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Journal of Chromatography A, 691 (1995) 81–89

JOURNAL OF CHROMATOGRAPHY A

Characterization of a new reversed-phase chromatographic column on a 2-μm porous microspherical silica gel

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Abstract

The physical and chromatographic properties of a reversed-phase column based on a 2-μm porous silica gel, TSKgel Super-ODS, were investigated. The Super-ODS column reveals higher column efficiencies (over 200 000 theoretical plates/m) at a higher flow-rate than that on a conventional column. High selectivity for a planar compound on Super-ODS implies that it possesses a polymeric ODS layer. Employing pure silica and a full end-capping procedure resulted in sharp peaks for basic and chelating compounds. To avoid the loss of column efficiency, the void volumes in an operating system such as connecting tubes, cell volume and sample volume, should be minimized as much as possible. The time constant of the detector also affected the column efficiency. The separation of peptides was achieved within 4 min.

1. Introduction

Octadecylsilylated silica gel (ODS) has been widely employed for separating many compounds. In general, the particle sizes utilized for fast LC and conventional columns are around 3 and 5 μm, respectively. The column efficiencies on these columns range from ca. 60 000 to 150 000 theoretical plates (TP)/m.

In the last decade, many researchers have emphasized the advantages of the use of small particles 1.5–3 μm in diameter [1–4]. These columns, based on non-porous materials, allow fast separations and good resolution for biological samples such as proteins and DNAs in several separation modes [5–7]. It is assumed that no diffusion of a solute into pores results in the avoidance of peak broadening. However, these columns show a low capacity for low-molecular-mass compounds because of the small surface area. On the other hand, Danielson and Kirkland [8] suggested the use of a short reversed-phase (RP) column packed with porous microparticles for separating large molecules such as peptides and proteins. They stressed that their short column was a useful tool for analysing biological macromolecules and reported high column efficiencies more than 130 000 TP/m).

Recently, a new reversed-phase chromatographic column, TSKgel Super-ODS, based on 2-μm silica gel, became commercially available from Tosoh (Tokyo, Japan). In this paper, we report the characterization of this RP column in comparison with conventional and fast LC ODS columns.

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0021-9673/95/$09.50
SSDI 0021-9673(94)00744-6

Table 1
HPLC columns employed

Column	Supplier	Column size (mm × mm I.D.)	Particle size (μm)	Functionality	End-capping
TSKgel Super-ODS	Tosoh	50 and 100 × 4.6	2	Polymeric C_{18}	Yes
TSKgel ODS-80Ts	Tosoh	150 × 4.6	5	Monomeric C_{18}	Yes
TSKgel Octadecyl-NPR	Tosoh	35 × 4.6	2.5	–	No
YMC-FL-ODS-3	YMC	50 and 100 × 4.6	3	Monomeric C_{18}	Yes
Capcell Pak $3C_{18}$	Shiseido	50 and 100 × 4.6	3	Monomeric C_{18}	Yes
Cosmosil $3C_{18}$	Nacalai Tesque	50 and 100 × 4.6	3	Monomeric C_{18}	Yes
Develosil ODS-K-3	Nomura Chemicals	50 × 4.6	3	Monomeric C_{18}	Yes

2. Experimental

2.1. Chemicals and materials

Perchloric acid, phosphoric acid and NaH_2Po_4 of guaranteed grade were purchased from Wako (Osaka, Japan). Organic solvents (methanol and acetonitrile) of HPLC grade were obtained from Kanto Chemicals (Tokyo, Japan). Peptides were purchased from the Peptide Institute (Kyoto, Japan) and antibacterial chemicals from Sigma (St. Louis, MO, USA). The HPLC columns (from LCC, Yamaguchi, Japan) are listed in Table 1 with regard to column size, supplier, particle size, functionality and end-capping procedure. Mobile phases were prepared by mixing deionized water obtained with a Milli-Q system (Millipore, Bedford, MA, USA) and an HPLC-grade organic solvent. Perchloric acid was added directly to an aqueous solution. Phosphate buffer was made by using NaH_2PO_4 (pH 6.8 adjusted with concentrated H_3PO_4). For peptide separations and determination of antibacterial chemicals, linear gradient elution was adopted.

2.2. Instrumentation

The HPLC system consisted of a Model CCPM-II dual computer-controlled multi-pump (Tosoh), a UV-8020 variable-wavelength UV detector equipped with a 2-μl microflow cell or a Model UV-8010 detector equipped with 10 μl flow cells with and without a heat-sink coil (Tosoh) and Model MCPD-3600 Spectro multichannel photodiode-array detector (Tosoh).

The physical properties of Super-ODS and ODS-80Ts were measured using an Autoscan-60 mercury penetration apparatus (Quantachrome, Syosset, NY, USA) and a JSM-5300 scanning electron microscope (Jeol, Tokyo, Japan).

3. Results and discussion

Table 2 gives the physical data for Super-ODS and ODS-80Ts gels measured by the mercury penetration method and scanning electron microscopy. Whereas ODS-80Ts has granules, Super-ODS possesses a smaller pore volume and

Table 2
Physical properties of ODS gels

Column	Pore volume (ml/g)	Specific surface area (m^2/g)	Mean pore diameter (nm)	Particle size ±S.D. (μm)
TSKgel Super-ODS	0.25	96.8	11.2	2.29 ± 0.27
TSKgel ODS-80Ts	0.63	312.8	8.2	5.06 ± 0.87

surface area. The small S.D. on Super-ODS (0.27 μm) implies that the Super-ODS gel is more uniformly sized than with ODS-80Ts (S.D. 0.87 μm). Fig. 1A and B show scanning electron micrographs of Super-ODS with different magnifications. Tables 3 and 4 give the column efficiencies on Super-ODS and commercial fast LC columns under the same conditions. For each column size, Super-ODS shows greater efficiency (over 200 000 TP/m) than the fast LC columns (110 000–170 000 TP/m). In spite of the high column performance with Super-ODS, the operating pressure drops were lower than those on the fast LC columns. It seems that the narrow particle distribution of the Super-ODS gel contributes to lowering the operating pressure drops. With respect to the retention ability, solutes on Super-ODS were eluted earlier than on the other columns. This is consistent with a small surface area of the base silica gel.

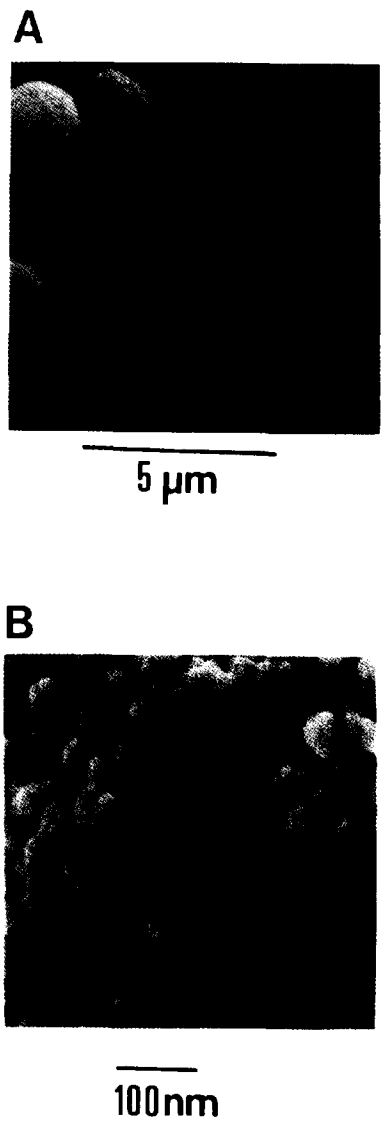

Fig. 1. Scanning electron micrographs of Super-ODS. Magnification: (A) ×5000; (B) ×100 000.

Fig. 2 shows a comparison of the elution profiles of a basic compound, pyridine, on Super-ODS and ODS-80Ts. In general, incompletely end-capped ODS gels give poor peak shapes for basic compounds owing to the strong adsorption [9,10]. The sharp peaks of pyridine on each column imply the existence of fewer ionic sites on each ODS surface contributing to the ionic interaction. Fig. 3 also demonstrates the inertness of the Super-ODS gel as a chelating reagent. Metal impurities on ODS gels affect on the elution profiles of chelating [11–13] and oxidizing compounds [14] owing to the formation of chelating complexes or to conversion into an oxidized form in an ODS column by an redox reaction. Consequently, the use of a purified silica gel containing a smaller amount of metal impurities leads to better peak shapes and reproducible chromatographic results.

Jinno and Kawasaki [15] emphasized that the selectivity for planar compounds relates to the manner of the introduction of a functional group on the silica surface, which is a monomeric and polymeric ODS layer. The separation factor, α(OT/TR), between *o*-terphenyl (OT) and triphenylene (TR) on a polymeric ODS column is generally greater than that on a monomeric ODS. As indicated in Table 5, the Super-ODS gel possesses a polymeric layer because it has a large α(OT/TR) of 1.98, whereas ODS-80Ts gel has a monomeric layer owing to the small α(OT/TR) of 1.27.

To obtain a high efficiency on a short column, there are a few factors to be considered, such as the void volume and the time constant, ν, of the detector [16]. The former factor consists of

Table 3
Comparison of column efficiencies on ODS columns

Column	Fluorene		Separation factor, α(NAP/FLU)	Pressure drop (MPa)
	Retention time (min)	TP/column		
TSKgel Super-ODS	3.71	10728	2.30	9.8
YMC-FL-ODS-3	6.10	7453	2.38	9.9
Capcell Pak $3C_{18}$	4.70	8701	2.27	12.6
Cosmosil $3C_{18}$	6.58	5893	2.39	11.8
Develosil ODS-K-3	6.61	7652	2.38	9.5

Mobile phase, methanol–water (70:30); detection, UV at 254 nm (detector equipped with a 2 μl flow cell); sample, naphthalene (NAP)–fluorene (FLU); flow-rate, 1 ml/min; column size, 50 mm × 4.6 mm I.D.

Table 4
Comparison of ODS column efficiencies

Column	Naphthalene		Pressure drop (MPa)
	Retention time (min)	TP/column	
TSKgel Super-ODS	4.06	20612	19.4
Capcell Pak $3C_{18}$	4.46	10651	26.5
Cosmosil $3C_{18}$	3.47	11685	19.4

Sample, naphthalene; column size, 100 mm × 4.6 mm I.D.; other conditions as in Table 3.

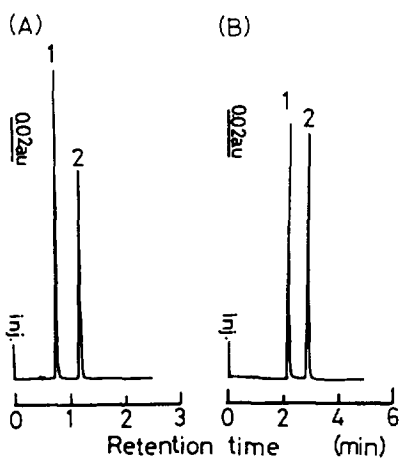

Fig. 2. Elution profiles of pyridine on RP columns. Column, (A) TSKgel Super-ODS (50 mm × 4.6 mm I.D.) and (B) TSKgel ODS-80Ts (150 mm × 4.6 mm I.D.); mobile phase, (A) acetonitrile–water (30:70) and (B) acetonitrile–water (50:50); detection, UV at 254 nm (ν = 50 ms); flow-rate, 1 ml/min; temperature, ambient. Peaks: 1 = pyridine; 2 = phenol.

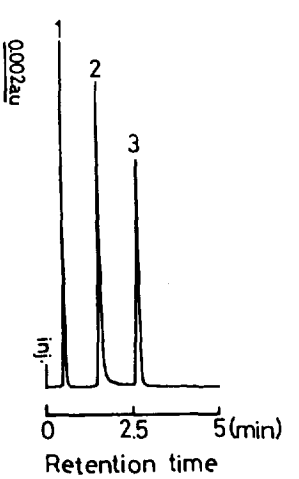

Fig. 3. Chromatogram of chelating reagent on Super-ODS. Column, TSKgel Super-ODS (50 mm × 4.6 mm I.D.); mobile phase, 20 m*M* Na_2HPO_4 (pH 6.8, adjusted with concentrated H_3PO_4)–acetonitrile (70:30); detection, UV at 245 nm; other conditions as in Fig. 2. Peaks: 1 = uracil; 2 = 8-quinolinol; 3 = methyl benzoate.

Table 5
Comparison of planar recognition ability on ODS columns

Column	o-Terphenyl		Triphenylene		Separation factor, α(OT/TR)	Resolution factor, R_s(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

Mobile phase, methanol–water (80:20) for Super-ODS and (85:15) for ODS-80Ts; detection, UV at 254 nm; sample, o-terphenyl (OT)–triphenylene (TR). The void volume of each column, v_0, was estimated from the elution volume of Uracil.

Table 6
Effect of void volumes in connecting tube on column efficiency of Super-ODS

(A) Injector–column			(B) Column–detector		
Tube length (cm)	Void volume (μl)	TP/column	Tube length (cm)	Void volume (μl)	TP/column
10	0.79	10741	10	0.79	10741
15	1.19	10635	15	1.19	10631
30	2.36	9569	30	2.36	10540
50	3.93	9076	50	3.93	9340
70	5.50	8496	70	5.50	9021

Stainless-steel tubes of 0.1 mm I.D. were used. In (A) the connecting tube length between the column and the detector was 10 cm and in (B) the tube length between the injector and the column was 10 cm. Other conditions as in Table 3.

connecting tubes, flow cell and sample volume injected. Table 6 shows the effect of the void volume in the connecting tubes on the number of theoretical plates. To prevent a decrease in column efficiency, the void volumes in the connecting tubes between the injector and column and the column and detector should be less than 2 μl.

The effect of the cell volume on column

Table 7
Effect of cell volume on column efficiency

Cell volume (μl)	TP/column	Decrease in column efficiency (%)
2	10767	0
10	10150	6
10[a]	3104	71

Detection, UV at 254 nm. Other conditions as in Table 3.
[a] The 10-μl flow cell was equipped with a heat sink coil of 1000 mm × 0.25 mm I.D.

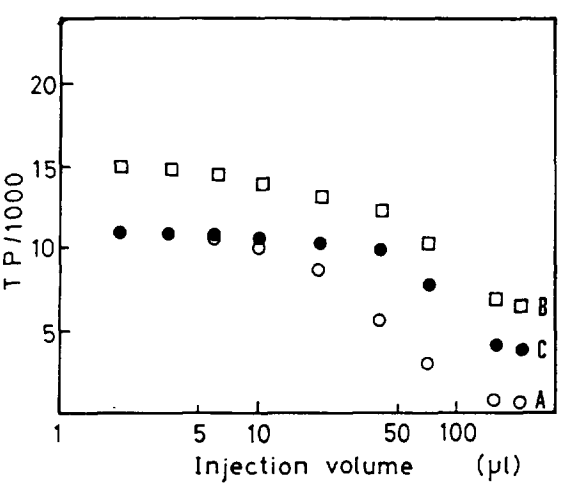

Fig. 4. Relationship between column efficiency and sample volume. Column, (A, C) TSKgel Super-ODS (50 mm × 4.6 mm I.D.) and (B) TSKgel ODS-80Ts (150 mm × 4.6 mm I.D.); mobile phase, (A, B, C) methanol–water (70:30); sample, naphthalene dissolved in (A, B) methanol–water (70:30) and (C) methanol–water (40:60); sample concentration, 0.1 mg/ml; other conditions as in Fig. 2.

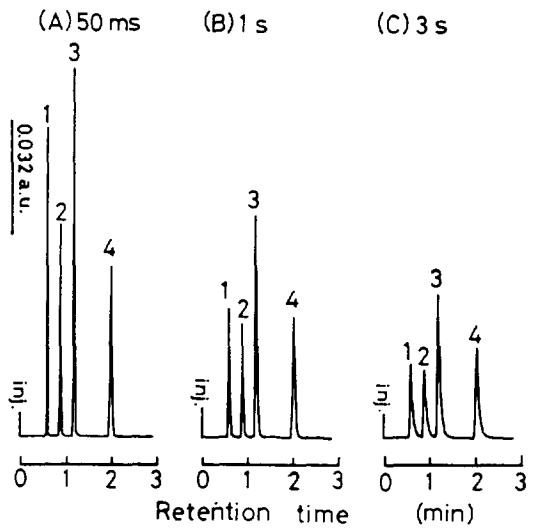

Fig. 5. Effect of time constant on peak shapes on Super-ODS. Mobile phase, acetonitrile–water (50:50); detection, UV at 254 nm [ν = (A) 50 ms, (B) 1 s and (C) 3 s]; other conditions as in Fig. 2. Peaks: 1 = uracil; 2 = benzene; 3 = toluene; 4 = naphthalene.

efficiency is summarized in Table 7. The use of a small cell volume is necessary to obtain high column efficiencies.

Fig. 4 shows the relationship between sample volume injected and column efficiency. The number of theoretical plates on Super-ODS decreased steeply with injection volumes over 10 μl (curve A). On the other hand, high column efficiencies on ODS-80Ts were maintained up to

Table 8
Effect of time constant on column efficiency

Time constant (ν)	TP/column	Resolution factor, *Rs* (TOL/NAP)
50 ms	10 529	13.37
1 s	6996	10.37
3 s	3420	6.87

injections of 50 μl (curve B). This is attributable to the difference in the surface areas of the packing materials. Although Giesche et al. [2] suggested that the sample volume must be 0.6 μl in their LC system, it is obvious that the surface area of their non-porous gel was too small to retain solutes adequately. With the injection of a sample solution that contains smaller amount of an organic solvent than that in the mobile phase, much larger sample volumes could be applied to the Super-ODS column without a decrease in column efficiency (curve C).

The time constant of the detector is another important factor, as reported by Giesche et al. [2]. The numbers of theoretical plates for the drugs on Super-ODS measured with different time constants are listed in Table 8. The TP measured with ν = 3 s decreased drastically. Fig. 5 illustrates the chromatograms of the drugs with different time constants (ν = 50 ms, 1 s and 3 s).

Fig. 6 shows the relationship between HETP and linear velocity, u (cm/min), using (A)

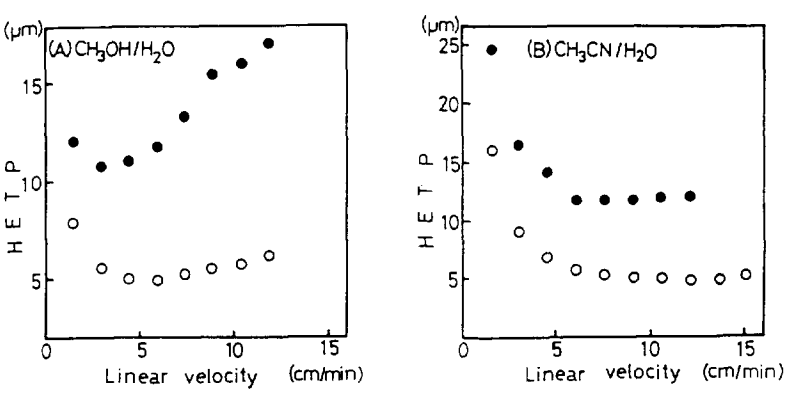

Fig. 6. H versus u curves on Super-ODS and ODS-80Ts with different mobile phases. Column, ○ = TSKgel Super-ODS; ● = ODS-80Ts; mobile phase, (A) methanol–water (70:30); (B) acetonitrile–water (50:50); sample, fluorene; flow-rate, 0.25–2.5 ml/min; other conditions as in Fig. 2.

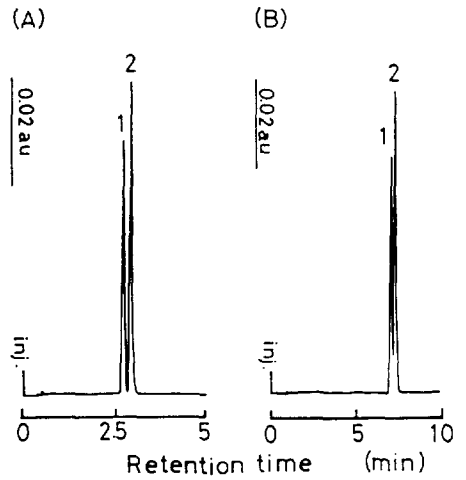

Fig. 7. Comparison of resolution between vitamin D_2 and D_3 on Super-ODS and ODS-80Ts. Mobile phase, methanol; other conditions as in Fig. 2. Peaks: 1 = vitamin D_2; 2 = vitamin D_3.

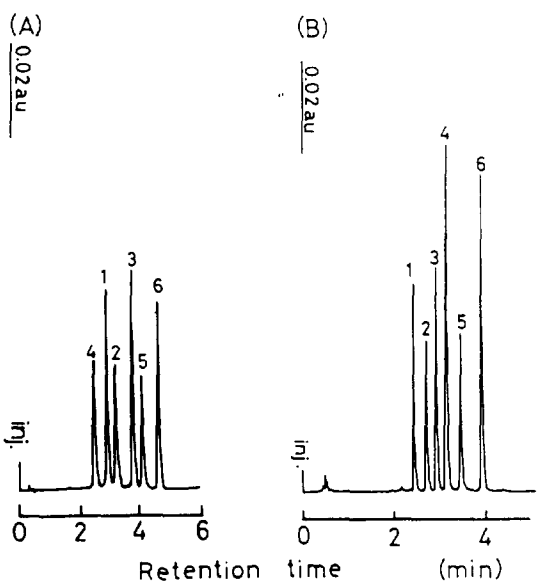

Fig. 8. Comparison of the separation of peptides on Super-ODS and Octadecyl-NRP. Column, (A) TSKgel Octadecyl-NPR (35 mm × 4.6 mm I.D.) and (B) TSKgel Super-ODS (50 mm × 4.6 mm I.D.); mobile phase, 13 m*M* perchloric acid–acetonitrile, with linear gradients of acetonitrile from 0% (for NPR) and 10% (for Super-ODS) to 50% in 10 min; detection, UV at 220 nm (ν = 50 ms); amounts of each solute injected, 0.1–0.2 μg; flow-rate, 1.5 ml/min on Octadecyl-NPR and 2 ml/min on Super-ODS; other conditions as in Fig. 2. Peaks: 1 = oxytosin; 2 = α-endorphin; 3 = bombesin; 4 = leu-enkephalin; 5 = γ-endorphin; 6 = somatostatin.

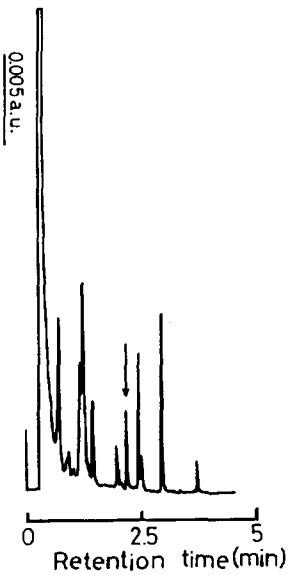

Fig. 9. Chromatogram of pork extract on TSKgel Super-ODS. Column, TSKgel Super-ODS (50 mm × 4.6 mm I.D.); mobile phase, 0.1% H_3PO_4–acetonitrile, with a 5-min linear gradient of acetonitrile from 10% to 40%; flow-rate, 2 ml/min; amount of sample injected, 10 μl of extract solution; detection, UV at 260 nm. The arrowed peak was investigated with the photodiode-array detector.

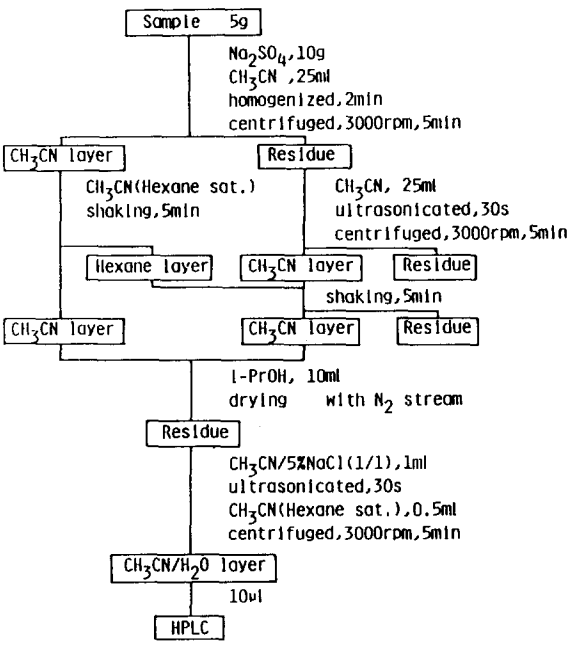

Fig. 10. Procedure for extraction of antibacterials in pork.

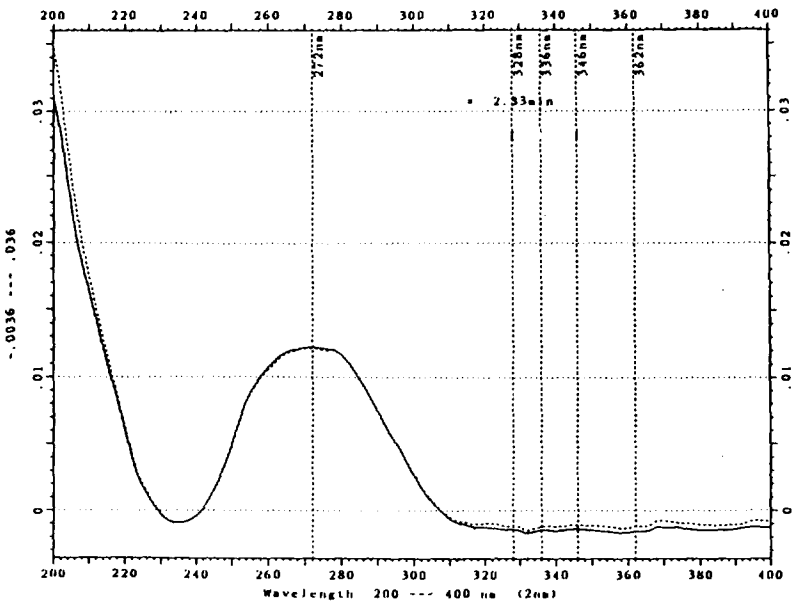

Fig. 11. Spectra of the arrowed peak in Fig. 9 (dashed line) and standard sulfadimethoxine (solid line). Detection with multi-channel photodiode-array detector. The similarity index was 960/1000. The *y*-axis represents absorbance units.

methanol–water and (B) acetonitrile–water systems. The smallest HETPs with methanol–water were obtained at 3 cm/min on ODS-80Ts and 6 cm/min on Super-ODS. Whereas the HETPs on each column increased with increase in linear velocity using methanol–water, slight changes in the HETPs on both columns were observed at more than 6 cm/min with acetonitrile–water. This is due to the difference in the viscosities of the mobile phases. Consequently, the use of the acetonitrile–water as the mobile phase would be convenient in fast LC.

Fig. 7 shows a comparison of the separation of vitamin D_2 and D_3 on Super-ODS and ODS-80Ts. The $\alpha(D_2/D_3)$ values were 1.10 on Super-ODS and 1.03 on ODS-80Ts. Evidently Super-ODS gives a fast and excellent resolution.

Fig. 8 compares peptide separation on Super-ODS and Octadecyl-NPR, which is an RP column based on a non-porous resin for separating biological macromolecules [17]. Under the optimum conditions, the peptide mixture was successfully separated on Super-ODS within 5 min and also on the non-porous column.

Fig. 9 shows the chromatogram for an extract from pork meat obtained as described in Fig. 10. By measurement of the arrowed peak on the chromatogram using the multi-channel photodiode-array detector, the presence of an antibacterial chemical (sulfadimethoxine) in the pork was recognized (Fig. 11).

4. Conclusions

The reversed-phase material, TSKgel Super-ODS, based on 2-μm porous microspherical silica gel, possesses a polymeric ODS layer on high-purity silica gel. This column showed less ionic and metal interactions than other columns. The use of the small particle size gives higher column efficiencies than other RP columns for many compounds. Therefore, fast separations and better resolutions can be achieved on Super-ODS. A few factors must be considered, however, when using this column: the retention ability is lower than that of other conventional ODS columns so that the content of organic modifier in the mobile phase should be lowered,

and the void volumes in the operating system must be minimized as much as possible.

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