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## The natural occurrence of toxigenic moulds and mycotoxins in Slovenian primary grain production

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### ABSTRACT

The aim of the present study was to determine the contamination of grains, grown in Slovenia and used for animal diets by Slovene farmers in year 2009. A total of 66 samples was examined on toxigenic moulds from genera *Fusarium*, *Penicillium*, *Aspergillus*, *Alternaria* and on 8 different mycotoxins. The leading contaminants among moulds were from *Fusarium spp.*, detected in 51 samples, mostly in barley (19). The average number of *Fusarium spp.* colony forming units (cfu) in different grains was from 5.5-23.3 x 10<sup>3</sup>/g, whereas the contamination of barley with *Penicillium*, *Aspergillus* and *Alternaria spp.* was 4.5, 19.3 and 5 x 10<sup>3</sup> cfu/g. Using liquid (HPLC) and gas chromatography (GC) methods, the presence of various mycotoxins (expressed for 12% of moisture content) was proved in 57.6% of all samples; mostly deoxynivalenol (DON, 54.5%) in concentrations of 130-2860 µg/kg, followed by zearalenone (ZON, 15.1%, 70-800 µg/kg), fumonisin B1 (3%, 120-210 µg/kg), while fumonisin B2, ochratoxin A, diacetoxyscirpenol (DAS), HT-2 and T-2 toxins were not detected. The results indicate that further control of toxigenic moulds and mycotoxins in Slovenian primary grain production is thus required and justified.

**Keywords:** animal feed, grains, grains-microbiology, mycotoxins-analysis, Slovenia

### IZVLEČEK

#### NARAVNA KONTAMINACIJA ŽIT IZ PRIMARNE PROIZVODNJE V SLOVENIJI S TOKSIGENIMI PLESNIMI IN MIKOTOKSINI

Z raziskavo smo želeli dobiti vpogled v kontaminacijo žit, ki so jih kmetje v Sloveniji pridelali in uporabili za prehrano živali v letu 2009. Na toksigene plesni iz rodu *Fusarium*, *Penicillium*, *Aspergillus*, *Alternaria* in 8 različnih mikotoksinov smo preiskali skupno 66 vzorcev. Najbolj razširjene so bile plesni iz rodu *Fusarium*. Izolirali smo jih iz 51 vzorcev, najpogosteje iz ječmena (19). Njihovo povprečno število kolonij (cfu) je bilo v različnih žitih od 5,5-23,3 x 10<sup>3</sup>/g, v ječmenu pa je bilo 4,5, 19,3 in 5 x 10<sup>3</sup> cfu/g kolonij plesni iz rodu *Penicillium*, *Aspergillus* in *Alternaria*. S tekočinsko (HPLC) in plinsko kromatografijo (GC) smo dokazali različne mikotoksine (rezultati so izraženi pri 12% vsebnosti vlage vzorca) v 57,6% vseh preiskanih vzorcev. Največ vzorcev je vsebovalo deoksinivalenol (DON, 54,5%) v koncentraciji od 130-2860 µg/kg, sledijo zearalenon (ZON, 15,1%, 70-800 µg/kg) in fumonizin B<sub>1</sub> (3%, 120-210 µg/kg). Fumonizinov B<sub>2</sub>, ohratoksina A, diacetoksiscirpenola (DAS), HT-2 in T-2 toksina nismo dokazali v nobenem vzorcu. Rezultati kažejo, da je nadaljnja kontrola toksigenih plesni in mikotoksinov v krmi iz primarne pridelave v Sloveniji vsekakor potrebna in upravičena.

**Ključne besede:** krma, žita-mikrobiologija, mikotoksini-analize, Slovenija

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## 1 INTRODUCTION

Ubiquitous microorganisms can be found in the air, water, soil and crops, on animals and human beings and in animal feed. Animal feed is thus being contaminated with microorganisms during its production, modification, storage and consumption (Marquardt, 1996; Maciorowski et al., 2007). Certain microbial groups (collective term: field flora of primary flora) can be found on plants predominantly at the time of harvesting. They are described also as product-typical microflora, including saprophytic bacteria (yellow pigmented bacteria, *Pseudomonas spp.*) and moulds (*Fusarium spp.*, *Cladosporium spp.*, *Verticillium spp.*, *Acremonium spp.*). During storage, a change in microflora can occur due to the reduction of the content of product-typical microorganisms or due to the reproduction of the spoilage-indicating microflora, adapted to the storage conditions. The main spoilage-indicating moulds are *Penicillium spp.*, *Aspergillus spp.*, *Scopulariopsis spp.*, and *Mucorales spp.* Microorganisms causing spoilage are also all species of yeasts (VDLUFA, 2007 d). The growth of moulds is greatly influenced by the water content of the substrate. At low moisture content (usually less than 14-16%) most storage fungi do not grow or grow very slowly (Smith and Yarrow, 1996).

Some species of mould are able to produce highly toxic compounds called mycotoxins. Different moulds can produce different mycotoxins on the same commodity. These toxins can adversely affect animal health and production and can have harmful effects on humans if transmitted to foods (Marquardt, 1996). However, it has been noted that mycoflora can colonize crops and produce mycotoxins without visible damage. Conversely, signs of fungal infestations do not necessarily correlate with the presence of mycotoxins (Patino et al., 2004).

Until now more than 300 mycotoxins have been identified. In grains, most important mycotoxins, affecting animals' health, are: aflatoxins, ochratoxins, fumonisins, zearalenone (ZON), deoxynivalenol (DON), T-2 toxin and other trichothecenes and ergot alkaloids (Kumar et al., 2008). The most commonly known mycotoxins are aflatoxins due to the fact that they represent one of the most potential carcinogenic

substance known so far. Aflatoxins are produced by the moulds of genus *Aspergillus*. Trichothecenes, most important among them are DON, T-2 toxin, HT-2 toxin and DAS, are in general potent inhibitors of eukaryotic protein synthesis (Bennett and Klich, 2003). Although DON is one of the least acutely toxic trichothecenes, it should be treated as an important food safety issue because of very common contamination of grains. Other trichothecenes, T-2 and HT-2 toxins may cause haematological changes and immune suppression, reduced feed intake and skin irritation as well as diarrhoea and haemorrhages of internal tissues. Trichothecenes are mainly produced by the moulds of genus *Fusarium*, which produce also ZON. ZON is a mycotoxin with strong hyper-estrogenic effects, resulting in impaired fertility, stillbirths of females offsprings and a reduced sperm quality in male animals. Ochratoxin A, which is produced by a number of *Aspergillus* and *Penicillium* species, has been listed as possibly carcinogenic to humans. It causes renal toxicity, nephropathy and immune suppression in several animal species, resulting in reduced animal production performance parameters. The most recently described mycotoxins with relevance in human and animal nutrition are fumonisins. They cause severe animal diseases such as equine leukoencephalomalacia (ELEM) in horses, and porcine pulmonary oedema (PPE) in pigs. Fumonisin are produced by a number species from genus *Fusarium*, as well as *Alternaria* (Binder, 2007). Recently, the mycotoxin analyses were performed on a total of 2798 samples sourced from Europe, Mediterranean and Asian-pacific region. More than a half of samples of grains and feeds from Europe were contaminated at levels above the limit of quantification of methods applied with DON, ZON and T-2 toxin (Binder et al., 2007).

In Slovenia, there is relatively little information related to the natural occurrence of fungi and mycotoxins in grains. In this context, the aim of this work was to investigate the contamination of grains on toxigenic moulds (*Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria*) and mycotoxins: ochratoxin A, ZON, DON, DAS, T-2 toxin, HT-2 toxin as well as fumonisins B1 and B2.

## 2 MATERIALS AND METHODS

### 2.1 Sample collection

The collecting of 66 samples of different grains, grown on Slovene farms, took place in the period between June and August 2009. Samples of raw material were taken directly at the farms. Several incremental samples were taken randomly from the whole lot and combined to the aggregate sample.

After homogenisation and grinding of the aggregate sample in the lab, a laboratory sample of 1.5 kg was taken for the examination. A total of 100 g was randomly selected for the analysis of mycoflora, which was done immediately while the other part of the sample was stored at 8 °C for mycotoxicological analyses.

## 2.2 Isolation and identification of moulds and yeasts

To examine the contamination of the samples on saprophytic moulds and yeasts, a slightly modified method of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) was used (VDLUFA, 2007a; b; c; d). To 20 g of each sample, 180 ml of 0.5% peptone water was added. The mixture was homogenised using linear shaker for 20 min and then diluted to final concentration of  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  (VDLUFA, 2007a). 1 ml aliquots of each dilution were spread (in duplicates) on the surface of solid medium, described by Schmidt et al. (1981). The composition of the medium was (per litre) as follows: 40 g malt extract, 2 g glucose, 2 g yeast extract, 1 ml Marlophen 810®, 60 mg Bengal Rose, 60 mg oxytetracycline-HCl (OTC), 12 g agar, 1000 ml distilled water. Plates were incubated for 3 days at 25 °C in the dark and in the normal atmosphere. Afterwards, plates were stored at room temperature for 2-3 days. Finally, the colonies were counted and the results were expressed as average colony forming units in thousands per gram of sample ( $10^3$  CFU/g) using the following formula (VDLUFA, 2007c):

$$N = \frac{\sum C}{V \times n \times d}$$

N = number of colony-forming units per gram of sample (CFU/g)

ΣC = sum of all colonies of the count plates

V = volume of the dilutions pipetted in the count plates in ml

n = number of count plates that can be evaluated

d = dilution factor

Taxonomic identifications of different genera of moulds were made visually and where applicable, by means of a magnifying glass or a stereomicroscope. Closer characterisation was possible using a light-optical microscope. In this case, selected parts of colonies were transferred onto a slide using adhesive film (Smith and Yarrow, 1996).

## 2.3. Mycotoxins analysis

### 2.3.1 Apparatus

Linear shaker IKA HS 501 digital (IKA Labortechnik, Germany) was used for the extraction. For liquid chromatography, the system Waters Alliance 2690 with the fluorescence detector Waters 474 (Waters, MA, USA) equipped with columns Prodigy 5μ ODS(2) and Synergy 4μ Hydro-RP 80A (Phenomenex, CA, USA) in the case of aflatoxin B<sub>1</sub> and fumonisins, respectively and with Hypersil ODS 5 μm (Thermo Scientific, MA, USA) in the case of ochratoxin A and ZON was used. For the post column derivatisation, either Kobra cell (Rhône diagnostics, Scotland) or post-column reactor RXN 1000 (Waters) was mounted between chromatographic column and fluorescence detector. Trichothecenes (DON, DAS, T-2, HT-2) were determined by

gas chromatography with mass spectrometry (GC-MS), using system HP 6890 Series (Hewlett Packard, CA, USA) with mass selective detector 5975B inert XL (Agilent Technologies, CA, USA). The column was HP-5MS, 30 m, 0.25 mm I.D., 0.25 μm (Agilent Technologies) and helium with a flow rate of 1 ml/min was used as the carrier gas.

### 2.3.2 Chemicals and reagents

Standard solutions of aflatoxin B<sub>1</sub>, ochratoxin A, ZON and fumonisins B<sub>1</sub> and B<sub>2</sub> were purchased from Biopure (Tulln, Austria) and a standard solution of trichothecenes (including DON, DAS, HT-2, and T-2) was purchased from R-Biopharm Rhône (Glasgow, Scotland). For sample clean-up, immunoaffinity columns (R-Biopharm Rhône) and Mycosep 227 Trich+ (Romer Labs, MO, USA) were used. Reagents purchased at Merck (Darmstadt, Germany) and Supelco (PA, USA) were of analytical or chromatography grade purity.

### 2.3.3 Analytical procedure

Aflatoxin B<sub>1</sub>, ochratoxin A, zearalenone, and fumonisins were determined following the instruction for use enclosed to immunoaffinity columns and a standard (R-Biopharm Rhône, 2003a, b, c; 2005; European Committee for Standardization, 2004). For the determination of trichothecenes, the procedure based on analytical methods described by Radová et al. (1998), Langseth and Rundberget (1998), Tanaka et al. (2000), Melchert and Pabel (2004), and Schothorst et al. (2005) was used.

Mycotoxins were extracted from samples with appropriate solvents. After sample clean-up, mycotoxins were determined by liquid chromatography or GC-MS. Before the detection, aflatoxin B<sub>1</sub> was derivatised with bromine in Kobra cell and fumonisins were derivatised with o-phthalaldehyde and N-acetyl-L-cystein in the post-column reactor.

The limit of detection (LOD) and the limit of quantification (LOQ) for aflatoxin B<sub>1</sub> were 0.2 and 0.6 μg/kg, respectively. LOD and LOQ for ochratoxin A were 10 and 30 μg/kg, for ZON 20 and 50 μg/kg, for fumonisins B<sub>1</sub> and B<sub>2</sub> 30 and 100 μg/kg, and for DON, DAS, T-2 and HT-2 50 and 100 μg/kg. To enable the comparison between samples and their estimation regarding recommended European guidance values (European Commission, 2006) the results of mycotoxicological analysis were recalculated to a sample moisture content of 12%. Samples with the concentration of mycotoxins higher than LOD were designated as positive.

### 2.4 Statistical analysis

The statistical analysis was performed using the statistical program SPSS (Statistical Package for Social Sciences, Version 12, November 2003). The number of positive samples, arithmetic mean and median of positive samples as well as the maximum level were calculated.

## 3 RESULTS

### 3.1 The contamination of grain samples with toxigenic moulds

The microorganisms, isolated from all 66 samples are shown in Table 1. Because of a small number of triticale (3), fodder pea (2), oats (1) and rye (1), these samples

were presented together under the label "other". The leading contaminant among moulds was *Fusarium spp.*, detected in 51 of 66 samples (77.2%). Maize had the highest average number of growth colonies of *Fusarium spp.* and *Penicillium spp.* The samples of barley

contained the highest average number of *Aspergillus spp.* and *Alternaria spp.* colonies.

**Table 1:** The presence of toxigenic moulds in grain samples.

| Number of samples  | Samples of grain   |                            |                          |                           | Total                             |
|--------------------|--|----------------------------|--------------------------|---------------------------|-----------------------------------|
|                    | Barley   | Maize                      | Wheat                    | Other                     |                                   |
| Micro-organisms    | (num. positive) <sup>a</sup><br>mean/median <sup>b</sup> ;<br>max.level (x 10 <sup>3</sup> cfu/g) <sup>c</sup> |                            |                          |                           | (num. positive) <sup>d</sup><br>% |
| <i>Fusarium</i>    | (19)<br>5.5/5.0;<br>17.0   | (12)<br>23.3/8.0;<br>128.0 | (15)<br>5.2/6.0;<br>12.0 | (5)<br>6.6/8.0;<br>11.0   | (51)<br>77.2                      |
| <i>Penicillium</i> | (11)<br>4.5/2.0;<br>12.0   | (6)<br>8.8/5.5;<br>30.0    | (9)<br>4.4/4.0;<br>11.0  | (1)<br>8.0                | (27)<br>40.9                      |
| <i>Aspergillus</i> | (11)<br>19.3/4.0;<br>100   | (9)<br>10.4/7.0;<br>38.0   | (5)<br>8.6/9.0;<br>16.0  | (3)<br>11.3/13.0;<br>20.0 | (28)<br>42.2                      |
| <i>Alternaria</i>  | (14)<br>5.0/2.0;<br>26.0   | (1)<br>5.0                 | (10)<br>1.5/1.5;<br>3.0  | (4)<br>2.0/2.0;<br>3.0    | (29)<br>43.9                      |

<sup>a</sup> Number of positive samples.

<sup>b</sup> Arithmetic mean/median of positive samples in x 10<sup>3</sup> cfu/g.

<sup>c</sup> Maximum level detected in x 10<sup>3</sup> cfu/g.

<sup>d</sup> Total number of positive samples regarding one microorganism.

### 3.2. The contamination with mycotoxins

A total of 66 grain samples was analysed, resulting in 528 analyses. Table 2 gives an overview of contamination grade of the tested samples, stating the total number of each sample tested, the number of positives, arithmetic mean and median of positive samples as well as the maximum level. In cases, where one sample was positive, the value is presented with one number. The samples of maize were most frequently contaminated and contained DON, ZON and fumonisin

B1. Generally, in most samples, DON was detected (54.5%). It was proven in 15 out of 18 samples of wheat, as well as in all other grain samples. DON was closely followed by ZON, which was detected in all grain samples. Fumonisin B1 was detected only in 2 maize samples.

Ochratoxin A, DAS, T-2 toxin and HT-2 were under the detection limit in all analysed samples.

**Table 2:** Occurrence of mycotoxin contamination in grain samples (results expressed for 12% moisture content).

|                            | Samples of grain       |   |                          |                        | Total                             |
|----------------------------|------------------------|---|--------------------------|------------------------|-----------------------------------|
|                            | Barley                 | Maize   | Wheat                    | Other                  |                                   |
| Number of tested samples   | 23                     | 18  | 18                       | 7                      | 66                                |
| Number of positive samples | 9                      | 12  | 15                       | 2                      | 38 (57.6%)                        |
|                            |                        | (num. positive) <sup>a</sup><br>mean/median <sup>b</sup> ;<br>max. level <sup>c</sup> |                          |                        | (num. positive) <sup>d</sup><br>% |
| Ochratoxin A               | n.d.                   | n.d.  | n.d.                     | n.d.                   | n.d.                              |
| Fumonisin B1               | n.d.                   | (2)<br>180/180;<br>210  | n.d.                     | n.d.                   | (2)<br>3.0                        |
| Fumonisin B2               | n.d.                   | n.d.  | n.d.                     | n.d.                   | n.d.                              |
| Fumonisin B1 + B2          | n.d.                   | (2)<br>180/180;<br>210  | n.d.                     | n.d.                   | (2)<br>3.0                        |
| Zearalenon                 | (2)<br>260/260;<br>390 | (4)<br>310/170;<br>800  | (3)<br>190/120;<br>370   | (1)<br>400             | (10)<br>15.1                      |
| Deoxynivalenol             | (9)<br>310/260;<br>600 | (10)<br>440/340;<br>920   | (15)<br>580/290;<br>2860 | (2)<br>845/845;<br>890 | (36)<br>54.5                      |
| DAS                        | n.d.                   | n.d.  | n.d.                     | n.d.                   | n.d.                              |
| T-2                        | n.d.                   | n.d.  | n.d.                     | n.d.                   | n.d.                              |
| HT-2                       | n.d.                   | n.d.  | n.d.                     | n.d.                   | n.d.                              |

n.d.= not detected

<sup>a</sup> Number of positive samples regarding individual mycotoxin.<sup>b</sup> Arithmetic mean/median of positive samples in µg/kg.<sup>c</sup> Maximum level detected in µg/kg.<sup>e</sup> Estimated value (LOD).<sup>d</sup> Total number of positive samples regarding individual mycotoxin.

#### 4 DISCUSSION

Microbiological examination proved that 77.2%, 40.9%, 42.2% and 43.9% of the samples of different grains contained moulds of genera *Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria*, respectively. As a result of this contamination, the 57.6% of samples were positive to at least one mycotoxin.

Slovenia, like all other EU countries, has no written legislative limits regarding the microbiological status of animal feed. There are no official methods for detection of the microbial contamination. With traditional culturing methods, used also in our study, it takes 5-7 days to get results. Molecular methods hold promise as a viable technology to reach these results, but will require considerable optimisation and standardisation before this becomes routine in the animal feed industry

(Maciorowski et al., 2007). Most of the countries tend to set general standards in the field of microbiological examinations, but agreement and setting of international regulatory standards is very difficult, as not only potential health benefits but also political and economic issues have to be considered.

The average contamination with colonies of *Fusarium spp.* in maize were lower than in previous years, when the average contamination was  $250 \times 10^3$  cfu/g and for *Penicillium* and *Aspergillus* 744 and  $7 \times 10^3$  cfu/g (Jakovac-Strajn et al., 2008). The moulds from genus *Alternaria* had the highest average number in barley. Based on the number of positive samples of all grains (43.9%), the potential presence of toxins such as

tenuazonic acid, alternariol and alternariol monomethyl ether may pose a contamination risk.

It is well known, that environmental conditions have great influence on the development and spread of moulds and consequently on the production of mycotoxins. Water stress, temperature stress and insect damage of a host plant are, under field conditions, the major determining factors of mould infestation and toxin production. With stored grain, factors which are likely to affect mycotoxin formation include moisture content and the composition of the substrate, environmental temperature, exposure time, damage to seeds, oxygen availability, carbon dioxide concentrations, fungal abundance, prevalence of toxic strains, spore loads, microbial interaction and invertebrate vectors, particularly insects. Spoilage, fungal growth and mycotoxin formation result from the complex interactions of these factors (Abramson et al., 1980; Santin, 2005).

There are also great differences in sensibility to mycotoxins among individual animal species. Factors such as breed, sex, environment, nutritional status, as well as other toxic entities can affect the symptoms of intoxication and may contribute to the significance of mycotoxin damage on economic output and animal health (Binder, 2007). Many countries have, therefore, established measures to control the contamination of these toxins in foodstuffs and animal feed. However, Slovenia and EU have, besides a Commission Recommendation on the presence of DON, ZON, ochratoxin A, T-2 and HT-2 and fumonisins (European Commission, 2006), placed legislative limits only for aflatoxin B1 in feed for different species and categories of livestock. Aflatoxin B1 concentrations in animal feed, grains being among them, may thus range up to 0.02 mg/kg of feed (European Commission, 2003a), but this mycotoxin was not the aim of our study. For DON, ZON, ochratoxin A and for the sum of fumonisins B1 and B2, the recommended highest concentration per kg in cereals and cereal products is 8 mg, 2 mg, 0.25 mg and 60 mg, respectively. Recommended maximum levels for T-2 and HT-2 toxin have not been set in Europe yet.

The levels of mycotoxins in our research did not reach the recommended levels, which is very satisfying. In general, our results are similar to those reported from around the world. DON is most widely spread mycotoxin, which is confirmed also by the investigation including samples from eleven European countries examined on trichothecenes contents. 57% of the samples were DON positive (0.003-3.7 mg/kg), mostly maize, and 20% contained T-2 toxin and HT-2 toxin (European Commission, 2003b; JECFA, 2001). Maize is known to be a good substrate for mould infection and production of potentially dangerous mycotoxins harmful to both humans and animals (Kumar et al., 2008), but in our investigation wheat was the most contaminated grain with DON, which has happened for the first time. Up to the present, also in Slovenia maize was the most contaminated grain (Jakovac-Strajn et al., 2008).

In neighbouring Croatia, a seven year long investigation of grains or animal feed samples (1998-2004) revealed 41% of DON, 16.8% of T-2 toxin and 27.6% of DAS (Sokolović and Šimpraga, 2006). In former investigations (Domijan et al., 2005), maize samples were tested on ochratoxin A, ZON and fumonisins B1 and B2. Most frequently fumonisin B1 was found (100%), followed by ZON (84%) and ochratoxin A (39%), while fumonisin B2 was found only in three samples. The concentrations (mean  $\pm$  SD) of fumonisin B1, ZON and ochratoxin A in positive samples were  $459.3 \pm 310.7$ ,  $3.84 \pm 6.68$  and  $1.47 \pm 0.38$   $\mu\text{g}/\text{kg}$ , respectively. In 2008, (Jajić et al., 2008) the results of the first examinations on DON in 139 samples of various grains were published. DON was found in 44.7% of maize samples, 37.5 % of wheat and 25% of barley samples. In positive samples, DON was found in concentration range between 40-2460  $\mu\text{g}/\text{kg}$ , but in our research in concentration at 130-2860  $\mu\text{g}/\text{kg}$ .

Taken together, the presence of toxigenic moulds in different grains grown in Slovenia showed and confirmed the presence of different mycotoxins, which were consumed by livestock. Although concentrations were not above the recommended levels, a relatively high percent of positive samples (57.6%) require a further control in Slovenian primary grain production.

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