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Detection of Zearalenone By Tandem Immunoaffinity-Enzyme-Linked Immunosorbent Assay and Its Application to Milk

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ABSTRACT

A hybridoma-based method utilizing tandem affinity chromatography and enzyme-linked immunosorbent assay (ELISA) was devised to detect zearalenone. A zearalenone specific monoclonal antibody was attached to Sepharose for initial sample clean-up. Zearalenone was eluted with methanol and then quantified by competitive direct ELISA. Average ELISA recoveries from the column for water spiked with zearalenone at levels of 1, 5, 10, 25, and 50 ng/ml were 107, 86, 95, 95, and 92%, respectively, with a mean recovery of 95%. Mean interwell and interassay coefficients of variation were 9.7 and 8.9%, respectively. Average recovery by the method from milk spiked with zearalenone at levels of 1, 5, 10, 25, and 50 ng/ml was 187, 113, 107, 110, and 112%, respectively, with a mean recovery of 126%. Mean interwell and interassay coefficients of variation were 14.5 and 9.1%, respectively. Zearalenone was not detectable in 12 commercial milk samples assayed by the tandem method.

Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-ß-resorcylic acid lactone] is a secondary metabolite produced by members of the genus *Fusarium* after colonization of corn and small grains both in the field and during storage (*9,16,24*). ZEA elicits estrogenic effects in mammalian reproductive systems and has specifically been associated with field cases of swine hyperestrogenism (*12,16*). There has also been reported positive evidence for carcinogenicity of this mycotoxin in rodents (*18*). A recent risk assessment of ZEA estimated a safe intake of 0.05 µg/kg body weight/day for humans (*14*). Although no adverse health effects would be anticipated due to ZEA exposure from corn cereals, the toxin could be present in other food sources such as milk and thus could increase exposure estimates.

Zearalenone is metabolized to α- and ß-zearalenol, and these metabolites have been detected experimentally at trace levels in cow's milk after oral administration of high zearalenone doses in feed (*7,17*). The presence of both metabolites in milk is important since α-zearalenol is three times more estrogenic than ZEA while ß-zearalenol has similar activity to ZEA (*8,29*). Thus, it is important to estimate the incidence of ZEA and its metabolites in milk and milk products because of the worldwide consumption of this commodity.

Conventional methods for analysis of ZEA in foods and feeds include thin-layer chromatography (TLC) (*1,5,25*), gas chromatography (*11,23,26*), and liquid chromatography (*2,13,22,28*). Regardless of the detection limits described, these procedures involve time-consuming extractions and clean-up steps, making the methods inconvenient for the routine screening of large sample numbers. Our laboratory has reported the application of competitive enzyme-linked immunosorbent assays (ELISA's) to detection of ZEA in corn, wheat, grain-based food products, and animal rations using polyclonal rabbit antisera (*15,30,31*) and, more recently, monoclonal antibodies (*4*). A new approach to improve aflatoxin analysis includes the use of a monoclonal antibody affinity chromatography column as preparative step prior to determination by liquid chromatography (*6*).

Here, we report the development of a method for detection of ZEA involving tandem immunoaffinity chromatography and ELISA and its application to milk.

MATERIALS AND METHODS

Materials

All inorganic chemicals and organic solvents were reagent grade or better. Chicken egg albumin (ovalbumin, grade III), Tween 20, 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS), thimerosal, hydrogen peroxide, horseradish peroxidase, zearalenone, pristane, and Sepharose 4B-CNBr were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco Laboratories (Grand Island, NY). Mice were purchased from Charles River Laboratories (Wilmington, MA). Zearalenone-horseradish peroxidase (ZEA-HRP) was prepared as described previously (*31*). Hybridoma cell line 2G3-6E3-2E2 which secreted anti-zearalenone monoclonal antibody was produced as ascites (*4*) and purified by ammonium sulfate precipitation (*10*). Cross-reactivity of that antibody towards zearalenone metabolites and analogues determined by an indirect ELISA was 107, 29, 35, and 25% for α-zearalenol, ß-zearalenol, α-zearalanol, and ß-

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zearalanol, respectively (*4*). Protein concentration was determined by the method of Bradford (*3*) using bovine plasma gamma globulin as standard.

Immunoaffinity gel

Antibody was coupled to Sepharose 4B as described in manufacturer's instructions (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Briefly, 2 g of freeze dried CNBr activated Sepharose-4B (S-4B) were washed and swollen in 1 mM HCl under slight vacuum on a sintered glass filter. The gel was washed with 50 ml of coupling buffer (CB) (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl). After suspending in 10 ml of CB, the gel was mixed with 70 mg MAb in 5 ml of CB and the suspension was mixed end-over-end for 2 h at room temperature. After filtration, the remaining active groups on the S-4B were blocked by placing the gel during 2 h at room temperature in 30 ml of glycine buffer (0.2 M, pH 8.0). This was filtered and the excess of blocking reagent and adsorbed protein was discharged by washing the gel with four alternating cycles of high and low pH; CB, followed by 0.1 M sodium acetate buffer, pH 4.0. Finally the immunogel ($\approx$ 7 ml) was washed with 0.01 M phosphate buffered (pH 7.4) saline (PBS), transferred to 15 ml of PBS containing 0.01% thimerosal, and stored at 4°C until required.

Immunoaffinity chromatography

Immunoaffinity columns consisted of 1 ml tuberculin syringes containing 200 µl of Sepharose-MAb slurry and held in place between porous polyethylene frits. Columns were equilibrated with PBS. The affinity chromatography studies were carried out at room temperature.

The immunogel was initially assessed for activity by passing an aliquot of 1 ml of distilled water containing standard ZEA (0-50 ng). Samples were pushed through the column using pressure supplied by a small peristaltic pump with a flow rate of 0.5 ml/min. The column was washed then with 5 ml of 10% (v/v) methanol in PBS (when 5 and 10 ml milk samples were assayed, the volume was increased) after which air was pumped during 1 min. ZEA bound to the immunogel was eluted with 2 ml of methanol followed by passing air for 1 min; this fraction was evaporated and then resuspended in 1 ml of PBS. Quantitation of ZEA in the eluate was determined by ELISA. Columns were re-equilibrated with 15 ml of PBS for reuse.

ELISA

Wells of polystyrene microtiter plates (Immunolon Removawells, Dynatech Laboratories, Alexandria, VA) were coated by air-drying (40°C) with 125 µl of 1:750 dilution zearalenone monoclonal antibody (MAb) (10 mg/ml) in sodium carbonate-bicarbonate buffer, pH 9.6. MAb-treated plates were washed four times with a microtiter plate washer (Dynatech Miniwash, Dynatech Laboratories, Alexandria, VA) by filling each well with 300 µl 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 300 µl of 1% ovalbumin (w/v) in PBS (OA-PBS) followed by four washes of PBS-Tween. Next, a 50 µl aliquot from samples or 50 µl of ZEA standard (0 to 50 ng/ml in PBS) and 50 µl of ZEA-HRP conjugate (diluted 1:800 in OA-PBS) were added consecutively to MAb coated wells. Plates were incubated for 1 h at 37°C and washed eight times with PBS-Tween, and bound ZEA-HRP was determined (*19*) using an enzyme immunoassay reader (Microelisa Minireader II, Dynatech Laboratories, Alexandria, VA).

Milk samples

Twelve samples representing different lots of dehydrated skim milk were purchased from mid-Michigan retail grocery stores. Samples (9.6 g) were reconstituted in 100 ml of distilled water (according to the proportions shown by the producers) and then filtered through Whatman No. 1 filter paper. Spiked samples were prepared by dilution of zearalenone stock (1 mg/ml in methanol) with distilled water and milk filtrates to give final concentrations of 1, 5, 10, 25, and 50 ng/ml. Spiked (1 ml) and unspiked (1, 5 and 10 ml) samples were subjected to tandem immunoaffinity-ELISA as described above.

RESULTS

A competitive direct ELISA was carried out by simultaneously incubating zearalenone with an appropriate dilution of ZEA-HRP over a MAb solid phase. A typical competition standard curve is shown in Fig. 1. Response

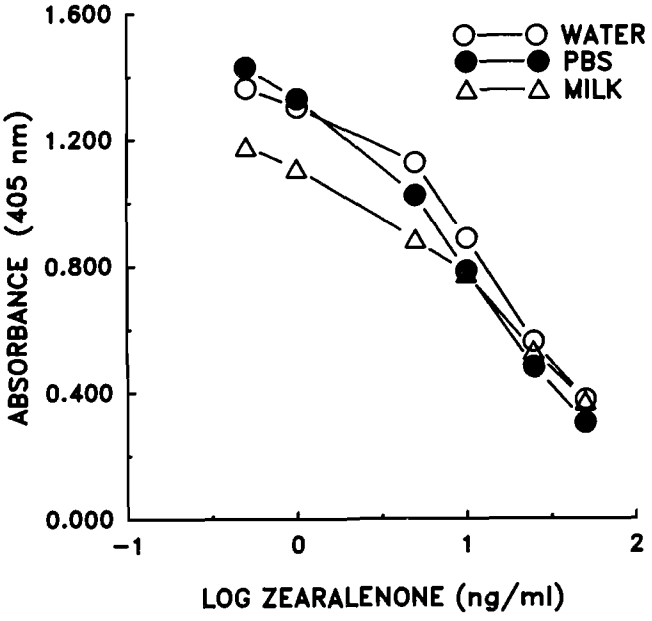

Figure 1. *Competitive ELISA standard curve of zearalenone in water, PBS, and milk. Each data point represents triplicate determinations in a single microtiter plate. Standard deviation values were always less than 3%. Milk used in standard curve was previously passed through an immunoaffinity column.*

range for this curve was between 0.5 and 50 ng/ml. The detection limit for ZEA (1 ng/ml or 50 pg/assay) was comparable to that previously described for competitive indirect (*4,15,20*) and direct (*31*) ELISA's and radioimmunoassay (*27*). Although the standard curve pattern of zearalenone in milk approximated those patterns obtained in distilled water and PBS, a decrease in absorbance values was observed at lower zearalenone levels in milk compared to the other diluents. This suggested that milk samples required a clean-up step prior to ELISA. An immunoaffinity clean-up procedure was therefore devised.

ELISA ZEA recoveries from immunoaffinity columns are summarized in Tables 1 and 2. Competition by chromatographic fractions was compared directly to standard

TABLE 1. *Recovery of zearalenone in distilled water from immunoaffinity columns by competitive direct ELISA.*

zearalenone added, ng/ml	sample[a]	recovery[b,c] ng/ml	recovery[b,c] %	interwell CV,[d,e]%
1	1	0.9 ± 0.4	92	46.3
1	2	1.1 ± 0.1	110	10.8
1	3	1.2 ± 0.1	120	10.1
5	1	4.4 ± 0.5	88	10.6
5	2	3.8 ± 0.2	77	6.1
5	3	4.6 ± 0.1	92	2.6
10	1	9.3 ± 1.5	93	16.5
10	2	7.6 ± 0.2	76	3.0
10	3	11.5 ± 0.9	115	8.1
25	1	23.3 ± 2.2	93	9.6
25	2	25.0 ± 0.7	100	2.8
25	3	23.2 ± 0.5	93	2.0
50	1	49.0 ± 5.7	98	11.6
50	2	45.1 ± 1.3	90	2.9
50	3	43.9 ± 1.1	88	2.5

[a]Each sample was spiked separately, passed through the immunoaffinity column, and then assayed in triplicate.
[b]Mean recoveries for samples containing 1, 5, 10, 25, and 50 ng/ml of zearalenone.
[c]Interassay coefficient of variation (n = 3) for 1, 5, 10, 25, and 50 ng/ml was 11.6, 8.0, 16.8, 3.5, and 4.7%, respectively. Mean interassay CV was 8.9%.
[d]Coefficient of variation.
[e]Mean interwell CV was 9.7%.

TABLE 2. *Recovery of zearalenone in spiked dehydrated skim milk from immunoaffinity columns by competitive direct ELISA.*

zearalenone added, ng/ml	sample[a]	recovery[b,c] ng/ml	recovery[b,c] %	interwell CV,[d,e]%
1	1	1.9 ± 1.2	190	60.5
1	2	2.0 ± 1.0	200	51.0
1	3	1.7 ± 0.2	170	14.1
5	1	6.0 ± 0.5	118	8.6
5	2	6.2 ± 0.7	123	11.6
5	3	4.9 ± 0.2	98	4.8
10	1	10.6 ± 1.1	106	10.6
10	2	9.3 ± 0.6	93	6.7
10	3	12.1 ± 1.9	121	15.9
25	1	27.7 ± 2.7	111	9.6
25	2	23.2 ± 2.5	93	10.6
25	3	31.8 ± 0.7	127	2.2
50	1	56.0 ± 2.9	112	5.1
50	2	59.2 ± 1.4	118	2.4
50	3	52.3 ± 2.0	105	3.9

[a]Each sample was spiked separately, passed through the immunoaffinity column, and then assayed in triplicate.
[b]Mean recoveries for samples containing 1, 5, 10, 25, and 50 ng/ml of zearalenone.
[c]Interassay coefficient of variation (n = 3) for 1, 5, 10, 25, and 50 ng/ml was 6.7, 10.0, 11.0, 12.7, and 5%, respectively. Mean interassay CV was 9.1%.
[d]Coefficient of variation.
[e]Mean interwell CV was 14.5%.

zearalenone competition curves in PBS. Recoveries for spiked water samples (Table 1) containing 1, 5, 10, 25, and 50 ng/ml were 107, 86, 95, 95, and 92%, respectively. The mean recovery was 95%. Recoveries for spiked milk (Table 2) at the same toxin concentrations were 187, 113, 107, 110, and 112%. The mean recovery was 126%.

No zearalenone was detected by ELISA in 12 marked samples of reconstituted dry milk. As a follow-up experiment, 5 and 10 ml milk samples were also applied to the immunoaffinity column. Eluates which were obtained reflected 5- and 10-fold concentration, respectively, thus enabling samples to be assayed with greater sensitivity (0.5-0.1 ng/ml). In spite of the concentration, ZEA was undetectable in these samples.

DISCUSSION

Extraction and clean-up procedures are sometimes required when zearalenone is analyzed in complex matrices such as foods and feeds. Current extraction procedures are time consuming and generally include use of organic solvents which are incompatible with immunoassay techniques and also present potential health risks to the analyst. However, rapid and reliable extraction procedures compatible with immunoassays involving organic solvents have been devised and are being currently used (*4,31*). Recently, monoclonal antibody-affinity chromatography columns have been utilized as preparative clean-up step for detection of aflatoxins by liquid chromatography (*6*). This procedure requires the availability of a high affinity antibody reactive with principal analogues and metabolites. Here, a high affinity zearalenone MAb that was cross reactive with ZEA analogues (*4*) was used in an immunoaffinity gel as a preparative step for ZEA detection. A simple scheme of the ZEA extraction using the MAb affinity columns is shown in Fig. 2.

ELISA recoveries in distilled water from immunoaffinity columns approached 100% (Table 1), whereas recoveries in spiked milk slightly exceeded 100% (Table 2). This might reflect presence at trace levels of ZEA or ZEA analogues in milk or from partial interference by the sample. Similarly, slightly higher ELISA ZEA recoveries have been reported in spiked corn (*4,31*).

The mean interwell coefficient of variation for spiked samples, 9.7 and 14.5% (6.5 and 7.6% excluding 1 ng data) in water and milk, respectively, reflects 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 similar to other previously found for ZEA (*4,31*) immunoassays, where zearalenone recoveries were carried out in spiked corn in the range of 50-500 ppb. The mean interassay coefficient of variation for spiked samples, 8.9 and 9.1% (8.2 and 9.6 excluding 1 ng data) in water and milk, respectively, reflects other sources of variation such as spiking, immunoaffinity extraction, and evaporation that may occur in the overall procedure.

Feeding studies carried out in cows administered with

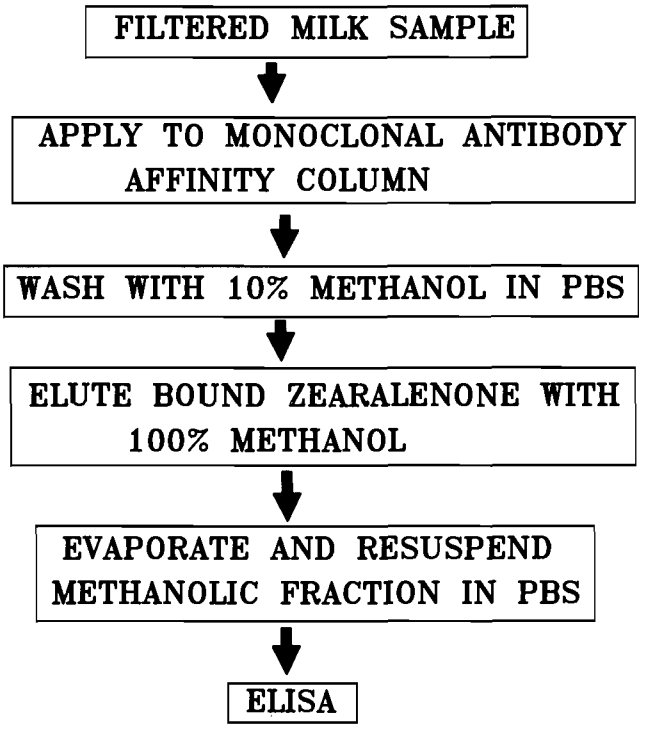

Figure 2. *Tandem immunoaffinity chromatography-ELISA procedure for zearalenone detection.*

200 mg/7 d, 1 g/1 d, and 1 g/2 d of ZEA estimated the presence of ZEA-α-zearalenol, and ß-zearalenol (either in free or conjugated form) in milk as 0.77, 0.12, and 0.17%, respectively, of the total ZEA consumed (*17*). A more recent feeding study in cows administered with 25 and 100 mg/toxin during 6 d has demonstrated that presence of ZEA and analogues in milk represented less than 0.1% of total ZEA consumed (*21*). Our inability to detect ZEA in the market samples is consistent with these reports, because, as predicted, unusually high levels of toxin in the feed would be required for detectable carryover in milk. Further large-scale surveys of the milk supply would be necessary to verify this result.

In summary, the ZEA immunoaffinity extraction method reported here was easy to perform, rapid, and reproducible. Estimated ELISA recovery using this procedure was 95% in water within the range of 1 to 50 ng and 110% in milk within the range of 5 to 50 ng. The affinity columns (0.2 ml volume size) remained active after eluting bound toxin with methanol and after re-equilibrating in PBS were ready for reuse. Typically, 10 samples could be passed through columns with no loss of activity being observable. We believe that the tandem procedure will be valid to detect ZEA in contaminated samples containing as low as 5 ppb (or less after sample concentration), while highly contaminated samples would be assayed by direct dilution of the chromatographic extracts before the ELISA analysis. Affinity columns would be potentially useful to detect conjugated forms of zearalenols in samples previously treated with ß-glucuronidase and aryl sulphatase (*17,22*) and also can be used in the isolation/concentration of zearalenone in clinical samples and other agricultural commodities prior to ELISA or other conventional methods such as HPLC, GC, or MS.

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