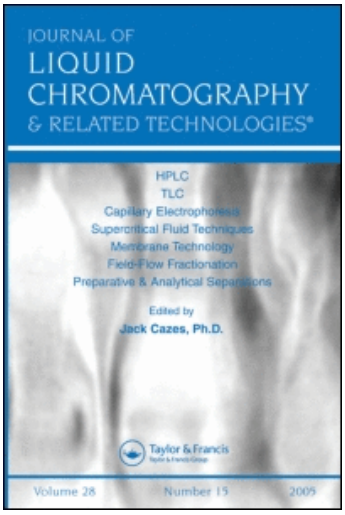

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SIZE EXCLUSION CHROMATOGRAPHY OF DEXTRANS IN DMSO AS ELUENT

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ABSTRACT

The elution conditions for an appropriate size exclusion chromatographic (SEC) characterization of dextrans were checked on a divinylbenzene grafted glucose column. Different eluents were tested. With neutral aqueous eluents, the dextrans were irreversibly adsorbed. In KOH, the separation was rather good but we could note some elution volume shifts when the KOH concentration was changed, and the solute was rapidly degraded by hydrolysis. The mixtures of DMSO/H_2O did not enable the dextrans to completely dissolve and large aggregates were present in the solution. In pure DMSO, even at 70°C, the polysaccharides were only partially eluted. The addition of a small quantity of salt to DMSO allowed total recovery of the samples. In DMSO + salt, the elution volumes shifted when we changed the nature of the salt but these differences were independent of the salt concentration. The secondary interactions were not relevant enough so that we could

consider that the dextrans were primarily eluted by means of size exclusion.

INTRODUCTION

Size exclusion chromatography (SEC) is a widely used and powerful technique to analyse polymer molar mass distributions. The microscopic properties ie average molar masses have a great influence on the macroscopic properties. The materials that are thus transformed also undergo macromolecular transformations and degradations which can be investigated upon by means of SEC.[1, 2]

Aqueous SEC is the most ancient technique to analyse naturally-occurring hydrophilic polymers such as starch or dextrans which are generally used to calibrate the columns. Various types of columns can be used for aqueous SEC: hydroxylated polyethers,[3,4] polyvinylic alcohol,[3] silicas,[5] styrene-co-divinylbenzene,[5] and methacrylates.[6] The eluents may vary from neutral aqueous solution[8, 9, 10] to alkalis[11, 12] and buffers.[13, 14] Moreover, additional peaks are often seen when pure H_2O is used and thus salts are often added to prevent polyelectrolytes chain expansion.[14, 15]

However, in many cases, polysaccharides are not totally water-soluble, this is why we also tested pure DMSO and blends of DMSO/H_2O. First of all, the DMSO offers numerous advantages: it is a strong hydrogen bond acceptor and, secondly, the interaction parameter between DMSO/dextran is 20 times as high as that of H_2O/dextran. The dextran dissolution enthalpy is also higher in DMSO than in H_2O.[16] DMSO can also be used at high temperature to promote the solubilization of crystalline polymers; indeed, some polysaccharides such as starch and starch derivatives, which do not well dissolve in H_2O, can be more easily dissolved in DMSO. Starch solutions in DMSO are stable and can be analysed several weeks after their preparation,[14] whereas, amylose retrogrades in neutral aqueous solvents, and starch degrades in alkalis.[14] The SEC columns available for DMSO-based SEC have to be relatively strong to withstand the high pressures due to the high viscosity of DMSO. Different column packages have been used: silicas grafted diol,[17, 18, 19, 20, 21] dextran bisacrylamide,[22] styrene divinylbenzene[23] normally used for organic SEC. As in aqueous SEC, secondary interactions can disturb normal elution. An additional retention is observed in the silica columns with blends of DMSO/H_2O as eluent,[24, 18, 20, 23] we can also notice additional peak.[23] Adding a small quantity of salt decreases these ionic interactions as in pure H_2O.[17,18,20]

So, the purpose of this study was to find the best elution conditions for analysing polysaccharides such as starch. We selected a very rigid column which could be used either in aqueous or polar organic eluents. We tested various aqueous eluents and DMSO-based eluents for their high dissolution power

to determine further starch characterization. The dextrans were used to check potential secondary interactions: electrostatic repulsion, adsorption, or hydrophobic interaction.

EXPERIMENTAL

Samples

The dextran samples were obtained from Sigma-Aldrich. We disposed of a range of weight-average molecular masses (M_w) varying from 10^4 to 2.10^6 $g.moL^{-1}$. The dextrans were designed by "T" followed by a number equal to $10^{-3} \times M_w$: T10, T40, T70, T500, and T2000. The saccharose from Prolabo was used as a pure (monodispersed) and small sample. The dimethylsulfoxide (DMSO) was also supplied by Prolabo (HPLC grade) and used without any further purification.

Chromatographic System

Because of the high viscosity of the solvent (DMSO), we had to choose a column which could withstand high pressures. The DMSO also had a high boiling point so SEC analyses could be carried out at 100°C or more. The column was a Jordi GBR (Interchim), 25 cm long, with particle size of 5 μm and mixed pore sizes (10^3 and 10^5 Å). Pure divinylbenzene (DVB) gel provided stiffness and chemical stability. Glucose units were attached to the DVB backbone through glucosamine bonds and it made the pack compatible with both polar samples and polar eluents. We had a wide range of eluents available and neutral aqueous, strong alkali and acid, polar organic (tetrahydrofuran, DMSO…), solutions could be used. The maximum pressure and temperature were, respectively, 140 bar and 150°C.

The chromatographic system was composed of an isocratic pump P100 (Spectra Physics) and a differential refractometer Shodex RI 71 for which the cell was regulated at 35°C. The samples were injected through an automatic injector with a variable loop volume AS300 (Spectra Physics). The sample vial volume was about 1mL.

Different eluents were tested: neutral aqueous solutions (NaCl), alkalis (KOH 0.1, 0.2, and 0.5 $mol.l^{-1}$), and DMSO solutions. The different DMSO-based eluents were: pure DMSO and salt-added DMSO (LiCl 0.03, 0.1, and 0.5 $mol.L^{-1}$, $NaNO_3$ 0.1 $mol.L^{-1}$, and CH_3COONH_4 0.1 $mol.L^{-1}$). Eluents were filtered through 0.45 μm filter before use. The eluent container was continuously stirred and heated at 30°C to prevent any degassing in the detector. It was also carefully kept under nitrogen flow to avoid any composition change during the analysis (DMSO is particularly hygroscopic).

The elution conditions were as follows: injected volume: 100 μL; injected solution concentration ≈ 1mg.mL^{-1}; flow rate: 1 mL.min^{-1}. The viscosity of DMSO/H_2O blends was higher than that of pure DMSO and needed decreasing the flow rate down to 0.7 mL.min^{-1} to keep a pressure below 140 bar. The flow rate was accurately controlled at each run by gravimetry, because small flow rate variations could be observed due to the high viscosity of the eluent. To obtain the exact elution volume we used the exact eluent density inside the column. When the analyses were achieved at 70°C, we had to take into account the DMSO dilatation coefficient: $(1/\rho)(d\rho/dT) = 8.8\ 10^{-4}\ K^{-1}$. To prepare the solutions we took the solvent directly from the eluent container so as to have minimum contact with the atmosphere. The solubilization was obtained by stirring the vials for only a few minutes. For the neutral aqueous eluents, we added sodium azide (NaN_3) to prevent any bacterial growth.

RESULTS AND DISCUSSION

Neutral Aqueous Eluents

In neutral aqueous eluents, the dextrans were irreversibly retained into the column. We did not notice any positive peak corresponding to the sample, there were only low intensity peaks due to some impurities appearing at the end of the chromatograms.

Alkali Eluents

On all the chromatograms, we could observe a negative peak at the end of the analysis. This peak was due to a change of composition in the solvent during the dissolution compared to that of the eluent. For low molecular weight samples (T10, saccharose) this negative peak slightly distorted the shape of the normal positive peak of the sample and we could not calculate the average molecular mass.

The column theoretical plate number (TPN) per meter was determined by measuring the width of the first half of the saccharose peak. TPN was stable either with KOH 0.1 mol.L^{-1}, 0.2 mol.L^{-1} or with 0.5 mol.L^{-1} and had a value around 36000.

It was well-known that polysaccharides degraded when remaining in prolonged conditions in alkali or when heated in a solution. The dextran solutions (in 0.1 mol.L^{-1} KOH) were analysed immediately after preparation and 24 hours later. We observed a shift toward higher elution volumes for the oldest dextran solutions. Because the saccharose peaks (fresh and old solutions) were superimposed, one could conclude that the shifts towards higher elution volume

observed on the dextran chromatograms were only caused by the polysaccharide degradation and not by a change in the flow rate.

We also compared the elution volumes of freshly prepared solutions in different KOH molarity solutions: 0.1, 0.2, and 0.5 mol.L^{-1}. The top positions of the peaks were delayed when we increased the KOH concentration. We checked the elution volume shifts according to the molecular mass and the eluent, drawing the dextran top elution volume (Vs) as a function of their average weight molecular mass (M_w). Nevertheless, because the samples were relatively wide MWD (molecular weight distribution), the highest top elution volumes did not exactly match any real average molecular mass. However, this procedure enabled us to qualitatively follow the elution volume dependence on the weight-average molecular mass. The saccharose peak was distorted by the end elution negative peak and so we did not use it in our study. We chose simple second order polynomials to best fit all the experimental points. The three curves were practically parallel (Figure 1) and could be explained accordingly: in neutral aqueous eluents, the polysaccharides were irreversibly adsorbed onto the column through hydrogen bonds. In alkalis, the hydrogen bonds were destroyed and the elution became possible. Additionally KOH could also involve negative charges into the glucose units, and consequently a repulsion between the solute and the column gel. By increasing KOH molarity the repulsion was more and

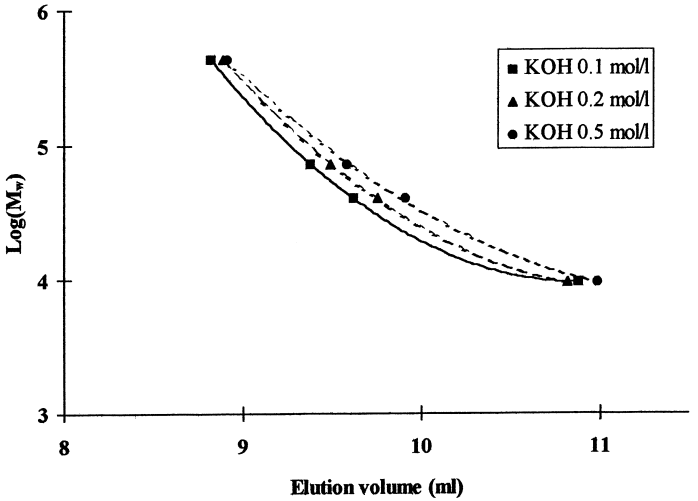

Figure 1. Jordi calibration curves in KOH made with the dextrans top peak volume and their log(M_w).

more screened and a slight tendency to hydrophobic interaction could lead to some elution delay. For the T500 sample, the width of the peak increased, so it seemed that for this sample, we had a higher MW part corresponding to some aggregation process.

Aqueous Eluent with Small Amounts of DMSO

As we knew, DMSO could be added to H_2O as a good hydrogen bond acceptor. This was the reason why we tested the following blends of DMSO/H_2O as an eluent:10% and 20% DMSO. A negative end elution peak appeared at 14.8 mL and 14.6 mL, respectively. But it was far enough on the scale to allow all the dextrans to elute appropriately. Only the saccharose peak was distorted. However, we observed a small premature peak near 7 mL when the eluent was at 10% DMSO and near 6.4 mL at 20% DMSO. When the saccharose or the pure eluent was injected, this small peak disappeared. These small peaks could correspond to the presence of very large molecules (aggregates).

The objective of this work was to determine SEC conditions for further starch molar mass determinations. As starch contained very large molecules, such very early peaks would not enable us to determine the average molecular mass of the starch samples. Therefore quasi elastic light scattering measurements were conducted with T70 in different solvents in order to check whether or not the premature peaks were induced by the presence of a very large population in the solution. The measurements were achieved in pure DMSO, pure H_2O, 10%, and 20% DMSO aqueous solutions (Table 1).

Table 1

Stokes Radii and Relative Intensities of T70 Measured by QELS in Different Solvents

Stokes Radii and Relative Instensities		Eluents H_2O	DMSO 10%	DMSO 20%	DMSO
Smallest population	Stockes radius (nm)	7	9	9	8
	relative intensity (%)	87	48	39	96
Largest population	Stockes radius (nm)	268	210	260	148
	relative intensity (%)	13	52	61	4

In pure DMSO and H_2O, one major population with 7 to 8 nm Stokes radius was present. In 10% and 20% DMSO-based solvents, 2 populations were observed : one of 9 nm radius which corresponded to the dextrans molecularly dissolved, and another one of a 210 to 260 nm Stokes radius, representative of large aggregates. The dextrans elution volumes were different in both eluents (10% and 20% DMSO). They were larger in 10% DMSO and the solvent effect might not be responsible for the shifts of elution volumes. In pure H_2O, the dextrans were irreversibly adsorbed. When we added a small quantity of DMSO it led to a complete elution of polysaccharides. Nonetheless, such blends of DMSO and H_2O were not suitable for analyses of large molar mass samples such as starch.

DMSO-Based Eluents

In pure DMSO and at ambient temperature, the dextrans were irreversibly adsorbed onto the column. When the analyses were conducted at higher temperatures (70°C), the adsorption became partially reversible but large secondary interactions kept polluting the chromatograms. Additional peaks, whose intensity did not depend on the either injected mass or the sample molar mass, appeared and the elution was not reproducible.

We tested other DMSO-based eluents: DMSO + LiCl, DMSO + $NaNO_3$ and DMSO + CH_3COONH_4. The first salt concentration was 0.1 mol.L^{-1} which was the current used concentration for aqueous SEC to avoid electrostatic interactions. The dextran chromatograms were not distorted by the end elution peak and no aggregate was seen. All the samples were well separated. We checked the void volume at the beginning of the elution volume of T2000 at 7.3 ml. It corresponded to the real void volume if polysaccharides were eluted only by pure size exclusion. The TPN was checked with the chromatogram of a pure solute: saccharose. In DMSO + LiCl 0.1 mol.L^{-1}, TPN = 28700 plates per meter. The column performances were much better in KOH 0.1 mol.L^{-1} and the eluents containing DMSO and salt enabled a very good separation without creating any additional peaks (Figure 2). The elution volumes were slightly dependent on salt concentration. On the other hand, when the analyses were conducted at 70°C, the head pressure fell to 62 bar. The elution volumes were quite close to each other at 20°C and 70°C and the peak width unchanged. However, we could notice that the salt nature greatly influenced the polysaccharides elution. Each dextran eluted earlier in DMSO + CH_3COONH_4 than in DMSO + LiCl and later in DMSO + $NaNO_3$ (Figure 3). The influence of the salt nature was important and quite surprising. In aqueous SEC, hydrophilic polymers were often charged and adding a small quantity of salt screened the charges. So, in this case, the salt nature did not influence the efficiency of the screening. But, in our case, the salt added could not only modify the charges distribution but also the DMSO structure. However, the difference between the elution volumes indicated the presence of secondary interactions. The first eventuality

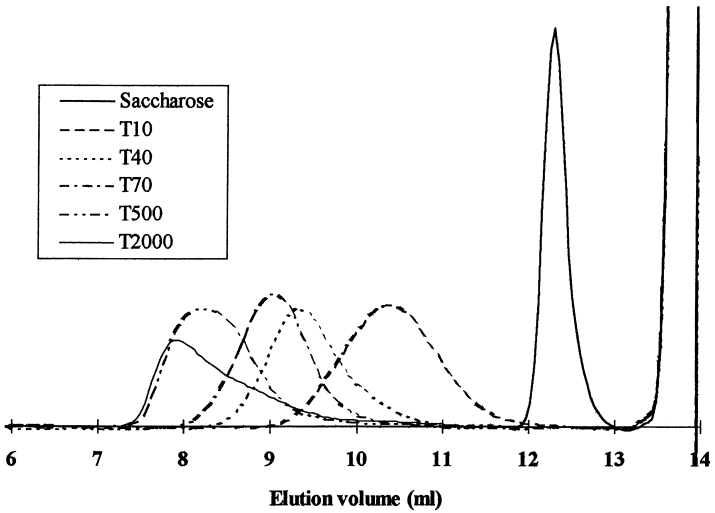

Figure 2. Dextran chromatogram in DMSO + LiCl mol.l^{-1} at 20°C. Flow rate: 0.99 mL/min, injected concentration: 1 mg/mL.

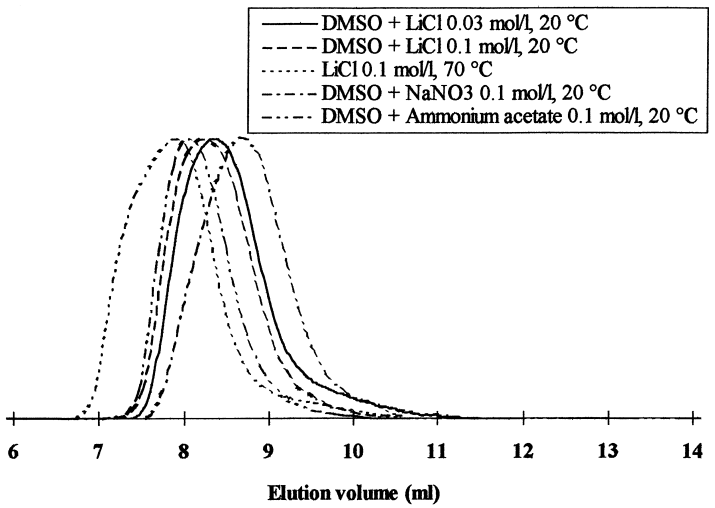

Figure 3. Chromatogram of T500 in DMSO + different salts.

could be a change in the solvent quality with the salt nature and so an expansion coefficient modification. We could rule out a gel swelling because of the high stiffness of the DVB column bead. However, the accessible volume could be reduced if a depletion layer was formed on the column or on the dextrans. In this case of electrostatic repulsion, a greater effect was expected for the smaller molecules. The last eventuality was a further retention due to reversible adsorption of the solutes. Here, the larger macromolecules should be more adsorbed than the smaller ones. Static light scattering measurements were made in order to determine whether or not the solvent quality, and consequently the macromolecule size, was changed when different salts were added to pure DMSO. The measurements were made with T500 solutions in DMSO + LiCl 0.1 $mol.L^{-1}$, DMSO + $NaNO_3$ 0.1 $mol.L^{-1}$ and in DMSO + CH_3COONH_4 0.1 $mol.L^{-1}$. The gyration volumes (Rg) and the second virial coefficient (A2) were determined and were not significantly distant in the different solvents (Table 2). Yet the Stokes radii were more accurate. The size of the dextrans remained the same in the different solvents and the elution volume shifts were caused by the secondary interactions between the solute and the column.

In order to check the nature of these interactions we calibrated the column more accurately than previously. The first pseudo calibration curves drawn with the top peak volume and Mw were quite rough because the dextrans were polydispersed so that a pure monodispersed solute with Mn = Mw would not elute at Vs. The real calibration curves were plotted with the polymolecular dextrans thanks to a software programme which we developed ourselves. This programme gave us the equation of the calibration curve that revealed the smaller difference between Mn and Mw based upon that calibration curve and the experimental average molar mass determined elsewhere. When KOH eluent was used, it was not possible to determine completely the molar mass distributions (because of the polluting peaks), and consequently this kind of pre-

Table 2

Gyration and Stokes Radii of T500 Measured in Different Solvents

Solvents	Gyration Radii (nm)	Stokes Radii (nm)
H_2O	21 ± 3	----
DMSO 10%	21± 3	14.0 ± 0.5
Pure DMSO	26 ± 3	14.0 ± 0.5
DMSO + LiCl0.1 $mol.l^{-1}$	25± 3	15.0 ± 0.5
DMSO + $NaNO_3$ 0.1 $mol.l^{-1}$	25 ± 3	15.0 ± 0.5
DMSO + CH_3COONH_4 0.1 $mol.l^{-1}$	22 ± 3	14.0 ± 0.5

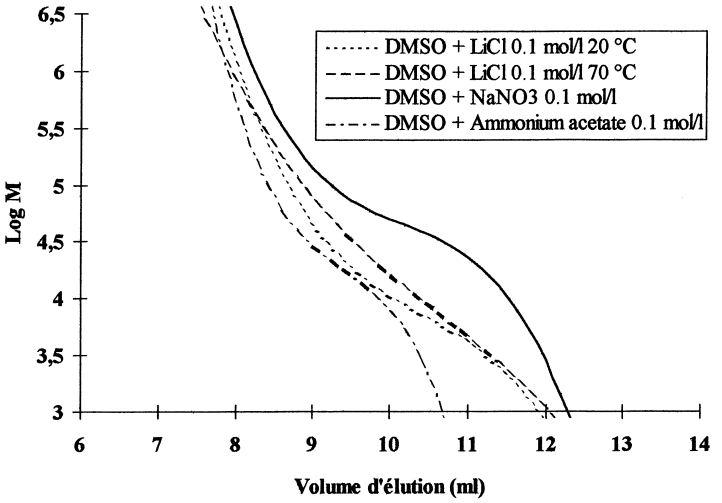

Figure 4. Real Jordi calibration curves in DMSO + different salts made with dextran complete chromatogram and their average molar masses Mn and Mw.

cise calibration had not been done. In DMSO-based eluents, the shifts between the elution volume were greater for the small macromolecules (Figure 4). It showed that the solutes were eluted owing to two processes: size exclusion and electrostatic repulsion. That is to say, that the same sign charges were present on the polysaccharides and on the column. The nature of the salt might play an important role because DMSO could capture the cations and release the nucleophile anions. These anions had the properties to fix themselves onto glucose units. In DMSO + $NaNO_3$ the charges did not seem screened. The asymmetry coefficients corroborated this supposition (Table 3). When the polysaccharides

Table 3

Peak Width and Assymetry Coefficient Measured at 10% of the Total Height for T40 andT70 in DMSO and Different Salts Added*

	DMSO + LiCl		DMSO + CH_3COONH_4		DMSO + $NaNO_3$	
Sample	$w_{0..1}$ (mL)	$f_{0.1}$ (mL)	$w_{0.1}$ (mL)	$f_{0.1}$ (mL)	$w_{0.1}$ (mL)	$f_{0.1}$ (mL)
T40	2.04	1.72	1.52	1.03	2.52	2.32
T70	1.77	1.11	1.63	1.06	2.28	2.80

were eluted, at the front between sample and solvent, the counter-ion diffused toward the eluent polymer-free and induced a coil expansion. The largest molecules eluted faster and the left slope of the chromatogram was flattened. The smallest molecules also expanded, eluted faster and were caught up by larger molecules so that the back slope of the peak became steep. Consequently, the asymmetry coefficient was lower than the measured one when the charges were screened.

CONCLUSION

We defined the elution conditions for which the dextrans were correctly and totally eluted. The dextrans were conveniently fractionated on Jordi DVB-GR when salt was added in pure DMSO. The polysaccharides were irreversibly adsorbed in neutral aqueous eluents and in pure DMSO at 20°C. In KOH, positive and negative additional peaks made the average molar mass determination impossible for small solutes. In 10 or 20% DMSO in H_2O, the dextrans were not completely dissolved and the presence of aggregates was shown in solution. In DMSO + salt, the elution was total, the resolution was correct but secondary interactions (electrostatic repulsion) induced shifts in the elution volumes. These shifts were dependent on the salt nature. However, we could assume that the polysaccharides were mainly eluted by size exclusion, so we could determine accurate calibration curves and elaborate classical molecular weight calculation with the chromatograms.

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