

Risk analysis and exposure assessment of Ochratoxin A in Serbia

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Abstract

Aim: The aim of this study was to check the current status of OTA contamination of the Serbian pork and chicken meat in naturally exposed animals which are part of chain production. The knowledge of these factors, their importance and significance, can be used as a tool from the food safety point of view to predict if OTA content in pork and chicken tissues poses a risk to human health and deleterious effects on animals' health and well-being.

Materials and Methods: A total of 540 samples of pigs blood, kidney, liver and chicken gizzard were randomly selected from slaughtered pigs (n=90) and chicken (n=90) and analyzed for the presence of ochratoxin A (OTA) by High-performance liquid chromatography (HPLC-FL).

Results: In porcine tissue samples, of the 90 liver samples, 26.6% contained OTA in the range of 0.22-14.5 ng/g. The incidence of OTA in serum and kidneys were very similar (31% and 33.3%, respectively), with a maximum concentration of 220.8 ng/mL, and 52.5 ng/g, respectively. Majority of chickens' tissues samples, were not found to contain measurable amounts of OTA. Moreover, the OTA levels found in analysed tissues were low in general.

Conclusion: The results of this preliminary research indicate that the content of OTA in the examined tissues are far below the values that represent hazard to the health of consumers. However, the results of this study show that in Serbia, consumers are nevertheless frequently exposed to ochratoxins.

Key words: ochratoxin A, tissues samples, residue, exposure assessment

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Introduction

Undesirable substances such as mycotoxins can be present in plants and derived products thereof. Depending on the nature and the concentration levels of the compound, these might be of concern for human and/or animal health. From the more than 300 mycotoxins isolated and described to the present day, OTA is recognized as a contaminant with a strong nephrotoxic activity [1]. The International Agency for Research on Cancer has classified OTA as possibly carcinogenic for humans (group 2B) [2], also teratogenic and carcinogenic effects have been described in some animal species [3].

Ochratoxin A is a secondary metabolite of fungi such as *Penicillium verrucosum* and *Aspergillus ochraceus* that occur in a number of food commodities like cereals, coffee, dried fruit, pulses, and derivatives products [4]. Apart from cereals products, that are

considered to be the most contaminated samples, meat and meat products are also frequently contaminated with OTA due to carry-over effect. Kidney is the most contaminated organ, followed by pig blood, liver, and meat from animals fed with contaminated feed [5-8]. Through the food chain OTA can appear in human fluids, such as plasma [9], urine [10] and milk [11].

Exposure to OTA is a potential human health hazard because of the frequent exposure, long-half life in the organism and its carcinogen properties in experimental animals. The risk is well recognized, but at present it has not been quantified accurately. In some Eastern European countries (in particular Bulgaria, Romania, Serbia, Croatia, Bosnia and Herzegovina) OTA has been associated with a high incidence of a kidney disease known as Balkan endemic nephropathy [12]. Consumption of foods contaminated with OTA during pregnancy and/or childhood is suspected of inducing lesions in testicular

DNA which, during puberty, can be promoted to testicular cancer [13]. Indeed, rates of incidence of testicular cancer in 20 countries correlated significantly with the per-capita consumption of coffee and pig meat. The dietary intake of OTA by the population of EU member states has been assessed and the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) has recently established a provisional tolerable weekly intake of 100 ng/kg b.w. per week [14].

Moreover, poultry and pork meat comprises a substantial portion of the Serbian diet (17.4 kg and 16 kg, respectively, per person in 2009). The aim of this study was to check the current status of OTA contamination of the Serbian pork and chicken meat in naturally exposed animals which are part of chain production. The knowledge of these factors, their importance and significance, can be used as a tool from the food safety point of view to predict if OTA content in pork and chicken tissues poses a risk to human health and deleterious effects on animals' health and wellbeing.

Materials and methods

Samples collection: Pilot study was carried out during 2009 and 2010. Swine and poultry commercial farms in the northern and central agricultural area of Serbia were randomly selected. Pigs and chicken were selected randomly in slaughterhouses that are important for overall meat production, at the end of fattening week, by random method. About 50 mL of blood per pig was sampled by jugular puncture. Blood samples remained at room temperature for 24 h to allow for clotting, and were subsequently centrifuged at $3,000 \times g$ for 20 min. Serum was decanted and stored at -20°C prior to analysis. Whole liver, kidney and gizzard (chickens) were sampled from each animal. No preservatives were added. A total of 540 samples of serum samples (pigs), liver, kidneys and gizzard (chickens) from slaughtered pigs ($n=90$) and chicken ($n=90$) were collected.

Chemical and Reagents: Ochratoxin A crystalline material was purchased from Sigma (St. Louis, MO, USA). Stock concentrated solution was prepared in toluene-acetic acid (99:1 v/v) at a final concentration of 1 mg/mL and kept in security conditions at -20°C , wrapped in aluminum foil, because OTA gradually breaks down under ultraviolet light. The OTA working solution was prepared by diluting the stock solution with toluene-acetic acid (99:1 v/v) to $\sim 10 \mu\text{L/mL}$. The actual concentration of OTA was calculated using the absorbance by the UV spectrophotometer set at 333

nm ($=5550$). After suitable dilutions by water-methanol-acetic acid (50:49:1 v/v/v), the working solution was used to prepare the external calibration curve. A working standard OTA for HPLC was prepared daily just before starting the injection of a series of samples. Other reagents were of HPLC grade. All chemicals were of HPLC grade, reagent grade or chemically pure.

Extraction and clean-up for ochratoxin analyses: Serum (0.8 mL) was extracted according to Curtui and Gareis (2001) [15] with 15% trichloroacetic acid (0.2 mL) and dichloromethane (1 mL), by vigorous vortexing for 30 s in a 2 mL safe-lock polypropylene conical-bottom centrifuge tube. The mixture was allowed to stand for 24 h at room temperature, and then centrifuged at $14,000 \times g$ for 10 min. The lower dichloromethane phase was carefully withdrawn by a Pasteur pipette and transferred to a 1.5 mL safe-lock polypropylene conical bottom centrifuge tube. The acidic phase and the compact precipitate layer formed between the two phases were re-extracted with dichloromethane (0.5 mL) for 30 s on a vortex mixer and then centrifuged for 5 min at $14,000 \times g$. The pooled dichloromethane extract was evaporated to dryness at 40°C under a gentle nitrogen flow. The remaining residue was reconstituted in methanol (500 μL) and transferred to a HPLC vial. The limit of detection (signal/noise: 3/1) was estimated at 0.1 ng/mL of OTA, and average recovery was 95%.

Kidney, gizzard and liver analyses were performed by the method of Matrella *et al.* [16], which briefly includes a double extraction with acidic ethyl acetate. The organic phase was removed and extracted with 0.5M NaHCO_3 , pH 8.4. The aqueous extract was acidified to pH 2.5 with 7M ortho-phosphoric acid. OTA was finally back extracted into ethyl acetate (3 mL). The organic phase was evaporated to dryness under N_2 steam, reconstituted in 500 μL of mobile phase and a 50 μL of aliquot was injected. The detection limit for OTA in organs was 0.01 ng/g with 61% (C.V. =14.5%) mean recovery from fortified samples at 3 ng/g ($n=3$).

Chromatographic conditions (HPLC): An aliquot of 20 μL for serum samples, 50 μL for kidney, gizzard and liver samples, and for the standards 10 μL , were injected onto a Waters Symmetry Shield RP (Reversed phase) 18, high pressure liquid chromatography column (length and inner diameter 150×4.6 mm, particle size 5 μm) on a Waters Alliance 2695 HPLC system. The OTA was eluted with 4% acetic acid and acetonitrile (32:68 v/v) at 25°C and a flow rate of 1 mL/min.

Table-1. Incidence and the mean concentration of ochratoxin A in tissues of slaughtered animals

	Pig tissues (N=90)			Chickens tissues (N=90)		
	Serum (ng/mL)	Liver (ng/g)	Kidneys (ng/g)	Liver (ng/g)	Kidneys (ng/g)	Gizzard (ng/g)
<LOD n (%)	62 (68.8)	66 (73.3)	60 (66.6)	67 (74.4)	73 (81.1)	75 (83.3)
$\bar{X}_1 \pm Sd$	3.69 ± 23.59	0.64 ± 1.87	1.24 ± 5.89	0.41 ± 0.92	0.36 ± 1.18	0.36 ± 1.49
$\bar{X}_2 \pm Sd$	11.87 ± 41.64	2.14 ± 2.93	4.14 ± 10.24	1.62 ± 1.16	1.19 ± 2.16	2.18 ± 3.13
Range	0.24 - 220.8	0.18 - 14.5	0.17 - 52.5	0.14 - 3.90	0.1 - 7.02	0.25 - 9.94
CV %	6.39	2.92	4.72	2.24	3.27	4.14

N-total number of analyzed samples, n- number of samples <LOD (limit of detection, see Materials and methods), \bar{X}_1 , arithmetic mean (conc. below LOD are regarded as zero) in all of analyzed samples, \bar{X}_2 -arithmetic mean in samples where OTA was detected, C.V.-coeff. of variation (x100).

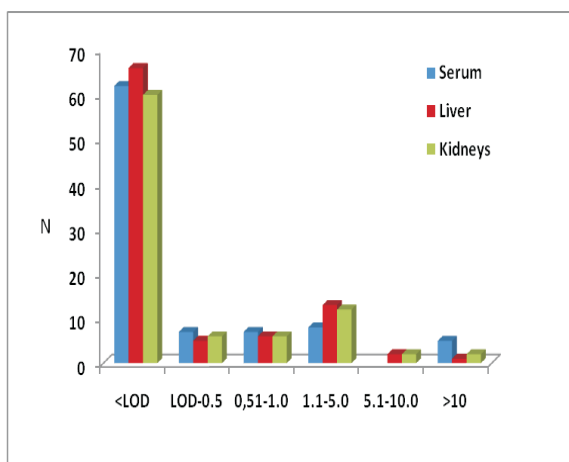


Figure 1. Distribution of OTA content in tissues of slaughtered pigs (ng/mL, ng/g), N-number of samples, LOD (limit of detection, see Materials and methods)

Measurements were performed by fluorescence detection at wavelengths of 334 nm (excitation) and 460 nm (emission), detector gain was set to 10. For more accuracy, 40 μ L of the serum samples, was re-injected in the case of the samples with an amount of OTA near the detection limit.

Statistical analysis: Descriptive data are presented as means, standard deviation (SD), and range, for continuous variables. Statistical differences in the mean levels of OTA contamination across the three groups of positive samples were determined by one-way ANOVA ($p < 0.05$). Significance was set at $p < 0.05$.

Results

A total of 540 tissues samples were analysed, 270 samples per pigs and 270 chickens, for the presence of OTA. The incidence of contamination, on serum, liver, kidneys and gizzard, by OTA is summarized in Table 1, Figure 1 and 2. Of the 90 liver, kidneys and gizzard samples, respectively, originating

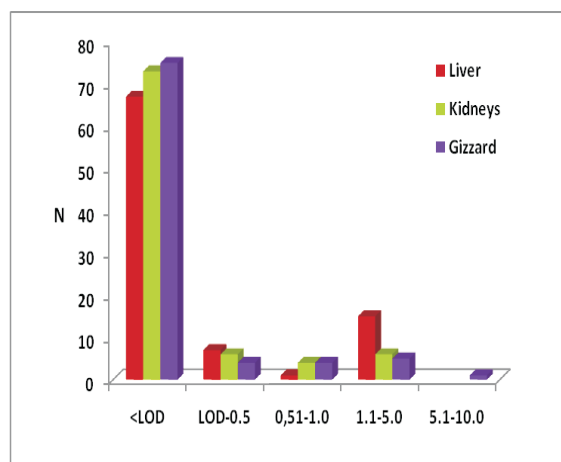


Figure 2. Distribution of OTA content in tissues of slaughtered chicken (ng/g), N-number of samples, LOD (limit of detection, see Materials and methods)

from chicken farms located in the different area of Serbia, OTA was reported in 23 (25.6%), 17 (18.9%) and 15 (16.7%) samples, respectively. The data in Table 1 show that majority (215) of these samples did not contain ochratoxin A and the rest largely had concentrations close to the detection limit. OTA levels in liver were slightly higher than those in kidneys and gizzard, but this difference was not statistically significant ($p < 0.05$).

The average level of OTA in liver was 0.41 ± 0.92 ng/g (range 0.14-3.9 ng/g), whereas OTA average value in kidneys and gizzard were 0.36 ± 1.18 ng/g (range 0.1 to 7.02 ng/g), and 0.36 ± 1.49 ng/g (range 0.25-9.94 ng/g) respectively. The high values of the standard deviation is due to two samples in kidneys, and one in gizzard that showed a concentration of OTA up to 7.02 ng/g and 9.5 ng/g, respectively (Fig. 2). All the analyzed tissues samples were below the limit established by JECFA (10 ng/g) [14]. Only one sample of gizzard contained OTA (9.94 ng/g), close to the maximum permissible levels of this toxin proposed by

the JECFA.

In the case of the OTA content in pigs tissues, OTA was detected in most frequently (33,4%) in kidney samples, whereas OTA was present in 31.2% and 26.7% of serum and liver samples, respectively. With regards to the concentrations of OTA, the highest level (220.8 ng/mL), was detected in serum samples. One samples of kidneys had 52.5 ng/g OTA, while maximum level of OTA in liver was 14.5 ng/g (Fig. 1). In 2.2% samples of kidneys, OTA levels was considerably higher and greatly exceed the permissible levels of this toxins established in Serbia and included those proposed (10 ng/g) by the Scientific Committee on Food [17], and JECFA [14].

Discussion

The exposure assessment was performed by a point-estimate deterministic approach, which is an evaluation built by combining concentration single levels, or mean contamination values, of analytes with food consumption data and divided by the body weight. In this study the Tolerable Daily Intake (TDI), calculated as nanograms of ochratoxin A per kilogram of body weight per week (ng/kg bw/daily), was evaluated using the equation; $CM \times P/M$.

CM is the concentration of mycotoxin in tissues (ng/g), P is the meat consumption (g/day), M is the mass of "average consumer" (60 kg body weight) [18].

Results found in this study suggest that, in general, OTA contamination in chicken meat originating from different part of Serbia is low, hence for the consumer the contribution to the total intake of ochratoxin A from chicken products is very small compared to other sources (0.28 ng/kg b.w. to 0.32 ng/kg b.w). Of 270 chicken tissues samples tested, measurable amounts of OTA were found in 55 (20.3%) tissues samples, whereas incidence of OTA in pigs tissues were higher, which accounts for 30.3% of all samples. Comparison the data obtained in this trial with other recently published data for the occurrence of OTA in pig edible tissues shows that the found levels are comparable with levels in other European countries, therefore the estimated daily intake of OTA through pork meat by a 60 kg adult is 0.46 ng/kg b.w. to 0.90 ng/kg b.w. respectively.

Currently, Ochratoxin A is receiving increasing attention for its toxic effects on human health and high incidence in a wide range of food commodities. Although the ochratoxin A amounts detected in meat from Serbia were relatively low, the levels may accumulate in the body of humans consuming contaminated food. Ochratoxin A is often not rapidly removed from the

body and it is frequently found in human blood due to its long elimination half-life (about 35 days in serum), as a consequence of its binding to plasma proteins, its enterohepatic circulation and its reabsorption from urine [19]. This makes of ochratoxin A the most detected mycotoxin in human blood all over the world [20]. Therefore, public health authorities in Serbia pay attention to ochratoxin A particularly by monitoring food and feedstuffs. At present, Serbia has not a maximum tolerable limits for ochratoxin A in meat and meat products, particularly pork meat. However, there is a tendency of authorities in Serbia to adopt the European Union limits for this mycotoxin.

Conclusion

The study provides the necessary knowledge information for updating the level of OTA exposure for the target products. Despite the limited number of samples examined in the present study, it is important to notice that even the low levels of OTA concentration found in tissues of slaughtered animals, may contribute to the OTA daily intake. On the basis of the risk assessment carried out in this study, the exposure to OTA, due to the consumption of pork and chicken products, is to be considered not a major concern. Therefore, it could be concluded that data arising from this study, adequate to align national legislation with the EU provisions. However, it should be remembered when align that factors such as climate conditions during harvest, practices for grain/feed storage etc have influence on the ochratoxin A level in edible tissues. Additionally, the study emphasized the role of management practices in the prevention of mycotoxin prevalence in animals farms. To protect consumer health and to reduce economic losses, surveillance and control of mycotoxins in food and feed has become a major objective for producers, regulatory authorities and researchers worldwide.

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Competing interest

Authors declare that they have no Competing interest.

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