Analysis of Deoxynivalenol and Its Derivatives (Batch and Single Kernel) Using Gas Chromatography/Mass Spectrometry

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This paper describes a method for the analysis of DON and its derivatives in wheat and barley using combination gas chromatography/mass spectrometry (GC/MS). The method has been designed for the analysis of large batch (100 g) samples and single kernels. The sensitivity of the method is 50 ppb (ng/g), and the precision in terms of percent standard deviation lies between 0.0 and 11.1. The percent recovery for 1, 5, and 10 μg/g (ppm) recovered from wheat is 97.2, 88.4 and 87.9% respectively. A comparison of methods was made between two laboratories using GC/EC and our method. There was no significant difference between the results of the two methods. The method is also applicable to 15-acetyldeoxynivalenol (15-ADON) as well as nivalenol (NIV). Standard curves constructed for DON, 15-ADON, and NIV show a linear relationship between 0.025 ng (limit of sensitivity) and 8 ng.

Keywords: *Deoxynivalenol; nivalenol; analytical method*

INTRODUCTION

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), also known as DON and vomitoxin, is one of the most common and abundant naturally occurring trichothecenes found in corn, wheat, and barley. It is found on cereal grains throughout the world.

DON is a mycotoxin produced by various species of *Fusarium* but most commonly identified with *Fusarium graminearum*. This fungus causes headblight of cereal grains such as wheat, barley, and oats and kernel and stalk infection in corn. DON affects swine and dogs by causing refusal of feed, weight loss, and vomiting (Mirocha et al. 1980). It also can cause vomiting in humans as well as headache, fever, upset stomach, and diarrhea (Kim et al. 1993).

Yoshizawa and Morooka (1973) reported on the isolation and characterization of deoxynivalenol after isolation of *F. graminearum* from red molded wheat and barley and growing it in laboratory culture. Its isolation and identification was prompted by a severe epidemic of *Fusarium* headblight of barley (red mold disease) in the Kagawa prefecture in Japan in 1970.

Trucksess et al. (1984) described a method of analysis of DON in wheat using TLC (limit of detection 40 ng/g). Fernandez et al. (1994) also used TLC with a detection limit of 40 ng/g. Chang et al. (1984) using HPLC described a method with a detection limit of 30 ng/g. Scott et al. (1986, 1981) used gas chromatography with electron capture detection (GC/EC) and mass spectral detection (GC/MS). Croteau et al. (1994) also described a GC/EC method with a limit of detection of DON in corn of 50 ng/g. We are confining our presentation to analyses by GC/MS using selected ion monitoring (SIM). The general method involved is based (especially the cleanup procedure) on that described by Tacke and Casper et al. (1996) using gas chromatography in combination with electron capture. It is our intention to describe a GC/MS method for the analysis of DON and its derivatives (batch and single-kernel analyses) in wheat and barley. The fragment ions used are specific for each DON derivative. The data are presented for both DON and 15-acetyldeoxynivalenol (15-ADON). The method described allows analysis of single kernels in studies of kinetics of DON synthesis during pathogenesis.

MATERIALS AND METHODS

Apparatus. All analyses were made on a Shimadzu QP-5000 combination GC/MS using CLASS 5000 software (version 1.24), splitless mode of injection of sample as well as pressure compensation for increase in column temperature. The autosampler used was a Shimadzu AOC-1400 with a AOC-17 Auto Injector. The chromatographic column used was a J&W DB-5MS, 0.25 μm film thickness, 250 μm i.d. and 30 m in length. The temperature program used: initial column temperature of 80 °C for 1 min and ramped at 25/min to 280 °C and isothermal for 6 min. The injection temperature is kept at 300 °C; column flow rate of 1 mL/min.

Sample Preparation. Wheat and barley samples are dried to a water content of approximately 15% before grinding. A 100 g aliquot of the sample is ground to an approximate 20 mesh size in a Stein Mill (Model M-2, Fred Stein Laboratories Inc., Atchison, KS) or any comparable mill. The sample is ground for 2 min. Twenty-five grams of the sample is used for extraction.

Extraction and Column Cleanup Procedure. Take 25 g of the finely ground wheat or barley and place into a 250 mL Erlenmeyer flask and cap with aluminum foil. Add 100 mL acetonitrile/water (84/16 v/v). Extract on a shaker for 1 h. Filter through Whatman no. 4 filter paper into a clean vessel, and pass 4.0 mL of the filtrate through a specially prepared column as described below. One milliliter of the filtrate is placed into each of two four milliliter screw top vials. The filtrate in each vial is evaporated to dryness using nitrogen (approximately 1−2 h); one of the vials is saved in a refrigerator for use in the future. Each of the vials is defined as a duplicate.

Column Packing Procedure. The column preparation procedure has been described by Tacke and Casper (1996) as follows: Press filter paper disk (S&S no. 740-E, 0.5 in.

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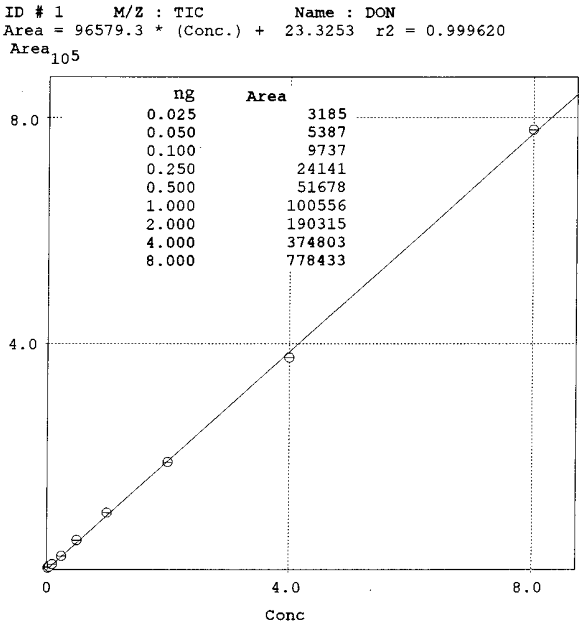
Table 1. Mass Spectral Molecular and Fragment Ions (Electron Impact) of the Trimethylsilyl Ether Derivatives of Deoxynivalenol and Derivatives and Mirex as well as Retention Times on a 30 m J&W DB-5MS Gas Chromatography Column

derivative[a]	diagnostic fragment ions, m/z values	GC retention time, min
DON	**103**, 197, **235**, 392, 422, **512**	9.56
7-ADON	**103**, 259, **289**, 317, **392**, 422, **482**	9.99
fusarenone-x	**103**, 191, 251, **480**, **570**	9.88
3-ADON	**117**, 103, 235, 289, 377, **392**, **482**	10.06
15-ADON	**193**, **235**, 350, **392**, 422, **482**	10.09
nivalenol	103, **191**, **289**, 379, 482, **600**	10.22
3,15-DADON	193, 289, **320**, 377, **540**	10.76
4,15-DADON	**117**, 235, 289, **408**, 450, 465, **540**	10.94
Mirex	235, 289, 333, **506**	11.63

[a] DON = deoxynivalenol; fusarenone-x = 4-acetylnivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; 3,15-DADON = 3,15-diacetyldeoxynivalenol. 4,15-DADON = 4,15-diacetyldeoxynivalenol. Mirex = 1,3,4-Metheno-1*H*-cyclobuta[*c*,*d*]pentalene, 1,1a,2,2,3,3a,4,5,5,5a,5b-undecachlorooctahydro-. Underlined m/z value = base peak and molecular ion, respectively. Highlighted m/z value = diagnostic major ion fragment.



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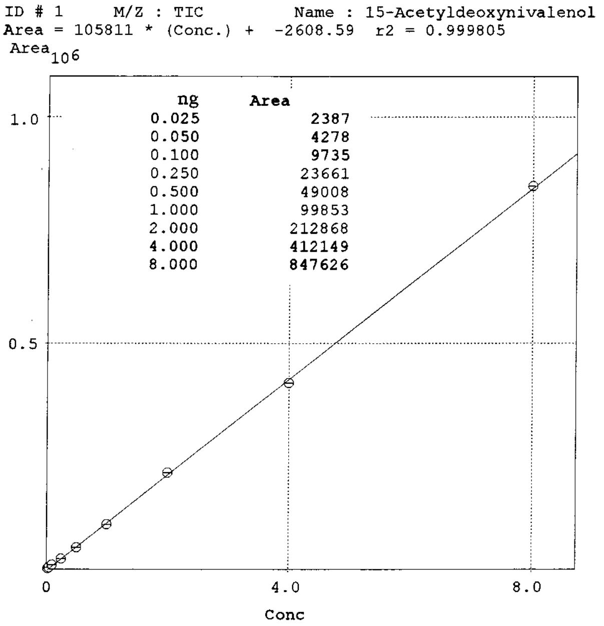
ID # 1      M/Z : TIC          Name : DON
Area = 96579.3 * (Conc.) +  23.3253  r2 = 0.999620
```

ng	Area
0.025	3185
0.050	5387
0.100	9737
0.250	24141
0.500	51678
1.000	100556
2.000	190315
4.000	374803
8.000	778433

Figure 1. Standard curve of deoxynivalenol run in a wheat extract matrix.



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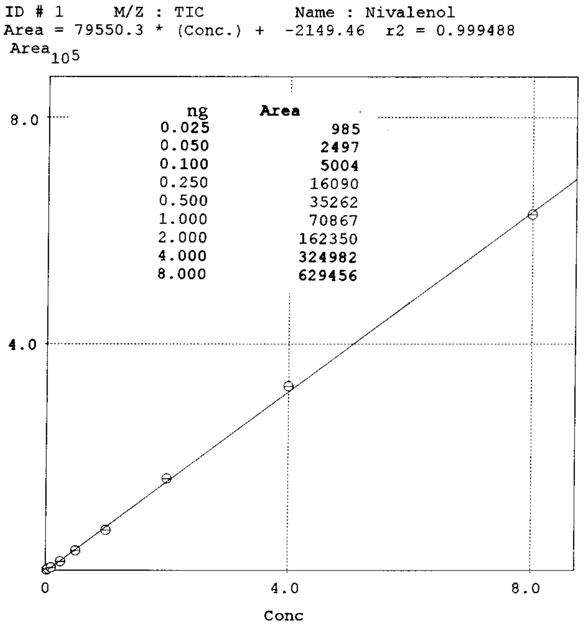
ID # 1      M/Z : TIC          Name : 15-Acetyldeoxynivalenol
Area = 105811 * (Conc.) +  -2608.59  r2 = 0.999805
```

ng	Area
0.025	2387
0.050	4278
0.100	9735
0.250	23661
0.500	49008
1.000	99853
2.000	212868
4.000	412149
8.000	847626

Figure 2. Standard curve of 15-acetyldeoxynivalenol run in a wheat matrix.



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ID # 1      M/Z : TIC          Name : Nivalenol
Area = 79550.3 * (Conc.) +  -2149.46  r2 = 0.999488
```

ng	Area
0.025	985
0.050	2497
0.100	5004
0.250	16090
0.500	35262
1.000	70867
2.000	162350
4.000	324982
8.000	629456

Figure 3. Standard curve of nivalenol run in a wheat matrix.

diameter) into the bottom of a 6 mL plastic syringe. Mix thoroughly one part C-18 packing material (Bakerbond no. 7025-01 octadecyl, 40 μm particle size packing, J. T. Baker Inc.) to 3 parts aluminum oxide neutral (EM Science #1077). Add 1 g of mixed packing material to the syringe and press filter disk gently on the top surface. Place in 60 °C oven overnight before use. Columns may be sealed in a plastic bag after the drying step.

Derivatization. Add 100 μL of the silylating reagent (TMSI/TMCS 100:1 obtained from Pierce Chemical Co.) to each vial, cap, and vortex gently to ensure that the reagent coats the side of the vial. Allow to react for 15 min at room temperature. Add 0.9 mL of isooctane and vortex gently. Add 1 mL of glass distilled water and vortex so that the milky isooctane layer becomes transparent. Transfer the upper layer (isooctane) into a 1.5 mL GC vial and cap. The 1 mL aliquot of filtrate passed through the column represents 0.25 g of sample in 1.0 mL of solution. One microliter of this is injected into the Shimadzu QP-5000 GC/MS and represents 0.25 mg equivalents of the original barley or wheat sample. To calculate parts per million (ng/mg), the absolute amount of DON found is multiplied by a factor of 4.0 assuming 87% recovery.

Preparation for Single Kernel Analysis. Single kernels of wheat or barley are weighed and then placed individually into culture tubes. One and a half milliliters of acetonitrile: water (86:14 v/v) are placed into the tubes and capped with Parafilm and allowed to extract by soaking and shaking for 24 h. Approximately 1.5 mL of the extract is passed through a minicolumn (1 mL tuberculin syringe packed with 150 mg of 1:3 (w/w) C-18:aluminum oxide and capped with 1/4 in. disks). One milliliter of the eluate is placed into a 0.5 dram vial and evaporated to dryness using nitrogen (approximately 1 to 2 h). Twenty microliters of TMS reagents (TMSI/TCMS; 100:1) is added to the vial. Rotate the vial so that the reagent makes contact with residue on the side of the vial (allow to react for 15 min); add 180 μL of isooctane followed by 200 μL of water to quench the reaction; vortex so that the milky

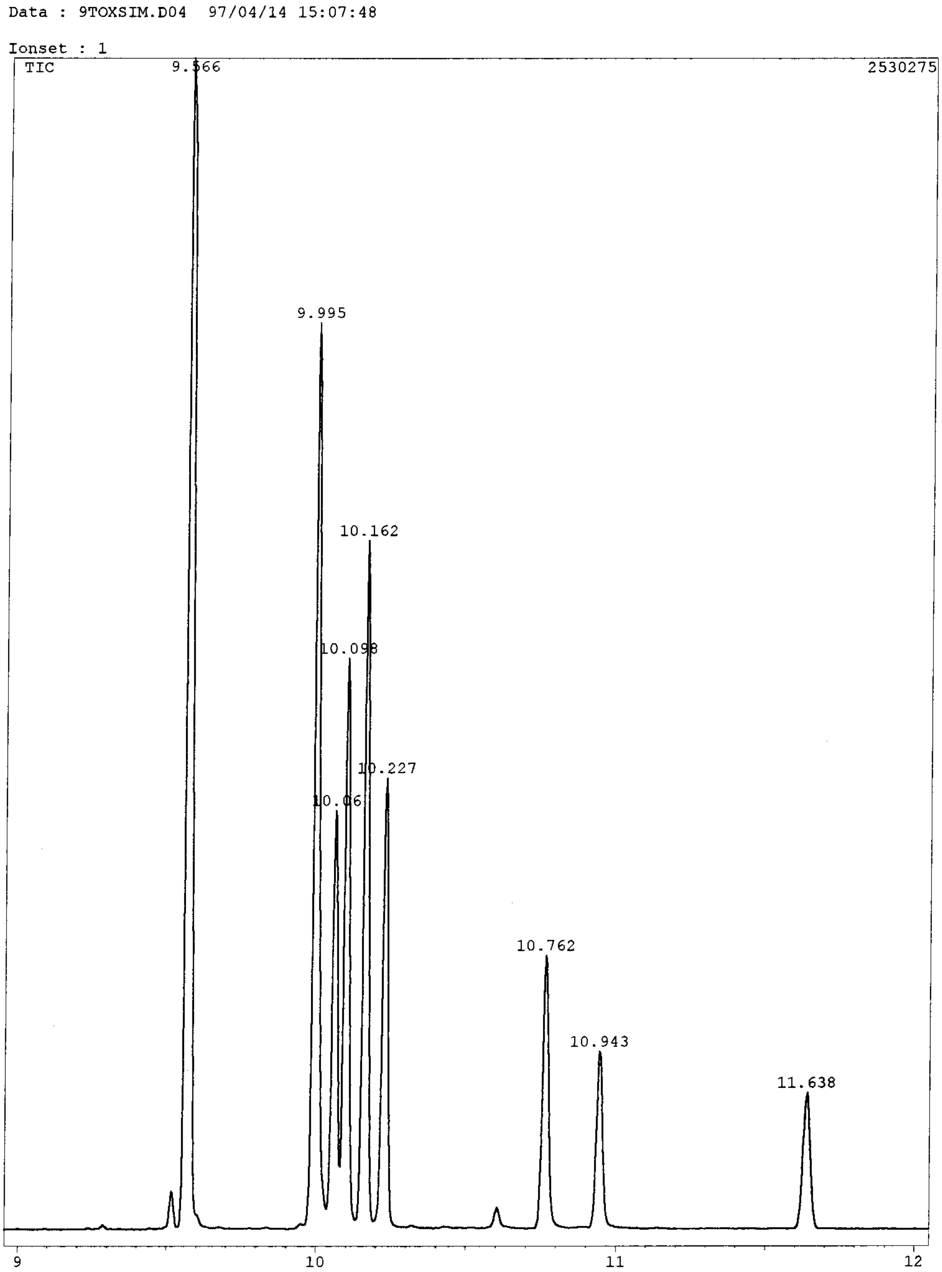

Figure 4. Gas chromatographic resolution of trimethylsilyl ether derivatives of deoxynivalenol derivatives separated on a DB-5 J&W column. The retention times are: deoxynivalenol (9.56), 7-acetyldeoxynivalenol (9.99), 4-acetyldeoxynivalenol (10.06), 3-acetyldeoxynivalenol (10.09), 15-acetyldeoxynivalenol (10.16), nivalenol (10.22), 3,15-diacetyldeoxynivalenol (10.76), 4,15-acetyldeoxynivalenol (10.94), and Mirex (11.63).

isooctane layer becomes transparent. Transfer the upper layer (isooctane) into a 1.5 mL GC vial (use 200 μL inserts) and cap.

One microliter is injected into the GC/MS; the latter represents 1/200 of the sample. The total is also multiplied by 1.5 to account for taking 1 mL of 1.5 mL total sample. The quantity of DON is expressed in ng/kernel. Weights of the individual kernels are taken, and then ppm are calculated.

Preparation of Standard Curve. Twenty-five grams of clean Norm wheat kernels are extracted as described above. Four milliliters of filtrate was passed through each of four (1:3, C-18:aluminum oxide) columns; the latter constitutes the wheat matrix into which the DON standards are added. The wheat matrix is pooled and nine 1 mL aliquots were taken and dried in 1 dram vials. The standard samples are interspersed among the analytical samples to eliminate any error encountered during the 6−8 h run.

A standard solution of DON and/or 15-ADON and/or NIV (10 ng/μL in methanol) is diluted so as to get 0.025, 0.05, 0.10, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ng/μL. A sufficient amount of standard solution was taken so that each of the latter concentrations were contained in 1 mL of solution. Example: 2.5 μL of the 10 ng/μL standard was added to obtain the 0.025 concentration, 5 μL for the 0.05 ng/μL concentration, etc. All of the solutions were dried before making the derivatives.

Analysis by GC/MS. All samples are analyzed on a Shimadzu QP-5000 GC/MS using selected ion monitoring (SIM). A standard curve of DON is run with each lot of wheat and/or barley samples. The standards (0.025, 0.05, 0.25, 0.50 1.0, 2.0, 4.0, and 8.0 ng) are interspersed among the samples being analyzed. Mirex (hexachloropentadiene dimer) is used to check on the quantitative precision of the instrument. A check sample (Norm wheat) with a known amount of naturally occurring DON (13 ppm) is used as a control for reproducibility of sample analysis.

All analyses are made by GC/MS with selected ion monitoring (SIM) and using fragment ions (*m/z* values) of 235.15,

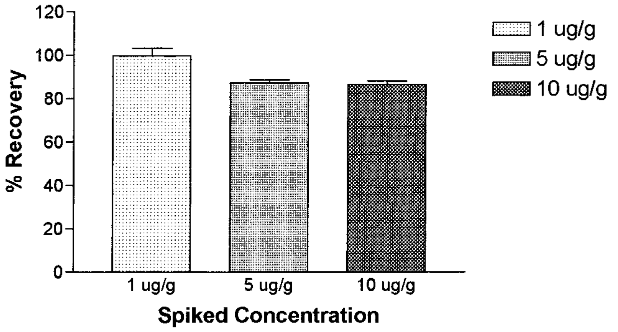

Figure 5. Percent recovery of deoxynivalenol after spiking wheat samples at 1, 5, and 10 μg/g.

422.25, 392.20, 482.30, 377.20, 480.30, 570.30, 585.30, 333.10, 289.15, and 151.15 as determinants. The total ion current generated from each analysis is used for quantitation. The *m*/*z* values used for each derivative is listed in Table 1. Although nominal masses can be used for SIM determination it is recommended that the observed mass defect be used.

RESULTS AND DISCUSSION

The standard curve of eight concentrations of DON ranging between 0.025 ng and 8 ng is shown in Figure 1. The area counts range between 3185 and 778433 from the lowest to the highest concentration when run in a wheat extract matrix as described. The *r* squared value is 0.999620. The same concentrations were run for 15-ADON in the same manner with similar results. The *r* squared value is 0.999805 (Figure 2). NIV is shown in Figure 3; its limit of detection is somewhat less than DON. The sensitivity value can vary depending on the column, condition of the filament, as well as instrument tuning. The values presented are those obtained with only normal (minimal) instrument tuning.

The separation of the various derivatives of DON analyzed as their TMS derivatives as well as the underivatized Mirex is shown in Figure 4. The chromatographic resolution obtained for all derivatives was suitable for analysis, quantitation, and full scan mass spectral analysis. DON itself was cleanly separated from all other compounds with a retention time of 9.56. The latter value will vary only slightly depending on column length, i.e., after cutting 5 cm off the leading edge for column maintenance.

The percent recovery of DON from wheat (batch analysis) after spiking with 1, 5, and 10 μg/g is shown in Figure 5. The percent recovery found with 1 μg/g (1 ppm) for four replicates is 87.2, 102.4, 99.2 and 100, respectively, with an average of 97.2%. The percent recovery for 5 μg/g (5 ppm) was 90.2, 87.58, 87.74, and 88.32 for an average of 88.46%. The percent recovery for 10 μg/g (10 ppm) was 87.06, 86.62, 88.76, and 89.37 for an average of 87.95%. The percent recovery of DON from single kernels (Figure 6) was conducted in the following manner: A 500 g sample of naturally infected wheat was thoroughly ground as described so as to get an even distribution of sample and analyzed as described. Twenty-five grams of unground kernels from the thoroughly mixed batch were analyzed by soaking as described for single-kernel analysis and their value compared to that obtained from the ground sample. It is awkward and perhaps impossible to evenly spike a hard kernel as we cannot deliver a measured amount of DON into the kernel. Although each kernel in a sample may contain different amounts of DON, we used this method to obtain an approximation of the recovery. The average concentration found in the ground batch sample was 10.0 (ng/g) ppm (11.3, 9.8, 11.3, 8.3, and 9.5). The average concentration found in the single kernels from this batch was 9.0 (ng/g) ppm (8.0, 8.0, 10.0, 9.4, and 9.4). We would expect at least 90% recovery and perhaps more depending on the concentration of DON in the single kernels.

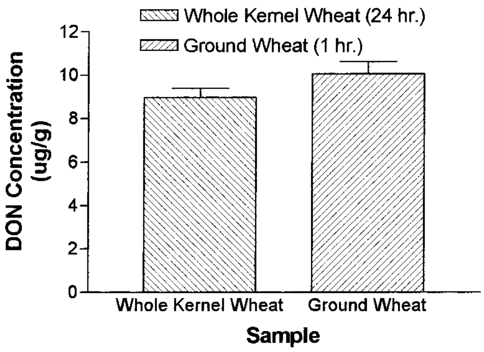

Figure 6. Comparison of recovery of deoxynivalenol from ground samples of wheat and whole kernels from the same evenly mixed batch of sample.

Table 2. Precision of the Method in Analysis of Samples of Barley for Deoxynivalenol

sample	analysis 1, μg/g (ppm)	analysis 2, μg/g (ppm)	analysis 3, μg/g (ppm)	mean	% SD
BRC.147	10	11.8	9.6	10.5	11.1
BRC.158	19.9	18.2	21.2	19.8	7.5
BRC.178	16.8	19.3	18.6	18.2	7.0
BRC.185	13.9	15.8	16.9	15.5	9.8
BRC.166	5.8	5.9	5.9	5.87	1.0
BRC.190	3.9	4	4.2	4.03	3.7
BRC.135	13.3	13.3		13.3	1.0
BRC.136	9.8	8.9		9.35	6.8
BRC.137	20.7	20.6		20.65	0.3
BRC.138	16.6	16.8		16.7	0.8
BRC.139	23	24.4		23.7	4.1
BRC.140	8.9	9.7		9.3	6.1
BRC.141	20.1	20. 6		20.35	1.7
BRC.142	42.9	44.4		43.65	2.4
BRC.183	11.1	11.7		43.65	2.4
BRC.143	3	2.9		2.95	3.6
BRC.144	3.7	3.3		3.5	2.3
BRC.146	1.5	1.3		1.4	4.3
BRC.148	3.2	3.3		3.25	2.1
BRC.149	4.6	4.9		4.75	4.4
BRC.150	0.6	0.7		0.65	10.7
BRC.160	4.1	3.9		4	3.5
BRC.161	0.8	0.8		0.8	0.0
BRC.175	2.2	2.2		2.2	0.0
BRC.182	4.3	4.3		4.3	0.0
BRC.190	3.9	4		3.95	1.7
BRC.192	3.4	3.1		3.25	6.4
BRC.193	2.2	2.2		2.2	0.0

The single-kernel analyses are used qualitatively for the most part to determine the kinds of toxins found as well as their first appearance after infection. The floret which contains the kernels at the time of flowering is soft and composed of the lemma and pelea. As the kernel develops, its texture, color, and hardness will vary depending on its maturity until it becomes firm at ripening. Single kernels and florets at all stages of maturity are amenable to analysis using this method.

The precision of the method was measured by analyzing at random 28 samples of barley (Table 2). The samples were ground and analyzed as described in the methods section. Six of the samples were analyzed three times independently and are shown in the top of

Table 3. Comparison of Two Analytical Methods for the Analysis of DON in Barley

			DON concn (ppm)				DON concn (ppm)					
			C. J. Mirocha				H. Casper					
sample no.	source	variety	anal. 1	anal. 2	av	SD	anal. 1	anal. 2	av	SD	*F*	*P* value
BRC5.135	2302	Robust	13.3	13.3	13.3	0	12.5	13.2	12.85	0.5	1.65	0.33[a]
BRC5.136	2303	Excel	9.8	8.9	9.35	0.64	10.2	11	10.6	0.57	4.31	0.17[a]
BRC5.139	2306	M80	23	24.4	23.7	0.99	21.2	23.2	22.2	1.41	1.51	0.34[a]
BRC5.140	2307	Chevron	8.9	9.7	9.3	0.57	10.1	11.2	10.65	0.78	3.94	0.19[a]
BRC5.143	2316	Zedhar#1	3	2.9	2.95	0.07	3	3.2	3.1	0.14	1.8	0.31[a]
BRC5.144	2317	Gobernadora	3.7	3.3	3.5	0.28	3.9	4.1	4	0.14	5	0.16[a]
BRC5.152	2344	GD2-27	10.8	12.6	11.7	1.27	10.9	12.5	11.7	1.13	0	1[a]
BRC5.156	QYT#2 462 (grad)	Stander	14.7	17.5	16.1	1.98	15.3	17	16.15	1.2	0.0009	0.98[a]

[a] Not significant at 0.10 level.

the table. Their percent standard deviation varied between 1.0 and 11.1. Two analyses were made on the remainder of the samples; the percent standard deviation ranged between 0.0 and 10.7.

A comparative study was initiated between the laboratory of Dr. Howard Casper (North Dakota State University) to determine the variation that might be expected between laboratories using different methods of analysis but on the same samples. Dr. Casper used gas chromatography/electron capture (Tacke and Casper 1996) whereas the method employed was the one described in this paper. The samples, shown in Table 3, were ground in our laboratory and then shipped to Dr. Casper for analysis.

Eight samples were analyzed using two separate analyses of the same sample. There was no significant difference found between the methods and the analyses.

Analyses were made of 10 subsamples of a large 22 kg bag of naturally infected wheat. The objective was to determine the variation one might expect from the diversity between subsamples, i.e., uneven distribution of infected kernels. The samples consisted of a simple grab aliquot taken at random from the bag. The results in ppm (ng/g) were: 9.2, 12.3, 9.9, 13.7, 11.8, 8.0, 10.8, 13.7, 11.3, and 9.8 ppm. The sample mean was 11.1 ppm whereas the standard deviation was 1.899. The 95% confidence limits for the mean (ppm) was $11.06 \pm 2.2622 \times 0.6006 = (9.7013, 12.419)$.

The results obtained herein indicate that indeed the method described has merit for the analysis of DON in cereals. The additional advantage of this method is that it can be expanded to include other DON derivatives such as 3-ADON, 15-ADON, nivalenol, 7-ADON, and others which might be present in the samples. SIM gives us the sensitivity needed (50 ppb) in order to analyze small samples.

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