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Occurrence of Aflatoxin M₁ in Raw and Pasteurized Goat Milk in Thailand

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1. Introduction

Aflatoxins are a group of structurally related mycotoxins produced by certain species of the genus *Aspergillus*, particularly *A. flavus*, *A. parasiticus* and *A. nomius*, which can grow on a variety of food and feed commodities [1]. Aflatoxin production is influenced by several factors: for example, temperature and humidity [2]. It has been shown that aflatoxin B₁ (AFB₁) is the most potent hepatocarcinogen of this group of mycotoxins. Aflatoxin M₁ (AFM₁) is a hydroxylated metabolite of AFB₁ produced by the hepatic microsomal cytochrome P450, and is secreted in the milk of mammals that have consumed AFB₁-contaminated foods. AFM₁ is also a hepatocarcinogen and is classified in Group 1 as carcinogenic to humans by the International Agency for Research on Cancer [3]. In terms of food safety and public health concerns, exposure to AFM₁ through milk products is considered to be a serious problem.

According to worldwide regulations for mycotoxins in food and feed compiled by the Food and Agriculture Organization of the United Nations, 60 countries have already established regulatory limits for AFM₁ in raw milk and milk products. The report also indicates that the limits vary from ND (not detectable) to 15 µg/L [4]. The values of 0.05 µg/L and 0.5 µg/L are the two most prevalent regulatory limits for AFM₁ in milk products, enforced in 34 and 22 countries, respectively. The maximum permitted level for AFM₁ established by the European Community is 0.025 µg/kg for infant formulae and follow-on formulae, including infant milk and follow-on milk, while the limit for raw milk and heat-treated milk is 0.05 µg/kg [5]. The U.S. regulatory standard for AFM₁ is 0.5 µg/L [4]. There are still several countries, including Thailand, that have not yet established regulatory limits for AFM₁ in dairy products.

The law that regulates the quality of milk products in Thailand is the Notification of the Ministry of Public Health No. 265, which regulates only cow milk products. However, the law does not specify the regulatory standards for AFM₁ but states that "...milk products may be contaminated with aflatoxins at a level that is not harmful to human health" [6]. The only guideline that regulates the quality of raw goat milk is the Thai Agricultural Standard TAS 6006-2008 of the National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives [7]. Like Notification No. 265 for cow milk products, the TAS 6006-2008 guideline does not specify the recommended limit for AFM₁ in goat milk.

In Thailand, the number of dairy goats is approximately 5% that of dairy cows [8–10]. Goat milk is consumed by only a small percentage of the country's population, particularly Thai people who have an allergy to cow milk. Goat milk has been shown to form finer and softer curds than cow milk following acidification under conditions similar to those in the stomach, thus making it more readily digested [11]. It has been reported that micellar caseins of human and goat milk were 96% hydrolyzed by pepsin and trypsin in *in vitro* studies, while the hydrolytic rate of cow milk was 76–90% [12]. With the knowledge that goat milk is more easily digested, some Thai adults prefer goat milk products. As a result, the number of dairy goats in Thailand has been gradually increasing in recent years. In 2009, the number of dairy goats in Thailand was 20,830; the numbers increased to 22,630 and 33,363 in 2010 and 2011, respectively [8–10].

Thailand is administratively divided into four regions: central, north, northeast and south. The central region was selected for this study, since this region has the highest number of dairy goats and the highest rate of goat milk production, accounting for approximately 60% of the national total [8–10]. There are no internationally published reports regarding the quality and levels of AFM₁ in goat milk produced in Thailand.

The purpose of this study was to investigate whether the concentrations of AFM₁ in raw and pasteurized goat milk produced in Thailand are within the acceptable level for consumption.

2. Materials and methods

2.1. Chemicals

AFM₁ reference standard (from *Aspergillus flavus*) was purchased from Sigma-Aldrich (St. Louis MO, USA). AflaM₁TM immunoaffinity columns were obtained from Vicam (Nixa MO, USA). Solvents (HPLC grade) – acetonitrile, methanol, and water – were purchased from Merck (Darmstadt, Germany).

2.2. Milk sample collection and sample preparation

Raw goat milk samples were collected from private farms, while pasteurized goat milk samples were purchased from supermarkets in the central region of Thailand. In Thailand, commercial pasteurized milk is produced by heat treatment, either at 63 °C for 30 min or at 72 °C for at least 15 s [6]. All milk samples were collected over three years: January–February of

the years 2009–2011. Both types of milk samples were frozen at –20 °C until analysis (within one month from the collection date for raw milk, or 2 months from the manufacturing date for pasteurized milk). A total of 90 milk samples were collected and analyzed in this study.

2.3. Extraction and determination of aflatoxin M₁

The extraction procedure was performed using the manufacturer's recommendations, as previously described by Ruangwises et al. [13]. Briefly, 50 ml of raw milk or pasteurized milk sample was pipetted into a 50-ml plastic centrifuge tube. Milk samples were defatted by centrifugation at 3,500 g for 20 min at 4°C. Fat was separated; the resulting skimmed milk was then transferred into a 50-ml plastic syringe with a Luer tip which was attached to an immunoaffinity column. The skimmed milk was allowed to flow into the column by gravity at a flow rate of approximately 1 ml/min. After the skimmed milk had run through, 20 ml of HPLC water was used to wash the column. AFM₁ was eluted from the column with 1.25 ml of acetonitrile:methanol (3:2) and 1.25 ml of HPLC water. The eluate (a total volume of 2.5 ml) was filtered through a nylon syringe filter for HPLC with pore size 0.45 µm (Whatman, UK). AFM₁ in the final solution was determined using HPLC. Each milk sample was extracted and analyzed for AFM₁ in duplicate.

2.4. Instrument

A complete liquid chromatographic system (ProStar; Varian, Palo Alto CA, USA) consisted of a HPLC pump (model 240), an auto injector (model 410), a column oven (model 510), and a fluorescence detector (model 363). The HPLC conditions for analysis of AFM₁ were as follows: column, Spherisorb ODS-2 (Waters, Milford MA, USA); column temperature, 40 °C; mobile phase, water:methanol:acetonitrile (57:23:20); flow rate, 1 ml/min; and detector, fluorescence spectrophotometer (excitation 360 nm; emission 440 nm).

2.5. Determination of limit of quantification

The Q2B procedure of the U.S. Food and Drug Administration [14] was used for determination of the limit of quantification (LOQ) for AFM₁. Milk samples (50 ml) were fortified with standard AFM₁ at four concentrations of 0.025, 0.050, 0.125 and 0.250 µg/L, while blank samples were not fortified with standard AFM₁. Concentrations of AFM₁ in AFM₁-fortified milk samples and blank samples were quantified as described above in Section 2.3 using AflaM₁TM immunoaffinity columns. All samples were analyzed for AFM₁ in duplicate.

Individual linear regression lines were obtained from least-square regression analyses of the residual peak areas versus the four concentrations of fortified AFM₁ (0.025, 0.050, 0.125 and 0.250 µg/ml). The residual peak areas were peak areas of AFM₁-fortified samples minus the peak area of blank sample. A total of 12 regression lines (six regression lines each for intraday and interday analyses) were obtained by least-square linear regression. The LOQ of the method was calculated using the equation $LOQ = 10 \sigma/S$, where σ is the standard deviation of y -intercepts and S is the average slope of the 12 linear regression analyses [14].

2.6. Statistical analysis

A randomized block experiment was used to evaluate the differences in AFM₁ concentrations in the two types of milk samples and among the three collection years. Duncan's multiple comparison test was applied to obtain significance levels between the raw milk and pasteurized milk, and among each year of individual milk products ($P < 0.05$). SPSS Statistics version 17.0 for Windows was used for statistical analysis.

3. Results and discussion

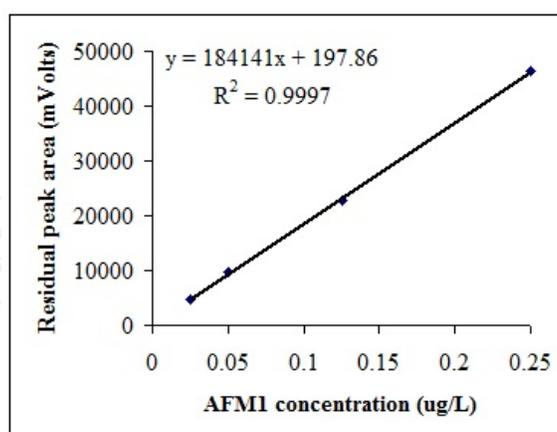
Table 1 shows the results of analysis and a regression line obtained from least-square analysis of Sample A, of which the slope and y -intercept were used for the calculation of LOQ. Twelve regression lines (six lines each for intraday and interday analyses) were performed in this study; slopes and y -intercepts of all 12 analyses are presented in Table 2. The calculation for LOQ was based on the equation $LOQ = 10 \sigma/S$, where σ and S are the standard deviation of y -intercepts and the average slope of the 12 regression lines, respectively. In this study, the standard deviation of y -intercepts was $173.69 \text{ mV} \times \text{L}/\mu\text{g}$ and the average slope was $180,518 \text{ mV}$. The calculated LOQ was $(10 * 173.69)/180,518 = 0.01 \mu\text{g/L}$. The accuracy of the method, expressed as % recovery, ranged from 88.8% to 94.1%, with an average value of 90.8%. The precision of the method, expressed as %RSD (percent relative standard deviation), ranged from 1.1% to 7.5%. Table 3 summarizes the accuracy and precision of determination of AFM₁ in goat milk samples fortified with AFM₁ at four concentrations, with intraday and interday analyses. HPLC chromatograms of standard AFM₁ ($10 \mu\text{g/L}$), a goat milk sample contaminated with AFM₁ ($0.05 \mu\text{g/L}$), and an uncontaminated goat milk sample are presented in Figure 1. The retention time for AFM₁ under the conditions in this study was approximately 6.8 min.

Table 4 shows the incidence and concentrations of AFM₁ in raw and pasteurized goat milk samples. The incidence of AFM₁ in raw goat milk collected in 2009, 2010 and 2011 was 46.7% (7/15), 66.7% (10/15) and 60.0% (9/15), respectively, while the incidence in pasteurized milk was 53.3% (8/15), 46.7% (7/15) and 53.3% (8/15), respectively. The total incidence of positive samples with respect to 90 samples analyzed in this study was 54.4% (49/90). Of the 49 positive samples, only 7 samples (14.3%) were contaminated with AFM₁ above the EU standard of $0.05 \mu\text{g/L}$. The three-year average concentrations of AFM₁ found in the raw and pasteurized milk samples were 0.043 and $0.040 \mu\text{g/L}$, respectively. The maximum concentration found in this study was $0.086 \mu\text{g/L}$, which was far below the U.S. regulatory limit of $0.5 \mu\text{g/L}$. In this study, statistical analysis showed that there were no significant differences in AFM₁ concentrations among the raw and pasteurized milk samples and across the two types of milk samples collected over a three-year period.

When compared to cow milk, goat milk has a lower percentage of positive samples and lower AFM₁ concentrations. Ghanem and Orfi [15] reported that the average concentration of AFM₁ in raw goat milk ($0.019 \mu\text{g/L}$, $n = 11$), collected from markets in Syria between April 2005 and April 2006, was less than that in raw cow milk ($0.143 \mu\text{g/L}$, $n = 74$); the percentage

of positive samples of goat milk (7 samples, 63.6%) was also less than that of cow milk (70 samples, 94.6%). Hussain et al. [16] found that 6 (20%) of 30 raw goat milk samples were contaminated with AFM₁ at an average concentration of 0.002 µg/L, while 15 (37.5%) of 40 raw cow milk samples were contaminated with an average AFM₁ level of 0.014 µg/L. Rahimi et al. [17] reported that the incidence of AFM₁ in raw goat and cow milk samples collected from Ahvaz in Khuzestan province, Iran, between November 2007 and December 2008, was 31.7% (19/60) and 78.7% (59/75), respectively. Concentrations of AFM₁ in raw milk samples of both species were 0.0301 and 0.0601 µg/L, respectively.

AFM ₁ added (µg/L)	Peak area ¹ (mV)	Residual peak area ² (mV)
0	6,410	-
0.025	11,126.5	4,716.5
0.050	16,144.5	9,734.5
0.125	29,251	22,841
0.250	52,773	46,363



slope = 184,141; y-intercept = 197.86

¹ Average value of two determinations

² Residual peak area = peak area of AFM₁-fortified sample – peak area of blank sample

Table 1. Linear regression analysis of AFM₁-fortified sample A for the determination of LOQ

Sample	Slope (mV × L/μg)	y-intercept (mV)
Intraday (n = 6)		
A	184,141	197.86
B	180,733	293.38
C	183,706	141.26
D	179,857	549.02
E	180,039	207.84
F	181,224	109.74
Interday (n = 6)		
G	181,454	127.39
H	175,861	432.76
I	185,285	223.45
J	179,462	442.02
K	175,904	339.74
L	178,545	639.60
Overall (n = 12)		
Mean	180,518 (S)	308.67
SD	2,955.5	173.69 (σ)

Table 2. Slopes and y-intercepts of 12 regression lines used for determination of LOQ for AFM₁

AFM ₁ added (μg/L)	Intraday (n = 6)			Interday (n = 6)		
	Found ^a (μg/L)	%RSD ^b	Recovery (%)	Found ^a (μg/L)	%RSD ^b	Recovery (%)
0.025	0.023 ± 0.001	4.3	92.1	0.024 ± 0.002	7.5	94.1
0.050	0.046 ± 0.001	2.2	91.9	0.046 ± 0.002	3.5	91.4
0.125	0.112 ± 0.003	2.7	89.3	0.111 ± 0.004	3.9	88.8
0.250	0.225 ± 0.002	1.1	89.8	0.222 ± 0.005	2.1	89.0

^a Values are mean ± SD

^b % RSD = percent relative standard deviation.

Table 3. Accuracy and precision of determination of AFM₁ in goat milk

Year	Samples analyzed	Positive ¹ (%)	AFM ₁ concentration (ng/ml) ²		AFM ₁ incidence ³	
			Mean	Range	0.010–0.050 µg/L	> 0.05 µg/L
<i>Raw milk</i>						
2009	15	7 (46.7)	0.042 ± 0.012	0.022–0.061	6 (85.7)	1 (12.5)
2010	15	10 (66.7)	0.049 ± 0.018	0.025–0.086	8 (80.0)	2 (20.0)
2011	15	9 (60.0)	0.036 ± 0.015	0.018–0.066	8 (88.9)	1 (11.1)
Total	45	26 (57.8)	0.043 ± 0.017	0.018–0.086	22 (84.6)	4 (15.4)
<i>Pasteurized milk</i>						
2009	15	8 (53.3)	0.039 ± 0.017	0.015–0.075	7 (87.5)	1 (12.5)
2010	15	7 (46.7)	0.045 ± 0.015	0.022–0.061	6 (85.7)	1 (14.3)
2011	15	8 (53.3)	0.035 ± 0.019	0.014–0.073	7 (87.5)	1 (12.5)
Total	45	23 (51.1)	0.040 ± 0.016	0.014–0.073	20 (87.0)	3 (13.0)
Overall	90	49 (54.4)	0.041 ± 0.016	0.014–0.086	42 (85.7)	7 (14.3)

¹Numbers in parentheses are percentages for each year

²Means and ranges of AFM₁ concentrations in the positive samples

³AFM₁ incidence of the positive samples

Numbers in parentheses are percentages with respect to the positive samples

Table 4. Incidence and concentrations of AFM₁ in raw and pasteurized goat milk samples collected within the central region of Thailand

High incidence and concentrations of AFM₁ in cow milk have also been found in Thailand. Ruangwises and Ruangwises [18] reported that all of 240 raw cow milk samples collected from 80 milk tanks at a milk collecting center in the central region of Thailand were found to be contaminated with AFM₁ at an average concentration of 0.070 µg/L. For pasteurized milk samples, our previous studies showed that AFM₁ was found in 349 (83.1%) of 420 pasteurized milk samples, collected from 40 provinces in all four regions of Thailand from May 2006 to January 2008, with AFM₁ concentrations ranging between 0.012 and 0.114 µg/L [13,19].

Table 5 shows the incidence and concentrations of AFM₁ in raw and pasteurized goat milk from various countries. For raw goat milk, Assem et al. [20] found that all of the three raw milk samples collected from markets in Lebanon between March–July 2010 contained AFM₁ less than the LOQ of 0.005 ng/ml. Ozdemir [21] found that the mean concentration of AFM₁ in 93 positive samples out of 110 raw milk samples collected from the city of Kilis, Turkey, from March–April 2006 was 0.019 µg/L. For pasteurized milk, Oliveira and Ferraz [22] determined the concentrations of AFM₁ in 12 pasteurized goat milk samples collected from the state of Sao Paulo, Brazil, and found that 7 samples (58.3%) were contaminated with an average concentration of 0.034 µg/L.

The levels of AFM₁ in goat milk are influenced by both feeding practices and the types of feedstuffs. Viridis et al. [23] determined the concentrations of AFM₁ in goat milk collected from two groups of farms with different feeding practices – extensive and intensive farms – in Sardinia, Italy, between the years 2003 and 2004. In extensive farms, goats were principally fed on grass and naturally growing bushes which were often present in marginal areas, supplemented with low levels of concentrates consisting of broad bean (*Vicia faba*) and garden pea (*Pisum sativum*). In intensive farms, goats were mainly fed silo maize, maize grains, and alfalfa (*Medicago sativa*). The incidence of AFM₁ in goat milk samples from extensive and intensive farms was 11.2% (9/80) and 71.4% (20/28), respectively. Concentrations of AFM₁ found in positive samples from both farms were 0.009 and 0.0177 ng/ml, respectively.

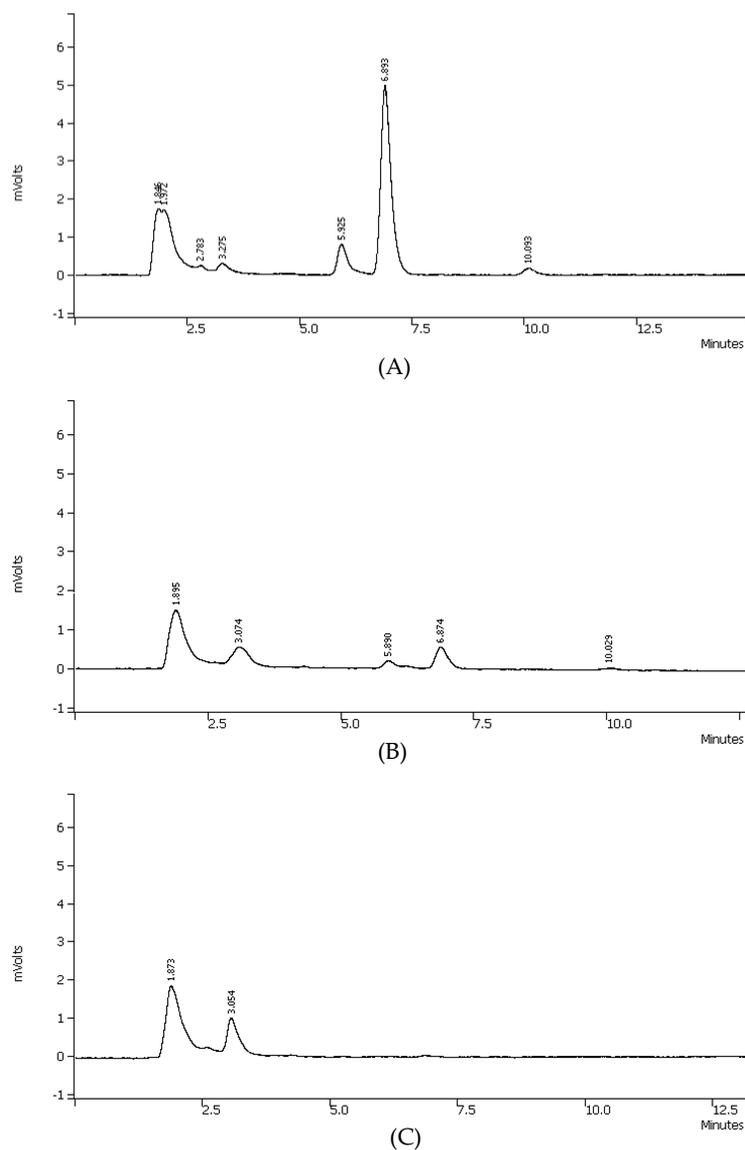


Figure 1. HPLC chromatograms of AFM₁ with a retention time of approximately 6.8 min: (A) standard 10 µg/L AFM₁, (B) goat sample contaminated with 0.05 µg/L AFM₁, and (C) uncontaminated goat milk sample

Country	Year	Samples analyzed	Positive (%)	Concentration (µg/L) ¹	Reference
<i>Raw milk</i>					
Lebanon	Mar–July 2010	3	0 (0)	< 0.005	Assem et al. (2011)
Iran	Nov 2007 – Dec 2008	60	19 (31.7)	0.0301 ± 0.0183	Rahimi et al. (2010)
Pakistan	Jan–Dec 2007	30	6 (20)	0.002 ± 0.005	Hussain et al. (2010)
Turkey	Mar–Apr 2006	110	93 (84.5)	0.019 (0.005–0.117) ²	Ozdemir (2007)
Syria	Apr 2005 – Apr 2006	11	7 (63.6)	0.019 ± 0.0138 (0.008–0.054)	Ghanem and Orfi (2009)
Thailand	Jan 2008 – Feb 2011	45	26 (57.8)	0.036 ± 0.015 (0.011–0.064)	Present study
<i>Pasteurized milk</i>					
Brazil	Oct 2004 – May 2005	12	7 (58.3)	0.072 ± 0.048	Oliveira and Ferraz (2007)
Thailand	Jan 2008 – Feb 2011	45	23 (51.1)	0.034 ± 0.014 (0.010–0.058)	Present study

¹ Concentrations of AFM₁ in positive samples

² Values in parentheses are ranges

Table 5. Incidence and concentrations of AFM₁ in raw and pasteurized goat milk in various countries

The observation that the incidence and concentrations of AFM₁ in goat milk are relatively lower than those in cow milk can be explained in terms of the feeding procedure and the carry-over rate of AFB₁ in feedstuffs to AFM₁ in the milk. Cows are generally fed with several major AFB₁-contaminated feedstuffs: corn, cotton seed, and concentrated feed. Unlike cows, goats are fed with fresh grass but not corn or cotton seed; the main AFB₁-contaminated feedstuffs fed to goats are concentrate feedstuffs. Motawee et al. [24] explained the different feeding patterns of cows and goats in Egypt. Cows are generally kept in enclosed areas and fed with a large proportion of AFB₁-contaminated feedstuffs, with a short period of time for grazing on pasture; while goats are allowed to graze on pasture in the morning and are brought back into the enclosed areas for concentrate feedstuffs in the evening. Hussain et al. [16] explained that goats in Pakistan are mainly fed by grazing on pasture. AFB₁-contaminated feedstuffs – corn, cotton seed, and concentrate feed – are not used to feed goats. In Thailand, the feeding procedures for cows and goats are similar to those in Egypt and Pakistan [25].

The carry-over rate of AFB₁ in feedstuffs to AFM₁ in milk is relatively lower in goats than in cows. The carry-over rates in cows have been reported to vary from 0.3% to 6.2%, with a mean value of 1.81% (n = 42) [26]. In Thailand, Ruangwises and Mhosatanun [27] determined the carry-over rates during the early lactation period (the first 4 weeks of lactation) in nine cows fed with feedstuffs naturally contaminated with AFB₁. The carry-over rates ranged between 1.96% and 3.12%, with an average value of 2.02%. For goats, Smith et al. [28] reported an average carry-over rate of 0.55% in three goats which were fed with feedstuffs containing 100 ppb AFB₁. Mazzette et al. [29] found an average carry-over rate of 0.26% in three goats within 72 h after receiving a single oral dose of 0.8 mg of AFB₁.

This study showed that 49 samples (54.4%) of the 90 goat milk samples collected within the central region of Thailand in January–February of the years 2009–2011 were contaminated with AFM₁ equal to or more than the LOQ of 0.01 µg/L. Concentrations of AFM₁ were not significantly different among the raw and pasteurized milk samples and across the two types of milk samples collected over three years. Of the 49 positive samples, 7 samples (14.3%) had AFM₁ greater than the EU regulatory limit of 0.05 µg/L. All 90 goat milk samples contained AFM₁ below the U.S. regulatory limit of 0.5 µg/L. This study presents the first internationally published report on the contamination of AFM₁ in raw and pasteurized goat milk produced in Thailand. The present study and our three previous reports on the occurrence of AFM₁ in cow milk products [13,18,19] suggest that regulatory standards be adopted for AFM₁ to ensure the quality of raw milk and milk products in Thailand.

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