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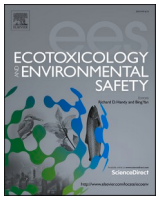

Establishment of a rapid and sensitive *ic*-ELISA for the detection of thiacloprid residues in honey and medicinal herbs using a novel highly specific monoclonal antibody

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ABSTRACT

Thiacloprid is one of the first generation of neonicotinoid insecticide with a chloropyridine structure like imidacloprid and acetamiprid. Recent studies have revealed its environmental and non-target organism toxicity, leading to restrictions on its use in many countries and regions. Despite limitations, thiacloprid has been detected in various environmental samples, food sources, and biological specimens, posing a significant threat to human health, necessitating advanced detection methods for monitoring. In this study, a highly specific monoclonal antibody against thiacloprid *via* a multi-immunogen strategy was prepared and a rapid and sensitive enzyme-linked immunosorbent assay for the detection of thiacloprid residues in honey and medicinal herbs was established. The half maximal inhibitory concentration (IC_{50}) of this method was 0.38 ng/mL, improving the sensitivity by 1.2−480.6 times compared to existing reports, and the limit of detection (IC_{20}) was 0.097 ng/mL. The method was successfully applied to the determination of thiacloprid residues in honey and medicinal herbs (*Crataegi fructus*, *Citri reticulatae pericarpium*), achieving recovery rates ranging from 87.50 % to 116.11 %. The obtained results were verified using the LC-MS/MS method. The multi-immunogen strategy proposed in this study provides an approach for the preparation of highly sensitive and specific monoclonal antibodies, and immunoassay established based on it has good application prospects in complex matrices.

1. Introduction

Neonicotinoid insecticides, once believed to possess the advantages of high efficiency, broad-spectrum, environmental friendliness, and low toxicity to mammals, have gradually replaced highly toxic pesticides since their launch, thereby occupying the majority of the pesticide market (Goulson, 2013). Thiacloprid, belonging to the first generation of neonicotinoid insecticides, has inhalation toxicity, gastric toxicity, and contact killing effects. It mainly acts on chewing and sucking mouthpart pests, and is commonly used to control pests on cotton, potatoes, and pear fruit trees (Deng et al., 2022), with a broad spectrum of insecticidal activity, and in some cases, it is more effective in controlling certain pests than other neonicotinoid insecticides (Su et al., 2020).

However, a large portion of thiacloprid will remain in the environment after application because of its high water-solubility (185 mg/L, 20 ℃) and good stability, causing serious pollution (Ying et al., 2022; Punniyakotti et al., 2022; Sousa et al., 2019), which also leads to food safety issues that cannot be ignored. Recent studies have shown that thiacloprid has toxicity to aquatic organisms (Zhong et al., 2022), non-target organisms (Airui et al., 2023), and even humans (Han et al., 2023). In response to its health risks, the European Union set maximum residue limits (MRLs) for thiacloprid in grains, animal-derived foods, and some plant-based products as early as 2005 ((EC) No 396/2005). Given the hazards of thiacloprid and its metabolites, France, the United

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Kingdom, and the European Union have successively banned the use or registration of thiacloprid ((EU) 2020/23). Although China has not banned the use of thiacloprid, a series of limit regulations have been introduced in recent years, covering food categories such as grains, vegetables, fruits, and animal derived foods, with the MRLs of thiacloprid ranging from 0.02−10 mg/kg (GB 2763–2021). Therefore, it is crucial to establish fast, stable, and efficient methods for detecting thiacloprid residue in food and the environment.

At present, the widely used methods for detecting thiacloprid mainly include high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). These methods have high sensitivity and selectivity, but require sophisticated pre-treatment, complex operations, and high costs. In recent years, immunoassays have experienced rapid development, characterized by their speed, simplicity, and sensitivity, addressing the limitations of traditional technologies and have proved suitable for on-site rapid detection (Han et al., 2022). Currently, some studies have established immunoassays for thiacloprid. Across the reported literatures, the sensitivity (IC_{50}) of these immunoassays for detecting thiacloprid without any added matrix ranges from 0.46 to 182.62 ng/mL (Yin et al., 2015; Li et al., 2014). However, a significant limitation in current research is the scarcity of applications of immunoassays for the detection of thiacloprid in real samples. Moreover, when dealing with complex samples, the matrix effect often necessitates the dilution of samples by several dozen or even hundreds of times to reduce potential interference. This dilution factor poses significant challenges for existing immunoassays in achieving the required detection sensitivity levels. The strictest MRLs of thiacloprid set in GB 2763–2021 (GB 2763–2021) is 0.02 mg/kg, while in the (EU) 2019/50 ((EU) 2019/50) is 0.01 mg/kg, further underscore the need for more sensitive and practical immunoassay methods.

As is well known, the quality of antibodies plays a crucial role in immunoassays, and challenges in producing high-quality antibodies have resulted in issues such as limited research and low analytical sensitivity in thiacloprid immunoassays. Our laboratory has demonstrated in previous study (Zhang et al., 2023) that multi-immunogen strategy has unique advantages in preparing broad-spectrum mAbs with high sensitivity. Based on the study, we further propose whether using molecules with similar structural fragments to prepare antibody *via* a multi-immunogen strategy can improve the sensitivity of single compound specific mAb compared to traditional immunization strategy, retaining its strong specificity at the same time. This strategy may provide a new research approach for the preparation of highly sensitive and specific mAbs.

Therefore, in the present study, a highly sensitive and specific mAb against thiacloprid was prepared using three first generation neonicotinoid insecticides with similar structural fragments based on our previously proposed multi-immunogen strategy (Zhang et al., 2023). A rapid and sensitive *ic*-ELISA for detection of thiacloprid residue in complex matrices was established and optimized using this mAb. Finally, honey and medicinal herbs samples were detected by this *ic*-ELISA method, and the results were verified by LC-MS/MS. This study provides a new strategy for preparing mAbs with high sensitivity and specificity, and improves the sensitivity of current thiacloprid immunoassays, meeting the strict limit standards. The established *ic*-ELISA method will satisfy the increasing detection demands.

2. Materials and methods

2.1. Reagents and instruments

The standard solutions of thiacloprid (THI), imidacloprid (IMI), acetamiprid (ACE), imidaclothiz (IMZ), thiamethoxam (TMX), flonicamid (FLO), dinotefuran (DIN), cycloxaprid (CYC), imidacloprid-olefin (IMI-olefin), guadipyr, piperidin, flupyradifurone, sulfoxaflor, thiacloprid-amid, clothianidin-urea, 4-(trifluoromethyl)nicotinamide were procured from Tianjin Beilian Fine Chemicals Development Co., Ltd. (Tianjin, China), and clothianidin (CLO) and nitenpyram (NIT) were obtained from First standard (Guangzhou, China). PSA and C18 were purchased from Agela Technologies (Delaware, USA), while GCB was from CNW Technologies GmbH (Düsseldorf, Germany). TMB, DMSO, bovine serum albumin (BSA), and Goat Anti-Mouse IgG-HRP were all acquired from Bairuiji (Beijing, China). Ovalbumin (OVA) was sourced from Sigma Aldrich (Missouri, USA), and the skimmed milk powder used was Yili (Inner Mongolia, China).

ic-ELISA results were obtained by Multisken ™ FC enzyme-labeled instrument from Thermo Fisher Scientific (Massachusetts, USA). The ELx50 automatic washer was produced by Bio Tek Instruments Inc. (Vermont, USA). The results were validated by Agilent (California, USA) 1260 Infinity II liquid chromatography system and QTRAP®4500 mass spectrometer produced by AB SCIEX (California, USA), and the chromatographic column was the ZORBAX SB-C18 column from Agilent (California, USA).

2.2. Experimental animals

The Balb/c female mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and raised in the Animal Experiment Center of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. All animals were kept in a pathogen-free environment and fed ad lib during the experiment. Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, followed by euthanasia through cervical dislocation to minimize the pain and distress experienced by the animals. After collecting blood and spleen, the dead mice were sent to the Animal Experiment Center of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. All animal experiments strictly complied with Chinese laws and guidelines, and was approved by the Animal Ethics Committee of the Institute of Medicinal Plants & Chinese Academy of Medical Sciences (SLXD-20220301028).

2.3. Preparation of antigens and antibodies

The antibodies utilized in this experiment were generated following the procedure for developing broad-specificity monoclonal antibodies against neonicotinoid insecticides in our laboratory (Zhang et al., 2023). In short, the chloridion bound to the pyridine groups of IMI, ACE, and THI were replaced by 4-mercaptobutyric acid to obtain haptens. By active ester method, the haptens were conjugated to KLH to obtain the immunogens, and conjugated to BSA to obtain the coating antigens respectively (Fig. 1). Three equal volumes of immunogens were mixed with Freund's complete adjuvant to achieve a final concentration of 1 mg/mL for the first immunization of 5-week-old female Balb/c mice. A total of 100 μL was injected intraperitoneally, and 50 μL was injected subcutaneously at each of the two points on the back. Two weeks after the first immunization, the same immunogens were mixed with Freund's incomplete adjuvant and injected every two weeks for enhanced immunization. One week after the third enhanced immunization, mice with high serum titers were selected to collect orbital blood for cell fusion. Positive hybridoma cells were screened and intraperitoneally injected into mice to induce ascites, then ascites was purified to obtain mAbs.

2.4. Screening of coating antigens and detected pesticides

The antibodies obtained by multi-immunogen strategy have uncertainty and may produce antibodies against multiple pesticide combinations, or even unexpectedly produce antibodies other than the pesticides used (Zhang et al., 2023). Considering that the mAb used in this study was prepared based on compounds which have similar structure, it is necessary to select the optimal coating antigen among structural analogues. Neonicotinoid insecticides (IMI, ACE, THI, IMZ, CLO, TMX) with structures similar to the selected three pesticides were

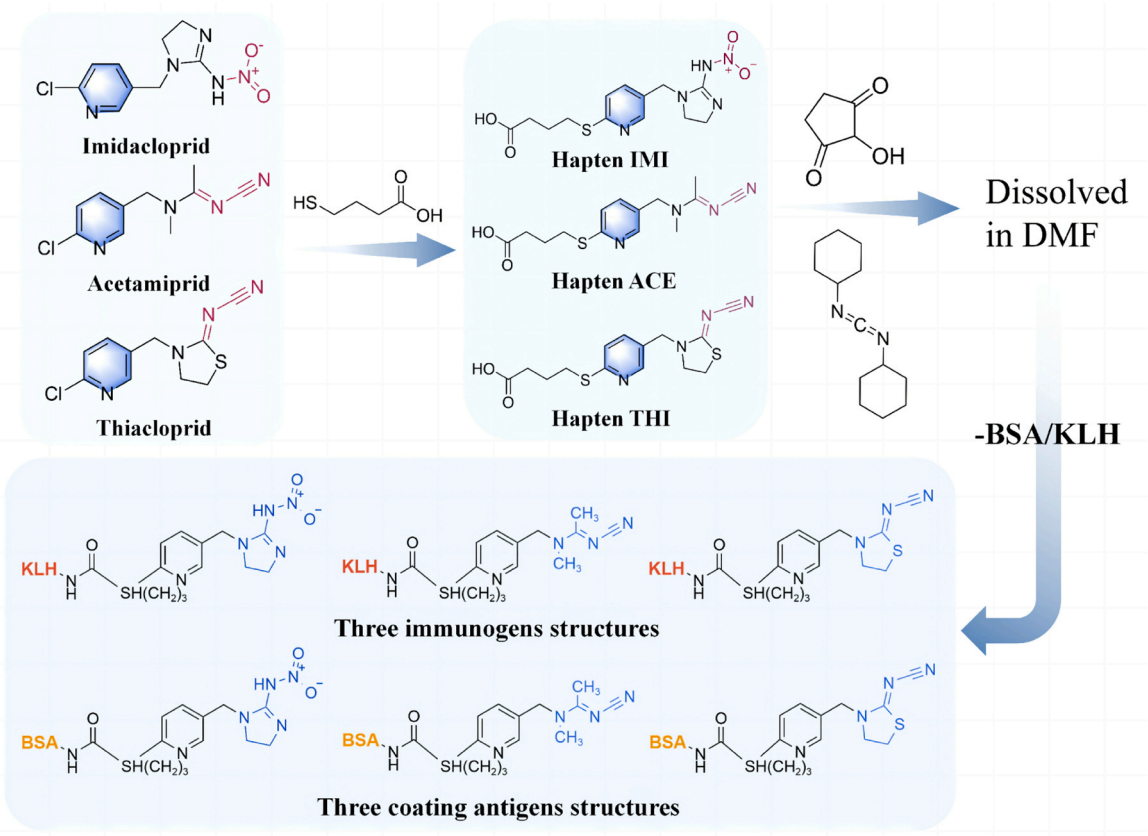


Fig. 1. The immunogens and coating antigens preparation process. The chloridion bound to the pyridine groups of imidacloprid, acetamiprid and thiacloprid were replaced by 4-mercaptobutyric acid, and then the three were conjugated to KLH or BSA to prepare immunogens or coating antigens, respectively. The three immunogens were mixed for immunization simultaneously.

modified into haptens as previously described, and then conjugated to BSA to generate coating antigens, namely IMI-BSA, ACE-BSA, THI-BSA, IMZ-BSA, CLO-BSA, and TMX-BSA. The mixed pesticides composed of IMI, IMZ, CLO, THI, IMI-olefin, ACE, CYC, and NIT were used to study the binding and inhibition of each group. Antigens that demonstrated strong binding to antibodies were chosen for pesticide screening. The selected antigens, at appropriate concentrations, were employed to assess the inhibition of each pesticide separately.

2.5. Establishment and optimization of ic-ELISA

The steps of *ic*-ELISA in this study were improved from previous research in our laboratory (Wang et al., 2022b). The antigen and antibody working concentration, type and concentration of blocking agents, and ionic strength were optimized to enhance sensitivity. The IC_{50} under each condition was compared to identify the optimal working conditions. Moreover, the sensitivity of the *ic*-ELISA method may be affected by the use of organic solvents, which are commonly necessary for pesticide extraction in the detection of pesticide residues in herbal medicines. Thiacloprid is soluble in chloroform and methanol, while chloroform is insoluble in water. If chloroform is used for extraction, the solvent needs to be evaporated to dryness before detection, which will not affect the results, thus eliminating the necessity to investigate the influence of chloroform on this method. The effect of introducing different concentration of methanol on the sensitivity of the method was investigated.

To ensure the accuracy and reliability of the established method, it is necessary to investigate the specificity of the mAb. In this study, 16 neonicotinoid insecticides including 3 metabolites were selected for investigation using the *ic*-ELISA under optimal conditions, and the inhibition curve was plotted and fitted to obtain IC_{50}. The half inhibitory concentration of thiacloprid was recorded as IC_{50}(A), while the half inhibitory concentrations of other compounds were recorded as IC_{50}(B). The formula for cross-reactivity (CR) is: $CR\ (\%) = IC_{50}(A)/IC_{50}(B)$ *100 %.

2.6. Real sample analysis

The detailed origin information of honey, *Crataegi fructus* and *Citri reticulatae pericarpium* is shown in Table S1. Blank samples were verified to be free of thiacloprid by LC-MS/MS before used to study matrix effect. A total of 40 batches of honey, 16 batches of *Crataegi fructus* and 43 batches of *Citri reticulatae pericarpium* were used to verify the feasibility of this method in real samples.

Crataegi fructus and *Citri reticulatae pericarpium*contain components like flavonoids, organic acids, and other substances that may influence antigen-antibody binding (Lyu et al., 2023; Zou et al., 2022). Therefore, it is necessary to develop a simple and effective pre-treatment method to mitigate the matrix effect on the sensitivity of *ic*-ELISA. The sample powder (1 g) was vortexed with 100 % methanol (5 mL), followed by sonication for 10 minutes. Subsequently, 2 mL of the supernatant was taken and mixed with 100 mg each of GCB, C18, and PSA. as well as setting up a control group without purification agent. All groups were vortexed for 2 min then centrifuged at 1644 g for 5 min, and the supernatant was diluted with PBS 10 times to obtain the matrix PBS solution. For honey, 1 g of honey was dissolved in 5 mL PBS. After centrifugation, 2 mL was taken out and the effectiveness of each purifying agent was also examined. The concentration inhibition curves were constructed through the *ic*-ELISA method, and the discrepancies between each curve and the standard curve without matrix were

assessed. The pre-treatment method employed for the curve exhibiting the least variation was considered optimal.

To evaluate the applicability of this method in these matrices, the accuracy and precision were studied. Low, medium, and high level of concentrations of thiacloprid standard solution were added in the blank sample respectively, then test solutions were prepared using selected pre-treatment method. For each addition level, three parallel samples were prepared and each sample was measured three times. Accompanying standard curves were prepared for each measurement simultaneously.

2.7. LC-MS/MS validation

The improved QuEChERS method was used to prepare the test solution, specifically: 1 g of tested sample was thoroughly vortexed in 5 mL of water containing 1 % acetic acid, then extracted by 5 mL of acetonitrile. Simultaneously, 1 g of NaCl and 2 g of $MgSO_4$ were added, and the extraction system was shaken for 15 min to fully mix, followed by centrifuging at 1644 g for 10 min. After centrifugation, 2 mL of supernatant was taken and mixed with 300 mg $MgSO_4$ and 50 mg PSA. The mixture was shaken for 2 minutes and then centrifuged at 1644 g for 5 minutes. The supernatant was then filtered through a 0.22 μm microporous filter membrane before being injected into the LC-MS/MS for analysis.

The chromatographic column was ZORBAX SB-C18 chromatographic column (2.1×150 mm, 3.5 μm), and mobile phase A was acetonitrile (containing 0.1 % formic acid), mobile phase B was water (containing 0.1 % formic acid), with a flow rate of 0.35 mL/min and an injection volume of 2 μL. Gradient elution program was as follows: 0−1 min, 10 % B; 1−2 min, 10 %−80 % B; 2−4 min, 80 % B; 4−5 min, 80 %−10 % B; 5−7 min, 10 % B. The ion source was ESI+, and multiple reaction monitoring (MRM) was employed with declustering potential (DP) of 80 V, entrance potential (EP) of 10 V, and cell exit potential (CXP) of 13 V. Curtain gas (CUR) was 35 psi, with nebulizer gas (GS1) and auxiliary gas (GS2) both at 55 psi, ion spray voltage (IS) at 5500 V, and the ion source temperature at 550 ℃. The monitoring ion mass charge ratio (m/z) was 253.1→126.1 and 253.1→186.1, and the collision energy (CE) was 23 V and 14 V, respectively.

3. Results and discussion

3.1. Preparation and performance of mAb

After cell fusion, a cell line that can stably secrete thiacloprid antibodies was obtained. According to the mouse monoclonal antibody Ig subtype identification kit (Biodragon, Beijing, China), the antibody's subtype produced by this cell line was IgG1 (Fig. 2A), which can be highly expressed in hybridoma cells with high stability and can be purified using efficient and economical methods. The successful screening of cell lines validates the hypothesis we proposed, that the specific mAbs can be prepared by structurally similar compounds *via* multi-immunogen strategy. IMI, ACE, and THI are all belong to the first generation of neonicotinoid insecticides which has a chloropyridine group (Wang et al., 2022a). Additionally, the structure attached to the other

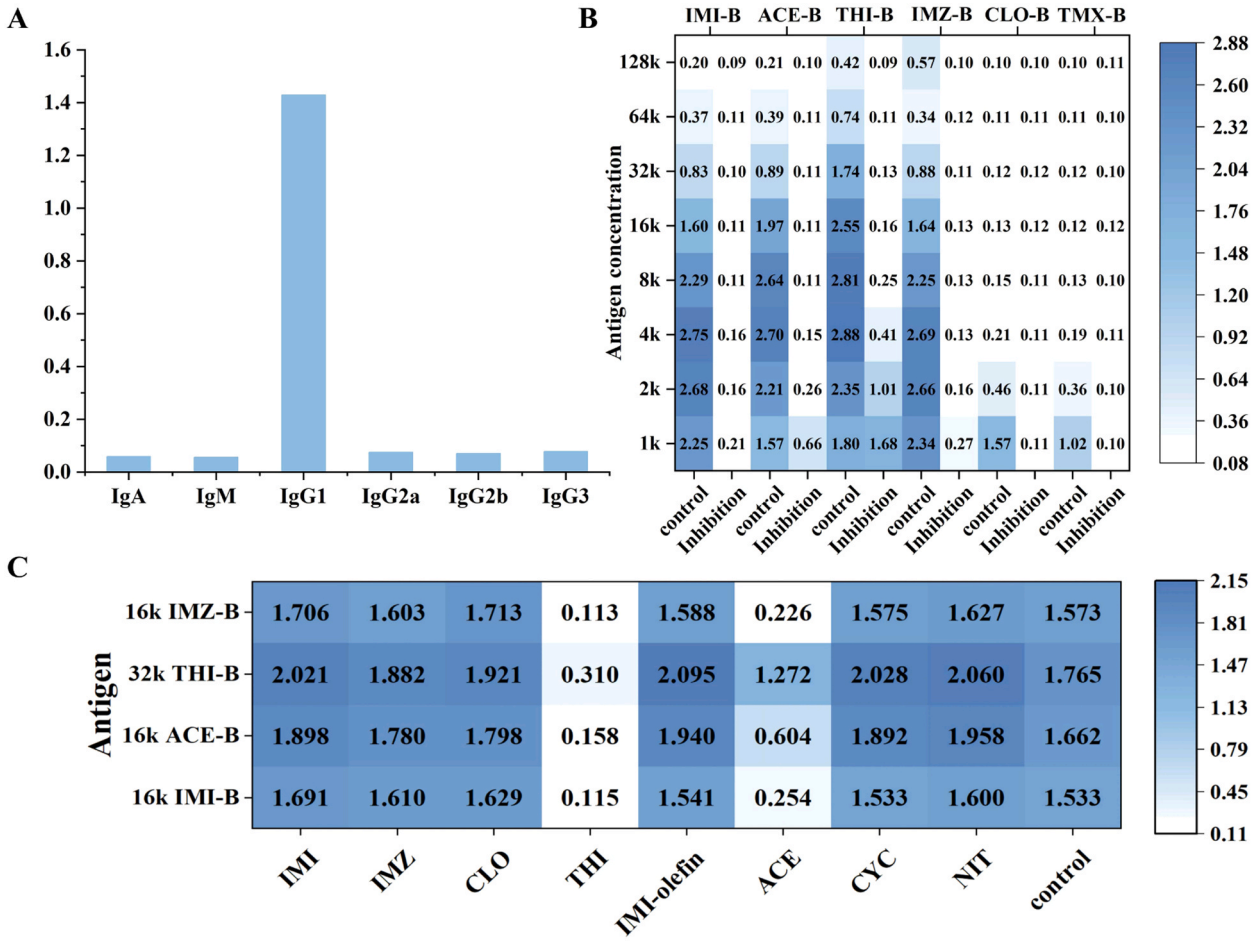


Fig. 2. Subtypes and performance of prepared antibody. (A) Detection of antibody subtypes generated by cell lines. (B) The binding of antibodies to various coating antigens and (C) the specific recognition of antibody with various neonicotinoid pesticides. *IMI-B, ACE-B, THI-B, IMZ-B, CLO-B and TMX-B refer to antigens made by conjugating haptens of imidacloprid, acetamiprid, thiacloprid, imidaclothiz, clothianidin and thiamethoxam to BSA, respectively.

end of the pyridine ring is derived from a guanidine moiety, with a strong electron-withdrawing group attached to its tail end (Fig. S1). These similarities provide a structural basis for the successful preparation of specific mAb *via* a multi-immunogen strategy. When these three antigens are simultaneously employed in immunization, the immune system may generate three distinct antibodies targeting individual compounds. Due to their structural similarity, the three immunogens may have a synergistic effect on the production of antibodies, resulting in the higher valence of mAb compared to single antigen immunization strategy (Andreano et al., 2021; Rodda et al., 2022). This method may also generate broad-specificity antibodies targeting various combinations of compounds simultaneously (Zhang et al., 2023), such as IMI−ACE antibody, IMI−THI antibody, ACE−THI antibody, and IMI−ACE−THI antibody, and even produce some unexpected antibodies.

The binding situation of antibody to various coating antigens is shown in Fig. 2B, indicating that CLO-BSA and TMX-BSA are hardly bind to antibodies. The structures of THI, IMI, ACE, IMZ, CLO and TMX are shown in Fig. S1. Antibodies can bind to IMZ-BSA which is other than immunogens, possibly due to the structural similarity between the long chain end of IMZ and IMI. In contrast, CLO and TMX also possess a nitro group that is identical to the one found in the IMI terminus, but they cannot effectively bind to antibodies, potentially due to their compact tail structure. Both TMX and CLO feature a five-membered N-hetero-aromatic heterocycle and a nitroso substituent in their molecular architectures. Notably, TMX further incorporates a six-membered N-hetero non-aromatic heterocyclic structure, while CLO boasts a methyl substitution alongside its nitroso substituent. As a category of nitrogen heterocyclic compounds with conformational constraints, nitrogen-bridged ring molecules exhibit intricate three-dimensional structures and stereochemical properties (Sovan et al., 2021). Structurally, the nitroso substituent connecting arm of TMX is relatively short, inhibiting the exposure of crucial functional groups. Similarly, the methyl group of CLO enhances steric hindrance at specific sites (Feng et al., 2020), further diminishing the accessibility of these functional groups. Consequently, both TMX and CLO encounter difficulties in binding to antibodies due to steric hindrance. (Marvin and Bruce, 1998).

To ensure the detection of the appropriate window value, the concentration of the antigens should be chosen based on the OD value of the control well that is closest to and greater than 1. The control wells' OD values of IMI-BSA, ACE-BSA, and IMZ-BSA were higher than 1 at 0.0625 μg/mL, while THI-BSA met the condition at 0.0312 μg/mL. In the subsequent study, the concentration of these four antigens were 0.0625, 0.0625, 0.0312 and 0.0625 μg/mL, respectively. When each pesticide was added to competitively inhibit antigen-antibody binding, the results in Fig. 2C demonstrated that THI and ACE exhibit a significant inhibitory effect on antigen-antibody binding. The inhibition rates of each group indicate that the thiacloprid exhibited a superior inhibitory effect. The inhibition rates of each antigen on thiacloprid ranged between 85.1 % and 97.8 %. Additionally, the dilution ratio of THI-BSA was twice that of the other antigens. Therefore, THI-BSA was chosen as the coating antigen, and the tested pesticide was THI.

3.2. Optimization of ic-ELISA

The optimal working concentration of antigen and antibody was determined through checkerboard titration, as illustrated in Table S2. To ensure the accuracy of the measurement, a combination with an OD value close to 1 and a high antibody dilution ratio was selected. When the antibody was diluted to 0.5 μg/mL, the antigen concentrations of 0.1250, 0.0625 and 0.0312 μg/mL satisfied the criteria, with the three combinations' inhibition rates being 85.09 %, 89.30 %, and 90.35 % respectively. By comparison, the combination of antigen concentration of 0.0312 μg/mL and antibody concentration of 0.5 μg/mL was optimal. The concentration of mAb utilized in this study is comparatively lower than reported in the majority of previous studies (Liu et al., 2016; 2013a; 2013b), suggesting a high antibody titer.

The effects of different blocking agents at four concentration levels of 2 %, 1 %, 0.5 %, and 0.1 % on this method was investigated. As shown in Fig. 3A−B, the IC_{50} of all curves decreased after adding the blocking agents. However, after adding OVA the IC_{20}−IC_{80} was 0.08−1.25 ng/mL, while it was only 0.12−0.82 ng/mL after adding skim milk powder, indicating excessively small detection ranges. What is more, OVA and skimmed milk powder were difficult to dissolve in water, and the OD value significantly decreased after the addition of these blocking agents. The average control group OD value after adding OVA was 0.79, and it was only 0.38 after adding skimmed milk powder, potentially impacting the accuracy of the detection. To ensure sufficient sensitivity and detection range, 0.5 % BSA was ultimately selected as the blocking agent. Four gradients of PBS concentration (0.2, 0.1, 0.05, and 0.01 M) were used to investigate the impact of ionic strength on the method by plotting the concentration inhibition curve (Fig. 3C−D). It can be seen that as the phosphate ion concentration increased, the IC_{50} gradually decreased, reaching its lowest point when the PBS concentration was 0.2 M. As shown in Fig. 3E−F, the IC_{50} increased when adding methanol, indicating that as the concentration of methanol increased, the sensitivity of the method gradually decreased. Upon reaching a methanol concentration of 15 %, the IC_{50} of this method nearly doubled compared to the absence of methanol. As depicted in Fig. 3F, the inhibition rate of the mAb against thiacloprid gradually decreased after the addition of methanol (5 %−25 %). Notably, when the methanol concentration attained 20 %, the inhibition rate decreased to 89.26 %, thereby affecting the efficacy of the antibody. To ensure the sensitivity of this method and preserve the inhibitory capability of the antibodies, it is recommended that the methanol concentration be maintained at or below 10 %.

To sum up, the optimal antigen concentration was 0.0312 μg/mL and the antibody concentration was 0.5 μg/mL. BSA was selected as the best blocking agent with the concentration of 0.5 %, and 0.2 M PBS was identified as the ideal choice for maintaining the desired ion concentration. Furthermore, it is recommended to control the methanol concentration within 10 %.

3.3. Specificity and applicability of mAb

The results of the cross reaction are shown in the Table 1. Only ACE and IMZ can bind to the mAb, but the cross-reactivity were only 0.40 % and 0.13 %, indicating strong specificity of the mAb. Among the reported thiacloprid antibodies, except for the antibody prepared by Yin et al. (2015) which has no cross reactions, all other antibodies showed higher cross-reactivity with ACE (0.42 %−35.67 %) than the mAb prepared in this study. In addition, antibodies prepared in previous studies can also react with IMZ, IMI, and other pesticides, indicating that the mAb prepared in this study has the advantage of high specificity.

The established *ic*-ELISA was used to detect thiacloprid residues in honey and medicinal herbs (*Crataegi fructus* and *Citri reticulatae pericarpium*). Fig. 4 shows a comparison between the inhibition curves of four pre-treatments in three matrices and the inhibition curves of matrix free PBS solution. As depicted in the Fig. 4A, the IC_{50} decreased after adding honey, concomitant with a reduction in the detection range, which may be related to the overall decrease in OD value. Despite the fact that PSA treatment resulted in the lowest IC_{50} value, it also coincided with the narrowest detection range. When comparing the trends between treatment methods with and without the inclusion of purification agents, minimal differences in trends were discernible among the various groups. Fig. 4B illustrates that after the addition of *Crataegi fructus*, the four treatment modalities underwent nearly negligible changes compared to the inhibition curve in the absence of the matrix. Remarkably, even the treatment without the addition of a purification agent achieved the lowest IC_{50} value. On the contrary, after the addition of *Citri reticulatae pericarpium*, the IC_{50} of all curves increased significantly. Among them, the curve treated with PSA exhibited the lowest

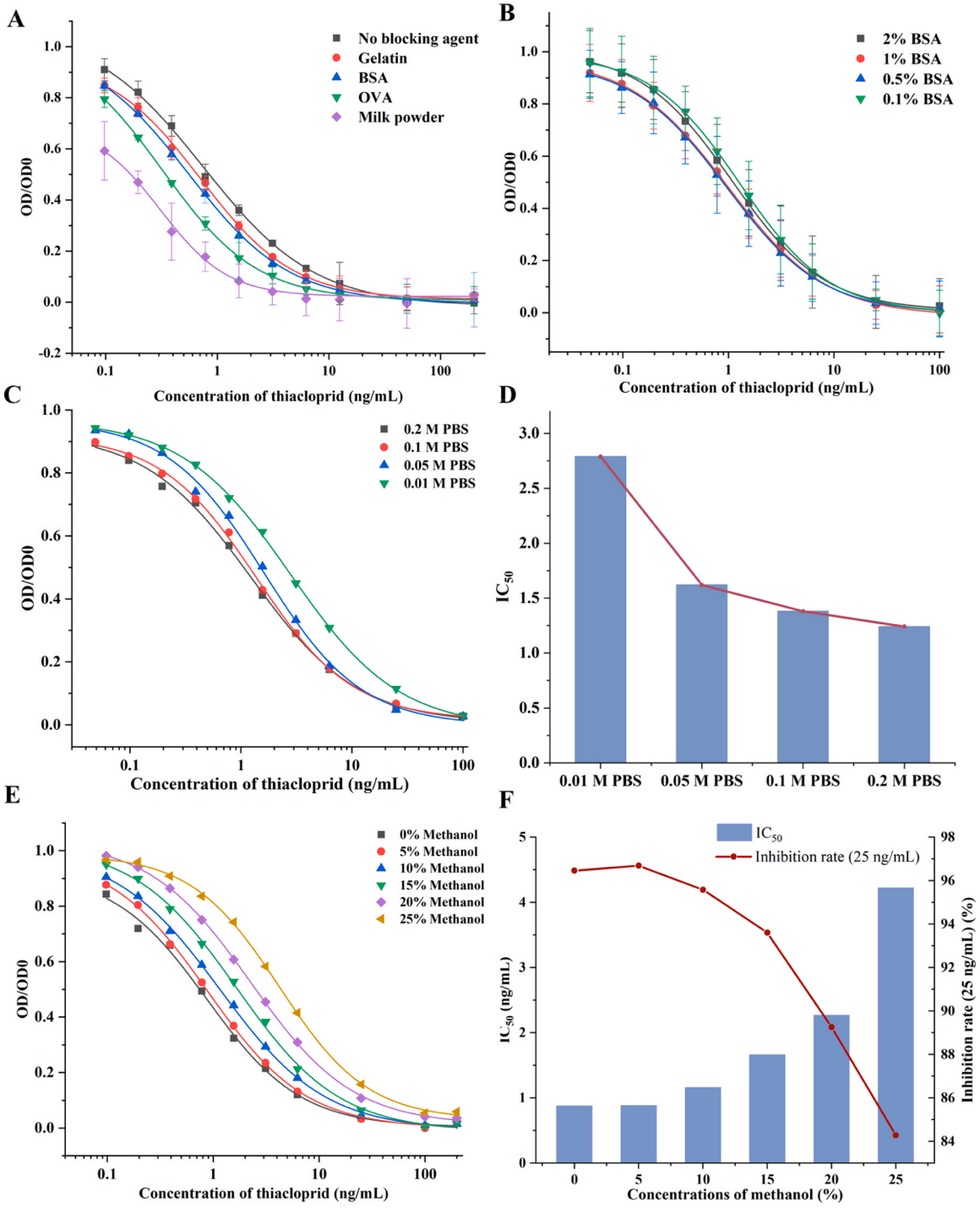
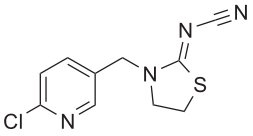
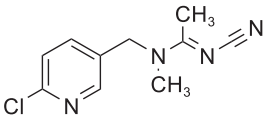
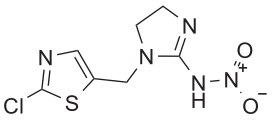
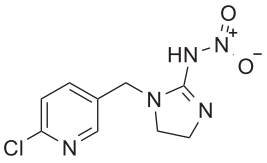
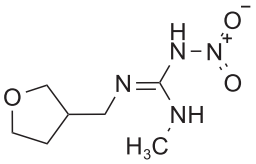
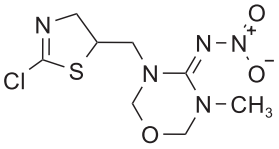
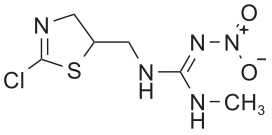
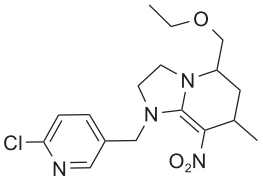
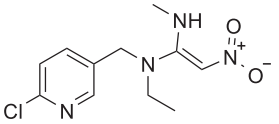
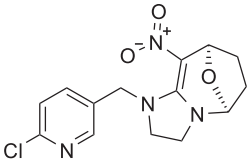


Fig. 3. Results of optimization of *ic*-ELISA. Selection of (A) types of blocking agent and (B) concentrations of BSA; (C) Inhibition curves obtained by adding different concentrations of PBS, and (D) the comparison of their IC_{50}; (E) Inhibition curves obtained by adding different concentrations of methanol and (F) impact of methanol on *ic*-ELISA.

IC_{50} and its trend was most consistent with the pure PBS curve. However, among the four treatment modalities, the inhibition curve without the addition of a purification agent had the widest detection range, while its IC_{50} was only 1.5 times higher than that of the PSA-treated curve. Considering the need for high efficiency, wide detection range, and simplicity in immunoassays, and following the principle of green chemistry, the extraction solution of each sample can be directly used for *ic*-ELISA after dilution without the need for any purification agent. The detection range of this method in honey was 0.003−0.06 mg/kg, in *Crataegi fructus* was 0.02−0.75 mg/kg, and in *Citri reticulatae pericarpium* was 0.12−2.87 mg/kg. The detection conditions are capable of fulfilling the threshold requirements set for thiacloprid in various matrices, including a limit of 0.2 mg/kg in honey as stipulated in the (EU) 2019/50, a limit of 0.5 mg/kg in stone fruits as specified in both the GB 2763–2021 and the (EU) 2019/50, and a limit of 0.5 mg/kg in citrus fruits as outlined in the GB 2763–2021.The recovery rates are shown in Table 2, which ranged from 87.50 % to 116.11 %, and the RSDs < 13.04 %, indicating the stability of the method meets the requirements.

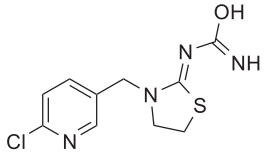
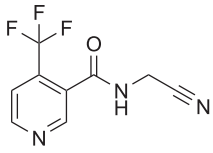
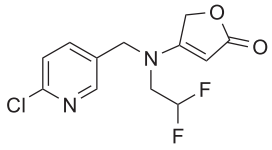
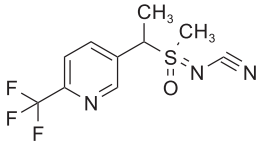
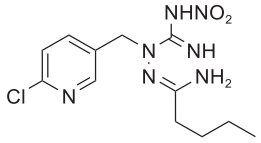
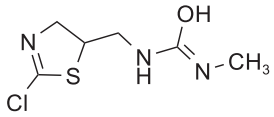
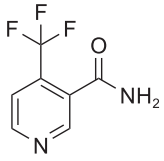
The detection results of *ic*-ELISA were validated by LC-MS/MS in

Table 1
Cross-reactivity of the neonicotinoid insecticides and metabolites with mAb.

Insecticide	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
Thiacloprid		0.38	100
Acetamiprid		95.14	0.40
Imidaclothiz		294.16	0.13
Imidacloprid		>1000	<0.038
Dinotefuran		>1000	<0.038
Thiamethoxam		>1000	<0.038
Clothianidin		>1000	<0.038
Piperidin		>1000	<0.038
Nitenpyram		>1000	<0.038
Cycloxaprid		>1000	<0.038

(*continued on next page*)

Table 1 (*continued*)

Insecticide	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
Thiacloprid-Amid		>1000	<0.038
Flunicamid		>1000	<0.038
Flupyradifurone		>1000	<0.038
Sulfoxaflor		>1000	<0.038
Guadipyr		>1000	<0.038
Clothianidin-Urea		>1000	<0.038
4-(trifluoromethyl)nicotinamide		>1000	<0.038

three matrices. The established LC-MS/MS method has undergone methodological validation. The recovery results are shown in Table 2, with a recovery range of 91.45 %−104.69 % and RSDs < 5.90 %. The linearity, range, detection limit (LOD), and quantification limit results (LOQ) are shown in Table 3. The representative chromatograms are shown in Fig. S2, indicating the reliability of the *ic*-ELISA method established in this study.

3.4. Real sample analysis

A total of 40 batches of honey, 16 batches of *Crataegi fructus* and 43 batches of *Citri reticulatae pericarpium* were tested both *ic*-ELISA and LC-MS/MS. The detailed results are shown in Table S1 and the detection situation are shown in Table 4. Three batches of honey samples were identified as positive for thiacloprid, while a single batch of *Crataegi fructus* tested positive. Conversely, no positive cases were detected in the samples of *Citri reticulatae pericarpium* and no false-positive results were detected, thus validating the reliability and accuracy of the proposed *ic*-ELISA method. It is noteworthy that all the positive samples remained within the MRLs set by the regulatory standards. A positive sample with 0.64 ng/g content was determined by LC-MS/MS in one batch of *Crataegi fructus*, whereas the *ic*-ELISA method failed to identify it because of its relative lower sensitivity in this matrix. This discrepancy is attributed to the fact that the *ic*-ELISA requires a 50-fold dilution of the *Crataegi fructus* samples before detection, leading to a final concentration that is significantly below the detection limit of the assay.

3.5. Comparison with reported immunoassays

The established method was compared with other reported methods (Table 5). The IC_{50} of the method established in this study reached 0.38 ng/mL. In previously reported *ic*-ELISA, the IC_{50} ranged from 0.46 to 26.3 ng/mL (Yin et al., 2015; Liu et al., 2020), while for other detection methods utilizing thiacloprid antibodies, the IC_{50} varied from 1.8 to 182.62 ng/mL (Liu et al., 2016; Li et al., 2014). Compared to the majority of reported *ic*-ELISA for detecting thiacloprid, this study demonstrates higher sensitivity, even surpassing that of other immunoassays. At the same time, there was no significant cross reaction between the mAb prepared in this study and the analogues of thiacloprid. Apart from the absence of cross-reactivity in the method developed by Yin

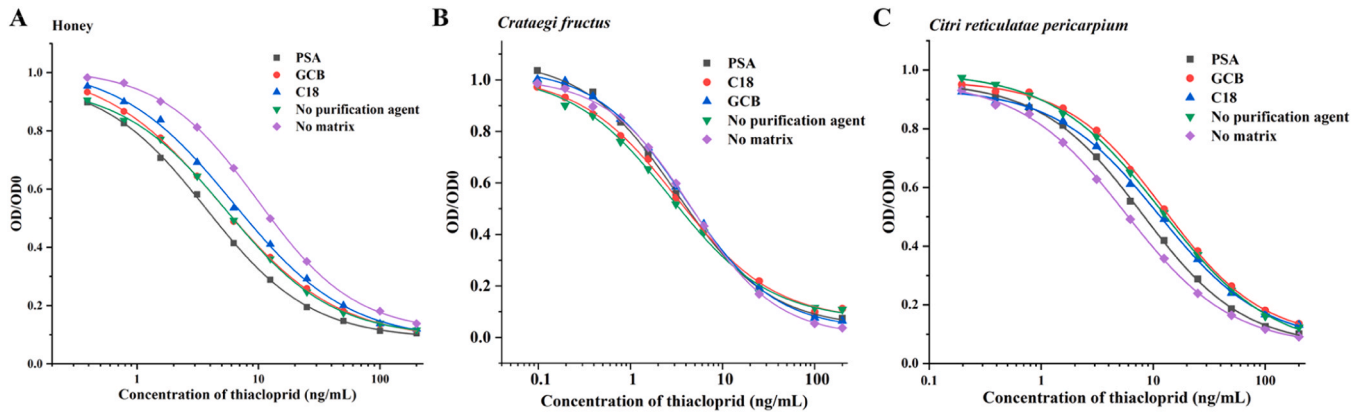


Fig. 4. Inhibition curves of different pre-treatment methods in different matrices. (A) Honey (B) *Crataegi fructus* (C) *Citri reticulatae pericarpium.*

Table 2
The recovery rates of ic-ELISA and LC-MS/MS methods.

Matrix	ic-ELISA				LC-MS/MS			
	Added (mg/kg)	Detected (mg/kg)	Average recovery±SD (% , n=3)	RSD (%)	Added (mg/kg)	Detected (mg/kg)	Average recovery±SD (% , n=3)	RSD (%)
Honey	0.05	0.048	96.20 ± 6.13	6.37	0.025	0.025	98.82 ± 5.83	5.90
	0.125	0.126	100.90 ± 6.80	6.74	0.05	0.052	104.14 ± 2.41	2.31
	0.25	0.26	103.81 ± 8.87	8.55	0.1	0.10	104.69 ± 2.76	2.64
Crataegi fructus	0.05	0.047	93.05 ± 4.75	5.11	0.025	0.025	103.88 ± 3.95	3.80
	0.125	0.125	100.03 ± 9.82	9.81	0.05	0.047	94.13 ± 5.41	5.74
	0.25	0.23	90.50 ± 11.80	13.04	0.1	0.09	91.45 ± 1.08	1.18
Citri reticulatae pericarpium	0.05	0.044	87.50 ± 8.09	9.25	0.025	0.024	95.73 ± 1.07	1.12
	0.125	0.131	105.13 ± 1.79	1.70	0.05	0.049	97.30 ± 4.41	4.53
	0.25	0.29	116.11 ± 6.41	5.52	0.1	0.10	100.03 ± 1.85	1.85

Table 3
Linearity, range, detection limit, and quantification limit results of LC-MS/MS.

Matrix	Regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)
Honey	y=133542x+72596	0.9995	0.06	0.19	0.19–50
Crataegi fructus	y=22831x+6749.8	0.9997	0.06	0.19	0.19–200
Citri reticulatae pericarpium	y=60755x+255189	0.9997	0.12	0.39	0.39–200

Table 4
The detection rates and detection level of thiacloprid in real samples.

Matrix	Detection rates (%)		Detection level (ng/g)	
	ic-ELISA	LC-MS/MS	ic-ELISA	LC-MS/MS
Honey	7.5 (3/40)	7.5 (3/40)	0.17–1.33	0.42–1.01
Crataegi fructus	0 (0/16)	6.25 (1/16)	/	0.64
Citri reticulatae pericarpium	0 (0/43)	0 (0/43)	/	/

/ : not applicable

et al. (2015), the specificity of our method surpasses that of methods established in other studies. Despite the fact that the specificity of our mAb is slightly lower than that prepared by Yin et al. (2015), the sensitivity of our mAb is nearly 70 times higher than theirs, and is more than 20 times higher than the antibody they modified. Therefore, the mAb developed in this study exhibits high sensitivity and specificity, and shows promising application prospects, highlighting the significant achievement in our immune strategies.

4. Conclusions

In conclusion, this study prepared a highly sensitive and specific thiacloprid mAb *via* a multi-immunogen strategy. The antibody type is IgG1, and there is almost no cross-reaction with thiacloprid analogues, demonstrating that this approach can enhance the sensitivity of single compound specific mAb while maintaining their strong specificity. This finding provides a new research concept for the production of mAbs with high sensitivity and specificity. Additionally, a rapid and sensitive *ic*-ELISA for detecting thiacloprid residues in complex matrix was successfully established and optimized using this mAb, with the IC_{50} of 0.38 ng/mL and the IC_{20} of 0.097 ng/mL, illustrating higher sensitivity than reported immunoassays. Moreover, the detection method was successfully applied to the honey, *Crataegi fructus* and *Citri reticulatae pericarpium*. The detection range of this method in honey was 0.003−0.06 mg/kg, in *Crataegi fructus* was 0.02−0.75 mg/kg, and in *Citri reticulatae pericarpium* was 0.12−2.87 mg/kg, meeting the MRLs testing requirements for thiacloprid as specified in GB (2021) and EU (2019/50). Real samples analysis and LC-MS/MS validation indicated that the developed method exhibits excellent specificity and accuracy, making it an ideal tool for rapidly detecting thiacloprid in complex matrices.

Table 5
Comparison of the immunoassay of thiacloprid.

Antibody type	IC_{50} (ng/mL)	Cross-reaction	Method	Reference
mAb	0.38	Acetamiprid (0.40 %), imidaclothiz (0.13 %)	ic-ELISA	This study
mAb	26.3	\	ic-ELISA	(Yin et al., 2015)
	8.3	\	phage ELISA[i]	
mAb	6.45	Acetamiprid (1.1 %), imidaclothiz (0.56 %)	FPIA[ii]	(Tao et al., 2019)
pAb	10	Acetamiprid (0.72 %), imidacloprid (0.23 %), dinotefuran (0.02 %), nitenpyram (0.12 %)	ic-ELISA	(Liu et al., 2013a)
	20	Acetamiprid (0.58 %), imidaclothiz (0.26 %), imidacloprid (0.26 %)	TRFIA[iii]	(Liu et al., 2013b)
	1.8	Acetamiprid (0.42 %)	ECL-EIA[iv]	(Liu et al., 2016)
mAb	15.34	Acetamiprid (2.5 %), imidacloprid (0.1 %), imidaclothiz (0.3 %), clothianidin (0.4 %)	FPIA	(Yuan et al., 2019)
rAb[vii]	4.268	Acetamiprid (19.72 %)	TRFIA-ICTS[v]	(Xu et al., 2023)
mAb	0.46	Acetamiprid (35.67 %), thiacloprid amide (2 %)	ic-ELISA	(Liu et al., 2020)
rAb	0.73	Acetamiprid (33.32 %), thiacloprid amide (4 %)		
pAb	182.62	Acetamiprid (5.4 %), imidacloprid (0.98 %)	dc-ELISA[vi]	(Li et al., 2014)

i. Antibody modified by a phage-displayed peptide was used to develop enzyme-linked immunosorbent assay (phage ELISA)
ii. Fluorescence polarization immunoassay (FPIA)
iii. Time-resolved fluoroimmunoassay (TRFIA)
iv. Enhanced chemiluminescent enzyme immunoassay (ECL-EIA)
v. Time-resolved fluorescent microsphere-immunochromatographic test strip (TRFIA-ICTS)
vi. Direct competitive enzyme-linked immunosorbent assay (dc-ELISA)
vii. Recombinant antibodies (rAb)

CRediT authorship contribution statement

Jing Zhang: Methodology, Investigation, Data curation. **Wanxuan Zhu:** Writing – original draft, Investigation, Conceptualization. **Huiru Zhang:** Methodology, Investigation. **Yuanyuan Zhang:** Methodology, Data curation. **Jiaoyang Luo:** Writing – review & editing, Supervision, Conceptualization. **Kun Miao:** Methodology. **Meihua Yang:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116911.

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