polymer resin in the H^+ form. The 0.0008 N H_2SO_4 mobile phase continuously regenerates the resin H^+ form during operation. Separation of organic acids occurs by ion exclusion and partitioning and organic acids elute in order of increasing pK_a (Jupille et. al., 1981). Heating the column to 75 °C speeds up analysis time and increases peak resolution. The function of the small ion exclusion guard cartridge is to bind irrevesibly the positively charged inorganic ions which would otherwise bind to the analytical column resin decreasing its separation capability of organic acids. Highly charged negative ions are excluded from the analytical column resin and elute in the void volume (Bio-Rad, 1981). The Aminex HPX-87H column has a long useable lifespan. More than 800 injections were made over a period of one year without any noticeable deterioration in separation capabilities.

Ultraviolet detection at 214 nm was able to detect organic acid concentrations less than 0.002%. An alternative method of detection, refractive index, was tried but resulted in several times less organic acid sensitivity.

Registry No. Oxaloacetic acid, 328-42-7; isocitric acid, 320-77-4; sucrose, 57-50-1; fructose, 57-48-7; ethanol, 64-17-5; chlorogenic acid, 327-97-9; caffeic acid, 331-39-5; maleic acid, 110-16-7; citric acid, 77-92-9; succinic acid, 110-15-6; oxalic acid, 144-62-7.

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Purification of Deoxynivalenol (Vomitoxin) by Water-Saturated Silica Gel Chromatography

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A simple procedure was developed for the laboratory production and purification of gram quantities of crystalline deoxynivalenol (DON). When *Fusarium graminearum* R6576 was grown on rice, concentrations of 600–700 ppm DON were obtained after 13–18 days of incubation. A DON derivative, 15-acetyl-DON (15-ADON), was also found at concentrations of 100–300 ppm after 7–10 days. Crude culture extracts were purified by low pressure liquid chromatography on a column of water-saturated silica gel which selectively extracted DON when methylene chloride was used as the mobile phase. After elution of DON with water and subsequent reextraction with ethyl acetate, DON could be readily crystallized. Purity of crystallized DON was verified by thin layer and high performance liquid chromatography.

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, DON), also known as vomitoxin, is a trichothecene mycotoxin produced by *Fusarium graminearum*, which has been associated with vomiting and feed refusal in swine (Vesonder et al., 1976; Forsyth et al., 1977). Recent examination of *Fusarium* infected grains, particularly in Canada and the Midwest, has confirmed the natural occurrence of DON (Scott et al., 1981; Eppley et al., 1984; Trenholm et al., 1983). Assessment of the hazards associated with exposure to DON has been hampered by a lack of the gram quantities of pure DON required for toxicological studies.

An efficient method for DON production requires a convenient, concentrated source of DON. Whereas concentrations found in naturally contaminated grains are not high enough for use of this material as a source, inoculation of growing corn with DON-producing *Fusarium* strains has provided a concentrated source of DON (Miller et al., 1983; Scott et al., 1984). DON production in lab culture provides a more convenient source of crude DON and both solid and liquid substrates have been investigated (Vesonder et al., 1982; Greenhalgh et al., 1983; Greenhalgh et al., 1984). Although several multistep schemes for purifying crude DON extracts involving solvent–solvent partitioning, column chromatography, preparative thin layer chromatography (TLC), and high pressure liquid chromatography (HPLC) have been reported (Pathre and Mirocha, 1978; Bennett et al., 1981; Ehrlich and Lillehoj, 1984; Scott et al., 1984), these have the disadvantage of requiring numerous, time consuming chromatography steps.

The results reported here describe an efficient method for producing and purifying gram quantities of crystalline DON on a laboratory scale. DON was produced at very high levels by *Fusarium graminearum* R6576 on rice and was purified in a single step by low pressure liquid chromatography using a water-saturated silica gel column that is selective for DON.

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MATERIALS AND METHODS

Inoculum Preparation. Potato dextrose agar plates were inoculated from stock soil cultures of *Fusarium graminearum* R6576 (*Gibberella zeae* U5373), a Michigan wheat isolate previously designated as W-8 (Hart et al., 1982), and incubated at 25 °C for 7 days in a 12-h light/dark cycle. Agar plugs (4 mm) removed from the growing edge of colonies were added to 500-mL Erlenmeyer flasks (3–4 plugs per flask) containing (carboxymethyl)-cellulose (CMC) medium (90 mL) (Cappellini and Peterson, 1965). These flasks were agitated on a rotary shaker (250 rpm) at 25 °C for 3–5 days. The suspension was

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filtered through sterile cheesecloth, and the macroconidia concentration was determined by counting on a hemacytometer.

DON Production. Fernbach culture flasks (2800 mL) containing dry white rice (350 g) and distilled water (150 mL) were stoppered with cotton plugs (foil caps were unsuitable) and autoclaved at 121 °C for 30 min. Flasks were inoculated with 10^6 macroconidia and the cultures were incubated in the dark at 28 °C, without shaking. Cultures were extracted after 7, 9, 11, 14, 17, 21, and 23 days of incubation to monitor the time course of DON production. An incubation period of 13–18 days was used for the routine production of gram quantities of DON.

DON Extraction. The extraction procedure was adapted from Pathre and Mirocha (1978). At the end of the incubation period, the contents of each flask were blended with 60% aqueous methanol (1400 mL). After soaking overnight, the mixture was filtered through Whatman No. 4 filter paper and methanol removed on a steam bath. The aqueous extract was saturated with sodium chloride and filtered to remove any precipitate. Saturated solutions were sometimes held overnight to allow complete precipitation. The aqueous solution was extracted 3 times with ethyl acetate with a volume ratio of 1:2 (ethyl acetate–water). Ethyl acetate from combined extracts was removed on a rotatary evaporator and the residue dissolved in either ethyl acetate for TLC quantitation or methylene chloride for silica gel chromatography.

Silica Gel Chromatography. Low pressure liquid chromatography was performed on silica gel (Adsorbosil, 200/425 mesh, Anspec) by using the following equipment (Ace Glass, Inc., Vineland, NJ): 37-mm i.d. Michel-Miller glass chromatographic column fitted with a safety shield, Teflon end fittings, connectors, and tubing (2-mm i.d.). A pump (Model RP-SY-1CSC; Fluid Metering, Inc., Oyster Bay, NY), adapted with low flow fittings and coupled to a pulse dampener, maintained a flow rate of about 5 mL/min (10–40 psi). Fractions (10 mL) were collected and monitored by TLC (see Analytical Procedures). The column was prepared by dry packing with silica gel (170 g). The packed column was equilibrated with distilled water (500–600 mL) and allowed to drain. Methylene chloride (400–500 mL) was pumped through the column until water no longer appeared in the effluent. A sample of crude extract (combined extracts from 10–20 flasks) in a minimum volume of methylene chloride was applied to the column and eluted with methylene chloride until metabolites (excluding DON) ceased to appear in the effluent, as indicated by TLC monitoring. The column was then eluted with distilled water to remove DON. Water fractions (including water layers in tubes containing both solvents) containing DON were combined and extracted 5–10 times with ethyl acetate (volume ratio 1:1). The water layer was analyzed by TLC to ensure removal of DON. Combined ethyl acetate extracts were concentrated on a rotary evaporator. After each run, the silica gel column was washed with water for reuse in DON purification.

DON Crystallization. Concentrated extract was dried under N_2 in a beaker (50 mL) and dissolved in a minimum volume of ethyl acetate. The solution was seeded with a few crystals of DON, and the beaker was covered tightly with aluminum foil and refrigerated (4 °C). When a precipitate had formed (several days were sometimes required), the supernatant was removed and saved. Solids were dissolved in ethyl acetate and filtered through Whatman No. 4 filter paper. DON was recrystallized in ethyl acetate as before, again reserving the supernatant. Methanol (0.5–1.0 mL) was mixed with the crystals to extract residual pigment. The mixture was refrigerated (4 C) for 8 h, then methanol removed and saved. This washing procedure was repeated with ethyl acetate (0.5–1.0 mL). The entire crystallization scheme was repeated with combined methanol and ethyl acetate supernatants. After residual solvent had been evaporated, purity of the white crystalline DON was checked by HPLC.

Analytical Procedures. Semiquantitative TLC was performed on precoated 20 × 10 cm silica gel G plates (Redi-Plates, Fisher Scientific Co.) with toluene–ethyl acetate (1:3). DON was detected by spraying the developed plate with a 15% aluminum chloride solution (15 g $AlCl_3 \cdot 6H_2O$ in 85 mL of ethanol + 15 mL of water) and then heating it for 5 min at 110 °C (Baxter et al., 1983). DON produces a blue fluorescent spot under longwave (365 nm) UV light at R_f 0.3. The location of 15-acetyl-DON (15-ADON) was identified by comparison with that of a qualitative 15-ADON standard (R_f 0.5) that had been previously prepared in this laboratory and confirmed with mass spectrometry by Dr. C. J. Mirocha (University of Minnesota). Both DON and 15-ADON were quantitated by visual estimation in a UV viewing cabinet using DON standard (Myco-Lab Co., Chesterfield, MO) dissolved in ethyl acetate.

Purity of crystalline DON was assessed with HPLC using a Model 2300 HPLC pump and V^4 variable wavelength absorbance detector (5-mm flow cell) (ISCO, Lincoln, NE). The system was equipped with RP-18 Spheri-10 MPLC analytical (22 cm × 4.6 mm i.d.) and guard (3 cm × 4.6 mm i.d.) cartridges (Brownlee Lab, Inc., Santa Clara, CA). Mobile phase was 7% (vol/vol) methanol in water with a flow rate of 2mL/min. Purity of DON dissolved in mobile phase was determined at 224 nm and 0.05 a.u.f.s. DON standard had a retention time of 16.9 min at 25 °C. A standard of 3,15-dihydroxy-12,13-epoxytrichotec-9-en-8-one (7-deoxy-DON) supplied by G. A. Bennet (Northern Regional Research Center, Peoria) had a retention time of 18.2 min in this solvent system.

Identity of crystalline DON was confirmed by comparison of its mass spectrum with that of the DON standard. Both spectra were obtained at 70 eV by using a direct probe, on a Finnigan 3200 gas chromatograph–mass spectrometer coupled to a Riber SADR data system. Ultraviolet (UV) spectrum of DON in ethanol (50 μg/mL) was determined on a Beckman Model 35 spectrophotometer. Partition coefficients were determined by adding either DON or 15-ADON to a mixture of water and methylene chloride. Equilibrium concentrations of toxins were determined by using the above HPLC procedure. Partition coefficient was defined as: toxin concentration in water/toxin concentration in methylene chloride.

Safety Note. Contact with DON and crude extracts was always avoided. Contaminated glassware was soaked in 10% bleach solution overnight before being washed (Thompson and Wannemacher, 1984). Safety shields were used with Michel-Miller glass columns and operating pressure did not exceed 300 psi.

RESULTS AND DISCUSSION

DON Production. Preliminary experiments indicated that optimal incubation conditions for DON production on rice by our strain were consistent with those reported by Vesonder et al. (1982) and Greenhalgh et al. (1983); that is, an incubation temperature of 28 °C and a initial substrate moisture content of 35–40%. *F. graminearum* R6576, however, required a shorter incubation time for maximum DON production. Maximum concentrations (600–700 ppm, dry rice basis) of DON occurred between 13 and 18 days of incubation (Figure 1), compared to

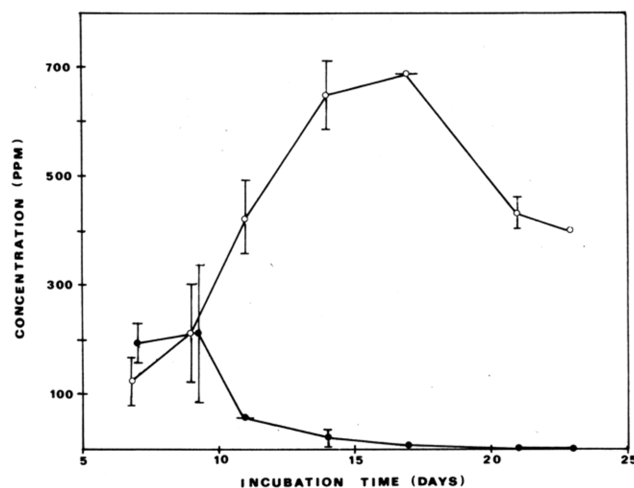

Figure 1. Time course of DON (○) and 15-ADON (●) production by *F. graminearum* R6576 on rice. Values are averages, with standard error bars, of 3 flasks, except for values at days 17 and 21 (2 flasks) and day 23 (1 flask). 15-ADON was not detected on days 21 and 23 (1 ppm detection limit). Concentrations are based on dry rice weights.

greater than 3 weeks for the above two studies. Shorter incubation time could be a result of our inoculum procedure or a characteristic of the isolate. A derivative of DON, acetylated on carbon 15 (15-ADON), was also produced; maximum concentrations (100–300 ppm) occurred between 7 and 10 days of incubation (Figure 1).

The order of appearance of DON and 15-ADON by *F. graminearum* R6576 on rice was qualitatively similar to that found in liquid culture in our laboratory (Pestka et al., 1985) where maximum DON levels occur at day 20. Field inoculation experiments by Miller et al. (1983) and Hart et al. (1984) in corn and wheat, respectively, also describe qualitatively similar behavior for both DON and 15-ADON production. Yoshizawa et al. (1975) have previously found that disappearance of 3-ADON was concurrent with DON production in the fermentation by *F. roseum* and it has been suggested that 3-ADON deacetylation is a step in the biosynthesis of DON. Miller et al. (1983) indicate that plant enzymes may be responsible for conversion of 15-ADON to DON and subsequent DON degradation. Our results suggest that these changes also may be caused by enzymatic or chemical reactions associated with the fungal culture.

DON Purification. Figure 2 shows the elution pattern, as visualized by TLC, for a typical chromatographic separation of crude DON on water-saturated silica gel. Crude extract (10.8 g) used in this run contained approximately 1.3 g of DON. Methylene chloride eluted most of the pigments and metabolites other than DON in the first 70 fractions while DON was retained on the column. When the solvent was changed to water at fraction 71, water first appeared in the effluent in fraction 82 and DON apeared in fraction 90. DON concentration peaked in the first few DON-containing fractions and then decreased rapidly. Fractions 90–110 were combined for DON crystallization, yielding 0.62 g of crystalline DON. Mass spectrometry confirmed the identity of the compound crystallized by this procedure. The UV spectrum showed an absorption maximum at 218 nm (ϵ 6805). Purity of the crystalline DON (mp 150–152 °C), as determined by HPLC, was equal to that of the analytical standard; a single peak was seen at 16.9 min. 7-Deoxy-DON was not detected in the purified DON.

We believe that DON separation on water-saturated silica gel was based on liquid-liquid partitioning rather

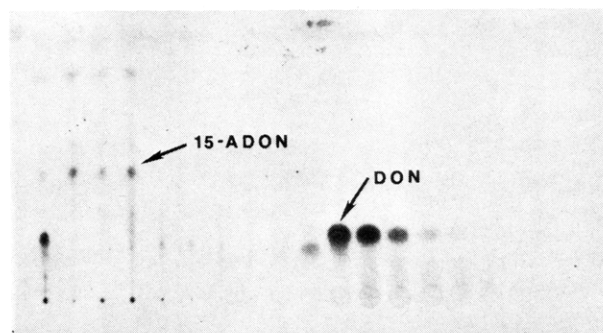

Figure 2. Elution pattern of crude extract on water-saturated silica gel column. The TLC plate was developed in toluene–ethyl acetate (1:3), then sprayed with 30% H_2SO_4, and heated for 5 min at 110 °C for photographic visualization. The first spot, left to right, is the crude extract before chromatography. Remaining spots, left to right, are fractions 15, 25, 35, 45, 55, 65, 75, 82, 88, 92, 98, 102, 108, 112, 118, 125, 135, and 145. Fractions 1–70 were eluted with methylene chloride and the remaining fractions with water.

than the usual adsorption mechanism of silica gel. Here, silica gel functioned as a support for a layer of adsorbed water. DON was extracted from crude preparations by partitioning into the water layer. Other metabolites, including most pigments, were eluted from the column with little or no retention. Elution with water quickly removed the water layer containing DON. 15-ADON was not retained with DON on the column, even though it differs from DON only in the replacement of a single hydroxyl group with an acetyl group. The difference in partition coefficients of DON and 15-ADON, 20 and 0.03, respectively, between the two liquid phases (water to methylene chloride) appears to account for the efficient separation of these two trichothecenes. The high solubility of DON in water has also been used by Terhune et al. (1984) in a procedure for quantitation of DON in grains by gas chromatography where samples were extracted with water instead of aqueous methanol. The aqueous extract was adsorbed onto a Clin Elut column and DON eluted with ethyl acetate. Scott et al. (1984) used an analogous step in the purification of gram quantities of DON. An aqueous extract of DON was adsorbed onto a ChemTube containing a hydrophilic matrix and DON was eluted with ethyl acetate. Several other steps, however including column chromatography and semipreparative liquid chromatography, were required to obtain crystalline DON.

In summary, *F. graminearum* R6576, when grown on rice, produced a yield of DON which was equivalent or better than reported levels produced in rice by other strains of this fungus (Vesonder et al., 1982; Greenhalgh et al., 1983). The crude DON extract prepared from this culture was rapidly purified by a single chromatographic step on water-saturated silica gel. Besides reduced chromatography requirements, the purification procedure described herein had significant advantages over other reported methods because it had minimal solvent requirements and did not require acetylation or hydrolysis steps (Pathre and Mirocha, 1978; Bennett et al., 1981; Ehrlich and Lillehoj, 1984; and Scott et al., 1984). Water-saturated silica gel might also be used as a simple cleanup step for the analytical determination of DON.

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Registry No. DON, 51481-10-8; 15-ADON, 88337-96-6.

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Identification of Four New Degradation Products of Aminocarb [4-(Dimethylamino)-3-methylphenyl *N*-Methylcarbamate] in Water

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In this study we have found that aminocarb, a forestry insecticide, gives four colored degradation products in purified water. They were identified as 6-(dimethylamino)-2-methyl-1,4-benzoquinone, 5-(dimethylamino)-2-methyl-1,4-benzoquinone, 6-(methylamino)-2-methyl-1,4-benzoquinone, and 5-(methylamino)-2-methyl-1,4-benzoquinone. The four chemicals were extracted from water, then separated, and purified by chromatographic methods. Identification was achieved by nuclear magnetic resonance, mass spectrometry, and X-ray crystallography.

INTRODUCTION

Aminocarb, [4-(dimethylamino)-3-methylphenyl *N*-methylcarbamate], also known as MATACIL, is a broad spectrum insecticide used throughout the world for the control of agricultural and forest pests. In Canada, it is used extensively to protect coniferous trees from *Choristoneura fumiferana* Clem., better known as the spruce budworm. Thus, the National Research Council of Canada (NRCC) is interested to know the fate of this chemical in the Canadian ecosystem and in a recent review of the subject (NRCC, 1982) it has recommended that further studies be done to establish the nature of all the degradation products of aminocarb.

Many researchers have studied the fate of aminocarb in various substrates (Abdel-Wahab et al., 1966; Krishna and Casida, 1966; Abdel-Wahab and Casida, 1967; Balba and Saha, 1974; Addison et al., 1974; Sundaram and Szeto, 1979; Sundaram et al., 1980; Cool and Jankowski, 1982). Several degradation products have been identified, namely, 4-(dimethylamino)-3-methylphenol, 4-amino-3-methylphenol, 4-(dimethylamino)-3-methylphenyl *N*-(hydroxymethyl)carbamate, 4-(methylamino)-3-methylphenyl *N*-methylcarbamate, and 4-(formamido)-3-methylphenyl *N*-methylcarbamate, to name but a few.

It is now generally accepted that aminocarb hydrolyzes readily in alkaline medium (Maguire, 1979) and under environmental conditions (Davidson and Dorais, 1981) to give the corresponding phenol. A recent study by Mallet and Levesque (1983) has established that several parameters such as pH, temperature, and dissolved oxygen may accelerate the degradation of aminocarb in water but no attempt was made to detect degradation products other than the corresponding phenol.

In an attempt to identify new degradation products of aminocarb in water, our attention was focused on a pink-reddish coloration that develops in aging aqueous solutions. This coloration has been reported by Vassilieff and Ecobichon (1982) who suggested that it could be caused by an equilibrium between protonated and unprotonated species. This tentative explanation seemed unsatisfactory since the

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