# EVALUATION OF REDUCED TOXICITY OF DEOXYNIVALENOL AND ZEARALENONE BY EXTRUSION PROCESSING AS DETERMINED BY THE MTT BIOASSAY USING MAMMALIAN CELL CULTURES

by

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# EVALUATION OF REDUCED TOXICITY OF DEOXYNIVALENOL AND ZEARALENONE BY EXTRUSION PROCESSING AS DETERMINED BY THE MTT BIOASSAY USING MAMMALIAN CELL CULTURES

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**University of Nebraska, 2003**

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The most sensitive mammalian cell lines to *Fusarium* mycotoxins, deoxynivalenol (DON), zearalenone (ZEN), fumonisin (FUM), and moniliformin (MON), were investigated for further toxicological investigations as alternatives to whole animal testing for screening extracts of *Fusarium* spp. contaminated foods and feeds. The Chinese hamster ovary (CHO-K1) for DON and FUM, hepatocellular carcinoma (HepG-2) for MON, and Balb/c mice keratinocyte (C5-O) for ZEN were found to be the most sensitive cell lines with $IC_{50}$ values of 0.27, 85.5, 26.8, and 24.1 μg/ml, respectively, as measured by the MTT bioassay. In the following study, reduced DON concentrations in contaminated corn grits after extrusion were analyzed by chemical (HPLC) and biochemical (ELISA) methods. The loss of toxicity of DON was confirmed by the MTT bioassay using the CHO-K1 cell line. Extrusion temperatures of 160, 180, 200 and 220°C and screw speeds of 50, 80, 110 and 140 rpm significantly reduced DON levels in contaminated corn grits by 22-35% ($P<0.05$). A second extrusion processing was designed to evaluate reduction of levels of both DON and ZEN in *F. graminearum* contaminated corn grits. This was analyzed by HPLC and ELISA, and reduced toxicity

was confirmed by the MTT bioassay. The CHO-K1 cells was used to evaluate the cytotoxicity of DON, whereas the adenocarcinoma human breast cells (MCF-7), which has estrogen receptors, was used to evaluate the estrogenic activity of ZEN. The effect of extrusion temperatures of 150, 175, and 200°C was found to be a linear, with DON reduced by 22-35%, whereas a quadratic response on reduced ZEN by 67-81%. The MTT bioassay results were more closely correlated with the HPLC results ($r = 0.90$ for DON, $r = 0.96$ for ZEN) than the ELISA results ($r = 0.78$ for DON, 0.83 for ZEN). The MTT bioassay was proved to be a useful method for quantification of DON and ZEN, as well as a potential toxicity screening method for contaminated extruded cereal based products.

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## GENERAL INTRODUCTION

The Food and Agriculture Organization (FAO) estimates that at least 25% of the world's cereal production is contaminated with mycotoxins (Dowling, 1997). Deoxynivalenol (DON) and zearalenone (ZEN) are the major *Fusarium* mycotoxins that occur on a worldwide basis in cereal grains, animal feeds and forages. Spontaneous outbreaks of *Fusarium* mycotoxicosis have been reported in Europe, Asia, New Zealand and South America (D'Mello et al., 1999). *Fusarium graminearum* causes *Fusarium* head blight or scab in wheat and barley, which has been a major problem in recent years in the upper Midwestern U.S. (McMullen et al., 1997). Cereal based processed foods for human consumption are staple foods in many countries and have received much attention because the raw cereals have often been found to be contaminated with mycotoxins (Solovey et al., 1999).

The removal of DON and ZEN from foods and feeds remains an important objective worldwide due to their common occurrence, and potential toxicity on humans and animals. Economic losses from contaminated foods and feeds are estimated to be annually in the billions of dollars worldwide (Pohland, 1993). Detoxification strategies of contaminated foods and feeds to reduce or eliminate the toxic effects of DON and ZEN are crucial to improve food safety, prevent economic loss and reclaim contaminated products. Extrusion cooking of cereal products is being used increasingly as a method of processing cereals into breakfast foods, snack foods and pet foods. Extrusion processes cause chemical changes and molecular transformations of food components and contaminants. A theoretical understanding of the chemical stability of DON and ZEN is

important not only for predicting the likely fate of the DON and ZEN during extrusion processing operations, but also for proposing modifications to the process that might reduce their concentrations in the finished products. The reductions in the levels of DON and ZEN are brought about by physical decomposition or by chemical modification. Thus knowledge of the identity of the transformation products is important for toxicological assessment. Previous studies in our laboratory and others indicate that extrusion processing offers a means of reducing the concentrations of DON and ZEN in cereal-based foods if the proper parameters are used. The levels of DON and ZEN in cereal based foods were reduced significantly by extrusion processing as determined by a chemical method, HPLC (Accerbi et al., 1999; Cazzaniga et al. 2001; Ryu et al., 1999). However, there remains a need to prove that the toxicity or biological activity of DON and ZEN has been reduced or completely eliminated in cereal based foods processed by extrusion cooking.

The purpose of this research was to determine whether extrusion processing could be a useful tool for detoxification of DON and ZEN in contaminated cereal grains using chemical and biological analytical methods to show loss. Detoxifying DON and ZEN contaminated cereal grains using extrusion processing could prevent considerable economic losses in contaminated agricultural products. This research will provide crucial information for risk assessment of extruded cereal-based foods, such as snack and breakfast foods, which are commonly consumed by children. Residual levels of DON and ZEN in extruded products may cause a long term chronic toxicity due to accumulation in target organs. Therefore, the use of suitable *in vitro* bioassays using sensitive

mammalian cell lines will provide certain toxicity data about DON and ZEN that will show whether chemical changes occurring during extrusion processing can be correlated to biological transformations and loss of toxicity.

# SECTION 1 - LITERATURE REVIEW

## DEOXYNIVALENOL

### Natural Occurrence of Deoxynivalenol

Deoxynivalenol (vomitoxin), a type B trichothecene (Figure 1-A), occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice sorghum, and triticale. The occurrence of DON is associated primarily with *Fusarium graminearum* (*Gibberella zeae*) and *F. culmorum*, both of which are important plant pathogens which cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize (JECFA47, 2001). DON occurs regularly throughout the world, at unacceptably high concentrations in cereal grains in the Upper Midwest region of the U. S. (Jones et al., 1999; Park et al., 1996; Prom et al., 1999; Salas et al., 1999; Wetter et al., 1999), Canada (Stratton et al., 1993; Scott, 1997; Usleber et al., 1996), South America (Gonzalez et al., 1999; Solovey et al., 1999; Furlong and Soares, 1995), Europe (Arseniuk et al., 1999; Curti et al., 1998; Gang et al., 1998; Kosiak et al., 1997; Munoz, et al., 1990; Patel et al., 1996; Stahle et al., 1999; Usleber and Martlbauer, 1998; Vrabcheva et al., 1996), Asia (Ali et al., 1998; Janardhana et al., 1999; Groves et al., 1999; Kim et al., 1993; Lee et al., 1986; Li et al., 1999; Park et al., 1992; Sohn et al., 1999), Africa (Thiel et al., 1982; Sydenham et al., 1990), and New Zealand (di Menna et al., 1997). In the U.S. and Canada, the most frequently encountered metabolite has been deoxynivalenol, along with zearalenone, in wheat, barley, corn, and sorghum (Pohland, 1993). Over six years (1993-1998), *Fusarium* head blight or scab of wheat was destructive to hard red spring wheat and barley production in Minnesota, North Dakota and South Dakota (Jones et al., 1999;

Prom et al., 1999; Salas et al., 1999). In 1993 and 1994, severe epidemics of *Fusarium* head blight severely damaged the hard red spring wheat and barley crops with mean concentrations of 8.3 and 10.4 μg/g, respectively, in Minnesota (Jones et al., 1999). Natural occurrence of DON in field samples from the 1992 Wisconsin Corn crop was found, with mean concentrations of 237.7 ng/g (Park et al., 1996). Unusually wet and cool weather conditions in Maryland and Delaware resulted in a severe outbreak of *F. graminearum* on sweet corn ears prior to harvesting and canning. Corn kernels from the visibly mouldy area of the ears contained average concentrations of approximately 446 mg/g DON (Wetter et al., 1999). DON has also been detected in processed breakfast cereals with an average concentration of 100 ng/g (Truckess et al., 1986) and in 50% of 92 snack food samples made from grain grown in the U.S. (Brumley et al., 1985). Monitoring of Canadian grain crops between 1979 and 1995 showed DON contamination of wheat and barley with mean values of 0.43 and 0.27 μg/g, respectively (Scott et al., 1997). In literature from around the world, mean DON concentrations in positive samples have ranged from 0.03 to 1.78 mg/kg in wheat with maximum levels up to 8.53 mg/kg. In grains from the Netherlands, Germany, Italy, and Australia, mean DON contents ranged from 0.5 to 64.6 mg/kg (Gang et al., 1998). DON was found in corn samples collected in South Africa at levels between 0.05 and 12.10 μg/g (Sydenham et al., 1990). In New Zealand, predominant *Fusarium* species produced DON in maize at levels between 8 and 25 mg/g (di Menna et al., 1997). Historically, severe epidemics of cereal scab occurred in the southern part of Korea in 1963. The data indicated that 90% of Korean cereals harvested in 1984 were contaminated with DON in concentrations that ranged from 3 to

2476 ng/g with a mean of 158 ng/g (Lee et al., 1986). The scab disease caused yield losses of cereals of 80 to 100% in some areas, and also caused mycotoxicoses in humans and farm animals (Kim et al., 1993). In 1991, a large outbreak of human intoxication attributed to the consumption of foods made from wheat and barley contaminated with DON occurred in China. A total of about 130,000 people were affected by gastrointestinal disorders, including abdominal pain and diarrhea, nausea, vomiting, fatigue, dizziness, headache, and fever (Huang, 1992). The acute symptoms were quite similar to those of human red-mold intoxications reported in Japan (Yoshizawa, 1983) and India (Bhat et al., 1989). DON was detected in all wheat, barley and corn samples collected from the human intoxication area in China at concentrations that ranged between 16 and 51,450 μg/kg (Li et al., 1999). In 1987, an acute outbreak of disease, affecting about 50,000 people in the Kasmir valley in India, was attributed to consumption of bread made from damaged wheat containing DON at concentrations that ranged from 0.34 to 8.4 mg/kg (Bhat et al., 1989).

**Toxicological Effects of Deoxynivalenol**

DON is capable of inducing both acute and chronic toxicity, which are related to dose levels and duration of exposure in animals and humans. DON reduces growth and feed consumption (anorexia) at low concentrations in the diet, whereas it induces vomiting (emesis) at higher acute doses (Rotter et al., 1992; Vesonder and Hesseltine, 1981; Forsyth et al., 1977; Prelusky and Trenholm, 1993). Both effects are thought to be mediated by affecting the serotoninergic activity in the central nervous system or via peripheral actions on serotonin receptors (Rotter et al., 1996). Acute/subacute toxicity of

DON to mice is characterized by vomiting (vomiting is seen in pigs, whereas delayed gastric emptying has been observed in rats and mice), feed refusal, weight loss and diarrhea. After acute intoxication, necrosis in various tissues such as gastrointestinal tract, bone marrow and lymphoid tissues has also been observed (Fioramenti et al., 1993; Rotter et al., 1996, IARC, 1993; Eriksen and Alexander, 1998). After oral administration of DON (10 mg/kg bw) to rats, the major metabolic pathway was de-epoxidation to the corresponding methylene derivative. This metabolite was found in feces, urine, plasma and milk of lactating cows. In sheep and cows, glucuronidation of DON was observed (Eriksen and Alexander, 1998). The biotransformation of DON by the normal bacterial gut flora of pigs was examined and showed to be de-epoxidation (Kollarzik et al., 1994).

DON inhibits protein synthesis in Vero cells (Ehrlich and Daigle, 1987; Thompson and Wannemacher, 1986), baby hamster kidney cells (BHK-21) (Scossa-Romano et al., 1987), rat spleen lymphocytes (Thompson and Wannemacher, 1986), and primary rat hepatocytes (Norred et al., 1990) at the ribosomal level, due to the presence of an intact 9,10-double bond and the C-12, 13 epoxide. Trichothecenes with substituents at both C-3 and C-4 predominantly inhibit polypeptide chain initiation, whereas those lacking one substituent at either site are inhibitors of chain elongation. Ehrlich and Daigle (1987) found a 50% protein synthesis inhibition by DON and its derivatives on murine erythroleukemia and Vero cells at concentrations of 1.6 and 0.9 μg/ml for 4-DON, 100 and 150 μg/ml for 7-DON, 0.8 and 10.0 μg/ml for 3,7-diDON, respectively, after 30 minutes exposure. The 50% protein synthesis inhibition effect of DON, as measured by [$^{3}$H]leucine uptake on a cloned thymoma (EL-4) T cell model, was estimated to be 280

ng/ml after 24 hours exposure (Dong et al., 1994). The inhibitory effects of DON on the clonal B cell model measured by both thymidine and leucine incorporation were found at concentrations of 120 and 110 ng/ml, respectively, after 3 days exposure (Minervini et al., 1993). The $IC_{50}$ values of DON on murine splenocytes and *Xeroderma pigmentosum* (XP) human fibroblast cells were found at concentrations of 131 and 252 ng/ml, respectively, after 48 hours exposure (Robbana-Barnat et al., 1988). Azcona-Olivera et al. (1995) found that the $IC_{50}$ value of DON in various tissues of mouse was 25 mg/kg of DON after 3 hours exposure. A dose related increase in the inhibition of DNA synthesis observed in L929 mouse fibroblast cells exposed to the mixture of DON, nivalenol (NIV), T-2 toxin and ZEN was interpreted as synergistic with respect to the inhibition of DNA synthesis (Groten et al., 1998; Tajima et al., 2002).

The cytotoxic effects of DON were evaluated using *in vivo* and *in vitro* methods. The comparison of *in vivo* and *in vitro* biological activity of DON demonstrated a significant correlation between these two tests (Terse et al., 1993; Abbas et al., 1984). The cytotoxic effect of DON on Swiss mouse 3T3 fibroblasts and human diploid skin GM3349 fibroblast cell lines correlated well with weight loss and feed refusal observed in rat feeding studies (Abbas et al., 1984). More than 20 cell cultures were screened to evaluate the cytotoxicity of DON using *in vitro* models, which showed different susceptibility to DON as measured by different cytotoxicity bioassays (Table 1). Cytotoxicological characterizations of *Fusarium* species contaminated extracts were screened to compare their toxin production with cytotoxicity on mammalian cell cultures (Abbas et al., 1984; Langseth et al., 1999; Langseth et al., 1997; Abeywickrama and

Bean, 1992; Robb et al., 1990). The cytotoxic properties of *Fusarium* mycotoxin contaminated grain samples, including high levels of DON, were examined with an *in vitro* MTT bioassay using swine kidney (SK), and VERO cells originating from African green monkey kidney cells as target cells. The SK monolayers proved to be more sensitive than the VERO cells (Langseth et al., 1997). The evaluation of cytotoxicity of *Fusarium* spp. contaminated Norwegian grains showed significant cytotoxicity with a large variation between species (Langseth et al., 1997). The cytotoxicity of DON on SK cells was found to be 0.8 μg/ml calculated as a 70% MTT cleavage activity (Langseth et al., 1999). Robb et al. (1990) examined the potential cytotoxicity of DON standards and extracts from fungi and feedstuffs to mouse myeloma cells (NS-1/I.AG4-I) as analyzed by fluorescent flow cytometry which were compared it to microscopic assessment of cytotoxicity on Giemsa's stained human epidermoid carcinoma (HEP-2) monolayers after 24 hours exposure. DON inhibited 84% of NS-1 and 25% of HEP-2 cell viability at concentrations of 2 and 5 ng/ml respectively (Robb et al., 1990). The cytotoxic effect of DON was also measured by a luciferase translation assay on a rabbit reticulocyte system and compared to the MTT assay using swine kidney PK15 cells. DON was found to inhibit 50% of rabbit reticulocyte and PK15 cells at concentrations of 1.47 and 0.757 μg/ml, respectively, after 72 hours exposure (Yike et al., 1999). A comparison among the cell lines SK, Madin Darby canine kidney (MDCK), and Helene-Langer (HeLa) cells showed different susceptibility to DON cytoxicity with 80% inhibition concentrations at levels of 0.8, >200, and 100 μg/ml, respectively, for 24 hour exposure (Hanelt et al., 1994). Reubel et al. (1989) also studied DON cytotoxicity at different exposure times,

including 2, 4, 16, and 24 hours, and at different concentrations of 0.1, 1, 10, and 100 µg/ml using four different mammalian cell lines, SK, MDCK, VERO, and bovine embryonic lung (BEL) cells. The MTT-cleavage activity of MDCK cells was scarcely influenced by DON, whereas cytotoxic effects were predominant in BEL cells at concentrations of 100 µg/ml and 10 µg/ml. A time-dependent reduction of the MTT-cleavage activity could also be seen in SK cells at a concentration of 100 µg/ml and in VERO cells at 1.0-100 µg/ml (Reubel et al., 1989). The cytotoxicity of *Fusarium* mycotoxins was evaluated using a trichothecene sensitive baby hamster kidney (BHK-21) cell line in combination with the MTT-cleavage test. The $LC_{50}$ values of T-2 and DON were found to be 1.6 and 112 ng/ml respectively, after 24 hours exposure (Rotter et al., 1993). In conclusion, the BHK-21 cell line was the most sensitive to evaluate DON cytotoxicity among the cell lines tested, followed by SK cells with an $IC_{50}$ at a concentration of 0.8 µg/ml. The $IC_{50}$ values of DON on a human erythroleukemia cell line (K-562), an EBV-transformed human lymphoid B-cell line (MIN-GL1), as well as its inhibitory effect on proliferation of phytohemagglutinin-stimulated human peripheral blood lymphocytes were found at concentrations of 0.3, 0.4 and 0.43 µg/ml respectively. In addition, $IC_{50}$ values of acetate derivatives of DON on K-562, MIN-GL1 cells and phytohemagglutinin-stimulated human peripheral blood lymphocytes were found at concentrations of 0.4, 2.0, and 0.45 µg/ml for 15-acetylDON and 2.0, 7.0, and 2.1 µg/ml for 3-acetylDON, respectively. A large decrease in cytotoxicity of DON compared with the other trichothecenes was observed because of the carbonyl group at the C-8 position, and decreased toxicity with acetylation at the C-3 and C-15 position (Visconti et al.,

1991). The effects of DON on the response of bovine peripherial blood mononuclear cells (PBM) *in vitro* to the mitogens concanavalin A (Con A), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were determined after 4 days incubation. The concentrations of DON required to reduce the proliferative response of PBM by 50% for Con A, PHA and PWM as measured by $^{3}$H-thymidine uptake and MTT bioassay were found to be 0.07, 0.09, 0.04 μg/ml, and 0.70, 0.50, 0.50 μg/ml respectively. Further cytotoxicity assays including the lactate dehydrogenase bioassay (LDH) and Trypan blue exclusion were performed only on Con A-stimulated PBM cells after 72 hours incubation. The 50% inhibition levels of DON with LDH bioassay and Trypan blue exclusion were 0.4 μg/ml and 2.3 μg/ml respectively (Charoenpornsook et al., 1998). Another study found a 50% inhibition of DON in PHA-stimulated lymphocytes proliferation for 5 days using the MTT assay with a concentration of 216 ng/ml (Meky et al., 2001). The B cell lymphoma line (CH12LX) was used to determine the effect of DON after 3 days exposure and the $IC_{50}$ value was found to be 130 ng/ml (Minervini et al., 1993). Ji et al. (1998) reported that DON at a concentration of 50 ng/ml or higher was found to significantly decrease proliferation and viability of murine macrophage RAW 264.7 cells as measured by the MTT bioassay after 24 hours exposure. Yang et al. (2000) showed close correlation between the cytotoxic and apoptotic capacities of DON using RAW 264.7 and human leukemic cell lines (U937). DON was found not toxic at concentrations of 5 and 10 ng/ml and did not cause apoptosis in either one of these cell lines after 6 hours exposure (Yang et al., 2000). Nagase et al. (2001) also found the same