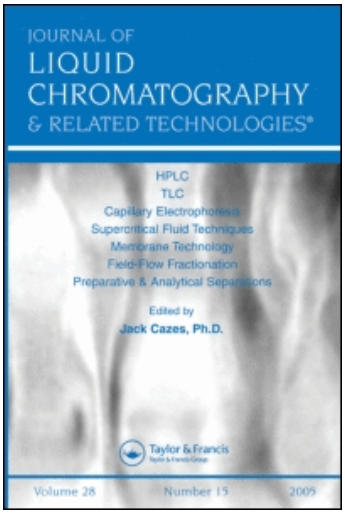

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EVALUATION OF NEW MICROPARTICULATE PACKINGS FOR AQUEOUS STERIC EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Two types of high performance aqueous size exclusion columns have recently been developed, one a rigid spherical silica-based packing containing a new hydrophilic bonded phase (MicroPak TSK Gel Type SW) and the other an organic-based, semi-rigid gel (MicroPak TSK Gel Type PW). Characteristics of MicroPak TSK SW and PW columns were compared to other commercially available aqueous SEC columns packed with similar supports. Chromatographic performance of pre-packed columns containing microparticulate support materials were compared for exclusion separations of water-soluble organic polymers, biopolymers, and small water-soluble oligomers. Amino acid probes were used to investigate non-exclusion effects of MicroPak TSK SW and PW columns.

INTRODUCTION

Steric exclusion chromatography (SEC) carried out in aqueous mobile phases, traditionally referred to as gel filtration chromatography, is widely used for the separation and characterization of natural and synthetic water-soluble polymers. The technique has been primarily employed to study biopolymers using crosslinked dextrans (e.g., Sephadex™) and agaroses (e.g., Sepharose™, Bio-Gel A™). Such

gels are of limited utility in HPLC because their inherently low compressive strengths require operation at low pressures and flow velocities.

Controlled-pore glasses (CPG) and silica packings allow analysis at pressures and flow velocities typical of HPLC. Microparticulate silica supports offer greatly reduced separation times compared to the soft gels traditionally used in aqueous SEC; however, their active surface sites often result in adsorption and other non-exclusion effects (2). Silica-based packings which contain a chemically bonded phase such as glyceryl propyl ether (diol) reduce adsorption (i.e., chemically deactivate the silica) but often exhibit low pore volumes compared to carbohydrate gels, thus reducing resolution.

New HPLC packings for aqueous SEC have utilized both microparticulate, hydrophilic polymer gels (3,4) and silica supports containing hydrophilic bonded phases (5,6). Several of these silica-based supports have recently been characterized by Pfannkoch, Lu, Regnier, and Barth (7) from the standpoint of both SEC performance and attendant non-exclusion effects. A comprehensive review of commercially available aqueous exclusion packings and chromatographic practices has been published by Barth (8).

Two types of microparticulate aqueous exclusion packings have been developed by Toyo Soda Mfg. Co. (Tokyo, Japan). One is a rigid, silica-based packing with a new hydrophilic bonded phase (TSK Gel Type SW) and the other a hydrophilic, polymer-based, semi-rigid gel (TSK Gel Type PW) (9, 10, 11).

In this report, the chromatographic performance of MicroPak TSK columns containing these two support materials has been compared for SEC separations of water-soluble organic polymers, biopolymers, and small water-soluble molecules. Column characteristics and chromatographic performance have also been contrasted with other commercially available aqueous SEC supports of similar nature. Amino acid probes were used to investigate non-exclusion effects on MicroPak TSK SW type and TSK PW type columns.

EXPERIMENTAL

Chromatography was performed on Varian Model 5000 LC systems equipped with a refractive index detector and a UV-50 variable wavelength absorbance detector. Chromatographic separations were carried out at 25°C using Varian MicroPak TSK SW and TSK Gel PW type columns (7.5 mm x 30 cm) at a mobile phase flow rate of 1 ml/min. Sample injection volumes were 100 µl using a Valco manual loop injector. Solvents used were 0.01 M KH_2PO_4 (pH 6.8) for amino acid probe samples, 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8) for proteins, and deionized water for polyethylene glycol standards.

Samples of polyethylene glycol (PEG) standards were obtained from Toyo Soda Mfg. Co. Ltd. (Tokyo, Japan) and Jefferson Chemical Co. (Austin, Texas). Aqueous solutions 0.1% w/v were used throughout this study. PEG standards above MW 10,000 were dissolved in aqueous solutions containing 0.5% ethanol to aid dissolution and retard chain scission of the standard (12). Detection of PEG standards was accomplished by a refractive index detector.

Proteins and amino acid probe samples were obtained from Sigma Chemical Co. (St. Louis, Missouri). Detection of proteins was at 280nm and detection of amino acids at 210nm in the UV.

RESULTS AND DISCUSSION

Characteristics of Commercially Available Aqueous SEC Microparticulate Supports

A. Surface-Modified, Silica-Based Packings

Tables 1 and 1a list all commercially available columns packed with surface-modified, silica-based aqueous SEC supports. Characteristics for each column type specify chemical nature of the bonded phase (or surface modification), packing name, available pore sizes, particle size and shape of the silica, molecular weight separation ranges, and a list of manufacturers and suppliers. Several of the support materials in this table are marketed by a variety of suppliers under different trade names. For example, SynChropak GPC

Table 1.
Characteristics of Microparticulate, Surface-Modified, Silica-Based Packings for Aqueous SEC

Chemical Type of Silica Surface Modification	Packing Name	Supplier
Glycerol propyl-type bonded phase; ($SiCH_2CH_2CH_2OCH_2CHOHCH_2OH$).	SynChropak GPC 100 SynChropak GPC 300 SynChropak GPC 500 SynChropak GPC 1000 SynChroPak GPC 4000	1,2 (sold as Aquapore), 3 (sold as Aquapore), 4 (sold as Bio-sil GFC).
	LiChrosorb Diol 100	5
	LiChrospher Diol 100 LiChrospher Diol 500 LiChrospher Diol 1000 LiChrospher Diol 4000	5
Polyether-type bonded phase; ($Si(RO)_nCH_3$); base silica μPorasil.	μBondagel E-125 μBondagel E-500 μBondagel E-1000 μBondagel E-High Å μBondagel E-Linear	6
Bonded phase structure has not been published.	Waters Protein Column I-60 Waters Protein Column I-125 Waters Protein Column I-250	6
Glycol ether-type bonded phase; surface covered with hydroxyl groups, exact structure has not been published.	TSK Gel Type 2000SW TSK Gel Type 3000SW TSK Gel Type 4000SW	10,4 (sold as Bio-Sil TSK), 7 (sold as MicroPak TSK SW), 8 (sold as μSpherogel SW), 9 (sold as Ultropak TSK SW).

Suppliers:
1. SynChrom Inc. (Linden, IN)
2. Brownlee Labs (Santa Clara, CA)
3. Chromatix (Sunnyvale, CA)
4. Bio-Rad Laboratories (Richmond, CA)
5. E. Merck (EM Laboratories, Elmsford, NY)
6. Waters Associates (Milford, MA)
7. Varian Associates (Walnut Creek, CA)
8. Beckman-Altex Inc. (Berkeley, CA)
9. LKB Instruments Inc. (Rockville, MD)
10. Toyo Soda Manufacturing Co. Ltd. (Tokyo, Japan)

Table 1a.

Packing Name	Pore Size (Å)	Particle Size (μm) and Shape	Molecular Weight Separation Range Proteins (Aqueous Mobile Phase)	Polystyrene (THF Mobile Phase)
SynChropak GPC 100	100	10 (spherical particles)	3,000-300,000	<5-80,000
SynChropak GPC 300	300	"	-	<1.5-300,000
SynChropak GPC 500	500	"	10,000-5 million	<3-600,000
SynChropak GPC 1000	1000	"	100,000-20 million	<0.6-1.4 million
SynChropak GPC 4000	4000	"	-	<2.5-8 million
LiChrosorb Diol 100	100	10 (irregular particles)	10,000-100,000	-
LiChrospher Diol 100	100	10 (spherical particles)	-	<80,000
LiChrospher Diol 500	500	"	-	<600,000
LiChrospher Diol 1000	1000	"	-	<1.4 million
LiChrospher Diol 4000	4000	"	-	<8 million
μBondagel E-125	125	10 (irregular particles)	-	2,000-50,000
μBondagel E-500	500	"	-	5,000-500,000
μBondagel E-1000	1000	"	-	50,000-2 million
μBondagel E-High Å	N.A.	20 (irregular particles)	-	15,000-7 million
μBondagel E-Linear	Blend	10 (irregular particles)	-	2,000-2 million
Waters Protein Column I-60	60	10 (irregular particles)	1,000-20,000	-
Waters Protein Column I-125	125	"	2,000-80,000	-
Waters Protein Column I-250	250	"	10,000-500,000	-
TSK Gel Type 2000SW	130	10 (spherical particles)	500-60,000	-
TSK Gel Type 3000SW	240	"	1,000-300,000	-
TSK Gel Type 4000SW	450	13 (spherical particles)	5,000-1 million	-

NOTE: Data obtained from manufacturer's literature.
N.A. = Not available,

packing is also sold under the names of Aquapore and Bio-Sil GFC columns, and TSK Gel SW packing is sold under names of μSpherogel TSK SW, MicroPak TSK SW, and other column names.

SynChropak high performance modified silica was the first commercially available glycophase-type packing for aqueous SEC. Silylpropylglycerol bonded phases of this type also include LiChrosorb Diol and more recently LiChrospher Diol. Although largely employed in the separation and characterization of water-soluble biopolymers, all of these packings can be used with organic solvents for analysis of synthetic polymers and other applications.

μBondagel packing is a silica-based support containing an aliphatic ether bonded phase. Its primary application has been the characterization of synthetic polymers and polysaccharides in both aqueous mobile phases and organic solvents such as tetrahydrofuran (THF). Recently, Waters Associates has developed a proprietary Protein Column designed specifically for characterization of biopolymers such as proteins. The nature of the silica surface modification has not been published although it is known to contain a neutral, hydrophilic phase covalently bonded to silica.

TSK Gel Type SW packing is believed to consist of a glycol ether-type bonded phase similar in nature to the glycophases. The surface of TSK SW packing is known to be highly hydroxylated, although the exact packing structure has not been published. This surface-modified silica support exhibits little tendency for adsorption and high efficiency for analysis and separation of biopolymers such as proteins and enzymes.

B. Organic Gel-Based Packings

Characteristics of commercially available organic gel-based packings for aqueous SEC are listed in Tables II and IIa. The chemical nature of the support, packing name, available pore sizes, particle size and shape, molecular weight separation ranges, and pH range of operation are listed for each material along with a list of manufacturers and suppliers.

Spheron packing is a poly(hydroxyl methacrylate) copolymerized with ethylene dimethacrylate. The packing particles are aggregated to form macroporous beads capable of withstanding pressures up to 3000 psi. The support is compatible with both water and organic solvents, allowing a wide range of applications (53).

Shodex OHpak support is believed to be composed of a glycerol methacrylate copolymer although the exact structure of the packing has not been published. Shodex OHpak B-804 packing is the only pore size available of this type and has been employed in the characterization of both polysaccharides and biopolymers.

Shodex IonPak is a sulfonated poly(styrene divinylbenezene) gel offering high efficiency and a wide variety of pore sizes for aqueous SEC. Applications of this gel have focused on characterization of polysaccharides and neutral, synthetic polymers.

TSK Gel Type PW packing is a crosslinked, hydroxylated polyether gel whose exact structure has not been published. Most of the pore sizes available with this packing are known to contain residual carboxyl groups and the smaller pore sizes (1000PW and 2000PW) additionally contain residual amino groups. This support material has been employed in a wide variety of aqueous SEC applications and has been found to be particularly well suited to the analysis of water-soluble synthetic polymers, including polycations.

Comparative Characterization of MicroPak TSK SW and TSK PW Columns

A comparison of the characteristics of MicroPak TSK SW and TSK PW columns serves to illustrate some of the fundamental differences between silica-based and organic gel-based supports for aqueous SEC as well as provide additional information on these two column types. MicroPak TSK SW column packing is a rigid, hydrophilic, spherical, and porous silica that contains a chemically bonded phase thought to be a polyether-type coating. The surface of the packing is covered with hydroxyl groups (13). MicroPak TSK PW column packing is a semi-rigid, hydrophilic, crosslinked polymer-based gel containing the group $-CH_2CHOHCH_2O-$ as the main backbone component (14).

Table 2.
Characteristics of Microparticulate, Organic Gel-Based Packings for Aqueous SEC

Chemical Type of Gel	Packing Name	Supplier
Glycol methacrylate gel copolymer; poly(2-hydroxyethylmethacrylate-co-ethylene dimethacrylate).	Spheron P40 Spheron P100 Spheron P300 Spheron P500 Spheron P700 Spheron P1000 Spheron P100000	1
Methacrylate glycerol copolymer.	Shodex OHpak B-804	2,3
Sulfonated poly(styrene divinylbenzene) copolymer.	Shodex IonPak S-801 Shodex IonPak S-802 Shodex IonPak S-803 Shodex IonPak S-804 Shodex IonPak S-805	2,3
Hydroxylated polyether copolymer; contains ($-CH_2CHOHCH_2O-$) groups as main backbone component. Also known to contain residual -COOH groups and $-NH_2$ groups (1000PW and 2000PW only); exact structure has not been published.	TSK Gel Type 1000PW TSK Gel Type 2000PW TSK Gel Type 3000PW TSK Gel Type 4000PW TSK Gel Type 5000PW TSK Gel Type 6000PW	7,4 (sold as MicroPak TSK PW), 5 (sold as μSpherogel PW), 6 (Bio-Gel TSK).

Suppliers:
1. LaChema (Brno, Czechoslovakia)
2. Perkin-Elmer Corp. (Norwalk, CT)
3. Showa Denko K.K. (Tokyo, Japan)
4. Varian Associates (Walnut Creek, CA)
5. Beckman-Altex Inc. (Berkeley, CA)
6. Bio-Rad Laboratories (Richmond, CA)
7. Toyo Soda Manufacturing Co. Ltd. (Tokyo, Japan)

Table 2a.

Packing Name	Pore Size (Å)	Particle Size (μm) and Shape	Molecular Weight Separation Range in Aqueous Mobile Phase Polysaccharides	Polyethylene Glycols (PEGs)	pH Stability
Spheron P40	40	10-20 (macroporous beads)	20,000-60,000	-	1-12
Spheron P100	100	"	40,000-100,000	-	
Spheron P300	300	"	60,000-300,000	-	
Spheron P500	500	"	80,000-500,000	-	
Spheron P700	700	"	250,000-700,000	-	
Spheron P1000	1000	"	800,000-5 million	-	
Spheron P100000	-	"	<100 million	-	
Shodex OHpak B-804	N.A.	10 (spherical particles)	<400,000	-	4-12
Shodex IonPak S-801	55	10 (spherical particles)	< 1,000	-	2-11
Shodex IonPak S-802	100	"	< 5,000	-	
Shodex IonPak S-803	160	"	< 50,000	-	
Shodex IonPak S-804	220	∿15 (spherical particles)	<500,000	-	
Shodex IonPak S-805	350	"	<5 million	-	
TSK Gel Type 1000PW	-	10 (spherical	-	∿100-1,000	2-12
TSK Gel Type 2000PW	50	10 particles)	-	200-5,000	
TSK Gel Type 3000PW	200	13	500-10,000	∿1,000-50,000	
TSK Gel Type 4000PW	500	13	1,000-700,000	2,000-300,000	
TSK Gel Type 5000PW	1000	17	10,000-2 million	4,000-800,000	
TSK Gel Type 6000PW	-	25	100,000-20 million	40,000-8 million	

NOTE: Data obtained from manufacturer's literature.
N.A. = Not available.

A comparison of the characteristics of MicroPak TSK SW and TSK Gel PW columns is shown in Table 3. Exclusion limits, typical efficiencies, ratio of pore volume (V_i) to interstitial volume (V_o), particle sizes, and pore sizes are listed for each column type. MicroPak TSK SW type columns have higher efficiencies and (V_i/V_o) ratios than the PW type columns, and therefore should offer higher resolution. The PW type columns operate over a larger molecular size range than do the SW columns due to the wider range of pore sizes available. MicroPak TSK PW columns also allow operation over a wider pH range (2 to 12) than the silica-based SW columns (2.5 to 7.5) (see reference 9).

Figure 1 displays a vanDeemter-type plot of linear velocity versus plate height (HETP) for some MicroPak TSK SW and PW type columns. Plate height increases with flow velocity and is found to plateau at high flow velocities. In comparing the 3000PW to the 4000SW column, it can be seen that the 4000SW column has a higher efficiency at all flow rates examined. The 3000SW column has a smaller particle size packing (10 μ) than either the 3000PW or 4000SW column packing (13 μ) and, consequently, lower plate height at all flow velocities. In practice, flow velocities of 0.04 to 0.06 cm/sec, roughly corresponding to 0.8 to 1.3 ml/min mobile phase flow rate, have been found to offer the best compromise between speed and efficiency for both column types.

Polyethylene glycol (PEG) calibration curves for SW and PW columns are shown in Figures 2 and 3. The slopes exhibited in the linear region of the calibration curve have been found to be indicative of the resolution attainable with steric exclusion columns (15). Lower slopes usually correspond to higher resolution values for a given column pore size.

Comparative Performance of Aqueous SEC Columns

Chromatographic column performance has been traditionally expressed in terms of the number of theoretical plates or efficiency:

$$N = 16\left(\frac{V_R}{W}\right)^2 \qquad (1)$$

TABLE 3.
Characteristics of MicroPak TSK Gel Type SW and TSK Gel Type PW Columns

Column Type	Particle Size*	Pore Size* (Å)	M.W. Exclusion Range PEG	Protein	Theoretical Plates/Meter (N/M)	Pore to Interstitial Volume Ratio (V_i/V_o)
2000SW	10 ± 2 μ	130	20,000	100,000	21,000	0.92
3000SW	10 ± 2 μ	240	40,000	400,000	19,000	1.33
4000SW	13 ± 3 μ	450	200,000	1 million (est)	17,000	1.52
1000PW	10 ± 2 μ	-	1,000	-	16,000	0.89
2000PW	10 ± 2 μ	50	4,000	15,000	17,000	0.87
3000PW	13 ± 2 μ	200	50,000	450,000	15,000	0.83
4000PW	13 ± 2 μ	500	200,000	-	14,000	0.78
5000PW	17 ± 2 μ	1000	1 million (est)	>1 million	13,000	0.98
6000PW	25 ± 5 μ	-	8 million (est)	-	8,100	1.06

NOTE:

1. Method for calculation of theoretical plates: Sample: 1% w/v solution Ethylene Glycol
   Mobile Phase: 1 ml/min H_2O
   Detector: RI
   Injection Volume: 100 μl

2. V_i = Pore Volume; V_o = Interstitial Volume.

* Data supplied by Toyo Soda. Estimated pore size values obtained by comparison with packings of known pore sizes from calibration curve data.

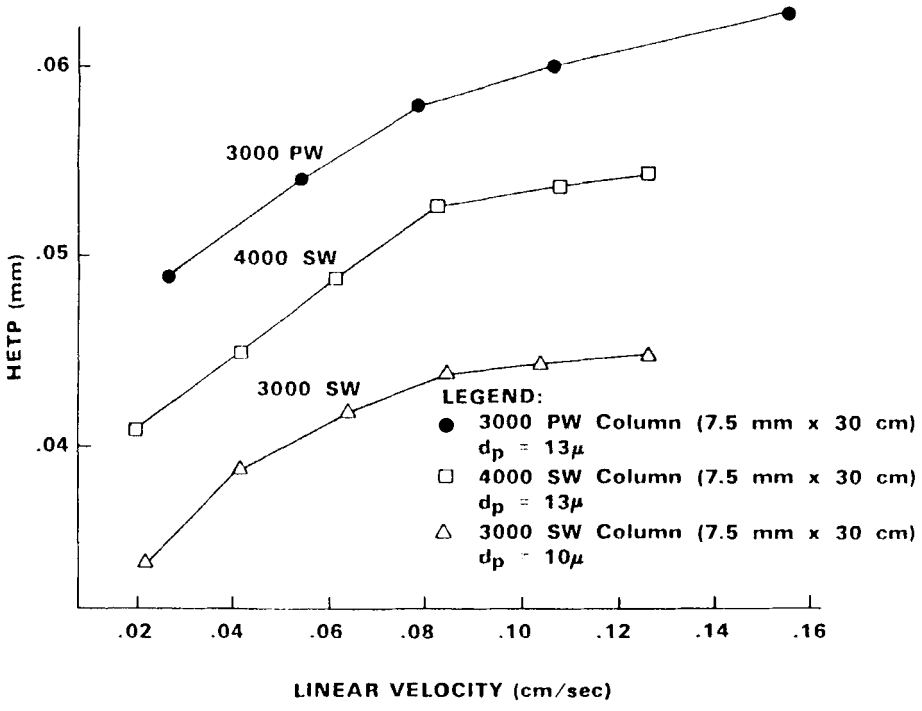

FIGURE 1. VanDeemter-type plot of linear velocity (cm/sec) versus plate height (HETP in mm) for MicroPak TSK 3000SW (d_p = 10 μ), 4000SW (d_p = 13 μ), and 3000PW (d_p = 13 μ) columns. Column dimensions: 7.5 mm x 30 cm; Mobile phase: H_2O

where V_R is peak retention volume and W the peak width at baseline as measured by a peak triangulation technique.

The resolution (or separation efficiency) of a two-component mixture has also been used as a column performance parameter as described by the following equation:

$$R_S = \frac{2(V_{R2}-V_{R1})}{W_1 + W_2} \qquad (2)$$

where V_{R1} and V_{R2} are the elution volumes of two solutes and W_1 and W_2 their respective peak widths.

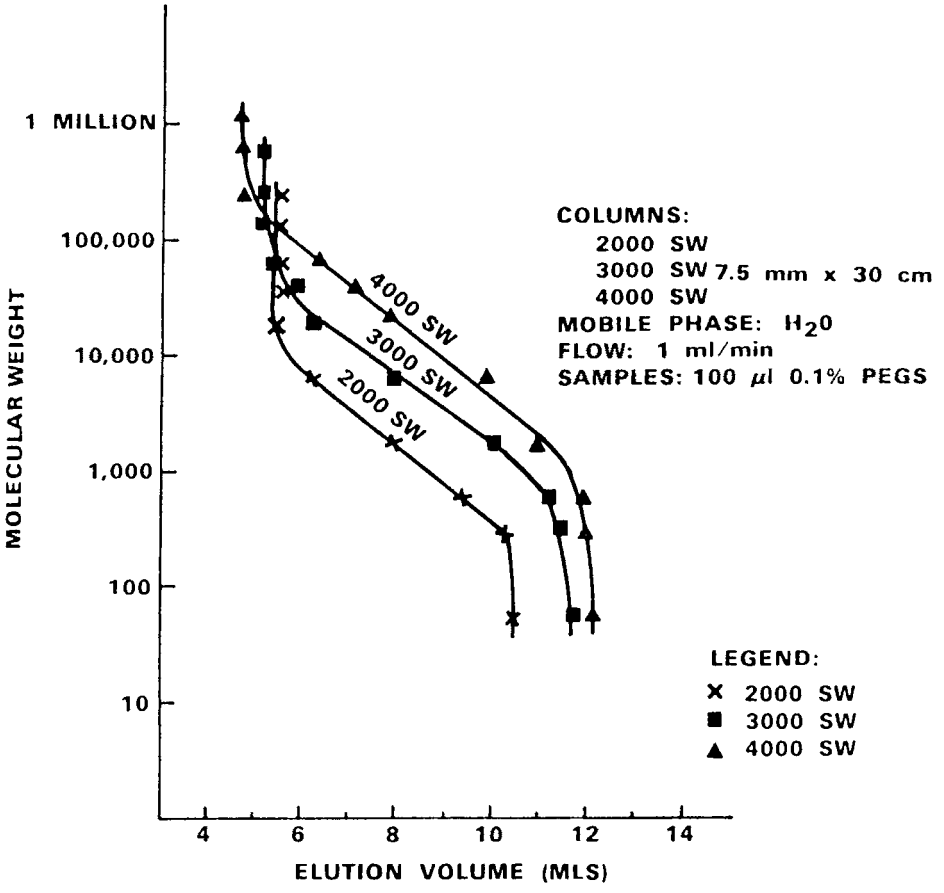

FIGURE 2. PEG standard calibration curves for MicroPak TSK SW type columns. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG. Column dimensions: 7.5 mm x 30 cm.

The values of R_S and N calculated by these equations, however, are highly dependent on column dimensions and on the solutes chosen to characterize performance. Additionally, in steric exclusion chromatography, it would be very desirable to relate chromatographic resolution to molecular weight since separation is based upon molecular size discrimination.

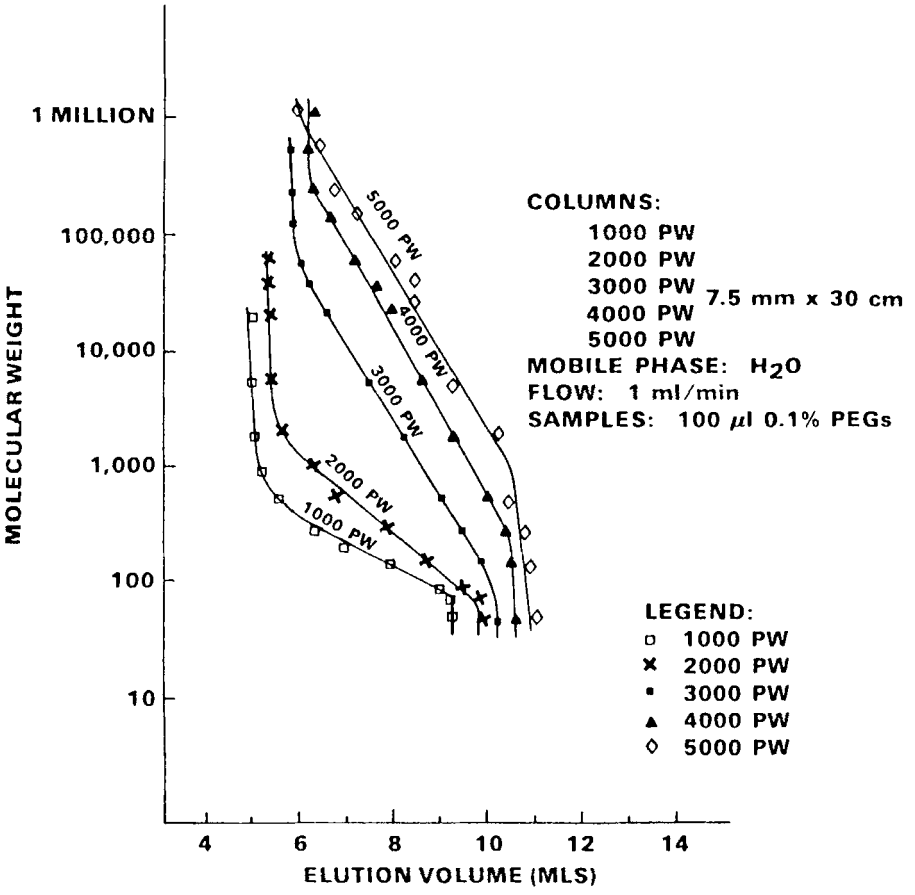

FIGURE 3. PEG standard calibration curves for TSK PW type columns. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG. Column dimensions: 7.5 mm x 30 cm.

The concept of specific resolution, R_{SP}, was introduced by Bly (16) who has shown that in the linear region of the calibration curve of log MW versus V_R for an exclusion column, resolution can be normalized and expressed as a function of molecular weights of a solute pair:

$$R_{SP} = \frac{2(V_{R2} - V_{R1})}{W_1 + W_2} \times \frac{1}{\log(MW_1/MW_2)} \qquad (3)$$

where MW_1 and MW_2 are the molecular weights of two solutes. For a pair of solutes with a decade difference in MW, equation (3) reduces to the expression for resolution. Additionally, outside the linear calibration range for a steric exclusion column, R_{SP} approaches zero.

Specific resolution, R_{SP}, is independent of the solute probes if the samples have very narrow molecular weight distributions (MWD) (17). Thus, R_{SP} is a more descriptive parameter for accurate performance comparison of steric exclusion column types. This parameter has been applied by Kirkland and Antle (18) to the performance characterization of high performance steric exclusion packings using organic mobile phases.

Table 4 displays a comparison of efficiency, specific resolution (R_{SP}), and pore volume data for several commercially available aqueous SEC columns packed with surface-modified, silica-based supports. In SEC, resolution is a function of both column efficiency and support pore volume (V_i). As can be seen from the data in this table, columns with high efficiency and large support pore volumes offer high resolution for SEC separations, as for example the TSK 3000SW column.

A comparison of pore volume and efficiency for several commercially available aqueous SEC columns packed with organic gel-based supports is shown in Table 5. Lack of published data on the performance of these packings precludes a more incisive comparison.

Specific resolution, R_{SP}, values were calculated with a series of narrow MWD polyethylene glycol standards and protein standards for both MicroPak TSK SW and PW columns. The average molecular weight for a pair of standards can be defined as follows:

$$\text{Average MW} = \frac{MW_1 + MW_2}{2} \qquad (4)$$

A plot of R_{SP} versus Average MW defines the molecular weight range of optimum resolution for a steric exclusion column and provides a practical performance criterion for column selection and comparison in steric exclusion analysis. Such plots have been used by Kato et

TABLE 4.
Comparison of Efficiency, Resolution, and Pore Volume for Commercially Available Columns Containing Surface-Modified, Silica-Based Supports for Aqueous SEC

Columns	Length	I.D.	(T.P./m)[a] Theoretical Plates/Meter	R_{SP}[b]	Pore Volume (V_i)
MicroPak TSK 2000SW	30 cm	7.5 mm	22,567	2.57	5.06 ml
MicroPak TSK 3000SW	30 cm	7.5 mm	30,720	3.32	6.78
MicroPak TSK 4000SW	30 cm	7.5 mm	17,000	-	6.84
SynChropak GPC 100	25 cm	4.6 mm	8,316	1.65	2.00
SynChropak GPC 300	25 cm	4.6 mm	16,800	2.08	-
Waters I-125	25 cm	7.8 mm	19,788	2.04	4.94
Waters μBondagel	30 cm	3.9 mm	⩾12,000	-	1.20

a) Measured using the peptide glycyltyrosine (K_D range 1.01 to 1.07 for columns tested) except TSK 4000SW (ethylene glycol) at aqueous mobile phase velocity of 0.33 mm/second, and μBondagel (from manufacturer's data measured at velocity of 1.8 mm/sec in THF).

b) Specific resolution factor computed for the peptide glycyltyrosine (MW=238) and the protein ovalbumin (MW=43,500).

NOTE: Based upon experimental data from reference 7.

Table 5.
Comparison of Efficiency and Resolution for Commercially Available Columns Containing Organic Gel-Based Supports for Aqueous SEC

Columns	Length	I.D.	(T.P./m) Theoretical Plates/Meter	Pore Volume
MicroPak TSK 1000PW	30 cm	7.5 mm	16,520[a]	4.54 ml
MicroPak TSK 2000PW	30 cm	7.5 mm	17,030	4.83
MicroPak TSK 3000PW	30 cm	7.5 mm	15,120	4.63
MicroPak TSK 4000PW	30 cm	7.5 mm	14,300	4.52
MicroPak TSK 5000PW	30 cm	7.5 mm	13,050	5.51
MicroPak TSK 6000PW	30 cm	7.5 mm	8,100	6.01
Shodex OHpak B-804	50 cm	8.0 mm	$\geq$10,000[b]	8.19
Spheron P-1000	25 cm	8.0 mm	9,000[c]	-
Shodex S-801/S	50 cm	8.0 mm	20,000[b]	-
Shodex S-802/S	50 cm	8.0 mm	20,000	-
Shodex S-803/S	50 cm	8.0 mm	20,000	-
Shodex S-804/S	50 cm	8.0 mm	14,000	-
Shodex S-805/S	50 cm	8.0 mm	14,000	-

a) Experimentally measured in aqueous mobile phase at a linear velocity of 0.4 mm/sec with ethylene glycol.
b) From manufacturer's literature.
c) Experimentally determined with 17 μm particles slurry packed into SS tubing. See reference 39.

al. to characterize protein separations on SW columns (19). Demonstration of the utility of these plots to chromatographic performance of MicroPak TSK SW and TSK PW columns has recently been performed (20).

Specific resolution curves for MicroPak SW columns using PEG standards are shown in Figure 4. In comparing the 2000SW and 3000SW columns, the 2000SW displays higher specific resolution values for solutes below MW 1000, while the 3000SW column displays higher resolution values for larger solutes. This fact has also been observed for protein separations using 2000SW and 3000SW columns (see reference 19). Although the 4000SW column operates over a wide range of solute molecular weights, specific resolution

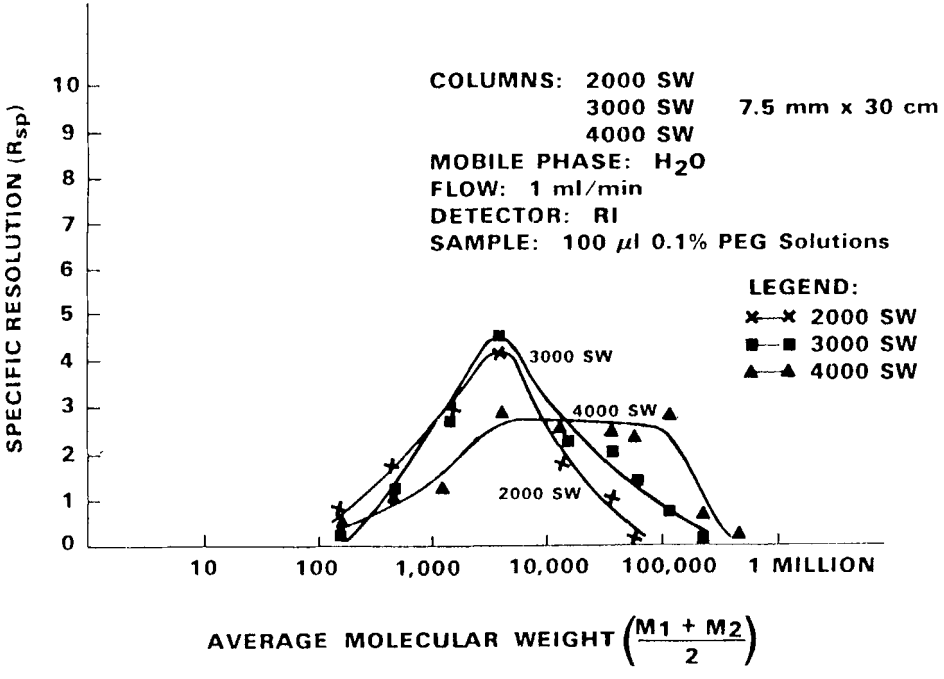

FIGURE 4. Specific resolution (R_{SP}) curves for MicroPak TSK Gel type SW columns using PEG standards. Mobile phase: H_2O; Flow rate: 1 ml/min; Injection volume: 100 µl; Loading: 0.1% PEG; Column dimensions: 7.5 mm x 30 cm.

values are much lower than those obtained with 2000SW and 3000SW columns for solutes less than 20,000 in molecular weight.

Specific resolution plots for MicroPak PW columns using PEG standards are displayed in Figure 5. As column pore size increases, specific resolution values decrease for PW columns. The extremely large values of specific resolution obtained with 1000PW and 2000PW columns reflect the advantage of these columns for analysis of small water-soluble molecules such as polyethylene glycol oligomers and oligosaccharides (21).

In comparing specific resolution curves for MicroPak TSK SW and PW columns, several points are noteworthy:

i. In molecular weight ranges applicable to both SW and PW columns, higher resolution is provided by operation with SW columns;

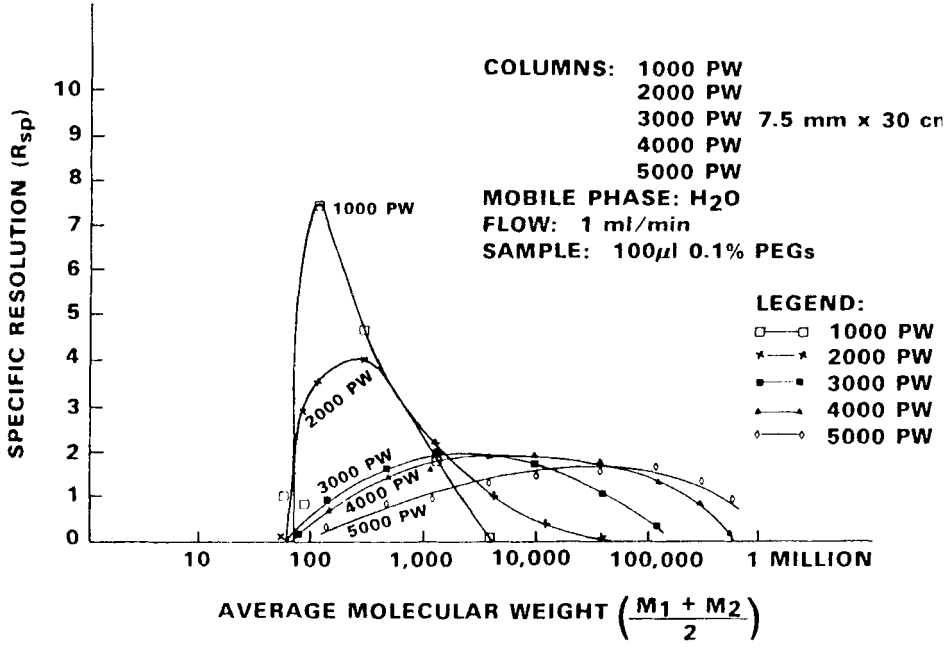

FIGURE 5. Specific resolution (R_{SP}) curves for MicroPak TSK Gel Type PW columns using PEG standards. Conditions same as in Figure 4.

ii. For solutes of molecular weight greater than 200,000 the PW columns offer a wider molecular weight range of operation and higher resolution values;

iii. 1000PW and 2000PW columns are best suited to the analysis of small water-soluble molecules.

Comparison of Microparticulate Aqueous SEC Columns for Analysis of Water Soluble, Synthetic Polymers

The influence of the mobile phase in aqueous steric exclusion chromatography is particularly important because of its effect on solute conformation and size. Ionic strength is critical in eliminating hydrophilic interactions between the column packing and charged solutes (polyelectrolytes) that lead to attendant problems of ion exclusion, ion inclusion, ion exchange, and adsorptive effects (22). For SW and PW type columns, ionic strengths greater than 0.1 μ are preferred, and polyvalent anions seem to be more effective in eliminating ionic effects for many polymers. Polysaccharides, poly(vinyl alcohol), and poly(vinyl pyrrolidone) polymers can be successfully chromatographed on both SW and PW type columns using low ionic strength mobile phases such as 0.02 M KH_2PO_4 (see reference 21).

Shodex IonPak columns have been shown to be effective in the characterization of high molecular weight polysaccharides, polyethylene glycols, and poly(vinyl alcohol) polymers utilizing water as a mobile phase (40). However, since the packing is ionic, the columns would be best employed in neutral to low pH mobile phases to avoid ion-exclusion or exchange separation mechanisms. In some cases ion-exclusion and hydrophobic interactions have been exploited to achieve separation of acidic from neutral compounds and small organic molecules (41).

μ-Bondagel and several glycophase-type packings have also been applied to the analysis of water-soluble polymers utilizing mobile phase ionic strengths of 0.1 μ or greater (42,43). Ionic interactions caused for example by residual silanol sites can be minimized

by higher ionic strength mobile phases; hydrophobic interactions with these supports can usually be controlled by adding organic solvent modifiers. Due to excellent mechanical strength of the supports, such packings can also be utilized for synthetic polymer analysis in organic solvents. μ-Bondagel has been used to characterize a number of water-soluble polymers, including sulfonated polystyrenes, poly(vinyl alcohol), and anionic polyelectrolytes (44).

For very polar synthetic polymers such as polyacrylamide, poly(acrylic acid), and polyethyleneimine, mobile phase ionic strengths greater than 0.3 μ give satisfactory results for MicroPak TSK PW type columns but SW type columns exhibit adsorption effects even at these ionic strengths, as do most other silica-based packings. The use of acetic acid as a mobile phase modifier has also been found to decrease non-exclusion interactions with PW type columns (23). In general, PW type columns give better performance than SW type columns for water-soluble organic polymers due to availability of larger pore size columns and minimal non-exclusion effects for polar, water-soluble polymers. Characterization of cationic polymers has also been reported on PW columns (45,46).

In exclusion chromatography, separation is achieved solely on the basis of effective molecular size of a solute only when no significant interactions take place between the stationary phase and the sample. For such a separation, the support material must be inert (i.e., contain no active surface sites) with the eluent used for analysis and the solute must be recovered with 100% efficiency. In practice, unwanted non-exclusion interactions or the inertness of a support material can be evaluated from solute elution volume and recovery in a given mobile phase. Recovery data for water-soluble polymers analyzed on MicroPak TSK 3000PW columns in various mobile phases is shown in Table 6 (24). Polymer mass recovery was defined as the peak area ratios of injections made with and without a column. Polymers studied were poly(vinyl alcohol) (PVA), poly(ethylene oxide) (PEO), poly(vinyl pyrrolidone) (PVP), poly(acrylamide) (PAM), and poly(acrylic acid) (PAA). Higher recoveries of

Table 6.
Recovery Data for Water Soluble Polymers on MicroPak TSK Gel PW Type Columns

Polymer	MW	3000PW (Water)	3000PW (0.08 M Tris)
Polyvinyl alcohol (PVA)	125,000	80%	98%
Polethylene oxide (PEO)	50,000	99	-
Polyvinyl pyrrolidone (PVP)	10,000	60	100
Polyacrylamide (PAM)	600,000	40*	80*
Polyacrylic acid (PAA)	90,000	-	84

Sample Loading: 50 to 300 μg
* 3000PW + 5000PW columns in series.

PVP and PAM polymers, both acidic polyelectrolytes, as well as PVA with 0.08 M tris eluent are due to reduction of hydrophilic interactions between packing and sample as a consequence of increased ionic strength of the mobile phase. No comparative synthetic polymer recovery data has been published utilizing similar microparticulate aqueous SEC supports.

Comparison of Microparticulate Aqueous SEC Columns for Biopolymer Analysis

High performance exclusion chromatography is a promising tool in protein chemistry for purification and molecular weight characterization of proteins. The utility of high speed SEC biopolymer separations depends upon adequate recovery of proteins in their native (e.g., active) form after passage through the column. Previous supports used for high speed SEC separations based on silica or controlled pore glass have suffered from poor recovery due to high densities of acidic silanol groups which contribute to adsorption and denaturation of proteins (25). Pfannkoch et al. (7) have measured recovery of enzyme activity from several new silica-based bonded phase support materials. Recovery of trypsin activity from such columns was greater than 86% for all supports tested and almost quantitative for some supports (LiChrosorb Diol; SynChropak GPC 100; and TSK 3000SW columns). Fukano et al. (26) using TSK 3000SW and 4000SW columns have reported almost quantitative mass

recovery of a number of proteins and quantitative recovery of activity for several enzymes.

Mass recovery of two proteins, cytochrome C (MW 13,500) and fluorescein conjugated rabbit anti-human IgG (MW 150,000) was determined using a MicroPak TSK 3000SW column (27). Protein recovery was defined as the peak area ratios of injections made with and without a column. As shown in Table 7, recoveries range from 84 to 88%.

To determine recovery of enzyme activity, β-galactosidase (grade IV from *E. coli*, Sigma Chemical Co.) was injected using the assay buffer (0.1 M KH_2PO_4 + 0.01 M KCl + 0.001 M $MgSO_4$ + 0.05 M β-mercaptoethanol, pH 7.0) as mobile phase. Enzyme activity was measured with and without a column using a modification of the ONPG hydrolysis procedure described by Miller (28). β-galactosidase, which has a molecular weight of about 520,000 daltons, elutes within the permeation volume from a MicroPak TSK 4000SW column. Activity recovery of ONPG units was 98%.

Recovery of cytidine kinase activity from MicroPak TSK 3000SW columns has also been determined (W. Kreis, Sloan-Kettering Institute, unpublished results). Two 7.5 mm x 30 cm 3000SW columns were used in series with 50 mM potassium phosphate (pH 6.8) as mobile phase flowing at 1.0 ml/min; 20 μl (containing 0.6 mg protein) of a

Table 7.
Protein Mass Recovery from MicroPak TSK 3000SW Column*

Sample	Detection	Chromophore	Recovery **
Anti-Human IgG, 31 μg	495nm	Fluorescein	88.1
Anti-Human IgG, 31 μg	280nm	Tyrosine, Tryptophan	84.1
Cytochrome C, 10 μg	280nm	Tyrosine, Tryptophan	84.9

* Conditions: Mobile Phase: 0.01 M KH_2PO_4 (pH 7.2) + 0.1 M KCl
Flow Rate: 1.0 ml/min
Temperature: 30°C

** Recovery = $\frac{\text{Total Area With Column}}{\text{Total Area Without Column}}$ X 100

mouse Ascites homogenate was injected and fractions were collected, held on ice, and assayed for cytidine kinase activity after the method of Kreis et al. (29). Activity of the pooled fractions was 70%, 89.3%, and 97.4% of the uninjected sample for three separate injections. (Collected fractions were held on ice for two hours prior to assay, and run-to-run variation may reflect technical variations in the assay).

Calibration curves for MicroPak TSK SW columns using protein standards are displayed in Figure 6. The utility of these columns for biopolymer separations has been demonstrated by Wehr and Abbott (30). Kato et al. (31) demonstrated the TSK 3000SW column to be the most useful SW column for protein separations.

Protein calibration curves for selected MicroPak TSK PW type columns are shown in Figure 7. The adsorption effects of proteins on 3000PW and 5000PW columns are slightly greater than the SW type columns; however, recovery of proteins from these PW type columns is greater than 80% in most cases (32). Hashimoto et al. (33) have shown TSK 3000PW and 4000PW columns to be suitable for biopolymer separations. In general, the 3000PW column has been found to be the most useful PW column pore size for protein analysis.

To compare MicroPak TSK SW and PW columns for protein separations, specific resolution curves using protein standards were constructed for 3000SW and 3000PW columns as shown in Figure 8. These plots clearly show the 3000SW column to provide higher resolution over most of the molecular weight region covered by these columns for protein analysis.

Hara et al. (34) have utilized a TSK 5000PW column and a 3000SW column in series for the analysis of serum lipoproteins. Such a column set takes advantage of the higher molecular weight region over which the larger PW column pore sizes operate and the efficiency gained by use of SW columns.

Spheron packing has been applied to the separation of glycoproteins and protoglycans (47). Although hydrophobic interactions with the packing were found to be significant, such interactions in

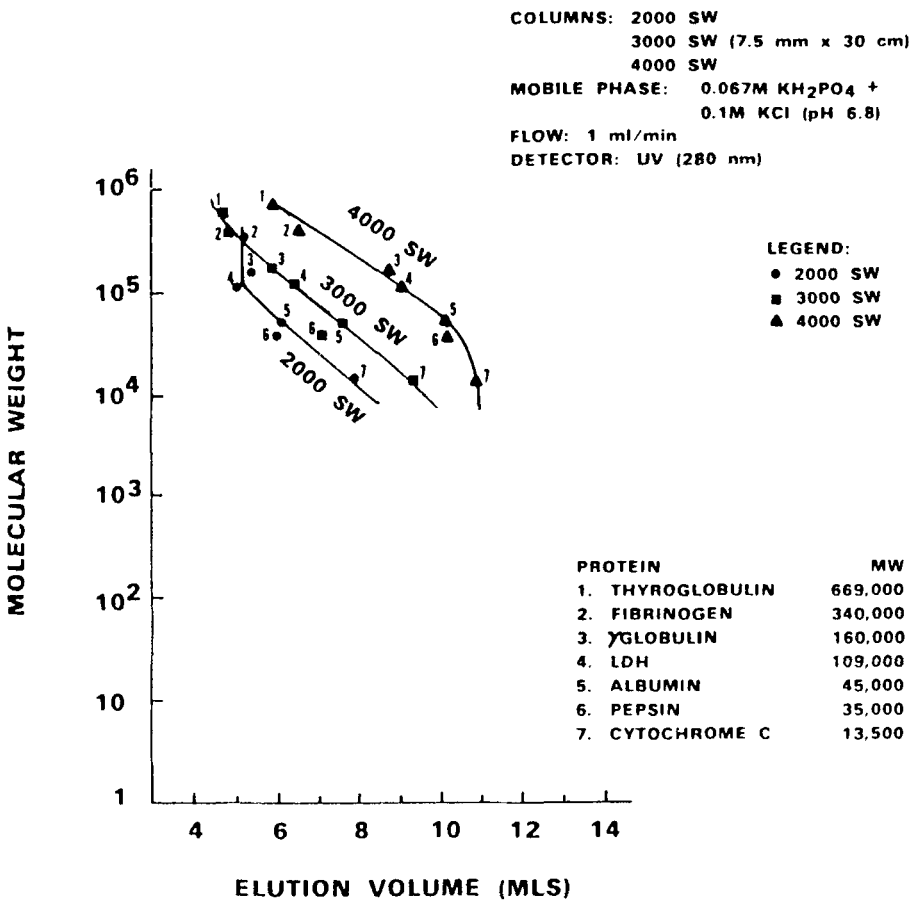

FIGURE 6. Protein standard calibration curves for MicroPak TSK gel type SW columns. Mobile phase: 0.067 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Injection volume: 20 µl; Column dimensions: 7.5 mm x 30 cm; Detector: UV (280nm).

some cases were exploited for the separation of peptides, proteins, and nucleic acids (48,49).

Significant hydrophobic interactions in aqueous mobile phases of Shodex OHpak has been employed in the separation of amino acids (50). This packing has also been applied to the analysis of some peptides.

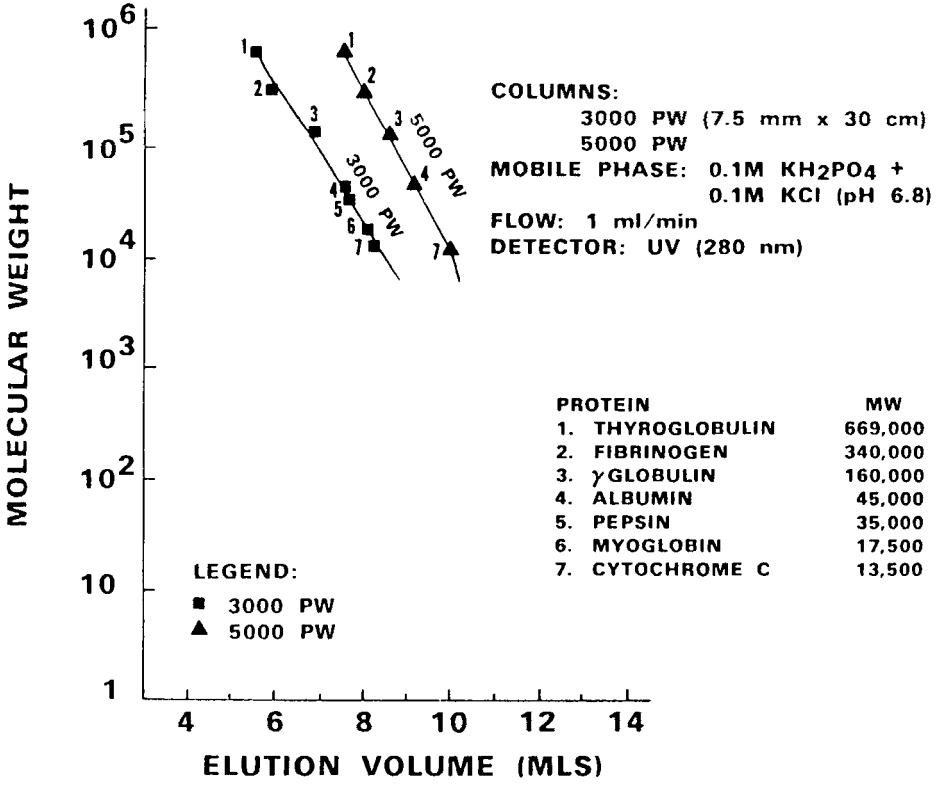

FIGURE 7. Protein standard calibration curves for MicroPak TSK 3000PW and 5000PW columns. Mobile phase: 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Column dimensions: 7.5 mm x 30 cm; Detector: UV (280nm).

Due to hydrophobic interactions, ethylene glycol or SDS has been used as a mobile phase modifier with μBondagel columns for protein characterization (44,51). The Waters Protein Columns have been employed for separation of proteins with acidic to mildly basic isoelectric points and enzymes (51,52,54). Apparently, proteins with higher isoelectric points (> 8) experience varying degrees of adsorption with these columns.

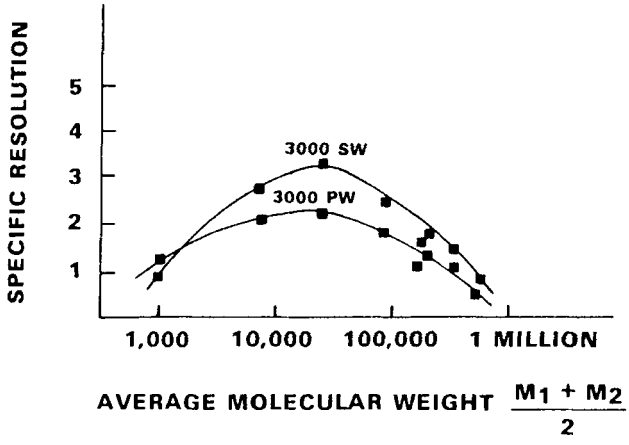

FIGURE 8. Comparison of specific resolution (R_{SP}) curves for MicroPak TSK 3000SW and 3000PW columns using protein standards. Mobile phase: 0.1 M KH_2PO_4 + 0.1 M KCl (pH 6.8); Flow rate: 1 ml/min; Injection volume: 100 µl; Column dimensions: 7.5 mm x 30 cm.

Characterization of Non-Exclusion Effects on MicroPak TSK SW and PW Columns

Non-exclusion effects resulting from solute-support interactions can be broadly classified as arising from hydrophilic interactions (ionic effects such as ion exchange, ion exclusion, ion inclusion) and hydrophobic interactions. Such interactions in aqueous SEC usually result in various degrees of adsorption (35,36).

Examples of hydrophobic interactions on a 2000SW column have been observed for xanthines (20) and pectins (37). In most cases, addition of small amounts of an organic modifier (5-10% MeOH) overcome adsorption effects of this type.

Hydrophobic and other non-exclusion interactions on SW and PW columns were characterized using a series of selected amino acids as test probes. These compounds were chosen in part due to a previously measured "hydrophobicity scale" defined by Rekker (38). Table 8 shows the summation of fragmental hydrophobicity constants for each

Table 8.

Hydrophobic Fragmental Constants of the Common Amino Acids

Amino Acid	Σf
Tryptophan	2.31
Phenylalanine	2.24
Leucine	1.99
Isoleucine	1.99
Tyrosine	1.70
Valine	1.46
Cystine	1.11
Methionine	1.08
Proline	1.01
Cysteine	0.93
Arginine	-
Alanine	0.53
Lysine	0.52
Glycine	0.00
Aspartic Acid	-0.02
Glutamine	-0.07
Histidine	-0.23
Threonine	-0.26
Serine	-0.56
Asparagine	-1.05
Glutamic Acid	-1.09

Where Σf = Summation of Fragmental Hydrophobic Constants.

amino acid. Negative numbers represent hydrophilic amino acids. Positive fragmental constants represent hydrophobic amino acids. The larger the positive summation, the "more hydrophobic" the amino acid. Tryptophan, phenylalanine, leucine, tyrosine, valine, and cysteine were chosen as test probes. To serve as controls, mono-, di-, tri-, and tetraglycine (MW ∿ 100 to 500) were analyzed on each column to ensure differences in amino acid retention were not due to molecular size differences. Glycine oligomers coeluted on all columns tested except the 2000PW column which exhibited some separation. It should also be noted that of the amino acid probes used, tryptophan, phenylalanine, and tyrosine are aromatic, and leucine, valine, and cysteine are non-aromatic compounds.

The amino acid probes were analyzed on MicroPak TSK 2000PW, 3000PW, and 5000PW columns as well as 2000SW, 3000SW, and 4000SW columns with a mobile phase of 0.01 M KH_2PO_4 (pH 6.8). Results are displayed in graph form in Figures 9 and 10. Summation of the hydrophobic fragmental constant is plotted versus k' for the amino acid probes. Several points are noteworthy:

i. A non-linear relationship exists between k' and hydrophobicity as measured by Σf;

ii. The PW columns seem very sensitive to aromatic compounds which exhibit greater retention (higher k's) than the non-aromatic

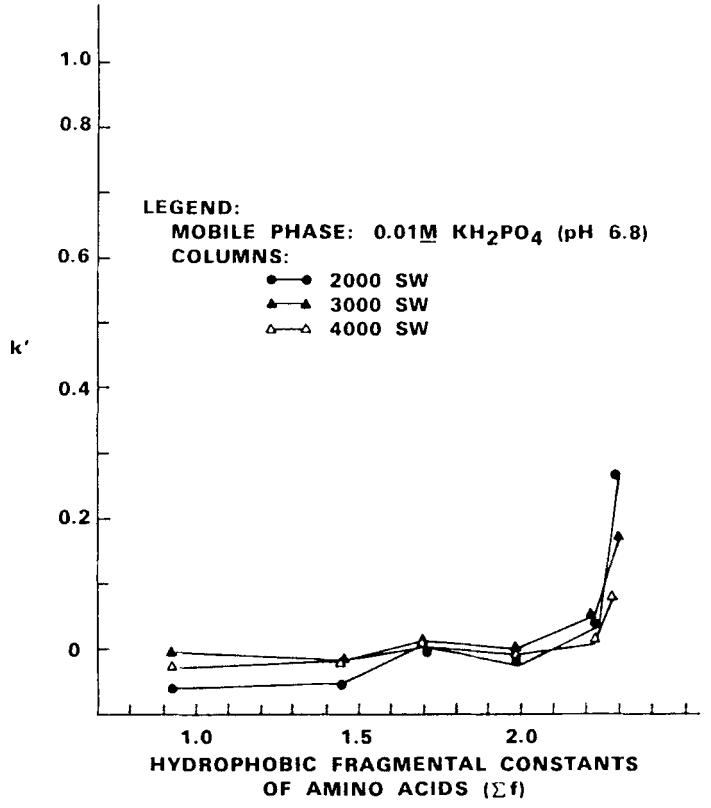

FIGURE 9. Comparison of hydrophobic interactions of MicroPak TSK SW type columns as measured with amino acid test probes. Mobile phase: 0.01 M KH_2PO_4 (pH 6.8); Flow rate: 1 ml/min; Injection volume: 100 µl; Column dimensions: 7.5 mm x 30 cm.

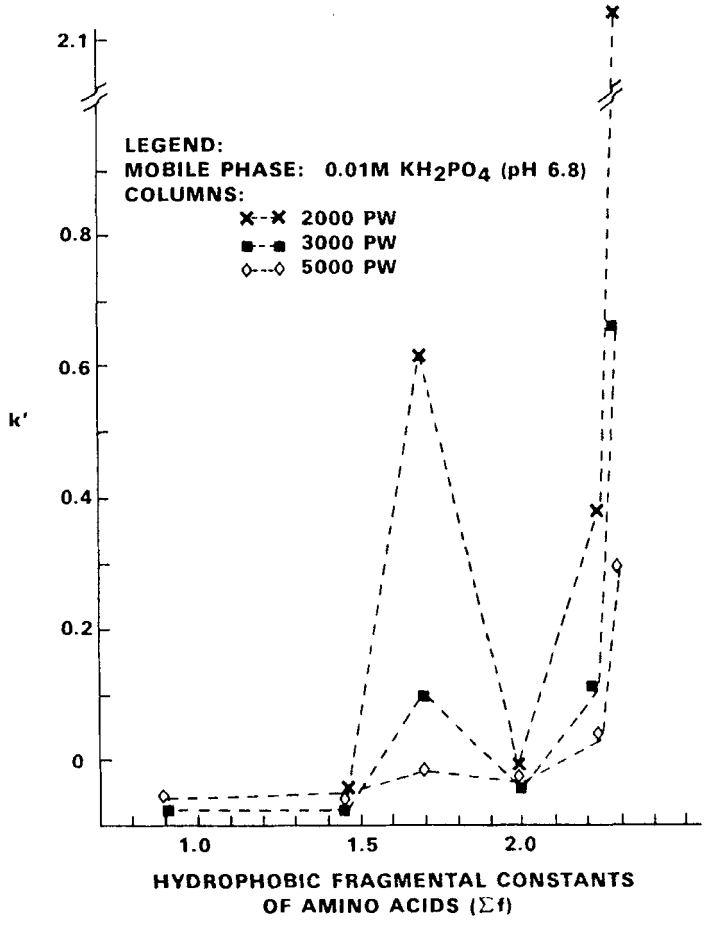

FIGURE 10. Comparison of hydrophobic interactions of MicroPak TSK Gel 2000PW, 3000PW, and 5000PW columns as measured with amino acid test probes. Conditions same as in Figure 9.

compounds. This is clearly demonstrated for tyrosine (Σf = 1.7);

iii. The sensitivity to aromatic compounds of the PW columns greatly increase with decreasing pore size.

Ion exchange and ion exclusion interactions were evaluated using charged and neutral amino acid test probes of similar molecular weights:

Pair 1: Methionine - neutral, MW 149
Lysine - net charge (+), MW 146
Pair 2: Threonine - neutral, MW 119
Aspartic acid- net charge (-), MW 133

These pairs of hydrophilic amino acid test probes were analyzed on PW and SW columns in mobile phases of increasing ionic strength. Ionic effects as measured by retention volume (k') as a function of ionic strength were negligible on both SW and PW columns using amino acid solute probes. However, Pfannkoch *et al.* (see reference 7) have shown TSK 3000SW column packing to have negative charge, resulting in ion-exclusion behavior with citric acid. Increasing mobile phase ionic strength above 0.12 M was shown to eliminate this effect.

CONCLUSION

Most commercially available microparticulate supports characterized and compared in this work exhibit varying degrees of non-exclusion effects in aqueous SEC separations. All the surface-modified, silica-based supports function as weak cation exchangers (see reference 7). All organic gel-based supports have some tendency toward hydrophobic interaction with a variety of solutes. In many instances, however, such non-exclusion effects can be exploited to achieve the desired chromatographic separation.

Chromatographic performance of commercially available aqueous SEC columns seems to be best judged by comparison of specific resolution values over the molecular weight range of operation. However, due to lack of comparative data of this type for most aqueous SEC columns, performance can be estimated based upon the slope of the calibration curve in the selective permeation range (linear molecular weight separation range) as well as efficiency and pore volume data.

A comparison of MicroPak TSK SW and PW columns reveals SW type columns have a more limited molecular weight separation range than PW type columns, although SW columns exhibit higher efficiency and resolution. Due to both limited pore size range and adsorption effects of polar, synthetic, water-soluble polymers on SW columns, PW

columns are recommended for the analysis of synthetic water-soluble polymers. SW type columns offer higher efficiency and resolution than PW type columns for protein analysis (3000SW most useful). Small pore size PW columns (1000PW and 2000PW) are best suited for small molecule analysis (MW 100 to 1000) as evidenced by large specific resolution values for PEG standards.

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