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Determination of aflatoxin B1 in food products in Thailand

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Aflatoxin B1 is a secondary metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*. It can be formed in commodities before and after harvest. This mycotoxin possesses a variety of toxic effects, potent carcinogen to both animal and human health. Aflatoxin B1 is generally found in feed and food stuff, such as cereal and all products derived from cereals, including processed cereals since it has been proven to be at least partly resistant to food processing methods. Hence, the aim of this study was to determine the possibility of contamination of aflatoxin B1 in food products in Thailand. The 100 food samples were purchased from markets around Bangkok. They were divided into five categories: seven samples of local fermented alcoholic beverages, five samples of imported blue cheese, 18 samples of fermented soybean products, 70 samples of raw peanuts (30) and peanut derived products (40). They were determined for aflatoxin B1 by ELISA method. The revealed rates of aflatoxin B1 contamination were 71.42, 100, 83.33, 86.67 and 90% for the alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived samples, respectively. The individual values with each category samples, ranged from 0.3 to 2.15 µg/kg (average 0.48 µg/kg), 0.5 to 1.25 µg/kg (average 0.95 µg/kg), 0.2 to 3.2 µg/kg (average 1.54 µg/kg), 0.2 to 8.05 µg/kg (average 6.83 µg/kg) and 0.1 to 73.85 µg/kg (average 5.6 µg/kg) for alcoholic beverages, blue cheese, soybean, raw peanuts and peanut derived samples, respectively.

Key words: Mycotoxins, aflatoxin B1, carcinogen.

INTRODUCTION

Mycotoxins are a group of toxic secondary metabolites with no apparent function in the normal metabolism of fungi. They are produced by certain fungi infesting agricultural products. They induce a variety of toxic response in humans and animals when foods or feeds containing these compounds are ingested. Therefore, human ingestion of mycotoxins mainly occurs from the consumption of mycotoxins in residues or metabolites in animal-derived foods or contaminated food products (Smith et al., 1995). Aflatoxins (B1, G1, B2, G2) are the

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best known and most widely studied mycotoxins. Since, Aflatoxins are found in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are optimum for growth of the molds and for production of the toxin. More than 300 to 400 classes of mycotoxins are identified, but the most significant and toxic group is aflatoxins. Aflatoxins have been considered as the most prevalent mycotoxins contaminating human food and animal feed (Bhat and Vashanti, 1999). They are highly toxic, mutagenic and carcinogenic compounds that have involved as a potential agent in human hepatic carcinogen (Wogan, 1999; 2004). Among aflatoxins, aflatoxin B1 (AFB1) is a natural toxin produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. AFB1 is a potent carcinogen, teratogenic and mutagenic and WHO-International Agency for Research on Cancer (IARC) have classified AFB1 as carcinogenic agent to humans as it is responsible for human primary hepatocellular carcinoma (IARC, 2002).

Aflatoxin M1 (AFM1) is the metabolite of AFB1 and has been reported that AFM1 was detected in milk within 12 to 24 h after the first ingestion of AFB1 (Asi et al., 2012). AFM1 is also classified as toxic like AFB1 and is resistant against heat, pasteurization and sterilization (Abdulrazzaq et al., 2003; Sadeghi et al., 2009). Aflatoxins are generally found in feed and foodstuffs, such as beans, cereals fruits and seeds. It has proven to be at least partly resistant to food processing methods meaning it is also present in derived products and thus, finds its way into humans. Contamination of foods and feeds by the aflatoxin-producing species *A. flavus* and *A. parasiticus* cannot be completely avoided and may lead to significant economic losses and health risks (Shane, 1994). As Thailand is in the tropical area, it is hard to avoid mold-contaminated food and feed. Therefore, mycotoxins production is unavoidable and depends on different environmental factors in the field or during storage. As a result, the high incidence rate of contamination of cereal grains and animal feed has been reported worldwide. It can be assumed that about 25% of food products, mainly from cereals, are substantially contaminated with aflatoxins (Magan et al., 2004). Mycotoxins are responsible for generating huge economic losses (Bhat and Vasanthi, 2003) and 25 to 40% of cereals consumed in the world are contaminated by these toxic compounds (Pitter, 1998). Due to this reason and their toxicity, the EU fixed the stringent maximum residue levels for total aflatoxins and AFB1 levels in human commodities to 4 and 2 µg/kg, respectively (Moss, 2002).

The Codex Alimentarius commission (Joint FAO/WHO) also adopted the total aflatoxins limit at 15 µg/kg in peanut (Codex, 2001), but the WHO prescribed the maximum limit for AFB1 in various food stuffs at 5 µg/kg (Papp et al., 2002). Owing to these adverse health effects of AFB1, a survey was conducted in various food products in Thailand. Thus, the main purpose of this study was to investigate the possible incident of AFB1 in food products by using a 96-well micro-titer plates ELISA test kit for AFB1 determination, and to compare the obtained levels to those set by the European commission.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, methanol, phosphate buffer saline (PBS) and phosphoric acid used, were of analytical grade and were purchased from Sigma (MO, USA) and Merck (Darmstadt, Germany). One milligram per milliliter of AFB1 standard was purchased from Supelco, USA, prepared as an aqueous stock and working solution in concentrations of 10,000 and 10 ng/ml, respectively, and stored at 4°C until analyses.

Sample collection

The 100 food samples were randomly purchased from different markets around Bangkok, Thailand during 2012 (November to December) and 2013 (February to April). They were divided into five groups: the first group was local alcoholic beverages, the second was imported blue cheese, the third was fermented soybean (pickled bean, curd soybean paste and salted soybean), the fourth was peanuts (raw peanuts with and without shells) and the last was peanut derived products (fried peanuts, roasted salted peanut, roasted ground peanuts, peanut sesame, crispy peanut cake, peanut cookies and etc.).

Aflatoxin B1 extraction

The extraction of AFB1 was carried out according to the AOAC method (AOAC, 1990) with a slight modification. Briefly, 20 g of each sample was mixed with 100 ml of 70% methanol and shaken for 300 rpm/10 min. After that, each sample was filtered through filter paper number 4 (Whatman International Ltd., Maid-stone, UK) and centrifuged. Then, the supernatant was taken to determine the aflatoxin B1 by using DOA-AFLATOXIN B1 ELISA test kit from Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives, Thailand. This test kit is the direct competitive ELISA with polyclonal antibody specific to AFB1 with the sensitivity of detection of 0.4 µg/kg.

ELISA determination

After the extraction and filtration as mentioned above, the sample was ready for the determination of AFB1 by following the test kit directions. The results were measured by micro ELISA Reader (Stat Fax 303 Plus, USA) at 450 nm. Briefly, AFB1 standard solutions, used for making the calibration curve, contained AFB1: 0, 4, 10, 20, 40, and 80 µg/l.

The samples were diluted with 200 µl of 0.01 M PBS-T and 50 µl of each diluted sample was dropped into a well of Micro ELISA plate which was coated with antibody and 50 µl of enzyme conjugate (AFB1-HRP) was added to the well afterward. The plate was allowed to stand in the dark for 30 min at room temperature. Thereafter, the wells were emptied by inverting the micro-plate upside down, tapped vigorously against absorbent paper and washed with 200 µl of 0.01 M PBS-T for 3 times. 100 µl of substrate (tetramethylbenzidine, TMB) was added to the wells and left in the

dark for 10 min at room temperature to produce yellow color. To stop the reaction, 100 µl of 0.5 M phosphoric acid was added. The intensity of the color is inversely proportional to the concentration of AFB1 in the sample or standard, which was measured at 450 nm. Each sample was analyzed in triplicates. The optical density of the standards from the standard curve, and the samples optical densities are plotted against the curve to calculate the exact concentration of AFB1.

Recovery detection

To test the sensitivity of the method, the AFB1 standard solution at different concentrations (2, 4, 10, 20 and 40 µg/kg) were added to the food samples suspected to contain less than 0.4 µg/kg of AFB1. Due to the fact that no certified sample was available for the recovery tests, the confirmation of recovery in various food samples was also done by spiking AFB1 at different concentrations (2, 4, 10, 20 and 40 µg/kg) into the food samples. The extraction of the spiked samples was done as the same as non-spiked samples which described above.

$$\% \text{ Recovery} = (A_s - B_n) \times 100 / C_{std}$$

A_s and B_n are the representative of the concentration of AFB1 in the food sample for spiked and non-spiked AFB1 standards, whereas C_{std} is the concentration of AFB1 standard.

The recovery tests were performed twice, and each instance included 5 replicates. Then, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated (Miller and Miller, 2000).

Statistical analysis

The data was analyzed using SPSS software IBM SPSS (PASW Statistics 19) and presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Food contamination with mycotoxins is a serious issue in developing countries that poses a significant health risks for humans as well as for animals. Mycotoxins are natural occurring toxin that cannot be 100% controlled. The presence of moulds in food stuffs could lead to the possible formation of mycotoxins, which is well known to be carcinogenic or gentoxic to both animals and humans' health and present a severe health hazard because these toxins are very stable even if passing through quite severe processes, especially AFB1, the most ubiquitous form and the most toxic (Moss, 1996; Creppy, 2002; Kamika and Takoy, 2011; Wild and Gong, 2010). Consequently, they can be a problem in processed foods and lead to health risk. Hence, the purpose of this study was to determine the concentration of AFB1 in various food product samples that are popular for Thai people. In this study, we assessed the natural occurrence of AFB1 in various food samples by using ELISA method because it has been shown to be reliable, simple, and it could be standardized for routine analysis of the mycotoxins present in food and feed materials. The LOD and LOQ estimated for the method were 0.36 and 1.2 µg/kg for local alcoholic beverages, 0.51 and 1.7 µg/kg for blue cheese, 0.33 and 1.1 µg/kg for fermented soybean, 0.36 and 1.2 µg/kg for raw peanuts and 0.45 and 1.5 µg/kg for peanut derived products, respectively (Table 1a). In our study, the average of % recovery rate in food samples (2 to 40 µg/kg) was 65.55 to 87.7% as shown in Table 1a and b. A total of 100 samples, consisting of 5 samples of imported blue cheese, 7 samples of local fermented alcoholic beverages, 18 samples of fermented soybean products, 30 samples of raw peanuts and 40 samples of peanut derived products, was analyzed to determine the amount of AFB1.

The results of the occurrence and level of AFB1 in the different food samples revealed that the rates of AFB1 contamination were 71.42, 100, 83.33, 86.67 and 90% for the alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived products, respectively (Table 2). The individual values, for each category sample, ranged from 0.3 to 2.15 µg/kg (average 0.48 µg/kg), 0.5 to 1.25 µg/kg (average 0.95 µg/kg), 0.2 to 3.2 µg/kg (average 1.54 µg/kg), 0.2 to 8.05 µg/kg (average 6.83 µg/kg) and 0.1 to 73.85 µg/kg (average 5.6 µg/kg) for alcoholic beverages, blue cheese, fermented soybean, raw peanuts and peanut derived products, respectively (Table 2). Our results showed that the concentration of AFB1 in the samples did not exceed the world accepted level (FAO, 2004; CAC, 2001) except raw peanuts and peanut derived products. The WHO prescribed the maximum limit for AFB1 in various food stuffs at 5 ug/kg (Papp et al., 2002). The limits of Aflatoxins (B1, G1, B2, G2) for human consumption were 15 µg/kg in raw peanuts and 10 µg/kgin processed peanuts (CAC, 2001). From the results, it can be concluded that because our country is in tropical area, the climate is suitable for fungal growth and peanuts are considered to be one of the most susceptible food materials for fungal growth and Aflatoxins productions. Several researchers have investigated peanuts for the presence of Aflatoxins, particularly AFB1 (Bankole et al., 2005; Barro et al., 2002; Mutegi et al., 2009). Iqbal et al. (2013) reported the level of AFB1 ranging from 2.4 to 12.3 µg/kg but Wagacha et al. (2013) reported AFB1 level in peanut products ranging from 0 to 1629 µg/kg.

Therefore, our study is in agreement with other previous studies in other countries on the natural occurrence of AFB1 in peanut samples. The contamination of aflatoxins in peanuts is related to climatic conditions (Nakai et al., 2008). It is known that the majority of food and feed products can allow the growth and development of toxic fungi during their processing, transport and storage (Frisvad and Samson, 1991). The ingestion of AFB1 by human occurs mainly through eating contaminated plant products and animal products (Smith et al., 1995). Keeping that in mind AFB1 can be found in a daily diet (nut, spices, dried fruits, wine, coffee, cereal, milk, cheese, meat, etc.) because they are able to contaminate a wide range of food commodities that are commonly consumed by all age groups including raw

Table 1a. Mean and %recovery test of AFB1 in spiked food samples using ELISA.

Type of food samples	LOD (µg/kg)	LOQ (µg/kg)	Spiked level (µg/kg)	Mean ± SD	Recovery (%)
Local alcoholic beverages	0.36	1.2	2	1.61±0.12	80.5
			4	3.04±0.22	75.9
			10	7.42±0.67	74.2
			20	16.65±0.84	82.8
			40	28.2±0.18	70.49
Blue cheese	0.51	1.7	2	1.58±0.17	79.1
			4	3.3±0.18	82.5
			10	7.57±0.24	75.7
			20	13.52±0.72	67.6
			40	28.23±0.67	70.58
Fermented soybean	0.33	1.1	2	1.51±0.11	75.4
			4	3.22±0.71	80.5
			10	8.08±0.45	80.8
			20	13.11±0.16	65.55
			40	28.05±0.58	70.12
Raw peanuts	0.36	1.2	2	1.75±0.12	87.7
			4	3.26±0.45	81.5
			10	7.47±0.37	74.7
			20	17.02±0.91	86.1
			40	32.59±0.27	81.48
Peanut derived products	0.45	1.5	2	1.65±0.15	82.6
			4	3.19±0.17	79.7
			10	8.59±0.18	85.9
			20	16.17±0.27	80.27
			40	31.8±0.98	79.5

7 replicates were done for 2 µg/kg samples, 3 replicates were done for 4, 10, 20 and 40 µg/kg samples (n = 3).

Table 1b. %recovery test of AFB1 in spiked food samples using ELISA.

Food sample	As (µg/kg)	Bn (µg/kg) (Mean±SD)	Cstd (µg/kg)	%Recovery (Mean±SD)
Local alcoholic beverages	3.65±0.51	1.9±0.12	2	85.5±8.41
Blue cheese	4.95±0.23	1.9±0.41	4	75.25±10.25
Fermented soybean	12.7±0.35	3.2±0.23	10	94.5±5.86
Raw peanuts	34.7±0.28	16.45±0.95	20	90.5±12.48
Peanut derived products	35±0.14	1.0±0.17	40	83.14±10.15

Values are mean±SD Limit of detection was 0.36. Five replicates were done. n=3, % Recovery= (As-Bn) x 100/Cstd, As and Bn are the representative of the concentration of AFB1 in the food samples that were spiked and not spiked with AFB1 standard, whereas Cstd is the concentration of AFB1 standard.

materials or cereal- based products, processed cereals and ready to eat foods such as snacks. So, total dietary AFB1 intake could likely be underestimated.

Conclusions

This study revealed the occurrence of AFB1 in various food products collected in Thailand. Approximately 87% of the food samples analyzed were positive but in most of the samples it was found that AFB1 levels did not exceed the maximum limit of 5 ug/kg prescribed by WHO except peanuts and peanut derived products and since they are used as an ingredient or snack, they must be considered for food safety. However, further research study should

Table 2. Occurrence and concentration of Aflatoxin B1 in food samples.

Food sample	Sample (n)	Positive sample	%	Min-Max (µg/kg)	Average, µg/kg (Mean±SD)
Local alcoholic beverages	7	5	71.42	0.3-2.15	0.48±0.71
Blue cheese	5	5	100	0.5-1.25	0.95±0.18
Fermented soybean	18	15	83.33	0.2-3.2	1.54± 0.25
Raw peanuts	30	26	86.67	0.2-8.05	6.83± 0.56
Peanut derived products	40	36	90	0.1-73.85	5.6± 0.12

Limits of detection was 0.36 ppb. Three replicates were tested. 1. The MRL of total Aflatoxins and AFB1in human foods are 4 and 2 µg/kg, respectively, (EU -regulation). 2. The total Aflatoxins of unprocessed peanut and ready to eat peanut are 15 and 10 µg/kg, respectively, (Codex, 2001). 3. WHO prescribed the maximum limit for AFB1 is 5 µg/kg in various foodstuffs.

be conducted by using more samples and using other methods such as HPLC together with immuno-affinity column to confirm results and reduce uncertain results so as to assess the risk of AFB1.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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