Annual and regional variations of aflatoxin B_1 levels seen in grains and feed coming from Croatian dairy farms over a 5-year period

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ABSTRACT

The aim of the study was to investigate annual and regional differences in the level of aflatoxin B_1 (AFB$_1$) in grains and dairy cattle feed. Maize ($n = 972$), wheat ($n = 201$), barley ($n = 147$), oat ($n = 136$), grain mixtures ($n = 168$), and dairy cattle feed ($n = 325$) were sampled from 2009 to 2013 on different farms and in different farm factories situated in four Croatian regions. The samples were analysed for AFB$_1$ using the validated ELISA immunoassay. AFB$_1$ was determined in 16.4% of all investigated samples, among which maize was proven to be the most contaminated, with 21.7% of the samples recovered during 2013 harbouring AFB$_1$ in concentrations over the permissible ones. Levels higher than permitted were observed in 17.9% and 12.3% of grain mixtures and dairy cattle feed, respectively, whereas concentrations of AFB$_1$ determined in other crops throughout the investigated period met the stipulated requirements. The results revealed the AFB$_1$ occurrence to be significantly ($p < 0.05$) dependent on the cultivation region, with the highest levels generally found in maize harvested in 2013 and consequently in grain mixtures and cattle feed that can most likely be associated with climatic conditions as the most critical factor for mould formation, and thus also AFB$_1$ production.

1. Introduction

Grains that are highly represented in human and animal diet, as also in industrial food and feed, may become contaminated by mycotoxin-producing moulds; such a contamination may occur either in the field or during storage. Aflatoxins are mycotoxins known to be produced by the species of *Aspergillus* genus, specifically *Aspergillus flavus* and *Aspergillus parasiticus*. They represent highly toxic, mutagenic, teratogenic and carcinogenic chemical compounds, causing both acute and chronic toxicity in humans and animals (Binder, Tan, Chin, Handl, & Richard, 2007; EFSA, 2004; Meggs, 2009).

Among the above, the most potent liver carcinogen known in mammals is aflatoxin B_1 (AFB$_1$), classified by the International Agency for Research on Cancer as Group 1 carcinogen (IARC, 2002). Contamination of food and feed is often unavoidable and still poses a serious problem affecting important agricultural goods, which emphasizes the need for suitable processing capable of inactivating the toxin. AFB$_1$ is a stable compound that cannot be destroyed during most of the food processing operations, therefore causing huge economic losses. However, AFB$_1$ presence could be minimized by making improvements in farming practices, such as by virtue of providing for better storage conditions or by virtue of using modified seeds, as well as by making improvements in manufacturing processes.

Factors that facilitate fungal infection and promote AFB$_1$ production are inoculum availability, weather conditions and pest infestation during crop growth, maturation, harvesting and storage (Lopez-Garcia & Park, 1998; Pleadin et al., 2014). Due to the fact that AFB$_1$ commonly contaminates grains, many studies have attempted to define multiple aspects of such a contamination. Maize, as the most widely grown crop, is a particular problem. Due to its nutritional value, a high percentage of the world maize production is destined to animal feeding.

The document issued by the European Food Safety Authority (EFSA, 2013) based on the analytical data on aflatoxin levels determined in food samples collected and analysed in 2007–2012 period, advises that the collection of data on the occurrence of

these toxins should be continued in order to gather a representative number of samples in different food and feed categories; in addition, the document draws attention to the need for harmonizing the reporting formats across the European countries (Pleadin et al., 2014). Given the fact that it has been proven that in dairy cattle fed on contaminated feed AFB_1 gets to be converted into aflatoxin M_1 (AFM_1), known as the "milk toxin" and subsequently excreted into the milk, concerns about the entry of this mycotoxin into the food chain through meat, eggs, milk and dairy products (carryover effects) have been raised (Markov et al., 2013; Prandini et al., 2009; Richard, 2007).

Respecting the above, the aim of this study was to obtain and summarize data on AFB_1 levels in different grains used in the production of dairy cattle feed and therefore present in the final feed products.

2. Materials and methods

2.1. Samples

The samples were collected from different farms and feed factories situated in four Croatian regions over a five-year period. The study made use of representative samples of grains used in dairy cattle feed production, as well as of those of final dairy cattle feed products, taken directly from dairy farms and feed factories situated in northern (Region 1), western (Region 2), eastern (Region 3) and central Croatia (Region 4) in 2009–2013 period. The grain samples included maize ($n = 972$), wheat ($n = 201$), barley ($n = 147$) and oat ($n = 136$) grains, as well as grain mixtures of different composition ($n = 168$). The feed samples ($n = 325$) were final feed products produced according to the manufacturer's specification, ultimately used for dairy cattle feeding.

The samples were sampled and prepared in full line with the ISO 6497:2002 and ISO 6498:1998, respectively. The prepared test portions were ground to a particle size of 1.0 mm using an analytical mill (Cylotec 1093, Tecator, Sweden) so as to obtain a fine, non-dried powder, and stored at $+4\ ^{\circ}C$ prior to AFB_1 analysis.

2.2. Chemicals and reagents

The ELISA method was performed using Ridascreen® kits (R-Biopharm, Darmstadt, Germany). Each kit contains a micro-titer plate with 96 wells coated with antibodies against AFB1, aqueous solutions of AFB_1 standard (0, 1, 5, 10, 20, and 50 μg/L), peroxidase-conjugated AFB_1, substrate/chromogen (urea peroxide), a stop-reagent (1 N-sulphuric acid), and the washing buffer (10 mM-phosphate buffer, pH = 7.4). AFB_1 standards employed in the analytical methods' validation were provided by Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals used for AFB_1 extraction and analysis were of an analytical grade.

2.3. Sample preparation

2.3.1. Grains
Samples were prepared using 5 g of a homogenized sample supplemented with 25 mL of 70%-methanol and shaken vigorously on head-over-head shaker for three minutes. The extract was then filtrated (Whatman, black ribbon); 1 mL of the obtained filtrate was further diluted with an appropriate volume of deionized water.

2.3.2. Feed
Five grams of a feed sample and 20 mL of (50% -) acetonitrile were shaken head-over-head for 60 min. After centrifugation (10 min, 5000 rpm, 10 °C), 4 mL of supernatant were diluted with 16 mL of deionized water and cleaned using ISOLUTE Myco 60 mg/

3 mL columns (Biotage, Sweden). The columns were conditioned with 2 mL of acetonitrile and water, respectively. A sample (4.5 mL) was then applied on the columns and first washed with 9 mL of water and then with 10%-acetonitrile. The columns were subsequently dried for 10 min under the maximal vacuum and eluted with 3 mL of 0.1%-formic acid in acetonitrile and 3 mL of methanol. The obtained eluate was evaporated in the nitrogen stream and dissolved in 1 mL of methanol/water solution (35/65).

2.4. Determination of AFB_1

The ELISA method was performed in full line with the kit manufacturer's instructions, and made use of an auto-analyzerChemWell 2910 (Awareness Technology, Inc, USA), as described earlier (Pleadin et al., 2014). When calculating the final AFB_1 concentration in the analysed sample, the applied dilution factor was duly taken into account. The obtained AFB_1 concentrations were calculated from a six-point calibration curve and finally corrected for recovery (maize, wheat and feed) or the mean recovery (other grain samples).

2.5. Validation of methods

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the mean value of ten control samples of grain/dairy cattle feed plus two- and ten-fold standard deviation, respectively. The recoveries were determined at three different levels (six replicates per concentration level per day) by virtue of spiking the control samples with the AFB_1 standard working solution (100 μg/L) correspondent to the assessed content levels. As regards the determination of intermediate precision, the same steps were repeated on two additional occasions within a month, using two different ELISA kits and chemicals lots, but under the same analytical conditions. The trueness was determined using the certified reference material–CRM (T04209QC, Fapas, Sand Hutton, York), AFB_1 thereby being certified in the range of 4.49–11.54 μg/kg.

2.6. Statistical analysis

Statistical analysis was performed using the Statistica Software Ver. 10.0 (StatSoft Inc. 1984–2011, USA), with the statistical significance set at 95%-level ($p = 0.05$).

3. Results and discussion

3.1. Validation data

Validation of the ELISA method resulted in LOD and LOQ values ranging from 1.0 μg/kg to 1.7 μg/kg, respectively. The mean recovery and the intermediate precision were determined to be 91.8% and 90.7%, respectively, with acceptable mean relative standard deviations of 5.8 and 7.5, respectively (Table 1). The mean value ($n = 8$) obtained with CRM-utilizing trueness determination equalled to 9.8 μg/kg. The AFB_1 concentration obtained is acceptable given the criterion established under the Commission Decision 2002/657/EC.

Earlier data showed that the development of immunological methods capable of detecting mycotoxins, especially that of ELISA, was a major step forward in the development of rapid, repeatable and sensitive assays, despite of the fact that only semi-quantitative data can be obtained (Bryden, 2012; Krska et al., 2008; Pleadin et al., 2012, 2013). Since this study investigated a large number of samples, the determination of AFB_1 was carried out using the validated ELISA method. Taking into account the obtained validation data, the applied method can be considered suitable for

Table 1
Results of the ELISA method validation.

Material[a]	LOD (µg/kg)	LOQ (µg/kg)	Spiked AFB$_1$ (µg/kg)	Mean recovery (%)	CV (%)	Intermediate precision (%)	CV (%)
Maize	1.0	1.5	5	92.7	6.1	91.6	8.1
			10	94.2	6.9	95.4	7.5
			20	92.6	5.4	94.7	7.8
Wheat	1.1	1.7	5	88.9	5.2	85.6	7.4
			10	86.5	5.9	83.9	7.2
			20	92.1	6.5	91.7	8.4
Feed[b]	1.0	1.4	2	90.9	4.8	89.4	6.7
			5	93.5	5.8	92.3	7.8
			10	95.1	5.2	91.8	6.4

[a] control samples (blank material).
[b] dairy cattle feed.

reliable and efficient determination of AFB$_1$ presence in grain and feed samples. In support of this choice of the study method, earlier investigation revealed a high positive correlation between AFB$_1$ levels determined in maize samples containing AFB$_1$ in concentrations surpassing the maximal permissible level (MPL) using the ELISA method and those determined using the LC-MS/MS (Pleadin et al., 2014).

3.2. AFB$_1$ in grains and feed

In the last decades, the issue of food and feed contamination with aflatoxins has received a great deal of attention all over the world. The presence of these toxins in feedstuffs comes as a consequence of contaminated raw materials used in their production; the use of such materials can also result in the contamination of food of animal origin. It is known that the contamination of crops with AFB$_1$ depends on climate and storage conditions (Bryden, 2012; Magan, Medina, & Aldred, 2011; Wu et al., 2011); however, investigations into the presence of mycotoxins in crops used as feed ingredients and in final feed products coming from the potentially contaminated crops are generally lacking, even on a global scale (Zinedine & Mānes, 2009).

In this study, crops and dairy cattle feed were analysed so as to investigate into their possible AFB$_1$ contamination. The investigation was carried out in Croatia over a five-year period. Summarized data on AFB$_1$ concentrations in grain/feed samples established throughout the investigated period across the Croatian regions are shown in Table 2. The comparison of the obtained AFB$_1$ crop levels proved maize to be the most contaminated, with AFB$_1$ determined in 31.4% of samples, as compared to 7.5% AFB$_1$-positive wheat, 6.1% AFB$_1$-positive barley, and 5.1% AFB$_1$-positive oat samples. Given that the AFB$_1$ MPL for all grains intended for feed production equals to 20 µg/kg (Commission Directive 2003/100/EC), levels higher than MPL were determined in 21.7% of maize samples, whereas all wheat, barley and oat samples had satisfied the given criterion. As for the grain mixtures analysed under this study, AFB$_1$ concentrations higher than permitted were found in 17.9% of the samples, all of them collected during 2013. As for the dairy cattle feed samples analysed within the frame of this research, 22.2% of samples harboured AFB$_1$ in concentrations surpassing the study method's limit of detection (i.e. were positive), out of which 12.3% in concentrations over the MPL of 5 µg/kg set for the dairy cattle feed (under the Commission Directive 2003/100/EC).

Data on AFB$_1$ occurrence displayed by the sampling year and the sampling region are presented in Tables 3 and 4, respectively. The analysis of variance (ANOVA) revealed statistically significant differences ($p < 0.05$) in concentrations of AFB$_1$ found in maize, grain mixtures and dairy cattle feed samples collected in 2013. The highest AFB$_1$ concentration was observed in the eastern region

Table 2
Total AFB$_1$ found in grains and feed in 2009–2013 period in four investigated Croatian regions.

Parameter	AFB$_1$ in grains and feed					
	Maize	Wheat	Barley	Oat	Mixtures[a]	Feed[b]
No of samples	972[e]	201	147	136	168	325
Mean (µg/kg)	38.46	1.65	1.54	1.18	10.98	8.41
SD (µg/kg)	75.68	1.70	1.24	0.84	12.43	11.48
Max (µg/kg)	2072	5.41	3.22	1.55	252.3	304.6
Positive[c] (%)	31.4	7.5	6.1	5.1	26.2	22.2
>MPL[d] (%)	21.7	ND	ND	ND	17.9	12.3

ND – not detected.
[a] Grain mixtures; grains present in different proportions as per manufacturer's specifications.
[b] Dairy cattle feed.
[c] % of samples in which AFB$_1$ was detected (>LOD).
[d] % of samples in which AFB$_1$ was detected in concentrations over the maximal permissible level (MPL) of 20 µg/kg for grains and 5 µg/kg for dairy cattle feed (according to the Commission Directive 2003/100/EC).
[e] For 633 out of 972 samples the results were already reported (Pleadin et al., 2014).

(R3), although elevated amounts of AFB$_1$ were determined in other study regions as well. Higher AFB$_1$ concentrations in grain mixtures and feed can be attributed to a substantial AFB$_1$ maize contamination determined in 2013. Significant differences in AFB$_1$ levels were not observed for other grains (wheat, barley and oat), either in the different study years or across the different study regions ($p > 0.05$). The lowest number of AFB1 – positive samples and the lowest average AFB$_1$ concentration were observed in oat, AFB$_1$ thereby being determined in only seven samples (5.1%) in concentrations approximating to or being slightly higher than the ELISA's limit of detection.

The first evidence of high AFB$_1$ contamination of maize used by Croatian milk producers was reported by Pleadin et al. (2014). In the maize sampled during 2013 (referred to the genus 2012), AFB$_1$ was detected in 38.1% of samples, with 28.8% of the samples containing this toxin in levels higher than the MPL. The highest percentage of samples containing AFB$_1$ in concentrations higher than the MPL was observed in the eastern Croatia (36.5%), which is the national leader not only in grain and feed production, but in farming and milk production as well. The maximal observed AFB$_1$ level was 2072 µg/kg, i.e. the level about 100 folds higher than the MPL; of note, the differences in maize contamination levels established between the investigated regions were significant, too. High maize contamination witnessed in 2013 was associated with weather conditions, as the preceding 2012 was extremely warm, dry, and characterised by a very low average rainfall, all of the aforementioned going in favour of mould formation and AFB$_1$ presence in maize (Pleadin et al., 2014).

Table 3
AFB$_1$ concentrations found in grains and feed in each sampling year.

Grain/Feed	Year/AFB$_1$ – mean ± SD (µg/kg)				
	2009	2010	2011	2012	2013
Maize	3.38 ± 2.15	4.15 ± 3.33	2.18 ± 2.44	3.87 ± 1.56	93.5 ± 213
Wheat	1.10 ± 0.61	1.21 ± 1.17	1.67 ± 1.11	1.76 ± 2.13	3.02 ± 2.15
Barley	ND	1.33 ± 1.24	ND	1.14 ± 1.27	2.11 ± 1.13
Oat	ND	ND	1.12 ± 0.15	ND	1.13 ± 0.53
Mixtures[a]	1.02 ± 0.35	1.11 ± 1.28	ND	1.33 ± 1.24	34.6 ± 51.4
Feed[b]	1.07 ± 1.23	1.09 ± 1.15	ND	1.12 ± 0.89	29.4 ± 41.5

ND – not detected.
[a] Grain mixtures; grains present in different proportions as per manufacturer's specifications.
[b] Dairy cattle feed.

Table 4
AFB$_1$ concentrations found in grains and feed in each sampling region.

Grain/Feed	Region/AFB$_1$ – mean ± SD (µg/kg)			
	R1	R2	R3	R4
Maize	18.25 ± 34.52	10.25 ± 28.31	132 ± 289	15.2 ± 52.4
Wheat	1.28 ± 1.53	1.41 ± 1.69	1.89 ± 2.44	1.64 ± 2.21
Barley	1.23 ± 1.14	ND	1.67 ± 1.53	1.75 ± 1.12
Oat	1.17 ± 0.81	ND	1.28 ± 0.31	ND
Mixtures[a]	5.52 ± 15.41	6.48 ± 20.12	28.5 ± 22.4	4.31 ± 3.38
Feed[b]	2.25 ± 11.14	1.89 ± 4.15	24.7 ± 21.4	3.12 ± 10.38

R 1 – Northern region, R 2 – Western region, R 3 – Eastern region, R 4 – Central region.
ND – not detected.
[a] Grain mixtures; grains present in different proportions as per manufacturer's specifications.
[b] Dairy cattle feed.

The results from that period (2013) revealed extremely elevated amounts of AFM$_1$ in cow milk produced in Croatia. High AFM$_1$ levels found in the cow milk coming from the eastern Croatia indicated the use of contaminated supplementary feedstuffs on some farms during the observed period (Bilandžić et al., 2014a, 2014b). Given the established high concentration of AFB$_1$ in maize and the identified elevated concentrations of the toxin in grain mixtures and dairy cattle feed, in which maize is usually represented in the amount of 20–30%, increased amounts of AFM$_1$ in milk were to be expected. The authors concluded that the frequency of control of feed and milk samples should be increased and that they should strive to educate breeders and those involved into the milk production on harmful effects aflatoxins might have on animal feed (Bilandžić et al., 2014a).

Given the fact that elevated mycotoxin concentrations are usually associated with weather conditions as the factor most critical for mould formation and thus also mycotoxin production, the explanation to this study's results should also be sought in that department. Aflatoxin contamination of crops is associated with high temperatures and a prolonged drought, and is often chronic in warm, humid, tropical, and subtropical maize-growing regions (Payne, 1998; Widstrom, 1996). Such weather conditions, witnessed during the maize-growing and maize-harvesting period, increase the growth of Aspergillus species and consequently the AFB$_1$ production, with optimal temperature facilitating the growth of these moulds ranging from 25 to 42 °C (Santin, 2005). On top of high temperature and drought that lead to mould colonisation, the above weather conditions favour the colonisation of various insects and lead to grain cracking and maize damaging, which additionally facilitate the production of the toxin in reference. Literature data have revealed that limitation of AFB$_1$ occurrence in grains before harvest can be achieved through the reduction of drought and temperatures, weed control, insect damage reduction, effective harvesting techniques and Aspergillus spore reduction in soil by virtue of crop turnover (Oyebanji & Efiuvwevwere, 1999; Widstrom, 1996).

AFB$_1$ levels obtained in this study per investigated year could be explained by weather conditions, best described by meteorological data provided by the Croatian Meteorological and Hydrological Institute (http://klima.hr/ocjene_arhiva.php). Based on the temperature data provided by the above institution, the years 2009, 2011 and 2013, i.e. the period of maize planting, growing and harvesting (April–September), was characterised as very (91–98%) to (rarely) extremely warm (>98). As for the precipitation seen in the years in question, it was reported to be normal (25–75%) to scarce (9–25%). The year 2010 was equally warm, but wet (75–91%) to highly wet (91–98%). As compared against the weather conditions described as favourable for AFB$_1$ production (Payne, 1998;

Widstrom, 1996), 2009–2013 weather conditions may be regarded as unfavourable for the toxin production, unlike the year 2012, when the weather went in favour of the latter.

As for Croatia, the part of the year 2012 of interest for this study was characterised by warm weather and the lack of precipitation. More specifically, the 2012 maize growth and harvesting period (May–August) was predominantly described as extremely warm (>98%) or warm (75–91%) to very warm (91–98%) (September), which unanimously applies for all investigated regions. The temperatures evidenced during the summer were close to 40 °C, with drought witnessed during the whole maize growth and harvesting period, in particular in August. As for the rainfall, the northern and the eastern region were very (2–9%) to extremely dry (<2%), with a substantially (even up to 71%) lesser average rainfall than the rainfall in the comparative period (1961–1990) (http://klima.hr/ocjene_arhiva.php). Such weather conditions seen during 2012 might have facilitated the formation of AFB$_1$ in maize, and consequently in grain mixtures and dairy cattle feed (analysed during 2013).

The AFB$_1$ levels determined under this study within 2009–2012 timeframe are comparable to those previously published for different years and different countries (Pietri, Bertuzzi, Pallaroni, & Piva, 2004; Rustom, 1997). Earlier extensive research of commodities, feedstuffs and feed ingredients revealed the maximal AFB$_1$ levels in samples coming from the Northern Europe to be 60 µg/kg, in samples coming from the Central Europe to be 311 µg/kg, and in samples coming from the Southern Europe and the Mediterranean region to be 656 µg/kg (Binder et al., 2007). As for this part of Europe, the research performed in Serbia during 2012 also pointed towards maize contamination with AFB$_1$ and concluded that weather changes might be held liable for such a contamination (Kos, Mastilović, JanićHajnal, & Šarić, 2013). Data have shown that, should a grain such as maize be grown at high ambient temperature, especially during drought, such a grain becomes more susceptible to aflatoxin formation. Grains stored under high moisture/humidity (>14%) conditions and at high temperatures (>20 °C) and/or inadequately dried, can potentially become contaminated. Grains have to be kept dry, free of damage and free of insects (Betran & Isakeit, 2004; Richard, 2007). It was observed that the risk of mycotoxin contamination is highly dependent on the country and region in which crops are grown (Bryden, 2012).

The fact remains that despite of all research efforts improper farming practices favouring AFB$_1$ contamination of feed and food are still in place (Prandini et al., 2009). In order to prevent them, it is necessary to implement the HACCP (Hazard Analysis and Critical Control Points) system and make every effort to improve agricultural practices, in particular grain production and storage environments. On top of that, screening and confirmatory analytical methods capable of rapidly and accurately detecting the contaminants of this sort should be further developed and widely implemented as a routine practice. Monitoring programs encompassing a sufficient number of representative food/feed samples analysed for mycotoxin presence and monitoring of risk factors identified as important for mycotoxin formation, should be carried out on an annual basis. Given the consequential economic losses and harm to human and animal health, a feasible strategy for reducing the risk of AFB$_1$ contamination of crops and feed should be developed, advocating a proper storage, a regular control of mould growth and the application of grain & feed decontamination methods so as to prevent or at least downsize the AFB$_1$ occurrence.

4. Conclusions

Significantly higher annual levels and frequencies of AFB$_1$ contamination were observed in maize, grain mixtures and dairy

cattle feed samples collected in 2013. Such a high level of AFB_1 contamination can be attributed to extreme warm and drought weather seen in 2012 during the maize growth and harvesting period. In other grains, significant AFB_1 levels failed to be determined throughout the investigated period. As for the sampling regions, significantly higher AFB_1 levels were determined in the samples coming from the eastern Croatia, which is a national leader in grain, feed and food production. In the future, in order to prevent the risk of AFB_1 contamination and the entrance of the toxin into the food chain, and subsequently also harm to human health and huge economic losses, a frequent AFB_1 monitoring should be established, encompassing a higher number of various cereal and feed samples, in particular maize and feed mixtures intended for dairy cattle feeding, which are to be sampled from all feed factories and all Croatian regions.

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