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Original Research Article

Multimycotoxin analysis of crude extracts of nuts with ultra-high performance liquid chromatography/tandem mass spectrometry

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ABSTRACT

A reliable, fast and simple method using ultra-high performance liquid chromatography with heated electrospray ionization triple quadrupole mass spectrometry (UHPLC/HESI-MS/MS) was developed for the simultaneous determination of aflatoxins B1, G1, B2 and G2, ochratoxin A (OTA), zearalenone (ZEA), HT-2 toxin, T-2 toxin and fumonisins B1 and B2 in crude extracts of various types of nuts. The procedure is based on the simultaneous extraction of selected mycotoxins with a mixture of acetonitrile/water/acetic acid (79:20:1, v/v/v) and defatting the obtained extract with hexane in order to remove the lipids. The validation data indicated that the analysis of different types of crude extracts of nuts is feasible and sensitive enough for determination of the majority of the studied mycotoxins. The recoveries of various nuts matrices ranged between 71.25% and 140.11% with relative standard deviation lower than 12%. The satisfactory recoveries were obtained for the most of mycotoxins using walnut matrix-matched calibration curves indicating the multi-matrix feasibility of the method. The applicability of the method was successfully demonstrated on 17 samples of nuts collected in a region of northern Serbian province of Vojvodina. Total frequency of the occurrence of the selected mycotoxins was 12%.

1. Introduction

Food contamination by mycotoxins is a continuous concern in food safety. The number of mycotoxins known to exert a toxic effect on human and animal health is constantly increasing, and more and more legislative provisions are taken to control their presence in food and feed (Zinedine and Mañes, 2009). These toxins occur naturally in plant products such as cereals, nuts and dried fruit and in their by-products as well (Bennett and Klich, 2003; Miraglia and Brera, 2002).

Nuts are among the most nutritious human foodstuffs because of their high content of proteins, carbohydrates, unsaturated lipids, vitamins and essential minerals (USDA, 2010). Nuts are commonly consumed by all age groups and across social strata in both developed and developing countries. Per capita consumption is expected to increase in the world wide with continuous promotion of their properties as healthy food. However, nuts have low water activity (a_w) so fungi are the major microbiological contaminants. Some of these molds are mycotoxigenic, thus high levels of mycotoxins have frequently been reported in nuts from the orchards and from the market (Bayman et al., 2002; Fernane et al., 2010). It is well known that nuts are among the commodities with the highest risk of contamination by aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). Reports on mycotoxin contamination of nuts have mainly focused on *Aspergillus* and *Penicillium* mycotoxins, such aflatoxins and OTA (Huang et al., 2010; Luttfullah and Hussain, 2011; Rubert et al., 2011) with scanty records on some other fungal metabolites including *Fusarium* mycotoxins like fumonisins B1 (FB1) and B2 (FB2), ZEA, HT-2, T-2, etc. (Abia et al., 2013; Varga et al., 2013).

Currently, only aflatoxins have been included in the European regulation for nuts, Regulation EC 165/2010 amending the Commission Regulation No. 1881/2006, which established the maximum levels (ML) for aflatoxins as follows: 10 μg/kg for

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aflatoxins (AFB1, AFG1, AFB2, AFG2) and 5 μg/kg for AFB1 for hazelnut; 4 μg/kg for aflatoxins and 2 μg/kg for AFB1 for walnut/peanut; 10 μg/kg for aflatoxins and 8 μg/kg for AFB1 for almond. Nevertheless, more information is needed on other mycotoxins as well, such as fumonisins, HT-2, T-2, ZEA, OTA, etc.

Hence the implementation of a reliable, rapid, cost-effective analytical strategy providing comprehensive data is an important task. The crucial condition for obtaining good recoveries is an efficient isolation of analytes from plant matrix. It should be noted that the physicochemical properties of mycotoxins vary widely, thus the choice of an efficient extraction procedure enabling high recoveries for all the target analytes is not an easy task (Zachariasova et al., 2010). Many laboratories routinely use preparatory methods based on extraction/cleanup/pre-concentration steps for only one or a small group of similar mycotoxins. For example, many authors have used methods employing clean-up for analysis of aflatoxins and OTA in nuts (Set and Erkmen, 2010; Huang et al., 2010; Luttfullah and Hussain, 2011; Rubert et al., 2011; Baquião et al., 2012). Although these methods are well established, and in some cases interlaboratory validated, the current trend is to introduce simple (one-step), broad-scope procedures which, thanks to the use of modern separation/detection instrumental technologies, allow accurate determination of as many as possible major mycotoxins even at low levels in crude extracts, and do not require a labor/cost-demanding clean-up step (Škrbić et al., 2011a, 2011b; Škrbić et al., 2012; Škrbić et al., 2013).

The Serbian population consumes nuts, mostly walnuts directly or as ingredients in cookies and other confectionary products. The Serbian regulation (28/2011) sets mycotoxins maximum levels at the same levels as the EC regulation (165/2010). However, until now there has been no information about the safety of nuts consumed by the Serbian population. Consequently, it is important to study the presence of mycotoxins, since there is a lack of information in the literature about their occurrence in these products.

Thus, the aim of this study was: (i) to develop a simple and simultaneous method for efficient extraction of regulated and non-regulated mycotoxins from nuts; (ii) to validate UHPLC/HESI-MS/MS multi-mycotoxin method using the obtained crude extracts from nuts; and (iii) to apply the method on samples collected within Novi Sad, the capitol of the northern Serbian province of Vojvodina.

As the crude extract method followed by UHPLC–HESI-MS/MS is a technique that has been frequently used in the literature as a routine analytical technique for mycotoxins in cereals, our intention was also to extend the scope of the previously developed method (Škrbić et al., 2011a, 2011b, 2012, 2013) and prove the availability of crude extract use for mycotoxin analysis of nuts matrices.

2. Materials and methods

2.1. Reagents and chemicals

Individual standard stock solutions of AFB1 (2 μg/mL), AFB2 (0.5 μg/mL), AFG1 (2 μg/mL), and AFG2 (0.5 μg/mL), OTA (10 μg/mL), HT-2 toxin (100 μg/mL), T-2 toxin (100 μg/mL), ZEA (100 μg/mL), FB1 (50 μg/mL) and FB2 (50 μg/mL) were purchased from Supelco Co. (Bellefonte, PA, USA). All standards dissolved in acetonitrile were stored at −20 °C in amber glass vials, and brought to room temperature before use. Composite working standard solutions were prepared by diluting the above-mentioned stock solutions in acetonitrile and they were added in appropriate dilution to the extract of the uncontaminated sample to prepare matrix-matched calibration standards in concentration ranges that include the maximum allowable concentrations and also the expected range of mycotoxin occurrence (in accordance to the available literature data). Ultra-pure water was produced by Milli-Q purification system (Millipore, Molsheim, France). Methanol, acetonitrile and ammonium acetate (all LC–MS grade) were supplied from J.T. Baker (Deventer, The Netherlands), glacial acetic acid (p.a.) was obtained from LTG Promochem (Wesel, Germany). Hexane (HPLC grade, ≥98.5%) was supplied from Sigma–Aldrich (Hamburg, Germany).

2.2. Collection of samples

Seventeen samples of different nuts were collected within Novi Sad, the capitol of the northern Serbian province of Vojvodina, in February 2013. Samples could be classified according to the origin as "domestic" (8 walnut (*Juglans*) and 2 hazelnut (*Corylus avellana*) samples were taken from private resources) and "commercial" (1 walnut, 1 hazelnut, 3 peanut (*Arachis hypogaea*) and 2 almond (*Prunus dulcis*) samples were selected randomly from different supermarkets within Novi Sad). The commercial packs of selected samples weighed from 75 to 500 g. Before analysis, each sample was ground and homogenized using a laboratory mill (A11 Basic, IKA, Germany). The samples were kept at 4 °C until analysis.

2.3. Sample preparation

Previously developed methods for the so-called crude extract of wheat flour (Škrbić et al., 2011b, 2012) and paprika (Škrbić et al., 2013) were slightly modified. Namely, since the previous studies of mycotoxin analysis in wheat flour (Škrbić et al., 2011b, 2012) showed that non-acidified extraction solvent could not recover FB1 and FB2 in a satisfactory manner (above 60%), acetic acid was added to the mixture of solvents for extraction (79:20:1, v/v/v, acetonitrile/water/acetic acid) (Sulyok et al., 2006, 2007, 2010; Abia et al., 2013; Varga et al., 2013), in order to enable the isolation of these toxins. Then, defatting of the obtained crude extracts with hexane was introduced into the sample preparation procedure in order to remove the lipids that might interfere with the mycotoxin analysis by UHPLC–HESI-MS/MS. Such prepared crude extracts of the samples were used for further analysis without any purification step.

Briefly, the samples were prepared as follows: 10 g of homogenized samples (walnuts, hazelnuts, peanuts or almonds) were extracted by shaking with 40 mL of acetonitrile/water/acetic acid mixture (79:20:1, v/v/v) for an hour using an automatic shaker (Promax 2020, Heidolph Instruments, Germany). After extraction, the suspensions were filtered through Whatman filter paper No. 4, and an aliquot (20 mL) of filtered crude extracts was transferred into a plastic flask. Then, 20 mL of hexane was added to the filtered crude extract (20 mL) and the content was thoroughly mixed for 2 min in order to remove the lipids. The mixture was centrifuged at 5000 rpm for 5 min. After separation of the two phases, hexane was eliminated. Before injection into the UHPLC/HESI-MS/MS, the crude extract in acetonitrile was passed through a 0.2 μm nylon syringe filter.

2.4. Instrumental conditions

Separation and detection were performed as described in previous studies (Škrbić et al., 2011b, 2012, 2013). The steps could be summarized as follows: ultra-high performance liquid chromatography (UHPLC) performed by Accela™ (Thermo Fisher Scientific, San Jose, United States) was used for separation of sample components. Hypersil GOLD™, 50 mm × 2.1 mm i.d., 1.9 μm column (Thermo Fisher Scientific) was used with a flow rate of 0.5 mL/min, and the column temperature was maintained at 25 °C. The injection volume was 10 μL. The mobile phase consisted

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