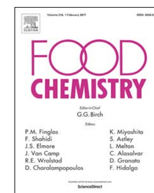
Food Chemistry 247 (2018) 8–15

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



# Preparation of a broad-spectrum anti-zearalenone and its primary analogues antibody and its application in an indirect competitive enzyme-linked immunosorbent assay

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ARTICLE INFO

*Keywords:*
Zearalenone
Monoclonal antibody
Indirect competitive enzyme-linked immunosorbent assay
Feed
Animal edible tissue

ABSTRACT

A broad-spectrum monoclonal antibody (mAb)-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has been developed for rapidly screening zearalenone (ZEN) and its primary analogues in various samples using an easy sample preparation procedure. Primarily, a group-specific mAb, 6C2, was produced, which had $IC_{50}$ values for ZEN, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone of 114.0, 127.4, 290.4, 114.9, 205.6 and 257.1 ng $L^{-1}$, respectively. The limit of detection and limit of quantitation of this method for ZEN and its five primary analogues in various matrix samples ranged from 114.2 to 812.3 ng $L^{-1}$ and 237.1 to 1653.9 ng $L^{-1}$, respectively. The recoveries of the above samples spiked with ZEN and its five primary analogues were in the range of 62.9–113.6%. The CVs were less than 13.2%. A good correlation ($R^2$ = 0.995) between the ic-ELISA results and the HPLC-MS/MS results for swine feeds supported the reliability of the developed ic-ELISA.

## 1. Introduction

Zearalenone (ZEN, also known as F-2 mycotoxin) is produced as a secondary metabolite by certain *Fusarium* fungi, and it has been recognized as one of the most frequent contaminants in feedstuffs and grains (Zaied, Zouaoui, Bacha, & Abid, 2012). The toxicity of ZEN is accompanied by typical signs of hyperestrogenism or hepatoxicity (Gonkowski, Obremski, & Calka, 2015), as well as precocious puberty (Massart, Meucci, Saggese, & Soldani, 2008), infertility in girls (Murphy, Hendrich, Landgren, & Bryant, 2006), and oestrogenic syndrome in swine (Tiemann & Dänicke, 2007). It is also a carcinogen that can damage DNA and has reproductive toxicity and immunosuppression effects in animals and humans (Berek, Petri, Mesterházy, Téren, & Molnár, 2001; Wang et al., 2015). In pigs, and likely in humans, ZEN can be quickly absorbed after oral administration, and is metabolized into α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zearalanone (ZAN) (Marin et al., 2011). These analogues and ZEN exhibit oestrogenicity, and α-ZAL has four times the oestrogenicity of ZEN (Shier, Shier, Xie, & Miroch, 2001). Hence, over the past few decades, α-ZAL has been widely used as a growth promoter in livestock production (Valenzuela-Grijalva et al., 2012). However, it has gradually been banned because of the potential risk for breast cancer and its strong and prolonged endocrine disruptive effects in humans at low concentrations (Matraszek-Zuchowska, Wozniak, & Zmudzki, 2013).

To protect the health of animals and humans, the European Union has set a strict limitation on ZEN in foods (100 μg $kg^{-1}$, unprocessed cereals) and in feeds (350 μg $kg^{-1}$, unprocessed maize) (Commission Regulation (EC) No 1126/2007 of 28 September 2007, 2007). China has established maximum residue limits (MRL) for ZEN in feeds (500 μg $kg^{-1}$) and in grains and grain-products (60 μg $kg^{-1}$) (Sun et al., 2014). Therefore, feedstuffs, grains and animal-derived foods should be guaranteed to be free of these residues.

To monitor trace ZEN and its metabolite analytes in complex matrices (feedstuffs, grains and animal-derived foods), it is essential to develop a simple, rapid, broad-spectrum, and sensitive analytical method. Presently, numerous physico-chemical methods have been developed for the determination of ZEN and its analogues, such as thin-layer chromatography (TLC) (Klarić, Cvetnić, Pepeljnjak, & Kosalec, 2009), gas chromatography tandem mass spectrometry (GC–MS/MS)

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https://doi.org/10.1016/j.foodchem.2017.12.016
Received 26 June 2017; Received in revised form 14 November 2017; Accepted 6 December 2017
Available online 07 December 2017
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(Rodriguez-Carrasco, Molto, Berrada, & Manes, 2014), high-performance liquid chromatography (HPLC) (Ok, Choi, Kim, & Chun, 2014), and liquid chromatography tandem mass spectrometry (LC–MS/MS) (Chen et al., 2013). These methods are highly sensitive and reliable, but they require expensive instruments, highly trained personnel and sophisticated sample pretreatments. As a result, they can only be applied under special laboratory conditions.

Immunoassays have been extensively used for food safety monitoring because of their sensitivity, reliability, rapidity, usability, and portability (Kong et al., 2016, 2017; Li et al., 2016; Peng et al., 2017). In the past few years, several monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assays (ic-ELISA) or ELISA methods have been developed to evaluate ZEN and its analogues (Burmistrova et al., 2009; Cha et al., 2012; Gao et al., 2012; Lee, Yuan, Hart, & Pestka, 2001; Pei, Lee, Zhang, Hu, & Eremin, 2013; Tang et al., 2014; Teshima et al., 1990). However, there are still some disadvantages due to the sensitivity or cross-reactivity of the majority of available monoclonal antibodies (mAbs). Some mAbs are highly specific to ZEN but are not suitable for monitoring its analogues in samples (Gao et al., 2012; Sun et al., 2014; Teshima et al., 1990), and some others may have sensitivity weaknesses (Cha et al., 2012; Lee et al., 2001; Liu et al., 2012; Pei et al., 2013; Shim, Kim, & Chung, 2009).

In the current study, hapten zearalenone-oxime (ZEN-CMO) was designed and synthesized to obtain a highly group-specific mAb against ZEN and its main analogues. Next, the mAb was used to develop a highly sensitive broad-spectrum ic-ELISA for analysing ZEN and its analogues in maize, swine feed, edible animal tissues and milk with a simple sample pretreatment.

## 2. Materials and methods

### 2.1. Chemicals

The standard analytes, including zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), zearalanone (ZAN), T-2 toxin, fumonisin $B_1$, fumonisin $B_2$, and ochratoxin A, O-carboxymethoxylamine hemihydrochloride (CMO), N-hydroxysuccinimide (NHS), N', N'-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), bovine serum albumin (BSA), albumin human serum (HSA), keyhole limpet haemocyanin (KLH), ovalbumin (OVA), tween-20, 3′,3′,5′,5′-tetramethylbenzidine (TMB), peroxidase-labelled goat anti-mouse immunoglobulins (HRP-IgG), Freund's adjuvants (complete and incomplete), polyethylene glycol 4000 (PEG 4000, 50%), dimethyl sulphoxide (DMSO), culture media RPMI-1640, hypoxanthine aminopterin thymidine (HAT) and hypoxanthine thymidine (HT) medium supplements, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Foetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All the other chemicals and organic solvents, such as sodium carbonate, N,N-dimethylformamide (DMF), and ethyl acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of analytical grade.

### 2.2. Experimental animals

Female Balb/c mice (6–8 weeks old, NO. 42000600014529) were purchased from the Hubei Centre for Disease Control and Prevention (Wuhan, China) and were raised in an environment with standard temperature and humidity conditions. All animal experiments in this study were performed in compliance with the Huazhong Agricultural University animal experiment centre guidelines and were approved by the Animal Ethics Committee.

### 2.3. Synthesis of ZEN-CMO hapten

The ZEN-CMO hapten was synthesized from ZEN to create a reactive group. Briefly, 2 mg of ZEN was dissolved in 1 mL of methanol and then 3 mg of CMO and 5 mg of $Na_2CO_3$ were added. The mixture was stirred at room temperature (RT) for 24 h and was dried with a flow of nitrogen. The residue was redissolved in 2 mL of HCl (0.05 M). The reconstitution fluid underwent three extractions with 2 mL of ethyl acetate, and all the ethyl acetate phases were pooled. The collected ethyl acetate phase was dried with a flow of nitrogen to obtain the ZEN-CMO product. The hapten was identified using ion trap and time-of-flight mass spectrometry coupled with an HPLC system (LC/MS-IT-TOF, Shimadzu, Kyoto, Japan). The $m/z$ calculated for $C_{20}H_{25}NO_7$ was M = 391.1558; for $[M-H]^-$, the $m/z$ was 390.1504.

### 2.4. Synthesis of antigens

The ZEN-CMO-BSA antigen was synthesized using the active ester method according to a modified procedure (Cha et al., 2012; Kong et al., 2016). Briefly, 1 mg of ZEN-CMO, 2 mg of DCC and 1 mg of NHS were dissolved in 0.4 mL of DMF and the mixture was stirred overnight at RT. The dicyclohexylurea precipitate was removed by filtration, and the clear solution was added dropwise to 4 mL of 0.1 mol $L^{-1}$ phosphate buffer (PBS, pH 8.0) containing 16 mg of BSA under continuous stirring. The mixture was kept in an ice-bath overnight and then centrifuged for 10 min at 5000 rpm. The supernatant was purified by exhaustive dialysis against PBS (0.01 mol $L^{-1}$, pH 7.4) at 4 °C, and the dialyzed solution was stored at −20 °C prior to use. The ZEN-CMO-HSA/KLH antigen was essentially prepared as described above except that the carrier proteins were HSA and KLH, respectively.

The ZEN-CMO-OVA conjugate was prepared based on a modified procedure that was previously described (Burkin, Kononenko, & Soboleva, 2002). Briefly, ZEN-CMO (1 mg) was dissolved in 0.2 mL of DMF, 3 mg of EDC and 1.5 mg of NHS. The reaction solution was stirred for 4 h at RT and added dropwise to 4 mL of PBS (0.1 mol $L^{-1}$, pH 8.0) containing 14 mg of OVA under continuous stirring. The remainder of procedure was the same as that described above.

### 2.5. Animal immunization

Twenty-four 6-week-old female BALB/c mice were randomly divided into three groups. The mice in each group were immunized with 50 µg and 100 µg of the antigens (including ZEN-CMO-BSA, ZEN-CMO-HSA and ZEN-CMO-KLH) on a one-to-one basis. The antigens were diluted with 500 µL of sterile isotonic saline and emulsified with an equal volume of Freund's adjuvant. The immunogen with complete adjuvant was subcutaneously injected at multiple sites on the back of each mouse for primary immunization and the incomplete adjuvant was used for two subsequence booster immunizations at 2 week intervals. Serum samples were collected from the mice at intervals starting 7 days after the last booster immunization, and the titres and specificity were assessed using an ELISA.

### 2.6. Antisera monitoring

Antisera titres were monitored using an indirect ELISA (Peng et al., 2016). MaxiSorp microtitre plates were coated with 100 µL per well of coating conjugates in CBS (0.05 mol $L^{-1}$, pH 9.6) at 4 °C overnight. The plates were blocked, 100 µL of antisera was added, and the plates were incubated for 0.5 h at 37 °C. After the plates were washed with PBST (PBS containing 0.1% tween-20), they were incubated with 100 µL of diluted HRP-IgG at 37 °C for 0.5 h. Then the TMB substrate solution was added and the reaction was terminated by adding 2 mol $L^{-1}$ $H_2SO_4$ after incubation at RT in the dark for 15 min. The absorbance was determined at 450 nm using a BioTek uQuant microplate reader (uQuant, USA).

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