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Size-exclusion chromatography of lignin- and carbohydrate-containing samples using alkaline eluents

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Abstract

Size-exclusion chromatography of carbohydrate- and lignin-containing samples, prepared from wood and pulp samples, was carried out using alkaline eluents. The elution of carbohydrate and lignin macromolecules can be monitored with good selectivity using a pulsed amperometric and a UV absorbance detector, respectively. However, the stability of the signals from samples stored in alkaline solutions requires careful consideration in the analysis because marked changes are observed in many cases. Furthermore, the interactions between aromatic molecules and the separation column are also dependent on the concentration of alkali in the eluent. Since there seem to be no adsorptive interactions between carbohydrate and lignin macromolecules during size-exclusion chromatography, the described method can be used to monitor chemical and enzymic modifications of the apparent molecular mass of these two classes of compounds, whether they occur in mixtures or covalently linked complexes.

Keywords: Mobile phase composition; Lignin; Carbohydrates

1. Introduction

Size-exclusion chromatography (SEC) is a common method for characterizing macromolecules. An estimate of their molecular mass (M_r) and M_r distribution is provided if elution rates are calibrated using appropriate M_r standards. Despite various limitations as a result of solute–solvent–matrix interactions, the technique has been successfully applied to both lignin [1–3] and carbohydrate [4–6] samples. However, additional difficulties could be encountered in the analysis of samples that contain both lignin and carbohydrate macromolecules because suitable eluents, separation matrices and detection systems would be required. The availability of convenient tools for the analysis of lignin–carbohydrate mixtures and lignin–carbohydrate complexes (LCCs) would be useful for on-going research on the structure of plant cell walls [7] and the removal of residual lignin from wood pulp [8]. This is because lignin–carbohydrate linkages are thought to have important roles in cell wall structure and in the difficulties encountered during pulp bleaching, respectively.

Caustic solutions, often used for the isolation

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and purification of lignin and carbohydrate samples, appear to be convenient eluents because 0.5 *M* NaOH can be used to dissolve nearly all lignin [2,9] and many carbohydrate [4,6] preparations. As a result, samples do not need to be derivatized for analysis. Although the highly alkaline pH might lead to some modifications of carbohydrates [10] and lignins [11,12], it would also dissociate the acidic groups in lignin to yield polyelectrolytes and prevent the agglomeration of lignin molecules that is observed at pH below 10.5 and in organic solvents such as dimethylformamide [1,12]. The high ionic strength also minimizes the tendency of these polyelectrolytes to swell in solution [13].

The chromatographic resins that have performed well in 0.5 *M* NaOH include Fractogel (Merck, Darmstadt, Germany) [9], Separon S HEMA 1000 (Tessek, Prague, Czech Republic) [6], Sepharose CL-6B (Pharmacia, Uppsala, Sweden) [2] and TSKgel G4000PW (Toyo Soda, Tokyo, Japan) [4]. Like Separon resins, Toyopearl HW resins (TosoHaas, Montgomeryville, PA, USA) are polymers based on methacrylate and ethylene groups. They are said to be stable from pH 2 to 14, and thus warrant consideration for the SEC of lignin–carbohydrate mixtures.

The continuous monitoring of lignin samples during chromatography has generally relied on UV absorbance at 280 nm, whereas that of carbohydrate samples has relied on refractive index (RI). The combination of these two detection systems has also been used to monitor independently both classes of compounds when they occurred in the same sample [14–17]. This convenient, dual detector system avoids the need for postcolumn reactions that have been suggested for the selective detection of carbohydrates [18–20]. However, it would not be effective in strong alkali owing to the intrinsically high RI of such eluents. An alternative has been made available with the recent introduction of pulsed amperometric detection (PAD) for routine carbohydrate analysis [21,22]. This highly sensitive detection method in fact requires alkaline eluents to oxidize carbohydrates at the surface of the working electrode. In this work, we evaluated the use of PAD, in combination with UV absorbance detection, for the SEC analysis of lignin–carbohydrate samples. This SEC system has been used in our laboratories to verify the presence of LCCs in kraft pulps [8].

2. Experimental

2.1. Molecular mass standards

Table 1 lists the various carbohydrate standards used to calibrate the SEC system along with their sources. The elution of other carbohydrates, and a series of aromatic compounds and macromolecules, was also used to evaluate the system. All of the commercially available materials were used without further purification. The number-average molecular mass (M_n) of the dextran and sodium polystyrene sulfonate (NaPSS) standards was used as their M_r. This is because the M_r distribution curve which was provided with each dextran purchased from Pharmacia indicated that the M_n is closer to the peak of each curve.

2.2. Polysaccharide and lignin samples

Carbohydrate and lignin samples were prepared from trembling aspen (*Populus tremuloides*) and black spruce (*Pinus piceae*), and their composition (Table 2) was determined as described previously [23,24]. Total lignin was determined by summing acid-insoluble and soluble lignin. Total carbohydrate was determined by summing the five neutral wood sugars (in their anhydroform) quantified in the acid hydrolysates of the samples by HPLC.

Two additional lignin samples were also analyzed. A dehydrogenative polymer of guaiacol was used as a model lignin, and it was prepared using horseradish peroxidase (Boehringer, Mannheim, Germany) [25]. Milled wood lignin (MWL) from lodgepole pine (*Pinus contorta*) was provided by Dr. S. Yokota (Utsunomiya University, Utsunomiya, Japan). To obtain this

Table 1
List of calibration standards (C) and samples (Nos. 1–16) that were used to calibrate and evaluate the size-exclusion system, respectively

No.	Standard or sample	$M_r \times 10^3$ [a]	Supplier
1	Guaiacol	0.124	Sigma (St. Louis, MO, USA)
2	Xylose	0.150	Fisher (Nepean, Ontario, Canada)
3	Vanillin	0.152	Sigma
4	Vanillic acid	0.168	Sigma
5	Tyrosine	0.181	Sigma
6	Catechin	0.290	Aldrich (Milwaukee, WI, USA)
7	Quercetin	0.301	Aldrich
C	Cellobiose	0.342	Sigma
C	Maltotriose	0.504	Sigma
8	Rutin	0.665	Aldrich
C	Dextran	1.01	Fluka (Ronkonkoma, NY, USA)
C	Dextran T10	5.5	Pharmacia (Uppsala, Sweden)
9	Na polystyrene sulfonate (NaPSS)	17.0	Scientific Polymer Products (Ontario, NY, USA)
10	*T. harzianum* xylanase	22.0	Maringer et al. [23]
C	Dextran T40	24.5	Pharmacia
11	Na polystyrene sulfonate	28.3	Scientific Polymer Products
C	Dextran T70	37.6	Pharmacia
12	Horseradish peroxidase	44.0	Boehringer (Mannheim, Germany)
13	Bovine serum albumin	67.0	Sigma
14	Na polystyrene sulfonate	103.2	Scientific Polymer Products
15	Na polystyrene sulfonate	167.0	Scientific Polymer Products
16	Dextran T500	195.3	Pharmacia

[a] The M_r values listed for the polydisperse polymers are the number-average values (M_n).

crude preparation of MWL, ball-milled wood meal (1 g) was extracted twice with 94% (v/v) dioxane (25 ml) for 1-day durations and the combined extracts were then freeze-dried.

2.3. Size-exclusion chromatography

Toyopearl HW-55S and HW-50S resins have separation ranges for dextrans of M_r 1000–

Table 2
Lignin and carbohydrate contents (%) of samples examined by SEC [23,24]

Wood	Source	Sample	Lignin	Carbohydrate
Aspen	Holocellulose	Acetylated xylan (DMSO[a] extract)	9.3	69.7
		Deacetylated xylan (alkaline extract)	10.8	61.8
	Kraft pulp	Enzyme lignin	81.3	7.8
		Kraft lignin	94.0	2.7
		Alkaline extract	5.0	71.0
		DMSO extract	5.5	79.0
Spruce	Kraft pulp	Enzyme lignin	82.5	7.7
		Alkaline extract	8.3	68.1

[a] Dimethyl sulfoxide.

200 000 and 500–20 000, respectively. Dual 20 × 0.5 cm I.D. HR columns (Pharmacia, Uppsala, Sweden), packed with the respective resins in series, were used for analytical chromatography. The samples were injected using a SpectraSYSTEM AS3500 autoinjector (Spectra-Physics, Fremont, CA, USA). Elution was carried out at 0.05 ml min^{-1} for 3 h using a Dionex (Sunnyvale, CA, USA) DX 500 HPLC system and monitored using a Dionex ED40 electrochemical detector (gold electrode, integrated amperometry mode, parameters set for detecting sugars as recommended by the manufacturer) and a Dionex AD20 absorbance detector (280 nm, path length 6 mm, set at low) in series. The HPLC system was controlled using PeakNet 4.10 software and the data were exported to a graphing program for manipulation and presentation.

The eluent initially used was 1 *M* NaOH, with the pump configuration set at pressure control. More recent experiments used 0.3 *M* or 50 m*M* NaOH as eluent, with the pump configuration set at flow control. These latter experiments also involved installing an old HPLC column and fine PEEK tubing between the pump and the autoinjector, for the purpose of keeping the backpressure at the pump near 200 p.s.i. After months of operation using 0.3 *M* NaOH as eluent, our experience indicated that nearly identical elution times are observed with freshly dissolved samples if the flow of eluent through the columns has not been interrupted. A day-long period was required to equilibrate the columns under running conditions.

2.4. Sample preparation

Polysaccharide and lignin samples were dissolved at 1 mg ml^{-1} in the desired eluent, filtered through 0.45-μm HV syringe filters (Millipore, Bedford, MA, USA), and 20 μl were injected for SEC analysis. Samples were also obtained by mixing a carbohydrate extract and an enzyme lignin (1 mg ml^{-1}) in a ratio of 4:1. These mixtures were compared with the carbohydrate extract (0.8 mg ml^{-1}) and the enzyme lignin (0.2 mg ml^{-1}), injected separately. All samples were analyzed at least twice, with time-dependent changes verified using three series of injections started on different days with freshly dissolved samples.

3. Results

3.1. Calibration of size-exclusion chromatography

Commercially available dextrans and oligosaccharides were used as M_r standards (Table 1), with their elution in 1 *M*, 0.3 *M* and 50 m*M* NaOH monitored using PAD (Fig. 1). Although the column bed swelled with decreasing caustic concentrations, linear calibration graphs could be achieved in each eluent for carbohydrates with M_r ranging from 340 to 37 600. The elution of the M_r 195 000 dextran (sample 16, Table 1) and xylose (2) appeared to be beyond the effective resolution range of the columns. It also seemed that maltotriose degraded in alkaline solutions as a trailing shoulder appeared in the chromatogram on storage for 12 h in 300 m*M* NaOH, which developed into a peak by 18 h.

The elution of a series of UV-absorbing samples, including phenolic compounds with low M_r (1,3–8), proteins (10,12,13) and NaPSS (9,11, 14,15), was compared with that of the carbohydrates after appropriate consideration of the delay between the UV and PAD detectors (Fig. 1). This delay was determined using tyrosine and vanillin because these compounds registered as single chromatographic peaks in both detectors. The results indicated that the elution of the UV-absorbing samples did not necessarily correspond to that of the carbohydrates. When 1 or 0.3 *M* NaOH was used as eluent, two of the phenolic monomers eluted relatively late during SEC, and this was particularly true for vanillin in 1 *M* NaOH. The elution of proteins was also delayed. In contrast, the elution of the low-M_r aromatics in 50 m*M* NaOH was quicker than that of the carbohydrates. These elution patterns indicate that only apparent M_r values could be estimated by the SEC system because of secondary separation effects.

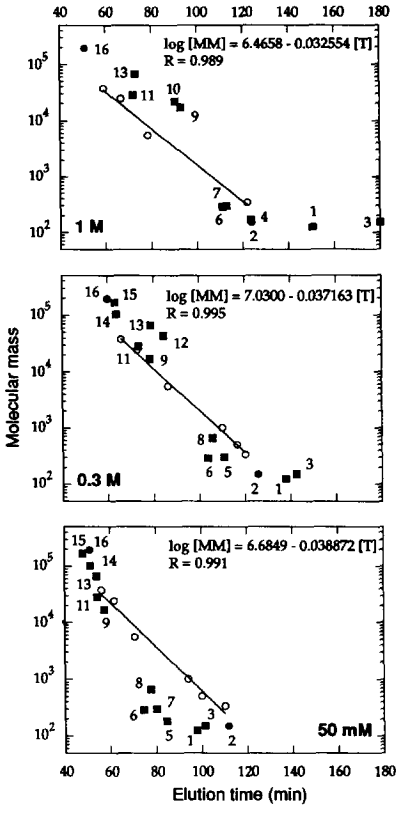

Fig. 1. Calibration of the SEC system using 1 *M*, 0.3 *M* and 50 m*M* NaOH as eluents, and carbohydrates as calibration standards (○). Solid symbols (●, ■) labelled with numbers (Table 1) represent other compounds used to evaluate SEC. The elution of carbohydrates and UV-absorbing samples was monitored using PAD and absorbance at 280 nm, respectively.

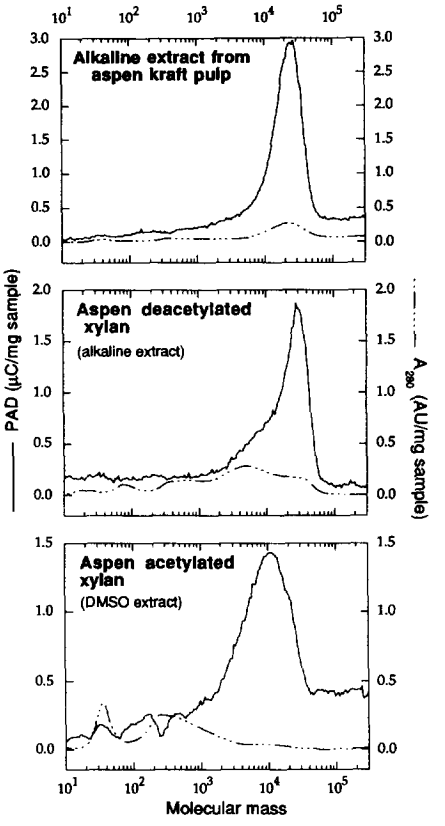

Fig. 2. SEC of carbohydrate samples extracted from aspen kraft pulp and aspen holocellulose. Elution was carried out using 1 *M* NaOH with the UV lamp set at high. The range of the ordinate for UV absorbance was chosen to provide a comparison with signals from lignin samples (Fig. 3).

3.2. Size-exclusion chromatography of carbohydrate and lignin samples

Strong alkali was required to redissolve alkaline extracts from lignocellulosic material, particularly those from softwood kraft pulp. When 1 *M* NaOH was used as the eluent for SEC, the bulk of the alkaline extract from aspen kraft pulp eluted with an M_r that was apparently between 10 000 and 40 000 (Fig. 2). This carbohydrate-containing material (Table 2) co-eluted with a small amount of UV-absorbing material. It had a smaller M_r than the alkaline extract from spruce kraft pulp (data not shown). A macromolecular peak was also observed with PAD during chromatography of the alkaline and DMSO extracts from aspen holocellulose (Fig. 2), with that of the former showing a higher M_r than that of the latter. In contrast to the extracts from kraft pulps, the M_r of the UV-absorbing material in the extracts from holocellulose was smaller than that of the material detected by PAD. The UV absorbance of all carbohydrate samples was an order of magnitude lower than that of lignin samples (Fig. 3), a difference similar to that seen between their respective lignin contents (Table 2).

When chromatography was carried out in 0.3 *M* NaOH, most of the PAD signals from the lignin samples were relatively small, particularly those in the macromolecular range (Fig. 3).

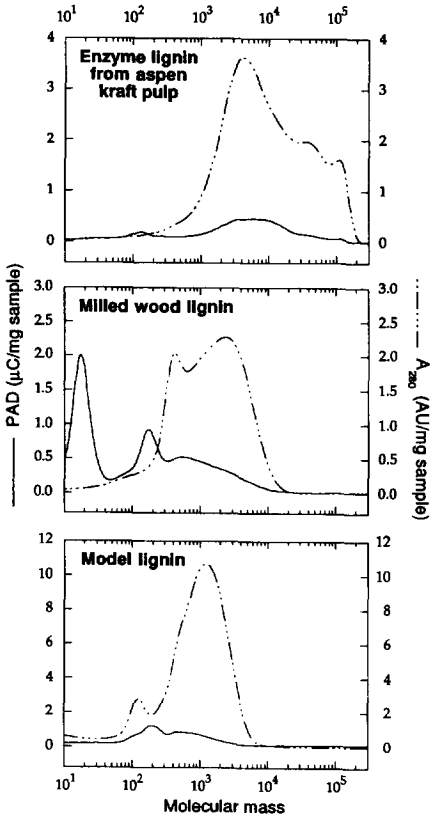

Fig. 3. SEC of lignin preparations. Elution was carried out using 0.3 *M* NaOH with the UV lamp set at low. The range of the ordinate for the PAD signals was chosen to provide a comparison with signals from carbohydrate samples (Fig. 2).

However, a strong PAD signal was seen in mill wood lignin for components having $M_r < 20$, and in certain lignin samples for components having M_r from 100 to 300. The latter signal was particularly strong in kraft lignins, where its magnitude was of the same order of magnitude as that of the polysaccharides (Fig. 4).

3.3. Effects of eluent concentration

The DMSO extracts from both kraft pulps and the lignin samples were soluble in 0.3 *M* NaOH, and the latter were also soluble in 50 m*M* NaOH. When the elution rates in the different eluent concentrations were compared, it appeared that in many cases the M_r of these samples increased with decreasing eluent concentration (Fig. 4). This was mainly true for the UV-absorbing materials. Marked differences were also observed in the PAD-positive peaks. For the DMSO extracts, the peak heights observed in 0.3 *M* NaOH were much higher than those observed in 1 *M* NaOH. For the lignin samples, the prominence of the PAD-positive component at M_r 100–300 appeared to depend greatly on the eluent concentration.

3.4. Effects of sample storage in eluent

The effects of storing the DMSO extracts, enzyme lignins and kraft lignins in 0.3 *M* NaOH were examined in some detail (Fig. 5). There was a time-dependent pattern in the changes that occurred in the elution profile of the samples, reflecting some of the effects observed with different eluent concentrations. For the DMSO extract from aspen and spruce kraft pulp, the height of their respective PAD-positive peak dropped to about 45 and 62% of the initial height after storage for 3 days in 0.3 *M* NaOH (Fig. 5). A decrease in the PAD signal was also observed with xylose on storage in 0.3 *M* NaOH (data not shown). These decreases in the PAD signals did not appear to be the result of a fouling of the PAD electrode because the peak heights were nearly identical for freshly dissolved samples. The major PAD-positive peak in the DMSO extracts also appeared to be shifting slightly toward higher M_r values, whereas the overlapping UV-absorbing peak was shifting slightly toward lower M_r. An additional change was observed in the UV chromatograms of the extract derived from aspen kraft pulp, this being an increase in the magnitude of a minor component having an M_r of 450.

Changes to the minor PAD-positive peaks in lignin preparations were also observed (Fig. 5). The magnitude of the high-M_r peaks (>1000) decreased with storage time, as did that of the M_r 150 peak in the kraft lignins. A distinct difference between the aspen and spruce kraft lignins is that a PAD-positive component, having an apparent M_r of 350, appeared with time in the former. A component with a similar M_r was also

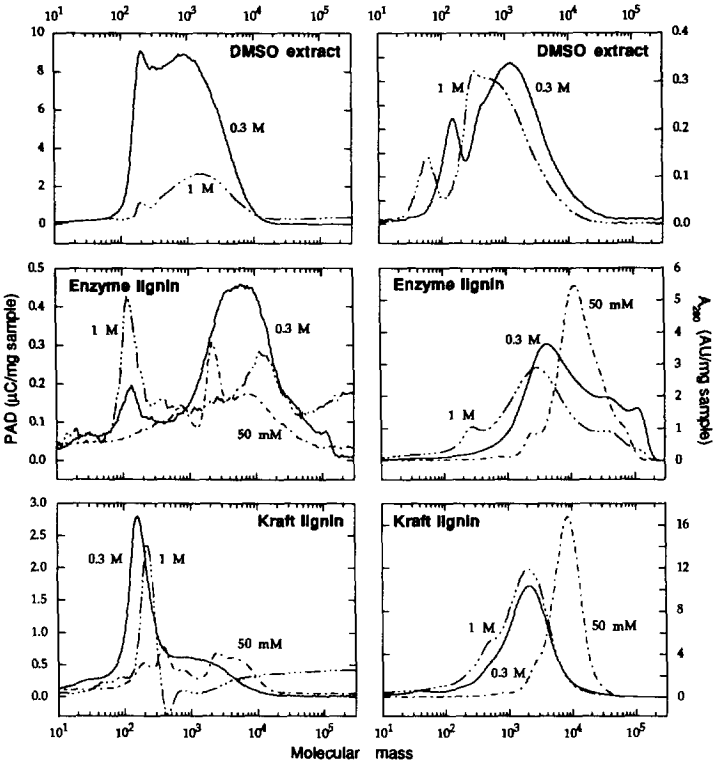

Fig. 4. Effect of eluent concentration on the SEC elution of carbohydrate and lignin samples isolated from aspen kraft pulp. Elution was carried out using 1 *M*, 0.3 *M* and 50 m*M* NaOH as eluents with the UV lamp set at low. The range for the ordinates was chosen to help show differences among minor peaks.

generated in both enzyme lignins on storage in 0.3 *M* NaOH.

The UV-absorbing components in the enzyme lignins were larger than those in the kraft lignins, with the lignins derived from spruce being larger than those from aspen. In both the enzyme and kraft lignins, there was an increase in the minor component with a low M_r of 400–450. For the enzyme lignins, there also appeared to be a decrease in the amount of material in the high-M_r range of their chromatogram.

3.5. Mixtures of lignin and carbohydrate

SEC was carried out on a mixture of the alkaline extract and enzyme lignin from aspen kraft pulp and a mixture of corresponding samples from spruce kraft pulp (Fig. 6). These chromatograms were compared with those calculated by summing the individual components. The results indicated that the chromatograms of the mixtures were well described by the sum of the chromatograms of the components. The only exception in the chromatograms shown was the UV peak observed at M_r 40 for the alkaline extract from aspen kraft pulp, a peak that was generally not seen in this sample (Fig. 2).

4. Discussion

The described SEC system is attractive for the analysis of lignin and carbohydrate samples because alkaline solutions are suited for the solubilization of these substances and for detection using PAD. The M_r estimated for wood xylans (10 000–40 000) corresponds to a degree of polymerization of 70–270, compared with

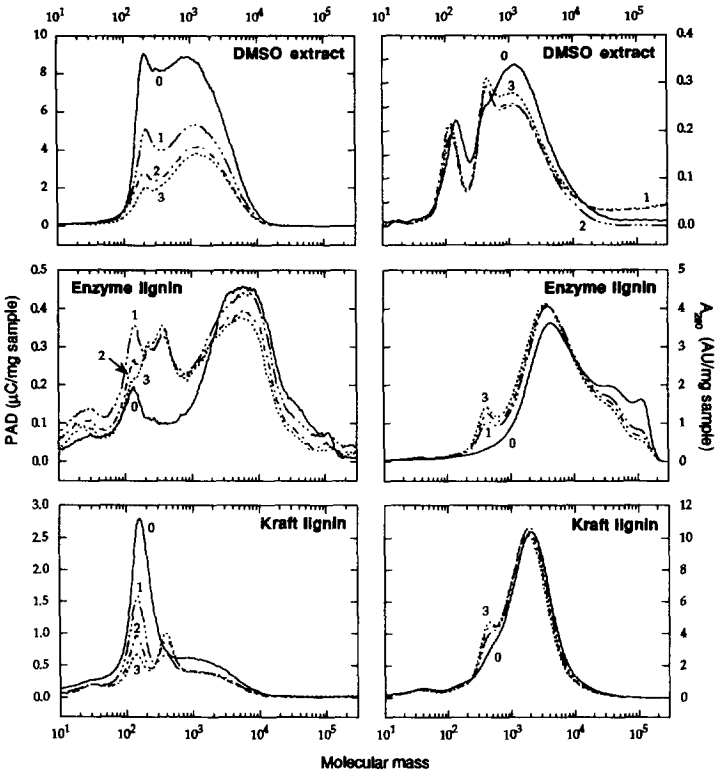

Fig. 5. Effect of sample storage in alkali on the SEC elution of carbohydrate and lignin samples isolated from aspen kraft pulp. Elution was carried out using 0.3 *M* NaOH before or after the indicated number of days of storage in the eluent, with the UV lamp set at low. The range for the ordinates was chosen to show differences among minor peaks.

reported values of >70 for softwood arabinoxylan and 200 for hardwood xylan [26]. Similarly, the M_r values estimated for kraft lignins (800–7000) agree in general with results found by other workers [1,3,16]. The dual detectors appear to offer a convenient method to monitor the elution of the lignin and carbohydrate components of a sample independently. However, caution is required in this analysis because of a number of limitations to the technique, such as the lack of absolute specificity of the detectors, the modification of the samples in the eluents and the interactions between samples and the separation matrix.

Although the UV absorbance in the carbohydrate samples (Fig. 2) may be attributable to the small amounts of lignin present (Table 2), the magnitude of the PAD signal in certain lignin samples (Figs. 3 and 4) cannot be accounted for by the small amounts of carbohydrate in these samples. Most of the PAD-positive components in lignin samples have apparent M_r values of less than 300, but a component of M_r 450 can be found in the aspen kraft lignin. Since several aromatic compounds having M_r below 400 are detected by PAD when set to monitor carbohydrates, it appears that similar low-M_r aromatic components in lignin, or other polyphenolics, may also be oxidizable and thus register in the PAD. The specificity of the dual detectors should therefore be further evaluated using known subunits of lignin, such as the stilbene and resinol type structures ($M_r \approx 300$–400) that have been found in kraft lignin [27]. It should be noted that such low-M_r compounds could be more fully analyzed using analytical HPLC or GC, with or without derivatization. Other compounds carrying sulfur functional groups may also be con-

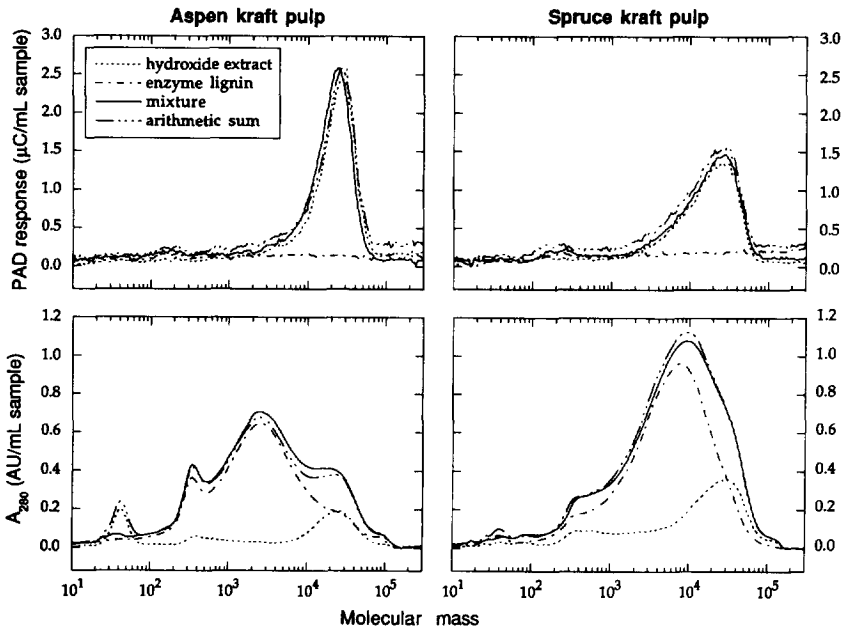

Fig. 6. SEC of the alkaline extract and the enzyme lignin isolated from kraft pulps. The elution of their mixture is compared with the arithmetic sum of the signals from the two individual components. Elution was carried out using 1 *M* NaOH with the UV lamp set at high.

tributing to the PAD signal [28], particularly since the low-M_r signal is exceptionally high in kraft lignin, which is a modified lignin solubilized from wood by hydrogen sulfide. More work on the characterization of the functional groups present in the low-M_r-fraction of the samples, and their behavior in pulsed amperometry, may help improve the specificity of detection [21,22].

When samples are more soluble in alkali, eluents having lower alkalinities seem preferable because they reduce degradation of the gold electrode in the PAD instrument and the silica window in the UV–Vis monitor. These eluents should permit the use of separation matrices that are less tolerant to extreme pH, but more tolerant to high pressures and flow-rates and thus decrease the analysis time. Lower alkalinity should also reduce the modification of samples. It was found to increase the magnitude of the PAD signal from carbohydrates, of which the observed gradual decrease (Fig. 5) was probably the result of carbohydrate modifications via the classical transformation of de Bruyn and van Ekenstein [10]. Changes can also be seen in the PAD and UV chromatograms for lignin samples on storage in 0.3 *M* NaOH, some of which might be attributed to a dissociation of macromolecular complexes or partial depolymerization of the samples [12], because low-M_r components appear with an apparent loss of high-M_r components. It is possible, however, that modifications of functional groups in lignin contribute to the apparent decrease in M_r by altering the UV absorbance of macromolecules.

Although the magnitude of the PAD signals from carbohydrates is affected by alkali concentration, the elution rates of these compounds are not greatly affected (Fig. 1). The apparently high M_r of lignin in 50 m*M* NaOH therefore seems to be the result of abnormally quick elution rates. This phenomenon may be due to an expansion of lignin macromolecules at low ionic strengths, a behavior known in such polyelectrolytes [1,13]. It could also be the result of an agglomeration of lignin at low pH, or ion exclusion that occurs when there are common charges in both the sample and the column matrix. Secondary separation mechanisms in

SEC have been extensively discussed in the literature [1,2,13,29]. They may also lead to a retardation of certain samples as a result of ion inclusion, ion exchange, hydrogen bonding or hydrophobic interaction. A high ionic strength is expected to increase adsorptive interactions between samples and the column matrix [1]. This may explain the slower elution of guaiacol and vanillin in 1 *M* NaOH as compared with that in 0.3 *M* NaOH. Another example of such adsorptive interactions is the elution of veratryl alcohol in 0.3 *M* NaOH, where the elution rate of this M_r 170 compound is less than half that of the M_r 150 xylose (data not shown).

The dual detector system described is highly sensitive for carbohydrate and lignin, with the chromatograms shown in this paper obtained using 20 μg of sample. Recently, Westermark and Gustafsson [17] used 1% LiCl in dimethylacetamide to dissolve hardwood pulp containing small amounts of lignin and carry out chromatography of 50–100 μg samples at 80°C. Although RI could be used to monitor the elution of carbohydrates, lignin was detected with lower sensitivity since it has to be monitored at 295 nm because dimethylacetamide absorbs at 280 nm. One disadvantage of the solvent was its inability to solubilize glucomannan fully [30]. Nevertheless, the method could be very useful for examining the association of lignin with cellulose.

Despite the limitations of using alkaline eluents and PAD as highlighted by the present work, the SEC system described is useful for a number of applications. Since there do not seem to be any adsorptive interactions between lignin and carbohydrate during SEC, the method could be used to characterize the apparent M_r distribution of lignin and carbohydrate macromolecules in mixtures containing these classes of compounds. For example, the materials solubilized from plant cell walls or wood pulp by enzymes could be conveniently analyzed. However, it has not been established that lignin and carbohydrate molecules of different sizes have equivalent UV and PAD signals, respectively. It would therefore seem that the described SEC system is more suited for monitoring changes that may occur in the apparent M_r distribution of the two components. These changes could be used to study and evaluate chemical or enzymic treatments of lignin- and carbohydrate-containing macromolecules [8].

5. Conclusion

There are currently few options available for selectively monitoring lignin and carbohydrate components of the same sample during SEC. In this work, we evaluated a convenient method that uses an HPLC system with UV detection and PAD; the latter has become more common in recent years owing to its applications for carbohydrate analyses. Although selectivity has not yet been achieved for molecules with apparent M_r smaller than 500, the method could be used for examining the association of lignin and carbohydrate macromolecules. Caution is required, however, in the analysis because considerations must be given to modifications of the samples in alkaline eluents and because adsorptive interactions can occur between aromatic materials and the separation matrix.

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