HPLC/Electrochemical Detection of Lignin Phenolics from Wheat Straw by Direct Injection of Nitrobenzene Hydrolysates

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Phenolics obtained by alkaline nitrobenzene hydrolysis of wheat straw were quantitatively determined by HPLC with electrochemical and UV detectors. Hydrolysates were injected both by direct means and after classical analytical procedure involving solvent partitioning. Results showed that solvent partitioning with CH_2Cl_2 was biased by losses of phenolics ranging from 10% for vanillin and syringaldehyde to 75% for *p*-coumaric acid. Yields improved by saturating the water phase with sodium chloride yet remained nonquantitative. The electrochemical detector was not affected by nitrobenzene interference and allowed the direct injection of nitrobenzene hydrolysates into HPLC, resulting in a simplified analytical procedure with quantitative results and improved detection limits.

Economical utilization of lignocellulosic wastes includes as a major effort the exploitation of cereal straw surpluses for conversion into inexpensive livestock feedstuffs (Sundstol and Owen, 1984). However, a major problem has been the high lignin content of the straws, since lignin and its constituent phenolics greatly reduce the ruminant digestibility of straw by removing structural carbohydrates as rumen enzyme substrates and/or inhibiting the enzymes themselves (Jung and Fahey, 1983).

A rapid and reproducible method for the quantitative determination of lignin constituent phenolics is highly desirable in the evaluation of lignocellulosic materials for both animal feed and industrial use (Reeves, 1985; Papadopoulos and Defaye, 1986). Nitrobenzene oxidative hydrolysis is one of the principal methods for the study of lignin composition (Chang and Allan, 1971).

Oxidative hydrolysis of lignin macromolecules in heterogeneous basic media containing nitrobenzene results in simple phenolic acids and aldehydes, consisting mainly of hydroxycinnamic and hydroxybenzoic moieties.

Although high-performance liquid chromatography (HPLC) (Hartley and Buchan, 1979) and gas chromatography (Reeves, 1986; Fritz and Moore, 1987) have been successfully applied to the separation and quantification of these compounds, the quantitative results of the workup of the nitrobenzene analytical procedure have been questioned (Reeves, 1986). The classical procedure involves elimination of excess nitrobenzene and its reduction products from the reaction mixture by means of solvent extraction, followed by acidification of the aqueous solution and extraction of the phenolic compounds. Incomplete removal of nitrobenzene and its byproducts can seriously interfere in the GC or HPLC elution profile, whereas the phenolic compounds can be underestimated due to losses during the extraction into the organic phase.

The use of electrochemical (ElCh) detection in the HPLC analysis of straw lignin hydrolysates eliminates the need for cumbersome and nonreproducible solvent extraction (Chiavari et al., 1988), because of selectivity toward electrochemically active compounds, such as phenolics (Chiavari et al., 1987). The scope of this work was to evaluate electrochemical detection for direct quantitative analysis of lignin constituent phenolics from wheat straw, prior to adoption for routine analyses. Both UV and electrochemical detection with HPLC will be used to compare direct analysis of lignin oxidative hydrolysates with analysis following extraction of actual samples and synthetic mixtures.

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MATERIALS AND METHODS

Sample Preparation. A sample of wheat straw, ground to pass a 2-mm screen, was carefully homogenized and divided in six subsamples prior to alkaline nitrobenzene oxidation.

Alkaline Nitrobenzene Oxidation. A 100-mg portion of straw was added with 5 mL of 2 N NaOH and 100 μL of nitrobenzene. The mixture was heated in a thick-glass tube with a Schott screw cap at 160 °C for 2 h in an oil bath under magnetic stirring. After cooling, the mixture was diluted to 25 mL with water and filtered.

Direct Analysis. A 2-mL sample of the filtrate was acidified with 2 mL of 1 N HCl, diluted to 25 mL with water, filtered through a cartridge filter (Millipore), and injected into the HPLC.

Classical Analysis. A 2-mL sample of the filtrate was extracted in a tube with CH_2Cl_2 (3 × 2 mL), discarding the organic phase. The aqueous residue was acidified with 1 mL of 1 N HCl and reextracted with CH_2Cl_2 (4 × 2 mL). The combined organic phases were dried over anhydrous Na_2SO_4, and CH_2Cl_2 was evaporated in rotary evaporator under vacuum. The residue was dissolved in 5 mL of MeOH, diluted to 25 mL with water, filtered through a 0.45-μm cartridge filter (Millipore), and injected into the HPLC.

Recovery Tests. Six phenolic standard solutions at different concentrations (1, 2×10^{-1}; 2, 4×10^{-2}; 3, 2×10^{-2}; 4, 4×10^{-3}; 5, 2×10^{-3}; 6, 4×10^{-4} g/L) were subjected to the procedure above described for the straw without heating.

Solvent extractions were performed both by the procedure described and with use of NaCl to saturate the acidified aqueous phase. The waste aqueous and organic phases were saved and analyzed for possible residual phenolics.

Apparatus. The liquid chromatograph system (Waters Associates, Milford, MA) consisted of an M45 pump, a Rheodyne 7010 sample injector (20-μL loop), and a 440 UV detector. A Metrohm (Herisau, Switzerland) equipped with a three-electrode detection cell (Model EA 1096/2) complete with a VA 641 potentiostat was used for the ElCh detection. The glassy carbon working electrode was polished daily with alumina powder (0.3 μm). UV and ElCh detectors were connected in series. UV absorbances were measured at 280 nm and displayed on a Leeds and Northrup Speedomax recorder. ElCh detector output was displayed on a Perkin-Elmer Model 561 recorder. A C_6 reversed-phase column (120 × 4.6 mm; Viospher C6, 5 μm; Violet, Rome, Italy) was employed for all separations. The mobile phase was methanol/0.1% perchloric acid in water (15/85, v/v). The flow rate was 1.00 mL/min, and all experiments were performed at ambient temperature. All solutions were filtered through 0.45-μm cartridge filters (Millipore) prior to HPLC analysis. A calibration curve with standard compounds was used for quantitation.

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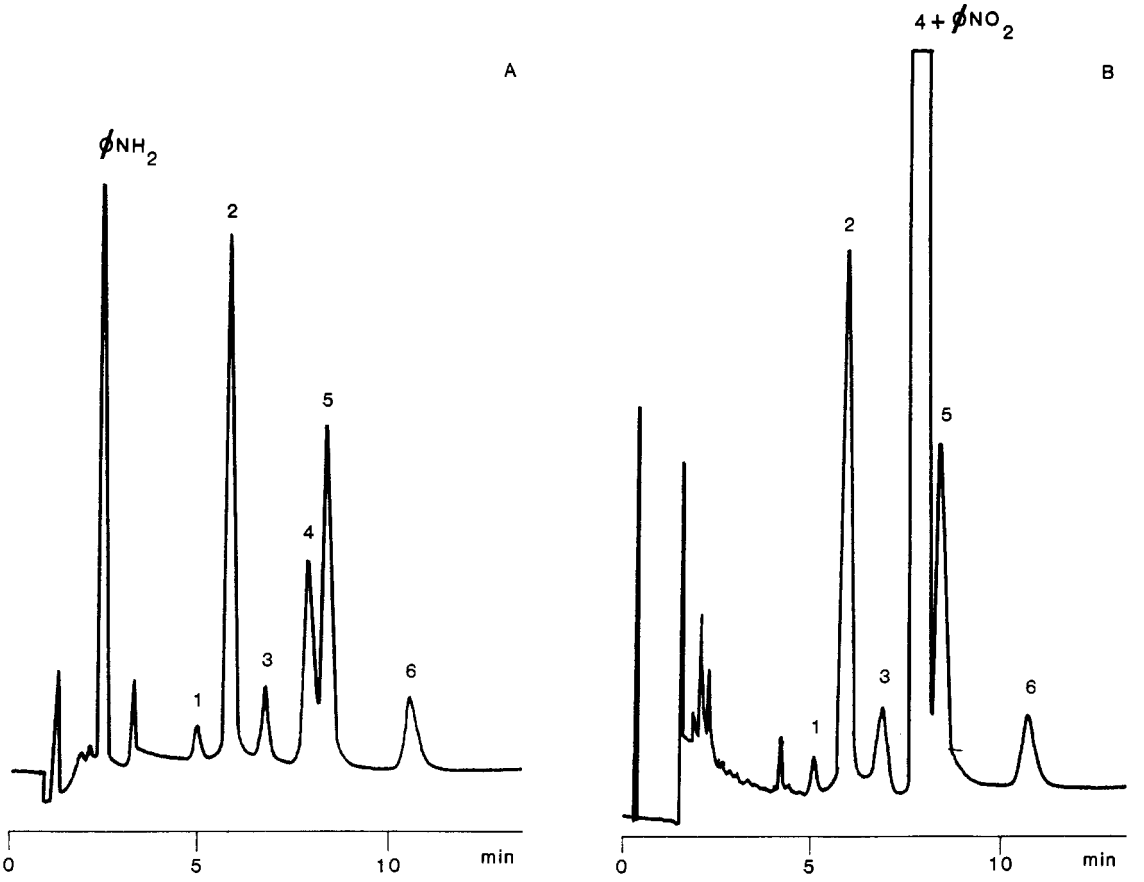

Figure 1. HPLC profiles of phenolics from wheat straw lignin obtained by direct analysis after nitrobenzene oxidation: (A) ElCh detector (+1.1 V); (B) UV detector (280 nm). Peaks: (1) vanillic acid; (2) vanillin; (3) syringic acid; (4) *p*-coumaric acid; (5) syringaldehyde; (6) ferulic acid.

Table I. Direct and Classical Analysis of Phenolics from Wheat Straw Lignin after Nitrobenzene Oxidation (Data Are the Average of the Six Samples Analyzed and Are Expressed as Percentages of the Original Material)

	direct analysis			classical analysis		
compound	ElCh	UV	var[a]	ElCh	UV	var
vanillic acid	0.069 ± 0.0081	0.084 ± 0.0069	ns	0.029 ± 0.0054	0.035 ± 0.0071	ns
vanillin	1.248 ± 0.0786	1.296 ± 0.0710	ns	1.111 ± 0.0728	1.135 ± 0.0651	ns
syringic acid	0.344 ± 0.0355	0.362 ± 0.0178	ns	0.217 ± 0.0137	0.264 ± 0.0166	**
p-coumaric acid	0.566 ± 0.0463			0.126 ± 0.0146	0.254 ± 0.1108	*
syringaldehyde	1.466 ± 0.1016	1.932 ± 0.0963	**	1.268 ± 0.0479	1.373 ± 0.0649	*
ferulic acid	0.413 ± 0.0324	0.414 ± 0.0582	ns	0.325 ± 0.0235	0.320 ± 0.0200	ns

[a] Variance analysis: ns, nonsignificant difference; *, significant difference at $P < 0.05$; **, significant difference at $P < 0.01$.

Table II. Recoveries (%) of Phenolic Standard Solutions Subjected to (A) Classical Extraction Procedure and (B) Extraction after Saturation of the Acidified Aqueous Phase with NaCl

	2×10^{-1} g/L		4×10^{-2} g/L		2×10^{-2} g/L		4×10^{-3} g/L		2×10^{-3} g/L		4×10^{-4} g/L	
	A	B	A	B	A	B	A	B	A	B	A	B
vanillic acid	57.0	90.0	47.9	75.5	45.5	72.1	46.8	77.9	50.0	66.9	54.6	77.3
vanillin	105.9	93.5	93.3	95.0	84.9	85.0	84.4	83.2	91.0	87.2	83.7	81.4
syringic acid	73.5	95.7	65.5	85.6	59.8	81.5	60.0	87.0	60.2	73.1	75.0	96.4
p-coumaric acid	25.9	61.4	19.0	43.2	19.3	53.7	19.2	51.4	23.4	40.4	25.4	45.1
syringaldehyde	102.8	99.2	94.3	93.9	84.6	86.5	85.9	88.3	86.1	81.0	80.7	83.9
ferulic acid	92.9	98.1	83.6	81.1	77.4	84.0	67.3	88.9	82.8	82.8	90.9	100.0

Statistical Analysis. A simple analysis of variance was employed to compare (a) UV and ElCh response of each phenolic both by direct and classical analyses (detector response comparison) and (b) direct and classical analysis results by both UV and ElCh detection. All data were the average of two chromatographic analyses.

RESULTS AND DISCUSSION

The ElCh UV chromatographic profiles obtained by direct analysis of a wheat straw hydrolysate are shown in Figure 1. The chromatographic and electrochemical conditions used in this work have been discussed elsewhere (Chiavari et al., 1988). These have been optimized for the quantification of the six major phenolics produced by nitrobenzene oxidation of wheat straw, namely syringaldehyde, vanillin, *p*-coumaric acid, ferulic acid, syringic acid, and vanillic acid.

The ElCh and UV detector quantitative results are reported in Table I for the direct analysis of a wheat straw hydrolysate.

Variance analysis indicated nonsignificant differences between the responses of the two detectors for all compounds with the exception of *p*-coumaric acid and syrin-

galdehyde. The former could not be detected in the UV chromatogram because it coelutes with the large peak of the UV-absorbing excess nitrobenzene. The latter was overestimated in the UV chromatogram ($P < 0.01$) due to its superimposition on the nitrobenzene peak tail.

When the two detectors were compared by the classical method of sample preparation (Table I), the UV results were again significantly higher than the ElCh results for *p*-coumaric acid and syringaldehyde, which are affected by the coelution of incompletely removed nitrobenzene, and nonsignificant differences for vanillic acid, vanillin, and ferulic acid were observed. The higher UV results for syringic acid cannot be easily explained other than due to a larger deviation between the two replications of the ElCh determinations ($P < 0.01$) than the UV one, although the trend is comparable for the analysis of all compounds. That is, UV data are slightly higher than ElCh data, the only exception being ferulic acid.

However, the main characteristic of the classical analysis data is that they were all significantly lower ($P < 0.01$) than those obtained by direct analysis. The classical analysis values range from about 25% of the direct analysis value for *p*-coumaric acid, to about 45% for vanillic acid, 65% syringic acid, 80% ferulic acid, 90% vanillin, and syringaldehyde (ElCh data). A reasonable explanation for this observation is incomplete extraction of the phenolics in the classical analysis.

To verify this hypothesis, six standard solutions of the phenolic compounds at the concentrations of 2×10^{-1}, 4×10^{-2}, 2×10^{-2}, 4×10^{-3}, 2×10^{-3}, and 4×10^{-4} g/L for each compound were subjected to the same extraction procedure as the nitrobenzene classical analysis. The phenolic recoveries are reported in Table II as determined by ElCh detector and expressed as percentage of the phenolics determined by direct analysis. For extractions without NaCl, the smallest recoveries were obtained for *p*-coumaric acid, from 19 to 26% depending on the concentration of the solution, whereas vanillic acid was recovered in percentages ranging from 46 to 57%. Syringic acid and ferulic acid recoveries were in the ranges 60–75% and 67–93%, respectively. The majority of the nonrecovered phenolics was found in the waste water phase.

Saturation of the acidified aqueous phase with NaCl generally improved the recoveries. However, these remained poor and nonconstant especially for *p*-coumaric acid (43–61%) and vanillic acid (67–90%). *p*-Coumaric acid can be quantitatively extracted with diethyl ether, but the use of this solvent is not recommended to improve sample stability (Reeves, 1985).

The method detection limit appeared to be fixed by the detector used. In fact, the most dilute standard solution was detectable only by the more sensitive ElCh detector (detection limit of about 1 pmol injected) after the extraction procedure.

ElCh detection appears to be a valuable means of determining the phenolics that originate from nitrobenzene alkaline oxidative hydrolysis of lignin-containing wheat straw. Samples can be analyzed rapidly, avoiding extraction procedures with any possible nonquantitative results. Unlike the UV detector, ElCh detector phenolic measurements are not affected by UV-absorbing nonphenolic compounds, predominantly nitrobenzene. In other cases, ElCh and UV quantitations are comparable. Column efficiency was not significantly affected by routine direct injections on a daily basis during a period of several months.

Registry No. Vanillic acid, 121-34-6; vanillin, 121-33-5; syringic acid, 530-57-4; *p*-coumaric acid, 7400-08-0; syringaldehyde, 134-96-3; ferulic acid, 1135-24-6.

LITERATURE CITED

Chang, H.-M.; Allan, G. G. Oxidation. In *Lignins Occurrence, Formation, Structure, and Reactions*; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley Interscience: New York, London, Sidney, Toronto, 1971.

Chiavari, G.; Vitali, P.; Galletti, G. C. Electrochemical Detection in the High-performance Liquid Chromatography of Polyphenols (Vegetable Tannins). *J. Chromatogr.* **1987**, *392*, 426–434.

Chiavari, G.; Concialini, V.; Galletti, G. C. Electrochemical Detection in the High-performance Liquid Chromatographic Analysis of Plant Phenolics. *Analyst* **1988**, *113*, 91–94.

Fritz, J. O., Moore, K. J. Separation and Quantification of Lignin-Derived Phenolic Monomers Using High-Resolution Gas Chromatography. *J. Agric. Food Chem.* **1987**, *35*, 710–713.

Hartley, R. D.; Buchan, H. High-Performance Liquid Chromatography of Phenolic Acids and Aldehydes Derived from Plants or from the Decomposition of Organic Matter in Soil. *J. Chromatogr.* **1979**, *180*, 139–143.

Jung, H. G.; Fahey, G. C., Jr. Nutritional Implications of Phenolic Monomers and Lignin: a Review. *J. Anim. Sci.* **1983**, *57*, 206–209.

Papadopoulos, J.; Defaye, J. Characterization of the Lignin Isolated from Birch Wood after Extraction with Hydrogen Fluoride. *J. Wood Chem. Technol.* **1986**, *6*, 203–218.

Reeves, J. B., III. Lignin Composition of Chemically Treated Feeds as Determined by Nitrobenzene Oxidation and Its Relationship to Digestibility. *J. Dairy Sci.* **1985**, *68*, 1976–1983.

Reeves, J. B., III. Use of Nitrobenzene Oxidation for Study of Lignin Composition with an Improved Method for Product Extraction. *J. Dairy Sci.* **1986**, *69*, 71–76.

Sundstol, F.; Owen, E. Introduction. In *Straw and Other Fibrous By-products as Feed*; Sundstol, F., Owen, E., Eds.; Elsevier: Amsterdam, Oxford, New York, Tokyo, 1984.

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