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Simple methodology for the determination of mycotoxins in pseudocereals, spelt and rice

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ABSTRACT

Nowadays the interest and consumption of pseudocereals is increasing due to their nutritional properties. Like cereals and oilseeds, pseudocereal seeds are susceptible to fungal growth and mycotoxin contamination; however these matrices have received little attention in literature. A sensitive, simple and rapid method for the determination of fifteen mycotoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, nivalenol, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in pseudocereals (buckwheat, quinoa and amaranth) has been developed and validated by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS), using a QuEChERS-based sample treatment. This study also includes cereals such as spelt and white, brown and red rice. Matrix-matched calibration curves were established and limits of quantification were below the maximum contents established by EU regulation in cereals. The precision (repeatability and intermediate precision) was lower than 12% in all cases and recoveries were between 60.0% and 103.5%, fulfilling the current legislation. Finally, the content of aflatoxin B_1 found in a red rice sample was confirmed by comparison of the result obtained by using immunoaffinity columns for extraction and clean-up.

1. Introduction

Pseudocereals are plants that produce fruits or seeds, which are used and consumed as grains, though botanically they are neither grasses nor true cereal grains, but since they produce starch-rich seeds like cereals they are called pseudocereals. Nowadays the interest and consumption of pseudocereals is increasing due to their nutritional properties, such as protein quality and amino acids balance of amaranth and quinoa or phytochemicals concentration of buckwheat (Schoenlechner, Siebenhandl, & Berghofer, 2008). In addition, pseudocereals are currently emerging as healthy alternatives to gluten-containing grains in the gluten-free diet necessary for celiac disease. Amaranth (*Amaranthus*), quinoa (*Chenopodium quinoa*) and buckwheat (*Fagopyrum esculentum*) are among the most consumed pseudocereals.

Like cereals and oilseeds, pseudocereal seeds are susceptible to fungal growth (Bresler, Rizzio, & Vaamonde, 1995; Danielsen, Bonifacio, & Ames, 2003; Nelson, Plattner, Shackelford, & Desjardins, 1992; Pappier, Fernández Pinto, Larumbe, & Vaamonde, 2008); however this issue has received little attention in literature. Therefore, there is an actual risk of pseudocereal contamination by mycotoxins as demonstrated in several studies (Aoyama et al., 2010; Krysińska-Traczyk, Perkowski, & Dutkiewicz, 2007; Kumagai et al., 2008; Schollenberger et al., 2005; Spanjer, Rensen, & Scholten, 2008; Sugita-Konishia et al., 2010; Veršilovskis, Bartkevičs, & Miķelsone, 2008). The analytical methods employed for these determinations were based mainly on liquid chromatography (LC) with fluorescence detection using a derivatization step (Sugita-Konishia et al., 2010), LC with mass spectrometry (MS) (Kumagai et al., 2008; Spanjer et al., 2008; Veršilovskis et al., 2008), thin layer chromatography (TLC) (Bresler, Vaamonde, & Brizzio, 1991; Bresler, Vaamonde, Degrossi, & Fernandez-Pinto, 1998; Kumagai et al., 2008), or, less commonly, gas chromatography-mass spectrometry (GC–MS) (Krysińska-Traczyk et al., 2007; Schollenberger et al., 2005).

Regarding the cereal market, rice (*Oryza sativa*) is one of the most consumed cereals in the world. Thus, the world rice trade in 2013 is forecast to reach 37.5 million tonnes (FAO, 2013). Moreover, rice is one of the matrices of interest in mycotoxin determination (Tanaka, Sago, Zheng, Nakagawa, & Kushiro, 2007). Among rice

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varieties, brown rice is increasingly been chosen by customers because of its health benefits, whereas red rice, obtained by the fermentation of normal rice with fungal from genus *Monascus* (Wang & Lin, 2007), has been traditionally used in China due to its medicinal properties (anti-hypertensive, anti-diabetic and blood circulation regulator), and nowadays it is an important component of the Chinese diet. However, several species of the genus *Monascus* also produce citrinin, thus this is a matrix of concern in the determination of mycotoxins (Samsudin & Abdullah, 2013).

Other cereal of interest is spelt (*Triticum spelta*), which was about to disappear due to its low yield; however, its nutritional properties, high resistance in unfavourable environmental factors and lower fertilization requirements compared to wheat, have made it increasingly valuable for food product manufacturers and consumers (Bonafaccia et al., 2000). Although mycotoxin contamination in spelt has not been extensively explored, spelt products have been included among others in several studies, as the determination of mycotoxins by enzyme-linked immunosorbent assay (ELISA) (Solarska, Marzec, Kuzdraliński, & Muszyńska, 2012) and by LC–MS (Juana, Ritieni, & Mañes, 2012; Maul et al., 2012; Serrano, Font, Ruiz, & Ferrer, 2012; Suchowilska, Kandler, Sulyok, & Krska, 2010; Sulyok, Krska, & Schuhmacher, 2007).

Regarding sample treatment, the analytical methods commented before have been based mainly on immunoaffinity columns (IAC) (Kumagai et al., 2008; Schollenberger et al., 2005; Sugita-Konishia et al., 2010), solid phase extraction (SPE) (Veršilovskis et al., 2008) or extractions with different solvents (Bresler et al., 1991, 1998; Juana et al., 2012; Maul et al., 2012; Spanjer et al., 2008; Suchowilska et al., 2010; Sulyok et al., 2007). However, some of these sample treatments, as IAC, are expensive and comprise complex purification; moreover, multiclass analysis is quite limited, due to their inherent selectiveness. As a consequence, simpler, more efficient, multiclass and environmentally friendly extraction systems are demanded.

An increasingly popular treatment is the so called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which has been widely used in the last years. QuEChERS methodology presents some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning-up complex samples (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). It involves two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE step that involves further clean-up using combinations of $MgSO_4$ and different sorbents, such as C_{18} or primary and secondary amine (PSA), to remove interfering substances. QuEChERS-based methods have been recently reported for the extraction of different mycotoxins in cereal products (Cunha & Fernandes, 2010; Desmarchelier et al., 2010; Sospedra, Blesa, Soriano, & Mañes, 2010; Vaclavik, Zachariasova, Hrbek, & Hajslova, 2010; Zachariasova et al., 2010), eggs (Garrido-Frenich, Romero-González, Gómez-Pérez, & Martínez-Vidal, 2011), wine (Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2011) and medicinal plants such as milk thistle (Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2013). It was also used for the multiresidue extraction of different contaminants, including mycotoxins, in organic food products and milk (Aguilera-Luiz, Plaza-Bolaños, Romero-González, Martínez-Vidal, & Garrido-Frenich, 2011; Mol et al., 2008; Romero-González, Garrido-Frenich, Martínez-Vidal, Prestes, & Grio, 2011).

In this paper, we propose a UHPLC–MS/MS method, very popular in the last years for the multiclass analysis of mycotoxins (Beltrán, Ibáñez, Sancho, & Hernández, 2009; Garrido-Frenich et al., 2011; Zachariasova et al., 2010), for the simultaneous determination of 15 mycotoxins in pseudocereals and spelt (as scarcely studied matrices) and different kinds of rice (an extensively consumed cereal) using a QuEChERS-based extraction. To the best of our knowledge, the proposed methodology has not been used for the determination of mycotoxins neither in pseudocereals nor in spelt, red and brown rice.

The studied mycotoxins are included in the European regulation (EC 1881/2006) (European Commission, 2006a), and subsequent amends (European Commission, 2007, 2010, 2012), such as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), in recommendations (Commission Recommendation, 2013) such as HT-2 toxin (HT-2) and T-2 toxin (T-2), and others which are not included, but are considered by the International Agency for Research on Cancer (IARC) as dangerous substances (IARC, 2012), such as nivalenol (NIV), fusarenon-X (F-X), citrinin (CIT) and sterigmatocystin (STE). The method was fully characterized in white rice, which was considered as representative matrix. The trueness of the method was then checked in pseudocereals, spelt, brown and red rice by means of recovery studies performed by spiking blank matrices.

In addition, among the studied samples, a sample of red rice obtained from a local market was found to be contaminated with a high concentration of AFB_1. The obtained result was statistically confirmed by a standard method, based on extraction by IACs (EN-ISO 16050:2011), obtaining similar results.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical reagent grade, solvents were LC–MS grade and mycotoxins were analytical standard grade. Formic acid eluent additive for LC–MS, methanol (MeOH) and ammonium formate were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany); acetonitrile (MeCN), phosphoric acid, potassium dihydrogen phosphate, disodium phosphate, potassium chloride, sodium chloride and sodium hydroxide were supplied by Panreac (Madrid, Spain).

Ultrapure water (18.2 MΩ cm^{-1}, Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

Individual standards of each mycotoxin were obtained from Sigma Aldrich. When standards were provided in dry powder form, the correct amount of solvent was injected through the septum vial. From these stock solutions, mycotoxins intermediate working solutions (used thorough all this work) were prepared at the following concentrations in MeCN: 1 μg mL^{-1} of AFB_1, AFB_2, AFG_1, AFG_2, STE and OTA; 2 μg mL^{-1} of CIT; 10 μg mL^{-1} of FB_1, FB_2, T-2, HT-2 and ZEN; and 1000 μg mL^{-1} of NIV and F-X. DON working solution was supplied at a concentration of 100 μg mL^{-1}. These solutions were stored at −20 °C and were stable at least three months. Since mycotoxins are highly toxic compounds, some general precautions should be followed for their manipulation and solutions preparation. Thus, safety glasses and disposable gloves were used thorough the work. Decontamination of laboratory glassware and laboratory surface was carried out by swabbing with 10% hypochlorite solution using disposable paper towels. Contaminated disposable material was properly stored and processed as biohazard residues.

Kits SampliQ QuEChERS consisted of buffered QuEChERS extraction packed (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) and were supplied by Agilent Technologies (Waldbronn, Germany).

Phosphate buffer used for purification of the samples in the IACs (AflaClean, LC Tech, Dorfen, Germany) was prepared by dissolving