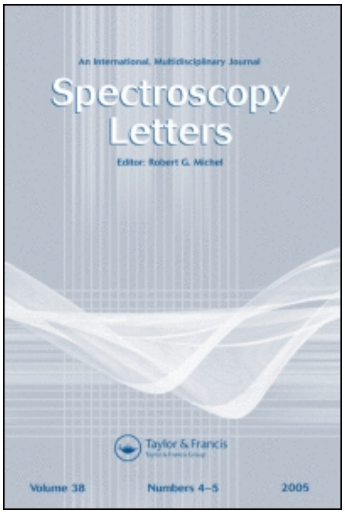

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Determination of Organophosphorus Pesticide Residues in Vegetables by Electrokinetic Sequential Injection Analysis

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Abstract: A fast and convenient analysis method based on a plant esterase reaction inhibited by organophosphorus pesticides was developed for the determination of the pesticide residues in vegetable samples. The proposed method was performed with an electrokinetic sequential injection analysis (ESIA) system, which consisted of a spectrophotometer, a homemade electro-osmosis pump, and four solenoid valves controlled by a Visual C program. The plant esterase extracted from fresh flour was adopted in the enzyme reaction. Several reaction parameters, such as the sample volume, the length of reaction coil, and the concentrations of reactants, are discussed in detail. The linear range of the calibration concentration was 0.03–0.5 μg/g dimethoate, which was used as a converted concentration of total organophosphorus pesticides. The detection limit of 0.01 μg/g dimethoate was achieved. The analytical throughput of the proposed method was about 24 samples per hour.

Keywords: Electrokinetic sequential injection analysis, flour esterase, inhibited enzyme reaction, organophosphorus pesticide residues, vegetable

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INTRODUCTION

In agriculture production, various pesticides have been used for protection against plant diseases and insect pests. Owing to the potential toxicity of organophosphorus pesticides for human beings, the monitoring of the pesticide residues in farm products has become an important item in food analysis. Therefore, more attention has been paid to the development of fast, convenient, reliable, and inexpensive analysis methods for pesticide multiresidues. Most of the pesticide analysis methods are based on chromatographic techniques, including gas chromatography, liquid chromatography, thin film chromatography, supercritical fluid chromatography and capillary electrophoresis, and so forth. These methods can provide high separation efficiency and individual pesticide analysis but require chromatographic instruments and highly trained operators. Different enzyme test papers and biosensors have been studied for pesticide detection. Some of the methods are based on the cholinesterase reaction inhibited by the pesticides. Acetylcholinesterase (AChE) is used frequently and obtained from electric eel,[1] *Drosophila melanogaster*,[2] bovine erythrocytes, and human erythrocytes.[3] Another adopted cholinesterase is butylcholinesterase (BuChE), which can be obtained from horse serum.[4] The organophosphorus pesticides can also inhibit plant esterase, such as flour esterase.[5,6] The esterase is inexpensive and can easily be extracted from fresh flour. Meanwhile, the sensitivity of the esterase reaction is similar to that of animal cholinesterase.[7]

Flow injection analysis (FIA) was introduced by Ruzicka and Hansen,[8] and has become a useful analysis technique. Following FIA, sequential injection analysis (SIA)[9] and multicommutation[10,11] were developed with the advantages of simplified manifold, reduced sample consumption, and improved controllability. As a suitable substitute of syringe pump, a homemade electro-osmosis pump was employed to deliver solutions in FIA.[12,13] The pump can provide relatively wide, stable, and pulseless flow rate. With the electro-osmosis pump in SIA, electrokinetic sequential injection analysis (ESIA)[14] was proposed, which mainly consists of an electro-osmosis pump, several solenoid valves, and a UV-Vis spectrophotometer.

In this work, the proposed analysis method for total organophosphorus pesticide residues was based on the flour esterase reaction inhibited by the pesticides and was performed by an ESIA system. Under the optimized conditions, the limit of detection (LOD) achieved 0.01 $\mu g/g$ dimethoate, which was used as a converted concentration for total organophosphorus pesticides. The limit of quantification (LOQ) was 0.03 $\mu g/g$ dimethoate and much lower than the maximum residue limits (MRLs) of vegetable samples established by the Ministry of Agriculture (China), in which most of the MRLs for vegetables were 0.1 $\mu g/g$.[15] The proposed method has been applied to the quantitative analysis of total organophosphorus pesticide residues in vegetable samples in this paper.

MATERIALS AND METHODS

Reagents and Solutions

The certified organophosphorus pesticides of phorate, phoxim, parathion, malathion, dimethoate, omethoate, trichlorfon, and methyl parathion were purchased from Accustandard Inc. (New Haven, CT, USA). Other certified pesticides were obtained from the Institute of Agro-Environmental Protection, Ministry of Agriculture (Tianjin, China), including the carbamates of carbaryl, fenobucarb, and carbofuran; the organochlorines of α-BHC, β-BHC, γ-BHC, δ-BHC, op′-DDT, pp′-DDT, pp′-DDE, pp′-DDD, dieldrin, aldrin, and endrin; and the pyrethroids of bifenthrin, fenpropathrin, cypermethrin, flucythrinate, and cyfluthrin. The calibration solutions were prepared in the concentration range 0.012–0.20 μg/mL dimethoate by diluting the dimethoate standard reagent with acetone. Other chemicals were of analytical grade and purchased from Chemical Reagent Co. (Shanghai, China). The color reagent solution was prepared by mixing 1.0 mmol/L 1-naphthyl acetate in 70% (v/v) acetone solution with 0.6 mmol/L fast blue B salt water solution in the volume ratio of 1 : 1. Fresh flour was obtained from a flour workshop.

Sample Preparation

Chinese cabbage samples were purchased from six local greengrocers. The samples were first washed to remove dirt, dried in the air, cut into small pieces, and milled in a mortar. Each treated sample was weighted (2.0 g), extracted with 10 mL acetic ether by an ultrasonic cleaner (35 kHz, 120 W) for 10 min, and the homogenate deposited for 10 min. Then the supernatant was filtered through a waterless Na_2SO_4 column, an active carbon column (lab-made), and a C_{18} purification column (TEDA, Tianjin, China) in turn, and eluted with 3 mL acetic ether additionally. The eluent was dried by a blower and dissolved with 5 mL acetone as the sample solution.

Apparatus

The ESIA system, as shown in Fig. 1A, consisted of one homemade electro-osmosis pump, four 3-way solenoid valves (161T031, Nresearch Inc., Caldwell, NJ, USA), and a 9100 UV-Vis spectrophotometer (Reili, Beijing, China). A DYY-III-4 electrophoretic power supply (10–1600 V; Liuyi, Beijing, China) was employed to provide the pump voltage. Detailed information about the electro-osmotic pump was described in Refs. [12 and 13]. Hexamethylene tetramine (HMTA) solution 0.5 mmol/L was used as the pump carrier to improve the stability of the pump flow rate. In this work, the pump flow rate was 0.6 mL/min at 400 V with its relative standard deviation (RSD) of 3.5% in 8 hr. The pump flow direction and flow rate

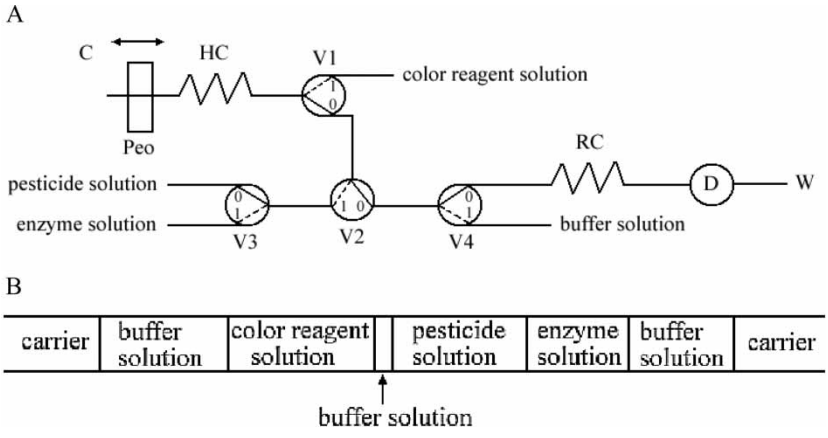


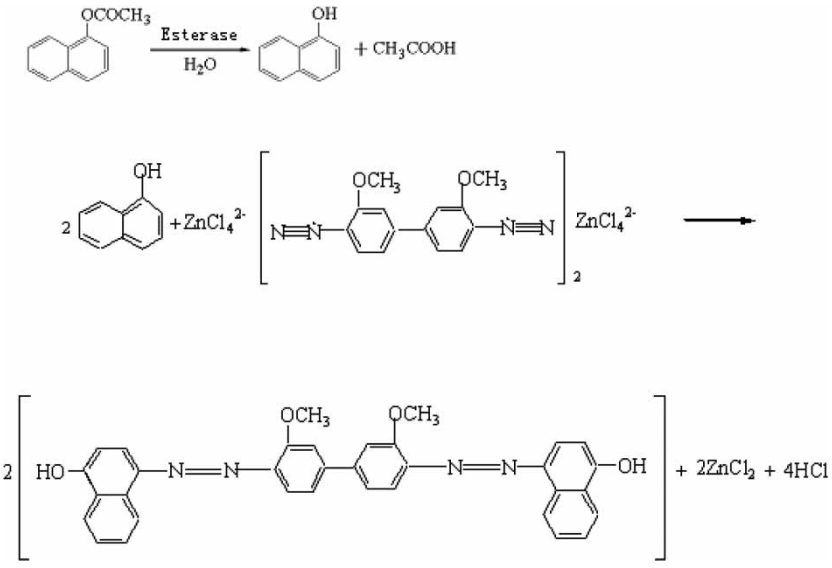
Figure 1. Schematic diagram of ESIA system (A) and zone distributions (B). C, carrier; Peo, electro-osmotic pump; HC, holding coil (2 m × 0.8 mm i.d. PTFE); RC, reaction coil (50 cm × 0.8 mm i.d. PTFE); $V_{1\text{-}4}$, solenoid valves; D, spectrophotometer; W, waste.

could be controlled by switching the polarity and varying the magnitude of the pump voltage, respectively. The injected reactant volumes could be regulated by the aspirating flow rate of the pump and the switching time of solenoid valves. The ESIA system was controlled by a personal computer with a homemade interface card and a Visual C program. The absorbance data of the color reaction were detected at 524 nm by the spectrophotometer equipped with a 10-mm flow cell and transmitted to the personal computer. PTFE tubing (0.8 mm i.d.) was used in the ESIA manifold.

Enzyme Extraction

Flour esterase was extracted from fresh flour obtained from a flour workshop. Fresh flour, 0.010 g, was added into 50 mL 0.05 mol/L phosphate buffer solution (pH 8.0) containing 0.15 mol/L NaCl, 2.0 mmol/L EDTA, and 5.0 mmol/L L-cysteine, and the solution in a 50-mL beaker was stirred inside an ice-water bath ($0 \sim 4^{\circ}C$) for 1 hr. Then the mixed solution was reserved under $4^{\circ}C$ for 12 hr. The supernatant solution was adopted as the esterase stock solution, which was prepared weekly. A working esterase solution was obtained by diluting the esterase stock solution to 1 : 60 (v/v) with pH 8.0, 0.05 mol/L phosphate buffer solution before use.

Flour esterase is a type of hydrolase. It can react with the substrate of 1-naphthyl acetate to produce 1-naphthol, and the latter combines with fast blue B salt to form an amaranth azo compound with the maximal absorption wavelength at 524 nm. According to the above-mentioned reactive processes, the esterase reactions in the substrate hydrolysis and the color reaction are similar to those of cholinesterase [16] and are described in Scheme 1.



Scheme 1.

When the organophosphorus pesticides are introduced, the pesticides can combine with the esterase and make the enzymatic activity decrease. The inhibition rate can be expressed as

$$IR = \frac{A_0 - A_1}{A_0}$$

where IR, A_0, and A_1 represent the inhibition rate and the absorbance of color reaction before and after inhibition, respectively.

Analytical Procedure

The analytical procedure of the proposed method is listed in Table 1 and described as follows (refer to Fig. 1). The conduits between the valves were as short as possible except for a 15-cm, 0.8-mm i.d. PTFE conduit between V2 and V4.

The first step was adopted to introduce a new sample solution into the ESIA system and the positions of the solenoid valves were V1(0), V2(1), V3(0), and V4(0), where V1(0) meant the position of value 1 at 0 (as shown in Figure 1). The pump aspirated the sample solution containing pesticide residues through V3 at -400 V for 5 s. Then the manifold was flushed with the pump carrier solution by the pump pushing forward at $+400$ V for 35 s and the valves positioned at V1(0), V2(0), V3(0), and V4(0). Successively, the pump aspirated the buffer solution into the system

Table 1. Analytical procedure of ESIA system

Step	Operation	Time (s)	Pump voltage (V)	Valve position			
				V_1	V_2	V_3	V_4
1	Aspirating sample solutions	5	−400	0	1	0	0
2	Washing main flow conduit	35	+400	0	0	0	0
3	Aspirating buffer solution zone	15	−400	0	0	0	1
4	Aspirating color reagent zone	7.5	−400	1	0	0	0
5	Aspirating pesticide zone	5	−400	0	1	0	0
6	Aspirating enzyme zone	7.5	−400	0	1	1	0
7	Pushing aspirated zones to detector	75	+400	0	0	0	0

ESIA, electrokinetic sequential injection analysis.

for 15 s with the valve positions of V1(0), V2(0), V3(0), and V4(1), so the manifold including V4, V2, V1, the conduits among the valves, and a small part of holding coil, which was adopted to prevent sample and reagent solutions from aspirating into the pump, was filled with the buffer solution. In the fourth step, the color reagent solution was inserted into the buffer zone for 7.5 s and the valve positions were V1(1), V2(0), V3(0), and V4(0). The sample solution was aspirated for 5 s in the fifth step with the same valve positions as the first step. There was a very small volume (2 μL) of buffer zone between the sample and color reagent zone due to the dead volume of V2 and the conduit between V2 and V4, which did not affect the repetition of the analysis. In the sixth step, the esterase solution followed the sample plug was inserted between the sample and buffer zone for 7.5 s with the valve positions of V1(0), V2(1), V3(1), and V4(0). From the third to sixth step, the pump voltage was kept at −400 V to aspirate each solution into the ESIA system. At last, the pump pushed the introduced zones to the detector with the pump voltage of +400 V, and the valve positions were V1(0), V2(0), V3(0), and V4(0). The consumed time of the last step was 75 s for detecting and washing.

RESULTS AND DISCUSSION

ESIA Conditions

The injection volume of the pesticide solutions was investigated in the range from 10 μL to 125 μL (i.e., from 1.0 s to 12.5 s) as shown in Fig. 2. Both the volumes of color reagent and esterase zone were 75 μL. The reaction coil length was 50 cm. The color reagent solution contained 1.0 mmol/L 1-naphthyl acetate in 70% (v/v) acetone solution and 0.6 mmol/L fast blue B salt water solution in the volume ratio of 1 : 1. The esterase stock solution was diluted to 1/60 (v/v) with pH 8.0, 0.05 mol/L phosphate buffer

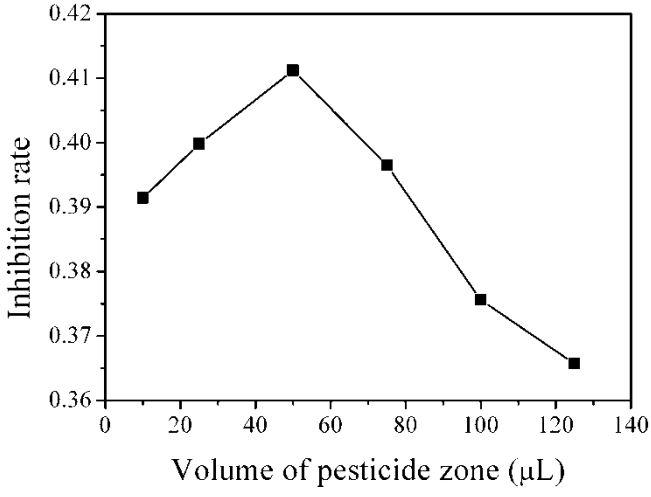


Figure 2. Effect of pesticide zone volume on inhibition rate.

solution. The buffer solution was pH 7.6, 0.05 mol/L phosphate solution. The inhibition rate increased with the pesticide volume up to 50 μL. In addition, an opposite variation was observed with the pesticide volume larger than 50 μL. The phenomena may result from the inhibited esterase reaction and the zone dispersion between the color reagent and esterase zone. When the pesticide volume was less than 50 μL and the zone dispersion was sufficient, the inhibition rate would be proportional to the pesticide volume approximately. However, when the pesticide volume was larger than 50 μL, the inhibition rate decreased with the increase of the pesticide volume, because the dispersion distance between the color reagent and esterase zone became longer and the color reaction could be restricted. Taking the experimental data into account, 50 μL pesticide solution was injected for the inhibiting reaction.

The reaction coil length was also studied for the esterase reaction. When the reaction coil length was shorter than 50 cm, the esterase reaction inhibited by the pesticide was not complete and the inhibition rate was enhanced with the reaction coil length. However, when the coil length was longer than 50 cm, the inhibition rate kept almost constant, because the inhibited esterase reaction had fulfilled. As a consequence, 50-cm reaction coil was selected in the ESIA system.

Enzyme Reaction Conditions

The pH value of the buffer solution can affect the enzyme activity. The pH values from pH 7.0 to 8.0 were examined with the absorbance of the color reaction, as shown in Fig. 3. The injection volumes of the color reagent,

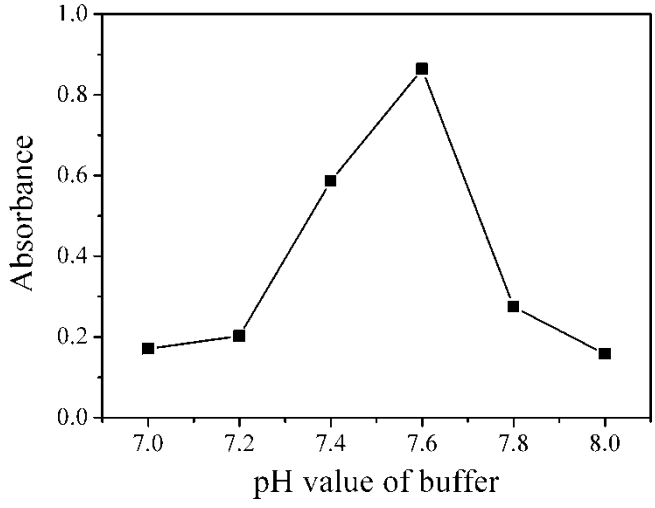


Figure 3. Effect of pH value on color reaction absorbance. Other conditions are the same as in Fig. 2.

enzyme, and pesticide zone were 75, 75, and 50 μL, respectively. It was found that a maximum absorbance could be achieved at pH 7.6, viz., the esterase possesses its maximal activity. When the pH value deviated from 7.6, the absorbance decreased rapidly. So the buffer solution for the esterase reaction was adjusted to pH 7.6 in this work.

To improve the stability of the esterase solution, its stock and working solution had to be prepared with pH 8.0, 0.05 mol/L phosphate buffer solution. The concentration of esterase working solution was studied on the absorbance of the color reaction, as presented in Fig. 4. When the dilution factor of the enzyme stock solution was higher than 60, the absorbance of the color reaction was reduced because of the insufficient hydrolysis reaction. However, when the dilution factor of the esterase stock solution was lower than 60, the increase of the esterase solution viscosity had a negative influence on the hydrolysis reaction. So the optimal sensitivity was obtained with the dilution factor of 60.

According to Michaelis–Menten equation of enzyme reaction, the increase of the substrate concentration has a limitation. When the substrate (1-naphthyl acetate) concentration was lower than 1.0 mmol/L, the amount of the hydrolysis product increased with the substrate concentration. However, when the concentration was higher than 1.0 mmol/L, the enzyme reaction was saturated and the absorbance curve became flat. Therefore, 1.0 mmol/L 1-naphthyl acetate as the substrate was introduced into the color reagent solution.

Under the reaction conditions of 1.0 mmol/L 1-naphthyl acetate in the color reagent solution and the dilution factor of 60 for the esterase

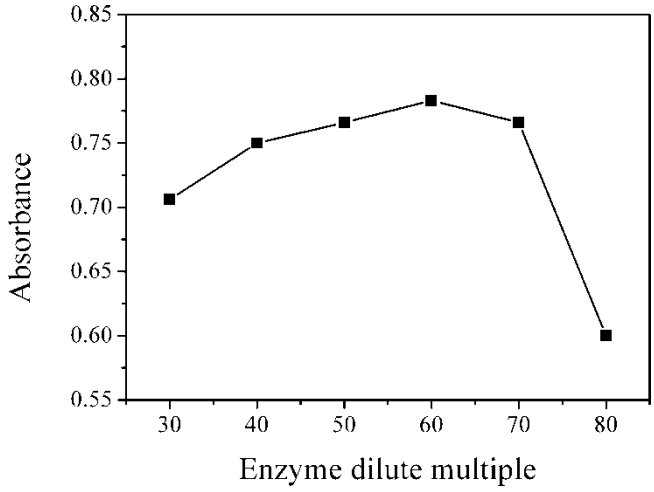


Figure 4. Effect of esterase concentration on absorbance. Other conditions are the same as in Fig. 2.

stock solution, the concentration of fast blue B salt was examined. The absorbance increased when the concentration of fast blue B salt increased up to 0.6 mmol/L. Then the absorbance changed a little by increasing the concentration of fast blue B salt further. It implies that the enzyme-catalyzed reaction has been completed and the color reagent is sufficient. Hence, 0.6 mmol/L fast blue B salt was used in this detection.

Interference

The organochlorines and pyrethroids with the concentration of 1.0 mg/mL did not inhibit the flour esterase, which is ten times MRLs in vegetables established by China.[15] Unfortunately, the carbamate pesticides could also inhibit the flour esterase. However, the carbamate pesticides displayed different hydrolysis degrees than the organophosphorus in water solution. By increasing the temperature, the hydrolysis degrees of carbamate pesticides were higher than those of the organophosphorus pesticides. It was found that the carbamate pesticides could not be detected by a treating temperature up to 80°C.

Sample Analysis

An external calibration was carried out with six dimethoate standard solutions under the selected conditions mentioned above. The logarithm regression equation of dimethoate was expressed as $Y = 31.5 \log C + 71.0$ in the logarithm range 0.012–0.20 μg/mL, where Y and C represent the inhibition

rate (%) and the dimethoate concentration, respectively. The coefficient of correlation (r) was 0.9979. The limit of detection (LOD) was 4 μg/L dimethoate, which was calculated as 3 times standard deviation of 11 successive noise peaks of the baseline. The inhibition rates of other organophosphorus pesticides can be converted to dimethoate, as shown in Table 2. For instance, the inhibition rate of 0.10 μg/mL omethoate was equal to that of 0.236 μg/mL dimethoate at 25°C but fell to that of 0.144 μg/mL dimethoate after increasing the temperature of the sample solution up to 80°C with a water bath and cooling down to room temperature. The inhibition rates of the organophosphorus pesticides decreased and their converted concentrations were reduced to those of (0.48–0.66) dimethoate concentration at 25°C, because of partial hydrolysis. The inhibition rate of dimethoate treated with 80°C also decreased to 61% dimethoate concentration at 25°C. Meanwhile, the residues of carbamate pesticides could not be detected. Thus the proposed method can be used to differentiate the organophosphorus and carbamate pesticides.

Six Chinese cabbage samples were determined by the proposed method. The residue concentrations of the pesticides were in the range 0.04–0.06 μg/g dimethoate. When the vegetable samples were spiked with 0.03, 0.05, and 0.07 μg/mL dimethoate, their recoveries were in the range 91–93%, as listed in Table 3.

Different washing methods were also investigated for the Chinese cabbage samples in this work in order to evaluate different washing methods in our daily life. The investigated samples were spiked with 0.10 μg/g dimethoate. After 1 hr, the spiked samples were washed by three different methods, viz. dipped in tap water, tap water with 1.0% detergent (Whitecat detergent, Whitecat Shareholding Ltd., Shanghai, China), and 0.25 mol/L $NaHCO_3$ for 10 min, respectively. Then the samples were washed with the tap water. The reagent blank, unspiked and spiked samples were determined by the proposed method.

Table 2. Converted pesticide concentrations at 25°C and treated with 80°C to dimethoate concentration at 25°C according to their inhibition rates

Pesticides	25°C	80°C
0.1 μg/mL omethoate	0.236 μg/mL dimethoate	0.144 μg/mL dimethoate
0.1 μg/mL trichlorfon	0.204 μg/mL dimethoate	0.098 μg/mL dimethoate
0.1 μg/mL methyl parathion	0.128 μg/mL dimethoate	0.068 μg/mL dimethoate
0.1 μg/mL phorate	0.107 μg/mL dimethoate	0.066 μg/mL dimethoate
0.1 μg/mL phoxim	0.139 μg/mL dimethoate	0.068 μg/mL dimethoate
0.1 μg/mL parathion	0.141 μg/mL dimethoate	0.072 μg/mL dimethoate
0.1 μg/mL malathion	0.100 μg/mL dimethoate	0.066 μg/mL dimethoate
0.1 μg/mL carbaryl	0.150 μg/mL dimethoate	Not detected
0.1 μg/mL fenobucarb	0.233 μg/mL dimethoate	Not detected
0.1 μg/mL carbofuran	0.177 μg/mL dimethoate	Not detected

Table 3. Dimethoate recoveries in six Chinese cabbage samples

Sample	Added concentration (μg/mL)	Detected concentration (μg/mL)	Recoveries (%)	RSD (%, n = 3)
Sample 1	0.030	0.028	93.0	3.0
Sample 2	0.030	0.027	91.0	3.2
Sample 3	0.050	0.045	91.2	2.4
Sample 4	0.050	0.046	92.6	2.2
Sample 5	0.070	0.064	91.4	4.6
Sample 6	0.070	0.063	91.3	2.3

RSD, relative standard deviation.

Table 4. Cleaning efficiency of different cleaning procedure

Cleaning solution	C_0 (μg/mL)	C_1 (μg/mL)	Cleaning efficiency (%)
Water	0.10	0.068	32
Water + detergent	0.10	0.031	69
0.25 mol/L $NaHCO_3$	0.10	ND	100

The cleaning efficiency can be define as: $X = (C_0 - C_1)/C_0$, in which X, C_0, and C_1 represent the cleaning efficiency, dimethoate concentration before and after cleaning, respectively. The cleaning efficiencies are presented in Table 4. It was found that the most efficient cleaning solution was 0.25 mol/L $NaHCO_3$.

CONCLUSIONS

The proposed method based on the inhibited esterase reaction was performed by an electrokinetic sequential injection analysis (ESIA) system and proved to be simple, fast, reliable, and inexpensive. The proposed method can be adopted to determine the residues of the organophosphorus pesticides in vegetable samples. The interference of carbamate pesticides can be eliminated by hydrolysis at 80°C, which offers the possibility to differentiate the residues of the organophosphorous and carbamate pesticides.

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