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Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry

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ABSTRACT

A reliable and rapid method has been developed for the determination of 10 mycotoxins (beauvericin, enniatin A, A1, B1, citrinin, aflatoxin B1, B2, G1, G2 and ochratoxin A) in eggs at trace levels. Ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) has been used for the analysis of these compounds in less than 7 min. Mycotoxins have been extracted from egg samples using a QuEChERS-based extraction procedure (Quick, Easy, Cheap, Effective, Rugged and Safe) without applying any further clean-up step. Extraction, chromatographic and detection conditions were optimised in order to increase sample throughput and sensitivity. Matrix-matched calibration was used for quantification. Blank samples were fortified at 10, 25, 50 and 100 $\mu g\,kg^{-1}$, and recoveries ranged from 70% to 110%, except for ochratoxin A and aflatoxin G1 at 10 $\mu g\,kg^{-1}$, and aflatoxin G2 at 50 $\mu g\,kg^{-1}$. Relative standard deviations were lower than 25% in all the cases. Limits of detection ranged from 0.5 $\mu g\,kg^{-1}$ (for aflatoxins B1, B2 and G1) to 5 $\mu g\,kg^{-1}$ (for enniatin A, citrinin and ochratoxin A) and limits of quantification ranged from 1 $\mu g\,kg^{-1}$ (for aflatoxins B1, B2 and G1) to 10 $\mu g\,kg^{-1}$ (for enniatin A, citrinin and ochratoxin A). Seven samples were analyzed and aflatoxins B1, B2, G1, G2, and beauvericin were detected at trace levels.

1. Introduction

Mycotoxins are secondary metabolites produced by many species of filamentous fungi. Currently, more than 400 mycotoxins have been identified in the world [1] and most of them can be categorized into *Aspergillus* mycotoxins (e.g. aflatoxins, ochratoxins), *Fusarium* mycotoxins (e.g. enniatins, beauvericin) and *Penicillium* mycotoxins (e.g. citrinin) [2,3]. The occurrence of these compounds depends on factors like strain of fungus, species, plant species, and environmental and ecological conditions such as humidity, temperature and presence of pests [4].

These compounds are toxic and pose a health hazard to humans and animals. This toxicity can range from the production of several hormonal disorders or immunosuppression to the induction of carcinogenic, teratogenic or mutagenic activities [5].

The presence of these contaminants and their metabolites in food of animal origin, such as meat, milk, eggs and cheese could be consequence of a carry over of these compounds into animal tissues after feeding of contaminated hay or corn [6]. Bearing in mind that egg is essential in diet and because the consumption is increasing worldwide [7], it is important to assure the safety of this product in terms of mycotoxins occurrence.

Among the different mycotoxins that can be found in eggs, aflatoxins are considered the most serious threat to public health due to the effects they can provoke. There are several compounds belonging to aflatoxins, such as B1, B2, G1 and G2. They have been detected at concentrations higher than 6 $\mu g\,kg^{-1}$ [8], and they are considered as Class 1 carcinogens by the International Agency for Research on Cancer (IARC). Aflatoxin B1 is considered the most toxic of them [2,9] and it can be metabolized by livestock into aflatoxin M1, which has also been detected at trace levels in eggs [8]. Ochratoxin A and citrinin can coexist in stored products and in cereal and cereal byproducts under optimal conditions, which may be part of feed and they can be found in animal origin products like eggs [10,11]. Despite their toxicity, ochratoxin A and citrinin are responsible of decreasing egg production and hatchability [12]. Other mycotoxins such as beauvericin and enniatins, including A, A1 and B1, are commonly present in harvested grains and because they are lipophilic contaminants, they can be bioaccumulated into egg yolks [13,14] at concentrations up to 7.5 $\mu g\,kg^{-1}$ [14].

Due to the high occurrence of these compounds in food and feed and their implication in pathologies, mycotoxins are a global concern and they are included in monitoring food program to minimise the levels in these products. Despite European legislation sets

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maximum levels of mycotoxin in foodstuffs [15], there is not specific legislation in eggs, and it should be established to assure food safety [5]. For this reason the development of analytical methods that allow unambiguous identification, quantification and detection at very low concentration levels is necessary. In this sense, it is difficult to develop an analytical method for the simultaneous determination of several mycotoxins, since they have different physicochemical properties. Thus, some of the developed analytical methods determine a single class of compounds [8,14], and very few multiclass methods for the determination of mycotoxins in egg have been proposed, analyzing simultaneously less than 7 compounds [16,17].

The analysis of mycotoxins in egg is a difficult task because this is a complex matrix, and the chromatographic analysis requires the application of previous extraction and/or clean-up steps in order to remove proteins and lipids [18]. Generally, the extraction of mycotoxins from egg is based on a simple extraction using acetonitrile or a mixture of methanol and water [17], which allows the precipitation of proteins. Because the amount of coextractive compounds typically present in eggs, a clean-up procedure is usually applied using immunoaffinity columns [16], or conventional sorbents such as OASIS [17] or silica [14], increasing the analysis time due to sample treatment. However, it is necessary to develop generic extraction procedures that reduce sample handling and increase sample throughput. In this sense, in the last few years QuEChERS procedure (acronym name for Quick, Easy, Cheap, Effective, Rugged and Safe) has been developed [19]. This method is based on an extraction with acidified acetonitrile followed by an induced liquid–liquid partition after the addition of salts. It has been used for the extraction of a wide variety of compounds, such as pesticides [20], veterinary drug residues [21], and mycotoxins [22], from variety of matrices such as fruits and vegetables [23], and cereal products [24]. However, it has not been checked for the extraction of mycotoxins from eggs.

For the detection and quantification of mycotoxins, chromatographic techniques like gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) can be used. LC–MS is a suitable technique for the analysis of polar substances like mycotoxins, because no derivatization step is required as in GC–MS [25–27]. For instance, LC using several analysers such a single quadrupole [28], time of flight (TOF) [29] or triple quadrupole enabling tandem mass spectrometry (MS/MS) [30] have been the most applied methods [25]. In this sense, LC–MS/MS provides the highest sensitivity and specificity, detecting low levels of mycotoxins in complex matrices, reducing sample preparation and analysis time [31,32]. Furthermore, the application of ultra high pressure liquid chromatography (UHPLC) has decreased the analysis time by means of the reduction of particle size of stationary phase (<2 μm). Therefore, it provides significant advantages in relation to conventional LC, such as higher speed of analysis, resolution, sensitivity and peak capacity. UHPLC has been used for the detection of several types of mycotoxins in different matrices [32–34].

The purpose of this study has been the development of a simple and efficient UHPLC–MS/MS multi-mycotoxin analytical method for the simultaneous determination of enniatins A, A1, B1, aflatoxins B1, B2, G1, G2, citrinin, ochratoxin A and beauvericin in eggs at trace levels using a simple extraction procedure avoiding further clean-up steps.

2. Materials and methods

2.1. Reagents and chemicals

Beauvericin, citrinin and aflatoxins B1, B2, G1 and G2 were purchased from Sigma–Aldrich (Madrid, Spain). Enniatin A, A1 and B1 were obtained from Santa Cruz (Santa Cruz, CA, USA). Stock solution of ochratoxin A (in acetonitrile) was purchased from Riedel de Haën (Seelze, Germany).

First, stock standard solutions were prepared by exact weighing of those mycotoxins obtained in powder and dissolved in 10 mL of HPLC-grade acetonitrile (Sigma). Then, a multicompound working solution (2 mg L^{-1}) was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with appropriate amounts of acetonitrile. These solutions were kept at 4 °C and renewed weekly.

Acetic acid (purity > 97%), formic acid (purity > 98%), ammonium formate and sodium sulphate anhydrous were obtained from Panreac (Barcelona, Spain). Sodium acetate anhydrous was purchased from J.T. Baker (Deventer, Holland). HPLC-grade methanol was supplied by Sigma. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA). C18 Sep-Pak 200 mg/3 cm^3 and Oasis HLB 200 mg/3 cm^3 cartridges (Waters, Milford, MA, USA) were used to evaluate a clean-up step during the development of the extraction procedure.

2.2. Apparatus and software

Chromatographic analyses were performed in an ACQUITY UPLC™ system (Waters, Milford, MA, USA), using an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm), with 1.7 μm particle size, from Waters. MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive and negative ion mode. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters). Centrifugations were performed in a high-volume centrifuge from Centronic (Barcelona, Spain). A Vortex mixer Heidolph, model Reax 2000 and an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland) were also used. A Reax-2 rotary agitator from Heidolph (Schwabach, Germany) was used for sample extraction.

2.3. UHPLC–MS/MS analysis

Chromatographic analyses were carried out using a gradient elution with eluent A being methanol and eluent B consisting on an aqueous solution of ammonium formate (5 mM). The analysis started with 25% of eluent A, which was increased linearly up to 100% in 3.75 min. This composition was held for further 1.25 min before being returned to 25% of eluent A in 0.5 min, followed by a re-equilibration time of 1 min, to give a total run time of 6.5 min. The flow rate was set at 0.30 mL min^{-1} and column temperature was maintained at 30 °C. Aliquots of 5 μL of sample extract were injected into the chromatographic system.

For MS/MS detection, the ionisation source parameters in positive mode were: capillary voltage 3.5 kV, extractor voltage 4 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 550 L h^{-1} (both gases were nitrogen). The ionisation source parameters in negative mode were the same except the capillary voltage, which was set at 2.5 kV. Collision-induced dissociation was performed using argon as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The optimum MS/MS conditions of the mycotoxins were performed by column injection of individual standards at 500 μg L^{-1}. Full-scan mass spectra and product ion scan were acquired in order to obtain at least one precursor and two product ions for each compound for both identification and quantification purposes, selecting the most abundant product ion for quantification and the second one for confirmation. The multiple reaction monitoring (MRM) transitions and