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.

REFERENCES

1. Sargeant, K., A. Sheridan, J. O'Kelly and R. B. A. Carnaghan, Nature *192*, 1096–1097 (1961).
2. Codner, R. C., K. Sargeant and R. Yeo, Biotechn. Bioeng. *5*, 185–192 (1963).
3. Hodges, F. A., J. R. Zust, H. R. Smith, A. A. Nelson, B. H. Armbrecht and A. D. Campbell, Science *145*, 1439 (1964).
4. Allcroft, R. and R. B. A. Carnaghan, Chem. Ind. (London), 50–53 (1963).
5. Loosmore, R. M., R. Allcroft, E. A. Tutton and R. B. A. Carnaghan, Vet. Rec. *76*, 64–65 (1964).
6. Hartley, R. D., B. F. Nesbitt and J. O'Kelly, Nature *198*, 1056–1058 (1963).
7. Asao, T., G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan, J. Am. Chem. Soc. *85*, 1706 (1963).
8. Chang, S. B., M. M. Abdel-Kader, E. L. Wick and G. N. Wogan, Science *142*, 1191–1192 (1963).
9. van Dorp, D. A., A. S. M. van der Zijden, R. K. Beerthuis, S. Sparreboom, W. O. Ord, K. de Jong and R. Keuning, Rec. Trav. Chim. *82*, 587–592 (1963).
10. Armbrecht, B. H., F. A. Hodges, H. R. Smith and A. A. Nelson, J. Assoc. Offic. Agr. Chem. *46*, 805–817 (1963).
11. Broadbent, J. H., J. A. Cornelius and G. Shone, Analyst *88*, 214–216 (1963).
12. Nabney, J., and B. F Nesbitt, Nature *203*, 862 (1964).
13. Nesheim, S., D. Banes, L. Stoloff and A. D. Campbell, J. Assoc. Offic. Agr. Chem. *47*, 586 (1964).
14. de Iongh, H., J. G. van Pelt, W. O. Ord and C. B. Barrett, Vet. Rec. *76*, 901–903 (1964).
15. King, W. H., J. C. Kuck and V. L. Frampton, JAOCS *38*, 19–21 (1961).
16. Pons, W. A., Jr., and L. A. Goldblatt, JAOCS, *42*, 471–475 (1965).

[Received January 29, 1965—Accepted February 19, 1965]

The Determination of Aflatoxins in Cottonseed Products[1]

W. A. PONS, JR., and L. A. GOLDBLATT, Southern Regional Research Laboratory, New Orleans, Louisiana[2]

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.
[2] A laboratory of the So. Utiliz. Res. and Dev. Div., ARS, USDA.

TABLE I

Selected Properties of Aflatoxins

Property	Aflatoxin			
	B_1	B_2	G_1	G_2
Molecular weight	312	314	326	328
Fluorescence	Blue	Blue	Green	Green
UV excitation maxima, mμ[a]	365	365	365	365
UV emission maxima, mμ[a]	425	425	450	450
Approx. R_f on silica gel G-HR[b]	0.5	0.45	0.4	0.35
Relative fluorescence intensity[a]	1	8	5	13
Relative toxicity-ducklings[a]	9.6	2	4.4	1
LD_{50} -day old ducklings, μg[a]	18	85	39	173

[a] From data reported by Carnaghan, R.B.A, et al., Nature *198*, 1101 (1963).
[b] Silica gel layer was 500 μ and developing solvent was 3% methanol in chloroform.

4. *Silica Gel Coated Plates.* Shake 45 g of Silica Gel G-HR with 90 ml of distilled water for 30 sec in a stoppered flask, pour into suitable applicator set for a 0.5 mm (500 μ) thickness coating and immediately coat five 20 × 20 cm glass plates. Allow plates to air dry for 2 hr, and heat in forced draft oven for 2 hr at 103–105C. Store dried plates in a suitable desiccator cabinet over silica gel or drierite desiccant.

5. *Microliter Syringe.* Hamilton 701-SN or equivalent, 10 μl capacity, with 26 gauge tip, 2 cm long. Any suitable micropipet is satisfactory.

6. *Spotting Template.* Construct of glass or other suitable material to hold microliter syringe in upright position so that tip of needle just touches surface of TLC plate.

7. *Fluorescent Viewer.* Blak-Ray B-100A, Ultra-Violet Products, Inc., San Gabriel, Calif., or equivalent, fitted with 100-watt long wave flood bulb. A UV chromatographic viewer equipped with four 15-watt long wave fluorescent bulbs similar to Blak-Ray C-4 is also satisfactory.

8. *Developing Tank.* Any suitable tank lined front and back with filter paper, and fitted with a tight cover. Add enough developing solvent to just cover the bottom of a TLC plate and allow to equilibrate 10–15 min before use.

Reagents

1. *Aqueous Acetone.* Mix 700 ml ACS grade acetone and 300 ml distilled water.

2. *Developing Solvent.* Mix 30 ml of ACS grade methanol and 970 ml of ACS grade chloroform. Keep in a stoppered bottle and protect from light.

3. *Silica Gel G-HR.* Brinkmann Instruments, Inc., Westbury, N.Y., or equivalent.

4. *Lead Acetate Solution.* Dissolve 200 g of ACS lead acetate trihydrate in distilled water, add 3 ml of glacial acetic acid, and dilute to one liter.

5. *Standard Aflatoxin Solution.* May be prepared from crystalline aflatoxins B_1 and G_1, accurately weighed, dissolved in, and suitably diluted with ACS chloroform to provide a standard solution containing from 0.0010 to 0.0015 μg of each aflatoxin per microliter. Alternatively, a secondary working standard, prepared from a purified mixture of all four aflatoxins dissolved in chloroform and assayed against crystalline B_1 and G_1 may be employed. The concn of the individual aflatoxins in the secondary working solution should be in the range of 0.0005 to 0.0015 μg/μl.

TABLE II

Solubility of Cottonseed Salad Oil in Aqueous Acetone Mixtures

Acetone concentration[a]	Weight of oil per 100 ml of solution
%	g
100	8.16[b]
95	3.46
90	0.64
85	0.18
80	0.061
70	0.010

[a] V/V mixtures of ACS acetone and water.
[b] Completely miscible.

Sample Preparation

1. *Cottonseed Kernels.* Dehull in a Bauer mill, and separate kernels from hulls and lint by hand screening. Grind kernels in a Wiley mill or equivalent to pass a 2 mm screen. Dry homogenization of kernels in a Waring Blendor is also satisfactory.

2. *Hulls and Lint.* Grind in a Wiley mill to pass a 1 mm screen.

3. *Meal or Cake.* Grind in a Wiley mill to pass a 1 mm screen.

Procedure

Weigh the sample, not to exceed 50 g, into a 500 ml Erlenmeyer flask and cover the bottom of the flask with a layer of 6 mm diameter solid glass beads. Add 250 ml of the 70% aqueous acetone, stopper the flask, and shake on a mechanical shaker for 30 min at such a rate that the sample material collecting in the neck of the flask is constantly washed back into the solvent. Filter the extract through an 18.5 cm circle of rapid filter paper (Whatman No. 4 or equivalent) folded to fit a 100 mm ID funnel, and cover the funnel with a watch glass to minimize evaporation. Collect at least 175 ml of the clear filtrate in a suitable stoppered container.

Measure 175 ml (graduated cylinder) of the extract into a 400 ml beaker marked to indicate 175 ml volume. Add 70 ml of distilled water, 20 ml of lead acetate solution, and several carborundum boiling chips. Boil on a steam bath until the volume is reduced to 175 ml; cool to room temp in an ice-water bath.

Transfer the contents of the beaker to a 250 ml centrifuge bottle and centrifuge for 10 min at 2000 rpm. Decant the centrifugate into a leak-proof Squibb type 250 ml separatory funnel, preferably fitted with a Teflon stopcock. Disperse the precipitate in the centrifuge bottle in 75 ml of acetone:water (20:80 V/V), stir well and recentrifuge for 10 min at 2000 rpm. Add the wash solution to the original centrifugate in the separatory funnel. Extract the combined solution with a 50 ml portion of ACS grade chloroform, shaking vigorously for 1 min. Allow the phases to separate, and filter the lower chloroform phase through the small sodium sulfate drying tube into a 250 ml beaker. Repeat the extraction with a second 50 ml portion of chloroform. After the second chloroform extract passes through the sodium sulfate tube, wash the column with ca. 20 ml. of chloroform.

Add several carborundum chips to the beaker, and evaporate the chloroform on a steam bath until the volume is reduced to ca. 1 ml. Transfer quantitatively to a small vial (1–2 dram cap) equipped with a tight-fitting polyethylene stopper, washing the sides of the beaker with a fine stream of chloroform to effect quantitative transfer. Evaporate to dryness on a steam bath, preferably under a slow stream of nitrogen, cool to room temp. Avoid overheating dry extracts on the steam bath. With a suitable micropipet add 500 μl (0.5 ml) of chloroform to the vial, and stopper the vial with a tight-fitting polyethylene stopper to minimize evaporation.

Spot 2, 5, and 10 μl portions of the sample extract and 1, 2, 3, 4, and 5 μl of the standard aflatoxin solution on a TLC plate, placing the spots along a line about 4 cm from the bottom of the plate. On the same plate, spot another 5 μl of the sample extract, and di-

rectly on top of it spot 3μl of the standard aflatoxin solution as an internal standard. Remove about 0.5 cm of the silica gel coating from the sides of the TLC plate to insure a uniform solvent front, place the plate in a suitable holder in the TLC developing tank, and develop in 3% methanol in chloroform until the solvent front has traversed about 14 cm beyond the origin. Remove the plate, air dry for about 5 min, and observe the sample and standard spots under an intense long wave UV source. Equate the intensity of the bluish fluorescent aflatoxin B_1 spots at R_f of approx 0.5 and the greenish fluorescent G_1 spots at R_f of approx 0.4, if present, with the appropriate B_1 and G_1 spots of the standards.

As R_f values in sample extracts may vary, use the internal standard to verify the presence or absence of aflatoxins in sample extracts. In cases where aflatoxins B_2 and G_2 are found in sample extracts, these are compared with the appropriate B_1 and G_1 spots of the standard aflatoxin solution, or with the B_2 and G_2 spots of the secondary working standard solution.

Should the TLC analysis indicate that the sample spots are too weakly fluorescent for reliable matching with the standards, dry the sample extract under a stream of nitrogen, dissolve in 250 μl (0.25 ml) of chloroform, and repeat the assay using 10 and 20 μl of the sample extract. In all tests where low levels of aflatoxins are encountered, 2–10 ppb, an intense long wave source such as the 100-watt flood bulb cited is recommended for accurate measurements.

Should the TLC analysis indicate that the sample spots are too intensely fluorescent for accurate matching with standards, dry the sample extract under a stream of nitrogen, dissolve in a suitable volume of chloroform (1–5 ml) and repeat the assay using 2, 3, and 5 μl of the sample extract.

Calculations

$$\text{Aflatoxin } B_1, \text{ ppb} = (V_s)(C_s)(\text{S.D.})(1000)/(X)(W)(0.7)$$

where,

(V_s) = μl of aflatoxin standard for which intensity of B_1 spot matches intensity of the B_1 spot of the sample.
(C_s) = Concn of aflatoxin B_1 in the standard, μg/μl.
(S.D.) = Volume to which sample extract is diluted for TLC analysis, in μl.
(X) = Volume of sample extract spotted, in μl.
(W) = Sample weight, grams.

(Valid only for 175/250 aliquot of sample extract. The same procedure is used to calculate the content of aflatoxins B_2, G_1 and G_2.)

Results and Discussion

Extraction Conditions

The choice of an analytical extraction system was governed by the dual requirements of a rapid and quantitative extraction of aflatoxins from cottonseed materials, coupled with minimum extraction of interfering substances. A number of solvents and solvent mixtures were evaluated for this purpose, and among them 70% acetone best met these requirements. A simple equilibrium extraction with this solvent on a mechanical shaker indicated that equivalent aflatoxin values were obtained when extraction time varied from 15 to 120 min. To insure an adequate safety factor, a 30-min extraction period was chosen for the procedure.

In addition to its property as an efficient solvent for aflatoxins, aqueous acetone has the desirable feature that lipids are practically insoluble in this solvent. This has obvious advantages as it obviates the necessity for removing excessive amts of lipid contaminants from sample extracts. To demonstrate this property, 5 ml portions of a winterized cottonseed salad oil were equilibrated with 50 ml volumes of aqueous acetone solutions where the concn of acetone varied from 100 to 70% by volume. After a 30 min equilibration on a shaker, the mixtures were centrifuged, and aliquots of the clear acetone extracts were evaporated to obtain the solubility data listed in Table II. From these results, it can be noted that below about 90% acetone concn the solubility of glycerides decreases rapidly, until at 70% acetone concn the solubility approaches 0.01%.

TABLE III

Number of Chloroform Extractions Required for Complete Removal of Aflatoxins

Volume of centrifugate extracted[a]	Successive 50 ml $CHCl_3$ extractions of centrifugate	Aflatoxin B_1 found in $CHCl_3$ extracts	
		Total weight	Percent of total
ml		μg	%
170	First	4.50	99.2
170	Second	0.038	0.8
170	Third	None	0

[a] 25 G cottonseed meal (260 ppb of aflatoxin B_1) extracted with 250 ml 70% acetone. After removal of lead precipitate by centrifugation in analytical procedure, the cenrtifugate was successively extracted with 50 ml portions of $CHCl_3$. Each $CHCl_3$ extract analyzed individually for aflatoxin B_1.

$$\left[\begin{matrix}\text{Partition coefficient} \\ \text{first extraction}\end{matrix}\right] = \frac{\text{amount in } CHCl_3 \text{ phase}}{\text{amount in aqueous phase}} = 118.5$$

The use of an equilibrium analytical extraction should allow reasonable variations in the ratio of sample to solvent without undue influence on the equilibrium attained. This variable was explored by varying the sample weight of an aflatoxin-containing cottonseed meal from 5 to 50 g, while holding the volume of aqueous acetone constant. Sample size was not found to be a critical variable over the range chosen, as equivalent values for aflatoxin content were obtained for meal to solvent ratios ranging from 1:5 to 1:50.

Removal of Gossypol Pigments

The presence of gossypol pigments in final extracts for TLC analysis on silica gel results in excessive streaking on the plate and tends to mask the fluorescence of the aflatoxins. Organic solvents which are efficient for the removal of aflatoxins are also excellent gossypol solvents. Aqueous acetone is no exception, as it has been employed as a solvent for the analysis of free gossypol pigments in cottonseed products (15). The effective removal of interfering gossypol pigments in aqueous acetone extracts was achieved by precipitating these pigments as insoluble lead salts. A number of other pigments are also precipitated as insoluble lead derivatives. These lead derivatives were found to be partially soluble in 70% acetone, but practically insoluble in 20% acetone. Accordingly,

TABLE IV

Comparison of Total Solids in Extracts of Cottonseed Products

Extraction		Total solids per 50 g sample			
		Raw meats		Screw-pressed meal	
Type	Solvent used	Crude extract	Final extract[a]	Crude extract	Final extract[a]
		g	g	g	g
Soxhlet	Methanol[b]	5.07	0.230	4.61	0.118
Shaker	Aqueous acetone[c]	4.90	0.007	6.01	0.016

[a] Thirty minute extraction by proposed procedure.
[b] Final purified extracts for TLC analysis.
[c] Samples defatted for 3 hr with petroleum ether prior to 6 hr methanol extraction (1,4).

TABLE V

Recovery of Pure Aflatoxins Added to Cottonseed Meats in Analytical Procedure

Substrate		Aflatoxin B_1		Aflatoxin G_1	
C/S Meats	70% Acetone	Added	Found[a]	Added	Found[a]
g	ml	μg	μg	μg	μg
25	250	3.80	3.81	3.20	3.19
25	250	1.90	1.96	1.60	1.64
25	250	0.76	0.65	0.64	0.58
25	250	0.38	0.33	0.32	0.26
Total		6.84	6.75	5.76	5.67

[a] Average values from TLC assay of several aliquots of each extract.

in the analytical procedure, to 175 ml of the reddish-brown 70% acetone extract water and lead acetate are added and the solution evaporated to 175 ml. This reduces the acetone concentration to about 20% by volume. After cooling, the lead precipitate is removed by centrifugation. The centrifugate is a pale lemon-yellow color and contains only trace quantities of gossypol derivatives. About 10–15% of the aflatoxins present in solution were found to be occluded in the lead precipitate obtained from extracts of cottonseed meals and meats, respectively. For complete recovery of aflatoxins, the precipitate is dispersed in 75 ml of 20% acetone, recentrifuged, and the wash solution combined with the primary centrifugate, prior to extraction of the aflatoxins into chloroform.

Chloroform Extraction

After prior removal of interfering gossypol-like pigments by precipitation with lead acetate, the aflatoxins are quantitatively separated from residual yellow pigments, excess lead salts, and soluble carbohydrate constituents by extraction into chloroform in a separatory funnel. From experimental result outlined in Table III where a typical centrifugate from the analysis of a cottonseed meal was extracted with four successive 50 ml portions of chloroform, it may be noted that more than 99% of the aflatoxins are removed in a single chloroform extraction. Complete recovery is attained in two extractions. The partition coefficient for a single extraction, 118.5, is so favorable that usually a single extraction would suffice. For greater accuracy, two extractions are recommended.

The final purified extracts for TLC analysis are quite low in total solids and residual pigmentation. A comparison of the level of total solids in aqueous acetone extracts with those obtained by application of a procedure proposed for peanut products (4,11) that involved 6 hr Soxhlet extraction with methanol is afforded by the data outlined in Table IV. Both methanol and aqueous acetone remove considerable amts of total solids in the crude primary extracts of cottonseed meats or meal. However, after removing interfering constituents by precipitation with lead acetate, and subsequent chloroform extraction, the aqueous acetone procedure yields final extracts for TLC analysis containing only 7 and 16 mg of total solids for meats and screw-pressed meal, respectively. These may be compared with values of 230 and 118 mg, respectively, for methanol extracts.

TABLE VI

Detection Limits for Aflatoxins in Cottonseed Meats and Meal

Weight dilution of C/S product[a]	Cottonseed meats		Screw-press meal	
	Aflatoxin B_1 parts per billion		Aflatoxin B_1 parts per billion	
	Calc.	Found	Calc.	Found
None	.....	260	.....	228
1:10	26.0	30.2	22.8	17.2
1:25	10.4	8.7	9.1	8.6
1:50	5.2	4.9	4.6	4.4
1:100	2.6	1.6	2.3	N.D.[2]
1:166	.....	.....	.....	N.D.[2]
1:200	1.3	0.7	1.3	

[a] Mixtures of aflatoxin-free meats or meal and aflatoxin-containing cottonseed meals with 260 and 228 ppb of B_1, respectively.
[b] Not detectable.

TABLE VII

Aflatoxin Content of Selected Cottonseed Products[a]

Sample No.	Product	Type of processing	Aflatoxins, parts per billion			
			B_1	B_2	G_1	G_2
A	Meats	.....	69	[b]	27	[b]
B	Hulls and Fine Meats	From sample A	129	Tr.[c]	99	Tr.[c]
C	Meal[d]	Unknown	290	104	58	29
D	Meal	Prepress solvent	175	29	....	....
E	Meal	Screw-press	300	128	....	....
F	Meal	Screw-press	26	.....	....	....
G	Meal	Solvent	260	87	....	....
H	Meal	Solvent	9	1	....	....

[a] Only samples in which aflatoxins were detected are reported here.
[b] None detected.
[c] Trace quantities.
[d] Meal C which had previously been estimated to contain of the order of 0.5 ppm of aflatoxin B_1 (16) was obtained through the courtesy of Dr. Ruth Allcroft.

Limits of Detection and Recovery

No significant loss or destruction of aflatoxins occurs in the extraction, lead precipitation and chloroform separation phases of the analytical procedure, as shown by the recovery experiments outlined in Table V. Aliquots of a primary standard solution of aflatoxins B_1 and G_1 of known concn were added to 25 g portions of cottonseed meats and aqueous acetone prior to the extraction step, and then carried through the entire analytical procedure. Each extract was individually analyzed by TLC using dilutions of the same aflatoxin primary standard for comparison. Adequate recoveries averaging 98% were obtained for both aflatoxins B_1 and G_1 in this experiment.

The detection limits of the technique were evaluated by preparing weighed mixtures of ground cottonseed meats, free of aflaoxin contamination, and cottonseed meal containing 260 ppb of aflatoxin B_1. The calculated values of B_1 in the mixtures and the values obtained by analysis show reasonably good agreement (Table VI). The lowest level which could be analyzed with confidence was about 1 ppb. In an analogous experiment with screw-pressed cottonseed meal (Table VI), calculated and determined levels were also in good agreement, but aflatoxin B_1 could not be detected with assurance below 4 ppb.

Application of Procedure

A number of cottonseed products were assayed for aflatoxin content by application of the proposed procedure. Tabulated in Table VII are values obtained on a selected group of cottonseed products found to contain aflatoxins. It should be emphasized that from the data reported in Table VII, no conclusion is warranted as to the incidence of aflatoxin contamination in domestic cottonseed products. A large number of cottonseed meats and meals have been analyzed and found to be free of aflatoxin contamination.

Precision

The precision of the method was evaluated by conducting 15 analyses for aflatoxin B_1 on a single sample of well-ground cottonseed meal over a period of 3 months. Three analysts working in the same laboratory conducted the analyses. The analytical values ranged from 228 to 300 ppb with a mean value of 253. The standard deviation was calculated to be $\pm$ 17 ppb, representing a coefficient of variation of $\pm$ 6.8%.

Although the analytical procedure reported here was designed primarily for the analysis of cottonseed products, it has been found to be applicable to the analysis of peanut products and a variety of other agricultural materials. These applications will be outlined in a subsequent communication.

ACKNOWLEDGMENT

G. A. Harper, National Cottonseed Products Association, supplied some of the experimental cottonseed samples; G. C. Cavanagh, Ranchers Cotton Seed Oil Co., Stanley Nesheim of FDA, and J. A. Robertson, Jr., of this Laboratory gave suggestions, analytical technique, and Mrs. Louise S. Lee and Mrs. Alva F. Cucullu of this Laboratory performed some of the analyses.

REFERENCES

1. Sargeant, K., A. Sheridan, J. O'Kelley and R. B. A. Carnaghan, Nature *192*, 1096–1097 (1961).
2. Allcroft, R., and R. B. A. Carnaghan, Chem. Ind., Jan. 1963, 50–53.
3. deIongh, H., R. O. Vles and J. G. Van Pelt, Nature *202*, 466–467 (1964).
4. Armbrecht, B. H., F. A. Hodges, H. R. Smith and A. A. Nelson, J. Assoc. Offic. Agr. Chem. *46*, 805–817 (1963).
5. Hodges, F. A., J. R. Zust, H. R. Smith, A. A. Nelson, B. H. Armbrecht and A. D. Campbell, Science *145*, 1439 (1964).
6. Asao, T., G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan, J. Am. Chem. Soc. *85*, 1706 (1963).
7. Wogan, G. N., E. L. Wick, C. G. Dunn and N. S. Scrimshaw, Federation Proc. *22*, No. 2, 611 (1963).
8. Chang, S. B., M. M. Abdel-Kader, E. L. Wick and G. N. Wogan, Science *142*, 1191–1192 (1963).
9. Hartley, R. D., B. F. Nesbitt and J. O'Kelley, Nature *198*, 1056 (1963).
10. Carnaghan, R. B. A., R. D. Hartley and J. O'Kelley, Nature *200*, 1101 (1963).
11. Broadbent, J. H., J. A. Cornelius and G. Shone, Analyst *88*, 214–216 (1963).
12. Nesheim, S., J. Assoc. Offic. Agr. Chem. *47*, 1010–1017 (1964).
13. Robertson, J. A., Jr., L. S. Lee, A. F. Cucullu and L. A. Goldblatt, JAOCS. In Press.
14. Pons, W. A., Jr., C. L. Hoffpauir and T. H. Hopper, Agric. Food Chem. *1*, 1115–1118 (1953).
15. Pons, W. A. Jr., and J. D. Guthrie, JAOCS *36*, 671–676 (1949).
16. Loosmore, R. M., R. Allcroft, E. A. Tutton and R. B. A. Carnaghan, Vet. Rec. *76*, 64–65 (1964).

[Received January 8, 1965—Accepted March 8, 1965]

Identification of Elementary Sulfur and Sulfur Compounds in Lipid Extracts by Thin-Layer Chromatography

SISTER MARY T. J. MURPHY and BARTHOLOMEW NAGY, Department of Chemistry, University of California, San Diego, La Jolla, California; and

GEORGE ROUSER and GENE KRITCHEVSKY, Department of Biochemistry, City of Hope Medical Center, Duarte, California

Abstract

Elementary sulfur, long chain thiols and sulfides in lipid mixtures can be separated and identified by thin layer chromatography (TLC), preparation of derivatives, development of typical flourescent colors with Rhodamine 6G under ultraviolet light, and colors with other spray reagents. Silica gel mixed with magnesium silicate and the same adsorbent plus silver nitrate are used for polar stationary phase and silver nitrate complexing chromatography, respectively.

Elementary sulfur yields a purple fluorescent spot with Rhodamine 6G in contrast to the yellow fluorescent of most lipids. Compounds isolated by means of TLC were further identified by spectroscopic methods. The sulfur bacterium (*Chromatium sp.*), and the Orgueil carbonaceous meteorite were analyzed by the new technique. Elementary sulfur was identified in both samples, but the lipid compositions of the bacteria and meteorite were found to be entirely different. The meteorite lipids and hydrocarbons were also different from the abiological hydrocarbons synthesized in a Miller high frequency spark discharge experiment.

The new analytical technique is suitable for the analysis of recent biological matter, petroleum, bitumens and organic matter from marine sediments.

Introduction

A STUDY OF LIPIDS in sulfur bacteria, petroleum, and carbonaceous stony meteorites resulted in the development of a chromatographic technique for the detection of trace quantities of elementary sulfur and organic sulfur compounds. The desirability of a technique to identify sulfur and sulfur compounds in lipid extracts was emphasized by the appearance of a number of well defined but unknown spots on the thin-layer chromatograms of lipid mixtures isolated from substances known to contain sulfur in some form. Standard methods of sulfur anaylsis did not appear to be applicable to sulfur determination in lipids.

Several of the standard analytical methods for determining trace quantities of sulfur and sulfur compounds are based on reaction with metal ions (1,2), oxidation (3,4,5,) and colorimetric determinations (6,7). Chromatographic methods have been used repeatedly in recent years. Sulfur compounds from large samples of petroleum have been identified by Smith et al. (8,9) by column chromatography. Ertel and Horner (10) separated a few sulfur compounds from microgram quantities of samples by TLC. Mangold (11) has reviewed some of the applications of TLC for lipids and quantitative TLC of lipids has been discussed in detail by Privett et al. (12,13,14).

Two approaches were used in the course of developing the present analytical procedure. First, experiments were performed with pure standards. Secondly, unidentified components of natural samples were separated by TLC, eluted from the adsorbent, and analyzed by suitable chemical means as well as IR and UV spectroscopy. TLC included 1) polar stationary phase and 2) polar stationary phase impregnated with silver nitrate. The overall technique permits identification of sulfides, thiols and elementary sulfur in the presence of hydrocarbons, fatty acids, fatty alcohols, esters and amines.

Materials and Methods

The following materials were used:

Methyl octadecanoate, Nutritional Biochemicals Corp, 99% purity.