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Mould contamination and co-occurrence of mycotoxins in maize grain in Croatia

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Maize grain samples (n = 15) collected during the autumn of 2002 were analyzed for the presence of moulds and mycotoxins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), zearalenone (ZEA), and ochratoxin A (OTA). Mycological analysis showed that all samples were contaminated with *Fusarium* spp. and *Penicillium* spp., while *Aspergillus* spp. were found in 5 samples. *F. proliferatum* and *F. verticilloides*, the producers of fumonisins, were found in 14 and 8 samples, respectively, while *F. graminearum,* the producer of ZEA, was present in all samples. The most frequent mycotoxins were FB_1 (15/15) and ZEA (12/15), followed by OTA (7/15), while FB_2 was found in only two samples. Seven samples were contaminated with two mycotoxins, seven with three, and one sample with only one mycotoxin. The concentrations (mean ± SD) of FB_1, ZEA, and OTA in positive samples were 459.5 ± 314.6, 1.70 ± 0.80, and 1.40 ± 0.55 μg kg^{-1}, respectively, and the concentrations of FB_2 in two samples were 68.4 and 3084.0 μg kg^{-1}. In general, such low mycotoxin concentrations are not a significant source of exposure to humans, but they may contribute to exposure from other commodities. A few samples with extreme values indicate that strict control is needed.

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Food and feed are commonly contaminated with moulds and their secondary metabolites mycotoxins. Ingested, mycotoxins cause a number of toxic effects in animals and humans (1, 2). Maize (*Zea mays*), one of the two most frequent crops in Croatia, is often contaminated with *Fusarium* and *Penicillium* spp. (3).

The most frequent fusaria found in maize are *F. verticilloides* (syn. *F. moniliforme*), *F. subglutinans*, and *F. proliferatum*. *F. verticilloides* and *F. proliferatum* are potential producers of fumonisins. The most frequent fumonisins are fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2). They cause mycotoxicoses in domestic animals (pulmonary oedema in swine, leu-

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koencephalomalacia in horses). Fumonisins are nephrotoxic in swine, rats, sheep, mice and rabbits, and hepatotoxic in rats and rabbits (4). There are some suggestions that human exposure to FB_1 in some regions of Africa and China may cause oesophageal cancer. The International Agency for Research on Cancer (IARC) has categorized toxins derived from *F. verticilloides* as possibly carcinogenic to humans (Group 2B) (5). The only investigation of FB_1 and FB_2 in naturally contaminated maize in this country revealed a high frequency of FB_1 positive samples (3).

Zearalenone (ZEA) is a mycotoxin produced mainly by *F. graminerarum, F. semitectum, F. equiseti, F. culmorum*, and *F. crookwelense*. ZEA has estrogenic effects in animals, which are a consequence of its binding to oestrogene receptors. It is also believed that exposure to high doses of ZEA in food causes precocious menarche in girls not only in tropical, but also in mild climates (6, 7). In Croatia, the first investigation of ZEA contamination of maize was performed on 191 samples collected in 1975, mostly in silos for animal consumption and a few samples from individual farmers (8). As 1975 was a particularly dry year, ZEA was found in 2.6% of samples, the mean ZEA concentration in positive samples was high (5.1 mg kg^{-1}), and the concentrations ranged from 0.043 to 10 mg kg^{-1}. In another study, ZEA was found in 5 of 26 maize samples collected in 1985 in the whole country (9). The mean ZEA concentration was 1.192 mg kg^{-1} and the concentration range was 0.56–3.0 mg kg^{-1}.

Moulds of *Penicillium* and *Aspergillus* spp. produce ochratoxin A (OTA), a common contaminant of cereals and other commodities all over the world (10). OTA is a nephrotoxic compound with carcinogenic properties in experimental animals and is classified by the IARC as possibly carcinogenic to humans (Group 2B) (5). It is considered to be involved in the aetiology of endemic nephropathy and is frequently associated with urothelial tumours in patients with endemic nephropathy (11). Since endemic nephropathy is present in Croatia, most investigations of OTA contamination of maize are focused on the endemic region. An investigation restricted to the endemic region included maize samples collected between 1972 and 1976 and analyzed using thin layer chromatography (TLC). The mean frequency of OTA-positive samples was 8.3%, ranging from 0 to 15.2% from year to year (12). A much higher frequency of OTA contamination (26%) was found in maize stored for animal consumption, collected during 1975 (8). In another study, OTA was analyzed in maize using high performance liquid chromatography (HPLC) (3). In samples collected from all maize-producing counties in 1996 and 1997, OTA was found in 10 and 25% of samples, respectively. In a recent study, OTA was detected in 17 of 51 maize samples (33.3%) using TLC and confirmed by HPLC (13). Maize OTA concentrations in samples collected from the three regions in 1999 were compared to OTA concentrations in samples collected in the endemic region. The mean OTA concentration was much higher in maize from the endemic region, but other regions had similar OTA concentrations in maize.

It is not unusual that cereals and other commodities are contaminated with two or more moulds and mycotoxins at the same time. The aim of this study was to determine mould contamination of maize in Croatia, with the emphasis on mycotoxin-producing *Fusarium* moulds, and to measure the concentration and the frequency of mycotoxins FB_1, FB_2, ZEA, and OTA in maize samples collected in 2002, a year with very high rainfall throughout the flowering period (July and August).

EXPERIMENTAL

Maize samples

Samples of apparently mould-free maize (n = 15) were collected immediately after harvest in several Croatian counties in the autumn of 2002. Each sample was stored at –20 °C until analyzed.

Chemicals

Standards of FB_1, FB_2, ZEA and OTA were purchased from Sigma, USA. Water (Merck, Germany) and methanol (Kemika, Croatia) used for the mobile phase were of HPLC-grade. All other chemicals were of *p.a.* grade.

Mycological analysis

One hundred corn kernels were taken from each sample. Kernels were surface-disinfected for 1 minute in NaOCl (1%) and rinsed twice with sterile water. Kernels were plated onto Petri dishes (10 kernels per dish) with a double blotter and incubated at 21 °C for 24 h. They were then frozen at –10 °C for another 24 h. Petri dishes were then placed in a thermostat and incubated at 24 °C for 7–10 days with a 12/12 hour light/dark cycle.

Penicillium and *Aspergillus* species that developed on kernels after incubation were determined according to Samson *et al.* (14) and *Fusarium* species according to Nelson *et al.* (15). Fungal colonies with sterile mycelium resembling *Fusarium* spp. were transferred to carnation-leave agar (CLA) according to Fisher *et al*. (16) and then determined according to Nelson *et al*.

Mycotoxin analysis

All mycotoxin analyses were performed on a HPLC (Varian, USA) with fluorescence detection. Chromatographic data were collected and processed using Star Chromatography Workstation software (Version 5.0, Varian).

Determination of FB_1 and FB_2

FB_1 and FB_2 were extracted from ground maize samples (50 g) and cleaned up using immunoaffinity columns (IAC, FumoniTest, Vicam, Watertown, MA, USA) according to the producer's instructions. The fumonisins were eluted from the IAC columns with an acetic acid/methanol solution (1:99) and the eluate was evaporated to dryness in a stream of nitrogen. Before injection, the sample was redissolved in methanol, and derived in the reaction with *o*-phthaldehyde (OPA). The injection volume was 20 μL. Mobile phase for HPLC analysis consisted of methanol and 0.1 mol L^{-1} NaH_2PO_4 x 2 H_2O (68:32) and pH was adjusted to 3.35 with *o*-H_3PO_4. The flow rate was 0.8 mL min^{-1}. FB_1 and FB_2 were separated on a 125.0 x 4.0 mm analytical column LiChrospher RP-18 (Merck) with 5 μm particles. Wavelengths of the detector were set at λ_{em} 336 and λ_{ex} 440 nm. The validation

of the method showed linear standard curves for FB_1 and FB_2 (with r^2 = 0.996771 and 0.996619, respectively). Detection limits for both mycotoxins were 10 μg kg^{-1}, and reproducibility, expressed as RSD, was below 10%.

Determination of ZEA

Preparation of samples for ZEA analysis was performed as described by Llorens *et al.* (17). Ground samples (4 g) were extracted three times with dichloromethane and the clean-up procedure was performed on C-18 solid phase extraction columns (Bond Elute, Varian). ZEA was eluted from C-18 columns with methanol and evaporated to dryness under a stream of nitrogen. The sample was redissolved in the mobile phase, and 20 μL was injected. The mobile phase consisted of methanol and water (80:20). The flow rate of the mobile phase was 0.5 mL min^{-1}. The guard column and analytical column were LiChrospher RP-18 (Merck) with 5-μm particles, and their sizes were 4.0 x 4.0 and 250.0 x 4.0 mm, respectively. Wavelengths of the detector were set at λ_{em} 274 and λ_{ex} 440. The linearity of the standard curve was 0.9992. The detection limit was 0.39 μg kg^{-1} and reproducibility, expressed as RSD, was 5.6%.

Determination of OTA

OTA was extracted from maize samples and cleaned up using IAC columns (OchraTest, Vicam). Ground samples (50 g) were extracted with methanol and water (80:20), filtered, and an aliquot of 10 mL was transferred to the IAC-column. OTA was eluted with methanol and the eluate was evaporated to dryness in a stream of nitrogen. Prior to HPLC analysis, samples were redissolved in the mobile phase. For OTA analysis, the mobile phase consisted of methanol, water, and acetic acid (70:30:2) with the flow rate 0.5 mL min^{-1}. The guard column and analytical column were LiChrospher RP-18 (Merck) with 5 μm particles. The sizes of the guard and analytical columns were 4.0 x 4.0 and 125.0 x 4.0 mm, respectively. Detector wavelengths were set at λ_{ex} 336 and λ_{em} 464 nm. The standard curve was linear (r^2 = 0.9982). The detection limit of the method was 0.25 μg kg^{-1}, and reproducibility, expressed as RSD, was 6.76%.

RESULTS AND DISCUSSION

It is known that the most frequent moulds growing on maize in Croatia are *Penicillium* and *Fusarium* (3). In mild climates, these moulds produce mycotoxins FB_1, FB_2, ZEA, and OTA. The growth of *Penicillium* and *Fusarium* spp. and the production of mycotoxins depends on the humidity and temperature in the maize flowering period, which in Croatia spans from the beginning of July until the end of August. According to the Meteorological and Hydrological Service of Croatia, this period was particularly rainy in 2002.

Table I shows the results of mycological analysis of maize samples. *Fusarium* spp. and *Penicillium* spp. were found in all the tested samples. Contamination with *Penicillium* spp. was the most severe, affecting more than 80% of kernels in three samples. *Trichoderma* spp., *Aspergillus* spp., and *Alternaria* spp. were found in 7, 5, and 5 samples, respectively. *F. proliferatum* and *F. verticilloides*, the producers of fumonisins, were found in

14 and 8 samples, respectively. Contamination with *F. proliferatum* affected a higher percentage of kernels than contamination with *F. verticilloides*. *F. graminearum*, the producer of ZEA, was found in all samples, but contamination was low.

Common FB_1 and FB_2 producers are *F. verticilloides* and *F. proliferatum*, endophytic moulds that usually do not interfere with the sprouting, growth, and development of maize. *F. proliferatum* was found in almost all the analyzed maize samples and *F. verticilloides* in 8 out of 15 samples, which is in agreement with the high frequency of FB_1--positive samples (Table I). FB_1 was found in all samples, which is similar to the study of Jurjević *et al*. (3) where 97% and 93% of maize samples contained FB_1 in two consecutive years. The mean FB_1 concentration in our study (459.5 μg kg^{-1}, Table II) was between the mean concentrations obtained in this study (645 and 134 μg kg^{-1}, respectively). In contrast to FB_1, in our study, as well as in the study by Jurjević *et al.* (3), the frequency of FB_2 positive samples was very low. These results are not surprising because it is known that contamination with FB_1 is usually higher than that with FB_2 (18).

ZEA was found in 12 samples, but the mean concentration was low (1.70 ± 0.80 μg kg^{-1}) (Table II). The low concentration of ZEA is in agreement with the low infection with *F. graminearum* (Table I).

In our study, contamination of maize samples with *Penicillium* spp. was high and that with aspergilli low (Table I). Some OTA-positive samples were contaminated with *Penicillium* spp. only. Although literature refers to different *Penicillium* species that produce OTA, Pitt (19) reported that the only OTA-producer in mild climates was *P. verrucosum.* As we did not test maize samples for *Penicillium* and *Aspergillus* spp. contamination, it may be reasonable to assume that OTA found in *Aspergillus*-free samples was

Table I. Mycotoxin contamination and moulds that may produce mycotoxins in individual samples

No. of sample	Mycotoxin contamination (μg kg^{-1})				Mould infection (%)				
	FB_1	FB_2	ZEA	OTA	F. *graminearum*	*F. proliferatum*	*F. verticilloides*	*Aspergillus* spp.	*Penicillium* spp.
1	250.8	n.d.	0.69	n.d.	2	14	n.d.	9	31
2	246.6	n.d.	2.07	n.d.	8	2	n.d.	n.d.	60
3	450.0	n.d.	2.43	1.27	4	28	n.d.	n.d.	27
4	238.2	n.d.	2.49	n.d.	4	20	1	n.d.	61
5	267.0	n.d.	1.40	2.54	2	27	n.d.	3	71
6	324.6	n.d.	0.62	0.73	8	39	9	n.d.	46
7	264.0	n.d.	n.d.	n.d.	2	5	n.d.	n.d.	78
8	320.4	68.4	n.d.	1.27	1	7	7	14	41
9	196.8	n.d.	2.04	n.d.	4	n.d.	1	n.d.	89
10	592.2	n.d.	0.89	1.27	4	6	n.d.	11	87
11	898.2	n.d.	1.70	n.d.	6	25	3	n.d.	35
12	1377.6	n.d.	3.22	n.d.	9	8	3	n.d.	97
13	572.4	n.d.	0.82	1.27	1	12	4	n.d.	55
14	454.8	n.d.	2.01	1.45	1	21	7	14	62
15	439.2	3084.0	n.d.	n.d.	6	40	n.d.	n.d.	28

n.d. – not detected

Table II. Concentrations of fumonisin B_1, fumonisin B_2, zearalenone, and ochratoxin A in maize[a]

	Mean concentration in positive samples ± SD (μg kg^{-1})	Range (μg kg^{-1})	Positive/total samples
FB_1	459.5 ± 314.6	196.8–1377.6	15/15
FB_2	[b]	68.4–3084.0	2/15
ZEA	1.70 ± 0.80	0.62–3.22	12/15
OTA	1.40 ± 0.55	0.73–2.54	5/15

[a] n = 15

[b] In two positive samples, concentrations of FB_2 were 68.4 and 3084.0 μg kg^{-1}.

Table III. Frequency of maize samples containing various combinations of mycotoxins (n = *15)*

Mycotoxin	Positive samples
FB_1	1
FB_1 + FB_2	1
FB_1 + FB_2 + OTA	1
FB_1 + ZEA	6
FB_1 + ZEA + OTA	6

produced by *P. verrucosum*. OTA was found in 7 samples (Table II). Compared to the studies of Jurjević *et al*. (3) and Puntarić *et al*. (13), our study showed a higher frequency of OTA-positive maize samples. Save for the endemic region, Jurjević *et al*. (3) did not find any difference in OTA concentrations in crops between two years of harvest, and Puntarić *et al*. (13) found no differences in OTA concentrations in maize between non-endemic regions of Croatia.

Mycological analysis instigated further analysis of the co-occurrence of FB_1, FB_2, ZEA and OTA in samples, the first study of the sort in Croatia. FB_1 was found in all samples, and ZEA in 12 samples, but the concentration of ZEA was low (Table II). The optimal temperature for fumonisin production is 20 °C and for ZEA production it is below 10 °C (20), which may explain high concentrations of fumonisins and low concentrations of ZEA in our study. Our results are similar to an earlier study on the co-occurrence of fumonisins and OTA in Croatia (3). The co-occurrence of two mycotoxins was found in seven samples, three mycotoxins were found in another seven samples, and one sample contained only one mycotoxin (Table III).

CONCLUSIONS

In Croatia, no maximum tolerable levels for mycotoxins in grain have been established. The European Union defined this limit for OTA contamination of raw cereal grains only (5 μg kg^{-1}), and no sample exceeded this limit in our study. Although there

are no EU limits for FB_1, FB_2 and ZEA in grains, some European countries have national legislation regarding these mycotoxins. The limits for ZEA concentration in cereals in Romania, Austria, France and Russia are quite different (30, 60, 200, and 1000 μg kg^{-1}, respectively) (21). Only Switzerland has defined a 1000 μg kg^{-1} maximum acceptable concentration of FB_1 + FB_2 in maize (21), which was exceeded in two samples in our study. The mean concentration of mycotoxins found in our study does not exceed the above limits, although some samples containing high concentration of FB_1 and FB_2 show that strict control of grain is needed.

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S A Ž E T A K

Kontaminiranost kukuruza u Hrvatskoj plijesnima i nekim mikotoksinima

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U uzorcima kukuruza (n = 15) sakupljenima u jesen 2002. godine determinirane su plijesni i određena koncentracija mikotoksina fumonizina B_1 (FB_1), fumonizina B_2 (FB_2), zearalenona (ZEA) i okratoksina A (OTA). Mikološkom je analizom utvrđeno da su svi uzorci kontaminirani s *Fusarium* i *Penicillium spp*., a *Aspergillus spp*. nađeni su u 5 uzoraka. *F. proliferatum* i *F. moniliforme*, koji proizvode fumonizine, pronađeni su u 14, odnosno u 8 uzoraka, dok je *F. graminearum*, koji proizvodi ZEA, nađen u svim uzorcima. U svim je ispitivanim uzorcima nađen FB_1 (15/15), ZEA je nađen u 12/15, OTA u 7/15 uzoraka, dok je FB_2 nađen samo u dva uzorka. U po 7 uzoraka nađeno je 2 odnosno 3 mikotoksina, dok je samo jedan uzorak sadržavao jedan mikotoksin. Koncentracija (srednja vrijednost ± SD) FB_1 bila je 459,5 ± 314,6 μg kg^{-1}, ZEA 1,70 ± 0,80 μg kg^{-1}, OTA 1,40 ± 0,55 μg kg^{-1}, dok su koncentracije FB_2 u dva pozitivna uzorka bile 68,4, odnosno 3084,0 μg kg^{-1}. Tako niske koncentracije mikotoksina nisu značajan izvor izloženosti ljudi. Međutim, nekoliko uzoraka koji sadrže vrlo visoke koncentracije mikotoksina ukazuju na potrebu kontrole mikotoksina u žitaricama.

Ključne riječi: fumonizin B_1, fumonizin B_2, kukuruz, okratoksin A, zearalenon