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ELISA AND HPLC ANALYSES OF DEOXYNIVALENOL IN MAIZE AND WHEAT

ABSTRACT: Deoxynivalenol (DON) is a part of the family of mycotoxins called trichothecenes which are produced by a number of different *Fusarium* mold species. The presence of DON in 25 wheat and 25 maize samples was examined by Enzyme Linked Immunosorbent Assay (ELISA) and High Performance Liquid Chromatography (HPLC) methods. The presence of DON was detected and determined in 5 (20%) maize and 6 (25%) wheat samples by both of the methods. Correlation between ELISA and HPLC results was established, with the correlation coefficients (r) of 0.9691 and 0.9735 for wheat and maize samples, respectively. The results obtained by ELISA method were significantly higher than those obtained by HPLC method. This fact can be explained by the presence of conjugated or masked mycotoxins in the samples, especially DON-3-glucoside (DON-3-Glc), which could not be determined by HPLC method due to the lack of external standards. Contrary to this, being insufficiently selective towards masked DON, ELISA method measures total DON content of a sample. According to the obtained results, ELISA can be used as a reliable screening method, but the confirmation of positive results must be done by HPLC method.

KEY WORDS: Deoxynivalenol, ELISA, HPLC, maize, wheat

INTRODUCTION

The presence of mycotoxins in agricultural products, mostly grains, causes a potential hazard to the health of humans and animals (Bennett et al., 2003). Mycotoxin presence depends on several factors, such as fungal strain, climatic and geographical conditions, cultivation technique, and crop protection, particularly during storage (Lo Curto et al., 2004).

Trichothecenes are a large group of agriculturally important mycotoxins produced by many *Fusarium* species. According to their chemical structure, they have been classified into four groups: A–D types; A and B are the most frequently found in cereals (W H O , 1990).

Deoxynivalenol (DON, vomitoxin) is the main representative of trichothecene B mycotoxins, as well as the most frequently detected trichothecene. This mycotoxin occurs predominantly in grains such as wheat, barley, oats, rye and maize, and less often in rice, sorghum, and triticale.

The occurrence of DON is associated primarily with *F. graminearum* (*Gibberella zeae*) and *F. culmorum*, both of which are important plant pathogens causing Fusarium head blight in wheat and Gibberella ear rot in maize (B e y e r et al., 2006).

In contaminated crops, deoxynivalenol-3-glucoside (DON-3Glc), 3-acetyl--deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) can co-occur in significant amounts with DON. These mycotoxins are also produced by *Fusarium* species, and they are equally or less toxic than DON (P e s t k a , 2008).

Maize and wheat cover about 1,200,000 and 570,000 ha, respectively, which makes them very important crops grown in Serbia. Due to heavy rain season (2009/2010) just before the harvest time, the expected quality of the wheat and maize crop was below average, since moisture level was up to 19 percent (H u y n h et al., 2010). Although the consumption of maize and wheat has been increased, there are no sufficient data about the contamination of crops with DON in Serbia. Moreover, the maximum permitted level of DON in food has not been set in Serbia yet.

On the other hand, allowed limits of DON in food have been established in many countries. Maximum tolerated levels of DON in food on the territory of European Union are regulated by uniform regulations for the entire European Union (EC, No. 1881/2006). According to the regulation, MTLs for different cereal products are as follows: unprocessed cereals other than durum wheat, oats and maize – 1250 μg/kg; unprocessed durum wheat, oats and unprocessed maize – 1750 μg/kg; cereal flour, including maize flour, maize grits and maize meal, pasta – 750 μg/kg; bread, pastries, biscuits, cereal snacks and breakfast cereals – 500 μg/kg; processed cereal-based food for infants and young children and baby food – 200 μg/kg.

As DON represents potential hazard to the health of humans and animals, there is a need to control the presence of the most common trichothecenes in cereals and cereal based food by sensitive and reliable methods. Several methods can be used for DON analysis: thin-layer chromatography (TLC), enzyme-linked Immunosorbent Assays (ELISA), gas chromatography (GC) and lipid chromatography with different detectors (W H O, 2001). The chosen method depends on several factors. Nowadays, ELISA and HPLC are the most ***commonly used methods*** for determination of DON in maize and wheat. Major advantages of ELISA method are minimal sample clean-up procedures, simple procedure and low prices. Major disadvantage of ELISA method is

possible cross-reactivity to similar compounds. Therefore, confirmation by HPLC based procedure is required (A n k l a m et al., 2002).

The aim of this work was to determine DON content in wheat and maize samples using ELISA and HPLC methods, and to compare the obtained results.

MATERIALS AND METHODS

Samples

Twenty five wheat and twenty five maize samples were collected in Vojvodina. Samples were collected during November 2010, and stored at 4 °C in refrigerator before analysis.

Determination of DON by ELISA

Neogen Veratox® DON 5/5 test kits were used, and the procedure provided by the manufacturer was applied for the analyses. Free DON in the samples and controls was allowed to compete with enzyme-labelled DON (conjugates) for the antibody binding sites. After washing, substrate was added, which reacted with the bound conjugate to produce blue color. More blue color meant less mycotoxin. The test was read in a microwell reader (Thermolabsystem, Thermo, Finland) to yield optical densities. The optical densities of the controls formed standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of mycotoxin. According to the manufacturer's description (Veratox®, Neogen), the detection limit for DON is 0.25 mg/kg.

Sample preparation. 10 g of ground sample was extracted with distilled water and shaken for 3 minutes. After filtration, the sample was ready for testing.

Determination of DON by HPLC-DAD

Determination of DON was carried out by HPLC Agilent 1200 model equipped with an Agilent diode array detector (DAD), Chemstation Software, a binary pump, a vacuum degasser and an auto sampler. The column was an Agilent column Hypersil ODS (100 x 4.6 mm, particle size 5 μm). The mobile phase consisted of an isocratic mixture of water/acetonitrile (86:14, v/v), with a flow rate of 0.8 ml/min. Fifteen microliters of standards and samples were injected onto the HPLC column. The spectra were recorded at 220 nm. Identification of DON was done by comparing the retention times and spectra of DON from samples with those of the standards.

Sample preparation. Around 25g of maize and wheat samples was extracted with water/acetonitrile (16:84, v/v) and shaken on ultraturrax for 3

minutes. After filtration through Advantec filter paper, extract was cleaned up on MycoSep column. The purified extract was evaporated to dryness under vacuum, redissolved in 3 ml of ethyl acetate and quantitatively transferred to an evaporation vessel by triple washing with 1.5 ml ethyl acetate. The eluate was evaporated to dryness, and redissolved in 300 µl of methanol.

RESULTS AND DISCUSSION

Twenty five wheat and twenty five maize samples were investigated. The first part of this study included determination of DON content by ELISA method. DON was found in 25.0% of wheat and 20.0% of maize samples. Positive samples were further analyzed by HPLC method which confirmed the presence of DON in all of them. The contamination frequencies and average values of mycotoxin content in the examined maize and wheat samples are shown in Table 1.

Tab. 1 – Contamination frequency (CF), interval (CI), and mean (CM ± SD) of maize and wheat samples

Samples (SN)	ELISA		HPLC	
Maize (25)	CF	20	CI	0.18 – 0.62
	CI	0.35 – 1.27		
	CM	0.75 ± 0.43	CM	0.39 ± 0.21
Wheat (25)	CF	25	CI	0.69 – 1.37
	CI	1.69 – 2.00		
	CM	1.83 ± 0.14	CM	1.00 ± 0.32

SN: sample number, SD: standard deviation, CF (%), CI (mg/kg), CM (mg/kg)

Content of DON in maize samples was 0.75 ± 0.43 mg/kg and 0.39 ± 0.21 mg/kg determined by ELISA and HPLC, respectively. Referring to the obtained results, content of DON in maize samples, determined by both of the methods, was in accordance with the European regulation (1.75 mg/kg). DON content in wheat samples was 1.83 ± 0.14 mg/kg and 1.00 ± 0.32 mg/kg determined by ELISA and HPLC methods, respectively. Positive results for wheat (3 out of 25) obtained by ELISA were higher than the maximum permitted value of 1.75 mg/kg. Contrary to this, positive results determined by HPLC method were lower than the maximum level allowed by European Regulation. J a j i ć et al. (2008) analyzed DON in maize (76 samples) and wheat (18 samples) during 2004 and 2005. The number of positive samples in maize and wheat was 44.7% and 37.5%, respectively.

The obtained results (Figure 1) indicate that ELISA results are significantly higher than the results obtained by HPLC method. This can be explained by the existence of conjugated or masked mycotoxins. The name of masked mycotoxins was derived as these substances escape routine mycotoxin detection methods, but can release their toxic precursors after hydrolysis

inside the gastrointestinal tract. DON-3-Glc, 3-ADON and 15-ADON appear quite often with DON in barley, maize, wheat, and other cereals. The similar molecular structure of DON and masked mycotoxins can be the reason for their competition in the antigen–antibody reaction. This phenomenon is called cross-reactivity and it can be the reason for overestimation and false results obtained by ELISA method (C a v a l i e r et al., 2005; A u r e l i et al., 2006; R u p r i c h et al, 2008; J a j i ć , 2008). B e r t h i l l e r et al. (2005) reported DON-3-Glc as the major form of masked DON, constituting up to 12% of total content of this mycotoxin in the examined samples of wheat and maize.

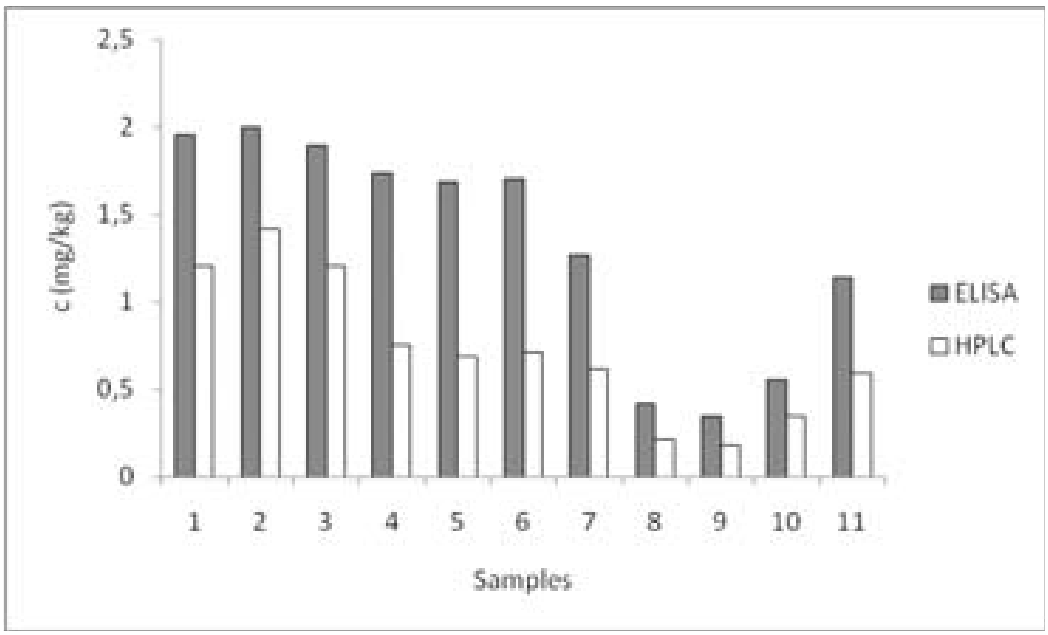

Fig. 1 – Comparison of ELISA and HPLC methods for DON determination in wheat (1-6) and maize (7-11) samples

R u p r i c h et al. (2008) found high level of cross reactivity of antibodies against DON mycotoxin with DON-3-Glc high, 82 and 98% in two repeated tests. They concluded that analytical results produced by ELISA could be interpreted as an approximate sum of DON and DON-3-Glc.

It should be emphasized that ELISA method used for determination of DON content cannot distinguish between DON, DON-3-Glc, 3-ADON, 15--ADON, and other similar compounds (J E C F A , 2001).

The correlation coefficients (r) between ELISA and HPLC data for wheat and maize samples were 0.9691 and 0.9735, respectively (Figure 2). The obtained values were in accordance with the results of other authors (A v a n t a g g i a t o et al., 2007).

Based on the given results, it can be concluded that immunoenzymatic methods are highly valuable for quantitative screening of DON presence in

food samples. Although ELISA gives overestimated results to some extent, we can fully rely on the results which are under the maximum allowed level. However, the confirmation of doubtful and/or positive ELISA results by HPLC must be done (A n k l a m et al., 2002).

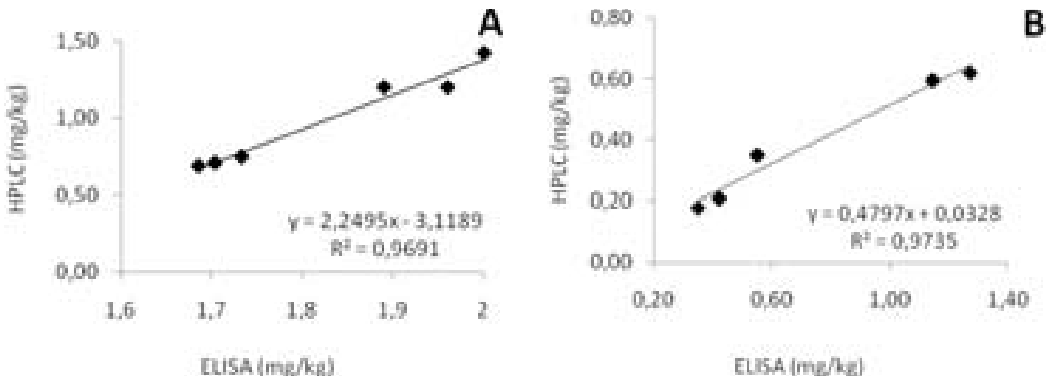

Fig. 2 – Correlation between HPLC and ELISA data for DON determination in wheat (A) and maize (B) samples

CONCLUSION

This study indicates the existence of risk related to the occurrence of DON in the food chain of Serbia, and importance of frequent monitoring of this mycotoxin. It is also important to establish maximum permitted level of DON in food at a national level.

The obtained results confirm that ELISA method can be used as a screening method for DON determination. However, confirmation of doubtful and/or positive ELISA results by HPLC method is required.

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АНАЛИЗЕ ДЕОКСИНИВАЛЕНОЛА У ПШЕНИЦИ И КУКУРУЗУ ПОМОЋУ ЕЛИСА И HPLC МЕТОДА

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Резиме

Деоксиниваленол (DON) припада групи микотоксина који се зову трихотецене, а које производе разне врсте плесни из рода *Fusarium*. Присуство DON-а у 25 узорака пшенице и 25 узорака кукуруза су испитани помоћу метода ELISA и течном хроматографијом под високим притиском (HPLC). Обе методе су потврдиле присуство DON-а у 5 узорака кукуруза (20%) и 6 узорака пшенице (25%). Утврђен је корелациони коефицијент (p) резултата добијених помоћу ELISA и HPLC метода, што је 0,9691 за узорке пшенице и 0,9735 за узорке кукуруза. Знатно виши резултати су добијени ЕЛИСА методом, у односу на HPLC метод. Ово се може објаснити присуством конјугованих микотоксина у узорцима, нарочито DON-3-глукозида (DON-3-Glc), који се не може утврдити HPLC методом због недостатка спољашњих стандарда. Насупрот томе, ELISA не прави довољну селекцију конјугованог DON-а, али мери његов укупан садржај у узорку. На основу добијених резултата утврђено је да је ELISA поуздан метод, али да се позитивни резултати морају добити HPLC методом.