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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYTICAL SYSTEM FOR AFLATOXINS IN WINES WITH FLUORESCENCE DETECTION

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SUMMARY

An improved procedure using reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection is described for the analysis of six aflatoxins (B_1, B_2, G_1, G_2, B_{2a}, and G_{2a}). The method provides greater analytical speed, reproducibility, and resolution, as well as better accuracy and sensitivity than the previously available thin-layer chromatographic methods. Aflatoxins B_1 and G_1 were hydrated to enhance their fluorescence response. Analyses of both hydrated and unhydrated compounds by HPLC provided simultaneous qualitative and quantitative information. A typical HPLC run was complete in 15 min. The recoveries of four aflatoxins (B_1, B_2, G_1, and G_2) from wines at the spiked level of 1 μg/l (1 ppb) were satisfactory. The method as applied to wine samples is sensitive at the 0.02 μg/l level.

INTRODUCTION

Aflatoxins, often found in trace quantities in a wide variety of agricultural and food products, are toxic metabolites of fungal origin. Chemical analysis of these compounds has been performed traditionally by a thin-layer chromatographic (TLC) procedure which is dependent on the blue or green fluorescence produced by these substances when they are excited by 365-nm radiation. The serious disadvantage in the TLC approach is the lack of quantitative accuracy due to the commonly used visual estimation technique. A possible measurement error of $\pm$ 30–50% is indicated when standard and unknown aflatoxin spots are matched, and $\pm$ 15–25% when the unknown is interpolated between two standards[1]. Other data established the precision limits of visual estimation at $\pm$ 20–28% under ideal conditions[1,2].

Fluorodensitometric measurement of TLC spots, on the other hand, provides an objective, precise, and accurate estimation of aflatoxins. However, this technique still suffers from the inherent limitations associated with any TLC procedure (*i.e.*, variability in spotting technique, in silica gel adsorption activity, in plate thickness, in silica gel particle size from batch to batch, and in temperature and humidity to which TLC plates are exposed during handling and spotting).

Aflatoxin analyses by TLC procedures present special problems due to their

susceptability to photochemical decomposition[3–6] and the sensitivity of aflatoxins to harmful vapors in the air[7].

These problems have led a number of investigators to examine high-performance liquid chromatography (HPLC), a powerful analytical tool recently added to the domain of separation sciences, for aflatoxin analyses[8–14]. The potential of HPLC has been demonstrated in the development of moderately successful procedures for aflatoxins using silica adsorption or Sephadex columns. However, investigation of reversed-phase systems for aflatoxin analysis has been largely neglected. One paper recently reported unsuccessful attempts to analyze aflatoxins with ODS-bonded or phenyl-bonded pellicular packings[14]. Furthermore, no reports to date have demonstrated the applicability of HPLC to the routine determination of the major aflatoxins in food samples where these toxins are likely to occur. Extremely low concentrations of aflatoxins sought in samples (often only trace quantities) and the background interferences from sample residues, combined with the close similarity in the chemical structures of aflatoxins, have made analyses of aflatoxins in samples very difficult. Although microparticle silica adsorption systems[12–14] provide a good resolution of the four aflatoxins (B_1, B_2, G_1, and G_2), the limits of detection are not better than 10 ppb (μg/kg) in actual sample analysis[14].

In this paper we describe a HPLC procedure for aflatoxins using reversed-phase columns and both UV absorbance and fluorescence detectors. When coupled to a hydration step which converts the non-fluorescent B_1 and G_1 to the highly fluorescent B_{2a} and G_{2a}, the assay provides quantitative accuracy at the subnanogram level. The advantage of the reversed-phase HPLC over a microparticle silica adsorption system is also discussed.

The applicability of the HPLC method combined with the previously developed preliminary clean-up procedure[15] to sample analyses was evaluated by determining the amount of recovery of aflatoxins B_1, B_2, G_1, and G_2 in spiked wines. Certain sweet wines were selected for this study since they were the types of wine suspected to be contaminated with aflatoxins[15]. The results obtained indicate that the method is capable of detecting aflatoxins in wines at the level of 0.02 μg/l, and is simpler, more rapid, sensitive, and more accurate than the TLC methods currently available[7,15].

Structural confirmation of aflatoxin B_1 or G_1 found in samples is achieved when these substances are converted to aflatoxins B_{2a} and G_{2a}. Comparative TLC analyses based on this principle have been reported[7] but require an additional time-consuming TLC separation. With the aid of HPLC, qualitative information is obtained swiftly, resulting in a shorter analytical time and in a more sensitive and accurate confirmation test.

MATERIALS AND METHODS

A Spectra-Physics Model 3500B liquid chromatograph equipped with a Spectra-Physics Model 770 spectrophotometer detector, a Laboratory Data Control Model 1209 fluoromonitor, and a Linear Instrument Model 285 recorder were used during this study. Two detectors were connected in series during HPLC operation for absorbance measurements at 365 nm and for fluorescence emission measurements (400–700 nm). Stainless-steel HPLC columns employed were: (1) a 25 cm × 3.2 mm I.D. 5-μm silica gel (Spherisorb; Spectra-Physics, Santa Clara, Calif., U.S.A.), (2)

a 30 cm × 4 mm I.D. 10-μm CN-bonded silica gel (μBondapak-CN; Waters Assoc., Milford, Mass., U.S.A.), and (3) a 25 cm × 3.2 mm I.D. 10-μm ODS-bonded silica gel (Spherisorb-ODS; Spectra-Physics). Columns 1 and 3 were slurry-packed at 9000 p.s.i.; column 2 was purchased from the manufacturer. The mobile phases and aflatoxin retention times for the three column packings studied are given in Table I. In all cases, standard mixtures (25 ng of each of aflatoxins in 10% aqueous acetonitrile for reversed-phase analysis or in dichloroethane for adsorption analysis) were applied to the column via a Valco rotary valve injector equipped with a 500-μl loop. The concentrations of these standard solutions were varied when linearity studies were made. The flow-rate was set initially at 2.0 ml/min, but was adjusted as needed.

TABLE I

AFLATOXIN RETENTION TIMES IN VARIOUS HPLC SYSTEMS

Stationary phase	*Mobile phase (v/v)*	*Flow-rate (ml/min)*	*Retention time* (min)*					
			B_1	B_2	G_1	G_2	B_{2a}	G_{2a}
Silica adsorption	chloroform–dichloroethane–methanol (40:10:1)	0.5	7.9	9.3	11	12	40	63
Silica adsorption	chloroform–dichloroethane–methanol (40:10:1)	0.9		4.1		5.5	19	30
Silica CN-bonded	water–acetonitrile (9:1)	1.7	23	17	20	15	10	8.6
Silica ODS-bonded	water–acetonitrile–methanol (15:3:2)	1.7	15	13	11	9.9	7.2	6.0
Silica ODS-bonded	water–methanol (3:2)	2.1	17	15	12	10	6.4	4.7
Silica ODS-bonded	water–acetonitrile (4:1)	1.6	22	17	15	11	7.3	5.0

* Flow-rates can be increased to shorten retention times if sc desired.

Test wine samples (blackberry, Madeira, and pomegranate) were divided into two equal portions (200 ml each), one of which was spiked with the four aflatoxins (1 μg/l each). The extraction and column chromatography for preliminary sample clean-up were carried out as previously described[15]. The column eluates from the spiked and unspiked samples were each divided into two equal parts and concentrated to *ca.* 1 ml. These concentrates were quantitatively transferred to 3-dram vials with a small amount of dichloromethane and the solvents removed just to dryness under a gentle stream of nitrogen at room temperature. To one vial of each pair was added 2.0 ml 10% aqueous acetonitrile (for reversed-phase analysis) or 2.0 ml dichloroethane (for adsorption analysis), and the solution was mixed well. To the second vial was added *ca.* 30 μl trifluoroacetic acid (Eastman-Kodak, Rochester, N.Y., U.S.A.) to completely wet the residue. After allowing the reaction mixture to stand for a few seconds, during which time compounds B_1 and G_1 were converted to B_{2a} and G_{2a}, respectively[16–19], 2.0 ml 10% aqueous acetonitrile or dichloroethane were added and the resulting solution mixed well. A third pair of vials each containing 100 ng each of the four aflatoxins were treated in a fashion identical to that used for the column

chromatography eluates. Prior to injection of each sample solution, the appropriate standard solution was analyzed. Peak heights were measured in mm and aflatoxin concentrations were calculated in μg/l.

RESULTS AND DISCUSSION

The HPLC separations obtained with the three columns using the standard aflatoxin mixtures (Fig. 1) with and without trifluoroacetic acid treatment are presented in Figs. 2–5. With silica adsorption chromatography, B_1, B_2, G_1, and G_2 separate with near-baseline resolution but under these conditions only G_1 and G_2 fluoresce (Fig. 2). The chromatographic tracing obtained after the trifluoroacetic acid treatment (Fig. 3) was not satisfactory since only G_2 and G_{2a} were detected by fluorescence under these conditions and the peaks for B_{2a} and G_{2a} were very broad. Contrary to the previous report indicating that aflatoxin B_{2a} was not eluted[12], both B_{2a} and G_{2a} were successfully eluted from this system. On the other hand, excellent separations were obtained with both the ODS-bonded and CN-bonded silica gel (Figs. 4 and 5). The combination of the normal and hydrated analyses provides a reversed phase-fluorescence assay for all four aflatoxins since the non-fluorescent B_1 and G_1 are converted to the fluorescent B_{2a} and G_{2a} molecules. The highly fluorescing compounds B_2 and G_2 remain unaffected by the hydration procedure.

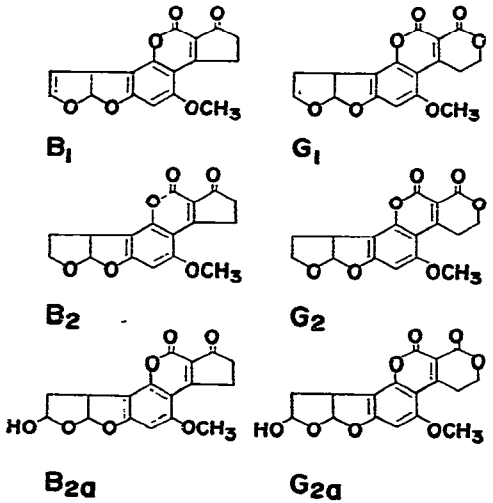


Fig. 1. Chemical structures of six aflatoxins.

The reversed-phase solvent systems used during this study were found to cause shift in the fluorescence maxima of aflatoxins to more useful longer wavelengths than the wavelengths observed in the silica adsorption solvent system (see Table II). Since the fluorescence detector used has the shorter wavelength limit of *ca.* 400 nm and has the optimum sensitivity near 450 nm[14], the advantage of the reversed-phase fluorescence determination of aflatoxins becomes readily evident. This is true of any other fluorescence detector which makes use of similar UV cut-off filters. The superiority of the

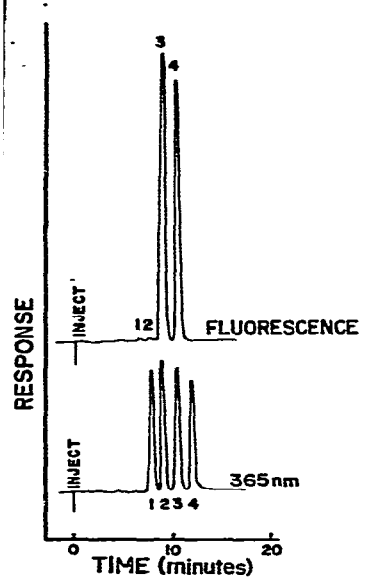


Fig. 2. Silica adsorption chromatogram of aflatoxins (25 ng each). Instrument, Spectra-Physics Model 3500B liquid chromatograph; column, 25 cm × 3.2 mm I.D. 5-μm Spherisorb; mobile phase, chloroform–dichloroethane–methanol (40:10:1); detector, at 365 nm UV absorbance (0.04 a.u.f.s. at 10 mV) and at 4A fluoromonitor setting (fluorescence at 365 nm excitation and above 400 nm emission); temperature, 25°; pressure, 430 p.s.i.; flow-rate, 0.5 ml/min. 1 = Aflatoxin B_1; 2 = B_2; 3 = G_1; 4 = G_2.

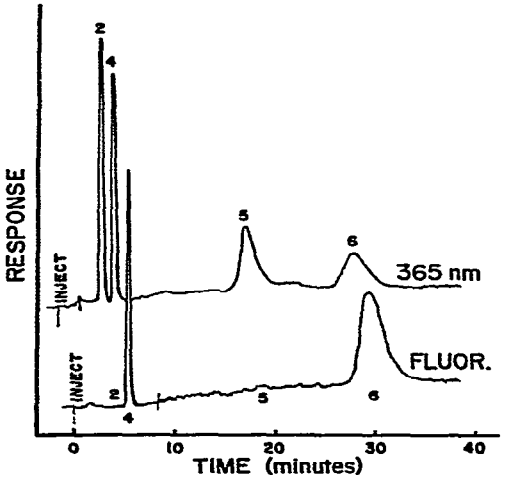
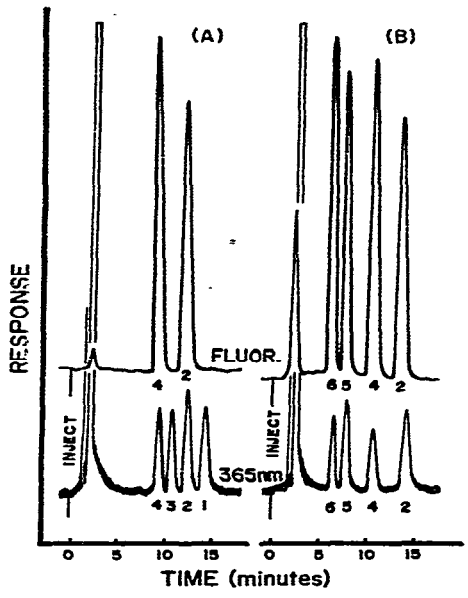


Fig. 3. Silica adsorption chromatogram of hydrated aflatoxins (25 ng each). HPLC operating parameters were identical as in Fig. 2 except the following conditions: detector, at 365 nm (0.02 a.u.f.s. at 10 mV). Fluorescence detectability increased from 8A to 2A setting at 8 min of chromatographic run for later eluting aflatoxins; pressure, 800 p.s.i.; flow-rate, 0.9 ml/min. 2 = Aflatoxin B_2; 4 = G_2; 5 = B_{2a}; 6 = G_{2a}.



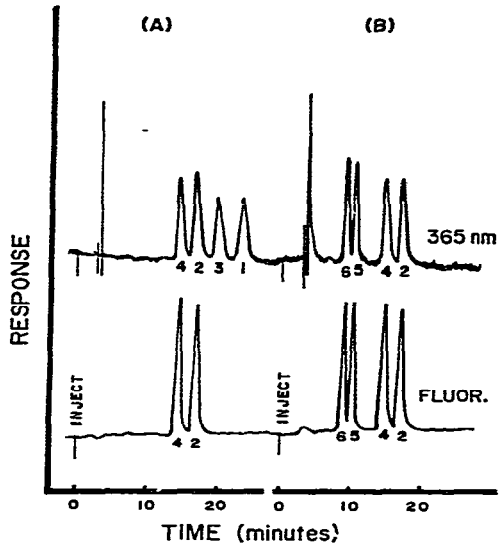


Fig. 4. Reversed-phase chromatogram of aflatoxins (25 ng each). (A) Unhydrated; (B) hydrated. Instrument and temperature, see Fig. 2; column, 25 cm × 3.2 mm I.D. 10-μm Spherisorb-ODS; mobile phase, water–acetonitrile–methanol (15:3:2); detector, at 365 nm (0.01 a.u.f.s. at 10 mV) and at 2A fluoromonitor setting; pressure, 1400 p.s.i.; flow-rate, 1.7 ml/min. 1 = Aflatoxin B_1; 2 = B_2; 3 = G_1; 4 = G_2; 5 = B_{2a}; 6 = G_{2a}.

Fig. 5. Reversed-phase chromatogram of aflatoxins (25 ng each). (A) Unhydrated; (B) hydrated. Instrument and temperature, see Fig. 2; column, 30 cm × 4 mm I.D. 10-μm μBondapak-CN; mobile phase, water–acetonitrile (9:1); detector, at 365 nm (0.01 a.u.f.s. at 10 mV) and at 8A fluoromonitor setting; pressure, 1400 p.s.i.; flow-rate, 1.7 ml/min. 1–6, *cf.* legend to Fig. 4.

TABLE II

FLUORESCENCE $\lambda_{max.}$ OF AFLATOXINS IN VARIOUS HPLC SOLVENT SYSTEMS

Solvent system	*Aflatoxin fluorescence ($\lambda_{max.}$, nm)* *					
	B_1	B_2	G_1	G_2	B_{2a}	G_{2a}
Chloroform–dichloroethane–methanol (40:10:1)	414	408	430	425	418	430
Water–acetonitrile (9:1)	440	438	460	460	442	465
Water–methanol (3:2)	440	435	462	460	440	465

* Determined with the Aminco spectrophotofluorometer at 365 nm excitation wavelength. UV absorption $\lambda_{max.}$ for all aflatoxins in the above solvent systems were in the range of 355–370 nm (Cary 14 spectrophotometer used).

reversed-phase fluorescence procedure over the silica adsorption UV procedure becomes even clearer when comparison of wine sample chromatograms is made. At 254 nm, absorption by sample impurities with the silica system made the detection of aflatoxins at the 1 μg/l level impossible. Late eluting components delayed subsequent sample injections considerably. Somewhat improved detectability was realized at 365 nm absorption and by fluorescence (Fig. 6), but sample impurities still obscured the

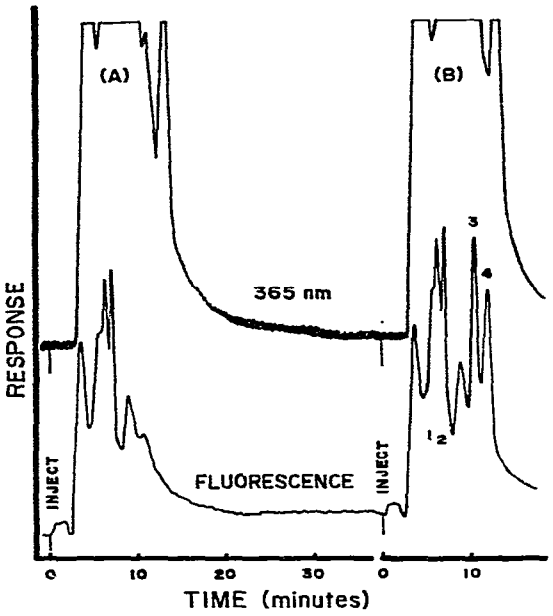


Fig. 6. Madeira wine extract. (A) Unspiked sample extract; (B) spiked with aflatoxins at 1 μg/l level. HPLC operating parameters were identical to those in Fig. 2. 1–4, *cf.* legend to Fig. 2.

aflatoxin peaks. The sensitivity of this system is estimated at 10–20 ppb. On the other hand, fluorescence detection of aflatoxins employing the reversed-phase systems routinely provided a ten-fold increase in sensitivity over 365-nm detection (see Table III). Interferences from fluorescing sample impurities in the reversed-phase system were negligible. (See Figs. 7–9 for examples of wine sample reversed-phase chromatograms).

Recovery studies were also undertaken. The three wines were spiked with aflatoxins B_1, B_2, G_1, and G_2 (1 μg/l each) and 200-ml samples of wine were carried through the entire clean-up and reversed-phase analytical procedures. The chromato-

TABLE III

APPROXIMATE DETECTABILITIES OF AFLATOXINS

Detection mode	*Detectability factor**			
	Adsorption HPLC		*Reversed-phase HPLC*	
254 nm absorption	1		1	
365 nm absorption	2		2	
Fluorescence (400–700 nm)	B_1, B_2, B_{2a}	G_1, G_2, G_{2a}	B_1, G_1	B_2, G_2, B_{2a}, G_{2a}
	0.1	9	0.1	17

* Factors are based on the 254-nm absorption peak height as 1 and are the average values of six aflatoxins. Arbitrary comparison of detectability was made with Spectra Physics Models 770 and 230 UV detectors and LDC Model 1209 fluoromonitor.

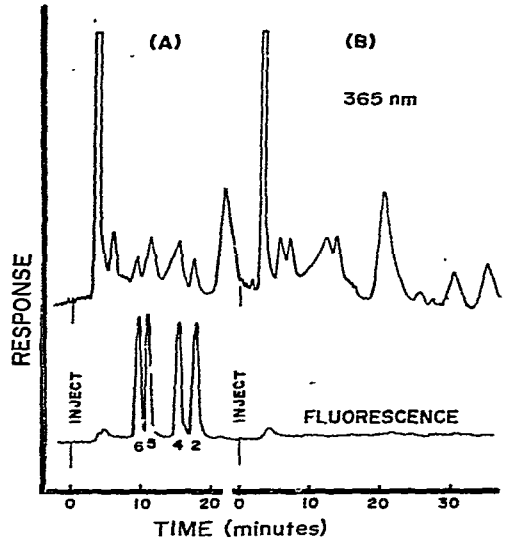


Fig. 7. Pomegranate wine extract. (A) Spiked with aflatoxins at 1 μg/l level; (B) unspiked sample extract. HPLC operating parameters were identical to those in Fig. 5, except the following condition: detector, at 365 nm (0.04 a.u.f.s. at 10 mV). 2, 4, 5, 6, *cf.* the legend to Fig. 3.

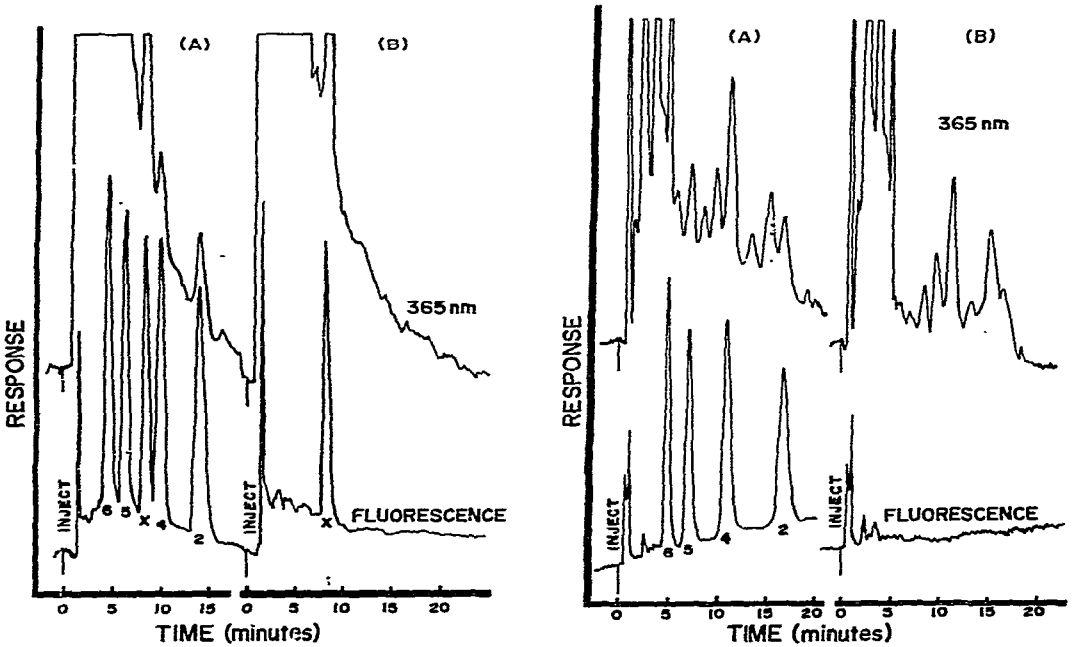


Fig. 8. Madeira wine extract. (A) Spiked with aflatoxins at 1 μg/l level; (B) unspiked sample extract. HPLC operating parameters were identical to those in Fig. 4, except the following conditions: mobile phase, water–methanol (3:2); pressure, 2300 p.s.i.; flow-rate, 2.1 ml/min. 2, 4, 5, 6, *cf.* the legend to Fig. 3; X = unknown.

Fig. 9. Blackberry wine extract. (A) Spiked with aflatoxins at 1 μg/l level; (B) unspiked sample extract. HPLC operating parameters were identical to those in Fig. 4, except the following conditions: mobile phase, water–acetonitrile (4:1); pressure, 1200 p.s.i.; flow-rate, 1.6 ml/min. 2, 4, 5, 6, *cf.* the legend to Fig. 3.

TABLE IV

RECOVERIES OF AFLATOXINS SPIKED AT 1 μg/l

Fluorescence determination. Aflatoxin B_1 was determined as B_{2a} and G_1 as G_{2a}.

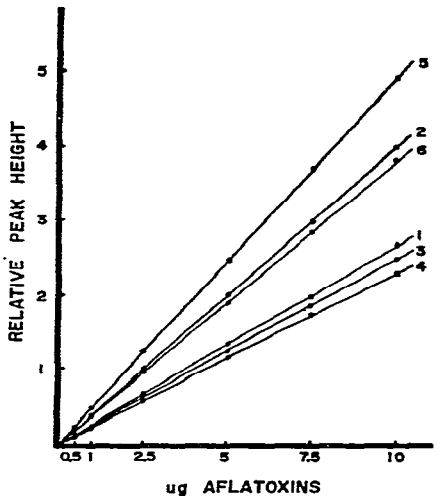
Wine	*HPLC*		*Recovery (%)*			
	Column	*Mobile phase*	$B_1(B_{2a})$	B_2	$G_1(G_{2a})$	G_2
Blackberry wine	25 cm × 3.2 mm I.D. 10-μm Spherisorb-ODS	water–acetonitrile (4:1)	99	101	96	100
Madeira wine (imported)	25 cm × 3.2 mm I.D. 10-μm Spherisorb-ODS	water–methanol (3:2)	100	92	106	103
Pomegranate wine	30 cm × 4 mm I.D. 10-μm μBondapak-CN	water–acetonitrile (9:1)	88	96	91	94

graphic characteristics of these samples are presented in Figs. 7–9 for pomegranate, Madeira, and blackberry wines, respectively. The recovery data are summarized in Table IV. As can be seen, recoveries were quite satisfactory and the quality of the fluorescence tracings is excellent. It should be noted that the UV (365 nm) tracings of three samples were singularly uninformative. A fluorescent peak, not corresponding to any standard aflatoxin, was observed in the analysis of the spiked and unspiked Madeira wine. Otherwise, no fluorescent materials were isolated from the wine samples analyzed.

As noted previously, aflatoxins B_1 and G_1 were hydrated to B_{2a} and G_{2a}, respectively, to increase their fluorescence response, since the fluorescence of these two aflatoxins was completely quenched in the reversed-phase system. However, the hydration process is particularly noteworthy since it did not produce any interfering fluorescent peaks in the sample extracts tested. Aflatoxin peaks were all sharp and symmetrical, permitting a quick and accurate peak height measurement for identification and quantitative calculation. An entire chromatographic run of six aflatoxins on the Spherisorb-ODS column (elution order: G_{2a}, B_{2a}, G_2, G_1, B_2, and B_1) was complete in *ca.* 15 min. On the μBondapak-CN column (elution order: G_{2a}, B_{2a}, G_2, B_2, G_1, and B_1), the analysis time was *ca.* 20 min.

The response *versus* concentration curves of all six aflatoxins demonstrated that the aflatoxin determinations were linear in the ranges tested (Figs. 10 and 11). Peak height measurements were used throughout. The observed linearity also established the reproducibility of retention times. The minimum detectable amount of aflatoxins in the reversed-phase fluorescence system was 0.5 ng. When calculated in terms of sensitivity for wines, this represents a detection of 0.02 μg/l.

No effort was made to establish the lowest limits of detection for aflatoxins in samples. If necessary, an increase in detection limits can be achieved by using larger sample injection volumes accompanied by further concentration of sample solution volumes. More efficient clean-up procedures of sample extracts could also be developed. The method described provides sensitivity and accuracy, since (1) interferences from sample impurities are minimal, (2) aflatoxin peaks are well resolved from each other and from sample impurities, (3) retention times and peak heights are reproducible, and (4) the absorption and fluorescence values are linear.



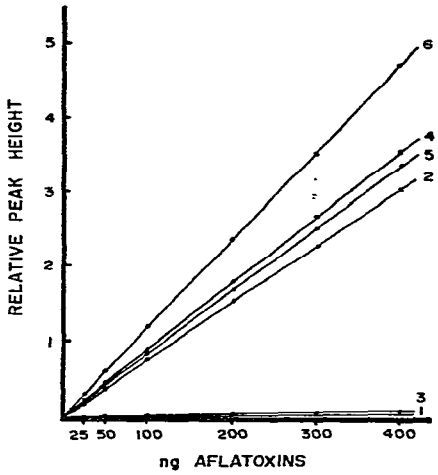


Fig. 10. Aflatoxin linearity in UV absorbance (365 nm). For HPLC parameters, see the legend to Fig. 2. 1–6, *cf.* the legend to Fig. 4.

Fig. 11. Aflatoxin linearity in fluorescence (above 400-nm emission). For HPLC parameters, see the legend to Fig. 4. 1–6, *cf.* the legend to Fig. 4.

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