https://doi.org/10.22319/rmcp.v12i2.4856

Technical note

Presence of aflatoxin B_1 in goat feed in goat milk production units of the Mexican Highlands

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Abstract:

The presence of aflatoxins in silage and grains intended for feeding lactating ruminants entails a problem for animal health and milk safety. The objective of this study was to determine the aflatoxin B_1 content in feed consumed by goats from four goat milk production units in the Mexican highlands (MHL). Samples (n= 47) of concentrates and 29 samples of silage from four goat milk production units in the Mexican Highlands (MHL) were analyzed by High Performance Liquid Chromatography (HPLC), using a reversed-phase column and fluorescence detection after the derivatization of aflatoxins. The results showed that 38.29 % and 31.02 % of the concentrate and silage samples, respectively, had AfB_1 levels above the maximum permissible limit established by the European Union (EU) (0.05 µg/kg); while 29.78 % and 10.34 % for concentrates and silage, respectively, presented values higher than the 20 µg/kg proposed by the official Mexican standard. The results obtained corroborate the current problem of the presence

of aflatoxins in the diet of lactating goats, as this toxin can be metabolized into aflatoxin M_1 and affect the safety of milk and milk derivatives.

Key words: Aflatoxin B_1, Concentrate, Silage, HPLC, Lactating goats.

Received: 13/04/2018

Accepted: 08/01/2020

Today, food safety and quality assurance is an important factor for many countries[1]. The health and productivity of an animal, along with the quality and safety of the milk it produces, depend on the quality and management of the feed it consumes. No feed intended for the nutrition of dairy animals should present any risk of physical, chemical or microbiological contamination. Both commercial feed and feed produced on the farm should be considered as a potential health risk; for this reason, before it is provided to dairy cattle such as goats, it should be carefully examined in order to ensure the absence of contaminants (soil, foreign bodies, wire, fungi, among others)[2].

Food contamination by fungi is a very frequent phenomenon, due to the fact that their spores are widely distributed in the environment (air, water, soil) so that agricultural production (mainly grains and seeds) is affected by more than 25 % by the presence of some type of mycotoxins[3]. Fungi can develop at any point in the food chain under conditions (pH, relative humidity, grain moisture, viability, storage time, and the presence of microflora) that favor their development[4]; it is worth mentioning that some species of fungi are able to colonize and produce aflatoxins in different media, such as food and animal feed.

Aflatoxins are toxic substances produced in the secondary metabolism of the fungi *Aspergillus flavus*, *Aspergillus parasiticus*, and *Penicillium puberulum*[5]. Eighteen (18) types of aflatoxins have been identified, of which six are significant food contaminants: B_1, B_2, G_1, G_2, M_1, and M_2; AfB_1 is the most carcinogenic and toxic of these[4]. Aflatoxins, when found in fodder, silage and concentrates, can be present in their original form, or metabolized in animal tissues when consumed by animals. Its metabolites include aflatoxin M_1 (AfM_1), which is excreted in milk[6,7].

The first studies on the determination of AfM_1 in Mexico were carried out in the state of Sonora by Esqueda *et al*[8] in samples of ultra-pasteurized cow's milk marketed in that state, and the presence of AfB_1 in feed for dairy goats in Mexican herds has not been reported either. Some of the countries in which research has been conducted on goat food, milk and dairy products are Egypt[9,10], Cuba[11], Portugal[12], Spain[13], Italy[14-17], Brazil[18], Turkey[19,20] Kenya[21,22], and South Africa[23]. Most of the researches determined the transfer of AfB_1 from food to AfM_1 in milk and cheese. They also evaluated the AfM_1 content in milk and cheese, finding up to 69 % of positive samples

with levels above the maximum permissible limit established by the European Union (EU), of 0.05 µg/kg for milk, and up to 19 % of positive samples with levels above the maximum permissible limit established by the EU for cheese. In Mexico, the current status of the presence of AfB_1 in goat feed is unknown, and so is the permanent application of methods and techniques to assess this mycotoxin considered as pathogenic.

The objective of this study was to determine the AfB_1 content in feed for goats from four goat milk production units of the Mexican Highlands (MHL). The methodology was developed as follows: 47 samples of concentrates (composed mainly of corn, sorghum and soybean) and 29 samples of corn silage were obtained from four goat milk production units (Unit 1, Unit 2, Unit 3 and Unit 4) of the MHL. Samples were taken simultaneously each month during the period from June 2008 to August 2009, according to the methodology proposed by the Norm NOM-188-SSA1-2002[24]. The minimum amount for each sample was one kilogram, taken from different points of the lot. Samples were transported to the laboratory in clean, labeled containers, protected against contamination and deterioration during transport. Samples were analyzed for AfB_1 content by HPLC with fluorescence detector according to AOAC Methods 968.22 (extraction and chromatographic column), 971.22 (preparation of solutions and determination of aflatoxin concentration), and 990.33 (derivatization)[25], as well as to the ISO 14718 method (high performance liquid chromatography)[26].

For sample processing, 500 g of sample were ground in a mill and passed through a sieve with an aperture of 1 mm. Subsequently, the sample was divided by the quarting procedure, taking the sample diagonally, mixed and stored in a nylon bag or tightly closed flask. For the extraction, 50 g of previously ground and sieved sample was weighed to the nearest 0.1 g and placed in an Erlenmeyer flask with a lid, and 25 g of Celite 545, 25 ml of water and 250 ml of chloroform were added to it. The cap was secured and mechanically agitated for 30 min. Subsequently, the solution was decanted by passing it through a fluted filter paper. The first 50 ml of the filtrate were collected and stored in an amber bottle at 4 ºC until analysis.

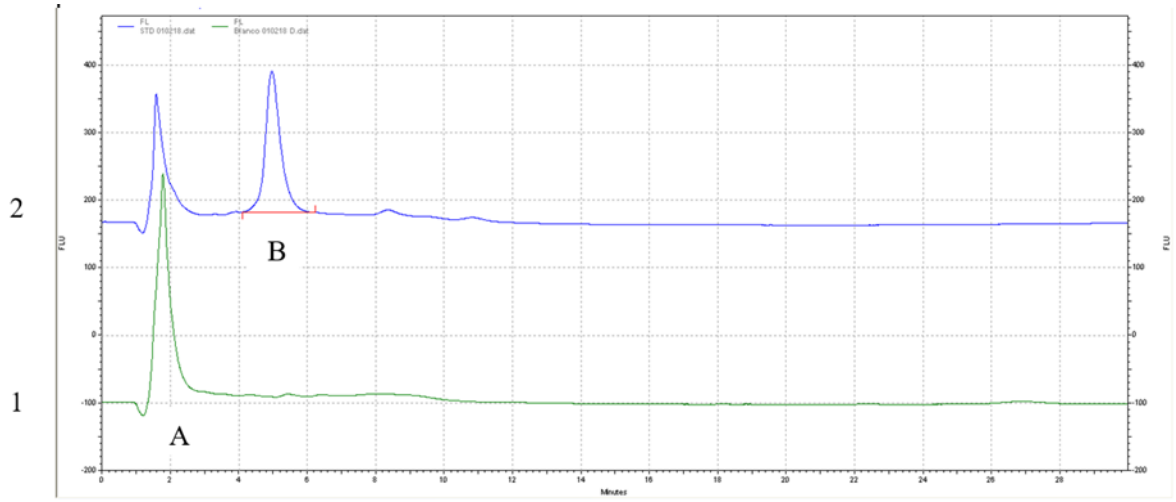
For purification, the bottom of a chromatographic column was filled with glass wool, 5 g of anhydrous sodium sulfate (J.T. Baker) and a sufficient amount of chloroform was added up to the middle of the column. Next, a solution of 10 g of silica gel (J.T. Baker) in chloroform was added by sliding it over the walls of the column. The column walls were washed with 20 ml of chloroform and allowed to drain. When 5 to 7 cm of chloroform remained above the silica, 15 g of anhydrous sodium sulfate were added leaving 1 to 2 cm of chloroform above the top of the sulfate. The chloroform was then drained to the top of the sulfate. The sample was added and drained until it reached the top of the column, which was washed with 150 ml of hexane and 150 ml of diethyl ether. Both washes were discarded and the aflatoxins were eluted with 150 ml of a mixture of chloroform and methanol (97:3 v/v). The chloroform:methanol eluate was rotoevaporated to near dryness under reduced pressure at a temperature of 30 ºC. The

residue obtained was transferred to a vial and recovered with 500 to 1000 µl of chloroform and evaporated to dryness under nitrogen. 200 µl of hexane were added to the dry residue and stirred with the vortex (ZX4 Advanced IR Vortex Mixer- VELP Scientifica) for 1 min. Subsequently, 50 µl of trifluoroacetic acid were added, capped and vortexed for 1 min and placed in a double boiler at 40 ºC for 30 min. After this time, they were evaporated under nitrogen. The residue was resuspended in 500 µl of the mobile phase (200 ml methanol, 200 ml acetonitrile, and 600 ml water, filtered and degassed) before being subjected to a chromatography. In order to derivatize the standard, 50 µl of the known concentration of the standard was taken, brought to dryness under nitrogen and proceeded in the same way as described above. A Merck-Hitachi high-performance liquid chromatograph with a LaCrhom-7480 fluorescence detector (excitation and emission length of 350 nm and 450 nm, respectively) and a C_{18} Lichrocart 100 reverse phase column (5µm 250 x 0.4 cm) were used. The flow rate was 1 ml/min. First, a mobile phase followed by the standard was applied to check the retention times. Subsequently, 50 µl of the eluate from the samples were applied.

Chromatograms were recorded using a Perkin Elmer NCI 900 interface and processed with TOTALCHROM version 6.2 software. Method validation was performed under the guidelines of the National Metrology Center (Centro Nacional de Metrología)[27]. A standard curve with known concentrations of 0.1, 0.25, 0.5, and 1 µg/ml was prepared from the working solution in order to establish the linearity, limit of detection, limit of quantification, and accuracy of the method.

The results were as follows: Figure 1 shows the chromatographic profile of AfB_1 when the standard is derivatized with trifluoroacetic acid. The retention time was 5.21 ± 0.10 min. The calibration curve (y=312869.71x+218056.30) showed significant linearity ($P<0.05$) over a range of 0.10 to 1 µg/ml with a regression coefficient of 0.99. The limits of detection and quantification were 0.241 and 0.43 µg/kg, respectively. Recovery was 87 %. The results obtained were satisfactory according to the criteria proposed in the Laboratory Guide for Method Validation[27], and they show that the method was efficient in assessing the presence of AfB_1 at the levels required by the EU[28] and the Norm NOM-188-SSA1-2002[24] for 5 and 20 µg/kg, respectively.

Figure 1: Chromatographic profile of the derivatized AfB_1 standard (2.19 µg/ml)



Chromatogram 1 is of the reagent blank where the signal (A) corresponds precisely to the solvent peak.

Table 1 shows a summary of the number of concentrate and silage samples at different AfB_1 content ranges, where it is observed that 38.29 and 31.0 % of the samples exhibited AfB_1 levels above the maximum permissible limit established by the EU (5 µg/kg) for concentrates and silage, respectively[28]. On the other hand, 29.78 and 10.3 % of the concentrate and silage samples, respectively, exceed the value established in the Mexican norm (NOM-188-SSA1-2002)[24].

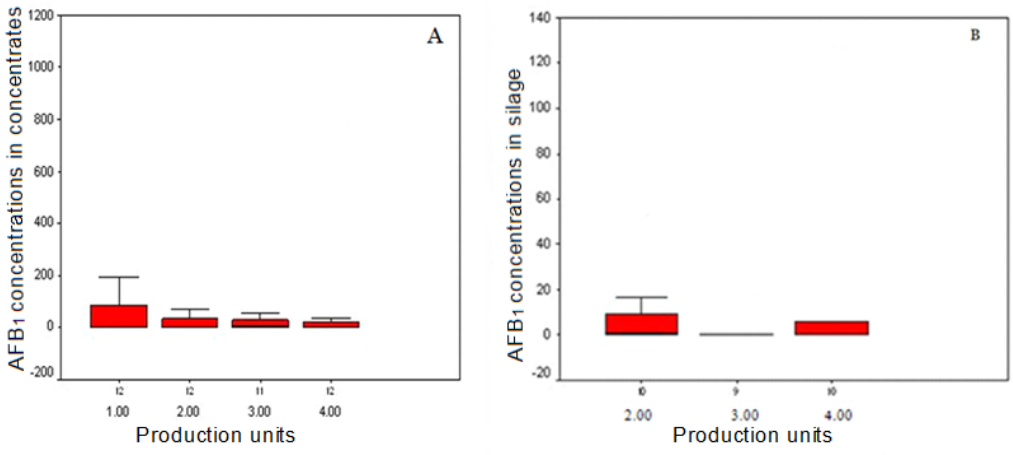
Table 1: Occurrence of aflatoxin B_1 in concentrate and silage samples in four goat milk production units

Frequency distribution in %			
Matrix	**Concentrate**	**Silage**	**Total**
Analyzed samples, No.	47	29	76
Positive samples exceeding the MPL established by the EU, %	38.3	31	69.3
Positive samples exceeding the MPL established by the NOM, %	29.8	10.3	41.1
nd-5 µg/kg	29 (61.7%)	20 (68.9%)	49 (64.4%)
5-20 µg/kg	4 (8.5%)	6 (20.6%)	10 (13.1%)
>20 µg/kg	14 (29.7%)	3 (10.3%)	17 (22.3%)

MPL = maximum permitted level; EU= European Union; NOM= Mexican Official Norm.

Figure 2 shows the ranges of aflatoxin B_1 concentrations per unit for concentrate and silage samples, where the concentration range ranged from 0.46-974 µg/kg and 0.44-123.98 µg/kg, respectively. Unit 1 for concentrate showed the highest aflatoxin values with a median of 125 µg/kg, while unit 2 for silage showed a median of 8 µg/kg. 22.3% of all samples (concentrates and silage) showed values above 20 µg/kg, reaching levels up to 50 times the permissible value, an aspect of great concern due to the risk it may pose to the safety of dairy products[29].

Figure 2: AfB_1 concentrations in concentrates (A) and silage (B) from four goat milk production units in the Mexican highlands



In graph B in Unit 1, the aflatoxin value is not provided because this product is not offered.

The presence of aflatoxins in corn in high concentrations in the order of thousands of µg/kg has been reported by several authors in the African region[30], where there is a predominance of a subtropical and tropical climate characterized by high temperature and humidity, which, together with poor agricultural and production practices, favors the growth of these toxins[31]. Reports in other regions such as Asia and Latin America[32], also report high aflatoxin levels in food, which corroborates the global problem of this toxin and has been alerted by the Food and Agriculture Organization of the United Nations (FAO) in different scenarios, where the risk of aflatoxin contamination is expected to increase in corn due to the effect of climate change[33].

Another factor that conditions the level and synthesis of aflatoxins is the substrate. Thus, foods with high concentrations of carbohydrates favor the production of toxins[29]. Carbohydrates are the most important part of cereals such as corn, oats, sorghum and soybeans, used for the production of concentrates that are fed to goats, and are therefore more susceptible to fungal contamination and the consequent synthesis of aflatoxins. The presence of aflatoxin B_1 in silage has also been reported when aerobic spoilage occurs during processing, which favors the growth of pathogenic microorganisms and the production of endotoxins and mycotoxins[34,35]. A study carried out in Brazil showed aflatoxin B_1 values in corn silage in a range of 0-100 µg/g in pre- and post-fermented samples[36], which are higher than those found in this study. Other studies also reflect

high counts of fungi, which could affect the palatability of feed and reduce nutrient absorption by animals[37]. However, the greatest causes for concern are the consumption of these foods by lactating animals and the presence of metabolic products in dairy products, which will eventually affect human health, mainly that of infants.

The presence of the aflatoxin M_1 in milk is a result of the metabolic transformation of aflatoxin B_1; thus, a contamination rate of 13.4 % in feed materials results in AfM_1 levels estimated between 0.22 to 3.47 %[38]. A study conducted on 17 goat farms in northeastern Italy showed a positive correlation (0.6) between the presence of aflatoxin B_1 in the concentrate and aflatoxin M_1 in milk, where concentrations of 5 µg/kg aflatoxin B_1 in feed exhibited an aflatoxin M_1 conversion rate of 0.5 % (25 ng/kg milk)[39]. These results alert regulatory agencies in predicting the presence of aflatoxin M_1 in milk in these units. Given that the median concentration of AfB_1 in concentrate and silage was higher than 20 µg/kg in this study and considering a conversion rate of 0.5%, it is possible to find more than 100 ng of aflatoxin per kilo, which is above the MPL established by the FDA. On the other hand, greater control of the feed used in livestock farms is required in order to minimize the impact of mycotoxins on the dairy industry and public health. This aspect has been corroborated in several studies when there is a continuous improvement of feeding techniques in dairy farms[40].

There was a high concentration of AfB_1 in the four goat milk production units of the MHL, above the maximum permissible limits established. Therefore, it is necessary to pursue further research and to develop a permanent detection program in these units in order to avoid batches of food contaminated with AfB_1, since it should be kept in mind that the absence of AfB_1 is very important for the dairy industry, because when an animal ingests food contaminated with AfB_1, between 1 and 3% of this aflatoxin is metabolized into the milk and dairy products in the form of AfM_1, which affects the quality and safety of milk and dairy products.

The presence of aflatoxin B_1 in silage in Mexico was reported herein for the first time, an aspect that should be further researched to detect the presence of other mycotoxins that also affect public health, such as ochratoxin, zearalenone and fumonisins, which have been reported in tropical countries such as Brazil. On the other hand, given that goat milk production in Mexico has increased in the last decade and due to the lack of information on this subject, longitudinal studies should be strengthened in order to understand the presence of AfB_1 and AfM_1 in goat production, adding as well other areas with great potential for goat milk production.

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