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# Multi-mycotoxins analysis in raw milk by ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry

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ABSTRACT

This paper presents the development and application of a sensitive and specific method for the analysis of multi-mycotoxins in raw milk using ultrahigh performance liquid chromatography quadrupole orbitrap mass spectrometry (UHPLC/Q-Orbitrap). Mycotoxins (a total of 14) included in this study were aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$, aflatoxin $M_1$, aflatoxin $M_2$, ochratoxin A, ochratoxin B, zearalenone, zearalanone, α-zeralanol, β-zeralanol, α-zeralenol, and β-zeralenol. Mycotoxins in milk were extracted and purified with a multi-mycotoxins AOZ immunoaffinity column in a single run, which made the matrix effect be negligible. The deliberate addition of small amount of acetonitrile was found to be beneficial for the extraction of mycotoxins from the complex milk matrix, especially for zearalenone and its derivatives. The final reconstituted extracts were analyzed using ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry (UHPLC Q-Orbitrap mass spectrometry). The extraction recoveries are in the range of 60–106%, with coefficient of variation <15%. The limits of detection for the analytes are in the range of 0.0003–0.008 μg/kg. Compared with a triple-quadrupole mass method in MRM mode, the Q-orbitrap analyzer provides accurate masses of both the precursor and product ions, thus offers a higher level of confidence in analyte identification without the sacrifice of analytical performance. The method was successfully applied to investigate the occurrence of the 14 mycotoxins in a total of two hundred and fifty raw milk samples collected from 5 different provinces of China.

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## 1. Introduction

Milk, as one of the most important sources of nutrients for human, is of particular importance for infants. When cattle ingests contaminated foodstuffs, mycotoxins may be metabolized and transferred to milk, thus becoming a risk to human health (Becker-Algeri et al., 2016; Flores-Flores, Lizarraga, De Cerain, & González-Peñas, 2015; Ketney, Santini, & Oancea, 2017). Mycotoxins as toxic secondary metabolites produced by certain fungal species potentially infest food and feed at all stages of production, processing, and storage (Picó, 2016; Selvaraj Wang et al., 2015). Aflatoxins (AFs) are produced by fungi belonging to several *Aspergillus* species and have been designated as a group 1 carcinogenic compound by the International Agency for Research on Cancer (IARC)(IARC, 2002). Aflatoxin $M_1$ ($AFM_1$) and aflatoxin $M_2$ ($AFM_2$) as the primary hydroxylated derivatives of aflatoxin $B_1$ ($AFB_1$) and aflatoxin $B_2$ ($AFB_2$)

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are formed in liver by the action of P450 cytochrome enzymes and are secreted into milk through the mammary gland of dairy cows (Flores-Flores et al., 2015; Mao et al., 2015). $AFM_1$ in milk has been the focus of research worldwidely (De Roma, Rossini, Ritieni, Gallo, & Esposito, 2017; Farah, Rosita, Norhaizan, & Redzwan, 2017; Iqbal, Asi, & Malik, 2017; Shuib, Makahleh, Salhimi, & Saad, 2017). Recently, it was reported that microbial ecosystem present inside the rumen is able to produce $AFB_1$ and $AFB_2$, which indicating that monitoring and regulating $AFB_1$ in cattle feed is not sufficient to ensure the absence of $AFM_1$ in milk (Nidhina, Bhavya, Bhaskar, Muthukumar, & Murthy, 2017). Ochratoxin and zearalenone have also been identified in milk and dairy products, although not been as extensively studied as $AFM_1$ (Becker-Algeri et al., 2016; Picó, 2016). Ochratoxins are toxic metabolites produced by several fungi of the genera *Aspergillus* and *Penicillium* and are commonly found in foodstuffs and feed (Mao, Lei, Yang, & Xiao, 2013; Zhu, Nie, & Xu, 2017). Ochratoxin A is considered as teratogenic, embryotoxic, genotoxic, immunosup-pressive, carcinogenic (IARC group 2B), and nephrotoxic (WHO, 2001). Zearalenone is a mycotoxin produced mainly by fungi belonging to the genus *Fusarium* (Xing et al., 2017). Although zearalenone and its derivatives (zearalanone, α-zeralanol, β-zeralanol, α-zearalenol, β-zearalenol) are considered non carcinogenic, they cause other adverse effects, in particular estrogenic effects. The estrogenic activity of α-zearalenol is even 3–4 times higher than that of the parent compound zearalenone (Huang et al., 2014).

In recent years, the number of reports of milk being contaminated by multiple mycotoxins has increased, raising concerns about whether synergetic effects of these coexisting mycotoxins could affect public health (Becker-Algeri et al., 2016; Flores-Flores et al., 2015; Huang et al., 2014; Picó, 2016). Therefore, methods by which multiple mycotoxins can be accurately quantified simultaneously will improve the efficiency of the analysis and the understanding about the prevalence of multiple mycotoxins contamination in milk (Berthiller et al., 2017). To evaluate the presence of multiple mycotoxins in milk, it would be laborious to use several single or single-class mycotoxin analysis methods as a routine practice. Therefore, increasing numbers of laboratories now use LC-MS for multi-mycotoxins analysis, which should outperform existing fluorescence- and UV-based methods due to its versatility, reliability and high throughput (De Girolamo et al., 2017; Miro-Abella et al., 2017; Selvaraj Zhou et al., 2015; Wang et al., 2016; Zhang, Wong, Krynitsky, & Trucksess, 2016; Zhang, Hu, Zhang, & Li, 2016). Also in the recent years, there are increasing interests in evaluating the use of high-resolution mass spectrometry (HRMS) for multi-mycotoxins analysis besides triple quadrupole-based methodologies (Anumol, Lehotay, Stevens, & Zweigenbaum, 2017; Berthiller et al., 2017; Dzuman, Zachariasova, Veprikova, Godula, & Hajslova, 2015; Perez-Ortega et al., 2016; Pesek et al., 2017). HRMS methods provide not only accurate mass information but also complementary structural information [e.g., tandem MS (MS/MS) data](Anumol et al., 2017; Berthiller et al., 2016; Knolhoff & Croley, 2016; Pesek et al., 2017; Picó, 2016; Shephard, 2016). Quadrupole orbital ion trap (Q-orbitrap) analyzer especially can be used for screening and quantifying for mycotoxins and aid in the structural elucidation, identification, and characterization of the chemicals on the basis of high -resolution and -accuracy mass results in MS/MS mode with improved method performance of analytical data (Jia, Chu, Ling, Huang, & Chang, 2014; Li et al., 2016; Liao et al., 2015; Martinez-Dominguez, Romero-Gonzalez, & Frenich, 2016; Wang, Leung, Chow, Chang, & Wong, 2015).

Herein, a multiclass method for the analysis of multi-mycotoxins in raw milk using ultrahigh performance liquid chromatography Q-orbitrap mass spectrometry (UHPLC/Q-Orbitrap) was developed. As milk presents a complex matrix with multiple components, complex interaction may exist between mycotoxins and matrix (Chavarría et al., 2017; Poor et al., 2017). Mycotoxins (a total of 14) including aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$, aflatoxin $M_1$, aflatoxin $M_2$, ochratoxin A, ochratoxin B, zearalenone, zearalanone, α-zeralanol, β-zeralanol, α-zeralenol, and β-zeralenol were extracted and purified using a multi-mycotoxins AOZ immunoaffinity column simultaneously. The developed method was validated in terms of matrix effect, overall recovery, and accuracy, *etc*., and was successfully applied on quantification of mycotoxins in raw milk samples.

## 2. Materials and methods

### 2.1. Materials

A total of 250 raw milk samples were collected from cattle ranches located in 5 different provinces (Sichuan, Shanghai, Neimeng, Hebei, and Shandong) of China in 2016. After collection at farms, milk samples were kept at 0 °C in incubator and upon arrival at the laboratory, samples were frozen at −20 °C in their original packs until analysis.

Mixed standard solution of aflatoxins (1.0, 0.3, 1.0 and 0.3 μg/mL for aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, and aflatoxin $G_2$, in 1.0 mL methanol) was got from Supelco (Bellefonte, PA, USA). Ochratoxin A, ochratoxin B, aflatoxin $M_1$, aflatoxin $M_2$, and zearalenone standards 10 μg/mL in acetonitrile or methanol were purchased from Pribolab (Pribolab Pte. Ltd., Singapore). Single standard solutions of aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, and aflatoxin $G_2$ 10 μg/mL in methanol were got from Clover (Clover Technology Group. Inc, China). Zearalanone, α-zeralanol, β-zeralanol, α-Zeralenol, and β-zeralenol in solid neat form supplied by Sigma–Aldrich (Sigma–Aldrich Co., MO, USA) were dissolved in methanol. Stock mycotoxins standard solutions in methanol with a concentration of 100 ng/mL (the contents of $AFB_2$ and $AFG_2$ are one third of other mycotoxins) were obtained by mixing corresponding standard solutions to 10 mL. Working standard solutions of 0.01–20 μg/L of mycotoxins were prepared by diluting stock standard with methanol: water (1:9 v/v). (**Caution**: Due to the extreme toxicity of mycotoxins, necessary protections must be taken in the whole experimental procedure. Safety goggles, respiratory mask and lab coats should be used throughout the experiment. All laboratory glassware and consumables contaminated with aflatoxins must be soaked with 10% sodium hypochlorite in a specified container for at least 24 h).

Multi-mycotoxins AOZ immunoaffinity columns for aflatoxins, ochratoxin and zearalenone were supplied by Clover (Clover Technology Group. Inc, China). These columns have a quoted capacity (the total amount of antibody that can be bound to the column gel) of 100, 100 and 1000 ng for aflatoxins, ochratoxin A and zearalenone, respectively, with at least 84% recovery. LC-MS grade acetonitrile and methanol, HPLC grade ammonium formate and formic acid were supplied by Sigma–Aldrich (Sigma–Aldrich Co., MO, USA). Pure water was obtained from a Milli Q water purification system (Millipore, Billerica, MA, USA).

### 2.2. Analytical procedure

#### 2.2.1. Sample preparation

Briefly, 30 g of raw milk was centrifugated for 10 min at 4500 rpm. Then after filtration with filter paper, 20 g of the supernatant was mixed with 20 mL water containing 5% (v/v) acetonitrile. Then the diluted sample was cleaned up through an AOZ immunoaffinity column at a flow-rate of about 1–2 drops per second. The column was washed with 20 mL pure water at a flow-rate of 1–2 drops per second. Mycotoxins were eluted with 3 mL

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