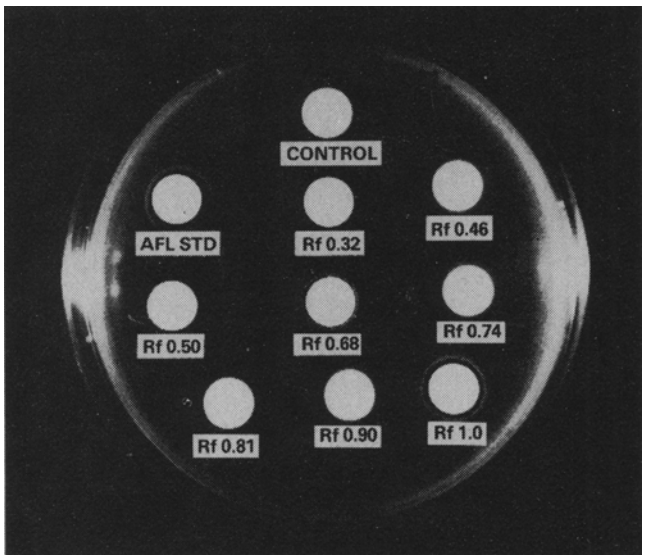

FIG. 7. Inhibitory effect of selected isolated decontamination by-products and aflatoxin B_1 on *B. megaterium* (see Table VII) (negative control—acetonitrile; aflatoxin B_1 standard 0.3 ng; R_f values of tested isolated component indicated for each disc).

by-products. Some compound(s) showed toxic responses to one test but were negative in others, emphasizing the need for battery testing when performing safety evaluations. Also, the level of sensitivity varied significantly with the test system: 5 ng, 1 μg and 50 ng for the animal cell, bacterial cell and chick embryo test systems, respectively. Compared to aflatoxin B_1, the relative concentrations and toxicities of the isolated decontamination-related compounds are inferior to unchanged aflatoxin remaining after the decontamination process by several orders of magnitude. Although some decontamination by-products may exhibit elevated responses to specific toxicity tests, the process significantly reduces the risk associated with the original contamination.

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REFERENCES

1. Goldblatt, L.A., and F.G. Dollear, in "Interactions of Mycotoxins in Animal Production," National Academy of Sciences, Washington, DC, 1979, pp. 176-184.
2. Jemmali, M., Pure Appl. Chem. 52:175 (1979).
3. Lee, L.S., E.J. Conkerton, R.L. Ory and J.W. Bennett, J. Agric. Food Chem. 27:598 (1979).
4. Lee, L.S., and A.F. Cucullu, Ibid. 26:881 (1978).
5. Helms, J.P., and A. Prevot, JAOCS 50:306A (1973).
6. Thiesen, J., Anim. Feed Sci. Technol. 2:67 (1977).
7. Giddey, C., R. Larroux, M. Jemmali and J. Rossi, "Detoxification of Aflatoxin-Polluted Peanut Cakes with Monomethylamine:$Ca(OH)_2$: Pilot Industrial Application, Nutrition Experiments; Toxicity Evaluation," presented at the IVth International IUPAC Symposium on Mycotoxins and Phycotoxins, Lausanne, Switzerland, Aug. 1979.
8. Jemmali, M., "Dossier Aflatoxine: Detoxification des Tourteaux d'Arachide par le Procede á la Monomethylamine-Chaux," Battelle Centre de Recherche de Geneva, Carouge-Geneva, Switzerland, 1979.
9. "Methods of Analysis of the AOAC," 13th Edn., edited by W. Horwitz, Association of Official Analytical Chemists, Washington, DC, 1980.
10. Pons, W.A., and A.O. Franz, Jr., J. Assoc. Off. Anal. Chem. 61:793 (1978).

Isolation and Purification of Deoxynivalenol and a New Trichothecene by High Pressure Liquid Chromatography

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ABSTRACT

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one) was extracted from corn with methanol/water (80:20, v/v) and purified by liquid:liquid partitioning and by preparative high pressure liquid chromatography (HPLC). This procedure was used to prepare mg quantities of toxin from field-inoculated corn for reference standards. Analysis of the isolated deoxynivalenol by analytical HPLC, gas liquid chromatography (GLC) and gas liquid chromatography/mass spectroscopy (GLC/MS) indicated the presence of a second compound similar to deoxynivalenol. This compound comigrates with deoxynivalenol on thin layer chromatography plates in chloroform/methanol (90:10, v/v), but can be separated by HPLC on a reverse-phase C_8 column with methanol/water (10:90, v/v). GC/MS of the compound and the trimethylsilyl ether derivative gave parent ions of m/e 280 and 424, respectively. These data and NMR data indicate that the compound is 3,15-dihydroxy-12,13-epoxytrichothec-9-ene-8-one, a previously unreported trichothecene.

INTRODUCTION

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene-8-one), also known as vomitoxin (1), is an important mycotoxin produced by certain species of Fusaria that invade grains in the field and in storage. This toxin is one of 4 known naturally occurring trichothecenes (the others are T-2, diacetoxyscirpenol and nivalenol) which singly or in combination are implicated as being responsible for animal disorders known as fusariotoxicoses (2-4). Recent documentation on the frequency of occurrence of deoxynivalenol (5,6) suggests that this toxin and other trichothecenes may cause greater health problems than aflatoxin in animals, especially in the midwest Corn Belt. Analytical methodology for trichothecenes is difficult due to the physicochemical properties of these toxins. Extensive clean-

up procedures are required for gas liquid chromatography (GLC) or thin layer chromatography (TLC) of deoxynivalenol, and no rapid, sensitive method is available for analysis of grains or feeds (7). Recent developments in HPLC make this nondestructive method of analysis an attractive adjunct to classical methods for isolating naturally occurring compounds. Normal and reverse-phase HPLC can be used in sequence to isolate natural products from interfering compounds of similar polarity. Development of methodology depends on the availability of an authentic reference standard, and this report outlines procedures to isolate and purify deoxynivalenol from contaminated corn by HPLC. During the course of these investigations, a new compound similar to deoxynivalenol was isolated and characterized.

MATERIALS AND METHODS

Contaminated Corn

Corn inoculated in the field was harvested and stored unprotected for one winter. The corn ears had been injected through the husks with Fusaria cultures (NRRL 6206 and NRRL 6207) known to produce zearalenone (8). In early spring, the highly damaged corn was dried to <15% moisture, shelled and stored at 0 C until processed. Analyses for zearalenone (9) and deoxynivalenol (5) showed the corn to be contaminated with 30 ppm and ca. 20 ppm of these toxins, respectively.

Extraction

A modified procedure of Pathre and Mirocha (10) was used to extract deoxynivalenol from 1-4 kg samples of ground corn. The corn was mixed with methanol/water (80:20) (2 L/kg of corn) for 1 hr with an air-driven impeller. The supernatant was filtered with vacuum through Whatman No. 1 filter paper and Celite (Hyflo Supercel). A second extraction was done for 30 min; the extracts were combined and methanol was removed on a steam bath. The aqueous solution of deoxynivalenol was partitioned with 500 mL ethyl acetate/L solution (3X). The ethyl acetate was removed on a rotary evaporator, and the oily residue was taken up in acetonitrile (200 mL) and defatted with an equal volume of petroleum ether. Acetonitrile was removed, and the residue was saved for HPLC analysis.

HPLC and TLC Procedures

The residue was dissolved in 200 mL methylene chloride/methanol (98:2) for preparative HPLC on a Prep LC/System 500 (Waters Associates, Milford, MA). A Prep Pak 500™ silica column was eluted with methylene chloride/methanol (98:2) at 250 mL/min, and the eluent was monitored with a refractive index detector at a sensitivity of 20. All mobile phases were sparged with helium to inhibit bubble formation. Sample extracts from less than 1 kg corn were chromatographed on a 3/8 in. × 4 ft column packed with Porasil A (Waters Associates) with the same eluting solvent at 8 mL/min. The eluant was monitored with a variable wavelength UV detector at 250 nm (Spectromonitor III, Laboratory Data Control). All fractions were evaporated to ca. 5 mL and assayed by TLC. Plates (Silica Gel 60-F_{254}, Merck) were developed in chloroform/methanol (90:10) and then sprayed with anisaldehyde reagent (11) to detect deoxynivalenol. Deoxynivalenol from normal-phase HPLC was purified further by reverse-phase HPLC on a 3/8 in. × 4 ft column (Bondapak C_{18}, Waters Associates) and a mobile phase of water/methanol (90:10) at 8 mL/min. The toxin was detected with a variable wavelength UV detector at 224 nm. Fractions (10-mL) were collected in a manner to eliminate contaminating peaks and solvent was removed on a rotary evaporator at 50 C. Deoxynivalenol from multiple runs was collected, solvent was removed and residue weight was determined.

ANALYTICAL PROCEDURES

UV spectrum of purified deoxynivalenol was determined in methanol (25 μg/mL) on a Beckman Model DB-G spectrometer. Analytical HPLC was done on a 4.6 mm × 25 cm reverse-phase, 6-μ particle size C_8 column (Zorbax C_8, Dupont, Wilmington, DE) operated at 30 C. A variable wavelength detector at 224 nm was used to measure deoxynivalenol. The mobile phase was water/methanol (90:10) (degassed and sparged with helium) at 1.5 mL/min. Deoxynivalenol and unknown peaks were collected and identities were determined by GLC, MS, GLC-MS and nuclear magnetic resonance (NMR). Deoxynivalenol and an unknown compound separated on the reverse-phase C_8 column were analyzed by GLC as the trimethylsilyl derivatives (TMS) (TRISIL-TBT, Pierce Chemical Co.) on a gas chromato-

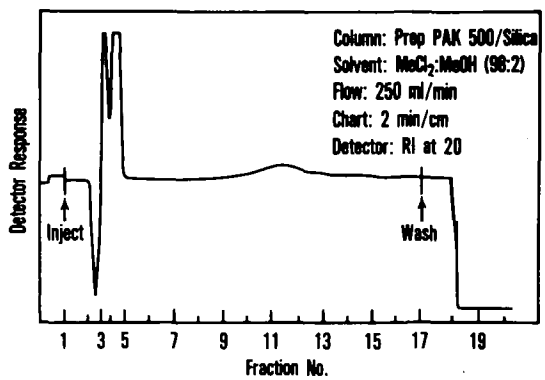

FIG. 1. HPLC of deoxynivalenol (DON) on Prep 500/LC system. Silica column (Prep Pak 500) eluted with methylene chloride/methanol (98:2) at 250 mL/min. Detector, RI at 20. Chart speed, 2 min/cm.

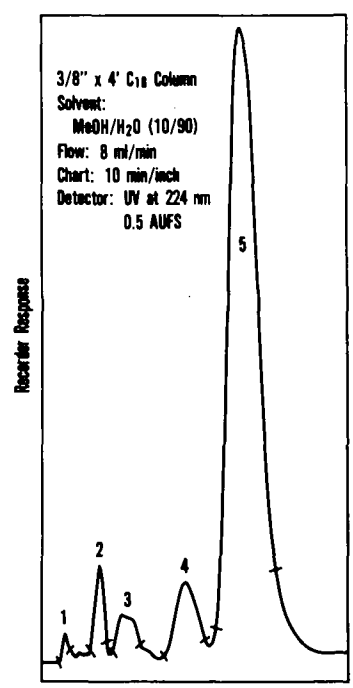

FIG. 2. HPLC of deoxynivalenol from preparative run. Column, 3/8 in. × 4 ft reverse phase C_{18}. Solvent: water/methanol (90:10) at 8 mL/min. Detector, UV at 224 nm; 0.5 AUFS. Sample size, 2 mL.

graph (Bendix Model 2600) equipped with a 4 ft x 2 mm glass column packed with 3% OV-1 on Gas Chrom Q 100/120 mesh and flame ionization detector. Column temperature was programmed for 150-250 C at 5 C/min. Other parameters were: injection port, 210 C; detector, 250 C; carrier gas, N_2 at 30 mL/min. Retention times and relative peak areas were measured with an electronic integrator (Hewlett/Packard Model 3370B). Mass spectra were obtained on a Kratos MS-30 at 70 eV. Spectra of deoxynivalenol and unknown compound were obtained by probe analysis, and spectra of TMS-derivatives were obtained by GC-MS. NMR spectra were obtained on a Brücker WH-90 instrument.

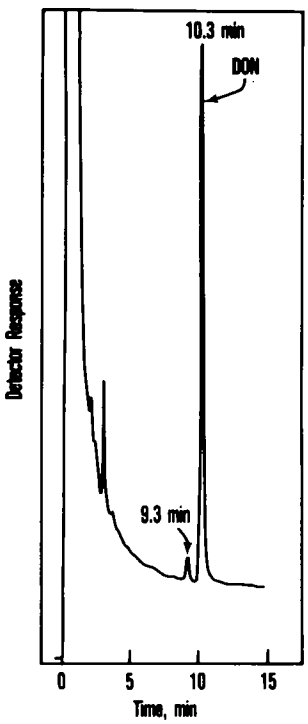

FIG. 3. GLC of purified deoxynivalenol (10.3 min) as TMS-derivative showing unknown compound at 9.3 min. 0.56 μg TMS-deoxynivalenol gives 80% full-scale deflection at attenuation of 5 x 10^{-10} AFS.

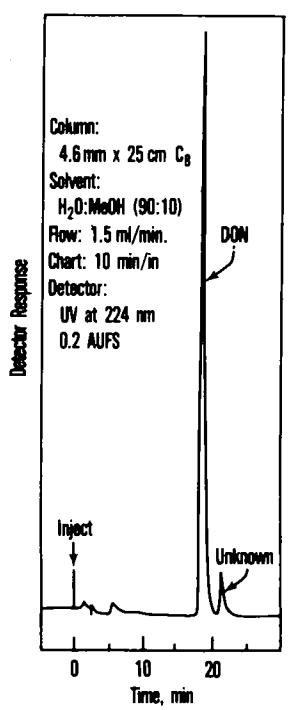

FIG. 4. Chromatogram of deoxynivalenol from C_{18} column on Zorbax C_8 column (4.6 mm x 25 cm). Solvent: water/methanol (90:10) at 1.5 mL/min. Detector, UV at 224 nm; 0.2 AUFS.

RESULTS AND DISCUSSION

Preparative HPLC has limited efficiency and resolution; however, large sample capacity and mobile phase velocity make this a useful tool for initial purification of crude extracts. The chromatogram shown in Figure 1 was obtained by chromatographing partially purified deoxynivalenol from 2 kg corn in 200 mL solvent. Fractions (500-mL) from the preparative runs were reduced to ca. 5 mL, and each fraction was spotted (10 μL) on TLC plates to determine the location of the toxin.

Fractions 9-14 contain deoxynivalenol, but also contain some interfering material of higher R_f value. The reference standard of 2 μg of pure deoxynivalenol is easily detected with the anisaldehyde reagent. Depending on concentration, deoxynivalenol turns yellow to yellow-brown after the plate is sprayed with anisaldehyde reagent and then heated at 105 C for 3-4 min. Chromatographing extracts from 0.5 kg corn on a semipreparative 3/8 in. x 4 ft Porasil A column uses much less solvent but requires more time and multiple runs to process quantities of crude extracts.

Reverse-phase chromatography of partially purified toxin is shown in Figure 2. Deoxynivalenol is separated from other compounds and is easily detected at its UV maximum of 224 nm. Peak 5 shows a single spot on TLC in chloroform/methanol (90:10) when examined under short-wave UV (254 nm) or sprayed with anisaldehyde reagent. However, GLC of the TMS-derivative shows a minor compound that appears consistently just ahead of deoxynivalenol (Fig. 3). Examination of the deoxynivalenol on an analytical reverse-phase column (Zorbax C_8) indicated that this compound could be separated from deoxynivalenol. A typical chromatogram is shown in Figure 4. Repeated runs were made to obtain sufficient quantities of this new compound for MS and NMR analysis. A comparison of mass spectra of the underivatized and TMS-derivatives of deoxynivalenol and the unknown are shown in Figures 5 and 6, respectively. The underivatized deoxynivalenol shows a parent ion at 296 m/e, which is relatively intense when compared to spectra of other trichothecenes (7,11). The unknown compound isolated by reverse-phase

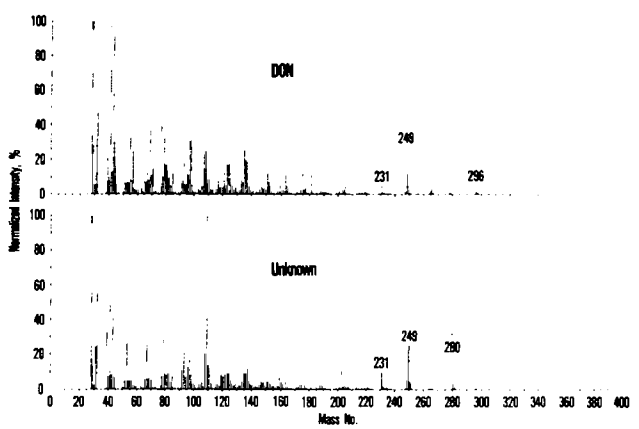

FIG. 5. MS (probe) of purified deoxynivalenol (upper) and new trichothecene (lower). Mass difference of 16 indicates 1 less hydroxy group for new trichothecene.

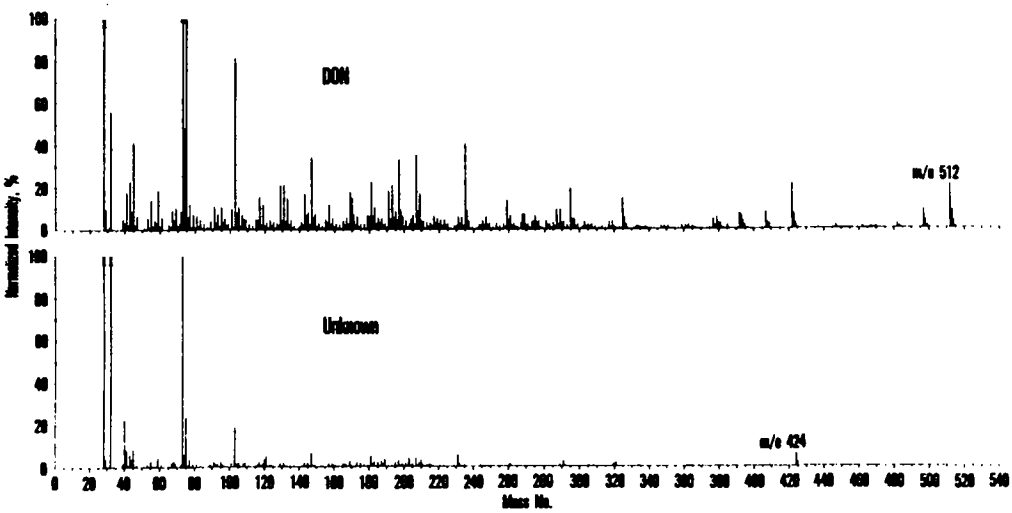

FIG. 6. GC-MS of TMS-derivatives of deoxynivalenol (upper) and new trichothecene (lower). Parent ions of TMS-deoxynivalenol (512) and TMS-unknown (424) indicate difference of 1 hydroxy group.

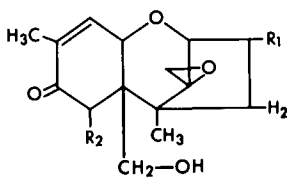

FIG. 7. Structure of deoxynivalenol (R_1 and R_2 = OH) and new trichothecene (R_1 = OH, R_2 = H).

C_8 chromatography shows a parent ion at 280 m/e, suggesting that the compound has one hydroxyl group less than deoxynivalenol. This conclusion is supported by the spectra obtained from TMS-derivatives of both compounds. TMS-deoxynivalenol shows a parent ion at 512 m/e and the unknown shows a parent ion at 424 m/e, one silylated hydroxyl less. NMR data are consistent with 3,15-dihydroxy-12,13-epoxytricothec-9-ene-8-one (Fig. 7). The spectrum is similar to deoxynivalenol with major exception of the resonance for H-7. A doublet at δ 2.87 (J=15.6 Hz) is observed in this study. The shift and coupling are similar to that found for H-7β in tricothecolone (12).

Recoveries of pure deoxynivalenol from field-inoculated corn were extremely low (ca. 20%) even for large-scale extractions. This estimated value is based on GLC assays of extracts (from 50-g samples of corn) which were cleaned up by silica gel column chromatography (5). However, sufficient quantities can be prepared to provide analytically pure reference standard of this scarce mycotoxin. Quantities of contaminated corn or wheat (13,14) could be processed with either preparative or semipreparative HPLC to provide scientists with pure deoxynivalenol to test analytical methods for sensitivity and percentage recoveries. Pure toxin is also needed to test possible synergistic effects that may occur with other trichothecenes. A new trichothecene has been isolated from field inoculated corn and characterized by MS and NMR. This compound was also detected in a standard previously thought to be pure deoxynivalenol. No toxicity studies have been done with the new compound, and the occurrence in substrates, other than field-inoculated corn, is unknown.

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REFERENCES

1. Vesonder, R.F., A. Ciegler and A.H. Jensen, Appl. Microbiol. 26:1008 (1973).
2. Forsyth, D.M., T. Yoshizawa, N. Morooka and J. Tuite, Appl. Environ. Microbiol. 34:547 (1977).
3. Mirocha, C.J., S.V. Pathre, B. Schauerhamer and C.M. Christensen, Ibid. 32:533 (1976).
4. Vesonder, R.F., A. Ciegler, H.R. Burmeister and A.H. Jensen, Ibid. 38:344 (1979).
5. Vesonder, R.F., A. Ciegler, R.F. Rogers, K.A. Burbridge, R.J. Bothast and A.H. Jensen, Ibid. 36:885 (1978).
6. Jemmali, M., Y. Ueno, K. Ishii, C. Frayssinet and M. Etiene, Experientia 34:1333 (1978).
7. Mirocha, C.J., in "Conference on Mycotoxins in Animal Feeds and Grains Related to Animal Health," edited by W. Shimoda, Bureau of Vet. Med., Rockville, MD, 1979.
8. Shannon, G.M., O.L. Shotwell, A.J. Lyons, D.G. White and G. Garcia-Aguirre, J. Assoc. Off. Anal. Chem. 63:1275 (1980).
9. Shotwell, O.L., M.L. Goulden and G.A. Bennett, Ibid. 59:666 (1976).
10. Pathre, S.V., and C.J. Mirocha, Appl. Environ. Microbiol. 35:992 (1978).
11. Stahr, H.M., A.A. Kraft and M. Schuh, Appl. Spectrosc. 33:294 (1979).
12. Bamburg, J.R., and F.M. Strong, in "Microbial Toxins," Vol. VII, edited by S. Kadis, A. Ciegler and S. Ajl, Academic Press, New York, NY, 1971.
13. Vesonder, R.F., and A. Ciegler, Eur. J. Appl. Microbiol. Biotechnol. 8:237 (1979).
14. Scott, P.M., J. Assoc. Off. Anal. Chem. 64:462 (1981).