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High-Performance Thin-Layer Chromatographic Determination of Deoxynivalenol, Fusarenon-X, and Nivalenol in Barley, Corn, and Wheat

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A thin-layer chromatographic (TLC) method for determining deoxynivalenol, fusarenon-X, and nivalenol in barley, corn, and wheat has been developed. The toxins were extracted witth acetonitrile–water–methanol and purified by lead acetate precipitation, filtration through an alumina–charcoal–Celite column, and separation on an octadecylsilane column. TLC on an aluminum chloride impregnated silica gel plate was used for quantitation. After the plate was developed with two different compositions of chloroform–acetone–2-propanol, it was heated to produce blue fluorescent spots, which were quantitated fluorodensitometrically. Average recovery of the three toxins at levels of 100 and 200 ng/g was 83%, and the limit of determination was 50 ng/g. Eight test samples naturally contaminated with nivalenol and/or deoxynivalenol were also analyzed. The identity of the three toxins was confirmed after trimethylsilyl derivative formation and gas chromatographic separation followed by matrix isolation/Fourier transform infrared spectrometric analysis.

The mold genus *Fusarium* contains many species that are pathogenic to cereal grains. Under appropriate conditions, these molds produce a variety of 12,13-epoxytrichothecene mycotoxinis (Scott et al., 1984), including fusarenon-X [FX, 4-(acetyloxy)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one], nivalenol [NIV, 3,4,7,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one], and deoxynivalenol [DON, 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one]. While over 60 trichothecenes have been isolated from pure fungal cultures, only a few of these mycotoxins have been isolated from contaminated foodstuffs. In North America, the most frequently detected tricothecene is DON, which has been found in corn, wheat, and barley. Recent surveys for DON have shown that it also occurs in other major cereal-growing regions of the world (Allen, 1984).

Several mold toxins may occur in the same naturally infested grain. For example, NIV and DON have been found in grain from Japan (Yoshizawa and Hosokawa, 1983), France (Jemmali et al., 1978), Korea (Lee et al., 1985), and Germany (Blaas et al., 1984). FX has been found in corn from several European countries (Bottalico et al., 1983). To date, neither FX nor NIV has been detected in grain from the United States.

DON, also known as vomitoxin, is associated with feed refusal and serious illness in livestock (Côté et al., 1984). However, the observation that corn naturally infested with *Fusarium spp.* including *Fusarium graminearum* can produce greater toxic effects in swine than can be accounted for by the amount of DON found by chemical analysis is an indication that other mycotoxins may also be present. Although DON at the levels found to date has not been associated with observable adverse effects in humans, the toxicity of scabby wheat to humans has been known since at least 1900 (Rodricks et al., 1977). In Japan, consumption of grain contaminated by *Fusaria* has been associated with outbreaks of human illness characterized by many of the symptoms observed in animals dosed with trichothecenes. The acute toxicity of FX and NIV in mice is greater than that of DON (Yoshizawa and Morooka, 1974), and the presence of FX and NIV in food could conceivably contribute to the observed mycotoxicoses in animals and humans.

Several analytical methods have been developed for determining DON (Kamimura et al., 1981; Scott et al., 1981; Trucksess et al., 1984, 1986) and NIV and DON

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(Steinmeyer et al., 1985; Tanaka et al., 1985; Yoshizawa and Hosokawa, 1983). There is only one report of a method for the simultaneous determination of FX, NIV, and DON (Visconti et al., 1984). However, recoveries of the three toxins at the 1 μg/g level varied from 20 to 70%. Since this method was not suitable for survey determination of all three toxins simultaneously, a method based on a previously published thin-layer chromatographic (TLC) procedure (Trucksess et al., 1986) has now been developed. The recoveries of the toxins at the 100 ng/g level were greater than 80%. Approximately 8 h is required for the analysis of eight samples.

EXPERIMENTAL SECTION

Materials and Reagents. All solvents were distilled in glass and were purchased from Burdick & Jackson Laboratories. DON, FX, and NIV were purchased from Myco-Lab Co.; Celite 545 was from Johns-Manville; alumina was from Matheson, Coleman and Bell; Regisil [BSTFA, bis(trimethylsilyl)trifluoroacetamide] and Derivisil [mixture of pyridine, BSTFA, trimethylchlorosilane, and (trimethylsilyl)imidazole] were from Regis Chemicals; activated carbon (Darco G 60), chromatographic tubes (6 mL), octadecylsilyl chromatographic columns (0.5 g, partially end capped by methylation using a proprietory process), column reservoirs (75 mL), adapters, and vacuum apparatus were from J. T. Baker Chemical Co. The precoated 20 × 10 cm silica gel 60 TLC plates were purchased from E. Merck, and the 0.5-μm PTFE Millex-SR filter unit was from Millipore.

Preparation of Standard Solutions. Stock solutions of FX, DON, and NIV were prepared in methanol–ethyl acetate (1:9) at 100 μg/mL. A mixed standard containing 20 μg/mL of FX, DON, and NIV was prepared by adding 2 mL of each of the stock solutions to a 10-mL volumetric flask and diluting to volume with ethyl acetate.

Preparation of Carbon Column. A 0.2-μm 13-mm Nylon 66 filter was placed on top of the frit of the chromatographic tube, which was secured on the vacuum apparatus manifold, and 1.5 g of alumina–charcoal–Celite (5:7:3) was added to the tube. A small ball of glass wool was placed on top of the adsorbent and compressed with a glass rod.

Preparation of TLC Plate. The TLC plate was impregnated with aluminum chloride by dipping it into a solution of aluminum chloride–water–ethanol (10:15:85, w/v/v). The plate was air-dried for 24 h, cleaned by development with chloroform–acetone–2-propanol (7:1.5:1.5), and then air-dried for 4 h.

TLC Apparatus. A Camag 20 × 10 cm twin-trough chamber was used for TLC development. A Camag TLC scanner set at 313-nm excitation and 400-nm emission and a Spectra Physics SP 4100 integrator were used for fluorodensitometric quantitation.

Gas Chromatographic (GC)/Spectrometric Instrumentation. A Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and a cross-linked methyl silicone fused silica column (0.33-μm film, 0.2-mm i.d., 25-m length) was interfaced with a Mattson Cryolect/Sirius 100 matrix isolation (MI)/Fourier transform infrared (FTIR) spectrometric system controlled by a Mattson Starlab data system.

Extraction. A 50-g portion of a ground and blended test sample was extracted with 200 mL of acetonitrile–water–methanol (70:25:5) for 60 min on a shaker. The extract was then filtered, and 40 mL of the filtrate was mixed with 2 mL of 20% lead acetate solution (200 g of lead acetate and 3 mL of glacial acetic acid, diluted to 1 L with water). After 5 min, the mixture was filtered, and 21 mL of filtrate was collected in a 25-mL graduated cylinder.

Column Chromatography. *Carbon Column.* A column reservoir was placed on top of the column with an adapter, and a 100-mL beaker was placed under the column in the vacuum apparatus. All unused positions in the manifold were plugged. The column was washed with 10 mL of acetonitrile–water (84:16) under vacuum (flow rate 6 mL/min). When the solvent reached about 0.5 cm above the glass wool, the vacuum was turned off. The manifold was lifted, and a clean 100-mL beaker was put under the column. The filtrate was applied to the column (flow rate 6 mL/min). When the column reservoir was empty, the graduated cylinder was rinsed with 25 mL of acetonitrile–water (84:16) and the rinse was added to the column reservoir. The vacuum was turned off as the solvent meniscus reached the top of the packed bed. The solution was transferred to a 250-mL round-bottom flask and evaporated to dryness on a rotary evaporator with a 35 °C water bath.

Octadecylsilyl (C_{18}) Column. The column was attached to the vacuum apparatus manifold and conditioned by washing with two 6-mL portions of methanol and then two 6-mL portions of water (flow rate 6 mL/min). The purified residue was dissolved in 3 mL of water and quantitatively transferred to the column. Additional water (3 mL) was used as required for transfer. The column was washed with an additional 1 mL of water and was air-dried under vacuum for 1 min. Water droplets on the wall of the column were removed. The column was then washed with 2 mL of hexane and air-dried under vacuum for 5 min. After the vacuum was turned off, a 2-dram vial supported in a test tube rack was placed under the column. The toxins were then eluted without vacuum with 6 mL of anhydrous ethyl ether–acetone (75:25). The eluate was evaporated to dryness on a 25 °C water bath under a stream of nitrogen.

Thin-Layer Chromatography. The dried eluate was dissolved in 100 μL of chloroform–methanol (8:2). A 10-μL Hamilton syringe was used to apply, at fixed intervals 1 cm from the bottom of the TLC plate, 10 μL of the eluate solution alongside 2-, 4-, and 8-μL aliquots of the mixed standard solution. The plate was developed first with chloroform–acetone–2-propanol (8:1:1). The solvent front was 9 cm from the the origin, with a developing time of ca. 20 min at room temperature. Afterward, the plate was air-dried for 5 min. The plate was developed again with chloroform–acetone–2-propanol (7:1.5:1.5). The solvent front was 2.5 cm from the origin. After air-drying, the plate was placed in a 120 °C oven for 8 min. After cooling, the toxins were observed as blue fluorescent spots under long-wave UV light: FX at R_f ca. 0.5; DON at R_f 0.4 (calculated from the beginning of the fine particle layer and the first development solvent front); NIV at R_f 0.5 (calculated from the beginning of the fine particle layer and the second development solvent front). The blue fluorescent FX, DON, and NIV spots were quantitated fluorodensitometrically with the TLC scanner.

Confirmation. To confirm the identities of the mycotoxins, a 20 × 10 cm silica gel 60 plate was divided into 3-cm channels by scoring. Extracts containing 100 or 200 ng of toxins were spotted in the middle channels, and 20 μL of the mixed standard was spotted in each side channel. The plate was developed in the same manner as for quantitation. The extract channels were covered with a glass plate, and then the standards were sprayed with the aluminum chloride solution (same composition as for dipping). The sprayed portion of the plate was placed on

Table I. Recovery of FX, DON, and NIV Added to Corn, Wheat, and Barley

grain[a]	toxin added, ng/g	av rec, % of added			std dev (n = 4)			CV, %		
		FX	DON	NIV	FX	DON	NIV	FX	DON	NIV
corn	200	85.0	87.5	77.5	4.7	6.4	2.7	5.5	7.3	3.5
wheat		92.5	81.5	67.8	4.1	5.7	3.7	4.4	7.0	5.5
barley		85.0	88.8	87.8	6.5	8.2	15.5	7.5	9.2	17.7
corn	100	81.8	89.3	90.8	11.2	6.5	13.5	13.7	7.3	14.9
wheat		98.0	85.0	76.0	3.7	6.8	4.3	3.8	8.0	5.7
barley		73.5	78.3	68.0	8.1	14.6	5.3	11.0	18.7	7.8
corn	50	78.3	102.6	87.5	11.5	6.5	5.5	14.7	6.4	6.3
wheat		56.8	61.3	55.8	4.6	12.3	8.3	8.1	20.1	14.9
barley		55.5	108.0	41.5	2.6	2.8	3.1	4.7	2.2	7.5

[a] Corn and barley were naturally contaminated with DON at 30 and 70 ng/g, respectively, and these values were subtracted from DON found in corresponding spiked test samples before recoveries were calculated; DON was not detected in wheat. Neither NIV nor FX was found in unspiked corn, wheat, or barley.

a hot plate at 105 °C for 2 min. Long-wave UV light was used to locate the position of each standard, which was then used to locate the toxins in the channels with extracts. A 1-cm band of the silica gel at the same R_f as the standards was removed by scraping each spot individually and placed in a 10-mL syringe coupled with a 0.5-μm PTFE Millex-SR filter unit and a 20-gauge needle. The toxin was eluted with chloroform–acetone–2-propanol (6:2:2). The eluate was collected in a 1.8-mL vial and then evaporated to dryness under a stream of nitrogen in a 35 °C water bath. Test residues and standards were treated with 50 μL of Regisil–Derivisil (1:2), and the vials were sealed with screw caps fitted with Teflon-faced silicone septa. The treated solutions were mixed on a Vortex mixer and heated at 60 °C for 30 min for derivatization.

The trimethylsilyl derivatives were subjected to capillary column GC analysis. The carrier gas was helium plus 1.5% argon. The effluent was split, with 20% going to the FID while the remainder was directed to the MI/FTIR spectrometer. The presence of toxins in the test samples was confirmed by comparison of unknown spectra with corresponding standard spectra.

RESULTS AND DISCUSSION

Recovery data for FX, DON, and NIV in corn, wheat, and barley are given in Table I. Mean recoveries of FX, DON, and NIV added to grains were 86, 85, and 78%, respectively (average 83%), at levels of 100 and 200 ng/g. Mean recoveries of FX, DON, and NIV were 64, 91, and 62% at 50 ng/g (the limit of determination) for the three commodities. Recovery of DON at 50 ng/g in corn and barley was >100%. The corn and barley used in this study were naturally contaminated with DON at about 30 and 70 ng/g, respectively.

The recoveries of NIV and DON depended on the degree of capping of the commercial C_{18} column used. The partially end-capped C_{18}-bonded silica gel must be used because the fully end-capped C_{18} silica gel retained only part of the DON and none of the NIV. A small amount of the packing material leached with the ether–acetone eluting solvent. After evaporation of the solvent, the residue appeared as a white film that dissolved readily in the spotting solvent and did not interfere with the TLC analysis. Alternatively, the toxins could be eluted with 5 mL of ethyl acetate. Evaporation of this solvent required greater time but gave less residue. Percent recoveries were comparable. NIV was more sensitive to heat than FX or DON. Prolonged heating at >65 °C resulted in low recoveries of the added NIV. For this reason acetonitrile–water was removed under vacuum at 35 °C and other solvents under a stream of nitrogen.

Figures 1 and 2 show that FX, DON, and NIV clearly separated from each other and from background fluorescent materials. FX, DON, and NIV are structurally similar, but their chromatographic properties on a silica gel TLC plate are very different with use of the same mobile phase. FX usually has the highest R_f and NIV the lowest. When a mobile phase capable of moving NIV to R_f 0.3 is used, FX is moved close to the solvent front. Therefore, a double-development technique with solvent systems of different composition but the same selectivity was used. FX and DON were separated with a solvent system that kept NIV near the origin spot. NIV was then separated from the origin spot with another more polar solvent system. The second development solvent front distance was one-third the first development solvent front distance. The second solvent front was about 0.5 cm below the DON spot. More reproducible results were obtained when the

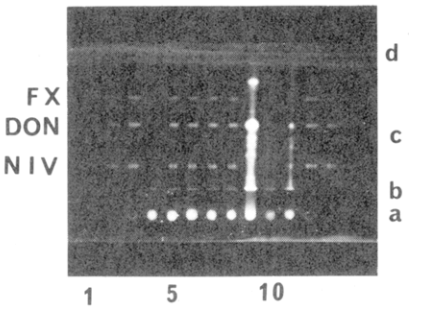

Figure 1. Photo of TLC plate. Plate: precoated silica gel 60 with concentration zone, impregnated with aluminum chloride. Mobile phases: first, chloroform–acetone–2-propanol (8:1:1); second, chloroform–acetone–2-propanol (7:1.5:1.5). Visualization: 120 °C, 8 min, excitation 313 nm, emission 400 nm. Channels: 1–3 and 12–14, standards 20, 40, and 120 ng; 4, corn (DON positive); 5–8, spiked corn; 9, mixed feed (DON positive); 10 and 11, wheat (DON and NIV positive). Key: a, origin spot; b, interphase of concentration zone and fine particle layer; c, second development solvent front; d, first development solvent front.

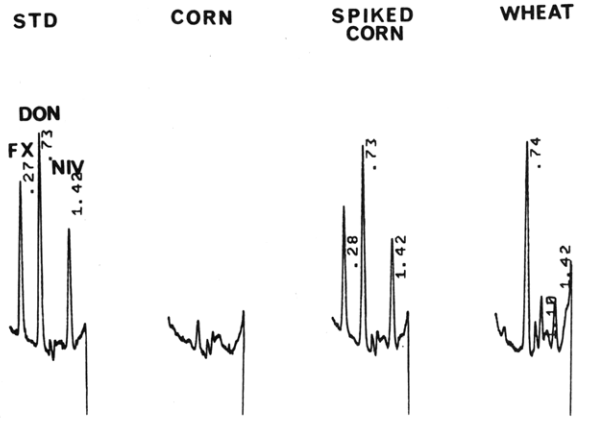

Figure 2. Fluorodensitometric scan (from top to bottom) of same TLC plate; from left to right, channels 2, 4, 5, and 11.

Table II. Analysis of Test Samples for FX,[a] DON, and NIV

grain	country of origin	anal., ng/g DON	NIV
wheat	England	188	103
wheat	England	20	78
wheat	England	20	232
wheat	England	41	30
rye	Japan	nd[b]	58
wheat	Japan	nd	63
polished barley	Japan	20	204
barley	Japan	110	347
barley	U.S.	618	nd
mixed feed	U.S.	25300	nd

[a] No FX was detected in any of these grains. [b] nd = not detected.

silica gel TLC plate was prewashed with the second developer.

To evaluate the applicability of the method to naturally contaminated grains, 10 laboratory samples of wheat and barley received from England and Japan were analyzed for FX, DON, and NIV. These laboratory samples had been previously analyzed in several laboratories and were reported to contain NIV and/or DON (Gilbert, 1985; Ueno, 1985). Results of the analyses are shown in Table II. No FX was detected. The identities of DON and NIV were confirmed by GC/MI/FTIR spectrometry (Mossoba et al., 1986). This multimycotoxin TLC method can be used to survey simultaneously for FX, DON, and NIV in corn, wheat, or barley and should be applicable to other grains.

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Registry No. FX, 23255-69-8; DON, 51481-10-8; NIV, 23282-20-4.

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Simultaneous Determination of Heavy Metals in *Chlorella* and Tea Leaves by High-Performance Liquid Chromatography

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Nickel, zinc, and copper in standard reference *Chlorella* and tea leaves were simultaneously determined by high-performance liquid chromatography (HPLC). The samples were ashed with mineral acids, and then the metals were extracted in chloroform as hexamethylenedithiocarbamate (HMDC) chelates. The metal chelates were separated with HPLC using a C_{18} column and detected at 260 nm (*Chlorella*) or 290 nm (tea leaves). Interference of manganese in the sample was removed with use of L-ascorbic acid. Results showed good agreement with certified values of the samples. The standard deviations based on 6-12 replicate determinations were 1.1-7.2%.

Heavy metals in plant samples are frequently determined by atomic absorption spectrometry (AAS) after undergoing an appropriate ashing procedure. Inductively coupled plasma atomic emission spectrometry (ICP) is also used if multielement analysis is necessary.

Simultaneous determination of heavy metals by HPLC has been studied in recent years (Willeford and Veening, 1983; O'Laughlin, 1984; Nickless, 1985). The HPLC ena-

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