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Separations of high-molecular-mass polystyrenes on different pore size and particle size reversed-phase columns in dichloromethane–acetonitrile

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Abstract

The effect of pore size and particle size of the silica support on the retention and resolution of high-molecular-mass polystyrenes was investigated in reversed-phase gradient elution high-performance liquid chromatography using a dichloromethane–acetonitrile mobile phase. An increase in pore size was found to increase retention slightly. A decrease in particle size was found to increase retention of all molecular masses. Both pore size and particle size had effects on resolution between polystyrenes of different molecular masses. Unretained polystyrene eluted at the solvent front for some of the conditions used but, this effect decreased with an increase in pore size and a decrease in particle size. A possible explanation for the greater effect on retention of particle size compared with pore size, is that the polystyrene molecules in a gradient elution experiment do not have access to the same number of pores as they do in an isocratic or size-exclusion experiment. It is proposed that this is due to a lag in the mobile phase composition in the pores compared with interstitial composition in the gradient experiment.

1. Introduction

Even though the analysis of polymers by reversed-phase chromatographic methods has received considerable attention in the last decade, very few molecular mass separations have been published. The first molecular mass separations using reversed-phase methods were published by Armstrong and Bui [1], who separated polystyrenes from molecular mass 2700 to 10^7 in a dichloromethane–methanol solvent system using octadecyl columns. Further separations were reported by the same workers where oligomers (from $n = 1$ to $n = 20$) and high-molecular-mass polystyrenes were separated using a single gradient [2]. Recent studies by Shalliker *et al.* [3] have shown that high-quality molecular mass separations of polystyrenes to a molecular mass of 10^6 are achievable using a variety of low-cost commercial octadecyl reversed-phase columns and a dichloromethane–acetonitrile gradient solvent system. These separations have been used to determine the polydispersity of commercial polystyrenes [4] and the method compared very favourably to conventional size-exclusion chromatography.

The behaviour of polystyrene in reversed-phase chromatography has been examined by a number of other workers in a variety of solvent systems and there appears to be little consensus

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on the mechanisms or the role of pore size. Larmann *et al.* [5] studied the behaviour of polystyrenes in a tetrahydrofuran–water solvent system for molecular masses up to 50 000. They concluded that retention followed normal chromatographic adsorption behaviour and that pore size had little effect because polymer chains could snake into excluded pores. Glöckner and Borth [6,7] studied the behaviour of polystyrene in solvent systems consisting of tetrahydrofuran–methanol and tetrahydrofuran with a number of alkanes. Glöckner [8] explained that the retention followed a precipitation redissolution mechanism. Quarry *et al.* [9] examined the retention mechanism of M_r 50 000 polystyrene in tetrahydrofuran–water and concluded that normal chromatographic behaviour, rather than precipitation–redissolution occurred, provided sample size was less than 200 μg. Northrop *et al.* [10] examined retention on a C_4 stationary phase using dichloromethane–methanol and tetrahydrofuran–acetonitrile solvent systems. They suggested that the pore size was important for the retention of the polystyrenes and showed that retention of the high-molecular-mass polystyrenes was reduced when excluded from the pores. They concluded that polystyrene retention behaviour depended on both the mobile phase and the exclusion properties of the column. Also, Lochmüller and McGranaghan [11], using a dichloromethane–acetonitrile solvent system reported a similar reduction in the expected retention of excluded polystyrenes. Studies by Shalliker *et al.* [12] in a dichloromethane–methanol solvent system and C_{18} columns of various pore sizes found that when the polystyrenes had access to the pores, elution occurred by adsorption or partitioning processes as elution occurred after the polymer solubility composition. However, when the polymers were excluded from the pores, the elution was complicated by polymer–solvent and polymer–polymer interactions that caused the polymer to elute prior to the polymer solubility composition or even before the solvent front. It is clear that the mobile phase is important for the elution of high-molecular-mass polystyrenes without unusual chromatographic effects. The best solvent systems reported to date appear to be dichloromethane–acetonitrile [3,4,11] and tetrahydrofuran–acetonitrile [10].

The development of small-diameter particles and improved methods for uniform column packing have lead to a great increase in the efficiency of the separations of small molecules. Columns packed with particles down to 2 μm in diameter have been used in protein separations [13]. The use of large diameter particles for all but preparative chromatography has generally become obsolete. Recently however large-diameter 20-μm non-porous particles have been employed for the rapid separation of protein mixtures [14]. These particles offered several advantages. They were easier to pack using the dry pack method. They operated at lower pressures and they were also less prone to clogging by strongly adsorbed species. Similar advantages are available with large particle size pellicular supports.

This paper reports the effect of pore size and particle size of the silica support on the reversed-phase retention behaviour of polystyrenes up to a molecular mass of 929 000 in the dichloromethane–acetonitrile solvent system. The effect of pore size is investigated by observing the retention on C_{18} reversed-phase supports with 10 μm particle size and three different pore sizes. For comparison, columns with a porous 6 μm particle size silica and a 35 μm particle size pellicular support were also used.

2. Experimental

All chromatographic experiments were performed using two M6000A pumps, a 660 solvent programmer and a U6K injector (Waters Associates, Milford, MA, USA). A Linear UVIS 200 variable-wavelength absorbance detector set at 262 nm was used (Linear Instruments, NV, USA). Data acquisition and analysis were done with a previously used [3] laboratory-built system and a 386 personal computer.

HPLC-grade acetonitrile and dichloromethane were used as mobile phases (Mallinckrodt Australia). The monodisperse polystyrene standards used were molecular masses 17 500, 50 000,

110 000, 410 000, 929 000 (Polyscience, Warrington, PA, USA) and 200 000 (Waters). A Zorbax ODS 150 mm packed column (DuPont, Wilmington, DE, USA) was purchased. Spherisorb 10 μm ODS (Phase Separation, Clwyd, UK) was purchased as a C_{18} packing. Perisorb A, LiChrospher SI500 and LiChrospher SI1000 (E. Merck, Darmstadt, Germany) were silylated. The reversed-phase packing materials were packed into identical column blanks of dimensions 150 mm × 4.6 mm I.D. The silylation method and packing methods are described elsewhere [15]. The details of all columns used are presented in Table 1. In this table, the surface areas were obtained from manufacturer's data. The carbon loading was obtained by drying the silylated silica in a vacuum oven at 50°C for 24 h, weighing, heating at 600°C for 24 h and reweighing. This method gave good agreement with elemental carbon and hydrogen analysis [15]. Surface ligand densities were estimated using the method of Berendsen and De Galan [16]. These surface ligand densities are all within 30% of the average. Size-exclusion calibration curves for each column were obtained by eluting the polystyrene standards in pure dichloromethane. The sample size was 10 μg and the flow-rate 0.5 ml/min. These curves are shown in Fig. 1.

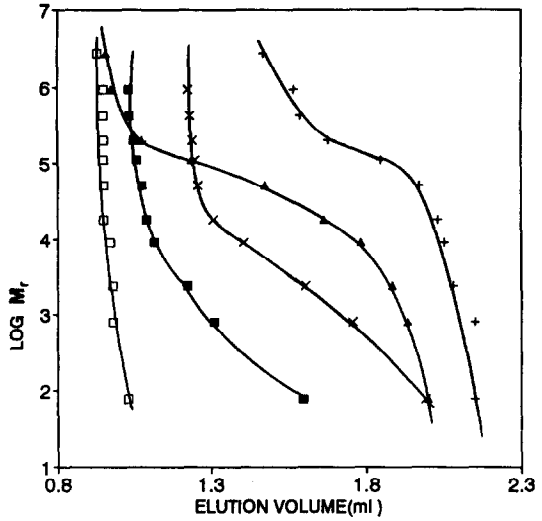

Fig. 1. Size-exclusion curves. Sample size, 10 μg; flow-rate, 0.5 ml min^{-1}; mobile phase, dichloromethane. Symbols: ■ = 7-nm pore size, 6-μm particle size column; × = 8-nm pore size, 10-μm particle size column; ▲ = 50-nm pore size, 10-μm particle size column; + = 100-nm pore size, 10-μm particle size column; □ = pellicular, 30–40-μm particle size column.

Gradient elution separations were obtained by injecting polystyrenes dissolved in pure dichloromethane. Sample size was 10 μg. Sample volume was 10 μl. The gradient started at 0.48 volume fraction of dichloromethane in acetonitrile and changed to a 0.64 volume fraction dichloromethane in acetonitrile over a 6 h period at a flow-rate of 1.0 ml/min. The gradient was stopped after elution of the last peak. For the pellicular column, the initial volume fraction of dichloromethane was 0.46. Curve 3 on the Waters 660 programmer defined the gradient profile at the column outlet. This profile is represented [18] by the equation

$$\varphi_e = \varphi_i + (\varphi_f - \varphi_i)\left(\frac{V_e - V_d}{V}\right)^k \quad (1)$$

Table 1
Column data

Silica	Pore size (nm)	Particle size (μm)	Surface area (m^2/g)	Carbon (%)	Ligand density (μmol m^{-2})	Reduced plate height[a]
Zorbax	7	6	350	14.5	2.7	2.08
Spherisorb	8	10	220	8.0	1.8	3.70
LiChrospher	50	10	60	4.6	3.8	0.34
LiChrospher	100	10	30	1.8	2.9	10.0
Perisorb	Pellicular	30–40	14	0.9	2.9	20.4

[a] Measured from phentole using conditions of Bristow and Knox [17].

where φ_e = volume fraction of dichloromethane at any elution volume; φ_i = initial volume fraction of dichloromethane; φ_f = final volume fraction of dichloromethane; V_e = elution volume; V = total volume of the gradient; V_d = delay volume; k = constant determined experimentally to be 0.31 compared with the literature value of 0.33 [18]. The experimental value was found by recording the gradient profile of dichloromethane spiked with benzene and fitting the curve to Eq. 1. Except for delay time at the detector, the gradient profile was the same with a column present as without. This gradient profile was used as it was found to give good resolution over the molecular mass range studied [3]. Linear gradients were found to give worse separation at higher molecular masses.

The value of the polymer solubility composition for each molecular mass, φ_s, was obtained by titrating the polymer dissolved in dichloromethane at 200 mg/l with acetonitrile at 25.0°C. The first appearance of a turbid suspension was used as φ_s. φ_s was expected to be concentration dependent and Glöckner [6] has shown that, for polystyrene in tetrahydrofuran–methanol, the amount of non-solvent required for precipitation increases as the polystyrene concentration decreases. The concentration of polystyrene was as low as practicable to approximate chromatographic conditions but the values of $\varphi_e - \varphi_s$ quoted in results are likely to be lower than the values expected under actual chromatographic conditions.

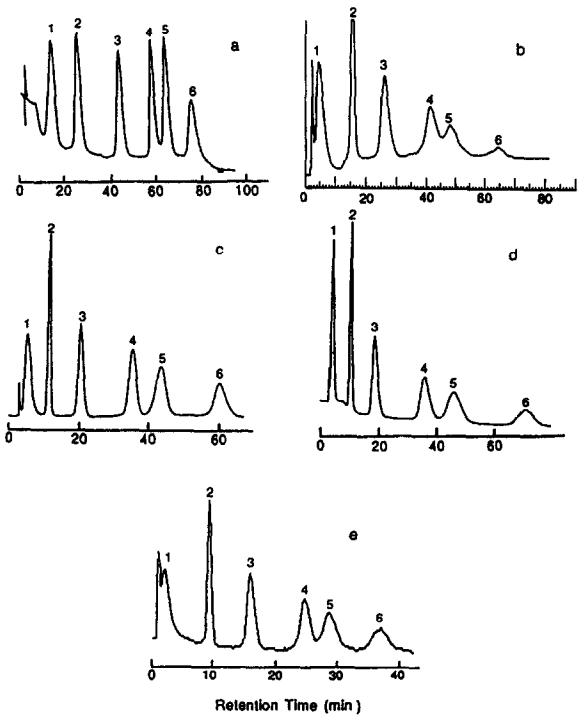

Fig. 2. Polystyrene separations. Sample size, 10 μg; injection solvent, dichloromethane; flow-rate, 1.0 ml min^{-1}; initial mobile phase, 0.48 volume fraction dichloromethane; final mobile phase, 0.64 volume fraction dichloromethane; gradient time, 6 h. (a) 7 nm pore size, 6 μm particle size column; (b) 8 nm pore size, 10 μm particle size column; (c) 50 nm pore size, 10 μm particle size column; (d) 100 nm pore size, 10 μm particle size column; (e) pellicular, 30–40 μm particle size column. Peaks: 1 = M_r 17 500 polystyrene; 2 = M_r 50 000 polystyrene; 3 = M_r 110 000 polystyrene; 4 = M_r 200 000 polystyrene; 5 = M_r 410 000 polystyrene; 6 = M_r 929 000 polystyrene.

3. Results and discussion

Fig. 2 shows the separations that were obtained on each of the columns. The high quality separations are similar to the separations obtained with commercial columns for the dichloromethane-acetonitrile solvent system [3]. No peaks eluted before the solvent front or before the polymer solubility composition φ_s, as reported for the dichloromethane–methanol solvent system [12]. However some columns did show elution at the solvent front. The important parameters describing these separations are considered to be as follows: (1) φ_e, the elution volume fraction of dichloromethane; (2) $\varphi_e - \varphi_s$, the difference between the elution volume fraction of dichloromethane and the polymer solubility volume fraction of dichloromethane (if, as in this study, this parameter is greater than zero then the polystyrenes must be eluting according to polymer stationary phase adsorption or partitioning interactions rather than a precipitation–redissolution mechanism; higher values of

$\varphi_e - \varphi_s$ indicate stronger interaction with the stationary phase); (3) R, the resolution between adjacent members of the polystyrene molecular mass series and (4) F, the area fraction of a polystyrene of given molecular mass eluting at the solvent front.

These parameters, for each column and polystyrene are shown in Table 2. The results show that gradient retention, whether determined by φ_e or $\varphi_e - \varphi_s$, increases as particle size of the silica support decreases for all molecular masses independent of pore size. Except for the M_r 17 500 polystyrene, an increase in pore size has a small but positive effect on retention.

Table 2
Polystyrene elution compositions, resolutions and fractions eluted at the solvent front

Column		M_r	φ_e [a]	$\varphi_e - \varphi_s$ [a]	R [a]	F [a]
Pore size (nm)	Particle size (μm)					
7	6	17 500	0.528	0.243	–	0
		50 000	0.543	0.148	2.23	0
		110 000	0.558	0.093	4.10	0
		200 000	0.567	0.082	3.38	0
		410 000	0.570	0.060	1.28	0
		929 000	0.575	0.035	2.14	0
8	10	17 500	0.480	0.195	–	0
		50 000	0.514	0.118	2.57	0
		110 000	0.537	0.072	2.50	0
		200 000	0.553	0.068	2.60	0
		410 000	0.558	0.048	0.71	0
		929 000	0.567	0.027	1.53	0.05
50	10	17 500	0.480	0.195	–	0
		50 000	0.519	0.124	2.50	0
		110 000	0.538	0.073	3.38	0
		200 000	0.554	0.069	4.09	0
		410 000	0.560	0.050	1.67	0
		929 000	0.570	0.030	2.74	0.02
100	10	17 500	0.480	0.195	–	0
		50 000	0.519	0.124	3.45	0
		110 000	0.535	0.088	3.27	0
		200 000	0.554	0.070	3.49	0
		410 000	0.560	0.050	1.38	0
		929 000	0.574	0.034	2.60	0
Pellicular	35	17 500	0.460	0.175	–	0.07
		50 000	0.499	0.101	3.45	0.03
		110 000	0.515	0.053	3.50	0.2
		200 000	0.529	0.047	3.00	0.2
		410 000	0.533	0.027	1.03	0.2
		929 000	0.541	0.005	2.15	0.4

Injection solvent, dichloromethane; sample size, 10 μg; initial composition, 0.46 volume fraction dichloromethane; final composition, 0.64 volume fraction dichloromethane; gradient time, 6 h, flow-rate, 1.0 ml min^{-1}.
[a] See text.

These results are surprising and this can be seen by considering the chromatographic behaviour of the M_r 50 000 polystyrene. Fig. 1 gives the size-exclusion data obtained by injecting the polystyrenes into pure dichloromethane mobile phase on each of the columns used for the reversed-phase experiments. From Fig. 1, it can be seen that the M_r 50 000 polystyrene is virtually excluded from the pores of the 7- and 8-nm pore size silicas but has access to about half the pores on the 50-nm and nearly all the pores on the 100-nm pore size silicas when eluted in pure dichloromethane. It is well known that the hydrodynamic volume of a polymer decreases on changing from a good solvent to a poor solvent. From viscosity measurements [15] it can be estimated that there is a decrease in the hydrodynamic radius of about 12% for M_r 50 000 polystyrene on changing from pure dichloromethane to 60% dichloromethane in acetonitrile. Hence similar pore volumes are accessible in the gradient elution experiment. By assuming (see Fig. 1), that the M_r 50 000 polystyrene has access to only the external surface area of the 8-nm silica but nearly all the surface area (external plus internal) of the 100-nm silica, it can be estimated that the increase in accessible surface area on changing from the 8-nm silica to the 100-nm silica is about 40 times. Yet $\varphi_e - \varphi_s$ only increases 1.05 times from the situation where the M_r 50 000 polystyrene is totally excluded on the 8-nm column to the situation where it has access to all the pores on the 100-nm column. Additionally, on changing from 10- to 6-μm particles, with similar pore size (7 to 8 nm), there is an increase in external surface area of about 1.5 times. There is a corresponding increase in $\varphi_e - \varphi_s$ of about 1.25. This increase in $\varphi_e - \varphi_s$ is essentially constant for all molecular masses used in this study as all only have access to the external surface on these small pore size particles. It appears that even when the polystyrene molecules have access to the pores, according to size-exclusion data, they have difficulty interacting with the internal pore surfaces in gradient elution. They only interact easily with the external surface of the particle, even though only a small portion of the C_{18} chains are on the external surface.

A possible reason for this is due to the gradient change in mobile phase composition. As the fraction of good solvent increases and moves down the column, the mobile phase composition in the pores will lag marginally behind the interstitial mobile phase composition. Although minor mobile phase composition variations are unimportant for small molecules this is not so for large molecules. High-molecular-mass polystyrenes have a large value of S, the slope of log k *versus* mobile phase composition [5,11,15,19]. Hence they are likely to interact with stationary phase at the pore entrance until the mobile phase composition in the pore increases in good solvent. Also, there will be a distribution coefficient for polystyrene between the pore composition and the higher interstitial composition which will decrease the chance of entry into the pores. These effects are not present in isocratic or size-exclusion experiments. The polystyrenes in a gradient elution experiment therefore may not have access to as many pores as they do in a size exclusion experiment. A number of other workers [5,12,19] have studied the effect of pore size in the gradient elution of polystyrenes. Larmann *et al.* [5] found decreased retention with increase in pore size and hence decrease in surface area for molecular masses up to 50 000. When molecular masses are increased to over 100 000, then the larger pore size packings show increased retention [12,19] even though a variety of mobile phases were used. This agrees with the results found here. There is no other work with which to compare our results which indicate that a decrease in particle size has a greater effect on retention than an increase in pore size.

Resolution between adjacent polystyrenes in the series used does not vary in a systematic way with silica size and pore size. The resolution between the M_r 17 500 and 50 000 polystyrenes is complicated by the fact that the M_r 17 500 polystyrene elutes during the isocratic portion of the run, before the gradient reaches the top of the column, on nearly all except the 6-μm particle diameter and the 100-nm pore size silicas. The two highest-molecular-mass polystyrenes show the highest resolution on the 100-nm pore size column. A plot of log resolution per 1000 molecular mass units *versus* log molecu-

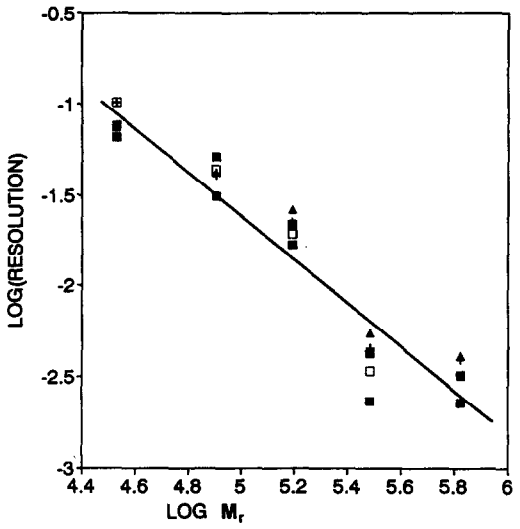

Fig. 3. Log resolution against log molecular mass. Symbols: ■ = 7-nm pore size, 6-μm particle size column; × = 8-nm pore size, 10-μm particle size column; ▲ = 50-nm pore size, 10-μm particle size column; + = 100-nm pore size, 10-μm particle size column; □ = pellicular, 30–40-μm particle size column.

lar mass (Fig. 3) shows a rapid fall in resolution with increase in molecular mass. There is little difference in resolution between columns, irrespective of pore size or particle size.

Some columns show unretained polystyrene eluting at the solvent front. This is an obvious disadvantage if molecular mass distributions are to be measured by reversed phase. The elution was most apparent on the pellicular column and injections of individual narrow disperse polystyrenes on this column showed that all tested molecular masses contributed to this peak. On the 8-nm and 50-nm pore size, 10-μm particle size columns, only the M_r 929 000 polystyrene showed elution at the solvent front. There was no indication of unretained polystyrene on the 100-nm pore size, 10-μm particle size column or the 6-μm particle size column. Re-examination of previous separations with the dichloromethane–acetonitrile solvent system and some commercial columns [3] indicates the same behaviour with the 10-μm columns, but again, a 5-μm column showed negligible solvent front peak. The fraction of unretained polystyrene, at constant injection mass, seems to be a function of available surface area and molecular mass.

The effect of injection mass on fraction of unretained polystyrene was not investigated. The injection mass was constant for all columns at 10 μg. We have previously shown that sample size up to 150 μg does not affect the chromatogram in a dichloromethane–acetonitrile solvent system [3]. This is unlike the dichloromethane–methanol system [12]. Previous workers [11,15] have shown that injection of polystyrenes dissolved in a mixture of the two solvents, rather than pure good solvent may overcome this problem. However, using a mixture of two solvents is complicated when a range of, possibly unknown, molecular masses is present because of different solubilities. Another method of eliminating elution at the solvent front is the recommendation by Lochmüller and McGranaghan [11] that a post-injection, pre-column mixer be used. This method adds considerable void volume to the system. The choice of a 5-μm particle size silica support seems a better solution.

4. Acknowledgement

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