FOOD CHEMICAL CONTAMINANTS

Determination of the *Fusarium* Mycotoxins Nivalenol, Deoxynivalenol, 3-Acetyldeoxynivalenol, and 15-*O*-Acetyl-4-deoxynivalenol in Contaminated Whole Wheat Flour by Liquid Chromatography with Diode Array Detection and Gas Chromatography with Electron Capture Detection

FRANK WALKER and BARBARA MEIER
University of Hohenheim, Institute of Phytomedicine, D-70599 Stuttgart, Germany

A rapid and sensitive method was developed for simultaneous detection of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-A-DON), and 15-*O*-acetyl-4-deoxynivalenol (15-A-DON) in wheat flour. Samples were extracted with acetonitrile–water (84 + 16), and the extract was filtered and purified by a column containing a combination of charcoal, celite, and other adsorbents. For screening analysis, the column eluate was only extracted with ethyl acetate. After evaporation of the solvent, the dried residue was redissolved in acetonitrile–water (2 + 8) and then analyzed by reversed-phase liquid chromatography (LC) with diode array detection. Recoveries of NIV, DON, 3-A-DON, and 15-A-DON from whole wheat flour spiked at 2 levels were 49–55, 92–97, 98–100, and 100–105%, respectively. To quantitate mycotoxin amounts lower than 1 ppm, purified column extracts were evaporated to dryness, derivatized with heptafluorobutyric anhydride, and analyzed by gas chromatography with electron capture detection (GC–ECD). Average recoveries of NIV, DON, 3-A-DON, and 15-A-DON from whole wheat flour spiked at 2 levels, were 45–52, 91–103, 81–85, and 84–92%, respectively. GC–ECD detection limits for all mycotoxins tested at a signal-to-noise ratio of 4:1 were <30 ng/g. Results of GC–ECD analysis for whole wheat flour samples spiked with mycotoxins at 3 and 10 ppm compared well with results (2.8 and 9.9 ppm) for the same samples analyzed by LC.

Trichothecene mycotoxins such as nivalenol (NIV), deoxynivalenol (DON), and the esters 3-acetyldeoxynivalenol (3-A-DON) and 15-*O*-acetyl-4-deoxynivalenol (15-A-DON) are produced by several *Fusarium* spp. These compounds are presumed to cause scabby wheat intoxication in humans and farm animals (2, 3). To determine the extent of mycotoxin contamination in whole wheat flour processed from grains that have been artificially inoculated or naturally contaminated with *Fusarium* spp., an assay was developed that could analyze a large number of samples quickly, reliably, and inexpensively.

Analysis of trichothecenes (4–12) typically involves solvent extraction and cleanup by solid-phase extraction (SPE), followed by thin-layer chromatography (4–6), capillary gas chromatography (GC; 7), liquid chromatography (LC; 8–10), or GC/mass spectrometry (11, 12). We describe here a screening procedure using LC with diode array detection (LC–DAD) to prove trichothecene contamination of 30 grain samples. Lower levels of NIV, DON, 3-A-DON, and 15-A-DON were confirmed by analyzing heptafluorobutyrate derivatives of the mycotoxins by GC with electron capture detection (ECD).

METHOD

Apparatus

(**a**) *Glass-stoppered Erlenmeyer flasks.*—250 mL.

(**b**) *Homogenizer.*—Ultraturrax (Janke & Kunkel, Staufen, Germany).

(**c**) *Filter paper.*—18.5 cm, prefolded (No. 595 ½, Schleicher & Schuell, Dassel, Germany).

(**d**) *Round bottom flasks.*—100 mL.

(**e**) *Volumetric flasks.*—10 mL.

(**f**) *Test tubes.*—1 mL, stoppered and graduated.

(**g**) *Test tubes for derivatization.*—Screw-capped with Teflon-lined caps.

(**h**) *Cleanup column.*—Mycosep No. 227 column (Romer Labs, Inc., Washington, MO).

(**i**) *Nitrogen evaporator.*

(**j**) *Shaker.*—IKA-Vibrax-VXR (Janke & Kunkel).

(**k**) *Variable microliter pipets.*—Model Research, 10–100 µL and 10–1000 µL (Eppendorf-Netheler-Hinz, Hamburg, Germany).

(**l**) *LC system.*—Pharmacia LKB gradient pump; Pharmacia LKB low-pressure mixer; Pharmacia LKB autosampler 2157 (Amersham Pharmacia Biotech, Freiburg, Ger-

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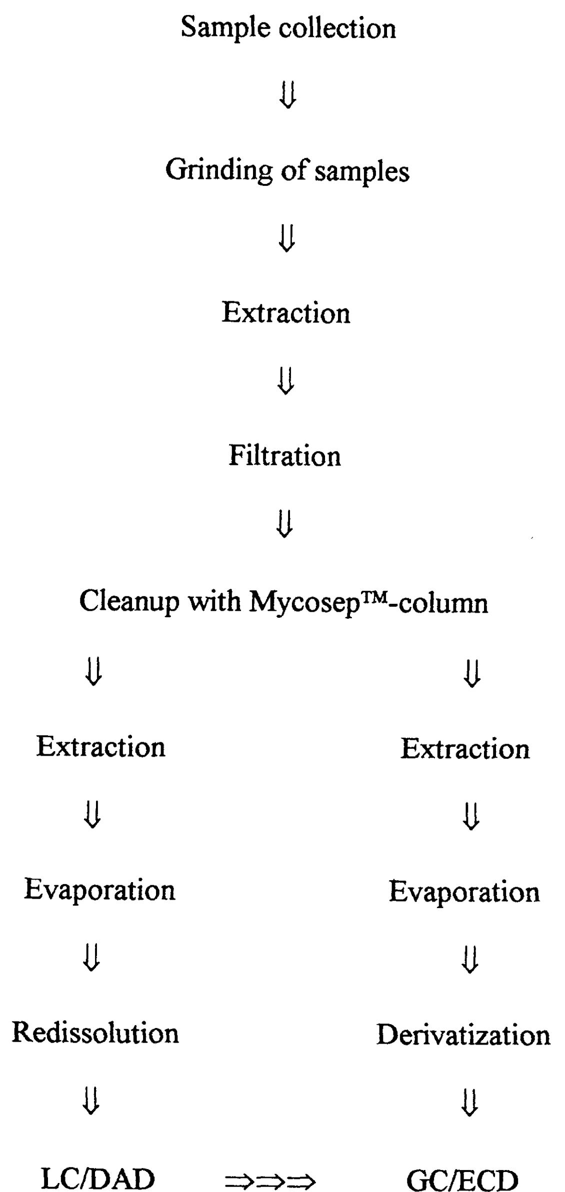

Figure 1. Scheme of the procedure for mycotoxins analysis.

many); Kontron Instruments diode array detector 440 set at 220, 230, and 240 nm; Kontron Instruments data system DS450-MT2/DAD (Neufahrn, Germany).

(**m**) *LC column*.—Nucleosil RP18, 250 × 4 mm, 5 μm (Macherey-Nagel, Duren, Germany).

(**n**) *GC system*.—Varian 3600 with Varian Star Chromatography workstation, Varian 8200 CX autosampler, split-splitless injector, ^{63}Ni electron capture detector and capillary column (Darmstadt, Germany).

(**o**) *GC column*.—Optima 1701, 25 m × 0.32 mm id, 0.35 mm (Macherey-Nagel) equipped with 5 m × 0.32 mm id deactivated guard column (Hewlett-Packard, Waldbronn, Germany). Guard column and Optima 1701 column were joined with column connector (Hewlett-Packard, No. 5041-2174).

Table 1. Gradient profile for LC determination

Step	Time, min	Flow, mL/min	Solvent A, %	Solvent B, %
1	0	0.650	8	92
2	7.0	0.650	8	92
3	7.1	0.750	8	92
4	28.0	0.750	20	80
5	32.0	0.750	100	0
6	36.0	0.750	99	1
7	40.0	0.750	8	92
8	40.1	0.650	8	92
9	45.0	0.650	8	92

Samples

Wheat samples were obtained through the Institute of Phytomedicine, University of Hohenheim, and various grain-processing farms.

Reagents

(**a**) *Solvents*.—Acetonitrile, pro analysis and gradient grade (Mallinckrodt-Baker, Griesheim, Germany); isooctane for residue analysis (Fluka-Chemie, Buchs, Switzerland), ethyl acetate (Merck, Darmstadt, Germany), and toluene p.a. (Carl Roth, Karlsruhe, Germany).

(**b**) *Extraction solution*.—Acetonitrile–water (84 + 16).

(**c**) *LC mobile phase*.—Solvent A, acetonitrile gradient grade; solvent B, water–acetonitrile (90 + 10).

(**d**) *Chemicals*.—Sodium chloride (Merck), 4-(dimethylamino)pyridine (Fluka), sodium hydrogen carbonate (Merck), and heptafluorobutyric anhydride (Fluka).

(**e**) *Saturated aqueous sodium chloride solution.*

(**f**) *DMAP/toluene mixture*.—2 mg 4-(dimethylamino) pyridine (DMAP)/mL toluene.

(**g**) *Aqueous sodium hydrogen carbonate solution*.—3%.

(**h**) *Mycotoxin standards*.—NIV, DON, 3-A-DON, and 15-A-DON certified trichothecene standards were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Purity was verified by comparison with compounds previously received from a different manufacturer or production lot.

(**i**) *Stock standard solutions*.—333 μg of each mycotoxin/mL; prepared by dissolving accurately weighed amounts of each standard in acetonitrile.

(**j**) *Mixed working standard solution*.—10 μg/mL; transfer appropriate amounts of individual mycotoxin stock standards to a 5 mL volumetric flask and bring to volume with acetonitrile.

Mycotoxin Analysis

Figure 1 is a scheme of the analytical procedure.

(**a**) *Extraction*.—Grind at least 300 g of each sample in a mill for 2 min. Weigh 5–10 g ground sample into a 250 mL Erlenmeyer flask that is equipped with a glass stopper. If fortifying samples, do so at this point with appropriate amounts of individual mycotoxin stock standards to fortify at the 0.1, 1.0, 2.0, and 10 ppm levels and wait 15 min. Add 50 mL extraction solution, stopper the flask, shake to suspend material, place in

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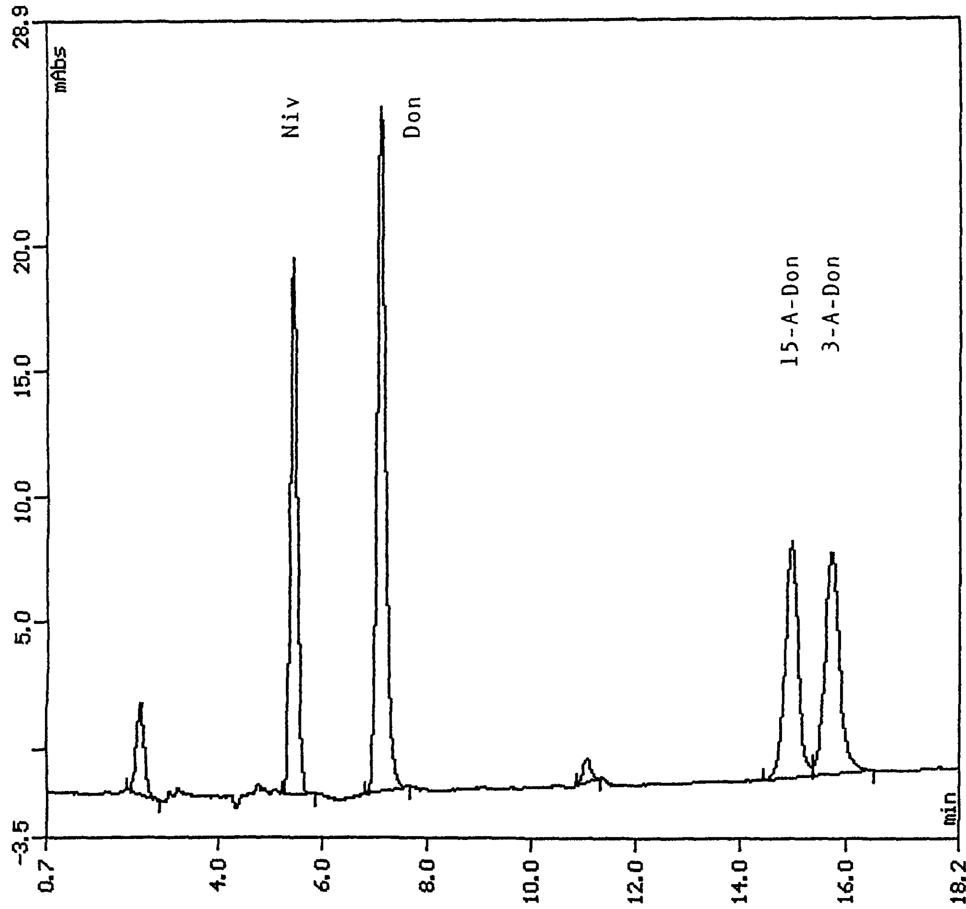

Figure 2. LC–DAD chromatogram of a mixed standard: NIV, 2.8 ppm; Don, 2.9 ppm; 3-A-DON, 3.0 ppm; and 15-A-DON, 2.9 ppm.

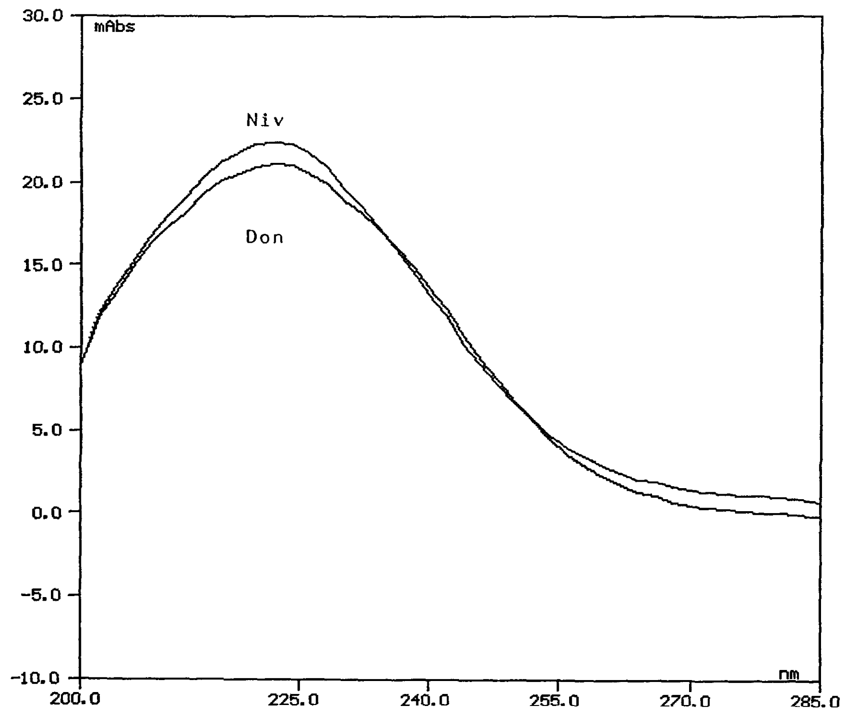

Figure 3. UV spectra of DON and NIV obtained from LC analysis of a mycotoxin standard.

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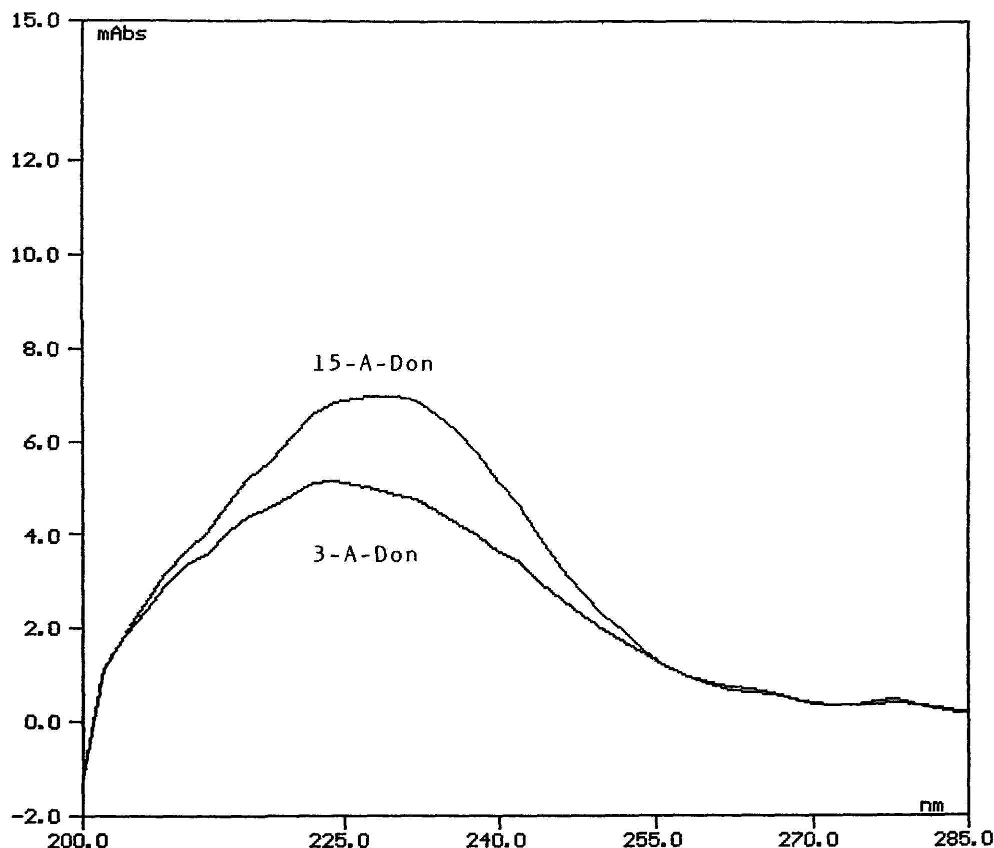

Figure 4. UV spectra of 15-A-DON and 3-A-DON standards.

rack, and stir with an Ultraturrax for 5 min. Filter mixture and collect extract. Transfer extract to 100 mL round-bottom flask and evaporate to near dryness on rotary evaporator. Redissolve extract in 2 mL acetonitrile–water (84 + 16), transfer to 10 mL volumetric flask, and fill up to mark with acetonitrile.

(**b**) *Column cleanup.*—Pipet 6 mL of sample extract into culture tube of SPE column (Mycosep column). Slowly push rubber flange end of cleanup column into culture tube, creating tight seal between rubber flange and glass wall of culture tube. As column is pushed farther into tube, force extract carefully through frit, one-way valve, and packing material successively (it should not take <25 s). Collect ca 2.2 mL purified extract in column reservoir. Using variable Eppendorf pipets, quantitatively transfer 1 mL purified extract from top of column to graduated test tube. For LC analysis, add 500 μL saturated aqueous sodium chloride solution, extract with 2 mL ethyl acetate, evaporate extract without drying over anhydrous sodium sulfate, and dissolve residue in 0.5 mL acetonitrile–water (2 + 8). Mix for 1 min and transfer into 2 mL microcentrifuge tube. Centrifuge for 2 min at 14 000 rpm. Transfer supernatant to vial.

(**c**) *LC–DAD determination.*—Start reversed-phase LC column chromatography by using gradient of water and acetonitrile. (Table 1). Inject 10, 20, and 40 μL mixed working standard solution (Figure 2) and prepare standard curve for mycotoxins tested each day. Inject 20–50 μL centrifugated test extract onto LC column. Dilute final extract to adjust response of mycotoxins to response range of standard curves.

Detect separated NIV, DON, 3-A-DON, and 15-A-DON at 2 wavelengths (220 and 230 nm) and confirm eluted compounds in all sample extracts by their UV spectra (Figures 3 and 4), obtained simultaneously by using a diode array detector during LC runs. These UV data are in accordance with published values.

(**d**) *Derivatization.*—Transfer 1 mL purified extract from Mycosep column into 5 mL test tube equipped with Teflon-lined screw cap. Add 500 μL saturated aqueous sodium chloride solution, followed by 2.5 mL ethyl acetate. Shake vigorously for 1 min on Vibrax shaker and then allow layers to separate for 2 min. Using Pasteur pipet, collect top (organic) layer and transfer it to another test tube. Repeat extraction of lower (aqueous) phase. Evaporate organic layer to dryness under gentle stream of nitrogen in 40°C water bath. Add 1 mL DMAP/toluene mixture and 50 μL heptafluorobutyric anhydride. Mix well. Derivatize sample in 60°C water bath for 20 min. After the mixture has cooled, add 1 mL 3% aqueous sodium hydrogen carbonate solution. Shake for 10 s on Vibrax shaker and allow layers to separate for 2 min. Discard lower aqueous layer. Wash organic phase with 1 mL water. Transfer top (toluene) layer to GC vial and analyze by GC–ECD with a series of standards. Dilute final extract further if response for injected extract is outside response range for standard curve.

(**e**) *Standard preparation.*—Transfer 100 μL mixed working standard solution to test tube and derivatize as described previously. Transfer appropriate amounts to 1 mL graduated

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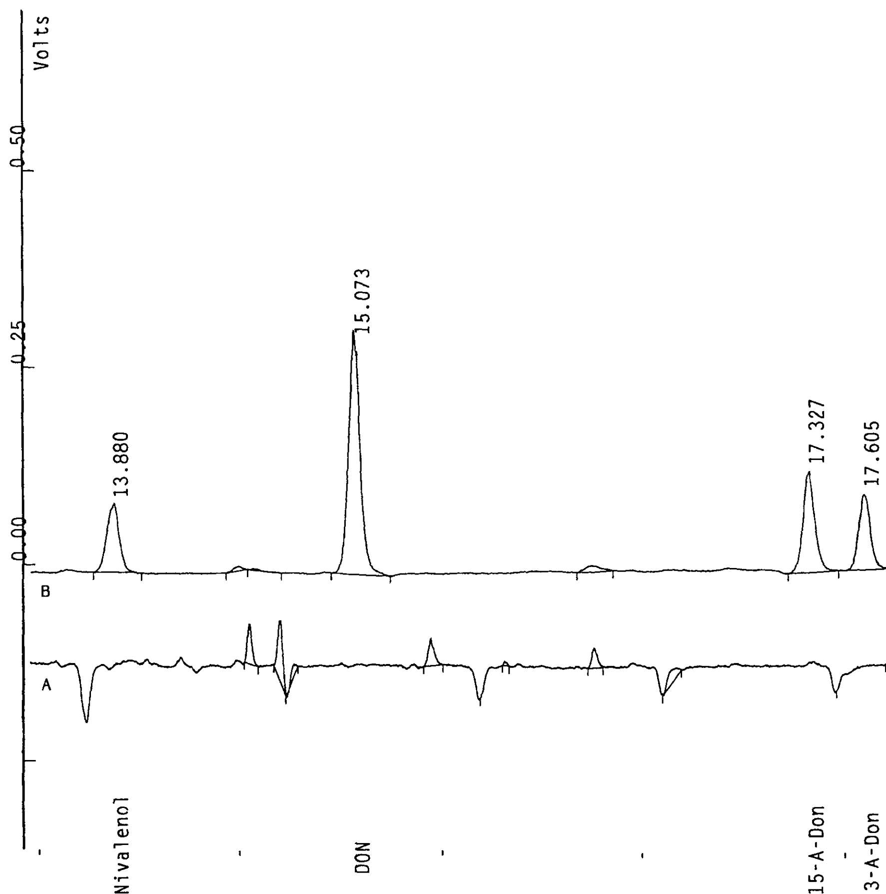

Figure 5. Typical GC–ECD chromatograms of heptafluorobutyrate derivatives of (A) blank and (B) a mixed standard containing 0.007 ppm NIV, 0.006 ppm DON, 0.006 ppm 3-A-DON, and 0.005 ppm 15-A-DON.

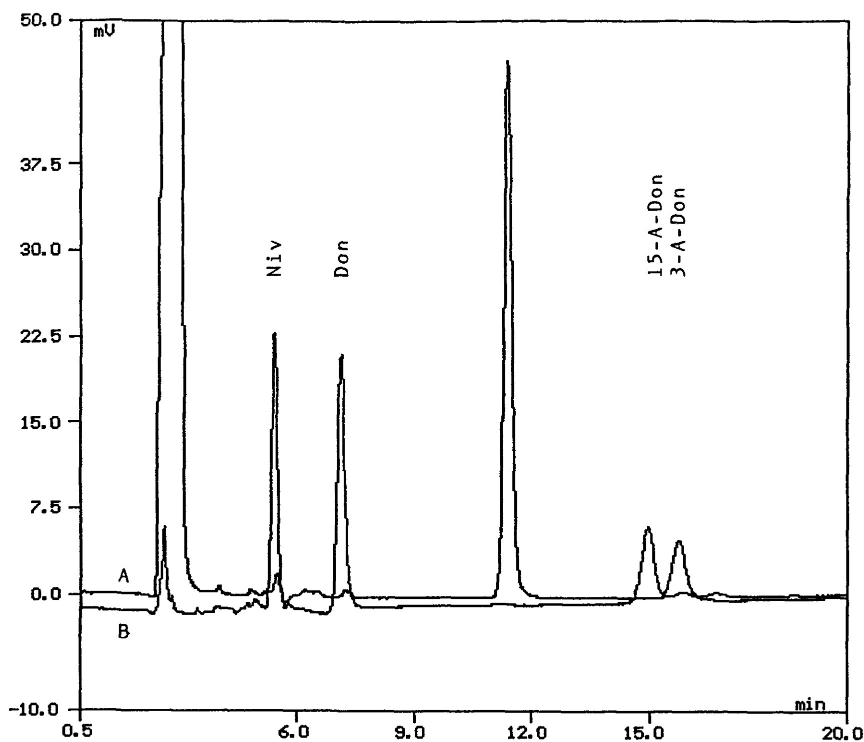

Figure 6. Typical LC–DAD chromatograms of (A) blank and (B) a wheat sample spiked with NIV, DON, 15-A-DON, and 3-A-DON, each at 10 μg/g.

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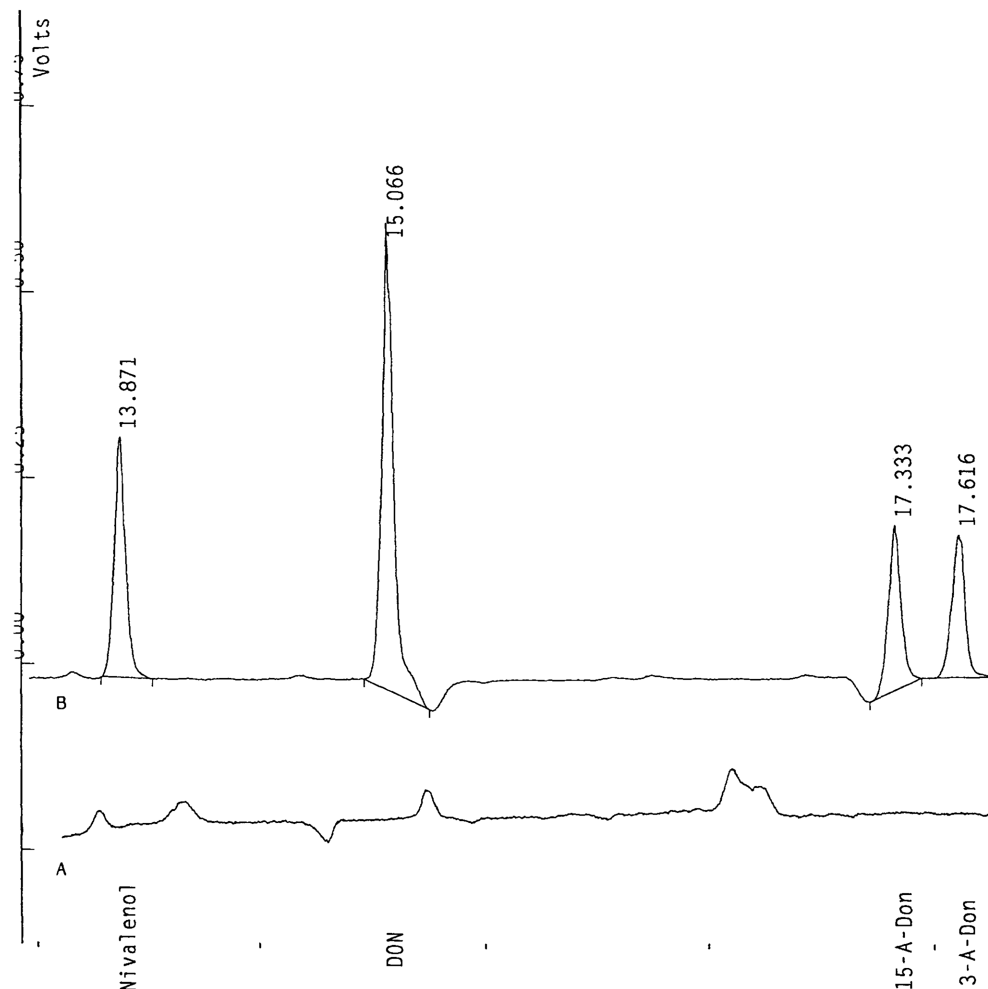

Figure 7. Typical GC–ECD chromatograms of heptafluorobutyrate derivatives of (A) blank and (B) a wheat sample spiked with NIV, DON, 15-A-DON, and 3-A-DON, each at 0.1 μg/g.

test tube and bring to volume with isooctane to make 0.002, 0.007, and 0.014 ppm derivatized standards.

(**f**) *GC determination*.—Inject 1 μL derivative solutions in splitless mode with injector at 250°C. Set GC oven temperature at 160°C, hold it for 3 min, then increase from 160° to 240°C at 6°C/min, then increase to 270°C at 30°C/min, with a 3 min hold at 270°C. Set detector at 320°C. Use nitrogen as carrier and makeup gas. Set head pressure to obtain linear velocity of 30 cm/s at 160°C (Figure 5).

Calculation of LC and GC Results

Use the following equations to calculate results:

$$\text{AT, ng/mL} = \frac{\text{AS} \times \text{area}_{\text{AT}}}{\text{area}_{\text{AS}}}$$

$$\text{AT, ng/kg} = \frac{\text{AT (ng/mL)}}{W} \times \text{df} \times X \times 1000$$

where AT = amount of test portion; AS = amount of standard (ng/mL); area_{AT} and area_{AS} = determined areas of test portion and standards in chromatogram, respectively; df = dilution factor; W = weight of ground sample (g); X = 0.5 mL for LC analysis and 1.0 mL for GC analysis.

Results and Discussion

The cleanup column was used to eliminate interferences such as fats, proteinaceous compounds, pigments, and carbohydrates extracted from the grain products by the extraction

Table 2. Results of LC–DAD and GC–ECD analysis of wheat spikes

	Recovery by LC–DAD, %		Recovery by GC–ECD, %	
Mycotoxin	Sample spiked at 2.0 ppm	Sample spiked at 10.0 ppm	Sample spiked at 0.1 ppm	Sample spiked at 1.0 ppm
NIV	49	55	45	52
DON	92	97	91	103
3-A-DON	98	100	81	85
15-A-DON	100	105	84	92

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solution. Aminopropyl, Florisil, and C_{18} columns were not as efficient as the Mycosep columns, giving low recoveries (<30%) of the mycotoxins (details not shown). The Mycosep cleanup columns were selected because of high purification efficiency of the packing, having no affinity for all the mycotoxins tested, which are eluted from the column. This procedure produces clean LC–DAD (Figure 6) and GC–ECD (Figure 7) chromatograms of whole wheat flour and wheat. Results of multiple LC–DAD and GC–ECD analyses of spiked samples are shown in Table 2.

Spike recoveries from mycotoxin-free whole wheat flour were run in triplicate at each level along with a separate blank. Recoveries of NIV, DON, 3-A-DON, and 15-A-DON spiked at 0.1 and 1.0 ppm and determined by GC–ECD analysis were 45–52, 91–103, 81–85, and 84–92 %, respectively (Figure 7). Recoveries of the same mycotoxins spiked at 2.0 and 10 ppm and determined by LC analysis were 49–55, 92–97, 98–100, and 100–105%, respectively (Figure 6). With ethyl acetate extraction as a further purification process, lower but reproducible recoveries of NIV have to be accepted because of the high polarity of the compound.

Method performance was demonstrated further by analysis of 30 whole wheat flour samples from various grain-processing farms. Sample analysis included reagent and matrix blanks, fortification recoveries, and calibration standards bracketing every 8–10 samples. In a first step, LC analysis of purified sample extract was performed to check for mycotoxin contamination levels >1 mg/kg. At lower levels of contamination, the identities of NIV, DON, 3-A-DON, and 15-A-DON in wheat flour samples had to be confirmed by derivatization with heptafluorobutyrate followed by GC–ECD analysis.

This LC selection process allows rapid sample screening and avoids derivatization of mycotoxin amounts >1 mg/kg.

For quantitation, percent change between bracketing standards should be ≤10 %. The average percent change in detector response between bracketing standards during analysis of the 30 whole wheat flour samples with accompanying blanks and fortification samples was well below this value.

Detector responses changed from 2 to 8% for all compounds with GC–ECD and from 0 to 3% with LC–DAD.

The 30 samples examined were whole wheat flours processed from grains that had been artificially or naturally contaminated by *Fusarium* spp. A summary of results is shown in Table 3. Among whole wheat flours from naturally contaminated grains, 20% contained DON at levels greater than the U.S. advisory level of 1 μg/g. The remaining samples were contaminated with DON at levels ≤1 μg/g. Samples processed from artificially inoculated grains showed significantly higher trichothecene contamination. DON, NIV, and 3-A-DON levels found in these samples were 3.10–8.33, 0.12–0.57, and 0.18–0.24 μg/g, respectively.

Preliminary runs clearly demonstrated that a 25 m × 0.32 id × 0.25 μm OV-1701 (14% cyanopropylphenyl, 86% dimethylpolysiloxane) column was necessary for obtaining baseline resolution of the mycotoxin heptafluorobutyrate derivatives.

Use of the catalyst 4-DMAP has been reported by Hofle et al. (14) to be effective in alcohol acylation. A mixture of 4-DMAP and heptafluorobutyric anhydride converts the mycotoxins to derivatives that are suitable and very sensitive for GC–ECD analysis.

Table 3. LC–DAD and GC–ECD analysis of 1997 whole wheat flours

	Amount of indicated mycotoxin found, μg/g			
Sample	NIV	DON	3-A-DON	15-A-DON
Whole wheat flours processed from naturally contaminated grains				
1	—[a]	—	—	—
2	—	—	—	—
3	—	—	—	—
4	—	—	—	—
5	—	—	—	—
6	—	0.52	—	—
7	—	1.14	—	—
8	—	—	—	—
9	—	—	—	—
10	—	0.94	—	—
11	—	—	—	—
12	—	4.06	—	—
13	—	1.68	—	—
14	—	0.65	—	—
15	—	1.88	—	—
16	—	0.51	—	—
17	—	—	—	—
18	—	—	—	—
19	—	0.36	—	—
20	—	—	—	—
Whole wheat flours, processed from artificially inoculated grains				
21	—	8.33	0.24	—
22	—	8.33	—	—
23	—	8.33	—	—
24	—	4.76	0.18	—
25	—	3.57	—	—
26	—	4.76	—	—
27	—	5.24	—	—
28	0.18	6.37	—	—
29	0.12	3.10	—	—
30	0.57	6.91	—	—

[a] —, no peak detected, or apparent peak area below limit of detection of 0.03 μg/g.

With the GC conditions described in the method, as little as 1 pg NIV, DON, 3-A-DON, and 15-A-DON standard can be detected. The limit of detection in whole wheat flour at a signal-to-noise ratio of 4:1 was <30 ng/g.

The sample cleanup procedure described is rapid, highly efficient, and reproducible. Because of the small elution volume of the column, significant time is saved in concentrating purified sample extract. Purification is efficient, as evidenced by the absence of interfering matrix peaks in either gas or liquid chromatograms (Figures 6 and 7).

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This method is an improvement over presently available workup procedures with respect to analysis time, cost, and consistency.

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