Objective Fluorometric Measurement of Aflatoxins on TLC Plates[1]

WALTER A. PONS, JR., JAMES A, ROBERTSON and LEO A. GOLDBLATT,
Southern Regional Research Laboratory,[2] New Orleans, Louisiana

Abstract

Measurement of the solid state fluorescence of aflatoxins on silica gel-coated TLC plates on a densitometer equipped for fluorescence measurements showed a linear relationship between peak areas and concentration over a range of at least 2 to 105×10^{-4} μg of aflatoxins per spot. Response of individual aflatoxins was in order of $B_2 > G_2 > B_1 > G_1$. Aflatoxins can be measured with a precision of ±2–4%.

Introduction

Most analytical procedures for the estimation of aflatoxins in agricultural products utilize thin-layer chromatography (TLC) of partially purified sample extracts on silica gel-coated plates for the separation and resolution of individual aflatoxins. Developed plates are examined under near ultraviolet radiation (long-wave, 365 mμ) and aflatoxin concentrations are estimated by visual comparison of the fluorescence intensities of the aflatoxin spots in sample aliquots with those of appropriate aflatoxin B_1 or G_1 standards chromatographed on the same plate (1–4).

The fluorescence of aflatoxins allows the detection of as little as 3 to 4×10^{-4} μg of aflatoxin B_1 or G_1 on a TLC plate (5), and thus comprises the basis of an extremely sensitive method. Due primarily to the difficulty of estimating small differences in fluorescence intensity with the eye, visual analysis is accurate to no more than about ±20% (1,6). Both the accuracy and precision of aflatoxin analyses would undoubtedly be enhanced by a more objective instrumental measurement of the solid state fluorescence of aflatoxins directly on a TLC plate. Recently Ayres and Sinnhuber (7) proposed an instrumental procedure for the determination of aflatoxin B_1 on TLC plates, using a densitometer equipped for fluorescence measurements. Their technique was applied only to aflatoxin B_1, and the logarithmic relationship between emitted fluorescence energy and concentration was found to be linear over a concentration range of about 2.5 to 15×10^{-4} μg of B_1 per spot (7). The present study was undertaken to explore more fully the parameters involved in the measurement of the solid state fluorescence of aflatoxins on TLC plates. The conditions for satisfactory resolution of individual aflatoxins, the linearity of emitted fluorescence to concentration, the relative response of individual aflatoxins, and the precision of solid state fluorescence measurements were investigated.

Equipment and Procedure

Densitometer

Photovolt Model 530 equipped with 320–390 mμ near UV source and primary fluorimetry filter, 6×19 mm inlet aperture, 6×0.1 mm exit slit, 445 and 465 mμ secondary filters, UV sensitive photomultiplier tube, and with TLC stage equipped for both manual (1 mm steps) and automatic (1 in./min) scanning. The stage was modified by replacement of the standard rounded tongue with an 8.5-in. long aluminum T-bar to allow better lateral alignment of plates. A Model 520-A multiplier-photometer, Varicord 42-B variable response recorder equipped with a 66-tooth motor gear for 3 in./min chart drive, and an Integraph Model 49 automatic integrator completed the assembly. Recorder leads from the multiplier-photometer were reversed to allow use of the recorder dark point control for the chart baseline setting, and for presentation of recorder traces ranging from 0 to 100 with increasing concentration. The equipment was operated in a dimly illuminated room.

TLC Conditions

Standard 20×20 cm plates coated with a 500-μ layer of silica gel G-HR (3) were used. Pure crystalline aflatoxins (8) were dissolved in ACS chloroform, and suitable aliquots representing concentrations in the range of 2 to 120×10^{-4} μg of individual aflatoxins were spotted 2 cm apart along a line 4 cm from the bottom of a plate. For measurements of all four aflatoxins a standard solution containing approximately 1.0 μg B_1, 0.3 μg B_2, 1.0 μg G_1 and 0.3 μg of G_2 per milliliter was found to yield satisfactory area responses for each aflatoxin. Aliquots of a standard solution containing 0.6 μg of B_1 and 0.4 μg of G_1 per milliliter were usually employed as plate reference standards. After spotting the aliquots, a line was scribed across the top of the plate 14 cm beyond the origin, about 0.5 cm of the gel coating was removed from the side edges of the plate, and the plate was developed in the dark in either chloroform:acetone (85:15 v/v), or chloroform:acetone:2-propanol (825:150:25 v/v), in an unlined and unequilibrated Desaga-Brinkmann glass tank. When the solvent front reached the scribed line (14 cm), the plate was removed, and air dried in the dark for 30 min prior to scanning.

Fluorescence Measurements and Calculations

About 0.5 in. of the gel was removed from the top and bottom edges of a developed plate, and small protective guides (made from 26-gauge aluminum, 7.5 in. long × 0.5 in. wide with a 5/32 in. channel) were slipped over the top and bottom of the plate. The plate was placed on the stage with the gel layer facing downwards, to minimize absorption of near UV radiation, and then securely butted against the T-bar. With the multiplier photometer at maximum amplification (Position 3) and the recorder at the linear response setting (Position 1), the aflatoxin B_1 spot of the plate reference standard was visually aligned over the inlet aperture, and the search unit was lowered until the exit slit was about 1 mm above the glass plate surface. Using the recorder full light control the recorder was quickly adjusted to about 60% full scale, the stage manually racked downwards until a blank zone just above the B_1 spot was

---

[1] Presented at the AOCS Meeting, Los Angeles, April 1966.
[2] So. Utiliz. Res. Dev. Div., ARS, USDA.

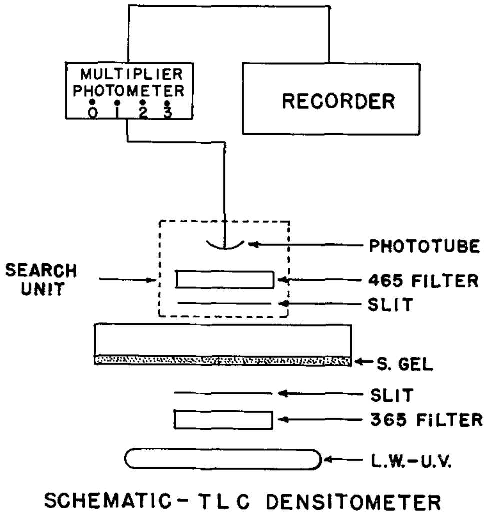

FIG. 1. Schematic diagram of the densitometer.

located over the exit slit, and the recorder baseline then adjusted to a chart value of 5 with the dark point control. The B_1 spot was again located over the exit slit and the stage manually racked backwards or forwards, in 1-mm increments, along the plate Y axis for maximum recorder response. If necessary, the full light control was used to readjust the recorder response to the approximate 60% full-scale setting. The plate was carefully moved laterally, by hand, to obtain maximum recorder response along the plate X axis, taking care that the plate was securely butted against the T-bar at all times. These critical adjustments served to align the spot for maximum response, and to ensure symmetrical recorder peaks. The plate was locked in place with the stage spring clips, and the recorder then adjusted to a chart value of 85% full scale with the full light control. A blank zone just above the B_1 spot was again located over the exit slit and, if necessary, the baseline was readjusted to a chart value of 5. The recorder chart drive was activated, the stage set for automatic scan, and the plate was scanned from just above the B_1 zone downwards toward the origin. All operations prior to the scan were performed as quickly as possible to ensure minimum exposure of aflatoxins to near UV radiation. Areas under the recorder peaks were determined by triangulation or by automatic integration. For the former, the chart drive was 2 in. per minute; for the latter it was 3 in. per minute.

Once the recorder scale expansion was set for the plate reference standard, no further recorder adjustments were made during the scan of other aflatoxin spots on the same plate. For other aliquots spotted on the plate, the B_1 spot in each case was manually aligned for maximum response, as outlined above.

TABLE I

R_f Values for Aflatoxins in Two Solvents

Aflatoxin	C:A[a]		C:A:P[b]	
	Lined tank[c]	Unlined tank[d]	Lined tank[c]	Unlined tank[d]
	R_f	R_f	R_f	R_f
B_1	0.44	0.61	0.54	0.73
B_2	0.39	0.53	0.48	0.66
G_1	0.34	0.46	0.42	0.59
G_2	0.31	0.39	0.37	0.52

[a] $CHCl_3$: acetone (85:15 v/v).
[b] $CHCl_3$: acetone: 2-propanol (825:150:25 v/v).
[c] Lined with filter paper and equilibrated, time = 40 min, 14-cm front.
[d] Without liner, unequilibrated, time = 60 min, 14-cm front. R_f values from recorder traces, measured from the midpoint of each peak. Silica gel G-HR, 500 μ, used for all plates.

The micrograms of aflatoxin in a given unknown solution, using B_1 as an example, were determined from the following relationship

$$Qx = \frac{(A_x)(V_s)(C_s)(S.D.)}{(A_s)(V_x)}$$

where Qx indicates μg of B_1 in the unknown solution; Ax, area under recorder peak for unknown B_1 spot; Vs, volume of B_1 reference standard spotted, in μl; Cs, concentration of B_1 in reference standard, in μg per μl; S.D., volume of unknown solution, in μl; A_s, area under recorder peak for B_1 reference standard spot; V_x, volume of unknown solution spotted on the plate, in μl.

The amounts of aflatoxins B_2, G_1 and G_2 were calculated as for B_1, using suitable B_2, G_1 and G_2 plate reference standards.

Experimental

A schematic outline of the basic system is shown in Figure 1. The plate is positioned over the UV source, with the gel layer facing down. Both the unabsorbed near UV excitation energy and the emitted visible fluorescence of the excited aflatoxin spots pass through the glass plate, the exit slit, and the secondary filter, where the near UV is screened out. The fluorescence energies of the aflatoxin spots are then suitably amplified and recorded.

TLC Separation

Preliminary experiments indicated that complete resolution of individual aflatoxins in the TLC step was a critical variable. With plates coated with a 500 μ layer of silica gel G-HR (6), chloroform: methanol (1,3) or chloroform:acetone development solvents in lined and equilibrated chambers yielded unsatisfactory resolution and subsequent overlapping of recorded peaks. However, using an unlined and unequilibrated chamber, a chloroform:acetone (85:15 v/v) solvent similar to that recommended by Engebrecht et al. (6), or a chloroform:acetone:2-propanol (825:150:25 v/v) solvent, yielded complete separation of individual aflatoxins, marked by return of the B_1, B_2, G_1 and G_2 peaks essentially to baseline (Fig. 2).

The effect of lined and unlined chambers on the resolution of aflatoxins is illustrated by the R_f values shown in Table I. The improved resolution in unlined chambers is in agreement with results recently reported by Eppley (9). In unlined chambers R_f values are higher and development time is increased from 40 min to 60 min for a 14-cm development. R_f values in unlined chambers usually exhibited more variability, as compared to those in lined and equilibrated chambers.

Area vs. Concentration

An essentially linear relationship between emitted fluorescence of aflatoxins and concentration, over a reasonable concentration range, is a desirable feature for analysis. This would obviate the necessity for chromatographing and measuring a series of standards on each TLC plate. To study this parameter, portions of an aflatoxin standard solution containing 30×10^{-4} μg of B_1 and 20×10^{-4} μg of G_1 per microliter were diluted with chloroform to provide four standards ranging from 3 to 15×10^{-4} μg of B_1 and 2 to 10×10^{-4} μg of G_1 per microliter. Aliquots of each diluted standard, ranging from 1 to

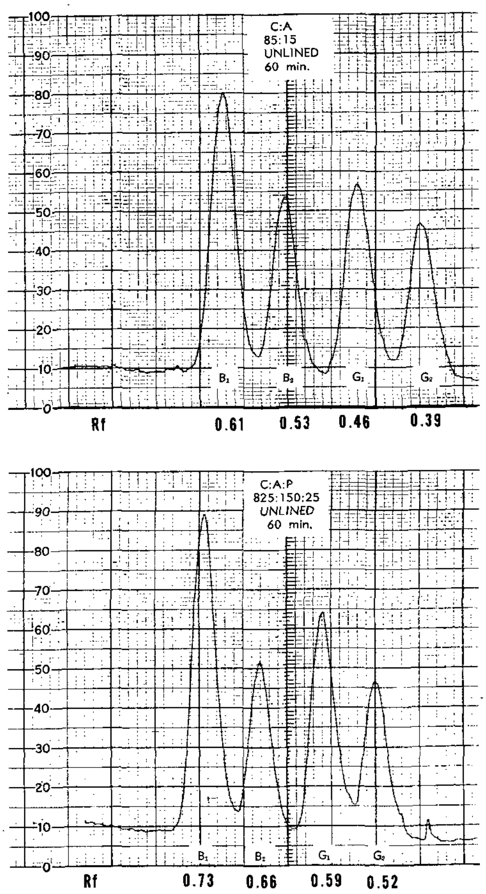

FIG. 2. Recorder traces of TLC plates. Aflatoxins developed in two solvents in unlined tanks.

8 µl, were spotted on TLC plates and developed as previously outlined. For each plate, the aflatoxin B_1 spot of the 8 µl aliquot was used as the plate reference standard, and the other seven aliquots were scanned in relation to this scale expansion. This was done since the useful concentration range on a given plate yielding recorder responses of 10–90% full scale represented about a 1 to 8 concentration ratio. Areas were determined by triangulation, and suitable factors were used to adjust the area response of each of the four 8-µl plate standards to a common basis. These factors were then employed to adjust the measured areas on the other aliquots, on each of the 4 plates, to a common reference standard basis. The adjusted areas for B_1 spots ranging from 3 to 105×10^{-4} µg are shown in Table II, where it may be noted that the average area per microliter is reasonably constant over this concentration range. Although not shown, similar calculations for G_1 over a range of 2 to 70×10^{-4} µg per spot exhibited a corresponding linear relationship. Inasmuch as these are adequate concentration ranges for analysis, no effort was made to investigate higher ranges although it is probable that the linear relationship would hold over a much wider range of concentrations.

TABLE II
Linearity of Area vs. Concentration for Aflatoxin B_1

µl/diluted standard on plate	Dilution of standard[a] 3×10^{-4} µg/µl	6×10^{-4} µg/µl	12×10^{-4} µg/µl	15×10^{-4} µg/µl	Average area	Area per µl diluted standard
	Adjusted area — mm²					
	Aflatoxin B_1					
1	242	175	174	186	194	194
2	383	331	370	414	375	188
3	542	549	521	578	548	183
4	740	678	717	712	712	178
5	1035	942	897	916	948	190
6	1180	1129	1166	1076	1138	190
7	1421	1342	1267	1330	1340	191
8	b	b	b	b	....	......

[a] 1 Ml aliquots of aflatoxin standard containing 30×10^{-4} µg B_1 and 20×10^{-4} µg of G_1 per µl diluted to 10, 5, 2.5 and 2.0 ml respectively.
[b] 8-µl Aliquot of each standard used to set instrument at full scale, 1600 mm².

TABLE III
Relative Response of Individual Aflatoxins on a TLC Plate

Aflatoxin	Amount spotted	445 Filter Area[a]	445 Filter Area/µg	445 Filter Rel. response	465 Filter Area[a]	465 Filter Area/µg	465 Filter Rel. response
	µg ($\times 10^{-4}$)		$\times 10^{-4}$			$\times 10^{-4}$	
B_2	33.8	102	3.020	4.9	105	3.108	3.3
G_2	36.1[b]	60	1.664	2.7	102.5	2.843	3.0
B_1	68.2	92	1.253	2.0	92.5	1.357	1.4
G_1	102.4	63	0.615	1.0	97.5	0.952	1.0
Response $B_2:B_1$ =			2.4 : 1			2.3 : 1	
Response $G_2:G_1$ =			2.7 : 1			3.0 : 1	
Response $B_1:G_1$ =			2.0 : 1			1.4 : 1	
Response $B_2:G_2$ =			1.8 : 1			1.1 : 1	

[a] Integrator counts.
[b] Corrected for purity (87%) from molar extinction coefficient.

A similar concentration experiment was conducted with a standard containing all four aflatoxins in which aliquots ranging from 1 to 6 µl and representing concentrations of 10–60 $\times 10^{-4}$ µg of B_1, 2–14 $\times 10^{-4}$ µg B_2, 11–66 $\times 10^{-4}$ µg G_1, and 3–20 $\times 10^{-4}$ µg of G_2 per spot were spotted on each of 9 TLC plates. Plates were developed as described above, and were scanned with reference to a common plate standard representing 60×10^{-4} µg of B_1. A plot of the area vs. concentration relationship, Figure 3, shows a linear relationship for all 4 aflatoxins. The slopes of the curves indicate a relative response in the order $B_2 > G_2 > B_1 > G_1$.

Relative Response

The magnitude of the relative response factors for individual aflatoxins was determined from scans of several TLC plates containing known concentrations of each aflatoxin. Highest purity, crystalline afla-

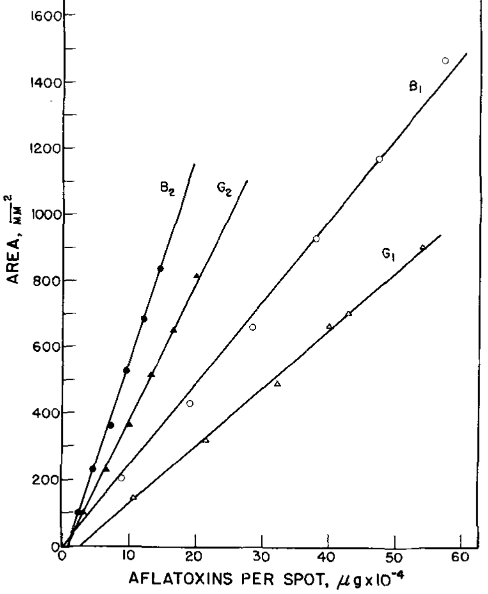

FIG. 3. Linearity of area vs. concentration relationship for aflatoxins B_1, B_2, G_1, and G_2.

TABLE IV

Precision of Fluorescence Measurements for Standard Containing Aflatoxins B_1 and G_1

Property	B_1	G_1
Number of plates, N	9	9
Duplicate aliquots spotted, µl[a]	10	10
Aflatoxin per spot, µg	60×10^{-4}	40×10^{-4}
Peak height as % of full scale	80	35
Standard deviation, µg $\times 10^{-4}$		
From adjusted areas	$\pm 1.3 \times 10^{-4}$	$\pm 1.6 \times 10^{-4}$
From difference between duplicates	$\pm 2.1 \times 10^{-4}$	$\pm 1.6 \times 10^{-4}$
Coefficient variation, %		
From adjusted areas	2.2%	4.1%
From difference between duplicates	3.5%	4.1%

[a] Standard containing 6×10^{-4} µg B_1 and 4×10^{-4} µg G_1 per µl.

toxins (8) were used for the experiment. Plates were scanned using both 445 mµ and 465 mµ secondary filters supplied as accessories with the densitometer. The results, listed in Table III, indicate that the magnitude of the relative response values is influenced by the transmission characteristics of the secondary filter. The 445 filter used was found to have a peak transmittance at 435 mµ with a UV cutoff at about 390 mµ, while the 465 filter exhibited peak transmittance at 460 mµ with UV cutoff at 400 mµ. Since aflatoxins B_1–B_2 have fluorescence maxima at about 425 mµ and G_1–G_2 at about 450 mµ (8,10), the 445 filter discriminates in favor of B_1–B_2 with reduction in the G_1–G_2 response. Inasmuch as the 465 filter gave a more nearly equivalent response of B_1:G_1 and B_2:G_2, it was selected for use in the present work. With either filter, the relative response is in the same order $B_2 > G_2 > B_1 > G_1$. The relative response values shown in Table III are not highly reproducible physical constants, as the magnitude of the values was found to vary with the type of silica gel. In experiments with 3 types of silica gels, the response ratio of B_2:B_1 varied from 1.6–2.8, G_2:G_1 from 2.2–3.7, B_1:G_1 from 1.3–1.5, and B_2:G_2 from 1.1–1.2. Thus, the type of gel had a marked effect on the B_2:B_1 and G_2:G_1 fluorescence ratios, while those of B_1:G_1 and B_2:G_2 were reasonably constant. These findings emphasize the necessity for employing suitable plate standards for each type of aflatoxin in quantitative analysis. They also emphasize that the common practice in visual analysis whereby the fluorescence intensity of B_2 and G_2 in unknowns is compared with B_1 and G_1 standards (1–4) introduces significant errors.

Precision

The precision of solid state fluorescence measurements can be influenced by a number of factors including errors in spotting aliquots, variations in day-to-day resolution of aflatoxins with different batches of silica gel, the associated errors in setting necessary *instrument* parameters prior to scanning, and errors in the measurement of peak areas. Precision of the technique under simulated analytical conditions was evaluated using duplicate 10-µl aliquots of a standard containing 0.6 µg of B_1 and 0.4 µg of G_1 per milliliter on each of 9 TLC plates. Each plate was prepared from different batches of gel, and each plate was spotted, developed, and scanned on different days. In each instance the B_1 spot of the first 10-µl aliquot was used as a plate reference standard. The areas of the B_1 and G_1 spots for the second aliquot were adjusted to a common plate standard basis, and the standard deviations of the measurements were calculated. Calculations were also made on the basis of the actual differences in the measured B_1 and G_1 areas of the duplicate aliquots, without regard to adjustment to a common plate standard basis. The results, set forth in Table IV, suggest that on either basis the precision of measurement is in the range of ±2–4% for either B_1 or G_1.

TABLE V

Precision of Fluorescence Measurements for Aflatoxins B_1 and B_2

µl Stand. on plate[a]	2	3	4	5	6
	Aflatoxin B_1				
Peak area as % of plate standard	27	40	53	67	80
Concn. per spot, µg $\times 10^{-4}$	19.0	28.4	37.9	47.4	56.9
Stand. Dev., µg $\times 10^{-4}$	±1.5	±2.5	±2.4	±3.1	±2.1
Coeff. var., %	7.8	8.8	6.4	6.7	3.7
	Aflatoxin B_2				
Peak area as % of plate standard	10	16	24	32	36
Concn. per spot, µg $\times 10^{-4}$	4.8	7.2	9.6	12.1	14.5
Stand. Dev., µg $\times 10^{-4}$	±1.0	±1.1	±1.3	±1.3	±1.6
Coeff. var., %	21.3	15.0	13.1	10.4	11.3
	Aflatoxins $B_1 + B_2$				
Concn. per spot, µg $\times 10^{-4}$	23.8	35.6	47.5	59.5	71.4
Stand. Dev., µg $\times 10^{-4}$	±2.2	±2.8	±2.8	±3.5	±3.4
Coeff. var., %	9.3	7.9	5.9	5.9	4.7

[a] Stand. containing 9.5×10^{-4}µg B_1, 2.4×10^{-4}µg B_2, 1.1×10^{-4}µg G_1, and 3.3×10^{-4}µg G_2 per µl.

TABLE VI

Precision of Fluorescence Measurements for Aflatoxins G_1 and G_2

µl Stand. on plate[a]	2	3	4	5	6
	Aflatoxin G_1				
Peak area as % of plate standard	21	32	43	53	
Concn. per spot, µg $\times 10^{-4}$	21.5	32.3	43.1	53.9	64.6
Stand. Dev., µg $\times 10^{-4}$	±2.8	±3.6	±4.6	±4.3	±5.6
Coeff. var., %	12.9	11.0	10.6	7.9	8.6
	Aflatoxin G_2				
Peak area as % of plate standard	8	13	22	30	
Concn. per spot, µg $\times 10^{-4}$	6.7	10.0	13.3	16.7	20.0
Stand. Dev., µg $\times 10^{-4}$	±1.0	±1.9	±1.2	±1.9	±3.8
Coeff. var., %	15.3	18.7	9.2	11.5	18.8
	Aflatoxins $G_1 + G_2$				
Concn. per spot, µg $\times 10^{-4}$	28.2	42.3	56.4	70.6	84.6
Stand. Dev., µg $\times 10^{-4}$	±3.2	±5.1	±5.2	±4.9	±7.0
Coeff. var., %	11.4	12.1	9.1	7.0	8.3
	Total ($B_1 + B_2 + G_1 + G_2$)				
Concn. per spot, µg $\times 10^{-4}$	52.0	78.0	104.0	130.0	155.9
Stand. Dev., µg $\times 10^{-4}$	±5.1	±7.0	±6.8	±7.5	±9.2
Coeff. var., %	9.9	9.0	6.5	5.8	5.9

[a] Stand. containing 9.5×10^{-4}µg B_1, 2.4×10^{-4}µg B_2, 1.1×10^{-4}µg G_1, and 3.3×10^{-4}µg G_2 per µl.

Similar precision measurements were conducted with a standard solution containing all 4 aflatoxins in which aliquots ranging from 2 to 6 µl were spotted on each of 8 TLC plates. A 10-µl aliquot of a B_1 standard representing 60×10^{-4} µg of B_1 was used as the plate reference standard for each plate. Areas for each aflatoxin measurement were adjusted to a common plate standard basis. The results, shown in Tables V and VI, indicate that generally the precision of measurement increases as the measured aflatoxin areas are 50% or more of the plate standard. It may be noted that the coefficients of variation are somewhat higher than those shown in Table IV where only B_1 and G_1 were measured. It is probable that small differences in day-to-day resolution of individual aflatoxins are responsible for this effect, as the precision of $B_1 + B_2$ and $G_1 + G_2$, as well as total aflatoxins, is generally better than that of the individual aflatoxin measurements.

Aflatoxin Stability

Aflatoxins on silica gel G-HR coated plates were markedly affected by exposure to near UV radiation. Exposure of aflatoxins to low intensity UV sources such as that incorporated in the densitometer used here was found to lead to an initial increase in the fluorescence intensity of both B_1 and G_1, with resultant increase in measured areas. Aflatoxins B_2 and G_2 decreased in fluorescence intensity. The rate of change was different for each aflatoxin, and was related to both intensity of near UV illumination and exposure time. With continued exposure to high intensity sources, all 4 aflatoxins decrease in fluorescence intensity. These effects are being investigated further. With the low intensity UV source used in the present work, about 100 micro watts per square centimeter, these changes are minimized during the momentary exposure necessary for the adjustment of instrument parameters. For most accurate solid state fluorescence measurements, TLC plates should be developed and dried in the dark, and should not be exposed to powerful near UV viewing sources prior to scanning. Once exposed and scanned, plates are no longer suitable for accurate quantitative measurements.

ACKNOWLEDGMENTS

Helpful suggestions on instrumentation provided by R. O. Sinnhuber: technical assistance by A. O. Franz, Jr.

REFERENCES

1. Nesheim, S., J. Assoc. Offic. Agr. Chemists *47*, 1010–1017 (1964).
2. AOAC Official Methods of Analysis. Sec. 25, Nuts and Nut Products, J. Assoc. Offic. Anal. Chemists *49*, 229–231 (1966).
3. Pons, W. A., Jr., and L. A. Goldblatt, JAOCS *42*, 471–475 (1965).
4. Huesinkveld, M. R., C. C. Shera and F. J. Baur, J. Assoc. Offic. Agr. Chemist *48*, 448–449 (1965).
5. Coomes, T. J., P. C. Crowther, B. J. Francis and L. Stevens, Analyst *90*, 492–496 (1965).
6. Engebrecht, R. H., J. L. Ayres and R. O. Sinnhuber, J. Assoc. Offic. Agr. Chemists *48*, 815–818 (1965).
7. Ayres, J. L., and R. O. Sinnhuber, JAOCS *43*, 423–424 (1966).
8. Robertson, J. A., W. A. Pons, Jr., and L. A. Goldblatt, in preparation.
9. Eppley, R. M., J. Assoc. Offic. Anal. Chemists *49*, 473–474 (1966).
10. Carnaghan, R. B. A., R. D. Hartley and J. O'Kelley, Nature *200*, 1101 (1963).

[Received August 17, 1966]