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Main lipophilic extractives in different paper pulp types can be removed using the laccase–mediator system

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Abstract Lipophilic extractives in wood and other lignocellulosic materials exert a negative impact in pulp and paper manufacturing causing the so-called pitch problems. In this work, the appropriateness of an enzymatic treatment using the laccase–mediator system for pitch biocontrol is evaluated. With this purpose, three pulp types representative for different raw materials and pulping processes—eucalypt kraft pulping, spruce thermomechanical pulping, and flax soda-anthraquinone pulping—were treated with a high-redox-potential laccase from the basidiomycete *Pycnoporus cinnabarinus* in the presence of 1-hydroxybenzotriazole as a redox mediator. The gas chromatography and gas chromatography/mass spectrometry analyses of the lipophilic extractives from the enzymatically treated pulps revealed that the laccase–mediator treatment completely or greatly removed most of the pitch-causing lipophilic compounds present in the different pulps including: (1) free and conjugated sitosterol in eucalypt paper pulp; (2) resin acids, sterol esters, and triglycerides in spruce pulp; and (3) sterols and fatty alcohols in the flax pulp. Different amounts of free and conjugated 7-oxosterols were found as intermediate products in the oxidation of pulp sterols. Therefore, the laccase–mediator treatment is reported as an efficient method for removing pitch-causing lipophilic compounds from paper pulps obtained from hardwood, softwood, and nonwoody plants.

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Introduction

Lipophilic extractives, the non-polar extractable fraction from lignocellulosic materials, cause technical, economic, and environmental troubles during pulp and paper manufacturing, commonly referred to as pitch problems (Back 2000). Different lipophilic extractives may cause pitch problems along the entire pulp and paper manufacturing processes (pulping, bleaching, and paper machine) depending on their chemical nature and on the process used. The use of totally chlorine-free bleaching in place of elementary chlorine-free bleaching is increasing the severity of pitch problems due to lower reactivity with pulp lipids (Gutiérrez et al. 2001b). Pitch deposition in pulp or equipment results in low-quality pulp and paper, and can cause the shutdown of mill operations (Hillis and Sumimoto 1989).

Traditionally, pitch deposits have been reduced by debarking and seasoning logs and chips, and by adding physicochemical agents. However, the cost is high and the results are often far from satisfactory (Back and Allen 2000). As an alternative, biological removal of extractives by treatment with enzymes (Fischer and Messner 1992; Fischer et al. 1993; Fujita et al. 1992) or microorganisms (Behrendt and Blanchette 1997; Farrell et al. 1993; Gao et al. 1994; Gutiérrez et al. 1999b, 2001b) has been suggested. Nevertheless, the commercially available preparations are not fully effective because they are based on enzymes (lipases) or organisms mainly hydrolyzing triglycerides. In addition to lipases, the use of sterol esterases has also been suggested (Calero-Rueda et al. 2002; Kontkanen et al. 2004). However, free sterols are as problematic as sterol esters. Therefore, new methods to solve pitch problems caused by non-easily hydrolyzable and biodegradable lipophilic extractives are still required.

Modification of some lipophilic extractives during laccase treatment of softwood pulping model process waters has been suggested (Buchert et al. 2002; Karlsson et al. 2001; Zhang et al. 2000). In contrast to lipases and sterol esterases, laccases are oxidative enzymes acting mainly on phenolic compounds. However, the use of the laccase–

mediator systems strongly expanded the potential of laccases in the degradation of lignin and other compounds (Bourbonnais and Paice 1990). Although the laccase–mediator treatment is considered as selective on lignin, it also slightly oxidizes cellulose. However, it has been demonstrated that this oxidation is only partial and reversible (a reductive stage allows to recover the initial viscosity) (Camarero et al. 2004). Many studies have been done on the laccase–mediator system for pulp delignification and bleaching (Bajpai 1999; Call and Mücke 1997). However, little is known about its eventual action on lipophilic extractives. In this work, the suitability of laccase–mediator treatment for the removal of lipophilic extractives from wood (including hardwood and softwood species) and nonwoody plant paper pulps (obtained by mechanical and chemical processes) is investigated using the laccase from the ligninolytic fungus *Pycnoporus cinnabarinus* (Sigoillot et al. 2005) in the presence of a synthetic redox mediator.

Materials and methods

Pulps

Eucalypt (*Eucalyptus globulus*) kraft pulp was obtained from the ENCE mill in Pontevedra (Spain). The unbleached (brown) eucalypt pulp, sampled after kraft pulping and oxygen delignification, had a kappa number of 14.2 and ISO brightness of 41.2% estimated following standard methods (International Organisation for Standardization Documentation and Information (ISO) 2003). Unbleached thermomechanical (TMP) pulp from Norway spruce (*Picea abies*) was obtained from the UPM-Kymmene pulp mill in Valkeakoski (Finland) after the primary refiner (63% ISO brightness). The unbleached flax (*Linum usitatissimum*) pulp from soda-AQ cooking was supplied by the CELESA mill in Tortosa (Spain) and had a kappa number of 11 and ISO brightness of 37%.

Fungal laccase and mediator

The laccase preparation used to treat the pulps was provided by Beldem (Andenne, Belgium). It was obtained from a laccase-hyperproducing strain of the fungus *P. cinnabarinus* (Herpoël et al. 2000). Laccase activity was measured by oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, from Roche) to its cation radical (ε_{436} 29,300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24°C. One activity unit was defined as the amount of enzyme transforming 1 μmol of ABTS per minute. 1-Hydroxybenzotriazole (HBT) was obtained from Sigma-Aldrich.

Pulp treatment with laccase–mediator

Pulp treatments were carried out in 4-liter stainless steel reactors using 200 g of pulp (dry weight) at 10% consistency under 6 kg/cm^2 O_2 pressure, except for the spruce TMP pulp that was treated under atmospheric pressure with oxygen bubbling. The treatments were performed using laccase (20 U/g pulp) and HBT (1.5%, referred to pulp dry weight) at pH 4, for 2 h at 50°C. Controls for evaluating the action of the enzymatic treatment were treated under the same conditions but without laccase and mediator. Additional controls including treatments with laccase without mediator and with mediator alone were also performed.

Lipid extraction from pulps and liquids

The enzymatically treated pulps and the corresponding controls were separated from their treatment liquid by filtration. Pulps were dried (40°C) and two samples were extracted with acetone for 8 h. Treatment liquid samples were extracted with methyl *tert*-butyl ether (MTBE) and analyzed to investigate the eventual release of lipids from pulps. All extracts were evaporated to dryness and redissolved in chloroform for analysis of the lipophilic fraction by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The whole acetone and MTBE extracts were completely soluble in chloroform revealing their lipophilic nature. Bis(trimethylsilyl)trifluoroacetamide (from Supelco) in the presence of pyridine was used to prepare trimethylsilyl derivatives.

GC and GC/MS analyses of lipophilic extracts

The GC analyses of the extracts were performed in an Agilent 6890N Network GC system using a short-fused silica capillary column (DB-5HT, 5 m × 0.25 mm I.D., 0.1-μm film thickness) from J&W Scientific. The temperature program was started at 100°C with a 1-min hold and then raised to a final temperature of 350°C at 15°C/min, and held for 3 min. The injector and flame-ionization detector temperatures were set at 300 and 350°C, respectively. The carrier gas was helium at a rate of 5 ml/min, and the injection was performed in splitless mode. Peaks were quantified by area in the GC chromatograms. A mixture of standard compounds (including octadecane, hexadecanol, palmitic acid, dehydroabietic acid, sitosterol, 7-oxocholesterol, sitosteryl 3-β-D-glucopyranoside, cholesteryl oleate, and triheptadecanoin) was used to produce calibration curves for the quantitation of the lipophilic extractives. The correlation coefficient was higher than 0.99 in all cases. The data from the two replicates were averaged. In all cases, the standard deviations from

replicates were below 10% of the mean values. When some peaks overlapped (e.g., fatty and resin acids in spruce extracts), separate integration in single-ion GC/MS chromatograms (see below) was used together with GC integration.

The GC/MS analyses were performed with a Varian model Star 3400 GC equipped with an ion trap detector (Varian model Saturn 2000) using a medium-length (12 m) capillary column of the same characteristics described above. The oven was heated from 120 (1 min) to 380°C at 10°C/min and held for 5 min. The transfer line was kept at 300°C. The injector was temperature programmed from 120 (0.1 min) to 380°C at a rate of 200°C/min and held until the end of the analysis. Helium was used as carrier gas

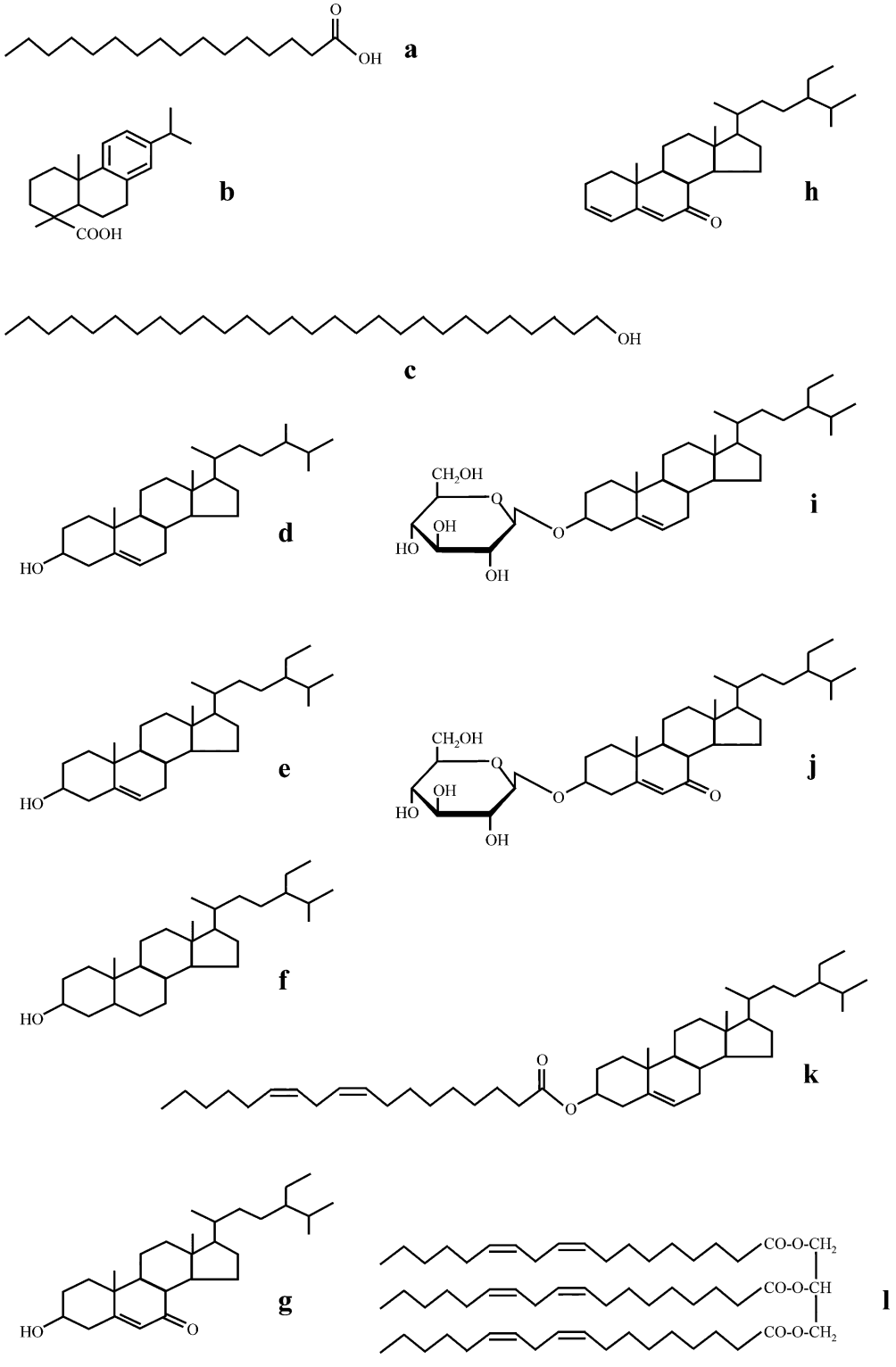

Fig. 1 Chemical structures of compounds representative of the main plant lipid classes present in the paper pulps analyzed: palmitic acid (**a**), dehydroabietic acid (**b**), *n*-octacosanol (**c**), campesterol (**d**), sitosterol (**e**), stigmastanol (**f**), 7-oxositosterol (**g**), stigmasta-3,5-dien-7-one (**h**), sitosteryl 3-β-D-glucopyranoside (**i**), 7-oxositosteryl 3-β-D-glucopyranoside (**j**), sitosteryl linoleate (**k**), and trilinolein (**l**)

at a rate of 2 ml/min. Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries, by mass fragmentography, and by comparing with standards.

Results

Three unbleached paper pulps representative for different hardwood (eucalypt), softwood (spruce), and nonwoody (flax) lignocellulosic raw materials and pulping processes (kraft, TMP, and soda-AQ, respectively) were treated with the high-redox-potential laccase from the basidiomycete *P. cinnabarinus* in the presence of HBT as a redox mediator. The laccase–mediator treatment affected all the main lipid classes found in the different pulp types (Fig. 1), as revealed by comparison with the corresponding controls without enzyme. In contrast, lipid modifications were not detected during pulp treatments with the laccase alone.

Treatment of eucalypt kraft pulp with laccase–mediator

The abundances of the main lipophilic compounds present in the control eucalypt (*E. globulus*) pulp and the enzymatically treated pulp are shown in Table 1. Sterols in free and conjugated form (as glycosides and esters) were the major compounds. Some steroid hydrocarbons and steroid ketones, such as stigmasta-3,5-dien-7-one and 7-oxositosterol, were also identified together with a series of free fatty acids.

Table 1 shows that the laccase–mediator treatment nearly completely removed the eucalypt-free sterols, sterol esters, and sitosteryl 3-β-D-glucopyranoside. Sitosterol that represents over 80% of free sterols in eucalypt pulp was completely removed, and only minor amounts of stigmastanol remained in the treated pulp. On the other hand, an increase in the amounts of stigmasta-3,5-dien-7-one, stigmastan-3-one, and especially 7-oxositosterol were observed after the laccase–mediator treatment. Together with 7-oxositosterol (72% of steroid ketones in the treated pulp), significant increases of the corresponding di-unsaturated ketone (stigmasta-3,5-dien-7-one) and glycoside (7-oxositosteryl 3-β-D-glucopyranoside) were also observed.

Treatment of spruce TMP pulp with laccase–mediator

The abundances of the main lipophilic compounds present in the control spruce (*P. abies*) TMP pulp and the enzymatically treated pulp are shown in Table 2. In the spruce TMP pulp, the composition of lipophilic extractives was different from that found in eucalypt pulp with the presence of high amounts of triglycerides, sterol esters, and resin acids. In addition, lower amounts of fatty acids and free sterols (campesterol and sitosterol) were present, together with minor amounts of fatty alcohols, steroid ketones, and sterol glycosides.

Table 1 Composition of the lipophilic extractives from the control eucalypt kraft pulp and the eucalypt pulp treated with the laccase–mediator system (mg/kg pulp)

	Control eucalypt pulp	Enzyme-treated eucalypt pulp
Total fatty acids	48.5	27.9
Myristic acid (FA-14)	2.1	1.7
Palmitic acid (FA-16)	4.8	4.8
Linoleic acid (FA-18:2)	2.9	0.7
Oleic acid (FA-18:1)	1.4	1.1
Stearic acid (FA-18)	2.7	2.1
Arachidic acid (FA-20)	2.1	0.7
Behenic acid (FA-22)	6.9	3.4
Lignoceric acid (FA-24)	9.4	4.2
Cerotic acid (FA-26)	9.2	5.3
Montanic acid (FA-28)	7.0	3.9
Total steroid hydrocarbons	14.2	4.9
Total free sterols	140.3	5.5
Sitosterol	111.0	–
Stigmastanol	25.8	5.5
Fucosterol	3.5	–
Total steroid ketones	9.0	97.2
Stigmastan-3-one	0.6	3.6
Stigmasta-3,5-dien-7-one	2.9	24.1
7-Oxositosterol	5.5	69.5
Total sterol glycosides	17.1	6.9
Sitosteryl 3-β-D-glucopyranoside	17.1	–
7-Oxositosteryl 3-β-D-glucopyranoside	–	6.9
Total sterol esters	95.4	–

All standard deviations were below 10% of the mean values presented
– Not detected, *tr* detected in trace amounts

Table 2 shows that the enzymatic treatment removed most of the triglycerides, sterol esters, and sterol glycosides. A significant removal of resin acids and free sterols was also produced by the enzyme-mediator treatment. The incomplete removal of some of the above lipids by laccase–mediator treatment could be related to the spruce pulp treatment conditions (carried out under atmospheric oxygen). Finally, the fatty alcohols and fatty acids were removed in a lower extent than the other lipophilic compounds. The oxidized 7-oxocampesterol and 7-oxositosterol as well as the respective di-unsaturated ketones ergosta-3,5-dien-7-one and stigmasta-3,5-dien-7-one increased after the laccase–mediator treatment, as also occurred in the enzymatically treated eucalypt pulp. 7-Oxodehydroabietic acid was detected in the treated spruce pulp, although it was not quantified separately.

The potential of the laccase–mediator system to remove lipophilic extractives during spruce TMP was confirmed by the analysis of the corresponding enzyme-treated liquids, in addition to the pulps. These analyses showed that only a small percentage of the pulp lipids was released to the treatment liquid in the control without laccase (5% of

Table 2 Composition of the lipophilic extractives from the control spruce pulp and the spruce pulp treated with the laccase–mediator system (mg/kg pulp)

	Control spruce pulp	Enzyme-treated spruce pulp
Total fatty acids	116.1	68.3
Palmitic acid (FA-16)	26.6	21.5
Margaric acid (FA-17)	21.0	14.1
Linoleic acid (FA-18:2)	33.7	6.0
Oleic acid (FA-18:1)	23.1	21.4
Stearic acid (FA-18)	11.7	5.3
Total resin acids	1278.4	400.9
Total fatty alcohols	18.6	14.9
n-Tetracosanol (FAL-24)	17.2	13.7
n-Hexacosanol (FAL-26)	0.4	0.3
n-Octacosanol (FAL-28)	1.0	0.9
Total free sterols	120.5	41.4
Campesterol	30.4	8.0
Sitosterol	90.1	33.4
Total steroid ketones	7.0	67.0
Ergosta-3,5-dien-7-one	2.0	8.0
Stigmasta-3,5-dien-7-one	3.3	27.0
Stigmast-4-en-3-one	1.7	1.7
7-Oxocampesterol	–	8.3
7-Oxositosterol	–	22.0
Total sterol glycosides	4.5	tr
Campesteryl 3-β-D-glucopyranoside	0.7	–
Sitosteryl 3-β-D-glucopyranoside	3.8	–
7-Oxocampesteryl 3-β-D-glucopyranoside	–	tr
7-Oxositosteryl 3-β-D-glucopyranoside	–	tr
Total sterol esters	1274.6	218.3
Total triglycerides	1982.5	151.2

All standard deviations were below 10% of the mean values presented
– Not detected, *tr* detected in trace amounts

sitosterol, 4% of sterol esters, and 4% of triglycerides) and that, moreover, these lipids were degraded during the enzymatic treatment. Similar results were obtained with the liquids from eucalypt and flax pulp treatment that contained a lower amount of lipophilic compounds.

Treatment of flax soda-AQ pulp with laccase–mediator

The abundances of the main lipophilic compounds present in the control flax (*L. usitatissimum*) soda-AQ pulp and the enzymatically treated pulp are shown in Table 3. The composition of the lipid fraction from flax pulp was also different from the two other pulps. The main compounds were fatty alcohols from docosanol to dotriacontanol. High amounts of free sterols and sterol glycosides were identified. Minor amounts of steroid ketones and waxes (esters of fatty acids with fatty alcohols) were also present.

Table 3 shows that the laccase–mediator treatment completely removed the three free sterols (sitosterol, campesterol, and stigmasterol) initially present in flax pulp, whereas some amounts of the corresponding oxidized compounds (7-oxositosterol, 7-oxocampesterol, and 7-oxostigmasterol as well as the respective di-unsaturated ketones, ergosta-3,5-dien-7-one, and stigmasta-3,5-dien-7-one) appeared in the treated pulp. Likewise, the three initial sterol glycosides (sitosteryl, campesteryl, and stigmasteryl

Table 3 Composition of the lipophilic extractives from the control flax pulp and the flax pulp treated with the laccase–mediator system (mg/kg pulp)

	Control flax pulp	Enzyme-treated flax pulp
Defoamer	849.5	125.2
Total fatty alcohols	121.3	49.7
n-Docosanol (FAL-22)	3.8	1.8
n-Tetracosanol (FAL-24)	4.0	1.5
n-Hexacosanol (FAL-26)	25.6	9.6
n-Octacosanol (FAL-28)	70.8	29.0
n-Triacontanol (FAL-30)	15.0	7.8
n-Dotriacontanol (FAL-32)	2.1	–
Total alkanes	12.8	8.0
n-Heptacosane (ALK-27)	2.7	1.5
n-Nonacosane (ALK-29)	8.1	5.5
n-Hentriacontane (ALK-31)	2.0	1.0
Total free sterols	81.5	–
Campesterol	11.6	–
Stigmasterol	3.7	–
Sitosterol	66.2	–
Total steroid ketones	2.4	19.1
Ergosta-3,5-dien-7-one	0.4	1.4
Stigmasta-3,5-dien-7-one	2.0	6.0
7-Oxocampesterol	–	2.9
7-Oxostigmasterol	–	tr
7-Oxositosterol	–	8.8
Total sterol glycosides	14.0	5.4
Campesteryl 3-β-D-glucopyranoside	2.4	–
Stigmasteryl 3-β-D-glucopyranoside	0.8	–
Sitosteryl 3-β-D-glucopyranoside	10.8	–
7-Oxocampesteryl 3-β-D-glucopyranoside	–	0.9
7-Oxostigmasteryl 3-β-D-glucopyranoside	–	tr
7-Oxositosteryl 3-β-D-glucopyranoside	–	4.5
Total waxes	2.6	0.6

All standard deviations were below 10% of the mean values presented
– Not detected, *tr* detected in trace amounts

β-D-glucopyranosides) were also completely removed, and minor amounts of the corresponding 7-oxosterol glucosides appeared. Fatty alcohols and *n*-alkanes were removed to a significant extent, although they were comparatively more resistant to the action of the laccase–mediator system than the other lipophilic compounds in this pulp. In addition, a mixture of non-well resolved peaks, corresponding to the defoamer used in the industrial process, was also removed during the laccase–mediator treatment.

Discussion

The present study shows that the laccase–mediator system can efficiently remove most of the lipid classes present in paper pulps from different origins. This was a relatively unexpected result because the ability of this enzymatic system to act on recalcitrant plant lipids had not been reported before.

The laccase–mediator system was very efficient in removing sterols (95–100% decrease), including free sitosterol and its glucosides and fatty acid esters, which are the most characteristic lipids in the eucalypt kraft pulp. These compounds arise from the lipophilic extractives present in eucalypt wood (Gutiérrez et al. 1999a) which survive the cooking and oxygen prebleaching and are at the origin of the pitch problems in pulping (del Río et al. 1998, 2000; Gutiérrez et al. 2001a,b). Among them, the saturated stigmastanol was more resistant to the action of the laccase–mediator system than the unsaturated sitosterol. Stigmastanol has also been reported to be more resistant to the action of chlorine dioxide (del Río et al. 2000; Jansson et al. 1995). The abundances of steroid ketones in the eucalypt pulp increased during the treatment suggesting that they are oxidation products of steroids. The minor amount of 7-oxositosterol already present in the initial eucalypt pulp was probably formed during pulping and oxygen delignification (Freire et al. 2006). The increased presence of these oxo-derivatives is congruent with the oxidative nature of the laccase–mediator treatment, which is a metalloenzyme-catalyzed O_2 reaction.

In the case of spruce TMP pulp, the three main lipid classes contributing to pitch problems (triglycerides, resin acids, and sterols) (Ekman and Holmbom 2003) were removed with variable efficiency (65–100%). As in the case of eucalypt pulp, the laccase–mediator system removed most of the sterols, which in spruce pulp were mainly present as fatty acid esters. The final abundance of steroid ketones was low, suggesting that they were mainly formed during the oxidative enzymatic attack to the free sterols. Among the two other lipid classes characteristic of softwoods and their mechanical pulps, triglycerides were affected to a higher extent (over 90% decrease) than resin acids (over 70%) by the laccase–mediator treatment. Resin acids are especially problematic due to their recalcitrance towards degradation and the high toxicity of some of them (Leach and Thakore 1976). Lipid removal from softwood pulping model white-waters by laccase has been reported (Zhang et al. 2000, 2002), although the effect was variable compared with the extensive removal obtained during the laccase–mediator treatment.

Although pitch problems in nonwoody plant pulps are less documented than in wood pulps, lipophilic extractives also cause pitch problems in the manufacturing of these pulps, especially when more environmentally sound strategies are adopted (Gutiérrez and del Río 2005). The laccase–mediator treatment nearly completely removed the sterols in flax pulp, mainly present in free and glycosylated forms, as previously discussed for eucalypt and spruce pulps. Moreover, an important percentage of fatty alcohols characteristic of this pulp (Gutiérrez and del Río 2003a,b) was also removed. It is interesting to note that most of the defoamer found in the flax pulp, which has been proven to be involved in pitch deposition (Allen 2000; Gutiérrez and del Río 2005), disappeared during the enzymatic treatment.

The efficiency of *P. cinnabarinus* laccase for delignifying and bleaching flax and eucalypt paper pulp in the presence of a redox mediator has been reported (Camarero et al. 2004; Ibarra et al. 2004). In the present paper we demonstrated for the first time that a simultaneous removal of pulp detrimental lipids and lignin can be obtained using the laccase–mediator system. The main lipid classes present in the different pulp types analyzed—including free and conjugated sitosterol, triglycerides, resin acids, and fatty alcohols—were removed in a large extent (often completely). The removal of lipids by laccase–HBT resulted in the formation of several oxidized derivatives that were absent or presented low abundances in the initial pulps. In spite of this, the total lipid content in pulps decreased significantly, and the most problematic compounds were completely removed. It is important to point out that the laccase–mediator system was effective in removing pulp lipids regardless of the pulping process, the raw material, or the chemical nature of the compound to be degraded. In spite of the high potential of this enzymatic system for the removal of both lipids and lignin from paper pulps, different issues are still to be solved before its industrial implementation becomes a reality including lowering the prices of laccases by genetic engineering and other means (Punt et al. 2002), and the search for safe, cheap, and efficient enzyme mediators (Camarero et al. 2005).

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