Survey of Corn for Aflatoxin, Zearalenone, and Ochratoxin[1]

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ABSTRACT

A total of 283 corn samples from commercial markets, including samples from all grades, from the crop year 1967, were assayed for the presence of aflatoxin, ochratoxin, and zearalenone. Samples were extracted and assayed by the procedure developed by the Food and Drug Administration for multitoxin analysis. The sensitivity limits of the analysis as carried out were 1 to 3 p.p.b. aflatoxin B-1 and G-1, 50 p.p.b. ochratoxin A, and 200 p.p.b. zearalenone. Six of the samples–five in sample grade (SG) and one in grade 3–contained aflatoxin in low levels. One SG corn contained ochratoxin A. Zearalenone was identified in two SG samples, and F-3, an estrogenic factor related to zearalenone, could also be present.

We initially surveyed 1,311 corn samples from commercial markets in the crop years 1964 and 1965 for the presence of aflatoxin (1). Although the results of the first survey did not appear to be alarming because of the low incidence and low levels of toxin detected, all in samples of poorest grade, it was decided to assay corn from the crop year 1967. During that year, because of rainy and cold weather, much corn remained in the fields for abnormally long periods. It was felt that conditions for mold growth accompanied by toxin formation might be ideal.

We decided to analyze these samples for aflatoxin; for zearalenone (F-2), an estrogenic factor (2,3); and for ochratoxins, a group of mycotoxins produced by *Aspergillus ochraceus* (4), one of which was described as a natural contaminant by us (5). The results are reported here.

MATERIALS AND METHODS

Collection and Preparation of Samples

Samples (1 kg.) of all grades of corn from the crop year 1967 were collected from commercial markets (from Des Moines, Iowa, and Peoria, Ill., with a few from Mobile, Ala., and Memphis, Tenn.) by the Grain Division of Consumer and Marketing Service, USDA. Samples were ground in a Raymond 6-in., stainless-steel laboratory mill equipped with a screen having 1/8-in. round-hole perforations.

Extractions and Column Chromatography

The method of extraction and column chromatography developed by Eppley (6) for determining aflatoxin, zearalenone, and ochratoxin in agricultural commodities was used. Ground corn (50 g.) was extracted with chloroform and water. The mycotoxins in extracts were separated and partially purified for

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[2]Responsible for the microbiological aspects of the survey.

thin-layer chromatography (TLC) on silica gel columns. Residues from eluates were dissolved in benzene (0.5 ml.) for TLC.

Thin-Layer Chromatography

Chromatoplates (20 by 20 cm.) were coated with a 0.5-mm. layer of Adsorbosil-1, dried about 30 min., heated 2 hr. at 105°C., and stored in a desiccating cabinet.

Benzene[3] solutions of unknowns (10 μl.) were applied to the plates alone and in admixture with standard benzene solutions of mycotoxins. Aflatoxin standards (1 and 2 μl.) (0.00249 γ B-1 per μl., 0.00056 γ B-2 per μl., 0.00248 γ G-1 per μl., and 0.000485 γ G-2 per μl.) were applied to plates on which unknowns that might contain aflatoxin were spotted. The plates were developed with acetone:chloroform:water (12:88:1.5 v./v./v.) (7). Plates containing zearalenone fractions and standard (5 μl.) (0.05 γ zearalenone per μl.) were developed with ethanol:chloroform (5:95 v./v.) (6). Plates containing ochratoxin fractions and standard (3 μl.) (0.01 γ ochratoxin A per μl.) were developed with glacial acetic acid:benzene:water (10:90:1 v./v./v.). Aflatoxins, ochratoxins, and zearalenone were detected on thin-layer plates by their fluorescence, and amounts were determined by visual comparisons with standards.

Samples that appeared to be positive by TLC were re-extracted to check assay. If the sample still appeared to be positive by TLC, identity of mycotoxin was confirmed by chemical and physical tests.

Confirmatory Tests

The identity of aflatoxin B-1 was confirmed by the formation of the water and acetate addition compounds according to the method of Pohland et al. (8). Corn samples (70 to 150 g.) were extracted, and extracts were chromatographed on silica gel columns as they were for original analyses. Partially purified extracts were further purified by preparative TLC (9) with the same adsorbent and solvent system used for detection. Products from TLC plates contained 0.5 to 1.1 γ B-1. To form water adduct, 0.25 γ B-1 in 0.25 ml. benzene:acetonitrile (98:2 v./v.) and 0.1 ml. water plus 1 drop of concentrated hydrochloric acid were heated on a steam bath for 10 min. Mixture was then evaporated to dryness on the steam bath. The acetate addition derivative was prepared by heating 0.25 γ B-1 in 0.25 ml. benzene:acetronitrile (98:2 v./v.) with 0.25 ml. acetic anhydride and 1 drop concentrated hydrochloric acid for 10 min. in a closed container, after which the mixture was evaporated to dryness. The addition compounds of unknowns were compared with the same derivatives of standard B-1 by TLC (9).

Ochratoxin A was confirmed by its solubility in sodium bicarbonate and formation of a methyl ester. Residues from extracts of corn samples (20 g.) purified on silica gel columns were dissolved in chloroform and extracted with 0.1M sodium bicarbonate (5). If fluorescing substances suspected to be ochratoxin A or B were soluble in the sodium bicarbonate layer, a methyl ester was prepared by treating residue from a chloroform extraction of an acidified aqueous layer with boron

[3]When this work was done, benzene was used to dissolve standards and unknowns to be applied to TLC plates. Presently benzene:acetonitrile (98:2) is recommended.

trifluoride (14%) in methanol. Methyl ester of the unknown was compared to methyl ester of the standard by TLC.

Zearalenone was confirmed by ultraviolet absorption studies and gas-liquid chromatography (GLC) (10). Extracts of corn (70 to 200 g.) were purified as before by chromatography on silica gel and preparative TLC. Ultraviolet absorption spectra were run on eluates dissolved in absolute ethanol from preparative plates. Ethanol was then removed on a steam bath under nitrogen. Trimethylsilyl ethers (TMS) were prepared by treating residues with methylene chloride (20 μl.), pyridine (20 μl.), chlorotrimethylsilane (20 μl.), and hexamethyldisilazane (40 μl.), shaking the reaction mixture 30 sec., and allowing it to stand 30 min. before GLC. The following conditions and equipment were used: F&M, Model 810 chromatograph, hydrogen flame detector, hydrogen flow rate 15 ml. per min.; Disc Model 227 integrator; injection port temperature, 265°C.; column temperature 240°C.; column, 1/8 in. by 6 ft. packed with 2.5% SE-30 (silicone gum rubber) coated on 80- to 100-mesh high-performance Chromosorb G (AW-DMCS); helium carrier gas, 20 ml. per min. flow rate; and Honeywell recorder, chart speed 4 min. per in.

Examination of Samples for Fungi

The methods used for microbiological examination of corn samples from the 1967 crop year were the same as those used in our survey on wheat, oats, and grain sorghum (11).

RESULTS AND DISCUSSION

Before the commercial samples were assayed, limits of detection were established by assay of artificially contaminated samples prepared by adding different amounts of aflatoxins B-1 and G-1, ochratoxin A, and zearalenone to ground corn shown to be free of the mycotoxins. The limits of detection for B-1, G-1, ochratoxin A, and zearalenone were 1 to 3, 1 to 3, 50, and 200 p.p.b., respectively. Recoveries of aflatoxins B-1 and G-1 were 92 to 100% and 50 to 70%, and of ochratoxin A, 40 to 60%. Zearalenone was quantitatively recovered, and sometimes TLC indicated more than 100%. Extracts of some of the artifically contaminated corn samples appeared to have substances that enhanced the fluorescence of zearalenone, although these substances did not appear as fluorescent zones on TLC plates in the absence of zearalenone. We found that the assay procedure would also detect ochratoxin B, if present, but not the methyl and ethyl esters of A and B.

The results of the survey of 283 corn samples from the crop year 1967 for aflatoxins, ochratoxins, and zearalenone are summarized in Table I. All positive samples were in sample grade (SG), except one which was in grade 3. The higher incidence of mycotoxins in poorer grades was to be expected from our previous survey (1). Also, grades of corn are determined by factors connected with mold growth. The percentage moisture and total damage in the samples assayed are plotted in Figs. 1 and 2.

Information on samples in which the presence of mycotoxin was confirmed is given in Table II. We received very few samples from Memphis and none of these was positive. There were positive samples from the other three locations. Levels of aflatoxin B-1 in the six positive samples ranged from 12 to 25 p.p.b. The percentage

TABLE I. CORN SAMPLES FROM 1967 CROP EXAMINED FOR THE PRESENCE OF MYCOTOXIN

Grade	Samples Tested	Positive Samples		
		Aflatoxins	Ochratoxin A	Zearalenone[a]
1	3	0	0	0
2	27	0	0	0
3	51	1	0	0
4	52	0	0	0
5	46	0	0	0
Sample grade	104	5	1	2
Total	283	6	1	2

[a]F-3 might have been present in positive samples, but no standard was available for direct comparisons.

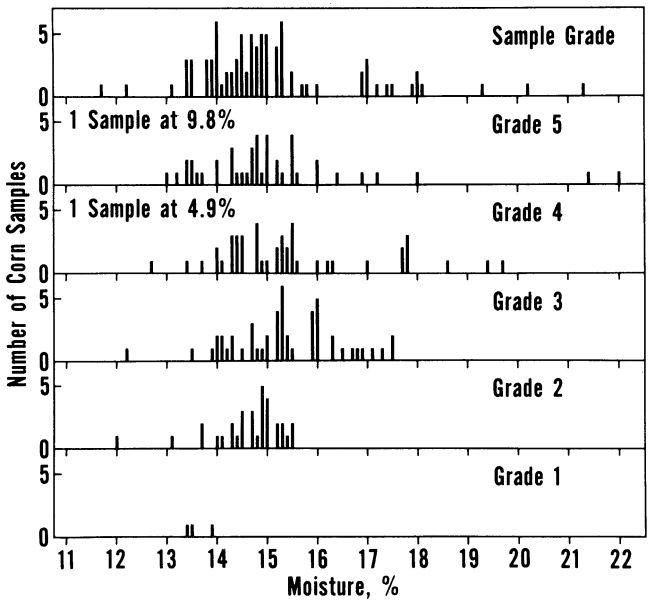

Fig. 1. Moisture in corn samples assayed for the presence of aflatoxin, ochratoxin, and zearalenone.

of moisture in these was not unusually high. Four of the six had high total damage (16 to 68%) and three had sour odors. Unfortunately, no grading information was given on the SG sample that also contained aflatoxin G-1 (12 p.p.b.), zearalenone, and possibly F-3, an estrogenic compound related to zearalenone. The presence of B-1 in the grade 3 corn was easily confirmed because of the lack of impurities in

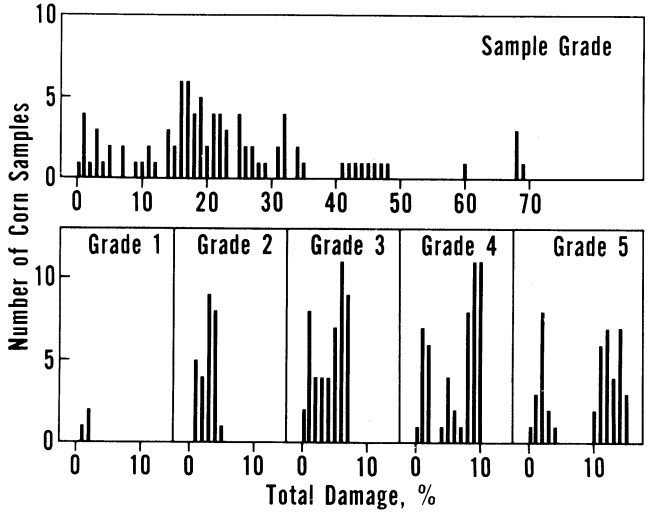

Fig. 2. Damage in corn samples assayed for the presence of aflatoxin, ochratoxin, and zearalenone.

extracts. One sample (SG) had a fluorescent substance with the same mobility as B-1 on TLC plates, but the compound did not form a water or acetate addition compound so could not be identified as aflatoxin B-1. Significantly, *A. flavus* was detected in all the aflatoxin-positive corn samples.

The most important fact disclosed by this survey is that percentage incidence of aflatoxin in 1967 was not greater than in previous years (Table III). Actually, incidence was lower than in 1964. Comparisons of grading factors are equally interesting (Table III). Eighty percent of the corn samples in 1967 had more than 14% moisture as compared with 32% in 1964 and 52% in 1965. Amount of total damage in samples from 1964, 1965, and 1967 did not differ greatly. In fact, more samples with higher total damage were collected in 1964. More 1967 samples than 1964 and 1965 samples were heating and had sour or musty odors.

Ochratoxin A was reported for the first time as a natural contaminant in one of the 283 corn samples (5). Attempts to isolate *A. ochraceus* from this sample were

TABLE III. CORN SAMPLES FROM DIFFERENT YEARS ASSAYED FOR AFLATOXIN

Samples %	Crop Year 1964	1965	1967
With more than 14% moisture	32	52	80
With more than 10% damage	44	37	38
With heating or heat damage	10	5	12
Having musty odor	13	13	16
Having sour odor	3	3	7
Containing aflatoxin	3.2	1.9	2.1

TABLE II. CORN SAMPLES (1967) CONTAINING MYCOTOXINS (TOTAL NUMBER ASSAYED, 283)

Sample Number and Market[a]	Grade	Mycotoxin	Level p.p.b.	Fungi Present			Grading Information			
				A. flavus	Fusarium	A. ochraceus	Moisture %	Total Damage %	Odor	Other
F-3916, M	SG	Aflatoxin B-1	25							
		Aflatoxin G-1	12	+	–	–				
		Zearalenone[b]	>1,250							
F-3931, DM	3	Aflatoxin B-1	25	+	+	–	14	6		2.1% FM[c]
F-4010, DM	SG	Aflatoxin B-1	6	+	–	–	14	16		3.3% FM
F-4015, P	SG	Aflatoxin B-1	10	+	–	–	14	47	Sour	2.5% FM
		Aflatoxin G-1	trace							
F-4020, P	SG	Aflatoxin B-1	12	+	+	–	16	21	Sour	0.7% heating
F-4195, P	SG	Aflatoxin B-1	12	+	–	–	14	68	Sour	2.5% heating
										6.0% FM
F-3972, DM	SG	Ochratoxin A	110–150	–	+	–	18	23	Musty	1.8% FM
F-4186, P	SG	Zearalenone[b]	800	+	+	–		26	Musty	1.8% FM

[a]M, Mobile; DM, Des Moines; P, Peoria.

[b]Ultraviolet absorption spectra of unknowns corresponded to that of zearalenone or F-3. Gas-liquid chromatography indicated that F-2 was present and F-3 could be present.

[c]FM, foreign material.

unsuccessful. Culture plates contained a high incidence of penicillia, which may have prevented the mold from producing conidia. Recently, however, the production of ochratoxin by *Pencillium viridicatum* has been reported (12). Six corn samples from 1967 had fluorescent factors with mobilities on TLC plates similar to that of ochratoxin A. Three of these compounds were insoluble in sodium bicarbonate and only one of the three soluble factors formed a methyl ester. Ochratoxin B was not detected in any of the samples.

Initially, five SG corns of the 283 samples appeared by TLC to contain zearalenone. Ultraviolet absorption studies indicated that only two contained compounds with peaks at 234, 276, and 314 nm. that could be attributed to zearalenone. The two samples contained compounds that formed TMS derivatives with the same retention time (12.5 min.) on GLC columns as standard TMS zearalenone. Another peak with a retention time (9.6 min.) that appeared to be like that of the F-3 derivative was also detected. We were not able to obtain a sample of F-3 for direct comparison and a more positive identification.

CONCLUSION

Six of the 283 corn samples collected from commercial sources in the crop year 1967 contained 12 to 25 p.p.b. aflatoxin B-1. All these were in SG, except one in grade 3. One SG sample also contained aflatoxin G-1 (12 p.p.b.), zearalenone, and possibly F-3. Unfavorable weather conditions in 1967 did not lead to more aflatoxin in the 283 samples assayed that were collected for the most part in the Midwest. However, colder temperatures in the Midwest when corn is harvested may have a greater effect on fungal population than a wet season. *Aspergillus flavus*, like many aspergilli, grows best at higher temperatures and producing strains from highest levels of aflatoxin at 24° to 34°C. Penicillia prefer lower temperatures. Considering all conditions, the growth of penicillia may have been favored over that of *A. flavus*. *Penicillium* was the most prevalent mold in all the corn samples collected. *Aspergillus flavus* was isolated from all the samples that contained aflatoxin. One SG sample contained ochratoxin A (110 to 150 p.p.b.). Two SG samples contained zearalenone and perhaps F-3, an estrogenic factor related to zearalenone.

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