Separation and Quantification of Lignin-Derived Phenolic Monomers Using High-Resolution Gas Chromatography

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Underivatized phenolic monomers commonly isolated from plant cell walls were separated and quantified on a 5% phenylmethyl-fused silica capillary column. Analysis of alkali-labile phenolics extracted from orchardgrass cell walls, which included vanillin and the cis and trans isomers of *p*-coumaric and ferulic acids, required less than 15 min to complete. Phenolic monomers were also separated as their trimethylsilyl derivatives. This system, utilizing high-resolution chromatography, allows rapid separation of phenolic monomers at a relatively lower cost with equal or improved sensitivity than other methods currently available.

Lignin-derived phenolic monomers commonly isolated from plant cell walls include derivatives of cinammic and benzoic acids, benzaldehyde, and acetophenone. Quantitative evaluation of the phenolic constituents of lignin is important as lignin–carbohydrate interactions limit the availability of cell wall polysaccharides to rumen microbial degradation (Van Soest, 1981). A negative correlation has been shown between in vitro digestibility and the concentration of *p*-coumaric and ferulic acids in grass cell walls (Hartley, 1972; Burritt et al., 1984). There is substantial evidence supporting the existence of chemical bonds between lignin and cell wall carbohydrates. Phenolic acids are known to form ester linkages with cell wall carbohydrates (Hartley, 1973; Hartley and Jones, 1977; Tanner and Morrison, 1983), and it has been suggested that they may serve as cross-links between lignin and cell wall polysaccharides (Morrison, 1974). Hartley and Keene (1984) have recently reported the presence of benzaldehyde derivatives covalently linked to polysaccharides of grass cell walls. Examination of lignin–carbohydrate complexes isolated from grass cell walls (Morrison, 1973; Tanner and Morrison, 1983) and from the rumen of grass-fed steers (Neilson and Richards, 1982) indicates the presence of ester and glycosidic linkages between lignin and cell wall carbohydrates.

Proper separation of phenolic monomers released from cell wall preparations is essential for their qualitative and quantitative analysis. Separation of various phenolic monomers has been accomplished by gas–liquid chromatography (GLC) with packed columns. Most of the proposed techniques utilizing GLC require derivatization of phenolic monomers to their trimethylsilyl ethers (Pellizzari et al., 1969; Cymbaluk and Neudoerffer, 1970), methyl ethers (Bicho et al., 1966), or ethyl ethers (Salomonsson et al., 1978). Several authors, however, have attempted separation of phenolic monomers on packed columns using GLC without prior derivatization (Hartley, 1971; Chaves et al., 1982). Generally, separation of phenolic monomers using GLC with packed columns can be limited by poor resolution of some peaks, a low signal to noise ratio resulting in decreased detector performance and sensitivity, and long analysis times (30–50 min). Hartley and Buchan (1979) proposed a technique utilizing high-performance liquid chromatography (HPLC) to separate phenolic acids and aldehydes. The use of HPLC eliminates the need for derivatization and is more sensitive than previous GLC techniques; however, it also is limited by long analysis times and by relatively higher cost.

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This paper presents a technique, utilizing high-resolution gas chromatography, developed for the rapid separation and quantitative analysis of underivatized phenolic monomers released by the alkaline and oxidative treatment of plant cell walls.

MATERIALS AND METHODS

Preparation of Plant Material. Orchardgrass (*Dactylis glomerata* L.) was hand-harvested at 4 weeks after boot stage (6 June) from plots established in southern Illinois. Plant material was dried in a forced-air oven at 60 °C and ground to pass a 1-mm screen. Neutral detergent fiber (NDF) was prepared from this material by the methods of Van Soest and Wine (1967); however, sodium sulfite was excluded (Hartley, 1972), and the resulting NDF was dried at 65 °C.

Alkaline Treatment of NDF. Approximately 500 mg of NDF was treated with 1 N NaOH (20 mL) under nitrogen at room temperature for 20 h. The mixture was filtered on a sintered glass crucible and the residue washed twice with 10 mL of water. *p*-Chlorobenzaldehyde (1 mg) was added to the combined filtrates as an internal standard. Alkali-labile phenolic monomers were extracted from the filtrate by methods described by Hartley (1971). Filtrate was acidified to pH 2.5 with 6 N HCl, saturated with NaCl (7.5 g), and the resulting solution extracted three times with 50 mL of ether. The combined ethereal extracts were dried over Na_2SO_4, and the solvent was evaporated. Residues isolated by alkaline treatment were dissolved in 2 mL of acetone and transferred to 2-mL vials, and 1-μL injections were analyzed directly (underivatized) by gas chromatography.

Gas Chromatography (GC) and Mass Spectroscopy (MS). Phenolic monomers were separated on a 5% phenylmethyl-fused silica capillary column (0.33-μm stationary phase thickness, 25 m × 0.2 mm (i.d.)) on a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless capillary inlet system and a flame ionization detector. Column oven temperature, for separation of underivatized phenolic monomers, was programmed to increase from an initial temperature of 180 °C to a final temperature of 230 °C with use of two temperature programs. Oven temperature was maintained at the initial temperature for 4 min after injection and then increased at 5 °C/min to an intermediate temperature of 200 °C. Oven temperature was maintained at the intermediate temperature for 2 min and then increased at 5 °C/min to the final temperature. Separations of underivatized phenolic monomers were also made isothermally (data not shown) at an oven temperature of 180 °C. Elution order for all phenolic compounds separated isothermally was identical with that done with the temperature programs;

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however, separation time was greatly increased. For separation of derivatized phenolic monomers (trimethylsilyl ether), column oven temperature was maintained at 195 °C for 6.5 min after injection and then increased at 6.0 °C/min to a final temperature of 250 °C. Other operating parameters were as follows: injector temperature, 220 °C; detector temperature, 240 °C; flow rate of nitrogen used as a carrier gas, 0.33 mL/min; split ratio, $^1/_{100}$. A Hewlett-Packard 3290A integrator was used to determine peak areas.

Mass spectra of phenolic monomers were obtained by a Hewlett-Packard 5985 GC/MS system operating in electron impact mode. Spectra were recorded at 70 eV with the source at 200 °C. Phenolics were separated on an SPB-7 (Supelco, Inc.) capillary column (0.25-μm stationary phase thickness, 15 m × 0.32 mm (i.d.)). Operating parameters for GC separation were similar to those previously described; however, initial column oven temperature was 150 °C and was increased at 5 °C/min to a final temperature of 250 °C.

Quantitative Analysis of Phenolic Monomers. Phenolic compounds used as reference standards included *trans*-ferulic acid, *trans*-*p*-coumaric acid, *trans*-sinapic acid, vanillin, *p*-hydroxybenzaldehyde, syringaldehyde, vanillic acid, *p*-hydroxybenzoic acid, syringic acid, acetovanillone *p*-hydroxyacetophenone, and acetosyringone. All phenolic standards were obtained commercially (Aldrich Chemical Co., Milwaukee, WI). Standards of *cis*-ferulic and *cis*-*p*-coumaric acid were commercially unavailable and were produced by exposing solutions of the trans isomers to ultraviolet radiation. Solutions of the phenolic standards, prepared in acetone, were analyzed directly (underivatized) by GC and combined GC–MS as previously described. Plots of weight vs. peak area were found to be linear for each standard in the range of 0.05–4.0 μg. Response factors were calculated from a calibration mixture by dividing the known weight of a phenolic standard by its corresponding peak area. Phenolic monomers in unknown plant extracts were identified by comparison of their retention times and mass spectra to those of known standards and quantified from peak areas from the calculated response factors. The cis isomers of *p*-coumaric acid and ferulic acid were quantified from the response factors generated for the trans isomers.

Derivatization of Phenolic Monomers. Derivatized phenolic standards were also analyzed by GC and combined GC–MS. Solvent was evaporated from the sample vials under a stream of nitrogen gas. Vials were placed in a desiccator, and residues were dried, in vacuo, for 3 h. Vials were capped after addition of 0.5 mL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heated at 100 °C for 15 min with periodic shaking to dissolve residues.

Deactivation of Split Inlet. Use of a packed split-inlet insert resulted in decomposition of underivatized ferulic, sinapic, and *p*-coumaric acids. Decomposition of ferulic and *p*-coumaric acids was eliminated by using an unpacked split inlet that had been deactivated by thorough silane treatment. After removal of all packing material, the insert was soaked for 12 h in 10 N hydrochloric acid and sequentially rinsed with distilled water, methanol, and acetone. The insert was then immersed for 20 min in a 30% solution of dimethyldichlorosilane in toluene, sequentially rinsed with toluene and methanol, and allowed to air-dry.

RESULTS AND DISCUSSION

Separation of Underivatized Standard. A typical chromatogram showing the separation of underivatized phenolic standards (0.5 μg of each component) is shown in Figure 1. Retention time for each peak is given in Table I. With the exception of *trans*-sinapic acid, all phenolic monomers in the standard mixture were eluted in less than 15 min. As a result of decomposition and formation of a second compound, underivatized sinapic acid was eluted as two peaks (peak numbers 8 and 14). It was determined that underivatized *p*-coumaric acid (PCA) and ferulic acid (FA) were also sensitive to the decomposition exhibited by sinapic acid; however, their decomposition was eliminated, as previously discussed, by simple modifications made to the split-inlet insert. Separations made without a modified inlet gave two additional peaks (not shown) with retention times of 3.84 and 4.70 min corresponding to the decomposition products of PCA and FA, respectively. Examination of mass spectra of both intact monomers and their respective decomposition products (Table II) indicated that the decomposition product was formed through loss of the carboxyl group from the cinnamic acid derivatives. Intact PCA and FA did not produce a molecular ion. The base peak for spectra of the intact monomers, as well as those of the decomposition products, was M – 44 (indicating loss of CO_2). However, an ion at *m/e* 44 is associated exclusively with the intact monomers, providing evidence that they remained intact during gas

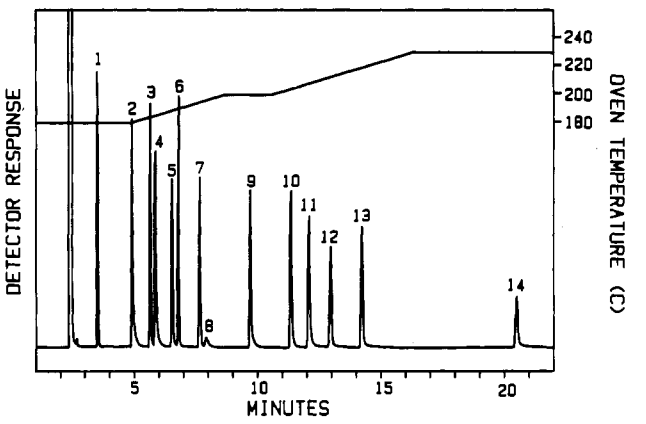

Figure 1. Chromatogram of underivatized phenolic monomer standards separated by high-performance gas chromatography on a 5% phenylmethyl-fused silica capillary column. Refer to Table I for peak number identification.

Table I. Retention Times of Underivatized and Derivatized (Trimethylsilyl Ether) Phenolic Monomer Standards[a]

	underivatized[b]		derivatized[c]	
phenolic compd	ret time, min	peak no.	ret time, min	peak no.
p-chlorobenzaldehyde	3.52	1	3.19	1
p-hydroxybenzaldehyde	4.92	2	4.20	2
vanillin	5.65	3	5.63	4
p-hydroxyacetophenone	5.86	4	4.93	3
p-hydroxybenzoic acid	6.56	5	6.63	5
acetovanillone	6.80	6	6.74	6
vanillic acid	7.68	7	8.97	8
sinapic acid[d]	7.90	8		
syringaldehyde	9.72	9	8.10	7
acetosyringone	11.36	10	9.31	9
trans-*p*-coumaric acid	12.10	11	12.34	11
syringic acid	12.99	12	11.53	10
trans-ferulic acid	14.25	13	15.20	12
trans-sinapic acid	20.52	14	18.41	13

[a] Phenolic monomers were separated on a 5% phenylmethyl-fused silica column. Chromatographic conditions are given in text. [b] Refers to Figure 1. [c] Refers to Figure 2. [d] Peak corresponding to the decomposition product produced from sinapic acid. Decomposition product was not formed from derivatized sinapic acid.

Table II. Mass Spectra of Underivatized *trans*-*p*-Coumaric Acid, *trans*-Ferulic Acid, and Their Respective Decomposition Products[a]

				trans-*p*-Coumaric Acid						
intact	*m/e*	120	105	91	75	65	55	44		
	RI, %	100	4	49	5	19	7	28		
dec	*m/e*	120	105	91	77	65	51	39		
	RI, %	100	3	46	3	15	6	12		
				trans-Ferulic Acid						
intact	*m/e*	150	135	120	107	91	77	63	44	
	RI, %	100	83	13	34	9	39	8	35	
dec	*m/e*	150	135	120	107	89	77	63	51	39
	RI, %	100	83	2	35	5	40	9	14	8

[a] Combined GC–MS conditions are given in text. Values represent mass to charge ratios (*m/e*) and relative intensities (RI) of the major ionic fragments. Molecular weights for *p*-coumaric and ferulic acid monomers are 164.17 and 194.19, respectively.

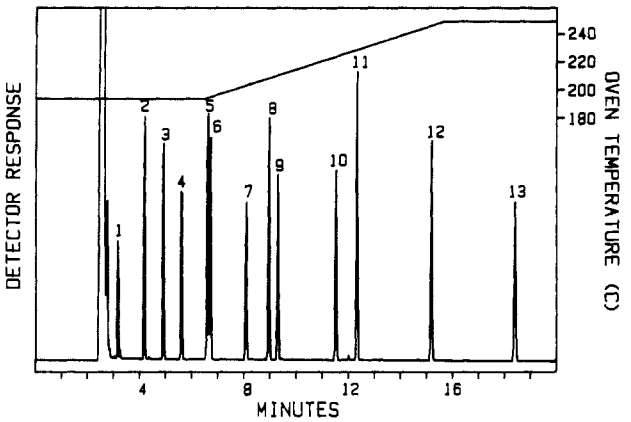

Figure 2. Chromatogram of derivatized (trimethylsilyl ether) phenolic monomer standards separated on a 5% phenylmethyl-fused silica capillary column. Refer to Table I for peak number identification.

chromatographic separation and were fragmented upon impact with the source (i.e, they arrived at the source as intact monomers). The lack of an *m/e* 44 ion in the mass spectra of the decomposition products indicates that they were produced by decarboxylation during gas chromatographic separation, most likely during volatilization in the unmodified inlet.

Separation of Derivatized Standard. A chromatogram showing the separation of trimethylsilyl (Me_3Si) derivatives of the phenolic standards (0.5 μg of each component) is given in Figure 2. The Me_3Si derivatives were eluted in a slightly different order than that of the underivatized monomers (Table I). Elution of all derivatized monomers, again with the exception of sinapic acid, required slightly more than 15 min. Base-line separation was not achieved between *p*-hydroxybenzoic acid and acetovanillone; however, this did not affect the integration of their peak areas. No decomposition of the derivatized cinnamic acid derivatives was detected. This result was expected as the Me_3Si derivatives are more thermally stable than their underivatized counterparts. Also, the minor tailing exhibited by some of the underivatized peaks was eliminated during separation of the more volatile Me_3Si derivatives.

Separation of Alkali-Labile Phenolics. Alkali-labile phenolic monomers extracted, separated, and quantified from orchardgrass cell walls included vanillin, *cis*-*p*-coumaric acid, *cis*-ferulic acid, *trans*-*p*-coumaric acid, and *trans*-ferulic acid (Figure 3; Table III). Trace amounts of syringaldehyde, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, and vanillic acid were also detected by the GC–MS system. The presence of the phenolic aldehydes in the alkaline extracted cell walls is in agreement with the work of Hartley and Keene (1984). Peaks 3 and 8 (Figure 3) were identified as long-chain hydrocarbon compounds derived from the ether used in the isolation procedure.

Table III. Retention Times of Alkali-Labile Phenolic Monomers Extracted from Orchardgrass Cell Walls[a]

phenolic compd	ret time, min	peak[b] no.
p-chlorobenzaldehyde	3.52	1
vanillin	5.65	2
hydrocarbon[c]	7.06	3
cis-*p*-coumaric acid	10.35	4
cis-ferulic acid	11.81	5
trans-*p*-coumaric acid	12.10	6
trans-ferulic acid	14.25	7

[a] Extraction procedures and chromatographic conditions are given in text. [b] Refers to Figure 3. [c] Peaks correspond to unidentified long-chain hydrocarbons.

Table IV. Concentration of Alkali-Labile Phenolic Monomers Extracted from Orchardgrass Cell Walls[a]

phenolic compd	concn,[b] g kg^{-1} cell wall	std error	
		extraction	injection
vanillin	0.15	0.004	0.006
cis-*p*-coumaric acid	0.75	0.029	0.019
cis-ferulic acid	0.48	0.034	0.007
trans-*p*-coumaric acid	6.63	0.240	0.047
trans-ferulic acid	14.15	0.405	0.097

[a] Procedures for extraction and quantification given in text. [b] Each value represents the mean of two separate injections for each of five samples.

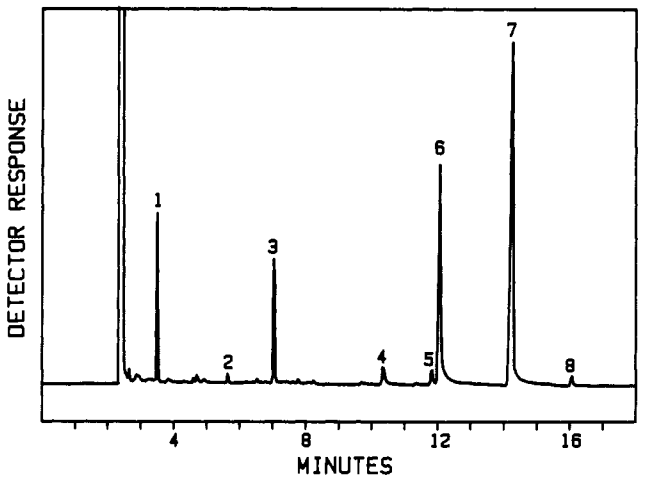

Figure 3. Chromatogram of underivatized alkali-labile phenolic monomers extracted from orchardgrass cell walls. Refer to Table III for peak number identification.

Concentrations of the quantified alkali-labile monomers are given in Table IV. The cis isomers of PCA and FA represent only minor components of the total alkali-labile phenolics when compared to the trans isomers. The sys-

tem offered excellent reproducibility as indicated by the low standard errors of injection for each of the monomers quantified.

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Registry No. Vanillin, 121-33-5; *cis*-*p*-coumaric acid, 4501-31-9; *trans*-*p*-coumaric acid, 501-98-4; *cis*-ferulic acid, 1014-83-1; *trans*-ferulic acid, 537-98-4; *trans*-sinapic acid, 7362-37-0; *p*-hydroxybenzaldehyde, 123-08-0; syringaldehyde, 134-96-3; vanillic acid, 121-34-6; *p*-hydroxybenzoic acid, 99-96-7; syringic acid, 530-57-4; acetovanillone, 498-02-2; *p*-hydroxyacetophenone, 99-93-4; acetosyringone, 2478-38-8; *p*-chlorobenzaldehyde, 104-88-1; sinapic acid, 530-59-6.

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Scanning Electron Microscopy of Mixed Hardwoods Subjected to Various Pretreatment Processes

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Mixed southern hardwood chips were subjected to rapid steaming, steam explosion, autohydrolysis, and wet oxidation pretreatments. Chips from each of these pretreatments were examined by scanning electron microscopy to help determine what had occurred in the components and morphology of these chips. Steam explosion was the most effective pretreatment for altering the morphology of the wood by causing separation of fibers at the middle lamella. Previous work has shown that all of the pretreatments altered the chemical components of wood, though in different ways, which was reflected in the electron micrographs of this work. Lignin tended to form beads on the surface of some of the fibers. Autohydrolysis and wet oxidation caused splitting of the cell walls parallel to the orientation of the microfibrils of the S-2 cell wall layer, while rapid steaming caused some splitting of the cell walls perpendicular to the long axis of the fibers.

Scanning electron microscopy is a valuable tool when studying changes in morphology of wood treated by processes such as pressure and heat refining (Short and Lyon, 1978; Koran, 1970; Murmanis et al., 1986). Considerable information may be obtained by scanning electron microscopy regarding the separation of fibers, the condition of the surface of the fibers, and separation of cell wall layers. It is now widely accepted that enzymatic hydrolysis of lignocellulosic materials depends on reducing the crystallinity of cellulose and removing the lignin sheath surrounding the cellulose fibers (Gencer and Mutharasan, 1979; Ryu et al., 1982; Saddler et al., 1982). Marchessault et al. (1980) showed that there was no detected loss of crystallinity as a result of steam explosion of aspen chips, while Tanahashi et al. (1983) even observed an increase in crystallinity after steam explosion of some species of wood; consequently, in pretreatment processes separation of the lignin from the cellulose is responsible for the increased rate of cellulase hydrolysis.

This study was undertaken to investigate the changes in morphology of wood induced by the pretreatments rapid steam hydrolysis, steam explosion, autohydrolysis, and wet oxidation. Although wood was the substrate for this study, the results should be useful for other substrates for biomass conversion such as sugar cane bagasse and corn stalks, which have compositions roughly the same as wood (Schultz et al., 1984).

EXPERIMENTAL SECTION

Mixed southern hardwood chips, predominantly oak and gum species, were pretreated according to previous methods: rapid steam hydrolysis (Biermann et al., 1984), steam explosion (Schultz et al., 1983), wet oxidation (McGinnis et al., 1983), and autohydrolysis (Biermann, 1983). In the case of rapid steam hydrolysis, wood chips

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