Non-Interference of Seed Coat Constituents of Cotton Seeds Using the Pons and Goldblatt Aflatoxin Procedure[1]

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Abstract

The normal constituents of seed coats and fibers of fuzzy cotton seeds were not found to interfere with aflatoxin analyses of embryos (meats). Several technique modifications of the Pons and Goldblatt (6) procedure for aflatoxin analyses in cottonseed are presented which aid not only in making better defined spots but also improve TLC resolution. Having assayed over 2,000 samples, we have found that the so-called blue spot of Ashworth et al. (2) was not found to either partially or totally obscure aflatoxin B_1 on the plates, as claimed by these workers. An improved method of aflatoxin estimation on TLC plates is presented.

Introduction

It was recently reported by Ashworth et al. (2) that "normal constituents of seed coats and fibers of fuzzy cotton seeds were found which can interfere with aflatoxin analyses of embryos (meats)." They further stated that "aflatoxins were partially or totally obscured in chromatograms prepared with extracts containing these constituents." Lest the conclusion of these authors give the possible erroneous impression that normal aflatoxin-positive samples determined on cottonseed could be in doubt, we wish to report that of at least 2,000 samples recently analyzed, we have not encountered any interference from this "blue spot." Since the photograph of the developed TLC plate of these workers indicated that their procedure was producing poor definition (and therefore resolution) we report modifications of the very excellent Pons and Goldblatt method (6) which may improve resulting interpretations. Furthermore, we obtained from the same source a sample of cottonseed hulls and fibers which Ashworth and coworkers used, and analyzed it according to our modifications of the Pons and Goldblatt procedure. We found no interference nor obscuring of aflatoxin B_1 by this "blue spot."

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Methods

Samples of whole cottonseeds were pre-ground in a Labconco Mill, followed by grinding in a Hobart grinder (Model 4812) using a 3/8 in. grid plate. Samples of hulls and fiber were sufficiently well-ground upon receipt and were further pulverized in a Waring Blendor prior to aqueous acetone extraction.

All extracts requiring solvent evaporation were evaporated on an angled flash evaporator instead of a steam bath because of faster evaporation and lower loss of aflatoxin due to heat.

Sample extracts were evaporated from vials using a stream of nitrogen delivered through disposable Pasteur pipettes, not reused, attached to a manifold by rubber tubing.

The method of sample application to the TLC plates was modified to give a more definite spot than obtainable with the Hamilton microliter syringes used in the Pons and Goldblatt procedure. Rather

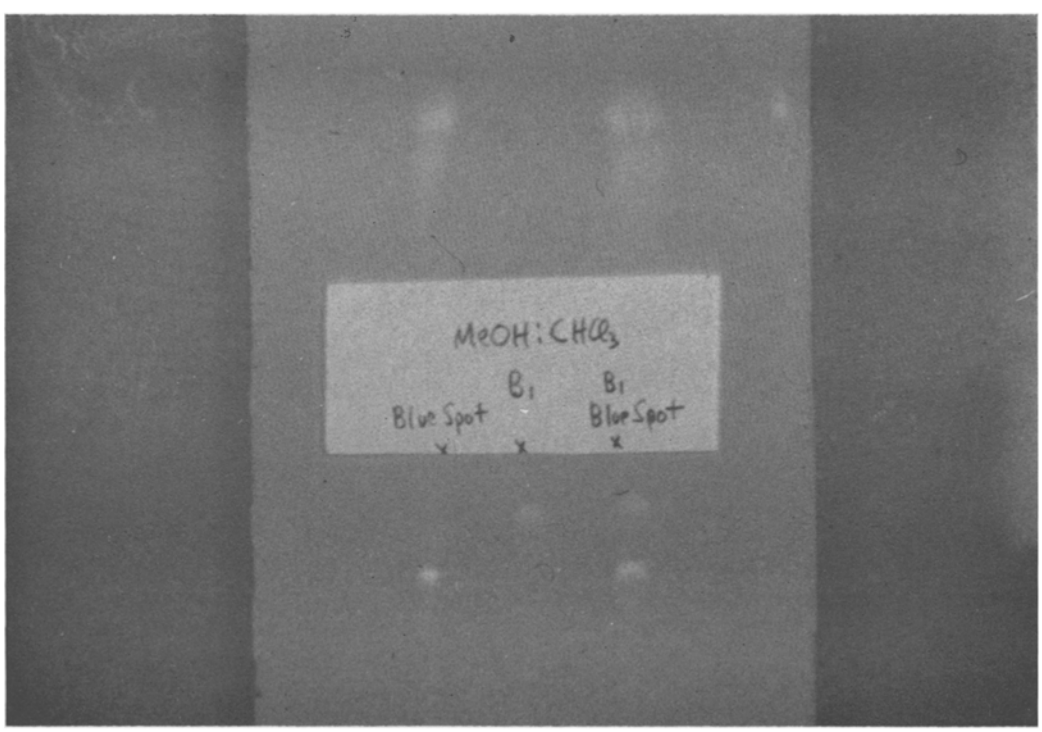

FIG. 1. Blue spot and aflatoxin B_1 in 3% methanol in chloroform developing solvent.

than using the syringes to apply three accurately measured extract spots and comparing the intensities with similarly applied standard spots, disposable Drummond microcaps (Preiser Scientific, Inc.) were used. Only the amount withdrawn by capillarity in the microcap was applied to the adsorbent. For each sample extract, two spots of single and double applications, respectively, were made; the fluorescent intensity of one of the spots was made to fall within the range of the intensities of the standard spots. This was accomplished by diluting or concentrating the extract solution and respotting onto another TLC plate. After assigning arbitrary values of 1.0 and 2.0 to the two standard spots, the B_1 content was calculated:

$$B_1 = \frac{(a)(b)(500)(c)}{(w)(0.7)(d)}$$

where a = intensity of one-application spot of sample B_1 relative to one-application spot of standard B_1 (expressed to two significant figures); b = conc. of standard B_1 in μg/ml; c = final dilution (if applicable) of 500 μl; d = concentration (if applicable) of 500 μl; w = wt. of sample in g; 500 = μl of sample extract volume; and 0.7 = correction for aliquot of original aqueous acetone extract analyzed.

For example, a sample set of results might yield: standard, 1.0 and 2.0; sample 1.3 and 2.6. This means that the sample B_1 concentration is 1.3 times that of the standard B_1 concentration.

Any one of three solvent systems are used for the TLC development: 3% methanol in chloroform (6), the benzene-rich phase of benzene-ethanol-water (45:36:19) (BEW) (3), or 10% acetone in chloroform (5). In addition, chromatography of the appropriately formed hydroxylated derivatives according to the method of Andrellos and Reid (1) can be used for final confirmation.

Because it had been noticed that R_f's generally increased and spots were less defined on more humid summer days, it was reasonable to assume that the adsorbent was becoming de-activated. Therefore, plates are not only activated just prior to use but also kept dry in the developing chamber by inserting two 100 ml beakers full of a commercial desiccating agent.

The photography of the TLC plates was undertaken with Ektachrome-X daylight film with an exposure of 10 sec at f8. Excess ultraviolet light is filtered with a Wratten 2A lens filter.

All other major extraction procedures of the Pons and Goldblatt method were not altered.

Results and Discussion

Although some of the above collection of modifications would not be expected to materially improve the resolution of the B_1 and the blue spot, other alterations are known to help.

The sample extract can be spotted more neatly and without disturbance of the silica gel when microcaps are used. Very small spots of 1–2 mm in diameter can be made and such a small size improves definition and resolution. Also, microcaps are disposed and eliminate the extreme care and time which must be exercised in being assured of clean Hamilton syringes. Accurate measurement of the amount applied is also not necessary since the only requirement is to apply the same amount for both standard and sample. Microcaps of 5-lambda size were used in these experiments, and it was found that the amount delivered by capillarity was 2.72 μl $\pm$ 4.0%. Reproducible volumes delivered by any instrument would be satisfactory; volumes do not need to be known as the only necessity is to be uniform. The 4.0% error in microcap delivery is reasonable and well-within the often quoted 10–20% variation encountered in the entire procedure.

To eliminate the possibility of cross-contamination between samples, separate Pasteur pipettes and sample vials are used for each sample since these two

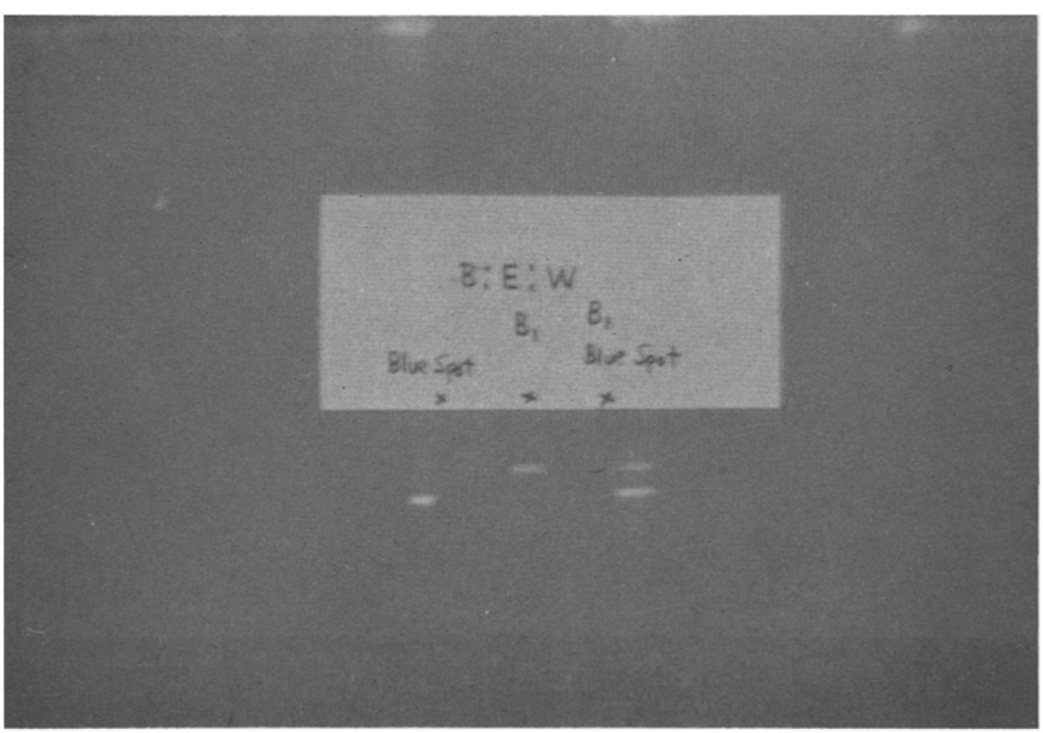

FIG. 2. Blue spot and aflatoxin B_1 in BEW developing solvent.

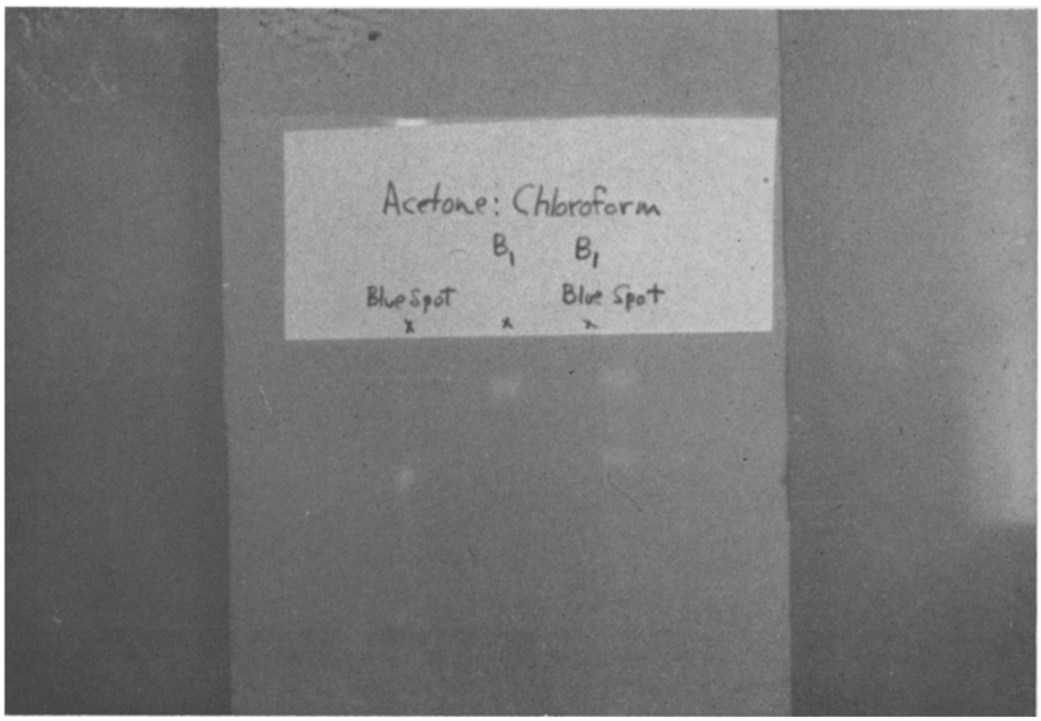

FIG. 3. Blue spot and aflatoxin B_1 in 10% acetone in chloroform.

pieces are the most easily contaminated with high concentrations of aflatoxin and equally the most difficult to clean.

Whereas the BEW solvent has been found to be superior for most products, the 3% methanol in chloroform is somewhat better for cottonseed samples. This is partially due to the occasional presence near B_1 of a yellow pigment which is easily distinguishable from B_1 on the basis of color alone. In a few exceptional samples (1–2%), we have encountered small amounts of a greenish-blue fluorescent compound not too different from the R_f of B_1. However, a redevelopment in either the BEW or the 10% acetone in chloroform has always confirmed or denied the true presence of B_1. In addition, the formation of hydroxylated derivatives and rechromatography of such derivatives (1) is available for final confirmation, although we have never had to resort to its use for field-collected samples of cottonseed.

The addition of a desiccant to the developing chamber aids in spot definition, resolution and R_f reproducibility. Also, measurement of the absolute moisture content of air (rather than relative humidity) aids in choosing the best time and place for plate development.

The blue fluorescing substance of Ashworth et al. was readily extracted from the hull samples; however, if the conditions set forth above are adhered to, there is no chance for misinterpretation. Presented in Figures 1, 2 and 3 are chromatograms of B_1 and the blue spot in each of the three solvent systems, respectively. Not readily shown in the black and white photographs is the fact that color rendition is additionally available to the naked eye to help in differentiating spots.

These results on the clear separation of the blue spot from aflatoxin therefore do not support the claim (2) that this material occurs in sufficient quantities to significantly affect aflatoxin analyses if seed coats are not removed.

It may be true that, as Ashworth et al. state, their blue spot is similar to that reported by Chen and Friedman (4); however, this latter blue spot is also easily distinguished from B_1, as personally observed by the senior author.

ACKNOWLEDGMENT

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