Journal of Chromatography, 116 (1976) 141–154
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 8644

SYSTEMATIC ANALYSIS OF MYCOTOXINS BY THIN-LAYER CHROMATOGRAPHY

ZDENA ĎURAČKOVÁ, VLADIMÍR BETINA and PAVEL NEMEC

Department of Technical Microbiology and Biochemistry, Faculty of Chemistry, Slovak Polytechnic University, Jánska 1, 880 37 Bratislava (Czechoslovakia)

(Received July 18th, 1975)

SUMMARY

The analysis of 37 mycotoxins and other fungal metabolites by thin-layer chromatography is described. Their R_F values in eight solvent systems, colour reactions after chemical detection and detection under UV light are presented. From the R_F values, "chromatographic spectra" which can be used as one of the characteristics for the characterization of unknown mycotoxins were constructed.

INTRODUCTION

Recently, increasing attention has been paid to the analysis of mycotoxins, of which over 100 are known at present, and screening methods for mycotoxins in contaminated products have been sought. Eppley[1] described a thin-layer chromatography (TLC) screening method for zearalenone, aflatoxin and ochratoxin. Steyn[2] developed a chromatographic separation and detection of eleven mycotoxins on thin layers. Fishbein and Falk[3] elaborated TLC procedures for five mycotoxins (aflatoxins, ochratoxins, aspertoxin, O-methylsterigmatocystin and sterigmatocystin) and some other fungal metabolites. The chromatography of eighteen mycotoxins in two different systems on thin layers and a combination of detection under UV light with spraying with *p*-anisaldehyde was applied by Scott *et al.*[4] in the screening of fungal extracts. Stoloff *et al.*[5] described selective extraction methods for aflatoxins, ochratoxins, zearalenone, sterigmatocystin and patulin and characterized these mycotoxins by TLC. The method was applied to different substrates and detection limits sufficient for the screening of mycotoxins were determined.

Analytical procedures for aflatoxins are best worked out in comparison with those for other mycotoxins. Jones[6] gave a survey of qualitative and quantitative TLC methods for aflatoxins. The most recommended material for TLC is silica gel.

The use of high-pressure liquid chromatography for the separation of aflatoxins[7] and gas chromatography for altenuene[8], patulin[9] and penicillic acid[10] has been described. However, TLC, owing to its simplicity, is the most frequently used chromatographic technique for mycotoxins.

In this paper, a systematic analysis of 37 mycotoxins and 6 other fungal metabolites by TLC in eight solvent systems combined with physical and chemical detection methods is described. Examples of the application of these procedures to the isolation and characterization of both known and unknown mycotoxins by use of chromatographic bioautography are described in the next paper[11].

MATERIALS AND METHODS

Mycotoxins and other fungal metabolites

We used the following mycotoxins and fungal secondary metabolites (lowercase letters in parentheses refer to the sources given below): 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (a), aflatoxin B_1 (b, c), aflatoxin B_2 (d), aflatoxin G_1 (c), aflatoxin G_2 (d), aflatoxin M_1 (c), altenuene (e), alternariol (e), alternariol monomethyl ether (AME) (e), aspergillic acid (r), citreoviridin (f), citrinin (r), curvularin (g), cyanein (r), cytochalasin A (h), cytochalasin B (h), cytochalasin D (h), diacetoxyscirpenol (i), N,N'-dibenzylethylenediamine (DBED) (e), fusaric acid (r), gliotoxin (r), griseofulvin (r), helvolic acid (r), kojic acid (r), luteoskyrin (i), monorden (k), mycophenolic acid (r), nivalenol (i), ochratoxin A (f, l, m), ochratoxin B (d), ochratoxin C (d), patulin (n), penicillic acid (n), rubratoxin B (l), rugulosin (r), secalonic acid (f), sterigmatocystin (f, i), terreic acid (r), T-2 toxin (a, m, o), trichothecin (r), vermiculin (p), viridicatum toxin (f) and zearalenone (d).

The sources of the metabolites were as follows: (a) Dr. S. G. Yates, U.S. Department of Agriculture, Northern Regional Research Laboratories, Peoria, Ill., U.S.A.; (b) Serva Feinbiochemica, Heidelberg, G.F.R.; (c) Dr. L. Shoetwell, U.S. Department of Agriculture, Northern Research Laboratories, Peoria, Ill., U.S.A.; (d) Dr. R. M. Eppley, Division of Food Chemistry and Technology, Washington, D.C., U.S.A.; (e) Dr. R. W. Pero, National Institute of Environmental Health Sciences, Research Triangle Park, N.C., U.S.A.; (f) Dr. P. S. Steyn, National Chemistry Research Laboratories, Pretoria, South Africa; (g) Dr. Richard, School of Chemical Research, Canberra, Australia; (h) Dr. Ch. Tamm, Institut für organische Chemie der Universität, Basle, Switzerland; (i) Dr. P. M. Scott, Food and Nutrition Division, Department of National Health, Ottawa, Ontario, Canada; (k) Dr. S. Wilkinson, Wellcome Research Laboratories, Beckenham, Great Britain; (l) Calbiochem, London, Great Britain; (m) Dr. R. D. Wyatt, Department of Poultry Sciences, North Carolina State University, N.C., U.S.A.; (n) Dr. H. J. Mintzlaff, Institut für Bakteriologie und Histologie, Kulmbach, G.F.R.; (o) Dr. H. R. Burmeister, ARS Culture Collection Investigations Fermentation Laboratory, 1815 North University Street, Peoria, Ill., U.S.A.; (p) Dr. J. Fuska, this Department; and (r) this Department.

Preparation of sample solutions

Stock solutions of fungal metabolites were prepared mostly at a concentration of 1 mg/ml. The numbers in parentheses below express the actual amounts of substances in micrograms applied on the chromatograms. The following solvents were used for the preparation of solutions: acetone for altenuene (5), alternariol (2), AME (2) and terreic acid (5); benzene for griseofulvin (5); benzene–acetonitrile (98:2) for

aflatoxins B_1 (2), B_2 (0.025), G_1 (2) and G_2 (0.025): chloroform for aflatoxin M_1 (0.01), 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (5), aspergillic acid (10), citreoviridin (5), cyanein (5), diacetoxyscirpenol (5), fusaric acid (50), gliotoxin (5), helvolic acid (5), luteoskyrin (2), mycophenolic acid (5), ochratoxin A (2), ochratoxin B (0.05), ochratoxin C (0.2), patulin (10), penicillic acid (2), rubratoxin B (10) sterigmatocystin (5), T-2 toxin (5), trichothecin (2), vermiculin (5) and zearalenone (2): chloroform–methanol (1:1) for secalonic acid (1) and viridicatum toxin (1); ethanol for nivalenol (5) and DBED (5), ethyl acetate for kojic acid (5) and rugulosin (2): and methanol for citrinin (0.5) curvularin (5), cytochalasins A (5), B (5) and D (5) and monorden (5).

Preparation of thin layers

Thin layers of silica gel G (Merck, Darmstadt, G.F.R.) of thickness 0.25 mm were prepared on glass plates (20 × 20 cm) employing an applicator. Silica gel for the application was prepared as given in ref. 12. When the plates were dry they were activated for 2 h at 110°. Before use, the plates were cleaned by developing them in the system that was subsequently used for their development with samples. After evaporating the solvents, the plates were stored for 24 h in a desiccator with dilute sulphuric acid (sp.gr. 1.440). The relative humidity in the desiccator was about 40%.

Pre-coated thin layers. Silufol sheets (Kavalier, Votice, Czechoslovakia) (20 × 20 cm) were cleaned and stored in the same manner as the glass plates with silica gel G.

Solvent systems.

A: benzene–methanol–acetic acid (24:2:1)[4].
B: toluene–ethyl acetate–90% formic acid (6:3:1)[4].
C: benzene–ethanol (95:5)[8].
D: chloroform–methanol (4:1).
E: chloroform–methyl isobutyl ketone (4:1)[2].
F: chloroform–acetone (9:1)[5].
G: chloroform–acetic acid–diethyl ether (17:1:3)[5].
H: *n*-butanol–acetic acid–water (4:1:4) (upper layer).

All solvents were purified before use by procedures described by Keil[13].

Method of application and development

After removal from the desiccator, the thin layer or the Silufol sheet was covered with a glass plate, except the area of the "origin", in order to prevent the absorption of moisture from the air. Thus, a relatively even moisture content of all of the plates was reached regardless of the moisture content of the ambient air[14]. The substances to be analyzed were applied to the plates from the stock solutions with micropipettes under orange light so that the resulting amounts were as given above. The spots were dried during application with a flow of cold air. The plates were developed in the dark in developing tanks (Zeiss, Jena, G.D.R.) saturated with solvent vapour. Each substance was chromatographed in two series in all eight solvent systems both on plates coated with silica gel G and on Silufol sheets. When the front of the system reached a height of about 10 cm above the origin, the development was interrupted, the chromatogram was dried in air and then detection was carried out.

TABLE I

$R_F \times 100$ VALUES OF MYCOTOXINS ON CLEANED SILICA GEL G PLATES AND CLEANED SILUFOL SHEETS IN 8 SOLVENT SYSTEMS AND RESULTS OF PHYSICAL AND CHEMICAL DETECTION

Colours: a = beige; b = blue; c = blue-grey; d = blue-green; e = brown-violet; f = dark pink; g = dark spot; h = dark yellow; i = grey; k = green-blue; l = grey-brown; m = green; n = green-grey; o = orange; p = pale beige; r = pale blue; s = pale brown; t = pale grey; u = pale green; v = pale pink; x = pale red; y = pale yellow; z = pink. α = red; β = violet; γ = yellow; δ = yellow-green; ε = yellow-orange. $^{+}$ = Tail; $^{++}$ = elongated spot. Fractions $\frac{12.0}{6.0}$ etc.: the numerators represent $R_F \times 100$ values on cleaned silica gel G plates and the denominators represent $R_F \times 100$ values on cleaned Silufol sheets.

$R_F \times 100$ values on $\frac{\text{silica gel G}}{\text{Silufol}}$

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
$\frac{12.0}{6.0}$	$\frac{11.5}{10.0}$	$\frac{2.5}{3.0}$	$\frac{57.0}{41.0}$	$\frac{5.0}{9.0}$	$\frac{8.0}{3.0}$	$\frac{7.0}{8.5}$	$\frac{55.0}{43.0}$
$\frac{28.0^{+}}{28.0^{+}}$	$\frac{31.5^{+}}{20.5^{+}}$	$\frac{19.5^{+}}{13.0^{+}}$	$\frac{80.5^{+}}{68.5^{+}}$	$\frac{22.5^{-}}{26.0}$	$\frac{32.5^{-}}{32.0}$	$\frac{31.5^{-}}{34.5^{+}}$	$\frac{61.5^{+}}{60.5^{+}}$
$\frac{30.0}{29.0}$	$\frac{18.0}{15.5}$	$\frac{14.0}{12.5}$	$\frac{80.0}{70.0}$	$\frac{9.0}{17.5}$	$\frac{33.0}{22.0}$	$\frac{20.0}{29.0}$	$\frac{61.0}{55.0}$
$\frac{19.0^{-}}{26.0^{-}}$	$\frac{19.0^{-}}{16.0^{-}}$	$\frac{13.5^{+}}{11.0^{-}}$	$\frac{84.5^{-}}{69.0^{-}}$	$\frac{12.0^{-}}{17.0}$	$\frac{25.0^{+}}{22.0}$	$\frac{20.0^{+}}{26.0^{+}}$	$\frac{50.0^{-}}{55.0^{+}}$
$\frac{22.5}{24.5}$	$\frac{8.0}{11.0}$	$\frac{8.0}{9.0}$	$\frac{78.0}{68.5}$	$\frac{4.5}{11.0}$	$\frac{17.0}{18.5}$	$\frac{11.0}{20.5}$	$\frac{51.5}{48.0}$
$\frac{21.5}{}$	$\frac{15.5}{}$	$\frac{10.0}{}$	$\frac{78.5}{}$	$\frac{0.0}{}$	$\frac{7.5}{}$	$\frac{6.5}{}$	$\frac{64.0}{}$
$\frac{24.0}{19.5}$	$\frac{33.0}{19.5}$	$\frac{7.0}{5.0}$	$\frac{66.0}{51.0}$	$\frac{3.5}{3.0}$	$\frac{6.5}{4.0}$	$\frac{12.0}{10.0}$	$\frac{83.0}{74.0}$
$\frac{30.5}{23.0^{+}}$	$\frac{65.5}{42.5}$	$\frac{17.0}{9.0}$	$\frac{83.0}{56.5}$	$\frac{32.0}{23.0}$	$\frac{28.5}{13.0}$	$\frac{58.0}{37.0}$	$\frac{95.0}{89.0}$
$\frac{57.5}{31.0}$	$\frac{72.5}{52.0}$	$\frac{33.0}{23.0}$	$\frac{88.0}{70.0}$	$\frac{67.0}{54.0}$	$\frac{56.5}{36.0}$	$\frac{73.5}{58.0}$	$\frac{93.0}{87.0^{+}}$
$\frac{67\text{–}80^{--}}{66.0^{-}}$	$\frac{62\text{–}82^{++}}{64.0}$	$\frac{35\text{–}57^{++}}{29\text{–}61^{++}}$	$\frac{74\text{–}94^{+-}}{88.5}$	$\frac{4\text{–}21^{++}}{70.0^{+}}$	$\frac{33\text{–}71^{++}}{69.0^{+}}$	$\frac{65\text{–}86^{++}}{74.5^{+}}$	$\frac{92.0^{++}}{95.5}$

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*$FeCl_3$*	*p-Anisaldehyde (UV, 366 nm)*	*$FeCl_3$ (UV, 366 nm)*
4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone	—	t	—	y	—
Aflatoxin B_1	b	m	—	o	b
Aflatoxin B_2	m	b	—	m	m
Aflatoxin G_1	b	—	—	v-o	b
Aflatoxin G_2	m	—	—	k	m
Aflatoxin M_1	b	—	—	z-o	r
Altenuene	z	e	β	m	r
Alternariol	b	s	s	b	b
AME	b	s	l	b	r
Aspergillic acid	g	o	γ	g	g

(Continued on p. 146)

TABLE I *(continued)*

$R_F \times 100$ values on $\frac{\text{silica gel G}}{\text{Silufol}}$

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
2–25^{+-}	0–48^{++}	0–5^{--}	0–24^{-+}	0–13^{+-}	0–20^{-+}	37.0^{+}	72.0^{+}
21.0^{+}	0–24^{++}	9.0	49.0^{-}	0–15^{++}	16.0	9–27^{++}	45.0
25.5^{-}	30.0^{-}	9.5^{+}	80.0^{-}	1.0^{+}	9.5^{+}	8.0^{-}	86.0^{-}
23.5^{+}	23.0^{-}	6.0^{-}	60.5^{+}	1.5	5.5^{+}	10.0^{+}	79.0^{+}
18.0	27.0	5.0	64.5	0.0	0.0	2.5	90.0
18.0	22.0	5.5	54.0	0.0	0.0	4.5	78.0
39.0	30.5	19.5	90.0	14.5	34.5	27.5	82.0
28.5	22.5	16.0	83.0	18.0	21.5	33.5	73.0
31.0	27–44^{-+}	2.0	31.0	0.0	0.0	21.0	69.0
23.5	11.5	0–11^{--}	70.0	0–12^{-+}	0–14^{+-}	12.5	58.0
17.0	7.0	11.0	53.0	6.5	8.5	11.0	57.0
50.0	42.5	31.5	85.0	18.5	32.0	30.5	81.0
34.0	41.5	24.5	80.0	22.5	23.0	43.5	88.0
40.5	46.0	19.0	91.0	24.0	38.0	43.0	66.5
28.0	24.0	20.5	71.0	28.5	34.5	41.0	71.5
52.5	53.0	28.0	89.0	15.0	29.0	50.0	93.5
34.5	39.5^{-}	24.5^{-}	83.5	28.0	23.0	44.0	0–100^{++}
12.5	15.0	2.5	52.0	0.0	2.0	6.0	65.0
4.0	5.0	0.0	31.0^{+}	0.0	0.0	1.5	44.0^{+}
0–45^{+-}	60.0^{+}	0–12^{--}	0–46^{--}	0–17^{++}	0–15^{++}	0–38^{++}	0–100^{--}
0–13^{++}	0–12^{-+}	1.5	62.0	0.0	0.0	0.0	94.0
7.0	5.0	0.0	50.0	0.0	0.0	0.0	67.5
7.0	0.0	0.0	36.5	0.0	0.0	0.0	50.0
52.0	59.5	34.5^{+}	79.0	0–11^{++}	0–23^{-+}	56.5	95.0
28.5^{+}	42.0	22.5^{-}	71.0^{+}	30.5^{+}	28.0^{-}	47.0^{+}	86.5

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*$FeCl_3$*	*p-Anisaldehyde (UV, 366 nm)*	*$FeCl_3$ (UV, 366 nm)*
Citrinin	γ	—	γ	u	γ
Citreoviridin	γ	i	γ	γ	γ
Cyanein	—	c	—	g	—
Diacetoxyscirpenol	—	β	—	ε	—
N,N′-dibenzylethylene-diamine	—	γ	p	u	—
Fusaric acid	y	—	—	y	—
Gliotoxin	—	r	—	g	—
Griseofulvin	b	a	—	γ	b
Helvolic acid	γ	b	—	v	—
Kojic acid	u	p	f	u	g
Luteoskyrin	h	o	h	o	—
Nivalenol	—	t	—	i	—
Ochratoxin A	d	—	f	m	m

(Continued on p. 148)

TABLE I *(continued)*

$R_F \times 100$ values on $\frac{\text{silica gel G}}{\text{Silufol}}$

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
41.0	46.0	12.0^{-}	65.0	0.0	2.0	33.5	79.0
80.0	72.0	75.0	91.0	53 0^{-}	73.5	86.0	87.0
34.0	37 0	22.0	72 0	20.5	25.0	30.0	78.0
24.0	27.0	14.0	61.5	19.5	17.0	24.0	70.5
32.0	43.0	18.5	76.0	18.5	22.5	31.0	88.5
27.5	31.5	14 5	66 5	17.5	15.5	26.5	76 0
0.0^{+}	0 0^{-}	0 0^{-}	34.0^{-}	0.0^{+}	0.0^{+}	0 0^{-}	84 0^{-}
0 0	0.0	0.0	28.0	0 0	0.0	0 0	88.0
0–34^{--}	51.5^{+}	1.5	0–38^{-+}	0–4^{++}	0–5^{+-}	27 5^{+}	92 0^{-}
28.0^{-}	37.0^{-}	16.0^{-}	62.5^{-}	13.5^{+}	16.0^{-}	27.5^{+}	87.0^{+}
45.0^{-}	55.5	0.0	54.0^{-}	0.0	5 0	20–37^{++}	72.0^{-}
28 0	32 5	0–9^{--}	68.5	0–6^{-+}	0–11^{+-}	12–35^{--}	85.0^{-}
67.5	71.0	59.0	93 0	71.5	74.0	78.0	84.5
51.0	49.5	46.0	81.5	80.5	56.5	73.0	83 0
55.0	66.0	55.0	79.0^{-}	51.0	49.0	63.0	90.5
31.0	43.5	29.5	62 5	51.5	39.5	44.0	81.0
43 5	32.0	16.5	92.0	16.0	38.5	38.5	81.0
34 5	46.0	26.5	80.0	36 5	33.5	47.0	88.5
68.5	55.5	43.0	92.0	63.0	73.5	69.5	81.5
52.0	43.0	52 0	83.0	64.0	55.0	64.0	83.0
0–12^{--}	0–41^{++}	0.0	0–12^{--}	0.0	0.0	0–17^{+-}	57.5^{+}
28.5^{-}	0–37^{-+}	14.0^{+}	61.0^{-}	14 5^{+}	20.0^{+}	0–24^{-+}	75–100^{++}
56 5	58.5	40.0	88.5	61.5	61.5	64.0	84.0
32.0	50.5	29.0	81.0	50.0	39.5	51.5	84.5
57 0	65.5	29.5	81.5	29.0	42.5	71.5	87.0
33.0	42.0	22.0	77.0	27.0	27.0	44.0	93.0

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*$FeCl_3$*	*p-Anisaldehyde (UV, 366 nm)*	*$FeCl_3$ (UV, 366 nm)*
Ochratoxin B	b	—	—	b	—
Ochratoxin C	k	—	—	k	—
Patulin	γ	β	a	δ	—
Penicillic acid	—	β	—	α	—
Rubratoxin B	o	a	—	o	—
Rugulosin	γ	γ	y	γ	—
Secalonic acid	o	l	a	g	—
Sterigmatocystin	α	b	i	α	x
Terreic acid	γ	β	γ	γ	g
T-2 toxin	b	β	—	γ	—
Trichothecin	—	a	—	m	—
Viridicatum toxin	o	—	γ	o	—
Zearalenone	b	n	—	g	u
Mycophenolic acid	b	i	l	r	r

Detection reagents

(1) A solution of *p*-anisaldehyde, consisting of a mixture of 0.5 ml of *p*-anisaldehyde, 85 ml of methanol, 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid was prepared just prior to use[4].

(2) A 1% solution of iron(III) chloride in ethanol[2].

Detection

The developed silica gel G plates were detected under UV light (UVIS, Desaga, Heidelberg, G.F.R.) at a wavelength of 366 nm and the Silufol sheets at wavelengths of 366 and 254 nm. One series of chromatograms was detected by spraying with *p*-anisaldehyde reagent[4]. The silica gel G plates were then heated for 15–20 min at 130° and the Silufol sheets for 15–20 min at 60° (Silufol sheets are bonded with starch and become black at higher temperatures).

The second series of chromatograms was detected by spraying with the 1% solution of iron(III) chloride in ethanol[2]. After spraying with the detection reagents, the chromatograms were checked again under UV light (as prior to the chemical detection).

RESULTS

When comparing R_F values on uncleaned silica gel G plates (plates not developed in the corresponding system but containing only a standard moisture content being placed in a desiccator) with R_F values on cleaned silica gel G plates and on cleaned Silufol sheets in parallel experiments, the highest variations in the R_F values were found for the uncleaned silica gel G plates, lower variations for the cleaned silica gel G plates and the lowest variations for the cleaned Silufol sheets. The silica gel G plates and Silufol sheets were developed prior to application of the sample as otherwise in some systems (especially systems A and D) a fluorescent strip appeared in one third or in the middle of the developed plate. This fluorescent strip stopped some spots at its front and thus the R_F values were distorted.

From the parallel experiments on cleaned silica gel G plates and Silufol sheets, average R_F values were calculated. In Table I, these average R_F values and results with all detection methods are given.

The R_F values of individual substances were plotted against the solvent systems and characteristic "chromatographic spectra" were thus obtained for each metabolite under defined conditions. Examples of the chromatographic spectra obtained on cleaned Silufol sheets for aflatoxin B_1, AME, kojic acid and trichothecin are shown in Fig. 1. These metabolites differ in their chemical structures and chromatographic spectra.

From the fungal secondary metabolites, macrolide antibiotics with known cytotoxicity[15–21] were also analyzed by the method described. The R_F values on previously developed silica gel G plates and Silufol sheets and the results of the physical and chemical detection of these metabolites are given in Table II.

DISCUSSION

Chromatographic systematic analyses of various compounds are known.

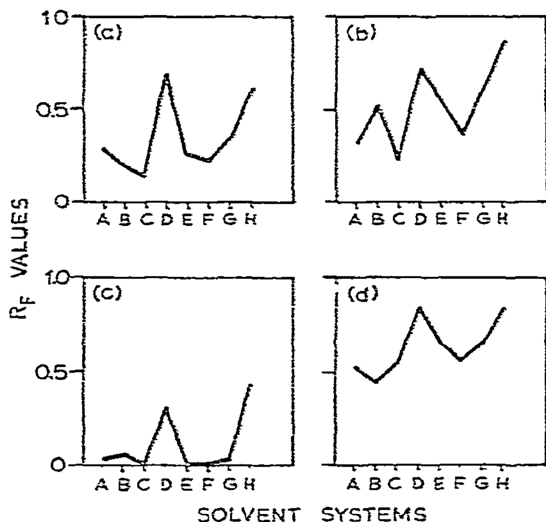

Fig. 1. Chromatographic spectra of secondary metabolites on cleaned Silufol sheets. (a) Aflatoxin B_1, (b) AME, (c) kojic acid, and (d) trichothecin.

Reio[22] described a paper chromatographic "reference system" for about 450 phenol derivates, mould metabolites and related compounds and for about 270 mono-, di- and trihydric phenol derivatives and various compounds of biochemical interest[23]. He developed the compounds in six solvent systems, and ten detection reagents and ultraviolet light were used for their detection. The R_F values of each compound were recorded on documentation reference cards in a form of "chromatographic spectrum" and the results of the detection were designated by a numerical index. Van Sumere *et al.*[24] obtained R_F values and described the detection of 93 phenolic substances and coumarins on thin layers using two solvent systems. They pointed out the advantages of using steamed plates in order to obtain a better separation of substances with similar polarities. A systematic analysis of antibiotics by paper chromatography has been described[25]. In the first stage, antibiotics were analysed simultaneously in four principal solvent systems and classified according to the results into 5 classes and 14 subclasses. Subsequently, supplementary systems were used and "summarized chromatograms" for 62 antibiotics were obtained. A systematic analysis of antibiotics by TLC was described by Aszalos *et al.*[26], in which 84 antibiotics were classified into four main classes according to their R_F values in three solvent systems. By the further application of 11 solvent systems, they obtained 15 sub-groups. For the routine identification of lichen-forming fungi by TLC, Culberson and Kristinsson[27] developed a systematic analysis of 104 products in three solvent systems. Spots of unknown substances were divided into eight classes according to the R_F values relative to the R_F values of two standard lichen substances. They recorded their results on cards in a similar manner to Reio. They consider that the advantage of using the R_F values for reference substances to which the R_F values of unknown substances are related, in comparison with the absolute R_F values of unknown compounds, consists in a smaller variation of the relative R_F values as a result of changes in the conditions of

TABLE II

$R_F \times 100$ VALUES OF MACROLIDES ON CLEANED SILICA GEL G PLATES AND CLEANED SILUFOL SHEETS IN 8 SOLVENT SYSTEMS AND RESULTS OF PHYSICAL AND CHEMICAL DETECTION

Notes as in Table I.

$R_f \times$ *100 values on* *silica gel G* / *Silufol*								*Macrolide*	*Detection*				
									Prior to chemical detection (UV, 366 nm)	*Chemical detection*		*After chemical detection*	
A	*B*	*C*	*D*	*E*	*F*	*G*	*H*			*p-Anisaldehyde*	*FeCl₃*	*p-Anisaldehyde (UV, 366 nm)*	*FeCl₃ (UV, 366 nm)*
23.0 / 26.0	53.0 / 36.5	15.0 / 10.0	72.0 / 63.5	18.0 / 12.0	16.0 / 11.0	31.0 / 22.5	98.0 / 94.0	Curvularin	—	z	—	m	—
18.0 / 18.0	27.0 / 22.0	5.0 / 5.5	64.5 / 54.0	0.0 / 0.0	0.0 / 0.0	2.5 / 4.5	90.0 / 78.0	Cyanein	—	c	—	g	—
41.0 / 34.0	57.5 / 42.5	25.5 / 23.5	85.0 / 83.0	17.0 / 20.0	33.5 / 31.0	46.5 / 44.0	99.5 / 95.0	Cytochalasin A	—	p	—	b	—
28.5 / 34.5	46.0 / 34.5	13.5 / 13.5	78.0 / 74.5	8.0 / 7.0	16.0 / 14.0	24.0 / 26.0	98.5 / 94.5	Cytochalasin B	—	β	—	b	—
19.5 / 32.0	24.5 / 27.0	10.0 / 10.5	75.5 / 74.5	0.0 / 4.0	8.0 / 9.0	14.5 / 20.0	90.0 / 85.0	Cytochalasin D	—	a	—	o	—
39.5 / 35.0	55.5 / 44.0	34.5 / 22.0	76.0 / 77.0	46.0 / 33.5	42.5 / 31.5	53.0 / 42.5	97.5 / 94.5	Monorden	—	l	—	γ	—
25.0 / 35.0	17.0 / 16.5	19.0 / 20.5	77.5 / 78.0	10.0 / 10.0	25.0 / 19.0	20.0 / 22.0	63.0 / 50.0	Vermiculin	—	s	—	γ	—
56.5 / 32.0	58.5 / 50.5	40.0 / 29.0	88.5 / 81.0	61.5 / 50.0	61.5 / 39.5	64.0 / 51.5	84.0 / 84.5	Zearalenone	b	n	—	g	u

the environment compared with the absolute R_F values. Different aspects of the systematic analysis of substances, especially those of pharmaceutical interest, by paper and thin-layer chromatography were discussed by Macek[28].

We investigated 43 mycotoxins and other fungal secondary metabolites by means of our systematic TLC analysis in eight solvent systems. From the results, a characteristic chromatographic spectrum for each substance was constructed (Fig. 1) which is used as one of the primary criteria when comparing unknown mycotoxins isolated from different materials.

The solvent systems chosen have different polarities. Systems of low polarity are E, C, and F, those of medium polarity are G, A and B and the most polar are D and H. Systems that give a large scatter of R_F values for different mycotoxins are advantageous. The greatest scatter of R_F values is obtained with the systems of medium polarity.

On comparing the average R_F values for all of the substances analyzed on cleaned silica gel G plates and cleaned Silufol sheets, the latter were found to give lower R_F values.

Table I includes metabolites which gave "tails" in certain systems. Other metabolites moved in the form of elongated spots in some systems. The numerical limits given in the table indicate the beginning and the end of these elongated spots. The greatest problem occurred in the chromatography of rubratoxin B[29], which gave long tails along the whole length of the silica gel G plates when systems B and D were used. The sample of rubratoxin was probably not homogeneous as in system D, for example, it was separated into eight spots on Silufol sheets.

In Table I, the results of chemical and physical detection are indicated by letters. When evaluating the colour of a spot after chemical detection, it is sometimes difficult to define the colour (*e.g.*, light brown, beige, grey-brown, etc.). Similar problems are encountered in the exact description of fluorescence in physical detection (*e.g.* light yellow, yellow-orange, etc.). With Silufol sheets, the spots were checked prior to the chemical detection under UV light at 366 nm (longwave UV light) and 254 nm (shortwave UV light). In Table I, results obtained under longwave UV light are given. Under shortwave UV light, the spots fluoresced at the same wavelength but with lower intensity and luminance.

When comparing the chromatographic spectra of the individual metabolites and their chemical structures, we can draw the following conclusions. Aflatoxins have similar chromatographic spectra but when sterigmatocystin with a related chemical structure (coumarin) is added to this group, its resulting chromatographic spectrum is different. Similarly, when comparing metabolites from the scirpens (diacetoxyscirpenol, T-2 toxin and trichothecin), their chromatographic spectra are also differentiated.

Our aim, however, was not to investigate the relationships between the chemical structures and chromatographic spectra of fungal metabolites. The significance of this systematic analysis is in the use of chromatographic spectra as one of the fundamental characteristics which, together with other data (melting point, molecular weight, mass and infrared spectra, etc.), helps to identify unknown mycotoxins. By means of the chromatographic spectrum of an unknown substance, it is possible to carry out a preliminary identification of the metabolite obtained simply and rapidly, without expensive equipment, by comparing it with the known chromato-

graphic spectra, or to eliminate the known metabolites from the unknown. We expect to use this system in the screening of mycotoxins, where it can be combined with the bioautographic detection of chromatograms employing an undemanding laboratory model, *Artemia salina* larvae[11]. The bioautographic detection of mycotoxins on thin-layer chromatograms is suitable especially at the stage where the unknown mycotoxins are available only in the form of crude extracts.

REFERENCES

1 R. M. Eppley, *J. Ass. Offic. Anal. Chem*, 51 (1968) 74.
2 P. S. Steyn, *J. Chromatogr.*, 45 (1969) 473.
3 L. Fishbein and H. L. Falk, *Chromatogr. Rev*, 12 (1970) 42.
4 P. M. Scott, J. W. Lawrence and W. van Walbeek, *Appl. Microbiol.*, 20 (1970) 839.
5 L. Stoloff, L. Nesheim, L. Yin, J V. Rodricks, M. Stack and A. D. Campbell, *J. Ass. Offic. Anal. Chem.*, 54 (1971) 91
6 B. D Jones, *Tropical Products Institute Report 56/62*, Tropical Products Institute, London, 1972.
7 R. C. Garner, *J. Chromatogr.*, 103 (1975) 186.
8 R. W. Pero, R. G. Owens and D. Harvan, *Anal. Biochem*, 43 (1971) 80.
9 A. E. Pohland, K. Sanders and C. W. Thrope, *J. Ass. Offic. Anal. Chem.*. 53 (1970) 692.
10 J. P. Snow, G. B. Lucas, D. Harvan, R. W Pero and R. G. Owens, *Appl. Microbiol.*, 24 (1972) 34.
11 Z. Ďuračková, V. Betina and P. Nemec, *J. Chromatogr.*, 116 (1976) 155.
12 "Changes in Official Methods of Analysis", *J. Ass. Offic Anal. Chem.*, 49 (1966) 229.
13 B. Keil, *Laboratorni Technika Organické Chémie*. Nakladatelství ČSAV, Prague, 1963.
14 K. Chmel, personal communication.
15 W. B. Turner, *Fungal Metabolites*, Academic Press, London, 1971.
16 S. B. Carter, *Nature (London)*. 213 (1967) 261.
17 V. Betina, P. Nemec, J. Dobiáš and Z. Baráth, *Folia Microbiol*, 7 (1962) 353.
18 D. Bačíková, V. Betina and P. Nemec, *Naturwissenschaften*, 51 (1964) 445.
19 D. Bačíkova, V. Betina and P. Nemec, *Nature (London)*, 206 (1965) 1371.
20 J. Fuska, L. P. Ivanickaja, K. Horáková and I. Kuhr, *Antibiotics*, 27 (1974) 141.
21 J. Fuska, L. P. Ivanickaja, L. V. Makucho and L. I. Volkova, *Antibiotiki*, 19 (1974) 890.
22 L. Reio, *J. Chromatogr.*, 1 (1958) 338.
23 L. Reio, *J. Chromatogr*, 4 (1960) 458.
24 C. F. van Sumere, G. Wolf, H. Teuchy and J. Kint, *J. Chromatogr.*, 20 (1965) 48
25 V. Betina, *J. Chromatogr.*, 15 (1964) 379.
26 A. Aszalos, S. Davis and D. Frost, *J. Chromatogr*, 37 (1968) 487.
27 Ch. F. Culberson and H.-D. Kristinsson, *J. Chromatogr.*, 46 (1970) 85.
28 K Macek, in K Macek (Editor), *Pharmaceutical Applications of Thin-Layer and Paper Chromatography*. Elsevier, Amsterdam, 1972, p. 116.
29 B. J. Wilson and R. D. Harbison, *J. Amer. Vet. Med. Ass.*, 163 (1973) 1274.