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Survey of Aflatoxin M_1 Contamination of Dairy Products in Taiwan

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ABSTRACT

Owing to their nutritional value, dairy products were popular in Taiwan. Aflatoxin M_1(AFM_1) was the metabolite of potential carcinogen aflatoxin B_1 and found in dairy products. An analytical method using immunoaffinty column for extraction and HPLC for quantification was developed for AFM_1. The detection limits for fresh milk (pasteurized milk), milk powder and drinking yogurt (0.002, 0.02 and 0.005 ppb, respectively) were 10 times lower than that in the former survey. The recoveries of AFM_1 from fresh milk were 83.3 ± 2.9 and 89.5 ± 2.9% at 0.5 and 0.05 ppb spiked levels, respectively. Spiked 5 and 0.5 ppb AFM_1 in milk powder, the recoveries were 86.0 ± 1.9 and 88.7 ± 1.9%, respectively. The recoveries of AFM_1 from drinking yogurt were 99.9 ± 1.4 and 94.8 ± 3.2% at 0.5 and 0.05 ppb spiked levels, respectively. It was the first time AFM_1 was tested in drinking yogurt in Taiwan in this survey, and the performance for detecting AFM_1 in drinking yogurt was also evaluated through attending an international proficiency test. Our laboratory got a satisfactory result. In order to survey AFM_1 contents in dairy products, 44 samples of fresh milk, 45 samples of milk powder and 24 samples of drinking yogurt were collected from supermarkets, convenience stores and drug stores located in 23 counties of Taiwan from June to August, 2002. The results showed that AFM_1 was detected in 40 samples of fresh milk at 0.002~0.083 ppb level. AFM_1 was not found in all milk powders. AFM_1 was detected in 3 samples of drinking yogurt, at the level of 0.007, 0.009 and 0.044 ppb. According to the food sanitary standard regulation in Taiwan, the action levels of AFM_1 were 0.5 ppb, 5 ppb and not-detectable for fresh milk, milk powder and infant formula products, respectively. The 113 samples collected in this survey all met the regulation requirements.

Key word: aflatoxin M_1, dairy product, immunoaffinity column, high performance liquid chromatography

INTRODUCTION

Aflatoxins, a group of several toxic secondary fungal metabolites produced by some *Aspergillus* spp., are found in a wide variety of foods and feeds around the world. Aflatoxin M_1 (AFM_1), a hydroxylated metabolite of the potential carcinogen Aflatoxin B_1 (AFB_1) (Figure 1), occurs in milk from lactating animals consuming feed contaminated with AFB_1[1]. About 0.3-6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk and a linear relationship has been found between intake of AFB_1 in contaminated feed and the AFM_1 content of milk in cows[2]. Following the withdrawal of the contaminated feed, AFM_1 levels in milk decrease to below the limit of detection within 72 hr[3].

The potential hazardous human exposure to AFM_1 via consumption of milk and milk products has been demonstrated[4,5,6,7]. Both AFB_1 and AFM_1 can cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalian cells *in vitro*, in insects, lower eukaryotes and bacteria[8]. However, AFM_1 is less carcinogenic and genotoxic than AFB_1[2].

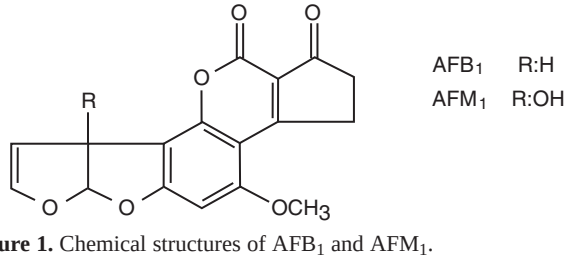

Figure 1. Chemical structures of AFB_1 and AFM_1.

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To protect consumers, particularly children, from contaminated dairy products, several countries have established legislation to regulate the levels of AFB_1 in feeds and AFM_1 in milk (Table 1). The Food and Drug Administration (FDA) of US has established an action level of 0.50 ppb in whole, low fat and skim milk[9], whereas the EU has set a maximum admissible level of 0.05 ppb in raw milk, heat-treated milk, and milk for the manufacture of milk based products[10]. In Taiwan, the action levels in fresh milk, milk powder and infant formula dairy products are 0.5 ppb, 5 ppb and not-

Table 1. Regulatory limits for AFM_1 in various countries

Country	Milk (ppb)	Infant formula (ppb)
Argentina	0.5[a]	0.1
Austria	0.05	0.01
Brazil	0.5	0.01
France	0.2	
Germany	0.05	0.01
Italy	0.05	0.05
Netherlands	0.05	0.05
Switzerland	0.05	0.01[b]
US	0.50	
EU	0.05	
Taiwan	0.5	Not-detectable

[a]Sum of B_1, B_2, G_1, G_2 and M_1.
[b]Sum of B_1 and M_1.

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detectable, respectively[11,12].

Monitoring surveys are frequently conducted in several countries all over the world to determine the levels of AFM_1 in milk and milk products. In Taiwan, 161 raw milk and milk powder had been examined according to the AOAC method in 1986, and no AFM_1 contamination in these samples was found[13]. Using a silica-gel column to extract and clean up all 110 dairy samples, 44 of 50 (88%) fresh milk, 15 of 25 (60%) milk powder, 6 of 10 (60%) condensed milk and 0 of 25 cheese samples were detected the contamination of AFM_1[14]. Fu determined the AFM_1 in milk and milk powder using an immunoaffinity column and fluorscence measurement. AFM_1 was detected in 3 of 25 (12%) fresh milk and 0 of 25 milk powder samples[15]. However, no survey has referred to the occurrence of AFM_1 in drinking yogurt. The objectives of this work were to establish a more sensitive method to determine the AFM_1 in dairy products (especially in drinking yogurt) and to understand the contamination of AFM_1 in fresh milk, milk powder and drinking yogurt in Taiwan.

MATERIALS AND METHODS

I. *Materials*

(I) *Sample collection*

The feed in Taiwan is much easier to be contaminated with AFB_1 due to the warm and humid weather in summer; therefore, this survey was conducted during June ~ August. The samples were collected from supermarkets, convenience stores and drug stores of 23 counties in Taiwan. Forty-four samples were fresh milk, 45 samples were milk powder (of which 21 were infant formula) and 24 samples were drinking yogurt. The minimum sample size was 500 mL or g. The samples were conserved in a refrigerator during the transfer to the laboratory. In the laboratory, fresh milk and drinking yogurt samples were stored in a refrigerator (5 ± 2˚C), and milk powder samples were stored at a temperature below 25˚C. All samples were analyzed before the expiration date.

(II) *Reagents*

AFM_1 standard was purchased from Supelco (Bellefonte, PA., U.S.A.). The immunoaffinity columns $AflaM_1^{TM}$ were purchased from Vicam (Watertown, MA., U.S.A.). Sodium hydroxide was reagent grade. Acetonitrile and methanol used for the liquid chromatographic mobile phases were of LC grade. Distilled, deionized water was used throughout the procedure.

II. *Methods*

(I) *Samples preparation*

The procedures were modified from Dragacci *et al.*[16] and Tuinstra *et al.*[17] Fresh milk was warmed before analysis to ca 37˚C in a water bath, and then stirred gently with a magnetic stirrer to disperse the fat layer. Ten grams of milk powder (including infant formula) were dissolved with 50 mL of warm water, and mixed with stirring until a homogeneous mixture was obtained. The solution of milk powder was transferred to a 100-mL volumetric flask and diluted to the mark. Liquid milk (fresh milk and the solution of milk powder) was centrifuged for 10 min at 2000×g to separate the fat, and the upper fat layer was discarded. The samples were filtered through one or more paper filters, and at least 50 mL of the filtrate was collected. Twenty-five grams of drinking yogurt was warmed as fresh milk and the pH was adjusted to 6.5 with 0.1N NaOH before passing samples through the clean-up column.

(II) *Immunoaffinity clean up*

Fifty mL of liquid milk and pH-adjusted drinking yogurt were passed at about 1-2 mL/min through an $AflaM_1^{TM}$ immunoaffinity column fitted with a 60-mL syringe reservoir. Twenty mL of water was used to wash the loaded immunoaffinity column at a steady flow rate. The column was blown to dryness with a stream of nitrogen, and AFM_1 was eluted with 4 mL of acetonitrile. The acetonitrile eluate was evaporated to dryness under a gentle stream of nitrogen, and the residue was redissloved by vortexing with 2 mL of mobile phase, which was then filtered through a 0.45 μm microfilter for HPLC analysis.

(III) *HPLC analysis*

The LC system consisted of a Shimadzu (Kyoto, Japan) $LC\text{-}10AT_{VP}$ pump, a $RF\text{-}10A_{XL}$ fluorescence detector, and a Waters 717_{plus} autosampler (Waters, MA., U.S.A.), all under the control of the Shimadzu $SCL\text{-}10A_{VP}$ system. Data acquisition was performed on an SISC program. The column (150 × 4.6 mm, Cosmosil 5C18-AR, 5 μm, Nacalai, Japan) was maintained at 30˚C. Injection volume was 200 μL and the detector wavelength settings were 365 nm (excitation) and 435 nm (emission). The mobile phase, water/acetonitrile/methanol (68/24/8, v/v/v), was pumped at a constant flow rate of 1.0 mL/min.

(IV) *Calibration*

AFM_1 standard was diluted with mobile phase to prepare a series of working solutions containing 0.002-10 ng AFM_1/200 μL. A calibration curve was constructed by plotting the peak area for each standard against the mass of AFM_1 injected. Slope and intercept data of the calibration curve were used to compute the quantity of the analyte in sample extracts.

(V) *Validation*

Analytical methods for AFM_1 determination from

three kinds of dairy products were validated in-house. Two each of AFM_1-free fresh milk and drinking yogurt samples were spiked with 0.05 and 0.5 ppb AFM_1 standards. Two AFM_1-free milk powders were spiked with 0.5 and 5 ppb AFM_1 standards, then dissolved with warm water. Then the following procedures were according to those described above. The method detection limit (MDL) of the chromatographic procedure was measured according to the Standard Operation Procedure for the MDL determination of Bureau of Food and Drug Analysis[29] as follows:

AFM_1 standards of serial concentrations were injected into and analyzed by the LC system. A curve was constructed by plotting the peak area for each standard against the mass of AFM_1 injected. The concentration where the slope of the standard curve changed markedly was defined as IDL (instrument detection limit). To obtain the MDL, an AFM_1 standard at a concentration equal to 1~5 times IDL was spiked into AFM_1-free samples, followed by the analytical procedures described above. Standard deviation (Sa) was calculated from 7 replicates. An AFM_1 standard at the concentration equal to 3 times Sa was spiked into AFM_1-free samples, followed by the analytical procedures described above. Standard deviation (S) was calculated from 7 replicates. The bigger one of Sa^2 and S^2 was chosen as the numerator. If the ratio was smaller than 3.05, the pooled standard deviation (S_{pool}) and MDL were calculated using the following regulations :

$$S_{pool} = [(6S^2 + 6Sa^2) / 12]^{1/2}$$
$$MDL = 2.681 \times S_{pool}$$

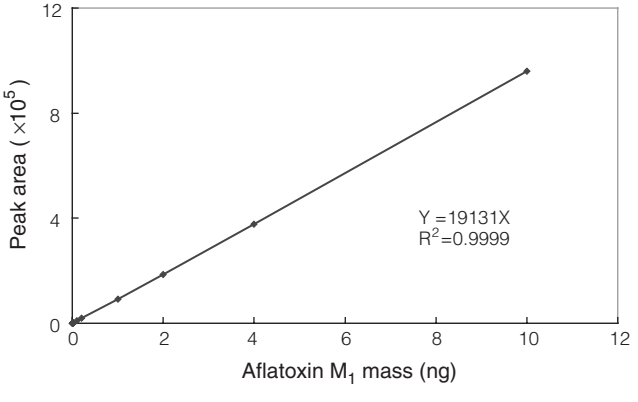

Figure 2. Calibration curve of AFM_1 analyzed by HPLC.

Table 2. Recoveries of AFM_1 in dairy products

Dairy product	Spiked levels (ppb)	Recovery[a] (%)	CV[b] (%)
Fresh milk	0.05	89.5 ± 2.9	3.2
	0.5	83.3 ± 2.9	3.5
Drinking yogurt	0.05	94.8 ± 3.2	3.4
	0.5	99.9 ± 1.4	1.4
Milk powder	0.5	88.7 ± 1.9	2.1
	5	86.0 ± 1.9	2.2

[a]Average of triplicate analysis ± standard deviation.
[b]CV: coefficient of variation.

(VI) *Quantification*

AFM_1 mass concentration of the test sample was calculated using the following equation:

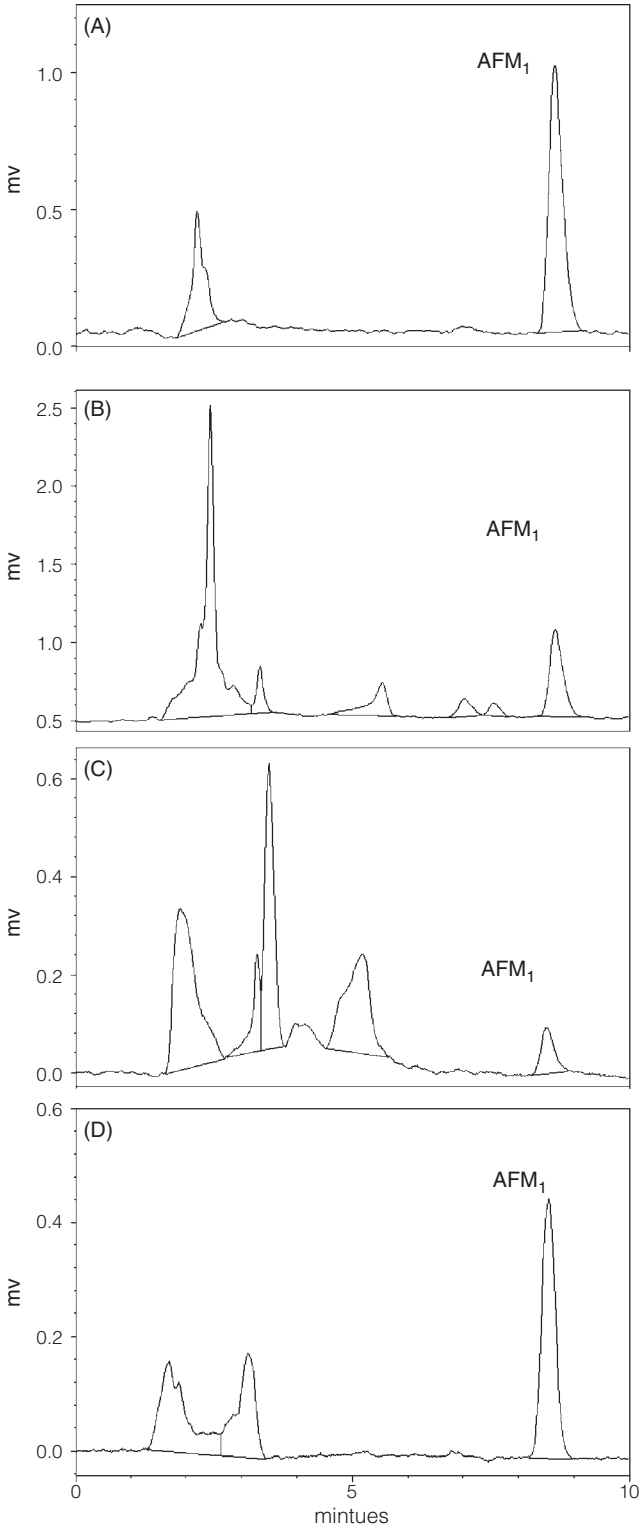

Figure 3. HPLC chromatograms of (A) AFM_1 standard, (B) AFM_1-spiked fresh milk, (C) AFM_1-spiked milk powder, (D) AFM_1-spiked drinking yogurt. HPLC conditions: Cosmosil 5C18-AR; mobile phase, water/acetonitrile/methanol (68/24/8, v/v/v) flow rate, 1.0 mL/min.

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$W_m = W_a \times (V_f/V_i) \times (1/V_s)$

Where W_m = the numerical value of AFM_1 mass concentration in the test sample in ppb (ng/g or ng/mL); W_a = the numerical value of the amount of AFM_1 corresponding to the area of AFM_1 peak of the sample extract (ng); V_f = the numerical value of the final volume of redissolved eluate (μL); V_i = the numerical value of the final volume of injected eluate (μL); V_s = the numerical value of volume or mass of prepared test portion passing through the column (mL or g).

Express the results to 3 significant figures.

(VII) *Proficiency test*

Our Laboratory participated in the "Analysis of Aflatoxin M_1 in yogurt" (Food Analysis Performance Test Assessment Scheme, Series 4, Aflatoxins Analysis, Round 4) proficiency test held by the Central Science Laboratory (Sand Hutton, York, U.K.) in February, 2002. The sample was analyzed as described above.

RESULTS

I. *Analytical Method Performance*

(I) *Calibration*

Within the calibration range of 0.002-10 μg/200 μL of AFM_1, the HPLC responses (peak area) positively regressed with injected AFM_1 mass and gave a correlation coefficient of $R^2 = 0.9999$, as shown in Figure 2.

(II) *Validation*

The MDLs of fresh milk, milk powder and drinking yogurt were 0.002, 0.02 and 0.005 ppb, respectively. Specificity of the AFM_1 peaks were clearly shown in HPLC chromatograms of Figure 3, which showed the absence of interfering signals at the AFM_1 retention area for fresh milk, milk powder and drinking yogurt. The recoveries of AFM_1 in fresh milk, milk powder and drinking yogurt were 83.3-89.5, 86.0-88.7, and 94.8-99.9%, respectively, as shown in Table 2.

(III) *Proficiency test*

Thirty-four of 43 participants delivered their detection results and 25 participants received the "satisfied result" ($|z| < 2$). The z-scores of all 34 participants were shown in Figure 4. Our laboratory number was 7, and the z-score was 0.7.

II. *Survey Results (Table 3)*

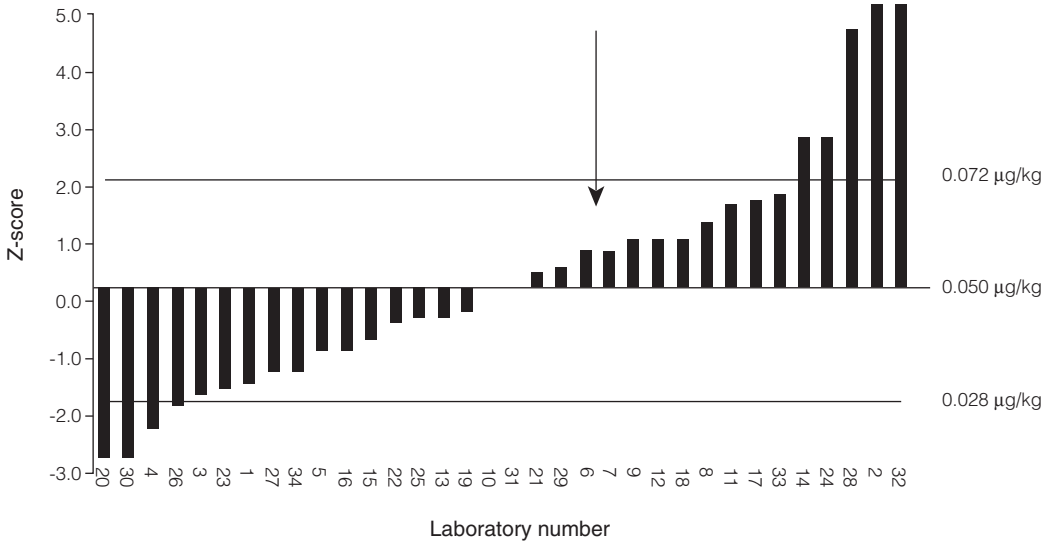

Figure 4. Z-score distribution of participants in the "Analysis of AFM_1 in yogurt" proficiency test. (The number of our laboratory was 7 as the arrow indicated)

Table 3. AFM_1 contents of dairy products

Dairy product	No. of samples	No. of positive samples (%)	AFM_1 contents (ppb)		
			0.002~0.01	0.01~0.05	0.05~0.1
Fresh milk	44	40 (90.9)	10	29	1
Milk powder	24	0 (0)	0	0	0
Infant formula	21	0 (0)	0	0	0
Drinking yogurt	24	3 (12.5)	2[a]	1	0
Total	113	43 (38.1)	12	30	1

[a]Between 0.007 and 0.01 ppb.

Table 4. Incidence and levels of AFM_1 contamination in fresh milk

Country	Year	No. of samples	No. of positive samples (%)	AFM_1 (ppb)
Brazil[19][a]	1992	52	4 (7.7)	0.073 ~ 0.370
Italy[20]	1995	159	136 (85.5)	< 0.001 ~ 0.11
Italy[21]	1996	161	125 (77.6)	< 0.001 ~ 0.024
Tailand[22]	1995~1996	250	249 (99.6)	47 more than 0.5 ppb
Taiwan[15]	1996	25	3 (12.0)	0.08 ~ 0.33
Korea[7]	1997	70	39 (55.7)	0.015 ~ 0.052
Kuwait[23]	1998	30	13 (43.3)	0.01 ~ 0.21
Spain[25]	1998	47	14 (29.8)	0.02 ~ 0.1
Portugal[26]	1999	101	85 (84.2)	2 more than 0.05 ppb (2%)
Greece[24]	1999~2001	136	113 (83.0)	0.005 ~ 0.05
Brazil[27]	1999~2000	139	29 (20.9)	0.05 ~ 0.24
Taiwan[b]	2002	44	40 (90.9)	0.002 ~ 0.083

[a]Reference.
[b]Data of this survey.

Table 5. Incidence and levels of AFM_1 contamination in milk powder

Country	Year	No. of samples	No. of positive samples (%)	AFM_1(ppb)
Italy[20][a]	1995	97	81 (83.5)	< 0.001 ~ 0.10
Italy[21]	1996	92	50 (54.3)	< 0.001 ~ 0.08
Tailand[22]	1995~1996	13	2 (15.4)	Not more than 0.5 ppb
Taiwan[15]	1996	25	0	0
Korea[7]	1997	50	35 (70.0)	0.032 ~ 0.342
Brazil[28]	1997	300	33 (11.0)	0.1 ~ 1.0
Kuwait[23]	1998	19	0	0
Taiwan[b]	2002	45	0	0

[a]Reference.
[b]Data of this survey.

Table 6. Incidence and levels of AFM_1 contamination in yogurt

Country	Year	No. of samples	No. of positive samples (%)	AFM_1 (ppb)
Italy[20][a]	1995	114	91 (79.8)	< 0.001 ~ 0.50
Italy[21]	1996	120	73 (60.8)	< 0.001 ~ 0.032
Korea[7]	1997	60	31 (51.7)	0.017 ~ 0.124
Kuwait[23]	1998	5	1 (20.0)	0.01 ~ 0.21
Taiwan[b]	2002	24	3 (12.5)	0.007 ~ 0.044

[a]Reference.
[b]Data of this survey.

(I) *Fresh milk*

Among the 44 fresh milk samples, 3 samples were manufactured by small scale farms and sold only in local supermarkets. The remaining 41 samples were manufactured by larger scale food companies and sold to other counties. AFM_1 above the MDL of 0.002 ppb was present in 40 of 44 (90.9%) fresh milk samples, among which 10 were between 0.002 and 0.01 ppb, 29 were between 0.01 and 0.05 ppb, 1 was between 0.05 and 0.1 ppb (0.083 ppb).

(II) *Milk powders and infant formula*

The milk powders and infant formula were all goods imported. No AFM_1 was detected in the 24 milk powders and 21 infant formula according to the MDL of 0.02 ppb.

(III) *Drinking yogurt*

Two of 24 samples of drinking yogurt were manufactured by small scale food processing companies and sold only in local supermarkets. The remaining 22 samples were manufactured by larger scale food companies and sold to other counties. The incidence of AFM_1 in drinking yogurt (12.5%) was lower than that in fresh milk. Three of 24 drinking yogurt samples were contaminated by AFM_1 at the concentrations of 0.007, 0.009 and 0.044 ppb.

DISCUSSION

The retention time of AFM_1 in the HPLC chromatograms was shorter in this survey (8~9 min) than that in the former survey (13~14 min)[15]. The MDL was 10 times lower.

Fu[15] surveyed the AFM_1 content in fresh milk, and found that 3 of 25 (12%) samples were positive. The column Fu used (AFLAPREP M, Vicam) was different from what we used in this survey, and the MDL was 0.05 ppb, which was much higher than those in this survey.

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Though the principles of these two surveys were similar, the sensitivity of the HPLC system in this survey was elevated and the MDL was lowered to 0.002 ppb. According to the MDL of 0.05 ppb, only 1 of 44 (2.3%) fresh milk was found to be positive in this survey. Hence, the AFM_1 contamination status in fresh milk was not serious. According to Visconti *et al.*[18], most of such results should be attributed to the poor sensitivity of the analytical methods used. Today, the increased efficiency of the extraction procedures and the increased accuracy of the analytical methods and better equipments resulted in much lower limits of detection, thereby a significant increase in the percentage of positive samples.

In comparison, the incidence of AFM_1 contamination in fresh milk in recent studies ranged from 7.7 to 99.6%, with means of AFM_1 contents from 0.01 to 0.5 ppb, as shown in Table 4[7,15,19,20,21,22,23,24,25,26,27]. In this survey, the incidence of AFM_1 contamination in fresh milk was very high, 90.9% of the samples were positive. However, Table 3 showed that AFM_1 was not detected in 9.1% of the fresh milk sample, detected at low levels (0.002 to 0.05 μg/L) in 88% of the samples and none of these positive samples were above the action level of 0.5 ppb. This supports the view that a high incidence of low-level contamination and infrequent high contamination levels in commercial milk could be caused by only a few contaminated samples entering the bulk milk supply[20].

The incidence of AFM_1 in milk powder in recent studies ranged from 0 to 84%, with AFM_1 mean contents from 0 to 1.0 ppb, as shown in Table 5[7,15,20,21,22,23]. The 45 milk powder samples (including 21 infant formula) collected in this survey were all imported from America, Japan, New Zealand and the Netherlands. The same as the result of a former survey held in 1996[15], no AFM_1 was detected in the milk powder samples. Though there were trace levels of AFM_1 contamination found in the milk powder samples in the recent survey, none of these positive samples was detected at levels above the action level of 5.0 ppb, and it seems that the milk powders exported to Taiwan were free of AFM_1. Since Taiwan became a member of WTO, there will be more and more milk powders imported from different countries to Taiwan. Therefore, related surveys should be continued in the future.

Owing to its nutritional value, yogurt, especially drinking yogurt, has become more and more popular in Taiwan. However, the content of AFM_1 is not reduced in the fermentation process if AFM_1-contaminated milk is used. The concentration of AFM_1 could even be increased due to the condensation process[20]. The incidence of AFM_1 in yogurt in recent studies ranged from 20 to 80%, with AFM_1 mean contents from 0 to 0.50 ppb, as shown in Table 6[7,20,21,22,23]. In this survey, it was the first time AFM_1 was tested in drinking yogurt in Taiwan. Though there was AFM_1 contamination in drinking yogurt, the content was low (0.007 to 0.044 ppb). Till now, the action level of AFM_1 in yogurt has not been set in Taiwan.

CONCLUSION

By combining immunoaffinity column for cleanup and LC with fluorescence for detection, the AFM_1 contents in dairy products could be measured with good recoveries and satisfactory MDL. Through participation in the "Analysis of Aflatoxin M_1 in yogurt" in 2002, our laboratory was among the 34 laboratories which received "satisfied result" in the proficiency test.

In this survey, it was revealed that trace levels of AFM_1 were detected in dairy products such as fresh milk and drinking yogurt in Taiwan. However, none of these positive samples was above the action level of 0.5 ppb.

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