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Improved Calibration Procedure for Gel Permeation Chromatography of Lignins

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Synopsis

In studying macromolecular properties of lignins, gel permeation chromatography (GPC) is a convenient method for the determination of lignin molecular weights and their distribution. A GPC analytical column was calibrated by preparing narrow molecular weight lignin fractions, then applying the method of Goring and co-workers to obtain molecular weights from the ultracentrifuge at zero concentration and zero field force. Diffusion and other curve-broadening effects in the GPC analytical column were taken into account by applying the correction method of Hamielec and co-workers to derive the coefficients of the calibration curve. The molecular weight of a lignosulfonate sample determined on the GPC was found to coincide closely with that obtained by ultracentrifugation.

INTRODUCTION

The principal approaches to an understanding of lignin structure have been through studies of degradation products, model compounds, synthetic products in vitro and in vivo, and macromolecular characteristics of lignin.[1] The subject of lignin macromolecular properties has been well reviewed recently by Goring,[2,3] and their understanding normally requires information on molecular weights and their distributions. However, even a cursory look at the literature reveals a lack of concordance among workers as to the molecular weight of lignins. The problem may be associated with the vastly different methods employed in their measurement. Problems in the determination of molecular weights also arise from the extent of molecular association and lignin–solvent interactions of soluble lignins. Another difficulty can be attributed to the polydispersity of lignins. This last factor must be considered very important because unless the full extent of polydispersity is recognized, experiments may be designed to yield information on either weight-average, number-average, or viscosity-average molecular weights, and on each account a rather lopsided indication of lignin molecular weight would result. Thus, a need arises to develop a relatively rapid, reliable procedure for the measurement of lignin molecular weights and their distribution.

Equilibrium ultracentrifugation has, thus far, been most successfully employed in lignin molecular weight measurements,[3] the length of time to

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attain equilibrium notwithstanding. Even so, it has been found necessary to develop a special procedure to take into account the anomalous behavior of lignins, which vary in molecular weights as the speed of the centrifuge is varied. Yean et al.[4] determined the molecular weights of lignosulfonate fractions at several speeds and concentrations. Molecular weights of the samples were then determined at zero concentrations by extrapolation at various speeds, and these were in turn plotted against the field force. The extrapolated values at zero field were taken as the true molecular weights. The results gave weight-average molecular weights, or at best yielded a polydispersity index without an indication of the molecular weight distribution.

Soundararajan and Wayman[5] sought to provide information on the molecular weight distribution of lignins by the use of a gel permeation chromatographic method. They calibrated their columns against synthetic lignin (DHP) fractions whose molecular weights had been determined by equilibrium ultracentrifugation. However, these determinations were made at single speeds.

The present method advances the Soundararajan and Wayman procedure by taking advantage of the result of determining molecular weights at various speeds to calibrate the gel columns. In addition, improved computer techniques have been used here in the corrections for the various curve-broadening operatives which distort gel permeation chromatograms.

EXPERIMENTAL

Preparative Chromatography

A spruce milled wood lignin sample (yield 57% of Klason lignin) was used in the preparation of narrow molecular weight fractions employed in the calibration of the gel columns. Details of isolation of the lignin sample are given in reference 6.

Two K25/45 (2.54 × 45 cm) columns from Pharmacia Canada Ltd. were packed, one with Sephadex G100 and the other with Sephadex G50 gels swollen in $0.2M$ NaOH solution and connected in series. The maximum flow rate obtainable from this series connection was 25 ml/hr, and this was used for the preparative fractionation.

Spruce milled wood lignin, 500 mg, was dissolved in 5 ml 0.5 N NaOH and cooked in a stainless steel bomb for 2 hr at 160°C. The product was filtered through a glass fiber filter to remove a very small amount of turbidity, and 2 ml of this was made up to 10 ml with the eluant. The sample was injected into the column and the flow was started. The highly colored soda-cooked lignin solution percolated the column, and its progress through the beds could be observed. The column outlet was collected in 10-ml fractions with the aid of a siphon. Each fraction was titrated with $0.05N$ HCl to pH 3, and the precipitated lignin was centrifuged at 15,000 rpm for 5 min, washed three times with water, and freeze dried from a dioxane–water solution.

Analytical Chromatography

The analytical system consisted of one K25/45 column packed with Sephadex G100 swollen in dimethyl sulfoxide (DMSO). The solvent reservoir was a Mariotte flask assembly and was connected to the column via a Hamilton three-way valve. The column effluent was continuously monitored at 280 nm using a Beckman DU spectrophotometer. An ultramicrocell of capacity 0.08 ml and 10 mm light path length was used for continuous flowthrough. The outlet was connected to a 2-ml siphon attached to a mechanical device which triggered an event marker each time the siphon was filled. Thus, a spike was recorded throughout the chromatogram at 2-ml eluant intervals.

The elution rate through the column was maintained at about 25 ml/hr by the control of the height of the Mariotte flask. Samples were injected at 5 mg/ml concentrations, and 0.4 ml of this solution was needed for an analysis. The injection of the sample was done using the syringe attached to the three-way valve, and injection was always in the direction of the reservoir, with the column closed off from the injection point. Flow was started as soon as the injection was over. The fractions obtained from the preparative chromatograph were run on the analytical system, and their chromatograms were obtained on a chart recorder. On the basis of their chromatograms, three fractions from the low, middle, and high molecular weight ranges were selected to be run on the ultracentrifuge for the calibration of the GPC column.

Equilibrium Ultracentrifuge

The short-column sedimentation equilibrium technique previously described by Van Holde and Baldwin[7] and Yphantis[8] and applied by Soundararajan and Wayman[5] and Yean et al.[4,9] was used to determine the weight-average molecular weights of the three fractions using a Beckman Spinco Model E analytical ultracentrifuge. The ultracentrifuge was equipped with a temperature control, and the experiments were carried out at a constant temperature of 20°C. Schlieren observations were made at phase-plate angles of 70°, 75°, 80°, and 85°. Exposures were taken on Kodak spectrographic plates, emulsion Type IIG.

An eight-channel centerpiece was used in these experiments. This made it possible for a concentration series to be run on each sample at any given speed. Freshly distilled DMSO was used in dissolving the lignin fractions. A solution of the highest concentration of a series was accurately made, the lignin sample being weighed from the freeze-dried fraction. The lower concentrations were then diluted from this stock solution. The filling of the cell reservoirs was carried out as recommended by the ultracentrifuge operating manual. The runs were made starting with the lowest speed. At the end of the run, the rotor speed was taken up to the next higher speed at which measurement was desired. For the lowest fraction, equilibrium was achieved after 8 hr, while for the highest fraction, it required running

overnight for equilibration. The photographic plates were measured with a Nikkon two-dimensional comparator. The height of the Schlieren pattern was read at the midpoint of the column, and this was converted into concentration terms after calibrating the Schlieren system with the calibration cell for the Model E.

The molecular weight of a sample of spruce lignosulfonate, RAS-II, supplied by Dr. D. A. I. Goring (Pulp and Paper Research Institute of Canada, Pointe Claire, Quebec), was also measured on the ultracentrifuge using the same technique.

Calculation

The calculation of molecular weights from the ultracentrifuge measurements is based on the formula of Van Holde and Baldwin[2] given as

$$M = \frac{1}{rC_0} \cdot \left(\frac{dc}{dr}\right)_{r=r'} \cdot \frac{RT}{\omega^2(1 - \bar{V}\rho)} \quad (1)$$

where M is the apparent molecular weight of dissolved lignin and r' is the position in the column at equilibrium where the concentration is equal to the original concentration, C_0. This equation can be written in terms of the measured linear displacement y' of the Schlieren pattern on the screen,

$$M = \frac{RT}{(1 - \bar{V}\rho)\omega^2} \cdot \frac{1}{r'C_0} \cdot \frac{y'\phi x}{\Delta n A_{\text{cal}}} \quad (2)$$

where r' is now approximated to be the column midpoint: Δn is the specific refractive index increment: x and ϕ are values obtained from the calibration cell, and A_{cal} is obtained using the value of the phase-plate angle at which the photograph was taken (see ref. 10); and $\bar{V}$, ρ, and ω have the usual meaning assigned to them in ultracentrifugation work. To obtain weight-average molecular weights, it is necessary that ϕ be expressed in radians, x in cm, C_0 in g/ml, y' in cm, A_{cal} in cm^2, and a, the cell thickness, in cm. For the alkali lignin fractions, $\bar{V}$ and Δn were determined to be 0.663 ml/g and 0.201 ml/g, respectively; ρ for DMSO is 1.0950; $\bar{V}$ and Δn for the lignosulphonate sample were taken as those obtained by Yean and Goring.[9]

Ultracentrifuge Results

The results of the ultracentrifuge sedimentation equilibrium experiments on the alkali lignin fraction designated 5 and the lignosulfonate sample RAS-II are presented in Figures 1 and 2. Similar results were obtained for the other lignin fractions designated 11 and 15. Measurements were made at several solute concentrations and rotor speeds as shown, and the M_w's, the weight-average molecular weights, were obtained by extrapolating to zero concentration and then to zero field force.

The straight lines in Figure 1 illustrate the behavior of lignin solutions in the ultracentrifuge as discussed by Yean and co-workers.[4] In the lower

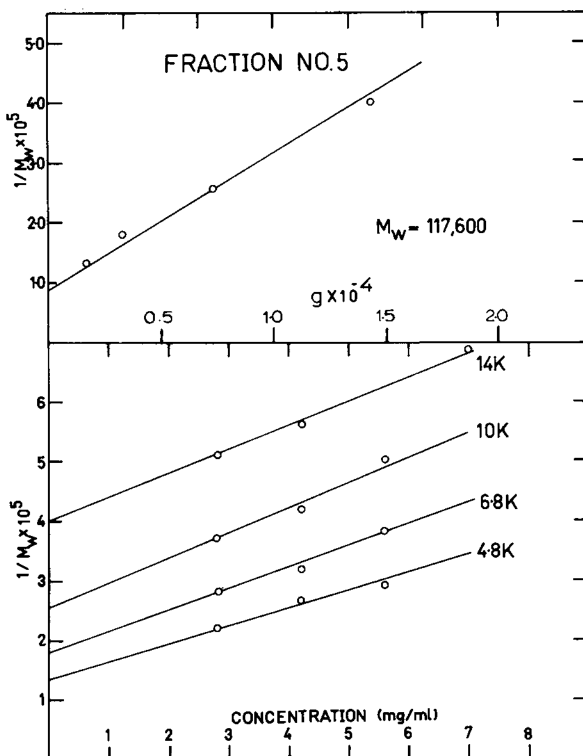

Fig. 1. Ultracentrifuge determination of molecular weight of lignin fraction 5.

half of the figure, the reciprocal of the weight-average molecular weight, $1/M_w$, is plotted against concentration. For each sample, a set of three or four straight lines is obtained, each line representing a different centrifuge speed. At each speed, the measured $1/M_w$ increased with increasing concentration, that is, the apparent molecular weight decreased with increasing concentration. This effect was due to the occurrence of a gel layer at the bottom of the cell the depth of which increased at higher fields and concentrations and was greater for the fractions of higher molecular weight. The loss of substance from solution into this gel layer gave a falsely high value for C_0 in eq. (2), and therefore a low M_w. There was very little concentration effect at the low molecular weight of 2900, fraction 15 (not shown).

Each line in Figure 1 was extrapolated to zero concentration, thereby obtaining a value for M_w independent of concentration at each centrifuge speed. In the upper half of the figure, $1/M_w$ was plotted against gravitational field. In all cases examined, the molecular weight was strongly dependent on the magnitude of the gravitational field. Extrapolation of each curve to zero field gave a value for M_w independent of both gravitational field and concentration. These are the values used in calibrating

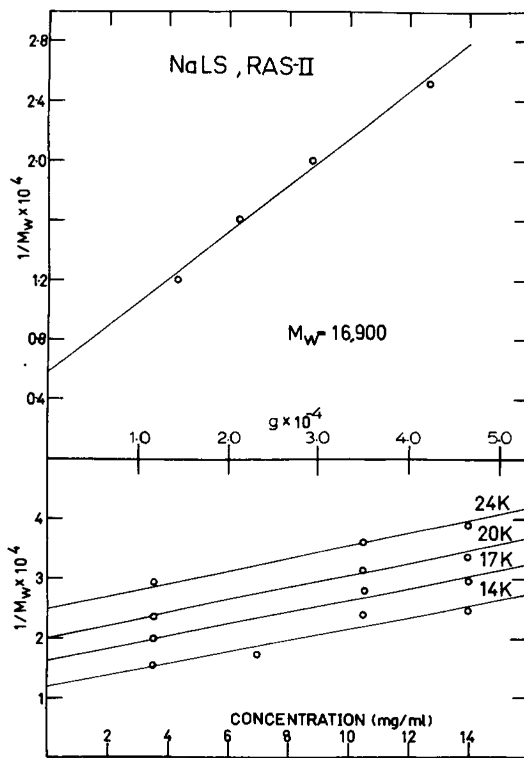

Fig. 2. Ultracentrifuge determination of molecular weight of sodium lignosulfonate RAS-II.

the GPC column. Fraction 5 had a M_w value of 117,600, while the values were 28,600 and 2,900 for fractions 11 and 15, respectively.

Calibration of GPC Column

The three lignin fractions whose molecular weights were determined by the short-column sedimentation equilibrium method were each injected into the Sephadex G100 analytical column, and their chromatograms are shown in Figure 3. These fractions, used for the purpose of calibration, were chosen such that their combined molecular weight distributions spanned the useful range of the gel column. The logarithms of their molecular weights were then plotted against their peak elution volume to obtain the calibration curve shown in Figure 4.

Gel permeation chromatography, like other chromatographic techniques, is limited in its resolution. When a monomeric compound is injected as a discrete pulse into a column, the chromatogram does not appear as a rectangle with a width of unit increment of molecular weight. Instead, spreading occurs owing to molecular diffusion and convection along the axis of the tube, and the chromatogram appears as a bell-shaped curve. The area under the curve is still proportional to the concentration of the entire sample but the height of the curve does not reflect directly the rela-

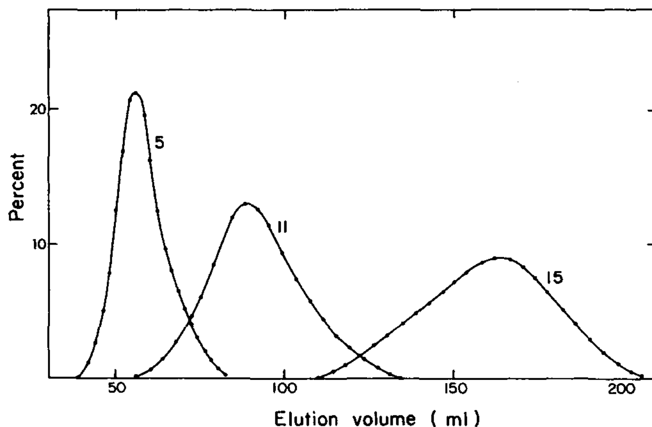

Fig. 3. Gel permeation chromatograms of lignin fractions designated 5, 11, and 15.

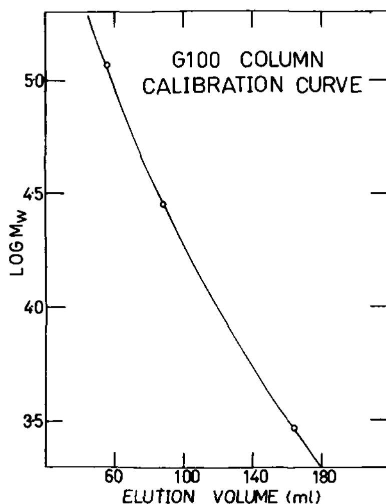

Fig. 4. Calibration curve for Sephadex G100 GPC analytical column.

tive abundance of the component at the corresponding elution volume as it depends also on the amount of the neighboring components.

A precise interpretation of the chromatogram must correct for this overlapping and diffused pattern. Tung[11] assumed a Gaussian chromatogram for a single species and argued that the total response of the multicomponent mixture is due to the summation of many Gaussian distributions yielding a continuous distribution. A mathematical expression of this is given as

$$F(v) = \sum_i A_i \sqrt{h_i/\pi} \exp\{-h_i(v - v_{0i})^2\} \tag{3}$$

and is called Tung's integral dispersion equation. In the equation, $F(v)$ is the chromatogram height at elution volume v, v_i is the elution volume of

the ith component at the peak position, A_i is a constant proportional to the mass of the ith component, and h_i is the resolution factor and is related to the standard deviation of the chromatogram. This equation is solved to find the corrected chromatogram $W(v)$.

To evaluate the curve-broadening operatives of a chromatogram, Smith[12] assumed a logarithm normal distribution of molecular weights of the single molecular weight species. This technique assumes a symmetrical pattern of axial dispersion and uses the standard deviation of the elution curve at different counts as a correction factor. Pickett et al.[13] took a phenomenological approach to account for skewing by measuring the shapes of the chromatograms of narrow standards. The assumption is made that normalized heights are independent of concentration and thus used. However, in a recent evaluation of these methods, Duerksen and Hamielec[14] found them to be subject to artificial oscillations in both the corrected chromatogram and corrected differential distribution.

To overcome some of these problems, Balke and Hamielec[15] first solved for moments of the corrected chromatogram and later for differential distribution using the corrected number-average and weight-average molecular weights along with the actual GPC chromatogram.

This method has been applied here. The Sephadex G100 column was calibrated using the ultracentrifuge molecular weights of the three lignin fractions to obtain the calibration curve shown in Figure 4. The chromatograms of these standards were used to search for an effective calibration curve equation which was then used to calculate corrected number-average, weight-average, and z-average molecular weights. (The computer program is given in ref. 16.)

The equation of the calibration curve may be represented by a general equation of the form

$$V_e = C_1 - C_2 \log M + C_3 (\log M)^2 \tag{4}$$

where C_2, C_2, and C_3 are constants and V_e is the peak elution volume. The computer program[16] accepts the initial guesses of the constants C_1, C_2, and C_3 along with the heights of the chromatogram and their elution volume. Also supplied are the weight-average molecular weights obtained from the ultracentrifuge. By shifting and rotating the calibration curve to correct for skewing and axial dispersion, corrected differential distributions are obtained from which the corrected molecular weight averages are calculated. The values of C_1, C_2, and C_3 are minimized at this point and are assigned to the calibration constants in the calibration curve equation. A series of initial values of the constants are imputed, and the convergence of the calculated values is taken as an indication that the true values had been obtained. The calibration curve of the Sephedex G100 column was found to be represented by the equation

$$V_e = 620.3 - 132.2 \log M_w + 13.5 (\log M_w)^2 \tag{5}$$

where M_w is the weight-average molecular weight.

TABLE I
Various Values of M_w for RAS-II in Various Solvents

Solvent	Method of detn.	M_n	M_w	M_w/M_n	Year	Ref.
0.1*M* NaCl	ultracentrifuge	—	60,000	—	1964	[a]
0.1*M* NaCl	ultracentrifuge	—	52,000	—	1967	[a]
DMSO	ultracentrifuge	—	43,000	—	1967	[a]
DMSO	GPC	3,700	17,600	4.7	1972	present data
DMSO	ultracentrifuge	—	16,900	—	1972	present data

[a] Personal communication from D. A. I. Goring.

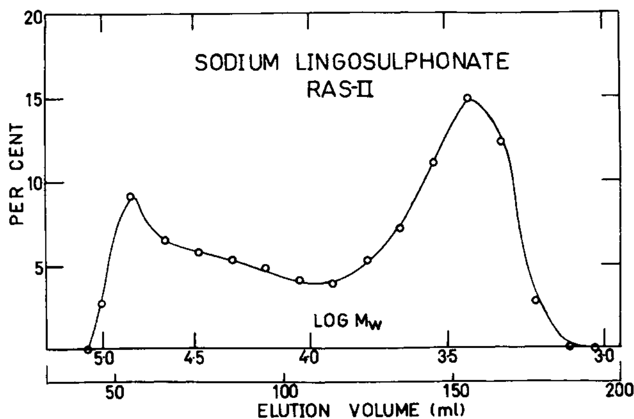

Fig. 5. GPC determination of molecular weight and its distribution for sodium lignosulfonate RAS-II.

To test the validity of the calibration curve, the molecular weight of the lignosulfonate sample RAS-II was determined by elution through the G100 column and found to have a weight-average molecular weight of 17,600. This is to be compared with a value of 16,900 obtained by the equilibrium sedimentation method. The number-average molecular weight computed from the gel chromatogram was 3,700, yielding a value of the polydispersity index, M_w/M_n, of 4.7. This depicts a rather broad distribution of molecular weight for the lignosulfonate sample, a fact which is borne out in the distribution curve of the sample as shown in Figure 5. Table I shows the various values of M_w obtained for RAS-II, in several solvents, over a period of years. Despite the claim[8] that the 13% deviation in molecular weight after four years of storage was within the range of experimental error of the short-column method, our significantly different data tend to suggest otherwise. Degradation may in fact be responsible for the consecutively lower molecular weight obtained for the same sample with the passage of time.

CONCLUSIONS

The molecular weights of lignin samples can be conveniently and rapidly determined by gel permeation chromatography. However, the confidence

to be reposed in the values so obtained must depend on the careful calibration of the chromatographic column. The criteria for this include the derivation of lignin molecular weights at zero concentration and zero gravitational field and the accounting for diffusion and axial dispersion in the GPC.

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