

Note on a Rapid Thin Layer Chromatographic Method for the Isolation of Aflatoxin B₁

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Since 1960, when the death of 100,000 turkey poults in England was traced to aflatoxin in the peanut meal (1), there has been a great amount of research on this toxin produced by the mold *Aspergillus flavus*. With the ever-increasing amount of research, there has been an increasing need for sufficient quantities of aflatoxin to work with. We have found a preparative layer TLC plate method that is faster and more economical than any method heretofore published. Our method deals with the production, isolation, and purification of aflatoxin B₁, which is the most potent and most prevalent component of the four aflatoxins (2, 3).

Experimental

Apparatus and Reagents

(a) *Sample applicator*.—Rodder streaker (Rodder Instruments, Los Altos, Calif.) or Desaga-Brinkmann electromechanical sample applicator (Brinkmann Instruments, Westbury, N.Y.).

(b) *TLC plates*.—20 × 40 cm glass plates.

(c) *Adsorbent*.—Silica gel PF₂₅₄ with CaSO₄ (Brinkmann) (the fluorescent indicator does not aid in identification of aflatoxins; no fluorescent-free product was available).

(d) *Mycological agar*.—Difco Laboratories, Detroit, Mich.

(e) *Sodium lauryl sulfate*.—0.01% aqueous solution.

(f) *Rice*.—Commercial brand, long grain ("White River", Riviana Foods).

Procedure

To prepare the aflatoxin, mycological agar slants were inoculated with *Aspergillus flavus* strain NRRL2999, obtained from the USDA Northern Regional Research Laboratory and allowed to grow for 1 week at 27°C. Three ml 0.01% sodium lauryl sulfate solution was added to the slant and shaken to prepare the spore suspension.

Prior to autoclaving, 50 g rice was soaked with 25 ml tap water in 250 ml Erlenmeyer flasks. A 0.5 ml aliquot of the above prepared spore suspension was added to inoculate the rice. After inoculation, the flasks were placed on a rotary shaker for 5 days. Each day the cultures were broken up by vigorous shaking and/or by the use of a sterile glass rod. We found that higher yields were usually obtained by not adding additional water during this period in spite of the fact that the cultures appeared quite dry. After 5 days of incubation the cultures were removed from the shaker and enough chloroform was added to cover the culture mat. The cultures were transferred to a large Soxhlet extractor and extracted with chloroform for 16 hr.

Since preliminary experiments indicated that the extraction in the presence of light gave smaller yields of B₁, a study was undertaken to evaluate this factor.

Ten culture flasks were prepared as described. After 5 days of incubation the cultures were removed from the shaker and enough chloroform was added to cover the culture mat. Five of the 10 cultures so prepared were covered with aluminum foil to keep out all light. The others were exposed to normal fluorescent room light. The contents of the covered flasks were combined in a large Soxhlet extraction apparatus and extracted with chloroform for 16 hr. The Soxhlet extraction apparatus was covered with aluminum foil and most of the extraction was performed during the night with room lights out. The other 5 were similarly extracted in a Soxhlet extraction apparatus, but this time they were exposed to normal room light and the extraction was performed during daylight hours. The contents of the two extractions were assayed by a modification of the Pons and Goldblatt method (4, 5):

TLC plates were coated with a 2 mm thick layer of silica gel PF₂₅₄, dried for 15 hr at room temperature, and then activated by heating 2 hr at 130°C. The entire extract from 50 g culture was dissolved in ca 15 ml chloroform and streaked on a plate with a mechanical streaker. The quantity of the total material applied was ca 270 mg/plate, containing from 20 to 40 mg B₁.

The following solvent systems were investigated relative to their resolving power on these preparative layer plates:

(1) 15% acetone in chloroform (6); (2) 10% acetic acid in chloroform; (3) chloroform:acetone:2-propanol (7); (4) benzene:ethanol:water (87.5:10:2.5) (upper phase) (8); (5) ether:chloroform:acetic acid (2:2:1) (9); (6) 1% methanol in chloroform; (7) 3% methanol in chloroform; and (8) 5% methanol in chloroform.¹

¹ Many methods use varying ratios of MeOH and CHCl₃.

Solvents 1, 3, 5, and 7 seemed to be the best solvent systems in their ability to separate this amount of aflatoxin. We chose Solvent 1 (15% acetone in chloroform) because of its resolution and convenience; an equilibrated chamber was not needed.

After a 15 cm development distance, the B₁ band was identified under long wave UV light and the spot was scraped off with the silica gel. Aflatoxin B₁ was eluted from the silica gel with absolute methanol and enough water and chloroform were added to the methanol solution to obtain a two-phase system. The B₁ was partitioned and separated into the chloroform-rich phase. After the B₁-chloroform solution was dried on a sodium sulfate column, the solvent was evaporated in a flash evaporator.

Results and Discussion

The sample extracted in the dark yielded 240 mg B₁ or 0.96 mg/g substrate. The sample extracted in the light yielded only 129.6 mg B₁ or 0.52 mg B₁/g substrate. Therefore, an 84% greater yield was obtained by working in the dark. In addition, we have found it best to develop the plates in the dark and remove the B₁ from the silica gel as soon as possible to avoid degradation.

It was generally necessary to rechromatograph the aflatoxin on another TLC plate as before to obtain B₁ with a purity of at least 95% relative to other extraneous fluorescent compounds. This degree of purity was estimated according to the widely used method of comparing fluorescent intensities. However, with care, it is often possible to obtain this degree of purity after one development, especially when the Rodder streaker is used.

An interesting problem relative to the separation of B₁ from certain pigments on the TLC plate was also studied. A flask of B₁ that had been allowed to sit around for several months was rechromatographed. On first observation, there did not appear to be very much B₁ on the plate after development but rather a dark greenish-yellow pigment in the general B₁ area. However, upon lightly scraping this pigmented band from the surface of the silica gel, we found the typical bright blue fluorescence characteristic of B₁ underneath. This greenish-yellow pigment covers the B₁ only on the surface of the silica gel. Most of this pigment can be removed by simply scraping the surface, and fairly pure B₁ is obtained. It is interesting to note that this pigment usually cannot be easily seen when a more dilute solution is spotted on a TLC plate as is done in the usual assay procedures. This is partly due to the fact that the amount of pigment

is small relative to the amount of B₁ as well as the fact that "rechromatography" necessitates the mixing of pigment with B₁ which makes it less obvious to the naked eye. However, such a pigment at high concentration may be misleading.

Although the column chromatographic technique of Shotwell *et al.* (10) has obviously been adequate for these workers, we have been unable in repeated attempts to reproduce the degree of resolution which is reported. Theoretically, of course, TLC is far superior to column chromatography in resolving power per unit weight of adsorbent. This TLC procedure is a rapid and efficient way of preparing pure aflatoxin B₁. For example, one technician should be able to extract and isolate from culture mats up to 1 g TLC-pure B₁ per day.

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