A highly sensitive bio-barcode immunoassay for multi-residue detection of organophosphate pesticides based on fluorescence anti-quenching

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1. Introduction

Organophosphate pesticides (OPs) are among the most widely used insecticides in agricultural production, protecting crops from pest damage and maintaining high and stable production yields.

OPs were developed to replace organochlorines; however, many of them are more toxic than the compounds they were meant to replace. Organophosphate residues can reach lakes, rivers, and other water bodies through direct runoff. Therefore, long-term exposure to large quantities of OPs may cause significant harm to ecosystems and human health [1]. Dietary exposure to OPs exceeding the maximum tolerated dose might inhibit acetylcholinesterase activity, causing the accumulation of the neurotransmitter acetylcholine, leading to prolonged overstimulation of cholinergic receptors and causing cholinergic toxicity, such as kinesthesia and respiratory failure [2]. Moreover, the widespread...
use of OPs in the environment may cause soil acidification and compaction, accelerating soil degradation [3]. The bioaccumulation of OPs may exert a tremendous toxic effect on human and animal health [4]. Pesticide residues are receiving much attention in agricultural safety studies. Balancing the risks and benefits of OPs relies partly on their accurate measurement to ensure human and environmental health. Thus, it is essential to develop simple, rapid, sensitive, and precise methods to determine OP residues in different matrices. So far, various techniques, such as immunoassays, spectroscopy, and chromatography, have been used for the qualitative and quantitative detection of residual OPs. Enzyme-linked immunosorbent assay [5] provides a reliable method for the qualitative and semi-quantitative detection of pesticide residues with simplicity, high selectivity, and reproducibility. Chemiluminescence immunoassay [6] has the advantages of being simple, rapid and safe, and having high sensitivity, short detection time, wide detection range and high sensitivity. Meanwhile, fluorescence immunoassay [7] has the advantages of having high specificity, high sensitivity, a wide range of standard curves, fast analysis, easy preparation of the markers, a long effective use period, and no radioactive contamination. The biosensor method has the advantages of having an excellent analytical performance with high analytical accuracy and specificity, but it can only perform semi-quantitative analysis [8]. Chromatographic methods can provide the required high sensitivity and accuracy according to the requirements of government agencies, such as the European Commission, The United States Department of Agriculture, and the Ministry of Agriculture of China. However, in many cases, they cannot detect trace residues due to high detection limits, expensive equipment, and lengthy analysis time [9].

The bio-barcode assay (BCA) was established to simultaneously detect multiple targets in a single run [10]. It is based on the relationship between the DNA barcoding strand and the target protein, which the bio-barcoding technology utilizes to perform multi-residue detection of macromolecular compounds. The corresponding DNA barcode can be designed according to the probe captured by the target. For instance, Stoeva et al. [11] designed nanoparticle probes (NP) to simultaneously detect three tumor protein markers. Additionally, Li et al. [12] designed probes with different types of fluorescence labels and used NP-DNA to detect five DNA sequences and their sources simultaneously. Further, Lin et al. [13] used bimetallic NP for a novel nanoenzyme-based fluorescence amplification method based on biological barcodes that allow simultaneous detection of the RNAs of human immunodeficiency virus and hepatitis C virus. This method has shown excellent detection characteristics. The studies mentioned above have shown that biological barcode detection can be applied to multi-residue detection of macromolecular compounds. In the last few years, the application of colloidal gold and biological barcode signal amplification in immunoassays has attracted much attention due to the unique optical, chemical, catalytic, and electronic properties of colloidal gold [14–17]. Gold nanoparticles (AuNPs) can easily function with biomolecules without affecting their biological activity [18,19]. Using biomolecules (including aptamers [20], amino acids [21], and proteins [22]), electrochemistry [23], colorimetry [24], and fluorescence quenching [25] are the current signal amplification strategies for AuNPs. Bio-barcodes have also been applied in ultra-sensitive immunoassays [26–28].

Fluorescence signals generated during the BCA are due to the activity of RNase H, which explicitly recognizes A-form RNA and B-form DNA, causing the fluorescent group to modify the RNA strand and subjecting it to the inhibitory effect of the quenching group, thus emitting fluorescence at a specific wavelength. A fluorophore is a label with relatively high fluorescence intensity that can also emit fluorescence at a specific wavelength without enhancing or inhibiting the fluorescence of other labels. The particles of the colloidal gold solution are made up of a gold core and a double ion layer around it. The inner layer is an anion layer dominated by tetrachloroaurate ions tightly connected to the surface of the gold core, while H⁺ forms the outer ion layer. Colloidal gold is produced by reducing chloroauroic acid (HAuCl₄) with trisodium citrate dehydrate. Biological barcoding uses covalent binding to label single-stranded DNA (ssDNA) chains and antibodies on colloidal gold. However, in labeling ssDNA and antibodies, the instability of charges can easily lead to the irreversible aggregation of gold particles. It has been reported that ultrasensitive immunoassay based on biological barcode nanoparticles and oligonucleotide signal amplification. For instance, Lin et al. [29] developed an ultrasensitive immunoassay for protein biomarkers based on electrochemiluminescent quenching of quantum dots by hemin bio-bar-coded nanoparticle tags. Moreover, Zhang et al. [30] developed a high-sensitivity method for detecting parathion using a competitive biological barcode immunoassay based on bimetallic nano enzyme catalysis. Additionally, our research group has successfully designed various detection and analytical techniques based on colloidal gold and biological barcodes based on the enzyme-linked immunoassay.

Herein, we developed a highly sensitive bio-barcode immunoassay based on fluorescence anti-quenching to simultaneously determine multi-residual levels of OPs, including triazophos, parathion, and chlorpyrifos in apples, turnips, cabbages, and rice. Three colloidal gold nanoprobes were synthesized using different sequences of ssDNA. Each probe hybridized with its complementary sequence and was labeled with a fluorescent moiety. The RNA strands were hybridized and paired, and the fluorescent groups were hydrolyzed by RNase H to generate a fluorescent signal at a specific wavelength. The developed bio-barcode was applied to detect low residual levels of OPs in agricultural products.

2. Experimental

2.1. Materials and reagents

Polyethylene glycol 20000 (PEG 20000), Tris-EDTA (TE) buffer (pH 7.4), ovalbumin (OVA), tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), and bovine serum albumin (BSA) were provided by Sigma-Aldrich (St. Louis, MO, USA). Institute of Pesticide and Environmental Toxicology (Zhejiang University, Zhejiang, China) donated antigens (triazophos, parathion, and chlorpyrifos) and their monoclonal antibodies at a concentration of 4.53, 7.57, and 6.31 mg/mL, respectively. Dr. Ehrenstorfer GmbH (Augsburg, Germany) supplied triazophos, parathion, and chlorpyrifos (purity, 98%) and their structural analogs (>95%) standards. RNase H was procured from Takara (Kusatsu, Japan). Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) supplied all oligonucleotides. Octadecyltrimethoxysilane (C₁₈, 40–60 μm) and primary-secondary amine (PSA, 40–60 μm) were acquired from Bonna-Agela Technologies (Tianjin, China).

2.2. Design of oligonucleotides

In a previous study, our group developed an immunoassay method for detecting triazophos based on DNA/RNA hybridization using barcoding identification [31]. Herein, we continue to explore the application of the technique for the multi-residue determination of OPs. The design of the oligonucleotide sequence was based on a previous protocol [32]. Table 1 shows three types of synthesized oligonucleotide sequences, three types of 5’-end modified sulfhydryl DNA strands, three types of 3’-end modified quenching
groups (black hole quencher-1, BHQ-1), and three types of 5’-end-modified 6-carboxyfluorescein (6-FAM) RNA strands.

2.3. Preparation of three different oligonucleotide-colloidal gold probes

The preparation of colloidal gold nanoparticles was modified based on a previously reported method [31]. The characterization of nanoparticles and their particle size distribution are shown in Fig. S1. The solution was cooled down to room temperature (25 °C) and then stored at 4 °C for later use. Three tubes with different ssDNA powder samples were centrifuged according to the manufacturer’s protocols. Then, 46 μL each of TE buffer and TCEP solution were added, and placed on an oscillator for activation for 3 h. The prepared colloidal gold solution was filtered through a 0.22-μm polyethersulfone membrane (Jintang Experimental Equipment Co., Ltd., Tianjin, China). Then, 1 mL of colloidal solution was pipetted into three centrifuge tubes, to which 30 μL of K2CO3 solution (pH 9.0) was added. After 15 min, antibodies against triazophos, parathion, and chlorpyrifos, respectively, were added, and the solutions were mixed thoroughly and slowly. One hour later, the corresponding activated oligonucleotide chain solution was added, and the solution was slowly and repeatedly blown to mix thoroughly, and was placed in a 4 °C refrigerator for 16 h. The mixture was equilibrated for approximately 1.5 h at room temperature, then 30% PEG 20000 was added at a final concentration of 0.5% and diluted 20 times. The solution was slowly and repeatedly blown to mix thoroughly. A volume of 0.1 mol/L phosphate-buffered saline (PBS) solution was added every 2 h for 6 times so to a final concentration of 0.01 mol/L. Then, the three solutions were kept at 4 °C for more than 8 h. After the aging process, the solution was placed at room temperature, and then a volume of 10% BSA blocking solution was added to a concentration of 1%. Blocking was done for 1 h. After centrifugation at 13,000 r/min for 15 min, the supernatant was removed, and the residue was redisolved in 400 μL of probe solution and then stored at 4 °C.

2.4. Sample preparation

The samples were prepared based on the modified quick, easy, cheap, effective, rugged, and safe “QuEChERS” method [32]. The tested samples, including apples, turnips, cabbages, and rice, were procured from the local market (Beijing, China). All samples were confirmed to be free from the tested OPs (triazophos, parathion, and chlorpyrifos) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, homogenized samples (5 g of sample + 5 mL of water) weighed into a 50 mL centrifuge tube were spiked with three different concentrations of the tested analytes (mixed quasi-solutions) at a concentration of 10, 50, and 100 μg/kg. The mixture was left to stand for at least 30 min. Afterwards, 10 mL of acetonitrile was added and mixed on a Vortex® (Scientific Industries, Bohemia, NY, USA) for 5 min. Next, 4 g of NaCl and 1 g of anhydrous MgSO4 were added and mixed for 5 min to remove water [31]. After that, the tubes were centrifuged at 5,000 r/min for 5 min. Subsequently, 3 mL of supernatant was added to a centrifuge tube containing 150 mg of PSA (40–60 μm), 150 mg of trimethoxyoctadecylsilane (C18, 40–60 μm), and 450 mg of MgSO4, and centrifuged at 5,000 r/min for 5 min. Finally, the extract was filtered through a 0.22-μm membrane filter (Jin Teng, Tianjin, China) for further detection. Half of the supernatant was analyzed by LC-MS/MS for residue analysis. On the other hand, 100 μL of the solution was concentrated under a stream of nitrogen, then reconstituted with 1 mL of 5% methanol-PBS solution, and assayed using the established bio-barcoding method [33].

2.5. Detection of multi-residual OP levels using a bio-barcode amplification strategy

The detection procedure for OPs (triazophos, parathion, and chlorpyrifos) was as follows: A volume of 100 μL (per well) of triazophos OVA-hapten, parathion OVA-hapten, and chlorpyrifos OVA-hapten at a dilution of 16,000, 8,000, and 8,000 times in 0.01 mol/L PBS solution was added into a 96-MicroWell™ black plate and then incubated at 4 °C overnight. Subsequently, the coated plate was washed with PBST (0.01 mol/L PBS, 0.05% Tween-20, 300 μL for thrice), and blocking buffer (300 μL, 1% BSA in 0.01 mol/L PBS) was added and incubated at 37 °C for 2 h. After blocking, the plate was washed three times with PBST. The probe solutions (for triazophos, parathion, and chlorpyrifos; 50 μL, diluted with 5% methanol-PBS or sample extract) and ssDNA were mixed. AuNPs–monoclonal antibodies (mAbs) complex (50 μL, diluted) was added to 0.01 mol/L PBS solution and incubated at 37 °C for 1 h. Then, the mixture was washed three times with PBST to remove the unbound mixed probe and ssDNA-AuNPs–mAbs complex. The ssDNA-AuNPs–mAbs complex coated on the plate was used in the subsequent reaction. Then, 100 μL of RNase H reaction solution (Tris-HCl buffer, pH 7.5), consisting of 10 mmol/L DTT, 6 mmol/L MgCl2, 0.1 μmol/L RNA probes (FAM-CCCAUGAGU-BHQ1, N,N’-(dipropyl)-tetramethyldi carbocyanine (Cy3)-CACUGUGGUGU-...
and BHQ1, and N,N’-(dipropyl)-tetrabenzyldiodocarbocyanine (Cy5)-
CUACAACGCU-BHQ1), and 6 U of ribonuclease H (RNase H) (Takara,
Kusatsu, Japan) were added to the system to generate a fluorescent
signal. The reaction was carried out at 37 °C for 90 min. Infinite
M200 PRO microplate reader (TECAN, Männedorf, Switzerland)
was used for detection. We investigated FAM, Cy3, and Cy5 as
fluorescent labels and measured their fluorescent signals at specific
wavelengths. The fluorescence intensity selected for detection was
Ex 460 nm/Em 520 nm, Ex 532 nm/Em 568 nm, and Ex 640 nm/Em
675 nm.

2.6. Spiked recovery experiment

To evaluate the feasibility of the developed method, the sam-
ple, including apples, cabbages, radishes, and rice, were secured
from a local farmer’s market. According to GB 2763-2021, the
maximum residue limits (MRLs) of triazophos and chlorpyrifos is
50 µg/kg. In contrast, the MRL for parathion is 10 µg/kg for all
vegetables. The samples were tested using LC-MS/MS to ensure
they were free from the tested analytes. The conditions for LC-MS/
MS are shown in the Supplementary data, and relevant MS pa-
rameters of the three pesticides are shown in Table S1. The samples
were processed as described in Section 2.4. Homogenized samples
were spiked with three different concentrations of the tested
analytes (mixed quasi-solutions of triazophos, parathion, and
chlorpyrifos) at 10, 50, and 100 µg/kg.

2.7. Statistical analyses

OriginPro 8.5 for Windows was used for data analysis. Each
experimental data obtained throughout the study was replicated at
least three times. Fluorescence intensity is calculated using the
following equation:

\[
F_i = \frac{F_{\text{max}} - F_x}{F_{\text{max}} - F_0} \times 100%
\]

Where \( F_i \) represents the inhibition rate of fluorescence intensity,
\( F_{\text{max}} \) represents the maximum fluorescence intensity (the reaction
system without adding triazophos, parathion, or chlorpyrifos), \( F_x \) is
the fluorescence intensity related to the concentration of the tested
analytes (triazophos, parathion, and chlorpyrifos), and \( F_0 \) is the
fluorescence intensity value of blank wells. The limit of detection
(LOD) was determined as the concentration of the tested analyte
that produced 10% inhibition of the maximum fluorescence.

![Fig. 2. Optimization of antibodies against the tested organophosphate pesticides.](image)

![Fig. 3. Cross-reactivity of the assay system.](image)

![Fig. 4. Optimizing the immunoreagent dilution of (A) ovalbumin (OVA)-haptens and (B) the ssDNA-gold nanoparticles (AuNPs)-monoclonal antibodies (mAbs) complex.](image)
3. Results and discussion

3.1. Fluorescence intensity of cross-reaction between fluorescent markers

We investigated FAM, Cy3, and Cy5 as fluorescent labels and measured their fluorescence signals at specific wavelengths. As shown in Fig. 1, these fluorophores had the highest fluorescence intensity at Ex 460 nm/Em 520 nm, Ex 532 nm/Em 568 nm, and Ex 640 nm/Em 675 nm, respectively, and their mutual influence was negligible. Hence, FAM, Cy3, and Cy5 were selected as labels on the RNA strand for the detection method.

3.2. Optimization of monoclonal antibody loading on gold nanoparticles

In this experiment, if there were few antibody labels on the colloidal gold, the antibodies would have a lower capacity to specifically bind to the coated antigen or small pesticide molecules on the 96-well plate. On the other hand, if there were too many antibodies, the colloidal gold might also be labeled. If the antibody occupied the colloidal gold, it would lower its affinity to the ssDNA chain, thereby reducing the sensitivity of the reaction. We optimized the preparation of colloidal gold probe and triazophos, parathion, and chlorpyrifos antibodies. We added 2, 4, 6, 8, 10, and 12 μL of antibodies to determine their respective optimal dosages. As shown in Fig. 2, the highest inhibition rate was observed at 4 μL for the parathion antibody. The inhibition rate decreased when the concentration was >4 μL. As the amount of chlorpyrifos antibody increased, the inhibition reaction first increased and then decreased. The inhibition rate was the highest at 8 μL, where sensitivity was also the highest. Thus, the optimal antibody dosages when preparing the colloidal gold probes were 4, 4, and 8 μL for triazophos, parathion, and chlorpyrifos, respectively.

3.3. Cross-reactivity of the assay system

While specific binding of antigen and antibody is a prerequisite to successfully developing immunoassays for multi-residue detection, particular hybridization of complementary DNA and RNA plays a vital role. The pesticide concentration could be too high or too low if the antibody cannot recognize the corresponding antigen in a mixed hapten system. If ssDNA and RNA cannot be hybridized according to the complementary base pairing rule, the hybrid strand cannot be synthesized, and RNase H cannot specifically hydrolyze the RNA strands in the hybrids. Under the effect of inhibitors, the fluorescent group cannot be excited to emit fluorescence even at the appropriate wavelength, resulting in false-negative results. Based on optimization experiments, the OVA-hapten of triazophos, parathion, and chlorpyrifos were diluted 16,000, 8,000, and 8,000-fold, respectively. After dilution, the OVA-hapten concentrations of triazophos, parathion, and chlorpyrifos were 0.28, 0.95, and 0.79 µg/L, respectively. The colloidal gold nanoparticles were diluted 30-fold, and 5 ng/mL OPs was added to each group. Since this cross-reaction was explored in a multi-residue detection system, the experimental work was divided into three groups, exploring the reaction of single half antigen with single and mixed DNA probes, single and mixed RNA strands, as well as mixed hapten. The wavelengths for detecting triazophos, parathion, and chlorpyrifos were 460/520, 532/568, and 640/675, respectively. The results from the parathion group showed that the fluorescence values detected by different combinations are generally similar (Fig. 3). In contrast, triazophos and chlorpyrifos groups with mixed OVA-hapten, mixed colloidal gold nanoprobe, and mixed RNA chain displayed slightly higher fluorescence values than a combination of single OVA-hapten, single colloidal gold nanoprobe, and single RNA chain. However, the

### Table 2

| Pesticides  | Linear equation | Correlation coefficient | Linear range (ng/mL) | IC₅₀ (ng/mL) | LOD (ng/mL) |
|------------|----------------|------------------------|----------------------|-------------|-------------|
| Triazophos | y = 22.277x + 51.347 | 0.977                 | 0.01–25             | 0.870       | 0.014       |
| Parathion  | y = 22.096x + 52.976 | 0.971                 | 0.1–50              | 0.733       | 0.011       |
| Chlorpyrifos | y = 23.283x + 30.976 | 0.968                 | 0.1–50              | 6.563       | 0.126       |

LOD: limit of detection.

### Table 3

| Pesticides | Samples | Spiked concentration (µg/kg) | Bio-barcode immunoassay | LC-MS/MS |
|------------|---------|-----------------------------|------------------------|----------|
|            |         | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| Triazophos | Apple   | 10 | 104.4 | 13.9 | 92.6 | 4.66 |
|            | 50      | 92.1 | 9.8 | 109.0 | 2.23 |
|            | 100     | 97.1 | 11.1 | 96.2 | 3.26 |
|            | Orange  | 10 | 96.3 | 13.7 | 104.0 | 6.22 |
|            | 50      | 86.4 | 8.62 | 104.0 | 3.71 |
|            | 100     | 92.5 | 10.4 | 101.0 | 6.98 |
|            | Pear    | 10 | 100.7 | 12.6 | 105.0 | 6.70 |
|            | 50      | 87.6 | 7.6 | 109.0 | 3.02 |
|            | 100     | 99.0 | 8.4 | 79.4 | 4.29 |
|            | Cabbage | 10 | 109.1 | 15.6 | 98.0 | 5.13 |
|            | 50      | 101.2 | 11.7 | 109.0 | 3.87 |
|            | 100     | 91.4 | 7.5 | 104.0 | 3.17 |
|            | Rice    | 10 | 105.8 | 10.4 | 89.0 | 8.80 |
|            | 50      | 96.1 | 9.4 | 94.4 | 9.62 |
|            | 100     | 86.3 | 12.2 | 87.4 | 4.35 |
|            | Orange  | 10 | 82.4 | 14.4 | 89.4 | 3.60 |
|            | 50      | 80.3 | 7.3 | 92.5 | 5.40 |
|            | 100     | 81.4 | 8.5 | 101.9 | 2.76 |
|            | Pear    | 10 | 83.7 | 13.5 | 90.4 | 7.81 |
|            | 50      | 86.6 | 15.2 | 93.1 | 6.69 |
|            | 100     | 83.2 | 7.4 | 89.7 | 5.36 |
|            | Cabbage | 10 | 86.2 | 17.6 | 95.8 | 4.68 |
|            | 50      | 80.3 | 9.0 | 95.6 | 4.17 |
|            | 100     | 88.5 | 9.2 | 93.7 | 2.31 |
|            | Rice    | 10 | 84.6 | 15.5 | 96.1 | 8.45 |
|            | 50      | 82.0 | 11.0 | 98.3 | 1.92 |
|            | 100     | 87.1 | 8.6 | 89.2 | 3.68 |
|            | Orange  | 10 | 94.4 | 16.0 | 90.0 | 3.34 |
|            | 50      | 102.1 | 10.9 | 97.9 | 7.76 |
|            | 100     | 89.9 | 7.6 | 101.8 | 5.18 |
|            | Pear    | 10 | 92.0 | 12.9 | 104.4 | 8.35 |
|            | 50      | 87.4 | 7.4 | 90.6 | 6.76 |
|            | 100     | 110.8 | 12.8 | 91.5 | 4.14 |
|            | Cabbage | 10 | 84.4 | 11.9 | 101.8 | 3.82 |
|            | 50      | 98.3 | 10.6 | 98.3 | 1.09 |
|            | 100     | 95.6 | 9.2 | 85.7 | 2.97 |
|            | Rice    | 10 | 90.6 | 14.3 | 88.6 | 7.56 |
|            | 50      | 95.0 | 8.5 | 99.1 | 8.47 |
|            | 100     | 88.2 | 10.5 | 92.7 | 5.22 |

RSD: relative standard deviation.
difference was insignificant, and the cross-reactivity can be considered to be within the acceptable range and the method can be applied to pesticide multi-residue detection.

3.4. Optimization of dilution factor for OVA-hapten and colloidal gold probe in multi-residue bio-barcode immunoassay

To establish a more efficient and accurate multi-residue detection system, the optimal dilution of immunoagents in various pesticide systems (triazophos, parathion, and chlorpyrifos) used a 10 mM PBS solution. The lower the IC50 value and the higher the maximum, the higher the sensitivity and accuracy of the experimental system. In Fig. 4, the various pesticide systems (triazophos, parathion, and chlorpyrifos) IC50 values reached the maximum. The lowest IC50 values (sensitivity) were obtained when triazophos OVA-hapten was diluted 16,000-fold, parathion and chlorpyrifos OVA-hapten were diluted 8,000-fold, and all three colloidal gold nanoprobes were diluted 30-fold.

3.5. Establishment of the standard curve for the multi-residue system

The stock solutions of the three pesticides were diluted to a series of concentrations from 0.01 to 50 ng/mL, using 5% methanol-PBS (0.01 mol/L) solution. Then, the experiments were carried out according to Section 2.3. The whole experimental system contains mixed OVA-hapten and pesticide. The colloidal gold nanoprobe explicitly recognizes the target OVA-hapten and pesticide small molecules. The plate wash solution did not wash away the probe bound to the OVA-hapten on top of the 96-well plate, thus remaining in the experimental system, participating in the hybridization reaction, and dissociating the fluorescent group on the RNA strand down by the action of RNase H. The fluorescence signal was detected at a specific wavelength. It is worth noting that the higher the pesticide concentration in the system, the probability of the probe binding to pesticide increases. The likelihood of binding to the OVA-hapten on the well plate decreases, resulting in a lower value of the detected fluorescence signal. The final detected fluorescence value in the experimental system was negatively correlated with the pesticide concentration. The standard curve is plotted using the log value of the pesticide concentration as the horizontal coordinate and the fluorescence inhibition rate as the vertical coordinate. From Table 2, it can be seen that the three pesticides, namely, triazophos, parathion, and chlorpyrifos, showed good linearity in the ranges of 0.01–25, 0.1–50, and 0.1–50 ng/mL, respectively. The established method showed a sensitivity of 0.870 ng/mL and LOD of 0.014 ng/mL for triazophos. The sensitivity for parathion was 0.733 ng/mL with the LOD of 0.011 ng/mL, and the sensitivity for chlorpyrifos was 6.563 ng/mL with the LOD of 0.126 ng/mL.

In summary, the established DNA/RNA hybridization-based multi-residue bioassay method could simultaneously detect the residues of the three pesticides in the range of 0.011–0.126 ng/mL. It could achieve the accurate detection of multiple pesticides with high sensitivity.

### 3.6. Spiked recovery experiment

To verify the accuracy of the DNA/RNA hybridization-based biological barcode method for detecting OPs in agricultural products, apples, oranges, pears, cabbages, and rice were purchased from local farmers’ markets and checked to be free from pesticide residues by LC-MS/MS. The samples were processed according to Section 2.6. The mixed standard solutions of the

| Method | Pesticide | Spiked samples | Recovery (%) | RSD (%) | Linear range (ng/mL) | LOD (ng/mL) | IC50 (ng/mL) | Ref. |
|--------|-----------|----------------|--------------|---------|----------------------|-------------|--------------|------|
| CFBBIA | Triazophos| Water, rice, cucumber, apple, and cabbage | 96.1 | 12.3 | 0.01–20 | 0.006 | – | [31] |
| LFIC   | Parathion, parathion-methyl, and fenitrothion | Cucumber, tomato, and orange | 67–120 | ≤19.54 | 0.98–250 | – | 3.44, 3.98, 12.49 |
| CCLEIA | Triazophos | Lettuce, apple, carrot, water, and soil | 100.7 | 19.8 | 0.04–5 | 0.063 | 0.87 |
| BI     | Tri chlorfon and chlorpyrifos | Orange and carrot | 77.8–92.0 | ≤4.0 | 1–100000 | 18.0, 19.0 | 5.860 | – |
| FPIA   | Parathion, phoxim, coumaphos, quinamphos, and triazophos | Water, cowpea, and leek | 103.1 | 10.1 | 16.09–512 | 11000, 9000 | – |
| ELISA  | Parathion-ethyl, fenamiphos, triazophos profenofos, and acephate | Cabbage and lettuce | 85.8–105.5 | ≤10.4 | – | 354, 527, 2218, 675, 261 |
| BA-IA  | Triazophos, carbofuran, and chlorpyrifos | Cabbage, carrot, and spinach | 84.5 | 12.3 | 0.02–50, 0.5–500, 1.0–1000 | 0.024, 0.93, 1.68 | – |
| CLEIA  | Parathion, parathion-methyl, and fenitrothion | Apple, Chinese cucumber, and rice | 73–118 | 3.35–10.12 | 0.19–100, 0.10–25 | 5.43, 1.34, 1.24 |
| BBC-IA | Triazophos | Apple, cabbage, orange, and rice | 89.5 | 15.5 | 0.04–10 | 0.020 | – |
| CIA-BBC | Triazophos | Apple, cabbage, orange, and rice | 92.1 | 14.0 | 0.015–4 | 0.014 | – |
| CFAQIA-BBC | Triazophos, parathion, and chlorpyrifos | Apple, cucumber, cabbage, pear, and orange | 80.3–110.8 | 7.3–17.6 | 0.01–25, 0.1–50, 0.1–50 | 0.014, 0.011, 0.126 | 0.870, 0.733, 6.563 | This work |

| CFBBIA: competitive fluorescence bio-barcode immunoassay; LFIC: lateral flow immunochromatographic assay; CCLEIA: competitive chemiluminescent enzyme immunoassay; BI: biomimetic immunoassay; FPIA: fluorescent polarization immunoassay; ELISA: enzyme-linked immunosorbent assays; BA-IA: bead-array competitive immunoassay; CLEIA: chemiluminescence enzyme immunoassay; BBC-IA: bio-barcode amplification immunoassay; CIA-BBC: colorimetric immunoassay based on bio-barcode; CFAQIA-BBC: competitive fluorescence anti-quenching immunoassay based on bio-barcode. |
three OPs (triazophos, parathion, and chlorpyrifos) were diluted with 5% methanol-PBS solution to final concentrations of 10, 50, and 100 µg/kg, respectively. The spiked recovery experiments were performed using the established method and LC-MS/MS. The results in Table 3 show that the spiked recoveries obtained using the established method ranged from 80.3% to 110.8%, with relative standard deviation (RSD) values ranging from 7.3% to 17.6%. The results obtained using LC-MS/MS ranged from 79.4% to 109.0%, with RSD values ranging from 1.09% to 9.62%. The results detected by the two methods were consistent, so the developed method was highly accurate for detecting OPs in agricultural products.

3.7. Comparison with bio-barcode immunoassay based on fluorescence anti-quenching

We established a bio-barcode immunoassay based on fluorescence anti-quenching. We set an intuitive correlation with the proposed method for detecting OPs. The standard curves of LC-MS/MS for five samples are shown in Table 4. The results for detecting OP residues in apples are shown in Fig. S2. The differences between the designed method and other immunoassays in terms of linear range, detection limit, IC50, spiked recovery, and RSD are summarized in Table 5 [33–42].

4. Conclusions

This study established a biological barcode multi-residue immunoassay based on DNA/RNA hybridization to simultaneously detect triazophos, parathion, and chlorpyrifos. Five agricultural products were selected for recovery to verify the accuracy of the developed method. The recovery rate obtained by the established method was 80.3%–110.8%, and the RSD value was 7.3%–17.6%. In contrast, the recovery rate and the RSD were 79.4%–109% and 1.09%–9.62% using LC-MS/MS. The results obtained by the two methods correlated well, indicating that the method has good accuracy and reliability. Therefore, this method can simultaneously quantify triazophos, parathion, and chlorpyrifos in one run.

**CRediT author statement**

**Lingyuan Xu:** Writing - Original draft preparation, Investigation, Conceptualization, Methodology; **Xiuyuan Zhang:** Conceptualization, Investigation, Methodology, Visualization; **A.M. Abd El-Aty:** Validation, Formal analysis, Writing - Reviewing and Editing; **Yuanshang Wang:** Investigation; **Zhen Cao:** Investigation; **Huiyan Jia:** Investigation; **J.-Pablo Salvador:** Validation, Writing - Reviewing and Editing; **Ahmet Hacimuftuoglu:** Investigation; **Xueyan Cui:** Investigation; **Yudan Zhang:** Investigation; **Kun Wang:** Investigation; **Yongxin She:** Investigation; **Fen Jin:** Investigation; **Lufei Zheng:** Supervision, Funding acquisition; **Baima Puja:** Investigation; **Jing Wang:** Funding acquisition, Supervision; **Maojun Jin:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision; **Bruce D. Hammock:** Validation, Writing - Reviewing and Editing, Funding acquisition.

**Declaration of competing interest**

The authors declare that there are no conflicts of interest.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2022.05.004.

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