A Bi-Directional HPTLC Development Method for the Detection of Low Levels of Aflatoxin in Maize Extracts

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Key Words

Thin layer chromatography
Bi-directional development
Pre-chromatographic clean-up
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Summary

A bi-directional high performance thin layer chromatography (HPTLC) method for the determination of low levels of aflatoxin in extracts of maize has been evaluated. The method, using diethylether and chloroform:xylene:acetone (6:3:1) as the two developing solvents was found to have a limit of detection of 0.8 (B_1), 0.4 (B_2), 1.7 (G_1) and 0.4 (G_2) µg/kg respectively, with CVs equal or less than 1.7% (B_1), 2.5% (B_2), 3.1% (G_1), and 3.1% (G_2). Inter-plate variation was significantly greater than intra-plate variation.

Compared with two-dimensional HPTLC methods, a 15 to 30 fold improvement in the sample capacity per plate is achieved and positioning errors are much reduced during densitometry.

1 Introduction

Aflatoxins have been detected in a variety of agricultural products and, as a consequence of their toxicity, 56 countries have either implemented, or are introducing, legislation to control the level of aflatoxin in foods and feeds. The regulations governing levels in certain feedstuffs specify aflatoxin concentrations of 10µg/kg or less [2]. In a recent publication [1] we evaluated the suitability of high performance thin layer chromatography (HPTLC) equipment for detecting low levels of aflatoxin and found that it was capable of operating at levels below 10µg/kg. However, the study did not evaluate the effect on the accuracy and precision of the method of interfering compounds which frequently occur in extracts of agricultural products.

Two-dimensional development on an HPTLC plate with a concentration zone has been used to separate interfering compounds from aflatoxins in extracts of poultry feed [3]. However, the low sample capacity of two samples per plate makes it economically unsuitable as a quality control procedure. Bi-directional HPTLC development, employing a pre-chromatographic development in diethyl ether, has been used in the detection of low levels of aflatoxin in almonds and groundnuts [4] and to remove ethoxyquin from extracts of poultry feed [5]. The advantage of this technique is the higher sample throughput of at least ten samples per plate.

The aim of this paper is to determine the suitability of bi-directional HPTLC development in the detection of low levels of aflatoxin in extracts of maize using a variety of solvent systems. The limits of detection, accuracy and precision over a range of aflatoxin concentrations, and the intra- and inter-plate precision of the most suitable system are evaluated to determine the suitability of the method for routine analysis.

2 Materials and Methods

2.1 Standards

Aflatoxin B_1 and G_1 (1µg/kg) and B_2 and G_2 (0.5µg/kg) were dissolved in benzene:acetonitrile (98:2) and stored in the dark at − 18°C until required for use.

2.2 Sample Solutions

Two types of benzene:acetonitrile (98:2) extract were used, namely, aflatoxin free extracts of maize, spiked with aflatoxin standard solution and extracts of maize naturally contaminated with aflatoxin.

2.3 HPTLC Plates

HPTLC aluminium-backed sheets (20 × 20cm, Merck 5547), without fluorescent indicator, were utilized. Prior to use, these were immersed in methanol for 60min, dried in a fan-assisted oven at 100°C for 5min and stored in a desiccator.

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2.4 Sample Application

In a darkened room, standard and sample solutions were applied to the HPTLC layer by an automated TLC sampler II (27200, Camag, Switzerland). The solutions were spotted 70mm from the bottom edge of the plate, at intervals of 5mm and at a flow rate of 200nl/sec.

2.5 Chromatogram Development

Chromatographic developments were carried out in the dark in an unsaturated TLC chamber (Universal Chromatank SAB 2842, Shandon, UK). A 30min pre-chromatographic development, to remove interfering compounds, was performed in a chamber filled to a depth of 40mm with anhydrous diethyl ether (previously stored over 5A molecular sieves, 15mm pellets, BDH Chemicals Ltd., Poole, UK). After the plates had been dried under a stream of nitrogen, the top portion (20mm) of each plate, containing the interfering compounds was removed by means of a sharp knife. The plates were rotated through 180° and the aflatoxins separated by two 20min developments in chloroform:xylene:acetone (6:3:1; 20ml). The solvent was removed between developments in a stream of nitrogen [1]. After the second development, the plates were dried (1min) in a fan-assisted oven at 100°C.

2.6 Densitometry

The aflatoxins were quantified by UV fluorescence in the reflectance mode using a Camag Scan II monochromatic densitometer (Camag, Switzerland) equipped with a Camag SP 4270 integrator linked to an Apricot PC microcomputer (Quadrant Scientific, UK). The parameters used were: light source, high pressure mercury lamp; wavelength 366nm; optical slit dimensions 4 × 0.2mm; scan speed 0.5mm/sec; peak evaluation by valley to valley baseline calculated peak heights; single level calibration using three external standards {containing a mixture of B_1 and G_1 (1.0ng) and B_2 and G_2 (0.5ng)}.

2.7 Evaluation of the Suitability of Solvent Systems when Used in Bi-Directional HPTLC

A known volume of aflatoxin standard was added to aflatoxin-free extract of maize to give a sample solution having the following concentrations of aflatoxin: 4.9 (B_1), 2.3 (B_2), 4.8 (G_1) and 2.3 (G_2) μg/kg.

Five replicate volumes (5000nl) of the sample solution were applied to five 10.0 × 4.0cm HPTLC aluminium sheets (cut from the same 10 × 20cm aluminium sheet) and each plate was subjected to bi-directional chromatography using diethyl ether followed by one of the following solvent systems:

1. CXA – chloroform:xylene:acetone (6:3:1)
2. CA – chloroform:acetone (88:12) [4, 5]
3. EMW – diethyl ether:methanol:water (94:4.5:1.5) [6]
4. CAA – chloroform:acetone:acetic acid (92:8:1) [7]
5. XEA – xylene:ethyl acetate:acetic acid (6:3:1)

The solvent migration distances, R_f-values, spot shape and efficiency of clean-up were evaluated visually under UV illumination (365nm). Each plate was scanned by a monochromatic densitometer.

Table I. Concentrations of aflatoxin added to extracts of maize

Level	aflatoxin (μg/kg)			
	B_1	B_2	G_1	G_2
A	0.00	0.00	0.00	0.00
B	4.98	2.53	4.91	2.57
C	10.02	5.10	9.98	5.18
D	25.21	12.82	24.87	13.03
E	75.03	38.15	74.01	38.76
F	199.87	101.62	197.15	103.25

2.8 Evaluation of the Accuracy, Precision and Limits of Detection of the Bi-Directional Method and its Comparison with One-Dimensional Chromatography

A benzene:acetonitrile extract of aflatoxin-free maize was divided into six aliquots (A to F) and solutions of aflatoxin standard were added to give the concentrations listed in Table I. Five replicate volumes of each aliquot were applied to two HPTLC plates. After either one-dimensional [1] or bi-directional development, the plates were scanned by a monochromatic densitometer.

3 Results and Discussion

3.1 Identification of a Solvent Suitable for Bi-Directional HPTLC

Comparison of the HPTLC plates (Table II) revealed that CXA and CA achieved the most effective sample clean-up, leaving the aflatoxins virtually free from interfering compounds. The other solvents investigated failed to separate the aflatoxin from the interfering compounds and aflatoxin G_1 was badly obscured after development in EMW and XEA. CXA effected the best resolution of the aflatoxins, and CA and EMW poor resolution.

With regard to spot shape, only CXA and XEA gave compact and circular spots. Densitometric evaluation of the chromatograms with respect to resolution and baseline stability confirmed the visual observations.

3.2 Evaluation of the Accuracy and Precision of the Bi-Directional HPTLC Method

Initially, the plates were compared visually under UV illumination (365nm). One-dimensional development gave slightly better separation of the aflatoxins than the bi-directional development, but it was difficult to differentiate between the aflatoxins and the interfering compounds at concentrations of 25μg/kg aflatoxin B_1 or less. After bi-directional development, the aflatoxins were clearly visible at the lowest concentration (5μg/kg B_1; B) while the control (A) appeared free from aflatoxin. Densitometric evaluation of the chromatograms (fig. 1) furnished results in agreement with the visual observations of the plates; bi-directional development revealed traces of aflatoxin B_1 not apparent by one-dimensional development. As the bi-direc-

Table II. Comparison of development solvents for bi-directional HPTLC

Solvent system	Migration dist (mm)	Clean-up	Spot shape	Rf-value			
				B_1	B_2	G_1	G_2
CXA	65	good	compact circular	0.40	0.35	0.29	0.25
CA	48	good	compact flattened	0.89	0.85	0.80	0.78
CAA	58	poor	diffuse circular	0.66	0.60	0.54	0.49
EMW	41	poor	compact flattened	0.92	0.88	0.82	0.73
XEA	65	poor	compact circular	0.31	0.25	0.20	0.18

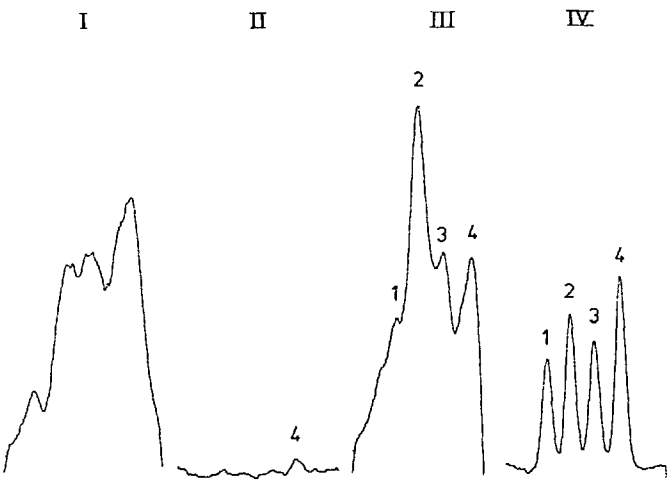

Fig. 1

Typical HPTLC chromatograms of aflatoxins present in maize extracts. (I) and (II) are one-dimensional and bi-directional chromatograms respectively of aflatoxin free maize extracts, and (III) and (IV) are the corresponding chromatograms after adding aflatoxin to the extracts (5.0μg/kg B_1, 2.5μg/kg B_2, 4.9μg/kg G_1, and 2.6μg/kg G_2). Peaks: 1 = G_2; 2 = G_1; 3 = B_2; 4 = B_1.

tional development was clearly the more sensitive, only these results were subjected to statistical analysis.

3.3 Statistical Analysis of the Results Obtained by the Bi-Directional Method

Both the measured levels of aflatoxin and the standard deviation (S.D.) were found to vary linearly with increasing concentration of aflatoxin; Fig. 2 shows the relationships obtained for aflatoxin B_1, and includes the confidence intervals (P = 0.05) which increase in width with increasing concentration.

The S.D.'s increased linearly by factors of 32 (B_1), 26 (B_2), 16 (G_1) and 21 (G_2) with increasing concentration of aflatoxin. However, the precision of the bi-directional method was considered acceptable since the coefficients of variation (CV) only varied between 0.7 and 1.7% (B_1), 1.6 and 2.5% (B_2), 1.0 and 3.1% (G_1) and 1.2 and 3.0% (G_2).

Linear and weighted regressions [1] were used to determine how the S.D. and the measured concentration varied with aflatoxin concentration (Table III). Correlation coefficients (r) varying between 0.993 and 1.000 demonstrate the closeness of fit for the regression curves.

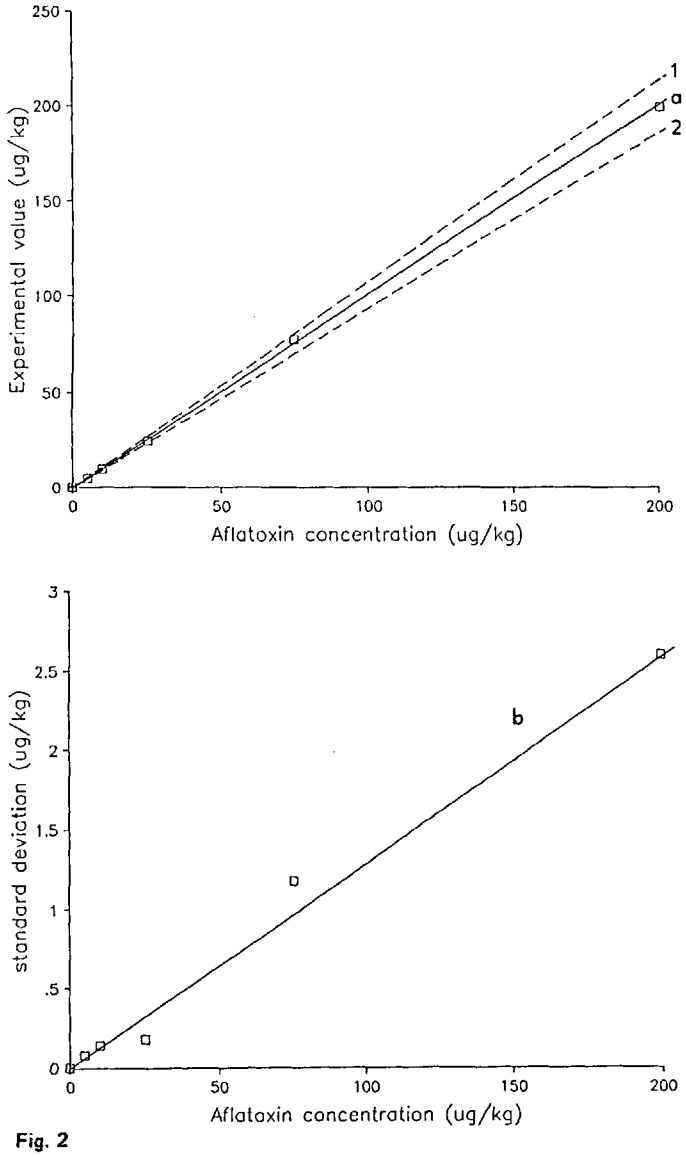

Fig. 2

Relationship between aflatoxin B_1 concentration and the experimental value (curve a), including the corresponding upper (1) and lower (2) confidence intervals (P = 0.05), and the standard deviation (curve b).

3.4 Errors in the Slope and Intercept of the Weighted Regression Curves

Relative and systematic errors in the bi-directional method were absent since the confidence interval (P = 0.05) for the slope and intercept overlapped with the values 1 and 0 respectively [8].

Table III. Bi-directional HPTLC: linear and weighted regression analysis

	a	b	r
Linear regression: S.D. against aflatoxin concentration (µg/kg)			
B_1	0.002	0.013	0.993
B_2	0.021	0.015	0.994
G_1	0.064	0.011	0.995
G_2	−0.009	0.014	0.996
Weighted regression: measured value against aflatoxin concentration (µg/kg)			
B_1	−0.175 ± 0.364	0.998 ± 0.037	1.000
B_2	−0.145 ± 0.197	0.983 ± 0.029	1.000
G_1	−0.413 ± 0.851	0.969 ± 0.049	0.999
G_2	−0.061 ± 0.219	0.971 ± 0.047	0.999

a = regression intercept; b = linear constant; r = correlation coefficient.

Table IV. Inter- and intra-plate variation in HPTLC

No.	aflatoxin B_1 (µg/kg)					
	plate 1	plate 2	plate 3	plate 4	plate 5	plate 6
1	72.2	71.0	67.0	67.4	67.3	64.1
2	79.9	75.2	70.1	72.0	67.0	65.8
3	75.9	70.2	68.7	68.1	66.5	63.9
4	73.1	69.9	68.7	65.8	66.0	64.3
5	73.1	71.1	67.6	65.9	65.5	66.1
mean	74.8	71.5	68.5	67.8	66.5	64.9
S.D.	3.13	2.15	1.41	2.60	0.70	1.03
CV	4.2	3.0	2.1	3.8	1.0	1.6

Analysis of variance (ANOVA)

Source	df	sum of sq.	mean sq.	F-ratio
between plates	5	327.469	65.494	16.43 ($P = 8.9 \times 10^{-6}$)
within plate	24	95.672	3.986	
Total	29	423.141		

Grand mean = 71.667, S.D. = 6.552, residual S.D. = 3.846, n = 60

3.5 Limit of Detection (l.o.d.)

The l.o.d. [1] of the aflatoxins were: 0.8 (B_1), 0.4 (B_2), 1.7 (G_1) and 0.4 (G_2) µg/kg respectively. These are below both the current and proposed legislative limits for aflatoxin in maize.

3.6 Evaluation of Inter- and Intra-Plate Variation

In HPTLC, it is important to evaluate both inter-plate and intra-plate variation, because each plate is individually calibrated using external standards. Table IV shows the results, and analysis of variance (ANOVA, P = 0.05) [1], obtained from six HPTLC plates, spotted with five replicate volumes (5000nl) of an extract of maize containing aflatoxin, following bi-directional development. A significant difference was found when the two sources of variation were compared using a one-sided F-test ($P = 8.9 \times 10^{-5}$).

4 Conclusions

Bi-directional development of extracts of maize on HPTLC plates using diethyl ether and chloroform:xylene:acetone (6:3:1) as the two developing solvents has been found to be an effective method for separating interfering compounds. Whilst one-dimensional chromatography was incapable of quantitatively detecting aflatoxin levels of 25µg/kg or less (B_1 and G_1) and 13µg/kg or less (B_2 and G_2), bi-directional development gave accurate results with excellent precision between the ranges investigated: 0 and 200µg/kg (B_1 and G_1) and 0 and 100µg/kg (B_2 and G_2). The l.o.d. of the method was 0.8 (B_1), 0.4 (B_2), 1.7 (G_1) and 0.4 (G_2) µg/kg respectively, with CV's varying between 0.7 and 1.7 (B_1), 1.6 and 2.5 (B_2), 1.0 and 3.1 (G_1) and 1.2 and 3.0 (G_2). Inter-plate variation was significantly greater than intra-plate variation. This might be reduced by using computer-controlled densitometry to minimize positioning errors [9]. The sample capacity per plate (10 × 20cm) can be as high as 30 samples including 3 standards. Compared with two-dimensional HPTLC methods, a 15 to 30 fold improvement in the sample capacity per plate is achieved and positioning errors are much reduced during densitometry.

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