COMPARISON OF DIFFERENT SPRAY REAGENTS FOR IDENTIFICATION OF TRICHOTHECENES

Y. RAMAKRISHNA and R.V. BHAT*

Food and Drug Toxicology Research Centre, National Institute of Nutrition, Hyderabad 500 007, India.

ABSTRACT

The efficacy of seven spray reagents for four naturally occurring trichothecenes has been comparatively evaluated. No single spray reagent was suitable for all the trichothecenes because of the variation in the structural conformity of the different trichothecenes. However, for each type of trichothecene a specific spray reagent such as aluminium chloride for B group trichothecenes and sulphuric acid spray for T-2 toxin etc, could be used.

INTRODUCTION

TRICHOTHECENES belong to a specific group of mycotoxins produced by a large number of fungi. They are the secondary metabolites and are sesquiterpenoid with a 12, 13 epoxy trichothec nucleus. Realizing their ill-effects in humans and animals, simple methods of analyses were evolved to identify, confirm and quantitate[1]. Further, they have been classified into 4 categories based on their chemical structure and fungi producing them viz *Type A* consisting of T-2 toxin, HT-2 toxin, diacetoxyscirpenol etc which are produced by *Fusarium tricinctum* and *F. sporotrichoides*; *Type B* consisting of Nivalenol and Deoxynivalenol produced by *F. graminearum*, *Type C* consisting of Crotocin produced by *Cephalosporium sps*, and *Type D* consisting of Verrucarins, Roridins and Satratoxins produced by *Stachybotrys sps.* However only T-2 toxin, Diacetoxyscirpenol, Nivalenol, Fusarenone-X, Deoxynivalenol and Acetyldeoxynivalenol occur naturally. Trichothecenes are largely unreactive chemical compounds and unlike aflatoxins do not show fluorescence under UV light, unless and until sprayed with specific chemical compounds. TLC is a rapid, simple and economical method for semiquantitative or quantitative (with the aid of instrument) analysis of mycotoxins. Spray reagents or derivatizing agents are used to fluoresce trichothecenes, thus aiding in their detection. Many compounds have been proposed as derivatizing agents for trichothecenes, namely, P-anisaldehyde[2], sulphuric acid[3], aluminium chloride[4], 4-(*p*-nitrobenzyl)pyridine[5], nicotinamide 2-acetyl pyridine[6], chromotropic acid[7] and phloroglucinol[8]. The present study relates to the comparative evaluation and trying their suitability for routine analytical work.

MATERIALS AND METHODS

Four trichothecenes namely Deoxynivalenol, Nivalenol (Wako Pure Chem. Industries Ltd., Osaka, Japan), T-2 toxin and Diacetoxyscirpenol (Makor Chem. Ltd., Jerusalem, Israel) were obtained. They were dissolved in chloroform and methanol (9 : 1) to give 50 μg/ml concentration (Deoxynivalenol and Nivalenol) and 100 μg/ml (T-2 and Diacetoxyscirpenol). Ground rice samples spiked with standard concentrations of the above five mycotoxins separately were then extracted by the method of Greenhalgh *et al*[9]. TLC plates (20 × 20 cm) were coated to a uniform thickness of 0.5 mm with silica gel G (BDH Chemicals), activated and used as and when required.

i. Sulphuric acid spray: 20 ml of conc. sulphuric acid in 80 ml ethanol.

ii. Aluminium chloride spray: 20 g of aluminium chloride to previously cooled 30% aq. ethanol.

iii. Chromotropic acid spray: 1 g of chromotropic acid in 10 ml water added to 50 ml of sulphuric acid and water (5 : 3).

iv. P-anisaldehyde reagent: 85 ml of methanol, 10 ml of glacial acetic acid and 5 ml of conc. sulphuric acid. Dissolve 0.5 ml of P-anisaldehyde in this mixture and use immediately.

v. Nitrobenzyl pyridine spray: 1% solution in chloroform and carbon tetrachloride (2 : 3).

vi. Nicotinamide 2-acetyl pyridine spray: 4% nicotinamide solution in acetone-ethanol (5 : 1).

vii. Phloroglucinol spray: saturated solution in conc. hydrochloric acid.

* For Correspondence.

The standard trichothecenes were spotted onto the TLC plates and developed with chloroform-methanol (93 : 7). The developed plates were air-dried and then the solvent was evaporated in a hot air oven for a minute or two. The same treatment was given to the spiked samples and all were spotted in duplicates. One plate was used separately for each spray reagent. The plate designated to each spray reagent was sprayed with the respective spray reagent except for nicotinamide 2-acetyl pyridine. In this case the dried plate was dipped in 4% nicotinamide solution in acetone-ethanol (5 : 1). The plate was taken out and the solvent evaporated in an oven (15 min/160°C). The plate was cooled to room temperature and then dipped into 2-acetyl pyridine solution (3% solution in hexane). It was then sprayed with potassium hydroxide solution (2 N solution in 80% aq. ethanol). The plate was allowed to stand for 30 min at room temperature and then dipped in formic acid solution (30% sol. in diethyl ether). The plates were then observed under visible and UV light (360 nm).

RESULTS AND DISCUSSION

The mycotoxins when treated with aluminium chloride, nitrobenzyl pyridine, nicotinamide acetyl pyridine and chromotropic acid exhibited fluorescent spots while those treated with P-anisaldehyde and phloroglucinol exhibited visible spots (table 1). Sulphuric acid showed charring effect on all the trichothecenes and fluorescence with T-2 toxin. There was not much difference between the spiked samples and standard mycotoxins. The spiked samples showed less intensity of fluorescence/colour development as compared to the standard for the same concentration and this may be due to losses in recovery.

Sulphuric acid spray (in 10–50% aq. methanolic or ethanolic solutions) has been reported to give greyish black colour for type A and brown colour for type B trichothecenes[1]. However in the present study it was difficult to distinguish between different colours and both A and B trichothecenes on spraying sulphuric acid (and charring) exhibited brownish charred appearance. It was observed that both types A and B trichothecenes gave a brownish charred appearance. Further, among the two trichothecenes tested of type A (T-2 and Diacetoxyscirpenol) only T-2 toxin was observed to give fluorescence at 360 nm, detecting about 0.5 μg/spot.

P-anisaldehyde was observed to be an effective spray reagent with type B trichothecenes, particularly Deoxynivalenol which gave yellowish brown colour. This agreed with the reports of earlier workers[1]. However, the problem faced with this reagent was the differentiation of the spot with the background and the care required in its preparation. Even a slight variation in the composition of the reagent was observed to give negative results.

4-(P-nitrobenzyl) pyridine gave a bluish violet-coloured spot on a whitish background. The technique was found to be laborious and time-consuming and hence cannot be used for regular screening of samples. However, this can be used for confirming the identity of trichothecenes.

Nicotinamide 2-acetyl pyridine gave bluish fluorescence with the trichothecenes studied. Although the reported sensitivity was 0.25 μg/spot for type A and 0.05 μg/spot for type B[6], it was observed that the sensitivity was enhanced by two

Table 1 *Action of different spray reagents on trichothecenes*

Spray reagent	Nature of reaction	DON	NV	T-2	DAS
Sulphuric acid	C/F	Charring	Charring	Charring	Blue green
P-anisaldehyde	VC	Yellow	Yellow		–
4 (P-nitrobenzyl) Pyridine	VC	Bluish violet	Bluish violet	Bluish violet	Bluish violet
Nicotinamide 2-acetyl pyridine	F	Bluish	Bluish	Bluish	Bluish
Chromotropic acid	VC	–	–	Purple	Brown
Phloroglucinol	VC	Pink	Pink	Pink	Pink
Aluminium chloride	F	Bluish	Bluish	–	–
R_f Values (Chloroform: Methanol, 97 : 3)		0.24	0.12	0.79	0.68

DON–Deoxynivalenol; NV–Nivalenol; T-2 – T-2 toxin; DAS – Diacetoxyscirpenol; C/F, Charring – fluorescence; VC; visible colour; F – fluorescence.

and half times for type A and reduced by 4 times for type B. The technique involves tedious and time-consuming procedure and the need for other accessory reagents. This spray reagent could also be ideal for confirming the identity of type A and B trichothecenes but cannot be put to use for routine analysis.

Chromotropic acid spray reagent gave purple colour, visible to the naked eye against a mauve background for T-2 toxin and brown for Diacetoxyscirpenol. The type B trichothecenes did not respond to this reagent since no colour/fluorescence was seen. Moreover, the differentiation of the spot from the background requires experience for regular handling.

Phloroglucinol spray reagent was reported to react with the allyl groups of the 12,13 epoxy D-9-trichothec nucleus giving a pink coloured spot[8]. The pink colour was specific for all trichothecenes with epoxy groups. All the five trichothecenes gave a pink colour making it difficult to differentiate between the trichothecenes. Further, the interference by the contaminating pigments which mimic the trichothecenes in fluorescence could not be eliminated.

Aluminium chloride spray reagent was found to be specific for type B trichothecenes namely Deoxynivalenol and nivalenol. A characteristic bluish fluorescence was observed[4]. However, the limit of detection was 0.1 μg/spot. The heating of the plate after spraying partially helped in charring the pigments in the spiked sample, while the toxins were more fluorescent. The reagent was ideal for routine analysis of type B trichothecenes.

5 May 1986; Revised 31 July 1986

1. Ueno, Y., (ed.) *Developments in food science*, Trichothecenes–chemical, biological and toxicological aspects, Kodansha Ltd., Tokyo and Elsevier Science Publ., Amsterdam, 1983.
2. Scott, P. M., Lawrence, J. W. and Walbeek, W. V., *Appl. Microbiol.*, 1970, **20**, 839.
3. Ueno, Y., Sato, N., Ishii, K., Sakai, H., Tsunoda, H. and Enomoto, M., *Appl. Microbiol.*, 1973, **25**, 699.
4. Kamimura, H., Nishijama, N., Yasuda, K., Saito, K., Ibe, A., Nagayama, T., Ushiyama, H. and Naoi, Y., *J. Assoc. Anal. Chem.*, 1981, **64**, 1067.
5. Takitani, S., Asabe, Y., Kato, T., Sujuki, M. and Ueno, Y., *J. Chromatogr.*, 1979, **172**, 335.
6. Sano, A., Asabe, Y., Takitani, S. and Ueno, Y., *J. Chromatogr.*, 1982, **235**, 257.
7. Baxter, J. A., Terhune, S. J. and Qureshi, S. A., *J. Liq. Chromatogr.*, 1983, **261**, 130.
8. Rao, G. V., Rao, P. S., Girisham, S. and Reddy, S. M., *Curr. Sci.*, 1985, **54**, 507.
9. Greenhalgh, R., Neish, G. A. and Miller, J. D., *Appl. Environ. Microbiol.*, 1983, **46**, 625.

NEWS

CANCER IS GENETIC

Cancer, the most dreaded disease that has afflicted mankind for centuries, still eludes an effective treatment. Huge sums are spent and thousands of scientists are engaged in the quest to find an effective remedy to combat this proliferating malignancy. Soviet scientists have broken new grounds in this field. The discovery of oncogenes, and the fact that oncogenes are present in any normal healthy cell, was a brilliant find. This amazing research led to the conclusion that the root cause of any malignant formation of a cell is due to the presence of oncogenes in cancer virus which transmits into the genes of a healthy cell, thus forever changing its hereditary character. (*Soviet Features*, Vol. XXVI, No. 41, April 7, 1987; Information Department, USSR Embassy in India, P.B. 241, 25 Barakhamba Road, New Delhi 110 001.)