Zearalenone in Cereal Grains

G.A. BENNETT and **O.L. SHOTWELL**, Northern Regional Research Center, Federal Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604

ABSTRACT

Zearalenone, a secondary metabolite with estrogenic properties, is produced by several *Fusarium* species that colonize cereal grains in the field and in storage. Recently, there have been reports of zearalenone contamination in corn, oats, barley, wheat, and grain sorghum. Corn and grain sorghum were examined for contamination due to obvious mold damage. Wheat, corn, and sorghum have been examined to determine the incidence of zearalenone in grains moving through commercial channels and stored on farms and at country elevators. Other grains such as oats and barley were analyzed because of associated estrogenic disturbances in farm animals. Steps in procedures for the determination of zearalenone are extraction of a representative sample, partial purification of the extract by column chromatography, alkali treatment, or liquid-liquid partitioning, and subsequent measurement of the isolated toxin. Zearalenone is measured in partially purified extracts by thin layer chromatography (TLC), gas liquid chromatography (GLC), and high pressure liquid chromatography (HPLC). Confirmation of zearalenone contamination can be accomplished by gas chromatography-mass spectroscopy (GC-MS). Multitoxin screening procedures have been deveoped for zearalenone in combination with one or more of the following mycotoxins: aflatoxin, T-2 toxin, diacetoxyscirpenol, patulin, ochratoxin, penicillic acid, citrinin, penitrem A, and sterigmatocystin.

INTRODUCTION

Although hyperestrogenic effects in swine were reported in the early 1900s (1), a cause-effect relationship between feeding "spoiled corn" and vulvovaginitis in gilts was first proposed in 1928. McNutt et al. (2) then hesitated to implicate directly the fungi in the corn to the estrogenic effects observed. In 1952 (3), *Fusarium graminearum (Gibberella zeae)*, which causes barley scab, was associated with hyperestrogenism in swine. The suggestion was made that this fungus might produce a toxic metabolite responsible for the adverse effect.

After a number of reports in 1957 and 1958 of swine herds exhibiting vulvar hypertrophy and other hyperestrogenic symptoms, an investigation (4) was initiated at Purdue University to establish a possible relationship between the microorganisms in the feed and the hyperestrogenic syndrome. An anabolic, uterotrophic compound (zearalenone) was isolated, characterized, and found to produce symptoms similar to those observed in the field. After unusually high incidence of estrogenic signs in swine in 1963 and 1964, Christensen et al. (5) isolated the same compound from *F. roseum* and named it "F-2." Zearalenone was shown to be an enantiomorph of 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone (Fig. 1) (6). Because of its structure, it has also been called RAL. Since then a number of derivatives have been described, and the chemistry of zearalenone has been summarized (7,8).

The natural occurrence of zearalenone in cereal grains has been established either as a result of investigations of field outbreaks of mycotoxicosis or from surveys of grains collected at different points in the marketing system. Some grains were tested because of obvious mold damage. Reviews by Eriksen (9), Hesseltine (10), Stoloff (11), Mirocha et al. (1), and Shotwell (12) include information on the occurrence of zearalenone in grains.

Corn has been the cereal grain most often implicated in cases of hyperestrogenism in farm animals, particularly swine. Zearalenone has been detected in such corn from Canada, England, France, Russia, United States, and Yugoslavia (Table I). One lot of freshly harvested corn was tested because of obvious *Gibberella zeae* damage (16). Zearalenone was also found in 1972 corn that had developed an area of *Aspergillus flavus* growth after storage in a bin in Illinois from January until June 1973 (19).

Zearalenone has been reported in corn harvested in Zambia, Lesotho, and Swaziland in Africa (20). Levels of 0.1-0.8 ppm were detected in 1974 corn used to produce opaque maize beer in Zambia (21). In this study, both home-brewed and commercial beers were examined and found to contain as high as 4.6 mg zearalenone/liter with a mean concentration of 0.92 mg/liter. Zearalenone was also detected in corn malt (0.8-4.0 ppm) used in the fermentations. In rural villages, levels of the mycotoxin in the opaque maize beer were correlated to levels in the corn fermented. In Swaziland, 55 samples of sour drinks, sour porridges, and local beers prepared by fermenting corn or sorghum meals were tested for zearalenone (20). Six of the 55 samples had zearalenone at concentrations of 0.8-5.3 ppm. Two moldy corn samples collected from the field were also found to contain the mycotoxin. Of 140 samples of local beers collected in Lesotho in 1974, 12% contained zearalenone (0.3-2.0 ppm) (20). Ingredients used in the fermentation are malted corn or sorghum, flour and hops, and sometimes fruits such as grapes and pineapples.

Other commodities that were tested because of adverse effects in farm animals and poultry and found to contain zearalenone are barley, grain sorghum, and sesame meal (Table II). Contaminated barley has been implicated in stillbirths, neonatal mortality, and small litters in swine and in decreases in egg production. Zearalenone (2.0-12.0 ppm) has been detected in grain sorghums associated with hyperestrogenism in swine. In 1973, two samples of grain sorghum with *Fusarium* head blight were found to contain zearalenone (25).

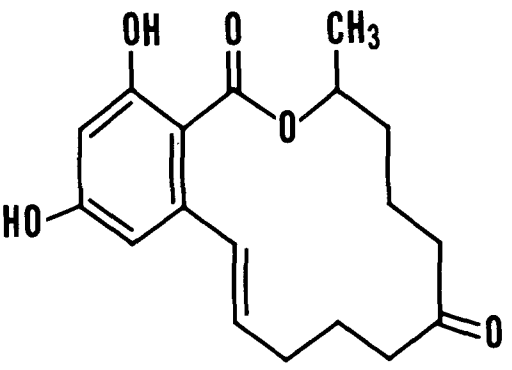

FIG. 1. Structure of zearalenone-6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone.

TABLE I

Natural Occurrence of Zearalenone in Corn

Examined because of:	Country	Levels (ppm)	Reference
Hyperestrogenism in farm animals	France	2.3	13
	Yugoslavia	18	10
Poisoning in swine	Yugoslavia	2.5-35.6	14
Severe mold damage and swine refusal	Yugoslavia	0.7-14.5	15
Gibberella zeae damage	United States	0.1-1.5	16
Porcine hyperestrogenism	Yugoslavia	35.6	17
Porcine hyperestrogenism	United States	2.7	17
Porcine absortion	United States	32.0	17
	England	306	17
Porcine feed refusal	United States	2.5	17
Hyperestrogenism in swine	United States	0.1-0.15	1
Hyperestrogenism in swine	Canada	0.2	1
Hyperestrogenism in swine	United States	0.12	1
Hyperestrogenism in swine[a]	United States	0.12	1
Hyperestrogenism in swine	United States	6.4	1
Vulvovaginitis, miscarriages, and infertility in pigs	Russia	Not stated	18

[a]Diethylstibesterol was also present in this sample.

TABLE II

Natural Occurrence of Zearalenone in Grains and Oilseeds

Commodity	Examined because of:	Country	Level (ppm)	Reference
Barley	Decreases in egg production	England	Not stated	22
Barley	Reduction in pig litters	Scotland	Not stated	22
Barley	Stillbirths, neonatal mortality, and small litters in swine	Scotland	0.5-0.75	23
Barley	Death in swine	England	Traces	24
Grain sorghum	Bovine abortion	United States	12.0	17
Grain sorghum	Hyperestrogenism in swine	United States	2.0-5.6	1
Sesame meal	Hyperestrogenism in swine	United States	1.5	1

TABLE III

Natural Occurrence of Zearalenone in Mixed Feeds

Feed	Examined because of:	Country	Levels (ppm)	Reference
	Infertility in cattle and swine	Finland	25.0	26
	Hyperestrogenism in cattle and swine	United States	0.1-2900	27
	Field problems in animals	United States	Not stated	28
Pig feed	Porcine hyperestrogenism	United States	50.0	17
Silage		United States	87.3	17
Dairy ration	Bovine feed refusal, lethargy, anemia	United States	1.0	17
Pig feed	Porcine internal hemorrhaging	United States	0.1	17
Pig feed	Porcine hyperestrogenism	United States	0.5	17
Porcine gestation ration	Porcine infertility, abortion	United States	0.01	17
Dry sow ration	Hyperestrogenism in swine	Canada	0.15	1
Farrowing ration[a]	Hyperestrogenism in swine	Canada	0.066	1
Dry sow ration[a]	Hyperestrogenism in swine	Canada	0.25	1
Lactation ration	Hyperestrogenism in swine	Canada	1.0	1
Gestation ration	Hyperestrogenism in swine	Canada	0.5	1
Commercial pelleted mixed feed	Hyperestrogenism in swine	United States	6.8	1
Feed components	Toxicosis in dairy cattle	Hungary	5-75	29

[a]Diethylstilbesterol was also present in these samples.

Zearalenone has been found in a number of mixed feeds implicated in hyperestrogenism in swine and cattle (Table III). Levels as high as 2000 ppm have been reported. Corn is probably the component in mixed feeds usually responsible for the contamination.

The problem of determining the compound or compounds responsible for mycotoxicosis in farm animals is complicated by the fact that several mycotoxins can be present in the same mixed feed or cereal grain (Table IV). A further problem is that mycotoxins not as yet discovered or described could be contributing to the adverse effects observed in farm animals. Zearalenone has been detected in corn that had aflatoxin (19,31,32) or ochratoxin A (15) present and in grain sorghum that had aflatoxin present. Much evidence exists that if one *Fusarium* toxin is present in a mixed feed or grain, others are likely to be (1,33).

Surveys have been made of corn, corn products, wheat, soybeans, and grain sorghum moving in commercial channels by the Northern Regional Research Center (NRRC) and the Food and Drug Administration (FDA). Results are summarized in Table V. Corn, in particular, has been examined at various points of the marketing chain–at farm and country elevators, food processing plants, terminal elevators, and at export markets. The incidence (17%) and

TABLE IV

Coexistence of Zearalenone with Other Mycotoxins

Commodity	Level zearalenone (ppb)	Mycotoxin	Level (ppb)	Reference
Corn	1200	Aflatoxin	37	31
Corn	600	Aflatoxin	6	32
Corn	14,500	Ochratoxin A	3100	15
Corn[a]	ND-92	Aflatoxin	ND-1700	19
Barley	Not stated	T-2 Toxin	Not stated	M.H. Formo unpublished info.
Corn	250	Deoxynivalenol[b]	1800	1
Corn	175	Deoxynivalenol	1000	1
Corn	1750	Deoxynivalenol	100	1
Commercial pelleted feed	3600	Deoxynivalenol	40-60	1
Mixed feed	700	T-2 Toxin	76	1
Mixed feed	500	Deoxyvinalenol	1000	1
Mixed feed	1000	Deoxynivalenol	1000	33
		T-2 Toxin	1300	
Mixed feed	15	Deoxynivalenol	1000	33
		T-2 Toxin	25	
Grain sorghum	1190	Aflatoxin	7	O.L. Shotwell unpublished info.

[a]Samples taken from various parts of bin and analyzed separately. Results are not representative of entire lot.
[b]Also known as vomitoxin.

levels (0.4-5.0) of zearalenone were understandably higher in the FDA survey of 1972 conducted in the spring of 1973 (34). Samples were collected in an area where known Fusaria damage had been reported or where the potential for Fusaria damage was considered to be higher. During the 1972 growing season, the Corn Belt had experienced unusually wet weather that delayed planting in the spring and harvesting in the fall. Some corn was not harvested until January. The FDA survey of 1973 corn collected from all over the United States (35) revealed that the Corn Belt, with a 10% incidence, experienced more problems with zearalenone contaminated than other areas (1% incidence). Levels encountered in the 1973 corn samples were low (<0.4 ppm). Zearalenone was not detected in prime products or byproducts collected from dry milling operations (36). In France, studies on zearalenone in corn are conducted by research workers and administrators from the public and private sectors. Since 1973, zearalenone has been found in 45% of the corn samples examined and accounts for the incidences of vulvovaginitis reported between 1973 and 1976 (38). Scott et al. reported the occurrence of zearalenone in a sample of corn flakes at a concentration of 14 μg/kg (39).

Wheat, soybean, and grain sorghum samples collected by the Agricultural Marketing Service (AMS) from the southeast and western part of the Corn Belt were analyzed by NRRC for zearalenone, aflatoxin, and ochratoxin A to determine the frequency of these mycotoxins (37; Shotwell, O.L., unpublished information). None of the three mycotoxins were found in soybeans (37). The wheat samples did not have detectable aflatoxin or ochratoxin A by the Eppley method (40), but 19 of 42 samples collected in Virginia had zearalenone levels ranging from 0.36-11.0 ppm (37). Half of these samples were selected because they were highly mold damaged. There was an unusually high incidence of scabby wheat (usually caused by *G. zeae*) in Virginia in 1975. The occurrence of zearalenone has been reported in three samples of wheat collected in Hungary at levels of 5-10 ppm (41). Zearalenone was detected in 57 of 197 grain sorghum samples collected in 10 states in the South, West, and Midwest (Shotwell, O.L., unpublished data).

A number of methods have been reported for screening agricultural commodities for mycotoxins, including zearalenone. Typically the multitoxin screening methods include the following steps: extraction, partial purification of the extract, and thin layer chromatography (TLC) (Table VI). Methods of purification have included liquid-liquid transfer, column chromatography, membrane dialysis, gel filtration, and precipitation with inorganic salts. Other mycotoxins screened for are aflatoxin, ochratoxin, sterigmatocystin, patulin, T-2 toxin, citrinin, penicillic acid, diacetoxyscirpenol, and penitrem A.

Three TLC schemes for differentiating and identifying a large number of mycotoxins have been reported. The first separated 11 mycotoxins and used Silica Gel G slurried with oxalic acid as the TLC adsorbant (51). The second has been used on extracts of foods and feeds and was designed to separated and identify 18 mycotoxins (52). A method has been published for the TLC of 37 mycotoxins and fungal metabolites, but no information was given as to the effectiveness when applied to extracts of cereal grains and feeds (53).

A number of sprays have been used to enhance the fluorescence of zearalenone on TLC plates or to form colored derivatives. An aluminum chloride spray was reported to enhance the fluorescence of zearalenone on TLC plates (34). Zearalenone forms a brown spot when TLC plates are sprayed with anisaldehyde in acidic methanol (52). Other reagents that have been used as sprays are potassium fericyanide-ferric chloride and then hydrochloric acid (17), 4-methoxy-benzene-diazonium fluoborate (54), and bis-diazotized benzidine (55). As little as 5 ng zearalenone can be detected on TLC plates with a spray of the diazonium salt reagent Fast Violet B (39).

The method (40) developed by Eppley for screening agricultural commodities for aflatoxin, zearalenone, and ochratoxin has been applied to surveys of 1162 corn samples collected at different points in the marketing system (31,32,34,35). This method has been validated for zearalenone in corn in a collaborative study with 22 participants from 10 countries (56). Average recoveries from spiked corn samples were 129% at 0.3 ppm, 101% at 1.0 ppm, and 88% at 2 ppm. The between-laboratories coefficients of variation were 53% at 0.3 ppm, 38% at 1.0 ppm, and 27% at 2.0 ppm. The mean level of zearalenone in the five naturally contaminated samples in the study ranged from 0.43 to 7.62 ppm. The mean coefficient of variation for all of the samples was 40.5%. The method was accepted in official first action by the Association of Official Analytical Chemists and the American Association of Cereal Chemists.

Collaborators used the following solvent systems with equal success: ethanol/chloroform (5:95 v/v), ethanol/

TABLE V

Surveys of Cereal Grains and Products for Zearalenone

Year	Cereal grain or product	Type of sample and source	Agency surveying	Number of samples assayed	Percent of samples with indicated level of zearalenone (ppm)					Reference
					ND[a]	0.4	0.4-0.9	1.0-5.0	>5.0	
1967	Corn	Grain Inspection, AMS	NRRC	283	99		1			31
1968-69	Corn	Export Grain Inspection, AMS	NRRC	293	98		2			32
1972	Corn	Elevator and food processing	FDA	223	83	9	4	4		34
1973	Corn	Farm and country elevators	FDA	315	94	6				35
	Corn	Mexican for human consumption	University of Minnesota	139[b]	96					27
1973-75	Corn products	Country dry milling operations	FDA	119[c]	100					36
1975	Wheat	Grain Inspection, AMS	NRRC	112[d]	83	1	2	12	2	37
1975	Soybeans	Grain Inspection, AMS	NRRC	180	100					37
1975-76	Grain sorghum	Grain Inspection, AMS	NRRC	197	71	2	8	18	1	Shotwell, unpubl.

[a]ND = not detected.
[b]Levels in 6 positive samples not reported.
[c]119 sets of two samples, including prime and byproducts from a mill run.
[d]Seventeen of 19 positive samples came from areas in which scabby wheat was a problem.

TABLE VI

Multitoxin Screening Methods for Zearalenone and Other Mycotoxins

Other mycotoxins	Extraction solvents	Purification of extracts	TLC solvent-zearalenone	Reference
Aflatoxin, ochratoxin	Chloroform/water (10:0 v/v)	Silica gel chromatography	Ethanol/chloroform (5:95 v/v)	40
Aflatoxins, ochratoxin, *sterigmatocystin, patulin*	Acetonitrile/4% potassium chloride (9:1 v/v)	Liquid-liquid transfer	Benzene/methanol/acetic acid (18:1:1 v/v/v) hexane acetone/acetic acid (18:2:1 v/v/v)	42
Aflatoxins	Methanol/water (6:1 v/v)	Liquid-liquid transfer and cupric carbonate precipitation	Acetone/chloroform (4:96 v/v)	43
Aflatoxins, ochratoxins, sterigmatocystin	Chloroform/water (10:1 v/v)	TLC development benzene/hexane (3:1 v/v)	Toluene/ethyl acetate/90% formic acid (6:3:1 v/v) benzene/acetic acid (9:1 v/v)	44
Aflatoxins, ochratoxin, sterigmatocystin, patulin, citrinin, T-2 toxin, penitrem A, diacetoxyscirpenol	Acetonitrile/1% potassium chloride (9:1 v/v)	Membrane cleanup	Toluene/ethyl acetate/90% formic acid (6:3:1 v/v/v)	45
Aflatoxins, ochratoxin, T-2 toxin, diacetoxyscirpenol	Acetonitrile/4% potassium chloride (9:1 v/v)	Ferric gel precipitation	Toluene/ethyl acetate/acetone (3:2:1 v/v/v)	46
Aflatoxins	Methanol	Liquid-liquid transfer	Benzene/chloroform/acetone (45:40:15 v/v/v)	47
Aflatoxins, ochratoxin, citrinin, penicillic acid	0.5 N phosphoric acid/chloroform (1:9 v/v)	Column chromatography	Glacial acetic acid/benzene (1:9 v/v)	48
Aflatoxins, ochratoxin, sterigmatocystin, patulin	0.1 M phosphoric acid/chloroform chloroform (1:10 v/v)	Gel filtration and TLC development benzene/hexane (3:1 v/v)	Benzene/ethyl acetate/formic acid (80:20:0.5 v/v/v)	49
Aflatoxins, ochratoxin A, sterigmatocystin, patulin	Acetonitrile/4% potassium chloride (9:1 v/v)	Liquid-liquid transfer	Benzene/methanol/acetic acid (18:1:1 v/v on Silufol plates)	50
Aflatoxins, ochratoxins, citrinin	Methanol/water (8:2 v/v)	Zinc sulfate-phosphotungstic acid precipitation	Chloroform/methanol (98:2 v/v) Ethyl acetate/chloroform/formic acid (60:40:1 v/v)	N. Vega unpublished data

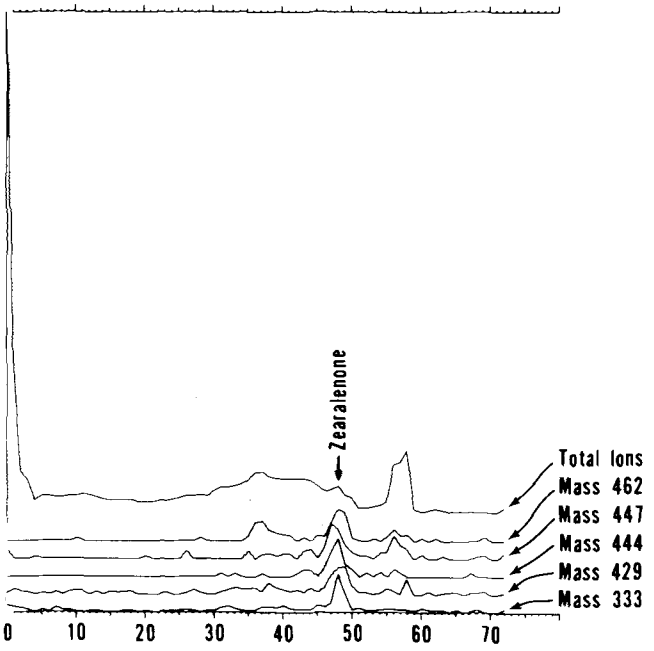

FIG. 2. Multi-ion scan of extract from naturally contaminated wheat showing the presence of zearalenone (TMS-derivative) by the simultaneous detection of ions:m/e$^+$ 462, 447, 444, 429, and 333.

chloroform (3.5:96.5 v/v), acetic acid/benzene (5:95 v/v), and acetic acid/benzene (10:90 v/v). The amounts of zearalenone on TLC plates were measured both visually and densitometrically. Jemmali (57) reported the evaluation of zearalenone on TLC plates by reflectance fluorodensitometry. A comparison (56) was made of measuring zearalenone in corn extracts both viasually and densitometrically on TLC plates. Excitation was at 313 nm and fluorescence at 443 nm. Results obtained densitometrically were more accurate than those obtained visually. The visual comparisons of the zearalenone from extracts with standards on TLC plates tended to give high results.

Detection and quantitation of zearalenone in cereal grains become difficult when the contamination level is low (50 ppb). This difficulty is due primarily to background interference from materials which extract with the zearalenone and mask the weak fluorescence of zearalenone on TLC plates. A versatile procedure to remove interfering compounds which extract with zearalenone was introduced by Mirocha et al. (17). This cleanup procedure involves partitioning the zearalenone from the organic extraction solvent into the aqueous alkaline phase, washing the aqueous phase with chloroform, and, after careful adjustment to pH 9.5, repartitioning the zearalenone back into chloroform. This procedure was used as a cleanup prior to quantitation by TLC, ultraviolet spectra, gas liquid chromatography (GLC), and combined GLC mass spectroscopy (GLC-MS). Extreme care must be exercised when partitioning the extraction solvent with aqueous sodium hydroxide to avoid emulsion formation. This cleanup procedure was used to isolate zearalenone from maize and barley extracts. Subsequent quantitation by GLC gave percent recovery (of added zearalenone) of 83.5 ± 7% and a detection limit of 50 ppb.

Analysis by GLC requires the formation of a derivative of zearalenone to increase this compound's volatility. The most common derivatives used in GLC analysis of zearalenone are the trimethylsilyl derivatives (TMS) (first reported in 1968 by Vandenheuvel) (58), dimethoxy derivative and the methyl oxime derivative (17). These compounds are readily detected with flame ionization detectors (FID), and 50 ng of standard zearalenone may be readily detected. More recently, Holder et al. (59) reported the detection of the pentafluoropropionyl derivative of zearalenone with an electron capture detector. These GLC procedures were developed to analyze contaminated samples where the zearalenone concentrations were 50-100 ppb or higher. Problems encountered in the GLC analysis of contaminated cereal grains are caused by grain components which extract with zearalenone, form similar derivatives, and thus interfere with both the identity of the proper GLC peak and accurate quantitation of the zearalenone peak.

A method for the unequivocal determination of zearalenone contamination is combined GLC-MS. The advantage of this method is the precise identification of small quantities of zearalenone in a complex matrix. The TMS derivative of zearalenone is resolved by gas chromatography and then subjected to analysis by mass spectroscopy. By using a technique known as multiple ion detection (MID), definitive identification of zearalenone is accomplished (17). The TMS derivative of zearalenone has characteristic ions that are selectively monitored. These characteristics and diagnostic ions are m/e$^+$ 462 (parent ion), 447, 444, 429, 333, 305, 260, and 151. Shotwell et al. (56) have reported the confirmation of zearalenone in wheat using a modification of this method. The characteristic ions monitored were m/e$^+$ 462, 447, 444, 429, and 333. These selected ions and their intensities were plotted under the total ionization pattern, and the simultaneous overlap of these five ions is used to confirm the presence of zearalenone (Fig. 2). Wheat extracts contain alkylresorcinols and fatty acids which confound the quantitation of zearalenone by GLC alone. The limit of detection in such a matrix was 0.2 μg zearalenone in 350 μg extract injected into the GLC column. GLC high resolution mass spectroscopy has also been used to detect low levels of zearalenone in corn flakes and corn-derived products intended for human consumption (39). Monitoring of total ion patterns did not permit detection of zearalenone; however, when only the parent ion (m/e$^+$ 462) was monitored, contamination was detected at 12.9 ppb zearalenone. The results agreed with values obtained by high pressure liquid chromatography (HPLC) analysis. One corn meal extract that contained a false positive for zearalenone by HPLC was clearly negative by the GLC-MS analysis.

Another technique known as field desorption (FD) mass spectroscopy has been investigated as a screening tool for mycotoxins (60). This technique involves ionizing a sample deposited on an activated field anode (emitter wire). An induced field causes quantum mechanical tunneling of an electron from the toxin molecule and produces positively charged ions. The ions are desorbed from the emitter, focused, and detected with a conventional mass spectrometer. Detection of zearalenone by this method depends on the detection of the parent ion (m/e$^+$ 318) in a complex matrix. Field desorption technique generally show higher molecular ion intensities and reduced fragmentation when compared to other ionization methods. This technique is not routine and work is continuing to improve its reliability. High cost and availability of sophisticated instruments such as GLC-MS greatly limit the use of these tools for routine detection of zearalenone in cereal grains.

The recent development of high efficiency packings and specialized detectors have made HPLC a desirable method of analysis for zearalenone contamination. Numerous reports on the use of HPLC for mycotoxin detection and quantitation have appeared since Sieber and Hsieh (61) adapted this technique to detect aflatoxins. Engstrom et al. (62) used HPLC to resolve up to seven different mycotoxin standards. Kovacs et al. (63) reported the potential for HPLC in zearalenone detection, and recently several reports have described the use of HPLC in conjunction with TLC, GLC, and GLC-MS to detect, quantitate, and confirm

TABLE VII

A Comparison of HPLC[a] Methods for the Determination of Zearalenone in Corn, Corn Products, and Feed

	Method			
Procedure	Malaiyanda and Barrette (Ref. 64)	Ware and Thorpe (Ref. 65)	Scott et al. (Ref. 39)	Holder et al. (Ref. 59)
Extraction	Chloroform/water/methanol (10:1:1 v/v/v)	Chloroform	Methanol	Methanol
Purification	Alkali treatment, silica gel column	Alkali treatment liquid-liquid partition into benzene	Hexane wash, alkali treatment, silica gel minicolumn	Liquid-liquid partition into benzene, Sephadex LH-20 column, silica gel column
Column packing	μBondapak C_{18}, reverse phase	Spherisorb ODS, reverse phase	Spherisorb silica	μBondapak C_{18}, reverse phase
Eluting solvent	Methanol	Methanol/water (58:42 v/v)	Cyclohexane/methylene chloride/methanol (300:90:10 v/v/v)	Methanol/water (65:35 v/v)
Detector	UV (280 nm)	Fluorescence	Fluorescence	UV (254 nm)
Detection limits	100 ppb	10 ppb	5-15 ppb	10 ppb
Recoveries (%)	62-81%	99-98%	84-104%	76-86%
Commodity assayed	Corn, corn oil, feed	Corn	Corn, cornmeal, corn flakes	Feed

[a]HPLC = High pressure liquid chromatography.

zearalenone contamination at low levels in corn and corn products. A summary of these methods is shown in Table VII.

The almost simultaneous appearance of these four publications reflects the utility of HPLC for zearalenone analyses. Malaiyanda and Barrette (64) used 1% aqueous sodium hydroxide to clean up the original extracts. After solvent exchange from chloroform into benzene, the extract was further purified on a silica gel G-60 column. After washing the column with benzene, zearalenone was eluted with benzene/ethyl acetate (90:10 v/v). After a solvent exchange into methanol, zearalenone was separated on μBondapak C_{18} column and detected by ultraviolet detector with a 280 nm filter. Quantitation was accomplished by constructing an analytical calibration curve with standard zearalenone. This paper reported that better recoveries of zearalenone were obtained when benzene instead of chloroform was used to transfer the sample extract to the silica gel column. Also, UV spectra and mass spectra of standard zearalenone and alkali-treated zearalenone showed no appreciable loss of toxin due to this clean-up procedure. However, low recoveries (67%) were obtained in analyses of pig feed; this was attributed to the problem of emulsion formation and partial hydrolysis of zearalenone during alkali treatment. Ware and Thorpe (65) reported recovery values of greater than 89% (for concentrations of 10-200 μg/kg) for zearalenone added to corn. This procedure involves extraction with chloroform, alkali treatment, liquid-liquid transfer to benzene, and direct analysis via HPLC using a fluorescence detector. Confirmation was accomplished by determining ratios of the following excitation wavelengths: 236:254; 236:274; 236:314. The peak height ratios of samples should agree within 5% of those obtained for standard zearalenone. This method was applied to 11 samples of corn meal from retail stores. Zearalenone was detected in nine samples of whole corn meal at levels from 12-60 ppb. Further confirmation was obtained in two of the positive samples by MS after isolation of zearalenone by HPLC.

Scott et al. (39) also used a fluorescence detector to quantitate zearalenone isolated by HPLC. Samples extracted with methanol are partially purified by alkali treatment and a silica gel minicolumn. Recoveries of 84-104% were obtained with corn meal, popcorn, and frozen corn spiked at 50 and 100 μg/kg. Detection limits were 5-15 μg/kg depending on the commodity. GLC high resolution MS was used to confirm TLC and HPLC results. Zearalenone was detected in corn flakes obtained from a retail store at levels of 14 μg/kg. Holder et al. (59) reported an HPLC method to detect zearalenone down to 10 ppb in animal chow. The clean-up procedure makes use of liquid-liquid partitioning, Sephadex LH-20 column chromatography, weak alkali treatment, and silica gel chromatography. Silica gel chromatography was required to separate zearalenone from zearalenol. A fluorescent detector was used, and recoveries of 76-86% were reported for feed spiked at 1.10, 1.0, and 10 ppm zearalenone and zearalenol. A confirmation procedure for zearalenone was described, which used GLC of the pentafluoropropionyl derivative and detection by electron capture detectors.

Although most of the corn produced is fed to livestock, over a half-billion bushels are processed to manufacture food and industrial products. The milling industries (dry milling and wet milling) screen incoming corn to eliminate mold-damaged corn; however, corn is purchased in large quantities from many lots of varying storage history. This permits the possibility of processing contaminated corn. To determine the extent of zearalenone contamination and the distribution of this contamination, dry milling and wet milling studies have been conducted on naturally contaminated corn. Dry milling studies were carried out using three lots of naturally contaminated corn (66). These corn lots were collected in the northern Corn Belt where the FDA reported a 17% incidence of zearalenone contamination at levels of 0.1-5.0 ppm. This high incidence and levels are not usual (35) but may occur whenever climatic conditions prevail which favor Fusaria outbreaks.

Results of the dry milling studies on corn lots containing 0.8, 3.5, and 8.1 ppm are shown in Table VIII. Prime product mix (Mix No. 1) contained only 10-22% zearalenone, although this mix accounted for 58-60% of total mill products. Highest levels of zearalenone were found in germ fractions (2.4-18.4 ppm), and these levels were 2 to 3 times the levels in the starting corn. Thoe most obvious hazard in dry milling zearalenon-contaminated corn occurs in the animal feed fractions produced. The germ and hominy feed fractions contained 60-70% of the zearalenone contamination. Wet milling studies (67) were conducted on samples of corn from the previously described lots (66) and the results are shown in Table IX. During this process, the toxin concentrated in the gluten fractions to a significant extent (49-56% of total zearalenone) even though gluten accounted for only 8-12% of milled corn fractions. Zearalenone concentrated in wet milled fractions in the order of

TABLE VIII

Product Yields, Zearalenone Levels, and Distribution among Milled Fractions from Contaminated Corn

Product	Yield, % n.p.[a]			Zearalenone, ppb			Zearalenone, % of total		
	28-2	14-2B	18-1	28-2	14-2B	18-1	28-2	14-2B	18-1
Grits	37	35	39	100	625	2100	5	8	13
Low-fat meal	18	19	19	150	775	2400	3	5	7
Low-fat flour	3	5	2	350	925	4600	2	2	2
Mix No. 1	58	59	60	125[b]	700[b]	2300[b]	10	15	22
High-fat meal	10	10	10	1200	6200	7900	16	22	13
High-fat flour	3	2	3	1200	3500	9200	4	3	4
Mix No. 2	13	12	13	1200[b]	5700[b]	8200[b]	20	25	17
Mix No. 1 + 2	71	71	73	300[b]	1600[b]	3400[b]	30	40	39
Hull	6	8	7	1200	5600	7900	10	16	9
Bran meal	4	3	4	600	4600	8500	3	5	5
Germ	17	14	15	2400	7000	18400	53	34	44
Degermer fines	2	3	1	1200	5600	14000	3	5	2
Composite calc'd				800[b]	2900[b]	6300[b]			
Corn, dry cleaned				800	3500	8100			

[a]n.p. = Net product (gross product less recycle fraction).
[b]Weighted average.

TABLE IX

Zearalenone Distribution among Wet Milled Corn Fractions[a]

Fraction	Control	28-2				14-2B				18-1			
			Zearalenone				Zearalenone				Zearalenone		
	% of Corn	% of Corn[b]	ppm[c]	Weight, μg	% of Sum	% of Corn	ppm	Weight, μg	% of Sum	% of Corn	ppm	Weight, μg	% of Sum
Corn as milled			0.9				4.1				9.4		
Germ	6.9	6.9	1.7	117	9.1	6.0	3.6	216	10.2	6.2	7.5	465	10.9
Fiber	7.1	9.0	2.7	243	19.0	8.6	3.6	310	14.7	9.7	6.8	660	15.4
Gluten	11.4	9.7	6.8	660	51.5	7.7	13.4	1032	48.8	11.8	20.4	2407	56.3
Starch	68.6	67.8	ND[d]	0	0	71.2	ND	0	0	65.2	Tr[e]	0	0
Solubles	6.8	6.7	3.9	261	20.4	6.1	9.1	555	26.3	7.0	10.6	742	17.4
Total	100.8	100.1		1281	100.0	99.6		2113	100.0	99.9		4274	100.0
				% Recovery 142%				% Recovery 52%				% Recovery 45%	

[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; ppm = parts per million.
[d]ND = not detected.
[e]Tr = trace, less than 0.1 ppm.

gluten>milling solubles>fiber>germ. Starch, which accounts for 65-71% of the milled products, contained no detectable quantities of zearalenone. However, feed fractions obtained from wet milling contaminated corn contained much higher levels of zearalenone than did the original corn. As in the case of feed fractions from dry milling contaminated corn, the selective distribution of zearalenone into feed fractions increases the potential for animal disturbances should contaminated corn be processed.

Proper management of zearalenone-contaminated grains should involve a rigid surveillance program to identify the contaminated lots of grain and to divert that grain to industrial purposes or to feed for feedlot animals only. One important factor that limits an effective surveillance or screening program is the lack of a truly rapid assay method for zearalenone for use on farms or at elevators.

Since prevention of zearalenone contamination is presently not feasible by current knowledge, methods to decontaminate infected grains must be developed. Limited information is availabel on detoxification of zearalenone-contaminated grains. Tamas and Wöller (68) reported that treating corn with 3-6% aqueous hydrogen peroxide (10 liters per 100 kg of corn) or ammonium hydroxide destroyed zearalenone contamination. However, this patented process did not report the levels of zearalenone in the contaminated corn. Experiments at NRRC have shown that zearalenone is very stable to heat and also is unaffected by the ammonia process developed to detoxify aflatoxin-contaminated corn (Bennett, unpublished data).

Previously described analytical procedures (HPLC and GLC-MS) have shown that zearalenone can be found in grain products processed for human consumption. The effects of zearalenone on humans is not known, but data from studies on nonhuman primates indicate that hormonal effects occur (69). Zearalenone, administered subcutaneously at 14 μg/kg body weight, depressed levels of serum luteinizing hormone in Rhesus monkeys. Also, Ruddick et al. (70) reported that pregnant rats receiving 1 mg/kg zearalenone produced litters which exhibited an increased incidence of fetal skeleton defects. The well known estrogenic response in mice has been extensively studied by Ueno and Yagasaki (71). They have shown that zearalenone causes an accelerating effect on RNA and protein synthesis in uterine tissue. Other studies, using a monolayer of cells from different tissues, have shown that zearalenone had cytotoxic effects on certain cell lines (72). Swine testicle cell cultures appeared to be most sensitive, followed by turkey and calf testicle cells. Other types of cell cultures showed no sensitivity to the toxin, and the method is proposed as a convenient model system to determine the sensitivity of various species of animals and tissues. Although only circumstantial evidence is available that suggests zearalenone may be harmful if consumed by

humans, adequate measures must be developed to prevent consumption of contaminated grain and processed cereal grain products.

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