Chemical, Physical, and Microscopical Studies of Scab-Infected Hard Red Winter Wheat

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Results from assays for the fungal component ergosterol and the mycotoxin 4-deoxynivalenol plus microscopy studies showed that the degree of fungal invasion (primarily *Fusarium graminearum*) varied greatly among kernels in samples of scabby wheat. As expected, kernels that appeared severely infected had the highest levels of ergosterol and DON. However, kernels that appeared lightly or moderately infected had ergesterol and DON contents that indicated significant fungal invasion. Microscopy showed that even lightly infected kernels had hyphae in the central endosperm and that hyphae were more prevalent in caryopsis coats than in central endosperm. From the results it appears improbable to avoid having DON in flour from scabby wheat. Ergosta-4,6,8(14),22-tetraen-3-one, zearalenone, nivalenol, and fusarenone-X were not found in severely infected wheat. Squalene content was higher in severely infected than in lightly infected kernels, but relative amounts of higher plant sterols were unaffected.

Wet weather during May and June of 1982 caused an unusually high occurrence of *Fusarium graminearum* Scwabe and its metabolite, 4-deoxynivalenol (DON, 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), in the wheat harvested in parts of Kansas, Nebraska, Missouri, and Iowa (Kansas Agricultural Experiment Station, 1983). *Fusarium graminearum*, a member of the *F. roseum* Link group, is usually the cause of scab in wheat grown in North America (Cook, 1981; Sutton, 1982).

Kernels with severe scab infection are easily identified because they are shriveled, soft, light in weight, and ivory or light grey with occasional pink or red patches. Other kernels with near normal size have various degrees of lighter-than-normal color suggesting various degrees of fungal infection. These variations, plus the extent of penetration of the fungus into the kernel, could significantly affect removal of DON during the cleaning and milling of scabby wheat.

We report results from ergosterol and DON assays, microscopy examinations, and other tests that provide significant information concerning fungal infection variations among kernels in samples of scabby wheat. Microscopy results also showed the extent to which the fungus penetrated into kernels, particularly those with moderate or severe infection. Unsaponifiable lipid extracts from lightly and severely infected wheat were extensively analyzed by high-pressure liquid chromatography (HPLC) to determine whether the fungus (1) produced ergosta-4,6,8(14),22-tetraen-3-one (ETO), a metabolite of many fungi (Turner and Aldridge, 1983), or (2) caused other significant compositional changes besides ergosterol. Severely infected wheat was also analyzed for other *Fusarium* metabolites, i.e., zearalenone, nivalenol, and fusarenone-X.

MATERIALS AND METHODS

Wheat Samples. All wheat samples analyzed were from the 1982 crop. Samples 1 and 2 were from north central Kansas and 3 was from south central Nebraska. Wheat samples 1–3 originally contained about 7 ppm DON. Sample 4, in which essentially all kernels appeared to be severely infected, was screenings from the cleaning of scabby wheat with an experimental cleaner at Iowa State University, Ames, IA.

Reference Standards. Ergosterol from Eastman Kodak Co. was recrystallized twice from absolute ethanol. Authentic ergosta-4,6,8(14),22-tetraen-3-one (ETO) was prepared from ergosterol by the method of Elks (1954). Practical grade squalene from J. T. Baker Chemical Co. was used without further purification. Samples of nivalenol, deoxynivalenol, and fusarenone-X were used as received from Wako Chemicals USA, Dallas, TX.

HPLC System. The chromatograph and the photodiode array detector used were obtained from the Hewlett-Packard Co. (Avondale, PA). The chromatograph was Model 1084B equipped with an autosampler, autoinjector, integrator, and column oven. The detector was Model 1040A consisting of a mainframe, master controller (HP85 computer), dual disk drive (HP82901M), and plotter (HP7074). An ultraviolet–visible (UV–vis) spectrum (190–600 nm) can be obtained at any time in the chromatogram without having to stop the flow of the mobile phase. Data for spectra and chromatograms were stored on flexible disks and then recalled later for plotting and comparisons. Chromatograms are formed by monitoring the difference between absorbances at sample and reference wavelengths. For all analyses the reference wavelength was centered at 550 nm with a 50-nm bandwidth. Sample wavelengths varied as indicated below, but all had a 4 nm bandwidth.

The analytical column (3.9 × 300 mm) contained C_{18} packing of 5-μm particle size (two 15-cm "Resolve" columns from Waters Associates, Milford, MA, placed in series). A guard column (C_{18}, 5 μm, 4.6 × 30 mm) from Brownlee Laboratories, Santa Clara, CA) was positioned between the analytical column and the injector. Column temperature was 50 °C. The mobile phase was methanol or methanol–water mixtures as indicated below. The flow rate was 1.2 mL/min.

Unsaponifiable Lipids. Unsaponifiable lipid extracts were prepared as described previously (Seitz et al., 1979) and then analyzed with HPLC by using 4% water in methanol as the mobile phase. Sample wavelengths were set at 200, 282, and 348 nm, which provided maximum sensitivity for detection of higher plant sterols, ergosterol, and ETO, respectively.

Sterols (4-demethyl) were isolated from the unsaponifiable lipids of lightly and severely infected kernels of sample 2 by using thin-layer chromatography (TLC). The extract was applied in a band to a glass-backed, precoated TLC plate (20 × 20 cm, coating 250 μm thick, Brinkmann G-25HR, Brinkmann Instruments, Waterburg, NY), and the plate developed in chloroform–acetone (95:5, v/v). To locate the sterol band a narrow section (about 3 cm) was removed (glass backing scored, then broken) from one edge of the plate, then sprayed with 50% sulfuric acid and heated. A rose-colored band at R_f 0.59 identified the

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sterols. The sterol band on the remaining section was scraped off, the silica gel extracted with methylene chloride–acetone (1:1, v/v), and the extract evaporated on a steam bath with a gentle stream of nitrogen directed into the vial. The residue was dissolved in methanol and analyzed by HPLC with methanol as the mobile phase. Sample wavelengths were 200 and 282 nm.

To identify squalene in unsaponifiable lipid extracts the silica gel TLC plate described above and the following solvents were used: chloroform; hexane–acetone (40:1, v/v); hexane–chloroform (32:1, v/v). Respective R_f values for squalene were 0.91, 0.86, and 0.44. Iodine vapor and 50% sulfuric acid were used as visualization agents.

DON, Nivalenol, and Fusarenone-X. DON in wheat was determined by the method of Scott et al. (1981) with minor modifications; also HPLC replaced gas chromatography for detecting DON in final extracts.

A 50-g sample taken from several hundred grams of wheat ground to about 20 mesh was extracted with methanol–water (1:1) in a blender for 5 min. The blended mixture was centrifuged at 3500 rpm for 5 min with a GSA rotor in a SS-3 Sorvall centrifuge. The centrifugate was treated with ammonium sulfate, filtered, and then extracted with four portions (one 100-mL and three 50-mL) of ethyl acetate. After drying over anhydrous sodium sulfate, the ethyl acetate extract was evaporated to dryness on a steam bath under N_2. The residue was dissolved in methylene chloride and transferred directly to a silica gel column. The column was washed with toluene–acetone (95:5) and the wash discarded; then DON was eluted with methylene chloride–methanol (95:5). The eluate was evaporated under N_2 until dry and then solubilized in methylene chloride. This solution was transferred to a vial, evaporated to dryness, and then redissolved with 1.5 mL of water–methanol (80:20). The final solution was forced by syringe through a 0.5-μm PTFE filter (Millipore Corp., Bedford, MA) directly into a 2-mL serum vial, which was capped with an aluminum seal having a Teflon-faced rubber septum, and placed in the autosampler of the HPLC instrument. The mobile phase was methanol–water (19:81, v/v) and the sample wavelength was set at 224 nm. DON was eluted in 10.4 min.

The modified method described above and the original, well-tested method of Scott et al. (1981) are essentially equivalent in extraction efficiency. Results from our method averaged only 5.4% lower than Scott's method when four samples of scabby wheat were analyzed. Results were respectively 0.67, 0.67; 6.68; 7.33; 6.81, 7.96; and 13.94, 13.70 μg/g.

Extracts of severely infected wheat for nivalenol and fusarenone-X analysis were prepared as described above for DON except that the silica gel column was omitted. These extracts were analyzed by HPLC by using the same conditions as for DON (standards of nivalenol and fusarenone-X were eluted at 6.5 and 16.7 min, respectively) and by TLC as follows. The extracts and a standard were spotted on two silica gel TLC plates (see unsaponifiable lipid analyses) and the plates developed in chloroform–methanol (7:1, v/v). One plate was sprayed with *p*-anisaldehyde reagent and the other with a saturated solution of aluminum chloride in ethanol (Takitani and Asabe, 1983). Both plates were heated to at least 120 °C before observing them under visible and ultraviolet lights.

Zearalenone. Severely infected wheats were extracted and analyzed by TLC as previously described (Seitz et al., 1975). Samples also were extracted by using the DON assay as described above, except for the following variations. Residues from evaporated ethyl acetate extracts and from evaporated toluene–acetone (95:5) wash from the silica gel column were dissolved in 0.5 mL of benzene–acetonitrile (95:5). Samples (25 μL) of each solution were analyzed by TLC (Seitz et al., 1975).

Table I. Ergosterol, DON, and Protein Contents and Weights of 1000 Kernels of Wheat Samples with Different Degrees of Visible Scab Infection

sample	degree of visible infection	ergosterol,[a] ppm	DON,[a] ppm	protein,[b] %	weight of 1000 kernels, g
1	light	2.8	0.4	13.1	29.9
1	moderate	29.0	22.7	11.4	25.6
1	severe	103.0	68.7	11.8	13.1
2	light	2.7	0.7	12.1	27.6
2	severe	86.8	68.5	10.8	15.8
3	light	2.6	0.8	13.7	34.6
3	severe	71.3	39.9	12.3	18.2
4	severe	118.0	71.9	12.7	15.2

[a] Each result is an average of duplicate HPLC analyses of a cleanedup extract from 50 g of ground, blended grain. [b] 14% moisture basis; averages of duplicates.

Protein. Protein was determined by Method 46-10 of the American Association of Cereal Chemists, St. Paul, MN.

Microscopical Methods. Micrographs of wheat kernel sections were obtained by using previously described methods (Bechtel et al., 1985).

RESULTS AND DISCUSSION

Apparent differences in degree of fungal invasion among kernels separated into lightly, moderately, and severely infected categories were confirmed by ergosterol and DON contents (Table I). As expected, kernels that appeared to be severely infected had the highest levels of ergosterol and DON. Moderately infected kernels (sample 1) appeared to have normal size and had only slightly lower kernel weights than normal, but ergosterol and DON contents indicated considerable fungal infection. Even kernels placed in the lightly infected category, which had normal kernel weights and nearly normal color, contained some DON.

Microscopy also clearly showed differences in the degree of fungal infection among the three categories into which wheat sample 1 was separated by visual inspection. Hyphae were sparse in lightly infected kernels but could be observed in both the outer kernel layers (Figure 1a) as well as in the central starchy endosperm (Figure 1b). Moderately inected kernels had a higher density of hyphae than the lightly infected ones, especially in the caryopsis coat layers (Figure 1c). The severely infected, shriveled kernels and the greatest amounts of hyphae. Little structure of the original wheat kernel was recognizable (Figure 1d). The fungus had removed most of the storage protein and cell walls, and starch granules were severely damaged (Figure 1e,f).

High-pressure liquid chromatograms obtained by monitoring light absorption at 200 and 282 nm clearly showed higher ergosterol levels in severely infected than in lightly infected wheat (Figure 2). An unexpected result was that the 348-nm chromatograms from extracts of all severely infected wheat samples indicated the absence of ETO. The small peak at the time expected for ETO in chromatogram C′ of Figure 2 did not have a UV spectrum like ETO. ETO is a metabolite of many fungi (Turner and Aldridge, 1983), including *Fusarium*, i.e., *F. moniliforme* (Price and Worth, 1974). ETO was also found in ergoty barley, rye, and other grasses (Seitz and Pomeranz, 1983).

Squalene content was notably higher in severely infected than in lightly infected wheat. This was evident from the

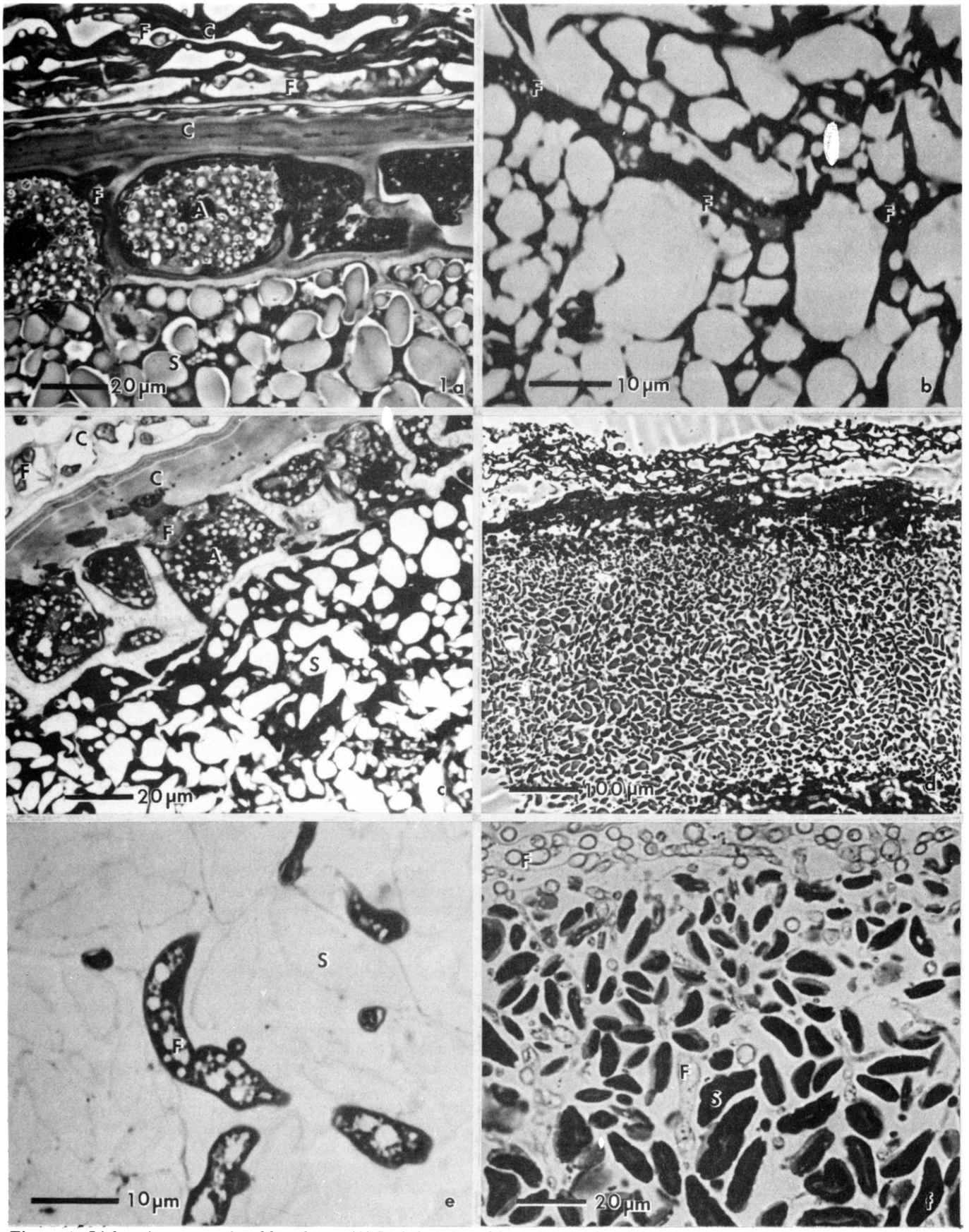

Figure 1. Light microscopy of scabby wheat. (A) Lightly infected kernel stained with basic fuchsin and toluidine blue O showing fungus (F) in caryopsis coats (C) and aleurone layer (A). S = starchy endosperm. ×650. (B) Central starchy endosperm of lightly infected wheat stained for protein with Coomassie Brilliant Blue showing fungus (F). ×1690. (C) Moderately infected wheat stained for protein showing numerous hyphae (F) in caryopsis coats (C) and aleurone layer (A). S = subaleurone starchy endosperm. ×650. (D) Shriveled kernel stained with basic fuchsin and toluidine blue O showing highly disrupted kernel. ×130. (E) Coomassie Brilliant Blue stained central starchy endosperm from severely infected kernel showing stained fungus (F), unstained starch (S), and lack of storage protein. ×1690. (F) Periodic acid Schiff's reaction for carbohydrates in severely infected kernel showing digested starch (S), lack of endosperm cell walls, and numerous hyphae (F). ×650.

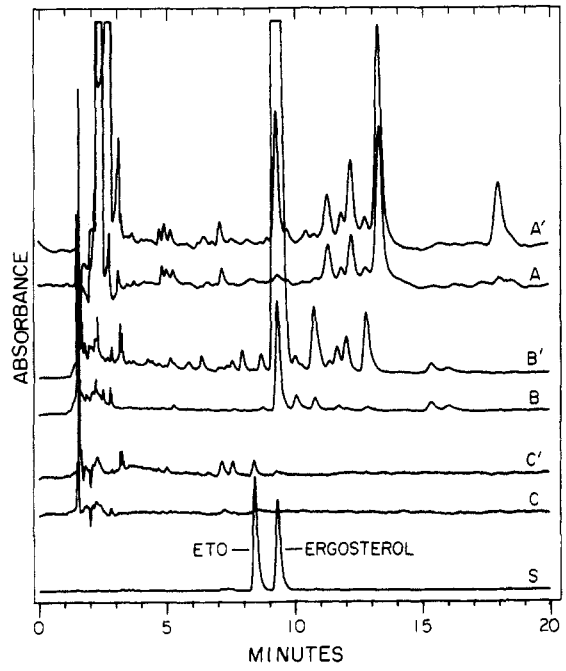

Figure 2. HPLC chromatograms of unsaponifiable lipid extracts of lightly (A, B, C) and severely (A′, B′, C′) infected kernels hand separated from sample 2 (Table I). Chromatograms A, B, and C were obtained by simultaneously monitoring 200-, 282-, and 348-nm wavelengths, respectively, after a single injection of sample. Chromatograms A′, B′ and C′ were similarly obtained. The chromatogram of standard (S) containing ETO and ergosterol was obtained by monitoring at 282 nm. Milliabsorbance units full scale are 400 for A and A′, 50 for B and B′, 25 for C and C′, and 1000 for S. The mobile phase was 4% water in methanol; other conditions are given in materials and methods.

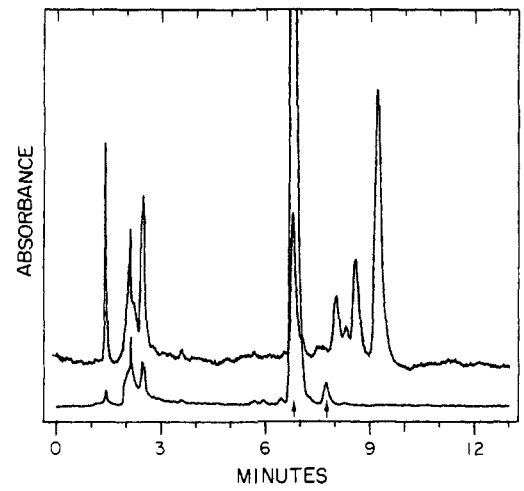

Figure 3. HPLC chromatograms of the band representing sterols after TLC fractionation of sample 2. The chromatograms were recorded simultaneously by monitoring 200- (upper) and 282-nm (lower) wavelengths. Milliabsorbance units full scale are 200 and 50 for the upper and lower chromatograms, respectively. Retention times at which spectra were recorded are indicated by arrows. The mobile phase was 100% methanol; other conditions are given in materials and methods.

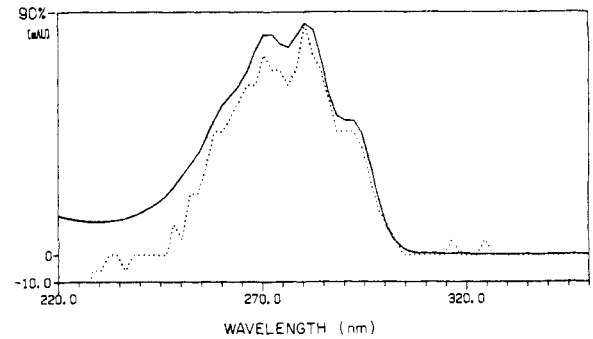

Figure 4. Ultraviolet spectra of ergosterol eluting at 6.8 min (–) and the other $\Delta^{5,7}$ sterol eluting at 7.7 min (··) in the 282-nm chromatogram shown in Figure 3. Milliabsorbance units full scale are 92.9 and 1.8 for ergosterol and the other sterol, respectively.

difference at 17.9 min in the 200-nm chromatograms (A and A′ in Figure 2). A squalene standard gave a peak at 17.9 min and had a UV spectrum (absorbance was maximum at 201 nm and declined sharply as wavelength increased to 230 nm) identical with the spectrum recorded at the apex of the 17.9-min peak in chromatogram A′ (Figure 2). TLC analyses of the extracts also indicated the presence of squalene. Squalene spots from sample and standard had identical R_f values in three solvent systems and identical colors after treatment with I_2 vapor and 50% sulfuric acid.

Squalene is a key intermediate in the formation of sterols in fungi and higher plants (Weete, 1980). The fact that ergosterol content increased significantly as the severity of fungal invasion increased suggests that squalene was a component of the fungal biomass. The possibility that squalene synthesis by the wheat was stimulated in response to the fungal attack cannot be ruled out, but it appears unlikely since relative amounts of the higher plant sterols were not significantly affected by the infection.

Most of the peaks between 9 and 14 min in the 200-nm chromatogram in Figure 2 were due to higher plant (Δ^5) sterols. Sterols isolated by TLC from nonsaponifiable lipids of severely infected wheat (sample 2) gave the same pattern of peaks when analyzed by HPLC (Figure 3, upper chromatogram). Peaks at 9.2, 8.6, 8.3, and 8.0 min in that chromatogram were found to be sitosterol, campesterol, stigmasterol, and avenasterols (Δ^5 and Δ^7, unresolved), respectively, by using chromatograms of standards and nonsaponifiable lipids of other grains for comparison. It is evident from chromatogram A and A′ in Figure 2 that *Fusarium* growth did not substantially affect the relative amounts of the higher plant sterols. Relative amounts of higher plant sterols indicated in our chromatograms are consistent with previously reported values (Weber, 1973). The minor component at 7.7 min in the 282-nm chromatogram (Figure 3, lower) had essentially the same UV spectrum as ergosterol (Figure 4). This fact and the elution position relative to ergosterol is consistent with the C_{24}-ethyl homologue of ergosterol. The minor component was apparently a fungal metabolite because it was not detected in similar extracts of lightly infected kernels (chromatogram not shown). Further identification of this component was not attempted.

Zearalenone, nivalenol, and fusarenone-X were not found in any of the wheat samples that were severely infected with *F. graminearum*. The detection limit for each compound was about 0.2 ppm.

Previous studies have shown that grain cleaners, such as those used in food processing plants and flour mills, will not completely remove DON from scabby wheat (Scott et al., 1983; Yamazaki et al., 1983; Young et al., 1984). We believe this is due to wide variation in degree of *Fusarium* infection among kernels, as indicated by results presented herein. Lightly or moderately infected kernels containing DON, but having size and weight similar to normal kernels, cannot be selectively removed by the cleaners. Cleaners would be most effective for wheat having most of the DON located in light-weight, severely infected kernels and least effective for wheat having most of the DON distributed among lightly or moderately infected kernels.

Our microscopy findings are consistent with reports indicating that all fractions from the milling of scabby wheat are contaminated with DON and that offals have higher DON contents than flours (Scott et al., 1983; Yamazaki et al., 1983; Young et al., 1984). The micrographs clearly showed that the fungus was distributed through the kernel with generally the highest concentration of hyphae in pericarp tissues. The presence of hyphae in endosperm tissues, especially in lightly and moderately infected kernels, would appear to make it improbable to avoid DON contamination in flours from scabby wheat.

Registry No. DON, 51481-10-8; ergosterol, 57-87-4.

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Characterization of Alkenylresorcinol in Mango (*Mangifera indica* L.) Latex

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The nonvolatile composition of mango latex was investigated. The major component (Ip) was isolated by thin-layer chromatography and characterized as 5-[2(*Z*)-heptadecenyl]resorcinol by a combination of infrared, ultraviolet, proton magnetic resonance, and gas–liquid chromatography–mass spectroscopy. It was identified for the first time in mango latex and considered as mango dermatitis allergen.

The latex of mango, a transparent fluid, that oozes out instantaneously from unripe (raw) fruit, as soon as the fruit is detached from the stalk, has long been known for its ability to produce allergic contact dermatitis among workers during harvesting of mature green (raw) fruit from the tree. Keil et al. (1946) demonstrated that the exudate of freshly picked unripe mango is resinous in nature and exhibits allergenic contact dermatitis similar to poison ivy. However, structural details of the compound in mango latex responsible for the allergic action on the skin is not known. Although the constituents of allergic noxious saps of various members of the *Anacardiaceae* family, such as poison ivy, cashew nut shell, oak, and other plant families have been classified as alkyl or alkenyl derivatives of catechol, resorcinol, and phenol (Keil et al., 1945; Symes and Dawson, 1953; Cirigottis et al., 1974; Billets et al., 1976; Reffstrup et al., 1982; Yamauchi et al., 1982). Besides the allergenic properties, these phenolics act as preservatives in necrotic organs of plants against microbial infection and undergo rapid resinification (Haslam, 1979).

Polyphenolic components such as mangiferin in mango stem bark and leaves (Bhatia et al., 1967) and β-glucogallin as well as gallotannin in pennicles and in mature green fruit of mango (El Ansari et al., 1967, 1971) were reported.

Mango latex is believed to contain tannins, enzymes, resins, and terpenes (Pantastico, 1975). The odorous principles of raw mango latex were characterized as *cis*-ocimene and β-myrcene (Gholap and Bandyopadhyay, 1977). The present paper relates to the isolation and identification of mango dermatitis allergen, a nonvolatile constituent of mango latex.

EXPERIMENTAL SECTION

Isolation of Allergen. Freshly picked, mature unripe Alphonso mangoes, a premier variety, with stalk (10–12-cm length) attached, were procured from a local market. Droplets of latex collected during destalking were extracted repeatedly with peroxide-free diethyl ether. The ether extract was washed with distilled water, dried over anhydrous sodium sulfate, and filtered. The bulk of the ether was removed in a flash evaporator at ambient temperature, and the extract was subjected to high-vacuum distillation to remove the essential oil components of the latex as described elsewhere (Gholap and Bandyopadhyay, 1977). The viscous residue left after high-vacuum distillation was light brown in color. The residue was dissolved in chloroform (10% solution), and suitable aliquots were subjected to preparative thin-layer chromatography (TLC) on silica gel G (E. Merck) plates (0.4-mm layer thickness, 20

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