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Reversed-phase gradient elution behaviour of polystyrenes in a dichloromethane–methanol solvent system

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ABSTRACT

A study was made of the gradient elution behaviour of the high-molecular-weight polystyrenes on C_{18} reversed-phase columns in methanol–dichloromethane solvent systems. The polymers were injected into mobile phase compositions which were expected to precipitate the polymer on the column and separation of the polymers was then expected as solvents able to dissolve the various molecular weights entered the column in the solvent gradient. The effects of column pore size, sample load and flow-rate were examined.

Results were determined mainly by polymer molecular weight and its relation to column pore size. When columns were chosen in which polymer had access to the pores, elution occurred after the expected solvent solubility composition indicating that normal adsorption processes were occuring. Polymers which were excluded from the pores underwent unusual elution processes which resulted in their elution at the solvent solubility composition, or before. The extent of this unexpected elution in a solvent of poorer solvating ability was more predominant on columns of small pore size.

The effects of these elution processes was to contribute to band broadening and to reduce selectivity between higher-molecular-weight polymers on small pore size adsorbents. An explanation as to the causes of such elution processes is presented, in which non-equilibrium solvation and precipitation of eluting polymer molecules occurs in the changing solvent gradient mixture.

INTRODUCTION

The molecular weight determination of macromolecules is usually carried out using size-exclusion chromatography (SEC). However, macromolecules have also been separated using reversed-phase high-performance liquid chromatography (RP-HPLC). As far as the chromatography of oligomers and lower molecular weights are concerned the separations achievable are at least as good and at times far exceed any SEC separation [1–6]. The RP-HPLC separation of polymers could become a powerful technique if the separation of the high molecular weights could be improved. Several workers [7–11] have studied separations of molecules with molecular weights in the order of several hundred thousand, however refinement is necessary to obtain separations comparable with those based on SEC.

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A mechanism for the gradient separation of polymers has been described by Glöckner [10], in which the molecules underwent a series of precipitation and redissolution processes along the column until finally eluting at a mobile phase composition equivalent to the solubility of the polymer. Boehm and co-workers [12–14] proposed a mechanism which considered that the polymer eluted at some "critical solution" composition. This mechanism has, however, been criticized by Quarry *et al.* [15] as being redundant and resembling the "on off" model where initially molecules stick to the column and are then eluted by the mobile phase with no reattachment. Snyder and co-workers [16,17] proposed that elution of polymers still obeys the principles of normal chromatographic adsorption and desorption processes. They showed in a study on polystyrene in tetrahydrofuran–water that elution was based on adsorption processes for sample loads less than 30 μg and for molecular weights up to 50 000 dalton. As the sample load was increased the mechanism was shown to change to that of the precipitation and redissolution process described by Glöckner, with corresponding changes in the peak shape. In addition, Lochmüller and McGranaghan [18] have presented evidence to support the adsorption mechanism argued by Snyder.

The improvement in the separation of higher-molecular-weight polymers on small-pore-size columns is hindered by strange behaviour often reported for the larger molecules [11,19,20]. All or part of the polymer may elute in the interstitial volume of the column, or the polymer may appear as a peak eluting within or just after the solvent front. This is largely a result of the necessity to introduce the polymer in a solvent of higher solvating strength than the running solvent. Problems in the separation of these macromolecules caused by the injection solvent are greater than in small-molecule adsorption LC as there is only a small change in polymer solubility for a large change in molecular weight, so that the selectivity which is obtainable in a system which is based largely on solvent–solute interactions is small. Interactions between the stationary phase and the solute play only a minor role as the larger molecules are generally excluded from the pores. Additionally there may be changes in polymer molecular configuration and solvent diffusion effects around the precipitation point [18]. These problems which occur for the higher molecular weights on the small pore size columns are unfortunate as they hinder the separation potential which these columns offer for lower molecular weights. Lochmüller and McGranaghan [18] have overcome some of these problems by recommending a premixing column to make sure the polymer which is dissolved in good solvent is thoroughly mixed with poor solvent before entering the column.

This work is mainly concerned with the elution of high molecular weight ($>50\,000$ dalton) polystyrenes and examines the effect of increasing the solute–stationary phase interactions by increasing the pore size, which provides the larger molecules with a greater surface area. The effects of pore size have previously been examined [1,21], but others have dealt only with molecular weights lower than 50 000 dalton. In this study we describe some unusual chromatographic behaviour that the larger molecules undergo in solvent compositions close to their solubility limit. Small sample loads (0.5 μg) were used to identify factors which contribute to band broadening of the polymer peaks and to a decrease in selectivity as molecular weight increases which may be less apparent at higher sample loads.

EXPERIMENTAL

All chromatographic experiments were performed using two M6000A pumps, a 660 solvent programmer and U6K injector (Waters Assoc., Milford, MA, U.S.A.). The detector was a variable-wavelength UV–VIS set at 262 nm (Activon Scientific, Thornleigh, Australia). Data acquisition and analysis were done with a home-built system. The columns used were a μBondapak C_{18}, 30 cm × 3.9 mm I.D., pore size 120 Å, particle size 10 μm, carbon load 10% (Waters Assoc.), a Serva 300 Å C_{18}, 5 μm particle size, 25 cm × 4.6 mm I.D., pore size 300 Å, carbon load 11.6% (Serva, Heidelberg, Germany) and a self-packed 4000 Å pore size, C_{18}, 10 μm particle size, 25 cm × 4.6 mm I.D. (Merck, LiChrospher SI4000), coated via an *in situ* process [22].

Methanol and dichloromethane (HPLC grade) were obtained from Merck. The monodisperse polystyrene standards used were molecular weights 3600, 110 000, 410 000, 929 000 and $2.7 \cdot 10^6$ dalton (Waters Assoc.) and 9000, 17 500 and 50 000 dalton (Polysciences, Warrington, U.S.A.).

The solvent composition at which each polymer exhibited insolubility, φ_s, was determined by dilution of a stock solution of each polymer (200 mg/l) in 100% dichloromethane. Aliquots of polymer stock solutions were added to various known ratios of methanol–dichloromethane prepared volumetrically in a total volume of 100 ml and these solutions were then shaken for 24 h at 25.0°C and allowed to settle. A 10-μl aliquot of the supernatant solution was then loaded onto a μBondapak C_{18} column and eluted isocratically in 100% dichloromethane to determine the amount of soluble polymer. For solvent compositions in which the polymer remained totally soluble the sample load was equivalent to 0.5 μg. The amount of soluble polymer was plotted against the solvent composition, and the midpoint of the curve between complete insolubility and complete solubility was then chosen as φ_s for each polystyrene standard.

All gradient elutions of polymers were carried out at flow-rates of 0.5 ml/min unless stated otherwise. Gradients were linear with a change of 2%/min, from φ_i to 100% dichloromethane, where φ_i represents the volume fraction of dichloromethane in the initial mobile phase of methanol and dichloromethane. The column was then re-equilibrated with a reverse gradient in 5 min to φ_i and then allowed to flush for a further 20 min. Polymer sample load was 0.5 μg using a 10-μl injection volume. Column temperature was maintained at 25.0°C in a thermostatted waterjacket. All size-exclusion data were recorded at flow-rates of 0.5 ml/min in an isocratic mobile phase of 100% dichloromethane.

To correct for possible pump flow and solvent mixing inaccuracies, gradient profiles at each φ_i composition were obtained by running UV absorbances on mobile phases of methanol, and methanol + benzene (0.012%, v/v) [23]. From these profiles the solvent composition at the time of peak elution (φ_e) for the various molecular weight polymer standards could be obtained.

The reproducibility of φ_e of the system was examined for a series of replicate injections ($n = 7$) of polystyrene molecular weight 410 000 dalton. Elution times for this series had a standard deviation of 0.7%. Performance was examined on a day-to-day basis, also with a standard deviation of 0.7% for the elution times.

Theoretical plate counts were determined by the method of Bristow and Knox [24] using the solute phenetol in water–methanol (60:40) mobile phase at a flow-rate of

0.5 ml/min. The number of theoretical plates on the μBondapak, Serva and 4000 Å columns were 8000, 5000 and 1100 plates/column, respectively.

RESULTS AND DISCUSSION

The elution of the polystyrene via gradient elution RP-HPLC was studied on different columns in order to examine the effects of pore size. The μBondapak column had a nominal average pore size of 120 Å and an exclusion limit of around 35 000 dalton, as shown in Fig. 1, curve A. The Serva column had a nominal average pore size of 300 Å and the exclusion limit was found to be slightly less than 110 000 dalton (Fig. 1, curve B). The 4000 Å column had an exclusion limit greater than $2.7 \cdot 10^6$ dalton thus allowing all the polymers tested access to the pores (Fig. 1, curve C). For each polymer, a range of initial mobile phase compositions (φ_i) was used. The solvent compositions at the times of peak maxima of polymer elution were used as an estimate of φ_e. The graphs in Fig. 2a–c show the relationship between peak elution composition φ_e and polymer molecular weight (curve A) for each column. The bars on the curves of φ_e show the range of the data as detailed in Table I, and curves are drawn through the means of the data. As expected φ_e was independent of φ_i except when φ_i was greater than φ_e. For comparison, the change in polymer solubility compositions φ_s with polymer molecular weight are also shown in Fig. 2 (curve B). The term φ_s refers to the solvent solubility composition for the polymer, the point at which the polymer undergoes a transformation from a soluble state to a solid or a gel phase in 24 h. The midpoint of the φ_s curve as described in the experimental was chosen so as to represent the mean molecular weight of the standard. The curve is typically very steep for the high-molecular-weight polymers changing 1% between complete solubility and insolubility for the 110 000-dalton polystyrene. The midpoint of this curve will vary depending on the concentration of the sample. However, the composition of φ_s was

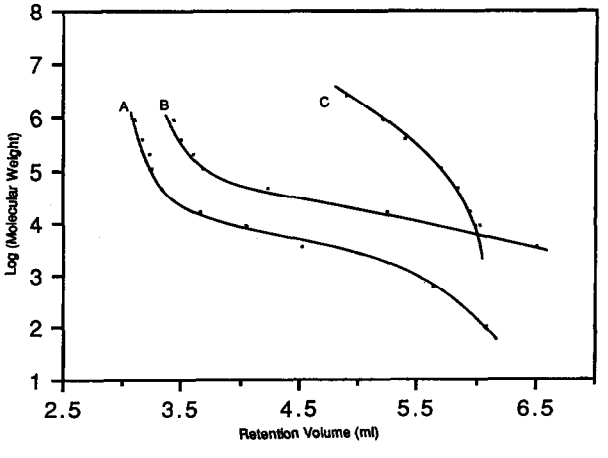

Fig. 1. Plot showing the size exclusion limit of the μBondapak column (A), the Serva 300 Å column (B) and the 4000 Å column (C) in dichloromethane.

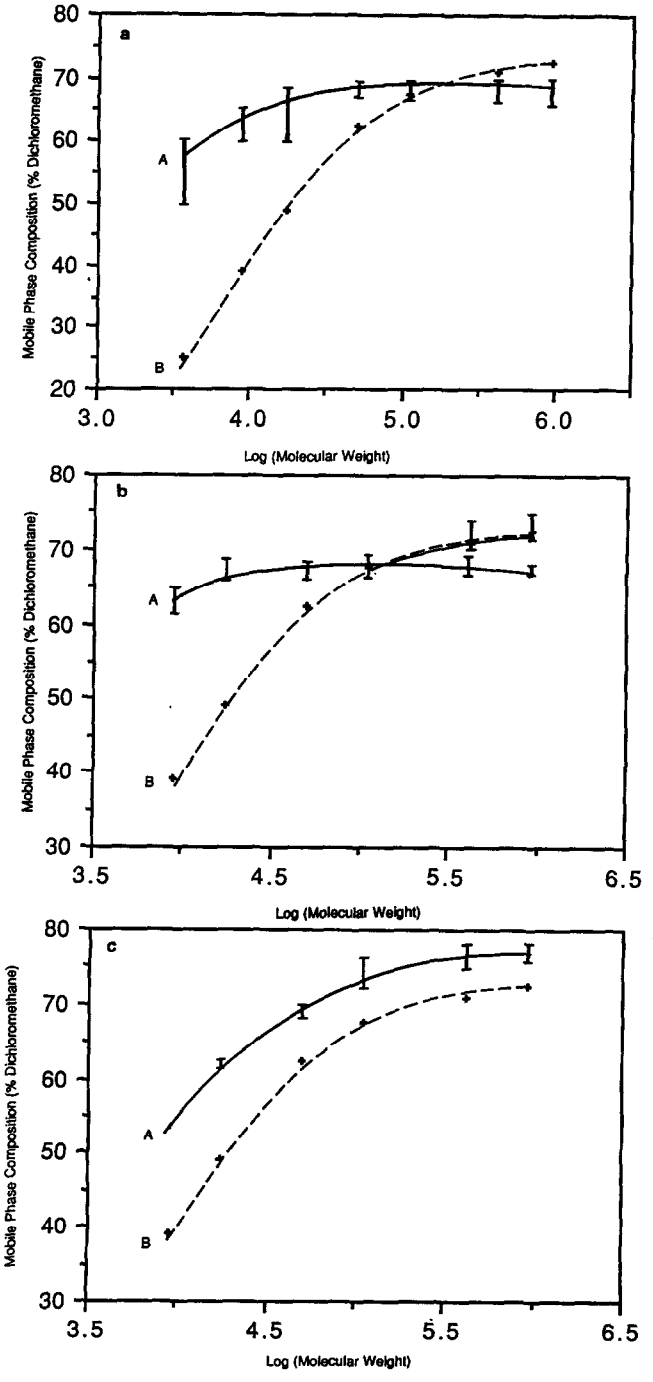

Fig. 2. Plots showing the relationship between elution composition, φ_e, and log molecular weight (A) and the relationship between the critical solubility composition, φ_s, and log molecular weight (B). (a) μBondapak column; (b) Serva 300 Å column; (c) 4000 Å column.

TABLE I

ELUTION COMPOSITION, φ_e, (% DICHLOROMETHANE), AT VARIOUS INITIAL MOBILE PHASE COMPOSITIONS, φ_i, FOR POLYSTYRENE ON THE 120 Å, 300 Å AND 4000 Å COLUMNS

Flow-rate, 0.5 ml/min, gradient 2%/min to 100% dichloromethane (see Experimental for details).

Molecular weight	φ_e (%)					
	φ_i = 60%	φ_i = 50%	φ_i = 40%	φ_i = 30%	φ_i = 20%	φ_i = 10%
120 Å column						
929 000	68.9	69.7	69.3	68.6	66.0	68.4
410 000	68.4	69.1	68.6	68.2	66.2	68.8
110 000	69.0	69.5	69.3	67.7	66.9	69.5
50 000	68.3	69.4	68.8	67.9	67.1	69.2
17 500	60[a]	68.5	67.8	66.2	65.8	67.9
9000	60[a]	64.8	65.2	63.4	63.0	65.0
3600	60[a]	50[a]	56.3	57.3	57.8	58.5
300 Å column						
929 000	71.6	71.9	72.5	72.5	73.3	74.8
929 000[b]	66.6	66.9	67.9	66.9	68.0	71.5
410 000	70.4	70.8	70.4	71.0	72.2	73.7
410 000[b]	66.8	68.1	67.2	67.2	69.0	71.0
110 000	66.8	66.9	66.5	68.7	67.2	69.3
50 000	66.0	66.9	67.5	67.2	66.5	68.2
17 500	66.6	67.3	64.7	67.6	67.2	68.7
9000	[c]	63.8	61.8	61.7	61.5	64.9
4000 Å column						
929 000	78.1	76.6	76.6	75.9	75.7	76.2
410 000	78.2	76.6	77.1	75.7	74.9	77.7
110 000	76.0	73.7	74.2	72.7	72.2	74.2
50 000	69.1	69.8	68.4	69.9	69.4	69.2
17 500	[c]	62.5	61.8	61.7	[c]	[c]
9000	[c]	[c]	[c]	53.5	[c]	[c]

[a] Peak eluted prior to the influence of the gradient.
[b] Elution composition of the pre-eluted polymer.
[c] Peak profile very broad.

determined at a concentration level similiar to that of the chromatographed polystyrenes. If elution of the polystyrene was to obey the precipitation redissolution mechanism then φ_e would be expected to occur at φ_s. In fact, three different types of elution occurred and they are all shown by the 120 Å column, the results for which are now considered in detail and are shown in Fig. 2a.

Elution of the lower-molecular-weight polystyrenes (up to 50 000 dalton) occurred at a higher concentration of dichloromethane than the solvent solubility composition (*i.e.*, $\varphi_e > \varphi_s$), indicating that there was significant adsorption occurring. Elution of the 110 000-dalton polystyrene occurred at the critical solubility composition (*i.e.*, $\varphi_e = \varphi_s$), but, as the molecular weight increased for these higher-molecular-weight polymers, elution occurred at concentrations of dichloromethane lower than the solubility composition (*i.e.*, $\varphi_e < \varphi_s$). The polymer eluted prior to its actual solubility and this unusual behaviour ($\varphi_e < \varphi_s$), shall be termed "pre-elution". This

indicates (1) that elution does not strictly follow a precipitation redissolution process and (2) that adsorption of high-molecular-weight polymers is not significant even given that small amounts of surface interaction may occur through polymer chain dissentanglement snaking into the pores [1] or because of the small surface area available for adsorption outside of the pores [10,25]. As molecular weight increased there was a decrease in the selectivity, to the point where retention times became constant. This decrease in selectivity has also been observed with oligomeric separations on μBondapak columns where the higher oligomers become increasingly difficult to resolve [23]. This was attributed to the limited access available for adsorption for the higher-order oligomers within the pores. Polystyrenes of molecular weight 50 000 dalton and above are all mainly excluded from the pores of the 120 Å column and adsorption effects would be expected to be minimal above this molecular weight limit. As the molecular weight increased beyond 110 000 dalton the peak profile changed from a narrow peak at 110 000 dalton to broad peaks with severe tailing at 410 000 and 929 000 dalton. This tailing extended until, at the extreme end of the peak, a small quantity of polymer eluted near φ_s (Fig. 3).

The 300 Å column had a higher exclusion limit than that of the 120 Å column and adsorption was again observed for polymers of molecular weights below 50 000 dalton.

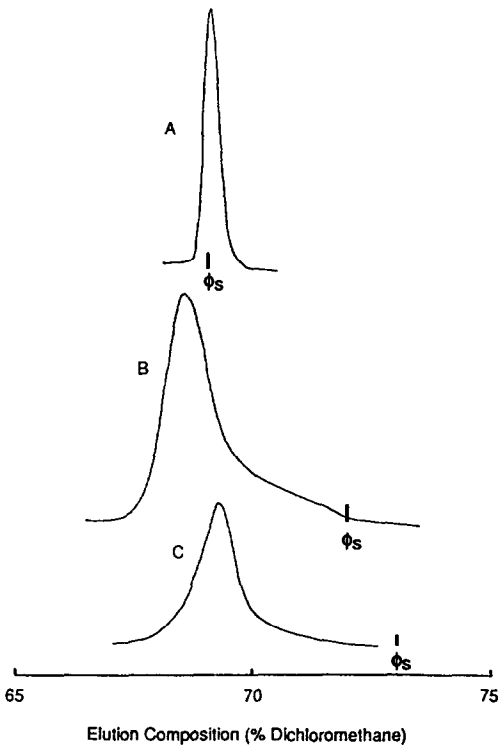

Fig. 3. Elution profiles for polystyrene on the μBondapak column; molecular weight A = $1.1 \cdot 10^5$ dalton, B = $4.1 \cdot 10^5$ dalton and C = $9.29 \cdot 10^5$ dalton. Mobile phase φ_i = dichloromethane–methanol (40:60), gradient elution 2%/min, flow-rate = 0.5 ml/min (note detector sensitivity, A and B = 0.005, C = 0.01 a.u.f.s.).

Elution of the 110 000-dalton polystyrene occurred at the critical solubility composition (*i.e.*, $\varphi_e = \varphi_s$), indicating adsorption processes were insignificant. Elution of the higher-molecular-weight polystyrenes (410 000 and 929 000 dalton) on the 300 Å column produced chromatographic profiles which had two distinct peaks (Fig. 4), as opposed to the tailing observed on the 120 Å column (Fig. 3). The later eluting peak was close to the expected solubility composition ($\varphi_e = \varphi_s$), whilst the other peak eluted with $\varphi_e < \varphi_s$, indicating pre-elution.

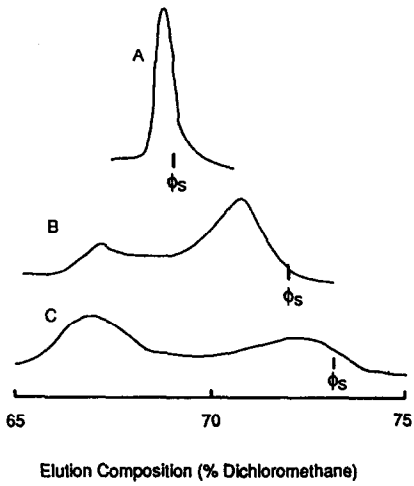

Fig. 4. Elution profiles for polystyrene on the Serva 300 Å column; molecular weight A = $1.1 \cdot 10^5$ dalton, B = $4.1 \cdot 10^5$ dalton and C = $9.29 \cdot 10^5$ dalton. Mobile phase φ_i = dichloromethane–methanol (30:70), gradient elution 2%/min, flow-rate = 0.5 ml/min.

The elution of the higher-molecular-weight polystyrenes was very different on the large-pore-size, 4000 Å C_{18} column. At no stage did the higher molecular weights exhibit pre-elution, and all the polystyrenes eluted with $\varphi_e > \varphi_s$, which indicated adsorption processes were likely to be occurring. The adsorption of these larger molecules is possible due to the larger accessible surface area within the pores. On this column lower-molecular-weight polystyrenes exhibited less adsorption compared with the 120 Å and the 300 Å columns, as the total surface area of the larger pore particles is less than that of the smaller pore size adsorbents.

A possible mechanism for the occurrence of pre-elution may be postulated by considering the behaviour of the high-molecular-weight polymer after it precipitates on the column by interaction with the solvent when it enters the poor mobile phase. If no precipitation occurred then the polymer would elute prior to the solvent front by the usual size-exclusion process. With the addition of the good mobile phase via gradient elution the polymer dissolves at the solvent solubility composition (φ_s), and the polymer begins to elute. While the polymer is in solution and moving down the column, solvent molecules have access to the pores whilst the polymer is excluded. The velocity of the polymer along the column is therefore greater than that of the solvent and the polymer again enters the poorer solvent where precipitation may again occur

as in the original Glöckner [10] model. However, as the molecular weight increases, an increasing time is required for the good solvent to diffuse out of the larger soluble polymer and thus allow precipitation to occur. This diffusion will be especially slow when the solvent composition is similiar to φ_s since the rate of diffusion is proportional to the concentration difference. Two extremes of behaviour can be distinguished. If the kinetics of polymer precipitation and redissolution are rapid the polymer elutes at the solvent solubility composition [10] ($\varphi_e = \varphi_s$). If the reprecipitation process is slow, the polymer may elute from the column in a solvent deficient in the stronger mobile phase by up to one column pore volume when access to the pores is denied ($\varphi_e < \varphi_s$). Hence, elution before φ_s can occur for higher molecular weights in a non-equilibrium process. Lochmüller and McGranaghan [18] also recently reported complications to elution based on a non-equilibrium process within the time domain of the chromatographic system. The consequences of polymer solvation and slow desorption of the solvent from within the larger molecules lead them to discuss non uniform sample distribution of polymer along the column which may lead to varied retention behaviour.

Support for our scheme is provided by the following observations.

(1) Broad tailing peaks were only observed above the column exclusion limit. The elution of the 110 000-dalton polystyrene on the 120 Å column occurred with a narrow peak profile as a result of an apparent adsorption free process and elution appears to be based solely on the solubility of the polymer, indicating a rapid precipitation redissolution process. In comparison, the peak profiles of higher molecular weights were broad with severe tailing (Fig. 3). The peak tailing of the 410 000-dalton polystyrene illustrates that both processes of pre-elution and elution based on the solubility occurred simultaneously. Most of the polymer was subject to pre-elution in the non-equilibrium process but there was peak tailing extending to near φ_s as some of the solvent diffused from within the polymer. Less tailing was observed with the 929 000-dalton polystyrene as more time was required for the good solvent to diffuse out and almost all of the polymer is pre-eluted in the non-equilibrium process.

Similiar effects were observed on the 300 Å column which resulted in the presence of multiple peaks for molecular weights of 410 000 and 929 000 dalton. Fig. 4 illustrates that the proportion of polymer undergoing pre-elution again increased with molecular weight.

(2) For molecular weights greater than the exclusion limit of the column the fraction of polymer eluting via the pre-elution process was dependent upon the pore size of the column. Fig. 5 compares the change in peak profile of polystyrene of molecular weight 410 000 dalton on the three columns again showing that the amount of pre-elution increased as the pore size decreases. For 410 000-dalton polystyrene, relatively more polymer eluted at the solubility composition on the 300 Å column compared with the 120 Å column. This may be a result of the increased surface area available for at least part of the polymer chains, thus increasing the polymer stationary phase interactions which allow a greater rate of diffusion of the solvent from within the polymer.

(3) The extent of pre-elution increased as the flow-rate increased. Fig. 6 illustrates these changes in peak profiles from 0.5 ml/min to 2.0 ml/min for the 410 000-dalton polystyrene on the 300 Å column. The decrease in the amount of polymer eluting at the solubility of the polymer at higher flow-rates indicates that the de-solvation of the polymer which causes pre-elution is a kinetic process. At the lower

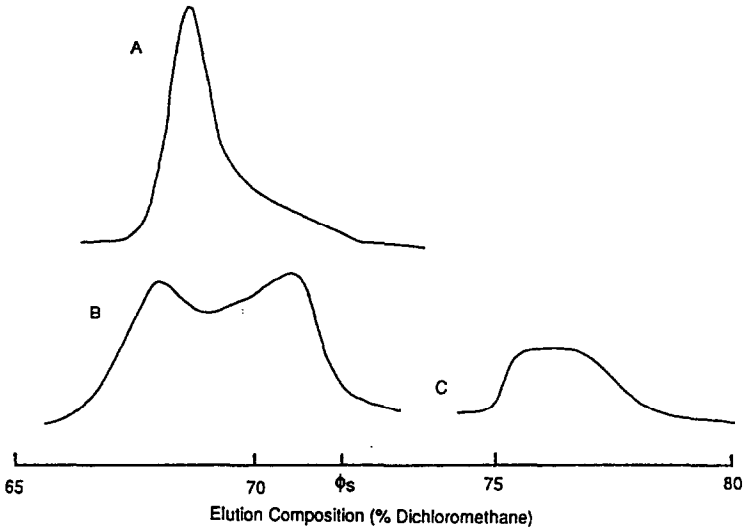

Fig. 5. Elution profiles for polystyrene molecular weight $4.1 \cdot 10^5$ dalton. Mobile phase φ_i = dichloromethane–methanol (50:50), gradient elution 2%/min, flow-rate = 0.5 ml/min. A = μBondapak column; B = Serva 300 Å column; C = 4000 Å column.

flow-rates there was more time for the good solvent to diffuse from the polymer chains.

(4) Pre-elution of the polystyrene always occurred within one column pore volume of φ_s. The maximum amounts of pre-elution calculated for one exclusion volume for the 929 000-dalton polystyrene on the 120 Å column and the 300 Å column correspond to solvent compositions of 6.0% and 8.0%, respectively. These compare to the actual amounts of pre-elution of 4.5% on the 120 Å column and 5.0% on the 300 Å column. The difference between the maximum quantity of pre-elution and that observed is an indication of the rate of diffusion of solvent from within the polymer.

(5) As the sample load was increased (with a constant injection volume of 10 μl) the pre-eluted polymer ceased to be the dominant form of the eluted polystyrene on

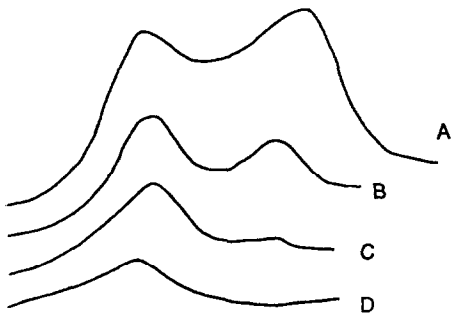

Fig. 6. Elution profiles for polystyrene molecular weight $4.1 \cdot 10^5$ dalton at various flow-rates. Mobile phase φ_i = dichloromethane–methanol (50:50), gradient elution 2%/min. Flow-rates: A = 0.5 ml/min; B = 1.0 ml/min; C = 1.5 ml/min; D = 2.0 ml/min.

both the 120 Å and the 300 Å columns (Fig. 7). At high mass loads the elution occurred predominantly at the solubility of the polymer, but pre-elution was still present (as shown by the assymetry at the front of the peak). The elution profile of the polymer on the 4000 Å column remained constant over the mass loads tested. In a study by Larmann *et al.* [1] it was found that the 50 000-dalton polymer also showed a variation in φ_e with differences in mass load. At low mass loads elution occurred such that adsorption was present and at higher mass loads elution occurred via a precipitation redissolution process. At these higher mass loads peak distortion also became noticeable.

The effect of mass load dependence shows that there was a limited quantity of polymer which could be solvated at any time. On the 120 Å and 300 Å columns this

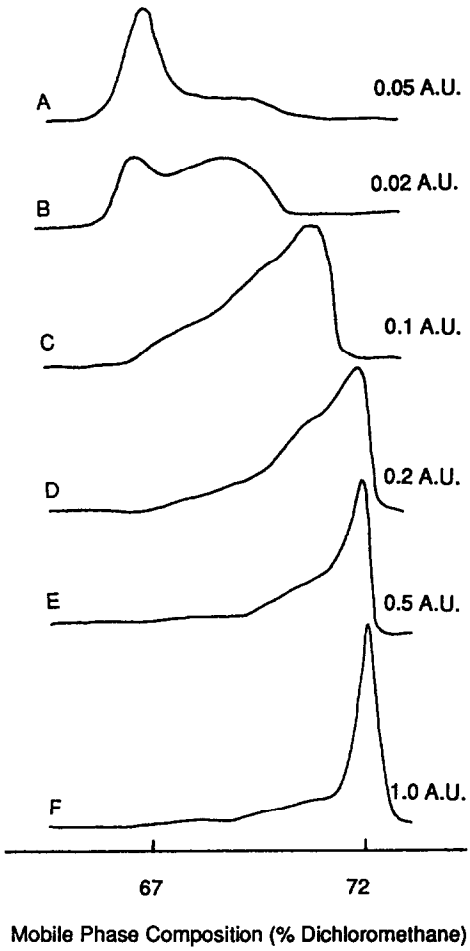

Fig. 7. Elution profiles of polystyrene $4.1 \cdot 10^5$ dalton at various mass loadings on the μBondapak column. A = 0.5 μg; B = 2.5 μg; C = 10.0 μg; D = 20.0 μg; E = 40.0 μg; F = 80.0 μg. Mobile phase φ_1 = dichloromethane–methanol (50:50), gradient elution 2%/min, flow-rate = 0.5 ml/min.

amount was less than 0.5 μg. At high sample loads the pre-eluted component was still present, but due to the change in detector sensitivity to correspond with the higher loadings it was often difficult to observe. The band broadening even at the higher mass loads would make the optimization of a separation difficult.

CONCLUSION

This study examines elution behaviour of high-molecular-weight polystyrenes under gradient elution conditions using methanol and dichloromethane solvents on C_{18} reversed-phase columns. The results indicate that the polystyrenes elute from the columns via an adsorption mechanism only when the polymer is enabled access into the pores, as is the case for the 4000 Å pore size column packing or for the low-molecular-weight polystyrenes on the small-pore-size columns. When exclusion of the polymer occurred, two extreme mechanisms of elution were observed. Elution may occur at the solubility of the polymer, or the polymer may elute at a solvent composition below the solubility limit by a process we term pre-elution. Elution at the solvent solubility composition requires rapid reprecipitation of the polymer in the changing solvent gradient. Pre-elution results when diffusion of good solvent from within the polymer is slow relative to the rate of change of the solvent composition. These combined effects contribute to band broadening of the eluting polymer and lead to a decrease in the selectivity to a point where the higher-molecular-weight polymers elute at the same time as the lower-molecular-weight polymers. A greater degree of selectivity was achieved by using the larger-pore-size column, however, band broadening was still a problem especially with the lower molecular weights. These problems indicate that the separation of polystyrenes on reversed-phase columns with a methanol–dichloromethane solvent system is unsuitable and future work will investigate the effects of other solvent systems.

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