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Paper pulp delignification using laccase and natural mediators

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Abstract

Three plant phenols, namely acetosyringone, syringaldehyde and *p*-coumaric acid, were selected as laccase redox mediators to investigate the enzymatic delignification of paper pulp (obtained from kraft cooking of eucalypt wood) in combination with peroxide bleaching. The effects of these natural mediators were compared with those obtained using the synthetic mediator 1-hydroxybenzotriazole. *p*-Coumaric acid only caused minor increase of pulp brightness and did not lower its kappa number (a rough estimation of the lignin content), whereas, the use of acetosyringone or syringaldehyde as laccase mediators enabled over 15% increase of final brightness and a similar decrease of final kappa number. Pulp delignification by laccase in the presence of the two latter natural mediators was demonstrated by analytical pyrolysis, which does not suffer from interferences by other pulp constituents as kappa number does, showing a preferential removal of lignin marker compounds compared with carbohydrate markers (up to 25% decrease of the corresponding ratio). This technique also revealed a modification of the residual lignin composition in terms of phenylpropane units after the laccase-mediator treatment. The use of laccase in combination with natural mediators, widely available from plant materials and pulping liquors, represents a promising alternative for environmentally friendly delignification of paper pulp.

Keywords: Laccase; Natural mediators; Analytical pyrolysis; Acetosyringone; Syringaldehyde; Paper pulp bleaching

1. Introduction

Lignin biodegradation, an extracellular oxidative process characteristic of wood-rotting basidiomycetes [1], has been largely investigated. This is mainly due to the interest on these fungi and their enzymes as industrial biocatalysts in paper pulp manufacture [2]. Wood pretreatment with *Ceriporiopsis subvermispora* and other basidiomycetes has been optimized for energy saving in mechanical pulping due to the preferential removal of lignin by this fungus [3]. Nevertheless, most interest has focused on ligninolytic enzymes to substitute chlorinated reagents in paper pulp bleaching [4]. Ligninolytic oxidoreductases include peroxidases, which are able to oxidize non-phenolic lignin, and laccases, whose direct action is in principle restricted to phenolic units that only represent a small percentage in lignin [5,6].

The interest on laccases strongly increased after discovering the effect of some synthetic compounds, including 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [7] and 1-hydroxybenzotriazole (HBT) [8], expanding the action of laccase to non-phenolic substrates. Since then, a variety of studies have confirmed the potential of the so-called laccase-mediator system for bleaching different pulp types [9–14], although several issues still remain to be solved before its industrial implementation. One of them is the cost of the synthetic mediators, which is difficult to lower enough for economic application. Moreover, a concern exists about the possible toxicity of some of the most powerful laccase mediators, such as the –N(OH)– compounds [15], or their reaction products.

A way to solve the above problems would be the use of natural mediators, including those compounds contributing to lignin degradation by some fungi that do not produce ligninolytic peroxidases but only laccases. In this way, a *Pycnoporus cinnabarinus* metabolite, 3-hydroxyanthranilic acid, has been reported as a natural laccase mediator [16] although its role in lignin biodegradation has not been confirmed [17], and the existence of other fungal mediators has also been suggested [18,19]. Recently, several lignin-derived phenols have been reported as efficient mediators for decolorizing different types of dyes using

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the *P. cinnabarinus* laccase [20]. In the present study, paper pulp delignification and bleaching using some of these natural phenols is shown for the first time.

2. Experimental

2.1. Pulp, enzyme and mediators

Unbleached eucalypt (*Eucalyptus globulus*) pulp with 14 kappa number, 41% ISO brightness and 1190 mL/g viscosity, and pulping black liquor were obtained from the ENCE kraft mill in Pontevedra, Spain. Laccase from *P. cinnabarinus* [21] was provided by Beldem (Belgium). One activity unit was defined as the amount of laccase transforming 1 μmol/min of ABTS to its cation radical (ε_{436} 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate, pH 5, at 24 °C.

Assays of decolorization of 25 μM Azure B (estimated at 647 nm) and Reactive Black 5 (estimated at 598 nm) (Fig. 1A and B) by *P. cinnabarinus* laccase (0.1 U/mL), as a preliminary test for mediator selection, were performed at 24 °C (160 rev/min) in 100 mM sodium citrate, pH 5. Syringaldehyde, acetosyringone, 2,6-dimethylphenol, 2,4,6-trimethylphenol, ethyl vanillin, acetovanillone, vanillin, vanillyl alcohol and *p*-coumaric acid (50 μM concentration) were assayed as mediators, and compared with HBT (Fig. 1C–L).

2.2. Pulp treatment with laccase mediator

Pulp treatments with laccase mediator were carried out in duplicate using 10 g (dry weight) of pulp at 3% consistency in 50 mM sodium tartrate, pH 4,

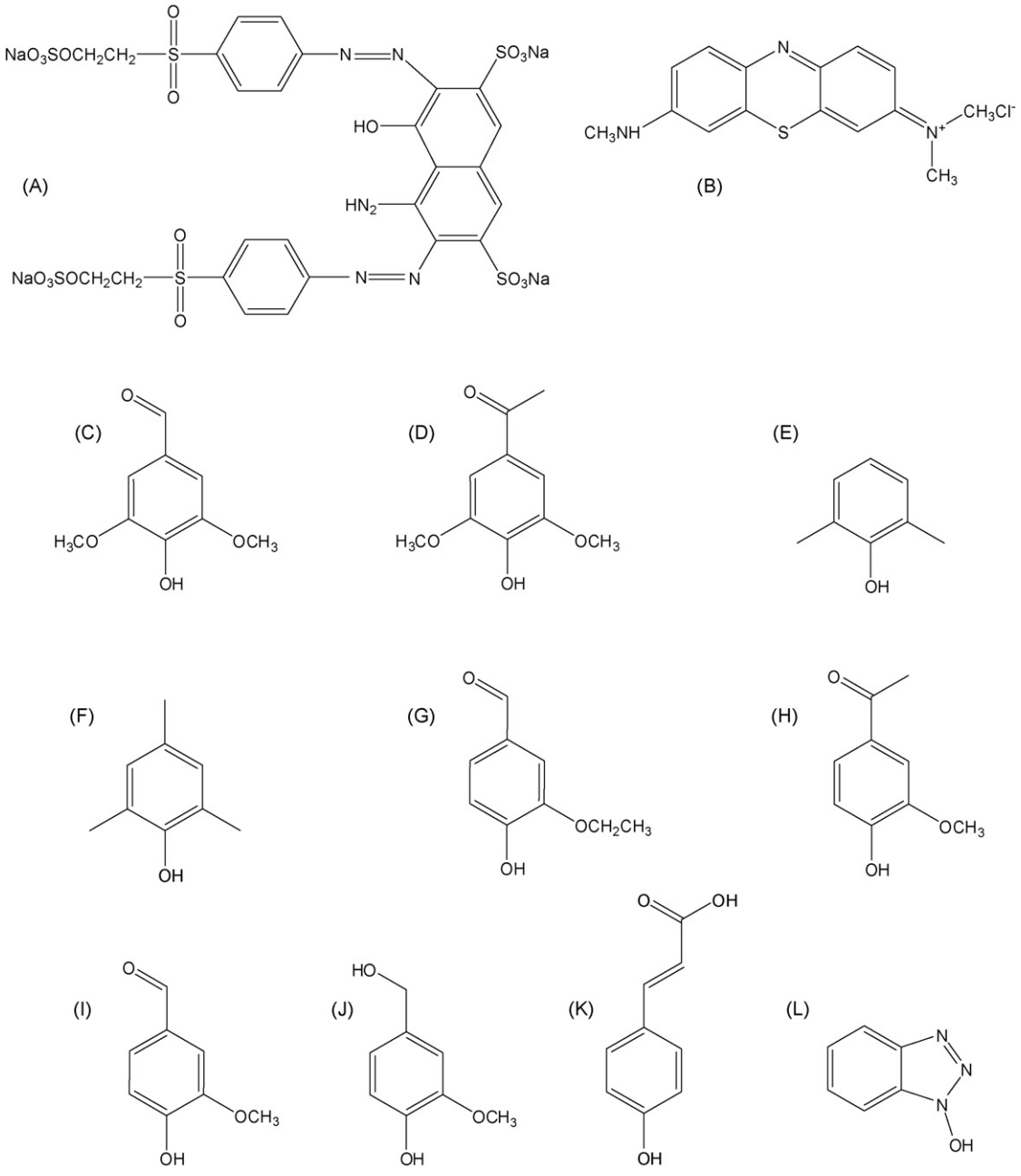

Fig. 1. Chemical structures of the two high redox potential dyes used as substrate for mediator screening ((A) Reactive Black 5; (B) Azure B) and the different natural phenols ((C) syringaldehyde; (D) acetosyringone; (E) 2,6-dimethylphenol; (F) 2,4,6-trimethylphenol; (G) ethyl vanillin; (H) acetovanillone; (I) vanillin; (J) vanillyl alcohol; (K) *p*-coumaric acid) assayed as laccase mediators, compared with HBT (L).

200 U of laccase, and 6.75 mM concentration of syringaldehyde, acetosyringone and *p*-coumaric acid. In the case of HBT, 1.5% concentration (referred to pulp weight) was used [22]. The treatments were carried out in 500-mL flasks with O_2 bubbling, placed in a thermostatic shaker at 170 rev/min and 50 °C, for 12 h. As controls, pulps were treated under the same conditions but without enzyme, mediator, or both. In a subsequent step, pulps (at 5% consistency) were submitted to: (i) an alkaline extraction stage using 1.5% NaOH (referred to pulp weight) at 60 °C for 1 h; or (ii) a bleaching stage using 3% H_2O_2 and 1.5% NaOH (both referred to pulp weight) at 90 °C for 2 h. Pulp brightness, kappa number and viscosity were evaluated following ISO 3688:1999, ISO 302:1981 and ISO 5351/1:1981, respectively [23].

2.3. Analytical pyrolysis conditions

Lignins in pulps were analyzed by pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS). Approximately 0.1 mg of dry pulp were introduced in quartz tubes, and placed in a CDS Pyroprobe AS-2500 autosampler. Pyrolyses were carried out at 550 °C for 10 s, and the camera (250 °C) was purged with He. The pyrolyzer was connected to an Agilent 6890 gas chromatograph, fitted with an in-column injector and a fused-silica capillary column (DV-1701, 60 m × 0.25 mm internal diameter, and 0.25 μm film thickness) coupled to a Agilent 5973 N mass spectrometer. The GC oven was heated from 45 °C (4 min) to 280 °C at 4 °C/min, and held for 15 min. The injector and transfer line were kept at 250 °C and 280 °C, respectively. Compounds were identified by mass fragmentography, and by comparing their spectra with those available from the literature [24,25] and the Wiley and NIST libraries. Py-GC/MS analyses were carried out in triplicate and variation coefficients were obtained.

2.4. Py-GC/MS analysis of lignin content and composition

For pulp lignin and carbohydrate analyses, reconstructed Py-GC/MS profiles were obtained using the molecular ions of selected markers [26]: *m/z* 124 (guaiacol), 138 (4-methylguaiacol), 150 (4-vinylguaiacol) and 164 (4-*t*-propenylguaiacol) for guaiacyl (G) lignin units; *m/z* 154 (syringol), 168 (4-methylsyringol), 180 (4-vinylsyringol) and 194 (4-*t*-propenylsyringol) for syringyl (S) lignin units; and *m/z* 96 (furfural), 98 (2,3-dihydro-5-methylfuran-2-one), 84, ((5*H*)-furan-2-one), 114 (4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one), 112 (2-hydroxy-3-methyl-2-cyclopenten-1-one) and 60 (levoglucosane) for carbohydrates. The relative lignin content in the final pulps was estimated from the lignin/carbohydrate (L/CH) index (expressed as a percentage), which provides only a comparative estimation of lignin removal because some carbohydrate breakdown products are non-chromatographied.

2.5. Chromatographic analysis of cooking liquor

Low molecular-mass compounds in industrial black liquor (obtained after kraft pulping of eucalypt wood) were isolated by liquid–liquid extraction using hexane–acetone (2:1) at pH 6. They were analyzed by GC/MS using an HP G1800A GCD System with a DB-5 column (30 m × 0.25 mm internal diameter, and 0.25 μm film thickness), which was programmed from 50 °C to 100 °C at 30 °C/min and from 100 °C to 300 °C at 5 °C/min. The final temperature was hold for 20 min. Compounds were identified by mass fragmentography, and by comparing their spectra with those available in the Wiley and NIST libraries.

3. Results and discussion

3.1. Selection of laccase mediators

High redox potential dyes including the heterocyclic dye Azure B and the diazo dye Reactive Black 5, whose chemical structures are shown in Fig. 1A and B, have been used to detect the activity of enzymes degrading lignin, such as lignin peroxidase [27] and versatile peroxidase [28], respectively. These

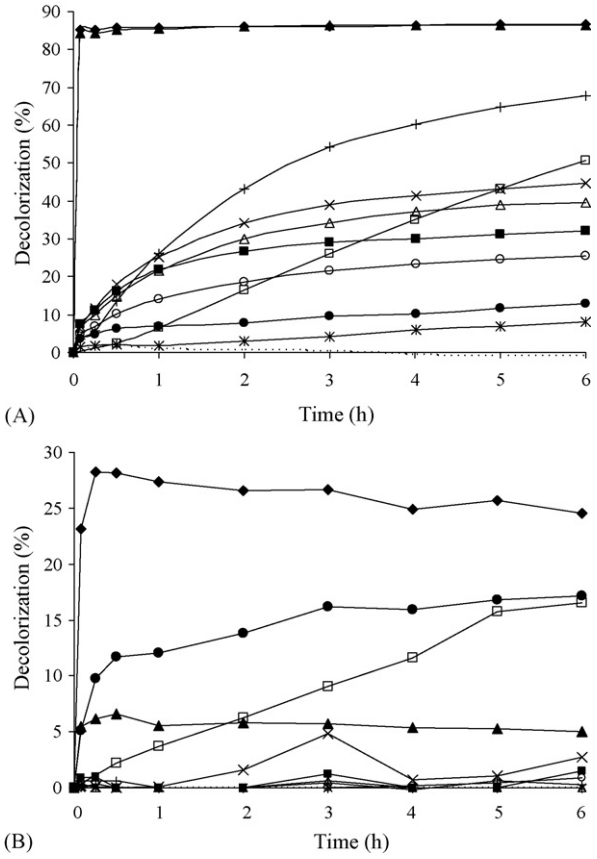

Fig. 2. Evaluation of potential laccase mediators. (A) Decolorization of Reactive Black 5. (B) Decolorization of Azure B. Twenty-five micromolar concentration of dyes and 50 μM concentration of the following mediators were used: syringaldehyde (▲), acetosyringone (♦), 2,6-dimethylphenol (◯), 2,4,6-trimethylphenol (+), ethyl vanillin (△), acetovanillone (■), vanillin (×), vanillyl alcohol (*), *p*-coumaric acid (●) and HBT (□) (no mediator, - - -).

two dyes were used here in a screening of natural phenols, whose chemical structures are shown (Fig. 1C–K), to identify those with the highest potential to act as laccase mediators for subsequent lignin removal from paper pulp (Fig. 2). The laccase from *P. cinnabarinus* was selected after comparison with other enzymes due to its thermal stability and high redox potential [22]. The best results were obtained with acetosyringone as laccase mediator since it produces maximal decolorization, near 90% for Reactive Black 5 and 30% for Azure B (using the same substrate/mediator ratio), in less than 30 min. In the case of Reactive Black 5, syringaldehyde showed similar efficiency as laccase mediator as acetosyringone. *p*-Coumaric acid also allowed up to 20% decolorization of the recalcitrant dye Azure B by laccase, although in longer reaction time. Less efficient decolorization of both dyes was obtained with HBT (Fig. 1L), one of the best laccase synthetic mediators described up to date [29,30], that required 6 h reaction time (a much longer treatment period than required when using the best natural mediators) to attain the maximal decolorization rates. Other phenols also acted as laccase mediators but with lower efficiencies than acetosyringone, syringaldehyde and *p*-coumaric acid.

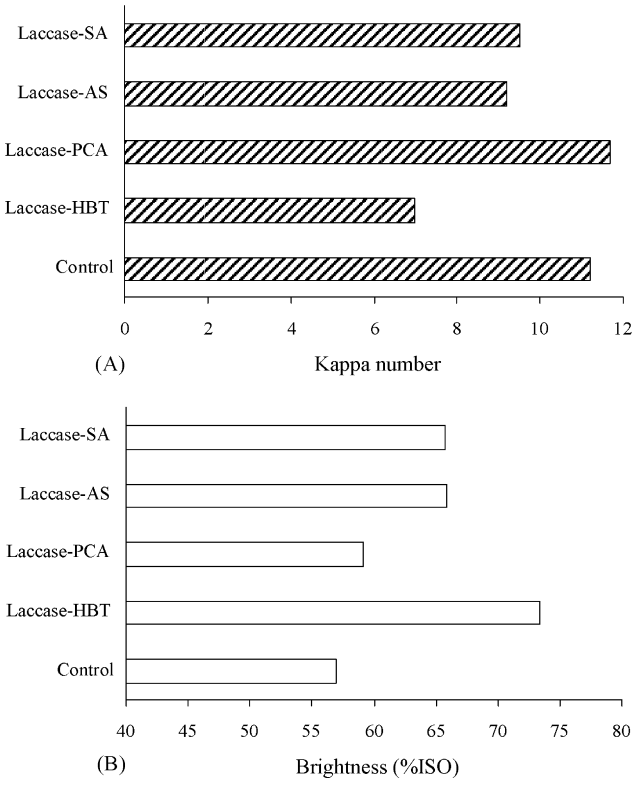

Fig. 3. Changes of kappa number (A) and brightness (B) of paper pulp treated with laccase in the presence of natural mediators (syringaldehyde, acetosyringone and *p*-coumaric acid) or HBT followed by hydrogen peroxide bleaching, compared with control (without both laccase and mediator). *Abbreviations*: AS, acetosyringone; SA, syringaldehyde; PCA, *p*-coumaric acid; HBT, 1-hydroxybenzotriazole. Variation coefficient values were below 5%.

3.2. Pulp treatment with laccase and three selected phenols

To evaluate the above mediators for pulp delignification, a sample of eucalypt pulp was treated with laccase in the presence of acetosyringone, syringaldehyde, *p*-coumaric acid or HBT, and then bleached with alkaline peroxide. The characteristics of the pulps after this simple totally chlorine free (TCF) bleaching sequence were evaluated in terms of kappa number (a rough estimation of the lignin content in pulp) and brightness, and the results are shown in Fig. 3 compared with a control pulp. Only a minor decrease in kappa number or brightness increase was observed in the pulps treated without enzyme or mediator, compared with the control (without both laccase and mediator). The treatment with laccase in the presence of *p*-coumaric acid did not improve significantly the eucalypt pulp properties with respect to the control. In fact, an increase in the kappa number, probably due to partial condensation of the *p*-coumaric acid phenoxy radical on pulp, was observed. However, pulps with lower kappa number and increased brightness were obtained after treatment with laccase in the presence of either syringaldehyde or acetosyringone revealing the capabilities of these natural mediators for delignifying and bleaching eucalypt kraft pulp. No further improvement of these pulp properties was obtained when the enzymatic treatment was followed by a simple alkaline extraction, revealing the need of a peroxide stage after the treatment with laccase and natural mediators. A small decrease of pulp viscosity (an estimation of cellulose integrity) was observed after the laccase-mediator treatment followed by a peroxide stage, although the decreases obtained using the natural mediators (10–13% of the initial value) were lower than those obtained using HBT (15%).

3.3. Py-GC/MS analysis of laccase-mediator action on pulp lignin

Since the estimation of the kappa number faces some interferences due to the presence of hexenuronic acids and oxidized lignin structures [31,32], the lignin in the enzymatically treated pulps was also analyzed by other means. However, the small amounts of pulp treated precluded the isolation of residual lignin by the procedure optimized for eucalypt pulps [33]. Py-GC/MS allows for *in situ* analysis of lignin by chromatographic separation and mass-spectrometric identification of the compounds released after the pyrolytic breakdown of the whole pulp [34]. However, the lignin content in pulps is often too low for direct detection of these breakdown products. Therefore, the relative abundances of S and G phenylpropane lignin units were analyzed here in single-ion chromatographic profiles corresponding to selected marker compounds [26], as illustrated in Fig. 4. In a similar way, the relative abundance of selected carbohydrate markers was estimated in single-ion chromatographic profiles, as illustrated in Fig. 5. From these profiles, the relative lignin content, as shown by the lignin/carbohydrate (L/CH) index, and composition, as revealed by the molar S/G ratio, in the enzymatically treated eucalypt pulps and controls, were calculated. The changes observed in the L/CH index (Fig. 6A) by the different laccase-mediator treatments confirmed those of the kappa number, although the decreases estimated by Py/GC-MS were more pronounced and attained 20–25% using acetosyringone or syringaldehyde as mediators and near 55% using HBT. This fact can be explained by the presence of hexenuronic acids in the pulp that hinders the estimation of the lignin content by kappa number measurement, as mentioned above. Hexenuronic acids account for up to 25% of the kappa number in unbleached eucalypt kraft pulp, and their relative abundance increases during TCF bleaching [14].

Py-GC/MS also provided information on lignin composition (Fig. 6B). An increase in the S/G ratio was observed after all the enzymatic treatments except when using *p*-coumaric acid, which did not seem to act as a laccase mediator for delignifying paper pulp. This increase would be explained if syringaldehyde and acetosyringone (two S-type compounds) radicals were incorporated into the pulp during the enzymatic treatment, and then computed for S/G estimation. However, the syringaldehyde and acetosyringone peaks only represented 10–14% of total S-compounds from the enzymatically treated pulps, and they were not computed as lignin markers for the S/G estimation to prevent interferences. Moreover, a similar increase in the S/G ratio was observed when using HBT as mediator, a compound that is not structurally related to lignin. The preferential removal of G-lignin units revealed by Py-GC/MS differs from the results obtained on flax pulp, which showed

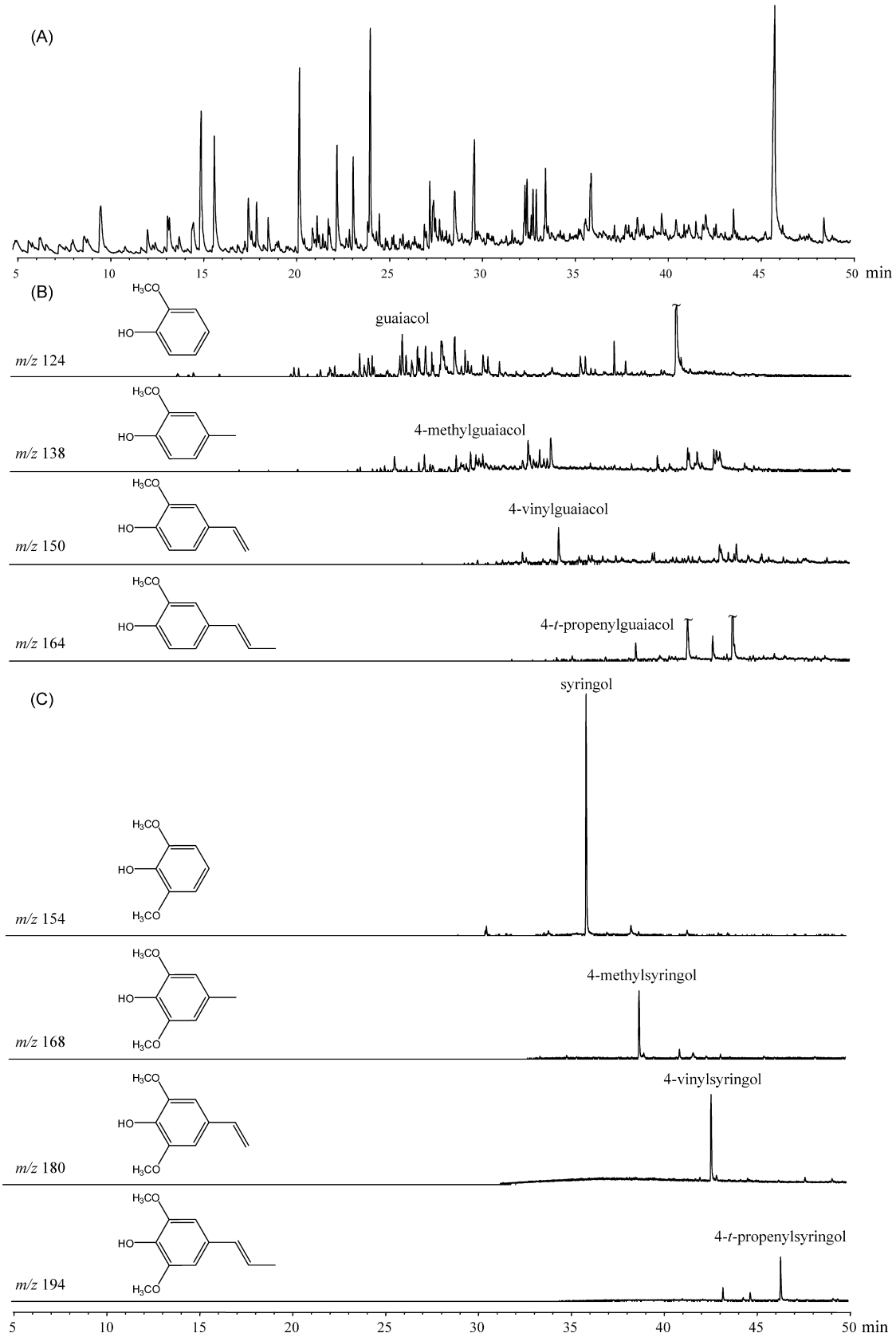

Fig. 4. Chromatographic traces used to estimate lignin content and composition after Py-GC/MS of paper pulp (control). (A) Total-ion chromatogram. (B) Single-ion traces of G-lignin markers: Guaiacol (*m/z* 124), 4-methylguaiacol (*m/z* 138), 4-vinylguaiacol (*m/z* 150) and 4-*t*-propenylguaiacol (*m/z* 164). (C) Single-ion traces of S-lignin markers: Syringol (*m/z* 154), 4-methylsyringol (*m/z* 168), 4-vinylsyringol (*m/z* 180) and 4-*t*-propenylsyringol (*m/z* 194). Same vertical scale was used in B and C chromatographic traces.

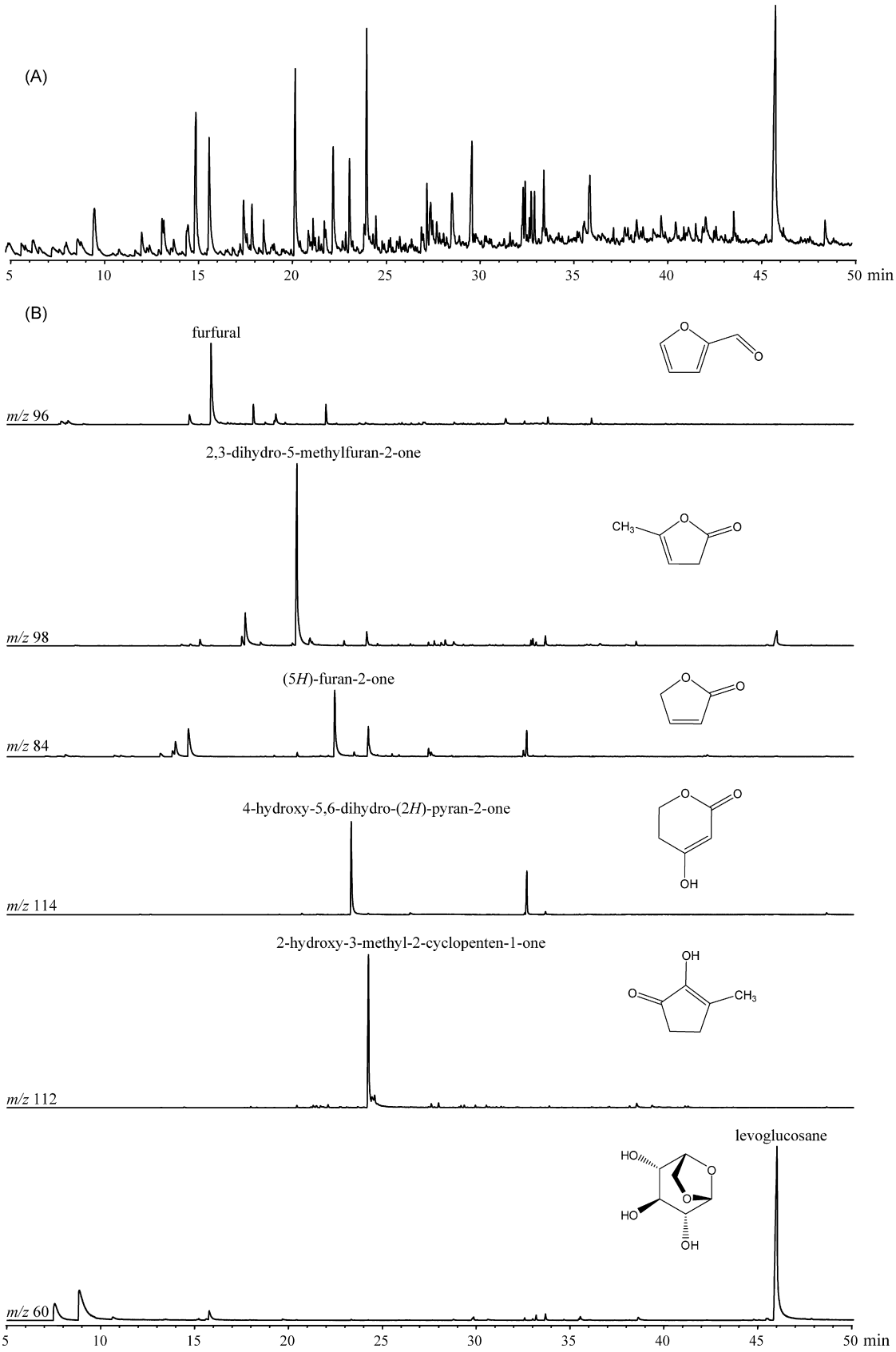

Fig. 5. Chromatographic traces used to estimate carbohydrate content after Py-GC/MS of paper pulp (control). (A) Total-ion chromatogram. (B) Single-ion traces of carbohydrate markers: Furfural (*m/z* 96), 2,3-dihydro-5-methylfuran-2-one (*m/z* 98), (5*H*)-furan-2-one (*m/z* 84), 4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one (*m/z* 114), 2-hydroxy-3-methyl-2-cyclopenten-1-one (*m/z* 112) and levoglucosane (*m/z* 60). Vertical scale in B was 30-fold higher than used in Fig. 4B and C.

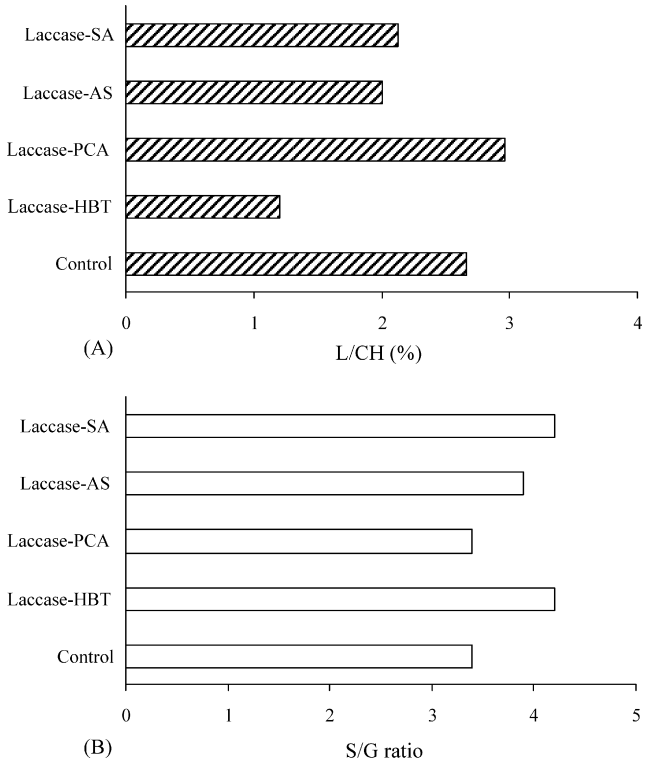

Fig. 6. Changes of lignin/carbohydrate (L/CH) index (%) (A) and S/G ratio (B) estimated by Py-GC/MS (see Fig. 4) in paper pulp treated with laccase and natural mediators (syringaldehyde, acetosyringone and *p*-coumaric acid) or HBT, followed by hydrogen peroxide bleaching, compared with control without laccase and mediator. Variation coefficient values were below 7%. For abbreviations see Fig. 3.

a nearly complete removal of S units after laccase-HBT treatment (followed by a peroxide stage) [13]. Differences between eucalypt (S-rich lignin) and flax (G-rich lignin), in terms of lignin composition and distribution, are probably related to these differences.

3.4. The potential of natural laccase mediators

The cost and potential toxicity of laccase mediators are the two major drawbacks for the industrial implementation of the mediators previously assayed for pulp bleaching, which are obtained by chemical synthesis. Natural phenolic compounds were firstly reported as laccase mediators for the oxidation of methoxylated benzyl alcohols [35], and more recently of polycyclic aromatic hydrocarbons [36], and other recalcitrant xenobiotics and dyes [20,37–39].

The present paper provides the first evidence of natural phenols as laccase mediators acting on paper pulp lignin. The delignification increase (up to 25%) and the improvement of pulp properties (around 15% increase of brightness and decrease of kappa number) was lower than those obtained with HBT but the pulp treatment conditions have still to be optimized, as previously done for HBT [14,22]. Taking into account the results obtained during the oxidation of recalcitrant dyes, shorter treatment periods can be envisaged for some of the natural mediators described, in comparison with HBT.

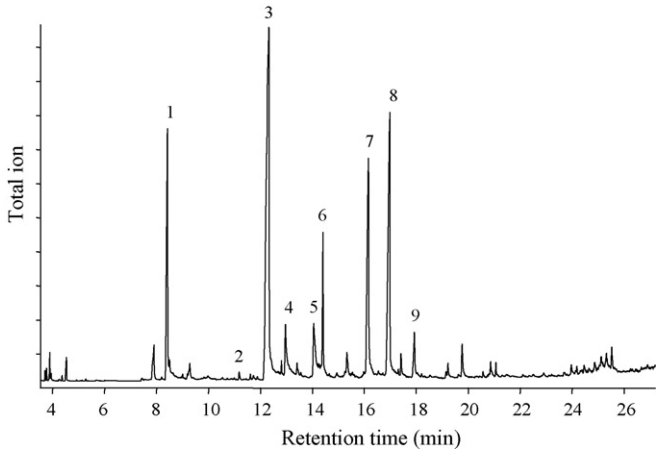

Fig. 7. GC/MS analysis of low molecular-mass polar compounds in the black liquor from kraft pulping of eucalypt wood. Peak identification: (1) guaiacol; (2) 4-ethylguaiacol; (3) syringol; (4) vanillin; (5) acetovanillone; (6) 4-ethylsyringol; (7) syringaldehyde; (8) acetosyringone; (9) propiosyringone.

According to the literature, the action mechanism of phenolic mediators should be similar to that of –N(OH)– type mediators (like HBT) the phenoxy radical acting as the reactive species abstracting one proton and one electron from the target substrate [40]. The relative abundance of the phenolate form and the ability to form relatively stable oxidized intermediates might determine the efficiency of a phenol as mediator together with steric issues [41,42]. In this context, the lower efficiency of *p*-coumaric acid as a laccase mediator could be related to both the higher pK_a of its phenolate group and the lower stability of its phenoxy radical (that can experiment condensation reactions at the C_3 and C_5 atoms with residual lignin) compared with syringaldehyde and acetosyringone that have lower pK_a values and form stable radicals [43].

The interest on these phenolic mediators increases as they can be easily obtained from natural substrates by organic extraction or alkaline treatment. This is illustrated in Fig. 7 that shows the GC/MS analysis of an extract from the black liquor of eucalypt kraft pulping. The high abundance of syringaldehyde (peak 7) and acetosyringone (peak 8) in this pulping liquor, together with other compounds, is related to the presence of a S-rich lignin in eucalypt wood [44].

The present study initiates a promising research area in environmentally friendly bleaching of paper pulps based on the use of some natural phenols, which are widely available at the paper pulp industry, as mediators for laccase delignification of pulp.

Acknowledgments

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