

## **A Review on Delineating the Distribution of *p*-Coumarate vs. *p*-Hydroxybenzoate as Acylating Groups in Lignin across Monocot Families**

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Lignin is a major component of plant cell walls that is essential to their function. However, the strong bonds that bind the various subunits of lignin, and its cross-linking with other plant cell wall polymers, make it one of the most important factors in the recalcitrance of plant cell walls against polysaccharide utilization. Lignin restricts the degradation of structural polysaccharides by hydrolytic enzymes, thereby limiting the bioconversion of forages and fibrous crops into liquid fuels and other industrial products. Over the past decade it has become apparent that the pretreatment of lignocellulosic materials to remove or modify the lignin, hence altering the metabolic malleability of lignification, provides potential for engineering the troublesome polymer to be more amenable to bioprocessing. The knowledge of the structure of the lignin polymer, including the strong binding between its various subunits, is thus important to develop appropriate pretreatment methods for lignin modification and/or removal. Acylation in lignin subunits provides a valuable distinguishing feature between monocot and dicot plants. A range of acylated monolignols, notably monolignol  $\gamma$ -acetates,  $\gamma$ -hydroxybenzoates, and  $\gamma$ -*p*-coumarates are known to be lignin precursor 'monomers' (monomer conjugates). Generally, in monocots, the monolignols are preacylated by *p*-coumaric acid (pCA) whereas in dicots the lignins are acylated by *p*-hydroxybenzoate. However, the Aracaceae family shows similarity with many of the dicots in containing *p*-hydroxybenzoate instead of *p*-coumarate as the units acylating the lignin. Hence there is differential distribution of lignin acylating groups across monocot families, which is the subject of present study.

*Keywords:* lignin; pCA; *p*-hydroxybenzoates; Monocots; Py-GC/MS; TMAH; HSQC; DFRC

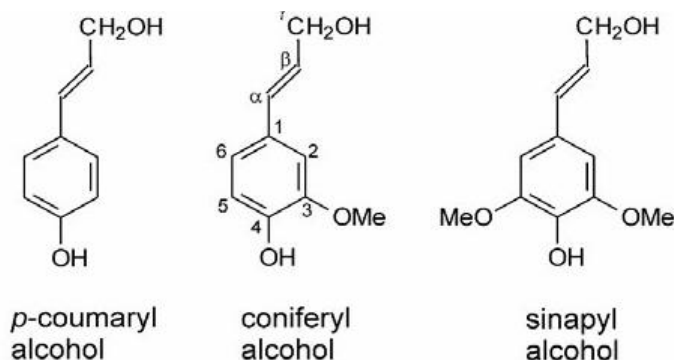
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### **INTRODUCTION**

Plants make lignin from a variety of monolignols including *p*-coumaryl, coniferyl, and sinapyl alcohols to produce the three primary lignin units: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively, when incorporated into the lignin polymer. The lignin composition depends on its botanical origin. Thus, hardwood lignins are composed of S and G units in varying ratios, softwood lignins are primarily composed of G units and a small amount of H units, and grass lignins include the three units (together with ferulates and *p*-coumarates). The H-unit content is usually small (typically <5%), but it is often reported as being higher because of conflation with various other *p*-

hydroxyphenyl units that do not arise from the incorporation of *p*-coumaryl alcohol into the lignin (Del Río *et al.* 2007)

The structures of the three traditional lignin precursors – *p*-coumaryl, coniferyl and sinapyl alcohol are shown in Fig 1.



**Fig. 1.** Structures of lignin precursors\*

\*(Sanna Koutaniemi, *Lignin biosynthesis in Norway spruce: from a model system to the tree*, University of Helsinki Finland)

Other typical monomers found in natural lignins include 5-hydroxyconiferyl alcohol, hydroxycinnamaldehydes, hydroxybenzaldehydes, arylglycerols, tyramine hydroxycinnamates, hydroxycinnamic acids, hydroxycinnamate esters, dihydrohydroxycinnamyl alcohols, arylpropane-1, 3-diols, and various acylated monolignols-hydroxycinnamyl acetates, hydroxycinnamyl-*p*-hydroxybenzoates and hydroxycinnamyl-*p*-coumarates. Hydroxycinnamaldehydes and their corresponding hydroxybenzaldehydes are found in all lignins. Hydroxycinnamyl acetates are found in most hardwoods and are present in high levels in kenaf and palms. Hydroxycinnamyl *p*-hydroxybenzoates are found in willows, palms, poplars, and aspens. Hydroxycinnamyl *p*-coumarates are found in all grasses. These monomers are polymerized into polymeric lignin by combinatorial radical coupling reactions. The pathway of Lignin biosynthesis is shown in Fig. 2.

The lignification of cell walls is also notable because it is likely the single most important factor that limits forage digestibility in ruminants and the “saccharification” of plant polysaccharides to simple sugars for use in biofuel or biochemical applications. Practically speaking, lignin is indigestible in the digestive tract of ruminants. The interfering presence of indigestible lignin limits the ability of ruminants to utilize otherwise digestible carbohydrates present in the forage they eat. Lignin also limits enzyme access to cell wall polysaccharides, inhibiting the release of monosaccharides for conversion to other products including biofuels. Thus, there remains a long-felt and unmet need to alter lignins in such a way that improves the digestibility/fermentability of the cell wall polysaccharides (Ralph *et al.* 2010).

### Presence of *p*-CA in Monocot Lignins

As suggested by Muyang Li *et al.* (2012) a major distinguishing feature of the monocot lignins is the considerable incorporation of the *p*-hydroxycinnamic acids including ferulic and *p*-coumaric acids (Nakamura and Higuchi 1978; Jeong *et al.* 2006; Jung and Phillips 2010). Monomers of *p*-coumaric acid are proposed to be esterified to

the lignin polymer at the  $\gamma$ -carbon of the side chain region of  $\beta$ -O-4 linked syringyl moieties (Hatfield and Chaptman, 2009) and to a lesser degree esterified to glucuronarabinoxylan. Grass lignins are significantly more condensed (i.e. contain more C-C linkages between monolignols) and have higher phenolic hydroxyl contents than the lignins of dicots (Jung and Phillips 2010; Iiyama and Lam 2001) and an important implication of this is that more than 50% of grass lignins can be solubilized by treatment with alkali (Hartley and Jones 1978) due to the destruction of alkali-labile ester linkages along with the high free phenolic content improving lignin solubility in alkali (Humphrey *et al.* 2007).

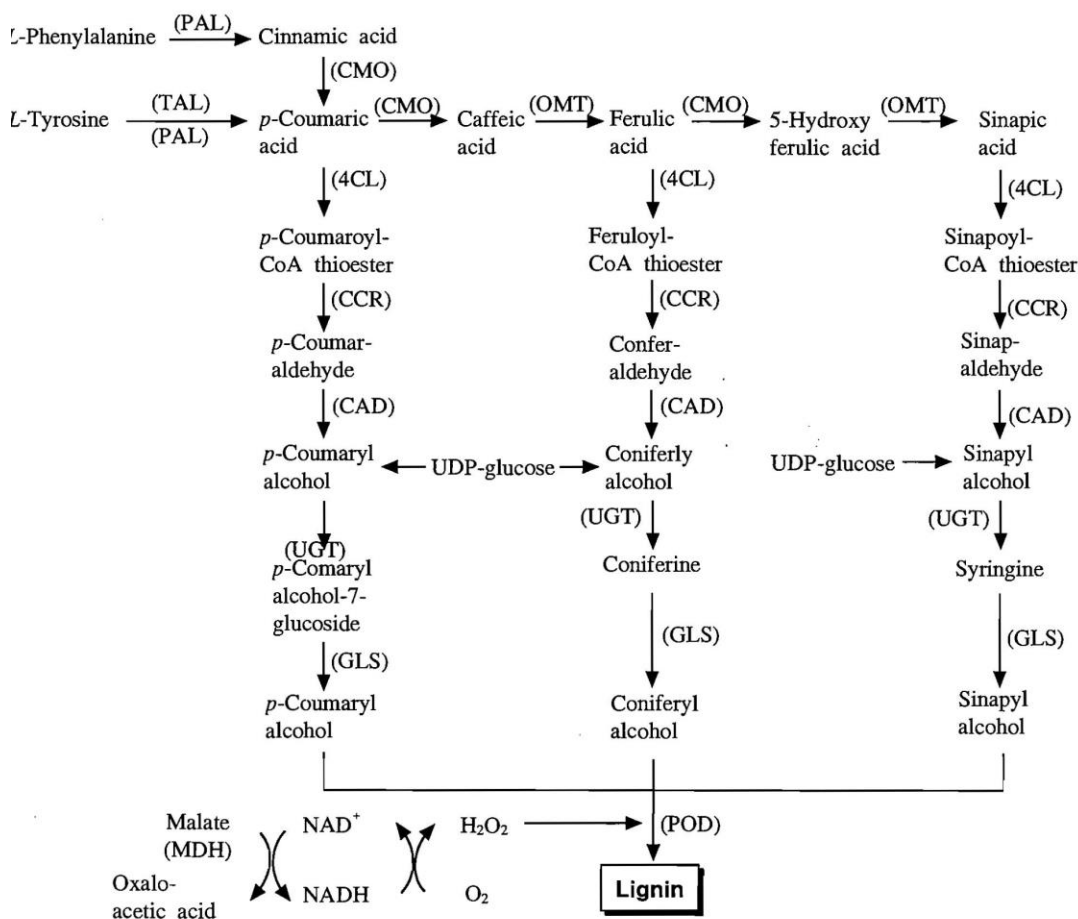


Fig. 2. Pathway of lignin biosynthesis

(Sanna Koutaniemi, *Lignin biosynthesis in Norway spruce*, University of Helsinki Finland)

Grass lignins are uniquely acylated with *p*-coumarates (pCA), resulting from the incorporation of monolignol *p*-coumarate conjugates into the growing lignin polymer within the cell wall matrix. The required acyl-transferase is a soluble enzyme (*p*-coumaroyl transferase, pCAT) that utilizes *p*-coumaroyl-Coenzyme A (pCA-CoA) as the activated donor molecule and sinapyl alcohol as the preferred acceptor molecule (Hatfield *et al.* 2009).

In grasses, these monolignols can be enzymatically preacylated by *p*-coumarates prior to their incorporation into lignin, and these monolignol conjugates can also be “monomer” precursors of lignin. Although monolignol *p*-coumarate-derived units may comprise up to 40% of the lignin in some grass tissues, the *p*-coumarate moiety from such conjugates does not enter into the radical coupling (polymerization) reactions of lignification (Withers *et al.* 2009).

The work of Del Río, Marques, Rencoret, Martínez and Gutiérrez extensively examines the occurrence of native acetylated lignin in a large set of vascular plants, including both angiosperms and gymnosperms, by a modification of the so-called Derivatization Followed by Reductive Cleavage (DFRC) method. Acetylated lignin units were found in the milled wood lignins (MWL) of all angiosperms selected for this study, including mono- and dicotyledons, but were absent in the gymnosperms analyzed. In some plants (like abaca, sisal, kenaf, or hornbeam), lignin acetylation occurred at a very high extent, exceeding 45% of the uncondensed (alkyl-aryl ether linked) syringyl lignin units. Acetylation was observed exclusively at the  $\gamma$ -carbon of the lignin side chain and predominantly on syringyl units, although a predominance of acetylated guaiacyl over syringyl units was observed in some plants. In all cases, acetylation appears to occur at the monomer stage, and sinapyl and coniferyl acetates seem to behave as real lignin monomers participating in lignification (Del Río *et al.* 2007).

The derivatization followed by reductive cleavage (DFRC) method cleaves alpha- and beta-ethers in lignins but leaves lignin gamma-esters intact (Lu and Ralph 1999). When applied to grasses, which contain *p*-coumarate esters on their lignins, esterified monolignol derivatives are released. Saturation of the *p*-coumarate double bond occurs during DFRC, so the released products are 4-acetoxycinnamyl 4-acetoxyphenyl-propionates. Synthesis of the esters allowed determination of response factors for the released products. Maize and bamboo lignins released 221 and 38 micromol/g of *p*-coumarate-derived esters. The sinapyl ester was much more abundant than the coniferyl one. The bamboo and maize lignin S/G ratios in the conjugates were 12 and 38 times greater than those of the normal monomers released by DFRC, evidence of a strong selectivity for acylation of syringyl units as seen in Table 1. Of three possible biochemical mechanisms for incorporating *p*-coumarates into lignin, evidence is mounting that the process involves incorporation of preacylated monolignols into the normal lignification process (Li, *et al.* 2012; Del Río and Gutiérrez, 2006; Ralph *et al.* 2010).

**Table 1.** Yields of Monomers and Acylated Monomers from DFRC of Bamboo, Maize, and Bromegrass Lignins

monomers ( $\mu\text{m/g}$ )	<i>p</i> -coumarate products ( $\mu\text{m/g}$ )				
	G	S	S/G	13a	13b
Sample 13b/13a					
bamboo lignin 5.33	504	222	0.44	6	32
maize lignin 13.73	342	124	0.36	15	206
bromegrass lignin	454	327	0.72	nd <sup>a</sup>	d <sup>b</sup>
<sup>a</sup> nd, not detected. <sup>b</sup> d, detected, by GC/MS					
*Lu F, Ralph, J					

Del Río and Gutiérrez (2006) thoroughly studied the chemical composition of leaf fibers of abaca, which are commonly used for high-quality paper pulp production (Del Río and Gutiérrez 2006). Their results revealed that the lignin content was 13.2% of the total fiber. The analysis of abaca fibers by pyrolysis coupled to gas chromatography-mass spectrometry (Py-GC/MS) released predominantly compounds arising from lignin and *p*-hydroxycinnamic acids, with high amounts of 4-vinylphenol. The latter compound was demonstrated to arise from *p*-coumaric acid by pyrolysis of abaca fibers in the presence of tetramethylammonium hydroxide, which released high amounts of *p*-coumaric acid (as the methyl derivative).

**Table 2.** Structural Characteristics from Integration of  $^{13}\text{C}$ - $^1\text{H}$  Correlation Peaks in the HSQC of the Wheat Straw MWL

Wheat straw MWL	
Lignin inter-unit linkages (%) (Expressed as a fraction of the total lignin inter-unit linkage types A-F)	
$\beta$ -O-4' aryl ethers (A/A')	75
$\alpha$ -oxidized $\beta$ -O-4' aryl ethers (Aox)	2
Phenylcoumarans (B)	11
Resinols (C)	4
Dibenzodioxocins (D)	3
$\alpha,\beta$ -diaryl ethers (E)	2
Spirodienones (F)	3
Lignin end-groups	
Cinnamyl alcohol end-groups (I)	4
Cinnamaldehyde end-groups (J)	4
Lignin side-chain $\gamma$ -acylation (%)	10
Lignin aromatic units (Molar percentages (H+G+S=100))	
H (%)	6
G (%)	64
S (%)	30
S/G ratio	0.5
<i>p</i> -Hydroxycinnamates (As percentage of Lignin Content)	
<i>p</i> -Coumarates (%)	4
Ferulates (%)	2
<i>p</i> -Coumarates/ferulates ratio	2.0

Products from *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) propanoid units, with a predominance of the latter (H:G:S molar ratio of 1.5:1:4.9), were also released after Py-GC/MS of abaca fibers. Sinapyl and coniferyl acetates, which are thought to be lignin monomer precursors, were also found in abaca. The extractives content of the abaca fiber (0.4%) was low, and the most predominant compounds were free sterols (24% of total extract) and fatty acids (24% of total extract). Additionally, significant amounts of steroid ketones (10%), triglycerides (6%),  $\omega$ -hydroxyfatty acids (6%), monoglycerides (4%), fatty alcohols (4%), and a series of *p*-hydroxycinnamyl (*p*-

coumaric and ferulic acids) esterified with long chain alcohols and  $\omega$ -hydroxyfatty acids were also found, together with minor amounts of steroid hydrocarbons, diglycerides,  $\alpha$ -hydroxyfatty acids, sterol esters, and sterol glycosides.

The HSQC spectrum of Wheat Straw (*Triticum durum* var. Carioca) as done by del Río *et al.* (2013) also reveals the presence of characteristic signals corresponding to the C $\gamma$ -H $\gamma$  correlations of  $\gamma$ -acylated  $\beta$ -O-4' (A') and other structures in the range from  $\delta$ C/ $\delta$ H 63.5/3.83 and  $\sim$ 4.30. Therefore, it is possible to conclude that the lignin of wheat straw is partially acylated at the  $\gamma$ -position of the lignin side-chain, as already observed in other grasses (Rencoret *et al.* 2013; Hatfield *et al.* 2009; Withers *et al.* 2012; Lu and Ralph, 1999). An estimate of  $\sim$ 10% for the percentage of  $\gamma$ -acylation of lignin side-chains was calculated by integration of the C $\gamma$ -H $\gamma$  correlation peaks corresponding to the hydroxylated (A $\gamma$ ) and acylated (A' $\gamma$ ) substructures in the HSQC spectrum of the isolated MWL (Table 2). The fact that the side-chain of the lignin in wheat straw is partially acylated at the  $\gamma$ -OH, together with the presence of significant amounts of *p*-coumarate moieties (4% with respect to lignin), which are known to acylate the  $\gamma$ -OH of the lignin side-chain in many plants, and particularly in grasses (Rencoret *et al.* 2013; Hatfield *et al.* 2009; Withers *et al.* 2012; Lu and Ralph 1999; Li *et al.* 2012), seems to indicate that *p*-coumarates also acylate the  $\gamma$ -OH in the lignin of wheat straw.

### Role of *p*-CA in Monocot Lignification

The participation of *p*-hydroxycinnamates in the cell wall composition and organization of mature lignified tissues is certainly the most specific feature of grass lignification (Barriere *et al.* 2007). However, the role of *p*CA in cell wall matrices is not fully understood. It appears the majority of *p*CA remains incorporated other than its attachment to monolignols via an ester linkage. Hence, *p*CA does not function as a cross linking agent between wall matrix polymers, different lignin polymer or between lignin and polysaccharides. It has been suggested that lignin may function as a radical transfer agent to aid in the formation of sinapyl alcohol and lignin radicals. There are reports of *p*CA being ester linked to arabinoxylans. However this level is quite low. Incorporation of *p*CA into grass walls appears to be primarily associated with the lignin fraction. Several studies have shown a positive relationship between *p*CA incorporation into cell wall matrices and levels of lignin formation (Grabber *et al.* 2005; Grabber 2005; Grabber and Lu 2007).

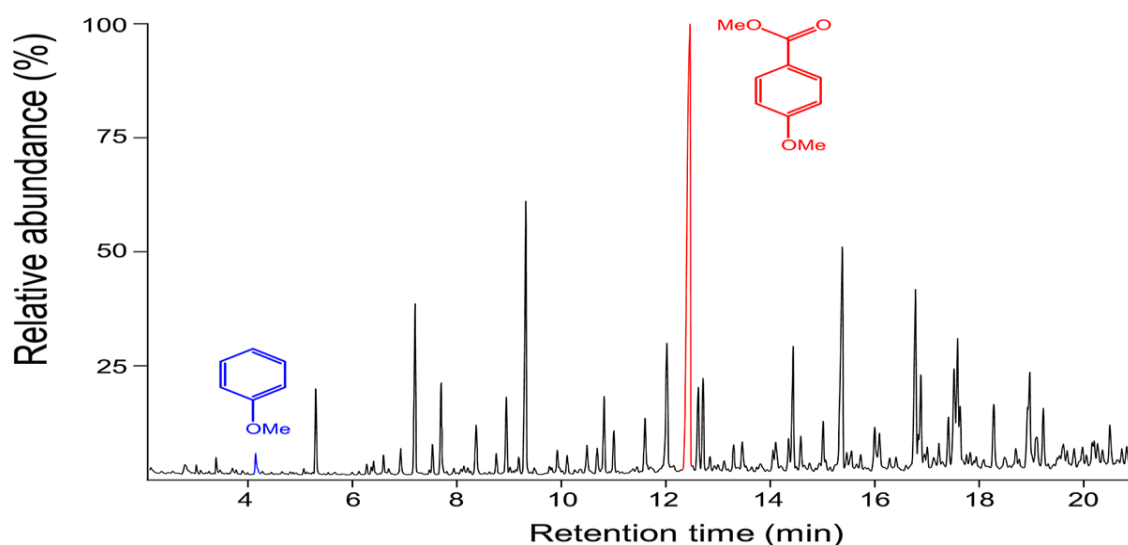
Works of Ralph *et al.* (1994) and Grabber *et al.* (1996) also confirmed that *p*CA is mainly esterified to the  $\gamma$ -position of the side chains of the S lignin units and lignified maize cell wall can contain up to 3% *p*-coumarate (Grabber *et al.* 1994). A small amount of *p*CA is esterified to hemicelluloses in immature tissues (Grabber *et al.* 1995; Grabber *et al.* 1996a,b; 1998). However most *p*-coumarate accretion occurs in tandem with lignification and *p*-coumarate accumulation is thus a relevant indicator of lignin deposition, particularly of S units (Grabber *et al.* 1994). Sinapyl alcohol, the precursor of S unit would be enzymatically preacylated with *p*-CA prior to their incorporation into lignin and sinapyl *p*-coumarate is thus the presumed precursor incorporated into lignin (Lu and Ralph 1999). In maize 25 - 50% of lignin subunit may be acylated by *p*CA (Barriere *et al.* 2007). This acylation has probably a marked influence on the bonding mode of S lignin unit, on the spatial organization of lignins and on their interacting

capabilities with polysaccharides. While sinapyl alcohol has a pronounced tendency to be involved as B-o 4 endwise coupling upon peroxidase polymerization in plant cell wall its *p*-coumaroylation at C $\gamma$  might change its coupling modes as well as the advancement of the peroxidase driven polymerization (Grabber *et al.* 2002; Grabber 2005; Grabber and Lu 2007). As a support to this hypothesis, a model study recently established that adding sinapyl *p*-coumarate with monolignols accelerated peroxidase inactivation and this suppressed lignification (Grabber and Lu 2007).

### ***p*-Hydroxybenzoates as Acylating Groups in Monocot Aracaceae members**

In coconut fibre, unlike in grasses, there is no significant *p*-coumarate component acylating the lignin. Like its palm (Arecales order, Arecaceae or Palmae family) relatives (but as also noted in angiosperm/dicot lines, *Salix* and *Populus*, both in the Salicaceae family), and unlike in grasses, the lignins are acylated by *p*-hydroxybenzoate (Rencoret *et al.* 2013).

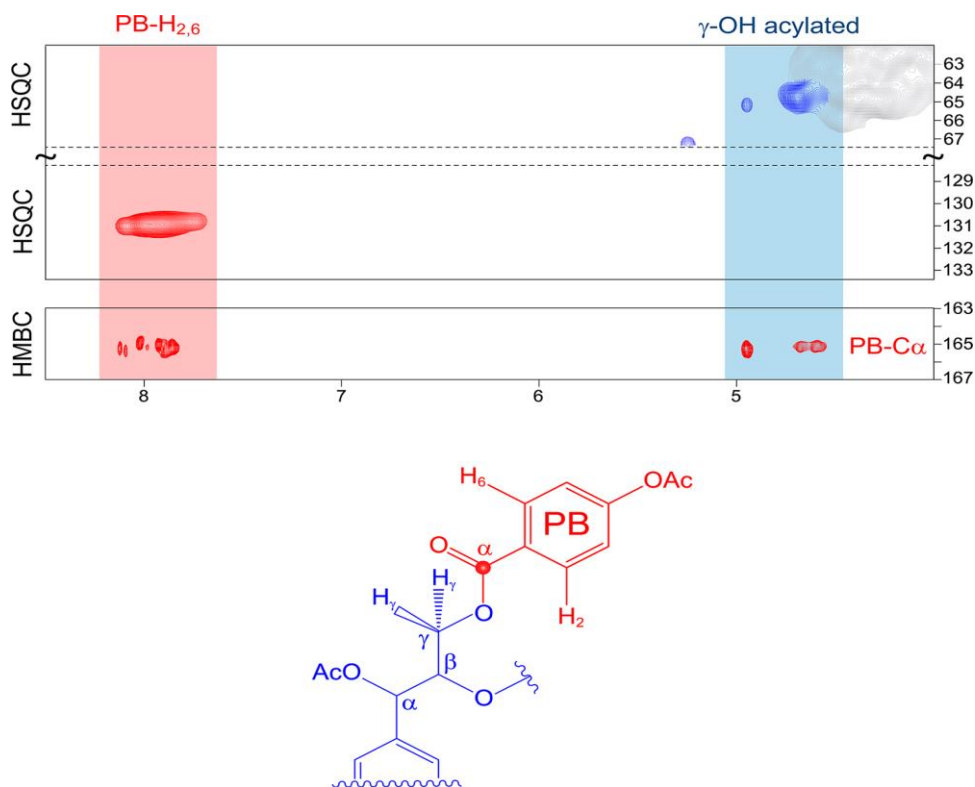
The MWL from coconut coir was analyzed by Py-GC/MS by Rencoret *et al.* (2013). The pyrolysis data indicate a predominance of G- over S-lignin units, with a S/G ratio of 0.29. In addition, high levels of phenol (27% of all phenolic compounds) were released upon pyrolysis from coconut coir MWL. This fact might suggest the presence of *p*-hydroxybenzoates in coir lignin, as also occurs in the lignin of other palms (Ishii *et al.* 1990). The occurrence of *p*-hydroxybenzoates in the lignin of coconut coir was assessed by pyrolysis in the presence of TMAH as methylating agent. Py/TMAH of coconut coir lignin (shown in Fig 3) released significant levels of 4-methoxybenzoic acid methyl ester, confirming the occurrence of *p*-hydroxybenzoates in this lignin. In contrast, the amounts methoxybenzene, which arises exclusively from H-lignin, were very low (only 2% of the 4-methoxybenzoic acid methyl ester peak area). This means that the great majority of phenols released after Py-GC/MS arise from *p*-hydroxybenzoates.



**Fig. 3.** Py-TMAH-GC/MS (b) chromatograms of the MWL isolated from coconut coir\*

\* Jorge Rencoret, John Ralph, Gisela Marques, Ana Gutiérrez, Ángel T. Martínez and José C. del Río, "Structural Characterization of Lignin Isolated from Coconut (*Cocos nucifera*) Coir Fibers", *J. Agric. Food Chem.* 2013, 61, 2434–2445

To obtain additional information on the structure of coir lignin, the MWL was analyzed by 2D NMR, which provides information on the interunit linkages as well as the lignin composition. The  $C_{\gamma}$ - $H_{\gamma}$  correlations in  $\beta$ -O-4' substructures were observed at  $\delta C/\delta H$  59.8/3.24 and 3.61 partially overlapped with other signals. In addition, the spectrum clearly showed the presence of signals in the range  $\delta C/\delta H$  63.3/4.46–4.30 corresponding to the  $C_{\gamma}$ - $H_{\gamma}$  correlations of  $\gamma$ -acylated units (substructure A', R = acyl). The HSQC spectrum therefore indicates that the coconut coir lignin is partially acylated at the  $\gamma$ -position of the lignin side chain. Strong signals for the C<sub>2,6</sub>-H<sub>2,6</sub> and C<sub>3,5</sub>-H<sub>3,5</sub> correlations of *p*-hydroxybenzoate units were observed at  $\delta C/\delta H$  131.2/7.68 and 114.3/6.61. To ascertain the nature of acylation, HMBC experiments were performed on acylated coconut coir MWL. Figure 4 shows the section of the HMBC spectrum of (acetylated) coconut coir MWL for the correlations of the carbonyl carbon of *p*-hydroxybenzoates acylating the lignin  $\gamma$ -OH.



**Fig. 4.** Section of the HMBC spectrum ( $\delta C/\delta H$  163–167/4.0–8.5) of acetylated coconut coir MWL (in  $CDCl_3$ ) showing the main correlations for the carbonyl carbon of the *p*-hydroxybenzoates acylating the  $\gamma$ -positions of the lignin side chains. Appropriate sections of the HSQC spectrum showing the  $C_{\gamma}$ - $H_{\gamma}$  correlations of the acylated lignin  $\gamma$ -carbon ( $\delta C$  62–68) and the C<sub>2,6</sub>-H<sub>2,6</sub> correlations of *p*-hydroxybenzoates ( $\delta C$  128–133) are also depicted.

\*Jorge Rencoret, John Ralph, Gisela Marques, Ana Gutiérrez, Ángel T. Martínez and José C. del Río, *J. Agric. Food Chem.* 2013, 61, 2434–2445.



The correlations of the carbonyl carbon at  $\delta C$  165.0 with the 2- and 6-protons at  $\delta H$  7.68 confirm that they belong to the *p*-hydroxybenzoates. In addition, the correlations of this carbonyl carbon with several protons in the range  $\delta H$  4.5–5.0 conclusively demonstrate that *p*-hydroxybenzoates are acylating the  $\gamma$ -position of the lignin side chains in coir lignin, as also occurs in the lignins of other plants (Ishii *et al.* 1990; Dien *et al.* 2005; Lu and Ralph 2005; Majcherczyk and Huttermann 1997; Murnen *et al.* 2007; Ralph *et al.* 1994).

Ken-ichi Kuroda, Tetsuo Ozawa and Takahiro Ueno of Institute of Agricultural and Forest Engineering and Institute of Applied Biochemistry, University of Tsukuba, Japan, prepared Dioxane lignin from Sago palm (*Metroxylon sago*) and characterized it by analytical pyrolysis coupled to gas chromatography–mass spectrometry. Large abundances of the *p*-hydroxybenzoates ester-linked to the lignin were proven by analytical pyrolysis as well as by mild alkaline treatment that produced *p*-hydroxybenzoic acid in 16.3% yield (Kuroda *et al.* 2001). Pyrolysis in the presence of tetramethylammonium hydroxide (TMAH) before and after alkaline treatment also showed the presence of ester- and ether-linked *p*-hydroxybenzoates. Quantitative results of pyrolysis showed that the sago palm lignin is of syringyl type. The relative abundances of TMAH/pyrolysis products derived from the syringyl  $\beta$ -aryl ether substructures were 4.9 times those of the guaiacyl equivalents. Proton nuclear magnetic resonance analysis also showed the presence of the *p*-hydroxybenzoates and the predominance of the syringyl moiety over the guaiacyl ones in the sago palm lignin.

## CONCLUSIONS

1. Natural lignins display a large structural variability, which is further increased by industrial and genetic transformations (and to a lower extent by environmental factors). An in-depth knowledge of lignin structure and organization opens avenues to rationally design lignocellulosics suitable for conversion processes.

2. Because DFRC leaves lignol  $\zeta$ -esters essentially intact, it is valuable for identifying esterified lignin components and for determining the sites of acylation. This study confirmed that grass lignins are acylated at the  $\zeta$ -position by *p*-coumarates; primarily syringyl units are acylated in maize, bamboo, and bromegrass

3. In case of Coconut fibre, the plant exhibited significant differences both from monocots and dicots. While coconut contains high lignin content (almost 50% of total cellulose) (Justiz –Smith *et al.* 2008) the acylating units were *p*-hydroxybenzoates instead of *p*-coumarates as found in most grasses. Also, there is a predominance of G units in coconut while grasses showed S units as dominant monolignol units. The S:G ratio in coconut was found to be low i.e. 0.29 as against other Monocot families. In case of Sago palm, similarity with grasses was seen as S units were predominantly acylated.

4. These variations as seen in case of Coconut plant require further study. The present findings may provide a template for future research correlating the unique features of Coconut lignin with its low digestibility. Also, the pretreatment methods for delignification of Coconut fibres may be suitably altered to obtain better results.

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