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GRADIENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ALKYLPHENONE RETENTION INDICES OF INSECTICIDAL EXTRACTS OF *PENICILLIUM* STRAINS

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SUMMARY

Purified extracts of four *Penicillium* strains which were active against the insect pest *Spodoptera littoralis* were analysed by gradient high-performance liquid chromatography (HPLC) for secondary metabolites using alkylphenone retention indices. HPLC of pure secondary metabolite standards detected previously in the extracts by thin-layer chromatography (TLC) was undertaken in order to obtain bracketed retention indices. More metabolites were detected by HPLC than by TLC, although some compounds detected by TLC in some strains were not detected by this HPLC method. A minority of metabolites were exclusive to each strain, and most were produced by more than one strain. The profiles were more characteristic of each strain when only the larger peaks were considered. This emphasizes the importance of detection limits in secondary metabolite analysis. Some of the implications of these analyses to fungus toxicity and systematic mycology are discussed.

INTRODUCTION

The development of alternatives to traditional insecticides is necessary because of increased levels of resistance in the insect population and concern about the effects of such compounds on the ecosystem. The potential of using secondary metabolites from microorganisms as insecticides is becoming increasingly apparent[1,2], including those from fungi. However, in many reports only uncharacterized crude extracts (*e.g.*, ref. 3), known mycotoxins[4,5] or compounds from known mycopathogens[6] have been tested. Very little is known about the complete metabolite spectrum of biologically active fungal extracts in general. However, the thin-layer chromatographic (TLC) profiles of seven crude ethanolic extracts of *Penicillium strains*, four of which were particularly active against the insect pest *Spodoptera littoralis* have been reported[7]. New toxicity was discovered when purified metabolites determined to be present in the

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crude extracts were tested. This work was progressed when brevianamide A, its photolysis product brevianamide D and ochratoxin A from two *Penicillium* strains were shown to be active against *S. frugiperda* and *Heliothis virescans* in dose response tests[8].

TLC has been used for over 20 years to detect fungal secondary metabolites, and diverse TLC methods for detecting mycotoxins have been employed[9]. An agar plug method with TLC for detecting secondary metabolites from cultures of fungi has been developed and the use of these compounds recommended as useful taxonomic characters[10]. A TLC database of fungal secondary metabolites in standard systems[11] has been used with the agar plug method in a multidisciplinary numerical taxonomic study of the terverticillate penicillia[12]. However, considerable variation in secondary metabolite production was observed, possibly owing to chemosyndromic variation, whereby the metabolite titre is sometimes below the detection limit of the analytical method employed.

High-performance liquid chromatography (HPLC) for the analysis of fungal secondary metabolites is particularly useful[13]. The specific detection systems associated with gas chromatography (GC) are either not available or less well developed for HPLC, but GC is less useful because most of the metabolites are difficult to volatilize. The use of alkylphenone retention indices with HPLC for mycotoxins has been recommended[14,15], and diode-array (DA) detection for HPLC is a significant advance[16], although (a) the sensitivity can be lower than that with more conventional detectors[17] (an unofficial figure being 10% lower), (b) UV spectra can vary in different solvent systems, as would be expected with gradient HPLC, especially those involving large pH changes, and (c) the equipment can be prohibitively expensive. However, databases of HPLC retention times[13] and bracketed retention indices (I_B)[16] can be used to identify tentatively the metabolites detected by HPLC used in other laboratories[15,16].

HPLC of purified extracts is described in this paper, using (a) alkylphenone retention indices and (b) the I_B of purified standards and also those provided by Frisvad and Thrane[16] to obtain additional information on the secondary metabolites produced by four of the most active insecticidal strains described in Paterson *et al.*[7] and also investigated in the taxonomic study of Bridge *et al.*[12].

EXPERIMENTAL

Fungi, growth and maintenance

Penicillium citrinum (IMI[a] 280297), *P. brevicompactum* (IMI 17456), *P. expansum* (IMI 232297) and *P. expansum* (IMI 297959) were stored at 4°C on Czapek-Dox agar (CZ)[18] and grown on yeast extract sucrose agar (YES)[19] at 25°C in the dark in 90-mm Petri dishes for 9 days.

Extraction and purification

Ten Petri dishes of each fungus were extracted three times with 90 ml of 90% aq. ethanol using a Waring blender at full speed. The extracts were filtered, pooled and

[a] IMI = the accession number of the culture collection of the CAB International Mycological Institute.

rotary evaporated to 80 ml. The remainder was defatted by the addition of 200 ml of hexane–90% aq. methanol (1:1, v/v), the hexane layer was discarded and the methanol layer was evaporated to dryness. Chloroform–water (1:1, v/v) was added and the chloroform layer removed and evaporated to dryness and the residue was dissolved in methanol (2 ml). Stock solutions of each alkylphenone standard were prepared in acetonitrile (20 μl/ml) and were mixed in equal volumes before use. A 2-μl aliquot of these was mixed with 10 μl either of fungal extract or pure metabolite.

Metabolite standards

These were obtained from a collection of metabolites held at the CAB International Mycological Institute[11].

Reagents

All solvents were of analytical-reagent grade, except those used for the elution of samples, where HPLC-grade acetonitrile and water were used. All alkylphenones were obtained from Aldrich (Gillingham, U.K.).

Chromatographic equipment

A Varian Model 5000 HPLC system with a variable-wavelength UV detector set to monitor at 254 nm was used for the analyses, which were performed on a 240 mm × 4.6 mm I.D. Spherisorb 5 ODS column (HPLC Technology, Macclesfield, U.K.) at 30°C with a flow-rate of 1.5 ml/min. Injections were made with an autosampler injector system (10 μl). A gradient solvent system with solvent A = acetonitrile and solvent B = water was used. The solvent programme was 10% A initially, raised to 50% in 30 min and 90% in 4 min, held for 4 min, then lowered to 10% in 7 min. The programme was based on that of Frisvad[13], except that trifluoroacetic acid was not added. The absorbance range was 0–0.1. A blank gradient was tested and only insignificant changes in absorbance were observed (chromatogram available).

Calculations

I_B values for secondary metabolite standards were calculated using the equation

$$I_B = \frac{(t_S - t_{P_1})\Delta z \cdot 100}{t_{P_2} - t_{P_1}} + z \cdot 100 \qquad (1)$$

where I_B = bracketed retention index, t_S = retention time of a secondary metabolite, t_{P_1} = retention time of the alkylphenone that elutes before the secondary metabolite, t_{P_2} = retention time of the alkylphenone that elutes after the secondary metabolite, z = number of carbon atoms in the alkylphenone that elutes before the secondary metabolite and Δz = the difference between the number of carbon atoms in the alkylphenones that elute before and after the secondary metabolite. The retention time (t) of acetophenone was used as t_{P_1} and that of propiophenone as t_{P_2} if t_S was less than the retention time of acetophenone.

The normalized retentions of alkylphenones and secondary metabolites were determined relative to butyrophenone and dodecanophenone to minimize coelution of secondary metabolites in extracts and alkylphenone standards. To calculate the

normalized retention index (I_N) of secondary metabolites in extracts, the normalized retention (N_i) was calculated with the equation

$$N_i = \frac{t_i - t_{C_{10}}}{t_{C_{18}} - t_{C_{10}}} \tag{2}$$

where N_i = the normalized retention of an alkylphenone (N_P) or a secondary metabolite (N_S), t_i = the retention time of the compound i being normalized, $t_{C_{10}}$ = the retention time of butyrophenone and $t_{C_{18}}$ = the retention time of dodecanophenone.

The retention time of each alkylphenone standard was obtained four times and the means and standard deviations were determined. The means were used for $t_{C_{10}}$ and $t_{C_{18}}$.

The following equation was used to calculate the normalized retention indices:

$$I_N = \frac{(N_S - N_{P_1})\Delta z \cdot 100}{N_{P_2} - N_{P_1}} + z \cdot 100 \tag{3}$$

where I_N = the normalized retention index, N_S = the normalized retention of a secondary metabolite, N_{P_1} = the normalized retention of the alkylphenone eluting before the secondary metabolite and N_{P_2} = the normalized retention of the alkylphenone eluting after the secondary metabolite.

If N_S was less than the normalized retention for acetophenone, then the N_P of acetophenone was used as N_{P_1} and the N_P of propiophenone was used as N_{P_2}.

Identification of peaks in extracts

The I_N values of peaks from the extracts were compared with the I_B values of the standards to identify the peaks in chromatograms. Other compounds were identified on the basis of published I_B data[16]. As the I_B values of the standards were consistently lower than those published[16] by a mean and standard deviation of 112 ± 24.9 (Table II), the I_N values of the peaks were correspondingly increased to permit comparisons with the published I_B data. This procedure sometimes provided an equivocal possible identification, and some metabolites were therefore identified by comparison with TLC data[7] and by analysis of the cultures using the agar plug–TLC method[12] (see Table III). The other peaks were named on the basis of the most likely metabolite to be produced by these strains from data provided in other publications[12,13,20–22]. For example, if a metabolite from *Aspergillus* or *Penicillium* had a similar I_B[16] to the corrected I_N for a peak, then the *Penicillium* metabolite was chosen for the peak identification.

Similarity analysis

Percentage similarities between strains were determined as $[X/(Y-X)] \cdot 100$, where X = number of secondary metabolites shared and Y = total number of metabolites produced by both strains. Average percentage linked similarities were determined and the dendrograms drawn in the usual manner.

TABLE I

MEANS AND STANDARD DEVIATIONS OF THE RETENTION TIMES (t) AND NORMALIZED RETENTION TIMES (N_i) OF THE ALKYLPHENONE STANDARDS ($n=4$)

Alkylphenone	$t \pm$ *S.D. (min)*	N_i
Acetophenone	17.2 ± 0.06	−0.944
Propiophenone	23.7 ± 0.01	−0.424
Butyrophenone	29.0 ± 0.01	0.000
Valerophenone	33.6 ± 0.15	0.368
Hexanophenone	35.3 ± 0.10	0.504
Octanophenone	37.2 ± 0.00	0.656
Decanophenone	38.9 ± 0.05	0.792
Dodecanophenone	41.5 ± 0.16	1.000

RESULTS

The standard deviations of the retention times for the alkylphenones were consistently low (Table I), and on this basis were satisfactory. The I_B values for the six secondary metabolites are given in Table II, together with the equivalent values obtained by Frisvad and Thrane[16].

In general, more peaks were obtained from the chromatograms of extracts obtained by the purification procedures described than those from the crude extracts. The two alkylphenone standards did not mask major metabolite peaks in either the *P. brevicompactum* or the *P. citrinum* strains, but two peaks with similar retention times were masked in each of the *P. expansum* strains, and in *P. expansum* IMI 232297 one of the peaks was of a considerable height [*i.e.*, approximately 5 percentage units (see Fig. 1)]. The chromatograms run without the alkylphenones were broadly similar to those

TABLE II

BRACKETED RETENTION INDICES (I_B) OF SEVEN FUNGAL SECONDARY METABOLITES ($n=2$)

The mean and standard deviation of the differences between the values is 112 ± 24.9, which was used as an adjustment factor to identify unknowns.

Metabolite	I_B	
	This work	*Ref. 16*
Brevianamide A	737	865
Citrinin	835	919
Cyclopenol	673	779
3,5-Dimethyl-6-methoxyphthalide	866	–
Ochratoxin A	929	1086
Patulin	588	684
Xanthocillin:		
Peak 1	928	1057
Peak 2	979	1067
Peak 3	1020	1149
Peak 4	1091	1182

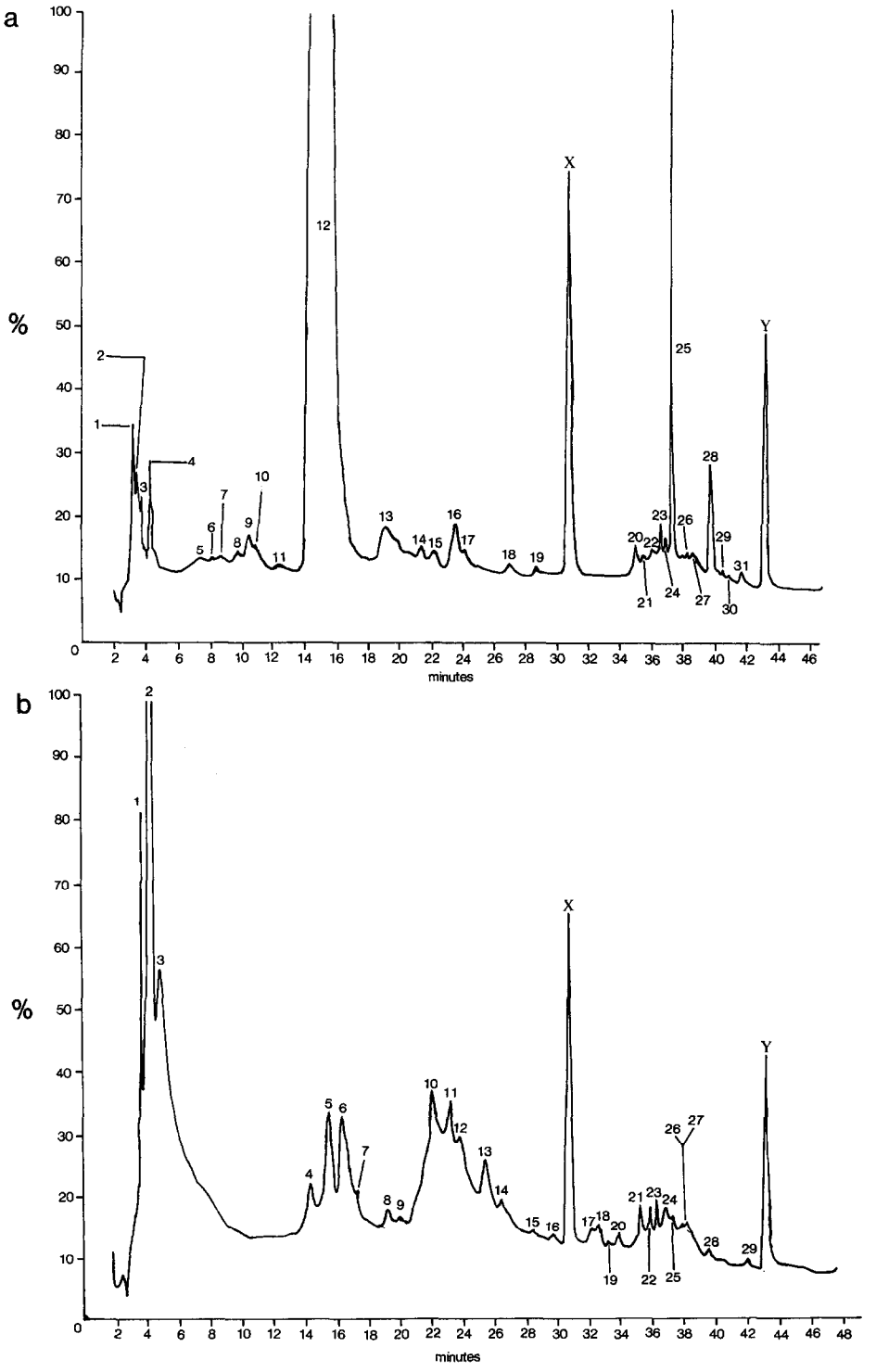

Fig. 1.

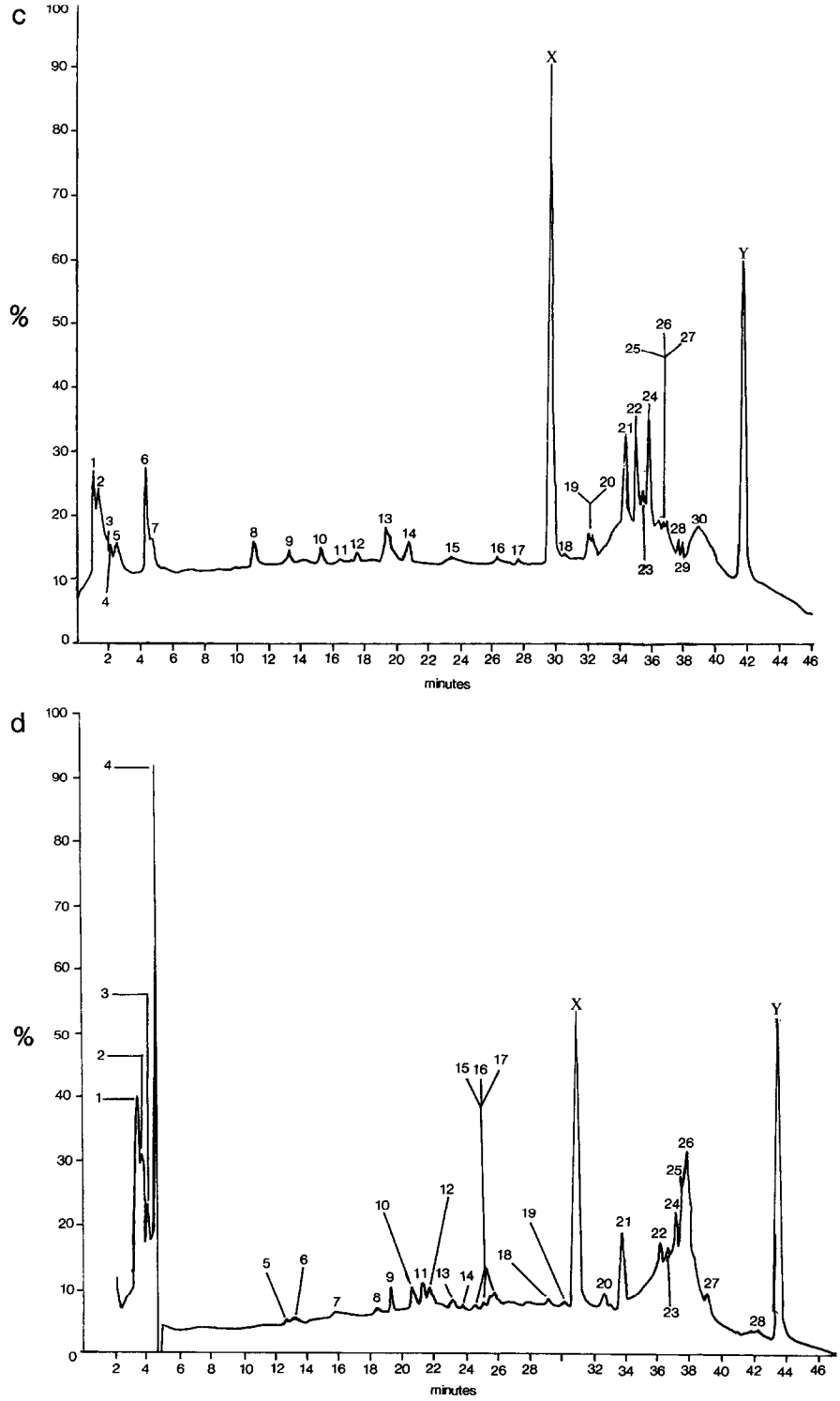

Fig. 1. HPLC of the extracts. For conditions, see Experimental. X = butyrophenone; Y = dodecanophenone. I_N values of peaks are given in Tables III and IV. (a) *Penicillium brevicompactum*; (b) *P. citrinum*; (c) *P. expansum* (IMI 297959); (d) *P. expansum* (IMI 232297).

with the alkylphenones, although possibly two peaks were missing from those of the *P. expansum* strains, which is indicative that the alkylphenones are not as completely unreactive as suggested[15], and might combine with certain metabolites and elute together. However, this phenomenon needs to be examined further, and is beyond the scope of the present work.

Peaks were more evenly distributed throughout the chromatogram and more metabolites were detected in the HPLC of the *P. brevicompactum* (IMI 17456) purified extract (Fig. 1a) than in that of the unpurified extract. It is possible that the first four compounds were eluted in the void volume and did not truly interact with the stationary phase, as indicated by the low I_N values for these peaks. Peak 12 could represent coelution of brevianamide A and mycophenolic acid, as preliminary work, indicated that the retention time of mycophenolic acid was similar to brevianamide A. Thirty-one peaks were detected from this extract, although some of the broad bands probably represent coelution of more than one metabolite (*e.g.*, 9 and 10, 13, 16 and 17).

The chromatogram of the purified extract was much more informative than that of the unpurified extract of *P. citrinum* (Fig. 1b), and numerous peaks were detected. The first three metabolites were possibly eluted in the void volume because of the low I_N values for these peaks. Most of the other peaks did not elute separately, and were connected at the lower section of the peaks, although the tops of the peaks were sharp and separated (*e.g.*, peaks 4–6 and 10–13). Twenty-nine peaks were detected in this extract. Resolution into separate peaks in some areas was not optimal.

More peaks were observed in the purified extract (Fig. 1c) than the unpurified extract of *P. expansum* (IMI 297959). Approximately the first four compounds were possibly eluted in the void volume. Many of the metabolites were well separated (*e.g.*, 6, 8, 9 and 10), although a shoulder (peak 7) was observed on peak 6; however, this was characteristic of patulin. Peak 7 was considered to be the second peak of roquefortine B (Table III) because the retention index data were similar to those of this metabolite. Thirty peaks were detected in this extract.

The metabolites appeared to be at a lower concentration in the pure extract of *P. expansum* (IMI 232297) than in the other strains (Fig. 1d), and in this instance the HPLC of the purified extract was similar to that of the unpurified extract. The first peaks coeluted in the first 3 min, and perhaps eluted in the void volume. Apart from these, the largest peaks were Nos. 21-26. Twenty-eight peaks were detected in this extract.

Peak assignments and TLC data are given in Table III, and an alphabetical list is given in Table IV; metabolite profiles, with minor peaks discounted, are given in Table V.

Fig. 2 shows an average linked percentage similarity dendrogram based on all the metabolites detected (Table III), indicating more similarity in the strains than when only the more major components were considered (Fig. 2, Table V). The two *P. expansum* strains had the most similar metabolite profiles, although large differences were observed, especially when minor peaks were discounted.

TABLE III

IDENTIFIED PEAKS IN THE CHROMATOGRAMS WITH THE ASSOCIATED NORMALIZED RETENTION INDICES (I_N)

The numbers in italics before the I_N values refer to the peak number as given in Fig. 1a–d. P.b. = *Penicillium brevicompactum*; P.c. = *P. citrinum*; P.e.[1] = *P. expansum* (IMI 232297); P.e.[2] = *P. expansum* (297959).

Metabolite	I_N[d]							
	P.b.		*P.c.*		*P.e.*[1]		*P.e.*[2]	
Gentisyl alcohol	*1*	546						
Penicillic acid	*6*	625						
Terrestric acid	*7*	636						
Orsellenic acid	*8*	652						
Roquefortine A	*9*	660						
Cyclopenol	*10*	666[a]			TLC[b]		TLC[b]	
5′-Hydroxyaspertin	*11*	691[b]						
Desacetylpebrolide	*14*	832[b]						
Rubratoxin B	*19*	961[c]	TLC[c]		TLC[c]		TLC[c]	
E	*30*	1642						
Mycophenolic acid	TLC[b]		*4*	728				
Cyclopenin			*6*	757				
Roquefortine C			*9*	815				
Viridicatin			*12*	873				
Cyclopiazonic acid			*18*	1034				
Mycelianamide			*19*	1053				
Lichexanthone			*26*	1279				
B			*28*	1471				
PR-toxin					*6*	710		
Frequentin					*10*	821		
Fulvic acid					*11*	831		
Griseophenone C					*13*	858	TLC[b]	
Purpurogenone	TLC[c]		TLC[c]		TLC[c]		*19*	1065[c]
Chrysophenol							*21*	1140
A							*28*	1453
Patulin					*4*	575[a,b,c]	*6*	603[a,b]
6-Methylsalicylic acid					*5*	702	*8*	704
Lapidosin					*9*	800	*12*	804[c]
Norlichexanthone					*16*	892	*15*	896
Islandicin					*26*	1240	*24*	1246
Roquefortine B:								
Peak 1	*2*	550					*1*	551
Peak 2	*5*	614					*7*	606
Erythroglausin	*26*	1346					*26*	1347
C	*28*	1507					*29*	1496
D	*29*	1610					*30*	1605
Roquefortine D			*3*	584			*5*	575
Palitantin	TLC[b]		*7*	769			*10*	771
Naphthaline anhydride			*27*	1292			*25*	1322
Ochratoxin A	*18*	923[ab]	*15*	923[a]				
Viridicatum toxin	*20*	1094	*21*	1093				
Skyrin	*25*	1226	*25*	1226				

(Continued on p. 162)

TABLE III *(continued)*

Metabolite	I_N[d]							
	P.b.		*P.c.*		*P.e.*[1]		*P.e.*[2]	
Pachybasic acid			*14*	907	*17*	898		
Carlosic acid			*2*	571	*3*	567	*4*	573
Xanthocillin:								
Peak 1	TLC[c]		*16*	934[a,c]	*18*	971[a,c]	*16*	950[a,c]
Peak 2					*19*	992[a]	*17*	977[a]
Peak 3			*17*	1028[a]	*20*	1038[a]	*18*	1031[a]
Peak 4			*20*	1066[a]	*21*	1060[a]	*20*	1071[a]
Kojic acid	*3*	556			*1*	557	*2*	556
Penitrem A	*24*	1205			*25*	1213[b]	*23*	1208
Aflatrem	*27*	1400			*27*	1387	*27*	1372
Griseofulvin	*17*	878[b]	*13*	899	*15*	885		
Viomellein:								
Peak 1	*21*	1107	*22*	1126	*22*	1129		
Peak 2	*22*	1148	*23*	1149	*23*	1157		
F	*31*	1706	*29*	1700	*28*	1699		
Terrein	*4*	564[c]	*1*	563[c]	*2*	562[c]	*3*	565[c]
Brevianamide A	*12*	733[a,c]	*5*	746[a]	*7*	744[a,c]	*9*	739[a,b]
3,5-Dimethyl-6-hydroxyphthalide	*13*	798[c]	*8*	803[c]	*8*	789[c]	*11*	789[c]
Citrinin	*15*	846[a]	*10*	848[a,b,c]	*12*	836[a,b,c]	*13*	834[a,c]
3,5-Dimethyl-6-methoxyphthalide	*16*	866[a,c]	*11*	865[a,c]	*14*	869[a,c]	*14*	854[a,c]
Xanthomegnin	*23*	1191	*24*	1178	*24*	1186	*22*	1176
Metabolites detected by TLC but not HPLC:								
P. brevicompactum canadensic acid[b]								
Unknown metabolites detected by TLC:								
P. brevicompactum P[c], "O"[b]								
P. citrinum P[c]								
P. expansum (IMI 232297) E[c], P[c]								
P. expansum (IMI 297959) P[c]								

[a] Compounds identified by comparisons with data for standards measured under the same conditions as given under Experimental.

[b] Compounds detected by the TLC–agar plug method[11]: Merck aluminium TLC sheets, silica gel 60, layer thickness 0.2 mm. TLC system 1: solvent system, toluene–ethyl acetate–90% aq. formic acid (5:4:1, v/v/v); spray reagent, 0.5% (v/v) anisaldehyde in methanol–acetic acid–concentrated sulphuric acid (17:2:1, v/v/v), heated for 8 min at 105°C. TLC system 2: solvent system, chloroform–acetone–propan-2-ol (85:15:20, v/v/v/); spray reagent, 1% (w/v) cerium (IV) sulphate in 3 *M* sulphuric acid. For full details see ref. 11.

[c] Compounds detected in ref. 7; TLC methods as for footnote *b* except 90% ethanolic extracts of agar cultures were used and TLC plates were developed in system 1 only.

[d] I_N values without a superscript letter *a–c* were identified on the basis of previously published I_B data[16]. Where TLC[b] or TLC[c] appears in the columns, this means detected by the particular TLC method only, and not detected by HPLC.

TABLE IV

ALPHABETICAL LIST OF METABOLITES IN EXTRACTS AND I_N VALUES

Column headings as in Table III.

Metabolite	I_N							
	P.b.		*P.c.*		*P.e.*[1]		*P.e.*[2]	
Aflatrem	*27*	1400			*27*	1387	*27*	1372
Brevianamide A	*12*	733	*5*	746	*7*	744	*9*	739
Carlosic acid			*2*	571	*3*	567	*4*	573
Chrysophenol							*21*	1140
Citrinin	*15*	846	*10*	848	*12*	836	*13*	834
Cyclopenin			*6*	757				
Cyclopenol	*10*	666						
Cyclopiazonic acid			*18*	1034				
Desacetylpebrolide	*14*	832						
3,5-Dimethyl-6-hydroxyphthalide	*13*	798	*8*	803	*8*	789	*11*	789
3,5-Dimethyl-6-methoxyphthalide	*16*	866	*11*	865	*14*	869	*14*	854
Erythroglaucin	*26*	1346					*26*	1347
Frequentin					*10*	821		
Fulvic acid					*11*	831		
Gentysyl alcohol	*1*	546						
Griseofulvin	*17*	878	*13*	899	*15*	885		
Griseophenone C					*13*	858		
5'-Hydroxyaspertin	*11*	691						
Islandicin					*26*	1240	*24*	1246
Kojic acid	*3*	556			*1*	557	*2*	556
Lapidosin					*9*	800	*12*	804
Lichexanthone			*26*	1279				
6-Methylsalicylic acid					*5*	702	*8*	704
Mycelianamide			*19*	1053				
Mycophenolic acid			*4*	728				
Naphthaline anhydride			*27*	1292			*25*	1322
Norlichexanthone					*16*	892	*15*	896
Ochratoxin A	*18*	923	*15*	923				
Orsellenic acid	*8*	652						
Pachybasic acid			*14*	907	*17*	898		
Palitantin			7	769			*10*	771
Patulin					*4*	575	*6*	603
Penicillic acid	*6*	625						
Penitrem A	*24*	1205			*25*	1213	*23*	1208
PR-toxin					*6*	710		
Purpurogenone							*19*	1065
Roquefortine A	*9*	660						
Roquefortine B:								
Peak 1	*2*	550					*1*	551
Peak 2	*5*	614					*7*	606
Roquefortine C			*9*	815				
Roquefortine D			*3*	584			*5*	575
Rubratoxin B	*19*	961						
Skyrin	*25*	1226	*25*	1226				
Terrein	*4*	564	*1*	563	*2*	562	*3*	565
Terrestric acid	*7*	636						
Viridicatin			*12*	873				

(Continued on p. 164)

TABLE IV *(continued)*

Metabolite	I_N[d]							
	P.b.		*P.c.*		*P.e.*[1]		*P.e.*[2]	
Viomellein:								
Peak 1	*21*	1107	*22*	1126	*22*	1129		
Peak 2	*22*	1148	*23*	1149	*23*	1157		
Viridicatum toxin	*20*	1094	*21*	1093				
Xanthocillin:								
Peak 1			*16*	934	*18*	971	*16*	950
Peak 2					*19*	992	*17*	977
Peak 3			*17*	1028	*20*	1038	*18*	1031
Peak 4			*20*	1066	*21*	1060	*20*	1071
Xanthomegnin	*23*	1191	*24*	1178	*24*	1186	*22*	1176
Unidentified:								
A							*28*	1453
B			*28*	1471				
C	*28*	1507					*29*	1496
D	*29*	1610					*30*	1605
E	*30*	1642						
F	*31*	1706	*29*	1700	*28*	1699		

TABLE V

METABOLITE PROFILES OF EXTRACTS EXCLUDING MINOR PEAKS OF LESS THAN 5%

Column headings as in Table III. + = Detection of metabolite.

Metabolite	*P.b.*	*P.c.*	*P.e.*[1]	*P.e.*[2]
6-Methylsalicylic acid				+
Purpurogenone				+
Chrysophenol				+
Penitrem A				+
Naphthaline anhydride				+
Erythroglausin				+
D				+
Gentisyl alcohol	+			
Roquefortine A	+			
Skyrin	+			
3,5-Dimethyl-6-hydroxyphthalide	+			
Xanthomegnin	+			
C	+			
Mycophenolic acid		+		
Cyclopenin		+		
Viridicatin		+		
Griseofulvin		+		
Lapidosin			+	
Frequentin			+	
Patulin			+	+
Xanthocillin			+	+
Xanthomegnin			+	+
Islandicin			+	+

TABLE V *(continued)*

Metabolite	*P.b.*	*P.c.*	*P.e.*[1]	*P.e.*[2]
Viridicatum toxin	+	+		
Brevianamide A	+	+		
3,5-Dimethyl-6-methoxyphthalide	+	+		
Carlosic acid		+	+	
Viomellein		+	+	
Roquefortine D		+		+
Citrinin		+		+
Roquefortine B	+			+
Kojic acid	+		+	+
Terrein	+	+	+	+

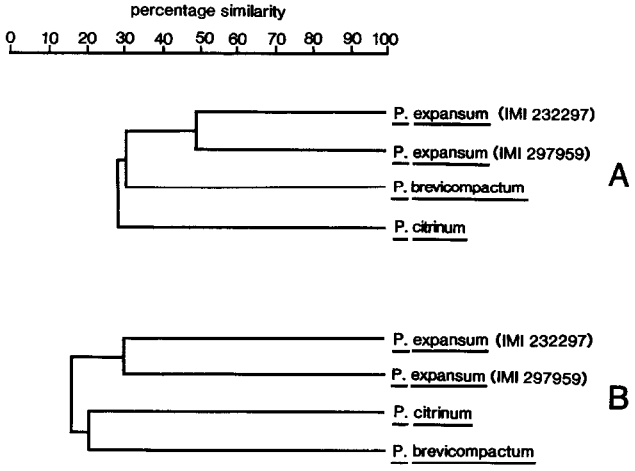

Fig. 2. Average linked percentage dendrograms. A, For all peaks; B, excluding minor peaks.

DISCUSSION

Separation is less linear for the higher molecular weight alkylphenones than for those described by Hill *et al.*[15] (Table I). However, the peaks were sharp, well separated, had a suitably large range of retention times (17.2–41.5 min) and separation was consistent, as shown by the low standard deviation of the retention times. Consequently, they were considered suitable for the standardization of the HPLC system described here. A different gradient might make the separation more linear.

The I_B values of secondary metabolites (Table II) suggested that there was sufficient consistency in the data with those of Frisvad and Thrane[16] for tentative identifications to be made, especially as the correction factor for the difference in the results was used. In addition, the peaks of the metabolites were sharp. However, mycophenolic acid might be an exception which requires further investigation (see

results under *P. brevicompactum*). The HPLC of the alkylphenones and the standard fungus metabolites indicated that the chromatographic conditions employed were suitable for the analysis of the fungus extracts.

Purification of extracts provided more informative chromatograms, except with *P. expansum* (IMI 232297) where it was not as marked. Compounds with a wide range of polarities can be separated using the method described here (Fig. 1a–d). However, optimum separation of all compounds was not obtained, although a comparative profile of the detected metabolites was achieved.

Numerous metabolites were produced by each fungus, and most appeared to be shared by at least one other strain if all peaks were considered (Table III, Fig. 1a–d). However, exclusive compounds were also produced by each strain (Table III). The metabolite profiles became more specific to each strain if minor peaks (*i.e.*, peak height less than 5%) were excluded. Secondary metabolites have been emphasized as taxonomic characters[10], but the data presented here (Tables I and III) indicate the importance of detection limits in the use of analytical methods for taxonomic characters, because when all peaks are considered the strains appear more similar than when only the larger peaks were considered (Fig. 2).

There was considerable dissimilarity in the metabolite profiles of the two *P. expansum* strains (Fig. 2), although the profiles were the most similar of the strains tested. Interestingly, the two strains grouped in separate clusters in the dendrogram from a numerical study of the terverticillate penicillia[12], which indicates consistency with the HPLC results presented here.

Comparisons of I_N data with published data does not give equivocal identifications of the metabolite peaks; however, useful tentative identifications and comparative metabolite profiles of fungi are obtained. Analysis of standard metabolites under conditions identical with those used for the extracts and TLC data help to improve the confidence in peak identification. The use of other techniques is necessary for complete structural identification, *e.g.*, HPLC–mass spectrometry, nuclear magnetic resonance spectrometry, diode-array detection.

The HPLC method described is of great use in providing information on the number and range of metabolites produced by fungi. However, the TLC method used by Paterson *et al.*[7] detected metabolites that were not detected by the HPLC method (Table III), indicating that TLC methods can sometimes be more specific than a generalized HPLC method. This is probably due to the more specific detection systems for certain metabolites that were used in the TLC method (other UV wavelengths, specific spray reagent, different solvents, etc.), and possibly coelution of compounds in HPLC. However, non-detection of metabolites can often be due to the inherent variability of fungal metabolite production in general[12,21,23], possibly because of the complex genetic constitution of some members of the penicillia[23].

The use of secondary metabolites as taxonomic characters is complicated by the fact that numerous compounds will almost certainly be produced and emphasis on any particular compound or compounds can sometimes be difficult to justify. Often great importance is attached to highly UV-absorbing compounds at unknown concentration (*e.g.*, refs. 13, 16 and 17). However, it is sometimes wrongly stated that they are produced at a high concentration *per se*. The issue of whether truly high-titre metabolites are more taxonomically significant than low-titre metabolites is unclear. At the level of the genetic control of regulation only a few genes are involved, and small

mutations could affect the titre significantly[24], and the situation is complicated by the probability that secondary metabolites are under less precise genetic control than primary metabolites[25]. Also, strains similar at the gross genetic level[26] can differ in other properties[12,23]. In general, the use of secondary metabolites in constructing taxonomies is valid only if considered together with other characters as undertaken in the numerical taxonomic study of Bridge *et al.*[12]. However, secondary metabolites can be useful in the identification of strains.

The results presented here indicate that a large number of metabolites are produced by the strains and the observed toxicity could be due to the combined effects of one or more of these compounds. Paterson *et al.*[7] showed that a number of the metabolites present in extracts can be toxic. It is therefore important to consider all toxic metabolites produced by fungi in areas where toxicity is important (*e.g.*, in foods) and not simply to consider a limited number of the most widely known, and easily detected, mycotoxins.

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