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Determination for multiple mycotoxins in agricultural products using HPLC–MS/MS via a multiple antibody immunoaffinity column

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ABSTRACT

Mycotoxins usually found in agricultural products such as peanut, corn, and wheat, are a serious threat to human health and their detection requires multiplexed and sensitive analysis methods. Herein, a simultaneous determination for aflatoxin B1, B2, G1, G2, ochratoxin A, zearalanone and T-2 toxin was investigated using high performance liquid chromatography coupled with tandem mass spectrometry in a single run via a home-made multiple immunoaffinity column. Four monoclonal antibodies were produced in our lab against aflatoxins, ochratoxin A, zearalanone and T-2 toxin, respectively, then combined as a pool and bound to Sepharose-4B for affinity chromatography. Seven mycotoxins were effectively extracted from the agricultural product samples by using acetonitrile/water/acetic acid (80:19:1, v/v/v) Then, the extraction was cleanup by multiple immunoaffinity column. This method demonstrated a considerable linear range of 0.30–25, 0.12–20, 0.30–20, 0.12–20, 0.60–30, 0.30–25, and 1.2–40 μg kg^{-1} and lower limits of detection at 0.1, 0.04, 0.1, 0.04, 0.2, 0.1 and 0.4 μg kg^{-1} for AFB1, AFB2, AFG1, AFG2, OTA, ZEN and T-2, respectively, in comparison with previously reported methods, as well as excellent recoveries. The mIAC capacity for AFB1, AFB2, AFG1, AFG2, OTA, ZEN, and T-2 were 187, 181, 153, 151, 105, 130, 88 ng, respectively. It was found that all of the 7 mycotoxins were present in 90 agricultural product samples. The proposed method meets the requirements for rapid sample preparation and highly sensitive identification of multiple mycotoxins in agricultural product and food safety. This method provides a promising alternative with high throughput and high sensitivity for rapid analysis of seven mycotoxins in the monitoring of food safety.

1. Introduction

Mycotoxins are harmful secondary metabolites from fungi. They contaminate agricultural products world-wide and are a threat to human health. Mycotoxins can be found throughout the food supply chain of agriculture production, including harvesting, storage, food processing and transportation [1,2]. Among these numerous mycotoxins, aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), ochratoxin A (OTA), zearalenone (ZEN) and T-2 toxin (T-2) have demanded attention, because that they are frequently co-occurring in agricultural products, including peanut, corn and wheat [3–6]. Most mycotoxins have been proved to be teratogenetic, carcinogenic, and mutagenic [7–11]. Different mycotoxins have various toxic effect. For example, AFB1 have been listed as the cancer cancerogen (class I) [12]. OTA has been proved to be nephrotoxic and is associated urinary tract tumours [13]. ZEN can lead to reproductive disorders of live stocks and unbalance hormone secretion of human beings [14]. There is evidence that ZEA and its metabolites possess oestrogenic activity in pigs, cattle, etc [14]. T-2 can cause both acute and chronic toxic effect and apoptosis in the immune system and fetal tissues [15]. The treatment of clinical disease due to multiple mycotoxins results in vast medical costs [16,17]. Additionally, the increasingly strict maximum residue limits set by EU, USA, and other nations, can lead to high costs for international trading due to the returned sales. Thus, it is important to have sensitive methods capable of multi-mycotoxin detection to simultaneously analyze these mycotoxins in a single run.

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Several strategies have been developed for mycotoxin determination, including thin layer chromatography (TLC) [18], high-performance liquid chromatography- fluorescence detection (HPLC-FD) [19], and gas chromatography-mass spectrometry (GC–MS) [20]. However, TLC and HPLC methods do not meet the requirement of multiple mycotoxins determination due to their comparatively low throughput. HPLC–MS/MS has dramatically enhanced sensitivity and identification ability because MS can simultaneously select and identify mycotoxin ions in a single run. Thus, HPLC–MS/MS techniques function in qualitative and quantitative determination of mycotoxins [21].

Recently, multiple determination methods have been arisen more and more interest [22–31], especially for agricultural products. This is probably because that the sample preparation in the complex matrices in agricultural products is hampered by the lipid solubility of solid samples, when compared with aqueous soluble body fluids from humans and animals [32]. For rapid and effective extraction of multiple mycotoxins, several studies were reported, including a two-step extraction in 4 min [33,34], homogenization, ultrasonic extraction [35] and matrix solid phase dispersion [36]. The uncertain combination and trace-level of multiple mycotoxins in agricultural products make it difficult to establish a simultaneous and sensitive determination method. To improve performance of HPLC–MS/MS, solid phase extraction (SPE) [35] and an immunoaffinity column (IAC) can be used in sample preparation. SPE clean-up method is typically used for mycotoxin determination, especially in the QuEChERS method [37]. However, the matrix effect affects sensitivity. Some commercialized IACs [38] were successfully employed in the determination of multiple mycotoxins. These efforts improved the determination of multiple mycotoxins using HPLC (or UPLC)-MS methods.

IAC method can dramatically reduce the matrix effect, even though multiple commercial antibodies increase the cost of the analysis [39]. To solve this dilemma, several home-made antibodies were used in the IAC method, allowing a low-cost IAC method. In this work, a simultaneous determination for the 7 mycotoxins, AFB1, AFB2, AFG1, AFG2, OTA, ZEN and T-2 was developed using multiple IAC (mIAC) combined with HPLC–MS/MS method in a single run. A one-step sample grinding and extraction was used for highly efficient sample preparation. With mIACs, 7 mycotoxins were recognized by specific mAbs in the same sample extraction. The HPLC–MS/MS method was conducted to identify the mycotoxins using the ESI interface and SRM mode. This approach has potential application in food safety assays.

2. Material and methods

Safety statement: These mycotoxins have been proven high toxic to human health and environment. It is suggested the experimenters should be have strict safety guidelines to prevent the direct contact with mycotoxins, and dispose the lab wastes in a proper way.

2.1. Regents and materials

CNBr-activated Sepharose 4B was purchased from GE Healthcare (Uppsala, Sweden). Standards solutions of AFB1, AFB2, AFG1, AFG2, OTA, ZEN, T-2, and HT-2 were purchased from Sigma-Aldrich. Methanol (MeOH, HPLC grade), acetonitrile (ACN, HPLC grade), formic acid, sodium acetate (NaAc), sodium chloride (NaCl), monopotassium phosphate, disodium hydrogen phosphate, hydrochloric acid, glacial acetic acid, and tris(hydroxymethyl) aminomethane (Tris) and sodium azide (NaN_3) were from Sinopharm Chemical Reagent (Shanghai, China) were used as received. All the other inorganic chemicals and organic solvents were of analytical grade. Water was obtained from Milli-Q system (Millipore, Billerica, Massachusetts, USA).

2.2. Preparation of monoclonal antibodies against mycotoxins

Monoclonal antibodies (mAbs) against AFT, OTA, T-2, and ZEN were generated in-lab using standard hybridoma technology [10,18,40–42]. In brief, antigens linked to Bovine Serum Albumin (BSA, AFT-BSA, OTA-BSA, ZEN-BSA and T-2-BSA) were injected subcutaneously into Balb/c mice. After four immunizations titers were determined by ELISA. mAbs were purified via the caprylic acid-ammonium sulfate method followed by protein-G IAC. mAbs were lypholized and stored at −20 °C. Titers of mAbs were determined before use by ELISA to verify effectiveness.

2.3. mIAC preparation

The gel-coupling method of mAb against Sepharose 4B was prepared by coupling the mAbs against mycotoxins with CNBr-activated Sepharose 4B. Sepharose 4B (1.3 g) was swollen thoroughly in 30 mL HCl (1 mM) for 15 min, and then washed by 200 mL HCl (1 mM) and 200 mL coupling buffer (a mixture containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) to remove their protecting groups. The gel was mixed with 15 mL coupling buffer containing 20 mg of each mAb generated against AFT, OTA, ZEN and T-2. The coupling reaction was conducted in a shaker at 200 rpm for 3 hrs at room temperature. The solution was then transferred to a sand funnel with the pore size of 2–5 μm. The unbound mAbs were removed by washing with the coupling buffer for 5 times. Further, the gently shaking was conducted to block the remaining active sites with the blocking buffer (0.1 M Tris–HCl, pH 8.0) for 2 hrs at room temperature. To remove excess of uncoupled ligand after coupling, the adsorbent was washed with buffer at pH 4.0 (0.1 M HAc–NaAc with 0.5 M NaCl) and 8.0 (0.1 M Tris–HCl with 0.5 M NaCl) for three times, respectively. Finally, the gel was equilibrated with phosphate buffered saline (PBS, 0.01 M, pH 7.4) and stored in PBS solution containing 0.02% NaN_3 at 4 °C before use. The gel-coupling method mAb against other mycotoxin with Sepharose 4B were in the same manner. The mIACs were then developed with a thorough mixing the gels coupled with mycotoxin. The wet gel of mycotoxin-specific mAb-bound Sepharose 4B (0.1 mL) was packed in 1 mL PBS, and stored at 4 °C before use.

2.4. Sample preparation

Crude samples of agricultural products (peanut, corn and wheat) were homogenized and extracted in a single step. A commercial homogenizer (Jiuyang soybean milk machine, Wuhan, Hubei, China) was used to simultaneously homogenize and extract agricultural products (20 g) in ACN/water/acetic acid (80:19:1, v/v/v, 100 mL) for 2 min. The supernatant of the resulting quiescent extraction was filtered and diluted with PBS at the ratio of 1:3. A total of 10 mL of the diluted extract was used for the mIAC.

2.5. mIAC clean-up purification optimization

First, for loading condition, ratios of ACN/PBS at 5:95, 10:90, 20:80, and 40:60 (v/v) for loading buffer were tested for optimization. mIACs were loaded with 10 mL of a mycotoxin standard solution containing AFB1, AFB2, AFG1, AFG2, OTA, ZEN and T-2 with a final concentration of 5 ng mL^{-1} each, respectively. After washing with 20 mL PBS, mycotoxins were slowly eluted with 1 mL MeOH. The eluent was collected and filtered with a 0.22 um filter membrane. Second, to reduce the matrix effect, the washing process of mIAC loaded multiple mycotoxins was studied. A series washing volumes of PBS and water, respectively were conducted at 5, 10, 15,