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Multi-mycotoxins analysis in *Pheretima* using ultra-high-performance liquid chromatography tandem mass spectrometry based on a modified QuEChERS method

Sisi Zhang[a,b], Jiwei Lu[b], Shaomin Wang[b], Dan Mao[b], Shui Miao[b], Shen Ji (Chief Pharmacist, PhD Supervisor)[b,*]

[a] School of Pharmacy, Fudan University, No. 826 Zhangheng Road, Pudong New Distr., Shanghai 201203, China
[b] Shanghai Institute for Food and Drug Control, No. 1500 Zhangheng Road, Pudong New Distr., Shanghai 201203, China

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ABSTRACT

A sensitive and high-throughput method was established and validated for the simultaneous determination of 22 mycotoxins in *Pheretima aspergillum* (E.Perrier) and *Pheretima guillelmi* (Michaelsen). A modified Quick Easy Cheap Effective Rugged and Safe (QuEChERS) method was used for sample preparation with recoveries ranging from 73% to 105% with relative standard deviations (RSDs) <8.0% for all target analytes. Ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC–MS/MS) was applied for separation and detection in ESI (+) and ESI (−) modes with the limits of detection (LOD) in the range of 0.05–10 μg kg^{-1}. The 22 compounds could be accurately quantified in the 0.5–1000 μg kg^{-1} concentration range with correlation coefficients >0.99. In all cases, the intra- and inter-day precisions were lower than 6% and 10%, respectively. Matrix-matched calibration was utilized for quantification purposes to compensate for the matrix effects. Furthermore, the established method was successfully applied in 17 batches of normal real samples collected from different areas of China and 2 batches of moldy samples due to improper storage, only mold-contaminated samples were confirmed to have fumonisin B1 (FB1) and fumonisin B2 (FB2) contamination at 2.54–3.78 μg kg^{-1}. The constructed method could serve as a practical application of the UHPLC–MS/MS method for the trace analysis of multiple mycotoxins in complex matrixes, especially for those with high lipid contents.

1. Introduction

Pheretima (dried products of *Pheretima aspergillum* (E. Perrier), *Pheretima vulgaris* Chen, *Pheretima guillelmi* (Michaelsen) and *Pheretima pectinifera* Michaelsen), a traditional animal-originated medicine, has been used for a long time in China; it is mainly distributed in the Zhejiang, Jiangsu and Guangdong provinces. Any one of the four species can serve as medicine individually, and these species are not usually used in combination with each other. In addition, its medicinal values have been proven in many clinical trials and pre-clinical studies for cardiovascular conditions, relieving asthma, antipyretic and diuretic effects, and it is recorded in the Chinese pharmacopoeia (2015 edition) as one of the most important traditional animal-originated medicines. Furthermore, lumbrokinase capsules are commercially available worldwide as dietary supplements to provide protection against allergic and vascular disorders. It was also demonstrated that the earthworm fibrinolytic enzyme (EFE), which is extracted from *Pheretima* possesses clot dissolving properties, anti-virus activity and anti-tumor potential [1–5]. Moreover, nucleobases and nucleosides that could be extracted from *Pheretima* have been verified to have anti-seizure and anti-platelet aggregation effects [6–8].

However, earthworms are typically active in moist soil, which is unfortunately also appropriate for fungi proliferation. In addition, the high hygroscopicity of this product due to the large amounts

Abbreviations: QuEChERS, quick easy cheap effective rugged and safe; FB1, fumonisin B1; FB2, fumonisin B2; FB3, fumonisin B3; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; OTA, ochratoxinA; OTB, ochratoxin B; DOM-1, de-epoxy-deoxynivalenol; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol; ZAN, zearalanone; ZEA, zearalenone; ^{13}C-ZEA, ^{13}C-zearalanone; α-ZAL, α-zearalanol; β-ZAL, β-zearalanol; DON, deoxynivalenol; 3-ADON, 3-acetyldeoxynivalenol, 15-ADON 15-Acetyldeoxynivalenol; Fus-X, Fusarenon X; HT-2, HT-2 toxin; T-2, T-2 toxin; FA, formic acid; AmAc, ammonium acetate; AmF, ammonium formate; AA, acetic acid; NaOAC, anhydrous sodium acetate; TCMs, traditional Chinese medicines; PSA, primary secondary amine; GCB, graphitized carbon black; d-SPE, dispersed solid-phase extraction.

* Corresponding author.
E-mail addresses: 14211030033@fudan.edu.cn (S. Zhang), lujiwei1011@163.com (J. Lu), wangshaomin2008@163.com (S. Wang), m21944@126.com (D. Mao), qmiao2008@163.com (S. Miao), jishen2016@126.com (S. Ji).

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of proteins and amino acids makes it susceptible to mycotoxin production during the processing and storage procedures. Therefore, the Chinese pharmacopoeia (2015 edition) set maximum levels for aflatoxin B1 (5 $\mu g\,kg^{-1}$) and for the total content of aflatoxins B1 + B2 + G1 + G2 (10 $\mu g\,kg^{-1}$). Nevertheless, in addition to the four mycotoxins mentioned above, other mycotoxins such as fumonisins, zearalenone, ochratoxin A and deoxynivalenol are not only notoriously carcinogenic and teratogenic but also highly stable and resistant to processing. Thus, they can reach consumers, which poses a serious threat to human health throughout the world. Although the existence of mycotoxins is potentially harmful, previous studies have mainly concentrated on food and feed. Therefore, few papers have been published on the monitoring of the mycotoxins in traditional Chinese medicines, especially for animal-originated medicines, which seems to be an understudied area in this field. Thus, there is an urgent need to establish a simple, quick, sensitive and rugged method to monitor the mycotoxins in traditional Chinese animal-originated medicines.

Currently, there are many different methods for mycotoxin determination such as thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) [9,10], high-performance liquid chromatography coupled with a UV or fluorescence detector or a mass spectrometry detector (MS) [11,12], gas chromatography mass spectrometry (GC–MS) and capillary electrophoresis (CE) [13–16]. TLC and ELISA are fast and inexpensive but only qualitative or semi-quantitative and are therefore usually used for screening samples. GC–MS also has limitations due to the usual needs for derivatization prior to injection into a GC column. As an emerging popular detection method, UHPLC–MS/MS stands out due to its high sensitivity and selectivity as well as its ability to perform multi-analyte determination in a single run, especially for the complex matrices such as traditional Chinese medicines (TCMs) [17–20]. Prior to UHPLC–MS/MS analysis, the low residue level of mycotoxins, complex chemical compositions of TCM samples and disparate physical and chemical properties of mycotoxins make a proper sampling method a key prerequisite for reliable detection, particularly for multi-class mycotoxin determination. At present, the high specificity and selectivity towards selected mycotoxins have made solid phase extraction with immune affinity column (IAC) cleanup one of the most commonly used procedures throughout the world. This technique is recorded in the United States pharmacopoeia 39, the European pharmacopoeia 8.0, the Chinese pharmacopoeia (2015 edition), etc. Moreover, some international organizations such as Association of Official Analytical Chemists (AOAC) and European Committee for Standardization (CEN) have also made the IAC method the most frequently performed sampling method in terms of mycotoxins determination. Nevertheless, the IAC method is not only complex and expensive but also unable to analyze multi-class mycotoxins. As a consequence, a simple, efficient, multiclass extraction system is emerging; this system is called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). QuEChERS, as an increasingly popular method, was first used for pesticide analysis on fruits and vegetables [21]. In recent years, its popularity has skyrocketed, and it has been applied to many disparate matrices such as feeds, rice, wine, milk and so forth [22–26].

Given the above facts, a modified QuEChERS clean-up method combined with UHPLC–MS/MS was established to simultaneously determine 22 mycotoxins in *Pheretima*, which was successfully put into use on 19 batches of *Pheretima aspergillum* (E.Perrier) and *Pheretima guillelmi* (Michaelsen) collected from different areas of China. To the best of our knowledge, this is the first study of mycotoxins determination in traditional animal-originated medicine using UHPLC–MS/MS, serving as a positive reference for further study on mycotoxins.

2. Experimental methods

2.1. Materials and reagents

Standards of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2(AFG2), ochratoxin A(OTA), de-epoxy-deoxynivalenol(DOM-1), α-zearalenol(α-ZOL), β-zearalenol (β-ZOL), zearalanone (ZAN), zearalenone (ZEA), ^{13}C-zearalanone (^{13}C-ZEA), α-zearalanol (α-ZAL), and β-zearalanol (β-ZAL) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and Fusarenon X (Fus-X) were obtained from Biopure (Tulln, Austria). HT-2, T-2 toxin, and ochratoxin B (OTB) were purchased from Alexis Corporation (Lausen, Switzerland). Atrazine-*d*5 was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). In addition, 2 batches of *Pheretima aspergillum* (E.Perrier) and 17 batches of *Pheretima guillelmi* (Michaelsen) were collected from the markets and stores in different areas of China including Shanghai, Henan, and Hainan to test the amount of mycotoxins.

Methanol, acetonitrile and formic acid of HPLC grade were purchased from Merck (Darmstadt, Germany). Formic acid, acetate acid, ammonium formate and ammonium acetate for LC–MS were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Ultrapure water was obtained from a Milli-Q Gradient Water System (Millipore, Bedford, MA, USA).

Anhydrous magnesium sulfate, anhydrous sodium acetate (NaOAc), sodium chloride, dispersed solid-phase extraction (d-SPE) sorbent C18, primary secondary amine (PSA) as well as graphitized carbon black (GCB) were obtained from Bonna-Agela Technologies (Tianjin, China).

2.2. Preparation of standard solutions

2.2.1. Preparation of reagent-only calibrators

Solid portions of each mycotoxin standard as well as the internal standards were weighed and dissolved directly in acetonitrile to prepare stock solutions of different concentrations. Stock solutions were stored in a refrigerator at −20 °C. Subsequently, each stock solution of mycotoxins was transferred into the combined mixed stock solution and diluted with acetonitrile step-by-step to prepare a sequence of reagent-only calibrators (0.5, 1, 2, 5, 10, 20 $\mu g\,L^{-1}$, the concentrations of each reagent-only calibrators were normalized to the concentration of the AFG1 in each reagent-only calibrators).

2.2.2. Preparation of matrix-matched calibrators

The *Pheretima guillelmi* (Michaelsen) (batch 9) (1 g) was weighed into a 50 mL centrifuge tube in six replicates. Internal standards (250 μL of 6 $mg\,L^{-1}$ atrazine-*d*5 and 10 μL of 20 $mg\,L^{-1}$ ^{13}C-zearalenone) were added into the tube and kept soaked for 30 min in 15 mL Milli-Q water. Then, the samples were extracted using 15 mL of aqueous acetonitrile containing 15% formic acid for 5 min by continuous high-speed shaking at 1500 strokes/min (SPEX ShaQer™ 1500, Metuchen, NY, USA). Subsequently, 4 g anhydrous $MgSO_4$ and 1 g NaCl were added into the mixture, and the tubes were shaken by hands immediately to prevent the formation of conglomerates before another 5 min of vigorous high-speed shaking at 1500 strokes/min. Then, the tubes were placed in an ice bath for 10 min and centrifugated for another 10 min at 18514 × g (Eppendorf, Hamburg, Germany). A total of 9 mL of extracts were transferred to the d-SPE tubes containing 900 mg anhydrous $MgSO_4$, 900 mg C18, 300 mg PSA, 60 mg GCB, and followed with vigorous shaking and centrifugation. Afterwards, the extracts were mixed into one centrifuge tube to guarantee the homogeneity of the matrix. Subsequently, 3 mL of the cleaned-up