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The validation of a solid phase clean-up procedure for the analysis of aflatoxins in groundnut cake using HPLC

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A solid-phase clean-up method for the determination of aflatoxins in groundnut cake has been statistically examined. The method involves the clean-up of an acetone and water (85 + 15) extract on a bonded-phase (PH) cartridge and quantification by HPLC with fluorescence detection following post-column derivatisation with iodine. Average recoveries were calculated as 84·1, 86·1, 88·0 and 82·1% with limits of detection of 2·7, 1·6, 2·5 and 3·2 μg/kg for aflatoxins B_1, B_2, G_1 and G_2 respectively. This method was compared with the official AOAC (CB) method for its ability to determine the aflatoxin B_1 and B_2 contents of groundnut cake samples. The precision of the two methods was found not to be significantly different at the 5% level, but the PH method recorded significantly more aflatoxin B_1.

The direct extraction of aflatoxins with aqueous acetone was also compared with a slurry extraction method. It was demonstrated that the slurry technique extracted significantly more aflatoxins B_1 and B_2; the precision of these two extraction methods was found not to differ significantly.

INTRODUCTION

Groundnuts, the seeds of *Arachis hypogaea*, are rich in protein. Groundnut cake, which is the product obtained when the oil has been removed from the kernels by compression, is widely utilised as a component of livestock feed. The contamination of the commodity by aflatoxins (toxic metabolites of the fungi *Aspergillus flavus, A. parasiticus* and *A. nomius*) poses serious problems for both producer and consumer.

Over 50 countries have in force, or have proposed, legislation for the control of aflatoxins in foodstuffs. Tolerances vary from country to country and are dependent upon the commodity and its intended usage. The level tolerated for aflatoxin B_1 in peanut products, intended as food for dairy cattle is most commonly accepted as 10 μg/kg (van Egmond, 1989). Current methods of analysis also vary, some being time-consuming and costly. Thin layer chromatography (TLC), based upon the official AOAC methods (Anon., 1980), is still widely used as the quantification procedure. Here we propose a solid-phase clean-up approach followed by high performance liquid chromatography (HPLC) as an accurate, precise and convenient method for the determination of aflatoxins in groundnut cake. A similar method has already been described for the determination of aflatoxin B_1 and B_2 in oil-free groundnut meal (Roch *et al.*, 1992).

Following extraction with acetone and water (85 + 15), the extracts are freed from interfering substances using a bonded-phase (PH) cartridge, and aflatoxins in the resulting residues are quantified by HPLC with fluorescence detection. The use of iodine as a post-column derivatising reagent (Shepherd & Gilbert, 1984; Jansen *et al.*, 1987) has been adopted. The validation of the whole methodology has been carried out according to a procedure devised by the Natural Resources Institute (NRI) whereby both spiked extracts of aflatoxin-

free material, and spiked solvent alone are subjected to the proposed analytical procedure and the results statistically examined (Nawaz *et al.*, 1992). The ability of the proposed method to extract and quantify aflatoxins from naturally contaminated samples of groundnut cake has been compared with the official (AOAC) CB method, which involves extraction of the cake with chloroform and a tedious chromatographic column clean-up procedure which uses unacceptably large volumes of organic solvents.

An additional investigation into the initial method of extraction of aflatoxins into the solvent has also been carried out. The direct extraction of the cake with a mixture of acetone and water (85 + 15) was compared with a method which involved first the slurrying of the cake with water and subsequent extraction of the slurry with acetone.

MATERIALS AND METHODS

Reagents — all chemicals used were either AnalaR or HPLC (Fisons) grade. Freshly distilled water was used throughout. Aflatoxin standards were purchased as solids from the Aldrich Chemical Co.

Validation for groundnut cake

Virtually aflatoxin-free groundnut cake (approx. 1 kg) was ground to a fine powder and mixed using a Hobart Vertical Cutter Mill (VCM 25). Then, 60 g of cake was extracted with acetone and water (85 + 15, 600 ml) using a Silverson overhead blender at high speed for 3 min with a fine (2 mm) mesh screen. The blended mixture was filtered through a Whatman No. 1 paper. To aliquots of both the extract and solvent alone, known amounts of aflatoxin standards were added to produce eight spike levels ranging from 0 to 300 $\mu g\ kg^{-1}$ of each aflatoxin, as indicated in Table 1.

Five aliquots of both the spiked extracts and solvents at each of the eight levels were freed from any interfering fluorescent materials following the solid phase extraction method of Tomlins *et al.* (1989):

The filtrate (5 ml), together with water–acetic acid–methanol (92·3 + 1·6 + 6·7, 60 ml) and lead acetate

Table 1. Evaluation data for the PH-HPLC method for determination of aflatoxin in groundnut cake

		Extract			Solvent		
	Spike ($\mu g\ kg^{-1}$)	Recovery ($\mu g\ kg^{-1}$) (%)	SD	% CV	Recovery ($\mu g/kg$) (%)	SD	% CV
Aflatoxin B_1	Blank	0·00(—)	—	—	0·00(—)	—	—
	10·8	8·93(83)	1·18	13·2	7·33(68)	0·41	5·6
	21·5	18·5 (86)	0·85	4·6	15·2 (71)	1·99	13·9
	32·3	27·9 (86)	1·03	3·7	25·6 (79)	0·93	3·5
	53·8	46·7 (87)	0·39	0·8	41·6 (77)	1·00	2·4
	107·6	89·4 (83)	1·35	1·5	86·7 (81)	0·74	0·9
	215·2	179 (83)	6·78	3·8	171 (80)	6·11	3·6
	322·8	260 (81)	8·53	3·3	260 (81)	8·13	3·1
Aflatoxin B_2	Blank	0·00(—)	—	—	0·00(—)	—	—
	7·5	6·29(84)	0·27	4·3	5·40(72)	0·39	7·2
	15·0	13·0 (87)	0·76	5·8	11·6 (77)	0·78	6·7
	22·5	19·2 (86)	0·69	3·6	18·7 (83)	1·11	5·9
	37·5	34·2 (91)	0·91	2·7	31·5 (84)	0·57	1·8
	75·0	64·9 (87)	1·29	2·0	63·6 (85)	1·37	2·2
	150	127 (85)	4·63	3·6	128 (86)	2·42	1·9
	225	186 (83)	4·71	2·5	191 (85)	3·55	1·9
Aflatoxin G_1	Blank	0·00(—)	—	—	0·00(—)	—	—
	8·82	7·48(85)	0·61	8·2	6·20(70)	0·46	7·4
	17·6	13·7 (77)	0·26	1·9	11·0 (62)	0·41	3·7
	26·5	23·8 (90)	0·44	1·9	22·0 (83)	1·34	6·1
	44·1	40·6 (92)	1·45	3·6	36·1 (82)	2·26	6·3
	88·2	80·7 (91)	1·97	2·4	76·9 (87)	2·59	3·4
	176	163 (93)	5·44	3·3	158 (90)	4·09	2·6
	265	233 (88)	8·97	3·8	189 (72)	11·22	5·9
Aflatoxin G_2	Blank	0·00(—)	—	—	0·00(—)	—	—
	11·0	8·40(76)	0·86	10·2	6·96(63)	0·72	10·3
	22·0	16·5 (75)	0·66	4·0	14·1 (64)	0·89	5·9
	33·0	26·1 (79)	0·80	3·1	25·1 (76)	0·96	3·8
	55·1	47·4 (86)	0·72	1·5	41·7 (76)	1·15	2·8
	110	96·8 (88)	2·70	2·8	88·1 (80)	1·93	2·2
	220	191 (87)	6·42	3·4	191 (87)	4·02	2·1
	330	277 (84)	9·98	3·6	242 (73)	10·9	4·5

SD, Standard deviation.
CV, Coefficient of variation; $n = 5$.

solution (20% in 0·3% acetic acid, 3 ml), was passed through a phenyl bonded column (PH, 500 mg) which had previously been solvated with methanol (10 ml) and water (10 ml) via a reservoir (75 ml) containing a frit and Hyflo Supercel filter aid (1·0 g). The flow through the column, under vacuum, was at a rate of approximately 10 ml/min. After washing the column with water (10 ml), the aflatoxins were eluted with chloroform (7 ml) and the eluate was dried by passage through a column of granulated anhydrous sodium sulphate (3 g).

The chloroform was removed at 45°C under a stream of nitrogen using a sample concentrator, and the residue retained for HPLC analysis.

The residues were dissolved in water and acetonitrile (70 + 30, 250 μl) immediately prior to quantification. Then, 100 μl was injected onto a Technicol Spherisorb 5 ODS reverse-phase column (25 cm × 4·6 mm) by means of a Rheodyne 7125 injector fitted with an inlet filter.

The mobile phase of water, acetonitrile and methanol (6 + 3 + 1) was delivered at 0·7 ml/min by a Severn Analytical Solvent systems (SA 6410B). A Waters (6000A) pump delivered saturated aqueous iodine solution at 0·6 ml/min. Mixing of the iodine solution with the column eluate was achieved via a Valco (1/16" × 0·25 mm) tee. The reaction coil (1/16" PTFE capillary, 5000 × 0·3 mm) was immersed in an oil bath maintained at 75°C. A Kratos (GM 970) monochromator was attached to a Kratos (FS 970L.C) Fluorometer fitted with a tungsten lamp. Excitation and emission wavelengths were 363 and 418 nm respectively. A Spectra-Physics integrator (SP 4290) collected and processed the output data. Concentrations of aflatoxins B_1, B_2, G_1 and G_2 were determined by comparison of peak areas with those of standard aflatoxin solutions. Chromatograms showing the separation of aflatoxins in standard solutions and in a sample extract are given in Fig. 2.

Comparison of the proposed (PH) and official AOAC (CB) methods

Two naturally-contaminated samples of groundnut cake (approximately 1 kg of each) were ground and thoroughly mixed using the VCM 25 mill. For the proposed method 5 × 400 g portions of cake were each mixed with 1·6 l water for 3 min at high speed in a Waring Blender to form a stable slurry. From each portion, 100 g slurry was extracted with acetone (420 ml) for 3 min in a Silverson overhead mixer used at high speed and fitted with a fine mesh. The filtered extract (5 ml) was then subjected to the proposed clean-up and HPLC quantification procedure. By dissolving the residue from the clean-up step in water and acetonitrile (70 + 30, 250 μl) and injecting 100 μl onto the HPLC column an effective weight of 0·2 g was achieved.

For the official (CB) method, five 50 g aliquots of mixed cake were each mixed with water (25 ml), chloroform (250 ml) and diatomaceous earth (25 g). Extraction of aflatoxins was achieved by shaking each for 30 min using a wrist-action mechanical shaker. The filtered extracts (50 ml) were subjected to the official CB method of clean-up as described in a recent publication (Roch *et al.*, 1992). By dissolving the residue in water and acetonitrile (70 + 30, 5 ml) and introducing 100 μl onto the HPLC column an effective weight of 0·2 g was again achieved.

Comparison of the direct extraction procedure with the slurry method

A further five 50 g aliquots of mixed cake were each extracted directly with acetone and water (85 + 15, 500 ml). The Silverson overhead mixer was used at high speed for 3 min to effect the extraction of the aflatoxins. Following filtration through a Whatman No.1 filter paper, the filtrate (5 ml) was treated as for the slurried material. The results of the analyses were statistically compared with those obtained using the proposed combination of slurry, clean-up and quantification methods.

RESULTS AND DISCUSSION

Method validation for groundnut cake

Table 1 lists the aflatoxin concentrations recorded at each spike level for the extract and solvent. The mean aflatoxin recoveries at each level of contamination, together with the standard deviation (SD) and percentage coefficient of variation (% CV) are also given in Table 1. The value for the coefficient of variation ranged from 0·8 to 13·2% (average value = 4·4) for aflatoxins in spiked extracts and from 0·9 to 13·9% (average value = 4·7) for aflatoxins in the spiked solvent. This clearly demonstrated that the presence of the sample matrix does not have a significant effect on the precision of the method.

Average recoveries for aflatoxin B_1, B_2, G_1 and G_2 were recorded at 84·1, 86·1, 88·0 and 82·1% from spiked extracts and 76·7, 81·7, 78·7 and 74·1, respec-

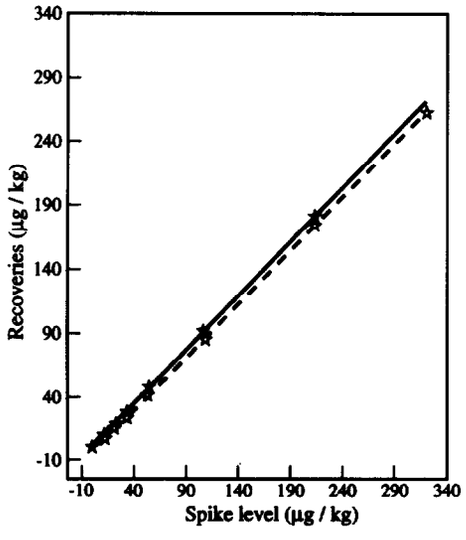

Fig. 1. Aflatoxin B_1 calibration. ——— Extract; - - - - - solvent

Table 2. Calibration of the proposed analytical method

	Y intercept	Slope of the line	Detection limit (μg/kg)	Correlation coefficient	Relative error (% Range)	
	(95% limits)					
B_1^E	1·35 ± 2·07	0·83 ± 0·04	2·7	0·9998	−20·6	−12·9
B_2^E	0·01 ± 0·92	0·86 ± 0·04	1·6	0·9998	−17·9	−10·0
G_1^E	−2·21 ± 2·11	0·94 ± 0·09	2·5	0·9994	−15·0	2·7
G_2^E	−2·28 ± 1·95	0·89 ± 0·05	3·2	0·9996	−14·1	−6·8
B_1^S	−1·45 ± 0·70	0·82 ± 0·01	1·4	1·0000	−19·7	−17·0
B_2^S	−1·00 ± 0·31	0·86 ± 0·01	0·6	1·0000	−14·9	−13·1
G_1^S	−2·84 ± 3·03	0·87 ± 0·13	6·2	0·9885	−26·3	−0·7
G_2^S	−3·03 ± 3·00	0·83 ± 0·06	5·7	0·9942	−23·2	−10·2

X^E, Spiked Extract; X^S, Spiked Solvent.

tively, from the spiked solvents (Table 1). This effect was evident in the calibration line for AFB_1 (Fig. 1). However, the differences are not significant, as shown in Table 2 (overlap of the confidence limits of the slopes). Calibration lines for AFB_2, AFG_1 and AFG_2 also exhibited similar trends.

The proposed method was calibrated using the weighted regression analysis (Miller, 1991) and the resultant data are summarised in Table 2. The regression analysis did not include the blank data since the blank produced zero response with zero standard deviations (see Fig. 2(c)).

The value of the *Y*-intercept is a measure of the systematic error in the method. The 95% confidence limits for the *Y*-intercept passed through zero in the case of aflatoxins B_1 and B_2 from spiked extracts and aflatoxin G_1 from spiked solvent, indicating absence of a systematic error. The rest of the calibration lines showed a negative systematic error inherent in the method. This effect was probably due to the presence of trace levels of a fluorescence quenching interference. The systematic errors can be corrected using the standard additions method (Miller & Miller, 1988). Table 2 also shows the 95% limits for the slope of the line, which

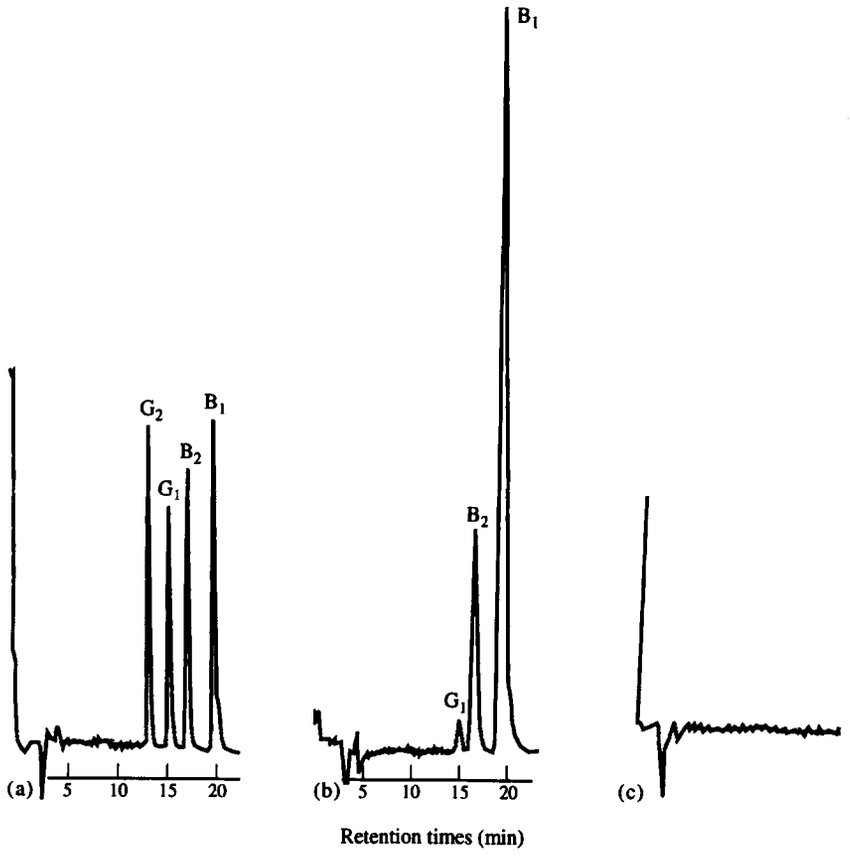

Fig. 2. Chromatograms showing the separation of aflatoxins G_2, G_1, B_2 and B_1 in: (a) a standard solution; (b) a typical sample extract containing G_1, B_2 and B_1 only; (c) an aflatoxin-free sample extract.

Table 3. Comparison of clean-up methods for analysis of aflatoxin B_1

	Analytical method								
	PH column method				Official (CB) method				
Sample	*X*	Variance	df	df*Var	*X*	Variance	df	df*Var	*t*-value
1	10·9	1·42	4	5·68	6·36	0·77	4	3·08	6·80
2	134	71·9	4	288	117	62·5	4	250	3·29
Sum			8	293			8	253	

S^2p PH method = 293·28/8 = 36·66 & CB method = 252·88/8 = 31·61.
Sp = 6·02 = 5·62.
Variance ratio (F_{obs}) 36·66/31·61 = 1·16.
t-statistic for eight degrees of freedom at 95% level = 2·31
Critical value for $F_{0.975}$ (8,8) is 4·433.

X, Mean recoveries $\mu g\ kg^{-1}$.
df, Degrees of freedom.
Var, Variance.
df*Var, Degrees of freedom multiplied by variance.
S^2p, Pooled variance.
Sp, Pooled standard deviation.

were used to calculate the presence of relative errors. This type of error is expected as it arises from the random nature of the data. The negative values signify losses of aflatoxins during the analytical procedures.

The limits of detection were calculated as recommended by the Analytical Methods Committee (1987) and are given in Table 2. These figures are within the legislative limit of 10 $\mu g\ kg^{-1}$ for this commodity.

Comparison of the proposed and the official AOAC (CB) methods

The ability of two methods to extract aflatoxins B_1 and B_2, from two naturally contaminated groundnut cake samples, was compared. The proposed method extracted significantly more aflatoxins B_1 and B_2 than the official method (Tables 3 and 4) when the slurry extraction method was employed (see below). The *t*-test confirmed this hypothesis, with the exception of aflatoxin B_2 from sample 2. The precisions of the two methods did not differ significantly as the calculated values for F-statistics of 1·30 and 1·16 were lower than the critical value (Tables 3 and 4).

Comparison of direct extraction and slurry extraction procedures

Direct extraction and slurry extraction procedures were compared for their abilities to extract aflatoxins from naturally contaminated samples. The two methods did not have significantly different precisions. However, the slurry method was shown to aid the extraction process and resulted in extraction of over 50% more aflatoxins B_1 and B_2. These findings were confirmed by the *t*-test (Tables 5 and 6). Similar improvements in the extractability of aflatoxins from slurried palm kernel samples has been reported (Nawaz *et al.*, 1992).

CONCLUSIONS

The proposed method for the detection of aflatoxins in groundnut cake and kernels which employs the extraction of a water slurry with acetone, followed by a combination of bonded-phase (PH) cartridge clean-up procedure and HPLC quantification (with post-column derivatisation using iodine), has been shown to be

Table 4. Comparison of clean-up methods for analysis of aflatoxin B_2

	Analytical method								
	PH Column method				Official (CB) Method				
Sample	*X*	Variance	df	df*Var	*X*	Variance	df	df*Var	*t*-value
1	5·04	0·74	4	2·96	3·82	0·08	4	0·32	3·02
2	20·36	0·40	4	1·60	21·96	1·40	4	5·60	2·66
Sum			8	4·56			8	5·92	

S^2p PH method = 4·56/8 = 0·57 & CB method = 5·92/8 = 0·74.
Sp = 0·76 = 0·86.
Variance ratio (F_{obs}) = 0·74/0·57 = 1·30.
t-statistic for eight degrees of freedom at 95% level = 2·31.
Critical value for $F_{0.975}$(8,8) is 4·43.

Table 5. Comparison of extraction methods for aflatoxin B_1

	Extraction method								
	Slurry method				Extraction without slurry				
Sample	*X*	Variance	df	df*Var	*X*	Variance	df	df*Var	*t*-value
1	10·9	1·42	4	5·68	6·22	0·49	4	1·96	7·50
2	134	71·9	4	288	85·7	113	4	450	7·88
Sum			8	293			8	452	

S^2p PH method = 293·28/8 = 36·66 & CB method = 452·36/8 = 56·55.
Sp = 6·02 = 7·52.
Variance Ratio (F_{obs}) 56·55/36·66 = 1·54.
t-statistic for eight degrees of freedom at 95% level = 2·31.
Critical value for $F_{0.975}(8,8)$ is 4·433.

Table 6. Comparison of extraction methods for aflatoxin B_2

	Extraction method								
	Slurry extraction				Dry extraction				
Sample	*X*	Variance	df	df*Var	*X*	Variance	df	df*Var	*t*-value
1	5·04	0·74	4	2·96	2·44	0·16	4	0·64	3·02
2	20·4	0·40	4	1·60	13·7	2·79	4	11·2	8·28
Sum			8	4·56			8	11·8	

S^2p PH method = 4·56/8 = 0·57 & CB method = 11·80/8 = 1·48.
Sp = 0·76 = 1·22.
Variance Ratio (F_{obs}) 1·48/0·57 = 2·60.
t-statistic for eight degrees of freedom at 95% level = 2·31.
Critical value for $F_{0.975}(8,8)$ is 4·43.

preferable to the official AOAC (CB) method for the determination of aflatoxins in groundnut cake. Whilst the precisions of the two methods were not significantly different, the ability of the proposed method to extract significantly more aflatoxin B_1 from naturally-contaminated samples is an important consideration. The reduction in the amount of solvents used provides not only a saving in the cost of purchasing the solvents but also in their disposal. The use of the post-column derivatisation technique avoids the use of the corrosive TFA and provides a method suitable for automation. In addition, the time taken for analysis is greatly reduced compared to the official method.

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