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Distribution of Aflatoxin and/or Zearalenone in Wet-Milled Corn Products: A Review

Glenn A. Bennett* and Roy A. Anderson

Aflatoxin and zearalenone are but two of the many mycotoxins that are metabolized in grains by field and storage molds. Corn appears particularly susceptible to these mycotoxin-producing fungi, and there have been several incidences of contamination in recent years. While most of the corn produced is fed to livestock, over a half-billion bushels are used to manufacture food and industrial products. Of this quantity, some 450 million bushels of corn are converted to starch, oil, and by-product feeds by the wet-milling industry. This industry requires high-quality, clean corn and does not purchase lower grade corn, especially that infected by molds. However, when corn is purchased in large quantity from many lots of varying storage history, the possibility of inadvertently getting contaminated corn does exist. To determine the fate of aflatoxin and zearalenone during the wet-milling process, studies have been carried out on artificially and naturally contaminated corn. The extent of contamination and the distribution of the mycotoxins in the milled products are reviewed.

Occurrence of Aflatoxins in Corn. Numerous surveys to determine the incidence and level of aflatoxin in corn have been conducted by the wet-milling industry, the Agricultural Research Service, and the Food and Drug Administration. A summary of these surveys is listed in Table I. Some of the first studies on aflatoxin in corn were done on 1964, 1965, and 1967 corn (all grades) from commercial channels in the Midwest (Shotwell et al., 1969, 1970). These surveys (>1300 samples) indicated both low incidence (2.1–2.3%) and low levels (3–37 ppb) of aflatoxins. Contaminated samples were mostly from poorest grades of corn. An extensive survey for aflatoxin by the wet-milling industry (Watson and Yahl, 1971) on grain inspection samples from 230 ears of shelled corn showed only four contaminated samples at levels of 3–5 ppb. In addition, corn lots in three plants were sampled daily for 1 year. Aflatoxin B_1 was reported in 6 of the 142 weekly samples at levels of 3–5 ppb. In 1971 and 1972, 525 samples collected from preharvest corn in Indiana showed no aflatoxin (Rambo et al., 1974). Extensive surveys were conducted by the Grain Division, AMS, USDA in 1972, 1973, and 1974 (Hunt et al., 1976). In 1972, 7913 samples were inspected for bright greenish-yellow (BGY) fluorescence, and suspect samples were assayed by CB method and minicolumn method. Only 1.1–1.5% of the

Northern Regional Research Center, Federal Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604.

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Table I. Incidence of Aflatoxin in Corn

year	origin of samples	no. of samples assayed	percent samples with indicated level of aflatoxin, ppb				
			ND[a]	20	20-49	50-100	100
1964-1965	Corn Belt	1311	98	2	0.1		
1965	Corn Belt	372	97	3			
1967	Corn Belt	283	98	1	1		
1968-1969	export cargo	293	97	2	1		
1969-1970	South	60	65	13	5	8	8
1971	southeast Missouri	1283	68	18	7	4	2
1972	Corn Belt	223	98	2			
1973	South Carolina[b]	297	49	19	17	8	7
1973	Corn Belt	169	98	2			
1973	South[c]	146	64	22	7	4	2
1975	Iowa[b]	214	83	15	1	1	

[a] ND = not detected. [b] Freshly harvested corn. [c] Stored ASCS white corn.

Table II. Surveys of Corn for Zearalenone

year	origin of samples	no. of samples assayed	percent samples with given level, ppm			
			ND	0.4	0.4-0.9	1.0-5.0
1967	Corn Belt	283	99		1	
1968-1969	export	293	98		2	
1972	Corn Belt	223	83	9	4	4
1973	Corn Belt	169	90	10		
1973	South	146	99	1		

samples had detectable levels of aflatoxin and only 0.3% had levels >15 ppb. Much higher incidence and levels of aflatoxin have been reported from surveys conducted in regions (southern and southeastern states) where incidence of *Aspergillus flavus* is expected to be high. In 1969 and 1970, 21 of 60 samples of white corn from Alabama, North Carolina, South Carolina, Tennessee, and Virginia contained aflatoxins (Shotwell et al., 1975). In 1973, freshly harvested corn (297 samples) was examined from South Carolina and 32% contained aflatoxin above 20 ppb. In 1975, freshly harvested corn (214 samples) from selected counties in Iowa was examined for BGY fluorescence and aflatoxin; 17% contained detectable levels of toxin, but only four samples contained >20 ppb (Lillehoj et al., 1976). Generally, these surveys show a low incidence and low levels of aflatoxin in the midwest Corn Belt; however, corn from selected regions may contain high levels of toxin.

Although *Aspergillus* spp. are generally considered to be storage fungi, a report as early as 1920 indicated that *A. flavus* invaded corn in the field (Taubenhaus, 1920). In 1971 and 1972, the presence of *A. flavus* in preharvest corn was reported in southern Indiana (Rambo et al., 1974). Other reports on *A. flavus* occurrence in corn at or before harvest have been published (Fennell et al., 1975; Lillehoj et al., 1976; Hesseltine et al., 1976). In related studies, evidence has been presented which shows that the formation of aflatoxin also occurs in the field (Lillehoj et al., 1975a,b). In a series of studies which covered the years 1971, 1972, and 1973, aflatoxin was found in the field in essentially all of the corn-producing areas of the United States (Anderson et al., 1975). The highest incidence of aflatoxin was found in warmer, more humid regions of the country. Numerous reports have related insect populations to *A. flavus* infection in corn, and these data are summarized in a recent review of aflatoxin in corn (Shotwell, 1977).

Occurrence of Zearalenone in Corn. Zearalenone is a metabolite of most known isolates of *Fusarium roseum* and of several other *Fusarium* species (Caldwell et al., 1970; Mirocha and Christensen, 1974). This estrogenic compound was first found in corn infected by *Fusarium graminearum* (syn. *F. roseum*), the asexual stage of *Gibberella zeae*, the pathogen responsible for corn ear rot disease (Stob et al., 1962; Mirocha et al., 1967). The surveys of corn for zearalenone have not been as extensive as those for aflatoxins. The *Gibberella* ear rot epidemics which occurred in Indiana in 1965 and 1972 demonstrated that large-scale outbreaks of zearalenone-producing strains may occur (Tuite et al., 1974). Large-scale production of zearalenone on corn appears to be a storage problem, because corn artificially inoculated in the field with *Fusarium roseum* produced <2% of the levels detected in stored corn (Caldwell and Tuite, 1970).

Surveys of corn from different regions of the United States for zearalenone have been reported by the Northern Regional Research Center and the Food and Drug Administration (FDA) (Table II). In 1967, 283 samples from the corn belt were assayed for aflatoxin, ochratoxin, and zearalenone. Only two samples contained zearalenone (0.4–0.8 ppm) and these were USDA Grade Number, Sample Grade (Shotwell et al., 1971). An FDA survey of 1972 corn crop was conducted in the spring of 1973 (Eppley et al., 1974). Samples were obtained from areas where known Fusaria damage had been reported and where the potential for damage was considered to be high. A 17% incidence of zearalenone occurred in the 223 samples assayed and the levels found were 0.1 to 5.0 ppm. An unusually wet season, which delayed both planting and harvesting of the 1972 crop, is suspected of causing the high incidence of zearalenone.

A survey for zearalenone in 1973 corn from different regions of the United States showed a 10% incidence in the Corn Belt and only a 1% incidence in corn from other regions (Stoloff et al., 1976). Of 169 samples of marketable corn (grades not given) from the Corn Belt, zearalenone was found in 17 samples at concentrations ranging from 0.04 to 0.2 ppm.

No truly rapid screening test (such as the black light or minicolumn used for aflatoxin) has been developed for zearalenone. A screening method that can be used to eliminate corn lots possibly contaminated with zearalenone is examination of corn for *Fusarium* damage. Evidence has been presented which indicates that other metabolites, which are produced by *Fusarium* spp. and therefore exist with zearalenone, are involved in mycotoxicosis of animals (Mirocha et al., 1976). Lack of methodology to detect these

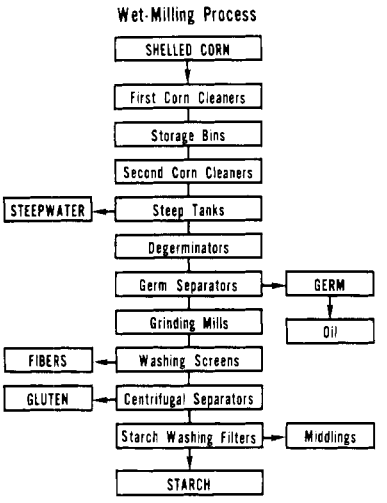

Figure 1. Flowsheet of corn wet-milling process.

metabolites limits the description of these toxins.

Wet-Milling Process. The recovery of starch and by-products by wet milling corn is a straightforward process starting with soaking the grain to prepare it for subsequent separation of the various kernel components. Separation is done by grinding, screening, and centrifuging operations. A flowsheet of the corn wet-milling process is shown in Figure 1. Corn is soaked or steeped in warm dilute solutions of sulfurous acid for a period of 36 to 48 h. The sulfurous acid is added partway in the steeping cycle, after the corn has been exposed to a mild lactic acid fermentation. After steeping, the water is drained from the corn, evaporated, and finally sold as-is for fermentation media or mixed with other corn fractions which make up the corn gluten feed. The corn is coarsely ground to free the germ, which is recovered by gravity separation in hydroclones. The macerated corn is ground once again to reduce the endosperm (starch and gluten) and loosen and free the fiber. Fibers are then separated from the starch and gluten by screening and become another ingredient of the feed fraction. The remaining fractions, starch and gluten, are separated by centrifugation. All fractions are dried. The starch has a multitude of food and industrial applications, whereas the gluten is a feed ingredient (Anderson, 1970).

The studies reported in this paper were conducted on a laboratory scale, essentially following the described commercial process and flowsheet. A complete description of the laboratory process has been published (Watson et al., 1951).

Analytical Methodology. The most commonly used methods of detection for both aflatoxin and zearalenone are dependent upon the fluorescence of these mycotoxins. Specific methods of analyses have been developed for different agricultural commodities and most follow a general sequence of events. These are sampling, sample preparation, extraction, removal of interfering substances, thin-layer or mini-column chromatography, and measurement of fluorescence. All these steps are included in specific procedures for aflatoxin or zearalenone measurements in wet-milled corn products.

Specific procedures had to be developed because accepted methods for determination of aflatoxin in corn are inadequate for wet-milled corn products. A procedure has been published (Barabolak et al., 1974) which combines the best features of three minicolumn procedures evaluated by a collaborative study (Shotwell and Stubblefield, 1973). Aflatoxins are extracted from corn and corn-derived products by high-speed blending in aqueous acetone. The filtered extract is purified with ammonium sulfate, and the aflatoxin is reextracted into benzene. The benzene extract is dried and the residue is dissolved in chloroform–acetone and then subjected to descending minicolumn chromatography. The fluorescence of the aflatoxin band at the top of the Florisil layer in the minicolumn permits detection of <5 μg of aflatoxins/kg of corn or <10 μg/kg of corn gluten, gluten feed, and steepwater. Analysis time is reported to be 35–40 min. This procedure was developed for rapid screening of corn and wet-milled products; however, the procedure is not usable for the quantitative determination of the different aflatoxins. The extraction, purification, and partitioning steps have been incorporated into a TLC method for quantitation (Barabolak, 1977). Interfering extraneous materials are removed from the benzene extract by column chromatography on silica gel and alumina. Extraneous material is washed from the column with benzene–acetic acid and then ether–hexane. The aflatoxins are eluted with methylene chloride–acetone, dried, dissolved in benzene–acetonitrile, and separated by TLC. This procedure showed good recoveries of aflatoxins B_1 and B_2 (88 to 100%) from corn, corn gluten feed, corn gluten meal, and steepwater which had been spiked with known quantities of aflatoxins.

Whenever aflatoxin is detected by TLC, additional confirmatory evidence should be obtained. A simple, rapid method for the confirmation of aflatoxin B_1 has been developed by Przybylski (1975), in which the B_1 is converted to a derivative (aflatoxin B_{2a}) directly on the TLC plate. The method is based on addition of water across the double bond of the terminal furan ring of aflatoxin B_1 by the catalytic action of trifluoroacetic acid. Results of a collaborative study using this test showed that aflatoxin was confirmed in 13 of 16 samples spiked at 5 μg/kg, and aflatoxin was confirmed in all samples spiked at 15 μg/kg (Stack and Pohland, 1975).

Analytical procedures for the detection and quantitation of zearalenone in corn and wet-milled products have been described (Bennett et al., 1978) and are adaptations of the Eppley procedure which was developed to screen agricultural commodities for zearalenone, aflatoxin, and ochratoxin (Eppley, 1968). Fifty-gram samples of ground, blended corn or wet-milled product are extracted with chloroform–water and filtered, and a portion (one-fifth of added chloroform) of the filtrate is taken for column chromatography on silicic acid column. The column is washed with hexane and then benzene to remove lipid materials. Zearalenone is eluted with benzene–acetone and then isolated on thin-layer chromatographic plates. Quantitation is accomplished by visually comparing the fluorescence of zearalenone in the sample to that produced by standard toxin.

In the analysis of fiber fractions and milling solubles fractions for zearalenone, the following modifications were made. The fiber fractions were extracted with 350 mL of chloroform instead of 250 mL due to excessive adsorption of the solvent by the fibers. Milling solubles were extracted by combining a volume of the solubles fraction which contained 50 g of corn with 250 mL of chloroform and 100 mL of aqueous sodium chloride (20%). This mixture was blended for 3 min in a high-speed blender and then centrifuged for 30 min at 3000 rpm. The chloroform layer (50 mL) was then taken for column chromatography and subsequent isolation of zearalenone.

The detection threshold for zearalenone by TLC is very high (50–100 μg/kg) when compared to aflatoxin because of the relatively weak fluorescence of zearalenone. The

Table III. Analysis of Corn Samples

	undamaged corn	inoculated corn	naturally contaminated corn
moisture, %	15	12.3	10.9
protein (N × 6.25), % MFB[a]	9.2	10.1	10.0
starch, % MFB	71.6	73.3	73.2
oil, % MFB	4.7	4.8	4.3
free fatty acids, % of oil	1.0	9.4	9.4
viability, %	95	24	32
aflatoxin B, ppb	0	638	120

[a] MFB = moisture-free basis.

relatively low toxicity, noncarcinogenic nature of this toxin, which acts primarily as an estrogen and whose visible effects are reversible, has resulted in little awareness for the potential economic harm this toxin might cause.

A collaborative study has been carried out to evaluate the Eppley method in determining zearalenone in white and yellow corn (Shotwell et al., 1976). Average recoveries from spiked samples were 129% at 0.3 ppm and 88% at 2 ppm. Large variations in analytical results of zearalenone determinations are probably due to interfering fluorescing materials which are difficult to remove completely from the assay sample. It is essential to confirm the identity of zearalenone, and the most unambiguous procedure is by gas chromatography–mass spectroscopy of the trimethylsilyl derivative of zearalenone.

Wet-Milling of Aflatoxin-Contaminated Corn. Yahl and co-workers (1971) have conducted laboratory wet-milling studies to determine the probable fate of aflatoxin should contaminated corn happen to be processed; they have also surveyed samples of finished products from several corn processing plants.

In their studies, three lots of corn (a control and two contaminated lots) were used. One corn was naturally contaminated, and the second was artificially infected with *Aspergillus parasiticus* NRRL 2999 under laboratory conditions. The naturally contaminated corn contained 120 ppb of aflatoxin, while the inoculated sample contained 638 ppb of aflatoxin. Analysis of the corn samples tested are given in Table III. Chemical analyses of the three samples showed no significant differences except for free fatty acid content. The two contaminated samples were considerably higher in free fatty acids than the control. Viability of the two contaminated corns was one-third to one-quarter of the control, probably due to degradation by the infection mold.

Duplicate wet-milling tests were carried out in each of the three corn samples. Yield and aflatoxin distribution are found in Table IV. Total dry substance recoveries were very good, in excess of 98%. Dry substance yields of milled fractions from the moldy corns were close to normal, but in several instances yields were undoubtedly affected by mold growth. Steepwater solids were higher and germ yields were less from the moldy corns. In the case of the germ, lower yields can be attributed to a more brittle germ being present in the moldy corn than in sound corn. Consequently, pieces of germ are recovered with the fibers and the gluten. This is indicated by higher gluten yield from the laboratory inoculated corn and is made more obvious by the higher fiber and gluten yields from the naturally contaminated corn sample. The higher gluten and middling yield from the moldy corns can also be attributed to poor starch–gluten separation resulting from mold damage to the starch and gluten particles. Starch yields are correspondingly reduced. Protein contents of the starches recovered from the moldy corns were in the normal range.

Each of the duplicate wet-milling fractions was assayed in duplicate for aflatoxin. In Table IV, aflatoxin is expressed as the weighted percentage of the sum of aflatoxins in the fractions. In both contaminated corn samples, the steepwater-solubles fractions contained the highest concentration of aflatoxin, about 40% of the recovered aflatoxin. The fiber fraction accounted for about one-third (30 and 38%) of the total aflatoxin and the germ for 6 and 10%. If no aflatoxin is lost when the germ is extracted for oil recovery, then the gluten feed, a blend of steepwater, fiber, and spent germ meal, would account for 81.5 and 83.5% of the aflatoxin from these two corn samples. The gluten fraction accounted for 17 and 13% of the total aflatoxin for the sample. Gluten, while not a constituent of corn gluten feed, is used as a poultry feed ingredient; thus, the two feed products accounted for over 97% of the aflatoxin contained in the two original corn samples.

The largest fraction, the most important product for food use, is the starch. Even though the corn lots were heavily contaminated, the starches had very low aflatoxin contents, 9 ppb from the artificially contaminated and 2.2 ppb from the naturally contaminated corn, which accounts for about 1% of the total aflatoxin. The middling fraction, being mostly starch, was low in aflatoxin.

These studies have shown that essentially all of the alfatoxin originally present in the corn will ultimately end in the feed fractions. This certainly is a fortuitous circumstance, because there are means available for decontaminating aflatoxin in corn by an ammonia process. In this process, corn at 17.5% moisture is treated with 1.5% ammonia for 14 days, reducing the aflatoxin B_1 content in corn from 750 ppb to less than 5 ppb. Feeding tests are currently underway to evaluate the effectiveness of the process on corn (Brekke et al., 1976). It is presumed that such a process could be used to decontaminate feed by-products from the milling industry.

Corn germ is always processed by the millers to extract the oil, a valuable by-product. It appears that some aflatoxin will be carried over into the oil when contaminated germ is expelled or solvent extracted for oil recovery. Studies have indicated that somewhat less aflatoxin carries over into expelled crude oil than into solvent-extracted crude oil. However, the process of alkali refining and bleaching of the crude oil renders the refined oil completely free of aflatoxin (Parker and Melnick, 1966).

As we have pointed out, the starch had an extremely low aflatoxin content and accounted for very little of the total aflatoxin in the corn. The very high levels of aflatoxin contamination of the initial corn were chosen deliberately to simplify the tracing of aflatoxin through the milling process. At lower levels of contamination (less than 30 ppb) it would not be possible to pick up the approximately 1% of original aflatoxin which was left in the starch.

Wet-Milling of Zearalenone-Contaminated Corn. Laboratory-scale studies have been conducted on naturally contaminated corn to determine the fate of zearalenone during the wet-milling process (Bennett et al., 1978). Three lots of corn containing 0.90, 4.10, and 9.40 ppm (dry basis) zearalenone were wet milled. These lots of corn were USDA Grade No. 2, 4, and Sample Grade, respectively, and *Gibberella zeae* was detected in 0, 12, and 16% of the kernels, respectively (Bennett et al., 1976). During the wet milling process, zearalenone concentrated in the product fractions in the order of gluten > milling solubles > fiber > germ. The starch fraction was free of measurable quantities of zearalenone even from a highly contaminated sample. Excellent recoveries of dry substance were ob-

Table IV. Yield and Aflatoxin Distribution among Wet-Milled Corn Fractions

fraction		yield, % of grain	aflatoxin B_1, ppb	% of sum
sound corn				
original corn		100		
steepwater and solubles		6.0		
germ		6.2		
fiber		9.2		
gluten		7.2		
middlings[a]		2.5		
starch		66.0		
	total	97.1		
inoculated corn (333)				
original corn		100	638	
steepwater and solubles		8.15	2450	41.5
germ		5.95	798	10
fiber		8.9	1600	30
gluten		10.65	768	17
middlings		4.8	71	0.5
starch		61.0	9	1
	total	99.45		100.0
naturally contaminated corn (41041)				
original corn		100	120	
steepwater and solubles		7.2	610	39.5
germ		5.2	140	6
fiber		12.5	340	38
gluten		10.7	140	13
middling		9.2	2.5	2.5
starch		53.7	2.2	1
	total	98.5		100.0

[a] Middlings are the washings from the starch table used in separating the starch from the gluten.

Table V. Zearalenone Distribution among Wet-Milled Corn Fractions[a]

	zearalenone								
	lot A			lot B			lot C		
fraction	% of corn[b]	ppb[c]	% of sum	% of corn	ppb	% of sum	% of corn	ppb	% of sum
corn as milled		900			4100			9400	
germ	6.9	1700	9.1	6.0	3600	10.2	6.2	7500	10.9
fiber	9.0	2700	19.0	8.6	3600	14.7	9.7	6800	15.4
gluten	9.7	6800	51.5	7.7	13400	48.8	11.8	20400	56.3
starch	67.8	ND[d]	0	71.2	ND	0	65.2	Tr[e]	0
solubles	6.7	3900	20.4	6.1	9100	26.3	7.0	10600	17.4
total	100.1		100.0	99,6		100.0	99.9		100.0

[a] Fractions from wet milling four to five 400-g portions from each lot. [b] Average of four determinations. [c] Assayed in duplicate; values reported on a dry basis. [d] ND = not detected. [e] Tr = trace, less than 100 ppb.

tained from all three contaminated lots of corn, >99%.

The distribution of zearalenone in mill products is shown in Table V. The germ fraction (from which edible oil is obtained) contained one to two times the level of zearalenone in the original corn. Fiber fractions had one to three times that in the original corn. Milling solubles contained one to four times the levels in starting corn, and gluten fractions were most highly contaminated with two to seven times the concentration found in the original corn. Milling solubles and gluten account for 14–19% of the corn; however, 72–75% of the zearalenone contamination was found in these two fractions. To date, no survey has been conducted to determine the incidence and level (if any) of zearalenone contamination in wet-milled corn products. In 1976, a survey on 1975 corn from 82 corn dry-milling establishments in 20 states failed to find zearalenone in any product. However, it was reported that there was little awareness of the potential for contamination by this mycotoxin (Stoloff and Dalrymple, 1977).

CONCLUSION

Potential problems cannot be minimized should widespread mycotoxin contamination occur on corn. So far, effective screening procedures and minimum occurrences of contamination have kept the wet-milling industry free from problems. Fortunately, in the case of aflatoxin and zearalenone, the contaminant concentrates in feed rather than in food products. Aflatoxin content of starch is extremely low, and oil is rendered free of aflatoxin during the refining process. With respect to feed contamination with aflatoxin, it now appears possible to decontaminate it by using the NRRC ammonia process.

In the case of wet-milled products from corn contaminated with zearalenone, again the starch is essentially free of the mycotoxin. Survival of zearalenone in oil refined from contaminated oil is being investigated. Evidence has been presented which shows that during the wet-milling process zearalenone concentrates in those fractions generally used for animal feed. This occurrence could create economic problems for the owners of animals, especially of breeding stock, which consume contaminated feed. At present, there is no proven process for the removal or destruction of zearalenone in corn. Today, proper management of mycotoxin contaminated corn involves a rigid surveillance program, using present analytical technology,

which permits the identity of the contamination and possible diversion of that corn to other uses.

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