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Identification of ochratoxin A in Chinese spices using HPLC fluorescent detectors with immunoaffinity column cleanup

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ABSTRACT

Spices can be heavily contaminated with *Penicillium verrucosum* and *Aspergillus ochraceus*, which develop during cultivation and after harvest if relative humidity is not controlled during storage. The present study was undertaken with the aim to provide an analytical method to detect OTA in spices commercialized in China, specifically to develop a simple and rapid method for the determination of Ochratoxin A content in spices via immunoaffinity column and high performance liquid chromatography with fluorescence detection (HPLC-FLD). The recovery (75.0–102.0%) and repeatability (RSD < 12) are acceptable according to the requirements of the Commission Regulation (EC) No. 401/2006. The method is accurate, sensitive and rapid. A total of 480 spice products were analyzed with the amended analytical methods used in our study, which involved the extraction of OTA, immunoaffinity cleanup and high performance liquid chromatography determination with fluorescence detection. This report is the first describing the OTA presence in retail spices (pepper, chili, prickly ash, cinnamon, aniseed, fennel, curry powder and cumin) in China. The percentage of OTA contamination in spice product is 9.6%, and two chili products were above the levels permitted by the EU for safe consumption. Surveillance of a large number of suspected spice products should be continuous and widespread.

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1. Introduction

The mycotoxin ochratoxin A (OTA) is a secondary metabolite resulting from different environmental conditions and is primarily produced by *Penicillium verrucosum* and *Aspergillus ochraceu* (Covarelli, Beccari, Marini, & Tosi, 2012; Fernandes, Barros, & Câmara, 2013; Tessini et al., 2010) during the storage of food products, including commercially available spices (Aziz, Youssef, El-Fouly, & Moussa, 1998; Elshafie, Al-Rashdi, Al-Bahry, & Bakheit, 2002; Hashem & Alamri, 2010; Jalili & Jinap, 2012; O'Riordan & Wilkinson, 2008; Petzinger & Ziegler, 2000; Shundo et al., 2009; Waśkiewicz, Beszterda, Bocianowski, & Goliński, 2013). OTA is highly stable and resilient to primary and further food processing, such as milling and baking (Haighton, Lynch, Magnuson, & Nestmann, 2012). OTA is one of the most toxic agents of mycotoxin, and numerous toxicology studies have shown that OTA is carcinogenic, genotoxic, immunotoxic, teratogenic and nephrotoxic; therefore, the International Agency for Research on Cancer (IARC) has classified OTA as a 2B cancer compound (Han et al., 2013; IARC, 1993). At the European Union (EU) level, maximum levels have been established for different mycotoxins in different food matrices. Because prevention is better than cure for the consumer, the European Union (EU) has set the tolerable weekly intake (TWI) of OTA at 120 ng/kg body weight, with the maximum level of OTA for infants and young children at 0.50 μg/kg, for all foodstuffs not including spices (EC, 2006b).

In 2010, the EU set additional limits for OTA in spices and licorice products. For spices, whole or ground, including chilies, chili powder, cayenne, paprika, white and black pepper, nutmeg, ginger, and turmeric, this limit was set at 30.0 μg/kg until mid-2012, when it was reduced to 15.0 μg/kg (EC, 2010). In the Treaty on the Functioning of the European Union, the EU amended the limits of OTA in spices again in 5 July 2012, setting the limit for Capsicum spp. at 30 μg/kg until 31.12.2014, when it will be reduced to 15 μg/kg (EC, 2012). Some European countries set national regulations restricting OTA levels in spices, including Bulgaria (10.0 μg/kg) and Switzerland (20.0 μg/kg) (Espejo, Vazquez, Patino, & Armada, 2010). Egypt and Bosnia and Herzegovina refer to the Commission Regulation for establishing their national limits for OTA. In Russia, OTA limits have been established for several cereals and cereal products, as well as for various products for children. The limit in wheat, barley, rye, oat and rice cereals and cereal products is

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set at 5 μg/kg, whereas the limit for specific products for children is set at 0.5 μg/kg. In the Codex Alimentarius Standard, an OTA limit of 5 μg/kg is set only for raw wheat, barley and rye (CAC, 1995). These limits have been adopted by GCC, Nigeria and Kenya. In India, a maximum limit for OTA is established for the same foodstuffs as in the Codex; however, the limit is set at 20 μg/kg. No specific limits for OTA in foodstuffs have been set in the USA, Canada, Australia, New Zealand, Japan, Mexico or South Africa (Kubo, 2012). In China, the maximum level of OTA in cereals and beans is 0.50 μg/kg (Ministry of Health, 2011). For many countries, there is still no OTA limit set for spices, but more and more countries are starting to notice the issue of OTA-contaminated foodstuffs and to establish a maximum level for OTA in spices to provide a high level of protection for human health.

To enforce the maximum levels established by the European Commission, it is important to develop analytical methods. The first studies concerning the detection of mycotoxins in spices and medicinal plants used thin-layer chromatography (TLC), which uses a silica gel adsorbent and an acidic solvent system. Visual detection of OTA was performed by greenish fluorescence under long-wave ultraviolet light, which changes to blue fluorescence upon spraying the plate with methanolic sodium bicarbonate solution or exposing it to ammonia fumes. The detection limit of this method is 10 μg/kg for grains and other commodities. Reversed-phase TLC applied to the analysis of cereals and animal feed did not improve much on the higher detection limit, although there was an increase in fluorescence intensity compared to normal-phase TLC (Scott, 2002). After TLC, the analytical methods for OTA have improved considerably in the last ten years; most notably, the detection limits have been lowered (Jorgensen, 2005). However, most of the analytical methods developed for OTA focus on red wine (Mao, Lei, Yang, & Xiao, 2013), beer (Saez, Medina, Gimeno-Adelantado, Mateo, & Jimenez, 2004), cereals and cereal-derived products (Ozden, Akdeniz, & Alpertunga, 2012) and coffee products (Amezqueta et al., 2012; Sibanda, De Saeger, & Van Peteghem, 2002), based on LC (Saez et al., 2004) or HPLC with a variety of detectors, and fluorescence detection (FLD) is still the main method of choice after immunoaffinity cleanup, for its stability and sensitivity (Kabak, 2012; Ozden et al., 2012). Furthermore, the method of reversed-phase high-performance liquid chromatography coupled to fluorescence detection has been reported in earlier publications, for detecting musts and sweet wines (Hernandez, Garcia-Moreno, Duran, Guillen, & Barroso, 2006). At the same time, other fast, sensitive and reliable methodologies such as enzyme linked immunosorbent assay (ELISA) have been used for rapid OTA screening (Turner, Subrahmanyam, & Piletsky, 2009; Zhang et al., 2011); however, the disadvantages of this method are false-positive results and occasionally unacceptable quantification accuracy, such that it requires additional confirmatory analysis (Perrotta, Arevalo, Vettorazzi, Non, & Fernandez, 2012). In recent years, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has become the universal approach for mycotoxin analysis. Although this method has high selectivity and allows the identification of several mycotoxins in one run (Beltran et al., 2011; Cao et al., 2013), the most important disadvantage of LC–MS/MS is that the co-eluting compounds may suppress or enhance the ionization of the analytes of interest, potentially considerably affecting the limit of detection (LOD), limit of quantification (LOQ), linearity, precision and accuracy of the method. This method's applicability to different food matrices still requires further investigation (Waśkiewicz et al., 2013). In a word, nevertheless, there are many methods to quantify OTA in food stuffs nowadays, the high performance liquid chromatography with fluorescence detection is still one of the most used analytical methods for OTA determination, as the results are precise and accurate in a wide range of concentrations for OTA samples.

Reliable analytical methods must be available to collect the exposure data that will supply a broader information base for establishing appropriate control measures for OTA in spices, as well as contribute to a reliable exposure risk assessment and risk management for governments to protect human health. However, because each type of spice forms a complex matrix, the ultimate aim should be to achieve optimal analytical methods for each type of matrix of spices. Therefore, the aim of the present study was 1) to establish an improved method for the analysis of OTA in spices in China based on immunoaffinity column (IAC) cleanup followed by high performance liquid chromatography coupled with fluorescence detection (HPLC–FD) and 2) to assess the occurrence of OTA in spice samples widely consumed in China.

2. Materials and methods

2.1. Samples

A total of 480 spice and processed spice product samples were collected between April and July 2009. These samples were selected randomly and purchased in amounts greater than 0.5 kg from various retail outlets (supermarkets, shops and market stalls) in eight cities in China. The samples were maintained at 0–4 °C until arrival at the laboratory, where all samples were ground into powder and stored in plastic bags at 4 °C until the analysis. The collected samples included pepper ($n = 80$), chili ($n = 80$), prickly ash ($n = 80$), cinnamon ($n = 80$), aniseed ($n = 80$), fennel ($n = 40$), cumin ($n = 29$), and curry powder ($n = 11$). Spice preparations were ground and mixed to a uniform consistency using a laboratory mill or mixer.

2.2. Chemicals and reagents

All chemicals were analytical reagent grade, except for methanol and acetonitrile (both of HPLC grade, Merck, Darmstadt, Germany). All solutions were prepared with deionized water. All other inorganic chemicals and organic solvents were of reagent grade or higher. OTA standard solution (5 μg/mL in methanol) was purchased from Sigma (Sigma–Aldrich) Chemicals Company, a stock standard solution of OTA (500 μg/L) was prepared by diluting 1 mL standard OTA solution to 10 mL with methanol in a brown flask. Working standard solutions of 0.5, 5, 10, 25, and 50 μg/L of OTA were prepared by diluting the stock standard with acetonitrile:water:acetic acid (49.5:49.5:1, v/v/v). OchraTest™ Immunoaffinity columns (IAC), glass microfiber filters, phosphate-buffered saline (PBS, pH 7.0), and Tween-20 were supplied by VICAM (Watertown, MA, USA). The solution of 0.1% Tween-20-PBS was prepared by adding 0.5 mL Tween-20 to 500 mL pH 7.0-PBS. The 1% sodium bicarbonate solution was prepared by diluting 10 g reagent-grade sodium bicarbonate (Sigma–Aldrich, ≥99.0%) with 1000 mL pure water. A series of extraction solutions were prepared as follows: A) methanol:water (60:40, v/v) and B) methanol:*x*% sodium bicarbonate solution (60:40, v/v), and extraction solutions were prepared daily from these standards.

2.3. Sample extraction and immunoaffinity cleanup

Samples were extracted and then purified using OchraTest™ immunoaffinity columns according to the manufacturer's instructions with some modifications. The extraction and IAC cleanup of spices was performed according to the AOAC official Method 200.03 and 999.07 (Entwisle, Williams, Mann, Slack, & Gilbert, 2000; Stroka, Anklam, Jorissen, & Gilbert, 2000) with some

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