

Fusarium Species and 8-Keto-Trichothecene Mycotoxins in Manitoba Barley¹

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ABSTRACT

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Fusarium head blight, induced primarily by *Fusarium graminearum*, resulted in widespread damage to the Manitoba barley crops of 1993 and 1994, with contamination by deoxynivalenol (DON) and other 8-keto-trichothecenes. Visible *Fusarium* mold in samples of 1994 barley had little relationship to DON levels in the kernel as determined by gas chromatography-mass spectrometry (GC-MS). While samples of 1993 and 1994 barley showed a weak correlation between the logarithm of DON level and percentage of kernels infected by *Fusarium graminearum* ($r = 0.79$ and 0.71 , respectively), the latter method is too lengthy and requires too much training for commercial application. A commercial

enzyme immunoassay for DON gave results that correlated well with GC-MS methods ($r = 0.95$ and 0.89 , respectively) in samples of 1993 and 1994 barley and afforded a rapid and convenient method for screening. In barley samples from 1994, DON, 15-acetylDON, 3-acetylDON and 3,15-diacetylDON were detected in the approximate ratio of 47:4:1:1. In view of the higher oral toxicities of 15-acetylDON and 3-acetylDON relative to DON, and the unknown oral toxicity of 3,15-diacetylDON, GC-MS assays might be advisable in samples positive for DON from enzyme immunoassay screening.

Wheat, corn, and barley, which together account for about two-thirds of the world production of cereals, are all susceptible to infection by *Fusarium* spp. and subsequent mycotoxin contamination. In addition to reducing quality and yield, *Fusarium* fungi can also produce various trichothecene toxins which contaminate the crop. Two of the most important species in this regard are *F. graminearum* Schwabe and *F. culmorum* (W. G. Smith) Sacc. These species typically develop during wet midsummer weather to produce fusarium head blight (known as scab in the United States) in barley and wheat. These fungi can also produce mycotoxins of the 8-keto-trichothecene group (Thrane 1989) at high enough levels to produce toxic effects in livestock. Disease outbreaks affecting only a fraction of the heads in a field are capable of producing a crop that exceeds accepted tolerances for mycotoxins in human food or animal feed (Miller 1994).

The mycotoxin most often associated with fusarium head blight is deoxynivalenol (DON), although acetylated analogs of DON may also be present. *F. graminearum* can produce both 3-acetyldeoxynivalenol (3-acetylDON) and 15-acetyldeoxynivalenol (15-acetylDON) in addition to DON in cultures (Mirocha et al 1989) and in field crops. This poses a potential problem for consumers since these acetylated analogs possess a higher oral toxicity than DON itself (Ueno and Ishii 1985, Forsell et al 1987). Both DON and acetylated DON analogs have been recently detected in barley from Japan (Yoshizawa and Jin 1995), from Poland (Perkowski et al. 1995), and from North Dakota (Schwartz et al 1995). Recently, 3,15-diacetyldeoxynivalenol (3,15-diacetylDON) (Fig. 1) has been found in Manitoba barley (Usleber et al 1996), and this is the first report of the trichothecene being detected as a natural contaminant.

In 1993 and 1994, southern Manitoba experienced exceptionally high midsummer rainfall, followed by an epidemic of fusarium head blight in barley and wheat. This resulted in deliveries to country elevators of grain frequently having visible *Fusarium* mold, and in numerous requests for DON testing in affected crops.

The purpose of this study was to determine the characteristics of *Fusarium* infection and trichothecene contamination in Manitoba barley affected by fusarium head blight. Also, results from enzyme immunoassay (EIA) and gas chromatography-mass spectrometry (GC-MS) methods were compared, so that the efficacy of EIA for rapid screening of trichothecenes in barley could be evaluated.

MATERIALS AND METHODS

Standards, Reagents, Media, and Materials

Commercial firms which supplied standards, reagents, media and materials included: Aldrich, Milwaukee, WI [1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (dichlorodiphenyltrichloroethane, DDT)]; Analtech, Newark, DE [bonded-phase C₁₈-silica for column chromatography, 35–75 μm dia]; Bio-Rad, Hercules, CA [Bio-Beads SX-3 3%-crosslinked styrene-divinylbenzene resin, 40–80 μm dia]; Difco, Detroit, MI [potato dextrose agar]; J & W Scientific, Folsom, CA [DB-1701 GC capillary column, 0.25 mm i.d. \times 30 m long, 0.25- μm phase thickness]; E. Merck, Darmstadt, Germany [neutral aluminum oxide, 63–200 μm dia]; Nunc, Weisbaden, Germany [Type I microtiter plates]; Pierce, Rockford, IL [heptafluorobutyrylimidazole (HFBI), heptafluorobutyric acid anhydride (HFBAA)]; R-Biopharm, Darmstadt, Germany [RIDASCREEN test kit for deoxynivalenol]; Shell Chemical, Toronto, Canada [1,3,4,5,6,7,8,8-octachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran (isobenzan, Telodrin)]; Sigma, St. Louis, MO [DON, 3-acetylDON, 15-acetylDON, pyridine, acetic anhydride].

The trichothecene 3,15-diacetylDON was produced by acetylation of 5 mg of DON in 1 mL of pyridine with 1 mL of acetic anhydride at 23°C for 160 min. The reaction product was assayed by liquid chromatography using a 4 mm i.d. \times 250 mm long column of Merck Lichrospher RP-18 (5- μm particle dia), eluted with a gradient of 12.5–35% (v/v) acetonitrile in water at 45°C, and monitored for absorbance at 220 nm. The reaction product consisted of 83% 3,15-diacetylDON and 17% 3,7,15-triacetyldeoxynivalenol (3,7,15-triacetylDON) and was not further purified for GC-MS purposes, since the latter compound would not interfere with determination of HFB-trichothecenes.

Collection of Samples

Samples of harvested barley (200–500g) were collected from crop districts in the Red River Valley and Interlake region of Manitoba during 1993 and 1994. Samples were sent to the Canadian Grain Commission by producers, grain companies, and elevator agents for grade determination, DON screening, or as part of a new crop survey.

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Visible *Fusarium* Mold

Test portions of ≈ 100 g were taken from the 1994 samples for assessing visible *Fusarium* mold. Kernels were examined using an illuminated magnifier with a $3\times$ lens surrounded by a circular 22-W cool white fluorescent tube. Weights of *Fusarium*-molded barley kernels were estimated in accordance with grading specifications recently established in Canada (CGC 1993), which define *Fusarium* mold in barley as orange sporodochia and black perithecia on the kernel, and which exclude other discolorations such as blackpoint.

Fusarium Species

Barley samples were stored at -15°C until analyzed. A test portion of 50 or 100 kernels was surface-sterilized with 0.3% sodium hypochlorite solution for 1 min, dried under aseptic laminar air-flow, and placed on potato dextrose agar petri plates (10 kernels per plate). Plates were incubated for five days at $22\text{--}25^{\circ}\text{C}$, and illuminated with a 4 + 1 mixture of cool white fluorescent and long-wave ultraviolet light from 40-W tubes at 48 cm distance on a 12-hr light and 12-hr dark cycle (Abramson et al 1987). *Fusarium* species were identified according to Nelson et al (1983).

GC-MS Assay for Mycotoxins

1993 barley. A modification of the sample preparation method of Ware et al (1986) was used. Barley was ground to pass through 1.0-mm apertures, and a 25-g test portion of ground barley was shaken for 60 min with 10 mL of water and 125 mL of chloroform + methanol 8+2. The extract was filtered through fluted Whatman no. 1 paper, centrifuged at $3200 \times g$ for 5 min, and passed through a cellulose membrane with $5\text{-}\mu\text{m}$ pores. A 5-mL aliquot of the filtered extract was purified by automated gel-permeation chromatography (model AS-2000, O-I Analytical, Columbia, MO) using a 2.5 cm i.d. \times 65 cm long column of Bio-Beads SX-3 eluted with methylene chloride + cyclohexane 15+85. The purified residue was derivatized with HFBA. GC-MS analysis of heptafluorobutyrate (HFB) derivatives, with electron-impact ionization and selected-ion monitoring, was performed using a Hewlett-Packard 5792 gas chromatograph with a Hewlett-Packard 5970B mass-selective detector. DON tri-HFB, isobenzan internal standard and 15-acetylDON di-HFB were resolved using a 0.20

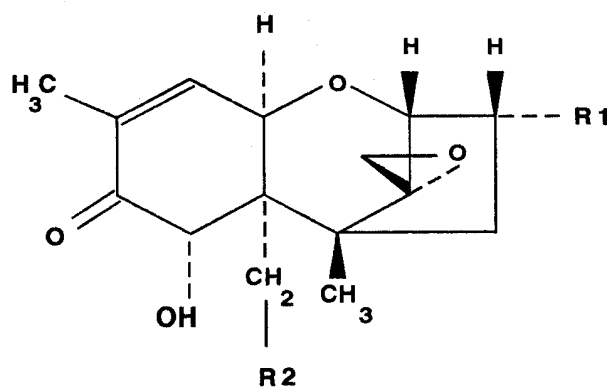
mm i.d. \times 12.5 m long capillary column of methyl silicone gum, with splitless injection, helium flow velocity of 45 cm/sec, and oven programming between 90 and 250°C . Electron-impact ionization was used to monitor: DON tri-HFB, 331 and 359; isobenzan (internal standard), 311; and 15-acetylDON di-HFB, 444 and 321. Generally, the first ion was used for quantitation, and the second ion for confirmation.

1994 barley. For sample preparation, a modification of the method of Tacke and Casper (1996) was used. Barley was ground to pass through 0.5-mm apertures, and 25-g test portions of ground barley were shaken for 2 hr with 100 mL of acetonitrile + water 86+14. After the solid material had settled, 12 mL of supernatant was transferred to a 1.8 cm i.d. \times 2.8 cm long column of alumina + C_{18} -silica 1+1 (w/w) contained in a disposable 20-mL polypropylene syringe barrel. Eluate (10 mL) was collected, and evaporated to dryness at 55°C under a nitrogen stream. Prior to analysis with a Hewlett-Packard 5988 GC-MS unit, residues were dissolved in 400 μL of benzene and derivatized with 200 μL of HFBI at 65°C for 1 hr. DON tri-HFB, 15-acetylDON di-HFB, 3-acetylDON di-HFB, DDT (internal standard), and 3,15-diacetylDON mono-HFB, were resolved (in that order) using a 0.25 mm i.d. \times 30 m long capillary column of DB-1701, with splitless injection, helium flow velocity of 41 cm/sec, and oven programming between 50 and 270°C . Electron-impact ionization was used to monitor: DON tri-HFB, 331 and 359; 15-acetylDON di-HFB, 444 and 321; 3-acetylDON di-HFB, 688 and 633; DDT (internal standard), 235; and 3,15-diacetylDON mono-HFB, 290 and 302.

EIA for Mycotoxins

Antisera from rabbits against 3-acetylDON and 15-acetylDON were prepared as previously described (Usleber et al 1991, 1993). Barley was ground to pass through 0.5-mm apertures. Test portions (2 g) of ground barley were stirred for 1 hr with 8 mL of methanol + water 7+3 at ambient temperature, filtered through Whatman no. 1 paper. A portion of the filtrate was diluted 1+6 with phosphate-buffered saline (0.01M phosphate, 0.1M NaCl, pH 7.2) and aliquots were taken for determination of 3-acetylDON (Usleber et al 1991) and 15-acetylDON (Usleber et al 1993).

Another 2-g test portion of ground barley was stirred for 2 hr with 10 mL of acetonitrile + water 84+16 at ambient temperature. A 1-mL portion was evaporated to dryness with a rotary vacuum evaporator, acetylated with acetic anhydride, and the DON content determined by the method of Schmitt et al (1996) using a commercial test kit from R-Biopharm.



	R1	R2
DON	OH	OH
3-AcetylDON	Acetyl	OH
15-AcetylDON	OH	Acetyl
3,15-DiacetylDON	Acetyl	Acetyl

Fig. 1. Structures of some 8-keto-trichothecene mycotoxins from *Fusarium* species.

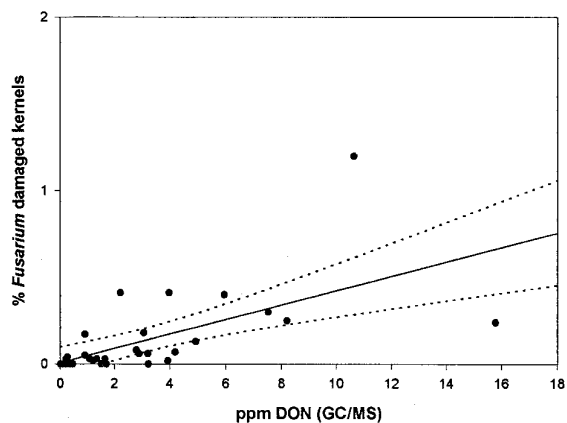


Fig. 2. Relationship between the percentage (by weight) of visibly *Fusarium*-molded kernels and deoxynivalenol (DON) content determined by gas chromatography-mass spectrometry in 1994 barley samples from Manitoba. Dotted lines represent 95% confidence limits.

RESULTS AND DISCUSSION

Visible *Fusarium* Mold

In Fig. 2, visible *Fusarium* mold on the barley kernel appeared to be an inadequate criterion for estimating the risk of DON contamination for *Fusarium* mold levels $\leq 0.5\%$ ($r = 0.59$), and there were not sufficient data to draw any conclusions for *Fusarium* mold levels $> 0.5\%$. The difficulty arises partly from the structure of the barley kernel. In barley after harvest, symptoms of *Fusarium* infection are often hard to see, because the lemma and palea are retained on the kernel and cover damage occurring to the caryopsis. Many barley kernels infected by *F. graminearum* displayed a dark discoloration at the basal end of the kernel called blackpoint, but this was not specific for *Fusarium* infection since it is also associated with infection of kernels by the field fungi *Helminthosporium sativum* and *Alternaria alternata* (Clear et al 1996).

Fusarium Species

Species identified in the 1993 and 1994 barley samples included *F. acuminatum* Ell. & Ev., *F. avenaceum* (Fr.) Sacc., *F. culmorum*, *F. equiseti* (Corda) Sacc., *F. graminearum*, *F. oxysporum* Schlecht. emend. Snyder & Hansen, *F. poae* (Peck) Wollenw., *F. sporotrichioides* Sherb., and *F. tricinctum* (Corda) Sacc. The incidence of these species in samples of 1993 and 1994 barley is given in Table I. *F. nivale* (Fr.) Cef. was identified only on a single kernel of 1993 barley, and is not listed.

Fusarium head blight may be caused in wheat and barley by several *Fusarium* species (Miller 1994). *F. graminearum*, the species found most often in affected crops, is not always the prime cause of fusarium head blight in every region, and there is usually some year-to-year variability in the relative abundance of the various *Fusarium* species (Tekauz et al 1996). *F. graminearum* is common in Manitoba, eastern Canada, and the United States, whereas *F. culmorum* is rarely encountered in western Canadian episodes of *Fusarium* disease (Abramson et al 1987).

In the barley samples from 1993 and 1994, the most abundant species was the ubiquitous *F. graminearum*, with *F. avenaceum* present to a lesser degree in almost all samples, and *F. culmorum* appearing in 2–6% of kernels in a few samples (Table I). Compared to *F. graminearum* and *F. culmorum*, the pathogenicity of *F. avenaceum* towards wheat and corn ranks low, and isolates of this species appear to be relatively weak producers of DON in culture (Abramson et al 1993). *F. poae* and *F. sporotrichioides* were also present in a large proportion of samples from both years, but the average infection level was $< 8\%$ of the kernels.

The logarithm of DON content was weakly correlated with the percentage of kernels infected by *F. graminearum* in the 1993 and 1994 samples ($r = 0.79$ and 0.71 , Fig. 3A and B, respectively). In a disease outbreak, it is often necessary to determine percentages of sample kernels infected by various *Fusarium* species, but these procedures require several days and call for highly skilled workers. In epidemics of fusarium head blight in barley, *F.*

graminearum infection levels would not predict the DON content of the barley crops with high accuracy, although infection levels and mycotoxin content are obviously related.

Measurement of Mycotoxins by GC-MS and EIA

The main trichothecene contaminants in barley samples from both 1993 and 1994 were DON and 15-acetylDON (Table II). 3-AcetylDON appeared as an additional contaminant in both years, although its presence in the 1993 samples was not confirmed by GC-MS assay. Recovery of DON from barley with the method of Ware et al (1986), as used in the 1993 barley samples, is assumed to be similar to recovery of DON from wheat, which is reported as 92% at 0.1–1.0 ppm. Recovery of DON from barley with the method of Tacke and Casper (1996), as used in the 1994 barley

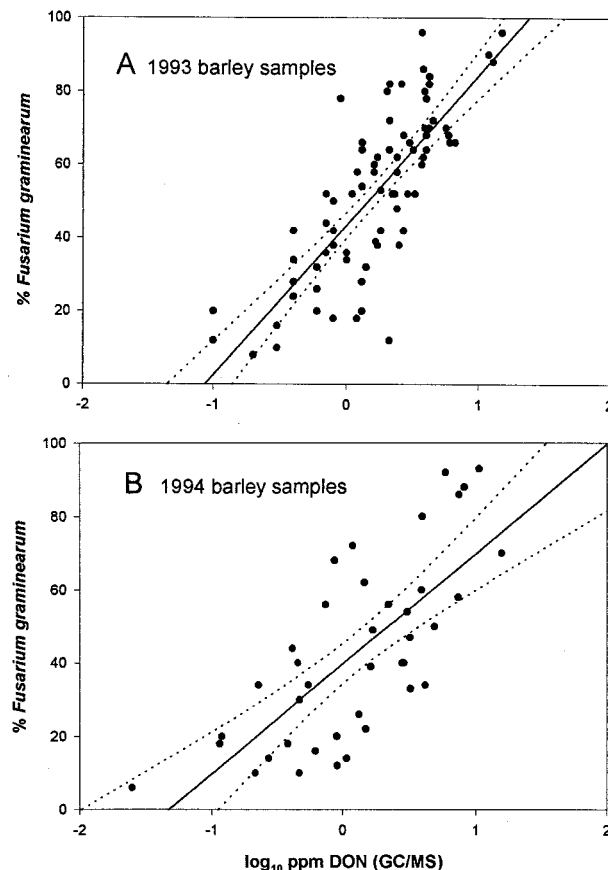


Fig. 3. Relationship between the percentage of kernels infected by *Fusarium graminearum* and the deoxynivalenol (DON) content determined by gas chromatography-mass spectrometry (GC-MS) in barley samples from Manitoba. A, 1993. B, 1994. Dotted lines represent 95% confidence limits.

TABLE I
Fusarium Species in Samples of Manitoba Barley Collected in 1993 and 1994

	1993 (77 samples)				1994 (40 samples)			
	Mean	Maximum	Minimum	Positives	Mean	Maximum	Minimum	Positives
<i>F. acuminatum</i>	3.2	24	0	47	0.7	4	0	11
<i>F. avenaceum</i>	23.0	62	0	76	10.2	38	0	36
<i>F. culmorum</i>	0.2	6	0	5	0.1	2	0	1
<i>F. equiseti</i>	1.5	12	0	36	0.9	4	0	17
<i>F. graminearum</i>	52.4	96	8	77	42.9	93	6	40
<i>F. oxysporum</i>	0.2	4	0	6	0	0	0	0
<i>F. poae</i>	1.9	12	0	31	4.7	17	0	34
<i>F. sporotrichioides</i>	7.4	42	0	67	3.1	10	0	32
<i>F. tricinctum</i>	0.1	2	0	5	0.1	2	0	2

samples, is 100-105% at ≥ 0.5 ppm. The compatibility between the GC-MS methods used to assay the 1993 and 1994 samples is underlined by the high degree of correlation between data from these GC-MS methods and from EIA, with $r = 0.95$ and 0.89 respectively.

3,15-diacetylDON, initially detected by immunochromatographic analysis in six samples of 1994 barley (Usleber et al 1996), was found in 25/40 samples of this crop by GC-MS. Remaining quantities of the 1993 crop samples were not sufficient to retest for this trichothecene. The presence of 3,15-diacetylDON in samples of 1994 Manitoba barley constitutes the first reported occurrence of this trichothecene as a natural contaminant. Previous efforts to detect this trichothecene in barley and corn from

Korea (Kim et al 1993) and in barley and wheat from Japan (Yoshizawa and Kim 1995) were not successful.

The EIA and GC-MS values for DON in the 1993 and 1994 barley samples (Fig. 4A and B) correlated very well ($r = 0.95$ and 0.89 , respectively). Compared to *F. graminearum* percentage (Fig. 3) or *Fusarium*-molded kernel percentage (Fig. 2), EIA serves as the best prediction of barley DON levels assayed by GC-MS. By both EIA and GC-MS, all barley samples contained detectable levels of this mycotoxin. In both crop years, mean DON levels in the barley samples (Table II) were >2 ppm tolerance specified for some cereals used as food in Canada and the United States, but <4 ppm tolerance for feed grains in the United States (Van Egmond and Dekker 1995).

For 15-acetylDON and 3-acetylDON, there was less correlation between EIA and GC-MS, and generally some overestimation of the values by EIA. This is not unanticipated, since the 15-acetylDON antiserum has a sixfold higher cross-reactivity toward 3,15-diacetylDON compared to 15-acetylDON, and the 3-acetylDON antiserum has a 23-fold higher cross-reactivity toward 3,15-diacetylDON compared to 3-acetylDON (Usleber et al 1996). In addition, the 3-acetylDON antiserum has a 22-fold higher cross-reactivity toward 3,7,15-triacetylDON compared to 3-acetylDON, but the 15-acetylDON antiserum has $<1\%$ cross-reactivity toward 3,7,15-triacetylDON compared to 15-acetylDON. Thus, small amounts of 3,15-diacetylDON in the barley will result in some overestimation of 3-acetylDON and 15-acetylDON. This was the case in the 1994 samples, and may account for the overestimation in the 1993 samples (Table II, Fig. 4). Detectable amounts of 3,7,15-triacetylDON were not evident in immunochromatograms of selected 1994 barley samples (Usleber et al 1996).

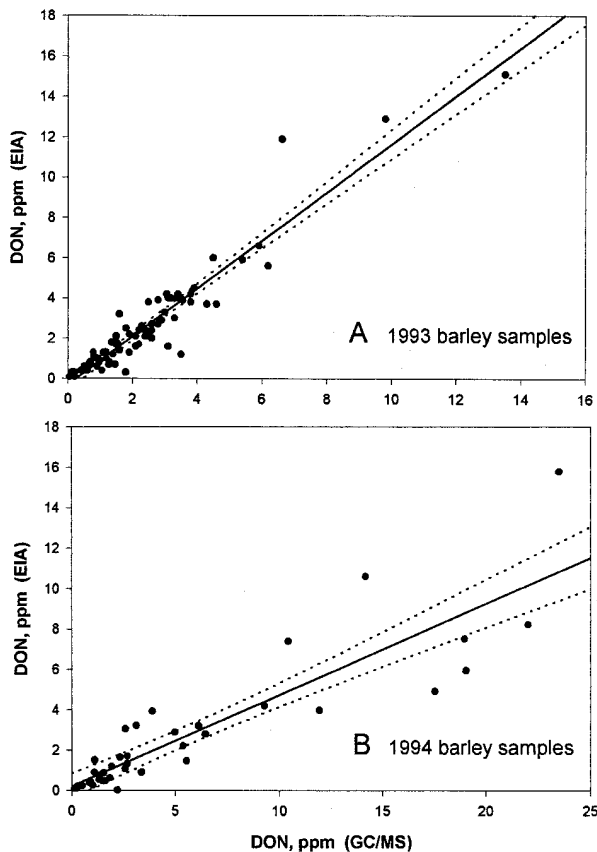


Fig. 4. Relationship between the deoxynivalenol (DON) content determined by enzyme immunoassay and the DON content determined by gas chromatography-mass spectrometry (GC-MS) in barley samples from Manitoba. **A**, 1993. **B**, 1994. Dotted lines represent 95% confidence limits.

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TABLE II
Fusarium Trichothecene Mycotoxins in Samples of Manitoba Barley Collected in 1993 and 1994

Mycotoxin ^a	Assay ^b	1993 (77 samples)				1994 (40 samples)			
		Mycotoxin Content (ppm)			Positives	Mycotoxin Content (ppm)			Positives
		Mean	Maximum	Minimum		Mean	Maximum	Minimum	
DON	GC-MS	2.56	15.10	0.10	77	2.69	15.79	0.03	40
	EIA	2.33	13.50	0.06	77	5.48	23.47	0.13	40
15-AcDON	GC-MS	0.04	0.40	0	24	0.21	1.24	0	39
	EIA	0.74	5.37	0	74	0.60	5.28	0	33
3-AcDON	GC-MS	0.04	0.35	0	24
	EIA	0.40	2.50	0	74	0.56	2.16	0	32
3,15-diAcDON	GC-MS	0.06	0.40	0	25

^a Deoxynivalenol (DON), 15-acetylDON, 3-acetylDON, and 3,15-diacetylDON.

^b Gas chromatography-mass spectrometry (GC-MS) and enzyme immunoassay (EIA).

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