Detection of aflatoxin M1 in powdered milk and sweetened condensed milk products in several cities in Java with HPLC-fluorescence method

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Abstract. Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 (AFB1) produced by lactating animals due to consuming AFB1-contaminated feed. AFM1 can be found in dairy products because it is resistant to heat during processing. This study aimed to detect AFM1 in powdered milk and sweetened condensed milk sold in several cities in Java. The amount of powdered milk sample was 20, while the amount of sweetened condensed milk sample was 16. AFM1 detection in powdered milk and sweetened condensed milk was conducted by HPLC-fluorescence method. The results showed that the concentration of AFM1 in powdered milk ranged from undetectable to 0.549 µg/kg and the highest data (55%) was distributed in concentration range of >0.05 µg/kg - 0.2 µg/kg. On the other hand, AFM1 levels in sweetened condensed milk ranged from undetectable to 0.056 µg/kg and 43.75% data was distributed in concentration range of >0.025 µg/kg - 0.05 µg/kg. All powdered milk and sweetened condensed milk samples have met the maximum level of AFM1 according to Indonesian regulation.

Keywords : Aflatoxin M, milk products, HPLC-fluorescence method

1. Introduction

Milk is one of many foods that consumed a lot by human, because it contains high nutrient that important for health and growth. However, milk also could be an agent for food contaminants, which can migrate into body and harm health. One of the harmful contaminants contained in milk is aflatoxin, which enters the human food chain through aflatoxin M1 (AFM1) residues. AFM1 in milk is stable through pasteurization and storage [1].

Aflatoxin is a secondary metabolite produced by various species of fungi from the genus Aspergillus, which, due to its genotoxic, mutagenic and carcinogenic characteristics, has the highest toxicity among all mycotoxins. Chemically, aflatoxin is a derived compound of difuranocoumarin (Figure 1) produced by two Aspergillus species, A. flavus and A. parasiticus. A. flavus grows in various places, especially on the heading of the plant (leaves, flowers) and produces only aflatoxin B, namely AFB1 and AFB2, while A. parasiticus produces aflatoxin B and G, namely AFB1, AFB2, AFG1 and AFG2. Aflatoxin M1 and M2 are the hydroxylated of aflatoxin B1 and B2 metabolites.
(addition of -OH groups) and can be found in milk and dairy products produced by livestock that consume contaminated feed [2].

![Chemical structures of aflatoxins](image)

**Figure 1.** Chemical structure of aflatoxin B, aflatoxin G and aflatoxin M1 [2]

International Agency for Research on Cancer (IARC) has categorized Aflatoxin M1 (AFM1) as a class 2B carcinogenic agent, which is very likely to affect humans, especially infants. The presence of AFM1 in milk, especially cow’s milk greatly affects the health condition of humans, where milk is a food that is often consumed, both by adults, and especially children. The existence of AFM1 should not be underestimated and neglected because of the uncertainty of current climatic and environmental conditions and the inability of agricultural systems to deal with and manage mycotoxin contamination [3]. In 2002, IARC has reclassified AFM1 as Group 1 carcinogenic agent, although its carcinogenicity is approximately 2 – 10 % of AFB1 [4].

According to ASEAN Food Reference Laboratories (2015), many countries have determined regulations regarding to the maximum limit of aflatoxin content in food products, especially AFM1 on dairy products. The maximum limit value for each country varies i.e undetectable or 0 μg/kg, max. 0.05 μg/kg, max. 0.2 μg/kg, max. 0.5 μg/kg, max. 5 μg/kg, and max. 15 μg/kg [5,6]. The Indonesian regulation governing the maximum limit of AFM1 in powdered milk can be found in the Regulation of the Head of Indonesia National Agency of Drug and Food Control No. HK.00.06.1.52.4011 about Determination of the Maximum Limit of Microbial and Chemical Contaminants in Food. Under this regulation, the maximum limit of AFM1 in powdered milk and powdered cream and analog (plain) is 5 μg/kg, while in sweetened condensed milk and its analog is 0.5 μg/kg [7].

Research and study about the content of AFM1 in dairy products has been widely conducted. In 2003, Widiastuti found that 78.38% of fresh cow’s milk samples in Darmaga Bogor were detected containing AFM1, with a concentration range of 0.001 to 1.200μg/l [1]. Celik reported his research on AFM1 contamination in pasteurized milk in Ankara, where 88.23% of samples were proven to be contaminated with AFM1, with 64% of it were above 0.05 μg/kg [8]. Jovana Kos has detected AFM1 content in 98.7% of cow’s milk sample in Serbia, with concentrations 0.01 to 1.2 μg/kg [9]. Langat has also examined the AFM1 content in milk and dairy products from Bomet County, Kenya, where 84.32% samples were positively contaminated with AFM1 in concentrations above 0.05 μg/l (52% raw milk, and 8.6% processed milk) [10].

This research aims to detect AFM1 content in powdered milk and sweetened condensed milk sold in mini market and traditional market in several cities in Java. These are the most dairy products consumed by the Indonesian population [11]. Data from Statistics Indonesia (BPS) based on Susenas
in March 2016, stated that consumption per capita per week for sweetened condensed milk and infant powdered milk is 0.013 kg and 0.542 ounces respectively [12].

2. Materials and methods

2.1 Materials and instruments

Samples were plain powdered milk according to SNI 2970:2015 and sweetened condensed milk according to SNI 2971:2011, which obtained from 4 cities in Java, namely Serang, Bandung, Semarang, and Surabaya. HPLC Brand Waters type e2695 Alliance, with Immunoaffinity column (IAC), coupled with fluorescence detector type 2475 FLR, was used to determine AFM1.

2.2 Methods

The powdered milk is weighed 10 g, added with 50 mL of 50 ºC distilled water, stirred, and put in to 50 ºC sonicator for 30 min, then cooled to 20-25 ºC. The sweetened condensed milk is heated in a water bath until the temperature of sample reach 35 - 37 ºC, centrifuged for 15 minutes (1000 rpm), filtered by Whatman paper no. 4 and put in a 50 mL flask. A total of 50 mL for each prepared samples, poured into immunoaffinity columns. Wash the column using PBS, and dissolve aflatoxin from the column using methanol, then place the eluate in the test tube. A total of 0.5 mL of eluate was mixed with 0.5 mL aquabidest, vortex mixed briefly and analyzed using HPLC (Fluorescence detector, 100 C18 chromosome column, and 25% acetonitrile as mobile phase). A total of two replicates for each standard solution and samples injected 50 μL into the HPLC system. The content of AFM1 (mg/kg) is calculated by this following formula:

\[
C_{sp} = \frac{A_{sp} \times C_{st} \times V_{st} \times V_{A}}{A_{st} \times W_{sp} \times V_{sp} \times f_{p}}
\]  

\( C_{sp} \) = concentration of sample (mg/kg)  
\( A_{st} \) = standard area (mAU)  
\( A_{sp} \) = sample area (mAU)  
\( C_{st} \) = concentration of AFM1 (mg/l)  
\( V_{st} \) = standard volume injected (μl)  
\( V_{sp} \) = sample volume injected, (μl)  
\( V_{A} \) = end volume (ml)  
\( f_{p} \) = dilution factor  
\( W_{sp} \) = weight of sample (gram)

3. Result and Discussion

Sample of sweetened condensed milk and powdered milk were taken randomly without considering any kind of variables, such as packaging material, packaging size, time of production, shelf life, storage area, distribution line, and selling location. Sampling was conducted during February - April 2016. Location, packaging and weather condition data of sampling are seen as Table 1.

Determination of AFM1’s content in powdered milk and sweetened condensed milk, focusing on enrichment extraction using immunoaffinity chromatography (immunoaffinity column) combined with quantification using HPLC. The HPLC system used in this research is kromosil 100 C18 columns, mobile phase acetonitrile 25 %, and Fluorescence detector.

Busman [13] said that HPLC based methods, often require several sample preparation steps, such as extraction, concentration, and isolation, to achieve desired method performance. Sample preparation can involve cleanup steps utilizing liquid-liquid extraction, solid-phase extraction and separation using immunoaffinity column. Enrichment extraction technique like immunoaffinity
chromatography provides opportunity for substantial analyte enrichment, while removing matrix components that could potentially attenuate analyte signal.

| Sampling Location | Sample | Packaging | Weather | Remark |
|------------------|--------|-----------|---------|--------|
| Surabaya         | powdered milk | sachet 40 g & box 200 g pouch & sachet | sunny | purchased in minimarket |
| Serang           | powdered milk | box 200 g & 400 g can & pouch | sunny | purchased in minimarket |
| Semarang         | powdered milk | sachet 40 g & box 200 g sachet | sunny | purchased in traditional market |
| Bandung          | powdered milk | box 200 g & 400 g pouch & sachet | sunny | purchased in minimarket |

Analysis results of AFM1 in powdered milk and in sweetened condensed milk are described on Table 2 and Table 3 respectively. According to Table 2, the concentration of AFM1 in powdered milk was at undetectable to 0.549 μg/kg. All powdered milk samples have met the requirement for maximum limit of AFM1 content in powdered milk, stipulated by Indonesian regulation which is 5 μg/kg [7]. While according to Table 3, the concentration of AFM1 in sweetened condensed milk is ranged from 0 – 0.056 μg/kg. All sweetened condensed milk sample have met the requirement from Indonesian regulatory for maximum limit of AFM1 content in sweetened condensed milk, which is 0.5 μg/kg [7].

| No. | Packaging | Size (g) | AFM1 (μg/kg) | Sampling location |
|-----|-----------|----------|--------------|-------------------|
| 1   | Sachet    | 40       | 0.064        | Surabaya          |
| 2   | Sachet    | 40       | 0.048        | Surabaya          |
| 3   | Box       | 200      | 0.072        | Surabaya          |
| 4   | Box       | 200      | 0.117        | Surabaya          |
| 5   | Box       | 200      | 0.087        | Surabaya          |
| 6   | Box       | 200      | 0.123        | Serang            |
| 7   | Box       | 400      | <0.025       | Serang            |
| 8   | Box       | 200      | 0.053        | Serang            |
| 9   | Box       | 400      | 0.064        | Serang            |
| 10  | Box       | 200      | 0.107        | Serang            |
| 11  | Box       | 200      | <0.025       | Serang            |
| 12  | Sachet    | 40       | 0.151        | Semarang          |
| 13  | Sachet    | 40       | 0.549        | Semarang          |
| 14  | Sachet    | 40       | 0.203        | Semarang          |
| 15  | Sachet    | 40       | 0.204        | Semarang          |
| 16  | Box       | 200      | 0.075        | Bandung           |
| 17  | Box       | 200      | 0.121        | Bandung           |
| 18  | Box       | 400      | 0.026        | Bandung           |
| 19  | Box       | 200      | 0.293        | Bandung           |
Table 3. Analysis results of AFM1 in sweetened condensed milk

| No. | Packaging | Size (g) | AFM1 (µg/kg) | Sampling location |
|-----|-----------|----------|--------------|------------------|
| 20  | Box       | 400      | <0.025       | Bandung          |

Referring to national and international regulatory [6], the data distribution of AFM1 concentration in powdered milk and sweetened condensed milk is shown in Table 4 and 5.

Table 4. Data distribution of AFM1 content in powdered milk

| AFM1 (µg/kg) | Amount of Data | Total |
|--------------|----------------|-------|
|              | Surabaya | Serang | Semarang | Bandung |
| Undetectable |         |        |          |         |
| <0.025 < x < 0.05 | 0 | 1 | 1 | 1 | 3 (15%) |
| 0.05 < x ≤ 0.2 | 4 | 4 | 1 | 2 | 11 (55%) |
| 0.2 < x ≤ 0.5 | 0 | 0 | 2 | 1 | 3 (15%) |
| x > 0.5 | 0 | 0 | 1 | 0 | 1 (5%) |

Table 5. Data distribution of AFM1 content in sweetened condensed milk

| AFM1 (µg/kg) | Amount of data | Total |
|--------------|----------------|-------|
|              | Surabaya | Serang | Semarang | Bandung |
| Undetectable |         |        |          |         |
| 0 < x < 0.05 | 3 | 1 | 0 | 3 | 7 (43.75 %) |
| 0.05 < x ≤ 0.2 | 0 | 3 | 1 | 0 | 4 (25%) |
| 0.2 < x ≤ 0.5 | 0 | 0 | 0 | 0 | 0 (0%) |
| x > 0.5 | 0 | 0 | 0 | 0 | 0 (0%) |

Based on Table 4, the percentage of powdered milk containing AFM1 consist of 15 % undetectable, 10 % ranged from <0.025 to 0.05 µg/kg, 55 % ranged from 0.05 – 0.2 µg/kg, 15 %
ranged from 0.2 – 0.5 µg/kg, and 5 % is above 0.5 µg/kg. These data show that the most AFM1 concentration contained in powdered milk is ranged from 0.05 – 0.2 µg/kg. On the other hand Table 5 show that the percentage of the most AFM1 concentration contained in sweetened condensed milk is ranged from <0.025– 0.05 µg/kg (43.75 %). Concentration AFM1 in sweetened condensed milk for undetectable is 31.25 % samples, ranged from <0.025 – 0.05 µg/kg were 43.75 % samples, and ranged from 0.05 – 0.2 µg/kg were 25 %.

AFM1 that already contaminated milk as raw material for powdered milk and sweetened condensed milk is difficult to control during processing, either by heat treatments, storage at low temperature, concentration and drying of milk, or, chemical and other biological treatment. Some of technologies that have involved in order to decreasing the level of AFM1 in milk processing are by the addition of oxidator agents and adsorber, but unfortunately, this technology has not been used commercially. The most likely effort to decrease the AFM1 levels is by controlling the liquid milk.

The presence of AFM1 in dairy products needs further attention, due to its high consumption, especially for infants and children, and its carcinogenic effect. Therefore it is necessary to apply a technology that can reduce AFM1 contaminants in dairy products. The study about preservative for milk has been conducted. Hypothiocyanite-rich-solution from lactoperoxidase system, obtained from bovine whey, was remarkably inhibited the growth of total bacteria in fresh milk [13,14]. But its activity to decrease the concentration of AFM1 in milk is still unclear. There is a review on prevention efforts to reduce aflatoxin content in animal feed and dairy products. The reduction of aflatoxin content in livestock feed is done indirectly, such as through monitoring of aflatoxin infections on farms, by protecting food from infectious sources, detain the spread of microorganisms, storage of raw materials, and control of humidity. The reduction of aflatoxin content in dairy products can be done through the use of absorbents such as bentonite, verticulate, etc., as well as the use of chemicals, such as hydrogen peroxide that used during the storage. The use of microorganisms such as Flavobacterium aurantiacum can also be used to reduce aflatoxin content in milk [15,16]. Other methods that have been used to reduce AFM1 levels is through fermentation, including the addition of antioxidant compounds such as carotenoids that found in carrots [17].

4. Conclusion

The content of AFM1 in powdered milk is ranged from undetectable to 0.549 µg/kg. According to data distribution, the most AFM1 concentration contained in powdered milk is ranged from 0.05 – 0.2 µg/kg, with the percentage is 55 %. The content of AFM1 in sweetened condensed milk is ranged from 0 – 0.056 µg/kg, and according to data distribution, the most AFM1 concentration contained in sweetened condensed milk is ranged from <0.025 – 0.05 µg/kg, with the percentage is 43.75 %. These results showed that all the samples have met the Indonesian regulatory requirement for maximum level of AFM1 in powdered milk and sweetened condensed milk.

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