

Isolation of high-purity residual lignins from eucalypt paper pulps by cellulase and proteinase treatments followed by solvent extraction

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Abstract

In the context of environmentally-friendly bleaching in paper pulp manufacturing, an enzymatic procedure was developed for the isolation of residual lignins from eucalypt pulp. The method was based on the hydrolysis and complete solubilization of pulp cellulose using a combination of *Trichoderma* cellulase and *Aspergillus* β -glucosidase, followed by lignin purification using *Bacillus* protease (for hydrolysis of contaminating cellulase) and extraction of lignin with dimethylacetamide (DMAC) and NaOH. This method was applied to both unbleached and totally chlorine free bleached eucalypt kraft pulps. The different fractions obtained were analyzed by Fourier-transform infrared spectroscopy and pyrolysis-gas chromatography/mass spectrometry to evaluate the effect of the different isolation steps, and optimize the lignin isolation procedure. Depending of the type of pulp and its delignification degree most of the lignin was recovered from the cellulase hydrolyzate or the non-hydrolyzable residue. High-purity lignins were obtained from pulps by a new isolation procedure that included double solvent purification after combining the two fractions from protease treatment. This method is being used to analyze the effect of chlorine-free reagents (including enzymes) in clean bleaching sequences.

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1. Introduction

Lignocellulose-degrading enzymes—which include hydrolases acting on cellulose and hemicelluloses, and oxidoreductases involved in lignin degradation—have high interest in different industrial sectors including paper pulp manufacturing. This includes the use of enzymes not only in environmentally-friendly production process, but also their formulation in commercial products and utilization as analytical tools [4,13,36].

High-quality paper pulps are obtained by chemical pulping processes (kraft cooking being the most common) the aim of which is to partially depolymerize and dissolve the lignin acting as a glue between wood fibers [17]. Due to its recalcitrant nature, a certain amount of residual lignin remains in pulp and, because of its oxidative alteration during

cooking, it is responsible for the dark color of pulps. Peroxide, oxygen, ozone, and lignocellulose-degrading enzymes are environmentally-friendly alternatives to chlorinated reagents (Cl_2 , ClO_2 , and NaOCl) in new totally chlorine free (TCF) bleaching sequences [3,4]. The analysis of pulp lignins is an important aspect for developing clean industrial processes. However, isolation of residual lignins from pulp is often a difficult task because lignin is chemically-linked to the carbohydrates.

Enzymatic isolation with cellulolytic enzymes [22] represents an attractive alternative to chemical isolation of residual lignin [20], which is generally based on pulp treatment with dioxane–water–HCl (acidolysis). During enzymatic isolation most of the pulp carbohydrates are hydrolyzed and lignin-enriched fractions are obtained. This type of isolation is the most suitable to release a residual lignin structurally representative for the in situ lignin in the pulp, but the preparations obtained are often heavily contaminated.

The aim of the present study was to optimize an enzymatic method for the isolation of residual lignin from

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unbleached and TCF-bleached kraft pulps from eucalypt wood. With this purpose an enzymatic cocktail containing endocellulase (randomly cutting cellulose chains), exocellulase (releasing terminal cellobiose units) and β -glucosidase (hydrolyzing cellobiose) activities was used [37]. Fourier transform-infrared (FTIR) spectroscopy and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) were used to estimate the purity of the different fractions. The mid-infrared region presents bands characteristic of lignin, and can provide an overall estimation of protein and carbohydrate contamination [15,28,35]. Py-GC/MS is a powerful analytical tool for the rapid analysis of complex polymer mixtures including lignocellulosic materials [9,18,29]. In the case of wood, lignin composition in terms of *p*-hydroxyphenylpropane (H), guaiacylpropane (G), and syringylpropane (S) units can be analyzed in situ by Py-GC/MS of the whole material [21,29]. However, a precise analysis of lignin in pulps, especially after bleaching, using Py-GC/MS and other techniques (e.g. NMR) often requires isolation of unaltered residual lignins as described in the present study.

2. Experimental procedures

2.1. Pulp samples

The eucalypt (*Eucalyptus globulus*) pulps were obtained after kraft cooking and TCF bleaching at the ENCE mill in Pontevedra (Spain). They included a brown (unbleached) pulp with a kappa number of 15.2 (that corresponds to approximately 2.2% lignin content) and 40% ISO brightness, and the same pulp bleached using an O-Q-PoP sequence (including oxygen, chelator, peroxide under pressurized oxygen, and atmospheric peroxide stages) resulting in a kappa number of 6.3 (that corresponds to approximately 0.9% lignin content) and 90% ISO brightness [24]. Samples were stored (-20°C) at the humid state (dry matter content approximately 30%).

2.2. Cellulolytic enzymes

The following commercial enzymes with cellulolytic activities were compared: cellulase R-10 from Onozuka; *Trichoderma reesei* Eiconase CEP from AB-Enzymes; *T. reesei* cellulase 22173 and *Aspergillus niger* β -glucosidase 49291 from Fluka; Celluclast 1.5L and *A. niger* Novozym 188 from Novozymes; and almond β -glucosidase G-0395 from Sigma. Their endocellulase (E.C. 3.2.1.4), exocellulase (E.C. 3.2.1.91) and β -glucosidase (E.C. 3.2.1.21) activities were compared on: Avicel (Merck), filter paper (Albet), brown and TCF-bleached eucalypt pulps (ENCE) for total cellulase activity (exocellulase); and the specific substrates carboxymethylcellulose (Serva) and *p*-nitrophenyl-glucoside (Sigma) for endocellulase and β -glucosidase activities, respectively [37]. The enzymatic activities were followed by

the release of reducing sugars estimated as glucose [30], or by the release of *p*-nitrophenol ($\epsilon_{412} 15\,200\text{ M}^{-1}\text{ cm}^{-1}$).

2.3. Optimization of enzymatic hydrolysis

For optimization of cellulase hydrolysis, samples (1.1 g dry weight) of brown and TCF-bleached pulps were incubated with Eiconase CEP at 50°C using three enzyme doses (40 mg, 200 mg and 1 g) and five hydrolysis times (6, 12, 24, 48 and 72 h) and centrifuged (11 000 rpm, 30 min, 5°C , using a LSS-34 Sorvall rotor). Cellulose hydrolysis was followed by weight loss after drying in an aerated oven at 60°C , and FTIR spectra of the non-hydrolyzable residue, and the release of reducing sugars [30].

2.4. Enzymatic isolation of residual lignin

After hydrolysis optimization, pulp samples (50 g dry weight) were treated with 10 g of cellulase and 3.6 ml of Novozym 188 (protein content 23 mg/ml) for 48 h. The enzymatic hydrolysis was performed at pH 5 (in 50 mM acetate buffer), 50°C , and 180 rpm, using 5% pulp consistency. The insoluble fraction was washed with the above buffer until no glucose was released. From the soluble fraction (cellulase hydrolyzate) a precipitate was obtained after addition of saturated NaCl (1:1) and acidification to pH 2.5 with HCl.

2.5. Enzymatic and solvent purification of residual lignin fractions

Two methods were compared for purification of the non-hydrolyzable residue and the acid-precipitable fraction from hydrolyzate, which contained contaminating protein. The first method was based on hydrolysis with 2% alkaline protease from *Bacillus licheniformis* (Subtilisin type VIII, Sigma) [33]. The reaction was conducted at pH 9.6 (0.5 M NaHCO_3 buffer) 37°C , with gentle shaking for 24 h. The solution was centrifuged (11 000 rpm, 5 min, 5°C) and both the insoluble part and the supernatant were recovered. The supernatant was acidified to pH 2.5 with HCl, and a precipitate was obtained.

The second method was based on lignin extraction using dimethylacetamide (DMAC) and NaOH [23]. Samples (50 mg) from enzymatic hydrolysis were dissolved at 60°C for 1 h in 1 ml of DMAC. Lignin was precipitated by pouring the DMAC solution into diethyl ether and isolated by centrifugation (13 000 rpm for 5 min at 5°C). The fraction obtained was dissolved in 15 ml of 0.5 M NaOH at room temperature (1 h). The solution was acidified to pH 2.5, and centrifuged (13 000 rpm, 5 min at 5°C) to obtain the purified residual lignin.

Finally, a combination of both purification methods was assayed by combining the two fractions obtained after protease hydrolysis, and submitting then to solvent purification under the conditions described above. In this way,

a single residual lignin sample was obtained from the cellulase hydrolyzate and a second one from the cellulase residue.

2.6. FTIR

FTIR spectra were obtained with a Bruker IF-28 spectrometer using 1 mg of lignin in 300 mg of KBr. A total of 50 interferograms were accumulated, and the spectra were corrected by baseline subtraction between valleys ca. 1850 and 900 cm^{-1} .

2.7. Py-GC/MS

Pyrolyses were performed with a Curie-point flash pyrolyzer coupled to a Varian Saturn 2000 GC/MS using a 30 m \times 0.25 μm DB-5 column (film thickness 0.25 mm). Approximately, 100 μg of finely divided sample was deposited on a ferromagnetic wire then inserted into the glass liner and placed immediately in the pyrolyzer. The pyrolysis was carried out at 610 $^{\circ}\text{C}$ into the glass liner for 4 s. The chromatograph was programmed from 40 $^{\circ}\text{C}$ (1 min) to 300 $^{\circ}\text{C}$ at a rate of 6 $^{\circ}\text{C}/\text{min}$. The final temperature was held for 20 min. The injector temperature was kept at 280 $^{\circ}\text{C}$ while the GC/MS interface was kept at 300 $^{\circ}\text{C}$. Compounds were identified by comparison with those reported in the literature [16,32] and in the Wiley and NIST computer libraries.

3. Result and discussion

Acidolysis is the most widely used procedure to isolate residual lignin from paper pulps [14,20,25]. However, acidolysis lignins cannot be considered as fully representative for lignin in pulp because the breakdown of inter-unit linkages in lignin [1,21], only partially-cleaved during pulping [19,26], is at the origin of the high yield attained. Enzymatic isolation using cellulases could be an alternative since it is based on mild hydrolysis of pulp polysaccharides, but lignin purification is required [6,22,23,33]. Enzymatic isolation has been used to isolate lignins mainly from softwood (woody gymnosperms) pulps. Previous studies have shown that enzymatic isolation from eucalypt and other hardwood (woody angiosperms) pulps is more complicate due to contamination troubles and low yield [10], and acidolysis has been used to investigate lignin during eucalypt pulp bleaching [11]. The conditions for enzymatic hydrolysis of eucalypt kraft pulp polysaccharides and subsequent purification of residual lignins are optimized here. This optimization included: comparison of cellulolytic enzymes; optimization of enzymatic hydrolysis conditions; FTIR and Py-GC/MS analyses of fractions after cellulase hydrolysis and residual lignin purification; and design of a final isolation protocol. Then, some conclusions on the chemical modification of lignin during pulping and bleaching are presented.

3.1. Comparison of cellulolytic enzymes

Seven commercial enzymes with different cellulolytic activities were compared for hydrolysis of eucalypt pulp (see Section 2). All the enzymes showed activity (0.1–1.6 U/mg) on the four cellulose preparations assayed, with the exception of the two β -glucosidases. The activity on brown pulp was approximately 40% lower than on bleached pulp suggesting that the higher lignin content in pulp before bleaching reduces the efficiency of cellulases. Nevertheless, the decrease of cellulose polymerization degree during bleaching could also improve the hydrolysis rate. Cellulase activity on filter paper was always the lowest (40% of bleached pulp activity) probably because of lower cellulose accessibility. Evaluation of β -glucosidase activity showed that Novozym 188 and Sigma β -glucosidase had specific activities 8–60-fold higher than the other enzymes as expected. The highest endoglucanase specific activities (3.4–6.8 U/mg) corresponded to Celluclast 1.5, Econase CEP, and Fluka cellulose.

Taking the above results into account, Econase CEP with high endoglucanase (4.6 U/mg) and medium exocellulase (0.2 U/mg using brown pulp as substrate) activities, and Novozym 188 with high β -glucosidase (80 U/mg) and also exocellulase (0.6 U/mg) activities, were chosen for isolation of residual lignins.

3.2. Optimization of enzymatic hydrolysis conditions

β -Glucosidase activity was not a limiting factor in the hydrolysis of pulp cellulose, and a relatively low dose of Novozym 188 was enough to guarantee the highest hydrolysis degree. By contrast, the dose of cellulase appeared as a key factor to attain a complete hydrolysis. Therefore, different amounts of Econase CEP were assayed on brown and TCF-bleached eucalypt pulp for a total hydrolysis period of 3 days (Fig. 1). The increased pulp weight immediately after mixing with the highest doses of enzyme suggested that cellulase is fixed on cellulosic fibers. This was confirmed by FTIR spectroscopy showing a strong 1655–60 cm^{-1} band (carbonyl stretching in amide bonds) in the spectrum of this sample. Therefore, some underestimation of pulp weight after hydrolysis could be produced.

No improvement of cellulose hydrolysis was obtained by increasing the dose of cellulase from 0.2 to 1 g (per gram of pulp). Moreover, when 0.2 g/g was used the hydrolysis rate did not increase by extending the hydrolysis period from 12 to 72 h. By contrast, no maximal hydrolysis of cellulose was obtained using the lowest cellulase dose (0.04 g/g), even after an extended hydrolysis period (72 h). Although the highest polysaccharide hydrolysis was produced during the first 12 h, the treatment was extended to 48 h as reported in other studies [23,33]. Therefore, 48 h hydrolysis with 10 g of Econase CEP and 3.6 ml of Novozym 188 (containing 0.5 g of enzyme) per 50 g of pulp was used in subsequent studies.

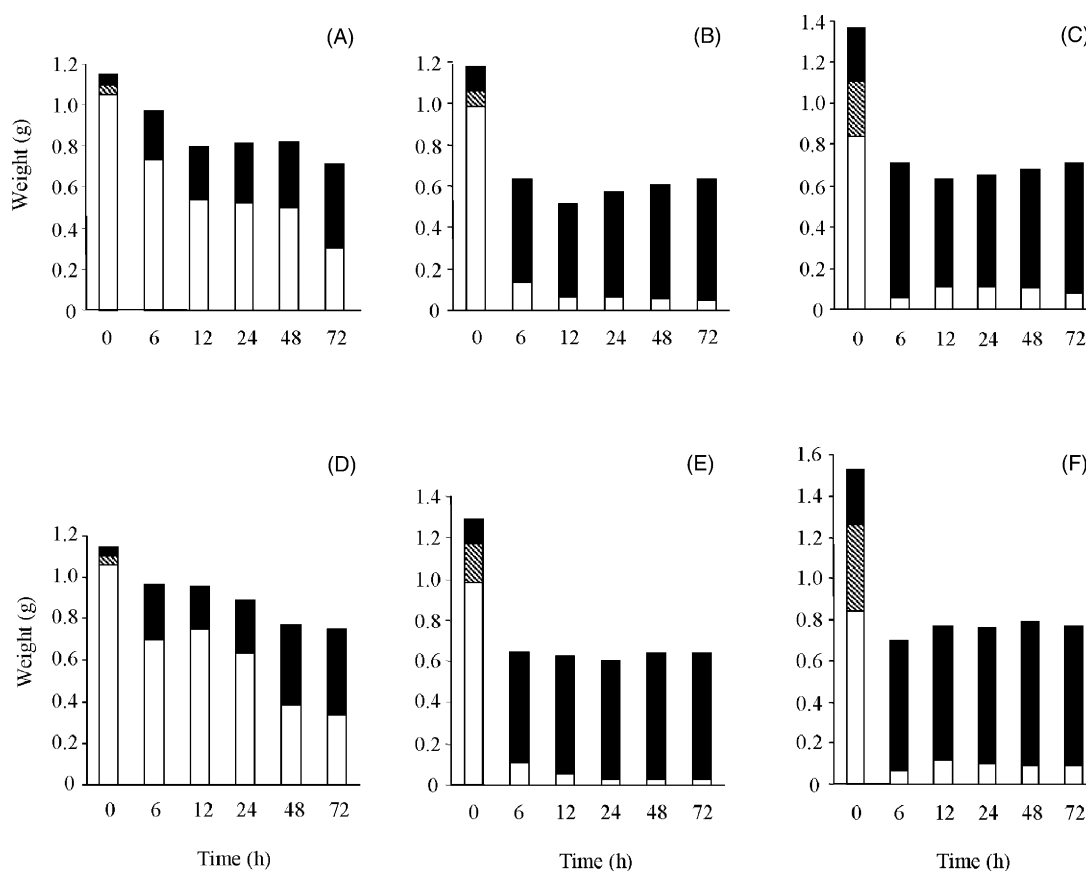


Fig. 1. Time-course (0–72 h) of enzymatic hydrolysis of eucalypt brown pulp (top) and TCF pulp (bottom) (1.1 g dry weight) using 40 mg (A and D), 200 mg (B and E) or 1 g (C and F) of Econase CEP. The total reducing sugars released (black bar) and the weight of non-hydrolyzed pulp (white bar) are indicated, together with an estimation of the enzyme fixed on the pulp at time 0 (dashed bar).

3.3. FTIR and Py-GC/MS analyses of fractions after cellulase hydrolysis

After hydrolysis of pulp with cellulases two fractions were obtained: a non-hydrolyzable residue, and a polymeric material recovered by acid precipitation of the hydrolyzate (Fig. 2A). FTIR analysis (Fig. 3 left, and Table 1) of brown pulp showed spectra dominated by cellulose bands, including the broad band centered on 1050 cm^{-1} . By contrast, the spectrum of the cellulase hydrolyzate showed high amount of protein with the two amide bands dominating the spectrum and only traces of polysaccharides and lignin [23,33], and a similar spectrum was obtained from the hydrolysis residue. The general picture provided by FTIR spectroscopy was completed by Py-GC/MS (Fig. 3 right, and Table 2). High amounts of cellulose-derived compounds were released after Py-GC/MS of brown pulp [8], with the presence of very low amounts of lignin markers. By contrast, the cellulase hydrolyzate was dominated by compounds arising from proteins (peaks 2, 12, 21, 37 and 47) and polysaccharides, particularly from hemicelluloses (peak 53), although some lignin-derived compounds (peaks 41, 65 and 75) were present in noticeable amounts. Similar results were

observed for the hydrolysis residue, with the lignin-derived compounds being the most prominent compounds in this fraction. The presence of protein was revealed by diagnostic products from phenylalanine (peaks 2 and 8), tyrosine (peaks 12, 21 and 30) and tryptophan (peaks 37 and 47) residues, since non-aromatic amino acids did not release suitable markers [2,7].

The above analyses showed that both the material recovered from the cellulase hydrolyzate of eucalypt pulp and the non-hydrolyzable residue contained some amount of lignin but they were strongly contaminated with protein and some polysaccharide. This protein derives from the enzymes used to hydrolyze cellulose, which remained linked to non-hydrolyzed cellulose in the residue, or co-precipitate with the lignin recovered from the enzymatic hydrolyzate.

3.4. Analyses of fractions after residual lignin purification

Alkali extraction, alone or after organic solvent extraction, was the first method used in the literature for residual lignin purification [23,38]. Protease hydrolysis was introduced later [34]. Both purification methods were compared

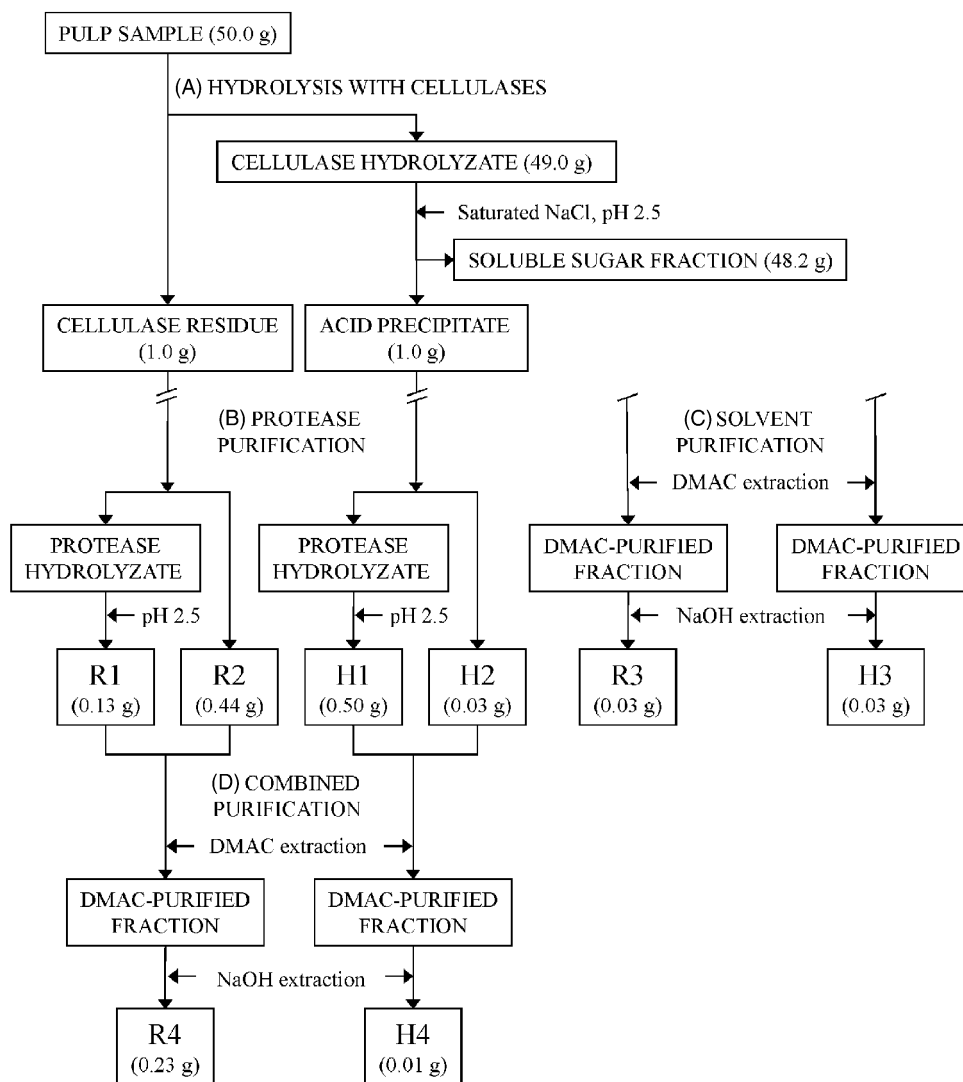


Fig. 2. Scheme for the isolation of residual lignin from eucalypt pulps by enzymatic hydrolysis (A), followed by purification based on protease hydrolysis (B), solvent extraction (C), and a combination of both (D) resulting in four lignin fractions derived from the hydrolysis residue (R1–R4) and four lignin fractions derived from the hydrolyzate (H1–H4) (yields in parentheses correspond to lignin isolation from brown pulp, and were calculated from its kappa number).

here on the residual lignin from the eucalypt pulps (Fig. 2B and C). The yield of the lignin fractions recovered after protease purification of the cellulase residue and hydrolyzate from brown pulp (fractions R1–R2 and H1–H2 respectively) was high (up to 45% for H1), the total lignin recovered being around 98% (Table 3). However, they still included some contaminant protein, as shown by the PR/(G + S) ratio, and their FTIR purity was only moderate. Solvent purification resulted in higher purity residual lignins (fractions R3 and H3) but the final yield (only 1–3%) was much lower than obtained by protease purification (Table 3).

The results were different during lignin isolation from the TCF pulp since the total yield after protease purification was only around 9%, and the fractions from the cellulase residue were strongly contaminated with calcium oxalate, as evidenced by strong 1616, 1316, 778 and 661 cm^{-1} FTIR

bands. Oxalate is formed during bleaching [12], and has been reported during lignin isolation from other TCF pulps [34]. Py-GC/MS of most lignin fractions isolated from TCF pulp also showed higher level of contamination with both polysaccharides (PO/(G + S) ratio) and proteins (PR/(G + S) ratio) compared with the brown pulp lignins (Table 3). The low lignin yield from TCF pulp could be due to isolation difficulties (e.g. due to oxalate presence). However, it is necessary to take into account that the lignin content in this pulp could be lower than estimated from the kappa number due to the presence of hexenuronic acids, which can be responsible for up to 40% of kappa number in some pulps [5,27].

The above differences in residual lignin behavior during its isolation from brown (high kappa) and TCF-bleached (low kappa) eucalypt pulps agree with information found in

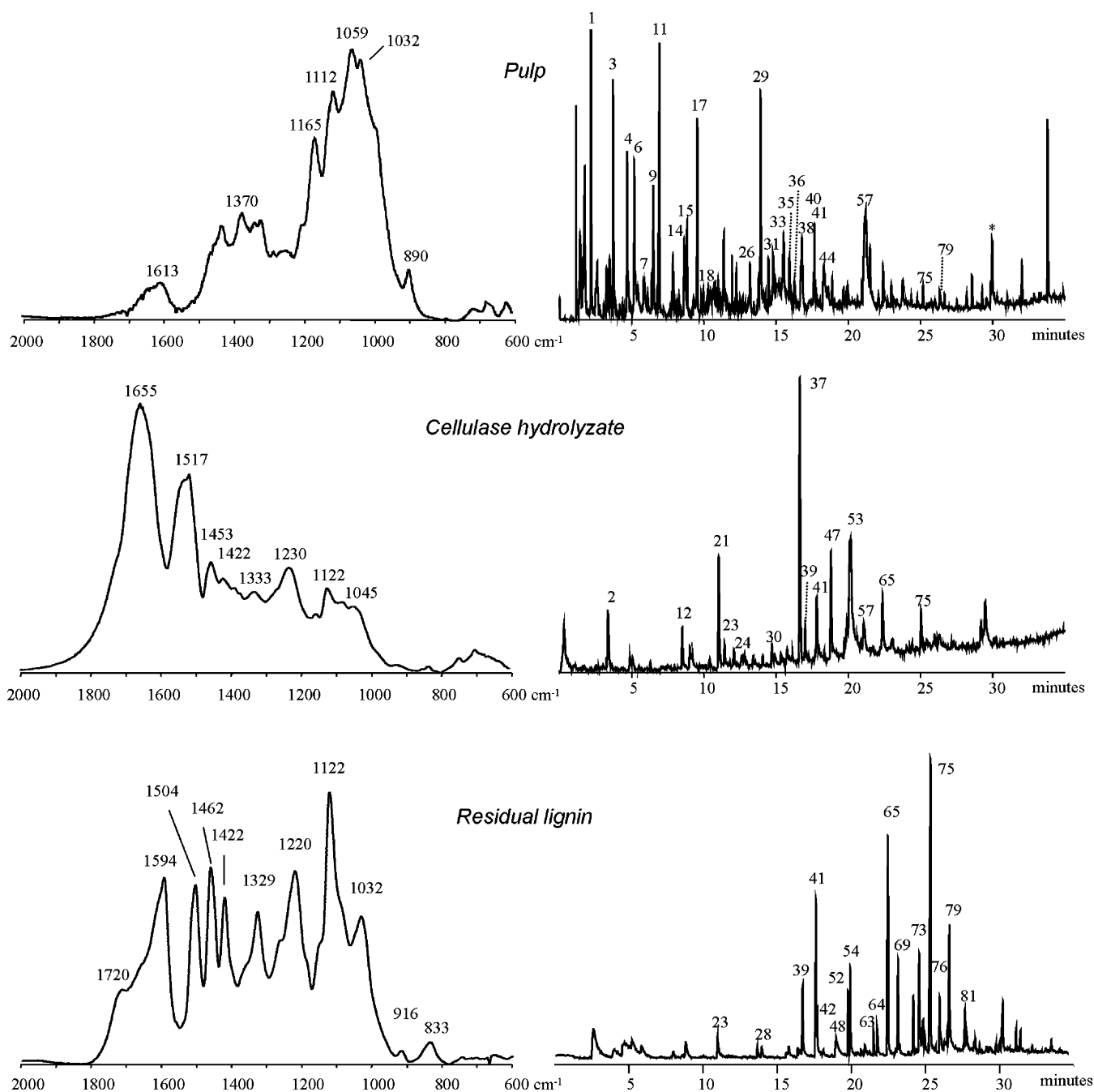


Fig. 3. Selected FTIR spectra, 2000–600 cm^{-1} region (left), and Py-GC/MS chromatograms (right) during enzymatic isolation of residual lignin, corresponding to eucalypt brown pulp (kappa number 15.2), cellulase hydrolyzate (acid-precipitable material), and residual lignin (fraction R4) (only the main FTIR signals and Py-GC/MS peaks are indicated). See Tables 1 and 2 for assignment of FTIR signals, and identification of lignin, cellulose and protein Py-GC/MS peaks (a lipid-derived compound, palmitic acid, is indicated with an asterisk).

the literature [22]. Previous results have shown that most of the lignin from high kappa pulps is recovered from the cellulase residue, but this amount decreases when the kappa number decreases, most of the lignin from TCF pulp being recovered from the hydrolyzate [23,34]. It has also been also reported that the distribution of residual lignin between cellulase residue and hydrolyzate depends on the origin of the pulp [33].

3.5. Design of a final isolation protocol

When both purification methods were applied separately to the cellulase residue and hydrolyzate material from eucalypt pulp, a total of six lignin fractions were obtained with different yield and composition as discussed above. With the purpose of reducing the fraction number and increasing purity a new purification strategy was applied (Fig. 2D). It

Table 1
Main assignments of lignin, polysaccharide and protein FTIR bands^a

Lignin	
1714–1725 cm ⁻¹	Stretching of C=O unconjugated to aromatic rings (oxidized side-chains)
1655 cm ⁻¹	Stretching of C=O conjugated to aromatic rings
1594–1609 cm ⁻¹	Aromatic ring vibrations and C=O stretching (S > G);
1504–1515 cm ⁻¹	Aromatic ring vibrations (G > S)
1462–1464 cm ⁻¹	Asymmetric C–H bending (in CH ₃ and –CH ₂ –)
1421–1424 cm ⁻¹	Aromatic ring vibrations
1329–1330 cm ⁻¹	Aromatic ring breathing (S and G _{condensed} units)
1270 cm ⁻¹ (shoulder)	Aromatic ring breathing (G units)
1216–1225 cm ⁻¹	C–C, C–O and C=O stretching (G _{condensed} > G _{etherified})
1114–1125 cm ⁻¹	Aromatic in-plane C–H bending (S units)
1030–1033 cm ⁻¹	Aromatic in-plane C–H bending (G > S)
913–929 cm ⁻¹	Out-of-plane aromatic C–H bending
833–834 cm ⁻¹	Out-of-plane C–H bending in S units
Polysaccharide	
1613 cm ⁻¹	Water
1370 cm ⁻¹	Symmetric bending of aliphatic C–H
1030–1170 cm ⁻¹	C–O stretching in alcohols
890 cm ⁻¹	β-Glycosidic linkages in pyranose units
Protein	
1655–1658 cm ⁻¹	C=O Stretching in amides (I)
1516 cm ⁻¹	C=O Stretching in amides (II)

^a See [15,28,35].

was based on combination of the protease residue and hydrolyzate material (fractions R1 and R2 on one hand, and H1 and H2 on the other hand), and application of a subsequent solvent purification step. In this way, two new lignin fractions (H4 and R4) were obtained, although only that derived from the cellulase hydrolyzate (H4) could be obtained from TCF pulp due to low lignin content in the cellulase residue (Table 3). On the other hand, fraction H4 from brown pulp could be discarded because of its low yield compared with fraction R4, which showed high-purity and a yield around 20%. Therefore, fractions R4 and H4 were selected as representative for residual lignins in brown and TCF-bleached eucalypt pulp, respectively.

The FTIR spectra of the purified residual lignins was dominated by lignin bands (Table 1), including the characteristic triplet at 1420–1510 cm⁻¹ (Fig. 3). Moreover, only lignin-derived products (G and S type) (Table 2) were released after Py-GC/MS of the residual lignins (Fig. 3), confirming the purity suggested by the FTIR spectra.

The main results obtained during optimization of lignin isolation from eucalypt brown and TCF-bleached pulps are summarized in Table 3 that shows yield, Py-GC/MS ratios (informing about lignin composition, and presence of con-

Table 2
Main pyrolysis products from lignin, polysaccharide and protein^a

G-lignin	
23 Guaiacol	55 Homovanillin
28 4-Methylguaiacol	56 Acetoguaiacone
34 4-Ethylguaiacol	64 Guaiacylacetone
39 4-Vinylguaiacol	67 Propiovanillone
42 Eugenol	77 <i>t</i> -Coniferaldehyde
48 Vanillin	78 <i>t</i> -Coniferyl alcohol
54 <i>t</i> -Isoeugenol	
S-lignin	
32 3-Methoxycatechol ^b	75 <i>t</i> -4-Propenylsyringol
41 Syringol	76 Acetosyringone
52 4-Methylsyringol	79 Syringylacetone
63 4-Ethylsyringol	80 Methyl syringate
65 4-Vinylsyringol	81 Propiosyringone
69 4-Allylsyringol	82 <i>c</i> -Sinapyl alcohol
71 <i>c</i> -4-Propenylsyringol	83 <i>t</i> -Sinapaldehyde
73 Syringaldehyde	84 <i>t</i> -Sinapyl alcohol
74 Homosyringaldehyde	
Polysaccharide	
1 3-OH-propanal	26 3,4-di-OH-benzaldehyde
3 Unknown	29 Catechol
4 2-Furaldehyde	31 5-OH-methyl-2-furaldehyde
6 2-OH-methylfuran	33 3-Methylcatechol
7 Cyclopent-1-ene-3,4-dione	35 1,4-Benzenediol
9 (5H)-furan-2-one	36 4-Methylcatechol
11 2,3-Dihydro-5-methylfuran-2-one	38 4-OH-benzaldehyde
14 5,6-Dihydropyran-2,5-dione	40 2-Methyl-1,4-benzenediol
15 4-OH-5,6-dihydro-(2H)-pyran-2-one	44 Pyrogallol
17 2-OH-3-methyl-2-cyclopenten-1-one	46 1,6-Anhydrogalactopyranose ^c
18 2,3-Dimethylcyclopenten-1-one	53 1,6-Anhydromannopyranose
24 Levoglucosenone	57 Levoglucosane
Protein	
2 Toluene	30 4-Vinylphenol
8 Styrene	37 Indole
12 Phenol	47 Methylindole
21 4-Methylphenol	
Others (from cooking reagents)	
85 Anthraquinone	

^a See [9,18,29].

^b From demethylated lignin (not included in S/G calculation of Table 3).

^c Observed only during TCF lignin purification (absent from Fig. 3).

taminating protein and polysaccharides), and FTIR purity in the wood, whole pulps, pulp fractions, and different residual lignins.

3.6. Chemical modification of lignin during pulping and bleaching

The FTIR spectra of residual lignins from both brown and TCF-bleached eucalypt pulps (Fig. 3, left) showed a typical lignin pattern [35] characterized by higher intensity of signals assigned to S-type units (1329 cm⁻¹ band) than to G-type units (1270 cm⁻¹ shoulder). In the same

Table 3
Residual lignin fractions from residue and hydrolyzate (precipitable material) from cellulase hydrolysis of brown and TCF eucalypt pulps after different purification methods (see Fig. 2): yield, Py-GC/MS molar ratios, and FTIR purity

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^a Lignin fractions from cellulase residue (R1–R4) and hydrolyzate (H1–H4).

^b Yield with respect to lignin content estimated from kappa number.

^c These samples include high amounts of *t*-sinapyl alcohol that was not included in calculations.

^d PO; polysaccharide.

^e PR, protein.

^f FTIR purity: intensity of lignin peaks with respect to protein and carbohydrate peaks (from – to +++++); na, not applicable; nd, not determined. Those compounds used for calculation of Py-GC/MS molar ratios are listed in Table 2.

way, the pyrograms of these residual lignins were dominated by syringol (peak 41), vinylsyringol (peak 65), *t*-4-propenylsyringol (peak 75), and *t*-sinapyl alcohol (peak 84) (Fig. 3, right) (the latter only in TCF lignin). The S/G ratio of the brown pulp lignin R4 (3.1) was only slightly higher than estimated in situ by Py-GC/MS of the whole pulp (2.6), but a stronger difference between S/G estimation in situ (1.1) and after residual lignin (H4) isolation (2.1) was found in the case of TCF pulp (Table 3). However, the latter ratio should be more reliable due to the extremely low lignin content in the TCF-bleached pulp, which strongly limits the possibility to estimate its S/G ratio by Py-GC/MS of the whole pulp even using single-ion chromatograms corresponding to selected lignin markers [8]. The eucalypt lignin S/G ratio, estimated after wood Py-GC/MS (Table 3), was similar to those reported in the literature [9,14]. A decrease of S/G ratio during kraft cooking of eucalypt wood has been suggested [11], however, no strong differences were found here. On the other hand, a higher intensity of the bands at 1594–1596 and 1714–1725 cm^{–1}, the latter assigned to non-conjugated carbonyls, was found in the TCF residual lignin. This suggests an increase of non-conjugated carbonyls during TCF bleaching of the eucalypt pulp, which included oxygen delignification and peroxide stages. The increase of carbonyl groups can be due to formation of muconic acid-type structures from aromatic-ring opening during bleaching [23,31].

We have shown here that residual lignins have been successfully isolated from eucalypt kraft pulps by cellulase hydrolysis followed by a combined purification using protease and lignin solvents. The high-purity of the residual lignins obtained was confirmed by FTIR and Py-GC/MS analyses. A similar enzymatic isolation procedure could be applied for residual lignin isolation from other hardwood pulps.

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