Research on a rapid detection method of pesticide residues in milk by enzyme inhibition

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Abstract. A rapid detection method of pesticide residues in milk by enzyme inhibition was researched. Construction of a quickly reaction system of enzyme inhibition for determination of organophosphorus and carbamate pesticide residues was achieved. The analysis of color reactions of milk showed a good correlation between color intensity and content of tolclofos-methyl, methamidophos and isoprocarb 1-naphthalenyl methyl carbamate. The detection limits of the four pesticide were 0.5~1.0 mg/kg, which were below the level of detection required to satisfy legislation in china. This method is simple and inexpensive and suitable for rapid detection of pesticide residues in milk.

1 Introduction

The detection card and determination meter photoelectricity system are two main methods for rapid detection of pesticide residues at present in China. The detection card is the most popular determination method. It is based on color reactions by inhibition of organophosphorus and carbamate pesticides against cholinesterase enzyme, and there is a positive correlation between pesticide concentration and inhibition rate [1-2]. The rapid detection card technology is to show the color change on the card to determine pesticide residues of sample through reaction of cholinesterase and the substrate.The sensitivity of this method for detecting pesticide residues has a certain relationship with the enzymes, as well as color and reaction time and temperature. Among them, the selected enzymes is the single most important factors in affecting the sensitivity and reliability of detection[3].The cholinesterase was selected from duck serum in this experiment , which was not only inexpensive but also widely available. Several reports[4,5] indicate that the cholinesterase from duck serum is stable and suitable to built for enzyme tablet.

Previous studies[6,7] in experiment have suggested that Indoxyl acetate can be rapidly hydrolyzed by cholinesterase to produce blue hydrazine. Using this reaction, we used milk as the test object, and immobilized cholinesterase on the membrane carrier to make of enzyme tablet. If the milk contains organophosphorus or carbamate pesticides, it will combine with cholinesterase to lose the ability to hydrolyze Indoxyl acetate, ultimately inhibiting the formation of blue products. It is a new way to detect organophosphorus and carbamate pesticide residues in milk,just judging by visual inspection alone.

2 Experimental method

2.1 Extraction of cholinesterase[4,8]

100ml fresh blood of duck was separated by centrifugation at 5000rpm for 15min, the supernatant was added an equal volume of PB buffer. After mixing, the corresponding amount of ammonium sulfate was addde into the supernatant to djust the saturation of 0.25, and was maintained on refrigerator at 4 ℃ for 24 h, then centrifuged 5000 r/min for 10 min to leave the supernatant. Taken the same type of steps, ammonium sulfate was added to adjust to a saturation of 0.6, was maintained on refrigerator at 4 ℃, then filterd to leave a precipitate, and dissolved the precipitate with PB buffer, which was the crude enzyme solution.

Since the crude enzyme had a large amount of impurities, it was necessary to carry on a purification of crude enzyme, and the secondary extraction was required. The corresponding amount of $(NH_4)_2SO_4$ was added to the crude enzyme in succession to achieve a saturation of 0.4 and 0.45. The crude enzyme was placed for a nigh, then was centrifuged and leaved the filtrate, the enzyme of two salting-out was obtained.

2.2 Determination of enzyme activity

According to the Ellman method[9] , 3mL of PB buffer, 50μL of DTNB（10mg/mL ）and 50μL of enzyme were taken into a centrifuge tube in turn, placed it 10min at 37℃ for reactiong. Before measurement, 50 μL BTChI (6.5 mg/ml) was added to form a 3.15 mL reaction system. After mixing, the OD value was measured at 412nm, and the 10s readings were read at intervals, recording 3-4min.

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With reaction time as abscissa, reaction OD value as ordinate, we drew a straight line. 10^5 times the slope of straight line is the value of enzyme activity.

2.3 Preparation and optimization of enzyme tablets

2.3.1 Selection of enzyme carrier

In this experiment, three membrane materials were selected as the carrier of the enzyme: cellulose acetate membrane, positively charged nylon membrane, and polyamide-6-film. The three membrane carriers were cut into small pieces of 1cm×1cm and the cellulose acetate membrane and the positively charged nylon membrane were directly fixed to the PVC substrate.10 μL of enzyme was loaded to the center of the membrane, and fixed for 30 min at 4℃. Then, 10 μL of indole acetate was add to the covering area of the enzyme for color reaction. The same step was fixed on the carrier membrane, and after 10 minutes, prepared by different membrane carriers, the color development effect of the enzyme membrane was observed and the enzyme membrane was measured the enzyme activity.

2.3.2 Enzyme recovery

Determination method of enzyme tablet activity referenced method 2.2. The enzyme tablet was immersed in the reaction solution, measured the enzyme activity, and calculated the enzyme recovery rate of the enzyme tablet.

Enzyme recovery of enzyme tablet(%)=(enzyme activity of enzyme tablet/ enzyme activity of the same volume of enzyme) ×100%

2.3.3 Selection of temperature and time for fixation of enzyme tablets

After 10 μL of the enzyme was spotted into the enzyme carrier, the fixed temperature were chosen for 4℃, -50℃ and 25℃ to compare, and the fixed time for 10, 20, 30, 60, 100 min. The color chang was observed and the enzyme tablet was measured the recovery rate.

2.3.4 Selection of fixed enzyme volume

8, 10, 15, 20, 25 μL of the enzyme was fixed on the membrane carrier, and measured the enzyme activity recovery rate. The substrate was added to the enzyme tablet, and the color development effect was observed after the color reaction.

2.4 Optimization of color reaction

2.4.1 The selection of substrate concentration

The substrate concentration has a large effect on the results of the enzymatic reaction. Within a certain range, the color change of the enzymatic reaction is dependent on the substrate concentration increases. 300, 600, 900, 1500, 3000, 4500 μg/mL were selected to substrate concentrations, we added different concentrations of the substrate to the enzyme tables, and observed the color effect.

2.4.2 The selection of inhibition time

Different concentrations of Tolclofos-methyl standard solution were added to the enzyme tablets, selected the suppression time of 5, 8, 10, 12, 15, 20min, The substrate was add to the enzyme tablet, and the color rendering effect was observed.

2.4.3 The selection of color time

The enzyme tablets were added different concentration of Tolclofos-methyl, then added substrate. 2, 3, 4, 5, 8, 10min color time were selected and the color rendering effect was observed.

2.5 Determination of detection limit

The organophosphorus and carbamate pesticides commonly used in Guangxi Province, such as Tolclofos-methyl, Methamidophos, Isoprocorb and 1-Naphthalenyl methyl carbamate, were detected in this experiment. Different concentrations of the standard solution was prepared with 0.2 mol/L PB buffer. Using enzyme tablets test, each concentration were repeated 3 times to determine the color intensity. Using buffer as a blank control, the color intensity is up to 100, the lowest is zero (the color of the intensive positive reaction is zero), and observe the specific color change of the enzyme tablet after the reaction.

The detection limit of the enzyme tablet is defined as the concentration at 1/2 of the color development intensity, that is, the IC_{50} when the pesticide inhibits the cholinesterase activity by 50%. the standard curve of color intensity with the concentration of pesticide standard solution was drawed by Origin, and then the IC_{50} was calculated.

2.6 Stability test

The enzyme tablets were placed in a refrigerator at 4℃ for storage and stored for 2, 5, 10, 15, 20, 30, and 50 days to detect the enzyme activity.

2.7 Milk sample test

5mL fresh milk was taken into the centrifuge tube, added equal amount of PB buffer and centrifuged at 10000rpm for 5min, then the supernatant was taken as the sample to be tested. 10 μL sample was added to the enzyme tablet, after 10 min of inhibition time, added substrate. After color development for 5min, the color change was observed to judge the residual pesticide.

2.8 Compared with commercially available pesticide residue test cards

Different amounts of Tolclofos-methyl were added to the milk, and then tested with a commercially available pesticide residue test card and did the further detection by gas chromatography.

3 Results and analysis

3.1 Extraction of enzyme solution

Enzyme activity is defined as an enzyme activity unit (U),which decompose 1 μmol of thioacetylcholine iodide per minute at 37 ℃, 0.2 mol /L pH 7.5-8.0 PB buffer.The enzyme activity of the duck serum after secondary extraction reached 995 U, which was reddish and met the detection requirements without affecting the subsequent experiments.

3.2 Preparation of enzyme tablets

3.2.1 Selection of enzyme carrier

After the cellulose acetate film was added with the color developer, there was a light blue color, and the blue color was more obvious after fixation. The positively charged nylon membrane plusing the color developer is fixed in light blue. The color of polyamide -6- film was obvious, as shown in Figure 1.

Fig. 1. Color results of three enzyme carriers.（From left to right, cellulose acetate film, positively charged nylon membrane and polyamide-6-film）

The initial enzyme activity of the enzyme was 995 U/mL, and the enzymatic activity of the cellulose acetate membrane and the polyamide-6-film was about 890 U, and the recovery rate was 90%. The enzymatic activity of the enzyme tablet with positively charged nylon membrane was 855U, and the recovery rate was 86%. It could be seen that the enzyme recovery rate of the enzyme tablets prepared by the three membrane materials was above 85%, which could be used to fix cholinesterase. Considering the color effect, the recovery of enzyme activity, the difficulty of preparation, the extensiveness of materials and the low cost, polyamide-6-film was chosen as the carrier of enzyme tablet.

3.2.2 Selection of fixed temperature and time for enzyme tablets

The initial enzyme activity of the fixed temperature enzyme solution was 995U. The recovery rate of enzyme activity was calculated according to the activity of enzyme tablet prepared at different fixation temperature and time. Table 1 presented information about the activity recovery rate of enzyme tablet at different fixeing temperatures and times. It could be seen that no matter what the fixing temperature was, the recovery rate of enzyme activity under 30min fixing time was higher. So 30min was chosen as the fixing time.

The Figure 2 below describes the color development results of enzyme tablets at different fixing temperatures at fixing 30min. Under the fixing time of 30min, the color of the enzyme tablets fixing at 4°C and -50°C was similar, and the color development result was ideal. However, the enzyme tablet placed at 25 ℃ had a general effect and the color was lighter. According to the above results, choose 4 ℃ was chosen as the fixing temperature.

Table 1. Activity recovery rate of enzyme tablet at different fixing temperatures and times.

Fixing temperature /°C	Fixing time /min				
	10	20	30	60	100
4°C	+	+	++	++	—
-50°C	+	++	++	++	—
Normal temperature	—	+	+	+	—

“—”：the recovery rate of enzyme activity was 0~35%;
“+”：the recovery rate of enzyme activity was 36~70%;
“++”：the recovery rate was 71~100%.

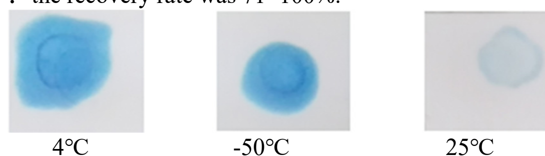


Fig. 2. Color development of enzyme tablets at different temperatures in fixing 30min

3.2.3 Selection of fixed enzyme volume

The fixed amount of different enzyme did not have much influence on the color development effect, and the color difference was not significant.

As shown in Figure 3 below, the enzyme recovery rate of the enzyme tablet shows a "厂" curve with the increase of the fixed amount of the enzyme solution, and gradually stabilized at 10 μL. Since the fixed amount of the enzyme solution had little difference in the color development effect, referring to the enzyme recovery rate of the enzyme tablet and the amount of the enzyme, a fixed amount of 10 μL of the enzyme was finally selected.

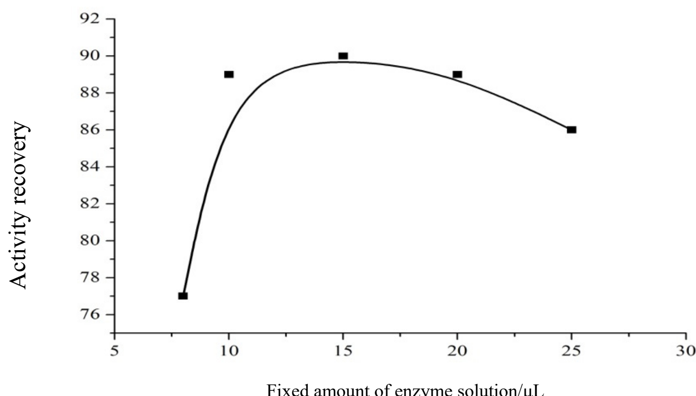
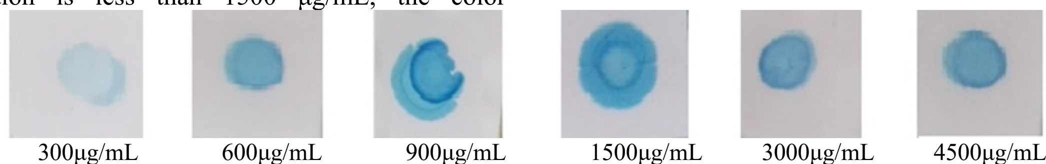


Fig. 3. Enzyme activity recovery rate in fixed amounts of different enzyme solutions.

3.3 Optimization of color reaction

3.3.1 Selection of substrate concentration

The Figure 4 below shows that when the substrate concentration is less than 1500 μg/mL, the color development effect increases with the substrate concentration, but when the substrate concentration is greater than 1500 μg/mL, the color development effect is weakened. Therefore, 1500μg/mL was chosen as the substrate concentration.



300μg/mL 600μg/mL 900μg/mL 1500μg/mL 3000μg/mL 4500μg/mL

Fig. 4 Color development effect at different substrate concentrations.

3.3.2 Selection of inhibition time

The inhibition time had a great influence on the color development effect. Too short a pesticide did not fully exert its inhibitory effect on the enzyme. Too long it would reduce the work efficiency and the effect of substrate dropping also has some influence. Moreover, the inhibition time had a large deviation from the study of the sensitivity and detection limit of subsequent enzyme tablets.

It could be seen from the Table 2 that with the increase of inhibition time, the inhibition of cholinesterase by tolclofos-methyl is enhanced, and the color of the enzyme tablet become lighter. When the inhibition time was above 10 min, the color of the enzyme tablet did not change substantially, indicating that the reaction had reached equilibrium. In order to ensure sufficient reaction, 10 min was chosen as the choice of inhibition time.

Table 2. Effect of different inhibition time on detection sensitivity.

Inhibition time/min	Tolclofos-methyl content /mg · L^{-1}					
	4	2	1	0.5	0.1	0
5	++	++	++	++	+	—
8	++	++	++	++	++	—
10	++	++	++	+	+	—
12	++	+	+	+	—	—
15	++	+	+	—	—	—
20	+	+	+	—	—	—

3.3.3 Selection of colour-developing time

If the color development time was too short, the color of the blank enzyme tablet would become lighter. If the time was too long, the color of the weakly positive enzyme tablet would be deepened, which affected the color contrast of the enzyme tablet[7].

From the observation of the color development time in Table 3, the color did not change substantially after 5 minutes, and the color became light after 10 minutes, indicating that the color reaction had ended at 5 minutes. Therefore, it was determined that the color development time was 5 min.

Table 3. Effect of different color development time on detection sensitivity.

Color development time /min	Content of Tolclofos-methyl /mg · kg^{-1}					
	4	2	1	0.5	0.1	0
2	++	++	++	++	+	—
3	++	++	++	+	+	—
5	++	+	+	+	+	—
8	++	+	+	+	—	—
10	++	+	+	—	—	—

3.4 Detection limit

The Figure 5 presents information about the detection limits for four pesticides by color development intensity of enzyme tablet. The detection limit of the four pesticides are 0.5~1.0mg/kg, which are compared with the maximum residue of pesticides in GB2763-2012 《Maximum residue limits for pesticides in food》, as shown in Table 4. The results shows that the detection limit for four pesticides of enzyme tablets is lower than the maximum residue limit in food and could be used to directly detect pesticide residues.

Table 4. Comparison of detection limits of four pesticides.

Pesticides	Detection limit of enzyme tablets(mg/kg)	Maximum residue limits for pesticides in food (mg/kg)
Tolclofos-methyl	1.0	2.0
Methamidophos	0.5	0.5
Isoprocorb	0.5	0.5
1-Naphthalenyl methyl carbamate	1.0	2.0

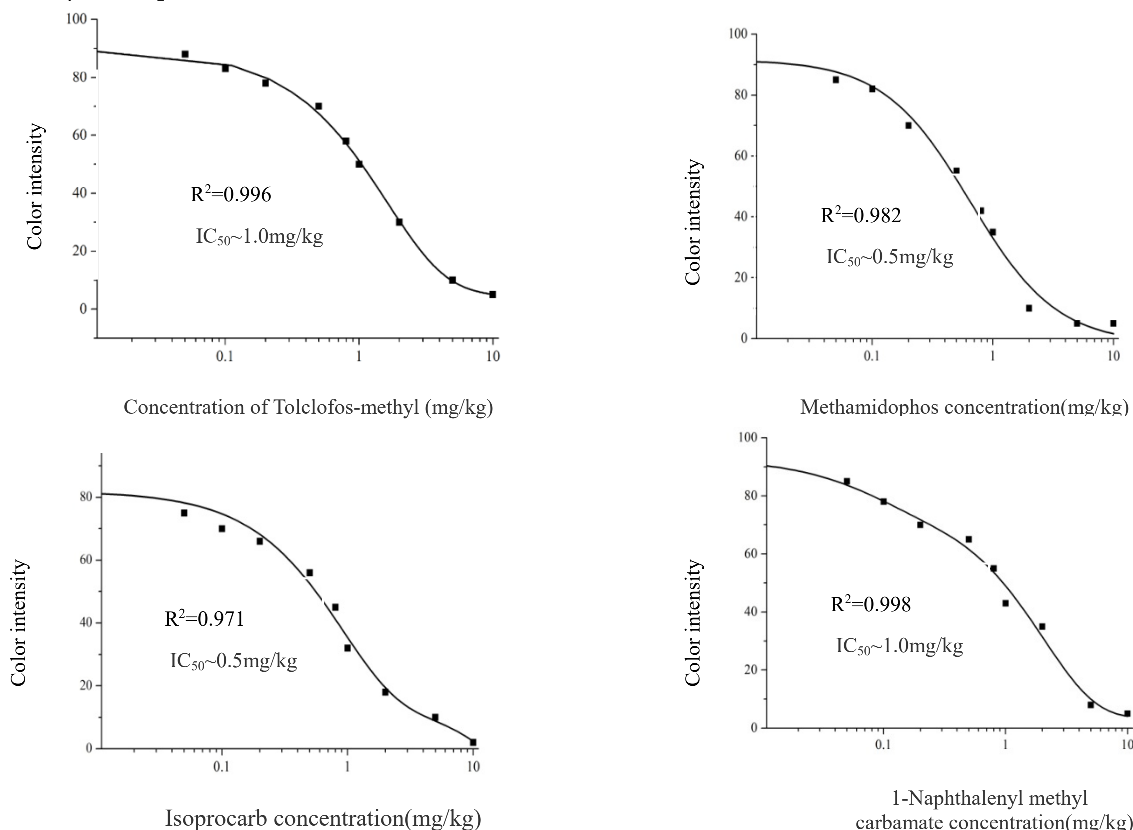


Fig. 5. Detection limits for four pesticides

3.5 Stability

The Figure 6 shows that the stability of enzyme tablet. When the enzyme tablets were stored in the refrigerator at 4°C for 10 days, the recovery rate of enzyme activity decreased by 25% and 35% after 30 days. The enzyme tablet placed for 30 days was used to detect the standard sample of tolclofos-methyl at 1 mg/kg, and the coloration result was still discernible. Therefore, although the enzymatic activity of the enzyme tablet was lost with the prolongation of the storage time, the enzyme tablet could be used for detection after being stored for 30 days.

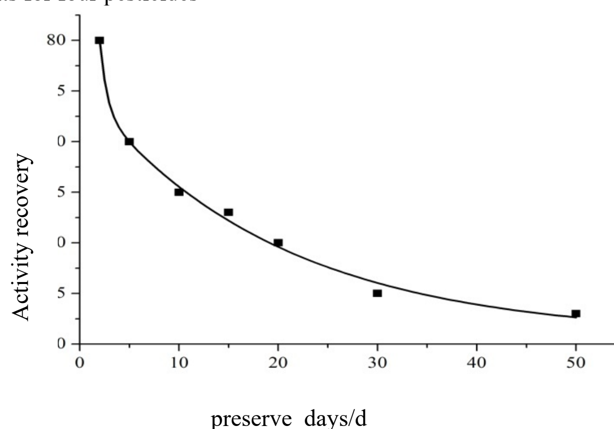


Fig. 6. The stability of enzyme tablet.

3.6 Compared with commercially available pesticide residue test cards and GC detection

The prepared enzyme tablets were compared with commercially available rapid test cards and GC, and the results are shown in Table 5. It could be seen that the enzyme tablets prepared by this method is consistent with the results of rapid detection card and GC, and there is no false positive in the detection, indicating that the method could be used to detect pesticide residues in milk. In addition, the method is simple to operate, does not need valuable instruments, low detection cost, suitable for rapid detection of milk on-site.

Table 5. Comparison of enzyme tablet with commercially available test cards and GC detection

Number	Tolclofos-methyl standard sample (mg/kg)	Enzyme tablet test	Commercial quick test card	GC test （mg/kg）
1	0	—	—	0.00
2	0.1	+	+	0.0998
3	0.5	+	+	0.412
4	1.0	++	++	0.892

4 Conclusion

The enzyme tablet in this study consisted of acetylcholinesterase from duck serum, indophenol acetate and polyamide-6-film.The enzyme activity was between 800-900U and could be kept for 30 days at 4 C. Color reaction of enzyme tablet could be used to quickly determine the pesticide residue in the milk.

The enzyme tablets could be used to detect four kinds of pesticides in milk. The detection limit was 0.5-1.0 mg/kg. The enzyme tablets could reach the maximum limit of pesticide residues in food. It could be used to detect organophosphorus and carbamate pesticides residues in milk quickly.

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