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CHROMATOGRAPHIC EVALUATION OF LARGE-PORE AND NON-POROUS POLYMERIC REVERSED PHASES

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SUMMARY

The polymeric reversed-phase packing material, PRP-1, a macroporous copolymer of styrene and divinylbenzene has been used for the liquid chromatographic analysis of proteins, peptides, oligonucleotides, pharmaceuticals and other biologically active materials. The PRP-1 phase has an average pore diameter of 75 Å which may limit its applicability to smaller molecules. Hence a complementary series of larger pore poly(styrene–divinylbenzene) packing materials has been prepared.

Chromatographic evaluation of 100-, 200- and 300-Å polymeric stationary phases showed identical small molecule selectivity through the series and similar sample load capacities. The 300-Å material showed the best resolution of protein samples during gradient elution. Its performance is compared to that of a non-porous PRP whose pore diameter may be considered infinite. Ghosting, recovery and column efficiency were also evaluated.

The chief benefit of the PRP packing is its hydrolytic stability from pH 1 to 13. Hydrolytic removal of protein contaminants from analytical PRP columns and packings was demonstrated.

INTRODUCTION

The polymeric reversed-phase packing materials PRP, specifically the porous poly(styrene–divinylbenzene) sorbents have been shown to be excellent alternatives to the alkyl-bonded silicas for reversed-phase high-performance liquid chromatography (HPLC) of small molecules. Their tolerance of extreme mobile-phase pH values allows selective chromatography of strong acids[1] and bases[2–4], enhanced detection sensitivity for protic samples[5,6], and exceptional mobile-phase selectivity[7,8]. An especially interesting example of mobile-phase selectivity was demonstrated by Sasagawa *et al.*[8] where tryptic digests of myglobin samples were chromatographed at pH 2 followed by rechromatography of the unresolved peaks at pH 9.6 to completely resolve the mixture.

The PRP-1 stationary phase used for the peptide separation has an average pore diameter of 75 Å[7]. Application of a PRP packing material to the separation of higher-molecular-weight peptides and proteins would require, according to the reports of others, a larger pore stationary phase[9–11] or a non-porous material[12]. Steric

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restrictions of reversed-phase packings for proteins are expected to be reduced upon increasing the average pore diameter from 100 to 300 Å and nearly eliminated when infinitely porous, namely non-porous materials are employed. This report contains the physical and chromatographic characterization of a series of PRP packings. The porous properties were determined by nitrogen adsorption and by gel chromatography. Small molecule capacity and selectivity was determined and the effect of average pore diameter on the chromatography of proteins was explored. Finally, the advantages of the chemical stability of a wide-pore PRP packing was demonstrated with a decontamination scheme for protein-fouled columns.

EXPERIMENTAL

Materials and equipment

The protein standards and the lyophilized bovine serum were obtained from Sigma (St. Louis, MO, U.S.A.). The polystyrene molecular weight standards were purchased from Goodyear Chemicals (Akron, OH, U.S.A.). Eluents were prepared from tetrahydrofuran (THF) and acetonitrile (Non-spectro, Burdick and Jackson, Muskegon, MI, U.S.A.), deionized water (Milli Q, Millipore, Bedford, MA, U.S.A.), and trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, U.S.A.).

The HPLC system consisted of two high pressure pumps (Model 510, Waters Chromatography Div., Millipore, Milford, MA, U.S.A.), a gradient controller and data system (Model 840, Waters), a sample injection valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.), and a variable-wavelength detector (Model 757, Kratos, Ramsey, NJ, U.S.A.). The poly(styrene–divinylbenzene) reversed phases (PRP-1, -2, -3 and -∞ used in these experiments were obtained from Hamilton (Reno, NV, U.S.A.). PRP-1 and PRP-3 are commercial products. PRP-2 and PRP-∞ are experimental materials and are included only for purposes of comparison. The PRP particles were packed into 150 × 4.1 mm columns except for those used in the gel chromatography experiments.

Sample preparation

Protein standards were prepared weekly at 2 mg/ml in 0.1% trifluoroacetic acid and stored at 4°C. The polystyrene samples were prepared at 1 mg/ml in tetrahydrofuran and stored at 4°C. The lyophilized bovine serum was reconstituted immediately before use with deionized water (4 g per 100 ml).

Analytical conditions

All gradient elution chromatography of proteins was run linearly from 0 to 60% B at 2 ml/min where solvent A is 0.1% (v/v) TFA in water (pH 2.0) and B is 0.1% (v/v) TFA in acetonitrile. The stainless-steel columns used were 150 × 4.1 mm I.D. The columns were packed as described earlier[7]. All PRP packing materials used consisted of 10-μm spherical particles except PRP-∞ which consisted of 5-μm particles.

The gel permeation chromatography (GPC) was performed with 1 ml/min THF and employed 100 × 7.0 mm columns. Retention time (volume) were duplicate or triplicate and agreed to within 1% of each other.

Nitrogen adsorption experiments were performed by Micromeritics (Norcross,

GA, U.S.A.). Elemental analyses were performed by Galbraith Labs. (Knoxville, TN, U.S.A.).

Chromatography of the three homologous series was performed at 1 ml/min at the stated mobile phase composition. Retention times were determined in duplicate using 10 μl of a 0.1% sample solution and 10 μl of a 0.01% solution and agreed to within 1%. Void volumes were determined for each mobile phase composition using sodium nitrate as the probe.

RESULTS AND DISCUSSION

Porous properties

Two methods were used to determine the pore size distribution of the PRPs: nitrogen adsorption and the GPC method of Warren and Bidlingmeyer[13]. The ideal pore size measurement method would be obtained under conditions identical to those experienced by the stationary phase during chromatography. Unfortunately, this method does not exist, but both methods used here show some similarities to those conditions. GPC was performed at room temperature, with the stationary phase wetted by an external liquid phase, providing dynamic measurements similar to the chromatographic experiment, but the stationary phase solute interaction is a very weak steric exclusion effect, while gradient elution of proteins involves strong stationary phase solute sorption and desorption. Pore measurements by nitrogen adsorption occur with probe stationary phase interaction similar to the chromatographic experiment, but are done at liquid nitrogen temperatures and the phase is not in contact with typical chromatographic eluents. Both methods were used to obtain an estimate of stationary phase porosity under chromatographic conditions.

Pore distribution by GPC involves the steric exclusion chromatography of solute molecules of different size. The relative retention (R) is plotted *versus* solute diameter ($\varnothing$). R is given by

$$R(\%) = \frac{V_e - V_{ex}}{V_{in} - V_{ex}} \cdot 100 \tag{1}$$

where V_e is the elution volume of the sample, V_{ex} is the excluded volume, *i.e.* the retention volume of a probe that cannot enter any of the pores, and V_{in} is the included volume or the mobile phase volume available to the smallest solute probe. Narrow-molecular-weight-distribution polystyrene standards served as the probes. The mobile phase was THF. Polystyrene of molecular weight (MW) $7 \cdot 10^6$ was employed to determine V_{ex}, and benzene was used to determine V_{in}. The solute probe diameter, $\varnothing$, was calculated by[13]

$$\varnothing = 0.62\ \mathrm{MW}^{0.59} \tag{2}$$

Pore distribution by nitrogen adsorption requires the measurement of the amount of nitrogen adsorbed *versus* relative pressure. These data are then transformed by the Brunauer-Emmett-Teller (BET) equations to incremental pore volume filled *versus* average pore diameter[14].

Nitrogen adsorption and GPC data have been plotted on the same coordinates

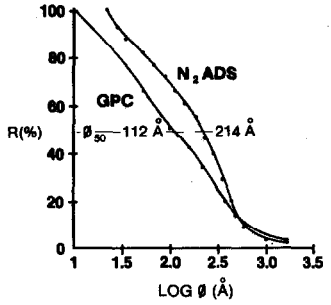


Fig. 1. Pore distribution of PRP-1 by GPC and nitrogen adsorption (N_2 ADS).

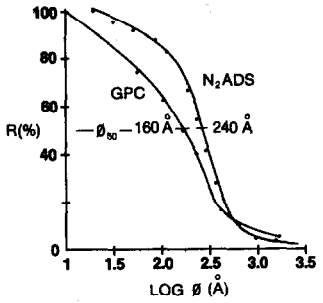


Fig. 2. Pore distribution of PRP-2 by GPC and nitrogen adsorption (N_2 ADS).

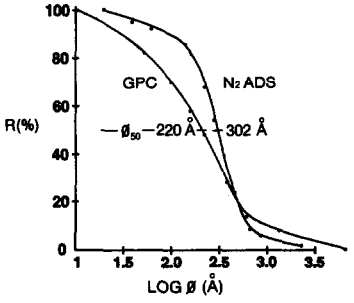


Fig. 3. Pore distribution of PRP-3 by GPC and nitrogen adsorption (N_2 ADS).

for the three PRP materials (Figs. 1–3). The GPC measurements generally produce lower average pore diameter values, $\varnothing_{50}$, than the nitrogen adsorption experiments. This may be because the THF mobile phase alters the pore structure relative to that existing during nitrogen adsorption or perhaps eqn. 2 incorrectly estimates solute probe diameter. Note that as the average PRP pore diameter increases, the distribution about this average decreases. While PRP-1 has a relatively broad distribution of pores, PRP-3 has a much narrower distribution centered around 200 or 300 Å. This is expected to allow efficient chromatography of large molecules.

Additional pore information is contained in Table I. The specific surface area

TABLE I

POROUS PROPERTIES OF THE PRP PACKING MATERIALS

Column	*BET surface area (m^2/g)*	*Pore volume (cm^3/g)*		*Pore diameter (Å)*		
		BET	*GPC*	*4V/A*	*GPC*	*BET*
PRP-1	396	0.81	0.78	82	110	220
PRP-2	154	0.45	0.45	119	160	240
PRP-3	104	0.40	0.38	155	220	300
PRP-∞	1.1 (calculated)	–	–	–	–	–

of the materials is seen to decrease as the pore diameter increases. The consequences of this will be explored later with some sample loading experiments and solute retention factor effects. Note that a fourth material, PRP-∞ has been added to the list. It is a totally non-porous poly(styrene–divinylbenzene). The average particle size was 5 μm. It is included to serve as a reference to the effect of porosity of any diameter on the chromatography of proteins.

Pore diameters are expressed in three ways in Table I, by GPC, nitrogen adsorption, and a third calculated average using pore volume and surface area data from the nitrogen adsorption experiments (4 V/A). This calculation assumes the pores to be cylindrical and their depth is four times the diameter. Depending upon which method is used, average pore diameter estimates may vary by as much as a factor of two. The pore volume measurements by nitrogen adsorption and GPC, agree remarkably well contrary to the pore distribution curves and suggests that PRP porosity is not altered upon going from a liquid nitrogen-filled pore to a THF-filled pore. This series of well characterized, porous PRP packings ranging in average pore diameter from 100 to 300 Å was then used to study the effects of porosity on the reversed-phase chromatography of small molecules and of proteins.

Small molecule chromatography

PRP-1, -2, and -3 were evaluated under isocratic mobile phase conditions using small solute probes to answer three questions:

(1) What is the effect of specific surface area on the maximum sample capacity?

(2) What is the effect of specific surface area on the solute retention factor, k'?

(3) Do these PRP packings of differing surface area and pore diameter and volume show the same selectivity?

The answers to these questions will aid in understanding the behavior of the PRP packings toward high-molecular-weight proteins during gradient elution.

In answer to the first question, the surface area seems to have no effect upon the maximum sample capacity except in the case of the very low surface area PRP-∞. A mixture of phenol, *p*-chlorophenol, and benzene was injected into separate 150 × 4.1 mm I.D. PRP columns. Acetonitrile–water (3:2, v/v) served as mobile phase. The maximum sample capacity was considered to be reached when the solute retention factor, k', changed by 10% of its original value. Sample capacities for PRP-1, -2 and -3 were 0.25 mg per gram of stationary phase for the phenols and 0.12 mg/g for benzene. The capacity of PRP-∞ was, however, less than could be detected at

TABLE II

CHROMATOGRAPHY OF HOMOLOGOUS SERIES

Homologous series	*Mobile phase*	*Slope (correlation coefficient)*		
		PRP-1	*PRP-2*	*PRP-3*
n-Alkylphenones	Acetonitrile–water (17:3)	0.135 (0.999)	0.129 (0.999)	0.136 (0.998)
n-Alkylcarboxylic acids	Acetonitrile–5.2 m*M* phosphoric acid, pH 2.45 (2:3)	0.31 (0.999)	0.32 (0.998)	0.36 (0.998)
n-Alkylamines	Acetonitrile–58 m*M* ammonia, pH 11.0 (1:3)	0.37 (0.999)	0.38 (0.999)	0.43 (0.999)

254 nm, less than 10 ng/g. The sample capacity of PRP-1, -2 and -3 for proteins is expected to be similar and very adequate, but, the applicability of PRP-∞ to any chromatography must at this point be considered to be doubtful.

Three homologous series were chromatographed on PRP-1, -2 and -3 in order to answer questions 2 and 3, *i.e.*, what are the effects of specific surface area and pore diameter on retention and selectivity? The *n*-alkylphenones, *n*-alkylamines and *n*-alkylcarboxylic acids were chromatographed according to the mobile phase conditions given in Table II and plots were made of log k' *versus* the number of carbons in the alkyl chain (Figs. 4–6).

The retention factor, k', by definition, equals n_s/n_m, where n_s is the amount of solute in the stationary phase and n_m is the amount in the mobile phase. These quantities can be expressed in terms of concentration of sample, [X], and volume V, of stationary, (s), and mobile (m) phases, respectively as $n_s = [X]_s V_s$ and $n_m = [X]_m V_m$. Therefore:

$$k' = \frac{[X]_s V_s}{[X]_m V_m} = \frac{K V_s}{V_m} \tag{3}$$

where K is the equilibrium distribution constant of X between stationary and mobile phases[15]. From eqn. 3, k' is proportional to the volume of the stationary phase, V_s. The value of V_m was nearly constant for PRP-1, -2 and -3, *i.e.*, 1.36, 1.32 and 1.50 ml, respectively. V_s is assumed to be the volume of the adsorbed layer at the stationary phase surface and should be proportional to the specific surface area. Thus, k' should be proportional to the specific surface area. This is not the case. While the surface area of PRP-1 is approximately four times that of PRP-3, retention, k' only differs by a factor of 3 for the acid series, 2.8 for the amine series and 1.5 for the alkylphenone series. Perhaps the assumption that V_s is proportional to surface area is incorrect. The general trend of increased retention with greater stationary phase surface area does however hold. Also, the methylene group selectivity of the PRPs is the same as evidenced by the slopes of the lines being equal in each homologous series.

The porous properties and reasonably predictable chromatographic properties of the PRP packing materials were explored. Their application to gradient elution chromatography of proteins will now be demonstrated and discussed.

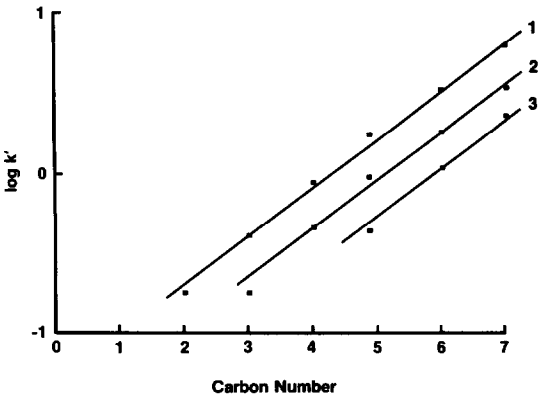


Fig. 4. Effect of chain length on retention of *n*-alkyl carboxylic acids. Curves: 1 = PRP-1, 2 = PRP-2, 3 = PRP-3.

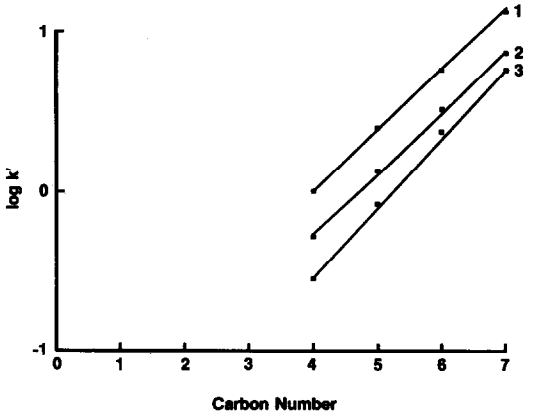


Fig. 5. Effect of chain length on retention of *n*-alkylamines. Curve identification as in Fig. 4.

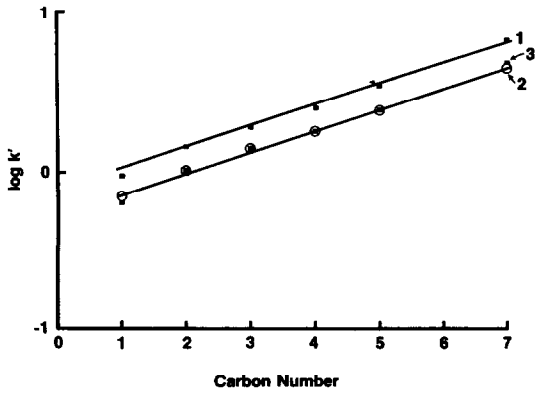


Fig. 6. Effect of chain length on retention of *n*-alkylphenones. Curve identification as in Fig. 4.

TABLE III

REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS

Columns, 150 × 4.1 mm I.D.; gradient: 0 to 60% B in 30 min at 2 ml/min; eluent A = 0.1% TFA in H_2O, pH 2.0; eluent B = 0.07% TFA in acetonitrile.

Protein	*MW*	*Total elution volume (ml)*			
		PRP-1	*PRP-2*	*PRP-3*	*PRP-∞*
Ribonuclease A	13 700	34.0	32.7	32.3	30.2
Cytochrome *c*	12 400	40.2	37.7	37.4	36.8
Trypsin	23 000	42.0	40.3	40.3	38.8
Lysozyme	14 000	42.3	40.8	40.9	38.5
Transferrin	80 000	43.5	41.9	42.3	41.6
BSA	67 000	45.5	43.5	44.2	42.9
Myoglobin	16 900	49*	46*	45*	44
Ovalbumin	43 000	54.6	53.0	53.3	52.0

* Peak appeared as doublet.

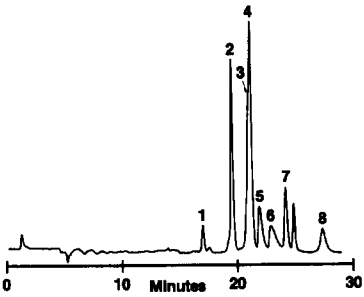
Chromatography of proteins

Eight proteins (MW 12 000–80 000) and ranging in hydrophobicity from ribonuclease A to ovalbumin were chromatographed at 2 ml/min from 0 to 60% B in 30 min, where solvent A was 0.1% TFA in water (pH 2) and solvent B was 0.1% TFA in acetonitrile. The retention times of the proteins on 150 × 4.1 mm columns of PRP-1, -2, -3 and -∞ are listed in Table III.

Small solute molecule behavior of the PRPs predicts that as surface area increases so does retention. This was generally found to hold true for the proteins. PRP-1 with the highest surface area generally shows the greater retention while PRP-∞ with a nominal surface area of 1.1 m^2/g showed the least retention. The effect is of course greatly attenuated in the gradient elution mode. There are three notable exceptions to this otherwise excellent correlation between surface area and retention. The three proteins with the highest molecular weight, ovalbumin, bovine serum albumin (BSA) and transferrin showed greater retention on PRP-3 as compared to the higher surface area PRP-2. Most likely steric exclusion phenomena are showing their effect. The larger average pore diameter of the PRP-3 phase allows for greater solute stationary phase interaction than PRP-2, the smaller pores of which probably partially exclude the larger proteins. Figs. 7–10 clearly show the greater ability of the larger pore PRP-3 to separate transferrin and BSA as well as some smaller proteins trypsin and lysozyme.

The non-porous PRP-∞, under gradient elution conditions had sufficient capacity to separate the mixture, but resolution was inferior to the porous 300-Å stationary phase due to differences in efficiency of the two packing materials.

The PRP columns were further evaluated for sample capacity, protein recovery and ghosting, the phenomenon where proteins from an earlier run are eluted by subsequent gradients. Sample capacity, *i.e.* the point at which increased sample load caused change in retention by 10%, occurred for each protein at 1 mg per g stationary phase for PRP-1, -2 and -3 and at 0.1 mg/g for PRP-∞. Small molecule chromatography supports this.



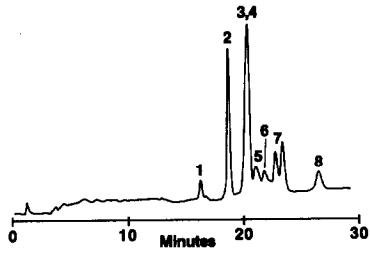
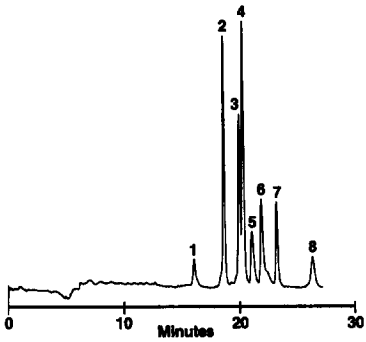


Fig. 7. Separation of proteins on a 150 × 4.1 mm PRP-1 column. Peaks: 1 = ribonuclease A, 2 = cytochrome *c*, 3 = trypsin, 4 = lysozyme, 5 = transferrin, 6 = bovine serum albumin, 7 = myoglobin, 8 = ovalbumin. Eluent described in Experimental.

Fig. 8. Same as Fig. 7, but with PRP-2 column.



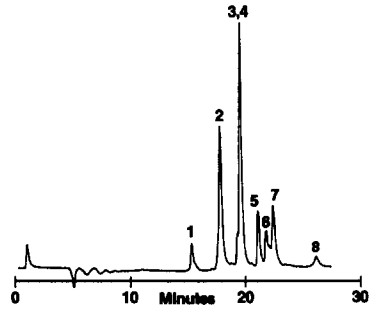


Fig. 9. Same as Fig. 7, but with PRP-3 column.

Fig. 10. Same as Fig. 7, but with PRP-∞ column.

Ghosting and sample recovery complemented one another, *i.e.* when recovery was poor, ghosting was high. Recovery was measured by comparing peak areas of single protein sample injections with and without the column in place. Ghosting was measured by comparing individual peak areas of an injected mixture to that from the chromatogram obtained from subsequent gradient elution without injection. Ghosting of PRP-1, -2 and -3 amounted to 1–2% and recoveries were all greater than 90% except when it fell to 80% for the small pore, PRP-1, large protein, BSA, case. PRP-∞ showed good recovery and little ghosting for some proteins, but, with others, notably trypsin and ovalbumin, recovery was only around 60% and ghosting was 10–60%. Care was taken in all recovery and ghosting experiments to work below the sample capacity level.

The porous and chromatographic properties of a series of PRP packings have been determined and shown, especially in the case of PRP-3, to be excellent alternatives to bonded-phase silicas for the reversed-phase chromatography of proteins. Their hydrolytic stability in contrast to the silicas should prolong column life and

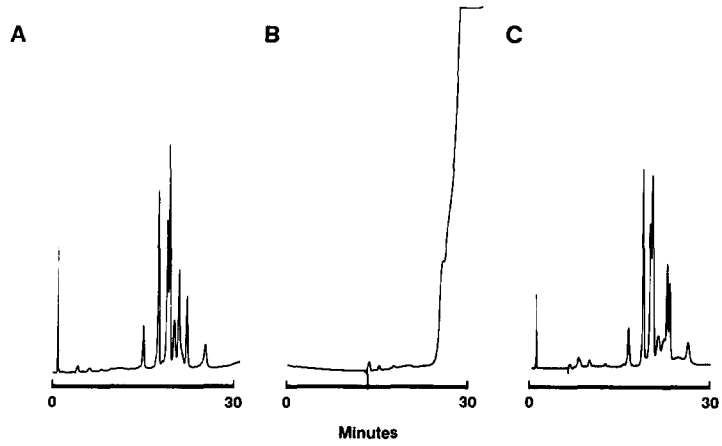


Fig. 11. PRP-3 clean-up. See text for description: (A) Separation of proteins before cleanup, for conditions see Fig. 9. (B) Fourth gradient elution after addition of 2 ml bovine serum into column. (C) Separation of proteins, after hydrochloric acid wash, see Fig. 9 for conditions.

assure indefinite stationary phase life. To test this, 2 ml of bovine serum was pumped into a 150 × 4.1 mm PRP-3 column at which point break-through occurred. Washing the column four times with the standard gradient profile did not adquately remove the contaminants (Fig. 11). A 1:1 mixture of 12 *M* hydrochloric acid and 0.1% TFA in acetonitrile was loaded into a 2-ml injection loop and injected into the column to effect protein hydrolysis and column decontamination. After an 8-h exposure, the gradient was again applied and 20 ml THF was pumped through the column. The result is shown in Fig. 11. The base line is clean and resolution is nearly the same as before except for a small loss between trypsin and lysozyme. Retention times are all within 2% of original values except for myoglobin which appeared as a doublet in all previous chromatograms and now appeared as a less well resolved doublet. Elemental analysis of the PRP-3 packing further confirmed the absence of any residual proteinaceous contaminants (Table IV). This hydrochloric acid wash procedure has been performed as many as five times to the same column with no ill effect.

TABLE IV

ELEMENTAL ANALYSIS OF PRP-3

Element	*Weight-%*	
	*Before**	*After***
C	90.27	90.01
H	8.27	8.22
N	0.013	0.029

* This sample of PRP-3 had never been packed into a column (unused).

** The entire contents of the column described in the test were vacuum dried and submitted for analysis.

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