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NEW SOLVENT SYSTEMS FOR THIN-LAYER CHROMATOGRAPHY OF AFLATOXINS

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SUMMARY

Three new solvent systems have been developed for the thin-layer chromatography (TLC) of aflatoxins B_1, B_2, G_1, and G_2. Solvent system "A" is an analytical adsorption chromatography system which changes the usual resolved sequence of aflatoxins to B_1, G_1, B_2, and G_2. Solvent "B" is a liquid partition chromatography system which is very good analytically and has the resolution needed to make its use for preparative TLC practical. The third TLC solvent system, "C", is an adsorption chromatography system which is excellent both analytically and preparatively. Solvent system "C" requires two developments or three hours of continuous development to give the maximum degree of resolution.

INTRODUCTION

Aflatoxins are toxic metabolites produced by some strains of the fungus *Aspergillus flavus*[1,2], which under the proper conditions of temperature and humidity will grow quite well on grains, peanuts, and other organic material. These metabolites have been implicated in intoxications of domestic animals which have been fed moldy feed[3,4]. Several papers have been published concerning aflatoxins which report techniques for the isolation and purification of sufficient quantities of the aflatoxins to meet other research requirements. The technique of thin-layer chromatography (TLC) has found extensive use in these research projects. The most important advantages of TLC are its resolving power, sensitivity, and speed. It is sensitive enough to detect nanogram quantities and has sufficient resolving power to separate milligram quantities of closely related aflatoxins into the following sequence: aflatoxin B_1, B_2, G_1 and G_2. Most of the solvent systems which have been reported in the literature relative to aflatoxin fractionation by TLC resolve these components into this same sequence. Chloroform–methanol systems with variations from 2 to 7% methanol have been reported by several laboratories[5–8]. Other solvent systems which provide an analytically effective separation include chloroform–acetone (90:10 or 85:15)[9], diethyl ether–

methanol–water (96:3:1)[10], the upper phase of benzene–ethanol–water (46:35:19)[11] and a partition system using benzene–formamide–water[12]. A special system for the purification of aflatoxin G_1 has been used to demonstrate that pure aflatoxin G_1 has a blue, rather than a green, fluorescence. This system of LIJINSKY AND BUTLER[13] is chloroform–diethyl ether–acetic acid (2:2:1). Recently, a two-dimensional system for TLC of aflatoxins has been reported by PETERSON AND CIEGLER[14] in which the plate is developed first in chloroform–acetone (90:10) and then in ethyl acetate–isopropanol–water (10:2:1). This system separates aflatoxin B_2 and G_1 better than the others and provides a way to purify aflatoxin G_2, which has been most difficult. All of these have been tried in this laboratory following the conditions described in the respective papers. The results of these comparison experiments have served to encourage publication of the solvent systems developed in this laboratory. The purpose of this paper is to report the effectiveness of three new solvent systems for TLC of aflatoxins.

METHODS

A thin layer of Silica Gel H* or HR* plus G-HR* (1:1, w/w) ($^1/_4$, $^1/_2$ or 1 mm) was applied to clean glass plates with an adjustable Desaga applicator*. The plates were air dried overnight and activated by heating to 120° for 1 h before being used or stored in a large desiccator. All the solvents were analytical reagent or reagent grade and the chloroform was redistilled before use. Most chromatograms were developed by the ascending method in the dark[15] at room temperature (25°) in unlined, unequilibrated glass tanks[16] containing freshly mixed solvent. Two variations of the continuous development technique were used with solvent system "C". The first procedure was with the B-N Chamber*,[17], in the horizontal position. Good results could also be obtained by placing the top of the regular development tank ajar after the solvent front has ascended about three-fourths of the way up the plate to allow for solvent evaporation from the top of the plate and continuous development. This was done in a hood. Fluorescence of the aflatoxins was visualized with the UV lamp** having a primary wave length of 365 mμ which corresponds with the excitation wave length for all four major aflatoxins[18]. UV absorption spectra of ethanol solutions of aflatoxins B_2 and G_1 were obtained by using a Beckman DB-G spectrophotometer equipped with a recorder. The crude aflatoxin mixture, obtained by Dr. A. C. PIER of this laboratory from Mr. PETER ROGOVIN, Northern Utilization Research and Development Division, Peoria, Ill., was part of the same batch used for studies of acute intoxication in swine[19].

RESULTS AND DISCUSSION

In the course of our mycotoxin research[19], three new solvent systems were developed which added to the value of TLC as a procedure for isolation and purification of aflatoxins.

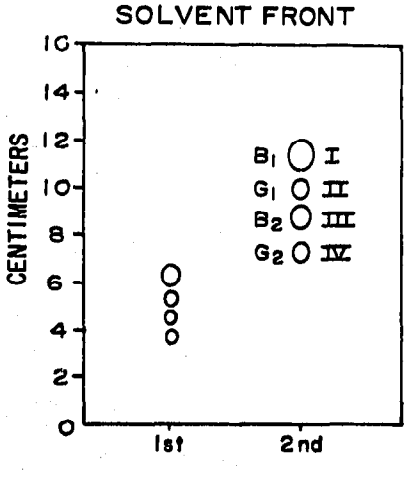
* Distributed by Brinkmann Instruments, Westbury, N.Y.
** Burton Ultraviolet Lamp, Model 19-0, Santa Monica, Calif.

Solvent system "A"

The high resolving power of TLC has proven very useful in characterization problems involving closely related compounds such as the aflatoxins. This was demonstrated again when it was found that the order or sequence of the aflatoxin series was different in this system from that in the others. Solvent system "A" consisting of methylene chloride–trichloroethylene–*n*-amyl alcohol–formic acid (80:15:4:1) changed the resolved order of the aflatoxins from B_1, B_2, G_1, G_2 to B_1, G_1, B_2, G_2. It required about 50 min for the solvent front to ascend a distance of 16 cm from the origin in an unlined, unequilibrated tank at room temperature (25°). The best chromatograms were obtained using $^1/_4$ mm layers of a mixture of Silica Gel HR and G-HR (1:1, w/w) as the adsorbent. The TLC plates were activated at 120° for 1 h and cooled before the sample was applied. Resolution of the four major aflatoxins was accomplished by one development, but was improved by a second development (Fig. 1). Reversal of aflatoxins B_2 and G_1 from their usual order was demonstrated by scraping off the four major fluorescent bands into separate tubes, eluting and rechromatographing with another TLC solvent system such as solvent system "C" or chloroform–acetone (90:10). Results of a typical experiment are illustrated in Fig. 2. In addition, the UV absorption spectra of fluorescent spot No. II from solvent system "A" had absorption maxima (in ethanol) at 243, 257, 264 and 362 mμ, which corresponded to those for aflatoxin G_1 standard[20]. Fluorescent spot No. III had absorption maxima (in ethanol) at 223, 265 and 362 mμ, which corresponded to those for aflatoxin B_2 standard[6].

Solvent system "B"

This is a liquid partition system which works well analytically. The TLC plates were sprayed with 12 ml of a solution of *tert.*-butyl alcohol–formic acid–water (10:1:25) per 8 × 8 in. plate and air dried again for 30 min. The plates were then ready for sample application and development in the ascending direction in an unlined, unequilibrated tank at room temperature (25°). One development required about 35 min and accomplished excellent resolution of the fluorescent toxins. The time of development varies



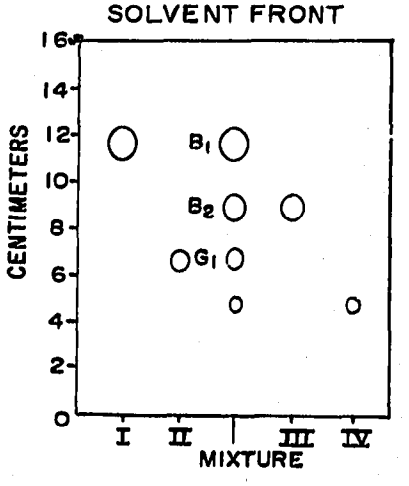
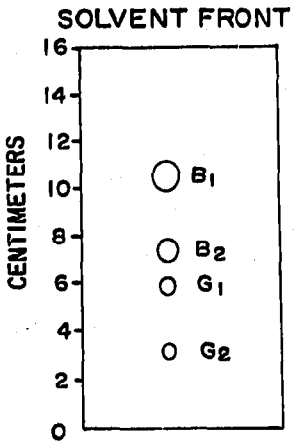


Fig. 1. Separation of aflatoxins on Silica Gel HR plus G-HR (1:1, w/w) after one and two developments with solvent "A".

Fig. 2. Separation of aflatoxins on Silica Gel H after two developments with solvent "C". Purified fractions are from TLC system "A", as in Fig. 1. I = aflatoxin B_1; II = G_1, mixture of four aflatoxins; III = B_2; and IV = G_2.



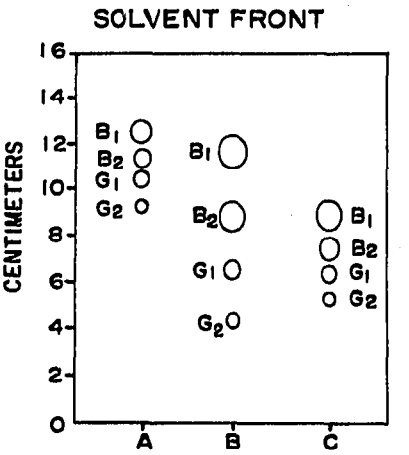


Fig. 3. Separation of aflatoxins on Silica Gel H after one development with solvent "B".

Fig. 4. Comparison of resolution of aflatoxins after two developments in the respective solvent systems. A = Chloroform–methanol (97:3, v/v) on Silica Gel G-HR; B = solvent "C" on Silica Gel H; C = chloroform–acetone (90:10, v/v) on Silica Gel G-HR.

with the thickness of the layer, the amount of spray used, the drying conditions, and the time between spraying and spotting sample. Best results were obtained with thin layers of Silica Gel H at $^1/_4$ mm thickness (Fig. 3). Solvent system "B" consisted of xylene–*tert.*-butyl alcohol–formic acid (94:5:1). The boiling points of *tert.*-butyl alcohol and xylene are low enough so that they can be removed from the aflatoxin by evaporation under vacuum with a Roto-Vap* at a temperature of 45°.

Solvent system "C"

Solvent system "C" provides a high level of resolution between all four major aflatoxins which makes preparative TLC much more practical and effective. In most of the TLC systems published to date for the separation of aflatoxins, the toxic fluorescent compounds are so close together (Figs. 4A and C) that it makes the use of these solvent systems for preparative TLC of aflatoxins rather tedious if not impractical. The highest quality chromatograms using solvent system ""C" were obtained with $^1/_4$ mm thin layers of Silica Gel H processed by continuous development in the B-N chamber for 3 to 4 h or by using the usual ascending technique and multiple development. Maximum resolution required two developments with each one taking about 75 min (Fig. 4B). Solvent system "C" consists of chloroform–trichloroethylene–*n*-amyl alcohol–formic acid (80:15:4:1). In 1962, VAN DER ZIJDEN *et al.*[15] reported using 72 large TLC plates to get 40 mg of aflatoxin B_1. Recently, HANNA AND CAMPBELL[21] reported a preparative procedure using 2 mm thick layers of Silica Gel PF_{254} with $CaSO_4$ as the adsorbent layer and developed with 15% acetone in chloroform. They applied 270 mg of solids per plate which contained 20 to 40 mg of aflatoxin B_1 and recovered an aflatoxin preparation of 95% purity. We were able to put 150 mg of crude solids on each large TLC plate of 1 mm thickness and after subsequent development in solvent "C" and elution obtained 60 to 70 mg of purified aflatoxin B_1. System "C" is very good, both analytically and preparatively, for the isolation and purification of aflatoxins B_1, B_2, G_1 and G_2.

* Buchler Instruments, Fort Lee, N.J.

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