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Note

Alkaline size-exclusion chromatography of lignins and coal extracts using cross-linked dextran gels

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Biochemists working with natural polymers have long been concerned with the challenge of fractionating these materials by chromatographic methods. However, many such polymers are difficult to disperse in aqueous solutions, even if made to relatively high ionic strengths at neutral pH. In the case of three classes of important biopolymers, coal extracts, humic acids and lignins, this behavior is often due to the presence of water-insoluble complexes containing aromatic or aliphatic polyphenols[1,2]. A successful solvent for these polymers was found by McNaughton *et al.*[3] and Sarkanen *et al.*[4] to be 0.1 *M* sodium hydroxide, which produces a pH of 13 at 20°C. One problem involved in adapting such a solvent–solute system to size-exclusion chromatography (SEC) is that of minimizing solute–solute interaction and solute–column gel interaction. Also, the chromatography gel must be stable for weeks in 0.1 *M* sodium hydroxide, and high-performance separations offering theoretical plates in the 10^3–10^4/m range are desirable.

Since the first descriptions of gel filtration chromatography (now more commonly referred to as SEC) in the mid-1950s by Lindqvist and Storgards[5], the separation and quantitation of a wide variety of biomacromolecules has been possible. This early work was performed with an insoluble, cross-linked polydextran gel, eventually called Sephadex™ by Pharmacia (Uppsala, Sweden). Many other "non-rigid" SEC separation gels soon became available, including the agarose gels Sepharose™ and Sepharose CL™ from Pharmacia, and Bio-Gel™ A series from Bio-Rad Labs. (Richmond, CA, U.S.A.); the dextran–bisacrylamide gels Sephacryl™ from Pharmacia; the acrylate gels Fractogel or Toyo-Pearl™ from Toyo Soda (Japan); the polyacrylamide gels Bio-Gel P series from Bio-Rad; and the agarose–acrylamide gels Ultrogel™ from LKB (Rockville, MD, U.S.A.). Since these gels are formed in the

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30–80-μm particle size range, free-packed column efficiencies are rather low. Also, only Sepharose CL is recommended[6] for use at pH values as high as 14.

Some "rigid" gels for high-pressure (HP) SEC available for the last decade include the native silica gels Porasil™ from Waters Assoc. (Milford, MA, U.S.A.), Fractosil™ from E. Merck (Darmstadt, F.R.G.) and CPG Electro-Nucleonics (Fairfield, NJ, U.S.A.); the hydrophilically bonded silica gels TSK-gel SW type from Toyo Soda, LiChrospher™ from E. Merck, Protein-Pak™ from Waters Assoc., and SynChropak™ from SynChrom (Linden, IN, U.S.A.); the acrylate polymer based gels TSK-gel PW type from Toyo Soda and OHpak™ from Showa Denko (Japan); the polyvinyl alcohol gels Ashipak™ GS series from Asahi Kasei (Japan) and the OHpak Q series from Showa Denko; the rigid polyacrylamide gel PL-aqua gel P series, Polymer Labs. (U.K.); and finally the cross-linked dextran gel Superose™ 6 HR and 12 HR from Pharmacia. Of these HPSEC gels, only TSK-gel PW and Superose offer extended stability at pH 14.

Prepacked columns of Superose gels were chosen in the study reported here, where neutral pH, aqueous extracts of lignite coal and ball-milled aspen lignin were analyzed in 0.1 *M* sodium hydroxide.

EXPERIMENTAL

Chromatography instrumentation

The system used in this study employed a Beckman Model 110B HPLC pump, a Waters Assoc. Model 440 UV detector with a 280-nm filter, and a Beckman Model 210 manual injection valve fitted with a 100-μl sample loop. Chromatograms were recorded and stored on floppy-disc media using Dynamic Solutions HPLC software and an MS-DOS compatible personal computer. The column set consisted of a Superose 6 HR (30 cm × 10 mm I.D.) and a Superose 12 HR (30 cm × 10 mm I.D.) connected in series. Samples were prepared in 0.1 *M* sodium hydroxide made with degassed, deionized water to a concentration of about 1 mg/ml. Samples were injected into the system immediately after preparation and fitration with 0.22-μm HPLC sample filters (Rainin Instruments).

Molecular weight standards

Sodium polystyrene sulfonate (NaPS) standards (peak-average molecular weight, M_p = 1200, 780, 400, 200, 100, 74, 34, 18, 8, 4.6 and 1.8 kilodaltons) were from Polymer Labs. (Church Stretton, U.K.) and sodium polyacrylate (NaPA) standards (weight-average molecular weight, M_w = 8300, 3800 and 1930) were from American Polymer Standards Corp. (Mentor, OH, U.S.A.). Tannic acid [formular weight (FW) = 1701], sodium azide (FW = 65), sodium 4,5-dihydroxynaphthalene-2,7-disulfonate (FW = 400), and sodium dihydroxybenzoate (FW= 154) were from Aldrich. Trisodium ethylenediaminetetraacetate (EDTA) (FW = 358) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) (FW = 168) were from Sigma.

Preparation of lignin and coal-extract samples

Texas lignite (coal) was oxidatively pretreated with 8 *M* nitric acid for 1 h. Resulting material was washed with deionized water until the pH of the wash water was greater than 5 and was then continuously extracted with water in a soxhlet

extraction apparatus for 6 weeks. This coal (1 g) was then repeatedly extracted with 10 ml of 50 m*M* phosphate buffer, pH 7. Initial additions of buffer resulted in immediate pH decreases, and little material was extracted. Subsequent extractions resulted in smaller decreases in pH and more material going into solution. The sample analyzed in this study resulted from an early extract. Materials from these extracts remain fully soluble at pH values above 7. Ball-milled aspen lignin was prepared following the procedure of Lundquist *et al.*[7]. The yield of purified milled wood lignin obtained was usually about 10% (w/w) that of ethanol–benzene-extracted aspen wood. The ball-milled aspen lignin was not esterified before analysis.

Analysis of chromatographic data

The elution profiles of aspen lignin and the coal extract were subjected to molecular weight distribution analysis [*e.g.*, for the number-average molecular weight (M_n), M_w and M_p] using the graphical method described by Yau *et al.*[8] after baseline correction. Values for molecular weight increment, M_i, along the elution envelope were determined from the linear portion of the column calibration curve (constructed from all standards from NaPS 400 000 through sodium tannate). Calculations for column theoretical plate analysis were from Fritz and Schenk[9].

RESULTS AND DISCUSSION

A HPSEC column system consisting of Superose 6 HR (30 cm × 10 mm I.D.) and Superose 12 HR (30 cm × 10 mm I.D.) columns connected in series was calibrated with newly available, sulphonated polystyrene and polyacrylate standards. For determination of the lower portion of the calibration curve several well characterized, alkaline soluble compounds were used, including sodium tannate, sodium vanillate and sodium dihydroxynaphthalene sulfonate. Sodium azide was used as a marker of V_t (Fig. 1). When plotted as the simple function log molecular weight *versus* elution time, the calibration standards appear to produce a reasonably linear curve over the molecular weight region $1 \cdot 10^3$–$1 \cdot 10^5$ (Fig. 1). Although many other relationships between solute molecular weight (or, more accurately, effective hydrodynamic volume) and elution volume can be proposed[8,10], the function presented here appears adequate for the scope of this preliminary study.

The lignin samples examined are irregular phenylpropane polymers that represent approximately 30% (w/w) (native lignin) of the available polymeric content of hardwood tree stems[1]. This material offers, therefore, a valuable resource that must be utilized as fully as possible if full value of harvested tree crops is to be attained. Understanding the relationship between lignin apparent molecular weight and various physical properties is of great concern. The ball-milled aspen lignin sample loaded on the alkaline Superose system was eluted as a multimodal envelope, which was graphically deconvoluted to remove the unknown component eluting after V_t (Fig. 2). The M_w was found to be 2450. This value corresponds well with numerous hardwood milled lignins studied by Faix and co-workers[11,12], where for spruce, aspen, beech and birch lignins, M_w values between 2800 and 5500 were found. Whereas previous studies of acetylated and unacetylated lignins on μStyragel columns with organic solvents show varying degrees of column–solute interaction[13,14], Superose, a non-aromatic, strongly hydrophilic chromatography gel, contributes little such interaction in the

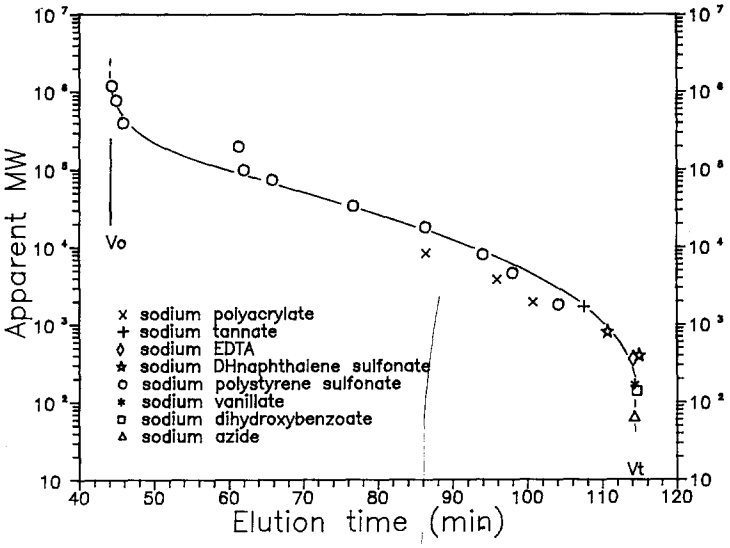

Fig. 1. Calibration of HPSEC column system consisting of one Superose™ 6 HR and one Superose 12 HR connected in series. The mobile phase was 0.1 *M* sodium hydroxide and the chromatography was conducted at 20°C. MW = Molecular weight; V_0 = the column elution volume corresponding to the interstitial volume between gel beads and V_t = the total liquid column volume; DH = dihydroxy.

presence of solvents like 0.1 *M* sodium hydroxide, which strongly dissociate both the solute and gel matrix[15], thus minimizing short-range adsorptive effects.

The coal-extract sample injected on the Superose system eluted as a narrow, symmetrical peak (Fig. 3). Graphical molecular weight distribution analysis produced values for M_n and M_w of 2800 and 3000, respectively. M_p was found to be 3900 by

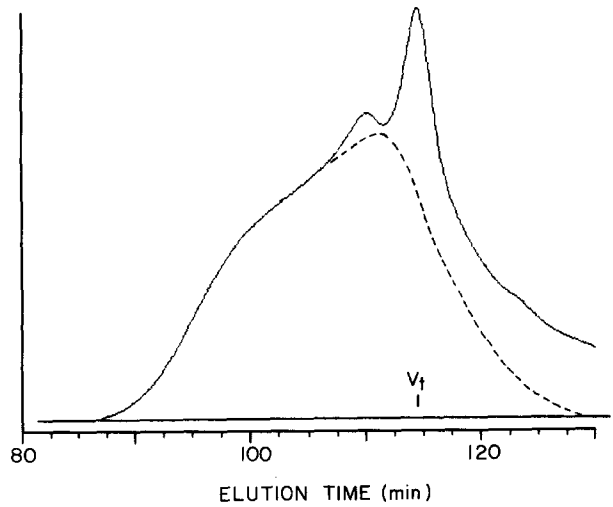

Fig. 2. Elution of ball-milled aspen lignin from Superose column system in 0.1 *M* sodium hydroxide. Column loading was 0.25 mg, the flow-rate was 0.3 ml/min and detection was at 280 nm. Dashed line shows result of graphical baseline subtraction and deconvolution of peak eluting after V_t (see text).

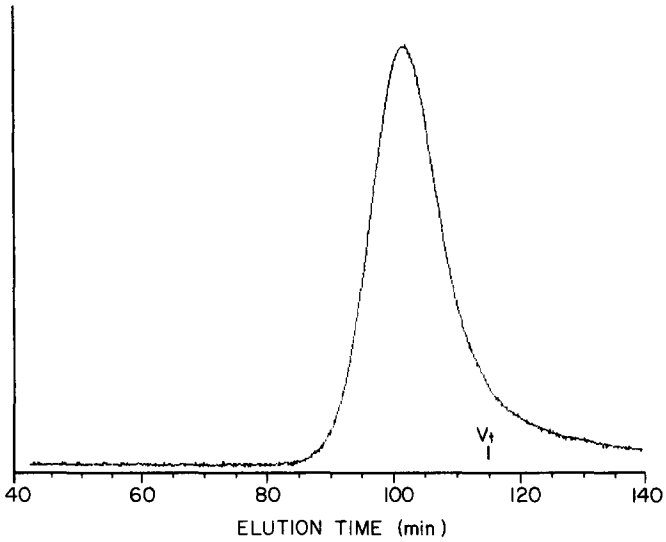

Fig. 3. Elution of acidified coal-extract from Superose column system in 0.1 *M* sodium hydroxide. Chromatography conditions as in Fig. 2.

comparison of the elution time of the peak-maximum-ordinate shown in Fig. 3 with the column calibration curve. The very low polydispersity found in the present study for this sample in sodium hydroxide (M_w/M_n = 1.03) may further reflect the low column–solute interaction available with this cross-linked dextran column packing material. Although the literature contains studies of molecular weight analyses of alkali-soluble, native coal extracts that yield molecular weights near $1 \cdot 10^6$ (ref. 16), studies describing the molecular weights of neutral pH, humic acid extracts of acid-hydrolyzed coal (as reported here) are not readily available.

In conclusion, "conventional gel permeation chromatographic" analysis was applied to Superose columns calibrated with alkali-stable standard polymers and low-molecular-weight acidic compounds. These compounds are similar in chemical structure to the polyphenolic or acidic biopolymers examined. Also, theoretical plate analysis for sodium azide elution showed the column system used in this study had $1.5 \cdot 10^4$ plates per meter, which is 10–100 times that possible with open-column type packing materials, such as Sephadex. Rigid, organic polymer-based packing materials for prepacked columns, such as TSK-gel PW series, although stable at pH 14, are quite hydrophobic and may retard the elution of polyphenol-containing polymers. Superose, the high-performance[17], alkali-stable, cross-linked dextran gel material, appears to be an ideal support matrix for SEC in strongly dissociating, high ionic strength solvents such as 0.1 *M* sodium hydroxide.

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REFERENCES

1 K. V. Sarkanen and C. H. Ludwig, *Lignins: Occurrence, Formation, Structure, and Reactions*, Wiley-Interscience, New York, 1971.
2 D. Fengel and G. Wegener, *Wood: Chemistry, Ultrastructure, Reactions*, Walter de Gruyter, Berlin, 1984.
3 J. G. McNaughton, W. Q. Yean and D. A. I. Goring, *Tappi*, 50 (1967) 548.
4 S. Sarkanen, D. C. Teller, E. Abramowski and J. L. McCarthy, *Macromolecules*, 15 (1982) 1098.
5 B. Lindqvist and T. Storgards, *Nature (London)*, 175 (1955) 511.
6 R. C. Montelaro, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 10, pp. 269–296.
7 K. Lundquist, R. Ohlsson and R. Simonson, *Sven. Papperstidn*, 78(1975) 390.
8 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size Exclusion Chromatography*, Wiley, New York, 1979.
9 S. Fritz and G. H. Schenk, *Quantitative Analytical Chemistry*, Allyn & Bacon, Boston, 1974, p. 377.
10 M. E. Himmel and P. G. Squire, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 1, pp. 3–22.
11 O. Faix, W. Lange and O. Beinhoff, *Holzforschung*, 34 (1980) 174.
12 O. Faix, W. Lange and E. C. Salud, *Holzforschung*, 35 (1981) 3.
13 H. L. Chum, D. K. Johnson, M. P. Tucker and M. E. Himmel, *Holzforschung*, 41 (1987) 97.
14 W. G. Glasser, P. C. Barnett, P. C. Muller and K. V. Sarkanen, *J. Agric. Food Chem.*, 31 (1983) 921.
15 P. L. Dubin, in P. L. Dubin (Editor), *Aqueous Size-Exclusion Chromatography*, Elsevier, Amsterdam, 1988, Ch. 3, pp. 55–75.
16 E. S. Olson, J. W. Diehl and M. L. Froehlich, *Fuel*, 66 (1987) 992.
17 T. Andersson, M. Carlsson, L. Hagel, P.-A. Pernemalm and J.-C. Janson, *J. Chromatogr.*, 326 (1985) 33.