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Evaluation of Reduced Toxicity of Zearalenone by Extrusion Processing As Measured by the MTT Cell Proliferation Assay

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The objective of this study was to determine loss of toxicity of zearalenone in extruded cereal-based products by the MTT (tetrazolium salt) cell proliferation assay using a sensitive MCF-7 human breast cancer cell line and to compare the results to chemical (high-performance liquid chromatography, HPLC) and biochemical (enzyme-linked immunosorbent assay, ELISA) methods of analysis. A split−split plot design was used for the extrusion process experiments at temperatures of 150, 175, and 200 °C and screw speeds of 70 and 140 rpm. The initial zearalenone concentration in the artificially contaminated corn grits with *Fusarium graminearum* was found at a mean concentration of 37.88 μg/g as measured by HPLC. The percent reductions of zearalenone in the contaminated corn grits upon extrusion processing were in the ranges of 67−81, 60−72, and 66−78% as measured by HPLC, ELISA, and the MTT cell proliferation assay, respectively. The MTT cell proliferation assay results were more closely correlated with HPLC results ($r = 0.96$) than ELISA results ($r = 0.83$). The MTT cell proliferation assay was demonstrated to be a useful method for quantification of zearalenone as well as a potential toxicity screening method for contaminated extruded cereal-based products.

KEYWORDS: Zearalenone; extrusion processing; detoxification; MTT bioassay; MCF-7 cell line

INTRODUCTION

A common feature of many *Fusarium* species is their ability to synthesize zearalenone and its co-occurrence with certain trichothecenes raises important issues regarding mycotoxicoses in humans and animals (*1*). The occurrence of zearalenone is associated primarily with *Fusarium graminearum* (telemorph *Gibberella zeae*) and *Fusarium culmorum*, both of which are important pathogens which cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize (*2*). Zearalenone occurs throughout the world in cereal grains such as maize, wheat, barley, oats, and rye (*3*) and also in processed cereal-based foods such as flakes and bread (*4*), corn pancakes (*5*), and beer (*6*). Zearalenone was shown to be produced on corn by *Fusarium* isolates from Europe (*7*) and North America (*8*) as well as in New Zealand (*9*) and Southeast Asia (*10*). Zearalenone was found at a mean concentration of 904.3 ng/g in Wisconsin corn (*8*). In Canada, annual levels of zearalenone in contaminated corn samples ranged from 23 to 215 ng/g, whereas it has been detected infrequently in wheat, barley, and soybeans (<75 ng/g) (*11*). Zearalenone was detected in 46% of infant cereal foods from the Canadian retail market (*12*). In Korea, zearalenone has been detected at mean levels up to 151 ng/g in corn and 287 ng/g in barley (*13*). In the United Kingdom, zearalenone was detected in 42% of maize containing >100 μg/kg (*14*).

Zearalenone is a nonsteroidal estrogenic mycotoxin that may be linked to mammary tumorigenesis. Its presence in feed causing hyperestrogenism, especially in swine, has adversely influenced the reproductive performance of breeding animals (*15*). There have been suggestions of the involvement of zearalenone in human cervical cancer and premature initial breast development (*16*). Zearalenone and its derivative, zearalenol, were suspected to be the causative agent in an epidemic of precocious pubertal changes in young children in Puerto Rico between 1978 and 1981 (*17*). Zearalenone has high binding affinities for the intracellular estrogen receptor (ER) and can enhance the proliferation of estrogen responsive tumor cells (*18*). It is able to stimulate the growth of ER positive cells, increase uterine weight, modulate the estrous cycle, and compete with estradiol for ER binding (*19*). A number of studies have investigated the estrogenic potency of zearalenone on ER-positive MCF-7 cell line (*20*−*22*). Mayr et al. (*20*) reported the relative estrogenic potency of zearalenone which was found to be 3−5% relative to 17β-estradiol. Zearalenone increased the proliferation of the MCF-7 cell line at a low concentration of 10 nM (*23*), whereas it was found to be moderately cytotoxic to several mammalian cell lines such as Mardin Darbin canine kidney (MDCK), swine kidney (SK) (*24*), baby hamster kidney (BHK-21) (*25*), and Balb/c mice keratinocyte (C5−O) cell lines at a high concentration of 100 μg/mL (*26*).

Zearalenone is considered to be a food safety issue due to its toxicity and widespread natural occurrence in cereals and also

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in processed cereal-based foods (*27*). On the basis of inadequate evidence in humans and limited evidence in experimental animals, zearalenone was allocated, together with other *Fusarium* toxins, in Group 3 (not classifiable as to their carcinogenicity to humans) by the International Agency for Research on Cancer in 1993 (*27*). Estimates of average dietary intakes are <2.1 μg/day (0.03 μg/kg bw per day) for American, <0.98 μg/day (0.02 μg/kg bw per day) for Canadian, 1.5 μg/day (0.03 μg/kg bw per day) for European, and 3 μg/day (0.06 μg/kg bw per day) for Middle Eastern consumers (*27*). The maximum tolerable levels of zearalenone ranging from 30 to 1000 μg/kg in food (mainly cereals) have been set in nine countries (*28*).

Detoxification strategies for contaminated foods and feeds to reduce or eliminate the toxic effects of zearalenone by chemical, physical, and biological methods are crucial to improve food safety, prevent economic losses, and reclaim contaminated products. Extrusion cooking of cereal products is being used increasingly in the food industry to convert cereals into breakfast foods, snack foods, and pet foods. Extrusion cooking is one of the fastest growing food-processing operations in recent years due to several advantages over traditional methods. In addition to improving the quality of intermediate and final processed products, it may also improve food safety because of having the potential to reduce mycotoxin levels in cereals (*29*−*31*), and it also reduced 83% of zearalenone in corn-based foods (*32*). The levels of zearalenone in cereal-based foods were reduced significantly by extrusion processing as determined by high-performance liquid chromatography (HPLC) (*32*). However, there remains a need to demonstrate that the toxicity or biological activity of zearalenone has been reduced or completely eliminated in cereal-based foods using extrusion processing.

In vitro MTT cell proliferation assay is one of the most used assays for preliminary screening of the mycoestrogen zearalenone using the ER positive MCF-7 human breast cancer cell line (*33*). The MTT cell proliferation assay measures the increase in cell number of target cells during the exponential phase of proliferation. It is a rapid, versatile, quantitative, and highly reproducible colorimetric assay for determining viable cell number in proliferation (*34*). The in vitro MTT assay has already been demonstrated to be a useful method for screening *Fusarium* contaminated grains to detect toxicity of zearalenone (*35*).

This study was done to determine whether zearalenone concentrations in cereal-based foods were destroyed or converted to other derivatives that might remain biologically active or toxic during extrusion processing. The objective of this study was to determine loss of toxicity of zearalenone in extruded cereal-based products by the MTT cell proliferation assay using a sensitive MCF-7 cell line and to compare the results to biochemical (enzyme-linked immunosorbent assay, ELISA) and chemical (HPLC) methods of analysis.

MATERIALS AND METHODS

Production of Zearalenone. *Fusarium graminearum* NRRL 5883 was obtained from the ARS Culture Collection National Center for Agricultural Utilization Research (USDA, Peoria, IL). The stock culture was maintained on Carnation leaf agar (CLA) slants at 5 °C and was grown on CLA plates for 10 days at room temperature under daylight to enhance sporulation (*36*). Spores were harvested by adding sterile 0.05% Tween 80 to the culture plates and filtering through several layers of cheesecloth to remove mycelial debris and collect the spores. The number of spores in the suspension was determined by serial dilution and total plate count on potato dextrose agar. The spore suspension was then adjusted to contain approximately 10^6 spores/mL for inoculation.

Corn grits were obtained from Lauhoff Grain Company, Danville, IL. A total of 1200 g corn grits were placed in jars and were inoculated with 10^6 spores of *F. graminearum* per 50 g corn grits. The final moisture content of the corn grits was adjusted to 35% on a dry basis (db). The jars were shaken to provide homogeneous distribution of the spore suspension and then incubated at room temperature for zearalenone production. After 15 days of incubation, the corn grits were autoclaved for 5 min to kill the spores and air dried for 24 h. The corn grits were not exposed to longer autoclaving process to prevent the loss of toxin levels and any changes in the food matrix. All corn grits were pooled and mixed in a low speed mixer to provide a homogeneous mixture of fermented corn grits and placed in an oven to dry at 65 °C overnight. The moisture content of the grits was adjusted to 20% (db) by adding distilled water and allowing corn grits to equilibrate 5 h before extrusion processing.

Extrusion Processing. A model CTFE-V laboratory scale conical twin-screw extruder (C.W. Brabender Instruments, Inc., Hackensack, NJ) with a barrel diameter of 1.9 cm and a length of 37 cm, compression ratio of 3:1, co-rotating mixing screws, and a 3 mm diameter cylindrical die was used. A split−split plot design was used for the extrusion process at temperatures of 150, 175, and 200 °C and screw speeds of 70 and 140 rpm at 20% moisture content (db) to evaluate the reduction of zearalenone concentrations in corn grits. All the extruded samples were dried at 60 °C for 24 h, finely ground with a household blender, and kept frozen at −20 °C in airtight sample bags until analyzed.

The zearalenone levels in *F. graminearum* contaminated but unextruded corn grit extract was used as a positive control, whereas an extruded corn grit extract without any zearalenone contamination was used as a negative control for HPLC and ELISA analysis. Zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone) standard was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in HPLC-grade methanol. A recovery experiment was performed in triplicate by spiking blank extruded corn grits with zearalenone at a concentration of 8 μg/g. The spiked samples were left for 1 h before following HPLC, ELISA, and MTT cell proliferation assay.

HPLC Analysis. Zearalenone in contaminated extruded corn samples was analyzed using immunoaffinity columns as described by Visconti et al. (*37*) with minor modifications. Zearala Test HPLC immunoaffinity columns (Vicam, Watertown, MA) were used to clean up zearalenone contaminated corn samples because of the high specificity of the antibodies. Contaminated extruded corn samples were extracted by adding 100 mL of acetonitrile:water (90:10, v/v) and 2 g of salt (NaCl) to 20 g of the sample and blending at high speed for 3 min using a household blender. The extracts were filtered through Whatman #1 filter paper. The filtered extracts were diluted at the ratio of 2:23 with deionized water and filtered through Whatman 9 mm GF/A glass microfiber filters (VWR International Inc., Bristol, CT), then 10 mL of filtrate of each sample (10 mL = 0.16 g equivalent) was passed through an immunoaffinity column at a rate of about 1−2 drops/s until air came through the column using a Visiprep solid-phase extraction vacuum (Supelco, Bellefonte, PA). Subsequently, the columns were washed with 10 mL of deionized water through the column at a rate of about 1−2 drops/s until air came through. The toxin was eluted by passing 1.5 mL of HPLC-grade methanol through the column. Eluant was collected in a 2-dram amber vial, and 1.5 mL of deionized water was added and then mixed on a vortex. Then 20 μL of each sample was injected into the HPLC system.

A model 510 HPLC (Waters Associates, Milford, MA) was equipped with a model 474 scanning fluorescence detector and an E60 injector (Valco Instruments Co., Inc., Houston, TX) coupled with a model 728 automatic sampler (Micrometritics, Norcross, GA). The fluorescence detector was set at 274-nm excitation and 440-nm emission wavelengths. A 150 × 3.9 mm inside diameter (i.d.), 4 μm, reverse-phase Nova-Pak C18 column (Waters Associates, Milford, MA) and a 10 × 3.9 mm i.d. Nova-Pak C18 guard column (Waters Corp., Milford, MA) were used for chromatographic separations. The mobile phase, which consisted of a mixture of acetonitrile:water:methanol (48:46:8, v/v/v), was used at a flow rate of 1 mL/min. Chromatographic separations were monitored with computer controlled Millennium 2010 software (Waters Associates, Milford, MA) connected to the HPLC instruments.

The detection limit for zearalenone was 10 ng/g under the conditions tested. Concentrations of zearalenone were determined based on retention times and peak areas compared to zearalenone standards dissolved in the mobile phase.

ELISA Analysis. Commercial Veratox quantitative ELISA test kits for zearalenone (Neogen Corp., MI) were used, following the standard protocols given. Extruded corn samples were extracted by adding 50 mL of 70% methanol solution (methanol:water, 70:30,v/v) to 5 g of ground sample and placing on a wrist-action shaker for 1 h then filtered through a Whatman #1 filter. The filtered crude extract of 100 μL (1 mL = 0.1 g equivalent) of each sample was analyzed with the ELISA test kits, which had a limit of detection at 50 ng/g.

Cell Culture and Conditions. The MCF-7 cell line was obtained from Dr. T. Lawson (Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE). Dulbecco's Modified Eagle Medium (DMEM), with nutrient mixture F-12 (Ham) (1:1), fetal bovine serum (FBS) heat inactivated, charcoal-stripped FBS, phosphate-buffered saline (PBS) pH 7.2, HEPES buffer solution 1 M, sodium pyruvate solution 100 mM, sodium bicarbonate 7.5% (w/v), nonessential amino acid (NEAA) 10 mM, trypsin-EDTA (0.25% trypsin, 1 mM EDTA.4Na), and antibiotic−antimycotic (10 000 units of penicillin, 10 000 μg of streptomycin, and 25 μg of amphotericin B as fungizone, antimycotic in 0.85% saline) were obtained from Life Technologies Gibco BRL Products (Rockville, MD). MCF-7 cells were grown as a monolayer in DMEM/F-12 in 80 cm^2 culture flasks and used at passage numbers between 40 and 50. DMEM/F-12 was supplemented with 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 1% NEAA, 25 mM HEPES, antibiotic−antimycotic (100 units of penicillin/mL, 100 μg of streptomycin/mL, and 250 ng amphotericin B/mL), 0.1% insulin (Sigma Chemical Co., St. Louis, MO), and 10% FBS. The MCF-7 monolayers were harvested when they reached 80% confluence to maintain exponential growth.

The MTT Cell Proliferation Assay. The MTT cell proliferation assay determines the ability of viable cells to reduce the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) to blue-colored formazan crystals by mitochondrial enzymes. The concentration of formazan crystals can be spectrophotometrically determined when dissolved in an organic solvent (*34*). Zearalenone standards and *F. graminearum* contaminated extruded corn extracts cleaned up with IAC were transferred to 2-dram amber vials and evaporated to dryness at 50 °C under a stream of nitrogen and then resuspended in phenol red free DMEM/F-12 media containing 10% charcoal stripped serum, 0.01% insulin, and 0.1% ethanol. The samples were filtered through an Acrodisc 0.22 μm syringe filter (Gelman Sciences, Ann Arbor, MI) to sterilize the samples and were diluted serially.

The MCF-7 cell monolayer in exponential growth was harvested using 0.25% trypsin and a single-cell suspension was obtained by repeated pipetting. The cells were counted with a hemacytometer (Hausser Scientific, Horsham, PA) under the microscope, and the MTT cell proliferation assay was performed concominantly. The MTT cell proliferation assay measures the increase in cell number of target cells during the exponential phase of proliferation. To determine the seeding cell number of MCF-7 (doubling time, 36 h) in exponential phase of growth throughout the experiment, the single cell suspension containing the cell densities ranging from 1×10^2 to 1×10^5 cells in 200 μL DMEM/F-12 medium/well were added to 96-well plates by serial dilution (*34*). The plates were incubated for 7 days at 37 °C in a humidified atmosphere of 5% CO_2. MTT, sodium dodecyl sulfate (SDS), and *N*,*N*-dimethylformamide (DMF) were purchased from Sigma Chemical Co. (St. Louis, MO). MTT was dissolved in PBS at a concentration of 5 mg/mL, filtered through a 0.22 μm filter to sterilize and remove insoluble residues and then stored in the amber vials at 4 °C up to one month. After 7 days of incubation, 25 μL of the MTT solution was added to each well of the 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. At the end of the incubation period, the media were discarded using a suction pump. The cell lysing solution of 20% w/v SDS in a solution of 50% DMF in demineralized water (50:50, v/v) was prepared and adjusted to pH 4.7, filtered through a 0.22 μm filter to sterilize and remove insoluble residues, and then stored at room temperature. The cell-lysing solution of 100 μL of 20% SDS was added into each well of the 96-well plate to solubilize the formazan crystals. The 96-well plates were placed on an orbital shaker at 37 °C overnight. The absorbance was measured at a test wavelength of 570 nm using a Sunrise absorbance microplate reader (Tecan, Phoenix, CA). The number of seeding cells was found to be 2×10^4 cells/well from the linear correlation between the number of seeded cells and the optical density (OD) values.

The proliferative effects of zearalenone in the corn samples were measured by performing the MTT assay. The MCF-7 cells were trypsinized and plated into a 96-well plate at a density of 2×10^4 cells/well in 100 μL DMEM/F-12 medium/well. They were allowed to attach to the wells for 24 h of incubation at 37 °C in a humidified atmosphere of 5% CO_2. The seeding medium was removed and replaced by fresh 100 μL of phenol red free DMEM/F-12 supplemented with 10% charcoal stripped serum to remove endogenous steroid hormones. Then 100 μL 17β-estradiol (Sigma Chemical Co., St. Louis, MO) at a final concentration of 1×10^{-9} M was added into the first column wells of the 96-well plate. The zearalenone standards ranged from 4×10^{-12} M to 1×10^{-6} M or the extracts of extruded corn samples (1 mL = 0.107 g equivalent) from each treatment were added in 100 μL of phenol free DMEM/F-12 medium containing 10% charcoal-stripped serum and 0.1% ethanol then added to the 96-well plates and incubated for 6 days at 37 °C in a humidified atmosphere of 5% CO_2. After 6 days of incubation, 25 μL of the MTT solution was added to each well of the 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2 then the media were discarded using a suction pump. The cell-lysing solution of 100 μL of 20% SDS was added into each well, and the 96-well plates were placed on an orbital shaker at 37 °C overnight. The absorbance was measured at a test wavelength of 570 nm using a microplate reader. The positive control contained contaminated unextruded corn extracts, and the MCF-7 cells in the medium contained 0.1% ethanol. The negative control contained the MCF-7 cells and uncontaminated extruded corn extracts in the culture medium containing 0.1% ethanol. The blank contained only MCF-7 cells in the medium containing 0.1% ethanol.

Quantifications of the zearalenone concentrations in the samples were determined using linear regression equation obtained from the dose−response curve of zearalenone. The effective concentration of 50% (EC_{50}) value of zearalenone was calculated by locating the *x*-axis value corresponding to one-half the absorbance value of the maximal effect.

Statistical Analysis. All trials were repeated three times. The Statistical Analysis System (SAS) was used to verify significant differences between treatments by analysis of variance in the mixed procedure, followed by the comparison of the least-squares means (LSM) at 95% confidence intervals for the extrusion study.

RESULTS

The effects of extrusion temperatures and screw speeds on reduction of zearalenone levels in *F. graminearum* contaminated corn grits were found to be significant when calculated from their differences of LSM values among HPLC, ELISA, and the MTT cell proliferation assay ($P < 0.05$) (**Table 1**). The effect of extrusion processing on zearalenone stability in contaminated corn grits was measured by HPLC, ELISA, and the loss of toxicity of zearalenone was confirmed by in vitro MTT cell proliferation assay using the MCF-7 cell line. The initial levels of zearalenone in *F. graminearum* contaminated corn grits used as a positive control were found at mean concentrations and standard deviations of 35.88 ± 3.57, 29.23 ± 2.14, and 36.57 ± 2.8 μg/g ($n = 3$) as measured by HPLC, ELISA, and the MTT cell proliferation assay, respectively. The zearalenone levels in negative control, extruded blank corn grits without any *F. graminearum* contamination, were found to be negative as measured by the all three methods of analysis. The other possible *F. graminearum* products of deoxynivalenol and its derivatives were not detected in the contaminated corn extracts cleaned up with IAC when analyzed by HPLC as described by Cahill et al. (*38*). The zearalenone metabolites, α-zearalenol and β-zearale-

Table 1. Levels of Zearalenone Remaining in *F. graminearum* Contaminated Corn Grits after Extrusion Processing As Measured by HPLC, ELISA, and the MTT Cell Proliferation Assay

extrusion temp and screw speeds		remaining levels of zearalenone in extruded corn grits μg/g[a] (% reduction)[b]		
°C	rpm	HPLC	ELISA	MTT bioassay
150	70	8.52 ± 0.56[c] (77.5)	9.76 ± 0.21[c] (66.6)	8.75 ± 0.86[c] (76.1)
	140	7.37 ± 0.71[c] (80.5)	8.35 ± 0.22[c] (71.4)	7.93 ± 0.78[c] (78.3)
175	70	8.32 ± 0.64[c] (78.0)	9.35 ± 0.16[c] (67.9)	9.39 ± 0.62[c] (74.3)
	140	7.90 ± 0.60[c] (79.1)	8.21 ± 0.59[c] (71.9)	8.38 ± 0.69[c] (77.1)
200	70	12.60 ± 0.54[d] (66.7)	11.59 ± 0.73[d] (60.4)	12.29 ± 0.74[d] (66.4)
	140	11.78 ± 1.02[d] (68.9)	10.33 ± 0.34[d] (64.7)	11.53 ± 0.93[d] (68.5)

[a] Means ± standard deviations ($n = 3$). [b] Means of three replications ($n = 3$) calculated from the initial levels of zearalenone. [c,d] Means with different superscripts in same columns were significantly different calculated from the least significant mean comparison at 95% confidence interval ($p < 0.05$).

nol, were not found in contaminated corn extracts when analyzed by HPLC. The percent recoveries of HPLC, ELISA, and the MTT bioassay were found at mean values and standard deviations of 95 ± 4.82, 96 ± 3.26, and 104 ± 6.55%, respectively. The limits of detection for HPLC, ELISA, and the MTT bioassay were found to be 10, 50, and 6 ng/g, respectively. The average of repeatabilities, relative standard deviations (RSDr), of HPLC, ELISA, and the MTT assay by analyzing triplicate samples ($n = 3$) were found to be 7.8 (ranging from 4.3 to 9.6%), 3.9 (ranging from 1.7 to 6.4%), and 7.9% (ranging from 3.5 to 10.9%), respectively.

The maximum reduction of zearalenone of 81% at 150 °C and 140 rpm was found to be significantly higher than the minimum reduction level of 67% at 200 °C and 70 rpm in contaminated corn grits measured by HPLC ($P < 0.05$). At 175 °C and 140 rpm, the zearalenone level was reduced 79% from the starting concentration. The effect of extrusion temperatures on loss of zearalenone was found to be a quadratic function. A temperature of 150 °C was found to reduce zearalenone significantly more than 200 °C but was not different from 175 °C. The percent reductions of zearalenone at screw speeds of 70 and 140 rpm were found to have mean values of 70 and 73%, respectively.

Zearalenone concentrations by extrusion processing at extrusion temperatures of 150, 175, and 200 °C were reduced by 69, 70, and 62%, respectively, as measured by ELISA (**Table 1**). The maximum reduction of zearalenone level of 72% at 175 °C and 140 rpm was found to be significantly higher than the minimum reduction level of 60% at 200 °C and 70 rpm in contaminated corn grits ($P < 0.05$). The levels of zearalenone reductions at 150 and 175 °C were found to be significantly higher than at 200 °C ($P < 0.05$).

The quantification of remaining zearalenone levels in contaminated corn samples after extrusion processing was analyzed by the MTT cell proliferation assay using the MCF-7 cell line. The proliferative effects of the zearalenone standards on the MCF-7 cell line at concentrations of 4×10^{-12} to 1×10^{-6} M are shown in **Figure 1**. The OD values of contaminated unextruded (positive control), extruded blank corn extracts (negative control), and contaminated extruded corn extracts are given in **Figure 1**. The zearalenone concentrations in the corn samples were calculated using a linear regression equation obtained from the dose−response curve. The zearalenone concentrations at extrusion temperatures of 150, 175, and 200 °C were reduced by 77, 76, and 67%, respectively. The reductions of zearalenone levels in contaminated corn grits at

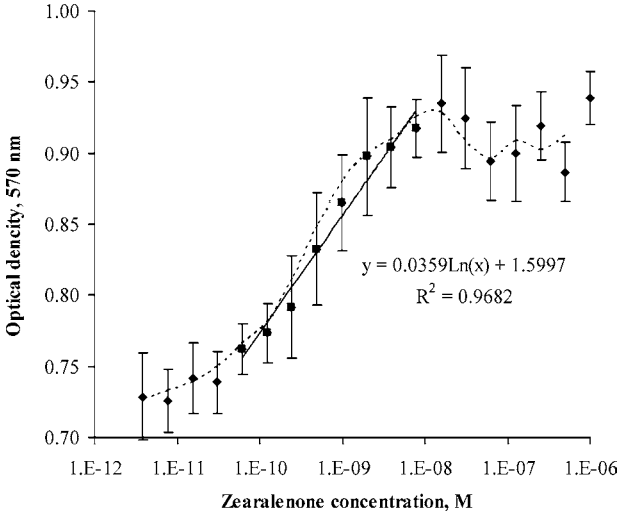

Figure 1. Dose–response curve of zearalenone at concentrations of 4×10^{-12}–1×10^{-9} M determined by the MTT cell proliferation assay using the MCF-7 cell line upon 6 days of exposure. The mean of OD ± SD values of the extruded samples ($n = 3$) at 150, 175, and 200 °C and screw speeds of 70 and 140 rpm were found to be 0.828 ± 0.012, 0.832 ± 0.010, 838 ± 0.009, 0.834 ± 0.011, 0.848 ± 0.013, and 0.845 ± 0.017, respectively, as measured by the MTT cell proliferation assay. The OD values of contaminated corn (positive control) and extruded blank corn extracts (negative control) were found to be 0.917 ± 0.048 and 0.746 ± 0.013, respectively.

extrusion temperatures of 150 and 175 °C was found to be significantly higher than the minimum reduction levels at 200 °C ($P < 0.05$) (**Table 1**). The MTT cell proliferation assay results were more closely correlated with the HPLC results ($r = 0.96$) than the ELISA results ($r = 0.83$). The estrogenic activity of zearalenone was also assessed by measuring the stimulatory effect on the MCF-7 cell proliferation comparing with 17β-estradiol (*21*, *22*). The relative proliferation potency of zearalenone was found to be 5 ((1×10^{-9} M 17β-estradiol/2 $\times 10^{-8}$ M zearalenone) $\times$ 100) calculated from the ratio between 17β-estradiol and zearalenone concentration needed to produce maximal cell yield. The EC_{50} value of zearalenone was found at a concentration of 1.06 nM after 6 days of exposure (*20*). The MTT cell proliferation assay was also used as a quantitative method with a limit of detection of 6 ng/g (the lowest limit of linear regression line reported as a detection limit) as well as screening to evaluate reduced toxicity of zearalenone to a mammalian cell culture.

DISCUSSION

A number of physical, chemical, and biological detoxification methods have been attempted to reduce zearalenone levels in naturally or artificially contaminated grains. The use of chemicals both pre- and postharvest is one strategy to detoxify zearalenone in contaminated grains; however, human and animal health concerns and the ecological and economic problems are quite high, in addition to altering the food physically, organoleptically, or nutritionally (*39*). Several heat treatments such as heating, baking, and roasting have been applied for zearalenone detoxification capabilities with varying degrees of success. The greatest reduction of zearalenone (up to 69%) was achieved by heating wheat flour cake spiked with zearalenone at 200 °C for 60 min (*40*), while baking at 190−200 °C and flame roasting at 110−140 °C for 6−15 min reduced zearalenone levels by 34−40 and 50%, respectively (*40*, *41*).

One of the more promising physical methods is extrusion processing under high temperature, high pressure, and high shear stress to detoxify zearalenone in cereal-based food products. The degree of detoxification depends on the combination of temperatures and screw speeds and the moisture content of the materials during extrusion processing. Extrusion processing has been shown to be quite effective in reducing 95% of fumonisin B_1 (*30*), 25% of aflatoxin, 95% of deoxynivalenol (*31*), and 83% of zearalenone (*32*) in corn-based foods. The application of extrusion processing might be very effective for detoxification of *Fusarium* toxins due to their widespread natural co-occurrence in cereals.

The effect of extrusion processing at temperatures of 150, 175, and 200 °C and screw speeds of 70 and 140 rpm on reductions of zearalenone levels in artificially contaminated corn grits was found to be effective, with 67−81% reduction as measured by HPLC. In general, the high efficiency extrusion cooking in reducing zearalenone is often attributed to the high temperature, high pressure, and severe shear stresses. However, in this study the results of extrusion processing showed higher reduction of zearalenone levels at lower temperatures of 150 and 175 °C than at the higher temperature of 200 °C. It could be speculated that the reduction of moisture content in food materials during extrusion at the higher temperature might reduce the heat transfer throughout the material, which might hinder complete exposure of zearalenone to heat. The corn grits were in continuous motion inside the barrel without prolonged exposure to heat. Therefore, zearalenone might be degraded to a lesser degree at the higher temperature than at the lower extrusion temperatures. At high temperatures, the residence time in the extruder might be reduced; consequently, zearalenone was extruded for a short time. Conversely, another study showed that the reductions of zearalenone levels in buffer solution heated at temperatures of 125 up to 225 °C were increased with increasing processing temperatures and time by 23 and 100%, respectively (*42*). In this study the different trend of the zearalenone reduction with increasing extrusion temperatures might be explained by the possibility of zearalenone binding to the food matrix increasing the resistance of zearalenone reduction. Therefore, in vivo studies are necessary to evaluate the bioavailability of zearalenone in extruded corn grits to examine toxicity of possible matrix-bound zearalenone.

Ryu et al. (*32*) also studied the effect of extrusion processing on 4.4 μg/g zearalenone spiked corn grits at temperatures of 120, 140, and 160 °C and moisture contents of 18, 22, and 26%. The percent reduction of zearalenone was found to be between 66 and 83%. The effects of extrusion temperatures showed the same decreasing trend on reduction of zearalenone, which was reduced more at either 120 or 140 °C than at 160 °C. Despite lower extrusion temperatures being used, slightly higher reductions in the zearalenone levels were observed. The different reduction ranges of zearalenone levels between these two studies might be attributed to the different zearalenone contamination processes of corn grits, which were contaminated artificially with *F. graminearum* instead of spiking with zearalenone. Another reason might be the higher level of zearalenone produced in corn grits contaminated with *F. graminearum* at the mean concentration of 37.88 μg/g than the spiked zearalenone concentration of 4.4 μg/g in the corn grits used for extrusion processing.

Zearalenone concentrations in extruded corn grits analyzed by ELISA were found to be a little higher than with HPLC results. This might be due to cross reactivity of zearalenone metabolites with the antibodies, although the metabolites (α-zearalenol and β-zearalenol) were not found in contaminated corn extracts when analyzed by HPLC. The MTT cell proliferation assay showed high sensitivity and a very low detection limit. The MTT cell proliferation assay results were more closely correlated with the HPLC results than with the ELISA results. The MCF-7 cell line was used as an in vitro model to evaluate the estrogenic activity of zearalenone due to the presence of ERs (*21*−*23*). Enhanced levels of formazans were interpreted as an increased metabolic activity of the MCF-7 cell line to show the potent proliferation activities of zearalenone. Mayr et al. (*20*) used relative estrogenic activity of zearalenone using the MCF-7 cell line to evaluate the content in cereal samples, and the results showed a good correlation with the HPLC results (*20*).

In this research, extrusion processing was demonstrated to be a useful heat treatment for detoxification of zearalenone in contaminated corn grits as measured by HPLC and ELISA, and the loss of biological activity was confirmed by the MTT cell proliferation assay using the MCF-7 cell line. The application of extrusion processing under optimized conditions might prevent considerable economic losses in contaminated agricultural products by detoxifying zearalenone contaminated cereal grains. The more rapid MTT cell proliferation assay is unlike many of the chemical analyses in that because it does not require high capital costs for equipment (e.g., gas chromatography, HPLC, and mass spectroscopy) nor a highly skilled technician. This study showed that the MTT cell proliferation bioassay is a useful method for quantification of zearalenone as well as a potential proliferation screening method for zearalenone contaminated cereal extracts to demonstrate reductions of toxicity and biological activity.

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