No. 095

TOSOH

SEPARATION REPORT

TSKgel SuperSW Series

Table of Contents

1. Introduction

High-performance liquid chromatography (HPLC) is widely used as a separation/purification method in the field of biopolymers due to its rapidness, ease of use and sensitivity. In particular, size-exclusion chromatography (SEC) has been used in protein separation/purification over the years because it separates samples by its molecular size as separation mode. While soft packing materials with reticulate structure such as dextran, agarose, etc. were employed as packing materials for early SEC, silica-type packing materials with high strength also have come to be employed for SEC in HPLC.

Our TSKgel SW series is a group of silica-type SEC packing materials with pore size distribution suited to protein separation, and is used throughout the world for its excellent resolution.

In recent years, speediness and high resolution are demanded in the field of HPLC as they have been in the present. However, demand for high sensitivity that is applicable to trace analysis is on the increase as sample grows smaller in size and lower in concentration. In other HPLC separation modes, for instance, semi-micro columns which are applicable to trace analysis have already been commercialized for reversed phase chromatography (RPC). Demand for high sensitivity, high resolution columns also grows in the field of SEC along with the application of HPLC to trace analysis.

This report describes the features, basic properties, and applications of TSKgel Super-SW series, in which particles have been made smaller than the conventional TSKgel SW series and column has been downsized to pursue high sensitivity and high resolution.

2. Features

Table-1 shows the specifications of TSKgel SuperSW and SW_{XL} series. In Table-2, separation ranges of TSKgel SuperSW series for polyethylene glycol (PEG), dextran, and protein are shown. The column size of TSKgel SuperSW series is 4.6mm I.D. × 30cm. Since the packing materials with smaller particle size compared to the conventional high performance column is employed, TSKgel SW_{XL}, the guaranteed theoretical plates is approximately 1.5 times higher than TSKgel SW_{XL} series as shown in Table-1.

In Figures-1 and -2, calibration curves of TSKgel SuperSW series for standard polyethylene glycols (PEG) and standard proteins are shown, respectively. Since TSKgel SuperSW series has the same calibration curve as the conventional TSKgel SW_{XL} series with equivalent grade, it has the same molecular weight separation range. In general, TSKgel SuperSW2000 is suited for separation of proteins with molecular weight of 70,000 or smaller, and TSKgel SuperSW3000 is suited for separation of proteins with molecular weight of 70,000 to 300,000.

Figures-3 and -4 show the chromatograms of standard proteins on TSKgel SuperSW series and the conventional TSKgel SW_{XL} series. UV detector with micro flow cell was used. In Table-3, resolution (Rs) calculated from these chromatograms are shown. It is clear from the table that TSKgel SuperSW series has approximately 1.2 times better resolution compared to TSKgel SW_{XL} series.

Figures-5 and -6 show comparison of analysis time under the condition that resolution is nearly equal between TSKgel SW_{XL} series and TSKgel SuperSW series. Since TSKgel SuperSW series has high resolution, the same resolution as conventional one can be obtained in a short time of about 1/2.

Table-1 Specifications of TSKgel SuperSW Series and TSKgel SW_{XL} Series

	Particle size (μm)	Column size	Guaranteed theoretical plates
TSKgel SuperSW2000	4	4.6mm I.D. × 30cm	30,000
TSKgel SuperSW3000	4	4.6mm I.D. × 30cm	30,000
TSKgel G2000SW_{XL}	5	7.8mm I.D. × 30cm	20,000
TSKgel G3000SW_{XL}	5	7.8mm I.D. × 30cm	20,000

Table-2 Molecular Weight Separation Range of TSKgel SuperSW Series

	Molecular weight separation range	
	SuperSW2000	SuperSW3000
Polyethylene glycol	500- 15,000	1,000- 35,000
Dextran	1,000- 30,000	2,000- 70,000
Protein	5,000-100,000	10,000-500,000

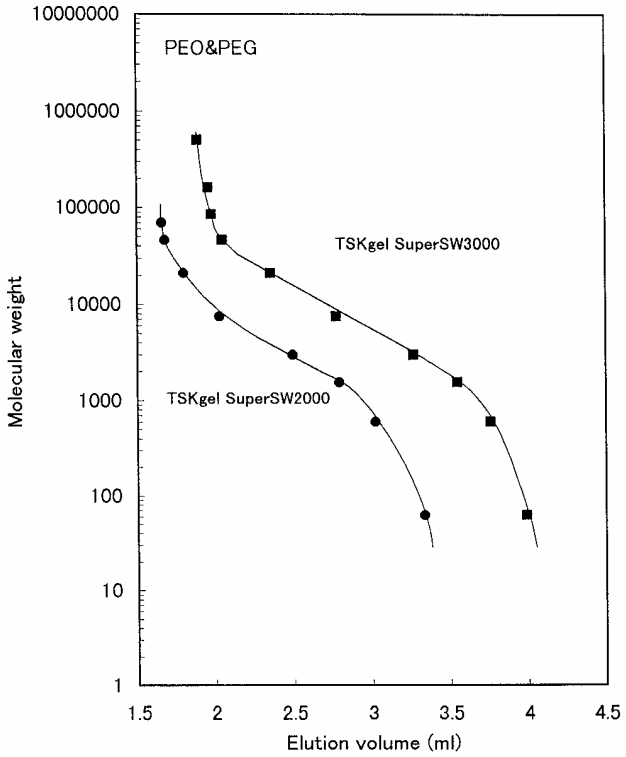

Figure-1 PEO&PEG Calibration Curves for TSKgel SuperSW Series

Column: TSKgel SuperSW Series
(4.6mm I.D. × 30cm)
Eluent: 0.05% sodium azide aqueous solution
Flow rate: 0.35mL/min
Temperature: 25°C
Detection: Refractive index detector
Samples: PEO, PEG (5μL)

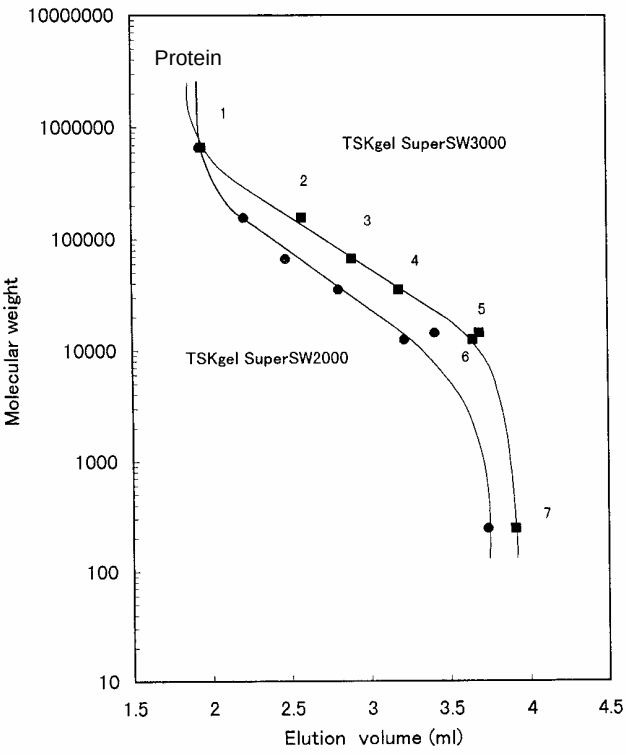

Figure-2 Protein Calibration Curves for TSKgel SuperSW Series

Column: TSKgel SuperSW Series
(4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm)
Samples: Standard proteins (5μL, 0.1g/L each)
1. Thyroglobulin
2. γ-globulin
3. Bovine serum albumin
4. β-lactoglobulin
5. Lysozyme
6. Cytochrome C
7. Glycine tetramer

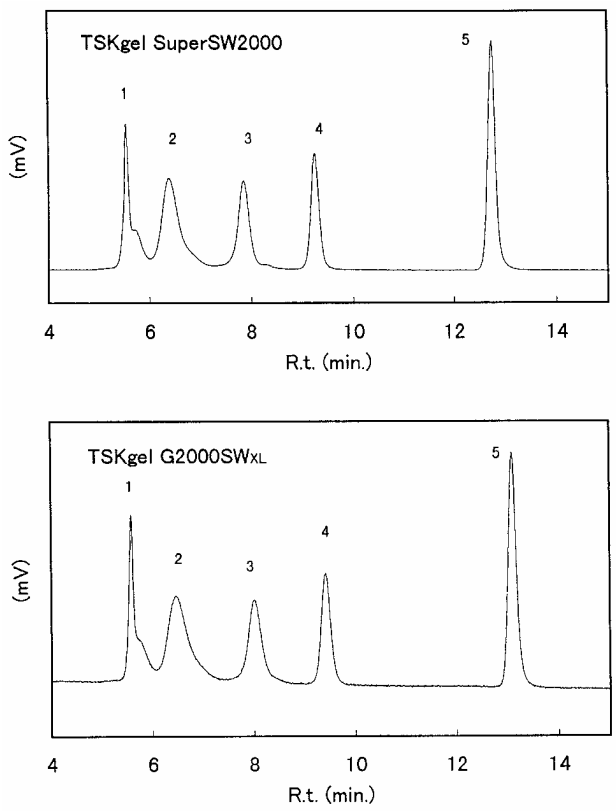

Figure-3 Comparison between TSKgel SuperSW2000 and TSKgel G2000SW$_{XL}$

Column: TSKgel SuperSW2000 (4.6mm I.D. × 30cm)
TSKgel G2000SW$_{XL}$ (7.8mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH6.7)
Flow rate: 0.35mL/min (TSKgel SupperSW2000)
1.00mL/min (TSKgel G2000SW$_{XL}$)
Detection: UV (280nm), micro flow cell
Samples: Standard proteins (5μL)
1. Thyroglobulin (0.5g/L)
2. γ-globulin (1g/L)
3. Ovalbumin (1g/L)
4. Ribonuclease A (1g/L)
5. p-aminobenzoic acid (0.01g/L)

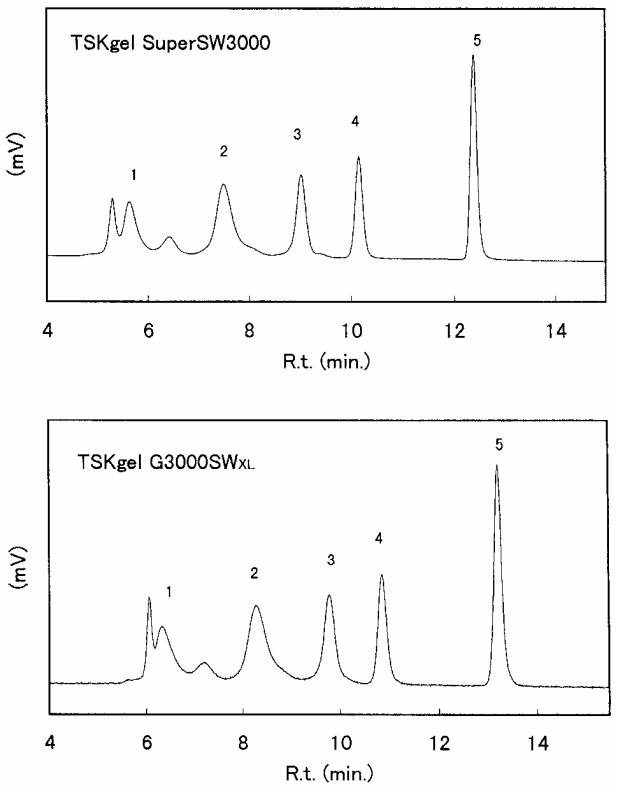

Figure-4 Comparison between TSKgel SuperSW3000 and TSKgel G3000SW$_{XL}$

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
TSKgel G3000SW$_{XL}$ (7.8mm I.D. × 30cm)
* Separation conditions are the same as Figure-3.

Table-3 Comparison of Resolution between TSKgel SupwerSW Series and TSKgel SW$_{XL}$ Series

	Resolution (Rs)	
	SuperSW2000	G2000SW$_{XL}$
Thyroglobulin		
	2.29	2.24
γ-globulin		
	3.15	2.85
Ovalbumin		
	4.15	3.55
Ribonuclease A		
	12.48	11.62
p-aminobenzoic acid		

	Resolution (Rs)	
	SuperSW3000	G3000SW$_{XL}$
Thyroglobulin		
	3.61	—
γ-globulin		
	3.39	2.79
Ovalbumin		
	3.73	2.94
Ribonuclease A		
	8.63	7.67
p-aminobenzoic acid		

* UV detector with micro flow cell

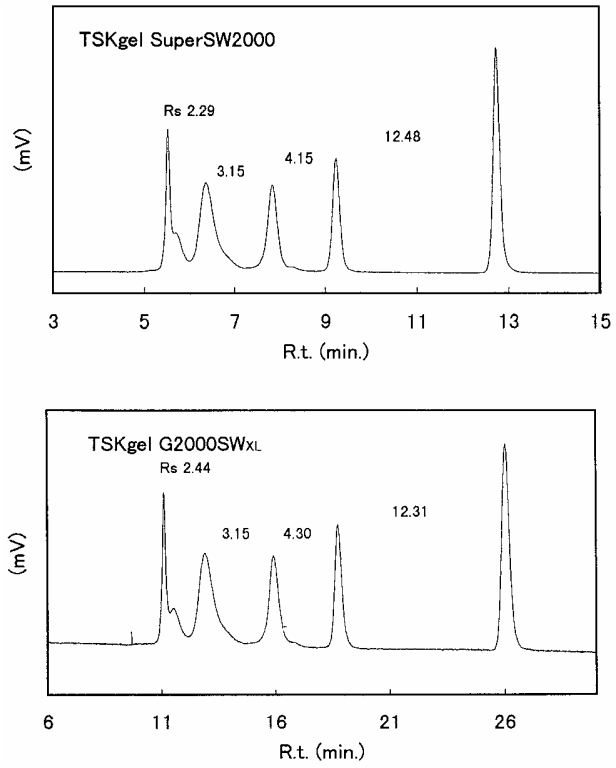

Figure-5 Comparison between TSKgel SuperSW2000 and TSKgel G2000SW$_{XL}$

Column: TSKgel SuperSW2000 (4.6mm I.D. × 30cm)
TSKgel G2000SW$_{XL}$ (7.8mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH6.7)
Flow rate: 0.35mL/min (TSKgel SupperSW2000)
0.50mL/min (TSKgel G2000SW$_{XL}$)
Detection: UV (280nm), micro flow cell
Samples: Standard proteins (5μL)
1. Thyroglobulin (0.5g/L)
2. γ-globulin (1g/L)
3. Ovalbumin (1g/L)
4. Ribonuclease A (1g/L)
5. p-aminobenzoic acid (0.01g/L)

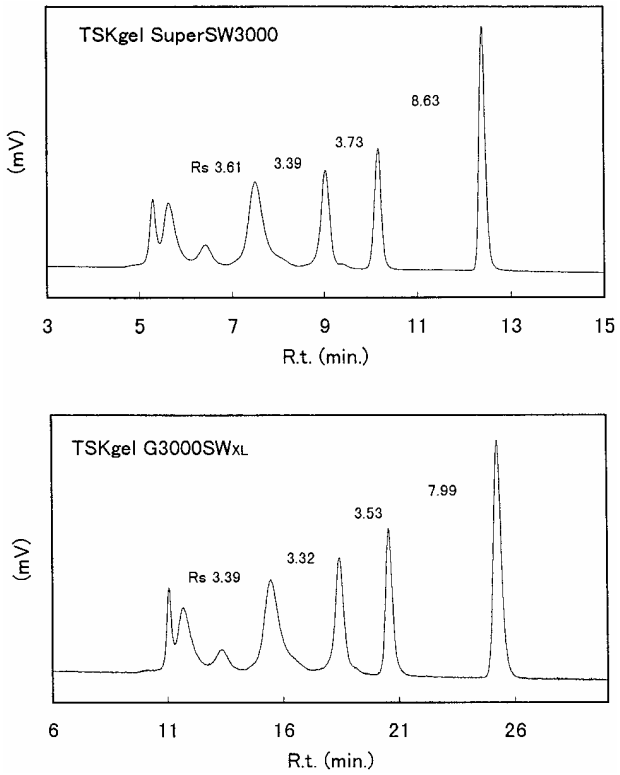

Figure-6 Comparison between TSKgel SuperSW3000 and TSKgel G3000SW$_{XL}$

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
TSKgel G3000SW$_{XL}$ (7.8mm I.D. × 30cm)
* Separation conditions are the same as Figure-5.

3. Basic Properties

3-1 Optimization of Equipment

Although TSKgel SuperSW series is a high-performance SEC column with high resolution and high sensitivity, it is necessary to optimize the equipment especially detector cell and tubing in order to maximize column performance. In this section, optimization of equipment is described.
In columns with small column volume such as TSKgel SuperSW series, the void volume of equipment has large influence on column efficiency. The following 3 factors are considered as the void volume of equipment.

- Void volume of tubing
- Cell volume of detector
- Void volume in injection unit

In TSKgel SuperSW series, it is necessary to suppress solute dispersion in these void volumes.

3-1-1 Void Volume of Tubing

In Figure-7, the effect of volume of tubing between injector/column and column/detector on column efficiency is shown. As the volume of tubing increases, dispersion of solute within the tubing increases and deteriorates the column efficiency. With TSKgel SuperSW series, column efficiency begins to deteriorate when volume of tubing exceeds 10μL (0.1mm I.D. × 150cm). It is desired that 0.1mm I.D. X 100cm or shorter tubing should be used between injector/column and column/detector with TSKgel SuperSW series. A set of two 0.1mm I.D. × 40cm pipes, “connection pipe set, type L,” (including two pipes, product No. 018186) is available from our lineup.

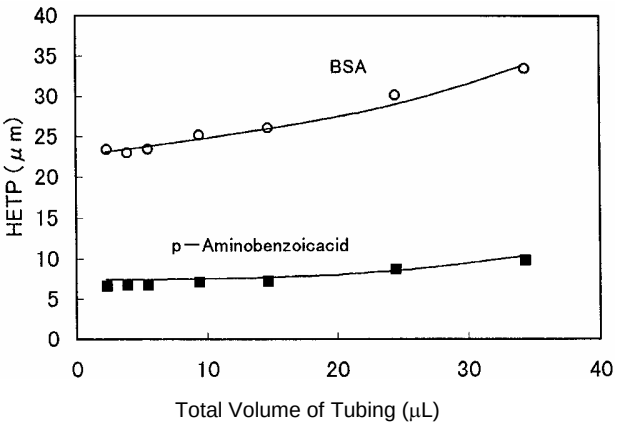

Figure-7 Effect of Total Volume of Tubing on HETP

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.1mol/L phosphate buffer + 0.1mol/L sodium sulfate + 0.05% sodium azide (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm)
Sample: Bovine serum albumin, p-aminobenzoic acid
* The volume of tubing is the total volume between injector/column and column/detector.

3-1-2 Cell Volume of Detector

Figure-4 shows the effect of detector cell. Although column efficiency deteriorates somewhat for low dead volume type cells (standard cells from which heat sink has been removed) because they have large volume compared to micro flow cell, rate of deterioration can be suppressed within 5%. However, since a standard cell has heat sink with approximately 30μL, column efficiency deteriorates by approximately 30%.
On the other hand, sensitivity is proportional to the length of light path in the cell. Figure-8 shows the chromatograms when micro flow cell or low dead volume type cell is used. With a low dead volume type cell with light path length of 10mm, 2.5 times sensitivity is obtained compared to micro flow cell with light path length of 4mm. In TSKgel SuperSW series, it is necessary that micro flow cell should be used when high resolution is required, and low dead volume type cell should be used when high sensitivity is required. Furthermore, sensitivity of TSKgel SuperSW series is improved by approximately 3 times compared to TSKgel SW_{XL} series even when a normal standard cell is used.

Table-4 Effect of Cell Volume on Column Efficiency

Cell volume	Theoretical plates of column (rate of deterioration in theoretical plates)
2μL (micro flow cell)	41,199 (0%)
10μL (low dead volume type cell)	40,189 (2.5%)
10μL (standard cell)	30,855 (25%)

Low dead volume type: Standard cells from which heat sink has been removed (type L tubing is used.)
Column: TSKgel SuperSW3000
Eluent: 0.2mol/L phosphate buffer (pH 6.7), Sample: p-aminobenzoic acid

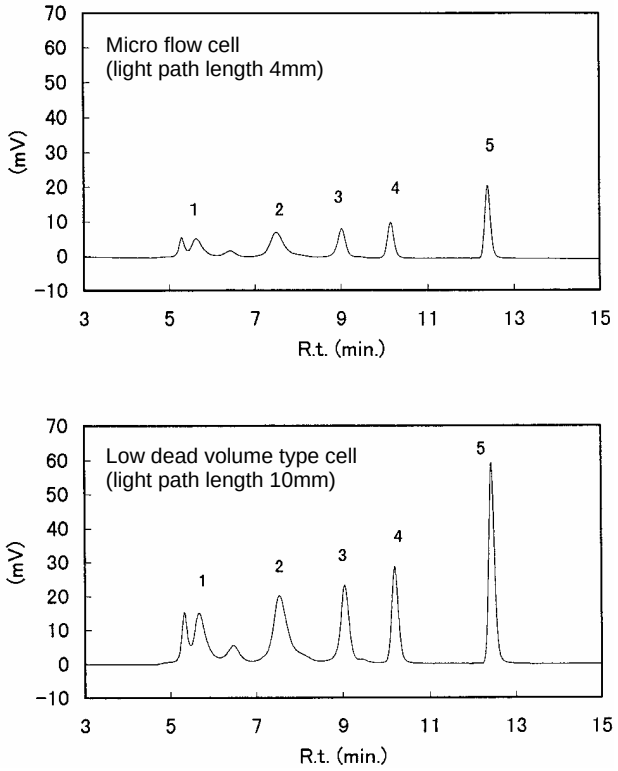

Figure-8 Comparison of Peak Heights between the Different Cell

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
* Separation conditions are the same as Figure-3.

3-1-3 Injector

Figure-9 shows the effect of injector and detector cell. The effect of injector and cell was examined, with the column efficiency when low-diffusion type injector (Rheodyne 8125) and micro flow cell are used, which is expected to have the smallest peak broadening, set to 100%. It is clear that dispersion of solute inside the injector was large with general-purpose injector (Rheodyne 7125) even when micro flow cell is used, and that the column efficiency deteriorates by approximately 10%. In the case of combining general-purpose injector and standard cell, the column efficiency deteriorates by 20% or more.

In order to exert the TSKgel SuperSW column performance sufficiently, it is desired that low-diffusion type injector is used. Furthermore, when auto-sampler is used, use auto-sampler for trace injection.

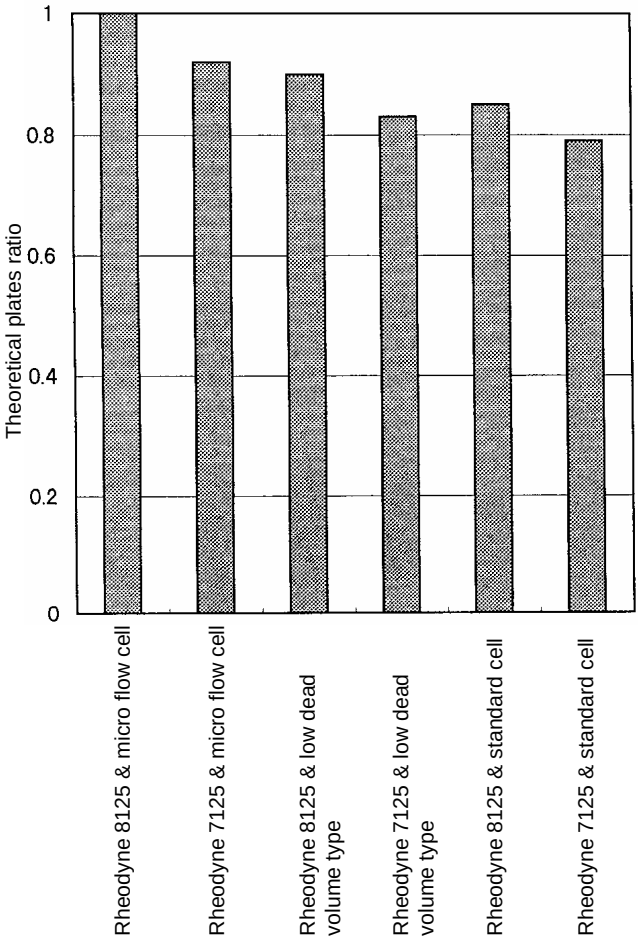

Figure-9 Effect of Injector and Detector Cell on Column Efficiency

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.1mol/L phosphate buffer + 0.1mol/L sodium sulfate + 0.05% sodium azide (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm)
Sample: p-aminobenzoic acid (5μL)

3-2 Sensitivity

Figures-10 and -11 show chromatograms to compare the peak height of standard proteins on TSKgel SuperSW series and TSKgel SW_{XL} series. It is evident that TSKgel SuperSW series can yield peak height approximately 4 times that of TSKgel SW_{XL} series due to downsizing in column and increased theoretical plates.

Table-5 shows the limits of detection for major proteins. Although limit of detection varies depending on sample, separation conditions, detection wavelength and light path length of the cell, it is approximately 1/2 – 1/3 of the SW_{XL} series when cell with light path length of 10mm (low dead volume type) is used with TSKgel SuperSW series, and analysis with nanogram order is also possible. TSKgel SuperSW series can be recommended as a SEC column that is suited for trace analysis.

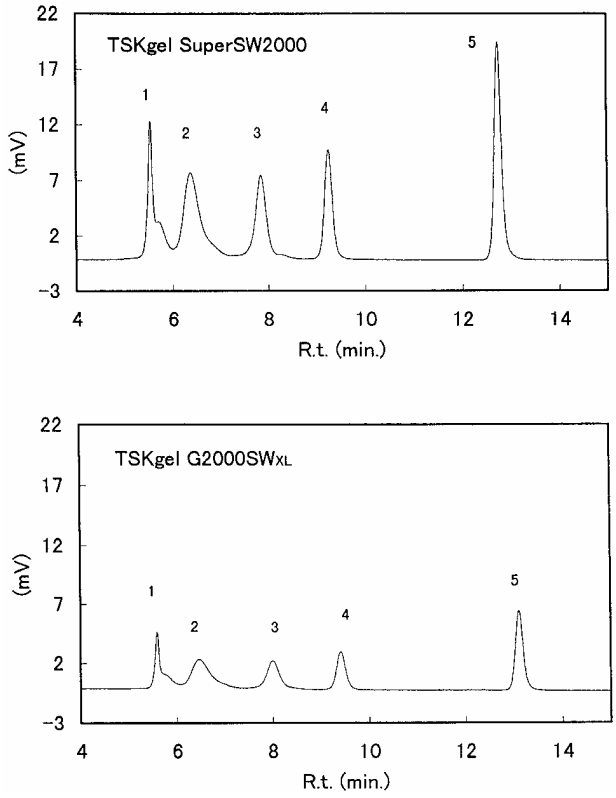

Figure-10 Comparison of Sensitivity between TSKgel SuperSW2000 and TSKgel G2000SW$_{XL}$

Column: TSKgel SuperSW2000 (4.6mm I.D. × 30cm)
TSKgel G2000SW$_{XL}$ (7.8mm I.D. × 30cm)
* Separation conditions are the same as Figure-3.

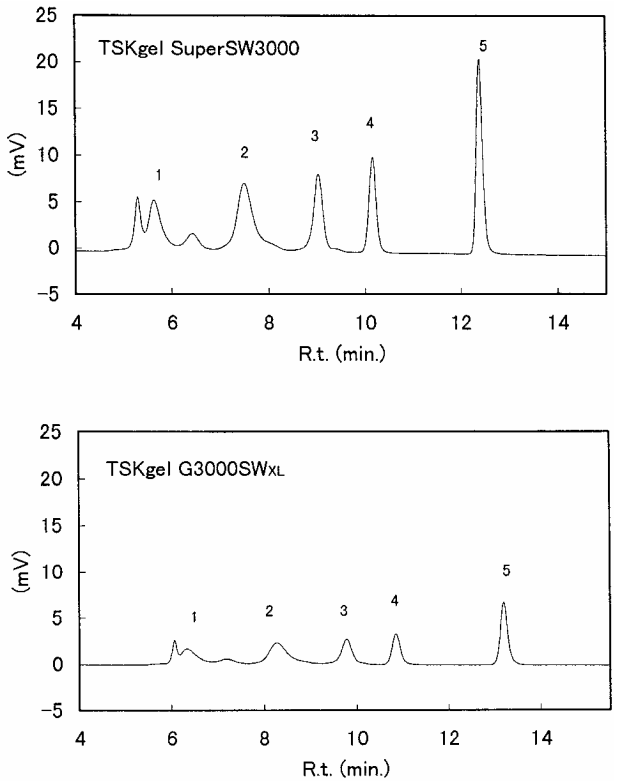

Figure-11 Comparison of Sensitivity between TSKgel SuperSW3000 and TSKgel G3000SW$_{XL}$

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
TSKgel G3000SW$_{XL}$ (7.8mm I.D. × 30cm)
* Separation conditions are the same as Figure-3.

Table-5 Limit of Detection for Proteins (S/N = 3)

	SuperSW3000		G3000SW$_{XL}$
	Standard cell (low dead volume type) Light path length 10mm	Micro flow cell 4mm	Standard cell (low dead volume type) 10mm
Thyroglobulin	70ng	300ng	200ng
γ-globulin	50ng	100ng	100ng
Bovine serum albumin	70ng	300ng	200ng
Ovalbumin	50ng	100ng	100ng
Myoglobin	15ng	50ng	30ng

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Detection: UV (280nm)

3-3 Flow Rate Dependence of Height Equivalent to a Theoretical Plate (HETP)

The effect of flow rate on height equivalent to a theoretical plate (HETP) depends on particle size of packing materials, sample molecular size, eluent viscosity, etc. A typical example of HETP's flow rate dependency on TSKgel SuperSW series and TSKgel SW_{XL} series using bovine serum albumin (BSA) and myoglobin is shown in Figure-12. It is clear that TSKgel SuperSW series has small HETP throughout the full flow rate range and small flow rate dependence compared to TSKgel SW_{XL} series since the particle size is small. The appropriate flow rate for TSKgel SuperSW series is 0.1 – 0.35mL/min.

In Figure-13, chromatograms of commercial molecular weight markers at respective flow rate are shown. Table-6 shows the resolution (Rs) calculated from the chromatograms. When flow rate is lower, separation of high polymer protein is improved, and resolution calculated at flow rate of 0.35mL/min is twice of that of 0.05mL/min. Although TSKgel SuperSW series have smaller flow rate dependency than conventional ones, please use it at lower flow rate when higher resolution is required.

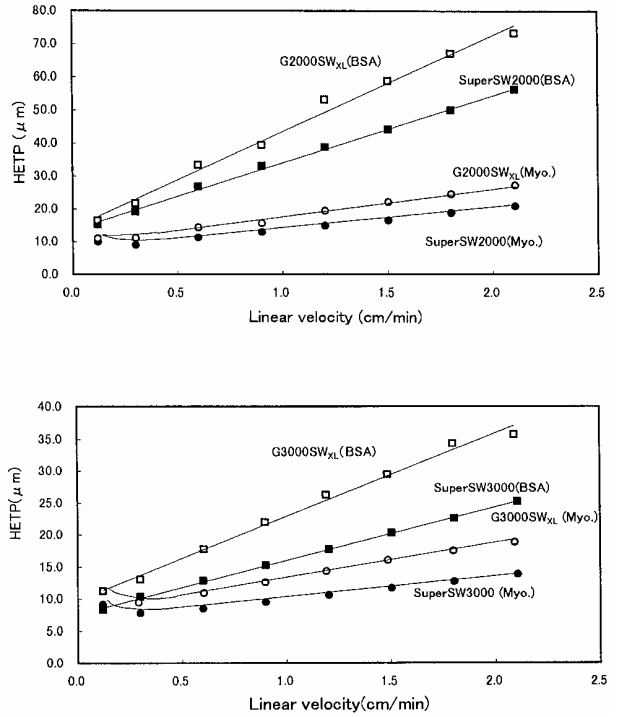

Figure-12 Relationship between Flow Rate and HETP in TSKgel SuperSW Series and TSKgel SW_{XL} Series

Column: TSKgel SuperSW series (4.6mm I.D. × 30cm)
TSKgel SW_{XL} series (7.8mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Detection: UV (280nm), micro flow cell
Samples: Standard proteins (5μL)
Bovine serum albumin (1g/L)
Myoglobin (1g/L)

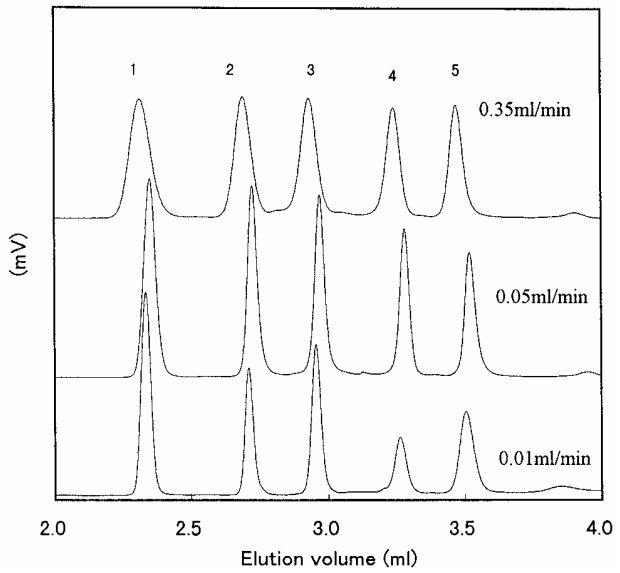

Figure-13 Effect of Flow Rate on Separation

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.1mol/L phosphate buffer + 0.1mol/L sodium sulfate + 0.05% sodium azide (pH 6.7)
Flow rate: 0.01, 0.05, 0.35mL/min
Temperature: 25°C
Detection: UV (280nm), micro flow cell
Samples: 1. Glutamate dehydrogenase
2. Lactate dehydrogenase
3. Enolase
4. Adenylate kinase
5. Cytochrome C

Table-6 Relationship between Flow Rate and Resolution

	Resolution (Rs)		
	0.35mL/min	0.05mL/min	0.01mL/min
Glutamate dehydrogenase			
	2.91	5.10	6.13
Lactate dehydrogenase			
	2.13	3.78	4.12
Enolase			
	2.97	4.79	4.75
Adenylate kinase			
	2.44	3.50	3.18
Cytochrome c			

3-4 Sample Load

Figure-14 shows the effect of sample load on HETP under a constant injection volume. Although HETP is small in TSKgel SuperSW series than in TSKgel SW_{XL} series, it is obvious that it increases drastically at load of 100μg or larger. It is also apparent that TSKgel SuperSW series should be used under load of 100μg or smaller.
In Figure-15, the effect of injection volume on HETP under a constant sample concentration is shown. It is obvious that the injection volume at which HETP starts changing is approximately 10μL for TSKgel SuperSW series, and that it is smaller than that of TSKgel SW_{XL} series.
It is surmised that the desired sample load of TSKgel SuperSW series are 100μg or smaller as total amount 10μL or smaller as injection volume.

3-5 Recovery of Protein

Table-7 shows the recovery of protein at sample concentration of 20μg/mL (sample load 100ng). With TSKgel SW_{XL} series, recovery of thyroglobulin at the sample load of 1μg was 70% level. In addition, recovery deteriorated with sample load of 1μg or smaller (see our separation report No.46). On the other hand, it was found that most protein was recovered quantitatively with TSKgel SuperSW series even under the sample load of 100ng. TSKgel SuperSW series is capable of obtaining high protein recovery even in trace analysis with sample load of 1μg or smaller.
While TSKgel SuperSW series has high recovery even with small sample concentration, sample might be adsorbed by HPLC system other than the column (tubing, etc.) in HPLC trace analysis. It is important that similar sample is injected several times before measurement so that the adsorption point within the system is inactivated in advance when trace analysis is performed.

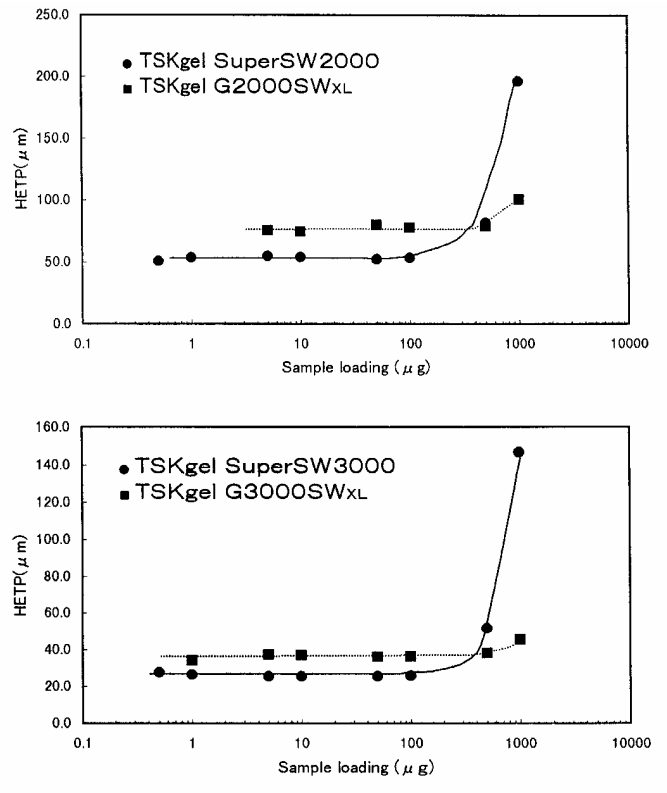

Figure-14 Relationship between Sample Load and HETP in TSKgel SuperSW Series and TSKgel SW_{XL} Series

Column:	TSKgel SuperSW series (4.6mm I.D. × 30cm) TSKgel SW_{XL} series (7.8mm I.D. × 30cm)
Eluent:	0.2mol/L phosphate buffer (pH 6.7)
Flow rate:	0.35mL/min (TSKgel SuperSW series) 1.00mL/min (TSKgel SW_{XL} series)
Detection:	UV (280nm), micro flow cell
Sample:	Bovine serum albumin (5μL)

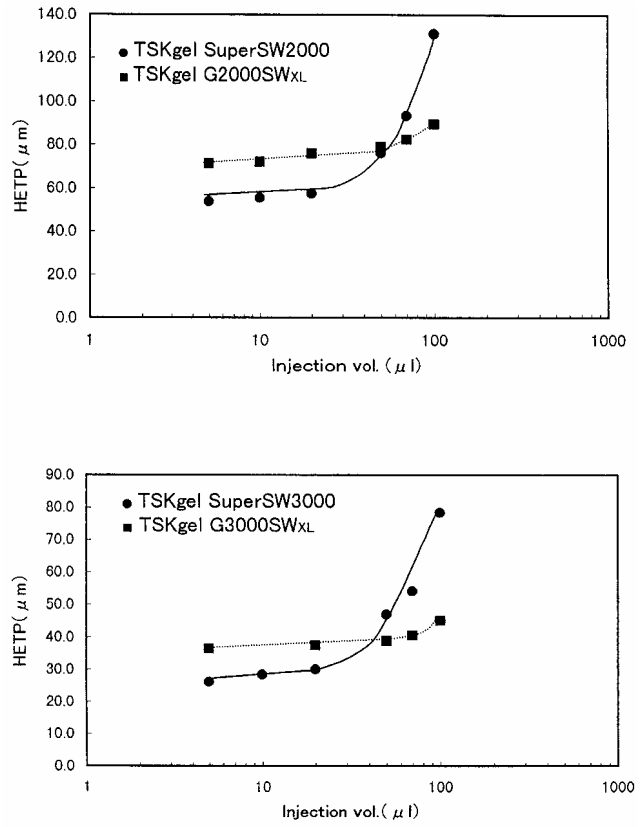

Figure-15 Relationship between Injection Volume and HETP in TSKgel SuperSW Series and TSKgel SWXL Series

Column: TSKgel SuperSW series (4.6mm I.D. × 30cm)
TSKgel SW_{XL} series (7.8mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min (TSKgel SuperSW series)
1.00mL/min (TSKgel SW_{XL} series)
Detection: UV (280nm), micro flow cell
Sample: Bovine serum albumin (0.2g/L)

Taible-7 Recovery of Protein

	SuperSW2000	SuperSW3000
Thyroglobulin	86%	97%
γ-globulin	90%	90%
Bovine serum albumin	99%	86%
Ovalbumin	97%	98%
Ribonuclease A	86%	87%
Myoglobin	93%	96%
Cytochrome C	85%	90%
Lysozyme	93%	89%

Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm), micro flow cell
Sample: 100ng (20mg/L, 5μL)

4. Applications of TSKgel SuperSW Series

Figure-16 shows an example of peptide mixture separation on TSKgel SuperSW2000. Figures-17, -18 and -19 show chromatograms of commercial glutamic acid-oxalacetic acid transaminase, mouse ascites monoclonal antibody (IgG1) and human serum on TSKgel SuperSW3000.

5. Conclusion

TSKgel SuperSW series is a group of columns in which particle size and column size of the conventional TSKgel SW_{XL} series have been reduced at the same time to improve resolution and sensitivity. Resolution has been improved to 1.2 – 1.5 times, and sensitivity to about 2 – 3 times compared to the conventional TSKgel SW_{XL} series. Furthermore, it maintains high recovery even for sample injection at a low concentration, and it is suited to trace analysis of biopolymers.
In order to exert the better performance of TSKgel SuperSW series, please use in a equipment with minimized dead volume. Peak broadening outside the column is a major cause of deteriorated separation performance. Table-8 summarizes the cautions in using TSKgel SuperSW series.

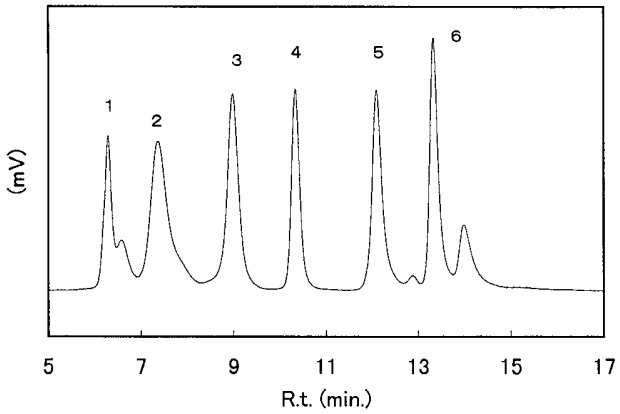

Figure-16 Separation of Mixture of Protein/peptide

Column: TSKgel SuperSW2000 (4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (220nm), micro flow cell
Sample: Protein/peptide mixture (5μL)
1. Thyroglobulin (0.1g/L)
2. γ-globulin (0.2g/L)
3. Ovalbumin (0.2g/L)
4. Myoglobin (0.1g/L)
5. Insulin (0.1g/L)
6. Oxytocin (0.1g/L)

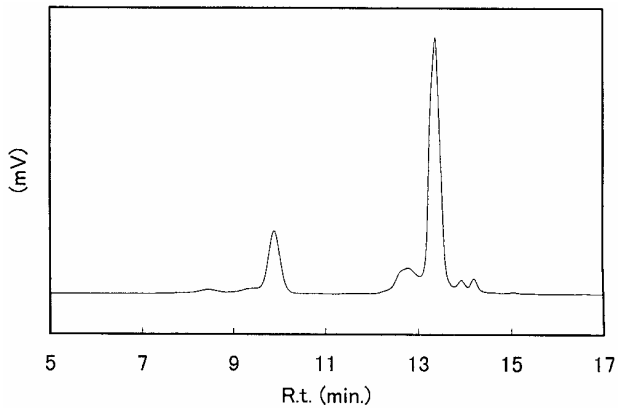

Figure-17 Separation of Commercial Glutamic Acid-Oxalacetic Acid Transaminase

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm), micro flow cell
Sample: Glutamic acid-oxalacetic acid transaminase (1g/L, 5μL)

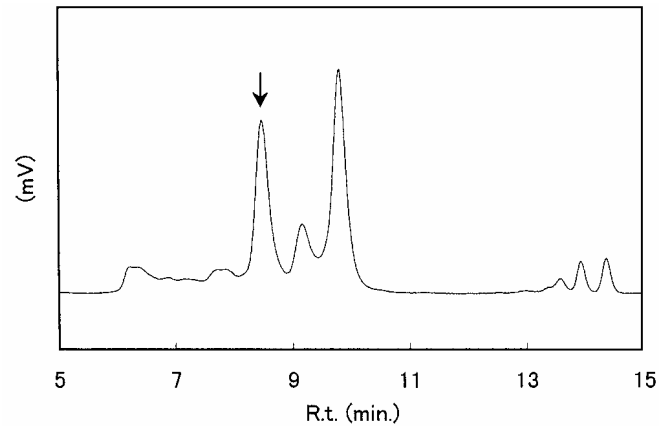

Figure-18 Separation of Mouse Ascites Monoclonal Antibody (IgG1)

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm), micro flow cell
Sample: Mouse ascites (5μL)

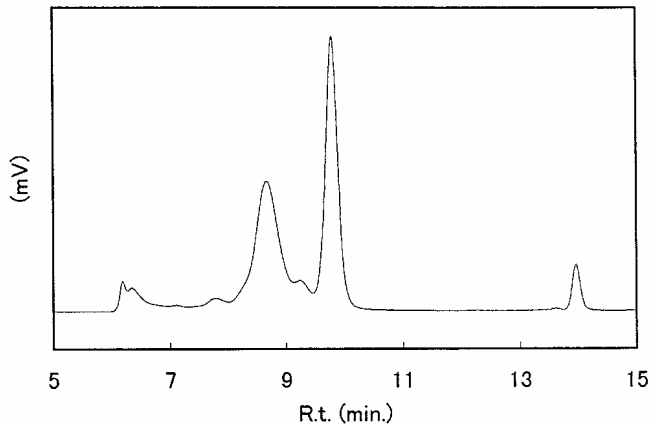

Figure-19 Separation of Human Blood Serum

Column: TSKgel SuperSW3000 (4.6mm I.D. × 30cm)
Eluent: 0.2mol/L phosphate buffer (pH 6.7)
Flow rate: 0.35mL/min
Detection: UV (280nm), micro flow cell
Sample: Human serum (5μL)

Table-8 Notes to Be Made in Using TSKgel Super SW Series

* Reduce peak broadening in tubing, detector, etc.
* Take care of sample overloading.
* Take care of flow rate of pumping system since its flow rate is low.

Tubing:

Use 0.1mm I.D. tubing. It is recommended that the total tubing length is 100cm or shorter.
Connection pipe set type L (product No. 018186: 0.1mm I.D. × 40cm, 2 pieces) available; connection surface (both ends) with fine-cut finishing
Sections requiring 0.1mm I.D. tubing
a) Between injection valve/column inlet, or auto-sampler/column inlet
b) Between column outlet/detector inlet (tubing on inlet side of the detector)

Pumping system:

Pumping system should be applicable to semi-micro HPLC.
Flow rate should be 0.1 – 0.35mL/min.

Injector:

Low-diffusion type injector (Rheodyne8125) is recommended.

Guard column:

Be sure to connect a guard column (product No. 18762) to protect the column.(A set of connection tubing is a standard accessory to the guard column.)

Detector:

For UV detectors, use micro flow cells or low dead volume type cells. Low dead volume type cells are effective in high-sensitivity analysis.
(Use of standard cell is also possible. However, theoretical plates will be approximately 80% of those with micro flow cell.)
Sample: Sample injection volume should be 1 – 10μL. Sample load should be 100μg or smaller.