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Identification of Phenolic Acid Composition of Alkali-extracted Plants and Soils

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ABSTRACT

Phenolic acids (PAs) released from plant residues have been implicated as important components in a variety of soil processes. To evaluate the role of plant PAs in soil processes, a quantitative alkaline extraction, solid-phase purification, and gas chromatographic protocol was developed for identification of the composition and concentration of plant and soil PAs. Water-soluble or EDTA-exchangeable PAs were not detected in soil. Alkaline hydrolysis (1 *M* NaOH) at ambient temperatures was required to extract ester-linked phenolics and alkaline hydrolysis (4 *M* NaOH) with heat extracted ether-linked PAs present in plant and soil material. Purification of NaOH-extracted PAs by polymeric solid-phase extraction with gas chromatographic flame ionization and mass spectral analysis of nonderivatized extracts resulted in a highly reproducible and accurate method for the saponifiable PAs. The method quantified plant and soil PAs as ethanone (acetylbenzene), benzaldehyde, and benzoic- and cinnamic-acid derivatives. The majority of soil PAs was identified as modified cinnamic acids originating from vascular plant tissue. Comparison of the described method with a standard acid digestion (12 *M* H_2SO_4) and gravimetric determination of lignin in plant residues found that interferences formed by strong acid digestion of plant residues such as soybean [Glycine max (L.) Merr.] or clover (*Trifolium pratense* L.) containing higher carbohydrate and protein contents resulted in an overestimation of plant lignin content when measured by the acid digestion-gravimetric method. Since the majority of soil PAs originate from vascular plants and are not microbial in origin, the composition of ester-linked PAs in soils may be an important indicator of the quantity of plant residue C present in soils under different management systems.

PHOTOSYNTHETIC PLANTS are the ultimate source of essentially all organic materials and are the direct contributors of biological substances to soils (Hedges and Mann, 1979). Of the organic plant components added to soil, lignin constitutes the second most abundant organic constituent in nature, next to cellulose (Harkin, 1973). Lignin is composed of polymerized PA monomers that can be cross-linked to carbohydrates and proteins. Phenolic acid monomers isolated from plant cell walls include derivatives of benzaldehyde, ethanone, and cinnamic and benzoic acids (Hedges and Mann, 1979). Phenolic acids are among the most widespread classes of secondary metabolites and are known to be of great significance in plant–soil systems (Siqueria et al., 1991). They function as part of the structural plant matrix (Siqueria, 1991), act as constitutive protection against invading organisms (Vidhyasekaran, 1988), affect cell and plant growth (Rice, 1984), and are structural and functional components of soil organic matter (Haider et al., 1975). Phenolic acids have also been shown to regulate the residue decomposition rate (Meentemeyer 1978; Berendse et al., 1987; Martens, 2000a), nutrient release from plant residues in soil (Tian et al., 1992) and increase long-term soil structure (Griffiths and Burns, 1972; Martens, 2000a,b).

The composition of lignin and other polyphenol polymers in plant tissue, soil, or sediment is not amenable to direct chemical analysis without prior isolation. Traditional theories (Chen, 1992) have reported lignin polymers to be composed of repeating units of coumaryl, coniferyl, and sinapyl alcohols (monolignols or phenylpropane-like structures). Qualitative total lignin analysis for wood content or forage digestibility has always centered on a proximate-gravimetric analysis of the strong-acid resistant fraction (12 *M* H_2SO_4) of plant biomass. Problems with this analysis include incomplete removal of carbohydrate and protein materials and condensation of carbohydrate products with lignin, both resulting in erroneously high lignin contents (Dence, 1992). Quantitative analysis of lignin polymers has been commonly characterized on the basis of their nitrobenzene or cupric oxide oxidation products as 4-hydroxybenzaldehyde, vanillin, and syringaldehyde (Chen, 1992). Interferences from reaction products and intensive sample preparation have limited the usefulness of the nitrobenzene and cupric oxide oxidations, respectively, for nonwoody plant or soil analysis (Chen, 1992). For a discussion of the extensive sample preparation for cupric oxide digestion and the limitation of the silylation derivatization procedure for gas chromatography of PAs, see Hedges and Ertel (1982).

Previous research provides substantial evidence supporting the involvement of covalent bonding by substituted hydroxycinnamic acids (Hartley and Buchan, 1979; Fritz and Moore, 1987) bridging plant lignin (monolignol structures) to cell wall carbohydrates (Hartley, 1973; Wallace et al., 1995) and proteins (Newby et al., 1980). The finding that the lignin of nonwoody plants has a more dynamic structure incorporating hydroxycinnamates ester-linked to polysaccharides from the lignin backbone was because of new extraction methodology for quantitative removal of ester-linked PAs using NaOH at ambient temperature or NaOH with heat and pressure to release the ether-linked lignin PAs (Provan et al., 1994). Sarakanen and Ludwig (1971) reported that *p*-coumaric [3-(4-hydroxyphenyl)-2-propenoic acid] and

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Abbreviations: DI, deionized; PA, phenolic acid.

ferulic [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid] compounds are essentially unique to vascular plants and the composition reflects plant taxonomy, tissue type, and lignin content suggesting that identification of modified cinnamic acids in soil can be used as an indicator of remaining particulate plant residue. The bifunctional nature of the hydroxycinnamates that allows them to link to other molecules through ester (carboxyl group) or ether linkages (aromatic hydroxyl group) warrants additional studies in that these compounds may be important precursors in the formation of stable organic materials as previously suggested by Martin and Haider (1976) and Haider and Martin (1975).

While a great deal of scientific interest has been devoted to understanding the structure and function of the PAs that compose the lignin fraction of woody species and forages, little effort has been directed to quantifying the phenolic composition of crop residues and the impact of the plant PAs on soil processes such as aggregate stability and organic matter sequestration. The importance of plant PAs in the processes of residue decomposition, nutrient release from plant residues and increasing long-term soil structure requires that a quantitative method of analysis be employed to determine the effect of the composition of plant PAs on the PA content of soil. The objectives of this study were to develop methodology for determination of the PA composition and concentration in plants and soils and to compare the new method with existing methods.

MATERIALS AND METHODS

Soil and Plant Samples

The Webster silty-clay loam soils (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) used in this study were obtained from a native prairie (Kelso Prairie, Pocahontas County, Iowa) and two different agricultural fields located directly across the county roads on private lands. All three sites are located within the same soil map unit delineation (Finnley and Lucassen, 1985) and three samples of each soil (0–15 cm) were collected from within a 1-m^2 grid. The soil was collected in April 1997 and stored moist at 4°C. A full description of soil properties is reported in Martens (2000a). The pH was measured in 0.01 *M* $CaCl_2$ (2.5:1 ratio) and organic C and total N (Table 1) were determined by dry combustion with a Perkin Elmer 2400 C/H/N analyzer (Perkin Elmer, Fullerton, CA)[1]. Alfalfa (*Medicago sativa* L.), oat (*Avena sativa* L.), canola (*Brassica napus* L. var. napus), corn (*Zea mays* L.), soybean, clover, and an unidentified prairie grass (April 1997) were harvested from field sites or from glasshouse pots at physiological maturity. Selected properties of the tissues are listed in Table 1. The plant samples included leaves and stems. A full description of the composition of the materials is reported in Martens (2000b).

Analytical

The following extraction methods can be used in sequence or individually. A total PA content (ester- and ether-linked PAs) can be obtained by extraction with 4 *M* NaOH.

[1] The use of product or trade names in this publication is for descriptive purposes only and does not imply a guarantee or endorsement by the USDA or the U.S. Government.

Table 1. Properties of soils and plant species used in the study.†

Material	pH	Organic C	Total N
		g kg^{-1}	
Soil			
Webster prairie	7.03	44.0	3.84
Webster corn	7.38	30.5	2.64
Webster soybean	7.28	28.8	1.86
Plants			
Corn	–	445	4.3
Oat	–	433	17.0
Prairie	–	453	3.7
Alfalfa	–	397	36.0
Soybean	–	397	10.7
Clover	–	464	44.9
Canola	–	410	12.8

† The soil identification indicates the plant residue decomposing in the Webster soil when the soils were sampled.

Water- and EDTA-Extractable Soil Phenolic Acids

Soil (2 g) was extracted with 10 mL of deionized (DI) water for 16 h on a reciprocal shaker followed by centrifugation and collection of the supernatant for purification. The soil pellet was then extracted with 10 mL of 50 m*M* EDTA (pH 7.5) for 16 h on a reciprocal shaker. After EDTA extraction, the samples were centrifuged and the supernatant saved for purification.

Ester-linked Phenolic Acids

Plant (50 mg) and soil (2 g) PAs were extracted with 5 mL of 1 *M* NaOH for 16 h on a reciprocal shaker at ambient temperatures as described by Kelley et al. (1994) and Provan et al. (1994). After extraction, the sample was centrifuged (5000 × *g*), rinsed with water, centrifuged again, and the supernatants were combined and placed in a disposable glass test tube and heated at 90°C for 2 h to release the conjugated PA (Whitehead et al., 1983). The heated extract was cooled, titrated with 4 *M* HCl to pH <2.0, diluted to 10 mL, with DI water, and centrifuged to remove the precipitate. The supernatant was saved for subsequent purification.

Ether-linked Phenolic Acids

Plant (50 mg) or soil (2 g) PAs were extracted with 5 mL of 4 *M* NaOH, heated to 160°C in Teflon microwave digesters (CEM Corp., Matthews, NC) in a CEM NMS-2000 microwave (CEM Corp., Matthews, NC) at 650 W (Provan et al., 1994) or in screw-top glass test tubes (16 by 125 mm) in an autoclave (15 min at 121°C; 104 kPa) as suggested by Dalton et al. (1987). After cooling, the microwaved samples were transferred to centrifuge tubes and the microwave and the autoclaved samples were centrifuged to sediment the material. Supernatants were collected and the soil pellets washed with water and centrifuged. The supernatants were combined and adjusted to pH <2.0 with 4 *M* HCl. The samples were diluted to 14 mL with DI water, centrifuged to remove the precipitate, and the supernatant was saved for purification.

Purification of Extracted Phenolic Acids

An aliquot (5–15 mL) of the various supernatants was passed through a conditioned Varian (Varian Assoc., Harbor City, CA) Bond Elut PPL (3-mL size with 200-mg packing) solid-phase extraction tube at ~5 mL min^{-1} attached to a Visiprep (Supelco, Bellefonte, PA). The tubes were then placed under a vacuum (−60 kPa) until the resin was thoroughly dried after which the PAs were eluted with 1 mL of ethyl acetate into gas chromatography autosampler vials. The PPL tubes were conditioned by first passing 2 mL of ethyl

Table 2. Names and structures of phenolic compounds investigated.

ID Number	Compound names		Structure†			
	Common	Chemical	R_1	R_2	R_3	R_4
1	Phenol	Phenyl hydroxide	H	OH	H	
2	Acetophenone	1-Phenylethanone	$COCH_3$	H	H	H
3	Cinnamadehyde	3-Phenyl-2-propenal	$(CH)_2CO_2H$	H	H	H
4	*p*-Hydroxy-benzaldehyde	4-Hydroxybenzaldehyde	COH	H	OH	H
5	Vanillin	4-Hydroxyl-3-methoxybenzaldehyde	COH	OCH_3	OH	H
6	*p*-Hydroxy-acetophenone	1-(4-Hydroxyphenyl ethanone	$COCH_3$	H	OH	H
7	Acetovanillone	1-(4-Hydroxy-3-methoxyphenyl)ethanone	$COCH_3$	OCH_3	OH	H
8	*p*-Hydroxy-benzoic acid	4-Hydroxybenzoic acid	COOH	H	OH	H
9	Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	COOH	OCH_3	OH	H
10	Syringaldehyde	3,5-Dimethoxy-4-hydroxybenzaldehyde	COH	OCH_3	OH	OCH_3
11	*c*-Coumaric acid	3-(4-Hydroxyphenyl)-2-propenoic acid	$(CH)_2CO_2H$	H	OH	H
12	Acetosyringone	1-(3,5-Dimethoxy-4-hydroxyphenyl)ethanone	$COCH_3$	OCH_3	OH	OCH_3
13	*t*-Coumaric acid	3-(4-Hydroxyphenyl)-2-propenoic acid	$(CH)_2CO_2H$	H	OH	H
14	Syringic acid	3,5-Dimethoxy-4-hydroxybenzoic acid	COOH	OCH_3	OH	OCH_3
15	Ferulic acid	3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid	$(CH)_2CO_2H$	OCH_2	OH	H
16	Sinapyl aldehyde	3-(3,5-Dimethoxy-4-hydroxyphenyl)-2-propenal	$(CH)_2COH$	OCH_3	OH	OCH_3
17	Sinapinic acid	3-(3,5-Dimethoxy-4-hydroxyphenyl)-2-propenoic acid	$(CH)_2CO_2H$	OCH_3	OH	OCH_3

† The structure of each compound is shown in Fig. 1 with the appropriate groups for R_1, R_2, R_3 and R_4.

acetate followed by 2 mL water (pH <2.0). The phenolic acid standards used were purchased from Aldrich (Aldrich Chemical Co., Milwaukee, WI).

Phenolic Acid Separation and Detection

The purified phenolic extracts (1 μL: 10:1 split) were analyzed for composition by comparison with authentic standards (Aldrich, Milwaukee, WI) and with cochromatography with standards on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Corp., Palo Alto, CA) equipped with a derivatized, nonpacked injection liner, a HP-5 (5% crosslinked phenylmethyl siloxane) capillary column (30-m length, 0.32-mm column id., 0.25-μm film thickness), and detected with a flame ionization detector (FID). The following conditions were employed for PA separation: injector temperature, 230°C; temperature ramp, 70°C for 2 min then ramped to 250°C at 10°C min^{-1}; and detector temperature, 250°C.

Mass Spectral Identification of Extracted Phenolic Acids

The phenolic compounds (1 μL: splitless injection) were separated with a Hewlett-Packard 1800A GCD gas chromatograph equipped with a HP-Ultra 1 capillary column (25-m length, 0.2-mm column i.d., 0.33-μm film thickness) and detected with a mass selective (MS) detector in the full scan (10 to 450 m/z) electron impact mode (70 eV, source temp. 170°C). The following conditions were employed for PA separation: injector temperature, 250°C; temperature ramp, 70°C ramped to 250°C at 10°C min^{-1}; detector temperature, 280°C. The mass spectra of standards and unknowns were compared with a NIST spectrum library and published results (Fritz and Moore, 1987; Meirer and Faix, 1992).

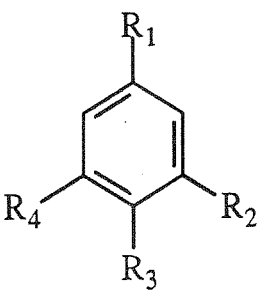

Fig. 1. Model chemical structure for the phenolic acids (PAs) studied here. The identities of the R_1 through R_4 groups for the various PAs are listed in Table 2.

RESULTS AND DISCUSSION

The large number of possible PA compounds present in plant and sediment samples (Hedges and Ertel, 1982) are represented by the modified benzaldehyde, ethanone, and cinnamic and benzoic acids shown in Table 2 and Fig. 1. Gas chromatography was employed because of higher resolution and decreased separation times of capillary columns for the many possible semivolatile phenolic compounds when compared with liquid chromatography. Gas chromatography resulted in baseline separation of the 17 phenolic compounds found to be present in different plant and soil samples (Fig. 2) and *r* values of 0.987 to 0.997 were determined for a four concentration standard curve for the 17 PA standards ranging from 10 to 75 mg PA L^{-1}. Relative standard deviations for five injections of the standards ranged from 2.3 to 3.6% for the 17 PAs tested. The *cis*-ferulic and *cis*-coumaric acid compounds (Compound 11) are not commercially available, but were prepared by exposure of the *trans* forms to daylight (ultraviolet radiation) for 5 h (Fritz and Moore, 1987). Exposure of *trans*-ferulic and coumaric acid standards to laboratory white fluorescent lighting (no daylight source) for 2 d did not yield detectable isomerization to the *cis* forms, so no special precautions were taken except all standards and samples were chromatographed within 15 h following extraction and purification. *Cis*-ferulic acid was not found in the samples analyzed, while *cis*-coumaric was found at low concentrations in several of the plant samples.

The PAs were not derivatized for this work to eliminate the problems and toxicity of the trimethylsilyl compound used in the derivatization process as described by Hedges and Ertel (1982) and because of the ease and success of chromatography of nonderivatized PAs reported by Fritz and Moore (1987). Fritz and Moore (1987) compared chromatography of derivatized and nonderivatized PAs and found only minor peak tailing with the nonderivatized acidic PAs that did not affect quantification. In this work, significant tailing of the acid phenolic compounds was noted after analysis of ~50 to 60 soil samples. This tailing was almost completely

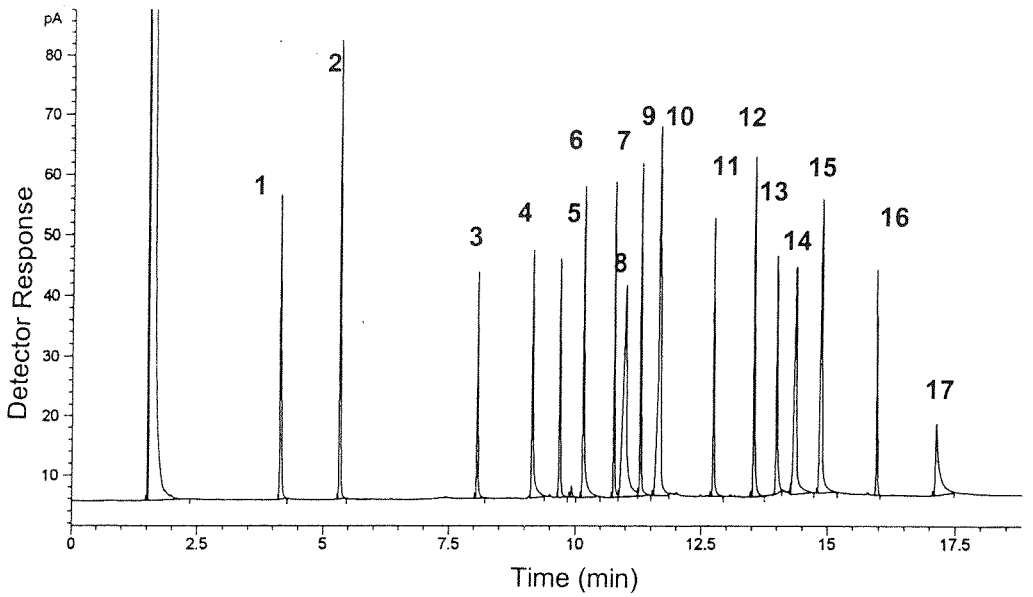

Fig. 2. Chromatographic trace of the 17 phenolic acid (PA) standards detected by gas chromatography with flame ionization detection (FID) under conditions givens given in the Materials and Methods section. The identities of the PA compounds are given in Table 2.

eliminated by (i) removal of ~7 cm of the front of the capillary GC column after 50 samples or (ii) the use of a 10-cm guard column that was changed after ~50 samples. Comparison of the peak area response from GC analysis of equal concentrations (10–75 mg PA L^{-1}) of derivatized versus nonderivatized PA standards (data not presented here) showed an r value of 0.998 suggesting that analysis of nonderivatized PAs was acceptable for the analysis. The lone problem encountered during GC analysis of nonderivatized PAs was a selective decomposition of the acidic phenolic compounds. Chromatography of coumaric and ferulic acid standards with a packed GC inlet liner resulted in identification of two peaks for each compound. The two peaks represented a decomposition product (loss of CO_2, m/z of 44) and the parent compound as determined by GC/MS. The decomposition was eliminated by use of a nonpacked, derivatized inlet as suggested by Fritz and Moore (1987).

Purification techniques used for preparing extracts of plant and soil samples containing PAs for chromatography have employed liquid-liquid extraction with ethyl acetate or ethyl ether (Fritz and Moore, 1987; Provan et al., 1994) or C_{18} solid-phase extraction (Lehmann et al., 1987). Liquid-liquid extraction represents a disposal and exposure hazard and C_{18} solid-phase extraction is of limited use for the ionized acid phenolic derivatives such as the cinnamic acid compounds because of the pH limitations of the C_{18} silica. The acidic phenolic compounds are not neutral molecules for quantitative C_{18} solid-phase extraction above pH 2.5 and the C_{18} silica backbone can dissolve at the low pH values. The Varian PPL polymeric solid-phase extraction phase used in this study is pH stable (0.5–13) (Varian brochure NJKS/MKTG/26-0796) and reproducible (standard deviation of 5 standard extractions was 3.3%).

Phenolic acids are present in plant biomass as ether-linked compounds requiring 4 M NaOH extraction with heat or ester-linked compounds requiring 1 M NaOH (ambient temperatures) extraction (Provan et al., 1994). Extraction of ester-linked PAs with NaOH at room temperature requires an additional step with heat to free the extracted PAs from the conjugate forms with carbohydrates and proteins that would not be detected by the GC analysis (Whitehead et al., 1983). In my study, a 2-h digestion of the 1 M NaOH supernatant at 90°C released conjugated PAs for subsequent purification and analysis. Sodium hydroxide is also the standard extactant for removal of humic substances from soils (Schnitzer, 1982). To determine if PAs were associated with the precipitate formed when the 1 M NaOH extracts were adjusted to pH <2.0, the precipitates were collected after centrifugation, microwave digested with 4 M NaOH and purified for analysis. If the 1 M NaOH extract was not heated to release the phenolic compounds from the conjugate forms, the precipitate contained the majority of the total extractable PAs. However, when the NaOH extract was heated before acidification, no PA monomers were detected in the dark precipitates. Extraction tests with samples following 1 or 4 M NaOH with the same solutions failed to detect additional PAs suggesting that the extraction solutions and conditions employed in this study resulted in complete extraction of the phenolic compounds in question. Only when the soil rinse step following extraction was omitted, did subsequent extraction detect remaining PAs. Shaking samples during extraction and suspending the soil pellet following collection of the first supernatant, yielded an average 30% increase in phenolic compounds as compared with extraction without shaking and without the soil rinse.

Microwave digestion using a temperature and pressure protocol has been proposed for increasing the extraction efficiency of total ester- and ether-linked PAs compared with ambient temperature (Provan et al., 1994). The commercial microwave digestion unit employed in my laboratory had a capacity of 12 digestion

Table 3. Composition of ester- and ether-linked phenolic acids (PAs) in plant residues.

Species	Compound†													
	4	5	6	7	8	9	10	12	13	14	15	16	17	Total
	g kg^{-1}													
							Ester-linked							
Corn	0.39	0.62	ND‡	ND	0.24	0.09	0.48	0.99	29.02	0.15	0.99	ND	ND	37.67 ± 1.3
Corn root	0.41	0.30	ND	ND	ND	ND	0.32	0.30	15.80	ND	2.02	ND	ND	19.46 ± 0.8
Oat	0.20	0.59	0.60	0.08	0.11	0.14	0.37	0.69	8.54	0.28	5.00	0.02	0.57	17.82 ± 1.2
Oat root	0.20	0.37	ND	ND	0.28	0.11	ND	ND	1.04	ND	2.61	0.02	ND	5.31 ± 0.3
Prairie	0.16	0.47	ND	0.08	0.09	0.16	0.15	0.57	3.89	0.13	2.58	0.02	0.28	9.32 ± 0.2
Prairie root	0.12	0.22	0.43	0.34	0.18	0.13	0.08	0.73	1.61	0.12	2.21	ND	0.46	6.48 ± 0.2
Alfalfa	0.13	0.29	0.45	0.36	0.19	0.13	0.09	0.77	1.26	0.12	2.34	0.08	0.48	6.82 ± 0.4
Soybean	0.08	0.27	ND	ND	ND	0.07	0.19	0.11	2.35	0.09	0.24	0.03	ND	3.40 ± 0.2
Canola	ND	0.04	ND	ND	ND	0.06	0.15	ND	0.09	ND	0.29	0.06	0.83	1.47 ± 0.1
Canola root	0.63	0.14	ND	ND	ND	ND	0.07	0.36	0.16	ND	0.07	ND	ND	0.36 ± 0.1
Clover	ND	0.06	0.20	0.07	0.10	0.06	ND	ND	0.32	ND	0.21	0.07	0.39	1.42 ± 0.2
Clover root	0.13	0.13	ND	0.12	ND	0.06	ND	ND	0.13	ND	0.13	ND	ND	0.70 ± 0.1
							Ether-linked							
Corn	0.38	0.84	ND	0.50	ND	0.10	3.20	7.89	ND	1.66	7.89	ND	ND	22.46 ± 1.2
Corn root	0.56	8.46	ND	0.32	ND	0.22	3.51	3.13	ND	0.63	1.34	ND	ND	18.17 ± 1.4
Oat	0.12	0.56	ND	0.26	ND	0.09	1.56	2.56	1.21	0.45	3.56	ND	ND	10.37 ± 1.1
Oat root	0.59	0.92	ND	0.39	ND	0.12	2.64	1.46	9.14	0.44	7.04	ND	0.44	23.18 ± 0.9
Prairie	0.23	0.97	ND	0.55	ND	0.15	1.76	4.76	1.49	0.63	3.75	ND	ND	14.29 ± 0.6
Prairie root	0.24	0.45	ND	ND	ND	0.12	0.92	0.57	5.61	ND	2.21	ND	ND	10.12 ± 0.4
Alfalfa	0.12	1.09	ND	0.23	ND	0.09	2.53	0.23	1.03	0.12	0.93	ND	ND	6.37 ± 0.2
Soybean	ND	1.22	ND	0.57	ND	0.21	1.80	1.36	ND	0.24	0.10	ND	ND	5.50 ± 0.3
Canola	ND	ND	ND	0.14	ND	0.49	ND	ND	0.34	ND	0.07	ND	ND	1.04 ± 0.1
Canola root	0.05	0.30	ND	0.16	ND	0.09	0.42	0.36	0.16	ND	0.24	ND	ND	1.78 ± 0.1
Clover	0.14	0.10	0.47	0.08	0.08	0.02	0.17	ND	ND	ND	0.25	ND	0.77	2.08 ± 0.1
Clover root	0.07	0.20	0.09	0.54	0.19	0.02	0.33	0.24	0.03	ND	0.17	ND	ND	1.88 ± 0.1

† The identities of the PA compounds are given in Table 2.
‡ ND, not detected.

vessels. Phenolic acid concentrations extracted when only 1–4 plant or soil samples were digested in the microwave unit could not be reproduced when the same four samples were included with eight additional samples (for a total of 12 cells), with a 50% decrease in PA concentration. The lack of reproducibility appeared to be different possibly because of the lack of equal distribution of heat and pressure with the use of the 12 vessels. In an attempt to decrease sample extraction variability and increase the number of samples that can be extracted, an autoclave extraction (Dalton et al., 1987) was compared with the microwave method (only four samples extracted per microwave cycle). A total of 30 samples were compared (data not shown) and the resulting total phenolic concentrations were correlated (r = 0.997), suggesting that both methods were very effective for extraction of plant and soil PAs.

Research has shown that the monolignols that compose the backbone of plant lignin are converted during oxidative extraction to the corresponding aldehyde compounds (Hedges and Mann, 1979; Chen, 1992), but little is known about whether other PAs present in plant biomass are also subject to decomposition during extraction and purification procedures. As previously found for cupric oxide oxidation, the coniferyl and sinapyl alcohol compounds were oxidized by the alkaline extraction procedure employed here to the corresponding aldehydes, vanillin, and syringaldehyde, respectively. Coumaryl alcohol is not commercially available, but has been reported to decompose to 4-hydroxy-benzaldehyde upon cupric oxide oxidation of woody plant tissue (Hedges and Parker, 1976). The remaining 10 spiked cinnamic, benzaldehyde, ethanone, and benzoic acid phenolic derivatives tested resulted in >95% recovery with the NaOH extraction, extract heating, purification, and gas chromatography conditions employed (data not presented) suggesting that the methodology produced a minimum of artifacts and was suitable for the wide range of PAs in the samples analyzed.

The ester- and ether-linked PAs extracted from different agronomic crops by sequential extraction of ester-linked PAs with 1 *M* NaOH (ambient temperatures) followed by 4 *M* NaOH (autoclave) for the ether-linked PAs are shown in Table 3, and chromatograms of the ester-linked PAs extracted from oat and alfalfa are presented in Fig. 3. It is apparent from Fig. 3 and Table 3 that different plant species have substantially different PA compositions and total phenolic contents in both the root and shoot biomass. As reported by Provan et al. (1994), the dicotyledonous plants, canola, soybean, alfalfa, and clover were substantially lower in PA content when compared with the monocotyledonous plants used in this study. The shoot biomass of the plant residues contained higher PA concentrations than the root biomass of the same species with the exception of the ether-linked PAs of canola roots. The cinnamic acid derivatives, coumaric and ferulic acids, accounted for the majority of the ester-linked PAs in the corn, oat, prairie grass, alfalfa, and soybean shoot as shown for other plant biomass (Provan et al., 1994). The majority of PAs reported in soil or sediments (Whitehead et al., 1981; Hedges and Ertel, 1982) are essentially unique to vascular plants (Sarkanen and Ludwig, 1971) suggesting the importance of determining the PA composition of plants contributing their biomass to the soil. The reported breakdown products of the lignin monolignols

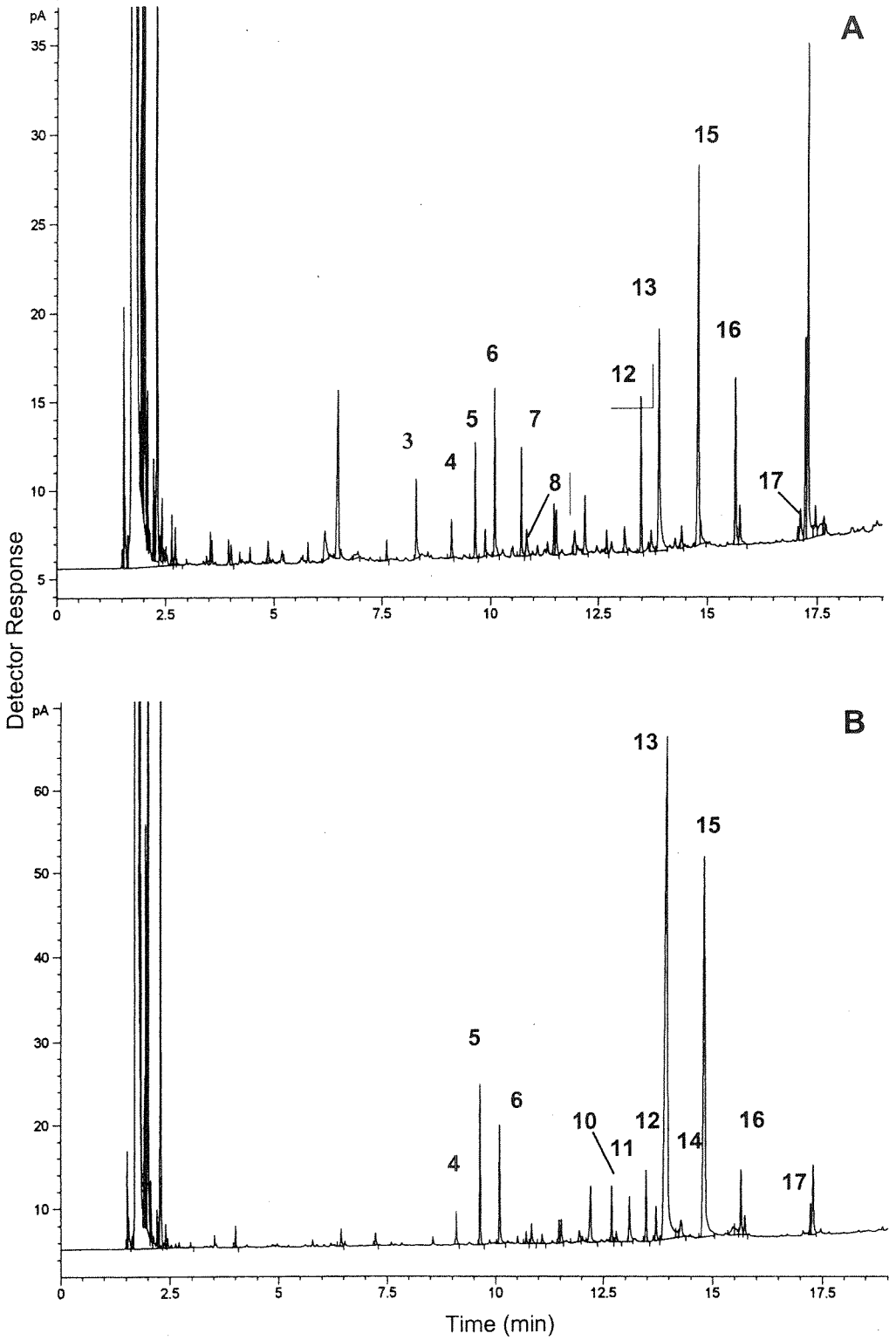

Fig. 3. Chromatographic traces of ester-linked phenolic acids (PAs) following alkaline extraction of (A) alfalfa biomass and (B) oat biomass. The identities of the PA compounds are given in Table 2.

(Hedges and Ertel, 1982), sinapyl (Compounds 10, 12, and 14), coumaryl (Compounds 4, 6, 8), and coniferyl alcohols (Compounds 5, 7, and 9) were substantially higher with the 4 *M* NaOH extraction supporting the current theories on the composition of ester- and ether-linked PAs in plant lignin (Hedges and Mann, 1979; Provan et al., 1994; Wallace et al., 1995).

To confirm the effectiveness of the proposed methodology for extraction of plant PAs, a proximate analysis used to determine lignin content for forage digestibility

Table 4. Composition of Webster soil ester- and ether-linked phenolic acids (PAs) sampled in April 1997.‡

Soil	Compound‡													
	4	5	6	7	8	9	10	12	13	14	15	16	17	Total
	mg kg^{-1}													
							Ester-linked							
Prairie	5.48	1.87	2.13	7.69	4.50	4.75	0.75	3.16	31.3	3.58	9.67	ND	8.73	83.6 ± 4.3
Corn	4.22	1.45	ND	2.83	ND	3.90	ND	3.62	31.7	3.61	4.47	6.26	ND	62.5 ± 3.2
Soybean	2.37	1.09	ND	ND	ND	2.35	ND	ND	16.8	ND	1.85	ND	ND	24.4 ± 1.8
							Ether-linked							
Prairie	12.40	9.25	22.4	19.2	44.8	41.2	12.0	27.9	75.1	30.4	51.4	ND	ND	365 ± 23.8
Corn	7.53	7.10	14.1	11.1	27.7	18.5	10.4	39.4	25.8	23.8	15.6	ND	ND	201 ± 19.3
Soybean	5.37	4.53	6.16	8.58	24.1	9.60	9.52	29.3	40.4	20.2	11.1	ND	ND	195 ± 15.6

† The notation prairie, corn, and soybean refer to the previous year's crop residue that was decomposing in the Webster soil when sampled.
‡ The identities of the PA compounds are given in Table 2.

(Goering and Van Soest, 1970) was used as a comparison with the soybean, clover (dicot species), prairie grass, and corn residues (monocot species). The proximate analysis method of Dence (1992) showed lignin contents of 12.5% for clover, 11% for soybean, 7.2% for corn, and 4% for the prairie grass residues (dry matter basis). Values found in the literature for the proximate analysis of lignin range from 6.8% lignin for the corn residue (Tian et al., 1992) to 13.5% lignin for the clover (Whitehead et al., 1979). The results based on the analysis of PA content (Table 3) show that corn had a lignin content of 6.0%, prairie grass 2.3%, soybean 0.89%, and clover of 0.35%. The large difference between the reported values for clover lignin content and the PA values reported (Table 3) may reflect varietal and maturity differences, but also may be because of the interferences that have been reported for plant biomass containing high amounts of carbohydrates and amino acids (Dence, 1992). Indeed, when the four plant residues were analyzed for C and N content (dry combustion) after digestion in the proximate analysis, high N values for the legume species suggest that the condensation of amino acids with the "lignin fraction" may have been a problem associated with the proximate procedure.

Previous research has been conducted on the extraction of PAs from soils spiked with various PAs (sterile or nonsterile) using a variety of extractants (Dalton et al., 1987) or extraction of soil organic matter using NaOH solutions (Hartley and Buchan, 1979; Whitehead et al., 1981; Dalton et al., 1987). However, the literature does not report a quantitative PA extraction and purification procedure for identification of ester- or ether-linked phenolic compounds from soil. To determine the extractability or state of PAs in soil, samples were sequentially extracted with water, EDTA (pH 7.5), 1 *M* NaOH (ambient temperatures), and 4 *M* NaOH (heat) to determine water-soluble, exchangeable, ester- and ether-linked PAs, respectively. No detectable water-sol-

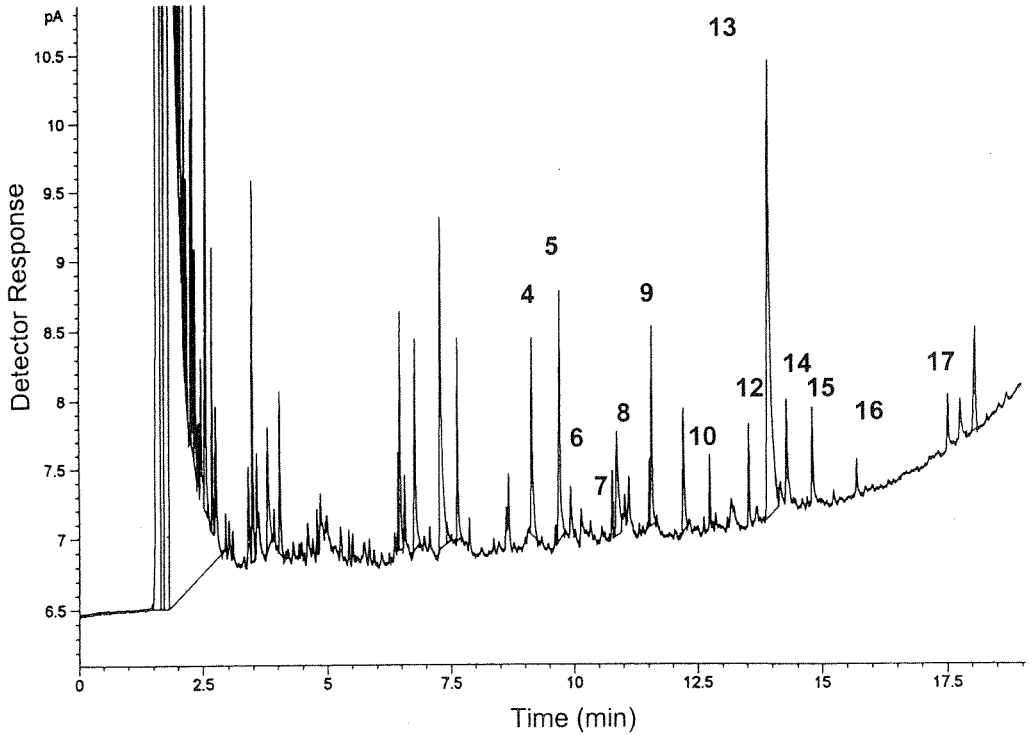

Fig. 4. Chromatographic traces of ester-linked phenolic acids following alkaline extractions of the Webster soil with decomposing soybean residue. The identities of the PA compounds are given in Table 2.

uble or exchangeable PAs were released from the soils tested as previously reported by Dalton et al. (1987). Increasing the soil mass for the water extraction from 2 to 20 g failed to yield detectable water-soluble PAs. For studies on the allelopathic effects of soil PAs, the Folin Ciocalteu colorimetric method has been proposed for determination of water-soluble PAs (Ohno and First, 1998). The results of the present study and others (Dalton et al., 1987) suggest that because of the extremely low concentrations of water-soluble phenolic compounds, colorimetric methodology may not be applicable for soil PA analysis as suggested by Ohno and First (1998).

The ester- and ether-linked PA contents of the ambient (1 *M*) and heated (4 *M*) NaOH sequential extractions for the Webster soil under native prairie or corn and soybean management are shown in Table 4. Of the various extractants tested in laboratories, only 1 or 2 *M* NaOH with or without heat has been reported to extract PAs from soil organic matter (Hartley and Buchan, 1979; Whitehead et al., 1981; Dalton et al., 1987) to which no PAs have been added (experiment controls). The Webster soil collected following the previous year's corn crop was lower in ester-linked PA content (1 *M* NaOH extraction) when compared with the prairie soil, but had greater than twice the amount of PAs when compared with the soil containing decomposing soybean residue (Table 4, Fig. 4). The ester-linked PA composition of the soils from corn and soybean (Table 4) closely reflects the PA composition of the respective decomposing plant residues (Table 3). The prairie soil contained 1.8 times more ether-linked phenolics (4 *M* NaOH extraction) compared with the corn and soybean soils and the soil ether-linked phenolic content for the corn, soybean, and prairie soils was closely related ($r = 0.998$) to the respective organic C content (Martens, 2000b). Identities of the PA peaks from the plant and soils were confirmed by mass spectrometry by searching the NIST library and by comparison with published results (Fritz and Moore, 1982; Meirer and Faix, 1992). The ester-linked cinnamic acid PAs comprised 49% of the prairie, 58% of the corn, and 76% of the soybean total ester-linked PAs, while the cinnamic acid PAs were <35% of the ether-linked extractable PAs. The synthesis of cinnamic acids is exclusively a function of vascular plants with no published reference of microbial cinnamic acid synthesis (Sarakanen and Ludwig, 1971; Hedges and Mann, 1979). The data suggest that ester-linked PAs may closely correlate with recent plant biomass additions, but the ether-linked soil PAs may reflect differences in long-term soil-crop management such as prairie compared with cultivation.

Phenolic acid analysis by cupric oxide oxidation results in soil PAs primarily in the aldehyde forms (Hedges and Ertel, 1982), suggesting that the cupric oxide method may underestimate the cinnamic acid derivatives as reported by publications using the cupric oxide extraction method. Guggenberger and Zech (1994) reported that vanillyl units were the major cupric oxidation products from soil with a minor contribution of cinnamyl derivatives and there was no mention of the syringyl units. Another recent study of lignin from 18 sites across a transect of North American grasslands, (Amelung et al., 1999) did not report amounts or mention the importance of cinnamyl phenolic cupric oxidation products. In contrast, this study found that the plant-derived cinnamic PAs accounted for the majority of the PAs released from similar native prairie vegetation (Table 3) and a grassland soil (Table 4). Chen (1992) reported that determination of cinnamyl PAs in grass tissues required an alkaline extraction procedure and analysis before cupric oxide oxidation of the remaining tissue because of possible destruction of cinnamyl PAs with the cupric oxide method.

Evaluation of the extraction and quantification method for PAs developed in this study was found suitable for the wide range of PAs previously reported in plant and soil analyses. Use of chromatography showed not only that the quantity of extracted PAs was different, but that the composition of the PAs present in roots or shoots varied considerably and the soil PA composition reflects the plant contribution. Phenolic acids have been implicated in many important processes in soil, thus continued research is needed to determine the impact of plant PAs in soil processes and to relate these processes to soil organic matter dynamics.

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