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Dual-wavelength fluorescence polarization immunoassay to increase information content per screen: Applications for simultaneous detection of total aflatoxins and family zearalenones in maize

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ABSTRACT

To increase information content per screen, a dual-wavelength, homologous and high-throughput fluorescence polarization immunoassay (DWFPIA) for simultaneous detection of total aflatoxins (AFs) and family zearalenones (ZENs) was developed. Aflatoxin B_1 (AFB_1) and zearalanone (ZAN) were labeled with different fluoresceins, and then combined with corresponding broad-specific antibodies to develop the DWFPIA. Under optimal conditions, the half maximal inhibitory concentrations of the FPIA in buffer were 2.68 μg/L and 4.08 μg/L for total four AFs (the weight ratio of AFB_1: Aflatoxin B_2: Aflatoxin G_1: Aflatoxin G_2 was 1: 1: 1: 1) and six ZEN analogs (the weight ratio of ZEN: ZAN: α-zearalenone: β-zearalenone: α-zearalanone: β-zearalanone was 1: 1: 1: 1: 1: 1) by mixing equal volumes (1: 1, v/v), respectively. The corresponding limit of detection values in maize flour samples were 4.98 μg/kg and 11.03 μg/kg, and the mean recoveries ranged from 78.6 to 103.6% with the coefficients of variation below 19.2%. The whole detection procedure, including sample preparation, took less than 30 min. Finally, the DWFPIA was applied to screen naturally contaminated maize flour samples, and the detection data were similar to those with HPLC-MS/MS.

1. Introduction

With the constant development of the feed economy, more and more industrial, academic and supervision departments have focused their attention on safety in the last decade. In this context, feed contaminated by mycotoxins, such as aflatoxins (AFs) and zearalenone (ZEN), is a very important issue. AFs are mainly produced by *Aspergillus parasiticus* and *Aspergillus flavus*, and aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) are considered the most toxic group and have been identified to have mutagenic and carcinogenic effects (Bakırdere et al., 2012; Santos et al., 2017; Yunus, Razzazi-Fazeli, & Josef, 2011). Due to their toxicity, the International Agency for Research on Cancer (IARC) has classified AFs as Group I mutagens (Wild, Miller, & Groopman, 2015). ZEN is a nonsteroidal estrogenic mycotoxin, mainly produced by several *Fusarium spp*, which usually co-occur with two other structurally similar mycotoxins α-zearalenol and β-zearalenol (J. Liu et al., 2014; N. Liu, Nie, Zhao, Meng, & Wu, 2015). ZEN exhibits hepatotoxic, immunotoxic and genotoxic effects, and has been classified as a Group III carcinogen by the IARC (Abbès et al., 2006; Organization & Cancer, 1993). Considering its toxicity, the European Union (EU) has applied the maximum

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permitted levels of 5.0 μg/kg for AFB_1 and 10.0 μg/kg for total AFs (AFB_1+AFB_2+AFG_1+AFG_2) in corn and rice.(EC, 2006; EU, 2010). In addition, the maximum residue level of ZEN was set at 350 μg/kg for unprocessed maize by European (EC, 2006). It has been reported that AFs contamination can cause losses to the corn industry of $52.1 million to $1.68 billion annually in the United States and 99% of corn samples were contaminated with ZEN in China with a maximum level of 1442.5 μg/kg (W. Li, Wen, et al., 2016; Li, Li, et al., 2016; Mitchell, Bowers, Hurburgh, & Wu, 2016). It should also be noted that co-occurrence of mycotoxins is common, as 48% of cereal samples were contaminated with two or more mycotoxins from a total of 7049 samples from North and South America, Europe, and Asia collected from 2009 to 2011 (Lee & Ryu, 2017; Rodrigues & Naehrer, 2012). It has been reported that the co-occurrence of mycotoxins results in increased additive or synergistic effects (Lee & Ryu, 2017; Speijers & Speijers, 2004). For example, maize contaminated with AFs and ZEN can result in reduced laying performance and egg quality in hens (Ru et al., 2016). More importantly, residues of these mycotoxins can persist in meat and eggs and ultimately be passed to humans (Iqbal, Nisar, Asi, & Jinap, 2014). Thus, screening for the co-occurrence of mycotoxins in maize is necessary to reduce the risk of exposure to these mycotoxins, for example total AFs and family ZENs. Considering the large number of samples, a high-throughput screening method that can simultaneously monitor a large number of samples, instead of individual samples, would be more practical.

Several instrumental methods for the simultaneous detection of total AFs and family ZENs have been reported, including high-performance liquid chromatography (HPLC) coupled with fluorescence detection or tandem mass spectrometry detection (Li, Wen, Chen, Xiao, & Ma, 2015; Ok, Chung, Lee, & Chun, 2015; Paschoal, Silva, Souza, Oliveira, & Pereira, 2016). As we all known, antibody-based analytical methods have recently emerged as an alternative to instrumental methods (Kong, Xie, Liu, Song, & Kuang, 2017; Kong, Xie, Liu, Song, Kuang, cui & Xu et al., 2016). In order to screen these two categories of mycotoxins in a large number of samples, some immunological analysis methods have been reported, such as fluorescent immunosorbent assays and lateral flow immunoassays (N. V. Beloglazova et al., 2014; Foubert et al., 2016; Kong, Liu, Song, Suryoprabowo, Li, Kuang, Wang, & Xu et al., 2016; Song et al., 2014; Suri et al., 2009). Recently, fluorescence polarization immunoassay (FPIA) has been used as a homogeneous analytical method which allows the determination of target analytes within several minutes and meets the requirement of simple, cost-effective and high-throughput, thus it is becoming an attractive and efficient method for on-site screening (Yang et al., 2015; Liu et al., 2017; Shen et al., 2017). Commercial FPIA kits for animal diseases, single mycotoxins, and therapeutic drugs have already been exactly developed by Ellie (formerly Diachemix) and Abbott.

To develop a highly accurate and sensitive FPIA for screening one or more analytes, antibodies with uniform affinity and appropriate fluorescent tracers are the two main factors which should be considered. The first study on FPIA for AFs was reported by Nasir et al. (And & Jolley, 2002). They prepared several tracers using the AFB_1-carboxymethoxylamine derivative (AFB_1-CMO) conjugated to fluoresceins such as fluorescein isothiocyanate isomer I, fluorescein isothiocyanate isomer II, 5-amino acetyl-amino fluorescein, 5-(5-aminopentyl) thioureidyl) fluorescein, fluorescein thiosemicarbazide, 4′-aminomethyl fluorescein (AMF) and 5-aminomethyl fluorescein. After selection of polyclonal antibodies, the FPIA was developed with the best tracer composed of AFB_1-CMO and fluorescein isothiocyanate isomer II, which achieved a detection range of 5–200 μg/kg. However, FPIA consistently underestimated the AFs contents in the naturally contaminated cereal samples. This was perhaps due to low cross-reactivity (CR%) of the used antibody with AFB_2, AFG_1 and AFG_2. Lobeau et al. developed a FPIA for AFB_1 using the conjugate of AFB_1-CMO and fluoresceinthiocarbamyl ethylenediamine (AFB_1-EDF) as the tracer with a very high IC_{50} value of 700 ng/mL in buffer (Lobeau, 2007). Beloglazova et al. optimized six antibodies and used AFB_1-EDF to develop a FPIA to detect only AFB_1 with an IC_{50} of 11 ng/mL (N. Beloglazova & Eremin, 2015). Recently, our group established a FPIA in order to detect total AFs based on monoclonal antibody (mAb) 3C10 and AFB_1-EDF with an acceptable detection spectrum but low sensitivity, which was not applied to real samples (Sheng et al., 2014). Therefore, these reported FPIAs were not sufficiently sensitive or accurate for screening total AFs due to the relatively low CR% with other AFs, in particular for AFB_2 and AFG_2 (CR% less than 30%) (N. Beloglazova & Eremin, 2015; Lobeau, 2007; Sheng et al., 2014). A similar situation occurred when attempting to establish a FPIA for the detection of family ZENs (35.3% ≤ CR%≤522.2% or 20% ≤ CR%≤ 195%) (Choi, Kim, Choi, Eremin, & Chun, 2011; Maragos & Kim, 2004). The high or low CR% of the FPIA could lead to false-positive or false-negative results for screening multi-mycotoxins, which were mainly determined by the specificity of employed antibodies (high IC_{50} or diverse CR% values). We recently prepared a new sensitive broad-specific monoclonal antibody (mAb) with uniform CR% for total AFs and one for family ZENs, and developed a FPIA for detecting family ZENs in maize (Zhang, Eremin, et al., 2017; Zhang, Song, et al., 2017). With an increasing desire to improve detection efficiency and reduce testing costs, efforts have been made to determine the efficacy of FPIA in screening multiple mycotoxins in a single run. The ability to monitor multiple mycotoxins is obviously preferable in order to gather more information on the sample with a single analysis. To the best of our knowledge, there is only one report on a multi-wavelength fluorophore-based FPIA for three mycotoxins, which have a high action level (ppm), for example, fumonisins, deoxynivalenol and T-2 toxin (C. Li, Wen, et al., 2016; Li, Li, et al., 2016). Here, we report the development of a highly accurate and sensitive dual-wavelength FPIA (DWFPIA) for the simultaneously screening of total AFs and family ZENs in maize by combining new mAbs and synthesized fluorescent tracers.

2. Materials and methods

2.1. Reagents and materials

AFB_1, AFB_2, AFG_1, AFG_2, aflatoxin M_1 (AFM_1), ZEN, zearalanone (ZAN), α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol, fumonisin B_1 (FB_1), ochratoxin A (OTA), deoxynivalenol (DON) and T-2 toxin were supplied by Sigma–Aldrich (St. Louis. MO, USA). *O*-Carboxymethyl oxime (CMO), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), pyridine and dimethyformamide (DMF) were obtained from Aladdin Chemistry Co., Ltd (Shanghai, China). Tetramethylrhodamine cadaverine (TRCA) and 4'-(aminomethyl) fluorescein (AMF) were supplied by Thermo Fisher Scientific Inc. (Waltham, MA). Ethylenediamine fluoresceinthiocarbamyl (EDF) was synthesized as described previously (Mi et al., 2014).

Specific mAb 2A8 and 5H3 against AFs, mAb 3D4 and 11C6 against family ZENs, AFB_1-CMO and ZAN-CMO were previously prepared in our laboratory (Zhang, Eremin, et al., 2017; Zhang, Song, et al., 2017).

2.2. Synthesis of the fluorescein-labeled mycotoxins

AFB_1-CMO was conjugated to amino-fluorescein derivatives (EDF/AMF/TRCA) using the active ester method (Fig. 1A) (Zhang, Song, et al., 2017). Briefly, AFB_1-CMO (2 mg), DCC (4 mg) and NHS