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UFLC-ESI-MS/MS analysis of multiple mycotoxins in medicinal and edible *Areca catechu*

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HIGHLIGHTS

- Mycotoxins co-occurrence in both medicinal and chewing *A. catechu* was investigated.
- A UFLC-ESI$^{+/-}$-MS/MS method was optimized and validated to evaluate multiple mycotoxins.
- A reliable, rapid and simple sample pretreatment method was optimized.
- The approach possesses a desirable level of sensitivity, recovery and precision.
- AFB_2 and ZON were respectively detected in medicinal and chewable *A. catechu*.

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ABSTRACT

A robust, sensitive and reliable ultra fast liquid chromatography combined with electrospray ionization tandem mass spectrometry (UFLC-ESI-MS/MS) was optimized and validated for simultaneous identification and quantification of eleven mycotoxins in medicinal and edible *Areca catechu*, based on one-step extraction without any further clean-up. Separation and quantification were performed in both positive and negative modes under multiple reaction monitoring (MRM) in a single run with zearalanone (ZAN) as internal standard. The chromatographic conditions and MS/MS parameters were carefully optimized. Matrix-matched calibration was recommended to reduce matrix effects and improve accuracy, showing good linearity within wide concentration ranges. Limits of quantification (LOQ) were lower than 50 μg kg^{-1}, while limits of detection (LOD) were in the range of 0.1–20 μg kg^{-1}. The accuracy of the developed method was validated for recoveries, ranging from 85% to 115% with relative standard deviation (RSD) ≤14.87% at low level, from 75% to 119% with RSD ≤ 14.43% at medium level and from 61% to 120% with RSD ≤ 13.18% at high level, respectively. Finally, the developed multi-mycotoxin method was applied for screening of these mycotoxins in 24 commercial samples. Only aflatoxin B_2 and zearalenone were found in 2 samples. This is the first report on the application of UFLC-ESI$^{+/-}$-MS/MS for multi-class mycotoxins in *A. catechu*. The developed method with many advantages of simple pretreatment, rapid determination and high sensitivity is a proposed candidate for large-scale detection and quantification of multiple mycotoxins in other complex matrixes.

1. Introduction

Fungal contamination was available in most of agricultural commodities during their growth, harvest, transportation or storage processes (Nácher-Mestre et al., 2015). Mycotoxins, the secondary metabolites, produced by various fungi have become an interesting topic over the past decade due to their wide range of

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toxic effects, such as carcinogenicity, teratogenicity and mutagenicity (Mézes et al., 2010). Hitherto, more than 400 mycotoxins have been identified in the world. The major mycotoxins are categorized into aflatoxins (e.g. aflatoxins B_1, B_2, G_1, G_2), trichothecenes (e.g. HT-2, T-2 toxin), fumonisins (e.g. fumonisin B_1, B_2), ochratoxins (e.g. ochratoxin A) and zearalenone (Cheli et al., 2014; Guo et al., 2014; Kimanya et al., 2014). The demonstrated toxic effects of these compounds has prompted the European Union and many other countries to set maximum limits of them in foodstuffs, feeds and other matrices intended for human or animal consumption (European Commission, 2006a; Food and Agriculture Organisation, 2003).

Areca catechu Linn. (family Arecaceae) is widely distributed in Southern and Southeast Asia including China, India, Malaysia and other countries (Lim and Kim, 2006; Peng et al., 2015). Traditionally, *A. catechu* is used to kill parasites and promote digestion (Anonymous, 1977; Peng et al., 2015). Currently, it is commonly used as one of the main traditional Chinese medicines (TCMs) in over 100 prescriptions based on its wide spectrum of biological and pharmacological activities, for treating various gastrointestinal, parasitic and edematous diseases (Chavan and Singhal, 2013). In addition, the immature fruit (areca nut) or pericarp of *A. catechu* is popularly edible (Lim and Kim, 2006; Benegal et al., 2008), which has been used as the natural "Plant gum" in daily life in Chinese Taipei and South and Southeast Asia, as well as the fourth most widely used addictive stimulant (Heatubun et al., 2012). Due to the high temperature and humidity conditions under which *A. catechu* grows, it is susceptible to the toxigenic molds growth and mycotoxins production. Hence, it is of great importance to routinely test the contamination levels of mycotoxins in *A. catechu* before being consumed.

Commonly, most matrices can be contaminated with a large group of mycotoxigenic fungi, producing more than one or one group of mycotoxin, which implies a risk of additional or even synergetic toxic effects (Pamel et al., 2011). Hence, a multi-mycotoxin analytical method for simultaneously detecting multi-group co-occurring mycotoxins is highly desirable. Over the last few years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the most extensively used method for mycotoxin analysis, because of unambiguous identification and confirmation, accurate quantification and no need of derivatization (Devreese et al., 2012; Åberg et al., 2013; Zheng et al., 2014), having an increasing impact on the development of multi-mycotoxin analytical methods (Monbaliu et al., 2010). Very recently, multi-residue LC-MS/MS analysis of mycotoxins in cereals, e.g. wheat and maize, has been successfully achieved by injecting diluted crude sample extracts without further clean-up (Sulyok et al., 2007; Aguilera-Luiz et al., 2011). The principle of this dilution method is to minimize matrix effects by reducing the concentration of interferences, which afforded a lot of significant references for high-throughput and economical analysis of multi-mycotoxin in *A. catechu*. Furthermore, liquid chromatography techniques have also improved with the development of ultra fast liquid chromatography (UFLC), in which columns with smaller packing particles are used (1.7 μ m instead of 5 μ m). UFLC with higher efficiency significantly reduces analysis time and allows the extension of new limits to peak capacity and speed.

The aim of this study was, therefore, to develop and validate a multi-mycotoxin UFLC-$ESI^{+/-}$-MS/MS method for simultaneous analysis of 11 mycotoxins including aflatoxin B_1, B_2, G_1, G_2 (AFB_1, AFB_2, AFG_1, AFG_2), HT-2, T-2 toxin (HT-2, T-2), fumonisin B_1, B_2 (FB_1, FB_2), Citrinin (CIT), ochratoxin A (OTA) and zearalenone (ZON) in medicinal and edible *A. catechu* on the basis of injection of diluted crude sample extracts. Matrix-matched standards calibration and internal standard were selected to compensate matrix effects for accurate quantification. To the best of our knowledge, this is the first report of employing UFLC-$ESI^{+/-}$-MS/MS method for simultaneous analysis of multi-class mycotoxins in *A. catechu* in one chromatographic run, which provide significant references and guidance for high-throughput and economical analysis of more classes of mycotoxins in other TCMs or complex matrices.

2. Materials and methods

2.1. Chemicals, products and reagents

The analytical standards of OTA, ZON, FB_1, FB_2, CIT, HT-2 and T-2 toxins in powder form (1 mg), together with AFB_1 and AFG_1 (each 2 μg mL^{-1}), AFB_2 and AFG_2 (each 0.5 μg mL^{-1}), as well as zearalanone (ZAN, 2,4-dihydroxy-6-(10-hydroxy-6- oxoundecyl) benzoic acid μ-lactone) as internal standard (IS) were all purchased from Pribolab (Singapore). Their chemical structures and other detailed information were summarized in Table S1, as Supplementary Material. Methanol and acetonitrile used as the mobile phase and extraction solvent, respectively, were of HPLC grade and provided by Burdick & Jackson (Morris, NJ, USA), while formic acid, acetic acid, ammonium formate and ammonium acetate of analytical grade were obtained from Beijing Chemical Works (Beijing, China). Purified water (Wahaha, Hangzhou, China) was used throughout the analysis.

A total of twelve medicinal *A. catechu* samples numbered from S1-1 to S1-12 were obtained from Anguo medicinal materials market (Hebei, China), which were originally produced in Vietnam, Malaysia and China. Meanwhile, twelve edible *A. catechu* samples numbered from S2-1 to S2-12 were purchased or collected according to their different manufacturers in local supermarkets in Beijing, China. All samples were homogenized by a laboratory mill and then passed through $\Phi = 250 \pm 9.9$ μm filter and, stored in ziplock bags at −20 °C before analysis.

2.2. Preparation of standard solutions

Stock solutions of OTA, ZON, FB_1, FB_2, CIT, HT-2, T-2 and ZAN (IS) were prepared in acetonitrile (1 mg mL^{-1}) and stored at −20 °C. Then, a mixed working standard solution (except IS) was prepared by combining suitable aliquots of each individual stock standard solution and diluting with appropriate amounts of acetonitrile. Meanwhile, the working solution of IS was similarly diluted to 10 μg mL^{-1}. All standard solutions were protected from light and stored in screw capped glass tubes, which were wrapped with parafilm and then stored at −20 °C until analysis.

2.3. Sample preparation

As for sampling, an aliquot of 2.0 g homogenized sample was weighed and 5 mL of extraction solvent (methanol/water (80: 20, *v*/*v*) was added. The mixture was let stand 20 min, then shaken with a vortex mixer for 3 min. After centrifugation at 12,000 rpm for 5 min, the supernatant extract was one-fold diluted with acetonitrile/water (20: 80, *v*/*v*). Then, the final extract was amended with 20 μL of internal standard solution at 10 μg mL^{-1}. The tube was subsequently capped and shaken about 30 s by vortex briefly. Finally, the solution was passed through a 0.22-μm syringe nylon filter and sealed in an auto-sampler vial and stored at −20 °C pending analysis.

2.4. Instrumentation

The chromatographic separation of the eleven mycotoxins was carried out on an ultra fast liquid chromatograph (UFLC)