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Note

Analysis of furanoacetylenic phytoalexins from the broad bean plant by high-performance liquid chromatography

A. E. A. PORTER and R. V. SMALLMAN*
Chemistry Department, University of Stirling, Stirling FK9 4LA (Great Britain)
and
J. W. MANSFIELD
Biology Department, University of Stirling, Stirling FK9 4LA (Great Britain)
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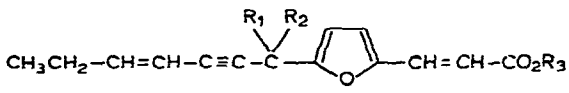
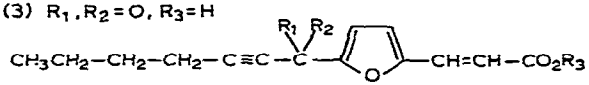
Phytoalexins may be defined as antifungal compounds produced by plants in response to microbial infection[1]. Tissues of the broad bean plant *Vicia faba* (L.) produce the furanoacetylenic phytoalexins wyerone (1), wyerol (2), wyerone acid (3), their dihydro derivatives (4), (5) and (6), and wyerone epoxide (7) when challenged by species of the fungal genus *Botrytis*[2–4].

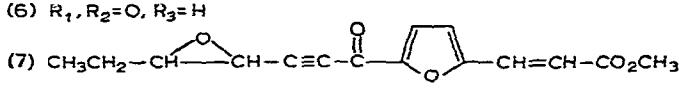
In earlier studies of the changes in the concentrations of phytoalexins in broad bean tissues following fungal infection, paper[5], thin layer[6] and gas–liquid[7] chromatographic methods were used. These techniques failed to resolve the dihydro derivatives (4–6) from their unsaturated analogues (1–3). We now report the application of high-performance liquid chromatography (HPLC) using the reversed-phase column packing ODS Hypersil to the quantitative analysis of seven wyerone derivatives from *Vicia faba* (L.).



(1) $R_1, R_2 = O$, $R_3 = CH_3$
(2) $R_1 = H$, $R_2 = OH$, $R_3 = CH_3$
(3) $R_1, R_2 = O$, $R_3 = H$



(4) $R_1, R_2 = O$, $R_3 = CH_3$
(5) $R_1 = H$, $R_2 = OH$, $R_3 = CH_3$
(6) $R_1, R_2 = O$, $R_3 = H$



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* To whom correspondence should be addressed. Present address: Standardising and Analytical Department, Imperial Chemical Industries Ltd., Organic Division, Grangemouth Works, Grangemouth, Scotland, Great Britain.

EXPERIMENTAL

Liquid chromatography

A pair of Waters Associates liquid chromatography pumps (Models 6000 and 6000A), controlled by a solvent programmer (Model 660), were used. The detector was a Cecil Model 272 variable-wavelength spectrophotometer fitted with a 10-μl flow-cell. For quantitative analyses, a Columbia Scientific Industries Supergrator computing integrator was employed.

A stainless-steel column (20 cm × 8 mm I.D.) was slurry-packed[8] with ODS Hypersil (5 μm) (Shandon, London, Great Britain) using methanol–0.1% aqeous sodium acetate solution as the packing support liquid. It was fitted with an on-column, needle-through-septum injector as described by Bristow[9] and connected to the detector flow-cell by 60 mm of microbore (0.015 mm) capillary. The column was tested (60% aqueous methanol at 4 ml/min, 2100 p.s.i.) with the following solutes (plate counts, height equivalent of theoretical plate): phenol (12,600, 0.015 mm), anisole (12,300, 0.0159 mm), phenetole (11,500, 0.017 mm).

The eluent reservoirs were contained in a water-bath controlled thermostatically at 30°, water from this bath was circulated through a jacket surrounding the chromatographic column.

Chemicals

All eluents were of HPLC grade. Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Great Britain), water was distilled in glass from potassium permanganate. Formic acid (BDH, Poole, Great Britain) was AnalaR grade. Butyl salicylate was obtained from Hopkin & Williams, Chadwick Heath, Great Britain.

Wyerone derivatives

Wyerone was separated from extracts of infected cotyledons by preparative TLC as previously described[10]. Even after repeated crystallisation from cyclohexane, the natural product was found to contain 10.6% of dihydrowyerone (4) when analysed on the ODS Hypersil column. Wyerol was synthesised[11] by reduction of the wyerone–dihydrowyerone mixture with sodium borohydride. Synthetic dihydrowyerone and dihydrowyerol were kindly provided by Mr. R. O. Cain (University of Stirling). Wyerone epoxide was isolated from infected cotyledons as described by Hargreaves *et al.*[4] and finally purified on pre-coated silica gel thin-layer chromatography (TLC) plates (Merck 5715) by repeated development in chloroform–light petroleum (b.p. 60–80°) (2:1).

Wyerone acid and dihydrowyerone acid were separated as a mixture from infected pod tissue extracts[6] on precoated silica gel TLC plates [diethyl ether–methanol (8:1), R_F 0.3]. The two acids were separated from each other by preparative HPLC using an ODS, Hypersil (5 μm) column (50 cm × 8 mm I.D.) (15,200 plates, $H = 0.033$ mm). Aliquots (3 mg) of the mixed acids (in 20 μl methanol) were injected and elution was with methanol–1% formic acid (50:50) at 6 ml/min. The identity of dihydrowyerone acid (not previously reported) was confirmed by mass spectroscopy: M^+ at *m/e* 246 (78%); *m/e* 165 (20%), 137 (100%) and 109 (19%).

For the development of quantitative analyses, concentrations of wyerone acid,

dihydrowyerone acid and wyerone epoxide were determined spectrophotometrically, the acids being measured using extinction coefficients published for their methyl esters[2,4]. Otherwise, standards were prepared using known weights of material.

RESULTS AND DISCUSSIONS

Development of the separation

The group of phytoalexins under examination all had strong UV absorbances in the region 310–350 nm, and were thus suitable for spectrophotometric detection. A wavelength of 312 nm ($\lambda_{max.}$ of wyerol) was used in early work but this was subsequently changed to 330 nm where a more satisfactory detector response for all compounds was obtained.

Scouting separations were performed with an ODS Hypersil column (10 cm × 5 mm I.D.). Isocratic elution of mixtures of the compounds with aqueous methanol or acetonitrile gave incomplete separations, but with each eluent mixture different regions of the chromatogram were well resolved. Fig. 1A shows that methanol–water (60:40) resolved wyerone acid (3), epoxide (7) and wyerol (2) but merged dihydrowyerol (5) and wyerone (1). Dihydrowyerol (5) and wyerone (1) were well separated by acetonitrile–water (50:50) but wyerone epoxide (7) and wyerol (2) were coincident using this eluent, as shown in Fig. 1B. Other mixtures of these eluents with water or the use of gradient elution did not improve the separation shown.

Combining these two eluents in a mixture of methanol–acetonitrile–water (30:20:50) gave a useful combination of the two effects. Synthetic mixtures of all of the phytoalexins were completely separated, but the chromatograms of real plant extracts run under these conditions contained other peaks which obliterated that early region of the separation occupied by wyerone acid (3) and dihydrowyerone acid (6).

The addition of 5% formic acid to the aqueous component of the eluent increased the retention of wyerone acid (3) bringing it clear of the interfering peaks, but it now eluted almost coincidentally with wyerone epoxide (7). Dihydrowyerone acid (6) was not resolved at all under these conditions.

The application of gradient elution conditions running linearly from methanol–5% formic acid (35:65) to methanol–acetonitrile–5% formic acid (40:10:50) finally effected resolution of all of the components, free from major interferences when the 20 cm × 8 mm I.D. column was used. The chromatograph obtained, and full conditions used are shown in Fig. 2. Mean elution times under these conditions were: wyerone epoxide (7), 23.1 min; wyerone acid (3), 23.9 min; dihydrowyerone acid (6), 25.9 min; wyerol (2), 29.1 min; dihydrowyerol (5), 33.5 min; wyerone (1) 36.5 min; and dihydrowyerone (4), 41.3 min.

Quantitative analysis

Butyl salicylate was selected as an internal standard for this analysis. It eluted with a retention time of 51.7 min, 10 min after the slowest eluting furanoacetylene, dihydrowyerone.

Methanolic solutions were prepared containing combinations of wyerone acid (3), dihydrowyerone (4), dihydrowyerol (5), dihydrowyerone acid (6) and wyerone epoxide (7). These solutions contained the dihydro compounds at concentrations of

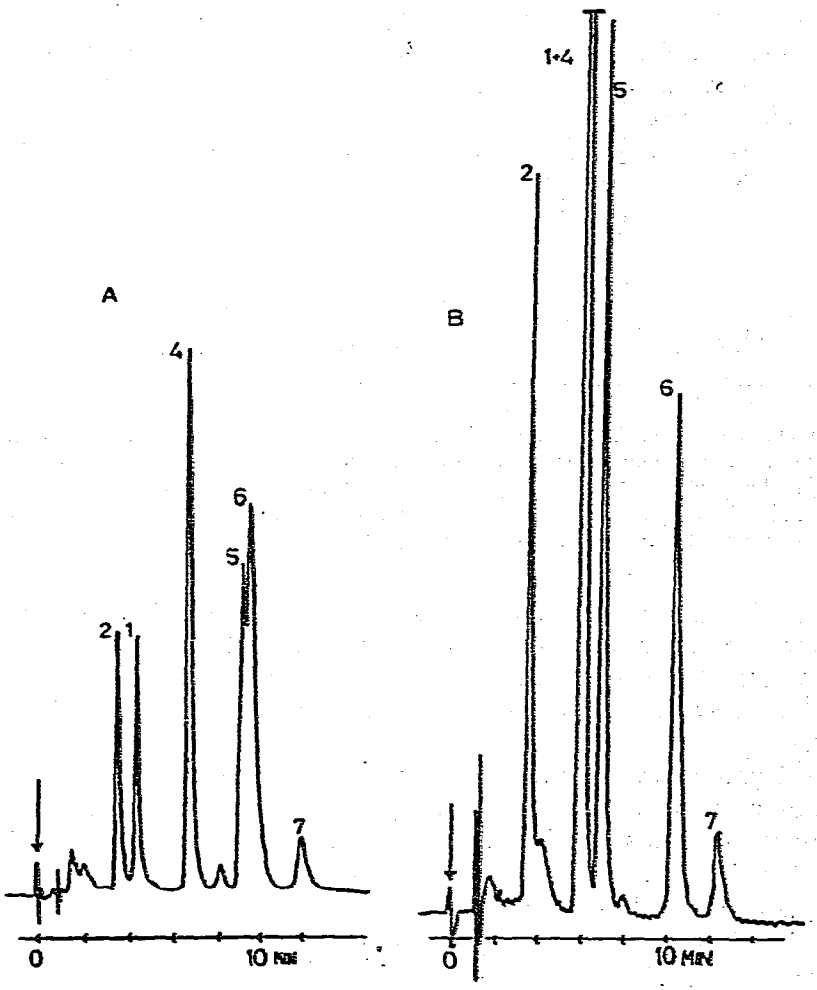

Fig. 1. Chromatogram of the separation of wyerone derivatives using a ODS Hypersil column (10 cm × 5 mm I.D.). Aliquots (5 μl) of a mixture of the phytoalexins in methanol were chromatographed using UV detection at 312 nm and 0.2 absorbance units for full scale deflection (a.u.f.s.). Eluent: A = methanol–water (60:40), flow-rate 1.5 ml/min; B = acetonitrile–water (50:50), flow-rate 1 ml/min. Peaks: 1 = wyerone epoxide; 2 = wyerone acid; 3 = dihydrowyerone acid (not resolved); 4 = wyerol; 5 = dihydrowyerol; 6 = wyerone; 7 = dihydrowyerone.

0.05–0.5 μg/10 μl and the other phytoalexins at 0.1–2.0 μg/10 μl. These levels very roughly reflected the range of concentrations found in plant extracts. Each solution contained 30 μg/10 μl of butyl salicylate standard.

Three analyses were made on each of these solutions using 10-μl injections. The areas of the phytoalexin peaks were expressed as the ratio to the area of the butyl salicylate peaks. Linear regression of the quantity of phytoalexin injected (as y) against the response ratio (as x) was carried out for wyerone epoxide (7), wyerone acid (3), dihydrowyerone acid (6), dihydrowyerol (5) and dihydrowyerone (4) and equations for the straight line calibrations were obtained.

The equations derived for the calibration of dihydrowyerol and dihydro-

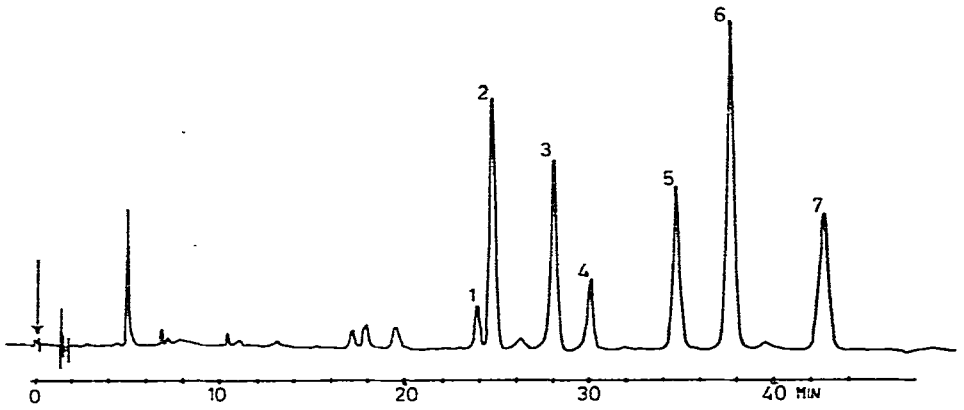

Fig. 2. Separation of wyerone derivatives using a ODS Hupersil column (20 cm × 8 mm I.D.). A 10-μl aliquot of a mixture of the phytoalexins in methanol was chromatographed using UV detection at 330 nm, 0.1 a.u.f.s., and gradient elution at a flow-rate of 5 ml/min. Initial solvent conditions were methanol–5% formic acid (35:65) running linearly over 30 min to final conditions methanol–acetonitrile–5% formic acid (40:10:50). Peak numbers as in Fig. 1.

wyerone were then used for wyerol and wyerone analyses. Thus the level of the dihydro derivatives present in the impure wyerol and wyerone samples was calculated at 10.6%. Linear regressions of the corrected wyerol (0.1–2.0 μg/10 μl) and wyerone (0.1–5.0 μg/10 μl) levels against the response ratios then gave calibration equations for these compounds.

The corrected calibration equations for all seven wyerone derivatives are given in Table I, the correlation coefficients demonstrate the good linearity obtained. From the standard deviations of the slopes and intercepts of these equations the expected 95% confidence limits for the determination of 1-μg quantity of each phytoalexin were calculated and are also given in the table.

Analysis of plant extracts

Extracts of bean cotyledon, leaf and pod tissues undergoing resistant reactions

TABLE I

CALIBRATION EQUATIONS FOR THE WYERONE PHYTOALEXINS

$y = ax + b$, where y is the amount (μg) of phytoalexin injected and x is the ratio of the phytoalexin peak area (330 nm) to that from 30 μg *n*-butyl salicylate.

Phytoalexin	*a (slope)*	*b (intercept)*	*Correlation coefficient*	*S.D. slope**	*S.D. intercept**	*S.D. for 1 μg*	*95% confidence limits for 1 μg (μg)*
Wyerone epoxide (7)	2.7276	−0.00883	0.9998	0.00698	0.00307	0.0033	±0.01
Wyerone acid (3)	3.6235	+0.01484	0.9995	0.02947	0.00980	0.0106	±0.02
Dihydrowyerone acid (6)	1.6931	−0.01094	0.9913	0.02788	0.00499	0.0097	±0.02
Wyerol (2)	3.0795	−0.01401	0.9983	0.02513	0.00767	0.0084	±0.02
Dihydrowyerol (5)	2.3964	−0.01559	0.9975	0.02243	0.00290	0.0056	±0.01
Wyerone (1)	2.7328	+0.03313	0.9997	0.00724	0.00634	0.0064	±0.01
Dihydrowyerone (4)	1.9720	−0.00167	0.9997	0.00592	0.00358	0.0069	±0.01

* $\sigma y = \sqrt{x^2 \sigma^2_{\text{slope}} + \sigma^2_{\text{intercept}}}$.

to infection by the fungus *Botrytis cinerea* Pers. have been analysed by this method. Tissues were extracted with diethyl ether as previously described[6] and the ethereal solution taken to dryness *in vacuo*. The solids were resuspended in an appropriate volume of methanol (1 ml methanol per 0.25–8 g original tissue) containing 3 mg/ml *n*-butyl salicylate. Aliquots of 10 μl were injected on to the ODS Hypersil column 20 cm × 8 mm I.D. Fig. 3 shows a typical chromatogram of a tissue extract.

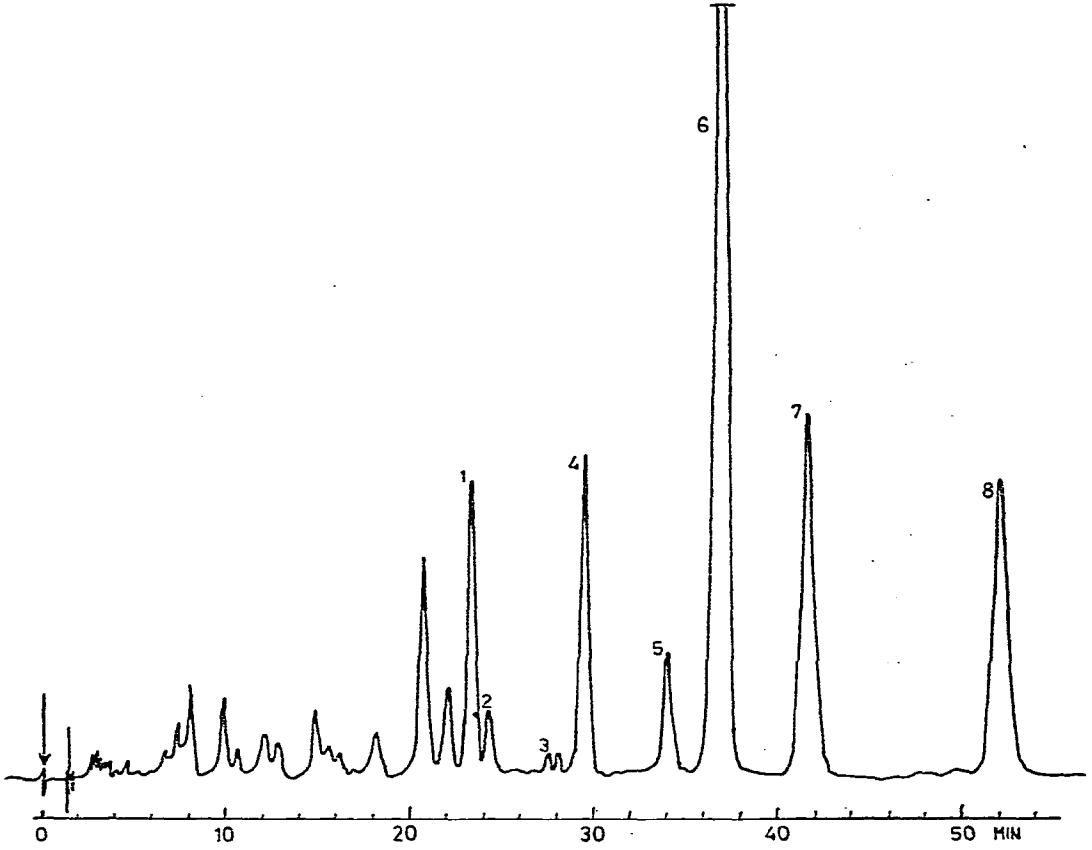

Fig. 3. Separation of wyerone derivatives from an extract of *Vicia faba* cotyledons prepared 6 days after their inoculation with *Botrytis cinerea*. A 10-μl aliquot of a methanolic solution of the tissue extract (0.5 g tissue per ml) was chromatographed as described in Fig. 2. Peaks numbers 1–7 as in Fig. 1; 8 = *n*-butyl salicylate (internal standard).

Even with thermostatic control of eluent and column temperatures, the precision of elution times of the phytoalexins was found to vary by 3–5% (for 95% confidence limits as a percentage of mean elution time). This means that the 95% confidence ranges for the elution times for the wyerone epoxide–wyerone acid and dihydrowyerone acid–wyerol pairs of peaks overlap. It was therefore found necessary to confirm the identity of peaks in the chromatogram of any previously unanalysed sample by "spiking" with authentic phytoalexins. Full details of the results obtained and their significance will be the subject of papers elsewhere.

CONCLUSIONS

Seven furanoacetylenic phytoalexins of the wyerone group, produced by the broad bean plant when challenged by *Botrytis* were separated by reversed-phase HPLC using gradient elution. Calibration with authentic standards gave a quantitative analysis applicable to plant extracts of biological interest.

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