Factors in Oats That Could Be Mistaken for Aflatoxin[1]

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ABSTRACT

In an examination of oat samples, initial chemical tests indicated that many of those tested contained fluorescing substances which behaved like aflatoxins B_1 and G_1 on thin-layer chromatographic plates. When these same samples were assayed in ducklings, symptoms typical of those caused by aflatoxin failed to develop. Several methods of purification, including solvent distribution, chromatography on silica gel, and lead acetate precipitation, failed to remove interfering substances. Analyses of groats and hulls of oats separately revealed that the fluorescing substances occur in the hulls. Aflatoxins B_1 and G_1 can be most conveniently differentiated from oat factors on thin-layer plates coated with Silica Gel G-HR and developed with 5 or 7% methanol in chloroform. Extracts of oats were purified on thin-layer plates and then treated with trifluoroacetic acid and with formic and acetic acids in the presence of thionyl chloride in an attempt to prepare derivatives. Thin-layer chromatograms of treated extracts showed that products formed were not similar to those obtained when aflatoxins were treated with the same reagents.

Since the discovery that the hepatotoxic metabolites, aflatoxins B_1, B_2, G_1, and G_2, were responsible for the deaths of 100 000 turkey poults in 1960 (1), much effort has been expended in determining the extent to which such toxins occur in agricultural commodities. In a survey of oat samples for the presence of aflatoxin, initial chemical assays indicated that many contained fluorescing substances that behaved like aflatoxins on thin-layer plates, but did not have the biological activity of aflatoxins. Japanese workers have observed that a number of molds used in food fermentations produce nontoxic compounds which resemble aflatoxins B_1 and G_1 in their chromatographic and fluorescent properties (2). Of the eight compounds they isolated, flavacol, deoxyhydroxyaspergillic acid, and deoxyhydroxymuta-aspergillic acid were identified. Other fluorescent substances difficult to differentiate from aflatoxins have been observed both in moldy peanut products (3) and in raw peanuts (4). This paper describes methods of differentiating aflatoxins B_1 and G_1 from interfering substances in oats.

MATERIALS AND METHODS

Collection and Preparation of Samples

Oat samples (1 kg.) from various grade levels were collected by the Grain Division of Consumer and Marketing Service, USDA. Samples were ground in a Raymond 6-in. stainless-steel laboratory mill equipped with a screen having ⅛-in. round-hole perforations, a small portion of unground oats being retained for further study. A number of the portions of unground oats from samples containing the interfering substances were combined and separated into hulls (366 g.), groats (525 g.) and miscellaneous particles (straw, dust, etc.) (57 g.).

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Extractions

Oat samples (50 g.) were extracted with methanol:water (55:45 v./v.) (250 ml.) by stirring in an explosion-proof Waring Blendor by the procedure recommended for determining aflatoxin in peanuts and peanut products (5). Aliquots (50 ml.) of the extract were mixed with acid-washed Celite 545 (55 g.) and water (5 ml.). The mixture was packed into a column and washed with hexane and eluted with hexane:chloroform (1:1 v./v.). Residues from eluates were dissolved in chloroform and subjected to thin-layer chromatography (TLC). This method was the one used in the over-all survey.

Several other methods developed for aflatoxin extraction were investigated in an effort to find a procedure that would not extract the interfering substances in oats simultaneously. Samples were extracted with chloroform and water by the method developed by Lee (6) for defatted peanuts. Another procedure tried involved extraction with acetone:water (70:30 v./v.) followed by precipitation of impurities with lead acetate and extraction of the resulting aqueous acetone solutions with chloroform (7). Still another method used for purifying extracts after treatment of oat samples with acetone:water (70:30 v./v.) was chromatography on cellulose columns washed with hexane and eluted with chloroform:hexane (1:1 v./v.) (8). Extraction of oat samples with acetone:hexane:water (5:48.5:1.5 v./v.) was also tried (9).

Partial Purification of Extracts

Extracts of oats were placed on silica gel (0.05–0.20 mm.) columns (35 mm. $\times$ 60 mm.) to remove impurities (7). Absolute diethyl ether (100 ml.) was used to wash impurities from the column, after which fluorescing substances from oats and aflatoxins were eluted with methanol:chloroform (3:97 v./v.) (150 ml.).

Thin-Layer Chromatography

Chromatoplates (20 $\times$ 20 cm.) were prepared for TLC by mixing 30 g. of Silica Gel G-HR with 64 ml. of distilled water in a Waring Blendor for 30 sec. and spreading a layer 0.250 mm. thick on the plates. Developing solvents were 3, 5, and 7% methanol in chloroform (7,3,5); the upper layer of benzene:95% ethanol:water (46:35:19 v./v.) (8); water-saturated methyl isobutyl ketone containing 2% *p*-toluenesulfonic acid; the upper layer of 1-butanol:acetic acid:water (20:1:19 v./v.) (10); acetone:chloroform (1:9 v./v., not equilibrated) (11); and 5% methanol in chloroform containing 0.5% acetic acid (12).

Plates were inspected with a high-intensity, long-wave ultraviolet Blak-Ray lamp or Chromato-Viewer to locate zones of fluorescence caused by the four aflatoxins and the oat factors.

Preparative Thin-Layer Chromatography

Extracts from oats (260 g.) were partially purified on silica gel (0.05–0.20 mm.) columns as described. The residue from the column eluate was dissolved in chloroform (0.250 ml.) in a vial. The solution was applied 10 μl. per spot at 5-mm. intervals along an origin line extending 2 cm. from one edge of the thin-layer plates to 2 cm. from the other edge. The plate had been coated with a 0.250-mm. layer of Silica Gel G-HR. When the entire

solution was applied, chloroform (0.250 ml.) was added to the vial to wash it out, and the wash solution was spotted on the origin line as before. Two washes of chloroform were used. A standard chloroform solution (5 μl.) containing 0.00244 γ/μl. aflatoxin B_1, 0.00061 γ/μl. B_2, 0.00218 γ/μl. G_1, and 0.00042 γ/μl. G_2 was spotted on the origin line, 1 cm. from each edge of the plate.

The plate was developed with methanol:chloroform (5:95 v./v.) for 40 min., and fluorescent areas caused by the two interfering substances in oats were marked with a needle. Silica gel in these areas was swept from the dried plate by suction. Fluorescent material was eluted from silica gel with six 5-ml. portions of methanol:chloroform (1:1 v./v.). Solutions were scanned with a spectrophotometer to give ultraviolet absorption spectra. Residues from the solutions were dissolved in 1 ml. chloroform and subjected to TLC separately and in admixture with aflatoxin standards on Silica Gel G-HR plates developed with methanol: chloroform (5:95 v./v.).

Attempts to Prepare Derivatives

The residue from eluates of Silica Gel G-HR from thin-layer plates containing interfering substances from oats was transferred to three vials, so that each would contain fluorescent material equivalent to 0.25 γ of aflatoxin B_1. Procedures used in the attempted preparation of derivatives were those developed by Andrellos and Reid (3).

Vial 1. After 3 drops of trifluoroacetic acid was added, the mixture was shaken and kept 15 min. before being evaporated to dryness on a steam bath under nitrogen.

Vial 2. Glacial acetic acid (0.2 ml.) was added with shaking followed by 1 drop of thionyl chloride. After being shaken, the mixture was held 5 min. and then evaporated to dryness.

Vial 3. The residue was treated as in the second vial, except that formic acid was substituted for acetic.

Treated extracts were compared by TLC with the three derivatives of aflatoxin B_1 prepared by the same procedures. Plates were coated with Silica Gel G-HR and developed with acetone:water (70:30 v./v.).

RESULTS AND DISCUSSION

When oat samples were analyzed by the procedures used in the survey of corn, sorghum, soybean, and wheat samples for aflatoxin, about one-third of the samples contained fluorescing substances, hereafter designated as compounds I and II, that behaved physically like aflatoxins B_1 and G_1, respectively. Results of the initial TLC are shown in Table I.

The extraction procedure was with methanol-water, followed by purification on acid-washed Celite 545 columns. Thin-layer plates for chromatography were developed with methanol:chloroform (3:97 v./v.). When the oat samples that appeared to contain aflatoxins B_1 and G_1 were tested biologically in ducklings by the Wisconsin Alumni Research Foundation, they did not exhibit aflatoxinlike activity. Aflatoxin cannot be identified by TLC alone; confirmatory tests are absolutely essential.

TABLE I
SUBSTANCES IN OATS INTERFERING WITH AFLATOXIN ASSAY
(Compounds I and II)

GRADE LEVEL[a]	NUMBER OF SAMPLES ASSAYED	SAMPLES CONTAINING COMPOUNDS I AND II	
			%
1	36	13	36
2	75	35	47
3	29	13	45
4	100	33	33
SG	77	16	21
Total	317	110	

[a]These are the grades of oats collected in commercial markets as determined by the Grain Division of Consumer and Marketing Service. Grades 3, 4, and SG (sample grade) represent oats of the poorest quality.

Chemical analysis of the hulls, groats, and miscelleanous particles from oat samples indicated that compounds I and II occurred in the hulls. Fermentation studies indicated that oat hulls are a poor substrate for aflatoxin production compared to groats (13). If, however, a large number of whole oat samples were to be assayed for the presence of aflatoxin, a chemical method had to be found that would differentiate compounds I and II from aflatoxins.

Although partial purification of oat extracts on silica gel (0:05–0.20 mm.) columns does not remove compounds I and II, treated extracts have fewer impurities that cause streaking on thin-layer plates. Separations of substances that move close together on thin-layer plates are easier after column chromatography.

Compounds I and II can be differentiated most efficiently from aflatoxins B_1, B_2, G_1, and G_2 on thin-layer plates coated with Silica Gel G-HR if they are developed with chloroform containing 5 or 7% methanol. If separations are not complete, plates can be dried after the first development and developed a second time in the same solvent system. In fact, very crude extracts can be chromatographed successfully with two developments. Separations are shown in Fig. 1. Compound I moves on thin-layer plates between aflatoxins B and G, and compound II moves slightly behind aflatoxin G. Although the following solvent systems separate the four aflatoxins on thin-layer plates, they did not differentiate between compounds I and II and the aflatoxins: upper layer of benzene:95% ethanol:water (46:35:19 v./v.) and acetone: chloroform (1:9 v./v.). Other solvent systems proved unsatisfactory: water-saturated methyl isobutyl ketone containing 2% *p*-toluenesulfonic acid; the upper layer of 1-butanol:acetic acid:water (20:1:19 v./v.); and 5% methanol in chloroform containing 0.5% acetic acid.

Compounds I and II are extracted as efficiently from oats as aflatoxins are from other products by all the procedures investigated. Because of the similarities in the solubilities of compounds I and II and the aflatoxins, it would be difficult to find a solvent system for the initial extraction of oats that would not remove these compounds if present.

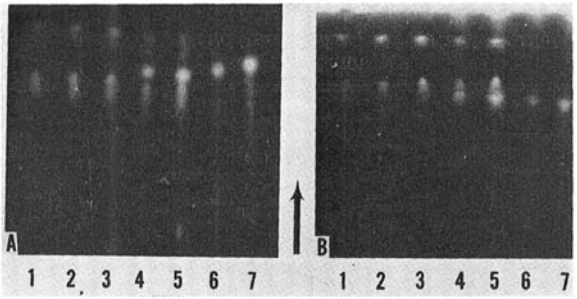

Fig. 1. Thin-layer chromatography of aflatoxins and compounds I (A) and II (B). The thin-layer plates were coated with Silica Gel G-HR and developed twice with 5% methanol in chloroform in the direction of the arrow. Standard aflatoxins (B_1, B_2, G_1, and G_2) were applied at spots 1, 2, and 3. Either compound I or II was applied at spots 6 and 7. Admixtures of compound I or II and aflatoxins were applied at spots 4 and 5.

The ultraviolet absorption spectra of eluates from preparative thin-layer plates containing compound I did not exhibit maxima typical of aflatoxin B_1 λ_{max} 224, 265, and 363 mμ (ε21,200, 12,100, and 22,700, respectively) (14). The eluate containing compound I has a spectrum with shoulders at 256 and 297 mμ. The spectrum of aflatoxin G_1 has maxima at 217, 245, 265, and 363 mμ (ε27,200, 11,000, 10,000, and 18,700, respectively) (14). The only peaks in the ultraviolet spectrum of eluates containing compound II are at about 238 and 270 mμ. Differences in the absorptivities of the two peaks were great. Both eluates probably contain compounds other than I and II.

Compounds I and II from eluates from preparative thin-layer plates treated with trifluoroacetic acid did not form products similar to those formed by aflatoxins B_1 and G_1 under identical conditions as determined by TLC, nor did eluates treated in the presence of thionyl chloride with formic or acetic acids. Andrellos and Reid (3) used the formation of addition compounds with trifluoroacetic, formic, and acetic acids as confirmatory tests for aflatoxins B_1 and G_1.

The most convenient procedure by which to differentiate compounds I and II from aflatoxins is by TLC with the solvent system of methanol:chloroform (5:95 v./v.) developed twice. If oat extracts contain too many impurities, they can be treated on silica gel (0.05–0.20 mm.) columns first. A survey of a large number of oat samples for the presence of aflatoxin is now being completed.

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