



Residue of ochratoxin A in swine tissues – risk assessment

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SUMMARY

Background: Samples of blood, kidney, and liver per animal were randomly selected from slaughtered pigs ($n=60$) and analyzed for ochratoxin A.

Methods: Determination of ochratoxin A concentration in samples of kidney and liver was performed by high-performance thin-layer chromatography after immunoaffinity column clean up, while for plasma samples, a spectrofluorometric procedure was used.

Results: Of the 60 plasma samples, 60% contained ochratoxin A in the range of 2.5–33.3 ng/mL (mean 3.05 ± 5.0 ng/mL), while the incidence of ochratoxin A in kidneys and liver were very similar (70% and 65%). The average ochratoxin A concentration in liver was 3.2 ± 4.35 ng/g (1.2–19.5 ng/g) and in kidneys was 3.97 ± 4.47 ng/g (1.3–22.0 ng/g). A statistically significant difference ($p < 0.01$) was found between region Bačka Topola and Kovilj for both liver and kidney samples. In kidney samples originating from region Kovilj and Senta, a statistically significant difference ($p < 0.01$) was found. Mean distribution followed the pattern: kidney > liver > serum (100 > 80.8 > 77%). The results from this survey indicated that there was a strong correlation between the ochratoxin A level in serum and liver as well as in the ochratoxin A serum in kidney ($r=0.884$ and $r=0.896$, respectively) while the strongest correlation was found between the ochratoxin A level in liver and in kidney ($r=0.970$).

Conclusion: The results of present study show that pork tissues as well as pork products are considered an important source of ochratoxin A in humans.

Key words: Ochratoxins; *Aspergillus ochraceus*; Tissues; Swine; Mycotoxins

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INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by fungi such as *Penicillium verrucosum* and *Aspergillus ochraceus* (1). This toxin has been detected in many countries around the world (2), predominantly found to occur in cereal and cereal products, as a result of poor agricultural practice during the drying of the product or poor storage (3). It was recognized that animal feed can be contaminated with ochratoxin A and can subsequently be transferred to animal products for human consumption such as pigs' kidneys and blood sausages (4,5). In fact, animal-derived products and tissues for human consumption, especially those that include blood and kidney (6), may well present OTA residues even if the animal has been nourished with feeds contaminated with low levels of OTA. It is a potent renal carcinogen in rodents, causes mycotoxicosis in animals, and mycotoxin plays a special role in the genesis of swine mycotoxic nephropathy, a common disease in Scandinavia (4,7). Its implication in an irreversible and fatal kidney disease in certain countries referred to as *Balkan endemic nephropathy* (8), raise great concern for human health. Animal studies have shown the toxin to be a potent nephrotoxin, immunosuppressant and teratogenic (9,10), and has been classified by the IARC (1993) (11) as group 2B at the account of its carcinogenicity. Surveys of pigs for OTA in blood and/or edible tissues have been carried out in several European countries (12-15). There are not enough data from Serbia. The purpose of this work was to monitor the presence of OTA in Serbian slaughtered swine, to investigate the regional distribution of OTA and to determine the distribution of OTA in edible tissues.

METHODS

Sample collection

During a three-month period of investigation, slaughtered pigs from each area were randomly sampled in the slaughterhouses line during meat inspection.

Samples of kidney, blood serum, and liver ($n=60$) from 659 slaughtered pigs without any sign of macroscopic changes of the kidneys were randomly collected. About 50 ml blood/pig was sampled when slaughtered pigs were bled by jugular puncture. Blood samples remained at room temperature for 10-12 h to allow clotting to occur, and then were centrifuged at 3000 g for 15 min. Serum was decanted and stored at -18 °C prior to analysis. About 70-100 g of liver and one whole kidney were sampled from each pig. The whole sample was homogenized and stored at -18 °C before analysis. No preservatives were added.

Standard solutions

Stock standard solutions of OTA (approximately 40 µg/ml) for TLC were prepared by dissolving the solid standard (Sigma Chemical Co., St Louis, MO, USA) in toluene/acetic acid (99:1 v/v) (16). A standard solution was prepared by diluting appropriate aliquots of standard solution with toluene/acetic acid (99:1 v/v). The solutions were stored below -18 °C and protected from light. The concentration was measured according to AOAC International (17).

A standard solution of ochratoxin A for spectrofluorimetric analysis was prepared from stock solution (10^{-4} M ochratoxin A) in buffer, which was stored frozen and protected from light. The concentration of the stock solution was determined spectrophotometrically at 380 nm, using the value of $5.680 \text{ M}^{-1} \text{ cm}^{-1}$ for the extinction coefficient.

Carboxypeptidase A. Carboxypeptidase A was obtained from Sigma Chemical Co. St. Louis, Mo. Carboxypeptidase A was prepared in 0.04 M tris(hydroxymethyl) aminomethane sulphuric acid buffer, pH 7.5, 1M sodium chloride (100 U/ml).

Extraction and clean-up for ochratoxin analyses

Serum. The spectrofluorometric procedure for ochratoxin A analysis (Hult et al. 1980) (18) has been applied. To a 50 ml nylon centrifuge tube, 2.5 ml

of plasma, 5.0 ml of chloroform, and 10 ml of a solution containing 0.05 M HCl and 0.1 M MgCl₂ were added for plasma. Extraction of ochratoxin A was carried out during 10 min in a tube-turning apparatus rotating 20 turns per min. The mixture was centrifuged for 10 min at 10000 g. From the bottom of the tube, 3.0 ml of the ochratoxin A, containing chloroform was transferred with a syringe into a 15 ml glass centrifuge tube. The chloroform was washed once with 1.5 ml of water on a tube shaker. The phases were separated by centrifugation with a table centrifuge A 3.0 ml portion of 0.04 M tris(hydroxymethyl) amino methane buffer, adjusted with sulphuric acid to pH 7.5 (20 °C), was added to the chloroform solution, and ochratoxin A was extracted into the buffer by using a tube shaker. The mixture was centrifuged to obtain a clear buffer solution. A 2.0 ml fraction of the buffer extract was transferred to a round borosilicate glass cuvette. A 100-td solution of carboxypeptidase A was added (100 U/ml in 0.04 M tris(hydroxymethyl) amino methane sulphuric acid buffer, pH 7.5, 1M sodium chloride). The sample was cooled in an ice bath for 10 min. The fluorescence excitation spectrum was recorded from 320 to 400 nm, at 450 nm emission. The sample was incubated at 37 °C for 2 h and cooled in an ice bath for 10 min. The fluorescence spectrum was recorded again. The loss of fluorescence at 380 nm was proportional to the concentration of ochratoxin A.

Kidney and liver. The detection of OTA in all samples was performed by high-performance thin-layer chromatography (HPTLC), following the methodology proposed by Jorgensen (2002) (6). A 25-g sample was extracted with 100 ml ethyl acetate and 10 ml 0.5 M phosphoric acid in 2.0 ml sodium chloride by blending in an Ultra-Turrax for 3 min, with a 10 min pause and again for 2 min. The extract was filtered through paper filter and evaporated to dryness. The residue was dissolved in 2 ml methanol, and 30 ml phosphate buffer (pH 7.3) was added. The clean up of OTA in the samples was carried out using standard, commercially available immunoaffinity columns (RIDA Ochratoxin A column, R-Biopharm, Darmstadt, Germany). This solution was centrifuged and passed through the immunoaffinity column. The column was washed with 20 ml water, dried by pressing air through the column, and the ochratoxin A was eluted by 5 ml methanol-acetic acid (98:2). This solution was evaporated under a nitrogen stream and the residue was redissolved in 200 µl HPTLC mobile phase (toluene/acetic acid (99:1 v/v) ready for analysis. Validations of the methods were performed in accordance with the protocol approved by the Association Official of Analytical Chemists (AOAC) (17).

Thin layer chromatography

The purified extracts were analyzed by instrumental high-performance thin-layer chromatography (HPTLC) on the Camag equipment, Switzerland. The 20 cm x10 cm silica gel 60 HPTLC plates (Merck) were used for separation with various mobile phases. One-dimensional TLC was sufficient for the separation and quantification of determined mycotoxins. The residues (20 ml) were spotted (LINOMAT IV), in triplicate, on a commercially precoated silica gel 60 glass plate. The glass plates were developed with toluene/ethyl acetate-88% formic acid (6:3:1 v/v/v) and let dry in a fume hood for 5 min. The TLC plates were examined visually under UV light at 365 nm (CAMAG UV Cabinet, 366/254 nm), the fluorescence intensity of standards

and samples were compared. The OTA concentration in the samples was calculated by comparing the area of chromatographic peak of the samples with that of the standard calibration curve by densitometry analysis (TLC SCANNER, λ-324 nm, filter 2, photo mode: fluorescence). The optimum spectral settings for densitometry were determined in the range 310-340 nm (17). In case the area of the samples was not within in the range of the calibration curve, the sample extract was quantitatively diluted and resotted. The confirmation of OTA was carried out by spraying the plates with sodium bicarbonate according to AOAC International (17). For every five regions of tissues analyzed, OTA free kidney and liver spiked with OTA at levels of 0.1, 1.0, 2.0 and 35 mg/l, extracted and analyzed sequentially. The detection limit for OTA in organs was 0.5 ng/g with an 83% mean recovery. All results were corrected for recovery by use of the actual recovery in the analytical series. For organs, the calculated limit of detection (LOD) (s/n 3/1) was 0.5 ng/g and limit of quantitation (LOQ) (s/n 7/1) was 1.0 ng/g. The linear equation of the standard calibration curve by densitometric analysis gave $R^2 = 0.978$

Apparatus

A Perkin Elmer LS-5 (USA) spectrofluorometer with scanning attachment was used. The spectrofluorometer was equipped with a polarizer in the emission pathway, which is necessary when measuring low amounts of ochratoxin. The fluorescence excitation spectrum was recorded from 320 to 400 nm, at 450-nm emission. The detection limit for OTA in plasma was 2 ng/mL with a 78% mean recovery. The linearity of the method from 2 to 200 ng/mL had a correlation coefficient of 0.988. Above 200 ng/mL, the method was not linear, and the recovery of ochratoxin A from plasma decreased to 68%.

Statistical analysis

Differences in the mean levels of OTA contamination across the four groups of positive samples were calculated by analysis of variance and then by a Student's t-test. Additional posttests were applied to evaluate differences between groups with statistically significant variation among means. The differences with p values, smaller than 0.05, were considered statistically significant.

RESULTS

The occurrence and distribution of ochratoxin A content into concentration ranges in tissues of slaughtered pigs in the region where samples were collected are summarized in Figure 1 and 2.

Serum. The results of this study show that 36 (60%) serum samples, out of 60 were found positive with toxin at levels ranged from 2.5-33.3 ng/mL (mean 3.05 ± 5.0 ng/mL). Out of the positive samples, the highest incidence (73.3%), and level were found in the samples originating from region Kovilj (mean 5.31 ng/mL, max 33.3 ng/mL), while the lowest incidence (46.6%) and level of OTA residue were found in the samples originating from region Šabac (mean 1.56 ng/mL, max 5.0 ng/mL). Figure 2 presents the results by the number of sample falling into specified concentration ranges. The majority of contaminated samples (48.3%) contained OTA between 2 and 5 ng/mL, while concentration in five (8.3%) were between 5 and 10 ng/mL. In only two (3.3%) samples, OTA concentration was greater than 10 ng/mL (max. 33.3 ng/mL). The results of this study show that the mean level of OTA throughout

the region where samples were collected is very similar, but the incidence of OTA is different (Figure 1).

Kidney. The results of this study reveal that the kidney was the most contaminated tissue. OTA was found in 70% of all samples in the range 1.3-22.0 ng/g (3.97 ± 4.47 ng/g). As in serum samples, the majority of contaminated samples (40%) contained OTA up to 5 ng/g, while in total 12 (20%) samples exceeded this concentration. In 6 (10%) samples of kidneys, OTA level was considerably higher and greatly exceeded the permissible levels of this toxin established in Serbia including those proposed by the European Commission (10 ng/g).

The highest concentrations of OTA were similar in kidney and liver (10% and 8.3% respectively) in contrast to serum (3.3%). With regard to regional distribution of OTA, the occurrence of OTA in these three regions where samples were collected is different and varied between 46.6% (region Senta) to 94% (region Kovilj) (Figure 1). Also, the mean level of contamination was very different and varied between 1.97 ng/g (region Senta) to 6.52 ng/g (region Kovilj).

Liver. OTA had almost the same incidence (65%) and mean value in liver as in kidneys. The incidence of OTA was in the range 1.2-19.5 ng/g (mean 3.2 ± 4.35 ng/g). Out of the positive samples, the same incidence was found in the range at 5-10 ng/g and >10 ng/g (8.3%), whereas about 48.3% showed concentration in the range at 0.5-5 ng/g (Figure 2). With regard to regional distribution of OTA, the occurrence of OTA throughout the region where samples were collected is different (46.6%, region Senta to 93%, region Kovilj) (Figure 1). The highest mean OTA level (mean 4.53 ng/g) and the highest OTA level (19.5 ng/g) was found in the samples originating from Šabac.

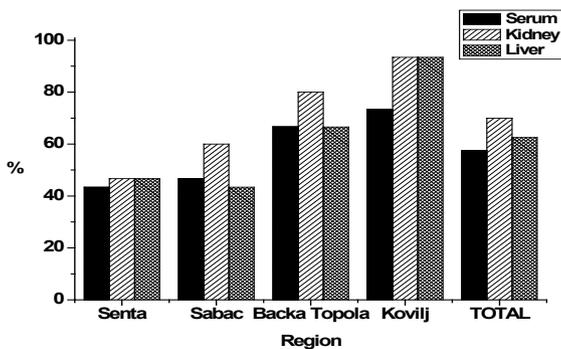


Figure 1. Occurrence of ochratoxin A in slaughtered pigs (n=60)

A statistically significant difference ($p < 0.01$) was found between region Bačka Topola and Kovilj for both liver and kidney samples. Also, in kidney originating from region Kovilj and Senta a statistically significant difference ($p < 0.01$) was found. The mean distribution followed the pattern kidney>liver>serum ($100 > 80.8 > 77\%$). The correlation between the OTA level in serum and in edible tissues is very different. The results from this survey indicated that there was a strong correlation between the OTA level in serum and in liver as well as in the OTA serum in kidney ($r = 0.884$ and $r = 0.896$, respectively) while the strongest correlation was found between the OTA level in liver and in kidney ($r = 0.970$). A similar correlation was found by Curtui et al. (2001) (19).

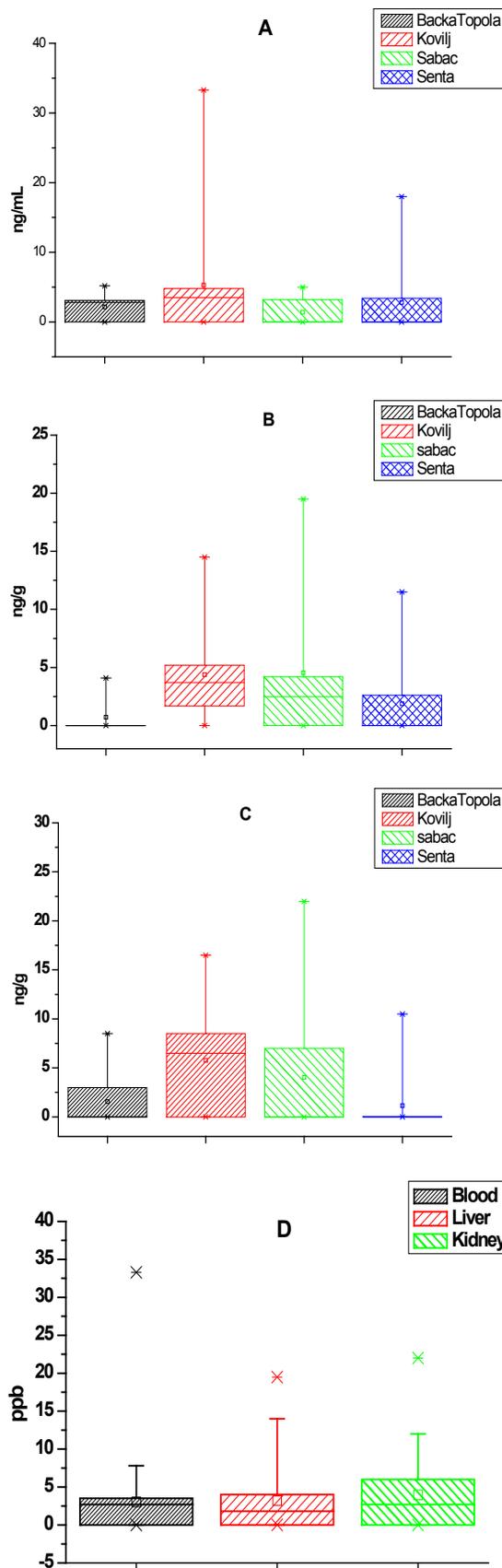


Figure 2. Distribution of ochratoxin A content into concentration ranges in tissues of slaughtered pigs in the region where samples were collected (A – blood, B – liver, C – kidney, D – Total).

DISCUSSION

The results of this study demonstrate the presence of OTA in tissues of Serbian slaughtered pigs with an incidence and a mean level of contamination comparable to other European countries (12-15, 19-21). A comparison with other published data for the occurrence of OTA in tissues of slaughtered swine and contamination level was generally not different from other European countries such as Sweden, Poland, and Germany, in areas of Balkan Peninsula or Canada. The present work indicates that regional differences and seasonal variations were observed. The regional differences and seasonal variations might thus explain the concentration differences in corresponding formulas partly, as could differences in the storage condition of feedstuff. Since the high OTA concentration is found in blood serum, it can be suggested that the serum is the most appropriate sample for the control of OTA in slaughtered pigs as well as in pig herds. The fact that ochratoxin A is heterogeneously distributed in a contaminated lot of feed material, makes the sampling problematic. It has been shown that an alternative method to monitor the ochratoxin A contamination in the feed is to analyze blood samples from pigs, which reflect the toxin content of the ingested feed. The pig can be looked upon as an *in vivo* sample collector, which forms an average, homogenous blood samples reflecting the ochratoxin A content in its feed. The amount of OTA in the feed given to pigs can be calculated from the values of the OTA concentrations in the pigs' blood. Hult *et al.* (1980) (18) have reported that the concentration in pig blood plasma (ng/ml) is 1.5-fold higher than in feed (ng/g). Later, other authors have found that the amounts of OTA in pig blood serum were 1.55 and 1.65 respectively, lower than the amounts in feed. Renal proteins have also been demonstrated to have a strong affinity for OTA. However, human renal proteins consistently displayed a 2–20-fold higher OTA binding capacity than any of the other species tested. The generated binding-capacity ranking of human>rat>pig>mouse correlates well with the biological half-lives determined by other authors (human>rat>pig>mouse) and with the toxicity ranking for experimental animals *in vivo* (22). Disappearance of OTA from blood was slower than from kidney, liver, and other tissues in pigs. Serum half-lives after oral administration of OTA were 72–120 h in pigs.

The higher incidence and concentration in kidney than other analyzed tissues is due to the fact that the kidney is the main target of OTA, although it has been shown that possible targets of OTA are the liver, the immune system, and the brain cells (23-26). This high susceptibility of the kidney is, at least in part, the result of OTA-toxicokinetics (27,28). Renal blood flow per tissue weight is extremely high, resulting in the delivery of relatively large amounts of OTA as compared to other organs. Furthermore, free OTA is secreted in the proximal tubule and subsequently reabsorbed, mainly in the proximal straight tubule, the thick ascending limb of the loop of Henle and the collecting duct (29,30). Mechanisms involved in reabsorption are, e.g., H⁺-dipeptide cotransporter(s) and nonionic diffusion (30,31). These toxicokinetic features result in an accumulation of OTA in renal tissue, where the highest concentrations have been detected in the papilla and the inner medulla (30). Whichever is the case, cellular accumulation probably plays an important role in OTA-mediated cytotoxicity. O'Brien and colleagues (2001) (32) demonstrated that primary renal epithelial cells of human and porcine origin rapidly accumulate 10–15-fold more ³H-OTA than their continuous cell line counterparts. O'Brien and co-workers (2001) (32) and Heussner and colleagues (2000) (33) investigated the cytotoxic effects of OTA in primary human, porcine and rat

renal epithelial cells as well as continuous epithelial cell lines from rat (NRK-52E) and pig (LLC-PK1). In these studies, primary cells of human origin were reported to be the most sensitive cell type to the antiproliferative/cytotoxic effects of OTA.

The inhibition of protein synthesis and the damaged energy production in the mitochondria could be considered as the most important factors for degenerative changes in the epithelial cells of proximal tubules where ochratoxin A was detected. The results of the present study show that pork tissues as well pork products are frequently contaminated with OTA. Therefore, pork products, especially those that include blood and kidney, are considered an important source of OTA in humans (6).

CONCLUSION

Considering the similarities with the occurrence of OTA in edible tissues reported in other countries, it seems that these findings in Serbian slaughtered pigs do not represent a particular situation. The content of the OTA analyzed was very variable in relation to the region where samples were collected and the different tissues examined. The risk assessment for OTA is based on exposure in adults and the continuous accumulation of OTA in the kidneys leading to tubular dysfunction. However, rodent bioassays do not and cannot provide data relevant to the generation of the nephropathy/progressive renal fibrosis assumed to be associated with chronic exposure to ochratoxin A in humans and pigs. Furthermore, profound differences in OTA distribution and elimination as well as in OTA sensitivity, between rodent and humans, suggest that the assessment or safety factor approach currently proposed by the IPCS (IPCS/WHO 2005) (34) may not suffice for the development of a reliable exposure dose-response model and consequently for a tolerable daily intake. However, the actual concentration in pork tissues was generally low compared with other sources and may not constitute a health hazard for consumers because the values are below the recommended daily intake of these toxins (35,36). Thus, it is important to keep the OTA levels in products aimed for human consumption as low as possible.

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Conflict of interest

We declare no conflicts of interest.

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