Seed Coat and Fiber Constituents of Cotton Seeds that May Influence Aflatoxin Analysis

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Abstract

Normal constituents of seed coats and fibers of fuzzy cotton seeds were found which can interfere with aflatoxin analyses of embryos (meats). Aflatoxins were partially or totally obscured in chromatograms prepared with extracts containing these constituents. A blue fluorescing substance with an R_F approximating that of aflatoxin B was found to be a normal constituent of seed coats and fibers. This substance differs from aflatoxins in several ways: it fades quickly from thin-layer chromatograms exposed to ultraviolet light, disappearing within an hour; it does not form complexes with acetic acid- and formic acid-thionyl chloride solutions or with trifluoroacetic acid; it does not have the characteristic ultraviolet absorption bands of aflatoxins.

Introduction

THE COMBINED SEED COAT and fibers portion of fuzzy cotton seeds represents about 40% of the weight of seeds and is a potential source of materials that may interfere with chemical analyses of embryos (meats). Perhaps for this reason, the authors of the commonly used procedure for analysis of aflatoxins in cotton seeds suggest removal of seed coats before seeds are extracted (8). It would be advantageous if whole seeds could be analyzed without removal of seed coats, mainly because the de-hulling step could be eliminated but also because seed coats can contain aflatoxins (6). This paper reports results of experiments to determine whether acetone/water-soluble substances, which may interfere with aflatoxin analyses, occur in fibers and seed coats of cotton, also whether aflatoxin-like artifacts occur in these tissues.

Materials and Methods

The aflatoxin analysis procedure described by Pons and Goldblatt (8) was used in all cases. Dried extracts were diluted with chloroform for spotting on 0.4-mm silica gel (Brinkman G-HR) plates. These chromatograms were developed with 3% methanol in chloroform (v/v basis). Aflatoxin standards included one prepared by H. W. Schroeder (Agricultural Research Service, USDA, Texas A&M University, College Station), which contained aflatoxins B_1 (about 0.0033 μg/μl), B_2 (about 0.001 μg/μl), G_1 (about 0.01 μg/μl), and G_2 (about 0.0008 μg/μl). It was used in making quantitative estimates of toxin concentrations. Two qualitative standards were used: a B_1 standard from Arthur D. Little Laboratories Inc. and a B_1-B_2 standard prepared in the authors' laboratory.

Tests of seed coat tissue for materials that interfere with aflatoxin analysis were made by spiking samples with aflatoxins prior to extraction. Seed coat tissue was first ground to pass a 20-mesh screen, and visible fiber fragments were removed by additional screening. In one experiment, duplicate 30-g samples of seed coats were spiked with aflatoxins so that they contained about 70 ppb total toxins (Schroeder standard). Qualitative observations initially were made to determine whether toxins were visible in 5-μl portions of extracts which had been concentrated to 200 μl. Then the remainder of the concentrates were subjected to a silica gel-sodium sulfate column clean-up (7), and the amount of toxins in the extracts were quantitatively estimated. A similar experiment was made, by using 5-, 10-, 15-, and 20-g samples of seed coats, to determine how much seed coat material could be present in an extract without obscuring aflatoxins in chromatograms. Triplicate samples of seed coat tissue from two sources were compared in this test.

Other tests were made in connection with an aflatoxin-like artifact which was observed in extracts of cotton fibers and seed coats. Seed coats, fibers, and meats were compared for occurrence of the artifact. Twenty-gram samples of seed coat tissue from each of 10 sources were used in the comparison. Twenty 15-g samples of fiber were analyzed: 10 from healthy unopened bolls and 10 from damage-free, newly opened bolls. Ten 30-g samples of meats, from the mature bolls described above, were analyzed. These tissue samples were believed to be free from aflatoxins because they came from bolls free from visible damage and because fibers and ginned seeds were free from the greenish-yellow fluorescence associated with infection by *Aspergillus flavus* Link (5) and with aflatoxins (2).

Extracts were prepared as described above. The artifact was chromatographically purified by streaking extracts containing it on silica gel plates. Following development, the blue fluorescing band was scraped from the plate, the material was eluted from the silica gel with chloroform, and the suspension was filtered. The filtrate was dried, then the residue was diluted to 100 or 200 μl with chloroform for qualitative chromatographic tests. The dilution of the extract varied when quantitative estimates of concentrations were made of the blue fluorescing material. Estimates of concentration of the blue material were made by comparing intensities of spots having known amounts of aflatoxin B_1 with spots having different volumes of solution containing the blue substance.

The chromatographically purified residues of 60 samples of 15 g of seed coats were bulked and diluted to 2.0 ml with chloroform for a spectrophotometric test. This solution had an amount of the blue fluorescent substance equivalent in fluorescent intensity to a solution containing approximately 20 ppm of aflatoxin B_1. The ultraviolet spectrum of the artifact was compared with the spectrum of the Schroeder standard. (The test was made with a Perkin-Elmer Model 202 spectrophotometer, using matched fused silica cells with a path length of 1 cm.)

Results and Discussion

Masking of Aflatoxins in Extracts Containing Soluble Components of Seed Coat Tissue. Tests of seed coat tissue for materials that interfere with aflatoxin analysis were made by spiking 30-g samples with aflatoxins prior to analysis. In two experiments,

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aflatoxins were not observed in seed coat samples spiked with approximately 70 ppb total aflatoxins. Instead, a dense bluish-grey material extended through R_F areas where aflatoxins were expected to occur. This material was removed from extracts by subjecting them to a clean-up process developed by Pons et al. (7), a method developed to remove a component of certain extracts of cottonseed products that caused streaking in chromatograms. Although the bluish-grey interfering component was eliminated when extracts were passed through the clean-up column, some toxins were lost in the process because only about one-third of the amount of toxins added to the samples, when extraction was begun, were found in the final extracts. In another experiment, the bluish-grey material of seed coat tissue was found to affect differentiation of aflatoxins on chromatograms, depending upon the amount of the interfering component in extracts. This was determined by extracting different amounts of seed coat tissue. The four aflatoxins were well differentiated in extracts of 5-g and 10-g samples of seed coats and in the control series in which the toxins were added to the solvent containing no seed coat tissue. In extracts of 15 g of seed coat tissue, aflatoxins B_1 and B_2 did not separate from each other, but the G toxins were well differentiated. All four toxins were obscured by the bluish-grey component of seed coats where 20 g of tissue were extracted, as was the case where 30 g of tissue were extracted in an earlier experiment.

Occurrence of an Aflatoxin-like Artifact in Fibers and Seed Coats of Cotton. A component of seed coats and fibers, which has chromatographic similarities with aflatoxins B_1 and B_2, was observed. It was first noted when 5-μl portions of extracts of seed coat tissue (the residue of 15 g of tissue diluted to 200 μl with chloroform) were chromatographed in pairs, one with and one without an internal aflatoxin standard (2 μl of Schroeder's standard per spot). The color of this material appears to be identical with the color of aflatoxins B, and initial observations indicated that it and the aflatoxins have similar R_F values. The R_F of the material was slightly greater than the R_F of aflatoxin B_2 when 1- to 10-μl portions of a solution, with the equivalent of about 50 ppb aflatoxin B_1, were spotted beside a standard solution containing all four toxins. But the R_F of the material was slightly lower than that of aflatoxin B_2 when 20- to 30-μl portions were spotted beside the standard solution.

Spots of the blue fluorescing substance and aflatoxin B_2 merged when 1- to 10-μl portions of the preparation were spotted with an internal standard of B_1 and B_2; and the B_1 and B_2 spots and the spot containing the blue fluorescing substance merged when 20- to 30-μl portions of the preparation were spotted in the same way. The R_F of the blue fluorescing substance, relative to those of aflatoxins B_1 and B_2, is shown in Figure 1.

The blue fluorescing substance was found to be quantitatively recoverable. Five-, 10-, and 15-g samples of two lots of seed coat tissue were carried through the procedure for analysis of aflatoxins, and the amount of was estimated, by using the aflatoxin B_1 standard. For one lot of seed coat tissue 5-, 10-, and 15-g samples had respectively a trace, trace plus, and 5 ppb of the substance, and another lot had respectively 6, 12, and 19 ppb of the substance if it is assumed that there is the same fluorescence intensity as for aflatoxin B_1.

An experiment was made to determine whether the blue fluorescing substance was a component only of seed coats. Extracts of seed coat tissue, meats of mature seeds, immature fibers, and mature fibers were compared. Results of this experiment indicate that the blue fluorescing substance is present in seed coats and fibers, but it is either absent from or present only in trace amounts in meats (Table I). The authors concluded that the blue fluorescing substance is a normal constituent of seed coats and fibers and is not a mold metabolite because, as discussed earlier, they are reasonably sure that the samples were free from *A. flavus*, or other mold, damage.

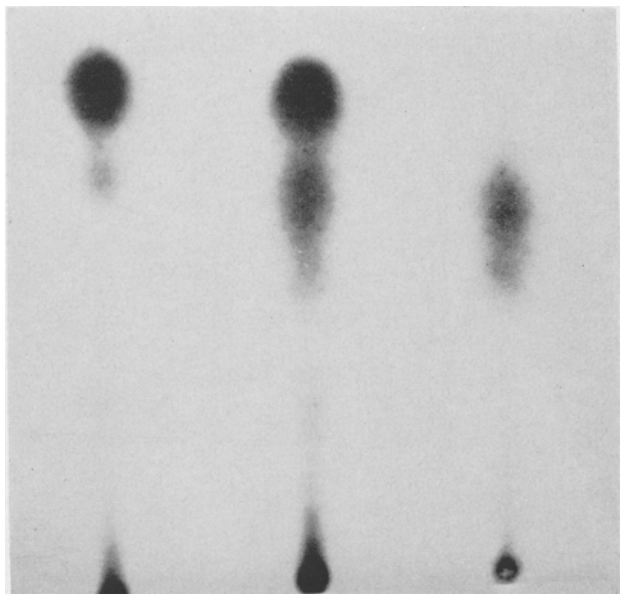

FIG. 1. Thin-layer chromatogram illustrating distribution of aflatoxins B_1 and B_2 and a blue fluorescing seed coat and fiber constituent of cotton with an R_F similar to aflatoxin B: left, aflatoxin B_1 (upper spot) and aflatoxin B_2 (lower spot); center, a mixture of B_1, B_2, and the blue fluorescing substance; and right, blue fluorescing substance.

Other comparative tests support this conclusion. The blue fluorescence of this material, at dilutions normally used to make estimates of concentration of aflatoxins in solution, fades within 15 to 60 min whereas similar spots containing aflatoxins remain visible for four or more hours in ultraviolet light. Dried samples however were stored in the laboratory for a month or more without apparent loss of the fluorescence. Also, unlike aflatoxins, this substance does not form complexes with acetic acid- and formic acid-thionyl chloride reagents, or with trifluoroacetic acid, as described by Andrellos and Reid (1). In two experiments a solution with an amount of the blue substance equivalent to about 2 ppm aflatoxin B_1, reacted negatively, and three sources of aflatoxins, with concentrations ranging from 0.5 to 1 ppm, reacted positively with these reagents. Lastly, the blue fluorescing substance did not have absorption bands like aflatoxins in the region of 265 and 362 mμ.

Results of these experiments indicate that seed coat tissue is the source for two or more materials that can affect aflatoxin analyses. One component appears to be similar to the streak-causing component of cotton seeds discussed by Pons et al. (7). The blue fluorescing component of seed coats and fibers appears to be chromatographically identical with the substance isolated from cottonseed meal by Chen and Friedman (3). The material occurs in sufficient quantities significantly to affect analyses for aflatoxins if seed coats are not removed. For example, if 50-g samples of whole seeds were extracted, 20-g of seed coat and

TABLE I

Distribution and Relative Amounts of an Aflatoxin-like Artifact in Certain Parts of the Cotton Fruit

Sample	Embryos (meats)	Seed coats	Fibers	
			Immature bolls	Mature bolls
	ppb[a]	ppb	ppb	ppb
1	ND[b]	40	64	110
2	ND	28	46	11
3	trace	38	9	11
4	ND	35	18	11
5	ND	28	18	22
6	ND	8	28	2
7	ND	14	4	28
8	ND	8	4	3
9	ND	14	28	5
10	ND	34	trace	trace

[a] Amounts estimated by using aflatoxin B_1 as the standard for color, size, and intensity of spots on chromatograms.
[b] Not detected.

fiber material would be present and could, alone, give an apparent aflatoxin B content of up to about 100 ppb (Table I). The blue fluorescing material also may account for the greater amount of aflatoxin isolated from whole seeds versus de-hulled seeds, as reported by Pons and Goldblatt (8). This view is supported by results (6) which indicate that seed coat material is a substrate for elaboration of aflatoxins by *A. flavus*.

Several avenues appear to be open for reducing or eliminating the influence of normal seed coat and fiber constituents on aflatoxin analyses of cotton seeds. The influence of aflatoxin-obscuring materials found in seed coats can be circumvented by removing seed coats prior to analysis or by adding a further clean-up step (7) to the analysis. Similarly the influence of the blue fluorescing component of seed coats and fibers on assessment of the B toxins can be eliminated by removing seed coats or by altering the chromatogram development procedures (3).

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