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DON, F-2 and T-2 mycotoxin assay of plant-based feedstock raw materials using the ELISA method

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1. Summary

In our study, plant-based raw materials, used for feeding different animal species, are investigated, using a competitive ELISA method. The raw materials most commonly used for feeding (soy and alfalfa pellets, as well as wheat, barley and maize) were used in the tests. Of Fusarium mycotoxins, deoxynivalenol (DON), zearalenone (F-2) and T-2 toxins were measured. Measurement results were evaluated using the mathematical-statistical program RStudio. In our experiment, we found that all three mycotoxins tested could be detected in all of the samples, but the values were not quantifiable with acceptable precision in each case. The average detected DON toxin result was an order of magnitude greater than the results of the other toxins. It has been shown in our study that the presence of the mycotoxins deoxynivalenol, zearalenone and T-2 poses a serious food and feed safety risk, since they are present in feedstock raw materials, even though only in small amounts. Today, these mycotoxins are present together in more and more cases, greatly increasing the above-mentioned risk.

2. Introduction

Contaminants of natural origin include mycotoxins, produced by microscopic fungi, which are secondary metabolic products of molds. The human and animal health significance of mycotoxins are outstanding **[1]**. Because of the climate change currently taking place, the risk of toxin occurrence on the food chain is of great importance. Based on the resolutions of the Intergovernmental Panel on Climate Change (IPCC) of the UN and the World Meteorological Organization (WMO), the research program named VAHAVA (Változás-Hatás-Válaszadás, Change-Effect-Response), and the National Climate Change Strategy adopted by the Parliament: „increased effects of climate change are expected in the Carpathian Basin" **[2]**. This process can affect adversely the domestic agriculture due to the expected loss in yields, may have a negative effect on food and feed safety because of the proliferation of harmful microorganisms, and can also have an indirect effect on human and animal health. Global climate change can promote the growth of mycotoxin-producing molds **[2]**. Animals fed on feeds contaminated with mycotoxins pose a serious food safety risk, so the consumption of products made from them may be risky as well, the development and weight gain of animals feeding on feeds containing toxins slows down, and their reproduction and animal health conditions deteriorate also. Adverse animal health characteristics affect livestock production, and so economic efficiency and production indicators can also decrease **[3]**.

In the last decade, interest has been focused more and more on mycotoxin research. Many experiments have been carried out in order to be able to reduce the risk of mycotoxins entering the food chain. There are also studies aimed at reducing the amounts of the contaminants that are within expected or allowed limit values (for example, in the case of raw materials) to even lower levels. The risk of toxins entering the food chain can be minimized by preventive

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agrotechnical operations applied during the growing of cereals **[4]**. Of these, the most widely used chemical plant protection can be highlighted, the efficiency and efficacy were investigated by Mesterházy et al. **[5]**. Also worth mentioning is biological plant protection, offering wider and wider possibilities, with the application of which pathogens can be suppressed by their natural enemies **[6]**. An important factor in preventive defense is variety selection, and in this respect, a pronounced role is played by plant breeding, i.e., the production of resistant or tolerant grain varieties **[7], [8]**.

The efficiency and efficacy of preventive agrotechnical operations depend greatly on the vintage effect and the technological discipline, as demonstrated by the experiences of recent years. In years with conditions favorable for the proliferation of *Fusarium* fungus, we should be prepared that wheat lots contaminated by the toxins to varying degrees will be harvested. In such years, there is also an increased risk that feeds will be more contaminated by the toxins than food industry raw materials. The main reason for this is that, in food production, raw materials must be selected in compliance with very strict legal regulations. Cereal lots that do not satisfy these criteria, for example, because of their toxin contamination, may be qualified as feeds. In view of this, a prominent role is played by research aimed at reducing toxin concentrations in the post-harvest period. In animal feeding one of the possibilities is the addition to feeds of such substances that can absorb toxins that are harmful to the animals. Bata **[3]** reports that toxin feeding experiments are performed on pigs, cattle, laying hens and turkeys of different age groups, and changes in their health status are monitored. Experiments have shown that fusariotoxicosis is manifested in mixed symptoms, and that the species most sensitive to fusariotoxins are pigs and poultry. In the case of these species, reproductive disorders and secondary diseases, the weakening of the immune system, are caused by them. Cattle are less sensitive to these toxins than pigs.

In the post-harvest period, it is possible to reduce the toxin content by cleaning grain using modern equipment. The efficiency of color sorting and surface cleaning is primarily investigated in the case of food raw materials **[9], [10]**, but it is also possible to use these methods during the preparation of materials used for feeding, if justified. It was pointed out by Kecskésné et al. **[11]** that the DON toxin content of the so-called byproduct, the fraction that is produced in addition to milling wheat during the color sorting, before grinding, of wheat intended for milling, increased in all cases, compared to the toxin content of the starting, unpurified wheat lot. However, the extent of the increase does not correlate with either the toxin content of the starting material, or the efficiency of the purification, because it can also be influenced by other factors that can be hard to determine in advance. It is important to know this, because the above-mentioned byproduct is used as feed or as an ingredient in feed mixtures. According to the experimental results, it is definitely recommended to measure the toxin content of such byproducts before use, to avoid the toxin contamination of feeds.

Humans, at the top of the food chain, can rightfully expect that the product reaching their table meet their health maintenance and disease prevention needs. Maintaining food safety, i.e., the detection of unsafe products and their removal from commercial circulation, is a major task.

The contamination of feeds and feed raw materials (primarily cereals) caused by DON, F-2 and T-2 mycotoxins has been a serious food safety challenge all over the world, and it still is **[12]**. In Germany, according to a previous study on wheat samples (n=84), DON mycotoxin values ranged from 4.0 to 20500 µg/kg, F-2 toxin values were between 1.0 and 8040 µg/kg, while T-2 could be detected in amounts between 3.0 and 250 µg/kg **[13]**. In Poland in 1990, when investigating wheat samples as well, the DON toxin contamination was between 2000 and 40000 µg/kg, while the amount of zearalenone toxin was lower, in the range of 10 to 2000 µg/kg **[14]**. In the case of maize, also in Poland, only deoxynivalenol toxin was tested, and the samples proved to be more contaminated than wheat samples. Values ranged from 4.0 to 320 mg/kg **[12]**. In Finland, the DON contamination of animal feeds and cereals (maize, wheat, barley) was between 7.0 and 300 µg/kg, while F-2 toxin values ranged from 22.0 to 95.0 µg/kg **[15]**, which were considerably lower than the above data. Data show that toxin concentrations may vary widely in a given area and period. Accordingly, food safety conditions can only be met reliably, if a toxin level monitoring system for feed raw materials is developed and toxin levels are continuously measured within its framework.

The contamination of feeds with molds does not always indicate the presence of mycotoxins as well **[16], [17]**. To judge the mycotoxin contamination accurately, sufficiently sensitive and specific analytical methods are needed. Generally speaking, detection of any of the mycotoxins is a very complex, time-consuming and costly process, the accuracy of which depends greatly on the correctness and efficiency of sampling **[16]**. Methods of determination were initially based on thin layer chromatography, and later on gas and liquid chromatography **[18], [19]**. These were supplemented by HPLC (High Performance Liquid Chromatography) methods **[19]**. In addition to these high performance analytical methods, routine immunoassays were also developed for the qualitative and quantitative determination of certain mycotoxins **[20], [21]**.

In recent years, the emergence of ELISA (Enzyme-linked immunosorbent assay) methods and high performance liquid chromatography methods with fluorescence or mass spectrometric detection could be

observed **[22]**. Currently used analytical methods are classified into three major categories: fast methods, separation techniques, immunological procedures.

However, the detection of mycotoxins has recently recognized limitations, primarily in the case of masked and bound toxins. The difficulties of masked mycotoxin determination come from their altered physicochemical properties, resulting in a changed extractability. Therefore, when using the usual analytical methods, their quantification is uncertain, and that of bound toxins is impossible **[23]**.

3. Materials and methods

3.1. Materials

For our investigations, feed raw materials were divided into two groups. In the first test group, a total of 20 samples soybean (*n*=10) and alfalfa (*n*=10) pellets made of green plant parts. Also 20 were included in the second test group. Here, the toxin contents of the grains of cereals, namely wheat (*n*=10), barley (*n*=5) and maize (*n*=5) were measured. So our total number of samples was *n*=40. Fast informative mycotoxin assay of the feed raw materials was performed by a competitive ELISA method, using Ridascreen Fast kits (R-Biopharm). The "souls" of the fast tests used by us were the standard solutions, contained by the kits according to the specific toxin measurement. DON kit (RIDASCREEN® FAST DON, Art. No.: R5902, 48 wells) standard solutions: „blank solution containing no mycotoxin", 0.222 mg/kg, 0.666 mg/kg, 2 mg/kg, 6 mg/kg. Zearalenon kit (RIDASCREEN® FAST ZEARALENON, Art. No.: R5502, 48 wells) standard solutions: „blank solution containing no mycotoxin", 50 µg/kg, 100 µg/kg, 200 µg/kg, 400 µg/kg. T-2 kit (RIDASCREEN® FAST T-2, Art. No.: R5302, 48 wells) standard solutions: „blank solution containing no mycotoxin", 50 µg/kg, 100 µg/kg, 200 µg/kg, 400 µg/kg. For the measurement, a Metertech-500 spectrophotometer (ELISA Reader) was used, with a measurement wavelength of 450 nm. Standard solutions necessary for method validation were from Sigma-Aldrich Chemie GmbH (Steinheim, Németország). Results were evaluated using the special software RIDA® SOFT WIN (Art. No.: Z9999). Statistical analysis was performed by the RStudio (Version 0.98.953) program.

3.2. Sample preparation

Samples were air-dry, so no further drying was necessary. Test samples were homogenized using a 1.0 mm mill crush size (Tecator, Sweden). Sample preparation for mycotoxins DON, F-2 and T-2 were performed according to the manufacturer's instructions (R-Biopharm). In the case of DON mycotoxin, 5 grams of the sample (ground, mixed) was weighed into a lockable glass crucible, then the solution was shaken intensely with 100 ml of distilled water for 30 minutes on a shaker (Tecator). The mixture was filtered into a 100 ml Erlenmeyer flask through a Whatman 1 filter placed in a glass funnel. 50 µl of the filtrate was used for the test: RIDASCREEN® FAST DON.

In the case of mycotoxins F-2 and T-2, the sample preparation protocols were the same, that is, aliquots of the same working solution were used for the tests. 5 grams of the sample was weighed, transferred into a 100 ml flask, 25 ml of 70% MeOH was added, and then it was shaken intensely for 30 minutes on a shaker. The mixture was filtered into a 100 ml Erlenmeyer flask through a Whatman 1 filter placed in a glass funnel. To 1 ml of the filtrate, 1 ml of distilled water was added, and 50 µl of the diluted sample was used for the tests: RIDASCREEN® FAST ZEARALENON, RIDASCREEN® FAST T-2.

3.3. Method validation

Suitability of the kits for the quantitative analysis of mycotoxins have been verified by the certificates of several relevant organizations: the *AOAC*, Association of Official Chemists (AOAC International/ Research Institute – PTM/Performance Tested Methods), the *FGIS* (Federal Grain Inspection Services - program of the Grain Inspection), and the *USDA/ GIPSA* (Packers and Stockyards Administration of the United States Department of Agriculture).

Validation of the DON toxin measurement method has been performed for the cereals and other plant-based raw materials listed by the manufacturer in the certificate, such as maize, wheat, barley, malt, wheat bran, sorghum, wheat flakes, wheat flour, soy flour, soy flakes, alfalfa, as well as cereal-based feeds. The limit of detection (LOD) specified by the manufacturer was 0.15 mg/kg , the limit of quantification (LOQ) was 0.20 mg/kg. Validation of the F-2 toxin measurement method was performed for cereals, such as maize, wheat, barley, oats, as well as mixed feeds. The detection range specified by the manufacturer was 17 to 41 µg/kg, the LOQ was 50 µg/kg. Validation of the T-2 toxin measurement method was performed for maize, pig and poultry feeds, as well as mixed feeds. The limits specified by the manufacturer were as follows: LOD: 20 µg/kg, LOQ: 50 µg/kg. Detection limits and limits of quantification, as specified by the certificates enclosed with the kits are summarized in ***Table 1***.

We also performed the validation of the measurement methods, during which n=10 wheat samples (blank) were used for the determination of each mycotoxin limit of detection (LOD), according to the sample preparation and measurement specified by the manufacturer. This was necessary, because concentration results exceeding the LOD were used during the evaluation and data analysis. LOD calculations were performed using the average concentration values calculated with the help of the calibration curves, based on the absorbance of the samples tested, and the corresponding standard deviation (SD) values.

LOD = (average of blank calculated concentration) + (twice the measurement results /SD/ standard deviation)

Recovery percentage determinations were performed at three different concentration levels (50, 100, 200 µg/kg). To the control wheat samples, 500 µg/l of the mycotoxin standard working solution, prepared by us, was added to set the estimated three concentration levels. For each concentration, six parallels were used each day.

Recovery % = 100 x measured content/adjusted level

To determine the intermediate precision or correctness, the same steps were repeated two more times. Each mycotoxin was measured 3 times at all concentration levels. Analyses were completed within two months, involving two different analysts, in the same laboratory, using the same instruments. Precision is value characteristic of the random changes of the measurement, which can be described by the within-laboratory variability, among other things.

$H = X_{meas} - X_{ref}$

Where H is the precision, equal to the difference between the measured (X_{meas}) and the reference value (X_{ref}), if a certified reference material (CRM) is available. In the absence of a CRM, the X_{ref} value was determined by us.

Wheat samples were used for the determination of the validation parameters of each mycotoxin tested (DON, F-2, T-2). Results are summarized in ***Table 2***.

Results were as follows: LOD for DON mycotoxin was 13 µg/kg, for toxin F-2 it was 17 µg/kg, and in the case of T-2 it was 12 µg/kg. Recovery ranged from 85.3 to 98.1%, and the coefficient of variation (CV) was 3.4- 5.7%. Intermediate precision was 86.9-96.9%, CV: 5.9- 7.1%. Calculations of the concentration values obtained in our analyses were based on the recovery of the given mycotoxin average value. According to the recovery acceptance criteria, the values should be between 60 and 115% in the case of a concentration value of 0.01 mg/kg, and between 80 and 110% in the case of 0.1 mg/kg (they have to comply with the provisions of Sections 1 and 2 of Annex III of Regulation (EC) No 882/2004 of the European Parliament and the Council). In our case, recovery was acceptable. However, in the case of the T-2 kit, cross-reactivity of toxin HT-2 has to be taken into consideration, and the cross-reaction specified by the manufacturer has to be taken into account. Test procedures and steps were carried out according to the instructions supplied by the manufacturer or the distributor (R-Biopharm, D.G.).

4. Results and evaluation

Prior to the mycotoxin analyses, a five-point standard calibration was performed, using the standard solutions supplied with the tests. The correlation coefficient (R^2) of the calibration curve in the case of DON toxin calibration was 0.9962; in the case of F-2 calibration it was 0.9998, and in the case of T-2 calibration it was 0.9943. The calibration is linear, if the correlation coefficient (R^2) is greater than 0.990. In the case of soy, for DON and T-2 toxins, test results of all of the samples were below the limit of quantification (<LOQ), but the toxins could be detected (>LOD). F-2 toxin could be quantified (>LOQ) in 60% of soy samples. In the case of the alfalfa pellet samples, concentrations were above the LOQ in 100% of the samples for all three toxins. Data evaluated with the help of the RStudio software (RStudio Inc.) **[24]** can be seen in ***Table 3***. This table shows the statistical evaluation of the mycotoxin concentrations of all of the samples tested, indicating that the median and mean values of DON mycotoxins are an order of magnitude higher than those of the zearalenone and T-2 mycotoxins, which were above the limit of detection in all cases (>LOD).

Due to the low sample number and the large difference between the median values for DON and the other two mycotoxins, analysis of the distributions and the normality cannot be performed, because of the high uncertainty. If a normal distribution cannot be demonstrated, then the median values can be compared using the Wilcoxon test. For the samples tested, median values of the F-2 and T-2 mycotoxins do not differ from each other significantly (p=0.0926; p> 0.05), but the values of the DON and F-2 (p=0.0069; p <0.05), and DON and T-2 toxins (p=0.0051; p<0.05) do. Evaluation of the quantifiable (>LOQ) concentrations is shown in ***Table 4***, where the average, SD, minimum and maximum values are given.

Before the measurement of wheat, barley and maize samples, a five-point calibration was performed for the Fusarium mycotoxins analyzed by us. The squares of the correlation coefficient (R^2) of the calibration curves for DON toxin calibration, F-2 calibration and T-2 calibration were 0.9974, 0.9977 and 0.9983, respectively.

In the case of wheat, 100% of the test results of the samples exceeded the LOQ value for F-2 toxin. For barley, DON values were above the LOQ in 20% of the samples. For maize, values were above the limit of quantification (>LOQ) in 50% of the samples for DON toxin, and in 20% of the samples for toxins F-2 and T-2. Data evaluated with the help of the RStudio software are given in ***Table 5***, where the statistical evaluation of the mycotoxin concentrations of all of the samples tested can be seen. Here again, similarly to group 1, all three mycotoxins were present in detectable (>LOD) amounts.

The mathematical-statistical program used by us made quick analysis of the data possible. It can be concluded from the evaluation that the mean value of deoxynivalenol is an order of magnitude higher than those of zearalenone and T-2 mycotoxins here again.

Due to the large differences, there was no point in this case either to perform distribution and normality analyses. If the distribution is not normal, the non-parametric Mann-Whitney U test can be used: there is no significant difference between the median values of the individual mycotoxins for the three sample groups (*p* values were above 0.05 in all cases). Performing the also robust Kruskal-Wallis ANOVA test, we found that even if all sample groups are examined together, there is still no significant difference between the median values of the mycotoxins. When examining the F-2 mycotoxin, the *p* value was bordering on the significance level (p=0.0498), and when examining the groups separately, there was no significant difference between the medians. Evaluation of the quantifiable concentrations is summarized in ***Table 6***, where the mean, standard deviation (SD), minimum and maximum values are given. In the case of DON, in maize samples, the mean value corrected by the standard deviation (± SD) was 297 ±24 µg/kg.

In Hungary, the mycotoxin contamination of feed raw materials, feeds and plant raw materials intended for human consumption is regularly checked by the National Food Chain Safety Office (NÉBIH). Based on results from previous studies **[22]**, in 2003, n=222 different agricultural raw materials were tested for DON toxin. The study shows that 30.3% of the samples contained the toxin in concentrations below 0.040 mg/kg, while 10.4% of them contained it in concentrations above 2.0. In 2008, in another study for DON toxin in feed raw materials, n=118 samples were analyzed, 50% of which showed a DON toxin contamination below 0.040 mg/kg, while 6.8% of them had DON concentrations above 2.0 mg/kg. In 2003, n=128 raw materials were tested for zearalenone (F-2), 77.3% of which contained the toxin in concentrations below 0.010 mg/kg, while concentrations were above 1.0 mg/kg in 0.8%. In 2008, during the same analysis for F-2 toxin *(n=56)*, concentrations below 0.010 mg/kg occurred in 80.4% of the cases, while no sample with a concentration above 1.0 mg/kg was found. In the case of T-2 toxin in 2003, n=147 samples were tested, 94.6% of which had concentrations below the limit of detection, while the number was 90% in 2006, and only 75% in 2008 (n=12). These results demonstrate that the amounts of mycotoxins in the different samples tested can change from year to year, as a result of the weather and climatic effects.

In our 2015 study, wheat, maize, barley and oat samples (n=116) were analyzed, and for DON, F-2 and T-2 toxins, maize proved to be the most contaminated cereal **[17]**.

The analysis of Fusarium toxins (DON, F-2, T-2) in agricultural raw materials and animal feeds is an important task, because that is where these contaminants can enter the food chain from.

5. Conclusions

One of the new threats emerging in connection with climate change is the increased frequency of mycotoxin occurrence in the food chain. In our study, the DON, F-2 and T-2 mycotoxin contamination of the most commonly used feed raw materials was measured using a competitive ELISA method. The three toxins tested could be detected in all of the samples (>LOD). Based on the data, it was found that the mean values for the DON toxin were an order of magnitude higher than the mean values of the F-2 and T-2 mycotoxins. From these data, the conclusion can be drawn that the toxin contaminating raw materials the most is DON, followed by F-2, and then toxin T-2.

Our results draw attention to the fact that the analysis of the mycotoxins deoxynivalenol, F-2 and T-2 is of the utmost importance in the fields of food and feed safety.

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