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Seasonal study of aflatoxin M_1 contamination in milk of four dairy species in Yazd, Iran

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ABSTRACT

This survey was conducted to determine the occurrence of aflatoxin M_1 (AFM_1) in samples of raw milk obtained from cow, sheep, goat, and camel herds in Yazd province during different seasons. Aflatoxin M_1 was analyzed using the competitive enzyme-linked immunosorbent assay technique for screening and high-performance liquid chromatography with fluorescence detection for confirmatory purposes. The detection rates of AFM_1 in cow, sheep, goat, and camel milk samples were 46.5%, 21.6%, 20.1%, and 4.03%, respectively. Levels of the toxin in 15.4% of cow milk, 11.5% of sheep milk, and 9.15% of goat milk samples exceeded the legal limit (0.050 μg/kg) recommended by the Institute of Standards and Industrial Research of Iran; while none of the camel milk samples exceeded the legal limit. The occurrence and levels of AFM_1 in cow milk samples from industrial dairy farms was significantly lower ($P \leq 0.05$) than those from traditional ones. Seasonal variations influenced the occurrence and levels of AFM_1 in cow, sheep, and goat milk; however, no statistically significant seasonal effect was found for camel milk. This study indicates a high occurrence of AFM_1 in cow milk especially those obtained from traditional dairy farms. Therefore, more supervision is required on these farms; and traditional dairy farms should be gradually replaced by industrial ones.

1. Introduction

Aflatoxins are secondary toxic metabolites of fungi produced by toxigenic strains of *Aspergillus* section *Flavi* species such as *A. flavus*, *A. parasiticus*, and *A. nomius* under critical conditions of temperature and humidity. These toxins are produced in various agricultural products during harvest and post-harvest processing. Due to their detrimental effects such as mutagenic, carcinogenic, and immunosuppressive effects, presence of aflatoxins in food and feed could be hazardous for health of humans and animals (Becker-Algeri et al., 2016; Binder, 2007; Fallah, Jafari, Fallah, & Rahnama, 2009; Fallah, Pirali-Kheirabadi, Rahnama, Saei-Dehkordi, & Pirali-Kheirabadi, 2014).

Up to now, more than 300 aflatoxins have been identified, and the most common occurring is aflatoxin B_1 (AFB_1). It is also the most powerful natural carcinogen in mammals (Creppy, 2002), which is classified as Group 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993) of World Health Organization (WHO).

Aflatoxin M_1 (AFM_1) is the monohydroxylated derivative of AFB_1, biotransformed at the hepatic level by microsomal cytochrome *P450* and excreted into the milk of lactating animals that have ingested AFB_1 contaminated feedstuffs. A linear relationship has been found between the content of AFM_1 in milk and AFB_1 consumption through feedstuffs. The conversion rate of AFB_1 to AFM_1 has been estimated between 0.5% and 6%. In lactating animals, 12–24 h after intake of AFB_1 contaminated ration, AFM_1 could be detected in milk, which decreases to an undetectable level within 72 h after stopping the intake of contaminated source (Fallah, 2010b; Felores-Felores, Lizarraga, de Cerain, & González-peñas, 2015; Galvano, Galofaro, & Galvano, 1996). Although, the toxicity of AFM_1 is less than its parent compound, AFB_1, the IARC (2002) of WHO classified AFM_1 as Group 1 human carcinogen due to its well-proved cytotoxic and carcinogenic effects.

Several countries have set up maximum residue levels (MRL) of AFM_1 in milk and dairy products to protect consumers, particularly children. The MRL of AFM_1 varies between 0.050 μg/kg approved by

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the European Commission (European Commission, 2001) to 0.50 μg/kg established by the US Food and Drug Administration (US FDA, 1996). The Institute of Standards and Industrial Research of Iran (ISIRI, 2002) has accepted 0.050 μg/kg as MRL for AFM_1, which is the same as the European Commission MRL.

Several surveys have been conducted on the occurrence of AFM_1 in milk and dairy products in different parts of Iran (Kamkar, Fallah, & Mozaffari Nejad, 2014). However, according to the scientific literature, no study was performed in this subject in Yazd province, located in central part of Iran. This study aimed to determine the occurrence and levels of AFM_1 contamination in raw milk of four dairy species (cow, sheep, goat, and camel) in Yazd province during different seasons.

2. Materials and methods

2.1. Sample collection

During year 2014, a total of 808 milk samples were collected from 78 cow, 52 sheep, 41 goat, and 31 camel herds in Yazd province of Iran. The samples (2000 ml each) were obtained from bulk tank milk of each herd during winter, spring, summer, and autumn, transported to the laboratory inside an icebox, and frozen at − 20 °C prior to analyses.

2.2. Chemicals and reagents

All reagents were of analytical grade unless otherwise stated. Acetonitrile (liquid chromatography grade), sodium chloride, sodium hydroxide, sodium phosphate dibasic, hydrochloric acid, methanol (liquid chromatography grade), potassium chloride, and potassium phosphate monobasic were purchased from Merck (Darmstadt, Germany). Deionized water (Milli-Q Millipore 18.2 MΩ/cm resistivity) was used throughout this study. Standard solution of AFM_1 (1000 ng/ml, in 6 ml acetonitrile) and AFLAPREP® M immunoaffinity column were obtained from R-Biopharm Rhône Ltd. (Glasgow, Scotland).

Stock solution of AFM_1 was diluted in acetonitrile to prepare the intermediate standard solution of 50 ng/ml. Working standard solutions of AFM_1 were individually prepared by diluting of intermediate standard solution in HPLC mobile phase.

2.3. Enzyme-linked immunosorbent assay (ELISA) analysis

Presence of AFM_1 in the milk samples was determined by competitive ELISA using RIDASCREEN® Aflatoxin M_1 (Art No.: R1121, R-Biopharm, Darmstadt, Germany) test kit. Preparation of the samples and ELISA test procedure were performed according to the instruction provided by the manufacturer. The detection limit of the method was 0.005 μg/kg for AFM_1 in milk. The recovery scores of the method at different spiking levels (0.010, 0.025, 0.050, and 0.075 μg/kg; $n = 6$ for each level) were in the range of 93.9–101% for cow milk, 92.5–97.1% for sheep milk, 91.9–98.4% for goat milk, and 90.7–95.3% for camel milk. The relative standard deviations were less than 10%.

2.4. High-performance liquid chromatography (HPLC) determination

After rapid screening by ELISA, presence of AFM_1 in the positive milk samples was confirmed and also quantified with the official HPLC reference method described by Institute of Standards and Industrial Research of Iran (ISIRI, 2010).

A milk sample (100 ml) was warmed up to 35 °C in a water bath under gentle stirring, and then centrifuged at 4000 rpm for 12 min. After centrifugation, the upper thin fat layer was discarded and a portion of skimmed milk (25 g) was passed through the immunoaffinity column at a flow rate of 2 ml/min. The column was washed with 20 ml of PBS (pH = 7.4), and AFM_1 was eluted from the column with 2.5 ml of a mixture containing methanol and acetonitrile (40:60, v/v) at a flow rate of 1 drop/s. The extract was evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved in 1 ml of mobile phase and filtered through a 0.45 μm syringe filter prior to HPLC analysis.

A HPLC system (Agilent 1100 chromatograph; Agilent Corporation, Santa Clara, CA, USA) equipped with a quaternary pump, a vacuum degasser, and a fluorescence detector (FLD) was used for quantification of AFM_1. The chromatographic separation was carried out on a Discovery® C18 HPLC column (250 mm × 4.6 mm i.d., 5 μm particle size) protected with a Discovery® C18 Supelguard column (2 mm × 4.6 mm i.d., 5 μm particle size) both from Supelco (Bellefonte, USA). The mobile phase was a mixture of water, acetonitrile, and methanol (60:20:20, *v/v/v*) with a flow rate of 1.0 ml/min. FLD was operated at wavelengths of 360 and 430 nm for excitation and emission, respectively. The injection volume of the samples or standard solutions was 100 μl.

2.5. Validation of the HPLC method

The validation parameters including sensitivity, accuracy, precision, and linearity were evaluated to ensure the method performance quality.

Sensitivity was expressed in terms of limit of detection (LOD) and limit of quantification (LOQ), which were calculated as signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. Accuracy of the method evaluated by analyzing of blank milk samples spiked with AFM_1 at levels of 0.025, 0.050, 0.075, and 0.100 μg/kg. For each type of milk, analyses were performed with 6 replicates at each level. The analyses were carried out on three different occasions with the same instruments, but with different batches of reagents and different operators. The recovery value was calculated by the following formula: (the measured concentration/the spiked concentration) × 100. The precision was expressed in terms of repeatability and within-laboratory reproducibility as relative standard deviation (RSD) of the recovery. To check the linearity, a six-point calibration curve was constructed with AFM_1 standard solutions in the concentration range of 0.05–0.2 ng/ml, each concentration injected in triplicate. The linearity was evaluated by linear regression analysis and expressed as squared correlation coefficient (R^2).

2.6. Statistical analyses

The data were statistically analyzed using the SPSS software version 20 for Windows (SPSS Inc., Chicago, IL, USA). The statistical tests were one-way ANOVA and *t*-test to evaluate the differences in AFM_1 levels among the seasons and between the traditional and industrial dairy farms, respectively. Moreover, chi-square test was used to compare the detection rates of AFM_1 among the seasons and between the traditional and industrial dairy farms. The differences were considered statistically significant at $P \leq 0.05$.

3. Results and discussion

3.1. HPLC method validation

The HPLC chromatograms demonstrated in Fig. 1 are related to AFM_1 standard solution (0.1 ng/ml), blank sample of milk, and spiked blank sample with AFM_1 (0.050 μg/l). It is evident that no interfering peaks were detected near the retention time of AFM_1