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Development of a Sensitive Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for the Analysis of Paclobutrazol Residue in Wheat Kernel

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ABSTRACT: An indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed with monoclonal antibody (mAb) mAb6H73C9 recognizing the plant growth regulator paclobutrazol (PBZ). The icELISA had a half-maximum inhibition concentration (IC_{50}) and working range of approximately 8.7 and 2.0−50.4 ng/mL, respectively. Average recoveries of PBZ in the wheat (*Triticum aestivum*) kernel samples were between 84.3 and 118.9% with relative standard deviations between 3.9 and 14.2%. As determined by the icELISA and further confirmed by liquid chromatography−electrospray ionization quadrupole Orbitrap mass spectrometry (LC−ESI-MS) analysis, the maximum residue concentration was about 0.07 mg/kg in the kernel samples, which indicated that PBZ could transfer from PBZ- treated seedlings to the kernel samples. The correlation coefficient (R^2) between icELISA and LC−ESI-MS results was 0.979, which manifested that the developed icELISA was sensitive enough for monitoring PBZ residues in wheat kernels.

KEYWORDS: *paclobutrazol, monoclonal antibody, ELISA, LC−ESI-MS, wheat kernel*

INTRODUCTION

Paclobutrazol, 1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol, is a triazole plant growth regulator inhibiting gibberellin and sterol biosynthesis, and hence it affects rates of cell division. It is applied as foliar spray, tree injection, and tree root drench, and a seed treatment for the reduction of terminal growth and pruning volume in fruit trees or crops.[1,2] PBZ can effectively suppress vegetative growth and improve yields of wheat and fruits.[3−5] PBZ has not been registered for uses on food crops in the US. The maximum residue limit (MRL) for PBZ in cereal grains has been set up to 0.5 mg/kg by China, while in the European Union (EU), the MRL is 0.02 mg/kg, which is 25 times lower. As food quality and safety have been becoming a priority issue in China, China intends to follow the EU or US MRL standards. A sensitive, high throughput and cost-effective analytical method is required to determine PBZ residues in foods.

Gas chromatography (GC) and high performance liquid chromatography (HPLC) are the traditional techniques for the determination of pesticide residues in foods.[6−8] The reported analytical methods for PBZ detection include GC coupled to mass spectrometry (GC−MS),[9] LC−MS[10] and LC coupled to tandem MS (LC−MS/MS).[11] Although the analytical techniques have a low limit of detection (LOD) adequate for PBZ residue analysis, these instrument methods are expensive and need highly qualified personnel.

Enzyme-linked immunosorbent assay (ELISA) is known to be cost-effective and sensitive for the analysis of a large number of samples.[12,13] ELISA has been extensively used in the detection of environmental small molecule contaminants.[14] To our knowledge, mAb-based ELISA has not been reported for the determination of PBZ. The purpose of the present study was to develop a sensitive and selective indirect competitive ELISA (icELISA) for PBZ residue analysis. The LOD pursued was 1 order of magnitude lower than the MRL of EU.

MATERIALS AND METHODS

Reagents. Reagents purchased from Sigma-Aldrich (St. Louis, MO, USA) included PBZ (99.6% purity), bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete Freund's adjuvant, cell freezing medium dimethyl sulfoxide (DMSO, serum-free), polyethylene glycol (PEG) 2000, HAT (hypoxanthine, aminopterin, and thymidine)/HT (hypoxanthine and thymidine) medium supplements, L-glutamine, penicillin, streptomycin, *o*-phenylenediamine (OPD), *N*-hydroxysuccinimide (NHS), and dicyclohexylcarbodiimide (DCC). 4-(Bromomethyl)benzoic acid was purchased from Tianjin Heowns Biochem LLC (Tianjin, China). Cell culture medium (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (PaisLey, Scotland). Goat anti-mouse IgG−horseradish peroxidase (IgG−HRP) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Mouse antibody isotyping kit was obtained from Pierce (Rockford, IL, USA). Polyethlene glycol 2000 was purchased from Fluka (Buchs, Switzerland). Triazole compounds used for cross-reactivity studies were provided by Professor Huizhu Yuan of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (Beijing, China). All other reagents were purchased from Beijing Chemical Reagents Co. (Beijing, China).

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Apparatus. Cell culture plates and 96-well polystyrene microtiter plates were purchased from Costar (Corning, NY, USA). A direct heat CO_2 incubator, an automated plate washer (Wellwash 4 MK2), and a microplate reader were purchased from Thermo (Franklin, MA, USA). An electric heating constant-temperature incubator was purchased from Tianjin Zhonghuan Experiment Electric Stove Co. Ltd. An ultrasonic cleaner (KH-500E, Jiangsu, China) was purchased from Kunshan Hechuang Ultrasonic Apparatus Co. Ltd. Syringe filters (25 mm, 0.2 μm, and 0.45 μm pore size, Acrodisc) and filter unit (Acrocap) were purchased from Pall (Ann Arbor, MI, USA).

Medium. DMEM containing 10−20% (v/v) FBS was supplemented with 0.2 M glutamine, 50000 units/L penicillin, and 50 mg/L streptomycin. The standard medium was used for growing of myeloma and hybridoma cells.

Buffers and Solutions. Buffers and solutions included the following: (1) coating buffer (0.05 M carbonate buffer, pH 9.6); (2) phosphate-buffered saline (PBS) (0.1 M phosphate buffer containing 0.9% NaCl, pH 7.5); (3) PBS with 0.1% (v/v) Tween-20 (PBST); (4) PBST containing 0.5% (w/v) gelatin (PBSTG); (5) PBSTG containing 10% (w/v) methanol; (6) citrate−phosphate buffer (0.01 M citric acid and 0.03 M Na_2HPO_4, pH 5.5); (7) substrate solution (4 μL of 30% H_2O_2 added to 10 mL of citrate−phosphate buffer containing 2 mg/mL OPD); (8) stop solution (2 M H_2SO_4).

Myeloma Cell Line. The HAT-sensitive BALB/c mouse myeloma cell line SP2/0-Ag14 obtained from the Chinese Institute of Veterinary Drug Control (Beijing, China) was used in fusion experiments.

Synthesis of Hapten−Protein Conjugate. The synthetic route of the immunogen and coating antigen is presented in Figure 1. The

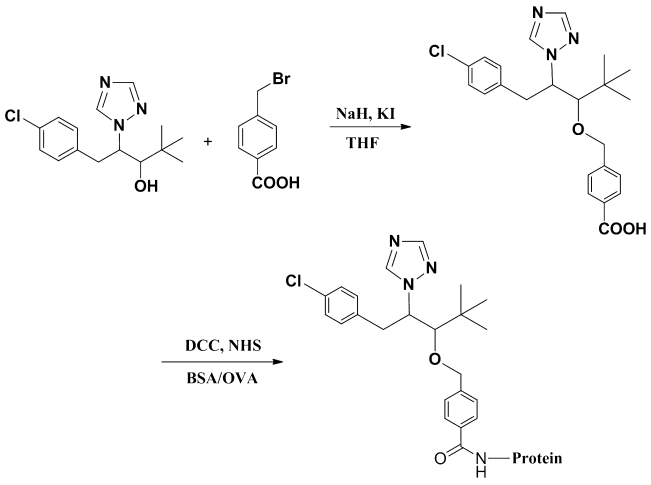


Figure 1. A general synthesis schematic and chemical structure of immunogen and coating antigen.

hapten, 4-(((1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-yl)oxy)methyl)benzoic acid, was synthesized through substitution reaction at the hydrogen group of PBZ. Briefly, 1.0 g (3.4 mmol) of PBZ and 0.73 g (3.4 mmol) of 4-(bromomethyl)-benzoic acid were dissolved in 20 mL of distilled tetrahydrofuran (THF) at 0 °C. The solution was stirred on an ice bath for 30 min after 0.3 g of NaH was added. After stirring at room temperature for an additional 30 min, 0.056 g of KI (0.34 mmol) was added. The solution was then heated to reflux overnight. The reaction solution was cooled to room temperature and quenched slowly by addition of 10 mL of distilled water. The mixture was acidified to pH 5 with 2 M HCl; and most THF was removed by rotary evaporation, followed by extraction of the organic layer three times with 20 mL of ethyl acetate. After the ethyl acetate extract had been dried over anhydrous Na_2SO_4, the solvent was evaporated. The residual product was eluted with a mixture of petroleum ether (boiling range: 60−90 °C)/ethyl acetate (4:1 v/v) from a silica gel column (30 g, 25 cm). The eluent was concentrated to give 0.70 g of hapten, in 48% yield. HRMS (ESI) calculated for $C_{23}H_{27}ClN_3O_3$: 428.17355, found m/z 428.17338 [M^+ + H] by Bruker Apex IV FTMS. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (s, 9, *tert*-butyl), 3.16−3.34 (m, 2, Ar-CH_2CH), 3.44 (d, 1, J = 2.7 Hz, CHC*H*C(CH_3)), 4.69 (s, 2, OCH_2-Ar), 4.84−4.91(m, 1, CH_2C*H*CH), 6.97 (d, 2, J = 8.3 Hz Ar-H_a), 7.19 (d, 2, J = 8.3 Hz Ar-H_b), 7.43 (d, 2, J = 8.2 Hz Ar-H_c), 7.86 (s, 1, Ar-H_e), 8.12 (d, 2, J = 8.2 Hz Ar-H_d), 8.53 (s, 1, Ar-H_f), 10.54 (s, 1, COOH). ^{14}C NMR ($CDCl_3$, 75 MHz): δ 26.5, 36.5, 40.4, 63.7, 76.3, 87.3, 126.8, 128.9, 129.9, 130.0, 130.4, 133.0, 135.0, 142.8, 144.2, 149.2, 169.4.

The hapten (54.5 mg), NHS (10.3 mg), and DCC (14.8 mg) were dissolved in 1 mL of dimethylformamide. The activation reaction was carried out for 1 h at ambient temperature and then 18 h at 4 °C. After centrifugation, the supernatant was divided into two equal aliquots and added dropwise to protein solution (130.0 mg of BSA or 88.0 mg of OVA in 10.0 mL of carbonate buffer, 50 mM, pH 9.6). The reaction mixtures were stirred overnight at 4 °C followed by dialysis against PBS with five changes in 3 days. The dialyzed conjugate solutions were lyophilized, and then the powders were stored at −20 °C. The conjugates of hapten−OVA and hapten−BSA were used as a coating and immunogen antigen, respectively. The molar ratios of hapten-to-BSA and hapten-to-OVA were 10:1 and 9:1, respectively, which were determined through the UV−vis method.[15]

Immunization Protocol, Monoclonal Antibody Production, Purification, and Characterization. Six female BALB/c mice, 8 weeks old, were immunized with the immunogen (hapten−BSA) .The protocols of immunization, fusion, antibody production, and purification were the same as described previously.[16] All the experiments were approved by Beijing Experimental Animal Management Office and performed in compliance with the regulation of Animals Welfare Act of the U.S. Department of Agriculture.

icELISAs. The procedure of icELISA was applied in the screening of the best-performance sera, positive clones, and assay condition. First, a microplate was coated with the coating antigen (100 μL per well in coating buffer), at 37 °C for 3 h. After four washes with PBS, each well was blocked with 200 μL of 3% nonfat dry milk in PBS for 30 min at 37 °C. After the plate was washed with PBST, to each well was pipetted 50 μL of analytes which were diluted in PBSTG containing 10% (w/v) methanol and 50 μL of antisera, supernatant, or mAbs, which were diluted in PBSTG; this was followed by incubation for 30 min at 37 °C. The plate was washed with PBST four times. An aliquot of 100 μL per well of goat anti-mouse IgG−HRP conjugate diluted in PBSTG was added. Likewise, after being incubated at 37 °C for 30 min, the plate was washed four times. To each well was added 100 μL of substrate solution for color development. Finally, the reaction was terminated with 50 μL of 2 M H_2SO_4. The absorbance was read at 492 nm on the microplate reader. The calibration curve data were imported into OriginPro 8.5 (OriginLab) and fit to a sigmoidal logistical equation.

Preparation and Extraction of Samples. PBZ (300.0 mg/L, a recommended use concentration of the commercial product) aqueous solution was sprayed on wheat seedlings which were cultivated in an outdoor experimental field of China Agricultural University. On the harvest day, sixty days after PBZ spraying, eight kernel samples were randomly collected at multiple sites in the experimental field and PBZ-free wheat kernels for recovery study were collected from the unsprayed control field. All kernel samples were stored under an ambient dry condition prior to test.

PBZ was fortified at varying concentrations in PBZ-free kernel samples (10.0 g) for recovery studies. The fortified samples (10.0 g) in triplicate were finely powdered in liquid nitrogen. After crushing, to 10.0 g of finely powered kernel was added 0, 0.4, 1, 2, 5, 10, 20, and 50 μL of 50.0 μg/mL PBZ standard solution in HPLC grade methanol to obtain fortification levels of 0, 2.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0 ng/g, respectively. The spiked samples were allowed to stand at room temperature overnight prior to extraction. The samples were then extracted with 20 mL of methanol under sonication (5 min, room temperature, ultrasonic frequency 40 Hz, ultrasonic power 500 W) followed by centrifugation at 10000g for 5 min. The extraction and centrifugation were repeated three more times. Solids were removed by filtration through filter paper. All supernatants were combined in a 100 mL triangular flask. Five grams of anhydrous Na_2SO_4 was added

to remove H_2O in the supernatants. After filtration through a filter paper the solution was evaporated to near dryness in a 40 °C water bath. The rest of the supernatants were then diluted in 4 mL of HPLC grade methanol and transferred to a 10 mL volume centrifuge tube. After storage at −40 °C for 30 min the supernatants were centrifuged at 10000*g* for 1 min at 4 °C to separate out most of the lipids. The supernatants were transferred to a 5 mL volume centrifuge tube and further dried under a gentle nitrogen stream. The residues reconstituted in 1 mL of PBSTG containing 10% methanol through vigorous vortexing for 2 min. The solution was further diluted 10-fold in PBSTG containing 10% methanol and then was analyzed with the icELISA.

The extraction procedure of kernel samples from PBZ-sprayed seedlings was the same as the recovery study. The residue dried under nitrogen was dissolved in 4 mL of HPLC grade methanol. The solution was divided into two equal aliquots. One aliquot was passed through a 0.22 μm Xiboshi cellulose filter (Envta, Beijing, China) prior to direct injection into the LC−electrospray ionization (ESI)-MS system. The other aliquot was dried with a gentle nitrogen stream and reconstituted in 1 mL of PBSTG containing 10% methanol for icELISA anlaysis.

LC−ESI-MS. PBZ in extracts was also analyzed on a Waters Acquity UPLC H-class bio system (Waters, MA, USA) interfaced to a Thermo Scientific Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher, Franklin, MA, USA) at the State Key Laboratory of Plant Physiology and Biochemistry of China Agricultural University. PBZ in extracts was separated on an Inspire C18 column (100 mm × 2.1 mm i.d., 3 μm) (Dikma, Beijing, China) by gradient elution. The mobile phase was started with 40% acetonitrile in distilled water containing 0.1% formic acid and gradually increased to 95% acetonitrile in 4 min. After 4 min at 95% acetonitrile, acetonitrile percentage was decreased to 40% in 6 s and remained at 40% acetonitrile for 4 min prior to the next sample injection. The flow rate was 300 μL/min. The sample injection volume was 5 μL. The retention time of PBZ was at 3.8 min.

RESULTS AND DISCUSSION

Characteristics of Monoclonal Antibody. Six monoclonal hybridomas which were positive to PBZ were obtained, and mAb6H73C9 was the best in sensitivity and selectivity (data not shown). The titer (the maximum serum dilution that gave an absorbance of 1.0 at the noncompetitive assay conditions) of the ascites was $2-4 \times 10^4$. The dissociation constant (K_d) of the mAb was determined with the method of Zhang et al.[15] The K_d value was 2.8×10^{-10} M. The mAb is IgG1 isotype that has κ light chains.

Optimization of icELISA. Organic solvents, ionic strengths, and pH values are the most frequently studied factors that affect the ELISA performance.[17−19] High concentrations of organic solvent were often used to assist the hydrophobic analytes to dissolve in ELISA sample buffer,[19] while 20% methanol in sample buffer, for example, can cause a decrease in sensitivity due to the nonspecific binding and higher background.[20] We assessed effects of methanol, acetone, and DMSO as described previously.[17] The result demonstrated that over 20% acetone caused measurement errors and severe sensitivity decrease and 10% DMSO led to serious antibody deactivation. Sensitivity was notably decreased as concentrations of methanol in PBSTG increased from 5%, 10%, to 20%, which IC_{50} values increased around 2-fold (data not shown). Similar tests were carried out to examine effects of ionic strengths (0.1−0.4 M Na^+), pH (4.5−9.5), and assay buffer concentrations (0.01−0.4 M PBSTG contain 0.14 M Na^+), respectively, and no significant effects were observed. Consequently, the optimal assay solution used in the study was 0.01 M PBSTG buffer containing 0.14 M sodium chloride (pH 7.4) and 10% methanol. MAb6H73C9 was characterized by icELISA using a two-dimensional checkerboard method. The optimum working dilutions for the coating antigen (1.0 mg/mL), purified mAb (1.0 mg/mL), and IgG−HRP conjugate (1.0 mg/mL) were at a ratio of 1:16,000, 1:32,000, and 1:1,000, respectively. Figure 2 shows

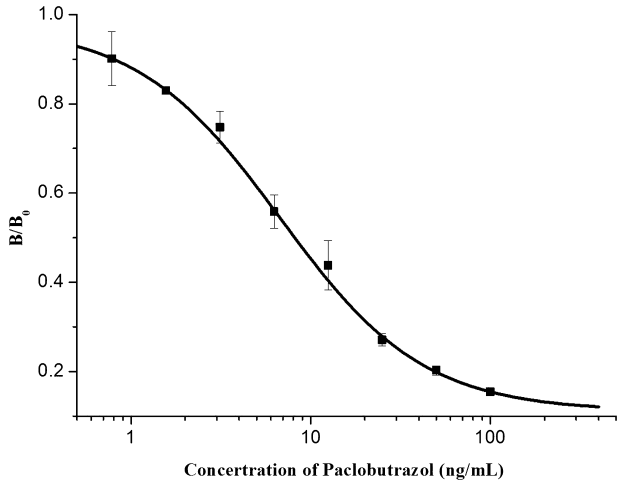


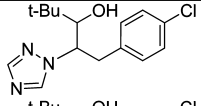
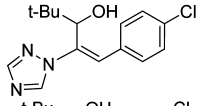
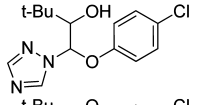
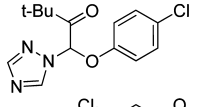
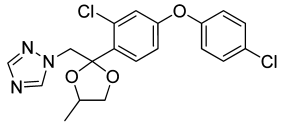
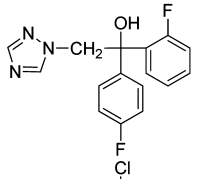
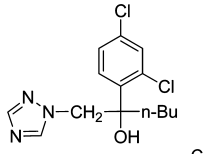
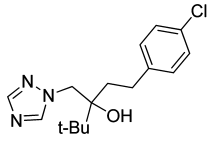
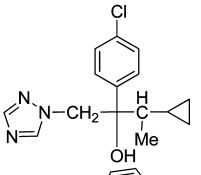
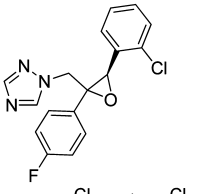
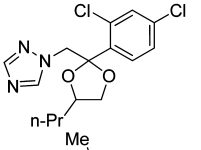
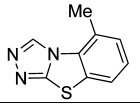
Figure 2. Standard inhibition curve of paclobutrazol by icELISA, obtained under optimized conditions. B_0 and B were absorbance in the absence and presence of competitors, respectively. Concentrations causing 20% and 50% inhibition by paclobutrazol were 2.0 and 8.7 ng/mL, respectively. Each value represents the mean of three replicates.

the standard sigmoidal inhibition curve of PBZ using the optimum reagent dilutions. The calibration curve showed good linearity with a correlation coefficient ≥0.999. The IC_{50} value and quantification detection range (between 80% and 20% B/B_0) were 8.7 ng/mL and 2.0−50.4 ng/mL, respectively. The IC_{50} value was lower than the reported LOD of GC−MS (10 ng/mL).[21]

Cross-Reactivity Studies. The choice and design of the hapten are critical for the production of highly selective antibodies. The strategy to link PBZ with a carrier protein through the hydroxyl group by O-alkylation or O-succinylation as described previously[22] failed, probably due to steric hindrance of the hydroxyl group. Table 1 shows the IC_{50} values and cross-reactivity of 12 triazole compounds. Although most triazoles share the same moieties: a 1,2,4-triazolyl group, a mono- or dichlorinated phenyl group, the cross reactivity of mAb6H73C9 with uniconazole and triadimefon was approximately 0.7% or less. No inhibition was observed up to 4,000 ng/mL of the other 9 analytes. The cross-reactivity result suggested good specificity of mAb6H73C9 to PBZ.

Analyses of Paclobutrazol in Spiked Kernel Samples. Table 2 shows the average recoveries of PBZ fortified in PBZ-free wheat kernels. The matrix interference was almost inevitable for ELISA analysis.[23] The quantitation of hydrophobic compounds such as triazoles requires minimizing matrix interference, which is often a bottleneck in foodstuff analysis by ELISA.[19,22,24] A common method to minimize matrix interference was dilution or appropriate sample pretreatment. In the present study, most lipids were separated out in a small volume of methanol at low temperature, and the −40 °C treatment of concentrated extract solution and centrifugation have most effectively decreased the matrix interference in the optimized icELISA. It is noteworthy that Szekacs et al.[25] studied further cleanup to minimize the interference effects, but

Table 1. Cross-Reactivity of Paclobutrazol and Other Triazole Compounds

Analyte	Structure	IC_{50} (ng/mL)	Cross-reactivity[a] (%)
Paclobutrazol		8.7 ± 0.6[b]	100 ± 6.9
Uniconazole		1,252 ± 14	0.7 ± 0.005
Triadimenol		> 4,000	< 0.2
Triadimefon		NI[c]	0
Difenoconazole		NI	0
Flutriafol		NI	0
Hexaconazole		NI	0
Tebuconazole		NI	0
Cyproconazole		NI	0
Epoxiconazole		NI	0
Propiconazole		NI	0
Tricyclazole		NI	0

[a]Cross-reactivity (%) = (IC_{50} of paclotrabuzol/IC_{50} of other compound) × 100. [b]Values were mean of triplicate determinations ± SD (standard deviation). [c]No inhibition was observed up to 4,000 ng/mL of the analytes.

the average recoveries of the target analytes were not significantly improved. Therefore, the sample extracts were not further cleaned up in the present study. The average recoveries were between 84.3% and 118.9% and the standard deviations were between 3.9 and 14.2% for the seven concentrations. Based on the quantification detection range of icELISA and the recovery study, the LOD of the assay was 2.0 ng/g, which was below the MRLs set by China (500 ng/g) and EU (20 ng/g).

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Table 2. Average Recoveries of Paclobutrazol from Wheat Kernels

spiked concns (ng/g)	mean recoveries (%, n = 3)[a]
0	0 ± 0[b]
2.0	118.9 ± 6.3
5.0	114.3 ± 6.3
10.0	101.3 ± 10.5
25.0	118.2 ± 6.2
50.0	99.2 ± 14.2
100.0	89.2 ± 3.9
250.0	84.3 ± 9.2

[a]Data were the means of triplicate samples. Each extract was diluted 10-fold in PBSTG containing 10% methanol before detection. [b]Not detected.

Analyses of Paclobutrazol Residues in Wheat Kernel Samples. The icELISA was applied to monitor PBZ residues in wheat kernels (Table 3). PBZ residues were detectable in all eight kernel samples of which the seedlings were sprayed with PBZ, indicating that PBZ could be transferred from vegetable organs to kernels. The results by icELISA agreed well with those by LC−ESI-MS. The PBZ concentration in the kernel samples varied from 1.5 to 7.3 ng/g, which may be due to uneven sprays of PBZ solution, different efficiencies of absorption and transportation, and metabolic rate in PBZ treated wheat seedlings.

Table 3. Comparison between icELISA and LC−MS Analyses for Paclobutrazol Residues in Wheat Kernels

	concns of PBZ residues (ng/g)	
samples	icELISA	LC−MS
1	7.4 ± 1.1[a]	7.3 ± 0.7
2	5.7 ± 0.8	5.6 ± 0.4
3	3.2 ± 0.7	3.4 ± 0.5
4	2.8 ± 0.4	3.1 ± 0.3
5	2.6 ± 0.7	2.7 ± 0.6
6	2.4 ± 0.8	2.3 ± 0.3
7	<2.0[b]	1.6 ± 0.1
8	<2.0	1.5 ± 0.2

[a]Data were means of triplicate samples. [b]Data were below the LOD of 2.0 ng/g.

We also analyzed PBZ residues with the developed icELISA in commercially available wheat flour samples purchased from different provinces of China including Beijing, Shandong, Shanxi, Anhui, Hebei, and Inner Mongolia. All residues were below the icELISA LOD level or not detected in these 20 samples (Figure 3). ELISA methods have advantages of high throughput and low costs, making it possible to analyze a large number of samples, and the assay we developed can be applied in routine analysis for screening of residue levels in large numbers of wheat flour samples. Samples that appear to exceed the MRL would then be subjected to a confirmatory analysis by HPLC−MS/MS methods.

According to the US Environmental Protection Agency (EPA) memorandum of Paclobutrazol Human Health Risk Assessment, PBZ is allowed for uses on nonfood crops and fruits, while in China, PBZ is widely used on crops or fruits such as wheat, rice, rape, soybean, peanut, orange, longan, litchi, and mango due to its obvious advantages on seedling height decrease while yield increases. Therefore, there is an urgent need to screen PBZ residues in agricultural produce. Our study proved that the developed icELISA could satisfactorily measure PBZ residues in wheat kernels and the LOD was 10-fold below the EU MRL.

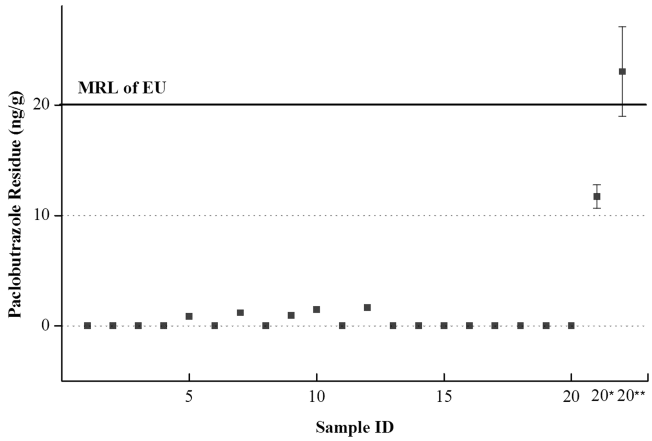

Figure 3. Result of paclobutrazol detection in twenty commercial wheat flour samples by icELISA. Samples 5, 7, 10, 12, and 19 contained paclobutrazol less than 2 ng/g. Residues were not detected in the other samples. Samples 20* and 20** represents sample 20 spiked with 10 ng/g and 20 ng/g paclobutrazol, respectively. Each value represents the mean of three replicates.

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Notes

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ABBREVIATIONS USED

PBZ, paclobutrazol; ELISA, enzyme-linked immunosorbent assay; icELISA, indirect competitive ELISA; mAb, monoclonal antibody; IC_{50}, half-maximum inhibition concentration; LC−ESI-MS, liquid chromatography combined with electrospray ionization quadrupole Orbitrap mass spectrometry; MRL, maximum residue limit; EU, European Union; GC, gas chromatography; LC, liquid chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; LOD, limit of detection; BSA, bovine serum albumin; OVA, ovalbumin; OPD, *o*-phenylenediamine; NHS, *N*-hydroxysuccinimide; DMSO, dimethyl sulfoxide; DCC, dicyclohexylcarbodiimide; IgG−HRP, IgG−horseradish peroxidase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PBST, PBS with 0.1% (v/v) Tween-20; PBSTG, PBST containing 0.5% (w/v) gelatin; THF, tetrahydrofuran

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