Absence of Aflatoxin from Refined Vegetable Oils[1]

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Abstract

The present investigation is the first definitive study of the fate of the aflatoxins in vegetable oils undergoing processing. Crude oils, obtained by solvent extraction or by hydraulic pressing of ground moldy peanuts (not suitable for human consumption), contained only small fractions of the aflatoxin originally present in the peanuts; the meals retained the bulk of the aflatoxin. Conventional alkali refining and washing of the oils reduced aflatoxin content to a range of 10 to 14 ppb. The subsequent bleaching operation essentially eliminated aflatoxin from the oils; the concentrations were now less than 1 ppb. The above results were confirmed using corn oils obtained from corn germ deliberately contaminated in the laboratory with *Aspergillus flavus*. The nonfluorescing forms of aflatoxins, capable of being produced during the alkali refining operations, are also absent from the refined vegetable oils; these aflatoxin derivatives are readily converted to their original form on acidification and thereby measurable by fluorescence, if present.

Introduction

SINCE THE DISCOVERY and isolation of aflatoxin by investigators at the British Tropical Products Institute in 1961, the subject of mycotoxins in foods and feeds has experienced a renewal of scientific investigation. Aflatoxin—as the name suggests—was found in these earlier studies (1) to be the toxic metabolite of a fungus identified as *Aspergillus flavus* Link ex Fries; the toxin was isolated from a sample of peanut meal that had been derived from moldy peanuts.

Aflatoxin is actually a complex (2), consisting of four compounds identified as B_1, B_2, G_1 and G_2. Under ultraviolet light the B compounds yield a blue fluorescence, while the G compounds exhibit a green fluorescence; this property of fluorescence has become the basis for the physicochemical method for quantitatively measuring the aflatoxins.

The B_2 and G_2 compounds are reduced products of the primary B_1 and G_1 compounds (3) and this slight change in chemical structures noticeably lowers the toxicity of the respective parent compounds (4). Most investigators are in agreement with Coomes et al. (5) in concluding that measurement of the B_1 compound (the principal and most toxic component) is normally adequate to define the magnitude of toxicity of a given sample with respect to total aflatoxin toxicity. All the aflatoxin compounds are relatively stable to heat and thus are resistant to destruction in the heat processing of foods (6).

Since fungi are common contaminants of practically every agricultural commodity, grains will become moldy under favorable conditions of temperature and humidity exposure. A variety of agricultural commodities, such as barley, rice, wheat, corn, oats, soybeans, cottonseed, peanuts, rye and buckwheat, have been shown to be capable of supporting the growth of *Aspergillus flavus* in varying degrees with resultant aflatoxin production (7,8). However, since aflatoxin was initially isolated from peanut meal, the bulk of the early work was based upon moldy peanuts (9–15).

In these published and oral reports, just cited in the preceding paragraph, statements have appeared to the effect that peanut oil which is derived from contaminated products should be essentially free of aflatoxin and that alkali refining should render such oil free of trace amounts of aflatoxin. However, all these reports (9–15) fail to give any laboratory or factual evidence to support the statement that refining eliminates aflatoxin from the oils derived from contaminated raw materials. Also, it must be emphasized that, since these earlier statements were made, the sensitivity of the analytical test has increased by a factor of at least 35 times. Thus, the sensitivity of the earlier methods for aflatoxin was 0.1 ppm (16); aflatoxin (when present) can be measured today *in routine work* in amount as little as 3 ppb, or 3 μg/kg. It has become necessary during the last few years to increase the sensitivity of the analytical procedure as new information revealed the high toxicity of the aflatoxins. Hence, what was regarded as a negligible or nonmeasurable amount in the period of 1961–63 may not be so regarded today.

It is apparent from the facts presented above, that there has been a need for an objective and definitive investigation of the fate of aflatoxin in the refining of vegetable oils for human consumption; the present report summarizes the results of such an investigation.

Materials and Methods

Source of Crude Peanut Oil

Since the aflatoxin content of peanuts used for human consumption in the United States ranges from zero to insignificant amounts, a special peanut sample was utilized to provide the test oils for investigation. The sample was obtained in the course of experimental work conducted on various methods of peanut harvesting. Three peanut types, comprising 49% oil stock peanuts, 3% damaged rejects and 48% immature (pee wee) peanuts, were composited to form the starting sample. Approximately 8 kg of the composited sample were thoroughly milled, blended, and an aliquot of the blended sample tested for aflatoxin. This was found to contain 5.5 ppm of aflatoxin B_1, a very high level of contamination. Hereafter, reference will be made to aflatoxin concentration in parts per billion, in keeping with the preferred method for expressing the concentration of aflatoxin.

Source of Crude Corn Oils

Since it was not possible to obtain naturally contaminated corn or corn germ having a significant or measurable level of aflatoxin, a sample of corn germ containing 35% residual moisture was inoculated with spores of a toxin producing strian of *Aspergillus flavus*. After an incubation period of 6 days at 30C, the germ was dried and one portion

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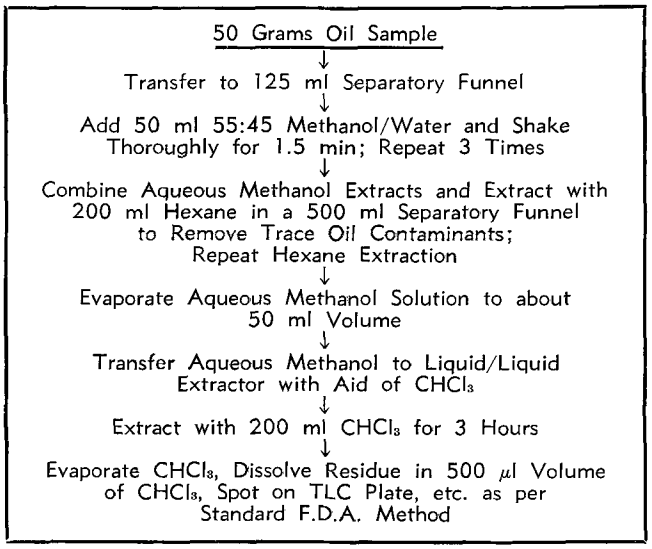

FIG. 1. Physiocochemical method for aflatoxin analysis of oil.

extracted with hexane and another portion extracted with chloroform to provide two samples of crude corn oil. Pertinent to this study was the selection of solvents for removal of the oil, since aflatoxin is regarded to be practically insoluble in hexane and soluble in chloroform.

Analytical Methods for Assay of Aflatoxins

The method applied to the starting raw materials was the fluorometric procedure that had been developed in collaborative studies involving both industry and the U.S. Food and Drug Administration (17). However, oils required special handling, as summarized in Figure 1.

The method involves selective extraction of the aflatoxin by a methanol-water solution. Emulsions may form during this extraction, but such interference can be eliminated by adding a small amount (viz. 0.3 g) of sodium chloride to the aqueous methanol solution. The aflatoxin, free of interfering substances, is transferred to chloroform solvent and spotted on a silica-gel thin-layer chromatographic plate, as outlined in the standardized method (17). Recovery tests, involving the analyses of vegetable oils deliberately contaminated with aflatoxin to the level of 100 ppb of the B_1 component, gave satisfactory results; 90% of the added aflatoxin was recoverable utilizing the test method developed for this study. As little as 1 ppb of aflatoxin B_1 in a test oil can be detected by this method; the size of the test sample is 5 times that called for in the Standard F.D.A. Method.

Some emulsion problems were encountered in the analyses of some *crude* oils, *viz.* the crude corn oils of very high free fatty acid content (the high values were undoubtedly due to lipolysis during mold propagation and metabolism). These emulsion problems were solved by adding hexane to the methanol/water solution in amount equal to the water present, using three times as much methanol/water solution (450 ml) now as a one-time solvent for the aflatoxin in place of the three extractions previously employed (see Fig. 1). Here also, the sodium chloride addition to the methanol/water solution reduced emulsion problems.

In the analyses of aflatoxin B_1 in pure solutions made alkaline or acid, the methanol/water solution was adjusted with added water to change the ratio from 55:45 to 55:165. These test solutions were each extracted twice in a separatory funnel with an equal volume of $CHCl_3$. The $CHCl_3$ solution was then evaporated to 500 µl and spotted on the thin-layer chromatographic plates.

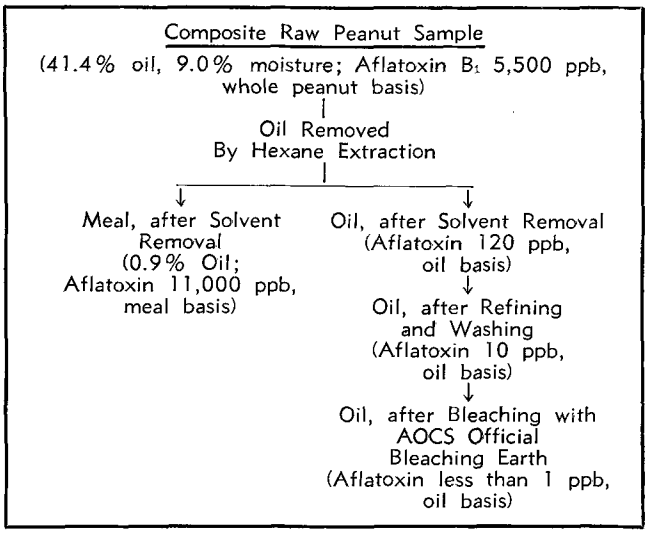

FIG. 2. Summary of results on extracted peanut oil.

Experimentation

Results with Peanut Oils

Approximately 2 kg of the ground peanut samples were defatted by means of solvent extraction with hexane (Skellysolve B). The hexane extraction produced a crude oil containing 120 ppb of aflatoxin (see Fig. 2). Most of the aflatoxin remained in the extracted meal and refining and bleaching eliminated all measurable traces of aflatoxin from the separated oil.

Figure 3 presents the results obtained with the oil following hydraulic (Carver) pressing of 3.5 kg of the same ground peanut sample. The crude oil in this case was found to be much higher in aflatoxin content than that obtained following the solvent extraction. The meal again contained the bulk of the aflatoxin. Undoubtedly, the high value of 812 ppb, found in this crude oil, was due to occlusion as some solids carried over into the oil by the pressing method.

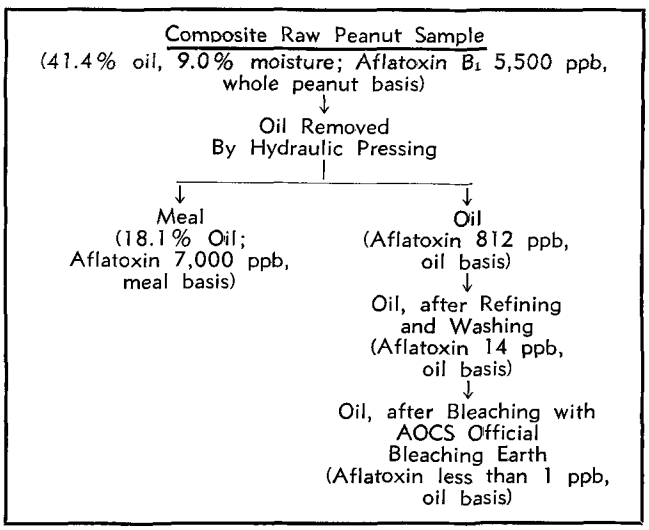

FIG. 3. Summary of results on pressed peanut oil.

TABLE I

Summary of Refining and Bleaching of Crude Peanut Oils

Processing of oil	Oil from solvent extraction	Oil from hydraulic pressing
Refining		
Quantity of oil refined, g	500	500
Free fatty acids in oil, %	4.0	4.3
NaOH required for neutralization, g	2.84	3.05
NaOH added in excess, g	0.75	0.75
Total NaOH added, g	3.59	3.80
16° Baume' caustic used, g	32.45	34.40
Oil recovered, g	434.9	450.9
Refining loss, %	13.0	9.9
Water washing (two times)		
Quantity of oil washed,	320	320
Water used, g	32	32
Bleaching		
Quantity of oil bleached, g	60	60
Oil temperature, °C	90	90
Bleaching earth, g	1.8	1.8
Bleaching time, min.	5	5

Here again, refining and bleaching eliminated all measurable traces of aflatoxin from the peanut oil.

The details of the overall processing steps in refining the oils are shown in Table I. The crude oils obtained by the two different methods from the ground peanut sample were refined using the middle cup procedure, essentially as outlined in the AOCS standard refining method; the amount of excess NaOH used in our test more closely duplicated plant practice and was equivalent to 0.15% based on the weight of the oil. After the oil had been refined and water washed, it was bleached as shown in Table I.

Results with Corn Oils

Figure 4 presents a summary of the processing of the corn germ to provide refined corn oils. Following the hexane extraction, the crude oil was found to contain only 8 ppb of aflatoxin B_1, while the oil recovered from the chloroform extract had 135 ppb of the B_1 component present. The spent corn germs retained the bulk of the aflatoxin. Both of the oils were refined according to conventional refining procedures simulating plant practices and then bleached with an acid earth ("Special Filtrol," the Filtrol Corporation, Los Angeles, Calif.). After such processing, both oil samples were analyzed and found to contain less than 1 ppb of aflatoxin B_1.

Data on the refining of the oils are given in Table II.

Re' Obscure Forms of Aflatoxin

Even though fluorescence under ultraviolet light is the basis of all physicochemical tests for measuring aflatoxin content, some investigators might challenge

TABLE II

Summary of Refining and Bleaching of Crude Corn Oils

Processing of oil	Oil from hexane extraction	Oil from $CHCl_3$ extraction
Refining		
Quantity of oil refined, g	500	500
Free fatty acids in oil, %	15.3	15.6
NaOH required for neutralization, g	10.90	11.15
NaOH added in excess, g	1.5	1.5
Total NaOH added, g	12.40	12.65
16° Baume' caustic used, g	112.0	114.0
Oil recovered, g	300	312
Refining loss, %	40.0	37.6
Water washing (two times)		
Quantity of oil washed, g	200	200
Water used, g	20	20
Bleaching[a]		
Quantity of oil bleached, g	100	100
Oil temperature, °C	82	82
Bleaching earth, g	0.6	0.6
Bleaching time, min.	30	30

[a] Note: Refined and washed oils were heated to 71 C and treated with 1% of a diatomaceous silica ("Hyflo Super-Cel," Johns-Manville Sales Corporation, New York, N.Y.) to remove trace turbidity before bleaching.

the conclusion that aflatoxin is eliminated as fluorescence decreases. Mild alkaline treatments at room temperature have been shown to decrease the fluorescence of aflatoxin B_1 but without a concomitant decrease in toxicity in the duckling tests (18). Figure 5 shows that the lactone ring structure of aflatoxin B_1, essential for fluorescence under ultraviolet light, is hydrolyzed in the presence of sodium hydroxide in aqueous solution (19). The sodium salt produced from this reaction is nonfluorescent. However, the original molecule and fluorescence is restored, as will be shown hereinafter, by treating the sodium salt with acid. Hence, it could be argued that the fluorescent free oils, following the tests for aflatoxin content in the present study, may have still contained the toxic sodium derivative. Even though there is little likelihood of such a water-soluble derivative being retained in the oil following the alkali refining and subsequent aqueous washing steps, this possibility was investigated.

Figure 6 is a photograph showing the loss of fluorescence on treating an aqueous methanol solution of 800 ppb of aflatoxin B_1 with sodium hydroxide in aqueous solution and the restoration of fluorescence following acidification with hydrochloric acid. The solution on the left, having the stronger fluorescence, was 3 ml of the initial aqueous methanol solution containing 800 ppb of the B_1 component. The beaker in the center contained 3 ml of the aqueous methanol solution to which had been added 0.3 ml of 1N NaOH solution at room temperature; this alkali treatment rapidly destroyed the fluorescence. The beaker

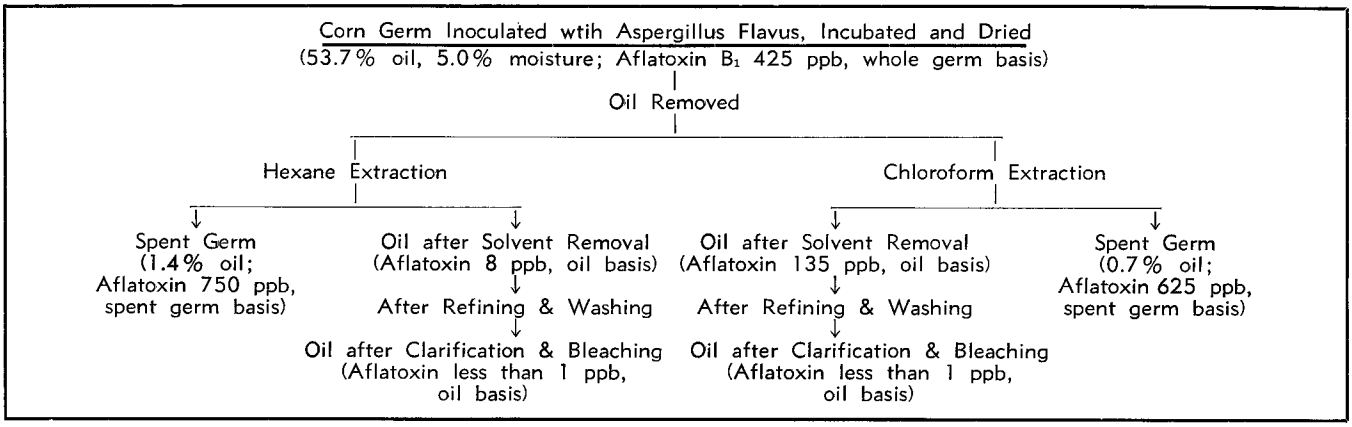

FIG. 4. Summary of results on extracted corn oil.

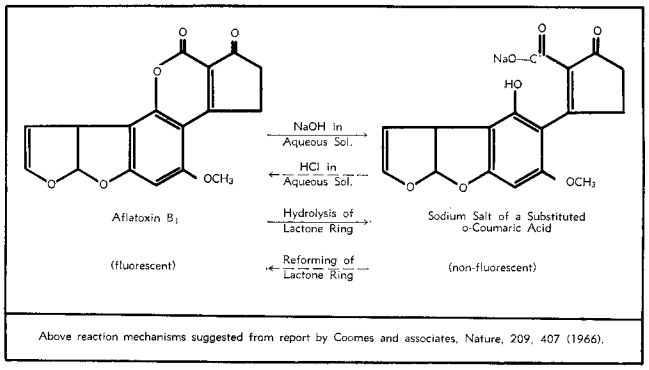

FIG. 5. Changes in the structural formula of aflatoxin B_1 on alkalinization and acidification.

on the right contained the same solution as the one in the center, but now acidified at room temperature by the addition of 0.4 ml of 1N HCl solution. About one-third of the original fluorescence was restored with correction made for the dilution factor.

Following quantitative thin-layer chromatographic tests on these particular samples, it was found that the B_1 component in all samples had been altered. Instead of the pure aflatoxin B_1 appearing on the plate, three derivatives were found in testing the solutions which exhibited fluorescence. Further experimentation demonstrated that the changes in the B_1 component were due to the prolonged exposures of the solutions to ultraviolet light in obtaining a number of photographic records.

This study was repeated, but this time the test solutions were never exposed to ultraviolet light until actual measurements were made on the final thin-layer chromatographic plates. The aqueous methanol sample, which received only the NaOH treatment at room temperature, yielded no recoverable aflatoxin; while the sample receiving the alkali treatment, followed by the acidification with HCl, yielded the original aflatoxin in 94% yield. The observed R_f factor,

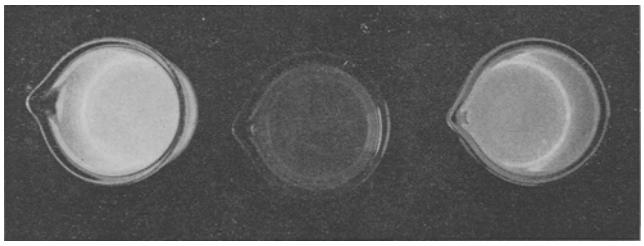

FIG. 6. Changes in fluorescence under ultraviolet light of aflatoxin B_1 on alkalinization and subsequent acidification; beaker on the left contains the original aflatoxin in aqueous methanol solution, the one in the center is now alkaline, and the one on the right has been brought from the alkaline to an acid reaction.

single spotting and quality of fluorescence all indicated no difference between the recovered aflatoxin and the control, which had not gone through the pH changes. The ease with which the aflatoxin was converted to the sodium salt derivative and back again to the original aflatoxin following acidification, with acid of the type and amount found in the normal stomach, supports Feuell's observation that a nonfluorescent form of aflatoxin obtained by mild alkaline treatment may still be toxic.

With the above as background, tests were conducted to determine whether any obscured nonfluorescent forms of aflatoxin remained in the test oils after alkali refining. Crude peanut oil, containing 800 ppb of aflatoxin B_1 (see Fig. 3 and Table I), was refined, water-washed and bleached in order to investigate this possibility. Acidification of the refined and washed oil and acidification following the bleaching step by the addition of HCl in aqueous solution to aliquots of these test systems after each stage of processing resulted in no increase in fluorescence over what had been found previously. Hence, it can be concluded that no measurable amount of aflatoxin in either its fluorescent or nonfluorescent forms remains in refined vegetable oils.

ACKNOWLEDGMENTS

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