

## SEPARATION OF PHENOLIC COMPOUNDS IN ALKALI HYDROLYSATES OF A FOREST SOIL BY THIN-LAYER CHROMATOGRAPHY

Wood and tissue of red alder (*Alnus rubra* Bong.) contain various phenolic compounds (2, 9, 11). Some of these inhibit or stimulate *in vitro* growth of *Poria weirii* Murr., a widespread pathogen of conifer roots in western America (5). Were the inhibitory compounds added to soil under alder through root secretion or decomposition of litter, they could reduce the longevity and spread of *P. weirii* in stands in which alder grows in mixture with susceptible conifers. In initial studies of this possibility, we immediately encountered difficulty in chromatographically separating and identifying phenolics in forest soils.

Vanillic, ferulic, p-coumaric, p-hydroxybenzoic, syringic, and probably caffeic acids are commonly occurring phenolic compounds in soil (7, 13, 14). They have been separated by paper chromatography with unidimensional, multiple-pass development. However, such methods did not work consistently in our examination of extracts from forest soils. Other methods, as recommended by Halmekoski [cited by Kirchner (4)], Van Sumere *et al.* (12), and Morris (8) failed to separate these compounds satisfactorily. The techniques we ultimately devised have resulted in consistently good separation of the compounds and are comparatively simple and timesaving.

The soil used in this study was collected at a depth of 0 to 15 cm, after removing litter, 60 cm away from the stem of a red alder tree at Cascade Head Experimental Forest near Otis, Oregon. The chemical and microbial properties of this soil have been described by Franklin *et al.* (1) and Lu *et al.* (6).

Phenolic acids were extracted from soil samples by the alkaline hydrolysis method of Pearl *et al.* (9), as applied to organic soils by Morita (7): Fifty grams of soil were hydrolyzed under nitrogen in 1500 ml of 4% NaOH for 4 hours at 90 C. After cooling to room temperature, the reaction mixture was centrifuged and the residue discarded after washing with distilled water. The supernatant and washings were combined, acidified to pH 2 with HCl, centrifuged, and the brown precipitate discarded. The supernatant was extracted with ethyl ether in a continuous liquid-liquid extractor (Kontes model K581500)<sup>2</sup> for 12 hours. The phenolic acids were taken up from the ether extract into 8% NaHCO<sub>3</sub>, and the alkaline solution obtained was then acidified and extracted with ether as above. Finally, the ether extract was dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to a small volume for thin-layer chromatography.

For thin-layer chromatographic separation of the phenolic compounds in the soil extract, 10 g of polyamide powder (E. Merck, Ag., Darmstadt, Germany) were mixed with 80 ml of 95% ethanol and homogenized at high speed in a Waring blender for 15 seconds. The suspension, enough for five 20×20 cm plates, was spread on the plates with a Desaga applicator set at 250 μ. Standards were spotted on the plate individually and in mixture, along with the soil ether extract, and developed at room temperature by the unidimensional stepwise ascending technique. The standards, ferulic, p-hydroxybenzoic, and vanillic acids were ob-

<sup>2</sup>Mention of trade names does not constitute endorsement by the U.S. Department of Agriculture.

tained from Sigma Chemical Co., St. Louis; p-coumaric and caffeic acids, from Mann Research Laboratories, New York; and syringic acid, from Eastman Organic Chemicals, Rochester.

Three initial developing reagents were compared: dibutyl ether-acetic acid (10:1), benzene-acetic acid (25:75), and ethyl acetate-acetic acid (95:5). Plates were developed to a height of about 17 cm. All plates were then dried and developed in the second solvent, acetic acid-water (30:70), up to the same height.

Locations of spots on the developed chromatograms were determined by ultra-violet examination (350-m $\mu$  wavelength) and by spraying with modified diazotized p-nitroaniline followed by 2 N NaOH in 50% ethanol. This chromogenic spray was prepared freshly for each use by mixing 3 ml p-nitroaniline (saturated in 0.33 N HCl-50% ethanol), 1.0 ml NaNO<sub>2</sub> (1% in 50% ethanol), and 1.0 ml urea (5% in 50% ethanol). After a few minutes, this mixture was diluted with 10 times its volume of 50% ethanol. The colors developed on the chromatogram were matched against Ridgway's (10) color standards. Since Ridgway's book is no longer generally obtainable, the Inter-Society Color Council's near-synonym color designation for Ridgway names (3) has been added to provide more widely understandable color definitions.

As indicated by the  $R_{fa}$  values in Table 1, the dibutyl ether-acetic acid system adequately separated all the phenolic acids tested, plus an additional two unknowns in the soil extract. Neither the benzene-acetic acid nor the ethyl acetate-acetic acid systems satisfactorily separated vanillic from ferulic acid or p-hydroxybenzoic from p-coumaric acid. Unknown 2 from soil did not separate well from ferulic acid in the benzene-acetic acid system, and did not even appear in the ethyl acetate-acetic acid system (Table 1).

Chromatographic comparison (dibutyl ether-acetic acid system) of extract from soil under the red alder with authentic compounds indicated the presence of syringic, vanillic, p-hydroxybenzoic, and p-coumaric acids in addition to two unknowns, while ferulic and caffeic acids were absent. The other two solvent systems confirmed these conclusions for the compounds that separated adequately, that is, syringic acid, caffeic acid, and unknown 1.

Table 1. Comparative  $R_{fa}$  values ( $R_f$  of compound/ $R_f$  of vanillic acid) of six authentic phenolic compounds and two unknowns from forest soil by three systems of stepwise, unidimensional, thin-layer chromatographic development

Compound	Solvent system*			Spot color†	
	1	2	3	Ridgway	ISCC-NBS
Syringic acid	1.20	1.17	1.22	Jay blue	Moderate blue
Vanillic acid	1.00	1.00	1.00	Dull dark purple	Strong reddish purple
Ferulic acid	0.73	0.94	1.00	Pale Russian blue	Very pale blue
p-Hydroxybenzoic acid	0.58	0.38	0.58	Thulite pink	Strong purplish pink
p-Coumaric acid	0.38	0.36	0.52	Etain blue	Very light bluish green
Caffeic acid	0.25	0.19	0.30	French gray	Light bluish gray
Unknown 1	0.43	0.28	0.38	Antimony yellow	Moderate orange-yellow
Unknown 2	1.40	0.92	Absent	Hermosa pink	Strong pink

\*No. 1: Dibutyl ether-acetic acid (10:1); 2: benzene-acetic acid (25:75); 3: ethyl acetate-acetic acid (95:5). After drying, all plates were redeveloped in acetic acid-water (30:70).

†After spraying with modified diazotized p-nitroaniline followed by 2 N NaOH in 50% ethanol. Color names from Ridgway (10) with the corresponding Inter-Society Color Council near-synonym (3).

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