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Mycotoxins in apples coming from organic production and integrated pest management

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Abstract: The aim of this study was to compare the occurrence of important mycotoxins often contaminating fruits, in particular aflatoxin B1, ochratoxin A, patulin, and an indicator of fungal metabolism – kojic acid, in dried apples from organic production and integrated pest management with origin in the Czech Republic. Regardless of the production management, both aflatoxin B1 and patulin concentrations were below the limit of quantification. Ochratoxin A was present in all samples examined in our study with concentrations ranging from 4.22 to 15.99 μg kg^{-1}. Kojic acid concentrations ranged from 3.57 to 9.44 mg kg^{-1}. However, no significant difference in ochratoxin A and kojic acid concentrations was found between samples coming from integrated pest management and samples coming from organic agriculture. The results of this study show that apples originating in organic production or integrated pest management have, under the same independent conditions, an equal probability of containing (or omitting) similar levels of the investigated mycotoxins. Moreover, these results, while demonstrating safe levels of some mycotoxins in different agricultural practices also highlight gaps in knowledge and legislation that may have direct and crucial effects on human health.

Keywords: orchards; fungicides; pesticides; *Aspergillus; Penicillium*

Mycotoxins are toxic secondary metabolites produced by different species of fungi, mainly of the genera *Aspergillus, Fusarium,* and *Penicillium* known for their adverse effects on human health (Madalena et al. 2018). A variety of biological or ecological factors may promote the growth of mycotoxin-producing fungi at any stage of the culturing, harvesting, or storage of agricultural products, leading to their contamination with mycotoxins (Marin et al. 2013).

The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the cereals produced worldwide are contaminated by mycotoxins (Rice and Ross 1994). Recently, this claim has been revised by Eskola et al. (2019), who reported that the mycotoxin occurrence above detectable levels in foodstuffs and feedstuffs is up to 60–80%. Apart from cereals, various types of food, such as nuts, spices, and fruits can also be contaminated. Apples are considered to be highly susceptible to fungal contamination at both pre- and postharvest stages (Köhl et al. 2018).

Maximum levels for mycotoxins are given in Commission Regulation (EC) No. 1881/2006. Concerning fruits (excluding nuts and dried figs), limits on maximum levels are given for aflatoxin B1 (AFB1) in dried fruits consumed without or with further sorting or treatment (2 and 5 μg kg^{-1}, respectively), ochratoxin A (OTA) in grape juice (2 μg kg^{-1}) and dried vine fruit (currants, raisins and sultanas; 10 μg kg^{-1}), and patulin in fruits juices (50 μg kg^{-1}) and solid apple products (25 μg kg^{-1}).

Out of 584 mycotoxin-related notifications listed in the Rapid Alert System for Food and Feed (RASFF)

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in 2019, 109 (18.7 %) were from the category of fruits, mainly dried raisins, figs, and grapes. In particular, 57 (9.8%) notifications were related to aflatoxins and 53 (9.1%) to OTA, with one sample containing both mycotoxins. AFB1 and patulin were also reported in apples and products thereof (Fernández-Cruz et al. 2010) with the most important producer of patulin being the apple-rotting fungus *Penicillium expansum*. Even though patulin and ochratoxin are produced by the same genera of moulds, little attention was paid to reports focused on the presence of OTA in apples and products thereof (Al-Hazmil 2010). Apart from the well-known and described mycotoxins (such as aflatoxins), a variety of fungal secondary metabolites including cyclopiazonic acid, aflatrem, aspertoxin, kojic acid, and others are produced by the genus *Aspergillus*. However, for these mycotoxins no maximum limits have been listed. Once food or feed is contaminated with mycotoxins, thermal, chemical, or physical treatment is not effective enough to destroy and remove them (Marin et al. 2013). Therefore, attention must be paid to prevent their occurrence in agricultural products. Fungicides are products to control and prevent the development of mycotoxins produced by fungi. However, frequent fungicide use is linked with elevated risks for farmers and consumers in addition to adverse impacts on soil, water quality and wildlife habitats (Baker et al. 2020).

Both integrated pest management (IPM) and organic agriculture reduce reliance on pesticides through balanced crop nutrition, biologically active soil, biodiversity, beneficial organisms, and many other tools. The principles of IPM and organic farming partly overlap. However, while both IPM and organic agriculture acknowledges the importance of biodiversity, soil biological activity and biological cycles, IPM, in addition, considers the importance of economic profit (Baker et al. 2020).

Even though the FAO report concerning the content of mycotoxins in crops showed no clear differences between organic and conventional farming systems (FAO 2000), it was stated that in certain circumstances, such differences might occur. Moreover, as no synthetic fungicides are used in organic production, some consumers are concerned that products of organic agriculture might be more susceptible to mycotoxin contamination.

Therefore, the aim of this study was to find out whether the occurrence of important mycotoxins often contaminating fruits differs in apples coming from organic production in comparison with IPM.

MATERIAL AND METHODS

Sample characterisation. For the analysis, ten samples of dried apples (approx. 200 g each) originating from one organic production system and ten samples from one IPM growing system were obtained from a Czech apple grower. Each of the twenty samples was created by blending apples from various parts of the farmer's orchards in order to exclude the impacts of locality and its microclimate on the occurrence of fungal diseases. The farm is located in the Bohemian region, northwest of the Czech capital of Prague. The area of the country is relatively dry and warm. The apples were sampled during the harvest of 2018. The year 2018 was exceptionally warm through spring, summer and autumn with annual total precipitation below average (76% of the normal value). After harvest, the apples were stored at 2 °C in an ultra-low oxygen atmosphere prior to processing. Next, the apples were sliced, spread on grids and dried by hot air in an industrial fruit dryer (60–70 °C; 6–8 h); finally, apples were stored at room temperature (22 °C) and relative humidity of 60% until further processing.

Based on the local knowledge, fruit culture, weather conditions, and threshold of pathogen harmfulness, the following registered active substances were used to ensure safe and economical production in IPM: copper oxychloride, polysulphide, sulphur, difenoconazole, fluxapyroxad, mancozeb, dithiadon, cyprodinil, captan, pyrimethanil, pirimicarb, thiram, methoxyfenozide, potassium bicarbonate, aluminium sulphate, thiacloprid, acetamipiride, spinosad, and chlorantraniliprole. In organic production, only copper oxychloride, sulphur, polysulphide, and spinosad were used. The active substances were applied with respect to their withdrawal periods in order to prevent any pesticide residues exceeding the maximum residue limit according to European Union Regulation (EC) No. 396/2005. No treatment against storage diseases was carried out.

Sample homogenisation and analysis of mycotoxins. All samples of sliced-dried apples coming from various parts of the IPM orchard were ground, thoroughly mixed, and divided into representative samples for the analysis of selected mycotoxins. The same procedure was repeated with apples coming from organic production. For the analysis of OTA, 10 g of the homogenised mixture was extracted with 40 mL of 50% methanol solution using an orbital shaker (KS 501 digital; IKA®-Werke GmbH & C, Germany) for 5 min. The resulting extract was filtered (15 µm,

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15s KA–1 filter paper; Pulp & Paper Mills Perštejn, Czech Republic) and collected. Finally, the analysis of OTA was performed by a competitive direct enzyme-linked immunosorbent assay (ELISA). This analysis was carried out at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic) using a commercial ELISA kit (Veratox for Ochratoxin 8610; Neogen, United Kingdom). The kit's limit of quantification (LOQ) was 2 μg kg^{-1}.

For the analysis of AFB1, part of the homogenised mixture of dried ground apples was sent to the State Veterinary Institute in Jihlava, Czech Republic. The analysis was carried out according to the accredited method described in the institutional standard operating procedure (SOP) 8.37 using high-performance liquid chromatography with fluorescence detection (HPLC/FLD) confirmed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS). The method's LOQ was 0.16 μg kg^{-1}.

The analysis of patulin was done at the State Veterinary Institute in Olomouc, Czech Republic, according to the accredited method described in the institutional SOP CHE 9/05 using HPLC-diode-array detector (HPLC/DAD) with LOQ 10 μg kg^{-1}.

The determination of kojic acid was conducted using liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC/ESI-MS/MS) and was based on a method described by Sulyok et al. (2020) with some modifications. Briefly, part of the homogenised mixture of dried ground apples (5 g) was extracted in a conical flask with solvent 1 (acetonitrile/water/acetic acid 79 : 20 : 1; v/v/v; 20 mL) for 60 min using a rotary shaker (Vibrax VXR Basic; IKA®-Werke GmbH & C, Germany). The resulting supernatant was filtered using paper filter (15 μm, 15s KA–1 filter paper; Pulp & Paper Mills Perštejn, Czech Republic) and diluted with solvent 2 (acetonitrile/water/acetic acid 20 : 79 : 1; v/v/v) (solvent 1/solvent 2; 2/1; v/v). The final solution was filtered through a 0.2 mm nylon filter (Millipore, USA) and used for LC/MS analysis. A Thermo Scientific UHPLC Accela 1250 chromatographic pump was connected to a Thermo Scientific TSQ Quantum Access MAX Triple Quadrupole Instrument (Thermo Scientific, USA) equipped with electrospray ionisation probe. A Kinetex C18 column (2.1 mm × 100 mm, 1.7 μm; Phenomenex, USA) was used at a constant flow rate of 300 μL min^{-1}. Mobile phase consisted of solvent A (methanol/water/acetic acid 10 : 89 : 1; v/v/v) and solvent B (methanol/water/acetic acid 97 : 2 : 1; v/v/v). The gradient used was: 0–1.0 min linear gradient from 20 to 50% B; 1.0–3.3 min held at 50% B; 3.3–4.0 min from 50 to 20% B and 4.0–4.3 min held at 20% B. The full-loop injection volume of the sample was set at 10 μL. The electrospray ionisation was operated in the positive mode under the following conditions: capillary temperature: 325 °C; vaporiser temperature 300 °C; sheath gas pressure 35.0 psi; auxiliary (drying) gas 10 a.u.; spray voltage 3300 V. The instrument was calibrated daily with multi-level calibration curves. The procedural blank was analysed for every set of 10 samples. The process efficiency of the method was 90.6%; extraction recovery was 96.5% and the matrix effect was –6.1%. The inter-day precision expressed as a relative standard deviation was 6.5% and the accuracy was 7.2%. The limit of detection (95 ng g^{-1}) was determined as a 3 : 1 signal versus noise value. Commercial standards of kojic and acetic acid were used (Sigma-Aldrich, USA). All solvents were of residual analysis purity (Chromservis, Czech Republic).

Statistical analysis. Statistical evaluation was carried out using the software Unistat 6.5 for Excel (United Kingdom). At first, all data were tested for normality of distribution using the Shapiro-Wilk test. Because data were normally distributed, parametric unpaired *t*-test was used. Differences between concentrations of individual mycotoxins in dried apples from organic production and IPM were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Apples represent a little over one third of the total area of fruit plantation in the European Union with Poland being the largest producer (European Commission 2020). Apples are the most important fruit crop produced in the Czech Republic with 113 000 t harvested in 2019 and 6 509 ha of apple trees (Ministry of Agriculture 2019). AFB1 is considered to be the most severe and frequent fruit contaminating toxin (RASFF). In our study, the concentration of AFB1 in all samples from both IPM and organic production was below the LOQ (< 0.16 μg kg^{-1}). In 2018, 90 notifications related to mycotoxins in fruits and vegetables were listed on the RASFF portal, out of those 41 concerned aflatoxins in concentrations above the limit (mainly in dried figs). In 2018, five positive samples containing AFB1 were reported in the Czech Republic. These were samples of raisins, dried apricots and dried figs originating from outside the Czech Republic. The AB1 levels measured

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in our study were below the European threshold. These results suggest that with regard to this toxin, Czech apples from either IPM or organic production do not pose a significant risk for human consumption. Unlike AFB1, OTA was present above the limit of detection in all the samples examined in our study with concentrations ranging from 4.22 to 15.99 μg kg^{-1} (Table 1). However, no significant difference in the OTA contamination level was found between apples coming from IPM and organic production ($P = 0.1432$). OTA, produced by the species of *Aspergillus* and *Penicillium*, is an important nephrotoxic mycotoxin with carcinogenic, teratogenic, immunotoxic, genotoxic, and possibly neurotoxic effects (Al-Hazmi 2010). OTA was reported to occur in cereal products, olives, beans, wine, coffee, cocoa products, raisins, figs, liquorice, spices, and tea (Malir et al. 2016). Occurrence of OTA in apples has been scarcely described in literature. However, isolates of several genera of fungi capable of producing OTA were found in pome fruits (Aziz et al. 2004). No limit for OTA has been laid down for dried apples in the European Union. The most related limit to be considered may be the one for dried vine fruit, which is established to be 10 μg kg^{-1} (Commission Regulation No. 1881/2006). Based on the values of OTA concentrations (Table 1), OTA levels in 3 out of the 10 tested samples exceeded this above-mentioned limit and, in this regard, may pose a risk to human consumption.

The concentration of patulin, a mycotoxin produced by some species of *Penicillium* and *Aspergillus*, in apples originating from both organic production and IPM in our study was below the limit of quantification (10 μg kg^{-1}). Patulin features different harmful functions such as toxic, antibiotic, carcinogenic, and mutagenic properties (Wichmann et al. 2002). The main mechanism associated with patulin toxicity involves the formation of covalent compounds containing sulphhydryls, such as glutathione, cysteine, and thioglycolate. These reactions also affect the activities of thiol-containing enzymes in many active groups, such as hexokinase, which plays an important role in glycolysis (Fliege and Metzler 2000). The maximum level for patulin contamination in solid apple products intended for direct consumption is 25 μg kg^{-1} (Commission Regulation No. 1881/2006). In 2018, no notification related to patulin in fruits and vegetables was listed on the RASFF portal. Regardless, patulin is said to be the most important fruit toxin globally (Vaclavikova et al. 2015). Major potential dietary sources that may contain patulin include apples and various apple-based products (e.g. juices, puree, ciders, concentrates and compotes) prepared from raw materials infested by moulds (Barkai-Golan and Paster 2008).

Kojic acid concentrations measured in our study ranged from 3.57 to 9.44 mg kg^{-1}. No significant difference between apples that originated in organic production and IPM was found (Table 1; $P = 0.7128$).

Kojic acid is found in very low concentrations in traditional Japanese foods such as miso, soy sauce, and sake. It is also used as an additive for preventing enzymatic food browning and for cosmetics. Although only very limited information about kojic acid toxicity is available, it was found to be a weak mutagen and it was able to induce sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells and *Salmonella typhimurium* (Wei et al.

Table 1. Concentration of ochratoxin A and kojic acid in dried apples coming from organic production and integrated pest management (IPM)

Production management	Sample No.	Ochratoxin A concentration (μg kg^{-1})	Kojic acid concentration (mg kg^{-1})
Organic production	1	7.38	6.56
	2	6.50	3.85
	3	6.30	6.00
	4	4.22	6.01
	5	7.62	5.97
	6	8.01	NA
	7	12.05	NA
	8	9.41	NA
	9	12.24	NA
	10	15.99	3.61
	mean ± SD	8.97 ± 3.51	5.33 ± 1.26
IPM	11	8.38	3.94
	12	7.36	3.57
	13	7.94	5.82
	14	6.34	5.14
	15	8.46	5.26
	16	8.53	6.89
	17	6.26	9.44
	18	6.80	4.91
	19	5.69	4.84
	20	5.54	5.37
	mean ± SD	7.13 ± 1.16	5.52 ± 1.66

No significant differences ($P < 0.05$) in mycotoxin contamination were found between the groups; NA – sample not available; SD – standard deviation

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1991; Bhatnagar et al. 2014). In current European and local legislation, there are no hygienic limits for kojic acid content in foodstuffs.

CONCLUSION

The aim of this study was to compare the occurrence of important mycotoxins often contaminating fruits, in particular AFB1, OTA, patulin, and an indicator of fungal secondary metabolism – kojic acid, in dried apples from organic production and IPM originating in the Czech Republic. Regardless of the production management, both AFB1 and patulin concentrations were below the LOQ of our analytic methods. Contrarily, OTA was present in all the samples examined in our study. However, OTA concentrations showed no significant difference between apples coming from IPM and organic production. Kojic acid showed no significant difference in concentrations between samples coming from IPM and samples coming from organic agriculture. No limit for kojic acid and OTA has been laid down for dried apples in the European Union. An important conclusion, additional to the main aim of this study, arises from the legislative perspective. Regarding OTA, the most related limit in EU legislation to be considered comparable is the one for dried vine fruit, which is established to be 10 μg kg^{-1} (Commission Regulation No. 1881/2006). When comparing this limit with the OTA concentrations measured in the samples in our study, it can be suggested that especially the samples from organic production contain significantly high values of OTA that may represent a potential risk for human consumption. Moreover, if the samples were intended for use as baby foods for infants and young children, none of either organic or IPM samples would meet the OTA limit of 0.5 μg kg^{-1}.

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