

Occurrence of ochratoxin A in animal tissues and feeds in Poland in 2014–2016

Katarzyna Pietruszka, Marta Piątkowska, Piotr Jedziniak

Department of Pharmacology and Toxicology,
National Veterinary Research Institute, 24-100 Pulawy
katarzyna.pietruszka@piwet.pulawy.pl

Received: September 14, 2017

Accepted: December 8, 2017

Abstract

Introduction: Ochratoxin A (OTA) is a toxic metabolite mainly produced by *Aspergillus* spp. and *Penicillium* spp. fungi. Research on the contamination of cereals, complete feeds, and tissues with this mycotoxin has indicated that it can be a toxicological problem impacting animal health and food safety in temperate climates. OTA contamination mainly besets the global pig industry, necessitating the monitoring of feeds and animal tissues. The aim of the study was to present the results of the official monitoring of OTA in animal tissues and feeds in Poland in 2014–2016 and determine the possible correlation between the presence of OTA in different types of samples. **Material and Methods:** The presence of ochratoxin A was determined using accepted procedures based on liquid chromatography with fluorescence detection after immunoaffinity column clean-up. Determination of OTA was afforded in the range of 0.3 µg/kg to 300 µg/kg in complete feeds and from 0.2 µg/kg to 150 µg/kg in the kidneys, liver, and muscles. **Results:** Over the three year span, about 23.5% of the animal tissues samples were contaminated by ochratoxin A. In the 2014 survey, 10% of the sample tissues contained 5–10 µg/kg (only one sample above 10 µg/kg), and in 2015 and 2016, 24% of samples showed levels above the limit of quantification 0.2 µg/kg, while none of the samples exceeded the established provisional action level of 5 µg/kg for animal tissues. The animal feed analysis showed that 9% was contaminated with ochratoxin A above the limit of quantification of 0.3 µg/kg. In 2% of feed samples the OTA concentration was greater than 50 µg/kg. **Conclusion:** The results confirm the appropriacy of OTA contamination monitoring and help to increase food safety.

Keywords: feeds, animal tissues, ochratoxin A, chromatography, national monitoring.

Introduction

Ochratoxin A (OTA) is a secondary metabolite containing chlorinated dihydroisocoumarin moiety in its molecule, produced by different species of *Aspergillus* spp. and *Penicillium* spp. (15). Based on sufficient evidence for carcinogenicity in experimental animal studies it has been recognised by the International Agency for Research on Cancer (IARC) as a possible human carcinogen and classified into group 2B (14, 17, 21).

OTA may be extensively distributed in agricultural commodities and in the environment. Contamination of foods with this mycotoxin, particularly of cereals, nuts, and coffee beans, has been noted in Eastern and Central Europe, North Africa, North America, and Japan (5, 14, 19). OTA accumulates in the body, especially in the kidneys as its target organ, where it exerts toxic and carcinogenic effects (1, 12, 13). In the light of the evidence of OTA's toxicity, its presence in food and feeds is undesirable.

To reduce the health risks posed by the mycotoxin contamination of food and feed, legal restrictions are placed on these commodities. Currently, EC Regulations No 1881/2006 and No 576/2006 (7, 9) and later amendments are in force in Poland, establishing recommended levels allowable for OTA presence in food and setting indicative values for feed ingredients. In Poland the recommended level (ML) adopted for animal tissues (kidneys, liver, and muscles) is set at 5 µg OTA per kg; and for feedstuffs, MLs are set at 50 µg/kg for complementary and complete feeds for pigs, 100 µg/kg for poultry feeds, and 250 µg/kg for cereals and cereal based products (6, 7).

As controlling the presence of OTA in the food chain is an important part of food safety, it was included in The National Residue Control Plan (NRCP) for Chemical, Biological, and Drug Residue in Animal Tissues and in the Food of Animal Origin mandatory in all countries of the European Union, according to Annex I of Council Directive 96/23/EC of 29 April (4).

The Polish monitoring programme for the presence of OTA in animal tissues according to Council Directive 96/23 has been implemented for many years only by the Department of Pharmacology and Toxicology of the National Veterinary Research Institute. In Poland, the presence of OTA in feeds and feed materials is monitored by the implementation of a feed control programme, fulfilled by six regional veterinary inspection laboratories. Feed samples for the control tests are collected by the Veterinary Inspectorate and sent to a designated laboratory. Subsequently the results of OTA testing of feeds and feed materials are reported to the National Reference Laboratory of the National Veterinary Research Institute.

In this paper the results of the Polish official monitoring of OTA residues in food of animal origin and feeds covering the years 2014–2016 are presented. The study is a continuation of the research carried out between 2003 and 2012 and published in 2013 (20).

Material and Methods

OTA was determined using validated and accredited procedures according to EU requirements (4, 8).

Sampling. In 2014–2016, a total of 300 feed samples and 430 samples of swine kidneys, poultry liver, and fish muscles from all over the country were tested for the presence of OTA. The sampling of the materials was performed under the NRCP in accordance with the internal instruction of the Chief Veterinary Officer. Tissue samples were kept frozen below -18°C until the day of analysis.

Reagents and chemicals. All chemicals were of HPLC grade. OchraTest WB immunoaffinity columns (IAC) were purchased from VICAM Inc (USA). Ochratoxin A standard and all other reagents obtained from Sigma-Aldrich (USA) were of minimum 99% purity. Methanol, acetonitrile, toluene, and acetic acid were obtained from J.T. Baker (USA). Water was obtained from a Milli-Q Advantage system (Millipore, USA). A stock solution of OTA (1,000 $\mu\text{g}/\text{mL}$) was prepared in a toluene–acetic acid mixture (99:1, v/v) and stored in the dark at -16°C or below for a maximum of six months. The OTA working standard solutions were prepared by appropriate dilution of the stock solution in mobile phase for LC analysis (acetonitrile–water–0.1% acetic acid, 495:195:10, v/v/v) and stored in the dark at 6°C or below for a maximum of three months.

Extraction and clean-up of animal tissues. A 10 g tissue sample (kidneys, muscles, or liver) was homogenised with 50 mL of chloroform. The sample was then centrifuged at 3,500 rpm for 15 min at -4°C and filtered. Next, the eluate was evaporated by rotary evaporator and the residue was dissolved in methanol. Whole extract was taken for clean-up using the SPE technique and OchraTest WB immunoaffinity columns. Afterwards, the eluate was dried under a gentle stream of nitrogen at $35 \pm 5^{\circ}\text{C}$ and subjected to chromatographic analysis.

Extraction and clean-up of feeds and feed materials. A 25 g feed sample was rotary shaken with methanol–water mixture, 80:20 (v/v), for 1 h. The solution was next centrifuged at 3,500 rpm for 15 min at -4°C . The extract was filtered and 15 mL of it was diluted with 60 mL of water. Subsequently, the diluted extract was taken for clean-up by SPE technique and OchraTest WB immunoaffinity columns. Then the eluate was dried under a gentle stream of nitrogen at $35 \pm 5^{\circ}\text{C}$ and subjected to chromatographic analysis.

Liquid chromatography–fluorescence detection. The liquid chromatograph (Agilent 1100 Series, Agilent, Germany) was coupled to a fluorescence detector with excitation wavelength of $\lambda_{\text{ex}} = 340$ nm and emission wavelength of $\lambda_{\text{em}} = 470$ nm. Chromatographic separation was performed using the column Inertsil ODS-3 (5 μ , 150 mm \times 4.6 mm, Phenomenex, USA). The mobile phase for LC analysis consisted of acetonitrile–water–acetic acid (495:495:10, v/v/v) mixture in isocratic elution with a flow rate of 1.4 mL/min at ambient temperature. The software used for data operation was ChemStation Rev.A.10.1 (Agilent, Germany).

Validation procedures. To perform the validation procedures, animal tissues and feed samples were spiked with OTA at the levels of 2.5, 5.0, and 7.5 $\mu\text{g}/\text{kg}$ and processed through the appropriate extraction procedures described above. The following parameters were established: linearity (working range), limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability (CV), within-laboratory reproducibility (CV), and uncertainty (U_c for spiking level C = 5 $\mu\text{g}/\text{kg}$) (Table 1).

Results

The validation results, which are in line with the requirements for mycotoxin assays in animal feed and tissues, are presented in Table 1.

The feed analysis showed that out of the 300 tested samples, 27 samples were contaminated with OTA above the limit of quantification (LOQ) of 0.3 $\mu\text{g}/\text{kg}$ (Table 2). Fifteen of them contained OTA in concentrations ranging from 0.3 to 10 $\mu\text{g}/\text{kg}$ and in seven samples the concentration ranged from 10 to 50 $\mu\text{g}/\text{kg}$. Five samples exceeded the established maximum level in feed set at 50 $\mu\text{g}/\text{kg}$ (maximum detected concentration of OTA: 222 $\mu\text{g}/\text{kg}$) (Fig. 1).

Over the three years under consideration, in Poland 23.5% of analysed tissue samples were contaminated with OTA (Table 3). Out of 430 tissue samples tested for the presence of OTA, 94 were contaminated at the levels of 0.2–5 $\mu\text{g}/\text{kg}$. The concentrations reached the provisional action level (set in Poland at 5 $\mu\text{g}/\text{kg}$) in seven samples (four samples with OTA at concentrations of 5–10 $\mu\text{g}/\text{kg}$ and three samples at concentrations above 10 $\mu\text{g}/\text{kg}$) (Fig. 2).

Table 1. Results of the method validation

| Parameter | Feed | | | Kidneys | | | Liver | | | Muscles | | |
|-------------------------------------------|---------------------------------|-----|-----|--------------------------------|-----|-----|---------------------------------|------|-----|---------------------------------|------|------|
| Working range ($\mu\text{g}/\text{kg}$) | 0.3–300 | | | 0.2–150 | | | 0.2–150 | | | 0.2–150 | | |
| LOD ($\mu\text{g}/\text{kg}$) | 0.1 | | | 0.13 | | | 0.12 | | | 0.11 | | |
| LOQ ($\mu\text{g}/\text{kg}$) | 0.31 | | | 0.2 | | | 0.21 | | | 0.26 | | |
| Spiking level ($\mu\text{g}/\text{kg}$) | 2.5 | 5.0 | 7.5 | 2.5 | 5.0 | 7.5 | 2.5 | 5.0 | 7.5 | 2.5 | 5.0 | 7.5 |
| Recoveries (%) | 93 | 83 | 84 | 98 | 72 | 67 | 72 | 65 | 73 | 75 | 78 | 70 |
| Repeatability (CV) | 5.2 | 1.4 | 3.7 | 5.2 | 1.4 | 3.7 | 9.5 | 4.0 | 3.9 | 10.1 | 7.0 | 1.5 |
| Reproducibility (CV) | 10.1 | 7.8 | 6.9 | 10.1 | 7.8 | 6.9 | 12.2 | 12.0 | 7.7 | 16.4 | 11.5 | 17.6 |
| Uncertainty ($\mu\text{g}/\text{kg}$) | $U_c(y) = 0.55; k = 2, U = 1.1$ | | | $U_c(y) = 1.1; k = 2, U = 2.2$ | | | $U_c(y) = 0.85; k = 2, U = 1.7$ | | | $U_c(y) = 0.77; k = 2, U = 1.5$ | | |

Table 2. Ochratoxin A levels in feeds determined in the national feed monitoring

| Year | Number of analysed samples | Number of positive results | Number of sample feeds with different levels of OTA residues ($\mu\text{g}/\text{kg}$) | | | Average concentration of OTA \pm SD ($\mu\text{g}/\text{kg}$) |
|-----------|----------------------------|----------------------------|------------------------------------------------------------------------------------------|---------------|-----------|-------------------------------------------------------------------|
| | | | ≥ 0.3 –10 | ≥ 10 –50 | ≥ 50 | |
| 2014 | 95 | 4 | 3 | nd* | 1 | 19.8 ± 2.0 |
| 2015 | 94 | 11 | 6 | 3 | 2 | 38.3 ± 3.9 |
| 2016 | 111 | 12 | 6 | 4 | 2 | 23.9 ± 24.1 |
| Total | 300 | 27 | 15 | 7 | 5 | 27.3 ± 2.8 |
| Total (%) | 100 | 9 | 5 | 3 | 2 | |

*nd – not detected

Table 3. Ochratoxin A levels in animal tissues determined in the national tissue monitoring

| Year | Number of analysed samples | Number of positive results | Number of tissue samples with different levels of OTA residues ($\mu\text{g}/\text{kg}$) | | | Average concentration of OTA \pm SD ($\mu\text{g}/\text{kg}$) |
|-----------|----------------------------|----------------------------|--------------------------------------------------------------------------------------------|--------------|-----------|-------------------------------------------------------------------|
| | | | ≥ 0.2 –5 | ≥ 5 –10 | ≥ 10 | |
| 2014 | 134 | 30 | 26 | 3 | 1 | 2.6 ± 0.5 |
| 2015 | 156 | 37 | 34 | 1 | 2 | 2.0 ± 0.4 |
| 2016 | 140 | 34 | 34 | nd* | nd* | 1.5 ± 0.3 |
| Total | 430 | 101 | 94 | 4 | 3 | 2.0 ± 0.4 |
| Total (%) | 100 | 23.5 | 22 | 1 | 1 | |

*nd – not detected

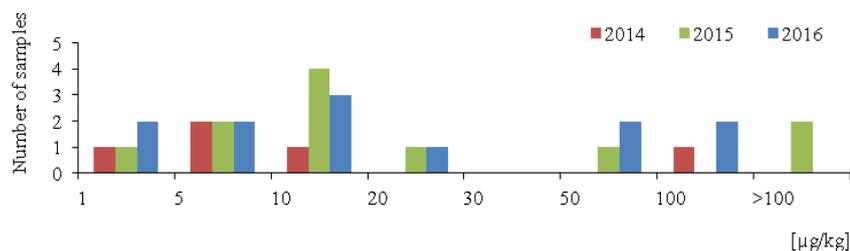
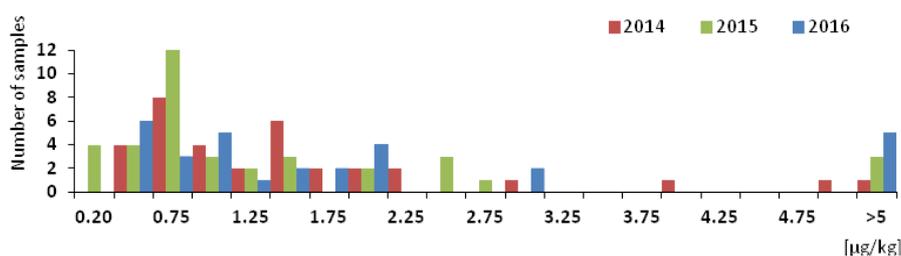
**Fig. 1.** Distribution of OTA contamination in feeds in Poland in the years 2014–2016**Fig. 2.** Distribution of OTA contamination in tissues in Poland in the years 2014–2016

Table 4. The residues of OTA in the kidneys after prior exposure to OTA in feed

| Authors | Animals | OTA dose (feed) | Exposure time (days) | Concentration of OTA (kidneys) |
|------------------------------|----------------------------|-----------------|----------------------|--------------------------------|
| Zhang <i>et al.</i> (22) | weaned pigs (weight 10 kg) | 800 µg/kg | 42 | 400 µg/kg |
| Bernardini <i>et al.</i> (2) | weaned pigs (weight 10 kg) | 181 ± 34 µg/kg | 43 | 21.91 ± 2.17 µg/kg |
| Perši <i>et al.</i> (16) | weaned pigs (weight 10 kg) | 300 µg/kg | 30 | 15.31 ± 3.11 µg/kg |

Discussion

Animals are continuously exposed to OTA-contaminated feeds, as OTA is one of the most often detected mycotoxins in animal feeds and feed components, whereas humans are mainly exposed to OTA *via* food and beverages (3, 11). The dietary exposure for adult consumers in the EU has been estimated at 0.015–0.06 µg/kg b.w. per week (10). Considering its toxic effects both to animals and humans, the standardisation of allowable OTA content in food and feed for the EU countries and the implementation of annual national monitoring programmes are crucial. A number of countries, particularly in Europe, have already implemented regulations governing the OTA presence in food and feed.

Most of the regulations set the maximum permitted or recommended levels for specific commodities. National legislation for OTA in products of animal origin exists in three countries. The Italian Ministry of Health has established an OTA maximum level of 1 µg/kg for pig meat and derived products (16). Estonia has accepted 10 µg/kg for pig liver. In Denmark, if OTA content in the kidneys is higher than 25 µg/kg, the whole carcass is rejected, because the meat is assumed to be highly contaminated; if the OTA concentration is between 10 and 25 µg/kg, edible offal is eliminated; but if the mycotoxin in kidney tissue is lower than 10 µg/kg, only the kidneys are discarded. However, most EU countries, including Poland, have adopted an action level of 5 µg/kg (6).

The experimental studies carried out on pigs demonstrated that the concentrations of OTA detected in the kidneys after the administration of OTA-contaminated feed varied from 5% to 50% of the dose given to animals (Table 4). These studies show the co-occurrence of OTA in feeds and in animal tissues in animals supplied feed contaminated with OTA (2, 16, 22). Therefore, it can be concluded that in Poland during the years 2014–2016 the persistently low, but constant level of OTA in swine tissues indicates the continuous presence of this mycotoxin in feeds. In the light of recent studies, even a low level of feed contamination (50 µg/kg) may activate the pig immune and oxidative stress response as well as the early carcinogenic events (12). However, pigs are very sensitive to mycotoxin intoxication and such effects need to be further investigated in humans (19).

OTA effects on the exposed organism are strongly dependent not only on OTA dose and exposure time, but

also on contamination type (natural or artificial). Some studies have shown that concentrations of OTA in serum, kidneys, liver, and muscle tissues in pigs fed naturally contaminated OTA feeds may be 3–5 times higher than in animals fed OTA-artificially contaminated feed (16). The results of the studies suggest that persistent exposure of pigs to OTA in feed may be the cause of nephropathy in the animals and may increase the risk of consumer exposure to OTA.

In our study, for the three years to the end of 2016 the average level of the OTA concentration in feeds was 27.3 µg/kg, whereas in animal tissues it was 2 µg/kg (below the action levels of 5 µg/kg in animal tissues and 50 µg/kg in feeds). The results of the studies show that there is a possible correlation between the level of OTA in feeds and residues in animal tissues. The continuous monitoring of OTA contamination of pig feed is necessary to guarantee the animals' health, reduce economic losses, and ensure the safety of animal-derived food.

In 2014, 28 European Union Member States reported results for mycotoxins in 1,968 target samples analysed in pigs under their national monitoring plans. Of 117 non-compliant results for mycotoxins (B3d group) 7 were reported as non-compliant for OTA. The non-compliant samples were reported by 19 Member States. The results of the Greek report in the EFSA of 2014 show that of the 24 analysed samples, 9 were non-compliant (37.5%) (18).

In Poland, OTA was determined using validated and accredited procedures, based on liquid chromatography with fluorescence detection after immunoaffinity column clean-up. Based on our obtained and published results from 2003 to 2012, the incidence of positive samples was from 24 to 41 in different years (20). In the study time frame the percentage of non-compliant samples (≥ 5 µg/kg) varied from 1% in 2011 to 9.9% in 2004. Only in 2010 were there no non-compliant samples. The percentage of positive kidney samples containing OTA over the limit of quantification (LOQ = 0.2 µg/kg) in 2006 was 5%, in 2007 it was 6%, in 2009 it was 10%, while the highest of 39% was in 2008 (20). In Poland in the most recent years OTA was detected annually on average in 22.3% of tissue samples (2014), in 23.7% of samples (2015), and in 23.7% of samples (2016). However, in about 2% of animal tissue samples the OTA concentration was greater than 5 µg/kg (Fig. 2).

Summing up, the results of our studies showed a low but constant level of feed contamination with OTA (average concentration 27.3 µg/kg, range from 1 to

222 µg/kg) and low and constant levels of OTA present in animal tissues (average concentration 2.0 µg/kg, range from 0.2 to 21.5 µg/kg). The results of the study are consistent with the results of the studies conducted between 2003 and 2012 and published in 2013.

Low levels of OTA in feed suggest low exposure of animals to this mycotoxin and low levels of OTA in tissues demonstrate that exposure of Polish consumers *via* food of animal origin approximates the average for consumer exposure for humans in the EU countries.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The investigation was supported by the Polish Ministry of Agriculture and Rural Development under the auspices of the Multiannual Programme “Control of the presence of prohibited substances in food of animal origin and undesirable substances in animal feed”.

Animal Rights Statement: None required.

References

- Aoudia N., Callu P., Grosjean F., Larondelle Y.: Effectiveness of mycotoxin sequestration activity of micronized wheat fibres on distribution of ochratoxin A in plasma, liver and kidney of piglets fed a naturally contaminated diet. *Food Chem Toxicol* 2009, 47, 1485–1489.
- Bernardini C., Grilli E., Duvinneau J.C., Zannoni A., Tugnoli B., Gentilini F., Bertuzzi T., Spinozzi S., Camborata C., Bacci M.L., Piva A., Forni M.: Cellular stress marker alteration and inflammatory response in pigs fed with an ochratoxin contaminated diet. *Res Vet Sci* 2014, 97, 244–250.
- BIOMIN Word Mycotoxin Survey 2016. Annual Report No.13. Available online: http://www.pigbusiness.nl/upload/files/1488236289MAG_MTXsurveyReport_2016_EN_0117_PKO.pdf.
- Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off J Eur Commun* 2002, 221, 8–36.
- Dragacci S., Grosso F., Bire R., Fremy J.M., Coulon S.: A French monitoring programme for determining ochratoxin A occurrence in pig kidneys. *Nat Toxins* 1999, 7, 167–173.
- EFSA, Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A (OTA) as undesirable substance in animal feed. Adopted on 22 September 2004. *EFSA J* 2004, 101, 1–36.
- European Commission Recommendation No 576/2006 of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2, and HT-2 and fumonisins in products intended for animal feeding. *Off J Eur Commun* 2006, 229, 7–9.
- European Commission Regulation No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the control of the levels of mycotoxins in foodstuffs. *Off J Eur Commun* 2006, 70, 12–35.
- European Commission Regulation No 1881/2006 of 16 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off J Eur Commun* 2006, 364, 5–24.
- Heussner A., Bingle L.: Comparative ochratoxin toxicity: a review of the available data. *Toxins* 2015, 7, 4253–4282.
- Krska R., Nährer K., Richard J.L., Rodrigues I., Schuhmacher R., Slate A.B., Whitaker T.B.: Guide to mycotoxins featuring mycotoxin risk management in animal production. BIOMIN, Austria 2012.
- Marin D.E., Braicu C., Gras M.A., Pistol G.C., Petric R.C., Berindan N.L., Palade M., Taranu I.: Low level of ochratoxin A affects genome - wide expression in kidney of pig. *Toxicon* 2017, 136, 67–77.
- Marin S., Ramos A.J., Cano-Sancho G., Sanchis V.: Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chem Toxicol* 2013, 60, 218–237.
- Mitchell N.J., Chen C., Palumbo J.D., Bianchini A., Cappozzo J., Stratton J., Ryu D., Wu F.: A risk assessment of dietary ochratoxin A in the United States. *Food Chem Toxicol* 2017, 100, 265–273.
- Naohiko A., Promsuk J., Hitoshi E.: Molecular mechanism of ochratoxin A transport in the kidney. *Toxins* 2010, 2, 1381–1398.
- Perši N., Pleadin J., Kovačević D., Scortichini G., Milone S.: Ochratoxin A in raw materials and cooked meat products made from OTA – treated pigs. *Meat Sci* 2014, 96, 203–210.
- Pfohl-Leszkowicz A., Manderville R.A. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol Nutr Food Res* 2007, 51, 61–99.
- Report for 2014 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. European Food Safety Authority (EFSA), 2016.
- Smith M.C., Madec S., Coton E., Hymery N.: Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins* 2016, 94, 1–36.
- Wiśniewska-Dmytrow H., Żmudzki J., Burek O., Pietruszka K.: Official control of ochratoxin A in food of animal origin in Poland between 2003 and 2012. *Bull Vet Inst Pulawy* 2013, 57, 519–523.
- Wiśniewska-Dmytrow H., Żmudzki J., Pietruszka K., Burek O.: Occurrence of ochratoxin A in organs, muscle tissue, and digestive tract content of wild game. *Med Weter* 2015, 71, 436–440.
- Zhang Z., Gan F., Xue H., Liv Y., Huand D., Khan A.Z., Chen X., Huang K.: Nephropathy and hepatopathy in weaned piglets provoked by natural ochratoxin A and involved mechanisms. *Exp Toxicol Pathol* 2016, 68, 205–213.