hypothesized include hydroxyanthraquinones as intermediates (Thomas, 1965; Aucamp and Holzapfel, 1970). Recently Heathcote et al. (1973) proposed versicolorin A (Figure 1, II) as a precursor of aflatoxin B_1 (Figure 1, I).

The isolation of versicolorin A from the mutant strain and chromatographic and mass spectral evidence of its presence in the nonmutant strain as well as reports of other hydroxyanthraquinones as secondary metabolites of *A. parasiticus* (Heathcote and Dutton, 1969; Lee et al., 1971) add credence to these theories.

However, little experimental evidence for the involvement of specific hydroxyanthraquinones had been obtained until 1973 when Lin et al. reported that *A. parasiticus* incorporated [^{14}C]averufin (Figure 1, IV) into aflatoxin B_1. Similar labeling experiments are now underway in this laboratory; we are using the yellow pigmented mutant and the nonmutant *A. parasiticus* to determine whether versicolorin A is also an intermediate in the biosynthesis of aflatoxin B_1.

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Rapid Screening Method for Aflatoxin in a Number of Products

Charles E. Holaday* and John Lansden

An improved qualitative minicolumn procedure for screening a number of different types of products for aflatoxin is presented. High-speed blending of the sample with aqueous methanol followed by purification with zinc acetate and re-extraction with benzene before subjecting to minicolumn chromatography is a simple, economical, and rapid method for aflatoxin detection. Sensitivities of 2 ppb can be achieved and the use of disposable plastic and glass items makes the method practical for field or in-plant application.

Since the introduction of the qualitative minicolumn technique by Holaday (1968), there has been a continuing interest in improving the range of commodities that can be analyzed with this technique and in minimizing the investment in time and equipment. During the past few years, a number of minicolumn methods have been proposed (Velasco, 1972; Pons et al., 1973; Holaday and Barnes, 1973; Shannon et al., 1975; McKinney, 1975) and the method described here utilizes some of the features of several of these methods. The purpose of this work was to develop a method that could screen a wide range of commodities and to provide a simpler and more rapid procedure which utilizes a number of plastic and glass disposable items that reduces the amount of maintenance and glassware cleanup, keeps the cost per determination at a low level, and decreases the danger of aflatoxin carry-over to a subsequent sample.

The simplicity of the method lends itself to field or in-plant use where laboratory facilities are limited or nonexistent. A subsequent report will describe a self-contained field unit which will require neither outside power nor running water.

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EXPERIMENTAL SECTION

Equipment used included a Chromatovue chamber equipped with long-wave uv, Ultra-Violet Products, Inc. (San Gabriel, Calif.); a Waring Blendor; vacuum source, either a water aspirator or small vacuum pump is satisfactory.

Supplies used included minicolumns packed with ca. 15 mm of Florisil (100–200 mesh) on bottom and ca. 15 mm of neutral alumina (100–200 mesh) on top (see Figure 1). For best results, the interface of the two materials should be as straight as possible. The alumina should have an activity grade of V. Some brands of alumina have a slight fluorescence which can cause interference. Two which do not fluoresce and which give excellent results are E. Merck and Woelm brands. The glass tubing is 5.5 mm i.d. and 160 mm long. Packing to hold the Florisil and alumina in place is made from paper pulp which provides a tight seal. This material is available from most chemical supply houses. Columns may be purchased from Tudor Scientific Glass Co. (Belvedere, S.C.).

Disposable items used included: culture tubes, 18 × 150 mm; plastic tube closures, 16 mm; pipets, 1 ml; plastic funnels, 2.25 in. top diameter.

Reagents used were: benzene; methanol–water solution (80:20, v/v); salt solution (600 g of sodium chloride, 600 g of zinc acetate, and 15 ml of glacial acetic acid dissolved in 4000 ml of distilled water); hexane–acetone solution (80:20, v/v). All reagents should be ACS grade.

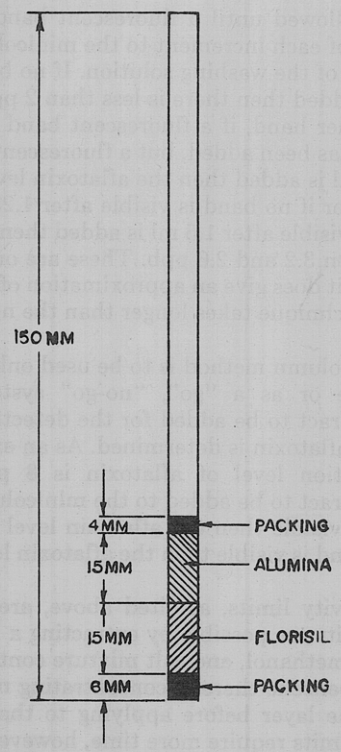

Figure 1. Diagram of minicolumn.

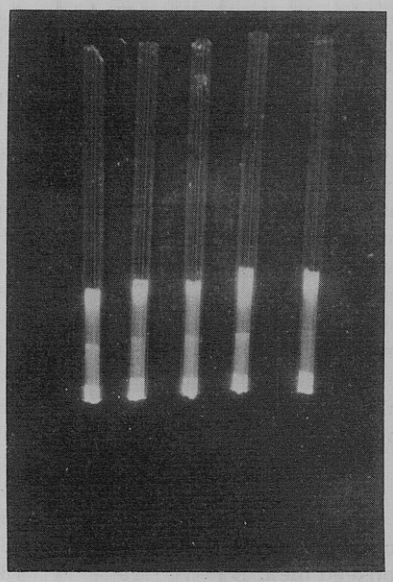

Figure 2. Detection of aflatoxin in samples (left to right) of peanuts (5 ppb), peanut butter (2 ppb), corn (3 ppb), cottonseed meal (10 ppb), and rice (0 ppb).

Procedure. Blend a 100-g sample with 200 ml of methanol–water solution for 1 min at high speed in the blender (any size sample can be used as long as the ratio of sample weight to solvent volume remains the same). Using a 15-cm filter paper (fast filtering type) folded into a funnel, filter 10 ml of extract into a culture tube. To this, add 10 ml of the salt solution. Close test tube with a plastic closure and shake vigorously for 5–10 sec. Filter 15 ml of contents through a glass fiber filter (9 cm) disk placed in the same funnel into a second culture tube. Add 3 ml of benzene, close the culture tube with the plastic closure, and shake contents vigorously for 10 sec. Let layers separate and pipet 1 ml of upper layer benzene into the top of a minicolumn the bottom of which is attached to a vacuum source. After the benzene has been pulled through, add 5 ml of the washing solution (hexane–acetone) to the top of the minicolumn and pull through. Continue pulling the vacuum for an additional 2 min or until all of the washing solution has evaporated from the minicolumn. Remove minicolumn and observe under long-wave uv. A blue fluorescent band in the center of the column or at the interface of the Florisil and alumina indicates at least 4 ppb of aflatoxin. Adding 2 ml of the benzene layer to the minicolumn increases the sensitivity to approximately 2 ppb. The addition of 0.1 ml decreases the sensitivity to approximately 40 ppb.

RESULTS AND DISCUSSION

The procedure for detecting aflatoxin to a sensitivity of 4 ppb on peanuts was timed on several occasions and the average time required for completion was 9.5 min on peanuts. When 2 ml of the benzene layer is added to the minicolumn to obtain a sensitivity of 2 ppb approximately another 30 sec to 1 min is required to remove a second milliliter of the benzene layer for application to the minicolumn. Adding less than 1 ml reduces time for an analysis to less than 9.5 min since less extract has to be pulled through the minicolumn. However, the sensitivity in this case is reduced.

The washing solution (hexane–acetone) not only washes the aflatoxin through the alumina layer onto the interface of the alumina and Florisil but also removes most of the remaining background fluorescence not removed by the salting-out step. On extremely dirty samples, if all of the washing solution is not evaporated by leaving the minicolumn on the vacuum for sufficient time some background fluorescence may be still present. Placing the minicolumn back on the vacuum for an additional time to evaporate the remainder of the washing solution usually removes this background fluorescence. (Some samples may require an additional 5 ml of washing solution.)

The most time-consuming step in the procedure is the filtration. Most extracts filter rapidly, but there are some, such as peanut butter, which take longer and the time required for an analysis of these products may be as much as 12 to 15 min. On the other hand, corn, rice, and other grain extracts filter very rapidly, more so than peanuts, and time for an analysis of these products is less than 9.5 min.

The technique of extracting the aflatoxin from the mixture of the salt and extraction solutions with benzene as recommended by Pons et al. (1973) is an effective way to concentrate the aflatoxin for application to the minicolumn. The mammalian toxicity of acetonitrile and lead acetate used for extraction and cleanup was a deterrent to routine use of Pons' minicolumn method (1973), however. The use of zinc acetate, on the other hand, was as effective as lead acetate and was considered safer. Earlier, McKinney (1975) recommended the substitution of zinc acetate for lead acetate and ferric chloride in the methods of Pons et al. (1972) and Velasco (1972a,b); however, these methods were based on TLC procedures and not minicolumn methods. Ammonium sulfate, as recommended by Shannon et al. (1975), was less effective as a cleanup agent on certain types of contamination. The method described herein is more sensitive and is considered faster (8–9.5 min) than the one described earlier by Holaday and Barnes (1973), which required 13–15 min for completion. It also requires much less manipulation and can be used in the field where little or no laboratory facilities are available. The minicolumn used in this method is of simpler design and is easier to prepare than the one proposed by Velasco (1972a,b).

The reliability of the procedure was tested by having ten inexperienced panelists select five minicolumns which had been prepared from five peanut butter extracts according to whether they either had or had not an aflatoxin band. The minicolumns represented 0, 2, 4, 6, and 10 ppb of all 4

Table I. Minicolumn Results on a Number of Spiked Samples

Sample identification	Amount of aflatoxin added, ppb	Minicolumn analyses, ppb
Peanut meal A	Neg.	Neg.
Peanut meal B	15	8–16
Peanut meal C	20	8–16
Peanut butter A	20	8–16
Peanut butter B	15	8–16
Peanut butter C	Neg.	Neg.
Corn A	15	8–16
Corn B	30	16–32
Corn C	Neg.	Neg.
Rice A	Neg.	Neg.
Rice B	8	4–8
Rice C	15	8–16
Cottonseed meal A	20	8–16
Cottonseed meal B	15	8–16
Cottonseed meal C	Neg.	Neg

of the aflatoxins ($B_1 + B_2 + G_1 + G_2$). In every case the panelists were able to determine which columns were positive and which one was negative. Figure 2 illustrates the sensitivities obtained with this procedure for the four aflatoxins ($B_1 + B_2 + G_1 + G_2$) on five types of samples. It is not necessary to prepare standards since false positives have not been a problem as long as the Florisil and the alumina do not have background fluorescence under uv.

The procedure was further checked by analyzing a group of spiked samples with the minicolumn method. Measured amounts of alfatoxin were added to the samples in order to give a range of known aflatoxin levels. The results are shown in Table I. This illustrates the applicability of the method to a wide range of different samples. The minicolumn results were obtained by adding the benzene extract to the minicolumns in increments of 0.25 ml. If no fluorescent band was visible after adding the first 0.25 ml, a second 0.25 ml of extract was added. If a fluorescent band was still not visible, a third increment was added. This procedure was followed until a fluorescent band was visible. The addition of each increment to the minicolumn was followed by 5 ml of the washing solution. If no band is visible after 2 ml is added then there is less than 2 ppb of aflatoxin. On the other hand, if a fluorescent band is not visible after 0.25 ml has been added, but a fluorescent band is visible after 0.5 ml is added then the aflatoxin level is between 8 and 16 ppb or if no band is visible after 1.25 ml is added but a band is visible after 1.5 ml is added then the aflatoxin level is between 3.2 and 2.6 ppb. These are only estimates, of course, but it does give an approximation of the aflatoxin levels. This technique takes longer than the normal screening procedure.

If the minicolumn method is to be used only as a screening procedure or as a "go", "no-go" system then the amount of extract to be added for the detection of the desired level of aflatoxin is determined. As an example, if the desired detection level of aflatoxin is 8 ppb then the amount of extract to be added to the minicolumn is 0.5 ml. If no band is visible then the aflatoxin level is less than 8 ppb, or if a band is visible then the aflatoxin level is at least 8 ppb.

The sensitivity limits, as cited above, are conservative and lower limits are possible by extracting a larger volume of the water, methanol, and salt mixture containing the aflatoxin with benzene thereby concentrating more aflatoxin in the benzene layer before applying to the minicolumn. These lower limits require more time, however, since larger volumes of liquid must be filtered.

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