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PRESENCE OF CITRININ IN GRAINS AND ITS POSSIBLE HEALTH EFFECTS

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Abstract

Background: Citrinin is a mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus* and it occurs mainly in stored grain. Citrinin is generally formed after harvest and occurs mainly in stored grains, it also occurs in other plant products. Often, the co-occurrence with other mycotoxins is observed, especially ochratoxin A, which is usually associated with endemic nephropathy. At the European Union level, systematic monitoring of Citrinin in grains began with the aim of determining its highest permissible amount in food. Thus, far the systematic monitoring of the above mentioned mycotoxin in Croatia is yet to begin.
Materials and Methods: The main goal of this study was to determine the presence of Citrinin in grains sampled in the area of Međimurje, Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County. For the purpose of identification and quantification of citrinin, high performance liquid chromatograph (HPLC) with fluorescence was used (Calibration curve $k \geq 0.999$; Intra assay CV = 2.1%; Inter assay CV = 4.3%; LOQ < 1 µg/kg).
Results: From the area of Međimurje County, 10 samples of corn and 10 samples of wheat were analyzed. None of the samples contained Citrinin (<1 µg/kg). From the area of Osijek-Baranja and Vukovar-Srijem County, 15 samples from each County were analyzed. The mean value for the samples of Osijek-Baranja County was 19.63 µg/kg (median=15.8 µg/kg), while for Vukovar-Srijem County the mean value of citrinin was 14,6 µg/kg (median=1.23 µg/kg). From 5 analyzed samples from Brod-Posavina County, one of the samples contained citrinin in the amount of 23.8 µg/kg, while the registered amounts in the other samples were <1 µg/kg.
Conclusion: The results show that grains from several Counties contain certain amounts of Citrinin possibly indicating a significant intake of citrinin in humans. It must be stated that grains and grain-based products are the basis of everyday diet of all age groups, especially small children, where higher intake of citrinin can occur. Consequently, we emphasize the need for systematic analysis of larger amount of samples, from both large grains and small grains, especially in the area of Brod-Posavina County, in order to obtain more realistic notion of citrinin contamination of grains and to asses the health risk in humans.

Key words: Citrinin, cereals, Balkan nephropathy, risk assessment

Introduction

Citrinin (CTN) is a nephrotoxic mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium* and *Monascus*. CTN occurs in different plant products, especially in grains (Flajs & Peraica 2009, Főllmann et al. 2014), and also in beans, fruit, vegetables, herbs and spices. Often, the co-occurrence with other mycotoxins is observed, especially ochratoxin A (OTA) (Ostyr et al. 2013, Wawrzyniak & Waśkiewicz 2014, Petkova-Bocharova et al. 1991, Dietrich et al. 2001, Tangni & Pussemier 2006, Roy & Kumari 1991). It is a known fact that CTN occurs during fermentation of red mould rice as a secondary metabolite of *Monascus purpureus*. After this kind of fermentation, red mould rice abounds with anti-hypercholesterolemic monacolin K and CTN. Red mould rice has been used for lowering lipoprotein levels in blood and also as a food dye for centuries (Klimek et al. 2009, Zhang et al. 2013, Wang et al. 2003, Hsieh et al. 2013).

Besides its nephrotoxicity, which has been proved by various studies, there is also proof that CTN is involved in induction of apoptosis through oxidative stress, although the precise regulatory mechanism is yet to be determined (Chen & Chan 2009). CTN induces micronucleus formation of centromeres while OTA damages chromosomes in HepG2 cells. Together these structurally related mycotoxins can significantly increase the possibility of carcinogenesis in humans even though their mechanism of action completely differs (Knasműller et al. 2009). Because of their cytotoxicity, it was considered that CTN and OTA can induce Balkan endemic nephropathy (BEN). Recent research shows that BEN often occurs after the exposure to phytotoxin aristolochic acid (AA), produced by *Aristolochia clematitis*. Although there are studies which connect aristolochic acid to BEN, clinical symptoms and Chinese herbs nephropathy (CHN), we cannot neglect the connection between OTA, CTN and BEN (Jelaković et al. 2012, De Broe 2012, Anninou et al 2014, Phillips et al 1980, Vrabcheva et al. 2000, Pepeljnjak & Klarić 2010).

Multiple animal experimental models show different toxicological effects of both CTN and OTA; nephrotoxicity, hepatotoxicity, embriotoxicity, cytotoxicity, immunotoxicity, carcinogenicity and the induction of

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reactive oxygen species (ROS) (Sugiyama et al. 2013, Singh et al. 2013, Wu et al. 2012, Kumar et al. 2014, Islam et al. 2012, Mehdi et al. 1984, Vaselá 1983, Siraj et al. 1981, Kuroda 2014, Singh et al. 2014, Arai & Hibino 1983). CTN is not water-soluble and has the ability to bind to serum proteins (Merck index 11th edition 1989, Damodaran 1977).

We suggest an existence of a weak, which, possibly changes the electrical charge of albumins, creating the possibility of passage through the glomerular filtration system. Therefore, we can assume that there are at least several available binding locations for CTN on albumin. It is quite possible that frequent inability to filtrate albumin and other CTN-bound serum proteins could cause immunocomplex-induced nephritis. This can result in possible scaring and creation of fibrous tissue in the Bowman's capsule and later cause glomerular filtration impairment (Gamulin et al. 1998,). The tubule is unable to reabsorb CTN through the epithelium. Therefore, CTN creates ROS in the tubules of the nephrone which furthers the inflammatory process. This dynamic environment enables profound effects of more potent toxins; AA and OTA (Klarić et al 2013, Cavin et al. 2009, Mally 2012, Grollman et al. 2007,)

The highest permissible amount of citrinin in food was most recently added to the European Union legislation. This indicates that there is much concern about this particular type of mycotoxin. It has been suggested to the Member States that they monitor the levels of Citrinin in grains and grain-based products. The obtained results are collected by the European Food Safety Authority (EFSA) for the purpose of health risk assessment and determination of highest permissible amounts of Citrinin in different food categories (European food safety authority 2012). In EFSA's opinion CTN is a nephrotoxin and its studies are still essential for the purpose of collecting new information concerning the contamination of grains an grain-based products in food or feed.

Given that in Croatia there are areas where incidence of BEN has been recorded BEN, this study analyzed the samples of corn and wheat from the areas of Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County, and also from Međimurje County which was used as a control group.

Materials and Methods

For the purpose of this study, samples were divided into two groups. The first group consisted of corn and wheat samples from Međimurje County and the second group consisted of corn and wheat samples from Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County. The total of 55 samples were analyzed. From the first group, 10 samples of corn and 10 samples of wheat were analyzed. From the second group, 35 samples were analyzed. Samples from Međimurje County represented the control group in relation to Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County, where, regarding the occurrence of endemic nephropathy, the presence of certain amount of Citrinin was expected. The samples from Međimurje County were collected from local corn and wheat producers, while the samples from Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County were collected from the siloses where they were stored. The samples were collected during the years 2013 and 2014.

Sample preparation included weighing 10g of previously homogenized sample and adding 50 ml of methenolone (70%) followed by a 30minute extraction using the magnetic stirrerThe sample was then centrifuged at 4000 RPM for 10 minutes. A volume of 1 ml of supernatant was measured with a pipette and mixed with 49 ml of 10mM phosphoric acid (pH=7.5). The solution was then filtrated through glass fiber filter paper and purified by Solid Phase Extraction column (CitriTest HPLC, Vicam).

For the identification and quantification of Citrinin, High Performance Liquid Chromatography with fluorescence detection (HPLC-FLD) was used which proved to be a good technique for determining this type of mycotoxin in similar researches (European Safety Authority 2012). Citrinin concentration was determined using liquid chromatography (HPLC) (Agilent 1200, Santa Clara, California, USA) with fluorescence detection (FD) (Calibration curve $k \geq 0.999$; Intra assay CV = 2.1%; Inter assay CV = 4.3%; LOQ < 1 µg/kg). For more efficient division of samples and better signal resolution, a C-18 column was used (Waters, Milford, USA), dimensions of 50 x 4.6 nm, filer particle size of 2.5 µm, and 30ºC temperature of column.the mobile phase comprised of 80% 10mM phosphoric acid (pH=2.5) and 20% acetonitrile, and the flow was set to 0.5 ml per minute. Purified sample (50 µl) was injected in the HPLC system. Wave lengths of the detector were set to 350 nm for excitation and 500 nm for emission. The duration of analysis was 10 minutes with 80-110 % yield, which is within the criteria prescribed by Directive (Commission Regulation (EU) No 519/2014).

Results

Results of this study are shown in Tables 1 – 5. Table 1 shows the determined Citrinin levels in Osijek-Baranja and Vukovar-Srijem County, while Table 2 shows the results for the analyzed samples from Međimurje County. Table 3 contains the results of the analyzed samples of wheat from Brod-Posavina County. Table 4 shows the result of the Kruskal-Wallis test for all of the samples. Figure 3 contains data on the sample distribution per county. Table 5 shows the results of the Kruskal-Wallis test for corn samples alone. Figures 1 – 2 show a spike in the chromatogram (standard and positive sample) in the correct retention time for CTN.

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Table 1: Amount of cintrinin in analyzed samples of corn from osijek-baranja and vukovar-srijem county

No	Sample type	County	Amount (μg/kg)	County	Sample type	Amount (μg/kg)
1	Corn	Osijek-Baranja	28.3	Vukovar-Srijem	Corn	12
2	Corn	Osijek-Baranja	10	Vukovar-Srijem	Corn	103
3	Corn	Osijek-Baranja	52.4	Vukovar-Srijem	Corn	7.24
4	Corn	Osijek-Baranja	24	Vukovar-Srijem	Corn	13.5
5	Corn	Osijek-Baranja	6.77	Vukovar-Srijem	Corn	0.9
6	Corn	Osijek-Baranja	19.5	Vukovar-Srijem	Corn	65.9
7	Corn	Osijek-Baranja	22.3	Vukovar-Srijem	Corn	<1
8	Corn	Osijek-Baranja	70	Vukovar-Srijem	Corn	<1
9	Corn	Osijek-Baranja	7.5	Vukovar-Srijem	Corn	3.5
10	Corn	Osijek-Baranja	<1	Vukovar-Srijem	Corn	<1
11	Corn	Osijek-Baranja	15.8	Vukovar-Srijem	Corn	5.9
12	Corn	Osijek-Baranja	<1	Vukovar-Srijem	Corn	<1
13	Corn	Osijek-Baranja	10.3	Vukovar-Srijem	Corn	<1
14	Corn	Osijek-Baranja	<1	Vukovar-Srijem	Corn	1.39
15	Corn	Osijek-Baranja	25	Vukovar-Srijem	Corn	1.23
Mean value			**19.64**	**Mean value**		**14.60**
Median			**15.8**	**Median**		**1.23**

Table 2: Amount of citrinin in analyzed samples of corn and wheat from međimurje and brod-posavina county

No	Sample type	County	Amount (μg/kg)
1	Corn	Međimurje	<1
2	Corn	Međimurje	<1
3	Corn	Međimurje	<1
4	Corn	Međimurje	<1
5	Corn	Međimurje	<1
6	Corn	Međimurje	<1
7	Corn	Međimurje	<1
8	Corn	Međimurje	<1
9	Corn	Međimurje	<1
10	Corn	Međimurje	<1
11	Wheat	Međimurje	<1
12	Wheat	Međimurje	<1
13	Wheat	Međimurje	<1
14	Wheat	Međimurje	<1
15	Wheat	Međimurje	<1
16	Wheat	Međimurje	<1
17	Wheat	Međimurje	<1
18	Wheat	Međimurje	<1
19	Wheat	Međimurje	<1
20	Wheat	Međimurje	<1

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Table 3: Amount of citrinine in analyzed samples of wheat from brod-posavina county

No	Sample type	County	Amount (µg/kg)
1	Wheat	Brod-Posavina	<1
2	Wheat	Brod-Posavina	<1
3	Wheat	Brod-Posavina	<1
4	Wheat	Brod-Posavina	<1
5	Wheat	Brod-Posavina	23.8

Tabel 4: Wheat and Corn (whole sample)

	County	**N**	**Minimum**	**Maximum**	**Percentiles**		
					25th	**50th (Median)**	**75th**
CITRININ (µg/kg)	Brod-Posavina	5	0,90	23,80	0,90	0,90	12,35
	Osijek-Baranja	15	0,90	70,00	6,77	15,80	25,00
	Vukovar-Srijem	15	0,90	103,00	0,90	1,39	12,00

	Kruskal Wallis	**df**	**P**
CITRININ (µg/kg)	6,460	2	,052

Table 5: Corn (only corn samples)

	County	**N**	**Minimum**	**Maximum**	**Percentiles**		
					25th	**50th (Median)**	**75th**
CITRININ (µg/kg)	Osijek-Baranja	15	0,90	70,00	6,77	15,80	25,00
	Vukovar-Srijem	15	0,90	103,00	0,90	1,39	12,00

	Kruskal Wallis	**df**	**P**
CITRININ (µg/kg)	3,500	2	,061

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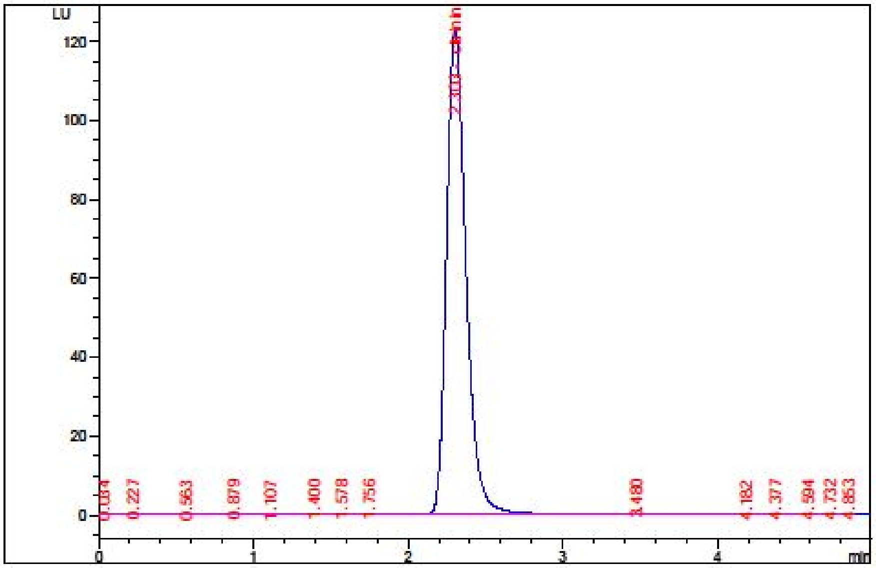

Meas. Ret.	Area	Amount	Compound Name
2.303	1071.2444	27.7538	citrinin

Figure 1: Chromatogram of a Citrinin standard

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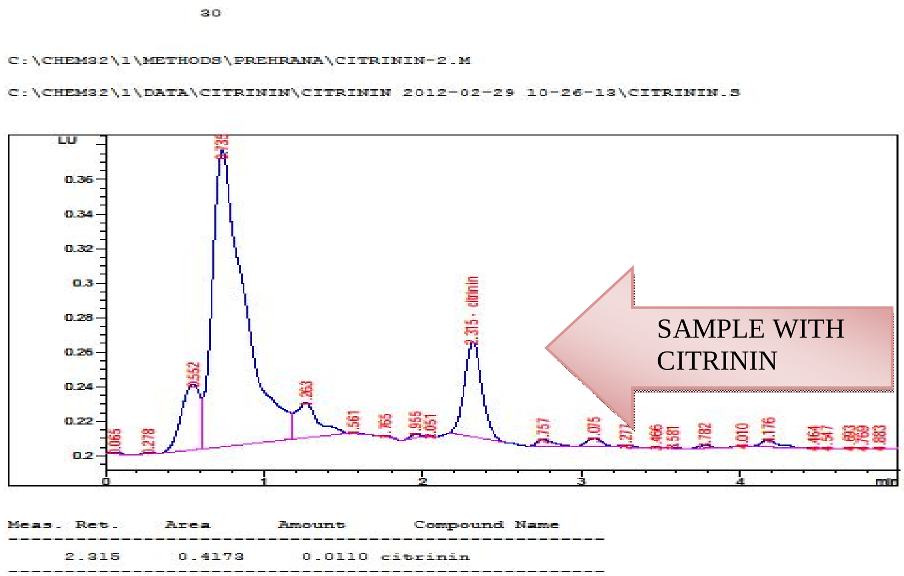

Figure 2: Chromatogram of A Sample Containing Citrini

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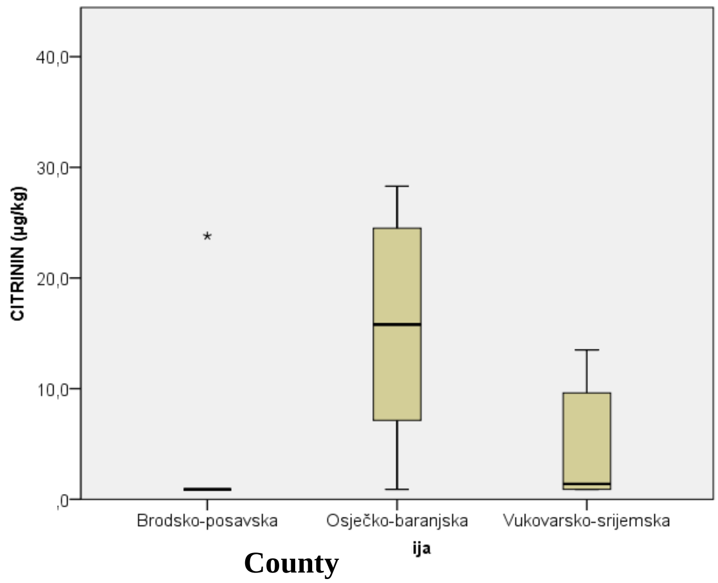

Figure 3: Kruskal-Wallis Test: Highest Values of Citrinin Have Been Found in Osijek-Baranja County.

Discussion

For the occurrence of mycotoxins in food, Citrinin included, suitable temperature, humidity conditions and toxigenic molds must be present. They occur in food during growth, storage, technological processes and transport (Flajs & Peraica 2009). Their toxicity and carcinogenicity has been proved and described in numerous professional and scientific studies (Sugiyama et al. 2013, Singh et al. 2013, Wu et al. 2012, Kumar et al. 2014, Islam et al. 2012, Mehdi et al. 1984, Vaselá 1983, Siraj et al. 1981, Kuroda 2014, Singh et al. 2014, Arai & Hibino 1983). For that reason, much attention is given to the monitoring of food for mycotoxin occurrence. For the purpose of determining the highest permissible amounts, the European Union has mandated the monitoring of Citrinin in Member States and submitting the data to EFSA. Croatia was not yet involved in this type of research, although Citrinin is a mycotoxin which could, for the reason of its toxicity and association to BEN, be of great importance for resolving its emergence in endemic and other areas. Recent research, conducted in endemic areas in Croatia, refer mostly to the monitoring of OTA (Puntarić et al. 2001), although in scientific papers it is often associated with CTN (Petkova-Bocharova et al. 1991, Vrabcheva et al. 2000) or presence of aristolochic acid (Stiborová 2013).

In this study three counties, associated with the emergence of BEN, were included; Osijek-Baranja, Vukovar-Srijem and Brod-Posavina County. The research included Međimurje County as well, with no BEN incidence, so its samples served as a control group. As could have been expected, none of the 10 samples of wheat and 10 samples of corn, collected from Međimurje County, contained the presence of Citrinin CTN (<1 µg/kg). Five samples of wheat were analyzed from Brod-Posavina County. One sample contained 23.8 µg/kg of citrinin, while other 4 samples contained no citrinin (<1 µg/kg). From Osijek-Baranja Count 15 samples of corn were collected, and 80% of the samples were positive for Citrinin. The values ranged from <1 µg/kg to 52.4 µg/kg, with the mean value of 19.64 µg/kg (median= 15,8 µg/kg). From Vukovar-Srijem County 15 samples of corn were collected and 66.7% of them contained Citrinin. The analysis determined the values ranging from <1 to 103 µg/kg, mean value being 14.6 µg/kg (median = 1.23 µg/kg). Recorded values confirm the presence of Citrinin in corn, in the areas of Croatia where the incidence of BEN has been recorded. Results of this study are in correlation with similar studies conducted in Bulgaria (Petkova-Bocharova et al. 1991), but also with the results of the French authors whose research proved the presence of Citrinin in food (Molinié 2005).

Conclusion

The results of the conducted study confirm the presence of Citrinin in grains from the sampled areas. Given that some of the areas researched are connected to BEN incidence, the studies must include larger number of grains and grain-based products and encompass larger geographical area for the purpose of more effective human health risk assessment.

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