eventually obtained from long-term [e.g., 84 days (11)] dietary studies with elaidate and other unusual fatty acids. In particular, some enzymatic basis may be found to indicate why the "cholesterol coefficient" of the *trans* monoenoic acids (+2.1) is so similar to that for the saturated acids (+2.7) whereas that for the *cis* isomers is essentially zero (12). At the present we can see an interesting similarity in the acyltransferase specificities at the 1-position of 2-acyl-GPC and these coefficients.

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Assay of Aflatoxin in Peanuts and Peanut Products Using Acetone-Hexane-Water for Extraction[1]

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Abstract

A quantitative method is described for the assay of aflatoxin in peanut products. The procedure involves extraction of aflatoxin from the sample with a homogeneous acetone-hexane-water solvent mixture followed by purification of the extract by phasic extraction of the aflatoxin with aqueous sodium chloride and then with chloroform. The purified chloroform extract is analyzed by thin-layer chromatography by comparison of the intensity of fluorescence of any aflatoxin with the intensity of a known standard. The aflatoxin analyses of peanuts were found to be very variable due to sampling, and this variability has been greatly reduced by finely grinding and thoroughly mixing 2 kg of the sample before removal of an aliquot for assay. The method is sensitive to approximately 2 parts per billion.

Introduction

AFLATOXINS ARE HIGHLY toxic metabolites initially found to be produced by species of the common mold *Aspergillus flavus* (1) and later found to be produced by other mold species (2,3). The aflatoxins were first found in a peanut meal which caused the death of a large number of farm animals (4). The toxins are not limited to peanut products but also have been found associated with the toxicity caused by other agricultural commodities (4,5).

Crude aflatoxin is a mixture of several fluorescent components, four of which have been isolated in a pure state and shown to be toxic in varying degrees to ducklings (6). These four components were designated aflatoxin B_1, B_2, G_1 and G_2 because of their origin as metabolites of *Aspergillus flavus* and from the blue and green fluorescence they exhibit when exposed to ultraviolet (UV) radiation. The structure of aflatoxin B_1 and G_1 has been established by Asao et al. (17). Aflatoxin B_2 and G_2 are dihydro derivatives of the respective B_1 and G_1 (6,8,9).

The aflatoxins are soluble in solvents such as methanol, and this solubility forms the basis for most of the present methods of assay (10–14). Some of these methods are long and time-consuming and lack sensitivity due to quenching of fluorescence by impurities.

Until quite recently all the procedures used for the determination of aflatoxin in peanut products called for initial separation of the aflatoxin by prolonged Soxhlet extraction. For this procedure a single solvent or a constant boiling mixture of solvents possesses obvious advantages. In this laboratory an azeotrope of acetone, hexane and water has been under investigation as a solvent for removal of lipids and gossypol from cottonseed for several years (15). The use of this azeotrope was investigated, and it was found to be an excellent extractant for aflatoxin from peanut products with the added advantage that it does not extract as much extraneous material as does methanol. Thus, equal amounts of aflatoxin were found in a commercial peanut meal when the aflatoxin was removed by 6 hr of Soxhlet extraction with methanol or with the acetone-hexane-water azeotrope. However, the residue obtained after evaporation of the methanolic extract from 100 g of commercial peanut meal amounted to 11.55 g, whereas that obtained after evaporation of the azeotrope amounted to only 3.13 g. Accordingly, the subsequent purification of the extract required for assay for aflatoxin by thin-layer chromatography (TLC) is much simpler if the azeotrope is used as the extractant.

Recently, it has been found in assaying for aflatoxin that equally satisfactory results can be obtained in much shorter extraction time by equilibrium extraction using a Waring Blendor or a shaker (16). The azeotrope is not completely miscible at room temp, and a small lower layer relatively richer in acetone and water separates out, thus making it somewhat inconvenient to handle in the laboratory. Since the constant boiling temp characteristic of an azeotrope is not important in equilibrium extraction, the utility, as an extractant for aflatoxin, of homogeneous mixtures of the solvents was investigated. A mixture of acetone, hexane and water in the proportion of 5:48.5:1.5 (v/v) was found to be homogeneous and to

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TABLE I

Variability of Aflatoxin Assay of Different 2 kg Subsamples from the Same 25 lb Sample of Peanuts[a]

2 kg Sample	Analyses	ppb Aflatoxin B_1
1	A	96
	B	72
2	A	160
	B	120
3	A	72
	B	96
4	A	160
	B	144
5	A	720
	B	600

[a] Each 2 kg subsample of ground peanuts was passed through a Jones riffle sampler successively to obtain the two aliquots used for analyses A and B.

give results by equilibrium extraction comparable to those obtained by Soxhlet extraction with the azeotrope. Although the azeotrope may be used and Soxhlet extraction with the azeotrope may be preferred with some materials, equilibrium extraction with the homogeneous solvent mixture generally will be preferred. This paper describes the method developed for the quantitative assay of aflatoxin in peanut products using this homogeneous mixture of acetone, hexane and water as the extractant.

Principle

The sample is extracted by equilibrium extraction using a Waring Blendor or a shaker with a homogeneous acetone-hexane-water solvent mixture. The resulting slurry is centrifuged, and an aliquot of the centrifugate is transferred to a separatory funnel. The aflatoxin is removed from the centrifugate by phasic extraction with 5% aqueous sodium chloride, and then removed from the aqueous phase with chloroform. After evaporation of the chloroform the residue, which contains the aflatoxin, is dissolved in a known volume of chloroform. The purified extract is spotted on a TLC plate and developed. The chromatogram is examined under long wave UV light, and the intensity of blue and green fluorescent spots of appropriate R_f's is compared visually with the intensity of known standards.

Experimental

Apparatus

1. Explosion-proof Waring Blendor with 1 qt jar and lid or Burrell Wrist-Action shaker or equivalent (see Note 1).
2. Centrifuge—equipped with head to hold 250 ml bottles.
3. Centrifuge bottles, 250 ml.
4. Separatory funnels, 250 ml with Teflon stopcocks.
5. Butt extractor tubes.
6. Microliter syringe, Hamilton 701-SN or equivalent, 10 μl capacity, with 26-gauge tip, 2 cm long.
7. Thin-layer chromatographic equipment—DESAGA/Brinkmann or equivalent.
8. Desiccating storage cabinet.
9. Long wave UV lamp—Spectroline Model C-3F Fluorescence Analysis Cabinet equipped with four 15-watt long wave UV lamps or Blak-Ray Model B-100A, Ultraviolet Products, San Gabriel, California, equipped with a 100-watt long wave flood bulb.
10. Vials, 2 dram with polyethylene stoppers (Kimble 60975-L).

TABLE II

Variability of the Aflatoxin Assay of a Finely Ground Peanut Sample

Analyses	ppb Aflatoxin B_1
1	246
2	246
3	234
4	258
5	264
6	274

Reagents

1. Chloroform, ACS grade.
2. Methanol, ACS grade.
3. *n*-Hexane, purified, boiling range 60–70C.
4. Acetone, ACS grade.
5. Anhydrous sodium sulfate, ACS grade.
6. Sodium chloride, ACS grade.
7. Silica Gel G-HR for TLC, Brinkmann Instruments.

Solutions

1. 5% Aqueous sodium chloride.
2. 3% Methanol in chloroform (v/v).
3. Acetone-hexane-water solvent mixture (50:48.5:1.5 v/v).
4. Aflatoxin standard—ca. 1.0 μg of B_1 and G_1 per ml chloroform. The aflatoxin standard may be prepared from pure, recrystallized aflatoxin or from purified extracts of known concn.

Procedure for Peanuts

Preparation of the Sample

1. Examine the sample and discard any sticks, stones, or other extraneous materials.
2. In the case of large samples of peanuts (over 2 kg), pass the whole sample successively through a Boerner Sampler or its equivalent and reduce to 2 kg.
3. Grind 2 kg of the sample in a Waring Blendor. This may be done by grinding the sample in 100 g batches in the blendor for 10–20 sec at full speed. This gives a particle size of approx 10–16 mesh.
4. Riffle the 2 kg of ground peanuts with a Jones riffle sampler or equivalent successively to a final sample size of 50 g.

Extraction

A. Waring Blendor

1. Weigh 50 g of the ground sample in a 100 ml beaker and transfer to a quart Waring Blendor container.
2. Add 200 ml of the acetone-hexane-water solvent mixture using some of the solvent to clean the beaker.
3. Blend for 4 min in an explosion-proof Waring Blendor using the low speed setting.

B. Mechanical Shaker

1. Weigh 50 g of the ground sample in a 500 ml standard taper Erlenmeyer flask and cover the bottom of the flask with 6 mm glass beads.
2. Add 200 ml of the acetone-hexane-water solvent mixture and stopper the flask with a polyethylene or glass stopper.
3. Attach the flask to a Burrell Wrist-Action shaker or its equivalent and shake for 30 min.

Purification of Extract and Preparation for Thin-Layer Chromatography

1. Transfer the slurry obtained in A or B above to a 250 ml centrifuge bottle as completely as possible and centrifuge at 1800–2000 rpm for 10 min. After centrifugation, decant the centrifugate into a 250 ml graduated cylinder.

2. Transfer 110 ml of the centrifugate to a separatory funnel, add 50 ml of 5% aqueous sodium chloride, and shake vigorously for ca. 30 sec (Note 2). Allow phases to separate and draw off the lower aqueous acetone layer into a 400 ml beaker. If necessary, place the separatory funnel under a stream of hot water to help break any emulsion which might form so that a clear lower layer is assured.
3. Repeat the extraction twice more with 50 ml portions of 5% aqueous sodium chloride adding 35 ml of acetone before each extraction. The added acetone reduces the tendency to form an emulsion. Discard the upper hexane layer.
4. Combine the aqueous acetone extracts in a 400 ml beaker, add a few boiling stones, and evaporate to approx 150 ml on a steam bath. This removes most of the acetone.
5. Allow the aqueous extract to come to room temp and quantitatively transfer to a separatory funnel, washing the sides of the beaker with a small amt of chloroform from a wash bottle.
6. Add 50 ml of chloroform to the separatory funnel, shake vigorously for ca. 30 sec, and allow the phases to separate.
7. Draw off the lower chloroform layer through a bed of anhydrous sodium sulfate into a 250 ml beaker (Note 3).
8. Repeat the extraction with another 50 ml portion of chloroform. After the last extraction, rinse the sodium sulfate with ca. 10 ml chloroform.
9. Add a few boiling stones to the beaker and evaporate the combined chloroform extracts almost to dryness on a steam bath.
10. Quantitatively transfer the residue to a 2 dram vial by washing with a small amt of chloroform. Evaporate to dryness on a steam bath under a stream of nitrogen.
11. Allow the vial to come to room temp, add 1.0 ml of chloroform, and stopper to prevent solvent loss.

Preparation of TLC Plates

1. Shake vigorously 50 g of Silica Gel G-HR with 100 ml of distilled water in a stoppered flask for no longer than 1 min and pour into the applicator. Immediately apply to five 20 × 20 cm clean dry glass plates using an adjustable applicator set at 500μ .
2. Allow the plates to air dry at room temp for 1 hr, and then dry the coated plates at 105C for 2 hr. Store the dried plates in a suitable desiccating cabinet until ready for use.
3. With a spatula or the edge of the finger, remove about 2 mm portion of the silica gel coating from along two parallel edges of the chromatoplate before spotting to reduce solvent front lag during development.

Chromatography

1. Using a 10 μl syringe, spot 1,3,5, and 10 μl of the chloroform extract on a line about 2 cm from the bottom of a plate coated with Silica Gel G-HR. On the same plate, spot 1,2,3, and 4 μl of the aflatoxin standard, and prepare an internal standard by spotting 5 μl of the sample solution and 4 μl of the aflatoxin standard on the same spot. Care should be taken to keep the spots as nearly equal in size as possible.

TABLE III

Extraction of Aflatoxin from Centrifugate with 5% Aqueous Sodium Chloride

Extractions	Ml of 5% aqueous sodium chloride	ppb Aflatoxin B_1	% of total aflatoxin
Sample A—1	25	180	65.7
2	25	70	25.5
3	25	18	6.6
4	25	6	2.2
Sample A—1	50	240	87.3
2	50	30	10.9
3	50	5	1.8
Sample B—1	50	360	86.5
2	50	50	12.0
3	50	6	1.5
4	50	trace	.....

2. Develop the plate immediately after spotting in an equilibrated tank with 3% methanol in chloroform. The tank should be lined with filter paper to aid in equilibration.
3. After the solvent front has traveled 10 cm from the base line, remove the plate from the tank, and air dry for 5 min before examination.

Estimation of Aflatoxin Concentration

1. Examine the plate under long wave UV light (365 mμ), observe the blue and green fluorescence of the standard aflatoxin B_1 and G_1 respectively, and observe the sample spots for corresponding fluorescence at the same R_f's as the aflatoxin B_1 and G_1. Compare the relative intensity of any aflatoxin spots with those of the known standard.
2. If the intensities of the sample spots are too weak or too strong to match the standard, adjust the concn of the sample extract and spot another chromatoplate.
3. Calculate the concn in parts per billion (ppb) of aflatoxin in the sample using the following formula:

$$\text{ppb } B_1 = (A)\ (B)\ (V)/(X)\ (25)$$

where A = μg/ml of aflatoxin B_1 standard
B = μl of aflatoxin B_1 standard spotted which matches intensity of B_1 spot in the sample (X)
V = volume in μl of final sample extract
X = μl of sample spotted for which B_1 spot matches intensity of B_1 spot of aflatoxin standard (B)
(25) = weight of sample in grams. Only 25 g or one-half of original sample is represented in the TLC assay (Note 2).

Calculate aflatoxin G_1 in like manner. Aflatoxin B_2 and G_2 are calculated using aflatoxin B_1 and G_1 as the standard for fluorescent intensity.

Notes

1. The ground sample may be extracted with a Waring Blendor or with a Burrell Wrist-Action shaker.
2. With peanuts and peanut butter, use 110 ml of the supernatant; but with peanut meal use 130 ml of the supernatant. These are the vol-

TABLE IV

Extraction of Aflatoxin from Aqueous Sodium Chloride Extract with Chloroform

Extractions	Ml of chloroform	ppb Afflatoxin B_1	% of total aflatoxin
1	50	252	99.2
2	50	2	0.8
3	50	0	0.0

TABLE V

Limits of Detection of Aflatoxin in Ground Peanuts

ppb Aflatoxin B_1	
ppb Added	ppb Recovered
12.0	20.0
6.0	5.0
2.4	1.8
1.2	0.9[a]
0.0	0.0

[a] Estimated using a fluorescent lamp higher in intensity than normally used, i.e. 100 watts.

umes estimated to represent 25 g or one-half of the sample, after making allowance for the oil extracted from peanuts and peanut butter.

3. Prepare by placing a glass wool plug in a Butt extractor tube and adding ca. 40 g of anhydrous sodium sulfate.

Procedure for Peanut Butter and Peanut Meal

Peanut Butter

A 50 g sample of peanut butter is extracted in the manner as described for peanuts. After extraction, the slurry is transferred to a centrifuge bottle and centrifuged at 2000–2200 rpm for 30 min. Then, proceed as described for peanuts.

Peanut Meal

The sample to be analyzed is reduced to 2 kg by quartering, and the 2 kg is riffled with a Jones riffle sampler or equivalent successively to the approx amt required for analysis.

To 50 g of the peanut meal, add 10 ml of distilled water and 250 ml acetone-hexane-water. Then, proceed as described for peanuts, extracting with mechanical shaker.

Results and Discussion

Variability of Aflatoxin Assay Due to Sampling

Procedures used at the present time recommend grinding from 40 to 200 g of whole peanuts. However, in the course of analyses using two of these methods it became obvious that consistent results could not be obtained using samples of this size. Four different assays on one 25 lb sample of peanuts using a 200 g subsample gave one strongly positive (375 ppb), a second moderately positive (38 ppb), and two negative results. This was probably due to the fact that all the toxicity may reside in a very small number of individual kernels. Therefore, the sample size must be large enough to include a representative amount of such peanuts. By grinding 2 kg subsamples of peanuts prior to the assay, consistently positive results were obtained; but there was still a wide variation in the aflatoxin content of different 2 kg subsamples. This variability in results of assays on five different 2 kg subsamples obtained by riffling a 25 lb sample of peanuts is shown in Table I.

In this case, the average duplicate analyses of each 2 kg subsample differed by about 20%, but the values obtained for the different 2 kg subsamples generally differed much more.

The reproducibility of the aflatoxin analyses that may be obtained within a 2 kg sample of ground peanuts is much better as may be seen from the results of six replicate analyses shown in Table II.

For this test a 2 kg sample of peanuts was finely ground in a Waring Blendor, thoroughly mixed, and 50 g aliquots for aflatoxin assay were obtained by riffling. The reproducibility of the analyses was good, varying from 234 to 274 ppb aflatoxin B_1 or a variability of about 15%.

The results shown in Table II indicate that a representative aliquot can be obtained from a well-mixed, finely ground sample. The results shown in Table I indicate that even a 2 kg subsample is too small to give a truly representative sample. However, a positive response was obtained in each case; and except for one subsample the amount of aflatoxin B_1 found ranged only from 72 to 160 ppb. Accordingly, although the proper size of subsample of peanuts to be taken for grinding cannot be stated at this time, at least 2 kg is recommended.

Effect of Sample Size on Aflatoxin Assay

The amts of 25 g, 50 g, 75 g, and 100 g of a well-mixed, finely ground toxic peanut sample were analyzed by the acetone-hexane-water procedure, keeping all of the conditions constant except the size of the sample. The final extracts were diluted in proportion to the original sample size, equal quantities spotted on a TLC plate, and the aflatoxin estimated. The aflatoxin concn of the peanuts was found to be 384 ppb aflatoxin B_1 for each of the different sample sizes used. Therefore, any sample size between 25 and 100 g may be used with the procedure. For ease in manipulation and simplicity of calculation, a 50 g sample was adopted.

Completeness of Extraction of Aflatoxin from the Centrifugate with 5% Aqueous Sodium Chloride

A 110 ml aliquot of the centrifugate from three separate samples, representing two well-mixed ground samples of peanuts, was extracted with different volumes of 5% aqueous sodium chloride. In each case the lower aqueous acetone layer was drawn off and carried through the remaining steps of the procedure to determine the aflatoxin concentration of each individual extraction.

Results tabulated in Table III show that the extraction of sample A with 25 ml of 5% aqueous sodium chloride removed aflatoxin in each of the four extractions; however, when the volume of aqueous sodium chloride was increased from 25 ml to 50 ml, aflatoxin was practically completely removed by three 50 ml extractions. In sample B, containing a higher concn of aflatoxin, similar results were obtained; only a trace of aflatoxin was detected in the fourth 50 ml extraction. Therefore, three extractions with 50 ml of 5% aqueous sodium chloride were adopted for the procedure.

Number of Extractions with Chloroform Required for Complete Removal of Aflatoxin from Concentrated Aqueous Acetone Extract

The conc aqueous acetone extract, obtained from the treatment of the centrifugate solution, was extracted with 50 ml portions of chloroform. The aflatoxins are preferentially soluble in chloroform and are partitioned into this phase. Each chloroform extract was assayed separately and the data reported in Table IV.

About 99% of the aflatoxin B_1 was extracted with the first 50 ml portion of chloroform and the remainder with the second 50 ml portion. Two extractions with chloroform are recommended in the analytical procedure although for most purposes a single extraction would probably suffice.

Limits of Detection of Aflatoxin in Ground Peanuts

The limits of detection of aflatoxin in ground peanuts were determined by preparing mixtures containing different proportions of ground peanuts free

of aflatoxin with ground peanuts containing a known concn of aflatoxin and assaying the mixture. Results are tabulated in Table V.

Results in Table V show that the sample which had 12 ppb added was estimated to contain 20 ppb. The difference probably is a reflection of sample variation. The limit of detection was found to be about 2 ppb. In normal practice, a concn lower than 2 ppb would not be estimated although traces as low as 0.5 to 1.0 ppb can be detected using a high intensity fluorescent lamp such as a 100-watt lamp.

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The Determination of Aflatoxins in Cottonseed Products[1]

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Abstract

A rapid and simplified procedure is proposed for the determination of aflatoxins B_1, B_2, G_1, and G_2 in cottonseed products. The method involves extraction of aflatoxins essentially free of lipid contamination by equilibrium extraction with 70% acetone. Interfering gossypol pigments are removed from the aqueous acetone extract by precipitation as insoluble lead salts. Aflatoxins in the centrifugate are quantitatively separated from excess lead salts, residual pigments and carbohydrates by extraction into chloroform to yield final extracts for thin-layer chromatographic (TLC) analysis on silica gel which are low in total solids and pigmentation. The procedure is sensitive to about 1 ppb in cottonseed meats and 4 ppb in cottonseed meal.

Introduction

THE DISCOVERY THAT CERTAIN strains of common molds such as *Aspergillus flavus* growing on agricultural commodities can elaborate a number of fluorescent, highly toxic, and carcinogenic metabolites has stimulated accelerated research on the problem of mold toxins in food and feeds (1–3). The collective term "aflatoxins" has been applied to this mixture of toxic metabolites, the composition of which varies with mold strain, substrate and the environmental conditions of mold growth (4). As the name implies, the aflatoxins were originally believed to be specific metabolites of certain *A. flavus* strains; however, they are now known to be elaborated by other common mold species (5). Four of these aflatoxins designated as B_1, B_2, G_1, and G_2 have been both structurally characterized (6–9) and evaluated for biological activity (10). At the present time these four difurano-coumarin derivatives are believed to be primarily responsible for the toxic and carcinogenic properties attributable to the aflatoxins (10). These findings have created the need for accurate and sensitive analytical methods for the estimation of aflatoxins in agricultural products at levels as low as several parts per billion.

The physical properties of the aflatoxins pertinent to the analysis of these compounds are outlined in Table I. Their nomenclature is obviously derived from the distinctive bluish and greenish fluorescence they exhibit when exposed to ultraviolet (UV) radiation. All four compounds are excited by long way (UV) light at 365 mμ and emit maximum fluorescence in the visible region between 425–450 mμ. The ability to separate the four compounds by TLC on silica gel forms the basis of most analytical methods for these materials. The inverse correlation between fluorescent and toxic properties emphasizes the need for separation and analysis of the individual compounds rather than an estimation based on total fluorescence.

Although a number of analytical procedures are available for the determinatoin of the aflatoxins in peanut products (4,11–13), relatively little attention has been given to methods for the estimation of these compounds in cottonseed products. When analytical methods satisfactory for the analysis of peanut products were applied to cottonseed kernels, extensive interferences due to the intense pigmentation of gossypol derivatives, which are present to the extent of 0.4 to 1.5% of the weight of the cottonseed kernel (14) imposed severe limitations on the use of these procedures. These considerations and the need for a sensitive and accurate procedure for use in research to evaluate the extent of the aflatoxin problem in cottonseed products prompted the development of the proposed method.

Analytical Method

Apparatus

1. *Mechanical Shaker.*—Burrel "Wrist Action" or equivalent, equipped to hold ℱ 32, 500 ml capacity Erlenmeyer flasks fitted with ℱ leak-proof polyethylene stoppers.

2. *Centrifuge.* Equipped with head to hold 250 ml roundbottom bottles.

3. *Sodium Sulfate Drying Tubes.* Prepare from standard Butt extraction tubes with a plug of glass wool in the constriction, and containing ca. 40 g of anyhydrous sodium sulfate.

[1] Honorable mention, Bond Award competition. Presented at the American Oil Chemists' Society, Chicago, October 11–14, 1964.
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