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SCREENING METHODS FOR THE DETECTION OF THIRTEEN COMMON MYCOTOXINS

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SUMMARY

A study of screening methods for thirteen mycotoxins showed that they can be separated as neutral and acidic metabolites. R_F values were determined in several solvent systems. The reactions of the mycotoxins with well known spray reagents were investigated, and their detection limits were established. A general procedure for the extraction of mycotoxins from contaminated samples is described.

INTRODUCTION

In recent years, extensive investigations of field and storage fungi have revealed many mycotoxins that show divergent toxicity patterns. Several of these compounds have been recognized in the etiology of human and animal malignancies[1–3], and as such their unambiguous detection and analysis is of prime concern. Apart from a limited knowledge of fungal ecology for deciding which mycotoxin to suspect in foodstuffs and feedstuffs (Table I), no principle exists for predicting the presence of a particular mycotoxin. The development of a multi-mycotoxin screening method, which could be introduced at some intermediate stage in the procedure for the clean-up of contaminated samples, would, therefore, be expedient. Only those compounds which show a positive response would then be subjected to methods specifically designed for the verification and quantitation of particular mycotoxins. Consequently, we have developed a detection procedure for thirteen important mycotoxins (Table I) using thin-layer chromatography (TLC). Previous screening methods[4–6] were not investigated as extensively as in this study.

EXPERIMENTAL

Mycotoxin standards

Pure samples of aflatoxin B_1, sterigmatocystin, penitrem A, fumitremorgen B, roridin A, ochratoxin A and citrinin were supplied by Dr. P. S. Steyn and roquefortin and α-cyclopiazonic acid by Dr. R. Vleggaar, National Chemical Research Laboratory, C.S.I.R., Pretoria, Republic of South Africa. Zearalenone was obtained from

TABLE I

MYCOTOXINS INVESTIGATED AND SOME OF THE FUNGI THAT PRODUCE THEM

Mycotoxin	*Fungal species producing mycotoxin*[1–3]
Aflatoxin B_1[7]	*Aspergillus flavus, A. parasiticus*
Sterigmatocystin[8]	*Aspergillus versicolor, A. nidulans, A. sydowi, A. rugulosus, A. flavus, Drechslera* sp., *Bioplaris* sp.
Zearalenone[9]	*Fusarium graminearum, F. roseum, F. nivale, F. tricinctum, F. sporotrichioides, F. oxysporum, F. moniliforme, Gibberella zea*
Patulin[10]	*Penicillium patulum, P. roqueforti, P. expansum, P. variable, P. claviforme, P. lapidosum, P. melinii, P. rugulosum, P. equinum, P. novaezeelandiae, P. divergens, P. griseofulvum, P. leucopus, P. cyclopium, P. chrysogenum, Aspergillus clavatus, A. giganteus, A. terreus, Byssochlamys nivea*
T_2-toxin[11]	*Fusarium tricinctum, F. roseum, F. lateritium, F. solani, F. rigidiusculum, Trichoderma viride*
Roquefortin[12]	*Penicillium roqueforti*
Penitrem A[13]	*Penicillium palitans, P. cyclopium, P. crustosum, P. spinulosum*
Fumitremorgen B[14]	*Aspergillus fumigatus, A. caespitosus, Penicillium lanosum*
Roridin A[15]	*Myrothecium roridum*
Ochratoxin A[16]	*Aspergillus ochraceus, A. ostianus, A. melleus, A. alliaceus, A. petrakii, A. sclerotiorum, A. sulphureus, Penicillium viridicatum, P. cyclopium, P. commune, P. palitans, P. purpurescens, P. variable, P. verrucosum, P. chrysogenum*
Citrinin[17]	*Penicillium citrinum, P. viridicatum, P. implicatum, P. fellutanum, P. citreo-viride, P. velutinem, P. canascens, P. purpurescens, P. jenseni, P. steckii, P. spinulosum, P. notatum, P. palitans, P. expansum, P. claviforme, P. roqueforti, Aspergillus niveus, A. terreus, A. flavipes*
α-Cyclopiazonic acid[18]	*Penicillium cyclopium, P. viridicatum, P. crustosum, P. puberulum, P. viridicatum, P. patulum*
Penicillic acid[19]	*Penicillium puberulum, P. piscarium, P. stoloniferum, P. viridicatum, P. cyclopium, P. martensii, P. thomii, P. verrucosum, P. suaveolens, P. chrysogenum, P. palitans, P. baarnense, P. expansum, P. madriti, P. paraherquei, Aspergillus ochraceus, A. sulphureus, A. quercinus, A. melleus, A. ostianus*

the IMC Chemical Group, Terre Haute, Ind., U.S.A. Patulin and penicillic acid were obtained from Mr. H. P. van Egmond, Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands, and T_2-toxin from Makor Chemicals, Jerusalem, Israel.

TLC procedure

Merck pre-coated silica gel F_{254} plates (Cat. No. 5715, thickness 0.25 mm) were used. Standard solutions of the mycotoxins (1 mg/ml in chloroform) were spotted on a baseline 2 cm from the bottom of the plate with a graduated 5-μl pipette, and the plate was then developed 16 cm in the appropriate solvent system in a tank lined with filter-paper. Analytical-reagent grade reagents were used throughout.

Detection procedure

The developed plates were examined under UV light at wavelengths of 254 and 366 nm using a Minuvis lamp. The following spray reagents were used: (a) 2,4-dinitrophenylhydrazine (2,4-DNP) (1 g), concentrated sulphuric acid (7.5 ml), ethanol (75 ml), water (170 ml); (b) 2-hydrazono-2,3-dihydro-3-methylbenzothiazole hydrochloride (MBTH) (0.5% aqueous solution); (c) iron(III) chloride (3% solution in

TABLE II

MEAN R_F VALUES OF NEUTRAL MYCOTOXINS

Mean value of six runs in different batches of the solvent mixture.

Mycotoxin	*Solvent system*					
	Chloroform–methanol (97:3)	*Chloroform–acetone–n-hexane (7:2:1)*	*Chloroform–acetone (9:1)*	*Ethyl acetate–n-hexane (1:1)*	*Chloroform–acetone–propan-2-ol (85:15:20)*	*Benzene–chloroform–acetone (45:40:15)*
Aflatoxin B_1	0.44	0.35	0.27	0.03	0.65	0.24
Sterigmatocystin	0.67	0.53	0.55	0.41	0.74	0.56
Zearalenone	0.40	0.51	0.38	0.41	0.71	0.44
Patulin	0.22	0.27	0.16	0.18	0.56	0.20
T_2-toxin	0.45	0.36	0.22	0.13	0.68	0.22
Roquefortin	0.03	0.01	0.02	0.01	0.13	0.02
Penitrem A	0.40	0.51	0.34	0.49	0.76	0.45
Fumitremorgen B	0.51	0.36	0.28	0.14	0.71	0.30
Roridin A	0.31	0.22	0.13	0.09	0.61	0.14

TABLE III

MEAN R_F VALUES OF ACIDIC MYCOTOXINS

Mean value of six runs in different batches of the solvent mixture.

Mycotoxin	*Solvent system*				
	Benzene–acetic acid (4:1)	*Ethyl acetate–methanol–ammonia (80:15:10)*	*Formic acid–diethyl ether (1:19)*	*Benzene–methanol–acetic acid (90:16:8)*	*Ethyl acetate–acetone–hydrochloric acid (1 N) (50:50:20)*
Citrinin	—*	—**	—*	0.20	0.34
Ochratoxin A	0.40	0.05	0.89	0.47	0.41
α-Cyclopiazonic acid	—*	0.24	—*	0.45	—*
Penicillic acid	0.18	0.41	0.67	0.41	—*

* Tailing prevents calculation of reliable values.
** Decomposes.

ethanol); (d) aluminium chloride (1% solution in chloroform); (e) Ehrlich reagent — solution (1), 4-dimethylaminobenzaldehyde (1% solution in ethanol), solution (2), hydrochloric acid (32%); (f) cerium(IV) sulphate (1% solution in 6 *N* sulphuric acid;

TABLE IV

MEAN R_F VALUES OF ACIDIC MYCOTOXINS USING TLC PLATES PRE-TREATED WITH OXALIC ACID

Mean value of four runs in different batches of the solvent mixture.

Mycotoxin	*Solvent system*	
	Chloroform–methanol (98:2)	*Chloroform–acetone (9:1)*
Citrinin	0.52	0.51
Ochratoxin A	0.32	0.34
α-Cyclopiazonic acid	0.52	0.44
Penicillic acid	0.16	0.20

and (g) vanillin (1% solution in 50% phosphoric acid). The plates were sprayed, the immediate effects noted, and then they were heated at 110° for 10 min. After development some of the plates were treated with iodine or ammonia vapour.

Extraction of contaminated maize

Sterilized maize samples were contaminated with *Aspergillus flavus*, *A. versicolor* and *Penicillium cyclopium*, known producers of the aflatoxins, sterigmatocystin and α-cyclopiazonic acid, respectively. The mouldered meal (100 g) was extracted in a Waring blender with methanol–chloroform (1:1) (400 ml) at 23,000 rpm for 4 × 1 min. The mixture was filtered and the filtrate was evaporated to dryness. The resultant brown residue was partitioned between *n*-hexane and 90% methanol (1:1) (200 ml), the *n*-hexane layer was discarded and the methanol layer was evaporated to dryness. The brown solid was partitioned between chloroform and water (1:1) (200 ml) and the chloroform layer was extracted with saturated sodium hydrogen carbonate solution (3 × 100 ml). The chloroform layer was concentrated, and con-

TABLE V

CHARACTERISTIC COLOURS WITH UV LIGHT AND SPRAY REAGENTS

Mycotoxin	*UV light*		*Cerium (IV) sulphate*		*2,4-DNP*		*Iron(III) chloride*	
	254 nm	*366 nm*	*a**	*b***	*a**	*b***	*a**	*b***
Aflatoxin B_1	Blue	Blue		Grey		Orange		Light yellow
Sterigmatocystin	Purple-black	Light red		Grey-green		Grey		
Zearalenone	Purple	White		Yellow-brown		Dark orange		Light purple
Patulin	Purple			Grey		Yellow		
T_2-toxin				Grey-black		Orange		
Roquefortin	Purple		Orange	Green-brown		Grey		
Penitrem A	Light purple		Green	Purple		Purple		Grey
Fumitremorgen B	Light purple		Yellow-brown	Yellow-brown		Light orange		Orange
Roridin A	Purple			Grey-black		Dark orange		
Citrinin	Purple	Yellow	Yellow	Yellow	Yellow	Brown-yellow	Light brown	Light brown
Ochratoxin A	Blue	Blue					Purple-brown	Fades
α-Cyclopiazonic acid	Purple		Red	Black	Light brown	Red-brown	Purple-brown	Brown
Penicillic acid	Light purple			Light orange				

* Immediate colour.
** Colour after 10 min at 110°.
*** Plate kept in iodine vapour for 15 min.
§ Plate kept in ammonia vapour for 15 min and examined under UV light (254 + 366 nm).

tained any of the so-called neutral mycotoxins (*viz.*, aflatoxin B_1, sterigmatocystin, zearalenone, patulin, T_2-toxin, roquefortin, penitrem A, fumitremorgen B and roridin A) that were present initially. The aqueous layer was carefully acidified to pH 2 (with 0.5 *N* hydrochloric acid) and extracted with chloroform (3 × 100 ml). The chloroform extract was concentrated and contained any of the so-called acidic mycotoxins (ochratoxin A, citrinin, α-cyclopiazonic acid and penicillic acid) present.

RESULTS AND DISCUSSION

Analysis of mycotoxins

The mean R_F values for the neutral mycotoxins in several solvent systems are given in Table II and those for the acidic mycotoxins in Table III. It is evident from Table II that no solvent system provides complete separations for all nine neutral compounds. However, the correct choice of systems, in combination with absorption and fluorescence properties and the use of chromogenic agents, allows the nine

Ehrlich reagent		*Vanillin*		*MBTH***	*Aluminium chloride***	*Iodine****	*Ammonia*§
*a**	*b***	*a**	*b***				
	Light grey		Green	Orange	Yellow	Orange	
			Light grey		Light yellow	Light yellow	
	Light pink		Brown	Light pink		Grey	
	White		Grey	Dark orange	Light brown	Light orange	
	White		Light grey				
	Grey-green		Purple			Light yellow	
	Blue-grey		Light purple	Light grey	Grey	Light yellow	
	Light grey		Yellow	Light orange	Light yellow	Yellow	
	White		Dark brown	Light yellow	Light orange	Dark yellow	
Yellow	Light purple	Yellow	Brown-yellow	Light-brown	Light yellow	Orange	Purple
							Purple
Light brown	Dark brown	Light yellow	Purple	Light yellow	Light brown	Orange	
			Brown-yellow	Light yellow		Light yellow	Blue

mycotoxins to be rigorously defined. As an example, zearalenone and penitrem A, which have identical R_F values in the first two solvent systems, are separable by using the third and fourth systems. In the third system zearalenone has a higher R_F value than penitrem A, and in the fourth system the opposite is true. Similarly, aflatoxin B_1 and T_2-toxin, indistinguishable by their mobilities in the first two systems, are separable in the third and fourth systems.

With the acidic mycotoxins, difficulty is experienced in obtaining clearly defined spots from which to calculate R_F values, as these compounds frequently tail. This problem can be overcome by using silica gel TLC plates that have been pretreated with oxalic acid[20]. The plates are immersed in a 10% solution of oxalic acid in methanol for 2 min. After heating at 110° for 2 min and cooling, the plates are immediately spotted, and developed in the appropriate solvent systems. R_F values obtained using this method are given in Table IV. In this way, well defined spots are obtained, and the four acidic compounds can be separated by the solvent systems shown in Table IV.

The results obtained from the different methods of detection are shown in Table V. A variety of colours arise for each mycotoxin, and this, coupled with the R_F values obtained previously, allows identification to be made. Direct comparison with standard samples increases the degree of confidence.

The extraction procedure described was tested on samples contaminated with *A. flavus*, *A. versicolor* and *P. cyclopium*. The maize meal infected with *A. flavus* and *A. versicolor* was spiked with 10 mg of each neutral mycotoxin, except aflatoxin B_1 and sterigmatocystin, respectively. After extraction and solvent partition, all of the compounds could be identified using the solvent systems and detection methods as described above. The use of pure standard samples permitted the comparison of R_F values and facilitated identification. The maize contaminated with *P. cyclopium* was

TABLE VI

DETECTION LIMITS

Mycotoxin	*Lowest amount detectable (g)*	*Most sensitive detection method*
Aflatoxin B_1	10^{-8}	UV (366 nm)
Sterigmatocystin	10^{-6}	UV (254 nm)
Zearalenone	10^{-6}	UV (254 nm)
Patulin	10^{-7}	UV (254 nm)
T_2-toxin	10^{-6}	$Ce(SO_4)_2$ spray reagent
Roquefortin*	10^{-7}	UV (254 nm), $Ce(SO_4)_2$ spray reagent
Penitrem A*	10^{-6}	UV (254 nm), $Ce(SO_4)_2$ spray reagent
Fumitremorgen B*	10^{-6}	UV (254 nm), $Ce(SO_4)_2$ spray reagent
Roridin A	10^{-7}	UV (254 nm)
Citrinin	10^{-7}	UV (366 nm)
Ochratoxin A	10^{-8}	UV (366 nm)
α-Cyclopiazonic acid	10^{-6}	UV (254 nm)
Penicillic acid*	10^{-6}	UV (254 nm), $Ce(SO_4)_2$ spray reagent

* In these instances both methods of detection give similar sensitivities.

spiked with pure citrinin, ochratoxin A and penicillic acid. Using the above procedures, all four acidic mycotoxins could be identified.

In some instances, other metabolites in the final extract caused interference. This could be reduced by initially developing the plates twice in benzene–*n*-hexane (3:1). In this system the mycotoxins do not move from the baseline, but most interfering compounds move with the solvent front.

Detection limits

The detection limits for the mycotoxins examined are shown in Table VI. The most sensitive methods of analysis are also indicated, but it is important to note that these are optimal values that apply to pure samples with no interference from other metabolites. It is unlikely that these values will be achieved in the analysis of naturally contaminated samples.

CONCLUSION

The above methodology allows material to be analysed for the mycotoxins aflatoxin B_1, sterigmatocystin, zearalenone, patulin, T_2-toxin, roquefortin, penitrem A, fumitremorgen B, roridin A, ochratoxin A, citrinin, α-cyclopiazonic acid and penicillic acid. A positive response could then be followed by isolation and characterization to permit definite identification.

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REFERENCES

1 K. Uraguchi and M. Yamazaki (Editors), *Toxicology, Biochemistry and Pathology of Mycotoxins*, Halsted (Wiley), New York, 1978.
2 I. F. H. Purchase (Editor), *Mycotoxins*, Elsevier, Amsterdam, 1974.
3 J. V. Rodricks, C. W. Hesseltine and M. A. Mehlman (Editors), *Mycotoxins in Human and Animal Health*, Pathotox Publishers, Park Forest South, 1977.
4 B. G. E. Josefsson and T. E. Möller, *J. Ass. Offic. Anal. Chem.*, 60 (1977) 1369.
5 L. Stoloff, S. Nesheim, L. Yin, J. V. Rodricks, M. Stack and A. D. Campbell, *J. Ass. Offic. Anal. Chem.*, 54 (1971) 91.
6 D. M. Wilson, W. H. Tabor and M. W. Trucksess, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 125.
7 T. Asao, G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan, *J. Amer. Chem. Soc.*, 98 (1965) 882.
8 E. Bullock, J. C. Roberts and J. G. Underwood, *J. Chem. Soc.*, (1962) 4179.
9 C. J. Mirocha, C. M. Christensen and G. H. Nelson, *Appl. Microbiol.*, 17 (1969) 482.
10 R. B. Woodward and G. Singh, *J. Amer. Chem. Soc.*, 71 (1949) 758.
11 J. R. Bamburg, N. V. Riggs and F. M. Strong, *Tetrahedron*, 24 (1968) 3329.
12 P. M. Scott, M. Merrien and J. Polonsky, *Experientia*, 32 (1976) 140.
13 B. J. Wilson, C. H. Wilson and A. W. Hayes, *Nature (London)*, 220 (1968) 77.
14 M. Yamazaki, H. Fujimoto and T. Akiyama, *Tetrahedron Lett.*, (1975) 27.
15 B. Böhner and Ch. Tamm, *Helv. Chim. Acta*, 49 (1966) 2527.
16 K. J. van der Merwe, P. S. Steyn and L. Fourie, *J. Chem. Soc.*, (1965) 7083.
17 A. C. Hetherington and H. Raistrick, *Phil. Trans. Roy. Soc. (London)*, B220 (1931) 269.
18 C. W. Holzapfel, *Tetrahedron*, 24 (1968) 2101.
19 J. H. Birkinshaw, A. E. Oxford and H. Raistrick, *J. Biochem.*, 30 (1936) 394.
20 P. S. Steyn, *J. Chromatogr.*, 45 (1969) 473.