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Fluorescence Polarization Immunoassay for *Alternaria* Mycotoxin Tenuazonic Acid Detection and Molecular Modeling Studies of Antibody Recognition

Feng Wang[1] · Jun Cai[1] · Sergei A. Eremin[2] · Zhi-Li Xiao[1] · Yu-Dong Shen[1] · Yuan-Xin Tian[3] · Zhen-Lin Xu[1] · Jin-Yi Yang[1] · Hong-Tao Lei[1] · Yuan-Ming Sun[1] · Hong Wang[1]

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Abstract
A simple and rapid method of fluorescence polarization immunoassay (FPIA) for detection of *Alternaria* mycotoxin tenuazonic acid (TeA) was described. In this study, the tracers TeAH-FITC and TeAH-DTAF were synthesized from hapten 5-(sec-butyl)-3-(1-hydrazonoethyl)-4-hydroxy-1H-pyrrol-2(5H)-one (TeAH) and different fluoresceins isothiocyanate isomer I (FITC) or 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride (DTAF), respectively. The established FPIA showed an IC_{50} value of 3.0 μg/mL with a detection limit of 0.13 μg/mL for TeAH based on the tracer of TeAH-FITC, and with the same properties of 7.3 and 0.93 μg/mL based on TeAH-DTAF. The former gave better assay sensitivity than the latter, that the 2,4-dichloro-s-triazine group of TeAH-DTAF may affect the specific recognition of tracer and antibody. Models of the lowest energy conformation and the molecular electrostatic potential isosurface studies revealed that the –C=N–N– and $–CHCH_3(CH_2CH_3)$ groups were the characteristic chemical groups in anti-TeAH antibody-analyte recognition via molecular modeling, which could create a theoretical guide for hapten design of small molecules and provide the traditional theory with empirical proof.

Keywords Tenuazonic acid · Fluorescence polarization immunoassay · Molecular modeling · Antibody recognition

Introduction

Tenuazonic acid (3-acetyl-5-s-butyl-4-hydroxy-3-pyrrolin-2-one, TeA) is a natural phytotoxin produced by species of *Pyricularia*, *Phoma*, and *Alternaria alternate*. Species of *Alternaria* are known to produce at least 70 secondary toxic metabolites. TeA was identified as having the highest toxicity in the family, which was the only one among *Alternaria* toxins that belongs to the list of toxic chemicals of the US Food and Drug Administration (Ostry 2008; Mikula et al. 2013). Firstly, it and its derivatives were used as a kind of commercial herbicide in fields (Sinning 1992). However, it was reported that TeA was acutely or subacutely toxic to mice, dogs, and shrimps (Yekeler et al. 2001; Rychlik et al. 2016; Patriarca 2016), and it could produce synergistic toxicity with alternariol, alternariol methyl ether, and other toxins (Lee et al. 2015; Vejdovszky et al. 2016). Recent researches have shown that TeA has strong brine shrimp cytotoxicity, and the mortality could approach 100%, often with high pollution levels varying from 1.76 to 520 μg/kg in wheat-based products, 10.2–1787 μg/kg in all tomato ketchup, and 7.4–278 μg/kg in tomato juice samples (Qin et al. 2009; Zhao et al. 2014; Zhao et al. 2015). TeA could be detected with high pollution levels in beers, potatoes, pepper, wines, and other food products (Siegel et al. 2010; Lohrey et al. 2012; Fontana et al. 2016; López et al. 2016a, b; Walravens et al. 2016). The risk assessment studies about TeA were also established given a scientific opinion on the risks for public health (Zhao et al. 2015; Patriarca 2016; López et al. 2016a, b).

By far, there is no sufficient data for system toxicology and no corresponding international standards. Current quantitative analysis methods of TeA were performed by a variety of

✉ Hong Wang
gzwhongd@163.com

[1] College of Food Science, South China Agricultural University, Guangdong Provincial Key Laboratory of Food Quality and Safety, National-Local Joint Engineering Research Center for Processing and Safety Control of Livestock and Poultry Products, Guangzhou 510642, People's Republic of China

[2] Faculty of Chemistry, M.V. Lomonosov Moscow State University, Leninskie gory 1, Building 3, Moscow 119991, Russia

[3] School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, People's Republic of China

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methods including high-performance liquid chromatography (HPLC) (Myresiotis et al. 2015; Fan et al. 2016), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Rasmussen et al. 2010; Fraeyman et al. 2015), stable isotope dilution assay (SIDA) (Asam et al. 2012; Asam et al. 2013), and enzyme-linked immunosorbent assay (ELISA) (Gross et al. 2011; Yang et al. 2012). But there were few fluorescence polarization immunoassays for TeA analysis. Fluorescence polarization immunoassay is a new, simple, rapid, and efficient immunoassay, and has been widely used in determination of pesticide drugs, veterinary drugs, and mycotoxins (Smith and Eremin 2008; Mi et al. 2013; Li et al. 2015a, b; Beloglazova et al. 2016). This study reported the development of fluorescence polarization immunoassay using polyclonal antibodies for detection of TeA. Firstly, two tracers TeAH-FITC and TeAH-DTAF with different fluorescent probes were prepared for FPIA performance (Fig. 1). Then the developed FPIA method was used to determine TeA in spiked drink samples with an acceptable recovery. To further reveal antibody-analyte recognition and key binding sites, the authors established models of the lowest energy conformation and the molecular electrostatic potential isosurface studies via molecular modeling, which laid the foundation of a better understanding of antibody recognition mechanisms.

Materials and Methods

Materials, Reagents, and Instruments

Fluorescein isothiocyanate isomer I (FITC), 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF), complete Freund's adjuvants, incomplete Freund's adjuvants, hydroxylamine hydrochloride, potassium borohydride, and tributylamine were purchased from Sigma (St. Louis, MO, USA). The hapten TeAH, complete antigen TeAHGA-BSA, and TeAH-OVA were prepared previously by our laboratory. Silica gel 60 aluminum sheets (type GF254, layer thickness 0.25 mm) for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). All other organic solvents and chemicals were of analytical grade. Borate solution (BB, 2.5 mmol/L, pH = 7.5, containing 1% sodium azide) was used as FPIA working buffer.

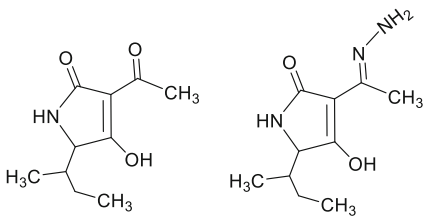


Fig. 1 Chemical structures of TeA and TeAH

The NanoDrop 2000c UV-Vis spectrophotometer was purchased from Thermo Scientific (Wilmington, DE, USA). The signal of fluorescence polarization and its intensity were measured on a TDx FLx analyzer (Abbott Laboratories, USA) in the Photo Check mode, and the analyzer was kept in Lomonosov Moscow State University.

Preparation of Hapten–Protein Conjugates and Polyclonal Antibody

Hapten–protein conjugates TeAHGA-BSA and TeAH-OVA were prepared and identified previously in our laboratory (Yang et al. 2012). A New Zealand white rabbit was firstly immunized by subcutaneous injection of 500 μg TeAHGA-BSA emulsified with the same volume of Freund's complete adjuvant. Then the immunogen with an equal volume of Freund's incomplete adjuvant was emulsified for booster injection at intervals of 2 weeks. The rabbit ear vein blood was collected at the interval of 7 days after each immunization to check the serum titer and specificity by indirect competitive ELISA (ic-ELISA) using the coating antigen TeAH-OVA. The ic-ELISA protocols were performed as described previously (Gross et al. 2011; Zhang et al. 2017). Until the titer was stable, all the blood was obtained and purified by the caprylic acid–ammonium sulfate precipitation method. The antibody concentration was determined by a NanoDrop 2000c UV-Vis spectrophotometer, and the polyclonal antibody was stored at −20 °C until use.

Synthesis and Identification of Fluorescent Conjugates (Tracers)

In the study, purified polyclonal antibodies were screened for specificity to the analyte of the TeA derivative TeAH via ic-ELISA, but not to TeA. And two tracers (TeAH-FITC and TeAH-DTAF) were synthesized according to the protocol with slight modifications (Li et al. 2015a, b). Schematic routes for the synthesis of TeAH-FITC and TeAH-DTAF are described in Fig. 2. More specifically, 2 mg TeAH and 3 mg FITC or 1 mg DTAF were dissolved in 500 μL anhydrous methanol. Then 50 μL trimethylamine was added into the solution. The mixture was stirred overnight at room temperature in the dark. The reaction was monitored by thin layer chromatography (TLC) with the mobile phase of trichloromethane/methanol (4:1, *v*/*v*). The main yellow bands for TeAH-FITC (R_f = 0.1 and R_f = 0.15) and TeAH-DTAF (R_f = 0.05 and R_f = 0.1) were collected separately in 100 μL methanol. Activities of all the bands were analyzed and identified, then the usable compositions with antibody-binding activity were stored as the working tracers for use.

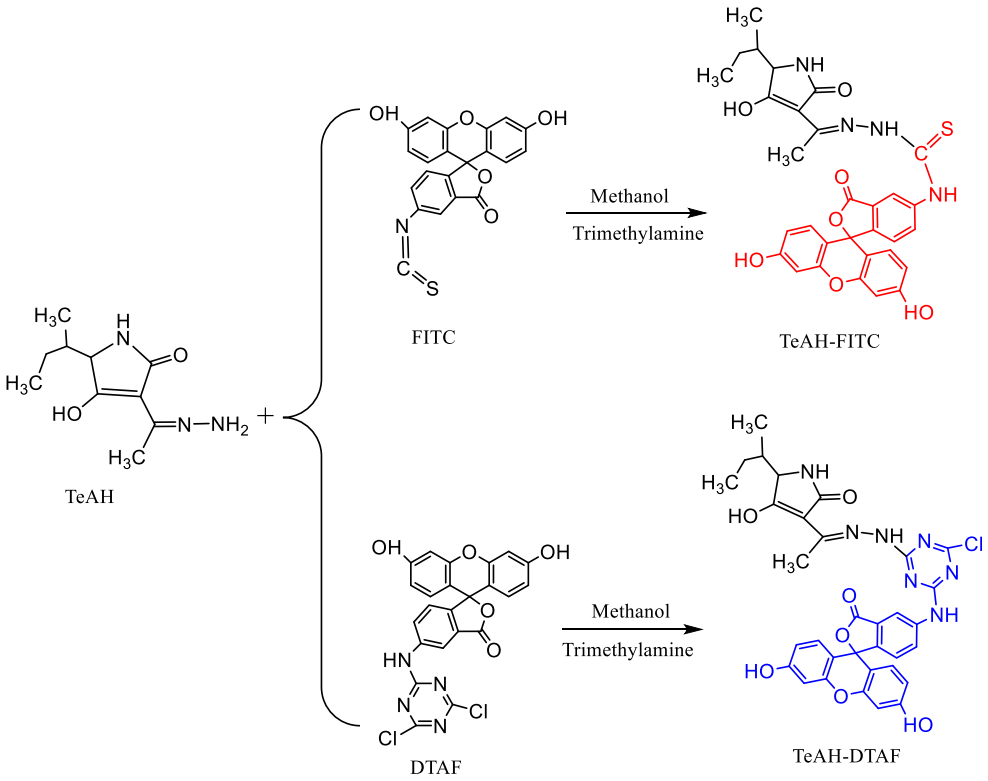


Fig. 2 Synthetic routes for the tracers TeAH-FITC and TeAH-DTAF

FPIA Procedure

The FPIA protocol was simply described as follows: 500 μL of trace working solution (TWS) was mixed with 50 μL of different concentrations of standard solution or sample extract in several glass culture tubes. Several concentrations of the standard solution (0, 0.1, 0.3, 1, 3, 10, 30, 100 μg/mL) were prepared for establishment of the FPIA standard curve. Afterwards, 500 μL of antibody working solutions (AbWS) was added and incubated for 2 min at room temperature, then the FPIA value (mP) was measured with the TDx FLx analyzer.

The antibody optimal dilutions for the two tracers TeAH-FITC and TeAH-DTAF were determined from the binding curves of the antibody with tracer in BB solution. The antibody dilution which has a response of approximately 150 of the mP value was chosen as the antibody working dilution.

Cross-Reactivity Determination

To evaluate the established FPIA method, the cross-reactivity (CR) with the common analogues was calculated as follows: CR = (IC_{50} of TeAH) / (IC_{50} of analogues) × 100%, where IC_{50} was the concentration at 50% of the maximum mP value.

Molecular Modeling Studies of Antibody Recognition

The lowest energy conformations and molecular electrostatic potential isosurfaces were operated on a SYBYL-X 2.1 program package (Tripos Inc., USA) running on an HP xw6600 molecular modeling workstation as reported (Zhang et al. 2017). The minimum energy conformations of all structures (TeAH, TeA, ITeA, ITeAH, ALT, and AME) were obtained using the Powell method. The charges of Gasteiger-Huckel were selected for energy. Tripos was used to minimize the total energy with a NB cutoff of 8.00 Å and a dielectric constant of 1.00. And then MOLCAD was used for revealing the molecular surface construction of the seven structures.

Analysis of Spiked Samples

The developed FPIA was applied for TeA detection in wheat beer and apple juice. Wheat beer and apple juice samples were purchased from a local supermarket in Lomonosov Moscow State University, Russia. As described previously with slight modifications (Yang et al. 2012), the pre-sample treatment procedures were summarized as follows: 5 mL wheat beer or apple juice was spiked with different concentrations of TeA (0.2, 0.6, 1 μg/mL), and the spiked samples were extracted twice with 2 mL chloroform in an ultrasonic bath for 5 min, followed by centrifugation at 4000 r/min for 10 min. The organic phase with an addition of 100 μL hydrazine hydrate was vortexed for 30 s,

and kept at 37 °C for 40 min. The reaction solvent was evaporated to dryness at 45 °C under nitrogen, and the residue was resuspended in 1 mL BB solution for analysis.

Results and Discussion

Characterization of Polyclonal Antibody and Tracers

The antiserum of rabbit was measured by ic-ELISA, which indicated that the titer of anti-serum was 8×10^3 with a high quality in specificity to TeAH. The polyclonal antibody was purified and stored with a concentration of 9.1 mg/mL for further research.

Different structures of fluorescein have a significant influence on antibody-tracer specific binding (Mi et al. 2013; Li et al. 2015a, b). The tracers TeAH-FITC and TeAH-DTAF were synthesized through the active ester method and isolated by preparative TLC. The main yellow bands were collected, then all the bands were respectively mixed with antibody buffer to measure the mP value. The identification results are shown as bar charts in Fig. 3. It was revealed that both TeAH-FITC with $R_f = 0.1$ and TeAH-DTAF with $R_f = 0.1$ could specifically bind to the antigen-binding site of the antibody, and then was stored at 4 °C in the dark until use.

Antibody Dilution Cures for TeAH-FITC and TeAH-DTAF

The antibody working concentration has a severe impact on the FPIA performance. As one of the key factors, different dilutions of the antibody could affect directly the sensitivity, accuracy, and suitability. In the study, a series of twofold dilutions of antibody was checked for antibody-tracer binding, and datas are shown in Fig. 4. According to empirical evidence

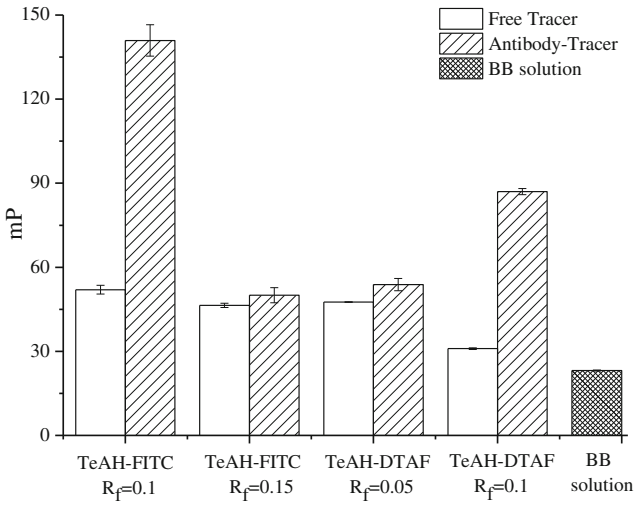


Fig. 3 Binding response of the tracers with antibody in BB solution

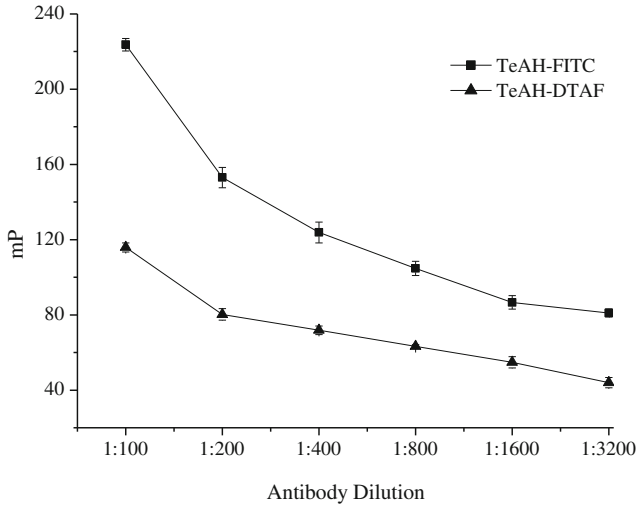


Fig. 4 Dilution curve for antibody with the tracers in BB solution

that nearly 150 of the mP value was selected for FPIA, the antibody dilution of 1:200 for TeAH-FITC with $R_f = 0.1$ and 1:100 for TeAH-DTAF with $R_f = 0.1$ were appropriated that it could guarantee the FPIA sensitivity and precision.

FPIA Standard Curves Based on Polyclonal Antibodies

The standard curves for the determination of TeA using TeAH-FITC and TeAH-DTAF tracers are shown in Fig. 5. The mP/mP_0 values were plotted against TeAH concentration, and fit to a four-parameter logistic equation using Origin 7.5 software. It was defined as follows: $y = (A - D)/[1 + (x/C)^B] + D$, where A is the maximal mP value, D is the minimal mP value, B refers to the steepness of the curve, and C is the concentration at 50% of antibody-tracer binding response (IC_{50}). The LOD (IC_{10}) for TeAH was 0.13 μg/mL with IC_{50} of 3.0 μg/mL, and the liner concentration range was calculated as 0.19–47.7 μg/mL ($r^2 = 0.998$) based on the

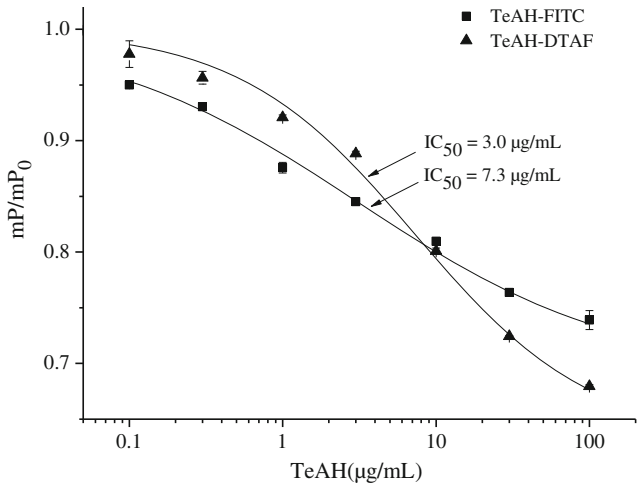


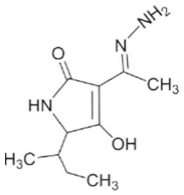
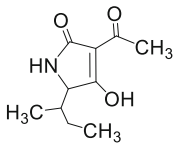
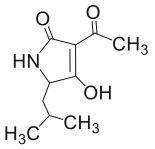
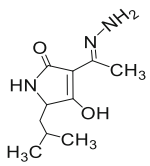
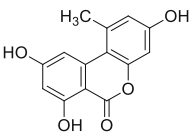
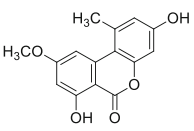
Fig. 5 FPIA standard curves based on the tracers TeAH-FITC and TeAH-DTAF

tracer TeAH-FITC. And the LOD and IC_{50} using TeAH-FITC were 0.93 and 7.3 μg/mL with the range of 1.2–45.8 μg/mL ($r^2 = 0.999$). The tracer TeAH-FITC gave a better assay sensitivity than TeAH-DTAF. It is speculated that the 2,4-dichloro-s-triazine group may affect the specific recognition of the tracer-antibody-binding domain, which decreased the detection sensitivity. The same research results were also observed in other small molecules (Wang et al. 2007; Zhang et al. 2014; Li et al. 2015a, b). Then the FPIA based on TeAH-FITC was applied to the cross-reactivity determination and recovery experiment of spiked samples.

Cross-Reactivity Determination

To evaluate the specificity of the FPIA method, cross-reactivity was calculated by IC_{50} of some analogues. As shown in Table 1, it was indicated that the antibody was specific to TeAH, and has low cross-reactivity (< 0.1%) with

Table 1 Cross-reactivity (CR) of the developed FPIA based on TeAH-FITC

Compounds	Structures	IC_{50} (μg/mL)	CR (%)
TeAH		3.0	100
TeA		>3000	< 0.1
ITeA		>3000	< 0.1
ITeAH		>3000	< 0.1
Hydrazine Hydrate	$H_2N—NH_2 \cdot H_2O$	>3000	< 0.1
Alternariol (ALT)		>3000	< 0.1
Alternariol Methyl Ether (AME)		>3000	<0.1

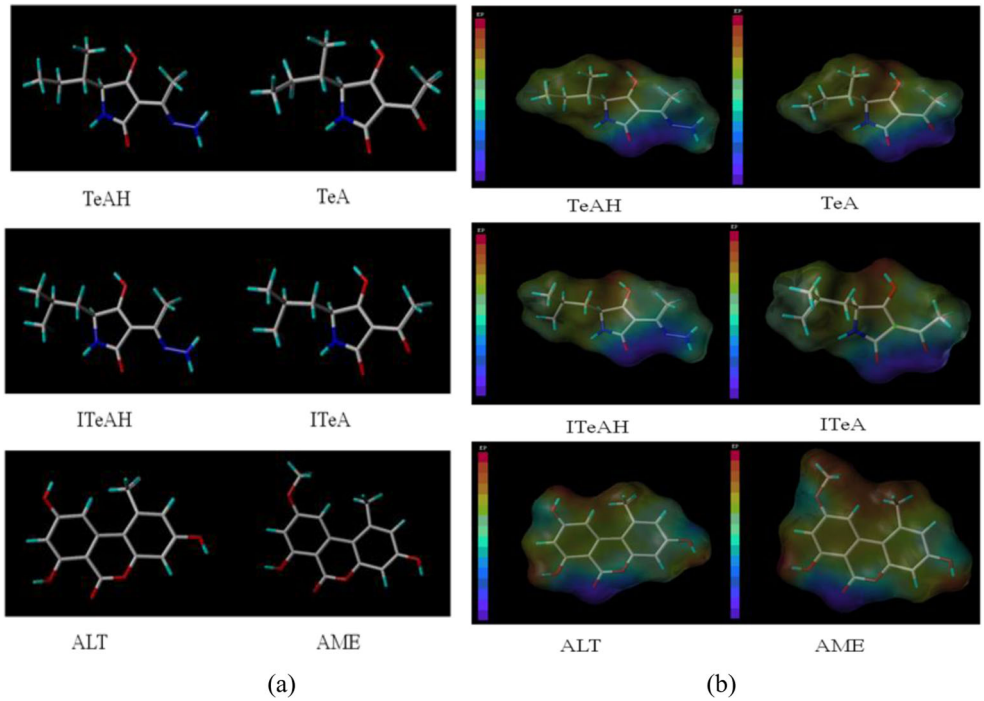


Fig. 6 Models of the lowest energy conformations (**a**) and the molecular electrostatic potential isosurfaces (**b**) of TeAH, TeA, ITeA, ITeAH, ALT, and AME

the analogues. In a word, the developed FPIA could specifically determine the content of TeA by the analysis of TeAH.

Molecular Modeling Studies of Antibody Recognition

Molecular modeling is a computer-aided method to obtain a deeper insight into antibody-analyte recognition. And from the simulation results, it could reveal to some extent the specificity of the antibody to the analogues and antibody–analyte-binding site (Monasterios et al. 2005; Xu et al. 2009; Zhang et al. 2017). Models of the lowest energy conformations and the molecular electrostatic potential isosurfaces of TeAH, TeA, ITeA, ITeAH, ALT, and AME are shown in Fig. 6. The models of the lowest energy conformations of TeAH and other analogues indicated that the –C=N–N– and $–CHCH_3(CH_2CH_3)$ were the characteristic chemical groups in antibody recognition. Compared with TeAH, TeA failed to be recognized by antibody for lack of –C=N–N–, while ITeA as a chemical isomer of TeA also cannot specifically bind to the antibody. Although ITeAH had a similar $–C=N–NH_2$ group, in the side chain the characteristic functional group was$–(CH_2)_2CH(CH_3)_2$. From the result, it may be speculated that the formation of the bridge –C=N–N– and the $–(CH_2)_2CH(CH_3)_2$ group was critical for antibody recognition.$–(CH_2)_2CH(CH_3)_2$ was greatly different from $–CHCH_3(CH_2CH_3)$ in lowest energy conformations and molecular electrostatic potential isosurfaces (Fig. 6). ALT and AME structures differed greatly from that of TeAH, which resulted in low cross-reactivity. In conclusion, the major structure of the analyte was the important locus of antibody recognition, but a small group on the side chain of the analyte also played an important role in antibody-antigen recognition. The difference of length or side groups of the chains varied in molecular electrostatic potential isosurfaces.

Analysis of Spiked Samples by FPIA Analysis

It was widely reported that TeA has been detected in apples, wheat, and the processed products (Zhao et al. 2015; Fontana et al. 2016; Walravens et al. 2016). Therefore, wheat beer and apple juice were taken as the spiked samples to evaluate the FPIA method. The content of TeA in the spiked samples was determined by FPIA, and the results are shown in Table 2. The derivative rate of TeAH derivatized from TeA with hydrazine hydrate was 64%. The acceptable recoveries using the FPIA method ranged from 76.0 to 112.4%. The average intra-assay CV and

Table 2 Recoveries of TeA from spiked samples by FPIA analysis

Aquatic products	Spiked level (ng/mL)	Found ± SD (ng/g)	Recovery ± CV (%)
Wheat beer	0	–	–
	200	152.0 ± 8.3	76.0 ± 4.2
	600	658.5 ± 6.5	109.8 ± 1.1
	1000	822.4 ± 7.1	82.2 ± 7.1
Apple juice	0	–	–
	200	199.1 ± 9.4	99.6 ± 4.7
	600	521.8 ± 1.3	87.0 ± 0.2
	1000	1124.2 ± 4.1	112.4 ± 4.1

SD standard deviation, *CV* coefficient of variation

inter-assay CV for TeA-spiked samples were 3.6 and 6.8%, respectively. The results indicated that the precision and accuracy of the FPIA method were capable of detecting TeA in drinks.

Conclusions

A rapid and inexpensive FPIA method was developed based on polyclonal antibody for determination of TeA in drinks. In this study, two kinds of tracers TeAH-FITC and TeAH-DTAF with different fluorescent probes were prepared. The established FPIA method was successfully suitable for analysis of TeA in foods with satisfactory specificity and recovery. This FPIA method provides an alternative for quantitative analysis of TeA in drink samples, which is particularly simple, rapid, and convenient. Additionally, this study provided a deeper insight into the antibody–analyte recognition via molecular modeling, which could reveal to some extent the specificity of antibody to the analogues and the antibody–analyte-binding site. Meanwhile, the study creates a theoretical guide for hapten design of small molecules and provides the traditional theory with empirical proof.

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Compliance with Ethical Standards

Conflict of Interest Feng Wang declares that he has no conflict of interest. Jun Cai declares that she has no conflict of interest. Sergei A. Eremin declares that he has no conflict of interest. Zhi-Li Xiao declares that she has no conflict of interest. Yu-Dong Shen declares that he has no conflict of interest. Yuan-Xin Tian declares that she has no conflict of interest. Zhen-Lin Xu declares that he has no conflict of interest. Jin-Yi Yang declares that he has no conflict of interest. Hong-Tao Lei declares that he has no conflict of interest. Yuan-Ming Sun declares that he has no conflict of interest. Hong Wang declares that She has 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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