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Quantification of deoxynivalenol in wheat using an immunoaffinity column and liquid chromatography

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Abstract

A simple and accurate method to quantify the mycotoxin deoxynivalenol (DON) in wheat is described. The method uses immunoaffinity chromatography for DON isolation and liquid chromatography (LC) for toxin detection and quantification. Wheat samples are extracted in water, filtered twice and applied to an immunoaffinity column. Following a water wash, DON is eluted from the column with methanol and injected onto an LC system with a UV detector for quantification. Test performance was evaluated in terms of antibody specificity, limit of detection, percentage recovery, precision, column capacity, assay linearity and comparison with the GC–electron-capture detection (ECD) method of Tacke and Casper. Specificity of the immunoaffinity column cleanup procedure was confirmed with only DON (>80%) and its 15-C derivatives (40–50%) being recognized by the antibody while 3-C DON derivatives, nivalenol, T-2 and fusarenon-X did not bind. The limit of detection is at least 0.10 μg/g. Percentage recovery for the entire assay range averages 90% with an average relative standard deviation of 8.3%. Naturally contaminated samples showed comparable precision. Column capacity was determined to be 3.3 μg. The assay showed a high degree of linearity (r^2=0.999) and an optimum assay range of 0.10 to 10.0 μg/g. Comparative analysis of 28 naturally or artificially contaminated wheat samples using DONtest-HPLC and the GC–ECD method of Tacke and Casper showed that DONtest-HPLC is a statistically significant predictor of the GC–ECD method (r^2=0.982). © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Deoxynivalenol (DON) is a mycotoxin produced by fungi of the *Fusarium* genus, in particular, *Fusarium graminearum* and *F. culmorum* [1]. It is also the most common trichothecene in North America followed by nivalenol, T-2 toxin, HT-2 toxin and diacetoxyscirpenol [2]. Wheat, corn and barley used for human and animal consumption are frequently contaminated with deoxynivalenol. Studies in the United States, Germany, the Netherlands, Bulgaria, Hungary, Russia, China, Korea and Argentina have shown that 60–100% of samples tested had DON contamination, at levels as high as 44 μg/g (44-times the US Food and Drug Administration advisory level of 1.0 μg/g for human consumption) [3–13]. Particularly alarming are recent studies of food samples for human consumption from

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retail stores. In 1991, a study in the USA showed DON levels as high as 19 μg/g with 50% of the samples at or greater than 1.0 μg/g [14]. A more recent study of food samples from retail stores in Argentina showed greater than 90% DON contamination with a mean content of 1.8 μg/g and samples as high as 9.3 μg/g [15]. These and other studies clearly demonstrate that DON is commonly found at high levels in the field in cereals intended for human and animal consumption and in at least some food products available in retail stores.

Even at low levels, deoxynivalenol may cause animals to refuse feed or, at higher levels, induce vomiting, causing growth depression [16,17]. DON has been shown to inhibit protein synthesis, alter brain neurochemicals, and, depending on the dose and duration of exposure, be immunosuppressive [17]. In addition, DON has been implicated in two large outbreaks of gastrointestinal illness in humans in India and China [16]. Since wheat, corn and barley frequently contain DON and constitute two-thirds of the world production of cereals [18], the health and economic ramifications of DON contamination necessitate an easy-to-use, rapid and quantitative detection method. In addition, since tolerance levels in countries vary widely [19], a procedure that covers a large range of DON concentrations is highly desirable. Current analysis methods reported in the literature include thin-layer chromatography (TLC) [20,21], gas chromatography (GC) with electron-capture detection (ECD) [22–25] or mass spectrometry [26], radioimmunoassay (RIA) [27,28], enzyme-linked immunosorbent assay (ELISA) [29,30], liquid chromatography with [31] or without derivatization [32–34], and fluorescent minicolumn [35]. Sensitivity and accuracy of these methods depend on their ability to selectively isolate toxins from interfering substances present in the complex matrices prior to detection. Clean-up procedures are normally used for toxin isolation, including supercritical fluid extraction (SFE) [36], solid-phase extraction (SPE) with various mixtures of solid phases including charcoal–alumina–Celite [21], cation-exchange resin and alumina–carbon [32], C_{18} [31], or Mycosep No. 225 (Romer Labs.) columns [33]. However, immunoaffinity chromatography is the only clean-up procedure that is toxin specific, minimizing the interference of co-extracted substances.

This paper describes conditions for determination of deoxynivalenol in wheat using an immunoaffinity column as a clean-up step followed by detection using LC with UV detection. This procedure is easy-to-use, rapid, accurate, toxin specific, and ideally suited for high throughput testing. This study presents some of the performance parameters of the DONtest-HPLC kit (VICAM, Watertown, MA, USA) including a comparison of this method with GC–ECD.

2. Experimental

2.1. Materials and reagents

DON was obtained from Sigma (St. Louis, MO, USA). Working standard solutions, ranging from 0.010 μg/ml to 1000 μg/ml, were prepared in 100% ethanol and stored at −20°C until use. DONtest-HPLC immunoaffinity columns, fluted and glass microfiber filter papers, and disposable cuvettes were from Vicam (Watertown, MA, USA). Analytical grade methanol and acetonitrile, polyethylene glycol (M_r 8000), nivalenol and fusarenon X were obtained either from Fisher Scientific (Pittsburgh, PA, USA) or Sigma. Tritiated T-2 was obtained from Dr. F.S. Chu (University of Wisconsin, WI, USA). Solutions of nivalenol and tritiated T-2 were prepared in methanol while fusarenon X was dissolved in ethylene chloride and stored at −20°C until use. DONtest-HPLC kits were used according to the instructions supplied by the manufacturer. Wheat samples were obtained from a local store or mills across the USA.

2.2. Apparatus

The Waters 2690 Alliance liquid chromatography system was used with a stainless steel, reversed-phase 150×3.9 mm, 4.0 μm spherical particle, 60 Å pore size, C_{18} Nova-Pak column (Waters, Milford, MA, USA). The acetonitrile–water (10:90, v/v) mobile phase was filtered through a 0.22-μm filter membrane (Micron Separations, Westboro, MA, USA), degassed, and used at a flow rate of 0.6 ml/min. DON was detected using a Waters 486 UV detector set to 218 nm. Data were analyzed with a Digital 575 Venturis equipped with Millennium 2010 Chromatography Manager system.

2.3. DONtest-HPLC method

Samples of wheat (50 g) were experimentally contaminated in a fume hood with DON standard and allowed to dry for at least 30 min before analysis. Following addition of 10 g polyethylene glycol (M_r 8000) to aid in filtration, samples were extracted with 200 ml distilled water by blending (Waring, New Hartford, CT, USA) for 1.0 min. Extracts were then filtered through both a DONtest fluted filter paper and a 1.5-μm glass microfiber filter. The filtered extract (1.0 ml=0.25 g equivalent) was applied to a DONtest-HPLC column, and the toxin bound specifically to the antibody. Subsequently, the column was washed with 5.0 ml water and the toxin was eluted by passing 1.0 ml methanol through the column and collecting this eluate in a glass cuvette. The single-use column was then discarded. The eluate was dried using a concentrator (Savant, Farmingdale, NY, USA), re-dissolved in 300 μl acetonitrile–water (10:90, v/v), and 50 μl were injected onto an LC system with a UV detector set to 218 nm.

2.4. Modified procedure to determine antibody specificity

A slightly modified procedure was used to evaluate the antibody specificity of the immunoaffinity column. One microgram of each toxin was added to 10 ml water, so that the organic solvent concentration was less than 0.0010%. The contaminated solution was passed across the column. Then the column was washed with 5.0 ml water followed by elution with 1.0 ml of methanol. For nivalenol and fusarenon X, 60 μl of eluate were diluted with 540 μl of distilled water (Milli-Q) and 250 μl of the diluted eluate was analyzed by LC. For T-2, a 500 μl portion of the eluate was read in a scintillation counter (Beckman Coulter, Fullerton, CA, USA).

3. Results and discussion

3.1. Antibody specificity

Detection of DON by LC requires thorough clean-up of sample extracts for optimal results. Immunoaffinity columns are a reliable clean-up method provided the antibodies used are specific for the target analyte. The DONtest-HPLC method uses immunoaffinity columns containing a monoclonal antibody to DON. The specificity of the antibody was evaluated by measuring the recovery of DON and other trichothecenes. The results showed high percentage recoveries (>80%) for DON with lower recoveries (40–50%) for 15-C DON derivatives. No binding was detected when 3-C DON derivatives, nivalenol, T-2, or fusarenon-X were passed across the column. Although the monoclonal antibody did show some reactivity with 15-C DON derivatives, these compounds are easily resolvable by LC and are only occasionally present in samples, at very low levels. When the mycotoxins produced by 42 isolates from seven different *Fusarium* species obtained from corn were analyzed, 15-acetyl-DON was not found while 3-acetyl-DON was isolated from seven of the 27 *F. graminearum* isolates [37]. A survey of 140 wheat samples intended for human consumption showed only a 0.70% contamination rate for 15-acetyl-DON and 2.1% for 3-acetyl-DON with a maximum concentration of only 0.10 μg/g [7]. Therefore, based on this information, results obtained using DONtest-HPLC accurately reflect the amount of DON in the samples.

3.2. Analytical performance

The limit of detection (LOD) of DON, defined here as the smallest amount reproducibly and accurately detected with at least a 3:1 signal-to-noise ratio, was determined by measuring experimentally contaminated DON-free wheat samples. The results, shown in Table 1, indicate that the method has an LOD of 0.10 μg/g or less. Samples containing less than 0.10 μg/g had lower accuracy and precision (data not shown).

Recovery using the DONtest-HPLC was determined using wheat samples experimentally contaminated with 0.10 to 10.0 μg/g DON. Recoveries ranged from 80 to 100%, with an overall average of 90% (Table 1).

Precision for the method was determined by assaying at least six replicates of wheat samples experimentally contaminated with DON at levels from 0.10 to 10.0 μg/g (Table 1). The average relative standard deviation (RSD) of this detection method was 8.3% across the assay range, reaching a

Table 1
Limit of detection, percentage recovery and RSDs of DON assays run on wheat samples by using the DONtest-HPLC method[a]

Added[b] (μg/g)	Recovery (%)	RSD (%)
0.10	100	16
0.25	100	12
0.50	102	10
1.0	85	5.9
2.5	84	3.0
5.0	80	5.8
10	81	5.1
20	59	1.4
40	36	3.5

[a] Wheat samples containing undetectable levels of DON by GC–ECD were experimentally contaminated with varying amounts of DON and analyzed using the DONtest-HPLC method.
[b] n=6, 8, 8, 12, 6, 8, 8, 3, 3 for the DONtest-HPLC 0.10 to 40.0 μg/g levels.

high of 16% at the LOD and leveling off at approximately 5.0% at and above 1.0 μg/g. In addition, a high degree of precision was demonstrated for analysis of naturally contaminated wheat samples. When ten aliquots of samples at 1.8 and 2.7 μg/g were independently tested the respective RSDs of 10% and 3.5% were comparable to those of the experimentally contaminated samples.

Column capacity was determined by comparing the amount of DON passed across the immunoaffinity column to the amount bound (Table 1). Good recoveries (≥81%) for DON were obtained when up to 2.5 μg (10 μg/g) was added. Above this level, only small amounts of DON bound to the column despite the large amounts added, resulting in a dramatic drop in percentage recovery. The point of intersection of the two linear fits of these different parts of the overall curve (0–2.5 μg added and 2.5 μg+ added) yields a column capacity of 3.3 μg.

Assay range was determined based on the linearity of the results in Table 1. Linear regression analysis shows the method is very linear (r^2=0.999) for the 0.10–10.0 μg/g range.

3.3. Comparison with the GC–ECD method of Tacke and Casper

Twenty-eight naturally or artificially contaminated wheat samples were analyzed at VICAM (DONtest-HPLC) and, separately, at North Dakota State University (GC–ECD) (Table 2 and Fig. 1). Paired t-test analysis comparing the DONtest-HPLC and GC–ECD results did not demonstrate a significant difference between the sample means or standard deviations. Linear regression analysis yielded the regression equation $y=0.916x+0.363$, a slope 0.916 ($P<$ 0.0001) with a 95% confidence interval (0.867, 0.966) and an r^2 value of 0.983 demonstrating that DONtest-HPLC is a statistically significant predictor of the GC–ECD method of Tacke and Casper.

Table 2
Comparison between DONtest-HPLC and GC–ECD methods[a]

Sample	DONtest-HPLC (μg/g)	GC–ECD (μg/g)	RSD (%)
1	0.15	0.2	20
2	0.20	0.2	0.0
3	0.29	0.5	38
4	0.32	0.5	31
5	0.51	0.7	22
6	0.51	0.9	39
7	0.77	1.0	18
8	1.0	1.1	6.7
9	1.2	1.4	11
10	1.3	1.5	10
11	2.0	2.0	0.0
12	2.1	2.3	6.4
13	2.2	2.3	3.1
14	2.5	2.5	0.0
15	2.8	2.9	2.5
16	2.4	3.1	18
17	3.0	3.3	6.7
18	3.2	3.5	6.3
19	3.5	3.7	3.9
20	3.3	4.0	14
21	3.4	4.0	11
22	4.3	4.3	0.0
23	5.2	5.6	5.2
24	7.4	6.5	9.2
25	6.5	6.8	3.2
26	7.5	7.0	4.9
27	8.8	7.8	8.5
28	8.5	8.3	1.7

[a] Naturally contaminated wheat samples were analyzed by DONtest-HPLC (VICAM) and GC–ECD (North Dakota State University).

4. Conclusions

The DONtest-HPLC method improves upon exist-

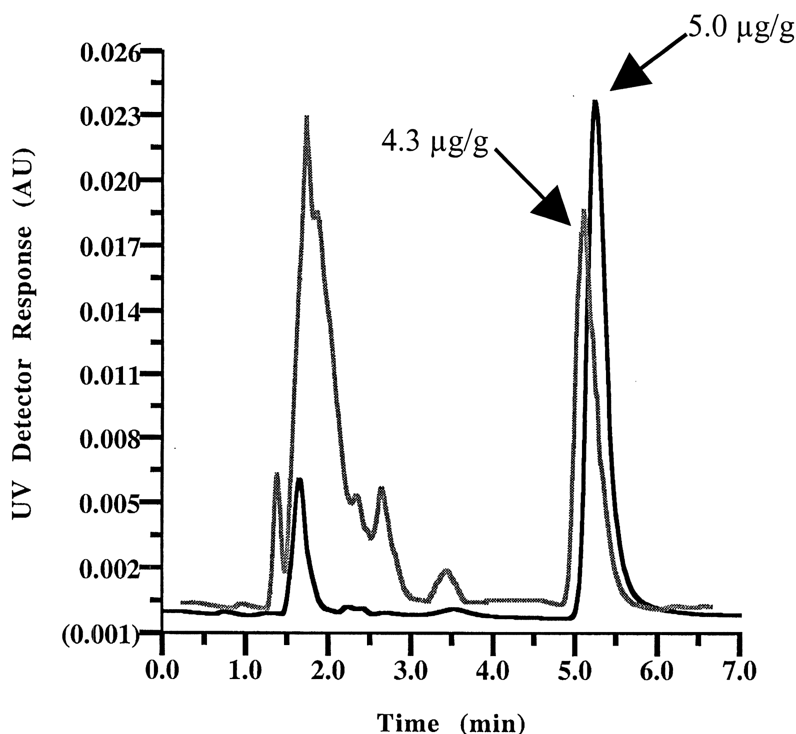

Fig. 1. Chromatograms of a naturally contaminated wheat sample (4.3 µg/g) analyzed by DONtest-HPLC and a 5.0 µg/g DON standard. Arrows indicate DON peaks. Under these experimental conditions DON retention time was ~5.3 min. Experiments using DONtest-HPLC immunoaffinity columns and columns without antibody have shown that the peak at ~3.7 min represent a compound that associates non-specifically to the Sepharose resin and not to the antibody.

ing technologies for the detection of DON in terms of ease-of-use, rapidity of analysis, specificity for the toxin, suitability for high throughput testing, assay range, and overall performance. Recent studies have clearly demonstrated that DON is commonly found at high levels in the field in cereals intended for human and animal consumption and in at least some food products available in retail stores. In addition, many studies have shown the substantial adverse effects of DON on animal and human health. This method offers a simple and reliable way of reducing or eliminating the risk of great economic loss in industry and the threat to human health due to exposure to DON.

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