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Aflatoxin in Raw Peanut Kernels Marketed in Malaysia

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ABSTRACT

The occurrence of aflatoxin in eighty-four samples of raw peanut kernels which are randomly collected from Malaysian supermarkets was examined. Analysis for aflatoxin was performed by solvent extraction and immunoaffinity clean-up followed by the determination using high performance liquid chromatography equipped with post-column photochemical reactor for enhanced detection and fluorescence detector. A detection limit of 0.01-0.09 ng/mL and a quantification limit of 0.04-0.30 ng/mL were obtained. The aflatoxin concentrations ranged from not detected to 97.28 ng/g in all samples investigated. About 78.57% of the samples were contaminated with aflatoxin, of which 10.71% exceeded the maximum tolerable limit of 15 ng/g set by the Codex. Average recoveries of the aflatoxin analysis were acceptable which were in the range of 74.85 ± 8.83% for AFG_2 at the concentration of 0.15 ng/mL and 103.91 ± 6.45% for AFB_2 at the concentration of 0.15 ng/mL. The average daily intake estimated for total aflatoxins was 10.69 ng/kg body weight. There was a significant difference ($P < 0.05$) in aflatoxin content between brands and locations.

Key words: Aflatoxin, peanut, food safety, HPLC with fluorescence detection, immunoaffinity clean-up

INTRODUCTION

Aflatoxins are secondary metabolites produced by many strains of *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nominus*[(1)]. After the death of 100,000 turkeys in the UK in 1960s owing to toxic metabolites coming from fungi[(2)], aflatoxins have been a major concern as a carcinogenic, mutagenic and immunosuppressive agent of feeds and foods[(3)]. Various agricultural commodities including peanut, corn, cottonseed, Brazil nut, pistachio nut, fig, spice and copra are likely to be contaminated by aflatoxins[(4)]. In comparison to the other agricultural commodities, peanut is very susceptible to aflatoxins contamination. It could be due to the kernels develop and mature beneath the surface, and domination of *Asp. flavus* of the peanut field soil[(5)].

Despite the fact that 20 aflatoxins have been identified, only 4 of them, the aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2), are fundamental contaminants of a diversity of foods and feeds[(1)]. AFB_1 has been classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen, mainly to cause liver cancer[(1)], whereas AFB_2, AFG_1, AFG_2 are classified as possible carcinogens to humans[(6)]. Since aflatoxins are potent source of health hazards to both human and animals and they are causing lots of economic losses, attempts have been made to study the aflatoxins occurrence in many parts of world and to completely annihilate the toxin or diminish its content in foods and feedstuffs[(7)].

The two probable reasons for aflatoxin contamination in peanut are severe late-season drought stress happening in the field (pre-harvest) and the existence of undesirable moisture and temperature conditions during storage (post-harvest)[(8)]. Peanut shell penetration by molds is facilitated by physical damage; hence, aflatoxins contamination will occur[(9)]. Malaysia, a tropical country with an average temperature of 28 to 31°C and heavy rainfall throughout the year, is appropriate for fungal growth. However, in the dry season the relative humidity is 50 to 60%, in contrast to 70 to 80% in wet seasons; hence mold growth and aflatoxins production in products such as peanut stored under these conditions will increase[(10)].

Therefore, the purpose of this study was to examine the occurrence and concentration of aflatoxins in packaged plastic bag raw peanut kernels marketed in

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Malaysian supermarkets located in four different areas using an immunoaffinity column $AflaTest_{WB}$ (IAC) clean-up and high performance liquid chromatography (HPLC) with fluorescence detector and to estimate the daily intake of this toxicant from peanut consumption.

MATERIALS AND METHODS

Mixed aflatoxin standards with AFB_1 and AFG_1 concentration of 300 ng/mL, AFB_2 and AFG_2 concentration of 1000 ng/mL, individual AFB_1 with 20000 ng/mL, and AFB_2, AFG_1 and AFG_2 with concentration of 3000 ng/mL were purchased from Supelco (Bellefonte, PA, USA). All solvents used for the experiments were of HPLC grade and supplied by Merck (Darmstadt, Germany). $AflaTest_{WB}$ immunoaffinity columns (IAC) with 1 mL volume were purchased from Vicam Company (Watertown, MA, USA).

I. *Sampling*

A total of 84 samples of packaged plastic bag raw peanut kernels were purchased randomly from different supermarkets in four locations (north, south, east and west region) of state of Selangor, Malaysia from April to August 2008. A wide range of brands were covered to ensure that the survey was representative of the range of products available to consumers in Malaysia. About 4 kg of samples were randomly collected from each supermarket. The samples were thoroughly mixed and went through quarter sampling to make a representative sample (100 g); the representative samples were then immediately transferred to dry clean polyethylene bags and stored at -18°C prior to analysis.

II. *Extraction and Clean up*

Mycotoxins should be separated from the solid phase of the matrix and distributed into the liquid phase[11]. Aflatoxins were extracted and determined using the AOAC official method 991.31[12] with minor modification. The representative sample (100g) was ground using a Waring blender (Vicam, Watertown, MA, USA) for about 3 min. Twenty-five grams of ground peanut samples and 5 g of NaCl were blended with 125 mL of methanol/water (70:30, v/v) for 2 min. Fifteen milliliters of the extract was diluted with 30 mL water after being filtered on a 24-cm fluted filter paper (Vicam, USA). Finally it was filtered on a Whatman glass microfiber filter (934-AH, Maidstone, UK). Fifteen milliliters of the filtrate was applied to the IAC containing monoclonal antibody specific for AFB_1, AFB_2, AFG_1, and AFG_2 to be purified. The column was washed with 20 mL of deionized water, then aflatoxins were eluted from the column with 1.0 mL of methanol and the eluted fraction was diluted twice with deionized water, and then stored in a vial at -18 to -20°C.

Aflatoxins extract in the methanol-water solution were determined by HPLC method with fluorescence detector after using a post-column photochemical reactor for enhanced detection (PHRED) (Aura Industries, N.Y, USA).

III. *HPLC Determination of Aflatoxins*

A HPLC method was used for aflatoxin analysis of all samples, using a reverse phase symmetry C18 column (Merck, Darmstadt, Germany) with a dimension of 25 cm × 4.6 mm, and 5 µm particle size, running on a Waters 2475 HPLC equipped with a fluorescence detector operated at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. The mobile phase was a mixture of water/methanol/acetonitrile (54:29:17, v/v/v) with a flow rate of 1 mL/min. A post-column PHRED was used to enhance the natural fluorescence of AFB_1 and AFG_1 and to improve detection[13]. The PHRED was located between the LC column and the detector; encompass a lamp holder, a 254 nm low-pressure mercury lamp, and a holder for the knitted reactor coils. During photolysis, AFB_1 and AFG_1 are converted to hemiacetals meaning AFB_{2a} and $AFG_{2a,}$ respectively. Due to the very low detectability of AFB_1 and AFG_1, the post column derivatization is used to make them detectable by the detector. The reactor coils are made of polytetrafluoroethylene (PTFE) that is transparent to the 254 nm UV light with 25m length[14].

For aflatoxin identification, linearity, accuracy, repeatability (RSDr), reproducibility (RSD_R), limit of detection (LOD) and limit of quantification (LOQ), linear equation and coefficient of regression (R^2) of the analytical method were determined. Linearity was estimated by injecting triplicate aflatoxin standards. Recovery studies were carried out by spiking aflatoxins in three replicated peanut samples at concentrations of 0.50, 5.00, 30.00 ng/mL and 0.15, 1.50, 9.00 ng/mL of AFB_1, AFG_1 and AFB_2, AFG_2 respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated using the 3xstandard deviation and 10xstandard deviation, respectively, calculated by 7 times injection of standards having the lowest concentration to be detected into the HPLC.

IV. *Estimated Daily Intake*

The estimated daily intake (EDI) values of aflatoxin by an adult (ng/kg body weight) were calculated using the average value by each type of peanut, i.e. EDI in ng/kg body weight = mean concentration of aflatoxin (ng/g) multiplied by the amount of peanut consumed/day (g) and divided by the average weight of an individual (60 kg)[15].

V. *Statistical Analysis*

The descriptive statistic (mean, standard deviation

and range) and analysis of variance (ANOVA) were employed using Minitab (Version 14, PA., State College, USA). A probability value of 0.05 was used to determine the statistical significance.

RESULTS AND DISCUSSION

I. *Method Performance*

For aflatoxin analysis, linearity was estimated by injecting triplicate aflatoxin standard solutions at concentrations of 2, 4, 6, 8, 10, 25, 50 and 100 ng/mL for AFB_1 and AFG_1, and 0.6, 1.2, 1.8, 2.4, 3.0, 7.5, 15.0 and 30.0 ng/mL for AFB_2 and AFG_2, respectively. Eight-point calibration curve was built for each individual aflatoxin used for quantification of aflatoxin in peanut samples. The correlation coefficient was more than 0.993 (R^2 > 0.993). The recoveries of aflatoxins in peanut samples are summarized in Table 1. The recovery for AFB_1 ranged from 95.53 ± 11.60 to 102.39 ± 7.64%, AFB_2 ranged from 88.73 ± 10.93 to 103.91 ± 6.45%, AFG_1 ranged from 91.72 ± 5.56 to 97.23 ± 10.96%, and AFG_2 ranged from 74.85 ± 8.83 to 78.00 ± 11.31%. The recoveries obtained for aflatoxins were in line with the legislated levels for aflatoxin determination methods described by commission regulation[16].

The chromatogram of the spiked peanut samples has well-separated peaks, as shown in Figure 1(a). The limit of detection (LOD) and limit of quantification (LOQ) are shown in Table 2. The LOD were found to be 0.03, 0.01, 0.09, and 0.06 ng/mL and the LOQ were 0.10, 0.04, 0.30 and 0.20 ng/mL for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Moreover, the repeatability (RSD_r) obtained for AFB_1, AFB_2, AFG_1 and AFG_2 were 1.62, 4.45, 5.10 and 9.91%, respectively. The reproducibility (RSD_R) found to be 7.92, 7.25, 9.62, and 10.94% for AFB_1, AFB_2, AFG_1 and AFG_2 respectively.

Table 1. Recoveries and relative standard deviations (%) of aflatoxin in spiked samples

Aflatoxins	Concentration of spiked aflatoxin (ng/mL)	Mean recovery [a] ± RSD (%)
AFB_1	0.50	100.03 ± 6.80
	5.00	102.39 ± 7.64
	30.00	95.53 ± 11.60
AFB_2	0.15	103.91 ± 6.45
	1.50	96.80 ± 3.91
	9.00	88.73 ± 10.93
AFG_1	0.50	91.72 ± 5.56
	5.00	92.74 ± 9.22
	30.00	97.23 ± 10.96
AFG_2	0.15	74.85 ± 8.83
	1.50	77.73 ± 10.50
	9.00	78.00 ± 11.31

[a] Mean recoveries were ascertained by assessing three replicate samples at each spiked level.

II. *Aflatoxin Occurrence in Analyzed Samples*

The concentrations of aflatoxins in all of the peanut

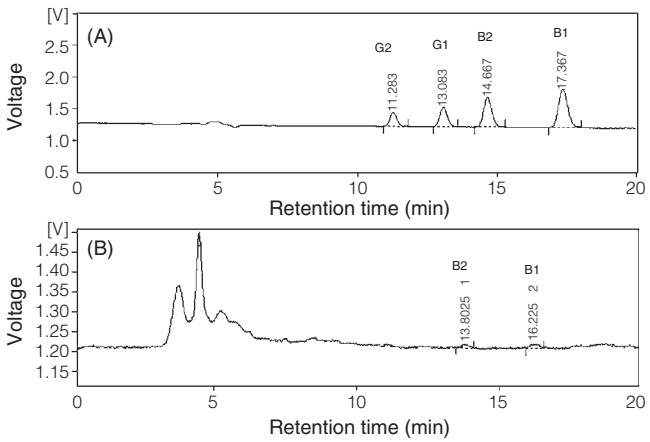

Figure 1. HPLC fluorescence chromatogram of spiked peanut samples and blank sample. (a) Spiked peanut samples with 2 ng/mL of AFB_1 and AFG_1, and 0.6 ng/mL of AFB_2 and AFG_2. (b) Blank sample.

Table 2. Linear equation, LOD and LOQ obtained for quantification of aflatoxin

Aflatoxins	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	Calibration curve	R^2
AFB_1	0.03	0.10	y = 8.97x - 11.65	0.9948
AFB_2	0.01	0.04	y = 20.99x - 8.33	0.9949
AFG_1	0.09	0.30	y = 3.18x - 4.33	0.9951
AFG_2	0.06	0.20	y = 6.88x + 0.18	0.9962

[a]limit of detection.
[b]limit of quantification.

samples used in the study are shown in Table 3. The results revealed wide variation in aflatoxins concentrations among the 84 samples analyzed. The study found that 78.57% of the samples were contaminated with total aflatoxins concentrations varying from 2.76 to 97.28 ng/g, whereas 10.71% of the samples exceeded the maximum tolerable limit (15 ng/g) set for total aflatoxins in nuts by the Codex[17]. It was interesting to note that only 10.71% of the samples were contaminated with AFG_1 and all samples were free from AFG_2. This might be due to the invasion of peanuts by *Asp. flavus* rather than *Asp. parasiticus*[1].

As shown in Table 4, 75, 67.85, and 10.71% of samples found to be contaminated with AFB_1, AFB_2, and AFG_1 with the mean concentration of 9.00 ng/g, 1.91 ng/g, and 0.38 ng/g respectively.

Samples of brand 1 were found to contain the highest level of aflatoxins contamination (total 48.95 ng/g) and brand 6 contained the lowest (2.89 ng/g). Moreover, the highest contamination of total aflatoxins was found in the south of Selangor state (19.71 ng/g) and the lowest was in the east (4.10 ng/g). This quite high contamination in the south might be due to the bad condition of its storage. The statistical analysis indicated significant difference ($p < 0.05$) between total aflatoxins, location and brands. The statistical differences are shown

Table 3. Concentrations of aflatoxins in 84 peanut samples analyzed by HPLC

Brands	Location	Sample number	AFB_1(ng/g) (mean ± SD)	AFB_2 (ng/g) (mean ± SD)	AFG_1 (ng/g) (mean ± SD)	AFG_2 (ng/g) (mean ± SD)	Total aflatoxins (ng/g) (mean ± SD)
B1		3	60.67 ± 4.46^a	8.75 ± 0.63^a	3.52 ± 0.47^a	ND	72.94 ± 5.39^a
B2		3	2.95 ± 0.11^b	1.45 ± 0.59^b	ND	ND	4.40 ± 0.68^b
B3		3	3.04 ± 0.27^b	1.21 ± 0.45^b	3.31 ± 0.40^a	ND	7.56 ± 1.12^b
B4	1	3	7.93 ± 0.50^c	1.58 ± 0.54^b	ND	ND	9.51 ± 0.51^b
B5		3	ND	ND	ND	ND	ND
B6		3	2.87 ± 0.10^b	ND	ND	ND	2.87 ± 0.10^b
B7		3	ND	3.47 ± 0.50^c	ND	ND	3.47 ± 0.50^b
B1		3	87.02 ± 4.63^a	10.26 ± 0.80^a	ND	ND	97.28 ± 5.43^a
B2		3	2.76 ± 0.06^b	ND	ND	ND	2.76 ± 0.06^b
B3		3	2.89 ± 0.21^b	ND	ND	ND	2.89 ± 0.21^b
B4	2	3	4.81 ± 0.37^b	2.86 ± 0.76^c	ND	ND	7.67 ± 1.08^c
B5		3	6.46 ± 0.29^b	2.69 ± 1.34^c	ND	ND	9.15 ± 1.62^c
B6		3	6.24 ± 0.56^b	2.46 ± 0.74^c	ND	ND	8.70 ± 0.34^c
B7		3	7.72 ± 0.59^b	1.81 ± 0.23^c	ND	ND	9.53 ± 0.40^c
B1		3	2.86 ± 0.15^a	0.95 ± 0.11^a	ND	ND	3.81 ± 0.25^a
B2		3	2.72 ± 0.08^a	0.98 ± 0.15^a	ND	ND	3.70 ± 0.24^a
B3		3	7.84 ± 0.41^b	2.41 ± 0.52^b	ND	ND	10.25 ± 0.92^b
B4	3	3	8.27 ± 0.66^b	2.71 ± 0.65^b	ND	ND	10.98 ± 0.48^b
B5		3	ND	ND	ND	ND	ND
B6		3	ND	ND	ND	ND	ND
B7		3	ND	ND	ND	ND	ND
B1		3	17.81 ± 0.97^a	3.98 ± 0.52^a	ND	ND	21.79 ± 1.45^a
B2		3	2.80 ± 0.10^b	0.98 ± 0.13^b	ND	ND	3.78 ± 0.22^b
B3		3	4.17 ± 0.33^c	1.88 ± 0.27^c	3.69 ± 0.66	ND	9.75 ± 1.25^c
B4	4	3	2.92 ± 0.20^b	0.99 ± 0.15^b	ND	ND	3.91 ± 0.32^b
B5		3	7.14 ± 0.67^d	1.94 ± 0.33^c	ND	ND	9.07 ± 0.99^c
B6		3	ND	ND	ND	ND	ND
B7		3	ND	ND	ND	ND	ND

1: North
2: South
3: East
4: West
ND: Not Detected
^{a,b,c,d}Similar letters in each column show insignificant differences.

Table 4. Prevalence of aflatoxins in 84 samples analyzed by HPLC

Aflatoxins	Positive samples (%)	Mean (ng/g)	Max (ng/g)	Median (ng/g)	Range
AFB_1	75.00	9.00	92.07	2.99	0-92.07
AFB_2	67.85	1.91	11.16	1.12	0-11.16
AFG_1	10.71	0.38	4.36	0	0-4.36
AFG_2	0	ND	ND	ND	ND
Total	78.57	11.28	103.23	4.18	ND

ND: Not detected.

in Table 3. The contamination which was detected in the samples is likely due to either the undesirable pre-harvest or adverse storage conditions[8].

The occurrence of aflatoxin in peanut has been reported by several authors from different countries. In the Philippines, a survey on peanut-based products showed 60% of the samples were contaminated by AFB_1 and AFG_1 in the ranges of 1-244 ng/g and 6-68 ng/g, respectively[18]. Juan *et al*.[1] reported a 0.3 ng/g aflatoxin contamination in peanut in the Rabat-Sale area, Morocco in 2008. Haydar *et al*.[19] analyzed the aflatoxin concentrations in Syrian foods and showed 2.7 ng/g AFB_1 contamination in 28.5% of raw shelled peanut. Farombi[20] reported 43-1099 ng/g aflatoxin in Brazilian peanuts in 1998, whereas Abdulkadar *et al*.[21] did not find any contamination in peanuts in Qatar in 2000. Chun *et al*.[6] showed 0.2 ng/g AFB_1 contamination in 25% of raw peanuts in South Korea and 20-200 ng/g in peanut samples, all from Argentina and Senegal. Due to the availability of peanut in all retail markets and supermarkets throughout Malaysia and its use in a variety of popular Malaysian foods such as satay (meat or chicken with peanut sauce) and rempeyek (traditional cracker), Malaysian people are at risk from the undesirable effects of aflatoxin on their health[22,23].

The differences of aflatoxin occurrence in different countries could be related to their different weather conditions, and pre-harvest and post-harvest practices; the phenomena is in agreement to what Akbas and Ozdemir mentioned that geographic location, agricultural practices and susceptibility of the products of fungal growth during harvest, storage and processing affect the occurrence of aflatoxin[3].

The recoveries from the spiked samples from the current study were different from other studies. Abdulkadar *et al*.[21] (2000) determined the amount of aflatoxin in different nuts, using HPLC and precolumn derivatization (trifluoroacetic acid), based on AOAC 990.33. The mobile phase was methanol/water/acetonitrile (13:74:13, v/v/v) at the flow-rate of 0.50 mL/min. The average recoveries for pistachio were 87, 95, 93 and 89% and the repeatability values (RSDr) were 6.12, 10.93, 6.97 and 8.31% for AFB_1, AFB_2, AFG_1 and AFG_2 respectively. The study reported a limit of detection of 0.1 ng/mL.

In Chun *et al*.[6] (2007) study, nine types of samples were analyzed using HPLC and precolumn derivatization (trifluoroacetic acid), based on AOAC method 990.33. The mobile phase was water/acetonitrile (3:1, v/v) at the flow-rate of 1 mL/min. The recoveries in peanut butter and walnut were 102, 84.8, 102.1 and 83.4% for AFB_1 and AFB_2 at spiking level of 20 ng/mL and AFG_1 and AFG_2 at spiking level of 10 ng/mL, respectively. The precision as determined by a multiple analysis of spiked samples was 7.11, 22.59, 5.42, and 27.75% for AFB_1, AFB_2, AFG_1 and AFG_2 respectively. The range of limit of detection was 0.08-1.25 ng/mL whereas for the limit of quantification it was 0.15-2.50 ng/mL.

Juan *et al*.[1] (2008) studied the occurrence of aflatoxins in dried fruits and nuts using HPLC and precolumn derivatization (trifluoraacetic acid). The mobile phase used was methanol/water/acetonitrile (17:54:29, v/v/v) with the flow rate of 0.8 mL/min. The recoveries were 83.6, 87.3, 88.5, and 89.5% for AFB_1, AFB_2, AFG_1 and AFG_2 respectively. The limit of detection was 0.006 ng/mL for AFB_1 and AFG_1 and 0.015 ng/mL for AFB_2 and AFG_2 whereas the limit of quantification was 0.02 ng/mL for AFB_1 and AFG_1 and 0.05 ng/mL for AFB_2 and AFG_2.

The differences in the recoveries value were possibly due to either interference of the fluorescence properties of the sample matrix in the detection process of this toxin component by HPLC[13] or different methods of extraction. The results gained in this study indicate that the HPLC method adopted in this research was acceptable.

III. *An Assessment of Aflatoxin Exposure in Humans*

The amount of peanut consumption may vary considerably from one individual to another. The daily intake of compounds from food consumption is dependent on the compound concentration in food and the amount of food consumed. Results of an official survey have shown that the average Malaysian consumes 56.90 g/day of peanut and the demand for peanut consumption is increasing over the years[24]. Based on this input

and the mean concentrations of total aflatoxins in peanut found in this study (11.28 ng/g), for 60 kg as the average of body weight, the ingestion of total aflatoxins was 10.69 ng/kg body weight per day.

CONCLUSIONS

This study showed as many as 78.57% of the 84 peanut samples were contaminated with aflatoxins, of which 10.71% of the samples exceeded the maximum tolerable limit for total aflatoxins of 15 ng/g set by the Codex regulation[17]. Considering the tropical weather in Malaysia, products such as peanut stored under this condition are very susceptible to aflatoxins contamination. Regular monitoring of aflatoxins content in peanut is recommended.

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