Extraction Partition and Column Chromatographic Separation of Aflatoxins B_1 and M_1[1]

MERLE SID MASRI, Western Regional Research Laboratory,[2] Albany, California 94710

Abstract

Extraction, partition and chromatographic elution characteristics of aflatoxin were studied. The novel use of aqueous dimethylsulfoxide or aqueous dimethylformamide for extraction from agricultural products was tested and found effective. Partition studies suggest advantages in analytical work of using solvent pairs in which benzene rather than (the usually employed) chloroform is used to transfer aflatoxin B_1 from primary extracts (aqueous phases). Tests of elution characteristics of aflatoxins B_1 and M_1 on silica gel columns with different developing solvents provided a basis for a procedure in which aflatoxins B_1 and M_1 of a test sample are recovered in separate eluates in which substances which interfere with TLC separation are minimized.

Introduction

In the course of work on aflatoxins, we were prompted to search for methods which would be particularly suitable for analysis of samples with low levels of aflatoxin. Procedures were also desirable for determining both aflatoxins B_1 and M_1 in the same test sample, especially when one was present in high concentration relative to the other, such as in experimental milk samples from cows receiving aflatoxin B_1 in the feed (1) and in our recent experiments of incubation of aflatoxin B_1 with liver microsomes (2; also, Masri and Page, submitted for publication), where the separation of the unused substrate from the metabolic products prior to thin layer chromatography (TLC) facilitated quantitative analysis. We describe here some procedures which we found to be helpful additions to the refined methods already in the literature (3–8) and which may suggest changes toward further refinement. Aspects which will be discussed are: (a) use of novel aqueous solvents for primary extraction of aflatoxins from test samples; (b) transfer of aflatoxin from primary extracts by partitioning with benzene or chloroform; and (c) column chromatography steps with elution programs for separating aflatoxins B_1 and M_1 into different eluates in which substances with R_f values close to those of the aflatoxins are minimized.

Experimental Procedures

Materials

Aflatoxin B_1 was isolated from cultures of *Aspergillus parasiticus* NRRL 2999 grown on rice (9). It was purified on silica gel columns and recrystallized. It had a molar absorptivity of 22,300 at 363 mμ in methanol which compares with values in the literature (10). Crystalline aflatoxin M_1 was obtained as described earlier (11).

All solvents were analytical reagent grade; chloroform, ACS, contained 0.75% ethanol; anhydrous diethyl ether, ACS contained 0.01% ethanol. Skellysolve F (Skelly Oil Co., Kansas City, Mo., bp 30–60 C) was used to defat aqueous extracts of aflatoxin. Merck Silica Gel, column chromatography grade, with particle size 0.05–0.2 mm, was used for column chromatography. Merck Silica Gel G-HR slurried in water, or a mixture of (25 g) Silica Gel HR plus (25 g) reagent grade anhydrous calcium sulfate (not gypsum) slurried in (100 ml) water were used for TLC (7). The slurries were spread on 20 × 20 cm glass plates at a wet thickness of 0.5 mm; the coated plates were air-dried then activated at 110 C for 1 hr. Chromatoplates were developed with 3–4% (volume) methanol in chloroform or 10–15% acetone in chloroform (7,12).

Two peanut meal samples were used; meal No. 1, spiked with aflatoxin [rice inoculated with *A. parasiticus* NRRL 2999; (9)], and meal No. 2, naturally contaminated with aflatoxin.

Primary Extraction, Partitioning and Quantitation

Peanut meal samples (about 25 g size) were homogenized for 4 min in a Waring Blendor with one of the following solvent mixtures (all by volume), using 6–7 ml solvent mixture per gram sample: (a) methanol-water (1:1); (b) dimethylsulfoxide (DMSO)-water (1:1); or, (c) dimethylformamide (DMF)-water (1:1). After blending, the suspensions were filtered (solvent a) or centrifuged solvents (b and c) at 7,000–8,000 × g in a Sorvall Model RC-2 Superspeed centrifuge using plastic bottles and the GSA rotor. Aliquots of the clear extracts were defatted with an equal volume of Skellysolve F. The defatted extracts were partitioned with chloroform (two equal volume extracts) or benzene (three equal volume extracts) to transfer the aflatoxin. Combined chloroform or benzene extracts were then washed three times with equal volumes of water. It is especially important to wash the DMSO or DMF from benzene extracts since traces of DMSO or DMF on chromatoplates may seriously affect the quality of TLC separations. The washed extracts were evaporated in a rotary film evaporator under reduced pressure at 60 C and the residue was dissolved in a small volume of chloroform in preparation for TLC (or column chromatography prior to TLC). An aliquot of the chloroform solution was applied as a streak across a chromatoplate. After development, the aflatoxin B_1 bands were scraped and the aflatoxin eluted by equilibrium extraction with a measured volume of methanol for spectrophotometric measurement. Determination of aflatoxins was made by measurement of absorbance in methanol at 363 mμ for B_1 and at 357 mμ for M_1 (7) with a Beckman Model DU spectrophotometer.

The effectiveness of chloroform and of benzene in removing aflatoxin B_1 from aqueous solutions of methanol, acetone, DMSO, or DMF was compared by studying the partition of crystalline aflatoxin B_1 using the following solvent pairs (all by volume): (a) methanol-water-chloroform (1:1:2); (b) methanol-water-benzene (1:1:2); (c) acetone-water-chloro-

[1] Presented at the AOCS Spring Meeting, San Francisco, April 1969.
[2] W. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Partition of Aflatoxin B_1

Solvent pair	Aflatoxin in extracts (μg)					Ext. coeff.,[a] %
	1st	1st washed	2nd	3rd	Total	
(a) Methanol-water-chloroform (1:1:2)	950	972	5	....	977	99.4
(b) Methanol-water-benzene (1:1:2)	835	870	107	12	989	87.9, 90.0
(c) Acetone-water-chloroform (2:4:3)	924	961	7	....	968	99.3
(d) Acetone-water-benzene (1:2:2)	920	937	25	1.5	964	97.2, 94.2
(e) DMSO-water-benzene (1:1:2)	850	880	109	11	1001	88.0, 90.8
(f) DMF-water-benzene (1:1:2)	780	820	132	18	970	84.5, 85.1

[a] Ext. Coeff., extraction coefficient. Only two extracts were obtained when chloroform was used for partition, but three extracts when benzene was used. When two values are shown for the same pair, the second coefficient was calculated as the ratio of aflatoxin in the second extract to that in the second plus third extracts.

form (2:4:3); (d) acetone-water-benzene (1:2:2); (e) DMSO-water-benzene (1:1:2); and (f) DMF-water-benzene (1:1:2). The mixtures in each pair gave approximately equal volumes of upper and lower phases. In each partition experiment with a solvent pair, 1 mg (gravimetric) of crystalline aflatoxin B_1 was equilibrated with 25 ml of upper plus 25 ml of lower phases. After separation of the phases, the aqueous phases were successively equilibrated with fresh 25 ml portions of the appropriate nonaqueous phases. The aflatoxin B_1 content of each of the benzene or chloroform extracts were determined spectrophotometrically on suitable aliquots reconstituted in methanol. The measurements were made directly on these extracts without TLC since crystalline aflatoxin was used. Aliquots of the first nonaqueous extracts in each experiment were analyzed before and after washing with one equal volume of water. In obtaining successive extracts of the aqueous phases, 25 ml fresh unequilibrated benzene or chloroform were used except with pairs c and d. With these two pairs (but not with the others), the use of unequilibrated solvents would have resulted in large changes in the volume ratios of upper to lower phases. Therefore, with these pairs, equilibrated chloroform or benzene phases were used for obtaining second or third extracts.

Column Chromatography

Chromatographic tubes, 30 cm long, 19 mm i.d. and fitted with a Teflon stopcock and a 250 ml reservoir (Kontes K 42028) were used. The columns were prepared in the usual manner (5–7), using 15 g silica gel, particle size 0.05–0.2 mm, slurried in chloroform and packed to a height of 10 cm. The sample was applied in chloroform (25 ml, including washes). The column was then washed with 225 ml hexane followed by 225 ml anhydrous diethyl ether. Slight positive pressure applied to the top of the column during the ether wash helps overcome excessive formation of bubbles. The use of hexane and ether as a mixture for washing the column as used by Pons et al. (6) may be preferable. The exact volume of chloroform used to apply the sample and the exact volumes of the hexane and ether washes are not critical since negligible movement of the aflatoxins occurs. After the washes, elution of aflatoxins was started using the elution programs discussed later under Column Separation of Aflatoxins B_1 and M_1.

TABLE II

Aflatoxin B_1 in Peanut Meals by Different Methods (ppb)

Meal[a]	Method (solvents used for extraction and partition)			
	DMSO-H_2O-benzene	DMF-H_2O-benzene	CH_3OH-H_2O-benzene	CH_3OH-H_2O-chloroform
1	5200	5050	4950	5260
2	737	.....	.....	787

[a] Meal No. 2 was analyzed by two methods only.

Results and Discussion

Partition of Aflatoxin

Results of analyses of the partition experiments with crystalline aflatoxin B_1 are shown in Table I. The extraction coefficients of aflatoxin B_1 were calculated, in per cent, as the ratios of aflatoxin in first (washed) extract to the total aflatoxin extracted; or as the ratios of aflatoxin in the second extract to the total in the second plus third extracts.

The coefficients with benzene clearly show that the three equal volume extracts transferred all the aflatoxin into benzene; further, it can be calculated that three ½ vol extracts would be sufficient. Coefficients obtained for benzene and chloroform point to the advantage of using benzene in analytical work, since chloroform appears superfluously too strong compared to benzene for the extraction of aflatoxin B_1. Our experience with a variety of test samples indicates that much cleaner extracts for TLC are obtained when benzene rather than chloroform is used. The high extraction coefficients with chloroform have other implications in both analytical and isolation work. Since a single chloroform extract removes more than 99% of aflatoxin B_1 from the aqueous media, a second and third chloroform extract could remove little additional aflatoxin but may add appreciable amount of extraneous materials. This is in agreement with other reports (13,14). In analytical work therefore, if chloroform should be used for partition, consideration should be given not only to limiting the number of extracts but also to using fractional volumes of chloroform. The implication in preparative work can be appreciated when dealing with large scale isolation of aflatoxin from large volumes of aqueous solutions such as liquid media of *A. flavus* cultures or aqueous methanol extracts of *A. flavus* grown on solid media such as rice (9). In such cases, extraction with only two $\frac{1}{10}$ vol for example of chloroform would be ample, rendering further operations, such as evaporation of chloroform, more manageable.

Repartitioning

The principle of repartitioning even when employing the same solvent pair used originally, is another useful procedure for purifying extracts. If extracts

from a first partitioning with chloroform or benzene are not sufficiently clean, considerable improvement may be achieved, as judged from reduction of residues and from quality of subsequent TLC by recycling the residues of these extracts. That is, the residue of a TLC extract from a first partitioning is dissolved in aqueous methanol (1:1 v/v) and the solution is defatted with Skellysolve F, then repartitioned with chloroform or benzene. This recycling procedure does not interfere with quantitation of aflatoxin B_1 but is not suitable for aflatoxin M_1 due to some loss of M_1 upon recycling.

Comparison of Extraction Coefficients of Aflatoxins B_1 and M_1

We reported earlier on the extraction coefficients of aflatoxin M_1 by benzene and chloroform from aqueous methanol (7). Comparison of the published data with those reported here for aflatoxin B_1 show that while aflatoxin B_1 is readily extractable with benzene, aflatoxin M_1 is not. However, aflatoxin M_1 is readily extractable with chloroform. Similarly, back-extraction (7) of aflatoxin M_1 (but not B_1) from benzene into small aqueous methanol washes is feasible. These differences in extractability can be utilized in preparative work to effect a preliminary rough separation of the two aflatoxins when present together in the same starting source. We have utilized this difference in isolating aflatoxin M_1 formed in vitro as a result of incubation of liver microsomes with aflatoxin B_1 substrate (2; also, Masri and Page, submitted for publication). Another example where this may apply is in the isolation of aflatoxin M_1 from cultures of *A. flavus* where small amounts of aflatoxin M_1 may be present together with relatively large amounts of aflatoxin B_1 (11).

Extraction with Aqueous DMSO or DMF

Partition results in Table I demonstrate suitability of using benzene to transfer aflatoxin B_1 from aqueous DMSO or DMF, and that washing the benzene extracts with water (to remove dissolved DMSO or DMF) does not result in loss of aflatoxin from the benzene. To use the solvent pairs DMSO-water-benzene, (1:1:2 v/v/v) or DMF-water-benzene, (1:1:2 v/v/v) in analysis of test samples, we checked the efficacy of aqueous DMSO or aqueous DMF (1:1 v/v) in extracting aflatoxin B_1 from agricultural products. Results in Table II for two peanut meal samples analyzed by different methods, illustrate the suitability of aqueous DMSO or DMF for primary aflatoxin extraction. These solvents were also used satisfactorily with other products such as cottonseed meal.

Column Separation of Aflatoxins B_1 and M_1

Column cleanup procedures in aflatoxin methodology (5–7) are very helpful analytical tools and are responsible in great measure for the refinement and sensitivity of current methods. These procedures however, have not been particularly aimed at analyzing for both aflatoxins B_1 and M_1 in the same test sample. Also, in these procedures eluates of aflatoxin B_1 are collected over an unnecessarily wide range. With these considerations in mind, we developed column procedures to accomplish two objectives: first, separation of aflatoxins B_1 and M_1 into different eluate collections, second, use of an elution program which collects the aflatoxins over a narrow but adequate range. For this, we studied the behavior of crystalline aflatoxins B_1 and M_1 admixtures (about 5 μg each) on silica gel columns using a variety of developing solvents. In these tests progress of the bands on the columns was visualized by long u.v. light and the positions of the bands were recorded. Tests to check on reproducibility of elution profiles showed remarkable consistency. Also, tests indicated satisfactory quantitative recovery of the added aflatoxins. We record in Table III data on elution profiles based on varying concentrations of methanol in chloroform as developing solvents. A number of elution programs with the objectives mentioned earlier can be formulated from these data. It appears that optimum separation occurs with 0.5% or 1% methanol in chloroform (0.73 and 0.75 cm separation for every 1 cm of column length used vs. 0.45 and 0.2 cm separation/cm column length used for methanol concentrations of 1.5% and 3%). In our work (1,2; also, Masri and Page, submitted for publication) we often used the following program which is based on the data in Table III. After the sample is applied and the column is washed with hexane and ether, elution is started with 225 ml of 0.5% methanol in chloroform followed by another 225 ml of 1.5% methanol in chloroform. As soon

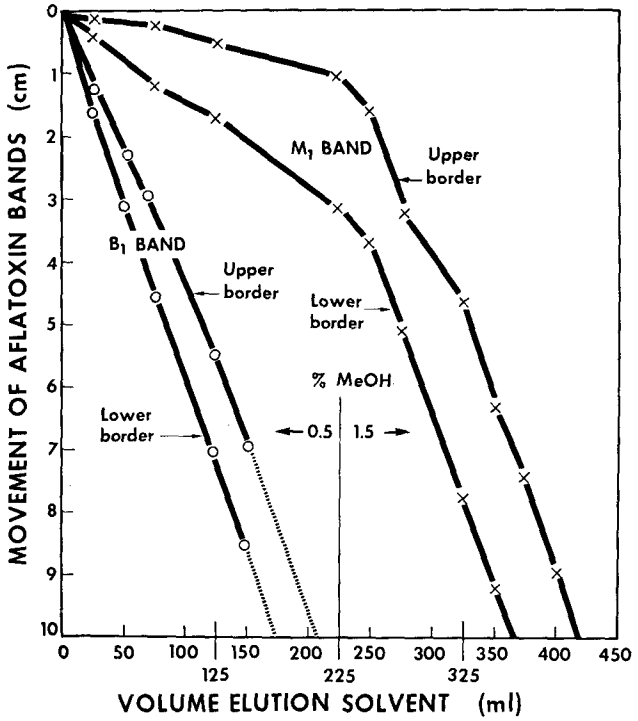

FIG. 1. Elution profiles of aflatoxins B_1 and M_1 on silica gel column with chloroform containing varying concentrations of methanol.

TABLE III

Elution of Aflatoxins B_1 and M_1 on Silica Gel Columns[a] With Varying Concentrations of Methanol in Chloroform[b]

Per cent methanol in chloroform (by volume)	Band movement (cm/25 ml solvent)		Separation, cm	
	B_1	M_1	Per 25 ml solvent[c]	Per cm of column length used[d]
0	Slight	Negligible	....	.....
0.5	1.5	0.4	1.1	0.73
1.0	2.4	0.6	1.8	0.75
1.5	2.7	1.4	1.3	0.48
2.0	3.0	2.3	0.7	0.23
3.0	4.5–5.0	3.6	1.0	0.2

[a] A 15 g particle size 0.05–0.2 mm; 19 mm i.d.; 10 cm high.
[b] ACS, containing 0.75% ethanol.
[c] Movement B_1/25 ml, minus movement M_1/25 ml.
[d] Separation (cm)/movement B_1 (cm).

as the ether reaches the top of the column, the following eluates are collected: (a) 0–125 ml, (b) 125–225 ml, (c) 225–325 ml and (d) 325–450 ml. Fraction b contains aflatoxin B_1 and fraction d contains aflatoxin M_1. Figure 1 depicts the elution profiles obtained using this program in an experiment with crystalline aflatoxins B_1 and M_1 in admixture.

ACKNOWLEDGMENTS

J. R. Page, V. C. Garcia Randall and L. Mehine assisted.

REFERENCES

1. Masri, M. S., V. C. Garcia and J. R. Page, Vet. Rec. *84*, 146–147 (1969).
2. Masri, M. S., J. R. Page and V. C. Garcia, "Metabolic Conversion of Aflatoxin B_1 to M_1 in vitro." Abstract, 153, AOCS Meeting, San Francisco, April 1969.
3. Nesheim, S., D. Banes, L. Stoloff and A. D. Campbell, J. Assoc. Offic. Anal. Chemists *47*, 586 (1964).
4. Pons, W. A., Jr., A. F. Cucullu, L. S. Lee, J. A. Robertson, A. O. Franz and L. A. Goldblatt, Ibid. *49*, 554–562 (1966).
5. Stoloff, L., Ibid. *50*, 354–360 (1967).
6. Pons, W. A., Jr., A. F. Cucullu, A. O. Franz, Jr. and L. A. Goldblatt, JAOCS *45*, 694–699 (1968).
7. Masri, M. S., J. R. Page and V. C. Garcia, J. Assoc. Offic. Anal. Chemists *51*, 594–600 (1968).
8. Masri, M. S., J. R. Page and V. C. Garcia, Ibid. *52*, 641–643 (1969).
9. Shotwell, O. L., C. W. Hesseltine, R. D. Stubblefield and W. C. Sorenson, Appl. Microbiol. *14*, 425–428 (1966).
10. Stubblefield, R. D., O. L. Shotwell and G. M. Shannon, JAOCS *45*, 686–688 (1968).
11. Masri, M. S., R. E. Lundin, J. R. Page and V. C. Garcia, Nature *215*, 753–755 (1967).
12. Epply, R. M., J. Assoc. Offic. Anal. Chemists *49*, 473–474 (1966).
13. Pons, W. A., Jr. and L. A. Goldblatt, JAOCS *42*, 471–475 (1965).
14. Waltking, A. E., G. Bleffert and M. Kiernan, Ibid. *45*, 880–884 (1968).

[Received August 13, 1969]