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DETERMINATION OF DEOXYNIVALENOL (VOMITOXIN) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid method for the analysis of deoxynivalenol (DON) was developed using high-performance liquid chromatography (HPLC) with reductive electrochemical detection (ED). Deoxynivalenol produced by *Fusarium roseum* growing on solid cornmeal and rice substrates and from naturally contaminated wheat was extracted and quantitated via ED. DON levels in wheat were verified by gas chromatography and structurally confirmed by mass spectrometry. DON was optimally resolved by HPLC employing a radially compressed octadecylsilane column and a mobile phase of deoxygenated methanol–40 m*M* borate buffer (35:65) at a flow-rate of 1.0 ml/min. Under these conditions DON exhibited an average retention time of 3.6 min. Reductive ED (−1.4 V) allowed a 12-fold increase in sensitivity and greater selectivity than classical UV absorption at 224 nm. A detection limit for DON of 25 pg/μl was achieved under these conditions. The determination of DON in crude grain extracts was hindered by extractable interfering substances, whereas ED was more functional-group selective (*i.e.* reduction of the carbonyl moiety). ED permits a direct quantitation of DON from crude grain extracts and may facilitate the determination of this agent and associated metabolites in biological samples.

INTRODUCTION

Deoxynivalenol (DON), generically known as vomitoxin, is a trichothecene mycotoxin produced by *Fusarium* spp. DON was first isolated and characterized from moldy barley in Japan[1] and subsequently found to be identical to the emetic factor from corn in the U.S.A.[2]. Aside from emesis and feed refusal in swine no other significant toxic effect has been reported for DON. Nevertheless, one report suggests

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that high levels of DON may be associated with esophageal cancer in humans[3]. Because the longterm effects of DON are unknown and because it has been detected at levels exceeding 5.0 ppm, the Canadian government has established a tolerance level of 0.1 ppm DON in wheat flour intended for human consumption. At this time there are no stringent U.S. guidelines, however the Food and Drug Aministration has suggested a level of concern of 1.0 ppm for processed wheat products and 4.0 ppm for animal feed ingredients[4].

Since DON poses a potential threat to human health and a certain threat to the swine industry, a selective and sensitive method of analysis is essential for a rapid survey of contaminated grain samples. Currently, the accepted method for DON determination involves gas chromatographic–mass spectrometric (GC–MS) analysis[5]. The method is both labor-intensive and time-consuming. Extensive sample purification and chemical derivatization are required before accurate quantitation of DON levels can be achieved. Simpler methods such as thin-layer chromatography (TLC)[6] and high-performance liquid chromatography (HPLC) with UV detection[7] have been employed. However, TLC fails to detect less than 20 ng and UV detection is only possible at wavelengths below 227 nm.

The polarographic behavior of several mycotoxins including aflatoxins[8], zearalenone[9] and various trichothecenes[10,11] has been described. A reductive potential (−1.4 V optimum) for DON was previously determined using steady state differential pulse polarography[10]. This is the first report of an HPLC method utilizing reductive electrochemical detection for any of the trichothecene mycotoxins. The method described was evaluated through the determination of DON produced in fungal cultures and naturally contaminated wheat samples.

EXPERIMENTAL

Intrumentation

A Waters Model ALC-204 HPLC system (Waters Assoc., Milford, MA, U.S.A.) equiped with a Model 6000A pump and WISP 710B automated sample injector was used. A modification of the HPLC apparatus consisting of a stainless-steel intake line from the sealed refluxed solvent reservoir to the pump was used to maintain a reductive environment. All analyses were performed using a Waters radial compression separation system consisting of RCM-100 radial compression module, reversed-phase C_{18} Radial-Pak cartridge (10 μm particle size) and a C_{18} Guard-Pak precolumn insert (Waters Assoc.). The Model LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and the Model 970A variable-wavelength UV detector (Tracor Instruments, Austin, TX, U.S.A.) served as the detectors. Electrochemical detector conditions were: applied voltage, −1.400 V applied; range, 1–100 nA; and offset, 0–10 nA. UV detector conditions were: absorption wavelength, 224 nm, and 0.005–0.05 absorbance units full scale (a.u.f.s.). The Hewlett-Packard Model 3390A (Hewlett-Packard, Avondale, PA, U.S.A.) and Model 730 Data Module (Waters Assoc.) prepared and quantified the chromatograms.

Chemicals

DON was obtained from Mycolab, (Chesterfield, MO, U.S.A.). Additional DON was derived from a DON-producing isolate of *Fusarium roseum* (NCPRL-A)

obtained from Dr. Charles Howell, (USDA-ARS, College Station, TX, U.S.A.) which was maintained on potato dextrose agar slants at 28°C. Media used for DON production consisted of Quaker yellow cornmeal and Uncle Ben's converted rice. Purity of the DON was confirmed by TLC and HPLC with structural integrity verified by proton-decoupled, ^{13}C NMR and mass spectal analysis (Hewlett-Packard 5970 hyperbolic quadrupole mass selective detector equipped with a 12-m cross-linked methylsilicone capillary column, Hewlett-Packard, Avondale, PA, U.S.A.)[12]. Aluminum chloride (Fisher, Fair Lawn, NJ, U.S.A.) was the TLC spray reagent used to visualize DON. HPLC-grade methanol, *n*-hexane, chloroform and acetone (Burdick and Jackson, Muskegon, MI, U.S.A.), triple distilled and demineralized 10 MΩ cm^{-1} water (MilliQ reagent grade water system, Millipore, Bedford, MA, U.S.A.) were used in all analyses. Boric, acid, sodium hydroxide and glacial acetic acid (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.) were used to prepare 40 m*M* borate buffer in 0.10 *M* acetic acid and adjust to pH 7.0 with 0.5 *M* sodium hydroxide.

The HPLC mobile phase consisted of deoxygenated methanol–40 m*M* borate buffer (35:65) and was held under prepurified nitrogen gas using a reflux apparatus consisting of a heating mantle and water-cooled condenser. The running solvent was refluxed at 100°C for 15 min and cooled to room temperature prior to use.

Sample preparation

DON was produced by laboratory culture of *Fusarium roseum* NCPRL-A as described previously[13]. Briefly, inoculum was prepared by adding 5 ml of sterile water to seven day-old agar slant cultures and 0.5-ml aliquots of the inoculum were added to 50-g samples of cornmeal or rice. Flasks of each medium were inoculated, incubated at either 14 or 28°C and analyzed at weekly intervals for a period of 12 weeks. Fungal cultures were extracted by agitating with 2 volumes of 25 ml of methanol–water (40:60) for 2 h and filtered sequentially through Whatman No. 3 and No. 1 paper. Aliquots ranging from 10 to 100 μl were analyzed directly via HPLC employing electrochemical detection (ED). Similarly, wheat samples weighing 2.0 g were extracted, filtered and analyzed by ED. Wheat samples naturally contaminated with DON were analyzed and also blended with uncontaminated wheat to produce Dilution 1 (2:1) and Dilution 2 (1:2) (see Table II).

When HPLC with UV detection was performed to confirm DON quantitation, crude extracts required additional purification prior to HPLC analysis. This was accomplished by removing the methanol by rotary evaporation and partitioning the resulting aqueous fractions against three volumes of *n*-hexane. The hexane was discarded and the aqueous portions concentrated to near dryness, quantitatively transferred to silica gel G preparative TLC plates (Analtech, Newark, DE, U.S.A.) and developed in chloroform–acetone (3:2). Standards of DON (50 ng) were co-chromatographed alongside the sample extracts and visualized by spraying with 20% aluminium chloride in methanol. The DON band (R_F = 0.65–0.70) was scraped and eluted from the silica with methanol–water (40:60) and analyzed by UV.

HPLC procedure

Levels of DON in crude extracts were determined by ED alone, while DON in TLC-purified samples and standard dilutions were mesured by ED and UV simultaneously. Calibration curves were constructed based upon peak area obtained

for the two detection methods. Linearity of response and minimum detection limits were determined by injection of the DON standard at concentrations ranging from 0.10 to 2500 ng. Fractions of HPLC-eluted DON from the standard solution and fungal extract were subjected to GC–MS analysis to confirm the molecular identity of the peak.

RESULTS AND DISCUSSION

DON standard

Under the conditions described, DON exhibited a retention time of 3.6 min. Optimal peak resolution was obtained at pH 7.0 with diminished detector responses observed below pH 7.0 and above pH 9.0. Maximal integrator response was achieved at settings of 224 nm and −1.400 V for UV and ED, respectively. The electrochemical response was linear for concentrations up to about 1000 ng and the detection limit equalled 250 pg of the standard. Peak area obtained by ED was 12-fold greater than that by UV at all concentrations tested. The regression lines describing the calibration curves for UV and ED were: $y = 0.508x + 0.00005$ and $y = 6.097x + 0.0001$, respectively.

Laboratory cultures

ED chromatograms of crude fungal cultures were virtually indistinguishable from those of defatted, TLC-purified samples. Spurious peaks present in the UV chromatograms were absent under electrochemical analysis. Representative chromatograms of UV and ED profiles of 12 ng DON extracted from contaminated wheat sample are shown in Fig. 1. DON produced by *F. roseum* NCPRL-A attained a maximum concentration of 120 ppm after 9 weeks growth on cornmeal and 185 ppm after 10 weeks growth on rice. More DON was elaborated in rice cultures than

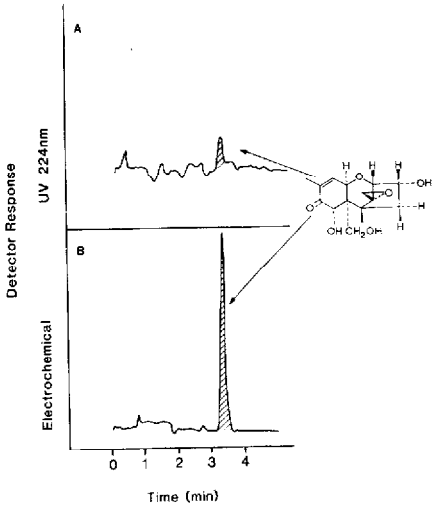

Fig. 1. Simultaneous determination of DON (12.0 ng) from contaminated wheat sample by HPLC with UV and ED. Octadecylsilane radial compression column with methanol–40 m*M* borate buffer, pH 7.0; flow-rate, 1.0 ml/min; detection at 224 nm and −1.4 V; sensitivity at 0.001 a.u.f.s. and 10 nA f.s.

TABLE I

DETERMINATION OF DEOXYNIVALENOL (ppm) ON SOLID MEDIA INOCULATED WITH *FUSARIUM ROSEUM* BY HPLC–ED

Entries represent mean ± S.D. (ppm) for six replicates per sample. See Experimental for details of analysis.

Week	*Corn*		*Rice*	
	14°C	*28°C*	*14°C*	*28°C*
1	–	2.1 ± 0.13	–	3.1 ± 0.09
2	–	19.3 ± 0.19	0.6 ± 0.07	27 ± 0.20
3	2.3 ± 0.28	42.4 ± 0.04	8.2 ± 0.21	61.5 ± 0.62
4	7.1 ± 0.47	53.2 ± 0.01	14.1 ± 0.33	76.1 ± 0.40
5	18.2 ± 0.33	64.8 ± 0.40	23.0 ± 0.02	87.1 ± 0.18
6	12.3 ± 0.26	74.2 ± 0.21	28.2 ± 0.46	101.2 ± 0.56
7	9.4 ± 0.36	81.2 ± 0.20	16.2 ± 0.19	128.3 ± 0.37
8	8.1 ± 0.09	93.4 ± 0.09	13.1 ± 0.14	149.2 ± 1.62
9	4.2 ± 0.26	120.4 ± 1.01	9.2 ± 0.37	180.3 ± 0.98
10	2.9 ± 0.82	113.4 ± 0.67	9.1 ± 0.09	185.4 ± 1.39
11	3.1 ± 0.46	97.1 ± 0.82	4.1 ± 0.09	170.1 ± 0.87
12	2.1 ± 0.45	92.4 ± 0.69	1.0 ± 0.03	114.2 ± 0.46

in the cornmeal with greater production overall at 28°C. This is consistent with the frequently high levels of DON reported in temperate regions. Weekly levels of DON produced on the solid media are shown in Table I. The increased sensitivity of ED permits an early detection of DON contamination, as is evidenced by the appearance of DON in both the cornmeal and rice cultures at 28°C after only one week of incubation.

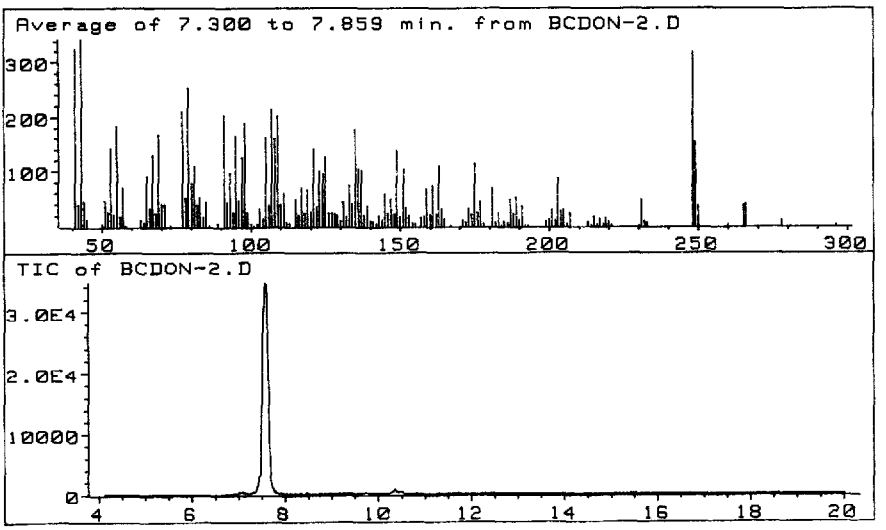

Fig. 2. Mass spectra (upper half) and total ion chromatogram (lower half) of DON derived from contaminated wheat sample. Splitless injection (1 μl acetonitrile containing approximately 10 ng DON) on a 12-m methylsilicone capilary column (0.2 mm I.D.); carrier gas, helium at 5.0 p.s.i.; injection temperature, 200°C; oven temperature ramped from 140 to 270°C at 15°C/min; electron impact ionization at 70 eV.

TABLE II

DETERMINATION OF DEOXYNIVALENOL LEVELS (ppm) IN WHEAT SAMPLES

Values represent the mean ± S.D. (ppm) for three replicates of three extractions/sample.

Sample	*Detection method*		
	UV	*ED*	*GC–ED**
Clean	0.0	0.0	0.0
Contaminated	36.1 ± 0.29	36.9 ± 0.18	26.0
Dilution 1**	22.9 ± 0.47	23.5 ± 0.06	18.1
Dilution 2***	11.8 ± 0.23	12.1 ± 0.11	11.2

* As previously determined via gas chromatography.
** Contaminated wheat blended 2:1 with clean wheat.
*** Contaminated wheat blended 1:2 with clean wheat.

Wheat samples

Clean wheat and DON-contaminated wheat were assayed in a blind study. The wheat samples constituted four different levels of DON as measured by gas chromatography with electron-capture detection: (1) clean wheat (0 mg/kg), (2) contaminated wheat (26 mg/kg), (3) Dilution 1 (18.1 mg/kg), and Dilution 2 (11.2 mg/kg). These data were subsequently compared to the UV and ED determinations obtained in this study (Table II). Recoveries of DON from spiked wheat samples demonstrated 94.6 ± 1.3% extraction efficiencies. Since these experiments were conducted, the extraction solvent has been modified to acetonitrile–water (90:10) which removes more than 98% of DON added to complex samples. Confirmation of the DON purified from the naturally contaminated wheat samples is presented in Fig. 2.

The selective nature of HPLC with reductive electrochemical detection provides an alternative method of DON determination which is more rapid, less labor-intensive and potentially more efficient than existing methods. In addition, the short retention time of DON (3.6 min) should allow for more thorough surveys of contaminated samples, since a greater number of samples per hour can be processed. Verification of the presence of DON in samples contaminated with multiple trichothecenes or other mycotoxins is also possible. DON in crude tissue extracts would be difficult to determine by differential pulse polarographic methods due to interferences from closely related compounds. However, HPLC separates DON from other Class B compounds, *e.g.*, acetyl-DON, nivalenol and fusarenon-X, which are more non-polar and thus exhibit longer retention times under reversed-phase HPLC. Class A trichothecenes, *e.g.*, T-2 toxin and diacetoxyscirpenol (anguidine), were found to be electrochemically inactive under reductive ED conditions.

Our laboratory is currently investigating the applicability of this assay to the determination of DON and its metabolites in animal tissues. As such, ED may prove to be useful in the rapid detection of DON contamination and facilitate the diagnosis of DON-related disorders.

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