Structural characterization of milled wood lignins from different eucalypt species

Jorge Rencoret[1], Gisela Marques[1], Ana Gutiérrez[1], David Ibarra[2,a], Jiebing Li[3], Göran Gellerstedt[3], J. Ignacio Santos[2], Jesús Jiménez-Barbero[2], Ángel T. Martínez[2] and José C. del Río[1,*]

[1] Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Seville, Spain

[2] Centro de Investigaciones Biológicas (CIB), CSIC, Ramiro de Maeztu 9, Madrid, Spain

[3] Department of Fiber and Polymer Technology, Royal Institute of Technology (KTH), Stockholm, Sweden

*Corresponding author.
Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Reina Mercedes 10, PO Box 1052, E-41080 Sevilla, Spain
E-mail: delrio@irnase.csic.es

Abstract

The chemical structure of milled-wood lignins from *Eucalyptus globulus*, *E. nitens*, *E. maidenii*, *E. grandis*, and *E. dunnii* was investigated. The lignins were characterized by analytical pyrolysis, thioacidolysis, and 2D-NMR that confirmed the predominance of syringyl over guaiacyl units and only showed traces of *p*-hydroxyphenyl units. *E. globulus* lignin had the highest syringyl content. The heteronuclear single quantum correlation (HSQC) NMR spectra yielded information about relative abundances of inter-unit linkages in the whole polymer. All the lignins showed a predominance of β-O-4′ ether linkages (66–72% of total side-chains), followed by β-β′ resinol-type linkages (16–19%) and lower amounts of β-5′ phenylcoumaran-type (3–7%) and β-1′ spirodienone-type linkages (1–4%). The analysis of desulfurated thioacidolysis dimers provided additional information on the relative abundances of the various carbon-carbon and diaryl ether bonds, and the type of units (syringyl or guaiacyl) involved in each of the above linkage types. Interestingly, 93–94% of the total β-β′ dimers included two syringyl units indicating that most of the β-β′ substructures identified in the HSQC spectra were of the syringaresinol type. Moreover, three isomers of a major trimeric compound were found which were tentatively identified as arising from a β-β′ syringaresinol substructure attached to a guaiacyl unit through a 4-O-5′ linkage.

Keywords: 2D-NMR; desulfuration; dimers; eucalypt wood; *Eucalyptus dunnii*; *Eucalyptus globulus*; *Eucalyptus grandis*; *Eucalyptus maidenii*; *Eucalyptus nitens*; heteronuclear single quantum correlation (HSQC); milled-wood lignin (MWL); pyrolysis-GC/MS; thioacidolysis; trimers.

[a] Current address: Department of Fiber and Polymer Technology, Royal Institute of Technology (KTH), SE-100 44 Stockholm, Sweden.

Introduction

Eucalypt species are cultivated in plantations as a fast-growing raw material for paper pulp production in Spain, Portugal, Brazil, South Africa, and other countries. The use of eucalypt wood for pulp has greatly increased during the last decades: world production with nearly 10 million tons per year is approximately one-third of the total hardwood pulp produced. *Eucalyptus globulus* Labill. provides the best raw material for kraft pulp in terms of high pulp yield (Rencoret et al. 2007). It is known that both pulping and bleaching performances are highly dependent on the relative content, structure, and reactivity of the biopolymers, cellulose, hemicelluloses, and lignin. In particular, different eucalypt species, or clones from the same species, might require different pulping conditions to achieve the same extent of delignification and yield of pulp (González-Vila et al. 1999; del Río et al. 2005). The lignin content and its composition in terms of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties and the different inter-unit linkages are important factors in pulp production affecting the delignification rate. It has been shown that higher S/G ratios in woods from the same eucalypt species implied higher delignification rates, less alkali consumption, and therefore higher pulp yield (del Río et al. 2005).

The wood from *E. globulus* has been widely characterized, including the lignin composition and structure (Ona et al. 1997; Rodrigues et al. 1999; del Río et al. 2001; Evtuguin et al. 2001; Ibarra et al. 2007a). The structure of *E. grandis* milled-wood lignin (MWL) has also been recently characterized by NMR techniques (Capanema et al. 2005). However, structural studies of lignins from other eucalypt woods used for papermaking are still scarce and mostly limited to their H:G:S ratios (Ona et al. 1997; Yokoi et al. 1999, 2001; Rencoret et al. 2007; Guerra et al. 2008).

More information on the structure of lignin in eucalypt species is needed to optimize their use for paper pulp manufacture. In this paper, we report the structural characterization of lignin from woods of *E. globulus*, *Eucalyptus nitens* (H. Deane & Maiden) Maiden, *Eucalyptus maidenii* F. Muell., *Eucalyptus grandis* W. Hill ex Maiden, and *Eucalyptus dunnii* Maiden, which are the species most frequently grown in eucalypt plantations for papermaking. This paper is a continuation of our previous work (Rencoret et al. 2007) about the chemistry of these eucalyptus species. The MWLs of the eucalypt trees were characterized by pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), thioacidolysis, and 2D-NMR.

Materials and methods

Samples

The wood was collected from 10–12-year-old trees of *E. globulus*, *E. nitens*, *E. maidenii*, *E. grandis* and *E. dunnii* which were provided by ENCE (Pontevedra, Spain). Klason lignin was estimated according to T222 om-88 (Tappi 2004). The MWL samples (Björkman 1956) were obtained from grounded wood prepared in a Retsch cutting mill to pass through a 100-mesh screen. This sawdust was successively extracted with acetone for 8 h in a Soxhlet extractor and with hot water (100°C) for 3 h. The extractive free sawdust was finely ball-milled in a Retsch S100 centrifugal ball mill with toluene (150 h). After this treatment, the milled wood was submitted to an extraction (4×24 h) with dioxane-water (9:1, v/v) (5–10 ml of solvent/g of milled wood). The solution was centrifuged and the supernatant subsequently evaporated at 40°C at reduced pressure until dryness. The residue obtained (raw MWL) was redissolved in a solution of acetic acid/water 9:1 (v/v) (20 ml of solvent/g of raw MWL). The solution was then precipitated in water and the precipitated residue was separated by centrifugation, milled in an agate mortar and subsequently dissolved in a solution of 1,2-diclorome-thane:ethanol (1:2, v/v). The mixture was then centrifuged to eliminate the insoluble material, the supernatant was precipitated in diethyl ether and the obtained residue was separated by centrifugation. This residue was then resuspended in petroleum ether and centrifuged again to obtain the final purified MWL fraction, which was dried under a N_2 current. The final yields ranged from 10% to 15% of the original Klason lignin content.

Py-GC/MS

Pyrolysis of MWL (approximately 100 µg) was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a HP 5MS 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 50°C (1 min) to 100°C at 30°C min^{-1} and then to 300°C (10 min) at 10°C min^{-1}. He was the carrier gas (1 ml min^{-1}). 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). Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized, and the data for two repetitive analyses were averaged and expressed as percentages.

Thioacidolysis

Thioacidolysis of 5 mg MWL was performed as described by Rolando et al. (1992) using 0.2 M BF_3 etherate in dioxane/ethanethiol 8.75:l. The reactions products were extracted with CH_2Cl_2, dried and concentrated. GC analysis of trimethylsilylated [using N,O-bis-(trimethylsilyl)-trifluoroacetamide, BSTFA] samples was performed with a Hewlett-Packard 6890 instrument using an Rtx 5 column from Restec Corporation (45 m×0.32 µm i.d., 0.25 µm film thickness) and a flame detector. The temperature was programmed from 180°C to 270°C (15 min) at 40°C min^{-1} and then to 300°C (5 min) at 4°C min^{-1}. Injector and detector were at 250°C and 280°C, respectively, and He was the carrier gas. Size-exclusion chromatography (SEC) of thioacidolysis products, acetylated using acetic anhydride/pyridine (Gellerstedt 1992), was carried out using three Styragel columns (HR4, HR2, and HR0.5 of 7.8×300 mm each) in series, tetrahydrofuran as mobile phase, and 280 nm detection.

Desulfurization of thioacidolysis degradation products

A total of 200 µl CH_2Cl_2 solution containing the thioacidolysis products was desulfurized as described by Lapierre et al. (1991). GC/MS analysis of the dimeric compounds was performed in a Varian Star 3400 equipment coupled to an ion-trap detector Varian Saturn 2000, using a DB-5HT fused-silica capillary column from J&W Scientific (30 m×0.25 mm i.d., 0.1 µm film thickness). The temperature was programmed from 50°C to 110°C at 30°C min^{-1} and then to 320°C (13 min) at 6°C min^{-1}. The injector and transfer line were at 300°C. Trimeric compounds were analyzed using a short (12 m) capillary column of the same characteristics, a Varian 3800 chromatograph coupled to an ion-trap detector Varian 4000 (with a range up to 1000 *m/z*), and a temperature program from 60°C (1 min) to 380°C (5 min) at 10°C min^{-1}. The transfer line was at 300°C, and the injector was programmed from 120°C (0.1 min) to 380°C at 200°C min^{-1}. In both analyses, He was the carrier gas (2 ml min^{-1}) and tetracosane was used as internal standard. Dimer identification was based on reported mass spectra of G-G compounds (Lapierre et al. 1991; Saito and Fukushima 2005) and mass fragmentography.

NMR spectroscopy

2D-NMR spectra were recorded at 25°C in a Bruker AVANCE 500 MHz, equipped with a z-gradient triple resonance probe. A total of 40 mg MWL was dissolved in 0.75 ml dimethylsulfoxide (DMSO)-d_6, and heteronuclear single quantum correlation (HSQC) spectra were recorded. The spectral widths were 5000 and 13 200 Hz for the ^{1}H and ^{13}C dimensions, respectively. The number of collected complex points was 2048 for ^{1}H dimension, with a recycle delay of 5 s. The number of transients was 64, and 256 time increments were always recorded in ^{13}C dimension. The $^1J_{CH}$ used was 140 Hz. The *J*-coupling evolution delay was set to 3.2 ms. Squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transformation, the data matrixes were zero filled up to 1024 points in the ^{13}C dimension.

The cross-signal intensity depends on the particular $^1J_{CH}$ value, as well on the T_2 relaxation time; therefore, a direct intensity analysis of the different signals is impossible. Thus, integration was performed separately for the different regions of the spectra, which contain signals corresponding to chemically-analogous C-H pairs, with similar $^1J_{CH}$ coupling values. In the aliphatic oxygenated region, inter-unit linkages were estimated from C_α-H_α correlations, except for structures **E** and **F** described below, where C_β-H_β and C_γ-H_γ correlations were used respectively, and the relative abundance of side-chains involved in different substructures and terminal structures were calculated (with respect to total side-chains), as well as the percentage of each inter-unit linkage type with respect to the linkage total. In the aromatic region, [13]C-^{1}H correlations from S and G units were used to estimate the S/G ratio.

Results and discussion

Py-GC/MS

Py-GC/MS is a rapid and sensitive technique for analyzing the composition of lignin (Faix et al. 1990; Ralph and Hatfield 1991). It has already been applied for the "*in situ*" calculation of S/G ratio in eucalypt wood (Rodrigues et al. 1999; Yokoi et al. 1999, 2001; del Río et al. 2005), including the above mentioned species (Rencoret et al. 2007).

All the eucalypt lignins yielded similar Py-GC/MS products, and a representative pyrogram is shown in Figure

1. The identities and relative molar abundances of the released lignin compounds are listed in Table 1. Among them, guaiacol and syringol-type phenols, derived from the G and S lignin units, respectively, were identified. Only traces (less than 0.1%) of phenol-type compounds from p-hydroxycinnamyl (H) units could be detected. The most important compounds identified were guaiacol (**1**), 4-vinylguaiacol (**5**), syringol (**6**), 4-methylsyringol (**11**), 4-vinylsyringol (**19**), syringaldehyde (**24**), (E)-4-propenylsyringol (**27**), and (E)-sinapaldehyde (**37**). In all the samples, the S phenols were released in higher abundances than the respective G phenols. The uncorrected molar S/G ratios obtained from the molar areas of all the lignin-derived compounds are shown in Table 2, ranging from 1.9 to 2.6. The lignin from *E. globulus* showed the highest S/G ratio, which makes it easier to be delignified under kraft cooking than the other eucalypt species (Rencoret et al. 2007) due to the higher reactivity of the S lignin in alkaline systems (Chang and Sarkanen 1973; Tsutsumi et al. 1995). A similar tendency of lignin S/G ratios was obtained by Py-GC/MS of the whole wood from these eucalypt species (Rencoret et al. 2007), although the values were on average 1.6-fold higher.

Concerning the S/G ratios, Sarkanen and Hergert (1971) pointed out that degradation methods in general overestimate the amounts of S units. This is due to the low condensation degree of S units within the macromolecule. Accordingly, all degradation techniques lead to an elevated amount of S units and, as a consequence, the uncorrected S/G ratios overestimate the true S/G ratios in lignin. This was also confirmed later for pyrolysis by Böttcher (1993), who presented correction formulae, and by Choi et al. (2006) and Rencoret et al. (2007), just to mention a few.

2D-NMR

2D-NMR provides information of the structure of the whole macromolecule and is a powerful tool for lignin structural characterization (Ralph et al. 1999, 2004b; Capanema et al. 2001, 2004, 2005; Balakshin et al. 2003;

Lu and Ralph 2005; Ibarra et al. 2007a,b) that enabled description of new lignin substructures during recent years (Karhunen et al. 1995; Zhang and Gellerstedt 2001; Zhang et al. 2006).

The lignin HSQC spectra showed three regions corresponding to aliphatic, side-chain, and aromatic ^{13}C-^1H correlations. The aliphatic (non-oxygenated) region included signals with no structural information in the case of lignin. The side-chain and aromatic regions of a representative eucalypt MWL are shown in Figures 2 and 3, respectively. Cross-signals were assigned by comparing with the literature (Ämmälahti et al. 1998; Ralph et al. 1999, 2004b; Capanema et al. 2001, 2004, 2005; Balakshin et al. 2003; Liitiä et al. 2003; Ibarra et al. 2007a,b) and the main assignments are listed in Table 3.

The side-chain region of the spectra yielded useful information about the inter-unit linkages present in the eucalypts lignins. All the spectra showed prominent signals corresponding to β-O-4′ ether linkages (substructure **A**). The C_α-H_α correlations in β-O-4′ substructures were observed at δ_C/δ_H 71.7–72.4/4.74–4.86 ppm, while the C_β-H_β correlations were observed at δ_C/δ_H 84.1–86.4/4.11–4.28 ppm. The C_γ-H_γ correlations in β-O-4′ substructures were observed at δ_C/δ_H 60.1/3.40 and 3.72 ppm, partially overlapped with other signals. In addition to β-O-4′ substructures, other linkages were also observed. Strong signals for resinol (β-β′/α-O-γ′/γ-O-α′) substructures (**B**) were observed in all spectra, with their C_α-H_α, C_β-H_β and the double C_γ-H_γ correlations at δ_C/δ_H 85.5/4.67, 54.1/3.06 and 71.7/3.83 and 4.19 ppm, respectively. Phenylcoumaran (β-5′/α-O-4) substructures (**C**) were also found, although in lower amounts, the signals for their C_α-H_α and C_β-H_β correlations being observed at δ_C/δ_H 87.4/5.47 and 53.7/3.46 ppm, respectively, and that of C_γ-H_γ correlation overlapping with other signals around δ_C/δ_H 62/3.8 ppm. Finally, small signals corresponding to spirodienone (β-1′/α-O-α′) substructures (**D**) could also be observed in the spectra, their C_α-H_α, $C_{\alpha'}$-$H_{\alpha'}$, C_β-H_β, and $C_{\beta'}$-$H_{\beta'}$ correlations being at δ_C/δ_H 81.7/5.09, 85.4/4.80, 60.3/2.75, and 79.8/4.11 ppm,
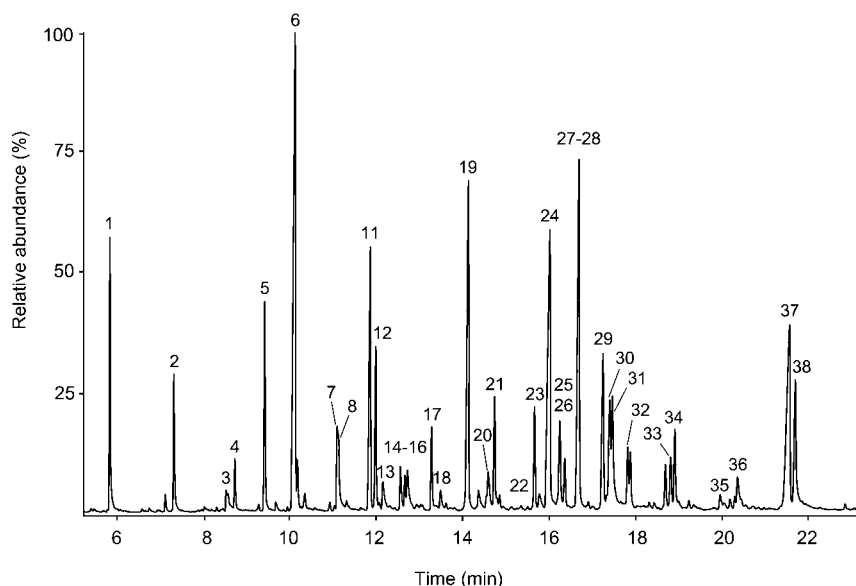

Figure 1 Py-GC/MS chromatogram of a representative MWL isolated from *E. dunnii* wood. The numbers refer to the compounds listed in Table 1.

Table 1   Identification and relative molar abundance of the compounds identified in the Py-GC/MS of MWL from wood of the different eucalypt species analyzed.

		E. globulus	E. nitens	E. maidenii	E. grandis	E. dunnii
1.	Guaiacol	5.5	5.5	6.3	5.7	5.1
2.	4-Methylguaiacol	3.8	3.5	4.1	4.6	2.6
3.	3-Methoxycatechol	1.3	2.4	1.7	0.2	1.3
4.	4-Ethylguaiacol	1.2	1.2	1.4	1.3	0.9
5.	4-Vinylguaiacol	4.8	4.6	5.1	5.9	4.0
6.	Syringol	13.8	13.5	14.2	11.8	14.5
7.	Eugenol	0.7	0.9	0.7	1.2	0.9
8.	4-Propylguaiacol	0.3	0.4	0.3	0.3	0.2
9.	Vanillin	1.5	2.6	2.8	2.1	2.4
10.	(Z)-Isoeugenol	0.3	0.1	0.3	0.8	0.4
11.	4-Methylsyringol	7.9	7.1	7.3	7.4	5.9
12.	(E)-Isoeugenol	2.6	3.9	3.4	3.7	3.1
13.	Homovanillin	1.2	1.0	1.1	0.9	0.8
14.	Propine-guaiacol	0.6	0.5	0.6	1.0	0.7
15.	Propine-guaiacol	0.5	0.4	0.5	0.7	0.5
16.	Acetoguaiacone	0.6	0.9	1.2	1.4	1.0
17.	4-Ethylsyringol	4.2	2.0	1.9	1.5	1.5
18.	Guaiacyl-acetone	0.6	0.5	0.5	0.4	0.4
19.	4-Vinylsyringol	9.9	8.2	7.5	8.1	7.9
20.	Guaiacyl vinyl ketone	0.6	0.6	0.7	0.8	0.8
21.	4-Allylsyringol	1.7	1.9	1.5	1.6	2.0
22.	Propylsyringol	0.2	0.2	0.2	0.2	0.2
23.	(Z)-Propenylsyringol	1.4	1.4	1.6	1.8	1.8
24.	Syringaldehyde	6.4	7.0	7.2	7.5	8.4
25.	Propine-syringol	1.7	1.4	1.2	1.3	1.5
26.	Propine-syringol	1.1	0.7	0.5	0.8	0.7
27.	(E)-Propenylsyringol	5.7	6.8	5.1	5.8	6.0
28.	Homosyringaldehyde	4.9	4.0	3.3	2.6	3.1
29.	Acetosyringone	2.6	2.7	3.0	3.4	3.4
30.	(E)-Coniferaldehyde	1.5	1.7	2.2	2.5	2.3
31.	(E)-Coniferyl alcohol	0.8	2.1	1.7	1.5	2.5
32.	Syringylacetone	1.5	1.2	1.1	0.9	1.0
33.	Propiosyringone	0.9	0.9	1.0	1.0	0.9
34.	Syringyl vinyl ketone	0.8	0.6	0.9	1.3	1.2
35.	Dihydrosinapyl alcohol	0.2	0.4	0.3	0.3	0.4
36.	(Z)-Sinapyl alcohol	0.3	0.6	0.5	0.3	0.8
37.	(E)-Sinapaldehyde	5.6	4.6	5.0	6.2	6.2
38.	(E)-Sinapyl alcohol	1.0	2.2	2.0	1.2	2.7

Table 2   Molar S/G ratios of the MWL from the different eucalypt woods estimated by different analytical techniques, and yields of thioacidolysis monomer obtained from SEC analyses.

	E. globulus	E. nitens	E. maidenii	E. grandis	E. dunnii
S/G ratio					
Py-GC/MS	2.6	2.1	2.0	1.9	2.4
2D-NMR	2.9	2.7	2.4	1.7	2.7
Thioacidolysis	2.5	2.1	1.8	1.6	2.3
Thioacidolysis monomers					
SEC (%)	47	45	44	42	48

respectively. Spirodienone substructures were previously reported in the lignin from *E. grandis* wood by Capanema et al. (2005). Other small signals in the side-chain region of the HSQC spectra corresponded to C_β-H_β correlations (at δ_C/δ_H 83.8/5.23 ppm) of β-O-4′ substructures bearing a C_α carbonyl group (**E**), and C_γ-H_γ correlation (at δ_C/δ_H 61.9/4.09 ppm) tentatively assigned to *p*-hydroxycinnamyl (**F**) terminal structures (although their olefinic correlations were not found in the corresponding region of the spectra). No dibenzodioxocin structures could be observed in the HSQC spectra of any of the eucalypt MWL samples selected for this study, in agreement with the results already reported for the lignins of *E. grandis*

(Capanema et al. 2005) and *E. globulus* (Ibarra et al. 2007a).

The percentage of lignin side-chains involved in the main substructures and terminal structures found in the eucalypt lignins (referred to total side-chains) are indicated in Table 4, which also shows (in parentheses) the relative abundance of each of the inter-unit linkages. In all cases, the main substructure was the β-O-4′ one (**A**) that corresponded to around 70% of all side-chains. *E. nitens*, *E. globulus*, and *E. maidenii* presented the highest proportion of β-O-4′ linkages, while *E. grandis* and *E. dunnii* presented the lowest proportion. The second most abundant linkage in the eucalypt lignin corresponded to
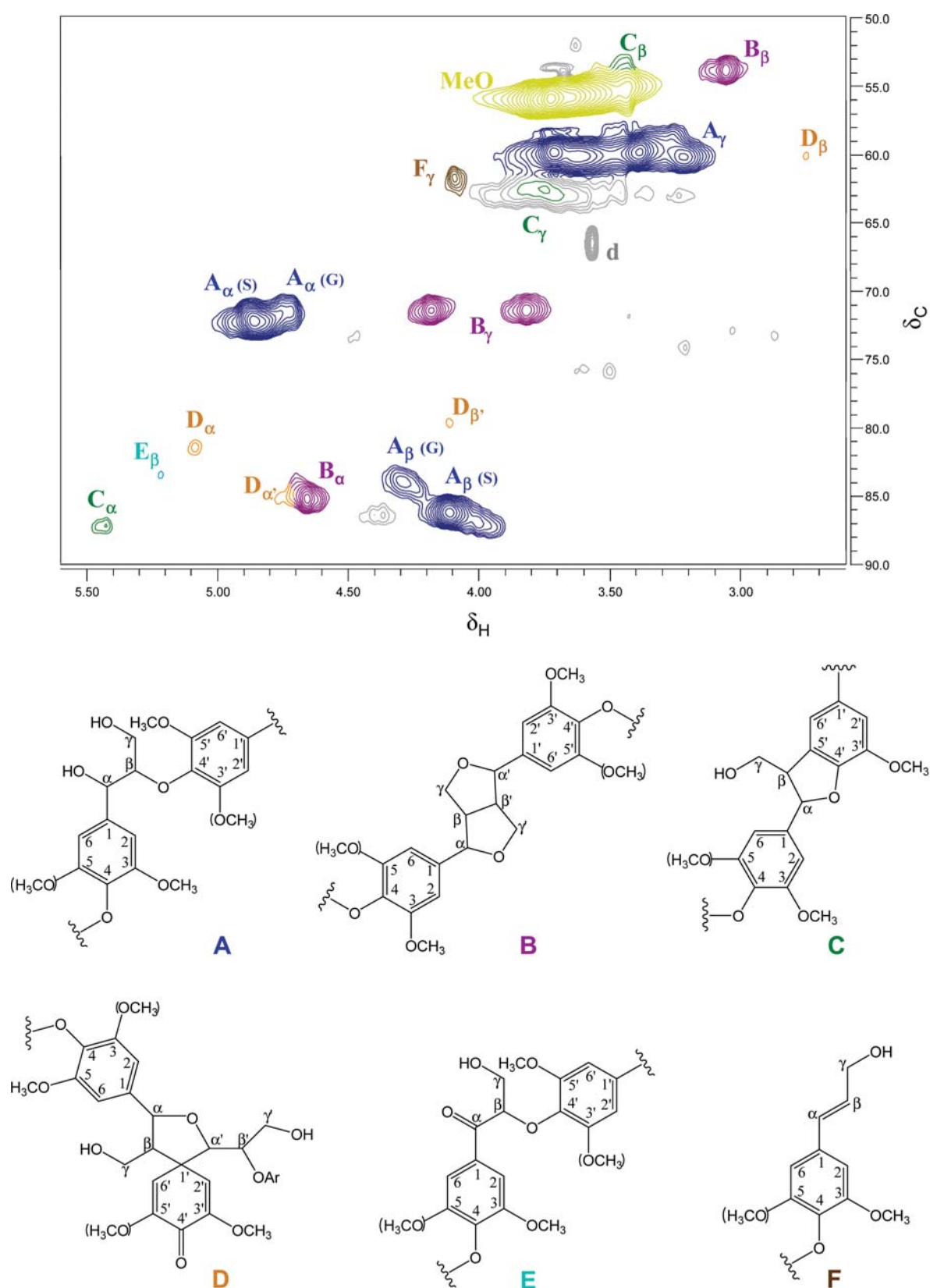
Figure 2   Expanded side-chain region, δ_C/δ_H 50–90/2.6–5.6 ppm, of the HSQC spectrum of a representative MWL (from *E. dunnii* wood). The main structures observed were: (**A**) β-O-4′ linkages; (**B**) resinol formed by β-β′, α-O-γ′ and γ-O-α′ linkages; (**C**) phenyl-coumarane structures formed by β-5′ and α-O-4′ linkages; (**D**) spirodienone structures formed by β-1′, α-O-4′ linkages; (**E**) C_α-oxidized β-O-4′ linkages; (**F**) *p*-hydroxycinnamyl alcohol terminal unit. See Table 3 for signal assignment. Different colors were used to identify the main cross-signals and their corresponding structures, and some unidentified signals are shown in gray. (d) dioxane impurity.
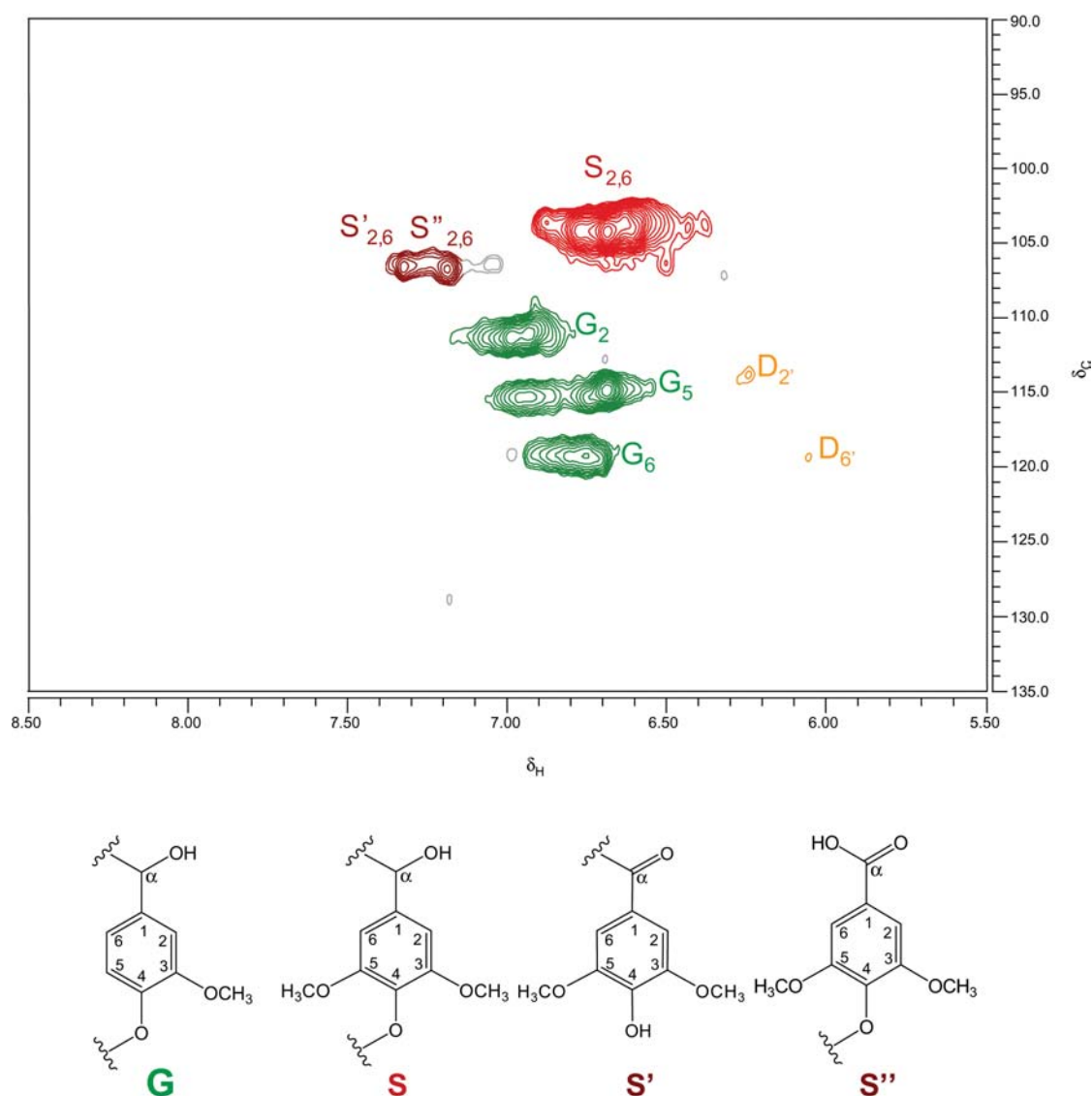
Figure 3 Expanded aromatic region, δ_C/δ_H 90–135/5.5–8.5 ppm, of the HSQC spectra of a representative MWL (from *E. dunnii* wood). The main aromatic structures observed were: (**G**) guaiacyl unit (etherified); (**S**) syringyl unit (etherified); (**S′**) oxidized syringyl unit bearing a carbonyl group at C_α (phenolic); (**S″**) oxidized syringyl unit bearing a carboxyl group at C_α: and (**D**) spirodienone (see Figure 2). See Table 3 for signal assignment. Different colors were used to identify the main cross-signals and their corresponding structures.

the resinol substructure (**B**) that involved around 17% of all side chains, the highest proportion being found in *E. dunnii*. The other linkages, such as phenylcoumaran (**C**) or spirodienone (**D**), were present in lower proportions. The lignin from *E. dunnii* had the lowest proportion of β-O-4′ ether linkages, and the highest proportion of condensed structures. This fact could explain why the wood from *E. dunnii* is the most difficult to delignify (Rencoret et al. 2007), despite having a relatively high S/G ratio. β-O-4′ linkages are cleaved during alkaline cooking, while several condensed linkages (such as β-β′, β-5′, and β-1′) resist cooking conditions (Gierer and Norén 1980; Gierer 1985; Ibarra et al. 2007a). The low content of phenylcoumaran and other condensed structures in *E. globulus* lignin has been reported previously (Xie 2004).

The main cross-signals in the aromatic region of the HSQC spectra (Figure 3) corresponded to the benzenic rings of lignin units. Signals from syringyl (**S**) and guaiacyl

(**G**) units could be observed in all the spectra. The S units showed a prominent signal for the $C_{2,6}$-$H_{2,6}$ correlation at δ_C/δ_H 104.7/6.69 ppm, while the G units showed different correlations for C_2-H_2 (δ_C/δ_H 111.6/6.99 ppm), C_5-H_5 (δ_C/δ_H 115.4/6.72 and 6.94), and C_6-H_6 (δ_C/δ_H 119.5/6.77 ppm). The double C_5-H_5 signal revealed some heterogeneity among the G units especially affecting the C_5-H_5 correlation, probably because it is due to different substituents at C_4 (e.g., phenolic or etherified in different substructures). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in C_α-oxidized S-lignin units (**S′** and **S″**) were observed at δ_C/δ_H 106.8/7.32 and 107.0/7.19, respectively. Signals of H units were not detected in any of the HSQC spectra, although a very minor signal with relatively similar δ_C/δ_H (130/7.2 instead of 130/7.0 ppm) was found in some of the MWL samples. A NMR estimation of the molar S/G ratios in the different lignins is included in Table 2, ranging from 1.7 to 2.9. The highest S/G value corresponded to the *E. globulus* lignin, while the lowest

Table 3 Assignments of ^{13}C-^{1}H correlation signals in the 2D-NMR spectra of MWL from wood of the different eucalypt species analyzed.

δ_C/δ_H (ppm)	Assignment
53.7/3.46	C_β-H_β in phenylcoumaran substructures (**C**)
54.1/3.06	C_β-H_β in resinol substructures (**B**)
56.2/3.73	C-H in methoxyls
60.1/3.40 and 3.72	$C_\gamma H_\gamma$ in β-O-4′ substructures (**A**)
60.3/2.75	C_β-H_β in spirodienone substructure (**D**)
61.9/4.09	C_γ-H_γ in p-hydroxycinnamyl (sinapyl/coniferyl) alcohol (**F**)
71.7/4.74	C_α-H_α in β-O-4′ linked to a G unit (**A**)
71.7/3.83 and 4.19	C_γ-H_γ in resinol substructures (**B**)
72.4/4.86	C_α-H_α in β-O-4′ linked to a S unit (**A**)
79.8/4.11	$C_{\beta'}$-$H_{\beta'}$ in spirodienone substructure (**D**)
81.7/5.09	C_α-H_α in spirodienone substructure (**D**)
83.8/5.23	C_β-H_β in β-O-4′ with C_α=O (**E**)
84.1/4.28	C_β-H_β in β-O-4′ linked to a G unit (**A**)
85.5/4.67	C_α-H_α in resinol substructures (**B**)
85.4/4.80	$C_{\alpha'}$-$H_{\alpha'}$ in spirodienone substructure (**D**)
86.4/4.11	C_β-H_β in β-O-4′ linked to a S unit (**A**)
87.4/5.47	C_α-H_α in phenylcoumaran substructures (**C**)
104.7/6.69	$C_{2,6}$-$H_{2,6}$ in etherified syringyl units (**S**)
106.8/7.32	$C_{2,6}$-$H_{2,6}$ in oxidized (C_α=O) phenolic syringyl units (**S′**)
107.0 /7.19	$C_{2,6}$-$H_{2,6}$ in oxidized (C_αOOH) syringyl units (**S″**)
114.1/6.26	$C_{2'}$-$H_{2'}$ in spirodienone substructure (**D**)
111.6/6.99	C_2-H_2 in guaiacyl units (**G**)
119.5/6.08	$C_{6'}$-$H_{6'}$ in spirodienone substructure (**D**)
115.4/6.72 and 6.94	C_5-H_5 in guaiacyl units (**G**)
119.5/6.77	C_6-H_6 in guaiacyl units (**G**)

value corresponded to that from *E. grandis*, as similarly observed by Py-GC/MS. Other signals in this HSQC region of the spectra are from spirodienone substructures (**D**) with $C_{2'}$-$H_{2'}$ and $C_{6'}$-$H_{6'}$ correlations at δ_C/δ_H 113.5/6.18 and 119.5/6.08, respectively.

The percentage of β-β′-linked side-chains found (16–19%) differed from that reported for *E. grandis* MWL (only 3% side-chains) (Capanema et al. 2005). However, higher resinol contents (13–15% side-chains) has been reported for *E. globulus* dioxane lignin, although with a very high S/G ratio of 6 (Evtuguin et al. 2001; Evtuguin and Amado 2003). A certain percentage of H units (up to 2%) has been reported in eucalypt lignin using NMR (Evtuguin et al. 2001; Capanema et al. 2005); however, we failed to confirm it by 2D-NMR of the different *Euca-*

lyptus lignins, in agreement with the very low Py-GC/MS relative abundances discussed above.

Thioacidolysis

Thioacidolysis (Rolando et al. 1992) has proven to be a selective method for degrading the most frequent inter-unit linkage in lignin, i.e., the β-O-4′ ether linkage. The total yields and relative distribution of the G and S thioacidolysis monomers reflect the amount and ring type of lignin units only involved in alkyl-aryl ether bonds. The main dimers recovered after thioacidolysis provide information on the various carbon-carbon and diaryl ether linkages, referred to as the "condensed" bonds (including 5-5′, 4-O-5′, β-1′, β-5′ and β-β′) (Lapierre et al. 1991, 1995).

While HSQC NMR yielded information on the main inter-unit linkages in the eucalypt lignins, it cannot provide information on the units attached to each other. The whole mixtures of thioacidolysis degradation products from the different eucalypt lignins were analyzed by SEC. A representative chromatogram is shown in Figure 4. The most prominent peak corresponds to monomeric compounds. Its high area (Table 2) was in agreement with the high extent of β-O-4′ ether linkages in the eucalypt lignins already observed by HSQC NMR. The two other fractions corresponded to dimeric compounds, and a mixture of trimers and oligomeric compounds.

The composition of the main monomers showed a predominance of S over G units in the etherified eucalypt lignin, and the nearly-complete absence of H units (less than 0.5% of the total) as observed by Py-GC/MS of the whole polymer (these low abundances are below the HSQC detection level). The thioacidolysis yield was 1000–1550 µmol g^{-1} lignin, and the molar S/G ratios obtained are provided in Table 2. It should be emphasized that the monomeric products released by thioacidolysis reflects only the lignin units linked by arylglycerol-β-aryl ether bonds. The differences in the S/G ratios were similar to those estimated by the two other methods, with the highest value for the *E. globulus* lignin and the lowest for the *E. grandis* and *E. maidenii* lignins. Also here, a hint is appropriate that the S/G ratios obtained by thioacidolysis may be elevated (see Sarkanen and Hergert 1971). It is interesting that the NMR method yielded similar results as those obtained by the two degradation techniques.

The dimeric compounds obtained by thioacidolysis of the eucalypt MWL were analyzed after Raney-Nickel

Table 4 Percentage of lignin side-chains forming different inter-unit linkages (**A–E**) and terminal structures (**F**) from integration of ^{13}C-^{1}H correlation signals in the HSQC spectra of MWL from wood of the different eucalypt species analyzed (referred to total side-chains).

	E. globulus	E. nitens	E. maidenii	E. grandis	E. dunnii
β-O-4′ (**A**)	69.3 (80.4)	71.7 (83.1)	69.7 (80.3)	66.9 (77.3)	65.9 (77.0)
Resinol (**B**)	18.2 (10.6)	16.1 (9.3)	16.4 (9.5)	16.5 (9.5)	19.0 (11.1)
Phenylcoumaran (**C**)	2.9 (3.4)	4.0 (4.7)	3.6 (4.2)	6.8 (7.9)	4.0 (4.7)
Spirodienone (**D**)	2.8 (3.3)	1.3 (1.5)	3.6 (4.2)	2.9 (3.3)	4.2 (4.9)
β-O-4′-C_α=O (**E**)	2.0 (2.3)	1.3 (1.5)	1.7 (1.9)	1.7 (1.9)	1.9 (2.2)
p-Hydroxycinnamyl alcohols (**F**)	4.7	5.7	4.9	5.3	4.9

The relative abundance of each inter-unit linkage type (taking into account that two side-chains are required to form one resinol structure) is indicated in parentheses.
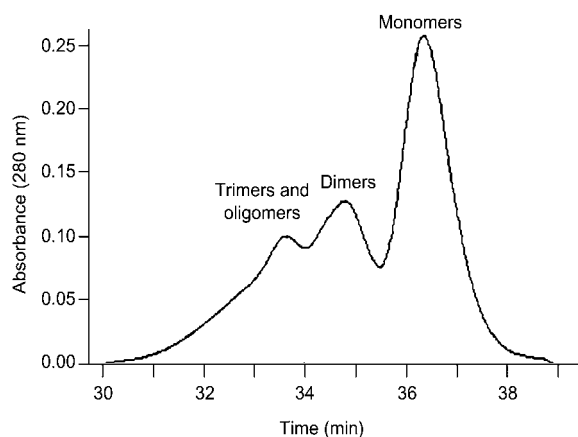
Figure 4 Size-exclusion chromatogram of the thioacidolysis degradation products of a representative eucalypt MWL (from *E. globulus* wood).

desulfurization. A representative chromatogram of the tri-methylsilylated products is shown in Figure 5. Their structures are shown in Figure 6, and all mass spectral data are summarized in Table 5. The main dimers obtained were of 5-5′ (dimers **3-5**), 4-O-5′ (**6** and **8**), β-1′ (**7**, **9**, **11**, **14**, **16** and **24**), β-5′ (**10**, **12**, **15** and **21**), β-β′ tetralin (**13**, **17**-**20** and **23**) and phenylisochroman (**22**, **25-27**; including β-1′/α-O-α′ bonds) types. Their relative proportions from the different lignins are shown in Table 6. The total abundances were much lower than those of monomers, and were roughly estimated as 400 μmol g^{-1} of initial lignin. The condensed-lignin S/G ratio (Table 6) was slightly lower than those obtained for the etherified or total lignins, revealing a higher presence of G units involved in condensed linkages (Lapierre et al. 1995). Many of the dimeric substructures in eucalypt lignin (**A–E**

in Figure 2) were partially modified during thioacidolysis resulting in breakdown of labile linkages and formation of new bonds (dimers **13**, **17–20** and **23**). In spite of this, the origin of these dimers could be established as follows: i) 5-5′ dimers from breakdown of α-O-4′ and β-O-4′ ethers in dibenzodioxocins and from other 5-5′ biphenyl structures; ii) β-1′ dimers from breakdown of α-O-α′ ether and side-chain lost in spirodienones; iii) β-5′ dimers from breakdown of α-O-4′ ether in phenylcoumaran substructures; and iv) tetralin dimers from breakdown of two α-O-γ ethers and subsequent reactions in resinol substructures.

Tetralin and β-1′ dimeric compounds were the most prominent thioacidolysis dimers from all the lignins and appeared in similar proportion (around 30% of each). Tetralins would be formed from the resinol substructures as described by Lapierre et al. (1995). The high proportion of β-β′ dimers (tetralin-type) was in agreement with the high amount (9–11%) of β-β′ substructures (resinol-type) in all the eucalypt lignins (Table 4) that would represent around 50% of the NMR structures with C-C linked side-chains (**B–D**). More interestingly, nearly all (93–94%) the β-β′ dimers from the different eucalypt lignins were of the syringaresinol type (pinoresinol being absent and the G-S structure appearing as a minor substructure) (Table 6). This is much more than expected from random coupling in a monolignol mixture with 2–3-fold higher concentration of sinapyl alcohol than coniferyl alcohol. The high predominance of syringaresinol among β-β′ substructures in *E. globulus* lignin has been suggested previously using electrospray-ionization MS (Evtuguin and Amado 2003).

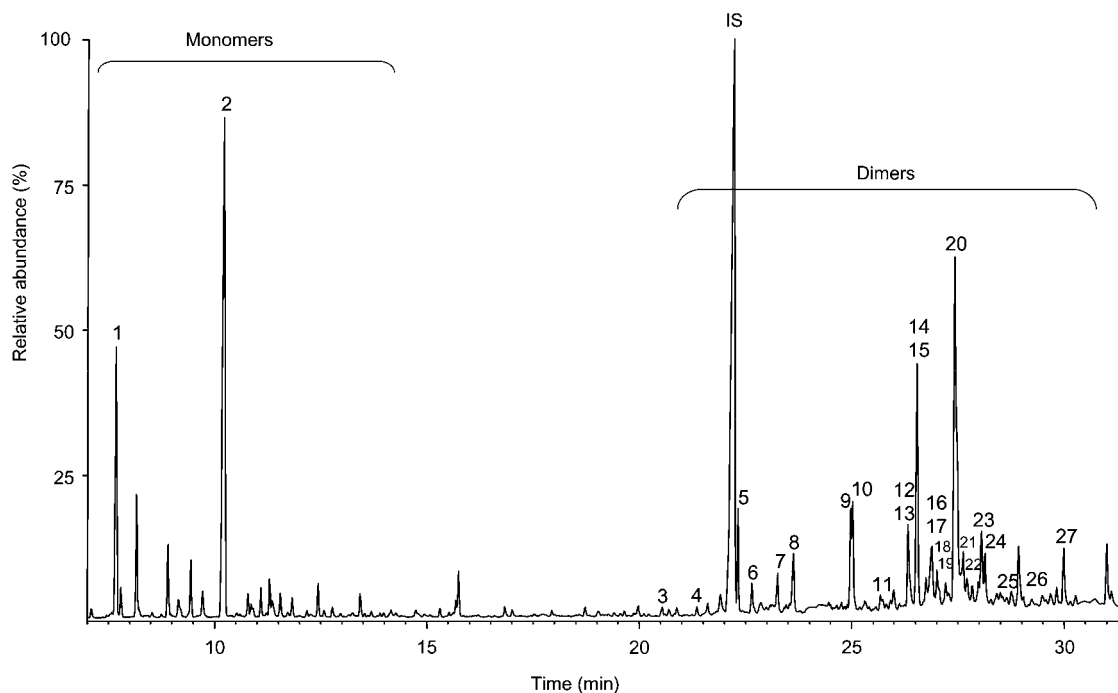β-1′ dimers were anomalously prominent, as reported for degradation products from other lignins (Lapierre et

Figure 5 Chromatogram of the thioacidolysis degradation products (after Raney-Nickel desulfurization) of a representative eucalypt MWL (from *E. maidenii* wood), as trimethylsilyl derivatives. The main structures observed include monomers (S and G), 5-5′, 4-O-5′, β-1′, β-5′, isochroman and β-β′ dimers (G-G, G-S, and S-S type). The numbers refer to the compounds (monomers and dimers) listed in Table 5, and the structures are shown in Figure 6. IS refers to tetracosane used as internal standard.
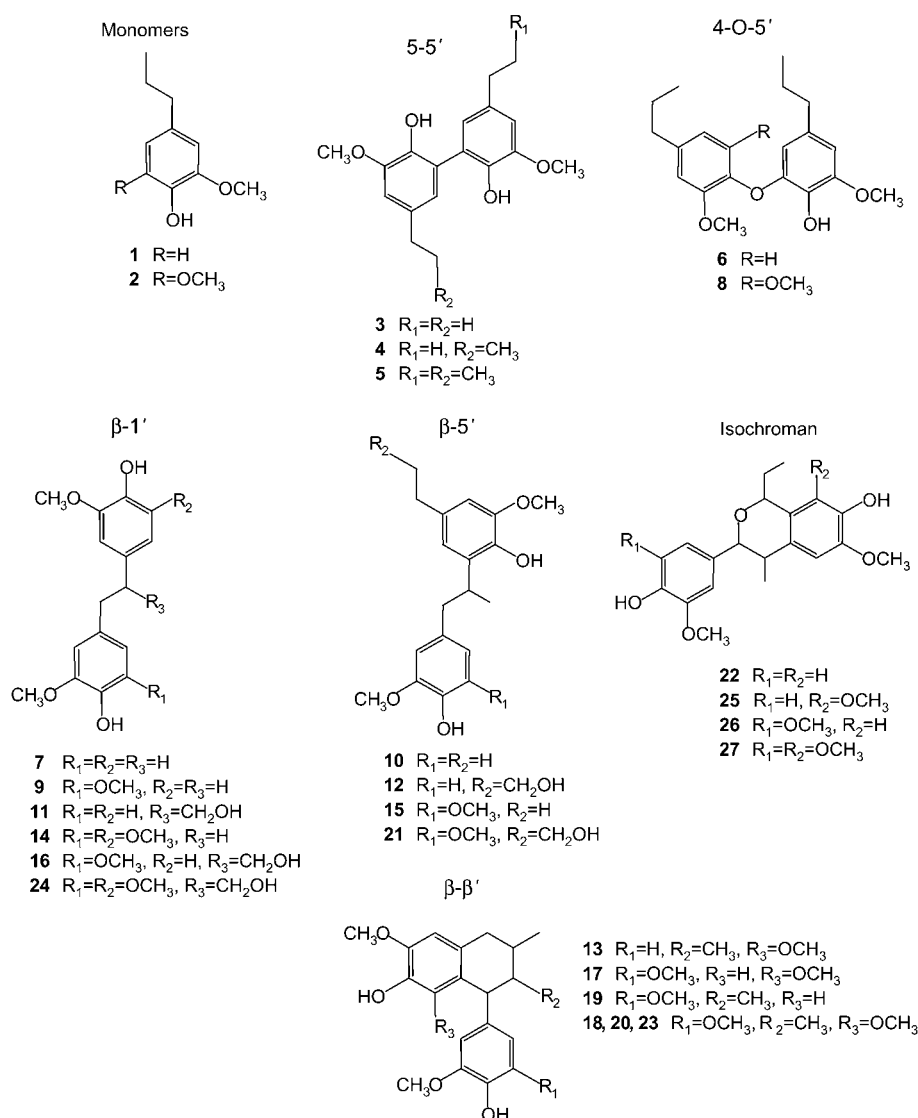
Figure 6   Structures of monomeric and main dimeric compounds obtained after thioacidolysis and Raney-Nickel desulfurization of the different eucalypt MWLs. All mass spectral data of the compounds (as trimethylsilyl derivatives) are listed in Table 5.

al. 1995; Ralph et al. 2004a). The existence of β-1′ dimeric substructures in lignin has been a matter of controversy for years. More recently, two β-1′ linked substructures, spirodienones, and phenylisochromans, were identified using 2D-NMR in combination with DFRC (derivatization followed by reductive cleavage) (Ralph et al. 1998; Zhang and Gellerstedt 2001). Phenylisochromans are resistant toward thioacidolysis; therefore, only spirodienones could be at the origin of the β-1′ dimers observed here. However, a comparison of the abundances of spirodienones (HSQC) and β-1′ dimers (thioacidolysis) suggested that other β-1′ structures, maybe including simple dimeric structures, are also present in eucalypt lignin. β-5′ dimers were also released in important amounts (11–17%) as corresponds to a phenylcoumaran (Lapierre et al. 1991) abundance near 25% of the condensed substructures in HSQC spectra (**B–D**). The three thioacidolysis dimers with the lowest abundance (5–10%), i.e., 5-5′, 4-O-5′, and isochroman, were not detected by NMR because the abundance of the original substructures was below the NMR detection level. Dibenzodioxocins are supposed to be the main biphenyl

structures in lignin (Karhunen et al. 1995; Ralph et al. 2004a); therefore, the 5-5′ dimers were considered mostly as being dibenzodioxocin degradation products, although simple biphenyl structures have also been reported in lignin (Capanema et al. 2004; Balakshin et al. 2005). The isochroman structure (Lapierre et al. 1991) is probably formed by side-chain migration (from C_1 to C_6) of a spirodienone intermediate during lignin biosynthesis (Ralph et al. 1998; Peng et al. 1999).

In addition to the above monomeric and dimeric compounds, several thioacidolysis trimers were obtained. Silylated desulfurized trimers were not detected under the chromatographic conditions used for analyzing the corresponding dimers. However, when a short (12-m length) column was used in combination with higher-temperature program (Gutiérrez et al. 1998), both dimeric and trimeric thioacidolysis products could be observed in the same chromatographic run as trimethylsilylated derivatives (Figure 7). Trimers were obtained with low abundance (below 50 μmol g^{-1} of lignin) but using selected-ion chromatographic profiles (Figure 7C) three main peaks (**a–c**) could be identified showing the same

Table 5   Identification and mass spectral fragments of the compounds (silylated monomers and dimers) released after thioacidolysis and Raney-Nickel desulfurization of MWL from wood of the different eucalypt species analyzed, whose structures are depicted in Figure 6[a].

Compound	Linkage	Fragments (m/z)	M_w
Monomers			
1	G	238, 223, 209, 179, 73	238
2	S	268, 253, 239,238, 209	268
Dimers			
3	5-5′ (G-G)	446, 431, 417, 416, 73	446
4	5-5′ (G-G)	460, 445, 431, 430, 73	460
5	5-5′ (G-G)	474, 459, 445, 444, 385, 357, 73	474
6	4-O-5′ (G-G)	402, 387, 373, 372, 357, 343, 73	402
7	β-1′ (G-G)	418, 209, 179, 73	418
8	4-O-5′ (G-S)	432, 417, 403, 73	432
9	β-1′ (G-S)	448, 433, 239, 209, 179, 73	448
10	β-5′ (G-G)	460, 445, 251, 236, 209, 207,179, 73	460
11	β-1′ (G-G,-OH)	520, 505, 417, 311, 223, 209, 179, 149, 73	520
12	β-5′ (G-G,-OH)	562, 472, 352, 263, 209, 191, 73	562
13	β-β′ (G-S)	502, 306, 269, 239, 209, 73	502
14	β-1′ (S-S)	478, 463, 239, 209, 73	478
15	β-5′ (G-S)	490, 239, 209, 73	490
16	β-1′ (G-S,-OH)	550, 535, 341, 73	550
17	β-β′ (S-S)	518, 503, 489, 488, 292, 73	518
18	β-β′ (S-S)	532, 517, 502, 445, 306, 291, 275, 73	532
19	β-β′ (G-S)	502, 487, 472, 415, 276, 73	502
20	β-β′ (S-S)	532, 517, 502, 445, 306, 291, 275, 73	532
21	β-5′ (G-S,-OH)	592, 502, 472, 239,209, 191, 73	592
22	β-1′/α-O-α′ (G-G)	488, 473, 459, 279, 251, 209, 73	488
23	β-β′ (S-S)	532, 517, 502, 445, 306, 291, 275, 73	532
24	β-1′ (S-S,-OH)	580, 565, 341, 239, 209, 73	580
25	β-1′/α-O-α′ (G-S)	518, 503, 489, 309, 239, 209, 73	518
26	β-1′/α-O-α′ (S-G)	518, 503, 489, 279, 251, 239, 209, 73	518
27	β-1′/α-O-α′ (S-S)	548, 533, 519, 309, 278, 281, 239, 209, 73	548

[a]Linkage type and molecular weight (M_w) are indicated, in addition to main mass fragments (the base peaks are underlined).

Table 6   Relative molar percentages of the different dimer types (see Table 5 and Figure 6) released after thioacidolysis and Raney-nickel desulfuration of MWL from wood of the different eucalypt species analyzed, and total S/G ratio of condensed lignin.

	5-5′	4-O-5′		β-1′			β-5′		β-β′		β-1′/α-O-α′			S/G
	GG	GG	SG	GG	SG	SS	GG	SG	SG	SS	GG	SG	SS	
E. globulus	6	3	6	4	8	19	10	5	2	30	1	1	5	1.8
E. nitens	10	3	7	3	5	24	7	4	2	30	1	1	3	2.0
E. maidenii	6	2	5	3	8	18	12	5	2	32	1	1	4	1.8
E. grandis	10	4	5	7	8	17	12	5	2	26	1	1	3	1.3
E. dunnii	11	4	8	3	4	24	8	5	2	25	1	1	4	1.7

mass spectrum. These above trimeric compounds presented a molecular (and base peak) at m/z 696, and a fragment at m/z 306 (characteristic of S-S tetralin dimers) and were tentatively identified as S-β-β′-S′-4′-O-5″-G″ trimers (Figure 7D). Three isomers of this trimeric structure were detected, as also occurred with the corresponding dimers (**18**, **20**, and **23**) (Figure 7B), which were among the main dimeric compounds released from eucalypt lignin (Figure 7A). The same trimeric compound has been identified as an acetylated derivative after thioacidolysis (and Raney-nickel desulfuration) of two other hardwoods (Önnerud and Gellerstedt 2003) and the corresponding G-G-G trimer has been reported in spruce (Önnerud 2003). In addition to these main trimeric compounds several other thioacidolysis trimers were detected in very minor amounts, being homologous to dimers **5**, **21**, **9**, **27**, **14**, **8**, and **12**. Due to their low abundance, the trimeric compounds did not provide useful information for comparing the different eucalypt lignins. How-

ever, they provided some additional clues on the structure of the condensed lignin in the eucalypt wood which completed that obtained from the analysis of dimers and the HSQC NMR studies. It is especially interesting that all the trimers detected were formed by addition to one of the main dimers previously described of one G unit linked by a condensed ether linkage being, therefore, of the type dimer-4-O-5′-G.

Conclusions

The MWL isolated from different eucalypt species (*E. globulus*, *E. nitens*, *E. maidenii*, *E. grandis*, and *E. dunnii*) was characterized by Py-GC/MS, 2D-NMR, and thioacidolysis (followed by Raney Ni desulfurization). A predominance of syringyl over guaiacyl lignin moieties was found in all eucalypt MWLs, with the highest S/G ratio observed in *E. globulus* lignin. The main inter-unit linkage present
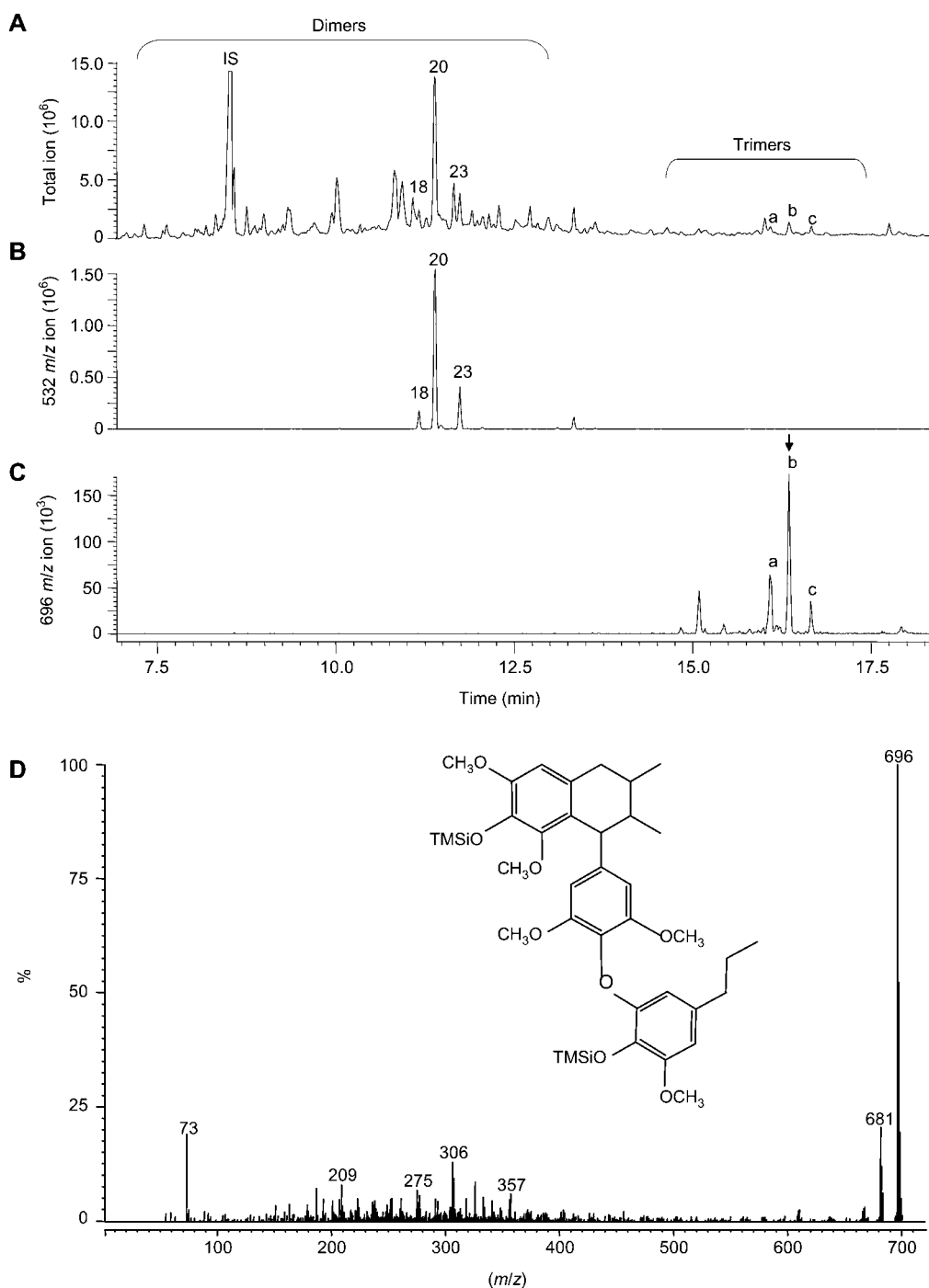
Figure 7 Short-column (12-m length) partial chromatographic profiles (7.0–18.3 min) enabling simultaneous analysis of silylated dimeric and trimeric compounds from thioacidolysis (and Raney-nickel desulfurization) of a representative eucalypt MWL (from *E. maidenii* wood). (A) Total-ion profile showing the dimer and trimer regions (and IS peak of tetracosane used as internal standard). (B) Selected-ion profile corresponding to the 532 *m/z* ion showing three isomers of the main dimer (peaks **18, 20**, and **23**). (C) Selected-ion profile corresponding to the 696 *m/z* ion revealing three peaks (**a, b**, and **c**) identified as three isomers of a trimeric compound. (D) Mass spectrum of trimer **b** and its tentative chemical structure (S-β-β′-S′-4′-O-5″-G type trimeric compound).

in the eucalypt MWL was the β-O-4′ aryl-ether bond followed by β-β′ resinol-type substructures, with lower amounts of β-5′ phenylcoumaran and β-1′ spirodienone substructures. Furthermore, analysis of desulfurated thioacidolysis products indicated that the β-β′ substructures present in the eucalypt MWL are almost exclusively of syringaresinol type. Some trimeric compounds made of a syringaresinol moiety attached to a guaiacyl unit through a 4-O-5′ linkage were also reported in the euca-lypt MWL. A conclusion to the whole lignin of the euca-lyptus species described is only possible with limitations, because the yields of the MWLs investigated amounted only to 10–15% of the original Klason lignin content.

Acknowledgements

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