Occurrence of aflatoxin M₁ in commercial powdered milk in Bangkok, Thailand

Manita Soontornjanagit¹, Osamu Kawamura¹

¹Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795, Japan

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Abstract
The contamination of milk with aflatoxin M₁ (AFM₁), a possible carcinogen to humans, is a serious problem. In Thailand, there are reports on AFM₁ contamination in powdered milk. In this study, we investigated the occurrence of AFM₁ in 79 commercial powdered milk products in Bangkok, Thailand over five years (2010–2014). An in-house immunoaffinity column-HPLC (IAC-HPLC) method was developed. Reconstituted powdered milk (10 mL) was applied to our IAC. The recoveries from 0.005–0.15 ng/mL AFM₁-spiked reconstituted milk were 73.6–86.3% with 5.2–7.4% of RSD. Twelve samples (15%) were contaminated with 0.005–0.135 ng/mL of AFM₁ (average 0.024 ng/mL, overall average 0.004 ng/mL) in reconstituted powdered milk. Our data indicated that the risk of AFM₁ in commercial powdered milk in Bangkok, Thailand was sufficiently low.

Aflatoxins, classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC), are produced principally by the fungi Aspergillus flavus and Aspergillus parasiticus, which infect food crops such as corn, peanuts, cottonseed and animal feed¹. Mammals that ingest aflatoxin B₁ (AFB₁), which is the most potent aflatoxin, from contaminated feed transfer its 4-hydroxylated metabolite known as aflatoxin M₁ (AFM₁) into milk². The occurrence of AFM₁, which is a possible carcinogen (Group 2B) classified by the IARC¹, in milk and milk products is considered a potential health risk to humans³. To decrease the risk, regulated limits of aflatoxins in dairy feed and AFM₁ in milk and milk products have been established in many countries. In the United States, the total aflatoxin limit in dairy cattle feed is 20 µg/kg⁴. In Thailand, the total aflatoxin limit in dairy feed is 200 µg/kg⁵. The regulated limit for AFM₁ is 0.05 µg/kg and 0.025 µg/kg in milk and infant milk, respectively, in the European Union⁵. The Codex Alimentarius Commission established a limit of 0.5 µg/kg for AFM₁ in milk⁶. However, Thailand has not yet established regulations for AFM₁ in milk and milk products⁷.

In Thailand, there were reports of AFB₁ in dairy feed in 2009. Of 90 dairy feed samples from western Thailand, 19 (21%) were contaminated with AFB₁ with an average of 13.9 µg/kg, and 3 samples (3.3%) exceeded the United States regulatory limit of 20 µg/kg⁸. All 131 dairy feed samples from southern Thailand were contaminated with AFB₁ with an average of 18.7 µg/kg⁹. Bantaokul reported that of 60 dairy feed samples from central Thailand, 50 (83%) were contaminated with AFB₁ with an average of 16.0 µg/kg, and 18 (30%) of the 60 samples exceeded 20 µg/kg (unpublished data). None of the dairy feed samples exceeded 200 µg/kg, which is the regulatory limit for cattle feed in Thailand. However, the data indicated that the level of AFB₁ contamination in dairy feed in Thailand was still very high.

There are a few reports on AFM₁ in liquid milk in Thailand. Ruangwises and Ruangwises¹⁰ reported that all 240 raw milk samples they tested were contaminated with AFM₁ with an average of 0.07 µg/kg, and 155 (65%) milk samples exceeded 0.05 µg/kg, which is the European Union regulatory limit. Ruangwises et al.¹¹ reported that 269 (75%) out of 360 commercial and school milk samples were contaminated with AFM₁ with an overall average of 0.041 µg/kg, and 147 milk samples (41%) exceeded 0.05 µg/kg.

There are a few reports on the occurrence of AFM₁ in powdered milk in Thailand. In 13 imported powdered milk products, 2 (15%) were contaminated with not more than 0.05 µg/L of AFM₁ in reconstituted milk¹². There is only one published paper on domestic powdered milk in Thailand. Ruangwises et al. analyzed 90 samples of powdered milk from 45 provinces in Thailand in 2011. Of the 90 samples, 19 (21%) contained 0.001 to 0.006 µg/L of AFM₁ in reconstituted milk.
milk with an overall average of 0.004 μg/L \(^1\). To clarify AFM\(_1\) contamination levels in commercial powdered milk in Thailand, we developed an in-house immunoaffinity column-HPLC (IAC-HPLC) method for AFM\(_1\) in powdered milk. Using this method, we analyzed samples of 79 commercial powdered milk marketed in Bangkok over five years from 2010 to 2014.

Seventy-nine commercial powdered milk products produced by 7 different companies (18 different brands) were purchased from conventional supermarkets and stores in Bangkok, Thailand, during the five years between 2010 and 2014. As a standard for AFM\(_1\), we used AFM\(_1\) solution (0.5 μg/mL acetonitrile solution) from Wako Pure Chemicals, Osaka, Japan. The AFM\(_1\) solution was diluted in steps with acetonitrile and the mobile phase of HPLC. Gibco Hybridoma-Serum Free Media (5 L). The culture supernatant of AM.3 hybridoma was collected and purified using the protein G column. The purified antibody was coupled to Affi-Gel 10 in the ratio of 5 mg of antibody per 1 mL of the gel, according to the manufacturer’s instructions. The gel-coupled AM.3 antibody was stored at 4°C in PBS containing 0.1% sodium azide until use. Gel (0.3 mL) was packed into a mini column (Muromac column size S; Muromachi Technos Co., Ltd., Tokyo, Japan).

The IAC was conditioned by passing through 10 mL of PBS. Sample solution (10 mL) was applied to the IAC at a flow rate of less than 1 mL/minute. The column was washed with 4 mL of PBS and then 4 mL of water. For elution of AFM\(_1\) from the IAC, we performed the following operation. The column was added to 3 mL of acetonitrile and closed the cap, and reversed 15 times of top and bottom upside down and leaved for two minutes, and then acetonitrile was eluted from the column. We repeated five times of these operations. The eluate was pooled and evaporated to dryness using a centrifugal evaporator at 40°C. The residue was dissolved in 1 mL of HPLC mobile phase, and analyzed using HPLC.

We used a Shimadzu (Kyoto, Japan) HPLC system with an LC-20AD pump, DGU-20A3 degasser, SIL-20AHT autosampler, CTO-10A column oven, and RF-20AXS fluorescence detector. A Shim-pack XR-ODS (2.2 μm particle size, 3.0 × 100 mm; Shimadzu, Kyoto, Japan) was used. The column oven was set to 50°C. The mobile phase was acetonitrile:methanol:water (10+10+80, v/v/v) as a mobile phase. The remnant of the HPLC sample (0.7 mL) was evaporated to dryness in an excitation wavelength of 360 nm and emission wavelength of 435 nm. Four concentrations of AFM\(_1\) standards (0.0125, 0.05, 0.10 and 0.25 ng/mL) were prepared to determine linearity. The calibration curve was linear with a determination coefficient (R\(^2\)) higher than 0.999, which revealed good correlation. The limit of (LOD), defined as a signal/noise (S/N) ratio of 3, was 0.0015 ng/mL. The limit of quantification (LOQ), defined as a S/N ratio of 10, was 0.005 ng/mL.

To confirm AFM\(_1\) in the powdered milk, trifluoroacetic acid (TFA) was used to change AFM\(_1\) to AFM\(_{2a}\). The remnant of the HPLC sample (0.7 mL) was evaporated to dryness and dissolved in 0.20 mL of n-hexane and 0.05 mL of TFA, and heated at 40°C for 20 minutes. After drying, the residue was redissolved in 0.7 mL of mobile phase and analyzed by HPLC using acetonitrile: methanol:water (10+10+80, v/v/v) as a mobile phase.

AFM\(_1\) solution (300 μL) in acetonitrile was spiked in 30 mL of reconstituted powdered milk. The final concentrations of AFM\(_1\) in the reconstituted milk were 0.005, 0.025, 0.05 and 0.15 ng/mL. Table 1 shows the results of the recoveries of AFM\(_1\) from spiked reconsti-
tuted powdered milk using our in-house IAC-HPLC method. The average recoveries were in the range 73.6 to 86.3% for spiking levels ranging from 0.005 to 0.15 ng/mL. Adequate recoveries were obtained with 60 to 120% at 0.01 to 0.05 ng/mL and 70 to 110% at more than 0.05 ng/mL. The precision of our method was also high, as estimated by relative standard deviations (RSD) of the recovery for AFM$_1$, which ranged between 5.2 and 7.4%. Therefore, our method is a reliable method for determining AFM$_1$ in powdered milk. HPLC chromatograms of the AFM$_1$ standard solution and the extract from non-spiked powdered milk, and spiked AFM$_1$ (0.05 ng/mL) in reconstituted powdered milk are shown in Fig. 1 A, B, and C. In the chromatograms of the extract from non-spiked powdered milk, there are few interference peaks around five minutes, which was the retention time of AFM$_1$ in Fig. 1 A and B, and there is no peak that overlaps AFM$_1$ in Fig. 1 C.

The results of the AFM$_1$ analysis of 79 powdered milk samples over five years (2010 to 2014) showed that 12 samples (15%) were contaminated with AFM$_1$ (Supplementary Table 1). The concentrations of AFM$_1$ in the powdered milk are the means of three estimates. A typical chromatogram is shown in Fig. 1 D. Contamination in all the samples was confirmed by TFA by converting AFM$_1$ to AFM$_{2a}$. After treatment with TFA, the AFM$_1$ peak disappeared and the AFM$_{2a}$ peak appeared. Four samples of powdered milk were contaminated at levels between the values of LOD and LOQ, while in the other contaminated samples, the range of AFM$_1$ was 0.005–0.135 ng/mL. The powdered milks of non-specified formula were highly contaminated with 0.066 and 0.135 ng/mL of AFM$_1$ (Supplementary Table 3). Because the density of these reconstituted powdered milks was approximately 1.03 g/mL, the concentration of AFM$_1$ in these samples were 0.064 and 0.131 ng/g, respectively. Two samples (2.5%) exceeded the European Union regulatory limit of 0.05 ng/g; however, none of the samples exceeded the Codex proposed maximum level for AFM$_1$ of 0.5 ng/g. The average was calculated as follows: LOD was 0 ng/mL and the samples contaminated between LOD and LOQ were 0.0025 ng/mL, which was 1/2 of LOQ. The mean of the positive samples was 0.024 ng/mL. The overall average was 0.004 ± 0.017 ng/mL. In a comparison by year, the overall average for 2010, 2011, 2012, 2013 and 2014 was 0.001, 0.007, 0.000, 0.007, and 0.004 ng/mL, respectively, and the positive ratio was 11%, 10%, 5%, 10%, and 60%, respectively. The reason for the high positive ratio in 2014 was that we selectively collected the powdered milk from potentially contaminated brands. However, the overall average in 2014 was not much different from the other four years. Therefore, our data indicated that there was no significant difference in the contaminated level of AFM$_1$ of powdered milk products in Bangkok over five years.

Powdered milk from 4 of 7 companies was contaminated with AFM$_1$, but products from other companies were not contaminated (Supplementary Table 1). Powdered milk from company F (a Thai company) was contaminated with AFM$_1$ in all 4 samples in 2010, 2011, 2013 and 2014 with an average of 0.059 ng/mL. Company F produced their products from raw milk from Thai dairy farmers. Two powdered milk products were...
contaminated with 0.066 and 0.135 ng/mL of AFM$_1$, which were higher than the European Union regulatory limit (0.05 ng/g). Powdered milk from company D (a Swiss company) was contaminated with AFM$_1$ in 5 of 15 samples (33%) with a mean of the positive samples of 0.008 ng/mL. 13% of powdered milk from company G (an American company, 16 samples) and 7% from company C (an international company, 15 samples) were contaminated with AFM$_1$ at levels below LOQ. However, no samples from company A (American company, 8 samples), B (international company, 16 samples) and E (Thai importing company, 5 samples) were contaminated with AFM$_1$.

The incidence of AFM$_1$ in powdered milk manufactured in different countries showed that of 34 samples of powdered milk made in Thailand, 9 (26%) were contaminated with AFM$_1$ with an average of 0.031 ng/mL (Supplementary Table 2). Of the powdered milk manufactured in Singapore (16 samples) and the Philippines (9 samples), 2 (13%) and 1 (11%), respectively, were contaminated with AFM$_1$ at levels below LOQ. However, none of the products manufactured in Malaysia (4 samples), Australia (5 samples) and European countries (Denmark, Germany, Ireland, Netherland and Spain, total 11 samples) was contaminated with AFM$_1$. These data indicated that the powdered milk made in Thailand was more highly contaminated than the powdered milk from other countries. The investigation of AFB$_1$ in cattle feed in Thailand showed that 21 to 83% was contaminated with AFB$_1$ with an average of 13.9 to 18.7 µg/kg (unpublished data). From 3.3 to 30% of these cattle feeds exceeded the United States regulatory limit (20 µg/kg), but none of the feeds exceeded the Thai regulatory limit (200 µg/kg). The concentration of AFM$_1$ in milk was on average 1–2% of the amount of AFB$_1$. The average of AFB$_1$ contamination in dairy feed in Thailand was 13.9 to 18.7 µg/kg. Accordingly, we could estimate that the average concentration of AFM$_1$ in cow milk was 0.139 to 0.374 ng/mL. The sample with the highest level of contamination in this study was 0.135 ng/mL of AFM$_1$, for powdered milk manufactured in Thailand. The estimated concentration of AFM$_1$ agreed with the actual concentration of AFM$_1$. Therefore, the result clearly suggested that the contamination level of AFB$_1$ in dairy feed affected the concentration of AFM$_1$ contamination in powdered milk. For 200 µg/kg of AFB$_1$ contamination in dairy cattle feed, 2–4 ng/mL of AFM$_1$ will be found in the cow milk. This AFM$_1$ level is 4–8 times higher than the limit set by the Codex Alimentarius Commission. We believe that the Thai regulatory limit is too high to ensure safe levels of AFM$_1$ in Thai milk. Based on these results, we think it is necessary to set a lower regulatory limit for AFB$_1$ in dairy feed in Thailand to reduce the risk of AFM$_1$ in milk and powdered milk manufactured in Thailand.

The incidence of AFM$_1$ in different types of powdered milk showed that none of the 29 samples of infant formula was contaminated with AFM$_1$ (Supplementary Table 3). Of 24 samples of the follow-on formula, 3 (13%) were contaminated with AFM$_1$ at levels below LOQ. In 22 samples of children to adults formula, 5 (23%) was contaminated with AFM$_1$ with an average of 0.008 ng/mL. All 4 samples of non-specified formula were contaminated with AFM$_1$ with an average of 0.059 ng/mL. The levels of AFM$_1$ in all samples of infant and follow-on formula were below the maximum limit of 0.025 ng/g set by the European Union for infants. Our results suggested that there is an increasing occurrence of AFM$_1$ in samples specified for higher-age children. However, the infant formula was totally free of AFM$_1$. Whey proteins are added to processed infant formula from dairy milk, which normally has a casein/whey protein ratio of about 80:20 (w/w) to achieve a ratio close to that of human milk of 40:60 (w/w). AFM$_1$ in milk is principally associated with caseins. Our data agreed with a study from Spain, which reported that the greater processing in premature and starter formula, versus follow-on formula, has a major role in the occurrence of AFM$_1$.

The incidence and average concentration of AFM$_1$ in powdered milk found in this study were almost similar to a previous report that describes a one-year study of powdered milk collected from 45 provinces in Thailand. Ruangwises et al. reported that 19 (21%) of 90 samples were contaminated with 0.001 to 0.006 ng/mL of AFM$_1$ in reconstituted powdered milk with an overall mean of 0.004 ± 0.009 ng/mL in 2011. In a recent study from Japan (2014), 36 of 108 (33.3%) samples of powdered formula were found to be contaminated with AFM$_1$ with an overall mean of 0.002 ng/mL in the range 0.003 to 0.025 ng/mL, and LOD was 0.003 ng/mL. The mean of the positive samples was 0.006 ng/mL. In another study from Spain (2010), 8 of 69 (11.6%) infant formula samples were contaminated with AFM$_1$ with a mean concentration of 0.001 ng/mL in the range 0.001 to 0.012 ng/mL, and LOD was 0.0018 ng/mL. The mean of the positive samples was 0.003 ng/mL. In India (2004), there was a high occurrence of AFM$_1$ contamination in 17 of 18 (94.4%) infant formula samples with an overall mean of 0.308 ng/mL in the range 0.143 to 0.770 ng/mL. The mean of the positive samples was 0.326 ng/mL. The overall average of AFM$_1$ in powdered milk in our samples was twice that in Japan and four times higher than in Spain, but was 77 times lower than that in India. Therefore, owing to the fact that only 2 samples exceeded the European Union regulatory limit (0.05 ng/g), but did not exceed the Codex Alimentarius Commission regulatory limit (0.5 ng/g), it is reasonable to say the risk of AFM$_1$ contamination in commercial powdered milk in Bangkok, Thailand is sufficiently low. However, it is necessary to continuously monitor AFM$_1$ in commercial powdered milk, because some powdered milk was highly contaminated with AFM$_1$. A review of the regulations for aflatoxins in dairy cattle feed, milk and its products is necessary in the near future.
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Supplementary Materials

Supplementary materials may be found in the online version of this article:

Supplementary Table 1 Occurrence of aflatoxin M1 in milk powder samples
Supplementary Table 2 Incidence of aflatoxin M1 in powdered milk manufactured in different countries
Supplementary Table 3 Incidence of aflatoxin M1 in different types of powdered milk

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**Supplementary Table 1  Occurrence of aflatoxin M$_1$ in milk powder samples**

| Year       | 2010 | 2011 | 2012 | 2013 | 2014 | 2010–2014 |
|------------|------|------|------|------|------|------------|
| Company    |      |      |      |      |      |            |
| A          | ND   | ND   | ND   | ND   | ND   | 0/8 (0%)   |
|            | ND   |      |      |      |      |            |
| B          | ND   | ND   | ND   | ND   | ND   | 0/16 (0%)  |
|            | ND   |      |      |      |      |            |
| C          | ND   | ND   | ND   | ND   | ND   | 1/15 (7%)  | 0.003      |
|            | ND   |      |      |      |      |            |
| D          | ND   | ND   | 0.007| ND   | 0.013| 5/15 (33%) | 0.008      |
|            | ND   |      |      |      |      |            |
| E          | ND   | ND   | ND   | ND   | ND   | 0/5 (0%)   |            |
| F          | 0.018| 0.066| 0.135| 0.018| 4/4 (100%)| 0.059      |
| G          | ND   | ND   | ND   | ND   | <LOQ  | 2/16 (13%) | 0.003      |
| Total      | 2/19 (11%) | 1/10 (10%) | 1/20 (5%) | 2/20 (10%) | 6/10 (60%) | 12/79 (15%) | 0.024      |

\(^a\) Mean of three estimates expressed as ready-to use form.

\(^b\) Mean of positive samples (<LOQ was calculated as LOQ/2 = 0.0025 ng/mL).

\(^c\) Not detected (Less than 0.0015 ng/mL).

\(^d\) <Limit of quantification (0.0015–0.005 ng/mL).
**Supplementary Table 2**  Incidence of aflatoxin M₁ in powdered milk manufactured in different countries

| Manufacturing country | Samples | Positives (%) | Aflatoxin M₁ (ng/mL) in reconstituted powdered milk |
|-----------------------|---------|---------------|----------------------------------------------------|
|                       |         |               | Mean \(^a\) ± SD | Min. | - | Max. |
| Thailand              | 34      | 9             | 26%               | 0.031 ± 0.044 | <LOQ\(^b\) | - | 0.135 |
| Singapore             | 16      | 2             | 13%               | 0.003 ± 0.000 | <LOQ | - | <LOQ |
| Philippines           | 9       | 1             | 11%               | 0.003        | <LOQ |   |      |
| Malaysia              | 4       | 0             | 0%                | -             |      |    | <LOQ |
| Australia             | 5       | 0             | 0%                | -             |      |    |      |
| Europe                | 11      | 0             | 0%                | -             |      |    |      |
| **Total**             | 79      | **12**        | **15%**           | **0.024 ± 0.039** | <LOQ | - | 0.135 |

\(^a\) Mean of positive samples (<LOQ was calculated as LOQ/2 = 0.0025 ng/mL).

\(^b\) <Limit of quantification (0.0015–0.005 ng/mL).
**Supplementary Table 3** Incidence of aflatoxin M<sub>1</sub> in different types of powdered milk

| Type of powdered milk                        | Samples | Positives (%) | Aflatoxin M<sub>1</sub> (ng/mL) in reconstituted powdered milk | Incidence in different manufacturing countries |
|----------------------------------------------|---------|---------------|---------------------------------------------------------------|-----------------------------------------------|
|                                              |         |               | Mean± SD Min. - Max.                                          | Southeast Asia Australia Europe               |
| Infant formula (for 0 to 1 year old)         | 29      | 0%            | -                                                              | 0/20 0/3 0/6                                  |
| Follow-on formula (for 6 months to 3 years)  | 24      | 13%           | 0.003 ± 0.000 <LOQ<sup>b</sup> - <LOQ                      | 3/18 0/2 0/4                                  |
| Children to adults formula (for 1 year to adult) | 22      | 23%           | 0.008 ± 0.004 <LOQ - 0.013                                   | 5/21 - 0/1                                   |
| Non-specified formula                        | 4       | 100%          | 0.059 ± 0.055 0.018 - 0.135                                  | 4/ 4 - -                                     |
| **Total**                                    | 79      | 15%           | 0.024 ± 0.039 <LOQ - 0.135                                   | 12/63 0/5 0/11                               |

<sup>a</sup> Mean of positive samples (<LOQ was calculated as LOQ/2 = 0.0025 ng/mL).

<sup>b</sup> <Limit of quantification (0.0015–0.005 ng/mL).