Densitometric Equipment for Rapid Quantitation of Aflatoxins on Thin Layer Chromatograms[1]

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ABSTRACT

A densitometric instrument (Spotmeter) has been designed for rapid measurement of aflatoxin on thin layer chromatographic plates. The Spotmeter measures the total amount of fluorescence of the aflatoxin spot and thus eliminates the need for scanning mechanisms, plotters and integrators incorporated into most commercially available densitometers used for aflatoxin analyses. In comparison with a Schoeffel SD 3000 Spectrodensitometer, the Spotmeter was more accurate in one test and at least equally accurate in another test. The Spotmeter made measurements in 1/4 the time required for the Spectrodensitometer and would cost about 1/6 as much as commercially available densitometers suitable for aflatoxin analyses.

INTRODUCTION

Thin layer chromatography (TLC) is the most common procedure used for routine analyses of agricultural commodities for aflatoxins. For quantification of aflatoxins on TLC plates, the amounts of fluorescence are compared between spots formed by unknown and known quantities of aflatoxins. Densitometers are commercially available for this purpose, but analysts in many laboratories continue to compare the fluorescent spots visually. Densitometric quantification of aflatoxins is more accurate than visual methods (1), but the high cost of equipment and the time required for quantification often preclude the use of such equipment.

With most commerical densitometers, the aflatoxin spot is exposed to long wave ultraviolet (UV) light (ca. 365 nm) and the fluorescent spot is scanned through a narrow slit. The quantity of light that passes the slit and 465-nm secondary filter is measured with a photomultiplier circuit. The output from the photomultiplier circuit may be plotted on a strip-chart recorder or summed with an electronic integrator. The peak height of the output curve or the area beneath the curve may then be used to estimate the amount of fluorescence of the aflatoxin spot. Such a procedure requires a mechanism to scan the spot and equipment to plot and integrate the output from the photomultiplier circuit.

We developed a specialized densitometer (Spotmeter) that enables the analyst to center the aflatoxin spot beneath a 4.8-mm circular aperture while the spot is exposed to long wave UV light. The light that passes the aperture and a 465-mm filter is measured with a photomultiplier circuit. Since the entire aflatoxin spot is exposed simultaneously, there is no need for a scanning mechanism, plotter, or integrator. We now describe the design and performance of the Spotmeter.

SPOTMETER DESIGN

Positioning Unit

Features of the Spotmeter positioning unit are illustrated by Figure 1. The TLC plate with aflatoxin spots (B) is placed in a holder (A) so the plate coating is beneath the plate. The holder keeps the coating about 1 mm above the table (D) so the holder and plate can be moved about on the table until the desired spot is over the illuminated opening (C) in the table. The illumination is provided by a 4-watt, long wave UV lamp (UVL-21 Lamp, Ultra-Violet Products, Inc., San Gabriel, CA) (E). A clear, plexiglass swivel plate (I) is mounted on a vertical shaft so it can be pivoted back and forth in a plane just above the TLC plate. Two adjustable stops (G and J) are provided to limit the swing of the swivel plate. A 4.8-mm target circle (F) and a 4.8-mm aperture (H) are located equidistant from the pivot point. The target circle is inscribed on the bottom side of the transparent swivel plate to reduce parallax when an aflatoxin spot is aligned beneath the target. The bottom of the triangular half of the swivel plate that contains the aperture is coated with an opaque paint to prevent light transmittance except through the aperture.

The swivel plate stops are adjusted so that when the swivel plate is against the stop (J) the target circle (F) is in precisely the same location as the aperture (H) will be when the swivel plate is against the opposite stop (G). This is easily accomplished by pivoting the swivel plate against stop J and adjusting the stop so the target circle is over the approximate center of the illuminated area (C). A piece of paper or cardboard with a dot or other target is then placed so that target is beneath the target circle. Without moving the target the swivel plate is pivoted against stop G. Stop G is then adjusted so the target is centered beneath the aperture H. A detector with a 465-nm secondary filter is then clamped to the swivel plate to filter and detect the quantity of light that passes the aperture.

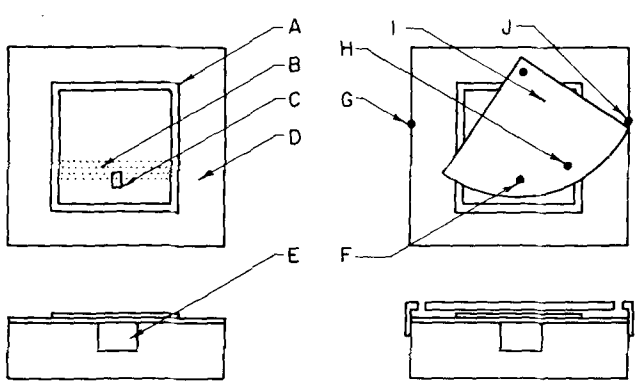

FIG. 1. Diagram of the Spotmeter positioning unit.

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Photometer

Figure 2 is a simplified diagram of the Spotmeter photometer. Light that emerges from the aperture and passes the UV filter is sensed by the photomultiplier tube in the detector. The signal from the detector is amplified and fed into a digital voltmeter. The digital printer provides a printed copy of the voltmeter readings.

Figure 3 is a schematic diagram of the Spotmeter amplifier circuit which has a logarithmic amplifier (Analog Model 755P) wired in the current-to-voltage configuration followed by 2 linear amplifiers (Model 233J). The first amplifier A1 provides zero offset (P1) with a gain of one while A2 provides positions for run and various test points within the circuit including one position (HV) to test the high voltage power supply for the photomultiplier detector.

Operation

During operation the swivel plate is first pivoted against stop J and the TLC plate is positioned so a selected fluorescent aflatoxin spot is directly beneath the target circle. Then, without moving the TLC plate, the swivel plate is pivotted against stop G such that the aperture is directly over the spot. The quantity of fluorescence of the spot is then recorded by the printer when a switch is manually closed. The quantity of fluorescence of a series of "unknown" spots and a series of "known" spots may be recorded in rapid sequence with this procedure. The amount of aflatoxin in each unknown spot may then be estimated by comparing the quantity of fluorescence of the unknown spots to the quantity of fluorescence of standard spots with known amounts of aflatoxin. Figure 4 shows a plot of Spotmeter readings vs the amount of aflatoxin B_1 in standard spots on a typical TLC plate. The amount of aflatoxin B_1 in unknown spots on the same TLC plate with Spotmeter readings between 53 and 225 can be estimated by straight-line interpolation between the data points on this curve. In order to evaluate Spotmeter readings slightly outside this range, the authors extrapolate the straight line between the lower 2 points on the curve to a point for 0.9 ng, and extrapolate the straight line between the upper 2 points to a point for 5.1 ng. When unknown spots have Spotmeter readings outside the range of readings for the standard spots, the extracts for the unknown spots must be diluted and respotted on another plate. Because TLC plates are variable, both the standard and unknown spots must be measured on the same plate.

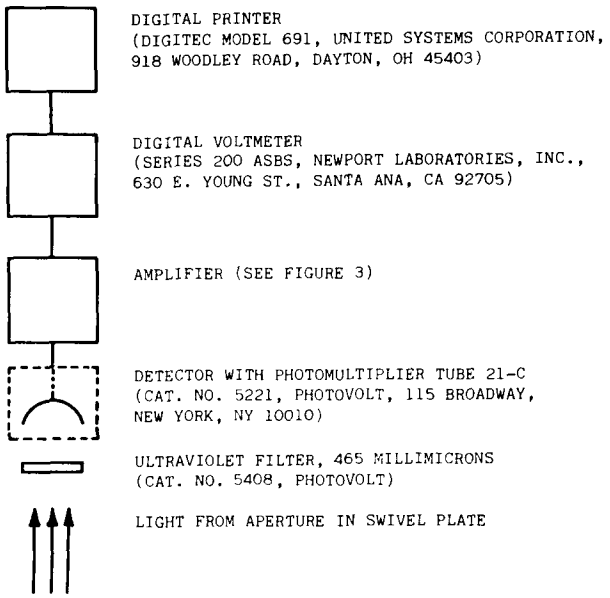

FIG. 2. Simplified diagram of the Spotmeter photometer.

EXPERIMENTAL

Procedure

For evaluation, accuracy was compared between the Spotmeter and a Schoeffel SD 3000 Spectrodensitometer (Schoeffel Instrument Corporation, Westwood, NJ).

Twenty-two 20 cm x 20 cm precoated TLC plates

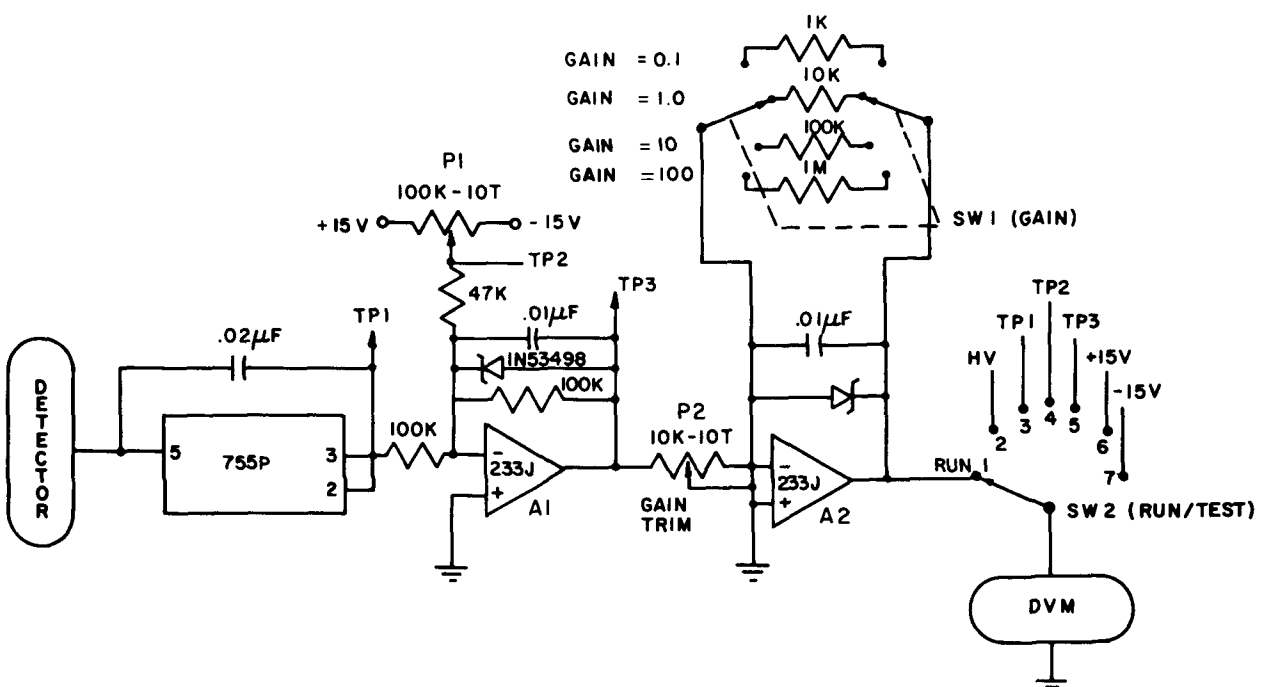

FIG. 3. Diagram of the Spotmeter amplifier circuit.

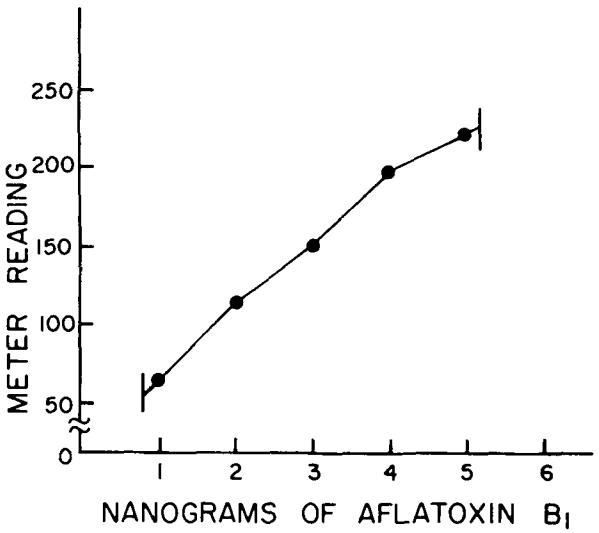

FIG. 4. Typical plot of Spotmeter reading vs amount of aflatoxin B_1 on TLC plate.

(Silica Gel GHR, 250 mm layer thickness, Analtech, Inc., Newark, DE) were scored with a Schoeffel SDA 320 scoring device to make 20 1-cm-wide lanes. Only the center 10 lanes were used. These lanes were numbered 1-10 from left to right. Lanes 1-5 and 6-10 were spotted with 1 μl, 2 μl, 3 μl, 4 μl and 5 μl, respectively, of standard aflatoxin solution. The standard aflatoxin solution was obtained from the Food Safety and Quality Service, United States Department of Agriculture. The standard solution consisted of benzene/acetonitrile (98:2) with 1 μg/ml each of aflatoxins B_1 and G_1 and 0.3 μg/ml each of aflatoxins B_2 and G_2. The spots were placed on the plates with a 10-μl syringe. The plates were developed in acetone/chloroform (5:95) according to procedures specified by Method II of AOAC (2). The developed plates were numbered 1-22.

The amount of fluorescence of each aflatoxin spot on the developed plates was measured with the Spotmeter and with a Schoeffel SD 3000 Spectrodensitometer. Output from the Spectrodensitometer was processed and recorded with a Shimadzu Chromatopac-E1A (Shimadzu Scientific Instruments, Inc., Columbia, MD). Plates 1-12 (test 1) were measured on the SD 3000 Spectrodensitometer in the mycotoxin laboratory of Food and Drug Protection, North Carolina Department of Agriculture. The remaining 10 plates (test 2) were measured on a different SD 3000 Spectrodensitometer in a laboratory at North Carolina State University. The same Spotmeter was used on all 22 plates. Persons with skill and experience in operating the Spectrodensitometer made the measurements with that instrument and persons with skill and experience in operating the Spotmeter made measurements with that instrument. Odd-numbered plates were first measured with the Spotmeter whereas even-numbered plates were first measured with the Spectrodensitometer.

Measurements with the Spotmeter and with the Spectrodensitometer were converted into amounts of aflatoxin using the following procedure: spots in lanes 1-5 on each plate were designated standard spots and calibration curves similar to the one shown in Figure 4 were determined for each of the 4 types of aflatoxin. The calibration curves for aflatoxins B_1 and G_1 were extrapolated to read between 0.9 ng and 5.1 ng and the calibration curves for aflatoxins B_2 and G_2 were extrapolated to read between 0.27 ng and 1.53 ng according to the procedure previously discussed in this paper. The spots in channels 6-10 were designated unknown spots and the amounts of aflatoxin in the spots were estimated from the appropriate calibration curve. Measurements outside the limits of the calibration curve were deleted from the data set. If a measurement by either the Spectrodemsitometer or the Spotmeter was deleted, the same measurement with the other instrument was deleted; the data set included only those estimates of aflatoxin that were made by both instruments on the same aflatoxin spots.

The percentage difference in the amount of aflatoxin estimated from the calibration curve and the amount of aflatoxin actually spotted (assuming no spotting error) was recorded as percentage error. Absolute values of percentage error were used. For test 1, measurements of both peak heights and integrated areas of the Spectrodensitometer output curves were evaluated. Peak height measurements were found to be more accurate so they were used in this study. For test 2, integrated area measurements were used since no difference was found between those measurements and peak height measurements.

RESULTS

Analysis of variance failed to show a significant effect of type of aflatoxin on the average percentage error by the Spotmeter or by the Spectrodensitometer. Therefore, the data for all types of aflatoxin were pooled. Comparisons of the average percentage error for the Spotmeter and for the Spectrodensitometer when different quantities of aflatoxin standard solution were spotted are given in Table I. In test 1, the average percentage error in estimating the amount of aflatoxin in 1 μl, 2 μl, 3 μl, 4 μl and 5 μl spots of aflatoxin standard solution were, respectively, 24.8%, 16.0%, 13.4%, 11.3% and 14.2% for the Spectrodensitometer and 10.9%, 10.0%, 7.9%, 8.1% and 3.8% for the Spotmeter. For all amounts spotted, the Spotmeter had significantly less error than the Spectrodensitometer. Test 2 failed to show a significant difference in the percentage error by the 2 instruments except that when 5 μl of standard was spotted the 5.8% error with the Spotmeter was significantly less than the 16.1% error with the Spectrodensitometer.

Data presented in Table I indicate that the Spotmeter was more accurate than the Spectrodensitometer in test 1 and as accurate as the Spectrodensitometer in test 2. Although Spectrodensitometer measurements in test 1 were made by experienced personnel, the measurements were made in a routine manner normally employed in the laboratory as were all measurements in both tests with the Spotmeter. Because of the large percentage error observed in test 1, test 2 measurements with the Spectrodensitometer were made much more carefully. The added care in proper alignment of the plates and adjustment of the Spectrodensitometer may account for the lack of significant differences between the Spectrodensitometer and the Spotmeter for 4 of the 5 comparisons in test 2.

As previously mentioned, computation of the percentage error using the 2 instruments was based on the assumption that there was no spotting error when the measured amounts of aflatoxin solution were put on the plates with the 10-μl syringe. Actually, the percentage errors reported in this study may have included errors in spotting, errors resulting from variability within each of the developed TLC plates and other types of error. Except for these types of errors, the standard spots and the unknown spots would have exhibited the same amount of fluorescence. Since this study was a comparison of the measured amounts of fluorescence of the 2 series of spots, consistent types of errors that affected both series of spots would not have caused an increase in percentage error. For example, if there were an error of -0.2 μl in any pair or pairs of standard and unknown spots, comparison of the amounts

TABLE I

Comparisons of Average Percentage Error (% E) for the Spectrodensitomer and for the Spotmeter when Different Quantities of Aflatoxin Solution Were Spotted[a]

	Quantity of aflatoxin solution spotted (μl)				
	1	2	3	4	5
			Test 1		
Spectrodensitometer (% E)	24.8	16.0	13.4	11.3	14.2
Spotmeter (% E)	10.9	10.0	7.9	8.1	3.8
No. observations	26	47	47	44	18
t Value	3.01[c]	2.76[c]	2.63[c]	2.07[c]	3.10[c]
Probability[b]	0.00	0.01	0.01	0.04	0.04
			Test 2		
Spectrodensitometer (% E)	7.6	8.9	9.2	9.9	16.1
Spotmeter (% E)	9.9	9.8	10.3	7.7	5.8
No. observations	18	36	40	40	24
t Value	0.95[d]	0.54[d]	0.57[d]	1.52[c]	4.83[d]
Probability[b]	0.35	0.59	0.57	0.13	0.00

[a]Observations for all 4 types of aflatoxin were combined. The aflatoxin solution consisted of benzene/acetonitrite (98:2) with 1 μg/ml each of aflatoxins B_1 and G_1 and 0.3 μg/ml each of aflatoxins B_2 and G_2.

[b]A statistically significant difference in percentage error for the 2 instruments was assumed when the probability was less than 0.05.

[c]Values for unequal variances.

[d]Values for equal variances.

of fluorescence of the 2 series of spots would not detect the error. Since the same spots were measured by both instruments, comparison between the percentages of error with the instruments indicates their relative accuracy.

The average time required to scan a lane (each lane contained 4 aflatoxin spots) was about 2 min with the Spectrodensitometer and 0.5 min with the Spotmeter. Therefore, only 1/4 as much time is required to read TLC plates with the Spotmeter as with the Spectrodensitometer. Although assembled units of the Spotmeter are not commercially available, we estimate that construction would cost about 1/6 as much as commercially available densitometers suitable for quantitation of aflatoxin on TLC plates.

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