Determination of organophosphate and carbamate pesticides based on enzyme inhibition using a pH-sensitive fluorescence probe

Shengye Jin a, Zhaochao Xu b, Jiping Chen a,∗, Xinmiao Liang a, Yongning Wu c, Xuhong Qian b

a Dalian Institute of Chemical Physics, The Chinese Academy of Sciences, Dalian 116012, China
b Department of Fine Chemical Engineering, Dalian University of Technology, Dalian 116012, China
c National Institute of Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, Beijing 100050, China

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Abstract

A flow injection system for the determination of organophosphate and carbamate pesticides is described. A sensitive fluorescence probe was synthesized and used as the pH indicator to detect the inhibition of the enzyme acetylcholinesterase (AChE). The percentage inhibition of enzyme activity is correlated to the pesticide concentration. Several parameters influencing the performance of the system are discussed. The detection limits of 3.5, 50, 12 and 25 μg/l for carbofuran, carbaryl, paraoxon and dichlorvos, in pure water, respectively were achieved with an incubation time of 10 min. A complete cycle of analysis, including incubation time, took 14 min. The detection system has been applied to the determination of carbofuran in spiked vegetable juices (Chinese cabbage and cole), achieving recovery values between 93.2 and 107% for Chinese cabbage juice and 108 and 118% for cole juice at the different concentration levels assayed.

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1. Introduction

Numerous chemical compounds, routinely used in agriculture and chemical industry, can form persistent toxic residues in air, soil, water and foods. Pesticides are one of the principal classes of such environmental pollutants widely encountered throughout the world, and millions of tonnes are used in agriculture, which results in serious food safety problems. Organophosphate and carbamate pesticides that are mainly used in agriculture show low environmental persistence but display high acute toxicity. Their presence in water and food poses a potential hazard to human health. In general these compounds inhibit the acetylcholinesterase (AChE) participating in nerve-impulse transmission. The inhibition mechanism, as described in Fig. 1, being very specific, has led to the development of several analytical techniques for the identification and quantification of such pesticides on the basis of the inhibition of cholinesterases [1–4]. Compared with the traditional procedures, based on gas chromatography (GC) and high-performance liquid chromatography (HPLC), which are reliable but expensive, complicated and time-consuming [5], these analytical techniques are rapid, inexpensive and need almost no sample pretreatment.

The development of biosensors based on enzyme inhibition is a matter of considerable interest, and many kinds of biosensors were reported in the past decade which could be mainly divided into three types according to the difference of transducers: potentiometric [7–10], amperometric [11–17], and optical biosensors [18–21]. However, these biosensors often have relatively short longevity, which greatly limit their uses in practice, and the detections often need more than half an hour and several milliliters of samples.

Flow injection analysis (FIA) [4,22–27] technique is also often used in the determination of the pesticides. FIA can offer advantages of high sample throughput and highly reproducible timing and eliminates the need to attain a
steady-state [28]. The determination of organophosphate and carbamate pesticides by flow injection analysis with a pH electrode has been reported earlier [4,26,27]. Most optical biosensors also use flow injection system to complete their analysis performance. A pH colorimetric indicator is often used in an optical biosensor. Andres and Narayanaswamy [29] developed a fiber optical biosensor using thymol blue as the pH colorimetric indicator covalently bound to aminopropyl glass and using a bioreactive layer consisting of AChE, covalently bound to isothiocyanate glass. The sensor displayed detection limits (I_{10\%}) of 3.1 and 24.7 \mu g/l, for carbofuran and paraoxon, respectively, using a sample volume of 5 ml. Xavier et al. [30] developed another fiber optical biosensor using porous glass with covalently bound chlorophenol red. The biosensor was applied to the determination of propoxur in spiked vegetables (onion and lettuce) using ultrasonic extraction, and the recovery values between 93 and 95% proved the good performance of the biosensor for this type of analysis. Nevertheless, in the case of lettuce samples the recoveries were lower (75%), indicating the presence of matrix effect originated from some vegetable pigments in the extracts such as chlorophyll which constituted an interference in the colorimetric detection of pesticides using a pH-indicator dye.

Some fluorescent compounds can also be used as pH indicators. In literature reports on this method are relatively few. Rogers et al. [31] reported the first fiber-optic anti-cholinesterase biosensor, immobilizing a fluorescein isothiocyanate-tagged acetylcholinesterase (FITC-AChE) on quartz fibers. The pH-dependent fluorescence signal generated by FITC-AChE was quenched by the protons produced during acetylcholine hydrolysis. Although, the carbamate insecticides bendiocarb and methomyl and the organophosphates ecomephate and paraoxon could be detected from the micromolar to nanomolar range, but malathion, parathion and dicrotophos were not detected even at millimolar concentrations. Höbel and Polster [32] described another acetylcholinesterase-based optical biosensor whose measurements were also based on the fluorescence changes brought by pH shifts during substrate hydrolysis, obtaining the determination ranges of 0.5-5 \times 10^{-7} \text{mol/l} for aldicarb and 0.1-2 \times 10^{-6} \text{mol/l} for carbofuran. Recently in 1999, Russell et al. [33] described the detection of organophosphorus neurotoxins using poly(ethylene glycol) hydrogel-encapsulated fluorophore-enzyme conjugates. Direct determination of enzyme-catalyzed neurotoxin hydrolysis is provided by the self-referencing, pH-sensitive dye seminapthofluorescein (SNAFL). Using spectrophotometry and paraoxon as a model organophosphate, paraoxon concentrations as low as 8 \times 10^{-7} \text{M} could be detected.

We herein describe the procedure of a flow injection system based on enzyme inhibition, using a sensitive fluorescence probe as a pH indicator in the carrier stream, for the determination of several kinds of carbamate and organophosphate pesticides. Free AChE is used and 7500 mm \times 0.5 mm capillary column is employed as the reactor. With the injection of 10 \mu l enzyme solution and the presence of the substrate in the carrier, the fluorescence intensity will be enhanced by the H^+ released by the hydrolysis of the substrate. The change between the peak heights before and after the enzyme inhibition is related to the carbamate and organophosphate pesticide concentrations in the sample solution. Factors affecting the analysis performance are discussed. Pesticide detection in spiked vegetable juice samples can be successfully accomplished using the described procedure without any pretreatment.

2. Experimental
2.1. Reagents

Acetylcholinesterase (AChE, E.C. 3.1.1.7 from electrophorus electricus, 235 U/mg) and acetylcholine chloride (99%) were purchased from Fluka (Buchs, Switzerland). HEPES (N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid) (99%) was purchased from Acros (Geel, Belgium). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranomethylcarbamate), paraoxon (diethyl-p-nitrophenyl phosphate), carbaryl (1-naphthyl-N-methylcarbamate), dichlorvos (2,2-dichlorovinyldimethyl phosphate) were pur-
chased from Accustandard, Inc. (New Haven, CT, USA). All other reagents were of analytical grade or better. Buffer solutions were prepared with Milli-Q water.

2.2. Synthesis of the fluorescence probe

The synthesis of the fluorescence compound used in the detection of pesticides 2-buty1-6-(4-methyl-piperazin-1-yl)benz[d]isoquinoline-1,3-dione, has been reported earlier [34]. Its excitation and emission maximum wavelengths are 390 and 530 nm, respectively.

2.3. FIA manifold

The configuration of the FIA manifold is shown in Fig. 2. The carrier stream was pumped through the FIA manifold by an HPLC pump (Waters 515). Samples were injected into the carrier stream using a six-way rotary injection valve (Rheodyne 7725i). A 750 mm × 0.5 mm capillary column was used as a reactor were AChE hydrolyzed acetylcholine to acetate and choline. The carrier consisted of an HEPES buffer, pH 7.5, containing 20 mM MgCl₂, 100 mM NaCl, 1.14 M fluorescence probe and 5 mM acetylcholine chloride. The changes of pH in the carrier stream caused changes in the fluorescence intensity which could be detected by the fluorescence detector (Waters 474) with Ex/Em at 390/530 nm and was recorded by DL-800 LC-recorder. The components of the system were connected with 0.02 in. i.d. stainless tubings (SS-316).

2.4. Measuring procedure

AChE catalyzes the hydrolysis of acetylcholine into choline and acetic acid, as shown below: The acid dissociates further to release H⁺ ions, which cause enhancement of fluorescence intensity of the fluorescence probe

acetylcholine + H2O ⇌ acetate + choline

Since most of the pesticides tested are insoluble in water, stock pesticide solutions were made with ethanol. It is noted that ethanol, up to 5% (v/v), does not inhibit the enzyme, and the ethanol concentrations in the final solutions were kept much less than 5%. Pesticide solutions were prepared with pure water. Enzyme solutions were prepared in 2.5 mM HEPES buffer (pH = 7.5) containing 20 mM MgCl₂ and 100 mM NaCl. The inhibition procedure was conducted by mixing 20 μl enzyme solution and 20 μl pesticide solution and after incubating for 10 min at 30 °C, 10 μl of the mixture was injected. It took only 4 min from sample injection to the recovery of the carrier line and the sample throughput was 15 per hour. Unlike the results reported in the previous papers [35,36], in which the measurements of the inhibition effect of pesticides in water sample consumed at least 20 U of AChE, only 1.88 U per sample was consumed in our pesticide analysis. Percentage inhibition of enzyme activity is determined by comparing the peak heights before and after the presence of pesticide. The operating conditions of the flow injection system for these experiments were: 2.5 mM HEPES working buffer (pH = 7.5); fluorescence probe concentration, 1.14 μM; substrate concentration, 5 mM; enzyme solution (pH = 7.5), 0.2 mg/ml (after mixing with pesticide solutions); pH of pesticide solutions, 7.5; flow rate, 1 ml/min; temperature, 30 °C.

The limit of detection of the pesticide was taken as that pesticide concentration that resulted in 10% inhibition of enzyme activity.

2.5. Analysis of pesticides in vegetable juices

Chopped Chinese cabbage and cole were blended with the aid of a mixer separately and 5 ml of crude juices was drawn from the homogenate samples. The pH of the juices was adjusted to 7.5 using NaOH and then the juice was centrifuged at 3000 rpm for 3 min and 3 ml of the supernatant juice was collected. In order to indicate the influence of pigments upon the recovery values, carbofuran was directly spiked in the supernatant juice samples. Each juice sample was spiked with known amounts of carbofuran (11.8 and 23.5 μg/l in Chinese cabbage and cole, respectively). Blank samples were prepared following the previous procedure without pesticides spiking.

3. Results and discussion

3.1. Selection of experimental conditions

3.1.1. Fluorescence probe concentration and pH

Fig. 3 shows the sensitivity of the fluorescence probe to pH changes. This fluorescence probe is very sensitive to pH changes from 8 to 7 with a good linearity (R² = 0.9944).
is noted that the sensitivity is improved with increasing concentrations of the fluorescence probe, as shown in Fig. 3(b), as shown in Fig. 5. However, higher fluorescence probe concentrations resulted in higher peak width at baseline. By injecting the same enzyme solution, the ratios of peak height to peak width at different probe concentrations in the carrier are shown in Fig. 4. According to the above results, a fluorescence probe of 1.14 μM was chosen in our experiments to maintain both a steady baseline and a high sensitivity.

As shown in Fig. 3(b), the pH-sensitive linear range of the fluorescence probe is from 7.0 to 8.5. Similar to the earlier reports [2], the autohydrolysis of acetylcholine is a great limitation of choosing the pH of HEPES buffer solution. At a pH of 8, the autohydrolysis of ACh was very great. In order to make sure that the variation in pH caused by enzyme injection would not overstep the pH-sensitive linear range of the fluorescence probe, a pH value much near to 7.0 was not suitable as well. So the pH value of 7.5 was selected in our experiment, at which not only the autohydrolysis of ACh was neutralized to some extent, but also the variation in pH happened in the linear range was allowed.

3.1.2. Flow rate and substrate concentration

With fixed length of the reaction column, flow rate was directly related to the reaction time of enzyme-catalyzed hydrolysis of the substrate. A lower flow rate resulted in more reaction time, and then a wider variation in pH that caused a higher peak height. As shown in Fig. 5(a), the peak heights decreased with the increasing flow rate. On the other hand, the analysis per sample injection consumes more time with lower flow rate, as shown in Fig. 5(b). So the choice of the operating flow rate was based on the following criteria:

- The chosen flow rate offered a sufficient enzyme–substrate reaction time to maintain a high sensitivity.
- The throughput of samples was satisfied by the lowest flow rate.
- The flow rate did not result in a wide variation in pH, which might overstep the pH-sensitive linear range of the fluorescence probe.
Finally, a compromise flow rate of 1 ml/min was chosen to conduct the experiments.

In order to provide an accurate measure of the enzyme activity, the concentration of the substrate (ACh) should be high enough and in the saturation concentration range for the enzyme, so that the hydrolysis rate of the substrate is only related to the enzyme activity. The reaction rates of AChE at various ACh concentrations are shown in Fig. 6. When the concentration of ACh is higher than 4 mM, the enzyme is saturated. A substrate concentration of 5 mM was chosen in our experiments.

3.2. Enzyme concentration

The reaction of cholinesterase inhibition in the presence of organophosphate or carbamate pesticides is slow, often requiring at least 30 min to several hours for the analysis [6]. However, using low enzyme concentrations cannot only reduce the enzyme inhibitor contact time, but also allow the detection of very low concentration of the inhibiting compounds [4]. On the other hand, lower concentration of the enzyme led to narrower linear detection range of pesticides. Furthermore, the concentration of the enzyme must give enough peak height. Fig. 7 shows the linearity of peak height to enzyme concentration obtained for various AChE concentrations at a carrier flow rate of 1 ml/min. An enzyme concentration range of 0.15–0.3 mg/ml was preferred. A concentration of 0.2 mg/ml (after mixing with pesticide solutions) was chosen in our experiments.

3.3. Temperature

For all the above-mentioned experiments the capillary column was placed in a water bath maintained at a controlled temperature of 25°C. The effect of varying the water bath temperature on the peak height was observed as indicated in Fig. 8. A maximum enzyme activity is noted at a bath temperature of 47°C. Higher temperatures resulted in enzyme inactivation.

In the same plot the effect of reactor bath temperature on the rate of inhibition of the enzyme is shown. An optimum temperature for conducting the inhibition tests is observed at a water bath temperature of 30°C, for the carbofuran solution. All other pesticides were analyzed with the reactor water bath maintained at 30°C.

3.4. Assay of organophosphate and carbamate pesticides

Calibration plots obtained for the determination of carbofuran, paraoxon, carbaryl, and dichlorvos, using this fluorescence probe, are shown in Fig. 9. The detection limits and the linear dynamic ranges are indicated in Table 1.

As can be seen in the calibration plots, the detection limits of the four pesticides (corresponding to a decrease of 10% enzyme activity) are all located in the linear portion of the calibration curves, because of the use of extremely small amounts of AChE. This analytical system with free enzyme and fluorescence probe allowed detection limits of 3.5 µg/l (1.6 × 10⁻⁶ M) for carbofuran and 12 µg/l (4.6 × 10⁻⁸ M) for paraoxon, which are favorable in comparing with those reported by other authors using similar pH-dependent fluorescence methods [31–33].
Fig. 9. (a) Peaks obtained by continuous injection of acetylcholinesterase inhibited by different concentrations of carbofuran. (b) Calibration plots showing the variation of percentage inhibition with pesticide concentration: carbofuran (●), paraoxon (○). Carrier, 2.5 mM HEPES working buffer; pH, 7.50; flow rate, 1 ml/min; temperature, 30°C; ACh, 5 mM; fluorescence probe, 1.14 μM; AChE, 0.20 mg/ml; incubation time, 10 min.

3.5. Reproducibility and precision

The relative standard deviation (RSD) for successive AChE (0.2 mg/ml) injections (n = 16) was 1.95%. An RSD (n = 7) for inhibition by 6.65 × 10⁻⁸ M carbofuran at 30°C was found to be 5.53%.

4. Analysis of pesticides in vegetable juices

The determination of carbofuran was carried out in spiked vegetable juice samples of Chinese cabbage and cole. Sample pretreatment is one of the main problems of the analytical procedure for pesticide determination of real samples [37]. Ordinarily, these compounds are extracted by organic media and concentrated afterwards, which is time consuming and liable to pesticide loss.

Table 1
Limits of detection and dynamic range of the various organophosphorous and carbamate pesticides in pure water

<table>
<thead>
<tr>
<th>Name of pesticide</th>
<th>Detection limit (μg/l)</th>
<th>Dynamic range (μg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbofuran</td>
<td>3.5</td>
<td>3.5-30</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>50</td>
<td>50-700</td>
</tr>
<tr>
<td>Paraoxon</td>
<td>12</td>
<td>12-40</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>25</td>
<td>25-250</td>
</tr>
</tbody>
</table>

Table 2
Recovery ratios of carbofuran in vegetable samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked (μg/ml)</th>
<th>Inh (%)</th>
<th>Calibration value (n = 3)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cabbage</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>11.8</td>
<td>28.5</td>
<td>12.7 ± 11</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>23.5</td>
<td>49.0</td>
<td>21.9 ± 0.2</td>
<td>93.2</td>
</tr>
<tr>
<td>Cole</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>11.8</td>
<td>24.0</td>
<td>12.8 ± 0.5</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>23.5</td>
<td>53.0</td>
<td>27.9 ± 0.9</td>
<td>118</td>
</tr>
</tbody>
</table>

In the present work, the vegetable samples were simply pretreated as described in Section 2 without using organic solvents and tedious preconcentration steps. Right after mixing 20 μl enzyme solution and 20 μl blank vegetable juice sample, 10 μl of the mixture was injected and another 10 μl was injected 10 min later for determining the pesticide residues in vegetables without pesticides spiked. Then the determination of carbofuran was carried in spiked samples. The injection of blank juice sample only resulted a little fluctuation, which indicate that this fluorescence detection method greatly eliminates the matrix effect originated by the presence of some vegetable pigments such as chlorophyll. The data summarized in Table 2 shows the recovery values being between 93.5 and 107% for Chinese cabbage juice samples, and between 108 and 118% for cole juice samples, at the two concentration levels assayed, proving a good performance of this detection method.

5. Conclusions

The development of a flow injection system using free AChE for the determination of organophosphate and carbamate pesticides is described in the present work. A highly pH-sensitive fluorescence probe was synthesized and used in the flow injection system allowing fluorescent monitoring of pH changes during the enzymatic reaction. Compared to other fluorescent compounds reported as pH-indicators, this system provides higher sensitivity. Satisfactory sensitivities (in the ppb range) and reproducibility of measurement were obtained in analyzing several organophosphate and carbamate pesticides in pure water. This flow injection system has been successfully applied to the analysis of carbofuran in vegetable samples (Chinese cabbage and cole) with good recoveries.

References


