Qualitative and Quantitative Assay of Trichothecein: a Myco-
toxin Produced by *Trichothecium roseum*

W. G. SORENSON,* M. R. SNELLER, AND H. W. LARSH

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73069

Received for publication 31 January 1975

Trichothecein (T-cin) is a member of a family of mycotoxins known as the 12,13-epoxytricho-
theceines. Members of this family share the
property of strongly inhibiting protein synthesis in eucaryotic cells, although at least two differ-
ent mechanisms of inhibition exist within the
group (3, 10). T-cin is one of the most potent
antifungal members of the group and one of the
least effective against mammalian cells (2).

Until recently, demonstration of the tricho-
theceines required relatively pure samples since
these compounds are not fluorescent and do not lend themselves easily to the usual methods of
assay. For these reasons, assay procedures ap-
licable to crude samples are badly needed (2).
Ueno et al. (12) described a procedure for
screening cultures and foodstuffs for *Fusarium*
trichotheceines based on both biological (animal
toxicity and inhibition of reticulocyte protein
synthesis) and chemical (thin-layer chromato-
graphy) properties. No such procedure is cur-
rently available for T-cin.

Ikediobi et al. (6) have developed a gas
chromatography system for the detection and
quantitation of several trichotheceines including
T-cin. Although their system has been used to
detect T-2 toxin in crude grain extracts, its use
with T-cin has so far been confined to mixtures
of pure crystalline samples.

Thin-layer chromatographic methods have
been described for all of the trichotheceines (2, 12). We have tried several of these with crude
extracts of *Trichothecium roseum* cultures but
were unable to achieve adequate separation.

The purpose of this communication is to
describe procedures currently being used in our
laboratory for the detection of T-cin in crude
culture extracts.

**MATERIALS AND METHODS**

T-cin was prepared by cultivation of *T. roseum* *(Pers.)* Link ex S. F. Gray (NRRL 1665) by the
method of Freeman and Morrison (5), except that
Fernbach flasks were used, each containing 750 ml of
medium. After 21 days of incubation, the mycelium
was removed by filtration, and the filtrate was ex-
ttracted three times with carbon tetrachloride (200
ml/liter of filtrate). The combined extracts were dried
by filtration through anhydrous NaSO₄ concentrated
in vacuo, and diluted to a final volume of 10.0 ml
in a volumetric flask. These crude extracts
were used for qualitative and quantitative assays and for
preparation of crystalline toxin.

Standard samples of crystalline T-cin were pre-
pared from crude CCl₄ extracts by column chromato-
graphy on Al₂O₃ (II) with the benzene-ether stepwise
eution method of Achilladelis and Hanson (1).
Column fractions were screened by bioassay, and
active fractions were pooled and concentrated in
vacuo. T-cin was crystallized from CH₃OH-H₂O
mixtures and recrystallized from petroleum ether. Its
identity was confirmed by nuclear magnetic reso-
nance analysis.

**Bioassay.** Qualitative and semiquantitative assay
was carried out by the agar diffusion method against
*Candida albicans* strain MI-023 obtained from the
Center for Disease Control in Atlanta, Ga. Inoculum
was prepared in a modified Wickerham medium
(13) consisting of Wickerham’s trace elements and
salts (ammonium sulfate omitted), 1% glucose, 0.5%
vitamin-free Casamino Acids (Difco), and 0.01% yeast
extract. A 10-ml basal agar layer was covered by a
6-ml seed agar layer containing 24-h cells at a density
of 2 × 10⁵ organisms/ml. Nutrient agar (Difco) was
used in both layers. A standard volume (0.1 ml) of
the extract or dilution to be tested was added to
Schleicher and Schuell antibiotic disks (12.7 mm),
and the disks were permitted to stand a minimum of
30 min at ambient room temperature to allow evapo-
rati on of the solvent. The disks were applied to the
plates 30 min after the seed agar layer was poured,
and the plates were incubated at 37 C for 24 h.

Colorimetric assay. Colorimetric assay of these extracts was accomplished by a modification of the procedure developed by Poltorak (7). Concentrated $H_2SO_4$ (1.5 ml) was added to 0.5 ml of extract (in CCl$_4$ or benzene) to be tested. The tube was then agitated immediately on a Vortex mixer and placed in a boiling-water bath for 4 min. The presence of T-cin or other $\alpha,\beta$-unsaturated ketones is indicated by the development of a red-brown color (7). After cooling for 1 to 2 min, this mixture was added cautiously to 7.5 ml of absolute methanol. Under these conditions, T-cin produces a blue color within a few minutes which is stable for several hours (7). The final solutions were read at 600 nm in a Beckman DB-G spectrophotometer after a minimum of 30 min.

Thin-layer chromatography. Silica Gel (Brinkman) at a thickness of about 375 $\mu$m was applied to plates (20 by 20 cm) with a Desaga spreader. Mallinckrodt Silicar 7G (250 $\mu$m) produced essentially similar results. The plates were activated at 110 C overnight and stored in a Lab Con Co vacuum desiccator (atmospheric pressure) prior to use. Separation of substances by chromatography can often be enhanced by repeated development in the same solvent system (11). The plates were spotted for either single or double development (12 cm) and were developed by the following solvent systems: (i) benzene-ethyl acetate (95:5), double development; (ii) benzene-acetone (95:5), double development; and (iii) chloroform-ethyl acetate (9:1), single development.

After development, the plates were sprayed with a 1:1 mixture of concentrated $H_2SO_4$ and absolute methanol and developed at 110 C for 15 min. T-cin appeared as a brown nonfluorescent spot (long-wave ultraviolet) which eventually began to fade and became surrounded by a white halo.

To enhance the value of thin-layer chromatography in identifying T-cin, 2,4-dinitrophenylhydrazine derivatives were prepared from crude extracts, crystalline T-cin, and acetone. The 2,4-dinitrophenylhydrazine reagent was prepared by the method of Roberts et al. (9). The reagent (1 ml) was mixed with 0.5 ml of extract on a watch glass and stirred occasionally until 2,4-dinitrophenylhydrazones were formed. These were collected by filtration through glass wool in a capillary pipette and washed repeatedly with ethanol. The product was then eluted into a test tube with about 1 ml of chloroform. Dinitrophenylhydrazine derivatives were used as a confirmatory test and were not used for quantitative purposes. The acetone-dinitrophenylhydrazone was used for relative $R_f$ determinations (not shown).

Semiquantitative estimation of T-cin concentrations in crude extracts was done by visual comparison with known concentrations of crystalline T-cin on the same plate.

To compare these procedures for the quantitative analysis of individual samples, a series of eight culture filtrates was prepared and analyzed as described above. In each case, dilutions were prepared with CCl$_4$ whenever required. Analysis of variance was done by standard methods for two factors and one observation per cell (4).

Several strains of $T. roseum$ were used to compare these methods of analysis and to determine whether different strains might present unique problems in extraction or analysis. These were obtained from C. W. Hesselteine of the Northern Regional Research Laboratory (NRRL 1665 and 2307), from J. L. Richard of the National Animal Disease Laboratory, Ames, Iowa (MC-156 and MC-176), and from the American Type Culture Collection (ATCC 13411 and 13442). Two of the strains were isolated from natural sources in Oklahoma (FL-1 from soil in Norman and RH-1 from a squash from Elk City). Each strain was cultured and extracted as described above.

RESULTS

Although the bioassay was intended initially only for qualitative purposes, it can be used semiquantitatively as well; a linear relationship holds between the log of concentration and the diameter of the zone of inhibition over the concentration range of 50 $\mu$g/ml to 2 mg/ml (Fig. 1). Since only 0.1 ml of extract is used per disk, a definite zone of inhibition is produced if about 2 to 5 $\mu$g of T-cin is present on the disk. The Poltorak colorimetric procedure yields a linear relationship between 0.2 to 3.0 mg/ml (Fig. 2), although certain unknown substances may cause interference in some cases. The presence of interfering $\alpha,\beta$-unsaturated ketones is indicated by a pink or purple color resulting from residual red-colored products of the first reaction.

$R_f$ values for T-cin, T-cin–dinitrophenylhydrazone, and acetone-dinitrophenylhydrazone are presented in Table 1. The minimum amount of T-cin or T-cin–dinitrophenylhydrazone which could be easily detected was 6 to 10 $\mu$g/spot. These solvent systems afford good separation of T-cin and an unknown contaminating sub-

![Graph](http://aem.asm.org/download/1994/039/03/fig1.png)

**Fig. 1.** Linear relationship between the diameter of the zone of inhibition of $C. albicans$ and the log of the T-cin concentration ($y = 14.0x + 7.0; r = 0.990$).
thin-layer chromatography is the least sensitive of the methods. A major disadvantage of the bioassay is its lack of specificity, since many naturally occurring substances inhibit *C. albicans*. Its convenience, simplicity, and economy make it a useful screening technique.

The specificity of the colorimetric test is currently not known. According to Poltorak (7), it is dependent upon a halochromatic reaction between concentrated H$_2$SO$_4$ and the $\alpha,\beta$-unsaturated keto group of T-cin. While we have not studied this point, we have tried quinhydrone (a mixture of quinone and hydroquinone) with H$_2$SO$_4$ under the conditions of the Poltorak test. Only a faint yellow color developed at a concentration of 1 mg/ml. Therefore, the red-brown color resulting from halochromation of T-cin is not a general phenomenon of $\alpha,\beta$-unsaturated ketones.

It would be helpful to know the structural requirements for this reaction and the subsequent reaction, i.e., the formation of the blue color with methanol. The Poltorak reaction is somewhat limited by its uncertain specificity and by the possibility of interference resulting from the occasional occurrence of turbid solu-

**DISCUSSION**

The minimum detectable levels in the bioassay, Poltorak colorimetric reaction, and thin-layer chromatography are 50 $\mu$g/ml, 200 $\mu$g/ml, and about 6 $\mu$g, respectively. Under the conditions employed, the bioassay is the most sensitive of the three methods. Because of the limited loading capacity inherent in analytical thin-layer chromatography, working samples must contain about 0.5 to 1.0 mg/ml. Therefore
grams and/or residual red-brown color from the 
H₂SO₄ step. It is a useful screening tool, how-
ever, and offers greater specificity than the 
bioassay. Interfering substances can be removed 
by column chromatography if desired, but we 
believe that this is rarely necessary. The Po-
torak colorimetric assay can be scaled down for 
qualitative purposes. This is helpful in testing 
column fractions or other samples when con-
ervation of material is desired. In this case, 
the volumes of samples, H₂SO₄, and methanol are 
reduced fivefold. Because the sample is in a 
volatile organic solvent which is readily driven 
off in the water bath, the sample volume may be 
increased five- to 10-fold for increased sensitiv-
ity. If desired, the final mixture can be poured 
onto a spot plate for easier visual examination.

The uncertainty of $R_\text{f}$ values in identifying 
organic compounds is well known (11), but by 
the use of multiple solvent systems and chro-
matography of derivatives (i.e., 2,4-dinitro-
phenylhydrazone-T-cin) the problem of speci-
ficity can be minimized. Although the spray 
used resembles the Poltorak reaction system, T-
cin does not form a blue spot on thin-layer 
chromatography plates. However, the spray is 
useful because it produces fluorescent spots 
with certain other compounds on the plates, 
which aids in the interpretation of chromato-
grams. Also it allows charring of organic com-
ounds present and gives a recognizable reac-
tion with T-cin.

Richard et al. (8) previously reported that T. 
roseum NRRL 2307 does not produce T-cin. 
However, our results indicate yields comparable 
to those of NRRL 1665. Perhaps the subculture 
used in their work had lost its ability to produce 
the compound. We were able to detect T-cin in 
all of the extracts reported herein, although the 
yields were often quite low. It is interesting that 
we have tried only two fresh isolates from 
Oklahoma, and that both produced the toxin 
under these conditions in yields comparable to 
those originally reported by Freeman and Mor-
rison (5).

Although each of the assay procedures used 
has certain inherent disadvantages, their use in 
combination allows qualitative determination of 
T-cin without purification, and quantitation by 
these methods yields results which are gener-
ally in close agreement.

ACKNOWLEDGMENTS

This investigation was supported by a U. S. Public Health 
Service training grant (#5 TOI AI00 123-AID) from the 
National Institute of Allergy and Infectious Diseases, and by 
the Faculty Research Committee of the University of Okla-
homa.

We also thank F. M. Strong of the University of Wisconsin, 
who kindly supplied a small sample of crystalline tricho-
thein, and Francis J. Schmitz of the University of Oklahoma 
for the nuclear magnetic resonance analysis. We also grate-
fully acknowledge receipt of T. roseum cultures from C. W. 
Hesseltine (ARS Culture Collection Investigations, U.S. 
Department of Agriculture) and J. L. Richard of the National 
Animal Disease Laboratory, Ames, Iowa.

LITERATURE CITED

1. Achilladelis, B., and J. R. Hanson. 1969. Minor ter-
penoids of Trichothecium roseum. Phytochemistry 
8:765-767.

2. Bambug, J. R., and F. M. Strong. 1971. 12,13-epoxytri-
chothecenes. In S. Sadas, A. Ciegler, and S. J. Ajl (ed.), 
Microbial toxins, vol. 7. Academic Press Inc., New 
York.

3. Cundiff, E., M. Cannon, and J. Davies. 1974. Mech-
anism of inhibition of eukaryotic protein synthesis by 
trichothecene fungal toxins. Proc. Natl. Acad. Sci. 
U.S.A., 71:30-34.

4. Dixon, W. J., and F. J. Massey, Jr. 1951. Introduction to 
statistical analysis. McGraw-Hill Book Co., Inc., New 
York.

5. Freeman, G. G., and R. L. Morrison. 1949. The isolation 
and chemical properties of trichothecin, an antifungal 
substance from Trichothecium roseum Link. Biochem. 
J. 44:1-5.

6. Ikediobi, C. O., I. C. Hsu, J. R. Bambug, and F. M. 
Strong. 1971. Gas-liquid chromatography of mycotox-
ins of the trichothecene group. Anal. Chem. 
43:327-340.

7. Poltorak, V. A. 1963. Colorimetric determination of 
trichothecin. (In Russian) Antibiotiki (Moscow) 8:636-
640.

8. Richard, J. L., G. W. Enstrom, A. C. Pier, and L. H. 
Tiffany. 1969. Toxicogenicity of Trichothecium roseum 
Link: isolation and partial characterization of a toxic
metabolite. Mycopathol. Mycol. Appl. 39:231-240.
9. Roberts, R. M., J. C. Gilbert, L. B. Rodewald, and A. S.
Wingrove. 1969. An introduction to modern experimental organic chemistry. Holt, Rinehart, and Winston, Inc., New York.
10. Schindler, D. 1974. Two classes of inhibitors of peptidyl transferase activity in eukaryotes. Nature (London) 249:38-41.
11. Stahl, E. 1969. Thin-layer chromatography, 2nd ed., p. 86, 100, 127, 217, 219, 900. Springer-Verlag, Inc., New York.
12. Ueno, Y., N. Sato, K. Ishii, K. Sokai, H. Tsunoda, and M. Enomoto. 1973. Biological and chemical detection of trichothecene mycotoxins of Fusarium species. Appl. Microbiol. 25:699-704.
13. Wickerham, L. J. 1946. A critical evaluation of the nitrogen assimilation tests as commonly used in classification of yeasts. J. Bacteriol. 52:293-301.