Densitometric measurement of aflatoxin

Intensive research on aflatoxin at the Northern Regional Research Laboratory has required a large number of quantitative determinations by thin-layer chromatography (TLC). The four aflatoxins (B_1, B_2, G_1, and G_2) are separated on chromatography plates by the use of Silica Gel G-HR (Brinkmann Instruments, Westbury, N.Y.)* as the adsorbent and $CHCl_3$–CH_3OH (97:3, v/v) as the developing solvent. A quantitative assay requires addition of several reference spots of various aflatoxin levels to each plate for visual comparison with samples of unknown concentration. Usually 1 μl to 5 μl of the reference standard are spotted and the fluorescence of the unknown is estimated to 0.5 μl of the standard. Within visual limits, the fluorescence given by aflatoxin under ultraviolet light (366 mμ) is directly proportional to the concentration. The validity of visual observation in a quantitative assay, however, is questionable since sensitivity can be influenced by several factors such as, stray light, proximity of spots being compared, and the observer's visual perception. Lack of sensitivity is illustrated by the observation that when a 1 μl portion of unknown is interpreted as being equivalent to 1.5 μl of reference standard, the actual range of estimation is 1.25 tot 1.75 μl, or a deviation of $\pm$ 17 %. This error becomes pragmatically significant with large dilutions of an unknown sample. The accuracy in visual comparisons is usually considered as within 20 %[1].

Recording densitometers can be purchased that give a quantitative graphic representation of the intensity of fluorescence from compounds on TLC plates. Because these units are expensive, we tried substituting a simple darkroom densitometer connected to an available recorder. This economical combination gave more reliable data for routine aflatoxin analysis than visual observation.

Experimental

Densitometer. An inexpensive darkroom densitometer (Science and Mechanics Darkroom Densitometer, Olden Camera and Lens Company, Inc., 1265 Broadway, New York, N.Y.) was modified to read fluorescence of aflatoxin components on TLC plates. A 1.27 cm disc was cut from a 2A (yellow) photographic gel filter and inserted into the densitometer probe housing. Because this filter transmits only wavelengths longer than 410 mμ, it eliminates the ultraviolet (366 mμ) illumination through the TLC plate (Fig. 1). The densitometer probe was positioned to completely cover the individual fluorescent areas and the ammeter values were recorded. The meter response was expanded by connecting two wires from the ammeter inputs of the densitometer to the millivolt (mV) inputs of a Model SRL Sargeant recorder.

The thickness of the adsorbent layer varied from plate to plate. The variable range control (0–100 mV) of the recorder therefore had to be adjusted to 100 (scale reading) for the highest strength of reference standard on each TLC plate to obtain the widest range of values between the background and reference standard.

The fluorescence of the individual spots decreased rapidly (50 % decrease in 3 min) when they were subjected to ultraviolet light. Exposure time was therefore limited to 5–10 sec per spot by masking the ultraviolet light (Fig. 1) and using a 1/2-in. diameter opening as the illuminating source.

* Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

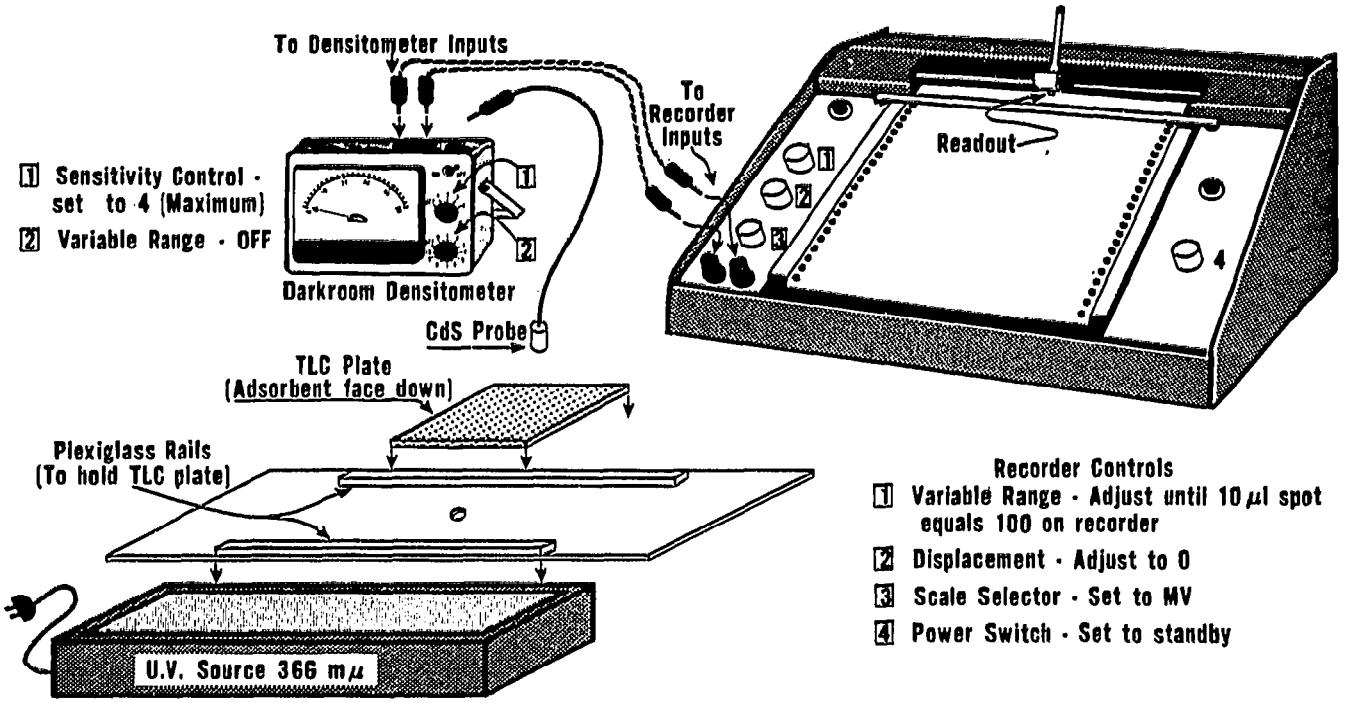

Fig. 1. Equipment for densitometric recording of aflatoxin concentrations on thin-layer chromatography plates.

Results and discussion

Densitometric measurements. Preliminary observations indicated a necessity for spotting two different levels of aflatoxin for both reference and unknown samples. This requirement was brought about by variations in both adsorbent layer thickness and glass transparency between plates that caused a variable recorder response per μl of sample. The recorder values of these two levels of aflatoxin standard, arbitrarily chosen for our experiments at 2 μl and 10 μl, determine the recorder response per μl of aflatoxin (Fig. 2) and provide a basis for judging the validity of the assay as explained later in this paper. A good linear relation was found between recorder reading and volume of aflatoxin standard applied.

Ideally, the reading of a single spot of a test solution could be used for an analysis, and the effect of background light corrected either by subtracting the reading for a portion of the plate containing no sample or by adjusting the instrument to zero response for the background. The latter approach was not possible with our simple equipment while the use of the blank reading was discarded because of irregularities of light transmission over each chromatographic plate.

The line corresponding to values for the two spots was used to obtain the instrument response (R) per μl of solution, while the intercept of this line on the axis representing the densitometer reading (Y axis) was estimated either graphically or algebraically. The concentration of an unknown, U (μg/μl) is then determined from the formula,

$$U(\mu g/\mu l) = R_u/R_s \times \text{concentration of the standard (in } \mu g/\mu l)$$

where R_u = recorder response per μl of unknown and R_s = recorder response per μl of standard.

The limits of variability of intercepts on the Y axis as applied to validity of a single assay depend on several factors, *e.g.*, the slopes of the two lines and the ratio of the two slopes. Pragmatically, the two lines should intersect within 0.5 μl of zero on the concentration axis (X axis) for the assay to be valid.

The standard error associated with the concentration of the unknown depends on the factors noted above. With an aflatoxin B_1 concentration of 0.0054 μg/μl in the standard and an R_s value of 4, the standard error is $\pm$ 0.10. This result gives a relative precision of $\pm$ 7 %.

The performance of this simple, low-cost unit (Fig. 1) proves that ultrasophistication in instrumentation, with its accompanying high cost, is unnecessary to secure data accurate enough for routine work.

Aflatoxin in fermentations. Densitometric and visual comparisons were made on extracts of fermentation broths in which aflatoxin B_1 and G_1 were being produced by *Aspergillus flavus*[2]. These comparisons (Fig. 3) indicate the accuracy of the two methods of estimation. The curve from densitometer measurements is the type to be expected for this fermentation, while the one from visual comparisons appears anomalous.

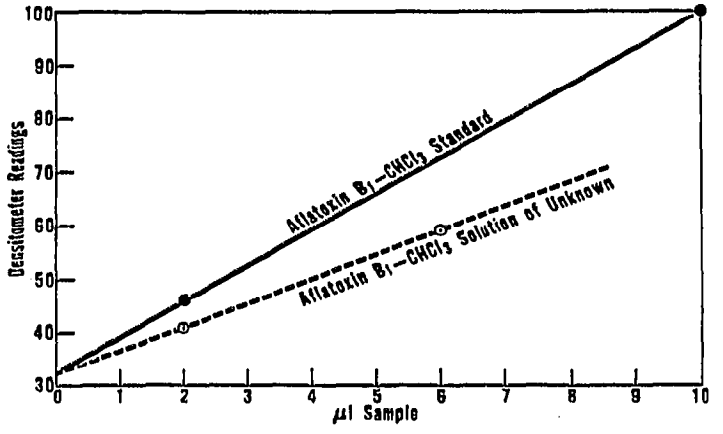

Fig. 2. Densitometer response to aflatoxin standard.

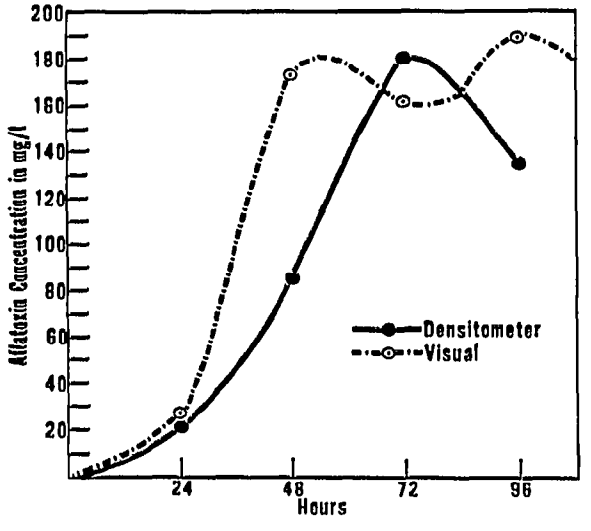

Fig. 3. Densitometer analysis *versus* visual observation.

Acknowledgement

We thank Dr. W. F. KWOLEK, Biometrician, Biometrical Services, Agricultural Research Service, U.S. Department of Agriculture, stationed at the Northern Regional Laboratory, for statistical analyses of the preliminary data.

Northern Regional Research Laboratory, Peoria, Ill. 61604 (U.S.A.)*

R. E. PETERSON
A. CIEGLER
H. H. HALL

1 O. L. SHOTWELL, C. W. HESSELTINE, R. D. STUBBLEFIELD AND W. G. SORENSON, *Appl. Microbiol.*, (1966) 425.
2 A. CIEGLER, R. E. PETERSON, A. A. LAGODA AND H. H. HALL, *Bacteriol. Proc.*, (1966) 4.

Received October 10th, 1966

* This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Resolution of complex purine and pyrimidine antagonist mixtures by thin-layer chromatography*

No rapid and sensitive microanalytical method has been described in the literature which permits complete resolution of complex purine and pyrimidine antagonist mixtures containing microgram amounts of individual compounds. Although WEMPEN AND FOX[1,2] have reported a paper chromatographic method for the determination of 6-fluorocytosine and a thin-layer chromatographic method and a paper electrophoresis method for the analysis of 6-fluorouracil, no R_F value has been reported. Recently, BAYER[3] reported a paper chromatographic method for the investigation of the decomposition products of 2,6-bis[bis(β-hydroxyethyl)amino]-4,8-dipiperidinopyrimido(5,4-*b*)pyrimidine. The method, however, is not applicable in the case of resolving a mixture of such compounds. In our laboratory over the past four-year period approximately 200 such compounds have been synthesized and evaluated under the auspices of the Cancer Chemotherapy National Service Center against various induced rodent tumors. It is now felt that a new sensitive and rapid chromatographic method by which complex antagonist mixtures can be separated and identified might be valuable in investigations concerning cancer research and metabolism of such antagonists in animal cells and tumor cells. This paper describes such a thin-layer chromatographic technique by which resolutions of complex purine

* This investigation was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute of the National Institutes of Health, the U.S. Public Health Service (Grant No. Ca-0649202).