Mycotoxins (Ochratoxin A, Citrinin, and Sterigmatocystin) and Toxigenic Fungi in Grains and Other Agricultural Products

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Ochratoxin A was detected in 18 out of 29 samples of heated grain from Saskatchewan farms at concentrations of 0.03 to 27 ppm. After development of an appropriate screening method, 13 of these samples were also found to contain citrinin (0.07 to 80 ppm). Sterigmatocystin was detected in one grain sample. Strains of *Penicillium viridicatum* Westling or *P. palitans* Westling, producing either ochratoxin A or citrinin or (usually) both toxins concomitantly, were isolated from 22 grain samples (including 16 of those containing ochratoxin A), from three samples of mixed feeds (one of which contained ochratoxin A), from four samples of dried white beans (three containing ochratoxin A), and from an ochratoxin A positive sample of moldy peanuts. *P. cyclopium* Westling that produced penicillic acid was isolated quite frequently, particularly from mixed feeds, although the mycotoxin itself was not found in the samples. Zearalenone was identified in a culture of *Fusarium equiseti* (Corda) Sacc. isolated from a wheat sample.

Ochratoxin A {(−)-*N*-[(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-3-phenylalanine}, one of six closely related metabolites (Steyn and Holzapfel, 1967), was first isolated from a strain of *Aspergillus ochraceus* Wilh. (van der Merwe *et al.*, 1965). Other members of the *A. ochraceus* group and also *Penicillium viridicatum* Westling have since been shown to produce the toxin (Lai *et al.*, 1970; van Walbeek *et al.*, 1969). Ochratoxin A has a high acute toxicity to ducklings, rats, chicks, and rainbow trout and causes kidney and liver damage (Doster *et al.*, 1971; Peckham *et al.*, 1971; Purchase and Theron, 1968; Theron *et al.*, 1966); in pregnant rats it induces fetal death and resorption (Still *et al.*, 1971). Subacute toxicity of ochratoxin A to chickens and rats is evident at levels as low as 0.2 ppm in the feed (Munro *et al.*, 1972; Tucker and Hamilton, 1971). Although ochratoxin A was not found carcinogenic to rats (Purchase and van der Watt, 1971), it produced liver tumors in rainbow trout when fed together with sterculic acid (Doster *et al.*, 1971).

Citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid) and penicillic acid (3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid) are toxic antibiotics produced by several species of *Aspergillus* and *Penicillium* (Korzybski *et al.*, 1967). Like ochratoxin A, citrinin causes kidney damage in experimental animals (Ambrose and DeEds, 1946; Krogh *et al.*, 1970), while penicillic acid has been found to cause local tumors on subcutaneous injection in rats and mice (Dickens and Jones, 1965).

Ochratoxin A has been detected in occasional samples of U. S. corn and barley (Nesheim, 1971; Shotwell *et al.*, 1971). We have reported the presence of ochratoxin A in a sample of heated wheat from Alberta (Scott *et al.*, 1970b). Strains of *Penicillium viridicatum* that produced ochratoxin A alone or together with citrinin predominated on the grain when incubated at 12°. We thus decided to routinely look for ochratoxin A, in addition to aflatoxins, in samples of heated grains, moldy feeds, and other foodstuffs. Zearalenone [(*S*)-(−)-3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7(8*H*)-dione], an estrogenic *Fusarium* mycotoxin (Mirocha *et al.*, 1967), and sterigmatocystin {3a,12c-dihydro-8-hydroxy-6-methoxy-7*H*-furo[3′,2′:4,5]furo-[2,3-*c*]xanthen-7-one}, a carcinogenic metabolite of *Aspergillus versicolor*, *A. nidulans*, *A. rugulosus*, *P. luteum*, and a *Bipolaris* sp. (Ballantine *et al.*, 1965; Dean, 1963; Holzapfel *et al.*, 1966; Purchase and van der Watt, 1970), were also included in the analytical screening procedures. Fungi isolated from samples were screened for their mycotoxin-producing ability (Scott *et al.*, 1970a), whereupon it became apparent that, in addition to ochratoxin A, citrinin and penicillic acid were significant potential problems requiring reanalysis of many of the samples for these mycotoxins.

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MATERIALS AND METHODS

Samples. Grain samples coded D (Table I) were associated with lung problems in farmers and elevator operators and were collected from farm storage bins in Saskatchewan (Dennis, 1972). The grain had been harvested in 1968, when an estimated 70% or more of the harvest was stored under damp conditions, resulting in heating and spoilage (Dennis, 1972). Dilution plate counts previously carried out on five of these samples (Wallace, 1970) indicated that *Penicillium viridicatum* predominated on samples D-29, D-43, D-36, and D-68 and *Aspergillus versicolor* on sample D-45. Two moldy grain samples (C70/661 and A 70/861; Table I) and all feeds listed in Table II were associated with the deaths of farm animals. Additional to samples listed in Tables I and II, seven other grain samples (four associated with sickness or death in farm animals), six more mixed feeds (all associated with veterinary problems), and samples of rapeseed–mustard pellets, rapeseed, and moldy peas were screened for mycotoxins and, in some cases, for mycotoxin-producing fungi.

Analysis of Mycotoxins in Agricultural Products. Samples of grains and other materials were ground (20 mesh) and analyzed for ochratoxin A, aflatoxins B_1, B_2, G_1, and G_2, sterigmatocystin, and zearalenone by either the "Best Foods" method (Waltking *et al.*, 1968), modified by using three chloroform extractions of the methanol–water phase to effect complete transfer of ochratoxin A (Method 1), or the multi-mycotoxin method of Stoloff *et al.* (1971), using 20- or 25-g samples without reduction of solvent volumes (Method 2). Method 2 was found to be preferable for wheat because of troublesome emulsions with Method 1. Most of the commodities from which citrinin-producing fungi were isolated were later reanalyzed for citrinin (and ochratoxin A) by Method 2 with the inclusion of 2 ml of 20% sulfuric acid in the 200-ml

Table I. Ochratoxin A, Citrinin, and Penicillic Acid in Grains and Isolated Strains of *Penicillium* spp.

Sample					Isolated fungi				
Type	Code	Ochratoxin A, ppm	Citrinin, ppm	Penicillic acid	Species	Strain no.	Ochratoxin A[a]	Citrinin[a]	Penicillic acid[a]
Wheat	C 70/661	ND[b]		ND	*P. cyclopium*[c]	335	ND[b]	ND	+
Wheat	A 70/786	0.13			*P. cyclopium*[c]	414	ND	ND	+
Grain	A 70/861	ND		ND	*P. viridicatum*[c]	489a	+	+	ND
Wheat	Birsay	0.18 (0.7)[d]	2.1		*P. viridicatum*	598	+	+	ND
Wheat	Landis	ND (0.03)[d]	0.1	ND	*P. viridicatum*	561	+	+	ND
Wheat	D-29	0.13 (0.62)[d]	2.0		*P. viridicatum*[c]	481	+	+	ND
					P. viridicatum[c]	482	ND	+	ND
Oats + barley	D-36	11 (22)[d]	60		*P. viridicatum*[c]	487	ND	+	ND
					P. viridicatum[c]	488	+	+	ND
Wheat	D-43	1.3 (1.6)[d]	2.4		*P. viridicatum*[c]	479	+	ND	ND
					P. viridicatum[c]	480	+	+	ND
Wheat[e]	D-45	ND			*P. viridicatum*[c]	484	+	+	ND
Oats	D-68	0.46 (3.0)[d]	10		*P. viridicatum*[c]	486	+	ND	ND
Wheat	D-16	ND (0.1)[d]	ND	ND	*P. viridicatum*	541	ND	+	ND
Wheat	D-18	ND (ND)[d]	ND	ND	*P. viridicatum*	560	+	+	ND
Wheat	D-23	ND (ND)[d]	ND		*P. viridicatum*	564	+	+	ND
Wheat	D-30	0.08 (0.27)[d]	0.28		*P. viridicatum*	543a	+	+	ND
Wheat	D-34	0.12 (0.20)[d]	ND		*P. viridicatum*	568	+	+	ND
Wheat	D-52	0.13 (0.14)[d]	ND		*P. viridicatum*	570	+	+	ND
Wheat	D-62	4.5 (3.2)[d]	1.7	ND	*P. viridicatum*	547	+	+	ND
Wheat	D-63	2.5 (3.3)[d]	1.0	ND	*P. cyclopium*[c]	548	ND	ND	+
					P. viridicatum	572	+	+	ND
Wheat	D-66	0.07 (0.21)[d]	0.07	ND	*P. cyclopium*	574	ND	ND	+
Wheat	D-67	2.1 (1.5)[d]	6.7	ND	*P. viridicatum*	597	+	+	ND
					P. cyclopium	551	ND	ND	+
Wheat + wild oats	D-69A	0.07 (0.07)[d]	ND	ND	*P. viridicatum*	596	ND	+	ND
					P. cyclopium	553	ND	ND	+
Rye	D-74	ND (ND)[d]	ND	ND	*P. viridicatum*	555	+	+	ND
Barley	D-76	ND (ND)[d]	ND	ND	*P. viridicatum*	556	+	+	ND
					P. cyclopium[c]	557	ND	ND	+
Rye	D-83	0.24 (0.72)[d]	0.96		*P. viridicatum*	558	+	+	ND
Wheat	D-85	19 (27)[d]	80		*P. viridicatum*	586	+	+	ND

[a] Toxins produced on yeast extract (2%)–sucrose (15%) medium. [b] ND = not detected. [c] Identified at the Centraalbureau voor Schimmelcultures, Baarn. [d] Determinations in parentheses refer to later reanalysis by Method 2A (same extract used for the citrinin determination). [e] Sample contained sterigmatocystin (approximately 0.3 ppm).

acetonitrile–aqueous KCl extraction mixture (Method 2A). A few samples (grains D-29, D-36, D-43, and D-68 and peanuts 107043 U) were analyzed for ochratoxin A, citrinin, and the other mycotoxins by Method 1, with the addition of the same proportion of sulfuric acid to the methanol–water extraction mixture (Scott *et al.*, 1970b) (Method 1A); this method did not give good recoveries of citrinin from spiked grains (Table III) and all results recorded for citrinin in Tables I and II (except for the peanuts) were determined by Method 2A. The mink feeds M-2 and M-3 (Table II) were analyzed by method 2A only. Penicillic acid and patulin were looked for by the method of Scott and Somers (1968) (wheat C 70/661 and feeds 49 FHR 49, 49 FHR 67, 49 FHR 181, and Medicine Hat), Method 2, or Method 2A, particularly in samples that harbored fungi producing these mycotoxins. Recovery experiments to assess the validity of Methods 1, 1A, 2, and 2A were carried out with powdered Selkirk wheat (or other foodstuff) spiked with appropriate toxins and analyzed immediately (Table III). Sample D-45 was analyzed for sterigmatocystin by the method of Stack and Rodricks (1971).

Thin-layer chromatography (tlc) of mycotoxins and sample extracts was performed on silica gel layers (Adsorbosil 5 or Analtech silica gel G) developed with toluene–ethyl acetate–90% formic acid (6:3:1, v/v/v; TEF) (Scott *et al.*, 1970a). Toxins were estimated by visual comparison with known amounts of standard spotted on the same tlc plate. Standard ochratoxin A solutions of 10 μg/ml or 1 μg/ml in benzene +0.25% acetic acid were used; the lowest standard concentration of citrinin was 10 μg/ml (in benzene). Estimation of ochratoxin A after ammonia treatment (Scott and Hand, 1967) was necessary if citrinin interfered; otherwise estimates before and after ammonia (green and blue fluorescent spots, respectively, under ultraviolet light) were generally averaged. Ochratoxin A was confirmed by tlc in the solvent system benzene–methanol–acetic acid (24:2:1, v/v/v; BMA), by solubility in sodium bicarbonate solution, and by formation of the fluorescent methyl ester on a microgram scale on heating the tlc-purified ochratoxin A 10 min with BF_3–methanol, followed by evaporation of the solvents and tlc.

Citrinin estimations were carried out without delay after extraction and concentration of the extract. Ether–methanol–water–90% formic acid (95:4:1:1, v/v/v/v; lined tank saturated with ether) was a preferable tlc solvent system for estimation of citrinin; confirmation of identity was obtained with ammonia, which washed out the yellow fluorescence, and then by spraying with fresh BF_3–methanol, which gave a green fluorescence. Sterigmatocystin was detected after tlc of sample D-45 extract by an aluminum chloride spray (Stack and Rodricks, 1971) and confirmed by formation of acid and acetate derivatives (Stack and Rodricks, 1971).

Two-dimensional tlc in the solvent systems ether and TEF was used to estimate penicillic acid recovered from spiked wheat. Acetone–chloroform (1:9, v/v) was an additional solvent system used for estimation of aflatoxin recoveries.

Isolation and Culture of Fungi. MEDIA. The following media were used: for identification of fungi, Czapek solution

Table II. Ochratoxin A, Citrinin, and Penicillic Acid in Feeds, Beans, Peanuts, and Isolated Strains of *Penicillium* and *Aspergillus* spp.

Sample					Isolated fungi				
Type	Code	Ochratoxin A, ppm	Citrinin	Penicillic acid	Species	Strain no.	Ochratoxin A[a]	Citrinin[a]	Penicillic acid[a]
Mixed feed	49 FHR 49	ND[b]		ND	*P. viridicatum*[c]	220	ND[b]	ND	+
(dairy ration)					*P. cyclopium*[c]	221	ND	ND	+
Horse feed	49 FHR 67	0.02		ND	*P. cyclopium*[c]	324	ND	ND	+
					A. ostianus[c]	325	ND	ND	+
Mixed feed (pelleted	49 FHR 181	0.53 (0.17)[d]	ND	ND	*P. viridicatum*[c]	490	+	+	ND
dairy ration)					*P. cyclopium*[c]	227	ND	ND	+
Chicken feed	(Riddell *et al.*,	ND			*P. viridicatum*[c]	475	+	+	ND
	1971)				*P. cyclopium*	354	ND	ND	+
Mixed feed (cattle)	Medicine Hat	ND		ND	*P. palitans*[c]	583	+	+	ND
Mink feed	M-2	ND	ND	ND	*P. cyclopium*	591	ND	ND	+
Mink feed	M-3	ND	ND	ND	*P. cyclopium*	592	ND	ND	+
Dried white beans	37852 U	0.05 (0.03)[d]	ND		*P. viridicatum*[c]	473	+	+	ND
Dried white beans	37790 U	0.50 (0.02)[d]	ND		*P. viridicatum*[c]	474	+	+	ND
					Penicillium sp.	590	ND	+	ND
					(white mutant)				
Dried white beans	141007 U	2.1 (1.9)[d]	ND	ND	*P. cyclopium*	565	ND	ND	+
(powdered)					*P. palitans*[c]	495	ND	+	ND
Dried white beans	Retail sample	ND			*P. viridicatum*[c]	469	+	+	ND
					P. cyclopium	387	ND	ND	+
Peanuts	107043 U	4.9	ND		*P. viridicatum*[c]	476	+	ND	ND
					P. cyclopium[c]	439	ND	ND	+

[a] Toxins produced on yeast extract (2%)–sucrose (15%) medium. [b] ND = not detected. [c] Identified at the Centraalbureau voor Schimmelcultures, Baarn. [d] Determinations in parentheses refer to later reanalysis by Method 2A (same extract used for the citrinin determination).

Table III. Recoveries of Ochratoxin A, Citrinin, and Other Mycotoxins Added to Powdered Grains and Beans

	Method 1		Method 1A		Method 2		Method 2A	
Mycotoxin	Concn added,[a] ppm	Recovery, %	Concn added,[a] ppm	Recovery, %	Concn added,[a] ppm	Recovery, %	Concn added,[a] ppm	Recovery, %
Ochratoxin A	0.50[b]	100	0.10	90	0.05	100	0.05	85
	0.19	80	0.05[c]	100[d]	0.20	93	0.20	94
	0.454	68			1.0	86	1.0	113
	3.16	88[d]						
	0.095[e]	100						
	0.454[e]	102						
Citrinin	1.0	0	0.25	0	5.0	0	0.50	63
	20.0	0.7	0.50	20			1.0	86
	100	0.8	1.0	40			5.0	100
			5.0[c]	10[d]			5.0	100
Sterigmatocystin	1.0[b]	60						
Zearalenone	5.0[b]	100						
Penicillic acid					4.0	60–80	4.0	30–60
Aflatoxin B_1			0.050[c]	100[d]	0.050	100	0.050	82
Aflatoxin G_1			0.050[c]	100[d]	0.050	100	0.050	98
Aflatoxins B_2, G_2			0.025[c]	100[d]	0.025	100	0.025	98

[a] Toxin added to powdered wheat except where indicated. [b] Added to powdered barley. [c] Added to rye flour. [d] Total of 200 ml of $CHCl_3$ used for partition step. [e] Added to powdered beans.

agar with and without 5% NaCl, malt agar (Difco products), and steep agar (Raper and Thom, 1968); for production of mycotoxins, 15% sucrose plus 2% yeast extract (YES) (Davis *et al.*, 1966); for isolation of single spore cultures and production of spore crops, potato dextrose agar prepared with fresh potatoes according to the Difco formula (FPDA); and for preparation of spore suspensions, sterile aqueous 0.05% polyoxyethylene sorbitan monooleate (Tween 80).

MONO-SPORE CULTURES. Light suspensions of spores were cross-streaked on FPDA plates (4 mm thick). After 24-hr incubation at 25°, plates were viewed on an inverted microscope to locate single germinating spores which were excised with sterile capillary tubes (1 mm diameter), inoculated on FPDA, and incubated at 25° until cultures sporulated heavily.

ISOLATION, SCREENING, AND IDENTIFICATION OF FUNGI. The samples examined for fungi producing known mycotoxins are listed in Tables I and II. The method for isolation of fungi was based on that of Wallace and Sinha (1962). Sterile Whatman No. 1 filter paper disks in sterile plastic petri plates were dampened with 2 ml of YES and seeded with 10 to 20 seeds, seed fragments, or 1 to 2 g of particulate samples. Individual plates were enveloped in polyethylene bags and incubated at 10°. After 14 days, plates were examined daily under a stereoscopic microscope for fungal growth. After 21 days, plates were transferred to a 25° incubator and examined at intervals dictated by the nature of the outgrowing fungi. As mycelial growth and sporulation occurred, spores from single sporophores of representative fungi were picked with a fine loop (1 mm diameter) coated with a film of Tween 80 solution to which spores readily adhered. This small inoculum was dispersed in 0.5 ml of Tween 80 solution using a vortex mixer. Part of this suspension was cultured on FPDA slopes and the remainder on 5 ml of YES (van Walbeek *et al.*, 1968). Chloroform extracts of the latter cultures were ini-

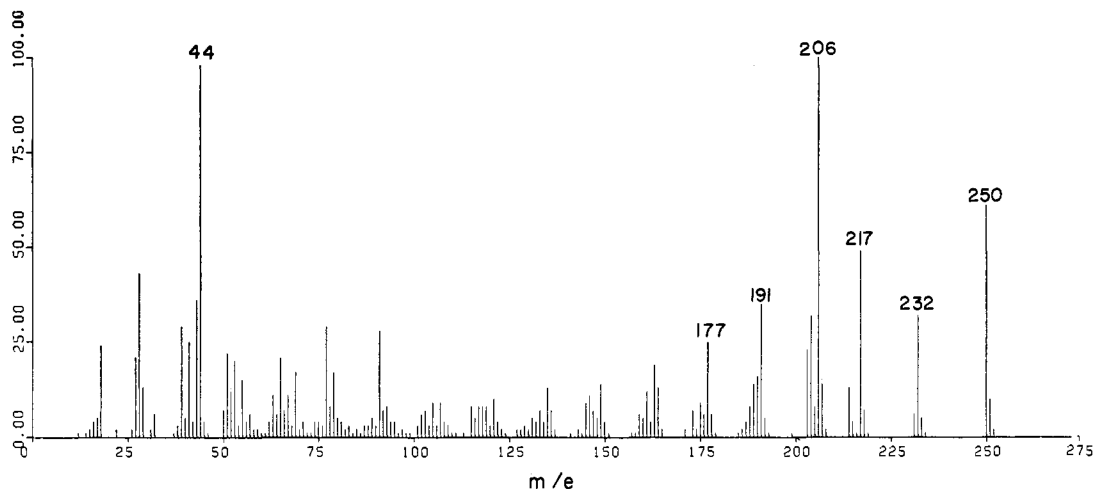

Figure 1. Mass spectrum of citrinin from *Penicillium palitans* isolate no. 495 recorded with a Perkin-Elmer RMS-4 instrument at 80 eV (source temperature 200°, probe temperature 95°)

tially screened for known mycotoxins by the method of Scott *et al.* (1970a). Those cultures producing mycotoxins were noted and the corresponding FPDA cultures were used to obtain mono-spore cultures, which were rechecked for toxin production (*vide infra*) after 7–9 days of incubation at room temperature, usually on 200 ml of YES in 2.8-l. Fernbach flasks. Culture liquids were decanted out of the Fernbach flasks and the mycelial mats were flooded with 250 ml of chloroform, heated in a 60° water bath for 10 min, and then allowed to soak for 18 hr at room temperature. Where necessary, culture extracts were concentrated under a stream of nitrogen. Spore suspensions from the mono-spore cultures were also preserved in sterile dry soil (Raper and Fennell, 1965). These were submitted to Centraalbureau voor Schimmelcultures, Baarn (CBS), for identification or were identified in this laboratory by comparison with the CBS cultures on the media specified above (Raper and Thom, 1968).

In addition to using the routine isolation procedure, detection of ochratoxin A and citrinin-producing fungi was greatly aided by briefly viewing the filter paper cultures over a Blak-Ray longwave ultraviolet transilluminator (model C-50) after 2 weeks at 10°. Green-blue and brilliant yellow fluorescing areas observed in the YES-impregnated filter paper were outlined on the plates, and spores from sporophores emerging from the marked areas were picked as described above. Although penicillic acid and patulin do not fluoresce under these circumstances, many strains producing these toxins frequently were observed to secrete in the filter paper fluorescent substances, which soon became associated with production of these toxins and hence aided detection of the responsible fungi.

Characterization of Mycotoxins in Fungal Extracts. Thin-layer chromatography of ochratoxin A and citrinin in fungal extracts was carried out by comparison with standards in three systems: TEF–silica gel, BMA–silica gel, and TEF–acidic alumina (Woelm). Confirmatory tests were ammonia treatment and BF_3–methanol spray (for citrinin). Additional confirmation by solubility in sodium bicarbonate solution and tlc of the methyl ester formed with BF_3–methanol was obtained for tlc-purified ochratoxin A (approximately 0.3 mg) isolated from *Penicillium palitans* strain no. 583 (mycelium). Crystalline ochratoxin A, mp 93–95° (from benzene), was obtained from the mycelium extract of *P. viridicatum* strain no. 476 after preparative tlc (TEF–silica gel), and identified by mixed melting point with authentic ochratoxin A and comparison of quantitative ultraviolet spectrum, infrared spectrum, and mass spectrum with those of the standard. Crystalline citrinin, mp 176–179° (dec), was isolated from the acidified culture liquid of *P. palitans* strain no. 495 after chloroform extraction, preparative tlc (TEF–silica gel), and recrystallization from ethanol, and identified by comparison with standard citrinin using the same criteria as for crystalline ochratoxin A. The mass spectrum, which has not been previously published, is shown in Figure 1. Samples of tlc-pure citrinin (not recrystallized) were obtained from the acidified culture liquids of *P. viridicatum* strains no. 541 and no. 561 and from the mycelium extract of *P. palitans* strain no. 583 following preparative tlc in 2–3 solvent systems, which included the ether–methanol–formic acid–water (95:4:1:1) system for the ochratoxin A-producing fungi (no. 561 and 583); identification of the citrinin was by ultraviolet and mass spectral comparison with standard.

Penicillic acid and patulin were detected in the tlc system TEF–silica gel by spraying and heating with acidic anisaldehyde (Scott *et al.*, 1970a). They were confirmed and visually estimated (Fernbach flask cultures) after development on silica gel with ether, spraying with 4% phenylhydrazine hydrochloride solution, and heating at 120° for 3 min; penicillic acid formed a yellow fluorescent spot under longwave ultraviolet light and patulin formed a yellow spot under visible light. Crystalline penicillic acid samples, mp 85–86° and 83.5–85°, respectively, were obtained from *Penicillium cyclopium* strain no. 551 (CH_2Cl_2 extract of culture liquid) and strain no. 557 (mycelium extract) after preparative tlc (ether–silica gel), recrystallization from benzene–*n*-hexane, and (557 sample) sublimation under reduced pressure; the samples were identified by comparison with authentic penicillic acid (mixed melting point, and infrared, mass, and quantitative ultraviolet spectra). Crystalline patulin was isolated from *P. patulum* strain no. 562 (Scott *et al.*, 1972). Griseofulvin was detected by its blue fluorescence under ultraviolet light at the same R_f as standard in the tlc systems TEF–silica gel and ether–silica gel.

Sterigmatocystin and zearalenone were detected as fluorescent spots by comparison with standards in ether or TEF–silica gel and BMA–silica gel; in addition aluminum chloride (Stack and Rodricks, 1971) and acidic anisaldehyde (Scott *et al.*, 1970a) were used as respective spray reagents. The identity of zearalenone isolated by preparative tlc (TEF–silica gel) from the mycelium extract of *Fusarium equiseti* strain no. 577 was confirmed by gas chromatography (gc) of the

trimethylsilyl ether [6 ft × 1/8 in. stainless steel column packed with 3% SE-30 on Chromosorb W (HP) 80/100, column temperature 260°, injector temperature 300°, flame ionization detector temperature 300°, 35 ml/min He].

RESULTS AND DISCUSSION

Mycotoxins in Grains and Other Agricultural Products. Ochratoxin A was detected and confirmed in 18 samples of grain (out of 32 samples examined) and two mixed feeds (out of 13 samples examined), in addition to three samples of dried white beans and one sample of peanuts previously found positive by Health Protection Branch Regional Laboratories (Tables I and II). No ochratoxin B or ochratoxin esters were observed in any sample. Concentrations of ochratoxin A in the 18 grain specimens ranged from 0.03 to 27 ppm and in 13 of these grains citrinin was also detected (at levels of 0.07–80 ppm). Differences in ochratoxin A concentrations found on reanalysis of the same sample by Method 2A are probably either due to sampling error (most of the samples were <100 g) or reflect the time interval between analyses (up to 1 year), even though samples were usually stored in a freezer. All grain samples (excluding three not listed in Table I, which were from Ontario or Alberta) were collected from farms in various parts of Saskatchewan. The highest concentrations of ochratoxin A found by Nesheim (1971) in commercial barley and by Shotwell *et al.* (1971) in commercial corn were 0.038 and 0.166 ppm, respectively, with incidences of 14 and 1%, respectively.

In only one sample (49 FHR 181) known to be associated with deaths of farm animals was an appreciable concentration of ochratoxin A found. This sample was from a moldy mixed feed suspected of causing the deaths of a cow and several calves, in addition to decreased milk production in the herd. The significance of this association cannot be determined. The experimental effect of ochratoxin A on cattle has not been studied.

Three samples of dried white beans that contained ochratoxin A (Table II) were satisfactorily analyzed by Method 1. The beans were from elevators in Southwestern Ontario. The ochratoxin A was concentrated in the beans with a darker surface; sample 37790 U (0.5 ppm of ochratoxin A) was separated by hand into groups of discolored and clean beans, which respectively contained 4.8 ppm and no detectable ochratoxin A.

The sample of moldy, unshelled peanuts (107043 U), imported from Mexico, had been stored for 6 months prior to sampling, at which time the storage room was cool and quite humid. Whether ochratoxin A was produced on secondary infection or whether the toxin and associated *Penicillium viridicatum* originated in Mexico is not known.

Other known mycotoxins that were looked for (see Materials and Methods Section) were not found, apart from sterigmatocystin in one wheat sample (D-45) and an unconfirmed trace (<0.01 ppm) of aflatoxin B_1 in one mixed feed (not listed in Table II). The concentration of sterigmatocystin in sample D-45 was too low (approximately 0.3 ppm) to be detected by a general screening method (1A, 2). Isolation of *Aspergillus versicolor* (Vuill.) Tiraboschi that produced sterigmatocystin prompted us to reanalyze the wheat specimen for this mycotoxin by a more specific method employing column chromatography (Stack and Rodricks, 1971). Despite fluorescent materials that interfered with estimation, the sterigmatocystin was now readily detectable with the aluminum chloride spray. This is the first published report of sterigmatocystin occurring naturally in an agricultural product.

No penicillic acid was detected in any of the samples analyzed for this mycotoxin, even where penicillic acid producing fungi were isolated. This is perhaps not too surprising in view of the instability of penicillic acid in wheat flour (Scott and Somers, 1968) and the tendency for its concentration in grains incubated with *Penicillium* spp. at 20° to generally decrease with time (Ciegler and Kurtzman, 1970).

Analytical Methods. The method (2A) developed for the analysis of grains for citrinin was satisfactory at fairly high levels of citrinin (≥1 ppm; Table III). Separation of citrinin from ochratoxin A on silica tlc plates was markedly better in the ether–methanol–water–formic acid (95:4:1:1) solvent system (respective R_f values approximately 0–0.27 and 0.48) than in TEF or BMA. Similarly the TEF–acidic alumina system used for fungal extracts readily separated citrinin (approximate R_f 0–0.16) and ochratoxin A (approximate R_f 0.38). This latter system, although useful for confirmation purposes, was not suitable for estimation of ochratoxin A or citrinin owing to diffuseness and shape of spots. It was found necessary to carry out tlc of grain extracts the same day, as the amount of citrinin decreased on standing a few days in the presence of sample residue, particularly when present at low levels. Boron trifluoride–methanol is a useful spray reagent for confirmation of the identity of citrinin on the thin-layer plate; a change to a green fluorescence is observed under longwave ultraviolet light whether or not the original yellow fluorescence of the citrinin area has been destroyed by ammonia fumes. On analysis by Method 2A, mixed feed 49 FHR 181 yielded a yellow fluorescent substance at a similar R_f to citrinin in the TEF–silica system. However, the material did not give changes expected for citrinin with ammonia or BF_3–methanol.

Production of Mycotoxins by Fungal Isolates. The particulate nature or degenerate condition of most samples precluded surface sterilization. Much of the superficial contamination could be eliminated from the grains by washing them in sterile Tween 80 solution; however, in general, most samples supported luxuriant fungal growth in culture. The low temperature at which cultures were initially incubated permitted outgrowth of strains of *Penicillium* that failed to compete successfully at 25° with more rapidly growing fungi such as strains of *Aspergillus* (Scott *et al.*, 1970b). The use of a Blak-Ray ultraviolet transilluminator to detect certain toxigenic fungi that secrete fluorescent substances in culture on YES-impregnated filter paper is being investigated further in this laboratory.

The strains listed in Tables I and II were selected for identification because they were representative of the isolates obtained from the corresponding samples. Isolates that failed to produce known mycotoxins by our methods are not included in this report.

Toxigenic *Penicillium* species were isolated from all the samples examined (Tables I and II). The majority of the strains producing ochratoxin A and citrinin or either toxin in YES were identified as *P. viridicatum* Westling. A novel observation was the production of both these toxins or citrinin alone by strains of *P. palitans* Westling. Strains of these two fungi producing either ochratoxin A or citrinin or both toxins concomitantly were isolated from 22 grain samples, 16 of which contained ochratoxin A, from three mixed feeds, one of which contained ochratoxin A, and from the samples of white beans and peanuts. We failed to isolate fungi that produced ochratoxin A from only 6 out of the 24 samples in which ochratoxin A itself was detected and citrinin-producing fungi from only 2 out of the 13 samples found to contain citrinin. Out of the 34 isolates of *P. viridicatum* and *P. palitans* listed on

Tables I and II, the majority (25) produced ochratoxin A and citrinin in the same culture, while three produced ochratoxin A alone and five produced only citrinin. Strain no. 476 was the best producer of ochratoxin A (31 mg was estimated in the extract from one mycelial mat of a Fernbach flask culture); and strains no. 495, 541, and 561 yielded the most citrinin (about 20 mg per mycelium extract).

With the exception of isolates identified as *Aspergillus ostianus*, from horsefeed sample 49 FHR 67, and *Penicillium viridicatum* from mixed feed 49 FHR 49, all the strains producing penicillic acid were identified as *P. cyclopium*. It is of interest that penicillic acid producing fungi were isolated from six feed samples, in addition to one grain sample, associated with deaths of farm animals. Of all 16 *P. cyclopium* strains isolated, strain no. 548 was the best producer, yielding 60 mg in the mycelium extract from one Fernbach flask. Penicillic acid has previously been reported from *P. cyclopium* (Korzybski *et al.*, 1967) but not from *P. viridicatum* or *A. ostianus*.

The amounts of ochratoxin A, citrinin, and penicillic acid found in fungal extracts are not recorded in Tables I and II. Soaking the mycelial mats in chloroform does not give a quantitative recovery of these mycotoxins. For example, experiments with three penicillic acid producing *Penicillium cyclopium* isolates grown in Fernbach flasks showed that only 49–58% of the penicillic acid in the mycelium was extracted by the soaking procedure; the remainder was extracted by blending the mycelium three times with chloroform. In addition, the culture medium contained 1.3–3.0 times more penicillic acid than the total present in the mycelium (similar ratios were observed for ochratoxin A and citrinin in experiments with three isolates of *P. viridicatum*).

The frequency with which toxigenic strains of *Penicillium viridicatum* and *P. cyclopium* were isolated from the various commodities suggests that these are common storage fungi in wheat, rye, barley, white beans, peanuts, and animal feeds. The occurrence of ochratoxin A and citrinin in these products (Tables I and II) reflects inferior storage conditions and also possibly the lengthy Canadian winter, which could permit the associated toxigenic strains of *Penicillium* to gain ascendency over other less cold tolerant microflora. Mislivec and Tuite (1970) have reported the growth minima of *P. viridicatum* and *P. cyclopium* to be −2°.

Stolk (1972), who identified many of our fungi, commented on the typical variation in color of our strains of *Penicillium viridicatum* and *P. cyclopium*. Since the penicilli of these organisms are almost identical, identification was mainly based on color of the colonies. Strains no. 495 and no. 583 showed blue shades in young colonies on Czapek agar, but with age they developed the characteristic dark green color typical of *P. palitans*. Strain 325 was tentatively identified as *Aspergillus ostianus* Wehmer. Unfortunately, cultures of this organism failed to develop sclerotia, the color of which is one of the more important criteria used to distinguish this organism from *A. ochraceus* Wilhelm.

Strains of *Penicillium patulum* (isolates no. 562 and 201) that produced patulin and griseofulvin in culture were isolated from two mixed feeds, "Medicine Hat" (Table II) and a corn–soybean meal from Almonte, Ontario, both of which were suspected of killing farm animals. Analyses of the feed samples for patulin (Scott and Somers, 1968) were negative and no other mycotoxins were detected.

A *Fusarium* sp. (strain no. 577) that grew luxuriantly on YES and produced zearalenone within 5 days at room temperature was isolated from wheat sample D-66. The fungus was identified as *Fusarium equiseti* (Corda) Sacc. (Booth, 1972), which has not previously been reported as a producer of zearalenone. A more polar fluorescent compound, tentatively identified by mass spectrometry as a hydroxyzearalenone, was also isolated from the culture by preparative tlc; the gc retention time of its trimethylsilyl derivative was 6.7 min and that of zearalenone bis(trimethylsilyl) ether was 5.4 min.

CONCLUSION

We do not claim our findings constitute an unbiased survey for mycotoxins and mycotoxin-producing fungi in prairie grains collected at the farm level nor that we have accounted for deaths of animals that ate certain feed and grain samples. We do believe, however, that our discovery of the co-occurrence of ochratoxin A and citrinin in grains is a problem area requiring further attention. Ochratoxin A is fairly stable in cereal products (Trenk *et al.*, 1971) and care should be taken that contaminated grains do not enter commercial food channels. The possibility that ochratoxin A and citrinin could be carried through from feeds into animal and poultry products (*e.g.*, eggs) ought to be investigated.

Penicillium viridicatum Westling has been shown to be an important natural source of ochratoxin A and citrinin. This fungus and its associated mycotoxins appear to be responsible for kidney disease in Danish pigs (Krogh *et al.*, 1970). Their role in mycotoxicoses of farm animals in North America has yet to be determined.

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Determination of Phenolic Wood Smoke Components as Trimethylsilyl Ethers

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A rapid and simple method, developed for qualitative and quantitative analysis of phenol mixtures, was applied to investigation of wood smoke condensates and model food components exposed to smoke vapor. Phenolic fractions were isolated by conventional methods based on acidity, followed by formation of trimethylsilyl (TMS) ethers by treatment with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at room temperature. An internal standard, 3,5-dimethylphenol, was added to eliminate volumetric errors in gas chromatographic (gc) injection. Mass spectra of TMS derivatives provide as much structural information as spectra of free phenols and the derivatives are more readily separated by gc. Phenol, guaiacol, 4-methylguaiacol, and syringol were the major components found in the wood smoke vapor phase. Their concentrations were determined in smoke vapor and in oil and water models exposed to the vapor for 4 hr.

Several previous studies of smoke or smoked foods included estimates of total phenolic compounds (Bratzler *et al.*, 1969; Foster and Simpson, 1961; Husaini and Cooper, 1957; Porter *et al.*, 1965; Simon *et al.*, 1966). No information was available, however, on quantities of individual phenolic compounds present because of the lack of an appropriate analytical method. Individual phenolic components of wood smoke condensate (Fiddler *et al.*, 1966; Lustre and Issenberg, 1969) and smoked pork (Lustre and Issenberg, 1970) have been investigated but without quantitative methods.

Phenols were isolated from aqueous smoke condensate by Lustre and Issenberg (1969) with a series of extractions to separate components by acidity. Recoveries ranging between 80 and 100% were achieved with a similar system for preparation of a weakly acidic or "phenolic" fraction from water and triolein solutions (Issenberg *et al.*, 1971).

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Gas chromatographic (gc) separation of free phenols is complicated by their polar nature and low volatility. Chromatographic peaks tend to be asymmetric; long times and high temperatures are required to elute some of the higher boiling phenols. Despite these difficulties, gc was the most promising method for separation and quantitative analysis of individual phenols in complex mixture.

Peak symmetry in gc of phenolic compounds can be markedly improved by formation of derivatives of higher volatility and lower polarity. Silylating reagents react with hydroxyl, carboxylic acid, amine, and mercaptan groups by replacing the active hydrogens with trimethylsilyl (TMS) groups. Blocking the hydroxyl groups of phenolic compounds inhibits the formation of intermolecular hydrogen bonds, thus reducing interaction with the solid support and apparatus surfaces which improves peak symmetry. Decreased interaction with the stationary phase provides shorter elution times (Bhattacharyya *et al.*, 1968).

Early procedures for silylation of phenolic compounds utilized a mixture of hexamethyldisilazane (HMDS), catalyst, solvent, and a drying agent (Langer *et al.*, 1958). Silylation by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is faster and more complete than by HMDS or *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (Stalling *et al.*, 1968). BSTFA