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Detection of muconic acid type structures in oxidised lignins using 2D NMR spectroscopy

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Abstract

The identification of muconic acid type structures (MATS) in oxidised lignins employing 2D NMR spectroscopy was carried out. The primary database for MATS detection by single bond ^{1}H-^{13}C (HSQC) and long-range ^{1}H-^{1}H and ^{1}H-^{13}C correlation (COSY and HMBC) NMR experiments was obtained with a series of model compounds: *cis, cis*- and *trans, trans*-muconic acids and their methylated analogues and MATS derived from monomeric lignin model compounds oxidised by ClO_2. Additional information was obtained from the study of oxygen-organosolv lignin obtained from spent liquor after the spruce wood delignification with oxygen in an acetone-water solution. This work allowed the reliable assignment of MATS signals in the HSQC spectra of oxidised lignin and provides a methodology for detection and distinguishing of MATS from structurally similar moieties.

Keywords: lignin; muconic acid structures; NMR spectroscopy; oxidation; structural analysis.

Introduction

The muconic acid pathway provides key routes in lignin biodegradation with fungi/lignolytic enzymes (Stanier and Ornston 1973; Higuchi 1993) and in oxidative delignification of lignocellulosics (Gierer 1982). In particular, oxidative delignification with oxygen, ozone, hydrogen peroxide, chlorine dioxide and with peroxy acids includes a cleavage of an aromatic ring predominantly between C-3 and C-4, thus resulting in monomethyl esters of hexadiendicarboxylic acid (muconic acid) structures (Figure 1).

The detection and quantification of muconic acid type structures (MATS) is an important but difficult analytical task in lignin chemistry. MATS are rather labile and present in oxidised lignin in small amounts either in open (**I** and **I'**) or in muconolactone (**II**–**IV**) forms (Figure 1). The presence of MATS in oxidised lignin is usually suggested based on analysis of its low molecular weight degradation products (Vilen et al. 2000; Brogdon et al. 2004), reactions with diazomethane (Sarkanen and Suzuri 1965), or based on FTIR and ^{13}C NMR spectra (Zarubin et al. 1989; Hortling et al. 1991; Evtuguin and Robert 1997; Runge and Ragauskas 1999).

^{13}C NMR spectroscopy is one of the most powerful tools for the detection/quantification of MATS, despite objective carbon assignment problems (Hortling et al. 1991; Evtuguin and Robert 1997). The presence of unsaturated moieties significantly hinders the detection of MATS in lignin by 1D ^{13}C NMR. Thus, C-2, C-3, C-4 and C-5 in structures **I**–**IV** possess the resonances in the same region (approximately 120–140 ppm) as aromatic carbons. The carbon resonances of carboxylic (approximately 167 ppm) and ester (approximately 171–172 ppm) groups attached to vinylic moieties in MATS are overlapped with those of cinnamic and benzoic acid type structures and their esters. The resonance at 51.6 ppm in ^{13}C NMR spectra of oxidised lignin was assigned to the carbon atom of methoxy groups in partially methylated structures **I** and **II** (Figure 1) and provides an indication of the presence of MATS (Evtuguin and Robert 1997). In particular, the successful MATS quantification may be carried out from CH_3 DEPT spectra using methoxy groups as an internal standard (Evtuguin et al. 1994). However, the detection of MATS based on the resonance at 51.6 ppm is possible if lignin oxidation occurs under acidic or neutral conditions only because under alkaline conditions methyl esters are readily saponified (Runge and Ragauskas 1999; Vilen et al. 2000). Therefore, the detection and quantification of MATS in those cases is impossible. Evidently, the reliable detection/identification of MATS in oxidised lignins is possible only by the combination of 1D and 2D NMR techniques.

The main goal of this work was to provide the methodology for MATS detection by conventional 2D NMR techniques.

Materials and methods

Model compounds

Trans, *trans*-muconic acid (**3**) and apocynol (**6**) were commercial products (Aldrich Chem. Comp., Madrid, Spain). *Cis*, *cis*-muconic acid (**1**) and 3-methyl-*cis,cis*-muconic acid (**5**) were synthesised using Fe(III)-catalysed peracetic acid oxidation of phenol and *p*-cresol, respectively (Pandell 1976). *Cis*, *cis*-muconic acid (b.p. 178°C) FTIR spectrum in KBr (ν, cm^{-1}): 3430 (w), 3070 (s), 1689 (s), 1590 (s) and 1250 (s); δ, 713/640 cm^{-1} (s). 3-Methyl-*cis,cis*-muconic acid, FTIR spectrum in KBr (ν, cm^{-1}): 3400 (w), 3050 (s), 2915 (w), 1680 (s), 1598 (s) and 1255 (s); δ, 720/650 cm^{-1} (s). Dimethyl esters of **1** and **3** were prepared by methyla-

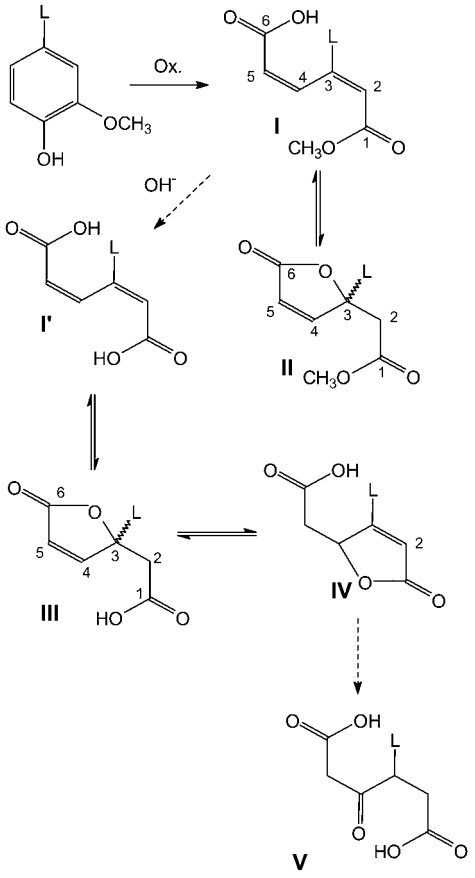

Figure 1 Schematic representation of lignin oxidation via muconic acid pathway.

tion with iodomethane. Dimethyl ester of *cis, cis*-muconic acid (b.p. 61°C) FTIR spectrum in KBr (ν, cm^{-1}): 3430 (w), 3073 (s), 1721 (s), 1589 (s) and 1220 (s); δ, 728 cm^{-1} (s). The NMR data for the model compounds **1**–**5** are shown in Table 1.

A phenolic model compound **6** was oxidised with an equimolar amount of ClO_2 at 0°C for 10 min to produce MATS. Typically, 0.15 mM of **6** dissolved in 0.25 ml of acetonitrile and 1.0 ml of water was oxidised with aqueous solution of ClO_2 in a Soverel tube. The conversion of starting **6** was approximately 90%. After the oxidation of compound **6** with ClO_2, the reaction mixture was separated using preparative ligand exchange size exclusion (LEX/SEC) chromatography (Reis et al. 2004), and the acidic fraction corresponding to MATS was isolated and freeze-dried (yield ~35% wt.).

Oxygen-organosolv lignin

Oxygen-organosolv lignin (OAL) was isolated from spent liquor after the delignification of spruce chips by oxygen in an acetone/water (60:40, v/v) medium under weakly acidic conditions as water insoluble fraction according to a previously published scheme (Zarubin et al. 1989). The saponification of OAL was carried out with 0.5 M NaOH at room temperature under a nitrogen atmosphere for 12 h. After neutralization with 0.2 M HCl to pH 4.5, lignin was precipitated in cold water, centrifuged, washed with distilled water until pH 5.0 and freeze-dried.

FTIR and NMR spectroscopy

FTIR spectra (KBr pellets) were recorded on a Mattson 7020 FTIR spectrometer. The spectra resolution was 4 cm^{-1} and 64 scans were averaged.

1D (^1H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR spectra were recorded on a BRUKER AMX 300 spectrometer operating at 300.13 MHz for proton and at 75.2 MHz for carbon, respectively, using standard pulse sequences. Samples were dissolved in DMSO-d_6 and the spectra were recorded at 303 (models) or at 318 K (lignins) with TMS as internal reference (δ 0.00) in a 5-mm diameter tube. Quantitative ^{13}C NMR spectra were recorded at 318 K using the following parameters for the inverse gated decoupling sequence: 4.1 ms pulse width (90° pulse angle); 12 s relaxation delay; 16 K data points and 18 000 scans.

2D ^1H NMR spectra (absolute-mode COSY spectra) were recorded on a BRUKER AMX 300 spectrometer operating at 300.1 MHz by acquiring 2 K×512 increments transformed to a 2 K×1 K data matrix after zero-filling, FT and squared sine-bell apodization applied to both dimensions. For each t_1 value, 400–600 scans were accumulated. The phase sensitive ^1H-detected HSQC spectra were acquired over a F1 spectral weight of 12 000 Hz and a F2 width of 2000 Hz with a 2048×1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarisation transfer was optimised for $^1J_{C\text{-}H}$=148 Hz. The heteronuclear multiple-bond correlation (HMBC) spectra (DMSO-d_6) were recorded using coupling evolution time of 110 ms ($^3J_{C\text{-}H}$=4.5 Hz).

Results and discussion

Study on the model compounds

The primary database for MATS detection in oxidised lignins by NMR was obtained from a series of model compounds (Figure 2). These were *cis, cis*- and *trans,*

Table 1 Chemical shifts (DMSO-d_6, 303 K) of model compounds **1**–**5** (Figure 2) in ppm.

Model	C-1/ H-1[a]	C-2/ H-2	C-3/ H-3	C-4/ H-4	C-5/ H-5	C-6/ H-6[a]	OCH_3 (CH_3 in **5**)
1	166.8/ 12.66	129.0/ 6.33	140.8/ 7.31	140.8/ 7.31	129.0/ 6.33	166.8/ 12.66	– –
2	165.8/ –	128.0/ 6.38	141.0/ 7.33	141.0/ 7.33	128.0/ 6.38	165.8/ –	51.7/ 3.68
3	166.6/ 12.76	125.2/ 6.01	136.7/ 7.73	136.7/ 7.73	125.2/ 6.01	166.6/ 12.76	– –
4	165.4 –	124.3/ 6.14	137.1/ 7.76	137.1/ 7.76	124.3/ 6.14	165.4/ –	51.5/ 3.69
5	167.2/ 12.48	121.4/ 5.98	155.4/- –	136.4/ 7.65	119.8/ 5.97	167.1/ 12.46	20.9/ 2.12

[a]Proton resonance in carboxylic group.

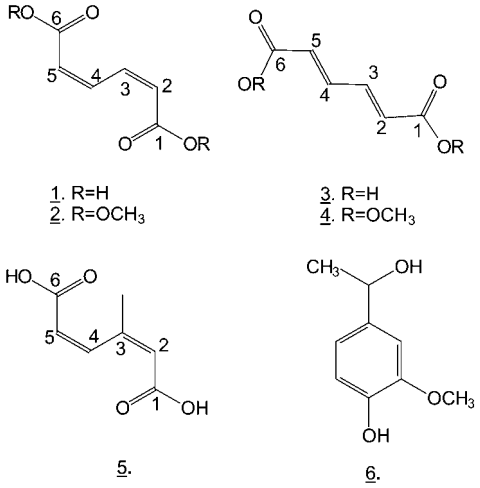

Figure 2 Model compounds.

trans-muconic acids (**1** and **3**, respectively), their dimethyl ester analogues (**2** and **4**), 3-methyl-*cis, cis*-muconic acid **5,** and MATS derived from a monomeric lignin model compound **6** in oxidation with ClO_2 at 0°C for 10 min. The signal assignments for models **1**–**5** are presented in Table 1. Data from these model compounds showed that *cis, cis*- and *trans, trans*-configurations of MATS in oxidised lignins could be easily distinguished due to the significant differences in carbon and proton chemical shifts of the vinylic moieties. Additionally, the proton-proton coupling constants $J_{2,3}$ and $J_{3,4}$: 8.0–10.5 Hz in *cis, cis*- isomers **1** and **2** and 11.4–13.4 Hz in *trans, trans*-isomers **3** and **4** allowed identification to be carried out. The *trans, trans*-configuration of MATS should be much less abundant in oxidised lignins than the *cis, cis*-configuration. However, the presence of *trans*-isomers cannot be excluded, especially not, if it is taken into consideration that *cis*-isomers can be converted into *trans*-isomers at increased temperatures under acidic conditions (Schmidt et al. 1980). MATS in oxidised lignins possess at least one substitution with an alkyl substituent, typically at C-3. NMR data for model **5** shows the importance of the substituting groups in MATS on proton and carbon chemical shifts (Table 1). Thus, electron-donor methyl substituent at C-3 shifts the vinylic protons and carbons downfield, $J_{4,5}$ was 9.8 Hz in **5**.

The chemical shifts of MATS were also estimated based on products of apocynol (**6**) arisen through oxidation with ClO_2 under mild conditions. In practice, a complex mixture of products was obtained, which were separated by preparative LEX/SEC. A low molecular weight fraction containing acidic products was analysed by single bond ^{1}H-^{13}C (HSQC) and long-range proton-proton and proton-carbon NMR techniques (TOCSY and HMBC). This fraction mainly contained MATS. The aliphatic region related to methoxy group and aromatic region in HSQC spectra of isolated acidic fraction and starting model **6** are shown in Figure 3. The major reaction product was assigned to the structure of type **I** (Figure 1), which have cross peaks in the HSQC spectrum at 122.3/5.92, 117.8/5.94 and 133.2/7.66 ppm. These were assigned to C-2/H-2, C-5/H-5 and C4-/H-4, respectively. The cross peak corresponding to the methyl group in partially esterified MATS was found at 51.6/3.64 ppm. In the ^{13}C NMR spectrum of oxidised products from apocynol, a series of signals at 166.6–166.9 ppm were assigned to carboxyl carbons in MATS.

The chemical shifts of protons in MATS are sensitive to cycloisomerisation. For example, α-vinyl protons in lactones of types **II** and **III** showed shifts of δ 0.1–0.3 to

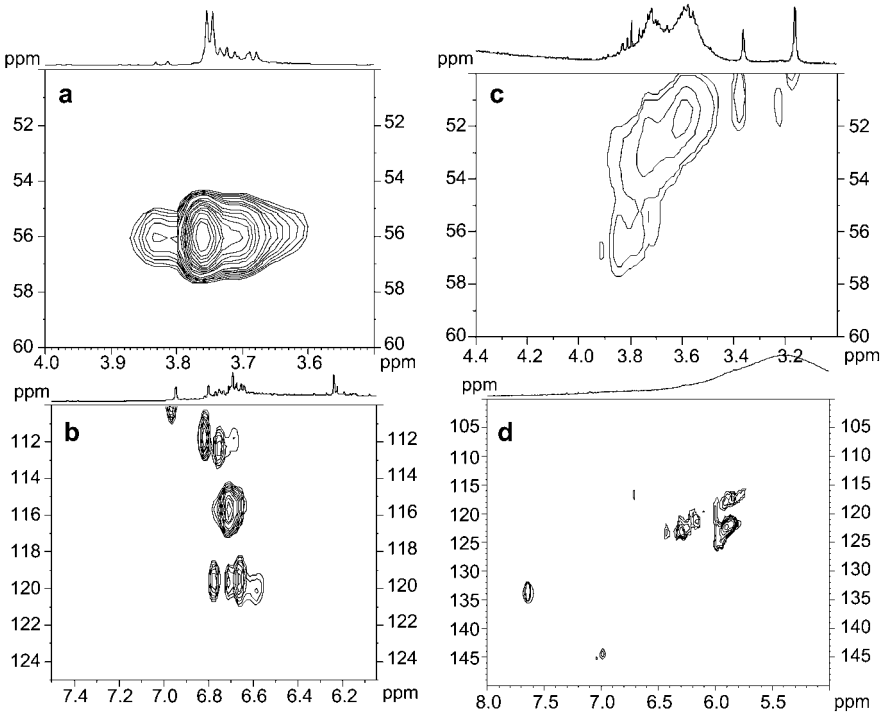

Figure 3 Expanded aliphatic (a,c) and aromatic (b,d) regions in HSQC spectra of apocynol (a,b) and MATS derived from the apocynol oxidation (c,d).

low field (Chen et al. 1996; Gesell et al. 2001) when compared to the parent non-cyclic structures. The proton-proton coupling constants $J_{\alpha,\beta}$ in vinyl moieties also decreases 2–3 Hz. For these reasons, cross signals at 121–123/6.15–6.30 ppm in HSQC spectrum of oxidised structures derived from apocynol were assigned to Hα/Cα of vinylic moieties in a series of muconolactones (Figure 3). The cycloisomerisation of MATS is favoured by medium acidity and by medium temperature (Catelani et al. 1971; Schmidt et al. 1980). The small signal at 122.8/6.45 ppm may be assigned to H-2/C-2 of vinylic moieties in MATS with partially oxidised side chain. In fact, the apocynol oxidation with chlorine dioxide also resulted in acetovanillone, which was further oxidised to MATS. The electron-acceptor substitute at C-3 in MATS normally favour to deshielding of Hα (Gesell et al. 2001). A very weak cross signal in HSQC spectrum of oxidised apocynol at 35.8/2.48 ppm was tentatively assigned to H-2/C-2 in γ-muconolactones of types **II** and **III** (Figure 1) based on reported data for cycloisomerisation of MATS (Chen et al. 1996; Gesell et al. 2001). Besides γ-muconolactones, δ-mucolactones were considered previously in oxidation of benzyl alcohol type lignin model compound with chlorine dioxide (Sarkanen et al. 1962). These δ-lactones are formed due to the reaction of α-hydroxyl group in side chain of lignin model and free carboxylic group of MATS. However, no clear evidences for the presence of δ-mucolactones after apocynol oxidation were found in this study (expected C/H cross signals in the spectrum region at 70.0–80.0/5.0–6.0 ppm were not detected).

The carbon chemical shifts of carboxylic/ester groups in lactones of types **II** and **III** are higher (170–172 ppm) than those in the parent non-cyclic structures (165–167 ppm) due to the loss of conjugation with vinylic moiety (Figure 1). These signals were also found in ^{13}C spectrum of oxidised products from apocynol (spectrum is not shown).

Based on studies of model compounds carried out in this work and published previously (Schmidt et al. 1980; Gesell et al. 2001), it may also be suggested that the chemical shifts of vinylic carbons/protons are strongly dependent on donor-acceptor character of substitutes in muconic structures. Therefore, for the lignin-derived MATS these chemical shifts can vary significantly. However, some specific signal areas in the 2D spectra of lignin may be indicative, anyway, of muconic structures.

Detection of muconic acid structures in lignin

The presence of characteristic resonances from MATS was verified by analysis of OAL isolated from spent liquor after oxygen delignification of spruce chips in acetone/water (60:40, v/v) medium. This lignin certainly contains high amounts of MATS since the oxidative C_3-C_4 cleavage via muconic acid pathway is one of the principal degradation routes during oxygen treatment in aqueous acetone solution (Zarubin et al. 1989; Evtuguin et al. 1994; Evtuguin and Robert 1997). As expected, the ^{13}C NMR spectrum of OAL (Figure 4) revealed characteristic signals at 51.6 and at 166.9 ppm assigned to the carbon atoms of the methoxy and carboxyl groups, respectively, of partially methylated MATS. These signals were also

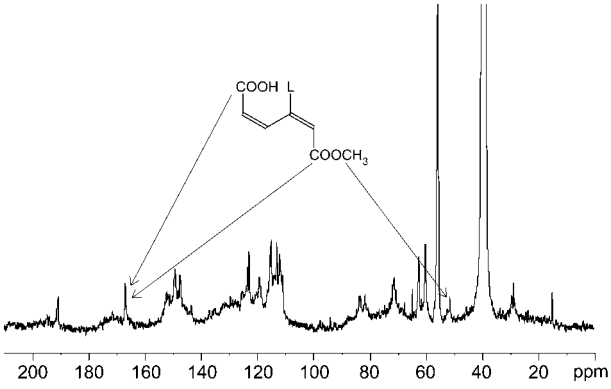

Figure 4 ^{13}C NMR spectrum (DMSO-d_6, 318 K) of oxygen-organosolv lignin.

found in ^{13}C NMR spectra of residual lignins from softwood kraft pulp after the D_0 bleaching stage (Runge and Ragauskas 1999). The amount of MATS in OAL, estimated based on the resonance at 51.6 ppm, was 0.12 per one aromatic ring. The notable signals at 120–123 ppm were assigned to vinylic moieties in MATS. These signals normally decrease after lignin reduction with hydrogen at room temperature with Pd/C as the catalyst (Evtuguin and Robert 1997). The signal centred at 171.5 ppm, assigned to a carboxyl carbon in a muconolactone that is not conjugated with a vinylic moiety, indicated the possible cycloisomerisation of MATS in lignin. The correctness of these assignments was verified by a ^{1}H-^{13}C single bond correlation experiment (HSQC).

The expanded regions of the HSQC spectrum of OAL, showing the aliphatic and aromatic regions, are presented in Figure 5. The cross peak from the methoxy group in the HSQC spectrum of partially methylated MATS was found at 51.6/3.65 ppm (Figure 5a), which is coherent with the data obtained for model compounds **2** and **4**, and MATS obtained after apocynol oxidation with ClO_2. For spruce lignin, the unusual group of cross peaks at 118–124/5.9–6.4 ppm were assigned to α-vinyl carbons/protons based on experiments carried out on model compounds. The group of cross signals at 131–138/7.5–8.3 ppm was assigned to β-vinyl carbons/protons in MATS (Figure 5b). All these cross peaks from vinylic moieties (Figure 5) were rather different from those in cinnamic acid (Cα/Hα at 144.6/7.64 ppm and Cβ/Hβ at 115.8/6.42 ppm), cinnamaldehyde (Cα/Hα at 153.3/7.62 ppm and Cβ/Hβ at 126.4/6.70 ppm) and cinnamyl alcohol (Cα/Hα at 128.6/6.49 ppm and Cβ/Hβ at 128.5/6.26 ppm) structures (Ralph et al. 1999). This fact shows that MATS in oxidised lignins can be reliably detected by 2D HSQC experiments.

The most intense resonances from the vinylic moieties in the HSQC spectra were observed at 123,1/6.21, 121.3/6.16, 131.3/7.81 and at 122.2/6.35 and 136.8/8.10 ppm, which may be assigned, based on experiments on model compounds, to C-2/H-2, C-5/H-5, C-4/H-4 in non-cyclic (types **I** and **I′**) and C-5/H-5 and C-4/H-4 in cyclic muconic structures of types **II/III**, respectively. The coupling constant found for the correlated in COSY spectrum protons at 6.16 ppm and 7.81 ppm (8.6 Hz) indicated a *cis*-configuration of the C_4-C_5 double bond in muconic structure. Hence, MATS were present in OAL essentially

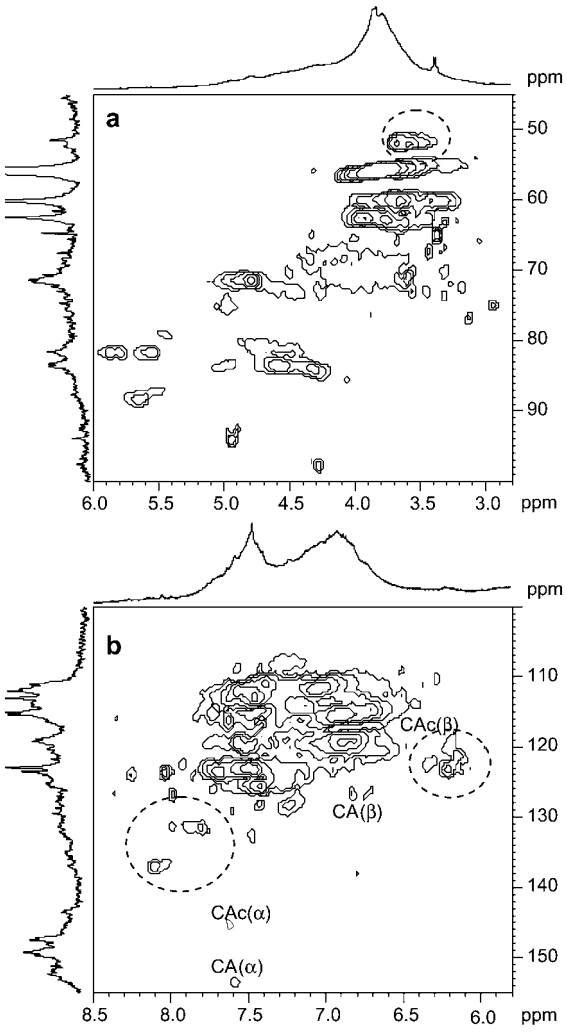

Figure 5 Aliphatic (a) and aromatic (b) regions in HSQC spectrum of oxygen-organosolv lignin (CA-cinnamaldehyde type structure, CAc-cinnamic acid type structures).

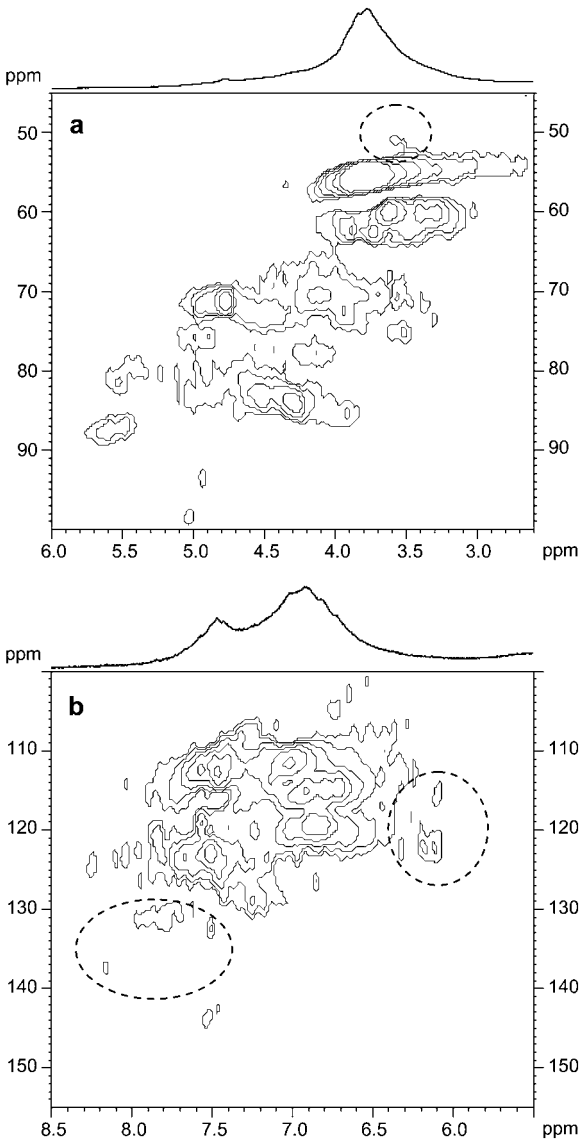

Figure 6 Aliphatic (a) and aromatic (b) regions in HSQC spectrum of saponified oxygen-organosolv lignin.

as *cis*-isomers. This, however, does not mean that MATS in a *trans*-configuration were completely absent in OAL, but certainly are present in much lower amounts than the *cis*-isomers.

A cross peak at 75.6/5.03 ppm was detected in the HSQC spectrum of OAL, which is rather unusual for spruce lignin. The carbon chemical shift of this signal is practically the same as for Cα in benzylic esters (Ralph et al. 1999), but the proton shift is substantially lower than in those structures and is very similar to the shift of protons at tertiary carbon, the hydroxyl group which is involved in the formation of muconolactons (Chen et al. 1996; Gesell et al. 2001). Accordingly, this cross signal in OAL was tentatively assigned to tertiary oxygenated carbon in muconolactones (both γ- and δ-lactones).

The alkaline treatment of OAL at room temperature (12 h) was carried out to confirm some MATS assignments and to prove their detection after oxidation under alkaline conditions. In the ^{13}C NMR spectrum (not shown) of saponified OAL (OALS), the signal at 51.6 ppm disappeared and the resonance at 167.0 ppm shifted to 166.7 ppm. The signal centred at 171.5 ppm decreased after OAL saponification but its intensity was still notable. The latter feature indicated the possible incomplete elimination of lactone structures from OAL or their formation in OALS after the acidification of the alkaline lignin solution during the sample isolation. The HSQC spectrum of OALS (Figure 6a) showed the disappearance of the cross peak at 51.6/3.65 ppm confirming alkaline hydrolysis of the methyl ester group in partially methylated MATS. The cross signals detected in HSQC spectrum of OAL at 122.2/6.35 and 136.8/8.10 ppm (Figure 5b) disappeared in the spectrum of OALS (Figure 6b). Simultaneously, new signals appeared in the spectrum of OALS at 116.6/6.12, 121.9/6.15 and 122.0/6.08 ppm and the relative intensity of the cross peak centred at 131.3/7.81 ppm increased. Taking into account the inferred assignments for the vinylic moieties in non-cyclic/cyclic MATS, as discussed above, these changes in the spectra were interpreted in terms of eventual saponification of muconolactones to non-cyclic MATS. The presence of low intensity cross peaks at 122.7/6.32 and 138.5/8.17 ppm in the HSQC spectrum of OALS indicate that a small proportion of muconolactone was still present after saponification as a result of structural rearrange-

ments after the acidification of lignin sample before isolation. Hence, the reliable detection of MATS in lignin oxidised under alkaline conditions is possible due to the presence of cross peaks in the HSQC spectra at 117–123/6.1–6.3 ppm and 131–139/7.5–8.2 ppm. These signals were assigned to α- and β-vinylic carbons/protons in MATS, respectively.

It is worth noting that in other series of experiments with dehydrogenation polymers, carried out in our laboratory, and their oxidation with chlorine dioxide or ozone always led to the appearance of characteristic MATS signals, thus confirming the possibility of their detection according to proposed methodology.

Conclusions

The results of this work allowed a database to be compiled for the reliable detection of MATS in oxidised lignins by 2D NMR. Thus, partially methylated MATS can be identified, in HSQC spectra, by a cross signal from the methoxy group at 51.6/3.65 ppm and by the carbon/proton signals from α-vinyl (117–123/5.9–6.3 ppm), and β-vinyl (131–138/7.5–8.2 ppm) moieties in non-cyclic and cyclic MATS. The oxidation of lignin under alkaline conditions or alkali treatment after the oxidation lead to the disappearance of a cross peak at 51.6/3.65 ppm, whereas the cross peaks of the vinylic moieties are shifted to higher field. All signals from the vinylic moieties of MATS were distinct from signals belonging to most known lignin structures with unsaturated moieties and can be used for the reliable detection of MATS in oxidised lignins. The carboxyl carbon resonance in the ^{13}C NMR spectra of MATS was detected at 166.6–167.0 ppm and the signals centred at 171.3–171.5 ppm were assigned to carboxyl carbons in muconolactones. It was suggested that most of the MATS in oxidised lignin are in a *cis*-configuration.

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