

CO-OCCURRENCE OF AFLATOXINS, OCHRATOXIN A, FUMONISINS, AND ZEARELENONE IN CEREALS AND FEED, DETERMINED BY COMPETITIVE DIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY AND THIN-LAYER CHROMATOGRAPHY*

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Aspergillus, *Penicillium*, and *Fusarium* species frequently contaminate crops. For this reason mycotoxins such as aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), and zearalenone (ZEA) are found in food and feed in a wide range of concentrations, depending on environmental and storage conditions. Consumption of mycotoxin-contaminated food and feed has been associated with acute and chronic poisoning and carcinoma. The aim of this study was to determine the incidence and co-occurrence of AFs ($B_1+B_2+G_1+G_2$), OTA, FBs ($B_1+B_2+B_3$), and ZEA in 37 samples of cereals and feed randomly collected in 2007 from households of an endemic nephropathy (EN) area in Croatia. The mycotoxins were determined using the competitive direct ELISA test (CD-ELISA) in combination with thin-layer chromatography (TLC). The most frequent mycotoxin was ZEA (92 %, mean $318.3 \mu\text{g kg}^{-1}$), followed by FBs (27 %, $3690 \mu\text{g kg}^{-1}$), AFs (24.3 %, $4.6 \mu\text{g kg}^{-1}$), and OTA (16.2 %, $9.8 \mu\text{g kg}^{-1}$). Levels of AFs, ZEA, and FBs detected by CD-ELISA significantly correlated with the TLC results. However, only one OTA-positive sample was confirmed by TLC due to its high limit of detection. The levels of these mycotoxins were below the permissible limit for animal feed. Twenty-nine percent of cereals were contaminated with FBs, OTA, or ZEA in mass fractions above the permissible limit for humans. Co-occurrence of two toxins varied between 4.2 % and 54 % and of three between 4.2 % and 7.6 %. Prolonged co-exposure to AFs, OTA, FBs, and ZEA might increase the risk of various chronic diseases.

KEY WORDS: *food contamination, mycotoxin synergism, mycotoxins, natural carcinogens*

Mycotoxins are secondary metabolites produced by various mould species. Aflatoxins ($AFB_1+B_2+G_1+G_2$) are mainly produced by *Aspergillus flavus* and *A. parasiticus*, while ochratoxin A (OTA) is produced by some *Penicillium* and *Aspergillus* species. These moulds usually contaminate stored crops. Fumonisins ($B_1+B_2+B_3$) and zearalenone (ZEA) are

produced by the *Fusarium* species, which are the most frequent pathogens of field crops in the mild climates. Mycotoxin contamination of food and feed depends on the distribution of toxigenic mould strains, micro-climate, harvesting techniques, and storage conditions. Sometimes this contamination may cause great economic loss (1-4).

From the public health point of view, AFs, OTA, FBs, and ZEA deserve special attention because they are frequently detected in cereals and feed

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and are known to have adversely affect human and animal health. AFs are well-known hepatotoxins and carcinogens for animals and humans, while OTA is a nephrotoxin with potential carcinogenic activity in humans. It has been associated with the development of endemic nephropathy (EN) (5, 6). Consumption of FBs-contaminated feed has been associated with various diseases in animals, including leukoencephalomalacia in horses, porcine pulmonary oedema, immunosuppression, liver and kidney toxicity, and liver cancer. In addition, fumonisin B₁ (FB₁) has been associated with human oesophageal carcinoma in South Africa and China. ZEA shows uterotrophic, oestrogenic, and anabolic activity in domestic animals, and has been implicated in hormonal interruption in humans (7, 8).

Techniques used to detect and measure mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), immunoassay, and their combinations. HPLC with fluorescence detection has increasingly been employed to detect OTA, AFs, or FBs due to very low limits of detection (9-11). TLC is still very popular in screening, despite its lower performance and relatively high limits of detection. Competitive direct enzyme-linked immunosorbent assay (CD-ELISA) is a very specific and very sensitive technique which makes use of monoclonal or polyclonal antibodies. The method makes it possible to analyse a large number of samples and does not require time-consuming procedures and sophisticated equipment (12).

The aim of this study was to determine the incidence and co-occurrence of AFs, OTA, FBs, and ZEA in cereals and feed collected in the EN region of Croatia combining CD-ELISA with TLC.

MATERIALS AND METHODS

Samples

Cereals and feed were randomly collected from households of four EN villages in the Croatian Brodsko-Posavska County (Oriovac District): Lužani (45°10'00"N, 17°42'28"E), Pričac (45°08'12"N, 17°40'51"E), Slavonski Kobaš (45°05'59"N, 17°44'37"E), and Živike (45°09'01"N, 17°40'51"E) in July of 2007. A total of 37 samples were analysed, including maize samples (N=12), maize-based feed (N=12) and one sample of oilseed rape that was mixed

in the feed (N=1), wheat (N=6), barley (N=4), and oat (N=2).

ELISA

AFs (B₁+B₂+G₁+G₂), OTA, FBs (B₁+B₂+B₃), and ZEA were analysed using CD-ELISA test kits (Veratox[®], Neogen Europe Ltd.). Ground samples (10 g) were extracted with 50 mL of 70 % methanol (Kemika, Croatia) and mycotoxins were analysed according to manufacturer instructions. The absorbance was measured at 650 nm using an ELISA reader (Awareness Stat Fax Reader, Neogen[®]). All samples and mycotoxin standards were analysed in duplicate. According to manufacturer descriptions, the detection limits (LOD) for AFs, OTA, FBs, and ZEA in cereals were 2 µg kg⁻¹, 1 µg kg⁻¹, 200 µg kg⁻¹, and 10 µg kg⁻¹ respectively.

TLC

Prior to TLC analysis, 30 mL of sample extracts in 70 % methanol were extracted with 30 mL of chloroform (Kemika, Croatia) and filtered through anhydrous Na₂SO₄ (Lach-Ner, Czech Republic). The samples were then evaporated to dryness and re-dissolved in 0.2 mL of chloroform for TLC analysis. Extracts were spotted on silica gel GF₂₅₄ (Macherey-Nagel GmbH, Germany) (for AFs, OTA, and ZEA) or 60 F₂₅₄ (Macherey-Nagel GmbH, Germany) (for FBs) along with mycotoxin standard solutions. For AFs, OTA, and ZEA analysis, plates were developed in toluene:ethylacetate:formic acid (5:4:1) (Kemika, Croatia). After air-drying, fluorescence intensities of toxin spots in samples were compared with mycotoxin standards under the UV light (366 nm). For FB₁ analysis, plates were developed in methanol:water (8:2) and after air-drying sprayed with 0.5 % *p*-anisaldehyde (Sigma-Aldrich Chemie GmbH, Germany) in methanol:acetic acid:sulphuric acid (85:10:5) (Kemika, Croatia) and heated at 110 °C for 10 min. FB₁ appears as a purple-red spot under the 366 nm UV light. The LODs for the analysed mycotoxins were as follows: 2 µg kg⁻¹ for AFB₁, 4.3 µg kg⁻¹ for AFB₂, AFG₁, and AFG₂, 60 µg kg⁻¹ for OTA, 1000 µg kg⁻¹ for FB₁, and 250 µg kg⁻¹ for ZEA.

Statistics

Mycotoxin concentrations detected by CD-ELISA were compared to TLC analysis using a correlation test to obtain linear regression equations and correlation coefficients.

RESULTS

Results of mycotoxin analysis in cereals and feed by ELISA and TLC method are shown in Table 1. The percentages of mycotoxins determined both by

ELISA and TLC were in order: ZEA>FB>AF>OTA. Concentrations of ZEA, FBs, and AFs detected by ELISA significantly correlated with results obtained with TLC. Correlation coefficients varied between 0.80 and 0.97 (Figure 1-3). We could not establish

Table 1. Determination of AFs, OTA, FBs and ZEA in cereals and feed using ELISA and TLC method

Mycotoxins	Samples	N	ELISA ¹			TLC ²		
			n	Mass fractions / µg kg ⁻¹		n	Mass fractions / µg kg ⁻¹	
				Mean	Range		Mean	Range
AF (B ₁ +B ₂ +G ₁ +G ₂) ³	Cereals (total)	24	5	3.2	2.0 to 4.5	1	-	5.2
	Maize	12	4	3.4	2.7 to 4.5	1	-	5.2
	Wheat	6	1	-	2.6	0	-	-
	Barley	4	0	-	-	0	-	-
	Oat	2	0	-	-	0	-	-
	Feed	13	4	6.9	4.2 to 10.3	3	5.7	5.0 to 6.8
	Total	37	9 (24.3 %)	4.6	2.0 to 10.3	4 (10.8 %)	5.5	5.0 to 6.8
OTA ⁴	Cereals (total)	24	4	10.1	2.5 to 31.7	1	133	133
	Maize	12	3	12.7	2.5 to 31.7 2.6	1	-	133
	Wheat	6	1	-	2.6	0	-	-
	Barley	4	0	-	-	0	-	-
	Oat	2	0	-	-	0	-	-
	Feed	13	2	9.2	5.4 to 12.9	0	-	-
	Total	37	6 (16.2 %)	9.8	2.5 to 31.7	1 (2.3 %)	-	133
FB (B ₁ +B ₂ +B ₃) ³	Cereals (total)	24	3	7600	200 to 20700	1	-	25000
	Maize	12	3	7630	200 to 20700	1	-	25000
	Wheat	6	0	-	-	0	-	-
	Barley	4	0	-	-	0	-	-
	Oat	2	0	-	-	0	-	-
	Feed	13	7	2300	200 to 5000	3	3900	2600 to 6100
	Total	37	10 (27.0 %)	3690	200 to 20700	4 (10.8 %)	9175	2600 to 25000
ZEA ⁴	Cereals (total)	24	21	112.0	12.5 to 1182	3	1481	1111 to 2222
	Maize	12	12	316.5	27.7 to 1182	3	1481	1111 to 2222
	Wheat	6	4	29.1	12.5 to 50.4	0	-	2222
	Barley	4	4	61.7	34.9 to 83.6	0	-	-
	Oat	2	1	-	18.4	0	-	-
	Feed	13	13	626.6	49.7 to 1168	6	1486	333.3 to 2222
	Total	37	34 (92.0 %)	318.3	12.5 to 118	9 (24.3 %)	1238	333.3 to 2222

¹LOD: AFs 2 µg kg⁻¹; OTA 1 µg kg⁻¹; FBs 200 µg kg⁻¹; ZEA 10 µg kg⁻¹

²LOD: AFB₁ 2 µg kg⁻¹; AFB₂, AFG₁, AFG₂ 4.3 µg kg⁻¹; OTA 60 µg kg⁻¹; FB₁ 1000 µg kg⁻¹; ZEA 250 µg kg⁻¹.

³AFG₁ and FB₁ was confirmed by TLC method

⁴Results of OTA and ZEA analysis by ELISA and TLC were published in Croatian in Krmiva 2008 (28)

N - number of samples; n - positive samples

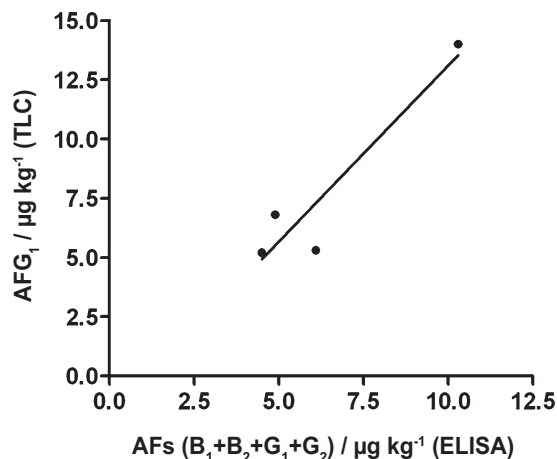


Figure 1 Correlation between CD-ELISA and TLC in determining AFG₁ ($r^2=0.8869$, $y=1.483x+1.173$)

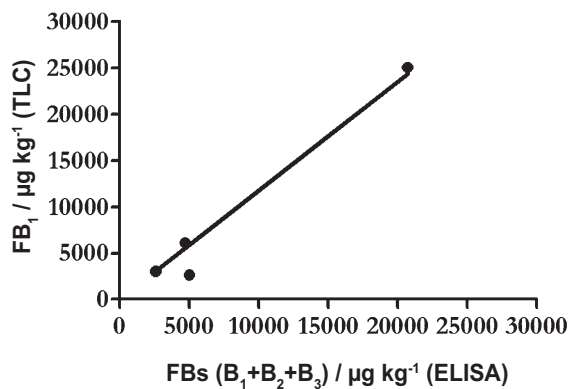


Figure 2 Correlation between CD-ELISA and TLC in determining FBs ($r^2=0.9736$, $y=1.258x+954.0$)

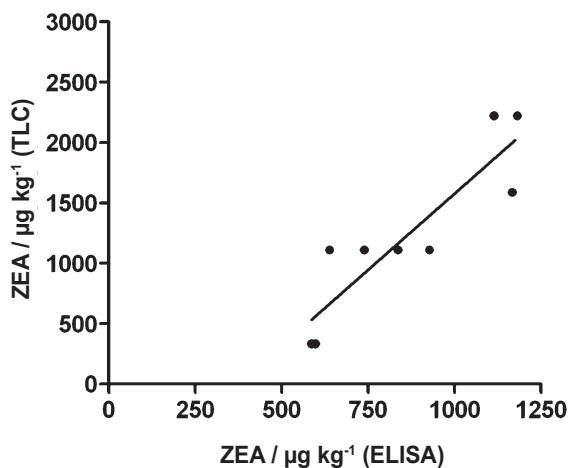


Figure 3 Correlation between CD-ELISA and TLC in determining ZEA ($r^2=0.8023$, $y=2.5205x+944.61.2$)

the correlation between the two methods for OTA because TLC detected OTA in one sample only. TLC confirmed the presence of FB₁ (Figure 4) and AFG₁

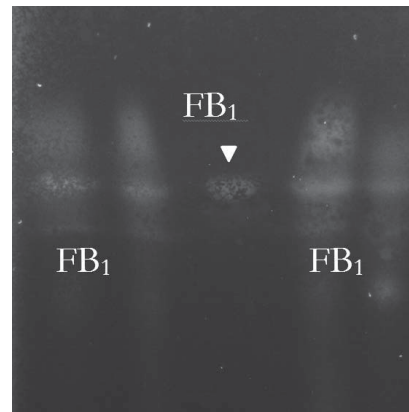


Figure 4 Chromatogram of FB₁ standard and FB₁ positive samples (UV 366 nm)

(Figure 5). It did not detect AFB₁, AFB₂ and AFG₂, and FB₂ and FB₃ were not analysed. FBs had the highest average concentrations in cereals and feed, followed by ZEA, OTA, and AFs. AFs determined by ELISA 16.2 % were under the LOD (1.1 µg kg⁻¹ to 1.4 µg kg⁻¹) (data not shown).

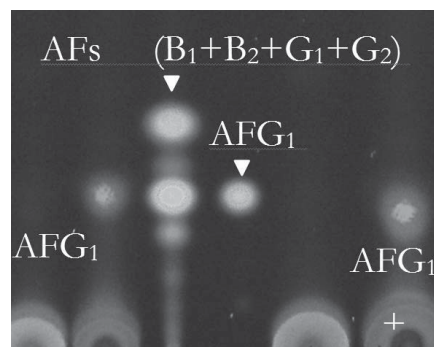


Figure 5 Chromatogram of AF, AFG₁ standards, and AFG₁ positive sample

Figure 6 shows the co-occurrence of two or three mycotoxins in cereal and feed samples. The most frequent two-toxin combination in the cereals was AFs+ZEA (17%) followed by AFs+OTA, OTA+ZEA and ZEA+FBs (12.5%). On the other hand, the most frequent two-toxin combination in the feed was ZEA+FBs (54 %), followed by AFs+ZEA (30 %) and OTA+ZEA (15 %). Three-toxin combinations were more frequent in cereals than in feed. All four toxins co-occurred in only one sample of maize at the following concentrations: 20700 µg kg⁻¹ of FBs, 639 µg kg⁻¹ of ZEA, 2.5 µg kg⁻¹ of OTA and 1.2 µg kg⁻¹ of AFs (data not shown)

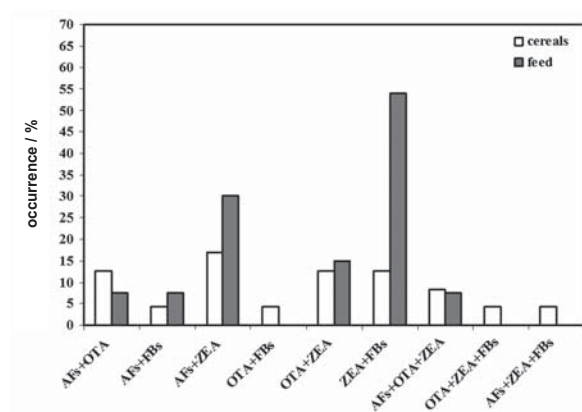


Figure 6 Co-occurrence of AFs, OTA, FBs, or ZEA in cereals and feed

DISCUSSION

Mycotoxin studies in Croatia over the past few decades showed that ZEA and FBs were more frequent in cereals (80 % to 100 %) than OTA (up to 40 %) or AFB₁, which was rare (11, 13-15). This study has confirmed ZEA's dominance with 92 % contamination of all analysed cereals and feed. FBs were detected only in maize and maize-based feed, and these findings are in accordance with most similar studies because *F. verticillioides* and *F. proliferatum*, which produce FBs, are principally maize pathogens (16). FBs have shown lower frequency (27 %) than in previous investigations. TLC confirmed FB₁ in only 10.8 % of samples, probably because of the high detection limit of the method (1000 µg kg⁻¹). AFs were detected in relatively many samples (24.3 %) in respect to earlier studies in Croatia. However, only AFG₁ (but not other AFs) was confirmed by TLC in this study, which is quite surprising because most of other studies detected (or analysed) only AFB₁. In a Turkish study using ELISA, total AFs were detected in all maize samples (mean 10.94 µg kg⁻¹) and in a Tunisian study in 50.5 % of cereals, dried fruit, and spices (mean 12.8 µg kg⁻¹) (17, 18). In Tunisian food, AFB₁ was confirmed in 37 % of samples. The frequency and levels of OTA contamination are in the range of previous reports based on HPLC analysis of OTA in cereals (11, 15). In general, mycotoxin levels reported in this study and their levels detected over the past few years are significantly lower than 20 to 30 years ago. This drop could be related to a relatively dryer weather in the past decade (13). Results of AFs, FBs, and ZEA obtained by ELISA significantly correlated with TLC, suggesting that these two simple methods

could be applied in combination for screening of these mycotoxins in cereals and feed. This however is not true for OTA, as TLC turns out not to be sensitive enough to determine low levels of OTA.

Nearly all mycotoxin mass fractions in our study were below the Croatian permissible levels for feed (5000 µg kg⁻¹ to 50000 µg kg⁻¹ for FB₁+FB₂ in a variety of feed, 3000 µg kg⁻¹ for ZEA, 250 µg kg⁻¹ for OTA, and 20 µg kg⁻¹ for AFB₁) (19). However, seven of 24 (29 %) cereal samples were contaminated with FBs, ZEA, or OTA in levels above permissible for humans (20). In one sample of maize intended for human consumption, FBs (20700 µg kg⁻¹) and ZEA (639 µg kg⁻¹) exceeded the national permissible level (4000 µg kg⁻¹ for FB₁+B₂+B₃ and 200 µg kg⁻¹ for ZEA). ZEA also exceeded the permissible level in five other samples (262.1 µg kg⁻¹ to 1182.0 µg kg⁻¹), while OTA (31.7 µg kg⁻¹) was above the permissible (5.0 µg kg⁻¹) in one maize sample. Total AF mass fractions were below the permissible limit for humans (4.0 µg kg⁻¹ to 10.0 µg kg⁻¹, depending on maize content in food).

Peraica and Domijan (21) reviewed the effects of AFs, OTA, FBs, and ZEA in humans. AFs and their metabolite M1 in cow's milk are true hepatocarcinogens (group 1A, IARC, 22), while FBs and OTA are proven nephrotoxins and carcinogens in animals (group 2B, IARC, 22, 23). Some studies suggest that OTA is associated with urinary tract tumours in the EN region, but this effect is still under debate (24). The carcinogenicity of ZEA has not been established, and IARC has classified it in group 3 (25). However, ZEA levels reported in cornflakes (13.0 µg kg⁻¹ to 20.0 µg kg⁻¹) stimulated proliferation of human carcinoma breast cells MCF7 (26), which suggests that ZEA could have a role in the development of breast cancer in women. Furthermore, ZEA contamination of maize products was associated with precocious sexual development (19). In addition, during sampling in Slavonski Kobaš, we observed symptoms of acute ZEA toxicosis in pigs, including oedema of the mammary and vulvar region (27). These pathological changes could be related to ZEA, which was detected in 80 % of feed in levels between 455 µg kg⁻¹ and 1168 µg kg⁻¹. Doses between 500 µg kg⁻¹ and 1000 µg kg⁻¹ in the feed have been reported to cause erythema and oedema in the mammary and vulvar region while higher doses (up to 5000 µg kg⁻¹) induce hyperoestrogenism (8).

From the public health point of view, this study points to an elevated risk for human and animal

health on two accounts. The first are concentrations of FBs, OTA, and ZEA above the permissible limits for humans and the second is the co-occurrence of two or three toxins in a high number of samples, particularly of feed. Jurjević et al. (14) detected higher levels and/or percent of co-occurrence of OTA and FBs in cereals in the EN region than in non-EN region in Croatia. Higher mycotoxin contamination in the EN region and Slavonia in general could be attributed to higher relative humidity in that area. On the other hand, FBs, OTA, and ZEA co-occurred in a high number of maize samples from several Croatian counties, but at lower concentrations (11). The established levels of mycotoxins and their co-occurrence, microclimatic conditions, and dietary habits of the EN residents suggest that people in the EN region are more exposed to mycotoxins than the rest of the Croatian population.

CONCLUSIONS

Chronic co-exposure to AFs, OTA, FBs, and ZEA and their possible synergism might affect the biochemical and immunological functions in animals and humans and lead to chronic diseases and cancer. Current permissible limits for these mycotoxins in food and feed do not take into account their possible cumulative effects. This calls for revision of current regulations, which would take into account the interaction of the most frequent mycotoxins in food and feed.

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Sažetak**ODREĐIVANJE AFLATOKSINA, OKRATOKSINA A, FUMONIZINA I ZEARELENONA U ŽITARICAMA I KRMIVU PRIMJENOM KOMPETITIVNOGA DIREKTOG IMUNOENZIMATSKOG TESTA (CD-ELISA) I TANKOSLOJNE KROMATOGRAFIJE (TLC)**

Vrste plijesni iz rodova *Aspergillus*, *Penicillium* i *Fusarium* česti su kontaminanti usjeva te na takvim supstratima tvore mikotoksine. Stoga su žitarice i krmiva često kontaminirana aflatoksinima (AFs), okratoksinom A (OTA), fumonizinima (FBs) i zearalenonom (ZEA) u različitim koncentracijama ovisno o mikroklimatskim uvjetima na polju i u skladištu. Konzumiranje hrane kontaminirane mikotoksinima često je povezano s akutnim ili kroničnim trovanjima, ali i s razvojem karcinoma. Cilj ovog rada bio je odrediti istodobnu pojavnost AFs ($B_1+B_2+G_1+G_2$), OTA, FBs ($B_1+B_2+B_3$) i ZEA u uzorcima žitarica i krme ($N=37$) koji su nasumično skupljeni u individualnim domaćinstvima na području endemske nefropatije (EN) u Hrvatskoj (2007). Za određivanje navedenih mikotoksina korišten je kompetitivni direktni ELISA-test (CD-ELISA) u kombinaciji s tankoslojnom kromatografijom (TLC). Najzastupljeniji mikotoksin bio je ZEA (92 %, srednja koncentracija $318.3 \mu\text{g kg}^{-1}$), nakon čega slijede FBs (27 %, $3690 \mu\text{g kg}^{-1}$), AFs (24.3 %, $4.6 \mu\text{g kg}^{-1}$) te OTA (16.2 %, $9.8 \mu\text{g kg}^{-1}$). Koncentracije AFs, FBs i ZEA određene CD-ELISA-testom statistički značajno koreliraju s rezultatima dobivenim s TLC. OTA je potvrđen metodom TLC samo u jednom uzorku zbog visokog limita detekcije. Dokazane koncentracije su ispod razina dopuštenih za krmiva, dok je 29 % uzoraka žitarica sadržavalo FBs, OTA ili ZEA u koncentracijama iznad dopuštenih u hrani za ljude. Kokontaminacija s dvama odnosno trima toksinima varirala je između 4.2 % i 54 % odnosno između 4.2 % i 7.6 %. Dugotrajni unos AFs, OTA, FBs i ZEA putem hrane može povećati rizik od razvoja različitih kroničnih bolesti zbog njihova mogućega sinergističkog djelovanja.

KLJUČNE RIJEČI: kontaminacija hrane, mikotoksini, prirodni karcinogeni, sinergizam mikotoksina

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