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Enzymatic grafting of simple phenols on flax and sisal pulp fibres using laccases

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ABSTRACT

Flax and sisal pulps were treated with two laccases (from *Pycnoporus cinnabarinus, Pc*L and *Trametes villosa*, *Tv*L, respectively), in the presence of different phenolic compounds (syringaldehyde, acetosyringone and *p*-coumaric acid in the case of flax pulp, and coniferaldehyde, sinapaldehyde, ferulic acid and sinapic acid in the case of sisal pulp). In most cases the enzymatic treatments resulted in increased kappa number of pulps suggesting the incorporation of the phenols into fibres. The covalent binding of these compounds to fibres was evidenced by the analysis of the treated pulps, after acetone extraction, by pyrolysis coupled with gas chromatography/mass spectrometry in the absence and/or in the presence of tetramethylammonium hydroxide (TMAH) as methylating agent. The highest extents of phenol incorporation were observed with the *p*-hydroxycinnamic acids, *p*-coumaric and ferulic acids. The present work shows for the first time the use of analytical pyrolysis as an effective approach to study fibre functionalization by laccase-induced grafting of phenols.

1. Introduction

The pulp and paper industry is under steady and ever-increasing pressure from global competition, stringent environmental regulations and new market demands. Enzymes are the most promising examples of new technologies designed to help the sector meet these challenges for their potential to supply specific reactions, to provide less environmentally deleterious processes, to reduce resources consumption and, ultimately, to decrease costs (Kenealy and Jeffries, 2003; Ragauskas, 2002). Since the raw materials employed for the manufacture of pulp and paper are natural fibres, the possibilities for introducing biotechnologies in this process are numerous (Bajpai, 1999).

Among the most investigated enzymes in the field of pulp and paper are laccases (EC 1.10.3.1), multi-copper oxidases, produced by microorganisms and plants, which participate in nature in both the biosynthesis and degradation of lignin (ten Have and Teunissen, 2001). Laccases catalyze the oxidation of various substrates, including phenols, diphenols, aminophenols, polyphenols and polyamines, with concomitant reduction of oxygen to water (Yaropolov et al., 1994). Their high availability at a reasonable price and broad oxidative capabilities make them suitable for a wide range of applications (Riva, 2006; Widsten and Kandelbauer, 2008).

Most of the earlier research focused on the potential of laccase for the biobleaching of pulp (Bourbonnais and Paice, 1990; Camarero et al., 2004; Fillat and Roncero, 2010; Valls and Roncero, 2009). In previous works (Aracri et al., 2009; Fillat et al., 2010) several lignin-derived phenols were assayed as laccase mediators for aiding pulp delignification in a bleaching sequence. Although no immediate improvement or even a slight loss of pulp properties, in terms of kappa number and brightness, was observed immediately after the enzymatic treatment, the delignification effect was observable at the end of the bleaching sequence.

When natural phenols are applied as laccase mediators to perform pulp bleaching, the delignification effect can be hindered by adverse reactions involving the phenoxy radicals generated upon the mediator enzymatic oxidation, such as depleting reactions (i.e. homopolymerization and cross-coupling reactions in the lignin structure) or fragmentations (d'Acunzo and Galli, 2003; Moldes et al., 2008). As a consequence, the treatment of lignocellulosic fibres and phenolic compounds with laccases are likely to result in a variety of oxidation and coupling products which are difficult to predict due to the complexity of the lignocellulosic matrix and the nature of free radical reactions (Kenealy et al., 2003).

On one hand, radical coupling reactions competing with delignification represent an adverse and undesirable phenomenon in biobleaching process (Camarero et al., 2007). On the other hand, they have been drawing increasing attention for being the key-mechanisms behind the laccase-assisted grafting of low-molecular weight phenols onto pulp fibres. This is a new approach

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to the use of these compounds, aiming at imparting better or novel properties to pulps and papers (Chandra and Ragauskas, 2002; Liu et al., 2009). Fibres modification, especially with the assistance of enzymes, is a rapidly growing field of research and interest (Viikari, 2002). Laccase-catalyzed bio-grafting is a versatile functionalization method due to the enzyme's nonspecific substrate requirements, which allow bonding a wide range of phenolic compounds and thus incorporating several desired properties into the fibre matrix (Chandra et al., 2004; Elegir et al., 2008; Grönqvist et al., 2006). The feasibility of this approach has been demonstrated in numerous studies; however, the interest has been focused mainly on wood materials and lignin-rich fibres.

In this work, a novel approach of analysis was adopted to gain an insight on the mechanism of the laccase-induced coupling of natural phenols onto flax and sisal pulps. In particular, the treated pulps were analyzed by analytical pyrolysis, a powerful and sensitive tool for the "in situ" analysis of residual lignin in pulps without the need of prior isolation (del Río et al., 2001). In addition, in order to avoid some of the analytical limitations of the pyrolysis technique, such as decarboxylation of carboxyl groups, the pyrolysis was also performed in the presence of tetramethylammonium hydroxide (TMAH) as methylating reagent (del Río et al., 1996), that allowed the detection of intact carboxylic acids as their methyl derivatives.

2. Methods

2.1. Raw materials, laccases and natural phenols

Flax (*Linum usitatissimum*) and sisal (*Agave sisalana*) alkaline pulps were obtained by soda-anthraquinone cooking and were supplied by CELESA pulp mill (Tortosa, Spain). Flax and sisal pulps had a kappa number of 7.00 and 7.75, a viscosity of 816 and 784 mL/g, and an ISO brightness of 38.8% and 47.3%, respectively. Both pulps were treated with laccases and different phenols, as reflected in Table 1. Both laccases are from white-rot fungi and have similarly high redox potential. Prior to the enzymatic treatments, the pulp was washed with acidified water (pH 4) for 30 min, at 2% pulp consistency, followed by filtration and extensive washing with de-ionized water. This procedure was necessary to remove contaminants and metals and to bring the pulp to the pH of the enzymatic treatments. *Pycnoporus cinnabarinus* laccase (*Pc*L) was produced by Beldem (Belgium) and *Trametes villosa* laccase (*Tv*L) by Novozymes (Denmark). Activity was followed by measuring the ABTS oxidation in 0.1 M sodium acetate buffer (pH 5) at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}). One activity unit was defined as the amount of laccase that transforms 1 μmol/min of ABTS at 25 °C. All measurements were carried out using a Shimadzu UV–vis 1603. The phenolic compounds (syringaldehyde, acetosyringone, *p*-coumaric acid, ferulic acid, sinapic acid, coniferaldehyde and sinapaldehyde) were purchased from Sigma–Aldrich.

2.2. Laccase treatments and Soxhlet extraction

Laccase treatments were carried out in an oxygen pressurized (0.6 MPa) reactor in 50 mM sodium tartrate buffer (pH 4) and 20 U/g of laccase at 50 °C. Tween 80 (0.05%, w/v) was added as a surfactant. The specific conditions for each pulp treatment are the following: 25 g odp of flax pulp were treated at 3% of consistency during 5 h at 60 rpm shaking with a mediator dose of 3% (w/w); 40 g odp of sisal pulp were submitted to the same treatment although the consistency, time, shaking and mediator dose were 5%, 4 h, 30 rpm and 1.5% (w/w), respectively. Pulp samples treated under identical conditions, but in the absence of the phenolic compound, were used as controls. After the enzymatic treatment, pulps were filtered and extensively washed with de-ionized water. Thereafter, they were extracted with acetone in a Soxhlet apparatus for 2 h and 15 min in order to eliminate the phenolic compounds adsorbed on the pulp.

Table 1
Laccase and natural phenols used in laccase treatments.

	Laccase	Natural mediators
Flax pulp	*Pycnoporus cinnabarinus*	Syringaldehyde (SA) Acetosyringone (AS) *p*-Coumaric acid (PCA)
Sisal pulp	*Trametes villosa*	Sinapic acid (SNC) Ferulic acid (FRC) Coniferaldehyde (CLD) Sinapaldehyde (SLD)

2.3. Evaluation of pulp properties

Brightness and kappa number of pulps before and after acetone extraction were assessed according to ISO 3688 and ISO 302, respectively. A straightforward method was developed to obtain an estimation of the amount of grafted phenol: kappa numbers of phenol solutions, where the presence of 1 g of totally bleached pulp was supposed, were measured for different phenol concentrations. Thus, a calibration line was originated, providing the amount of grafted phenol in correspondence to the increase of kappa number produced by this compound with respect to laccase control (samples coming from the acetone extraction). Calibration lines were:

$y = 166.55x + 0.6554$ (PCA),
$y = 141.77x + 0.2969$ (SA),
$y = 130.65x + 0.2876$ (AS),
$y = 150.75x + 0.0404$ (SLD),
$y = 153.72x + 0.0336$ (SNC),
$y = 147.36x + 0.1416$ (CLD), and
$y = 150.68x + 0.2774$ (FRC).

If the coexistence of grafting and delignification reactions is supposed, this method will provide the *minimum* amount of phenol onto fibres.

The optical properties of pulp were analyzed using a reflectance measuring Technidyne Colour Touch apparatus at standard illuminant D65 (LAV/Spec. Excl., d/8, $D_{65}/10°$). The colour of the samples was described according to the CIE $L^*a^*b^*$ colour system, where L^*, a^* and b^* are the coordinates of the colour in the cylindrical colour space, based on the theory that colour is perceived as L^* (Lightness, which varies from 100 for a perfect white to 0 for absolute black), a^* (which varies from greenness to redness), and b^* (which varies from blueness to yellowness, from negative to positive values) (Hunt, 1998).

Other optical parameters used were:

- Chroma (C^*):
  Perpendicular distance from lightness axis, measure of colour saturation,

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

- Dye Removal Index (DRI) (Fluet and Shepperd, 1997):
  The percentage of original colour removed by the treatment,

$$DRI = -100[\Delta R^2/R_1^2]$$

where,

$$R^2 = a^2 + b^2 + (100 - L)^2$$

is the geometric distance from the pulp CIE $L^*a^*b^*$ location to the ideal bleach point where $a^* = b^* = 0$, and $L^* = 100$,

$$\Delta R^2 = R_2^2 - R_1^2$$
$$= R^2 \text{ (for treated pulps)} - R^2 \text{ (for reference pulp)}$$

Pulps treated with laccase alone (in the absence of phenolic compounds) were used as reference, therefore positive values represent colour removal and negative ones represent colouration.

2.4. Pyrolysis–gas chromatography/mass spectrometry (Py–GC/MS)

Pyrolysis of pulps (approximately 1 mg) was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a DB-5MS (Agilent J&W) fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The oven temperature was programmed from 40 °C (1 min) to 300 °C at 6 °C min^{-1} (10 min) and the carrier gas (He) was set at 1 mL min^{-1}. In addition, pulp samples were analyzed by pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), as a base and methylating reagent. For the Py/TMAH, 1 mg of pulp sample were mixed with approximately 0.5 μL TMAH (25%, w/w, aqueous solution) and the pyrolysis was carried out as described above. The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature (Faix et al., 1990; Ralph and Hatfield, 1991).

3. Results and discussion

3.1. Flax pulp treated with PcL and natural phenols

The flax pulp was treated with the laccase from *P. cinnabarinus* (*Pc*L) in the presence of syringaldehyde (SA), acetosyringone (AS) and *p*-coumaric acid (PCA), as reflected in Table 1. After the laccase treatments, flax pulps showed an increase in kappa number (Fig. 1) when treated with SA, and especially, when treated with PCA (more than 10 points increase). In the case of laccase treatment with AS, the kappa number obtained was very similar to the control pulp. In order to eliminate the low molecular-mass phenols contribution to kappa number, a Soxhlet extraction with acetone was carried out to remove these compounds adsorbed on the pulp. After acetone washing, the kappa number decreased in all cases, although they were still higher than the control pulp when using SA and PCA. These results suggest the occurrence of crosslinking or cross-coupling reactions of these phenolic compounds into the fibres. The highest increase of kappa number was observed with PCA (*ca.* 3 points higher than the control pulp), indicating a higher degree of PCA incorporation into the flax fibres. The minimum amount of grafted compound was estimated from the increase of kappa number with respect to the control pulp in the laccase treated pulps after acetone extraction. The minimum amount, determined by the calibration line was estimated to be 12.3 μmol/g of pulp for PCA treatments and 2.1 μmol/g of pulp for SA treatments. Interestingly, in a previous study (Fillat et al., 2010), we demonstrated the capacity of some of these phenolic compounds (AS and SA) to delignify flax pulp, when a subsequent hydrogen peroxide stage was applied.

Besides the increase in kappa number, the pulp treatment also showed a decrease of pulp brightness (5–14% decrease), which suggests the formation of chromophores groups due to the oxidative action of the enzyme treatment and/or the grafting of the phenolic compounds onto the pulp. After acetone extraction, the brightness remained lower than the control pulp, similarly to kappa number results, except in the case of the SA treated pulp, in which the brightness increases just a 6% respect to the non-extracted sample. This may be explained on the basis of the radical coupling reactions between the natural phenols and the fibres.

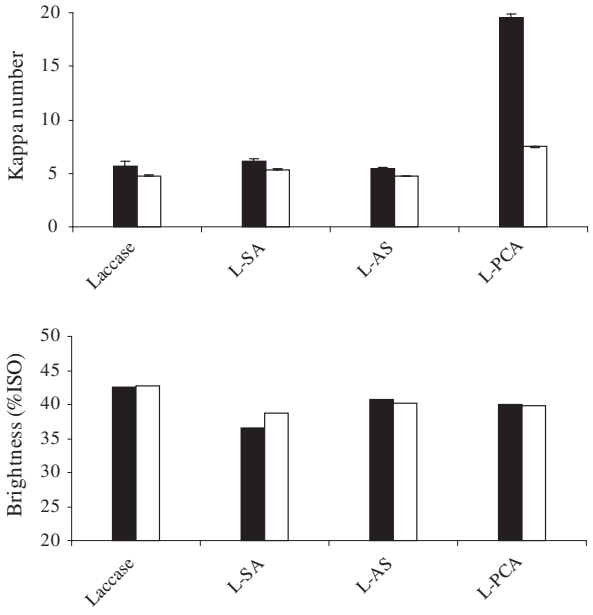

Fig. 1. Kappa number and brightness of flax pulps after the enzymatic treatment (black bars) and the subsequent Soxhlet extraction with acetone (white bars). Laccase control sample was treated in the absence of phenolic compounds. The dashed line indicates the value of the initial pulp.

Additional optical properties, such as Chroma (C^*, that indicates the colour saturation) and Lightness (L^*, that indicates the light amount of a colour), were also analyzed. Fig. 2a shows two groups of C^* and L^* data corresponding to pulps treated only with laccase and to pulps treated with laccase and the different phenolic compounds. The assayed compounds led to a clearly C^* increase (around a 20% increase), while the control pulp only showed an L^* enhance (2 points higher than the starting pulp). This C^* increase is in agreement with the previously suggested coupling reactions and the possible generation of quinones. The Dye Removal Index (DRI) is another optical parameter providing information about the loss of colour associated to an enzymatic process. This index indicates how much colour is removed during a bleaching process. All the phenols assayed here showed negative DRI values that denotes a formation or coupling of coloured products (Table 2). The more negative value of DRI after LMS treatment, and following extraction with acetone, for SA in comparison with the other samples, matched up with the lower brightness values obtained and indicated a colouration process.

In order to confirm the incorporation of the different simple phenols assayed here into the flax pulp fibres, the acetone extracted laccase treated pulps were analyzed Py–GC/MS, both in the absence and in the presence of tetramethylammonium hydroxide (TMAH). Py–GC/MS is a powerful and sensitive tool to analyze residual lignin in pulp without the need of previous isolation (del Río et al., 2001). Among the different laccase treated flax pulps, the Py–GC/MS of the pulp treated with PCA released high amounts of 4-vinylphenol, a compound arising from the decarboxylation of PCA during pyrolysis (del Río et al., 1996), and which was absent in the control pulps (Table 3). In addition, pyrolysis in the presence of TMAH (Py/TMAH) of this pulp released high amounts of intact PCA as its methyl derivative (i.e. the methyl ester of 4-methoxycinnamic acid), and which is absent in the control pulp (treated with laccase alone) (Table 3). These data clearly demonstrate that PCA is covalently bound to the pulp fibres, and are in agreement with the high increase observed in the kappa number of this fibre. In

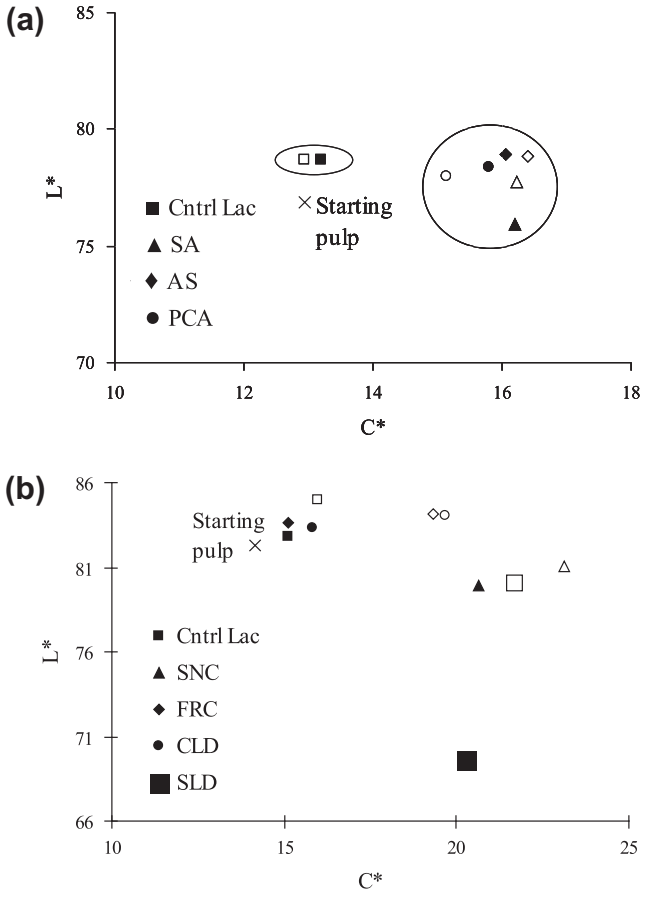

Fig. 2. Chromatic coordinates (L^* and C^*) of flax pulp (a) and sisal pulp (b) treated with different enzymatic systems (black spots) and after a subsequent Soxhlet extraction with acetone (white spots). Laccase represents the control pulp sample: pulp treated only with laccase.

Table 2
Dye Removal Index of flax and sisal pulps after treatment with laccase and natural phenols, and after subsequent Soxhlet extraction.

	SA	AS	PCA
Flax pulp			
LMS treatment	−33.89	−11.39	−14.01
Soxhlet extraction	−20.80	−14.08	−13.67
	SNC	**FRC**	**CLD**	**SLD**
Sisal pulp				
LMS treatment	−58.72	5.07	−0.64	−155.26
Soxhlet extraction	−85.13	−29.33	−32.73	−80.27

Laccase control pulp used as reference (DRI = 0%).

the case of pulps treated with SA and AS, the analysis by Py–GC/MS and Py/TMAH also demonstrated the grafting of a part of these phenolic compounds onto the pulp fibres (Table 3). However, the lower kappa number of the pulps treated with *Pc*L and AS or SA indicates a lower degree of grafting in comparison with that treated with PCA.

3.2. Sisal pulp treated with TvL and natural phenols

The sisal pulp was treated with the laccase from *Trametes villosa* (*Tv*L) and ferulic acid (FRC), sinapic acid (SNC), coniferaldehyde (CLD) and sinapaldehyde (SLD). All of these phenolic compounds

Table 3
Products released upon Py–GC/MS and Py/TMAH of the flax and sisal pulps treated with laccases and different phenolic compounds.

Phenolic compound	Py–GC/MS products	Py/TMAH products
Flax pulp		
Acetosyringone (AS)	Acetosyringone	Acetosyringone (methyl derivate)
Syringaldehyde (SA)	Syringaldehyde	Syringaldehyde (methyl derivate)
p-Coumaric acid (PCA)	4-Vinylphenol	*p*-Coumaric acid (methyl derivate)
Sisal pulp		
Ferulic acid (FRC)	4-Vinylguaiacol	Ferulic acid
Sinapic acid (SNC)	n.d.	Syringic acid (methyl derivate)
Coniferaldehyde (CLD)	4-Vinylguaiacol	Vanillin (methyl derivate) + ferulic acid (methyl derivate)
Sinapaldehyde (SLD)	n.d.	n.d.

n.d.: No phenolic markers detected.

employed in the laccase treatments of sisal pulps resulted in increased kappa numbers, with respect to the control pulp (Fig. 3), suggesting the occurrence of grafting copolymerization reactions of the phenolic compounds with the fibres. After acetone extraction, a slight decrease of kappa number was observed in all pulps; however, all the laccase treated pulps still presented a kappa number higher than the control pulp, as already observed in the case of the flax pulp treated with *Pc*L and other phenols, thus indicating that a binding of these simple phenols onto fibres effectively occurred.

The kappa numbers of the acetone-extracted pulps indicated a higher degree of grafting for the guaiacyl-type phenolic compounds, FRC and CLD (causing 42% and 36% kappa number increases, respectively) than for the syringyl-type phenolic compounds, SNC (23% increase) and SLD (16% increase). This difference may be due to the steric effects of the structure of the phenols. SNC and SLD are of syringyl-type, bearing two methoxy

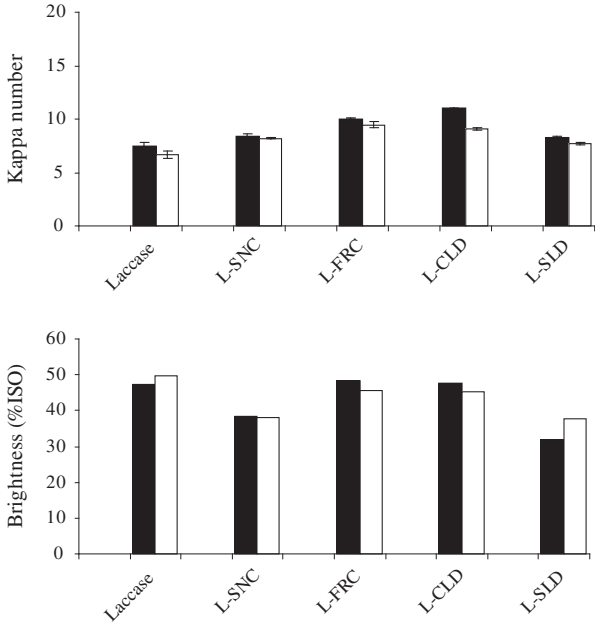

Fig. 3. Kappa number and brightness of sisal pulps after the enzymatic treatment (black bars) and the subsequent Soxhlet extraction with acetone (white bars). Laccase control sample was treated in the absence of phenolic compounds. The dashed line indicates the value of the initial pulp.

groups, and present more steric hindrance than FRC and CLD, of guaiacyl-type (monomethoxylated), which increases the long-living of the phenoxy radicals and prevents coupling reactions, thus resulting in less grafting onto the fibres (Astolfi et al., 2005). The minimum amount of grafted phenol for the laccase treated sisal pulps was estimated to be 6.6, 9.6, 17.3 and 16.9 μmol/g of pulp for SLD, SNC, CLD and FRC, respectively.

A marked decrease of brightness, respect to the control pulp, was observed in the pulps treated with the syringyl-type phenols, SNC (18% decrease) and SLD (32% decrease), while this parameter was barely modified after treatment with the guaiacyl-type phenols, FRC and CLD. After acetone extraction, no improvement of brightness was obtained in the treated pulps, by exception of that treated with SLD, in which the removal of the adsorbed products of oxidation resulted in 18.5% increase of brightness compared to the non-extracted sample.

The optical properties corresponding to L^* and C^* data of the laccase treated sisal pulps are shown in Fig. 2b. Similarly to brightness, Chroma and Lightness properties of FRC and CLD treated pulps did not result in significant difference with respect to control pulp, whilst a marked increase of colour saturation was induced by SNC and SLD (26.6% and 25.4%, respectively), the latter being accompanied by a pronounced loss of L^* (16%). The removal of the adsorbed phenol from pulps by acetone extraction resulted in enhanced Lightness in all enzymatically treated pulps. However, no Chroma decrease was observed in any case, indicating that the quinone-like oxidized compounds were still present in the extracted pulps. DRI values (Table 2) followed the same trend as C^* data, being more negative after acetone extraction than after the L stage, by exception of SLD-treated pulp, which also experimented the most important Lightness gain after the extraction.

The analysis of the laccase treated sisal pulps (after acetone extraction) by Py–GC/MS and Py/TMAH also demonstrated, as in the case of flax pulp, the incorporation of some of these phenolic compounds into the fibres (Table 3). Py–GC/MS of the sisal pulp treated with FRC released high amounts of 4-vinylguaiacol, a compound arising from the decarboxylation of the FRC during pyrolysis (del Río et al., 1996), and which was completely absent in the control pulps. This data was further confirmed by Py/TMAH of this pulp that released intact FRC as its methyl derivative (i.e. the methyl ester of 3,4-dimethoxycinnamic acid), which was absent in the control pulp (Table 3), indicating that FRC is covalently bound to the pulp. In the case of sisal pulp treated with CLD, Py–GC/MS released some structurally related compounds, such as 4-vinylguaiacol, but not CLD as such. The analysis of this pulp by Py/TMAH also released other structurally related compounds (i.e. vanillin and ferulic acid, as their methyl derivatives) but not intact CLD. This seems to indicate that CLD does not incorporate as such into the sisal pulps but in a different or modified form, probably as vanillin and/or ferulic acid. In the case of SNC, Py–GC/MS and Py/TMAH did not release any compound that could clearly indicate its incorporation as such into the pulps, although a significative increase of syringic acid (as its methyl derivative) was observed by Py/TMAH. This could indicate that SNC is probably incorporated into the pulps as syringic acid. In the case of SLD, neither Py–GC/MS nor Py/TMAH could detect any phenolic marker indicative of its incorporation into the pulp, and therefore, in this particular case, we could not firmly demonstrate the incorporation of SLD to the pulp fibres.

4. Conclusions

The enzymatic treatment of non-wood pulps with laccases in the presence of simple phenols resulted in their incorporation into the pulps. This assertion is based on the pulp properties obtained, and on the Py–GC/MS and Py/TMAH results. This enzymatic system could be regarded as a method for the grafting of phenols onto the pulp fibres, which may give them improved or novel properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.05.080.

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