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A simple sample pretreatment method for multi-mycotoxin determination in eggs by liquid chromatography tandem mass spectrometry

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ABSTRACT

In this study, a reliable and fast method using a quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction procedure without any clean-up step was developed for simultaneous extraction of 15 mycotoxins, i.e., aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, aflatoxin M1, aflatoxin M2, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, de-epoxy-DON, zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, and β-zearalanol, from eggs. High-performance liquid chromatography tandem mass spectrometry was used to separate and detect all of the analytes. Electrospray ionization at both negative and positive modes and multiple reaction-monitoring mode were applied to detect these analytes. The main factors, such as extraction time, extraction solvent, evaporation temperature, and pH of the solvent, were carefully optimized to improve the extraction efficiency. The coefficients of determination of the calibration curves ranged from 0.9884 to 0.9998. The recoveries of most of the analytes were between 71.3% and 105.4% at three concentration levels, except for AFB1 that showed recovery rates of not more than 67.5% in all concentrations. The repeatability and intra-lab reproducibility of this method were both lower than 15% and 25%, respectively. The limit of quantification ranged from 0.2 μg/kg to 5 μg/kg. The matrix effect was evaluated and reduced by the use of matrix-matched calibration curves. The validated method was applied in a pilot study to analyze mycotoxin contamination in 12 eggs, and trace amounts of deoxynivalenol, 15-acetyldeoxynivalenol, aflatoxin B1, aflatoxin G2, zearalenone and β-zearalenol were detected in these samples.

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1. Introduction

Egg is an essential protein source for humans. The egg production and consumption worldwide have increased by nearly four times from 1960 to 2003. An increase by another 30% has been forecasted for the next 20 years [1,2]. Egg consumption in industrialized countries grows even faster. The General Administration of Customs of the People's Republic of China reported that the total egg consumption in China mounted to 2.4×10^6 t in 2010, indicating that approximately 300 billion eggs were consumed in that year. However, a wide range of chemical contaminants, biological agents, and natural toxins are often found in eggs; thus, eggs can potentially cause food poisoning [3,4]. Among the possible contaminants, mycotoxins are believed to be dangerous contaminants because of their widespread occurrence.

Mycotoxins are a heterogeneous group of toxic secondary fungal metabolites and are commonly found in cereals, such as maize, wheat, and barley, at favorable temperature and humidity. The toxic effects of mycotoxins include immunosuppression and endocrine disorder; mycotoxins are also carcinogenic, teratogenic, or mutagenic. In recent decades, mycotoxins have increasingly

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attracted attention worldwide because of their widespread occurrence and high frequency of contamination [5]. Aside from mycotoxicoses caused by direct consumption of contaminated food and feed, the "carry over" of these compounds into edible animal tissues, milk, or eggs is also possible.

Researchers have proven that feeding blends of grains contaminated with *Fusarium* mycotoxins may lead to the transfer of a certain proportion of mycotoxins from hens to eggs. For example, deoxynivalenol (DON) and its metabolite de-epoxy-DON (DOM) were detected in home-produced eggs in Belgium [6], and trace amounts of aflatoxin (AF) B1, B2, G1, G2, β-zearalenol (β-ZOL), and beauvericin were also detected in eggs from the local supermarket [7,8]. The European legislation has set maximum levels of mycotoxin in food [9], and some research has underscored that compliance to this rule in poultry husbandry will prevent mycotoxin contamination in eggs. By contrast, mycotoxin contamination is widespread in less developed countries [10]. The mycotoxins in the liver or other organs of hens biotransform into a variety of metabolites, which may be transferred into their eggs [11]. Given that the concentration of mycotoxins or their metabolites in egg reflects the contamination levels of mycotoxins in feed, the egg is a possible target for evaluation of animal exposure to mycotoxins [12,13].

Chromatographic-based methods, such as liquid chromatography tandem mass spectrometry (LC-MS/MS), have been extensively used as highly selective and sensitive confirmatory methods for mycotoxin determination [6,7,11,14]. However, simultaneous extraction of mycotoxin from the matrix is difficult because mycotoxins belong to a large group of heterogeneous compounds exhibiting a wide range of chemical properties. Therefore sample preparation remains the bottleneck in all mycotoxin detection protocols. Solid phase extraction (SPE) using various cartridges is currently the most widely used method in sample preparation. The limitations of SPE, however, include low recovery, which results from the interaction between the sample matrix and analytes, and plugging of the cartridge or blocking of the pores in the sorbent by solid impurities and oily components [15,16]. Although multifunctional clean-up columns or immuno-affinity columns have been widely used, these approaches are expensive, which limit their extensive application in analyzing large amounts of samples [17].

Highly efficient extraction systems that require minimum sample treatment are increasingly desired in food contaminant analysis. Thus, a quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based method prior to LC–MS/MS analysis is attracting more attention [18]. This method has been successfully applied in detecting various compounds, such as veterinary drug residues [19], antibiotics [20], pesticides [21], and mycotoxins [7,22,23], in various matrices. Frenich et al. [7] performed a pilot research on mycotoxin determination in eggs, where 10 mycotoxins were included in their study but excluding the highly polar compounds, such as DON and its metabolites. The present study aimed to develop a modified QuEChERS preparation method for simultaneous determination of 15 analytes, DON, zearalenone (ZEA), AFB1, and their possible metabolites in eggs. Coupled with an optimized LC–MS/MS method, this method can successfully detect mycotoxins in real samples.

2. Experimental

2.1. Reagents and standards

All organic solvents, acids, and salts were HPLC or analytical grade. Acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ammonium acetate (AA), formic acid (FA, 98%), $MgSO_4$ anhydrous, and NaCl were provided by Aladdin Co. (Shanghai, China). Ultrapure water was obtained from Millipore (Bedford, MA, USA). The solid standards of AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2 were obtained from Alexisa (San Diego, CA, USA). DON, 3-acetyldeoxynivalenol (3-Ac DON), 15-acetyldeoxynivalenol (15-Ac DON), DOM, ZEA, α-zearalenol (α-ZOL), β-ZOL, α-zearalanol (α-ZAL), and β-zearalanol (β-ZAL) were obtained from Sigma Aldrich (St. Louis, Mo, USA).

The stock standard solution of each compound was prepared in ACN at 100 μg/mL, and the commercially certified DOM was used directly as stock standard solution. All of the stock solutions were kept in brown glass vials and stored at −20 °C. Two different working solutions were prepared in this experiment. The first was prepared from 1 μg/mL of each toxin in ACN and the other was set at the following concentrations: AFB2, α-ZAL, β-ZAL, and ZEA (50 ng/mL); AFB1 (150 ng/mL); AFM1, AFM2, AFG1, AFG2, DOM, α-ZOL, and β-ZOL (250 ng/mL); 3-Ac DON and 15-Ac DON (500 ng/mL); and DON (750 ng/mL). All working solutions were freshly prepared. Blank egg samples in spiking and validation studies were produced by hens feed in our lab, which were previously analyzed. No analytes of interest were found. The samples in the pilot study were purchased from local supermarkets in Shanghai.

2.2. Sample preparation

Two sample preparation protocols were investigated in the study.

2.2.1. Matrix solid phase dispersion

The matrix solid phase dispersion (MSPD) method was performed according to the published method with some modification [24,25]. Briefly, the fresh eggs were homogenized, and 1.0 ± 0.05 mL of the homogenous mixture was placed in a glass beaker (25 mL) and then gently blended with 1 g of C_{18} for 5 min. The mixture was allowed to stand at room temperature for 2 h before further extraction. The mixture was then introduced to a 6 mL SPE empty column and eluted dropwise with 20 mL of ACN/MeOH (50/50, v/v) with 1 mM AA. The eluent was transferred into a 25 mL glass tube and then evaporated to dryness at 50 °C with a gentle stream of nitrogen. The residue was re-dissolved in 600 μL of mobile phase and filtered through 0.22 mm nylon filters (Millipore, 13 mm diameter) before LC–MS/MS analysis.

2.2.2. QuEChERS method

The fresh eggs were first thoroughly mixed manually, and then 1.0 ± 0.05 mL of the homogenized egg sample was transferred into a 50 mL polypropylene centrifuge tube. After adding 4 mL water into the tube, the tube was vortexed for 1 min. Thereafter, 5 mL of acidified ACN (1% FA) was added and the system was subjected to extraction using a horizontal electric shaker for 30 min at 120 rpm. $MgSO_4$ (2.0 g) and NaCl (0.5 g) were subsequently added into the solution. In case of agglomeration, the mixture was vortexed vigorously for 2 min immediately. Furthermore, after centrifugation at 4500 × *g* for 10 min, the supernatant ACN layer of the mixture was transferred into a 10 mL test tube and then evaporated at 50 °C under a gentle stream of nitrogen. Before analysis, the residue was processed following the procedure described in Section 2.2.1.

2.2.3. Experimental design for the QuEChERS method optimization

The QuEChERS method was optimized using a D-optimal design consisting of 27 experiments [26]. Four factors with three levels each were considered in the design. These factors include (1) extraction time (15–60 min), (2) FA concentration in the extraction solvent (0–1%), (3) evaporation temperature (30–70 °C), and (4) extraction volume (1–10 mL). The response (peak area) was used in statistical analysis. All analyses were performed using

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