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SCREENING OF MYCOTOXINS IN ANIMAL FEED FROM THE REGION OF VOJVODINA

ABSTRACT: This paper shows the results of screening of mycotoxins in animal feed originating from the region of Vojvodina. Permanent screening is needed on all levels of production and storage, as well as the use of known methods to reduce mould contamination or toxin content in feedstuffs and feed. A total of 56 representative samples were collected from feed companies from the region of Vojvodina. Samples were collected during February 2009. The collected samples included 41 samples of feedstuffs (soybean, soybean meal, soybean grits, soybean cake, maize, sunflower meal, barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, alfalfa meal, yeast, dried whey, fish meal, meat-bone meal) and 15 samples of complete feedingstuffs. The amounts of aflatoxins, ochratoxin A, zearalenone, fumonisin and deoxynivalenol were determined. Screening method for the analysis was done using Neogen Veratox® testing kits. The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Mycotoxins were present in 71.4% of the samples, but the values determined were below the maximum allowed limits for both Serbian and EC reference values. Zearalenone was found with the highest incidence (57.1% of samples), followed by ochratoxin A (37.5%), fumonisin (33.9%), deoxynivalenol (14.3%) and aflatoxins (3.6%).

KEYWORDS: animal feed, ELISA, mycotoxins, screening

INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi that cause a toxic response (mycotoxicosis) when ingested by higher animals. Many fungi of the genera *Fusarium*, *Alternaria, Penicillium, Aspergillus*, *Cladosporium* and others are well known as producers of a great number of various toxic metabolites. The produced mycotoxins are thermo resistant and lose none of their toxicity during thermal processing (Stojanović et al., 2005).

Moulds that produce toxins may contaminate human foods and animal feeds through fungal growth prior to and during harvest, or during (improper) storage (Bhatnagar et al., 2004). Plants may be contaminated by mycotoxins in two ways: fungi growing as pathogens on plants, or growing saprophytically on stored plants. However, not all fungal growth results in mycotoxin formation, and the detection of fungi does not necessarily imply the presence of mycotoxins (Binder et al., 2007). The formation of mycotoxins is affected by biological, physical and chemical factors (D'Mello and Macdonald, 1997). The same toxin may be formed by a variety of species of fungi, but not necessarily by all the strains of the same species. Similarly, in certain instances, the same species of fungi may produce several forms of mycotoxins.

For practical consideration, in the feed manufacturing process, aflatoxins, trichothecenes, zearalenone, ochratoxins, and fumonisins are of particular interest (Table 1), though the extent of harm each toxin (group) can cause is highly species-dependent (Binder, 2007). Mycotoxins, when present in the diet, cause acute and/or chronic adverse health effects in animals and humans, depending upon the level consumed (Thieu et al., 2008).

Tab. 1 — Overview of the most relevant mycotoxins in animal production (Binder, 2007)

Major classes of mycotoxins	Most relevant representatives in grains and feed	Examples of mycotoxin-producing fungi	Effects observed in animals
Aflatoxins	Aflatoxin B1, B2, G1, G2	*Aspergillus flavus, Aspergillus parasiticus*	Liver disease (hepatotoxic, hepatocarcinogen), carcinogenic and teratogenic effects
Trichothecenes	Deoxynivalenol, T-2 toxin	*Fusarium graminearum, Fusarium sporotrichioides, Fusarium poae, Fusarium equiseti*	Immunologic effects, hematological changes, digestive disorders (emesis, diarrhea, reduced feed intake) dermatitis, oral lesions, hemorrhages of intestinal tissues, edema
Zearalenone	Zearalenone	*Fusarium graminearum*	Estrogenic effects (edema of vulva, enlargement of uterus), atrophy of ovaries and testicles, abortion
Ochratoxins	Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum, Penicillium viridicatum*	Nephrotoxicity, porcine nephropathy, mild liver damage, immune suppression
Fumonisins	Fumonisin B1, B2, B3	*Fusarium verticillioides* (syn., *moniliforme), Fusarium proliferatum*	Pulmonary edema, leukoencephalomalacia, nephrotoxicity, hepatotoxicity

Mycotoxin-producing mould species are extremely common and can grow on a wide range of substrates under a wide range of environmental conditions. For agricultural commodities, the severity of crop contamination tends to vary

from year to year, based on climate and other environmental factors. Mycotoxins occur, with varying severity, in agricultural products all around the world. The estimate usually given is that one quarter of the world's crops are contaminated to some extent with mycotoxins (Fink-Gremmels, 1999; Mannon and Johnson, 1985).

Mycotoxins can enter the food chain in field, during storage, or at later points. Mycotoxin problems are exacerbated whenever shipping, handling, and storage practices are conducive to mould growth. Animal feeds are an essential part in the farm animal to human food chain; therefore, infectious and non-infectious hazards present in animal feeds pose a threat to human health. Mycotoxin contamination of feeds results in economic loss and transmission of toxins into the food chain.

Since it is normally impracticable to prevent the formation of mycotoxins, the food industry has established internal monitoring methods. Similarly, government regulatory agencies survey the occurrence of mycotoxins in foods and feeds and establish regulatory limits. Maximum tolerated levels of mycotoxins in animal feed have been established in many countries. Allowed limits for mycotoxins in feed on the territory of the European Union are regulated by the regulations of the European Union (EC 32/2002, EC 100/2003, EC 576/2006). Guidelines for establishing these limits are based on epidemiological data and extrapolations from animal models, taking into account the inherent uncertainties associated with both types of analysis. Estimations of an appropriate safe dose are usually stated as a tolerable daily intake (Kuiper-Goodman, 1998; Kuiper-Goodman, 1994; Smith et al., 1995). Countries that are members of the European Union have harmonized their regulations while other countries, like Serbia, have their own regulations. Allowed limits for mycotoxins in animal feed in Serbia are determined by official regulations of Serbia (Official Gazette of SFRY, 2/90, 27/90). The main differences between the EU and Serbian regulations for the feedstuffs and feedingstuffs are as follows: different categories of feedingstuffs; different values for allowed limits; in the EU, complete and complementary feedingstuffs categories are separated as opposed to the Serbian regulations, and in Serbia, the maximum allowed limits for FUM have not been determined. In Serbia, monitoring of mycotoxins is not obligatory at present, but the approval of a new law has been awaited, which will be in accordance with the EU law. By the new law, monitoring will be compulsory.

The aim of our work was to screen the presence of mycotoxins in animal feed originating from the region of Vojvodina. Permanent screening is needed on all levels of production and storage, as well as the use of known methods to reduce mould contamination or toxin content in feedstuffs.

MATERIAL AND METHODS

A total of 56 representative samples (1—2 kg per sample) were collected from the feed companies in Vojvodina. Samples were collected during February 2009. The collected samples included 41 samples of feedstuffs (soybean,

soybean meal, soybean grits, soybean cake, maize, sunflower meal, barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, alfalfa meal, yeast, dried whey, fish meal, meat-bone meal) and 15 samples of complete feedingstuffs.

The amounts of aflatoxins (AFS), ochratoxin A (OTA), zearalenone (ZEA), fumonisin (FUM) and deoxynivalenol (DON) were determined. Screening method for the analysis was done using Neogen Veratox® testing kits with limits of detection of 1 μg/kg (ppb) for ochratoxin A, 2 μg/kg (ppb) for aflatoxins, 10 μg/kg (ppb) for zearalenone, 50 μg/kg (ppb) for fumonisin and 0.1 mg/kg (ppm) for DON.

The test itself is a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Free mycotoxins in the samples and controls are allowed to compete with enzyme-labelled mycotoxins (conjugates) for the antibody binding sites. After a wash step, substrate is added, which reacts with the bound conjugate to produce blue colour. More blue colour means less mycotoxin. The test is read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities are plotted against the curve to calculate the exact concentration of mycotoxin.

RESULTS AND DISCUSSION

A total of 56 samples of feedstuffs and complete feedingstuffs were analyzed. Mycotoxins were found in 71.4% of the samples, but the values determined were below the maximum allowed limits according to both Serbian and EC reference values. ZEA was found with the highest incidence (57.1% of samples), followed by OTA (37.5%), FUM (33.9%), DON (14.3%) and AFS (3.6%). Incidence rate of aflatoxins was very low (3.6%) which was expected since aflatoxins are rarely found in Serbia (Jajic et al., 2008). The obtained results were compared with available literature. No results were found regarding the presence of FUM in feedstuffs and complete feedingstuffs in Serbia.

Tab. 2 — Occurrence of mycotoxins in maize samples

No. of samples	Feedstuff	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
4	Maize	—[a]	—	—	438	—
		—	—	—	350	0.46
		—	—	—	543	—
		—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in the maize samples are given in Table 2. FUM and DON were found in 75 and 25% of the samples, respectively, but none of the samples was contaminated with AFS, OTA and ZEA.

Jajić et al. (2008) analyzed DON in maize samples collected in 2004 (10 samples) and 2005 (66 samples). The number of positive samples for

DON in maize was 50% in 2004 and 43.9% in 2005. Mycotoxicological analyses of maize in the previous investigations showed significant contamination with AFS, OTA and ZEA. In 2000, Jajić et al. (2001) analyzed 38 samples of maize and found AFS in 73.6%, OTA in 78.9% and ZEA in 86.8% of the analyzed samples. Two years later, in the study conducted on the samples collected from the region of Vojvodina, Mašić et al. (2003) analyzed 53 samples of maize and found lower incidence of mycotoxins; AFS were found in 16.9%, OTA in 24.5% and ZEA in 35.8% of the analyzed samples. This shows that AFS, OTA and ZEA concentrations in maize may vary considerably, probably as a result of different drying techniques and weather conditions.

Tab. 3 — Occurrence of mycotoxins in soybean, soybean meal, soybean grits and soybean cake samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
5	Soybean	—[a]	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	2.63	36.5	—	—
		—	—	26.9	—	—
7	Soybean meal	—	5.12	55.3	—	—
		—	3.97	56.3	—	—
		—	3.52	61.0	—	—
		5.53	3.75	56.9	97.4	0.25
		5.20	2.61	69.5	—	—
		—	—	74.3	—	—
		—	4.87	61.8	—	—
2	Soybean grits	—	3.32	48.9	—	—
		—	—	50.2	—	—
1	Soybean cake	—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in soybean, soybean meal, soybean grits and soybean cake samples are given in Table 3. 3 out of 5 analyzed samples of soybean did not contain mycotoxins. OTA was detected in one and ZEA in two samples. AFS, FUM and DON were not present in any soybean sample, which is in accordance with the previous results of Jakić et al. (2005) who investigated 63 samples of soybean collected in the period from 1999 to 2004, and their results showed that 0% of soybean samples were contaminated with aflatoxins. In soybean meal samples, ZEA was found with the highest incidence (100% of samples), followed by OTA (85.7%), AFS (28.6%), FUM (14.3%) and DON (14.3%). Jajić et al. (2001) analyzed 10 samples of soybean meal collected in 2001 and, according to their results, 100, 90 and 100% of the samples were contaminated with AFS, OTA and ZEA, respectively. This is in contrast to the data reported by Mašić et al. (2003) who investigated 43 samples of soybean meal from the region of Vojvodina in 2002, and found that 2.3, 6.9 and 4.6% of the samples were contaminated with AFS,

OTA and ZEA, respectively. Jajić et al. (2008) analyzed DON in soybean and soybean meal samples collected in 2004 (13 samples) and 2005 (11 samples). The number of positive samples for DON was 7.7% in 2004 and 9.1% in 2005, which is in accordance with our results.

Tab. 4 — Occurrence of mycotoxins in sunflower meal samples

No. of samples	Feedstuff	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
7	Sunflower meal	—[a]	2.58	48.4	168	0.27
		—	—	41.3	—	—
		—	2.24	35.0	—	0.33
		—	3.82	32.8	—	—
		—	—	38.9	—	0.33
		—	2.62	44.2	—	—
		—	2.37	38.8	67.5	0.28

[a] toxin was not detected

The results of screening of mycotoxins in sunflower meal samples are given in Table 4. Of the 7 analyzed samples, ZEA was found with the highest incidence (100% of samples), followed by OTA (71.4%), DON (57.1%) and FUM (28.6%). None of the samples was contaminated with AFS. In the period between 1999 and 2001, Jajić et al. (2001) investigated sunflower meal (21 samples) for the presence of ZEA. The percentage of samples contaminated with ZEA during a 3-year period was 100%, which is in accordance with our results. Jajić et al. (2008) analyzed DON in sunflower and sunflower meal samples collected in 2004 (9 samples) and 2005 (10 samples). The number of positive samples for DON was 44.4% in 2004 and 50% in 2005. Our results show that the percentage of samples contaminated with DON was somewhat higher and was 57.1%. The incidence of OTA in sunflower meal was registered in 71.4% of the analyzed samples. Previous analyses (Jajić et al., 2001) showed that in years 1999, 2000 and 2001, the percentage of samples contaminated with OTA was higher (100, 92.3 and 100%, respectively). Analyses of sunflower meal showed that no samples were contaminated with aflatoxins, which is in accordance with the results reported by Mašić et al. (2003) who analyzed 19 samples of sunflower meal in 2002 from the region of Vojvodina.

Tab. 5 — Occurrence of mycotoxins in fish and meat-bone meal samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
5	Fish meal	—[a]	6.83	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
		—	—	—	—	—
2	Meat — bone meal	—	—	—	—	—
		—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in fish and meat-bone meal samples are given in Table 5. A total of 5 samples of fish meal were analyzed. Only one sample was positive for the presence of OTA and other mycotoxins were not detected. In 2 samples of meat-bone meal mycotoxins were not detected.

Tab. 6 — Occurrence of mycotoxins in various feedstuffs samples

No. of samples	Feedstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
1	Barley	—[a]	—	—	—	—
1	Wheat feed flour	—	4.79	35.7	—	0.60
1	Rapeseed meal	—	—	—	—	—
1	Dehydrated sugar beet pulps	—	—	—	—	—
2	Alfalfa meal	— —	9.48 3.49	177 159	— —	0.28 —
1	Yeast	—	2.26	31.8	—	—
1	Dried whey	—	—	—	—	—

[a] toxin was not detected

The results of screening of mycotoxins in various feedstuffs samples are given in Table 6. One sample of barley, wheat feed flour, rapeseed meal, dehydrated sugar beet pulps, yeast, dried whey and two samples of alfalfa meal were analyzed. Mycotoxins were not detected in barley, rapeseed meal, dehydrated sugar beet pulps and dried whey. Analyses showed that wheat feed flour, alfalfa meal and yeast were contaminated with OTA and ZEA, while DON was found in wheat feed flour and in one sample of alfalfa meal. None of the samples was contaminated with AFS and FUM. The number of analyzed samples was small and cannot be interpreted as the actual situation in the field conditions.

Tab. 7 — Occurrence of mycotoxins in complete feedingstuffs samples

No. of samples	Complete feedingstuffs	AFS (ppb)	OTA (ppb)	ZEA (ppb)	FUM (ppb)	DON (ppm)
15	Complete mash for piglets — pre-starter	—[a]	—	—	—	—
	Complete mash for piglets from 1 to 15 kg — starter	—	—	—	270	—
	Complete mash for piglets from 1 to 15 kg — starter	—	—	37.7	439	—
	Complete mash for piglets from 15 to 25 kg — starter	—	—	27.2	232	—
	Complete mash for pigs growth and fattening from 25 to 60 kg	—	—	—	555	—
	Complete mash for gilts	—	—	—	291	—
	Complete mash for gestating sows	—	—	—	288	—
	Complete mash for lactating sows and boars	—	—	37.8	479	—

Complete mash for dairy cows over 20 l milk per day	—	—	52.6	335	—
Complete mash for cattle fattening from 250 to 350 kg	—	—	40.2	415	—
Complete mash for cattle fattening from 250 to 350 kg	—	5.24	129	500	—
Complete mash for broilers I	—	8.89	36.7	430	—
Complete mash for broilers I	—	—	52.0	286	—
Complete mash for layers	—	2.66	58.4	270	—
Complete mash for trout	—	—	33.6	—	—

[a] toxin was not detected

The results of screening of mycotoxins in complete feedingstuffs samples are given in Table 7. A total of 15 samples of complete feedingstuffs were analyzed. FUM was found with the highest incidence (86.6% of samples), followed by ZEA (66.6%) and OTA (20%). None of the samples was contaminated with AFS and DON.

The difference in contamination level in our samples and samples analyzed in previous years could be attributed partly to agricultural factors and partly to variations in the susceptibility to different *Fusarium, Aspergillus* and *Penicillium* species in interaction with climatic factors.

CONCLUSION

Although this screening showed that 71.4% of the samples were contaminated with mycotoxins, concentrations were lower than the maximum level adopted by Serbian and European Commissions' regulations. Aflatoxins were found with the lowest incidence (3.6%) followed by deoxynivalenol (14.3%), fumonisin (33.9%), ochratoxin A (37.5%) and zearalenone (57.1%). Since all mycotoxins were found in the analyzed samples, it can be concluded that the monitoring is necessary. Given the vast diversity of commodities that may be infected by fungi, it is important to acknowledge the fact that the presence of specific fungi does not necessarily mean that a fungal toxin is present. It is, therefore, pertinent to analyse the presence of mycotoxins in all cases as far as possible. The results will help ensure better quality assurance in the feed, as well as develop the tools for management decision on the fate of feeds that do not meet the required standards. Based on the given results for the presence of fumonisins in feed, inclusion in the National regulation should be considered. Also, there is a need for harmonization of the National regulations with those of EU.

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СКРИНИНГ МИКОТОКСИНА У ХРАНИ ЗА ЖИВОТИЊЕ СА ПОДРУЧЈА ВОЈВОДИНЕ

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Резиме

Рад приказује резултате скрининга микотоксина у храни за животиње са подручја Војводине. Перманентан скрининг је потребан на свим нивоима производње и складиштења, као и коришћење познатих метода за смањење контаминације плеснима или токсинима у хранивима и храни за животиње. Прикупљено је укупно 56 репрезентативних узорака из фабрика за прозводњу хране за животиње на подручју Војводине. Узорци су прикупљени током фебруара 2009. Прикупљени узорци су обухватали 41 узорак хранива (соја, сојина сачма, сојин гриз, сојина погача, кукуруз, сунцокретова сачма, јечам, пшенично сточно брашно, сачма уљане репице, суви резанац шећерне репе, брашно од луцерке, сточни квасац, сурутка у праху, рибље брашно, месно коштано брашно) и 15 узорака потпуних смеша. Одређена је количина афлатоксина, охратоксина А, зеараленона, фумонизина и деоксиниваленола. Скрининг метода за анализу је изведена помоћу Neogen Veratox® тестова. Овај метод подразумева директну компетитивну ензимску имуноафинитетну методу (CD-ELISA). Микотоксини су детектовани у 71,4% узорака, али су утврђене вредности испод максималне дозвољене границе прописане правилником Србије и ЕУ. Зеараленон је пронађен у највећем броју узорака (57,1% узорака), затим охратоксин А (37,5%), фумонизин (33,9%), деоксиниваленол (14.3%) и афлатоксини (3.6%).