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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENOLIC ACIDS AND ALDEHYDES DERIVED FROM PLANTS OR FROM THE DECOMPOSITION OF ORGANIC MATTER IN SOIL

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SUMMARY

Methods are described for the estimation by high-performance liquid chromatography of *p*-hydroxybenzoic, vanillic, and syringic acids, of their corresponding aldehydes, and of the *cis* and *trans* isomers of the substituted cinnamic acids, *p*-coumaric, ferulic, sinapic and caffeic. As little as 0.1 μg can be estimated by these methods. Application of the methods is illustrated by estimation of several of the acids and aldehydes in extracts, prepared with 1 *M* sodium hydroxide, of cell walls of Italian ryegrass (*Lolium multiflorum* L.) and of soil.

INTRODUCTION

The substituted benzoic acids, *p*-hydroxybenzoic, vanillic and syringic, and the substituted cinnamic acids, *p*-coumaric, ferulic, sinapic and caffeic, are widely distributed in plants and are often combined with sugars as glycosides or esters[1,2]. The *cis* and *trans* isomers of *p*-coumaric and ferulic acids are bound to the cell walls of grasses and are released by treatment with sodium hydroxide; the amount of these acids released is related to the degradability, by carbohydrases, of the structural polysaccharides of the walls in the ruminant animal, both *in vitro* and *in vivo*[3,4]. Some of the substituted benzoic and cinnamic acids are formed during decomposition of organic matter in soil[5,6] and may reduce plant growth[6] possibly by inhibiting nutrient uptake[7,8]; they may also inhibit nitrification in soil[9].

The present work was undertaken to provide an improved method for estimating several of these acids, together with the aldehydes of the substituted benzoic acids. Although the phenolic acids can be separated as their trimethylsilyl ethers by gas–liquid chromatography (GLC)[10–13], the work reported here shows that high-performance liquid chromatography (HPLC) is a more sensitive technique and does not require preliminary preparation of derivatives of the acids before separation.

MATERIALS AND METHODS

Materials

The sources of the *trans* isomers of *p*-coumaric, ferulic, sinapic and caffeic acids were reported previously[13]. *cis-p*-Coumaric acid was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). *cis*-Ferulic acid is not available commercially.

Cell walls of primary growth of shoots of Italian ryegrass (*Lolium multiflorum* L.) cv RVP, were separated as reported previously[14] from material harvested in June.

The soil was a clay loam of the Swanmore series taken from a field which had been under grass (mainly perennial ryegrass) for at least 20 years. Soil cores (2.5 cm diameter) were taken in November to a depth of 15 cm and the combined sample passed through a 4 mm sieve. Any readily available roots were removed by hand. The water content of the soil, determined by heating at 105°, was 27% of the dry weight and its pH measured in 0.01 *M* calcium chloride (25 ml plus 10 g soil) was 6.3.

Exposure of the trans isomers of ferulic, sinapic and caffeic acids to UV radiation

Each of the *trans* isomers of the phenolic acids (5.0 mg in 1.0 ml methanol) was exposed to UV radiation for 3 h by the method reported previously[13], to produce mixtures of *cis* and unchanged *trans* isomers.

Treatment of cell walls with sodium hydroxide

Cell walls (30 mg) were shaken with 5 ml *M* sodium hydroxide under nitrogen (containing < 5 ppm oxygen) at 20° for 20 h. The mixture was filtered (No. 1 porosity glass sinter) and the residue washed with water (3×0.5 ml). *p*-Anisic acid (0.20 mg), the internal standard, was added as a solution in 1.0 ml of methanol and the combined solution acidified with 12 *M* hydrochloric acid to pH 2.5. The solution was made up to 5.0 ml with water and subjected to HPLC. All manipulations of *cis* and *trans* phenolic acids were carried out in "white" fluorescent light to prevent isomerization[13,15,16].

Treatment of soil with sodium hydroxide

Moist soil (25 g) was shaken with 40 ml *M* sodium hydroxude under nitrogen (containing < 5 ppm oxygen) at 20° for 20 h. The suspension was centrifuged (1000 *g*) then filtered (Whatman No. 1 filter paper) to remove floating particles of roots and other debris. A 10.0-ml aliquot was acidified with 12 *M* hydrochloric acid to pH 2.5 and centrifuged (1800 *g*) to separate "humic" acid. *p*-Anisic acid (0.56 mg), the internal standard, was added in 2.8 ml methanol and the solution made up to 20.0 ml with water. The humic acid precipitate obtained after centrifuging was washed with methanol (4×2 ml). The methanol washes were combined, *p*-anisic acid (0.28 mg) in 1.4 ml methanol added as internal standard, and the solution made up to 10.0 ml with methanol. The two solutions were subjected to HPLC.

Separation and estimation of reference mixtures of phenolic acids and aldehydes

Mixtures of *p*-hydroxybenzoic, vanillic, the *cis* and *trans* isomers of *p*-coumaric, ferulic, sinapic and caffeic acids were separated by HPLC using a high-pressure pump (Waters 6000A), a loop injector (Water U6K), a variable wavelength UV detector (Pye Unicam LC3) set at 275 nm, with 10-μl flow cell, a recorder (Houston Instrument Omni-scribe) and an integrator (Laboratory Data Control 308). Reversed-phase

chromatography was carried out using a steel column (25 cm × 4.6 mm I.D.) containing Spherisorb C_{18} bonded-phase on silica (Phase Separations, Queensferry, Great Britain; S-50DS) and isocratic elution with water–acetic acid (BDH, Poole, Great Britain; AnalaR)–*n*-butanol (BDH AnalaR) (342:1:14, solvent 1). The flow-rate was 1.2 ml/min and the column pressure $1.3 \cdot 10^7$ N/m^2. Calibration curves of *p*-hydroxybenzoic acid, vanillic acid, *cis*-*p*-coumaric acid, each of the *trans* acids, and *p*-anisic acid were linear in the range 0–1.5 μg.

A mixture of *p*-hydroxybenzoic, vanillic and syringic acids, of their corresponding aldehydes, of the *cis* and *trans* isomers of *p*-coumaric and ferulic acids, and of *p*-anisic acid, was separated by the same method except that water–acetic acid–*n*-butanol (347:1:11, solvent 2) was used as eluent. Calibration curves of the acids and aldehydes (excluding *cis*-ferulic acid which was unavailable) were linear in the range 0–1.2 μg.

Separation and estimation of phenolic acids and aldehydes in solutions from cell walls and from soil

The solution obtained from treatment of grass cell walls was subjected to HPLC by the above method using solvent 1 while the solutions from soil were analysed similarly using solvent 2. The volume injected was 40 μl each time. The separated compounds were estimated by reference to the calibration curves. *Cis*-ferulic acid was estimated by reference to the curve of the *trans* isomer.

RESULTS AND DISCUSSION

The separation of *p*-hydroxybenzoic acid, vanillic acid, and of the *cis* and *trans* isomers of *p*-coumaric and ferulic acids is shown in Fig. 1a and the separation of the isomers of sinapic and caffeic acids is shown in Fig. 1b. The *cis* isomers of ferulic, sinapic and of caffeic acids are not available commercially and were obtained by exposure of the *trans* isomers to UV radiation. A separation of reference phenolic acids and aldehydes that are often found in extracts from soil is shown in Fig. 1c. Application of these methods for estimating phenolic compounds in a solution obtained from treatment of grass cell walls with sodium hydroxide is illustrated in Fig. 1d and Table I.

When phenolic compounds in soil were estimated by a similar method to that used for grass cell walls, treatment with sodium hydroxide followed by acidification of the aqueous solution led to the precipitation of humic acid. The amounts of the compounds in the aqueous solution after separation of humic acid and the amounts of the compounds in a methanol solution obtained by combining the extracts from four solvent extractions of the humic acid, are shown in Table II; the separation is illustrated in Fig. 1e. In a separate experiment it was shown that the first two methanol extracts contained more than 90% of the phenolic compounds extracted with the solvent, the fourth extract containing only traces of the compounds.

The HPLC method is approximately ten times more sensitive than GLC and will enable small amounts of phenolic acids and aldehydes in soils and in plant materials to be estimated more rapidly and with greater accuracy. There is less interference from other compounds with the HPLC method as detection is by UV absorption at 275 nm.

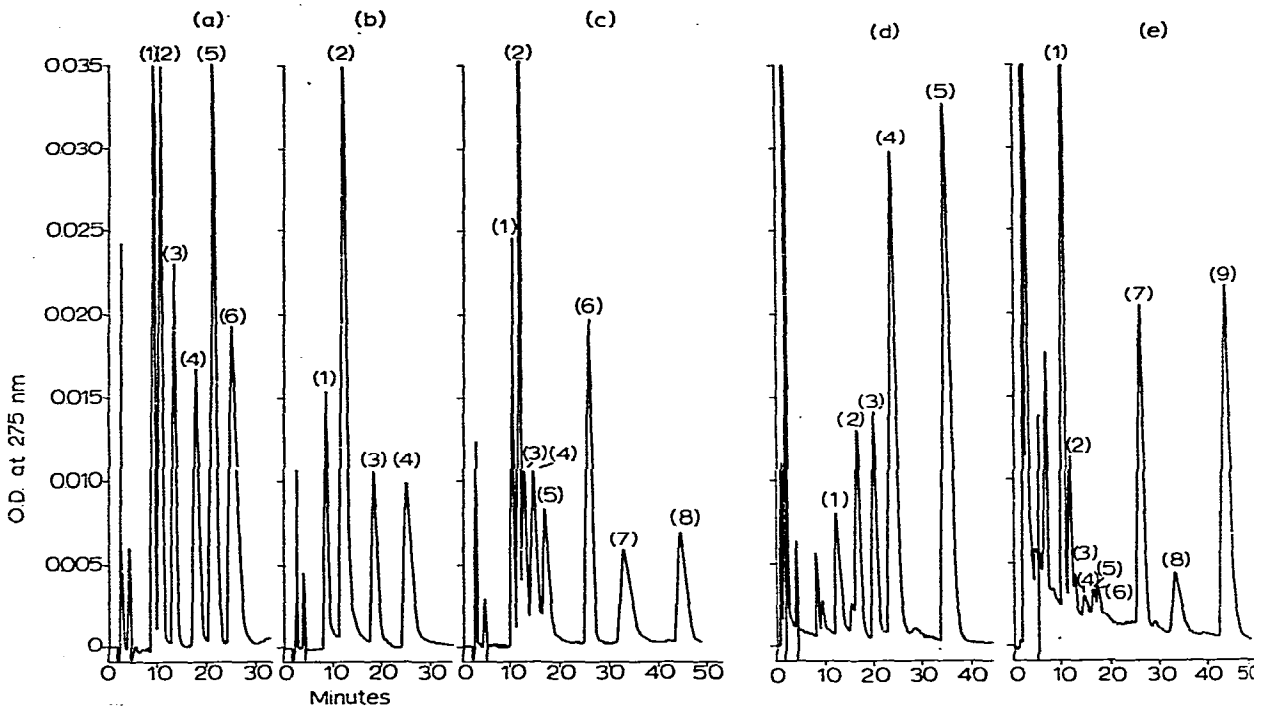

Fig. 1. Separation of phenolic acids and aldehydes by HPLC (experimental conditions in the text). (a) Solvent 1: a reference mixture of (1) *p*-hydroxybenzoic, (2) vanillic, (3) *cis*-*p*-coumaric, (4) *cis*-ferulic, (5) *trans*-*p*-coumaric and (6) *trans*-ferulic acids (1 μg of each); (b) solvent 1: a reference mixture of (1) *cis*-caffeic, (2) *trans*-caffeic, (3) *cis*-sinapic and (4) *trans*-sinapic acids (1.5 μg of each); (c) solvent 2: a reference mixture of (1) *p*-hydroxybenzoic acids (0.4 μg), (2) *p*-hydroxybenzaldehyde (0.2 μg), (3) vanillic acid (0.4 μg), (4) syringic acid (0.4 μg), (5) vanillin (0.2 μg), (6) *trans*-*p*-coumaric acid (0.4 μg), (7) *trans*-ferulic acid (0.4 μg) and (8) *p*-anisic acid (0.4 μg); (d) solvent 1: a NaOH extract of cell walls of *Lolium multiflorum* (1) *cis*-*p*-coumaric, (2) *cis*-ferulic, (3) *trans*-*p*-coumaric, (4) *trans*-ferulic and (5) *p*-anisic (internal standard) acids; (e) solvent 2: a NaOH extract of soil (aqueous solution) (1) *p*-hydroxybenzoic acid, (2) *p*-hydroxybenzaldehyde, (3) vanillic acid, (4) syringic acid, (5) *cis*-*p*-coumaric acid, (6) vanillin, (7) *trans*-*p*-coumaric acid, (8) *trans*-ferulic acid and (9) *p*-anisic acid (internal standard).

TABLE I

PHENOLIC ACIDS RELEASED FROM GRASS CELL WALLS BY TREATMENT WITH SODIUM HYDROXIDE

Analyses were in triplicate on the same sample.

Compound	*Retention time (min)*	*Amount (mg/g cell wall)*	
		Mean	*S.E.M.*
cis-*p*-Coumaric acid	13.2	0.71	*
cis-Ferulic acid	17.6	1.50	±0.038
trans-*p*-Coumaric acid	21.0	0.86	±0.007
trans-Ferulic acid	24.8	4.89	±0.030

* Error too small to measure.

TABLE II

PHENOLIC ACIDS AND ALDEHYDES RELEASED FROM SOIL BY TREATMENT WITH SODIUM HYDROXIDE

Analyses were in triplicate on the aqueous solution and on the methanol solution.

Compound	*Retention time (min)*	*Amount (μg/g dry soil)*				
		Aqueous solution		*Methanol solution*		*Total**
		Mean	*S.E.M.*	*Mean*	*S.E.M.*	
p-Hydroxybenzoic acid	9.8	152.08	±0.471	11.84	±0.037	163.92
p-Hydroxybenzaldehyde	11.1	8.49	±0.025	0.55	±0.004	9.04
Vanillic acid	12.2	9.10	±0.029	0.67	**	9.77
Syringic acid	14.0	4.56	±0.149	0.38	±0.011	4.94
cis-p-Coumaric acid	16.0	2.02	±0.149	0.42	±0.031	2.44
Vanillin	16.5	4.10	±0.394	0.36	±0.033	4.46
trans-p-Coumaric acid	24.5	46.72	±0.306	9.71	±0.061	56.43
trans-Ferulic acid	31.7	23.84	±0.946	6.05	±0.239	29.89

* Calculated by summation of the mean amounts.
** Error too small to measure.

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