Computerized System to Quantify Aflatoxin Using Thin Layer Chromatography[1]

T. B. Whitaker*, J. W. Dickens, and A. B. Slate[2]

ABSTRACT

A microcomputer was interfaced to an instrument (spotmeter) previously designed to measure the fluorescent intensity of aflatoxin spots on thin layer chromatography (TLC) plates. Software was developed that uses a cubic regression equation to describe the relationships between the spotmeter readings and the known quantities of aflatoxin in standard spots on TLC plates. The regression technique also provides methods to detect spotting and/or measurement errors. Based on the regression equation and measurements of sample extract spots on the same TLC plate, the system computes and records the amount of aflatoxin in the sample extract spots and the concentration of aflatoxin that was in the extracted sample. The percent error associated with computed amounts of aflatoxin in sample extract spots is affected by the amount of aflatoxin in the sample extract spots and standard spots on the plate. The average percent error ranged from 14.9% for a 2.6 ng spot of 4.1% for a 13 ng spot.

Key Words: Peanuts, aflatoxin, TLC, computer system

The aflatoxin concentration of a commodity may be determined by solvent extracting the aflatoxin from a known mass of commodity and using thin layer chromatography (TLC) to measure the amount of aflatoxins B_1, B_2, G_1, and G_2 extracted (1). For the TLC procedure, the aflatoxins are dissolved in a known volume of an appropriate solvent and a measured volume of the solution is placed on a TLC plate (sample spot) along with measured volumes of a standard solution which contains known concentrations of aflatoxin B_1, B_2, G_1, and G_2 (standard spots). One procedure is to use 1, 2, 3, 4, and 5 µL spots of a standard solution which contains 1.0 ng/mL of aflatoxins B_1 and G_1 and 0.3 ng/µL of aflatoxins B_2 and G_2 (2). When the TLC plate is developed, sample and standard spots separate into four separate spots representing aflatoxins B_1, B_2, G_1, and G_2. The aflatoxin spots fluoresce when excited with ultraviolet (UV) light at a wavelength of approximately 365 nm. The fluorescent intensity of a given aflatoxin spot is proportional to the quantity of aflatoxin in that spot. The quantity of aflatoxin in each sample spot may be determined by visual or densitometric comparison of the fluorescent intensity of the sample spot to the fluorescent intensities of the standard spots. The ng of aflatoxin per gram of product in the sample is then computed.

Visual methods are not as precise as densitometric methods of comparison. However, the high cost of densitometric equipment and the time required for analysis has discouraged the use of such equipment in many aflatoxin laboratories. A relatively inexpensive densitometric instrument (spotmeter) has been designed to rapidly and accurately measure the fluorescent intensity of both unknown and standard spots on TLC plates (3). A printer is attached to the instrument to provide a printed copy of the spotmeter readings. Fig. 1 shows a plot of spotmeter readings versus the ng of aflatoxin B_1 in five standard spots on a TLC plate. The amount of aflatoxin B_1 in sample spots on the same TLC plate with spotmeter readings between approximately 35 and 125 can be estimated by straight line interpolation between data points on the curve in Fig. 1. Then the ng of aflatoxin per gram of product, S, can be calculated using the equation below.

$$S = (A * V * C)/(D * M) \quad (1)$$

where A is the concentration of aflatoxin in the standard solution spotted on the TLC plate in ng/µL, V is the µL standard in the spot with a fluorescent intensity equivalent to the sample spot, C is the µL of sample solvent, D is the µL of the sample solution spotted on the TLC plate, and M is the grams of sample represented by the sample solution. The values of A, C, D, and M are defined by the AOAC extraction procedure (1) while the value of V (µL of standard solution spotted) is determined from spotmeter data in Fig. 1 and the following equation.

$$V = Q/A \quad (2)$$

where Q is the ng of aflatoxin spotted and A is defined above in equation 1.

The spotmeter can quickly provide meter readings proportional to the fluorescent intensity of both sample and standard spot, but considerable time is required to compute the values of V and to compute the concentration of all four aflatoxins in each sample. The objective of this study was to develop equipment and procedures to automatically store spotmeter readings, calculate values of V, and then calculate the aflatoxin concentration for each sample.

Materials and Methods

Equipment - The spotmeter uses a photomultiplier tube (PMT) that produces an output signal proportional to the intensity of the fluorescent spot. The output signal from the PMT is amplified and fed into a digital voltmeter (DVM). An IBM or compatible PC microcomputer with 640 k bytes of memory, two disk drives, and printer were interfaced to the spotmeter. An interface unit was constructed to accept the binary coded decimal (BCD) signal from the DVM on the spotmeter and pass the BCD signal to the proper addresses on the microcomputer bus.

Linear Interpolation and Regression Techniques - Linear interpolation and regression techniques were compared to determine which technique would best describe the relationship between the quantities of aflatoxin B_1, B_2, G_1, and G_2 in the standard spots and spotmeter readings. With linear interpolation techniques, adjoining points were connected by linear segments as shown in Fig. 1. The slope of each linear segment was determined by the values of the adjoining points. With the regression technique, a single equation was developed to describe the relationship between spotmeter readings and the ng of a given type of aflatoxin across all five standard spots (1 µL - 5µL). In order to determine the form of regression equation for each of the four types of aflatoxin, the following procedure was used for each type. The spotmeter readings associated with a series of five standard spots (1, 2, 3, 4, and 5 µL) on each of 103 TLC plates were analyzed. The 103 meter readings for each volume spotted on the TLC plate were averaged and a polynomial regression equation fit to the

[1]Paper No. 12250 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7643. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service nor the United States Department of Agriculture of the products named, nor criticism of similar ones not mentioned.

[2]Agricultural Engineers, United States Department of Agriculture, Agricultural Research Service, North Carolina State University, Box 7625, Raleigh, NC 27695-7625.

*Corresponding author.

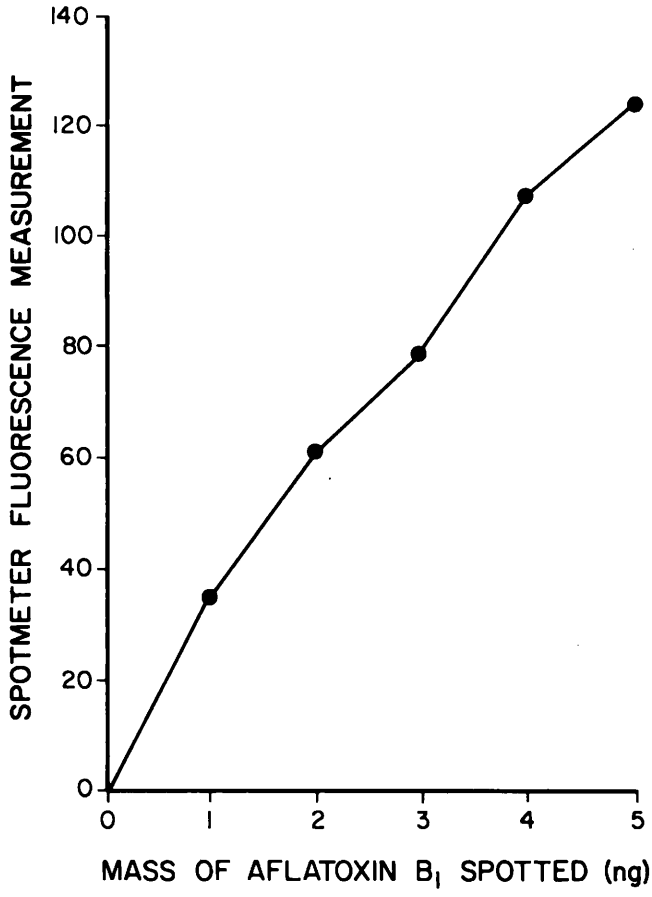

Fig. 1. Typical plot of spotmeter fluorescence measurements versus mass of aflatoxin B_1 on TLC plates.

five averaged data values.

$$V = \alpha_0 + \alpha_1 X + \alpha_2 X^2 + \alpha_3 X^3 \quad (3)$$

where V is the μL of standard solution spotted on the TLC plate, α_i are regression coefficients, and X is the spotmeter reading. Both a quadratic and a cubic regression equation were fitted to the five averaged spotmeter readings. The intercept value and the sum of squared residuals were used to determine which equation (quadratic or cubic) best fit the observed values.

The error associated with predicting the quantity of aflatoxin in a sample spot was estimated when using both the linear interpolation technique and a regression equation to describe the relationship between the spotmeter readings and the volume of standard solution spotted on a TLC plate. Using a standard solution, a series of five standard spots (1, 2, 3, 4, and 5 μL) were placed on a TLC plate. A second series of nine spots using nine different volumes of standard solution (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 μL) to stimulate sample spots were also placed on the same plate. The total quantity of aflatoxin ($B_1+B_2+G_1+G_2$) in each of the nine sample spots was estimated using both the regression technique and the linear interpolation techniques. The percent error, PE, was calculated for each of the nine sample spots on each TLC plate.

$$PE = (OI - TI)*100/TI \quad (4)$$

where OI is the computed ng of aflatoxin and TI is the true quantity of aflatoxin in the sample spot. The test was repeated on 25 TLC plates giving 25 PE estimates for each of the 9 different sample spots.

Software - The software program was written in BASIC. The program consisted of four major sections: (a) administrative or record keeping section, (b) aflatoxin standards computation section, (c) sample concentration computation section, and (d) analytical quality control section.

Results

The average of 103 spotmeter reading for the 1, 2, 3, 4, and 5 μL standard spot for B_1, B_2, G_1, and G_2 aflatoxin and curves representing cubic regression equations are plotted in Figs. 2, 3, 4, and 5, respectively. Quadratic and cubic regression equations were fit to the data for each type of aflatoxin. As shown in Table 1, the sum of squared residuals is lower for the cubic equation than for the quadratic equation for all four types of aflatoxin. Also, unlike the quadratic equation, the intercept term of the cubic equation was not significantly different from zero at the 95% confidence level for each of the four types of aflatoxin. These results suggest that the cubic equation fits the observed data better than the quadratic equation for all four types of aflatoxin. The three regression coefficient α_1, α_2, and α_3 in the cubic equation were all positive ($\alpha_0=0$); so there were no points of inflection in the curves over the range of spotmeter measurements for each of the four types of aflatoxin.

The PE associated with predicting the amount of aflatoxin in the sample spots when using both regression measurements and linear interpolation techniques for spotmeter readings of the five standard spots on the same TLC plate are shown in Fig. 6. As seen in Fig. 6, the PE associated with using the cubic regression is approximately the same as the PE associated with the linear interpolation technique. For both

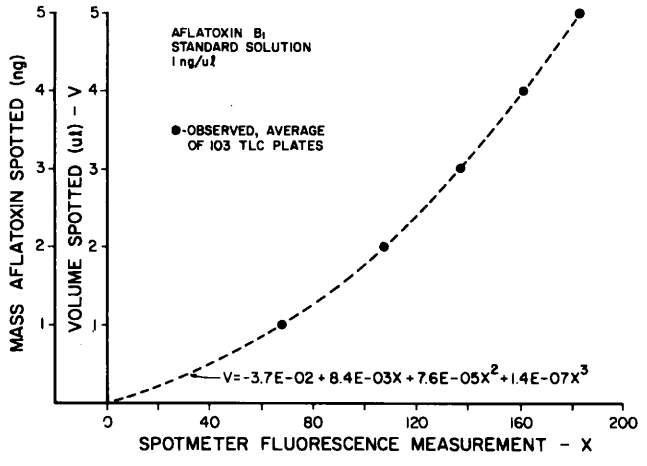

Fig. 2. Average spotmeter fluorescence measurements of aflatoxin B_1.

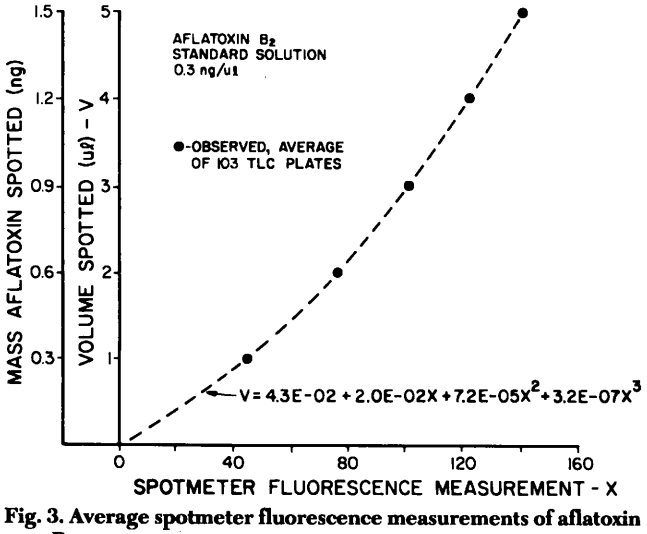

Fig. 3. Average spotmeter fluorescence measurements of aflatoxin B_2.

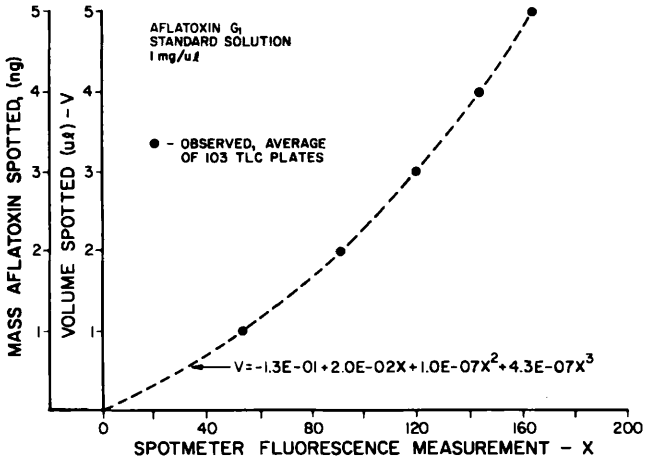

Fig. 4. Average spotmeter fluorescence measurements of aflatoxin G_1.

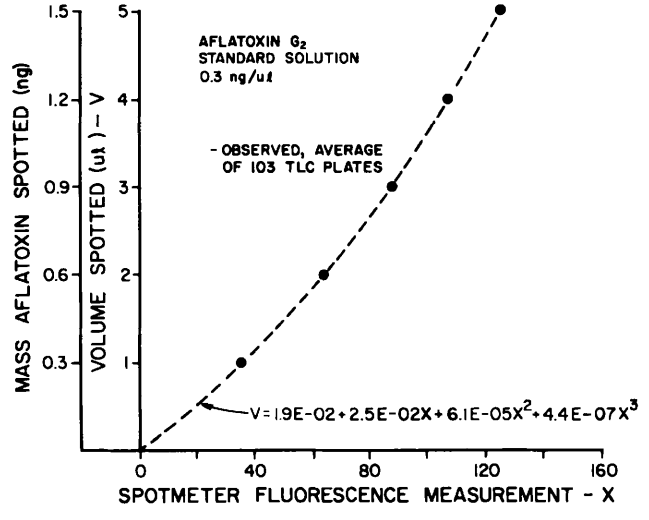

Fig. 5. Average spotmeter fluorescence measurements of aflatoxin G_2.

techniques, the PE decreased as the quantity of aflatoxin increased. Because the PE associated with the 1 µL (2.6 ng) spot is large, larger volumes of sample and standard solutions should be used to quantify aflatoxin on the TLC plate.

Regression techniques provide the capability to test spotmeter measurements on standard spots for outliers. Outliers may be caused by errors in spotting and/or measurement; so the regression technique is preferable to linear interpolation. In the regression analysis, the intercept term α_0 is forced to be zero and the sign of the remaining three regression coefficients α_1, α_2, and α_3 in the cubic equation are tested to see if they are all positive. If the three regression coefficients are not all positive, one or more of the five data point is suspected to be an outlier. An outlier is identified by removing one data point at a time, starting with the 1 µL spot measurement and progressing to the 5 µL spot. Each time a point is deleted, the cubic regression equation is fitted to the remaining four data points. If all three coefficients are positive after a point is deleted, the deleted point is assumed to be an outlier. The above point deletion process and a procedure to identify and record all suspect spotmeter measurements were incorporated into the application software.

After obtaining positive regression coefficients, the degree of scatter of the spotmeter measurements about the regression equation is also tested by computing the squared deviations between the observed and predicted values. While

Table 1. Sum of squared residuals (SSD) when fitting a quadratic and a cubic equation to five standard spots of B_1, B_2, G_1, and G_2.

Regression Equation	SSD x 10^5			
	B_1	B_2	G_1	G_2
Quadratic[a/]	31.0	47.8	105.3	69.4
Cubic[b/]	15.3	25.1	3.1	35.0

$$\underline{a}/V = \alpha_0 + \alpha_1 X + \alpha_2 X^2$$

$$\underline{b}/V = \alpha_0 + \alpha_1 X + \alpha_2 X^2 + \alpha_3 X^3$$

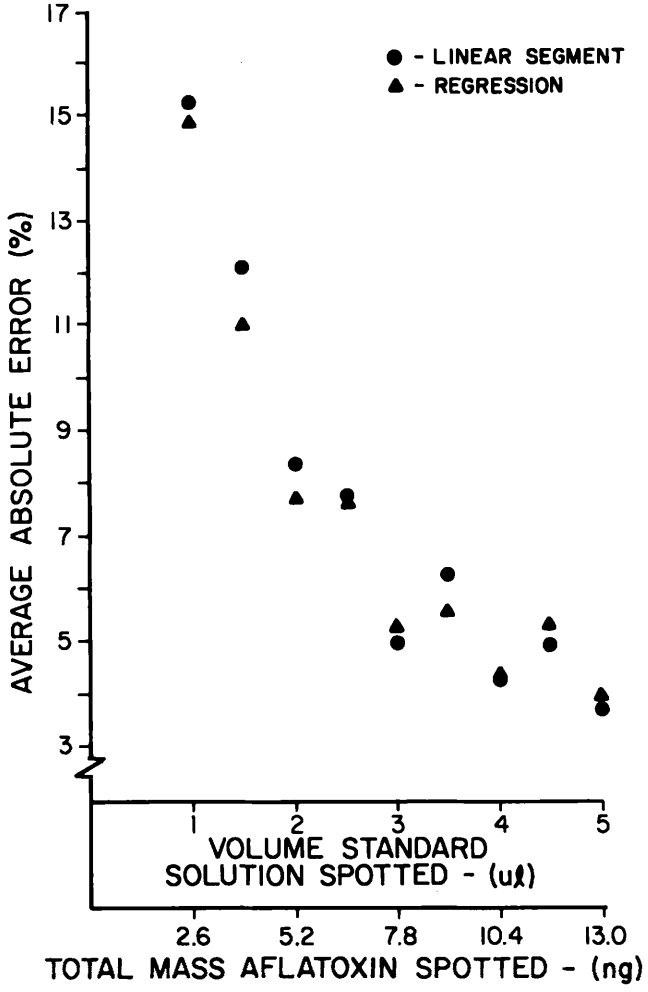

Fig. 6. Percent error associated with predicting the total amount of aflatoxin spotted on TLC plates when using linear interpolation and regression techniques.

a small deviation of the points about the regression equation is expected, a large deviation indicates errors in spotting and/or measurement. The deviation for each measurement is determined and recorded. If any deviation is greater than a predefined maximum value established by the analyst, the associated measurements are identified.

If three positive coefficients are not obtained initially or with the point deletion process or if two or more residuals exceed the defined maximum, computation stops and the analyst is given the following options: (a) respot the TLC plate (assumes a spotting error), (b) make the spotmeter measurements again (assumes a reading error), or (c) continue the quantification process with questionable data.

The outlier identification and deletion process is demonstrated by the data shown in Fig. 7 which are spotmeter measurements associated with five standard spots of aflatoxin B_1. The spotmeter measurement of 79 associated with the 3 µL spot appears to be out of line with the other four spotmeter measurements on the same TLC plate. As shown in Table 2, when the cubic regression equation was run of the five values shown in Fig. 7, one of the regression coefficients was negative. As each point is deleted, the sign of the three regression coefficient is checked. One deletion of the spotmeter measurement associated with the 3 µL spot gave all positive coefficients indicating that this measurement was in error. The regression equation with the measurement of the 3 µL spot deleted is shown in Fig. 7.

To test the cubic regression and point deletion method, the cubic regression analysis was performed on the five spotmeter measurements for aflatoxin B_1 associated with each of the 103 previously mentioned TLC plates. No measurements were deleted for 41 of the regression analyses, and one point was deleted for 39 of the regression analyses. The cubic regression and point deletion process rejected 23 of the 103 TLC plates because all positive coefficients were not obtained even after deletion of one measurement. The 23 rejections resulted from a combination of spotting errors by the analysis and possible imperfection in the coating on the glass plate.

An example of the screen display and printed output associated with quantifying aflatoxin on a TLC plate is shown in Fig. 8. The first information requested of the analyst is the value of the maximum residual. The program allows the analyst to use one value for B_1 and G_1 and another value for B_2 and G_2. The date and TLC plate identification number

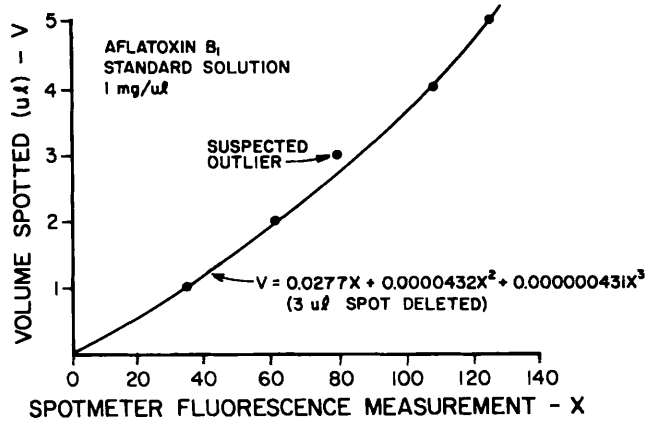

Fig. 7. Cubic regression equation fitted to spotmeter fluorescence measurements with a suspected outlier at 3 µL.

Table 2. Sum of squared residuals (SSD) and signs associated with cubic regression equation used to fit five standard spots of B_1 using the point deletion method[a].

Point Deleted	Sign for α_1	α_2	α_3	SSD x 10^3
None	+	+	-	60.7[b]
1	+	+	-	56.3
2	+	+	-	56.4
3	+	+	+	6.3
4	+	+	-	11.1
5	+	+	-	17.9

[a] $V = \alpha_0 + \alpha_1 X + \alpha_2 X^2 + \alpha_3 X^3$

[b] 5 data points instead of 4.

are requested next. After the spotmeter measurements associated with the standard spots are made and automatically entered, a table of measurements are printed for each type of aflatoxin and volume spotted. If suspect spotmeter measurements are identified after the regression analysis, a table is printed using asterisks to show the suspect measurement(s) and the analyst is given the option to repeat the plate, remeasure the spots, or continue. Asterisks printed for all standard spots of aflatoxin B_1 and aflatoxin G_2 indicate that the cubic equation could not be fitted to the spotmeter measurements. Asterisks also indicate that for B_2, the 2 and 3 µL spot exceeded the maximum residual of 0.2.

MAXIMUM RESIDUAL ALLOWED FOR B_1 AND G_1 = 0.2
MAXIMUM RESIDUAL ALLOWED FOR B_2 AND G_2 = 0.2

DATE: 1/11/89 PLATE ID: 16

METER READINGS FOR STANDARD SPOTS

TYPE SPOT	VOL. STANDARD SOLUTION SPOTTED (µL) 1	2	3	4	5
B_1	79	105	120	143	160
B_2	33	57	65	81	104
G_1	54	81	103	123	139
G_2	14	35	43	69	79

SUSPECTED OUTLIERS

TYPE SPOT	VOL. STANDARD SOLUTION SPOTTED (µL) 1	2	3	4	5
B_1	*	*	*	*	*
B_2		*	*		
G_1					
G_2	*	*	*	*	*

SUSPECTED OUTLIERS ARE SHOWN ABOVE BY AN ASTERISK
SELECT OPTION BELOW FOR DESIRED ACTION:
1) RESPOT TLC PLATE (ABANDON PLATE)
2) MEASURE SPOTS AGAIN
3) CONTINUE QUANTIFICATION, PROCEED TO UNKNOWNS

Fig. 8. Microcomputer screen display showing date, plate id, and spotmeter readings for standard spots.

If no problems are found with the measurements of the standard spots, the analyst then measures the sample spots. Spotmeter measurements associated with a sample spot (sample 1) and the computed aflatoxin concentrations are shown in Fig. 9. If the spotmeter measurements of sample spots are outside the range of spotmeter measurements of the standard spots, an additional table is printed which identifies those measurements, whether they are greater than (H) or less than (L) the range of standard spot measurements and how far out of range are the sample spots. In the example shown, the sample spot measurement of 25 for aflatoxin G_1 (Fig. 9) is identified as being lower than the lowest standard spot measurement of 54 (Fig. 8). Also the sample spot measurement of 90 for aflatoxin G_2 (Fig. 9) is higher than the highest standard spot measurement of 79 (Fig. 8).

METER READINGS FOR SAMPLE SPOTS

SAMPLE ID	TYPE SPOT B_1	B_2	G_1	G_2	SAMPLE WT.	SOL. VOL	ALIQ. VOL	DIL. VOL	AMT SPOT	WT. FACT
1	100	75	25	90	50	250	50	300	1	10

PARTS PER BILLION (PPB) FOR SAMPLE SPOTS

SAMPLE ID	PPB AFLATOXIN TOTAL	B_1	B_2	G_1	G_2
1	85.7	39.0	15.9	5.7	25.1

******** % OUT OF RANGE (L = LOW, H = HIGH) ********

SAMPLE ID	TYPE SPOT B_1	B_2	G_1	G_2
1			62L	11H

Fig. 9. Printer output showing calculated aflatoxin concentration and sample spot measurements that were outside the range of standard spot measurements.

Summary and Conclusions

A microcomputer was interfaced to a spotmeter to take the PMT output signals from the spotmeter and compute the aflatoxin concentrations in sample extracts spotted on TLC plates. A cubic regression was used to describe the relationship between the spotmeter measurements and the quantity of aflatoxin standard solution spotted on the plate. The PE associated with predicting the quantity of aflatoxin in unknown solutions is about the same for both the cubic regression and linear interpolation techniques. The PE varied from a high of 14.9% for a 1 μL spot to a low of 4.1% for a 5 μL spot. The computer program that uses the regression technique checks the spotmeter measurements of standard spots for deviations from the regression equation which indicate measurement and/or spotting errors. The computerized densitometer has been used successfully in a USDA, ARS research laboratory where a large number of samples are quantified and most samples contained aflatoxin. The computerized system provides an automated procedure to quantify aflatoxin on TLC plates, to compute aflatoxin concentration in samples, and to provide quality control for some TLC analytical procedures.

Literature Cited

1. AOAC: Natural Poisons, Chapter 26 from Official Methods of Analysis. 1984. 14th Edition, Assoc. Off. Anal. Chem., Arlington, VA Sec 26.026-.048.
2. United States Department of Agriculture Inspector's Instructions for Aflatoxin. 1988. USDA, Agricultural Marketing Service, Washington, DC, pp. 7-18.
3. Dickens, J. W., W. F. McClure, and T. B. Whitaker. 1980. Densitometric equipment for rapid quantitation of aflatoxins on thin layer chromatograms. J. Amer. Oil Chem. Soc. 57:205-208.

Accepted October 22, 1990