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**A Ultrasensitive Immunoassay based on Weakly Charged AuNPs orientated
Antibody for Triazophos Detection in Vegetables**

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Abstract: Immunoassays based on gold nanoparticles (AuNPs) often suffer from limited sensitivity. Herein, we synthesized weakly charged AuNPs using ascorbic acid and establish a competitive immunoassay for the detection of triazophos. The weak electronegativity of these AuNPs reduces electrostatic repulsion during antibody adsorption, leading to increased antibody loading and favorable antibody orientation, thereby enhancing detection sensitivity. The triazophos antibody and horseradish peroxidase (HRP) were electrostatically adsorbed onto the AuNP surface to form an antibody-AuNP-enzyme probe complex. In the immunoassay, triazophos in the sample competes with the coated antigen for binding to the Ab@AuNPs@HRP probe, and quantification is achieved through an HRP-catalyzed colorimetric reaction. The proposed method demonstrated a linear range of 1.08–22.48 $\mu\text{g/L}$, a sensitivity of 4.93 $\mu\text{g/L}$, and a detection limit as low as 0.06 $\mu\text{g/L}$. This approach eliminates the need for enzyme-labeled secondary antibodies, offering advantages such as lower cost, simpler operation, higher sensitivity, and good specificity.

Keywords: AuNPs; HRP; Triazophos detection; Competitive Immunoassay; Ab@AuNPs@HRP

Introduction

Triazophos is a broad-spectrum organophosphorus insecticide that was once widely used in agricultural production [1]. However, its neurotoxicity in mammals and persistent residues in the environment have raised serious concerns regarding food safety and ecological health [2-4]. Consequently, numerous regulatory agencies worldwide have implemented bans or strict restrictions on its use. Nonetheless, the risks associated with its residues remain complex and persistent in practice [5-7]. Accordingly, rapid, sensitive, and robust analytical tools for tracing triazophos residues are now indispensable to protect consumer health and reinforce environmental surveillance.

Conventional pesticide-residue analysis relies on chromatographic and chromatography-mass-spectrometry platforms [8-11]. Although these methods offer high sensitivity and accuracy, they typically require complex sample pretreatment, expensive instrumentation, and specialized operators, making them unsuitable for onsite, rapid, and high-throughput screening. Immunoassays, particularly ELISA [12-14], have considerable advantages because of their simple operation, low cost, and suitability for rapid screening of large sample batches. AuNPs are extensively used as immunosensing probes owing to their unique optical properties, high surface-to-volume ratio, good biocompatibility, and ease of functionalization. However, traditional AuNP-based ELISA often lacks sufficient sensitivity [15, 16]. To address the sensitivity limitation, researchers have developed various strategies, such as the use of novel nanolabels or the introduction of signal amplification systems [17-21]. Nonetheless, these amplification strategies often require additional steps, reagents, or equipment and involve complex preparation processes. Balancing improved sensitivity with maintained convenience,

cost-effectiveness, and time efficiency remains a key challenge in current research.

The preparation of antibody-conjugated gold nanoparticles is central to nanoparticle-based immunoassays, and the pH of the reaction buffer governs the coupling efficiency. When the buffer pH is markedly below the isoelectric point (pI) of the antibody, the protein carries a high positive charge. Under these conditions, a single IgG molecule can interact with several AuNPs, and each nanoparticle can bind multiple antibodies, resulting in extensive cross-linking and the formation of inactive aggregates. At the pI, the solubility of the antibody is minimal; intermolecular electrostatic repulsion collapses, promoting precipitation. Conversely, when the pH exceeds the pI, the antibody is strongly negatively charged and experiences pronounced electrostatic repulsion with the citrate-capped AuNPs, which are also negatively charged. This repulsion lowers the labeling efficiency and, under extreme conditions, can completely prevent protein adsorption[22]. Consequently, the buffer pH is routinely set slightly above the antibody pI, imparting a moderate net negative charge that preserves structural integrity, prevents over-conjugation-induced aggregation, and maintains a level of electrostatic attraction compatible with efficient surface loading [23].

Conventional immunoassays rely on citrate-stabilized AuNPs that present a highly ionized, strongly charged interface at neutral pH. Because the pI of most murine and rabbit IgG subclasses lies near pH 8, the coupling reaction is typically performed at pH approximately 8.5 to minimize charge-driven aggregation. However, the dense negative layer generated by citrate ligands repels the antibody, weakening its affinity for the gold surface and yielding a random, disordered orientation that partially masks the antigen-binding sites—an effect widely recognized as a principal limitation on assay sensitivity.

76 Here we replaced citrate with ascorbate, a mild reductant and low-charge stabilizer, to generate
77 AuNPs bearing a loosely charged interface. The weakly anionic ascorbate layer forms
78 spontaneously at neutral pH and creates a low-density electrostatic barrier. Under these
79 conditions, the Fc region of the antibody carries a slight positive charge, whereas the Fab
80 regions are weakly negative, giving the molecule an overall neutral dipole. This charge
81 distribution favors initial electrostatic docking of the Fc domain, followed by hydrophobic
82 interactions that anchor the antibody to the gold surface. The negatively charged Fab fragments
83 are electrostatically repelled from the particle, forcing them to project outward and leaving the
84 paratopes fully accessible. The residual surface negative charge of the conjugate prevents self-
85 aggregation, ensuring colloidal stability without additional pH adjustment. This method
86 streamlines the fabrication process and, while preserving convenience and cost/time efficiency,
87 markedly enhances sensitivity. By dispensing with conventional enzyme-labeled secondary
88 antibodies, HRP was directly immobilized on gold nanoparticles to form Ab@AuNPs@HRP
89 conjugates that generate the analytical signal. This approach addresses the complexity and cost
90 issues of conventional assays, offering a promising route for the rapid, sensitive, and on-site
91 screening of pesticide residues in food safety monitoring (Fig. 1).

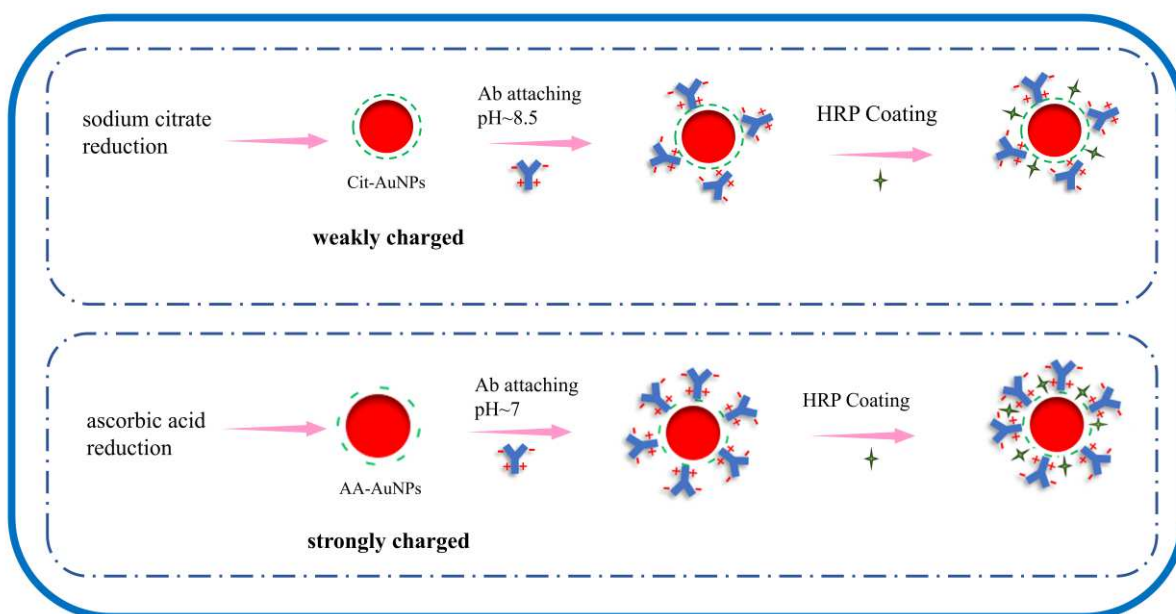


Fig. 1. Schematic illustration of antibody attachment on Cit-AuNPs and AA-AuNPs.

Experimental

Materials and reagents

Trisodium citrate, ascorbic acid, and chloroauric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Triazophos monoclonal antibody and OVA-hapten (triazophos) were obtained from Hangzhou Biosunshine Biotechnology Co. (Hangzhou, China). The triazophos pesticide standard was purchased from Beijing Huawei Sike Technology Co., Ltd. (Beijing, China). Acetonitrile (chromatographic grade) was acquired from Merck AG (Darmstadt, Germany). TMB single-component chromogenic solution, phosphate-buffered saline (PBS), and horseradish peroxidase (HRP) were supplied by Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Tween-20 was secured from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Immunotransparent 96-well microplates were procured from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

The microplate reader was from Tecan Group Ltd. (Männedorf, Switzerland). A DEM-3 plate washer was purchased from Beijing Top Instrument Co., Ltd. (Beijing, China). A microplate thermostatic oscillator was obtained from Langjieke Technology Co., Ltd. (Beijing, China).

Characterization and instruments

The morphology and size of the samples were characterized via transmission electron microscopy (TEM, FEI Tecnai G2 F30, USA). The elemental composition and distribution were analyzed via energy-dispersive X-ray spectroscopy (EDS, FEI Tecnai G2 F30, USA). The absorbance values were measured with a multifunctional microplate reader (Tecan Spark, Salzburg, Austria). UV–visible (UV–vis) absorption spectra were recorded via a UV–vis spectrophotometer (TU-1810, China). Fourier transform infrared (FT-IR) spectroscopy (Nicolet iS5, Thermo Scientific, USA) was employed to identify the functional groups present in the materials. The surface elemental composition and semiquantitative analysis were conducted via X-ray photoelectron spectroscopy (XPS, ESCALAB 250Xi, Thermo Scientific, USA).

Synthesis of AA-AuNPs

All glassware used in the synthesis was soaked in aqua regia overnight. Fifty milliliters of a 1 mM HAuCl₄ aqueous solution was placed in a 250 mL round-bottom flask and heated to 90 °C under stirring. Subsequently, 3 mL of a 1 % (w/v) ascorbic acid solution was rapidly added, and the reaction was allowed to proceed for 20 min [24]. Upon completion, a wine-red solution was obtained, which was then naturally cooled to room temperature and filtered through a 0.22 µm membrane filter. The resulting dispersion was centrifuged at 8000 rpm for 10 min at 4 °C to remove unreacted reagents. The precipitate was finally redispersed in deionized water to the original volume. The synthesis of AA-AuNPs is described in the Supporting Information.

Preparation of Ab@AA-AuNPs@HRP

Triazophos antibody was added to the AA-AuNP dispersion to achieve a final antibody concentration of 6 µg/mL. The mixture was incubated on a shaker for 30 min. Subsequently, 5 µL of a 1000 µg/mL HRP solution was added, followed by another 30-min incubation on a shaker. Then, a 2 % bovine serum albumin (BSA) solution was added, and the mixture was further incubated for 30 min to block nonspecific binding sites on the AA-AuNP surface. After the incubation steps, the mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended and appropriately concentrated in 0.01 M PBS. The final conjugate was stored at 4 °C for subsequent use. The preparation method of Ab@Cit-AuNPs@HRP is described in the Supporting Information.

Establishment of a AuNP-based ELISA

The antigen diluted in PBS was coated onto a 96-well electrostatic adsorption plate (100 µL per well) and incubated at 37 °C for 2 h. The plate was then washed three times with phosphate-buffered saline containing 0.1 % Tween-20 (PBST, pH 7.4), followed by blocking with 2 % BSA (300 µL per well) at 37 °C for 1 h. After another three washes, 50 µL of triazophos pesticide standard solutions (gradient concentrations: 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 µg/L) were added to each well, along with 50 µL of reconstituted Ab@AuNPs@HRP. The competitive reaction was carried out at 37 °C for 2 h, after which the plate was washed three times with PBST. Then, 100 µL of TMB substrate was added to each well, and the absorbance was measured at 650 nm [25]. To ensure reproducibility and reliability, each experiment was independently repeated three times.

Specificity evaluation of Ab@AuNPs@HRP

Fifty microliters of 50 µg/L methanol solutions (10 %) of triazophos, parathion, chlorpyrifos, malathion, phoxim, or fenthion were separately added to the precoated microplate wells, followed by the addition of 50 µL of HRP-Ab-AuNP immunoprobe to each well for competitive reactions. The mixture was incubated at 37 °C with shaking for 2 h and then washed three times with PBST. Subsequently, 100 µL of single-component TMB chromogenic solution was added to each well and allowed to react at room temperature for 15 min. The absorbance at 650 nm was immediately measured via a microplate reader.

Sample preparation

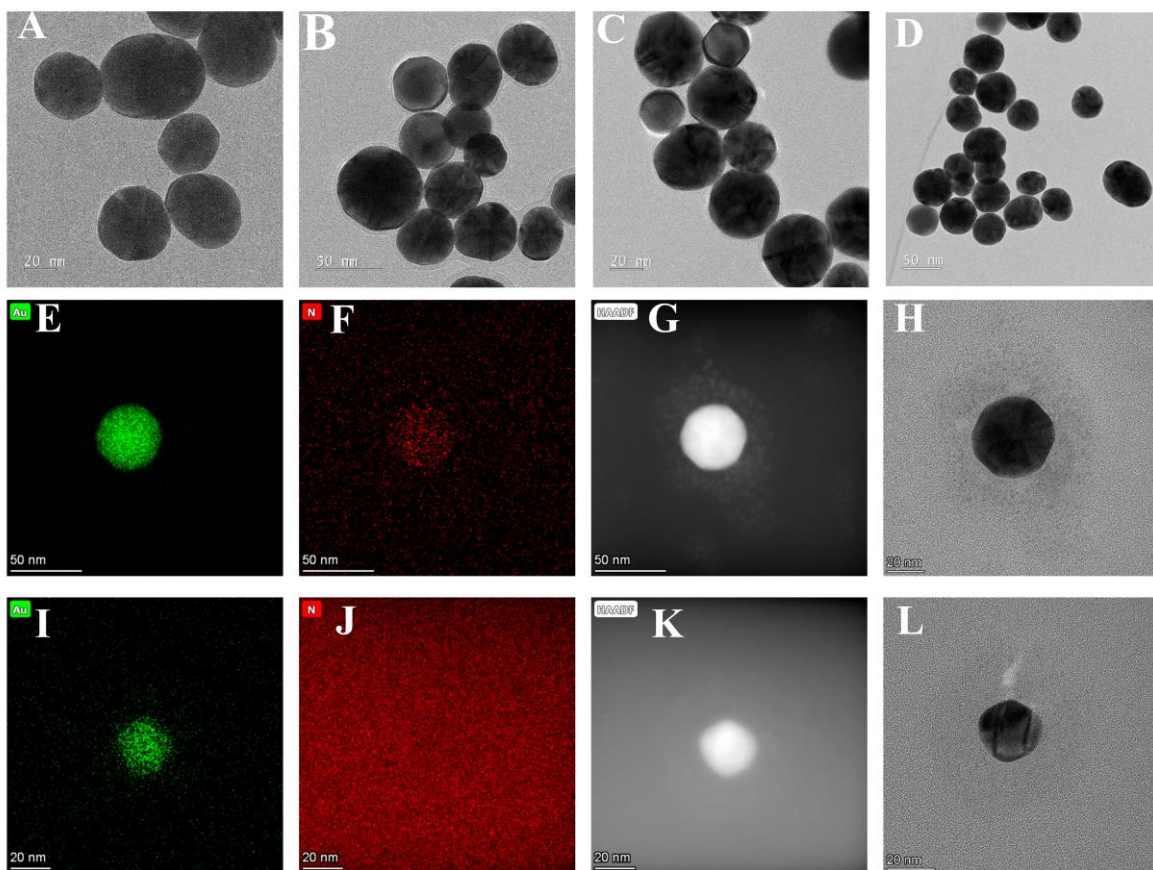
Cabbage, Celery cabbage, and cucumber samples purchased from a supermarket in Haidian District, Beijing, were used as spiked samples to evaluate the method's performance (LC–MS/MS analysis confirmed the absence of triazophos residues). A 10 g portion of homogenized sample was mixed with 10 mL of acetonitrile as the extraction solvent and vortexed vigorously for 1 min. Then, 4 g of anhydrous magnesium sulfate and 1 g of sodium chloride were added to the mixture, followed by vortexing for 1 min and centrifugation at 6,000 rpm for 5 min at 4 °C. A 2 mL aliquot of the supernatant was transferred to a dispersive solid-phase extraction (dSPE) tube containing 52 mg of PSA, 52 mg of C18, and 26 mg of graphitized carbon black. After vortexing for 1 min and centrifuging again at 6,000 rpm for 2 min, the supernatant was filtered through a 0.22 µm nitrocellulose membrane and stored at 4 °C [26].

Results and discussion

Characterization of AuNPs

AA-AuNPs and Cit-AuNPs (for comparative testing) were synthesized via a one-pot reaction. TEM and DLS characterization revealed that the synthesized Cit-AuNPs were nearly spherical

173 with an average particle size of approximately 36 nm (Fig. 2A-B); the size distribution is shown
174 in Fig. 3D. To ensure that the sizes of the two types of AuNPs were comparable, the uniformity
175 and size of the synthesized AA-AuNPs were optimized. This was achieved by varying the
176 volume of the 1% ascorbic acid solution used during synthesis (1.5, 3, 4.5, and 6 mL) to adjust
177 the particle size and uniformity. The average particle sizes and PDI values of the resulting
178 AuNPs, which were determined via TEM and DLS characterization, are shown in Fig. 3A and
179 3B. The results indicated that the AuNPs synthesized with both 1.5 mL and 3 mL of 1% ascorbic
180 acid presented similar average particle sizes of 40 nm. However, the AuNPs synthesized with 3
181 mL of ascorbic acid had a PDI of 0.066, whereas those synthesized with 1.5 mL of ascorbic
182 acid had a PDI of 0.089. Consequently, 3 mL of the 1% ascorbic acid solution was selected for
183 the preparation of the weakly charged AuNPs. The resulting weakly charged AuNPs had an
184 average particle size of 40 nm (Fig. 2C-D), and the size distribution is shown in Fig. 3C. These
185 AuNPs exhibited good uniformity and were comparable in size to the Cit-AuNPs (Fig. 3E).



186
 187 Fig. 2. (A-B) TEM image of Cit-AuNPs; (C-D) TEM image of AA-AuNPs; (E-F) TEM-EDS
 188 elemental mappings of AA-AuNPs; (G-H) HAADF-HRTEM image of AA-AuNPs; (I-J) TEM-
 189 EDS elemental mappings of Cit-AuNPs; (K-L) HAADF-HRTEM image of Cit-AuNPs

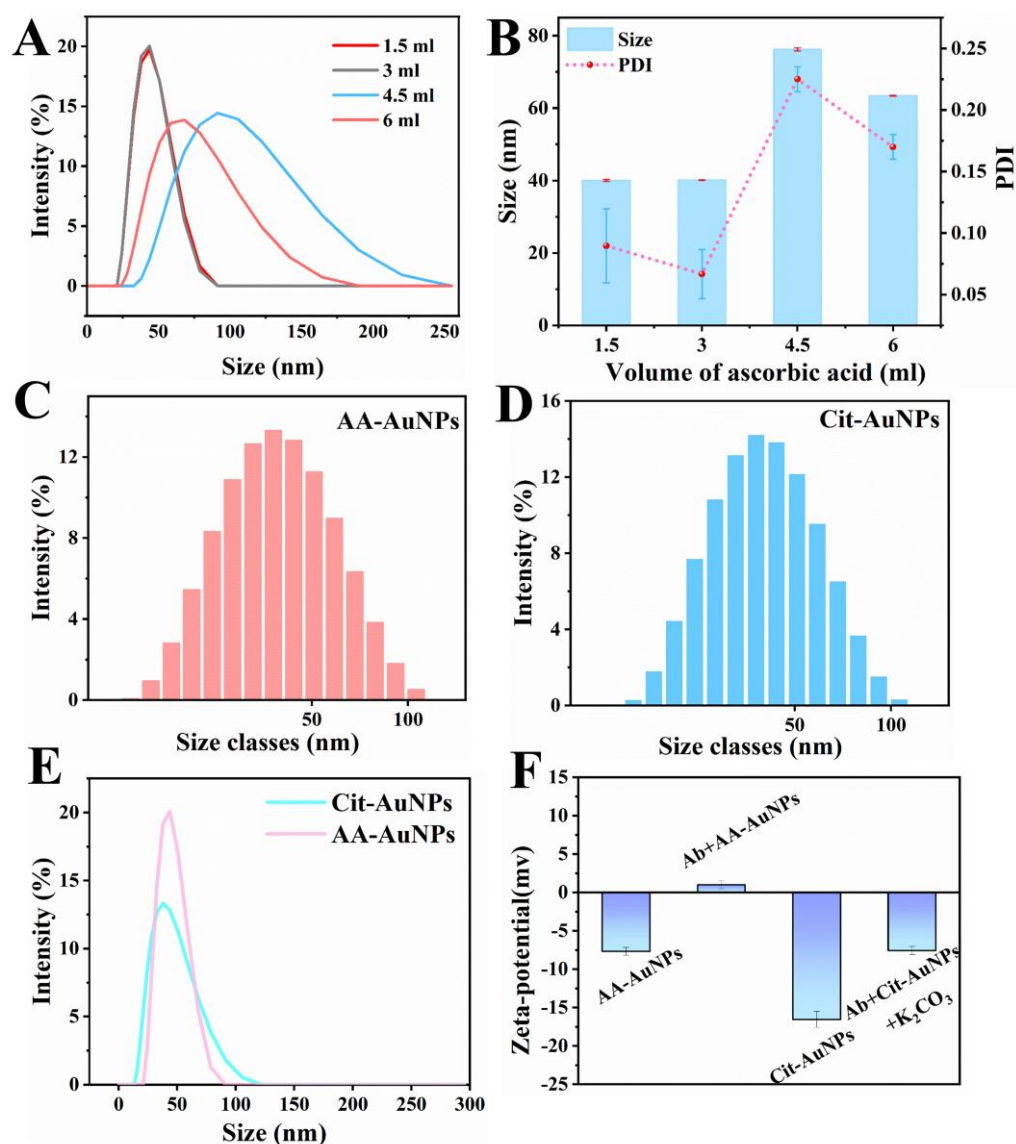


Fig. 3 (A-B) Particle size distributions and PDIs of AA-AuNPs synthesized with different volumes of ascorbic acid; (C-E) Particle size distributions of AA-AuNPs and Cit-AuNPs; (F) Zeta potential values of Cit-AuNPs, AA-AuNPs, the Ab@Cit-AuNP complex, and the Ab@AA-AuNP complex.

The ionization of ligands imparted different surface zeta potentials to the AuNPs. At neutral pH, the zeta potential was -15.96 mV for Cit-AuNPs and -7.43 mV for AA-AuNPs (Fig. 3F). Compared with strongly ionized citrate ligands (Cit^{3-}), ascorbic acid ionizes weakly (AA^-). Therefore, AA-AuNPs are defined as weakly charged AuNPs, which are distinct from strongly

199 charged Cit-AuNPs. To prepare Ab@AuNP complexes, the concentrations of AA-AuNPs and
200 Cit-AuNPs were first normalized by dilution to achieve equal absorbance at 522 nm. The UV-
201 Vis absorption spectra after dilution revealed similar absorption peaks at 522 nm for both the
202 AA-AuNPs and Cit-AuNPs, indicating successful normalization of their concentrations (Fig.
203 4A-B). Direct addition of antibodies to the AuNP colloid caused aggregation of the strongly
204 charged Cit-AuNPs, as evidenced by a redshift or disappearance of the absorption peak (Fig.
205 4B). This aggregation is attributed to strong electrostatic interactions between the highly
206 negatively charged Cit-AuNPs and the positively charged antibodies (Cit: $pK_a \sim 6.40$; Antibody:
207 $pI \sim 8$). To successfully prepare colloidal Ab@Cit-AuNPs, the pH was adjusted using a base
208 such as K_2CO_3 . When the pH exceeds 8, the net charge of the antibody becomes slightly
209 negative, although local positive charges remain. This adjustment eliminated the aggregation
210 of negatively charged Cit-AuNPs caused by electrostatic interactions. The resulting colloidal
211 Ab@Cit-AuNPs presented a decrease in absorbance but no significant redshift or aggregation
212 behavior (Fig. 4B). Antibody adsorption reduced the zeta potential of the Cit-AuNPs (Fig. 3F).
213 For the weakly charged AA-AuNPs, the direct addition of an antibody at neutral pH was
214 straightforward and efficient, yielding colloidal Ab@AA-AuNPs. A decrease in absorbance was
215 observed, but no significant redshift, color change, or aggregation occurred (Fig. 4A), similar
216 to that of the colloidal Ab@Cit-AuNPs. The weakly charged nature of the AA ligands prevented
217 charge interaction-mediated aggregation of the AA-AuNPs during antibody incubation.
218 Concurrently, antibody attachment at neutral pH reversed the zeta potential of the AA-AuNPs,
219 resulting in a positive charge for the colloidal Ab@AA-AuNPs (Fig. 3F).
220 XPS analysis was performed to characterize the two different AuNPs and their Ab@AuNP

complexes. The Au 4f spectra showed no significant changes for either the AA-AuNPs or Cit-AuNPs before and after antibody incubation (Figure 4C). The appearance of peaks in the N 1 s spectra indicated protein adsorption on the AuNP surfaces, as nitrogen is characteristic of proteins, whereas AA and citrate contain no nitrogen atoms (Fig. 4D). Semiquantitative analysis on the basis of the relative intensity of the N 1 s peaks suggested that more antibody was adsorbed on the surface of the AA-AuNPs than on the surface of the Cit-AuNPs.

HRTEM-EDS and HAADF characterization were employed to analyze the distributions of Au (from the AuNP core) and N (characteristic element of antibody proteins) in Ab@AA-AuNPs and Ab@Cit-AuNPs. The results for Ab@AA-AuNPs clearly revealed the Au core and a uniform coating of nitrogen surrounding it, indicating successful antibody conjugation to the AuNP surface (Fig. 2E-H). In contrast, a distinct nitrogen coating was not readily observable for Ab@Cit-AuNPs. This suggests that the weakly charged AA-AuNPs are more favorable for antibody adsorption (Fig. 2I-L).

FT-IR analysis further confirmed the successful conjugation of the antibodies to the AA-AuNPs (Fig. 4E). The FT-IR spectrum of the bare AuNPs synthesized with ascorbic acid showed characteristic absorption peaks of the ascorbic acid stabilizer: a broad peak at $\sim 3200\text{--}3500\text{ cm}^{-1}$ attributed to O–H stretching vibrations; a sharp, strong peak at $\sim 1745\text{ cm}^{-1}$ assigned to the C=O stretching vibration of the lactone ring in ascorbic acid; and a peak at $\sim 1650\text{ cm}^{-1}$ potentially related to C=C vibrations. For the Ab@AA-AuNP conjugate, the peak intensity in the $\sim 1650\text{ cm}^{-1}$ region significantly increased, and the band broadened, which was consistent with the characteristics of the protein amide I band (primarily C=O stretching). Although the ascorbic acid background contributes to absorption in this region, the marked intensity change indicates

243 the superposition of a new amide I component. A distinct new absorption peak appeared at
244 $\sim 1545\text{ cm}^{-1}$, which is unequivocally assigned to the protein amide II band (N-H bending/C-N
245 stretching vibrations). This peak is typically absent or very weak in the spectrum of bare AuNPs
246 and serves as direct evidence for successful antibody conjugation. Additionally, the intensities
247 of the C-H stretching vibration peaks at $\sim 2950\text{ cm}^{-1}$ and $\sim 2850\text{ cm}^{-1}$ noticeably increased,
248 further confirming coverage by organic macromolecules. Collectively, the FT-IR results,
249 specifically the emergence of the amide II band, the significant enhancement of the amide I
250 band, and the changes in the C-H region, provide strong evidence for the successful conjugation
251 of antibodies to the surface of the ascorbic acid-synthesized gold nanoparticles.

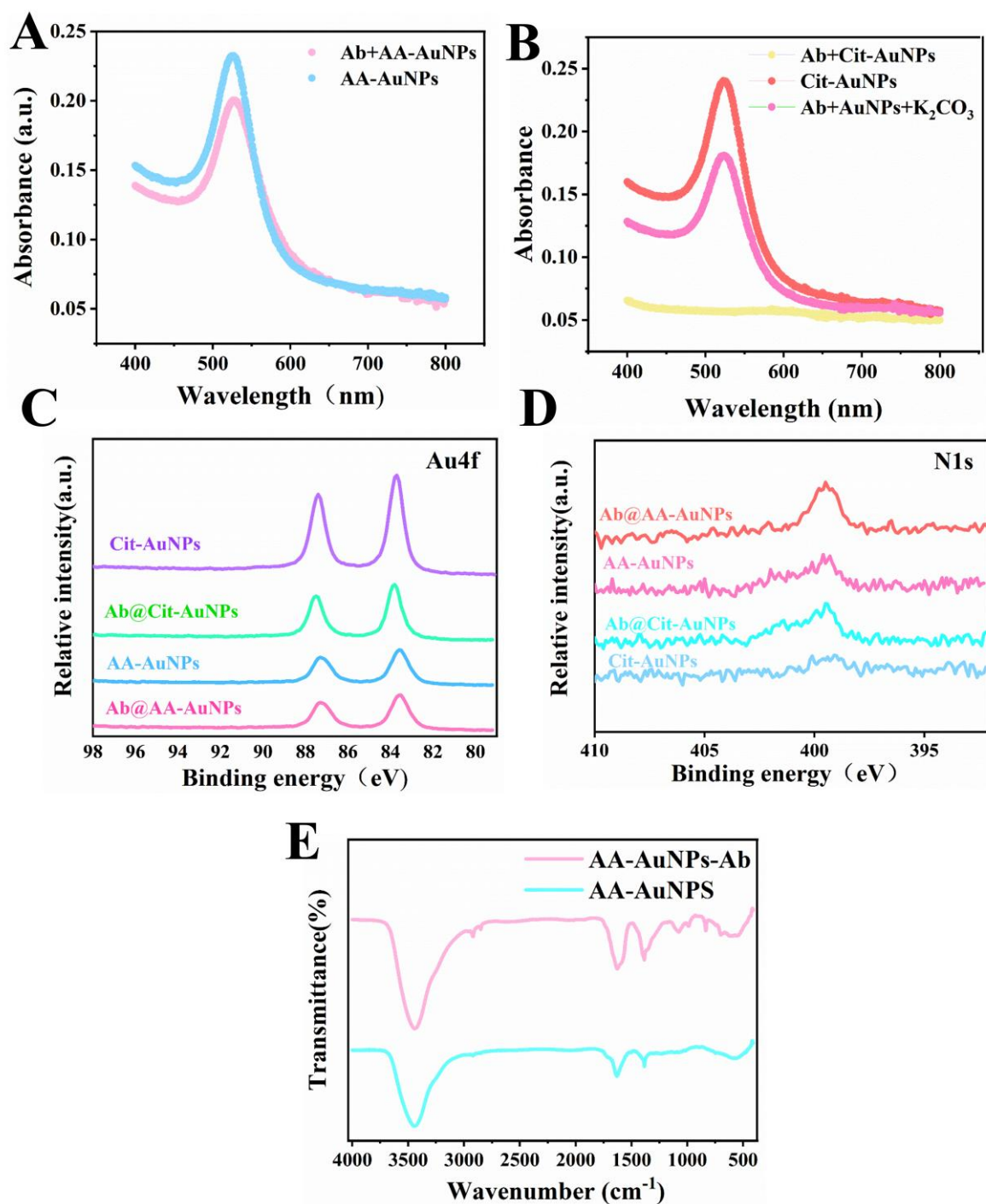


Fig. 4 (A) UV-Vis absorption spectra of AA-AuNPs and Ab@AA-AuNPs; (B) UV-Vis absorption spectra of Cit-AuNPs, Ab@Cit-AuNPs and Ab@Cit-AuNPs + K₂CO₃; (C-D) the Au4f and N1s XPS binding energy profiles of AA-AuNPs and Cit-AuNPs before and after incubation with Ab, respectively; (E) FT-IR spectra of AA-AuNPs and Ab@AA-AuNPs

Establishment of AuNP-based ELISAs

Optimization of the working concentrations of OVA-Ag and Ab

The concentrations of the coating antigen (OVA-hapten) and the detection antibody play critical roles in determining the sensitivity of the immunoassay. To achieve high detection performance, the working concentrations of these two components were systematically optimized via a checkerboard method. In this experiment, the OVA-hapten concentrations were set at 1, 2.5, 5, 7, and 9 $\mu\text{g/mL}$, while the antibody concentrations were set at 2, 4, 6, 8, and 10 $\mu\text{g/mL}$. To balance sensitivity with reagent economy, an absorbance value of 1.0 was used as the criterion for evaluating all 25 combinations.

As shown in Fig. 5A, the absorbance initially increased but then decreased with increasing antibody concentration, which can be attributed to the saturation of antibodies on the surface of the gold nanoparticles. Similarly, as the OVA-hapten concentration increased, the absorbance also initially increased but then decreased. This trend may be due to steric hindrance caused by excessive antigen coating, which could impede efficient immunorecognition. At an antibody concentration of 6 $\mu\text{g/mL}$, the absorbance values corresponding to OVA-hapten concentrations of 1 $\mu\text{g/mL}$ and 2.5 $\mu\text{g/mL}$ were both greater than 1.0 and closely matched. To further determine the optimal antigen concentration and ensure analytical sensitivity, the inhibition rates of different combinations were compared.

The results demonstrated that when the coating antigen concentration was 2.5 $\mu\text{g/mL}$ and the antibody concentration was 6 $\mu\text{g/mL}$, the system not only achieved high signal intensity (absorbance > 1.0) but also exhibited the highest inhibition rate (Fig. 5B). This finding indicates an optimal balance between immunoreaction efficiency and signal response under these conditions. Therefore, this combination was selected as the optimal working concentration for

subsequent experiments.

Optimization of the HRP concentration

Horseradish peroxidase (HRP) is central to the signal output of the immunoprobe, and its concentration directly affects the detection sensitivity and signal-to-noise ratio. To determine the optimal labeling concentration, the influence of a series of HRP concentrations (2.5, 5, 7.5, 10, and 12.5 $\mu\text{g}\cdot\text{mL}^{-1}$) on the assay performance was evaluated. The results (Fig. 5C) revealed that the signal absorbance increased with increasing HRP concentration in the range of 2.5--10 $\mu\text{g}\cdot\text{mL}^{-1}$, peaking at 10 $\mu\text{g}\cdot\text{mL}^{-1}$. However, no significant difference in absorbance values was observed between 5 and 10 $\mu\text{g}\cdot\text{mL}^{-1}$. Further competitive assays revealed that the system achieved the highest inhibition rate for the target analyte at an HRP concentration of 5 $\mu\text{g}\cdot\text{mL}^{-1}$, indicating optimal immunoreaction efficiency under these conditions. In summary, considering both its high detection sensitivity and cost-effectiveness, 5 $\mu\text{g}\cdot\text{mL}^{-1}$ was selected as the optimal working concentration of HRP for subsequent experiments.

Optimization of the blocking agent concentration

Proteins can adsorb onto the surface of AuNPs via electrostatic attraction and hydrophobic interactions. However, such nonspecific adsorption can also lead to the binding of nontarget proteins in the detection system, thereby increasing the background signal and reducing the detection sensitivity and specificity. Therefore, after the conjugation of antibodies and enzymes is complete, a blocking agent is required to block the remaining unbound sites on the AuNPs. This study optimized the concentration of bovine serum albumin (BSA) as a blocking agent (1–5 %) (Fig. 5D). The results indicated that as the BSA concentration increased, the background absorbance initially increased but then decreased continuously, which can be attributed to its

effective steric hindrance effect. However, high concentrations of BSA might also partially shield the active sites of the target antibodies, leading to excessive signal suppression. At a BSA concentration of 2 %, the system maintained a high signal intensity (absorbance>1) while achieving the highest inhibition rate, demonstrating an optimal balance between a high signal-to-noise ratio and high immunoreaction efficiency. Consequently, 2% BSA was chosen as the optimal blocking concentration.

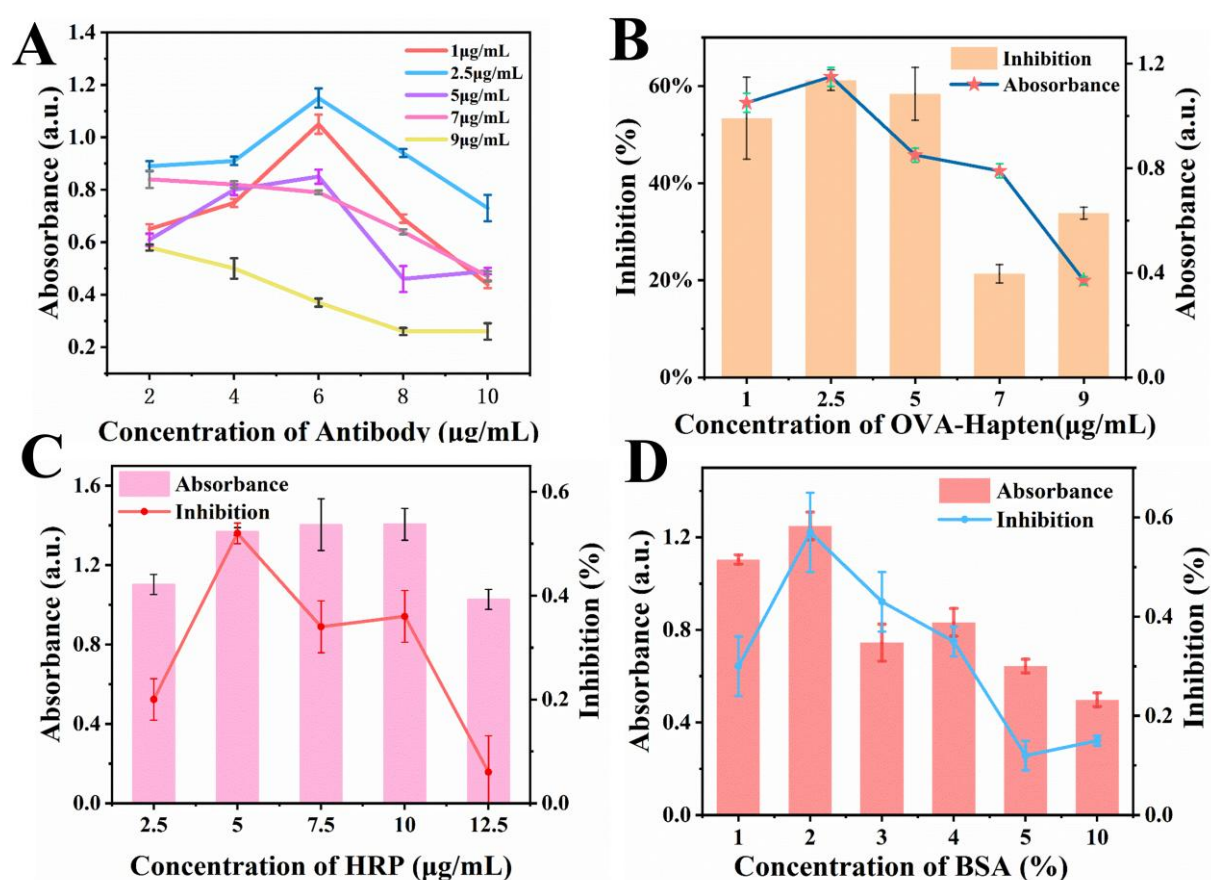


Fig. 5. Optimization of the experimental parameters. (A-B) Absorbance values for different combinations of antigen and antibody concentrations; (C-D) Absorbance values and inhibition of Ab@AuNPs@HRP immunoreactivity at different concentrations of BSA and HRP.

Establishment of the standard curve

Under the optimized conditions, a standard curve was established for the indirect competitive

immunoassay assisted by weakly charged gold nanoparticles. The results showed that as the pesticide concentration increased, the absorbance decreased while the inhibition rate increased, which is attributed to the competition between the pesticide and the coating antigen for binding to the antibodies conjugated on the gold nanoparticles. The calibration curve and regression equation were constructed by plotting the inhibition rate (%) on the y-axis against the logarithm of the pesticide concentration on the x-axis.

As shown in Figure 6A, the immunoassay standard curve for triazophos based on AA-AuNPs exhibited a wide linear range from 1.08 to 22.48 $\mu\text{g/L}$ with excellent linearity ($R^2 = 0.9947$), a low limit of detection ($\text{LOD} = 0.06 \mu\text{g/L}$), and satisfactory sensitivity ($\text{IC}_{50} = 4.93 \mu\text{g/L}$). In comparison, the competitive immunoassay based on Cit-AuNPs showed an IC_{50} value of 124.27 $\mu\text{g/L}$ and a limit of detection of 25.22 $\mu\text{g/L}$ (Fig. 6B). The immunoassay based on AA-AuNPs offered a significantly lower detection limit and an IC_{50} value that was approximately 25-fold lower, indicating a substantial improvement in sensitivity. Moreover, the linear range of the proposed method covered lower concentration regions that are more relevant for trace residue analysis. These findings demonstrate that the developed immunoassay provides superior sensitivity and a lower detection limit for triazophos residue analysis compared to the conventional approach based on Cit-AuNPs.

Specificity analysis

Specificity is a critical parameter for evaluating the ability of an immunoassay to recognize target molecules. To validate the selectivity of the established competitive immunoassay for triazophos detection, structural analogs (including parathion, chlorpyrifos, malathion, fenthion, and phoxim) were selected as potential interferents for cross-reactivity testing (Fig. 6C). The

concentration of each tested compound was 50 µg/L.

As shown in Fig. 6D, the absorbance value was significantly inhibited in the sample spiked with triazophos. In contrast, the absorbance signals of the other structural analogs did not obviously change and were comparable to those of the blank control (without pesticide), with all values remaining at approximately 1.0. These results demonstrate that the established immunoassay based on weakly charged gold nanoparticles has high specificity for triazophos, effectively distinguishing them from structural analogs and showing good anti-interference ability.

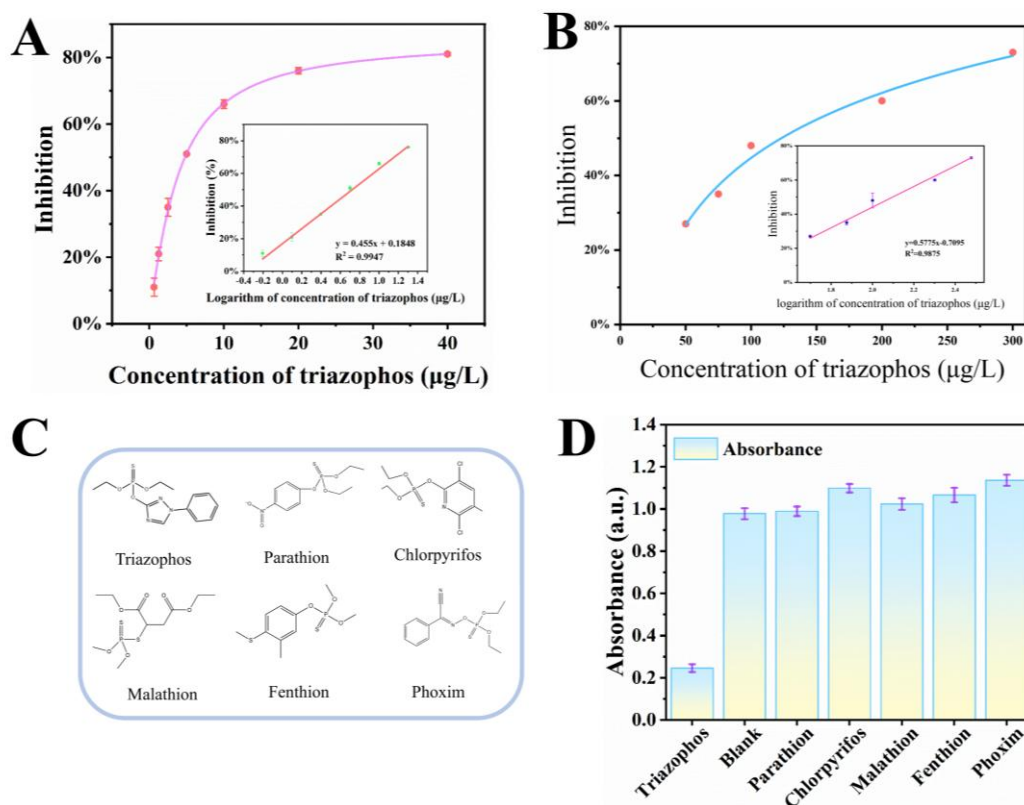


Fig. 6. Calibration curves and specificity of the Ab@AuNPs@HRP immunoassay for triazophos. (A) Calibration curves for triazophos using the Ab@AA-AuNPs@HRP indirect competitive immunoassay. (B) Calibration curves for triazophos using the Ab@Cit-AuNPs@HRP indirect competitive immunoassay. (C) Chemical structures of triazophos and its

structurally similar compounds. (D) Specificity of the immunoassay toward triazophos and its structural analogs.

Sample analysis

To systematically evaluate the reliability and applicability of the established AA-AuNP immunoassay, spiked recovery experiments were conducted using three vegetable samples—Celery cabbage, cucumber, and cabbage—to validate the accuracy, precision, and detection performance of the method. All the samples were confirmed to be free of triazophos residues by LC–MS/MS analysis prior to analysis.

Standard curves for each vegetable matrix were established (Fig. 7). The regression equations for Celery cabbage, cucumber, and cabbage were $Y = 0.4138X + 0.1088$ ($R^2 = 0.9893$), $Y = 0.2427X + 0.1123$ ($R^2 = 0.9902$), and $Y = 0.1757X + 0.2555$ ($R^2 = 0.9632$), respectively, all of which demonstrated good linearity. In terms of sensitivity, the limits of detection (LODs) for Celery cabbage, cucumber, and cabbage were 0.80, 1.34, and 0.16 $\mu\text{g/L}$, respectively, while the IC_{50} values were 24.64, 1.59, and 1.47 $\mu\text{g/L}$, respectively. Notably, the vegetable matrix had a certain influence on the detection sensitivity, as the sensitivity of the matrix-standard curve was lower than that of the solvent-standard curve. This may be attributed to interference from complex components in the sample matrices during the immuno-recognition process. Some matrix components may compete for antibody binding sites or cause steric hindrance, thereby affecting the efficiency of antigen–antibody binding [27].

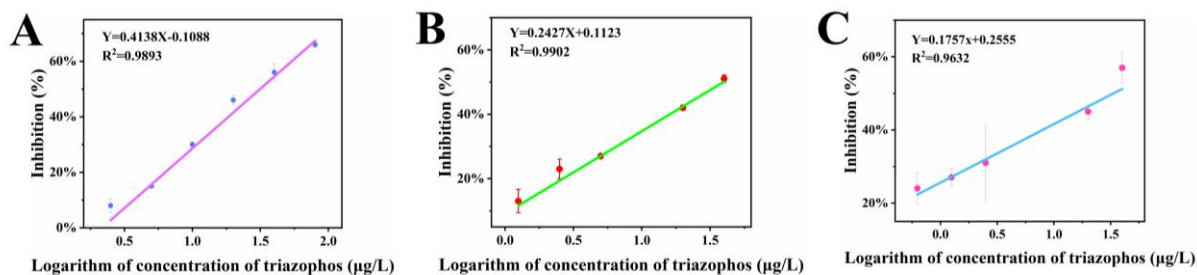


Fig. 7. (A) Triazophos standard curve in cabbage samples of the immunoassay. (B) Triazophos standard curve in cucumber samples of the immunoassay. (C) Triazophos standard curve in celery cabbage samples of the immunoassay.

As shown in Table 1, all three vegetable matrices presented satisfactory recovery rates (76.82–115.83 %) and precision ($\text{RSD} \leq 5.69\%$) at different spiked concentrations (5, 10, and 20 $\mu\text{g/L}$).

These results comply with the method validation criteria specified in both the Chinese (GB 2763-2021) maximum residue limits for pesticides in food and the European Union guideline SANTE/12682/2021 (required recovery range: 70-120%, $\text{RSD} \leq 20\%$), indicating that the method maintains high accuracy and robustness even in complex vegetable matrices.

Furthermore, the LODs for all samples were below the maximum residue limits (MRLs) for triazophos set by Chinese national standards (Celery cabbage: 1 mg/L ; cucumber and cabbage: 0.05 mg/L) and below the uniform MRL of 0.01 mg/kg (approximately 10 $\mu\text{g/L}$) stipulated by the European Union. These results demonstrate that the proposed method is suitable for detecting triazophos residues in practical samples.

The Ab-AuNP-HRP immunoprobe developed in this study has significant advantages in terms of performance. As shown in Table 2, the immunoassay established with this probe achieved a lower detection limit than existing methods for triazophos detection.

Table 1. Detection and recovery of triazophos in spiked vegetable samples.

Sample	Spiked sample concentration ($\mu\text{g/L}$)	Found ($\mu\text{g/L}$)	Recovery (%)	RSD (%)
Celery cabbage	5	5.39	107.87	3.16
	10	8.40	84.05	0.60
	20	18.15	90.78	3.47
Cucumber	5	4.61	92.10	3.02
	10	7.68	76.82	1.80
	20	18.61	93.06	3.38
Cabbage	5	5.79	115.83	1.09
	10	9.22	92.27	0.95
	20	22.10	110.53	5.69

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389 Table 2. Comparison of the published methods for triazophos detection.

Method	Linear rangr($\mu\text{g/L}$)	LOD ($\mu\text{g/L}$)	Reference
LFIA (AuNPs)	/	250	[28]
ELISA (AuNPs)	4.47-170.19	1.94	[29]
LFIA (AuNPs)	/	50	[30]
SERS(UiO-66@TiO ₂ /Au)	50-10000	0.66	[31]
SERS(AuNPs@MIL-101)	1-10000	1	[32]

GC–MS/MS	3.4-5.4	20	[33]
Fluorescence (Tb-MOF)	3-800	0.12	[34]
LFA (THBu-IgG-FITC)	20-500	20	[35]
Chemiluminiscence (Fe ₃ O ₄ @COF-AuNP)	1.57-94.0	0.31	[36]
Ab@AuNPs@HRP	1.08-22.48	0.06	This work

Conclusions

This study successfully established an indirect competitive immunoassay based on ascorbic acid-functionalized gold nanoparticles (AA-AuNPs) for the highly sensitive detection of triazophos pesticide residues in vegetables. Compared with conventional immunoassays, the constructed Ab@AuNP@HRP immunoprobe has a larger specific surface area and superior antibody-loading capacity, effectively enhancing the detection sensitivity. This method eliminates the need for a secondary antibody by directly conjugating HRP onto the gold nanoparticle surface, which reduces costs while significantly streamlining the experimental procedure.

The proposed method demonstrated a good linear relationship within the range of 1.08–22.48 µg/L, with a sensitivity of 4.93 µg/L and a limit of detection (LOD) of 0.06 µg/L. In practical sample analyses, the average recovery rates ranged from 72.5 % to 115.18 %, indicating satisfactory accuracy and reliability. Notably, the LOD of 0.06 µg/L (approximately 0.06 µg/L) reflects the high detection sensitivity of the method.

In summary, the AA-AuNP-based indirect competitive immunoassay offers distinct advantages,

including high sensitivity, cost-effectiveness, and operational simplicity. Without the need for complex synthesis steps or time-consuming procedures, this method shows considerable potential for the detection of small-molecule pesticide residues in fruits and vegetables, thereby providing new technical support for agricultural product quality and safety monitoring.

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