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Title: USE OF ALKALI IN THE ISOLATION AND PREPARATION OF AFLATOXINS

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The biological preparation of aflatoxins was accomplished by culturing the mold Aspergillus flavus on various media. Aflatoxin was isolated by chloroform extraction of the culture, and purified by column fractionation and crystallization. Rice, wheat, soya bean, peanut and YES media were found to give high yields of aflatoxin. It was also found that A. flavus could grow and produce toxin on fresh tomatoes and grapes.

The pure aflatoxins B_1 and G_1 were treated separately with standardized NaOH and NH_4OH solutions. Changes in solvent system, exposure time and alkali concentration were found to affect the stability of aflatoxin. Solutions of aflatoxins B_1 and G_1 in ethanol and methanol were quite unstable when treated with strong base even after a short exposure period. The aflatoxins were quite stable, however, if treated in a chloroform solution at a lower base concentration.

Several crude chloroform extracts containing aflatoxins were prepared from various culture media with different mold strains. These extracts were then shaken gently with an equal volume of 0.25 N NaOH solution for several seconds. This treatment produced final chloroform extracts which were lower in pigments, total solids, and lower R_f-value compounds than similar extracts which received no base treatment. The decolorizing ability of the alkali treatment was demonstrated by comparison of the ultraviolet absorption spectra of the treated samples and control samples. This base treatment shortened the preparation time and increased the yield of aflatoxins from crude chloroform extracts.

Photodegradation of aflatoxins B_1 and G_1 was observed in both ethanol and chloroform solutions. After an induction period, the aflatoxin was degraded to low R_f-value compounds, which were then converted to non-fluorescent products. Aflatoxin G_1 was found to be more stable than aflatoxin B_1 to light exposure. Base treatment as described above was found suitable for purifying aflatoxin which contained photodegraded contaminants.

USE OF ALKALI IN THE ISOLATION AND PREPARATION OF AFLATOXINS

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USE OF ALKALI IN THE ISOLATION AND PREPARATION OF AFLATOXINS

I. INTRODUCTION

Another chapter in mankind's struggle against his hostile environment is being written today in what can be called the aflatoxin story which started with the death of more than 100,000 turkeys in England in 1960. The cause was a toxic material produced by the common mold, Aspergillus flavus, in peanut meal. The substance was named aflatoxin for A. flavus toxin. At about the same time, a high incidence of liver tumors (hepatoma), was found in hatchery-reared rainbow trout in the United States. In 1963, the cottonseed meal in the commercial pelleted feed was found to be the source of the carcinogen, and in 1965, aflatoxin was isolated from several commercial cottonseed meals used as trout feed.

Aflatoxin has been reported to be photosensitive and alkali labile. The alkali refining of crude vegetable oil which might be contaminated with aflatoxin is the basis for the interest in this reaction.

In this work, the effect of alkali on the stability of aflatoxins was investigated and found to be dependent on solvent employed, the concentration and exposure time. The alkali treatment was found to be useful in the preparation and isolation of aflatoxins, and

also in the removal of degradation products during the determinative procedure.

Certain strains of A. flavus were found to produce aflatoxins when grown on fresh tomatoes and grapes.

II. LITERATURE REVIEW

In England during 1960, more than 100,000 turkey poults exhibited certain characteristic symptoms of toxemia and died. This condition, for lack of a better name, was called "Turkey X" disease (13). Concurrently, several outbreaks of a similar "new" disease appeared in ducklings (10). Reports that turkeys and ducklings in other countries, including the United States, were also afflicted with this malady soon appeared (2, 46).

Moldy peanut meal in the rations was implicated as the cause and presence of the common mold Aspergillus flavus or some factor associated with it seemed to correlate with all toxic samples (48). Aflatoxin was the name given by the British Government Interdepartmental Working Party on Groundnut Toxicity Research to the total toxic material elaborated by certain strains of A. flavus even before its complex nature was known (31).

Aflatoxin is not produced by all strains of A. flavus (39) and certain fungi besides A. flavus can also produce aflatoxin. These include Aspergillus parasiticus and Penicillium puberulum (11, 30, 39).

In the Spring of 1960, a high incidence of hepatoma was observed in hatchery-reared rainbow trout in Idaho (45). The disease in trout seemed to be correlated with the recent use of commercial pelleted

feeds. In 1963, Wolf and Jackson (59) found that the cottonseed meal in a commercial pelleted feed was the source of the carcinogen although the identity of the carcinogen was then unknown. Engebrecht, Ayres and Sinnhuber (27) isolated aflatoxin from several samples of commerical cottonseed meal used for trout feed. These meals and their extracts were demonstrated by Sinnhuber et al. (52) to produce hepatoma in rainbow trout and bile ductal proliferation in ducklings.

The carcinogenic property of aflatoxin has been found to extend to rats, dogs and other animals (32, 34, 46, 49). The possibility that these cancer producing substances may be present in human foods has made this a matter of public health concern.

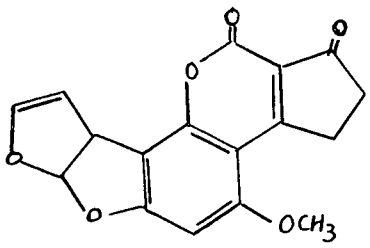
Aflatoxin has been found in samples of African maize (17) and it has been shown that the mold can grow and produce toxin on rice, wheat, corn, soya bean, cottonseed and bread. Furthermore, it has been found that the toxin can pass through the rumen into the milk of cows fed rations containing toxic peanuts (5).

Chemical and Biological Properties of Aflatoxin

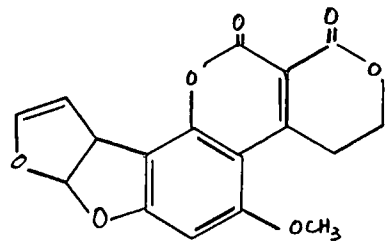
Initially, aflatoxin was considered to be a single substance having a blue fluorescence under ultraviolet light. By using more refined chromatographic techniques, it was found that several substances were involved. The major aflatoxin was called B because of its blue fluorescence. Another component was designated G

because of its green fluorescence. Each of these toxins has been resolved further into two components, all of which are termed aflatoxins B_1, B_2, G_1 and G_2. They have been reported to occur in the approximate ratio of 40 B_1:1 B_2:50 G_1:1 G_2 (29). According to our results, as will be described later, these ratios vary with the culture conditions.

The structures of the aflatoxins were reported in 1963 by Asao et al. (8), Chang et al. (19), and by Hartley, Nesbitt and O'Kelly (29). Aflatoxins B_2 and G_2 were reported to be the reduction products of the respective B_1 and G_1 (29). The structures of the aflatoxins are shown in Figure 1. It is of interest to note that the aflatoxin molecule has a coumarin nucleus as does the mold metabolite found to be responsible for sweet clover poisoning (6).



Aflatoxin B_1



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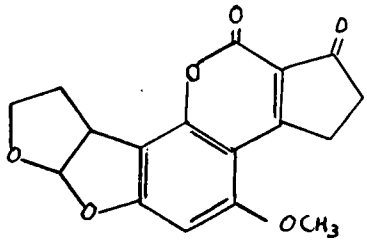
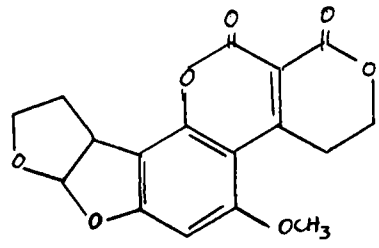


Aflatoxin B_2



Aflatoxin G_2

Figure 1. The structure of aflatoxins.

The most useful physical characteristic of the aflatoxins is their distinct fluorescence in ultraviolet light and this has been employed in their quantitative determination (48). When excited with 365 mμ light, aflatoxins B_1 and B_2 have an emission maximum at 435 mμ, and 450 mμ for aflatoxins G_1 and G_2 (18).

Aflatoxins are extremely heat stable. Austwick and Ayerst (11) reported that heating of toxic peanut meal at 150°C for several hours did not reduce toxicity. The heat stability of aflatoxins is an important consideration for heat processed food products.

The physical characteristics of aflatoxins as reported by van der Merwe, Fourie and Scott (55) are summarized in Table 1.

Table 1. The physical characteristics of aflatoxins.

Aflatoxins	Structure formula	Melting point	λ Max (mμ)
B_1	$C_{17}H_{12}O_6$	265°C	224, 265, 363
B_2	$C_{17}H_{14}O_6$	305°C	220, 265, 363
G_1	$C_{17}H_{12}O_7$	257-259°C	217, 245, 265, 363
G_2	$C_{17}H_{14}O_7$	230°C	217, 245, 265, 363

The biological effect of aflatoxin varies with the species of animals. By controlled feeding trials, turkeys, ducklings, cattle, swine, rats, mice, guinea pigs, rabbits, dogs, monkeys and rainbow

trout have been found susceptible to aflatoxin toxicity (3, 28, 47, 54, 58).

In chickens, aflatoxin, though producing definite pathological disturbances, is rapidly metabolized (40). Generally, the ingestion of the toxin causes severe liver damage and is fatal on continuous ingestion.

The duckling is the most susceptible species to the toxin with the possible exception of the rainbow trout. In the duckling, the histopathological hepatic lesions developed extremely rapidly, within three to four days of feeding. The proliferation of bile ductal epithelial cells is visible and marked by seven days (2). The extreme toxicity of aflatoxins is demonstrated by the LD_{50} values for the day-old duckling. For aflatoxin B_1, it has been reported to be 30 μg (29) and 28.2 μg (8); for aflatoxin G_1, 60 μg (29), and 90 μg (8); and over 200 μg for both B_2 and G_2 (29). Usually, young animals are more susceptible to aflatoxin toxicity than older animals. The relative carcinogenicity of these four aflatoxins has not been established.

The Biological Preparation and Isolation of Aflatoxins

For studies of the chemistry and toxicology of aflatoxins, large quantities of toxin are required. The total chemical synthesis of racemic aflatoxin B_1 has recently been reported by Büchi et al. (15).

However, it may more easily be prepared by biological procedures. This has been accomplished by growth of A. flavus on sterilized agricultural products such as peanuts (21), crushed wheat (8), corn meal (55) and rice (51). The production has also been accomplished on semisynthetic (26) and synthetic media (35). Adye and Mateles (1) demonstrated that significant labeling of aflatoxins can be achieved by the addition of labeled precursors to a synthetic medium.

The toxin may be conveniently prepared by growing the mold, A. flavus on suitable media for the prescribed time and temperature (51, 26, 35). After culturing, the four aflatoxins are extracted from the media with chloroform and precipitated by adding ten volumes or more of petroleum ether.

The fractionation of crude aflatoxin into its individual components is achieved by thin-layer chromatography (8). The major components are easily located by inspection of the chromatoplates under ultraviolet light. However, the preparation of large quantities by this method is time-consuming and laborious.

The use of column chromatography for isolation and fractionation of crude aflatoxin has been reported by Shotwell et al. (51). The sample is applied to a silicic acid column and eluted with ethanol: chloroform (1:99 v/v). The elutes are collected as 20 ml fractions and aliquots chromatographed on a thin-layer plate to determine their composition.

The aflatoxin fractions obtained both from thin-layer and column chromatographic methods must be recrystallized in order to remove additional impurities. Six recrystallizations from chloroform-methanol for aflatoxin B_1 and five recrystallizations for aflatoxin G_1 were reported as necessary by Asao et al. (8). A pentane-chloroform mixture was used for recrystallization as described by Shotwell et al. (51).

A. flavus grown on rice produced the following component ratios of aflatoxins: B_1-100; B_2-0.15; G_1-0.22; G_2-0.02 (51). Since the proportions of B_2 and G_2 are small, it was found that it would be more convenient to prepare these two components by catalytic hydrogenation of B_1 and G_1. This was accomplished by use of palladized charcoal catalyst (8).

For optimum mold growth and maximum aflatoxin production in submerged cultures, zinc is required at a level of 0.8 mg per 1 of medium. The production of aflatoxin is inhibited by the presence of barium ions in the medium and stimulated by cadium ions (33).

Chemical and Biological Assay of Aflatoxin

A number of methods for the isolation and determination of aflatoxin in feed and oilseed products have been recently reported (16, 20, 25, 36, 43, 44, 53). Most of these published procedures are based on the original method proposed by the Tropical Product

Institute (14). Briefly, the sample is defatted with petroleum ether or diethyl ether, then extracted with a Soxhlet apparatus using methanol for a few hours. The water diluted methanol extract is extracted with chloroform and the separated chloroform layer is dried with anhydrous sodium sulfate.

The extracted toxin is separated either by paper (22) or thin-layer (36, 42, 53) chromatography and estimated by the intensity of fluorescence after exposure to ultraviolet light. A visual comparison is made using standards of approximately the same concentration. This subjective procedure has certain limitations as discussed by Ayres and Sinnhuber (12), who described an objective method based on the use of fluorodensitometry.. This procedure, probably the most accurate and sensitive, is still limited by the presence of interfering materials which may be present in the extracts.

Quantitative ultraviolet spectrophotometry (8, 29, 56) has also been useful for the determination of aflatoxins (1, 17). This method requires a rather large amount of sample and is more accurate than the subjective measurements (16, 43, 53) but less suitable for routine analysis.

In 1965, a rapid method for the extraction of aflatoxin from the cottonseed products was proposed by Pons and Goldblatt (42). A solvent mixture of acetone and water was used to extract the aflatoxin followed by lead acetate treatment to remove the gossypol pigments.

This procedure still leaves some pigments and components which cause streaking on the thin-layer plate. A silicic acid column purification step was proposed by the same group (43). Aflatoxin isolated by this method is lower in total solids and pigments but still may contain some interfering materials.

In addition to the chemical analysis of aflatoxin, several biological methods have been proposed and are used by many investigators. The biological assay of aflatoxin based upon liver lesions in ducklings has been used quite widely (4, 10). Early experimental studies and field observations suggested that the duckling was extremely susceptible to the toxic effects of aflatoxin. The LD_{50} value for the day-old duckling is much lower than for other animals, according to Armbrecht and Fitzhugh (7). They fed the suspected toxic extract to day-old ducklings by stomach tube over seven days, the animal was sacrificed and the livers were sectioned, stained and examined. Bile duct proliferation score was used as an index of aflatoxin in the suspected toxic extract. The basic pathologic change in ducklings when fed aflatoxin consists of hepatic parachyma necrosis and proliferation of bile ductule cells, the liver appears pale or yellowish-green in color and is smaller in size (37).

Rainbow trout has been found to be an extremely sensitive test animal to the effects of aflatoxin. Levels of only a few parts per billion in the trout ration will induce liver cancer in less than a year

according to Sinnhuber et al. (52) and Ashley, Halver and Wogan (9). Cell culture techniques have also been used to assay aflatoxins, the results of which were summarized by Wogan (58) in 1966.

The chicken embryo has been used in the assay of aflatoxin and probably is the most sensitive in vivo test described (41, 57). Injections of test solutions are made into fertile White Leghorn eggs and the development of the embryo is observed for the full 21 day incubation period. The data of Verret, Marliac and McLaughlin indicated that the LD_{50} for aflatoxin B_1 was 0.048 and 0.025 μg for the yolk and air cell routes, respectively.

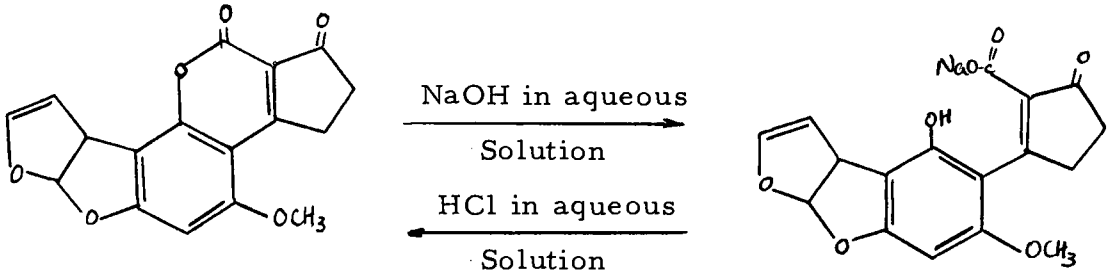
Aflatoxin also affects plant tissues. Schoental and White (50) reported that 25 μg of aflatoxin per ml inhibits the germination of cress seeds, and interferes with chlorophyll synthesis.

Decomposition of Aflatoxin

Aflatoxins have been reported to be photosensitive and altered by alkali (24, 56). The compounds appear partially to decompose, for example, upon standing in methanolic solution, and this process is greatly accelerated in the presence of light or heat. Substantial degradation also occurs on chromatograms exposed to air and ultraviolet or visible light (58).

The treatment of aflatoxin B_1 with alkali has been reported by van der Zijden et al. (56) to induce a shift of λ_{max} from 360 mμ

to 370 mμ and that the shift is reversible on immediate acidification. In 1966, Coomes et al. (24) suggested that the lactone ring structure of aflatoxin B_1, which is essential for fluorescence under ultraviolet light is hydrolyzed in the presence of sodium hydroxide in aqueous solution and the sodium salt produced from this reaction is not fluorescent. This reaction is shown in Figure 2.



Aflatoxin B_1

Sodium Salt of Aflatoxin B_1

Figure 2. The structure of aflatoxin B_1 in basic and acidic media.

Mild alkaline treatment of aflatoxin B_1 at room temperature was shown by Feuell of Tropical Products Institute, London, to decrease the fluorescence without affecting the toxicity according to duckling tests (38).

The treatment of aflatoxin B_1 in methanol solution with NaOH was recently described by Parker and Melnick (38). They stated that the aqueous methanol sample, which received only the NaOH treatment at room temperature, will yield no recoverable aflatoxin; while the sample receiving the alkali treatment followed by the acidification with HCl yielded the original aflatoxin in 94% yield.

III. EXPERIMENTAL

Biological Preparation of Aflatoxin

Mold Strains

Aspergillus flavus strains M-93, M-3, A-12-353, and 15517 were used to produce aflatoxin. Strains M-93 and M-3 were obtained from the Food and Drug Administration of the U. S. Department of Health, Education and Welfare, Washington, D. C. Strain A-12-353 was obtained from the U. S. Department of Agriculture, Western Utilization Research and Development Division, Albany, California, and strain 15517 was obtained from the American Type Culture Collection, Washington, D. C. These mold strains were maintained on mycological agar slants.

Media Preparation

YES (Yeast Extract-Sucrose): The basal medium contained 2% yeast extract (Difco) and 20% sucrose (26). For a 2.8 l Fernbach flask, 500 ml of media were used.

Rice: This medium was prepared by mixing one part of rice with three parts of tap water (w/v). For a 2.8 l Fernbach flask, 100 gm of rice and 300 ml of tap water were used. A rice medium which contained 300 gm of rice and 150 ml of tap water in a 2.8 l

Fernbach flask as described by Shotwell et al. (51) was also used.

Wheat: For each 50 gm of crushed wheat, 20 ml of tap water were added. A 2.8 l Fernbach flask culture may contain 300 gm of wheat.

Others: Agricultural commodities such as soybeans, peanuts, fresh sliced tomatoes, fresh sliced grapes, defatted wheat middlings and peanut meal were used.

Culture

The flasks, containing media, were stoppered with cotton plugs and autoclaved for 15 min at 20 psig in an autoclave. After cooling to room temperature, the media were inoculated with a spore suspension of A. flavus and incubated at 28°C as a stationary culture for seven to ten days.

Isolation

Solid media: The cultures were covered with chloroform (USP redistilled) and allowed to stand overnight. The mixture was heated under reflux for two hours and the extracts were filtered through cheesecloth, washed with distilled water, and dried over anhydrous sodium sulfate. This extraction was repeated three times. The sodium sulfate was removed by filtration and the chloroform extract concentrated in vacuo to a small volume (10 to 15 ml). Crude

aflatoxin was precipitated by adding ten volumes of hexane to the concentrate and collecting the precipitate by centrifugation.

Liquid media: The cultures were extracted by adding chloroform and filtering through cheesecloth to separate the mycelial mats. The aflatoxin was then removed from the aqueous phase by refluxing the two phase system for 2 hr, separating the chloroform layer and re-extracting the filtrate by shaking with chloroform several more times. The chlorcform extracts were dried, concentrated, and the crude aflatoxin precipitated in the same manner as previously described. The prepared crude aflatoxin is a yellow colored precipitate which contains some low R_f value fluorescent compounds as shown by thin-layer chromatography.

Purification

The fractionation of crude aflatoxin into its individual components can be accomplished by thin-layer chromatography, but more easily by column chromatography.

An important factor in the column fractionation of aflatoxin was the packing of the silicic acid column. The column was prepared by packing 100 mesh silicic acid (Mallinckrodt A. R. grade) as a slurry with ethanol:chloroform (1:99 v/v) into 2.4 cm I. D. column. Nitrogen at 3 psig was used to pack the column. The height was 31 cm and about 80 gm of the silicic acid were used for each column. One liter

of solvent was passed through the column prior to sample application.

The crude aflatoxin was dissolved in a small volume of ethanol: chloroform (1:99 v/v) and applied to the column. The column was developed and eluted with the same solvent under 3 psig and 20 ml fractions were collected. A 2 μl portion of each fraction was spotted on a thin-layer plate to determine its aflatoxin composition, and similar fractions were combined. Often the aflatoxin fractions were somewhat colored and contained trace impurities.

The fractionated aflatoxin was dissolved in chloroform:methanol (1:5 v/v) in a 15 ml centrifuge tube. An acetone heating bath was used for the heat transfer. The crystals were collected by centrifugation after the mixture was cooled overnight at -20°C. The colored liquid phase was removed by a small pipette and saved for a second crop of crystals. The crystallization procedure was repeated and the solvent system was changed to chloroform:methanol (1:3 v/v) after the third recrystallization. As purity increased, cooling time was decreased and finally white aflatoxin crystals were obtained. Chloroform solutions were used for the qualitative and the quantitative assays.

Qualitative Analysis

Melting point determination: The melting points of the prepared aflatoxins were measured by using a Fischer-Johns Block and were uncorrected.

Thin-layer chromatographic analysis: Thin-layer plates were prepared by spreading a MN-Silica Gel G-HR water slurry (1:2 w/v) on clean dry glass plates. The adjustable TLC spreader and the stationary phases were obtained from Brinkmann Instruments, Inc., Westbury, N. Y. Plates of 0.25 mm thickness were air dried for 30 min and activated for 1 hr by heating in an oven at 110°C. The plates were stored over indicator silicic acid in a desiccator. For analysis, diluted samples were spotted on a line about 1 cm from the bottom edge of the plate, and the plate placed in a developing chamber equilibrated with chloroform:methanol (97:3 v/v). The plate was withdrawn after the solvent front reached 15 cm above the origin. The plate was observed under ultraviolet light, and a single spot indicated a pure compound. The R_f values of the compounds were compared with suitable standards.

Quantitative Analysis

Fluorodensitometry: The recording Photovolt fluorodensitometer was used for the measurement of aflatoxins on a developed thin-layer plate. The procedure described by Ayres and Sinnhuber (12) was followed.

Spectrophotometry as described above was also used to effect quantitative analysis by observing absorbances at the various wavelength maxima.

Alkali Treatment of Aflatoxins

Exposure Time

The following series of experiments were designed to study the effects of strong and weak alkali on the aflatoxins. Standard solutions of sodium hydroxide and ammonium hydroxide were prepared in both water and ethanol and were titrated against standard potassium acid phthalate.

Aflatoxin was dissolved in chloroform to give an absorbance ranging from 0.5 to 1.0 at 362 mμ. Ten ml of this solution were placed on a 60 ml separatory funnel and 10 ml of 0.9880 N NaOH solution added. The funnel was stoppered and placed on a wrist-action shaker (Burrell Corp., Pittsburgh, Pa.). The mixture was shaken at maximum speed for various times (Figure 4). At the maximum shaking speed, five exchanges of the solution per second were observed. The alkali-treated chloroform layer was separated and dried over anhydrous sodium sulfate. The ultraviolet spectra of these samples were measured using a Beckman DK-1 Spectrophotometer, and compared with an untreated blank. Thin-layer chromatographic analysis was used for qualitative verification.

A similar set of experiments was conducted using 0.9998 N NH_4OH.

Effect of Different Alkali Concentrations

This experiment was done in the same manner as the one in the previous section, except that the shaking time was set for one minute, and the base concentration was varied. Five concentrations (Figure 3) for both NaOH and NH_4OH solutions were used.

Also prepared and tested were ethanolic aflatoxin solutions, for which six samples were prepared by mixing 4 ml of ethanolic aflatoxin with 1 ml of ethanolic alkaline solution at different concentrations. The ultraviolet spectra of these mixtures were measured immediately after mixing. Analysis by thin-layer chromatography was also carried out. Methanolic aflatoxin was also treated with aqueous alkaline solution.

In order to check the regeneration of aflatoxin, some neutralization experiments were performed on the base-treated samples. The ultraviolet spectra were measured and thin-layer chromatography performed on the neutralized mixtures.

Purification of Aflatoxin by Treatment with Alkali

Several chloroform extracts of crude aflatoxin were prepared from the different culture media with several mold strains. These extracts contained a yellow pigment with an absorption maximum at 440 mμ. Ten ml of the extract were mixed with 10 ml of 0.25 N NaOH

in a separatory funnel. The chloroform layer was separated after shaking by hand for a few seconds. After drying over anhydrous sodium sulfate, the chloroform solution was analyzed for aflatoxin and the presence or absence of the colored compound(s) noted using spectrophotometry and thin-layer chromatography.

A crude aflatoxin extract was prepared from the M-93 wheat culture. This contains an X component (see Figure 5) with a lower R_f value than aflatoxin G_1. Ten ml of the prepared extract were treated with 10 ml of the freshly prepared NaOH solution. The percent NaOH concentrations used were 0.0, 0.5, 1.0, 10.0, 20.0, 30.0 and 40.0 (w/v). The chloroform layers which had been dried over anhydrous sodium sulfate were analyzed by thin-layer chromatography. The fluorescence of these samples was measured with the Photovolt fluorodensitometer. The loss of the aflatoxin B_1, G_1, and the low R_f value compounds (X) was followed by plotting log peak area versus alkali concentration.

Determination of Aflatoxin

Meal Samples

Cottonseed meals were obtained from the National Cottonseed Products Association, Memphis, Tennessee.

Toxic peanut meal was obtained from Dr. G. C. Solomon,

Armed Forces Institute of Pathology, Washington, D. C.

Extraction and Purification of Samples

The procedure developed by Pons and Goldblatt (42) was used for this work. The sample was extracted with 70% aqueous acetone and the gossypol pigment was removed by lead acetate treatment. The filtrate was transferred into a separatory funnel and extracted with chloroform after dilution with water.

For some very viscous extracts, the chromatographic column cleanup procedure described by Pons et al. (43) was used.

Application of Alkali Treatment on the Aflatoxin Determination

One ml of the prepared sample extracts was diluted to about 10 ml. This mixture was treated with 10 ml of 0.25 N NaOH solution by shaking the mixture for 10 or 15 sec by hand in a 60 ml separatory funnel. The chloroform layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness with a rotary evaporator. The residue was then taken up in 1 ml of chloroform for analysis.

The treated and untreated samples were compared qualitatively and quantitatively by thin-layer chromatography.

Photodegradation of Aflatoxins

Stock solutions of aflatoxins B_1 and G_1 were prepared in both chloroform and ethanol solutions at a concentration of approximately 10 μg/ml. Duplicate samples of each stock solution were placed in 25 ml volumetric flasks and brought to volume. One sample was wrapped in aluminum foil and the other was exposed to room illumination at room temperature in the laboratory.

At intervals of 0, 3, 5, 7 and 9 days, the illuminated and the non-illuminated samples were checked by means of thin-layer chromatography for aflatoxin.

Solutions of aflatoxin in chloroform which had been exposed to light were treated as described earlier with NaOH and NH_4OH solutions. The NH_4OH layer was collected and concentrated. The aflatoxin and photodegraded products were examined by means of thin-layer chromatography and fluorodensitometry.

IV. RESULTS AND DISCUSSION

The Production of Aflatoxins

Mold Strains

Among the strains of Aspergillus flavus which we have in the laboratory, strain A-12-353 was found to be negative in aflatoxin production. Strain M-93 gradually lost its ability to produce toxin after it had been transferred several times on mycological agar slants. Strains M-3 and 15517 were found to give high aflatoxin production in all media and after many transfers.

Aflatoxin Yield on Various Media

The substrates, rice, wheat, soya bean, peanut and YES media, as described in the experimental section, all gave high yields of aflatoxin. Diethyl ether defatted wheat middlings and peanut meal produced lesser quantities. These results suggested that some diethyl ether soluble components may stimulate aflatoxin production.

Non-sterilized fresh sliced tomatoes and fresh sliced grapes were found to give high toxin production. It is of particular interest that A. flavus will grow and produce toxin in fresh vegetables and fruits which are low in protein. The possible contamination to tomatoes and grape products by A. flavus could make this a problem

of public health concern.

The production of aflatoxin B_1 on YES media by A. flavus strain 15517 was found to be greater than 1.86 gm of aflatoxin B_1 per kg of culture, or more than 7,250 μg of aflatoxin B_1 per gm of rice. This yield was higher than that of the rice media as described by Shotwell et al. (52). They used an A. flavus strain which gave a maximum production of 1.02-1.12 gm of aflatoxin B_1 in 1 kg of culture.

Aflatoxin Proportion on Various Media

The proportions of the aflatoxins on thin-layer plates were measured by fluordensitometry as reported by Ayres and Sinnhuber (12). The relative responses of the aflatoxins were calculated to be B_1-1.0, B_2-2.2, G_1-1.5, G_2-2.0 from peak area versus concentration curves. The aflatoxin ratios of the various media are summarized in Table 2.

The results indicated that soya beans and fresh sliced and non-autoclaved tomato media gave a much higher aflatoxin G_1 production than the other media described in this experiment. The peanut medium had higher levels of aflatoxins B_2 and G_2. These substrates could be useful for the production of aflatoxin components other than aflatoxin B_1.

Table 2. The component ratios of crude aflatoxins from various media.

A. flavus strain	Culture media	Areas on Fluodensitometer measurement				Relative composition			
		B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
M-93	Rice	428	17	276	4	100	21	61	1
M-93	Wheat	420	12	171	1	100	18	57	Tr.
M-93	Peanut	581	53	228	49	100	28	57	31
M-93	Soya bean	50	--	480	24	100	--	105	40
M-93	Tomato	60	--	326	10	100	--	97	29
M-93	Grape	420	--	72	11	100	--	47	--
M-3	Wheat	819	18	456	8	100	20	61	2
M-3	Soya bean	125	--	532	42	100	--	87	39
15517	Rice	399	40	80	12	100	28	49	22
15517	Wheat	390	21	195	8	100	22	57	2
15517	Peanut	452	18	286	32	100	21	62	28
15517	YES	308	12	63	4	100	20	48	1

Fractionation and Isolation of Aflatoxin

Thin-layer chromatography has been used by many investigators for the fractionation and isolation of the four aflatoxins; however, preparative thin-layer techniques are time-consuming, require a great number of plates, and the yields are small. Frequently, the separated components decompose on the plates before they can be removed from the stationary phase. Because of these factors column chromatography was found to be a useful tool for the preparation and isolation of aflatoxins. This procedure may be considered a fractional chromatographic method, and by repeated column chromatography and combination of like fractions, relatively large quantities of pure aflatoxins may be prepared. Fairly large amounts of crude toxin were required, usually about 100 mg, and the procedure was found to be ineffective if less than 5 mg of aflatoxin B_1 was available in the mixture. The richer aflatoxin G_1 fractions usually contained some aflatoxin B_2 which could be eliminated by repeated crystallization of the G_1. Since it is difficult to obtain quantities of aflatoxins B_2 and G_2 by column separation of the crude aflatoxin extract, aflatoxins B_2 and G_2 can be most conveniently prepared by catalytic hydrogenation of aflatoxins B_1 and G_1.

Colored impurities were removed by repeated crystallizations. In our experimental procedure, chloroform:methanol (1:5 v/v) mixture

was used for the first two crystallizations with this ratio being changed to 1:3 v/v beginning with the third crystallization.

Qualitative Assay of the Prepared Pure Aflatoxin

Aflatoxin B_1 crystals were found, by microscopic examination, to be colorless prisms, while the aflatoxin G_1 crystals appeared as colorless needles. The decomposition points of the prepared crystals were measured as 268°C for aflatoxin B_1 and 253°C for aflatoxin G_1.

Both visual and fluordensitometric measurements of the thin-layer plates indicated that no fluorescent impurities were present. The ultraviolet spectra of these compounds in ethanol agree with the data of Asao et al. (8).

Effect of Alkali Treatment on Aflatoxin

It was found that the stability of aflatoxins toward base was affected by the solvent system, type of base, time of exposure and base concentration. When treated with strong alkali (NaOH), solutions of aflatoxins B_1 and G_1 in ethanol and methanol were unstable even if they were exposed for short periods of time.

The mixture of aflatoxin B_1 and sodium hydroxide in ethanol exhibited a yellow color, and the solutions showed a dark green fluorescence when irradiated with ultraviolet light. The ultraviolet spectrum of these mixtures was no longer the same as that of aflatoxin B_1.

On the thin-layer plate, the ethanolic mixture of aflatoxin B_1 and sodium hydroxide gave a blue fluorescent streak. The fluorescent intensity decreased as the NaOH concentration was increased. Similar results were observed with an ethanolic mixture of aflatoxin G_1 and sodium hydroxide except that the fluorescent streak was green instead of blue in color. No regeneration of the fluorescence of these mixtures was observed on neutralization.

The mixture of aqueous sodium hydroxide and methanolic aflatoxin B_1 had a tailing blue fluorescent spot on thin-layer chromatographic plates. The R_f values of these spots were lower than that of the aflatoxin B_1 standard and the fluorescent intensity of these spots was also weaker.

In contrast to the degradative changes caused by exposure to alcoholic strong bases, NH_4OH had little effect. Ethanolic NH_4OH at concentrations below 1.0 N left the ultraviolet spectrum of aflatoxin B_1 unchanged.

When aflatoxin G_1 was treated with alcoholic NH_4OH a shift in the absorption peak from 363 mμ to 355 mμ was observed. Partial regeneration was obtained after neutralization with acid. By thin layer chromatography, the neutralized mixture had the same green fluorescent spot as the aflatoxin G_1 standard.

The most important point concerning the treatment of aflatoxin with base is that aflatoxins are quite stable if treated in chloroform

solution with aqueous base. Chloroform is the usual solvent used, both in the preparation and in the determination of these toxins. The aflatoxin-chloroform solution was treated by adding equal volumes of aqueous base at different concentration levels. The mixture was shaken with a shaker for 1 min. The results for both the strong and the weak base treatments were similar. At lower base concentration, aflatoxin B_1 was unaffected, and only a small amount of destruction of aflatoxin G_1 was observed. The basic reason for the application of alkali in the preparation and determination of aflatoxin is removal of impurities from the chloroform solution.

A typical stability curve for the treatment of an aflatoxin-chloroform solution with aqueous alkali is shown in Figure 3. Treatment time with aqueous alkali had no effect on aflatoxin B_1 in a chloroform solution. For aflatoxin G_1, a very small effect after prolonged contact was observed (Figure 4). A study of Figures 3 and 4 indicated that a relatively small amount of aflatoxin was destroyed when aqueous base with a concentration less than 0.25 N was used in the treatment of aflatoxin in a chloroform solution.

Impurities in the Crude Aflatoxin Extract and Their Removal

A yellow impurity which was diethyl ether soluble was found in the chloroform extract of the A. flavus culture. The silicic acid

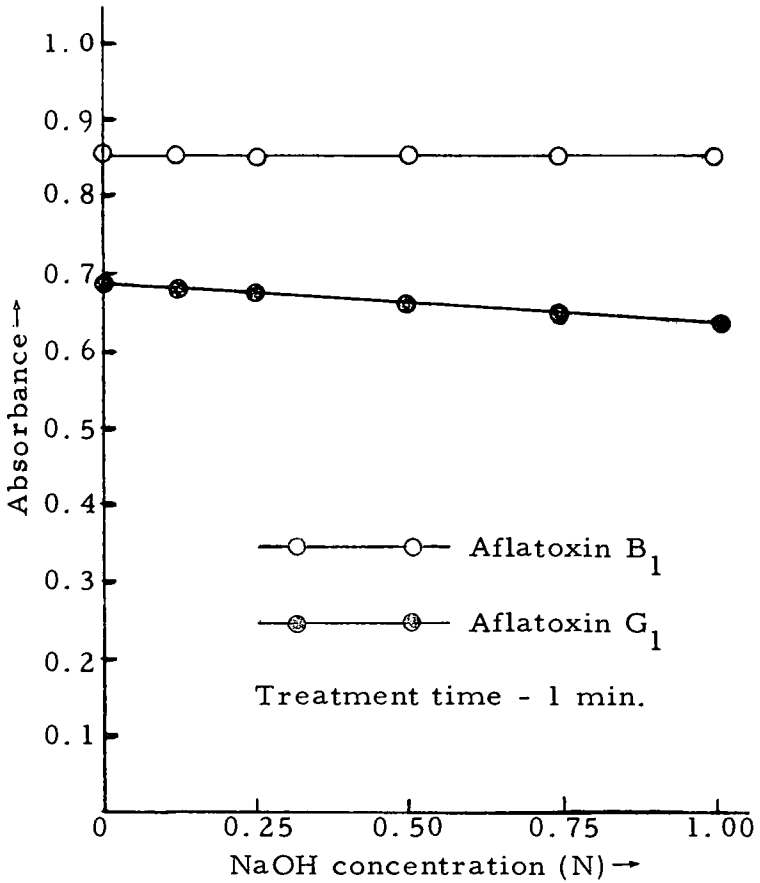


Figure 3. Effect of aqueous NaOH on the stability of aflatoxins in chloroform.

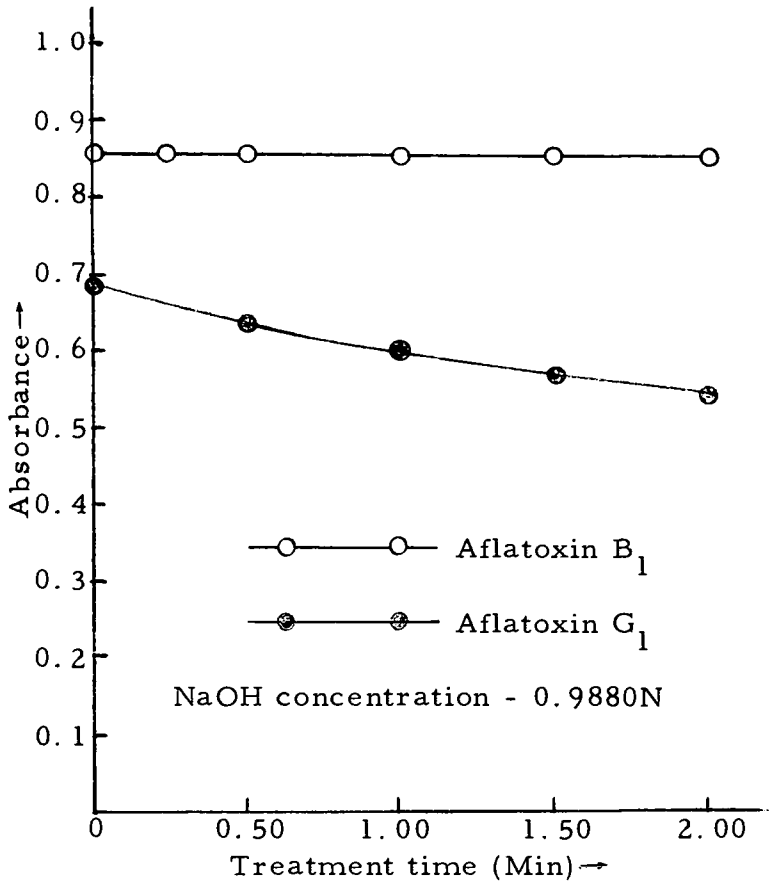


Figure 4. The stability of aflatoxin solutions in chloroform after exposure to alkali.

column purification or hexane precipitation procedure removed part of this material. The prepared crude aflatoxin, as shown in Figure 5, was a yellow extract with several unknown fluorescent components.

These low R_f value compounds are often seen and vary in quantity depending on the substrate and the strain of A. flavus. These low R_f value compounds are believed to be metabolites of A. flavus, and may be designated as unknown compounds (X), nothing is reported about their chemical nature or biological properties.

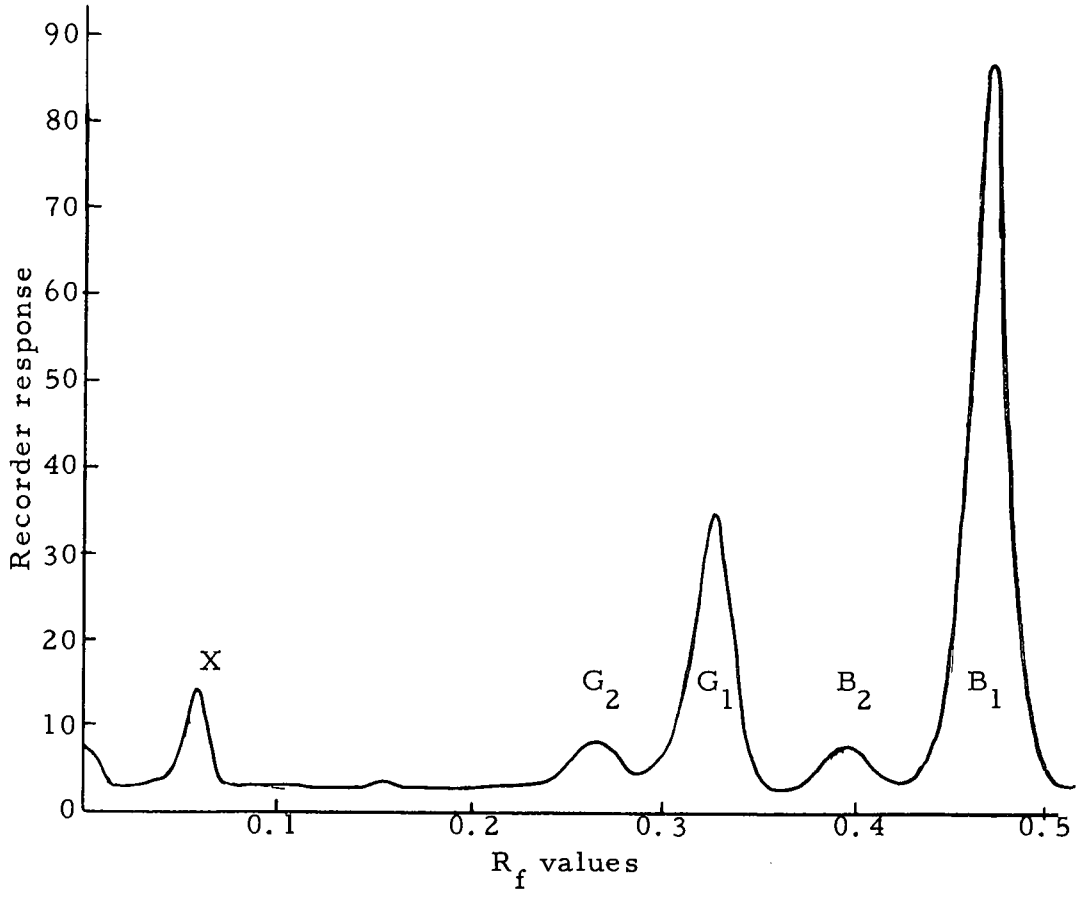


Figure 5. Fluorodensitometric scan of crude aflatoxin from an M-93 crushed peanut culture.

Several crude aflatoxin extracts obtained from various culture media were used to verify the effectiveness of the base treatment in removing these colored compounds. An absorption maximum at 440 mμ has been found to characterize these materials. The spectra of the controlled samples were compared with those of the base-treated samples (Table 3). It was found that the base treatment was effective in removing the colored impurities from the crude chloroform aflatoxin extract.

Table 3. The effect of the alkali treatment of crude aflatoxin extract on the removal of colored compounds.*

Crude aflatoxin extract		Absorbance at 440 mμ	
Media	Mold strain	Before treatment	After treatment
Rice	M-93	0.393	0.010
Wheat	M-93	0.415	0.026
Tomatoes	M-93	0.085	0.015
Grapes	M-93	0.098	0.005
Rice	M-3	0.705	0.095
Rice	15517	0.165	0.063

* Equal volume of extract and 0.25 N NaOH.

By a second treatment with fresh aqueous base, the absorbance at 440 mμ of the treated samples could be lowered still further. For some sample extracts, the presence of diethyl ether soluble substances caused formation of an emulsion during the base treatment.

Therefore, it was desirable to remove these substances by column or hexane precipitation prior to treatment. Thin-layer chromatographic analysis indicated that there was no loss of aflatoxin in the treated samples.

Removal of the Low R_f Value Unknown Compounds (X) From a Wheat Culture

Usually the concentration of X compounds is quite low in aflatoxin extracts; however, an M-93 crushed wheat culture extract was found to contain a very high amount of the X component shown in Figure 5. This crude aflatoxin was isolated by the acetone extraction method, and this sample, the area of the X component by fluorodensitometry measurement, was found to be greater than that of aflatoxin G_1. In order to test the effect of the NaOH solution on the aflatoxin mixture, the concentration of alkali was varied from 0.1% to 40%. The results of this treatment are shown in Figure 6. It was found that the X component could easily be removed by treatment with a dilute NaOH solution without the destruction of the aflatoxin.

Modification of the Pons and Goldblatt Procedure by the Alkali Treatment

In the Pons and Goldblatt (42) method, the 70% aqueous acetone extract of the sample, after addition of 60 ml of distilled water and 20 ml of lead acetate solution is boiled to remove 60 ml of the solvent.

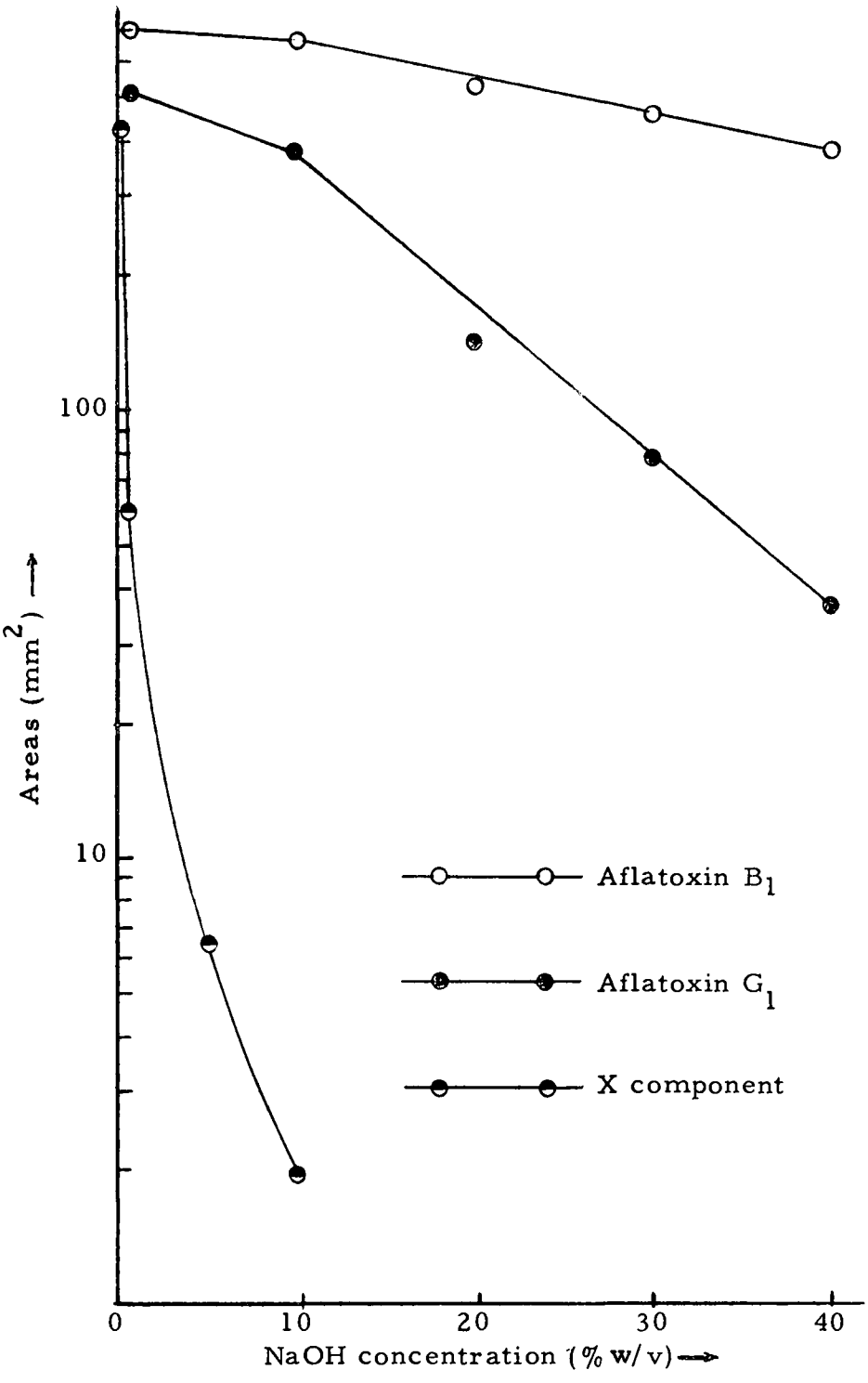


Figure 6. NaOH treatment of the crude M-93--crushed wheat crude aflatoxin in chloroform solution.

Evaporation was found time-consuming and a foaming problem was frequently observed if rotary evaporation was attempted in the case of cottonseed sample extracts. Instead of evaporation, chloroform extraction of the mixture can be performed after further dilution of the filtrate with distilled water. This modification will not alter the quality of the chloroform extract and shortens the procedure and conveniently prepares it for the alkali treatment step.

Shaking the diluted final chloroform extract very gently with a low concentration of base solution, such as 0.25 N NaOH, gave a final extract with fewer pigments and less residual solids. For example, by the Pons-Goldblatt procedure, the Ralston E cottonseed meal final extract weighed 22 mg. Alkali treatment reduced this to 2.3 mg. The weight of the original sample was 21 gm. In toxic peanut meal, sample extracts were reduced from 40 mg to less than 4 mg. A comparison between the fluorodensitometric scans of the treated toxic peanut meal extract and that of the control sample, are shown in Figure 7. For some cottonseed meal extracts, a stable emulsion was obtained during the treatment. In this case, a silicic acid column purification prior to the alkali treatment was performed.

For some meals, such as Proflo cottonseed flour, a blue fluorescent spot with an R_f value between those of aflatoxins B_1 and G_1 was found in the extract. This unknown compound can easily be mistaken for aflatoxin B_2. When this extract is treated with a very

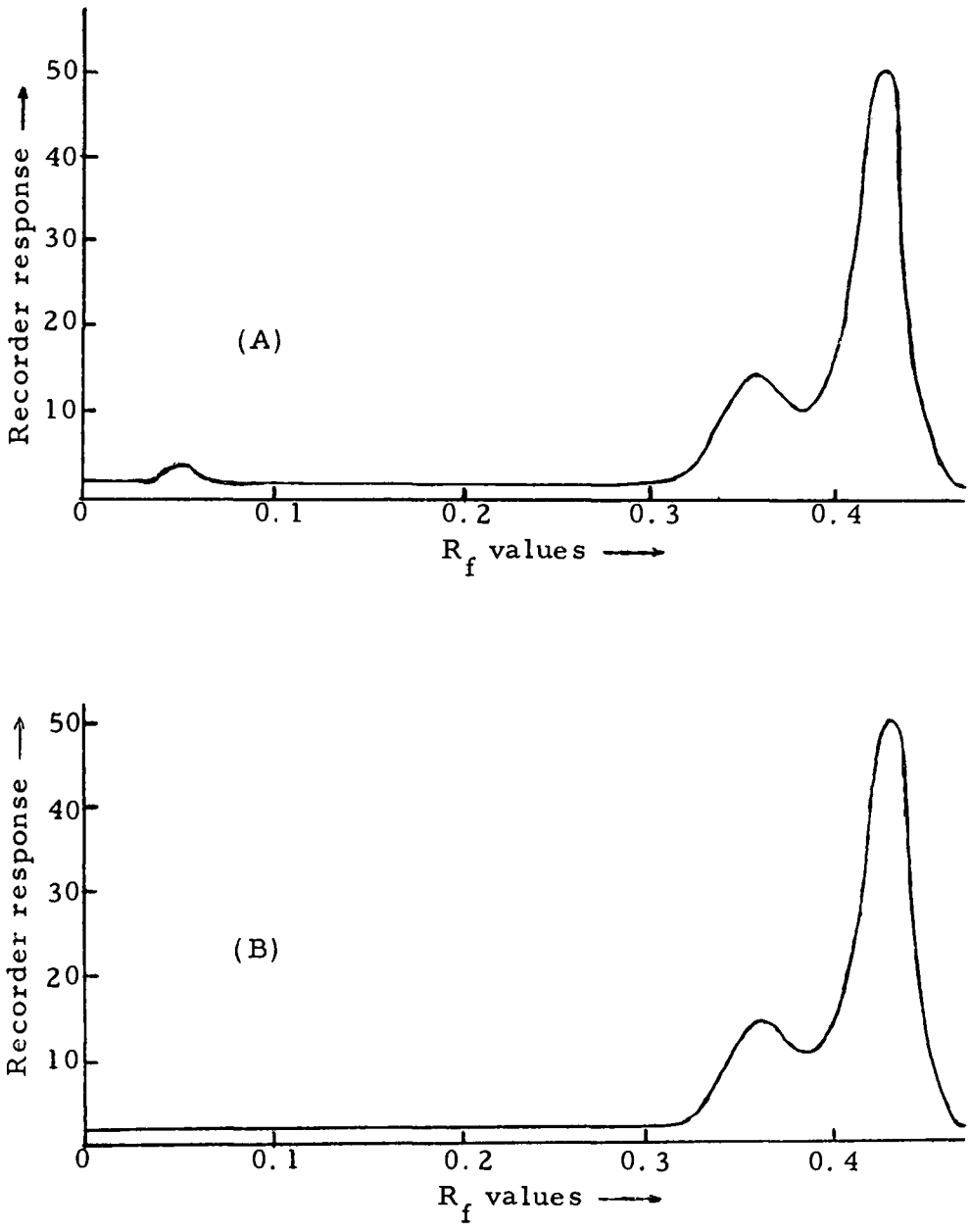


Figure 7. The fluorodensitometric scan of the toxic peanut meal extract: (A) Before alkali treatment; (B) After alkali treatment.

low concentration of aqueous base, this blue spot can be removed completely and separated from B_2.

The Photodegradation of Aflatoxin

An induction period was observed during photodegradation of aflatoxin. Once the reaction had reached a certain level, the rate increased sharply. As shown in Figures 8 and 9, the photodegradation of aflatoxin appears to be a two-step reaction. The aflatoxin was degraded to low R_f value compounds as indicated by thin-layer chromatographic analysis. These compounds were further converted to the nonfluorescent products which were undetectable on the thin-layer plate. Generally speaking, after aflatoxin had been exposed to light for three days, only a small photo-induced peak was found by fluorodensitometry. From our observations, it seems that crude aflatoxin or the aflatoxin mixture is more stable to light illumination than the purified single components.

It is interesting to observe that aflatoxin G_1 is much more stable than aflatoxin B_1 in both the chloroform and the ethanol solutions during illumination. The cause of this difference is unknown, but the presence of a second lactone ring in the aflatoxin G_1 structure seems to be a reasonable explanation.

A chloroform solution of aflatoxin is very unstable on long term exposure to light. Chloroform can be oxidized by air and sunlight to

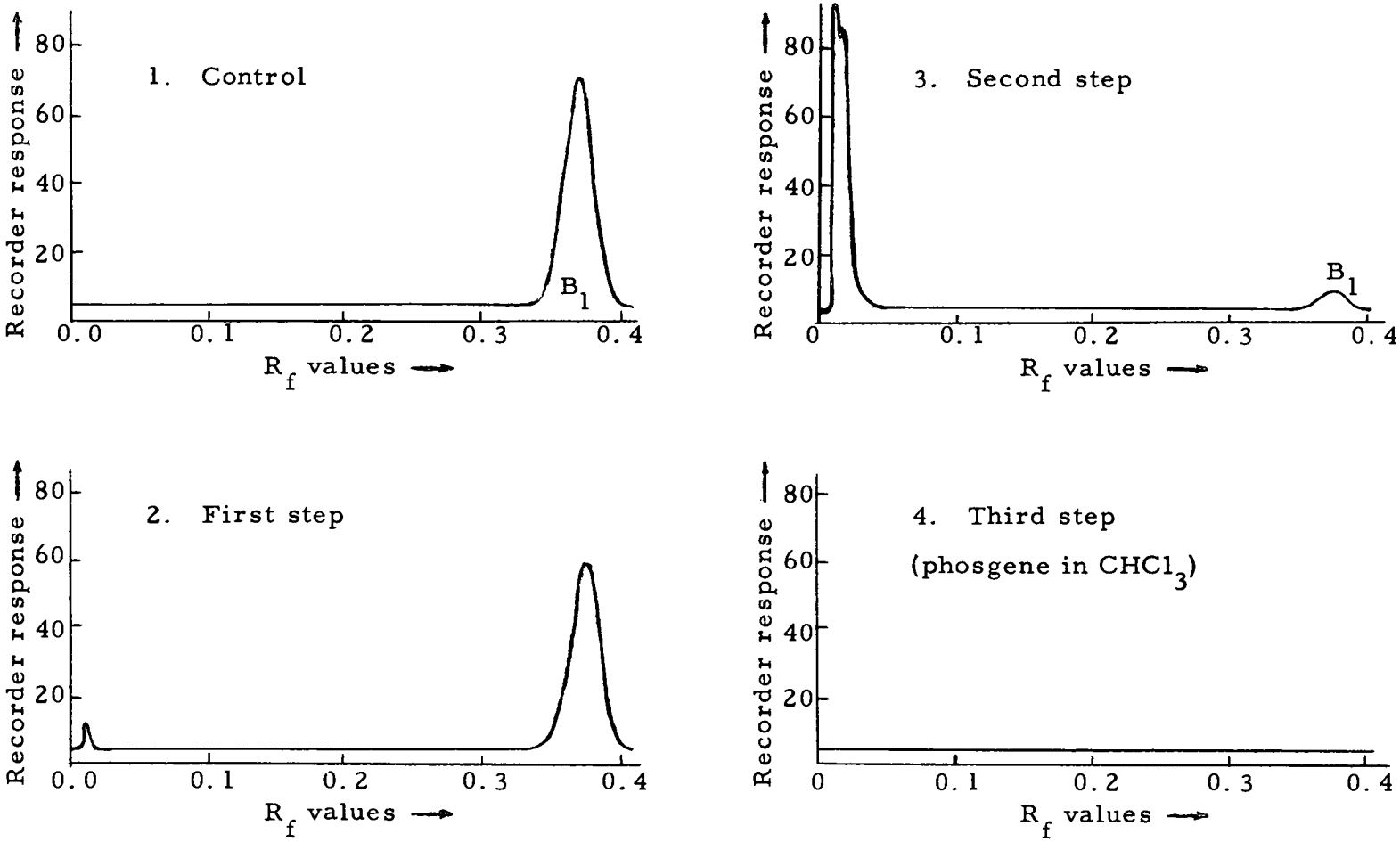


Figure 8. The photodegradation of aflatoxin B_1 in chloroform solution.

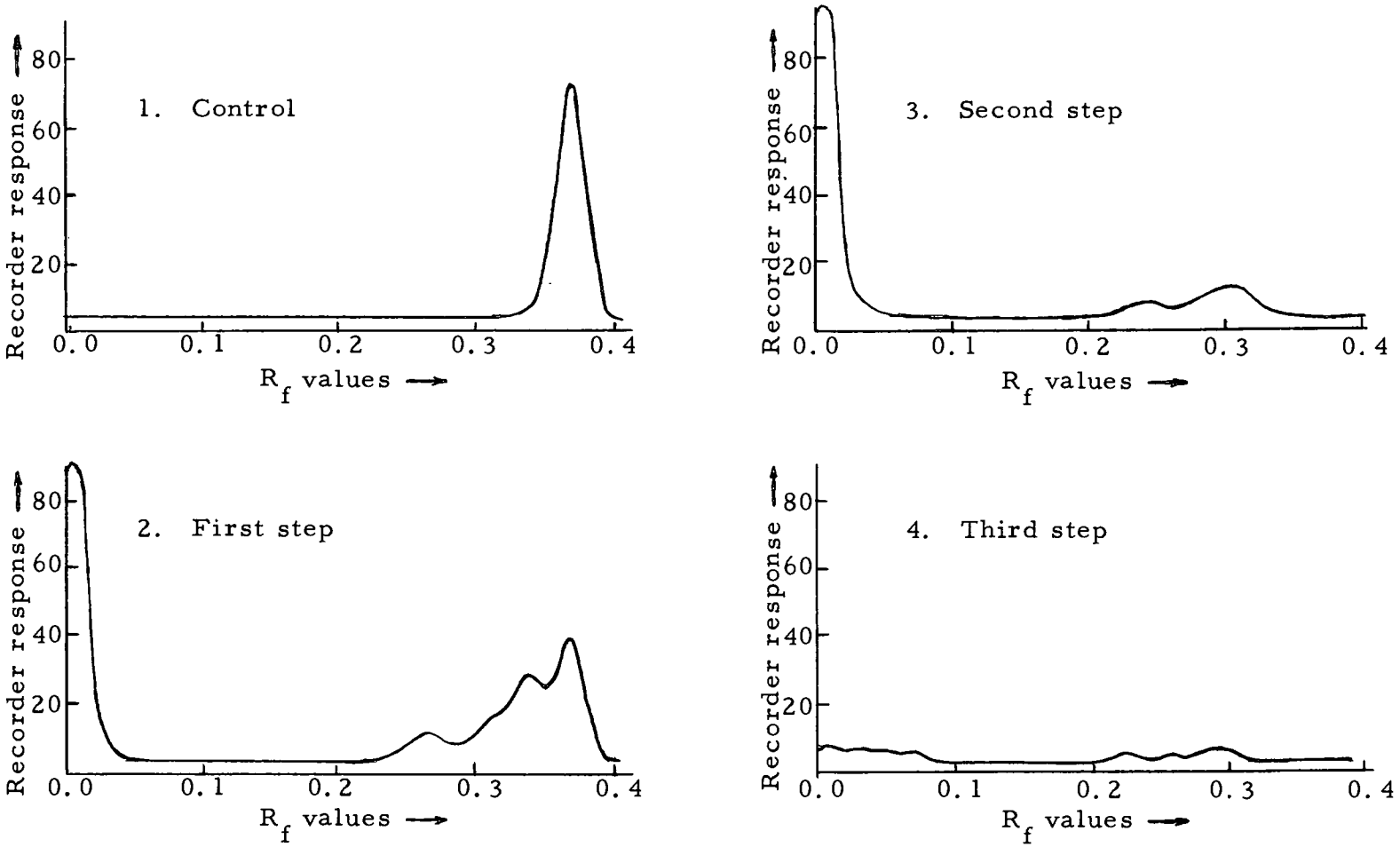


Figure 9. The photodegradation of aflatoxin B_1 in ethanol solution.

phosgene and it was believed that aflatoxin was destroyed by phosgene formed in chloroform solution. The degradation of aflatoxin in ethanol follows a different course from that of aflatoxin in a chloroform solution as shown in Figure 9.

Removal and Isolation of the Photodegraded Aflatoxin by Alkali Treatment

It was found by thin-layer chromatography that the pure prepared aflatoxin solution was contaminated by trace impurities having low R_f values after frequent usage for several weeks. These low R_f value compounds have also been found in the aflatoxin standard obtained from the Southern Utilization Research Laboratory.

Since there was no detectable difference between the ultraviolet spectra of the samples containing trace degraded compounds and those of the pure ones, the degraded component could not be detected by using the ultraviolet spectra. This component is present on the thin-layer plate as a tiny blue fluorescent spot.

From the ultraviolet spectra and the thin-layer chromatographic data, it was shown that this component can be removed by a very mild base treatment. As shown in Figure 10, the impurity may be responsible for as much as 9% of the total ultraviolet absorption value, which would result in an erroneously high value for the calculated concentration of aflatoxin.

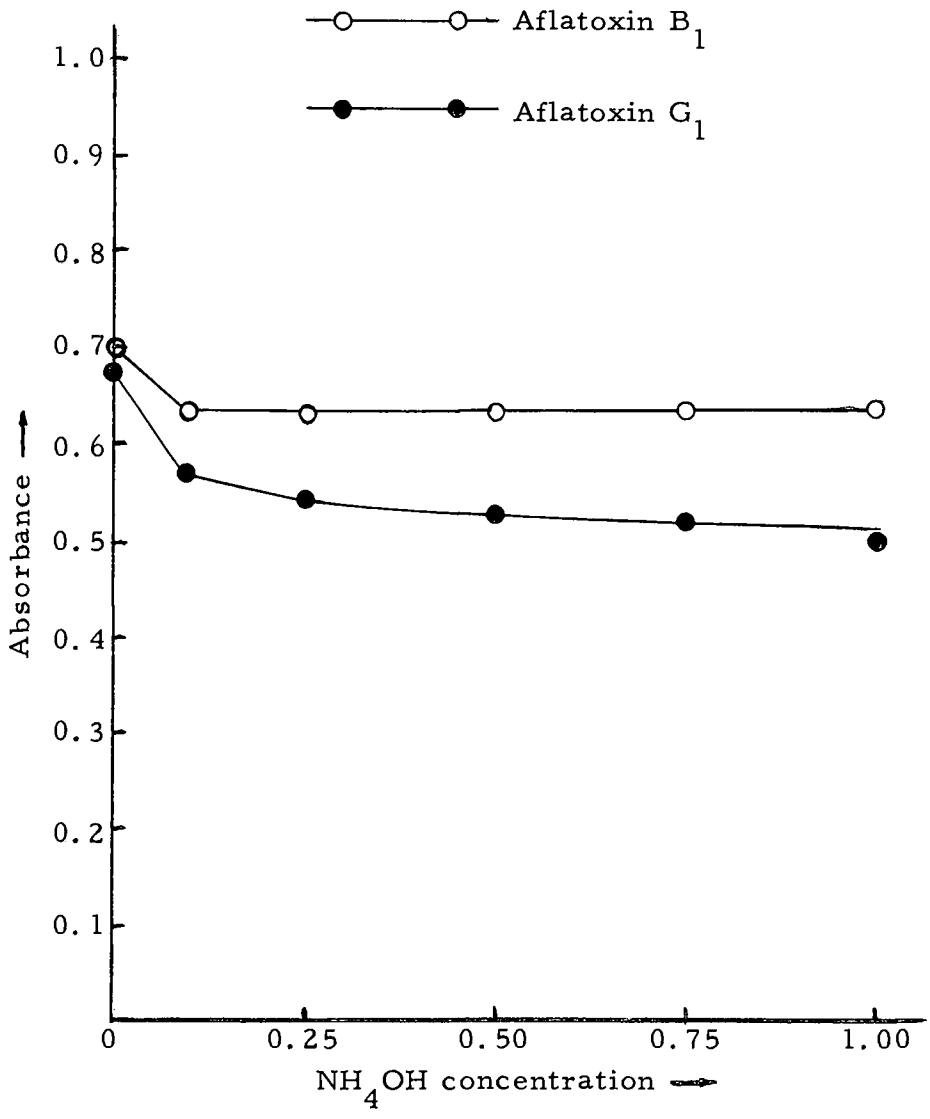


Figure 10. Effect of alkali treatment in the removal of the photodegraded compounds from aflatoxin chloroform solution.

It is possible to isolate the photodegraded compounds by use of a modified alkali treatment. Chloroform solutions were treated with mild NH_4OH and the alkali solutions collected and concentrated on a steam bath. Ammonia was released by heating, leaving the degraded products. Fluorodensitometric scans of the thin-layer plate of the concentrates are shown in Figure 11.

The photodegraded compounds of aflatoxin B_1 continued to have a blue fluorescence under ultraviolet light on the thin-layer plate. A yellowish-green fluorescence was observed for photodegraded aflatoxin G_1.

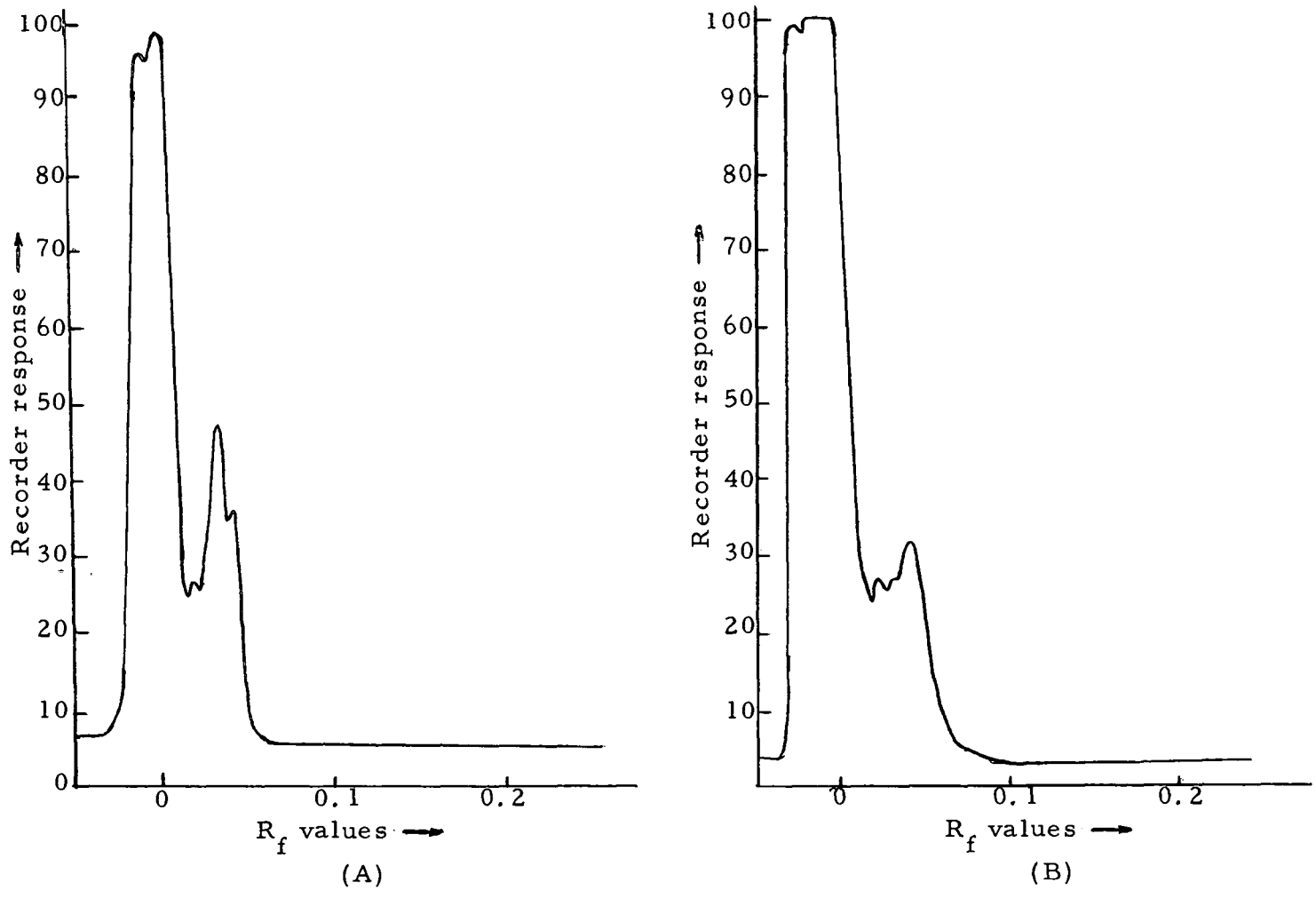


Figure 11. Development scans of the aflatoxin degraded products from NH_4OH aqueous concentrate: (A) from aflatoxin G_1; (B) from aflatoxin B_1.

V. SUMMARY AND CONCLUSIONS

A procedure for the biological preparation, purification and determination of aflatoxins was developed which was an alkali treatment to effect removal of pigments and a variety of interfering substance including photodegraded products of aflatoxins. The stability of aflatoxins toward alkali was influenced by the solvent system, type of alkali, time of exposure, and alkali concentration. Solutions of aflatoxins B_1 and G_1 in ethanol and methanol were quite labile when treated with strong base even for a short period of time. The aflatoxins were quite stable if treated in a chloroform solution with aqueous base. At lower base concentrations, aflatoxin B_1 was not affected and the destruction of aflatoxin G_1 was negligible. When the chloroform extract containing aflatoxin was treated very gently with a low concentration of base solution, such as 0.25 N NaOH, the resultant sample contained much less residual material. The pigment, low R_f value unknown compounds, and total solids of the final extract were greatly reduced. This should permit a greater specificity and accuracy in the final determination of aflatoxin.

Biological preparation of aflatoxin, rice, wheat, soya bean, peanut and YES media was found to give a high yield of aflatoxin. It was found that Aspergillus flavus can grow and produce toxin in fresh vegetables and fruits such as tomatoes and grapes.

The photodegradation of aflatoxin appeared to be a two-step reaction. After an induction period, the aflatoxin was degraded to yield low R_f value compounds, which in turn were further altered to non-fluorescent products which were undetectable on thin-layer plates. Aflatoxin G_1 was much more stable than aflatoxin B_1 both in the chloroform and in the ethanol solution during light irradiation. The mechanism of this difference is not clear. However, the function of the other lactone ring in the aflatoxin G_1 structure seems to be responsible.

The low R_f value fluorescent compounds could not be detected by ultraviolet spectrophotometry, but could be removed by a very mild base treatment. This procedure is easily applied and found to be effective in removal of interfering and degradative products associated with aflatoxin.

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