Usefulness of the analytical control of aflatoxins in feedstuffs for dairy cows for the prevention of aflatoxin M$_1$ in milk

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Abstract

Aflatoxin M$_1$ (AFM$_1$) is a hydroxylated metabolite of aflatoxin B$_1$ (AFB$_1$) and can be excreted in milk of cows after consuming aflatoxin (AF)-contaminated feed. The aim of this research was to assess the levels of total AFs in samples of feedingstuff for dairy cows ($n = 193$) and the levels of AFM$_1$ in raw bulk tank milk samples ($n = 375$), in order to estimate the ratio of “AFB$_1$ feed input” versus “AFM$_1$ milk output” in four specific regions of Spain. Moreover, the correlation between the raw materials used as ingredients of the total mixed ration (TMR) and the presence of AFs was studied. About one-third (34.7%) of the feed samples were positive for total AFs in a range of 0.05–6.45 μg/kg, and 12.4% were positive for AFB$_1$. AFM$_1$ was detected in 18.9% of bulk milk samples, with concentrations ranging from 0.009 to 1.36 μg/kg. While none of the feed samples exceeded the European Union (EU) maximum content for AFB$_1$ in feedingstuff for dairy animals of 5 μg/kg, three bulk milk samples exceeded the EU maximum level for AFM$_1$ in milk of 50 ng/kg. The transfer ratio AFB$_1$/AFM$_1$, which was derived from AFB$_1$ levels in feed, AFM$_1$ levels in bulk tank milk, feed intake, and milk yield data, was 0.6–6%, which corresponded well with the range of published carry-over data for aflatoxins. Statistical analyses showed that the main sources of AFB$_1$ in TMR were maize silage, bagasse, soya bean husk, maize, alfalfa hay, cotton seed and compound feed, thus special attention should be paid in controlling these raw materials when used in TMR preparation. Although the analysis of AFs in feed did not correlate with the presence of AFM$_1$ in milk, monitoring feedstuffs is a useful tool in order to try and minimise AF-contamination of milk.

Keywords Aflatoxin B$_1$ · Aflatoxin M$_1$ · Aflatoxin transfer ratio · Total mixed ration · Raw bulk milk

Introduction

The most common mycotoxins that may occur in feedstuffs are aflatoxins (AFs), fumonisins (FBs), zearalenone (ZEN), ochratoxin A and trichothecenes (Driehuis et al. 2008). AFs are mycotoxins produced by Aspergillus species, mainly by A. flavus and A. parasiticus. As a consequence of fungal contamination before or after harvest, these toxins may be found in products such as nuts, dried fruits, cereals, spices, seeds, cocoa or coffee (Juan et al. 2007; Garrido et al. 2012). Up until now, more than 20 different types of AFs have been described; however, the most important in foods are aflatoxin B$_1$ (AFB$_1$), aflatoxin B$_2$ (AFB$_2$), aflatoxin G$_1$ (AFG$_1$), aflatoxin G$_2$ (AFG$_2$) and aflatoxin M$_1$ (AFM$_1$).

AFs are highly toxic, carcinogenic, mutagenic and teratogenic compounds (Bakirdere et al. 2012). AFB$_1$ is the most toxic AF and it is considered as the most powerful natural hepatocarcinogenic agent in mammals. The International Agency for Research on Cancer (IARC) has classified this toxin as a Group 1 human carcinogen (IARC 2012).

AFM$_1$ is a hydroxylated metabolite of AFB$_1$ and can be excreted in milk of cows that have been fed with aflatoxin-contaminated feed (JECFA 2001). AFM$_1$ has been also classified by IARC as Group 1 human carcinogen (IARC 2012). AFM$_1$ can be detected in milk of animals that have consumed feedstuff with AFB$_1$ within 24–48 h and it almost disappears after 72 h (Van Egmond 1989; JECFA 2001). The transformation of AFB$_1$ into AFM$_1$ and its presence in the secreted milk depends on several factors related with feed, metabolism, weather and geographical location of dairy farms (Masoero et al. 2007; Iqbal et al. 2013). The extent of carry-over is influenced by various nutritional and physiological factors,
including regimes, rate of ingestion, health of the animal, hepatic biotransformation capacity, and milk production. Consequently, the rate of absorption of AFs and the excretion of aflatoxins varies between animal, from day to day, and also from one milking to the next. It has been reported that carry-over from feed to cows’ milk appears to increase exponentially with milk yield (Britzi et al. 2013), while published carry-over rates vary from 0.3% to about 6% in high-yielding animals (Applebaum et al. 1982; Veldman et al. 1992; Britzi et al. 2013). To avoid violent levels of AFM$_1$ in milk, maximum levels for AFB$_1$ in feedingstuffs for lactating animals of 5 μg/kg have been set within the European Union by directive 2002/32/EC (European Union 2002). In addition, in order to reduce human exposure to AFM$_1$, the European Commission Regulation 1881/2006 sets a maximum permissible limit of 0.05 μg/kg for AFM$_1$ in raw milk, heat-treated milk, and milk for the manufacture of milk-based products and of 0.025 μg/kg in infant milk and follow-on milk (European Commission 2006). Precisely because of the small amounts of toxin allowed, it is necessary to develop sensitive and specific analytical methods which allow the detection of very small amounts of AFs in animal feed, as well as their quantification.

Nowadays, dairy cows are usually fed using the total mixed ration (TMR) method, that consists on the supply of forages together with different kinds of concentrates or by-products (such as whole cottonseed or compound feed), grains, protein supplements, minerals and vitamins in specific quantities which are mixed thoroughly making up a balanced ration. TMR used to feed dairy cows is usually enriched with concentrates to achieve a high milk yield. Concentrates are low-fibre, high-energy material used as components of the ration for dairy cows in order to raise the energy level and to increase the level of proteins, minerals (macro and micro) and fat-soluble vitamins, compensating any other deficiencies of the total ration derived from the use of the forage portion. These concentrates may be an important source of mycotoxins, as well as other materials such as cereal grains or soybean products, press cakes from oil plants (Nawaz et al. 2005; Mansfield and Kuldau 2007). The aim of this work was to evaluate the occurrence of AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ in different dairy cow TMR samples from farms located in four Spanish areas during 2016–2018 using an in-house validated method. Moreover, the level of AFM$_1$ in bulk milk samples from cows fed with the mentioned feed was assessed in order to evaluate the usefulness of monitoring AFB$_1$ in the TMR as a means of minimising AFM$_1$ content in the milk. The possible relation between the TMR composition and the presence of AFs in feed was also studied.

### Materials and methods

#### Sampling

From February 2016 to January 2018, a total of 193 different feedstuff samples and 375 raw bulk milk samples were collected from dairy farms located in different areas of Spain (Cantabria, Castilla-León, Cataluña and Galicia). Farms with previous problems related to AFs (milk with AFM$_1$ concentrations over 0.015 μg/kg occasionally found), due to weak food safety management systems, were specifically selected. The mean size of these farms was 116 Holstein Friesian lactating cows (range from 25 to 426). Taking into account the pooled data from each farm, it resulted that each cow consumed an average of 45.36 kg of TMR (20.50–77.07 kg) and produced 36.63 L of milk (20.92–90.70 L). The mean content of fat was 3.54% (2.69–4.16%) and 3.19% of protein (2.96–3.59%).

At the beginning of the day, farmers formulated the TMR batch to be consumed in that day using a “unified system”. During the day, at least 40 subsamples of 100 g were taken by specialised technicians. Finally, each farm provided a bulk sample of 4–5 kg of TMR in order to have a representative fraction of the whole. Farms were asked to provide a report containing the composition of each TMR sample and also the composition of the compound feed used as an additional TMR ingredient, however, this information was available for 163 out of the 193 TMR samples received. Data related to the most common ingredients of the feed samples are shown in Table 1. In addition, 37 raw materials used as TMR components including cotton seed (n=12), maize (n=6), compound feed (n=5), soya bean (n=2), okara (n=2), maize silage (n=2), dried mixture (n=1), barley silage (n=1), ryegrass silage (n=1), sugar beet pulp silage (n=1), alfalfa silage (n=1), immature corn silage (n=1), sugar beet pulp (n=1) and soya bean husk (n=1) were received; and they were analysed separately. Bulk milk samples were collected in the dairy farms from the milk refrigeration tank containing milk obtained 24 and 48 h after the bulk TMR was prepared and delivered to the cows. All samples were stored at −18 °C until analysis.

#### Reagents and solutions

The analytical standards of AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$ and AFM$_1$ were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile and methanol were both HPLC grade and were obtained from Scharlab (Sentmenat, Spain). Acetic acid glacial was supplied by Fisher Scientific (Loughborough, UK). Filter paper (Whatman No. 113) was purchased from Whatman ( Maidstone, UK). Immunofluor chromatography columns (IAC) for AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ (Easi-
Table 1  Main ingredients of TMR samples, number of samples that contained each of them and percentage of inclusion in the feed (n = 163). In AFB₁-positive samples (n = 18), it is indicated the range of toxin found in samples that contained each ingredient.

| TMR component (origin)* | Total set of samples | AFB₁-positive samples |
|-------------------------|----------------------|-----------------------|
|                         | No. of samples (%) total | Percentage (%) | No. of samples (%) total | Percentage (%) | Range (μg/kg) |
| Compound feed (N/I)     | 145 (88.96%) | 1.61–59.18 | 17 (94.44%) | 9.52–48.39 | 0.14–4.66 |
| Maize silage (N)        | 107 (65.64%) | 9.92–76.92 | 11 (61.11%) | 18.29–56.45 | 0.21–4.66 |
| Straw (N)               | 95 (58.28%) | 0.68–10.71 | 8 (44.44%) | 1.19–5.82 | 0.14–4.66 |
| Dehydrated alfalfa (N) | 78 (47.85%) | 2.28–25.66 | 7 (38.89%) | 3.32–12.31 | 0.14–4.66 |
| Alfalfa hay (N)         | 63 (38.65%) | 1.63–32.65 | 9 (50.00%) | 3.32–21.81 | 0.25–1.47 |
| Maize (N/I)             | 51 (31.29%) | 0.11–22.83 | 4 (22.22%) | 3.74–14.29 | 0.34–1.04 |
| Grass silage (N)        | 47 (28.83%) | 9.89–89.29 | 5 (27.78%) | 13.48–85.65 | 0.14–1.94 |
| Bagasse (N)             | 40 (24.54%) | 3.56–25.81 | 6 (33.33%) | 13.29–18.59 | 0.25–1.47 |
| Mature corn silage (N)  | 33 (20.25%) | 3.76–19.05 | 1 (5.56%) | 15.58 | 0.98 |
| Sugar beet pulp (N)     | 30 (18.40%) | 0.93–22.79 | 3 (16.67%) | 0.93–10.77 | 0.27–0.98 |
| Cotton seed (N/I)       | 30 (18.40%) | 0.68–5.51 | 2 (11.11%) | 1.66–4.67 | 0.98–1.04 |
| Ryegrass silage (N)     | 28 (17.18%) | 7.60–75.95 | 4 (22.22%) | 18.29–34.29 | 0.34–1.25 |
| Alfalfa silage (N)      | 21 (12.88%) | 5.71–44.78 | 2 (11.11%) | 8.43–22.58 | 0.31–1.47 |
| Defatted soya flour (I) | 21 (12.88%) | 3.04–14.47 | 4 (22.22%) | 3.43–4.02 | 0.25–1.25 |
| Barley (N)              | 21 (12.88%) | 1.13–8.10 | 1 (5.56%) | 2.37 | 1.04 |
| Vetch hay (N)           | 15 (9.20%) | 2.06–13.64 | 3 (16.67%) | 2.37–13.64 | 0.14–4.66 |
| Oat silage (N)          | 13 (7.98%) | 2.00–47.46 | 2 (11.11%) | 9.35–13.33 | 0.34–0.67 |
| Dehydrated ryegrass (N) | 12 (7.36%) | 1.90–8.44 | – | – | – |
| Soybean husk (I)        | 10 (6.13%) | 1.49–2.54 | 1 (5.56%) | 2.42 | 1.47 |
| Barley silage (N)       | 8 (4.91%) | 16.01–23.23 | 3 (16.67%) | 18.29–23.24 | 0.25–1.25 |
| Wheat silage (N)        | 5 (3.07%) | 18.60–32.38 | – | – | – |
| Okara (N)               | 4 (2.45%) | 3.72–8.85 | – | – | – |

* N, national
I, imported

extract® Aflatoxin) and for AFM₁ (Aflaprep® M Wide) were purchased from R-Biopharm (Rhône LTD Glasgow, UK). Pure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (Panreac), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (Panreac) in 1 L of pure water; the pH was brought to 7.4.

Preparation of stock standard solutions

Standard solutions of AFB₁, AFB₂, AFG₁ and AFG₂ were dissolved in methanol at a concentration of 250 mg/L and stored at 4 °C in a sealed vial until use. The concentrations in the stock solutions were checked by UV spectroscopy according to AOAC official methods of analysis (Horwitz and Latimer 2006). Working standard solutions (0.1–20 μg/L for AFB₁ and AFG₁, and 0.05–10 μg/L for AFB₂ and AFG₂) were prepared by appropriate dilution of known volumes of the stock solution with methanol:water (50:50, v/v) and used to obtain calibration curves in the chromatographic system. The standard of AFM₁ was dissolved in acetonitrile at a concentration of 1.0 mg/L and stored at 4 °C in a sealed vial until use. Working standards (0.1, 0.25, 0.5, 1, 2, 5, 10, 25, 50 μg/L) were also prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the chromatographic system.

Feed sample extract preparation

Before the analysis, the samples were dried during 24 h at 60 °C and ground into fine powder. TMR samples were extracted and cleaned up according to the Easi-extract® Aflatoxin manual slightly modified in order to obtain higher recoveries. Briefly, 5 g of feed sample were extracted with 40 mL of acetonitrile:water solution (90:10, v/v) and kept in an ultrasonic bath for 10 min. The sample was...
then centrifuged for 10 min at 4676×g. Three millilitres of supernatant were diluted with 72 mL of PBS and drained through the IAC column. The column was washed with 20 mL of PBS and AFs were eluted by applying 1 mL of methanol and 1 mL of water, consecutively. Before the injection in the chromatographic system, the final extract was filtered through a 0.22-μm PTFE disposable syringe filter (Kinesis, Cambridgeshire, UK).

**Milk sample extract preparation**

Milk analysis was carried out with immunoaffinity columns, according to the ISO official method (ISO 2007). Milk samples were placed into 50-mL centrifuge tubes and were warmed to 37 °C for 30 min. Then, they were centrifuged for 10 min at 4534×g. The upper cream layer was removed and the defatted supernatant was filtered with No. 113 Whatman filter papers. Forty millilitres of this fraction were passed through the IAC Aflaprep® M Wide column. After this, the column was washed with 20 mL of PBS and, finally, eluted twice with 1.25 mL of acetonitrile:methanol (60:40, v/v). The eluent was dried under nitrogen stream and then it was resuspended with 300 μL of mobile phase. Before the injection in the chromatographic system, the final extract was filtered through a 0.22-μm PTFE disposable syringe filter.

**Analysis of aflatoxins by UHPLC with fluorescence detection**

The equipment used for the detection of AFs was an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Santa Clara, California, USA) with a quaternary pump, an auto sampler, a vacuum degasser and a fluorescence detector. The chromatographic separation was carried out in a Poroshell 120 EC-C18 UHPLC column (2.7 μm particle size, 4.6 × 50 mm; Agilent Technologies) protected with a Poroshell 120 EC-C18 UHPLC Guard 3PK (2.7 μm particle size, 4.6 × 5 mm; Agilent Technologies). In order to enhance and confirm AFB1 and AFG1 detection, a post-column derivatization was performed with a LCTech UVE photochemical system (LCTech GmbH, Obertaufkirchen, Germany) was performed. The fluorescence detector was set at wavelengths of 365 nm and 440 nm for excitation and emission, respectively. The mobile phase consisted of acetonitrile:methanol:water (10:20:70, v/v/v) and the flow rate was 1.2 mL/min. The temperature of the column was set at 40 °C, and the injection volume was 50 μL.

For the detection of AFM1, excitation and emission wavelengths were set at 360 nm and 450 nm, respectively. The mobile phase consisted of methanol:acetonitrile:0.1% acetic acid (5:15:80, v/v/v) and the flow rate was 0.8 mL/min.

**Quality control of analytical method**

The analytical method used for the detection of AFs in TMR samples was assessed for precision, calculating the apparent recovery, which is the ratio between the measured concentration and the spiked concentration. For this purpose, fifteen blank feed samples were spiked with known amounts of AFs mixtures at three different concentration levels (low, medium and high). As each TMR sample had a unique composition, it was not possible to validate the method for every single feed sample. Accordingly, a TMR sample, with an average composition, was considered as a blank sample to calculate the apparent recovery of the method. Method performance for AFB1, AFB2, AFG1 and AFG2 is summarised in Table 2.

**Estimate of the relationship between aflatoxin B1 content in feed and aflatoxin M1 levels in bulk milk**

In order to assess the ratio between the measured AFB1 content in the feedingstuffs sampled from the dairy farms and the AFM1 levels determined in bulk tank milk of these farms, the correlation was estimated by using the following Eq. 1.

\[
\frac{AFB1 \, \mu g \, \text{TMR kg}}{milk \, L \, cow} = \frac{AFB1 \, \mu g \, milk \, L}{milk \, L}
\]

\[
\text{Ratio } AFM1_{\text{in milk vs AFB1 in feed }} (\%) = \frac{AFM1_{\mu g \, milk \, L}}{AFB1_{\mu g \, milk \, L}} \times 100
\]

**Statistical analysis**

The analysis of the data was performed using JMP Pro 13. Firstly, ANOVA was used so as to know if there were significant effects of year, farm and composition of TMR samples on aflatoxin levels in both feed and milk samples. A multivariate partial least square regression (PLS) was carried out in order to determine which components of the feedstuffs could explain the presence of AFs in feed. PLS is a multiple regression method which produces a model for the mycotoxin by combining all TMR components at a time. VIP (variable importance in projection) scores were also calculated, which are a measurement of a variable’s importance in the PLS model. They summarise the contribution of a variable to the model; higher VIP scores allow to identify the most contributory variables in class discrimination in the PLS model.
Results

AFs occurrence in feed and milk samples

From February 2016 to January 2018, a total of 193 different TMR samples and 375 bulk milk samples were analysed. AFs were detected in 67 samples (LOD for AFB1 and AFG1 0.1 μg/kg; LOD for AFB2 and AFG2 0.05 μg/kg) and 71 milk samples were positive for the presence of AFM1 (LOD 0.009 μg/kg). A summary of the occurrence and the range of concentrations of AFs in feed and AFM1 in milk is given in Tables 3 and 4.

Out of the 67 positive feed samples, 24 contained AFB1, 9 were positive for AFB2, 47 for AFG1 and 13 for AFG2. Therefore, AFB1 and AFG1 were more frequently detected than AFB2 and AFG2. In none of the AF-positive feed samples, the concentration of AFB1 exceeded the EU maximum content of 5 μg/kg for complete feedingstuffs for dairy animals (EU 2002). For one sample, an elevated level of AFG1 of 6.45 μg/kg was found, but AFG1 is not included by regulations for feedingstuffs with European Union legislation. The number of AFB1-positive samples was higher in 2016, while in 2017 the most frequent toxin found was AFG1. Moreover, there seemed not to be significant differences among the four different locations under study (data not shown).

Regarding ingredients that were studied separately, AFs were not found in any of the dried mixture (n = 1), maize silage (n = 2), barley silage (n = 1), sugar beet pulp silage (n = 1), immature corn silage (n = 1), sugar beet pulp (n = 1), okara (n = 2), soya bean husk (n = 1) and ryegrass silage (n = 1) samples. On the other hand, positive results for the presence of AFs were obtained from the analysis of maize, cotton seed, compound feed and soya bean samples (Table 5).

AFM1 was present in 18.4% of bulk milk samples. Significant differences were not found between samples obtained 24 h and 48 h after feeding, 19.2% and 18.3% being positive for the presence of AFM1, respectively. Figure 1 shows the distribution of AFM1 during the length of the study. The number of positive samples was clearly higher in 2016 than in 2017 and the season when more positive results were obtained was from May to July in 2016. Three bulk milk samples exceeded the EU limit of 0.05 μg/kg.

Relationship between TMR composition and AFs occurrence in feed and milk samples

PLS multivariate regressions were carried out in order to assess the impact of the TMR components in AFs, AFB1 and AFG1 presence (Fig. 2). The first model explained 49% of variability, the second 64% and the third one 37%.

AF positive samples usually contained maize silage, barley silage, bagasse, okara, dehydrated ryegrass, straw and maize. Moreover, it has to be pointed out that the compound feed was related to the presence of group B aflatoxins in feed samples (Fig. 2a). AFB1-positive feed samples presented maize silage, bagasse, soya bean husk, maize, alfalfa hay, cotton seed and compound feed as ingredients (Fig. 2b). As for AFG1, maize silage, alfalfa silage, barley silage, okara, soya bean husk, straw, dehydrated alfalfa and cotton seed were positively related to the presence of the toxin (Fig. 2c).

Due to the weight of the compound feed in the presence of AFB1 in feed samples, a PLS analysis was carried out taking into account the components of the compound feed separately, instead of considering it as a unique element. It was not possible to get from all farmers the complete information of each TMR sample (including compound feed composition), for this reason, the analysis was performed considering only those...

| Mycotoxin | LODa (μg/kg) | N | Spiking level (μg/kg) | Recoveryb (%) | RSDrc (%) |
|-----------|--------------|---|----------------------|---------------|-----------|
| AFB1      | 0.1          | 5 | 2                    | 97.05 ± 6.66  | 6.86      |
|           |              | 5 | 20                   | 81.07 ± 1.45  | 1.79      |
|           |              | 5 | 50                   | 79.25 ± 1.17  | 1.48      |
| AFB2      | 0.05         | 5 | 2                    | 97.36 ± 2.90  | 2.97      |
|           |              | 5 | 20                   | 92.04 ± 1.88  | 2.05      |
|           |              | 5 | 50                   | 90.03 ± 1.51  | 1.68      |
| AFG1      | 0.1          | 5 | 2                    | 88.36 ± 19.55 | 22.13     |
|           |              | 5 | 20                   | 73.05 ± 2.04  | 2.80      |
|           |              | 5 | 50                   | 70.35 ± 3.32  | 4.72      |
| AFG2      | 0.05         | 5 | 2                    | 76.22 ± 5.66  | 7.43      |
|           |              | 5 | 20                   | 80.96 ± 2.72  | 3.36      |
|           |              | 5 | 50                   | 81.40 ± 1.24  | 1.52      |

a LOD, limit of detection  
b Mean value ± standard deviation  
c RSDr, relative standard deviation
samples whose information was available \((n = 33)\). In this subset of samples, barley silage, defatted soya flour, maize, barley and alfalfa hay were the ingredients present in AFB1-positive samples. They also contained maize, cotton seed and wheat as ingredients coming from the compound feed (Fig. 2d). With this PLS model a 91% of the variability was explained.

As it is shown in Table 1, among the ingredients possibly related with the presence of AFs, maize, cotton seed, defatted soya flour and compound feed used in the formulation of TMR samples may have been imported from other countries.

**Ratio AFM1/AFB1**

A total of 15 AFM1-positive milk samples came from cows that had been fed with AFB1-positive feed. However, eight feed samples contained AFB1, but bulk milk samples obtained from cows fed with these feed turned out to be negative for the presence of AFM1. Conversely, 27 AFM1-positive samples came from cows fed with feed samples that were not contaminated by AFB1.

Taking into account the cases in which AFs were present in both feed and milk, the daily feed intake and the milk yield by each cow, the ratio AFM1 in milk/ AFB1 in feed varied from 0.6 to 6%.

### Discussion

**Occurrence of AFs in feed**

The presence of AFB1 in animal feeds has been legislated in different countries in order to avoid the possible toxic effects related with its ingestion both in animals and in humans. The EU has set an upper limit of 5.0 \(\mu\)g/kg in complete feedstuffs for dairy cows with a moisture content of 12% (European Union 2002). However, other non-EU countries are less restrictive and have established higher limits, such as USA, which have set an action level of AFs of 20.0 \(\mu\)g/kg in feeds and ingredients for dairy animals (FDA 1996).

The number of AF-contaminated samples found in this study was low, with only one sample containing a relatively high level of AFG1 (Table 3). Our results show that 34.7% of the samples studied were contaminated by AFs in a range of 0.05–6.45 \(\mu\)g/kg. The most frequently found toxins were AFB1 (12.4%) and AFG1 (24.4%).

In agreement with our research, similar results have been obtained (EFSA 2004), although it is worth noting that the farms included in our study were selected specifically due to their previous problems with AFs. Many studies found AFs in dairy feedstuffs, although only a small proportion of the contaminated samples exceeded the maximum tolerable EU limit of 5.0 \(\mu\)g/kg. Also in Spain, Hernández-Martínez and Navarro-Blasco (2015)
found that 90% of the dairy cow feedstuff samples under study showed detectable concentrations of AFs, without exceeding the legal limit. Decastelli et al. (2007) in Italy detected also a low number of contaminated samples (8.1%) but with concentrations above the EU limit.

By contrast, other studies found a relatively low number of contaminated feed samples but some of them presented high values of AFs, widely exceeding the established legal limit. In Portugal, Martins et al. (2007) carried out a 10-year study finding 37.4% positive samples with a level of contamination ranging from 1 to 74 μg/kg. Levels of AFB1 above the EU limit (from 5.1 to 74 μg/kg) were observed in 6.2% of the samples. Similarly, Pleadin et al. (2015) obtained 22.2% AFB1-positive samples and 12.3% had concentrations over the limit, showing, occasionally, very high values being the maximum found 304.6 μg/kg.

Conversely, other studies, specifically from African, Middle East and Asian countries, found higher frequency and levels for AFs in feedingstuffs for dairy animals (Kocasari et al. 2013; Ehsani et al. 2016; Gizachew et al. 2016; Mohammed et al. 2016; Ismail et al. 2017). Contamination by AFs is more common in Asian and Pacific regions than in Europe (Binder et al. 2007). Moreover, these elevated levels of AFs in animal feed samples might be associated with the lack of facilities to store dairy animal feed, favourable conditions for the growth and production of toxins, illiteracy of dairy feed suppliers and insufficient monitoring by the government agencies (Ismail et al. 2017).

**Occurrence of AFM1 in milk**

Although the problem of AFM1 in milk has been known for a long time, recently several cases of milk contaminated with this toxin in Europe have revealed the extent of the problem, both from the point of view of public health and from an economic angle. For example, in a single case of milk contamination in Southern Spain in 2013, two million litres of raw milk had to be destroyed because of AFM1 exceeding the EU maximum level, causing a total economic loss of more than 760,000 Euro (Caravaca 2013).

The present study analysed bulk milk samples obtained after 24 and 48 h, respectively, from cows that had been fed with the sampled TMR. Results showed that 71 out of 375 milk samples (18.9%) contained AFM1 in concentrations above the limit of detection in a range from 0.009 to 1.36 μg/kg, with only three samples exceeding the EU limit.

These results are in line with data reported in other researches carried out in Europe about the occurrence of AFM1 in milk in recent years. In these studies, a low number

| Raw material   | No. of total samples | No. of positive samples (range, μg/kg) |
|----------------|----------------------|----------------------------------------|
|                |                      | AFB1 | AFB2 | AFG1 | AFG2 |
| Maize          | 6                    | 3 (0.24–1.05) | 1 (0.06) | 3 (0.18–0.76) | 2 (0.11–0.78) |
| Cotton seed    | 12                   | 5 (0.08–1.97) | 2 (0.16–0.21) | 4 (0.27–3.68) | 3 (0.23–0.77) |
| Soya bean      | 2                    | 1 (0.26) | 0 | 0 | 0 |
| Compound feed  | 5                    | 2 (0.17–2.44) | 2 (0.05–0.21) | 3 (0.11–3.25) | 2 (0.16–0.2) |

*Fig. 1 Percentage of AFM1-positive milk samples from February 2016 to January 2018*
Fig. 2 Importance of the TMR components (VIP) on the presence of AFs a, of AFB b and of AFG c in feed samples and on AFB d presence in feed taking into account the ingredients of the compound feed d
of AFM₁ contaminated samples were reported, with similar ranges of toxin concentration. Moreover, samples with AFM₁ levels above the UE established limit were rarely detected (EFSA 2004; Prandini et al. 2009; Flores-Flores et al. 2015). There were also several Italian authors that reported very low levels of contamination in cow milk and usually no sample exceeded the EU regulation limit (Decastelli et al. 2007; Armorini et al. 2016; De Roma et al. 2017).

By contrast, a wide number of authors detected higher percentages of contaminated samples with concentrations that frequently exceeded the legal values. Bakirci (2001) in Turkey found AFM₁ in 87.8% of the samples examined and with 44.3% of the cases surpassing the EU limit. In Ethiopia, Gizachew et al. (2016) reported that all the milk samples analysed were positive for the presence of AFM₁ and only 8.2% contained less than or equal to 0.05 μg/kg of AFM₁. Mohammed et al. (2016) in Tanzania detected that 83.8% of the milk samples were contaminated with AFM₁, exceeding all of them the EU established limit. These differences could be attributed to several factors including different analytical techniques, sample size and composition, season of the year, livestock management and dairy processing systems.

**Relationship between results for AFB₁ in feedingstuff and results for AFM₁ in bulk tank milk**

The results obtained in this study show that there is only a very weak qualitative and quantitative relationship between positive findings for AFB₁ in feedingstuff and AFM₁-positive results for bulk tank milk. For only 15 out of 60 AFM₁-positive bulk tank milk samples, the corresponding feedingstuff was also positive for AFB₁, meaning a qualitative agreement between both parameters of just 25%. For 27 AFM₁-positive bulk tank milk samples, no AFB₁ was found in the corresponding feedingstuff. Vice versa, eight cases in which AFB₁-positive feedingstuffs were determined, negative results for AFM₁ in the corresponding bulk tank milk were obtained, thus the presence of AFB₁ in feed samples was not always reflected in milk.

This fact has also been previously reported by other authors (Battaccone et al. 2003; Gizachew et al. 2016; Han et al. 2013). A possible explanation could be the heterogeneous distribution of mycotoxins in raw materials and the problems derived from the sampling procedure. Only some sections of the feedstuffs are likely to be highly contaminated whereas most of the feed will probably not present any mycotoxin (Miraglia et al. 2005). Consequently, the amount of toxin could be underestimated if the contaminated fractions are not analysed. In a like manner, if only the heavily AF-contaminated fraction is examined, the mycotoxin content could be overestimated. Therefore, the sampling step represents one of the main sources of variability in mycotoxin analyses (Miraglia et al. 2005). This procedure is carried out using a small proportion of the test sample (in our study 5 g from a bulk sample of 4–5 kg previously ground and homogenised) which is assumed to have the same mycotoxin concentration, but, even these subsamples could be different one from each other. Both, subsampling and grinding are also sources of error, although much smaller. The sampling error in toxin quantification has been shown to depend on the size of the sample. The error is reduced with bigger samples and subsamples (Armorini et al. 2015) and also with a smaller size of the ground particle (Whitaker 2003; Miraglia et al. 2005; Whitlow and Hagler 2010). Consequently, in order to try to minimise the sample preparation error as much as possible, feed samples were dried and ground before being analysed and each sample was tested in triplicate.

However, feed analyses are still useful to help minimising the contamination of milk, although monitoring cannot prevent that occasionally positive AFM₁ samples occur despite AFB₁—negative results obtained for feedingstuff. Consequently, it should be considered that, in order to prevent AFM₁ contamination in milk, a possible solution would consist of including a performance objective for AFB₁ in feedingstuff of, for example, 3 μg/kg. This would solve the problem linked to the heterogeneous distribution of aflatoxins in the TMR.

**TMR composition**

Concentrates used in order to enrich feed for dairy cows can represent up to a 70% of the daily feed ration, in our case, they accounted for up to 40%. As a consequence, these ingredients may be an important source of mycotoxins due to its possible contamination with AFs. These toxins can also be found in cereal grains or soybean products as well as in by-products from oil plants such as peanuts, sunflower seed, cotton seeds, palm kernels and copra (Nawaz et al. 1997; Scudamore and Livesey 1998; Placinta et al. 1999). Another source of AFs in the diet of dairy cows comes from the consumption of preserved feeding stuffs like silage, hay or straw (O’Brien et al. 2005; Mansfield and Kulda 2007). After a long storage period, silage can be spoiled by some fungal species, being Aspergillus species (A. fumigatus and A. flavus) some of them (Cole et al. 1977). However, AFs only have been sporadically detected at low levels in forages. Maize has been traditionally related with the presence of AFs in dairy cows diet, too (Whitlow and Hagler 2005), and it is usually one of the main components of animal feed (EFSA 2013; Battilani et al. 2012).

The multivariate PLS regression carried out led us to identify which components of the total ration could have influenced on the presence of AFs in the feed (Fig. 2). Table 5 shows those raw materials that were positive for the presence of AFs and the range of concentrations. Positive samples of maize and compound feed could confirm the results of the PLS analysis in which it was
observed that these ingredients seemed to be related with the presence of AFB\(_1\) in the feed samples (Fig. 2b and d). The highest concentration of AFB\(_1\) (2.44 \(\mu\)g/kg) was found in one of the compound feeds which was also positive for AFB\(_2\) (0.21 \(\mu\)g/kg), AFG\(_1\) (3.25 \(\mu\)g/kg) and AFG\(_2\) (0.20 \(\mu\)g/kg). In this case, the correspondent TMR was also analysed and it was found out to be positive for the presence of AFB\(_1\), AFB\(_2\) and AFG\(_1\). In addition, milk samples from cows fed with this feed were contaminated with AFM\(_1\). Some samples of cotton seed and maize were also positive for the presence of AFs. However, although cotton seed presented sometimes high concentrations of AFs, the toxins did not appear in the correspondent TMR sample. It may be explained because compound feed used to represent a relatively high proportion of the whole whereas the cotton seed represented normally a small portion (0.68–5.51\%) of the total ration (Table 1).

Our positive outcomes in cotton seed and in maize are in agreement with other studies related to the analysis of feed ingredients. Liu et al. (2016) found that cottonseed was the most seriously contaminated feed ingredient. They also found that a high percentage of maize samples presented AFB\(_1\). Sadegh et al. (2013) in Iran found out that cotton seed and sunflower meal samples were the most contaminated materials with AFB\(_1\). The toxin was also present in alfalfa, straw, rapeseed, cotton seed, maize silage and soybean meal samples although the highest concentration was in cotton seed. For Pleadin et al. (2015), maize was the most contaminated component although they also had positive AFB\(_1\)-samples of wheat, barley and oat. Whole cottonseed, as well as other oilseed such as soybean, flaxseed and sunflower, could be used as fat sources in dairy cows feed. These ingredients allow a slow release of lipids in the rumen. Whole cottonseed has 22\% crude protein, 20\% ether extract and 83\% of total digestive nutrients, being, therefore, an excellent source of supplemental fat to increase diet energy density. Moreover, it seemed to be highly effective in maintaining milk fat. Despite all of these advantages, it could be related to the presence of AFs, therefore, it should be studied if it would be possible the replacement of this ingredient with others with similar nutritional properties (De Almeida et al. 2017).

Despite of the fact that the farms included in the study had presented problems related to AFs in previous years, the level of contamination in feed and milk samples collected from February 2016 to January 2018 was relatively low. These results were in agreement with other studies carried out recently and this is probably related to the increasing surveillance measures and quality control of raw materials used for manufacturing feeds as well as the application of Good Agricultural and Storage Practices. However, in recent years, there are still outbreaks of AFM\(_1\) in milk over the legal limit and this may be linked to the raw materials used in the formulation of feedstuffs. Hence, it is important to have information about the contribution of the ingredients on the AFB\(_1\) content of the total mixed ration (TMR) given to cows. Moreover, it is important to remember that, besides AFs, there are other mycotoxins that can occur in dairy feedstuffs and feed ingredients, including deoxynivalenol, zearalenone and fumonisins that may be also harmful to animals and humans (Driehuis et al. 2008). Thus, mycotoxin contamination in feed and in milk should be regularly monitored, in particular the most susceptible ingredients, in order to reduce their incidence and their effects.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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