APPLIED MICROBIOLOGY, Jan., 1967, p. 186-190

Vol. 15, No. 1
Printed in U.S.A.

Production of Aflatoxin on Wheat and Oats: Measurement with a Recording Densitometer

R. D. STUBBLEFIELD, O. L. SHOTWELL, C. W. HESSELTINE, M. L. SMITH, AND H. H. HALL

Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois

Received for publication 12 September 1966

ABSTRACT

A method has been developed for the production of aflatoxin by growing *Aspergillus flavus* NRRL 3145 on solid substrate wheat. Optimal yields of 900 μg of aflatoxin G_1 and 900 μg of aflatoxin B_1 per g of substrate were obtained in 4 to 5 days at 28 C. A study of aflatoxin production on hulls and groats of oats and on whole oats by *A. flavus* strains NRRL 2999, NRRL 3000, and NRRL 3145 revealed that aflatoxin was produced on all three substrates, although production was very slight on hulls. Strain NRRL 3145 grown on solid substrate groats produced the largest amounts of aflatoxin: 580 μg of B_1 and 450 μg of G_1 per g of substrate. A densitometric method for reading thin-layer chromatographic plates is described; this is more objective and more accurate than the visual methods previously used for the determination of all four aflatoxins.

The hepatotoxic metabolites known as aflatoxins (B_1, B_2, G_1, and G_2) have been produced on several agricultural commodities by certain strains of *Aspergillus flavus*. Feeds containing aflatoxin caused the deaths of approximately 100,000 turkey poults in 1960 (2) and hepatoma formation in trout (1). Consequently, there is concern as to the effects of aflatoxin on larger domestic animals. A method was needed to produce the large quantities required to carry out the necessary feeding trials. Peanuts (6), crushed wheat (5), and corn meal (11) have been used as substrates in aflatoxin production. Hesseltine et al. (9) reported production on rice, wheat, corn, soybeans, and sorghum. They found that rice was the best substrate for producing aflatoxin B_1 and that wheat was the best for aflatoxin G_1. As a continuation of these studies, Shotwell et al. (13) described a method for obtaining large quantities of B_1 on rice; the moldy rice could be used either as is for feeding trials or for isolating the aflatoxin.

Since the production of aflatoxin on oats had not been investigated and since wheat is a good substrate for the production of aflatoxin G_1, we extended our studies to include these agricultural commodities. This paper describes the production of aflatoxin on oats and a method for making large quantities of G_1 on wheat.

In connection with these studies, a method for reading thin-layer chromatographic plates was developed; this method was more objective and more accurate than visual comparison methods. The densitometric method of Ayres and Sinnhuber (4) was the starting point for the procedure we developed that permits accurate determination of all four aflatoxins.

MATERIALS AND METHODS

Cultures. The three strains of *A. flavus* used in this study came from the Commonwealth Mycological Institute (CMI) and were isolated from peanuts. NRRL 2999 was CMI 91019b (Austwick's strain V. 3734/10, isolated from Uganda peanuts). NRRL 3000 was CMI 89717 and came from Tropical Products Institute as T-12, isolated from Uganda peanuts. NRRL 3145 (also NRRL A-11,613) was CMI 93080 (Austwick's V. 4065/4 from South African peanuts).

Fermentation. Conley hard red spring wheat, used as the substrate for NRRL 3145, was pearled to get maximal growth of the fungus. This mechanical treatment abraded the surface of the kernels but did not completely remove the bran nor break the kernels. The treated wheat was weighed dry into 300-ml Erlenmeyer or 2.8-liter Fernbach flasks, and tap water (50 ml per 100 g of wheat) was added. The mixture was allowed to stand covered at room temperature overnight. The wheat absorbed all the water, but the kernels were still readily separated from one another. Flasks were sterilized for 20 min at 15 psi in an autoclave, cooled, and inoculated with a spore suspension (0.5 ml per 25 g of wheat). The spore suspension was prepared by adding 6 ml of sterile tap water to a sporulated culture incubated for at least 7 days on a Malt Extract Agar slant at 25 C.

After inoculation, the flasks were placed on a Gump shaker (200 rev/min) in an incubator (28 C). Sterile

water was added to the Erlenmeyer flasks at 24 and 48 hr (1 ml per 25 g of wheat) and to the Fernbach flasks at 24, 48, 68 (1 ml per flask), and 76 hr (0.5 ml per flask). The flasks were harvested on the fourth day, and were briefly steamed to destroy the fungus.

Whole oats (50 g), groats (50 g), and hulls (25 g) were weighed into 300-ml Erlenmeyer flasks and treated with water as described for wheat. Substrates were inoculated, in duplicate, with spore suspensions (0.5 ml per 25 g of substrate) of NRRL 2999, NRRL 3000, and NRRL 3145. The spore suspensions were prepared like those for wheat, except that NRRL 2999 and NRRL 3000 were grown on potato-dextrose-agar slants (13). The flasks were incubated on the Gump shaker (200 rev/min) at 28 C. Sterile tap water (1 ml) was added to each flask at 24, 48, and 72 hr. Flasks were harvested on the sixth day and steamed.

Assay procedure. Moldy wheat (25 g) was extracted by mixing for 5 min with 250 ml of water in a Waring Blendor, followed by mixing for 5 min with 250 ml of chloroform (10). Moldy groats (25 g), whole oats (25 g), and hulls from 25 g of substrate were extracted by the same procedure and by Lee's (10) alternate method (used for samples that form emulsions with chloroform-water). In the alternate method, each oat sample was blended for 5 min with 25 ml of water and 250 ml of chloroform. The blended mixtures from both methods were centrifuged for 15 min at 3,000 rev/min. After filtration through cheesecloth into separatory funnels, the chloroform layers were treated with anhydrous sodium sulfate (20 to 25 g each) to remove water. Sodium sulfate was collected on Büchner funnels, and the extracts were concentrated in vacuo on a rotary evaporator. Residues were made up to 10 ml with chloroform for thin-layer chromatography.

Thin-layer chromatographic plates (20 by 20 cm) were prepared by the method of Pons and Goldblatt (12). Plates were coated by mixing 30 g of Silica Gel G-HR (Brinkmann Instruments, Inc., Westbury, N.Y.) with water (64 ml) in a blendor for 30 sec and spreading to 0.250-mm thickness. The solvent system was a modification of that described by the Association of Official Analytical Chemists (3). Benzene-95% ethyl alcohol-water (46:35:19; v/v) was shaken vigorously for 5 min in a separatory funnel and allowed to settle for 30 to 60 min. The upper layer of solvent was equilibrated in an unlined chromatography tank (20 by 20 by 10 cm) for 30 min. Samples (5 μliters) of several dilutions of chloroform extract and standard aflatoxin solutions (5 μliters) were spotted on thin-layer plates with microsyringes (Hamilton Co., Inc., Whittier, Calif.) and developed 10 cm in the upper layer. The dried plate was viewed under a Chromato-Viewer (Ultraviolet Products, Inc., San Gabriel, Calif.) to determine the proper dilution. Diluted extract (2, 3, and 4 μliters) and standard aflatoxin (2, 3, and 4 μliters; 2.4, 0.61, 2.1, 0.42 μg of B_1, B_2, G_1, and G_2 per ml, respectively) were developed 12 cm on thin-layer plates in the same solvent and read by densitometry.

Densitometry. A TLC Densitometer (model 530; Photovolt Corp., New York, N.Y.) was used to read the thin-layer plates. This unit consisted of the following apparatus: densitometer (model 520-A) equipped with an automatic-scanning thin-layer plate stage composed of a search unit (model 52-C) with an ultraviolet light source (320 to 390 mμ), a primary filter (365 mμ), a secondary filter (445 mμ maximum), and a primary slit (0.1 by 15 mm); a varicord Recorder (model 42-B) equipped with a 66-tooth gear to drive the chart; and an Integraph automatic integrator (model 49). Distance between plate and search unit was adjusted to 1 mm, and the response setting was set at 1.

The developed plate was placed face down on the stage, with the origin on the left. The densitometer was adjusted by hand to the center of the 4-μliter standard B_1 zone containing 9.8×10^{-3} μg for the highest reading and peak. The height of this peak was arbitrarily set so that other samples which had B_1 zones of slightly greater intensity would be completely recorded on the chart. The densitometer was adjusted to the lowest background at which no fluorescence occurred, to obtain a base line. The fluorescent zones were centered inside the 15-mm slit of the search unit so that the path of development would be scanned. The stage was set at the origin of the plate, and the plate was scanned automatically toward the solvent front. All experiments were carried out in a darkened room to protect the aflatoxins from decomposition and to prevent erratic readings of the densitometer.

From the tracings on the chart, the integrated areas of the unknowns were evaluated by comparison with the integrated areas of the standards. Routinely, two of the three paths of each unknown and standard were scanned. The third path was scanned if the other two were not in close agreement. Unknowns were calculated for aflatoxin concentration in the following manner.

(i) The intersect of tangents for slopes of adjacent curves were determined, and lines were dropped perpendicular to the integration line. Units between the perpendicular lines were counted to measure the area under each curve.

(ii) The average number of units per microliter spotted for the unknowns was calculated and compared to the same value for the standards. The following formula was used to determine the concentration of aflatoxin B_1 in the unknowns: micrograms of aflatoxin B_1 per gram of substrate = $(U_u/U_s) \times C_s \times (V/W_e) \times (W_p/W_0)$, where U_u = average number of units of B_1 in unknown extract per μliter; U_s = average number of units of B_1 in standard per μliter; C_s = concentration of standard B_1 (micrograms per microliter); V = final volume (microliters) of diluted unknown extract; W_e = weight (grams) of unknown extracted; W_p = weight (grams) of moldy product; W_0 = weight (grams) of substrate. Concentrations of aflatoxins B_2, G_1, and G_2 were calculated similarly.

Isolation. A product was obtained by growing *A. flavus* NRRL 3145 on wheat [3,600 g in 12 Fernbach flasks (2.8 liter)] and recovered by extraction with chloroform. The moldy wheat was steeped twice in chloroform (1.5 ml per g of wheat) for 24 hr. Extracts were filtered through cheesecloth, combined, and concentrated in vacuo to 450 ml. Anhydrous sodium sulfate (100 g) was added to remove water. After removing sodium sulfate by filtration, the clarified filtrate was concentrated in vacuo to 150 ml and added

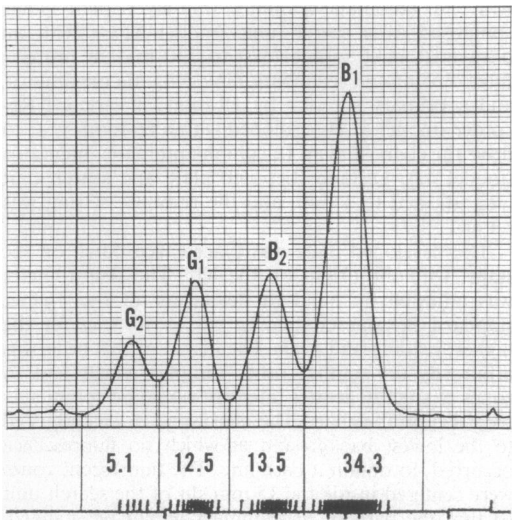

FIG. 1. *Typical densitometric recording of an aflatoxin sample, showing the four aflatoxin peaks and the integrated areas under each.*

TABLE 1. *Comparison of aflatoxin concentrations determined by densitometric and visual methods*

Aflatoxin	Aflatoxin concn (μg/ml)	
	Densitometer	Visual
B_1	379 ± 5.5[a]	360 ± 56[a]
B_2	11 ± 0.2	11 ± 2.2
G_1	34 ± 0.7	33 ± 4.8
G_2	1.7 ± 0.1	<3.25

[a] These values represent average deviation.

to 10 volumes of *n*-hexane to precipitate crude aflatoxin. The precipitate was dried overnight in a hood and finally in vacuo.

RESULTS AND DISCUSSION

A typical densitometric recording of an aflatoxin sample (Fig. 1) shows the four aflatoxin peaks and a small peak preceding G_2 (attributed to an impurity). This impurity would not be seen under conditions of the usual visual methods or would appear as part of G_2 if resolution was incomplete. Integrated areas are easily counted with the 66-tooth gear in the recorder. Base-line variation caused by varying silica gel thickness across the plate was negligible. Resolution of the aflatoxins is a necessary requirement in both the densitometric and visual methods. The upper layer of the benzene-ethyl alcohol-water solvent proved superior to the various methanol-chloroform solvents now being used.

In Table 1 is shown a comparison of the aflatoxin concentrations in chloroform extracts as determined by densitometric and visual methods. The data reveal the greater accuracy achieved by the densitometric method. Usually, average deviation in values determined for B_1 and G_1 is 2 to 3%. Since concentrations of B_2 and G_2 tend to be quite low, the smaller counts lead to larger average deviations (5%). Visual methods commonly used are said to be accurate within 20%. Low concentrations can be accurately deter-

TABLE 2. *Production of aflatoxin on wheat in 300-ml Erlenmeyer flasks by Aspergillus flavus NRRL 3145*[a]

Time	Aflatoxin production (μg/g of substrate)[b]			
	B_1	B_2	G_1	G_2
days				
0	ND[c]	ND	ND	ND
1	0.27, 0.20	0.024, ND	0.56, 0.49	ND, ND
2	220, 182	ND, ND	272, 269	35, ND
3	695, 781	73, 74	671, 614	76, 52
4	930, 677	96, 80	875, 691	62, 45
5	774, 739	86, 107	878, 632	54, 41
6	657, 602	61, 65	647, 427	16, 10
7	647, 663	85, 111	517, 584	18, 44
8	636, 693	90, 100	409, 465	19, 42
9	428, 581	42, 72	249, 457	10, 16

[a] Wheat (25 g) incubated at 28 C on a New Brunswick rotary shaker, 200 rev/min (in duplicate).

[b] As determined by thin-layer chromatography and densitometry.

[c] Not detected.

TABLE 3. *Production of aflatoxin on wheat in 2.8-liter Fernbach flasks by Aspergillus flavus NRRL 3145*[a]

Time	Aflatoxin production (μg/g of substrate)[b]			
	B_1	B_2	G_1	G_2
days				
0	ND[c]	ND	ND	ND
1	0.68, 1.54	ND	0.45, 0.77	ND
2	177, 184	ND	256, 224	ND
3	526	61	549	98
4	514	60	524	15
5	555	90	397	49
6	418	45	182	8
7	212	73	87	20
8	171	36	61	ND

[a] Wheat (150 g) incubated at 28 C on a New Brunswick rotary shaker, 200 rev/min.

[b] As determined by thin-layer chromatography and densitometry.

[c] Not detected.

TABLE 4. *Production of aflatoxin by strains of Aspergillus flavus on oats*[a]

Substrate	Extraction method[b]	NRRL 3145				NRRL 3000				NRRL 2999			
		B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Hulls	1	3.5	0.8	5.5	1.0	0.15	0.08	0.11	0.08	1.2	0.3	0.5	Trace
	2	4.2	0.8	6.2	1.0	0.37	0.15	0.42	0.15	1.9	0.3	0.9	0.2
Groats	1	563	174	374	84	37	9	11	Trace	440	168	172	36
	2	593	278	510	247	35	11	3	Trace	481	124	181	Trace
Whole oats	1	270	63	429	103	77	14	93	7	202	74	213	40
	2	282	5	483	143	77	22	85	11	288	58	272	54

[a] Hull (25 g), groats (50 g), and whole oats (50 g) were incubated, in duplicate, in 300-ml Erlenmeyer flasks for 6 days at 28 C on a Gump shaker, 200 rev min. Production (micrograms per gram of substrate) was determined by thin-layer chromatography and densitometry.

[b] Method 1: sample blended 5 min each with 250 ml of water and 250 ml of chloroform; method 2: sample blended 5 min with 25 ml of water and 250 ml of chloroform.

mined by the densitometer, as shown for G_2 in Table 1. As long as the peaks can be fully scribed on the chart, concentrations can be calculated. All four aflatoxins can usually be determined from one dilution by the densitometer, whereas two or more dilutions may have to be chromatographed for the visual method.

Yields of aflatoxin produced on wheat by NRRL 3145 in 300-ml Erlenmeyer and 2.8-liter Fernbach flasks are shown in Tables 2 and 3. Peak yields of aflatoxin G_1 were obtained in 4 to 5 days in Erlenmeyer (875 μg per g of substrate) and Fernbach flasks (549 μg per g of substrate). Maximal production of aflatoxin B_1 occurred in 4 to 5 days in both Erlenmeyer and Fernbach flasks (930 and 555 μg per g of substrate, respectively). A total of 1,950 μg of aflatoxin per g of substrate was produced in Erlenmeyer flasks and 1,250 μg of aflatoxin per g of substrate in Fernbach flasks. Yields of aflatoxin G_1 decreased in Erlenmeyer flasks (500 μg per g of substrate) and in Fernbach flasks (200 μg per g of substrate) when the substrate was increased to 75 g and 300 g, respectively; however, the yield of aflatoxin B_1 was not affected. Indented Fernbach flasks had no effect on aflatoxin production.

Amounts of aflatoxin G_1 produced by NRRL 3145 on wheat (1,000 μg per g of substrate) were equal to those of aflatoxin B_1 produced by NRRL 2999 on rice (13). More total aflatoxin (1,950 μg per g of substrate) was produced on wheat than on rice (1,510 μg per g of substrate). Wheat could easily be used to produce mixtures of aflatoxin for feeding-trials with animals or for isolating quantities of aflatoxin G_1.

Production studies of aflatoxin by *A. flavus* strains NRRL 3145, NRRL 3000, and NRRL 2999 on oats (Table 4) show uniformity among duplicate flasks. The two methods of extraction removed the same amounts of material, but the method using less water was more convenient. Groats inoculated with NRRL 3145 produced the largest amounts of aflatoxin B_1 (578 μg per g of substrate). Highest yields of aflatoxin G_1 (440 to 450 μg per g of substrate) were produced by NRRL 3145 on groats and whole oats. Lowest yields were obtained when flasks were inoculated with NRRL 3000. Although aflatoxin was not expected to be produced on hulls, small quantities were detected. These quantities are considered to range from toxic to very toxic (7). All three strains produced more aflatoxin on groats than on whole oats. Large quantities of aflatoxin B_2 or G_2 were not produced on oats. None of the strains produced on oats the quantities of aflatoxin B_1 that NRRL 2999 produced on rice or of aflatoxin G_1 that NRRL 3145 produced on wheat.

ACKNOWLEDGMENTS

We appreciate the assistance of Carl R. Martin in pearling the wheat and in supplying the hulls, groats, and whole oats for the fermentations. We also thank Gail M. Shannon and Elsie E. Vandegraft for technical assistance.

LITERATURE CITED

1. ANONYMOUS. 1964. Hepatomas in trout. Nutr. Rev. **22**:208–210.
2. ALLCROFT, R., AND R. B. A. CARNAGHAN. 1963. Toxic product in groundnuts. Biological effects. Chem. Ind. (London), p. 50–53.
3. ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS. 1966. Changes in official methods of analysis. J. Assoc. Offic. Anal. Chemists **49**:231.
4. AYRES, J. L., AND R. O. SINNHUBER. 1966. Fluorodensitometry of aflatoxin on thin-layer plates. J. Am. Oil Chemists' Soc. **43**:423–424.
5. CHANG, S. B., M. M. ABDEL KADER, E. L. WICK, AND G. N. WOGAN. 1963. Aflatoxin B_2: chemical identity and biological activity. Science **142**:1191–1192.
6. CODNER, R. C., K. SARGEANT, AND R. YEO. 1963.

Production of aflatoxin by the culture of strains of *Aspergillus flavus-oryzae* on sterilized peanuts. Biotechnol. Bioeng. **5**:185–192.

7. COOMES, T. J., P. C. CROWTHER, B. J. FRANCIS, AND L. STEVENS. 1965. The detection and estimation of aflatoxin in groundnuts and groundnut materials. Part IV. Routine assessment of toxicity due to aflatoxin B_1. Analyst **90**:492–496.
8. HESSELTINE, C. W. 1965. A millennium of fungi, food, and fermentation. Mycologia **57**:149–197.
9. HESSELTINE, C. W., O. L. SHOTWELL, J. J. ELLIS, AND R. D. STUBBLEFIELD. 1966. Aflatoxin formation by *Aspergillus flavus*. Bacteriol. Rev. **30**:795–805.
10. LEE, W. V. 1965. Quantitative determination of aflatoxin in groundnut products. Analyst **90**: 305–307.
11. MERWE, K. J. VAN DER, L. FOURIE, AND DE B. SCOTT. 1963. On the structure of the aflatoxins. Chem. Ind. (London), p. 1660–1661.
12. PONS, W. A., JR., AND L. A. GOLDBLATT. 1965. The determination of aflatoxin in cottonseed products. J. Am. Oil Chemists' Soc. **42**:471–475.
13. SHOTWELL, O. L., C. W. HESSELTINE, R. D. STUBBLEFIELD, AND W. G. SORENSON. 1966. Production of aflatoxin on rice. Appl. Microbiol. **14**:425–428.