

# A Rapid Quantitative Method for the Analysis of Sesquiterpene Phytoalexins by High Performance Liquid Chromatography

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A new high performance liquid chromatographic technique has been developed for the rapid analysis of sesquiterpene phytoalexins such as capsidiol, rishitin, lubimin and phytuberol. This method employs a cyanopropyl-bonded phase column with an isocratic mixture of hexane and isopropanol. When used with a flame ionization detector the lower limits of detection are about 0.1  $\mu\text{g}$  and the response of the detector is linear in the range 0.1–30  $\mu\text{g}$ . With the UV detector at 205 nm, the lower limits of detection are about 0.1  $\mu\text{g}$  for capsidiol and rishitin and 0.5  $\mu\text{g}$  for lubimin, phytuberol and debneyol. The response of the UV detector is linear in the range 0.5–30  $\mu\text{g}$ . Although both detectors proved to be useful, the signal response with the flame ionization detector was proportional to the mass of each of the phytoalexins, while the signal with the UV detector was proportional to the number of carbon–carbon double bonds in each of the compounds.

*Keywords:* High pressure liquid chromatography; phytoalexins; tobacco; pepper.

## INTRODUCTION

Many plants have been shown to synthesize phytoalexins (low molecular weight antimicrobial compounds) upon infection by pathogenic fungi or microbes or upon treatment with biotic elicitors such as cellulase or pectinase (Bailey, 1982). Most of the phytoalexins which are produced by members of the Solanaceae family (which includes potatoes, tomatoes, peppers and tobacco) are sesquiterpenes (Elakovich, 1987). Traditionally these sesquiterpenes have been analysed by gas chromatography or thin layer chromatography (Guedes *et al.*, 1982). Our laboratory has recently published a technique (Moreau *et al.*, 1990) for the analysis of lipid classes from plant tissues via high performance (pressure) liquid chromatography (HPLC) with a flame ionization detector (HPLC-FID). This study was undertaken to develop a similar technique for the analysis of sesquiterpene phytoalexins.

## EXPERIMENTAL

Cholesterol, stigmaterol, 2,4-dinitrophenylhydrazine and vanillin were obtained from Sigma Chemical Co. (St. Louis, USA). Acylated sterol glycoside was obtained from Matreya Chemical Co., (Little Gap, Pennsylvania, USA). Acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) was obtained from Aldrich Chemical Co. (Milwaukee, USA). HPLC-grade solvents were obtained from Burdick and Jackson (McGraw Pk., Illinois, USA). Cellulase (*Trichoderma viride*, type RS, Onozuku) was obtained from Karlan

Chemical Co. (Torrance, California, USA). Capsidiol, rishitin, and lubimin were generously provided by Drs. G. Spencer and H. Gardner, Peoria, Illinois, USA. Phytuberin, phytuberol and debneyol were purified from tobacco suspension cells and their identity was verified by gas chromatography–mass spectrometry (GC–MS).

Bell pepper (*Capsicum annuum*) fruit were obtained from a local market. The fruit was elicited as previously described (Watson and Brooks, 1984) by injecting with 20 mL cellulase from *Trichoderma viride* (1 mg/mL) and incubating for 3 days at 25°C. The fruit was cut in half and rinsed three times each with distilled water and with diethyl ether. The extracts were pooled and the diethyl ether phase was collected, dried under a stream of nitrogen and the residue redissolved in chloroform.

Cell suspension cultures of *Nicotiana tabacum* Ky 14, were obtained from Dr. Joseph Chappell, University of Kentucky, and were grown in a modified Murishige–Skoog media as previously described (Vogeli and Chappell, 1990). Cells were grown for 7 days and elicited with cellulase from *T. viride* (0.1  $\mu\text{g}/\text{mL}$  cell suspension) for 24 h. The cells were removed by filtration and the culture filtrate was extracted twice with an equal volume of diethyl ether.

HPLC analyses were performed on an ISCO Model 2350 HPLC pump equipped with a Valco manual injector, 10  $\mu\text{L}$  sample loop, an ISCO Model V<sub>4</sub> UV–visible detector and a Tracor Model 945 flame ionization detector. The column was a Spherisorb CN (100 mm  $\times$  3 mm i.d.; 5  $\mu$  particle size) and the solvents were hexane:isopropanol (97:3 v/v) with a flow rate of 0.5 mL/min.

Thin layer chromatography (TLC) of the sesquiterpenes was carried out on silica gel HPTLC–GHLF plates (Analtech, Newark, USA) developed in cyclohexane:ethyl acetate (1:1, v/v). After drying for 10 min, the plates were sprayed with vanillin–H<sub>2</sub>SO<sub>4</sub> (2.8 g vanillin in 100 mL MeOH containing 0.5 mL conc. H<sub>2</sub>SO<sub>4</sub>) and heated to 100°C for 10 min. In other experiments plates were sprayed with 2,4-dinitrophenylhydrazine and heated to 110°C for 10 min (Krebs *et al.*, 1969).

**Table 1. Separation of sesquiterpene phytoalexins and related compounds by HPLC, GC-MS and TLC**

Component	HPLC retention time (min)	GLC retention time (min)	TLC, $R_f$ (colour reaction) <sup>a</sup>
Phytuberin	1.5	—	0.81 (m)
Phytuberol	3.6	20.17	0.48 (m)
Debneyol	4.3	14.32	0.41 (p)
Rishitin	4.5	—	0.28 (b)
Lubimin	7.1	—	0.37 (b)
Capsidiol	11.7	8.47	0.17 (b)
Acetosyringone	14.0	—	—
Acylated sterol glycoside	18.2	—	0 (b)

<sup>a</sup> Colours after spraying with vanillin-sulphuric acid: m = magenta, b = blue, p = purple.

GC-MS analyses were performed on an Hewlett-Packard Model 5995B GC-MS. The column was a 15 m Me Silicone (Hewlett-Packard) and the temperature program started at 125°C and increased to 250°C at a rate of 4°/min.

MS [principal ions at 70eV;  $m/z$  (relative intensity)] were: phytuberol—309 (3), 266 (2), 205 (2), 189 (3), 133 (6), 131 (B); debneyol—292 (8), 207 (4), 202 (6), 189 (20), 187 (15), 162 (32), 147 (31), 145 (30), 131 (68), 119 (27), 105 (B); capsidiol—380 ( $[M]^+$ , 19), 365 (B), 290 (61), 275 (58), 219 (14), 147 (28).

## RESULTS AND DISCUSSION

For the development of the method, standards of capsidiol, rishitin and lubimin were separated by normal-phase HPLC using a cyanopropyl bonded-phase column. A silica gel column could have also been used, but the instability problems which are often encountered with silica gel columns (Moreau *et al.*, 1990) were not observed with the cyanopropyl column. After studies with several mobile phase compositions it was established that a mixture of hexane:isopropanol (97:3 v/v) gave suitable retention times of 4.5, 7.1 and 11.7 min for rishitin, lubimin and capsidiol, respectively (Table 1).

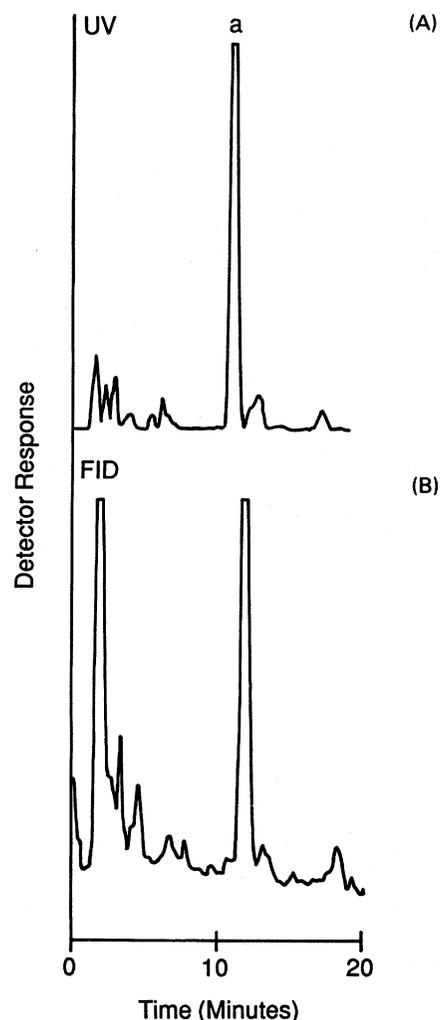
This HPLC method was then applied to the analysis of the phytoalexins accumulated by bell pepper fruit and by tobacco cell suspensions when both were elicited with cellulase. For bell pepper, treatment with cellulase resulted in the accumulation of phytoalexins in the locular space. The chromatograms obtained with either the flame ionization detector or the UV detector (205 nm) were similar (Fig. 1). A single major peak at 11.7 min co-chromatographed with capsidiol standard and confirmed earlier reports that this was the major phytoalexin produced by this plant tissue (Watson and Brooks, 1984). Capsidiol was absent in chromatograms of extracts from unelicited peppers (data not shown).

For tobacco cell suspensions, cellulase treatment resulted in the accumulation of several compounds in the culture medium, as previously reported (Tanaka and Fujimori, 1985; Watson *et al.*, 1985; Threlfall and Whitehead, 1988; Vogeli and Chappell, 1990). The HPLC chromatogram, obtained with either detector, contained three major peaks (at 3.6, 4.3 and 11.7 min), and several minor ones, with the 11.7 min peak co-chromatographing with capsidiol (Fig. 2). These peaks

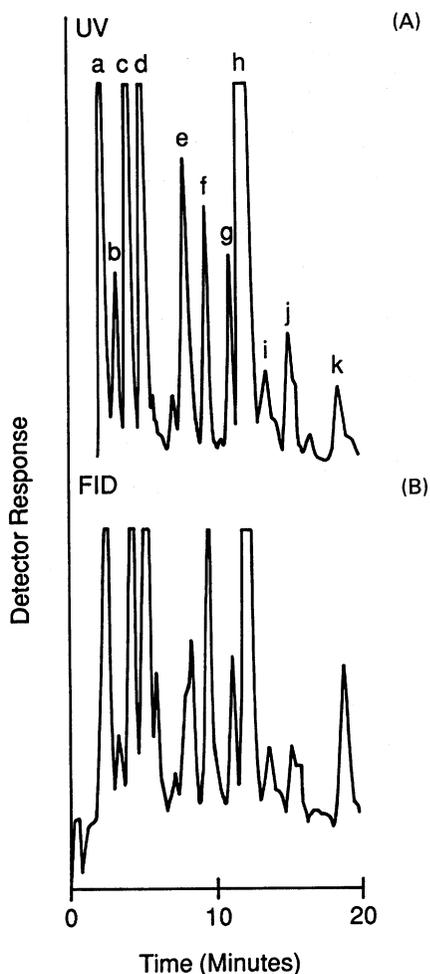
were absent in chromatograms of media extracts from unelicited tobacco cells (data not shown). The FID was then disconnected and each of the major peaks was collected for chemical identification. Material thus collected was used for further characterization by TLC and analysis by GC-MS. Tentative identification of the three major peaks from the tobacco samples and the one major peak from the bell pepper sample was made using TLC on silica gel layers followed by spraying with vanillin-sulphuric acid (Guedes *et al.*, 1982).

Material from the 3.6 min peak had an  $R_f$  of 0.48 and appeared magenta in color, suggesting that it was phytuberol (Guedes *et al.*, 1982). Confirmation of the identity of phytuberol was further indicated by the change in  $R_f$  to 0.9, with retention of the magenta color, upon acetylation of this material to phytuberin (Christie, 1982). Finally, GC-MS analysis of the TMS derivative of the compound comprising the 3.6 min peak gave the same major ion fragments (see Experimental) as those previously reported for phytuberol (Watson *et al.*, 1985).

The compound represented by the 4.3 min peak had an  $R_f$  of 0.41 and appeared purple when chromatographed by TLC and sprayed with vanillin-sulphuric acid. When the TMS derivative of the material from the



**Figure 1.** Chromatograms of phytoalexins produced by cellulase-elicited bell pepper fruit: (A) obtained with a UV detector at  $\lambda = 205$  nm, (B) obtained with a flame ionization detector. The major peak in both chromatograms was identified as capsidiol (a).



**Figure 2.** Chromatograms of phytoalexins produced by cellulase-elicited tobacco suspension cells: (A) obtained with a UV detector at  $\lambda = 205$  nm, (B) obtained with a flame ionization detector. The peaks in both chromatograms were identified as phytuberin and perhaps non-polar lipids (a), phytuberol (c), debneyol (d), capsidiol (h) and acylated sterol glycoside (k). The minor peaks (b), (e), (f), (g), (i), and (j), were not identified.

4.3 min peak was subjected to GC-MS analysis, it exhibited major ion fragments (see Experimental) which were consistent with the previously published direct probe MS of underivatized debneyol (Burden *et al.*, 1985). However, others have reported a fragment of  $m/z$  279 (which was not present in our spectrum) as the base peak for the di-TMS derivative of debneyol when analysed by GC-MS (Watson *et al.*, 1985). This discrepancy in fragmentation patterns may be due to experimental differences in obtaining the mass spectrum, and hence on the basis of all of the data obtained, the 4.3 min peak has been tentatively identified as debneyol. Further work will be necessary to identify this compound conclusively.

GC-MS analysis of the TMS derivative of the material of the 11.7 min peak (which had a blue colour in TLC) revealed the same major ion fragments and molecular ion (see Experimental) as those previously reported for capsidiol (Watson *et al.*, 1985).

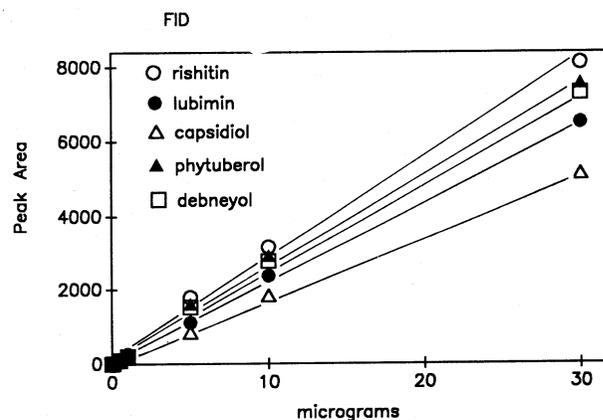
When the crude extract from elicited tobacco cell suspensions was subjected to GC-MS analysis, it was also found to contain, in addition to the three major components mentioned above, a small peak with major ion fragments identical to those previously reported for phytuberin (Watson *et al.*, 1985). Because phytuberin is

less polar than phytuberol its presence was sought in the small 1.5 min fraction (Fig. 2) eluting from the HPLC column. Using the TLC vanillin-sulphuric acid system, which produces a characteristic red colour for phytuberin and phytuberol, it was confirmed that a small amount of phytuberin was in the 1.5 min fraction (data not shown). In addition to phytuberin, a small amount of free sterol and other non-polar lipids were also detected in the 1.5 min fraction. The small peak at 18.2 min (Fig. 2) was coincident with that of standard acylated sterol glycoside (ASG), a common sterol component in most plant tissues (Moreau *et al.*, 1990). To our knowledge ASG has never been reported as being secreted into the growth media of suspension cells.

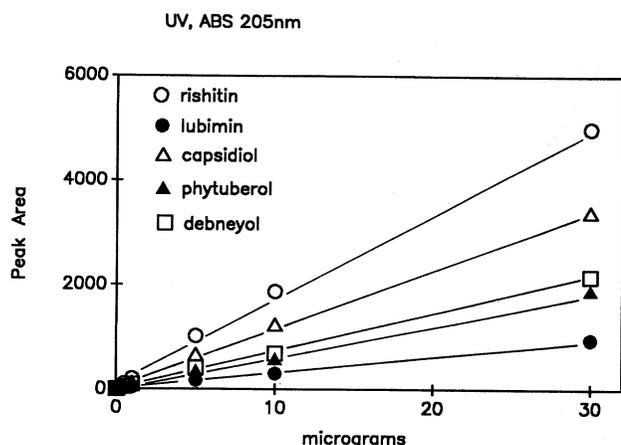
Table 1 shows the HPLC retention times of the six components identified above as well as ASG and acetosyringone. Apart from debneyol ( $T_r = 4.3$  min) and rishitin ( $T_r = 4.5$  min), each of the stress metabolites normally encountered in the tissues of Solanaceous plants are well resolved in this HPLC system. TLC and GC-MS analyses revealed that tobacco suspension cells produced debneyol but not rishitin and so we are confident that the chromatogram in Fig. 2 did not contain any rishitin. However, when using this technique in another tissue or cell type one would have to beware of this potential problem. The combination of these chromatographic analyses (HPLC, GC-MS and TLC) indicated that lubimin and solavetivone were not accumulated by the elicited tobacco cells. This was confirmed by using TLC followed by spraying with 2,4-dinitrophenylhydrazine and heating (Christie, 1982) which would have given a characteristic yellow colour if one of these two compounds had been present (data not shown).

Although others have reported that elicited tobacco cells produce acetosyringone (Threlfall and Whitehead, 1988), our chromatogram (Fig. 1) did not reveal its presence at 14.0 min. However, our analysis of the culture filtrate (Fig. 1) confirms earlier reports that these cells produce capsidiol (Threlfall and Whitehead, 1988; Vogeli and Chappell, 1990), phytuberin (Tanaka and Fujimori, 1985; Watson *et al.*, 1985), phytuberol (Tanaka and Fujimori, 1985; Watson *et al.*, 1985), and debneyol (Watson *et al.*, 1985; Threlfall and Whitehead, 1988).

In order for this HPLC method to be useful as a quantitative technique, it is necessary to relate peak



**Figure 3.** Calibration curves of the response of the FID detector with various amounts of phytuberol, debneyol, rishitin, lubimin and capsidiol.



**Figure 4.** Calibration curves of the response of the UV detector at  $\lambda = 205$  nm with various amounts of phytuberol, debneyol, rishitin, lubimin and capsidiol.

area to mass of sesquiterpene phytoalexins. Standards of phytuberol, debneyol, rishitin, lubimin and capsidiol in the range 0.1–30  $\mu\text{g}/10 \mu\text{l}$  injection were weighed and injected. The signal response of each of the five sesquiterpenes was similar ( $\pm 20\%$ ) at each mass value with the flame ionization detector (Fig. 3). The detector response was linear over the entire mass range 0.1–30  $\mu\text{g}$ . With FID, the sensitivity was inversely proportional to the retention time of each component, with capsidiol having a much longer retention time than the other components and a correspondingly lower sensitivity. We believe that the normal peak broadening that occurs with longer retention times appears to be accompanied by a decline in the response (of the FID) (data not shown).

With the UV detector, the signal output was essentially a function of the number of carbon–carbon

double bonds, which are the only UV chromophores present in sesquiterpenes (Fig. 4). The sesquiterpenes that contained two carbon–carbon double bonds (rishitin and capsidiol) produced stronger signals than each of the other three compounds which each contained only one carbon–carbon double bond. The minimum limits of detection of the UV detector were about 0.1  $\mu\text{g}$  for rishitin and capsidiol and about 0.5  $\mu\text{g}$  for the other sesquiterpenes. The signal response with the UV detector was linear from 0.5 to 30  $\mu\text{g}$  for each of the five sesquiterpenes.

A comparison of the two detectors indicates that the FID produces signals which are, for the most part, only dependent on the mass of sample injected, and the UV detector responds as a function of the degree of unsaturation of each compound, as expected. The major advantage of the FID is that one can more easily compare peak areas of the various components (since the standard curves of each of the components are quite similar, Fig. 3). For example, if one compares the peak area obtained with 10  $\mu\text{g}$  of capsidiol with that produced by 10  $\mu\text{g}$  of lubimin, they differ by about 20% with the FID and by about 500% with the UV detector. However, because the minimum limits of detection and linearity of response of the two detectors were quite similar, many researchers may find sesquiterpene analysis with the UV detector to be acceptable as long as its limitations are understood.

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