Specific monoclonal antibody based enzyme immunoassay for sensitive and reliable detection of Alternaria Mycotoxin Iso-Tenuazonic Acid in food products

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ABSTRACT: In this paper, we report the development of a sensitive and specific monoclonal antibody based immunodiagnostic method for the detection of iso-tenuazonic acid (ITeA), an *Alternaria* mycotoxin, in food samples. The ITeA was derivatized with hydrazine hydrate to produce the antigen (E)-3-(1-hydrazonoethyl)-4-hydroxy-5-isobutyl-1H-pyrrol-2(5H)-one (ITeAH) which was further reacted with glyoxalic acid to generate the hapten (E)-2-((Z)-(1-(4-hydroxy-5-isobutyl-2-oxo-2,5-dihydro-1H-pyrrol-3-yl)ethylidene) (ITeAHGA) which was used as an immunogen after conjugation to bovine serum albumin (BSA). A highly specific monoclonal antibody selectively binding to ITeAH was generated via the hybridoma technique and subsequently used to construct a heterologous indirect competitive enzyme-linked immunosorbent assay (icELISA) using ITeAH as the competitive antigen for the detection of ITeA with a limit of detection (LOD) of 0.5 ng/mL. Under the optimum conditions, the developed icELISA is highly sensitive (IC_{50}= 7.8 ng/ml) with recovery rates ranged from 82.3 to 109.8% for spiked food samples. The comparative analysis of results revealed a good correlation between the icELISA and the standard HPLC-MS/MS method, confirming the suitability of the developed icELISA for screening and detection of mycotoxin ITeA in food samples.

KEYWORDS: *iso-tenuazonic acid*; *monoclonal antibody*; *enzyme-linked immunosorbent assay*; *mycotoxin*

1. Introduction

Tenuazonic acid (TeA) and its isomer, iso-tenuazonic acid (ITeA), are major metabolic toxic products of *Alternaria* and other fungal species such as *Aspergillus flavus*, *Pyricularia oryzae*, and *Phoma sorghina* (Qiang et al. 2008; Marin et al. 2013). Owing to their ability of growth at low temperature, *Alternaria* species are responsible for spoilage of food plants during refrigerated transport and storage, whilst some *Alternaria* mycotoxins are heat-resistant even at relatively high temperature of 230ºC and thus can not be detoxicated by cooking (Siegel et al. 2010). TeA-producing fungi are ubiquitous in many biological environments and capable of infecting most plant species including food crops. In fruits and vegetables, TeA has the highest contamination frequency and is present in higher concentrations compared to other *Alternaria* toxins (EFSA 2011). In spite of being cautious pathogens of many plant diseases, genotoxic and fetotoxic in rats, as well as being linked to the development of oesophageal cancer, currently there are no regulations on *Alternaria* toxins in food and feed in Europe or in other regions of the world. Furthermore, TeA is considered the most acutely toxic *Alternaria* mycotoxins (Shephard et al. 2012). Because of the similarities in chemical structure, it is speculated that ITeA and TeA are of similar toxicological relevance. For instance, they both exhibit remarkable toxic effects on *Artemia salina* with mortality rates of 68.9% and 73.6%, respectively (Qin et al. 2009). Not only the antibacterial activity of ITeA is identical to TeA (Gitterman 1965), ITeA also exhibits significant phytotoxicity inhibiting plant growth and promoting leaf browning (Lebrun et al. 1988).

It was reported that some naturally contaminated food commodities contained only 4% ITeA in their total TeA content (Asam et al. 2013), but the high level of ITeA in

sorghum based infant food has raised increasing concerns and more samples should be analyzed to elucidate if there is a general tendency related to sorghum (Qiang et al. 2008). Nevertheless, TeA has been found to be the predominant *Alternaria* mycotoxin detected in China in all tomato ketchup (10.2-1781 μg/kg) and tomato juice samples (7.4-278 μg/kg) and in 99.4% of wheat flour (1.76-520 μg/kg) (Zhao et al. 2015a,b). Therefore, the total exposure of ITeA can not be neglected due to its acute toxicity and potential harmful effects on human and animal health.

Subsequently, it is necessary to continually monitor ITeA and TeA in fruits, vegetables, cereals and oleaginous plants intended for human consumption and feed production (Qiang et al. 2008). Although several instrumental methods exist for measuring of TeA and its analouges (Noser et al. 2011; Siegel et al. 2009; Asam et al. 2011; Prelle et al. 2013), LC-MS is the only instrumental technique available for ITeA (Asam et al. 2013). The method simultaneously detects both TeA and ITeA after their derivatization with 2,4-dinitrophenylhydrazine. While instrumental methods can offer a high level of precision and accuracy, the sophistication aspect of such analytical tools rendering their limited applications in routine and high throughput analysis. Immunochemical methods, on the other hand, are simple and cost effective, yet sensitive and rapid, enabling for a large array of sample screening. Immunoassay for TeA has recently been described in a couple of studies (Gross et al. 2011; Yang et al. 2012). Production of an antibody to the analyte is essential to an immunoassay. Compared with polyclonal antibody which is widely used in immunoassay, monoclonal antibody is more specific and homogenous, also more difficult to produce. There is no report on ITeA immunoassay based on monoclonal antibody available to date to the best of our knowledge. The present study therefore aimed to develop a sensitive and specific immunochemical screening method and monitor ITeA in food

products.

In the present study, two novel ITeA haptens, ITeAH and ITeAHGA (Fig.1), were adopted to develop a specific anti-ITeAH antibody, using ITeAHGA as a hapten to prepare the immunogen by coupling to a carrier protein (BSA). A highly specific monoclonal antibody (mAb) selectively binding to ITeAH, was generated via the hybridoma technique and was subsequently used to develop an icELISA for the detection of ITeA. Various ELISA conditions were optimized and performance of the assay was evaluated by measuring ITeA in real food samples.

2. Materials and methods

2.1 Reagents and Chemicals

Leucine, bovine serum albumin (BSA), dicyclohexylcarbodiimide(DCC), N-hydroxysuccinimide(NHS), polyethylene glycol (PEG) 2000, ovalbumin (OVA), 3,3',5,5'-tetramethylbenzidine(TMB), complete and incomplete Freund's adjuvants, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT), culture media RPMI-1640, and pristane were obtained from Sigma (St. Louis, MO, USA). The mouse SP2/0 myeloma cell line was sourced from the Sun Yat-sen University (Guangzhou, China). Tween-20, *N*,*N*-Dimethylformamide (DMF), sodium ethylate, diketene, benzene, ethyl acetate, chloroform, hydrazine hydrate, methanol, glutaric dialdehyde, and 4-hydroxybenzaldehyde were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Horseradish peroxidase-labeled goat anti-mouse IgG (IgG-HRP) was obtained from Boster Biotech Co., Ltd. (Wuhan, China). Polystyrene microtiter plates were sourced from Jiete Biotech Co., Ltd. (Guangzhou, China). Microwell plates for cell culture were obtained from Corning Incorporated (New York, USA). All organic solvents and chemicals used were of analytical grade. Female Balb/c mice were purchased from Guangdong Medical

Laboratory Animal Center. The mycotoxin standards of AOH, AME were purchased from Taileqi Technology Co., Ltd. (Beijing, China) and TeA, ITeA, ITeAH were synthesized in Guangdong Provincial Key Laboratory of Food Quality and Safety (Guangzhou, China).

2.2 Buffers and Solutions

Buffers were prepared and used as follows: 10 mmol/L PBST (PBS buffer containing 0.1% Tween-20) for washing; 50 mmol/L carbonate buffer (pH 9.6) for coating, 5% of skimmed milk powder in PBS buffer for blocking, sodium phosphate buffers (pH 5.4) as general diluent, and 2 mol/L H_2SO_4 was used as the stop solution. Chromogenic reagent was prepared using 150 μL of the TMB solution (15 mg/mL in DMF) and 2.5 μL of 6% (w/v) H_2O_2 in 10 mL of 0.1 mol/L citrate.

2.3 Instruments

Centrifuge (5810R) was purchased from Eppendorf Company, USA. The LC-MS/MS analysis was carried out using a 1,200 series LC system (Agilent, USA) equipped with the Agilent 6410 Triple Quad LC-MS System. The analytical column was 2.1 mm×150 mm, 3.5 μm Zorbax SB-C18. Nuclear magnetic resonance (NMR) spectra were obtained with DRX-600 NMR spectrometers (Bruker, Germany-Switzerland). Ultraviolet-visible (UV-vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). Microtiter plates were washed using a Multiskan MK2 microplate washer (Thermo Labsystems, USA). The Optical Density (OD) of ELISA signals were measured using a Perkin Elmer 1420 Multi-label Analyzer (USA). Wrist-action shaker (Vortex Genius3) was a product of IKA Company, Germany.

2.4 Synthesis and Characterization of Haptens

2.4.1 ITeA synthesis

ITeA was synthesized according to the method previously described (Yang et al. 2012) (Fig. 2). After recrystallization in chloroform, a white needle solid was obtained with a 38.6% yield. The ITeA structure was confirmed by APCI-MS and NMR analysis. Two haptens namely, ITeAH (*(E)-3-(1-hydrazonoethyl)-4-hydroxy-5-isobutyl-1H-pyrrol-2(5H)-one*) and ITeAHGA (*(E)-2-((Z)-(1-(4-hydroxy-5-isobutyl-2-oxo-2,5-dihydro-1H-pyrrol-3-yl)ethylidene)hydrazono)acetic acid*) were subsequently synthesized following the procedures shown in Fig. 2.

2.4.2 ITeAH synthesis

The synthesis was carried out by the Wolff-Kishner reaction. Briefly, ITeA (1.85 g, 10 mmol) was dissolved in 20 mL chloroform and added dropwise into the flask containing 20 mL of hydrous hydrazine hydrate. After mixing for 1h, 20 mL distilled water was added, and the mixture was then extracted twice with chloroform. The organic phase was then washed with water and dried over anhydrous magnesium sulfate. The solvent was removed to obtain a gray solid of ITeAH with a 65% yield.

2.4.3 ITeAHGA synthesis

The mixture of ITeAH (1.99 g, 10 mmol) and 2-oxoacetic acid (0.89 g, 12 mmol) was dissolved in 20 mL chloroform and agitating for 2h to produce a white solid of ITeAHGA, with a 48% yield.

2.5 Preparation and characterization of hapten-protein conjugates

The ITeAH hapten was conjugated to OVA via the glutaraldehyde method (Hamajima et al. 1995) in the following procedures and used as a coating antigen: OVA (1.66 μmol/L) and ITeAH (166 μmol/L) were first prepared in PBS (pH7.4), and 60 μL of glutaric dialdehyde was then added dropwise. The mixture was gently stirred for 12h at 4℃ and purified by dialyzes against PBS (10 mmol/L, pH 8.0) for two days.

167 The dialyzed product was centrifuged for 10 min and the supernatant was collected
168 and stored at 4℃. The structures of the final conjugates were confirmed by a UV-vis
169 (200-500 nm) spectroscopy.

170 The ITeAHGA hapten was conjugated to BSA and OVA via the active ester method
171 (McAdam et al. 1992) to prepare the immunogen and coating antigen, respectively.
172 Briefly, ITeAHGA (0.166 μmol), DCC (0.122 μmol), and NHS (0.122 μmol) were
173 dissolved in 1.0 mL of DMF and the mixture was gently stirred at 4℃ overnight.
174 After centrifugation for 10 min, 500 μL of the supernatant was collected and added
175 dropwise to 10 mL of PBS (10 mmol/L, pH 8.0) containing BSA or OVA (with mole
176 ration of carrier protein to antigen at 1:60). The mixture was agitated at 4℃ for 12h
177 and purified by dialyzes against PBS (10 mmol/L, pH 8.0) for two days. After
178 centrifugation for 10 min, the supernatant was collected and stored at 4℃. Formation
179 of the conjugate was confirmed with a UV-vis spectroscopy.

180 *2.6 Production of mAb*

181 Six-week-old female Balb/c mice were immunized at multiple sites with 50 μg of
182 ITeAHGA-BSA conjugate emulsified in complete Freund's adjuvant. Booster
183 injections were given at 2-week intervals with the same amount of conjugate
184 emulsified in incomplete Freund's adjuvant. Mice were tail bled, and the quality of the
185 antiserum was assessed using an indirect ELISA. The mouse with the highest titer
186 received a final intraperitoneal injection of 100 μg of immunogen conjugate (without
187 adjuvant) three days prior to cell fusion.

188 Cell fusion procedures were performed as described by Moreno et al. (2001). The
189 spleen cells (10^8 cells) from the selected mouse were mixed with SP2/0 myeloma cells
190 (10^7 cells) at a 10:1 ratio in 50% (w/v) PEG 2000. The fused cells were distributed in
191 96-well plates and cultured in HAT selection medium at 37℃ in a humidified 5% CO_2

192 incubator.

193 When the hybridoma cells reached around 30-40% confluence, culture supernatants
194 were screened for their binding activities to ITeAHGA-OVA with an indirect ELISA.
195 The hybridomas showing the desired specificity were sub-cloned for multiple rounds
196 by the limiting dilution method until a pure and stable antibody-producing clone was
197 obtained. The positive clones were injected into female Balb/c mice to obtain ascitic
198 fluid for antibody production. Antibodies in the fluid were purified by the caprylic
199 acid-ammonium sulfate precipitation method (Zhao et al. 2002) and stored at -20℃.

200 *2.7 Indirect Competitive ELISA* (icELISA)

201 *2.7.1 icELISA Protocol*

202 Ninety-six well microtiter plates were coated with 100 μL/well of ITeAH-OVA
203 overnight at room temperature. The plates were washed and incubated with 120
204 μL/well of blocking solution for 3 h at 37℃. After washing, 50 μL of the standard
205 solution or sample extracts along with 50 μL of antibodies were added. Plates were
206 incubated for 40 min and washed. Goat anti-mouse IgG-HRP was added (100 μL/well)
207 and incubated for 30 min at 37℃. After washing, 100 μL of the chromogenic reagent
208 was added and incubated for 10 min. The reaction was stopped by adding 50 μL of
209 stop solution, and the absorbance was measured at 450 nm using a Plate Reader.

210 The results were expressed as the percentage of inhibition (B/B_0), where B and B_0
211 are the absorbance values of the wells with and without standard solution, respectively.
212 The competitive standard curve was constructed by plotting the B/B_0 values against
213 the logarithm of analyte concentration. Sigmoid curve was obtained using OriginPro
214 8.5 software (OriginLab Corp., Northampton, USA). The limit of detection (LOD)
215 was determined as the 10% inhibiting concentration (IC_{10}) (Henniona and Barcelob
216 1998). The linear range was defined as the detection regime between the lower and

upper limits of quantification, i.e., the IC_{20}-IC_{80} working range.

2.7.2 Optimization of Assay Conditions

The most sensitive reaction condition of the icELISA assay was achieved when using ITeAHGA-BSA, ITeAH-OVA, and ITeAH as the immunogen, coating antigen, and competition analyte, respectively. Other experimental parameters were also optimized to further improve the assay sensitivity including checkerboard titrations of coating antigens and antibody dilutions, different incubation time of antigen-antibody and secondary antibodies, as well as various buffer systems.

2.8 Cross reactivity

The specificity of the generated monoclonal antibody was assessed for its cross-reaction rate (CR) with a group of structurally similar analogues based on the IC_{50} data calculated according to the following equation (Cui et al. 2011):

$$CR(\%) = \frac{IC_{50\,ITeAH}}{IC_{50\,structural\,analogue}} \times 100 .$$

2.9 Sample Collection and Preparation

Twenty samples were obtained from the local supermarket, apple juice (n=5), beer (n=5), tomato ketchup (n=4), and dried fruit (n=6). The liquid samples (1mL) were extracted with 2 mL of chloroform on a wrist-action shaker for 1 min. This was repeated two times followed by centrifugation (1,000×g, 10 min). The dried fruit samples were extracted with 2:3:3 methanol-acetonitrile-water (v/v/v) for 25 min and 4:1 chloroform-ethanol (v/v) for 1 min successively at room temperature (Stinson et al. 1981). ITeA in the samples was first reduced to ITeAH using hydrazine hydrate prior to detection using the following procedures. The organic phase containing the ITeA was mixed with 100 μL hydrazine hydrate and vigorously agitated for 30 min at room temperature. The reaction was stopped by addition of 500μL of H_2O and the mixture was transferred into a 25 mL round-bottom flask where the solvent was

evaporated to dryness in a rotary evaporator at 60℃ under reduced pressure. The residue was then resuspended in 1 mL H_2O. To eliminate sample matrix effects, the apple juice, beer solution and the tomato ketchup was further diluted 35-45 times with the assay buffer prior to icELISA analysis. All samples were subject to analysis by both icELISA and HPLC-MS/MS.

2.10 Recovery tests

ITeA was added to apple juice (1 mL) to give the final concentrations at 30, 150, 300 ng/mL, respectively. For the beer sample (1 mL) and tomato ketchup (1 g), the final concentrations were 150, 300, 720 ng/mL or ng/g, respectively. All of the spiked samples were prepared as described in 2.9 and measured with the developed icELISA. Calibration curve was constructed with a serial dilutions of ITeAH (0, 0.064, 0.32, 1.6, 8, 40, 200, and 1,000 ng/mL) and used to measure the concentration of ITeA from different extracted samples based on the reduction rate of 65% (ITeA to ITeAH).

2.11 HPLC-MS/MS analysis

The mobile phase was a mixture of the ammonium formate solution (5 mmol/L in water, adjusted to pH 7.8 with ammonia) (A) and acetonitrile (B), which was used in the following linear binary gradient: 0-3min, 5% B; 3-5 min, 5-15% B; 5-8 min, 15%-100% B; and 8-11 min, 100% B. The injection volume and flow rate were 50μL and 0.4 mL/min, respectively. Analytes were determined by ESI-MS/MS in the positive mode. Other parameters were as follows: gas temperature, 350℃; gas flow, 10 L/min; nebulizer gas, 50 psi; and capillary voltage, 3.5 kV.

3. Results and discussion

3.1 Hapten Synthesis and Conjugate Preparation

The design and production of functional haptens is the first and a critical step in any immunoassay development. Similar to many other small molecules, ITeA (197 Da) is

not immunogenic itself and lacks an available chemical group for protein conjugation. In this work, two novel ITeA haptens is illustrated (Fig.2). An intermediate hapten ITeAH was first prepared by condensation of hydrazine hydrate to the ketone group of ITeA. It was then reacted with glyoxalic acid to introduce the carboxyl group to obtain the tentative hapten ITeAHGA with a short aliphatic spacer arm. It has been suggested that a linear interval arm with aliphatic linkers comprised of a semi-rigid unsaturated double bond structure with three to six carbon atoms is generally good for producing the desired antibodies (Shen et al. 2007; Mercader et al. 2008). Using the same strategy, we previously reported the successful production of anti-TeA antibody and subsequently development of an ELISA for TeA (Yang et al. 2012). The successful syntheses of ITeA, ITeAH, and ITeAHGA were confirmed by MS and NMR data.

ITeA: APCI-MS, m/z 196.4 ($[M-H]^-$). ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ0.96 (d, J=6.37 Hz, 3H, CH_3), 0.98 (d, J=6.46 Hz, 3H, CH_3), 1.45 (m, 1H, CH), 1.84-1.67 (m, 2H, CH_2), 2.46 (s, 3H, CH_3), 3.85 (ddd, J= 9.80, 3.59, 0.88 Hz, 1H, CH), and 6.03 (s, br, 1H, NH).

ITeAH: The APCI-MS was m/z 212.1($[M+H]^+$). The ^{1}H NMR (600 MHz, $CDCl_3$, TMS): δ0.96 (d, J=6.43 Hz, 3H, CH_3), 0.95 (d, J = 6.34 Hz, 3H, CH_3), 1.33-1.41 (m, 1H), 1.72 (m, 2H), 2.67 (s, 3H, CH_3), and 3.48-4.04 (m, 1H).

ITeAHGA: APCI-MS, m/z 266.0 ($[M-H]^-$). The ^{1}HNMR (600MHz, DMSO-d_6, TMS): δ 0.88 (d, J=6.59 Hz, 6H, $2CH_3$), 1.29 (ddd, J=13.90, 9.47, 4.73 Hz, 1H, H_aCH_b), 1.48 (ddd, J=13.50, 9.31, 4.08 Hz,1H, H_aCH_b), 1.86-1.75 (m, 1H, CH), 2.61 (s, 3H, CH_3), 3.73 (dd, J=9.10, 4.00 Hz, 1H), 7.69 (s, 1H), 6.40 (s, br, 1H, NH), and 13.13 (s, br, 1H, COOH).

The production of immunogen and the homologous coating antigen was carried out by coupling the hapten ITeAHGA to the carrier protein (BSA/OVA) via the common

292 N-hydroxysuccinimide active ester method, while ITeAH was conjugated to OVA and
293 used as the heterologous coating antigen through the cross-linking agent
294 glutaraldehyde. Successful conjugations were confirmed by the UV-vis data (data not
295 shown). The antigen was added in molar excess over that of carrier protein in order to
296 bind to sufficiently (Hamajima et al. 1995). Most reported hapten:protein ratios are
297 between 50:1 to 100:1 which resulted in ideal artificial antigen and subsequently
298 produced ideal antibodies (Hamajima et al. 1995; McAdam et al. 1992; Shen et al.
299 2007).

300 *3.2 mAb Production and Identification*

301 In our experiment, the mAb raised against ITeAH was successfully produced from
302 a selected single hybridoma (2E8) and used to establish icELISA detection system for
303 ITeA. The derivation strategy that is based on the special antibody to the derivative of
304 the determinants, has been successfully used by other researchers to establish
305 immunoassays for several haptens including 1-aminohydantoin (AHD) (Jiang et al.
306 2012), furaltadone metabolite AMOZ (Shen et al. 2012) and TeA (Prelle et al. 2013).
307 This strategy is particularly useful when production of antibody to toxic compound is
308 in question, whether it is due to the extremely toxicity or the lack of toxic reagent
309 itself. Considering the homologous coating antigens for which antibodies generally
310 have weaker affinity towards (Xu et al. 2012; Galve et al. 2002), two coating antigens
311 ITeAHGA-OVA and ITeAH-OVA were compared in the present study and the results
312 (Fig. 3) clearly indicated that the heterologous coating antigen ITeAH-OVA (IC_{50} =
313 14.5 ng/mL) was superior to the homologous coating antigen ITeAHGA-OVA (IC_{50} =
314 27.2 ng/mL). This is in accordance with other research finding that the use of an
315 appropriate heterologous coating antigen can significantly improve sensitivity of the
316 assay (Qi et al. 2012). Subsequent experiments were therefore carried out based on the

317 coating antigen of ITeAH-OVA.

318 *3.3 ELISA Optimization*

319 To optimize the developed ELISA, we tested various concentrations of the coating
320 antigen and antibody, reaction time of antigen with antibody, incubation time of HRP
321 labeled secondary antibody, different analyte/antibody ratio and buffering system. The
322 A_{max}/IC_{50} ratio (A_{max} is the maximum value of absorbance) from the competition
323 curves of ITeAH was used to estimate the influence of each condition on assay
324 performance, and the higher ratio suggests the higher sensitivity of the assay (Liang et
325 al. 2007). Figure 4(A) shows an optimal combination of a coating antigen at
326 concentration of 15.6 ng/mL and an antibody at 1:2,000 dilution, exhibiting the lowest
327 IC_{50} value of 5 ng/ml. Other optimized assay conditions include 40-min reaction time
328 (Fig 4(B)) for incubation of the antigen-antibody, 30 min for the anti-IgG-HRP
329 antibody (Fig 4(C)), and H_2O was found as the most suitable diluent for the analyte
330 (Fig 4(D)). Under these conditions, a calibration curve was constructed for ITeAH at
331 concentration ranged from 0.064 to 1,000 ng/mL with a linear working range between
332 1.7 and 36.4 ng/mL (R^2=0.9944) (Fig. 5). The established icELISA system is highly
333 sensitive with an IC_{50} value of 7.8 ng/mL and a LOD of 0.5 ng/mL for ITeAH. An
334 enzyme immunoassay has been reported for TeA in apple and tomato products with an
335 IC_{50} of 320 ± 130 ng/ml for TeA, but a much lower IC_{50} of 23.3 ± 7.5 ng/ml for the
336 TeA acetate. When TeA acetate was employed as the standard in the EIA to measure
337 the acetylated TeA, an IC_{30} of 5.4 ± 2.0 ng/ml was resulted (Gross et al. 2011).
338 Ackermann et al.(2011) described the development of an EIA for rapid determination
339 of alternariol, another *Alternaria* mycotoxin, with a detection limit of 1-2 μg/kg.

340 *3.4 Cross-reactivity Studies*

341 The specificity of the developed monoclonal antibody was examined by testing the

cross-reactivity (CR) rates of several *Alternaria* mycotoxin analogues (Table 1), including iso-tenuazonic acid (ITeA), tenuazonic acid (TeA), alternariol (AOH), alternariol methyl ether (AME), and hydrazine hydrate. The results were all less than 0.1%, suggesting the high specificity of the produced monoclonal antibody towards ITeAH, which is vital in the developed ELISA system.

3.5 Analysis of Spiked Samples

Spiked apple juice, beer, and tomato ketchup samples were analyzed using the developed ELISA. It is generally recognized that the reasonable sample preparation can effectively reduce the matrix effect (Sheng et al. 2012). A simple H_2O dilution of samples was used in this study, i.e., 35 times dilution for the extracts of apple juice and beer, and 45-fold dilution for extract of tomato ketchup. Samples were spiked with ITeA at different concentrations to evaluate the recovery rates of the developed immunoassay. As shown in Table 2, the recoveries of ITeA ranged from 93.3% to 109.8% for apple juice, 82.3% to 93.2% for beer, and 93.2% to 107.3% for tomato ketchup, respectively, and that was within the general requirement of 70-120% for screening immunoassays (Kondo et al. 2012; Wang et al. 2011). The coefficients of variation (CV) were all found to be less than 15%, indicating a good level of precision of the developed ELISA.

3.6 Comparison of the ELISA and HPLC-MS/MS Method

The ELISA results were compared and confirmed by the HPLC-MS/MS method. The linear relationship between the two methods was at $y=0.7660x+45.52$ with a squared coefficient of correlation (R^2) of 0.9557 for the spiked food samples (Fig. 6), suggesting a good agreement between the screening ELISA and confirmatory HPLC-MS/MS methods. These results also demonstrated the suitability of the developed ELISA for the detection of trace levels of ITeA in food samples. Asam et al.

(2013) reported the development of analytical methods for detection of TeA and its analogues in foods with an LOD of 1 μg/kg (TeA) and 3 μg/kg (ITeA) for derivatized samples and 60 μg/kg (TeA and ITeA) for samples without derivatization, while the ELISA method we developed here has a significantly lower LOD of 0.5 μg/L for ITeA. Taking consideration of the generally lower level of ITeA than TeA in fruits and vegetables, the present ITeA ELISA possesses lower detection limit and higher sensitivity.

3.7 Detection of ITeA in real samples

The commercial food samples (n=20) were analyzed using both ELISA and HPLC-MS/MS methods. As shown in Table 3, results revealed a highly conformity between the two methods, i.e., all samples were found to be positive by the developed ELISA and their quantified results are in consistence with the HPLC-MS/MS data. This further confirms the reliability of the established ELISA method as a fit-for-purpose screening tool for quantitative analysis of ITeA in food samples. ITeA was detected with varying concentrations in all samples tested, i.e., 39.2-110.3 ng/mL in apple juice, 45.4-79.3 ng/mL in beer, 41.5-81.1 ng/mL in tomato ketchup and 43.4-157.2 ng/mL in dried fruits. Using the UPLC-ESI-MS/MS, Walravensa et al. (2014) reported around 71% of rice samples and 31% of oat flake samples obtained in Belgium were contaminated with TeA at concentrations ranging from 1.90-113 μg/kg and 2.13-39 μg/kg, respectively. Such high frequency and levels of contamination of *Alternaria* mycotoxins and TeA in particular has highlighted the importance of continued monitoring of TeA in food and feed. Likewise, similar occurrence will also be applied to ITeA contamination scenario.

Currently, there are no details of accepted daily intake (ADI) and maximum residue limits (MRL) available for ITeA. Moreover, due to a lack of information on occurrence and toxicity, the European Food Safety Authority (EFSA) stated that a risk assessment for *Alternaria* mycotoxins in feed was not possible (EFSA, 2011). This may partially due to the lack of rapid methods such as immunoassays that are capable of screening a large number of samples within a relatively short period of time. Consequently, the liquid chromatography coupled to (tandem) mass spectrometry is the method of choice for quantification of *Alternaria* toxins in foods and feeds. The sensitive and reliable ELISA described in this study would therefore contribute greatly to the effective and efficient monitoring of ITeA in food and the environment.

4. Conclusions

In this paper, we report the development of a sensitive ELISA using a specific monoclonal antibody for reliable detection of ITeA in food samples. The optimized ELISA has an IC_{50} value of 7.8 ng/mL and a detection limit of 0.5 ng/mL with good extraction efficiency for apple juice, beer, and tomato ketchup samples. The established immunoassay was subsequently implemented in a mini-survey of commercial food samples with results revealing a potentially widespread contamination of ITeA (probably alongside TeA) in processed commercial foods. The close agreement between the ELISA result and HPLC-MS/MS data has confirmed the reliability of this newly developed ELISA as a versatile screening tool for monitoring ITeA in different foods. Future studies will seek its applications in a wider range of foodstuffs including animal feeds to facilitate the collection of occurrence data and estimation of dietary exposure for this *Alternaria* mycotoxin.

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Conflict of Interest: All authors declare no conflict of interest.

Ethical approval: All procedures involving animals were approved and performed in accordance with the relevant protective and administrative guidelines for laboratory animals of China.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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547 of an enzyme-linked immunosorbent assay based a monoclonal antibody for the
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553 spectrometric method for the simultaneous determination of free and conjugated
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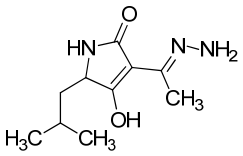
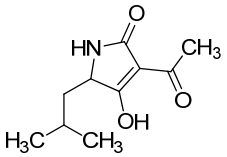
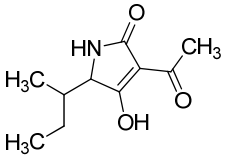
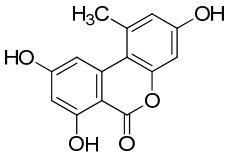
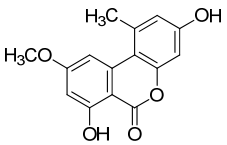
555

556

557 **Table 1.** Cross-reactivity (CR) of the novel mAb with ITeAH and other compounds

558 using the indirect competitive ELISA developed.

559

Compound	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
(E)-3-(1-hydrazonoethyl) -4-hydroxy-5-isobutyl-1H -pyrrol-2(5H)-one (ITeAH)		7.4	100
Iso-tenuazonic acid (ITeA)		>8000	<0.1
tenuazonic acid (TeA)		>8000	<0.1
Alternariol (AOH)		>8000	<0.1
Alternariol methyl ether (AME)		>8000	<0.1
Hydrazine hydrate	NH_2-NH_2	>8000	<0.1

560

561 **Table 2.** Recoveries of ITeA from spiked apple juice, beer, and tomato ketchup by

562 icELISA (n=3).

Matrix	ITeA spiked concentration (ng/mL or ng/g)	icELISA found (ng/mL or ng/g)	
		Recovery± SD (%)	CV (%)
apple	30	93.3 ± 10.2	10.9
juice	150	98.7±9.8	9.9
	300	109.8±10.6	9.7
beer	150	89.1±7.9	8.8
	300	93.2±8.2	8.8
	720	82.3±3.6	4.4
tomato	150	94.3±9.7	10.3
ketchup	300	93.2±11.4	12.2
	720	107.3±6.5	6.1

563

564

565 **Table 3.** Concentrations of ITeA in food samples determined by icELISA and

566 HPLC-MS/MS.

Sample	Number	icELISA (ng/mL or ng/g)	HPLC-MS/MS (ng/mL or ng/g)	Sample	Number	icELISA (ng/mL or ng/g)	HPLC-MS/MS (ng/mL or ng/g)
apple juice	1	39.2±3.1	44.6±5.2	tomato ketchup	1	41.5±4.1	39.5±4.4
	2	98.5±9.4	103.5±8.1		2	81.1±8.5	96.4±11.4
	3	110.3±10.2	102.8±13.9		3	28.1±2.4	39.4±3.8
	4	68.4±5.1	76.6±6.4		4	30.6±1.9	38.1±5.9
	5	82.9±7.5	79.0±8.6	dried fruit	1	48.4±2.2	43.5±1.6
beer	1	45.4±6.7	40.8±3.9		2	83.8±10.6	92.1±6.4
	2	76.2±7.9	83.5±9.7		3	76.6±6.9	84.7±9.4
	3	69.8±4.3	80.7±7.3		4	157.2±1.3	156.9±2.7
	4	79.3±6.7	81.9±10.5		5	52.2±0.7	47.4±0.5
	5	73.5±8.9	68.4±9.5		6	43.4±1.3	44.1±0.7

567

568

569 **Figure captions**

570 **Fig. 1**. Chemical structures of TeA, ITeA and its derivatives, ITeAH and ITeAHGA.

571 **Fig. 2.** Synthesis route of hapten ITeA and the derivatives of ITeAH and ITeAHGA.

572 **Fig. 3**. Dose-dependent indirect competitive ELISA curves for ITeAH against two
573 coating antigens. The error bar represents the standard deviation of the mean (n=3).

574 **Fig. 4.** Optimization of assay conditions: (A) coating antigen concentration and
575 antibody dilution; (B) competition time of antigen-antibody; (C) IgG-HRP incubation
576 time; and (D) the diluting factor of the analyte.

577 **Fig. 5.** Calibration curve for the detection of ITeAH by icELISA. Each point
578 represents the mean results of four replicates. The vertical bars indicate the mean
579 results of the standard deviation.

580 **Fig. 6.** Comparison of icELISA and HPLC-MS/MS results for ITeA quantification.

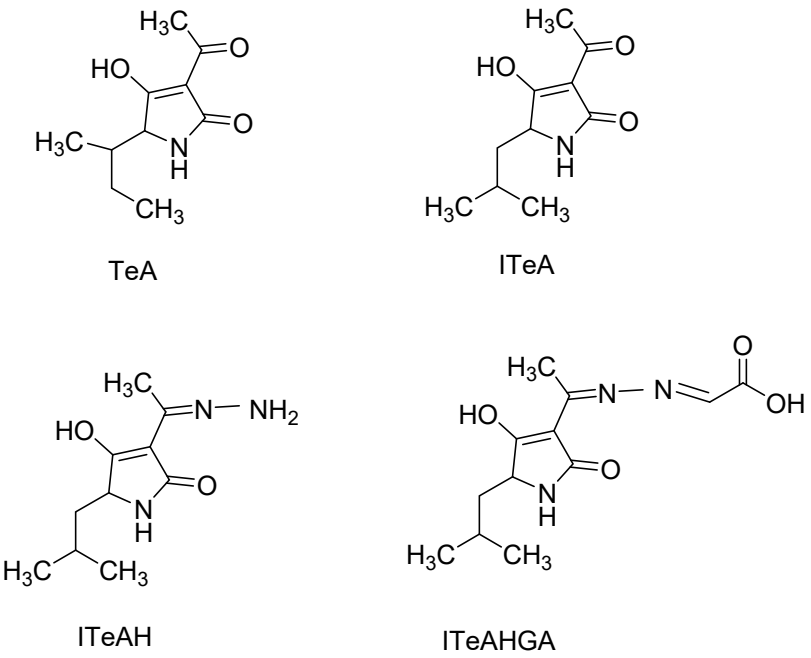


Fig. 1

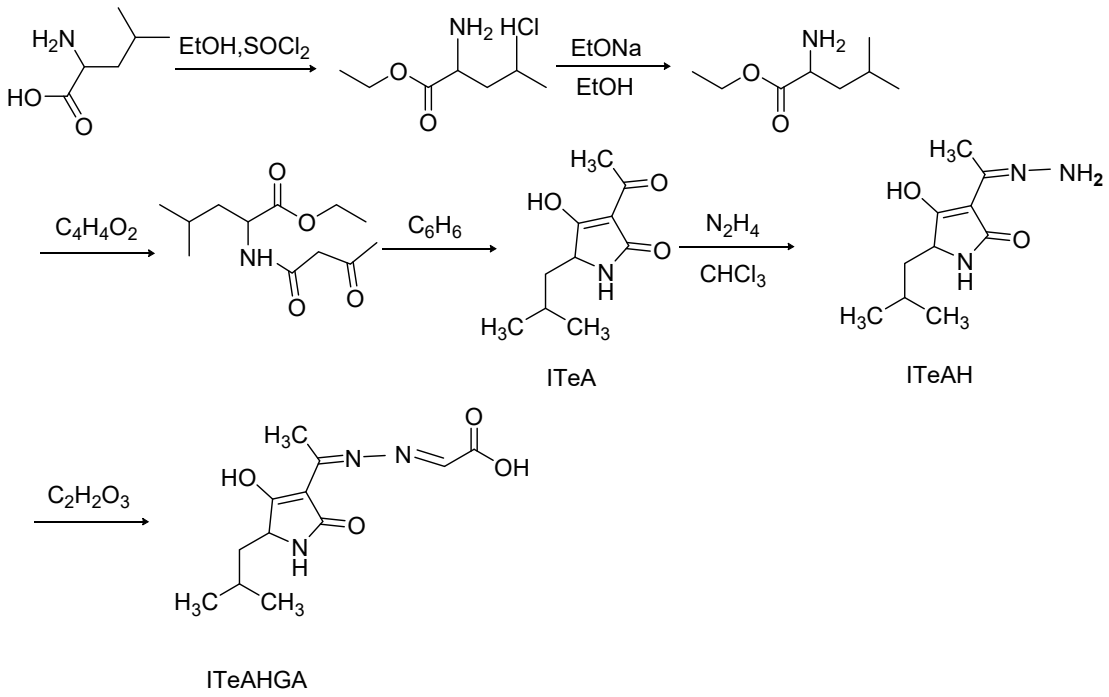

EtOH,SOCl2
EtONa
EtOH
C4H4O2
C6H6
N2H4
CHCl3
C2H2O3
ITeA
ITeAH
ITeAHGA

Fig. 2

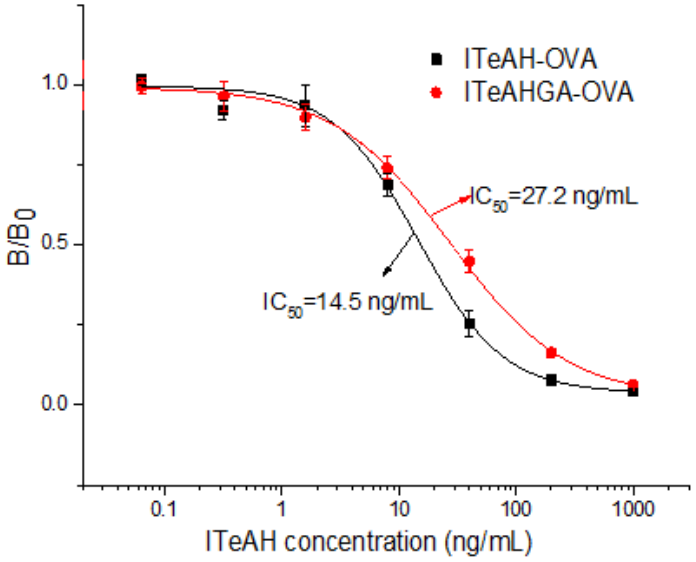

ITeAH-OVA
ITeAHGA-OVA
IC50=27.2 ng/mL
IC50=14.5 ng/mL
B/B0
1.0
0.5
0.0
0.1
1
10
100
1000
ITeAH concentration (ng/mL)

Fig. 3

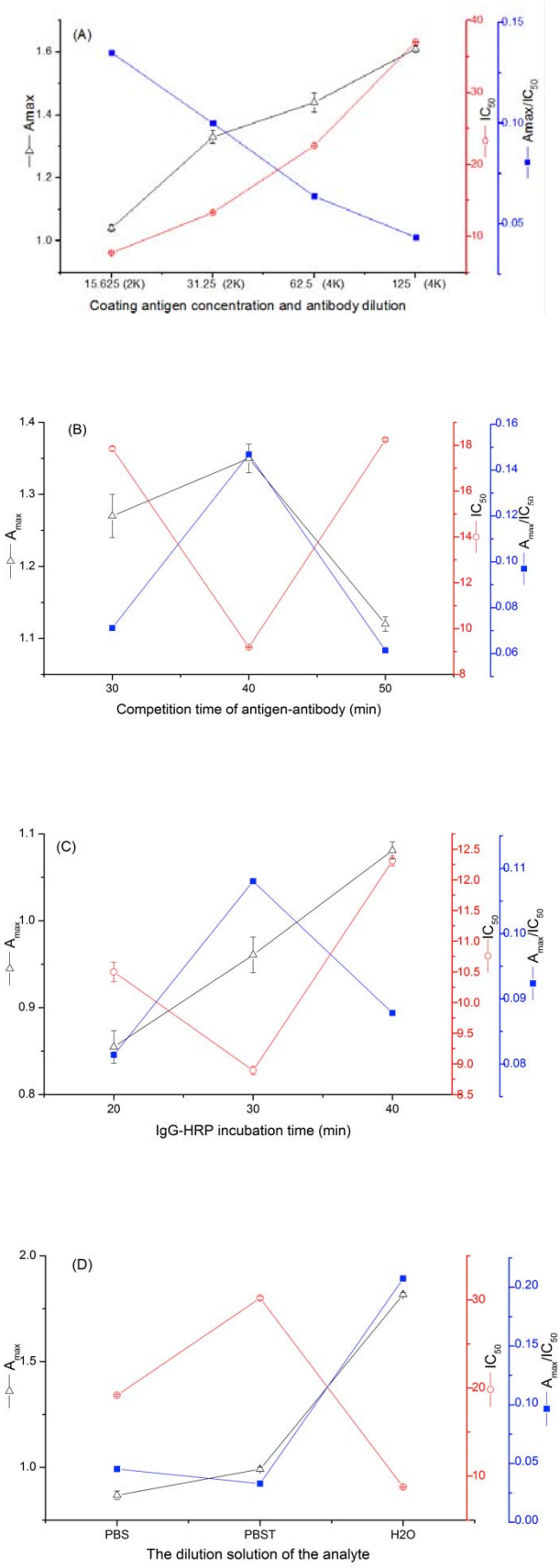

(A)
Amax
IC50
Amax/IC50
15.625 (2K)
31.25 (2K)
62.5 (4K)
125 (4K)
Coating antigen concentration and antibody dilution
(B)
Competition time of antigen-antibody (min)
(C)
IgG-HRP incubation time (min)
(D)
PBS
PBST
H2O
The dilution solution of the analyte

Fig. 4

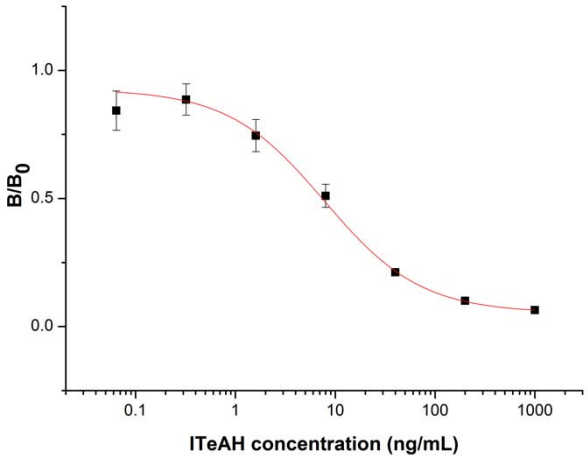


Fig. 5

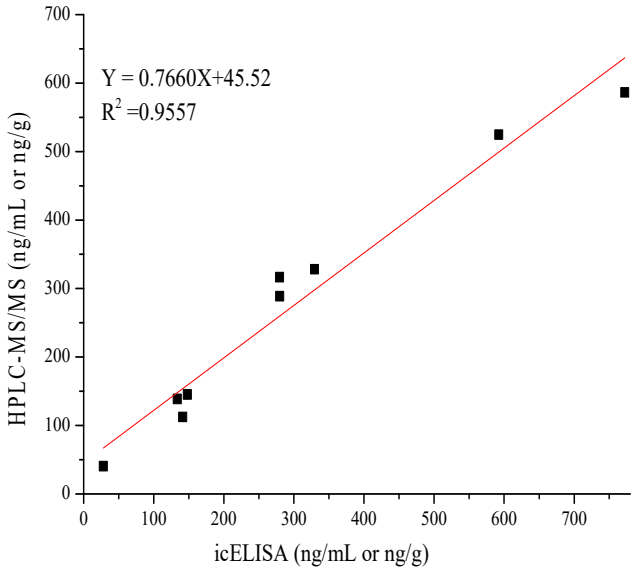

Y = 0.7660X+45.52
R2 =0.9557
HPLC-MS/MS (ng/mL or ng/g)
icELISA (ng/mL or ng/g)
0
100
200
300
400
500
600
700

Fig. 6