Determination of aflatoxin M₁ in powdered formula: an inter-laboratory study and the surveillance in Japan

Hisako SAKUMA¹, Yoshiko SUGITA-KONISHI² *, Toshitsugu TANAKA³, Toshihiro NAGAYAMA⁴, Shige Hiro NAITO⁵, Masakazu HORIE⁶, Eiichi ISHIKURO⁷, Masahiro NAKAJIMA⁸, Tomoya YOSHINARI¹, and Hiroshi KAWAKAMI⁹

¹National Institute of Health Sciences, 1-18-1 Kamiyogo, Setagaya-ku, Tokyo 158-8501, Japan
²Azabu University, 1-17-1, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
³Kobe Institute of Health, 4-6 Minatojo inakamachi, Chuo-ku, Kobe Hyogo 650-0046, Japan
⁴Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
⁵National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan
⁶Otsuna Women’s University, 12 Sanbanchyo, Chiyoda-ku, Tokyo 102-8357, Japan
⁷Japan Scientific Feeds Association, 821 Yoshikura, Narita, Chiba 286-0133, Japan
⁸Nagoya City Public Health Research Institute, 1-11, Hagiyama-cho, Mizuho-ku, Nagoya, Aichi 467-8615, Japan
⁹Kyoritsu Women’s University, 2-2-1 Hitotsubashi, Chiyoda-ku, Tokyo 101-8437, Japan

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Abstract

A method to determine aflatoxin M₁ (AFM₁) levels by using an immunoaffinity column-based clean-up procedure and HPLC with fluorescence detection was validated by an inter-laboratory study among ten laboratories in Japan. Using the validated method, we surveyed AFM₁ contamination in powdered formula. Samples for validation included a blank, three levels (blind pairs) of AFM₁ spiked into liquid milk, naturally contaminated liquid milk, and naturally contaminated powdered formula. All samples were frozen and sent to the ten participating laboratories. For the liquid milk spiked at 1.0, 0.5, and 0.05 µg/kg levels, recoveries were 89.9, 91.6, and 88.2%, respectively. The repeatability relative standard deviation (RSDr) and reproducibility relative standard deviation (RSDR) were less than 7.4 and 8.1%, respectively. The recovery, RSDr, and RSDR of the powdered formula were 94.5, 8.9, and 11.9%, respectively. The RSD, and RSDR of the naturally contaminated milk were 13.3 and 20.9%, respectively. The Horwitz ratio (HorRat) values of all six samples were less than 1.0. For surveillance, 108 commercial powdered formulae were obtained in Japan. The average value of AFM₁ in the powdered formulae was 0.002 µg/L, as ready-for-infant liquid milk (14 g powdered formula in 100 mL water). The highest contamination was 0.025 µg/L.

Introduction

Aflatoxins, a group of potent genotoxic carcinogenic compounds, are secondary metabolic products of Aspergillus flavus, A. parasiticus, and A. nomius that may contaminate various agricultural commodities⁴. Aflatoxin M₁ (AFM₁) is an aflatoxin B₁ (AFB₁) metabolite that is readily transferred to mammalian milk². 

Corresponding Author

* Azabu University, 1-17-1, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan. Tel: +81-42-754-7111; Fax: +81-42-754-7661 E-mail: y-konishi@azabu-u.ac.jp

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Aflatoxins containing AFM₁ are classified as possibly carcinogenic to humans (Group 1) by the International Agency for Research on Cancer⁴. In 2001, the Codex Alimentarius Commission established a maximum residue level (MRL) of 0.5 μg/kg for AFM₁ in milk¹. Many countries have established regulations for AFM₁ levels in bovine milk. The MRLs for bovine milk are 0.5 and 0.05 μg/kg in the United States⁵ and the European Union⁵, respectively. For infants, the MRL for bovine milk is 0.025 μg/kg in the European Union⁵.

In Japan, the surveillance of AFM₁ have been carried out in raw bulk milk in 2004 and in commercial liquid milk in 2001 and 2002. The average level of AFM₁ in raw bulk milk was less than 0.011 μg/kg and that in commercial liquid milk was 0.009 μg/kg. These levels were below those deemed permissible by the Codex Alimentarius Commission. However, the surveillance of powdered formula has not yet been undertaken in Japan. Before such monitoring takes place, the analytical methods for powdered formula and liquid milk must be validated with an inter-laboratory study. Several validated analytical methods that include TLC, HPLC, and lateral flow assay have been reported for the determination of AFM₁. Immunoaffinity columns (IACs) are the most popular method for the clean-up of AFM₁ samples from milk. Regulations regarding AFM₁ have not existed in Japan yet. In this study, we conducted an inter-laboratory study for the validation of AFM₁ in powdered formula and liquid milk. Using this method, the surveillance of 108 powdered formulae was conducted for the first time in Japan.

Materials and Methods

**Standard and reagents**  An AFM₁ (Wako Pure Chemicals, Osaka, Japan) standard stock solution (1.0 μg/mL) was in a sealed amber glass bottle. The AFM₁ concentration was determined according to the molar absorptivity of AFM₁ in acetonitrile (19,000) at the maximum adsorption near 350 nm. AFM₁ standard stock solutions were stored at −20°C until use. HPLC grade acetonitrile and water were used. The IACs (Horiba, Kyoto, Japan) were stored at 4°C.

**Fortification procedure and samples for inter-laboratory study**  To evaluate recovery, AFM₁ solutions at three different concentrations were added to liquid milk (20.0 g, blank), which was purchased from a supermarket located in Tokyo, and stirred gently. The final concentrations of AFM₁ in the liquid milk were 1.0 μg/kg (A), 0.5 μg/kg (B), 0.05 μg/kg (C), and blank (D). Naturally contaminated raw milk was prepared by feeding cows with AFB₁ contaminated feed. Naturally contaminated powdered formula (0.473 μg/kg), which was a surplus sample of the food analysis performance assessment scheme (FAPAS), was purchased from GSI Creos Corporation (Tokyo, Japan).

**Pretreatment**  The artificially and naturally contaminated liquid milk samples and the blank were warmed to 37 °C, stirred gently to mix using a glass rod or magnetic stirrer, and sonicated for 5 min. At least 40 mL of milk was transferred to a 50 mL plastic centrifuge tube. After 5 min centrifugation at 3000 rpm at 25 °C or room temperature, an upper layer of fat was removed. The milk was filtered through a glass fiber filter in a glass funnel and transferred to an Erlenmeyer flask or beaker. Exactly 20.0 g of filtrate was weighed for purification by IAC. To spike, AFM₁ solutions (20 μL) were added and gently stirred and sonicated in 5 minutes and loaded onto an IAC.

Powdered formula (5.0 g) was weighed, mixed with water (30 mL, 50°C), and sonicated for 5 min to
obtain a homogeneous mixture. This was allowed to cool to room temperature (\(\sim 25^\circ C\)). The sample was diluted to 50 mL with water. The solution was filtered through a glass fiber filter. If necessary, the milk was centrifuged for 5 min at 3000 rpm at 25°C or room temperature. The filtrate sample (20mL) was loaded on an IAC immediately after filtration.

**Purification by IAC** The loaded samples of pretreated liquid milk and powdered formula solution were dropped at a flow rate of 1-2 drops s\(^{-1}\). The IAC was then washed with water (15 mL). AFM\(_1\) was eluted with acetonitrile (3 mL) and the eluate was collected in a silanized amber screw top vial. After solvent evaporation under nitrogen gas, HPLC injection solution (1 mL, acetonitrile:water (2:8, v/v)) was added and agitated using a mixer. The solution was transferred to a silanized amber vial for HPLC injection. The silanized amber screw top and HPLC vials were washed with 20-30% acetonitrile solution before use.

**HPLC conditions** The HPLC column was octadecyl silylated gel (3-5 \(\mu\)m particle size; diameter: 3-4.6 mm; length: 150-250 mm) maintained at 40°C in a column oven. The mobile phase was acetonitrile:water (25:75, v/v), used at a flow rate of 0.6-1.0 mL/min. The injection volume was 20-100 \(\mu\)L, and detection was with a fluorometric detector by an excitation wavelength of 365 nm and emission wavelength of 435 nm.

**AFM\(_1\) standard solution for HPLC** The AFM\(_1\) standard solution (1.0 \(\mu\)g/mL) was diluted in acetonitrile, and dried with a nitrogen gas stream or an evaporator. One mL of acetonitrile: water (2.8, v/v) was added to the residue and mixed well for AFM\(_1\) calibration standard solutions. A seven point calibration curve covering the range of interest for the test sample (0.1-20.0 ng/mL) was established. The calibration curve was to be linear.

### Table 1. Inter-laboratory study results of aflatoxin M\(_1\) in milk with limits of detection (LOD) and limits of quantification (LOQ)

<table>
<thead>
<tr>
<th>laboratory</th>
<th>A : 1.0 (\mu)g/kg</th>
<th>B : 0.5 (\mu)g/kg</th>
<th>C : 0.05 (\mu)g/kg</th>
<th>D : blank</th>
<th>Powdered formula (0.473 (\mu)g/kg)</th>
<th>Naturally contaminated milk</th>
<th>LOD ((\mu)g/kg)</th>
<th>LOQ ((\mu)g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.767</td>
<td>0.868</td>
<td>0.426</td>
<td>0.448</td>
<td>0.039</td>
<td>0.042</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>2</td>
<td>0.932</td>
<td>0.918</td>
<td>0.507</td>
<td>0.483</td>
<td>0.049</td>
<td>0.042</td>
<td>0.006(t) (t)</td>
<td>0.017(t) (t)</td>
</tr>
<tr>
<td>3</td>
<td>0.976</td>
<td>1.014</td>
<td>0.500</td>
<td>0.489</td>
<td>0.054</td>
<td>0.044</td>
<td>0.010(t) (t)</td>
<td>0.010(t) (t)</td>
</tr>
<tr>
<td>4</td>
<td>0.903</td>
<td>0.946</td>
<td>0.473</td>
<td>0.473</td>
<td>0.046</td>
<td>0.045</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>5</td>
<td>0.854</td>
<td>0.855</td>
<td>0.427</td>
<td>0.440</td>
<td>0.042</td>
<td>0.044</td>
<td>0.010(t) (t)</td>
<td>0.010(t) (t)</td>
</tr>
<tr>
<td>6</td>
<td>0.868</td>
<td>0.913</td>
<td>0.450</td>
<td>0.450</td>
<td>0.044</td>
<td>0.046</td>
<td>0.010</td>
<td>0.011</td>
</tr>
<tr>
<td>7</td>
<td>0.859</td>
<td>0.916</td>
<td>0.455</td>
<td>0.468</td>
<td>0.041</td>
<td>0.042</td>
<td>0.009(t) (t)</td>
<td>0.009(t) (t)</td>
</tr>
<tr>
<td>8(^a)</td>
<td>0.941</td>
<td>0.882</td>
<td>0.417</td>
<td>0.321</td>
<td>0.020</td>
<td>0.278</td>
<td>--(^b)</td>
<td>0.000</td>
</tr>
<tr>
<td>9</td>
<td>0.887</td>
<td>0.510</td>
<td>0.342</td>
<td>0.404</td>
<td>0.039(t) (t)</td>
<td>0.153</td>
<td>0.000</td>
<td>0.032(t) (t)</td>
</tr>
<tr>
<td>10</td>
<td>0.861</td>
<td>0.929</td>
<td>0.424</td>
<td>0.417</td>
<td>0.045</td>
<td>0.042</td>
<td>0.011</td>
<td>0.011</td>
</tr>
</tbody>
</table>

(t): trace  
(a) IAC choked  
(b) sample vial breakage  
(c) all data eliminated for statistical analysis  
d) data of 1.0, 0.5, 0.05 \(\mu\)g/kg were raw data minus 0.010
Calculation
The AFM concentration of the test sample was calculated using the following equation for liquid milk:
\[
W_m = \frac{W_a}{W_s}
\]
Where, \(W_m\) is the numerical value of the AFM in the test sample (µg/kg), \(W_a\) is the numerical value of the AFM in the HPLC injection test sample (ng/mL), and \(W_s\) is the weight of the test sample.

For the powdered milk sample, the AFM concentration of the test sample was calculated using the following equation.
\[
W_m = \frac{W_a}{(W_s/2.5)}
\]

The AFM concentrations in the surveillance samples as liquid milk (powdered formula/water = 14 g/100 mL by the conventional manufacturer’s manual) were calculated using the following equation.
\[
W_l = \frac{W_a}{(W_s/2.5)} \times 14/100.
\]

The limit of detection (LOD) was calculated with a signal/noise (S/N) ratio of 3:1, and the limit of quantification (LOQ) was calculated with an S/N ratio of 10:1.

Inter-laboratory study
To validate the method, an inter-laboratory study was carried out using six samples (a blank, three spiked liquid milks, one naturally contaminated liquid milk, and one naturally contaminated powdered formula) with duplicate blind samples, according to the protocols of the Association of Official Analytical Chemists (AOAC). Ten laboratories participated in the inter-laboratory study: the Kawasaki City Institute for Public Health, Kewpie Corporation, Food Analysis Technology Center SUNATEC, Japan Ecotech Co., Ltd., Japan Food Research Laboratories, Hamamatsu City Health and Environment Research Institute, Mie Prefecture Health and Environment Research Institute, Meiji Dairies Corporation, Morinaga Corporation, and Snow Brand Milk Products Co., Ltd.

Surveillance of powdered formula
The 108 samples for the surveillance comprised 24 brands with different product lot numbers that were purchased or obtained from six manufacturers of infant powdered formulae in 2010 in Japan. The samples were dissolved in hot water in the same manner as in the validated inter-laboratory method.

Statistical analysis
The precision parameters; the inter-laboratory relative standard deviations for repeatability (RSDr) and for reproducibility (RSDr) were deduced as recommended by the AOAC.

Results and Discussion
Validation of method for detection of AFM
In Table 1, the results of the inter-laboratory study are shown. Ten laboratories returned results, but because of the incomplete results from laboratory 8, its data were omitted from the statistical analysis. Outliers were determined by the Cochran and the Grubbs tests. For samples A, B, C (spiked AFM in liquid milk), and D (blank), laboratory 9 was an outlier by the Cochran test. For the powdered formula, laboratory 3 was an outlier by the Cochran test. For the naturally contaminated milk, the results from laboratory 3 were omitted because only one result for duplicate samples was reported.
The data for the blank sample (D) showed that the AFM$_1$ concentration of commercial liquid milk in Japan was 0.010 µg/kg (average). This value was nearly equal to the average for Japanese commercial liquid milk reported by Nakajima et al. (0.009 µg/kg)$^7$. All spiking data (A: 1.0; B: 0.5; C: 0.05 µg/kg) were subtracted from the blank (0.010 µg/kg). The LODs for the majority of the participants were under 0.010 µg/kg or equal to, except for laboratory 3, 5 and 9.

Table 2 shows the average levels, precision parameters, and Horwitz ratio (HorRat) values for this method. The recoveries of the spiked samples (A, B, and C) were acceptable, in the range 88.2-91.6%. The recovery for the powdered formula (94.5%) was also acceptable. The RSD$s$ of samples A, B, and C were very low, <7.4%. The RSD$s$ of the naturally contaminated powdered formula and liquid milk were 8.9% and 13.3%, respectively. The RSD$s$ of the spiked samples (A, B, and C) were less than 8.1%, whereas those for the naturally contaminated powdered formula and milk were 11.9% and 20.9%, respectively. The HorRat values were calculated using RSD$_R$/22$^{14}$. HorRat values for the spiked samples (A, B, and C) were less than 0.37, whereas those of the naturally contaminated powdered formula and liquid milk were 0.54 and 0.95, respectively. According to the criteria of the EU$^{15}$, the precision parameters and HorRat values in this method are adopted as the method for the surveillance of powdered formula as well as liquid milk.

In another inter-laboratory study of an analytical method for the determination of AFM$_1$ using an immunoaffinity column as a clean-up procedure and HPLC with fluorescence detection, the recovery of a µg/L. The distribution of AFM$_1$ concentration range of 0.08-0.6 µg/kg was 11-23%. Compared to the other two inter-laboratory studies, this method is superior in terms of the analysis for both liquid milk and powdered formula as a validated method.

**Surveillance** Using the method validated in this study, a survey of AFM$_1$ levels in powdered formulae was performed. The LOD was 0.003 µg/L (as ready-for-infant liquid milk; 14 g powdered formula in 100 mL water). The average value was 0.002 µg/L. The distribution of AFM$_1$ contamination showed that 72 samples were lower than the LOD; 16 samples were at the LOD ~0.05 µg/L; 12 samples were 0.005-0.010 µg/L; 6

<table>
<thead>
<tr>
<th>Sample</th>
<th>A: 1.0 µg/kg</th>
<th>B: 0.5 µg/kg</th>
<th>C: 0.05 µg/kg</th>
<th>D: Blank</th>
<th>Powdered formula (0.473 µg/kg)</th>
<th>Naturally contaminated milk</th>
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<td></td>
<td>10</td>
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<td></td>
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<tr>
<td>Average (µg/kg)</td>
<td>0.899</td>
<td>0.458</td>
<td>0.044</td>
<td>0.010</td>
<td>0.447</td>
<td>0.477</td>
</tr>
<tr>
<td>True value, %</td>
<td>89.9</td>
<td>91.6</td>
<td>88.2</td>
<td>94.5</td>
<td>94</td>
<td>94.5</td>
</tr>
<tr>
<td>Repeatability SD [S,$_m$]</td>
<td>0.038</td>
<td>0.010</td>
<td>0.003</td>
<td>0.040</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>Repeatability relative SD [RSD,$_m$, %]</td>
<td>4.3</td>
<td>2.1</td>
<td>7.4</td>
<td>8.9</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>Repeatability value [r (2.8S,)]</td>
<td>0.107</td>
<td>0.028</td>
<td>0.009</td>
<td>0.111</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>Reproducibility SD [S,$_r$]</td>
<td>0.059</td>
<td>0.029</td>
<td>0.004</td>
<td>0.053</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>Reproducibility relative SD [RSD,$_r$, %]</td>
<td>6.6</td>
<td>6.3</td>
<td>8.1</td>
<td>11.9</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>Reproducibility value [R (2.8S,)]</td>
<td>0.166</td>
<td>0.081</td>
<td>0.010</td>
<td>0.149</td>
<td>0.279</td>
<td></td>
</tr>
<tr>
<td>HorRat [RSD$_R$/22]</td>
<td>0.30</td>
<td>0.29</td>
<td>0.37</td>
<td>0.54</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>
samples were 0.010-0.015 µg/L; one sample was 0.020-0.025 µg/L; and one sample was 0.025 µg/L. The strictest regulatory limit is 0.025 µg/kg of the EU for infant milk. This study demonstrates that the powdered formula supplied in Japan contains extremely low levels of AFM₁, as determined using the analytical method validated by inter-laboratory study.

Acknowledgements

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