FOOD CHEMICAL CONTAMINANTS

Determination and Survey of Ochratoxin A in Wheat, Barley, and Coffee—1997

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Ochratoxin A (OA) is a nephrotoxic and nephrocarcinogenic mycotoxin produced by *Aspergillus* and *Penicillium* species. It has been found mainly in cereal grains and coffee beans. The purpose of this study was to investigate the occurrence of OA in cereal grains and in coffee imported to the United States. A modified liquid chromatographic (LC) method for determining OA in green coffee was applied to wheat, barley, green coffee, and roasted coffee. The test sample was extracted with methanol–1% $NaHCO_3$ (7 + 3), and the extract was filtered. The filtrate was diluted with phosphate-buffered saline (PBS), filtered, and passed through an immunoaffinity column. After the column was washed with PBS and then with water, OA was eluted with methanol. The eluate was evaporated to dryness, and the residue was dissolved in acetonitrile–water (1 + 1). OA was separated on a reversed-phase C_{18} LC column with acetonitrile–water–acetic acid (55 + 45 + 1) as eluant and quantitated with a fluorescence detector. Recoveries of OA from the 4 commodities spiked over the range 1–4 ng/g were 71–96%. The limit of detection was about 0.03 ng/g. OA contamination at >0.03 ng/g was found in 56 of 383 wheat samples, 11 of 103 barley samples, 9 of 19 green coffee samples, and 9 of 13 roasted coffee samples. None of the coffee samples contained OA at >5 ng/g; only 4 samples of wheat and 1 sample of barley were contaminated above this level.

Ochratoxin A (OA) is a secondary metabolite produced by several species of *Aspergillus* and *Penicillium* that has been found in a wide variety of cereal grains and coffee beans. Recently, OA was found in raisins produced in several countries (1). It also has been identified in tissues and blood of animals fed contaminated feed, and in human blood in the Balkans, Scandinavia, Germany, France, and Canada (2). OA consists of a dihydroisocoumarin moiety linked through its 7-carboxyl group by an amide bond to L-phenylalanine. OA induces nephrotoxic effects in pig, chicken, mouse, rat, and dog in chronic toxicity studies (3). A maximum limit of 5 ng OA/g for cereals and cereal products intended for direct human consumption is being proposed (4). OA has not been reported to be a problem in U.S. grains, and this may be partly due to the fact that the determination limit of the method used for surveillance is >10 ng/g (5). Human nephropathy is not as common in the United States as it is in some Balkan countries where OA commonly has been found in food, especially food derived from barley. The purpose of our study was to evaluate or modify an existing method for the investigation of OA occurrence at ≤5 ng/g in U.S. wheat, barley, and imported coffee.

Analytical methods for determining OA include thin-layer chromatography, liquid chromatography (LC), and immunochemical techniques (6–9). Three commercial immunoaffinity columns (IACs) for cleanup of OA are available: Easi-Extract from Rhone-Poulenc Diagnostics (Biocede, Ltd., France), OchraTest from Vicam (Watertown, MA), and Rida ochratoxin A from R-Biopharm (Darmstadt, Germany; 10). To save time and organic solvent, we modified the OchraTest procedure for OA in coffee (11) by optimizing solvent extraction, LC separation, and quantitation steps as applied to OA determination in barley, wheat, and coffee at levels of 1–5 ng/g. This resulting method was applicable to these commodities and was used for the 1997 survey reported here.

METHOD

Samples

Samples (ca 2 kg size each) were collected by the U.S. Department of Agriculture (USDA) Grain Inspection, Packers, and Stockyard Administration (GIPSA) from 25 states. They consisted of 383 winter wheat and 103 barley samples randomly collected from carriers (rails or trucks) within each state according to the USDA sampling plan and not according to production volume of the state. Each sample was mixed for preparation of a homogenous composite. Approximately 400 g of each composite was sent to the U.S. Food and Drug Admini-

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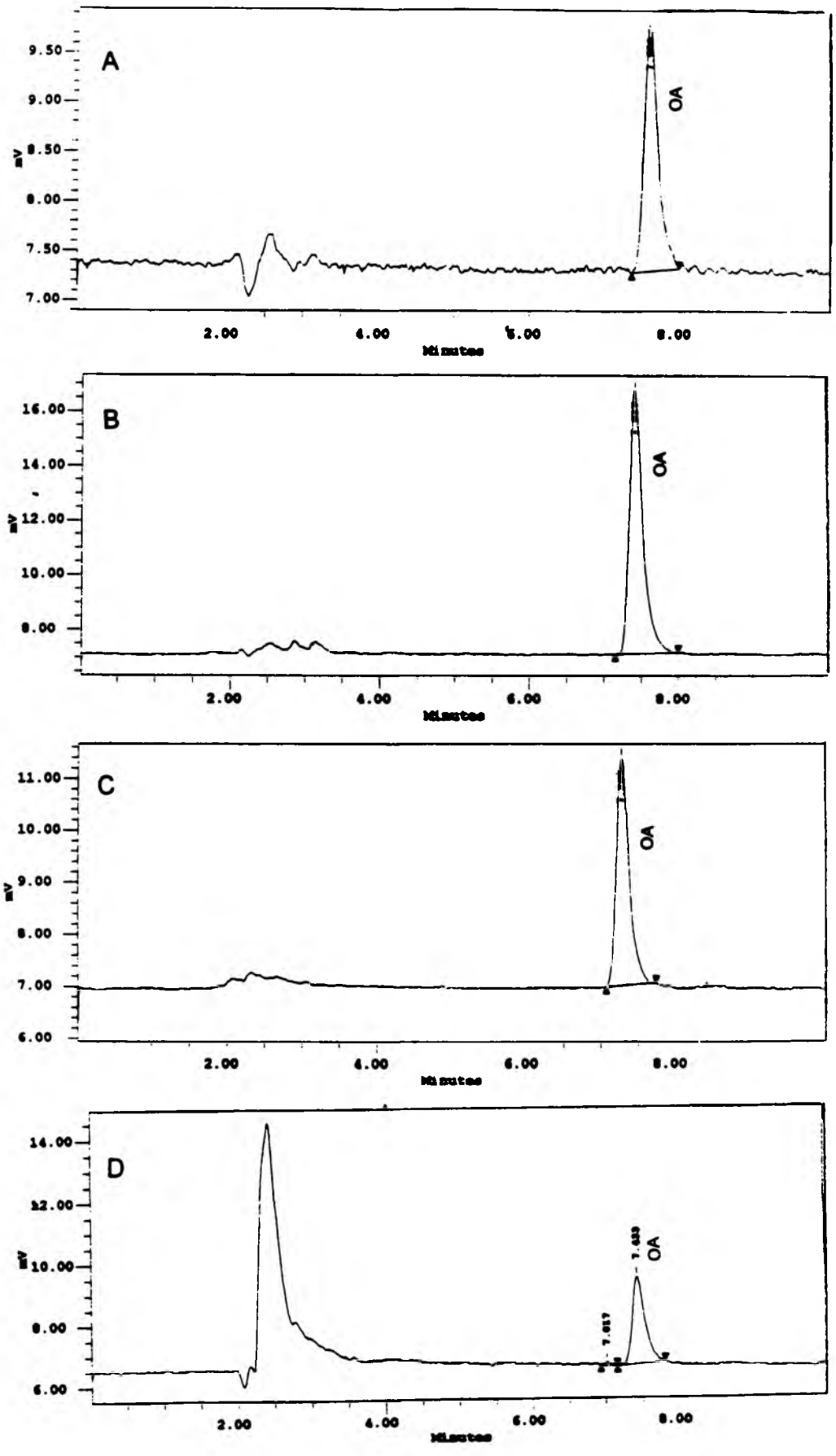

Figure 1. Liquid chromatograms of (A) OA standard (200 pg), (B) naturally contaminated wheat extract (OA at 8.4 ng/g), (C) naturally contaminated barley extract (OA at 3.5 ng/g), and (D) naturally contaminated green coffee (OA at 2.2 ng/g).

Table 1. Recovery of ochratoxin A added to wheat, barley, green coffee, and roasted coffee

Product	OA added, ng/g	Recovery, % (n = 3)	SD, %	RSD, %
Wheat	0	0	—	—
	1	93	5.9	6.3
	2	96	3.2	3.3
	4	95	2.1	2.2
Barley	0	0	—	—
	1	71	1.2	1.7
	2	82	13.6	16.6
	4	90	14.6	16.2
Green coffee	0	0	—	—
	1	89	4.4	4.9
	2	90	2.1	2.3
	4	86	7.6	8.8
Roasted coffee	0	0	—	—
	1	75	2.7	3.6
	2	81	2.5	3.1
	4	81	3.1	3.8

Table 2. Ochratoxin A in wheat, barley, green coffee, and roasted coffee

Product	No. of samples	OA concentration, ng/g	
		Range	Average
Wheat	327	<0.03	<0.03
	8	0.03–0.1	0.07
	26	0.2–1.0	0.45
	11	1.1–2.0	1.49
	3	2.1–2.9	2.50
	2	3.1–4.0	3.55
	2	4.7	4.70
	4	5.6–31.4	15.41
Barley	92	<0.03	<0.03
	4	0.03–0.2	0.13
	3	0.3–0.8	0.53
	3	1.5–3.5	2.80
	1	17.0	17.0
Green coffee	10	<0.03	<0.03
	9	0.1–4.6	1.41
Roasted coffee	4	<0.03	<0.03
	9	0.1–1.2	0.41

stration (FDA) for OA analysis. The entire 400 g was ground with a Retsch mill with a 2 mm screen to pass a No. 20 sieve. A 25 g portion was taken for analysis.

Thirty-two samples of coffee (ca 500 g each) imported from South America were provided by a coffee importer. Coffee was ground with a Waring blender and then with a Retsch mill with a 2 mm screen to pass a No. 20 sieve.

Apparatus

(a) *Mill.*—Retsch mill ZM1 with 2 mm screen (Dietz-Motoren GmbH & Co., Haan, Germany).

(b) *Explosion-proof blender.*—Waring Model EP-1 with 200 mL jar and cover.

(c) *Immunoaffinity column.*—OchraTest column (Vicam, Watertown, MA).

(d) *Filter paper.*—18 cm, prefolded (Whatman 2 V, Whatman, Inc., Clifton, NJ).

(e) *Glass microfiber filter paper.*—11 cm (Whatman 934AH).

(f) *Column reservoir.*—15 mL, polypropylene (Alltech Associates, Deerfield, IL).

(g) *Solid-phase extraction (SPE) manifold.*—12 ports (Alltech Associates).

(h) *LC column.*—Beckman No. 235335, Ultrasphere, 4.6 × 250 mm, 5 µm (Beckman Instruments, Inc., Fullerton, CA).

(i) *LC system.*—Waters Model 510 pump, Wisp 710 autoinjector, Model 540 fluorescence detector, and Millennium data system (Waters, Milford, MA). Operating conditions: flow rate, 1.0 mL/min; excitation, 333 nm; emission, 477 nm.

(j) *LC/MS system.*—Finnigan TSQ-7000 triple-quadrupole mass spectrometer (ThermoQuest Corp., Austin, TX), electrospray ionization (ESI) interface, HP 1050 liquid chromatograph (Hewlett Packard, Wilmington, DE), YMC column, J'sphere ODS-M80, 2.0 × 250 mm, 4 µm, 80A (YMC, Inc., Wilmington, NC). Mobile phase, acetonitrile–water–formic acid (60 + 40 + 0.1); flow rate, 200 µL/min.

Reagents

(a) *Solvent and reagents.*—Distilled-in-glass methanol; LC grade acetonitrile and methanol; ACS grade acetic acid, sodium bicarbonate, NaCl, sodium dibasic phosphate, sodium monobasic phosphate, Tween 20; and Milli-Q water (Millipore Corp., Bedford, MA).

(b) *Extracting solution.*—Methanol–1% sodium bicarbonate (7 + 3).

(c) *Diluting solution.*—Prepare pH 7.4 0.1M phosphate-buffered saline (10× PBS) by dissolving 2.56 g $Na_2HPO_4 \cdot 2H_2O$, 22.5 g $NaH_2PO_4 \cdot 7H_2O$, and 87.9 g NaCl in ca 900 mL water, adjusting to pH 7.4, and diluting with water to 1000 mL. Make dilution solution (1× PBS) by adding 100 mL 10× PBS to 900 mL water and 2 drops of Tween 20.

(d) *Ochratoxin A standard solutions.*—OA was isolated from cultures and purified in the FDA laboratory. (*1*) *OA stock standard solution.*—100 µg/mL. Dissolve 5 mg OA in 50 mL acetonitrile–water (55 + 45). (*2*) *Working standard solutions.*—Transfer 100 µL OA stock standard solution to 5 mL volumetric flask and dilute to volume with acetonitrile–water (55 + 45) to prepare OA solution at 2 µg/mL; make serial dilutions with the same solvent to prepare OA solutions at 4, 2, 1, and 0.5 ng/mL. Prepare all working standard solutions weekly.

(e) *LC mobile phase.*—Acetonitrile–water–acetic acid (55 + 45 + 1), degassed.

(f) *Derivatization reagent.*—14% boron trifluoride in methanol (Sigma).

Extraction of Samples

Weigh 25 g test samples in blender jar. Add 100 mL extracting solution and blend for 3 min at high speed. Filter through prefolded paper. Pipet 10 mL filtrate into a 125 mL Erlenmeyer flask. Add 40 mL diluting solution, mix, and filter through glass microfiber paper. Collect 20 mL filtrate into a 25 mL graduate cylinder and proceed immediately with IAC chromatography.

IAC Chromatography

Insert 12 gauge needle into top cap of IAC. Remove top cap from column. Cut off tip and use cap as connector between column and reservoir. Remove end cap from column and connect column onto SPE manifold. Immediately add second filtrate (equivalent to 1 g test portion) into reservoir. Let filtrate flow through column. If flow stops, use a Pasteur pipet to transfer some of the filtrate from the reservoir to fill the column and force air out of the top of the column. Let column run by gravity. Wash column with 5 mL diluting solution followed by 5 mL water. Use vacuum to dry column for 1 s. Remove reservoir and top cap and then place column in 4 mL vial. Add 0.8 mL methanol onto column directly. Use a 10 mL syringe coupled with the top cap and column and slowly push eluate through the column (ca 2 drops/s). Add additional 0.8 mL methanol to column and repeat elution. Evaporate eluate to dryness in heating block at 60°C under a stream of nitrogen.

LC Analysis

Dissolve residue in 200 μL acetonitrile–water (55 + 45). Mix 30 s. Inject 50 μL acetonitrile–water, working standards, or test sample into LC column. Identify OA peak in test sample by comparing retention time (ca 7.5 min) with those of standards. Construct standard curve. Determine concentration of OA in test solution from standard curve. If test sample gives response that is out of range of the standard curves, dilute with acetonitrile–water or concentrate and reinject to bring sample response within range of standard curve.

Calculations

Calculate concentration of OA in sample with the following formula:

$$\text{OA, ng/g} = (A \times F)/5 \text{ g/mL}$$

where A = ng OA/mL test solution, 5 g/mL = 1 g/0.2 mL, and F = dilution factor.

Chemical Confirmation

Evaporate to dryness the test extract remaining after LC analysis and containing OA at >1.0 ng/g. Add 500 μL derivatization reagent and heat at 60°C for 10 min, according to AOAC Official Method **991.44** (11). After evaporation of reagent, dissolve residue in 100 μL acetonitrile–water (55 + 45). Inject 50 μL into LC column. The retention time of the OA methyl ester is ca 17 min.

LC/MS/MS Analysis

Collect MS/MS data after LC separation. MS ionization conditions are as follows: needle spray voltage, 4.5 kV; heated capillary, 230°C; and tube lens, 100 V. Use nitrogen at a sheath gas pressure of 70 psi and also through the sheath liquid inlet to assist in nebulization. Operate the mass spectrometer in the positive-ion MS/MS mode with Q1 set to transmit *m/z* 404. Use argon for the collision gas at a pressure of 1.5 millitorr. Set collision offset voltage at –15 V. Scan Q3 from 230 to 406 daltons in 0.5 s.

Results and Discussion

The extraction method for OA in coffee (11) was modified. The solvent/sample ratio was increased to 4 to provide better extraction of OA in barley and wheat. LC chromatograms of OA standard, wheat, barley, and coffee are shown in Figure 1. The retention time (RT) for OA was about 7.5 min, and OA clearly was separated from background fluorescent materials. Chromatograms of the 4 commodities were obtained on different days, resulting in minor difference in RTs. Because the LC mobile phase was isocratic, it was recycled for at least a week before a new mobile phase was made. Recent studies in our laboratories have demonstrated that this method is also applicable to raisins.

Recoveries of OA from wheat, barley, and coffee spiked at 1, 2, and 4 ng/g are shown in Table 1. Recoveries were 71–96%, with standard derivations of 1.2–16.6. Method sensitivity was about 0.03 ng/g (inject 0.5 g test sample containing 0.015 ng OA into LC, signal-to-noise ratio = 5).

The method was used to survey OA in 383 wheat, 103 barley, 19 green coffee, and 13 roasted coffee samples (Table 2). Of 383 wheat samples, 327 were negative and 56 were positive for OA contamination. Levels in the 56 positive samples ranged from 0.03 to 31.4 ng/g; 4 samples (1%) contained >5.0 ng/g. Of 103 barley samples, 92 were negative and 11 were positive. OA levels in 9 samples ranged from 0.1 to 17.0 ng/g; 1 sample (1%) contained >5.0 ng/g (17.0 ng/g). Of 19 green coffee samples, 10 were negative and 9 were positive. OA levels ranged from 0.1 to 4.6 ng/g. Of the 13 roasted coffee samples, 4 were negative and 9 were positive. OA levels ranged from 0.1 to 1.2 ng/g. None of the coffee samples contained OA at >5.0 ng/g.

The identity of OA in a test sample extract (1–4 ng/g) was confirmed by forming the methyl ester that gave an RT of about 17 min. A test sample extract containing OA at >4.5 ng/g was analyzed by LC/MS. Confirmation was based on comparison of the mass spectrum of the presumptive OA in the sample extract and the mass spectrum of the OA standard. The presence of molecular ions at *m/z* 404, 239, 341, 358, and 386 confirmed the identity of OA in the samples.

For the 1997 crop year, about 1% of the winter wheat and barley contained OA at >5 ng/g. Additional studies are required to determine year-to-year variations.

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