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# Monitoring of selected mycotoxins in maize and grass silages

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**Abstract.** The research aimed to quantify the occurrence of selected mycotoxins: AFB1 (aflatoxin B1), DON (deoxynivalenol) and ZEN (zearalenone) in maize and grass silages. The silage was prepared in foil bales without the addition of differentiating factors. Silages derived from 36 farms located in Wielkopolskie voivodeship. The total number of mould fungi and the pH value were determined in the collected samples. The level of contamination with selected mycotoxins was determined by high-performance liquid chromatography (HPLC). Based on the obtained results, it was found that the total number of mould fungi in the examined silage samples was in the range 5.00–6.90 log CFU $g^{-1}$ fresh weight, pH value in the range 3.34–4.73. It was shown that maize silage is more contaminated with mycotoxins than grass silage. In the tested samples, statistically significant differences ($P < 0.05$) were found only for the mycotoxin ZEN. ZEN content was 74% higher in maize silage compared to grass silage. Contamination of silages with mycotoxins AFB1 and DON was at a similar level, and it was 4.22 and 354.14 µg $kg^{-1}$ for maize and 4.98 and 330.25 µg $kg^{-1}$ for grasses, respectively.

**Keywords:** aflatoxin B1, deoxynivalenol, zearalenone, silage

## INTRODUCTION

The method of silage production for cattle should ensure its high nutritional and hygienic value. In the daily ration for cows, the proportion of high-quality silage should be between 50–75% (Driehuis, 2013). Several factors can cause the decline in the quality of silage: inappropriate plant selection and moisture content, ensiling technology (poor compaction of ensiled matter), mould growth, mould production of mycotoxins (Vaičiulienė et al., 2021), and inadequate pH. The pH value is the primary determinant of silage quality and is conditioned by the amount of lactic acid produced by lactic fermentation bacteria. The lactic acid dominates the total pool of acids produced during ensiling of plant material. It is 10 to 12 times more potent than acetic or propionic acid. The silage's final pH is determined by the amount of acid produced and the buffering capacity of the ensiled mass (Kung et al., 2018). Such an acidic environment can be an excellent site for the growth of mould fungi, which are capable of producing low molecular weight secondary metabolites such as mycotoxins. Numerous filamentous fungi *Aspergillus*, *Fusarium*, *Penicillium*, or *Alternaria*, can produce substances mentioned above under silage spoilage influenced by yeast and aerobic bacteria activity. Such compounds can be made before, during, or after crop harvest (Kukier et al., 2014). In silage, the most commonly determined mycotoxins are aflatoxin B1 (AFB1), deoxynivalenol (DON), zearalenone (ZEN), and T-2 (Driehuis, 2013). Maximum levels for aflatoxin B1 in the feed are regulated by Directive 2002/32/EC of the European Parliament and the Council and for DON and ZEN toxins by Commission Regulation (EC) No 1881/2006 (Table 1). The production of fusarium mycotoxins mainly occurs during the growing season. *Fusarium* spp. does not find favourable conditions for growth during ensiling in the acidic and anaerobic environments (Driehuis et al., 2010). The highest concentrations of DON and ZEN are observed in the outer layers of silages, where aerobic conditions dominate (Cavallarin et al., 2004). The danger of silage contamination by *Aspergillus* spp. moulds and their production of aflatoxin B1 increase with plant damage during the growing season, leakage of silos and foils, and during silage picking (Kukier et al., 2014). Feed, and thus food contaminated with mycotoxins, can significantly affect animal and human health. The ingestion of even small amounts of mycotoxins can consequently lead to dangerous mycotoxicoses in animals with a wide variety of clinical symptoms (Chebutia et al., 2020). The main symptoms of excessive intake of mycotoxins are digestive disorders, histomorphological abnormalities of the

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Table 1. Limit values of selected mycotoxins in cattle feed in the EU (Vaičiulienė et al., 2021).

| Mycotoxin | Products intended for animal feed | Maximum level or guidance value [μg kg$^{-1}$]# |
|---|---|---|
| Aflatoxin B1 | Feed materials with the exception of: | 50 |
| | – groundnut, copra, palm kernel, cotton seeds, maize and products derived from the processing thereof | 20 |
| | Complete feedingstuffs for cattle except for: | 50 |
| | – dairy cattle | 5 |
| Deoxynivalenol | Feed materials# | |
| | – maize by-products | 12,000 |
| | – compound feed | 5000 |
| Zearalenone | Feed materials# | |
| | – maize by-products | 3000 |
| | – compound feed of calves, dairy cattle, sheep (including lamb) and goats (including kids) | 500 |

# Relative to a feedingstuff with a moisture content of 12%

intestines, damage to the integrity of the intestinal barrier, decreased mucin production, and alterations in the gastrointestinal biota composition. Furthermore, neurotoxic, hepatotoxic, nephrotoxic, genotoxic, immunomodulatory, developmental, and reproductive effects may arise (Reisinger et al., 2019).

The research hypothesis assumed that toxic secondary metabolites of mould fungi could be demonstrated in silage samples intended for cow feed irrespective of the site of collection (within the voivodeship) and the plant material used for ensiling. The study aimed to quantify the occurrence of selected mycotoxins: AFB1 (aflatoxin B1), DON (deoxynivalenol), and ZEN (zearalenone) in maize and grass silages produced in the Wielkopolskie voivodeship.

## MATERIALS AND METHODS

### Samples

During the study period (2020–2021), 150 silage samples exhibiting no mould growth were collected from 36 farms in the Wielkopolskie voivodeship. Maize silages constituted 50% (n=75) and grass silages 50% (n=75). Silages were prepared in plastic bales without any differentiating factors. The sampling date was November–December 2020–2021. Sampling (4 samples per foil bale) was accomplished with a silage density auger (Pioneer, 4.5 cm Ø, 45 cm length). Samples of 5 kg were transported to the laboratory in PVC bags and stored for 12 h at 21 ˚C ± 2 until analysis.

### Mycological quantitative analysis

The total number of mould fungi was determined by serial dilution using OGYE Agar (OXOID) with oxytetracycline-glucose-yeast-extract agar. A 10 g initial weight was homogenised in 90 cm$^3$ saline. The material was incubated for 5–7 days at 24 ˚C. Results were expressed as log CFU g$^{-1}$ fresh weight of silage.

### pH value

The pH values were determined using a pH meter Hi 98128, 0.01 pH (Hanna Instruments). For such purpose, a 25 g sample was homogenised with 100 cm$^3$ of distilled water for 20 min. After establishing a constant measurement value with an accuracy of 0.05, a reading of the results was taken in triplicate.

### Determination of mycotoxins

The concentration of mycotoxins AFB1 and ZEN in the silage samples tested was measured using the high-performance liquid chromatography (HPLC) method with a fluorescence detector (FLD) 1260 Infinity II Fluorescence Detector Spectra (Agilent) based on the PN-EN 15850:2010 (2010) and PN-EN 15792:2012 (2012) standards. For the determination of DON concentration, the HPLC method with Ultraviolet (UV-ViS) Detector: SPD-20A/20AV (Shimadzu) was used according to the standards PN-EN 15891:2010 (2010) and PN-EN 15791:2012 (2012). The silage samples were dried at room temperature (21 ˚C ± 2) and sieved through a sieve with a mesh diameter of 1 mm. For DON determination, extraction was performed in distilled water, and for AFB1 and ZEN, methanol: distilled water mixture (75:25 v/v) was applied. After extraction, samples were centrifuged (RCF) 3500 × g for 15 min (Frontier 5515 Ohaus). The supernatant was filtered through syringe filters with a pore diameter of 0.22 μm (Syringe filters, VWR) and diluted with phosphate buffer salt (PBS). A Multi-Mycotoxin Column CrossTox immunoaffinity column (LcTech-Germany) was installed

according to the manufacturer's guidelines to purify the samples. Mycotoxins were identified by comparing retention time peaks in the tested extracts with those obtained from standard solutions.

### Statistical analysis

The results obtained during the study were expressed as mean values from three replications and as the standard error of the mean, followed by the descriptive statistics analysis (SAS, 2012). The significance of differences was tested by the Tukey HSD test at α=0.05.

## RESULTS AND DISCUSSION

The total number of moulds in the silage samples (n=144) remained within the range of 5.00–6.90 [log CFU $g^{-1}$ fresh weight] (Table 2). The highest fungal counts were determined in maize silages and, as logarithms, were 6.25% higher than in grass silages. The pH value was between 3.34 and 4.73 (Table 3), qualifying them as good quality silages. A higher pH characterised grass silages on average of 12.04% compared to maize silages.

Mycotoxins were detected in all the silage samples analysed. The AFB1, DON, and ZEN concentrations are compiled in Table 4. The mean values for mycotoxin-contaminated samples were calculated by omitting samples in which mycotoxin concentrations were determined at the detection limit. Based on the analyses, it was found that most maize silages were contaminated with ZEN toxin (32%) and grass silages with DON toxin (20%). When analysing the content of individual mycotoxins in the samples, statistically significant differences ($P<0.05$) were observed only for the ZEN mycotoxin. The ZEN level was 74% higher in maize silages than in grass silages. The contamination of the samples with mycotoxins AFB1 and DON was at similar levels.

Table 2. Total count of mould fungi in silages [log CFU $g^{-1}$ fresh weight].

| Samples | Mould fungi | | | |
|---|---|---|---|---|
| | No. of positive samples (%) | Range | Mean | SD |
| Maize silage (*n* = 75) | 71 (94.70) | 5.00–6.90 | 5.95 | 0.37 |
| Grass silage (n = 75) | 73 (97.30) | 5.10–6.10 | 5.60 | 0.36 |

SD – standard deviation

Positive samples – samples in which the presence of mould fungi was found

Table 3. pH values of maize and grass silage.

| Samples | pH | | |
|---|---|---|---|
| | Range | Mean | SD |
| Maize silage (*n* = 75) | 3.34–4.25 | 3.80 | 0.38 |
| Grass silage (n = 75) | 3.91–4.73 | 4.32 | 0.35 |

SD – standard deviation

The mould fungi produce mycotoxins as by-products of metabolic processes or as defence compounds under environmental stress conditions, for example, as a result of applying substances aggressive towards the fungi (Barabasz, Pikulicka, 2017). The abundance of mould fungi in silage increases with rising oxygen availability. According to Kukier et al. (2014), the average mould occurrence in maize silages is in the range of 3.30–4.12 [log CFU $g^{-1}$ fresh weight]. The values obtained in our study for maize silage were higher and amounted to 5.00–6.90. Jatkauskas and Vrotniakiene (2013) in silages made of a mixture (70:30) of perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.) determined the abundance of mould fungi at 3.0, while in maize silages it was 2.01. The results of our study show a higher abundance of these fungi in grass and maize silages, 5.00–6.90 [log CFU $g^{-1}$ fresh weight]. Low pH values in silage indicate good forage quality due to fermentation during ensiling (Chen et al., 2018; Miguel et al., 2021). The production of mainly lactic acid causes low pH values by lactic fermentation bacteria (Shao et al., 2005). Furthermore, it should be noted that low pH and high acid content (e.g., lactic and acetic) are among the factors limiting the growth of unfavourable microorganisms in silages (Muck, 2010). The silage pH value determined in the present study averaged 3.80 for maize silage and 4.32 for grass silage. Jatkauskas and Vrotniakiene (2013) achieved similar values, and they were 3.74 and 4.55 for maize and grass silages, respectively.

Analysis of the results corroborated a statistically significant ($p<0.05$) difference in the contamination of silages with the mycotoxin ZEN, whose average content for maize and grass silages was 505.27 and 290.10 μg $kg^{-1}$, respectively. It should be emphasised that the toxins produced by fungi of the genus Fusarium (DON and ZEN) were among the most common in silages. Similar results were achieved by Panasiuk et al. (2019), who determined the concentration of DON in 82% of samples at 447 μg $kg^{-1}$ and ZEN in 57% of samples at 82.4 μg $kg^{-1}$. Similar results regarding the high frequency of DON and ZEN in maize silage were reported by Kosicki et al. (2016), who determined them in 86% and 88% of samples, respectively. However, Storm et al. (2014) identified the presence of DON in only 6% of silage samples tested.

Similar results to those obtained in our study regarding the concentration of mycotoxins are found in Vaičiulienė et al. (2021), who determined the ZEN content in maize and grass silages at 505.00 and 286.67 μg $kg^{-1}$. The same

Table 4. Concentration of mycotoxins in maize and grass silages [μg kg$^{-1}$].

| Samples | Mycotoxin | No. of positive samples (%) | Range | Mean | SD |
|---|---|---|---|---|---|
| Maize silage (n = 75) | Aflatoxin B1 | 13 (17.33) | 0.58–9.00 | 4.22 | 2.12 |
| | Deoxynivalenol | 16 (21.33) | 98.95–442.00 | 354.14 | 201.21 |
| | Zearalenone | 24 (32.00) | 56.02–685.01 | 505.27 A | 215.12 |
| Grass silage (n = 75) | Aflatoxin B1 | 8 (10.67) | 1.78–8.31 | 4.98 | 2.14 |
| | Deoxynivalenol | 15 (20.00) | 98.34–475.23 | 330.25 | 125.21 |
| | Zearalenone | 10 (13.33) | 128.00–350.00 | 290.10 B | 74.12 |

The values in the columns marked with different letters differ significantly at the level of $P < 0.05$
Positive samples – samples in which the presence of mould fungi was found.
SD – standard deviation

authors determined the concentration of AFB1 and DON at 3.15 and 362.50 μg kg$^{-1}$ for maize silages and 5.50 and 321.25 μg kg$^{-1}$ for grass silages. Such a low concentration of the mycotoxin AFB1 may be related to the geographical latitude of the crops grown. A higher frequency of AFB1 determination is recorded in Europe in the Mediterranean regions, and the discrepancy of results regarding the occurrence of significant mycotoxins, e.g., DON, is determined by the climate of Central and Eastern Europe (Panasiuk et al., 2019). However, papers report also extreme low contamination of maize silage with AFB1 toxin. Garon et al. (2006) determined the content of AFB1 in maize silages at 1 μg kg$^{-1}$, and Schmidt et al. (2015) detected its presence in only 0.92% of maize silage samples. It should be pointed out that the incidence and content of AFB1 toxin in well-prepared silages are low (Vaičiulienė et al., 2021), or its presence is not detected at all (Dagnac et al., 2016).

Maize and grass silages demonstrated certain quantitative and qualitative differentiation concerning mycotoxin contamination. The higher contamination of maize silage may be due to the different chemical composition of the ensiled plants. Maize contains higher amounts of protein and polysaccharides, which are beneficial for the growth of mould fungi (Driehuis et al., 2008; Zachariasova et al., 2014). The appearance of fusarium toxins in grass silage is not a common phenomenon. If they are determined, it is at low levels. Skladanka et al. (2013) reported concentrations of DON and ZEN in grass silages at 167 μg kg$^{-1}$ and 66.9 μg kg$^{-1}$. Such results differ from our study, where DON and ZEN were determined at considerably higher concentrations. The low concentration of ZEN (53 μg kg$^{-1}$) is also reported in McElhinney et al. (2016). Such discrepancies may be due to the different levels of production of these toxins by *Fusarium* sp. already under field conditions (Panasiuk et al., 2019). In contrast, Cavallarin et al. (2004) present the conception that ZEN can be produced in grass silages at high levels, even above 300 μg kg$^{-1}$, confirming the results obtained in this study.

Summarising the results, it was considered that the values obtained for the contamination of silage with mycotoxins did not exceed the limits covered by the Directive 2002/32/EC for AFB1 and by Commission Regulation No 1881/2006/EC for DON and ZEN. Simultaneously, the investigated feeds were found to be a potential source of mycotoxins. The selected mycotoxins belong to a group of five (out of 500) that are considered to be of global toxicological and economic significance. The inability to avoid contamination by mycotoxins of crops, and thus of fodder produced from them, should force producers to develop methods of eliminating and inactivating them. Accurate fodder ensiling can prevent the formation of mycotoxins, but it does not fully inactivate metabolites already produced under field conditions. To avoid exposing animals to the adverse effects of mycotoxins in feed, special attention should be paid to the feed quality and storage. The critical issue is to minimise conditions that favour mould growth.

## CONCLUSIONS

1. A similar abundance of mould fungi characterised the analysed maize and grass silages.
2. Maize silages provided a lower pH in comparison with grass silages. The pH values determined in the silages allowed to consider them as good quality feeds.
3. Based on the results, more samples were contaminated with mycotoxins for silages made from maize.
4. The average concentrations of aflatoxin B1 and deoxynivalenol in maize and grass silage samples were similar. Zearalenone concentration was higher in maize silages by 74% compared to grass silages.

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