NOTE ON A TWO-DIMENSIONAL TLC PROCEDURE FOR DETERMINING AFLATOXINS IN CORN[1]

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Recently, we encountered a problem of determining aflatoxins in extremely moldy samples of corn grits. The grits had been improperly stored and were extensively contaminated with a variety of unidentified microorganisms.

For normal corn we had been employing the Seitz and Mohr (1) procedure, which we had modified for quantitative determinations of aflatoxins. However, this tlc procedure was unsatisfactory with the moldy samples because of interfering substances. These substances appeared as spots of various colors and hues, which overlapped and interfered with both visual detection and quantitation of the aflatoxins.

These problems eventually led to our examination of two-dimensional tlc. With this technique, we were able to affect complete separation of the aflatoxins from interfering substances. Details of the procedure and results obtained with spiked and moldy corn samples are described.

MATERIALS AND METHODS

Aflatoxin Standards

Aflatoxins B_1, B_2, G_1, and G_2 were obtained from Applied Science Laboratories, Inc., State College, PA 16801. Aflatoxin solutions of various concentrations were prepared in 2:98 v/v acetonitrile–benzene.

Corn Samples

Corn used in this study was whole corn ground to pass through a 20-mesh screen. Samples were examined by the procedure to verify the absence of aflatoxins.

The three moldy corn samples used in this investigation were prepared from corn which was inoculated with aflatoxin-producing mold and maintained for 6 weeks at 25°–27° C at 18–20% H_2O to promote mold growth and production of aflatoxin. After 6 weeks, the samples were air-dried to about 8% H_2O and ground to pass a 20-mesh screen.

tlc Procedure

1. Place 50 g of corn ground to pass a 20-mesh screen and 150 ml of methanol[2] into a Waring Blendor, and blend at high speed for 2 min.
2. Gravity filter through a qualitative filter paper[3], and collect 60 ml of filtrate in a 100-ml graduated cylinder.
3. Transfer filtrate to a 250-ml separatory funnel. Using the same graduate, add 120 ml of a 20% ammonium sulfate solution at 45° C and 60 ml of hexane.

[1]Paper presented in part at the 60th Annual Meeting, Kansas City, Mo., Oct., 1975.
[2]It was recently brought to our attention that aqueous methanol does a more thorough job of extracting naturally occurring aflatoxins. Preliminary work with an 85:15 v/v mixture seems to confirm this suggestion.
[3]We found Reeve Angel 802 filter paper to be satisfactory.

Vigorously shake the separatory funnel for 30 sec, with venting. Allow layers to separate.

4. Drain lower layer into a beaker; discard the upper layer.

5. Return lower layer to separatory funnel and add 10.0 ml of reagent-grade chloroform[4]. Shake funnel, with venting, for 1 min and allow layers to separate for 10 min. While layers are separating, dry drain tube of funnel using a Q-tip.

6. Carefully drain the chloroform layer into a 10-ml graduated cylinder and record volume.

7. Quantitatively transfer the chloroform to a 2-dram vial, and evaporate to dryness by placing vial in a shallow pan containing water at 50°–60°C or by passing a stream of air over the vial.

8. When dry, add 1.0 ml of chloroform to the vial, cover, and swirl to dissolve the residue.

9. Spot 10, 15, and 20 μl of sample onto separate 10 × 5-cm Brinkman SILG-HR-25 precoated plates[5]. Each plate is scored 8 cm horizontally and 4-1/2 cm vertically. No activation of tlc plates is necessary.

10. Also, spot plates containing known quantities of each aflatoxin. Aflatoxin levels should be adjusted so that intensities are similar to those of the unknown samples.

11. Place plates in an unlined developing chamber containing 0.5 cm of 12:88 v/v acetone–chloroform, and develop them vertically until the solvent front reaches the score.

12. After development, dry plates with a stream of air, rotate them 90°, place them in a second developing solvent, 0.5 cm of 95% denatured ethyl alcohol, such as Mallinckrodt 7006, and develop them until the solvent front reaches the score.

13. Examine the dried plates under long-wave uv light. If aflatoxin spots are present, visually estimate the intensity of unknown spots against known aflatoxin spots.

14. Calculate the concentration of each aflatoxin using the following equation:

$$\text{ppb aflatoxin} = ABCD/E \times (150/60) \times (10/F) \times 20$$

$$= 500 \times ABCD/EF$$

where A = numerical factor of unknown spot relative to standard spot
B = volume of standard spot in μl
C = concentration of aflatoxin standard in ng/μl
D = volume of chloroform used to dissolve residue in μl (usually 1000 μl)
E = volume of unknown sample spotted in μl
F = volume of chloroform recovered after liquid-liquid extraction in ml
10 = original volume of chloroform added for extraction in ml
150/60 = ratio of volume of methanol added to volume of filtrate recovered
20 = factor used to convert results from μg/50 g to μg/kg or ppb

[4]Initial work employed methylene chloride as the final extraction solvent. Recoveries of 130–160% of theoretical were obtained, indicating probable loss through evaporation. Chloroform was eventually selected as the better solvent.

[5]The one-dimensional technique as described by Seitz and Mohr (1) is satisfactory for normal corn samples and may be used to replace steps 9 through 12 if extraneous colors are not a problem.

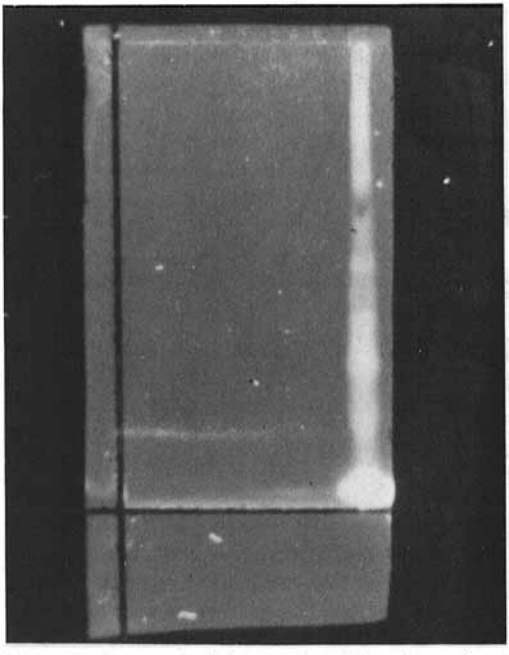

Fig. 1. One-dimensional development with acetone–chloroform showing no separation of aflatoxins from interfering substances. The corn sample contained naturally occurring aflatoxin B_1 and was spiked with 20 ppb each of B_1, B_2, G_1, and G_2.

TABLE I
Visual Determination of Various Aflatoxins in Spiked Corn Samples by Two-Dimensional tlc

Aflatoxin Present	Aflatoxin, ppb		% of Aflatoxin Added
	Added	Estimated[a]	
B_1	15.0	14.4	96
B_2	5.0	4.4	88
G_1	15.0	12.4	83
G_2	5.0	4.0	80
B_1	80.0	87.2	109
B_2	10.0	9.1	91
B_1	5.0	5.3	106
B_2	5.0	4.7	94
G_1	15.0	15.9	106
B_1	10.0	9.9	99
G_1	10.0	9.5	95
G_2	5.0	4.1	82

[a]Average of three investigators.

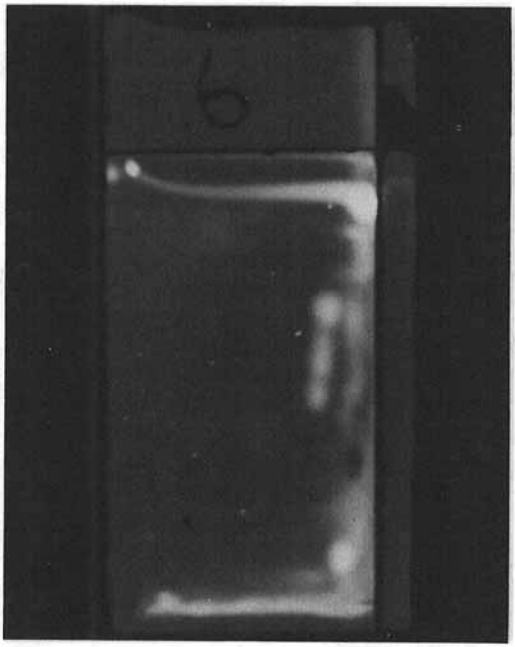

Fig. 2. Separation of aflatoxins from interfering substances using a second development with 95% ethanol. The corn sample contained naturally occurring aflatoxin B_1 and was spiked with 20 ppb each of B_1, B_2, G_1, and G_2. Location of aflatoxins in descending order: B_1, B_2, G_1, and G_2.

TABLE II
Reproducibility of Estimating Aflatoxins in One Moldy Corn Sample by Two-Dimensional tlc

Sample Number[a]	Aflatoxin B_1, ppb		
	Investigator		
	1	2	3
1	353	379	293
2	299	336	341
3	374	347	340
4	343	353	342
5	345	361	372
6	362	382	368
Average, ppb	346	360	343
Std. deviation, ppb	25.7	18.1	28.2
Coef. of variability, %	7.4	5.2	8.1

[a]Six subsamples were taken from one large sample of moldy corn, which had been thoroughly mixed and ground.

TABLE III
Comparison of Aflatoxin Values Obtained in Two Moldy Corn Samples by Two Aflatoxin Procedures

Sample	Aflatoxins Observed	Aflatoxin, ppb	
		2-D tlc procedure[a]	Eppley procedure[b]
A	B_1	299	326
	B_2	16	18
B	B_1	191	242
	B_2	7	15

[a]Average of three investigators.
[b]Average of two determinations. Procedure performed by NRRL in Peoria, Ill.

RESULTS AND DISCUSSION

The advantage of using this procedure can be seen by comparing Figs. 1 and 2. After one-dimensional development with acetone–chloroform (Fig. 1), aflatoxins were difficult to detect because of interfering colored substances. After a second development with reagent alcohol (Fig. 2), aflatoxins were well separated from interfering substances and could easily be detected and quantitative values determined.

Once the utility of the procedure had been demonstrated, a measure of the accuracy and precision of the procedure was obtained. Results with spiked corn samples (Table I) indicated that an accuracy of about ±20% of the theoretical amounts present could be expected.

Reproducibility or precision of the procedure was determined by analyzing a moldy corn sample six times. Results shown in Table II indicated agreement among the three investigators within 5% of the 350 ppb average. The lowest and highest estimates were 84 and 109% of the average, respectively, indicating similar accuracy to that obtained with spiked corn samples (Table I).

The procedure was then compared to results obtained with a second aflatoxin method. Two samples of moldy corn were submitted to O. L. Shotwell of the Northern Regional Research Laboratory in Peoria, Ill. Shotwell employed a modification of the procedure of Eppley (2). The data shown in Table III indicated that fairly comparable values were obtained by the two methods.

Visual estimation of the intensity of aflatoxin B_2 in the presence of high levels of B_1 was somewhat difficult. Possibly this accounted for the low estimate of B_2 with sample B in Table III. The lower values obtained for both B_1 and B_2 may be related to our use of 100% methanol rather than aqueous methanol as the extraction solvent.

CONCLUSION

The data we have presented here suggest that a useful procedure for measuring aflatoxins in moldy cereal products has been developed. The procedure is fairly rapid, taking about 1 hr to obtain quantitative values with one sample; about 2 hr

with four or five samples.

The basic method is also fairly versatile. It can be used with one-dimensional development to detect aflatoxins in normal corn products and with two-dimensional development with samples containing high levels of interfering substances. In addition, we have preliminary data suggesting that zearalenone can be determined using the method.

It is our intention to further examine this procedure and compare it more extensively with existing approved methods for mycotoxins.

Acknowledgments

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