HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY OF LOW-MOLECULAR-WEIGHT LIGNINS AND MODEL COMPOUNDS

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SUMMARY

An experimental high-performance size exclusion chromatographic procedure to obtain apparent molecular weights and their distribution for low-molecular-weight lignins was developed. Polystyrenes and lignin model compounds were utilized for calibration of the molecular weights. Values found in this study for weight and number average molecular weights for steam-exploded aspen lignins agree well with values reported by other workers using different solvent systems and calibration methods for similar lignins. In addition, possible associative effects were estimated by using one model compound with a free phenolic group and a carbonyl group, acetovanillone. The mobile phase and concentration range were optimized to minimize associative effects. The procedure used proved to be reproducible and to give good resolution of paucidisperse components. An examination of the application of Kirkwood–Riseman theory to the elution behavior of low-molecular-weight polystyrenes and lignin model compounds, is also included.

INTRODUCTION

Lignins[1–6] are irregular phenylpropane polymers which comprise, together with cellulose and hemicelluloses, the cell wall of plant materials. Their precursors are the compounds 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, 4-hydroxy-3,5-dimethoxycinnamyl (sinapyl) alcohol and *p*-hydroxycinnamyl (*p*-coumaryl) alcohol. The proportions of each alcohol depend on the species and on the location in the cell wall. In this work we investigated hardwood lignins which are composed mainly of polymers of coniferyl and sinapyl alcohols. The major type of bond present links the central carbon atom (β-position) of the side chain to the aryl group through an ether (β-O-4-alkyl aryl ether), although many other types of bonds are also found linking monomeric precursors. Unlike other natural polymers, lignins cannot be degraded to

give structurally intact precursors. Due to the presence of many reactive sites in the molecule, hydrolysis reactions are often coupled to condensation reactions.

Lignins are the major renewable source of phenolic compounds. There are methods of separation of the three major polymeric components of wood which lead to lignin polymers of low molecular weight, high solubility in simple organic solvents and high reactivity[7,8]. Such materials may be of industrial importance in the future. A more detailed knowledge of the relationship between the structural features of lignins and their physical and chemical properties is a prerequisite for commercial application, whether as a source of phenolic compounds or as a replacement for phenol in thermosetting resins. The understanding of the macromolecular properties of lignins requires a detailed knowledge of the molecular weights of these polymers and of their distribution[9,10].

The lignins investigated in this work were obtained from a process called steam explosion. The basic method was discovered by Mason[11] and is applied commercially to the manufacture of hardboards. More recently, however, it was found that the steam explosion method could be applied in the separation of the polymeric components of hardwoods such that the resulting cellulosic fraction was almost totally nutritive to ruminants[12] or could easily be converted by enzymatic or acid hydrolysis into glucose[13]. In this method, chips of hardwood are subjected to steam at pressures of 500–1000 p.s.i., and temperatures of around 200°C, for very short residence times (less than 1 min), after which the steam pressure is suddenly released, turning the chips to a mass of brown fibers. Chemically this is an acid hydrolysis process, the acid (mainly acetic) being generated by the hydrolysis of the acetyl groups of the xylan hemicelluloses. Coupled to the chemical process, the mechanical shock aids the depolymerization and enables most of the repolymerization reactions to be avoided. The product is a finely divided mass of fibers consisting of depolymerized and now water-soluble hemicellulose, aqueous-alcohol-soluble lignin and low-molecular-weight, but crystalline, cellulose[14,15].

The products of steam explosion have been characterized by several researchers. Marchessault and St.-Pierre[15] have investigated lignin obtained by methanol extraction of moist (50% water) exploded aspen (*Populus tremuloides*), followed by aqueous fractional precipitation. These authors characterized the lignin using elemental analysis, infrared, proton and carbon-13 NMR of unacetylated materials, as well as size exclusion chromatography. Chum *et al.*[16] have investigated lignin obtained by ethanol extraction of moist exploded aspen after removal of the hemicelluloses by aqueous extraction. A complete functional group analysis (phenolic hydroxy, aliphatic hydroxy, methoxy, carbonyl) based on chemical analyses and on quantitative carbon-13 NMR of the acetylated materials was described. Also, Glasser *et al.*[17] have reported the characterization of a sodium hydroxide-extracted lignin from steam exploded aspen.

Size exclusion chromatography (SEC) has been employed to yield information on the molecular weights of lignins and their distribution[9,10]. In many instances, data from different authors in different solvent systems do not agree. McCarthy and co-workers[18,19] have investigated associative effects, which have been found to mask the molecular weight distribution. Such effects and a resulting bimodal elution profile were eliminated by increasing the ionic strength of the solvent, *e.g.*, by adding LiCl or LiBr to dimethylformamide or aqueous sodium hydroxide as mobile phase. Obi-

aga and Wayman[20] have also improved the calibration procedures for lignins, using equilibrium ultracentrifugation in conjunction with SEC. However, compared to high-performance SEC (HPSEC), the procedures mentioned above are quite time consuming.

MATERIALS, METHODS AND THEORETICAL

Instrumentation

The HPSEC of lignins and elution standards was performed on a Beckman/Altex 100A solvent pumping system with a Beckman/Altex Model 420 system computer control and manual injection using a Beckman/Altex Model 210 injection valve (Beckman/Altex, Berkeley, CA, U.S.A.). Detection was made at 280 nm with a Waters Model 450 variable wavelength detector. A bimodal column system was constructed by connecting in series three μSpherogel columns (300 × 8.0 mm I.D.) obtained from Rainin Instrument Company (Woburn, MA, U.S.A.). These columns were connected to the injection port in the order of increasing pore size, 100, 100 and 500 Å. The mobile phase consisted of ultra-high-purity dioxane and chloroform from Burdick & Jackson Labs., (Muskegon, MI, U.S.A.). Because of the likelihood of peroxide formation, the dioxane was used fresh for each chromatographic series. The mobile phase was mixed in the appropriate proportions, filtered and deaerated by bubbling with helium before chromatography. The mobile phase flow-rate was 1.0 ml/min, with UV detector attenuation at 0.02–0.40 a.u.f.s. Chromatography was performed with 50-μl injections of 0.2–0.4 % (w/v) lignin solutions. Elution data were recorded on the Beckman/Altex Model C-R1A data processor with electronic integration. The hard copy elution diagrams were manually entered into the computer memory and stored on tape using a Tektronix Model 4052A computer (Tektronix, Beaverton, OR, U.S.A.) and digital tablet. Extended BASIC computer programs were written to allow superposition of chromatographic data and elution data plotted with molecular-weight calibration.

Elution standards and model compounds

The polystyrene standards were obtained from Beckman/Altex. Acetovanillone (AV) (4-hydroxy-3-methoxyphenylethanone) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and was purified by recrystallization. 1-Benzyloxy-2-methoxybenzene (BMB) (O-benzylguaiacol) was prepared from guaiacol and benzyl chloride (Aldrich) following the procedure of Leopold[21], and was recrystallized from hexane. [1-(4-Benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)ethanone] (BMME) (ω-guaiacylacetovanillone-O-benzyl ether) was prepared by boiling under reflux in acetone ω-bromoacetovanillone-O-benzyl ether (obtained by bromination of O-benzylacetovanillone), guaiacol and potassium carbonate for 1.5 h. The mixture was added to water, and the emulsion extracted with chloroform. The combined chloroform extracts were washed with 1 M sodium hydroxide solution to remove excess of unreacted guaiacol. After removal of the solvent under reduced pressure, the remaining oil crystallized upon standing. Purification was carried out by recrystallization from acetone or ethyl acetate (calc. for $C_{23}H_{22}O_5$; C 73.0%, H 5.82%, OCH_3 16.4%. Found: C 72.05%; H 5.82%; OCH_3 15.9%). 1-(4-Benzyloxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propan-1-one (BMHMP) was ob-

tained by reacting the previous compound, BMME, with formaldehyde and purifying it according to Weinstein and Gold[22].

Lignin samples

The aspen lignin used in this work was isolated from steam-exploded aspen wood supplied by Iotech Corp. (Canada). Aspen chips were subjected to steam explosion at 240°C for 55 sec of residence time. A modification of the procedure described by Marchessault and St.-Pierre[15] was used to separate the lignin fraction from the other polymers and contaminants (extractives). Water extraction for 3 h removed the soluble hemicellulosic fraction (wood:water ratio 11:1). After filtration, the insoluble lignocellulosic fraction was washed twice with water, and the solid stirred with ethanol under nitrogen (same wood:liquid ratio). The filtrate and ethanol washings were combined, and the ethanol removed under reduced pressure. The yield of ethanol-extracted steam-exploded aspen lignin (EESEAL) was 18 % based on the original dry weight of the wood (78 % yield based on Klason lignin). The crude lignin extract was purified by continuous carbon tetrachloride extraction to remove the fatty acids and alcohols which were also extracted by ethanol. The statistical functional group distribution of this lignin was: $C_{9.0}H_{6.7}O_{1.5}(OH)_{1.2}(OCH_3)_{1.2}$[16].

The EESEAL was subjected to fractionation based on differential solubility as a function of pH. EESEAL (3 g) was dissolved in 150 ml of methanol containing 0.1 M tetraethylammonium perchlorate (supporting electrolyte for prior electrochemical studies) at pH 7.0 or 12.0 (adjusted by adding sodium methoxide). The solutions were stirred under nitrogen for 2 h. The methanolic solution was added to 300 ml of deaerated aqueous 0.5 M hydrochloric acid at about 2°C. The very fine precipitate of the acid-insoluble (AI) fraction was then isolated by centrifugation and the supernatant was retained. The acid-insoluble fraction was washed several times by resuspension in water followed by centrifugation and drying in a vacuum desiccator over P_2O_5. The supernatant solution was saturated with KCl and then extracted three times, with ethyl acetate (100 ml each time). The combined ethyl acetate extracts were washed with saturated aqueous sodium chloride solution, dried with $MgSO_4$ and the solvent evaporated under reduced pressure. The final product, the acid-soluble lignin fraction, was a glassy brown material. Yields of the acid-insoluble lignins were between 73 and 67 %, whereas the yields of the acid-soluble lignins were 14 and 25 %, for fractionations carried out at pH 7 and 12, respectively.

Mobile phase selection

The mobile phase chosen was a binary system of dioxane and chloroform. These solvents are considered good in high pressure liquid chromatography (LC) because of their low viscosity, low UV cutoff (< 240 nm) and a low vapor pressure. They are also known to be compatible with cross-linked styrene–divinylbenzene copolymer packing materials like μSpherogel[23]. Also, the solvent strength and solubility parameters are similar to those of benzene[24], a known "good" solvent for polystyrene[25]. It was imperative that the mobile phase chosen was a good solvent for both the polystyrenes and the lignins (see Discussion). Lignin solubitility was examined as the parameter of choice for the dioxane–chloroform proportion. Dioxane and chloroform solutions were prepared in ratios from 10:90 to 90:10 (v/v), in steps of 10 %; the maximum solubility of the lignin crystals was obtained between 70:30 and 50:50. The 50:50 mixture was chosen as the mobile phase.

Calculation of column parameters

The elution volume external to the column bead pores is defined as V_0, and that volume accessible to all molecules is V_t. The volume occupied by the pores is $V_i = V_t - V_0$ and the volume displaced by the bead material, V_s, is equal to the geometrical column volume, V_g, minus V_t[26]. For the chromatography system discussed here these parameters were: $V_t = 35.80$ ml, $V_0 = 15.98$ ml, $V_g = 41.90$ ml, $V_i = 19.82$ ml and $V_s = 6.1$ ml.

Elution theory

The classical approach to the treatment of elution behavior by SEC is to attempt to correlate some function of molecular weight $f(M)$, or molecular size, to a function of the solute elution volume, $f(V)$. The elution volume parameter is thought to reflect, in part, the geometry and volume of the pores in the column packing material. When defined as K_d, this parameter is equal to $(V_e - V_0)/(V_t - V_0)$[26]. The elution volume parameter $F(v)$, defined as

$$F(v) = (V_e^{1/3} - V_0^{1/3})/(V_t^{1/3} - V_0^{1/3})$$

has been suggested by Himmel and Squire[27,28] for native proteins eluting from TSK semi-rigid organic gels. Here the gel pores are described as an assembly of cones, crevices and cylinders. When considered simply, the parameter $f(M)$ may be chosen as $\log M$ or $M^{1/3}$. Data calculated from several empirical relationships based on these parameters are shown in Table II.

Treatment of small molecule elution

The correlation of gel elution data with small molecules to some appropriate parameter of molecular size is both difficult and poorly addressed in the literature. Molecules of very small size (less than $M = 300$) that are rigid and similar in shape can be treated according to a constant molecular parameter such as the molecular weight, molecular volume[29] or the longest axis length. The latter approach was shown to work well with polynuclear aromatics[30]. However, as a result of the complexity of the solution conformation of polystyrenes greater than distyrene, a direct examination of the molecular dimensions is difficult. When small molecules and oligomers are non-rigid and non-uniform in molecular conformation, as well as in the degree of and the effect of solvation and solvent draining, these parameters must be considered in evaluating elution behavior.

As a first approximation, polystyrenes of degree of polymerization, DP < 200 appear to be excellent SEC models for the low-molecular-weight lignins. Both series of polymers are polyaromatic with similar solubilities. The basic chemical repeating unit of polystyrene is styrene ($M = 104$). However, the widely recognized unit for computing polymer dimensions for polystyrene is the C–C single bond[31]. The basic unit length, l_{av}, is then 1.54 Å. Since there are two of these units per styrene monomer, the unit molecular weight is $M_0 = M/2 = 52$. The value σ is defined as equal to M/M_0 and is the number of mass elements (or bonds) per polymer chain.

The statistical theory of linear polymer systems has been widely discussed[31] and allows the calculation (by summing dimensions over all possible conformations) of important macromolecular parameters such as the average end-to-end distance,

h_{av}, and the radius of gyration, R_G. The radius of gyration is an excellant molecular parameter on which to compare the elution behavior of unlike solutes by SEC[32]. Furthermore, the effective hydrodynamic radius, R_e, may provide a further improvement in elution prediction as this parameter includes the contribution of the associated solvent to R_G. Here, $R_e = \xi_f R_g^{33}$.

We will develop this parameter R_e for polystyrenes of $2 < DP < 30$ ($4 < \sigma < 60$), as this is the range of interest here for SEC elution behavior. From the Kirkwood–Riseman[34–36] theory the frictional coefficient, f, described for random coil macromolecules is dependent upon, in part, the frictional contributions of each mass element, ζ; then

$$f = \frac{(3\pi^{1/2}/8)6\pi\eta R_G}{1 + (9\pi^{3/2}\eta R_G)/4\sigma\zeta} \qquad (1)$$

where η is the viscosity of the solvent.

The term $(9\pi^{3/2}\eta R_G)/4\sigma\zeta$ is assumed small in the treatment of large polymers (*i.e.*, $\sigma \geqslant 100$); however, we must include it to consider the low-molecular-weight polymers of interest here. If one assumes the frictional contribution of each element is $6\pi\eta l_{av}$, then

$$f = \frac{(3\pi^{1/2}/8)6\pi\eta R_G}{1 + 0.153 \, (\pi/\sigma)^{1/2} \, \alpha\beta/l_{av}} \qquad (2)$$

where α is the interaction parameter which describes the segment effective exclusion volume and β is the effective length per segment (usually $\approx 3l_{av}$). Also $f = 6\pi\eta R_e$, where R_e is the effective hydrodynamic radius, therefore:

$$R_e = \frac{(3\pi^{1/2}/8)R_G}{1 + 0.153 \, (\pi/\sigma)^{1/2} \, \alpha\beta/l_{av}}$$

The relation parameter, ξ_f, is equal to R_e/R_G. For high polymers ($\sigma > 1000$), $\xi_f = 0.665$[34]. However, for the low DP oligomers here, we find:

$$\xi_f = \frac{0.665}{1 + 0.271\alpha\beta/\sigma^{1/2}l_{av}} \qquad (3)$$

For polystyrenes in ideal solvents, $l_{av} = 1.54$ Å, $\beta = 5.02$ Å and $\alpha = 1$[25]. Eqn. 3 becomes:

$$\xi_f = \frac{0.665}{1 + 0.564/\sigma^{1/2}} = \frac{0.665 \, \sigma^{1/2}}{0.564 + \sigma^{1/2}} \qquad (4)$$

Continuing[37], we can write

$$R_G = \alpha\beta\sigma^{1/2}/6^{1/2} \qquad (5)$$

and:

$$R_e = \frac{(3\pi^{1/2}/8)\alpha\beta\sigma^{1/2}/6^{1/2}}{1 + 0.153 \ (\pi/\sigma)^{1/2} \ \alpha\beta/l_{av}} \qquad (6)$$

Collecting terms and solving for R_e, we obtain:

$$R_e = \frac{\sigma}{1/l_{av} + (\sigma^{1/2}/0.271\alpha\beta)} \qquad (7)$$

For polystyrenes in all solvents we expect $l_{av} = 1.54$ Å and $\beta \approx 3 \ l_{av} \approx 4.62$ Å. A specific form of eqn. 7 is:

$$R_e = \frac{1.25 \ \alpha\sigma}{0.814 \ \alpha + \sigma^{1/2}} \qquad (8)$$

For polystyrenes in ideal solvents, $\alpha = 1$ and:

$$R_e = \frac{1.25 \ \sigma}{0.814 + \sigma^{1/2}} \qquad (9)$$

The interaction parameter α is also equal to $A M^x$ where x is not greater than 0.10 and A is determined experimentally. For polystyrenes in a good solvent[25], $\alpha = 0.521$ $M^{0.086} = 0.734\sigma^{0.086}$. Eqn. 8 becomes:

$$R_e = \frac{0.918\sigma^{1.086}}{0.597\sigma^{0.086} + \sigma^{0.5}} = \frac{0.0125 \ M^{0.586}}{(0.424/M^{0.414}) + 0.139} \qquad (10)$$

Ideally, values for α must be determined experimentally. Here, values have been estimated from literature data for polystyrenes in solvents similar to that chosen for this study. It is also important to note that portions of Kirkwood–Riseman theory are based on the earlier work of Flory[38], where the system is considered ideal ($\alpha = 1$) and ξ_f is constant only for polymers of $\sigma > 1000$. It has been suggested[33], however, that the limit of fit is unknown and may apply to very low values of σ.

It now becomes important to compare "actual" values of R_e found through molecular volumetric estimation with those found from Kirkwood–Riseman treatment (eqn. 10). As this is only possible for reasonably small oligomers, we will use PS 1 and PS 2.

Steam exploded lignin is believed to be essentially linear (DP < 5) since acid hydrolysis cleaves the branching α-ether bonds[7]. This generates a significant problem for calculation of statistical solution conformations for high DP lignins, however, as the concept of end-to-end distance is altered[39]. Also, the question of appropriate values for M_0 is not insignificant. For the lignin model compounds BMB, BMME and BMHMP, eqn. 3 must be used to describe ξ_f. R_e is then calculated from R_G. If we assume that l_{av} for these compounds is also 1.54 Å (the C–C bond distance) then $\beta \approx 3 \ l_{av} \approx 4.62$ Å. As a first approximation, we will assume that for BMB, BMME and BMHMP the number per molecule of C–C or C–O single bonds separat-
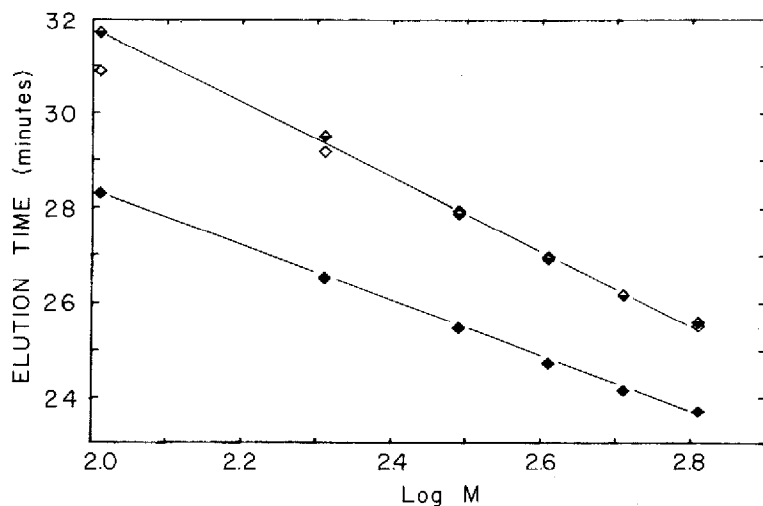
Fig. 1. Comparison of elution time and log M of low-molecular-weight polystyrenes at a flow-rate of 1.0 ml/min in different solvent systems. Closed squares, open squares and half-open squares correspond to data from dioxane–chloroform (10:90, 50:50 and 90:10, v/v).

TABLE I

ELUTION STANDARDS

No.	Name	$M \times 10^{-3}$	$V_e = t_e$ (min)
1	PS	3000.00	15.97
2	PS	275.0	15.95
3	PS	148.00	15.98
4	PS 288	30.10	16.05
5	PS 87	9.000	17.95
6	PS 27	2.820	20.50
7	PS 9	0.936	24.35
8	PS 7	0.728	24.15
9	PS 6	0.624	25.60
10	PS 5	0.520	26.18
11	PS 4	0.416	26.94
12	BMHMP	0.408	26.38
13	BMME	0.378	27.45
14	AV 2	0.332	25.44
15	PS 3	0.312	27.89
16	BMB	0.214	29.08
17	PS 2	0.208	29.18
18	AV 1	0.166	27.60
19	PS 1	0.104	30.89
20	Benzene	0.078	33.10
21	Methanol	0.034	33.78
22	water	0.020	35.80

TABLE II

DATA FROM LINEAR REGRESSION

Function	Relative error*	Correlation** coefficient
$\log M$ vs. K_d	0.100	-0.9967
$M^{1/3}$ vs. $K_d^{1/3}$	0.134	-0.9957
$M^{0.50}$ vs. $K_d^{1/3}$	0.361	-0.9838***
$M^{1/3}$ vs. $F(v)$	0.320	-0.9767***
$M^{1/3}$ vs. K_d	0.395	-0.9643***

   * Calculated as the standard deviation, $\sigma = [1/(N - 1)]^{1/2} [(M_{calc.} - M_{true})/M_{true}]^{1/2}$.

  ** Calculated for the linear portion of the elution curve (Nos. 4–19, except AV 1 and AV 2).

 *** Distinct curvature is observed.

ing the aromatic rings is equal to σ. This argument is similar to that developed for polystyrenes (above). For BMB, BMME and BMHMP, σ and M_0 are: 3 and 71, 7 and 54 and 7 and 58 respectively. We will also compare values of R_e found for lignin model compounds (BMB, BMME and BMHMP) from direct molecular volumes to values of R_e for polystyrenes found from statistical treatment. Calculations from molecular models (Framework Molecular Models; Prentice-Hall, NY, U.S.A.) led to the estimation of the molecular volume of model compounds with $M < 400$. Assuming these small oligomers may be treated as spheres, the geometrical radius is $R = (3V/4\pi)^{1/3}$ and the radius of gyration is defined as $R_G = (3/5R^2)^{1/2}$ (ref. 40). R_e is then found from R_G and ξ_f.

RESULTS

Polystyrene oligomers of DP 1–6 were chromatographed in three solvent systems for a comparison of column packing swelling and elution aberrations. These solvent systems were dioxane–chloroform (10:90, 50:50 and 90:10, v/v). Examination of these elution data (Fig. 1) shows that changes in elution time are minimal with over 50% dioxane mixtures. This may reflect an equilibrium condition for solvent interaction with the styrene–divinylbenzene copolymer gel, resulting in pore size stability. Dioxane–chloroform (50:50, v/v) was therefore chosen as the mobile phase.

The elution behavior of 14 polystyrene standards and 5 model compounds is presented in Table I. These data were plotted as various functions of molecular weight (M, $M^{1/3}$, $M^{0.50}$) vs. functions of elution volume [K_d, $K_d^{1/3}$, $F(v)$]. Calculated data from the five plots showing the highest correlation coefficients are included in Table II. A standard deviation of 10% in M was found for $\log M$ vs. K_d. This function, and $M^{1/3}$ vs. $K_d^{1/3}$, showed no evidence of curvature throughout the included volume. The other three functions showed severe curvature over this molecular weight range. A plot of $\log M$ vs. K_d for the data in Table I is shown in Fig. 2. The solid squares indicate the elution of the model compound acetovanillone, which exhibits association. This anomalous behavior is examined in the Discussion. The excluded volume appears to be 15.98 ml from the chromatographic data of the three high-molecular-weight polystyrenes examined. The estimation of V_t was made from the detection of water in water-saturated mobile phase injected as a sample. Also,
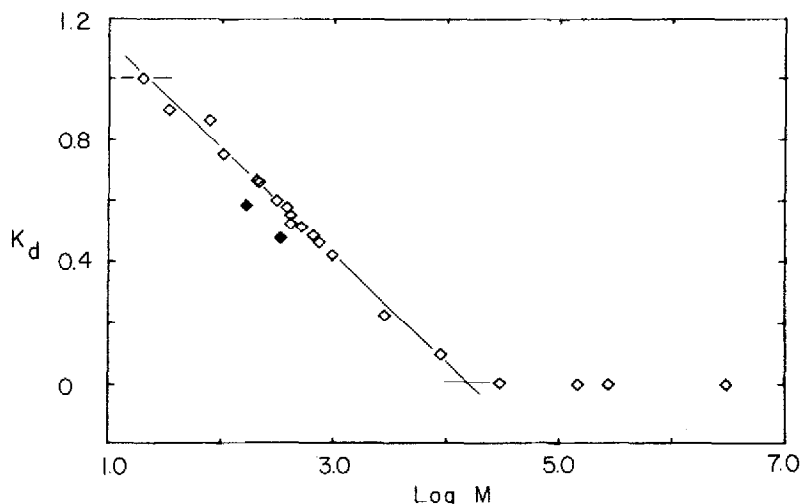
Fig. 2. Plot of log M and elution volume parameter, K_d. Open squares from left to right are the elution standards (1–22) from Table I. The linear regression equation for these data is $K_d = 1.47 - 0.324 \log M$. Closed squares represent acetovanillone monomer and dimer.

elution at this volume, 35.80 ml, was observed routinely with lignins and some model compounds.

Acetovanillone (AV), a possible lignin degradation product, was subjected to a series of experiments designed to test for association behavior in this solvent system. The concentration dependence of association was found by injecting 50 μl of acetovanillone from serial dilutions in mobile phase, yielding 500–5 mM samples.
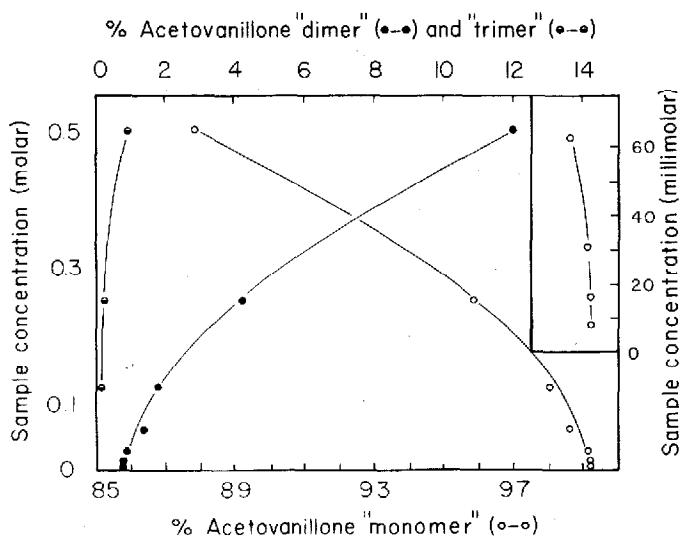

Fig. 3. Association study of acetovanillone from molar to millimolar sample concentration. The sample percentage was taken from electronic peak integration.
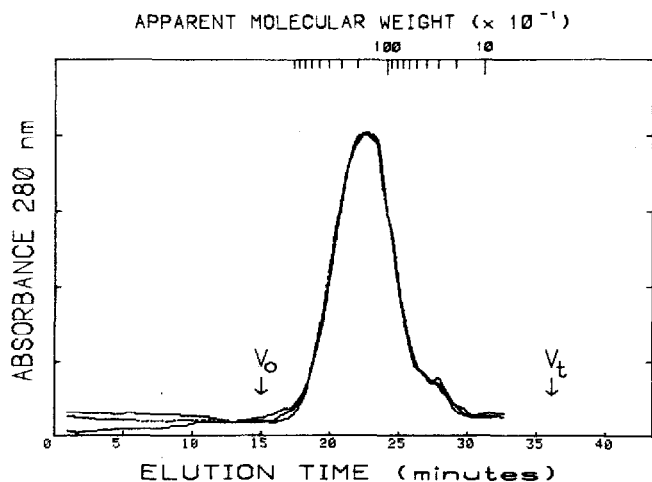
APPARENT MOLECULAR WEIGHT (× 10⁻¹)

Fig. 4. The apparent molecular weight distribution and elution profile of EESEAL upon serial dilution in the dioxane–chloroform (50:50, v/v) solvent. Molecular weight calibration alignment is taken from the linear regression data in Fig. 2.

These data are shown in Fig. 3. It is apparent that AV is 99.3 % monomeric at sample concentrations below 10 mM.

The chromatography of EESEAL is shown in Fig. 4. This figure also illustrates the superposition of chromatograms from three serial dilutions of this lignin from 0.45 to 0.15 % (w/v). With an injection volume of 50 μl, the sample loading is 125–4.1 nmoles (assuming $M = 2000$). The excellent agreement in the elution curves indicates that chromatography was performed at sample loads low enough to eliminate associative effects. Lignin curves are presented with molecular calibration generated
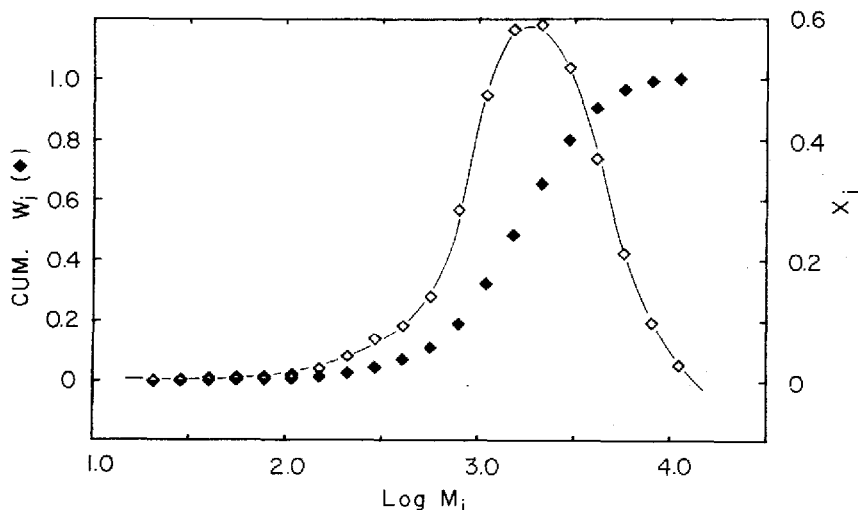

Fig. 5. The cumulative weight average (■) and molecular weight frequency distribution (□) of the EESEAL shown in Fig. 4.
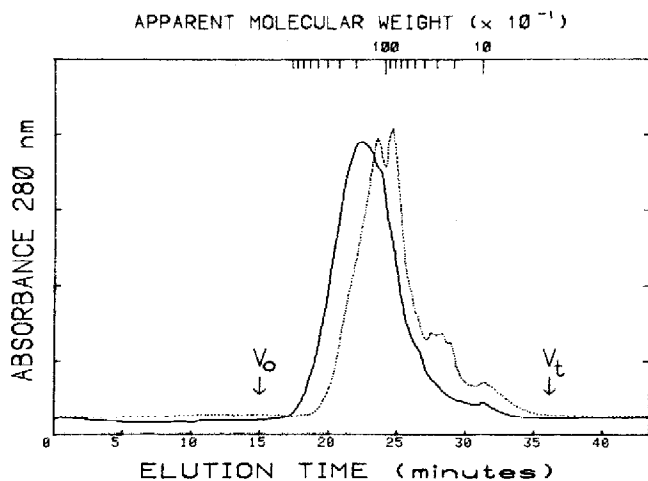
APPARENT MOLECULAR WEIGHT (x 10^{-1})

Fig. 6. The elution profile of fractionated EESEAL into acid-insoluble (———) and acid-soluble (· · · ·) lignin fractions, from methanolic solution at pH = 7.

from the plot of log M *vs.* K_d for the compounds in Table I. Fig. 5 shows the results from hand integration[41] of the lignin curve shown in Fig. 4. The weight-average molecular weight, $\bar{M}_w$, and the number-average molecular weight, $\bar{M}_n$, were found to be 1980 and 925, respectively. The ratio $\bar{M}_w/\bar{M}_n = 2.14$.

The chromatograms of the two fractions of EESEAL —the acid-insoluble and acid-soluble lignins, prepared as described in Materials, methods and theoretical, from methanolic solutions at pH = 7 and pH = 12 are shown in Figs. 6 and 7, respectively. The fractionation procedure employed separates the high-molecular weight acid-insoluble fraction from the low-molecular-weight fraction, which demonstrates paucidispersity. These components are seen to increase when the fractionation is carried out
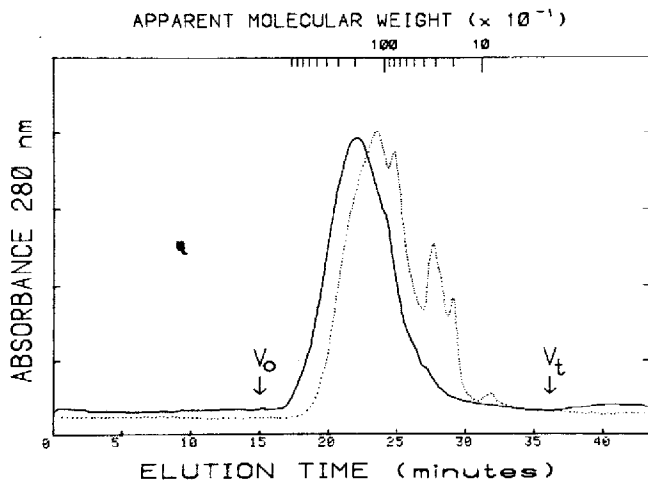
APPARENT MOLECULAR WEIGHT (x 10^{-1})

Fig. 7. The elution profile of fractionated EESEAL into acid-insoluble (———) and acid-soluble (· · · ·) lignin fractions, from methanolic solution at pH = 12.
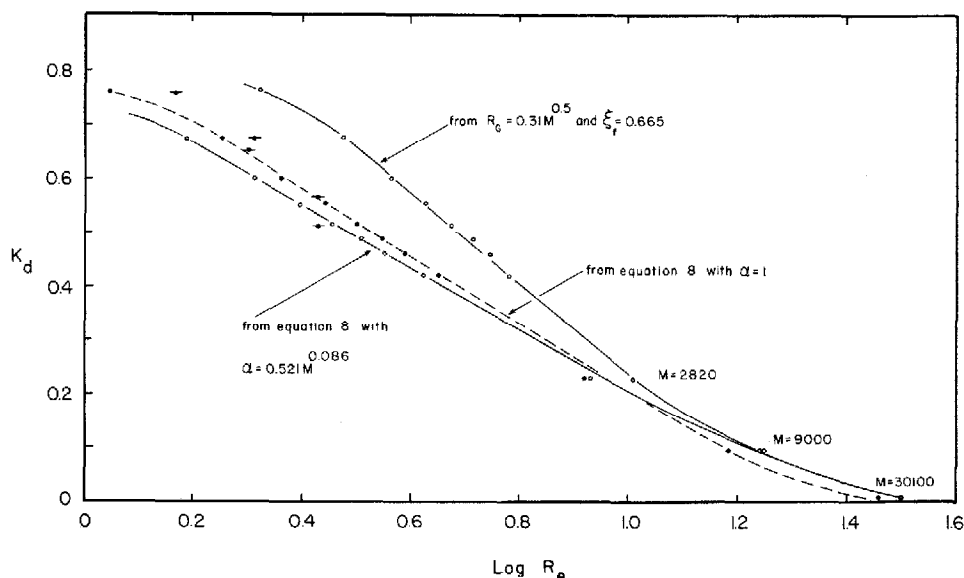
Fig. 8. Comparison of calculated values for R_e found statistically with elution volumes for the polystyrene standards (PS 1 to PS 288 from left to right). Several model compounds are also plotted with values found by direct volume measurement (solid circles with error bars for uncertainty in molecular volume). These model compounds, from left to right, are: PS 1, BMB, PS 2, BMME and BMHMP.

at pH = 12. Average molecular weight values found for the AI fraction are shown in Table III.

Although the lignins examined in this study were described in terms of apparent molecular weights found from direct polystyrene calibration, it is worthwhile to compare the elution of lignin model compounds when described in terms of the effective hydrodynamic radius, R_e. Fig. 8 shows the comparison of values of R_e calculated by several methods from the known molecular weights of polystyrenes of $\sigma < 600$ when plotted against the elution parameter, K_d. R_e values for styrene and distyrene, as well as BMB, BMME and BMHMP, are also plotted in Fig. 8 (as solid circles). For these compounds, values of R_e were found by volumetric approximation of R_G, followed by the definition of ξ_f in terms of several specific assumptions (see Theoretical). The agreement observed in Fig. 8 for these values of R_e is quite good.

DISCUSSION

The HPSEC of polystyrenes and lignin model compounds on μSpherogel columns in dioxane–chloroform (50:50, v/v) proved to be well-behaved and reproducible. The highest correlation coefficients from these data were obtained by plotting log M vs. K_d. The standard deviation from this function was $\pm 0.10 M$. Acetovanillone was found to elute early (the solid squares in Fig. 2). Unlike the model compounds BMB, BMME and BMHMP which contain protected phenolic hydroxy groups and benzyl groups and appear to chromatograph predictably, acetovanillone behaves as if the solvent is a "poor" solvent. Because acetovanillone contains an unprotected phe-

TABLE III

COMPARISON OF HPSEC DATA ON STEAM-EXPLODED ASPEN (*POPULUS TREMULOIDES*) LIGNINS

THF = Tetrahydrofuran; DMF = dimethylformamide. N.A. = Not available.

Isolation of lignin (gun conditions)	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_w$	Mobile phase	Lignin concentration (%, w/v)	Column material	Detection technique	Ref.
Methanol–water extraction, reprecipitation with water (234°C, 45 sec)	700	1700–1900	2.4–2.7	THF	0.5	TSKG4000H8 + G2000H8 or G2500H8 × 2 (Toyo-Soda)	Differential refractometry and light scattering	8
Ethanol–water* extraction; EESEAL (240°C, 55 sec)	925	1980	2.1	Dioxane–chloroform (50:50, v/v)	0.15–0.45	μSpherogel 100 × 2 + 500 Å	UV	This work
AI fraction from EESEAL (pH = 12)	1070	2130	2.0	Dioxane–chloroform (50:50, v/v)	0.15–0.45	μSpherogel 100 × 2 + 500 Å	UV	This work
NaOH extraction** 800–1200*** (N.A.)		2600	3.25–2.2	DMF–0.1 M LiBr	N.A.	μSpherogel 500 + 1000 +	UV	10
NaOH extraction* (N.A.)	600	1900	3.5	DMF–0.1 M LiBr	N.A.	μSpherogel 500 + 1000 + 10,000 + 100,000 Å	UV	10

* Sample from Iotech Corp., 1981.

** Sample from Iotech Corp., 1978.

*** Obtained by osmometry.

nolic hydroxy group and an α-carbonyl group, the segment–segment contacts are preferred to solvent–segment contacts. This condition may lead to an increased effective molecular volume and early elution. However, we have also shown that at low molar concentrations these associative effects are largely minimized (see Fig. 3).

The steam-exploded aspen lignins examined in refs. 15–17 are different due to the different methods of isolation and purification, and also to the different explosion conditions employed. The results of the various molecular-weight distributions from those references are assembled in Table III, and are compared with our present data. The data of Marchessault and St.-Pierre[15] and ours agree reasonably well. The more severe explosion conditions in our case justify the higher $\bar{M}_w$ and $\bar{M}_n$ observed. As expected, the method of extraction of lignin greatly influences its polydispersity. Solvent extraction leads to materials with polydispersities of 2–2.5, whereas larger values (≈ 3) are found in the higher-yield extraction by sodium hydroxide.

It does seem from all of these data (Table III) that on the column materials employed and in the various mobile phases used, association is not a major effect. The good resolution of the various oligomers achieved in the chromatograms of the acid-soluble fractions in the present work is gratifying.

Although the empirical relationship log M vs. K_d describes reasonably well the elution behavior of intermediate-molecular-weight polystyrenes, i.e., S.D. $= \pm 10\%$ M, the comparison of polymers of different structures by molecular weight alone cannot always allow correct elution prediction. A more generic parameter for elution behavior, such as R_e, should be a better comparative measure. However, the calculation of values of R_e for polymers of model compounds of lignins or even polystyrenes of low σ is not straightforward. Fig. 8 demonstrates the results of one such comparison. From light scattering data, the relationship $R_G = 0.31 M^{0.50}$ was established for polystyrenes from $M = 40,000$ to 5×10^6 in a good solvent at 25°C[42]. At high values of $\sigma, \xi_f = 0.665$. The upper solid line in Fig. 8 was then calculated from the values of M in Table I. A statistical approach for the calculation of R_G (eqn. 5) and ξ_f (eqn. 3) yields eqn. 8 (see Theoretical). The lowest solid curve in Fig. 8 was found using eqn. 8 with $\alpha = 0.521 M^{0.086}$. This value for α was determined from viscosity data for polystyrenes of $40,000 < M < 5 \times 10^6$ in benzene[25], which is considered a "good" solvent for the polystyrenes. The upper and lower solid curves in Fig. 8 superimpose at high values of M, e.g., 9000 and 30,100. This is very satisfactory as the upper curve is generated largely from experimental data and the lower from a statistical solution-conformation treatment. The dashed line in Fig. 8 indicates elution data plotted with R_e found from one limiting condition for R_e, where $\alpha = 1$. This condition reflects the maximum contraction of the polymer in an "ideal" solvent, and is observed in Fig. 8 at values of $M > 2000$. A crossover point of the curve from eqn. 8 with $\alpha = 1$ and the curve with $\alpha = AM^x$ occurs at approximately $\sigma = 36$. This point may reflect the limit in one or several assumptions in extending the high polymer theory to values of σ lower than 36. It is doubtful that l_{av} or β are in error here as these parameters are primarily molecular weight independent and only slightly temperature dependent. However, failure in these assumptions at values of $\sigma = 1$ or 2 probably leads to the deviation seen at low values of r_e, i.e., for styrene. The parameter α is highly dependent, however, on molecular weight and solvent. The assumption that $\alpha = 0.521 M^{0.086}$ at σ values less than 36 may prove incorrect. The solid circles with error bars in Fig. 8 represent the elution behavior of PS 1 and PS

2 as well as BMHMP, BMME and BMB. Here R_e was calculated from volumes found from geometrical models and with ξ_f calculated using eqn. 4. The error bars represent the level of confidence in estimating possible conformations obtainable in solution. A difficulty arises in the assignment of σ (or M_0) for BMHMP, BMME and BMB. Clearly, from Fig. 8, the fit of R_e found volumetrically for PS 1 and PS 2 (for which M_0 is known) is good. However, the spread of data here shows the probable compounding of small errors which render distinction between good or ideal solvent conditions impossible.

Although some observations have been made on the correlation of values of R_e to elution volume of intermediate polystyrenes, more primary data are needed to find values of R_e for the low-molecular-weight lignins. Light scattering measurements made in HPSEC solvents and values of intrinsic viscosity are needed to substantiate R_G and α, respectively. Alternatively, the lignin polymer may be fractionated by HPSEC, followed by the direct determination of molecular weight averages for these fractions by sedimentation equilibrium. We are presently fractionating these lignin polymers and plan to subject them to sedimentation equilibrium studies as well as careful characterization (analytical and spectroscopic). However, the absence of curvature in the data plotted with the empirical relationship log M vs. K_d indicates that this approach is a good first approximation to the true lignin molecular weights. Furthermore, good agreement was shown in values of R_e found for low DP polystyrenes and lignin model compounds by molecular volumetric estimation and statistical polymer theory. This indicates the validity of the application of Kirkwood–Riseman theory to polymer solution dynamics and HPSEC elution behavior. Eventually, this approach may lead to the determination of polymer molecular weights from elution behavior based on column calibration in terms of R_e. For now, the relationship of molecular weight to R_e, specific for each polymer–solvent system, would be determined by a direct method. Hopefully, future extensions of Kirkwood–Riseman treatment will allow prediction of polymer solution conformation from chemical composition alone.

ACKNOWLEDGEMENTS

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