Determination of trichlorfon residues in vegetables using a quantum dot-labeled biomimetic immunoassay method followed by capillary electrophoresis

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ABSTRACT
In this study, a molecularly imprinted polymer that could selectively recognize trichlorfon was synthesized. Using the imprinted polymer as biomimetic antibody, a novel biomimetic immunoassay-capillary electrophoresis method with improved sensitivity was developed for the detection of trichlorfon. This method was based on the competitive reaction between quantum dot-labeled trichlorfon and free trichlorfon with the biomimetic antibody. Under optimal conditions, the limit of detection (IC_{15}) and the sensitivity (IC_{50}) of the method was 0.35 ± 0.021 μg/L and 0.81 ± 0.025 mg/L, respectively. To evaluate the accuracy of the method, trichlorfon spiked in the carrot and zucchini samples was determined, and the recovery was in the range of 80.1–95.3%. This method was also applied to the quantitative detection of trichlorfon residues in the edible rape and leek samples, and the results had a good correlation with those obtained using a gas chromatography method.

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Introduction
Organophosphorus pesticides are widely used for prevention and control of plant diseases and insect pests, and they can easily enter the human body through skin and the respiratory tract. In recent years, organophosphorus pesticides have been paid much more attention globally because of their highly toxic and potentially carcinogenic properties (Zhao et al., 2014). To prevent these uncontrolled effects on human health, it is crucial to establish a rapid, accurate analysis method to detect organophosphorus pesticide residues.

There have been some reports regarding the detection methods for organophosphorus pesticide residues, such as gas chromatography (GC) coupled with tandem mass spectrometry (Chen, Chen, Feng, & Li, 2009; Pang et al., 2006), GC with flame photometric detection (Dugo, Bella, Torre, & Saitta, 2005; Liu et al., 2014), GC with nitrogen-phosphorus detection (Shen, Su, Zhu, & Gao, 2007; Tian, Liu, Fang, An, & Duan, 2014), high performance liquid chromatography (He & Lee, 2006; Seebunrueng,
Santaladchaiyakit, & Srijaranai, 2014), liquid chromatography coupled with tandem mass spectrometry (Ingelse, van Dam, Vreeken, Mol, & Steijger, 2001; Li et al., 2013; Mol, van Dam, & Steijger, 2003), chemiluminescence (Chen, Lin, Cai, Chen, & Wang, 2008; Li, He, & Xu, 2007; Li, Hu, Huo, & Xu, 2006; Wang, Zhang, Wang, Yang, & Zhang, 2001), capillary electrophoresis-quantum dot/laser induced detection (CE-QD/LIF) (Chen & Fung, 2010) and biosensor (Sun & Wang, 2010). However, these methods require expensive instruments and lengthy analytical times. The enzymatic inhibition method is also one of common methods to detect organophosphorus pesticide residue (Akkad & Schwack, 2010; Duford, Xi, & Salin, 2013; Salam et al., 2016), but the sensitivity of this method is not high, which needs the further qualitative and quantitative analysis by the GC, and the inhibiting degree of enzyme is affected by the amount of pesticides. As an alternative, immunoassay method has been developed and extensively used for rapid detection of organophosphates (Kim et al., 2011; Le, Zhu, & Yu, 2016; Liang, Liu, Liu, Yu, & Fan, 2008; Skerritt, Guihot, Asha, Bea, & Ngk, 2003; Wang, Tang, Fang, Pan, & Wang, 2011; Zhang, Wang, Yang, Du, & Lin, 2013).

Immunoassays, methods to determine trace levels in samples, have many advantages based on the specificity of the antigen–antibody reaction, such as high sensitivity, specificity, rapidity, low cost, and applicability to large numbers of samples (Li et al., 2014; Liu et al., 2012; Song et al., 2011). However, traditional immunoassay methods are difficult to perform because of the use of laboratory animals, the high cost and difficulties associated with antibody production, especially the sensitivity and instability of the biological antibody for the temperature and pH (Wang et al., 2009).

To overcome the difficulties of traditional immunoassay methods, many studies have been devoted to synthesizing antibody-like receptors. One of the most promising methods is molecular imprinting technology (Wang, Liu, Xu, Zhang, & Wang, 2007). The resulting molecularly imprinted polymer (MIP) has many advantages, such as good stability and high specific recognition ability. In recent years, certain biomimetic enzyme-linked immunoassay methods based on MIP have been studied (Fang et al., 2011; Lee et al., 2006; Meng, Qiao, Xu, Xin, & Wang, 2012; Sun, Xu, Ma, Qiao, & Xu, 2014; Tang et al., 2017; Tang, Fang, Wang, Sun, & Qian, 2013; Wang, Zhang, et al., 2011; Whitcombe, Kirsch, & Nicholls, 2014). However, natural enzymes are macromolecules, which have complicated structures and mutable characteristics. The selective recognition of MIP toward the enzyme-labeled antigen or antibody is very low, which weakens the competitive reaction and lowers the sensitivity of the method. Therefore, a marker that has a relatively small structure is necessary.

QDs, one of the nanometer-scale particles that consist of a semiconductor core, have garnered great scientific attention because of their narrow, symmetric, bright, and photo-stable fluorescent emission. Therefore, it is a better choice to make QDs as markers instead of enzymes for immunoassays. Furthermore, CE has proven to be an attractive analysis technique for various applications because of its major advantages including high efficiency and resolution in addition to low consumption of reagents and solvents (Gallartayala, Núñez, Moyano, & Galceran, 2010; Takeda et al., 2003; Zhao et al., 2014). Therefore, coupling CE with the biomimetic immunoassay technique can take advantage of the high separation efficiency of CE with the specificity of biomimetic immunoassay, and promote the sensitivity and accuracy of the biomimetic immunoassay (Li, Lu, Qiao, & Xu, 2016).
As one of the important organophosphorus pesticides and potent inhibitor of choline esterases, trichlorfon can over stimulate the nervous system and cause serious conditions including nausea, dizziness, and confusion, and respiratory paralysis and death at very high exposures (Sun et al., 2008). The target of this study was to present a new biomimetic immunoassay-capillary electrophoresis (BI-CE) method for determination of trichlorfon using MIP as the biomimetic antibody and QDs as the marker. The factors affecting the performance of this method were studied in detail, and the applicability and advantages of this developed method were evaluated.

Material and methods

Chemical and reagents

Fused-silica capillaries with 100 μm i.d. × 375 μm o.d. were purchased from the Yongnian Optic Fiber Plant (Hebei, China). The carrot and zucchini samples were purchased from the Aijia supermarket (Tai’an, China), and the edible rape and leek samples were purchased randomly from a vegetable market of Tai’an (Shandong, China), in January 2017.

Ethylene glycol dimethacrylate (EGDMA) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Methacrylic acid (MAA) and 2,2-azobisisobutyronitrile (AIBN) were purchased from Tianjin Chemical Reagent Factory (Tianjin, China), and MAA was vacuum distilled and AIBN was recrystallized before use. QDs were purchased from Jiayuan Quantum Dots Co., Ltd. (Wuhan, China). The analytical standard trichlorfon (99%) was obtained from the Institute for the Control of Agrochemicals of Ministry of Agriculture (Beijing, China). Double distilled water (DDW, 18.2 MΩ/cm) was obtained from an Aike ultrapure water instrument (Tangshi Kangning Technology Company, Chengdu, China) and used throughout the experiments. All other reagents and solvents used in this study were of the highest available purity and of analytical grade.

Solutions

The buffer solutions involving phosphate buffer saline (PBS), phosphate buffered saline with Tween-20 (PBS/T), and borate buffer saline (BBS) were prepared in DDW, and they were filtered through a 0.22-μm filter before use.

Apparatus

The CE experiments were performed on a P/ACE MDQ CE system (Beckman-Coulter, Fullerton, CA, USA) equipped with a photo-diode array detector. Data processing and acquisition were performed by Beckman 32 K software. The detection was operated at 195 nm, and the separation voltage was 25 kV. Before use, the capillary column was rinsed with 0.1 mol/L NaOH for 5 min and DDW for 5 min, and then with the running buffer solution, sodium borate solution for 20 min. Sample injection was conducted at 0.5 psi for 5 s, unless stated otherwise.

FT-IR spectra (4000–500 cm⁻¹) in KBr were recorded using a Vector 22 spectrometer (Bruker, Karlsruhe, Germany). A DTG-60AH thermogravimetric analyzer (Shimadzu, Kyoto, Japan) was also used in this study.
A Shimadzu 2010 GC equipped with a flame photometric detector (Shimadzu, Kyoto, Japan) was used for the separation and determination of trichlorfon residue in vegetables. The separation was conducted on a RTX-1701 capillary column (30 m × 250 μm i.d. × 0.1-μm film thickness). Nitrogen was used as the carrier gas at a constant flow rate of 1.0 mL/min, and the injection volume was 1.0 μL. The injection port temperature was set at 180°C at the split mode with a split ratio of 2:1. The detector temperature was held constant at 250°C. The makeup flow rate was 30.0 mL/min.

**Synthesis of trichlorfon hapten**

Trichlorfon hapten was prepared following our previous study (Meng et al., 2012). Firstly, 5.664 g of trichlorfon (22 mmol) and 2.002 g of succinic anhydride (20 mmol) were dissolved with 5 mL of anhydrous pyridine, and the yellowish-brown dope formed after magnetic stirring for 10 min and placed in a dark room for 18 h. After purging pyridine with nitrogen, 50 mL of DDW was added, and the pH was adjusted to 8.0–9.0 with a NaHCO₃ saturated solution. After extraction with ethyl acetate (3 × 30 mL), the organic fractions were evaporated to dryness under reduced pressure. The obtained crude product was dissolved in 50 mL of DDW.

**The conjugate of trichlorfon hapten and QDs**

The conjugate was prepared as follows: 72 μL of BBS (10 mM, pH 7.4) and 40 μL of trichlorfon hapten were added to the reaction container. The mixture was stirred for 5 min at room temperature. Then, 32 μL of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (10 mM, dissolved in BBS) was added, and the mixture was stirred for 15 min. When 10 μL of NH₂-QDs were added, the mixed solution reacted for 2 h at room temperature. Finally, the solution was ultrafiltered through a 0.22-μm filter for 5 times, and the end product was preserved at 2–8°C.

**Synthesis of MIP**

The MIP was prepared as follows: 0.257 g of trichlorfon (1.0 mmol) and 0.172 g of MAA (2.0 mmol) were dissolved in 3.0 mL of chloroform. After stirring for 30 min at room temperature, 0.754 mL of EGDMA (4 mmol) and 20.0 mg of AIBN were added, and the mixed solution was ultrasonicated and purged with nitrogen for 15 min, respectively. Then, the solution was incubated in a water bath at 58°C for 18 h. When the polymerization process ended, the rigid polymer was crushed and sieved with a 200-mesh sieve. The MIP particles were extracted with methanol/acetic acid (120/40, v/v) for 24 h, followed by 160 mL of methanol for 12 h. Finally, the polymer was dried in a vacuum oven at 60°C for 12 h.

For comparison, a non-imprinted polymer (NIP) was synthesized in the same way except for the addition of trichlorfon.

**Procedure of QD-BI-CE**

In this study, trichlorfon (Ag) competed with trichlorfon hapten labeled with QDs (Ag⁺) to bind to a limited amount of antibody, MIP (Ab). The reaction process was as follows: 5
mL of Ag⁺ (diluted to 1:300 with BBS) was mixed with 5 mL of trichlorfon standard solution or sample extract. When 5.0 mg of MIP was added, the mixture solution was shaken for 80 min at room temperature. After centrifugation (5000 r/min) for 20 min, the supernatant was filtered with a 0.22-μm filter membrane and injected into CE for analysis.

For a comparison, a NIP was used in place of the MIP for the above procedure.

**Sample preparation**

To check the accuracy of the QD-BI-CE method, the carrot and zucchini samples spiked with trichlorfon were detected by this method. The specific steps were as follows: the carrot and zucchini were cut into slices, and 1.0 g of each was separately weighed into 100-mL beakers, and then spiked with 1.0 mL of trichlorfon standard solution (0.5 mg/L, 1.0 mg/L, and 5.0 mg/L). After incubation for 1 h at room temperature, the spiked samples were ultrasonicated with 3 × 10 mL DDW for 30 min. After filtration with a 0.22-μm filter membrane, the extractions were transferred to a 50-mL volumetric flask, and then diluted by DDW to 50 mL. Finally, the resulting extractions were used for the QD-BI-CE procedure, and the recoveries were calculated.

To detect the concentrations of trichlorfon in edible rape and leek samples, 1.0 g of samples were separately weighted into 100-mL beakers and prepared according to the above process except for the addition of trichlorfon. Finally, the trichlorfon level was calculated.

**GC analysis**

To identify the accuracy of the QD-BI-CE method, the edible rape and leek samples were determined by GC according to the method described in GB/T 5009.20–2003 (China). Briefly, 50.0 g of the edible rape and leek samples were separately weighed into a 300-mL beaker and mashed. Then, 50 mL of DDW and 100 mL of acetone were added. The mixture was ultrasonicated for 20 min. After filtration, 100 mL of the filtrate was transferred to a 500-mL separatory funnel. After 10 g of sodium chloride was added, the separatory funnel was shaken vigorously for 3 min. The aqueous phase was separated again by adding 2 × 50 mL of dichloromethane. All of the organic phases were collected. After being condensed by a rotary evaporator, the resulting extraction was diluted to 10 mL using chromatography grade dichloromethane. Finally, the extractions were filtered using a 0.22-μm filter membrane, and 1.0 μL of the filtrate was injected into the GC for analysis.

**Statistical analysis**

The ANOVA (SAS Institute, Cary, NC, USA) test was performed to evaluate the difference between the results obtained by the QD-BI-CE and GC methods.

**Results and discussion**

**Characterization of MIP**

**FT-IR spectra analysis**

The FT-IR spectra of trichlorfon (a), the NIP (b), the imprinted polymer without extraction (c), and the imprinted polymer after extraction (d) are compared in Figure 1. For the
imprinted polymer without extraction and imprinted polymer after extraction, the observed features around 2957 and 2954 cm$^{-1}$ indicated a C–H stretch, and the features around 1723 and 1725 cm$^{-1}$ indicated a C=O stretch, which resulted from the COOH of MAA. For the FT-IR spectra of trichlorfon and the polymer without extraction, the features around 1030 and 1040 cm$^{-1}$ indicated the presence of a P=O bond of trichlorfon (Meng et al., 2011), and the stretch shift in the position of the spectrum can be attributed to the hydrophobic interaction between the –OH group of MAA and the P=O group of trichlorfon (Xu, Fang, & Wang, 2010). These results showed that trichlorfon reacted with MAA, and the MIP was synthesized.

**Thermogravimetric analysis**
Thermogravimetric analysis of the MIP is shown in Figure 2. When the temperature was increased from 0°C to 338°C, the weight of the MIP did not have an obvious decrease. However, the weight of the MIP did decrease with increasing temperature when the heating temperature was higher than 338°C. These results demonstrated that the synthesized MIP had good thermal stability at room temperature.

**Evaluation of adsorption ability of MIP**
To evaluate the adsorption ability of the MIP, the adsorption capacity (Q) of the MIP and NIP was calculated according to the following equation (Gong, Yu, Meng, Hu, & He, 2004):

$$Q = \frac{(C_0 - C_1)V}{M},$$

where $C_0$ and $C_1$ are the concentrations of trichlorfon in solution before and after adsorption, respectively, $V$ is the volume of the solution, and $M$ is the mass of the MIP or NIP. The adsorption isotherms of the MIP and NIP are displayed in Figure 3. As seen in Figure 3, the

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**Figure 1.** FTIR spectra of trichlorfon (a), the non-imprinted polymer (b), the imprinted polymer without extraction (c), and the imprinted polymer after extraction (d).
adsorption capacities of MIP and NIP template molecules increased with increasing concentration of trichlorfon, respectively. The MIP exhibited a higher adsorption capacity towards trichlorfon, which was approximately 1.79 times more than that of NIP at 600 mg/L. These results showed that the MIP had specific adsorption ability for trichlorfon.

**Optimization of the QD-BI-CE method**

To improve the precision and sensitivity of the QD-BI-CE method, the experimental conditions, such as the diluted concentration of Ag⁺, competitive reaction time, and buffer solution composition, were optimized.
The absorption of MIP for $\text{Ag}^*$ was affected by the diluted concentration of $\text{Ag}^*$. In this study, 5.0 mL of different concentrations of $\text{Ag}^*$ (diluted to 1/100, 1/200, 1/300, 1/400, and 1/500) were mixed with 5 mL of trichlorfon standard solution (0.1 mg/L) to carry out the competitive reaction. As shown in Figure 4(a), the peak area of $\text{Ag}^*$ decreased with decreasing concentration of $\text{Ag}^*$. A relatively moderate concentration (dilution ratio at 1/300) was chosen as the experimental work concentration.

The competitive reaction time can affect the combination of $\text{Ag}$ and $\text{Ab}$. The effect of different competitive reaction times (20, 40, 60, 80, 100 and 120 min) on the QD-BI-CE method was investigated. The inhibition ratio became larger as the reaction time increased until the reaction time was 80 min (Figure 4(b)), which indicated that the adsorption had reached equilibrium at 80 min. Therefore, 80 min was selected as the reaction time.

The buffer solution used for the preparation of the standard solution and samples affected the recognition ability and adsorption capacity of the MIP toward the target molecules, which directly affected the sensitivity of the method. In this study, different buffer solutions involving PBS, phosphate buffered saline with Tween-20 (PBS/T), BBS and DDW were investigated (Figure 4(c)). Experimental results indicated that in the DDW system, we obtained a better inhibition ratio and higher sensitivity. Therefore, DDW was chosen as the buffer solution.

**Selectivity of the QD-BI-CE method**

With the MIP and NIP respectively as the antibody, the QD-BI-CE standard curves for different trichlorfon concentrations from 0.1 to 10,000 $\mu$g/L were established under optimal experimental conditions (Figure 5). The results showed that the MIP had a higher inhibition rate compared to the NIP at the same concentrations of trichlorfon. With the MIP as $\text{Ab}$, the inhibition rate reached 64.45% when the concentration of trichlorfon was 10,000 $\mu$g/L, while the inhibition rate was only 34.37% when the NIP was used as $\text{Ab}$.

Cross-reactivity experiments can evaluate the specificity of the QD-BI-CE method. Monocrotophos and acephate, two structurally analogous compounds of trichlorfon, were chosen for the cross-reactivity experiments. The results in Figure S1 and Table 1 indicated that MIP had a higher selectivity for trichlorfon than the other structural analogous compounds, monocrotophos and acephate, and lower cross-reactivities were obtained, which were 14.2% and 13.7% for monocrotophos and acephate, respectively.

**Analytical parameters of the QD-BI-CE method**

The analytical parameters of the developed method for the determination of trichlorfon were evaluated under optimal conditions. The limit of detection (LOD, $\text{IC}_{15}$) and sensitivity ($\text{IC}_{50}$) of the method were $0.35 \pm 0.021 \mu$g/L and $0.81 \pm 0.025$ mg/L, respectively. According to the “Regulation (EC) No 396/2005 of the European Parliament and of the Council”, the maximum residue level of trichlorfon in primary products is 50 $\mu$g/L. Therefore, this method is sensitive enough to be used for the determination of trichlorfon.
Figure 4. (a) The peak area of Ag* at different Ag* diluted concentration ratios, (b) the influence of the competitive reaction time on trichlorfon inhibition, (c) the QD-BI-CE standard curves of trichlorfon using the MIP as the antibody at concentrations of 0.1–10,000 μg/L in PBS, PBS/T, BBS, and DDW solutions.
Accuracy and applicability evaluation of the QD-BI-CE method

To evaluate the accuracy of the proposed method, the carrot and zucchini samples spiked with trichlorfon at 0.5, 1.0, and 5.0 mg/L levels were extracted and analyzed by this method. For each concentration, measurements were performed in triplicate. The analytical data are shown in Table 2. Good recovery was achieved, which was from 80.1% to 95.3%.

To evaluate the practical application of the QD-BI-CE method, trichlorfon residues in edible rape and leek samples were detected (Figure S2). Trichlorfon was quantitatively

Table 1. The pesticide structure, IC50 and cross-reactivity (CR) of the trichlorfon and structural analogous compounds of monocrotophos and acephate.

| Pesticides      | Pesticide structure | IC50 (mg/L) | CR (%) |
|-----------------|---------------------|-------------|--------|
| Trichlorfon     | ![Pesticide Structure](https://via.placeholder.com/150) | 0.81        | 100    |
| Monocrotophos   | ![Pesticide Structure](https://via.placeholder.com/150) | 5.70        | 14.2   |
| Acephate        | ![Pesticide Structure](https://via.placeholder.com/150) | 5.91        | 13.7   |

**Table 2.** The recoveries of QDs-BI-CE method for the determination of spiked trichlorfon in the carrot and zucchini samples (n = 3).

| Samples | Spiking levels of trichlorfon (mg/L) | RSD | Recovery (%, ±RSD) |
|---------|-------------------------------------|-----|--------------------|
| Carrot  | 0.5                                 | 2.2 | 95.3 ± 2.2         |
|         | 1.0                                 | 4.6 | 87.4 ± 4.6         |
|         | 1.5                                 | 5.1 | 85.6 ± 5.1         |
| Zucchini| 0.5                                 | 4.0 | 85.5 ± 4.0         |
|         | 1.0                                 | 4.4 | 92.5 ± 4.4         |
|         | 5.0                                 | 2.7 | 80.1 ± 2.7         |
detected to be $0.027 \pm 0.001$ mg/kg and $0.507 \pm 0.025$ mg/kg in the edible rape and leek samples, respectively. In addition, a comparative analysis of the edible rape and leek samples was performed by GC (Figure S3), which detected $0.023 \pm 0.001$ mg/kg and $0.541 \pm 0.022$ mg/kg of trichlorfon, respectively. There was no significant difference between the results obtained by the above two methods ($P > 0.05$), which proved that the QD-BI-CE method had good accuracy and application for the determination of trichlorfon in agricultural products.

**Advantages of the proposed method**

Compared with the reported methods, the results obtained in this study suggested that the QD-BI-CE method has many advantages. First, the structure of QDs is small, so using QDs as a marker can enhance the competitive reaction, subsequently improving the sensitivity of the proposed method, which was much higher than that of the biomimetic enzyme-linked immunosay studied by Meng et al. (6.8 mg/L). Second, the LOD of this method is comparable to that of GC (Shen et al., 2007) and is lower than that of the reported CE-QD/LIF method (Chen & Fung, 2010). Therefore, our method has potential to be used as a tool to detect trace levels of trichlorfon in food samples. Third, a cycle of this method lasts 110 min, while a complete procedure of the traditional chromatography methods is usually 180 min, so our procedure is more efficient. Finally, MIP used as the antibody is stable and can be reused for eight times without a loss in sensitivity, which also reduces the costs of analysis.

**Conclusion**

In this study, we developed a BI-CE method based on QD labels, and this method was successfully applied to the determination of trichlorfon in vegetables. With good accuracy and improved sensitivity, our study has provided a new analytical method for the quick determination of organophosphorous pesticide residues in food.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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