No. 089

TOSOH

SEPARATION REPORT

Packed Column for Ultra-Fast Reversed-Phase Liquid Chromatography, TSKgel Super-ODS

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TOSOH CORPORATION

1. Introduction

Reversed-phase packed column is accepted as an important method of separation analysis due to its features of high resolution, wide range of target samples, ease to use, etc. The particle size of Reversed-phase packed column that is most frequently used at present is approximately 5μm, indicating high theoretical plates. In addition, it has advantages that allow separation conditions to be set up to match the sample properties, such as varying selectivity depending on the density of introduced C_{18} layer and organic solvent composition that can be selected optionally from 0 to 100%.

In recent years, demand for energy-saving analysis techniques including separation in low flow rate range (semi-micro application) and increased efficiency in analysis by time reduction (micro-particle application) is increasing. In the case of the former technique (semi-micro application), the volume of solvent used can be reduced to 1/5 to 1/10 by reducing the internal diameter of analysis column. Furthermore, in the case of the latter technique (micro-particle application), equivalent column efficiency can be obtained by reducing the particle size to 3μm and column length.

Tosoh Corporation has leaped over the conventional 3μm particle size to develop an ultra-fast Reversed-phase packing material, TSKgel Super-ODS, with the basis of 2μm silica particles, to achieve high speed and high resolution. This report mainly describes the features of this packed column.

2. Column Specification

Table-1 shows the specifications of TSKgel Super-ODS column. The column size is 4.6mm I. D. × 5cm or 10cm are available. In addition, a guard filter for analysis column protection is also available.

3. Features of Packing Materials

Table-2 shows the physical properties of TSKgel Super-ODS and TSKgel ODS-80Ts. TSKgel Super-ODS, which is a conventional reversed-phase packing material, has both pore volume and specific surface area that are about 1/3 of TSKgel ODS-80Ts. On the other hand, pore size has been set larger in TSKgel Super-ODS. The reason why pore volume and specific surface area are set small is to ensure sufficient pressure resistance under high pressure, and the reason why the pore size is set large is to ensure retention and selectivity by introducing C18 poly-layer.

The average particle size is about 2.3μm, which is nearly half of conventional packing materials. Employing such fine particles, high theoretical plates can be achieved. Moreover, the fact that the standard deviation of particle size distribution is smaller than the conventional products is also an essential factor for achieving high theoretical plates under relatively low pressure.

Table-1 Specifications of TSKgel Super-ODS

Product name	Product no.	Column size	Guaranteed theoretical plates/column
TSDgel Super-ODS	18154	4.6mmI.D. × 5cm	8000
TSDgel Super-ODS	18197	4.6mmI.D. × 10cm	16000
Guard filter (4-4)	18206	(for 4mmI.D. × 4mm)	—
G filter	18207	4mmI.D.× 4mm	—

Table-2 Physical Properties of Reversed-phase Packing Materials (After introducing octadecyl groups)

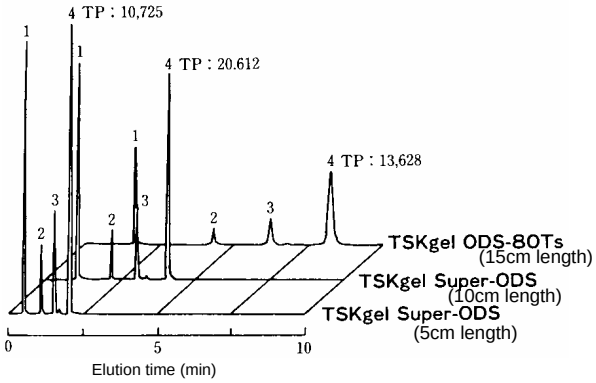
Packing material	Pore volume (mL/g)	Specific surface area (m^2/g)	Average pore size (nm)	Average particle size x, SD (um)	Carbon content (C%)
TSKgel Super-ODS	0.25[1)]	96.8[1)]	11.2[1)]	2.29, 0.27[2)]	Approx. 8
TSKgel ODS-80Ts	0.63	312.8	8.2	5.06, 0.87	Approx. 15

1) Measurement with mercury porosimeter, 2) measurement with SEM

4. Chromatographic Characteristics

4-1 Column Efficiency

Table-3 shows the comparison of column theoretical plates with commercial 3μm packed columns. It is evident that commercial 3μm columns have theoretical plates of about 6000 to 8000/5cm, while TSKgel Super-ODS has 10,000 or more plates. In addition, it is indicated that TSKgel Super-ODS has high mechanical strength of the packing material, because its pressure drops at the operation is lower or equivalent to that of 3μm columns while retaining high theoretical plates.
Table-4 shows a comparison of 10cm columns, and it is clear that TSKgel Super-ODS has low pressure drops and high theoretical plates at the same time.
While it is evident that TSKgel Super-ODS has smaller retention than commercial ODS packing materials, this is due to the difference in specific surface area, as mentioned in section 3.
In Figure-1, comparison of retention among Super-ODS 5cm and 10cm columns and ODS-80Ts 15cm column is shown.



Column: TSKgel Super-ODS (4.6mm I.D.×5cm)
TSKgel Super-ODS (4.6mm I.D.×10cm)
TSKgel ODS-80Ts (4.6mm I.D.×15cm)
Eluent: 70% CH_3OH
Flow rate: 1.0mL/min Temperature: 25°C
Detection: UV (254nm), micro flow cell
Samples: 1. uracil, 2. benzene, 3. toluene,
4. naphthalene

Column	Rs (1/2)	Rs (2/3)	Rs (3/4)
Super-ODS (5cm)	16.44	8.09	7.56
Super-ODS (10cm)	24.42	11.43	10.70
ODS-80Ts	21.88	9.53	7.39

Figure-1 Comparison with Conventional Columns (Isocratic Elution)

4-2 Steric Selectivity

Table-5 shows the results of separation between o-terphenyl (OT)/triphenylene (TR) on TSKgel Super-ODS and TSKgel ODS-80Ts. Compared to TSKgel ODS-80Ts, TSKgel Super-ODS indicated large values for resolution and separation factor while yielding a small value of capacity factor (k'). This is ascribable to the fact that TSKgel Super-ODS has been introduced with octadecyl base in a poly-layer form and has high steric selectivity.

Table-3 Comparison of Column Efficiency in 5cm ODS Columns

Column	Particle size (μm)	Fluorene		Resolution Rs α (NAP/FLU)	Pressure drops* (kg/cm²)
		RT (min)	TP/column		
TSKgel Super-ODS	2	3.71	10728	2.30	97
ODS by company A	3	6.10	7453	2.38	98
ODS by company B	3	4.70	8701	2.27	124
ODS by company C	3	6.58	5893	2.39	116
ODS by company D	3	6.61	7652	2.38	94

* 70% methanol, 1mL/min, NAP: naphthalene, FLU: fluorene

Table-4 Comparison of Column Efficiency in 10cm ODS Columns

Column	Particle size (μm)	Naphthalene		Pressure drops* (kg/cm²)
		RT (min)	TP/column	
TSKgel Super-ODS	2	4.06	20612	191
ODS by company A	3	4.46	10651	262
ODS by company B	3	3.47	11685	191

* 70% methanol, 1mL/min

Table-5 Comparison of Steric Selectivity

Column	o -terphenyl		triphenylene		Separation factor α (OT/TR)	Resolution Rs (OT/TR)
	k'	TP	k'	TP		
TSKgel Super-ODS	2.19	9596	3.84	6059	1.98	13.53
TSKgel ODS-80Ts	6.65	14163	8.00	14571	1.27	5.53

Eluent: 80% methanol (TSKgel Super-ODS)
85% methanol (TSKgel ODS-80Ts)
Flow rate: 1mL/min Detection: UV (254nm)

4-3 Interaction with Residual Silanol Group

One disadvantage of silica gel support is the interaction between residual silanol group and ionic substances. In general, if there are residual silanol groups on the packing material surface, acidic substances cause ionic repulsion and basic substances are adsorbed, causing difficulty in obtaining normal chromatogram. Figure-2 shows a comparison of elution of pyridine, which is a basic substance, with TSKgel ODS-80Ts. In either packing material, it is evident that pyridine is eluted normally. Therefore, it is apparent that sufficient end-capping has been achieved in either column.

4-4 Elution of Chelate Compound

Existence of chelate compounds may cause deterioration in sample recovery or large distortion of peak shape by forming metallic complex with metal species on packing material surface (such as iron, copper ion, etc.). In addition, substances that can easily be oxidized or reduced may become decomposed and cause change in peak shape or deterioration in recovery, leading to results with low reproducibility in either case.
TSKgel Super-ODS and TSKgel ODS-80Ts use high-purity silica gel that has been adjusted with a manufacture method in which metal species do not mix in from silica gel materials or production process in order to prevent this interaction between metal species and substances. Therefore, they do not have interaction with metal species even in chromatography of chelate compounds or oxidizable substances and yield favorable reproducibility. Figure-3 shows a chromatogram of 8-quinolinol, which is a metal chelate compound. It is apparent that elution is made normally.

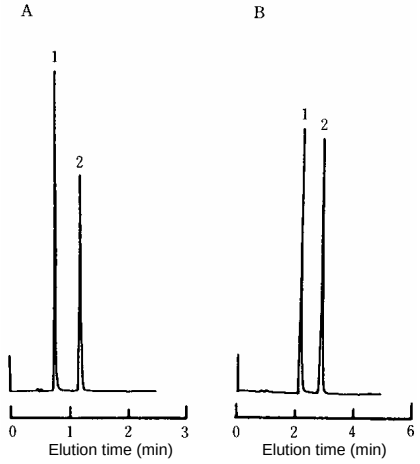


Figure-2 Comparison of Elution in TSKgel Super-ODS and TSKgel ODS-80Ts

Column: A. TSKgel Super-ODS (4.6mm I.D.×5cm)
B. TSKgel ODS-80Ts (4.6mm I.D.×15cm)
Eluent: A. 30% acetonitrile
B. 50% acetonitrile
Flow rate: 1.0mL/min Temperature: 25°C
Detection: UV (254nm), micro flow cell
Samples: 1. pyridine, 2. phenol

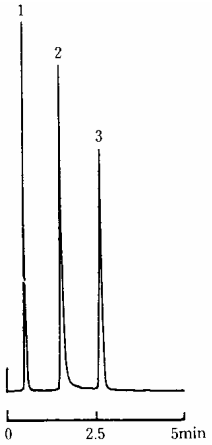


Figure-3 Chromatogram of Chelating Agent

Column: TSKgel Super-ODS (4.6mm I.D.×5cm)
Eluent: 20mmol/L phosphate buffer (pH 6.8)/ acetonitrile = 70/30
Flow rate: 1.0mL/min
Temperature: 40°C
Detection: UV (245nm), micro flow cell
Samples: 1. uracil, 2. 8-quinolinol, 3. methylbenzoic acid

4-5 Relationship between Flow Rate and Column Efficiency

Column efficiency is expressed as the contribution by various sample dispersion within the column as shown in the formula below. That is,

Theoretical plate height of column H = Hp + Hd + Hs + Hm

Here, Hp indicates dispersion by eddy diffusion, Hd indicates dispersion by diffusion in longitudinal direction within the mobile phase, Hs indicates dispersion by delay in mass transfer within the stationary phase, and Hm indicates dispersion by delay in mass transfer within the mobile phase. The terms that are related to particle size are Hd and Hm, and the effect of reducing particle size becomes especially large with Hm, which contributes by the square value of particle size. Furthermore, although the terms related to flow rate are Hd, Hs and Hm, Hd becomes smaller and Hs and Hm become larger when the flow rate is increased, and column efficiency generally deteriorates in high flow rate range. However, the effect of Hm becomes large when particle size is reduced, suppressing the column efficiency deterioration even in high flow rate range.

Figure-4 shows the relationship between flow rate and column efficiency in TSKgel Super-ODS and TSKgel ODS-80Ts under different eluent compositions. In the eluent containing methanol, optimal flow rate range is found near linear velocity of 4cm/min (approximately 0.6mL/min) for TSKgel ODS-80Ts, while it is found near 6cm/min (approximately 1mL/min) for TSKgel Super-ODS. Although column efficiency starts to deteriorate rapidly at 4cm/min or faster with TSKgel ODS-80Ts, column efficiency for TSKgel Super-ODS deteriorates only gradually.
Meanwhile, in the eluent containing acetonitrile system, optimal flow rate range lies near 6cm/min (1mL/min) for TSKgel ODS-80Ts and near 12cm/min (2mL/min) for TSKgel Super-ODS, and optimal flow rate range shifts toward higher flow rate compared to the eluent containing methanol on either column. This is due to the difference in the speed of mass transfer which comes from solvent viscosity. Column efficiency deterioration by flow rate is small when separation is made in solvent system with low viscosity, enabling analysis in high flow rate range. The relationship between the flow rate in various solvent compositions and pressure drops is shown in Figure-5.

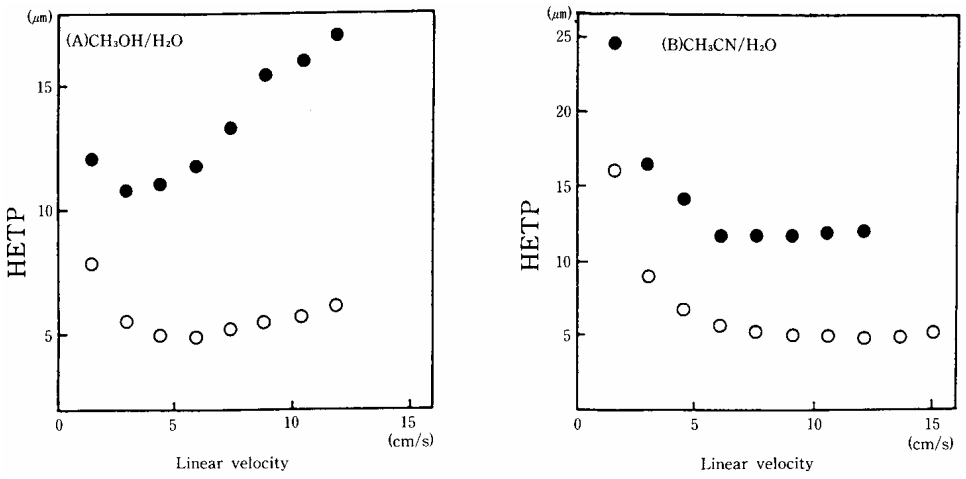


Figure-4 H/u Curve in Various Eluent Compositions

Column: (❍) TSKgel Super-ODS (4.6mm I.D.×5cm)
(●) TSKgel ODS-80Ts (4.6mm I.D.×15cm)
Eluent: (A) 70% methanol (B) 50% acetonitrile
Flow rate: 0.25 to 2.5mL/min
Detection: UV (254nm) Temperature: 25°C
Sample: Fluorene

5. Factors Affecting Separation

Although the factors affecting separation are similar to those for conventional analysis columns, various factors begin affecting the column efficiency when the column volume becomes small. In this section, these factors are examined.

The factors affecting the column efficiency are largely divided into the following.

I) Void volume
II) Detector response
III) Sample injection volume

I) is further divided into dispersion outside the column and inside the column. Dispersion inside the column is a specific problem of column structure, and columns employed in TSKgel Super-ODS have been designed with intention for thorough low dead volume.

There are factors of ① tubings, and ② detector cell volume outside the column.

Table-6 shows the effect of volume of tubing between injector/column and between column/detector on column efficiency. As seen clearly in the table, column efficiency deteriorates by approximately 10% when volume of tubing exceeds 2μL. It is also evident that the effect of void volume between injector/column on column efficiency is larger than that of void volume between column/detector.

Table-6 Effect of Volume of Tubing on Column Efficiency

Injector/column*			Column/detector**		
Length of the tubing (cm)	Volume of the tubing (μl)	HETP (μm)	Length of the tubing (cm)	Volume of the tubing (μl)	HETP (μm)
10	0.79	4.66	10	0.79	4.66
15	1.19	4.70	15	1.19	4.70
30	2.36	5.23	30	2.36	4.74
50	3.93	5.51	50	3.93	5.35
70	5.50	5.89	70	5.50	5.54

Tubings with 0.1mm I. D. were used.
*: Distance between column/detector 0.1mmI.D.×10cm
**: Distance between injector/column 0.1mmI.D.×10cm
Column: TSKgel Super-ODS (4.6mm I.D.×5cm)
Eluent: 70% methanol Flow rate: 1mL/min
Detection: UV (254nm), micro flow cell
Sample: Fluorene

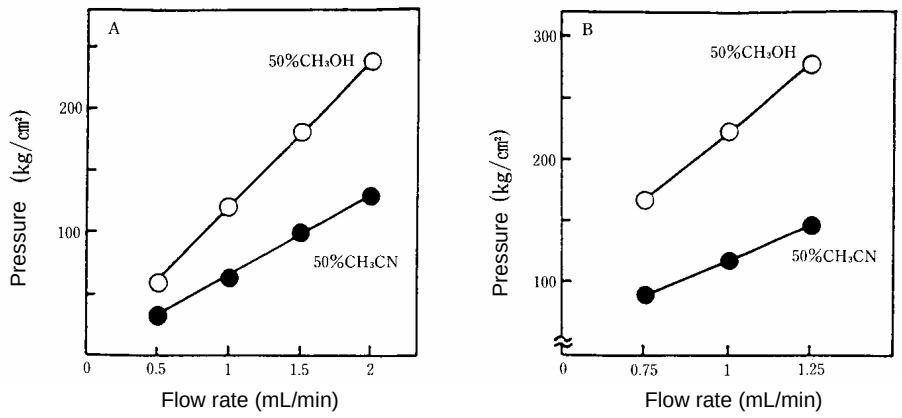


Figure-5 Relationship between Eluent Composition and Pressure

Column: A. TSKgel Super-ODS (4.6mm I.D.×5cm)
B. TSKgel Super-ODS (4.6mm I.D.×10cm)
Eluent: 50% CH_3OH, 50% CH_3CH
Flow rate: 0.5 to 0.2mL/min
Temperature: AMBIENT

Table-7 shows the effect of detector cell volume on column efficiency. For detector, 2μL microcell, 10μL standard cell, or low dead volume type cell was used.
Although column efficiency decreased by 6% in the low dead volume type cell compared to microcell, it decreased by as much as 70% in standard cell because the volume of heat sink section reached approximately 30μL. As it is evident, the cell volume needs to be minimized when short columns such as TSKgel Super-ODS is used.
Detector response in II) also has large effect on column efficiency in high-speed separation. Table-8 shows the relationship between detector response and column efficiency.
It is apparent that resolution deteriorates and theoretical plates decrease drastically when the time constant becomes large. Therefore, it is necessary that the time constant should be selected so that it becomes as small as possible. In Figure-6, chromatogram measured with various time constant values are shown. It is clear that the peak width becomes enlarged at 3 sec, causing extreme deterioration in resolution.

Table-7 Effect of Detector Cell Volume on Column Efficiency

Cell volume (μL)	Column theoretical plates (rate of deterioration in theoretical plates) TP/5cm column
2 (micro flow cell)	10769 (0%)
10 (low dead volume type)	10150 (6%)
10 (standard flow cell)	3104 (71%)

Eluent: 70% methanol, Sample: Fluorene

Table-8 Relationship between Detector Response and Column Efficiency

Time constant	Naphthalene theoretical plates TP/column (rate of deterioration)	Resolution α (TOP/NAP)
50msec	10529 (0%)	13.37
1sec	6996 (34%)	10.37
3sec	3420 (68%)	6.87

Eluent: 70% methanol, Samples: Toluene (TOL), naphthalene (NAP)

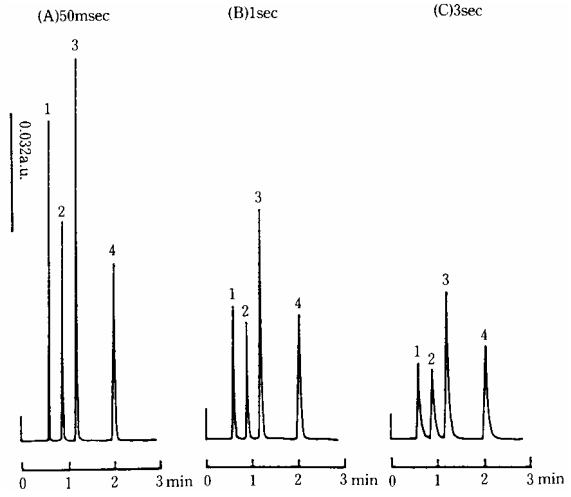


Figure-6 Effect of Detector Time Constant on Theoretical Plates

Column: TSKgel Super-ODS (4.6mm I.D.×5cm)
Eluent: 70% methanol
Flow rate: 1.0mL/min
Sample: Fluorene Temperature: 25°C
Detector: UV (254nm), micro flow cell
Time constant: (A) 50msec, (B) 1sec, (C) 3sec

III) is related to the limit of injection volume that can maintain the column performance. The injection volume with an allowance relates to eluent composition and the composition of sample solution. Figure-7 shows the relationship between sample injection volume and column efficiency. When sample dissolved to a similar solution to the solvent as composition is injected, the column efficiency begins deteriorating at a small injection volume on TSKgel Super-ODS (5cm column) due to the facts that its gel volume is 1/3 of TSKgel ODS-80Ts and that its specific surface area is small. In TSKgel Super-ODS, 10μL or smaller is recommended as injection volume. However, it is evident that injection volume can be increased up to about 5 folds without deterioration of column efficiency by setting the content of organic solvent in the sample solution to 40% (when the content of organic solvent in eluent is 70%).

6. Applications

Figure-8 shows some applications of high-speed chemical separation. When flow rate is changed from 1mL/min to 4mL/min, analysis time can be reduced from 3 minutes to 1 minute or less, enabling high-speed separation. Although resolution at each flow rate deteriorates gradually along with the flow rate, analysis time reaches 1/4.

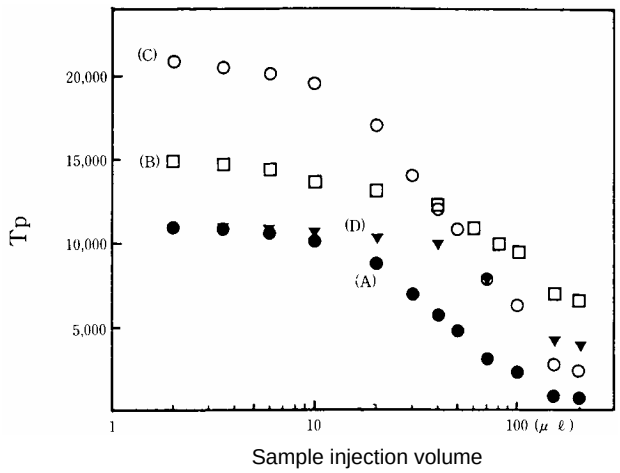


Figure-7 Sample Injection Volume and Column Efficiency (Theoretical Plates)

Column: (A), (D) TSKgel Super-ODS (4.6mm I.D.×5cm)
(B) TSKgel ODS-80Ts (4.6mm I.D.×15cm)
(C) TSKgel Super-ODS (4.6mm I.D.×10cm)
Eluent: 70% methanol
Flow rate: 1.0mL/min Temperature: 25°C
Detection: UV (254nm)
Samples: Naphthalene (0.1g/L), (A) (B) (C), dissolved in 70% methanol, (D) dissolved in 40% methanol, (0.1 g/L)

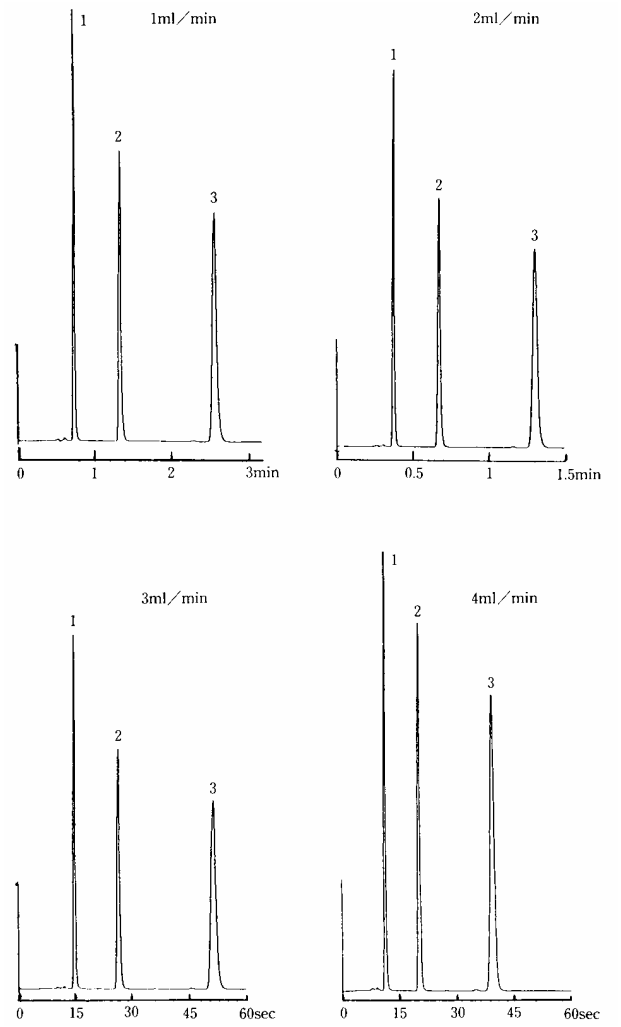


Figure-8 Relationship between Flow Rate and Separation on TSKgel Super-ODS

Column: TSKgel Super-ODS (4.6mm I.D. × 5cm)
Eluent: 20mmol/L phosphate buffer (pH 2.5)/ acetonitrile = 80/20
Flow rate: 1 to 4mL/min Temperature: 25°C
Samples: 1. caffeine, 2. salicylamide, 3. phenacetin
Detection: UV (254nm), micro flow cell

Flow rate	Elution point (min)			Resolution	
	Sample 1	Sample 2	Sample 3	Rs (1/2)	Rs (2/3)
1mL/min	0.73	1.33	2.58	14.45	16.17
2mL/min	0.37	0.67	1.30	13.04	15.08
3mL/min	0.25	4.44	0.86	12.01	14.20
4mL/min	0.19	0.33	0.66	10.34	12.95

Figure-9 shows the applications of vitamin D_2 and D_3 separation. In monolayer TSKgel ODS-80Ts, sufficient separation is not achieved. However, in poly-layer TSKgel Super-ODS, rapid and better separation is obtained.

An application of peptide separation is shown in Figure-10. Although TSKgel Octadecyl-NPR, a polymer-type non-porous Reversed-phase packing materials (2.5μm), is available for high-speed separation of peptides or proteins, it has a disadvantage of weak retention to hydrophilic peptides because hydrophobicity of the packing material is weak. On the other hand, since TSKgel Super-ODS has sufficient hydrophobicity and is porous, it shows favorable separation of peptides with high hydrophilicity.

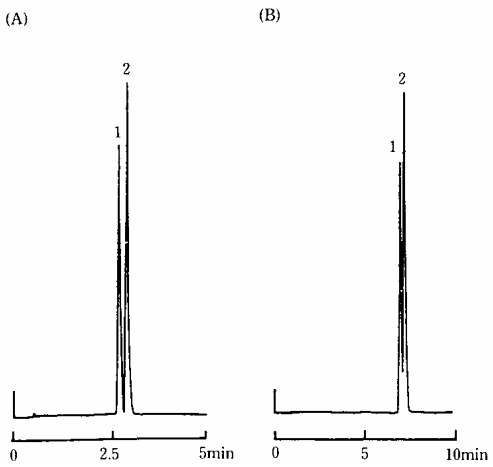


Figure-9 Comparison of Vitamin D_2 and D_3 Separation

Column: (A) TSKgel Super-ODS (4.6mm I.D. × 5cm)
(B) TSKgel ODS-80Ts (4.6mm I.D. × 15cm)
Eluent: Methanol
Flow rate: 1mL/min Temperature: 25°C
Detection: UV (254nm), micro flow cell
Samples: 1. vitamin D_2, 2. vitamin D_3

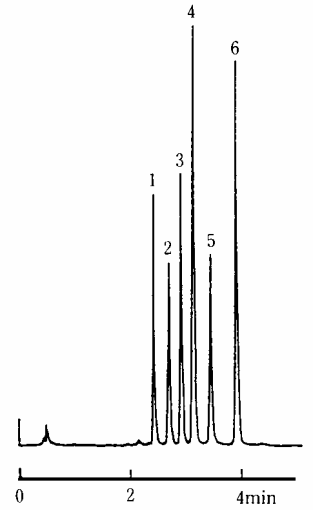


Figure-10 Comparison of Peptide Separation

Column: TSKgel Super-ODS (4.6mm I.D. × 5cm)
Eluent: 13mmol/L HClO4/acetonitrile
Linear gradient from 10% acetonitrile to 50% for 10 minutes
Flow rate: 2mL/min Temperature: 25°C
Detection: UV (220nm), micro flow cell
Samples: 1. oxytocin, 2. α-endorphin, 3. bombesin, 4. Leu-enkephalin, 5. gamma-endorphin, 6. somatostatin
All peptides are injected at 0.1 to 0.2μg each.

7. Operating Instructions in HPLC System, etc.

As described in section 5, some remarks must be taken for HPLC system, etc. in order to deliver sufficient performance of TSKgel Super-ODS. These precautions are shown in Table-9.

8. Conclusion

TSKgel Super-ODS is a Reversed-phase packed column which is capable of achieving high-speed, high-resolution separation by employing micro-particle silica gel. It is a packing material in which C18 layer has been introduced in poly-layer based on high-strength, high-purity silica gel, and separation without large influence by residual silanol group or metals can be obtained.
In order to exert the best performance by TSKgel Super-ODS, please perform separation in an equipment in which dead volume is minimized as much as possible. Dispersion outside the column may become a serious cause of resolution deterioration.

Table-9 Handling Instructions When Using TSKgel Super-ODS

The following remarks must be taken in HPLC system.

* Suppress peak expansion in tubings, detector, etc.
* Prevent the sample from overloading.
* Use caution in setting up detection and data processing since analysis time is short (5 minutes or less).

Tubings:

Use 0.1mmID tubing. Length of 30cm or less is desired.
Connection pipe set, type L is available; the liquid contact surface (both ends) has fine-cut finishing.
(Product no. 018686, 0.1mmID×40cm, 2 sets included)
Sections requiring 0.1mmID tubing

a) Between injection valve/column (guard filter) inlet
or between auto-sampler/column (guard filter) inlet
b) Between column outlet/detector inlet (tubing on detector inlet side)

Gradient mixer:

Static mixer A (product no. 08407) is applicable to 5 to 20 minutes of gradient
Dynamic mixer (product no. 08410) is applicable to 10 to 20 minutes of gradient

Auto-sampler (sample injection):

Sample injection volume should be 5 to 10μl. Sample concentration should be approximately 50μg/L.

Column:

Always connect a guard filter to protect the column.
(Guard holder: product no. 18206, G filter: product no. 18207)
Connection tubing set is a standard accessory to the guard holder.

Column oven:

Available at 25C or higher; Pressure drops decreases and theoretical plates are improved at 40C compared to room temperature.

Detector:

UV detector requires micro flow cells or low dead volume type cells.
Response: set to 50msec or 150msec.

Data processing:

Sampling pitch: 50msec