Journal of Chromatography A, 654 (1993) 247–254
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 529

Determination of aflatoxins by reversed-phase high-performance liquid chromatography with post-column in-line photochemical derivatization and fluorescence detection

Henry Joshua*

Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065 (USA)

(First received May 4th, 1993; revised manuscript received August 26th, 1993)

ABSTRACT

Post-column in-line photochemical derivatization permits fluorescence detection of all four common aflatoxins B_1, B_2, G_1 and G_2. Chromatographic evidence indicates that the photolysis causes the hydration of the non-fluorescent B_1 and G_1 components to the B_{2a} and G_{2a} components respectively. Analysis of naturally contaminated corn samples show no interfering peaks and permits the determination of 1 and 0.25 ppb (w/w) of B_1 and B_2, respectively.

INTRODUCTION

Aflatoxins are a major concern as toxic, carcinogenic and mutagenic contaminants in feeds and foods [1] and are closely monitored by commercial and governmental bodies [2]. The four major aflatoxins produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* are B_1, B_2, G_1 and G_2 (Fig. 1). The B_1 component is usually predominant [3] and is also the most toxic on a mass basis [1]. The determination of aflatoxins in food products by chromatography has recently been reviewed [4]. Reversed-phase HPLC affords separation of the components using C_{18} columns and water–acetonitrile–methanol as mobile phase. Whereas UV detection at 365 nm affords peaks for all four components, fluorescence detection (365 nm excitation, >415 nm emission) is more selective and has greater sensitivity for the B_2 and G_2 components. Sensitivity for components B_1 and G_1, however, is quite poor due to the quenching of their fluorescence by eluents used in both normal- and reversed-phase HPLC. Two basic approaches have been used to increase the fluorescence detectability of the B_1 and G_1 components in HPLC: (a) methods to decrease the quenching of fluorescence by modifying the eluent and/or the detector and (b) pre- or post-column derivatization to compounds whose fluorescence is not quenched.

Methods of reducing fluorescence quenching include, for normal-phase HPLC, switching from chloroform, dichloromethane or methanol as major components of the eluent to a mobile phase consisting mainly of toluene [5] and packing the fluorescence flow cell with silica gel [6].

For reversed-phase HPLC the addition of cyclodextrins to the mobile phase causes a decrease in the quenching of fluorescence of B_1 and G_1 [7–9].

Pre-column derivatization includes the re-

* Present address: Aura Industries, Inc., P.O. Box 898, Staten Island, NY 10314, USA.

0021-9673/93/$06.00

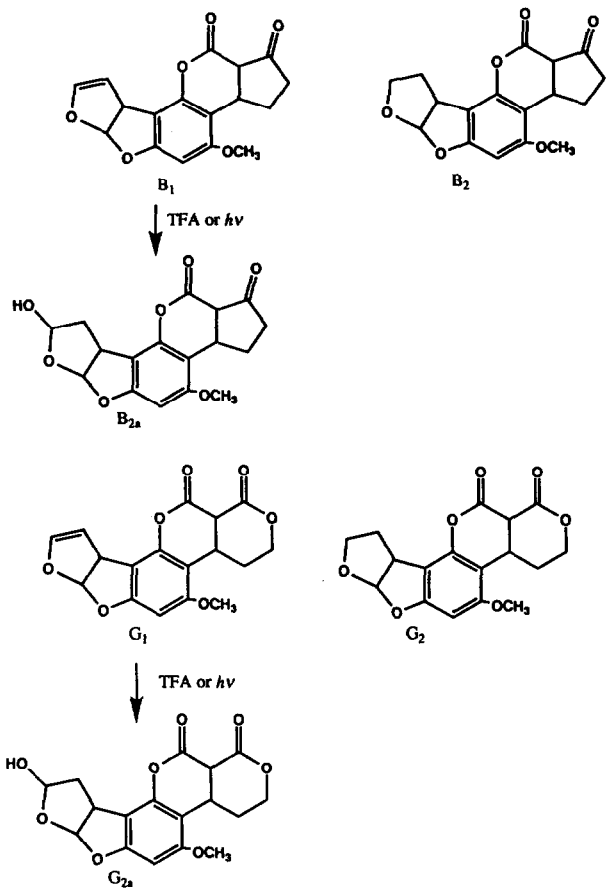

Fig. 1. Structure of the four common aflatoxins and the hydration products of B_1 and G_1.

action of B_1 and G_1 in aqueous trifluoroacetic acid (TFA) to form B_{2a} and G_{2a} (Fig. 1) whose fluorescence is not quenched under HPLC conditions [10,11]. Unfortunately, pre-column derivatization requires chemical manipulations which are time consuming, involve aggressive reagents and are difficult to automate. The reactions are not always complete and side reactions occur. Furthermore, the derivatives are usually more polar than the starting components and consequently elute earlier in reversed-phase chromatography with retention times which are similar to many polar compounds unrelated to the aflatoxins.

Similarly, B_1 and G_1 form non-quenched derivatives when reacted with iodine. A post-column application of this procedure requires the mixing of the column effluent with a stream of water saturated with iodine followed by the reaction of the flowing mixture in a heated capillary reactor [12]. The disadvantages of this procedure include the requirement to prepare the iodine solution daily, the necessity for two pumps, dilution of the eluent stream, the need to thermostat the reactor coil and insufficient day-to-day reproducibility [13]. Post-column split flow iodine addition from a solid-phase iodine reservoir to derivatize aflatoxins was reported requiring only one pump [14].

Post-column derivatization of aflatoxins with electrochemically generated bromine also produces compounds which are well detected by fluorescence detectors but requires a KOBRA electrochemical cell [13].

Post-column photochemical derivatization procedures have been reported to successfully enhance the sensitivity and selectivity of response of many analytes by a variety of detectors [15,16].

The application of photochemical derivatization for the determination of aflatoxins by HPLC with fluorescence detection has not been reported to our knowledge. We report the use of post-column photochemical derivatization to increase the fluorescence response of aflatoxins B_1 and G_1. Coupling post-column photochemical derivatization with fluorescence detection thus permits the sensitive detection of all four common aflatoxins with the advantages of simplicity, linearity of response and reproducibility without requiring chemical reagents, additional pumps or electrochemical cells. We also present chromatographic evidence which suggests that the initial photolysis reaction product of G_1 is G_{2a} (Fig. 1). It is highly probable that similarly photolysis of B_1 generates B_{2a}.

Application of this method to the analysis of naturally contaminated corn samples shows no interfering peaks for the B_1 component based on the comparison of chromatograms with and without photolysis. B_1 and B_2 can be determined at 1 and 0.25 ppb (w/w), respectively.

EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of a SP 8700 XR pump, SP 4200 computing inte-

grator, SP 8780 autosampler, Spectra FOCUS rapid scanning UV detector and Spectra-Physics WINNER software (Spectra-Physics Analytical, San Jose, CA, USA). Further a Kratos FS 970 LC fluorometer set for 365 nm excitation and >415 nm emission, 0.2 μA range and 6 s time constant a Microsorb-MV 25 cm × 4.6 mm I.D. C_{18} column (Rainin, Woburn, MA, USA) thermostated at 40°C with a CJB-14 column jacket and a "PHRED" photochemical reactor with low-pressure mercury lamp were used. Knitted reactor coils: KRC 5-25, 5 m × 0.25 mm, KRC 10-25, 10 m × 0.25 mm; KRC 15-25, 15 m × 0.25 mm I.D.; KRC 5-50, 5 m × 0.5 mm; KRC 10-50, 10 m × 0.5 mm I.D.; KRC 15-50, 15 m × 0.5 mm I.D. (AURA Industries, Staten Island, NY, USA). The lengths and I.D. dimensions for these coils are nominal and before knitting. The calculated void volumes based on these values differ from those observed for the knitted coils (see text).

The flow-rate of eluent was 1.0 ml/min and 20-μl full loop injections were made. The eluent composition was water–acetonitrile–methanol (63:22:15) or where noted aqueous 0.1% TFA–acetonitrile–methanol (63:22:15).

Chemicals and solvents

An aflatoxin mixture containing components B_1 and G_1 at 1 μg/ml and B_2 and G_2 at 0.3 μg/ml in methanol (aflatoxin mixture M, cat. No. 4-6303) was purchased from Supelco, Bellefonte, PA, USA). Aflatoxins G_{2a} (cat. No. A-9151), B_1 (cat. No. A-6636), B_2 (cat. No. A-9887), G_1 (cat. No. A-0138) and G_2 (cat. No. A-0263) were purchased from Sigma, St. Louis, MO, USA). Solvents were HPLC-grade Omnisolv, EM Science, Gibbstown, NJ, USA). Water was purified through a Milli-RO4 and Milli-Q water purification system (Millipore, Milford, MA, USA).

The aflatoxin mixture was serially diluted with methanol to give B_1, G_1, and B_2, G_2 concentrations of 400, 120; 200, 60; 100, 30; and 50, 15 ng/ml. For the study of fluorescence response as a function of UV irradiation time 20-μl injections of the solution containing B_1 and G_1 at 200 ng/ml and B_2 and G_2 at 60 ng/ml was used.

The dried purified extracts of three naturally contaminated corn samples, prepared according to Wilson and Romer [17], supplied by Romer Labs. (Union, MO, USA), were each taken up in 1 ml of methanol. Each extract represented 4 g of corn. A knitted reactor coil of 15 m × 0.25 mm I.D. was used in the determination of these samples.

RESULTS AND DISCUSSION

Fluorescence response after photoirradiation

Preliminary to the photoirradiation–fluorescence experiments, a commercial mixture of aflatoxins was chromatographed using a UV detector. A 20-μl injection of a solution containing B_1 and G_1 at 1 μg/ml and the B_2 and G_2 components at 0.3 μg/ml with detection at 360 nm gave the chromatogram shown in Fig. 2.

A 15 m × 0.25 mm I.D. knitted reactor coil was attached to a polished support plate, positioned in the bottom of the photochemical reactor housing and the UV lamp assembly placed on top of the coil (Fig. 3). One end of the knitted reactor coil was attached to the outlet of the column and the other end to the flow cell of the fluorescence detector. The residence time in the coil is defined as the void volume in ml divided by the flow-rate in ml/min. The irradiation time is equivalent to the residence time when the UV light is on. The determination of the void volumes is discussed subsequently. A 20-μl injection of the aflatoxin mixture containing B_1 and G_1 at 200 ng/ml and B_2 and G_2 at 60 ng/ml with the "PHRED" light off gave the chromatogram shown in Fig. 4a, while the same injection made with the "PHRED" light on gave the chromatogram shown in Fig. 4b.

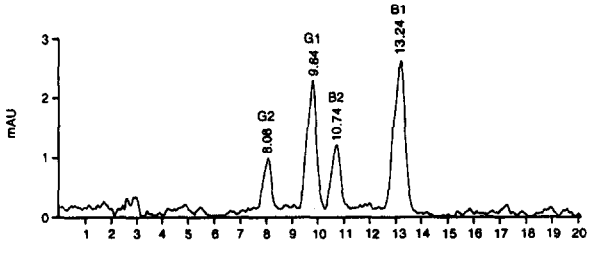

Fig. 2. Chromatogram of aflatoxins with UV detection at 360 nm, using a reversed-phase C_{18} column at 40°C and eluent consisting of water–acetonitrile–methanol (63:22:15) at 1 ml/min. The peaks at 8.08, 9.84, 10.74 and 13.24 min correspond to G_2, G_1, B_2 and B_1 respectively.

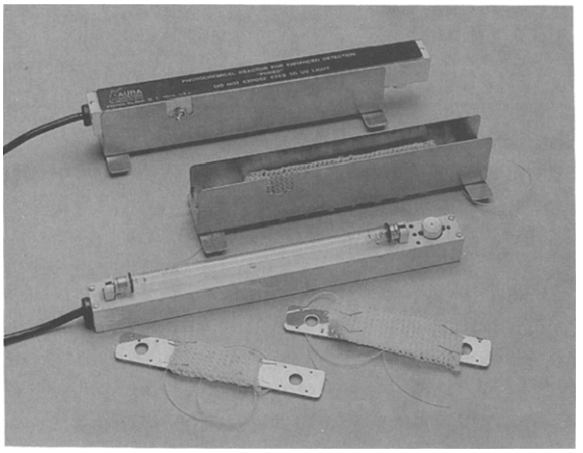

Fig. 3. Photochemical reactor components from front to rear, knitted reactor coils attached to polished support plates, UV lamp attached to lamp fixture, reactor housing with reactor coil placed inside and assembled photochemical reactor.

In Fig. 4a (light off) components G_2 (9.52 min) and B_2 (12.36 min) give significant peaks. In contrast components G_1 (*ca.* 11.4 min) and B_1 (*ca.* 15.0 min) are barely discernable. In Fig. 4b (light on), however, all four components show significant well resolved peaks. The UV irradiation consequently modified the B_1 and G_1 components to fluorescent entities.

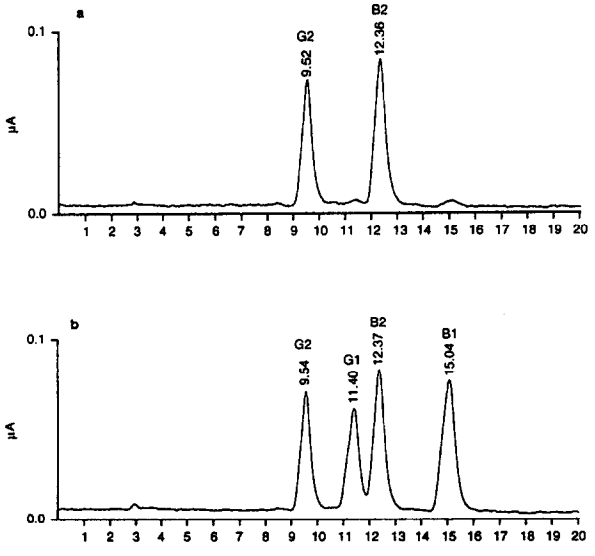

Fig. 4. Chromatogram with fluorescence detection of aflatoxins B_1, B_2, G_1 and G_2, using photochemical reactor with (a) light off and (b) light on. Time scale in min.

Linearity of fluorescence response

The linearity of response was studied with a 15 m × 0.5 mm I.D. knitted reactor coil in the reactor housing and injecting 20-μl samples of the serially diluted aflatoxin solutions starting with one containing B_1 and G_1 at 400 ng/ml and B_2 and G_2 at 120 ng/mol. The peak areas for the four components at the indicated concentrations are plotted in Fig. 5. Linear least squares analysis of the data points yields the intercept, slopes, the coefficient of determination (R^2) and the relative response (peak area/ng/ml). They are listed in Table I. No deviation from linearity was observed within the range studied for any of the four components.

Fluorescence response as a function of UV irradiation time

The irradiation time for each of the coils was determined by subtracting the retention time for G_2 with the column connected directly to the fluorescence detector from the retention time observed for G_2 with a knitted reactor coil interposed between the column and the detector. The retention time differences were also determined for B_2. The retention time differences for each KRC obtained for the G_2 and B_2 components as well as the average of these differences are collected in Table II. As a com-

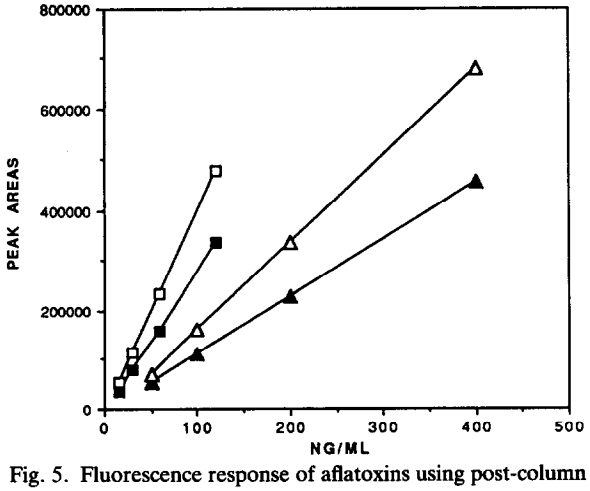

Fig. 5. Fluorescence response of aflatoxins using post-column in-line photolysis. KRC 15-50, light on, 20-μl injections. ■ = G_2; ▲ = G_1; □ = B_2; △ = B_1.

TABLE I

LINEARITY OF RESPONSE AND RELATIVE RESPONSE FOR AFLATOXINS G_2, G_1, B_2 AND B_1

Component	Intercept	Slope	R^2	Relative response
G_2	−8416.3	2858.6	1.000	0.708
G_1	−3107.8	1151.0	1.000	0.285
B_2	−6468.3	4036.7	1.000	1.00
B_1	−12401.4	1735.4	1.000	0.430

parison, Table II also lists the nominal dimension values for the coils before knitting.

Fig. 6a plots the peak areas obtained from B_2 and G_2 for identical 1.2-ng injections as a function of irradiation time in knitted reactor coils of 0.25 and 0.5 mm I.D. The response is essentially constant. In Fig. 6b the peak areas for 4-ng injections of B_1 and G_1 are similarly plotted as a function of UV irradiation time in knitted reactor coils of 0.25 and 0.5 mm I.D. There is a considerable and consistent increase in fluorescence response with irradiation time up to 1.8 min. A smaller increase between 1.8 and 3.1 min indicates that an equilibrium has been reached or that the original photolysis product(s) decomposes further to less fluorescing compounds. Experiments indicating the fate of the photolysis product(s) will be presented below.

The relative B_1/B_2 and G_1/G_2 fluorescence response normalized for equal-mass injections as a function of irradiation time is plotted in Fig. 7. The calculated B_1/B_2 and G_1/G_2 relative response values at 1.8 min are 0.39 and 0.38, respectively and at 3.1 min they are 0.43 and 0.43, respectively. The similarity in response indicates similar photolysis reactions and products for both B and G sets of components. The relative response factors listed above for the post-column photochemical irradiation derivatization can be compared to the B_1/B_2 relative response factors extracted from reports on post-column derivatizations with electrochemically generated bromine [13,18]. Fig. 3a of ref. 13 shows a B_1/B_2 fluorescence response ratio of *ca.* 0.5 for pure standards. Fig. 1b of ref. 18 shows a chromatogram of a maize sample spiked with

TABLE II

NOMINAL DIMENSIONS AND RESIDENCE TIME AT A FLOW-RATE OF 1 ml/min IN KNITTED REACTOR COILS

Coil	Length[b] (m)	I.D.[b] (mm × 100)	Volume[b] (ml)	RTG_2[c] (min)	ΔRTG_2[d] (min)	RTB_2[e] (min)	ΔRTB_2[f] (min)	AVEΔRT[g] (min)
[a]	0.5	25		8.39		11.15		
1	5	25	0.25	8.74	0.35	11.50	0.35	0.35
2	10	25	0.50	9.33	0.94	12.11	0.96	0.95
3	15	25	0.75	9.54	1.15	12.37	1.22	1.185
4	5	50	1.0	9.12	0.73	11.76	0.61	0.67
5	10	50	2.0	10.20	1.81	12.89	1.74	1.775
6	15	50	3.0	11.55	3.16	14.30	3.15	3.155

[a] Column connected directly to fluorescent detector with stainless-steel capillary.
[b] Nominal values before knitting of coil.
[c] Retention time of G_2.
[d] Difference in retention time for G_2 due to insertion of coil.
[e] Retention time of B_2.
[f] Difference in retention time for B_2 due to insertion of coil.
[g] Average of the differences in retention times due to the insertion of coil for G_2 and B_2.

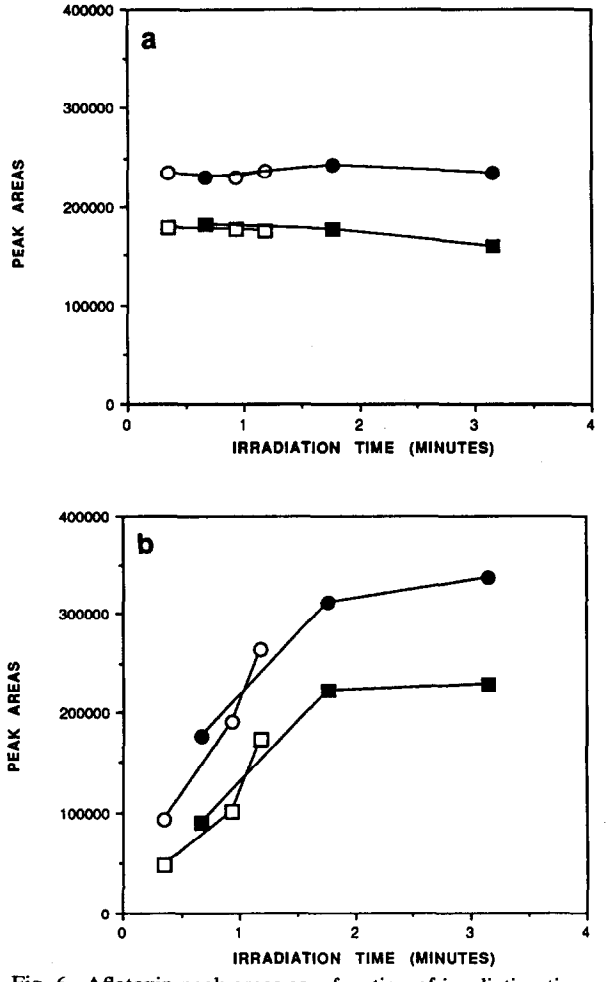

Fig. 6. Aflatoxin peak areas as a function of irradiation time in reactor coils of 0.25 mm (open symbols) and 0.5 mm I.D. (closed symbols). (a) B_2 (circles) and G_2 (squares); (b) B_1 (circles) and G_1 (squares).

aflatoxins. The B_1/B_2 relative fluorescence response calculated from peak heights after normalization for mass of injected components was *ca.* 0.40.

Characterization of photolysis product

The conversion of B_1 and G_1 on silica gel thin-layer plates by irradiation with UV light to new fluorescent more polar compounds was reported in 1967 [19]. A number of subsequent articles have also reported the photoactivation of B_1 [20–24].

For this study 500 μl of a solution containing

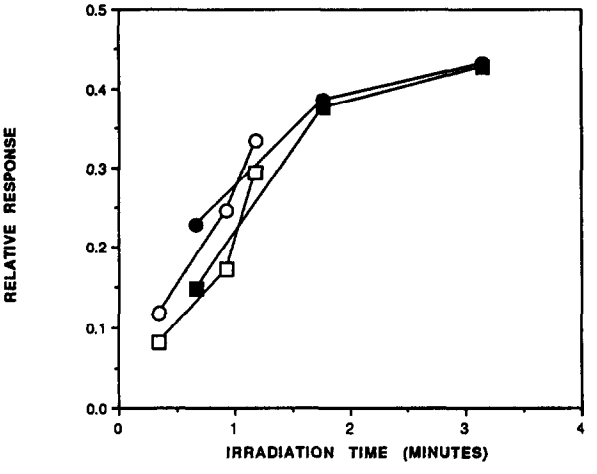

Fig. 7. Relative B_1/B_2 (circles) and G_1/G_2 (squares) fluorescence response as a function of irradiation time. Open symbols, 0.25 mm coils; closed symbols, 0.50 mm coils.

250 μg G_1/ml in methanol was injected directly to a 15 m × 0.5 mm I.D. long irradiated knitted reactor coil using a manual injection valve. The pump, eluent composition and flow rate conditions for the analytical HPLC analysis were used to cause the photolysis in a flow injection mode. The photolysis reaction mix obtained by the 3.15 min irradiation of G_1 in the flow injection mode was collected and analyzed by analytical HPLC using the same 15 m × 0.5 mm I.D. coil in the photochemical reactor. The chromatogram (Fig. 8) shows a peak for G_{2a} (8.01 min), residual G_1 (13.53 min) and an unknown (14.48 min). The retention time of a G_{2a} standard under these conditions was 8.01 min. The G_{2a}/G_1 peak area ratio is 0.69. In order to ascertain whether the G_{2a} and G_1 components interconvert under the photolysis conditions, the photolysis reaction mix obtained

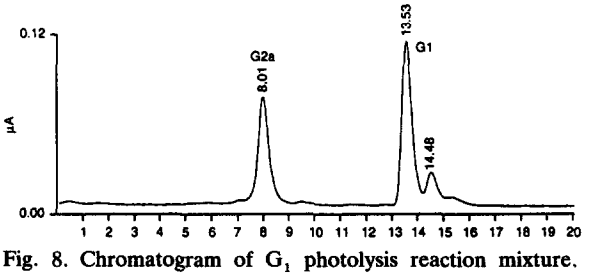

Fig. 8. Chromatogram of G_1 photolysis reaction mixture. G_{2a} retention time 8.01 min, residual G_1 at 13.53 min.

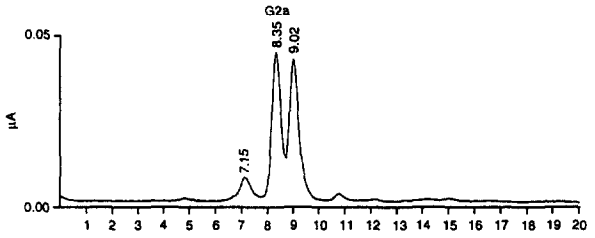

Fig. 9. Chromatogram of G_{2a} photolysis reaction mixture. Residual G_{2a} retention time 8.35 min.

by the 3.15 min irradiation of G_{2a} in a flow injection mode was analyzed as above. The chromatogram (Fig. 9) shows residual G_{2a} (8.35 min) and unknowns at 7.15, 9.02 and 10.8 min. However, no peak for G_1 was observed. It is highly probable that under the photolysis conditions B_1 undergoes the parallel reaction to B_{2a} (Fig. 1).

The effect of low pH on fluorescence response

The conversions of B_1 to B_{2a} in aqueous TFA is well established [10,11]. It was hoped therefore, that adding TFA to the eluent might increase the conversion of B_1 to B_{2a} and G_1 to G_{2a} during the photolysis. Using aqueous 0.1% TFA–acetonitrile–methanol (63:22:15) as eluent and the 15 m × 0.5 mm I.D. coil the relative peak areas normalized for mass of components injected was 0.790, 0.206, 1.000 and 0.259 for G_2, G_1, B_2 and B_1, respectively. The B_1/B_2 relative fluorescence response was lower using the eluent containing TFA than when the neutral eluent was used (Table I).

Analysis of naturally contaminated corn samples

The application of this method to "real life" samples of corn was demonstrated by the analysis of three naturally contaminated corn samples, which had been extracted and the extracts forced through Mycosep multifunctional cleanup columns [17], which allow aflatoxins to pass through while retaining interfering compounds. The samples A, B and C had nominal concentrations for B_1 of 15, 30 and 100 ppb. Using 20-μl injections, each representing 80 mg of corn, gave the chromatograms shown in Fig. 10A, B and C, respectively. Quantitation of the B_1 peaks and comparison with those obtained from B_1 standards indicated that samples A, B and C assayed at 23, 37 and 65 ppb, respectively. These values did not take into account possible sampling problems, losses in the extraction and cleanup processes, nor degradations in transit. However, a comparison of the chromatogram of sample C with the UV light of the photochemical reactor on (Fig. 10C) with the chromatogram of the same sample with the UV light off (Fig. 10D) shows that no naturally fluorescing compounds elute at the same retention time as B_1, and that the B_2 peak is unaffected by the photolysis.

Aflatoxins B_1 and B_2 concentrations of 1 and 0.25 ppb, respectively, can readily be determined since at these levels the signal-to-noise ratio is approximately three. However, increasing the milligrams of feed equivalence for each injection, by using larger injection volumes and/or higher equivalence of mass of feed per injection volume as well as the use of more sensitive fluorescence detectors would significantly increase the sensitivity of this method.

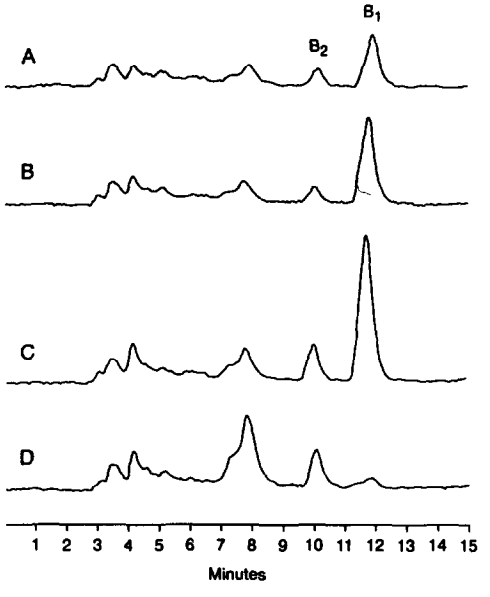

Fig. 10. Chromatograms of purified extracts of naturally contaminated corn samples with nominal B_1 concentrations of (A) 15, (B) 30 and (C) 100 ppb with photolysis light on. (D) Chromatogram of sample C with photolysis light off.

CONCLUSIONS

The HPLC analysis of aflatoxins using post-column photochemical UV irradiation and fluorescence detection is sensitive, simple and robust even for the G_1 and B_1 components whose fluorescence detectability is normally low or absent. This method obviates the need for pre- or post-column chemical derivatizations which require chemical reagents, pumps, heated reactors, electrochemical reagent generators and/or related apparatus. The demonstrated linearity of response over a wide range of concentrations is not affected by the number of photolysis products formed. No interfering peaks were observed in the analysis for B_1 in naturally contaminated corn samples.

ACKNOWLEDGEMENTS

Helpful suggestions by Mr. Edward Vecchione in the use of the Spectra-Physics instrumentation and software are very much appreciated. Mr. Tom Romer of Romer Labs. Inc. provided the purified extracts of naturally contaminated corn samples. They are gratefully acknowledged.

REFERENCES

1 W.O. Ellis, J.P. Smith, B.K. Simpson and J.H. Oldham, *Crit. Rev. Food Sci. Nutr.*, 30 (1991) 403.
2 H.P. van Egmond, *Food Addit. Contam.*, 6 (1989) 139.
3 S. Tabata, H. Kamimura, A. Ibe, H. Hashimoto, M. Iida, Y. Tamura and T. Nishima, *J. Assoc. Off. Anal. Chem.*, 76 (1993) 32.
4 M. Holcomb, D.M. Wilson, M.W. Truckess and H.C. Thompson, Jr., *J. Chromatogr.*, 624 (1992) 341.
5 M. Manabe, T. Goto and S. Masuura, *Agric. Biol. Chem.*, 43 (1978) 2003.
6 T. Panalaks and P.M. Scott, *J. Assoc. Off. Anal. Chem.*, 60 (1977) 583.
7 O.J. Francis, G.P. Kirschenheuter, Jr., G.M.G. Ware, A.S. Carman and S.S. Kuan, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 725.
8 M.L. Vazquez, A. Cepeda, P. Prognon, G. Mahuzier and J. Blais, *Anal. Chim. Acta*, 255 (1991) 343.
9 M.L. Vazquez, C.M. Franco, A. Cepeda, P. Prognon and G. Mahuzier, *Anal. Chim. Acta*, 269 (1992) 239.
10 D.M. Takahashi, *J. Chromatogr.*, 131 (1977) 147.
11 H. Engelhardt and P. Maas, *J. Chromatogr.*, 31 (1993) 13.
12 M.J. Shepherd and J. Gilbert, *Food Addit. Contam.*, 1 (1984) 325.
13 W.Th. Kok, Th.C.H. van Neer, W.A. Traag and L.G.M.Th. Tuinstra, *J. Chromatogr.*, 367 (1986) 231.
14 H. Jansen, R. Jansen, U.A.Th. Brinkman and R.W. Frei, *Chromatographia*, 24 (1987) 555.
15 J.R. Poulsen and J.W. Birks, in J.W. Birks (Editor), *Chemiluminescence and Photochemical Reaction Detection in Chromatography*, VCH, New York, 1989, Ch. 6, pp. 149–230.
16 I.S. Lurie, D.A. Cooper and I.S. Krull, *J. Chromatogr.*, 629 (1993) 143.
17 T.J. Wilson and T.R. Romer, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 651.
18 C. Dunne, M. Meaney, M. Smyth and L.G.M.Th. Tuinstra, *J. Chromatogr.*, 629 (1993) 229.
19 P.J. Andrellos, A.C. Beckwith and R.M. Eppley, *J. Assoc. Off. Anal. Chem.*, 50 (1967) 346.
20 J.C. Shieh and P.S. Song, *Cancer Res.*, 40 (1980) 698.
21 G. Buchi, K.W. Fowler and A.M. Nadzan, *J. Am. Chem. Soc.*, 104 (1982) 544.
22 K. Nagaya, B.H. Way, R.M. Tran and P.S. Song, *Cancer Lett.*, 18 (1983) 191.
23 B.L. Sergeyev, S.P. Bogovsky, R.Kh. Tanner, T.I. Veidebaum and I.N. Shevchuk, *Eksp. Onkol.*, 11 (1989) 76.
24 A.A. Stark and D.F. Liberman, *Mutat. Res.*, 247 (1991) 77.