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COMPARISON OF METHODS FOR AFLATOXIN ANALYSIS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

LARRY M. SEITZ

U.S. Department of Agriculture, Agricultural Research Service, U.S. Grain Marketing Research Center, 1515 College Avenue, Manhattan, Kansas 66502 (U.S.A.)

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SUMMARY

Reversed-phase columns packed with octadecyl and phenyl reversed phases did not provide adequate separation of aflatoxins. A pellicular adsorption column provided partial separation, *i.e.* B_1 and B_2 from G_1 and G_2, but not B_1 from B_2 nor G_1 from G_2. A microparticulate adsorption (Micro-A) column completely separated aflatoxins B_1, B_2, G_1, and G_2. Detection was more selective at 350 nm (or 365 nm) than at 254 nm. A Fluoro Monitor Model 1209 detector (Laboratory Data Control Corp.) was more sensitive for aflatoxins G_1 and G_2 than for B_1 and B_2. Aflatoxin B_1 at the 30-ppb level in yellow corn was detected with the Micro-A column and the 350-nm photometer. The limit of detection was estimated at about 10 ppb**.

INTRODUCTION

There is considerable interest in high-pressure liquid chromatography (HPLC) for aflatoxin analysis[1–4]. This technique is attractive as a possible alternative to thin-layer chromatography (TLC) because of its potential to make separations faster and provide improved quantitative accuracy and precision. Realization of the potential, however, requires optimum column and detector performance. Research is presently in a stage of selecting among available columns and detectors to determine optimum conditions. In this paper the separation of aflatoxins B_1, B_2, G_1, and G_2, the use of 350 nm *versus* 254 nm UV absorption and fluorescent detection, and the detection of aflatoxin in yellow corn are discussed.

EXPERIMENTAL***

A Varian Associates 4200 series solvent delivery system was used. A Varian

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** Throughout this article the American billion (10^9) is meant.

*** Mention of specific instruments or trade names is made for identification purposes only and does not imply endorsement by the U.S. Government.

UV detector was modified by Laboratory Data Control (LDC) Corporation to allow operation at either 254 nm or 350 nm. A Fluoro Monitor Model 1209 from LDC Corporation was used to detect fluorescence.

The types of column packings compared in this study are described in Table I. Commercial packings were chosen only as being representative of the four types. Other commercially available packings of each type may be equivalent or superior to those mentioned.

TABLE I

TYPES OF COLUMN PACKINGS USED FOR COMPARISON

Column packing type	*Description*	*Approximate particle size (μm)*
Octadecyl reversed phase (ORP) (ODS-Permaphase*)	Octadecyl groups permanently bonded to pellicular packing (Zipax*)	50
Phenyl reversed phase (PhRP) (Diphenyl Corasil**)	Phenyl groups permanently bonded to pellicular packing (Corasil II**)	50
Pellicular adsorption (PA) (Corasil II**)	Solid glass bead core with porous silica crust	37–50
Microparticulate adsorption (Micro-A) (μPorasil**)	Porous silica	10

* Trademark of DuPont, Wilmington, Del. U.S.A.
** Trademark of Waters Ass., Framingham, Mass., U.S.A.

The PA and ORP columns were dry-packed according to a vertical bumping procedure recommended by Randau and Schnell[5]. The packing was introduced into 2.1-mm-I.D. precision-bore stainless-steel tubing in about twenty consecutive portions per each 61 cm of length. After each addition, the column was forcefully bumped, in a vertical direction, about fifteen times. The PA column, 61 cm long, was used in a straight configuration at ambient temperature. The ORP column was 244 cm long and coiled, after packing, to fit into a water-bath held at 50°. A straight 61-cm, prepacked PhRP column was fitted with a water jacket to allow temperature control at 50°. A prepacked Micro-A column was used at ambient temperature. The column was 6.35 mm O.D. × 30 cm, packed with fully porous 10-μm silica gel, and the interior surface of the tubing was finished to eliminate longitudinal striations which could cause channeling of solvent.

Solvents were spectrograde quality and degassed before introduction into the pumps. Solvents used with the PA column contained 0.1 ml water per 100 ml solvent. Chloroform and methylene chloride used with the Micro-A column were adjusted, to what is termed here "50% water saturated", as follows: The respective solvent (reagent grade in this case) was partitioned by a separatory funnel with three separate portions of distilled water. After removal of the third portion of water, the solvent was slowly drained into another dry separatory funnel. About 5 min were allowed for excess water to collect on the side before slowly draining the solvent into a dry

flask or bottle. The wet solvent was divided into two equal portions. One portion was dried over anhydrous calcium chloride (8 mesh) for about 10 min. The calcium chloride was removed by filtration and the dried solvent combined with the remaining portion of wet solvent.

A synthetic mixture of aflatoxins B_1, B_2, G_1, and G_2 was used in the investigation of the ORP column. Aflatoxin standards from the USDA-ARS Southern Regional Research Center, New Orleans, La., were used in all other tests. Assignments of chromatographic peaks were confirmed by TLC.

The yellow corn extracts were prepared by AOAC Method I (CB Method)[6].

RESULTS AND DISCUSSION

Columns packed with ORP or PhRP did not adequately separate aflatoxins B_1, B_2, G_1, and G_2. The best separation from a 244-cm ORP column is shown in Fig. 1B. After continued use, the performance deteriorated to partial separation (Fig. 1C). Practical use of the ORP column was limited because of frequent overloading due to slight solubility of aflatoxins in water and because the long column made quick analysis impossible. Elution of aflatoxins from a 61-cm PhRP column required 45 to 50% methanol in water. This solvent minimized overloading problems but reduced column efficiency. Neither of these reversed-phase systems appear to warrant further consideration for aflatoxin separation.

An Application Bulletin published by a chromatography supply firm[7] described good separation of aflatoxins B_1, B_2, G_1, and G_2 with a 2-mm × 500-mm glass column packed with PA, 20% isooctane in chloroform, and 0.7 ml/min flow-rate. When we used the described procedures, except that stainless-steel tubing was substituted for glass, aflatoxins were not eluted from the column. Solvent mixtures of greater polarity than 20% isooctane in chloroform were required and separation of aflatoxins was not complete. Aflatoxin B_1 was separated from G_1, whereas B_2 was not separated from B_1 nor G_2 from G_1. Results obtained with 3% acetone–97% chloroform are illustrated in Fig. 2. Similar chromatograms were obtained with either 45% tetrahydrofuran–isooctane (45:55) or ethanol–isooctane (25:75). The complete separation of aflatoxins reported earlier with another lot of PA has not, to my knowledge, been confirmed.

Complete separation of aflatoxins was achieved with the Micro-A column (Figs. 3 and 4). The order in which B_2 and G_1 eluted was reversed when the solvent was changed from chloroform–methylene chloride (75:25) containing 0.5% methanol (Fig. 3) to methylene chloride containing 0.6% methanol (Fig. 4). Performance was not deteriorated by continuous use for several days. Isooctane–tetrahydrofuran was not a useful solvent system with this column.

The Micro-A column, which is twice as large in diameter and one-half the length of the PA column, requires a flow-rate of at least twice that required for the PA column to obtain comparable retention times. The rather high flow-rates are a disadvantage, from a routine analytical standpoint, because large amounts of solvents are required.

A system designed for routine use should include convenient recycling of the solvent. It would, therefore, seem advantageous to use either a methylene chloride–methanol or chloroform–methanol solvent system for routine aflatoxin analyses. The

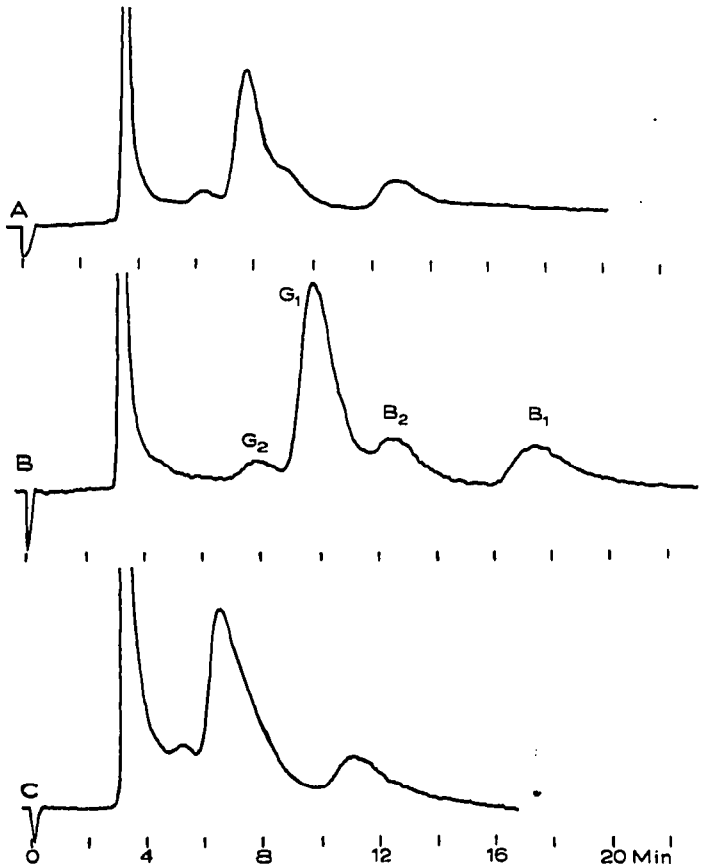

Fig. 1. Chromatograms obtained from an ORP column at 50°, 100% water mobile phase, 0.67 ml/h flow-rate, 1500 p.s.i. and 254-nm detector at 0.04 absorption units full scale (a.u.f.s.). The 6 μl of aflatoxin solution, in methanol, injected, contained about 65, 55, 200, and 30 ng of B_1, B_2, G_1, and G_2, respectively. Chromatograms illustrate separation achieved at the following stages: (A) Immediately after preparation and conditioning of the column. (B) After the column had been eluted with 100% methanol overnight for the first time. (C) After the column had been used continuously for several days. Performance deteriorated to that shown here and eluting with 100% methanol did not restore the resolution to that in B.

used solvent could be cleaned by partioning with water–methanol and adjusting to "50% water saturation" as described above. Solvents containing mixtures of methylene chloride and chloroform could also be handled this way, but it would be difficult to keep the ratio of the two solvents constant.

The sensitivity of UV absorption detection at 350 nm is illustrated in Fig. 3. Detection is more effective at 350 nm (or at 365 nm used by other available detectors) than at 254 nm because it provides greater selectivity for aflatoxins, is easier to establish and maintain a steady baseline, and tolerates a variety of injection solvents. Solutions of aflatoxins in aromatic solvents can be injected with little distortion of the chromatogram, whereas much of the early part of the chromatogram is masked at 254 nm. Advantages of 365-nm detection have been discussed by Williams *et al.*[3].

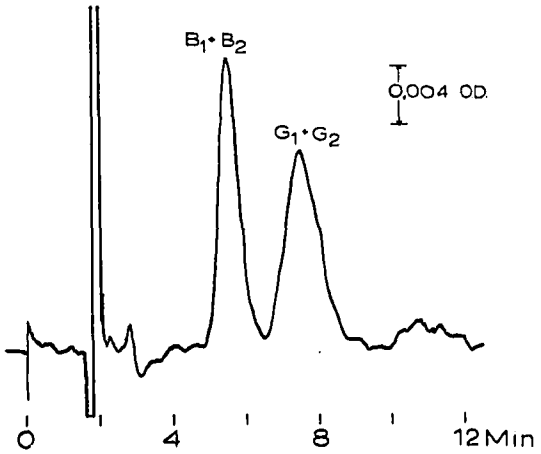

Fig. 2. Chromatogram illustrating partial separation of aflatoxins on a PA column, 3% acetone in choroform, 0.83 ml/min flow-rate, 350-nm detector at 0.04 a.u.f.s., approximately 150 p.s.i. The sample was 20 μl of a benzene–acetonitrile (98:2) solution containing 5, 1.5, 5, and 1.5 ng/μl of B_1, B_2, G_1, and G_2, respectively.

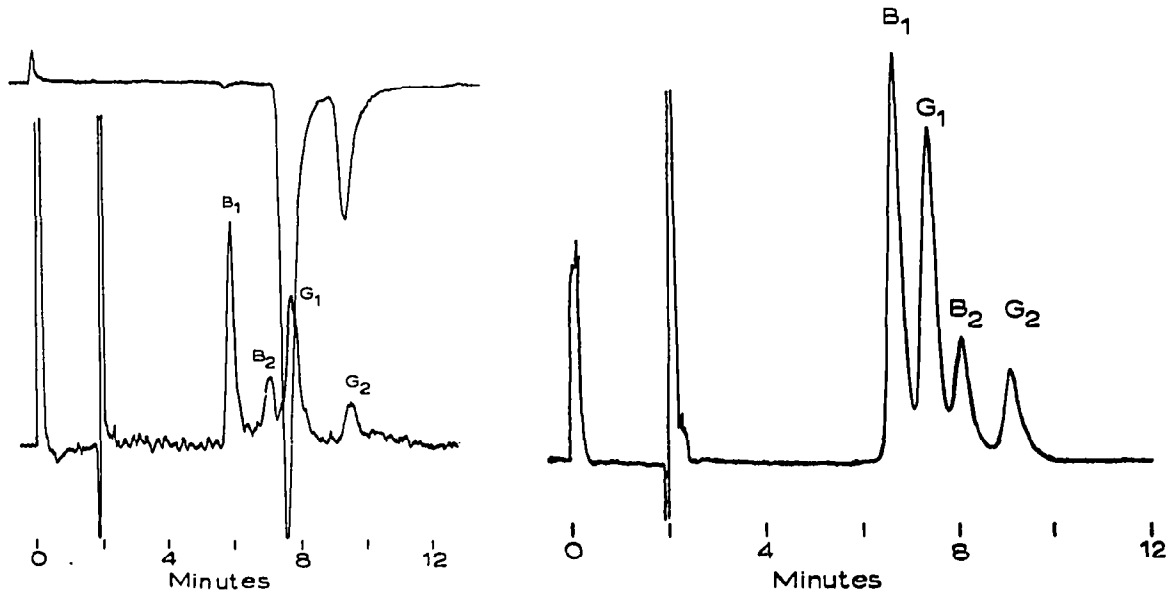

Fig. 3. Separation of aflatoxins using a Micro-A column, chloroform–methylene chloride (75:25) containing 0.50% methanol, 2 ml/min flow-rate, about 450 p.s.i. The fluorescence detector (upper curve) was set at 64× attenuation and the 350-nm photometer (lower curve) at 0.01 a.u.f.s. The chromatogram represents 25 ng each of B_1 and G_1, and 7.5 ng each of B_2 and G_2.

Fig. 4. Separation of afltoxins using a Micro-A column, methylene chloride (50% water saturated) containing 0.6% methanol, 2 ml/min flow-rate, about 450 p.s.i., 350-nm photometer at 0.04 a.u.f.s. The chromatogram represents 150 ng each of B_1 and G_1, and 45 ng each of B_2 and G_2.

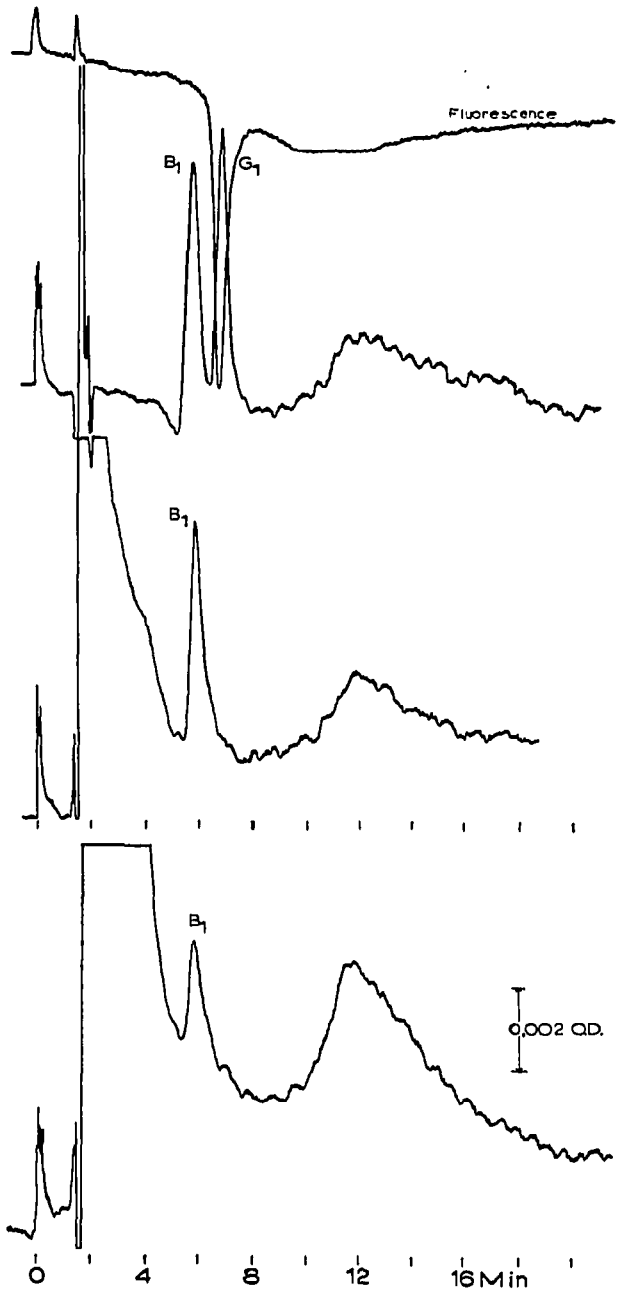

Fig. 5. Chromatograms obtained using the PA column and the following program at 1 ml/min flow-rate, about 200 p.s.i.: hold at 25% tetrahydrofuran (THF) for 2 min; increase THF 6%/min for 6 min; hold at 61% THF. The 350-nm detector was set at 0.01 a.u.f.s. and the fluorescence detector at 8 × attenuation. The upper chromatogram represents 50 ng each of B_1 and G_1. The middle and lower chromatograms represent 20-μl injections of extracts of yellow corn containing 150 and 50 ppb, respectively.

The fluorescence detector exhibited much greater sensitivity for aflatoxins G_1 and G_2 than for B_1 and B_2 (Figs. 3 and 5). Emission from G_1 and G_2 (about 450 nm) is nearer the wavelength at which the sensitivity of the detector is optimum. Emission from aflatoxin B_1 and B_2, on the other hand, is closer to the lower wavelength limit (approximately 400 nm) of the detector. Consequently, the usefulness of the LDC fluorescence detector for aflatoxin analysis is limited. Similar problems have been noted with another detector (ref. 4, paper 24).

Chromatograms of yellow corn extracts (50 and 150 ppb B_1) obtained by the PA column are shown in Fig. 5. Solvent programming was used to sharpen the aflatoxin peak and achieve resolution from early eluting components. The limit of detection was estimated at about 20 ppb.

Under the conditions in these tests, the Micro-A column provided better sensitivity than other columns tested, without the need for solvent programming (Fig. 6). The extract injected into the Micro-A column was from yellow corn which contained 30 ppb B_1 as determined by TLC. Comparison of aflatoxin peak areas in Fig. 6 indi-

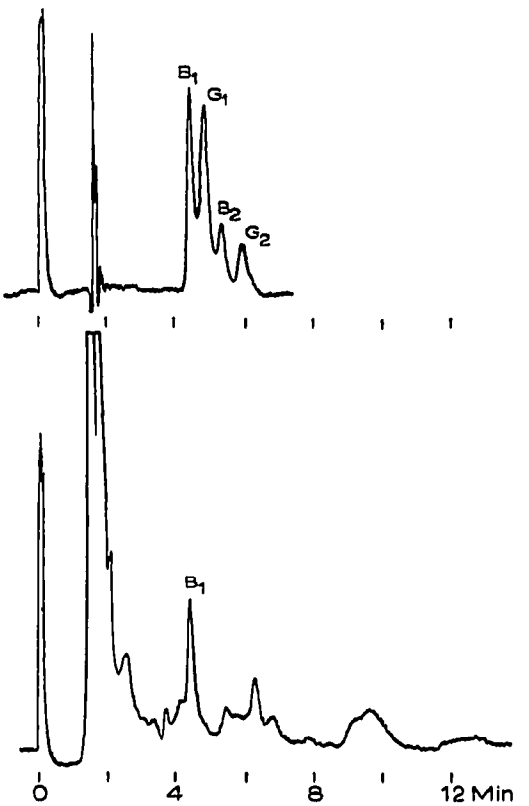

Fig. 6. Chromatograms obtained using the Micro-A column, methylene chloride containing 0.8% methanol, 2.7 ml/min flow-rate, about 550 p.s.i., 350-nm photometer at 0.02 a.u.f.s. The upper chromatogram represents 50 ng each of B_1 and G_1, and 15 ng each of B_2 and G_2. The lower chromatogram represents a 50-μl injection of extract from yellow corn containing 30 ppb (as determined by TLC) aflatoxin B_1.

cates a B_1 content of 36 ppb. At the 10-ppb level, one may have difficulty in deciding whether or not aflatoxin is present. TLC methods will allow detection at the 1- to 2-ppb level.

Extensive studies will be required to determine if HPLC provides better quantitation than TLC.

Solvent programming in the methylene chloride–methanol or chloroform–methanol systems caused fairly large changes in baseline, especially at the higher-sensitivity settings of the photometer, *i.e.* 0.01 or 0.02 absorption units full scale. If isocratic conditions were to be used for routine analyses, it would be necessary to occasionally flush the column with a polar solvent mixture to remove strongly adsorbed components.

TABLE II
COMPARISON OF HPLC SYSTEMS FOR AFLATOXIN ANALYSIS

Column and solvent	*Advantages*	*Disadvantages*
ORP (244 cm × 2.1 mm I.D.) Water	Solvent (water) is inexpensive and non-toxic. Column can be dry-packed.	Inadequate resolution. Poor sensitivity. Above ambient temperature required. Frequent overloading.
PhRP (61 cm × 2.1 mm I.D.) Water–methanol	Column can be dry-packed.	Inadequate resolution. Poor sensitivity. Above ambient temperature required. Frequent overloading. Re-use of solvent difficult.
PA (61 cm × 2.1 mm I.D.) Isooctane–THF	Column can be dry-packed. Minimal baseline drift in 350-nm detector when solvent programming. Ambient temperature.	Aflatoxin B_1 not resolved from B_2, nor G_1 from G_2. Fair sensitivity. Re-use of solvent is difficult.
PA (61 cm × 2.1 mm I.D.) Acetone–chloroform or ethanol–isooctane	Column can be dry-packed. Ambient temperature.	Aflatoxin B_1 not resolved from B_2, nor G_1 from G_2. Fair sensitivity. Re-use of solvent is difficult. Moderate to severe baseline drift from 350-nm detector when solvent programming.
Micro-A (30 cm × 6.35 mm O.D.) Chloroform–methylene chloride–methanol	Complete resolution of aflatoxins B_1, B_2, G_1, and G_2. Improved sensitivity. Ambient temperature.	Re-use of solvent is difficult. Higher flow-rates required. Column must be packed by high-pressure slurry methods; so must purchase expensive prepacked column. Moderate baseline drift from 350-nm detector when solvent programming.
Micro-A (30 cm × 6.35 mm O.D.) Methylene chloride–methanol	Complete resolution of aflatoxins B_1, B_2, G_1, and G_2. Improved sensitivity. Methylene chloride solvent can be recycled and is less toxic than chloroform. Ambient temperature.	Higher flow-rates required. Column must be packed by high-pressure slurry methods; so must purchase expensive prepacked column. Moderate baseline drift from 350-nm detector when solvent programming.

Advantages and disadvantages of the HPLC systems tested in this study are summarized in Table II. When choosing a packing, the emphasis should be placed on one made from porous silica with spherical particles of 10 μ or less in diameter. This is necessary to achieve the high efficiency required to separate aflatoxins B_1, B_2, G_1, and G_2.

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