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Analytical Methods

Determination of mycotoxins in plant-based beverages using QuEChERS and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A method was developed for the simultaneous determination of 11 mycotoxins in plant-based beverage matrices, using a QuEChERS extraction followed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry detection (UHPLC–(ESI)MS/MS). This multi-mycotoxin method was applied to analyse plant-based beverages such as soy, oat and rice.

QuEChERS extraction was applied obtaining suitable extraction recoveries between 80 and 91%, and good repeatability and reproducibility values. Method Quantification Limits were between 0.05 $\mu g\ L^{-1}$ (for aflatoxin G_1 and aflatoxin B_1) and 15 $\mu g\ L^{-1}$ (for deoxynivalenol and fumonisin B_2). This is the first time that plant-based beverages have been analysed, and certain mycotoxins, such as deoxynivalenol, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, T-2 toxin and zearalenone, were found in the analysed samples, and some of them quantified between 0.1 $\mu g\ L^{-1}$ and 19 $\mu g\ L^{-1}$.

1. Introduction

Mycotoxins are natural secondary metabolites produced by some species of filamentous fungi of the *Aspergillus*, *Penicillium* and *Fusarium* genera (Richard, 2007). Over 400 types of mycotoxins are reported, classified by their structure, their biological source or the moment of production from preharvest on the plant culture to storage, transport or processing stages (Bhat, Rai, & Karim, 2010). Modern techniques and good practices of handling and preserving food and feed reduce the presence of mycotoxins. Nevertheless, these species also grow in cereals, fruit and milk (Bhat et al., 2010). Of all mycotoxins, aflatoxin B_1 (AFB_1) is the most potent carcinogen, but all mycotoxins are harmful in different ways, displaying acute and chronic toxicity, such as genotoxicity, carcinogenic toxicity, immunotoxicity (immunostimulatory or immunosuppressive), mutagenicity, nephrotoxicity and teratogenicity attributes (EFSA, 2007, 2014).

The main foods affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and several types of fruit, particularly apples, or sub-products produced from contaminated raw materials, such as wine and beer (EFSA, 2013). Mycotoxins are a serious health risk present throughout the entire food chain as they display stability at high temperatures and withstand cooking processes (Bullerman & Bianchini, 2007). People can be intoxicated if they eat either contaminated food or products, such as eggs, meat and milk from animals that previously consumed these toxins. In order to reduce the effects of mycotoxin ingestion, the European Union Commission Regulation establishes the maximum levels allowed in certain kinds of food for the major mycotoxins, such as aflatoxins (AFG_1, AFG_2, AFB_1, AFB_2), fumonisins (FB_1, FB_2), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) (EC, 2007), and recommends the maximum levels for the sum of T-2 toxin (T-2) and HT-2 toxin (HT-2) (EC, 2013). For example, the maximum level allowed in the case of AFB_1 in all cereals and all derivatives is 2.0 $\mu g\ kg^{-1}$. Consequently, this might be the maximum level permitted for oat- and rice-based products. However, this regulation does not consider the mycotoxin levels that may exist in legumes, such as soybeans. Soybeans are not a product that favours the production of certain mycotoxins. However, there is still a risk as the presence of the main fungi contributor to aflatoxin production has been reported in this type of legume (Nesheim & Wood, 1995).

Over the last few years, the consumption of beverages of plant origin has increased for medical reasons (e.g. due to intolerances and allergies), or as part of an alternative lifestyle (Lawrence,

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Lopetcharat, & Drake, 2016; Mårtensson, Öste, & Holst, 2000). If the raw material contains mycotoxins, the resulting beverage will also probably contain these toxins. To analyse these mycotoxins during beverage production, it is important to note that, depending on the raw plant material composition, the beverage might be very different (Mäkinen, Uniacke-Lowe, O'Mahony, & Arendt, 2015), which results in different interferences between matrices when determining the analytes of interest. Considering these differences, finding a common method to determine different mycotoxins for all of the different types of beverages is challenging.

There are different extraction techniques suitable for mycotoxin isolation, such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) for liquid samples, and pressurized liquid extraction (PLE) and solid–liquid extraction (SLE) for solid samples, among others (Capriotti et al., 2012; Köppen et al., 2010). The method selection depends on the nature of the matrix, its characteristics and complexity. However, some of these methods are expensive, complex, and/or involve considerable consumption in terms of time and solvent. In order to minimize the sample treatment but prevent exposure to matrix effects, a Quick, Easy, Cheap, Effective, Rugged and Safe method (QuEChERS) is a suitable alternative. The QuEChERS method has been used for mycotoxin extraction from food, both in solid samples, such as dried fruit (Azaiez, Giusti, Sagratini, Mañes, & Fernández-Franzón, 2014), pseudocereals, spelt and rice (Arroyo-Manzanares, Huertas-Pérez, García-Campaña, & Gámiz-Gracia, 2014), and in liquid samples, such as wine (Pizzutti et al., 2014) and beer (Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015). However, plant-based beverages have not previously been analysed and QuEChERS extraction could be a proper choice.

The aim of this study is to develop a method for the simultaneous determination of 11 mycotoxins in soy, oat and rice plant-based beverages, using QuEChERS extraction followed by UHPLC–(ESI)MS/MS.

2. Materials and methods

2.1. Reagents and chemicals

The target mycotoxins, which are restricted or subject to recommendations by the European legislation (EC, 2007, 2013), were four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), OTA and six Fusarium toxins (DON, ZEA, T-2, HT-2, FB_1 and FB_2). They were purchased (>99% purity) from Trilogy Analytical Laboratory (Washington, WA, USA). AFB_1, AFB_2, AFG_1 and AFG_2 were in acetonitrile (ACN) at 25 mg L^{-1}; ZEA, DON and OTA were in methanol (MeOH) at 25 mg L^{-1}, 100 mg L^{-1} and 10 mg L^{-1}, respectively; T-2 and HT-2 were in ACN at 100 mg L^{-1}; and a mixture of FB_1 and FB_2 was in ACN/water (50:50, v/v) at 100 mg L^{-1} and 30 mg L^{-1}, respectively. A mixed solution of all of the analytes was prepared at 1 mg L^{-1} for all of the analytes, except in the case of FB_2 at 0.3 mg L^{-1}, in MeOH/H_2O (1:1, v/v). Mixed solutions were stored at 4 °C for six months.

MeOH and ACN, both for LC–MS, were purchased from Panreac (Barcelona, Spain). Ultrapure-grade water was obtained from a MilliQ water purification system (Millipore, Darmstadt, Germany). Formic acid (HCOOH) ~98% and 10 M ammonium formate (NH_4HCOO) aqueous solution were purchased from Fluka (St. Louis, MO, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. QuEChERS extraction packets (4 g $MgSO_4$, 1 g NaCl) were obtained from Agilent Technologies (Waldbronn, Germany).

Real samples were soy, oat and rice plant-based beverages obtained from local supermarkets. Three different commercial brands were selected for each cereal.

It is important to take certain security measures when handling mycotoxins, such as wearing double gloves (latex underneath and nitrile on top) and cleaning all laboratory materials that have been in contact with mycotoxins, including old solutions, with 20% commercial sodium hypochlorite (NaClO).

2.2. Liquid chromatography-mass spectrometry

Chromatographic analyses were performed in an Agilent 1290 Infinity LC Series coupled to a 6495 iFunnel Triple Quadrupole MS/MS with an electrospray ionisation (ESI) interface, all from Agilent Technologies, operating in positive ion mode. Chromatographic separation was performed using a Cortecs UHPLC C_{18} column (100 mm × 2.1 mm, 1.6 μm) from Waters (Wexford, Ireland).

The chromatographic separation was performed by gradient elution using a binary mobile phase constituted of water (solvent A) and MeOH (solvent B), both with 5 mM NH_4HCOO and 0.1% HCOOH. The elution started at 10% of B and increased up to 50% in 4.5 min, then to 95% in 7.5 min, remaining in isocratic mode for 2.5 min. The injection volume was 10 μL, the flow rate was fixed at 0.45 mL min^{-1} and the column temperature was held at 40 °C. Samples were kept in the autosampler at 4 °C until analysis.

The source parameters were a capillary voltage of 4000 V for aflatoxins and 3500 V for the rest of compounds, desolvation gas flow and temperature of 18 L min^{-1} and 160 °C, nebulizer pressure of 35 psi, nozzle voltage of 500 V, fragmentor voltage of 380 V, cell acceleration voltage of 5 V, and sheath gas flow and temperature of 11 L min^{-1} and 350 °C. The high and low pressure funnel parameters were, respectively, 180 and 150 V for aflatoxins and 150 and 90 V for the rest of compounds. The acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive polarity. For each analyte, three characteristic MRM transitions were monitored, in accordance with the European Commission guidelines (SANTE, 2015). Four different time segments were also established in order to improve sensitivity. All of these parameters are specified in Table 1.

2.3. Sample preparation

For the extraction of soy, oat and rice plant-based beverages, the original QuEChERS extraction method (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003) was used just with the addition of formic acid in the extraction buffer. Briefly, 10 mL of sample was added to a 50 mL centrifuge tube with 10 mL ACN with 1% HCOOH and shaken for 3 min. Then, 4g of $MgSO_4$ and 1g of NaCl were added to the solution, and shaken vigorously for 3 min. Afterwards, the tubes were centrifuged at 10,000 rpm at 20 °C for 5 min. Finally, 1 mL aliquot of the supernatant phase (organic layer) was diluted 1:1 (v/v) with solvent A of the mobile phase, and filtered with a 0.2 μm nylon filter (GVS Filter Technology, Indianapolis, IN, USA). The extracts were stored at 4 °C until analysis in order to preserve their stability.

3. Results and discussion

3.1. Instrumental optimisation

With the aim of identifying the optimal conditions for the ESI of mycotoxins, different concentrations of HCOOH (0–0.3%) and NH_4HCOO (0–10 mM) on mobile phase were tested, since the addition of buffers to the mobile phase allows a reduction in sodium adducts, improving analyte ionisation (Campone et al., 2015). The addition of HCOOH is important, especially in the case of fumonisins (FB_1 and FB_2), because it increases their sensitivity and improves their peak shape (Zöllner & Mayer-Helm, 2006). However, higher buffer concentrations cause ion suppression (Beltrán,