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On the propensity of lignin to associate: A size exclusion chromatography study with lignin derivatives isolated from different plant species

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Abstract

Despite evidence that lignin associates under both aqueous and organic media, the magnitude and nature of the underlying driving forces are still a matter of discussion. The present paper addresses this issue by examining both solution properties and size exclusion behaviour of lignins isolated from five different species of softwoods, as well as from the angiosperms *Eucalyptus globulus* and wheat straw. This investigation has used the recently described protocol for isolating enzymatic mild acidolysis lignin (EMAL), which offers lignin samples highly representative of the overall lignin present in the wood cell wall. The molecular weight distributions of these EMALs were found to be dependent upon the wood species from which they were isolated and upon the incubation conditions used prior to size exclusion chromatography. While the chromatograms of EMALs isolated from softwoods displayed a bimodal behaviour, the elution profiles of EMAL from *E. globulus* and straw were nearly unimodal. A marked tendency to dissociate prevailed under incubation at room temperature for all examined species with the exception of the straw lignin preparation; furthermore, lignin solutions incubated at 4 °C showed an associative behaviour manifested by an increase in the weight and number average molecular weights for some species. The extent of such association/dissociation, as well as the time needed for the process to reach completion, was also found to depend upon the wood species, i.e. lignins from softwoods were found to associate/dissociate to a greater extent than lignins from *E. globulus* and straw. The origin of such effects within the lignin structure is also discussed.

Keywords: Enzymatic mild acidolysis lignin; Milled wood lignin; Association; Aggregation; Lignin; Southern pine; *Pinus palustris*; Norway spruce; *Picea abies*; Douglas fir; *Pseudotsuga menziesi*; White fir; *Abies concolor*; *Pinaceae*; Redwood; *Sequoia sempervirens*; *Taxodiaceae*; *Eucalyptus globulus*; Myrtaceae; Wheat *Triticum aestivum*; Gramineae

1. Introduction

Lignin is a complex natural polymer built up of different interunit linkages such β-*O*-4′, β-β′, β-5′, β-1′, 5-5′, 4-*O*-5′, etc. (Fengel and Wegener, 1989). Furthermore, lignin is covalently linked to carbohydrates forming a lignin–carbohydrate network (Yaku et al., 1976; Lawoko et al., 2006). Most softwood lignins consist predominantly of guaiacyl (G) units, whereas the structure of hardwood lignins is more complex due to the presence of both guaiacyl (G) and syringyl (S) units (Fengel and Wegener, 1989).

While the lignin interunit linkage pattern is relatively well known, the three-dimensional structure of lignin and its ultrastructural assembly with in the carbohydrate matrix of plant cell walls remain poorly understood (Besombes and Mazeau, 2005a). Recent experimental observations have suggested the existence of a certain level

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of coherence in the ultrastructure of lignin in native woody tissues, as a probable consequence of an ordered and controlled process of assembly with the other polysaccharide components during lignin deposition (Terashima and Seguchi, 1988; Besombes and Mazeau, 2005a). Such observations are in agreements with the work of Agarwal and Atalla (1986) and Atalla and Agarwal (1985), which indicated that the aromatic rings of lignins are oriented preferentially parallel to the surface of the cell wall in spruce. Based on these studies and on the general agreement that the cell wall is formed via successive deposition of cellulose, hemicelluloses and lignin, Atalla (1998) suggested the existence of a strong associative interaction between precursors and the polysaccharide matrix as the dominant organizing influence upon lignin ultrastructure. Nevertheless, the present knowledge of the existence and significance of these associative forces during lignin deposition on the cell wall has been limited mainly to computational studies (Houtman and Atalla, 1995; Besombes and Mazeau, 2005a,b). Progress toward understanding such associative interactions have been hindered by the formidable difficulties of the field.

Most experimental evidence that lignin components tend to associate with one another have been obtained by evaluating the behavior of kraft lignin derivatives under alkaline conditions (Lindström, 1979; Sarkanen et al., 1982, 1984; Norgren et al., 2002; Bikova et al., 2004; Gidh et al., 2006). Despite the fact that kraft lignins have undergone significant structural modifications compared with the native polymer (Sarkanen et al., 1982), these efforts have been used to reveal the intricacy of lignin association, which can be further complicated by aggregation between the resulting complexes (Lindström, 1979; Norgren et al., 2002). For example, Lindström (1979) evaluated the colloidal behavior of kraft lignins and emphasized the importance of the hydrogen bonding between the carboxylic groups and ether oxygens in the association process. It is likely though that these changes could have been induced as a result of the kraft delignification processess. He also concluded that the association is thermally irreversible and that prolonged storage of the samples results in the formation of a three-dimensional network. In contrast to his findings, Sarkanen et al. (1982) have reported that the associative/dissociative process is reversible and apparently governed by nonbonded orbital interactions. Furthermore, Sarkanen et al. (1984) have also hypothesized that the associative process, occurring within kraft lignin, is stoichiometrically constrained, i.e. each associated complex possesses a locus that is respectively complementary to only one type of component.

Additional efforts to evaluate association in lignin samples which are less severely modified than kraft lignins include those of Connors et al. (1980), Sarkanen et al. (1981) and Cathala et al. (2003), which used Braun's native lignin; organosolv lignins; milled wood lignin (MWL) and synthetic lignin (DHP), respectively. By using organosolv lignins isolated under relatively mild conditions from different angiosperms, Sarkanen et al. (1981) have reported varying degrees of association, whose extent was dominated by preferential interactions between their lower and higher molecular weight components. They have also confirmed the reversibility of the association/dissociation process described for kraft lignins and have concluded that it involves at least two kinetically distinct steps. On the other hand, Cathala et al. (2003) assessed the association behavior of MWL and lignin model compounds in organic media and concluded that the association of the starting material was not the result of an equilibrium between associated and molecularly dispersed species.

Despite the various studies that point to a prevailing consensus that lignin associates in both aqueous and organic media, the magnitude and the underlying driving forces behind these processes are still a matter of discussion. Accordingly, experiments aimed at supplementing our knowledge of the lignin association process with samples highly representative of native lignin would offer new insights into these processes. Such understanding is of presumed significance as far as the process of lignin deposition in the plant cell wall is concerned. This paper addresses this topic and focuses on the associative behaviour of lignins isolated from different wood species. To overcome the limitations of structural modification and inherent low molecular weights associated with the use of kraft, organosolv and MWL, the recently developed protocol for isolating enzymatic mild acidolysis lignin (EMAL) (Wu and Argyropoulos, 2003; Guerra et al., 2006a) in high yield and purity was used for the first time to address this issue. The combination of derivatization followed by reductive cleavage (DFRC) with quantitative ^{31}P NMR (DFRC/^{31}P NMR) was also applied in an attempt to better understand the lignin association process.

2. Results and discussion

Recent progress toward isolating lignin preparations from woody plant material has shown that the combined application of cellulolytic enzymes followed by mild acidolysis affords lignin samples (EMAL) more representative of the overall lignin present in milled-wood (Guerra et al., 2006a,b). Since mild acidolysis can liberate lignin from lignin–carbohydrate complexes, known to limit lignin isolation in high yields, it can be combined with low severity of milling, facilitating the isolation of a less modified lignin in high yields from milled-wood (Wu and Argyropoulos, 2003; Guerra et al., 2006a,b). Low intensity milling refers to the milling conditions optimized and described in our previous publications (Guerra et al., 2006b). Moreover, it has been recently suggested that the EMAL isolation procedure offers significant opportunities for studying lignin association phenomena (Guerra et al., 2006a). This suggestion was based on the molecular weight distribution of EMAL isolated from Norway spruce, which showed an unusual highly polydisperse behavior when compared to other lignin preparations isolated from the same batch of milled-wood (Guerra et al., 2006a). Consequently, we embarked on adopting the combination of enzymatic hydrolysis and mild acidolysis with low severity milling to isolate lignin (EMAL) from different wood species. Such isolated lignins were used in the present work to investigate lignin association. Norway spruce, Southern pine, Douglas fir, white fir and redwood were used to evaluate the aforementioned effects on lignins from different species of softwoods, while *Eucalyptus globulus* and wheat straw were selected as sources of lignin from hardwood and non-woody species, respectively. More details about the EMAL isolation procedure as well as the yields, purities and structures of the examined EMALs isolated from different wood species can be found in our earlier publications (Guerra et al., 2006a,b). It is important to mention at the onset of this work that the yields of the EMALs described in the present work were about 3–5 times greater than the corresponding MWL isolated from the same batch of milled-wood.

2.1. Molecular weight distribution of lignins from different woody and non-woody species

Native, underivatized, lignin samples are usually sparingly soluble in tetrahydrofuran commonly used for size exclusion chromatography (SEC) with the EMAL samples studied here being of no exception, regardless of whether they were acetylated in pyridine or phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Under these circumstances, we embarked in adopting the recently described procedure for lignin that involves derivatization in acetyl bromide. It has been reported that acetobromination represents a facile and rapid alternative to the complete solubilization of sparingly soluble lignin samples, while still allowing for an accurate analysis (Guerra et al., 2006a). By dissolving a lignin sample in neat acetyl bromide diluted with glacial acetic acid (8:92, v/v), the primary alcohol and phenolic hydroxyl groups are acetylated, while the benzylic α-hydroxyls are displaced by bromide (Lu and Ralph, 1998). Similarly, benzyl aryl ethers are quantitatively cleaved to yield aryl acetates and acetylated α-bromide products (Lu and Ralph, 1998). The concerted effect of acetylation when coupled with the polarity induced by the selective α-bromination caused every lignin sample examined so far to become highly soluble in THF, allowing rapid SEC analyses. Comparison between acetobromination and acetylation with acetic anhydride/pyridine has shown only minor differences in the UV responses and elution profiles, facts supportive of the viability of using acetobromination as derivatization technique to sparingly soluble lignin (Guerra et al., 2006a).

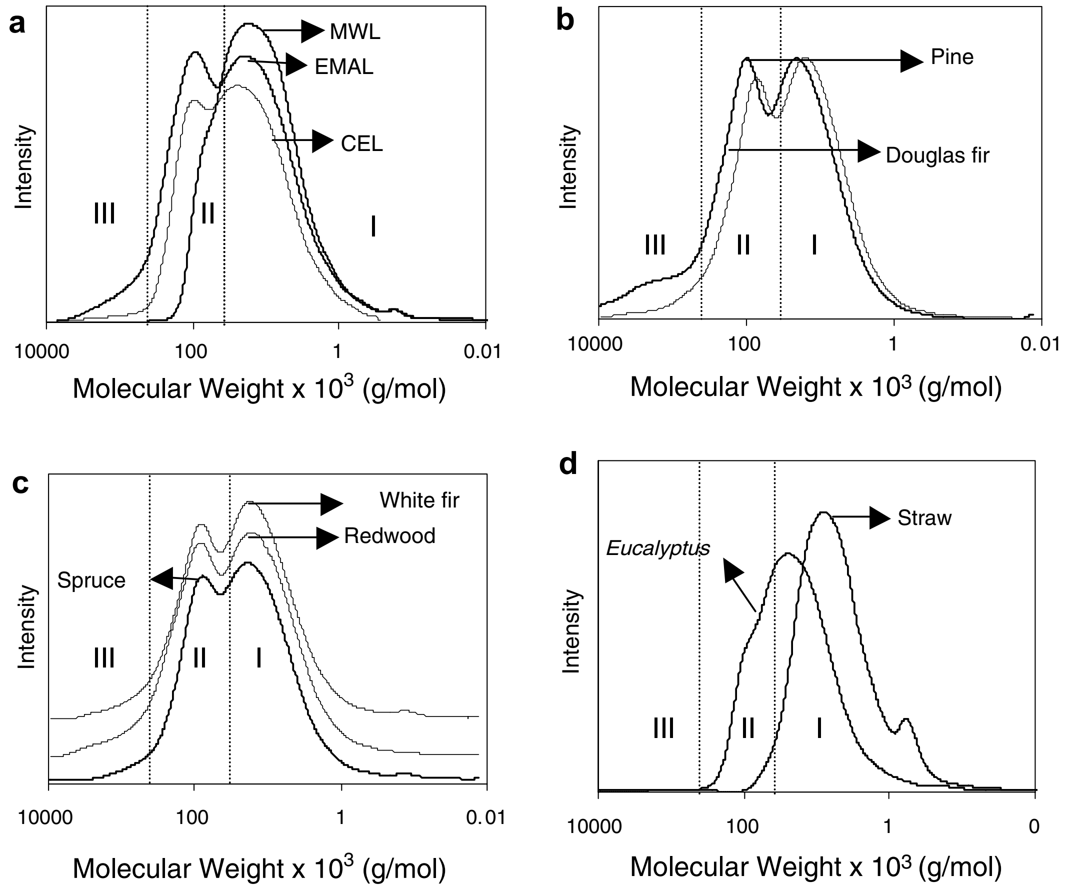

Fig. 1. Typical size exclusion chromatograms (SEC) of lignin samples isolated from the same batch of milled Norway spruce (a); SEC of lignins isolated from softwoods (b,c) and SEC of lignins isolated from *Eucalyptus globulus* and wheat straw (d). All SEC were obtained after acetobromination.

Fig. 1a shows a typical size exclusion distribution obtained for acetobrominated EMAL derivatives from Norway spruce softwood. For comparative purposes, the SEC chromatograms obtained from milled wood lignin (MWL) and cellulolytic enzyme lignin (CEL) isolated from the same batch of milled-wood and acetobrominated as described above are also included. To ensure that the derivatization technique (acetobromination) would not lead to a misinterpretation of the SEC data, all samples were derivatized under the same conditions, immediately dissolved in THF after acetobromination and analyzed by size exclusion chromatography using THF as the mobile phase with UV detection at 280 nm. Invariably unimodal elution profiles were obtained for MWLs isolated from all woody species examined in this effort, while the chromatograms of acetobrominated EMAL and CEL displayed a bimodal behavior (Guerra et al., 2006b). In addition to the high- and low-molecular weight peaks, a high molecular weight fraction (albeit in low abundance) was apparent in the chromatograms of EMAL from softwoods. To facilitate data interpretation, the chromatograms in Fig. 1 were arbitrarily divided into three zones. The lowest molecular size zone corresponds to material with estimated molecular weights ranging from oligomers to 50×10^3 g mol^{-1}. The central zone (II) includes fragments higher than 50×10^3 g mol^{-1} but lower than 500×10^3 g mol^{-1}, while the high molecular weight fraction, extending from 500×10^3 g mol^{-1} to several million, appears as a discernible tail (zone III) in the chromatograms of Fig. 1. Based on the data of Fig. 1a it becomes apparent that EMAL derivatives are significantly enriched in such higher molecular weight species, which appeared in significantly lower abundance in CEL and were completely absent in MWL. Based on these findings and in the yields previously reported for EMALs (Guerra et al., 2006a,b), one should note that the EMAL isolation procedure offers significant opportunities for studying lignin association phenomena with lignin samples more representative of the total lignin present in the wood.

The question that emerges at present is whether the material that causes formation of the aforementioned high molecular weight fractions (zones II and III) consists of covalently bonded lignin, lignin–lignin associated species, or both. The molecular weight distribution of freshly acetobrominated EMALs was found to be strongly dependent upon the woody species from which they were isolated. As anticipated, highly polydisperse behavior is apparent in the SEC chromatograms of the acetobrominated EMAL derivatives as far as their molecular weight distributions are concerned (Fig. 1b–d). The elution profiles, however, were found to be different amongst the EMAL isolated from different woody species. While the chromatograms of acetobrominated EMAL isolated from softwoods (Fig. 1b and c) displayed a bimodal behaviour, the chromatogram of EMAL isolated from *E. globulus* (Fig. 1d) showed only a low-M_w peak and a small shoulder extending over 100×10^3 g mol^{-1} (zone II). Moreover, the high molecular weight fraction (zone III), extending into about 500×10^3 g mol^{-1}, was apparent in the chromatograms of EMALs from softwoods. Such a fraction, however, was absent in the lignin from *E. globulus*. Fig. 1d also illustrates the nearly unimodal elution profiles obtained for EMAL from wheat straw. In addition to the absence of the high molecular weight fractions (zones II and III), a small peak due to species with low molecular weight is apparent in the chromatogram of wheat straw (Fig. 1d).

2.2. The effects of incubation on the molecular weight distribution of the acetobrominated lignin derivatives

The data in Fig. 2 show the effect of incubation on the molecular weight distribution of acetobrominated EMALs. Incubation in the present work refers to the aging of the acetobrominated lignins conducted at 2.0 g L^{-1} in THF at 25 ± 3 °C without stirring. As illustrated in Fig. 2a, for the EMAL isolated from pine, the three different molecular weight zones were clearly observed only in the chromatograms of acetobrominated EMALs from softwoods carried

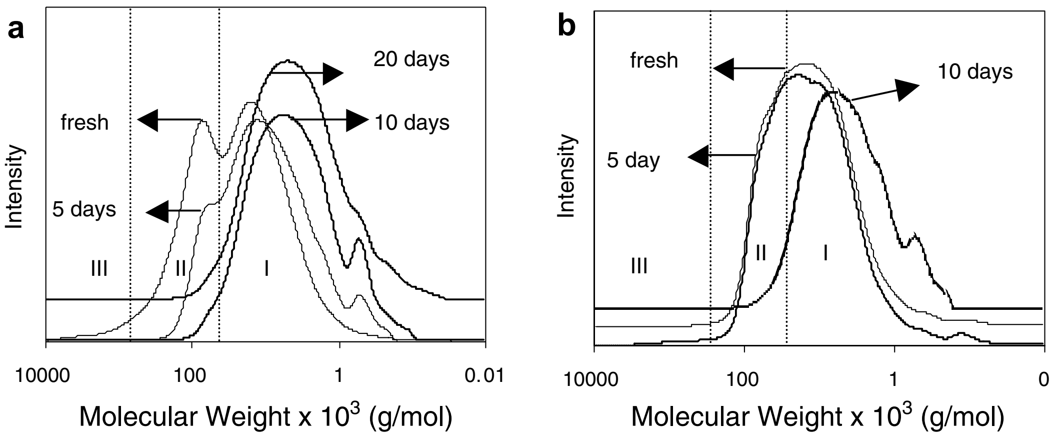

Fig. 2. Effects of incubation at room temperature on the molecular weight distribution of EMAL from Southern pine (a) and *Eucalyptus globulus* (b). All the lignins were previously completely acetobrominated.

out on freshly prepared lignin solutions (analyzed immediately after derivatization). After 5 days of incubation, the species displaying high hydrodynamic volumes appearing in the aforementioned tail (zone III) disappeared, while the high-M_w peak (zone II) became a shoulder, which was no longer observed after 10 days. Furthermore, the low-M_w peak (zone I) was found to shift toward longer retention times (after 10 days of incubation), demonstrating the accumulation of species with smaller hydrodynamic volumes. In addition, one well-resolved peak appearing at longer retention times (i.e. lower molecular mass) was discernible after 10 days (Fig. 2a). This peak was found to be due to a significant increase in the proportion of dimers. This finding was deduced directly from the calibration curve and confirmed by co-elution of 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-methoxy-phenyl)-propanediol-1,3 with an EMAL sample (SEC not shown). After longer incubation times, however, the bimodal elution pattern observed for acetobrominated EMALs from softwoods was replaced by a single broad elution peak and the absorption due to dimers was no longer resolved. This could be rationalized on the basis of the accumulation of lignin oligomers released during incubation. This in turn replaces the bimodal elution pattern by a broad peak eluting at the low molecular weight zone (zone I). This finding is supported by the work of Evtuguin et al. (1999), who evaluated lignin molecular weight by electrospray ionization mass spectrometry (ESI-MS) and distinguished lignin oligomers ranging from trimers to octamers as prominent fragments in the structure of lignins from spruce and *E. globulus*. Furthermore, it should be noted that the above data have recently been further confirmed in our laboratory. This was done by using an 18 angle light scattering detector, in a static mode (i.e. without using size exclusion columns) that allowed for accurate Zimm plots to be derived for identical lignin samples examined in this paper. This effort has confirmed that our findings are real and not artifacts induced by the fractional preferential elution of material through the size exclusion columns. This will be the subject of an additional communication in the near future.

The observed dependency of molecular weight distribution upon incubation time is not totally surprising when viewed in the light of the earlier conclusions of Sarkanen et al. (1981), where a pronounced reduction in the apparent weight-average molecular weight has been reported for organosolv, synthetic, kraft, and Braun's native lignins during incubation in alkaline conditions. It is of significance, however, that such notable incubation effects were not observed for the size exclusion chromatograms of acetobrominated EMAL from *E. globulus* (Fig. 2b). The lignin derivative from *E. globulus* displayed an almost unimodal elution profile for freshly prepared samples. After 10 days of incubation, the peak eluting in zone I shifted toward longer retention times (lower molecular weights) and the peak due to the accumulation of oligomeric species also appeared. Longer incubation times, however, had negligible effects on the molecular weight distribution of acetobrominated EMAL from *E. globulus*. Strikingly, the molecular weight distribution of acetobrominated EMAL isolated from wheat straw was found not to be affected by the incubation time and the chromatogram of freshly prepared solutions (Fig. 1d) completely overlapped with those of samples incubated for up to 10 days.

In an effort to ensure that the aforementioned effects on the molecular weight distribution of acetobrominated EMALs were not due to degradation of covalent linkages within lignin, the incubation of such EMALs derivatives was also monitored by quantitative ^{31}P NMR. ^{31}P NMR spectroscopy is a reliable method to accurately determine the amounts of various hydroxyl groups within the lignin macromolecule (Argyropoulos, 1994; Granata and Argyropoulos, 1995; Akim et al., 2001). Such hydroxyl groups can be detected and quantified after phosphitylating lignin with 2-chloro-1,3,2-dioxaphospholane or 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. In order to analyze the same samples by SEC and ^{31}P NMR spectroscopy, the EMALs were first acetobrominated and dissolved in THF. After examining their SEC profiles the THF was immediately removed under nitrogen and the remaining lignin was dissolved in pyridine/$CDCl_3$ and phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. As mentioned before, by dissolving a lignin sample in neat acetyl bromide diluted with glacial acetic acid (acetobromination), the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α-hydroxyls are displaced by bromide (Lu and Ralph, 1998). Such acetylation of the hydroxyl groups precludes them from being detected by ^{31}P NMR spectroscopic analysis, since they can no longer be phosphitylated. As a result, no signals due to phenolic or aliphatic hydroxyl groups were detected in the ^{31}P NMR spectra (spectra not shown) of the freshly prepared lignin solutions. For the purposes of the present investigation, one should note that absence of such signals in the spectra of the starting acetobrominated lignins facilitates the monitoring of the incubation process. Accordingly, aryl ether linkage cleavage or oxidative reactions taking place within these lignin preparations would be promptly recognized by the appearance of the corresponding signal in the ^{31}P NMR of such samples after incubation. When the aryl ether linkages are cleaved, the corresponding phenolic hydroxyls released can be quantified by ^{31}P NMR spectroscopy, while oxidation reactions may result in oxidative fragmentation of the lignin macromolecule with concomitant creation of carboxylic acid groups, which are also detectable by ^{31}P NMR (Argyropoulos, 1994). However, under the incubation conditions evaluated in this work no such reactions were apparent and the OH and COOH contents of the incubated EMALs derivatives were found to be remarkably constant throughout the incubation period extending into 30 days (spectra not shown). This finding supports the contention that the observed effects of the incubation on the molecular weight distribution of the acetobrominated EMALs isolated from different softwoods and *E. globulus* are due to the disruption of physical association rather than the cleavage of covalent bonds within the lignin macromolecules. The fact that the molecular weight distribution of EMAL derivatives from wheat straw has not been affected by the incubation process further supports the validity of our conclusions, pointing to the absence of artifacts that may be caused by the hydrolysis or oxidation of covalent bonds during the incubation of acetobrominated lignins in THF.

The accompanying change in the apparent weight-average molecular weight of the acetobrominated lignins isolated from the different woody and monocotyledon species as a function of incubation time is shown in Table 1 and Fig. 3. Noteworthy in Fig. 3a is the significant difference in the dissociation behaviour among softwoods (illustrated by spruce and pine), *E. globulus* and wheat straw. In an attempt to compare such dissociation behavior in numerical terms, the M_w reduction factor, *F*, reported in Table 1 was calculated from the relation between the M_w of the EMAL freshly acetobrominated and after complete dissociation in THF. For the purposes of calculating *F*, we ensured that when the molecular weight distribution became stable the dissociation process had been completed. This was done by examining the SEC chromatograms of samples incubated for 20–30 days in the presence of LiCl. The latter eliminates potential residual association by the shielding of dipole effects (Cathala et al., 2003). No alteration in the molecular weight distribution in the presence of LiCl was apparent, supporting our contention of complete dissociation. The data of Fig. 3a and Table 1 show that the apparent weight-average molecular weight for acetobrominated lignins isolated from various softwoods decreased as a whole by *F* factors ranging between 4.9 and 8.9, while the *F* factor was found to be less than 3.0 for *E. globulus* and 1 (no alteration) for wheat straw. This finding indicates that lignins from different woody species have different propensity to associate in THF.

The time taken for the dissociation process to reach completion was clearly dependent upon the woody species from which the lignins were isolated. As shown in Fig. 3 and Table 1, while the M_w of white fir and redwood (Fig. 3b) became stable after 10 days of incubation, Southern pine and spruce (Fig. 3a) were found to take 20 and 30 days, respectively, to dissociate completely under these conditions. The Douglas fir curve, which was omitted to prevent data over-crowding in Fig. 3b, would have overlapped with that of the white fir curve. Moreover, a considerable variation was observed in the M_w reduction factors *F* calculated for the softwoods. These were found to

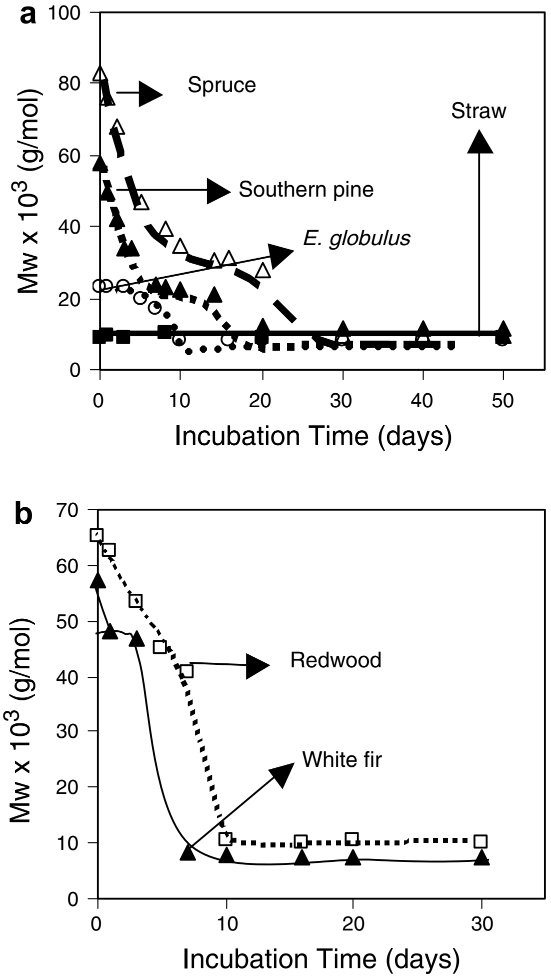

Fig. 3. Effects of incubation at room temperature on the apparent weight-average molecular weight (M_w) of lignins isolated from different woody species and wheat straw: (a) Norway spruce (open triangles), Southern pine (black-filled triangles), *Eucalyptus globulus* (open circles) and wheat straw (black-filled squares) and (b) redwood (open squares) and white fir (black-filled triangles). All the lignins were previously completely acetobrominated.

Table 1
Effect of dissociation on apparent weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity (*D*) of acetobrominated EMALs isolated from different woody species and wheat straw

EMAL derivatives from	Time (days)[a]	M_w (g mol^{-1})	M_n (g mol^{-1})	*D*	M_w reduction factor ($M_{w\ initial}/M_{w\ final}$)
Norway spruce	0[b]	83,200	10,000	8.3	8.9
	30	9350	3350	2.8	
Redwood	0[b]	65,200	7760	8.4	6.5
	10	10,000	3700	2.7	
Douglas fir	0[b]	49,500	7700	6.4	4.9
	10	10,100	3740	2.7	
White fir	0[b]	57,000	7700	7.4	7.6
	10	7500	2800	2.7	
Southern pine	0[b]	57,600	9760	5.9	5.0
	20	11,400	4200	2.7	
Eucalyptus globulus	0[b]	23,400	6500	3.6	2.9
	10	8100	2890	2.8	
Wheat straw	0[b]	10,100	2730	3.7	1
	10	10,090	2650	3.8	

[a] Dissociation under these conditions was complete within time specified.

[b] Analysed immediately after acetobromination ($t = 0$ h).

decrease in the following order: spruce (8.9), white fir (7.6), redwood (6.5), pine (5.0), and Douglas fir (4.9). These data are supportive of the existence of different propensities to associate even amongst lignins from different species of softwood. This finding is in good agreement with our recent conclusions that different softwood species offer different lignin yield, structure and molecular weight when isolated with the same method (Guerra et al., 2006b).

Our work has shown that incubation in THF at 4 °C induced some (albeit small) association between components of lignins from all evaluated woody species with the exception of lignins from spruce and straw. As illustrated in Fig. 4, both the extent and pattern of the associative process appears to depend upon the woody or non-woody species. For example, the extent of association observed for lignin from redwood was smaller than those for the other evaluated wood species. After 1 day of incubation at 4 °C, the apparent weight-average molecular weights for acetobrominated lignins isolated from *E. globulus*, redwood, white fir and Douglas fir as a whole increased by factors of 1.3, 1.1, 1.3, and 1.5, respectively. The behavior observed for lignin from spruce at 4 °C, however, was quite unexpected. As shown in Fig. 4, no evidence of association has been observed for such lignin for up to 16 days of incubation at 4 °C. This may indicate that for the lignin from this species the initial sample was already fully associated under the conditions evaluated in the present work. Comparison of the M_w reduction factor F among different woody species (Table 1) is supportive of this assumption, since F was much higher for lignin from spruce (8.9) than for any other wood species (1–7.6).

Incubation at 25 °C of those samples previously associated at 4 °C (cf. Fig. 4) allowed subsequent dissociation to occur and the apparent weight-average molecular weights after prolonged incubation were identical with those for samples fully dissociated without prior association at 4 °C (cf. Fig. 3). This finding indicates that the observed association phenomenon in THF is reversible. On the other hand, however, incubation at 4 °C of samples previously fully dissociated at 25 °C has not been followed by reassociation (data not shown). Sarkanen et al. (1981) investigated the association/dissociation phenomena among organosolv lignin components in alkaline system and obtained results similar to the present data. As such they concluded that the dissociation of the associated complexes involves at least two kinetically distinguishable steps: a rapid equilibrium between associated complexes and dissociated components and a slow change, possibly conformational in nature, during which the dissociated species undergo conversion to forms for which reassociation is not directly accessible. Moreover, it should be noted that the dissociation of polymer aggregates is usually much more rapid than their association (Doty et al., 1947; Strauss et al., 1956; Sarkanen et al., 1981). As far as lignin is concerned, the time taken for such a theoretical reassociation to occur is completely unknown. In this manner, possible re-association of fully dissociated species after much longer incubation time at 4 °C cannot be ruled out but has not been observed in our hands. Additional incubations at higher lignin concentrations and at ambient temperatures may further supplement the present experiments.

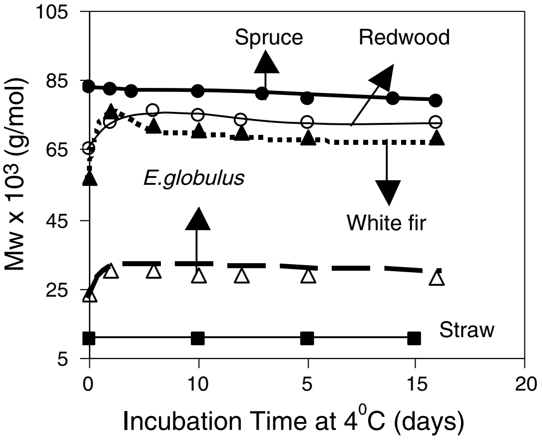

Fig. 4. Effects of incubation at 4 °C on the apparent weight-average molecular weight (M_w) of lignins isolated from spruce (black-filled circles), redwood (open circles), white fir (black-filled triangles), *E. globulus* (open triangles) and straw (black-filled squares). All the lignins were previously completely acetobrominated.

2.3. Relationship between structural characteristics and association behavior

As far as the lignin association phenomenon is concerned, it is significant to note that different efforts have been conducted in order to better understand the operational driving forces (Lindström, 1979; Sarkanen et al., 1982, 1984; Norgren et al., 2002; Bikova et al., 2004; Gidh et al., 2006). Despite these efforts, the reasons for such association processes are still a matter of discussion. For example, Cathala et al. (2003) investigated the molecular weight distribution of MWL and DHP and concluded that the original bimodal distribution pattern of such samples in DMF is not caused by an equilibrium process of molecularly dispersed and associated species. They also concluded that association effects cannot be driven exclusively by molar mass and speculated that it might be affected by different degrees of branching and cross-linking. It was also speculated that such association effects could be the result of irregular distribution of OH, CO and COOH groups in lignin leading to different molecule-to-molecule interactions. In contrast to their findings, Sarkanen et al. (1981, 1982, 1984) investigated association phenomena by using organosolv, synthetic, kraft, and Braun's native lignins in aqueous and organic media and concluded that association is reversible and governed by nonbonded orbital interactions. While Lindström (1979) suggested that long-range van der Waal forces play an important role together with intermolecular and intramolecular associations of the carboxylic groups, Bikova et al. (2004) attributed association to the ionization of functional groups, formation of intermediates and oxidation of lignin. Our results, as we will show, are supportive that two different forces, at least, govern lignin association in THF.

In an effort to examine for the presence of any correlation between the observed molecular weight dissociation effects and specific functional groups within the lignin macromolecules, the underivatized EMALs (natural EMALs without acetobromination) isolated from the different woody species and wheat straw were analyzed by quantitative ^{31}P NMR spectroscopic analysis (Argyropoulos, 1994; Granata and Argyropoulos, 1995; Akim et al., 2001). Furthermore, the condensed and uncondensed β-aryl ether linkages were evaluated by the combination of DFRC with ^{31}P NMR (Tohmura and Argyropoulos, 2001; Guerra et al., 2006a).

Fig. 5 shows the M_w reduction factor F due to dissociation plotted as a function of the different functional groups within the lignin macromolecule. The data indicates that there is no clear correlation between the extent of dissociation and total amount of hydroxyl groups (Fig. 5a), carboxylic acids (b) and condensed phenolic groups within lignin (c). Extensive, multiple linear regression efforts did not provide statistically significant models ($P \leqslant 0.1$) capable of predicting the F factor as a function of these functional groups. The lack of correlation between the extension of dissociation and the total amount of hydroxyl and carboxylic acids groups (Fig. 5a and b) indicates that ionization and conjugation of various functional groups cannot explain the large differences observed in the dissociation behavior of lignins from different woody species and straw in THF (Figs. 2 and 3). This is in contrast to the report of Bikova et al. (2004), who studied the incubation of kraft lignin in alkaline media. These authors observed that the ionization of various lignin functional groups is followed by the formation of the corresponding intermediates and its oxidation (Bikova et al., 2004). Based on these observations, the same authors concluded that ionization and conjugation govern both elution behaviour and the molecular weight distribution patterns of kraft lignins in aqueous media. The observed differences may reside in the nature of the lignins examined between those of Bikova et al. (2004) (kraft lignins) and ours (acetobrominated EMALs). The actual disparity may also indicate that the association forces that operate in aqueous and organic media are not essentially the same. Furthermore, it is worth emphasizing that the EMAL lignins evaluated in the present work have been previously acetobrominated and dissolved in THF, which preclude ionization effects and

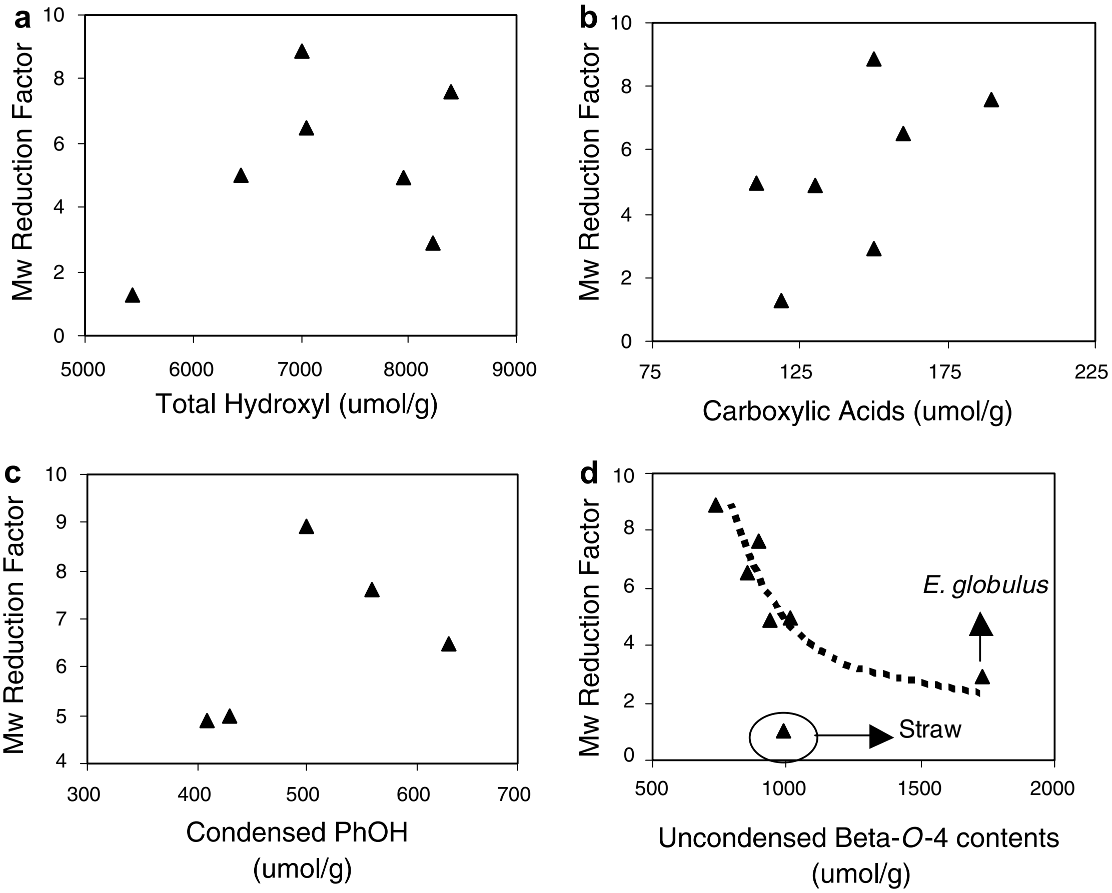

Fig. 5. Molecular weight reduction factor ($M_{\mathrm{w\ initial}}/M_{\mathrm{w\ final}}$) as a function of the total amount of hydroxyl (a), carboxylic acids (b), condensed phenolic hydroxyl groups (c) and uncondensed β-aryl ether contents (d).

inter and intramolecular hydrogen bonds to occur. Furthermore, it should also be kept in mind that kraft lignin components in 0.10 M NaOH are polyanionic in nature and therefore they may behave differently from acetobrominated lignin derivatives in THF.

Additional efforts to better understand and clarify the lignin association process included the acquisition of SEC chromatograms under conditions of multi-wave UV/VIS detection. These experiments were aimed at further clarifying the effect of lignin's chemical composition distribution (CCD) curves, which are reflected in the concentration profile of specific functional groups and subunits via their molecular weight distributions (Bikova et al., 2004). This method, which is based on acquiring SEC chromatograms at different wavelengths, allows the differentiation of some specific chemical transformations occurring in the high- and low-M_w peaks during incubation in THF (Bikova et al., 2004). During our work, any possible chemical changes in the high- and low-M_w peaks were monitored at 280, 320 and 350 nm. These wavelengths may provide information on changes in the concentration of methoxylated phenolic rings (280 nm), aromatic carboxylic acids and α-carbonyl groups (320 nm) as well as carbonyl- and double-bond-conjugated phenols (350) (Lin, 1982; Bogolitsyn and Lindberg, 1986).

This approach was driven by the hypothesis that the irregular distribution of the aforementioned groups might lead to different molecular interactions and consequently different associative behaviour (Bikova et al., 2004; Cathala et al., 2003). However, as far as the CCD curves are concerned, no significant differences were observed in the elution profiles of the EMALs when detected at different wavelengths (Fig. 6). The similarities among the chromatograms of the freshly acetobrominated EMALs detected at 280, 320 and 350 nm (Fig. 6a) indicate a uniform distribution of methoxylated phenols, carbonyl- and double-bond-conjugated phenols over the whole molecular weight range for the examined lignin samples. Furthermore, incubation in THF was found not to disturb the uniform distribution of such functional groups (Fig. 6b).

The total amount of uncondensed β-aryl ether bonds present within the underivatized lignins was also correlated with the extent of dissociation factor *F* (Fig. 5d). The data obtained by DFRC/^{31}P NMR shows that *E. globulus* contains much more uncondensed β-aryl-ether structures than any of the softwoods evaluated. This is clearly reflected in the value of 1730 μmol g^{-1} of uncondensed β-aryl-ethers obtained for *E. globulus*, which is 62.2% of the total amount of β-aryl ether linkages present within such lignin, considering that the total amount of β-aryl-ether structures for this sample was found to be 2780 μmol g^{-1} (Guerra et al., 2006b). Amongst softwoods, Southern pine was found to contain slightly higher contents of such linkages (1015 μmol g^{-1}), while spruce and redwood were seen to contain the lowest values (740 and 851 μmol g^{-1}, respectively). These data corroborate previous efforts showing that lignin isolated from *E. globulus* is more linear (Evtuguin et al., 2001; Guerra et al., 2006b) than lignins from softwoods (Adler, 1977; Guerra et al., 2006b), which are expected to be more branched. Consequently, the data of Fig. 5d indicate that the total amount of uncondensed β-aryl ether linkages correlates with the extent of dissociation when one compares softwoods and *E. globulus*, i.e. the higher the contents of uncondensed β-aryl ether linkages are, the lower the extent of dissociation observed during the incubation of the lignin. This finding is indicative that the observed effects may have their origin, at least in part, in chain entanglements operating within different macromolecules. Such effects are anticipated to manifest themselves differently in lignins with different degrees of branching (branched in softwood lignin versus more linear in *E. globulus* lignin).

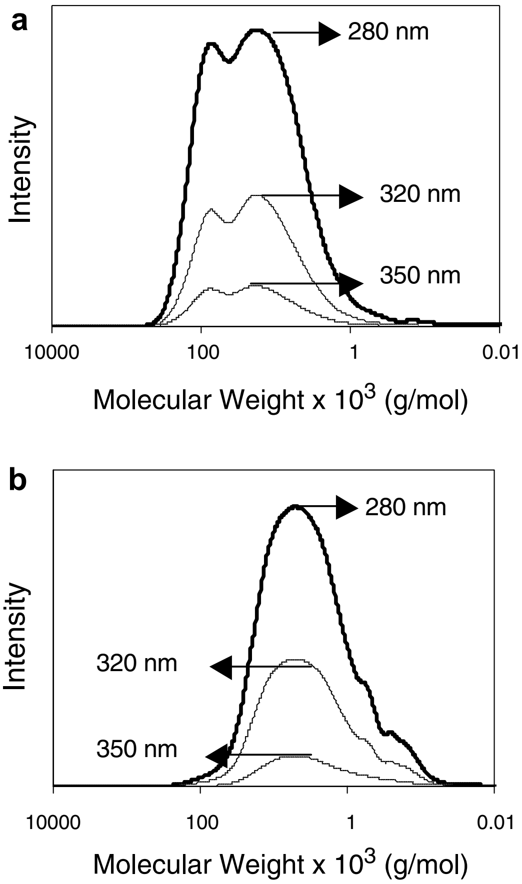

Fig. 6. Combined UV chromatograms of freshly acetobrominated (a) and dissociated lignins (b) detected in different wavelength.

The dissociation behavior, however, for the lignin from wheat straw (circled in Fig. 5d), did not match those observed for neither *E. globulus* nor any of the examined softwood lignins. Wheat straw lignin, despite the fact that it contained lower amounts of uncondensed β-aryl ether linkages than *E. globulus*, was found to associate less in THF than *E. globulus*. While this is certainly a fact requiring further investigation, it is not totally surprising since lignins from straw are known to be significantly different from those of softwoods and hardwoods (Higuchi et al., 1967; Ralph et al., 1992; Crestini and Argyropoulos, 1997). Earlier efforts have shown that wheat straw contains high amounts of *p*-hydroxyphenyl units, most of them due to the presence of esterified *p*-coumaric acid (Higuchi et al., 1967; Ralph et al., 1992, 1994). Such *p*-coumarates residues are bound to the γ-position of the lignin side-chain, while C-5 substituted phenolic units are selectively esterified (Crestini and Argyropoulos, 1997; Ralph et al., 1992, 2001). To date the effects of such unusual amounts of *p*-coumarate residues on the association behaviour of lignin from wheat straw are unresolved.

Though the exact conformation that represents proposed entanglement is not yet known, different models have been used to describe the nature of chain entanglements in bulk systems (Si et al., 2005). Since such phenomena probably involve looping of at least one polymer backbone around another (Tonelli, 1970) with physical forces keeping the chains in contact, it seems reasonable that dissociation of entangled chains may differ under statical and dynamic incubation conditions. Such an assumption was attempted in the present work through evaluation of the effects of stirring on the apparent weight-average molecular weights of lignins from different woody species (Fig. 7). More specifically, the dissociation behavior of lignin solutions incubated without stirring (static) were compared to the profile of the same samples incubated under vigorous magnetic stirring (dynamic). As mentioned before, both the extension of disassociation and the time needed for such a process to reach completion were found to be dependent upon the woody species, regardless of whether the incubation was performed under static or dynamic conditions. The effects of stirring, however, are apparent as far as the dissociation profiles are concerned (Fig. 7). While the apparent weight-average molecular weight, after complete dissociation, was found not to be dependent on stirring, the dissociation process of both softwoods and *E. globulus* was faster when the incubations were conducted under vigorous stirring. For example, the dissociation of redwood (*Sequoia sempervirens*) without stirring was completed after 10 days, while it needed only 5 days to reach completion under stirring. Despite the anticipated effect of the woody species on the time needed for the dissociation to reach completion, the dissociation behavior of redwood (*Sequoia sempervirens*) may be considered as typical for all examined softwoods; i.e., the dissociation was faster under stirring. This finding corroborates the data shown in Fig. 5d, reinforcing the argument that chain entanglements, may also operate in the underlying mechanisms of lignin association. An alternative possibility that may also operate during the stirring experiments is that stirring perturbs any possible equilibrium that may occur between various lignin components enhancing their effective rate of diffusion away from one another and/or reducing the probability of re-association. Additional experiments to further clarify these effects are currently in progress.

Intermolecular orbital interactions, dominated by those of the HOMO–LUMO type, have been invoked as being responsible for the lignin association phenomena in acetylated lignins (Sarkanen et al., 1981). If such interactions govern the associative behavior of lignin in organic solvents then addition of iodine to the solution of acetobrominated lignin dissolved in THF should diminish its propensity to associate. This assumption is based on the ability of iodine to form iodine-aromatic hydrocarbon complexes with different compounds characterized by intense absorption peaks in the 280–400 nm region (Benesi and Hildebrand, 1949). The presence of iodine in the lignin derivatives samples should theoretically dislocate the aromatic rings from each other effectively eliminating such associative interactions. It is also important to note at this point that the iodination work has been carried out in the absence of light and overall carefully selected experimental

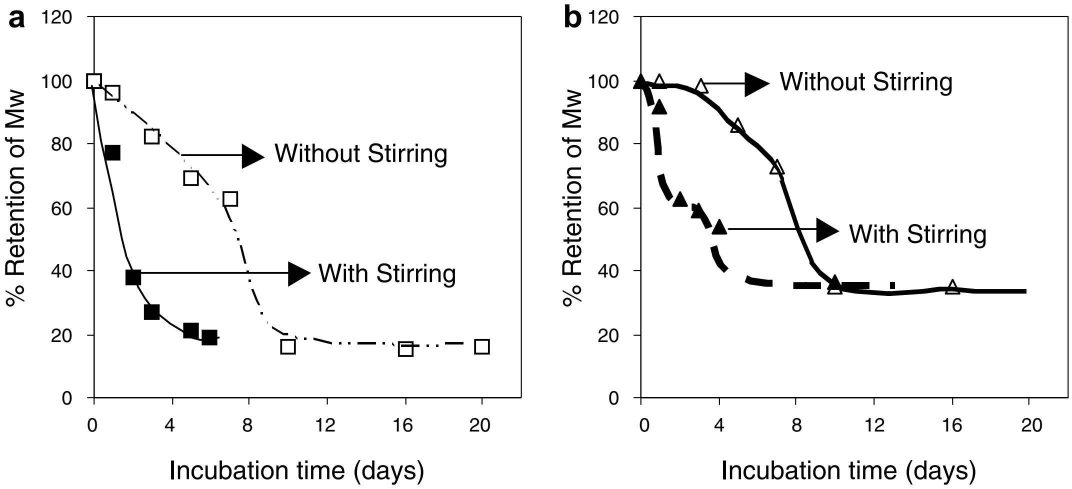

Fig. 7. Effect of stirring on the apparent weight-average molecular weight of lignin isolated from redwood (a) and *E. globulus* (b). Incubation performed without (open symbols) and under vigorous stirring (black-filled symbols). All the lignins were previously completely acetobrominated.

conditions (absence of weak bases) in order to preclude iodination of the aromatic rings.

Fig. 8a shows that when different amounts of molecular iodine were added to solutions containing freshly acetobrominated EMAL from Norway spruce, significant changes in the SEC chromatograms occurred. Furthermore, Fig. 8b shows a consistent reduction in the apparent weight-average molecular weights as a function of the amount of iodine for all different lignins examined. The similarities between the effects of iodine (Fig. 8a) and incubation (Fig. 2a) on the molecular weight distribution of the acetobrominated lignins are noteworthy. As shown in Fig. 8a the addition of 1 mmol of iodine per mmol of lignin reduces the amount of high molecular weight species eluting in zone III of the chromatogram. As observed before, the high-M_w peak decreases and becomes a shoulder when the amount of iodine was increased up to 4 mmol per mmol of lignin. Higher amounts of iodine, however, were found to precipitate in THF prohibiting the process to reach completion with further addition of iodine. In accordance with most of our previous data, fresh solutions of softwood lignins were affected more by addition of iodine than the lignin from *E. globulus* and wheat straw (Fig. 8b).

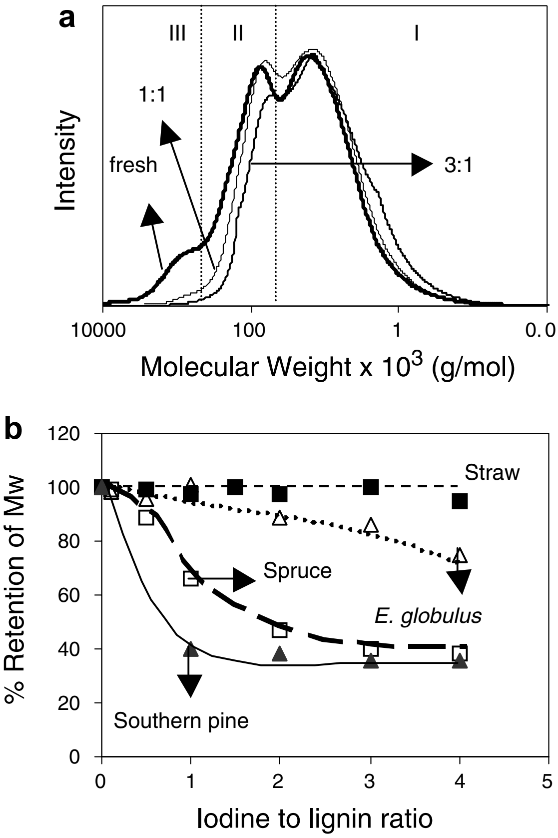

Fig. 8. Effects of different amounts of iodine (mmol of iodine per mmol of lignin) on the molecular weight distribution (a) and on the apparent weight-average molecular weight (b) of lignins from different woody species and wheat straw. All the lignins were previously completely acetobrominated.

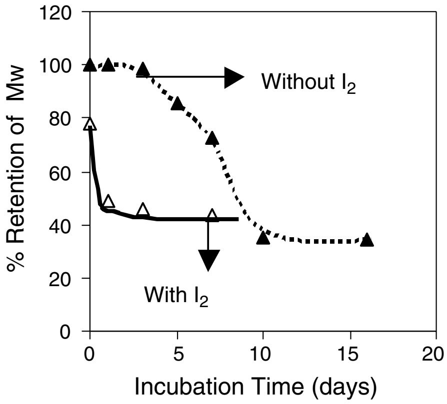

Fig. 9. Effects of incubation in the presence of iodine (4 mmol per mmol of lignin) on the apparent weight-average molecular weight (M_w) of lignins isolated from *E. globulus*. All the lignins were previously acetobrominated.

Fig. 9 illustrates the synergistic effect of iodination and incubation on the apparent weight-average molecular weight of lignin isolated from *E. globulus*. As anticipated, the addition of iodine (4 mmol of iodine per mmol of lignin) into the fresh solution of acetobrominated lignin reduced the apparent weight-average molecular weight of this lignin preparation by at least 20%. A comparison of the data of Fig. 9 with the data of Fig. 7b show that at least 1.5 days are needed to reach a similar degree of reduction in the M_w under stirring in the absence of iodine. In the absence of rapid stirring it took more than 7 days to attain the same percentage of molecular weight reduction as observed by adding iodine into the fresh solution (Fig. 9). Furthermore, the lignin derivative solution when incubated in the presence of iodine was found to dissociate rather rapidly. As shown in Fig. 9, the M_w of acetobrominated EMAL from *E. globulus* decreased by about 32% after 1 day of incubation in the presence of iodine. Further incubation, however, had negligible effect on the M_w, which stabilized after 1 days of incubation in iodine. Fig. 9 also shows that the dissociation profile in the presence of iodine is different from that in its absence, i.e.; the dissociation was found to be faster in the presence of iodine. This finding may indicate that non-bonded intermolecular orbital interactions prevail over entanglements as far as the operating association forces are concerned. After disrupting such intermolecular interactions, chain entanglement can be easily dislocated and the dissociation process is completed easier.

3. Concluding remarks

Our laboratory has recently developed a new method for lignin isolation termed enzymatic mild acidolysis lignin (EMAL). The fact that this procedure affords lignins of hight purity and yields to be obtained has allowed, for the first time, for a thorough insight into the propensity of lignin to associate. By using acetobrominated EMAL samples that were completely soluble in tetrahydrofuran, a series of plant species were examined. Our data are indicative of evidence that such lignin derivatives associate in fresh THF solutions and the magnitude of the de-association process changes from species to species.

4. Experimental

4.1. Isolation of EMALs, MWLs and CELs

Enzymatic mild acidolysis lignins (EMALs) and milled wood lignin (MWL) were isolated from Norway spruce (*Picea abies*), Douglas fir (*Pseudotsuga menziesi*), white fir (*Abies concolor*), redwood (*Sequoia sempervirens*), eucalyptus (*Eucalyptus globulus*), Southern pine (*Pinus palustris*) and wheat straw according to the procedures described before (Guerra et al., 2006a,b; Björkman, 1956, 1957). Cellulolytic enzyme lignin (CEL) was isolated from the insoluble material obtained after isolating MWL according to the method of Chang et al. (1975) modified by Ikeda et al. (2002). Both preparations were purified as described elsewhere (Björkman, 1956). More details about such isolation procedures as well as the yields, purities and structures of the examined lignins isolated from different species can be found in our previous publications (Guerra et al., 2006a,b).

4.2. Acetobromination derivatization procedure

Acetobromination was carried out following the procedure described elsewhere (Lu and Ralph, 1998; Guerra et al., 2006a,b). Specifically, a lignin sample (∼10 mg) was added into a solution of AcBr:AcOH (2.5 mL, 8:52 v/v); followed by stirring for 2 h at 50 °C, the solvent was evaporated in vacuum (using a high vacuum pump and a cold trap) and the resulting residue was immediately dissolved in THF (5 mL) and subjected to size exclusion chromatographic analyses (SEC).

4.3. Incubation of acetobrominated EMALs in THF

After complete acetobromination, an aliquot (4.5 mL) of each solution of lignin derivative in THF was removed and split equally into three different vials. One vial was sealed to avoid evaporation and kept at room temperature (25 ± 3 °C) without stirring for periods of up to 30 days, while the second was sealed and maintained at 4 °C. Samples were used to evaluate the effects of incubation at room temperature and 4 °C, respectively, on the molecular weight distributions. To evaluate the effects of stirring, the third aliquot of each lignin sample was kept at room temperature under vigorous magnetic stirring (5000 rpm).

4.4. Iodination of the acetobrominated EMALs

The acetobrominated lignins dissolved in THF ($2\ \mathrm{mg\ mL^{-1}}$) were iodinated (Benesi and Hildebrand, 1949), with an iodine solution (Reagent grade, Fisher Chemical Co.) containing $12.69\ \mathrm{mg\ I_2\ mL^{-1}}$. The amount of added solution was calculated to add a range of iodine onto the lignin varying from 0 to 4 mmol per mmol of lignin present in the final solution. The mixtures were stirred for 5 min and then injected into the chromatographic system without treatment. All solutions of lignin in iodine were freshly prepared 5 min prior to size exclusion measurements.

4.5. Size exclusion chromatography

SEC of EMAL samples were performed on a size exclusion chromatographic system (Waters system) equipped with a UV detector set at 280 nm. Analyses were carried out at 40 °C using THF as eluent at a flow rate of $0.44\ \mathrm{mL\ min^{-1}}$. Aliquots (120 μL) of each sample dissolved in THF ($2\ \mathrm{mg\ mL^{-1}}$), were injected into HR5E and HR 1 columns (Waters) connected in series. The HR5E column specifications allow for molecular weights up to $4 \times 10^6\ \mathrm{g\ mol^{-1}}$ to be reliably detected. The SEC system was calibrated with polystyrene standards in the molecular weight range of $890–1.86 \times 10^6\ \mathrm{g\ mol^{-1}}$ and Millenium 32 GPC software (Waters) was used for data processing. To determine the chemical composition distribution (CCD) curves the same conditions were applied, except that the UV detector was set at 320 and 350 nm.

4.6. Quantitative ^{31}P nuclear magnetic resonance

Quantitative ^{31}P NMR spectra of lignin preparations were obtained using published procedures (Argyropoulos, 1994; Granata and Argyropoulos, 1995). To improve resolution, a delay time of 5 s was used and a total of 256 scans were acquired.

4.7. DFRC/^{31}PNMR

The DFRC was performed as described by Lu and Ralph (1998). The precise amounts of the lignin and precautions due to the ensuing ^{31}P NMR steps were nearly identical to those reported elsewhere (Tohmura and Argyropoulos, 2001; Guerra et al., 2006a).

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