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SURVEY OF MYCOTOXIN FEED CONTAMINATION INCROATIA

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Abstract: The aim of this study was to investigate the contamination of feed with mycotoxins. A total of 103 samples of feed for poultry, pig and calves was collected at different animal feed factories in the central, eastern and north region of Croatia. The quantitative determination of aflatoxin B1 (AFB_1), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisins B_1, B_2 and B_3 (FUM) and T-2 toxin (T-2) concentration was done using the validated ELISA method. The observed results indicated an increased contamination of pig feed with *Fusarium* mycotoxins DON and ZEA with mean concentrations of 1454±1444 µg/kg and 153±161 µg/kg, higher than recommended in 20% and 5% of the samples, respectively. Higher DON concentrations than recommended were also observed in 7% of calves feed with a mean concentration of 1140±1288 µg/kg. The concentrations of AFB_1, OTA and FUM in all the investigated samples in all country regions were according to the maximum allowed or recommended values in the European Union.

Key words: mycotoxins, feed, Croatia, ELISA

Introduction

As livestock production is an important part which plays a significant role in providing the high quality of food, the fact that many types of moulds commonly contaminate feed and food with mycotoxins has caused an increased concern about the effects of mycotoxins on animal health and well being. The presence of moulds and mycotoxins in feed, as their secondary metabolites, results in the raw materials used in their production. The relatively high intake of raw materials with the diet of livestock can lead to nutrient losses and have adverse effects on animal health and on productivity *(Biagi, 2009)*. Several authors have classified mycotoxins as the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives, or pesticide residues *(Zinedine and Mañes, 2009)*.

Mycotoxins are mainly produced by moulds of the genera *Aspergillus*, *Penicillium* and *Fusarium,* commonly found in cereal grains, with different physical and chemical properties *(Binder et al., 2007)*. Traditionally, mycotoxigenic fungi have been considered to fall into two groups: field or plant pathogenic and storage or saprophytic fungi *(Placinta et al. 1999)*. During rainy years the percentage of mould contamination rises considerably, which frequently leads to the contamination of feed with mycotoxins *(Pleadin et al., 2012a)*. Factors that influence the mycotoxin production include temperature, moisture, oxygen, substrate aeration, inoculums concentration, microbial interaction, mechanical damage and insect infestation. When conditions are favourable, they can germinate, grow and produce the toxin *(CAST, 2003, Sforza et al., 2006)*.

Although acute ingestion of high levels of mycotoxins can be very harmful to animals, long term consumption of low concentrations of mycotoxins can also be damaging *(Chaytor et al., 2011)*. Aflatoxin B1, ochratoxin A, zearalenone, fumonisins, deoxynivalenol are the major mycotoxins that are commonly found in feed. Animals consumption of a mycotoxin contaminated diet may induce acute and long-term chronic effects resulting in teratogenic, carcinogenic, oestrogenic and immune-suppressive effects. The direct consequences of consumption of such contaminated feed include reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, increased disease incidence and reduced reproductive capacities *(Binder et al., 2007; Morgavi and Riley, 2007)*.

Among farm animals, pigs are especially sensitive to mycotoxin feed contamination *(Pietri et al., 2006)*, the most to aflatoxin B1, deoxynivalenol and zearalenone *(Chaytor et al., 2011)*. Aflatoxin B1 is the most potent hepatocarcinogen known in mammals and is classified by the International Agency of Research on Cancer as Group 1 carcinogen *(IARC, 1993)*. Due to the established toxicity of mycotoxins, it is necessary to determine their levels through national control programs in different feeds *(Köppen et al., 2010)*. Of particular concern is the co-occurrence of several *Fusarium* mycotoxins in the same sample of grain or animal feed, often in the presence of aflatoxin B1 *(Placinta et al., 1999)*.

The aim of this study was to evaluate the mycotoxins occurrence, namely aflatoxin B1 (AFB_1), ochratoxin A (OTA), zearalenone (ZEA), fumonisins B_1, B_2 and B_3 (FUM), deoxynivalenol (DON) and T-2 toxin (T-2) in poultry, pig and calves feed during the 2011 year in regions that have the highest feed production in Croatia.

Materials and Methods

Feed samples. A study was performed on 103 representative feed (feedingstuffs) samples from different animal feed factories (in total 30 factories) located in three regions of Croatia (central, eastern and north). All the samples were collected during the year 2011 and divided into three groups, according to the animal species for which they were used and the division of samples stated in Commission Recommendation 2006/576/EC. Poultry feed (n=34), pig feed (n=40) and calves feed (n=29) samples were sampled and prepared completely according to ISO 6497:2002 and ISO 6498:1998, respectively. Feed samples were ground in an analytical mill to a fine powder (Cylotec 1093, Tecator, Sweden). All the collected samples were stored at 4 ºC prior to analysis.

Mycotoxins determination. The concentration of mycotoxins was determined using the competitive ELISA method according to the kits manufacturer's instructions. Ridascreen kits were provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with antibodies; standard solutions with different concentrations of mycotoxins; enzime conjugate; anti-antibody; substrate and chromogen solution (urea peroxide/tetramethylbenzidine); stop solution, washing and dilution buffers. The standards of mycotoxins for the validation of analytical methods were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals used in the analysis were of analytical grade. The ELISA test was performed using the auto analyzer ChemWell (Awareness Technology Inc. 2910, USA). Absorbance was measured at 450 nm, considering the standard curve; the calculation of the mycotoxins concentration was done precisely taking into account the dilution factors. Statistical data analysis was performed using the Statistica Ver. 6.1 software (StatSoft Inc. 1984-2003, USA) with statistical significance set at a level of 95% (P =0.05).

Results and Discussion

Contamination of feed with mycotoxins is often a worldwide problem since there is no universal procedure that removes most of the mycotoxins without any affect on the nutritional value or not make it more expensive to produce. With the aim of minor losses in the livestock industry, considerable attention is paid to the prevention of mycotoxins contamination, and studies on different types of raw materials and compound feed, depending on various factors, are of great importance. The problem of mycotoxins is particularly expressed during rainy years, when the percentage of mould contamination which leads to subsequent formation of mycotoxins significantly increase *(Pepeljnjak et al., 2008)*. Among farm animals pigs are specially sensitive to mycotoxins, while ruminants are able to degrade the toxins in the rumen *(Pietri et al., 2006)*. In general, there is a lack of investigations on the presence of mycotoxins in animal feed *(Zinedine and Mañes, 2009)*.

Earlier published data in Croatia revealed that due to the specificity of climate in this region, it can be supposed that particular mycotoxins are often the contaminants of grains and feed as well. Results have shown that *Fusarium* and *Penicillium* species equally dominated in crops (40-60%), which is the reason for the frequent contamination of cereals and manufactured feed with mycotoxins *(Domijan et al., 2005; Pepeljnjak et al., 2008; Pleadin et al., 2012a)*. Whereas some European countries regarding many mycotoxins in animal feed have national legislation, in Croatian legislation there is no defined maximum allowed level except for aflatoxin B1 *(Official Gazette of the Republic of Croatia 80/2010)*.

This study investigated the contamination of feed for poultry, pigs and calves with mycotoxins during 2011 in samples from different feed factories, located in central, eastern and north region of Croatia. In the determination of their concentrations the ELISA method was used. Data of methods validation for each analyzed mycotoxin in feed samples was earlier published *(Pleadin et al., 2012b)*. The obtained results of mycotoxins concentration according to country regions (central, eastern and north) and types of feed (poultry, pig and calves) are shown in Table 1 and Table 2, respectively.

Table 1. Mycotoxin concentrations (μg/kg) in feed samples of central, eastern and north region of Croatia

Item	Central			Eastern			North		
	Mean[a]	Min[b]	Max[c]	Mean[a]	Min[b]	Max[c]	Mean[a]	Min[b]	Max[c]
AFB_1	1.15	0.88	3.11	2.11	0.93	4.33	3.28	1.12	5.36
OTA	1.31	1.15	1.88	1.41	1.20	1.62	1.44	1.35	1.72
ZEA	122	1.83	452	243	1.55	558	124.3	1.91	436
DON	1170	144	3272	1503	311	4716	1398	169.2	4107
T-2	12.1	7.20	14.2	25.2	14.3	35.1	19.4	7.7	32.5
FUM	81.2	21.4	140	558	446	670	1271	32.2	1939

[a]Arithmetic mean
[b]Minimum value detected
[c]Maximum value detected

Literary data shows that AFB_1 ingestion by animals may result in decreased growth rates, liver damage and immune suppression, carcinogenicity and other problems which are often greater in younger animals *(Chaytor et al., 2011)*. Only two papers reported the contamination of poultry feed with aflatoxins *(Zinedine and Mañes, 2009)*. The authors reported that the level of contamination ranged from 20 to 200 μg/kg, except in 4 samples which had higher levels of AFB_1 (2000-5625 μg/kg). These cases were associated with clinical aflatoxicosis in broiler chickens *(Kichou and Walser, 1993)*. *Zinedine et al. (2007)* reported that the percentage of contamination with aflatoxins is about 66.6%, while the contamination levels of poultry feed samples ranged between 0.05 and 5.38 µg/kg for AFB_1. In this study the determined mean AFB_1 concentrations were 3.22±2.21 µg/kg in poultry feed, 2.32±1.26 µg/kg in pig feed and 3.45±1.42 µg/kg in feed for calves. Maximum values of AFB_1 were lower than the maximum allowed level according to the Official Gazette of the Republic of Croatia 80/2010 in all the analyzed samples of different feed and there were no significant differences ($p>0.05$) in the values according to country regions.

During an extremely wet 1978 in Croatia maximum concentrations ranged up to 68900 µg/kg OTA and during 1980 up to 4700 µg/kg OTA *(Pepeljnjak et al., 2008)*. OTA has nephrotoxic properties, causing both acute and chronic lesions of the kidneys, and it is hepatotoxic, carcinogenic, teratogenic and immunotoxic to several animal species *(Pfohl-Leszkowicz and Manderville, 2007)*. Investigations show that feed contamination with OTA results in an increased mortality, poor feed conversion, poor growth rates and feed refusal *(Surai et al. 2008)*. The mean and maximum concentrations of OTA determined in this study, however, were according to the recommended values, significantly lower than stated in these earlier studies, and were lower or around the method limit of detection. *Binder et al. (2007)* as in this study found that the concentrations of AFB_1 and OTA were significantly lower in comparison to the concentrations of DON, ZEA and FUM.

Studies revealed that contamination of pig feed with DON caused decreased body weight in animals *(Harvey et al., 1989; Doll et al., 2003)*, increase in serum IgA and IgM *(Swamy et al., 2002; Pinton et al., 2010)*, cytokine tumor necrosis factor alpha *(Chaytor et al., 2011)* and caused damage including necrosis, blood vessel thickening and hemorrhage *(Chen et al., 2008)*. In Croatia, earlier investigation performed on feed samples during 2010 pointed to 96% contamination with a mean DON concentration of 1432.15±1749.68 µg/kg, significantly higher in comparison to 2009 when DON was determined in 25% of samples with a mean concentration of 22.07±40.5 µg/kg *(Mitak et al., 2011)*. Results of this study pointed to mostly similar concentrations in comparison to those obtained in 2010 with a mean value of 1089±862 µg/kg in poultry feed, 1454±1444 µg/kg in pig feed and 1140±1288 µg/kg in feed for calves.

Table 2. Mycotoxin contamination (µg/kg) of poultry, pig and calves feed in investigated regions

Item	Poultry feed				Pig feed				Calves feed			
	Mean	SD	Max level	n Positive samples[c]	Mean	SD	Max level	n Positive samples[c]	Mean	SD	Max level	n Positive samples[c]
AFB_1	3.22	2.21	20[a]	-	2.32	1.26	20[a]	-	3.45	1.42	10[a]	-
OTA	1.42	1.33	100[b]	-	1.33	1.25	50[b]	-	1.48	1.17	-	-
ZEA	112	147	-	-	153	161	250[b]	2	213	185	500[b]	-
DON	1089	862	-	-	1454	1444	900[b]	8	1140	1288	2000[b]	2
T-2	18.2	8.31	-	-	19.4	11.5	-	-	32.4	15.1	-	-
FUM	643	768	20000[b]	-	32.4	16.8	5000[b]	-	1556	344	20000[b]	-

[a]Maximum allowed level according to the Official Gazette of the Republic of Croatia 80/2010
[b]Maximum recommended level according to the Commission Recommendation 2006/576/EC
[c]Number of samples that exceeded the allowed or recommended maximum levels

The mean concentrations can be also compared with data of other studies of *Fusarium* mycotoxins published by several European authors *(Tanaka et al., 1990; Placinta et al., 1999, Binder et al., 2007)*. *Binder et al. (2007)* reported the results of research of *Fusarium* mycotoxins, AFB_1 and OTA in Europe with regard to geographical origin. The highest concentrations were determined for DON and amounted to 5510 µg/kg in the region of Northern Europe, 8020 µg/kg in the region of Central Europe, and 3036 µg/kg in the region of Southern Europe with the Mediterranean. In the research *Mankevičiene et al. (2007)*, conducted on grain samples that were used as fodder (from harvests 2004 and 2005) and focused on *Fusarium* mycotoxins, the most common mycotoxin DON was detected in 62.5% to 100% of cases with concentrations ranged up to 1121 µg/kg. As both years covered by the survey were extremely rainy, high concentrations of DON were expected.

In the study by *Binder et al. (2007)* the highest concentrations of ZEA and FUM were determined in Southern Europe and the Mediterranean at levels of 2348 µg/kg for ZEA and 3120 µg/kg for FUM. High concentrations of ZEA, in the range of 2200 to 22090 µg/kg of feed in breeding gilts, resulted with harmful effects on their reproductive performance by altering embryo development and reducing the number of live born piglets *(Tiemann and Danicke 2007)*. In Croatia, *Pepeljnjak* *and Šegvić (2004)* made a six-year survey in which they found a high incidence of *Fusarium* mould species (70%), production of ZEA in 14.8% of *F. graminearum* strains, and 3.2% in strains of *F. moniliforme*. In a research made *by Domijan et al. (2005)* the maximum found concentration of ZEA was 3.22 µg/kg which indicates the low level of maize contamination with this mycotoxin.

In relation to the results of previous research for DON and ZEA in Croatia *(Domijan et al., 2005; Mitak et al., 2005; Pepeljnjak et al., 2008)* and also with the published data worldwide *(Binder et al., 2007)*, it can be concluded that a certain number of feed samples in this research had significantly high concentrations. Also, comparing the obtained concentrations of DON and ZEA with the maximum recommended concentrations for these mycotoxins in feed *(EC 576/2006*) the results indicated an increased contamination of pig feed with DON and ZEA, with mean concentrations of 1454±1444 µg/kg and 153±161 µg/kg, higher than recommended in 8 (20%) and 2 (5%) of the 40 samples of pig feed, respectively. Higher DON concentrations than recommended were also observed in 2 of 29 (7%) samples of feed for calves with a mean concentration of 1140±1288 µg/kg. DON and ZEA were determined with great variations of concentrations between the samples. The concentration of DON was generally (103 samples analyzed) higher than the recommended in about 10% of samples with a maximum concentration of 4107 µg/kg. A higher ZEA concentration than the maximum recommended was determined in about 2% of the total number of feed samples, with a maximum concentration of 558 µg/kg determined in feed for calves in the eastern part of the country. In this study it was also observed that the samples in which the low concentrations of DON were determined have also low concentrations of ZEA, or both mycotoxins were not detected, or mostly the results indicate on both higher concentrations as in our earlier study performed on feed for fattening pigs *(Pleadin et al., 2012b)*.

In Europe, there is very little research conducted on the occurrence of T-2 in feed, which bearing in mind the explicit toxicity of this mycotoxin needs further data collection and the legal values of the maximum allowed level need to be defined *(Vulić et al., 2011)*. T-2 is a very potent cytotoxic and immunosuppressive toxin, which can cause acute intoxication and chronic diseases in both humans and animals *(Peraica et al., 1999)*. The major effect of T-2 is considered to be the inhibition of protein synthesis, which leads to a secondary disruption of DNA and RNA synthesis *(Bennett and Klich, 2003)*, and also affects the immune system *(Creppy, 2002)*. The maximum allowed levels of T-2 toxin in feed have not been set in Europe nor in Croatia yet. Earlier performed investigations of T-2 toxin in Croatia by *Sokolović and Šimpraga (2006)*, over the period 1998 to 2004, determined the values of 100 to 500 µg/kg whereas in an investigation by *Vulić et al. (2011)* the highest concentrations of T-2 was determined in cattle feed (67.68 µg/kg). The highest concentration of T-2 toxin (1776 µg/kg) was determined in Northern Europe *(Binder et al., 2007)*. In this study, the lower mean concentration was determined in poultry feed (18.2±8.31 μg/kg) and the highest mean concentration in feed for calves (32.4±15.1 μg/kg), which points to generally not significantly high concentrations in comparison to earlier published data.

Contamination of feed with FUM resulted in a diverse range of damage to animal tissues, including lesions to the esophagus, gastrointestinal tract, liver, lungs, and brain *(D'Mello et al., 1999)*. *Domijan et al. (2005)* analyzed samples of corn from different parts of Croatia collected after the autumn harvest and observed high concentrations of fumonisin B_1, which ranged from 196.8 to 1377.6 μg/kg, and fumonisin B_2 from 684 to 3084 μg/kg. Fumonisin B_1 was the most common mycotoxin and was detected in all the samples. FUM concentrations determined in this study in some samples were significantly higher than the method limit of detection with a maximum concentration of 1939 μg/kg determined in the northern part of the country in pig feed, although in all the analyzed samples the obtained values were according to the recommended values.

In eastern Croatia the highest mean concentrations of ZEA and DON, significantly higher ($p<0.05$) than in central and north region, were determined, whereas the highest mean concentration of FUM was observed in the northern country region. In the central part, the lower mean concentrations of all the analyzed mycotoxins were determined which means that this region has a lower level of feed contamination.

Conclusion

The results indicated an increased contamination of pig feed with *Fusarium* mycotoxins DON and ZEA and higher DON concentrations in particular samples of calves feed than recommended. The determined T-2 concentrations pointed to not significantly high values in comparison to earlier published data. Concentrations of AFB_1, OTA and FUM in all the investigated samples in the central, eastern and north region of Croatia were according to the maximum allowed or recommended values.

Pregled kontaminacije hrane za životinje mikotoksinima u Hrvatskoj

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Rezime

Cilj ovog istraživanja bio je da se utvrdi kontaminacija hrane za životinje mikotoksinima. Ukupno 103 uzorka hrane za živinu, svinje i telad prikupljeno je u različitim fabrikama stočne hrane u srednjem, istočnom i severnom području Hrvatske. Kvantitativno određivanje koncentracija aflatoksina B_1 (AFB_1), ohratoksina A (OTA), zearalenona (ZEA), deoksinivalenola (DON), fumonizina B_1, B_2 i B_3 (FUM) i T-2 toksina (T-2) vršeno je korišćenjem validnih ELISA metoda.

Dobijeni rezultati pokazali su povećanu kontaminaciju hrane za svinje *Fusarium* mikotoksinima DON i ZEA, s prosečnim koncentracijama od 1454±1444 µg/kg i 153±161 µg/kg, većim od preporučenih za DON u 20% i za ZEA u 5% uzoraka. Veće koncentracije DON od preporučenih određene su i u 7% uzoraka hrane za telad, sa prosečnom koncentracijom od 1140±1288 µg/kg. Koncentracije AFB_1, OTA i FUM u svim ispitivanim uzorcima, u svim područjima zemlje, bile su u skladu sa dozvoljenim ili preporučenim vrednostima Evropske Unije.

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