Title: Surveillance of aflatoxin content in dairy cow feedstuff from Navarra (Spain).

Abstract: Aflatoxins (B_1, B_2, G_1 and G_2) are produced by the fungi *Aspergillus (A. flavus and A. parasiticus)* in substrates used in cattle feed manufacturing. Aflatoxin M_1 (AFM_1) is a major metabolite of Aflatoxin B_1 (AFB_1) which may be present in milk from animals that consume contaminated feed. Levels of aflatoxins in 78 dairy cow feedstuff samples from 40 farms located in Navarra were determined by HPLC-FLD (High Performance Liquid Chromatography with fluorescence detection) and post-column derivatization. The influence of geographical location, season and type of feeding system on aflatoxin content was studied. The climatic profile of AFB_1 pointed to spring as the season with the highest aflatoxin level (0.086 µg/kg), followed by winter and summer (0.075 and 0.030 µg/kg, respectively), and to a lesser degree, autumn (0.017 µg/kg). Moreover, wet and dry TMR (Total Mixed Ration) feeding systems (i.e. AFB_1: 0.076 and 0.068 µg/kg; Aflatoxin G_1 (AFG_1): 0.050 and 0.011 µg/kg, respectively) showed a greater content of the analyzed aflatoxins in comparison with compound feed (i.e. AFB_1: 0.039 µg/kg; AFG_1: 0.007 µg/kg). The fact that the majority of the samples collected were based on compound feed shows that this type was preferred by most dairy farmers. The undetectable levels of aflatoxins in the organic homemade compound feedstuff are also worth mentioning. While none of the feedstuff samples contained amounts over those permitted under European legislation (5 µg/kg), the theoretical extrapolation of the carryover rate suggested in previously published experiments of AFB_1 to AFM_1 in secreted cow's milk predicts that only one of the feed samples studied had a positive aflatoxin level (53.4 ng/kg) higher than the legal limit for raw cow's milk.

Keywords: aflatoxins, dairy cattle, feed contamination, feed crops, feed analysis, high-performance liquid chromatography.

1. Introduction

Mycotoxins constitute a potential threat to international public health (Méndez-Albores et al., 2007) because of their frequent occurrence in foodstuffs for humans and animals. These compounds are a heterogeneous group with very diverse origins. Aflatoxins (AFs) are produced by mainly *Aspergillus flavus* and *Aspergillus parasiticus*. These mycotoxins may occur during harvesting, storage (and transport), production technology, processing and preparation of food. Moreover, the occurrence of AFs is enhanced by several factors such as stress due to drought before harvesting, insect activity, soil type and inadequate storage conditions.

There are more than twenty distinct, but structurally related, aflatoxin compounds. Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) appear in many food products, but especially in those with a high carbohydrate and lipid content such as nuts (peanuts, pistachios, walnuts), dried fruits (figs), cereals (maize), spices (pepper), seeds, cocoa and beer, as a result of fungal contamination before or after harvest (Garrido et al., 2012; Oruc et al., 2006). Most of the other AFs described in the reference literature come from hydroxylation at different points in the molecular structure of these AFs. In this respect, aflatoxins M_1 (AFM_1) and M_2 (AFM_2), 4-hydroxy derivative of AFB_1 and AFB_2 respectively, are found in mammals secretions (urine and milk). AFM_1 mammary excretion begins approximately 12-24 hours after animals have ingested AFB_1 contaminated food and disappears about 24 to 72 hours after its absence in the diet (Zinedine et al., 2007a).

AFs are extremely toxic: these compounds are immunosuppressive, mutagenic, teratogenic and carcinogenic in most organisms. The International Agency for Research on Cancer (IARC) has classified AFB and AFG in group 1 as human carcinogens, the

liver being the main target organ for toxicity (IARC, 2012; Zain, 2011; Giray et al., 2007).

The transformation of AFB_1 from feedstuffs to AFM_1, consumed by cows, and subsequently carried over into secreted milk, depends on several feed-related factors (quantity, characteristics of the food consumed and the dose level of AFB_1), metabolism (milk yield, lactation stage, species, breed, time of day) and other factors such as weather and/or geographical location of dairy farms (Masoero et al., 2007). Taking into account all these relevant considerations, the predicted rate of AFB_1/AFM_1 carry-over from feedstuff into milk is approximately 0.3 to 6.0% (Heshmati and Milani, 2010). Van Eijkeren et al. (2006) proposed a steady-state model for predicting the correlation between AFB_1-contaminated feedstuff consumed by a dairy cow and AFM_1 excreted into milk.

Due to the toxicity of AFB_1, Directive 2002/32/EC provided a limit for undesirable substances in animal feedstuffs with 12% moisture content, setting an upper limit of 5 μg/kg for AFB_1 in complete feedstuffs for dairy cattle (EC, 2002). In addition, the EFSA CONTAM Panel (Scientific Panel on Contaminants in the Food Chain) has recently concluded that the currently established maximum levels for AFB_1 in animal feed not only provide adequate protection from adverse health effects in target animal species, but more importantly, appear to successfully prevent undesirable concentrations of AFM_1 in milk. Therefore, there is no need to modify the existing maximum levels for AFB_1 (EFSA, 2004).

However, the occurrence of different AFs in animal diet during its production or storage is quite heterogeneous and depends on many factors: the environmental conditions during fungal growth, the different feeding patterns depending on the season, agricultural practices, etc. It therefore seems reasonable to ask for closer surveillance

and monitoring of food products, cereals and fodder for animal consumption (Signorini et al., 2012).

At this respect, the AFs production is not particularly restricted to any ingredient of the animal feeding but the AFs levels vary, as mentioned above, with location and climatic profile which determine the risk of contamination in the dairy cow feeding (Bryden, 2012). As the aflatoxin-contaminated dairy cow feed is intrinsically related to a deficient dairy farming, any threat to feed security could involve a significant impact on the economic vitality of the dairy cow farm (Cheli et al. 2013). Cow milk farmers have often attempted different strategies to reduce feed costs. The evaluation of the cost-effectiveness of different types of dairy cow feeding systems is a common practice. In this regard, the total mixed rations (TMRs) are widespread based on economics and practicality. Nonetheless, an adequate choice of the dairy cow feeding system is crucial to avoid the potential risk of aflatoxin contamination of feedstuffs, contributing with a negligible aflatoxin exposure of the dairy cows fed on. Therefore, a complete description of different feeding systems based on AFs content will be useful to provide satisfactory data for dairy cow farmer to develop a traceability system with the purpose of minimizing a potential hazardous exposure. Taking into account these points, the rationale for the current work is the assessment of the AFs concentration levels supplied by different dairy cow feeding systems: i) based on compound feed (conventionally and organically produced) supplied together with alfalfa, hay and straw to complete the TMR; ii) wet- and iii) dry- TMR feeding systems combining all forages, grains, protein feeds, minerals, vitamins and feed additives, manufactured with different moisture. As it is evidenced by their qualitative composition, all studied feeding systems might supply a similar source of aflatoxin contamination. Hence the similarity in these compounds allows a helpful statistical comparison of different groups of cow feedstuff in relation to

the well-known factors of mycotoxin contamination (Driehuis et al. 2008; Cheli et al. 2013). Specifically, the aims of the present study are to evaluate: a) the occurrence of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in different dairy cow feedstuff samples; b) the potential relationship between the degree of contamination with these mycotoxins and the influence of seasonal factors, geographical location and animal feeding systems; c) to assess the exposure of dairy cattle to AFB_1; and d) to estimate, based on the theoretical intake, its biotransformation into AFM_1 and the subsequent carryover into raw cow's milk.

2. Materials and methods

2.1 Dairy cow feeding sampling

The animal feed study was carried out in 2008 in collaboration with the Danone cow milk collection center (Ultzama, Navarra, Spain); and included several dairy farms from 5 different sampling areas (Baztán: 43.15ºN, 1.50ºW; Malderreka-Leitza: 43.14ºN, 1.77ºW; Aralar-Ultzama: 42.96ºN, 1.76ºW; Erro: 42.97ºN, 1.42ºW; and Zona Media: 42.52ºN, 1.71ºW), representing the overall production in Navarra (Spain).

A stratified random sampling with proportionate stratification for a total of 40 dairy farms (Baztan: n=15, Malderreka-Leitza: n=4, Aralar-Ultzama: n=12, Erro: n=6 and Zona Media: n=3) was carried out. The guidelines for sampling for the official control of mycotoxins in foodstuffs imposed by Regulation No. 401/2006 from the European Commision (EC) were strictly followed (EC, 2006a). Trained professionals from Danone cow milk collection center carried out the dairy cow feed sampling. In this respect, the sampling plan was adapted to the routine collection of Danone quality control samples. Dairy cow feedstuff samples (2 kg per each sample) were taken monthly from conventional and organic farms. To obtain representative samples, these

primary samples were homogenized and quartered to obtain a 250 g sample for laboratory analysis. Samples were stored at −20ºC until analyzed. A total of 6-7 samples per month were collected (n=78), taking into consideration the study factors.

The sampling includes different samples of dairy cow feedstuff provided as a TMR. The rations of different feeding systems were formulated or balanced correctly to be an effective and nutritionally appropriate way to feed dairy cow; classified as follow: i) Complete ration mix based on dairy cow compound feed (n=59; the TMR is made up of concentrate feed – containing mainly as an example: maize (252 g/kg fresh weight, FW), soya bean meal (177 g/kg FW), barley (134 g/kg FW), dried maize distillers grains (129 g/kg FW), maize gluten feed (103 g/kg FW), palm kernel meal (63 g/kg FW), mineral salts (50 g/kg FW), sorghum (49 g/kg FW), rapeseed oil (21 g/kg FW), molasses (17 g/kg FW) and unmolassed sugarbeet pulp (5 g/kg FW) –, formulated in pellets and provided together with alfalfa hay and straw to meet the specific nutritional requirements for dairy cattle), ii) wet TMR of silage (n=10; a TMR based on corn silage (250 g/kg FW), alfalfa hay (118 g/kg FW), feed barley (101 g/kg FW) and other materials such as grass silage (97 g/kg FW), wet grains (maize, 92 g/kg FW and sorghum 19 g/kg FW), fresh orange pulp (64 g/kg FW), dried maize distillers grains (63 g/kg FW), maize gluten feed (63 g/kg FW), soya bean meal (34 g/kg FW), palm kernel meal (20 g/kg FW), cassava flour (15 g/kg FW), forage wheat flour (13 g/kg FW) mineral salts (17 g/kg FW), sorghum (19 g/kg FW), molasses (6 g/kg FW), unmolassed sugarbeet pulp (5 g/kg FW) and rapeseed oil (4 g/kg FW), with a high water content), dry fodder-cereal TMR (n=6; it corresponds with a complete mixed ration based on dry cereal, straw or fodder mixed with cereals, and protein supplements; as an example a typical dry mixture contains: maize (350 g/kg FW), soya bean meal (134 g/kg FW), alfalfa hay (123 g/kg FW), dried maize distillers grains (113 g/kg FW), palm kernel

meal (40 g/kg FW), cottonseed (49 g/kg FW), mineral salts (39 g/kg FW), barley (35 g/kg FW), molasses (33 g/kg FW), dried orange pulp (20 g/kg FW), maize gluten feed (26 g/kg FW), soya bean (21 g/kg FW) and unmolassed sugarbeet pulp (17 g/kg FW)); and, iv) organic homemade compound feed-based TMR (n=3, a homemade balanced formulation carried out as a combination of several organic ingredients: forage, hay, cereal straw, maize and other cereals, supplemented with vitamins and minerals, to provide all the nutritional needs for the dairy cow. Maize, barley, alfalfa hay and straw were organically grown by the dairy cow farmer).

2.2 Dairy cow feedstuff consumption estimate

The dairy cow is fed according to very specific feeding guidelines. The predicted food consumption depends on several factors: body weight, milk yield, ration diet quality (the ability to metabolize food expressed by means of the q value: the ratio between metabolizable energy (ME) and gross energy (GE)) and physiological animal needs, lactation and gestation stages. The total nutritional requirement was estimated as the sum of the requirements for appropriate bodily functions. Thus, the calculation base will use two initial assumptions. The first assumption includes the following: i) a dairy cow weight estimated average of 600 kg, ii) a theoretical daily milk production of 20 liters, iii) feeding by a high performance diet with a q value of 0.6, and iv) application of a correction factor on consumption of the dry substance, after the twelfth week of lactation, corresponding to the unit. Therefore, a predictive value of intake of 17.0 kg of dry diet, which is 19.32 and 32.08 kg of total diet for those feeding systems based on compound feed or dry mixture and wet mixture, respectively (taking into account an average moisture contents of approximately 120 g/kg FW -880 g/kg dry matter basis-

and 470 g/kg FW -530 g/kg dry matter basis-, respectively) was established (Chamberlain and Wilkinson, 2002).

The feed intake estimates created by the above methods were generally consistent with the observations of the dairy farmers, cow milk collection center specialized professionals and dairy cow feeding manufacturers we spoke to.

The second assumption is that the median and maximum aflatoxin values, representative of the overall distribution and the extreme situation, respectively, should be used.

In short, the theoretical dietary aflatoxin intake was estimated using the median and maximum aflatoxin values obtained for the different feeding systems studied and the theoretical value of feeding intake above assumed.

2.3 Chemicals and Reagents

A commercial solution of AFB_1, AFB_2, AFG_1 and AFG_2 in methanol, 1000 ng/mL (250 ng/mL AFB_1, 250 ng/mL AFB_2, 250 ng/mL AFG_1, 250 ng/mL AFG_2, Aflastandard, P22A, R-Biopharm, Madrid, Spain), methanol and acetonitrile (ACN) HPLC gradient grade (Merck, Barcelona, Spain), ultrapure deionized water Type I reagent grade (Wasserlab, Noain, Spain), nitric acid 65% (Merck, Barcelona, Spain) and potassium bromide (Merck, Barcelona, Spain) were used for standards and mobile phase.

Sodium chloride and PBS (phosphate buffered solution pH=7.4) containing potassium chloride, sodium phosphate dibasic anhydrous, and potassium phosphate monobasic, purchased from Panreac (Barcelona, Spain), were used during the extraction and purification of AFs.

2.4 Analytical procedure

2.4.1 Extraction and purification

A total of 50 g of cattle feed sample, 4 g of sodium chloride and 250 mL of extracting agent ACN/H_2O (60/40, v/v) were mixed in a blender jar for 2 minutes at high speed. Next, the extract was filtered through Whatman No. 4 filter paper (Whatman International, Maidstone, UK). An aliquot of 25 mL was evaporated at a temperature of 30ºC and a rotation speed of 65 rpm for 8 minutes in a rotavapor (Buchi R-3000 Rotavapor; Büchi Labortechnik AG, Postfach, Switzerland). The evaporation residue was collected, mixed with 500 µL ACN and transferred into a 50 mL volumetricflask; and finally diluted to volume with a solution of PBS.

Next, 10 mL of the reconstituted extract were passed through the immunoaffinity column (Aflaprep, P07, R-Biopharm, Madrid, Spain) to carry out the clean-up of all AFs in order to avoid any type of interference, at a flow rate of 2 mL/min. The column was washed with two 10 mL aliquots of ultrapure water at a flow rate of 5 mL/min, and all analyzed AFs were slowly released from the antibody using 1 mL of methanol and then eluted with 1 mL of ultrapure water. The last step consisted of filtering the eluted samples with a PVDF (Polyvinylidene Fluoride) syringe filter (13 mm, 0.22 µm, Tecnokroma, Barcelona, Spain), and collecting them in vials for HPLC analysis.

2.4.2. HPLC determination of AFs

A Luna C18 (2) column of 4.6 x 150 mm, 5 µm particle size, 100Å (Phenomenex, Torrance, CA, USA), protected by a pre-column (Phenomenex, Torrance, CA, USA): pre-column holder, analytical guard cartridge system (4.6 x 10 mm), cartridge guard column and C_{18} cartridges security guard (4 x 3 mm), was used for the separation of AFs in a 1100 Series HPLC system (Agilent Technologies, Barcelona, Spain) equipped

with a quaternary pump (G1311A), autosampler (G1313A) and a fluorescence detector FP-2020 Plus communicated via a LC-Net II/ADC (Jasco, Madrid, Spain). A post-column derivatization with a Kobra Cell system (R-Biopharm, Madrid, Spain) was used to quantify AFB_1 and AFG_1. The chromatographic conditions which were previously optimized for determining AFs in sample extracts are specified in the reference literature (Hernández-Martínez and Navarro-Blasco, 2010).

2.4.3 HPLC method performance

In order to verify and validate the analytical method, aflatoxin-free dairy cow feed samples were spiked with standard aflatoxin solutions at the levels of 2.50, 6.25 and 12.50 μg/kg. Six replicates of each sample were tested to assess the recovery. The intra-day (RSD_r) repeatability and inter-day (RSD_R) reproducibility were also ascertained at spiking levels as mentioned above. The reference ranges of accuracy (Recoveries according to AFB_1 concentration levels: lower than 1 μg/kg: 50-120%, from 1 to 10 μg/kg: 70-110%, and higher than 10 μg/kg: 80-110%) and precision (RSD_r and RSD_R according to AFB_1 concentration levels: lower than 1 μg/kg: ≤40% and ≤60%; from 1 to 10 μg/kg: ≤20% and ≤30%; and higher than 10 μg/kg: ≤15% and ≤20%, respectively), set by the report UNE-CR 13505 (UNE, 2003), are imperatives. The results were corrected by mean recovery rates obtained from the recovery experiments.

In addition, the accuracy of the method was checked by evaluating several naturally contaminated reference materials (Animal feed P64-ASF3 and P64-ASF4, and Ground corn P64-A227, R-Biopharm, Madrid, Spain). Moreover, the laboratory participated in a FAPAS® (Food Analysis Performance Assessment Scheme, organized by The Food and Environment Research Agency, Sand Hutton, York, United Kingdom, in accordance with ISO 5725-2) inter-laboratory proficiency test (# 04124), where 70

participants of 27 different countries analyzed an animal feed material based on cereals and determine the content of AFB_1, AFB_2, AFG_1 and AFG_2 and total aflatoxin. These profiency testing qualify a satisfactory result when z-score is less than 2. The proficiency testing is an independent check of the laboratory procedures providing the assurance of accurate aflatoxin results with the analytical method used. Limits of detection (LODs) and quantification (LOQs) were determined at a signal-to-noise (S/N) ratio of 3/1 and 10/1, respectively.

2.4.4 Safety

Extreme caution, using goggles, mask and gloves of category III, must be exerted when handling pure aflatoxin solution within a laminar flow extractor hood.

2.5 Statistical analysis

All the statistical analyses of the data were carried out using an SPSS (Statistical Package for the Social Sciences) program, version 15.0.1. At first, the Kolmogorov-Smirnov statistic established whether or not the data followed a normal distribution. All data are taken into account for the statistical study; samples under the limit of detection (LOD) and with a detected signal were assumed to be at a concentration value of half of the LOD. Different groups of dairy cattle feed samples classified by geographical area, season and feeding system were compared using a non-parametric Kruskal-Wallis test and a Mann-Whitney U-test with a statistical significance set at $P<0.05$, due to the lack of normal distribution and the limited samples in some of the groups.

3. Results

3.1 Quality assurance assays

Table 1 shows the recoveries and both relative standard deviation for within-day (RSD_r) and between-day (RSD_R) samples at different assayed levels. These values fall within the reference ranges of accuracy and precision set by the report UNE-CR 13505 (UNE, 2003). The overall recoveries of AFB_1, AFB_2, AFG_1 and AFG_2 were (mean±s.d.): 83.7±3.8%, 83.2±3.2%, 81.9±2.4% and 70.2±1.1%, respectively.

In addition, the accuracy of the method (Table 2) was also demonstrated by the good agreement with the results obtained by the reference material samples. Besides, during this study, the results of an inter-laboratory study of AFB_1, AFB_2, AFG_1 and AFG_2 and total aflatoxin, (FAPAS® programme), indicated a satisfactory z-score and a suitable performance of the analytical methodology (data are shown in Table 2).

3.2 Content of AFs in dairy cow feedstuff

Table 3 shows the distribution of aflatoxin content in the feed samples assayed; that is, the percentage of samples which are above the limit of the quantification, between the two limits, below the detection limit, and finally, the non-detected levels. The experimental results for the content of AFs (B_1, B_2, G_1, G_2 and total) for feed samples collected from the different farms are also summarized (Table 3).

A detailed analysis of these data indicates that 90% of the analyzed feed samples contain detectable aflatoxin (70 samples detected out of a total of 78). Among these, 74%, 32%, 58% and 22% of dairy cow feedstuff samples exhibit a higher content than the limit of detection for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Quantitatively, the feed samples analyzed have an overall upper concentration level (median (First quartile, Q1); Third quartile, Q3)) for AFB_1 (0.040 (0.005;0.099) μg/kg), intermediate for AFG_1 (0.007 (non-detected.(n.d.);0.044) μg/kg) and very low for both AFB_2 (n.d. (n.d.;0.013) μg/kg) and AFG_2 (n.d. (n.d.;0.003) μg/kg).

3.2 Influence of geographical areas, seasons and feeding system

Table 4 shows the concentration of aflatoxin in bovine feedstuff from the regions under study. In view of these data it is worth pointing out: i) the slightly higher content found in samples coming from Baztan (AFB_1: 0.061 μg/kg), and Zona Media (AFB_1: 0.054 μg/kg and AFG_1: 0.028 μg/kg); ii) the low levels found in Erro (AFG_1: 0.003 μg/kg); and iii) the almost unchanging values found for both AFB_2 and AFG_2 distributions, except in the case of a few specific samples (AFB_2: 0.444 μg/kg and AFG_2: 1.002 μg/kg).

The seasonal profile of AFs in the analyzed samples of dairy cow feedstuff is shown in Fig. 1. AFB_1 and AFG_2 content differs significantly ($P<0.005$, Kruskal-Wallis test), which points to spring as the most disadvantaged season with the highest aflatoxin level found, as shown by the homogeneous subsets displayed in Fig. 1.

The evaluation of AFs content regarding the type of feedstuff consumed, allows assessment in relation to the dietary pattern (Table 5). Wet and dry TMR feeding systems show a greater occurrence of the analyzed AFs in comparison with compound feed. It is worth highlighting the non-detected levels of AFs in organic homemade compound feed samples.

4. Discussion

4.1 Incidence of AFs in dairy cow feedstuff

The AFs content shown in Table 3 is in agreement with the overall presence of aflatoxin B_1 in different food matrices (Van Eijkeren et al., 2006). In this sense, these aflatoxin levels are comparable to those found in countries with more restrictive legislation. Baydar et al. (2005) evaluated the content of aflatoxin in samples of seeds

and cereal flours in Turkey. A total of 64% and 72% of the samples studied exhibited an AFB_1 and AFG_1 content between 0.03-1.61 μg/kg and 0.03-2.79 μg/kg, respectively. Zinedine et al. (2007b) and Simas et al. (2007) reported concentration ranges of AFB_1 (0.05-5.38 and 1-3 μg/kg, respectively) and analogous incidence (66.6%) in poultry feed and dairy cattle feed samples, respectively, that were also similar to the findings obtained in this study. In China, Han et al. (2013) analyzed 200 dairy cow feed samples, which they found to contain AFB_1 in the range of 0.05-3.53 μg/kg.

Animal feed samples from Kuwait, Brazil, Indonesia, Thailand, Philippines, Sudan and Asian-Oceania region show a notable level of AFB_1: 0.64-19.9 (Dashti et al., 2009), n.d.-29.04 (Sassahara et al., 2005), 54 (Goto et al., 1999), 72 (Yoshizawa et al., 1996), 369 μg/kg (Arim et al., 1999), 5.94-327.73 μg/kg (Elzupir et al., 2009) and 13.9 μg/kg (Borutova et al., 2012), respectively. This pattern is observed in those geographic areas characterized by high temperature and humidity.

Maize is the main component of animal feed (EFSA, 2012, 2013) and unfortunately, the literature links it to aflatoxin in the dairy cow diet (Whitlow and Hagler, 2002). Bankole and Mabekoje (2004), and Fu et al. (2008) found approximately 20% of aflatoxin incidence in corn samples and obtained levels of AFB_1 in the range of 3-130 and 2.41 μg/kg; AFB_2: 4-26 and 0.68 μg/kg; AFG_1: 5-11 and 1.72 μg/kg and AFG_2: 7 and 0.86 μg/kg, respectively. Whitlow and Hagler (2002) reported higher AFB_1 content in maize silage and grain corn in samples from North Carolina, with a mean concentration of 28 and 170 μg/kg, respectively.

In this respect, AFB_1 value found in this study did not exceed the level legislated by the European Union (EU) for dairy cattle feed of 5 μg/kg, in line with data reported by Han et al. (2013). However, a previous study of dairy cow feed carried out over a period of ten years (1995-2004) in Portugal showed that 6.2% of the samples exceeded

the EU limit. It should be noted that in the last two years of research, none of the studies reached this upper limit, probably related to the increasing surveillance measures and quality control of raw materials used for manufacturing the feeds in this country during the past few years (Martins et al., 2007). A similar situation has been shown by Decastelli et al. (2007) who found that in 2004 the occurrence of AFB_1 in cow feed was higher than the maximum allowable in 8.1% of feed samples while in 2005 the presence of this aflatoxin was below the limits of EU regulations.

4.2 Influence of geographical areas, seasons and feeding system on aflatoxin content

Cattle exposure to aflatoxin has been extensively reported in situations where the basic cereal food comes from nearby regions or where a large number of the bovine concentrate feed components is imported from different geographical areas with tropical or subtropical climates (Giray et al., 2007). Taking into account this initial reasoning and the geographic differentiation with regard to ochratoxin content in cereals (Araguás et al., 2005), a statistical study of AFs in dairy cow feed samples collected from the five different geographical areas of study might be relevant. Using the Kruskal Wallis test, no significant differences were found in the mycotoxin levels among the studied samples of dairy cow feed collected from the different geographic areas. Therefore, the dietary supply provided by the different dairy milk farms does not appear to establish a pattern of dependence between the geochemical environment and the levels of the studied AFs, a pattern which is consistent with that reported by Han et al. (2013). The limited influence of the geographical concentration of aflatoxin in animal diet is supported by a previous study (Gómez-Arranz, 2008) which established that the location of the dairy cow farm is not considered to be relevant with regard to the level of AFM_1 in raw cow's milk.

Climate changes or extreme climatic events are affecting the mycotoxin content in human food and animal feed. In this regard, many researchers have ascribed an impact on the proved seasonal variability of AFM_1 in raw cow milk to the nutritional feeding systems, specifically the seasonal ingredients and dietary supplements used for dairy cow diet in the course of the climatic seasons (Gómez-Arranz, 2008; Zinedine et al., 2007a). The sequence of AFB_1 seasonal content (median (Q1;Q3), spring: 0.086 (0.030;0.130), winter: 0.075 (0.037;0.138), summer: 0.030 (0.005;0.082) and autumn: 0.017 (<LOD;0.031) µg/kg) is comparable and seems to reflect with the reported levels of AFM_1 found in cow's milk from the study region by Gómez-Arránz (2008), where the highest values were obtained in the milk collected in spring and winter (AFM_1: 11 (6;18) and 7 (n.d.;17) ng/kg, respectively), followed by an intermediate level in summer samples (AFM_1: 3 (n.d.;10) ng/kg) and lastly, the lowest content was found in autumn (AFM_1: n.d. (n.d.;3) ng/kg). The analysis of 256 samples of feed, including cattle feedstuff, from northern India showed higher contamination during the monsoon (April-July) and post-monsoon seasons, reaching an incidence of 74.26%. Reasonably high temperature, fairly high relative humidity and non-seasonal rains and floods in different regions where crops were located during the harvest season clarify the origin of the contamination. Likewise, an increased concentration of aflatoxin in animal feedstuff has also become evident in those countries with less adverse weather throughout the wet periods (Dalcero et al., 1998).

Nowadays, dairy cow farmers have developed quality management models to establish the best guarantees concerning the origin and quality of dairy cow feed and to ward off the presence of contaminants as a result of poor conservation practices in the area of raw materials, fodders or cereals. The balance between food safety and the economic cost of ensuring the traceability of cattle feed leads to the use of prepared

compound feedstuffs as a routine dietary practice carried out on most of the dairy farms that have been studied (Driehuis et al., 2008). Nevertheless, other feeding systems may also be worth considering. It is reasonable to assume that the samples of dairy cow feedstuff would show uneven fungal development as a direct consequence of the different raw materials, manufacturing methods, degrees of industrialization, and morphological and physicochemical characteristics of the different feeding systems. In this sense, the contents of all the studied AFs differ significantly (AFB_1 P=0.049; AFB_2 P=0.037; AFG_1 P=0.045; AFG_2 P=0.031, Kruskal-Wallis test) for the different groups of dairy cow feedstuffs. Most dairy farmers mainly used the TMR based on compound feed as their chosen feeding system. Moreover, the findings shown in Table 5 make it clear that aflatoxin production differs in wet and dry TMR feeding systems despite the similar composition of the raw materials; this fact might be due to the varying levels of moisture, the uneven colonization by *A. flavus* and *A. parasiticus*, and the different storage periods (Klich, 2007).

The influence of dairy cow feeding on the carryover of AFB_1 to AFM_1 in milk is well-known. The evaluation of the incidence of AFM_1 can be categorized according to the different types of feedstuff supplied. A higher level of contamination was reported by Gómez-Arranz (2008) in milk from the dairy cows fed on wet TMR (AFM_1 7 (3;14) ng/kg); an intermediate level of AFM_1 corresponded to dry TMR and compound feedstuff-based TMR (5 (n.d.;16) and 4 (n.d.;12) ng/kg, respectively); and, finally, the TMR based on organic homemade compound feedstuff was found to have no detectable concentrations over the entire study period, in agreement with the above-mentioned AFB_1 content in the feeding systems. The good practices of the organic cow milk farm meant that we were unable to detect any level of AFM_1. Organic milk production provides environmental benefits because of the reduction of pesticides and phosphate

fertilizers related to the acidification of the surroundings (Cederberg and Mattson, 2000). On the other hand, several studies suggest that there is significant fungal growth and, consequently, mycotoxin contamination in various feeding products (cereal grains, corn and milk) that are organically produced in comparison with their conventional counterparts (Ghidini et al., 2005), pointing to the lack of the inhibitory role in the toxin synthesis in those pesticide-free farms.

The need to establish appropriate animal dietary guidelines is reflected in the fact that the studies regarding aflatoxin content in milk that is organically produced versus that which is conventionally produced are scarce or their results are inconclusive.

4.3 Carry-over to AFM_1 in milk

Assuming the theoretical dairy cow food consumption established above in section 2.2, the daily AFB_1 intake for dairy cows fed on different studied feeding systems has been estimated according to the median and maximum concentration levels of AFB_1 obtained in order to evaluate both the global position and the extreme circumstance, respectively.

Table 6 shows the daily amount of AFB_1 supplied by the different groups of feedstuff studied. AFB_1 intake was expressed as a percentage of the threshold value currently in force. This limit was established by the European Directive 2002/32/EC on undesirable substances in animal feed in order to adopt measures to reduce, or even eliminate, the potential sources of fungal contamination (EC, 2002).

This fact leads to an uncomplicated calculation for the theoretical estimate of the carryover rate of AFB_1 to AFM_1 excreted in cow milk after three to six days (Masoero et al., 2007). Several models (Masoero et al., 2007; Van Eijkeren et al., 2006; Veldman et al., 1992) have been proposed for evaluating the biotransformation of AFB_1. A linear

model with different variations, taking into account different types of feedstuffs and milk yields in all the adjustment equations, is accepted. Usually, a transfer rate ranging from 0.3 to 6% is assumed (Heshmati and Milani, 2010). Table 6 shows the comparison between the different estimated AFM_1 values found in dairy cow milk for each type of feed analyzed.

The difference accounted for the type of feeding system is quite low. The organic homemade compound feedstuff-based TMR does not show any contribution to daily AFB_1 intake. However, the wet and dry TMRs contribute the highest intakes (rates of 0.015 and 0.014 in relation to the threshold value in force, respectively), followed by, to a lesser degree, the complete ration based on compound feedstuff, which supplies an intermediate intake (a rate of 0.008 in respect to the threshold value). Anyway, it is noted that this estimated intake is lower than those reported in the bibliography (Sassahara et al., 2005; Arim et al., 1999; Goto et al., 1999; Yoshizawa et al., 1996), showing that the dairy cow farmers in this study used good practices and took special care with regard to aflatoxin contamination, especially in the case of the organic farmer.

Theoretical extrapolation of the AFB_1 content supplied by studied feed samples according to Van Eijkeren's model, only predicts a hypothetical positive value of AFM_1 (53.43 ng/kg) in secreted cow milk. Therefore, with this exception, no other sample would exceed the statutory level of AFM_1 (50 ng/kg) in raw cow's milk (EC, 2006b), which suggested that the current legal limit of AFB_1 in dairy cow feed does not guarantee that the AFM_1 content will remain within the limit in force for raw cow's milk (Han et al., 2013).

Additionally, the theoretical concentration ranges obtained after biotransformation in this study (Table 6) suggest that the likelihood of AFM_1 contamination by means of the raw cow's milk from the farms studied is not very likely to occur. Nevertheless, this

fact cannot be completely excluded due to the changes in the raw materials of the feed, the intake of an unusually high amount of feed concentrates or the adaptive physiological alterations which occur particularly in high-yielding cows (EFSA, 2004). In this sense, the appraisal presented here is a good tool for predicting potentially hazardous situations regarding aflatoxin contamination.

On this subject, the World Health Organization specifies that raw materials and components used in animal feed should not pose a risk to the final consumer. Thus, it is necessary to: i) implement a control system of critical points, ii) verify compliance with the legislation in force by means of a national sampling plan and lastly, iii) apply the management systems (Hazard Analysis Critical Control Points, HACCP; and Good Manufacturing Practices, GMP) in the feed production chain in order to reduce aflatoxin contamination (Bryden, 2012; Binder et al., 2007; Kan et al., 2007; Vlachou et al., 2004).

5. Conclusion

The low aflatoxin content found in dairy milk feedstuff samples, which in no case exceeded the statutory AFB_1 level in force of 5 μg/kg, is noteworthy. But, the 90% incidence of aflatoxin in feed samples, however small the concentrations, indicates that toxigenic Aspergillus is present in Spain. That finding calls for continued care and vigilance on the part of the dairy farmers in order to maintain the very low AFM_1 content of Spanish milk.

The limited influence of the geographical location on the level of AFs in dairy cow feeding suggests other factors such as seasons, weather, or breed, as the cause of the wide variety of mycotoxins found in some of the feedstuffs included in this study. The seasonal pattern of AFs in dairy cow feed samples, pointing to spring as the most

disadvantaged climatic season, is in accordance with previous studies on AFM_1 in cow's milk. Nevertheless, the good farming practices carried out on the organic dairy farm which provided its own compound feedstuff with no detectable levels of aflatoxin showed proper control of fungal growth. Wet and dry mixing feeding systems had the highest aflatoxin contents. The extrapolation of the carry-over rate of AFB_1 to AFM_1 in secreted cow's milk predicts that only one among the feed sample studied would give a positive level (53.4 ng/kg) higher than that stipulated by law for raw cow's milk. In view of these findings, it appears reasonable to ask that more efforts be made to carry out stricter control regarding cattle nutritional system, especially during the vulnerable seasons.

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Highlights

- Aflatoxins (AFs) content was analyzed in bovine feedstuff from dairy farms in Spain.
- None of the samples exceeds AFB_1 legislation but a high incidence (90%) was found.
- Geographical location, unlike season or feeding system, had limited influence on AFs.
- Organic homemade feedstuff showed non-detected level of AFs throughout the study.

Table 1. Accuracy and precision of the method for aflatoxin determination applied to aflatoxin-free cow feed samples spiked at different concentration levels.

Concentration level spiked (µg/kg)	**Recovery** n=15 (%)	**RSD_r** n=9 (%)	**RSD_R** n=15 (%)
2.50	70.0–87.9	0.54–6.66	3.72–18.51
6.25	69.2–80.4	2.73–3.86	4.48–5.80
12.50	71.4–84.4	3.92–7.59	5.36–9.09

RSD_r: With-day relative standard deviation (Repeatibility)
RSD_R: Between-day relative standard deviation (Reproducibility)

Table 2. Detection and quantification limit (ng/kg) of the method and quality control parameters (aflatoxin content found in certified reference material assayed and the FAPAS interlaboratory test, µg/kg) for aflatoxin determination.

Aflatoxin	LOD	LOQ	Animal feed low level n=27		Animal feed high level n=18		Ground corn n=6		FAPAS interlaboratory proficiency test	
			Obtained [a]	Reference [b]	Obtained [a]	Reference [b]	Obtained [a]	Reference [b]	Obtained [a]	Assigned [c]
AFB_1	3	12	6.68±0.44	6.6±1.6	19.21±1.73	19.5±3.6	9.66±0.54	9.7±1.5	9.99±0.27	9.99
AFB_2	2	9	-	-	1.57±0.06	1.0±0.9	-	-	2.81±0.13	2.64
AFG_1	2	11	-	-	-	-	-	-	4.80±0.28	4.04
AFG_2	2	10	-	-	-	-	-	-	1.81±0.32	1.71
AF_{Total}	-	-	-	-	-	-	-	-	19.41	18.08

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AF_{Total}, sum of aflatoxins
LOD: Limit of detection
LOQ: Limit of quantification
[a] mean±standard deviation
[b] mean±standard deviation at 95% confidence interval
[c] assigned value at z-score=0.

Table 3. Incidence of aflatoxins in feedstuff samples analyzed.

Aflatoxin	No detected (%)	Detected (%)		Median [d] (µg/kg)	(Q_1;Q_3) [e] (µg/kg)	Outliers [f] (µg/kKg)
	n.d. [a]	<LOD [b]	>LOD [c]			
AFB_1	15	10	74	0.040	(0.005;0.099)	3.19
AFB_2	53	14	32	n.d.	(n.d.;0.013)	0.44
AFG_1	33	9	58	0.007	(n.d.;0.044)	0.65
AFG_2	67	12	22	n.d.	(n.d.;0.003)	1.01
AF_{Total}	7.7	2.6	89.7	0.102	(0.034;0.211)	-

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AF_{Total}, sum of aflatoxins

[a] percentage of samples where AF was non detected

[b] percentage of samples with a AF level lower than LOD (limit of detection)

[c] percentage of samples with a AF level higher than LOD (limit of detection)

[d] median of all detected samples

[e] Q_1 and Q_3: First and third quartile values of the distribution of aflatoxins

[f] concentration level of samples that lie outside the overall pattern of a distribution

Table 4. Regional variability of aflatoxins in dairy cow feedstuff.

Aflatoxin	Area	n [a]	Mean [b] (µg/kg)	s.d. [c] (µg/kg)	Median [d] (µg/kg)	(Q_1;Q_3) [e] (µg/kg)
AFB_1	Baztan	27	0.182	0.605	0.061 [n.s.]	(0.026;0.109)
	Malderreka-Leitza	8	0.053	0.066	0.032 [n.s.]	(0.010;0.070)
	Ultzama-Aralar	25	0.067	0.093	0.030 [n.s]	(0.005;0.085)
	Erro	12	0.060	0.060	0.039 [n.s]	(<LOD;0.103)
	Zona Media	6	0.073	0.079	0.054 [n.s.]	(0.005;0.118)
AFB_2	Baztan	27	0.021	0.050	<LOD [n.s.]	(n.d.;0.012)
	Malderreka-Leitza	8	0.015	0.024	0.004 [n.s.]	(n.d.;0.019)
	Ultzama-Aralar	25	0.028	0.059	n.d. [n.s.]	(n.d.;0.024)
	Erro	12	0.026	0.069	n.d. [n.s.]	(n.d.;0.009)
	Zona Media	6	0.074	0.181	n.d. [n.s.]	(n.d.;n.d.)
AFG_1	Baztan	27	0.033	0.043	0.007 [n.s.]	(<LOD;0.050)
	Malderreka-Leitza	8	0.012	0.016	0.007 [n.s.]	(n.d.;0.018)
	Ultzama-Aralar	25	0.060	0.140	0.007 [n.s.]	(n.d.;0.047)
	Erro	12	0.007	0.013	0.003 [n.s.]	(n.d.;0.009)
	Zona Media	6	0.063	0.087	0.028 [n.s.]	(n.d.;0.096)
AFG_2	Baztan	27	0.028	0.077	n.d. [n.s.]	(n.d.;0.003)
	Malderreka-Leitza	8	0.013	0.034	n.d. [n.s.]	(n.d.;0.003)
	Ultzama-Aralar	25	0.081	0.214	n.d. [n.s.]	(n.d.;0.032)
	Erro	12	0.028	0.081	n.d. [n.s.]	(n.d.;<LOD)
	Zona Media	6	0.011	0.026	n.d. [n.s.]	(n.d.;n.d.)

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2

n.s.: no significant differences in aflatoxin content among samples collected from different geographical areas (Mann-Whitney U-test, P>0.05)

n.d.: non detected

[a] total number of samples analysed

[b] arithmetic mean of all samples tested

[c] standard deviation

[d] median of all samples tested

[e] Q_1 and Q_3: First and third quartile values of the distribution of aflatoxins

Table 5. Aflatoxin content (µg/kg) in dairy cow feeds according to the feeding system used.

Aflatoxin	TMR	n [c]	Mean [d] (µg/kg)	s.d. [e] (µg/kg)	Median [f] (µg/kg)	(Q_1;Q_3) [g] (µg/kg)
AFB_1	Comp. feed-based	59	0.119	0.415	0.039 [a]	(0.009;0.092)
	Wet TMR	10	0.075	0.050	0.076 [a]	(0.024;0.118)
	Dry TMR	6	0.066	0.049	0.068 [a]	(0.030;0.112)
	O.H.C.F.-based	3	n.d.	.	n.d. [b]	(n.d.;n.d.)
AFB_2	Comp. feed-based	59	0.029	0.074	n.d. [a,b]	(n.d.;0.018)
	Wet TMR	10	0.003	0.009	n.d. [a,]	(n.d.;n.d.)
	Dry TMR	6	0.068	0.102	0.010 [b]	(0.003;0.144)
	O.H.C.F.-based	3	n.d.	.	n.d.[a,]	(n.d.;n.d.)
AFG_1	Comp. feed-based	59	0.037	0.097	0.007 [a,b]	(n.d.;0.027)
	Wet TMR	10	0.061	0.055	0.050 [a,]	(0.002;0.096)
	Dry TMR	6	0.023	0.035	0.011 [a,]	(0.003;0.021)
	O.H.C.F.-based	3	n.d.	.	n.d.[b]	(n.d.;n.d.)
AFG_2	Comp. feed-based	59	0.021	0.066	n.d.[a]	(n.d.;0.003)
	Wet TMR	10	0.060	0.118	<LOD [a, b]	(n.d.;0.064)
	Dry TMR	6	0.236	0.389	0.068 [b]	(n.d.;0.280)
	O.H.C.F.-based	3	n.d.	.	n.d.[a, b]	(n.d.;n.d.)

TMR: Total mixed ration; Comp. feed-based: Compound feed-based; O.H.C.F.-based: Organic homemade compound feed-based; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2

n.d.: non detected

[a, b] Homogenous subsets from Mann-Whitney U-test. Different letters denote significant differences in aflatoxin content among samples belonging to different types of feeding system ($P<0.01$)

[c] total number of samples analyzed

[d] arithmetic mean of all samples tested

[e] standard deviation

[f] median of all samples tested

[g] Q_1 and Q_3: First and third quartile values of the distribution of aflatoxins

Table 6. Daily AFB_1 intake supplied by dairy cow feedstuff and theoretical carryover estimation to AFM_1 in milk.

TMR	n [a]	Daily intake rate		AFB_1 dietary intake (µg/day)		Carryover to AFM_1 (ng/L)			Positive [i] n (ng/L)
		Median [b]	Max [c]	Median [d]	$(Q_1;Q_3)$ [e]	Median [f]	$(Q_1;Q_3)$ [g]	0.3-6% [h]	
Comp. Feed-based	59	0.008	0.640	0.71	(0.09;1.74)	0.61	(0.08;1.51)	0.01-5.41	1 (53.43)
Wet TMR	10	0.015	0.032	1.48	(0.7;2.13)	1.73	(0.82;2.49)	0.11-6.61	-
Dry TMR	6	0.014	0.021	1.31	(0.59;2.13)	1.13	(0.51;1.84)	0.09-6.60	-
O.H.C.F.-based	3	-	-	-	-	n.d.	n.d.	n.d.	-

TMR: Total mixed ration; Comp. feed-based: Compound feed-based; O.H.C.F.-based: Organic homemade compound feed-based; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFM_1, aflatoxin M_1

n.d.: non detected

[a] total number of samples analyzed

[b] AFB_1 daily intake rate, calculated to the median content, supplied by feed stuff samples analyzed expressed in relation to the threshold value in force

[c] AFB_1 daily intake rate, calculated to the maximum content, supplied by feed stuff samples analyzed expressed in relation to the threshold value in force

[d] AFB_1 daily intake, calculated to the median content, supplied by feed stuff samples analyzed expressed in $\mu g \cdot day^{-1}$

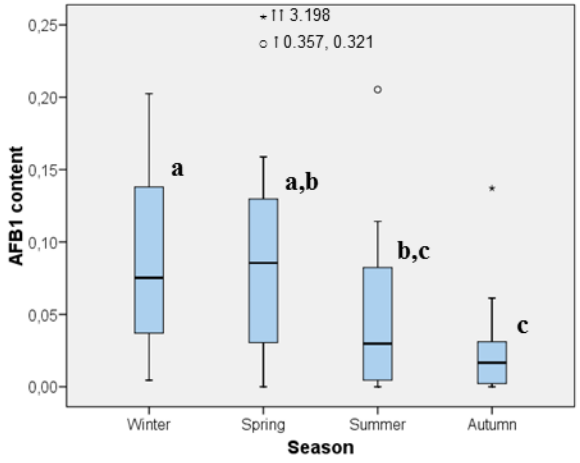
[e] AFB_1 daily intake, calculated to the first and third quartile values, supplied by feed stuff samples analyzed expressed in µg/day

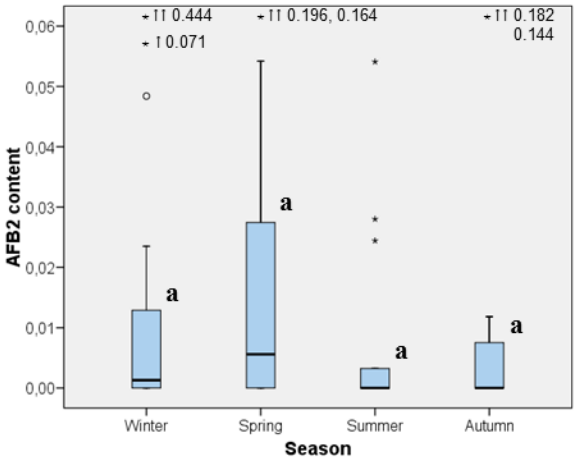
[f] Carryover of AFB_1 to AFM_1 calculated to the median content according to the model proposed by Van Eijkeren et al., 2006

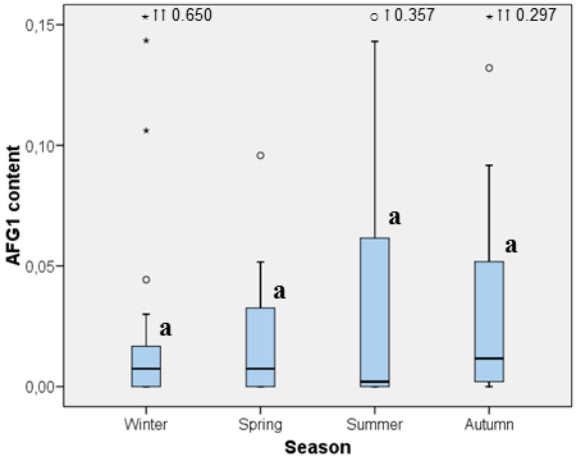
[g] Carryover of AFB_1 to AFM_1 calculated to the first and third quartile values according to the model proposed by Van Eijkeren et al., 2006.

[h] Carryover of AFB_1 to AFM_1 calculated to the median content according to a transfer rate ranging from 0.3 to 6.0% (Heshmati and Milani, 2010)

[i] number of positive samples with a AFM_1 content higher than 50 ng/L (concentration level of the positive sample in ng/L)


3.198
0.357, 0.321
AFB1 content
0,25
0,20
0,15
0,10
0,05
0,00
a
a,b
b,c
c
Winter
Spring
Summer
Autumn
Season


0.444
0.071
0.196, 0.164
0.182
0.144
AFB2 content
0,06
0,05
0,04
0,03
0,02
0,01
0,00
a
a
a
a
Winter
Spring
Summer
Autumn
Season


0.650
0.357
0.297
AFG1 content
0,15
0,10
0,05
0,00
a
a
a
a
Winter
Spring
Summer
Autumn
Season

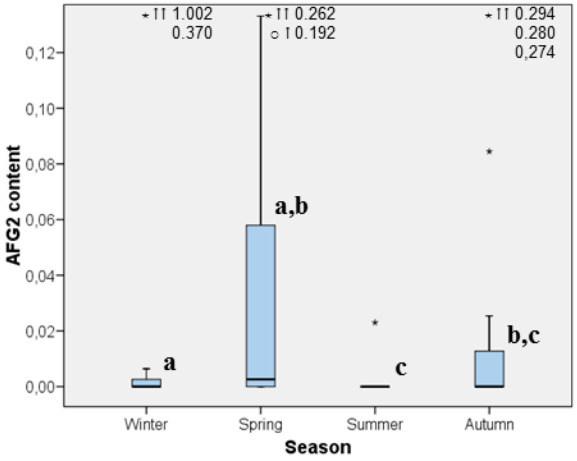

1.002
0.370
0.262
0.192
0.294
0.280
0,274
AFG2 content
0,12
0,10
0,08
0,06
0,04
0,02
0,00
a
a,b
c
b,c
Winter
Spring
Summer
Autumn
Season

Fig. 1. Box-plot (the bottom and top of the box correspond with the first and third quartile, respectively; while the band inside is the median value, and the small circle or star are the outliers) of aflatoxin content (μg/kg) in dairy cow feedstuff depicting the seasonal variability. Aflatoxin abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2. [a, b, c] Homogenous subsets from Mann-Whitney U-test. Different letters denote significant differences in aflatoxin content among samples collected at different seasons ($P<0.05$)