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ARTICLE

Sensitive colorimetric detection of cyromazine in cucumber samples by using label-free gold nanoparticles and polythymine

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Jinchuan Liu[a,b], Wenhui Bai[a,b], Chao Zhu[a,b], Mengmeng Yan[a,b], Shuming Yang[a,b] and Ailiang Chen[a,b]*

Cyromazine (CYR) can cause serious damage to the organs of animals or human beings, and it was found to bind to polythymine (polyT10) via multiple hydrogen bonding interactions. Based on this novel finding, a highly sensitive and simple colorimetric method was developed for CYR detection by using label-free gold nanoparticles (AuNPs) and polyT10. Under the optimized conditions, excellent linearity was acquired for CYR within the range of 1-500 ng/mL. In addition, the spectra and color changes of the AuNP solution were measured by spectrophotometry and observed by the naked eyes, and the results showed as low as 1 and 5 ng/mL of CYR could be detected, depending upon the measurement methods. Afterwards, cucumber was selected to investigate the sample matrix effect and a sample pretreatment procedure was developed with simple homogenization and filtration. Even after 200 times dilution, the limit of detection (LOD) and limit of quantitation (LOQ) reached 252 ng/g and 500 ng/g, respectively. The LOD and LOQ satisfied the Chinese requirement for the maximum residue limit (MRL), which is 0.5-1 μg/g of CYR in most vegetables. The assay also showed a good average recovery of 83.7-104.8% with the RSD of less than 7% and good selectivity for cyromazine over other pesticides that may exist in vegetable samples. The method proposed in this study was simple, fast, and highly sensitive and accurate, and the test result with this method was visible to the naked eyes. Therefore, it could be used for routine determination of CYR residues in cucumber samples.

1. Introduction

Cyromazine (N-cyclopropyl-1, 3, 5-triazine-2,4, 6-triamine, CYR) is a triazine pesticide used as an insect growth inhibitor for fly control in cattle manure, field crops, vegetables, and fruits[1, 2]. A portion of cyromazine can be degraded via dealkylation reactions to melamine, which is toxic by high-dose exposure and causes renal failure[3]. In recent years, the use of CYR has been proved to cause potential environmental and human health problems, for example, mammary tumors in mice[4]. China has set maximum residue limits (MRLs) for CYR in the range of 0.5-1 μg/g, which is associated with the vegetable type. For cucumber, a common fresh-cut vegetable, there are some potential safety risks of CYR residues because of some illegal operations in planting, deep processing and circulation of the product. Therefore, to develop accurate and reliable methods for determination of CYR in cucumber is required to ensure food safety.

CYR analysis is usually performed in various samples using chromatographic methods including gas chromatography-mass spectrometry (GC-MS) with the limits of quantification (LOQs) ranging from 10-100 μg/kg[5-7] and liquid chromatography-mass spectrometry (LC-MS) with the LOQs from 0.05 mg/kg to 40 mg/kg[8-12]. Although many methods have been established for CYR determination, the approaches based on these techniques involve time-consuming sample preparation steps and require sophisticated equipment and trained personnel, which discourage the applications of these methods to the analysis of food samples in common laboratories. Therefore, it is critically important to develop a simple and rapid screening methodology for the detection of CYR in cucumber with good sensitivity and reliability.

In view of a huge amount of samples to be screened, visual detection methods would be extremely attractive because of the possibility of readily reading out with the naked eyes, in some cases at the point of use. Gold nanoparticles (AuNPs) have been widely used to develop various colorimetric assays including the commonly used human chorionic gonadotropin test paper for its good biocompatibility, stability and especially excellent optical properties[13, 14]. AuNPs have strong optical effects of particle space and could induce a color change from red to blue when dispersed nanoparticles aggregate[14]. In 2004, Rothberg first found that single-strand DNA (ssDNA) could be directly adsorbed onto the unmodified AuNP surface and stabilize AuNPs against the aggregation induced by salt addition and that the solution retained its wine-red color[15]. Based on this, some ssDNA aptamer/AuNP based

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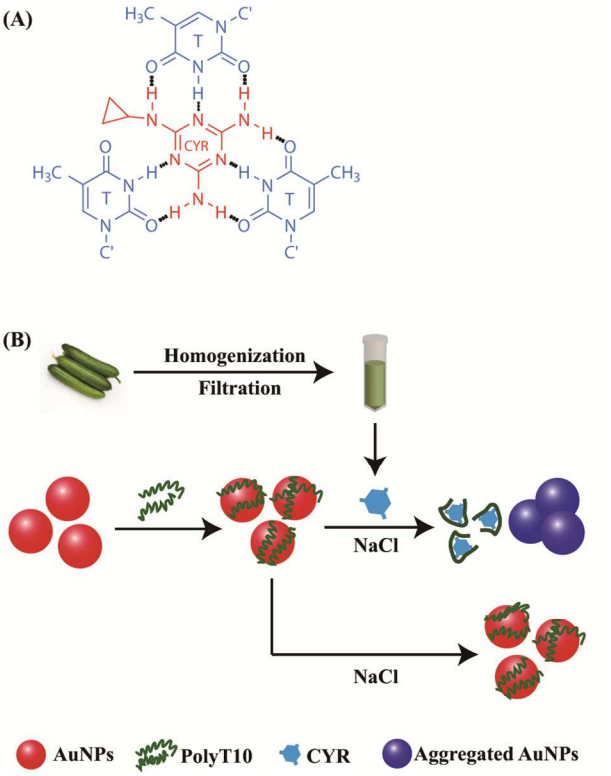
colorimetric assays have been developed[16-20]. For these assays, upon addition of targets, the conformation of aptamers is transited to the folded state by binding to the targets and desorbed from the surface of AuNPs, which resulted in subsequent aggregation of AuNPs and the solution's color change from red to purple-blue. This method has advantages over the chromatographic methods as it requires no expensive and complicated instruments, making onsite and real-time CYR sensing possible. To utilize the approach, however, the ssDNA with high affinity and selectivity to CYR must be provided.

The hydrogen bonding between thymine and 2,6-diamino-5-methylpyrimidin-4-one, which has a similar molecular structure with CYR, has been reported[21]. With the hydrogen bonding, polythymine (polyT10) has been designed for melamine detection using an unmodified AuNP based colorimetric assay, in which single-strand polythymine adsorbed onto the AuNP surface was desorbed by binding to melamine and resulted in a color change of the AuNP solution[22]. Inspired and encouraged by the similar structure of CYR with 2,6-diamino-5-methylpyrimidin-4-one and melamine, here for the first time, we reported a sensitive AuNP-polythymine based colorimetric assay (Scheme 1A) for CYR detection, in which CYR could also form multiple hydrogen bonds with thymine, as shown in Scheme 1B.



Scheme 1. (A) The structure of the hydrogen-bonding recognition between CYR and thymine. (B) The working principle of CYR detection. Label-free AuNP sensor for optical analysis of CYR.

2. Experimental

2.1 Reagents and chemicals

The single-strand polythymine was synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China), and the lyophilized powder was dissolved in pure water and stored at 4°C before use. The concentration of the oligonucleotide was determined by measuring the UV absorbance at 260 nm. Chloroauric acid ($HAuCl_4$) and sodium citrate ($C_6H_5Na_3O_7$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cyromazine, cypermethrin, esfenvalerate, dimethoate, parathion-methyl and methamidophos were purchased from J&K Chemical Ltd. (Beijing, China). Other common chemicals, including methanol and sodium chloride (NaCl), were all from Beijing Chemical Reagent Company (Beijing, China).
All of the chemicals were at least analytical grade. A 96-well polystyrene microplate (12 strips of 8 wells) was purchased from Corning (Corning, NY). The water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The ultraviolet-visible (UV-vis) absorption spectra of polyT10 and AuNPs were measured using a NanoDrop 2000c Scan UV-vis spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) with a 10 mm path length fused-silica cuvette at room temperature.

2.3 Preparation of the standard solutions and spiked cucumber samples

The stock solution containing CYR (1.0 mg/mL) was prepared with methanol. The standard solutions were prepared by diluting the stock solution with deionized water. Fresh cucumber was purchased from a local supermarket, and accurately weighed cucumber of 5.0 ± 0.05 g was homogenized with a household cutter (Joyoung Ltd., Jinan, China). Then, the CYR stock solution was added to the homogenate to prepare spiked cucumber samples as CYR is a water-soluble pesticide. After incubation for 5 min, the homogenate was filtered through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) and 1.5 g filtrate was collected. After diluted 200 times with pure water, the samples were used for assay.

2.4 Synthesis of the citrate-protected AuNPs

AuNPs were synthesized in the classical citrate reduction method[23]. Briefly, colloidal AuNPs with an average diameter of 13 nm were prepared by rapidly injecting a sodium citrate solution (2 mL, 194 mM) into a boiling aqueous solution of $HAuCl_4$ (100 mL, 1 mM) with vigorous stirring. After boiling for 20 min, the reaction flask was removed from the heat to allow the reaction solution to cool at room temperature. The concentration of the AuNPs was approximately 14 nM, which was determined according to Beer's law by using the extinction coefficient of 2.01×10^8 M^{-1} cm^{-1} at 520 nm for AuNPs of 13 nm in diameter[24].

2.5 Procedure of CYR detection

In a typical experiment, 50 μL of AuNPs was mixed with 50 μL of 2.5 μM polyT10 and incubated for 5 min at room temperature. Then, 50 μL of CYR at different concentrations was added and allowed to stand for another 5 min. Subsequently, 10 μL of 0.9 M NaCl was transferred to such a solution to give a final volume of 160 μL and they were mixed thoroughly. After the solution was equilibrated for 5 min, the resulting solution was transferred to a 10 mm quartz cuvette. The UV-vis absorption spectrum was measured with respect to water over the wavelength range of 450-750 nm, and finally photographs were taken with a Sony TX20 digital camera.

3. Results and discussion

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3.1 Principle of colorimetric detection of CYR with label-free AuNPs and polyT10

To develop an ssDNA-AuNP based colorimetric assay for CYR detection, polythymine (TTTTTTTTTT, polyT10) was designed as a specific recognition molecule for CYR binding. The principle of the label-free AuNP based colorimetric detection of CYR using polyT10 was illustrated in Scheme 1B. The random coil single-strand polyT10 could be easily adsorbed on the surface of AuNPs through coordination between Au and N atoms in DNA bases[25, 26]. Then, the AuNPs were stabilized against the aggregation upon salt addition and the solution remained wine-red. However, when CYR was added, polyT10 was desorbed from the surface of AuNPs by binding to CYR. The unprotected AuNPs readily aggregated in the presence of salt and provoked a red-to-blue color change due to the interparticle coupled plasmon excitons in the aggregated state[27]. Based on this principle, a sensitive, simple and rapid method for screening CYR was developed by optimizing the key parameters such as the concentrations of NaCl and polyT10.

3.2 Optimization of the NaCl concentration

The sensitivity of an AuNP based colorimetric assay is closely related with the state change (dispersion/aggregation) of AuNPs, which depends on the concentration of the added salt. To get the best performance, 10 μL NaCl at different concentrations was added to a 150 μL system that contained 50 μL of 13 nm AuNPs and 100 μL pure water (instead of 50 μL polyT10 and 50 μL CYR) and the absorbance at 520 nm was measured. As shown in Fig. 1, with the increase of the NaCl concentration, the AuNP solution's color changed from red to blue. The UV–vis absorbance values showed that 0.9 M NaCl was suitable for aggregation of nearly all the AuNPs. Thus, 0.9 M NaCl was chosen for the following experiments.

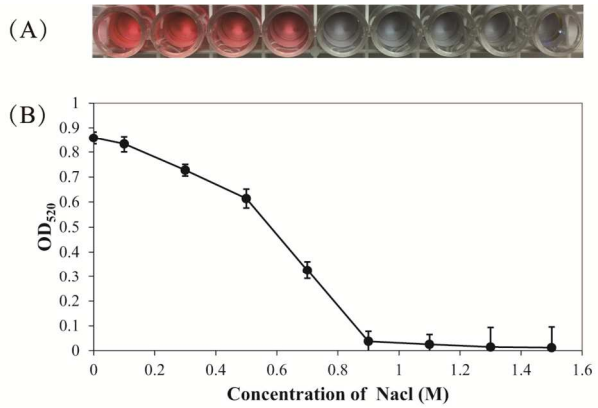

Fig. 1 AuNP aggregation status upon treatment with different concentrations of NaCl (0, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3 and 1.5 M). (A) Visual color changes. (B) Absorbance value changes at 520 nm.

3.3 Optimization of the polyT10 concentration

The concentration of polyT10 was studied with the fixed concentration of 0.9 M NaCl. First, polyT10 of varied concentrations (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 μM) was incubated in 50 μL AuNP solution for 5 min, and then 50 μL pure water and 10 μL 0.9 M NaCl were added. As shown in Fig. 2, the result showed that 2.5 μM polyT10 could protect AuNPs from aggregation induced by 0.9 M NaCl. Even higher concentrations of polyT10 provided more protection, 2.5 μM polyT10 was chosen as an optimum concentration to detect CYR since a low concentration of polyT10 means high sensitivity in view of the competitive polyT10 binding between AuNPs and CYR.

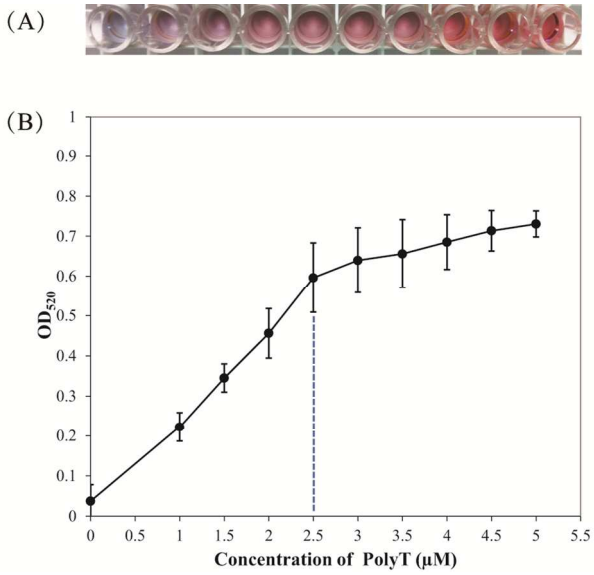

Fig. 2 Stabilization effect of different concentrations of polyT10 on AuNPs under 0.9 M NaCl. (A) Visual color changes. (B) Absorbance value changes at 520 nm.

3.4 Sensitivity and linearity of the colorimetric assay

The optimized sensor was applied to the detection of CYR in water, and the sensitivity and linearity of the colorimetric assay were determined. As shown in Fig. 3A and 3B, with the increase of the CYR concentration from 1 ng/mL to 500 ng/mL, the AuNP solution's color showed a red-to-blue change and a decrease in the absorbance value at 520 nm. For convenient analysis of the spectra and color changes, the absorbance value at 520 nm was plotted to the logarithm of the CYR concentration and found to be well correlated with the spectra and visual observation. As can be seen in Fig. 3C, excellent linearity was acquired from 1 ng/mL to 500 ng/mL. As low as 1 or 5 ng/mL of CYR could be respectively measured by spectrophotometry or the naked eyes to indicate the spectra and color changes of the AuNP solution. The linear relationship of $y = -0.053x + 0.5735$ ($R^2 = 0.9789$) for 1-50 ng/mL CYR was used for subsequent cucumber sample recovery analysis.

3.5 Cucumber sample pretreatment and matrix effects

Tedious and time-consuming sample pretreatment is always a bottleneck of a rapid screening method. To simplify sample extraction and investigate the matrix effects, cucumber samples were simply homogenized and filtered, and then the filtrate was diluted for 10, 50, 100 and 200 times, respectively. After the dilution, 50 μL of the diluent was added to the 100 μL mixture of 50 μL polyT10 and 50 μL AuNPs and incubated for 5 min. Subsequently, 10 μL NaCl was added and the absorbance value at 520 nm was recorded. As shown in Fig. 4, the cucumber sample was somewhat effective in increasing the A520 of the AuNP solution and the matrix effects decreased with the increase of the dilution folds. After diluted 200 folds, the cucumber matrix effects were almost negligible.

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Therefore, 200-fold dilution was selected for the following CYR recovery tests of the cucumber samples.

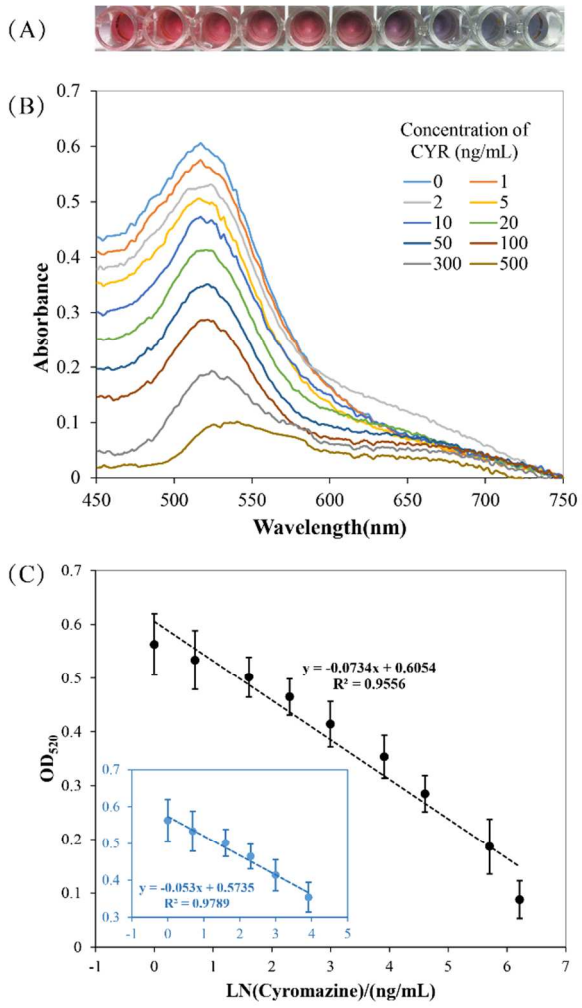

Fig. 3 Sensitivity and linearity of the polyT10-AuNP based colorimetric assay for CYR detection. (A) Visual color changes of AuNPs in the water solution containing different concentrations of CYR. (B) The UV-vis absorbance spectra changes of AuNPs. (C) The absorbance value changes of 520 nm in linear with the logarithm of the CYR concentrations in the range from 1 ng/mL to 500 ng/mL. Inset: from 1 ng/mL to 50 ng/mL.

3.6 Specificity of the colorimetric assay

The specificity of the assay protocol relies on the selectivity of polyT10 for CYR through hydrogen bonds. In order to determine the specificity of this method, we tested the sensing platform against several other common pesticides in water as well as the cucumber diluent such as cypermethrin, esfenvalerate, dimethoate, parathion-methyl and methamidophos at the same concentration of 300 ng/mL. The five pesticide structure was listed in Fig. 5. As shown in Fig. 6, the pesticides other than CYR had little effects on the detection of CYR and there was only about 19% decrease in the A520 value even for parathion-methyl, which had the highest cross-reaction. These results clearly indicated that the polyT10-based colorimetric method is highly specific for CYR determination. Surely, as mentioned in the "Introduction" section, the polyT10 has also been designed for melamine detection, which has a similar structure with CYR[22]. Since a portion of cyromazine can be degraded to toxic melamine via dealkylation reactions, the proposed method is more suitable for CYR screening in cucumber even though its metabolite melamine could also be detected. Besides, Hg^{2+} has also been reported to bind to polyT[28]; however, it does not affect the practicability of the method because there is no Hg^{2+} in cucumber in general. Anyway, since the cucumber matrix effects were almost negligible and as a screening method for possible CYR contamination, any positive results should be validated using chromatography-mass methods.

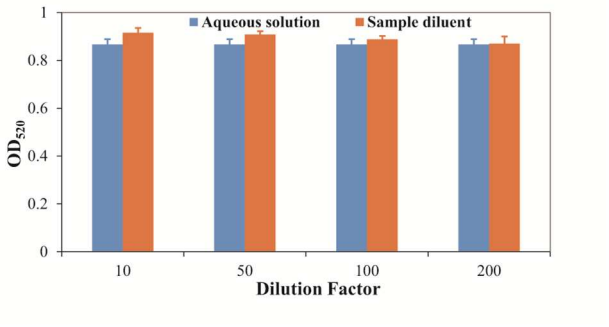

Fig. 4 Cucumber sample matrix effects with the dilutions of 10, 50, 100 and 200 folds.

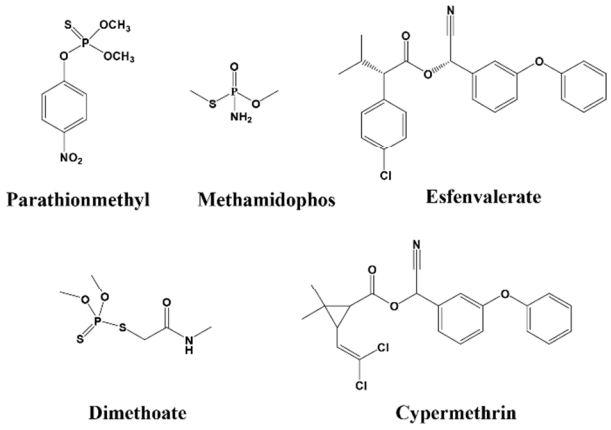

Fig. 5 Five pesticide structures used for specificity evaluation.

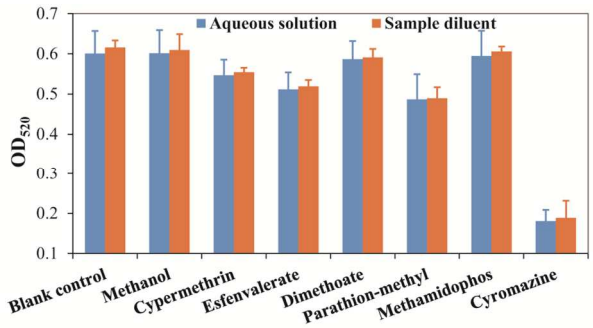

Fig. 6 Specificity evaluation of the proposed method for CYR against other common pesticides in water as well as in the cucumber diluent with the same concentration of 300 ng/mL.

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Control experiments were performed by using polyC10 and 8 mer random sequence (CGGTGGTG) as relevant ssDNAs. Under 0.9 M NaCl, the required minimum concentrations of polyC10 and 8mer random sequence for stabilizing AuNPs were determined as 0.5 μM and 0.3 μM, respectively. The lower adsorption concentration of polyC10 or 8 mer random sequence than polyT10 may attribute to the lower affinity of thymine than the other three DNA bases[29]. As could be observed in Fig. 7, the absorbance of the AuNP solutions shows no obvious decrease in all different concentrations of CYR after the addition of salt. It indicates that polyT10 plays a specific role in the colorimetric assay for CYR and supports the proposed binding mechanism between polyT10 and CYR.

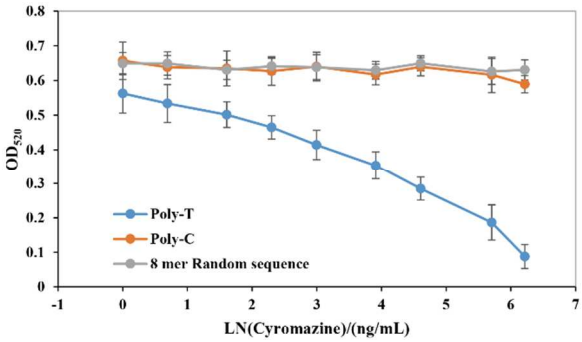

Fig. 7 Control experiments for verifying the polyT10 binding mechanism to CYR in the colorimetric method for CYR detection.

3.7 Method validation in cucumber samples

Cucumber samples known to be free of CYR residues were analyzed to determine the limit of detection (LOD) of the method. The average concentration of CYR in negative cucumbers was found to be 84 ng/g with an SD of 56 ng/g using the proposed method after being multiplied by 200 times of the dilution ratio. Then, the LOD was determined as 252 ng/g with the calculation method of mean+3SD. To determine the limit of quantitation (LOQ) and recovery of the method, the fortified cucumber homogenates containing CYR at 5 different concentration levels were analyzed. The recovery was from 83.7 to 104.8% with an RSD less than 7% (Table 1). The LOQ was determined as 500 ng/g since it can be detected with good repeatability. The recovery experiment results demonstrated that the proposed visual detection of CYR by using AuNPs and polyT10 had good precision and accuracy.

Table 1. The accuracy and precision of the polyT10-AuNP based visual method for determination of CYR in cucumber samples (n=3)

Spiked concentration (ng/g)	Concentration after 200 times dilution (ng/g)	Found concentration (ng/g)	Recovery (%)	RSD (%)
500	2.5	487.0	97.4	3.22
1000	5	1016.5	101.6	3.77
2000	10	2095.1	104.8	6.57
5000	25	4184.7	83.7	3.30
10000	50	9967.9	99.7	6.95

Conclusions

In this study, we have successfully developed a label-free AuNP based visual detection method for CYR by using polyT10. The assay combined the selectivity and affinity of polyT10 to CYR with the spectroscopic advantages of gold nanoparticles to allow for sensitive detection of CYR. The red-to-blue color change of AuNPs in the presence of CYR was easily observed by the naked eyes or measured by a UV-vis spectrometer. The limit of detection and limit of quantitation reached 252 ng/g and 500 ng/g respectively, meeting the Chinese MRL for CYR in most vegetables. The sensitivity could be further improved by optimization of pH and salt concentration in the reaction system, which has important effects on the ssDNA adsorption on the AuNPs[29, 30]. On the basis of these qualities, polyT10-AuNPs could become a powerful tool for onsite screening of CYR residues in vegetables.

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Notes and references

[a] Key Laboratory of Agro-product Quality and Safety, Institute of Quality Standards and Testing Technology for Agro-products, Chinese Academy of Agricultural Sciences, Beijing, 100081, China.
[b] Key Laboratory of Agri-food Quality and Safety, Ministry of Agri-culture, Beijing, 100081, China.

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