

MONOLITHIC DISK FOR THE FAST CHROMATOGRAPHIC SEPARATION OF PROTEINS

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ABSTRACT

Poly(styrene/divinylbenzene) (PS/DVB) monolithic disk was prepared by in situ free-radical copolymerization of styrene and divinylbenzene in the presence of decanol and tetrahydrofuran as porogens. PS/DVB monolithic disks were produced in two different lengths 1.5 mm and 3 mm. The disks were used in reversed phase chromatography of proteins with 0.2 % trifluoroacetic acid (TFA) and 0.2 % TFA in acetonitrile as mobile phase A and B, respectively. The effect of gradient rate, flow rate, temperature and disk length on the separation of proteins were also studied. PS/DVB monolithic disks allow the rapid separation of proteins in reversed phase chromatography.

Keywords: *monolithic disk, poly(styrene/divinylbenzene), proteins*

INTRODUCTION

During the last seventeen years, a tremendous increase in the speed of chromatographic separations of large molecules having low mobility, such as proteins, DNA, and even viruses, can be noticed. These fast separations, with concomitant reduction in the contact time between the labile biomolecules and the surface of the stationary phase, have enabled a considerable increase in both the specific activity and yield various therapeutics. These benefits make HPLC one of the key analytical techniques requested by regulatory authorities [1, 2].

Chromatography researchers continue to make improvement in chromatography packings and sorbent for biomolecules. Each generation of chromatographic sorbent has provided better efficiency and increased mechanical stability in their resistance to back pressure generated by flow. The four distinct generations of sorbent materials have been polysaccharide-based, cross-linked and coated, monodisperse and monoliths [3]. Monoliths are the fourth generation and the latest development to increase the chromatographic performance. Reversed phase HPLC on hydrophobic stationary phases is one of the most common and powerful chromatographic separation modes for proteins and peptides [4].

The speed of the separation depends on the length of the column that determines the residence time of the analyte molecules within the separation device. Since, in general, the residence times in shorter columns are shorter at the same flow velocity, the separation cycle is faster. However, such short columns must possess sufficient separation power to afford the desired resolution. Chen and Horváth [2] have shown that for proteins this could be achieved using gradient elution. Particle diameters of less than 1 μm and column lengths shorter than 1 cm were suggested to be optimal for the separation on these short columns and, therefore, this approach was not suitable for preparative work [1].

From the point of view of fast and efficient chromatographic separations of large biomolecules such as proteins, the short monolithic disk are characterized by some very interesting features such as low flow resistance, the absence of a gradient in the mobile phase composition between the inlet and the outlet of the column as well as improved mass transfer characteristics [5]. Monolithic disk was first produced and commercialized by Knauer Saelentechnik (Berlin, Germany) in 1990 [6,7]. Eight years later, BIA separation d.o.o (Ljubljana, Slovenia) made it under the trade name Convective Interactive Media (CIM). Belenkii and co-workers [8] for reversed phase chromatography together with Tennikova *et al.* for ion-exchange chromatography [9] developed the concept of short monolithic column (disk). The main conclusion was that it is possible to use ultra-

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short columns for the separation of proteins and other molecules that traditionally have been separated using steep gradients, with appropriate resolution and at low gradient times. Short columns can be efficiently used for the separation of proteins and probably for other large molecules as well, especially in reversed phase chromatography [5].

The major difference in the treatment of column (conventional particle-based, but also monolithic columns) chromatography in general and disk chromatography is the significance ascribed to any individual adsorption/desorption step. Multiple interactions and hence (statistical) accumulation of the effects are assumed in column chromatography. Consequently, the effect of the initial step is considered as too insignificant to deserve or require an individual evaluation. In disk chromatography, on the other hand, the separation is interpreted as one-act desorption process even in such cases where the theoretical separation layer thickness does not exactly correlate with the physical thickness of the chromatographic disk [10].

This study described the preparation PS/DVB monolithic disks and their application as stationary phases for the reversed phase chromatographic separation of proteins.

EXPERIMENTAL SECTION

Materials

Ribonuclease A bovine pancreas (min. 90 %), cytochrome C equine heart (min. 95%), α -lactalbumin Type I bovine milk (≥ 85 %), myoglobin equine heart (min. 90 %), 1-decanol (≥ 98 %), 1-decanol (purum) and trifluoroacetic acid 99 % (TFA) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile gradient grade ($\geq 99.9\%$), divinylbenzene (synthesis grade) and tetrahydrofuran (≥ 99.9 %) were purchased from Merck (Darmstadt, Germany). α , α' -Azobisisobutyronitrile (AIBN, ≥ 98 %) was from Fluka (Buchs, Switzerland), styrene (synthesis grade) was from J.T. Baker (Deventer, Netherland) and Argon gas were from AGA (Vienna, Austria).

Styrene and divinylbenzene were distilled before use to remove inhibitor. The tetrahydrofuran (THF) used as porogen was distilled over sodium metal to remove the inhibitor 2,6-di-tert-butyl-4-methylphenol (BHT). For preparation of all aqueous solutions, high purity water (Epure, Barnstead Co., Newton, MA, USA) was used.

Procedure

Preparation of the PS/DVB monolithic disk

A mixture containing 600 μ L or 546.0 mg styrene, 600 μ L or 558.0 mg divinylbenzene, 1560

μ L or 1294.8 mg 1-decanol, 240 μ L or 213.6 mg THF, and 30.0 mg AIBN were sonicated and purged with argon for 5 minutes. The polymerization mixture was filled to the PEEK cylinder covered with the PEEK cap (the outer diameter of the pipe is 16 mm and corresponds to that in the disk, the inner diameter of the hole is 10.6 mm and corresponds to the inner diameter the disk).

The polymerization took place in the water bath at 70°C for 24 hours and then the disk was cut in 3 mm and 1.5 mm thick. The disk was put into housing, washed with 80 % Acetonitrile for 10 minutes, in order to remove remaining monomers and porogen.

High-performance liquid chromatography (HPLC) using PS/DVB monolithic disk

HPLC was carried out using a Merck-Hitachi pump model L-7120 controlled by a personal computer with HPLC System Manager model D-7000 software. The samples were injected through an autosampler from Merck-Hitachi L-7200, and monitor at 214 nm with an L-7400 Merck-Hitachi UV detector. The column oven was an L-7300 Merck-Hitachi.

Solutions consisting of 0.2 % TFA and 0.2 % TFA in acetonitrile were used as mobile phase A and B for the gradient elution, respectively.

RESULT AND DISCUSSION

Preparation of the PS/DVB monolithic disk

Preparation of the monolithic disk mostly involves three major steps: (i), polymerization of a monolithic material in a suitable format of sheets (membranes) or rods, (ii) cutting disks from them, and, (iii) if necessary, their chemical modification. Once the preparation of monolithic disk is completed, a short monolithic column is obtained by inserting them in an appropriate cartridge or other device [1].

PS/DVB monolithic disk was prepared by a free-radical copolymerization of styrene as a monomer with divinylbenzene as a cross-linking monomer in the presence of 1-decanol and THF as porogens and AIBN as an initiator. The reaction is illustrated in Figure 1. PS/DVB monolithic disks were produced in our group with two different lengths 1.5 mm and 3 mm (Figure 2).

High performance liquid chromatography using a PS/DVB monolithic disk was performed for the separation of standard proteins (Figure 3). An excellent separation of proteins within a short time was achieved using monolithic disk as shown in this figure.

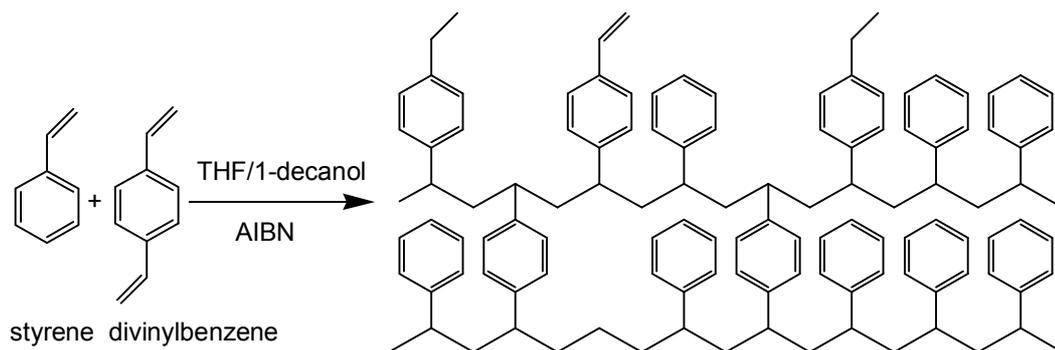


Figure 1 Free-radical copolymerization of PS/DVB monolithic

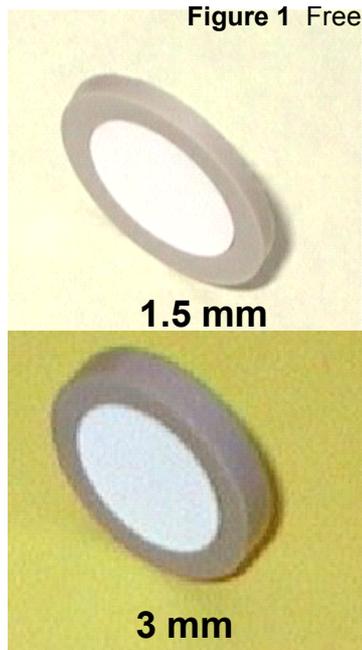


Figure 2 PS/DVB monolithic disk (Upper, PS/DVB monolithic disk with dimension: 1.5 x 10.6 mm, Lower, PS/DVB monolithic disk with dimension: 3 x 10.6 mm)

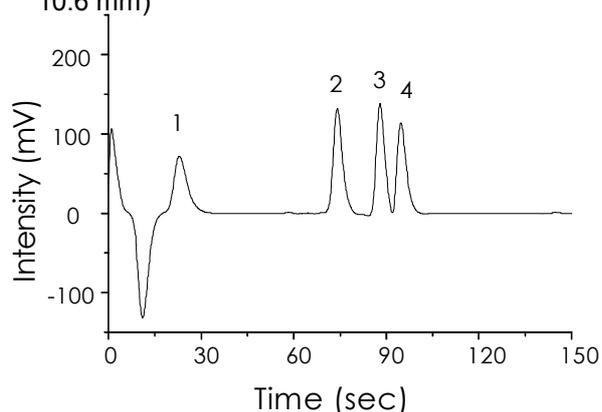


Figure 3 Separation of proteins on PS/DVB monolithic disk. Conditions: Disk 1.5 x 10.6 mm I.D.; mobile phase A = 0.2% aqueous TFA, B = 0.2% TFA in acetonitrile; gradient 80-10% A in 2.5 minutes, flow rate, 2 mL/min; detection UV, 214 nm; temperature, 55° C, injection volume, 5 μ L, sample :1=ribonuclease A, 2=cytochrome C, 3= α -lactalbumin and 4=myoglobin.

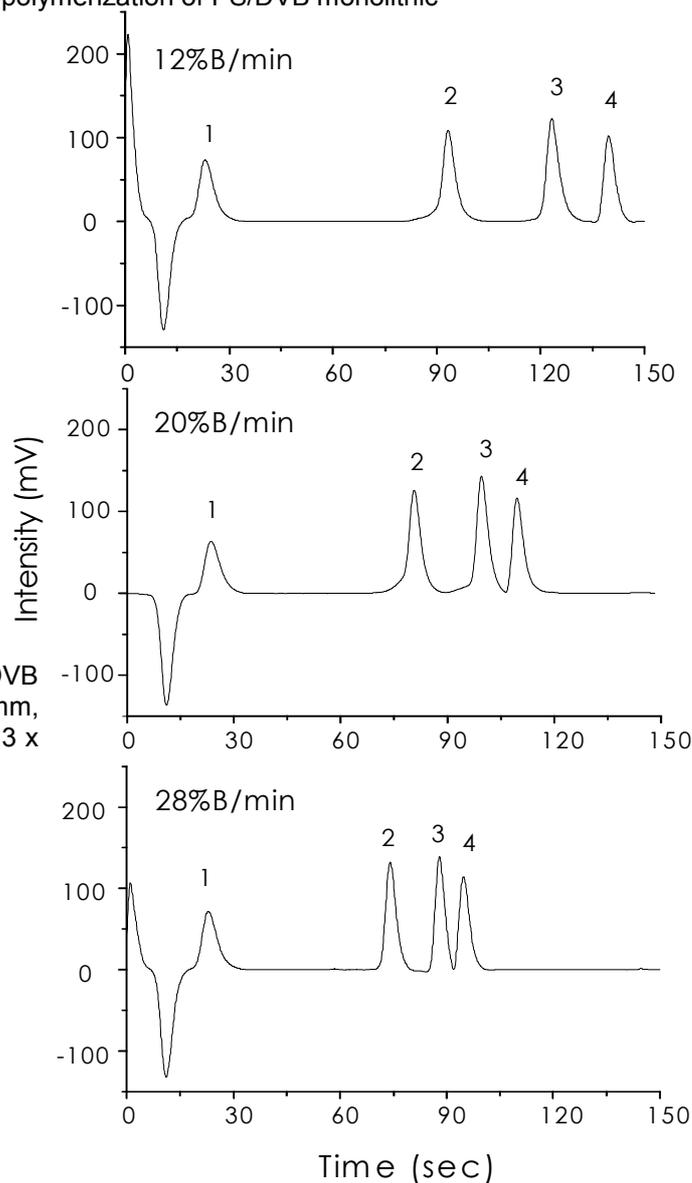


Figure 4 The effect of varying gradient-rate on separation of proteins using PS/DVB monolithic disk. Conditions: Disk 1.5 x 10.6 mm I.D.; mobile phase A = 0.2% aqueous TFA, B = 0.2% TFA in acetonitrile; flow rate, 2 mL/min; detection UV, 214 nm; temperature, 55° C, injection volume, 5 μ L.

The effect of gradient-rate on separation of proteins

Figure 4 demonstrates the effect of varying gradient-rate on the separation of proteins using a monolithic disk. An indirect correlation between retention times and increase of the gradient rate from 12% B/min to 28% B/min is evident. However a good separation can still be achieved with the highest gradient-rate (28% B/min), although resolution value are lower (Figure 5).

The effect of flow rate on sensitivity and resolution of proteins

Figure 6 shows the separations of proteins obtained at different flow rate, i.e. 1 mL/min (A) and 2 mL/min (B) at a constant gradient-rate of 12.5% B/min. As expectable, a higher flow rate shortens

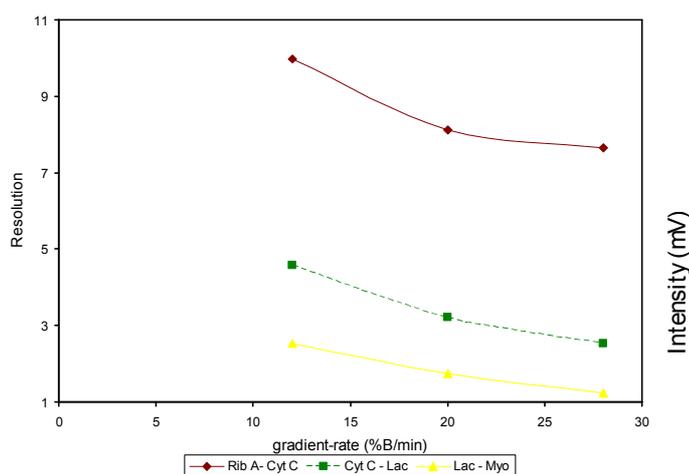


Figure 5 Dependence of resolution between standard proteins on gradient-rate. Column and conditions are the same as described in Figure 4.

the retention time of standard proteins, e.g. the retention time for protein 1 (ribonuclease A) is 133.2 and 72 seconds at flow rate 1 and 2 mL/min, respectively. Surprisingly, peak shape and as a consequence of that also resolution was improved, owing to lower longitudinal diffusion (Table 1). On the other side, the difference in retention time between protein peaks is independent on the flow rate.

The effect of flow rate on peak height and area of proteins are illustrated graphically in Figure 7 A and B respectively. From this figure, it can be summarized as follows:

1. Peak height increases with decreasing flow rate at constant gradient rate of 12.5% B/min, as already reported [11, 12].
2. Peak area decreases with increasing flow rate at constant gradient rate, owing to the detector response time and sample dilution [13].

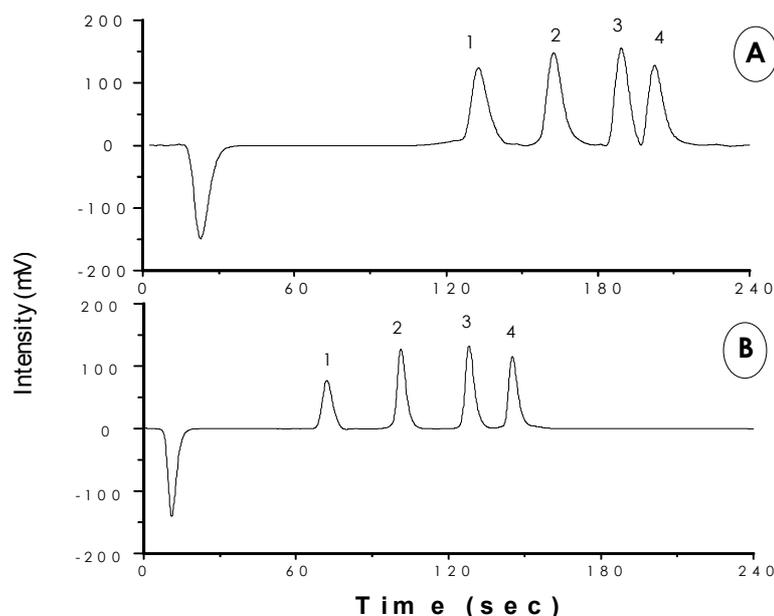


Figure 6 Effect of the flow rate to the separation of protein on PS/DVB monolithic disk (A; 1 mL/min, and B; 2 mL/min). Conditions: Disk 1.5 x 10.6 mm I.D.; mobile phase: A = 0.2% aqueous TFA, B = 0.2% TFA in acetonitrile, gradient: 80-30% A in 4 min; detection UV, 214 nm; temperature, 30°C

Table 1 Effect of flow rate on the resolution of proteins using PS/DVB monolithic disk

Flow rate (mL/min)	Resolution (R)		
	Rib A – Cyt C	Cyt C- Lac	Lac - Myo
1	2.53	2.41	1.21
2	4.05	4.12	2.58

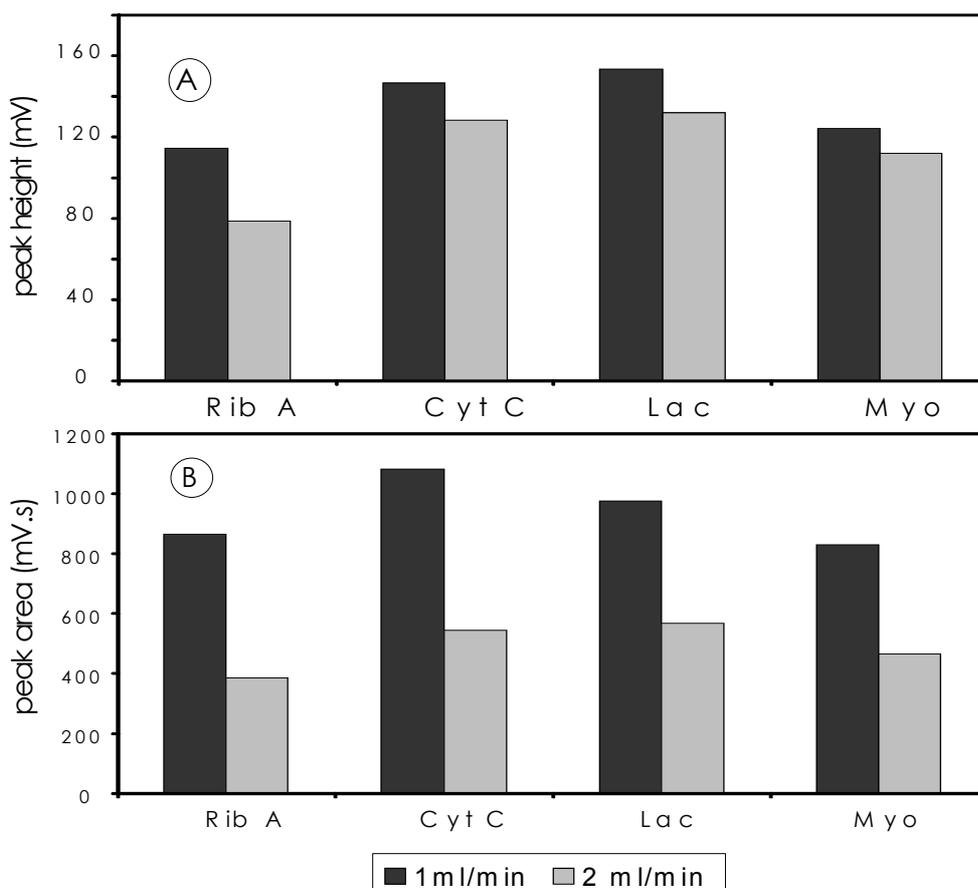


Figure 7 Graphical representation of effect flow rate on sensitivity of peak detection in reversed phase chromatography of proteins. Effect of flow rate on peak height of proteins (A) and peak area of proteins (B). Column and conditions are the same as described in figure 6.

Table 2 Influence of the temperature to the separation of proteins on PS/DVB monolithic disk

Ret. time (sec)	Temperature (°C)		
	30	40	55
ribonuclease A	66.0	59.4	23.4
cytochrome C	86.4	85.5	81.0
α -lactalbumin	103.8	103.2	99.6
myoglobin	114.6	114.0	109.8

The effect of temperature on proteins separation using monolithic disks

The effect of temperature on the separation of proteins using monolithic disks was studied in the range of 30° C, 40° C and 55° C (figure 8). It can be seen from figure 8 that increasing the temperature will shorten the retention time of the proteins, mainly ribonuclease A, accelerated from 66 seconds to 23.4 seconds retention time (Table 2). This data agree with already published investigation on conventional RP-HPLC, where a general reduction in protein or peptide retention time with increasing temperature was shown, due

to increasing solubility of the solute in the mobile phase [14, 15].

The effect of disk length

Previous study indicates that the column length often has only a small effect on the resolution of large molecules such as proteins in all modes of retentive chromatography [16]. In this study, two different disk lengths were used for protein separation i.e. 3 mm x 10.6 mm I.D. and 1.5 mm x 10.6 mm I.D. The evaluation showed that the disks with shorter length (B) delivered shorter retention times than a longer one (A), e.g. retention time for

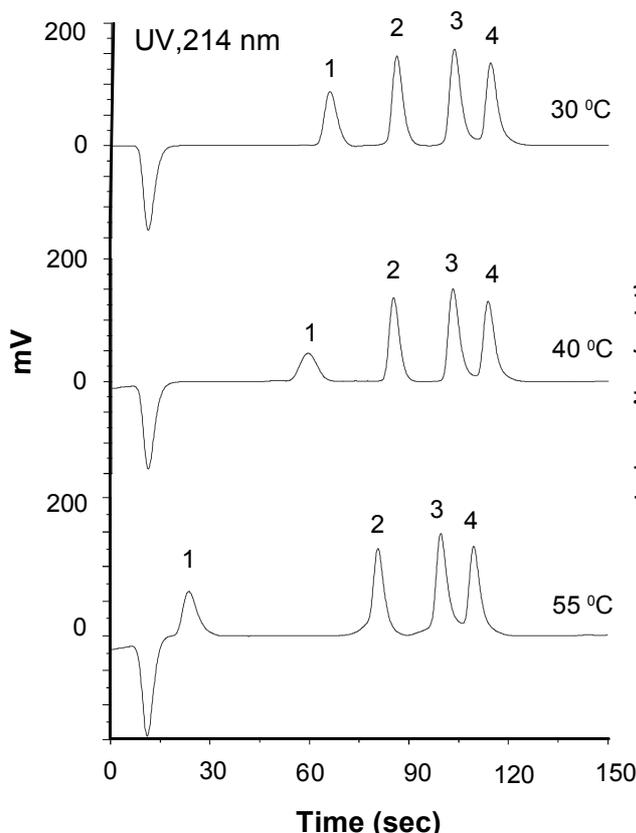


Figure 8 Influence of the temperature on the separation of proteins using a PS/DVB monolithic disk. Conditions: Disk, monolithic (1.5 x 10.6 mm I.D.); mobile phase A = 0.2% aqueous TFA, B = 0.2% TFA in acetonitrile; gradient, 80-30% A in 2.5 min; detection UV, 214 nm; flow rate, 2 mL/min.

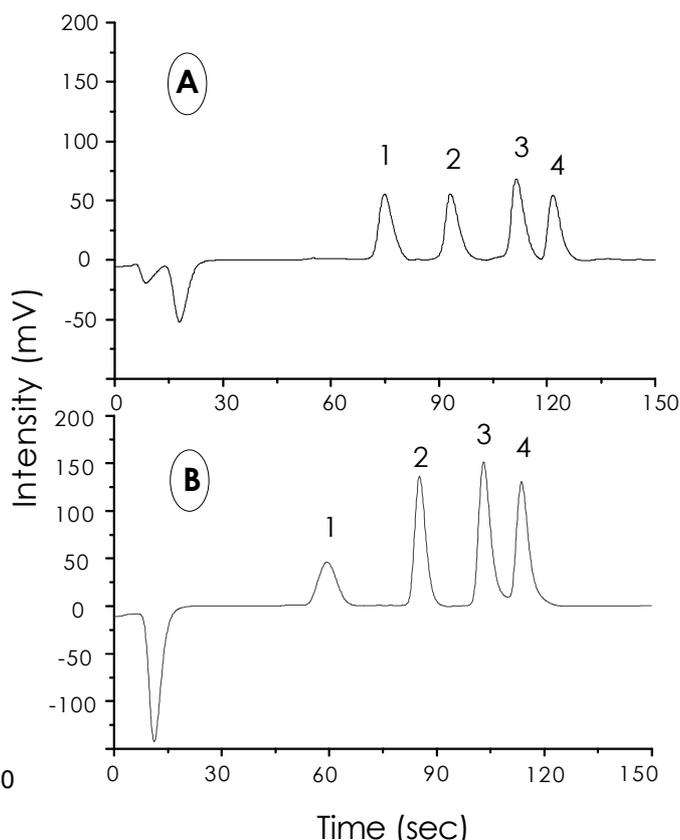


Figure 9 Separation of (1) ribonuclease A, (2) cytochrome C, (3) α -lactalbumin and (4) myoglobin on PS/DVB monolithic disk (A) 3 x 10.6 mm I.D. and (B) 1.5 x 10.6 mm I.D. Conditions: mobile phase A: 0.2% TFA in water, B: 0.2% TFA in acetonitrile; gradient, 80-30% A in 2.5 min, flow rate, 2 mL/min, detection, UV 214 nm, temperature, 40°C, injection volume, 5 μ L.

Table 3 The effect of disk length to the retention factor (k)

Protein	k	
	1.5 x 10.6 mm I.D.	3 x 10.6 mm I.D.
ribonuclease A	4.21	7.93
cytochrome C	6.47	10.07
α -lactalbumin	8.05	12.29
myoglobin	9.00	13.50

ribonuclease A is 59.4 seconds for 1.5 mm disk and 76.8 seconds for 3 mm disk (Figure 9). With the shorter length, separation times may decrease somewhat due to the smaller void volume, but under the slow gradient rates, which gave the best resolution; this difference becomes negligible [17]. However, the retention factor (k) is higher at least 1.5 times when using 3 mm disk than 1.5 mm disk (Table 3). In general, shorter disk is favored in term of fast separation of proteins.

CONCLUSION

The preparation of PS/DVB monolithic disks were simple by *in situ* free-radical copolymerization of styrene and divinylbenzene in the presence of decanol and THF as porogens. The disks enable fast separation of proteins in reversed phase chromatography.

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