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The use of fluorescent probes in the characterization of lignin: the distribution, by energy, of fluorophores in *Eucalyptus grandis* lignin

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Abstract

Four fluorescent probes (biphenyl, naphthalene, pyrene and phenanthrene) were used to map the energy distribution of the structural units present in lignin fragments from *Eucalyptus grandis* wood. This distribution shows that these fragments present two regions with a high concentration of chromophores, one between 418 and 385 kJ/mol, and the other below 322 kJ/mol. When this lignin was treated with $NaBH_4$, the two more intense regions occurs between 418 and 346 kJ/mol, followed by a significant increase in the concentration of chromophores in almost all the studied energy range. Lifetime distributions present a bimodal pattern, with two typical peak lifetime values, the first of 1.36 ± 0.17 ns, with a relative amplitude above 80%, and the second of 8.48 ± 2.32 ns, for both species, with some fluctuation for different λ_{em}. The synchronous spectra indicates, for this lignin, at least three broad spectral envelopes, with a large superposition of the emission maxima. The results indicate the existence of at least three most representative fluorophores, most probably due to biphenyl, coniferyl alcohol and stilbene structures, with varying substituents. The majority of the fluorescence complexity of this lignin seems to be associated with the inhomogeneous emission decay kinetics associated with ground state heterogeneity, due to the complex mixture of the different fluorophores, on which are superimposed different distributions of environments.

Keywords: *Eucalyptus grandis* lignin; Characterization; Fluorescence lifetime

1. Introduction

Fluorescent probes have been used for the investigation of complex systems [1]. For example, its use in the characterization of petroleum gives a good view of the distribution of non-aliphatic components in this complex mixtures [2]. In this case, the logarithm of the concentration of aromatic and polar components is proportional to the singlet energy of the probes [2]. These results give useful information not readily obtained by other methods and increase the knowledge of the photochemical environmental degradation of petroleum.

The fluorescence of lignins has been investigated by a number of researchers [3–12]. It is known, e.g., that all the luminescence observable for lignin (in wood as in liquid solutions) at the ambient temperature is due to fluorescence [7,13]. Much of the accumulated knowledge is due to studies, in which lignin is compared with models designed to mimic the lignin in wood [8,11,12]. Fluorescent emission in lignin has been attributed to aromatic structures such as conjugated carbonyl, biphenyl, phenylcoumarone and stilbene groups [11,12]. In spite of this, no work on the distribution of chromophores, nor on the nature and photophysical properties of the fluorescent species present in lignin has been done.

In this work we have studied the energy distribution of the fluorophores units present in lignin fragments from *Eucalyptus grandis* wood. This was done using fluorescent probes to evaluate the energy distribution of the fragments. These fragments were recovered from the spent liquor of a peroxyformic acid pulping process [14]. *E. grandis* and related hybrids are the major wood sources for production of chemical pulp in Brazil. Time-resolved and stationary fluorescence measurements were performed with the object of obtaining a better understanding of the fluorophores present in this liquor. The study was also performed on sodium borohydride reduced lignin (REL) by comparison with the non-reduced lignin (EL), in order to determine the influence

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of the presence of carbonyl chromophores on the energy distribution and the fluorescence properties of this mixture.

2. Experimental

2.1. General

Lignin from *E. grandis* wood was obtained from peroxyformic acid cooking effluent. The methodology of isolation is described in a previous work, in which it was also characterized [14]. The fluorescent probes were biphenyl, naphthalene, pyrene and phenanthrene, all suitable to be used as photosensitizer. They were purchased from J.T. Baker, and were used as received. Solutions of ca. 40–50 mg l^{-1} of these probes were prepared using spectroscopic grade toluene as solvent (VETEC).

To prepare the solutions of lignin, it was firstly diluted in a portion of 1,4-dioxane (Merck). After that, the volume was completed with toluene until the final composition was toluene with 20% 1,4-dioxane and 30 mg l^{-1} in lignin. Aliquots of that solution were used to prepare the solutions for the quenching experiments. In this case, 2 ml of the solution of the probe plus a variable volume of the lignin solution were added to a 10 ml volumetric flask, and the difference was completed with toluene. All solvents were spectrophotometric grade.

Reduced lignin was prepared by the reaction of lignin and sodium borohydride in aqueous alkaline solution. The mass of $NaBH_4$ was five times the mass of lignin, and the mixture was left to react for 24 h. After this, the solution was acidified, using HCl, and the reduced lignin was separated by centrifugation, washed several times with lightly acidified water and dried before use. A CARY 1-E ultraviolet–visible spectrophotometer was used for electronic absorption measurements.

2.2. Lifetime suppression and lifetime measurements

Time-resolved fluorescence measurements were performed using an FL900CDT fluorescence lifetime spectrometer from Edinburgh Analytical Instruments. This equipment was employed with a front face accessory. An nF900 flash lamp filled with hydrogen gas was used for the excitation of the samples. For data analysis, a software of the equipment was used to perform lifetime distribution analysis (LEVEL2). These experiments were done using a timebase of 100 ns, and at least 1000 counts per experiment.

The solutions containing the fluorescent probe and different concentrations of lignin were bubbled with analytical grade nitrogen (White Martins) before each measurement.

2.3. Steady-state fluorescence experiments

Steady-state fluorescence experiments were performed using an SPEX FLUOROLOG 1681 fluorescence spectrometer. Excitation and emission spectra were performed for lignin solutions. When necessary, excitation and emission spectra were also measured for the fluorescent probes. All the spectra were instrument and solvent corrected. The measurements were made between 26 and 28°C.

3. Results and discussion

3.1. Steady-state fluorescence of lignin and sodium borohydride reduced lignin

It is well established that the luminescence of emission of the wood is due to lignin fluorescence [7]. Fig. 1 shows emission spectra of lignin (EL) for different excitation wavelengths. As expected, the fluorescence emission spectrum of lignin varied in intensity and in shape with the excitation

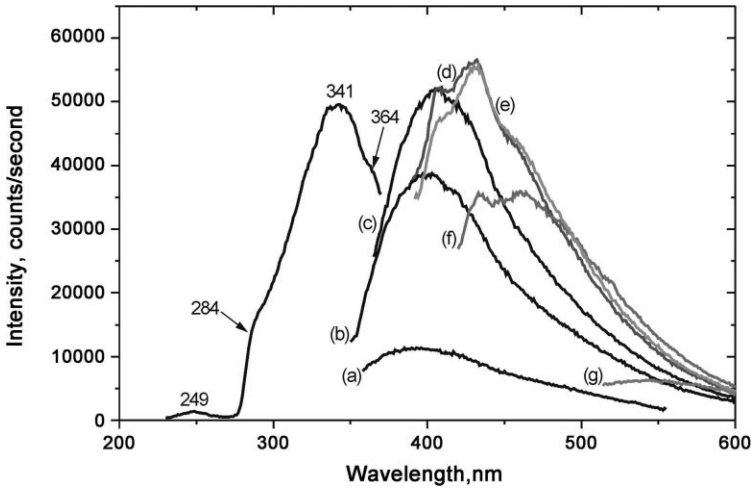

Fig. 1. Fluorescence spectra of EL in toluene/dioxan solutions (see Section 2): excitation spectrum $\lambda_{em} = 400$ nm; emission spectra: (a) $\lambda_{exc} = 284$ nm; (b) $\lambda_{exc} = 320$ nm; (c) $\lambda_{exc} = 336$ nm; (d) $\lambda_{exc} = 357$ nm; (e) $\lambda_{exc} = 364$ nm; (f) $\lambda_{exc} = 390$ nm; (g) $\lambda_{exc} = 474$ nm.

wavelength. This evidences a complex structure, characteristic of these composites: different "active" species must contribute to its fluorescent behavior. Lundquist et al. [3,4,12] using steady-state fluorescence measurements verified that the presence of stilbene structures and aryl-conjugated carbonyl groups must exert a strong influence on the lignin fluorescence. On the other hand, the occurrence of different behavior for a same species in concentrated or solid lignin may be due to different physical environments, as proposed by Castellan et al. [11]. However, in this case, in dilute fluid solutions this is not likely and the observed distribution is more likely due to variable substitution of the same basic fluorophores. The proximity between the potential fluorophores and different functional groups present in the lignin fragments also must impose variations in the behavior of these fluorophores.

The excitation wavelengths of the spectra shown in Fig. 1 were very close to the wavelengths used to excite the probes in the lifetime suppression experiments. It is pointed out that above 400 nm the shape of the emission spectrum begins to change with a trend toward structuration of the band.

The treatment of lignin with sodium borohydride results in an increase in the fluorescence intensity. This is probably due to the reduction of carbonyl groups present in lignin, which are essentially non-fluorescent, resulting in the formation of more fluorescent reduced products [5]. Fig. 2 shows a comparison of the fluorescence spectra of the lignin and reduced lignin.

The study of lifetime suppression of different fluorescent probes induced by lignin can indicate the energy distribution of the different chromophores present in lignin. This can help us to have a better understanding of the lignin photophysics. Some of them should be responsible by the fluorescent behavior of the lignins. In this study, all the fluorescent probes were suppressed by lignin. Because of technical limitation, it was not possible to extend this study to regions with singlet energies higher than 418 kJ/mol, associated to non-conjugated aromatic structures.

The observed lifetime quenching can be considered to be mainly due to the exothermic energy transfer from the probe to aromatic structures, which are part of the complex lignin polymer.

Fig. 3 shows a typical Stern–Volmer plot of the probe fluorescence quenching by lignin, in which the values of k (the decay rate constants of the probe) are plotted against the concentration of lignin. Each value of k is the result of at least five experiments, and the correlation between the points was always higher than 0.98.

Table 1 shows the data obtained for the quenching induced by lignin for the different probes. We considered that the quenching rate constants, k_q, calculated for the probes lifetime, are diffusion limited, and that the quenching is basically through energy transfer. In order to obtain bimolecular rate constants, from these data, the molecular weight of lignin must be known. However, this composite is highly disperse [14]. This problem can be overcome using the ratio between the values of the diffusion rate constant ($k_{diff} = 3.1 \times 10^{10}\,l\,mol^{-1}\,s^{-1}$), calculated in toluene [16], with our experimental k_q. This ratio gives us the mole fraction of the chromophores present in the energy distribution [2].

Below 418 kJ/mol, it is expected that the chromophores in lignin be associated to polyaromatic structures. As can be seen in Table 1, unreduced lignin (EL), has a concentration of 4.03 mol/kg of chromophores with singlet energies below 418 kJ/mol. The next value, 1.26 mol/kg, corresponds to chromophores with singlet energies below 385 kJ/mol. The difference between these two values gives us the concentration of chromophores with singlet energy between 418 and 385 kJ/mol, named Region I in the distribution. Region I have the major concentration of these components in the distribution: 2.77 mol/kg. This result suggests that nearly 70% of the quenchers in the unreduced lignin possess singlet energy in this range. Since a large content of biphenyl-like structures is expected for these lignin fragments, is possible that these structures are acting as fluorescence quenchers in this region. Biphenyl structures have been qualitatively

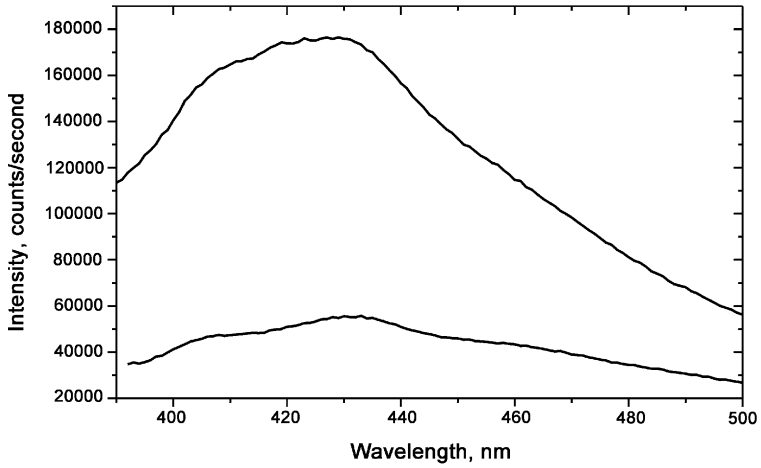

Fig. 2. Fluorescence emission spectra of EL and REL ($\lambda_{exc} = 336$ nm) EL in toluene/dioxan solutions (see Section 2) at a same concentration (44 mg l^{-1}).

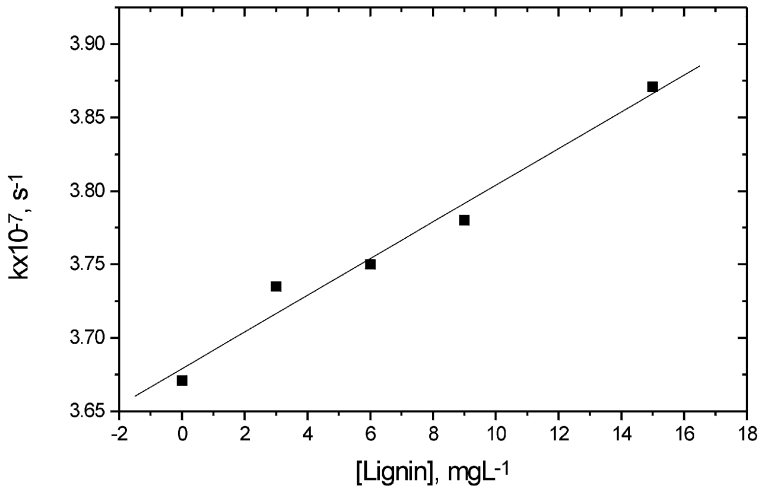

Fig. 3. Stern–Volmer of the quenching of biphenyl by lignin ($R = 0.9865$).

Table 1
Parameters obtained from lifetime suppression of fluorescent probes, promoted by EL and REL

Probe	E_s (kJ/mol) [15]	λ_{exc} (nm)	λ_{em} (nm)	k_q (EL) ($l\,kg^{-1}\,s^{-1}$)	k_q (REL) ($l\,kg^{-1}\,s^{-1}$)	k_{diff}/k_q (EL) (kg/mol)	k_{diff}/k_q (REL) (kg/mol)
Biphenyl	418	284	320	1.25×10^{11}	2.53×10^{11}	4.03	8.16
Naphthalene	385	310	355	3.89×10^{10}	1.22×10^{11}	1.26	3.94
Phenanthrene	346	334	364	3.69×10^{10}	1.97×10^{10}	1.19	0.64
Pyrene	322	334	390	3.49×10^{10}	8.90×10^{9}	1.13	0.29

identified by ^{13}C and ^{31}P NMR spectroscopy for these lignin fragments [14].

Using the same procedure, it is possible to assign the Region II, with components having singlet energies between naphthalene ($E_s = 385\,kJ/mol$), and phenanthrene ($E_s = 346\,kJ/mol$), Region III, with components having singlet energies between phenanthrene and pyrene (322 kJ/mol), and Region IV, with components having singlet energies below 322 kJ/mol. Regions II and III have the lowest concentrations of fluorophores in the distribution, respectively, 0.07 and 0.06 mol/kg, corresponding to 2% of the total amount. The Region IV has also a large concentration of chromophores (1.13 mol/kg), corresponding to 28% of the total amount present in this lignin. These results are resumed in Table 2.

For the reduced lignin, a different pattern was obtained. A considerable increase in the concentration of chromophores in the two first regions was observed. Probably with the reduction of the carbonyl groups with borohydride, new chromophores became available in the lignin fragments. For the first region, this represented an increase of 52%. For the second region, an increase from 0.07 to 3.30 mol/kg, giving a positive variation of 4600%. The next region also showed an expressive increase: 480%. Below 322 kJ/mol an inverse trend was observed: the concentration of quenchers diminished from 1.13 to 0.29 mol/kg, representing a reduction of 74%. In this region for EL some carbonylated structures must be expected, like as quinonoid and other carbonyl-conjugated structures, which are usually present in oxidized lignins [17,18].

Table 2
Distribution, by energy range, of the fluorescence quenchers present in EL and REL

Energy range (kJ/mol)	Region	Mole fraction (kg/mol)	
		EL	REL
$385 < E \leq 418$	I	2.77	4.22
$346 < E \leq 385$	II	0.07	3.30
$322 < E \leq 346$	III	0.06	0.35
$E \leq 322$	IV	1.13	0.29

These fluorescent probes are, in principle, quenched by any lignin component with a singlet energy below that of the probe. They will therefore "see" both fluorescent and non-fluorescent components. Thus, a correlation must be expected between the fraction of the components, which are fluorescent, and the total concentration of components with a specified energy.

The observed profile for this distribution is in a good agreement with that observed for the emission spectra of this lignin: a displacement to the blue with a significant increase in the intensity is observed for the fluorescence spectrum of the lignin after treatment with $NaBH_4$ (see Fig. 2). After this treatment most of the carbonyl fluorophores were reduced, as can be shown in Fig. 4.

It was observed that the presence of carbonyl derivatives leads to fluorescence suppression in lignin [5]. The

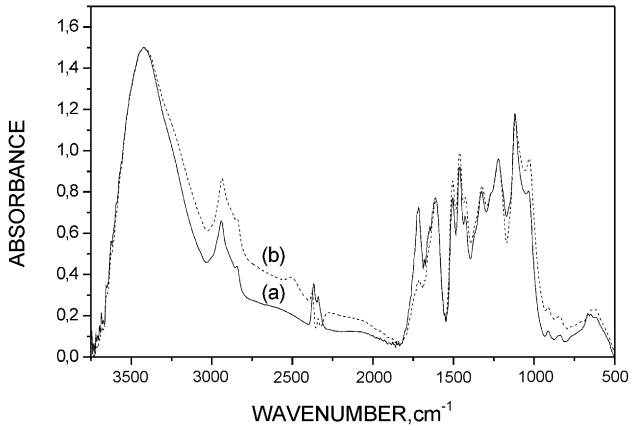

Fig. 4. Infrared spectra for (a) unreduced lignin and (b) reduced lignin.

reaction of lignin with sodium borohidride should bring an increase in the fluorescence intensity due to the formation of fluorophores. The increase of fluorescence in Region I, observed after the reduction with borohidride, might indicate that the new fluorophores formed by reduction could be biphenyl structures. Region II could be attributed to stilbene structures. Fig. 5 shows a better view of the distribution.

The profile of the energy distribution agrees with the proposition of that after the treatment with $NaBH_4$, a great part of the carbonylated components was reduced, and new fluorescence quenchers possessing higher values of singlet energy are acting in other regions of the energy distribution. Because carbonyl derivatives induce the fluorescence suppression of lignins [5], its reduction must result in an increase in the fluorescence intensity, with some shift to higher energies, due to the participation of components before quenched by carbonyl groups in its neighborhood.

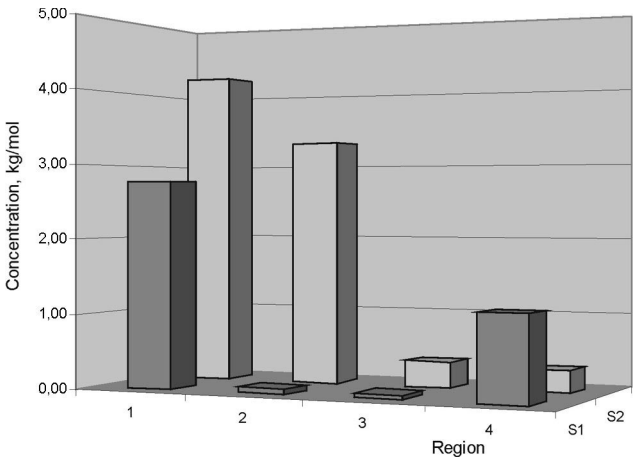

Fig. 5. Histogram showing the energy distribution (kJ/mol) of the fluorophores of the *E. grandis* lignin fragments. S1: unreduced lignin; S2: reduced lignin.

3.2. Lifetime distribution

The lifetime distribution for EL and REL was measured. The typical profile of their fluorescence lifetime distribution is reported in Fig. 6. The analysis of the histogram indicates the presence of at least two important groups of fluorophores and bimodal profiles for fluorescence lifetime distribution were obtained for different excitation wavelengths for both samples. The first peak of 1.36 ± 0.17 ns has a relative weight above 80% for the two lignin samples. Castellan [10] has reported a lifetime of 1.6 ns for a phenolic biphenyl lignin model in methanol solution, very close to this value. The second peak possesses a lifetime of 8.48 ± 2.32 ns, and must be due to a different type of fluorophore.

The reasonable invariance of the peak values indicates that the fluorescence kinetics of these lignin fragments is weakly dependent on the excitation wavelength. Apparently, the same fluorophores contribute to the evolution in time of the emission spectra, whatever the excitation wavelength was used. The same trend was also observed in other solvents. For example, in acetonitrile/water 4:1, the peak values found were 1.33 ± 0.21 ns (rel. wt. 80–85%) and 9.10 ± 0.95 ns, respectively, for the first and second peaks [19].

Castellan and Davidson [7] found biexponential decay for the time-resolved fluorescence of Abies wood reduced by $NaBH_4$. According to their interpretation, the decays indicates that there are two emitting species, which may be due to two different chemical species or to one species present in different physical environments [7].

3.3. Synchronous spectra

The synchronous spectra were recorded for EL and REL samples (Fig. 7). The spectrum of the reduced lignin is less structured than the one of unreduced lignin, but both spectra present the same trend. The minor structuration noted for the REL emission must be associated with an increase in the content of the two most important fluorophores after the reduction induced by sodium borohydride. A general increase in the emission intensity is observed, except for the band with a maximum at 393 nm which had a lower enhancement.

This result indicates the existence of at least three important fluorophores in this lignin, with emission peaks at 346, 365 and 393 nm, corresponding, in energy, respectively, to 345.5, 327.5 and 304.2 kJ/mol. The treatment of lignin with $NaBH_4$ induced a substantial increase in the observed fluorescence, with a preferential displacement of the emission band to high energies (Fig. 3), which agrees with the energy distribution shown in Fig. 5.

The synchronous spectra were recorded using an offset of 40 nm between the excitation and emission wavelengths. The spectra indicates that the lignin chromophores absorbing above 300 nm such as coniferyl alcohol, stilbenes and biphenyl structures are very good fluorophores in the lignin

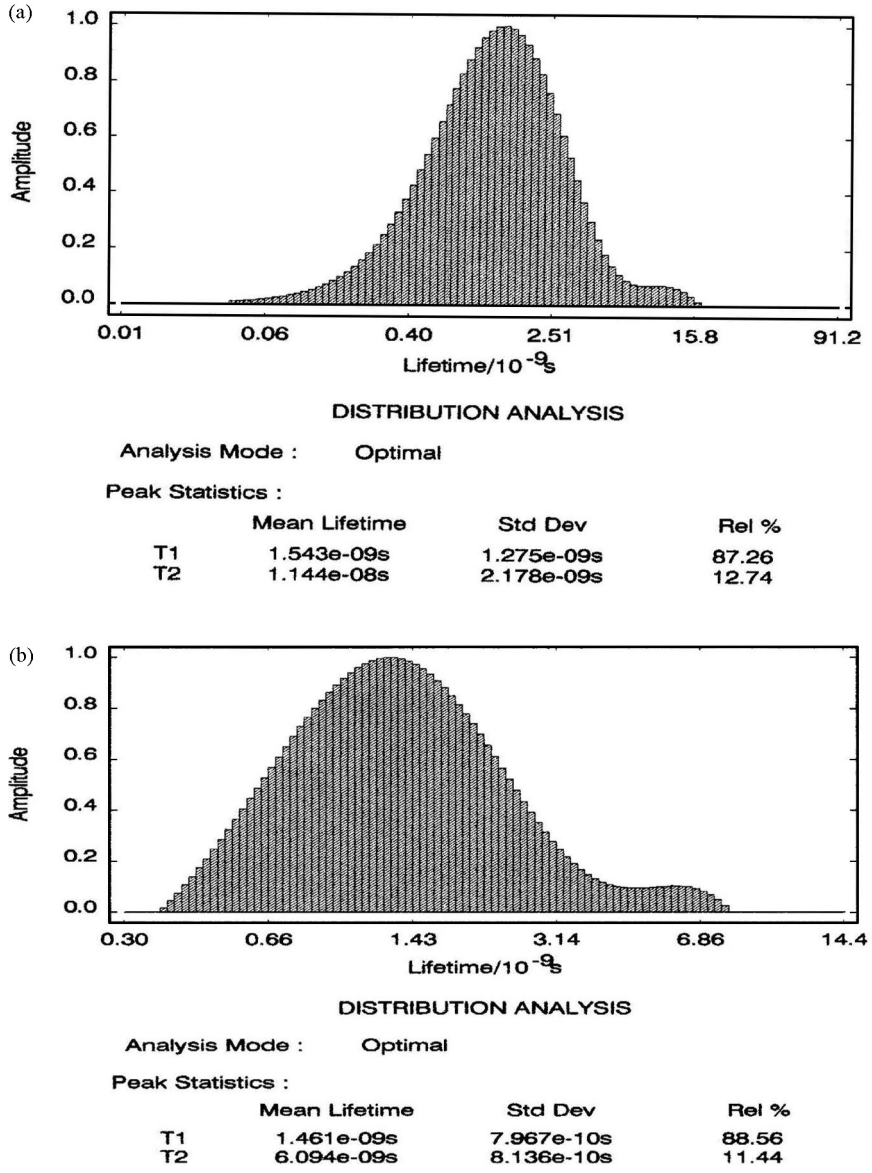

Fig. 6. Fluorescence lifetime distribution: (a) *E. grandis* lignin — $\lambda_{exc} = 295\,nm$; $\lambda_{em} = 335\,nm$; $\chi^2 = 1.10$; (b) reduced *E. grandis* lignin — $\lambda_{exc} = 341\,nm$; $\lambda_{em} = 405\,nm$; $\chi^2 = 1.07$.

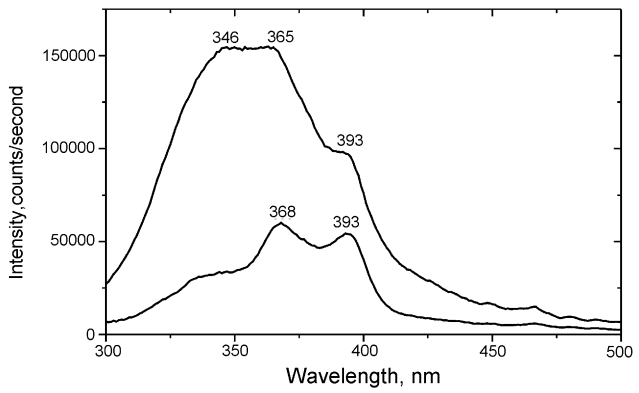

Fig. 7. Synchronous spectra for *E. grandis* lignin fragments: (a) unreduced; (b) reduced ($\Delta\lambda = 40\,nm$).

samples [4]. They are probably the origin of the most important part of the fluorescence in these lignin fragments.

4. Conclusion

A bimodal distribution with peak decay time values at 1.36 ± 0.17 ns for the first peak, and at 8.48 ± 2.32 ns for the second peak, was found. The fluorescence kinetics for both samples was little dependent on the excitation wavelength.

A large part of the fluorescence complexity seems to be due to the inhomogeneous emission decay kinetics associated to the ground state heterogeneity. That heterogeneity can be expected due to the complex mixture of different fluorophores, which compose the lignin structure.

The results indicate that the emission spectrum of this lignin may be regarded as the superposition of at least three broad spectral envelopes with slightly different emission maxima and widths. It is tempting to attribute the shortest decay time component to biphenyl structures, in the blue edge of the spectral range, where its amplitude was most pronounced. Also stilbenes and coniferyl alcohol structures can be influencing the fluorescence profile. The fluorophores in these lignin fragments must be heterogeneously distributed into the structure, some of them with carbonyl structures in the neighborhood. It was found that the energy distribution of this complex mixture could be successfully studied using the lifetime quenching of long-lived fluorescent probes.

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