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

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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 few 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 *A. 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 humans²⁾. 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/kg³⁾. In Thailand, the total aflatoxin limit in dairy feed is 200 µg/kg⁴⁾. 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 some recent reports of 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 with an overall average of 0.004 $\mu\text{g/L}$ ¹¹⁾. To clarify AFM₁ contamination levels in commercial powdered milk in Thailand, we developed an in-house immunoaffinity column-HPLC (IAC-HPLC) method for AFM₁ 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 AFM₁, we used AFM₁ solution (0.5 $\mu\text{g/mL}$ acetonitrile solution) from Wako Pure Chemicals, Osaka, Japan. The AFM₁ solution was diluted in steps with acetonitrile and the mobile phase of HPLC. Gibco Hybridoma-Serum Free Media was purchased from Life Technologies Corporation (New York, United States). Protein G column, a ready-to-use column for purifying mouse IgG₁ antibody, was purchased from GE Healthcare (Uppsala, Sweden). Affi-Gel 10, an activated affinity medium to provide coupling of ligands via primary amines, was purchased from Bio-Rad (Hercules, United States). HPLC-grade acetonitrile and methanol were from Wako Pure Chemicals, Osaka, Japan. Ultra-pure water was produced on a Milli Q E-Pure-4 system from Barnstead (Los Angeles, United States). Other reagents used the special grade chemicals from Wako Pure Chemicals (Osaka, Japan).

Powdered milk of 4.2 g was dissolved in hot water (65°C) to make 30 mL according to the manufacturer's instruction. When powdered milk was dissolved in water, the pH of the solution was approximately 6.3. This pH was slightly low to optimum pH of antigen-antibody reaction. When powdered milk was dissolved in 0.1 M Tris-HCl buffer pH 7.5, the pH of the solution was approximately 7.4, which was optimum pH of antigen-antibody reaction. When 1.80 g of NaCl was added, removal of fat was carried out effectively. We pre-treated the sample by the following methods for reasons of the above. A mixture of 4.20 g of milk powder, 1.80 g of NaCl, and 18 mL of 0.1 M Tris-HCl buffer pH 7.5 (pre-heated at 65°C) was heated at 65°C for 3 minutes, and then vigorously mixed for 30 seconds and sonicated for 10 minutes. The solution was adjusted to 30 mL with 0.1 M Tris-HCl buffer pH 7.5. Then, this reconstituted powdered milk (30 mL) was shaken at 200 rpm for 30 minutes using a shaker (AS-1 Almighty Shaker; AS ONE Corporation, Osaka, Japan). After centrifuging at 3,000 rpm for 20 minutes, the fat layer was removed. Because the IAC was often clogged without the de-fatting, to prevent the clog, we performed de-fatting in every sample. The middle layer was collected and filtered using a GA-200 (Advantec Toyo, Tokyo Japan) glass fiber filter. The filtrate was filtered again using a GA-55 (Advantec Toyo, Tokyo Japan) glass fiber filter, and 10 mL of the filtrate was applied to the IAC.

The in-house IAC was prepared with an anti-AFM₁

monoclonal antibody named AM.3¹³⁾. The AM.3 hybridoma was cultured in 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 dialyzed against Dulbecco's phosphate buffered saline (PBS). 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 AFM₁ 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 $\times$ 100 mm; Shimadzu, Kyoto, Japan) was used. The column oven was set to 50°C. The mobile phase was acetonitrile:methanol:water (10+30+60, v/v/v) at a flow rate of 0.4 mL/minute. The injection volume was 10 μL . The fluorescence detector was used at an excitation wavelength of 360 nm and emission wavelength of 435 nm. Four concentrations of AFM₁ 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 detection (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 AFM₁ in the powdered milk, trifluoroacetic acid (TFA) was used to change AFM₁ 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.

AFM₁ solution (300 μL) in acetonitrile was spiked in 30 mL of reconstituted powdered milk. The final concentrations of AFM₁ 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 AFM₁ 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/mL¹⁴. The precision of our method was also high, as estimated by relative standard deviations (RSD) of the recovery for AFM₁, which ranged between 5.2 and 7.4%. Therefore, our method is a reliable method for determining AFM₁ in powdered milk. HPLC chromatograms of the AFM₁ standard solution and the extract from non-spiked powdered milk, and spiked AFM₁ (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 AFM₁ in Fig. 1 A and B, and there is no peak that overlaps AFM₁ in Fig. 1 C.

The results of the AFM₁ analysis of 79 powdered milk samples over five years (2010 to 2014) showed that 12 samples (15%) were contaminated with AFM₁ (Supplementary Table 1). The concentrations of AFM₁ 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 AFM₁ to AFM_{2a}. After treatment with TFA, the AFM₁ 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 AFM₁ 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 AFM₁ (Supplementary Table 3). Because the density of these reconstituted powdered milks was approximately 1.03 g/mL, the concentration of AFM₁ 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 AFM₁ 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 AFM₁ of powdered milk products in Bangkok over five years.

Powdered milk from 4 of 7 companies was contaminated with AFM₁, but products from other companies

Table 1 Recoveries of aflatoxin M₁ from spiked reconstituted powdered milk using our in-house immunoaffinity column-HPLC method

Spiked aflatoxin M ₁ in reconstituted powdered milk (ng/mL)	Recovery (% mean ± SD ^a) ^b			RSD ^c (%)
0.15	73.6	±	4.3	5.8
0.05	78.2	±	4.7	6.0
0.025	83.9	±	4.4	5.2
0.005	86.3	±	6.4	7.4

^a Standard deviation.

^b Average of 3 determinations.

^c Relative standard deviation.

Fig. 1 HPLC chromatograms of aflatoxin M₁ standard and the extracts from powdered milk samples.

A) Aflatoxin M₁ standard (0.50 ng/mL).

B) Extract from non-spiked powdered milk.

C) Extract from spiked aflatoxin M₁ in reconstituted powdered milk 0.05 ng/mL.

D) Extract from sample naturally contaminated with 0.066 ng/mL of aflatoxin M₁ in reconstituted powdered milk.

were not contaminated (Supplementary Table 1). Powdered milk from company F (a Thai company) was contaminated with AFM₁ 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 AFM₁, which were higher than the European Union regulatory limit (0.05 ng/g). Powdered milk from company D (a Swiss company) was contaminated with AFM₁ in 5 of 15 samples (33%) with a mean of the positive samples of 0.008 ng/mL. Thirteen percent of powdered milk from company G (an American company, 16 samples) and 7% from company C (an international company, 15 samples) were contaminated with AFM₁ 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 AFM₁.

The incidence of AFM₁ in powdered milk manufactured in different countries showed that of 34 samples of powdered milk made in Thailand, 9 (26%) were contaminated with AFM₁ 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 AFM₁ at levels below LOQ. However, none of the products manufactured in Malaysia (4 samples), Australia (5 samples) and European countries (Denmark, Germany, Ireland, Netherlands and Spain, total 11 samples) was contaminated with AFM₁. These data indicated that the powdered milk made in Thailand was more highly contaminated than the powdered milk from other countries. The investigation of AFB₁ in cattle feed in Thailand showed that 21 to 83% was contaminated with AFB₁ with an average of 13.9 to 18.7 µg/kg^{8,9)} (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 AFM₁ in milk was on average 1–2% of the amount of AFB₁²⁾. The average of AFB₁ contamination in dairy feed in Thailand was 13.9 to 18.7 µg/kg. Accordingly, we could estimate that the average concentration of AFM₁ 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 AFM₁, for powdered milk manufactured in Thailand. The estimated concentration of AFM₁ agreed with the actual concentration of AFM₁. Therefore, the result clearly suggested that the contamination level of AFB₁ in dairy feed affected the concentration of AFM₁ contamination in powdered milk. For 200 µg/kg of AFB₁ contamination in dairy cattle feed, 2–4 ng/mL of AFM₁ will be found in the cow milk. This AFM₁ 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 AFM₁ in Thai milk. Based on these results, we think it is necessary to set a lower regulatory limit for AFB₁ in dairy feed in Thailand to reduce the risk of AFM₁ in milk and powdered milk manufactured in Thailand.

The incidence of AFM₁ in different types of powdered milk showed that none of the 29 samples of infant formula was contaminated with AFM₁ (Supple-

mentary Table 3). Of 24 samples of the follow-on formula, 3 (13%) were contaminated with AFM₁ at levels below LOQ. In 22 samples of children to adults formula, 5 (23%) was contaminated with AFM₁ with an average of 0.008 ng/mL. All 4 samples of non-specified formula were contaminated with AFM₁ with an average of 0.059 ng/mL. The levels of AFM₁ 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 AFM₁ in samples specified for higher-age children. However, the infant formula was totally free of AFM₁. 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)¹⁵⁾. AFM₁ in milk is principally associated with caseins¹⁵⁾. 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 AFM₁¹⁵⁾.

The incidence and average concentration of AFM₁ 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 Thailand¹¹⁾. Ruangwises *et al.* reported that 19 (21%) of 90 samples were contaminated with 0.001 to 0.006 ng/mL of AFM₁ 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 AFM₁ 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/mL¹⁶⁾. In another study from Spain (2010), 8 of 69 (11.6%) infant formula samples were contaminated with AFM₁ 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/mL¹⁵⁾. In India (2004), there was a high occurrence of AFM₁ 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/mL¹⁷⁾. The overall average of AFM₁ 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 AFM₁ contamination in commercial powdered milk in Bangkok, Thailand is sufficiently low. However, it is necessary to continuously monitor AFM₁ in commercial powdered milk, because some powdered milk was highly contaminated with AFM₁. 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

References

- 1) The International Agency for Research on Cancer: IARC monographs on the evaluation of carcinogenic risks to humans, Volume 100F, pp. 225–248 (2012), The International Agency for Research on Cancer, Lyon, France
- 2) Mohammadi, H.: "Aflatoxins - Biochemistry and Molecular Biology" (ed. Guevara-Gonzalez, R. G.), pp. 397–414 (2011), In Tech, Rijeka, Croatia
- 3) United States Food and Drug Administration Compliance Policy Guide: Action levels for aflatoxins in animal feeds. Sec. 683.100. (1994)
- 4) Ministry of Agriculture and Cooperatives: Royal Thai Government Gazette, **112**, 10–11 [Accessed 18 July 2015]. Available at http://www.dld.go.th/th/images/stories/law/announce_feed2537.pdf (1994) (in Thai)
- 5) European Commission: Commission regulation (EC) No 1831/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union, **L364**, 5–24 (2006)
- 6) Codex Alimentarius Commission: Codex general standard for contaminants and toxins in food and feed. CODEX-STAN 193-1995 [Accessed 18 July 2015]. Available at http://www.fao.org/fileadmin/user_upload/agns/pdf/CXS_193e.pdf (Amended 2010)
- 7) Ministry of Public Health: Royal Thai Government Gazette, **120** [Accessed 18 July 2015]. Available at <http://newsser.fda.moph.go.th/food/file/Laws/Notification%20of%20Ministry%20of%20PublicHealth/Law03P265.pdf> (2003) (in Thai)
- 8) Arunvipas, P., Tungmunkong, P., Jala, P.: A study on risk factors of aflatoxin B₁ contamination in dairy feed in Kanchanaburi, Nakhon Pathom and Ratchaburi provinces. J Thai Vet Med Assoc, **60**, 39–48 (2009) (in Thai)
- 9) Songsang, A., Dejsupa, S., Ritto, R., Suwanpugdee, A., Pasitkul, P.: The contamination of aflatoxin in feedstuffs and complete feed in Phattalung province. Thaksin Univ Annu Conf, **19**, 1–11 (2009) (in Thai)
- 10) Ruangwises, N., Ruangwises, S.: Aflatoxin M₁ contamination in raw milk within the central region of Thailand. Bull Environ Contam Toxicol, **85**, 195–198 (2010)
- 11) Ruangwises, N., Saipan, P., Ruangwises, S.: "Aflatoxins - Biochemistry and Molecular Biology" (ed. Guevara-Gonzalez, R. G.), pp. 439–446 (2011), In Tech, Rijeka, Croatia
- 12) Saitanu, K.: Incidence of aflatoxin M₁ in Thai milk products. J Food Prot, **60**, 1010–1012 (1997)
- 13) Okumura, H., Okimoto, J., Kishimoto, S., Hasegawa, A., Kawamura, O., Nakajima, M., Miyabe, M., Ueno, Y.: An improved indirect competitive ELISA for aflatoxin M₁ in milk powders using novel monoclonal antibodies. Food Agric Immunol, **5**, 75–84 (1993)
- 14) European Commission: Commission regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off J Eur Union, **L70**, 12–34 (2006)
- 15) Gómez-Arranz, E., Navarro-Blasco, I.: Aflatoxin M₁ in Spanish infant formulae: occurrence and dietary intake regarding type, protein-base and physical state. Food Addit Contam Part B, **3**, 193–199 (2010)
- 16) Sakuma, H., Sugita-Konishi, Y., Tanaka, T., Nagayama, T., Naito, S., Horie, M., Ishikuro, E., Nakajima, M., Yoshinari, T., Kawakami, H.: Determination of aflatoxin M₁ in powdered formula: an inter-laboratory study and surveillance in Japan. JSM Mycotoxins, **64**, 15–21 (2014)
- 17) Rastogi, S., Dwivedi, P. D., Khanna, S. K., Das, M.: Detection of aflatoxin M₁ contamination in milk and infant milk products from Indian markets by ELISA. Food Control, **15**, 287–290 (2004)

Supplementary Table 1 Occurrence of aflatoxin M₁ in milk powder samples

Year	2010	2011	2012	2013	2014	2010–2014		
Company		Aflatoxin M ₁ (ng/mL) ^a				Positives/total (%)	Mean of Aflatoxin M ₁ (ng/mL) ^b	
A	ND ^c	ND	ND	ND	ND	0/8 (0%)	-	
	ND			ND				
				ND				
B	ND	ND	ND	ND	ND	0/16 (0%)	-	
	ND	ND	ND	ND				
	ND		ND	ND				
	ND		ND					
			ND					
			ND					
C	ND	ND	ND	ND	ND	1/15 (7%)	0.003	
	ND	ND	ND	ND	<LOQ ^d			
	ND		ND		ND			
	ND		ND					
D	ND	ND	0.007	ND	0.013	5/15 (33%)	0.008	
	ND		ND	0.010	0.005			
			ND	ND	<LOQ			
			ND	ND				
				ND				
E	ND	ND	ND	ND		0/5 (0%)	-	
	ND							
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	
	ND	ND	ND	ND				
	ND		ND	ND				
	<LOQ		ND	ND				
				ND				
Total	Positives/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 M ₁ (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%	-			
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 M₁ in different types of powdered milk

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

^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).