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HIGH-SPEED STERIC EXCLUSION CHROMATOGRAPHY OF BIOPOLYMERS*

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SUMMARY

Biopolymer separations were studied on Micropak TSK type SW columns, which contain an aqueous compatible steric exclusion support. Columns of two different pore sizes, designated 2000SW and 3000SW, were compared for separation of proteins and nucleic acids covering a molecular weight range of 13,500 to 340,000 and the effect of molecular shape and denaturation upon elution volume was investigated. Use of high-speed steric exclusion chromatography as a prefractionation step prior to ion-exchange chromatography in biopolymer purification schemes is discussed.

INTRODUCTION

The essential characteristics of steric exclusion supports for use in biopolymer separations are:

(1) A predominantly hydrophilic character. Hydrophobic interactions can irreversibly adsorb and denature proteins.

(2) A pore size sufficient to allow permeation by macromolecules. Most protein work requires 200–1500 Å pore size supports. Permeation by viruses and nucleic acids can require larger pore sizes (see Table I). As a general rule, the support pore size should be several times that of the major axis of the macromolecule.

(3) An inert surface, to avoid ion exchange adsorption interactions with the macromolecules.

(4) Mechanical strength.

(5) Geometric insensitivity to changes in mobile phase pH and ionic strength.

(6) Stability over a wide pH range.

Classical steric exclusion separations of biopolymers have utilized relatively soft, hydrophilic gels such as the synthetic polyacrylamides (*e.g.*, Bio-Gel P) and the

* *Editor's note:* A similar study was submitted on April 3rd, 1979: S. Rokushika, T. Ohkawa and H. Hatano, *J. Chromatogr.*, 176 (1979) 456–461.

TABLE I

MOLECULAR WEIGHT AND MAJOR AXIS DIMENSIONS OF BIOPOLYMERS

Protein	*Major axis (Å)*	*Molecular weight*
Blood proteins		
Fibrinogen	700	340.000
γ-Globulin	235	160.000
Albumin	150	69.000
Hemoglobin	57	68.000
α-Bence Jones protein	43	35.000
Lipoproteins		
α-Lipoprotein	300	200.000
β-Lipoprotein	185	1.300.000
Enzymes		
Pepsin	84	35.000
Lysozyme	60	14.100
Ribonuclease	—	12.700
Viruses		
Bushy stunt virus	250	7.600.000
Tobacco mosaic virus	2700	40.000.000

polysaccharide dextrans (*e.g.*, Sephadex) and agaroses (*e.g.*, Sepharose, Bio-Gel A). Polystyrene–divinylbenzene resins have been avoided due to irreversible adsorption and denaturation effects of the hydrophobic aromatic matrix and to the relatively low pore sizes of these supports ($\leqslant 50$ Å).

The polyacrylamide and polysaccharide gels are hydrophilic and can be prepared in large pore sizes. Polyacrylamide and dextran supports have been used for separation of biopolymers of mol.wt. up to 10^6. Agarose has been used to separate viruses and nucleic acids of mol.wt. up to $1.5 \cdot 10^8$. The extremely high exclusion limits of the agarose supports (Table II) has allowed separation of subcellular particles[1].

Unfortunately, these gels are characterized by a relatively low compressive strength and must be operated at low pressures and flow velocities. Thus, equilibration separation and washing times are long, and sample throughput is low. Cross-linked agarose, the most mechanically stable of these gels, is operated at flow velocities of 0.002–0.02 cm/sec. This is an order of magnitude slower than typical flow velocities employed in high-speed liquid chromatography (HSLC).

The need for a rigid, microparticulate support for biopolymer HSLC has long been evident. Controlled-pore glasses (CPGs) developed by Haller[2] in 1965 and available[3] in pore sizes from 40 to 1500 Å and particle diameters of 5–10 μm, have been used for steric exclusion of biopolymers of mol.wt. up to 10^6. However, both controlled pore glasses and silica gel surfaces are characterized by high densities of highly polar, weakly acidic silanol (Si–OH) groups. Silanols can behave as cation exchange sites and result in adsorption and denaturation of biopolymers[4,5], especially proteins with isoelectric points above pH 7.5. This problem has seriously inhibited the use of uncoated CPG or silica gel for biopolymer HSLC.

Coating of CPG and silica gel supports with polyethylene glycol (PEG) of mol.wt. $\approx 10^4$–10^5 significantly reduces these adsorption effects[5–7]. Although PEG-coated CPG has been used successfully in the separation of high-molecular-weight

TABLE II

CHARACTERISTICS OF SUPPORTS FOR STERIC EXCLUSION CHROMATOGRAPHY OF BIOPOLYMERS

Name	*Support*	*pH Range*	*Available particle size (μm)*	*Available pore size (Å)*	*Corresponding exclusion limits*	*Calibration standard*
Bio-Gel P	Cross-linked polyacrylamide	2–9	10–37		2500–5000	Peptides
			37–75	—	17,000	Proteins
			75–150	—	50,000–400,000	Proteins
Sephadex	Cross-linked dextran	2–12	40–120	—	700–800,000	Peptides, proteins
Bio-Gel A	Agarose	4–13	37–75	—	500,000–150,000,000	Dextrans
			75–150	—	50,000–150,000,000	Dextrans
CorningCPG	Controlled-pore glass	2–8	5–10	40 thru 1500	8000–1,000,000	Dextrans
LiChrospher	Spherical silica glass	2–8	10	100 thru 4000	80,000–8,000,000	Poly-styrenes
TSK-SW	Spherical, rigid gel, hydrophilic stationary phase	3–8	10	—	20,000 and 150,000	Dextrans
			13	—	600,000	Dextrans

viruses[6], the coating process is a reversible one and the PEG phase elutes under continuous use[7]. In addition, adsorption effects are still observed for certain proteins[6].

The coating "bleed" problem can be avoided by covalently bonding an inert hydrophilic phase onto CPG or silica gel supports. Regnier and co-workers bonded a "diol" phase (Fig. 1) onto both CPG[8] and micropaticulate silica gel[9], obtaining HSLC exclusion bonded phases with negligible adsorption of biopolymers. These phases are often called "glycophases". Regnier has also bonded polydextran onto silica through an alkylamine intermediary. However, enzyme recoveries from the dextran–silica supports were significantly lower than those obtained from a diol–silica support.

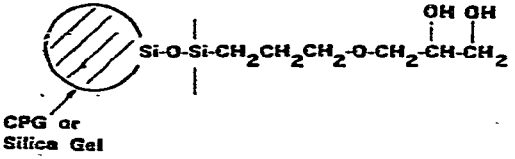

Fig. 1. "Diol" HSLC bonded exclusion phase for biopolymers.

Tayot *et al.*[11] succeeded in preparing "bleed-less" ion exchange supports for biopolymer HSLC by impregnating silica gel with cross-linked DEAE-dextran polymers. This technique may also be promising for HSLC exclusion of biopolymers, incorporating the separation characteristics of a polysaccharide with the mechanical strength of a microparticulate silica gel.

Another type of HSLC exclusion support, TSK-SW gels, was recently developed by Toyo-Soda (Tokyo) Japan. The support —a rigid gel (silica gel) whose surface is

covered with hydroxyl groups— has a pH range of 3–8. Particle size is 10 μm and at a typical flow velocity of $\approx$ 0.05 cm/sec, efficiencies of 20,000–40,000 plates per meter are obtained. Toyo-Soda has demonstrated quantitative recovery of proteins and of enzyme activity from the supports. The exact structure of this support has not been published. Biopolymer separations were obtained on pre-packed Varian MicroPak TSK-2000 and 3000 SW columns (8 mm $\times$ 30 cm).

EXPERIMENTAL

Chromatography was performed on a Varian Model 5020 gradient HPLC system equipped with a Varichrom variable wavelength absorbance detector.

The following protein and transfer RNA standards were obtained from Sigma (St. Louis, Mo., U.S.A.) and solutions were prepared in the mobile phase buffer: cytochrome *c* from horse heart, Type VI; human fibrinogen, fraction I; pepsin from hog stomach mucosa; ovalbumin; bovine α-globulins, Cohn fraction IV; lactic dehydrogenase from rabbit muscle, Type II; bovine γ-globulins, Cohn fraction II; tyrosine specific transfer RNA and N-formylmethionine specific transfer RNA from *Escherichia coli*. Rabbit liver transfer RNA, prepared by the method of Anandaraj and Roe[12] (extraction with phenol in pH 4.5 acetate buffer followed by chromatography on DEAE-cellulose) was kindly provided by Dr. R. D. Kudrna. Human serum was filtered through a 0.8-μm filter prior to use.

RESULTS AND DISCUSSION

Biopolymer separations were studied on MicroPak TSK 2000 SW and MicroPak TSK 3000 SW columns, having exclusion limits for dextran standards of 20,000 and 150,000 respectively. Exclusion volume is dependent on hydrodynamic volume of a solute rather than simple molecular weight. Exclusion limits will thus be greater for proteins, which are not as linear as dextrans and thus penetrate the support pores more readily. Fig. 2 shows calibration plots of a series of protein standards on 2000 and 3000 SW columns. Exclusion limits for the proteins are seen to be $\approx$ 100,000 and $>$ 350,000 respectively.

Fig. 3 is a calibration plot of the same series of protein standards on a 3000 SW column with hemoglobin added. Although hemoglobin and albumin have similar molecular weights (68,000 *vs.* 69,000) the hemoglobin (57 $\times$ 34 Å) is more globular than albumin (150 $\times$ 38 Å) and thus permeates the pores to a greater extent. The larger hemoglobin elution volume is characteristic of a more linear (albumin-like) protein of mol.wt. 28,000. For accurate molecular-weight determination of proteins by steric exclusion chromatography (StEC), one can denature the proteins by addition of guanidinium hydrochloride[13] or sodium dodecylsulfate (SDS)[14] to the mobile phase.

The value of a proper choice of pore size for biopolymer separations is shown in the protein mixture separations of Fig. 4. The separations are obtained in 15 min, at a flow velocity of $\approx$ 0.04 cm/sec. Note that the γ-globullins (mol.wt. $\approx$ 160,000) which elute as one peak at the 2000 SW exclusion volume are split into three peaks on the 3000 SW column. The 3000 SW column is optimum for separation of most proteins of mol.wt. 40,000–400,000. The 2000 SW column is optimum for most proteins of mol.wt. $<$ 40,000.

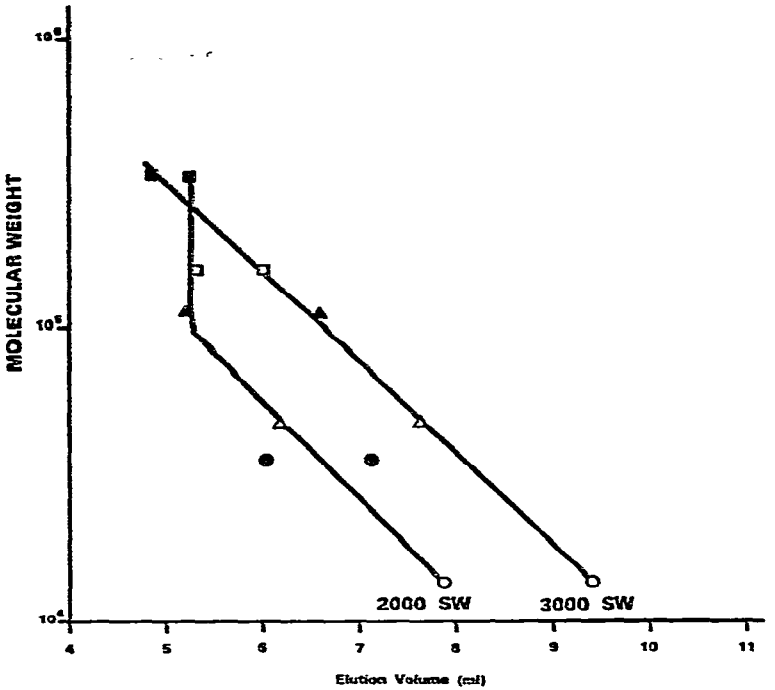

Fig. 2. Calibration curves for MicroPak TSK Type SW gels. Conditions: solvent, 0.067 *M* KH_2PO_4 + 0.1 *M* KCl + $6 \cdot 10^{-4}$ *M* sodium azide, pH 6.8; flow-rate; 1.0 ml/min; temperature, 30°; column dimensions, 30 cm × 7.5 mm. ■, Fibrinogen (340,000); □ γ-globulin (160,000); ▲, LDH (109,000); △, ovalbumin (43,500); ●, pepsin (35,000); ○, cytochrome *c* (13,500).

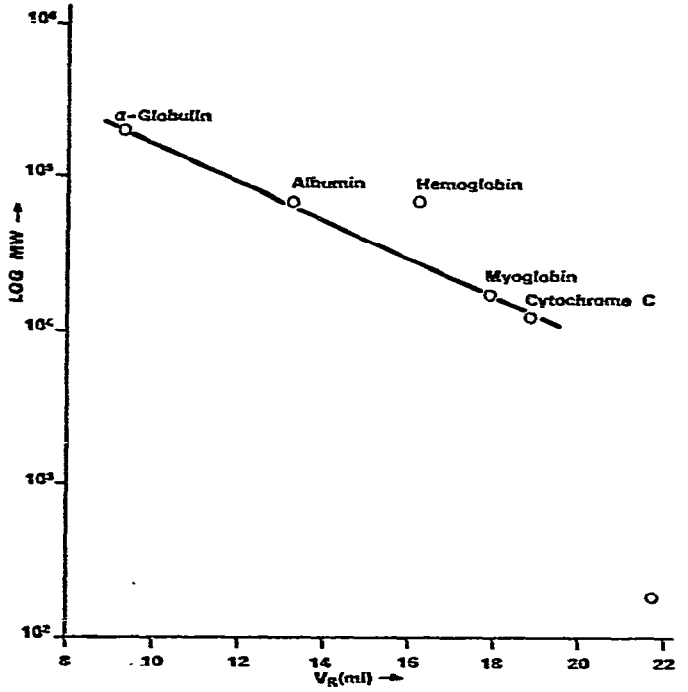

Fig. 3. Calibration curve for MicroPak TSK-3000. Conditions: solvent, 0.067 *M* KH_2PO_4 (pH 6.8) + 0.1 *M* NaCl; flow-rate, 1.0 ml/min; column dimensions, 7.5 mm × 60 cm.

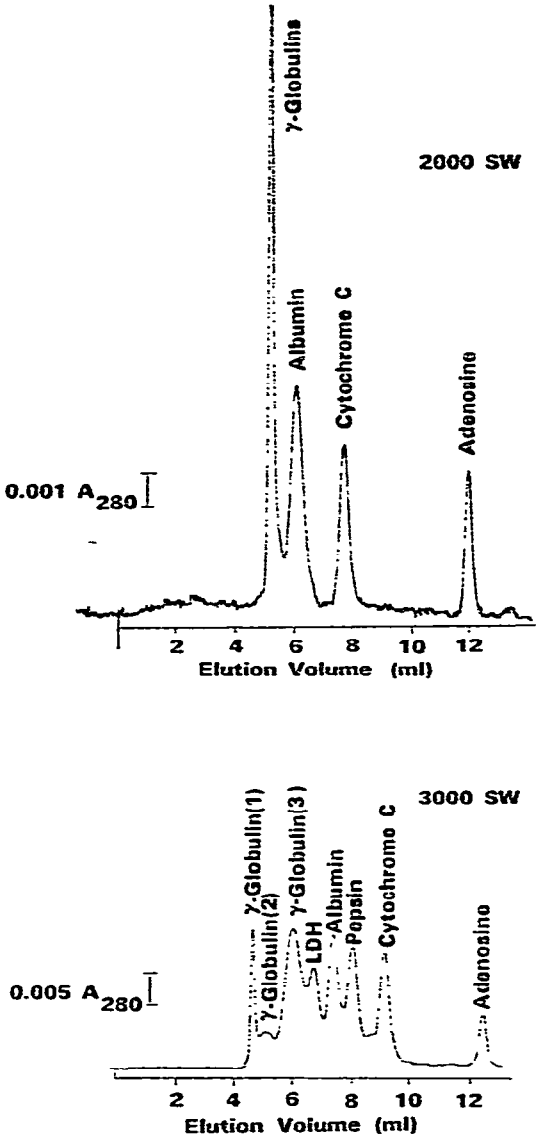

Fig. 4. Separation of protein standard mixtures. Conditions as in Fig. 2.

The 3000 SW column provides the best separation of components in human serum, as shown in Fig. 5. The first peak probably contains high-molecular-weight immunoglobulins partially excluded from the gel; the next peak should include γ-globulins (160,000 daltons). The large peak at $V_R \approx 7$ ml matches the elution volume of albumin (69,000 daltons). The last two peaks to elute are unknowns.

The hydrodynamic volume of a protein is a function of its conformation. Pepsin, a globular protein with unusual stability in acidic solutions, is denatured at pH values above 5. Consequently, it behaved as a linear molecule under the conditions

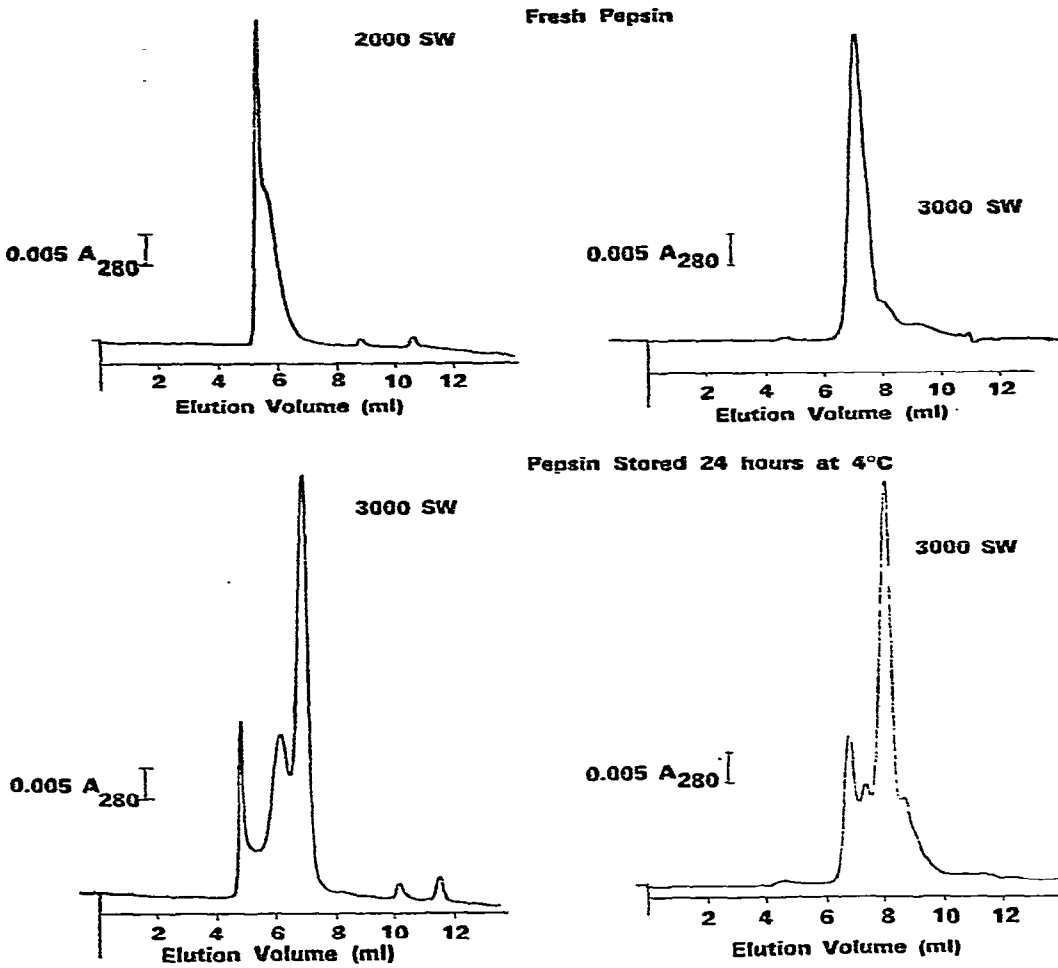

Fig. 5. Separation of human serum. Conditions as in Fig. 2.

Fig. 6. Separation of pepsin and degradation products. Conditions as in Fig. 2.

used in Fig. 2 (eluent pH 6.8). Upon incubation in the cold, a new peak appeared, corresponding to a species with a mol.wt. of 35,000 daltons (Fig. 6), suggesting either that the denatured pepsin was degraded into fragments or underwent partial renaturation to a more globular conformation.

The influence of hydrodynamic volume upon permeation is also evident in the elution of transfer RNAs (Fig. 7). These are relatively small ribonucleic acids with molecular weights in the range of 25,000–30,000 daltons, yet they elute from the 2000 SW column in the same volume as would a globular protein with a mol.wt. of 60,000 daltons. This reflects the relatively linear tRNA structure. Note that the two species (tyrosine specific and N-formylmethionyl specific) are partially resolved on the 3000 SW column. Comparison of the elution volumes with those of dextran polymers[15] indicates that the tRNAs elute as would linear dextrans with mol.wts. of 23,000 and

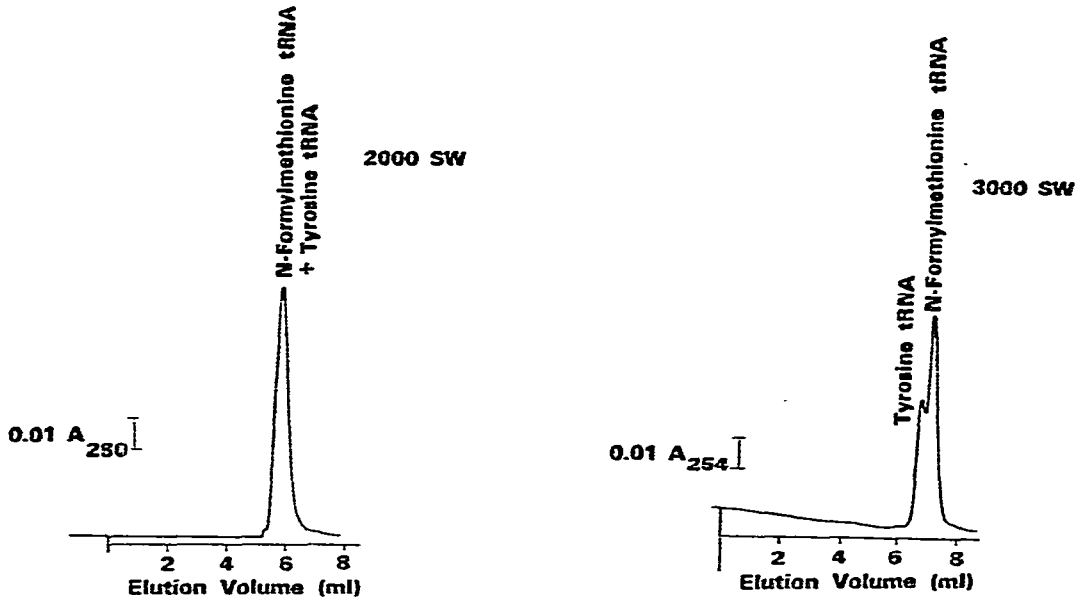

Fig. 7. Separation of tRNA standards. Conditions as in Fig. 2.

30,000 daltons. Separation of a tRNA extract from rabbit liver (Fig. 8) reveals a major peak corresponding to material in the range of 24,000 daltons (as determined from a dextran calibration curve), another component of about 30,000 daltons and smaller amounts of material of greater than 100,000 daltons (possibly ribosomal RNA).

Organic solvent compatible StEC is often used in tandem with a reversed-phase column[16] for resolution of complex mixtures. The StEC acts as a rapid pre-fractionation and clean-up step. Aqueous compatible StEC on the TSK-SW columns has great potential for coupling to a subsequent large-pore ion-exchange column. One can thus capitalize on the high speed and efficiency of the StEC columns along with the fact that the same mobile phase required for the ion exchange separation (typically a Tris buffer with NaCl added) can be used for the StEC separation. The future use of such a technique should be enhanced by the ability of microprocessor-controlled chromatographs to automate such a coupled column-"heart cutting" scheme.

Work in our laboratory is aimed at developing such HSLC ion-exchange columns for coupling to an StEC pre-fractionation. Fig. 9 shows the separation of tRNA standards on an experimental MicroPak anion exchange bonded phase column; here, the tyrosyl tRNA standard which elutes as a single peak from the steric exclusion column is separated into at least three UV-absorbing species. Application of such columns for analysis of tRNA preparations is quite promising, as suggested by the rapid separation of the rabbit liver tRNA extract shown in Fig. 10. Use of StEC pre-fractionation should greatly enhance the separating power of the technique and extend ion exchange column life by reducing its exposure to extraneous matrix material.

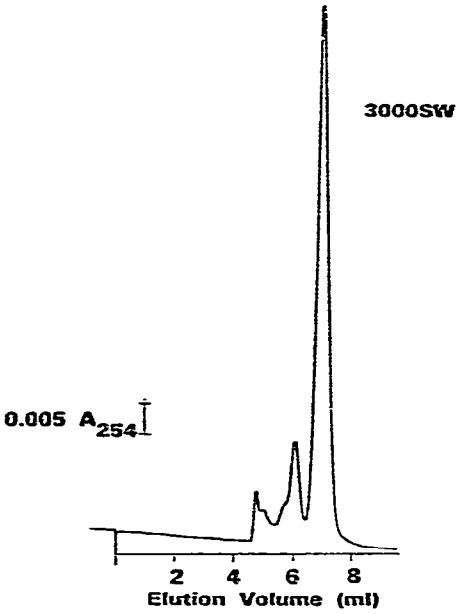

Fig. 8. Separation of rabbit liver tRNA extract. Conditions as in Fig. 2.

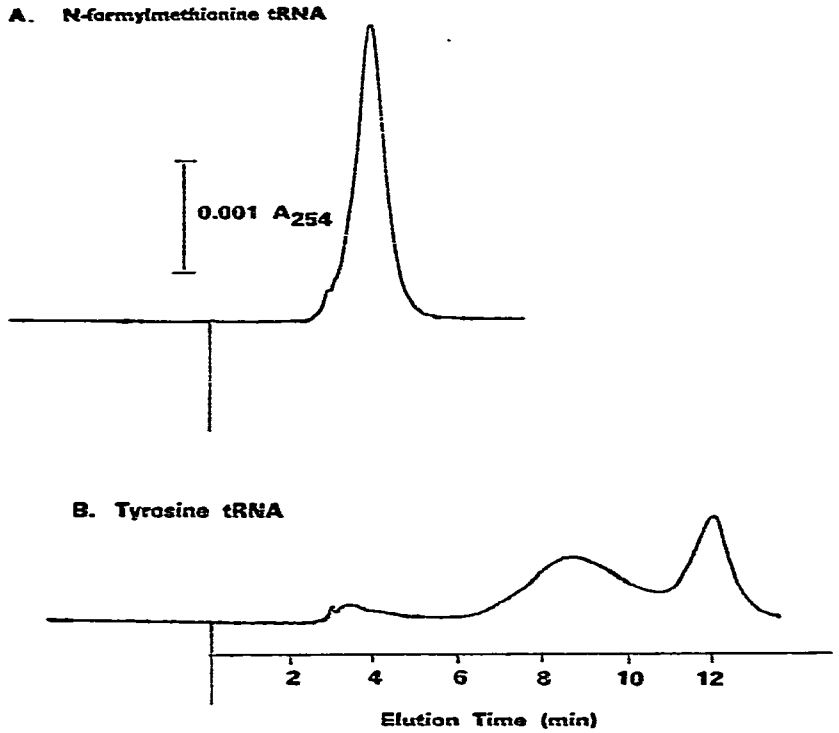

Fig. 9. Separation of transfer RNA on MicroPak MAX-500. Conditions: solvent A, 0.1 *M* Tris, pH 6.8; solvent B, 0.1 *M* Tris, pH 6.8 + 1 *M* NaCl; Gradient: 0% B 5 min, then 0–45% B in 15 min; flow-rate, 1.0 ml/min; temperature, 30°.

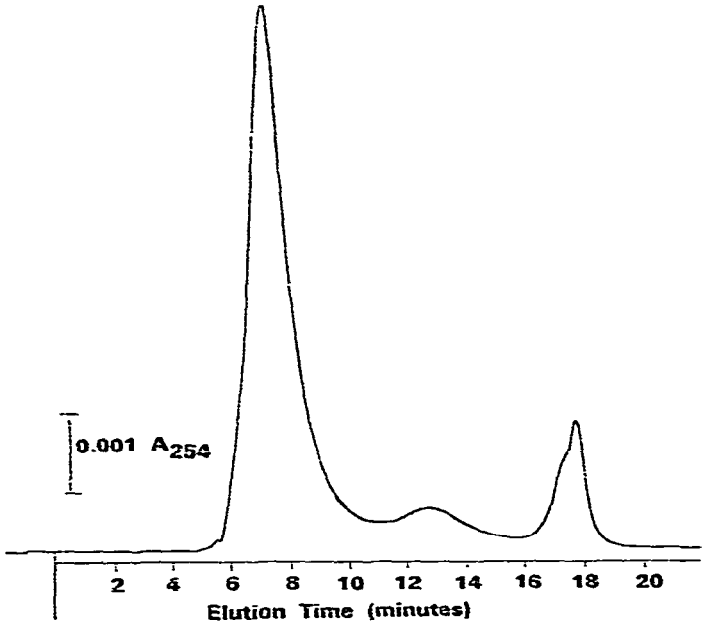

Fig. 10. Separation of rabbit liver tRNA extract on MicroPak MAX-500. Conditions as in Fig. 9 except flow-rate is 0.5 ml/min.

REFERENCES

1 J. Borovanský, P. Hach and J. Duchoň, *J. Chromatogr.*, 134 (1977) 230.
2 W. Haller, *Nature (London)*, 206 (1965) 693.
3 *Pierce Chemical Company Catalog*, Pierce, Rockford, Ill., 1978, p. 274.
4 T. Mizutani and A. Mizutani, *Anal. Biochem.*, 83 (1971) 216.
5 C. W. Hiatt, A. Shelokov, E. J. Rosenthal and J. M. Galimore, *J. Chromatogr.*, 56 (1971) 362.
6 T. Darling, J. Albert, P. Russell, D. M. Albert and T. W. Reid, *J. Chromatogr.*, 131 (1977) 383.
7 I. Shechter, *Anal. Biochem.*, 58 (1974) 30.
8 F. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
9 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 125 (1976) 103.
10 F. E. Regnier, *U.S. Pat.* 3,983,299 (1976).
11 J. L. Tayot, M. Tardy, P. Gattel, R. Plan and M. Roumiantzeff, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers*, Vol. 2, Wiley, New York, 1978, p. 34.
12 M. P. J. S. Anandaraj and B. A. Roe, *Biochemistry*, 14 (1975) 5068.
13 R. J. Blagrove and M. J. Frenkel, *J. Chromatogr.*, 132 (1977) 399.
14 R. C. Collins and W. Haller, *Anal. Biochem.*, 54 (1973) 47.
15 *SW Type Technical Data*, Toyo-Soda Mfg. Co. Ltd., Tokyo, 1978.
16 E. Johnson, R. Gloor and R. E. Majors, *J. Chromatogr.*, 149 (1978) 571.