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Original research paper

DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE CITRININ DETERMINATION IN RED RICE

Gorica Vuković[1,*], Marina Đukić[1], Vojislava Bursić[2], Tijana Stojanović[2], Aleksandra Petrović[2], Slobodan Kuzmanović[3], Mira Starović[3]

[1]Institute of Public Health, Bulevar despota Stefana 54a, 11000 Beograd, Serbia
[2] University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
[3]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Beograd, Serbia

*Corresponding author:
E-mail address: goricavukovic@yahoo.com

ABSTRACT: The liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the citrinin (CIN) determination in red rice was developed. The mycotoxin was extracted from red rice using anetonitrile/water/acetic acid mixture followed by the clean-up step on Captiva EMR cartridges. The developed method was validated according to the Commission Regulation No. 401/2006/EC. The validation data were evaluated in terms of recoveries, reproducibility, limits of quantification (LOQ), limit of detection (LOD), specificity, linearity and matrix effects for CIN in matrix using 13C20-OTA as an internal standard. The obtained method performance parameters indicate that the method is suitable for the CIN routine analysis.

Key words: *citrinin, validation, LC-MS/MS*

INTRODUCTION

The attention drawn by mycotoxins has been increasing due to their extensive occurrence and highly frequent cases of contamination including severe toxic effects on animal and human well-being (Zöllner and Mayer-Helm, 2006; De Girolamo et al., 2017). More than 400 mycotoxins have been detected in different agricultural commodities. The fungal production of mycotoxins depends on the weather conditions, damage caused by insects during the different stages of production, as well as on the environmental conditions including temperature and moisture content during the storage and the transportation of products (Donga et al., 2019).

CIN is a polyketide mycotoxin with mutagenic and carcinogenic properties and the molecular formula $C_{13}H_{14}O_5$, (IUPAC: (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid). It has been synthesized by several fungal species of the genera *Aspergillus*, *Monascus* and *Penicilium* during the products storage. It can be detected in a wide variety of cereals (corn, wheat, barley, rye, rice, oat and linseed) and their products, sunflower seeds, black olives, fruit juices and apples, almonds, hazelnuts, pistachio nuts, peanuts, spices (black pepper, coriander, cardamom, cumin, turmeric and fennel) along with red yeast rice-based food supplements (rice fermented with red microfungi *Monascus purpureus*) (Ostry et al., 2013; Vuković et al.,

2017). The highest concentrations of CIN discovered in food and feed were 420 and 998 µg/kg, respectively (EFSA, 2012). CIN was also found in the samples of dried ginger in the concentration of 85.1 ng/g (Jaswal and Kumar, 2015) and also in the human urine (Meerpoel et al., 2018). The concentration of CIN in different products may vary from 0.1 to 500 mg/kg (Li et al., 2012).

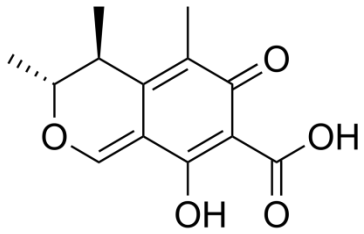

Figure 1. Structural formula of citrinin

According to the Commission Regulation (EC) 1881/2006, as amended by the Regulation (EU) 212/2014, the maximum level of 2000 µg/kg of citrinin in red yeast rice-based food supplements is to be reviewed before 1 January 2016 considering the new information about the exposure to CIN from the other food products and its toxicity, primarily its carcinogenic and genotoxic properties.

CIT disrupts renal function which leads to the necrosis of the distal tubule epithelium and degeneration and function alteration of the renal tubules. The cereals and their products are considered to be the main source of the dietary exposure to CIT (EFSA, 2012).

Some of the methods used for the mycotoxin extractions so far include a liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), solid phase extraction (SPE), pressure liquid extraction (PLE), matrix solid-phase dispersion (MSPD) and ultrasound and homogenizing extraction with the various mixtures of organic solvents. "Green Analytical Chemistry" is a trend derived from the desire and need for the environmental friendlier chemical analyses which are evolving very quickly (Breidbach, 2017). Because of that fact QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) has been identified as the most modern extraction and extract purification method (Cunha and Fernandes, 2010; Capriotti et al., 2012). It has drawn attention in the research field of mycotoxins because of its simplicity and effectiveness for the isolation of mycotoxins from complex matrices and has also gained the popularity as an environmental friendly technique (Vuković et al., 2017a).

The aim of this study was to establish a sensitive and accurate LC-MS/MS method for the determination of citrinin in read rice, through the validation according to the Regulation (EU) 212/2014.

MATERIAL AND METHODS

Chemicals and apparatus

Citrinin and 13C20-Ochratoxin A (13C20-*OTA*) (used as an internal standard) reference standards were obtained from Biopure (RomerLab, Inc. America, Cat). Stock standard solution of CIN was obtained in acetonitrile EITH CONC 100,4±0,6 µg/mL (100). The working standard solution was prepared at 5 µg/mL with acetonitrile and stored in the

dark at -20 C. HPLC grade methanol and acetonitrile (100% purity) were obtained from J.T. Baker Chemicals (Netherlands). Formic and acetic acid were purchased from Fisher Scientific UK (Loughborough, UK). The Captiva EMR-Lipid 3 ml cartridges Agilent part No. 5190-1003 were purchased from Agilent (Agilent Technologies Inc. Folsom).

An Agilent series 1200 HPLC system (Agilent Technologies) equipped with a G1312B binary pump, a G1367D autosampler, a G1379B degasser, a G1316B column compartment temostat, The HPLC system was coupled to an Agilent triple quadrupole mass spectrometer (6410B) coupled to an electrospray ionization source (ESI+). A Zorbax XDB C18 column (50x4.6 mm, 1.8 µm particle size) from Agilent (San Jose, CA, USA) was employed for the separation. The chromatographic determination of CIN was carried out employing a binary mobile phase with methanol (0.1% HCOOH, v/v - A) and an aqueous solution of formic acid (0.1%, v/v - B). A gradient elution started at 60% of B at flow rate of 0.4 mL/min. This composition was reduced to 30% B in 10 min, and held for 5 min. The system was equilibrated during 5 min. The injection volume was 5 µL and column temperature was kept at 30 ºC. The ESI source values were as follows: drying gas (nitrogen) temperature 350 °C, drying gas flow rate 10 L/min, nebulizer pressure 40 psi and capillary voltage 4000 V. The detection was performed using the multiple reactions monitoring mode (MRM). The Agilent MassHunter software (version B.06.00 Agilent Tehnologies, 2012) was used for the optimization and quantification.

Validation parameters

The proposed method was validated by determining the limits of quantification (LOQs), and the limit of detection (LODs). The lineary was estimated using four concentration levels 200; 400; 1000 to 2000 µg/kg (corresponding four fortification levels on 10, 20, 50 and 100 ng/mL). The recoveries was done at two concentration levels on LOQ limit of 200 µg /kg and MRL level of 2000 µg /kg in three replicates. The method precision is expressed as the repeatability (RSD%) based on the recovery experiments. The LOD was estimated from the chromatogram of the lowest level of calibration using the Agilent MassHunter software (Agilent Technologies, B.06.00) for those concentrations that provide a signal to noise ratio of 3:1. The LOQ was defined as the reference value 200 µg/kg in consideration of Commission Recommendation (EU) 1881/2006, as amended by Regulation (EU) 212/2014.

Sample preparation

A 5 g of homogenized and spiked red rice samples were placed into erlenmayer flasks of 250 mL. A 100 mL of solution of anetonitrile/water/acetic acid (79/20/1, v/v/v) was added to each erlenmayer flasks and extracted in ultrasound bath for 30 min. After the extraction, the samples were filtrated (Wathman No 41) and cleaned-up through Captiva EMR cartridges. The 0.5 mL of eluate was evaporated to dryness and dissolved in the mixture of methanol/water (81/1, v/v) with 0.1% of HCOOH. Transfer 100 µL in glass insert, add 25 µl of IS 13C20-*OTA* and do the LC-MS/MS analysis.

RESULTS AND DISCUSSION

The LC-MS/MS (ESI+) fragmentation of the protonated molecular ion of CIN, which yielded three product ions and 13C20-OTA with one product ion are given in Table 1.

Table 1. MRM transitions, fragmentation and colision energies

Analite	Rt (min)	Transitions (*m/z*)	CE (V)	Frag. (V)	Analite	Rt (min)	Transitions (*m/z*)	CE (V)	Frag. (V)
CIN	9.95	251.1 >233.3	17	66	13C20-*OTA*	15.29	424.2>250.1	25	120
		>205.2	29	66					
		> 91.3	40	66					

Validation parameters

Specificity, LOD and LOQ. Limit of quantification (LOQ) were taken as the lowest fortification level and it was set on 200 µg/kg and the limits of detection (LOD) were calculated by MassHunter software from signal/noise ratio at the same fortification level. No interference peaks (S/N>3) were detected in the blank sample in the range of the retention time ± 0.1 min for the CIN, conforming the method specificity.

Linearity

Linearity in the predefined ranges was evaluated by the coefficient of correlation (R^2>0.99). The coefficient of correlation of CIN in the mobile phase was 0.9981 (Figure 2), while in the matrix it was 0.9973, respectively (Figure 3).

The calculated matrix effect, which can be caused by the competition between the analytes and co-eluting matrix compounds with ions formed in the LC-MS/MS interface, was 130% (calculated from slope of calibration curve). The quantification of CIN as an analyte can be strongly affected by matrix effects of red rice. So, in the routine analyses of CIN in this matrix, the matrix effects cannot be ignored and the quantification must be done using the matrix match calibration.

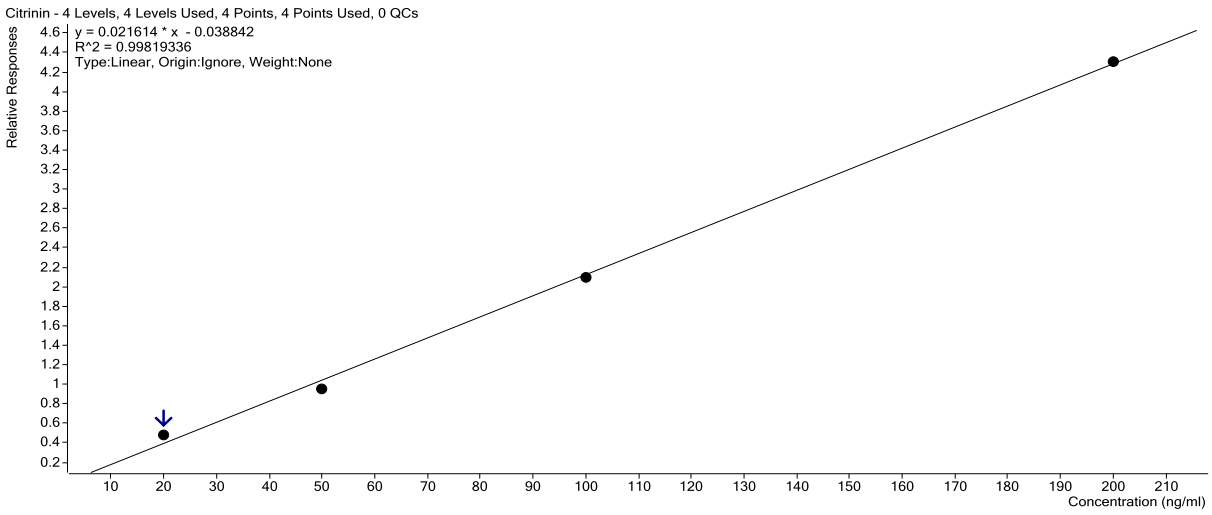

Figure 2. CIN calibration in mobile phase

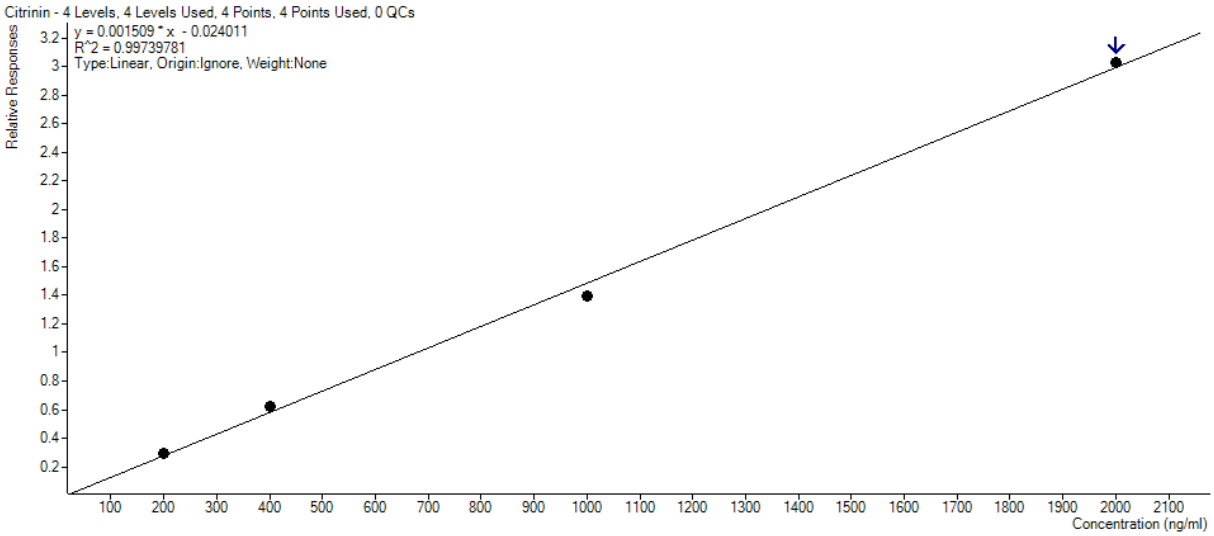

Figure 3. Matrix match calibration

The spiking LC-MS/MS TIC chromatogram with MRM transition of the internal standard (13C20-OTA) and the investigated analyte – CIN at the concentration level of 10 ng/mL was shown in Figure 4.

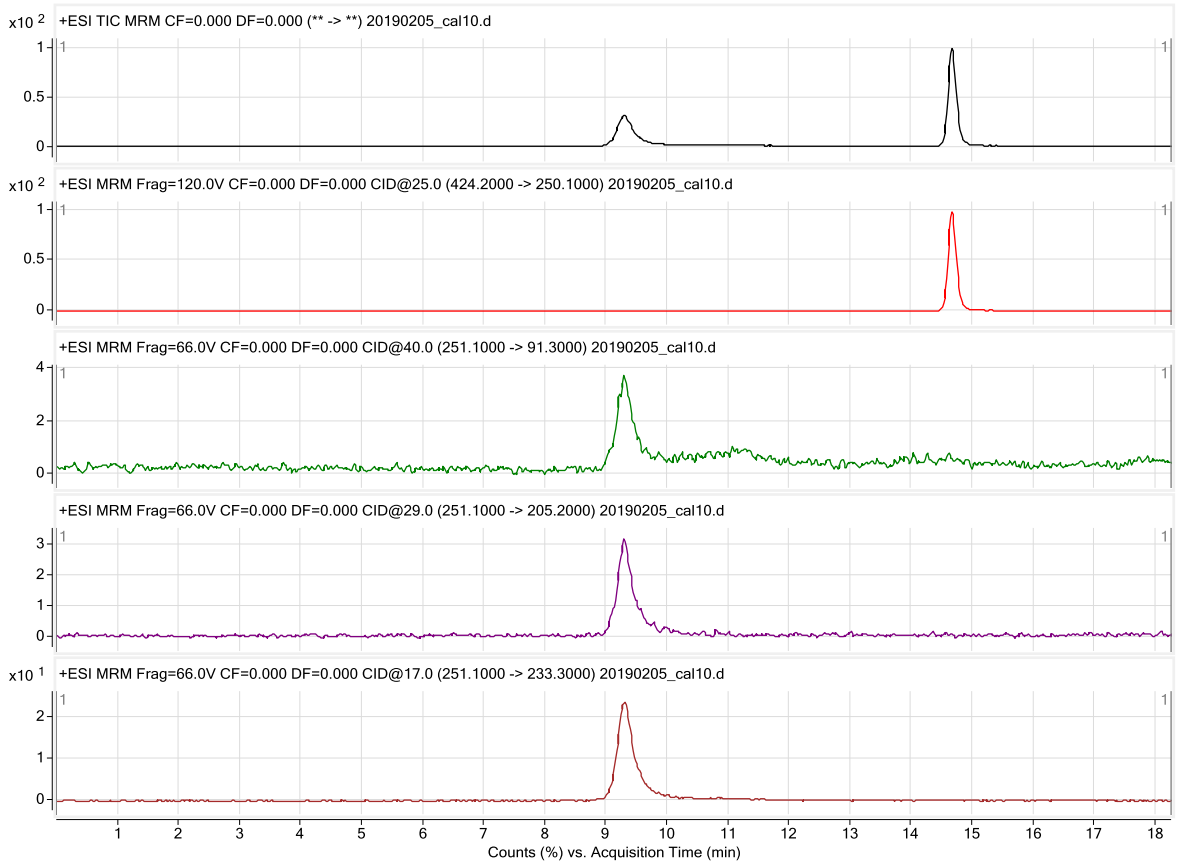

Figure 4. TIC and MRM chromatograms of CIN and 13C20-OTA

Recovery

The accuracy was investigated through the recovery trials and spiked blank samples. According to the followed guidelines (EC 401/2006), the gain average recovery of 102.8% was in the range according to guidelines, which is 70-120%. The recovery was obtained at two fortification levels (200 and 2000 µg/mL) in three replicates. The precision, expressed as a relative standard deviation (RSD, %), was evaluated in terms of repeatability and the obtained value was 6.27%. The recovery and RSD, % are given in Table 2.

Table 2. Validation parameters

Spike samples	Final Conc. (µg/mL)	Exp Conc. (µg/mL)	Recovery (%)	Average Rec. (%)	Average Rec. (%)	RSDr (%)	RSD_R (%)
1.	219.81	200	109.9				
2.	217.12	200	108.6	109.9		1.15	
3.	222.18	200	111.1		102.8		6.27
1.	2041.13	2000	102.1				
2.	1810.27	2000	90.5	95.6		6.16	
3.	1884.85	2000	94.2				

The spiking LC-MS/MS chromatogram with MRM transition of the internal standard (13C20-*OTA*) and investigated analyte – CIN at the concentration level of 1000 µg/kg was shown in Figure 5.

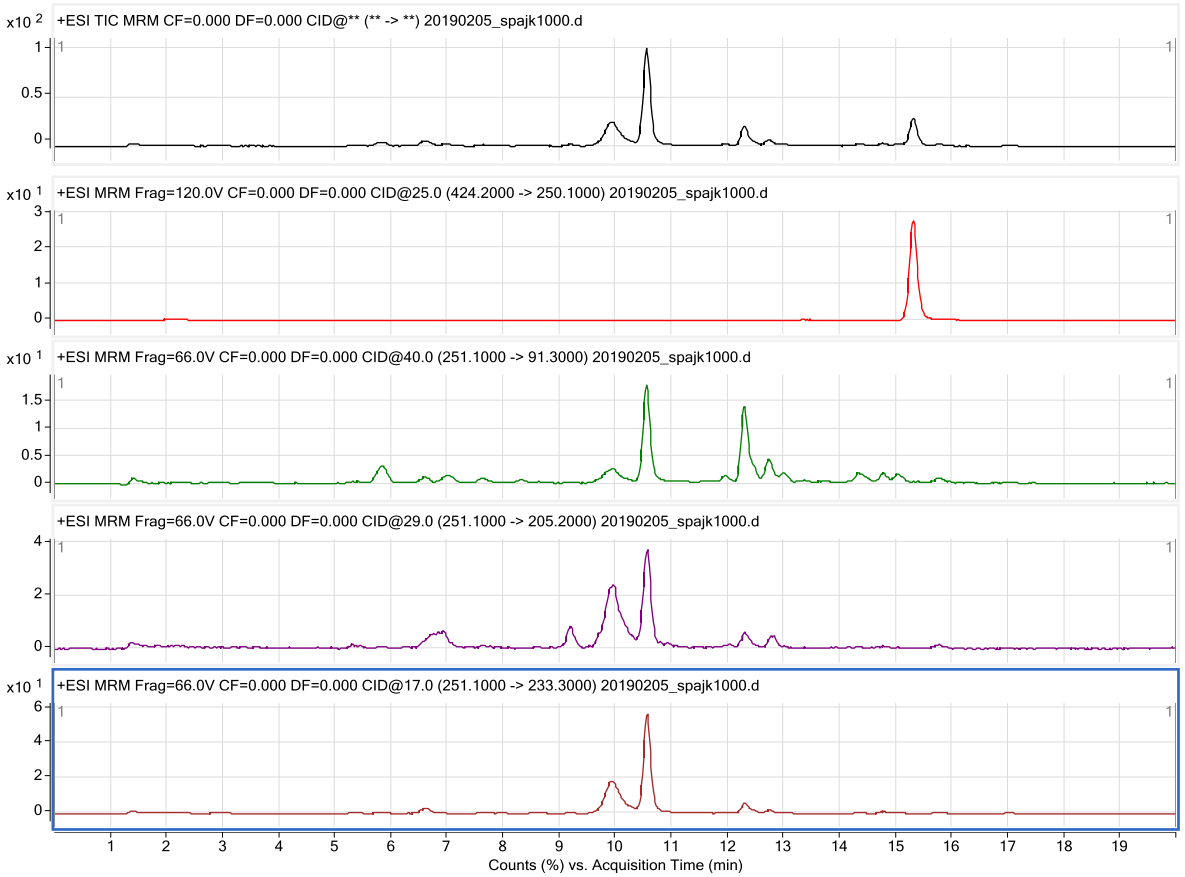

Figure 5. TIC and MRM chromatograms of spiking samples with CIN and 13C20-*OTA*

CONCLUSIONS

The validation of the LC-MS/MS method using Captiva EMR column clean up for the extraction of CIN has been done. The proposed method represents the successful example of CIN determination in red rice. The need to reach low level of LOQ and satisfactory performances for all the considered validation parameters for the CIN determination was achieved by means of a robust matrix matched validation experiments performed on the red rice as selected matrix. The method provides a very high sensitivity, good reproducibility, appropriate linearity and can be applied with a high reliability to the analysis of the CIN content in real red rice samples.

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