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Separation and identification of phytoalexins from leaves of groundnut (*Arachis hypogaea*) and development of a method for their determination by reversed-phase high-performance liquid chromatography

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ABSTRACT

Leaves of groundnut, *Arachis hypogaea*, infected with the early leaf spot fungus, *Cercospora arachidicola*, were extracted in aqueous ethanol and the phytoalexins partitioned into ethyl acetate. Flash chromatography of the ethyl acetate extract on silica gel yielded fractions with one to five compounds from which the phytoalexins could be isolated by semipreparative reversed-phase high-performance liquid chromatography (HPLC). The major phytoalexins were demethylmedicarpin, formononetin, 7,4′-dimethoxy-2′-hydroxyisoflavanone and medicarpin. Minor components were 7,2′-dihydroxy-4′-methoxyisoflavanone and daidzein. Compounds were identified by cochromatography and comparison of their ultraviolet and mass spectra with authentic samples using an HPLC system equipped with a diode-array detector, HPLC mass spectrometry and gas chromatography–mass spectrometry of their trimethylsilyl derivatives. A solid-phase extraction method was developed for processing large numbers of samples. Acetonitrile eluates from C_{18} cartridges were separated by reversed-phase HPLC and the phytoalexins quantified by reference to external standards of the authentic compounds.

INTRODUCTION

Phytoalexins are antimicrobial compounds which accumulate in plants in response to challenge by parasites and other traumas. In some interactions of plants and microbes there is good evidence that they are determinants of resistance [1,2]. We previously reported that the pterocarpan, medicarpin, was the predominant phytoalexin present in groundnut leaves infected by the fungus, *Cercospora arachidicola* [3]. However, further experiments showed that other antifungal compounds were also present. Before the role of medicarpin and these other phytoalexins in resistance to fungal leaf spot diseases could be assessed, it was necessary to identify them and devise means for their accurate determination.

In this paper, we report methods for phytoalexin isolation in quantities suffi-

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cient for structural determination and a technique for their quantitative analyis in small samples involving solid-phase extraction and reversed-phase high-performance liquid chromatography (HPLC). Since low concentrations of phytoalexins were elicited by abiotic agents such as UV light or the salts of heavy metals, leaves infected with *C. arachidicola* were used as a source of the antifungal compounds.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile and methanol were obtained from Fisons (Loughborourgh, UK). Ethyl acetate, methanol and cyclohexane (GPR) were purchased from BDH (Poole, UK). Chloroform and acetic acid (AR) were bought from May and Baker (Ongar, UK). Pure water was obtained from an Elga (High Wycombe, UK) pure-water system.

All other chemicals were of analytical grade and were purchased from BDH or Sigma (Poole, UK). Spherisorb ODS 1 (10 μm) was obtained from Phase Separations (Deeside, UK). Silica gel 60 and thin-layer chromatography (TLC) plates were obtained from BDH.

The phytoalexin standards demethylmedicarpin, medicarpin and 7,2′-dihydroxy-4′-methoxyisoflavanone were gifts from Professor P. M. Dewick (Department of Pharmacology, University of Nottingham, Nottingham, UK). Medicarpin, formononetin and daidzein were gifts from Professor W. Barz (Lehrstuhl für Biochemie der Pflanzen, Universität Münster, Münster, Germany).

Extraction of phytoalexins

Method 1. Groundnut leaves from the cultivars Flamingo and Egret (1 or 125 g fresh weight), infected with *C. arachidicola*, were collected from experimental plots near Harare, Zimbabwe and air-dried. They were vacuum-infiltrated with 60% ethanol and agitated at intervals for 24 h. After removal of the ethanol *in vacuo* at 40°C, the remaining aqueous solution was partitioned three times against ethyl acetate. The ethyl acetate fractions were combined and dried over anhydrous sodium sulphate.

Method 2. For analytical samples, leaves infected with *C. arachidicola* (1 g fresh weight) were extracted by facilitated diffusion[4] in 60% ethanol. The ethanol was decanted and diluted to 25% before being applied to a solid-phase cartridge. This consisted of 500 mg Techoprep C_{18} (25–40 μm: HPLC Technology, Macclesfield, UK), conditioned with methanol and washed with water. After application of the sample, the cartridge was washed with 5 ml of 25% ethanol, and the phytoalexins were eluted with 1 ml of acetonitrile.

The efficiency of these methods was examined by applying them to samples treated with silver nitrate, a poor elecitor of phytoalexins in groundnut leaves. Ten 5-μl droplets of silver nitrate solution ($10^{-3}M$) were applied to fully expanded, detached leaves and incubated for 48 h. Samples (1 g each), spiked with medicarpin (100 μg per sample) or not, were extracted by method 1 or method 2. In method 2, the cartridges were additionally eluted with a second millilitre of acetonitrile which was analysed separately. Material not retained on the cartridges was concentrated *in vacuo* and also analysed for the presence of medicarpin.

Isolation and purification of phytoalexins

Phytoalexins extracted by method 1 from large samples (125 g) were separated by a two-stage process consisting of flash chromatography [5] and semipreparative HPLC.

The ethyl acetate solution was evaporated to dryness *in vacuo*, the residue dissolved in 10 ml of chloroform and the chloroform solution separated on a column of silica gel 60 (Merck, 40–60 μm, 230–400 mesh, obtained from BDH; 150 mm × 20 mm). After conditioning the column with cyclohexane, the chloroform sample (5 ml) was applied and the phytoalexins eluted with a stepwise gradient of ethyl acetate in cyclohexane starting with 100% cyclohexane. At each step of the gradient (100 ml) the ethyl acetate content was increased by 10% (v/v), and two 50-ml fractions were cut. After the ethyl acetate concentration of the eluent had reached 50%, a final fraction consisting of 100 ml of 100% ethyl acetate was collected. All fractions were examined by analytical HPLC or TLC.

Phytoalexins in fractions from the silica column were isolated by semipreparative HPLC. The apparatus consisted of an Altex pump (Beckman Instruments, Berkeley, CA, USA), a column of Spherisorb ODS 1 (Phase Separations, 250 mm × 10 mm I.D.; 10 μm particle size), a Pye-Unicam UV detector (Philips Analytical, Cambridge, UK) set at 290 nm and a Tekman chart recorder (Tekman Electronics, Bicester, UK). Samples (250–500 μl) were introduced to the column via a 2-ml loop attached to an Altex valve and eluted in acetonitrile–water (1:1, v/v). Fractions corresponding to absorption peaks were collected manually. Purity of the compounds was checked by TLC using iodine vapour to visualize the spots and by HPLC using a system equipped with a diode-array detector (see below).

Thin-layer chromatography

Samples were run on TLC plates (silica gel 60 on an aluminium support; Merck No. 3554) in a tank saturated with a solvent system consisting of ethyl acetate–cyclohexane (1:1, v/v). Spots were detected by exposure to iodine vapour or spraying with diazotized *p*-nitroaniline [16], and those that were antifungal by spraying with a spore suspension of *Cladosporium cucumerinum* made up in half strength Czapek-Dox medium at a density of 0.8 absorbance units at 620 nm. After spraying, the plates were incubated at high humidity in the dark for 48 h [7].

Analytical HPLC

A Philips apparatus was used, consisting of two pumps (PU 4100), an automatic sampler (PU 4700) and a diode-array detector (PU 4021) interfaced to a trivector data system (Philips Analytical). Compounds were separated on a column of Spherisorb ODS 1 (250 mm × 4.6 mm I.D.; 10 μm particle size) protected by an Upchurch low-volume guard column (20 mm × 2 mm I.D., Anachem, Luton, UK) packed with Techoprep C_{18} (25–40 μm; HPLC Technology). The mobile phase was a gradient of acetonitrile in 1% acetic acid in which the acetonitrile concentration was increased from 35% to 40% over the first 12 min and then to 75% over the next 23 min. A reequilibration time of 5 min was allowed to elapse before the next sample was injected. The flow-rate was 1.5 ml/min and the eluent was monitored at 290 nm at an absorbance range of 0.04 a.u.f.s. Compounds were identified on the basis of retention time and comparison of their UV spectra with those of authentic samples. They were

quantified by comparison of peak areas with those of external standards. Purity of isolated compounds was tested by determining the similarity of several spectra cut during the elution of a peak.

Identification of phytoalexins

Ultraviolet spectroscopy. The UV spectra of purified compounds were obtained on a Varian-Cary spectrophotometer (Varian Assoc., Palo Alto, Ca, USA). Methanolic solutions were scanned from 220 to 340 nm.

Purified compounds were quantified by their absorbance using previously calculated molar extinction coefficients: medicarpin (287 nm), 2 log $\varepsilon = 3.90$ [8]; isoflavanones (277 nm), log $\varepsilon = 4.00$; formononetin (250 nm), log $\varepsilon = 4.35$ and demethylmedicarpin (287 nm), log $\varepsilon = 3.93$ [9].

Gas Chromatography–mass spectrometry (GC–MS). A Carlo Erba Strumentazione HPGC 5160 (Fisons Instruments, Crawley, UK) was fitted with a BP1 fused-silica/quartz capillary column (50 m × 0.32 mm I.D. with 0.5 μm film: SGE). The carrier gas, helium, was introduced into the system with a column head pressure of 130 KPa. Dried samples were derivatised in a mixture of trimethylchlorosilane (Sigma) and pyridine (1:1) and introduced into the instrument with a splitless on-column injector. The components were separated using the following GC programme: 50°C increasing to 250°C at 40°C/min with a further increase to 300°C at 5°C/min where it was maintained for 15 min. Ions were detected by a Hewlett-Packard 5970 series mass-selective detector (Hewlett Packard, Wokingham, UK) using an electron energy of 70 eV.

LC–MS. Samples were separated by gradient elution with a flow-rate of 1 ml/min on a Hichrom ODS 2 column (3 μm particle size; 150 mm × 4.9 mm I.D.). Solvent A was 20% acetonitrile in 0.1 *M* ammonium acetate and solvent B was 80% acetonitrile in 0.1 *M* ammonium acetate. Solvent B was maintained at 15% for 1 min, increased to 25% over the next 12 min and to 90% over the following 18 min. The column was reequilibrated by decreasing solvent B to 15% over 4 min and maintaining it at this percentage for the next 3 min. Analytes were monitored at 254 nm, and the mass spectra of the compounds giving rise to the peaks were determined by means of a Vestec 201 thermospray mass spectrometer with positive-ion discharge (1000 V: Vestec, Houston, TX, USA). Start conditions were: control, 144°C; tip heater, 288°C; source block, 326°C and vapour, 343°C.

HPLC–UV spectrophotometry. Samples were run on the analytical HPLC system and the retention times and spectra obtained from the diode-array detector compared with those of authentic compounds.

RESULTS

At least six antifungal zones that corresponded with areas of UV absorption were visible on TLC plates which had been spotted with extracts from diseased groundnut leaves and sprayed with spore suspension of *C. cucumerinum*. Flash chromatography of extracts resulted in fractions containing one to five major components which could be separated by semipreparative HPLC (Fig. 1).

Medicarpin had an R_F of 0.71 on TLC and gave a pale orange spot when

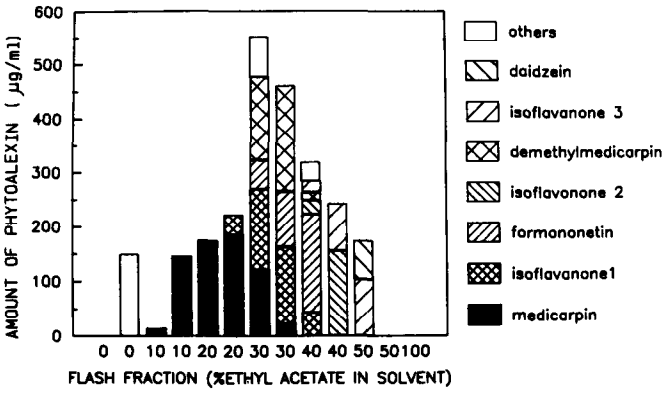


Fig. 1. Distribution of phytoalexins in fractions from flash chromatography on silica gel. Two 50-ml fractions were collected for each concentration of ethyl acetate in the mobile phase apart from the final fraction which was 100 ml and was eluted with 100% ethyl acetate. 'Others' were unidentified compounds. Isoflavanone 3 was a compound with a UV spectrum and mass fragmentation pattern corresponding to an isoflavanone but was not characterized further. Isoflavanone 2 was identified as 7,2'-dihydroxy-4'-methoxyisoflavanone by GC–MS of the TMSi derivative, cochromatography by HPLC and matching of the UV spectrum with that of an authentic sample of the compound. Isoflavanone 1 was identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone by GC–MS and LC–MS.

sprayed with diazotized *p*-nitroaniline. The absorption maxima in methanol were at 282 nm and 286.5 nm. GC–MS of the trimethylsilyl (TMSi) derivative gave m/z – 342 (M^+) and prominent peaks at 327 ($M^+ - 15$), 219 ($M^+ - 123$), 206 ($M^+ - 136$), 164 ($M^+ - 178$), 148 ($M^+ - 194$) and 73 (TMSi). On LC–MS the compound had a retention time of 24.72 min and gave an MH^+ of 271. The retention time on the analytical HPLC system was 21.12 min compared with 21.10 min for an authentic sample, and good agreement of UV spectra was obtained.

Formononetin had an R_F of 0.42 on TLC and gave a very pale orange–yellow derivative when sprayed with diazotized *p*-nitroaniline. The absorption maxima in methanol were at 249 and 299 nm. GC–MS of the TMSi derivative gave m/z – 340 (M^+) with prominent peaks at 325 ($M^+ - 15$), 208 ($M^+ - 132$), 163 ($M^+ - 177$), 132 ($M^+ - 208$) and 73 (TMSi). An MH^+ of 269 was obtained at a retention time of 21.72 min on LC–MS. On analytical HPLC an authentic sample eluted at 19.02 min and a compound with a similar spectrum from infected leaf samples at 19.10 min.

Demethylmedicarpin had an R_F value of 0.54 on TLC and gave a bright orange derivative on reaction with diazotized *p*-nitroaniline. The absorption spectra in methanol was similar to that of medicarpin with λ_{max} at 282 and 286.5 nm. GC–MS of the TMSi derivative gave m/z 400 (M^+) with prominent peaks at 385 ($M^+ - 15$), 219 ($M^+ - 181$), 206 ($M^+ - 194$), 185 ($M^+ - 215$) and 73 (TMSi). LC–MS gave a peak at retention time 12.60 min with MH^+ of 257. The authentic compound eluted at 11.20 min and a compound with a similar spectrum obtained from infected leaves at 11.10 min on the analytical HPLC.

An isoflavanone, referred to as isoflavanone 1, was identified as 7,4'-dimethoxy-2'-hydroxyisoflavanone, had an R_F value of 0.46 on TLC and produced a yellow–orange derivative when sprayed with diazotised *p*-nitroaniline. The absorption maxi-

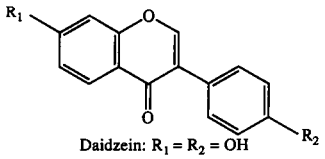
ma in methanol were at 277 and 311 nm. GC–MS of the TMS derivative gave *m/z* 372 (M^+) with prominent peaks at 357 ($M^+ - 15$), 209 ($M^+ - 163$), 193 ($M^+ - 179$), 164 ($M^+ - 208$), 149 ($M^+ - 223$) and 121 ($M^+ - 251$). LC–MS gave a peak at retention time 23.34 min with an MH^+ of 301. No authentic material was available for chromatographic and spectral comparison.

A second isoflavanone, referred to as isoflavanone 2, was identified as 7,2'-dihydroxy-4'-methoxyisoflavanone by co-chromatography and matching of the UV spectrum with that of authentic material. GC–MS of the TMS derivative gave *m/z* 430 (M^+) with prominent peaks at 415 ($M^+ - 15$), 281 ($M^+ - 149$), 222 ($M^+ - 208$) and 207 ($M^+ - 223$). LC–MS gave a peak at a retention time of 15.14 min with MH^+ 287. The retention times of the authentic compound and samples from infected leaves on analytical HPLC were 13.38 and 13.42 min, respectively, and their spectra corresponded with absorption maxima at 275 and 311 nm.

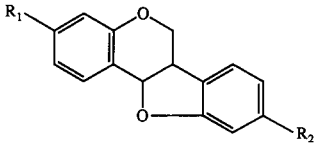
An isoflavone was identified as daidzein. GC–MS of the TMS derivative gave *m/z* 398 (M^+) with prominent peaks at 383 ($M^+ - 15$), 281 ($M^+ - 117$) and 207 ($M^+ - 191$). LC–MS gave a peak at a retention time of 9.39 min with an MH^+ of 255. The authentic compound eluted at 9.10 min on the analytical HPLC system and a compound with a similar spectrum (λ_{max} 245 and 2989 nm) obtained from infected leaves eluted at 9.13 min.

Structures of the six compounds are presented in Fig. 2.

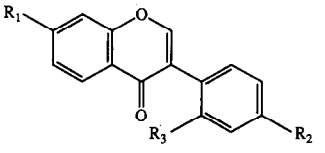
Analytical reversed-phase HPLC with a gradient of acetonitrile in 1% acetic acid on a column of Spherisorb ODS 1 (10 μm; 250 mm × 4.6 mm I.D.) gave good separation of the principal phytoalexins (Fig. 3). Demethylmedicarpin, formonone-



Daidzein: $R_1 = R_2 = OH$
Formononetin: $R_1 = OH$; $R_2 = OCH_3$



Medicarpin: $R_1 = OH$; $R_2 = OCH_3$
Demethylmedicarpin: $R_1 = R_2 = OH$



Isoflavone 1: $R_1 = R_2 = OCH_3$; $R_3 = OH$
Isoflavone 2: $R_1 = R_3 = OH$; $R_2 = OCH_3$

Fig. 2. Structures of the phytoalexins identified in leaves of groundnuts infected by the fungal parasite *C. arachidicola*.

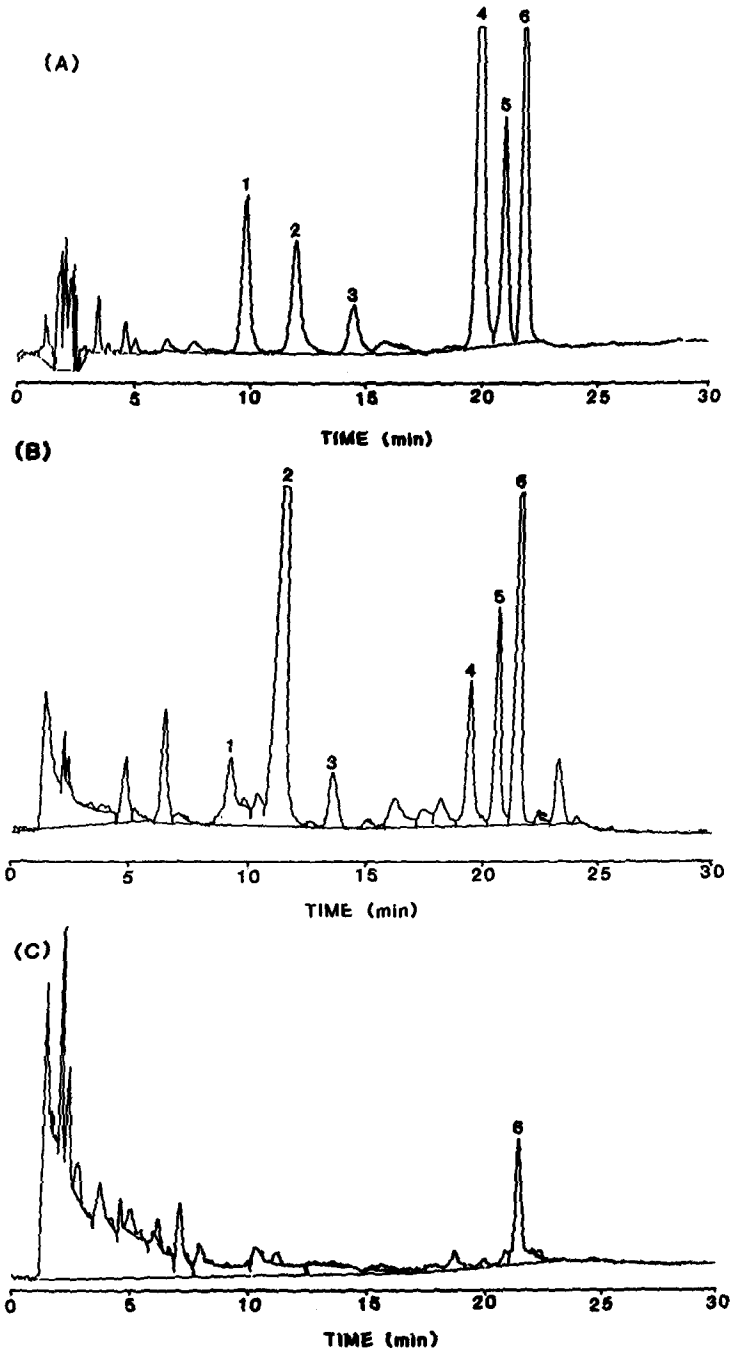


Fig. 3. HPLC of phytoalexins found in leaves of groundnuts infected by the fungal parasite *C. arachidicola*. (A) Chromatogram of six phytoalexin standards; a 10-μl sample containing the following concentrations of phytoalexins was injected: 1, daidzein, 50 μg/ml; 2, demethylmedicarpin, 25 μg/ml; 3, isoflavanone 2 (7,2'-dihydroxy-4'-methoxyisoflavanone), 10 μg/ml; 4, formononetin, 150 μg/ml; 5, isoflavanone 1 (7,4'dimethoxy-2'-hydroxyisoflavanone), 65 μg/ml; 6, medicarpin 100 μg/ml. (B) Typical chromatogram of an extract from infected groundnut leaves with peaks labelled as in (A). (C) Typical chromatogram of an extract from uninfected groundnut leaves showing traces of medicarpin only.

TABLE I

CONCENTRATIONS OF PHYTOALEXINS ACCUMULATED IN UNINFECTED AND INFECTED GROUNDNUT LEAVES (CV. EGRET) 18 DAYS AFTER INOCULATION WITH *CERCOSPORA ARACHIDICOLA*

Compound	Concentration (mean ± S.D., $n=5$) (μg/g fresh weight)	
	Infected	Uninfected
Daidzein	8.45 ± 3.98	5.48 ± 2.24
Demethylmedicarpin	57.50 ± 26.25	2.69 ± 2.48
Isoflavanone 2[a]	3.70 ± 1.84	0.37 ± 0.52
Formononetin	127.29 ± 7.79	4.79 ± 1.56
Isoflavanone 1[b]	53.82 ± 14.62	1.63 ± 1.32
Medicarpin	122.10 ± 25.75	14.32 ± 9.87

[a] Isoflavanone 2 was identified as 7,2′-dihydroxy-4′-methoxyisoflavanone by GC–MS of the TMSi derivative, co-chromatography by HPLC and matching of the UV spectrum with that of an authentic sample of the compound.
[b] Isoflavanone 1 was identified as 7,4′-dimethoxy-2′-hydroxyisoflavanone by GC–MS and LC–MS.

tin, 7,4′-dimethoxy-2′-hydroxyisoflavanone and medicarpin were prominent compounds in extracts of groundnut leaves (cv. Egret) infected with *C. arachidicola* and harvested 18 days later. Daidzein and 7,2′-dihydroxy-4′-methoxyisoflavanone were minor components. The phytoalexins were essentially absent from uninfected leaves apart from low concentrations of medicarpin (Fig. 3 and Table I).

Method 2, using solid-phase extraction, was as efficient as method 1 for the extraction of phytoalexins and more appropriate for the analysis of large numbers of samples (Table II). In tests of samples spiked with medicarpin, most of the compound

TABLE II

COMPARISON OF RECOVERY OF MEDICARPIN FROM LEAF EXTRACTS BY LIQUID–LIQUID AND SOLID-PHASE EXTRACTION

Treatment	Medicarpin (mean ± S.D., $n=3$) (μg/ml)
Liquid–liquid extraction (method 1)	
Unspiked sample [a]	12.55 ± 4.79
Spiked sample[b]	114.71 ± 4.68
Solid-phase extraction (method 2)	
Unretained eluate (unspiked)	0.00
Unretained eluate (spiked)	0.00
First ml of acetonitrile eluate (unspiked)	22.74 ± 5.57
First ml of acetonitrile eluate (spiked)	120.98 ± 5.65
Second ml of acetonitrile eluate (unspiked)	0.00
Second ml of acetonitrile eluate (spiked)	3.99 ± 3.92

[a] Unspiked samples were extracts of leaves which had been treated with silver nitrate (see text for details).
[b] Spiked samples were extracts of leaves which had been treated with silver nitrate and spiked with 100 μg of medicarpin (see text for details).

was eluted in the first millilitre of acetonitrile from the solid-phase cartridge and <4% in the second millilitre. In view of this result and the good recovery in the first millilitre of eluate a single millilitre would seem appropriate for routine work.

DISCUSSION

Accumulation of isoflavone and isoflavanone phytoalexins is a common response of legumes to challenge with phytopathogenic fungi [10,11]. The identification of the compounds reported in this paper (Fig. 2) was therefore not surprising although they contrast with the stilbenes elicited in groundnut cotyledons [12,13]. Formononetin, daidzein and 7,2′-dihydroxy-4′-methoxyisoflavanone are precursors of medicarpin [14,15], 4′-methylation of daidzein results in the formation of formononetin which is 2′-hydroxylated and reduced to the isoflavanone. Cyclisation of this compound leads to the formation of medicarpin. In contrast, demethylmedicarpin by analogy with the demethylation of pisatin [2] and comparison with the modification of medicarpin by the fungus, *Ascochyta rabiei* [16], is probably a degradation product.

With regard to the techniques described in this paper, the use of flash chromatography was a helpful first stage in the purification of phytoalexins and resulted in fractions containing one to five compounds. These were easily separated on semipreparative HPLC with an isocratic mobile phase. For the routine analysis of phytoalexins in small samples from infected groundnut leaves, solid-phase extraction proved to be a practical and reliable alternative to liquid–liquid extraction. Although reasonable separation was achieved on Sherisorb ODS 1 (10 μm) there is no doubt that the use of a smaller size of particle in the stationary phase would enhance resolution.

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