Distribution of Deoxynivalenol and Zearalenone in Milled Fractions of Wheat[1]

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ABSTRACT

Wheat samples collected from various locations in Kansas were milled at 16% moisture content using a Buhler mill. Wheat bran, shorts, and flour from samples invaded with *Fusarium graminearum* and several noninvaded samples were analyzed for deoxynivalenol and zearalenone using thin-layer chromatography. The presence of deoxynivalenol and zearalenone on random samples was confirmed using gas chromatography. When present in milled fractions, the levels of deoxynivalenol and zearalenone were generally highest in the bran and lowest in the flour. Ten of 27 bran samples (37%) and six of 27 shorts samples (22%) contained deoxynivalenol levels that exceeded 1 ppm. Three of 27 samples (11%) yielded flour with deoxynivalenol levels that exceeded 1 ppm. Zearalenone levels exceeded 1 ppm in five of 27 bran samples (19%) and four of 27 shorts samples (15%). None of the flour fractions contained >1 ppm zearalenone.

Deoxynivalenol (DON) and zearalenone are major metabolites produced by *Fusarium graminearum* Schwabe. This fungus causes scab or head blight on wheat and other cereals. Growth and toxin production by *F. graminearum* is favored by low temperatures, high rainfall, and high relative humidity.

DON is one of the most common trichothecene toxins produced by *F. graminearum*. It causes feed refusal and emesis in pigs and experimental animals (Marasas and Nelson 1987). Cases of vomiting, nausea, abdominal pain, and diarrhea in humans following consumption of scabby grain have been reported in Japan (Marasas and Nelson 1987). In September 1993, the Food and Drug Administration (FDA) issued new "advisory levels" for DON that range from 1 ppm for finished wheat products such as flour, bran, and germ destined for human consumption to 5–10 ppm for grains and grain by-products for animal feeds depending on the animal and ration (*personal communication*, Ronald G. Chesemore, Associate Commissioner for Regulatory Affairs, FDA, to state agricultural directors, state feed control officials and food, feed, and grain trade organizations, September 16, 1993).

Zearalenone, an estrogenic metabolite, commonly occurs with DON in cereal crops in the United States (Bennett and Shotwell 1979, Wood 1992). Zearalenone induces feminization at dietary concentrations of less than 1 ppm, whereas higher concentrations interfere with conception, ovulation, implantation, fetal development, and the viability of newborn animals (CAST 1989).

Previous studies conducted on the effect of milling on DON in wheat were mostly on eastern Canadian wheats: hard white wheat (Hart and Braselton 1983), hard spring wheat (Scott et al 1983), Ontario soft white winter and Quebec red hard spring wheat (Young et al 1984), and durum and red spring wheat (Nowicki et al 1988). Milling studies conducted so far on U.S. wheats were on hard red winter wheat (Hart and Braselton 1983, Seitz et al 1986) and soft wheat (Seitz et al 1985). The effect of milling on zearalenone in Korean wheat was reported (Lee et al 1987).

The crop year from July 1992 through June 1993 for all districts in Kansas was characterized by above-normal total precipitation. For most of the spring and summer of 1993, heavy and frequent rains saturated fields in central and eastern Kansas (Roozeboom et al 1993). Such a condition favors growth and toxin production by *F. graminearum*, the fungus that produces DON and zearalenone.

Our major objective was to determine the distribution and level of DON and zearalenone in milled fractions from the 1993 Kansas hard red winter wheat milling performance study.

MATERIALS AND METHODS

Wheat Samples

Fifty-two large-scale samples of 1993 hard red winter wheat from east central, northeast, north central, northwest, and south central Kansas (Fig. 1) were tempered to 16% moisture content for 18 hr and then milled using a Buhler mill (Satumbaga 1994). Bran, shorts, and flour samples were placed in plastic bags and stored at 5°C until analyzed.

Determination of Fungal Invasion

Wheat samples were surface-disinfected by shaking for 1 min in 2% NaClO (Clorox) and rinsed in sterile distilled water. One hundred kernels were placed on plates of malt agar (MS6T) containing 6% NaCl and 200 ppm Tergitol NPX (Sigma Chemical Co., St Louis, MO). The plates were incubated at room temperature (25–27°C) for five to seven days. The number of kernels yielding fungi was counted, and fungi were identified using a dissecting microscope. Identification of *F. graminearum* was confirmed using carnation leaf agar based on Nelson et al (1983).

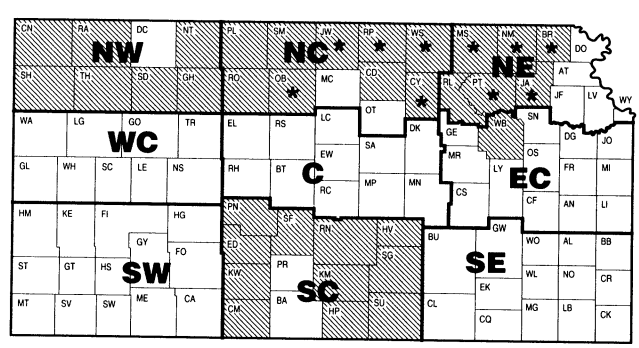

Fig. 1. Kansas map showing counties (shaded) where hard red winter wheat samples were collected. Counties with an asterisk (*) had samples that contained deoxynivalenol or zearalenone, or both. N = north, W = west, C = central, E = east, S = south.

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Mycotoxin Analyses

Bran, shorts, and flour samples from the 15 wheat lots invaded by *F. graminearum* and 12 randomly selected wheat lots of those apparently noninvaded were analyzed. DON and zearalenone were determined using Mycotest three-toxin quantitative test kits (Romer Labs Inc., Union, MO) for thin-layer chromatography (TLC).

Extraction. Twenty-five grams of wheat, bran, shorts, or flour sample and 100 ml of acetonitrile (CH_3CN) and water (84:16) were blended for 3 min at high speed in a Waring Blendor. About 5 ml of filtered extract was placed in a 15- × 85-mm culture tube. Ten microliters of concentrated acetic acid was added using a microliter capillary pipet. After being shaken for a few seconds, more than 2 ml of the filtrate was pushed through a Mycosep no. 224 multifunctional cleanup column (Romer Labs, Inc.). A 2-ml aliquot of purified extract was transferred to a 10-ml culture tube and evaporated to dryness using a hot water bath at 60°C and vacuum.

TLC. With a 500-µl syringe, 100 µl of toluene and CH_3CN (97:3) was added to the residue in the culture tube. The tube was stoppered and mixed on a vortex mixer for approximately 30 sec. With capillary pipets, 20 µl each of sample along with 5, 10, and 20 µl of DON and zearalenone standards were spotted on a 10-cm-high silica gel TLC plate. The plate was placed into a developing tank containing 50 ml of toluene and acetone (1:1) until the solvent traveled to about 1.0 cm from the top of the plate. The plate was blown dry using a hair drier and then dipped into 20% aluminum chloride ($AlCl_3$) in methanol. The plate was allowed to air dry, then was observed under long-wave UV light for a zearalenone light blue fluorescent spot at 0.7 R_f value. The TLC plate was placed on a hot plate (140–150°C) and viewed under long-wave UV light while being heated. The plate was removed from the hot plate when DON standard spots appeared at 0.3 R_f. The levels of DON and zearalenone were estimated visually by comparison with standards. The amount (in nanograms) of toxin in the sample spot was divided by 0.1 g sample equivalent and by 1,000 to obtain parts per million (ppm) of toxin in the initial sample.

Confirmation of Mycotoxins

DON confirmation. Random samples were analyzed using a gas chromatograph (GC) with an electron capture detector based on the procedure described by Richard et al (1993) with minor modifications. The detection limit was 0.01 ppm. Briefly, evaporated extracts in culture tubes, obtained as described above, were dissolved in 1 ml of toluene and CH_3CN (95:5) then mixed for 5 sec on a vortex mixer. Fifty microliters of heptafluorobutyrylimidazole (HFBI) was added; the mixture was agitated for 5 sec on a vortex mixer and heated at 60°C in a sand bath for 1 hr. After the mixture was cooled to room temperature, 1 ml of 3% sodium bicarbonate was added and the mixture agitated vigorously on a vortex mixer for 30 sec to form fine emulsions. The phases were allowed to separate. A syringe was used to transfer 50 µl of the upper phase into a 1.8-ml crimped vial along with 950 µl of hexane. The DON standard (100–125 pg/µl) in CH_3CN was evaporated and derivatized similarly.

TABLE I
***Fusarium graminearum* Invasion and Deoxynivalenol and Zearalenone Concentrations in Whole Wheat**

Range of Infection, %	Number of Samples		Mean Toxin Concentrations, ppm	
	Tested	With Toxin	Deoxynivalenol	Zearalenone
0	12	5	0.6	0.4
1–10	9	3	0.9	0.3
11–20	3	3	1.3	0.2
21–30	0	0	0.0	0.0
31–54	3	3	3.8	3.7

A Varian 3600 gas chromatograph with a ^{63}Ni electron capture detector and a megabore (0.53 mm) DB-5 capillary column, 15 m, and 1.5-µm film thickness was used. The carrier and make-up gas used was carrier grade nitrogen. The gas flow was set at 6.5 ml/min. The column temperature was programmed from 150 to 210°C at 5°C/min. The initial column hold time was 1 min, and the final column hold time was 2 min. The injector and detector temperatures were set at 220 and 300°C, respectively. The retention time for the DON-heptafluorobutyryl derivative at this setting was approximately at 10.5 min.

The amount of DON in the sample was calculated based on the formula by Ware et al (1986), which was adopted as an official method by the Association of Official Analytical Chemists (AOAC 1990). Briefly, 2 µl of sample extract was injected into the GC under the same conditions used for preparing a standard curve. The amount of DON in the sample was calculated by comparing the peak area of the sample with the peak area of the derivatized DON standard, using the formula:

$$\text{DON, ng/g} = (C'/C) \times (V'/V) \times (\text{PA}/\text{PA}')$$

where C' = concentration of DON standard (ng/µl), V' = volume of DON standard injected (µl), PA = peak area of sample, PA′ = peak area of standard, C = concentration of sample (0.0002 g/µl since 25 g sample was used), and V = volume of sample extract injected (µl). A standard curve was prepared before injecting the sample extracts into the GC. Derivatized DON (1–5 µl) was injected into the column to obtain a peak response. A standard curve was constructed by plotting the amount of derivatized DON versus detector response for a 100–500 pg range.

Zearalenone confirmation. Random samples were confirmed using gas chromatography based on the procedures described by Mirocha et al (1974) and Richardson et al (1985).

Evaporated extracts obtained as for DON-GC analysis were derivatized for 20 min at room temperature using 100 µl of Tri-Sil TBT (Pierce, Rockford, IL). A similar derivatization procedure for zearalenone standard solution (2 µg/µl) in CH_3CN was used.

A Varian 3600 GC with a flame ionization detector was used. The column was a capillary, DB-5, 0.25 mm × 30 m with 0.25-µm film thickness. The carrier and make-up gas was carrier-grade helium with flow adjusted at 30 ml/min. Injector and detector temperatures were set at 220 and 300°C, respectively. The oven was programmed from 150 to 260°C at 6°C/min. The final column temperature was held for 5 min. The retention time for the trimethylsilyl-zearalenone derivative at this setting was ≈19.5 min.

Determination of Ash and Protein

Since ash and protein contents of bran, shorts, and flour vary, we determined whether they were correlated with toxin level. Ash, protein, and moisture content of the bran, shorts, and flour were determined following AACC methods 08-01, 46-16, and 44-15A, respectively (AACC 1995).

RESULTS AND DISCUSSION

Level of *F. graminearum* Invasion

F. graminearum was detected in 29% of the hard red winter wheat samples. The percentage of infected kernels ranged from 1 to 54% (Table I). Invaded samples were collected from northeast and north central Kansas (Fig. 1). Three samples were highly invaded; two (with 47 and 48% infection) were from northeast Kansas and one (with 51% infection) from north central Kansas. These areas had unusually high levels of precipitation during flowering and harvest in the 1993 crop year (Trigo-Stockli et al 1995). Cook (1981) reported that moisture during flowering is the most important factor affecting incidence of *Fusarium* head blight.

Levels of DON and Zearalenone in Whole Wheat Samples

Of the 15 samples in which *F. graminearum* was detected, nine samples (60%) contained DON or zearalenone. Five of the 12 samples (42%) in which *F. graminearum* was not detected during plating contained DON or zearalenone (Table I). This may have resulted from the extensive growth of other molds, which obscured the growth of *F. graminearum* and made them undetectable during plating (Shotwell et al 1980). The same samples were graded U.S. no. 5 by the Federal Grain Inspection Service in Kansas City, indicating that the samples were of low quality.

The level of DON in whole wheat samples increased with an increase in *F. graminearum* invasion (Table I). This observation agrees with that reported by McMullen et al (1993) and Trigo-Stockli et al (1995). We did not observe the same trend with zearalenone. However, the highest level of zearalenone was observed on samples that were highly invaded with *F. graminearum* (Table I).

The levels of DON and zearalenone in whole wheat samples were generally lower than the levels observed in the bran and shorts (Fig. 2). This finding is similar to a previous report by Hart and Braselton (1983). Although they speculated that it may have been due to the growth of fungi during a week-long tempering process, we attribute it to the concentration of toxins in the milled fractions other than flour. This indicates further that DON and zearalenone were generally not uniformly distributed in the wheat kernels.

Levels of DON and Zearalenone in Milled Fractions

Of the 27 samples each of bran, shorts, and flour tested, 52, 44, and 15%, respectively, contained DON, zearalenone, or both (Table II). The levels of DON and zearalenone ranged from <1 to >5 ppm.

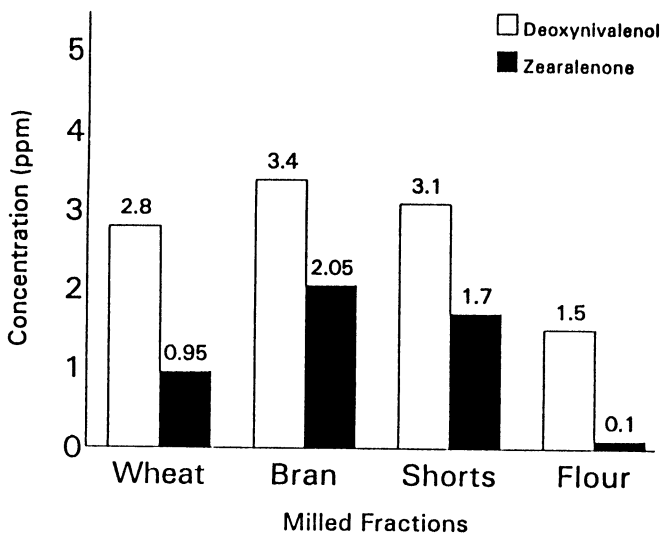

Fig. 2. Levels of deoxynivalenol and zearalenone in milled fractions of wheat. Data are averages of toxin levels in samples that contained deoxynivalenol, zearalenone, or both.

TABLE II
Milled Fractions of Hard Red Winter Wheat Tested and Levels of Deoxynivalenol (DON) and Zearalenone Detected

Milled Fraction	Number of Fractions Tested	Number of Fractions with Toxin and Toxin Level, ppm		
		DON No. (Level)	Zearalenone No. (Level)	DON and Zearalenone No. (Level)
Bran	27	4 (<1–2)	3 (0–<1)	7 (2–>5)
Shorts	27	4 (<1–2)	3 (0–<1)	5 (0–>5)
Flour	27	2 (0–2)	0 (0)	2 (<1–5)

As in previous studies (Scott et al 1983; Young et al 1984; Seitz et al 1985, 1986; Lee et al 1987), DON and zearalenone were present in the bran, shorts, and flour. Seitz and Bechtel (1985) showed that *F. graminearum* hyphae are present in the central endosperm even in lightly infected wheat kernels. Moreover, they reported that DON is usually associated with the fungal hyphae, as indicated by the high correlation between ergosterol and DON levels.

DON and zearalenone levels were highest in the bran and lowest in the flour (Figs. 2 and 3). This observation is similar to previous reports on wheat (Young et al 1984, Seitz et al 1985, Nowicki et al 1988) and corn (Bennett et al 1976). The high level of toxin in the bran may be attributed to the prevalence of the *F. graminearum* fungus in the aleurone and pericarp tissues (Bechtel et al 1985).

Since ash and protein content are highest in the bran and lowest in the flour, correlation analyses were made. DON and ash ($r = 0.319$, $P < 0.05$) and DON and protein ($r = 0.313$, $P < 0.05$) were significantly correlated. Similarly, our analyses showed significant correlations between zearalenone and ash ($r = 0.431$, $P < 0.05$), as well as zearalenone and protein ($r = 0.499$, $P < 0.05$). Ash and protein were also highly correlated ($r = 0.855$, $P < 0.001$). This shows that DON and zearalenone positively correlated with ash and protein, which are highest in the bran due to aleurone and pericarp tissues. As discussed above, *F. graminearum* is prevalent in the aleurone and pericarp tissues of an infected wheat kernel. This result is consistent with that reported by Seitz et al (1985) on soft wheat mill streams.

Several of the milled fractions from this study contained DON levels higher than the FDA advisory limit of 1 ppm for processed wheat products for human consumption. Fractions with DON levels >1 ppm were bran, 37%; shorts, 22%; and flour, 11%. Fractions with zearalenone levels >1 ppm were bran, 19%, and shorts, 15%. None of the flour fractions contained >1 ppm zearalenone.

Based on the FDA advisory levels, fractions with DON levels higher than 1 ppm cannot be utilized for bakery and other processed products for human consumption. DON is stable at baking (Scott et al 1983) and cooking temperatures (Nowicki et al 1988).

Presently, no advisory levels are set for the estrogenic metabolite zearalenone. However, concentrations above 1 ppm cause reproductive problems in animals (CAST 1989).

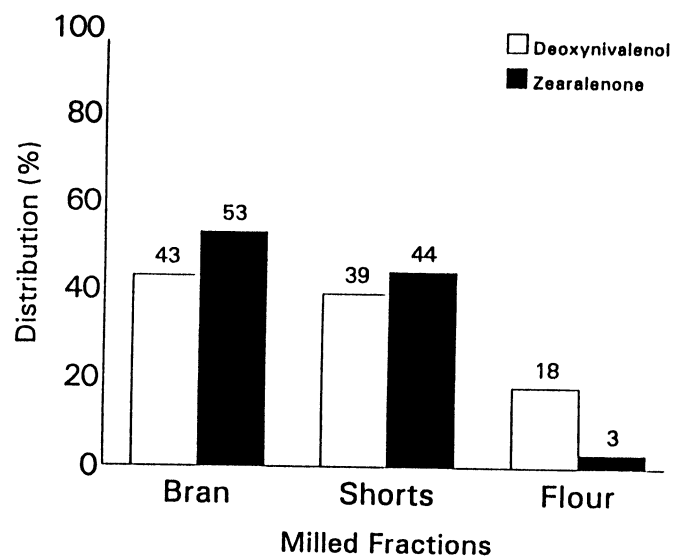

Fig. 3. Distribution of deoxynivalenol and zearalenone in milled fractions of wheat. Data are based on toxin levels in samples that contained deoxynivalenol, zearalenone, or both.

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