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Survey of Cereal Grains and Soybeans for the Presence of Aflatoxin. II. Corn and Soybeans[1]

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ABSTRACT

A total of 1,311 corn samples and 866 soybean samples, including samples from all grades and two different crop years, 1964 and 1965. were assayed for the presence of aflatoxin. Samples were extracted by the procedure used in the survey of wheat, grain sorghum, and oat samples, and extracts were tested for the presence of aflatoxin by thin-layer chromatography (TLC). The sensitivity limit of the analysis as carried out was 2 to 5 p.p.b. of the metabolite. Of the 35 corn samples that were positive by TLC, 30 gave aflatoxinlike responses in ducklings. Five of these were in grade 5, and 25 were in sample grade. Two of the 866 soybean samples tested contained aflatoxin by TLC and the duckling test. These were in sample grade.

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Corn and soybeans were included as part of a survey of agricultural commodities from commercial markets for the presence of aflatoxin. A total of 1,368 samples of wheat, grain sorghum, and oats were assayed and results are reported in Part I of this series (1). Aflatoxin was not detected by the duckling test in any grain sorghum samples, although thin-layer chromatography (TLC) of extracts suggested that the toxin could be present. Biological tests on the two wheat samples and three oat samples positive by TLC indicated that, although traces of toxin could be present, the tests were probably negative.

Corn was shown to be a good substrate for aflatoxin production by three strains of *Aspergillus flavus* (2). The same three strains did not produce nearly so much toxin on soybeans as on corn, but dangerous levels (8 to 22 γ/g.) were obtained in a 6-day fermentation. It is, therefore, possible that the toxins could occur as natural contaminants in both corn and soybeans. The analysis of these samples of corn and soybeans for the presence of aflatoxin is reported in this paper.

MATERIALS AND METHODS

Collection and Preparation of Samples

Samples (1 kg.) from all grades of corn and soybeans were collected from commercial markets by the Grain Division of Consumer and Marketing Service, USDA. As in the preceding paper (1), 300 samples were requested from each location. Samples were collected during two different crop years. The samples requested and those actually received are shown in Tables I and II.

Samples (1 kg.) were ground in a Raymond 6-in. stainless-steel laboratory mill equipped with a screen having 1/8-in. round-hole perforations.

Extractions

Corn and soybean samples were extracted by the same method used for wheat, grain sorghum, and oat samples (1,3). The sample (50 g.) was blended for 3 min. in a Waring Blendor with methanol:water (55:45 v./v.) (250 ml.). A 50-ml. aliquot of the extract was mixed with acid-washed Celite 545 (55 g.) and water (5 ml.) and packed in a column to be washed with hexane and eluted with hexane:chloroform (1:1 v./v.). Residues from eluates were dissolved in chloroform (1 ml.) and subjected to TLC.

TABLE I. CORN SAMPLES EXAMINED IN A SURVEY FOR THE PRESENCE OF AFLATOXIN

Samples	Grade						Total
	1	2	3	4	5	SG	
No. requested[a]	100		26	26	48	100	300
Source							
Omaha 1964	8	41	29	24	66	62	230
Peoria 1964	4	70	50	50	26	65	265
Peoria 1965	12	48	59	46	23	36	224
Des Moines 1964	11	51	35	20	79	92	288
Des Moines 1965	5	43	37	34	87	98	304
Total received	40	253	210	174	281	353	1,311
Positive by TLC[b]	0	2N	1N	2N	5WP	25P	35
Aflatoxin[c](%)	0	0.8	0.5	1.1	1.8	7.1	

[a]No. requested from each location.

[b]By duckling test: N, negative; WP, weakly positive; P, positive.

[c]Incidence in given grade.

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TABLE II. SOYBEAN SAMPLES EXAMINED IN A SURVEY FOR PRESENCE OF AFLATOXIN

Samples	Grade 1	2	3	4	SG	Total
No. requested[a]	36	60	26	100	80	302
Source						
Decatur 1964	6	60	41	83	55	245
Decatur 1965	3	60	63	64	89	279
Memphis 1964	32	60	26	90	89	297
Memphis 1965	10	16	9	6	4	45
Total received	51	196	139	243	237	866
Positive[b]	0	0	0	0	2	2

[a]No. requested from each location.

[b]By both TLC and duckling tests.

Partial Purification of Soybean Extracts

Of the 866 soybean samples tested, 21 contained fluorescing substances that interfered with TLC. Two could be further purified on silica gel (0.05 to 0.20 mm.) columns (35 by 60 mm.) (4). The column was washed with anhydrous diethyl ether and eluted with methanol:chloroform (3:97 v./v.) (150 ml.). The other 19 samples were assayed only by the duckling test.

Thin-Layer Chromatography

Extracts (20 microliters) were spotted on Silica Gel G-HR plates, alone and in admixture with a standard solution in chloroform of aflatoxins B-1, B-2, G-1, and G-2. Plates were developed with 3% methanol in chloroform, and fluroescent zone were detected as before (1).

Samples for Duckling Tests

Duckling tests were conducted by the Wisconsin Alumni Research Foundation (WARF) (1). Corn samples in grades 2, 3, and 4, in which TLC revealed detectable amounts of aflatoxin, were combined to make possible a significant response in the duckling test. All the positive samples in grade 5 were combined for the biological test, and positive corn samples in Sample Grade (SG) were combined five at a time for the assay. The two soybean samples (SG) positive by TLC were combined for biological testing, and five to six samples at a time were extracted when samples contained substances that interfered with TLC and could not be removed on silica gel columns.

Examination of Samples for Fungi

The methods used for the microbiological examination of soybean and corn samples were the same as those used in our survey on wheat, oats, and grain sorghum (1).

RESULTS AND DISCUSSION

No difficulty was experienced in preparing extracts of corn suitable for TLC. The sensitivity limit of the assay as carried out was 2 to 3 p.p.b Of the 1,311 samples analyzed, 35 (2.7%) appeared by TLC to contain aflatoxin B-1. Most of the samples were in the poorer grades with 25 in SG (7.1% incidence) and five in grade 5 (1.8% incidence) (Table I). Chi-square computed for Table I was used to test the hypothesis of a constant percent incidence for all grades. The value computed

TABLE III. CORN SAMPLES IN GRADES[a] 2, 3, 4, AND 5 CONTAINING AFLATOXINS BY TLC (TOTAL NUMBER ASSAYED, 918)

Sample No. and Market[b]	Grade[c]	Aflatoxin by TLC		*A. flavus*	Grading Information	
		B-1 *p.p.b.*	G-1 *p.p.b.*	Present[d]	Moisture %	Total Damage %
F-967, P	2	13	2	-	14	4
F-2707, P	2	13	ND[e]	-	15	2
F-2963, DM	3	6	ND	-	13	7
F-292, DM	4	7	ND	+	15	8
F-2836, P	4	6	ND	-	20	...
F-659, DM	5	19	ND	+	18	14
F-803, DM	5	7	ND	...	12	12
F-959, DM	5	3	3	+	13	14
F-1292, O	5	13	trace	+	13	12
F-1167, DM	5	3	ND	+	13	12

[a]Aflatoxin was not detected in any grade 1 sample.

[b]P, Peoria; DM, Des Moines; O, Omaha.

[c]Aflatoxin in grades 2, 3, and 4 not detected by duckling test. Duckling test was weakly positive in grade 5.

[d]+, Present; -, not found.

[e]ND, not detected.

(36.28 with 5 degrees of freedom) has a probability of less than 0.005. The low probability of chi-square leads us to conclude that percent incidence is correlated with grade rather than being constant. This result confirms one of the assumptions made in designing the survey. The incidence was essentially the same in the three locations where samples were collected. Aflatoxin occurred in 3.2% of the samples collected in 1964 and 1.9% of those collected in 1965. Actually, weather conditions in the 1965 crop year should have been more favorable for mold growth accompanied by aflatoxin formation.

More information about the samples of corn positive by TLC including facts from grading slips, levels of aflatoxins present, and results from the examination of samples for fungi are presented in Tables III and IV. Very few samples contained aflatoxin G-1. The most typical strains of *A. flavus* that produce aflatoxin do not form G-1 or G-2, only B-1, B-2, and M (5). Strains of *A. parasiticus* produce all the aflatoxins, but these strains were not detected in the examination of corn samples for fungi. Strains of *A. parasiticus* probably do not occur as contaminants in the areas where we collected our samples. The levels of aflatoxin observed in positive samples were low, the highest levels being 19 p.p.b. B-1 and 8 p.p.b. G-1.

Results from tests conducted by WARF in ducklings on corn samples positive by TLC showed that no aflatoxin was present in samples from grades 2, 3, and 4, but the test on grade 5 samples was weakly positive or indicated traces. Samples from SG gave definite aflatoxinlike responses in ducklings, but biological tests indicated the presence of lower levels of toxin than did TLC.

Moisture, total damage, and heat-damage in the corn samples tested for the presence of aflatoxins are depicted in Figs. 1, 2, and 3, respectively. Comparisons of the positive samples in Table III and particularly those in Table IV that were positive by both biological and physical chemical tests with Figs. 1, 2, and 3 indicate that grading factors used to evaluate these samples would probably serve to eliminate, from human usage, corn that is likely to contain aflatoxin. Of the 25 positive samples in SG, 15 contained more than 14% moisture and 22 had more than 10% damage. More than a third of these samples showed spontaneous heating

TABLE IV. CORN SAMPLES (SG) CONTAINING AFLATOXINS[a] (TOTAL NUMBER ASSAYED, 353)

Sample No. and Market[b]	Aflatoxin by TLC B-1 p.p.b.	Aflatoxin by TLC G-1 p.p.b.	*A. flavus* Present[c]	Grading Information: Moisture %	Total Damage %	Odor	Other
F-70, P	14	ND[d]	+	21	3	Sour	Heating
F-147, DM	14	ND	+	17	1	Musty	Heating
F-556, DM	19	8	+	13	58	Musty	...
F-606, P	7	ND	-	13	18	...	...
F-654, O	19	ND	+	...	22	...	...
F-701, DM	19	ND	+	16	34	Musty	...
F-869, O	13	ND	+	15	36	Musty	...
F-961, DM	3	ND	+	14	27	Musty	...
F-963, DM	7	ND	+	14	32	Musty	...
F-1083, P	7	ND	+	13	60	Sour	5% HD[e]
F-1139, DM	13	ND	+	14	23	...	...
F-1166, P	3	ND	+	17	11	Musty	0.5% HD
F-1316, O	13	ND	+	...	23	Musty	...
F-1395, P	13	ND	+	17	18	Sour	2.3% HD
F-1818, P	7	ND	+	15	56	Sour	4.0% HD
F-1879, P	13	ND	+	15	64	Sour	4.0% HD
F-2251, O	13	ND	+	15	30	...	...
F-2599, DM	3	ND	...	13	19	Musty	...
F-2649, P	13	ND	...	22	5	Sour	Heating
F-2669, P	5	ND	...	17	...	Sour	...
F-2823, DM	6	ND	...	24	10	Musty	...
F-3018, DM	7	3	...	13	18	...	...
F-3184, DM	10	ND	...	14	13	Musty	...
F-3239, P	13	ND	...	19	48	Sour	Heating
F-3375, DM	6	6	...	13	27	Musty	...

[a]Confirmed by duckling test.

[b]P, Peoria; DM, Des Moines; O, Omaha.

[c]+, Present; -, not found in sample

[d]ND, not detected.

[e]HD, heat damage.

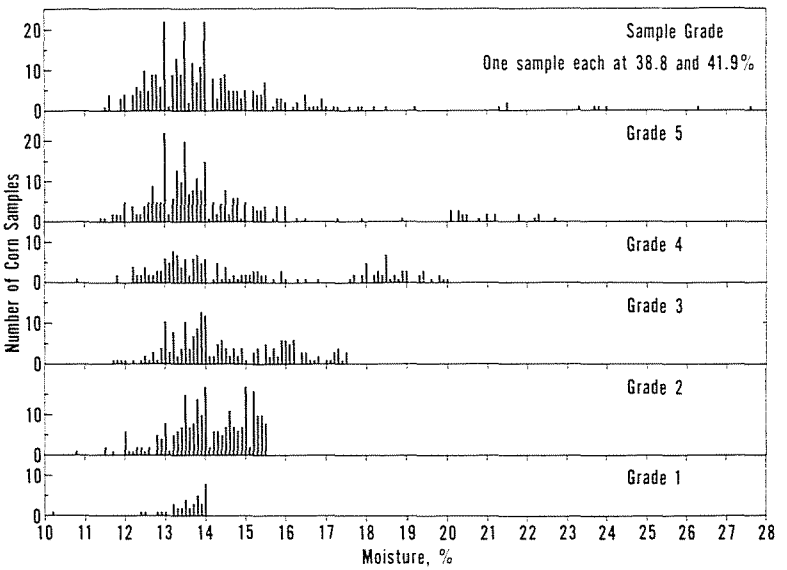


Fig. 1. Moisture in corn samples assayed for the presence of aflatoxin.

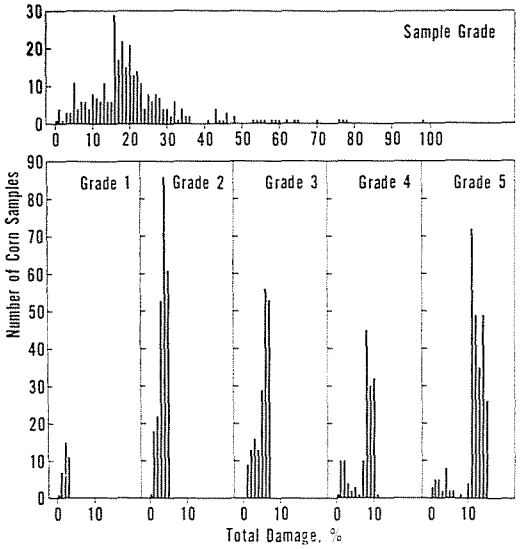


Fig. 2(left). Damage in corn samples assayed for the presence of aflatoxin.

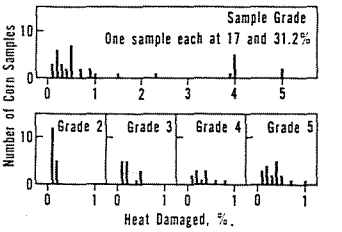


Fig. 3(right). Heat-damage in corn samples assayed for the presence of aflatoxin. None of the grade 1 samples were heat-damaged. Of the 1,311 corn samples assayed, 98 were heat-damaged.

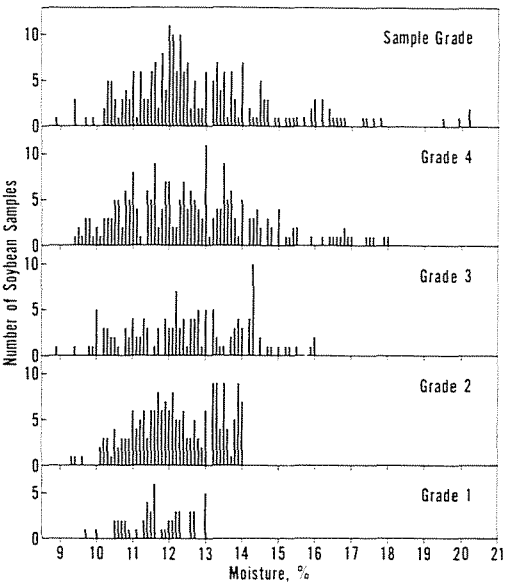
or had heat-damage. Of the 353 samples of corn assayed in SG, 164 had a musty odor, 4 had a musty odor and were heating, 37 had a sour odor, and 6 had a sour odor and were heating. Twenty of the 25 positive SG samples had musty or sour odor. Odor and spontaneous heating are typical symtoms of mold growth that might lead to the formation of aflatoxin.

Two soybean samples of the 866 analyzed contained low levels of aflatoxin by both TLC and duckling tests. Both samples were in SG (Table II). With such a low incidence, comparisons cannot be made between crop years 1964 and 1965 and locations collected. Grading information on positive samples is given in Table V and information on moisture, splits, total damage, and heating in the soybean samples tested is shown in Figs. 4, 5, 6, and 7, respectively. Comparisons of the grading information on positive samples and that in Figs. 4, 5, and 6 are not very significant

TABLE V. SOYBEAN SAMPLES (SG) CONTAINING AFLATOXINS (TOTAL NUMBER ASSAYED, 866)

Sample No. and Market	Aflatoxin by TLC		Grading Information			
	B-1 *p.p.b.*	G-1 *p.p.b.*	Moisture %	Splits %	Damage %	Other
F-2678 Memphis	10	ND[a]	16	12	7	Heating
F-3346 Decatur	7	4	13	19	16	...

[a]ND, not detected.



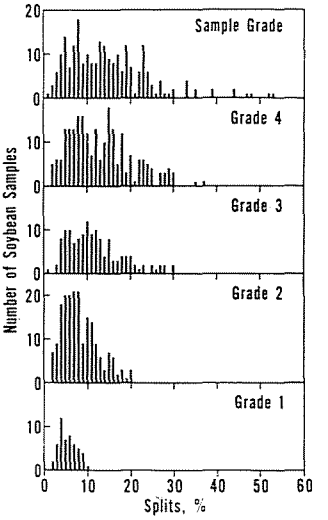


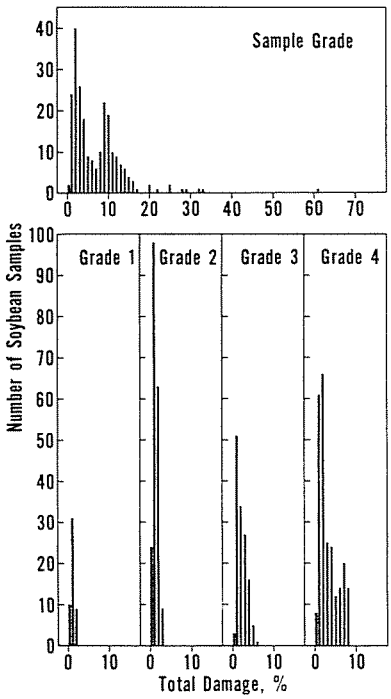
Fig. 4(left). Moisture in soybean samples assayed for the presence of aflatoxin.

Fig. 5(right). Splits in soybean samples assayed for the presence of aflatoxin.

because of the low incidence of aflatoxin. The two positive samples did have relatively high moisture content, had a number of splits, and showed high total damage.

Nineteen samples had to be tested only in ducklings because of impurities that fluoresced yellow and interfered with TLC. Attempts to remove impurities by silica-gel columns, lead-acetate precipitation (4), and hexane precipitation were unsuccessful. Five of these samples combined gave a response similar to that of aflatoxin in the ducklings, but without further confirmation, chemical or physical-chemical, one cannot assume that the causative factor is aflatoxin. The level of aflatoxin necessary for the observed response would be 2 to 4 p.p.b. Grading information (Table VI) indicates that three of these samples had a very high percentage of total damage.

The results of the examination of grains and soybeans for the presence of *A. flavus*, series organisms that produce aflatoxin, are given in Table VII. Chi-square computed from data was 15.62 with 4 degrees of freedom. The probability of a value as large as this is less than 0.005 and, therefore, we conclude that incidence of *A. flavus* is dependent on commodity. Twenty-three of the corn samples that contained aflatoxin were examined, and 22 of these contained *A. flavus* (Tables III and IV). *A. flavus* could have been present in the remaining sample (F-606), but became nonviable, perhaps by drying out, the toxin level remaining the same. This sample contained *Fusarium* and *Absidia corymbifera*. Only 9 of the 34 corn samples examined in which aflatoxin was not present contained *A. flavus*. Almost pure culture plates of *A. flavus* were obtained from several of the positive and negative samples. Corn samples had a high incidence of *Fusarium*, as well as considerable numbers of *Mucor, Rhizopus*, and *Absidia* spp.



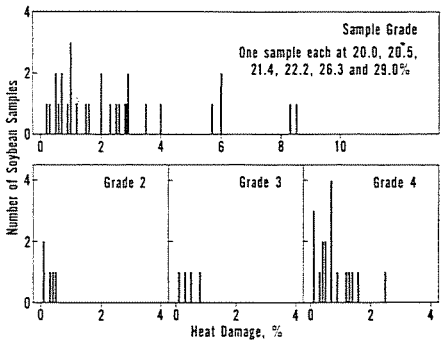


Fig. 6(left). Damage in soybean samples assayed for the presence of aflatoxin.

Fig. 7(right). Heat-damage in soybean samples assayed for the presence of aflatoxin. None of the grade 1 samples were heat-damaged. Of 866 samples tested, 63 had heat-damage.

Soybean samples in which aflatoxin was not present often had an abundance of the *A. flavus* group, but as mentioned before, soybeans are not a good substrate for aflatoxin production. In 15 out of 28 samples examined, *A. flavus* was detected. In one of these, the only mold present was *A. flavus*, and in four others more than half of the colonies were *A. flavus*. Note in Table VII that 50% of the soybean samples had one or more colonies of this mold. The only other commodity with a higher incidence of *A. flavus* was corn, and yet there were very few soybean samples containing aflatoxin. Of the 32 soybean samples examined, 17 contained *Penicillium* spp. and 6 contained *Fusarium*.

TABLE VI. SOYBEAN SAMPLES (MEMPHIS MARKET) GIVING AFLATOXINLIKE REACTION IN DUCKLINGS

Sample No.	Grade	Grading Information				
		Moisture %	Damage %	Splits %	Heat-Damage %	Odor
F-1713	4	12	1	12	1	...
F-2083	SG	13	29	5	26	Sour
F-2086	SG	12	25	3	21	Sour
F-2211	SG	13	28	4	22	Sour
F-2337	4	14	2	8	...	...

TABLE VII. OCCURRENCE OF ASPERGILLUS FLAVUS GROUP IN GRAIN SAMPLES

Item	Corn	Wheat	Oats	Sorghum	Soybean
Total samples examined	57	45	7	28	32
Presence of A. flavus					
No. positive	31	9	1	12	16
No. negative	26	36	6	16	16
A. flavus present, %	54	20	14	43	50

CONCLUSIONS

Of the 1,311 corn samples assayed, 35 contained aflatoxins, primarily B-1, by TLC. Tests in ducklings confirmed the presence of aflatoxin in 30 of these positive samples. The 30 definitely positive samples contained aflatoxins at very low levels and were in the poorer grades (5 and SG). Levels of aflatoxin B-1 were 3 to 19 p.p.b., and the levels of G-1 in the five samples in which it was detected were 2 to 8 p.p.b. Our results indicate that the factors used to grade these corn samples would probably exclude from food markets samples likely to contain aflatoxin.

Including the five samples for which we have only a biological response (equivalent to 2 to 4 p.p.b. B-1), the incidence of aflatoxin in soybeans was at most 0.8%, and positive samples were in grade 4 and SG. The two samples positive by TLC and the duckling test had 7 to 10 p.p.b. B-1 and 0 to 4 p.p.b. G-1.

According to recent publications, the levels at which we detected aflatoxin in corn and soybeans would not be injurious to swine (6,7) or cattle (8). Coomes et al. (9) have stated that samples having up to 50 p.p.b. are of zero to low toxicity when considered in animal feed. Nothing definite is known about toxicity levels for man, but in a study by Tulpule et al. (10) of the effect of feeding aflatoxin to young monkeys, liver lesions, very like bilary cirrhosis, could be induced in 3 weeks when the monkeys were fed 1 mg. aflatoxin daily. Monkeys were not, however, as susceptible to the toxin as ducklings or guinea pigs. Wogan (11) has attempted to evaluate the possible effects of aflatoxins in humans. Circumstantial evidence such as epidemiologic patterns of incidence of primary liver cancer in man and knowledge of the effects of the compounds on animals may, in the future, implicate aflatoxins as causative agents of the disease. The joint FAO-WHO committee on nutrition has recommended that the level of aflatoxin in protein supplements, of which 100 g. is eaten per day, should not exceed 30 p.p.b. (12). FAO-WHO wanted to establish lower levels, but felt a compromise was necessary because of existing world food shortages. The U.S. Food and Drug Administration has not established a tolerance level for aflatoxin.

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