

Natural Occurrence of Deoxynivalenol, 15-Acetyl-Deoxynivalenol, and Zearalenone in Refusal Factor Corn Stored since 1972†

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Two samples of "refusal factor" corn, one stored frozen in Minnesota and one stored dry in Indiana since 1972 or 1973, were analyzed for the presence of *Fusarium* spp. and *Fusarium* toxins. Both samples were from corn refused by swine in Indiana from 1972 to 1973. Sample FS 808 (stored in Indiana) contained 20 ppm of deoxynivalenol (20 µg/g), 16 ppm of 15-acetyl-deoxynivalenol, 5 ppm of zearalenone, and 0.2 ppm of α-zearalenol. Sample FS 362 (stored in Minnesota) contained 3 ppm of deoxynivalenol, 1 ppm of 15-acetyl-deoxynivalenol, and 0.3 ppm of zearalenone. The presence of 15-acetyl-deoxynivalenol is significant because it is the first report of it occurring naturally in refusal factor corn, and it may account in part for the refusal that could not be solely attributed to deoxynivalenol.

Interest in the trichothecene mycotoxins has increased in recent years as a result of the widespread occurrence of deoxynivalenol (DON) in corn (12) and cereal grains (1). Many mycotoxins produced by *Fusarium* spp., such as T-2 toxin, diacetoxyscirpenol, DON, zearalenone (ZEA), and nivalenol, have been reported as naturally occurring in grains and animal feeds (5, 10, 11, 14, 16). Mirocha et al. (9) reported that a sample of corn (FS 362) contained *Fusarium* mycotoxins such as DON and ZEA. Vesonder et al. (15) reported various concentrations of DON in different naturally *Fusarium*-infected corn samples collected from Ohio, Indiana, Missouri, and Illinois. The role of these mycotoxins as causal agents of mycotoxicosis is well established, and they have often been found in both shelled corn kernels and corn kernels on the cob. Miller et al. (7) reported the production of DON, 15-acetyl-deoxynivalenol (15-ADON), and ZEA in liquid culture of *Fusarium graminearum* isolated in North America. Miller et al. (8) reported various concentrations of DON, 15-ADON, and ZEA in field corn inoculated with *F. graminearum*. Pestka et al. (13) reported the production of 15-ADON and DON from *F. graminearum* R6576 in glucose-yeast extract-peptone liquid medium. This study describes the first report of the natural occurrence of 15-ADON in corn associated with feed refusal by swine. The samples in question were collected in 1972 and 1973 in Indiana. Analysis at that time revealed DON and ZEA. In 1985, they were reanalyzed after freezer storage for 13 years in Minnesota and Indiana and were found to contain 15-ADON, DON, and ZEA, as well as an isolate of a *Fusarium* sp. from one of the samples that produces all of the mycotoxins described above.

MATERIALS AND METHODS

Apparatus. The following apparatus and procedures were used: (i) high-performance liquid chromatography (HPLC) with a Model 440 absorbance detector for DON and 15-ADON (Waters Associates, Inc., Milford, Mass.); (ii) a µBondapak reverse-phase C₁₈ liquid chromatography column (inside diameter, 3.9 mm; height, 30 cm) for HPLC (Waters

Associates); (iii) to detect ZEA and α-zearalenol, a Model RF530 fluorescence detector with variable wavelengths, 276-nm excitation, and a 425-nm emission flow cell (Shimadzu, Columbia, Md.); (iv) a pre-column filter (Waters Associates); (v) Hewlett-Packard Model 5840 gas chromatograph and Model 5987 mass spectrometer (Hewlett Packard Co., Palo Alto, Calif.); (vi) for thin-layer chromatography (TLC), prepared silica gel plates E. Merck AG, Darmstadt, Federal Republic of Germany).

Corn samples. In 1972, a 20-kg sample of refusal factor corn (FS 362) was obtained from Purdue University and promptly placed in cold storage at -8°C at the University of Minnesota and kept there for 13 years. This lot of corn had a history of being refused by swine on a farm (5) and experimentally (4) in Indiana, hence our interest in the possible mycotoxins that it contained. An analogous sample (FS 808, 20 kg) collected in Indiana in 1973 from the same lot as FS 362 and stored at Purdue University for 13 years at 4°C was analyzed for mycotoxins in the Mycotoxicology Laboratory at the University of Minnesota. A random sample (1 kg) was taken from each lot of corn and analyzed as described below.

Isolations. One hundred corn kernels were split longitudinally, surface sterilized for 10 s with a 5% NaOCl solution, rinsed in sterile water, transferred to lactic acid-potato-dextrose agar in petri dishes, and incubated under fluorescent lamps (5,300 lx) at 22°C. At regular intervals, the dominant fungi growing on the agar were examined, and the *Fusarium* species were isolated.

Reference standards. DON was purchased in analytically pure form (Myco-Lab Co., Chesterfield, Mo.). ZEA and α-zearalenol were produced and purified in this laboratory by the method of Mirocha et al. (9). 15-ADON standard was the gift of H. Cohen, Agriculture Canada, Ottawa, Canada.

Extraction of DON and 15-ADON. A sample of corn kernels (1 kg) was ground to the consistency of flour and shaken well to mix uniformly. The resulting sample was analyzed for DON and 15-ADON by the following method. Of the ground corn sample, 50 g was extracted three times with methanol and water (3:1, vol/vol) to a total volume of 300 ml. The extracts were filtered through Whatman no. 2 filter paper. The filtrates were combined and defatted twice with *n*-hexane to a total volume of 200 ml. The methanol-

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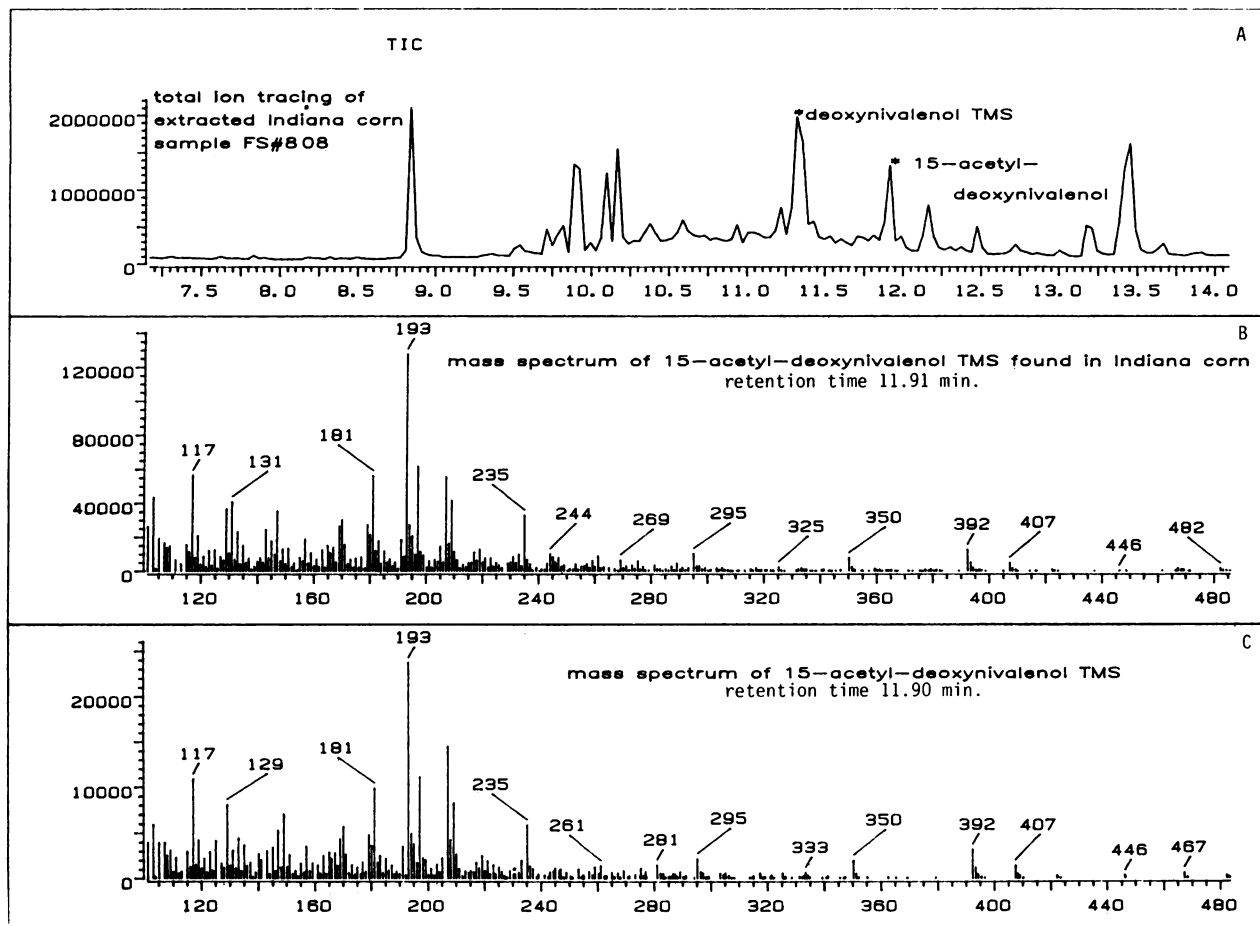


FIG. 1. (A) Total-ion chromatogram of 15-ADON-TMS and DON-TMS derivatives with retention times of 11.91 and 11.39 min, respectively. (B) Mass spectrum of a 15-ADON-TMS derivative from Indiana corn. (C) Mass spectrum of the 15-ADON-TMS standard with a retention time of 11.90 min. Mass spectrum confirmed its presence.

water layer was concentrated on a rotary evaporator to a volume of 10 ml and applied onto a column of Amberlite XAD-2 (1 by 15 cm, 20/50 mesh; Mallinckrodt, Inc., Paris, Ky.). The column was washed with 100 ml of water, and then the sample was eluted with 100 ml of 90% methanol. XAD-2 resins were purified before use by extraction with acetone overnight in a Soxhlet apparatus and a rinsing with 200 ml of distilled water immediately before use. The XAD-2-methanol eluate was evaporated to dryness on a steam bath, redissolved in 10 ml of chloroform-methanol (9:1, vol/vol), and added to a column of Florisil (1 by 12 cm) packed in the same solvent. The Florisil column was capped with 10 g of anhydrous Na_2SO_4 . DON and 15-ADON were eluted with 200 ml of chloroform-methanol (9:1, vol/vol), and the eluate was collected in a 500-ml round-bottom flask and evaporated to dryness. The residue was dissolved in 4 ml of methanol. A 1-ml portion of each sample was used for HPLC and gas chromatography-mass spectrometry (GC-MS) analyses, and 3 ml of each sample was used for the TLC studies.

ZEA and α -zearalenol extractions. Samples of ground corn were extracted and analyzed by the method of Bennett et al. (3).

Quantitation of mycotoxins by HPLC. The residues of the four mycotoxins were dissolved in 100 or 500 μl of mobile phase solvents of methanol-water (30:70, vol/vol) for quantitation of DON and 15-ADON or in methanol-acetonitrile-

water (1.0:1.6:2.0, vol/vol/vol) for quantitation of ZEA and α -zearalenol and were immediately mixed on a Vortex mixer for 1 min. The HPLC quantitation of the mycotoxins used absorbance for DON and 15-ADON (1) and fluorescence detection for ZEA and α -zearalenol (3). Concentrations of the four mycotoxins were calculated by the peak height method.

Confirmation of mycotoxins by TLC and GC-MS. The four mycotoxins were confirmed by TLC by the visual comparison method with standards and by GC-MS with both full scans and selected ion monitoring (1, 2, 9). Mass spectral confirmation (positive chemical ionization in methane) was

TABLE 1. Concentration of various mycotoxins in naturally infected field corn samples

Compound	Concn of toxin (ppm) ^a	
	FS 808	FS 362
15-ADON	16.3 \pm 1.51	1.20 \pm 0.91
DON	19.5 \pm 2.10	2.90 \pm 1.07
α -Zearalenol	0.21 \pm 0.92	0.00 \pm 0.00
ZEA	4.81 \pm 1.62	0.31 \pm 0.07

^a Each value is the mean of the analysis of four portions of a sample plus or minus standard deviation. Compounds were quantitated by HPLC by the peak height method of quantification and were confirmed by TLC and GC-MS.

done on a Hewlett-Packard 5987 quadrupole mass spectrometer after resolution of the mycotoxins as their trimethylsilyl (TMS) ethers on a DB-5 capillary gas chromatographic column (height, 30 cm).

RESULTS

F. graminearum and *F. moniliforme* were the only *Fusarium* species isolated from corn samples FS 362 and FS 808, respectively. Species of the genera *Aspergillus*, *Mucor*, and *Papulospora* were also isolated from FS 362. *F. graminearum* grew from 60% of the kernels of the corn sample FS 362, and *F. moniliforme*, *Penicillium* spp., and *Aspergillus glaucus* grew from 5, 6, and 4%, respectively, of the kernels of the corn sample FS 808.

Initial determination of the mycotoxins was done by HPLC. The percent recovery (spiked sample, 1 ppm) was $68.3 \pm 0.9\%$, $73.2 \pm 1.2\%$, and $76.8 \pm 1.5\%$ for DON, 15-ADON, and ZEA, respectively; the percent recovery of α -zearalenol was not determined. DON, 15-ADON, and ZEA were found in both samples, whereas α -zearalenol was found only in the corn sample FS 808. The concentrations of these mycotoxins were higher in the corn sample FS 808 than in the corn sample FS 362 (Table 1).

It is evident that results obtained by TLC and GC-MS (selected ion monitoring) refer to DON, 15-ADON, ZEA, and α -zearalenol and that no interference was present. The retention times of the mycotoxins DON, 15-ADON, ZEA, and α -zearalenol in corn samples FS 808 and FS 362 were identical to the retention times of their standards and were observed at 11.3, 11.9, 14.4, and 15.3 min, respectively. The total-ion chromatogram and mass spectrum of the 15-ADON-TMS derivative are shown in Fig. 1. The toxins 3-acetyl-deoxynivalenol, nivalenol, and fusarenon-X were not detected in either corn sample.

DISCUSSION

F. graminearum grew from 60% of the kernels of corn sample FS 362 after 13 years of storage at below-freezing temperature. We assume that this is the same *Fusarium* isolate that originally infected this lot of corn in Indiana for the following reasons. (i) *F. graminearum* survives well in corn seed stored frozen. (ii) This isolate was tested for mycotoxin production and found to produce all of the toxins found in the lot of corn. Only *F. moniliforme* grew from corn lot FS 808, stored at Purdue University (it grew from 5% of the kernels). Although *F. moniliforme* may have survived for 13 years, it did not produce ZEA or DON and thus is not important to the documentation of this case.

The occurrence of 15-ADON in these corn samples is important because it demonstrates for the first time the occurrence of this toxin in corn associated with refusal by swine under field conditions. It is also of importance because the mycotoxins described were found in samples of corn from the same field, although the samples were harvested at different times. Both analyses confirm one another and help to authenticate the findings. The presence of DON in the corn analyzed in 1972 could not account for the entire degree of refusal reported then. DON, when added to feed, reduced feed consumption by 20 to 45 kg in pigs, ranging from a 20% decrease with 3.6 ppm to a 90% decrease with 40 ppm. Loss in weight was associated with feed refusal. Feed refusal, however, was much greater for naturally infected corn samples than for feeds with equal concentrations of the pure compound added, indicating the involvement of an additional factor(s) in the swine refusal response (4). DON and

3-acetyl-deoxynivalenol caused 50% feed refusal by rats when they were present in the diet at levels of 100 and 150 $\mu\text{g/g}$, respectively (17); no experiments on 15-ADON and feed refusal have been carried out. It is possible and reasonable to assume that 15-ADON accounts for a portion of animal refusal.

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