Screening for Zearalenone in Corn by Competitive Direct Enzyme-Linked Immunosorbent Assay

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A simple protocol was devised for the routine immunochemical assay of zearalenone in corn. Essential features of the procedure were (1) blending corn in methanol–phosphate-buffered saline–dimethylformamide with a volume ratio of 70:29:1, (2) filtering of the blended sample, and (3) analysis of filtrate by competitive direct enzyme-linked immunosorbent assay (ELISA). Average ELISA recoveries of zearalenone in corn spiked at levels of 57, 151, and 307 μg/kg were 50, 119, and 123%, respectively. Mean interwell and interassay coefficients of variation for the spiked samples were 21.6 and 5.9%, respectively. The ELISA cross-reacted with α-zearalenol and to a lesser extent with β-zearalenol, α-zearalanol, and β-zearalanol. Levels of zearalenone detected in three naturally contaminated samples were comparable to those determined by liquid chromatography. The results suggested that the procedure was suitable for rapid semiquantitative screening of zearalenone in corn at levels considered to be at the threshold required for swine hyperestrogenism.

INTRODUCTION

Zearalenone is a β-resorcylic lactone that is naturally produced by *Fusarium* in corn, wheat, and other cereal grains (Mirocha and Christensen, 1974; Mirocha et al., 1977; Shotwell et al., 1977). The mycotoxin elicits estrogenic effects in mammalian reproductive systems and has specifically been associated with field cases of swine hyperestrogenism (Mirocha et al., 1977). Zearalenone is metabolized to α- and β-zearalenol, and these metabolites can be excreted in milk (Mirocha et al., 1981). Conventional analytical methods for zearalenone include thin-layer chromatography (TLC) (AACC, 1983; AOAC, 1984; Mirocha et al., 1974; Scott et al., 1978; Gimeno, 1983; Swanson et al., 1984), liquid chromatography (Holder et al., 1977; Scott et al., 1978; Ware and Thorpe, 1978; Cohen and LaPointe, 1980; Trenholm et al., 1981; James et al., 1982; Turner et al., 1983), and gas–liquid chromatography (Holder et al., 1977; Scott et al., 1978; Thouvenot and Morfin, 1979). The limit of detection for approved TLC detection (AACC, 1983; AOAC, 1984) is 200 μg/kg. Recently a liquid chromatography method for zearalenone and α-zearalenol with a detection limit of 10 μg/kg has been approved by the Association of Official Analytical Chemists (Bennett et al., 1985). Regardless of the zearalenone detection procedure used, time-consuming extraction and cleanup steps are required, making the methods inconvenient for the routine screening of large sample numbers. It has been demonstrated that analysis of mycotoxins by enzyme-linked immunosorbent assay (ELISA) allows greater sample throughput without a concurrent loss in sensitivity (Lawellin et al., 1977; Pestka et al., 1980, 1981a, 1981b, 1982; Chu, 1984; Gendloff et al., 1984; Liu et al., 1985). In this report, we describe the application of a competitive direct ELISA to the screening of zearalenone in corn after only a single extraction with methanol–phosphate-buffered saline–dimethylformamide.

MATERIALS AND METHODS

Apparatus. Enzyme immunoassay (EIA) reader used was Model EL 307 (Biotek Instrument Inc., Burlington, VT).

Materials. All inorganic chemicals and organic solvents were reagent grade or better. Bovine serum albumin (fraction V, fatty acid free) (BSA), chicken egg albumin (ovalbumin, grade III), 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), hydrogen peroxide, horseradish peroxidase, *N,N*-dimethylformamide, *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxymethoxylamine and dimethylformamide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Complete and incomplete Freund's adjuvant were purchased from Difco Laboratories (Detroit, MI). Rabbits (New Zealand white does) were purchased from Bailey Rabbitry (Alto, MI). Zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol standards were provided by International Minerals and Chemical Corp. (Terra Haute, IN).

Preparation of Protein Conjugates. Zearalenone was converted to 6'-(carboxymethyl)zearalenoneoxime (zearalenone oxime) as described by Thouvenot and Morfin (1983). Zearalenone oxime was conjugated to bovine serum albumin for use as immunogen and to horseradish peroxidase for use as enzyme marker by the *N*-hydroxysuccinimide procedure of Kitagawa et al. (1981). Conjugates were kept at −20 °C in 0.5-mg aliquots (0.5 mg/mL).

Antisera Preparation. Antiserum specific for zearalenone was produced against zearalenone oxime–bovine serum albumin conjugates in rabbits as described by Chu et al. (1979). Resulting serum was purified by ammonium sulfate precipitation (Hebert et al., 1973) and reconstituted to the original volume with 0.1 M sodium phosphate buffer (PBS, pH 7.5). Antisera was dialyzed against the same buffer for 48 h at 4 °C and titered by ELISA as described below.

Spiking and Extraction of Corn Samples for ELISA. Naturally contaminated, homogeneous ground corn samples containing 7, 114, 290, and 2910 μg/kg of zearalenone were provided by the Northern Regional Research Center, Peoria, IL. For spiking studies, zearalenone was dissolved in methanol (100 μg/mL) and was added to 10 g of ground corn (7 μg/kg of zearalenone) to give final concentrations of 7, 57, 157, and 307 μg/kg. The corn was shaken in a stoppered bottle and the methanol allowed to evaporate overnight in a fume hood. The entire sample was then blended with methanol–0.01 M PBS–dimethylformamide at a volume ratio of 70:29:1 for 5 min. Blended corn was filtered through Whatman No. 4 filter paper and the filtrate sued for ELISA analysis. Corn extracts prepared in this manner could be stored at 4 °C for as long as 3 days without effect on ELISA recoveries.

ELISA. For determination of ELISA titer, pooled antisera were serially diluted in 0.01 M PBS and 100-μL aliquots air-dried to wells of a microtiter plate (Immulon

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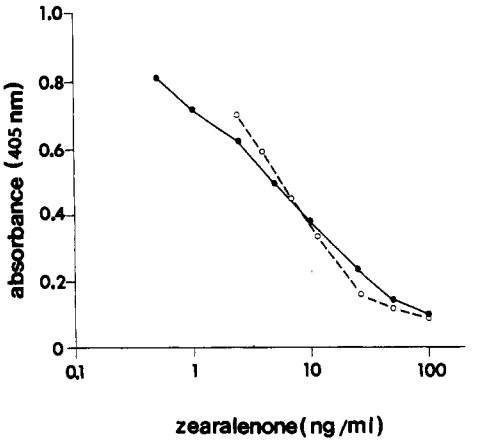

Figure 1. Competitive direct ELISA standard curve for zearalenone in extractant and corn extracts: (●) extractant standard curve; (○) corn extract standard curve. Corn initially had 7 μg/kg of zearalenone before extraction.

Removawells, Dynatech Laboratories, Alexandria VA) as described by Pestka etal. (1981c). Antisera-treated plates were washed three times by filling each well with 0.3 mL of 0.02% (v/v) Tween 20 in PBS (PBS–Tween) and aspirating the contents. Nonspecific binding was decreased by incubating each well for 30 min at 37 °C with 0.3 mL of 1% (w/v) ovalbumin in 0.01 M PBS (ovalbumin–PBS) followed by four washes of PBS–Tween. Zearalenone–peroxidase conjugate (diluted 1:300 in ovalbumin–PBS) was added in 50-μL aliquots to each well. Plates were incubated for 1 h at 37 °C and washed six times with PBS–Tween, and bound peroxidase was determined as described by Pestka et al. (1982). The serum dilution yielding visually distinct color from preimmune serum controls at the same dilution was arbitrarily designated as the titer end point (Pestka et al., 1980).

For competitive assays, sample extracts (or standard) and zeralenone–peroxidase conjugate (diluted 1:300 in ovalbumin–PBS) were mixed in a 1:1 ratio (100 μL + 100 μL), and a 100-μL aliquot was then added to antisera-coated (diluted 1:750) wells. Zearalenone standards were prepared on day of assay in methanol–PBS–dimethylformamide (70:29:1). Plates were incubated for 1 h at 37 °C, and bound enzyme was determined as described above.

RESULTS

Direct Competitive ELISA. Rabbits began to produce sera with detectable antizearalenone titers at approximately 10 weeks after the initial immunization. Antisera from three rabbits were pooled (titer 1500) for subsequent competitive ELISA. Response range for a typical direct competitive ELISA standard curve was between 0.5 and 50 ng/mL (Figure 1). Standard curves prepared in spiked methanol–PBS–dimethylformamide extracts of corn approximated those curves prepared in spiked extractant alone (Figure 1), suggesting that corn samples could be screened for zearalenone in ELISA by direct comparison to an extractant standard curve. The relative specificity of this ELISA toward zearalenone and its analogues is summarized in Table I.

ELISA of Spiked Corn. The ELISA results obtained on corn containing 7 μg/kg of zearalenone spiked with additional zearalenone are summarized in Table II. Competition by methanol–PBS–dimethylformamide extracts of the spiked corn was compared directly to standard competition curves in extractant for zearalenone quantitation. Recoveries for samples containing 57, 157, and 307 μg/kg were 50, 119, and 123% respectively. The mean recovery was 97%, and mean interwell coefficient of variation was 21.6%. The mean interassay coefficient of variation was 5.9%. Zearalenone was not detectable by ELISA in the samples containing 7 μg/kg of the mycotoxin.

Table I. Reactivity of Zearalenone Analogues in Competitive Direct ELISA

analogue	amt req for 50% inhibn,[a] ng/mL	cross-react. rel to zearalenone, %
zearalenone	5.8	100
α-zearalenol	2.1	280
β-zearalenol	16.5	35
α-zearalanol	26	22
β-zearalanol	60	10

[a] Represents mean of four replicate trials.

Table II. Recovery of Zearalenone from Spiked Corn by Competitive Direct ELISA

zearalenone added, μg/kg	init zearalenone content, μg/kg	sample[a]	recovery[b,c] μg/kg	recovery[b,c] %	interwell CV,[d,e] %
0	7	1	ND[f]		
0	7	2	ND		
0	7	3	ND		
50	7	1	26.8 ± 11	47	41
50	7	2	29.4 ± 5	52	17
50	7	3	28.5 ± 10	51	35
150	7	1	176 ± 36	117	20
150	7	2	174 ± 33	111	19
150	7	3	202 ± 44	128	22
300	7	1	337 ± 53	112	16
300	7	2	401 ± 50	131	13
300	7	3	387 ± 66	126	17

[a] Each sample was spiked separately and then extracted and assayed in replicates of eight. [b] Mean recoveries for sample containing 57, 157, and 307 μg/kg of zearalenone. [c] Interassay coefficient of variation (n = 3) for 50, 150, and 300 μg/kg were 3.5, 6.9, and 7.3%, respectively. Mean interassay coefficient of variation was 5.9%. [d] Coefficient of variation. [e] Mean interwell CV was 21.6% [f] None detected.

Table III. Comparison of Zearalenone Levels Detected in Three Samples of Naturally Contaminated Corn by Competitive Direct ELISA and by HPLC

zearalenone levels by HPLC,[a] μg/kg	zearalenone levels by ELISA,[b,c] μg/kg
114[d]	127 ± 12
290	357 ± 40
2910[e]	2813 ± 407

[a] Blended homogeneous samples were prepared, and zearalenone content was determined using HPLC as part of the collaborative study of Bennett et al. (1985). [b] ELISA performed in replicates of eight on a single 10-g sample. [c] Mean coefficient of variation was 11.6%. [d] Contained 7 μg/kg of α-zearalenol. [e] Contained 20 μg/kg of α-zearalenol.

ELISA of Naturally Contaminated Corn. Three samples of corn that were naturally contaminated with zearalenone were analyzed by ELISA (Table III). Again zearalenone content was determined by directly comparing competition caused by methanol–PBS–dimethylformamide extracts of the samples to standard competition curves in extractant. Samples previously determined to contain 114, 290, and 2910 μ/kg of zearalenone by liquid chromatography were estimated to contain 127, 357, and 2813 μg/kg by ELISA, respectively.

DISCUSSION

Our laboratory has previously described the use of competitive indirect ELISA for detecting zearalenone (Liu et al., 1985; Pestka et al., 1985). In the indirect ELISA, zearalenone antibody competes with free toxin for binding to a solid-phase zearalenone–poly-L-lysine conjugate. A

second anti species enzyme conjugate is then required to determine total bound antibody. In the competitive direct ELISA described here, zearalenone–peroxidase conjugate is simultaneously incubated with free toxin over solid-phase zearalenone antibody. Of the two approaches, the direct assay is preferable because it requires one less incubation step and one less washing step than the indirect ELISA. Sensitivity for zearalenone detection by the direct ELISA (1 ng/mL) was comparable to that previously described for indirect ELISA (Liu et al., 1985; Pestka et al., 1985) and radioimmunoassay (Thouvenot and Morfin, 1983).

Pooled antisera showed greater apparent specificity for α-zearalenol (2.8 times) than zearalenone in the competitive direct ELISA (Table I). Thouvenot and Morfin (1983) have also reported that zearalenone and α-zearalenol cross-reacted to the same extent in a radioimmunoassay using porcine antisera raised against zearalenone oxime protein conjugates. However, as found previously in other zearalenone immunoassays (Liu et al., 1985; Pestka et al., 1985; Thouvenot and Morfin, 1983), the competitive direct ELISA described here showed reduced ability to recognize either the β configuration at C6′ of zearalenol and zearalanol or the single bond at the C1′ to C2′ position found in α- and β-zearalanol. The ability of α-zearalenol to react to a greater extent than zearalenone in this competitive direct ELISA may have arisen because the antibody recognized a preferred steric configuration in the immunogen produced by the *N*-hydroxysuccinimide ester procedure. This conjugation procedure contrasts with the mixed-anhydride (Liu et al., 1985) and carbodiimide procedures (Thouvenot and Morfin, 1983) that have been used previously. Any future application of this immunoassay to the screening of zearalenone must therefore take into account antibody specificity. The ability to detect α-zearalenol is advantageous because this compound is more estrogenic than zearalenone and it has both been shown to occur both in culture (Hagler et al., 1979) and as a metabolite in biological samples (Mirocha et al., 1981). However, because it reacts more strongly than zearalenone in the present competitive direct ELISA, presence of α-zearalenol in a sample (Table III) could bias total zearalenone estimation. Development of appropriate monoclonal antibodies to zearalenone could possibly circumvent this problem.

Since zearalenone is a frequent low-level contaminant of midwestern corn, we were required to use a homogeneous ground corn sample already containing trace (7 μg/kg) amounts of zearalenone for our spiking study. Results of the spiking study (Table II) revealed that recoveries at the 57 μg/kg level were incomplete. This could be due to incomplete sample extraction or extract interference (Figure 1). In contrast, recoveries exceeded 100% at the 157 and 307 μg/kg level. Similarly, ELISA estimates for naturally contaminated ground corn were slightly higher by ELISA than estimates by liquid chromatography (Table III). In order to ensure consistency in future studies, we chose to prepare ELISA standard competition curves in methanol–PBS–dimethylformamide extractant rather than in "blank" corn extract that may contain variable trace levels of zearalenone. Thus, recoveries exceeding 100% may have indeed resulted from partial interference by the sample corn extract as might be predicted from Figure 1. Here, sample extracts containing greater than 10 ng of zearalenone/mL would be overestimated whereas samples containing less than 10 ng of zearalenol/mL would be underestimated when read off the extractant curve. Further investigation of potential extract effects and alternative solvent extractants might improve accuracy of the ELISA.

The mean interwell coefficients of variation for spiked samples (21.6%) reflect variability of antibody coating to the polystyrene solid phase and nonspecific binding of the peroxidase conjugate during the course of the assay. These values are consistent with those previously found for mycotoxins (Pestka et al., 1981a, 1981b) and hormone (Vogt, 1984) immunoassays. Interassay coefficients of variation (5.9%) reflect other sources of variation such as spiking, extraction, and filtration that may occur in the overall procedure.

In a recent evaluation of the immunoassay procedures, Vogt (1984) indicated that ligand binding assays were superior to other analytical procedures for determining the presence of single chemical components but that chromatographic methods offered greater advantages in the determinations of chemical profiles. The average cost of a mechanized enzyme immunoassay for a single analyte was approximately 5.9 and 2.5% of that required for doing the same analysis by gas chromatography or gas chromatography–mass spectrometry, respectively. The competitive direct ELISA described herein has distinct advantages in terms of extraction ease, assay time, and sample throughput. However, the disadvantages of specificity, slightly excessive recoveries, and high interwell coefficients of variation make this assay essentially semiquantitative. Nevertheless, this approach should be readily applicable to the rapid screening of zearalenone in cereals and animal feeds at levels greater than 150 μg/kg, an approximate threshold at which hyperestrogenism might be expected (Mirocha et al., 1977). Thus, highly contaminated commodities could be diverted from the food and feed chain and, if necessary, subjected to further procedures to confirm the presence of zearalenone.

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Residues of Aldicarb and Fenamiphos in Soil, Leaves, and Fruit from a Treated Vineyard

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Residues of aldicarb and fenamiphos [parent compound, sulfoxide, and sulfone metabolites analyzed as sulfone and expressed as total aldicarb (TA) or total fenamiphos (TF)] in the roots, soil, leaves, and fruit from vineyards were determined with a gas chromatograph equipped with packed columns. Temik (aldicarb) granules at 5 kg/ha and Nemacur (fenamiphos) granules at 20 kg/ha were applied either in scatter over the entire area (broadcast) or in 20-cm bands on either side of the rows of vines. No residues of TA or TF were present in any of the samples taken before application, showing that there were no residues from previous treatments. Twenty-eight days after application no TA residues were present in any soil samples at the depths sampled; TF residues were, however, still present in the 30–60-cm layer. In spite of residues of TA and TF in the soil, all fruit samples contained less than 0.01 mg/kg of TA and less than 0.02 mg/kg of TF at harvesting. Band application in contrast to broadcast application gave rise to a lower maximum leaf concentration of TA and a higher maximum leaf concentration of TF. The control samples were without any TA or TF residues.

No nematicide has as yet been registered specifically for use on wine grapes, although research on residues and persistence of aldicarb [2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime] and fenamiphos [ethyl 4-(methylthio)-3-methylphenyl isopropylphosphoramidate] has been done in vineyards in the U.S. (Raski, 1955; Raski and Schmitt, 1964; Hafez and Raski, 1981; Hafez et al., 1981). As there is a nematode problem in vineyards of the Vaalharts area in South Africa, several compounds are being tested there as soil systemic nematicides. This paper describes the total residues (parent compound, sulfoxide, and sulfone analyzed as sulfone) of aldicarb and fenamiphos found in soil, root, leaf, and fruit samples at various times after application. The objectives were to determine whether unacceptably high concentrations of total aldicarb or fenamiphos were present in harvested fruit after applications at dosages used on other crops and to compare the leaching and persistence of the two compounds in the soil.

In soil and plants, aldicarb and fenamiphos are converted into many metabolites of which the sulfoxide and the sulfone are highly toxic to man (Waggoner, 1972); oral LD_{50} (rats) of aldicarb, i.e. the sum of aldicarb and its sulfoxide and sulfone expressed as aldicarb, is 0.5–1 mg/kg and that of fenamiphos, i.e. the sum of fenamiphos and its sulfoxide and sulfone expressed as fenamiphos, is 15–19 mg/kg. A soil or plant sample contains many of the aldicarb or fenamiphos metabolites. The extraction process oxidizes any residues of the parent compound as well as the sulfoxide to sulfone. All the results of aldicarb or fenamiphos in this paper therefore signify the total residues of the parent compound, the sulfoxide metabolite, and the sulfone metabolite analyzed, determined together, and expressed as total aldicarb or total fenamiphos. Aldicarb is registered in South Africa for use on tobacco,

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