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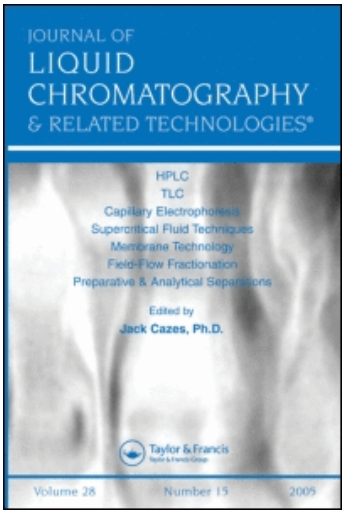

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Aflatoxin Analysis by Reverse Phase HPLC Using Post-Column Derivatization for Enhancement of Fluorescence

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AFLATOXIN ANALYSIS BY REVERSE PHASE HPLC USING POST-COLUMN DERIVATIZATION FOR ENHANCEMENT OF FLUORESCENCE

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ABSTRACT

The application of a technique for the determination of aflatoxins by reverse phase HPLC and fluorescence detection incorporating post-column derivatization with iodine, is described. The procedure proved to be extremely sensitive and reproducible. Chromatograms of extracts from maize, peanut butter, sorghum malt and duckling mash are presented illustrating the value of the procedure for confirming the presence of aflatoxins B_1 and G_1.

INTRODUCTION

The potent carcinogenic effect of the aflatoxins on several animal species (1) and the considerable evidence that they may play a key role in human liver cancer in certain areas (2), motivated the establishment of regulations controlling the levels of

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aflatoxin in foods and feeds. A sensitive and accurate analytical procedure is essential to implement such regulations as well as to study the exposure of populations to aflatoxins.

Various TLC procedures for aflatoxin analysis are in use worldwide (3). Thin layer chromatography is however considered to be only semi-quantitative and cannot be automated. Since the advent of HPLC various excellent procedures have been published which use UV detection after separation of the aflatoxins by either normal or reverse phase chromatography. UV detection has limited sensitivity so that low concentrations of aflatoxins (< 10 µg/kg) cannot be detected without considerable sample cleanup. HPLC separation combined with fluorescence detection would be ideal except that aflatoxins B_1 and B_2 fluoresce weakly in normal phase solvents and likewise B_1 and G_1 in reverse phase solvents. The sensitivity for aflatoxins B_1 and G_1 by fluorescence detection can be increased by derivatization of samples with mild acid treatment forming the strongly fluorescing aflatoxins B_{2a} and G_{2a} (4,5) or by using silica-packed flow cells, enhancing the fluorescence of aflatoxin B_1 and B_2 in normal phase solvents (6). Thorpe et al. (7) however, offered an alternative solution whereby the fluorescence of aflatoxin B_1 and G_1 could be enhanced after reverse phase separations by post-column derivatization with aqueous iodine. In their hands an approximately 50 fold increase in fluorescence of aflatoxins B_1 and G_1 could be obtained without affecting the response of aflatoxins B_2 and G_2. Apart from the advantage of the drastic increase in fluorescence of aflatoxins B_1 and G_1 the presence of these two aflatoxins could be dramatically confirmed by making sequential injections with and without post-column reagent addition.

This detection technique has since only been applied by Tuinstra and Haasnoot (8) who described a rapid procedure for the analysis of animal feeds which includes a TLC cleanup procedure. They included data on the analysis of cattle feed only.

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This paper reports the results on the optimization of this procedure in our laboratory and the application to several different types of samples.

MATERIALS AND METHODS

Aflatoxins B_1, B_2, G_1 and G_2 were obtained from Makor Chemicals Ltd., (Jerusalem, Israel). Individual solutions of each toxin in methanol were prepared and calibrated spectrophotometrically (9). Sample extracts were prepared and purified by the C.B. procedure of the AOAC (10).

Sample components were separated on a reverse phase column (Altex Ultrasphere, 3 µ ODS, 4.6 mm ID x 7.5 cm) using a mobile phase of 0.01 M KH_2PO_4 + acetonitrile + methanol (39:9:7).

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Post-column derivatization was done with a saturated aqueous solution of iodine. A fresh iodine solution was prepared daily by shaking 0.25 g iodine with 250 ml distilled water in the dark for 30 minutes, filtering and degassing.

Two Waters HPLC pumps (Model 510) were used to deliver respectively the mobile phase and the saturated iodine solution. The iodine flow was added to the effluent from the chromatographic column by means of a T-junction with a low dead volume.

The combined flow was passed through a Teflon reaction coil (0.343 mm ID x 10.67 m) before entering the fluorescence detector. The chromatographic column, T-junction and reaction coil were maintained at 60°C by immersion in a thermostatically controlled water bath.

Samples were injected by means of a Waters U6K manual injector. Aflatoxins were detected using a Perkin Elmer Fluorescence Detector (Model 650S) at an excitation wavelength of 365 nm and an emission wavelength of 440 nm with slit width settings of 20 for both excitation and emission. The output from the detector was monitored and the peak areas integrated using a Waters Data Module (Model 730).

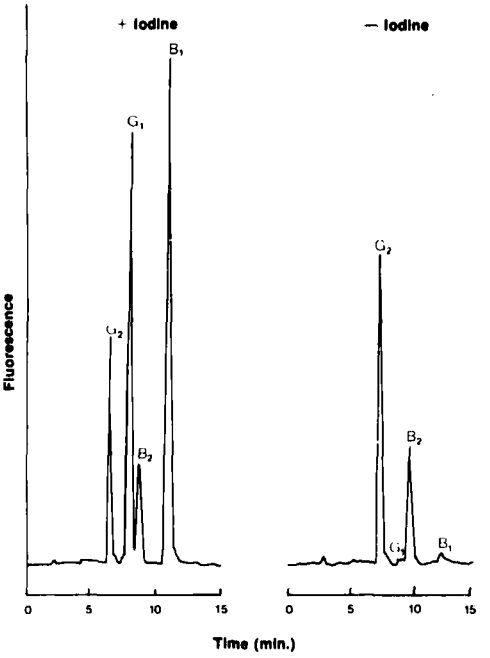

FIGURE 1. Chromatograms of a standard mixture of aflatoxins containing 290, 80, 390 and 135 picograms of aflatoxin B_1, B_2, G_1 and G_2 respectively in 5 µl analysed with and without iodine derivatization.

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RESULTS AND DISCUSSION

Optimal derivatization of aflatoxins B_1 and G_1 was achieved with a mobile phase flow of 0.6 ml/min and a flow of derivatizing agent of 0.3 ml/min. At these flow rates using a short column the analysis could be done in less than 12 minutes although aflatoxins B_2 and G_1 were not completely resolved (Fig. 1).

The dramatic increase in fluorescence of aflatoxins B_1 and G_1 by switching on the iodine flow is shown in Fig. 1 and is of the same order (50 times) as reported by Thorpe et al. (7).

Flow rates were at a later stage increased to 1.0 and 0.4 ml/min for the mobile phase and the derivatization agent respectively, decreasing the analysis time to less than 7 minutes. Only a small loss in fluorescence of derivatized aflatoxins B_1 and G_1 occurred because of the decrease in heating time in the reaction coil.

The reproducibility of retention times and quantification by peak area was excellent as determined by 10 consecutive injections of only 5 µl of an aflatoxin standard mixture (Table 1).

The low injection volume could be used because of the sensitivity of fluorescence detection and had two distinct advantages namely prolonging the life of the column and improving resolution because of the narrow band of sample injected.

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Fluorescence detection of the aflatoxins using the Perkin Elmer fluorescence detector proved to be extremely sensitive. Injection of a standard mixture containing respectively 5.8, 1.6,

TABLE 1

Reproducibility of Retention Times and Peak Areas for Aflatoxins B_1, B_2, G_1 and G_2 analysed by HPLC with Post-Column Derivatization

	Aflatoxin							
	B_1		B_2		G_1		G_2	
	RT	PA	RT	PA	RT	PA	RT	PA
Average	6.40	36256	5.37	7201	4.94	24566	4.24	12308
Std deviation	0.062	660	0.054	87	0.051	431	0.041	134
Std error	1.0	1.8	1.0	1.2	1.0	1.8	1.0	1.1

RT = Retention Time

PA = Peak Area

7.8 and 2.7 picograms of aflatoxins B_1, B_2, G_1 and G_2 in 5 µl gave peak heights well above the noise level (Fig. 2).

It would however not be possible to detect such low amounts in extracts prepared from agricultural commodities because of the presence of interfering substances. It is an indication of the ultimate sensitivity of the procedure should the extracts be extensively cleaned up.

The fluorescence response as measured by peak areas was linear with aflatoxin concentrations up to 5.8, 2.4, 7.4 and 4.2 ng/5µl respectively for aflatoxin B_1, B_2, G_1 and G_2.

Aflatoxins could be determined accurately and sensitively in maize using the CB procedure for extraction and cleanup. The chromatogram obtained for an extract prepared from a maize sample containing only 0.7 µg aflatoxin B_1/kg is given in Fig. 3. The reproducibility of the procedure was determined by performing eight replicate analyses of a maize sample containing only aflatoxins B_1 and B_2 (Table 2).

The reproducibility was good considering that eight completely separate analyses on eight portions of the sample were performed. The reproducibility of the aflatoxin B_2 determination was not as good as that of aflatoxin B_1 as the concentration level was relatively low (0.81 µg/kg).

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TABLE 2

Reproducibility of Aflatoxin Analysis using the CB Cleanup Procedure in Combination with HPLC Separation, Post-Column Derivatization and Fluorescence Detection

	Aflatoxin B_1	Aflatoxin B_2
Number of analyses	8	8
Average concentration µg/kg	6.26	0.81
Std deviation	0.35	0.17
Std error (%)	5.6	21.0

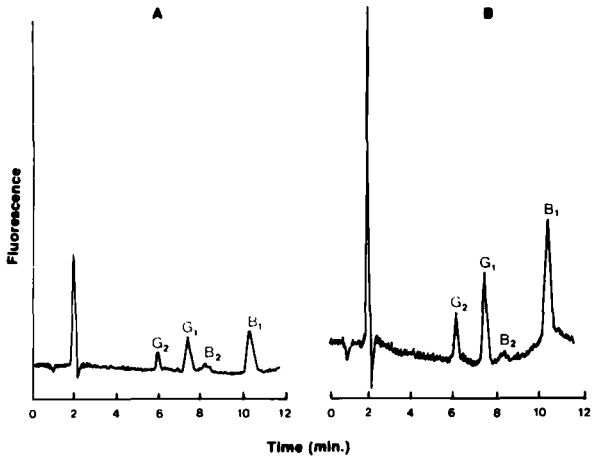

FIGURE 2. Chromatograms of a standard mixture of aflatoxins containing 5.8, 1.6, 7.8 and 2.7 picograms of aflatoxins B_1, B_2, G_1 and G_2 respectively in 5 µl.

A : Range setting 3
B : Range setting 10

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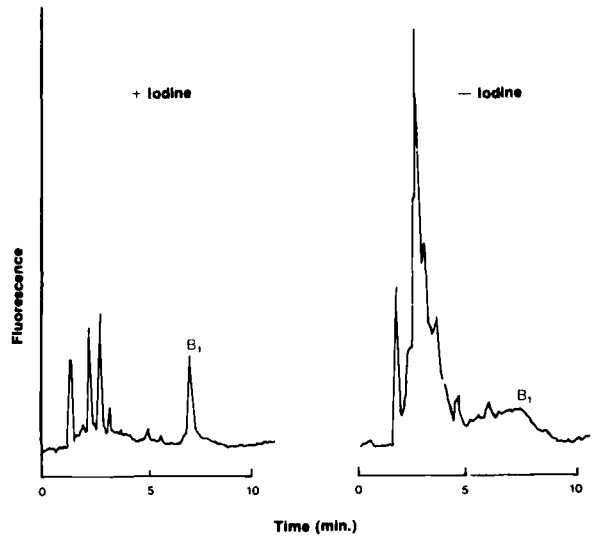

FIGURE 3. Chromatograms of an extract from maize containing 0.7 µg aflatoxin B_1/kg analysed with and without iodine derivatization.

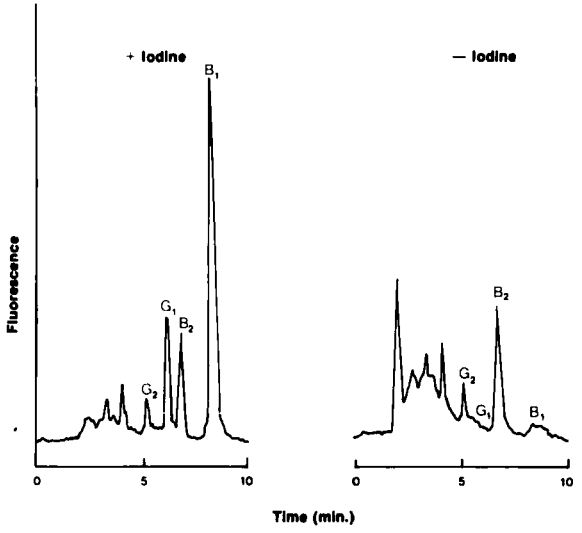

FIGURE 4. Chromatograms of a peanut butter extract analysed with and without iodine derivatization. This sample contained 13.7, 4.7, 8.5 and 1.6 µg/kg aflatoxins B_1, B_2, G_1 and G_2 respectively.

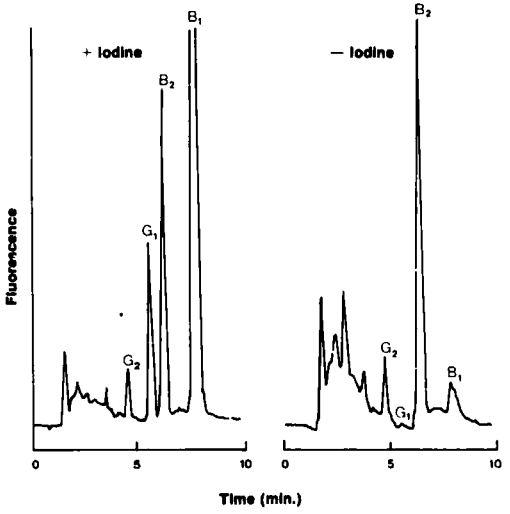

FIGURE 5. Chromatograms of an extract of sorghum malt analysed with and without iodine derivatization. The concentrations of aflatoxins B_1, B_2, G_1 and G_2 in the malt sample were respectively 36, 12, 7 and 2 µg/kg.

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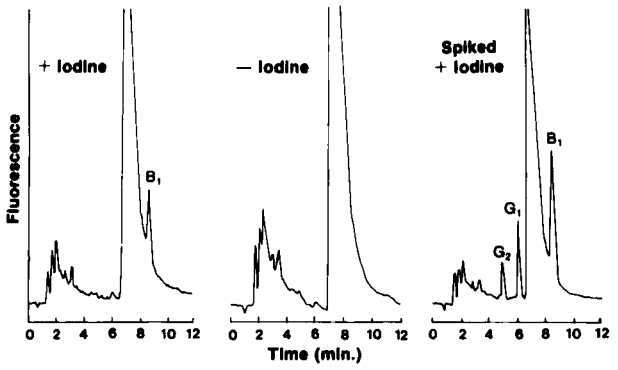

FIGURE 6. Chromatograms of an extract from duckling mash containing 12 µg/kg aflatoxin B_1 analysed with and without post-column derivatization and spiked with 58 pg B_1, 16 pg B_2, 78 pg G_1 and 27 pg G_2.

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The procedure was also shown to be suitable for the analysis of peanut butter (Fig. 4) again demonstrating the value of the derivatization procedure for the enhancement of the sensitivity for aflatoxins B_1 and G_1 as well as proof of the identity of the peaks for aflatoxins B_1 and G_1.

The procedure was also satisfactorily applied to the analysis of sorghum malt (Fig. 5) for which the extract was sufficiently clean to be able to quantify all four naturally occurring aflatoxins.

Aflatoxins B_1, G_1 and G_2 could be detected and quantified in an extract prepared from commercial duckling mash (Fig. 6). An interfering peak, however, prevented the quantification of aflatoxin B_2. It is unlikely that improved resolution on the HPLC column would be able to solve this problem and the only solution would be to remove the interfering compound before the chromatographic step.

Post-column derivatization with iodine in conjunction with fluorescence detection therefore provides an extremely sensitive technique for the analysis of aflatoxins in a variety of commodi-

ties. In addition this technique enables the confirmation of the presence of aflatoxins B_1 and G_1.

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