Journal of Chromatography, 346 (1985) 440–445
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 003

Note

A rapid method for the separation and quantification of simple phenolic acids in plant material using high-performance liquid chromatography

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(First received May 3rd, 1985; revised manuscript received July 4th, 1985)

In the past few years there has been a proliferation of methods for the determination of phenolic acids and other phenolic compounds in plant material and plant products[1–8]. Interest has centred around the effects of this group of compounds on the flavour of foods[9–14] and beverages[15–17] and on their possible significance for the diet selection of certain agricultural pests[18–21].

Phenolic acids can be adequately separated using reversed-phase high-performance liquid chromatography (RP-HPLC) providing that the aqueous solvent is of sufficiently low pH to suppress any ionisation of phenolic hydroxyl or carboxyl groups[3,7–8]. Most workers have adopted the use of 25–30 cm C_{18} (5- or 10-μm packing)[1,2,5–8] columns with aqueous phases containing either acetic or formic acid although a novel technique employs a polystyrene–divinyl benzene column[22]. Separations of the complex mixtures of phenolics arising from plant products have been achieved with gradient run times of approximately 1 h.

This paper describes an analogous method based on the use of a 10-cm C_{18} (3-μm packing) column eluted with a gradient of 5% (v/v) aqueous formic acid and 75% (v/v) aqueous methanol. Separations of the phenolic acids commonly found in plant products can be achieved in under 25 min. Because the column is of conventional internal dimensions (4 mm I.D.), not small- or micro-bore but uses 3-μm packing, it provides a useful compromise between micro-bore columns which require modifications to sample injection and detection systems and conventional columns. The separations achieved are comparable with those obtained with larger columns but with a considerable saving of solvent usage and analysis time.

The method described in this paper has been used to look at the phenolic content of two varieties of orchard pear bud. Pear buds are susceptible to severe damage by bullfinches[23] and in an attempt to reduce this, a multi-disciplinary project has been set up to study the chemistry and biology of diet selection in this species in situations where it is a pest. The variety "Conference" is particularly susceptible to disbudding by bullfinches whilst "Doyenné du Comice" is less favoured. Previous work has shown that gross nutritional factors such as protein or carbohydrate levels cannot adequately explain the preferences of the birds[24] thus this method was developed to look at the levels of potentially organoleptic phenolic acids in pear buds.

EXPERIMENTAL

Apparatus

The chromatograph consisted of two Waters Model 6000 A pumps, a Waters Model 710 B Wisp autosampler, a Waters Model 481 variable-wavelength UV detector set at 280 nm (detector sensitivity 0.10 a.u.f.s.), a Waters Model 730 data module printer/plotter and a Waters Model 721 system controller (Millepore-Waters, Harrow, U.K.). The column was maintained at 35°C using a Jones Chromatography 30-cm column block heater. (Anachem, Luton, U.K.). Separations were achieved on a Rainin Short-one 10 × 0.4 cm, 3-μm C_{18} packed reversed-phase column supplied by Anachem.

Elution

The two solvents employed were: (A) 5% (v/v) aqueous formic acid and (B) 75% (v/v) aqueous methanol. Both solvents were filtered through 0.45-μm membrane filters, degassed under reduced pressure and stored under helium throughout their use. Elution was carried out at 1.0 ml/min using the complex gradient described in Table I.

TABLE I

GRADIENT CONDITIONS USED TO ELUTE SIMPLE PHENOLIC ACIDS FROM A RAININ SHORT-ONE 3 μm C_{18} REVERSED-PHASE COLUMN

Solvent A: 5% (v/v) aqueous formic acid; solvent B: 75% (v/v) aqueous methanol. Flow-rate 1 ml/min throughout.

Time (min)	*A (%)*	*B (%)*	*Curve**
0	97	3	
0.5	97	3	6
5	92	8	6
10	61	39	4
16	43	57	6
18	0	100	6
22	0	100	6
25	97	3	6

* Curve shape as designated by Millepore-Waters system controller, 6-linear gradient, 4-convex gradient.

Standards

The compounds named in the results were obtained from a variety of commercial sources, with the exception of isochlorogenic acid which was kindly donated by Prof. J. B. Harborne (Reading University, U.K.). All standards were 0.1 mg/ml in 5% (v/v) aqueous formic acid excepting isochlorogenic acid which was used as a qualitative standard only. In order to identify peaks in the standard mixture (Fig. 1), compounds were first chromatographed individually or in mixtures of two or three where identification would not be confusing. The tentative identification of compounds in the plant material extracts was achieved by co-chromatography with the appropriate standards.

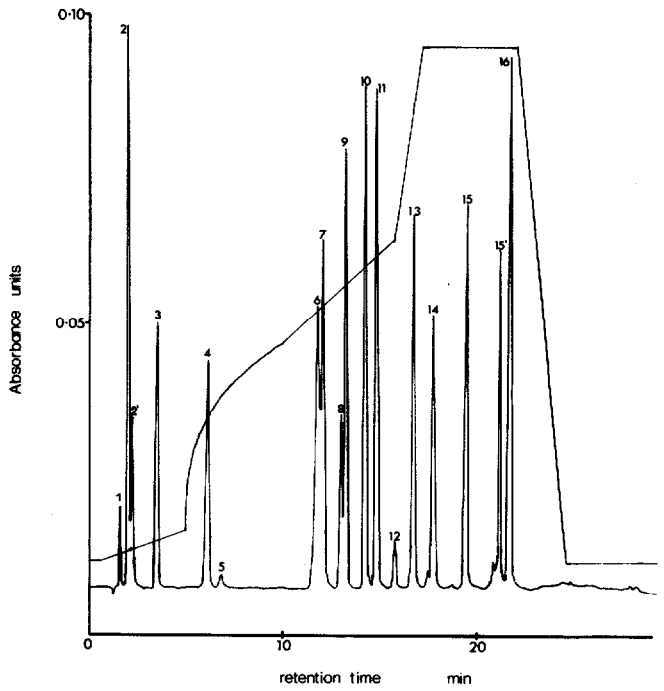

Fig. 1. Separation of 17 phenolic acid standards (0.1 mg/ml) on a Rainin short-one 3 μm C_{18} reversed-phase column. The gradient is as depicted and described in the text. The compounds are: 1, arbutin (retention time 1.65 min); 2, gallic acid (2.05 min); 2', hydroquinone (2.15 min); 3, protocatechuic acid (3.59 min); 4, *p*-hydroxybenzoic acid (6.25 min); 5, gentisic acid (6.97 min); 6, vanillic acid (11.94 min); 7, caffeic acid (12.21 min); 8, homovanillic acid (13.16 min); 9, chlorogenic acid (13.40 min); 10, syringic acid (14.49 min); 11, *p*-coumaric acid (15.05 min); 12, benzoic acid (16.03 min); 13, ferulic acid (16.96 min); 14, synapic acid (18.03 min); 15 + 15', isochlorogenic acid (19.44 min and 21.23 min); 16, cinnamic acid (22.06 min).

Plant extractions

Plant material collected from an orchard site in south-east England was lyophilised immediately on return to the laboratory and stored desiccated at 4°C until required for analysis. Prior to analysis the outer scale-like tissue (not consumed by the bird) was removed from the developing flower initial and the latter finely macerated. A portion of the chopped initial (50 mg) was extracted in ethanol–methanol (1:1) (5 ml) at 80°C for 1 h. After cooling the alcoholic solvent was removed by evaporation under a stream of nitrogen. A volume of 5 ml 5% (v/v) aqueous formic acid was added and the resultant solution extracted into 4 × 5 ml diethyl ether. The bulked ether extracts were evaporated under nitrogen and the residue redissolved in 5% (v/v) aqueous formic acid (3 ml) prior to HPLC analysis. All samples were stored under nitrogen in sealed amber vials to prevent excessive phenolic oxidation.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of 17 phenolic components commonly found in plant material. This separation was achieved in less than 25 min and represents a

TABLE II

THE RELATIVE LEVELS OF THE SIX PRINCIPAL PHENOLIC COMPONENTS OF PEAR FRUIT BUD FLOWER INITIALS AS DETERMINED BY RP-HPLC

All results are quoted in arbitary peak area units as determined at 280 nm. The retention times are quoted in parenthesis.

	Doyenne du Comice	*Conference*
1,4-Dihydroxybenzene (2.15 min)	3.21	2.15
X (9.77 min)	0.84	0.59
Chlorogenic acid (13.41 min)	3.28	3.14
X (13.94 min)	1.31	0.19
Syringic acid (14.41 min)	1.20	0.84
Isochlorogenic acid (19.46 min)	8.58	6.03

considerable saving of both time and solvents, etc., when compared with similar methods employing conventional columns. It should be noted that the "pure" isochlorogenic acid gives rise to two peaks (15 and 15') which as this compound is the di-caffoyl ester of quinic acid presumably represent two isomeric forms. There appears to be no practical reason why this rapid separation technique could not be employed to study other plant phenolics such as flavonoids, etc.

The method described in this paper was applied to the analysis of pear flower initials in the bud collected in mid-January 1985, the period when maximum bird damage is likely to occur[24,25]. Table II shows the levels of the major phenolic acids in the two varieties studied and a chromatogram for one variety, "Doyenné du Comice" is shown in Fig. 2. The chromatogram demonstrates that the bud flower initials contain three principal components, hydroquinone (1,4-dihydroxybenzene), chlorogenic acid and isochlorogenic acid. It appears likely that the hydroquinone detected is the breakdown product of arbutin (the glucoside of hydroquinone) produced during extraction, particularly as arbutin, chlorogenic acid and isochlorogenic acids have long been recognised as the major phenolic constituents of pear leaves and bark[26]. Fig. 2 also shows the presence of three additional components, one which co-chromatographs with syringic acid, and two, as yet are unidentified peaks (X).

The bullfinch shows a marked preference for buds of "Conference" pears whilst avoiding those of "Doyenné du Comice". Although physical factors such as shape, size and texture may have a role in these preferences[27], it would appear that there is also a chemical basis for diet selection and knowledge of the compounds involved will assist in the development of suitable pest management techniques. There seems to be little or no difference between varieties in terms of their gross nutritional value or in the levels of high-molecular-weight polyphenolic materials (tannins). It is interesting to note, however, that the preferred variety, "Conference" has the lower levels of the phenolics determined in this study particularly isochlorogenic acid and the unidentified peak at 13.94 min. No attempt has been made to quantify fully the data primarily because of the lack of standards for isochlorogenic acid and the two unknowns. Work is in hand to see if these chemicals affect dietary selection of birds in the laboratory.

Although there are a great number of phenolic substances known to occur in plant material[7], this method offers the analyst a rapid and economical technique for

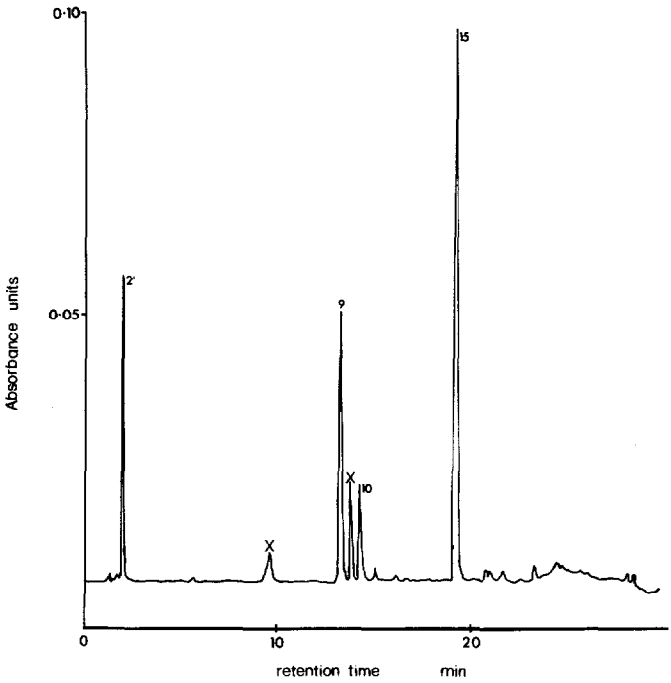

Fig. 2. Separation of the diethyl ether soluble phenolic acids from fruit bud flower initials of "Doyenné du Comice". The gradient is as described in the text and the numbering system is the same as that used in Fig. 1 (X = unidentified components).

the quantification of such substances. Naturally, the final identification of any component within a particular plant tissue must be based not solely on a chromatographic separation but on what might reasonably be expected in a specific plant and on other confirmatory analytical techniques.

ACKNOWLEDGEMENTS

I would like to thank Miss C. A. Blunden for her assistance in this work and Prof J. B. Harborne for his kind gift of isochlorogenic acid.

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