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Influence of operation conditions on laccase-mediator removal of sterols from eucalypt pulp

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ABSTRACT

The way how sterols, the main lipophilic compounds present in eucalypt kraft pulp, are eliminated by an enzymatic stage using the laccase-mediator system was evaluated. With this purpose laccase-mediator stage (L) was applied on an *Eucalyptus globulus* pulp under different operation conditions following a three-variable (laccase dose, mediator dose and reaction time) sequential statistical plan, to optimise the removal of sterols. The decrease in pulp sterol content during the enzymatic treatment was related to the decrease in kappa number and to brightness increase, as well as with the increase in some oxidation products of sitosterol (namely 7-oxositosterol and stigmasta-3,5-dien-7-one). The increase in reaction time from 1 to 5 h strongly reduced the sterol content, while no more sterols were eliminated during the 5–7 h period. Increasing the laccase dose from 1 to 20 U g^{-1} of pulp produced a high reduction in pulp sterols, whereas the increase in mediator (1-hydroxybenzotriazole) dose (from 0.5 to 2.5% of pulp weight) had only a slight influence in removing sterols. Therefore, at 16 U g^{-1} laccase dose, 0.5% mediator dose, 4 h of reaction, practically all the sterols were removed. Finally, it was demonstrated that sterols were more sensitive to a L stage (practically 100% of sterols were eliminated) than to a chlorine dioxide stage (54% of sterols eliminated).

1. Introduction

The so-called pitch deposits formed during the pulp and paper manufacturing processes cause drastic decreases in the final product quality, and affect negatively the runnability of the paper machine. Moreover, they are responsible for shutdowns of mill operations, resulting in important economical losses in this industrial sector [1]. Pitch deposits have their origin in the lipophilic components of the raw material (wood) which are not removed during the cooking and bleaching processes. Pitch problems have increased with the introduction of totally chlorine free (TCF) bleaching sequences since oxygen and hydrogen peroxide used in TCF sequences are not as effective as chlorine-based reagents, such as chlorine dioxide, in removing these lipophilic compounds [2–4]. Moreover, pitch problems have also been aggravated in mills with a high degree of closed water systems resulting in higher concentration of pitch compounds.

Eucalypt kraft pulp is of high strategic interest for Spain, Portugal, Brazil, South Africa and other countries because high quality writing and printing paper are manufactured with this pulp due to its peculiar properties. Sterols are the predominant lipophilic compounds in eucalypt wood [5,6], pulp [2] and process waters [7] and the main responsible for pitch problems in the processing of this cellulosic material [8,9].

The use of biotechnology in the bleaching processes as well as in the control of pitch has gained ground during the last years [10–18]. Laccases are multicopper oxidases that are able to oxidize phenolic compounds. However, the use of laccase in the presence of redox mediators [19] strongly expands their potential for degradation of lignin and other aromatic compounds. Much work has been done on the laccase-mediator system for delignification and bleaching of different paper pulps [20-25]. Recently, it has been demonstrated the high efficiency of the laccase-mediator system in removing sterols from *Eucalyptus globulus* pulp using both synthetic and natural mediators [18,26–28]. However, several issues remain to be solved before the industrial implementation of laccase-mediator systems, namely the high cost of synthetic mediators and the possible toxicity problems associated with their reaction products.

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The present work aims at the optimization of the laccase-mediator treatment on *E. globulus* kraft pulp including the reduction of mediator dose. With this purpose, a sequential statistical plan of three variables was applied to evaluate how the laccase and mediator doses, as well as the reaction time, affected the elimination of sterols from *E. globulus* pulp. In addition to the sterol removal, the evolution of kappa number and brightness properties was also evaluated. Finally, the elimination of sterols by the laccase-mediator system and by a chlorine dioxide stage were compared.

2. Materials and methods

2.1. Raw material

The raw material used in this study was an oxygen delignified *E. globulus* kraft pulp produced by the Torraspapel mill (Zaragoza, Spain). Before applying the treatments, the pulp was washed with 50 mM Tris–HCl buffer (pH 7) at room temperature for 30 min. The initial characteristics of the washed pulp were: 8.4 kappa number, and 51.2% ISO brightness.

2.2. Laccase-mediator treatment

High redox-potential laccase from *Trametes villosa* (NS-51002) was supplied by Novozymes (Bagsvaerd, Denmark). The mediator was 1-hydroxybenzotriazole (HBT) from Sigma–Aldrich. The laccase-mediator stage (L) was performed in 50 mM sodium tartrate buffer (pH 4) at 5% consistency. The treatments were performed in a pressurized reactor with oxygen at 590 kPa, 30 °C, and 60 rpm stirring. Tween 80 (0.05% w/v) was added as surfactant. The laccase dose, the mediator dose and the reaction time were the three variables of the experimental design. After the L stage, the pulp was efficiently washed with distilled water before characterization.

2.2.1. Experimental design

The enzymatic treatments were carried out following a 2^3 sequential statistical plan (two levels and three variables) plus three repetitions at the central point. The three independent variables were varied over the following ranges: 1–20 U g^{-1} odp (oven-dried pulp) for the laccase dose (X_1), 0.5–2.5% odp for the HBT dose (X_2), and 1–7 h for the reaction time (X_3). The different experiments performed are shown in Table 1. The results obtained were analyzed with the Excel program using the "stepwise backward regression" method. After a first analysis of the statistical plan, it was determined that the quadratic term was significant and, therefore, two additional experiments were carried out.

2.3. Chlorine dioxide bleaching

The chlorine dioxide stage (D) was carried out at 10% consistency and 56 °C, for 60 min and with 3% odp chlorine dioxide as active chlorine. These conditions corresponded to the application conditions of an industrial D stage.

2.4. Analysis of free sterols

Free sterols were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) following the methodology previously developed [29].

The GC analyses were carried out 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 the final temperature of 350 °C at 15 °C min^{-1}, and held for 3 min. The injector and flame-ionization detector (FID) temperatures were set at 300 °C and 350 °C respectively. The carrier gas was helium at a rate of 5 mL min^{-1}, and the injection was performed in splitless mode. Peaks were quantified by area in the GC chromatograms. The data from three replicates were averaged. All standard deviations were below 7% of the mean values presented.

The GC/MS analyses were performed with a Varian 3800 gas chromatograph equipped with an ion-trap detector (Varian 4000) using a medium-length (12 m) capillary column of the same characteristics described above. The oven was heated from 120 °C (1 min) to 380 °C at 10 °C min^{-1} and held for 5 min. The transfer line was kept at 300 °C. The injector was temperature programmed from 120 °C (0.1 min) to 380 °C at a rate of 200 °C min^{-1} and held until the end of the analysis. Helium was used as carrier gas at a rate of 2 mL min^{-1}. Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries, by mass fragmentography, and by comparing with standards.

Table 1
Application conditions of the different experiments performed.

X_1	X_2	X_3	Laccase dose (U g^{-1} odp)	HBT dose (% odp)	Reaction time (h)
−1	−1	−1	1	0.5	1
1	−1	−1	20	0.5	1
−1	1	−1	1	2.5	1
1	1	−1	20	2.5	1
−1	−1	1	1	0.5	7
1	−1	1	20	0.5	7
−1	1	1	1	2.5	7
1	1	1	20	2.5	7
0	0	0	10.5	1.5	4
0	0	0	10.5	1.5	4
0	0	0	10.5	1.5	4
1	0	0	20	1.5	4
0	−1	0	10.5	0.5	4

2.5. Kappa number and brightness properties

For pulp characterization, kappa number and brightness were determined according to ISO 302 and ISO 3688 standards, respectively. For each experiment kappa number and brightness were measured twice and by quadruplicate, respectively, to obtain a standard deviation of 0.1.

3. Results and discussion

The main lipophilic extractives present in the initial eucalypt pulp were free sterols (Fig. 1a, peak 1), with sitosterol predominating (around 80%) followed by stigmastanol (20%) and fucosterol (5%). The peaks of these three compounds overlapped during GC analysis, but they could be separately quantified using specific ions

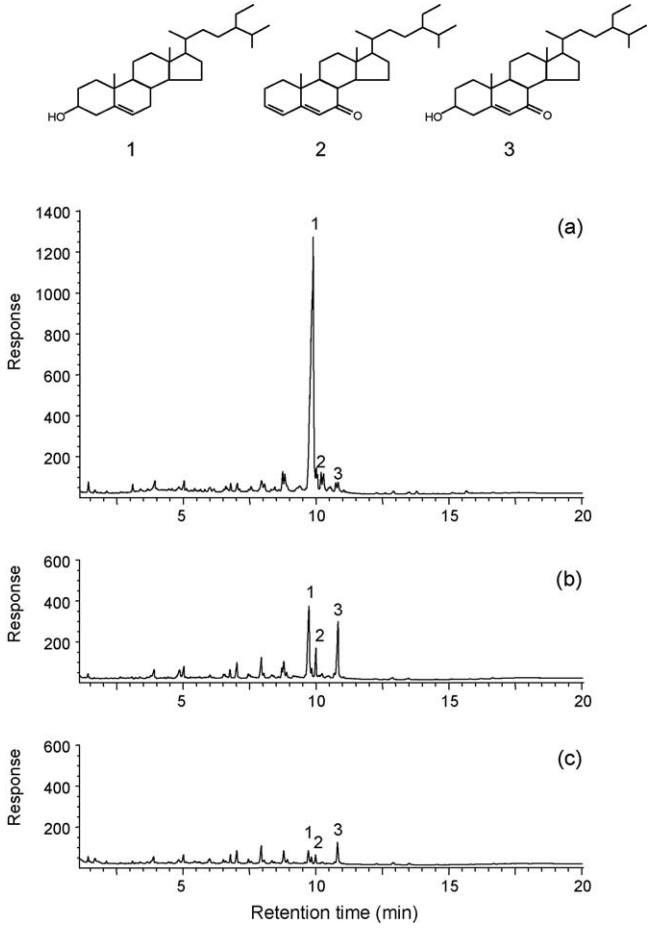

Fig. 1. GC analyses of lipophilic extractives in *E. globulus* pulp before (a) and after laccase-mediator treatment using the highest enzyme and mediator doses for 1 h (b) X_1-laccase dose = 20 U g^{-1} odp; X_2-HBT dose = 2.5% odp; and X_3-reaction time = 1 h) and 7 h (c) X_1-laccase dose = 20 U g^{-1} odp; X_2-HBT dose = 2.5% odp; and X_3-reaction time = 7 h). The chemical structures of the main compounds identified are also shown: sitosterol (1), stigmasta-3,5-dien-7-one (2) and 7-oxositosterol (3).

Table 2
Free sterol content after the L stage in the different experiments performed (values for the X_1, X_2 and X_3 variables are shown in Table 1).

Experiment	Laccase dose (X_1)	HBT dose (X_2)	Reaction time (X_3)	Free sterols (mg kg^{-1} odp)	Stigmasta-3,5-dien-7-one (mg kg^{-1} odp)	7-Oxositosterol (mg kg^{-1} odp)
Initial	–	–	–	326.2	6.7	21.9
1	−1	−1	−1	198.9	6.8	12.2
2	1	−1	−1	111.2	14.8	65.5
3	−1	1	−1	169.3	7.8	23
4	1	1	−1	51.2	13.1	56.5
5	−1	−1	1	63.9	5.4	19.7
6	1	−1	1	19.3	13.8	59
7	−1	1	1	65.2	12.7	55.5
8	1	1	1	8.7	5.4	22.6
9	0	0	0	20.2	20.3	90.6
10	0	0	0	17.7	20.9	89.2
11	0	0	0	19.1	26.4	93.3
12	1	0	0	11.5	9.6	44.3
13	0	−1	0	35.5	12.2	48.3

in GC/MS analysis. Minor amounts of fatty acids and products of sitosterol oxidation, such as 7-oxositosterol (peak 3) and stigmasta-3,5-dien-7-one (peak 2) were also present (Table 2).

The experimental design to optimize the removal of sterols from eucalypt kraft pulp gave rise to 13 experiments, where the laccase-mediator system was applied at different conditions of laccase dose, mediator dose and reaction time (Table 2). Free sterol content (with sitosterol predominating) as well as the content of two sterol oxidation products, after the L stage are also shown in Table 2. The evolution of free sterols during the different experiments was compared to the kappa number decrease and the brightness increase for the same pulps (Fig. 2a and b). According to these figures, the decrease in sterols after the L stage was linked to a decrease in kappa number and an increase in brightness. The enzymatic treatment of pulp gave rise to some minor oxidation products, namely 7-oxositosterol and stigmasta-3,5-dien-7-one (Fig. 3), the first one produced in higher amounts. The increase in these oxidation products was strongly related to the decrease in sitosterol. Similar oxidation products were also identified in laccase-mediator reactions with model compounds including sitosterol [30].

Once it was observed that sterol removal was influenced by the different application conditions of the laccase-mediator system, the analysis of how each variable affected sterols was carried out. This effect was also compared with the effects on pulp kappa number and brightness. Second, a statistical analysis was performed to obtain a preliminary model to predict the variation of sterol content as a function of these variables. Finally, the efficiency of the L stage in sterol removal was compared with that of a D stage.

3.1. Influence of variables on sterol removal, kappa number and brightness

3.1.1. Effect of the laccase dose (X_1)

The effect of increasing the laccase dose, from 1 to 20 U g^{-1} odp, in the removal of pulp sterols is shown in Fig. 4(a). An increase in laccase dose caused a reduction in the sterol content in pulp, whose extent depended on the other variables. The effect of laccase dose was greater at shorter times, and the highest effect was produced at the lowest time and when the dose of mediator was the highest (B) resulting in 70% sterol removal at the maximal enzyme dose. The increase in laccase dose also produced a decrease in kappa number (Fig. 4b). At low mediator dose and short reaction time (condition A) it was not important to increase the dose of laccase, while the increase was significant under conditions B, C and D. The highest effect of laccase on kappa number (1.35 units decrease) was produced when the mediator dose was low and the reaction time high (C). Brightness was also affected by the increase of the laccase dose from 1 to 20 U g^{-1} odp (Fig. 4c) that always caused a brightness increase of about 5% ISO, independently of the other variables.

Finally, the formation of the oxidation products of sitosterol was also influenced by the laccase dose (Table 2). When the laccase dose increased, both 7-oxositosterol and stigmasta-3,5-dien-7-one also increased under the different application conditions. When all the application conditions were maximal (Table 2, Experiment 8), this effect was not observed since these products could have been removed. These effects are also shown in Fig. 1, where the high peak of free sterols (peak 1 in Fig. 1a) strongly decreased after 1-h treatment and its oxidation products (peaks 2 and 3 in Fig. 1b)

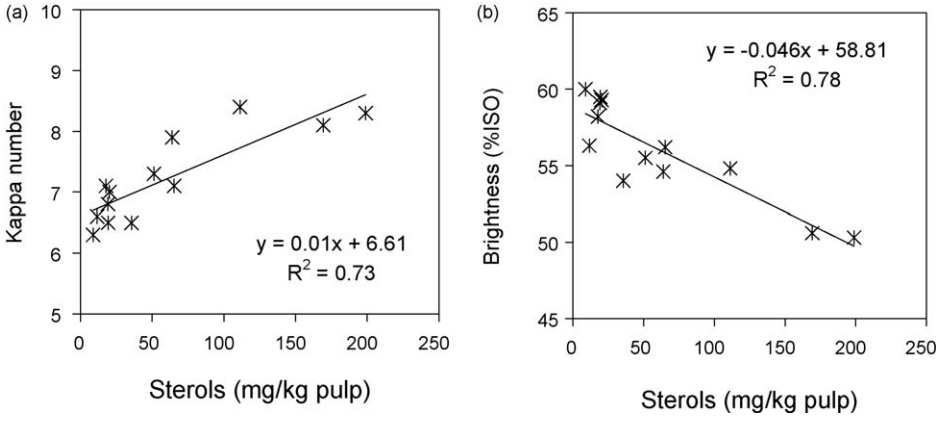

Fig. 2. Sterol content versus kappa number (a) and brightness (b) of pulp in L experiences.

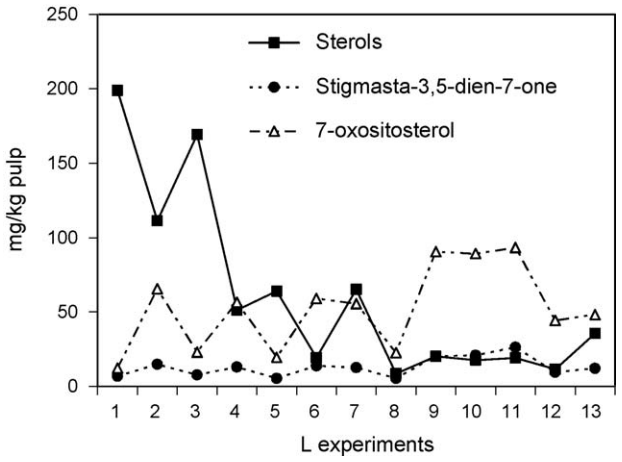

Fig. 3. Sterol content in pulp versus oxidation products, namely 7-oxositosterol and stigmasta-3,5-dien-7-one (note that the same compounds amounted for around 326, 7 and 22 mg kg^{-1} odp, respectively, in the initial *E. globulus* pulp).

increased. However, when reaction time increased (Fig. 1c) all the peaks practically disappeared.

Therefore, the increase in laccase dose caused a reduction in sterols that implied an increase in its oxidation products, and a reduction of kappa number depending on the other variables. However, brightness always increased independently of the other variables.

3.1.2. Effect of the mediator dose (X_2)

The effect of increasing the mediator dose, from 0.5 to 2.5% odp, on the reduction in pulp sterols is shown in Fig. 5(a). This increase in mediator dose produced significant effects only at short times (A and B). When the dose of laccase was at its highest level (B), the highest reduction in sterols (54%) due to the increase in the mediator dose was attained. The increase in mediator dose reduced slightly the kappa number (Fig. 5b) when the other two variables were both at minimal or maximal conditions (A and D). The highest effect of the mediator dose in reducing the kappa number (1.02 units) was produced when the laccase dose was maximal and the reaction time minimal (B). An increase in brightness (1.6% ISO) was also produced in C (when the laccase dose was minimal and the reaction time maximal) whereas in other combinations of variables, the brightness increase was very low (Fig. 5c). Finally, the oxidation products of sitosterol were no significantly affected by the increase in mediator dose.

Thus, the increase in mediator dose only caused slight effects on pulp sterol removal, kappa number and brightness. This fact suggested that the mediator dose could be maintained at the lowest level (0.5% odp) during the enzymatic treatment of pulp. This result was of great relevance since lowering the HBT dose will reduce not only the potential toxicity problems but also the costs of the process making its industrial implementation more feasible.

3.1.3. Effect of the reaction time (X_3)

Fig. 6(a) illustrates the effect of increasing the reaction time, from 1 to 7 h, on pulp sterol removal. An increase in the reaction time always caused a reduction in the pulp sterol content, which

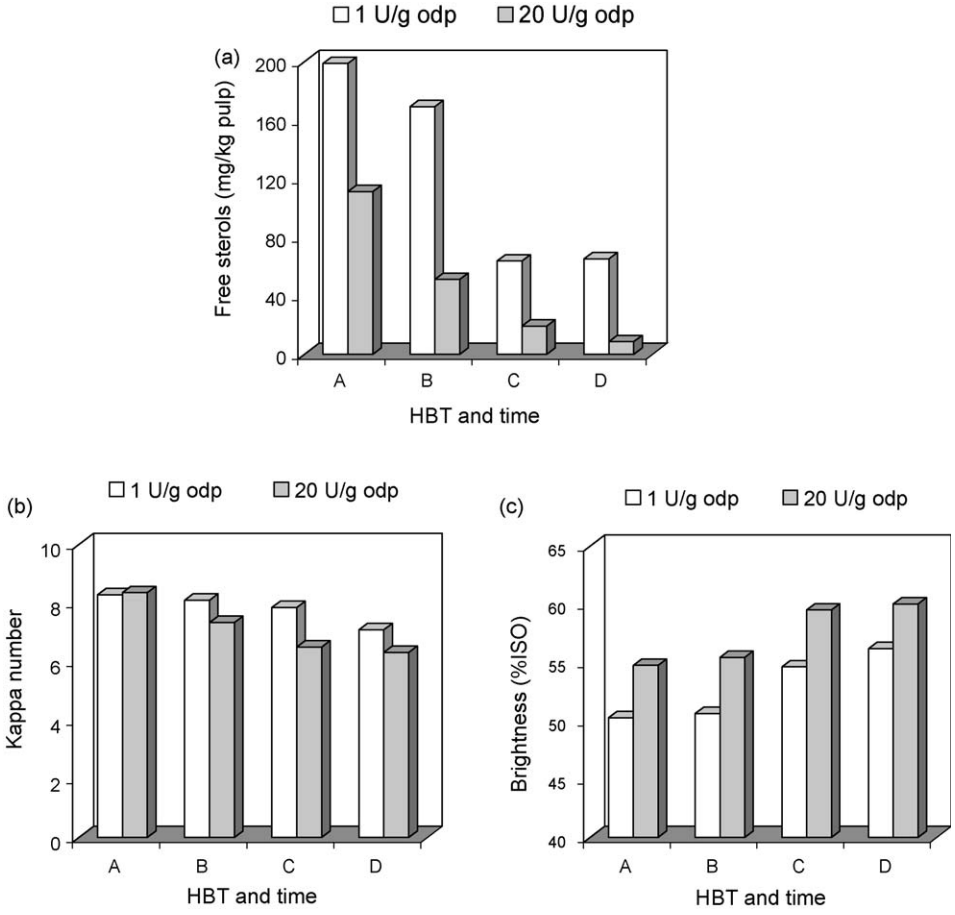

Fig. 4. Effect of increasing the laccase dose (X_1) from 1 U g^{-1} odp (□) to 20 U g^{-1} odp (■) on sterol content (a) kappa number (b) and brightness (c) during stage L. Influence of the conditions of the other two variables: (A) (HBT dose = 0.5% odp; reaction time = 1 h), (B) (HBT dose = 2.5% odp; reaction time = 1 h), (C) (HBT dose = 0.5% odp; reaction time = 7 h) and (D) (HBT dose = 2.5% odp; reaction time = 7 h).

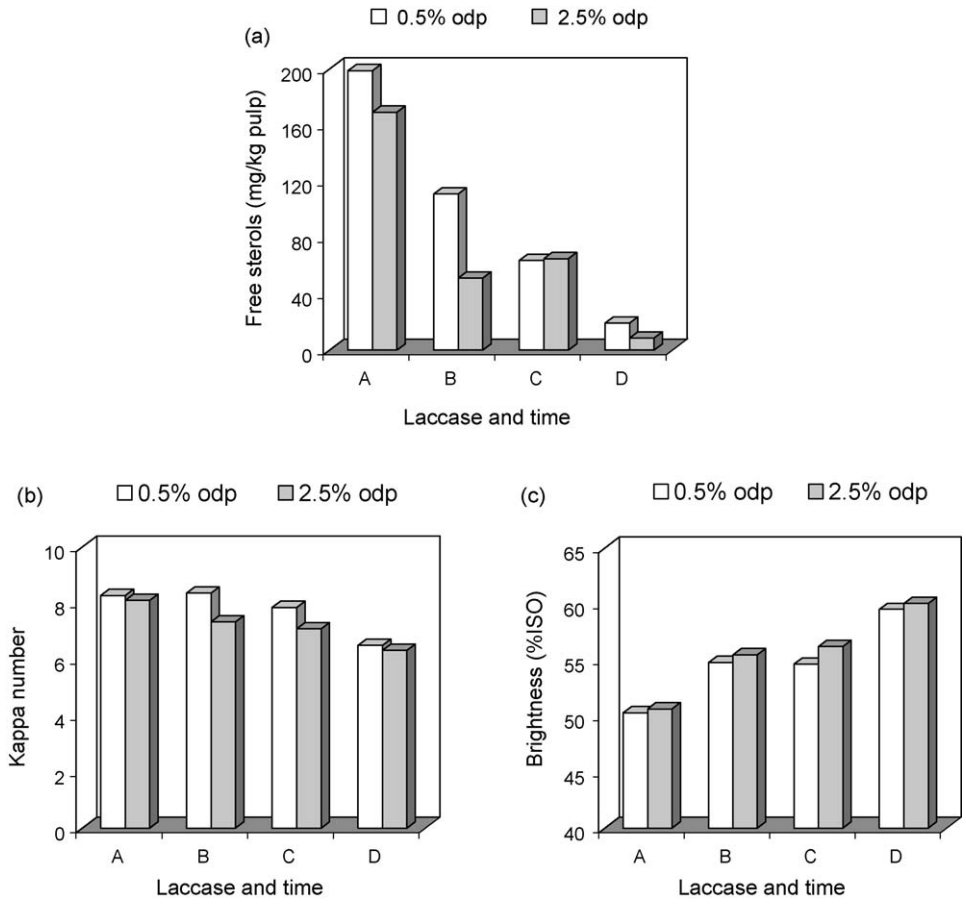

Fig. 5. Effect of increasing the mediator dose (X_2) from 0.5% odp (□) to 2.5% odp (■) on sterol content (a) kappa number (b) and brightness (c) in stage L. Influence of the conditions of the other two variables: (A) (laccase dose = 1 U g^{-1} odp; reaction time = 1 h), (B) (laccase dose = 20 U g^{-1} odp; reaction time = 1 h), (C) (laccase dose = 1 U g^{-1} odp; reaction time = 7 h) and (D) (laccase dose = 20 U g^{-1} odp; reaction time = 7 h).

depended on the other two variables. The effect of the reaction time was higher when laccase was at its lowest dose (A and C). The highest effect (a decrease of 68%) was found when the mediator was also at the lowest dose (A). An increase in the reaction time always caused a decrease in kappa number (Fig. 6b). This decrease was minimal when the other two variables were at low levels (A) and was maximum (1.86 units decrease) when laccase dose was high and the mediator dose minimal (B). Finally, brightness significantly increased in all cases (Fig. 6c), with the highest effect (5.6% ISO) when the laccase dose was minimal and the mediator dose was maximal (C). In spite of sitosterol decreased with the increase in reaction time, the products of its oxidation did not always increase, since they were removed at long reaction times.

According to the effects obtained by increasing the different variables, reaction time was the most influential variable in reducing sterol content as well as in decreasing kappa number and brightness. Laccase dose was the second variable with more influence and finally, mediator dose only had slight influence.

3.2. Representation of the model obtained for sterol removal

A statistical analysis was carried out from the experimental points. A model was obtained for the removal of free sterol. However, it was not possible to obtain a model from the formation of the sitosterol oxidation products. The model obtained predicted variations in sterol content from the system variables (Eq. (1)).

$$Y_{\text{Free sterols}} = 28 - 36X_1 - 47X_3 + 58X_3^2 \quad (1)$$

with R^2 = 0.90

In the above equation, $X_1 = (L - 10.5)/9.5$ (L denoting the laccase dose, in U g^{-1} odp), and $X_3 = (t - 4)/3$ (t being the reaction time, in h). As can be seen from the R^2 values obtained, the fit was quite good.

According to the model obtained, laccase dose (X_1) and reaction time (X_3) had a strong influence in diminishing the free sterols present in pulp whereas mediator dose (X_2) did not show a significant influence. Moreover, reaction time showed a quadratic effect since variable X_3^2 also showed influence on the model. In Fig. 7a, the variation in sterol content is plotted as a function of the reaction time at constant laccase doses. It was observed that an increase in reaction time from 1 to 5 h produced a reduction of sterols while from 5 h to 7 h the sterol content did not decrease anymore. As previously said, the oxidation products of sitosterol did not increase with the reaction time. This fact could be explained because at long reaction times no more sterols were oxidized and, moreover, 7-oxositosterol and stigmasta-3,5-dien-7-one were slowly destroyed. This fact made also difficult to obtain a model from the experimental points.

It was observed that reaction time showed two distinct stages in the elimination of sterols. Similar results were obtained in recent works where high delignification and brightness increases were observed at an initial stage, while in a second stage delignification was slow or absent [15,31].

Increasing the laccase dose (from 1 to 20 U g^{-1} odp) entailed a decrease in the sterol amount independently of the reaction time (Fig. 7b). An important change in sterol content was produced when going from short (1 h) to medium reaction times (4 h).

Thus, in order to obtain the lowest content of sterols in the pulp with a laccase-mediator treatment stage, the conditions of

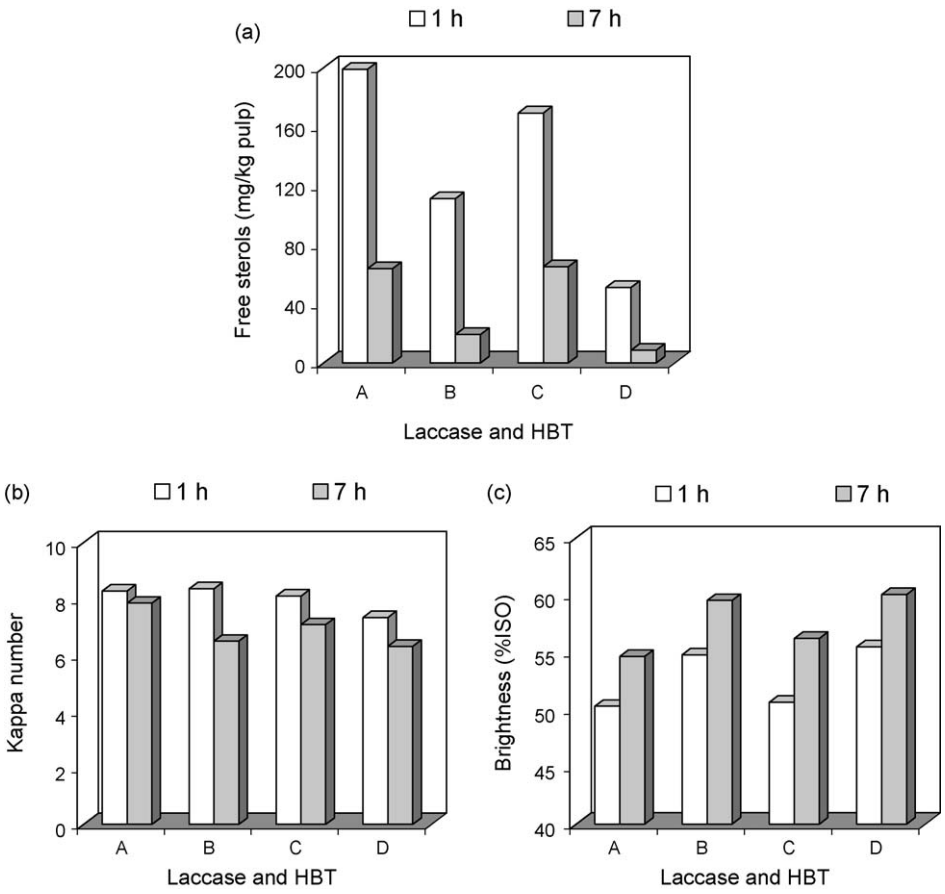

Fig. 6. Effect of increasing the reaction time (X_3) from 1 h (□) to 7 h (■) on sterol content (a) kappa number (b) and brightness (c) during stage L. Influence of the conditions of the other two variables: (A) (laccase dose = 1 U g^{-1} odp; HBT dose = 0.5% odp); (B) (laccase dose = 20 U g^{-1} odp; HBT dose = 0.5% odp); (C) (laccase dose = 1 U g^{-1} odp; HBT dose = 2.5% odp); (D) (laccase dose = 20 U g^{-1} odp; HBT dose = 2.5% odp).

L application would be: 16 U g^{-1} odp of laccase dose, 0.5% odp of mediator dose and 4 h of reaction time (Fig. 7b). According to the model, at this point almost all the sterols present in the pulp would be eliminated. Moreover, it has to be pointed out that the mediator dose was reduced to a minimal value (0.5% odp) and also the reaction time was reduced in 3 h. In relation with previous studies [26,27], the laccase dose was reduced by 20% and mediator dose by 66%. These facts are of great relevance because the reduction of both mediator dose and reaction time were very important for the feasibility of the industrial implementation.

3.3. Is the L stage comparable to a D stage?

It is known that chlorine dioxide degrades efficiently unsaturated sterols [32]. In this work, the elimination of free sterols in an L stage with the laccase-mediator system was compared with the removal attained in a D stage under typical industrial conditions (Table 3). At maximum application conditions of L stage (Table 2, Experiment 8) almost all (97%) the initial sterols were eliminated from the pulp. However, with a D stage an important amount of sterols still remained in the pulp (only 54% were eliminated). Moreover, the sterol elimination in the L stage was higher under

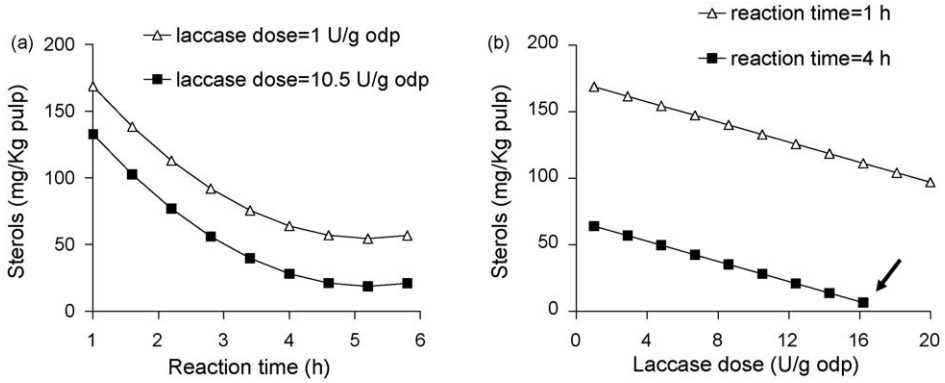

Fig. 7. Variation of sterol content as a function of reaction time for a constant laccase dose (a) and as a function of the laccase dose for a constant reaction time (b) (note that total sterols amounted for 326 mg kg^{-1} odp in the initial *E. globulus* pulp).

Table 3
Free sterol content of initial, D and L pulps.

	Free sterols (mg kg^{-1} odp)
Initial pulp	326.2
D	149.5
L	8.7

almost all the operation conditions assayed, with the exception of Experiments 1 and 3 (Table 2), where laccase and time were at minimal conditions. It has been reported [2-4] that the oxygen and hydrogen peroxide used in TCF sequences are not as effective as chlorine dioxide (ECF sequences) in removing these lipophilic compounds. It was demonstrated here that free sterols were more sensitive to the L stage than to the D stage, and this fact will be very interesting for the introduction of these TCF sequences (including an L stage) in the industry.

4. Conclusions

The effect of the laccase-mediator treatment variables (laccase dose, mediator dose and reaction time) in reducing the sterol content of an eucalypt kraft pulp was evaluated. A three-variable sequential statistical plan was carried out. Sterols decreased during application of the laccase-mediator treatment under different conditions, and this decrease was related with a decrease in kappa number and an increase in brightness. The oxidation of sitosterol, first produced an increase of its oxidation products, however, they were removed at longer reaction time. The decrease in sterols was strongly influenced by the increase in reaction time up to 5 h while from 5 to 7 h sterols did not decrease to any further extent. The increase in the laccase dose from 1 to 20 U g^{-1} odp also produced a reduction of sterols, and the increase in HBT dose from 0.5 to 2.5% odp had only a slight influence. At 16 U g^{-1} odp of laccase, 0.5% odp of mediator dose and 4 h of reaction time, almost all free sterols in pulp were eliminated being these conditions of great relevance for industrial application. Moreover, it was demonstrated that sterols were better eliminated with an L stage than with a chlorine dioxide stage. This will be also very interesting for the industrial introduction of TCF sequences applying an L stage. The reduction of the mediator dose attained in the present study will contribute to the industrial feasibility of the laccase-mediator system. Future trends will include the use of natural mediators since some promising results have been obtained with phenolic compounds in the removal of sterols from paper pulp.

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