Zearalenone in feed, urine and meat from three pig farms in Croatia

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Abstract

Zearalenone (ZEN) is a mycotoxin of the genus Fusarium which belongs to the group of macrocyclic lactones. ZEN contamination occurs during cereal harvest or in the early phase of storage if drying was insufficient. The aim of this study was to determine the level of ZEN in feed mixtures given to pigs during the fattening period at three different farms in the Republic of Croatia, as also to determine ZEN levels in urine and meat taken from the same animals. The study also examined correlations between ZEN concentrations in urine and meat with the estimation of ZEN intake in the human body through meat consumption, expressed as a percentage of the Tolerable Daily Intake (TDI). In total, 9 feed mixtures (3 samples per farm), 45 urine and 45 meat samples (from 15 animals per farm) were taken during 2021 from three pig farms located in eastern and central Croatia. ZEN concentrations were determined by the competitive enzyme ELISA method. All values in feed from all three farms were within maximum recommended limit (MRL) given in EU Recommendation, i.e., 250 µg/kg, though at one far, the levels recorded were just under the MRL. Monitoring of ZEN levels in urine can be used as an indicator for the detection of feed contamination with this mycotoxin. Although pigs were fed with feed with near the MRL level of contamination, a negligible percentage of TDI value was obtained for this mycotoxin for humans through meat consumption. However, since meat is just one component of the human diet, and in view of the fact that ZEN can be present in a number of foodstuffs, especially cereals, its total intake could be significantly higher than estimated herein.

Key words: *zearalenone; feedstuffs; urine; meat; contamination; pig farms*

Introduction

Mycotoxins are secondary fungal metabolites, most often found in cereals and cereal by-products intended for human consumption and animal feeding. They can enter the human body directly through contaminated food or indirectly through contaminated feed used in the farm animal's diet, which are then used

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to produce food of animal origin. The biosynthesis of mycotoxins is influenced by microclimatic factors, *i.e.*, moisture in the substrate, relative air humidity, the presence of oxygen, pH, temperature, physical damage and the presence of fungal spores. It occurs during harvesting, drying and storage of different foodstuffs and feedstuffs (Janssen et al., 1997; Hoffmans et al., 2022). Studies have shown that during extremely wet years, *Fusarium* fungi development and occurrence of their mycotoxins are common (Pleadin et al., 2018).

Zearalenone (ZEN) is a mycotoxin of the genus *Fusarium* that has an endocrine-modulating effect. It belongs to the group of macrocyclic lactones, isolated from the mould culture of *Giberella zeae*, as the sexual stage of the mould *Fusarium graminearum*. Further, it was found that ZEN can be also produced by *F. culmorum*, *F. equiseti* and *F. verticilioides* as fungal species that develop on plants (Chelowski, 1998; Bennet and Klich, 2003; Nahle et al., 2021). ZEN contamination occurs at harvest or during early storage when the cereals are insufficiently dried, and represents one of the most prevalent mycotoxins in animal feed (Tolosa et al., 2021). The optimum temperature for the development of ZEN mycelium is reported to be about 20–25°C and occurs at high water activity (> 0.90) (Llorens et al., 2004). ZEN is insoluble in water but soluble in organic solvents, and stable at high temperatures during the process of food grinding, processing and storage (Zollner et al., 2002; Alexander et al., 2004). It acts as a phytoestrogen and shares a common chemical similarity with synthetic and natural oestrogen hormones (Metzler et al., 2010; Kriszt et al., 2015).

In humans and animals, exposure to ZEN results in disorders of the urogenital system, while in the case of stronger acute or chronic poisoning it can leave high consequences on the organs of the reproductive system in the form of degenerative changes on the testicles, ovaries and prostate, and inhibition of the anterior lobe of the pituitary gland and hypothalamus. The effect of this mycotoxin leads to damage to the sex cells of domestic animals, and to hyperoestrogenism in cattle, pigs and poultry (Mitterbauer et al., 2003). Symptoms of exposure also include abortions, prolongation of the duration of oestrus, infertility, reduction of libido and mummification (Visconti and Pascale, 1998; Minervini and Dell'Aquila, 2008).

Some authors have concluded that due to the rapid biotransformation and excretion of ZEN witnessed in animals, human dietary intake of this mycotoxin originating from the consumption of meat and meat products is considered to be of low significance (Creppy, 2002). However, some studies have indicated that humans are often exposed to ZEN via ingestion of different contaminated foodstuffs and that risk assessments of concerning exposure to this mycotoxin raised concerns for certain products and populations (Aldana et al., 2014; Zhang et al., 2020). In recent decades, the European Food Safety Authority (EFSA) has launched a series of dietary exposure assessments on ZEN and its modified forms (EFSA, 2011, 2014, 2016) to evaluate the chronic dietary exposure to ZEN for different age and consumer groups in Europe. The conclusion is that the risk is low for all age groups, based on analytical results on ZEN occurrence in food performed by 19 European countries and food consumption data recorded in the EFSA Comprehensive European Food Consumption Database (EFSA, 2011).

The aim of this study was to determine the level of ZEN in feed mixtures

given to pigs during the fattening period at three different farms in the Republic of Croatia, as secondly to determine ZEN levels in urine and meat taken from the same animals. The study also examined the correlation of ZEN concentrations in urine and meat of these pigs and estimation of ZEN intake through meat consumption expressed as a percentage of the Tolerable Daily Intake (TDI).

Materials and methods

Sampling and sample preparation

Samples of feed mixtures for pigs, urine and meat were collected during the period October to December 2021 from three pig farms, two of which are located in eastern and one in central Croatia. From each farm, three 1 kg samples of feed mixtures used during pig fattening were taken. Upon slaughter, from each farm and from the same animals, 15 urine samples were taken by removing the urinary bladder content and 15 meat samples were taken in the quantity of 250–300 g from different parts of the animals. In total, 9 feed mixture, 45 urine and 45 meat samples were taken from the three pig farms.

Upon arrival at the laboratory, feed mixtures were thoroughly ground in an analytical mill (Cylotec 1093, Tecator, Sweden) so as to achieve a particle size of 1.0 mm and stored at 4 °C until analysis. Urine and meat samples were transferred into plastic cuvettes or bags and stored at -20 °C immediately after sampling until analysis.

Reagents and equipment

A Ridascreen ZEN kit for the competitive enzyme ELISA method was provided by R-Biopharm (Darmstadt, Germany). The kit contained a microtiter plate with 96 wells coated with specific antibodies, standard solutions of ZEN in concentrations of 0, 50, 150, 450, 1350 and 4050 µg/mL, peroxidise-conjugated ZEN, anti-ZEN antibody, substrate/chromogen solution (urea peroxide/tetramethylbenzidine), a stop solution and sample/conjugate dilution buffers. ZEN standards used for the method validation were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

An auto analyzer ChemWell Awareness Technology Inc. 2910 (Palm City, FL, USA) was used for ELISA method performance.

Extraction of ZEN

Feed: Each feed sample (5 g) was extracted using 100 mL distilled water and 25 mL methanol/water solution (70/30). The extraction was performed by vigorous 3-minute shaking on a shaker, following which the extracts were filtered through a filter paper (Whatman, Black Ribbon, GE Healthcare, Buckinghamshire, United Kingdom). The supernatants obtained with both analytes were appropriately diluted and used for the ELISA.

Urine: Urine samples (50 µL) were diluted with 3 mL 50-mM acetate buffer (pH = 4.8). After dilution, 8 µL glucoronidase/sulphatase of Helix pomatia (Sigma, Steinheim, Germany) were added and incubated for 3 h at 37 °C. Hydrolysed urine samples were loaded into SPE Isolute cartridges (Biotage, Uppsala, Sweden). The sample preparation procedure was described earlier in detail by Vulić et al. (2012). Obtained solutions were used for determination of ZEN with ELISA kit.

Meat: Homogenised muscle sample (2 g) was mixed with 3 mL 50-mM acetate buffer (pH = 4.8) and 8 µL glucuronidase/arylsulphatase (Sigma, Steinheim, Germany) and incubated for 3 h at 37 °C. Methanol (7 mL) was added and stirred

for 20 min and centrifuged (320AR, Hettich, Tuttlingen, Germany) at 2300 g for 15 min at room temperature. The supernatant (2 mL) was diluted with distilled water (2 mL), dichloromethane (3 mL) was added, shaken for 60 sec, and centrifuged. The upper aqueous layer was removed completely and the lower dichloromethane layer was reduced to dryness at 60°C in a nitrogen stream. This content was dissolved with 2 mL of the sample dilution buffer from the ELISA kit, mixed with 1 mL isooctane and shaken for 30 sec. After centrifugation, the upper layer was completely removed, while 50 µL methanol was added to 450 µL of the lower layer. The obtained solution was applied to wells provided in the ELISA kit.

ELISA method performance

The analytical method for ZEN determination was performed in line with the ELISA kit manufacturer's instructions. The concentrations of ZEN in the analysed samples were calculated from the calibration curve so that the means of absorbance values obtained for the standards and the samples were divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus equal to 100% and the absorbance values are quoted in percentages (absorbance standard (or sample) / absorbance zero standard x 100 = % absorbance). In the 50–1350 µg/mL range, the calibration curve was virtually linear. The ZEN concentration corresponding to the absorbance of each sample was red from the calibration curve using a mathematical interpolation and multiplied by the corresponding dilution factor of the particular sample. The calibration curve and calculation of results were performed using the software of the ELISA auto analyzer. ZEN concentrations were expressed in µg/kg for pig feed and meat samples and in µg/L for urine.

ELISA method quality control

The ELISA method used for the quantitative determination of ZEN in different materials was previously validated and published by Pleadin et al. (2015). The limit of quantification (LOQ) was 2.8 µg/kg for pig feed, 0.1 µg/L for urine and 0.4 µg/kg for meat. Quality control in this study was performed using the CRM of feed (cereal based), with the assigned value of 147±9 µg/kg (TYG087RM, Fapas, Sand Hutton, York., UK). The control found that ZEN concentrations in all performed analyses of this study fell within the CRM assigned range, with an obtained mean value of 144 µg/kg.

Data analysis

The distribution of data was tested using the Shapiro-Wilk W-test. ZEN concentrations in pig urine and pig meat were not normally distributed. In order to test animal gender-based differences, the Wilcoxon Mann-Whitney rank sum test was applied. In order to assess the relationship between ZEN values in pig urine and pig meat, the Kendal tau rank correlation coefficient was calculated, with statistical significance set at 95% (P=0.05). Statistical analysis was performed using Stata 13.1 Software (StataCorp. 2013: Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.).

Results and discussion

Multi-year research carried out in Croatia points to the frequent occurrence of ZEN in different cereals and feed (Pepeljnjak and Šegvić, 2004; Domijan et al., 2005; Pleadin et al., 2012a, 2013, 2015). With respect to ZEN, some studies have reported that animal tissues and by-products especially reflect the animals' expo-

sure to contaminated feed (Tolosa et al., 2021). This is due to the rapid absorption of ZEN in the digestive tract and the intensive enterohepatic cycling of ZEN and its derivatives (Dänicke and Winkler, 2015). However, some studies have shown that meat and other edible tissues may not be contaminated with this mycotoxin even if the animal is exposed in high concentrations (Goyarts et al., 2007; EFSA, 2011). An earlier study in Croatia found ZEN concentrations in meat to be low and safe for human consumption (Pleadin et al., 2015). In this study, pig feed was collected from three farms in the Republic of Croatia and from animals that were fed with these feed mixtures, after the final stage of fattening urine and meat samples were taken at slaughter.

The mean values of ZEN measured in pig feed were 160.13±3.30 μg/kg on Farm 1, 239.05±8.25 μg/kg on Farm 2, and 47.60±4.71 μg/kg on Farm 3 (Figure 1). The Croatian legislation stipulates no maximum limits for ZEN in any kind of feedstuffs, so all results are interpreted according to European Commission Recommendations 2006/576/EC (EC, 2006). All values in pig feed from three farms were within the maximal recommended limit (MRL) of the EC Recommendation, of 250 μg/kg for pig feed.

Mean ZEN concentrations determined per farm varied significantly (P<0.05). The maximum concentration determined on Farm 2 was 248.53 μg/kg, and was just under the MRL (250 μg/kg). In this study, feed samples were produced from cereals harvested in 2021. According to the percentile distribution, the thermal conditions in Croatia in 2021 were described as warm (Eastern and Central Croatia) with normal precipitation levels in all growing areas related to this research. Such weather conditions can be associated with the moderate level of cereal and feed mixture

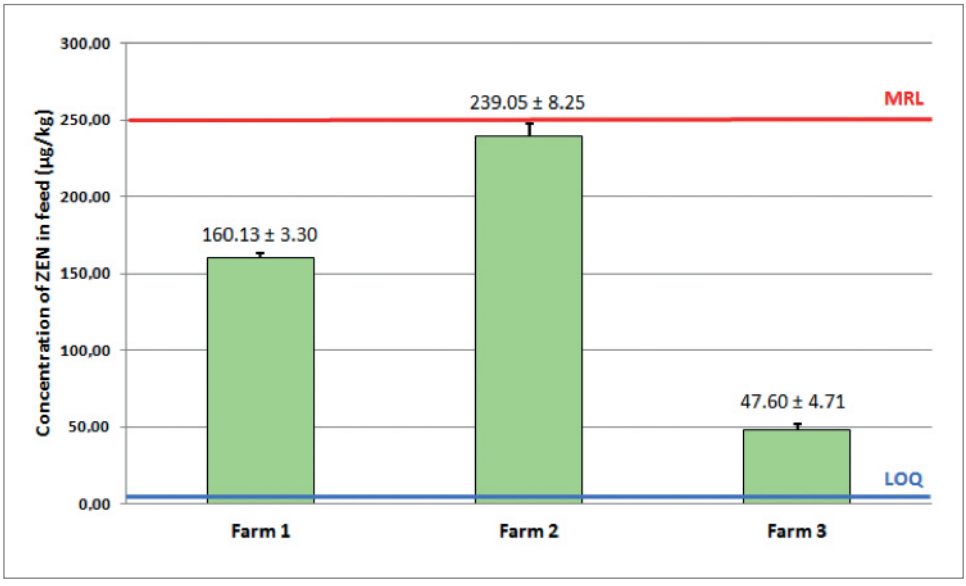

Figure 1. Concentrations of zearalenone (ZEN) determined in pig feed from three farms in the Republic of Croatia

MRL – maximal recommended limit for pig feed (250 μg/kg); LOQ – limit of quantification (2.8 μg/kg)

contamination, of which the lowest ZEN values in general among farm animals are recommended for pigs.

Earlier studies performed in Croatia also found varying concentrations of ZEN in pig feed and the level of contamination by year was associated with weather conditions observed during the cereal cultivation, concluding that extremely wet periods resulted in the highest ZEN concentrations that were significantly higher than MRLs (Pleadin et al., 2012a, 2012b, 2015). Earlier the highest determined concentration of ZEN in feed was 1949 µg/kg and the occurrence of hyperoestrogenism was recorded (Pleadin et al., 2015). The data have indicated that among cereals, maize is the most commonly contaminated cereal with the highest risk of frequent and high-level ZEN contamination, while contamination of wheat, oat and soybean products has only been sporadic (Placinta et al., 1999; Zinedine et al., 2007; Pleadin et al., 2015). A high ZEN occurrence in feeding materials was based on maize contamination and explained by cold weather and extremely high precipitation observed during 2014. It is important to point out that in Croatia, feedstuff production relies heavily on maize, while wheat is used very rarely or not at all. The same applies to other countries situated in this part of Europe.

Concentrations of ZEN determined in urine from three farms in the Republic of Croatia are shown in Figure 2.

It is known that the main route of ZEN elimination from the organism is through urine, so the monitoring of ZEN levels in urine can be used as an indicator for the detection of feed contamination with this mycotoxin (Döll et al., 2003; Thieu and Pettersson, 2009; Takagi et al., 2011). Observed maximal mean urine ZEN concentration of 92.46±12.98 µg/L, with a maximum absolute value of 116.32

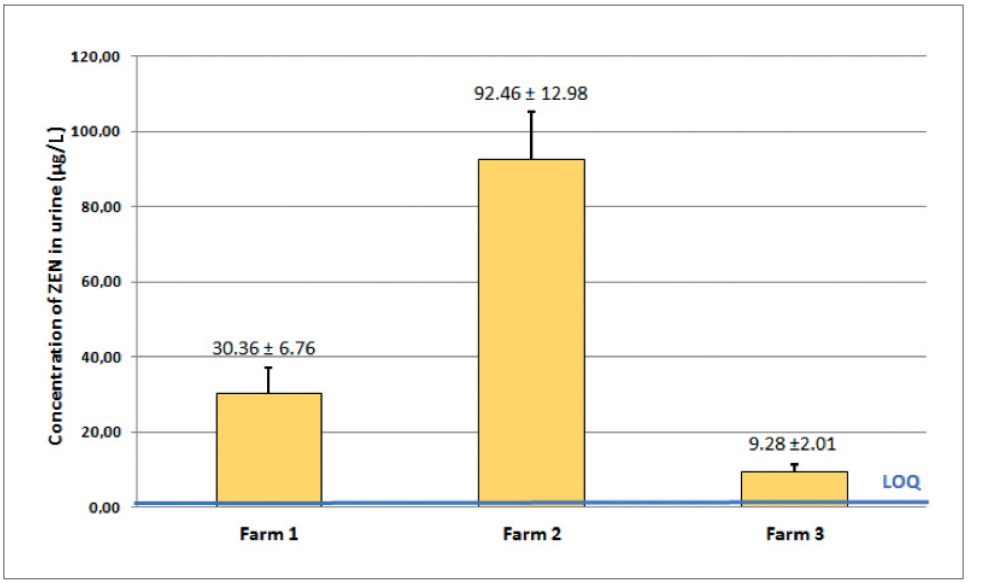

Figure 2. Concentrations of zearalenone (ZEN) determined in urine from three farms in the Republic of Croatia

LOQ – limit of quantification (0.1 µg/L)

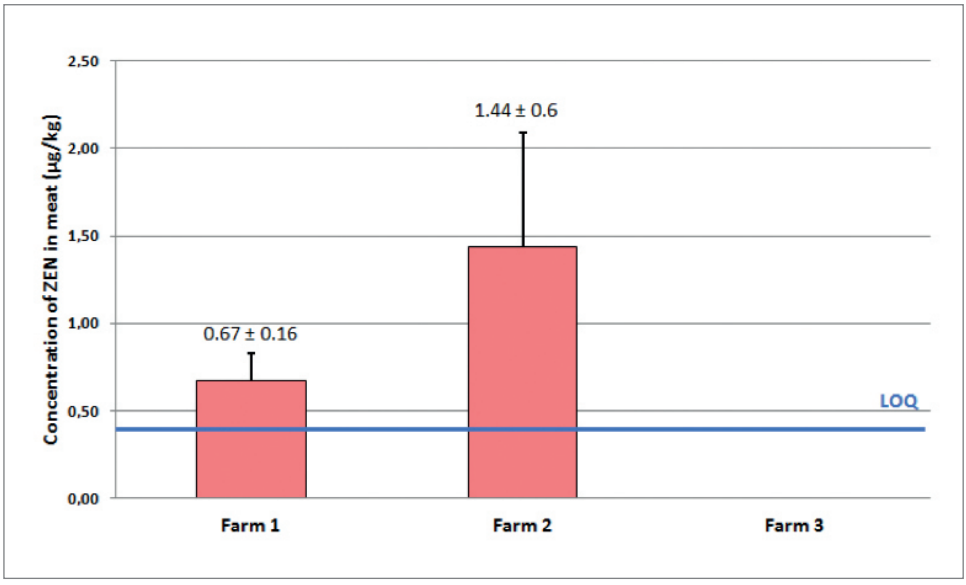

Figure 3. Concentrations of zearalenone (ZEN) determined in meat from three farms in the Republic of Croatia

LOQ – limit of quantification (0.4 µg/kg)

µg/L, was observed on Farm 2, where the highest contamination of feed was also determined.

Earlier studies within the frame of which urine was randomly sampled from pigs farmed in different regions and fed on ZEN-contaminated feeding stuffs showed the mean ZEN concentration in pig urine was 40.45±68.25 µg/L with a maximum of 241.1 µg/L (Vulić et al., 2012). Further, at farms in which high feed contamination and pig hyperoestrogenism were observed, the mean ZEN concentration in pig urine was 206±20.6 µg/L (Pleadin et al., 2015). In gilts fed on ZEN low-dose (192 µg/kg b.w./day for 4 days), ZEN urine concentration reached a maximum level of 158.9 µg/L (Minervini and Dell'Aquila, 2008). In this study generally lower values of ZEN concentrations were determined in urine, as also in feed.

Concentrations of ZEN determined in meat from all three farms are shown in Figure 3.

The ZEN mean value of positives in meat at all three farms was 0.64 µg/kg, with the highest mean value of 1.44±0.60 µg/kg determined on Farm 2. In an earlier study in Croatia, a similar mean ZEN concentration in meat was determined (0.62 µg/kg) (Pleadin et al., 2015).

The correlation of ZEN concentrations determined in urine and meat samples from all three investigated pig farms is shown in Figure 4.

Correlation coefficients with a magnitude between 0.7 and 0.9 indicate variables that can be considered highly correlated (https://www.andrews.edu/~calkins/math/edrm611/edrm05.htm). In this study, the correlation coefficient for the concentration of ZEN in urine and meat samples from three investigated pig

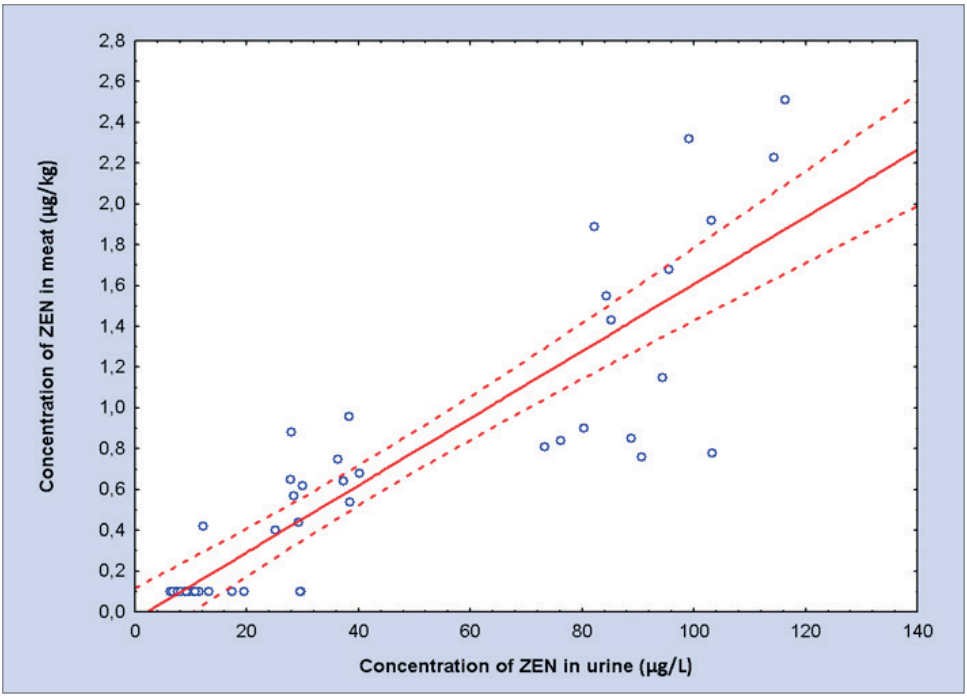

Figure 4. Correlation of ZEN concentrations in urine and meat determined on investigated pig farms

farms was r =0.88229, yielding a high correlation between these two matrices.

Tolerable daily intake (TDI) gives an estimate of the amount of a substance in food that can be taken in daily over a lifetime without appreciable health risk. TDIs are calculated on the basis of laboratory toxicity data to which uncertainty factors are applied and they are used for substances that do not have a reason to be found in food. Based on recent data in the most sensitive animal species, the pig, and taking into account comparisons between pigs and humans, the Panel on Contaminants in the Food Chain established a TDI for ZEN of 0.25 µg/kg b.w. (EFSA, 2011). The estimation of ZEN intake in humans through meat consumption expressed as a percentage of TDI as determined in this study is shown in Table 1.

The latest data show that the average weight of an adult in Croatia is 83 kilograms (91.3 kg for male and 74.7 kg for female) (https://www.worlddata.info/average-bodyheight.php). Therefore, the maximum amount of ZEN daily intake according to the average weight is 20.75 µg. In this study, the highest concentration of ZEN in meat was 2.51 µg/kg, which means that by consuming a 200 gram portion of meat, 2.42% of the TDI value for ZEN is introduced into the human body. This value represents the maximum percentage of TDI determined in this study on all three investigated farms. It can be concluded that when pigs are fed with near the maximum recommended level of ZEN contamination, a negligible percentage of TDI value for this mycotoxin is obtained. However,

Table 1. Estimation of ZEN intake in humans through meat consumption expressed as a percentage of the Tolerable Daily Intake (TDI)

Origin of meat	Mean concentration (µg/kg)	% of TDI	Maximum concentration (µg/kg)	% of TDI
Farm 1	0.67	0.65	0.96	0.93
Farm 2	1.44	1.39	2.51	2.42
Farm 3	0	0	0.42	0.40

TDI for ZEN: 0.25 µg/kg b.w./day; average weight in Croatia for adult human male and female: 83 kg

since meat represents only one component of the human diet and in view of the fact that ZEN can be present in a number of food groups, especially in cereals, its total intake (*i.e.*, the percentage of the TDI actually entering the body) could be significantly higher than estimated herein. When it comes to specific population such as children, ZEN intakes could also be higher than stated here.

Conclusion

All values in feed from the three farms were within the MRL, though the ZEN level approached the MRL at one farm. Monitoring of ZEN levels in urine can be used as an indicator for the detection of feed contamination with this mycotoxin. Although pigs were fed with feed with this level of contamination, a negligible percentage of TDI value for this mycotoxin was obtained for humans through meat consumption. However, meat is just one component of the human diet and ZEN can be present in a number of food groups. Therefore, prevention of contamination with ZEN is of great importance for the protection of public health by identifying key critical control points. Measures taken to the above effect require the application of effective techniques capable of reducing the mycotoxin presence in food and feed or of decontaminating the latter. In further research, with the aim of collecting the most precise data possible, it is necessary to have an understanding about the duration of exposure of individuals to ZEN and about the time that has passed before the sampling of urine and meat from the same exposed individuals.

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Zearalenon u stočnoj hrani, urinu i mesu s tri svinjogojske farme u Hrvatskoj

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Zearalenon (ZEN) je mikotoksin iz roda *Fusarium* koji pripada skupini makrocikličkih laktona. Kontaminacija ZEN-om javlja se tijekom žetve žitarica ili ukoliko nije provedeno dostatno sušenje u ranoj fazi skladištenja. Cilj je ovog istraživanja bio utvrditi razinu ZEN-a u krmnim smjesama koje su davane svinjama tijekom tova na tri različite farme u Republici Hrvatskoj i utvrditi njegovu razinu u urinu i mesu tovljenih životinja. Istraživanjem je ispitana i korelacija koncentracija ZEN-a u urinu i mesu svinja i napravljena je procjena njegovog unosa u organizam konzumacijom mesa, izražena kao postotak podnošljivog dnevnog unosa (TDI). Ukupno je, tijekom 2021. godine, s tri farme svinja u istočnom i središnjem dijelu Hrvatske uzorkovano 9 krmnih smjesa (3 uzorka po farmi), 45 uzoraka urina i 45 uzoraka mesa (od 15 životinja po farmi). Koncentracije ZEN-a određene su kompetitivnom enzimatskom ELISA metodom. Sve vrijednosti u hrani za svinje s tri farme bile su unutar najveće preporučene količine (MRL) definirane u Preporuci EU, koja za hranu za svinje iznosi 250 µg/kg, a razina ZEN-a na jednoj farmi bila je gotovo jednaka MRL. Praćenje razine ZEN-a u urinu može se koristiti kao pokazatelj za detekciju kontaminacije hrane tim mikotoksinom. Iako je svinjama tijekom hranidbe davana hrana koja je sadržavala razinu kontaminacije ZEN-a oko MRL, konzumacija mesa rezultirala je zanemarivim postotkom vrijednosti TDI-a za ovaj mikotoksin u ljudi. Međutim, budući da je meso samo jedna komponenta ljudske prehrane i budući da ZEN može biti prisutan u različitim namirnicama, posebice u žitaricama, njegov ukupni unos mogao bi biti znatno veći od procijenjenog u ovom istraživanju.

Ključne riječi: *zearalenon, stočna hrana, urin, meso, kontaminacija, farme svinja*