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# Highly sensitive simultaneous detection of major ochratoxins by an immunochromatographic assay

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ABSTRACT

Ochratoxins are highly toxic contaminants and considerable threat to the health of humans and animals. Ochratoxins, mainly include ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC), always exist in the food raw materials with the form of the mixture and they can convert to each other easily under special environment. The co-occurrence of three ochratoxins and conversion between different types of major ochratoxins make the specific detection for a separate kind of ochratoxin can not fully meet the rapid analysis demand of ochratoxins. Thus, the simultaneous detection for three major ochratoxins is urgent and indispensable. Here, a monoclonal antibody (mAb) which could simultaneously recognize OTA, OTB and OTC was screened out and then based on the mAb, an immunochromatographic assay (ICA) for rapid detection of the three major ochratoxins was first developed. After optimization, the visible limits of detection (vLODs) were 0.05, 0.025, 0.1 ng/mL and the cut-off concentrations were 0.5, 0.25, 0.5 ng/mL for OTA, OTB and OTC, respectively. Finally, the assay was evaluated using ochratoxins spiked maize samples, and results showed that the developed ICA could provide a practical method for the detection of three major ochratoxins on-site.



## 1. Introduction

Food safety is very important to human's health, but many factors impact on food safety, such as pesticide and veterinary drug residues, food additives abuse, microbial contamination and so on. Among these factors, microbial contamination poses the most risk for food safety, because it can not be addressed by formulating relevant laws and regulations as other factors. Except for aflatoxins, the most common and harmful mycotoxins in food and foodstuff are ochratoxins, which are secondary metabolites produced by several fungi of the *aspergillus* and *penicillium* families. Ochratoxins have carcinogenic, teratogenic, nephrotoxic and mutagenic to humans and animals (Majdinasab et al., 2015; Sharma, Manderville, & Wetmore, 2014), and are mainly include ochratoxin A (OTA), ochratoxin B (OTB) and ochratoxin C (OTC) (Meulenberg, 2012). Ochratoxins naturally occur in many kinds of food such as cereals, beans, coffee, fruits and spices, and their products such as bread, beer, and wine because of its chemical stability against heat treatments and hydrolysis, they also occur in animal products such as milk and meat due to the transfer and accumulation of toxins in animals through the food chain (Amezqueta, Gonzalez-Penas, Murillo-Arbizu, & De Cerain, 2009; Dall'asta et al., 2010; Han, Zheng, Luan, Ren, & Wu, 2010; Meulenberg, 2012; Monaci, Tantillo, & Palmisano, 2004; Pitt, 2000). OTB and OTC are the non-chlorinated and ethyl ester of OTA, respectively (Van Der Merwe, Steyn, & Fourie, 1965), they are frequently found in contaminated products accompanied by occurrence of OTA. Even though OTC exists at a lower concentration and has a similar or weaker toxicity than OTA under different conditions (Abbas, Valez, & Dobson, 2009; Davis, Searcy, & Diener, 1969; Harris & Mantle, 2001; Hult, Hökby, & Gatenbeck, 1977; Valero, Marin, Ramos, & Sanchis, 2008), yet a study indicates that OTC is more toxic than OTA on the respect of the destruction of immune cell functions (Heussner & Bingle, 2015; Muller et al., 2004; Muller, Rosner, Rohrmann, Erler, Geschwend, Grafe, et al., 2003). Some reports mentioned that usually a mixture of fungal metabolites were detected under natural conditions in many types of mycotoxin contaminated food (Remiro, Ibanez-Vea, Gonzalez-Penas, & Lizarraga, 2010). Except for co-occurrence, what is more serious is that the three major ochratoxins can convert to each other. Galtier and Alvinerie showed

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that OTA could transform easily to OTC in vitro with the presence of rumen fluid (Galtier & Alvinerie, 1976). If OTA transformed into OTC, the superabundant OTC in food of animal origin might lead to public health problems due to accumulation (Galtier, Alvinerie, & Charpenteau, 1981). Some researches also showed the OTC could convert into OTA under the special environment. For example Fuchs and co-workers had reported that OTC was easy to be hydrolyzed to OTA after oral or intravenous injection, and the conversion in vivo was very fast (Fuchs, Hult, Peraica, Radić, & Plestina, 1984). Peckham, Doupnik and Jones first reported the toxicity of OTB, then the toxicity of OTB was researched (Peckham, Doupnik, & Jones, 1971). Subsequently, some studies reported that OTB could be transformed to OTA even though at a lower level (Abbas et al., 2009). Mally and co-workers suggested that OTA and OTB had a similar potency to induce cytotoxicity in vitro (Mally, Keim-Heusler, Amberg, Kurz, Zepnik, Mantle, et al., 2005). Besides, other studies also indicated that OTB could cause pronounced inhibition of cell at a much lower concentration (Knasmuller et al., 2004). Based on the above statements, OTA, OTB and OTC can all cause harm to the health of human and animals, so the co-occurrence and conversion between different types of major ochratoxins in foodstuff should be noted. In order to evaluate the total intake of ochratoxins, the simultaneous detection of the three major ochratoxins is very important and necessary.

Over the last few decades, many methods have been used for the detection of ochratoxins in food and feed. The most common used methods are instrument analysis methods, chemical analysis methods and immunoassays. Instrumental methods are the most important and common detection methods, mainly including high performance liquid chromatography (HPLC) (Mishra, Catanante, Hayat, & Marty, 2016) and liquid chromatography-tandem mass spectrometry (LC-MS) (Toffa et al., 2013). HPLC is most frequently used as a reference method because of its high accuracy, e.g., Han and co-workers used ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to determine OTA and OTB simultaneously in traditional Chinese medicines (TCMs), the quantitation limit was 0.03–0.19 ng/mL (Han et al., 2010). Remiro and co-workers used HPLC with fluorescence detection for the simultaneous quantification of OTA and its analogues OTB, OTC and methyl ochratoxin A (MeOTA), the limits of detection (LODs) were 0.16, 0.32, 0.27 and 0.17 ng/mL for OTA, OTB, MeOTA and OTC respectively (Remiro et al., 2010). So, HPLC is sensitive and accurate, and can satisfy the detection demand of major ochratoxins at the same time. But this method is high-cost and time-consuming, and is not suitable for on-site detection in food safety monitoring. Thin layer chromatography (TLC) is the most common used method of chemical analysis, and is also the earliest testing method for mycotoxin detection. Before 2009, the TLC method was as a national standard test method in China, but now it's rarely used because of its low sensitivity and specificity. Immunoassays, based on the antibody-antigen recognition, are another kind of frequently used methods, they mainly contain enzyme-linked immunosorbent assay (ELISA) (Liang, Huang, Yu, Zhou, & Xiong, 2016; Zou et al., 2016), fluorescence immunoassay (Hayat, Paniel, Rhouati, Marty, & Barthelmebs, 2012; Huang et al., 2016), chemiluminescent immunoassay (CL-IA) (Yu, Vdovenko, Wang, & Sakharov, 2011) and immunochromatographic assay (ICA) (Majdinasab et al., 2015; Maragos, 2004) and etc. Among these methods, ICA is frequently used in recent years because of its incomparable field applicability and other obvious advantages such as easy performance, immediate results, low cost, short time and no skilled technicians or expensive equipment requirements. Besides, ICA is the most popular and ideal method at present in food safety monitoring for toxic analytes. Most ICA methods are for single analyte, still there are some ICAs for multi-analytes simultaneously in recent years, e.g., Li and co-workers proposed a method of multi-component immunochromatographic assay to detect aflatoxin $B_1$, ochratoxin A and zearalenone, among three analytes the visual detection limit (vLOD) for OTA was 0.5 ng/mL (Li, Li, Zhang, Li, Zhang, Zhang, et al., 2013). In all ICAs reported previously for ochratoxins detection, the methods for OTA testing have been perfected, the vLOD has been as low as 0.2 ng/mL, but there are no reports for the detection of OTB and OTC, much less any ICAs for the detection of three ochratoxins. To fully meet the rapid and on-site analysis demand of major ochratoxins in the case of co-occurrence and conversion of different ochratoxins, in this work, a highly sensitive immunochromatographic assay was developed to detect the three major ochratoxins simultaneously for the first time. Results showed that this method could detect the three ochratoxins sensitively and the vLOD for OTA was lower than those of most conventional ICAs reported previously.

## 2. Materials and methods

### 2.1. Reagents

Ochratoxin A and ochratoxin B were obtained from Qingdao pribolab biotech co., ltd. Ochratoxin C was purchased from Shandong Lvdu Bio-science & Technology Co., Ltd. Bovine serum albumin (BSA) was purchased from MP Biochina. Ovalbumin (OVA) was purchased from Solarbio® Life Sciences. OTA-BSA conjugate was purchased from Beijing Longrun Biological Technology Co., Ltd. Goat anti-mouse IgG was purchased from Luoyang Bai Aotong Experimental Materials Center. Anti-ochratoxin mAb (11B9) was prepared in our laboratory, characterized by ELISA method, the sensitivities (expressed as 50% inhibition concentration) of 11B9 for OTA, OTB and OTC detection were 0.7874 ng/mL, 0.9391 ng/mL and 1.3466 ng/mL, respectively, there were almost no cross-reactions of 11B9 with other interfering mycotoxins, and the subtype of 11B9 was IgG2a. Nitrocellulose membrane was purchased from Millipore Corp. Glass fibers, sample pads, and absorbent pads were purchased from Shanghai Kingdiag-Biotech Co., Ltd. Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot xH_2O$) was purchased from Sigma-Aldrich. Unless otherwise stated, all other inorganic chemicals and organic solvents were of analytical reagent grade or better.

### 2.2. Instrumentation

Guillotine cutter (HGS-201) and Dispensing platform (HGS510-2D) were purchased from Hangzhou Autokun Technology Co., Ltd. The high speed refrigerated centrifuge (HC-3018R) was obtained from Anhui USTC Zonkia Scientific Instruments Co., Ltd. Freeze dryer (FDS-2.5E) was purchased from SIM International Group Co., Ltd.

### 2.3. Preparation of gold nanoparticles (GNPs)

The GNP was prepared using the method of sodium citrate reduction reported previously with some slight modifications (Zhang, Li, Zhang, & Zhang, 2011). The experiment procedure was as follows: 100 mL of distilled water was heated until boiling, then 1 mL of 1% $HAuCl_4$ was quickly added under intense strring, after 2 min, 4 mL of 1% trisodium citrate solution was added quickly and kept strring. The color of solution changed from gray to black, then to purple, and finally to burgundy, then the solution was kept boiling for 10 min. The distilled water was added to GNPs solution to restore the original volume after cooling at room temperature. The GNPs solution was characterized with UV–vis spectra from 400 nm to 700 nm and then stored at 4 °C for further use.