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DEVELOPMENT OF A SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTION OF AFLATOXINS IN PISTACHIO NUTS

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SUMMARY

A very sensitive method is described for the detection of aflatoxins in pistachio nuts. The method employs the extraction procedure used in the BF method (described in the Association of Official Analytical Chemists' Book of Methods) followed by an acid alumina column clean-up to remove sample components which would otherwise interfere in the final determinative step. The quantitation of the aflatoxins is carried out by high-performance liquid chromatography on a C_{18} reversed-phase column after conversion of aflatoxins B_1 and G_1 to their more fluorescent derivatives, B_{2a} and G_{2a}. The limit of detection was found to be 0.5 μg/kg. The chromatograms were found to be free of interfering peaks and multiple injections showed excellent reproducibility.

INTRODUCTION

Most methods commonly used for analysis of pistachio nuts for aflatoxins are based on a clean-up procedure originally developed for grains and employ silica gel thin-layer chromatography (TLC) in the final determination step. The dependence of these methods upon quantitation can lead to variations in the results, presumably because the resolution and fluorescent intensity of aflatoxins on thin-layer plates varies with the type of silica gel and with the environmental conditions prevailing in a given laboratory[1–4]. Intralaboratory collaborative studies where TLC was used to quantitate the aflatoxin content have reported coefficients of variation ranging from 20 to 60% (refs. 5–7).

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In recent years instrumentation for high-performance liquid chromatography (HPLC) and high-resolution columns has become available. These advances have resulted in several applications of this technique to the analysis of foodstuffs for the presence of aflatoxins and other mycotoxins with the advantages of speed, good resolution and high degree of accuracy and precision. HPLC has been applied to the detection of aflatoxins in ground *Aspergillus*-infested grain samples[8], mold extracts[9], cottonseed[10], wine[11], corn[12] and some other mycotoxins including patulin in apple juice[13]. Recently we have also applied this technique successfully to the determination of ochratoxin A in corn[14].

Application of the HPLC technique to semipurified extracts of naturally contaminated pistachio nuts obtained from Official Methods, AOAC[15] showed that residual non-aflatoxin artifacts in the extract interfere with the resolution of aflatoxins. In this article a very sensitive HPLC method for aflatoxin determination in pistachio nuts is proposed in which the sample extract from Official Methods (BF method) is further purified on a small acid alumina clean-up column designed to completely remove the non-aflatoxin impurities followed by conversion of aflatoxins B_1 and G_1 to their hydroxylated derivatives B_{2a} and G_{2a}. The fluorescence characteristics of the aflatoxins B_{2a} and G_{2a} have been reported, and they were found to be more fluorescent in polar solvents than B_1 and G_1 (refs. 12 and 16).

EXPERIMENTAL

Equipment

A DuPont Model 840 liquid chromatograph was used with a Valco septumless injector and a Model 836 fluorescnece detector with a Corning CS-7-54 excitation filter (UV range 328–385 nm) and a CS-3-72 emission filter (UV cut-off 451 nm) connected to a 1-mV recorder. The stainless-steel HPLC column employed was 30 cm × 4 mm I.D., and contained 10-μm ODS-bonded silica gel (μBondapak; Waters Assoc., Milford, MA, U.S.A.). A Schoeffel Model SD 3000 fluorodensitometer was employed in TLC mode. A 300 × 15 mm I.D. glass column with a PTFE stopcock and a 125-ml reservoir was used for acidic alumina column chromatography.

Reagents

Acidic alumina (Fisher A-948, 80–200 mesh) was prepared by adding 3% water (w/w), shaking for several minutes and allowing to stand overnight to equilibrate. Crystalline aflatoxins B_1, B_2, G_1 and G_2 were obtained from the Food and Drug Administration (Washington, DC, U.S.A.) and standard solutions were prepared containing 0.030, 0.045, 0.060, 0.075 and 0.090 μg/ml of each aflatoxin. The HPLC elution solvent employed was distilled water–methanol–acetonitrile (67:20:13) degassed before use.

Sample preparation and extraction

Pistachio nut samples were prepared and 50 g of each sample was extracted according to Official Methods, AOAC, 26.020–26.023[15] and evaporated to dryness.

Column chromatography

A ball of glass wool was placed in the bottom of the column and 2 g anhydrous

sodium sulfate was added as a base for the acidic alumina. Benzene was added until the column was about half full, then 5 g of acidic alumina was added. The sides of the column were washed with about 10 ml of benzene and stirred to disperse the alumina. The benzene was drained to about 3–5 cm above the alumina, then 5 g of anhydrous sodium sulfate was slowly added and the benzene was drained to the top of the packing. The sample extract was dissolved in 2 ml of chloroform–benzene (1:4) and quantitatively transferred onto the column. The sample beaker was rinsed with an additional 5 ml of chloroform–benzene (1:4) and the contents added to the column. To the column, 50 ml of benzene was added, drained at maximum flow-rate and discarded. Finally, the aflatoxins were eluted with 50 ml of chloroform–methanol (97:3) into a 100-ml beaker. This solution was evaporated to about 2 ml on a steam bath, transferred to a 5-ml vial and evaporated to dryness under a stream of nitrogen.

Derivative formation

The conversion of aflatoxins B_1 and G_1 to their water adducts B_{2a} and G_{2a} is catalyzed by acid. A 1-ml volume of each of the standard solutions was pipetted into separate 5-ml vials and evaporated to dryness under a stream of nitrogen. To each vial of standard and sample 100 μl of trifluoroacetic acid and 100 μl of water were added. The vials were heated on a steam bath (at about 50°C) for 30 min. Then the solutions were evaporated to dryness under nitrogen. Each sample or standard was dissolved in 1 ml of mobile phase. If there were any particles in the sample solution, these were filtered through a 5-μm filter prior to injection into the HPLC column.

HPLC determination

Pressure was applied to the HPLC column to give a flow-rate of 1 ml/min. A 20-μl volume of the most dilute standard solution was injected and the detector was set to give about "½" pen deflection for the first two peaks (B_{2a} and G_{2a}).

A standard curve was prepared for each aflatoxin by injecting 20 μl of each standard solution into the HPLC column and plotting peak height *vs.* standard concentration (in ng/ml). The four aflatoxins were eluted in the order G_1, B_1 (as G_{2a} and B_{2a}), G_2, and B_2.

A 20-μl volume of the sample extract was injected into the HPLC column and the aflatoxin peaks were identified by comparing the retention times with those of the stanards. The peak heights were measured and the concentrations were determined in sample from the standard curves. The amount of each aflatoxin in the original sample was calculated using the equation $\mu g/kg = C_s \times V_s/5$, where V_s is the final sample volume (ml) and C_s is the aflatoxin concentration as read from the standard curve (ng/ml).

If the aflatoxin peaks in the sample are larger than the most concentrated aflatoxin standard solution, the samples should be diluted and re-injected.

RESULTS AND DISCUSSION

Several HPLC mobile-phase solvent mixtures were tried before deciding on the mixture of water–methanol–acetonitrile (67:20:13). This solvent elutes the four aflatoxins in from 6.8 to 15.8 min at a flow-rate of 1.0 ml/min. Fig. 1 is a chromatogram of 1.7 ng of each of the four aflatoxins. It was found that the solvent mixture

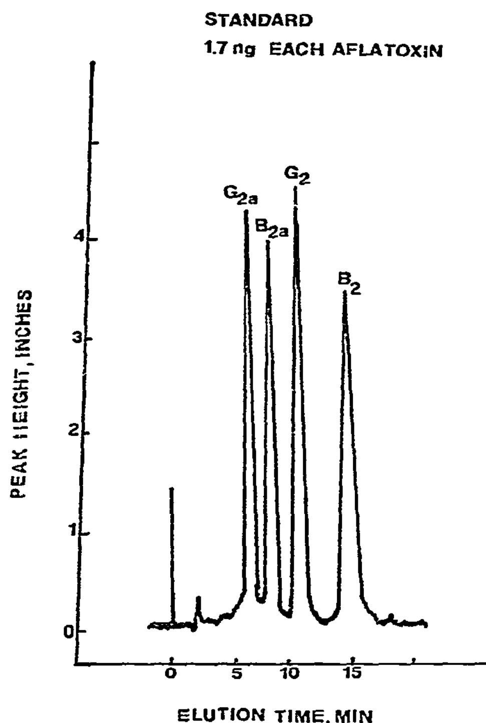

Fig. 1. Resolution of aflatoxins in reference standards, 1.7 ng each B_1, B_2, G_1 and G_2. Chromatographic conditions: column, C_{18} (octadecyl reversed phase), detector, fluorescence at 8 NAFS; pressure, 1200 p.s.i. to give a flow-rate of 1 ml/min; chart speed: 0.1 in./min; mobile phase, water–methanol–acetonitrile (67:20:13).

had to be varied slightly in order to achieve the same separation of the aflatoxins on a series of C_{18} reversed-phase columns from the same manufacturer. With a new column the solvent mixture described above was used as a starting point and a comparable separation of the aflatoxins was usually achieved by altering the methanol:water ratio by 1 or 2%. Once a satisfactory solvent mixture was found the separation was found to be reproducible over several months.

TABLE I

REPRODUCIBILITY OF RETENTION TIMES OF AFLATOXINS IN STANDARD SOLUTIONS AND SAMPLE EXTRACTS

For standard solutions 20-µl injections of solutions containing 0.3–1.8 ng of each aflatoxin were made. For sample extracts 20-µl injections of pistachio nut sample extracts which contained 10 µg/kg of each of the aflatoxins were made. Number of injections was 6 in each case.

	Aflatoxin standards				*Sample extracts*			
	G_{2a}	B_{2a}	G_2	B_2	G_{2a}	B_{2a}	G_2	B_2
Mean retention time (min)	6.81	8.79	11.39	15.8	6.82	8.79	11.21	15.88
Standard deviation	0.036	0.026	0.026	0.017	0.02	0.02	0.028	0.028
Coefficient of variation (%)	0.4	0.3	0.24	0.10	0.3	0.2	0.25	0.2

To demonstrate the reproducibility of the retention times 20 μl of six standard solutions ranging in concentration from 0.015–0.090 ng/μl were injected into the HPLC column and six 20-μl injections of a pistachio nut extract to which each of the four aflatoxins had been added at levels of 10 μg/kg were also made. The mean retention times, standard deviations and coefficients of variation for these injections are given in Table I. The low values for the standard deviations and coefficients of variation (0.1–0.4% for standards and 0.2–0.3% for samples) demonstrate that the retention times

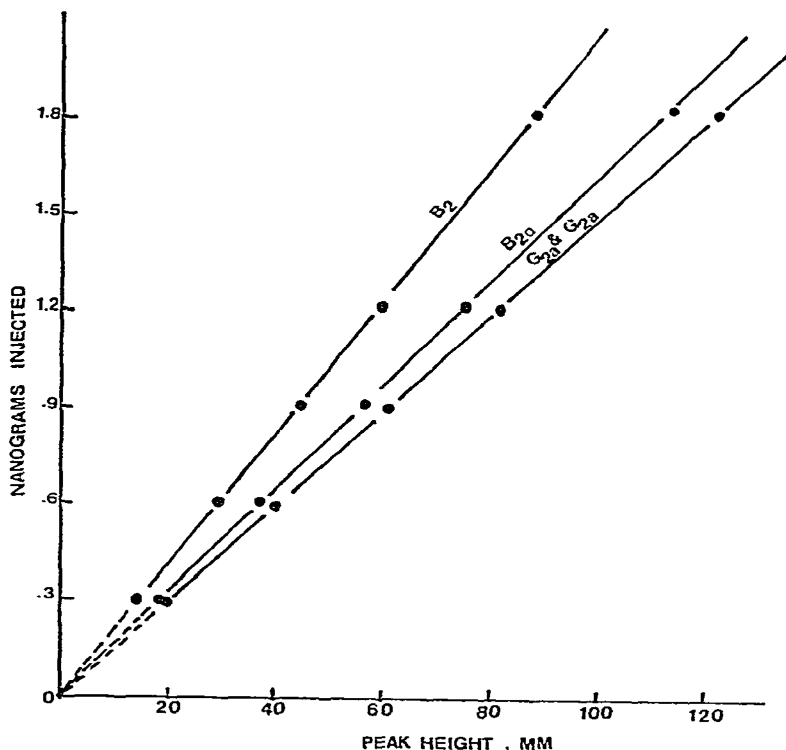

Fig. 2. Typical standard curves of aflatoxins B_{2a}, B_2, G_{2a} and G_2. Chromatographic conditions are the same as those in Fig. 1.

are reproducible within the concentration range studied and are not affected by other components in the sample extract.

The linearity of the HPLC fluorescence detector response for the four aflatoxins was investigated over the range of 0.3–1.8 ng. The response was found to be linear for all four aflatoxins. Typical standard curves of peak height *vs.* amount injected (in ng) are reproduced for B_{2a}, B_2, G_{2a} and G_2 (Fig. 2).

Before development of a new clean-up procedure, both BF (26.020–26.024) and CB (26.014–26.019) methods of the AOAC methods[15] for pistachio nuts were modified by substituting HPLC for TLC in the determinative steps. In Fig. 3 are reproduced the HPLC chromatograms which were obtained following clean-up of a pistachio nut sample by the BF and CB methods. Although sample clean-up by the CB method was

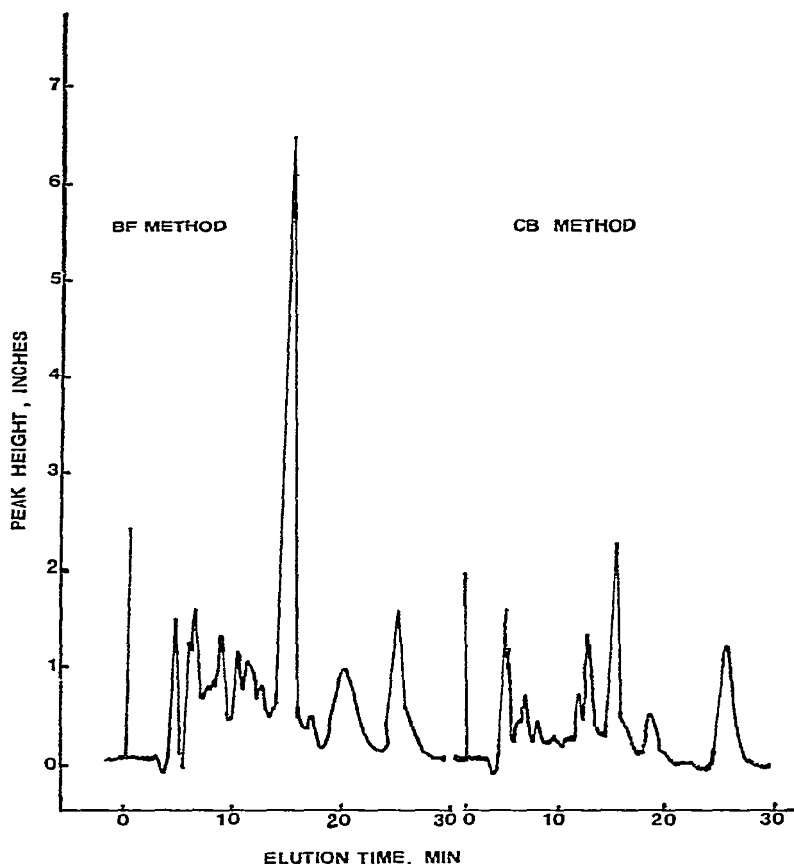

Fig. 3. High-performance liquid chromatograms of pistachio nut extracts prepared by the BF and CB methods. Chromatographic conditions are the same as those in Fig. 1.

more satisfactory, it was concluded that other components in the sample would interfere with the analysis for aflatoxins.

In Fig. 4 three chromatograms are reproduced of the HPLC analysis of pistachio nut extracts after sample clean-up by the procedure described earlier. The first chromatogram is of a sample to which each of the four aflatoxins has been added at the 10 μg/kg level. The second chromatogram is of a naturally contaminated pistachio nut sample which contained a high level of B_1 and smaller amounts of G_1, G_2 and B_2. The third chromatogram is from a sample in which no aflatoxins were detected. These chromatograms illustrate the effectiveness o the clean-up achieved; in no case was an appreciable background due to the sample matrix observed.

A total of nine pistachio nut samples, one sample to which no aflatoxins were added, six samples to which each of the four aflatoxins was added at levels ranging from 0.5–18.0 μg/kg, and two naturally contaminated samples, were cleaned up by the procedure described earlier. The aflatoxins were determined first by TLC using a fluorodensitometer, then by HPLC; in each case the same extracts were used. The results of these analyses are tabulated in Table II. The total aflatoxins recovered ranged

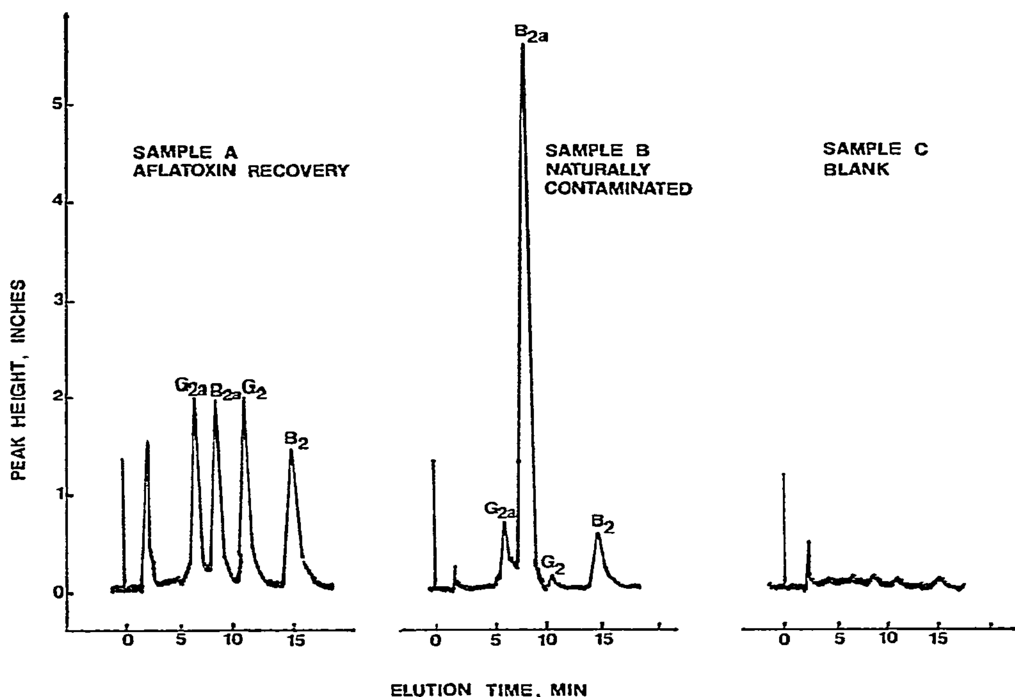

Fig. 4. High-performance liquid chromatograms of pistachio nut extracts prepared by the procedure described in the Experimental section. Chromatographic conditions are the same as those in Fig. 1.

TABLE II

COMPARISON OF THE DETERMINATION OF AFLATOXINS IN PISTACHIO NUTS BY TLC AND HPLC

NC = Naturally contaminated.

Aflatoxins added (µg/kg)	B_1		B_2		G_1		G_2		*Total*	
	Found		*Found*		*Found*		*Found*		*Found*	
	µg/kg	*%*	*µg/kg*	*%*	*µg/kg*	*%*	*µg/kg*	*%*	*µg/kg*	*%*
HPLC analysis										
0	0		0		0		0		0	
0.5	0.46	92	0.22	44	0.47	94	0.27	54	1.42	71
2.0	1.43	72	1.2	60	1.4	70	1.2	60	5.23	65
6.1	5.0	82	3.09	51	4.83	79	2.8	46	15.72	64
10.0*	8.9	89	6.77	68	8.82	88	6.75	67	31.24	78
10.0**	6.04	60	7.0	70	6.8	68	6.6	66	26.44	66
18.0	13.07	73	8.15	45	12.51	70	7.29	41	41.02	57
NC	2.06		0.3		0.83		0		3.19	
NC	100.4		13.8		11.4		1.91		127.5	
TLC analysis										
0	0		0		0		0		0	
0.5	0		0		0		0		0	
2.0	1.46	73	1.26	63	1.31	66	1.12	56	5.15	64
6.1	4.78	78	3.66	60	3.23	53	3.26	54	14.93	61
10.0**	5.4	54	7.1	71	7.32	73	6.9	69	26.7	67
18.0	12.6	70	9.54	53	12.8	71	8.46	47	43.4	60
NC	1.88		0		0		0		1.88	
NC	101.5		14.0		11.0		1.4		122.9	

* First sample.

** Second sample. Tgis sample was used for TLC analysis in this table and in Table III.

TABLE III

DETERMINATION OF AFLATOXINS IN PISTACHIO NUTS BY TLC PRIOR TO ALUMINA COLUMN CLEAN-UP STEP

NC = naturally contaminated.

Aflatoxins Added (μg/kg)	*B_1*		*B_2*		*G_1*		*G_2*		*Total*	
	Found		*Found*		*Found*		*Found*		*Found*	
	μg/kg	%	*μg/kg*	%	*μg/kg*	%	*μg/kg*	%	*μg/kg*	%
0	0		0		0		0		0	
6.1	4.8	79	3.54	58	3.42	56	2.9	48	14.7	61
10.0*	5.7	57	7.4	74	8.3	83	8.3	83	29.7	74
18.0	12.06	67	9.72	54	10.26	57	12.6	70	44.6	62
NC	1.42		0		0		0		1.42	
NC	97.4		14.4		13.0		1.32		126.1	

* See footnote ** to Table II.

from 57–78% for the HPLC analyses and from 60–67% for the TLC analyses although recoveries of individual aflatoxins showed greater variations. The agreement between the HPLC and TLC analyses was excellent. To determine if there was any loss of aflatoxins during the acid alumina column clean-up step, the same samples were analyzed by TLC after the initial extraction. The results of these determinations are given in Table III. A comparison of these results with those in Table II indicates that there is no loss of aflatoxins in the alumina column clean-up step.

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