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Validation of a method for determination of mycotoxins subjected to the EU regulations in spices: The UHPLC–HESI–MS/MS analysis of the crude extracts

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ABSTRACT

Validation study of the method, previously developed for multimycotoxin analysis of cereals crude extracts by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS), was performed to establish a high-throughput multi-matrix procedure for simultaneous determination of mycotoxins included in the European Union's (EU) regulations for spices. Two kinds of the spice matrices were included: red pepper (*Capsicum* species) and black pepper (*Piper nigrum*). They were fortified with aflatoxins (AFB1, AFB2, AFG1 and AFG2) and ochratoxin A (OTA) at levels that are equal or below the maximum allowable contents for spices according to the Commission Regulations (EU) No. 165/2010 and No. 105/2010. The obtained validation data suggested that the analysis of red pepper crude extracts is feasible and at the same time sensitive enough for determining AFs and OTA levels set in the EU Regulations. The analysis of 17 spice samples by the validated method did not reveal the occurrence of AFs and OTA in the products from the local markets in Novi Sad, capitol of the Vojvodina Province. To confirm the results obtained by UHPLC–MS/MS extracts were also screened by a method based on accurate mass measurements using high-resolution MS (HRMS) system with Orbitrap technology. However, validation parameters for analysis of some mycotoxins in black pepper crude extracts did not comply with the EU regulation, particularly with respect to the recoveries and LODs for AFG1, AFG2 and OTA, due to significant matrix interferences.

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1. Introduction

Even though spices constitute a natural medium in general not favorable for the growth of molds and the production of mycotoxins, they are largely produced in countries where tropical and subtropical climates are favorable for mycotoxin contamination. Concerning the most frequent mycotoxin contamination of spices, it comes from the genera *Aspergillus* and *Penicillium*, some of which are known as producers of different aflatoxins (AFs), ochratoxins and sterigmatocystine, i.e. mycotoxins that exhibit toxic, mutagenic, teratogenic and carcinogenic effects in humans and animals (Zinedine & Manes, 2009). Due to toxic effects on human and animals, the assessment of the spices contamination by mycotoxins is of high relevance. Therefore, monitoring and control programs for mycotoxins in spices have been implemented in many countries, especially in the European Union (EU). For the first time, the European Commission (EC) set a legal limit for AFs in spices in 2002, while after several attempts it just recently regulated the maximum presence of ochratoxin A (OTA). According to the latest Commission Regulations No. 165/2010 (EU, 2010a) and No. 105/2010 (EU, 2010b), both amending Commission Regulation No. 1881/2006 (EC, 2006a), the following limits of AFs and OTA in spices have been set: maximum level for total AFs, representing a sum of AFB1, AFG1, AFB2 and AFG2, is 10 μg/kg, with maximum level of AFB1 of 5 μg/kg (EU, 2010a) while for OTA it is 30 μg/kg (EU, 2010b).

To enforce the EU legal limits, it would be preferable to determine all mycotoxins by routine analysis in one single (sample) extract, and, if possible, in a single analytical run. However, several difficulties are found to reach this objective, since development of multitoxin method is often impeded by the chemical diversity of the analytes and high variability of matrix composition. For the analysis of mycotoxins in peppers the preferred option has been the cleanup procedures of the extracts based primarily on rather expensive immunoaffinity column-based preparatory methods, followed by the high performance liquid chromatography with fluorescence detection (HPLC–FLD) (Brera et al., 2011).

In contrast to usual preparatory methods based on extraction/cleanup/pre-concentration steps used by many routine laboratories

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for analysis of single toxin or multiple toxins belonging to the same group in one particular sample matrix, several authors prefer to analyze the crude matrix extracts (Beltrán, Ibáñez, Sancho, & Hernández, 2009; Spanjer, Rensen, & Scholten, 2008; Sulyok, Berthiller, Krska, & Schuhmacher, 2006).

In this study, the possibility to broad the scope of the multi-mycotoxin method developed for analysis of crude cereal extracts (Škrbić, Malachova, Živančev, Veprikova, & Hajslová, 2011; Škrbić, Živančev, Đurišić-Mladenović, & Godula, 2012) was explored toward the analysis of EU-regulated mycotoxins in spices. The methodology has been investigated in two different matrices, powdered red pepper and black pepper, representing ones of the most important and favorite spices all over the world. The method was also applied to the analysis of powder samples of red pepper, black and white pepper collected from the markets in Novi Sad. In order to confirm the results obtained by UHPLC–MS/MS, and to determine the occurrence of other, non-regulated mycotoxins in spices, extracts were also screened by a method based on accurate mass measurements using high-resolution MS (HRMS) system with Orbitrap technology as a powerful tool for fast qualitative analysis. To our knowledge, no simple and high-throughput method based on crude extract analysis by UHPLC–MS/MS method for simultaneous determination of AFs and OTA has been reported for pepper powders yet. This is also the first study of mycotoxins in spices on the Serbian market, which stands highly among the European red pepper producers, with about 170,000-t production in 2005, being behind Italy, Spain, The Netherlands and Romania (Santos, Marín, Sanchis, & Ramos, 2008).

2. Experimental

2.1. Reagents and chemicals

Aflatoxins (AFB1, AFB2, AFG1, and AFG2) and OTA certified standard solutions were purchased from Supelco Co. (Bellefonte, PA); aflatoxins concentrations in standard solutions were: AFB1 2 μg/ml, AFB2 0.5 μg/ml, AFG1 2 μg/ml, AFG2 0.5 μg/ml, while ochratoxin A was 1000 μg/ml. All standards dissolved in acetonitrile were stored at −20 °C in amber glass vials and brought to room temperature before use. For calibration purposes, starting from the purchased AFs and OTA certified standards, stock mix solution at 50 ng/ml in acetonitrile was prepared by combining suitable aliquots of each individual standard.

Ultra-pure water was produced by Milli-Q system (Millipore, Molsheim, France). Methanol, acetonitrile and ammonium acetate (all LC–MS gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands), glacial acetic acid (p.a.) was obtained from LTG Promochem (Wesel, Germany).

2.2. Samples

In order to validate the method, 17 samples of different pepper types (red pepper powder, white and black pepper) were collected during October 2010 in Novi Sad, the capitol of the northern Serbian province of Vojvodina, where the biggest producers of spices in Serbia are located. Selection was made in order to analyze different qualities and brands of pepper spices available in Serbia. Seven samples of red pepper powder, 2 samples of black pepper and 2 samples of white pepper were purchased from the local retail shops, while 6 more samples of red pepper powder intended for the production of the traditional meat product (sausage) were obtained from the local meat industry. Packs with red pepper (100 g and 200 g weight) and white pepper (50 g weight) powder, produced by one of the leading Serbian food producers, were bought at the local supermarkets. The pepper (either red or black/white) powders were also collected in local markets selling goods in bulk. All the red pepper powders were domestically produced, while the black and white pepper samples were produced in Vietnam and repacked in Serbia.

2.3. Sample preparation

Method used to prepare the crude extracts of the selected spices was previously used by Škrbić et al. (2011, 2012), for preparation of wheat extracts for *Fusarium* mycotoxins analysis by HPLC–MS/MS. Briefly, 5 g of homogenized spice powdered samples were extracted by shaking with 20 ml of acetonitrile/water mixture (86:14, v/v) for an hour using an automatic shaker (Promax 2020, Heidolph Instruments, Germany). After that, the suspensions were filtered and an aliquot (1 ml) of filtered crude extracts were transferred into glass vials and diluted with 3 ml of the UHPLC mobile phase of the initial content (95% A and 5% B; explanation of the composition of the eluent A and B is given hereafter). Thus, the final extracts contained 0.0625 g sample per milliliter. Before injection into the UHPLC–MS/MS (or UHPLC–HRMS) system, the extracts were passed through the 0.2 μm nylon syringe filter.

2.4. UHPLC–MS/MS

Ultra-high performance liquid chromatography (UHPLC) performed by Accela™ (Thermo Fisher Scientific, USA) consisting of a quaternary gradient pump (Thermo Fisher Scientific, USA) was used for analytical separation of the sample components. Hypersil GOLD™, 50 × 2.1 mm i.d., 1.9 μm column (Thermo Fisher Scientific, USA) was used with a flow rate of 0.5 ml/min, and the column temperature was maintained at 30 °C. The injection volume was 10 μl. The mobile phase consisted of eluent A containing water/acetic acid (99:1, v/v), and eluent B consisting of methanol/acetic acid (99:1, v/v). Both eluents contained 5 mM ammonium acetate. The gradient started with 95% A and 5% B and was kept until 0.5 min, afterward a linear gradient was applied, reaching 95% B after 3.04 min (holding time 2.1 min) and then switch back (in 6.20 min) to 95% A (holding time 1.80 min), which was maintained till the end of the run at 8 min.

For analytes detection, triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, USA) equipped with heated-electrospray ionization probe (HESI-II, Thermo Scientific, USA) was used. Parameters of the ion source were as follows: spray voltage – 3.4 kV, vaporizer temperature – 350 °C, sheath gas pressure – 40 arbitrary units, auxiliary gas pressure – 10 arbitrary units, capillary temperature – 270 °C. Other source parameters were automatically tuned for maximal intensity of particular analyte in the respective time window. Argon pressure in the collision cell (Q2) was set to 1.2 mTorr. Mass resolution at the first quadrupole (Q1) was set to 0.2 Da at full width half maximum (FWHM) and to 0.7 Da FWHM at the third quadrupole (Q3). Instrument control and data collection were handled by computer equipped with Xcalibur 2.1.0 (Thermo Fisher Scientific, USA).

2.5. UHPLC–HRMS

Ultra-high performance liquid chromatograph (Accela™, Thermo Fisher Scientific, USA) coupled to high-resolution mass spectrometer with Orbitrap technology (Exactive™, Thermo Fisher Scientific, USA) was used to screen the obtained extracts in order to look for mycotoxins currently not regulated by the relevant EU legislations. The chromatographic column, mobile phases, gradient and injection volume were the same as in the UHPLC system described above, except the mobile flow rate which was set at 0.4 ml/min to allow adequate sensitivity with the used ESI source of Orbitrap MS.

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