TOSOH

SEPARATION REPORT NO. 97

APPLICATION OF TSKgel SEMI-MICRO COLUMNS FOR HIGH-SENSITIVITY ANALYSES

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1. Introduction

Semi-micro columns, also referred to as narrow bore columns, are columns with an internal diameter smaller than conventional 4.6mm ID columns. In comparison to conventional columns of the same length, the flow rate of semi-micro columns is scaled down by the ratio of the cross-sectional surface areas of the columns according to the formula in equation (1), in which F is the flow rate and D the column diameter.

(1) $F_{semi\text{-}micro} = \{0.25*\pi*D^2_{semi\text{-}micro} / 0.25*\pi*D^2_{conv}\} * F_{conv}$

Narrow bore columns are the columns of choice when faced with a limited sample mass, as in trace analysis, or when using a mass spectrometer or other detector that performs more optimally at lower flow rates.

Since peaks elute in smaller volumes from a narrow bore column, on a unit-mass basis, peaks are higher than on wider columns, which results in better sensitivity. High sensitivity analysis of trace amounts of samples is often required when analyzing low molecular weight compounds in reversed phase chromatography (RPC) or in Hydrophilic Interaction chromatography (HILIC). The demand for semi-micro columns has increased in recent years due to the need to analyze or purify smaller and smaller amounts of biological samples.

The TSKgel columns shown in the table below can all be used in a wide variety of applications due to their excellent performance and chemical stability.

This document describes the basic properties, as well as several applications, utilizing these semi-micro columns.

2. Features

Table 1 lists the semi-micro TSKgel columns available through Tosoh Bioscience.

The semi-micro columns for the separation of biological samples contain the same packing materials as the packing material used in the more conventional 4.6mm ID format.

The new semi-micro columns deliver the following features:

- Compared to conventional 7.5mm ID columns, approximately a 14-fold increase in detection sensitivity can be achieved. Approximately a 5-fold increase in sensitivity can be obtained compared to conventional 4.6mm ID RPC columns.
- Since the same packing materials as the conventional columns are used, they can be used under identical mobile phase conditions, achieving very similar separation patterns.
- Components present in trace amounts can be separated at high concentration and recovery.

Table 1 Specifications of semi-micro columns for the separation of biological samples

Separation mode	Product name	Resin type	Column size	Part no.
IEC	TSKgel DEAE-5PW	Polymer	2.0mm ID × 7.5cm	18757
	TSKgel SP-5PW	Polymer	2.0mm ID × 7.5cm	18758
	TSKgel DEAE-2SW	Silica	2.0mm ID × 25cm	18761
HIC	TSKgel Phenyl-5PW	Polymer	2.0mm ID × 7.5cm	18759
	TSKgel Ether-5PW	Polymer	2.0mm ID × 7.5cm	18760
RPC	TSKgel ODS-80Ts	Silica	2.0mm ID × 15cm	18150
			2.0mm ID × 25cm	18151
	TSKgel ODS-80Ts QA	Silica	2.0mm ID × 15cm	18768
			2.0mm ID × 25cm	18769
	TSKgel ODS-120T	Silica	2.0mm ID × 15cm	18152
			2.0mm ID × 25cm	18153
	TSKgel Phenyl-5PW RP	Polymer	2.0mm ID × 7.5cm	18756
	TSKgel Octadecyl-4PW	Polymer	2.0mm ID × 15cm	18755
	TSKgel Octadecyl-2PW	Polymer	2.0mm ID × 15cm	18754

3. Basic Properties

3-1 Comparison between Semi-micro Columns and Conventional Columns

When the total amount of sample load is identical, peak height increases inversely to the column's cross-sectional area. Figures 1 and 2 show a comparison of sensitivity between a conventional column and a semi-micro column using a TSKgel DEAE-5PW and a TSKgel SP-5PW column. The cross-sectional area of the semi-micro column used in this study was 1/14 compared to the conventional column and is, therefore, capable of detecting various proteins at 10-fold or higher sensitivity.

Figure 3 shows the sensitivity comparison between a conventional column and a semi-micro column on TSKgel Phenyl-5PW RP. Since the cross-sectional area of the semi-micro column is one-fifth that of the 4.6mm ID conventional column, it is capable of detecting proteins at approximately 5-fold higher sensitivity.

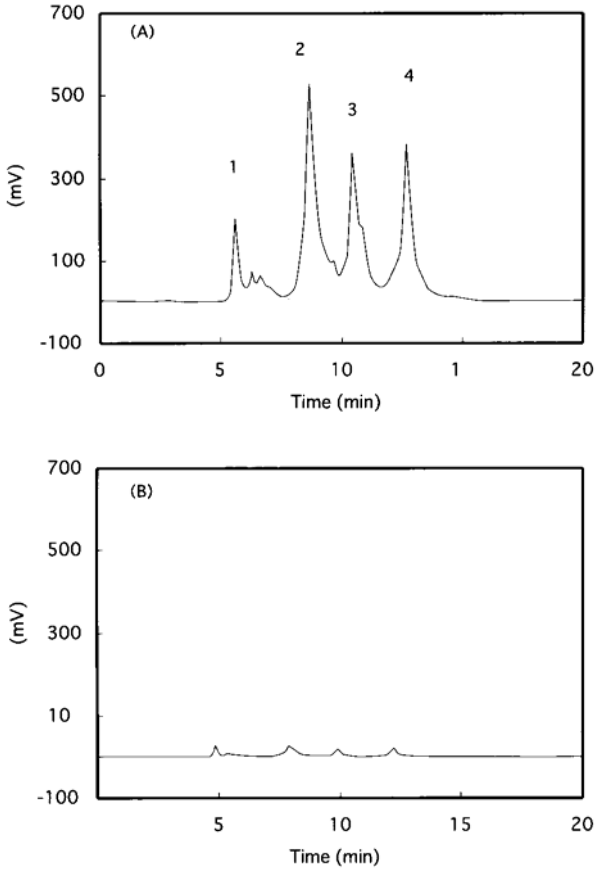


Figure 1 **Comparison of sensitivity between semi-micro column and conventional column**

Column: (A) TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
(B) TSKgel DEAE-5PW (7.5mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard protein (10μL)
1. carbonic anhydrase (2.4g/L)
2. transferrin (4g/L)
3. ovalbumin (5g/L)
4. soybean trypsin inhibitor (5g/L)

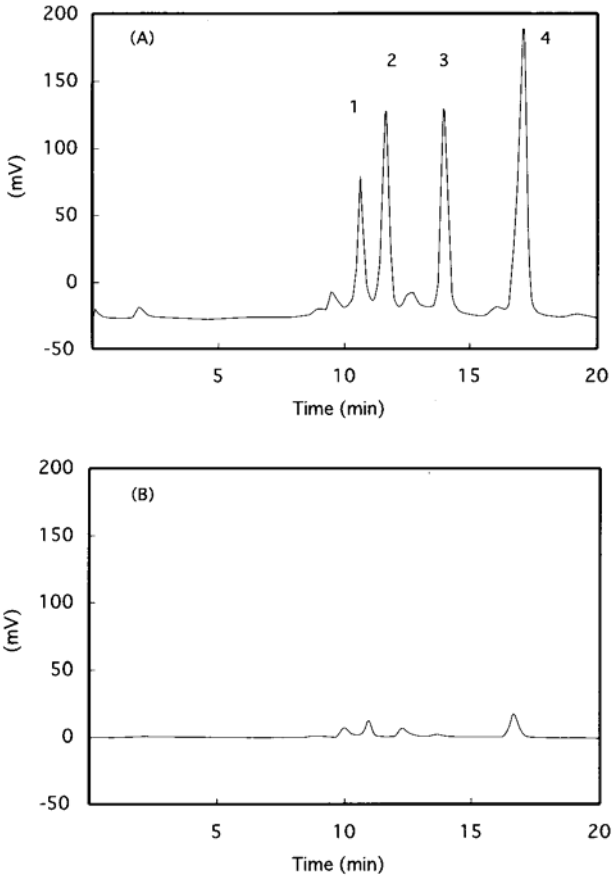


Figure 2 **Comparison of sensitivity between semi-micro column and conventional column**

Column: (A) TSKgel SP-5PW (2.0mm ID × 7.5cm)
(B) TSKgel SP-5PW (7.5mm ID × 7.5cm)
Eluent: A: 20mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard protein (10μL)
1. ribonuclease A (2g/L)
2. α-chymotrypsinogen A (1g/L)
3. cytochrome C (5g/L)
4. lysozyme (1g/L)

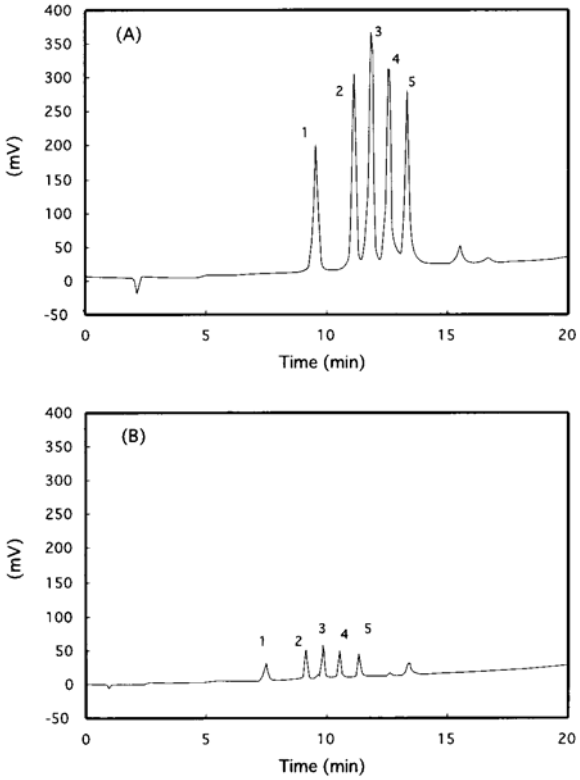


Figure 3 **Comparison of sensitivity between semi-micro column and conventional column**

Column:	(A) TSKgel Phenyl-5PW RP (2.0mm ID × 7.5cm)
	(B) TSKgel Phenyl-5PW RP (4.6mm ID × 7.5cm)
Eluent:	A: 5% acetonitrile + 0.1% TFA
	B: 80% acetonitrile + 0.1% TFA
	A→B linear gradient (20 min.)
Flow rate:	(A) 0.10mL/min
	(B) 1.0mL/min
Temperature:	25°C
Detection:	UV@280nm, micro-cell
Sample:	standard protein (10µL)
	1. ribonuclease A (0.2g/L)
	2. cytochrome C (0.2g/L)
	3. lysozyme (0.2g/L)
	4. α-lactoglobulin (0.2g/L)
	5. myoglobin (0.2g/L)

3-2 Flow Rate Dependency

In general, peak separation and peak height in HPLC vary according to the flow rate and the gradient steepness in gradient elution. The relationship is as follows:

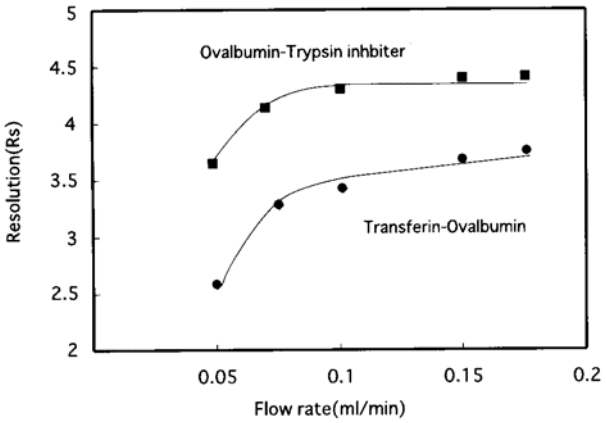
When the flow rate is higher,
- peak separation is better.
- peak heights are lower.

When the gradient is shallower,
- peak separation is better.
- peak heights are lower.

Figures 4 and 5 show the effects of resolution and peak height when varying the flow rate using a TSKgel DEAE-5PW and a TSKgel SP-5PW column.

As the flow rate increases, resolution improves and remains approximately constant at a flow rate of 0.10mL/min and higher. On the other hand, peak height tends to increase as the flow rate decreases.

Based on this, a flow rate of about 0.10mL/min can be considered optimal for the trade-off between resolution and peak height.



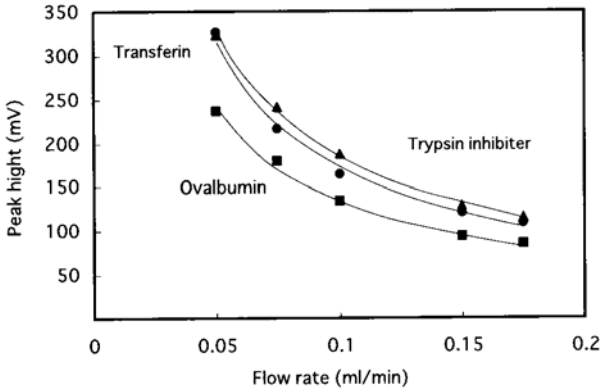
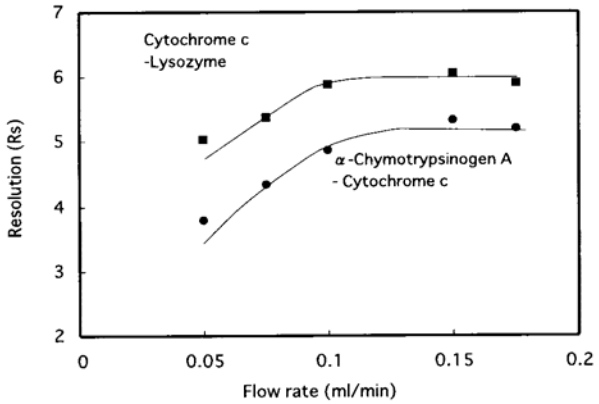


Figure 4 **Flow rate dependency on a TSKgel DEAE-5PW (semi-micro) column**

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard protein (10μL)
transferrin (4g/L)
ovalbumin (5g/L)
soybean trypsin inhibitor (5g/L)



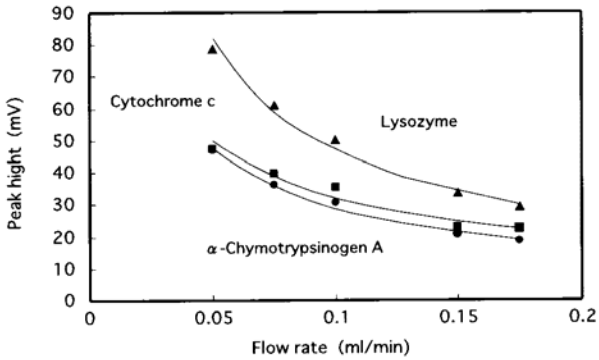


Figure 5 **Flow rate dependency on a TSKgel SP-5PW (semi-micro) column**

Column: TSKgel SP-5PW (2.0mm ID × 7.5cm)
Eluent: A: 50mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard protein (10μL)
α-chymotrypsinogen A (1g/L)
cytochrome C (1g/L)
lysozyme (1g/L)

3-3 Effect of Gradient

Figure 6 shows the effect of changing the gradient time on the resolution of standard proteins on a TSKgel DEAE-5PW column.

Though separation is greatly improved by increasing the gradient time from 20 minutes to 60 minutes, the peak height decreases by approximately 50%.

3-4 Sample Load

Figures 7 and 8 show the sample load on TSKgel DEAE-5PW and TSKgel SP-5PW columns. It is evident that the resolution gradually decreases at sample loads of 200µg and higher, although the resolution is constant up to a sample load of 100µg.

Figure 9 shows the calibration curves for trypsin inhibitor under low loading conditions on semi-micro and conventional columns. For the conventional column, a deviation from a straight line was observed when injecting less than 250µg protein. In contrast, a linear calibration curve was obtained on the semi-micro column when injecting up to 100µg protein.

This indicates that conventional columns are suitable for sample loads of 200µg and larger, while semi-micro columns are ideal for analysis of less than 200µg protein.

3-5 Sample Injection Volume

It is necessary when operating under isocratic conditions to minimize the sample volume for semi-micro columns and suppress band broadening in the column as the sample already starts moving through the column during the injection of the sample. It was determined separately that the optimal injection volume for semi-micro columns under isocratic conditions is 10µL for gradient analysis and less than 5µL for isocratic analysis.

It is known that the effect of sample injection volume on band broadening can be virtually eliminated by concentrating the injected sample on the top of the column. This can be achieved by injecting the sample in a solvent that is weaker than the (starting) mobile phase.

Figure 10 shows an example of eliminating the band broadening effect of sample injection volume by using gradient elution. It shows that the resolution is not affected, even when the sample injection volume is 1000µL, because the injected sample is concentrated on the top of the column. Under such conditions, semi-micro columns are capable of concentrating diluted samples to a higher degree compared to conventional columns. Semi-micro columns allow efficient and highly sensitive analysis while also promoting effective concentration and purification of dilute samples.

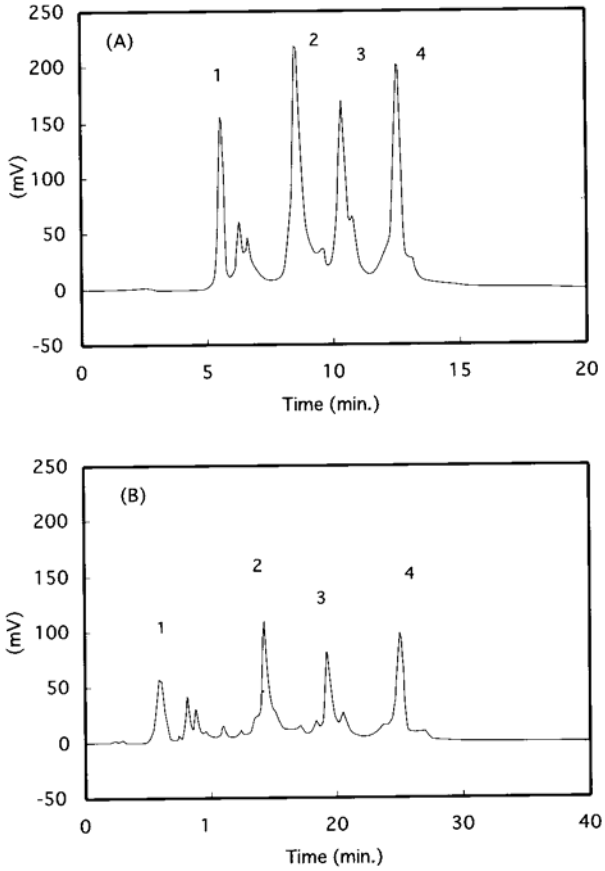


Figure 6 **Effect of gradient time on peak resolution**

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
(A): A→B linear gradient (20 min.)
(B): A→B linear gradient (60 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard protein (10µL)
1. carbonic anhydrase (2.4g/L)
2. transferrin (4g/L)
3. ovalbumin (5g/L)
4. soybean trypsin inhibitor (5g/L)

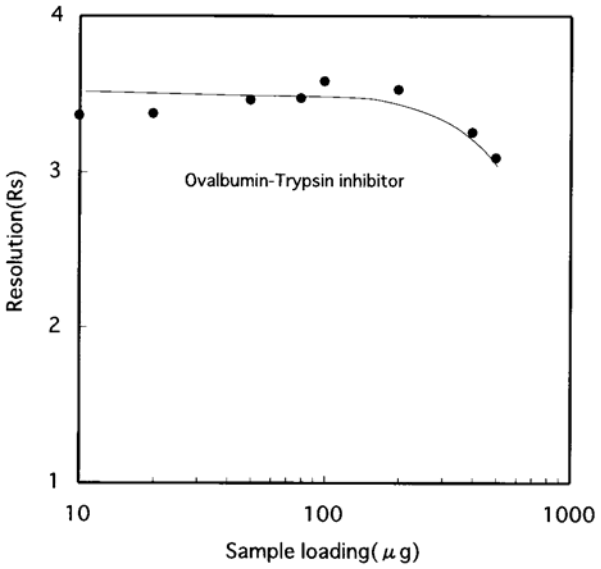


Figure 7 **Relationship between resolution and sample load on TSKgel DEAE-5PW**

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard proteins (50μL inj. volume)
ovalbumin
soybean trypsin inhibitor

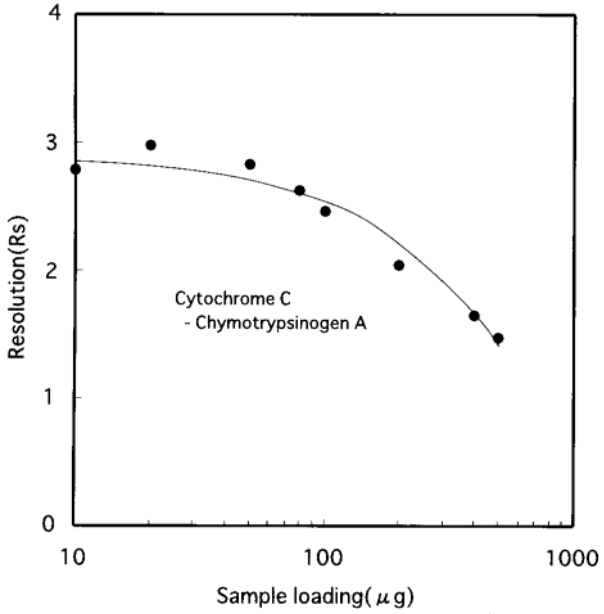


Figure 8 **Relationship between resolution and sample load on TSKgel SP-5PW**

Column: TSKgel SP-5PW (2.0mm ID × 7.5cm)
Eluent: A: 20mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard proteins (50μL)
α-chymotrypsinogen A
cytochrome C

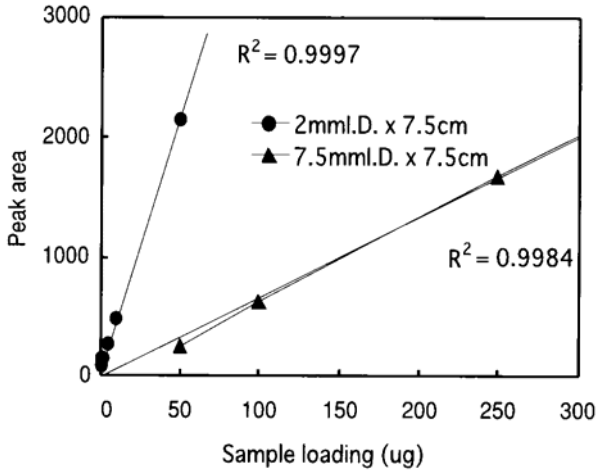


Figure 9 **Comparison of calibration curves between semi-micro and conventional columns with trace amount sample loads**

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
TSKgel DEAE-5PW (7.5mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min (2.0mm ID × 7.5cm)
1.0mL/min (7.5mm ID × 7.5cm)
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: soybean trypsin inhibitor (10μL)

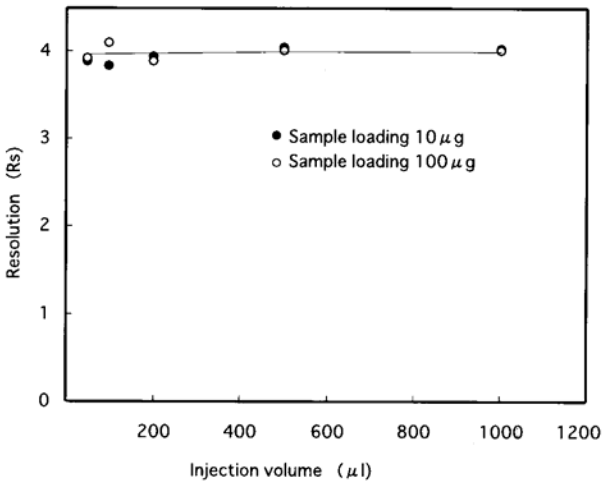


Figure 10 **Relationship between resolution and sample volume on TSKgel DEAE-5PW**

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard proteins
ovalbumin
soybean trypsin inhibitor

3-6 Recovery

Table 2 shows the protein recovery on semi-micro and conventional-sized TSKgel DEAE-5PW columns.

For the conventional column, high recovery was obtained until the sample load reached about 20µg. However, recovery deteriorated drastically for most proteins when the sample load was 5µg.

Conversely, the semi-micro column delivered high recoveries of all but one sample protein, soybean trypsin inhibitor, even with a sample load of 2µg.

Table 2 Comparison of protein recoveries on semi-micro and conventional columns

	Semi-micro column (2.0mm ID × 7.5cm)		Conventional column (7.5mm ID × 7.5cm)	
Sample load	20µg	2µg	20µg	5µg
Bovine serum albumin	88%	82%	87%	75%
Ovalbumin	96%	93%	94%	81%
Myoglobin	98%	93%	94%	79%
Soybean trypsin inhibitor	89%	72%	84%	62%

Column: TSKgel DEAE-5PW (2.0mm ID × 7.5cm)
TSKgel DEAE-5PW (7.5mm ID × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.5)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min (2.0mm × 7.5cm)
1.0mL/min (7.5mm × 7.5cm)
Injection volume: 20µL

4. Other Columns and Applications

Figures 11 to 14 show the comparison of relative sensitivity on conventional columns and semi-micro columns when separating biological samples. Figure 15 shows an analysis example of a trypsin digest on a TSKgel ODS-80T$_s$ semi-micro column.

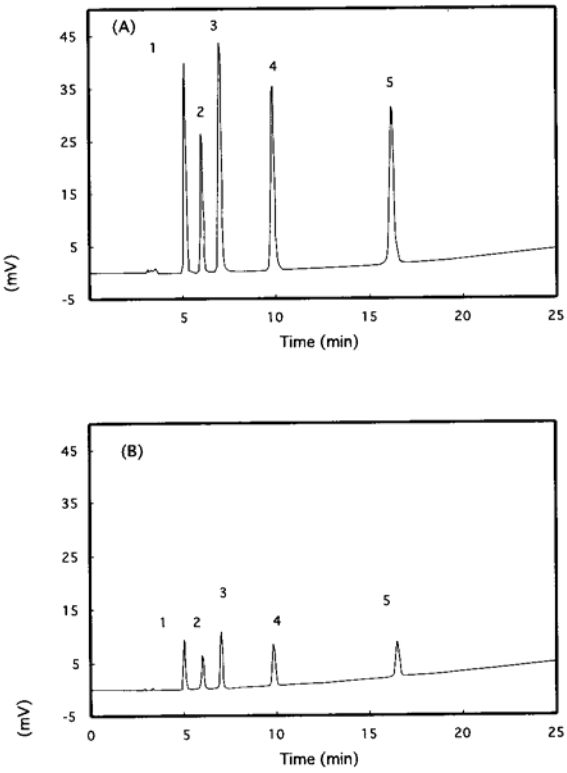


Figure 11 Comparison of sensitivity on semi-micro and conventional columns

Column: (A) TSKgel DEAE-2SW (2.0mm ID × 25cm)
(B) TSKgel DEAE-2SW (4.6mm ID × 25cm)
Eluent: A: 10mmol/L phosphate buffer (pH 3.0)/ acetonitrile = 80/20
B: 50mmol/L phosphate buffer (pH 3.0)/ acetonitrile = 80/20
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@260nm, micro-cell
Sample: nucleotide mixture (2µL)
1. AMP (45g/L)
2. IMP (90g/L)
3. GMP (90g/L)
4. ADP (90g/L)
5. ATP (90g/L)

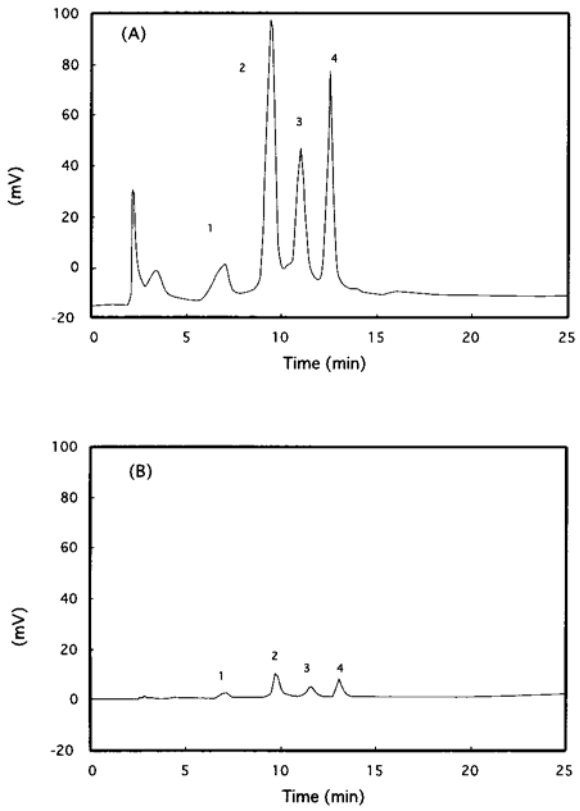


Figure 12 Comparison of sensitivity on a semi-micro column and a conventional column

Column: (A) TSKgel Ether-5PW (2.0mm ID × 7.5cm)
(B) TSKgel Ether-5PW (7.5mm ID × 7.5cm)
Eluent: A: 0.1mol/L phosphate buffer + 1.8mol/L ammonium sulfate (pH 7.0)
B: 0.1mol/L phosphate buffer (pH 7.0)
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@280nm, micro-cell
Sample: standard proteins (10µL)
1. ribonuclease A (0.5g/L)
2. lysozyme (0.5g/L)
3. α-chymotrypsin (0.5g/L)
4. α-chymotrypsinogen (0.5g/L)

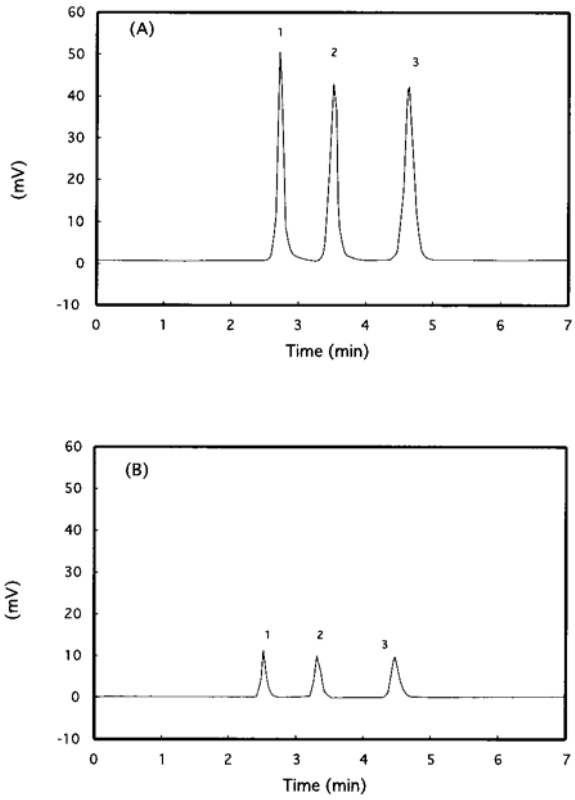


Figure 13 Comparison of sensitivity on a semi-micro column and a conventional column

Column: (A) TSKgel Octadecyl-2PW (2.0mm ID × 15cm)
(B) TSKgel Octadecyl-2PW (4.6mm ID × 15cm)
Eluent: 20mmol/L phosphate buffer (pH 7.0) / acetonitrile = 50/50
Flow rate: (A) 0.19mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@254nm, micro-cell
Sample: amines (1µL)
1. aniline (13.8mg/L)
2. N-methylaniline (30mg/L)
3. N,N-dimethylaniline (30mg/L)

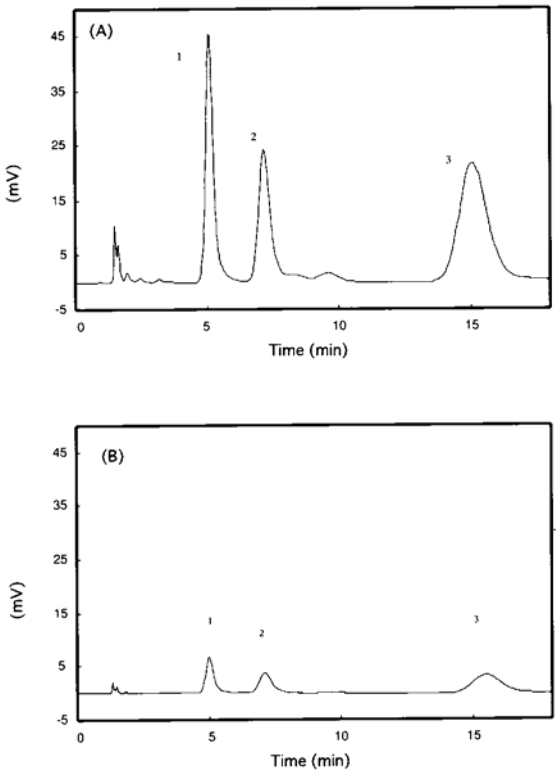


Figure 14 Comparison of sensitivity on a semi-micro column and a conventional column

Column: (A) TSKgel Octadecyl-4PW (2.0mm ID × 15cm)
(B) TSKgel Octadecyl-4PW (4.6mm ID × 15cm)
Eluent: 50mmol/L phosphate buffer (pH 7.0) / acetonitrile = 90/10
Flow rate: (A) 0.19mL/min
(B) 1.0mL/min
Temperature: 25°C
Detection: UV@215nm, micro-cell
Sample: peptide sample (1.4µL)
1. methionine-enkephalin (30mg/L)
2. leucine -enkephalin (30mg/L)
3. oxytocin (30mg/L)

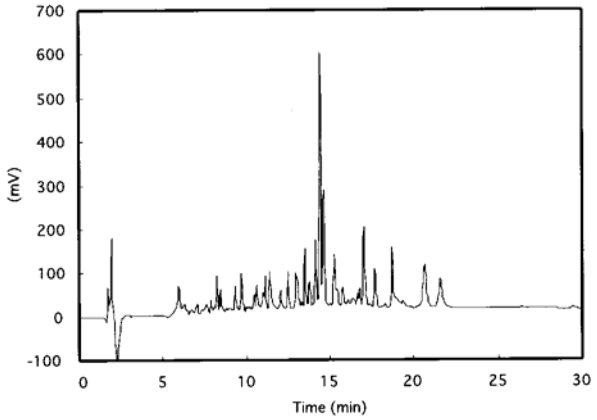


Figure 15 Analysis of a trypsin digest of β-lactoglobulin

Column: TSKgel ODS-80T_s (2.0mm ID × 15cm)
Eluent: A: 0.1% TFA solution
B: Acetonitrile + 0.1% TFA
A (100%) → A (30%) linear gradient (30 min.)
Flow rate: 0.20mL/min
Temperature: 25°C
Detection: UV@215nm, micro-cell
Sample: trypsin digest of β-lactoglobulin (10µL

Table 3 summarizes the recommended precautions that should be taken when working with semi-micro columns.

Table 3 Recommended operating conditions when using TSKgel semi-micro columns

In general:
- Suppress peak broadening by minimizing the dead volume in the tubing between the injector, guard column, analytical column, and detector.
- Prevent the sample volume from causing extra-column band broadening due to volume overloading. Test this by injecting half the sample volume and measuring peak efficiency. There should be very little loss in peak efficiency.

Tubing:
- Use 0.005" ID (0.125mm) tubing, when available. This is most important when working with small MW samples and silica-based reversed phase columns. A total length of 30cm or less is desired. When using slightly larger ID tubing (for example 0.01" ID), make sure to reduce the length of the tubing beyond the recommendations given in the text.
- Sections requiring 0.005" ID tubing
    - Between injection valve and guard column (see below) and between guard column outlet and analytical column
    - Between the analytical column outlet and the detector inlet

Autosampler (sample injection):
- Sample injection volume should not be more than 10µL. Larger volumes are allowed when the strength of the injection solvent is weaker than that of the starting mobile phase composition.

Column protection:
- When possible and available, use a guard column containing the same packing material as the analytical column.

Detector:
- Replace the UV detector cell with a micro-flow cell or low dead-volume type cell. Set the response time of the detector to 50msec or 150msec.

Additional precautions when using semi-micro silica-based RPC columns:

Column oven:
- Use a constant temperature of 25°C or higher. Pressure decreases and theoretical plates increase at 40°C compared to room temperature.

Data processing:
- Use a sampling rate of 50msec.

5. Conclusion

The analysis of biological samples utilizing semi-micro columns enables high sensitivity as a result of reduced column diameter. Semi-micro columns are suitable for analysis of trace sample amounts since an increase in sensitivity of about 14-fold is possible when scaling down from 7.5mm ID to 2mm ID and injecting the same sample load. Similarly, about a 5-fold increase in sensitivity is possible when scaling down from 4.6mm ID to 2mm ID when the same sample load is injected.

In addition, semi-micro columns exhibit higher recovery when analyzing trace amounts of samples, while trace components can be recovered in higher concentrations. It is possible to implement high sensitivity analysis and purification of diluted samples under gradient conditions by concentrating the samples on the top of the column.

Since the same packing materials are used for the semi-micro and conventional columns, the same selectivity can be obtained when operating the columns under identical mobile phase conditions, given that the HPLC system has been optimized to prevent efficiency losses due to extra-column band broadening. With regard to the HPLC system, it is recommended to minimize the dead volume to fully benefit from the performance characteristics of the TSKgel semi-micro columns as extra-column band broadening can be a major cause of lower than expected column efficiency.