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ADAPTATION OF GLASS COLUMNS FOR CLEAN-UP IN RP-HPLC DETERMINATION OF AFLATOXINS WITH POST-COLUMN DERIVATISATION WITH BROMINE AND FLUORESCENCE DETECTION

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ABSTRACT

Consumption of aflatoxin-contaminated foods in developing countries may not only cause cancer in the general population, but is also associated with stunting and immune suppression in children. Accurate determination of aflatoxins in foods is important for the prevention of the above health risks. This study aimed to evaluate the possibility of using cheap florisil-packed glass columns instead of relatively complex and expensive pre-packed cartridges for afltoxin clean-up prior to HPLC analysis. Additionally, effects of aflatoxin extract storage temperature, and the moisture content of florisil on aflatoxin recovery were assessed. Results showed that the standard curve was highly linear as depicted by the high correlation coefficient for all four aflatoxins [0.99, 0.99, 0.97 and 0.98, respectively for AFG_1, AFB_2 and AFB_1 and AFG2. The retention times over a total run time of 20 minutes were 14.8 (SD 0.4), 12.4 (SD 0.1), 10.7 (SD 0.3) and 9.1 (SD 0.3) minutes, respectively for AFB_1, AFB_2, AFG_1 and AFG_2. Florisil powder that had been dried for 12 hours at 100 ^oC, then hydrated with water at 5% (w/v) before packing on glass columns gave slightly higher recovery for all aflatoxins (100.3% AFB_1, 93.1% AFB_2, 83.8% AFG_1 and 71.1% AFG_2) compared to the pre-packed cartridges (96.4% AFB_1, 91.5% AFB_2, 80.6% AFG_1 and 66.9% AFG_2). There was 10% and 20% aflatoxin loss at 4 ^oC and 22 ^oC, respectively. After 6 hours of storage, no aflatoxin degradation was observed at –18 ^oC. There was 0-5% aflatoxin loss at –18 ^oC after 12 hours, while at 4 ^oC and 22 ^oC, the loss range was 20-50% and 30-55%, respectively. Aflatoxin recoveries ranged from 120-160%, 90-110%, 80-98%, and 55-74% for florisil which had been hydrated with water at 2.5%, 5% and 10%, respectively. Glass columns are cheap and can be used many times and are a feasible and sustainable alternative to pre-packed florisil cartridges for aflatoxin clean up to ensure food safety, especially in resource poor settings.

Key words: Aflatoxins determination, glass column, HPLC

INTRODUCTION

Aflatoxins, secondary metabolites produced by moulds, are known for their acute toxicity and carcinogenicity [1-3].The most common aflatoxins are B_1, B_2, G_1 and G_2. They are associated with immune function modification [4] and disorders in protein and enzyme synthesis and lipid metabolism [5]. Maize and groundnuts, the main ingredients used for complementary foods in most African countries, are most vulnerable to aflatoxin contamination [6, 7]. Application of relatively cheap methods for aflatoxin determination in foods in developing countries may help in the evasion of adverse health effects associated with aflatoxin exposure.

The stability of aflatoxins in reversed phase high performance liquid chromatography (RP-HPLC) solvents such as methanol and acetonitrile is of concern, as samples may spend several hours in these solvents prior to analysis [8]. Aflatoxin detection can be affected by extract storage conditions such as the temperature and length of storage. Further, derivatisation reagent factors such as flow rate may also influence aflatoxin detection. Even though the solubility of many mycotoxins in water is low wet apparatus and reagents may result in significant losses of aflatoxins [9].

Most aflatoxin clean up procedures prior to high performance liquid chromatography (HPLC) determination employ pre-packed commercial cartridges, followed by C18 clean up. These include aluminium oxide-packed minicolumns [10], phenyl-packed minicolumns [11], and Sep-Pak Florisil cartridges [12, 13, 14]. Other studies [15, 16] have reported the use of immunoaffinity columns for clean up in RP-HPLC using post-column derivatisation with bromine. A major disadvantage of pre-packed columns is that they are expensive and must be disposed of after single clean up procedures. In order to efficiently promote optimum public health, the application of relatively inexpensive glass columns may be a feasible and more sustainable alternative as this may help in the evasion of adverse health effects associated with aflatoxin exposure.

This paper presents the assessment of the applicability of a florisil clean up procedure using glass columns (laboratory burettes) for aflatoxin determination by RP-HPLC with post-column derivatisation using pyridinium bromide perbromide and fluorescence detection. The paper further evaluates effect of different analytical conditions including, extract storage temperature; length of extract storage prior to analysis; PBPB flow rate; the moisture content of florisil on aflatoxin recovery.

The findings of this study are likely to inform the choice of most cost-effective methods of aflatoxin determination, especially in developing countries, where a great proportion of the population is likely to be exposed to risk of immune dysfunction and cancer attributable to aflatoxin contamination of staple foods

MATERIALS AND METHODS

Raw peanuts were purchased from Gikomba Market, Nairobi and transported by air to the Department of Food Technology & Nutrition, Ghent University, Belgium. The peanuts were held at room temperature until analysis. Black pepper and nutmeg spices were purchased from a local supermarket in Ghent, Belgium. Ampoules containing 5mg of pure AFB_2, AFG_1 and AFG_2, respectively, were purchased from Makor Chemical Limited (Israel). A-5mg ampoule of pure AFB_1 was obtained as a gift from the RIKILT laboratory, Netherlands. All solvents used were of HPLC grade.

RP-HPLC determination of aflatoxins

The mobile phase consisted of water: acetonitrile; methanol (55:18:27, v/v) and was filtered through 0.45µm HPLC-certified cellulose filter paper (Watman). The mobile phase was delivered at 1ml/min by a Gilson pump (model 307). The injection solution was water: acetone (85:15, v/v). Aflatoxin solution samples were injected using a Rheodyne 7725I manual injector via a microlitre syringe (Hamilton 1ml 1001LTN, 22GA). The RP-HPLC column was a Chrompack C18RP 290*4.6mm (3µ). PBPB solution was delivered to the effluent by a Waters HPLC pump (Model 510) via a T-junction with a low dead volume. The combined flow passed through a Teflon coil (50cm x 0.5mm i.d) before entering a Gilson (Model 122) fluorescent detector with excitation and emission at 360nm and 425nm, respectively.

Effect of PBPB flow rate on aflatoxin detection

The post-column derivatisation reagent was prepared by dissolving 25mg of pyridinium hydrobromide perbromide (PBPB) in 500ml distilled water and shaking vigorously. The solution was stored in an amber bottle wrapped in aluminium foil. A fresh solution was prepared every three days. To study the effect of PBPB on aflatoxin detection, samples were injected with or without PBPB. The derivatisation pump was set at different flow rates i.e., 0.05ml/min, 0.1ml/min and 0.3ml/min and the effect on aflatoxin recovery evaluated.

Aflatoxin standard curves

Contents (5mg) of each aflatoxin ampoule were dissolved in 50ml chloroform to give a stock solution of 0.1mg/ml for each aflatoxin. Oneml of the stock solution was diluted 1000-folds to a 0.1ng/µl from which 2000µl were drawn and evaporated under a gentle stream of nitrogen in a fume hood. The residue was dissolved in 100ml water: acetone (85:15) (v/v) solution resulting in aflatoxin concentration of 2ng/ml. Subsequent dilutions were prepared from this solution namely, 0.25ng/ml, 0.5ng/ml, 1.0ng/ml and 1.5ng/ml.

Spiking of food samples

Fifty grams of ground food sample were weighed out into a conical flask. 50µl was drawn from the 0.1ng/µl stock solution of each aflatoxin and transferred to the conical flask. The contents were shaken thoroughly by hand before shaking on a mechanical shaker (Certomat) for 30 minutes before extraction. The resulting aflatoxin-spiking rate was 100µg/kg.

Aflatoxin extraction and clean-up
Aflatoxins were extracted by adding 250ml chloroform and 25 ml water and shaking on a mechanical shaker (Certomat) for 30 minutes. The suspension was filtered through fluted filter paper (Watman) and 50 ml filtrate collected.

Effect of type of clean up column on aflatoxin recovery
Pre-packed commercial Sep-Pak Florisil cartridges were compared with glass columns (Pyrex 50ml/0.1ml division volumetric burettes with PTFE (Teflon) stop-cock) packed with 1g Florisil powder hydrated with water at 5% (w/v). The Florisil powder for glass column packing was dried overnight for 12 hours in an air oven at 100 ^{o}C and cooled in a dessicator.

Effect of water addition to Florisil powder on aflatoxin recovery
To study the effect of water on aflatoxin recovery, florisil powder was hydrated by adding distilled demineralised water at 2.5%, 5% and 10% ensuring uniform water dispersion and drying overnight at 100 ^{o}C.

Florisil Clean-up procedure
Pre-packed cartridges were primed by rapidly passing 8ml chloroform via a stopcock attached to the shorter stem and draining by gravity. A glass column was attached to the longer stem of the cartridge and 2ml chloroform added via the glass column into the cartridge. The florisil-packed glass columns were primed by passing 8ml chloroform by gravity. The 50ml filtrate obtained from extraction was introduced into either the pre-packed or glass columns and drained by gravity. The column was rinsed with 5ml chloroform followed by 20ml methanol. The eluates were discarded. Aflatoxins were eluted with 40 ml acetone: water (98:2, v/v) and collected in a 250ml round bottomed flask and evaporated on a rotary evaporator (Buchi RE11) at 45 ^{o}C under vacuum.

C18 Sep Pak clean-up
A rubber stopper was attached to the longer stem of a C18 cartridge. An air-tight syringe with teflon plunger was connected to the stopper. The cartridge was primed and air removed by rapidly passing 10ml methanol from the syringe followed by 10ml distilled water. The evaporated residue of aflatoxin eluates from Florisil clean-up was re-dissolved in 1ml methanol and 4ml water and transferred quantitatively to the C18 column, twice rinsing the flask with 5ml water: methanol (80:20, v/v). 25ml water: methanol (80:20, v/v) was passed through the cartridge and eluates discarded. Aflatoxins were eluted with 50ml water: acetone (85:15, v/v) and the whole eluate collected in a volumetric flask.

Effect of extract storage temperature and storage duration on aflatoxin recovery
Different 50ml samples of 1.0ng/ml mixed aflatoxin standard solution were subjected to the Florisil clean-up, followed by C18 clean-up. The extracts were stored in water: acetone (85:15, v/v) in amber bottles wrapped with aluminium foil at –18 ^{o}C, 4 ^{o}C and 22 ^{o}C for 6, 12 and 24 hours in the dark before HPLC determination.

RESULTS

Effect of derivatisation with PBPB on afaltoxin detection

To study the effect of PBPB on aflatoxin detection at a concentration of 10 ng/ml, samples were injected with and without derivatisation by setting the PBPB delivery pump flow rate at 0.1ml/min and 0ml/min, respectively. Table 1 shows the increment or decrement factors in detection with and without PBPB. The detection of AFB_1 and AFG_1 was enhanced 15 and 20 times, respectively in the presence of PBPB, but both peaks disappeared in the absence of PBPB. The addition of PBPB had little effect on the detection of both AFB_2 and AFG_2.

Effect of PBPB flow rate on aflatoxin detection

To study the effect of PBPB flow rate on aflatoxin recovery from standard aflatoxin solution prepared at 1.0 ng/ml, the PBPB delivery pump was adjusted to 0.05, 0.1 and 0.3ml/min. Peak areas were calculated for 10 runs at each flow rate. Table 2 gives the average peak areas and the standard deviations for aflatoxins at different flow rates. Aflatoxin peak areas tended to decrease with increasing flow rate. Reproducibility as measured by coefficient of variation was highest at 0.1ml/min. The CV% ranged from 2.7%-6.1%, 0.8%-7.9% and 5.5%-23.8% at 0.05ml/min, 0.1ml/min and 0.3ml/min, respectively as presented in table 3.

Calibration curve

Calibration solutions were prepared with mixed aflatoxins and ranged from 0.25ng/ml-1.5ng/ml for AFG_1, AFB_2 and AFB_1 and 0.5ng/ml-2.0ng/ml for AFG2. The standard curve was highly linear as depicted by the high correlation coefficient for all four aflatoxins [0.99, 0.99, 0.97 and 0.98, respectively for AFG_1, AFB_2 and AFB_1 and AFG2. The retention times over a total run time of 20 minutes were 14.8 (SD 0.4), 12.4 (SD 0.1), 10.7 (SD 0.3) and 9.1 (SD 0.3) minutes, respectively for AFB_1, AFB_2, AFG_1 and AFG_2.

Effect of type of clean up column on aflatoxin recovery

Table 4 shows a comparison of percent aflatoxin recovery obtained by using glass columns packed with florisil powder dried overnight and hydrated with water at 5% (w/v) and pre-packed cartridges. There was a trend towards greater recovery of aflatoxins with glass columns compared with pre-packed florisil cartridges.

Effect of water addition to Florisil powder on aflatoxin recovery

Florisil powder for glass column packing was dried in an air oven at 100 ^{o}C overnight and moistened by adding water at 2.5%, 5% and 10% (w/v) and shaken vigorously for 30 minutes and allowed to rest for 30 minutes. Figure 1 shows the influence of addition of water to florisil powder on aflatoxin recovery from standard solutions. It was observed that the addition of 2.5% water resulted in increased recovery (90 - 120%) for all the aflatoxins. However, above 2.5% water aflatoxin recovery was reduced as the amount of water increased. The recoveries ranged from 80-98% and 55-74% for florisil with water at 5% and 10%, respectively. Addition of 5% water resulted in 10-20% loss in recovery, while at 10% water, the loss of recovery ranged from 27-66%.

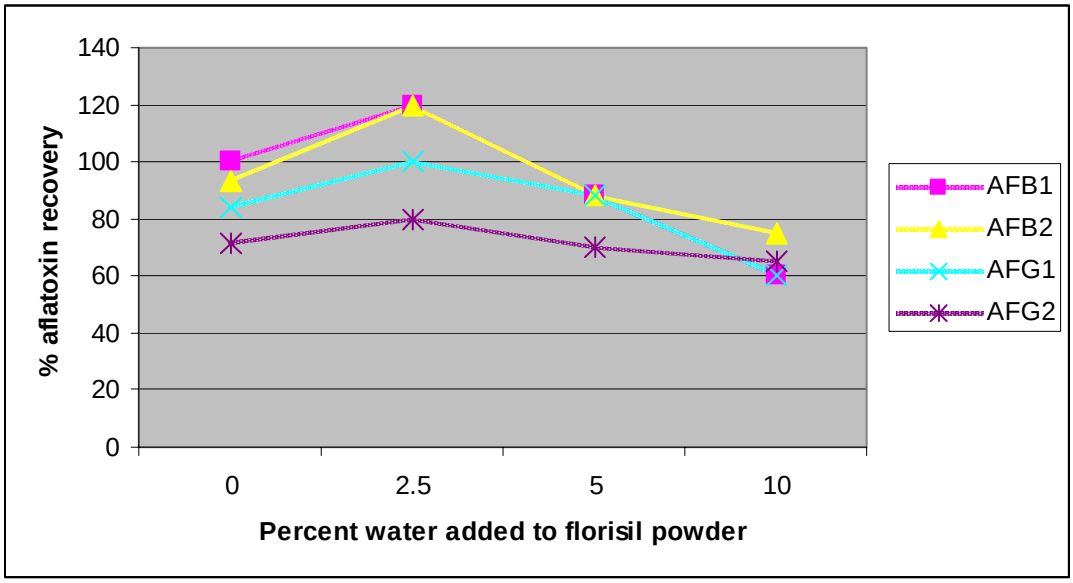

Figure 1: Effect of water addition to florisil powder on aflatoxin recoveries.

Effect of extract storage temperature and storage duration on aflatoxin recovery

Even though HPLC method for aflatoxin determination is accurate and sensitive, it requires time-consuming sample clean up and several hours elapse between extraction and analysis [9, 17]. Dilute solutions of aflatoxins are not particularly stable, especially to light and must be renewed regularly [10]. All standard solutions of mixed aflatoxins that had been subjected to the clean-up procedure were stored in water: acetone (85:15, v/v) in amber bottles wrapped with aluminium foil at –18 ^{o}C, 4 ^{o}C and 22 ^{o}C for 6, 12 and 24 hours in the dark before HPLC determination. Aflatoxin recovery was evaluated against the temperature and duration of storage. Figure 2 shows the influence of holding time at -18 ^{o}C on the stability of AFB1, AFB2 and AFG1 standard solutions. There was 10% and 20% aflatoxin loss at 4 ^{o}C and 22 ^{o}C, respectively. After 6 hours of storage, no aflatoxin degradation was observed at –18 ^{o}C. There was 0-5% aflatoxin loss at –18 ^{o}C after 12 hours, while at 4 ^{o}C and 22 ^{o}C, the loss range was 20-50% and 30-55%, respectively. It was observed that AFB_2 was more stable at all temperatures followed by AFB_1. AFG_1 was the least stable. At 4 ^{o}C, the losses were 20%, 30% and 50% for AFB_2, AFB_1 and AFG_1, respectively. At 22 ^{o}C only 30% AFB_2 had been lost compared to about 50% for both AFB_1 and AFG_1 after 12 hours. After 24 hours the loss ranges were 25-55%, 40-50%, and 40-50%, respectively for –18 ^{o}C, 4 ^{o}C and 22 ^{o}C with AFB_2 still showing the lowest degradation.

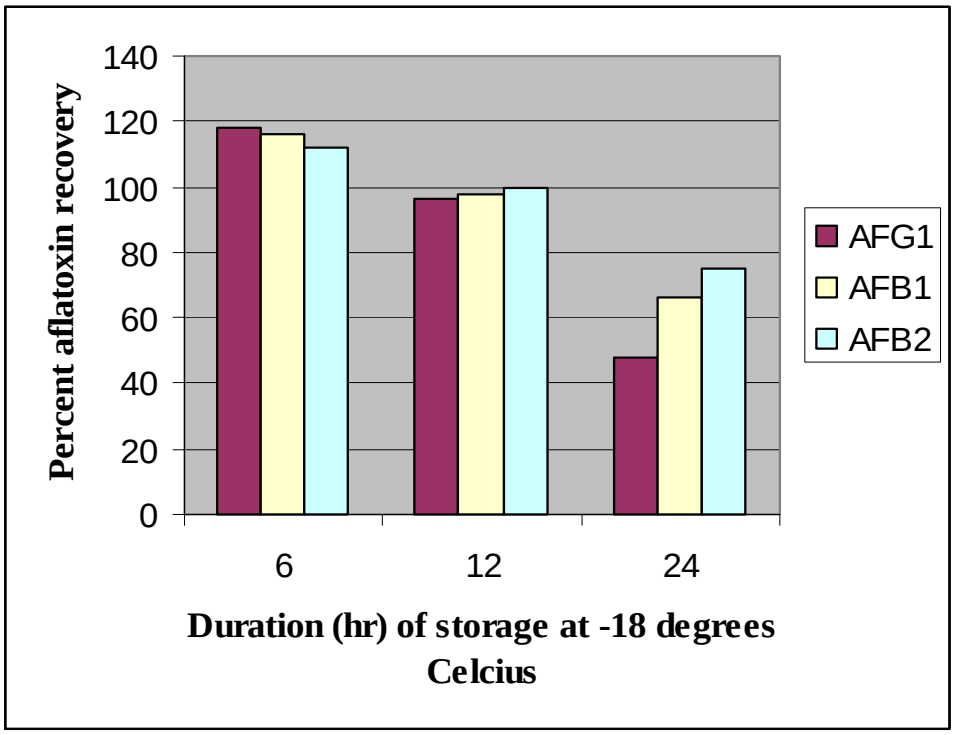

Figure 2: Effect of holding time on AFB_1, AFB_2 and AFG_1 recovery at 18 °C.

DISCUSSION

This study was designed to assess the applicability of glass columns (laboratory burettes) for aflatoxin determination by RP-HPLC and, to evaluate effect of extract storage temperature; length of extract storage prior to analysis; PBPB flow rate; and the moisture content of florisil on aflatoxin recovery.

The results showed that the addition of PBPB enhanced the detection of AFB_1 and AFG_1, but had little effect on the detection of AFB_2 and AFG_2. Aflatoxin peak areas tended to decrease with increasing flow rate. There was a trend towards greater recovery of aflatoxins with glass columns compared with pre-packed florisil cartridges. The addition of water to florisil was beneficial in peak enhancement up to 2.5% w/w of water. There was lesser aflatoxin detection as storage temperature and time increased, but this observation was more pronounced for AFB_1 and AFG_1.

The enhancement of AFB_1 and AFG_1 peaks could be explained by the fact that these two aflatoxins possess a double bond at position 8,9 on the terminal furan ring [18]. The first step of derivatization is the bromination of the 8,9-double bond to form a high fluorescent product. This bond does not exist in AFB_2 or AFG_2 and hence they do not react with bromine [18]. Thus reduction in AFB_2 and AFG_2 detection is as a result of a dilution effect of PBPB solution. The disappearance of AFB1 and AFG1

without PBPB and their appearance with PBPB is a good confirmatory test for the presence of these aflatoxins [1].

That aflatoxin peak areas decreased with increasing flow rate can be explained by the fact that when an excess of bromine is present, the first reaction (bromination of 8,9-double bond of AFB_1 and AFG_1) is followed by a second reaction (bromination of the phlorogucinol nucleus), which yields a low fluorescent product [18]. The decrease in peak areas for AFB_2 and AFG_2 at higher PBPB flow rate could be attributed to dilution effect.

The relatively higher recovery with the glass column can be explained by the fact that the overnight drying of florisil powder resulted in air and moisture expulsion. The CV% for recoveries on pre-packed columns ranged from 17-41% compared with 11-22% for glass columns. The presence of air bubbles in florisil could have resulted in the large variations observed in the recoveries. This effect was partially eliminated by drying glass columns overnight at 100 ^{o}C. Secondly, gravity elution through the pre-packed cartridges took longer time than with glass columns. This could have resulted in aflatoxin degradation. A higher solvent head in the glass columns resulted in faster elution. The average aflatoxin recoveries, 83.9% and 87.1% for Sep-Pak Florisil cartridges and glass columns, respectively are within the range of average recovery of 84% reported previously [12]. Another study [13] found an average recovery of 91% for pre-packed florisil column clean up. Studies using immunoaffinity columns have reported recoveries of the range 71-92% total aflatoxin, 82-109% for AFB_1 and 92-101% for AFB_1 [15, 16].

The observed decrement of aflatoxin peaks when more than 2.5% water was added to florisil shows that hydration of aflatoxin molecules to some extent may enhance detection; however, the mechanism of this effect cannot be explained in this paper.

The existence of a double bond on position 8,9 at the terminal furane ring of AFG_1 and AFB_1 may explain their more reactivity during storage compared to AFB_2 and AFG_2 both of which lack the double bond [10, 18].

CONCLUSIONS

Glass columns are cheap and can be used many times and are a feasible and sustainable alternative to pre-packed florisil cartridges for aflatoxin clean up. The glass columns also present a more rapid clean up alternative. Even though the method described in this study was originally meant for the determination of AFB_1, this study has shown that it is also applicable to AFB_2, AFG_1 and AFG_2 between concentrations from 0.25ng/g to at least 10 ng/g. It is recommended that all aflatoxin extracts be analysed within 6 hours of clean up. However, extracts can be held for up to 12 hours at –18 ^{o}C. All apparatus used for aflatoxin analysis need to be dried to remove air and water that would otherwise cause variation in results and loss of aflatoxins. PBPB flow rate has an effect both on aflatoxin detection and method reproducibility and 0.1ml/min seems to be the optimum value. These findings may be used as the basis for the choice of most cost-effective methods of aflatoxin determination, especially in

developing countries, where a great proportion of the population is likely to be exposed to risk of immune dysfunction and cancer attributable to aflatoxin contamination of staple foods.

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Table 1: Increment or decrement factors of aflatoxin peaks with or without PBPB at a standard mixed aflatoxin solution concentration of 10 ng/ml.

Aflatoxin	With PBPB	Without PBPB
AFB_1	15 (+)	ND
AFB_2	0.69 (-)	1.44 (+)
AFG_1	20 (+)	ND
AFG_2	0.62 (-)	1.60 (+)

ND – not detected, (+) =increment, (-) = decrement

Table 2: Average peak areas of aflatoxins at different PBPB flow rates obtained by injecting standard aflatoxin solutions at 1.0 ng/ml[1]

Aflatoxin	PBPB flow rate (ml/min)		
	0.05	0.1	0.3
AFB_1	9355.7 (532.2)	7188.9 (244.6)	7884.3 (437.5)
AFB_2	18977.9 (517.5)	18391.0 (155.1)	20505.4 (1486.9)
AFG_1	5818.2 (356.0)	4793.7 (378.5)	4517.7 (1076.0)
AFG_2	7576.9 (466.0)	7428.8 (374.3)	6031.3 (564.9)

[1]Figures in parentheses are standard deviations for 10 determinations

Table 3: Percent coefficient of variation for aflatoxins at different PBPB flow rates for standard solutions prepared at 1.0 ng/ml.

Aflatoxin	PBPB flow rate (ml/min)		
	0.05	0.1	0.3
AFB_1	5.7	3.4	5.5
AFB_2	2.7	0.8	7.2
AFG_1	6.1	7.9	23.8
AFG_2	6.1	5.0	9.4
Average	**5.2**	**4.3**	**11.5**

Table 4: Comparison of percent aflatoxin recovery obtained by using glass columns packed with florisil powder dried overnight water addition at 5% (w/v) and pre-packed cartridges[1].

	Sep-Pak Cartridge	Glass Column
AFB_1	96.4 (16.0)	100.3 (21.0)
AFB_2	91.5 (13.5)	93.1 (9.9)
AFG_1	80.6 (33.0)	83.8 (18.2)
AFG_2	66.9 (14.4)	71.1 (8.3)

[1]Figures in parentheses represent standard deviations of ten determinations

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