Quantitative Determination of Hydroxycinnamic Acids in Wheat, Rice, Rye, and Barley Straws, Maize Stems, Oil Palm Frond Fiber, and Fast-Growing Poplar Wood

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A new method has been developed for the quantitative determination of hydroxycinnamic acids participating in ester or ether linkages to the cell wall polymers. The method is based on mild alkaline hydrolysis followed by acid hydrolysis or mild alkaline hydrolysis, which partially removed esterified phenolic acids, and high-temperature concentrated alkaline treatment, which cleaved both the ester and ether linkages. It was found that traditional mild alkaline hydrolysis and acid hydrolysis released only part of the ester- and ether-linked phenolic acids, respectively. Approximately half (44.0−47.9%) of the total ester-linked *p*-coumaric acid and 18.2−32.6% of the total esterified ferulic acid remained ester-linked to the mild alkali-soluble lignin polymers, and 55.0−72.0% of the total ether-linked *p*-coumaric acid and 37.5−53.8% of the total ether-linked ferulic acid remained ether-linked to the solubilized lignin molecules after the acid hydrolysis. To correct this, a second mild alkaline hydrolysis of the alkali-soluble lignin preparations and acid hydrolysis of the solubilized lignin fractions, obtained from the first acid hydrolysis of the cell wall materials, was investigated. On the basis of this new method, a majority of the cell wall *p*-coumaric acid (55.8−81.5%) was found to be ester-linked to cell wall components, mainly to lignin, and about half of the cell wall ferulic acid is etherified through its phenolic oxygen to the cell wall lignin component, whereas the remainder is esterified to the cell wall hemicelluloses and/or lignin in different plant materials.

Keywords: *Ferulic acid; p-coumaric acid; lignin; wheat straw; rice straw; rye straw; barley straw; maize stems; oil palm frond fiber; fast-growing poplar wood*

INTRODUCTION

Hydroxycinnamic acids, particularly ferulic acid and *p*-coumaric acid, occur widely in cell walls of graminaceous plants such as wheat straw and maize stems (*1*, *2*). They are principal components governing cell wall integrity, shape, and defense against pathogenic ingress (*3*). As bifunctional molecules with carboxylic and phenolic bonding sites, it is believed that ferulic acid is laid down in ester linkages to primary cell wall polysaccharides and provides ether linkage initiation sites for lignin (*4*). Such a cross-linking has been reported to have a profound influence on the growth of the plant cell wall and its mechanical properties and biodegradability (*5*−*7*). Using model compounds it has been demonstrated that ferulic acid has the potential to cross-link polysaccharides and lignin through covalent ester−ether bridges (*8*, *9*), but there is only circumstantial evidence that these structures occur in cell walls (*10*−*12*) suggested by the presence of ester−ether bridges through ferulic acids between lignin and polysaccharides, based on the distribution of ester- and ether-linked ferulic acids in fractions containing the lignin of cell walls of temperature grasses. They suggested that all of the ferulic acid etherified to lignin also is esterified to polysaccharides, but *p*-coumaric acids are not involved in the ester−ether bridges. Small amounts of *p*-coumaric acid are esterified to arabinoxylans early in primary wall development in much the same way as ferulic acid, but, later in wall development, *p*-coumaric acid is found to be more extensively esterified to lignin in the cell wall of maize stems (*13*).

The ester or ether linkages between hydroxycinnamic acids and other cell wall components can be determined by treatment of the cell wall fraction with 1 M NaOH at room temperature that cleaves ester bonds or by hot concentrated alkali (4 M NaOH at 170 °C for 2 h) that cleaves both ester and ether bonds. Ether-linked hydroxycinnamic acids were estimated as the difference between total and ester-linked *p*-coumaric acid and ferulic acid released by the two alkaline hydrolysis treatments or determined by treatment of the 1 M NaOH extracted residue with dioxane/2 M HCl (9:1, v/v) at 87 °C or reflux for 1 h (*12*, *14*). However, in this commonly used procedure for cell wall hydroxycinnamic acids analysis, a portion of the phenolic acids is not extracted and remains in the residue or is still linked to solubilized lignin, hemicelluloses, or the lignin−hemicellulosic complex after such treatment. In other words, during the analysis the hydroxycinnamic acids are released by alkali treatment; the hydrolysate should

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be acidified to pH 1−2 and then extracted with an organic solvent such as diethyl ether or ethyl acetate. This acidification often results in the formation of a precipitate of released lignin or lignin−hemicellulosic complex that also contains significant amounts of phenolic acids linked to polymers by ether bonds and some ester linkages, which are not cleaved by the alkali at room temperature. In addition, treatment at different temperatures has also a significant effect on the values of hydroxycinnamic acids released (*15*). Owing to this methodological problem, various values have been reported from the cell walls of same grasses or agricultural residues. Our experiments showed that the values of esterified ferulic and *p*-coumaric acids released depend on the dilute alkali treatment temperature used and, in particular, that ferulic and *p*-coumaric acids attached to the precipitated lignin fractions are substantial and cannot be ignored. Therefore, this paper reports the effect of dilute alkali treatment temperature on the release of ferulic acid and *p*-coumaric acid and their content in isolated lignin fractions from wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood in our newly developed process.

MATERIALS AND METHODS

Materials. Wheat, rice, rye, and barley straws and maize stems were obtained from the experimental farm of The North-Western Sciences and Technology University of Agriculture and Forestry (Yangling, People's Republic of China). These were dried in sunlight and then cut into small pieces. The cut straw was ground to pass a 1.2 mm screen. A fast-growing poplar tree, 12 years old, was harvested in December of 1997 at the above University Forest. After the outer and inner barks were peeled off, the remainder was chipped and dried, and the chips were ground to pass a 1.2 mm screen. Oil palm frond fiber supplied by the South University of Agriculture was cut by a hammer mill into lengths of 0.8−1.2 mm and screened to remove fines and dusts. All of the ground samples were further dried at 55 °C for 16 h and dewaxed with toluene/ethanol (2:1, v/v) in a Soxhlet apparatus for 6 h. All standard chemicals, such as *trans*-ferulic acid and *trans*-*p*-coumaric acid, were purchased from Sigma Chemical Co. (Beijing, China).

Isolation of Total (Ester- and Ether-Linked) Hydroxycinnamic Acids. A 0.025 g portion of the dewaxed samples was saponified at 170 °C for 2 h with 7 mL of 4 M NaOH. After filtering and washing (2 × 7 mL of water), the filtrate was adjusted to pH 2 with 6 M aqueous HCl. The acidified solution was extracted with 3 × 30 mL of chloroform. The combined organic extracts were dried by removal of the solvent under reduced pressure at 40 °C. This crude final residue containing the hot concentrated alkali-released phenolic acids and aldehydes was redissolved in 2 mL of methanol and stored in the dark prior to analysis by high-performance liquid chromatography (HPLC). All analyses were performed at least in triplicate.

Isolation of Ester-Linked Hydroxycinnamic Acids. The extractive-free and ground samples (3.0 g) were saponified with 300 mL of 1 M NaOH for 18 h under N_2 with magnetic stirring at 18, 25, and 30 °C, respectively. Upon completion, the residue was filtered off and washed thoroughly with water until the filtrate was neutral and then dried in an oven at 60 °C for 16 h. The combined supernatant fluid was neutralized to pH 6.0 with 6 M HCl, and the solubilized hemicelluloses were isolated by precipitation of the concentrated filtrates with 3 volumes of 95% ethanol. After filtration, the isolated hemicelluloses were thoroughly washed with 70% ethanol and then air-dried. The solubilized lignins were obtained by reprecipitation at pH 1.5 adjusted with 6 M HCl from the corresponding supernatants after evaporation of ethanol. The isolated lignin preparations were washed with acidified water (pH 1.5−2.0) and freeze-dried overnight. The combined supernatants were extracted with 3 × 100 mL of chloroform. The chloroform extracts were then evaporated under reduced pressure. A 5 mg portion of the dried extract was redissolved in 2 mL of methanol and analyzed by HPLC. The scheme for isolation of alkali-soluble lignin fractions is illustrated in Figure 1. The remaining ester-linked hydroxycinnamic acids in the isolated lignin and hemicellulosic fractions were cleaved by mild alkaline hydrolysis of the lignin samples (1 M NaOH, 30 °C, 18 h). After acidifying the hydrolysates with 6 M HCl to pH 2.0, the released ester-linked hydroxycinnamic acids were extracted with chloroform as above and analyzed by HPLC.

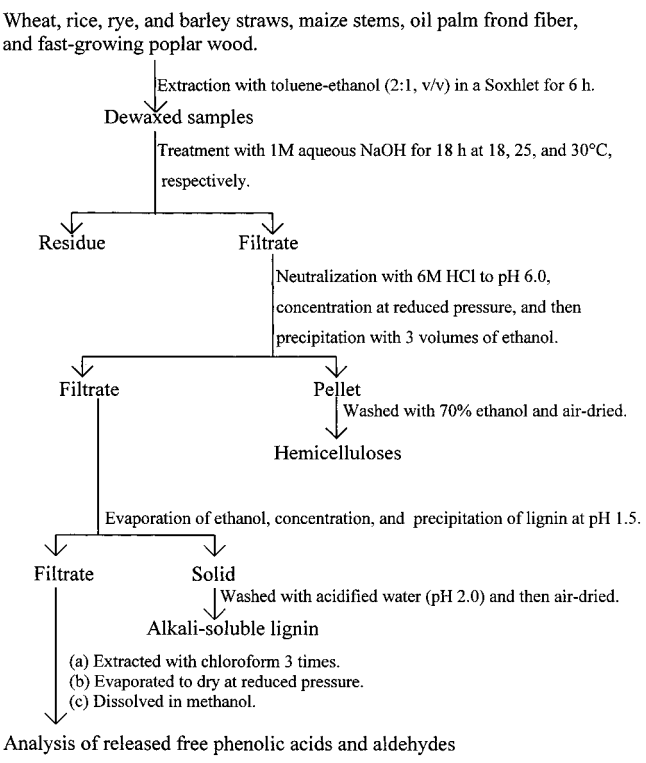

Figure 1. Scheme for isolation of alkali-soluble lignin, hemicelluloses, and free phenolic acids and aldehydes from wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood.

Isolation of Ether-Linked Hydroxycinnamic Acids. The above mild alkali-treated residues were refluxed with dioxane/2 M HCl (9:1, v/v) for 1 h at a solvent/straw ratio of 100 mL/g. After cooling, the residues were filtered off and the filtrates were concentrated under reduced pressure to evaporate the solvent and then neutralized with 2 M NaOH to pH 6.0. The same procedure for isolation of solubilized hemicelluloses, lignin, and released hydroxycinnamic acids in supernatants, as well as combined hydroxycinnamic acids in isolated lignin samples, was then followed as described in the mild alkaline hydrolysis.

HPLC Analysis. The phenolic acids and aldehydes obtained from the above procedures were separated on a Hichrom H5ODS (250 × 4.6 mm i.d., Phenomenex Co., Beijing, China) HPLC column at room temperature. The samples were eluted by a linear gradient consisting of solvent A (water/methanol/acetic acid 89:10:1) and solvent B (methanol/water/acetic acid 90:9:1) run over 31 min from 0 to 40% B at a flow rate of 1 mL/min. Quantitative data were obtained from the chromatograms recorded at 320 nm with an ultraviolet monitor. Calibration curves were established with appropriate mixtures of authentic phenolic acids and aldehydes.

Sugar Analysis and Spectroscopic Characterization of Isolated Lignin Fractions. The neutral sugar composition of the associated hemicelluloses in the lignin fractions was determined by gas chromatography (GC) according to the method of Blakeney et al. (*16*). FT-IR spectra were obtained on an FT-IR spectrophotometer (Nicolet 510) using a KBr disk

Table 1. Total Content (Percent Dry Sample, w/w) of Phenolic Acids and Aldehydes Released in 4 M NaOH Aqueous Solution at 170 °C for 2 h from Wheat, Rice, Rye, and Barley Straws, Maize Stems, Oil Palm Frond Fiber (OPFF), and Fast-Growing Poplar Wood (FGPW)

	sample/content						
phenolic acids and aldehydes	wheat straw	rice straw	rye straw	barley straw	maize stem	OPFF	FGPW
p-hydroxybenzoic acid	0.062	0.012	0.017	0.012	0.046	0.040	0.060
p-hydroxybenzaldehyde	0.061	0.081	0.013	0.031	0.19	0.011	0.080
vanillic acid	0.15	0.097	0.027	0.050	0.069	0.068	0.052
syringic acid	0.34	0.23	0.051	0.10	0.24	0.078	0.038
vanillin	0.34	0.13	0.12	0.24	0.30	0.078	0.11
syringaldehyde	0.28	0.12	0.10	0.18	0.39	0.13	0.12
acetovanillone	0.19	0.14	0.051	0.072	0.013	0.020	ND[a]
p-coumaric acid	0.66	0.86	0.36	0.36	1.08	0.075	0.10
ferulic acid	1.24	0.87	0.68	0.66	0.75	0.24	0.17
sinapic acid	ND	ND	ND	0.021	0.013	0.053	0.026
total	3.32	2.54	1.42	1.73	3.09	0.77	0.75

[a] ND, not detectable.

containing 1% finely ground lignin samples. The ^{13}C NMR spectrum was recorded on a Bruker MSI-300 spectrometer at 74.5 MHz from 200 mg of sample dissolved in 1.0 mL of DMSO-d_6 after 20000 scans. A 70° pulse flipping angle, a 10 pulse width, and a 15 s delay time between scans were used.

RESULTS AND DISCUSSION

Total Content of Hydroxycinnamic Acids. The total yield (based on percentage of dry sample, w/w) of ester- and ether-linked phenolic acids and aldehydes released by alkali hydrolysis at high temperature (4 M NaOH, 170 °C, 2 h) from dewaxed wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood is given in Table 1. Higher amounts of phenolic acids and aldehydes were released from wheat straw (3.32%), maize stems (3.09%), and rice straw (2.54%). Treatment of barley and rye straws yielded 1.73 and 1.42% phenolic acids and aldehydes, respectively. The alkaline hydrolysis of oil palm frond fiber and fast-growing poplar wood under the conditions used released much lower amounts of combined phenolic acids and aldehydes, 0.77 and 0.75%, respectively. Ferulic and *p*-coumaric acids were the dominant hydroxycinnamic acids, which together comprised 59.2, 63.8, 68.1, 59.0, 73.3, 40.9, and 36.0% of the total phenolic acids and aldehydes released from maize stems, wheat, rice, barley, and rye straws, oil palm frond fiber, and fast-growing poplar wood, respectively. This indicated that higher proportions of covalently bound ferulic and *p*-coumaric acids are located mainly in Gramineae cell walls such as cereal straws and stems. Similar results have been reported by Iiyama et al. (*12*), Lozovaya et al. (*15*), and Salomonsson et al. (*17*). These authors stated that the walls of graminaceous monocots typically contain larger amounts of hydroxycinnamic acids than dicotyledons, and *p*-coumaric acid tends to be the main hydroxycinnamic acid in the stems/stalks of cereals, whereas ferulic acid is enriched in the cereal brans. In addition, as seen from Table 1, the content of *p*-coumaric acid in rice (0.86%) and wheat straws (0.66%) is significantly higher than that in rye (0.36%) and barley straws (0.36%) but considerably lower than in maize stems (1.08%). The level of *p*-coumaric acid in cell walls of grass may reflect a classification of tropical (e.g., maize and sorghum), subtropical (rice), and temperate (wheat, rye, and barley) plants. Occurrence of substantial amounts of ferulic acids in the cell walls of straw (0.66−1.24%) or stems (0.75%) implied that the esterified and etherified ferulic acid may form covalent association between lignin and polysaccharides, reinforcing the mechanical strength of stalk or stems (*18*).

In addition to ferulic and *p*-coumaric acids, considerable amounts of vanillin (0.078−0.34%) and syringaldehyde (0.10−0.39%) and noticeable quantities of *p*-hydroxybenzaldehyde (0.011−0.19%), but much less than those of ferulic and *p*-coumaric acids, were also identified in the mixture of hydrolysates (Table 1). This indicates that high-temperature alkaline hydrolysis under oxidative conditions (4 M NaOH, 170 °C, 2 h) also cleaves 4-*O*-β' and 4-*O*-α' ether-linked structures in lignin because vanillin and syringaldehyde would be formed from terminal uncondensed lignin monomer units through quinonemethide intermediates during the alkali hydrolysis. These results imply that vanillin and syringaldehyde are etherified probably to α- and/or β-positions of the lignin side chains, because of the great stability of any other structural units in lignin with the alkaline hydrolysis condition used (*19*). Occurrence of noticeable amounts of syringic acid (0.038−0.34%), vanillic acid (0.027−0.15%), and *p*-hydroxybenzoic acid (0.012−0.062%) suggested that these phenolic acids may be also involved in ester−ether linkages between lignin and polysaccharides and/or between lignin fragments in the cell walls of wheat, rice, barley, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood. Such observations have been already found from the cell walls of fast-growing poplar (*Populus maximowiczii* Henry) by Kim and co-workers in 1995 (*19*). They revealed that *p*-hydroxybenzoic acids are esterified mainly to lignin, not to wall polysaccharides. The ether-linked *p*-hydroxybenzoic acid was identified in the cell wall of fast-growing poplar, but the amounts were much less than ester-linked *p*-hydroxybenzoic acids. Similarly, substantial amounts of esterified *p*-hydroxybenzoic acids in the cell wall of oil palm trunk fiber were also detected in our previous study (*20*). Previous investigations also indicated that considerable amounts of vanillic and syringic acids were in both ester- and ether-linked forms, probably to lignin, but not involved in ester−ether bridges between lignin and polysaccharides. However, vanillic acid and/or syringic acid may form bridges between lignin fragments in the cell wall of fast-growing poplar (*19*). Furthermore, trace amounts of sinapic acid were also identified in the hydrolysates of barley straw, maize stems, oil palm frond fiber, and fast-growing poplar wood at 170 °C. The occurrence of a small portion of sinapic acid was previously detected in wheat, barley, oat, rye, and rice

Table 2. Content (Percent Dry Sample, w/w) of (Esterified) Ferulic and *p*-Coumaric Acids Released in 1 M NaOH Aqueous Solution at 18, 25, and 30 °C for 18 h from Wheat, Rice, Rye, and Barley Straws, Maize Stems, Oil Palm Frond Fiber (OPFF), and Fast-Growing Poplar Wood (FGPW)

	ferulic acid			*p*-coumaric acid		
sample	18 °C	25 °C	30 °C	18 °C	25 °C	30 °C
wheat straw	0.52	0.55	0.58	0.26	0.35	0.38
rice straw	0.34	0.50	0.53	0.27	0.41	0.48
rye straw	0.27	0.32	0.37	0.16	0.21	0.25
barley straw	0.35	0.36	0.38	0.18	0.20	0.24
maize stem	0.40	0.43	0.44	0.75	0.85	0.88
OPFF	0.13	0.15	0.15	0.061	0.062	0.065
FGPW	0.088	0.089	0.092	0.069	0.072	0.072

straws extracted with aqueous alkali by Salomonsson and co-workers as early as 1978 (*17*). However, no sinapic acid was identified from wheat, rice, and rye straws in the present investigation. It is presumed that sinapic acid in these straws could be converted into syringaldehyde under the alkaline hydrolysis conditions (*17*).

It should be noted that hydroxycinnamic acids, such as ferulic and *p*-coumaric acids, were found in both cis and trans forms, but the trans forms in every case predominated, and it is reasonable to suppose that, if light had been excluded during the treatments and analysis, only the trans forms would have been present. In the present study, all of the treatments and analyses were performed in the dark. The cis forms, therefore, are ignored in all of the analyses. A similar phenomenon was observed for the determination of free and bound phenolic acids of lucerne (*Medicago sativa* cv. Europe) by Newby et al. (*21*). Additionally, ester-linked di- and trihydroxy phenolic acids are not expected to survive to any significant extent during the alkaline hydrolysis conditions. Caffeic acid, for example, could not be detected after the alkaline hydrolysis conditions used in the present investigation. If such alkali-labile ester-linked acids need to be analyzed, enzymatic hydrolysis could be the best method (*17*).

Content of Ester-Linked Hydroxycinnamic Acids. The commonly used procedure for cell wall hydroxycinnamic acids ester-linked to wall polymers of the samples is extraction by dilute alkali (1 M NaOH) at room temperature, and this is the only fraction analyzed (*12*, *19*, *22*, *23*). However, in our experiments, it was found that treatment temperature played a very important role in the release of esterified hydroxycinnamic acids such as ferulic acid and *p*-coumaric acid. As shown in Table 2, increase of the treatment temperature from 18 to 25 °C, and subsequently to 30 °C, resulted in an increment of esterified *p*-coumaric acid and ferulic acid of wheat straw from 0.26 and 0.52% to 0.35 and 0.55% and then to 0.38 and 0.58%, respectively. A similar increasing trend was observed for other samples. An increment in temperature from 18 to 30 °C led to an extra release of 31.6% ester-linked *p*-coumaric acid and 10.3% esterified ferulic acid, based on the total amounts of ester-liked hydroxycinnamic acids, from wheat straw, 43.8% *p*-coumaric acid and 35.8% ferulic acid from rice straw, 36.0% *p*-coumaric acid and 27.0% ferulic acid from rye straw, 25.0% *p*-coumaric acid and 7.9% ferulic acid from barley straw, 14.8% *p*-coumaric acid and 9.1% ferulic acid from maize stems, 6.2% *p*-coumaric acid and 13.3% ferulic acid from oil palm frond fiber, and 4.2% *p*-coumaric acid and 4.3% ferulic acid from fast-growing poplar wood, respectively. Evidently, an increase in alkaline treatment temperature has a greater effect on the release of ester-linked *p*-coumaric and ferulic acids from straw and stems than from oil palm frond fiber and fast-growing poplar wood. An optimum temperature of 30 °C should therefore be used if 1 M aqueous NaOH is used to detect esterified hydroxycinnamic acids.

More significantly, our results also showed that a substantial portion of the esterified hydroxycinnamic acids was not extracted by organic solvents and remained ester-linked to the solubilized lignin fraction. To verify the content of remaining ester-linked hydroxycinnamic acids in isolated lignin fractions, all of the precipitated lignin preparations, which are relatively free of bound hemicelluloses (0.9% neutral sugar content), were hydrolyzed with 1 M NaOH at 30 °C for 18 h and the released hydroxycinnamic acids were analyzed by HPLC. The data as given in Table 3 showed that 44.0−47.9% of the total ester-linked *p*-coumaric acid and 18.2−32.6% of the total esterified ferulic acid remained ester-linked in the solubilized lignin fractions. Thus, the values of *p*-coumaric acid and ferulic acid shown in Tables 2 and 3 represent the total amounts of ester-linked *p*-coumaric and ferulic acids obtained from both the alkaline hydrolysates and the isolated lignin preparations. It should be noted that a small portion of esterified hydroxycinnamic acids is also still linked to the solubilized hemicellulosic fractions as determined by mild alkaline hydrolysis. However, the values are not significant as compared to those in the alkaline hydrolysates and isolated lignin preparations and they are, therefore, ignored in this study. The above results indicated that substantial amounts of *p*-coumaric acids are tightly esterified to lignin in the cell walls of samples and were not significantly cleaved by mild alkali such as 1 M NaOH at room temperature or even at 30 °C, whereas the majority of ferulic acids are ester-linked to hemicelluloses and easily removed during the mild alkali hydrolysis at a temperature of 30 °C.

It is well-known that ferulic acid and *p*-coumaric acid are linked to lignin and/or hemicelluloses through ester or ether bonds in monocotyledonous plants, for example, cereal straws. Obviously, only the molecules, except for dimerized ones, that are associated both with lignin by ether bonds and with hemicelluloses by ester bonds contribute to the cell wall cross-linking. On the basis of the difference in stability of the ester and ether bonds in mild alkaline hydrolysis, the ester- and ether-linked ferulic acid and *p*-coumaric acid can be separated by a sequential sample treatment. In general, mild alkaline hydrolysis serves to remove ester-linked ferulic acid and *p*-coumaric acid, whereas acid hydrolysis cleaves the ether bonds of hydroxycinnamic acids (*24*). However, it should be kept in mind that this method cannot differentiate between esterified and etherified phenolic acids because only part of the ester-linked hydroxycinnamic acids was cleaved by the mild alkaline treatment at room temperature. To correct this, a second procedure of mild alkaline hydrolysis of the precipitated lignin fractions was introduced in this study to determine the remaining esterified hydroxycinnamic acids in solubilized lignins.

As discussed previously, two different procedures have been proposed to determine the ester- and ether-linked hydroxycinnamic acids. The first (*25*) was mild alkaline hydrolysis followed by acid hydrolysis. The second procedure (*12*) was based on the difference between mild alkaline hydrolysis (1 M NaOH at room

Table 3. Content (Percent Dry Sample, w/w) of Total and Ester- and Ether-Linked *p*-Coumaric and Ferulic Acids in Wheat, Rice, Rye, and Barley Straws, Maize Stems, Oil Palm Frond Fiber (OPFF), and Fast-Growing Poplar Wood (FGPW)

	p-coumaric acid				ferulic acid			
			ether-linked				ether-linked	
sample	total[a]	ester-linked[b]	I[c]	II[d]	total[a]	ester-linked[b]	I[c]	II[d]
wheat straw	0.66	0.46[b] (0.22)[e]	0.28	0.25[d] (0.18)[f]	1.24	0.58[b] (0.11)[e]	0.66	0.62[d] (0.25)[f]
rice straw	0.86	0.48 (0.23)	0.38	0.33 (0.22)	0.87	0.53 (0.10)	0.34	0.32 (0.17)
rye straw	0.36	0.25 (0.11)	0.11	0.09 (0.05)	0.68	0.37 (0.08)	0.31	0.28 (0.14)
barley straw	0.36	0.24 (0.11)	0.12	0.09 (0.06)	0.66	0.38 (0.07)	0.28	0.26 (0.14)
maize stems	1.08	0.88 (0.39)	0.20	0.20 (0.11)	0.75	0.44 (0.08)	0.31	0.31 (0.15)
OPFF	0.075	0.065 (0.030)	0.010	0.009 (0.005)	0.24	0.15 (0.04)	0.09	0.08 (0.03)
FGPW	0.10	0.072 (0.033)	0.028	0.026 (0.016)	0.17	0.092 (0.03)	0.078	0.068 (0.02)

[a] Total contents of *p*-coumaric and ferulic acids were determined with 4 M NaOH at 170 °C for 2 h. [b] Contents of total ester-linked *p*-coumaric and ferulic acids were determined by alkaline hydrolysis of dewaxed samples and alkali-soluble lignin fractions with 1 M aqueous NaOH at 30 °C for 18 h. [c] Contents of total ether-linked *p*-coumaric and ferulic acids were calculated as the difference between total and ester-linked *p*-coumaric and ferulic acids. [d] Contents of total ether-linked *p*-coumaric and ferulic acids were determined by acid hydrolysis of the alkali-treated residues with dioxane/2 M HCl (9:1, v/v) at reflux for 1 h and acid hydrolysis of the dioxane-soluble lignin fractions with dioxane/2 M HCl (9:1, v/v) at 87 °C for 2 h. [e] Represents the content of esterified *p*-coumaric and ferulic acids in alkali-soluble lignin preparations precipitated at pH 1.5; values were determined by alkaline hydrolysis of the lignin samples with 1 M aqueous NaOH at 30 °C for 18 h. [f] Represents the content of etherified *p*-coumaric and ferulic acids in dioxane-soluble lignin preparations precipitated at pH 1.5; values were determined by acid hydrolysis of the lignin samples with dioxane/2 M HCl (9:1, v/v) at 87 °C for 2 h.

temperature), which released only the esterified phenolic acids, and high-temperature alkaline treatment (4 M NaOH at 170 °C), which cleaved both the ester- and ether-linked hydroxycinnamic acids. The etherified phenolic acids, therefore, were the difference in amounts of hydroxycinnamic acids recovered from these two alkaline treatments. However, a very low value of etherified ferulic acid and *p*-coumaric acid was obtained by acid hydrolysis, and it was speculated that substantial amounts of those substances might be lost by discarding the aqueous layers (*24*). In fact, substantial amounts of etherified ferulic acid and *p*-coumaric acid were still bound to the isolated lignin fraction after acid hydrolysis of the alkali-treated residues. In the present study, the precipitated lignin preparations, which contained minor quantities of associated polysaccharides (1.1% neutral sugars), underwent a second procedure of acid hydrolysis with dioxane/2 M HCl (9:1, v/v) at 87 °C for 2 h, and the results are listed in Table 3. Clearly, the lignin fragments contained 55.0−72.0% of the total ether-linked *p*-coumaric acid and 37.5−53.8% of the total ether-linked ferulic acid, indicating again that the acid hydrolysis of the alkali-treated residues with dioxane/2 M HCl (9:1, v/v) under reflux for 1 h only partially cleaved the etherified hydroxycinnamic acids, and the majority of *p*-coumaric acid and substantial amounts of ferulic acid remained ether-linked to the precipitated lignins.

On the basis of these results it can be concluded that acid hydrolysis under reflux released only a portion of hydroxycinnamic acids that are etherified to cell wall components as a result of cleavage of the ether bond. This treatment also solubilized a portion of lignins that contained substantial amounts of etherified hydroxycinnamic acids linked to lignin molecules. To correct this, the values given in Table 3 for the ether-linked ferulic acid and *p*-coumaric acid include both those detected from the acid hydrolysates and those found in the precipitated lignin fractions even though they are still slightly lower than those obtained by the second procedure. This newly developed method also explained why only 40% of the ferulates incorporated into lignin was recovered following acid hydrolysis of ether linkages, that is, why only a portion of ferulates in lignified tissues was measurable by current solvolytic methods in previous studies (*22*, *25*). Similar observations have been reported by Iiyama and co-workers (*12*) in the study of phenolic acid bridges between polysaccharides and lignin in wheat internodes with acid hydrolysis. These authors stated that the unpurified dioxane/water extract, which contains large amounts of lignin−carbohydrate complex, is rich in etherified ferulic acid. In this study, however, we found that the majority of the ether-linked ferulic acid and *p*-coumaric acid remained bound to the solubilized lignin fraction after acid hydrolysis of the alkali-treated residues, whereas the solubilized hemicellulosic fractions contained only a small portion of the etherified hydroxycinnamic acids. This indicated that large amounts of ether-linked ferulic acid and *p*-coumaric acid are associated with lignin molecules rather than with hemicellulosic polymers in the cell walls of the materials investigated.

On the basis of the data in Table 3, we also considered that a predominant amount of *p*-coumaric acid is ester-linked to the cell wall components (69.7% in wheat straw, 55.8% in rice straw, 69.4% in rye straw, 66.7% in barley straw, 81.5% in maize stems, 86.7% in oil palm frond fiber, and 72.0% in fast-growing poplar wood), mainly to lignin. About half of the ferulic acid is esterified to the cell wall hemicelluloses and/or lignin (46.8% in wheat straw, 60.9% in rice straw, 54.1% in rye straw, 57.6% in barley straw, 58.7% in maize stems, 62.5% in oil palm frond fiber, and 54.1% in fast-growing poplar wood), and another half of the ferulic acid can also be etherified through the phenolic oxygen to the cell wall lignin component. This finding was consistent with the investigation of cinnamic acid bridges between cell wall polymers in wheat and *Phalaris* internodes for Gramineae by Lam et al. (*26*), which revealed that all of the etherified ferulic acid in the dioxane/water-soluble fractions is also ester-linked. Also, the current results are in good agreement with the hypothesis that the hydroxycinnamic bonding between components of the cell walls shifts from a predominantly ferulic acid ester linkage during the development of the cell wall to a predominantly ether linkage as lignification begins and to a predominantly *p*-coumaric acid ester linkage late in the lignification process (*4*).

FT-IR Spectra. Figure 2 illustrates the FT-IR spectra of two lignin fractions extracted with 1 M aqueous NaOH at 30 °C for 18 h from maize stems (spectrum A) and rye straw (spectrum B). A broad band at 1164 cm^{-1}

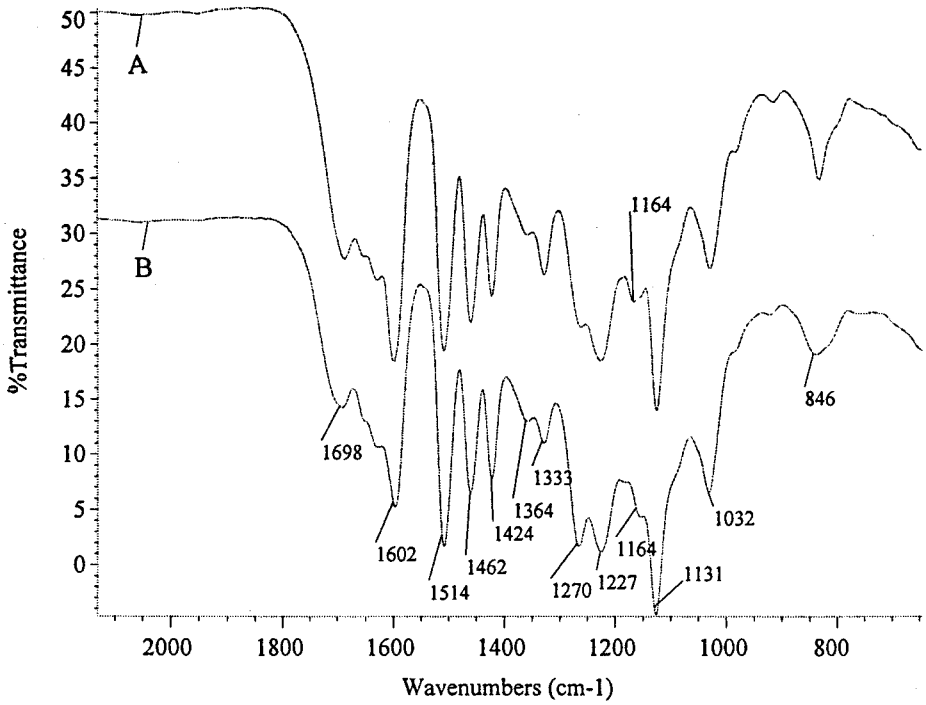

Figure 2. FT-IR spectra of alkali-soluble lignin fractions extracted with 1 M aqueous NaOH at 30 °C for 18 h from maize stems (A) and rye straw (B).

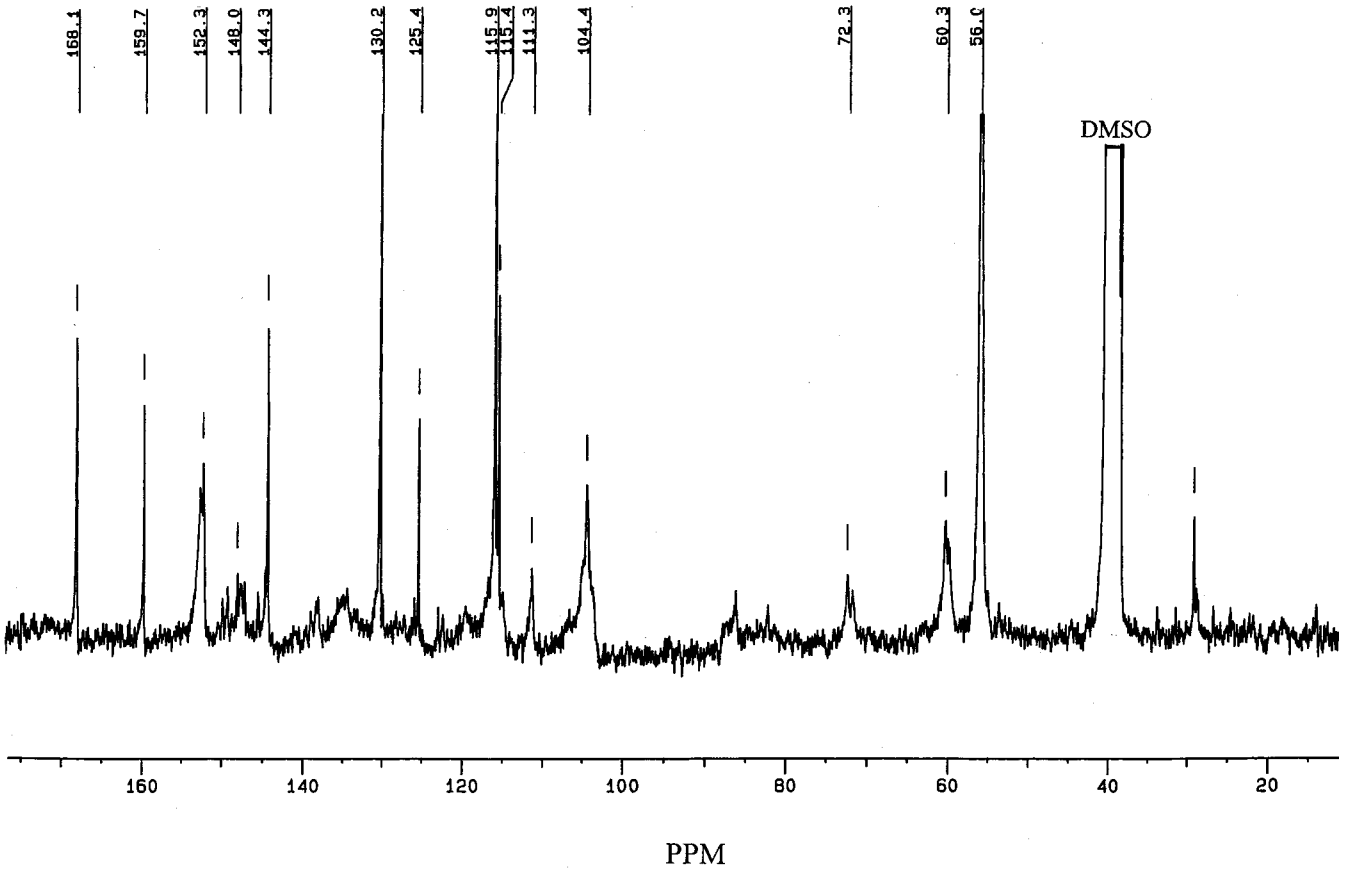

Figure 3. ^{13}C NMR spectrum of the alkali-soluble lignin preparation solubilized during 1 M NaOH treatment at 30 °C for 18 h from dewaxed maize stems.

in spectrum A and a shoulder in spectrum B are characteristic of the carbonyl in ester groups, indicating residual ester-linked hydroxycinnamic acids, such as esterified *p*-coumaric acid or ferulic acid in lignin preparations. The intensity corresponds to the values of ester-linked *p*-coumaric acid in the alkali-soluble lignin fractions from maize stems and rye straw (Table 3). The band at 1698 cm^{-1} is attributed to the conjugated carbonyl stretching in lignin.

^{13}C NMR Spectrum. To verify the residual ester-linked hydroxycinnamic acids in mild alkali-soluble lignin fractions, the lignin preparation solubilized dur-

ing 1 M NaOH treatment at 30 °C for 18 h from dewaxed maize stems was investigated by ^{13}C NMR spectroscopy (Figure 3). The most striking characteristic of the spectrum is the occurrence of strong signals for significant amounts of hydroxycinnamic acids, particularly *p*-coumarate ester. As shown in the spectrum, the strong signals at 168.1 (C-γ in esterified *p*-coumaric acid), 159.7 (C-4 in esterified *p*-coumaric acid), 130.2 (C-2/C-6 in esterified *p*-coumaric acid), 125.4 (C-1 in esterified *p*-coumaric acid), and 115.9 and 115.4 ppm (C-3/C-5 in esterified *p*-coumaric acid) are attributed to the esterified *p*-coumaric acid. Etherified ferulic acid gives signals at 144.3 (C-α in etherified ferulic acid) and 122.3 ppm (C-6 in etherified ferulic acid), whereas the esterified ferulic acid exhibits a signal at 122.5 ppm (C-6 in esterified ferulic acid). These observations strongly supported the chemical analysis that *p*-coumaric acid is linked to lignin mainly by ester bonds, whereas ferulic acid is linked to lignin by both ether and ester bonds. These chemical shifts observed in the alkali-soluble lignin fraction are consistent with those of *p*-coumarate esters in which the phenolic hydroxyl is unetherified. That is, the relative sharpness of the peaks also implied that the *p*-coumarate unit has not been incorporated into the lignin structure and remains as a pendant, terminal group on the polymer (*13*). On the basis of the extensive studies on the pathway of *p*-coumaric acid incorporation into maize lignin by NMR, they also unambiguously revealed that *p*-coumaric acid is attached exclusively at the γ-position of lignin side chains by an ester bond and not at the α-position.

In summary, the wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood contained 1.24, 0.87, 0.68, 0.66, 0.75, 0.24, and 0.17% ferulic acid and 0.66, 0.86, 0.36, 0.36, 1.08, 0.075, and 0.10% *p*-coumaric acid, respectively. A dominant amount of the *p*-coumaric acid is ester-linked to the cell wall components, mainly to lignin. About half of the ferulic acid is esterified to the cell wall hemicelluloses and/or lignin, and another half of the ferulic acid is etherified through their phenolic oxygen to the cell wall lignin component. This newly developed analytical procedure can be applied to determine both ester- and ether-linked hydroxycinnamic acids in lignified walls of other species and to follow changes in these associations during plant growth and after chemical or biological treatments.

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