# Original Article

# Development of polyclonal antibody based enzyme-linked immunosorbent assay for the analysis of the agricultural insecticide imidacloprid: food quality and safety

Zhixiong Li MSc[1], Yingli Liu DSc[1], Yuanming Sun PhD[1,2], Qing Wu DSc[1,2], Hongtao Lei PhD [1,2], Hong Wang PhD [1,2] and Zhili Xiao PhD [1,2]

[1]*College of Food Science, South China Agricultural University, Guangzhou, Guangdong, China*
[2]*Key Laboratory of Food Quality and Safety of the Education Department of Guangdong, China*

In order to develop an indirect competitive enzyme-linked immunosorbent assay (ELISA) for the insecticide imidacloprid, the anti-imidacloprid polyclonal antibody was produced and the affinity of three different coating antigens with the polyclonal antibody was compared. The antigenic determinant in antigen aimming at the polyclonal antibody was raised and analyzed. The standard curve for imidacloprid had been developed. The effects of organic solvents and buffer ionic strength on the ELISA for insecticide were studied and conditions of analysis were also optimized in this research. Experiment result showed that the coating antigen IMMP-OVA ,whose binding ratio was 14:1, had stronger affinity with the anti-imidacloprid polyclonal antibody than IMMB-OVA and IMEB-OVA ; the $IC_{50}$ value in the standard curve was 995.4ng·mL$^{-1}$ and the limit of detection (LOD) was 30ng·mL$^{-1}$; the cross reaction ratios with nitenpyram and acetamiprid were 5.73% and 11.31% respectively.

**Key Words: insecticide, imidacloprid, ELISA, polyclonal antibody, residue detection**

## Introduction
Imidacloprid ((E)-1-((6-chloropyridin-3-yl)methyl)-N-nitroimidazolidin-2-imine) is a new kind of nicotinamide insecticide,which has been widely used as soil, seed and foliar plants application for control of sucking and chewing insects including rice hopper, aphid, thrips, whiteflies, and so on. The yield of imidacloprid is about 30~31 million tons per year and about 6 million tons is used to export in addition in China.[1] However, it has been reported that imidacloprid is high toxic for silkworm, bee and shrimp recently.[2] Thus the safety of imidacloprid application has been more and more concerned. In the recent "Positive List" System from Japan, imidacloprid residue is limited at 0.02ppm in agricultural products.[3] much lower than the standard of China, which has made Chinese grangers suffer economic losing badly and affected the export trade with Japan. Therefore, in the research report to the "Positive List" System of Japan, the General Administration of Quanlity Superision, Inspection and Quanrantine of the People's Republic of China has placed imidacloprid on the list of "Chemicals have great influence on Chinese exported agricultural products" and appealed to improve our detecting technology.[4]

However, at present, the mainly detecting methods for imidacloprid residues were large-scale instruments, including gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) at home and abroad. These instrumental methods are accurate but expensive and time-consuming, requiring complex sample extraction and procedures. In contrast, enzyme-linked immunosorbent assay (ELISA) is one of the most extensive-applied immunoassay methods in rapid detection field because it is more sensitive, simpler, faster, and needs lower cost. ELISA has been widely application for detecting transfers gene foods and veterinary drug residue in animal tissue. But it is still infrequently used in measurement of insecticide residues. So, developing a rapid and accurate measurement of imidacloprid is very important in modern food science.

## Materials and methods
### Drugs and reagents
Imidacloprid (>96% purity) was obtained from Chemspec Agro-Chemical Corp (Jiangsu, China). Standard clothanidin, nitenpyram, nicotine and acetamipridwere obtained from Jingpeng Bio-Pesticide Co.,Ltd.(Shandong, China). Mercaptopropionic acid and ethyl-4-bromobutyrate were purchased from Alfa Corporation. Methyl-3-mercaptopropionate was purchased from Fluka Corporation. Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma Corporation.

Three haptens IMMP, IMMB and IMEB were synthesized by combining imidacloprid with mercaptopropionic acid, methyl-3-mercaptopropionate and ethyl-4-bromobutyrate,[5,6] respectively (Fig 1).

**Corresponding Author:** Professor Yuanming Sun, College of Food Science, South China Agricultural University, Guangzhou, Guangdong, China 510642
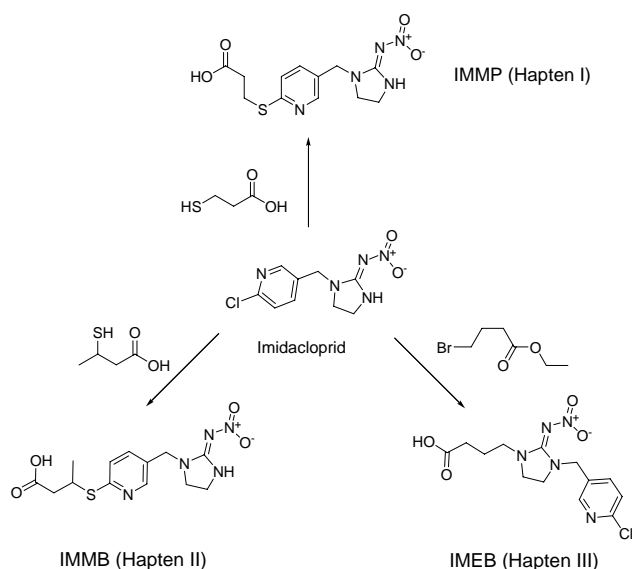Tel: 86 020 8528 3448; Fax: 86 020 8528 0272
Email: ymsun@scau.edu.cn

**Figure 1.** Synthesis of three haptens

Then, one immunogen IMMP-BSA and three coating antigens IMMP-OVA, IMMB-OVA, IMEB-OVA were obtained by coupling hapten IMMP, IMMB or IMEB with bovine serum albumin (BSA) or ovalbumin (OVA) using the active ester method. The productions were identified by UV and conjugation molar ratios of hapten to BSA or OVA were calculated, [7] which were 17:1, 14:1, 12:1 and 21:1, respectively.

### Preparation of polyclonal antibody for imidacloprid
Female Balb/c mice of 6~8 weeks were immunized with 100μg immunogen (IMMP-BSA) conjugate emulsified with an equal volume of Freund's complete adjuvant by intraperitoneal injections. The injections were carried out every 2 weeksand 10 days after each injection, blood sample was taken from the tail vein. The blood was centrifuged for 10 min (6 000 r/min) and the anti-imidacloprid polyclonal antibody serum was collected and stored at -20℃ for further analysis.

### Indirect competitive ELISA
Standard imidacloprid and its structurally related compounds were diluted to $10^5$, $10^4$, $10^3$, $10^2$, 10 and 1ng·mL$^{-1}$ with PBS before the ELISA assay. Relative absorbance was calculated using the formula B/B$_0$,[6] where B$_0$ is the absorbance of the well in which the antibody was not challenged with imidacloprid. The standard curves were constructed by plotting B/B$_0$ values against the concentration of imidacloprid. The inhibition percentage was calculated as inhibition rate = (1-B/B$_0$) ×100.

### Precision and evaluation of standard curves
The precision was expressed by the within-run precision and the between-run precision following the method of Dong Guowei.[8] And then, each calibration point of standard curves and each sample value was determined by calculation of the mean of the data (n = 3). Standard curves were obtained as follow by fitting calculated means to logistic model, a four parameter function:
$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2$$
Where $x$ is the concentration of toxins (ng·L$^{-1}$), $A$ is the absorbance at 450 nm ($A_{450nm}$),

$A_1$ is the maximum absorbance (upper asymptote, $x\rightarrow 0$), $A_2$ is the minimum absorbance (lower asymptote, $x\rightarrow \infty$), $x_0$ is the midpoint or inflexion (IC$_{50}$ value) (ng·L$^{-1}$), $p$ is relative to the slope of tangent at inflexion.

### Cross-reactivity (CR)
The ability of the obtained polyclonal antibody to recognize several structurally related compounds were tested by performing competitive assays and determining their respective IC$_{50}$ values (50% inhibition of control). Cross-reactivity was calculated as (IC$_{50}$ of imidacloprid /IC$_{50}$ of related compound) ×100%.[9] The IC$_{50}$ value was determined after fitting a 4-parameter sigmoid curve to data points.

### Potential ELISA interferences
Methanol, acetonitrile and acetone, were tested for their effects on the ELISA. The effects of organic solvents were tested by dissolving the analyte in PBS containing various proportions of solvent (0~2.0mol·L$^{-1}$ final concentration) and incubating these with polyclonal antibody in PBST on the coating plate.

## Results
### The optimum ELISA conditions
The polyclonal antibody was used to screen against three different coating antigens by using indirect competitive ELISA. The concentration of all coating antigens were 2.0μg·mL$^{-1}$ and the working dilution of polyclonal antibody was 1:6400, then the curve's axis X was made the logarithm transformation during the data processing (Fig 2). According to the result, the curve's slope of the coating antigen IMMP-OVA was the best and the IC$_{50}$ value was the lowest. Therefore, IMMP-OVA was chosen to be the fitting antigen for the following detection.

### Precision and evaluation of standard curves
The intraassay coefficient of variation (CV) was 3.25%. After duplicating measurement of these samples in 3 different runs and taking the average, the between-run precision was 11.45%. So, the within-run and between-run CVs were 3.25% and 11.45%.

The assay affinity for imidacloprid represented by the IC$_{50}$ value was 995.4ng·mL$^{-1}$ (Fig 3).The detection limit of the method was corresponded to the concentration of toxins yielding 20% inhibition 30ng·mL$^{-1}$. The
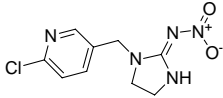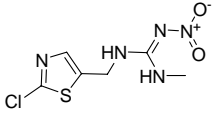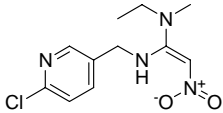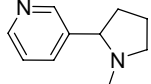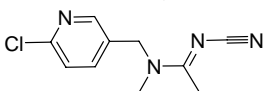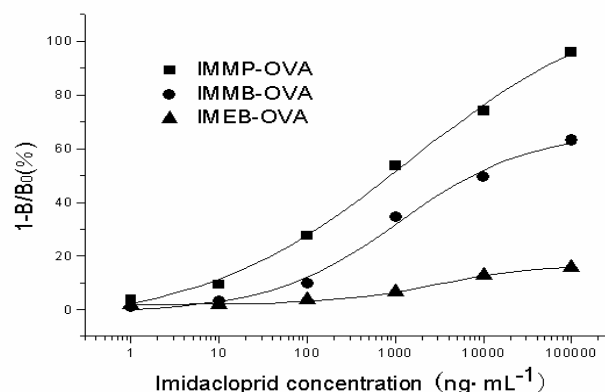


**Figure 2.** Inhibition rate of three coating antigen for the imidacloprid ELISA

**Table1.** Cross-reactivity (CR) of imidacloprid and related compounds with polyclonal antibody

| Compound | Structure | $IC_{50}(ng\cdot mL^{-1})$ | CR(%) |
|---|---|---|---|
| Imidacloprid | | 1045.8 | 100 |
| Clothianidin | | $>10^6$ | <0.1 |
| Nitenpyram | | 18251.3 | 5.73 |
| Nicotine | | $>10^6$ | <0.1 |
| Acetamiprid | | 9246.68 | 11.31 |

When the cross-reactivity is lower than 0.1%, it can be considered that there is no cross-reactivity with the related compound.
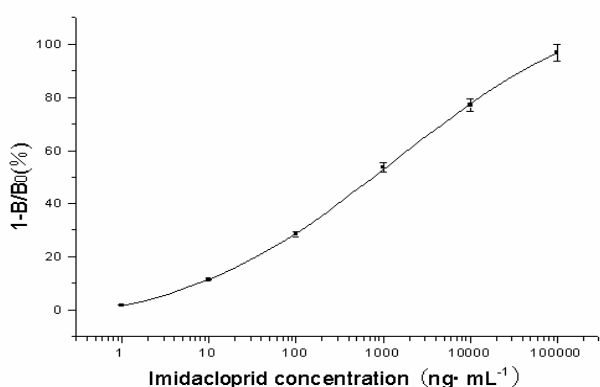


**Figure 3.** The standard curve of imidacloprid ELISA
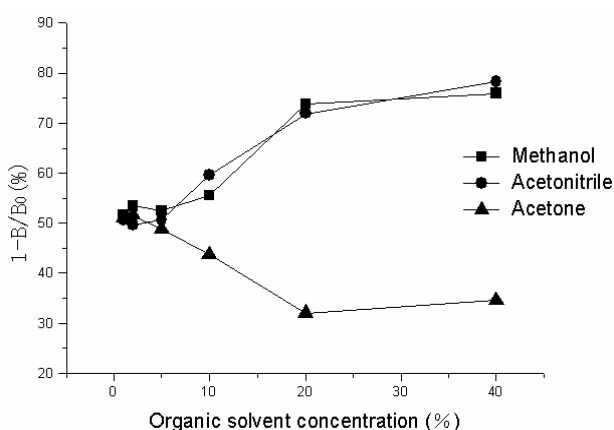


**Figure 4.** Influence of organic solvent to imidacloprid ELISA

quantitative detection range defined as the concentration of toxins yielding from 80% to 20% inhibitions, and which should be a linear range.

### Cross-reactivity (CR)
CR values for each compound are given in Table 1. The polyclonal antibody showed 5.73% and 11.31% CR with nitenpyram and acetamiprid, which had the same chloro-phridine functional group as imidacloprid, indicating that the chloro-phridine caused relatively certain binding with the polyclonal antibody. By contrast, clothianidin and nicotine compounds showed very lower cross-reactivity. These results showed that ELISA was sensitive enough for practical detection of imidacloprid.

### Potential ELISA interferences
Amounts of the organic solvents that are less than 5% in the assay buffer had little effect on the sensitivity of the ELISA (Fig 4). The assay sensitivity was higher in the buffer containing higher concentration methanol and acetonitrile. For acetone, it was reversed. That is because organic solvents with high concentration would decrease the affinity between antibody and antigen and destroy the Van der Waals force and the hydrophobic interaction in the antibody-antigen conjugation, or even make the complex substance break down.

In the range tested, the ionic strength value affected the assay (Fig 5). The sensitivity of the assay was a little higher under slightly NaCl concentration ($<0.5mol\cdot L^{-1}$), as shown by the $IC_{50}$ values. But, as the concentration escalated, the inhibition rate brought down, indicating that the high concentration of ionic strength could cause some hindrance of antibody-antigen binding.

### Discussion
In this research, the polyclonal antibody was used to screen against three different coating antigens by using indirect competitive ELISA and the result shown that the coating antigen IMMP-OVA was the best. This could be explained that IMMP-OVA had stronger affinity with the imidacloprid polyclonal antibody than the others, so it could compete with the imidacloprid for the polyclonal antibody in the ELISA system and obtain a better sensitivity. It was probably because the antibody aiming at imidacloprid was in accord with the antigenic determinant N-(1-methylimidazolidin-2-ylidene) nitramide group
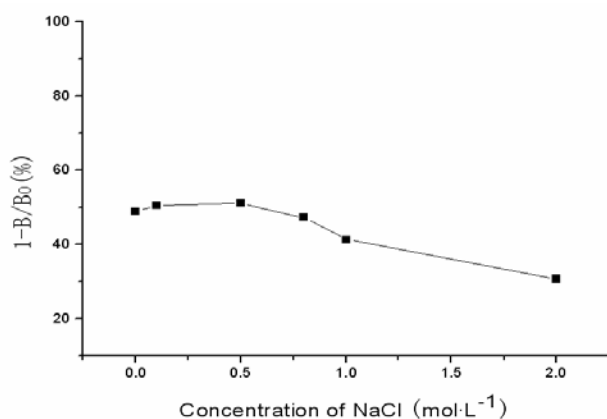
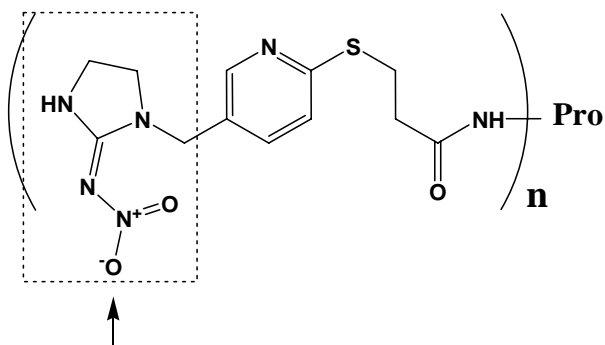**Figure 5.** Influence of ionic strength to imidacloprid ELISA



**Figure 6.** The antigenic determinant of antigen IMMP-OVA

(Fig.6). When the -COOH of hapten conjugated with the carrier protein (OVA), in contrast with coating antigen IMEB-OVA, the antigenic determinant of IMMP-OVA exposed more completely and combined the antibody easier.

The $IC_{50}$ value in the standard curve was 995.4ng·mL$^{-1}$ and the limit of detection (LOD) was 30ng·mL$^{-1}$, the cross reaction ratios with nitenpyram and acetamiprid were 5.73% and 11.31%, but there was no cross-reactivity with clothianidin and nicotine. All these data shown that the anti-imidacloprid polyclonal antibody with high specificity was produced. The repeatability of this research was good (the within-run and between-run CVs were 3.25% and 11.45%) and it is useful for the preparation of monoclonal antibody for imidacloprid and ELISA kits.

Various matrixes may interfere with the antigen–antibody interaction, causing differences between expected and observed ELISA results, and poor correlation with instrumental analysis. Accordingly, we studied concentration of organic solvents, and the buffer ionic strength as possible sources of interference in ELISA with polyclonal antibody. Indirect competitive ELISA for imidacloprid was affected obviously by organic solvents and the buffer ionic strength. Lower concentration of organic solvents (<5%) and the ionic strength (<0.5mol·L$^{-1}$) were little affected the assay, even advantageous to increasing the detecting sensitivity. Research on these factors can be helpful to optimize the condition of analysis. It is important in terms of the practice.

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