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# Structural insights on laccase biografting of ferulic acid onto lignocellulosic fibers

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ABSTRACT

Treatment of high-kappa sisal pulp with *Trametes villosa* laccase and ferulic acid resulted in strong increases of kappa-number and acid-group content due to biografting of this phenolic acid, as shown by pyrolysis in the presence of tetramethylammonium hydroxide. The coupling linkages were investigated by 2D NMR of the lignin isolated from pulps. The aromatic region of the spectra showed incorporation of the cinnamic molecule, representing ~4% of the lignin content, that according to the displacement of its olefinic $^{13}C_{\beta}$–$^{1}H_{\beta}$ signal to 117.0/6.40 ppm would be $C_4$-etherified. The aliphatic region of the spectra showed that ferulic acid also incorporates as the corresponding β–β′ dilactone (another ~4% of the total lignin) with characteristic $^{13}C_{\alpha}$–$^{1}H_{\alpha}$ and $^{13}C_{\beta}$–$^{1}H_{\beta}$ correlations at 81.8/5.69 and 47.9/4.19 ppm, respectively. The sisal lignin in the treated pulps was only slightly modified (including a small increase of $C_{\alpha}$-oxidized units) revealing that the main effect of the treatment was ferulic acid biografting.


## 1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases widely distributed in fungal and plant species, where they play multiple functions [1]. Laccases present a broad substrate specificity including substituted phenols, aromatic amines and thiols and many others, which are converted into reactive radicals using oxygen as the electron acceptor (and releasing water) [2]. By virtue of these characteristics, laccases are being intensively investigated as eco-friendly biocatalysts for a wide array of biotechnological applications [3]. Within the pulp and paper industry, laccases have arisen great interest, especially in combination with chemical mediators for delignifying (and bleaching) pulp [4–6]. Due to its molecular size and low redox potential, laccases can directly oxidize only the exposed phenolic moieties in the lignin polymer. The advantage of the use of mediators [7] is their ability to expand the activity toward the more recalcitrant non-phenolic lignin and overcome the accessibility restrictions of pulp cell walls. In addition to synthetic mediators, with some environmental risks due to potential toxicity, the so-called natural mediators have been largely investigated during recent years including phenolic compounds related to lignin [8].

A novel subject of research in the pulp and paper field is the application of laccase-catalyzed radical coupling reactions to modify lignocellulosic fiber chemistry with a view to altering paper properties [9]. Two main approaches are used for laccase-assisted modification of lignocellulosic fibers: (i) laccase-mediated cross-linking of lignin molecules in situ; and (ii) coupling of low molecular weight (generally phenolic) compounds onto fibers (*biografting*). The former approach has proven effective for the manufacture of binderless wood boards, where the bonding mechanism involves the enzymatic activation of lignin on fiber surfaces through the production of phenoxyl radicals which couple when the fibers are pressed into boards [10]. Other studies showed combination of laccase with lignin-rich extractives or different mediators to improve pulp wet strength, which was ascribed to both polymerization of added lignin and to production of phenoxyl radicals forming water-resistant linkages between fibers [11,12].

The second approach, which has been intensively investigated and coincides with that of the present study, provides a versatile method for functionalizing lignocellulosic fibers and imparting

*Abbreviations:* 2D NMR, two-dimensional nuclear magnetic resonance; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate); DMSO, dimethylsulfoxide; FA, ferulic acid; G, guaiacyl; GC/MS, gas chromatography/mass spectrometry; HSQC, heteronuclear single-quantum correlation; S, syringyl; TMAH, tetramethylammonium hydroxide.

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desirable properties to pulps. Biografting of phenolic acids to kraft pulp fibers was found to increase dry strength properties in the resulting papers, which was ascribed to the ability of carboxyl groups to promote inter-fiber hydrogen bonding and fiber swelling [13]. Other studies have reported the development of antibacterial properties in different lignocellulosic substrates treated with laccase and simple phenols or tannins [14–16]. Laccase-catalysed grafting has also been applied to impart hydrophobicity to wood veneers and kraft pulp by using fluorophenols and lauryl gallate, respectively [17,18]. Although much research has been carried out to explore the potential of biografting for tailoring the properties of lignocellulosic materials, few of them have assessed the mechanistic aspects of this process and the nature of the chemical bonds formed [16,17] and they mainly involved the use of lignin model compounds due to the complexity of the lignin polymer.

In previous works [15,19,20] laccase biografting of simple phenolic compounds (such as syringaldehyde, acetosyringone and *p*-coumaric acid) on flax and sisal pulps was observed by the increase of both kappa number and Klason lignin content and, in the case of phenolic acids, by the increase of fiber anionic charge due to the presence of the carboxylic functionality. The covalent binding of these compounds on fiber components was confirmed by using an analytical approach based on pyrolysis of the whole treated fibers in the presence of tetramethylammonium hydroxide (TMAH), a method also known as thermochemolysis [21]. In the present study, a high-kappa pulp from sisal was treated with laccase and (*trans*) ferulic acid (FA) according to the conditions reported by Aracri et al. [20] as those providing the highest degree of grafting of this phenolic acid. After extensive washing, the treated pulp was directly analyzed by pyrolysis (in the absence/presence of TMAH) to confirm the FA incorporation. Then, in order to gain additional information on the amount of FA incorporated with respect to pulp lignin, and identify the lignin-FA linkages formed in the biografting reaction, analysis of the lignin isolated from treated pulp was performed by HSQC (heteronuclear single quantum correlation) 2D NMR spectroscopy. The results obtained are highly novel and relevant to the understanding of the biografting mechanisms during laccase-assisted modification of lignocellulosic materials.

## 2. Materials and methods

### 2.1. Enzyme and chemicals

Laccase from *Trametes villosa* was kindly provided by Novozymes (Bagsvaerd, Denmark) and frozen until use. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) per minute to its cation radical ($\varepsilon_{436\,nm}$ = 29,300 $M^{-1}$ $cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 25 °C. Chemicals for enzyme assay were purchased from Sigma–Aldrich and used as received.

### 2.2. Pulp

Pulp was obtained from a laboratory cooking of sisal (*Agave sisalana*) fiber bundles kindly supplied by Celesa mill (Tortosa, Spain). The raw material was placed into a stainless steel rotating digester, where white liquor consisting of de-ionized water and NaOH was added to reach a liquid/solid ratio of 3.5. The digester was sealed, heated from room temperature to 160 °C in 60 min and kept isothermally for 45 min. Decreasing concentrations of NaOH were assayed (from 15% to 9% active alkali expressed as $Na_2O$) until obtaining a pulp with the highest possible lignin content. After cooling, the resulting products in the digester were filtrated with an 80 mesh screen for recovering the liquor and pulp. The pulp was thoroughly washed with tap water, defibrated in a defibrator chamber and then screened on a flat screen using a 0.2 mm slot screen.

Prior to initial characterization, pulps (2% consistency) were washed with diluted $H_2SO_4$ (pH 4) for 30 min, which was followed by filtration and extensive washing with de-ionized water. This step ensured removal of contaminants and metals, and brought the pulp to the pH required for the enzyme treatment.

Acid-washed pulps were disintegrated for 50,000 revolutions (ISO 5263), which was followed by filtration through a Buchner funnel, pre-refining for 5000 revolutions and refining for 4500 revolutions according to ISO 5264-2. The application of a pre-refining step, carried out using a 2 mm gap between the working surfaces of the PFI mill, was considered necessary to obtain a more homogeneous material and ensure an easier running of the PFI mill during refining.

### 2.3. Enzymatic treatments of pulps

Refined pulp samples were treated in an oxygen-pressurized (0.6 MPa) reactor at 5% consistency [20], using 50 mM sodium tartrate (pH 4), 40 U/g laccase and 3.5% (w/w) FA (all relative to pulp dry weight). Tween 80 (0.05%, w/v) was added as surfactant. Treatments were conducted for 4 h at 30 rev/min shaking, and 50 °C. Pulp samples treated under identical conditions in the absence of FA were used as controls. After treatment, the pulp samples were filtered in a fritted glass funnel and washed with de-ionized water until a colorless, neutral filtrate was obtained.

### 2.4. Analysis of pulp properties

Pulp properties were analyzed after Soxhlet extraction with acetone aimed at removing the fraction of FA that failed to covalently bind to fibers [19]. Kappa number and brightness were determined according to the standard methods ISO 302 and ISO 3688, respectively. The bulk acid group content was determined by conductimetric titration as described elsewhere [22]. In short, 1.5 g pulp was stirred in 300 ml of 0.1 M HCl for 1 h, followed by rinsing with deionized water in a finely fritted funnel. The sample was resuspended in 250 ml of 1 mM NaCl, spiked with 1.5 ml of 0.1 M HCl and titrated against 0.05 M NaOH in 0.25 ml increments, with conductivity measurement after each addition. All the reported results were the averages of two measurements.

### 2.5. Enzymatic isolation of residual lignin from pulps

Cellulolytic enzyme lignins were isolated by enzymatically saccharifying polysaccharides as described by Chang et al. [23]. Cellulysin (Calbiochem), a crude cellulase preparation from *Trichoderma viride* also containing hemicellulase activities, was used. Its activity was ≥10,000 units/g, estimated as reducing sugars (glucose equivalents) released from paper filter at 40 °C, pH 4. The extractives free ball-milled material (200 mg) was suspended in 20 mM NaOAc buffer (30 ml, pH 5.0) in a 50 ml centrifuge tube, 8 mg of Cellulysin was added, and the reaction slurry was incubated at 30 °C for 48 h. The solids were pelleted by centrifugation (8000 rpm, 4 °C, 20 min), and the process was repeated with fresh buffer and enzyme, two times. Finally, lignin was recovered by filtration, washed with ultrapure water and then lyophilized.

### 2.6. Analytical pyrolysis

Pyrolysis coupled to gas chromatography/mass spectrometry (GC/MS) of pulp samples was performed in the absence and the presence of TMAH. The pyrolysis was carried out using an EGA/PY-3030D micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 7820A gas chromatograph using a

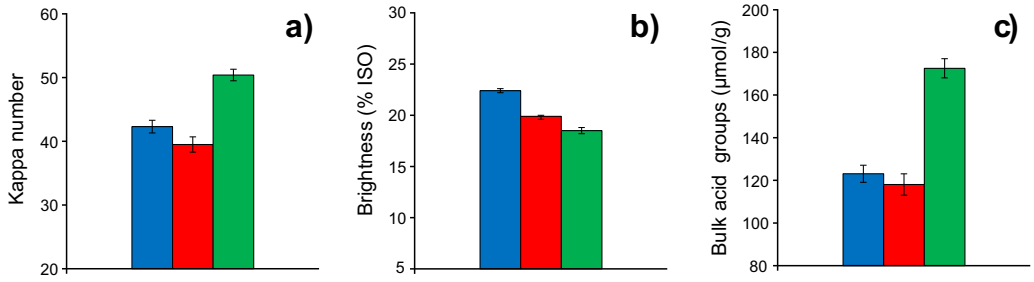


**Fig. 1.** Kappa number (a), brightness (b) and bulk acid group content (c) of sisal pulp before (blue) and after treatments with laccase (red) and laccase-FA (green). Means and standard deviations are shown.

DB-1701 fused-silica capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The oven temperature was programmed from 45 °C (4 min) to 280 °C (10 min) at 4 °C $min^{-1}$. Helium was the carrier gas (2 mL $min^{-1}$). For pyrolysis/TMAH, approximately 0.1 mg of milled pulp was mixed with 1 μL of TMAH (25%, w/w, methanol solution) and pyrolysis was performed as described above. In addition to total-ion chromatograms, selected-ion chromatographic profiles were also analyzed.

### 2.7. NMR analysis

For NMR analysis of isolated lignins, around 20 mg of the isolated lignin was dissolved in 0.75 mL of deuterated dimethylsulfoxide (DMSO-$d_6$). 2D NMR HSQC spectra were acquired at 25 °C on a Bruker AVANCE III 500 MHz spectrometer fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D $^{13}C$–$^{1}H$ correlation spectra were carried out using an adiabatic HSQC pulse program (Bruker standard pulse sequence 'hsqcetgpsisp2.2′) and the following parameters: spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 ($^{1}H$) using 1000 data points for an acquisition time of 100 ms, an interscan delay of 1 s, and from 200 to 0 ppm (25,168) in F1 ($^{13}C$) using 256 increments of 32 scan, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in $^{1}H$ and a squared cosine bell in $^{13}C$. The central solvent peak was used as an internal reference ($\delta_C/\delta_H$ 39.5/2.49). 2D NMR cross-signals were assigned as in previous publications [24–27]. Integration of the lignin and FA cross-signals was performed separately for the different regions of the HSQC spectrum, which contain signals that correspond to chemically analogous carbon-proton pairs. For these signals, the $^{1}J_{CH}$ coupling value is similar and integrals can be used semiquantitatively to estimate the relative abundance of the different species. In the aliphatic-oxygenated region, the relative abundances of side-chains involved in inter-unit linkages were estimated from the $C_\alpha$–$H_\alpha$ correlations to avoid possible interference from homonuclear $^{1}H$–$^{1}H$ couplings. In the aromatic region, the $S_{2,6}$, $G_6$ and $FA_\beta$ signals were used to estimate the relative abundances of the different lignin and FA units.

## 3. Results and discussion

### 3.1. Laccase-FA modification of pulp properties

Recent works demonstrated the successful functionalization of (non-wood) low-lignin content pulp fibers (sisal and flax) through biografting of phenolic compounds [15,19,20]. This resulted in improved or new papers properties including an increase of fiber surface anionic charge when FA was used. In this work, a laboratory cooking process was carried out whose conditions were investigated and selected in order to obtain a high-kappa sisal pulp. Prior to the enzymatic treatments, pulp samples obtained from the cooking process at 9% active alkali were mechanically refined in order to increase the fiber surface area and the accessibility of the functionalizing reactant. Both the use of high-lignin content pulp and its refining were aimed at achieving high biografting efficiency and thus facilitating NMR characterization.

As shown in Fig. 1a, the laccase-FA treatment increased the kappa number of sisal pulp by 11 units relative to control treatment with laccase alone (which was slightly lower than that of the initial pulp). The more marked increases of kappa number compared with Aracri et al. [20] is probably due to the use of lignin-rich fibers providing a higher number of available sites for the cross-linking of FA-derived phenoxyl radicals.

Fig. 1b shows the effects of the enzymatic treatment (with laccase and laccase-FA) on pulp brightness. Phenoxyl radicals from laccase oxidation of phenolic substrates are very unstable and reactive species, susceptible to undergo many reactions (besides cross-coupling with lignin). These include formation of quinones [28] whose incorporation into pulp is often responsible for the change of optical properties [19,29]. As expected, laccase-FA treatment of the (high lignin content) sisal pulp caused a brightness loss with respect to the laccase-treated sample, although to a lower extent than reported for low-lignin content sisal pulp [20]. A possible reason may be the prevalence in the present study of cross-coupling reactions of the added phenol with lignin over quinone formation leading to chromophoric species. Interestingly, treatment with laccase alone resulted in a certain brightness loss (with respect to initial pulp) in spite of the slight decrease of kappa number, which can be attributed to the formation of chromophoric structures (C-alpha carbonyls, quinones, etc.) during lignin degradation by the enzyme.

Similar to kappa number, titration of acid groups in fibers is a valid approach to estimate the amount of phenolic acids attached to pulp, and hence to assess the grafting efficiency [13,14]. As can be observed from Fig. 1c, the increase in kappa number in laccase-FA treated pulp echoes that in the bulk acid group content, which resulted 40% higher than the value observed for the initial sample. Similar percentage increases are reported in the literature [13,14] for the biografting of phenolic acids onto high-yield kraft pulp fibers. In the absence of FA, no significant modification of the bulk acid group content was produced.

### 3.2. Demonstration of FA biografting by pyrolysis/TMAH of whole fibers

In order to confirm the enzymatic incorporation of FA into sisal fibers, the acetone extracted pulps were analyzed by pyrolysis in the absence (pyrolysis-GC/MS) and the presence of TMAH, as a base and methylating reagent. Pyrolysis-GC/MS was unable to demonstrate the presence of FA in the pulp treated with laccase-FA, although some differences in the aromatic products

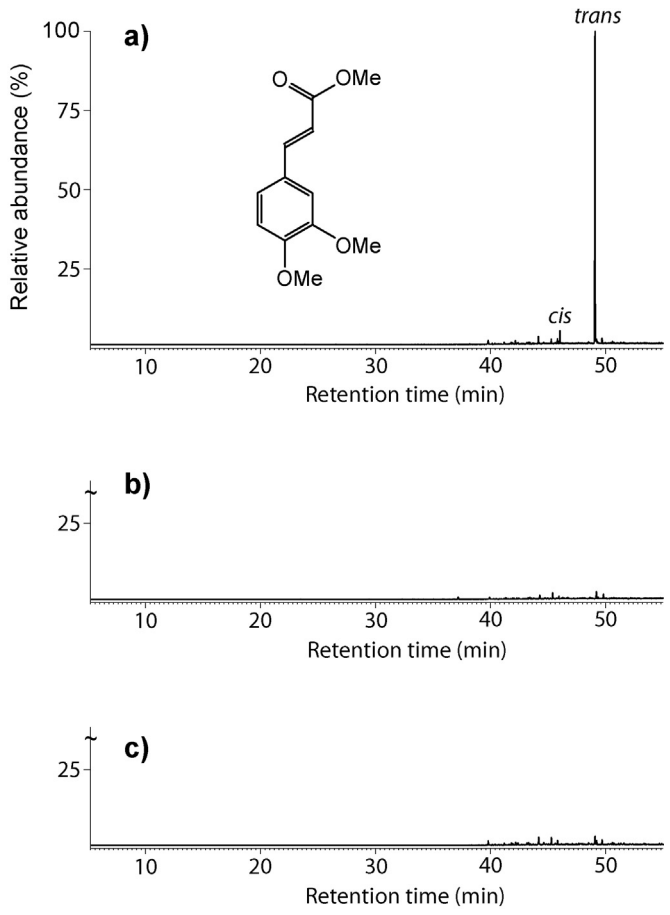


**Fig. 2.** Pyrolysis/TMAH of sisal pulp treated with laccase-FA (a), laccase alone (b) and control (c). Selected-ion (*m/z* 222) chromatographic profiles, normalized to the same ion numbers (275 000 ions correspond to 100% in the three analyses), showing the presence of *trans* (and *cis*) FA in a, and its absence from b and c.

released were observed when compared with the pulp treated with laccase alone and the control pulp (both yielding identical pyrolysis-GC/MS profiles) providing a lower syringyl-to-guaiacyl (S/G) ratio (as shown in Supporting Fig. S1 and Table S1). This is because FA under pyrolysis-GC/MS conditions yields some of the same products obtained from G lignin (such as 4-vinylguaiacol, 4-ethylguaiacol and 4-methylguaiacol) [24]. However, detection of the grafted FA could be achieved by pyrolysis/TMAH of the whole treated fibers. This analytical method prevents decarboxylation of acid groups that are converted into their methyl esters, a crucial aspect to identify the grafted FA on treated pulps, and causes alkaline depolymerization of lignin (and methylation of phenolic products) providing information on its constitutive units [21]. Pyrolysis/TMAH of laccase-FA treated sisal pulp (after extensively washing with acetone to remove adsorbed and non-covalently bound FA) released important amounts of the methyl derivative of FA (methyl 3,4-dimethoxycinnamate), which was detected in the *m/z* 222 ion chromatographic profile (Fig. 2c) but which was absent from the pyrograms of both the untreated (control) pulp and the pulp treated with laccase alone (Fig. 2a and b, respectively). This fact confirms that the cinnamic FA molecule was successfully incorporated by the laccase (through covalent bonds) into the sisal pulp, in agreement with previous results [19]. Its relative abundance (estimated from the total-ion chromatograms) was ~10% of the total degradation products in the laccase-FA treated pulp.

### 3.3. Aromatic/unsaturated signals in 2D NMR spectra of pulp lignins

Since the aim of the study was to get further information of the laccase biografting of phenols onto pulp (including identification of the main linkages formed), the lignins were enzymatically isolated from the laccase-FA treated sisal pulps (where grafting of the FA molecule had been shown by Py/TMAH, after the indirect evidence provided by kappa and total acid group estimations) and subsequently analyzed by 2D NMR spectroscopy. 2D NMR (using HSQC experiments) represents an important tool for the structural analysis of complex lignin, since the large signal overlapping produced in 1D NMR spectra is overcome when $^{13}C–^{1}H$ correlations are analyzed [30]. The HSQC spectra of the lignins isolated from the laccase-FA treatment (and the respective untreated control pulp) are shown in Fig. 3. The main signals identified in the aliphatic oxygenated ($\delta_C/\delta_H$ 45–95/2.5–6.0 $ppm^{-1}$) and aromatic/unsaturated ($\delta_C/\delta_H$ 98–148/5.2–8.7 ppm) regions of the HSQC spectra of the two lignins are discussed below.

The main cross-signals in the aromatic/unsaturated region of the spectra of the lignins isolated from the laccase-FA treated sisal pulp, and the untreated control (Fig. 3d and b, respectively) corresponded to the aromatic rings of the G and S lignin units, and the FA attached to the lignin (in the laccase-FA treated pulp) (Fig. 4, bottom line). The S-lignin units gave a single prominent signal for the $C_{2,6}–H_{2,6}$ correlation at $\delta_C/\delta_H$ 104/6.6 ppm, and a smaller one for the same correlation in minor $C_\alpha$-oxidized S-lignin units (S′) at $\delta_C/\delta_H$ 106/7.2 ppm. The G units showed three different signals corresponding to the $C_2–H_2$ ($\delta_C/\delta_H$ 110/7.0 ppm), $C_5–H_5$ ($\delta_C/\delta_H$ 115/6.7 ppm) and $C_6–H_6$ ($\delta_C/\delta_H$ 119/6.8 ppm) correlations.

Signals for the $C_\alpha–H_\alpha$ and $C_\beta–H_\beta$ correlations in the FA cinnamic structure were observed at $\delta_C/\delta_H$ 144/7.5 and 117/6.4 ppm, respectively. Interestingly, the latter olefinic signal was displaced with respect to the position found in the spectrum of free FA (116/6.4 ppm) and exactly matched with that reported for 3,4-dimethoxycinnamic acid [31]. The presence of this signal (and the absence of the above-mentioned $C_\beta–H_\beta$ correlation signal characteristic of free FA), clearly indicated that FA is $C_4$-etherified during its incorporation onto the sisal lignin. The etherified-FA $C_6–H_6$ correlation signal ($\delta_C/\delta_H$ 123/7.1 ppm) appeared with low intensity, while those of $C_2–H_2$ and $C_5–H_5$ correlations ($\delta_C/\delta_H$ at 110/7.1 and 111/6.9 ppm, respectively) would overlap with the lignin $G_2$ signal.

### 3.4. Side-chain signals in 2D NMR spectra of pulp lignins

The main cross-signals in the aliphatic-oxygenated region of the spectra of the lignins isolated from the laccase-FA treated sisal pulp, and the untreated control (Fig. 3c and a, respectively) corresponded to side chains of lignin and FA-derived structures (in the laccase-FA treated pulp) forming different inter-unit linkages and end-units (Fig. 4, structures A–I). These signals correspond to: (i) $C_\alpha–H_\alpha$ ($\delta_C/\delta_H$ 72/4.8 ppm), $C_\beta–H_\beta$ ($\delta_C/\delta_H$ 86/4.1 and 83/4.3 ppm when the second unit is a S or a G unit, respectively) and two $C_\gamma–H_\gamma$ ($\delta_C/\delta_H$ 59/3.4 and 3.7 ppm) correlations in the main β-O-4′ ether substructures (A); (ii) $C_\alpha–H_\alpha$ ($\delta_C/\delta_H$ 87/5.4 ppm) and $C_\beta–H_\beta$ ($\delta_C/\delta_H$ 52/3.3 ppm) correlations in phenylcoumarans (B); (iii) $C_\alpha–H_\alpha$ ($\delta_C/\delta_H$ 85/4.6 ppm), $C_\beta–H_\beta$ ($\delta_C/\delta_H$ 54/3.1 ppm) and two $C_\gamma–H_\gamma$ ($\delta_C/\delta_H$ 71/3.8 and 4.2 ppm) correlations in resinols (C); (iv) $C_\alpha–H_\alpha$ ($\delta_C/\delta_H$ 72/4.8 ppm) and $C_\beta–H_\beta$ ($\delta_C/\delta_H$ 81/51 ppm) correlations in spirodienones (F); and (v) $C_\gamma–H_\gamma$ ($\delta_C/\delta_H$ 63/4.1 ppm) correlation in cinnamyl alcohol end-groups (I).

Moreover, a comparison of the aliphatic region of the 2D NMR spectra revealed that, in addition to the intact cinnamic molecule, FA also incorporates to lignin (in the laccase-FA treated pulp) as the corresponding β–β′ dilactone dimer (structure CL in Fig. 4) with characteristic $C_\alpha–H_\alpha$ and $C_\beta–H_\beta$ correlations at $\delta_C/\delta_H$ 82/5.7

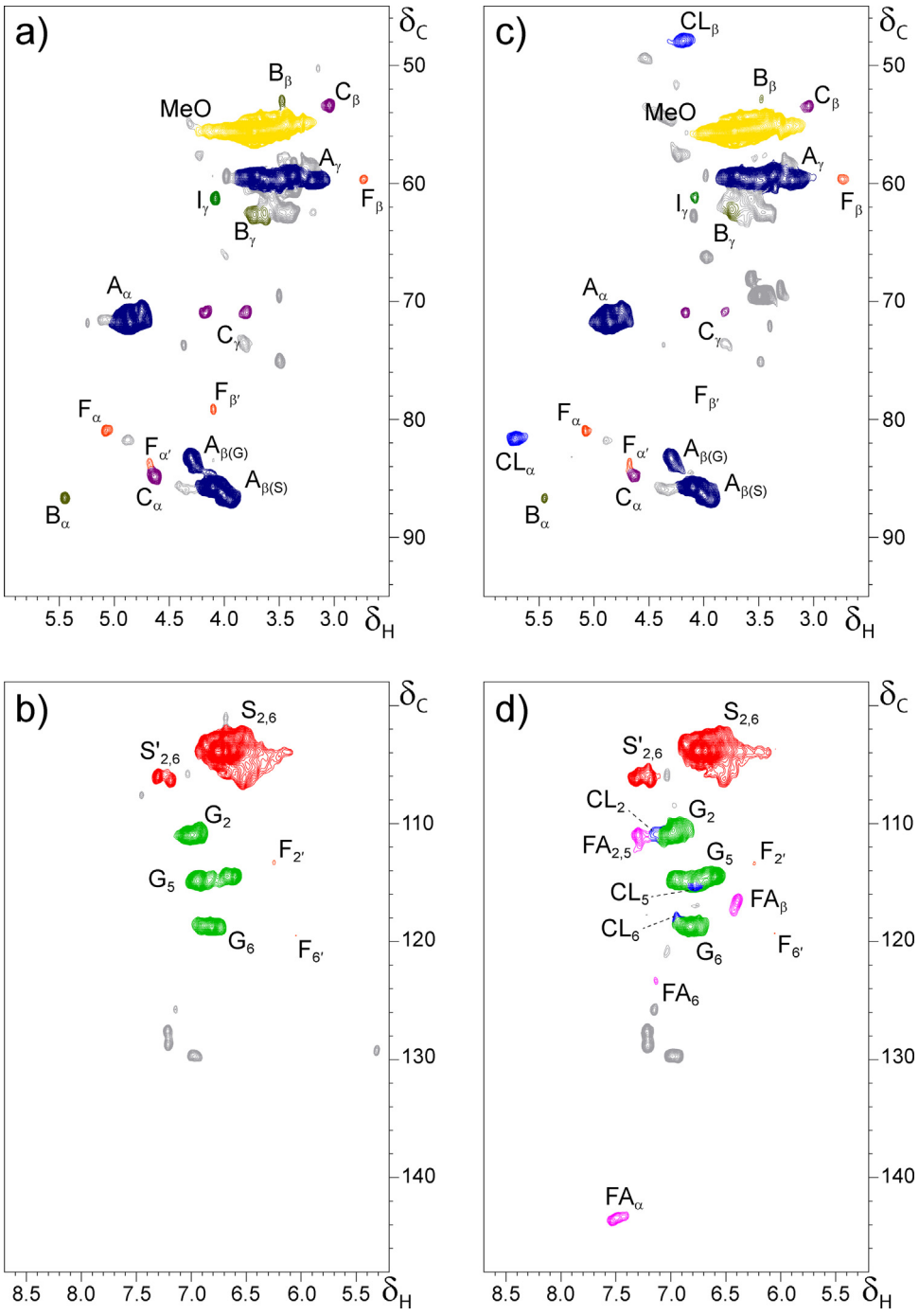


**Fig. 3.** HSQC spectra of the lignins isolated from sisal pulp treated with laccase-FA and the respective control without enzyme. The aliphatic-oxygenated and aromatic/unsaturated regions of the control pulp are shown in a and b, respectively. The aliphatic-oxygenated and aromatic/unsaturated regions of the laccase-FA treated pulp are shown in c and d, respectively. See Fig. 4 for the main structures identified, and Table 1 for their relative abundances.

and 48/4.2 ppm, respectively [31]. The aromatic signals for this FA-derived structure were not mentioned above because they overlap with those of normal (G2, G5 and G6) lignin units (Fig. 3d), and no olefinic signals appear because of the β–β′ linkage and subsequent side cyclization.

### 3.5. Laccase treatment: FA grafting vs lignin modification

A semiquantitative estimation of the different (i) lignin (S, S′ and G) and etherified FA units, from volume integrals of the corresponding aromatic/unsaturated signals, and (ii) lignin (A to I) and FA dilactone side-chain structures, from volume integrals of the corresponding aliphatic-oxygenated signals, in the HSQC spectra of control (Fig. 3a and b) and laccase-FA treated (Fig. 3c and d) pulps is provided in Table 1.

The values obtained showed over 4% etherified FA (estimated from the olefinic $FA_\beta$ signal) referred to the lignin content (estimated from the S, S′ and G signals) in the lignin isolated from the laccase-FA treated pulp. The FA aromatic signals are consistent with FA coupling (onto lignin) through a $C_4$-ether linkage maintaining the olefinic side chain. Moreover, the aliphatic region of the spectrum of the lignin isolated from the laccase-FA treated pulp

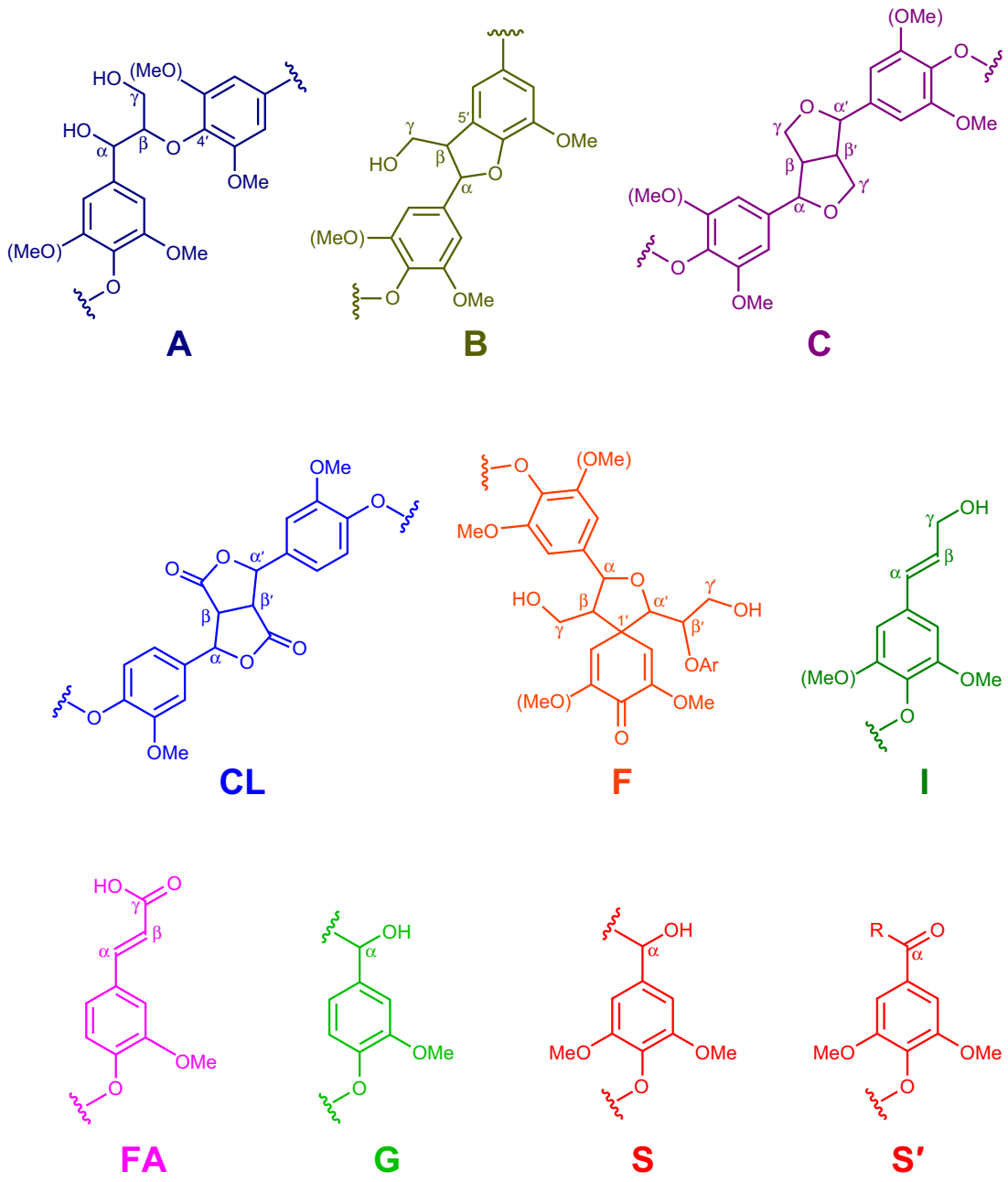


**Fig. 4.** Main substructures identified in HSQC spectra (Fig. 3) of isolated sisal lignins: (A) β-O-4′ ether structure; (B) phenylcoumaran; (C) resinol; (CL) FA dilactone; (F) spirodienone; (I) *trans* cinnamyl end-group; (FA) etherified *trans* ferulic acid; (G) guaiacyl unit; (S) syringyl unit; (S′) $C_\alpha$-oxidized syringyl unit.

revealed that a similar percentage of the lignin-linked FA (~4% referred to the lignin content estimated by NMR) is coupled to a second FA molecule forming a dilactone structure (CL in Fig. 4, resulting from FA radical β–β′ coupling followed by side chain cyclization through α-O-γ′ and α′-O-γ ether linkages). These lactones represent 7% when referred to lignin side-chain signals (A to I) and 4.5% when referred to lignin aromatic signals (S + S′ + G).

On the other hand, although much less efficient than *p*-coumaric acid and synthetic compounds such as 1-hydroxybenzotriazole, FA has also been reported as a ("natural") redox mediator in laccase oxidation of different compounds [32]. Therefore, the semiquantitative analysis of the HSQC spectra also considered the eventual modification of lignin that might occur in parallel to FA biografting. The percentages of lignin substructures (characterized by different side-chain interunit linkages) were practically identical in the laccase-FA treated pulp and the control pulp (2% difference in β-O-4′ relative abundance and only 1% difference in the abundances of all the other substructures). However, slight differences in lignin composition, in terms of S and G units were observed, including higher amounts of S′ and G units and lower amounts of S units in the lignin from the laccase-FA treated sisal pulp, in agreement with the decrease of S/G ratio observed by pyrolysis-GC/MS (Table S1). The former difference (S′ increase from 3% to 6%) agrees with the general tendency found in previous NMR studies on lignocellulose treatment with other laccase-mediator systems, although the extent of $C_\alpha$-oxidation of lignin S units found here is much lower than observed when typical mediators (such as HBT or methyl syringate) are used [33,34]. The 5–6% increase of G units, and equivalent decrease of total S units, would suggest a slight preference of the laccase-FA system degrading S vs G units. However, formation of typical (non-cinnamic) G-type structures during FA coupling (such as in the dilactone structures discussed above) would also

**Table 1**
Quantification of main lignin structures and units, and FA-derived structures in control and laccase-FA treated sisal pulps, as estimated from HSQC spectra (Fig. 3).

| | Control pulp | Treated pulp |
|---|---|---|
| *Lignin substructures* (%)[a] | | |
| β-*O*-4′ (A) | 88 | 90 |
| Phenylcoumaran (B) | 2 | 1 |
| Resinol (C) | 4 | 3 |
| Spirodienone (F) | 3 | 2 |
| Cinnamyl alcohol end-group (I) | 3 | 3 |
| *Lignin units* (%)[b] | | |
| Syringyl units (S) | 85 | 77 |
| Oxidized syringyl units (S′) | 3 | 6 |
| Guaiacyl units (G) | 11 | 17 |
| *Ferulic structures/units* (%) | | |
| FA dilactone structures (CL)[a] | 0 | 7.0 (4.5[b]) |
| Etherified ferulic acid units (FA)[b] | 0 | 4.2 |

[a] Percentage of side-chains (A + B + C + F + I).
[b] Percentage of lignin (S + S′ + G).

contribute to the relative increase of the G units. Concerning the different interunit linkages in sisal lignin, the percentage of side chains involved in β-O-4′ linkages (88–90%), resinols (3–4%), spirodienones (2–3%) and phenylcoumarans (1–2%) were very similar in the treated and the control pulps, and the same happened for the cinnamyl end-groups (3%) (Table 1), while modification of linkage percentages have been reported using other laccase-mediator systems [33].

Taking the above results together, it is therefore possible to conclude that lignin composition in the treated pulps was only slightly modified with respect to the controls (showing a small increase in $C_\alpha$-oxidized S units) and that the main effect of the enzymatic treatment on high kappa-number sisal pulp was FA biografting. Oxidative coupling of FA esters in oligomeric structures has been investigated as a model for lignin-ferulate cross-links in grasses [35–37]. However, this is the first study where coupling of free FA directly on the pulp residual lignin, with the aim of functionalizing lignocellulosic fibers by enzymatic grafting, is investigated using 2D NMR.

## 4. Conclusions

Laccase is highly efficient in incorporating FA on high lignin sisal pulp, as suggested by the increases of kappa number (11 points) and bulk acid-group content (40%) and demonstrated by Py/TMAH. 2D NMR of the enzymatically isolated lignins confirmed that grafting is produced on the lignin component of the pulp. Moreover, information on the linkage types formed during laccase grafting of phenols onto pulp was provided by both the aromatic/unsaturated and aliphatic-oxygenated regions of the NMR spectra. While the former showed significant amounts of FA etherified (to lignin) at its C4 position, the latter revealed formation of a similar amount of FA dilactone structures from coupling at the side chain level. Although minor modification of the sisal lignin composition was observed after the laccase-FA treatment, we could conclude that biografting is the predominant modification during sisal pulp treatment with laccase-FA.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2014.02.013.

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