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Size-exclusion chromatography of solubilised low-rank coal

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Abstract

Solubilised brown coal is a polydisperse and chemically heterogeneous polyanion. The parameters appropriate for estimation of its molecular mass (M_r) by size-exclusion chromatography (SEC) using a stationary phase comprising glyceryl-propyl silica were investigated. Ion exclusion occurred when the ionic strength (I) was zero, being most pronounced for coal soluble at low pH. Reversed-phase partitioning appeared at low ionic strength. Non size-exclusion interactions were minimised when I was between 0 and 0.033. The estimates for the modal M_r of solubilised coal ranged between 355 000 and 33 000. These values are consistent with ultrafiltration data, suggesting that SEC is a practical method for determining coal M_r.

Keywords: Coals; Mobile-phase composition

1. Introduction

Low-rank coal is one of the world's most abundant yet under-utilised fossil resources. Biological treatments are potentially cost-effective and clean methods for converting coal to liquid and gaseous fuels, commodity and fine chemicals [1,2]. Brown coal is solubilised by a range of microorganisms [3]. The product is a polydisperse hydrosol composed principally of high-molecular-mass humic acids and is chemically similar to the native material and to coal solubilised with alkaline and chelating agents [4,5]. This material has limited utility and the microbial option for its production may not be cost competitive with chemical routes. However, chemically and biologically solubilised coal (SC) is a useful starting material for more aggressive biological treatments designed to yield value-added products. Bacteria and fungi have already been shown to convert SC to products of higher and lower M_r [6–8].

Since extracellular depolymerisation of SC is anticipated to be an important initial step in its bioconversion, reduction in M_r has commonly been used as an indicator of the extent of SC decomposition. Unfortunately, determination of the M_r of SC has proved difficult. Diverse analytical methods applied to a single sample of SC can yield apparent M_r values covering several orders of magnitude [9]. Low-angle laser light-scattering photometry yields absolute M_r values and appears to have been successfully used to determine the M_r of reductively acetylated lignite-derived humic acids [10,11]. While this technique may be equally successful in determining the M_r of biotransformed SC, it is expensive and not commonly available.

The simplicity and low cost of aqueous size-

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exclusion chromatography (SEC) makes it possibly the most widely used analytical method for estimating the M_r of SC. SEC of solubilised Mississippi Wilcox lignite with a SynChropak GPC 300 HPLC column is reported to give reproducible M_r determinations unaffected by solute pH, freeze–thaw, autoclaving and the presence of typical growth medium components and microbial metabolites [12]. This column has been used to demonstrate depolymerisation of lignite by aerobic and anaerobic bacteria [13–15]. We have shown that polyvalent metal cations cause the apparent M_r of alkali-solubilised Morwell brown coal to increase, although acid-washing the coal effectively removes these ions [8].

Ideally, the only interaction between gel and solute during SEC is exclusion of solute molecules from the stationary phase determined by their hydrodynamic volume ($[\eta]M$). Measurement of the molecular mass of the solute depends upon using molecular mass standards having similar conformation, charge and hydrophobicity. In practice, coulombic and Van der Waals interactions may occur between polyelectrolytes and the packing, electrostatic forces within solute molecules can change $[\eta]M$ and M_r standards with comparable properties may not be available [16,17].

M_r standards composed of low-rank coal do not exist and SEC can only provide an apparent M_r for solubilised coal. The extent to which SC macromolecules differ from commonly used M_r standards (e.g. proteins, dextrans and polystyrenes) in their shape and non size-exclusion behaviour is largely unknown. Non size-exclusion partitioning of low M_r solutes, including ion exclusion of charged species and hydrophobic adsorption of aromatic compounds, occurs on the SynChropak GPC 100 column and is dependent on the pH and ionic strength of the mobile phase and the chemical nature of the solute [18]. The GPC 300 column is expected to perform similarly as it possesses the same stationary phase. Low-rank coal is chemically heterogeneous, containing both hydrophobic and hydrophilic functionalities [19], suggesting that it will exhibit non size-exclusion behaviour when solubilised.

We report here the influence of the ionic strength of the eluent on the elution behaviour and apparent M_r of alkali-solubilised Morwell brown coal. Optimal conditions and limitations of SEC for determination of the molecular mass of solubilised coal are discussed.

2. Experimental

2.1. Coal solubilisation

Solubilised coal was prepared from run-of-mine brown coal mined at Morwell, Victoria, in 1982. The native coal had been stored, dried and ground to pass a 75-μm screen as described in Ref. [7]. Prior to solubilisation, a coal fraction insoluble in tetrahydrofuran (THF) was prepared by Soxhlet extraction of 20 g of native coal for 72 h with 200 ml of THF and dried as described in Ref. [7]. The THF-insoluble (THFI) coal was then alkali solubilised, acid precipitated and the precipitate sequentially resolubilised to yield coal fractions soluble below pH 3, between pH 3 and pH 4.5 and between pH 4.5 and pH 6 as described in Ref. [6]. Coal solutions were sterilised by filtration through 0.22-μm filters and stored at 4°C. The concentration of stock solutions was determined by weighing coal residue after drying 10-ml aliquots at 80°C to constant weight and, for very dilute solutions, from standard curves of optical density of SC at 400 nm.

2.2. Chromatography

Size-exclusion chromatography of alkali-solubilised THFI coal was performed using a Waters (Millipore, Milford, MA, USA) Model 600E high-pressure pump with a U6K injection valve and 2-ml sampling loop coupled to a SynChropak GPC-300 column (250×4.6 mm I.D.) which consists of a hydrophilic glycerylpropyl phase covalently bonded to a silica support as shown below:

$$\text{silica support} \equiv \text{Si} - (\text{CH}_2)_3 - \text{O} - \text{CH}_2 - \text{CHOH} - \text{CH}_2\text{OH}$$

The manufacturer (SynChrom, Lafayette, IN, USA) states that the inclusion range of the column is 4000 to 200 000 for linear polymers and 6 000 to 1.5 000 000 for globular proteins. Eluate was passed

through a Waters Model 486 UV–Vis variable-wavelength spectrophotometer and data acquisition and analysis was performed by 810 Baseline (Millipore–Waters) software. The mobile phase was 10% methanol adjusted to pH 6.9 with 10 *M* NaOH. The ionic strength of the mobile phase was adjusted by the addition of KH_2PO_4 at constant pH. Sample volumes of 20 μl containing 9 μg of SC were eluted at a flow-rate of 0.3 ml/min and the absorbance of the effluent was recorded at 280 nm for 20 min. Calibration of the column at each ionic strength was performed with 20 μl of a 2 mg/ml solution of each of the following globular proteins (Sigma) (protein source, M_r and p*I* in parentheses): cytochrome *c* (horse heart, 12 400, 9.4); carbonic anhydrase (bovine erythrocyte, 29 000, 5.4); alcohol dehydrogenase (yeast, 150 000, 5.4); β-amylase (sweet potato, 200 000, 4.77); apoferritin (horse spleen, 443 000, 4.27); and thyroglobulin (bovine, 669 000, 4.58). Protein M_r and isoelectric points were obtained from Sigma. The apparent permeation volume of the column was determined with benzoic acid (M_r 122). Eluents and protein solutions were filtered through 0.22-μm filters prior to use.

Ionic strength (*I*) of the phosphate buffer mobile phase was calculated using the equation:

$$I = \frac{1}{2} \sum M_i Z_i^2$$

where M_i is the molarity of the ion and Z_i is the total charge on the ion [20]. Concentrations of KH_2PO_4 (corresponding *I* in parentheses) were 0 *M* (0), 0.02 *M* (0.033), 0.05 *M* (0.083) and 0.1 *M* (0.166).

SEC of SC was performed after dilution of stock solutions with water to a final coal concentration of 0.045% and filtration through a 0.22-μm filter.

2.3. Effect of ionic strength on A_{400} of solubilised coal

Solubilised coal was diluted with water to an A_{280} of ca. 1.0. The absorbance at 400 nm was then measured following the addition of 1, 2, 3 and 4 volumes of 200 m*M* KH_2PO_4, pH 6.9. Coal solutions diluted with water were used as a control.

2.4. Ultrafiltration

SC, at a concentration of 0.045% in 100 m*M* KH_2PO_4, pH 6.9, containing 10% methanol was ultrafiltered through YM membranes (Amicon) with nominal molecular mass cut-offs of 30 000 and 100 000 as described in Ref. [7]. The retentate was sequentially resuspended in buffer and refiltered until no colour was evident in the filtrate.

2.5. Reagents

Methanol was chromatographic grade (Hypersolv, BDH) and KH_2PO_4 and NaOH were analytical grade (Ajax). All water was deionised, distilled and filtered through a Milli-Q system (Millipore) before use.

3. Results and discussion

3.1. Yields of solubilised coal

Almost 25% of THF-insoluble Morwell brown coal was recovered when alkali-solubilised and acid precipitated material was resolubilised below pH 6 (Table 1). About two thirds of the resolubilised coal went into solution below pH 4.5 but only 15% of the SC was soluble below pH 3. The yield of SC is much higher if the coal is autoclaved in the presence of alkali [7]. However, we deliberately chose mild conditions since high temperature may oxidise, hydrolyse, polymerise or induce other changes to the coal. Solubilisation at ambient temperatures still gives a high yield of a product that is expected to closely represent the native coal. To maintain mild conditions, we filter-sterilise solubilised coal for inclusion in microbial growth media.

3.2. Column calibration

With the exception of cytochrome *c*, the retention time of globular proteins and benzoic acid on the SEC 300 column increased with an increase in ionic strength of the mobile phase. The largest changes in

Table 1
Effect of pH of solubilisation on the yield of solubilised Morwell brown coal

pH of solubilisation	Yield[a]	
	% of starting material[b]	% of total product yield[c]
≤3	3.8	15.2
3–4.5	11.4	46.8
4.5–6	9.3	38.0
Total	24.5	100

[a] Mean of three replicates.
[b] Initial mass of coal was 20 g.
[c] Total mass of coal solubilised was 4.9 g.

retention time occurred when I was increased from 0 to 0.033 (Fig. 1). These results are consistent with those previously obtained using SynChropak and LiChrosorb Diol (E. Merck, Darmstadt, Germany) SEC columns [18,21,22] and are attributed to neutralisation of ion exclusion of solute molecules with increase in I. Since the ionisation constant of benzoic acid and the isoelectric points (pI) of the proteins

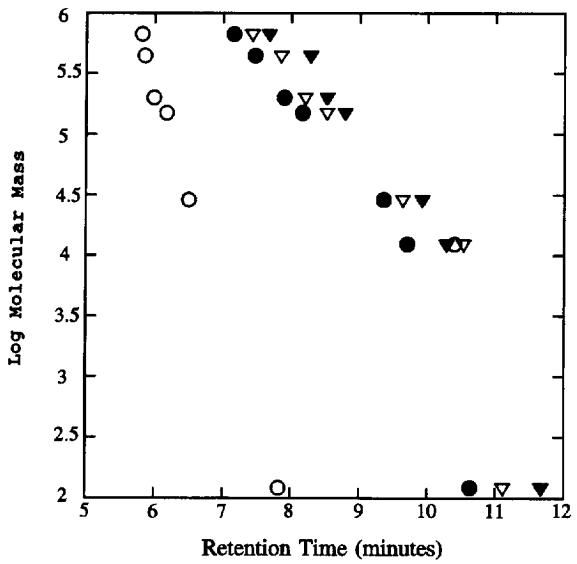

Fig. 1. Correlation of ionic strength of the mobile phase with retention time of benzoic acid and globular protein molecular mass standards. The M_r of standards is given in Section 2. Ionic strengths were: (○) 0; (●) 0.033; (▽) 0.083; (▼) 0.17. All values are the mean of three replicates. HPLC conditions: column, SynChropak GPC-300 (250×4.6 mm I.D.); eluent, 10% methanol containing KH_2PO_4, pH 6.9; flow-rate, 0.3 ml/min; detection, UV at 280 nm.

(cytochrome c excepted) are all less than the pH of the mobile phase (pH 6.9), these molecules possess a net negative charge under the conditions described. The bonded silica gel packed columns (LiChrosorb columns also possess a glycerylpropylsilyl bonded phase) bear a net negative charge due to underivatised silanol groups [18]. Thus, the anionic nature of the solutes and packing gives rise to ion exclusion.

Ionic interactions were almost completely neutralised when I was increased to 0.083 although small additional increases in retention time of the M_r standards occurred when I was further increased to 0.166 (Fig. 1). Minor increases in retention time are probably due to suppression of residual repulsion between anions rather than hydrophobic adsorption of the M_r standards onto the column because proteins have not been observed to hydrophobically partition onto a glycerylpropyl-silyl phase at near neutral pH until I exceeds 0.3 [21] and, for low M_r acids, until I exceeds 0.6 [18].

Cytochrome c ($\log M_r = 4.09$) possesses a high isoelectric point (pI=9.4) and exists as a cation at pH 6.9. Consequently, it is not subject to anionic exclusion at low ionic strength. Cytochrome c was not used to calibrate the column when $I=0$ because of its aberrant permeation behaviour relative to the other protein standards.

3.3. SEC of solubilised coal

With few exceptions, each fraction of solubilised Morwell brown coal eluted with a single absorbance peak at all ionic strengths (Fig. 2). When I was 0.033, coal solubilised below pH 3 eluted with a

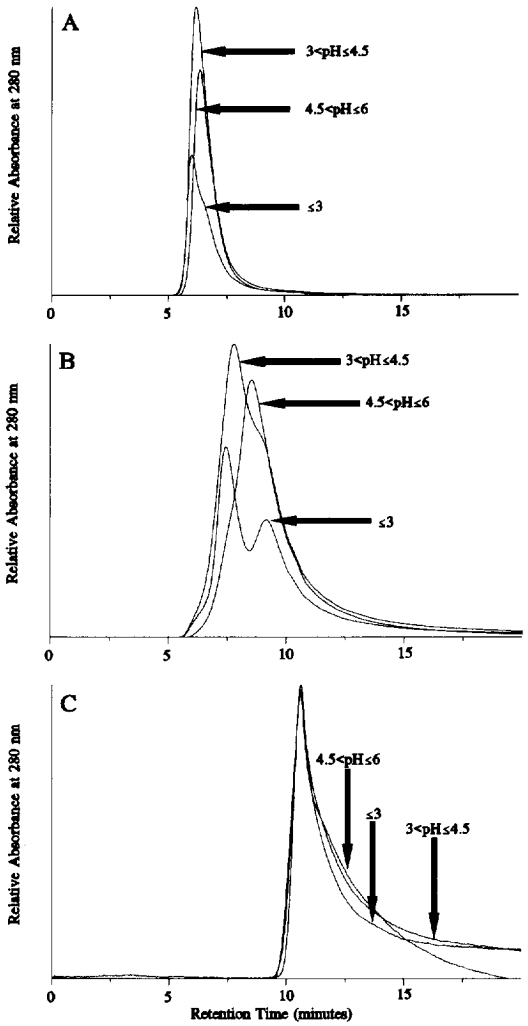

Fig. 2. Influence of the ionic strength of the mobile phase on elution behaviour of alkali-solubilised Morwell brown coal. For each elution profile, arrows indicate the pH used for coal solubilisation. Ionic strengths were: (A) 0; (B) 0.033; (C) 0.083. HPLC conditions as in Fig. 1.

bimodal absorbance distribution and coal solubilised between pH 3 and pH 4.5 possessed an absorbance shoulder. Peak "tailing" was most pronounced at high ionic strength. The elution profiles of the coal fractions when I was 0.166 (data not shown) were almost identical to the profiles obtained for $I=0.083$. The modal retention time of SC increased when I was raised from 0 to 0.083, irrespective of its pH of solubilisation (Fig. 3) but a further increase in I to

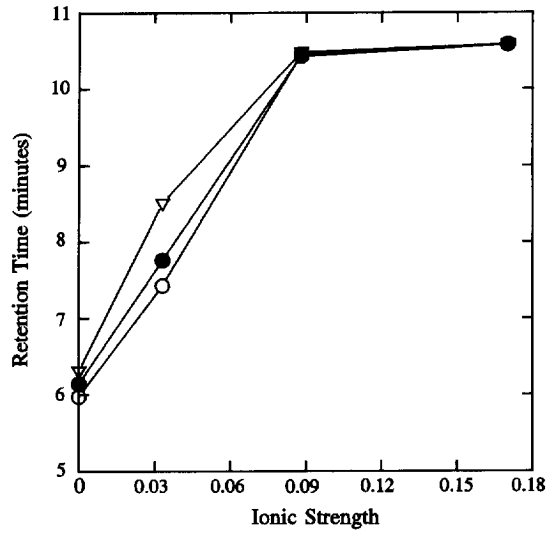

Fig. 3. Influence of ionic strength of the mobile phase on modal retention time of alkali-solubilised Morwell brown coal. The pH used to solubilise coal was: (○) less than pH 3; (●) between pH 3 and pH 4.5; (▽) between pH 4.5 and pH 6. When $I=0.033$, coal solubilised below pH 3 exhibited two modal retention times. The retention time of the first mode is plotted here. All values are the mean of three replicates. HPLC conditions as in Fig. 1.

0.166 caused almost no additional change in retention time.

The structure of low-rank coal is extraordinary amongst natural organic polymers in its chemical complexity and heterogeneity [19]. Its behaviour on a size-exclusion column can, however, be attributed to broad chemical properties of the coal. Low-rank coal is solubilised predominantly by deprotonation of acidic groups (carboxylic acids and phenols) and by removal of polyvalent metal cations which bridge these acidic groups [4,5]. Consequently, SC is a polyanion and is subject to ion exclusion at low ionic strength. This explains the rapid elution of the three coal fractions when $I=0$ and, because size exclusion is inhibited, molecular masses are overestimates. When I was increased from 0 to 0.033, each of the coal fractions eluted later and over a greater period of time. Of particular interest are the two distinct absorbance modes exhibited by coal solubilised below pH 3. This behaviour suggests that ion exclusion was suppressed, at least in part, and that the coal consists of highly polydisperse material.

Since ionisation of acidic functional groups in low-rank coal is required for its dissolution, the abundance of such groups is anticipated to be correlated inversely with the minimum pH at which the coal solubilises. Therefore, a coal fraction soluble at low pH will possess more anionic character and be more susceptible to ion exclusion than less soluble fractions. This hypothesis is supported by the observation that, when $I=0$ and $I=0.033$, the modal retention time of SC increased with an increase in the minimum pH at which it was soluble (Fig. 3). It appears, however, that none of the coal fractions are fully excluded from the stationary phase when $I=0$. At this ionic strength, the bulk of each coal fraction eluted later than apoferritin (M_r 443 000) and, indeed, coal solubilised between pH 3 and pH 4.5 eluted after β-amylase (M_r 200 000) and coal solubilised between pH 4.5 and pH 6 eluted after alcohol dehydrogenase (M_r 150 000).

Adsorption of SC to the stationary phase is a potential source of retardation of movement of the coal through the column. This will lead to increased retention times and underestimates of the M_r of the coal. Comparison of peak areas of the coal with the peak areas obtained with the column by-passed shows that hydrophobic bonding is a dominant non size-exclusion interaction between SC and bonded phase, even at relatively low ionic strength (Table 2). Each of the solubilised coal fractions behaved similarly. No adsorption of coal was observed when the mobile phase consisted only of 10% methanol but ca. 35% of each coal fraction bound to the column when I was 0.033. Almost 100% of the coal in each fraction adsorbed to the column when I was 0.083. The decrease in peak area was not contributed to by any change in the chromophoric properties of SC. When SC was diluted with phosphate buffer, the progressive increase in ionic strength did not result in deviation from the Beer–Lambert law (data not shown).

3.4. Apparent molecular mass of SC

The influence of ionic strength on the modal apparent molecular masses (MAM_r) of the SC fractions, based on the retention time of globular protein M_r standards, is shown in Fig. 4. The three coal fractions exhibited a MAM_r of about 12 000 when I was 0.083 and 9000 when I was 0.166. At lower ionic strength, the coal fractions exhibited larger and significantly different MAM_rs. When I was 0, the MAM_r of the coal ranged from 737 000 for coal solubilised below pH 3 to 248 000 for coal solubilised between pH 4.5 and pH 6. These estimates are uncertain for several reasons. Proteins are not ideal molecules for calibration of a column used to fractionate coal because of structural dissimilarity. Brown coal is probably more like lignin, consisting

Table 2
Effect of the ionic strength of the mobile phase on the area of 280 nm absorbance peaks of alkali-solubilised brown coal

pH of solubilisation of coal	Mobile-phase ionic strength	Total peak area (%)[a]
≤3	0	100
	0.033	67.7
	0.083	4.9
	0.17	3.5
3–4.5	0	100
	0.033	61.5
	0.083	4.2
	0.17	2.3
4.5–6	0	100
	0.033	61.5
	0.083	5.1
	0.17	3.9

[a] Relative to total peak area when $I=0$ and column is by-passed. Mean of three replicates.
HPLC conditions as in Fig. 1.

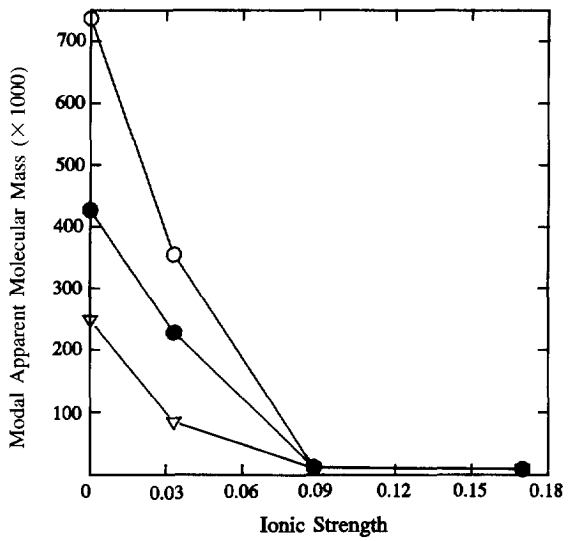

Fig. 4. Influence of ionic strength of the mobile phase on modal apparent molecular mass (MAM_r) of alkali-solubilised Morwell brown coal. The pH of solubilisation of coal was: (○) 3; (●) 3–4.5; (▽) 4.5–6. When I=0.033, coal solubilised below pH 3 exhibited two modal retention times. The MAM_r corresponding to the retention time of the first mode is plotted here. MAM_rs were derived from calibration curves constructed with protein M_r standards at the same ionic strength (see Fig. 1). All values are the mean of three replicates. HPLC conditions as in Fig. 1.

of a three-dimensional lattice of aromatic ring systems linked by methylene bridges and polyvalent metal ions [19]. The data presented here demonstrate that SC is more hydrophobic than protein and it may prove difficult, if proteins are to be used as M_r standards, to identify an ionic strength at which ion exclusion of proteins and hydrophobic adsorption of coal are both inhibited.

3.5. Ultrafiltration of solubilised coal

Since solvophobic effects probably lead to underestimation of the M_r of solubilised coal, an independent measure of M_r was used to estimate the extent of error. When each of the three SC fractions was ultrafiltered using diluent with an ionic strength of 0.166, about 90% of each fraction was retained by a membrane with a molecular mass cut-off of 30 000 (Table 3). A 100 000 molecular mass cut-off membrane retained 54% of coal solubilised between pH 4.5 and pH 6 and about 78% of the other coal fractions.

Table 3
Effect of pH of solubilisation on ultrafiltration of alkali-solubilised Morwell brown coal

pH of coal solubilisation	Proportion of coal (%) passing through membrane with nominal M_r cut-off of[a]	
	30 000	100 000
≤3	13.3	23.17
3–4.5	10	20.83
4.5–6	10.5	46.4

[a] Solubilised coal at a concentration of 0.045% was suspended in 20 ml of 10% methanol containing 100 mM KH_2PO_4 adjusted to pH 6.9. All values mean of three replicates.

Ultrafiltration, which may provide a better estimate of M_r than SEC for poorly characterised solutes [23], indicates that almost all of the SC molecules possess a M_r greater than 30 000 and a significant fraction possesses a M_r greater than 100 000, particularly those molecules soluble at low pH. These results contrast markedly with those obtained by SEC at the same ionic strength (0.166) where all of the coal fractions had a MAM_r of 9000. This very low apparent M_r is attributable to the higher susceptibility of SC to solvophobic effects than proteins and benzoic acid. This implies that SC is less anionic in character (or possesses a more diffuse net negative charge) and that a large proportion of its structure comprises hydrophobic moieties such as unsubstituted aromatic rings, long alkyl chains and methylene bridges.

It has been suggested that the hydrodynamic volume of humic acid molecules is increased by intramolecular coulombic repulsion between ionised functional groups. Addition of electrolytes (i.e. increasing I) which associate with and neutralise these groups can cause a decrease in $[\eta]M$ and permit greater permeation of the gel by the humic acid molecules [23]. It follows that, if the influence of ionic strength on the hydrodynamic volume of solute molecules differs sufficiently between various molecular species, they may fractionate in a manner that has little relevance to their M_r. Rather, charged species experiencing a high degree of intramolecular coulombic repulsion may elute faster than more

neutral molecules with smaller hydrodynamic volume, even if the latter molecules possess a larger M_r. In such circumstances, the diversity in chemical properties of the solute molecules may preclude the formulation of a suitable set of molecular mass standards required for calibration of a SEC column.

The data presented here, however, indicate that contraction of the hydrodynamic volume of coal molecules alone is not sufficient to explain their low apparent molecular masses obtained by SEC at high ionic strength. The high rates of retention of all of the SC fractions by the ultrafiltration membranes when I was 0.166 suggests that reversed-phase partitioning is a more important cause underlying these small M_r values.

4. Conclusions

The optimum ionic strength for SEC of solubilised Morwell coal (i.e. the ionic strength at which maximum suppression of non size-exclusion interactions occurs) lies between 0 and 0.033. This is consistent with the results of Polman and Quigley [12]. The best estimates of the MAM_r of SC, based on the retention time of globular proteins when I=0.033, are 228 000 for coal solubilised between pH 3 and pH 4.5 and 84 000 for coal solubilised between pH 4.5 and pH 6. Coal solubilised below pH 3 possesses two MAM_rs of 355 000 and 36 000. Since some reversed-phase partitioning of the SC occurs at this ionic strength, the true M_r values may be higher. It should be noted that, under similar chromatographic conditions, the M_r of a solubilised North American lignite, Mississippi Wilcox lignite, was found to be very low (approximately 27 000) and no coal-column binding was observed [12]. The low apparent M_r of this coal may be a good indication of its actual M_r. Alternatively, diversity in the structure of low-rank coals may result in significant differences in their non size-exclusion behaviour. Thus, the optimal conditions for SEC of solubilised coals may vary between coal samples and would need to be individually determined to minimise errors in M_r determination.

Molecular masses of SC obtained by SEC must be treated with caution because the structure of low-rank coal is not fully understood and no suitable M_r standards are available. The latter problem can be avoided if only relative changes in retention time between SC samples are considered. However, biotransformed coal presents a unique problem. Its chemistry may be sufficiently different to the native coal, particularly in aspects which affect its non size-exclusion behaviour, that even relative changes in retention time are not indicative of corresponding changes in M_r. In a worst-case scenario, reduction in M_r of SC may not be detected if it is offset by increase in its anionic character (e.g. by loss of hydrophobic groups) which enhance ion exclusion. It is clear that the size-exclusion behaviour of biologically modified SC should be interpreted in conjunction with functional group analyses.

Acknowledgments

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