Estimated Daily Intake of Aflatoxin M_1 in Thailand

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1. Introduction

Aflatoxins are a group of mycotoxins produced by certain species of *Aspergillus*. These molds grow on a variety of food and feed commodities and produce aflatoxins under appropriate temperature and humidity (Jay et al., 2005). Aflatoxin B_1 (AFB_1) is the most potent hepatocarcinogen of this group of mycotoxins. Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1 and is secreted in the milk of mammals that have eaten contaminated foods. AFM_1 is also a hepatocarcinogen and is classified in Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2002). Exposure to AFM_1 through milk products is considered to be a serious public health problem. Several countries have established regulatory limits for AFM_1 in raw milk and milk products, which vary from country to country. According to the Food and Agriculture Organization of the United Nations (FAO), there are 60 countries that have established regulatory limits for AFM_1; the values vary from ND (not detectable) to 15 µg/L (FAO, 2004). The two most prevalent limits are 0.05 µg/L (34 countries) and 0.5 µg/L (22 countries). The European Community has set the maximum permitted level for AFM_1 in infant formulae and follow-on formulae, including infant milk and follow-on milk, at 0.025 µg/kg, and in raw milk and heat-treated milk at 0.05 µg/kg (European Commission, 2006). The U.S. regulatory limit for AFM_1 is 0.5 µg/L (FAO, 2004). However, several countries, including Thailand, have not yet established regulatory limits for AFM_1. The Notification of the Ministry of Public Health No. 265 – the law that regulates the quality of milk products in Thailand – only states that ''…*milk products may be contaminated with aflatoxins at a level that is not harmful to human health*'' (Ministry of Public Health, 2003).

A national food consumption survey was conducted in Thailand during the years 2002–2004; 18,746 participants were divided into five age groups (Groups 1–5): 0–3, >3–9, >9–19, >19–65, and >65 yr (National Bureau of Agricultural Commodity and Food Standards, 2006). The survey showed that the consumption amounts of four types of milk products – milk powder, school milk, commercial pasteurized milk, and UHT (ultra-high-temperature) milk – comprised approximately 93% of all milk products consumed by the Thai population

(National Bureau of Agricultural Commodity and Food Standards, 2006). In Thailand, in the morning on every school day, students in Grades 1–6 (age range, 6–12 yr) are served pasteurized milk (200 ml) provided by the School Milk Project (Ruangwises & Ruangwises, 2009). Thailand is administratively divided into 76 provinces; the 45 provinces with the highest population were selected for milk sample collection. The purposes of this study were to investigate whether the concentrations of AFM_1 in milk powder, school milk, commercial pasteurized milk, and UHT milk products consumed in Thailand are within the acceptable level for consumption, and to estimate the daily intake of AFM_1 for the Thai population.

2. Materials and methods

2.1 Chemicals

AFM_1 reference standard (from *Aspergillus flavus*), trifluoroacetic acid, methylene chloride, *n*-hexane, and silica gel 60 were purchased from Sigma-Aldrich (St. Louis, MO, USA). $AflaM_1$™ immunoaffinity columns were purchased from Vicam (Nixa, MO, USA). Spherisorb ODS-2 HPLC columns (5 µm, 4.6 × 250 mm) and C_{18} Sep-Pak columns were obtained from Waters Corporation (Milford, MA, USA). Solvents (HPLC grade) – acetonitrile, methanol, isopropyl alcohol, and water – were obtained from Merck (Darmstadt, Germany).

2.2 Milk sample collection and sample preparation

Milk powder samples were purchased from supermarkets (2 samples/province), while school milk samples were collected from 180 elementary schools (4 schools/province). Commercial pasteurized and UHT milk samples were purchased from supermarkets (2 samples each of pasteurized and UHT milk/province). All milk samples were collected between January 2007 and January 2008. Pasteurized milk samples were frozen at –20 °C until analysis (within one month from the manufacturing date). A total of 450 milk samples were analyzed in this study.

Milk powder sample (31.25 g) was reconstituted in 200 ml of distilled water in a 250-ml volumetric flask, mixed well, and adjusted to 250 ml with distilled water (dilution 1: 8). Concentrations of fat, protein, and solid-not-fat (SNF) in reconstituted milk powder samples, analyzed using a MilkoScan 133B (Foss Electric, Hillerød, Denmark), were 4.12 ± 0.36, 3.21 ± 0.08, and 8.59 ± 0.09 g/100 ml (n = 30), respectively. These milk compositions conformed with the Notification of the Ministry of Public Health No. 265, which states that fluid milk with full butter fat must contain fat, protein, and SNF of at least 3.2, 2.8, and 8.25 g/100 ml, respectively (Ministry of Public Health, 2003). The densities of reconstituted and liquid milk samples were determined using 50 ml of milk sample.

2.3 Extraction and determination of Aflatoxin M_1

AFM_1 was extracted from milk samples using an $AflaM_1$™ immunoaffinity column. The extraction procedure was according to the manufacturer's recommendations, as previously described by Ruangwises & Ruangwises (2009). In brief, an aliquot of 50 ml of reconstituted milk powder or liquid milk sample was transferred to a 50-ml plastic centrifuge tube and defatted by centrifugation at 3,500 *g* for 20 min. After the fat was separated, the resulting skimmed milk was transferred into a 50-ml plastic syringe which was attached to an immunoaffinity column. The skimmed milk was allowed to flow into the column by gravity at a rate of 1 ml/min. The column was then washed with 20 ml of water. AFM_1 was eluted with 1.25 ml of acetonitrile: methanol (3: 2), followed by 1.25 ml of HPLC water. A total volume of 2.5 ml of eluate was filtered through a nylon filter (0.45 μm) and used for analysis of AFM_1 using HPLC. All milk samples were analyzed in duplicate.

The complete chromatographic system (Class-LC10; Shimadzu, Kyoto, Japan) consisted of a HPLC pump (model LC-10AD), an auto injector (model SIL-10A), a column oven (model CTO-10A), and a fluorescence detector (model RF-10AXL). The HPLC conditions for analysis of AFM_1 were as follows: column – Spherisorb ODS-2; column temperature – 40°C; mobile phase – water: methanol: acetonitrile (57: 23: 20); flow rate – 1 ml/min; detector – fluorescence spectrophotometer (excitation 360 nm; emission 440 nm).

The AOAC Official Method 986.16 for detection of aflatoxins M_1 and M_2 in fluid milk (Cunniff, 1995) was performed to confirm the AFM_1 analysis using the immunoaffinity column. In brief, an aliquot of fluid milk or reconstituted milk powder sample (20 ml) was mixed with 20 ml of hot water (80°C), and AFM_1 was extracted using a C_{18} Sep-Pak column. AFM_1 was eluted from the column with ether. The ether phase was then cleaned up using a silica gel 60 mini-column; AFM_1 was eluted with a mixture of methylene chloride: ethanol (95: 5). The eluate was evaporated to dryness under nitrogen gas; the residue was then dissolved in 0.2 ml of *n*-hexane. To convert AFM_1 to AFM_{2a}, which has a higher extinction coefficient, the solution was derivatized with 0.2 ml of trifluoroacetic acid at 40°C and then evaporated to dryness under nitrogen gas. The residue was dissolved in 2 ml of water: acetonitrile (3: 1). The final solution was filtered through a nylon filter (0.45 μm) and used for analysis of AFM_1 by HPLC. The HPLC conditions for analysis of AFM_{2a} were as follows: column – Spherisorb ODS-2 (5 μm, 4.6 × 250 mm); mobile phase – water: acetonitrile: isopropanol (80: 12: 8); flow rate – 1 ml/min; detector – fluorescence spectrophotometer (excitation 365 nm; emission 455 nm).

A combination of the two methods was used to confirm the quantification of AFM_1 extracted using the $AflaM_1$™ immunoaffinity column. The eluate (2.5 ml) from the immunoaffinity column was evaporated under nitrogen gas to dryness. The residue was dissolved in 0.2 ml of *n*-hexane and was quantified as described in the AOAC Official Method 986.16, with the addition of 0.2 ml of trifluoroacetic acid.

2.4 Determination of limit of quantification

The limit of quantification (LOQ) for AFM_1 was determined using the Q2B method of the U.S. Food and Drug Administration (U.S. FDA, 1996). Milk samples (50 ml) were fortified with standard AFM_1 at concentrations of 0.0125, 0.025, 0.05, 0.25, and 0.5 μg/L; blank samples were not fortified with standard AFM_1. Concentrations of AFM_1 in fortified milk samples and blank samples were quantified as described in Extraction and Determintion of AFM_1 using the $AflaM_1$™ immunoaffinity column. Calibration curves (n = 12) were obtained by least-square linear regression analysis of the residual peak heights versus fortified AFM_1 concentrations. The calculation for LOQ was based on the standard deviation of *y*-intercepts of linear regression analysis (σ) and the slope (S) using the equation LOQ = 10 σ/S. The LOQ of the method was 0.01 μg/L and the overall recovery across the five concentrations of fortified AFM_1 was 85.6%. The precision of the method, expressed as %CV (coefficient of variation), ranged from 2.8 to 5.6%, as previously discussed by Ruangwises & Ruangwises (2009).

2.5 Statistical analysis

A randomized block experiment was used to assess the differences between AFM_1 concentrations. Duncan's multiple comparison test was applied to obtain significance levels between the four types of milk samples ($P < 0.05$). SPSS Statistics version 17.0 for Windows was used for statistical analysis.

3. Results and discussion

Concentrations of AFM_1 extracted using an immunoaffinity column with HPLC quantification were comparable to those analyzed using AOAC Official Method 986.16, and also comparable to those obtained from a combination of the two methods (extraction with an immunoaffinity column, derivatization with trifluoroacetic acid, and quantification with HPLC). Comparative results for AFM_1 concentrations in liquid milk and milk powder samples obtained from the three procedures are presented in Table 1. In this study, the average density of reconstituted milk powder and liquid milk samples was 1.03 ± 0.027 g/ml (n = 90), which was used for unit conversion. Concentrations and incidence of AFM_1 in 450 milk samples are presented in Table 2. Of the 450 samples, 288 (64.0%) were found to be contaminated with AFM_1 equal to or above the LOQ of 0.01 μg/L. The incidence of AFM_1 in milk powder, school milk, commercial pasteurized milk, and UHT milk samples was 21.1% (19/90), 71.1% (128/180), 78.9% (71/90), and 77.8% (70/90), respectively. Average concentrations of AFM_1 found in the four types of milk samples were 0.004 ± 0.009, 0.035 ± 0.028, 0.048 ± 0.034, and 0.045 ± 0.034 μg/L, respectively. In this study, statistical analysis showed that the average concentration of AFM_1 in milk powder samples was significant lower than those found in the other three milk products. The average concentration of AFM_1 in school milk samples was significantly lower than those found in the commercial pasteurized and UHT milk samples. Of the 19 positive milk powder samples, only 2 samples were contaminated with AFM_1 above the EU limit for infant milk products of 0.025 μg/kg. For school milk, commercial pasteurized milk, and UHT milk samples, 68/180 (37.8%), 25/90 (27.8%), and 29/90 (32.2%) samples, respectively, were contaminated with AFM_1 within the EU limit of 0.05 μg/kg. Concentrations of AFM_1 found in all milk samples were within the U.S. regulatory limit of 0.5 μg/kg.

Sample	AFM_1 concentration (μg/L) *		
	AOAC Official Method 986.16	Immunoaffinity column + TFA	Immunoaffinity column
Milk Powder (dilution 1:8)			
Sample A	0.021	0.024	0.026
Sample A + 0.05 μg/L AFM_1	0.027	0.028	0.033
Sample A + 0.1 μg/L AFM_1	0.119	0.120	0.125
Sample A + 0.25 μg/L AFM_1	0.291	0.302	0.299
Sample B	0.046	0.047	0.042
Sample C	0.031	0.040	0.038
Sample D	0.022	0.028	0.023
Liquid Milk			
Sample 1	0.035	0.032	0.033
Sample 1 + 0.05 μg/L AFM_1	0.079	0.078	0.076
Sample 1 + 0.1 μg/L AFM_1	0.127	0.122	0.129
Sample 1 + 0.25 μg/L AFM_1	0.246	0.247	0.244
Sample 2	0.062	0.060	0.065
Sample 3	0.040	0.043	0.039
Sample 4	0.089	0.085	0.091

*All samples were analyzed in duplicate.

Table 1. AFM_1 concentrations in milk powder and liquid milk samples obtained from three procedures

Sample	Samples Analyzed	Positive (%)	AFM_1 concentration (µg/kg)		AFM_1 incidence[1]			
			Mean ± SD	Range[2]	0.010- 0.050 µg/kg	0.051- 0.075 µg/kg	0.076- 0.100 µg/kg	≥ 0.101 µg/kg
Milk powder	90	19 (21.1)[3]	0.004± 0.009[4] A[5]	0.011-0.048	19 (21.1)	-	-	-
Liquid milk								
Pasteurized school milk	180	128 (71.1)	0.035±0.028 B	0.012-0.104	68 (37.8)	50 (27.8)	7 (3.9)	3 (1.7)
Pasteurized commercial	90	71 (78.9)	0.048± 0.034 C	0.014-0.109	25 (27.8)	29 (32.2)	14 (15.6)	3 (3.3)
UHT commercial	90	70 (77.8)	0.045± 0.034 C	0.012-0.112	29 (32.2)	26 (28.9)	11 (12.2)	4 (4.4)
Overall	450	288 (64.0)	0.034± 0.031	0.011-0.112	141 (31.3)	105 (23.3)	32 (7.1)	10 (2.2)

[1]AFM_1 incidence of the positive samples
[2]Ranges of AFM_1 concentrations of the positive samples
[3]Numbers in parentheses are percentages of each milk product
[4]Concentrations of AFM_1 in reconstituted milk powder samples (1: 8)
[5]Different letters (A, B, C) denote significant differences between means of each milk product ($P < 0.05$)

Table 2. Concentrations and incidence of AFM_1 in milk powder and liquid milk samples

Since each of the five Thai population groups consumed different types of milk products, calculation of the daily AFM1 intake for each population group was based on the mean AFM1 concentrations in the corresponding milk products. The mean daily AFM1 intake by each group was calculated as follows: mean AFM1 intake (ng AFM1/day) = (mean daily milk intake, kg/day) x (mean AFM1 concentration in the corresponding milk products, µg/kg) x 1,000 (a unit conversion factor, ng/µg). The mean consumption amounts of milk products for the five Thai population groups were 0.429, 0.220, 0.138, 0.059, and 0.036 kg/day, respectively, while the average body weights were 10.1, 20.0, 46.2, 59.4, and 54.5 kg, respectively (National Bureau of Agricultural Commodity and Food Standards, 2006).

For Group 1 (age range, 0–3 yr), a mean AFM1 concentration in milk powder samples of 0.004 µg/kg (n = 90) was used for the calculation of daily AFM1 intake. A mean AFM1 concentration of 0.024 µg/kg in milk powder and school milk samples (n = 270) was used for the calculation of daily AFM1 intake for Group 2 (>3–9 yr), while a mean AFM1 concentration of 0.040 µg/kg in school milk, commercial pasteurized milk, and UHT milk samples (n = 360) was used for Group 3 (>9–19 yr). As school milk is consumed by students in Grades 1–6 (6–12 yr), concentrations of AFM1 in school milk samples were used for both Groups 2 and 3. For Group 4 (>19–65 yr) and Group 5 (>65 yr), a mean AFM1 concentration of 0.045 µg/kg in commercial pasteurized and UHT milk samples (n = 180) was used for the calculation of daily AFM1 intake. The estimated daily intakes of AFM1 for the five Thai population groups were 1.63, 5.29, 5.50, 2.63, and 1.62 ng/day, respectively. The estimated daily intakes of AFM1 with respect to body weight for the five population groups were 0.16, 0.26, 0.12, 0.04, and 0.03 ng/kg BW/day, respectively.

Daily intakes of AFM_1 in various regions and countries are presented in Table 3. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) calculated that the daily AFM_1 intakes in Africa, the Middle East, Latin America, Europe, and the Far East (using the mean AFM_1 concentrations in four different milk products) were 0.002, 0.005, 0.022, 0.023, and 0.36 µg/kg, respectively (JECFA, 2001). The amounts of milk products consumed in the five regions were 0.042, 0.12, 0.16, 0.29, and 0.032 kg/day, respectively. The estimated intakes of AFM_1 in the five regions were 0.1, 0.6, 3.5, 6.8, and 12 ng/day, respectively. When AFM_1 intakes were calculated with respect to body weight (assuming 60 kg), the estimated daily intakes of AFM_1 were 0.002, 0.1, 0.058, 0.11, and 0.20 ng/kg BW/day, respectively (JECFA, 2001).

Region/Country		Milk consumption[1] (kg/day)	AFM_1 concentration (µg/kg)	Daily AFM_1 intake ng/day	Daily AFM_1 intake ng/kg BW/day	Reference
Africa		0.042	0.002	0.1	0.002	JECFA (2001)
Middle East		0.12	0.005	0.6	0.1	JECFA (2001)
Latin America		0.16	0.022	3.5	0.058	JECFA (2001)
Europe		0.29	0.023	6.8	0.11	JECFA (2001)
Far East		0.032	0.36	12	0.20	JECFA (2001)
France	age 3-14 yr	0.312	0.005-0.05[2]	NA[3]	0.22	Leblanc et al. (2005)
	age >15 yr	0.229	0.005-0.05[2]	NA[3]	0.09	
Brazil	children	0.360[4]	0.061	23.92	1.04[5]	Shundo et al. (2009)
	adults	0.412[4]	0.031	11.28	0.188[5]	
Spain	age 4-9 yr	0.532	0.00969[6]	2.63	0.21	Cano-Sancho et al. (2010)
	age 10-19 yr	0.404	0.00969	2.01	0.07	
	age 20-65 yr	0.305	0.00969	1.44	0.04	
	age >65yr	0.407	0.00969	1.94	0.05	
Thailand	age 0-3 yr	0.429	0.004[7]	1.63	0.16	This study
	age >3- 9yr	0.220	0.024[7]	5.29	0.26	
	age >9-19 yr	0.138	0.040[7]	5.50	0.12	
	age >19-65 yr	0.059	0.045[7]	2.63	0.04	
	age >65yr	0.036	0.045[7]	1.62	0.03	
	Overall	0.176	0.032	3.33	0.12	

[1]Average milk consumption for each region or country
[2]AFM_1 concentrations in five milk products which were used for the calculation of AFM_1 intake for each population group (see text for details)
[3]NA = Data not available
[4]The values are average milk consumption amounts for both sexes calculated from milk consumption amounts by males and females in each of four population groups presented in the report
[5]Daily AFM_1 intakes were a summation of individual AFM_1 intakes from five milk products
[6]A single value of average AFM_1 concentration in three milk products was used for the calculation of daily AFM_1 intake for all four Spanish population groups
[7]Average AFM_1 concentrations in corresponding milk products for each Thai population group were used for the calculation of daily AFM_1 intakes (see text for explanation)

Table 3. Daily AFM_1 intakes in various regions/countries

Leblanc et al. (2005) used individual means of AFM_1 concentrations of five milk products – butter (0.05 μg/kg), desserts (0.05 μg/kg), cheeses (0.05 μg/kg), milk (0.005 μg/kg), and ultra-fresh dairy products (0.047 μg/kg) – and daily milk consumption to calculate daily AFM_1 intakes for two French population groups: children (3–14 yr) and adults (≥ 15 yr). Daily intakes of AFM_1 for each population group were the summation of daily AFM_1 intakes from the five milk products; the estimated AFM_1 intakes by French children and adults were 0.22 and 0.09 ng/kg BW/day, respectively. Shundo et al. (2009) used AFM_1 concentrations in different milk products to estimate daily AFM_1 intake by Brazilian children and adults in the city of Sao Paulo. Based on the mean AFM_1 concentration in powder milk samples (61 ng/kg) collected from municipal day-care centers and elementary schools, an average milk consumption of 0.412 kg/day, and a body weight of 23 kg, the estimated daily AFM_1 intake for children was 1.04 ng/kg BW. For adults, the estimated AFM_1 intake was 0.188 ng/kg BW/day, which was calculated using the mean AFM_1 concentrations in milk powder and fluid milk samples purchased from supermarkets (31 ng/kg), a daily milk consumption of 0.361 kg, and a body weight of 60 kg.

Cano-Sancho et al. (2010) used an average AFM_1 concentration of 9.69 ± 2.07 ng/kg found in three milk products (UHT milk, cheese, and yogurt samples) to estimate daily AFM_1 intake by four population groups in Catalonia, Spain. Average milk consumption for each of the four population groups – children (4–9 yr), teenagers (10–19 yr), adults (20–65 yr), and elderly (>65 yr) – was 0.532, 0.404, 0.305, and 0.407 kg/day, respectively; while the body weights were 26.2, 54.1, 73.7, and 73.3 kg, respectively. The estimated AFM_1 intakes for the four Spanish population groups were 0.21, 0.07, 0.04, and 0.05 ng/kg BW/day, respectively.

This study showed that 288 (64.0%) of the 450 milk samples collected from 45 provinces of Thailand were contaminated with AFM_1 equal to or more than the LOQ of 0.01 μg/L. Daily intakes of AFM_1 in five Thai population groups, calculated using corresponding AFM_1 concentrations in milk products consumed by each population group, were comparable to those of other regions and countries. Thai children (3–9 yr) had the highest AFM_1 intake, 0.26 ng/kg BW/day, which was comparable to those in the Far East (0.20 ng/kg BW/day), France (3–14 yr; 0.22 ng/kg BW/day), Brazil (adults, 0.188 ng/kg BW/day), and Spain (4–9 yr, 0.21 ng/kg BW/day). Thailand is one of several countries that have not yet established regulatory limits for AFM_1 in raw milk and milk products. The present study and our two previous reports (Ruangwises & Ruangwises, 2009, 2010) suggest regular monitoring of raw milk and milk products, and regulatory limits for AFM_1 to ensure the quality of raw milk and milk products in Thailand.

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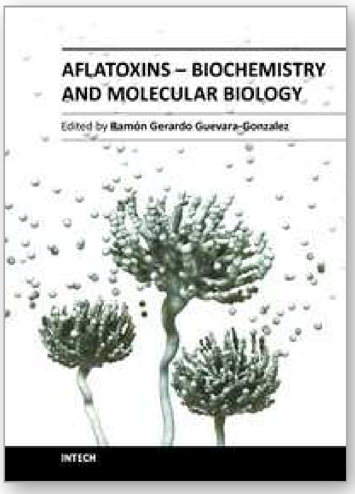

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Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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