Å); mobile phase: A, 0.01 *M* sodium phosphate buffer; B, 0.5 *M* NaCl in "A"; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; detection: UV 254 nm at 0.05 AUFS.

behavior of proteins over a very broad pH range. Fig. 6 shows retention vs. pH plots of some representative proteins over the pH range 2–12. The elution order of these proteins is directly related to their isoelectric points (p*I*). A protein with a higher p*I* is retained longer, as expected in cation-exchange mode. However, a few remarks can be made on this data. (i) The pH at which the protein starts to be retained is not always equal to the p*I* of the protein. This is consistent with the results reported by Kopaciewicz et al. [1], even though the results for some specific proteins (e.g. cytochrome c, myoglobin and lysozyme) are not identical. Table 1 shows that lysozyme, cytochrome c, ribonuclease A and α-chymotrypsinogen A start to be retained at a pH of 1–2 units above their p*I*s, while myoglobin was not retained until the pH dropped 2 units below its p*I*. This can be due to differences in the charge distribution on the protein surface, as explained by Kopaciewicz et al. [1]. Only the accessible surface charges contribute to the retention. (ii) The separation selectivity changes with the pH of the mobile phase. The most significant change is between ribonuclease A and α-chymotrypsinogen A which have similar p*I*s. The elution order switches at pH 7. This can be used as an advantage in selecting the optimized separation condition [3]. (iii) At a pH below 6,

Table 1
The p*I* of proteins and the pH at which proteins start to be retained

Protein	Source	M_r^a	p*I*[a]	pH of retaining
Myoglobin	Horse muscle	17 500	6.47–7.76	4.5
Ribonuclease A	Bovine	13 683	8.8	10.2
α-Chymotrypsinogen A	Bovine pancreas	25 000	8.8, 9.2, 9.6	10.2
Cytochrome c	Horse heart	12 200	9.0, 9.4	11
Lysozyme	Chicken egg	13 930	11	12

[a] Data from Refs. [1,28,29].

cytochrome c can not be eluted within a reasonable time. This behavior of cytochrome c at low pH was also described before [2].

3.5. Non-specific protein adsorption

We did not find non-specific protein adsorption on this new strong cation exchanger for the proteins used (and some other proteins not mentioned in this paper). Particularly hydrophobic interaction on this new material was investigated. Two representative tests are discussed below.

Protein recovery

Protein mass recovery was examined using five proteins. For testing conditions see Experimental section. The results are listed in Table 2. A recovery of more than 95% was obtained for most proteins, except for myoglobin. It was noted that myoglobin was eluted first with almost no retention. This may affect the accuracy of the measurement.

Influence of organic solvent on protein elution

Iso-propanol (IPA) was used as an additive in the mobile phases to detect any hydrophobic adsorption between the solutes and the packing material. If hydrophobic interactions exist, adding a few percent IPA to the mobile phase should reduce the retention significantly. Fig. 7 shows chromatograms obtained with 0%, 1% and 2% IPA added to both A and B eluting buffers.

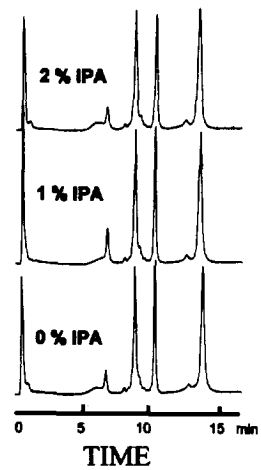

Fig. 7. Influence of organic solvent in the mobile phase on column performance. Column: 5.0 × 0.46 cm I.D. packed with 400VHP5; mobile phase: A, 0.01 *M* tris-HCl, pH 7.34; B, 0.5 *M* NaCl in "A"; iso-propanol (IPA) was added to both "A" and "B" with percentage as indicated in the figure; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; peaks: 1 = myoglobin, 2 = conalbumin, 3 = α-chymotrypsinogen A, 4 = cytochrome c, and 5 = lysozyme.

Table 2
Protein mass recovery

Protein	Myoglobin	Conalbumin	α-Chymotrypsinogen A	Cytochrome c	Lysozyme
Amount (μg)	6.25	62.5	50	18.75	37.5
Recovery (%)	87.1	106.7	99.9	99.9	96.9

For conditions see Experimental section.

Almost the same retention was observed for all proteins with and without IPA added. It has been reported that α-chymotrypsinogen A is strongly retained in hydrophobic interaction chromatography (HIC) at pH 7 [27]. Because of being closer to the isoelectric point (p*I*: 8.8), α-chymotrypsinogen A should be more hydrophobic at the pH of 7.34 used in the current experiment than at pH 7. The chromatograms in Fig. 7 show no change for this hydrophobic protein. This result indicates that the new modified surface is hydrophilic and shows no hydrophobic interaction with these bio-macromolecules.

3.6. Protein loading capacity

The dynamic protein loading capacity was measured by frontal loading using lysozyme at four different concentrations. The results are listed in Table 3. At 10 mg/ml concentration, a total loading capacity of 48.75 mg per ml column volume was obtained. If an amount of protein equivalent to 10% of the total capacity is injected, one can separate more than 10 mg of protein per run using a 5.0 × 0.75 cm I.D. column.

3.7. Comparison of the new column with other commercially available columns

The new strong cation-exchange column was compared with several commercially available strong cation-exchange columns using a group of standard proteins. Because of the differences in column dimensions (particularly in column diameter), the flow-rate was adjusted to give the same linear flow velocity for all the columns. To ensure the reliability of the comparison, we used the same protein mixture solution and the same elution buffers, and performed the experiments on the same day using fresh columns. The results are shown in Fig. 8. Under the conditions used, some differences were observed. Even though they are all strong cation-exchange columns

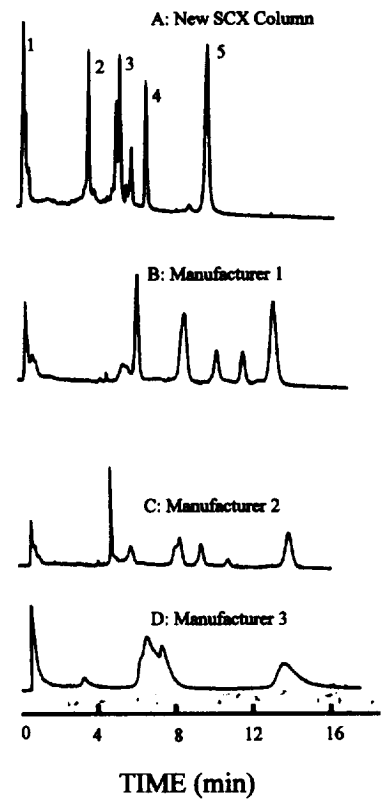

Fig. 8. Comparison of the new SCX column with other commercial columns. Columns: (A) SCX, 5.0 × 0.46 cm I.D. (400VHP5 series), (B) 5.0 × 0.5 cm I.D. (from manufacturer 1), (C) 7.5 × 0.75 cm I.D. (from manufacture 2), and (D) 5.0 × 0.5 cm I.D. (from manufacturer 3); mobile phase: A, 0.01 *M* tris-HCl, pH 7.34; B, 0.5 *M* NaCl in "A"; flow-rate: 1.0 ml/min (column A), 1.2 ml/min (column B), 2.66 ml/min (column C) and 1.2 ml/min (column D) with linear velocity kept the same; gradient 0–100% B in 20 min; peaks: 1 = myoglobin, 2 = conalbumin, 3 = α-chymotrypsinogen A, 4 = cytochrome c, and 5 = lysozyme.

Table 3
Total loading capacity of lysozyme at different concentration

Concentration (mg/ml)	20	10	5	2.5
Capacity (mg/ml of col. vol.)	56.6	48.75	45.1	41.5

Column: 5.0 × 0.46 cm I.D.; loading buffer (used to prepare the lysozyme solution): 0.01 *M* tris-HCl, pH 7.35; elution buffer: 0.5 *M* NaCl in the loading buffer; flow-rate: 1 ml/min.

designed for protein separations, the comparison reveals some differences in their resolution and selectivity characteristics. This reflects possible differences in the native properties of the matrix, in the derivatization chemistry to connect the ligand functional groups to the matrix and even in the ligand itself.

In most cases, the detection sensitivity was lower on columns with larger internal diameter (see chromatograms B, C and D), as one would expect, particularly when the linear velocity was kept the same. Fig. 8 clearly shows that narrower peak widths can be obtained on the new SCX column. This is particularly noticeable when comparing peaks eluting at the same time on columns from other manufacturers. The narrower peak widths result from the combination of the unique surface chemistry and the smaller particle size.

The tested proteins appeared to elute faster from the new SCX column than from the other columns at the elution conditions used. This means that on the new SCX column a lower salt concentration can be used for protein elution. For example, lysozyme elutes at about a 25–29% lower salt concentration on the new column than on the other columns. This difference cannot be directly related to the column loading capacity (data not shown). One may use this feature to reduce the analysis time without deteriorating the separation. More important is that a specific protein can be eluted at a lower salt concentration. This can be an advantage if removal of salt from the collected proteins is required for further analyses. On the other hand, if longer retention is required to resolve a protein mixture, some of the other columns may have certain separation advantages, particularly when slightly hydrophobic interaction is involved. One way to compensate for this difference is to lower the pH of the mobile phase used to elute the new SCX column. The pH should be lowered by 1.5 units, or more if possible, as shown in Fig. 6.

3.8. Influence of separation speed on the column

Fig. 9 shows a rapid separation of the protein mixture using the HPLC system described in the Experimental section without making any modification. Using a high flow-rate helped to reduce the effect of the system's dead volume originating mainly from the large volume mixer. Complete baseline separation of the five proteins was achieved in less than 3 min. This chromatogram indicates that only very small changes in performance are observed when very short columns are used for high-speed separations.

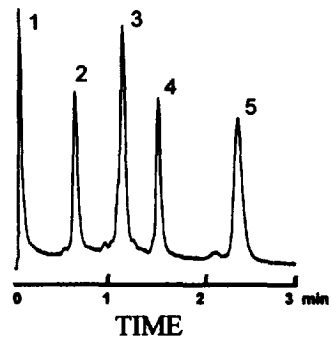

Fig. 9. Example of rapid protein separation. Column: 2.0 × 0.46 cm I.D. packed with 400VHP5; mobile phase: A, 0.01 *M* tris-HCl, pH 7.21; B, 0.5 *M* NaCl in "A"; flow-rate: 4 ml/min; gradient: 0–100% B in 5 min; sample identification as in Fig. 8.

4. Conclusion

A novel PS–DVB-based HPLC strong cation-exchange packing material was evaluated for its chromatographic properties. The columns packed with this new packing material exhibit both high chemical stability and high column efficiency. The column efficiency achieved on the polymer-based cation-exchange column is equivalent to that of silica-based packing materials. This demonstrates that the limitations of polymer-based packing materials in obtaining high column efficiency can be minimized. Using this new SCX column, protein separations can be performed with elution at lower salt concentrations than those used on other commercially available columns. Because of its hydrophilic nature, a high protein mass recovery could be obtained on these new SCX columns.

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