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Ochratoxin A detection by HPLC in target tissues of swine and cytological and histological analysis

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Abstract

Ochratoxins are fungal secondary metabolites that may contaminate a broad variety of foodstuff, such as grains, vegetables, coffee, dried fruits, beer, wine and meats. Ochratoxin A (OTA) is a potent nephrotoxin, carcinogen, teratogen and immunotoxin. Samples of kidney, urinary bladder, intestine, stomach, liver, lymph nodes and muscles were obtained from 5 swine fed with OTA-contaminated feed. In the 5 swine, microscopical lesions were evidenced exclusively in the kidneys and in the urinary bladder, that displayed the highest concentrations of OTA by HPLC-FLD analysis, 23.9–27.5 μg/kg and 9.8–11.5 μg/kg, respectively.
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Keywords: Ochratoxin A; Kidney; Urinary bladder; HPLC

1. Introduction

Ochratoxins are fungal secondary metabolites that contaminate grains, legumes, coffee, dried fruits, beer and wine, and meats (Frisvad, 1995; Romani, Sacchetti, Chaves López, Pinnavaia, & Dalla Rosa, 2000). Ochratoxins are to be considered potent nephrotoxins, carcinogens, teratogens, and immunotoxins in rats and likely also in humans.

Ochratoxins may be produced by members of two genera of fungi: the Aspergillus species, *A. ochraceus* (Kuiper-Goodman & Scott, 1989), *A. melleus*, *A. auricomus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum* and *A. sulfureus*, all of which are classified in the section *Circumdati* (also called the *A. ochraceus* group); *A. alliaceus* and *A. albertensis*, formerly placed in the section *Circumdati* and recently reclassified in the section *Flavi* (Peterson, 2000); *A. niger* and *A. carbonarius* (in the section *Nigri*); *A. glaucus* (or *Eurotium herbariorum* section *Aspergillus*); *Penicillium verrucosum* (Abarca, Bragulat, Sastellá, & Cabañes, 1994; Ciegler, 1972; Heenan, Shaw, & Pitt, 1998; Peterson, 2000; Téren, Varga, Hamari, Rinyu, & Kevei, 1996; Varga, Kevei, Rinyu, Téren, & Kozakiewicz, 1996).

The presence of ochratoxins in food has been reported in several countries, worldwide.

Mycotoxic porcine nephropathy (MPN) has been comprehensively reviewed by Krogh (1976). The toxic effects by ochratoxin A (OTA) are regarded as the possible cause of this nephropathy (Krogh, Axelsen, & Elling, 1974; Szczech, Carlton, & Tuite, 1973).

OTA is highly nephrotoxic and may cause both acute and chronic lesions of kidneys. Accordingly, OTA has been suspected to be involved in the aetiology of Balkan endemic nephropathy (BEN), a human disease characterized by progressive renal fibrosis and by tumors of the urinary tract (IARC, 1993; Krogh, 1980; Marquardt & Frohlich, 1992; Pfohl-Leszkowicz, Petkova-Bocharova, Chernozemsky, & Castegnaro, 2002), such as carcinoma of the renal pelvis, ureters and bladder (Radavanovic, Jankovic, & Jeremovic, 1991).

Similarly, neoplasia (fibroma, adenoma and fibroadenoma) in kidney have been reported in the Bulgarian cases of MPN (Stoev, Hald, & Mantle, 1998a, 1998b, 2002).

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OTA is a moderately stable molecule that remains unaltered during most processes of food transformation. In addition, OTA may undergo bio-concentration in some animal tissues/organs and reach concentrations in meat products that are not acceptable for human consumption (Jorgensen, 1998).

In 2005, during a pilot programme to survey the presence of mycotoxins in animal feed, OTA was detected in the feed given to large white pigs in a herd from the Apulia region, southern Italy.

The aim of this study was to investigate the presence of OTA throughout the chain production of the pig meat. OTA was detected in animal feed and in the tissues of the animals and OTA-related lesions were reported in slaughtered pigs from the herds.

2. Materials and methods

2.1. Samples

A total of 20 samples of swine feed were obtained from 10 herds in the Apulia region and analysed to estimate the presence of OTA. Five 10-month-old large white swine, that were approximately of the same body weight (95 kg) were selected from two herds, where the highest amount of OTA was detected. The content of OTA was estimated in various tissues and organs (kidney, urinary bladder, intestine, spleen, liver, lymph nodes and muscles).

2.2. Analytical reagents

Supelco Ochratoxin A (product no. 46912), packaged in sealed ampules at a concentration of about 50 ng/μl in benzene:acetic acid (99:1) was used as a standard. This standard was prepared according to the AOAC official methods. The standards for the OchraTest by HPLC were prepared as follows:

(a) Ochratoxin-working solution 1: dilution 1:50 of Supelco ochratoxin A standard (50 ng/μl).
(b) Ochratoxin-working solution 2: prepared by diluting Ochratoxin-working solution 1 (1 ng/μl) 10 times.

The calibration curve was obtained by diluting Ochratoxin-working solution 2 with methanol in order to obtain the following concentrations: 1.0, 10.0, 20.0 and 50.0 (ng/g).

1.5 ml water were added to all our standards and samples before injecting onto the HPLC to make the solvent for the standards and samples similar to the mobile phase.

2.3. Apparatus and chromatographic conditions

For liquid chromatography analysis, an Agilent 1100 Series instrument equipped with pumps, a Rheodyne Model 7125 injector (100 μl loop) and a fluorescence detector was used. A LC Restek column C18 (5 μm) (250 mm × 4.6 mm internal diameter) was used with a mobile phase consisting of a mixture of water:acetonitrile:acetic acid (49.5:49.5:1 by volume), degassed at a flow rate of 0.9 ml/min. Detection of OTA was carried out using 333 and 477 nm as wavelengths for excitation and emission, respectively.

2.4. Immunoaffinity clean-up

To measure ochratoxin levels, samples were prepared by mixing with an extraction solution (see below), followed by blending and filtering. The extract was then applied to the OchraTest WB column (VICAM), which contained specific antibodies for ochratoxin A. Using monoclonal affinity chromatography, OchraTest is the only ochratoxin test that produces precise numerical results. At this stage, the ochratoxin binds to the antibody on the column. The column is then washed to rid the immunoaffinity column of impurities. By passing methanol through the column, the ochratoxin is removed from the antibody. The methanol can then be injected into an HPLC system.

2.5. Sample extraction and clean-up of animal feeds

A 50 g aliquot of animal feed was extracted with 100 ml of acetonitrile:water (60:40) for a few minutes. A 10 ml aliquot of the homogenate was filtered and diluted with 40 ml of PBS buffer (pH 7.0). The diluted extract was loaded onto an OchraTest WB column. After washing with 10 ml PBS Buffer and 10 ml of water, the mycotoxin was eluted with 1.5 ml of methanol. 1.5 ml water were added to all samples before injecting onto the HPLC to make the solvent for the standards and samples similar to the mobile phase.

2.6. Sample extraction and clean-up of tissues and organs

A 20 g aliquot of pig tissue was homogenised with 6 ml of 1 M phosphoric acid in a Ultra Turrax T25 homogeniser for a few minutes. A 2.5 g aliquot of the homogenate was transferred into a Pyrex centrifuge tube, extracted twice with 5 ml of ethyl acetate, and centrifuged for 5 min at ca. 350*g*. The organic phases were combined, reduced to approximately 3 ml, and back-extracted with 3 ml of 0.5 M $NaHCO_3$ (pH 8.4). The aqueous extract was loaded on to an OchraTest WB column. After washing with 10 ml of PBS Buffer and 10 ml of water, the mycotoxin was eluted with 1.5 ml of methanol. 1.5 ml water were added to all samples before injecting onto the HPLC to make the solvent for the standards and samples similar to the mobile phase (Monaci, Tantillo, & Palmisano, 2004).

2.7. Samples for cytological and histological test

At the slaughterhouse, slaughtering of the five selected swine was followed and the gross lesions were recorded. Samples of kidney, urinary bladder, intestine, stomach, liver, lymph nodes and muscles were obtained. For cyto-

logical examination, samples were collected by aspiration with a needle.

Slides for cytological analysis were prepared at the slaughterhouse, fixed in methanol for 15 min and stained by the May Grunwald Giemsa method.

For histological examination, blocks of tissue were collected and fixed in 10% neutral buffered formalin. The samples were embedded in paraffin wax, sectioned at 4 μm and stained by Haematoxylin Eosin.

3. Results and discussion

OTA was detected in animal feed samples and ranged between 149 and 327 μg/kg dry weight. The limit of detection and quantization of OTA in the samples of feed and tissues, by HPLC-FL were 0.10 μg/kg and 0.30 μg/kg, respectively. Recovery was 85 + 15%, as evaluated on spiked samples at the 1 μg/kg level and day-to-day RSD was 10%.

In all the samples, the levels of OTA were higher than allowed by European Legislation by Commission Decision (2000/77/CE; 2001/46/CE; 2001/102/CE; 2002/32/CE; 2003/57/CE), i.e. 50 μg/kg. The high amounts of OTA detected in the feed samples led us to consider the animals from the herds where the highest mycotoxin contaminations were detected. Kidneys, urinary bladder, intestine, spleen, liver, lymph nodes and muscles of these animals were analysed by the HPLC-FL method to assess the presence of OTA.

OTA was detected only in kidneys, urinary bladder and spleen. The highest levels were found in the kidneys of the five swine analysed (23.9–27.5 μg/kg, average 25.6 ± 1.56 μg/kg). The levels found in the urinary bladder and in the liver were two- and five-fold lower, respectively, than in kidneys (urinary bladder: 9.8–11.5 μg/kg, average 10.5 ± 0.6 μg/kg; liver: 3.2–5.3 μg/kg, average 4.4 ± 0.8 μg/kg). Finally, the lowest contents were found in the spleen (0.3–0.5 μg/kg, average 0.4 ± 0.1 μg/kg).

In the five animals included in our analysis, gross and microscopical lesions were evidenced exclusively in the kidneys and in the urinary bladder.

The gross lesions of kidneys showed the presence of cortical hyperemia of nephritic tissue and an enlargement of pelvis. It was possible to observe many red streaks arranged radially, that suggested an infiltration of the cortex (Fig. 1).

Cytological examination of kidneys showed, in four out of five subjects, the presence of plurinucleate cells, with hyperchromic nucleus and vacuolar degeneration of cytoplasm (Fig. 2). The major histo-pathological lesions in the kidneys were observed in the epithelium of the proximal tubules, with karyomegaly and granular degeneration, hypercellularity and thickening of the capillary walls of a glomerulus (Fig. 3). Glomerular sclerosis was also observed (Fig. 4).

The gross lesions of urinary bladder showed hyperemia of mucosa and thickening of the wall. (Figs. 5 and 6). The presence of turbid urine demonstrated possible damage of the kidney Cytological examination of the urinary bladder showed karyorexis, large nuclei and hyperchromic nucleoli, features that are usually regarded as precancerous changes (Fig. 7). The histopathological examination showed limited proliferation of the fibroblastic connective tissue and focal infiltration by mononuclear inflammatory cells (Fig. 8).

The findings of our study demonstrate that swine are highly susceptible to OTA, notably in the case of high feed contamination. Also, these findings demonstrate that innovation is necessary in *postmortem* visits to the slaughter-

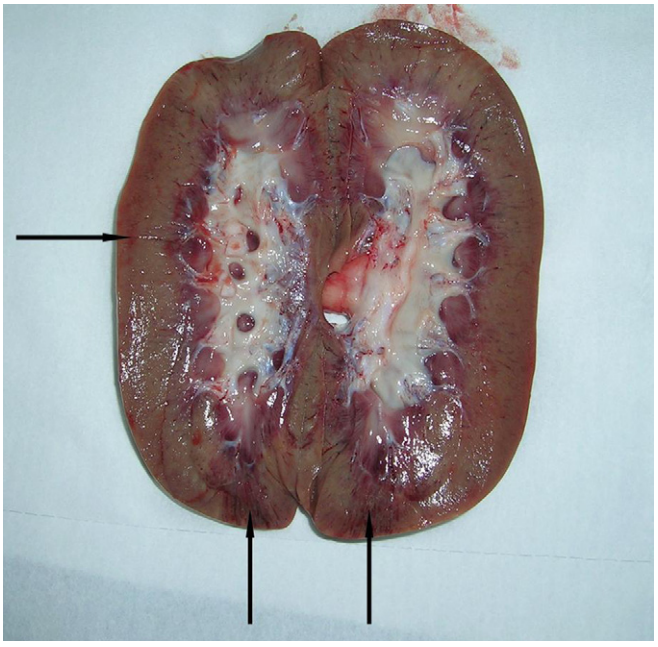

Fig. 1. Kidney. Many red streaks arranged radially, that suggest an infiltration of the cortex.

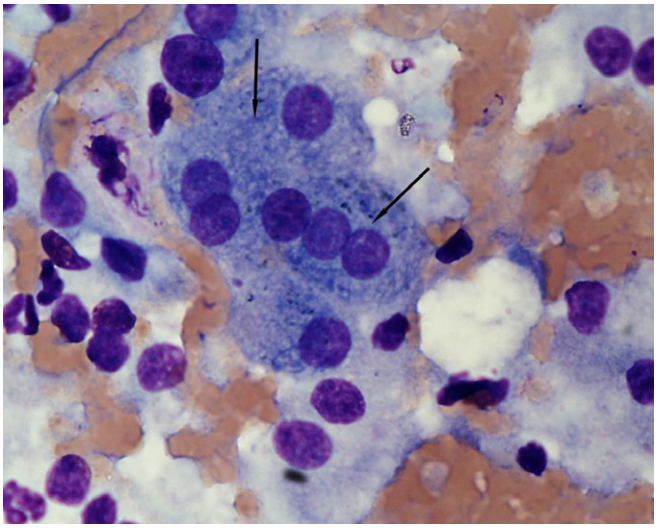

Fig. 2. Kidney. Presence of plurinucleate cells, with hyperchromic nucleus and vacuolar. M.G. (May Grunwald Giemsa) × 100.

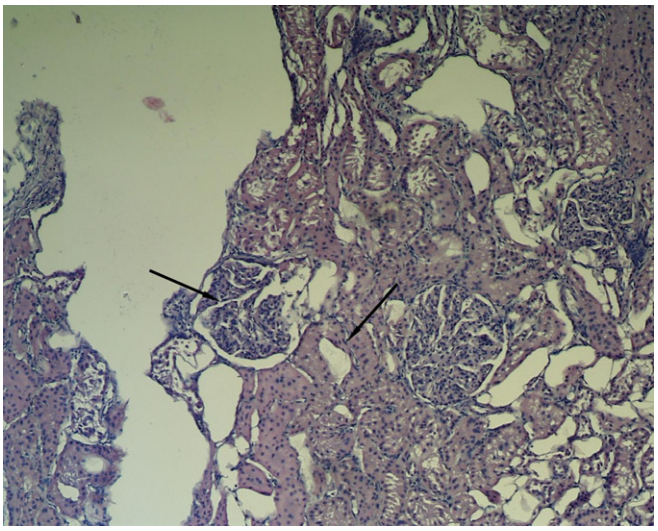

Fig. 3. Kidney. Epithelium of the proximal tubules, with karyomegaly and granular degeneration, hypercellularity and thickening of the capillary walls of a glomerulus. HE (Hematoxylin eosin) × 20.

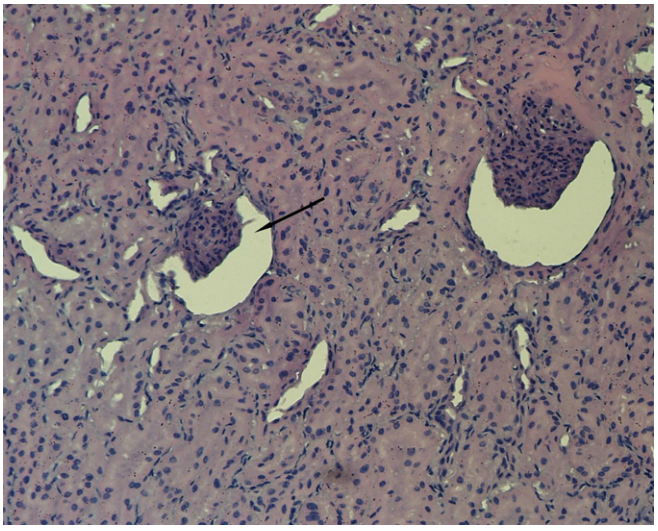

Fig. 4. Kidney. Glomerular sclerosis. HE (Hematoxylin eosin) × 40.

house, notably for the pathologies elicited by toxic substances.

Altogether, in agreement with the guidelines of the European community (Commission Regulation (EC) No. 178/2002), our study demonstrates that monitoring the quality of animal feed is a priority to decrease the possibility of "carry over" to humans. To guarantee the food safety, it is necessary to control all the steps of the food chain. All the controls must be integrated in the correct execution of the HACCP system in the farms, in order to actuate a preventive control of the hazards. Accordingly, along with the various risks factors throughout the process of transformation and distribution of food, it is important to consider the risks in the early phase of the food chain, i.e. the risks inherent in animal feeding.

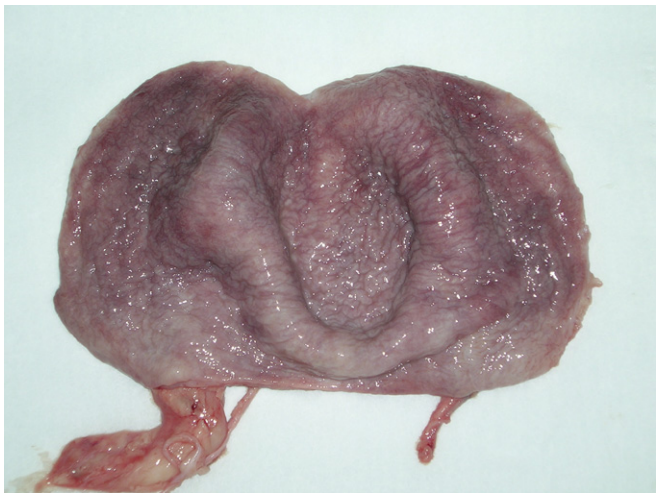

Fig. 5. Urinary bladder. Hyperemia of mucosa and thickening of the wall.

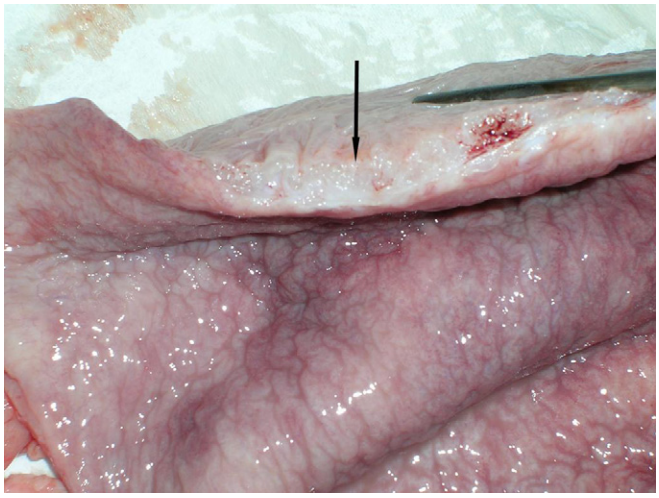

Fig. 6. Urinary bladder. Thickening of the wall.

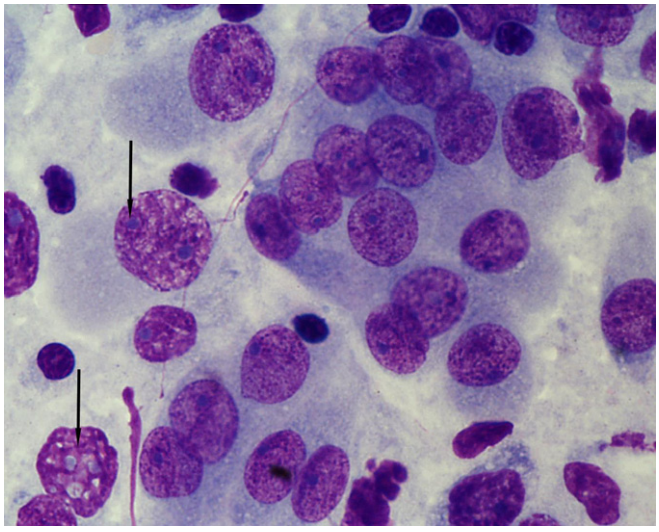

Fig. 7. Urinary bladder. Karyorexis, large nuclei and hyperchromic nucleoli. M.G. (May Grunwald Giemsa) × 100.

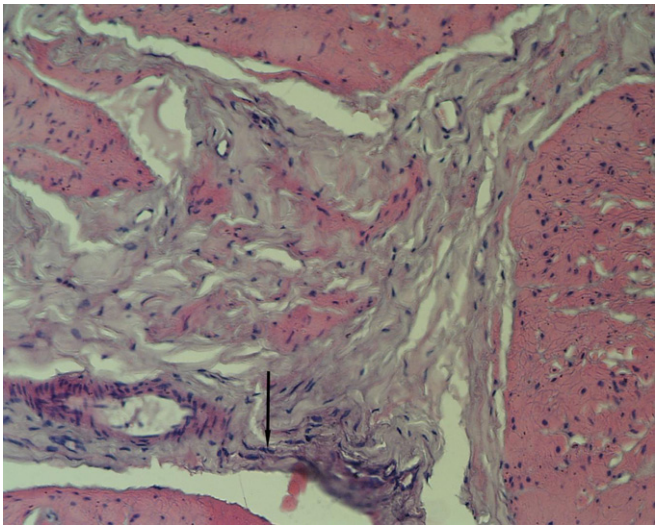

Fig. 8. Urinary bladder. Limited proliferation of the fibroblastic connective. HE (Hematoxylin eosin) × 40.

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References

Abarca, M. L., Bragulat, M. R., Sastellá, G., & Cabañes, F. J. (1994). Ochratoxin A production by strains of *Aspergillus niger var. niger*. *Applied and Environmental Microbiology, 60*, 2650–2652.

Ciegler, A. (1972). Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Canadian Journal of Microbiology, 18*, 631–636.

Commission Decision (EC) No. 77/2000; No. 46/2001; No. 102/2001; No. 32/2002; No. 57/2003.

Commission Regulation (EC) No. 178/2002 of 28 January (2002).

Frisvad, J. C. (1995). Mycotoxins and mycotoxigenic fungi in storage. In D. S. Jayas, N. D. White, & W. E. Muir (Eds.), *Stored grain ecosystems* (pp. 251–288). New York, NY: Marcel Dekker.

Heenan, C. N., Shaw, K. J., & Pitt, J. I. (1998). Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *Journal of Food Mycology, 1*, 67–72.

IARC (1993). Ochratoxin A. In: Some naturally occurring substances; food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the evolution of carcinogenic risks to humans, Vol. 56. Geneva: International Agency for Research on Cancer, pp. 489–521.

Jorgensen, K. (1998). Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. *Food Additives Contaminants, 15*, 550–554.

Krogh, P. (1976). Mycotoxic nephropathy. *Advances in veterinary science and comparative* (vol. 20, pp. 147–170). New York: Academic Press.

Krogh, P. (1980). Ochratoxins: occurrence, biological effects and casual role in disease. In E. Eaker & T. Wadstrom (Eds.), *Natural toxins* (pp. 673–680). Oxford: Pergamon Press.

Krogh, P., Axelsen, N., & Elling, F. (1974). Experimental porcine nephropathy: Changes of renal function and structure induced by ochratoxin A – contaminated feed. *Acta Pathologica et Microbiologica Scandinavica Section A, 246*(Suppl), 1–21.

Kuiper-Goodman, T., & Scott, P. M. (1989). Risk assessment of the mycotoxin ochratoxin A. *Biomedical and Environmental Sciences, 2*, 179–248.

Marquardt, R. R., & Frohlich, A. A. (1992). A review of recent advances in understanding ochratoxicosis. *Journal of Animal Science, 70*, 3968–3988.

Monaci, L., Tantillo, G., & Palmisano, F. (2004). Determination of ochratoxin A in pig tissues by liquid–liquid extraction and clean-up and high-performance liquid chromatography. *Analytical and Bioanalytical Chemists, 378*, 1777–1782.

Peterson, S. W. (2000). Phylogenetic relationships in Aspergillus based on rDNA sequence analysis. In R. A. Samson & J. I. Pitt (Eds.), *Classification of Penicillium and Aspergillus: Integration of modern taxonomic methods* (pp. 323–356). Reading, United Kingdom: Harwood Publishers.

Pfohl-Leszkowicz, A., Petkova-Bocharova, T., Chernozemsky, I. N., & Castegnaro, M. (2002). Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. *Food Additives Contaminants, 19*(3), 282–302.

Radavanovic, S., Jankovic, S., & Jeremovic, J. (1991). Incidence of tumours of urinary organs in focus of Balkan endemic nephropathy. *Kidney International, 40*(Suppl. 34), 75–77.

Romani, S., Sacchetti, G., Chaves López, C., Pinnavaia, G., & Dalla Rosa, M. (2000). Screening on the occurrence of ochratoxin A in green coffee beans of different origins and types. *Journal of Agricultural and Food Chemistry, 48*, 3616–3619.

Stoev, S. D., Grozeva, N., & Hald, B. (1998b). Ultrastructural and toxicological investigations in spontaneous cases of porcine nephropathy in Bulgaria. *Veterinarski Arhiv, 68*, 39–49.

Stoev, S. D., Hald, B., & Mantle, P. G. (1998a). Porcine nephropathy in Bulgaria: a progressive syndrome of complex of uncertain (mycotoxin) etiology. *Veterinary Record, 142*, 190–194.

Stoev, S. D., Paskalev, M., MacDonald, S., & Mantle, P. G. (2002). Experimental one year ochratoxin A toxicosis in pigs. *Experimental and Toxicologic Pathology, 53*, 481–487.

Szczech, G. M., Carlton, W. W., Tuite, J., et al. (1973). Ochratoxin A toxicosis in swine. *Veterinary Pathology, 10*, 347–364.

Téren, J., Varga, J., Hamari, Z., Rinyu, E., & Kevei, F. (1996). Immunochemical detection of ochratoxin A in black *Aspergillus strains*. *Mycopathologia, 134*, 171–176.

Varga, J., Kevei, É., Rinyu, E., Téren, J., & Kozakiewicz, Z. (1996). Ochratoxin production by *Aspergillus* species. *Applied and Environmental Microbiology, 62*, 4461–4464.