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The Occurrence of Ochratoxin A in White and Parboiled Rice

Jakub TOMAN[1], František MALÍŘ[1], Vladimír OSTRÝ[2], Yann GROSSE[3], Vladimír DVOŘÁK[1], Tomáš ROUBAL[4] and Lucie NEUCHLOVÁ[1]

[1]Department of Biology, Faculty of Science, University of Hradec Králové, Hradec Králové, Czech Republic; [2]Center of Health, Nutrition and Food, National Institute of Public Health, Brno, Czech Republic; [3]International Agency for Research on Cancer, Lyon, France; [4]Regional Branch Hradec Králové, Institute of Public Health in Ústí nad Labem, Hradec Králové, Czech Republic

Abstract

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The levels of ochratoxin A (OTA), one of the five agricultural and toxicological most important mycotoxins, have been determined by HPLC with fluorescence detection (HPLC-FLD) or enzyme-linked immunosorbent assay (ELISA) in 60 samples of white and parboiled rice, purchased on the Czech food market. In all 60 samples of rice tested by HPLC-FLD. OTA levels were below the limit of quantification (LOQ), i.e. < 0.2 ng/g. The same samples were also tested by ELISA (LOQ = 0.05 ng/g), and 58 samples (96.7%) were found to be positive. OTA levels in white and parboiled rice ranged from 0.05 to 0.17 ng/g; the arithmetic mean for all samples was 0.15 ng/g, the median 0.15 ng/g, and the 90% percentile 0.17 ng/g. These findings confirm the necessity of monitoring OTA in different foodstuffs and food, due to the precautionary principle and the uncertainty in the genotoxicity status of OTA.

Keywords: mycotoxins; white rice; foodstuffs; determination; monitoring; HPLC; ELISA

Rice (*Oryza sativa* L.) is a semi-aquatic annual grass that can be grown under a broad range of climatic conditions. The unique ability of the rice plant to grow on all kinds of land and water regimes, combined with its adaptation to a wide variety of climates and agricultural conditions, makes rice the world's most important cereal crop (Owens 2001; Sales & Yoshizawa 2005). Rice is a staple cereal food for over half of the world's population, the most consumed worldwide after wheat. Worldwide production of rice reached 741 mil. t in 2013, more than that of wheat (716 mil. t), soybeans (276 mil. t), and potatoes (376.5 mil. t) (FAOSTAT 2015). In the tropics, rice is the primary source of human nutrition, and is one of the cheapest sources of food energy and protein (Meija 2003). According to the Food and Agriculture Organization, the worldwide rice production is predicted to be 743 mil. t (FAOSTAT 2015). In Europe, rice is mainly produced in France and Spain (Suarez-Bonnet *et al.* 2013).

Rice is frequently produced in suitable climatic environmental conditions favourable for infections by fungi and their growth, followed by possible mycotoxin contamination (Sales & Yoshizawa 2005). Fungal spoilage of food causes significant global economic losses (Iqbal *et al.* 2013). Under inappropriate storage conditions, rice can be an ideal substrate for mycotoxin-producing fungi (Lai *et al.* 2014a), e.g. *Fusarium* (Gomes *et al.* 2015; Rofiat *et al.* 2015), *Aspergillus* (Aydin *et al.* 2011; Rofiat *et al.* 2015; Zhihong *et al.* 2015), *Penicillium* (Aydin *et al.* 2011; Zhihong *et al.* 2015), and *Alternaria* (Rofiat *et al.* 2015). These fungi usually produce the mycotoxins

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fumonisin (Makun *et al*. 2011; Ortiz *et al*. 2013; Rofiat *et al.* 2015), aflatoxins (Nguyen *et al.* 2007; Rahmani *et al*. 2010; Aydin *et al*. 2011; Makun *et al*. 2011; Ortiz *et al*. 2013; Lai *et al.* 2014b; Rofiat *et al.* 2015), but also beauvericin (Nazari *et al.* 2015; Rofiat *et al.* 2015), citrinin (Nguyen *et al.* 2007; Rofiat *et al.* 2015), patulin (Makun *et al*. 2011), sterigmatocystin, moniliformin, fumonisin B_3, cytochalasins (Rofiat *et al.* 2015), enniatins (Nazari *et al.* 2015), zearalenone (Rahmani *et al*. 2010; Makun *et al*. 2011; Lee *et al*. 2015), deoxynivalenol (Dors *et al*. 2011; Makun *et al*. 2011; Ortiz *et al*. 2013), and ochratoxin A (Pena *et al*. 2005; Ghalia *et al.* 2008; Juan *et al.,* 2008; Frelund *et al*. 2009; Buyukunal *et al*. 2010; Rahmani *et al*. 2010; Aydin *et al*. 2011; Bansal *et al*. 2011; Almeidaa *et al.* 2012; Mosayebi & Mirzaee 2014; Rahimi 2014; Venkataramana *et al.* 2015; Xianwen *et al.* 2015). The rice parboiling process increases the migration of mineral salts by about 30% into parboiled rice (compared to white rice) during the soaking and boiling steps (Dors *et al.* 2009). Coelho *et al.* (1999) demonstrated that this process also allowed the migration of mycotoxins from the outside, through the inner layer of rice grain, to the amylaceous endosperm (Coelho *et al.* 1999).

The purposes of our study were to identify which type of rice (white or parboiled) is more contaminated by Ochratoxin A (OTA), and – through quantification – facilitate the assessment of the risk resulting from the intake of OTA-contaminated rice available on the Czech market.

Ochratoxin A is one of the five agriculturally most important mycotoxins (i.e., OTA, deoxynivalenol, aflatoxins, fumonisins, and zearalenone) (IARC 1993). OTA is considered as one of the major toxins produced in food, mainly in subtropical and tropical areas by the moulds *Aspergillus* section *Circumdati* (*A. ochraceus*, *A. westerdijkiae*, *A. stenii*) and *Aspergillus* section *Nigri* (*A. carbonarius*, *A. foetidus*, *A. lacticoffeatus*, *A. niger*, *A. sclerotioniger*, *A. tubingensis*), but also in the temperate and colder zone by moulds of the *Penicillium* species (*P. verrucosum*, *P. nordicum*) (Ostry *et al.* 2013). OTA is both acutely and chronically toxic, and chronic exposure to low OTA doses could be even more hazardous than acute exposure to a high dose (Pfohl-Leszkowicz *et al*. 2007). Indeed, nephrotoxicity is a consequence of acute, sub-acute, but also chronic exposure to OTA. Moreover, exposure to low OTA doses initiates nephrotoxicity (Gekle *et al*. 2005). In this regard, chronic exposure (even to low doses) of OTA seems to be a significant public health problem (Malir *et al*. 2013a). OTA has been shown in experimental and farm animals to have other toxic properties such as hepatotoxicity, reprotoxicity, embryotoxicity, teratogenicity, neurotoxicity, immunotoxicity, and carcinogenicity. OTA was classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC 1993). OTA is one of the major contaminants of foodstuffs, food and feed (Malir *et al*. 2013b), with cereals being viewed as the major source of daily OTA intake in humans (58%), before wine (15%) and pork meat (3%) (JECFA 2008). It is a fact that OTA remains a controversial topic, with opinions differing on its metabolism, genotoxicity, and mechanism of renal carcinogenicity. Nonetheless, and for the reasons cited above, the precautionary principle warrants monitoring ochratoxins – especially OTA and its metabolites – with a view to protecting public health and preventing economic losses (Malir *et al*. 2013a).

MATERIAL AND METHODS

Sampling of rice. A total of 60 randomly chosen samples of rice (white rice, n = 30; parboiled rice, n = 30) were purchased on the Czech food market from 24 locations in 2014 (Beroun, Odolená Voda, Kostelec nad Labem, Libeznice – January; Ostrava, Jihlava – February; Písek – March; Kladno, Litovel, Lutin, Slatinice, Brno – April; České Budějovice – May; Jičín – June; Chrudim, Mikulov, Hrušovany nad Jeviškou, Drnholec – September; Vlašim, Votice, Trhový Štepánov, Prague – October; Šumperk, Kyjov – November) (Figure 1).

Determination of OTA in rice by HPLC-FLD. Purchased rice samples were first well-homogenised and properly tested for their homogeneity according to ISO 13528:2005 (Annex B: Homogeneity and stability checks of samples) (EN ISO/IEC 17025:2005). 40 ml of acetonitrile/water (60 : 40) were added to 10 g of a homogenised white or parboiled rice sample. Samples were shaken for 2 min (at a rotation speed of 11 000/min), filtrated and then centrifuged for 10 min (at a rotation speed of 3500/min) using a multifunctional cooled centrifuge B4i (Jouan, Saint Herblain, France). Four ml of supernatant (deriving from 1 g of sample) were diluted with 44 ml of phosphate buffer saline (PBS) pH 7.4 (Skarkova *et al*. 2013). The solution was put through an immunoaffinity column (IAC)

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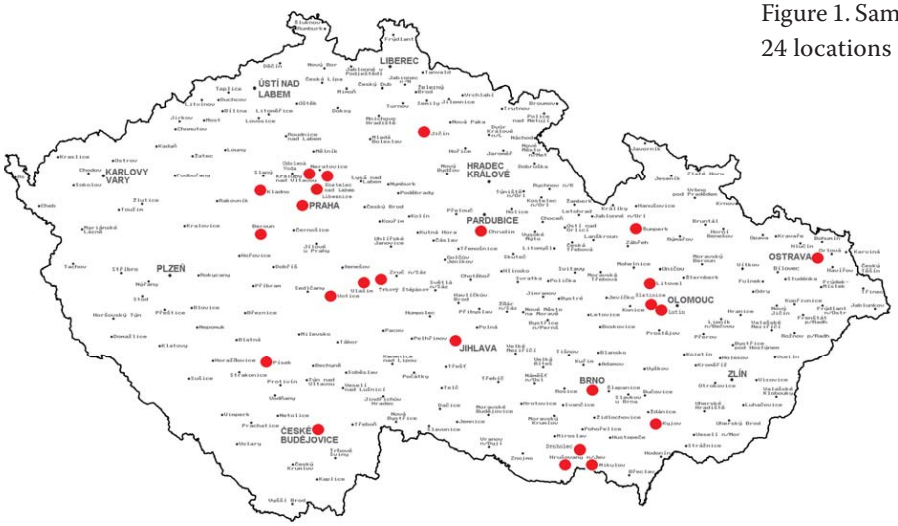

Figure 1. Samples of rice randomly chosen from 24 locations in the Czech Republic

(OCHRAPREP® columns, R-Biopharm, Darmstadt, Germany) using a Baker spe-12G vacuum unit (Baker, London, UK) with a Millipore pump (Millipore, Billerica, USA). The IAC was washed with 10 ml PBS twice. OTA was eluted with 1.5 ml of methanol/acetic acid (98 : 2). The solution was evaporated under nitrogen stream using an EVATERM evaporator (Ing. Hanuš, Brno, Czech Republic). The residue was dissolved in 0.5 ml of methanol/acetic acid (98 : 2), and – after thorough dissolution – 0.5 ml H_2O was added (Skarkova *et al.* 2013).

The validated and accredited method (EN ISO/IEC 17025:2005) of reversed-phase high-performance liquid chromatography with fluorescence detection (HPLC-FLD) was applied for OTA detection and quantification in the rice samples (HPLC; SpectraSYSTEM™, San Jose, USA; FLD detector 920 FP; Jasco, Tokyo, Japan). Validation of the method was performed according to the protocol approved by the AOAC (Official method 2000.23, 2002; Igarashi *et al.* 2008) and to the principles of the ICH Guideline for HPLC. The analytical column Inertsil was filled with ODS-3V, 150 × 4.6 mm of 5-µm particle size (Hichrom, Reading, UK), and coupled with the pre-column Inertsil ODS-3, 10 × 4 mm of the same particle size (Hichrom). The injection volume of samples was 100 µl. Acetonitrile/deionised water/acetic acid (51 : 48 : 1, v/v/v) was used as the mobile phase. Parameters of the fluorescence detector were: excitation wavelength – 333 nm; emission wavelength – 465 nm. The OTA concentrations were calculated using the calibration curve method. The limits of OTA detection (LOD) and quantification (LOQ) were 0.07 ng/g and 0.2 ng/g, respectively (Skarkova *et al.* 2013).

Determination of OTA in rice by ELISA. Rice samples were prepared following a process also using an IAC and similar to the above, except that OTA was eluted with 4 ml of methanol, the obtained solution was evaporated under nitrogen stream, and the residue was dissolved in 500 µl of 0.13 M $NaHCO_3$ pH 8.1 buffer for enzyme-linked immunosorbent assay (ELISA).

The ELISA was performed using the RIDASCREEN Ochratoxin A 30/15 kit (R-Biopharm 2014). Fifty µl of standard solutions (0, 50, 100, 300, 900, 1800 pg OTA/g) were added to the wells of the first row of a microtiter plate, and 50 µl of each sample were sequentially added to the adjacent wells. Fifty µl of diluted enzyme conjugate were then added to all standard's and sample's wells. The plate was gently shaken and incubated in the dark for 30 min at room temperature. The supernatant was removed from all wells, which were then washed twice with 250 µl washing buffer on a Microplate Strip Washer ELx50 (BioTek Instruments, Inc., Vermont, USA). One hundred µl of substrate/chromogen was added to each well and gently mixed for 15 min in the dark at room temperature. Finally, 100 µl of stop solution was added to each well. Absorbance was measured at 450 nm on a microplate spectrophotometer ELISA (BioTek Instruments, Inc.) (RIDASCREEN). The Analysis Software GEN 5TM (Version 1.10) and the Microplate Software for the Way you Work (BioTek Instruments, Inc.) were used for processing and evaluation of measured data. The ELISA had lower limits of detection and quantification (0.05 ng/g) compared to HPLC-FLD.

RESULTS AND DISCUSSION

Both HPLC and ELISA methods for OTA detection and quantification from various food matrices belong

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to basic methods. Therefore the presence of trace amounts of OTA in samples of white and parboiled rice has been investigated by using immunoaffinity chromatography (IC) with a commercially available IAC because the combination of IC with HPLC-FD or with ELISA increases the selectivity and sensitivity of these methods. A total of 60 rice samples were measured using both methods. The determination of OTA levels by ultra-trace HPLC-FLD in all 60 samples of both types of rice demonstrated levels below the LOQ (≤ 0.2 ng OTA/g). The results were more accurate for ELISA testing, due to a lower LOD (0.05 ng/g) (Table 1). The possibilities of false positive results due to antibody cross-reaction with matrix components or other ochratoxins (OTC 44%, OTB 14%, OTα < 0.1%), or of false negative results due to inadequate sensitivity, were eliminated by cleaning the sample on an IAC before the ELISA, and also by using the reference materials P64/OW 806 (OTA-naturally contaminated wheat, 7.7 ng/g) and P64/OW 815 (OTA-naturally contaminated wheat, 4.9 ng/g). These reference materials were used for validation of both methods. Moreover, in addition to testing the 60 samples of white and parboiled rice, some of these rice samples were also spiked with OTA and analysed concurrently as test materials to confirm the quality of the laboratory results.

The samples of white rice and parboiled rice consumed in the Czech Republic were analysed and found contaminated with OTA at an average of 0.14 and 0.16 ng OTA/g (range for both types, < 0.05–0.17 ng/g), respectively (Table 1). These levels are clearly below the maximum limit set by the European Union (EU) legislation (Regulation 1881/2006): 5 ng OTA/g for unprocessed cereals and 3 ng OTA/g for all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption (European Union 2006). Our results are similar to data on rice sold in Canada (0.05–0.20 ng/g; Bansal *et al.* 2011), and Turkey (0.16–0.20 ng/g; Ozden *et al*. 2012). In Brazil, the analysis of mycotoxins was carried out in 2007–2009 on 230 samples of rice and rice products, and OTA was detected in 40% of samples, with 28% of samples contaminated in the range of 0.20–0.24 ng/g; the highest levels of contamination were demonstrated in rice hulls and rice bran (Almeidaa *et al.* 2012). In other studies, OTA levels reached higher concentrations, which could cause toxicity in humans, with levels of 0.02–32.4 ng OTA/g in Morocco (Juan *et al.* 2008) and 0.09–3.52 ng/g in Portugal (Pena *et al.* 2005). In Iran, 182 rice samples were analysed by HPLC-FLD; and OTA was detected in 6% of rice samples, with a mean value of 1.6 ng/g in positive samples (range, 0.2–4.8 ng/g) (Feizy *et al.* 2011). In another study from Turkey, OTA levels in unpackaged rice samples were found by ELISA to be higher than the legal limits of the EC regulation in 30% of rice samples; the highest OTA level was 80.7 ng/g in one sample (Aydin *et al*. 2011).

Yet, in comparison with other cereals (e.g. wheat and maize), the frequency of mycotoxin (including OTA) contamination is lower in rice (Tanaka *et al*. 2007). Margo *et al*. (2006) reported higher levels of mycotoxins in paddy rice and brown rice compared to white and long rice that had undergone technological processing.

The dietary exposure of humans to OTA can be assessed by calculating the daily intake of OTA in food using: the amount of OTA in the analysed diet, and the estimated amount of consumed diet (Malir *et al*. 2006). According to the Joint Expert Committee on Food and Additives (JECFA), the provisional tolerable weekly intake (PTWI) of OTA (based on nephrotoxicity studies in pigs) is 100 ng/kg body weight/week, equivalent to 6 mg/week for a person weighing 60 kg. If we consider the EU regulation (based on studies of carcinogenicity in rodents), the

Table 1. Determination of OTA in rice samples from the Czech Republic by ELISA

Samples	*n*	*n*+	Arithmetic mean	Median	Perc. 90%	Min	Max
	(%)		(ng/g)				
White rice	30 (100)	28 (93.3)	0.14	0.15	0.17	0.05	0.17
Parboiled rice	30 (100)	30 (100)	0.16	0.15	0.17	0.05	0.17
Total	60 (100)	58 (96.7)	0.15	0.15	0.17	0.05	0.17

n – number of samples; *n*+ – numbers of positive samples ≥ 0.05 ng/g (limit of quantification)

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tolerable daily intake (TDI) is twenty times lower (5 ng/kg body weight/day); this corresponds to the consumption of 300 ng OTA/day for a person weighing 60 kg. Knowing that the consumption of rice was only 4.5 kg/person/year in 2010 (around 87 g per person per week or 12.4 g/person/day) in the Czech Republic (ČSÚ 2012), and based on our findings, it can be calculated that the consumption of the maximally contaminated rice leads to an intake of 2.28 ng/person/day. This is much lower than the EU regulation and the JECFA (2008) recommendation.

Nonetheless, and due to the precautionary principle, we find necessary to maintain the lowest possible concentrations of ochratoxin A in foodstuffs and food, knowing that the OTA genotoxicity status is still unknown, though controversial (Mally 2012; Pfohl-Leszkowicz & Manderville 2012). Indeed, direct-acting genotoxic effects postulate that OTA could act through a non-threshold mechanism (triggering the application of highly conservative low-dose modelling to quantitatively assess the risk), whereas an epigenetic mode of action would give ground to the assumption that OTA acts through a threshold mechanism (Malir *et al*. 2012). In this perspective, we note that both mechanisms could actually operate (in parallel or not). If the non-threshold genotoxic effects of OTA are confirmed, risk management and exposure mitigation measures will have to be implemented to ensure the lowest possible human exposure (Dohnal *et al*. 2013). From this point of view, it is reassuring that our findings indicate a very small contamination of white rice and parboiled rice on the Czech market.

The hypothesis that parboiled rice would have been more contaminated by OTA than white rice because OTA could pass into the rice seed during the boiling process, has not been confirmed nor disproved.

CONCLUSIONS

The objective of this study was to obtain information on the degree of contamination of white and parboiled rice by OTA, which would serve as a basis for the evaluation of the dietary exposure to and health risk assessment of OTA in the Czech Republic. Our findings indicate that the exposure of the Czech population from the occurrence of very low OTA concentrations in rice available on the Czech market is minimal, and it seems that rice does not represent an important health hazard for the Czech population. Nevertheless, the OTA occurrence may be different from one year to another. Therefore, it is important to monitor OTA occurrence in rice samples regularly, e.g. once every other year. Prevention of contamination at source is considered to be the most effective public health measure, but the overall situation may change due to the increasing globalisation of the food market. In order to reduce the risks associated with OTA and other mycotoxins, and minimise their overall impact on public health in the Czech population, continuous monitoring of their presence in rice is necessary, in conjunction with a strict respect of the EU legislation. In this respect, our next study will focus not only on white and parboiled rice, but also on brown rice (with lemmas) available on the Czech market.

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Corresponding author:

Mgr. Jakub Toman, Univerzita Hradec Králové, Přírodovědecká fakulta, Katedra biologie, Rokitanského 62, 500 03 Hradec Králové, Česká republika; E-mail: jakub.toman.1@uhk.cz