Journal of Chromatography, 235 (1982) 257–265
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,298

FLUORODENSITOMETRIC DETERMINATION OF TRICHOTHECENE MYCOTOXINS WITH NICOTINAMIDE AND 2-ACETYLPYRIDINE ON A SILICA GEL LAYER

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(Received August 17th, 1981)

SUMMARY

A fluorodensitometric method for the determination of trichothecene mycotoxins on silica gel thin-layer plates based on a fluorescence reaction of the epoxy group with nicotinamide and 2-acetylpyridine is described. The limits of detection for the five trichothecenes examined were 20–50 ng per spot with visual inspection under UV light (360 nm). Further, these trichothecenes could be determined fluorodensitometrically in the range from *ca.* 10–25 ng per spot to 1500 ng per spot with a coefficient of variation of about 10%.

INTRODUCTION

Thin-layer chromatography (TLC) is one of the most useful methods for trichothecene mycotoxins with a characteristic 12,13-epoxytrichothec-9-ene nucleus, which are fungal metabolites produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma*, *Cepharosporium*, etc., and are among the most interesting mycotoxins studied in the fields of foods and feeds. Sulphuric acid[1–3], *p*-anisaldehyde[1,4], aluminium chloride[5] and 4-(*p*-nitrobenzyl)pyridine[6] have been used as the detection reagents on thin-layer plates. However, these reagents, except for the last one, have a poor structural selectivity for the trichothecene nucleus.

We have previously investigated the analysis of trichothecene mycotoxins[6–9] and in the last paper[6] we reported a densitometric determination using 4-(*p*-nitrobenzyl)pyridine as the chromogenic reagent. Although our method is the most convenient and has the highest selectivity towards trichothecenes in comparison with other methods, it has some defects such as low colour stability and low sensitivity.

This work was aimed at establishing a fluorodensitometric method for determining these mycotoxins on thin-layer plates by making use of a fluorescence reaction of the 12,13-epoxy group in the trichothecene nucleus with nicotinamide and 2-acetylpyridine.

0021-9673/82/0000–0000/$02.75

EXPERIMENTAL

Chemicals

The trichothecene mycotoxins used are listed in Table I. The stock standard solutions of the toxins were prepared at a concentration of 5 μg/μl in chloroform, except for deoxynivalenol and nivalenol, which were dissolved in methanol.

Nicotinamide (Tokyo Kasei Co., Japan) was used as a 4% solution in acetone–ethanol (5:1). 2-Acetylpyridine (Nakarai Chemical Co., Japan; re-distilled under reduced pressure) was used as a 3% solution in *n*-hexane. Potassium hydroxide (Kanto Chemical Co., Japan) was used as a 2 *N* solution in 80% ethanol. Formic acid (98–100%; Nakarai Chemical Co.) was used as a 30% solution in diethyl ether.

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

Apparatus

A Shimadzu CS-910 dual-wavelength chromatoscanner (Shimadzu, Kyoto, Japan) equipped with an integrator was employed in the reflectance and linear scanning modes.

A 2-μl volumetric micropipette (Microcaps; Drummond, Broomall, PA, U.S.A.), was used to apply the sample to the TLC plates, except for recovery examination in which a 5-μl micropipette was used.

Thin-layer chromatography

A 2-μl volume of the sample solution was spotted at 2 cm from the lower edge of the plate and the plate was developed by the ascending technique until the front had reached a height of 10 cm using an appropriate solvent system. The solvent systems examined were chloroform–methanol (97:3, 95:5 and 7:1). The developed plate was dried in an air stream and was dipped in the nicotinamide solution*. After evaporation of the solvent, the plate was heated in an oven for 15 min at 160 C in method I, which was used for the simultaneous analysis of both type A and B trichothecenes, or for 10 min at 140 C in method II, which was used only for type B trichothecenes, and then cooled to room temperature. The cooled plate was dipped in the 2-acetylpyridine solution and then sprayed with the potassium hydroxide solution. The plate was allowed to stand for 30 min at room temperature, then dipped in the formic acid solution. After evaporation of the solvent, the acidified plate was heated in an oven for 4 min at 100 C and then cooled to room temperature.

The trichothecene mycotoxins could be observed as light blue fluorescent spots on a very weak dark blue fluorescent background under UV light (360 nm).

Densitometry

A CS-910 chromatoscanner was used under the following conditions: scanning speed, 20 mm/min; chart speed, 20 mm/min; wavelengths, λ_{ex} = 380 nm, λ_{em} = 450 nm (interference filter); and beam slit, 10 × 0.5 mm. Scanning was also carried out in the direction of development.

* Dipping was carried out in the direction of development.

RESULTS AND DISCUSSION

By analogy with the fluorescence reaction of N^1-methylnicotinamide with an active methylene compound[10], the fluorophore obtained by our method is assumed to be a stable naphthylidine derivative, which resulted from (1) N-alkylation of nicotinamide with the epoxy group and (2) subsequent condensation of N^1-alkylnicotinamide with 2-acetylpyridine in the presence of an alkali and followed by (3) treatment with an acid.

Trichothecene mycotoxins are classified into two groups (types A and B) according to the structural variations at the C-8 position of the trichothecene nucleus (see Table I). Various experimental conditions were examined, using T-2 toxin as a typical sample of type A and fusarenon-X as type B because the reactivity of type A trichothecenes in the reaction with nicotinamide differed distinctly from that of type B in our preliminary examinations.

N-Alkylation reaction conditions

Constant integrated values were obtained at concentrations above 1% for fusarenon-X in the examination of the influence of nicotinamide concentration on the N-alkylation reaction. On the other hand, the integrated values for T-2 toxin increased gradually within the 6% nicotinamide concentration range examined. Higher nicotinamide concentrations caused a higher fluorescent background, and also more polar solvent was necessary to dissolve the reagent, which resulted in diffusion of the spots. Hence a 4% acetone–ethanol (5:1) solution was chosen.

The relationship between fluorescence intensity and reaction time was studied at 140 and 160°C (Fig. 1). When the heating was carried out at 140°C, the reaction of T-2 toxin (type A trichothecenes) was incomplete after 30 min, but an approximately constant intensity was obtained after heating at 160°C for 10–30 min.

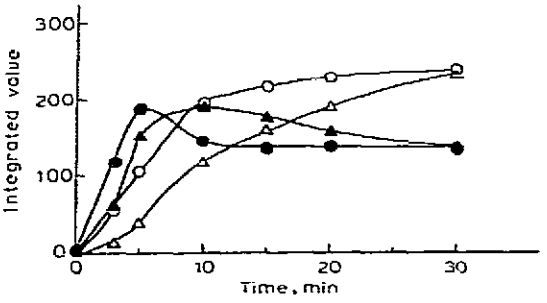


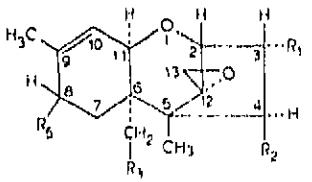
Fig. 1. Effect of reaction time and temperature on the N-alkylation reaction. Open symbols, T-2 (0.5 μg per spot); closed symbols, F-X (1.0 μg per spot). △, ▲, 140°C; ○, ●, 160°C. Abbreviations as in Table I.

On the other hand, with fusarenon-X (type B trichothecenes), an almost constant and maximal intensity was obtained after heating at 140°C for 10 min. Moreover, when the heating temperature was elevated to 160°C, the fluorescence intensity reached the maximal value after heating for 5 min and, after decreasing rapidly, the intensity reached a constant value (correspond to *ca.* 74% of the maximal intensity) after 10–30 min, whereas heating over 30 min resulted in a brown spot. As the maximal intensity after heating at 140°C for 10 min was *ca.* 1.4 times higher than

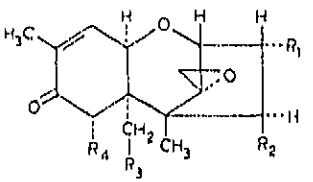
TABLE I

STRUCTURES OF TRICHOTHECENE MYCOTOXINS

Type A:



Type B:



Type A

Mycotoxin (abbreviation)	R_1	R_2	R_3	R_5
T-2 toxin (T-2)*	OH	$OCOCH_3$	$OCOCH_3$	$OCOCH_2CH(CH_3)_2$
Neosolaniol (NS)*	OH	$OCOCH_3$	$OCOCH_3$	OH
Diacetoxyscirpenol (DAS)**	OH	$OCOCH_3$	$OCOCH_3$	H

Type B

Mycotoxin (abbreviation)	R_1	R_2	R_3	R_4
Fusarenon-X (F-X)***	OH	$OCOCH_3$	OH	OH
Nivalenol (Niv)‡	OH	OH	OH	OH
Tetraacetylnivalenol (TAN)‡	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
Deoxynivalenol (DON)‡‡	OH	H	OH	OH

* Cultured *Fusarium solani*.

** Purchased from Makor Chemicals Ltd. (Israel).

*** Cultured *Fusarium nivale*.

‡ Prepared from fusarenon-X.

‡‡ Supplied by T. Yoshizawa (Kagawa University, Japan).

the constant value at 160°C, heating at 140°C seemed optimal for the analysis of fusarenon-X.

Therefore, heating at 160°C for 15 min was selected in method I, which was used for simultaneous analysis of the both type A and B trichothecenes, and heating at 140°C for 10 min was also selected in method II, which was used exclusively for the analysis of type B trichothecenes.

As the best result was obtained when the subsequent reaction was done without heating, the plate was therefore cooled to room temperature before proceeding to the next step.

Conditions of the condensation reaction of N^1-alkylnicotinamide with 2-acetylpyridine

Various active methylene compounds were examined as reagents in the condensation reaction. As shown in Table II, 2-acetylpyridine gave the highest fluorescence intensity; the intensities were twice and four times as great as that of acetophenone used as the reagent for T-2 toxin and fusarenon-X, respectively, whereas with common epoxy compounds such as glycidyl phenyl ether, the intensity obtained with 2-acetylpyridine was only 1.4 times as great as that of acetophenone[11]. Therefore, 2-acetylpyridine was chosen in the present method to increase the analytical sensitivity for mycotoxins. As almost constant fluorescence intensity was obtained at a concentration above 1 % of 2-acetylpyridine for both type A and B trichothecenes, a 3 % solution was used.

TABLE II

EFFECT OF ACTIVE METHYLENES ON THE CONDENSATION REACTION

T-2 toxin, 0.5 μg per spot; fusarenon-X, 1.0 μg per spot. Results obtained by method I.

*Compound**	*Relative fluorescence intensity***		*Compound**	*Relative fluorescence intensity***	
	T-2	*F-X*		*T-2*	*F-X*
Acetone	0.3	0.9	*p*-Hydroxyacetophenone	0	0
Methyl ethyl ketone	0.9	2.4	*p*-Acetylacetophenone	41	16
Cyclopentanone	11	24	1-Acetylnaphthalene	2.7	3.4
Cyclohexanone	18	37	2-Acetylnaphthalene	6.0	3.7
1-Indanone	2.6	2.9	2-Acetylpyridine	100	100
α-Tetralone	4.3	5.6	3-Acetylpyridine	38	43
Acetophenone	57	24	4-Acetylpyridine	21	33
p-Chloroacetophenone	42	23	2-Acetylfuran	7.7	13
p-Bromoacetophenone	30	22	2-Acetylthiophene	5.1	11
p-Nitroacetophenone	0	0	3-Acetylindole***	0	0
p-Methylacetophenone	34	15			

* Reagent concentration: 5 %.

** 2-Acetylpyridine arbitrarily taken as 100.

*** Saturated solution.

Potassium hydroxide was chosen as the alkali in the condensation reaction because it gave superior fluorescence intensity, signal-to-noise ratio and solubility in aqueous ethanol compared with other alkalis and organic bases. Although water or

an aqueous solvent is desirable for dissolving potassium hydroxide, it has an unfavourable effect on the silica gel layer. Therefore, an 80% ethanol solution was used in this step. Next, the effect of potassium hydroxide concentration on this reaction was studied; the formation of the fluorophore was affected by the alkali concentration, as shown in Fig. 2. The concentration used was 2 *N* because some destruction of the silica gel plate was observed when solutions of concentration higher than 3 *N* were used, although the maximal intensity was obtained with a 2.5 *N* solution.

For addition of the alkali solution, spraying was chosen in order to prevent peeling of the silica gel layer; in the other steps the reagent solutions were added to the silica gel plate by dipping.

The relationship between fluorescence intensity and the standing time after spraying with the potassium hydroxide solution was studied at room temperature. As a constant integrated value was obtained when the standing times were longer than 10 min for T-2 toxin and 20 min for fusarenon-X, a standing time of 30 min was therefore chosen.

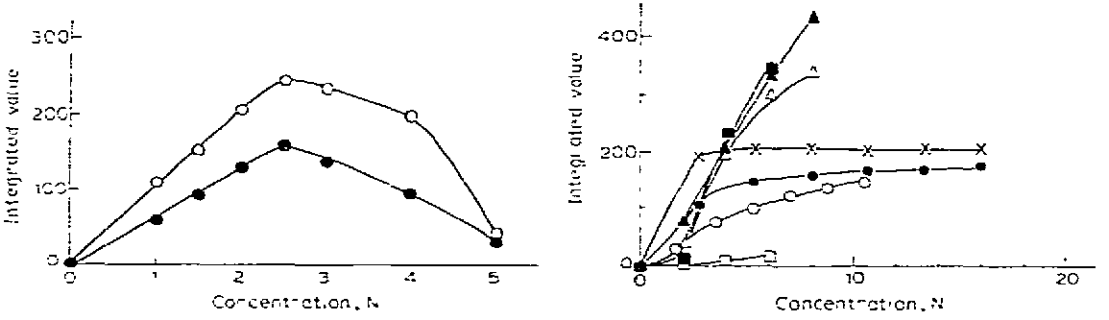


Fig. 2. Effect of potassium hydroxide concentration on the condensation reaction (method I). ○, T-2 (0.5 μg per spot); ●, F-X (1.0 μg per spot).

Fig. 3. Effect of acid species and their concentration on the acid treatment (method I). T-2 (0.5 μg per spot): ○, formic acid; ○, acetic acid; ▲, citric acid; △, malonic acid; ■, sulfuric acid; □, phosphoric acid. F-X (1.0 μg per spot): ●, formic acid.

Conditions of acid treatment

Various acids were examined (Fig. 3). Formic acid was chosen because it was superior in both stability and intensity of the resulting fluorescence and in solubility for non-polar solvent such as diethyl ether compared with the other acids examined, *e.g.*, acetic, malonic, citric, sulphuric and phosphoric acids.

As almost constant intensities were obtained at concentrations above 10% (2.65 *N*) for T-2 toxin and 20% (5.3 *N*) for fusarenon-X, 30% (7.95 *N*) formic acid solution was used.

The heating step for the conversion of the reaction intermediate into the final product was studied; it was found that the reaction rate was slow at room temperature but the reaction was complete within a few minutes at 100 C. Therefore, heating at 100 C for 4 min was selected.

The fluorescences obtained by both methods I and II were stable at least for 4 h at room temperature.

Satisfactory results were obtained in the TLC of seven trichothecenes, as shown in Fig. 4.

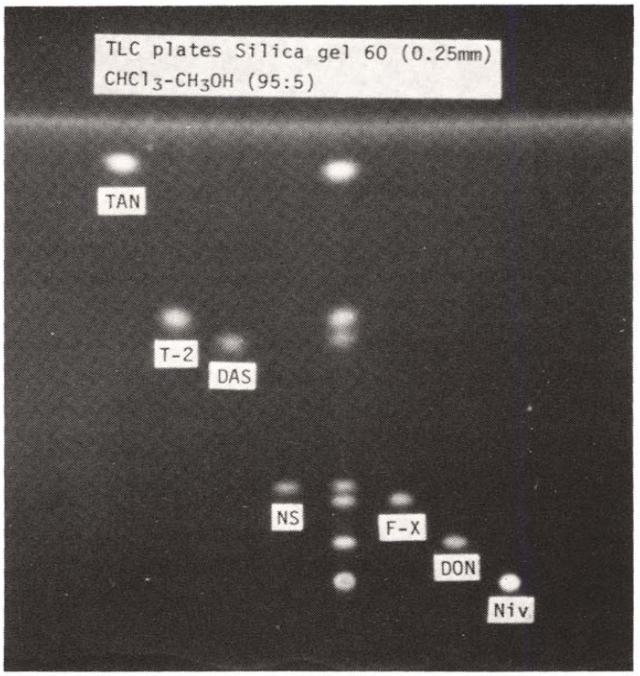


Fig. 4. Thin-layer chromatogram of trichothecene mycotoxins. Developing solvent: chloroform–methanol (95:5); method 1.

Limits of detection and calibration graphs

The analytical data for T-2 toxin, diacetoxyscirpenol, fusarenon-X, deoxynivalenol and nivalenol obtained by the proposed methods are summarized in Table III; the limits of detection with visual inspection under UV light (360 nm) were 20–50 ng per spot, and the calibration graphs for these mycotoxins were straight lines in the concentration ranges given in Table III.

The coefficients of variation of the measurements were satisfactory in most instances.

Detection of T-2 toxin, fusarenon-X, deoxynivalenol and nivalenol added to a corn sample

The proposed method has a high selectivity for epoxy compounds but is seriously affected by other alkylating agents because the reactivity of trichothecenes in the N-alkylation reaction was considerably lower than that of common epoxy compounds. An attempt to apply our method directly to trichothecenes in agricultural samples was unsuccessful because several interfering materials prevented the detection of the spots. Hence, it is necessary to use some clean-up step prior to practical analyses.

The clean-up method of Kamimura *et al.*[12] was tested using a corn sample to which T-2 toxin, fusarenon-X, deoxynivalenol and nivalenol had been added. This method was effective for analysis by the integrated area method of T-2 toxin, fusarenon-X and nivalenol but not for deoxynivalenol, as shown in Figs. 5 and 6. Further,

TABLE III

ANALYTICAL DATA FOR TRICHOTHECENE MYCOTOXINS

Method	*Developing solvent*	*Mycotoxin*	R_f *value*	*Determination range* (ng per spot)*	*Coefficient of variation* (%)*	*Limit of detection (ng per spot)*
I	$CHCl_3$–CH_3OH (97:3)	T-2 toxin	0.55	10–1500 (10–1250)	1.0 μg, 2.8 (7.4) 25 ng, 8.8 (12.5)	25
		Diacetoxyscirpenol	0.50	10–1500 (10–1000)	1.0 μg, 5.6 (3.9) 25 ng, 8.6 (9.3)	25
		Fusarenon-X	0.14	25–1500 (25–1250)	1.0 μg, 3.8 (5.8) 50 ng, 7.8 (13.0)	50
		Deoxynivalenol	0.08	25–1500 (25–1250)	1.0 μg, 4.4 (7.7) 50 ng, 9.0 (11.8)	50
		Nivalenol	0.02	25–1500 (25–1250)	1.0 μg, 5.2 (4.7) 50 ng, 7.3 (5.4)	50
II	$CHCl_3$–CH_3OH (7:1)	Fusarenon-X	0.64	20–1500 (20–1250)	1.0 μg, 5.4 (8.3) 50 ng, 8.8 (10.2)	25
		Deoxynivalenol	0.47	20–1500 (20–1250)	1.0 μg, 2.4 (4.7) 50 ng, 7.4 (7.3)	25
		Nivalenol	0.22	10–1500 (10–1250)	1.0 μg, 3.2 (9.7) 50 ng, 4.9 (3.3)	20

* Values without parentheses, peak-area method; values in parentheses, peak-height method.

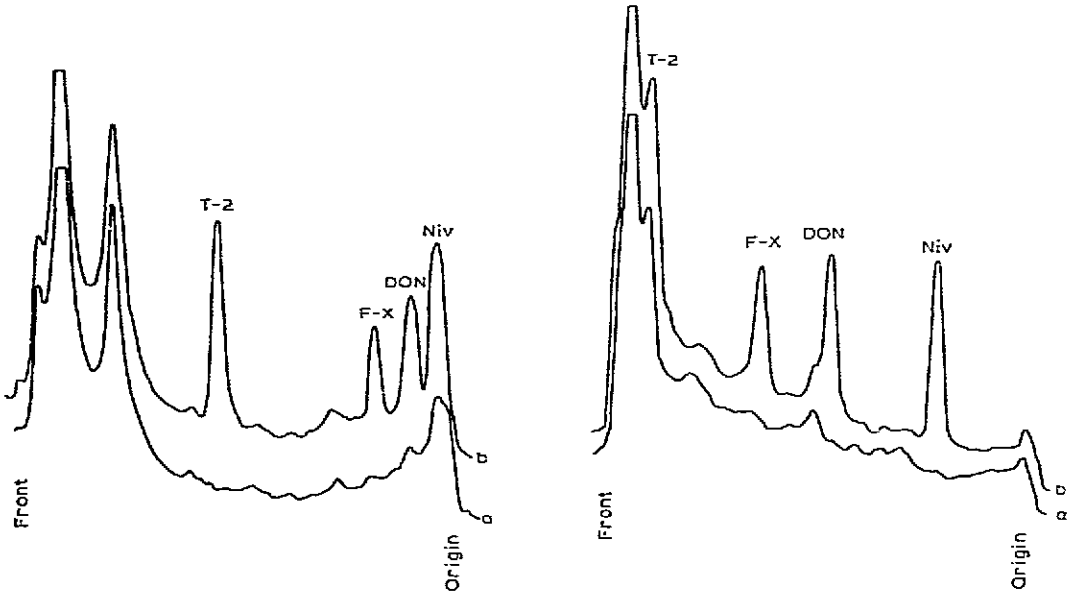


Fig. 5. Fluorodensitograms of extract from corn after fluorescent reaction (method I). (a) Corn; (b) corn + trichothecenes (corresponding to 0.5 ppm). Developing solvent: chloroform–methanol (97:3).

Fig. 6. Fluorodensitograms of extract from corn after fluorescent reaction (method II). (a) Corn; (b) corn + trichothecenes (corresponding to 0.5 ppm). Developing solvent: chloroform–methanol (7:1).

deoxynivalenol could be determined by the peak-height method; about 60–70% recoveries were obtained by the method II after development with chloroform–methanol (7:1) when 1 ppm of deoxynivalenol and nivalenol were added to an original corn sample.

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

ACKNOWLEDGEMENT

The authors thank Dr. T. Yoshizawa for supplying the sample.

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