

Determination of Deoxynivalenol and Nivalenol in Corn and Wheat by Liquid Chromatography with Electrospray Mass Spectrometry

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The fungus *Fusarium graminearum* is a pathogen of both wheat and corn. Strains of the fungus from the United States produce a toxin, deoxynivalenol (DON); strains of the fungus from Asia and Europe produce DON or a related toxin, nivalenol. These toxins can cause disease in livestock, and their potential presence in feed and foods is a concern for animal and human health. A method was developed to detect both toxins in corn and wheat by liquid chromatography/mass spectrometry of an extract of ground grain. The method requires no sample cleanup and can detect the toxins at 0.05 µg/g.

Deoxynivalenol (DON), 3,7,15-trihydroxy-12-13-epoxy-trichothecene-9-en-8-one, also known as vomitoxin, is a sesquiterpenoid secondary fungal metabolite of a family known as trichothecenes. DON is an important mycotoxin which is associated with vomiting and feed refusal in swine (1). DON is produced by *Fusarium graminearum*, and related *Fusarium* species, which are ubiquitous fungi that are important pathogens of cereal crops (2). These fungi are also frequently found in crop residues and soil debris. DON is the predominant trichothecene mycotoxin produced by strains of *F. graminearum* in North America (3). Nivalenol (NIV), which has an additional hydroxyl group at carbon 4, is the predominant trichothecene produced by some strains of *F. graminearum* from Asia and Europe, whereas other strains make principally DON (4, 5). NIV-producing strains of *F. graminearum* have not been reported in North America (6).

F. graminearum is the principal fungus identified as the cause of the wheat disease called Fusarium head blight, also called scab (7). Fusarium head blight disease occurs sporadically in most wheat-growing areas worldwide and has caused devastating losses over the past decade in many wheat-grow-

ing areas of the Midwest (Illinois, Indiana, Ohio, and Michigan) and especially the upper Midwest winter wheat-growing areas of Minnesota and North and South Dakota. In addition to losses in crop yield caused by the disease, the production of mycotoxins, particularly DON in scabby wheat, is an important concern to both livestock and human health. This fungus is also implicated in ear rot diseases in preharvest corn and can accumulate in corn kernels (2).

The need to ensure that the food supply is safe has necessitated considerable expenditure of resources in testing of grain for DON and other trichothecenes. Conventional chemical methods for analysis of trichothecenes involve extraction with organic solvents such as acetonitrile–water followed by cleanup of the extract to remove interfering matrix contaminants and to concentrate the sample for analysis. The determinative step generally uses a chromatographic separation technique such as thin-layer chromatography (TLC), gas chromatography (GC), or liquid chromatography (LC) coupled with one of a variety of detection techniques. Because useful separations can be obtained without the need for derivatization, LC has become the method of choice for the separation technique. Trucksess et al. (8) reported a method for determination of DON in white flour, whole wheat flour, and bran based on LC with UV detection at 220 nm. This method has been accepted by AOAC as a Peer-Verified Method for DON (9) and is widely used.

Three papers published in 1999 reported methods for detection of DON and NIV using LC coupled with mass spectrometry (MS; 10–12). Berger et al. (12) reported the use of positive atmospheric pressure chemical ionization (APCI) LC/MS for quantitative determination of trichothecenes, including DON and NIV in spiked wheat and wheat flour prepared from *Fusarium*-infected and contaminated wheat batches. Razzazi-Fazeli et al. (11) reported the determination of NIV and DON in wheat extracts by LC/MS with negative ion APCI. Both methods required cleanup of extracts to minimize source-cleaning intervals and achieve required detection limits. Plattner (10) reported a rapid screening method for DON in wheat and corn extracts using LC/MS with negative electrospray ionization (ESI) that was not optimized for sensitivity.

This study presents a rapid and sensitive method to detect and quantify DON and NIV in grain extracts by LC/MS/MS

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with ESI. DON and NIV are identified and measured by their retention times on reversed-phase LC columns and their MS/MS spectra in a diluted aliquot of the crude grain extract. No sample cleanup was required to achieve detection limits for DON or NIV of 0.05 $\mu\text{g/g}$ (ppm) in wheat or corn.

Experimental

Apparatus

(a) *LC system*.—Thermo Separations (San Jose, CA); consisting of a Model P4000 solvent delivery system and an AS3000 autosampler.

(b) *Column*.—Intersil 5 μm ODS-3, 150 \times 3.0 mm (MetaChem Technologies, Inc., Torrance, CA); used for separations. The flow rate was 0.3 mL/min with column effluent directly coupled to a Thermal Instrument/Finnigan LCQTM (San Jose, CA) mass spectrometer.

(c) *Vaporizer oven*.—(1) *For APCI*.—Operated at 350°C, capillary at 150°C, and the sheath gas was nitrogen. The discharge current was 5 mA (typically at ca 4.5 kv). The instrument was operated either in positive or negative ion mode. (2) *For ESI*.—The capillary was operated at 220°C, the sheath gas was nitrogen, and the spray voltage was 5.0 kv. The instrument was operated in the negative ion mode.

Reagents

(a) *Extraction solvent*.—Acetonitrile–water (84 + 16, v/v).

(b) *Tetrabutylammonium dihydrogen sulfate (TBAHS)*

Sample Preparation and Extraction

Ground grain samples (corn or wheat) were extracted with 4 mL/g extraction solvent (acetonitrile–water, 84 + 16, v/v) in Erlenmeyer flasks with shaking for 2 h. The extraction solvent was filtered through Whatman 2V filter paper. Extracts were stored in capped vials at 4°C until analyzed. For LC–UV comparisons, the sample cleanup method of Trucksess et al. (8) was used. In most LC/MS and LC/MS/MS experiments, an aliquot of the filtered extract was diluted 10-fold with acetonitrile–water, and a portion (10–30 μL) was injected directly into the LC system for analysis. In other experiments, the extract was concentrated by evaporating 2 mL extract under nitrogen to dryness. The residue was redissolved in 200 μL extraction solvent and transferred to an autosampler vial. To determine whether sample cleanup improved the analysis for some experiments, cleanup was performed as follows: A 6 mL portion of extract was placed into a 15 \times 85 mm test tube. A MycoSep 225 column (Romer Labs, Inc., Washington, MO) was inserted into the top of the test tube and slowly pushed to the bottom of the tube. A 4 mL portion was then taken from the top of the column, and an aliquot was used for the LC/MS analysis, as with the crude extract. It was determined that, for detection of DON at levels >0.05 $\mu\text{g/g}$, sample cleanup was not needed.

LC/MS and LC/MS/MS Analysis

Isocratic liquid chromatograms were used to evaluate the spectra obtained for DON or NIV with different modes of op-

eration of the mass spectrometer. The solvent system was water–methanol (75 + 25, v/v) with 0.25% acetic acid. For identification and quantitation of DON or NIV in sample extracts, a gradient was required. The column was equilibrated with water–methanol (99 + 1, v/v) with 0.3% acetic acid. After injection, the solvent was held for 1 min, programmed to water–methanol (67 + 33, v/v) with 0.3% acetic acid over 6 min, and held for 17 min. The solvent was then returned to the starting solvent over 1 min and held for 25 min. Under these conditions, the injection of up to 50 μL extraction solvent did not affect the chromatography; DON eluted at ca 15 min, and NIV eluted at ca 13 min.

Quantitation was performed by the external method. For LC/MS, the peak area of the signal at $(\text{M}+\text{acetic acid}-\text{H})^-$ for DON (m/z 355) or NIV (m/z 371) at the appropriate retention time was used. For LC/MS/MS, the acetic acid adduct was isolated and the daughter fragment ion at $[\text{M}-\text{H}]^-$ (m/z 295 for DON or m/z 333 for NIV) at the appropriate retention time in the chromatogram was used. To detect both DON and NIV in a single analysis by LC/MS/MS, the LCQ experiment was set up to select the respective parent adduct m/z values for either DON or NIV in every other scan, and the 2 respective scanning segments were separated into segments for each toxin and quantified separately by software control. Spiked extracts of clean corn or wheat were used as external standards for analysis of corn or wheat extracts, respectively. A multipoint response curve was constructed with extracts of clean corn or wheat matrix, containing <0.1 $\mu\text{g/g}$ spiked with DON (or NIV) at levels ranging from 0.1 to 5 $\mu\text{g/g}$. A linear response was obtained across the concentration range.

LC–UV Analysis

Corn extracts were analyzed by LC–UV as reported by Maragos and Plattner (13). Essentially, it is the method of Trucksess et al. (8) with a modification to the mobile phase and column. The column was a reversed-phase analytical column (ODS-120T, 4.6 \times 25 cm, 2 μm ; TosoHaas, Montgomeryville, PA) and the UV detector was a Spectra System UV2000 (Thermo Separations). The mobile phase was a binary gradient of methanol and acetonitrile–water with TBAHS at a flow rate of 0.7 mL/min. The latter solution was prepared by adding 10 mL concentrated ion-pair reagent to 1 L 8% (v/v) acetonitrile in water, with pH adjusted to 6.5 with 5N potassium hydroxide. The concentrated ion-pair reagent consisted of 0.114 g/mL TBAHS and 0.114 g/mL (anhydrous) monobasic potassium phosphate in water. The initial LC condition was 0% methanol. The methanol was ramped to 5% for 17 min, when it was increased to 40%. At 25 min, the mobile phase was returned to the initial condition and the column was allowed to equilibrate for 20 min before the next injection. Under these conditions, DON eluted at 17 min.

Safety

DON and NIV are mycotoxins, and proper and relevant safety procedures for handling them should be followed.

Results and Discussion

Several procedures have been reported on the determination of trichothecenes in grain extracts using LC/MS on a single quadrupole mass spectrometer. Razzazi-Fazeli et al. (11) reported a method for the determination of DON and NIV in wheat using LC/MS with negative ion APCI detection. Their method used an acetonitrile–water extract followed by cleanup with 2 multifunctional columns (MycoSep 227 and 216; Romer Labs), concentration, and separation by isocratic reversed-phase LC. The column eluant was transferred into the MS system by an APCI interface operated in the negative ion mode, and DON and NIV were detected in the selected ion monitoring mode. They reported detection limits of 0.04–0.05 µg/g.

Berger et al. (12) reported a similar method for the determination of trichothecenes, including DON and NIV along with Fusarinone X, 3-acetyl DON, diastoxyscerpenol H-T2 toxin and T-2 toxin by LC/MS, using an ion trap. Their method used the same acetonitrile–water extraction with cleanup on the same MycoSep columns, and concentration. A C₁₈ LC column with a binary solvent gradient was used for analysis. The eluant was transferred to the MS system with an APCI interface operated in the positive ion mode. The trichothecene verrucarol was used as internal standard for quantitation and detection limits were 0.1 µg/g. Both of these methods required considerable cleanup and concentration of the extract to achieve the reported detection limits.

We used LC/MS with both APCI and ESI interfaces to detect DON in acetonitrile–water extracts of wheat samples from field and greenhouse tests (10) and compared our results with those of the method of Malone et al. (14). The ESI interface gave similar sensitivity for DON, was more rugged, and required less cleaning and maintenance to achieve better performance than the APCI interface. A detection limit of <2 µg/g for DON was achieved by injection of 10 µL sample extract with no sample cleanup.

In mixtures of water–methanol, negative ESI yielded less abundant signals for DON and NIV than are seen in APCI conditions. The negative ESI spectrum of DON was similar to that reported for negative APCI. The base peak in the spectrum was the proton abstracted molecule [M–H][–] at *m/z* 295. A small fragment ion was observed at *m/z* 265. However, the addition of acetic acid to the solvent system resulted in formation of intense acetate adduct ion for type B trichothecenes (10, 12). For DON, this was observed at *m/z* 355, whereas the adduct ion for NIV was seen at *m/z* 371. Addition of even trace amounts of acetic acid to the solvent resulted in a large increase in the signal for DON or NIV, making the overall sensitivity for them approximately equal to that observed with APCI. ESI also has the advantage of lower background signals from solvents and sample matrixes than are observed with APCI. Therefore, we found the best full scan signal-to-noise (S/N) ratios for DON or NIV present in naturally contaminated extracts were obtained with the gradient solvent program and ESI. Although detection limits were similar for both APCI and ESI, we also found that the long-term performance of the MS interface upon injection of a

Table 1. Comparison of DON levels in naturally contaminated corn extracts by LC/MS/MS and LC–UV

Sample ^a	DON by LC/MS/MS, ppm	DON by LC–UV, ppm
1a	3.57	3.5
1b	4.44	3.76
1c	3.60	3.57
2a	4.56	3.98
2b	4.63	4.09
2c	4.51	3.76
3a	4.11	4.13
3b	4.32	3.61
3c	4.44	4.11
4a	3.77	3.39
4b	4.57	3.80
4c	4.38	3.82
5a	3.89	— ^b
5b	3.72	3.3
5c	4.49	4.03
6a	1.72	1.68
6b	2.17	1.49
6c	1.80	1.61
7a	2.24	2.14
7b	2.28	1.79
7c	1.82	1.56
8a	1.19	1.10
8b	1.50	1.20
8c	1.42	1.22
9a	1.24	1.10
9b	1.25	1.16
9c	1.39	1.19
10a	3.14	2.75
10b	2.96	2.40
10c	2.84	2.40
11a	3.12	2.61
11b	3.32	2.71
11c	3.09	2.84
12a	5.12	4.67
12b	5.12	4.67
12c	5.44	5.47
13a	0.74	0.59
13b	0.66	0.70
13c	0.79	0.80
14a	1.10	0.73
14b	0.90	0.89
14c	0.96	0.90
15a	0.82	0.55
15b	0.63	0.69
15c	0.73	0.84

^a a–c are replicate extracts of the same naturally contaminated sample.

^b — = Sample not run.

series of extracts was significantly better with the ESI interface. The APCI interface required rigorous cleaning every few days to maintain sensitivity, whereas the ESI interface gave satisfactory results with no significant loss in sensitivity for several weeks, over which hundreds of injections of crude extracts were made.

Under isocratic conditions (water–methanol, 75 + 25, v/v), when standard solutions of DON in the elution solvent were injected, DON had a retention time of approximately 5 min. However, under these same conditions, injection of as little as 10 μ L standard solutions of DON in extraction solvent (water–acetonitrile, 16 + 84, v/v) greatly affected the retention time for DON, causing it to co-elute with NIV at or just after the column void volume (3 min).

When sample extracts were injected, detection of DON was obscured by its co-elution with polar matrix components in the extract which eluted at the void volume, and the abundance of its signal was reduced by matrix effects. Therefore, for analysis for DON, one of 3 procedures was necessary: Either the sample was diluted with a solvent that did not affect chromatography (such as the elution solvent); the sample was evaporated under nitrogen and the residue extracted with the elution solvent; or a suitable gradient was needed to separate DON from these polar matrix components. A gradient system was chosen as the most practical solution because it offered the advantages of simplifying the sample preparation and allowed NIV and DON to be cleanly separated and measured directly from extracts of wheat, corn, and other small grains without cleanup. With the gradient system, detection limits in the full scan mode were similar to those obtained under the isocratic system.

When 500 pg DON or NIV was injected into the LC system with the ESI interface operated in the full scan mode, the negative signal at m/z 355 or 371 (M +acetic acid–a hydrogen) for both mycotoxins could be reliably detected above the chemical noise. This allowed a detection limit of about 2 μ g/g for DON or NIV in extracts of corn or scabby wheat samples from field and greenhouse studies when a 10 μ L aliquot of the crude extract was injected (10).

In the present study, the detection limit was dramatically improved by operation of the mass spectrometer in the MS/MS mode. In this mode, the quadrupole ion trap collects ions at m/z 355 (for DON) or m/z 371 (for NIV) while ejecting all other signals. For a time, an excitation signal is applied to the poles of the ion trap to excite selected ions, which collide with the helium buffer gas and fragment. The resulting fragment ions are subsequently expelled from the trap and acquired as the spectrum. In this mode, only fragments arising from the selected parent are detected and most of the chemical noise from other signals in the matrix is eliminated, improving the S/N ratio. Injection of as little as 10 pg DON into the LC system resulted in a spectrum for DON with detectable fragments at m/z 295 [M –H] $^-$ and m/z 265, an improvement in S/N of >50-fold. In the MS/MS mode, DON was reliably detected in extracts of grain samples at levels as low as 0.05 μ g/g when a 10 μ L aliquot of a 10-fold diluted extract was injected.

To assess performance of the method, we extracted a series of naturally contaminated corn and wheat samples for analysis by LC/MS/MS and by the method of Trucksess et al. (8), slightly modified to improve performance for corn extracts (13). Table 1 shows the results of the analysis of 3 replicate extracts of 15 naturally contaminated corn samples by the 2 methods. The DON levels in the samples ranged from 0.6 to 5.4 μ g/g, which is of interest based on allowable levels of DON in grains intended for human consumption. Excellent agreement was obtained between the 2 methods. Similar good agreement between the methods was obtained for a series of wheat samples at levels between 0.5 and 15 μ g/g (data not shown).

The use of MS rather than UV absorbance at 220 nm to detect DON in grain extracts following the extraction procedure of Trucksess et al. (8) has the advantage of allowing simple detection of the presence of NIV as well as DON without modification of the extraction or chromatography. NIV is quite rare in naturally contaminated wheat or corn from the United States and has not been reported to be produced by North American strains of *F. graminearum*.

The ability of LC/MS to detect and measure either DON or NIV in grain samples was evaluated by using greenhouse-grown wheat plants artificially infected with 51 strains of *F. graminearum* collected from Nepal. Plants were infected by injection of spores into the developing heads of 3 spikes. Seed from these 3 heads harvested at maturity several weeks after inoculation were combined, ground, and extracted. Because the levels of toxin in these artificially infected wheat samples were high, a higher detection limit was acceptable; therefore, the extracts were diluted 20-fold with acetonitrile–water and analyzed by LC/MS. This resulted in a detection limit of about 5 μ g/g for DON or NIV. NIV was detected as the predominate toxin in 27 of the strains examined, while 11 produced only DON in the infected wheat, and 13 of the isolates produced >5 μ g/g toxin in the wheat. The level of toxin detected in harvested grain ranged from the detection limit (about 5 μ g/g) to 150 μ g/g. Although very small traces of DON (<1% of the level of NIV) could sometimes be detected in NIV-producing isolates, no NIV was detected in any strain that produced high levels of DON. The full results of this study will be reported elsewhere.

Conclusions

The LC/MS/MS method to measure DON or NIV in corn or wheat represents an alternative detection method to the method developed by Trucksess et al. (8). The method gives essentially identical results but is much more rapid because sample cleanup is not necessary. Because this LC/MS/MS method uses MS for detection, which is more specific and selective than UV absorbance at 220 nm, it could be used to confirm positive samples detected in that method, or even replace the UV detection method. It also has the advantage of being able to separate and simultaneously measure DON and NIV, which would be an improvement over the Trucksess et al. (8) method, which does not address NIV at all. This improvement can be important for the analysis of naturally contaminated

grains that come from areas where the potential for the presence of either DON or NIV should be considered.

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