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SPECTRODENSITOMETRIC DETERMINATION OF TRICHOTHECENE MYCOTOXINS WITH 4-(*p*-NITROBENZYL)PYRIDINE ON SILICA GEL THIN-LAYER CHROMATOGRAMS

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SUMMARY

A simple method for the detection and spectrodensitometric determination of a number of trichothecene mycotoxins on silica gel layers based on a colour reaction between 4-(*p*-nitrobenzyl)pyridine and the 12,13-epoxy group in the trichothecene nucleus is described. The detection limits for the twelve trichothecenes examined were 0.025–0.2 μg per spot. Further, six of the twelve trichothecenes could be determined spectrodensitometrically in the range from *ca.* 0.05–0.2 to 10 μg per spot with a coefficient of variation of *ca.* 5%.

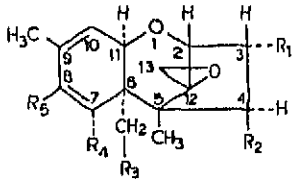
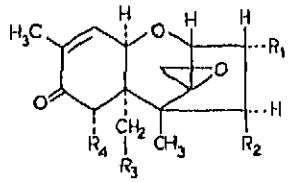
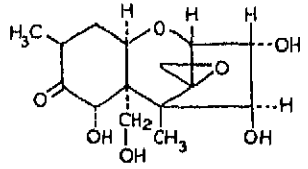
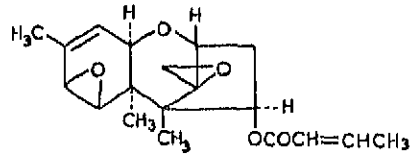
INTRODUCTION

Trichothecene mycotoxins with a 12,13-epoxytrichothec-9-ene nucleus are fungal metabolites produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma* and *Cepharosporium*, and about 40 homologues of the toxin have so far been reported. There have been many reports that these toxins cause a number of illnesses in man and animals, including Akakabi diseases of wheat in Japan and alimentary toxic aleukia in the U.S.S.R. Gas chromatography[1–3] and thin-layer chromatography (TLC)[4–7] have been widely employed for the analysis of trichothecene mycotoxins, and sulphuric acid, *p*-anisaldehyde and aluminium chloride have been used in the latter method as detection reagents. These reagents, however, have a poor structural selectivity for the trichothecene nucleus.

We have previously investigated the analysis of trichothecenes[8,9], and the present work was aimed at establishing a spectrodensitometric method for determining these mycotoxins on TLC plates by making use of the colour reaction of the 12,13-epoxy group in the trichothecene nucleus with 4-(*p*-nitrobenzyl)pyridine (NBP), which has been used as a chromogenic reagent for epoxides[10,11], alkylating agents[12–14], ethyleneimines[12] and organophosphates[15].

TABLE I

STRUCTURES OF TRICHOTHECENE MYCOTOXINS

Structure	*Mycotoxin (Abbreviation)*	R_1	R_2	R_3	R_4	R_5
	T-2 toxin (T-2)*	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
	HT-2 toxin (HT-2)**	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
	Neosolaniol (NS)*	OH	$OCOCH_3$	$OCOCH_3$	H	OH
	Diacetoxyscirpenol (DAS)***	OH	$OCOCH_3$	$OCOCH_3$	H	H
	7,8-Dihydroxydiacetoxyscirpenol (DiOHDAS)§	OH	$OCOCH_3$	$OCOCH_3$	OH	OH
	Fusarenon-X (F-X)§§	OH	$OCOCH_3$	OH	OH	
	Nivalenol (Niv)§§§	OH	OH	OH	OH	
	Diacetylnivalenol (DAN)§§§	OH	$OCOCH_3$	$OCOCH_3$	OH	
	Tetraacetylnivalenol (TAN)§§§	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	
	Deoxynivalenol (DON)†	OH	H	OH	OH	
	Dihydronivalenol (DHN)§					
	Crotocin (Crtn)††					

* Cultured *Fusarium solani*.
** Prepared from T-2 toxin.
*** Purchased from Makor Chemicals Ltd. (Israel).
§ Supplied by T. Tatsuno (Institute of Physical and Chemical Research, Japan).
§§ Cultured *Fusarium nivale*.
§§§ Prepared from fusarenon-X.
† Supplied by T. Yoshizawa (Ehime University, Japan).
†† Supplied by E. T. Glaz (Medical University, Budapest, Hungary).

EXPERIMENTAL

Chemicals

The trichothecene mycotoxins used are listed in Table I. Stock standard solutions of the toxins were prepared at a concentration of 100 μg/μl in chloroform, except for nivalenol, which was dissolved in water.

NBP (Tokyo Kasei Co., Japan; recrystallized from *n*-hexane) was used as a 1% (for detection) or a 3% (for quantitative determination) solution in chloroform–carbon tetrachloride (2:3) and tetraethylenepentamine (TEPA; Tokyo Kasei Co.) as a 10% solution in the same solvent mixture.

Pre-coated silica gel 60 TLC plates (layer thickness 0.25 mm) were purchased from E. Merck (Darmstadt, G.F.R.). All other chemicals were of analytical-reagent grade.

Apparatus

An Ozumor 82 densitometer (Asuka Kogyo, Tokyo, Japan) equipped with an integrator was used for determining the conditions of the colour reaction, and a Shimadzu CS-910 dual-wavelength chromatoscanner (Shimadzu, Kyoto, Japan) equipped with an integrator was employed in the reflectance and zig-zag scanning modes for quantitative determinations. Reflectance spectra of the chromatographic spots were measured with a Shimadzu UV-210A double-beam spectrophotometer equipped with a reflectance accessary. A 2-μl volumetric micropipette (Microcaps; Drummon, Broomall, Pa., U.S.A.), was used for application of the sample on the TLC plates.

Thin-layer chromatography

A 2-μl volume of the standard solution was spotted 2 cm from the lower edge of the plate and developed by the ascending technique until the front reached a height of 10 cm using an appropriate solvent system. The solvent systems examined were chloroform–methanol (9:1), benzene–acetone (1:1), ethyl acetate–*n*-hexane (5:1) and chloroform–acetone (3:2). The developed TLC plate was dried in an air stream and sprayed with 1% NBP solution for detection and dipped in 3% NBP solution for determination. After evaporation of the solvent, the plate was heated in an oven for 30 min at 150° and then cooled to room temperature. The cooled plate was sprayed with 10% TEPA solution for detection and dipped in the same solution for determination. The trichothecene mycotoxins could be observed as blue spots on a white background.

Densitometry

The Ozumor 82 was used under the following conditions: scanning speed, 20 mm/min; chart speed, 40 mm/min; and wavelength, 610 nm (filter). Scanning was carried out in the direction of the development. The CS-910 chromatoscanner was used under the following conditions: scanning speed, 20 mm/min; chart speed, 20 mm/min; wavelength, λ_1 (sample) = 610 nm, λ_2 (reference) = 710 nm; and beam slit, 1.25 × 1.25 mm. Scanning was also carried out in the direction of the development and the linearizer was operated on channel 2.

RESULTS AND DISCUSSION

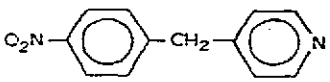
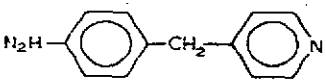
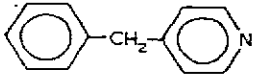
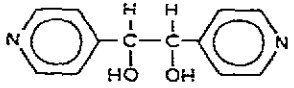
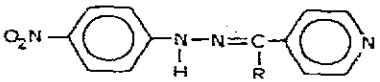
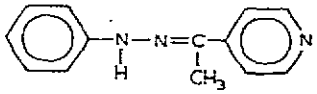
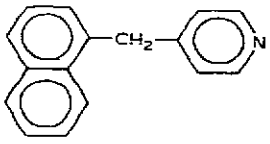
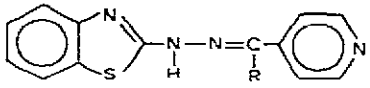
Colour reaction of trichothecenes with NBP

Although several 4-substituted pyridines were found to give coloured products by the reaction with trichothecenes on a silica gel layer, other reagents for detection of common epoxides, such as $Na_2S_2O_3$, $MgCl_2$, HCl and picric acid, gave negative results. NBP was the best among the 4-substituted pyridines (see Table II). TEPA was chosen as the base in our method because it was superior, in the stability and intensity of the colour produced, to the other amines and alkalis, such as aliphatic amines, aromatic amines, KOH and Na_2CO_3. After examining various reaction conditions using T-2 toxin, the reagent concentrations were chosen to be 1% NBP and 10% TEPA for detection, and 3% NBP and 10% TEPA for quantitative determination.

TABLE II

REACTION OF T-2 TOXIN WITH 4-SUBSTITUTED PYRIDINES ON SILICA GEL LAYER

T-2 toxin: 100 μg per spot.

*Reagent**	*Colour*	
	Spot	*Background*
	Blue	Colourless
	Colourless	Colourless
	Colourless	Colourless
	Pink	Yellow
	Green	Yellow
	Reddish brown	Yellow
	Colourless	Colourless
	Reddish brown	Yellow

* Reagent concentration: 1% in chloroform–carbon tetrachloride (2:3). R = H, CH_3.

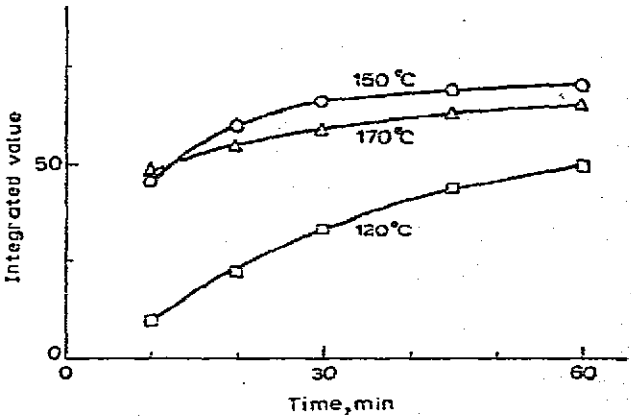


Fig. 1. Effect of reaction temperature and time. T-2 toxin: 10 μg per spot.

A higher reaction temperature was necessary for the reaction of NBP with the trichothecenes on the silica gel layer than that of the substituted pyridine with common epoxides in order to obtain satisfactory results. As is shown in Fig. 1, a constant integrated value was obtained on heating at 150° for *ca.* 30 min, whereas heating at temperatures higher than 170° resulted in a pale blue background.

As trichothecene-like compounds lacking the 12,13-epoxy group did not give a colour reaction with NBP, it was concluded that this reaction was specific to the 12,13-epoxy group in the trichothecene nucleus.

In the quantitative measurement of each spot, dipping of the plate into the reagent solution gave a better result than spraying of the reagent solution with regard to the stability of the colour and reproducibility of the results. Chloroform–carbon tetrachloride (2:3) was chosen as the solvent for NBP because it did not cause diffusion

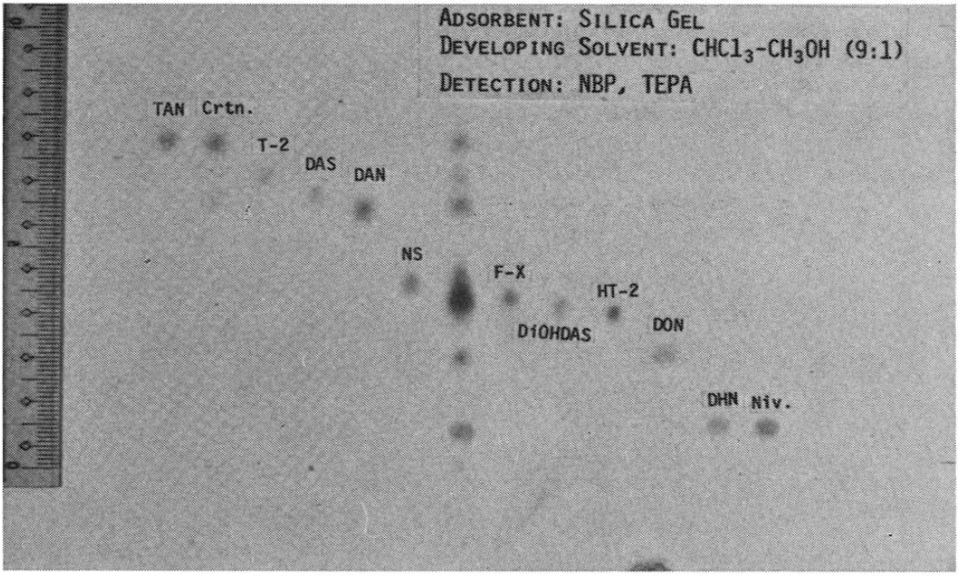


Fig. 2. Thin-layer chromatograms of trichothecene mycotoxins. Abbreviations as in Table I.

TABLE III

CHROMATOGRAPHIC DATA FOR TRICHOTHECENE MYCOTOXINS

Mycotoxin	R_F *value**				*Detection limit*** *(μg per spot)*	*Determination range*** *(μg per spot)*	*Colour stability**** *(h)*
	A	*B*	*C*	*D*			
Tetraacetylnivalenol	0.72	0.62	0.53	0.63	0.1	—	3
Crotocin	0.72	0.59	0.43	0.65	0.05	—	1
T-2 toxin	0.64	0.55	0.38	0.52	0.1	0.1 –10	3
Diacetoxyscirpenol	0.60	0.52	0.34	0.50	0.2	0.5 –10	3
Diacetylnivalenol	0.57	0.51	0.34	0.44	0.05	—	3
Fusarenon-X	0.36	0.41	0.24	0.29	0.05	0.1 –10	2
Neosolaniol	0.39	0.38	0.14	0.29	0.1	0.2 –10	1.5
HT-2 toxin	0.31	0.30	0.13	0.21	0.02	0.05–10	3
7,8-Dihydroxydiacetoxyscirpenol	0.32	0.35	0.14	0.24	0.2	—	1.5
Deoxynivalenol	0.23	0.31	0.16	0.20	0.1	—	1.5
Dihydronivalenol	0.07	0.13	0.05	0.05	0.1	—	1.5
Nivalenol	0.07	0.09	0.03	0.03	0.05	0.2 –10	3

* A, $CHCl_3$–CH_3OH (9:1); B, C_6H_6–$(CH_3)_2CO$ (1:1); C, $CH_3COOC_2H_5$–*n*-C_6H_{14} (5:1); D $CHCl_3$–$(CH_3)_2CO$ (3:2).

** Solvent system A was used, and a dash indicates that the sample was not tested.

*** By the dipping method.

of the spots. Scanning was carried out at 610 nm because the absorption maxima in the reflectance spectra of the spots obtained with all trichothecenes, except for crotocin (580 nm), are in the range 600–620 nm.

Satisfactory results were obtained in the TLC of twelve trichothecenes as is shown in Fig. 2. Their R_F values in several solvent systems and detection limits together with the stabilities of the colours are given in Table III.

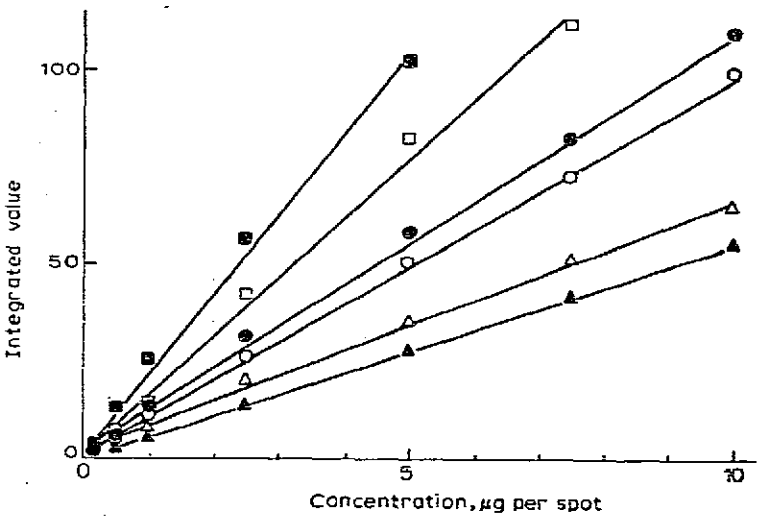


Fig. 3. Calibration graphs for trichothecene mycotoxins. ■, HT-2 toxin; ●, fusarenon-X; ▲, diacetoxyscirpenol; □, nivalenol; ○, T-2 toxin; △, neosolaniol.

Calibration graphs

Calibration graphs were constructed for T-2 toxin, HT-2 toxin, diacetoxyscirpenol, fusarenon-X, nivalenol and neosolaniol. As is shown in Fig. 3, the integrated values of the reflectance were proportional to the trichothecene concentration for all spots. Therefore, these six trichothecenes could be determined by this method in the range from *ca.* 0.05–0.2 to 10 μg per spot with a coefficient of variation of *ca.* 5%.

Detection of fusarenon-X or T-2 toxin added to a polished rice

The direct application of our method to trichothecenes in agricultural samples was unsuccessful because of several interfering substances which prevented the spots of trichothecenes from being correctly measured. Therefore, the clean-up method of Naoi *et al.*[5] was tested, using a sample of polished rice to which fusarenon-X or T-2 toxin had been added. It was found that this method is probably effective for fusarenon-X, as is shown in Fig. 4 (*ca.* 50–60% recoveries were obtained when 1–2.5 ppm of fusarenon-X was added), but not for T-2 toxin. Other clean-up methods should be applied to trichothecenes whose R_F values are higher than that of fusarenon-X (see Table III).

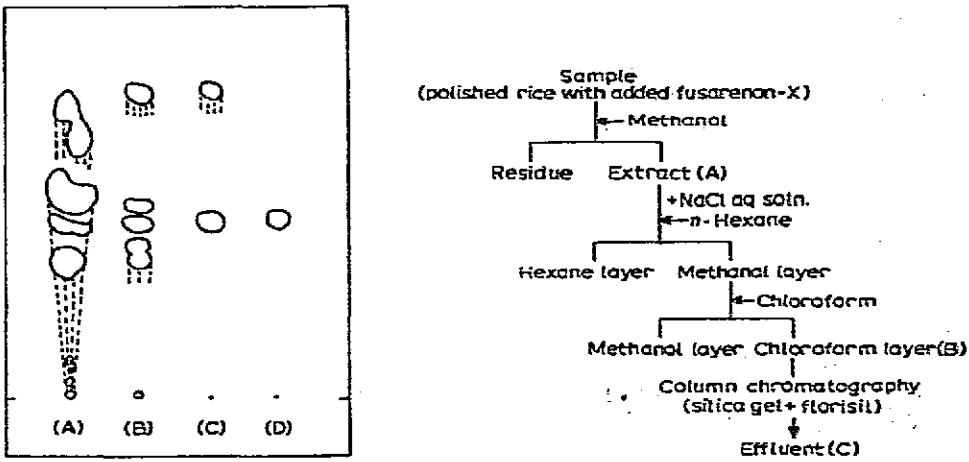


Fig. 4. Thin-layer chromatogram of the samples obtained in each step of the clean-up of Naoi *et al.*[5]. (A) Methanol extract; (B) chloroform layer; (C) effluent; (D) standard fusarenon-X. Developing solvent: $CHCl_3$–CH_3OH (9:1).

As agricultural products are contaminated with various mycotoxins, further studies of clean-up procedures and investigations of the systematic analysis of mycotoxins are required. Nevertheless, the present method seems to be useful for the analysis of trichothecene mycotoxins.

ACKNOWLEDGEMENTS

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REFERENCES

1 C. O. Ikediobi, I. C. Hsu, J. R. Bamburg and F. M. Strong, *Anal. Biochem.*, 43 (1971) 327.
2 Y. Nakahara and T. Tatsuno, *Chem. Pharm. Bull.*, 21 (1973) 1267.
3 K. Tanaka, T. Amano, K. Kawada and H. Tanabe, *Shokuhin Eiseigaku Zasshi*, 15 (1973) 195.
4 Y. Naoi, K. Saito, E. Kazama, H. Ogawa and Y. Kimura, *Ann. Rep. Tokyo Metr. Res. Lab. P. H.*, 23 (1971) 175.
5 Y. Naoi, E. Kazama, K. Saito, H. Ogawa, K. Shimura and Y. Kimura, *Ann. Rep. Tokyo Metr. Res. Lab. P. H.*, 25 (1974) 203.
6 N. Nakano, T. Kunimoto and K. Aibara, *Shokuhin Eiseigaku Zasshi*, 14 (1973) 56.
7 Z. Ďuračková, V. Betina and P. Nemec, *J. Chromatogr.*, 116 (1976) 141.
8 T. Kato, Y. Asabe, M. Suzuki and S. Takitani, *Bunseki Kagaku (Jap. Anal.)*, 25 (1976) 659.
9 T. Kato, Y. Asabe, M. Suzuki and S. Takitani, *Bunseki Kagaku (Jap. Anal.)*, 26 (1977) 422.
10 L. G. Hammock, B. D. Hammock and J. E. Casida, *Bull. Environ. Contam. Toxicol.*, 12 (1974) 759.
11 J. H. Brewer and R. J. Arnsberger, *J. Pharm. Sci.*, 55 (1966) 57.
12 J. Epstein, R. W. Rosenthal and R. J. Ess, *Anal. Chem.*, 27 (1955) 1435.
13 O. M. Friedman and E. Boger, *Anal. Chem.*, 33 (1961) 906.
14 E. Sawicki, D. F. Bender, T. R. Hauser, R. M. Wilson, Jr., and J. E. Meeker, *Anal. Chem.*, 35 (1963) 1479.
15 R. R. Watts, *J. Ass. Offic. Anal. Chem.*, 48 (1965) 1161.