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STANDARDIZED ONE- AND TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC METHODS FOR THE IDENTIFICATION OF SECONDARY METABOLITES IN *PENICILLIUM* AND OTHER FUNGI

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SUMMARY

Standardized thin-layer chromatographic (TLC) data in two solvent systems are presented for secondary metabolites of *Penicillium* and other fungi to assist in the identification of products of *Penicillium* species, based on a study of 304 strains. Rapid identification aids are needed to check for activity during preservation for mycotoxin production, and for screening systematic and biotechnological purposes. The data are stored on a microcomputer which permits flexible storage, retrieval, and updating of information. Of 107 metabolites detected with TLC system 1 [solvent: toluene–ethyl acetate–90% formic acid (5:4:1, v/v/v)], 80 (75%) are named and 27 (25%) as yet unidentified compounds are alloted reference numbers; in the case of the 97 metabolites detected by system 2 [solvent: chloroform–acetone–propan-2-ol (85:15:20, v/v/v)] the equivalent figures are 79 (81%) and 18 (19%) respectively.

INTRODUCTION

Approximately 3000 fungal metabolites have been listed of which many have biological activities of potential commercial and/or medical significance[1–3]. Over 300 mycotoxins are known, formed by 350 species: when variations within species are considered, it has been estimated that there are 10 000 mycotoxin producers which threaten foodstuffs[4], although it is not clear if each is distinct genotypically. Approximately 30 mycotoxins have been listed recently produced by *Penicillium*, although that list is admittedly incomplete[5]. In a recent survey of scientists from 30 countries it was revealed that the most needed research on mycotoxins was rapid and improved methods for analysis[6]. Several animal and/or human health problems can arise if links within the food chain are contaminated with mycotoxins. On the other hand, the potential of useful metabolites has not been fulfilled with the notable exception of β-lactams (cephalosporins and penicillins). Considerable ambiguity exists in the biochemical literature because of uncertainties and errors of identification in particular groups within *Penicillium*. The above facts prompted an integrated multidisciplinary approach to the systematics of *Penicillium* within which the rapid detection of the metabolites produced by numerous strains was required[7,8]. The present

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study was devised primarily for this purpose and the results will be included in the final report of this project. However, the results will also assist in (a) screening for mycotoxins, antibiotics, insecticides, etc., (b) checking for reported activities before, during, and after preservation procedures of strains, and (c) the identification of compounds detected by thin-layer chromatography (TLC) in other fungi.

TLC methods have been used to detect fungal secondary metabolites for over 20 years but in most cases published reports have been concerned with only a few metabolites or species[9–12]. A comprehensive review of diverse TLC methods for mycotoxins is available[13], but this does not include the complete range of non-toxic products of potential or actual economic significance and does not present standardized TLC data.

TLC has been used in chemotaxonomic studies of secondary metabolites for many years in the lichen-forming fungi[14] and a recent publication of standardised data is available[15]. In addition, a standardised TLC method has been reported recently as an identification aid for *Cortinarius*[16]. In *Penicillium* certain secondary metabolites detected by TLC have been employed for taxonomic purposes[17–20], although all detectable metabolites have not been considered previously.

Data are provided here on the TLC characteristics of 79 purified metabolites, most of which have been reported to be produced by *Penicillium*[1,2], together with other metabolites detected from 304 *Penicillium* strains studied. The TLC characteristics of unnamed metabolites are also given to facilitate reference to them by future workers. Results are presented in a standardized tabular form suitable for continuous updating, and form a reference data base for future chemotaxonomic and biochemical work in filamentous fungi as this is the largest set of such data hitherto compiled.

MATERIALS AND METHODS

Isolates were stored at −20°C on Czapek Dox agar (Cz)[21] slopes and inoculated onto malt extract agar (MEA)[21] slopes and incubated at 25°C for 7 days. Petri dishes containing either yeast extract sucrose (YES)[22] or Czapek yeast autolysate (CYA)[21] media were inoculated from the MEA slopes and analysed after 7 days growth at 25°C.

For system 1, agar plugs were removed from the colonies grown on YES using a 4-mm flamed cork borer and the agar ends of the plugs were placed at the origin of the TLC plates (20 × 20 cm Merck aluminium sheet, silica gel 60, layer thickness 0.2 mm) with a flamed dissecting needle; each strain represented at a unique position on the origin. The plugs were held in position for 10 s with slight pressure applied by the dissecting needle and then removed. The plates were developed in toluene–ethyl acetate–90% formic acid (5:4:1, v/v/v) (TEF) and examined by the visualization methods described in Table I.

In system 2, the plugs were removed from colonies grown on CYA and a drop of chloroform–methanol (2:1, v/v) was placed on the colony end of the plug which was placed quickly onto the origin of a TLC plate and removed. These plates were developed in chloroform–acetone–propan-2-ol (85:15:20, v/v/v) (CAP) and examined using the visualisation techniques shown in Table I. All purified metabolites were dissolved in chloroform–methanol (2:1, v/v) before analysis and were studied at least in triplicate to obtain standard deviations. All TLC plates were developed with a

griseofulvin standard and occasionally other standards. Strong natural and artificial light was avoided throughout the extraction and development procedures.

The agar plug method was used in a two dimensional (2D) TLC system in the case of some citrinin and ochratoxin A producing strains which had been grown on YES under the standard conditions given above. TLC plates were cut to produce 10 × 10 cm plates. The extraction procedure was as described above for system 1. The origin was situated at the lower left side of the plate (Fig. 1). Development in the first dimension was with TEF and in the second dimension with CAP; visualization was by UV light (365 nm). To confirm the identity of ochratoxin A after development in CAP, the spot was removed from the plate by scraping, ochratoxin A was dissolved in distilled water, and redeveloped using TEF.

The identification of metabolites for both 1D and 2D TLC was based on a comparison of TLC characters to those of purified metabolites and to lesser extents from (a) known characteristics from published results, and (b) by comparison with isolates known to produce specific metabolites.

RESULTS

Table I presents the TLC characteristics of 107 and 97 metabolites analysed by systems 1 and 2 repectively; of these, 80 (74%) and 79 (81%) are named. Examples of isolates which produce the unidentified metabolites detected by system 1 and system 2 are presented in Table II. Systems 1 and 2 can detect the same 43 (54%) of the

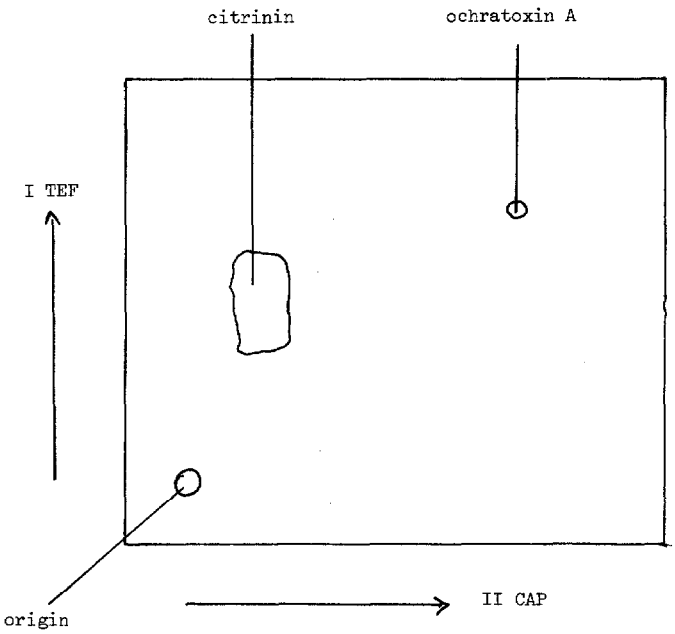

Fig. 1. Diagram of a TLC plate developed in toluene–ethyl acetate–90% formic acid (5:4:1, v/v/v) (TEF) in the first dimension then chloroform–acetone–propan-2-ol (85:15:20, v/v/v) (CAP) in the second. Plates were examined under UV Light (365 nm). Citrinin and ochratoxin A give bright green and turquoise spots respectively.

TABLE I

THIN-LAYER CHROMATOGRAPHY CHARACTERISTICS OF THE METABOLITES IN SYSTEMS 1 AND 2

Visualisation methods: System 1. 1, white light; 2, UV light (365 nm); 3, UV light (254 nm); 4, UV light (254 nm) for 30 s, return to 365 nm; 5, 0.5% (v/v) anisaldehyde in methanol–acetic acid–concentrated sulphuric acid (17:2:1, v/v/v); heat for 8 min at 105°C + white light; 6, anisaldehyde spray + UV light (365 nm); 7, anisaldehyde spray + UV light (254 nm); 8, anisaldehyde spray + UV light (254 nm) for 30 s, return to 365 nm. System 2. 1, white light; 2, UV light (365 nm); 3, 1% (w/v) ceric sulphate in 6 *N* sulphuric acid + white light; 4, ceric sulphate spray + UV light (365 nm). Colours/shape: b, blue; bk, black; br, brown; c, cream; d, dark; f, faint; fl, fluorescent; g, green; gy, grey; l, light; o, orange; p, purple; pk, pink; r, red; t, turquoise; v, violet; y, yellow; (t), tail. Column headings: *a* is the number given to metabolites for the coded data subjected to cluster numerical analysis; *b* represents present/absent in *Penicillium* strains studied; *n* is the number of measurements of R_F, ( ) appears after the value for *n* indicating the number of times the R_F was measured; *s.d.* is the standard deviation of R_F; $\bar{x}R_F$ is the mean of R_F; unidentified metabolites are given a letter code; " " means also detected by Frisvad and Filtenborg[20].

No.	*a*	*Metabolite*	*System 1*					
			b	*n*	*S.D.*	$\bar{x}R_F \times 100$	*1*	*2*
1	1	Austdiol	–	7	1	19	–	–
2	4	Brefeldin A	+	6	3	26	–	–
3	2	Brevianamide A	+	19	2	22	–	g
4	8	Canadensic acid	+	4	2	41	–	g
5	13	Canadensolide	+	3	1	49	–	–
6	15	Canescin	–	3	4	43	–	v
7	16	2-Carboxy-3,5-dihydroxyphenyl acetyl carbinol	Multiple spots					
8	5	Carlosic acid	+	3	1	10	y	–
9	11	Carolic acid	+	4	1	18	–	–
10	12	Chaetoglobosin A	+	5	5	48	–	–
11		Cinnamic acid	–	3	0	53	–	–
12	7	Citrinin	+	6	5	43	–	g(t)
13	9	Compactin	+	4	4	32	–	–
14	14	Cyclopenin	+	5	1	35	–	–
15	6	Cyclopenol	+	4	1	25	–	–
16	10	α-Cyclopiazonic acid	+	10	6	49	p/r(t)	y(t)
17	19	11-Deacetoxywortmannin	+	4	1	35	–	g/gy(t)
18	17	Desacetylpebrolide	+	3	2	15	–	–
19		Dechlorogriseofulvin	–	4	2	37	–	b
20		Dehydrocanadensolide	–	3	2	46	–	–
21		Dehydrocarolic acid	–	3	6	49	–	–
22	21	3,5-Dimethyl-6-hydroxyphthalide	–	5	2	57	–	v
23	22	3,5-Dimethyl-6-methoxyphthalide	–	7	2	56	–	v/p
24	24	Dipicolinic acid	–	4	2	23	–	–
25	18	Duclauxin	+? Multiple spots					
26		Epoxysuccinic acid	Too variable					
27	23	Ergosterol	Multiple spots					
28		Ethisolide	Not detected					
29	25	Frequentin	+	4	1	35	–	br/y
30	24	Fulvic acid	+	4	1	44	y	b
31	28	Gentisic acid	+	4	1	44	y	t
32	27	Gentisyl alcohol	+	4	1	26	–	–
33		Gladiolic acid	Multiple spots					
34		Glauconic acid	Not detected					

						System 2								
3	*4*	*5*	*6*	*7*	*8*	*Code*	*b*	*n*	*S.D.*	$\bar{x}R_F + 100$	*1*	*2*	*3*	*4*
–	–	p	br	–	–		+	5	3	51	–	b	v(fl)	b
–	–	p	b	–	–		+	1(5)	–	42	–	–	–	b(d)
–	–	–	g	–	–		+	7	2	60	br	g	br	g
–	–	p/b	b	–	–	Too variable								
–	–	t	v	–	–	Not detected								
–	–	t	v	–	–		+	3	7	79	–	b	–	–
							+	3	1	43	–	v/g	–	–
br	–	y	b	–	–	Multiple spots								
–	–	y(t)	br	–	–		–	1(5)	–	23	p(l)	–	–	–
–	–	gy/br	d	–	–		+	6	5	69	–	–	o/br	br
bk/br	–	o	–	–	–		–	1(5)	–	73	–	br	–	–
–	–	–	g(t)	–	–		+	12	1	11	br(f)	g	–	g
–	–	br/p	b	–	–		+	3	4	72	–	v	v	b
–	–	y(f)	b/p	–	–		+	4	10	73	–	g	–	y
–	–	br	d	–	–		+	3	5	64	–	–	o(fl)	d
–	–	p(t)	d	–	–		+	6	3	21	–	y(t)	br/r	br/r
–	–	t	–	–	–	Not detected								
–	–	p/bk	b	–	–	Not detected								
–	–	o	b/o	–	–	Multiple spots								
y	–	y(l)	–	y	–	Not detected								
–	–	y(t)	g(t)	–	–	Multiple spots								
–	–	r(f)	p	–	–		+	4	4	75	–	v	v(fl)	v
–	–	–	p(l)	–	–		+	4	2	74	–	v	v(fl)	p
–	–	b	–	–	–	Not detected								
							+	3	5	81	–	y	g	o
						Not detected								
							+	3	5	66	–	g/b	v	g/b
						Not detected								
–	–	bk/gy	bk	–	–	Not detected								
–	–	y	b	–	–		+	3	3	54	y	t	y	y/g
–	–	g	b	–	–		+	3	4	19	–	b/v	–	v
–	–	br	bk	–	–		–	2(6)	–	51	–	–	v(fl)	–
						Not detected								
						Not detected								

(Continued on p. 254)

TABLE I *(continued)*

No.	*a*	*Metabolite*	*System 1*					
			b	*n*	*S.D.*	$\bar{x}R_F \times 100$	*1*	*2*
35	29	Gliotoxin	+	7	3	38	–	–
36	26	Griseofulvin	+	22	3	33	–	v/b
37	30	Griseophenone C	+	5	1	48	–	–
38		Hadacidin	Not detected					
39	31	5′-Hydroxyaspertin	+	5	1	25	–	t
40	32	*m*-Hydroxybenzoic acid	–	4	1	50	–	v/b
41		*p*-Hydroxybenzoic acid	Not detected					
42		Hydroxyisocanadensic acid	Multiple spots					
43		Itaconic acid	–	1(5)	–	76	–	br
44		Kojic acid	Multiple spots					
45		Lapidosin	–	3	1	17	y	–
46		Lichexanthone	–	3	3	67	–	br/o
47	34	6-Methylsalicylic acid	–	4	1	57	–	b
48		Monorden	–	3	3	47	–	b
49		Mycelianamide	–	4	3	32	br(t)	br
50	33	Mycophenolic acid	+	11	4	41	–	v(f)
51	35	*β*-Nitropropionic acid	+	4	3	38	–	–
52	36	Norlichexanthone	+	4	1	53	y	y/o
53	37	Ochratoxin A	+	10	2	48	–	t
54	38	Orsellenic acid	–	3	2	52	–	–
55	42	Palitantin	+	3	1	19	–	–
56	40	Patulin	+	8	3	35	–	y
57	39	Penicillic acid	+	10	4	34	–	–
58	41	Penitrem A	+	4	2	62	–	b
59		Phoenicin	–	5	1	41	y/br	br/bk
60	43	PR toxin	+	3	4	48	y(t)	–
61	45	Purpurogenome	–	5	4	67	pk/r	o/r
62		2-Pyruvoylaminobenzamide	Multiple spots					
63	47	Roqufortine B	+	6	1	2	–	–
64	48	Roqufortine C	+	1(10)	–	6	–	–
65	50	Roscopurpurin	Multiple spots					
66		Rubratoxin A	–	1(4)	–	5	–	–
67		Rubratoxin B	–	2(5)	–	11	–	–
68	46	Rugulovasine A	+	5	1	17	–	–
69		Secalonic acid	–	5	4	42	y/br	br
70	51	Scytalone	+	4	1	34	–	g
71	52	Spinulosin	–	3	3	43	p	br
72		Sterigmatocystin	–	4	1	57	–	o/r
73		Tenuazonic acid	Multiple spots					
74	54	Terrein	+	7	2	16	–	–
75	53	Terrestric acid	+	13	3	32	–	–
76	55	Viomellein	+	5	1	37	y/br	d
77		Viridicatum toxin	–	2(5)	–	34	–	o/y
78	57	Wortmannin	–	6	2	35	y	y/o
79	59	Xanthocillin	+	3	1	54	y/br	g
80*	58	Xanthomegnin	+	7	2	23	y/br	d
81	61	"m"		19	3	22	–	–
82	62	"r"		1	–	22	–	–

						System 2								
3	*4*	*5*	*6*	*7*	*8*	*Code*	*b*	*n*	*S.D.*	$\bar{x}R_F + 100$	*1*	*2*	*3*	*4*
–	–	g/br	d	–	–		–	4	6	67	br	v	b(f)	v(d)
–	–	p	v/b	–	–		+	16	3	62	–	v	–	v
–	–	o/r	d	–	–		+	3	3	65	–	–	v(fl)	b/g
							Not detected							
–	–	y	t	–	–		+	4	3	56	–	b/g	v(fl)	g/y
–	–	–	v/b	–	–		+	4	3	52	–	b	v(fl)	–
							Too variable							
							Not detected							
–	–	–	–	–	–		Not detected							
							Too variable							
–	–	g	br	–	–		Too variable							
–	–	y/o	o	–	–		–	4	7	77	–	o/br	–	–
–	–	pk	b	–	–		+	4	7	39	–	v	v(fl)	–
–	–	bk/gy	–	–	–		–	2(4)	–	67	–	gy	–	o/br
–	–	br/r	br	–	–		Too variable							
v	–	gy	–	–	–		+	5	4	57	–	v	–	–
–	–	c	g	–	–		Not detected							
–	–	y	y	–	–		–	3	4	70	–	br/o	y	–
–	–	–	t	–	–		Multiple spots							
–	–	r	bk	–	–		+	2(5)	–	45	–	v	v(fl)	–
–	–	br/bk	br	–	–		+	1(4)	–	36	–	–	–	y/g
–	–	y/br	o/br	–	–		–	5	8	59	br/y	b/gy	–	b/g
–	–	g/br	v	–	–		Not detected							
–	–	v	bk	–	–		+	5	9	75	y/br	g	g/gy	br/g
–	–	br	br	–	–		–	4	1	19	pk	b	g	g
–	–	br(t)	br(t)	–	–		Multiple spots							
–	–	o/r	r/o	–	–		+	8	7	84	y	o	–	–
							Multiple spots							
–	–	p	br	–	–		+	5	2	5	–	b	br/r	br/y
–	–	b/gy	–	–	–		+	61	9	34	–	–	o	d
							+	5	11	73	y	o	–	–
–	–	pk	y/g	–	–		Not detected							
–	–	br	b	–	–		Not detected							
–	–	gy/p	y(f)	–	–		Multiple spots							
–	–	br	br	–	–		–	2(5)	–	85	–	o(t)	o	o
–	–	p	bk	–	–		–	3	2	57	–	g	p(fl)	–
–	–	gy	p	–	–		+	3	5	22	p(t)	br	p	br
–	–	gy	–	–	–		–	3	4	77	–	o/r	–	o
							–	1(4)	–	56	–	–	–	v(f)
–	–	bk/br	bk	–	–		+	5	11	49	–	–	v(fl)	b(d)
–	–	y	d	–	–		Not detected							
–	–	br	v/b	–	–		+	3	4	71	g/br	–	br	–
–	–	–	–	–	–		+	1(5)	–	8	–	o	–	–
–	–	gy/bk	bk	–	–		+	4	1	9	y	o	–	–
–	–	br	b	–	–		Multiple spots							
–	–	g(f)	bk	–	–		No data							
–	–	r/p	r/o	–	–	a		1	–	8	–	–	–	br(f)
–	–	p(f)	br(l)	–	–	b		1	–	13	–	gy(t)	–	–

(Continued on p. 256)

TABLE I *(continued)*

No.	*a*	*Metabolite*	*System 1*				
			b	*n*	*S.D.*	$\bar{x}R_F \times 100$	*1*	*2*
83	3	"M-Brevianamide B?"		5	1	18	–	g
84	64	"P"		6	3	33	–	b(f)
85	65	"R"		1	–	19	–	–
86	66	"S"		3	1	20	–	b
87	68	"U"		9	1	4	–	v
88	69	"0"		1	–	25	–	–
		Code						
89	70	A		2	–	3	y/br	br
90	71	B		17	1	3	–	–
91	72	C		6	1	5	–	g
92	74	E		1	–	9	–	–
93	75	F		6	1	10	–	r
94	76	G		6	5	18	–	–
95	77	H		1	–	19	–	–
96	78	I		3	1	19	–	–
97	80	K		5	2	29	–	–
98	81	L		2	–	44	–	y
99	82	M		2	–	76	–	–
100	83	N		1	–	79	–	–
101	84	O		1	–	82	–	b(f)
102	85	P		2	–	86	–	–
103	86	Q		2	–	92	–	–
104	87	R		1	–	5	–	–
105	88	S		1	–	26	–	–
106	89	U		1	–	36	–	–
107	91	V		1	–	45	–	–

* Identified from published results alone.

named metabolites; 22 (28%) of the named metabolites can be detected by system 1 but not system 2; 5 (6%) of the named metabolites can be detected by system 2 but not system 1; and 9 (11%) of the named metabolites cannot be detected satisfactorily by either system because (a) spots were not detected, (b) multiple spots were produced, or (c) the standard deviation of R_F values was excessive. Of the 66 (82%) of named metabolites which can be detected satisfactorily with system 1, 41 (62%) were detected also from extracts of the *Penicillium* study group; the equivalent figures for system 2 are 47 (60%) and 35 (74%) respectively. Of the purified metabolites which gave satisfactory results in at least one TLC system, 61 (77%) were detected in the *Penicillium* study group. The proportion of unidentified metabolites from system 1 which are the same metabolites as the unidentified and/or unsatisfactorily detected metabolites from system 2 is uncertain at this stage; and *vice versa* for unidentified metabolites from system 2.

From Table I it can be observed that, in general, lower standard deviations in R_F values were obtained with system 1. Inconsistent detection of spots was apparent from certain metabolites when examined by the various visualisation techniques (*e.g.* carolic acid, system 2).

						System 2								
3	*4*	*5*	*6*	*7*	*8*	*Code*	*b*	*n*	*S.D.*	$\bar{x}R_F + 100$	*1*	*2*	*3*	*4*
–	–	–	g	–	–	c		2	–	17	–	br	–	–
b	–	–	v	–	–	d		1	–	13	–	–	–	v
b	–	–	–	–	–	e		1	–	12	–	–	r	–
–	–	–	b	–	–	f		3	2	27	–	b	–	–
–	–	–	b	–	–	g		1	–	29	–	–	–	g
–	–	t	bk(f)	–	–	h		1	–	27	–	–	–	b(f)
–	–	–	–	–	–	i		6	1	28	–	g	–	g
–	–	r	o	–	–	j		1	–	43	–	b	o/r	b
–	–	–	g	–	–	k		1	–	44	–	gy	–	v
–	–	–	–	b	–	l		5	2	52	–	g	–	g
–	–	–	o	–	–	m		1	–	51	–	–	g(f)	–
–	–	br	pk	r	–	n		1	–	64	y	–	–	–
–	v	–	–	–	–	o		2	–	65	–	y(t)	–	y
–	–	–	o	–	–	p		3	1	71	–	–	p/r	–
–	–	p	b	–	–	q		6	2	75	y(f)	y(f)	–	y(f)
–	–	–	–	–	–	r		1	–	76	r/br	–	–	bk
–	–	p	r	–	–									
–	–	b	–	–	–									
–	–	–	–	–	–									
–	–	p	–	–	–									
–	–	–	gy(f)	–	–									
–	–	y	–	–	–									
y(l)	–	–	–	y(l)	–									
–	–	–	g	–	–									
y(l)	–	–	–	–	–									

In order to provide an aid to the identification of unknown TLC spots for *Penicillium* isolates and other fungi, the data from Table I are re-ordered in Table III where the metabolites which gave unsatisfactory TLC data are presented first, and the remainder is listed in ascending R_F value order. The data are presented in two formats (Tables I and III) to enable the metabolite which corresponds to a particular R_F value to be cross-referenced in Table I to obtain the metabolite characteristics expected from the visualization procedures employed.

A diagram of a TLC plate used in the 2D TLC analysis of citrinin and ochratoxin A producing *Penicillium* strains is provided in Fig. 1. When the plate was developed in the first direction with TEF, citrinin and ochratoxin A are eluted to a $R_F \times 100$ value of 44, with ochratoxin A being masked by citrinin. Citrinin was eluted to a $R_F \times 100$ value of 10 in the second direction using CAP, whereas the equivalent value for ochratoxin A was 70.

DISCUSSION

In general, the standard deviations of the R_F values are acceptable, at least for

TABLE II

EXAMPLES OF ISOLATES IN THE CAB INTERNATIONAL MYCOLOGICAL INSTITUTE WHICH PRODUCE UNIDENTIFIED METABOLITES

System 1		
a in Table I	*Metabolite*	*Isolate*
61	"m"	285522 (*P. viridicatum* II), 285523 (*P. viridicatum* III)
62	"r"	291199 (*P. verrucossum* var. *cyclopium*)
63	"M"	94149 (*P. brevicompactum*), 40225 (*P. brevicompactum*)
64	"P"	285220 (*P. brevicompactum*), 94149 (*P. brevicompactum*)
65	"R"	39767 (*P. nigricans*)
66	"S"	910206 (*P. cyclopium*), 39807 (*P. clavigerum*)
68	"U"	286753 (*P. raistrickii*), 27831 (*P. camembertii*)
70	A	89312 (*P. cyclopium* var. *album*), 296927 (*P. aurantiogriseum* var. *neochinulatum*)
71	B	91944 (*P. frequentans*), 89372 (*P. cyclopium*)
72	C	224935 (*P. echinulatum*), 291190 (*P. verrucosum* var. *cyclopium*)
74	E	194564 (*P. claviforme*), 291202 (*P. roqufortii*)
75	F	285512 (*P.hirsutum* III), 285528 (*P. claviforme*)
76	G	285526 (*P. concentricum* I), 194564 (*P. claviforme*)
77	H	297895 (*P. olivinoviride*)
78	I	285525 (*P. griseofulvum*), *285527* (*P. concentricum* II)
80	K	284410 (*P. viridicatum* II), 68236 (*P. echinulatum*)
81	L	40221 (*P. raistrickii*)
82	M	68234 (*P. olivinoviride*), 293186 (*P. aurantiogriseum* II)
83	N	68234 (*P. olivinoviride*)
84	O	91944 (*P. frequentans*)
85	P	280215 (*P. cyclopium*), 68234 (*P. olivinoviride*)
86	Q	173209 (*P. viridicatum*), 280297 (*P. citrinum*)
87	R	285507 (*P. camembertii* II), 151748 (*P. hordei*)
88	S	297991 (*P. italicum*)
89	U	34913 (*P. puberulum*)
91	V	293209 (*P. concentricum*), 299046 (*P. expansum*)

a tentative identification of the metabolites, especially if distinctive colours were produced from the visualization techniques. Variations can be attributed to, for example, variation in spot size because the application of constant concentrations of metabolites was not attempted. However, this will more closely imitate the situation in living cultures where wide variations in concentrations of the same metabolites are apparent between certain strains. The reasons for the relatively large standard deviations of some R_F values, in the case of system 2 is unclear; although it might be related to greater solvent volatility compared to system 1. However, it is worth emphasizing that one R_F unit is equivalent to a length on the TLC plate of 1.7 mm, therefore at relatively high standard deviations, identifications can be made especially if distinctive colours are produced.

In some cases more than one attempt was necessary to detect a spot from a particular metabolite (e.g. carolic acid in system 2) due to difficulty in visualizing the spot. More effective visualization techniques would be advantageous in these cases

System 2

Metabolite	*Isolate*
a	285507 (*P. camembertii* II)
b	39752 (*P. atramentosum*)
c	40225 (*P. brevicompactum*), 151748 (*P. hordei*)
d	285527 (*P. concentricum* II)
e	297897 (*P. viridicatum*)
f	285220 (*P. brevicompactum*), 51355 (*P. cyclopium*)
g	17456 (*P. brevicompactum*)
h	285508 (*P. echinulatum*)
i	285528 (*P. claviforme*), 284408 (*P. viridicatum* I)
j	293197 (*P. concentricum*)
k	284415 (*P. viridicatum*)
l	39758ii (*P. viridicatum*), 284415 (*P. viridicatum*)
m	39752 (*P. atramentosum*)
n	297546 (*P. claviforme*)
o	40028 (*P. cyclopium* var. *echinulatum*), 297900 (*P. hordei*)
p	285510 (*P. crustosum*), 297959 (*P. expansum*)
q	205652 (*P. patulum*), 39812 (*P. commune*)
r	39758ii (*P. viridicatum*)

but must be restricted in number for a rapid standardized system. For example, spots were observed occasionally from certain metabolites which fluoresced under white light after being treated with a reagent (*e.g.* gentisyl alcohol in system 2); this phenomenon often was difficult to observe.

System 1 was found to be more useful generally than system 2 for the metabolites considered here because of the (a) lower standard deviations in R_F values, (b) more consistent metabolite detection, (c) larger number of both named and unidentified metabolites detected and (d) distinctive colours from more metabolites. However, system 2 is valuable because of distinctive colours produced by the ceric sulphate spray reagent, particularly with alkaloids[23] (*e.g.* the orange colour produced with roqufortine C).

The extraction methods for system 1 and 2 have been considered to give extracellular and intracellular metabolites respectively[20]. However, both extracellular and intracellular metabolites can be extracted in system 2, particularly if fluid is

TABLE III

METABOLITES ARRANGED BY ASCENDING $R_F \times 100$ VALUE (METABOLITES WHICH GAVE UNSATISFACTORY RESULTS ALSO PROVIDED)

System 1		*System 2*	
$\bar{x}R_F \times 100$	*Metabolite*	$\bar{x}R_F \times 100$	*Metabolite*
Too variable	Epoxysuccinic acid	Not detected	Canadensolide
Not detected	Ethisolide	Not detected	11-Deacetoxywortmannin
Not detected	Glauconic acid	Not detected	Desacetylpebrolide
Not detected	Hadacidin	Not detected	Dehydrocanadensolide
Not detected	*p*-Hydroxybenzoic acid	Not detected	Dipicolinic acid
Multiple spots	2-Carboxy-3,5-dihydroxyphenyl acetyl carbinol	Not detected	Epoxysuccinic acid
		Not detected	Ethisolide
Multiple spots	Ergosterol	Not detected	Frequentin
Multiple spots	Duclauxin	Not detected	Gladiolic acid
Multiple spots	Gladiolic acid	Not detected	Glauconic acid
Multiple spots	Hydroxyisocanadensic acid	Not detected	Hadacidin
Multiple spots	Kojic acid	Not detected	Hydroxyisocanadensic acid
Multiple spots	2-Pyruvoylamino benzamide	Not detected	Itaconic acid
		Not detected	β-Nitropropionic acid
Multiple spots	Roseopurpurin	Not detected	Penicillic acid
Multiple spots	Tenuazonic acid	Not detected	Rubratoxin A
2	Roqufortine B	Not detected	Rubratoxin B
3	A	Not detected	Terrestric acid
3	B	Multiple spots	Carlosic acid
4	"U"	Multiple spots	Dechlorogriseofulvin
5	C	Multiple spots	Dehydrocarolic acid
5	R	Multiple spots	Ochratoxin A
5	Rubratoxin A	Multiple spots	2-Pyruvoylaminobenzamide
6	Roquefortine C		
9	E	Multiple spots	PR-Toxin
		Multiple spots	Rugulovasine A
10	Carlosic acid		
		Multiple spots	Xanthocillin
10	F		
		Too variable	Canadensic acid
11	Rubratoxin B		
		Too variable	*p*-Hydroxybenzoic acid
15	Desacetylpebrolide		
		Too variable	Kojic acid
16	Terrein		
		Too variable	Lapidosin
		Too variable	Myceliamide
17	Lapidosin		
		No data	Xanthomegnin
17	Rugulovasine A	5	Roqufortine B
18	Carolic acid	8	a
18	G	8	Viridicatum toxin
18	"M-Brevianamide B?"	9	Wortmannin
19	Austdiol	11	Citrinin
19	H		

System 1		*System 2*	
$\bar{x}R_F \times 100$	*Metabolite*	$\bar{x}R_F \times 100$	*Metabolite*
19	I		
19	Palitantin		
19	"R"	12	e
20	"S"	13	b
22	Brevianamide A	13	d
22	"m"	17	c
22	"r"	19	Gentisic acid
23	Dipicolinic acid	19	Phoenicin
23	Xanthomegnin	21	α-Cyclopiazonic acid
25	Cyclopenol	22	Spinulosin
25	5-Hydroxyaspertin	23	Carolic acid
25	"0"	27	f
26	Brefeldin A	27	h
26	Gentisyl alcohol	28	i
26	S	29	g
29	K	34	Roqufortine C
32	Compactin	36	Palitantin
32	Mycelianamide	39	6-Methylsalicyclic acid
32	Terrestric acid	42	Brefeldin A
33	Griseofulvin	43	2-Carboxy-3,5-dihydroxyphenyl-acetyl carbinol
33	"P"		
34	Penicillic acid	43	j
34	Scytalone	44	k
34	Viridicatum toxin	45	Orsellenic acid
		49	Terrein
		51	Austdiol
35	Cyclopenin	51	Gentisyl alcohol
35	Frequentin	51	m
35	Patulin	52	*m*-Hydroxybenzoic acid
35	11-Deacetoxywortmannin	52	l
35	Wortmannin	54	Fulvic acid
36	U	56	5′-Hydroxyaspertin
37	Dechlorogriseofulvin	56	Tenuazonic acid
37	Viomellein	57	Mycophenolic acid
38	Gliotoxin	57	Scytalone
38	β-Nitropropionic acid	59	Patulin
41	Canadensic acid	60	Brevianamide A
41	Mycophenolic acid	62	Griseofulvin
41	Phoenicin	64	Cyclopenol
42	Secalonic acid	64	n
43	Canescin	65	Griseophenone C
43	Citrinin	65	o
43	Spinulosin	66	Ergosterol
44	Fulvic acid	67	Gliotoxin
44	Gentisic acid	67	Monorden
44	L	69	Chactoglobosin C
45	V	70	Norlichexanthone
46	Dehydrocanadensolide	71	p
		71	Viomellein

(Continued on p. 262)

TABLE III *(continued)*

System 1		*System 2*	
$\bar{x}R_F \times 100$	*Metabolite*	$\bar{x}R_F \times 100$	*Metabolite*
47	Monorden	72	Compactin
48	Chaetoglobosin A	73	Cinnamic acid
48	Griseophenone C	73	Cyclopenin
48	Ochratoxin A	73	Roseopurpurin
48	PR-toxin	74	3,5-Dimethyl-6-methoxyphthalide
49	Canadensolide		
49	α-Cyclopiazonic acid	75	3,5-Dimethyl-6-hydroxyphthalide
49	Dehydrocarolic acid		
50	*m*-Hydroxybenzoic acid	75	Penitrem A
52	Orsellenic acid	75	q
53	Cinnamic acid	76	r
53	Norlichexanthone	77	Lichexanthone
54	Xanthocillin	77	Sterigmatocystin
56	3,5-Dimethyl-6-methoxyphthalide	79	Canescin
57	3,5-Dimethyl-6-hydroxyphthalide	81	Duclauxin
57	6-Methylsalicylic acid	84	Purpurogenome
57	Sterigmatocystin	85	Secalonic acid
62	Penitrem A		
67	Lichexanthone		
67	Purpurogenome		
76	Itaconic acid		
76	M		
79	N		
82	O		
86	P		
92	Q		

produced by the culture during growth. For this reason, equating system 2 with intracellular metabolites exclusively should be avoided. However, system 1 will detect extracellular metabolites exclusively.

Another example of the flexibility of both the material and methods is the ease by which development of 2D TLC systems can be achieved for the 43 (54%) of the named metabolites which are detected in both systems (Tables I and III), as demonstrated with citrinin and ochratoxin A producing strains (Fig. 1). The use of 2D TLC can give increased resolution of metabolites from complex substrata (*e.g.* grains) where development in the first or second dimension can act as a purification step to remove interfering compounds (*e.g.* lipids) which can obscure the fungal metabolite of interest. Tables I and III can be used to develop 2D TLC methods for improved identification of unknowns in a manner similar to that described above for 1D TLC.

Some of the metabolites in Tables I–III are also produced by genera apart from *Penicillium* (*e.g.* ochratoxin A by *Aspergillus ochraceus*) while a few have not yet been detected from *Penicillium* strains (*e.g.* sterigmatocystin from *Aspergillus* species and scytalone from *Verticillium* species)[1,2] and were included as an aid to identify the unidentified products of the strains studied. It was important to include as many metabolites as possible because, *inter alia*, (a) under certain conditions par-

ticular strains might produce metabolites which were previously unrecorded, (b) strains with new metabolite producing capabilities could be discovered, (c) it is important to differentiate between metabolites with similar TLC characteristics, and (d) it increases the utility of the data to other fungi. The TLC data provided in this paper will be useful in studies of other genera than *Penicillium* and can be built up using new strains and purified metabolites to make it more comprehensive.

The lists of TLC characters provided here are a reference data base for future chemotaxonomic and biochemical research to enable the characterization and/or preliminary identification of unnamed secondary (and primary) metabolites. The preliminary identifications will provide valuable information for proper structural identification of these compounds using, for example, nuclear magnetic resonance and mass spectrometry. These are aids to the identification of certain species difficult to distinguish by morphology alone and the methods are also effective for the rapid detection of known mycotoxins from material which may enter the food chain or for the checking for the occurrence of known metabolites from cultures during preservation and subculturing. They will therefore be of particular value to culture collections.

Additional utility is achieved by the development of 2D TLC methods which will be useful for resolution of metabolites difficult to detect by 1D TLC.

The method and data base has also been used to detect secondary metabolites in dried cultures in herbaria as a part of screening for new sources of particular compounds or as a part of chemotaxonomic investigations[24].

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