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Note

Use of chromotropic acid for improved thin-layer chromatographic visualization of trichothecene mycotoxins

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Trichothecene mycotoxins (with a 12,13-epoxytrichothec-9-ene nucleus), including T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (vomitoxin) and others have been detected in fungal contaminated grain[1,2] and are associated with a variety of diseases in animals and man[3]. Current thin-layer chromatographic (TLC) methods for these toxins lack sensitivity mainly because trichothecenes are difficult to visualize on a TLC plate since they neither fluoresce nor absorb UV light appreciably. Therefore, visualization of trichothecenes requires chromogenic reagents. Current procedures employ reagents such as aluminium chloride[4,5], sulphuric acid[6], *p*-anisaldehyde[7], 4-(*p*-nitrobenzyl) pyridine (NBP)[8], and nicotinamide–2-acetylpyridine (N-A)[9].

In our current research on trichothecene methodology, we have found that aluminium chloride was relatively specific for visualizing type-B trichothecenes such as vomitoxin (Fig. 1). The type-A trichothecenes such as T-2 or DAS, do not react

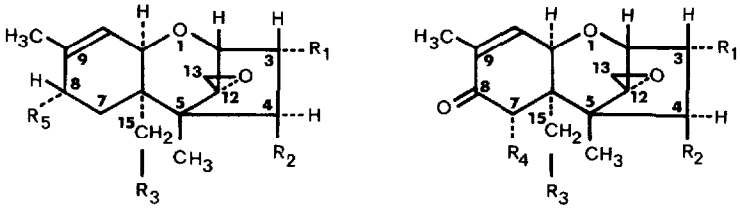

Fig. 1. Structures of some trichothecene mycotoxins.

	R_1	R_2	R_3	R_4	R_5
Type A					
T-2	OH	OAc	OAc		$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpenol	OH	OAc	OAc		H
HT-2 toxin	OH	OH	OAc		$OCOCH_2CH(CH_3)_2$
T-2-tetraol	OH	OH	OH		OH
T-2-triol	OH	OH	OH		H
Type B					
Deoxynivalenol	OH	H	OH	OH	

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with aluminium chloride but can be visualized by any of the other reagents mentioned above. These reagents, however, react with a wide range of extraneous compounds which often produce spots that obscure the toxins. Thus, with these reagents, high sensitivity cannot be obtained with TLC unless the extracts are subjected to extensive and impractical cleanup procedures.

Recently, Kato *et al.*[10] have proposed the use of chromotropic acid for the spectrophotometric determination of several trichothecene mycotoxins. From this work, we have developed a new procedure employing chromotropic acid as a sensitive and specific spray reagent to detect trichothecenes on TLC plates. The major advantage of this reagent lies in the contrasting colours produced by the trichothecenes in comparison to those of extraneous compounds in plant extracts.

EXPERIMENTAL

Materials

Reference standards of T-2, DAS, HT-2, T-2 triol, and T-2 tetraol were purchased from Sigma. Vomitoxin was obtained from Mycolabs. All standards were stored in sealed vials at −20°C.

Chromotropic acid (4,5-dihydroxynaphthalene-2,7-disulphonic acid, disodium salt, dihydrate) was purchased from J. T. Baker, and aluminium chloride (hydrated) was obtained from Fisher. We employed pre-coated TLC, plates with silica gel 60 (0.2 mm layer thickness) on aluminium foil, from E. Merck. Sulphuric acid, ethanol, benzene, acetonitrile, toluene, ethyl acetate and 90% formic acid were all analytical grade.

Spray reagents

Aluminium chloride reagent. A 20-g amount of aluminium chloride was dissolved in 100 ml of water–ethanol (1:1). The solution was stable at room temperature. Fresh reagent was prepared monthly.

Chromotropic acid reagent. One part of 10% aqueous solution of chromotropic acid was mixed with 5 parts of concentrated sulphuric acid–water (5:3). The reagent is clear initially but darkens with age. Fresh reagent was prepared after 3–4 weeks.

Mycotoxin standard solutions

Individual standard solutions were prepared containing 0.1 mg each of T-2, DAS, HT-2, vomitoxin, T-2 triol and T-2 tetraol per 1 ml of benzene–acetonitrile (98:2). Standard solutions were stored at −20°C.

Visualization procedure

Following TLC development, the plate was dried, lightly sprayed with aluminium chloride reagent, heated for 5 min at 110°C and then viewed under longwave UV light (365 nm) to determine the presence of vomitoxin.

The same plate was then sprayed with chromotropic acid reagent and heated at 110°C until all mycotoxin reference standards appeared as dark spots against a light mauve background (usually 5–15 min). After cooling, the plate was re-examined under longwave UV illumination.

Determination of detection limits

From 0.02 to 1.0 μg of mycotoxin reference standards were applied to the TLC plate. The spots were developed in toluene–ethyl acetate–90% aqueous formic acid (5:4:1) and were visualized as described above.

RESULTS AND DISCUSSION

Chromotropic acid reacts with formaldehyde released from the trichothecenes, upon heating with acid, to form a purple monocationic dibenzoxanthylium dye[11]. It has been postulated that the bond between carbon atoms 6 and 15 is cleaved oxidatively by sulphuric acid so that the hydroxymethyl group is oxidized to formaldehyde[10]. The minimum detectable levels and colour characteristics of trichothecenes are shown in Table I. Although extraneous phenols and aromatics can also be expected to release formaldehyde under these conditions, extracts processed by current cleanup methods for trichothecenes[4,5] seldom produce purple spots at R_F values similar to those of trichothecenes. Most interfering compounds appear as various shades of yellow or brown.

It is very advantageous that chromotropic acid can be applied to plates previously treated with aluminium chloride as vomitoxin (and other type-B mycotoxins) can be detected at levels as low as 50 ng/spot using aluminium chloride. Although chromotropic acid provides somewhat less sensitivity for vomitoxin, it does serve as a valuable confirmatory reagent.

At present, the type-A trichothecenes such as T-2 can be detected at levels ranging from 200 ng per spot with *p*-anisaldehyde[7], 100–200 ng per spot with sulphuric acid[6], 100 ng per spot with NBP[8], and 25 ng per spot with N-A[9]. Chromotropic acid permits visualization at levels comparable to most of these reagents but offers several practical advantages not inherent in other methods.

Unlike NBP or N-A, only one reagent is required in a simple one-step spraying procedure. The reagent is relatively stable and does not have to be prepared fresh

TABLE I

CHROMATOGRAPHIC DATA FOR TRICHOTHECENE MYCOTOXINS

Mycotoxin	R_F	*Aluminium chloride/ UV (365 nm)*		*Chromotropic acid*			
				Visible light		*UV (365 nm)*	
		Colour	*Detection limit (ng per spot)*	*Colour*	*Detection limit (ng per spot)*	*Colour*	*Detection limit (ng per spot)*
T-2	0.43	–	–	Purple	100	Bright blue	50
DAS	0.385	–	–	Brown	200	Blue-white	100
HT-2	0.275	–	–	Purple	100	Bright blue	50
Vomitoxin	0.18	Bright blue	50	Grey	100	Intense black	100
T-2 triol	0.15	–	–	Purple	100	Bright blue	50
T-2 tetraol	0.085	–	–	Purple	100	Bright blue	50

each time of use as is the case for *p*-anisaldehyde, NBP, or N-A. Colours remain visible for weeks unlike those from *p*-anisaldehyde or NBP. The presence of trichothecenes can be determined under visible as well as UV illumination to corroborate toxin identity. The reagent is compatible with pretreatment by aluminum chloride, permitting a valuable substantiation for type-B trichothecenes. The greatest advantage, however, is seen in the contrasting colours of the toxins and extraneous compounds. This feature makes chromotropic acid especially suited for use in simple screening procedures in which extensive cleanup steps are not practical.

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