Colloidal Gold Immunochromatographic Assay for Rapid Detection of Carbadox and Cyadox in Chicken Breast

Lingling Guo,§ Xiaoling Wu,*,†,§ Gang Cui,‡ Shanshan Song,*,†,§ Hua Kuang,*,†,§ and Chuanlai Xu*†,§

*State Key Lab of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, People’s Republic of China
†School of Food Science and Technology, Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, Jiangnan University, Wuxi, Jiangsu 214122, People’s Republic of China
‡Yancheng Teachers University, Yancheng 224100, People’s Republic of China

ABSTRACT: Abused or misused carbadox (CBX) and cyadox (CYA) in animal feed may cause food safety concerns, threatening human health. Here, we describe the design of a novel hapten for preparation of a monoclonal antibody against CBX and CYA simultaneously. Using this antibody, colloidal gold immunochromatographic assay (GICA) was developed for screening of CBX and CYA residues in chicken breast. Under optimal conditions, semiquantitative analysis results were visible by eye, with a visual limit of detection of 8 μg/kg for CBX and CYA, and cut-off values of 20 μg/kg for CBX and 40 μg/kg for CYA in chicken breast. Quantitative analysis could be performed using a hand-held strip scanner, with a calculated limit of detection of 2.92 μg/kg for CBX and 2.68 μg/kg for CYA in chicken breast. Validated by liquid chromatography−MS/MS, the developed GICA provides a useful tool for rapid on-site CBX and CYA residue screening in chicken breast.

INTRODUCTION

Carbadox (CBX) and cyadox (CYA) belong to the class of compounds known as quinoxaline 1,4-dioxides, which are widely used as antibacterial growth-promoting agents in animal feed. Because CBX has mutagenic, teratogenic, and carcinogenic properties, many countries have forbidden its use in food animals. CYA is a novel species of quinoxaline and is considered to be safer than CBX, and thus, has replaced other quinoxalines in some countries. However, some studies recently reported that CBX might have potential mutagenicity and liver toxicities at certain doses. Thus, it is necessary to establish a screening method for CBX and CYA residues for animal-origin food.

Several instrumental methods have been established for detection of CBX and CYA, such as high-performance liquid chromatography with ultraviolet (UV) detection and high-performance liquid chromatography tandem mass spectrometry (HPLC−MS/MS). Because of its high accuracy and sensitivity, HPLC−MS/MS is used as the standard method for actual sample detection. However, such methods usually need complex sample pretreatment, expensive instruments, long detection times, and professional technicians. These disadvantages restrict their application for the rapid screening of large numbers of samples.

Compared with these instrumental methods, immunoassay methods have advantages of simple sample preparation, low cost, time-saving, and convenient operation. For this reason, immunoassays, including enzyme-linked immunosorbent assay (ELISA), colloidal gold immunochromatographic assay (GICA), and fluorescence immunoassays, have been widely applied in food safety on-site detection. Recently, some research studies about immunoassays for the rapid detection of quinoxalines had been established. As shown in Table 1, ic-ELSA and immunochromatographic assays have been developed to simultaneously detect five quinoxalines: CBX, CYA, olaquindox (OLA), quinocetone (QCT), and mequindox (MEQ). However, no immunoassays have been reported for simultaneous detection of CBX and CYA in animal tissues.

In this work, we first designed a novel hapten for CBX and CYA monoclonal antibody (mAb) preparation. Based on this antibody and the visualization of gold nanoparticles (GNPs), a strip sensor was fabricated for rapid detection of CBX and CYA residues in chicken breast.

RESULTS AND DISCUSSION

Hapten Design. CBX (MW = 262.23) and CYA (MW = 271.24) are both of low-molecular weight and have no immunogenicity. Thus, they need to be coupled to a carrier protein to induce an immune response by the mouse. However, CBX and CYA have no active groups. As shown in Figure 1a, such as −NH2 or −COOH that can react directly with a carrier protein. In order to prepare a mAb which can identify CBX and CYA simultaneously, we kept the shared structural element of CBX and CYA, as the hapten (Figure 1b). Besides, the −NH2 of the hapten can conjugate with a carrier protein...
using the GA method. GA is a common protein coupling method and can introduce a five carbon chain as the spacer arm that is beneficial to exposure of the antigenic determinant. This results in conditions that are favorable for the mouse’s immune system to produce antibodies against CBX and CYA.

The LC−MS/MS spectrum (Figure 2a,b) revealed a molecular ion at \( m/z \) 205.1 \( [M + 1]^+ \) at a retention time of 2.287 min, which supported a molecular formula of \( C_9H_8N_4O_2 \) (MW 204.19). The structure of the hapten in this work was also further confirmed by \( ^1H \) NMR spectrometry (400 MHz, DMSO-\( d_6 \)) (Figure 2c): \( \delta \) 8.40–8.54 (m, 5H), 8.18 (s, 1H), 7.86–7.93 (m, 2H).

**Antigen Characterization.** Antigens, including hapten−ovalbumin (OVA), hapten−BSA, and hapten−keyhole limpet hemocyanin (KLH), were characterized by UV spectroscopy. As shown in Figure 3, the characteristic UV absorption peaks of hapten and carrier proteins were at 378 and 280 nm. The antigens simultaneously had the absorption peak of hapten at

| methods                        | target analytes                  | LOD       | matrix                  | references               |
|-------------------------------|----------------------------------|-----------|-------------------------|--------------------------|
| immunochromatographic assay   | QCA                              | 25 ng/g   | pig tissues             | Le et al. 2012\(^{22}\)   |
| ic-ELISA and time-resolved     | QCA                              |           | porcine muscle and liver| Le et al. 2015\(^{20}\)   |
| fluorooimmunoassay            | CBX, MEQ, OLA, QCT, and CYA      | 10, 15, 20 and 20 ng/mL | animal feeds            | Le et al. 2015\(^{10}\)   |
| immunochromatographic strip   | MQCA                             | 0.25 ng/mL| fish                    | Liu et al. 2017\(^{11}\)  |
| immunochromatographic assay   | OLA                              | 0.68 μg/kg| swine feeds             | Peng et al. 2019\(^{27}\) |
| GICA                          | CBX and CYA                      | 2.92 and 2.68 μg/kg | chicken breast          | this work                |

**Figure 1.** (a) Chemical structure of CBX, CYA; (b) synthetic route of hapten.

**Figure 2.** LC−MS/MS and \(^1\)H NMR spectra of hapten. (a) Positive ions LC spectrum of hapten with a retention time of 2.287 min; (b) mass spectrum of hapten with a \( m/z \) ratio of 205.1 confirmed the formula of hapten (\( C_9H_8N_4O_2 \), MW 204.19). (c) \(^1\)H NMR spectra of hapten.
345 nm and carrier proteins at 280 nm, and the obviously shifted peaks indicated these antigens were successfully produced.

**mAb Characterization.** The sensitivity of a mAb determines to a great extent the sensitivity of the associated immunoassay. The assay buffer plays a vital role in immunoassay analysis. The pH value, ionic strength, and organic solvent content of assay buffer have an effect on protein configuration, which will influence the conjugation of the antibody and antigen.\(^{31,32}\) Besides, different analytes have different dissolved conditions; for example, dibutyl phthalate could be sufficiently dissolved at a certain concentration of organic solvent; tetracycline could undergo hydrolysis under acidic and basic conditions, and remain stable under neutral conditions. In this work, NaCl content ranging from 0.4 to 6.4% was tested to assess the effect of ionic strength. As shown in Figure 4a, the absorbance value decreased significantly along with the increasing NaCl content. The maximum absorbance value \(A_{\text{max}}\) was less than 1.0 when the NaCl content was greater than or equal to 3.2%. The biggest \(A_{\text{max}}/IC_{50}\) value was obtained when the NaCl content was 0.4% in assay buffer. The methanol content and pH value of assay buffer have few effects on \(A_{\text{max}}\) values. The biggest \(A_{\text{max}}/IC_{50}\) value was obtained when the methanol content was 10% and the pH value was at 7.4 (Figure 4b,c). Thus, assay buffer with 0.4% NaCl content, 10% methanol content, and pH 7.4 was used to establish the standard curve. Under these optimum conditions, the IC\(_{50}\) values of CBX and CYA were 1.84 and 1.85 ng/mL (Figure 4d), respectively.

Identification of the mAb subtype benefits the selection of the mAb purification method.\(^{33}\) Figure 4e shows that the mAb against CBX and CYA was subtype IgG2a, which can be purified using the salting out method (caprylic acid-saturated ammonium sulfate precipitation method), protein A, or protein G method.\(^{34}\) We used the protein G method for ascites purification to obtain the mAb.

In general, a large \(K_{\text{aff}}\) value indicates high mAb affinity. A high affinity antibody, with a \(K_{\text{aff}}\) value between \(10^7\) and \(10^{12}\) L/mol, can limit the consumption of antibody and antigen in immunoassay development. Through fitting the curve in Figure 4f, \([\text{Ab}]_t\) values of \(2.14 \times 10^9\), \(4.94 \times 10^9\), and \(2.50 \times 10^9\) mol/L were obtained at the corresponding coating concentrations of 1, 0.3, and 0.1 \(\mu\)g/mL, respectively. Substituting into the calculating formula, the \(K_{\text{aff}}\) value of our mAb was \(3.19 \times 10^9\) L/mol. In addition to establishment of the GICA, this antibody was also applied in immunoaffinity column development for the pretreatment of positive samples containing CBX or CYA.\(^{35}\)

The cross-reactivity of our mAb is shown in Table 2. As shown, it has little cross-reactivity with QCA, with an IC\(_{50}\) value of 25.5 ng/mL and cross-reactivity (CR) of 7.3%. However, it shows no cross-reactivity with other quinolones (CR < 1.0%), including OLA, MEQ, QCT, and MQCA. This indicated that the methyl group had a significant influence on antibody generation in the mouse.

**GICA Principle and Establishment.** The schematic diagram showing the GICA principle is shown in Figure 5. The GNP-labeled mAb was first conjugated with target analytes (CBX or CYA) to form the GNP-labeled-mAb—

![Figure 3. UV spectrogram of hapten-KLH, hapten-BSA, and hapten-OVA.](image)

![Figure 4. Characterization of mAb. Optimization of assay buffer for ic-ELISA, (a) NaCl content, (b) methanol content, (c) different pH; (d) standard curve for CBX and CYA with ic-ELISA; (e) subtypes determination; (f) affinity detection.](image)
The CBX (or CYA) complex. The complex flowed with the standard or sample solution from the sample pad to the absorption pad due to the capillary action of the absorption pad. When the solution reached the T zone on the nitrocellulose (NC) membrane, the unconjugated GNP-labeled mAb conjugated with the hapten−OVA on the T line. When it reached the C zone, the GNP-labeled mAb always conjugated with the goat antimouse IgG antibody. If the sample was negative, the GNP-labeled mAb conjugated with the hapten−OVA and generated a red line in the T zone. If the sample was weakly positive, some of the GNP-labeled mAb could also react with the hapten−OVA sprayed on the T line. Therefore, the T zone was colorless. Meanwhile, the C zone would always appear as a red line because of the conjugation between the GNP-labeled mAb and the goat antimouse IgG antibody. If the C line was colorless, this indicated that the strip was invalid.

The coating antigen on the T line and the amount of the mAb used for GNP labeling was optimized. As shown in Figure 6a,b, when the coating antigen was hapten−OVA and the amount of mAb was 8 μg/mL GNP solution, the T line color was relatively lighter at a CBX concentration of 5.0 ng/mL, which showed that the inhibition to CBX was relatively better.

The sample resuspension solution plays an important role in actual sample analysis. Three surfactant species (Tween-20, ON-870, and Triton-100) at 3% in 0.01 M pH 7.4 phosphate-buffered saline (PBS) were optimized. From Figure 6c, it can be seen that the GNPs clumped on the sample pad, which resulted in the T line and C line color being really light. Compared with PBS, the addition of the surfactant increased the flow of sample solution on the strip. However, it also caused visible GNP aggregation on the sample pad when the surfactant was Tween-20 or ON-870. Consequently, 0.01 M PBS pH 7.4 with 3% Triton was chosen as the chicken breast sample resuspension solution for analysis of CBX and CYA in this work.

Under the above optimal conditions, a series of fortified samples (0, 2.0, 4.0, 8.0, 10, 20, and 40 μg/kg) were tested to establish the GICA method for detection of CBX and CYA residues in chicken breast. As shown in Figure 7a,c, the cut-off values when the T line became colorless were 20 μg/kg for CBX and 40 μg/kg for CYA. The lowest concentrations when the T line color become too light to see with the naked eye, defined as the visual limit of detection (LOD), were 8 μg/kg for both CBX and CYA. These results can be used for semiquantitative analysis of unknown samples. If the CBX (or CYA) content of a sample was equal to or greater than 20 μg/kg (or 40 μg/kg), it was considered a positive sample. If the CBX (or CYA) content was between 8 and 20 μg/kg (or 40 μg/kg), it was a weakly positive sample, while if the CBX (or CYA) content was below than 8 μg/kg, the sample was negative.

For quantitative analysis, results were evaluated using a hand-held strip scanner. The standard curves established with the chicken matrix are shown in Figure 7b,d. Twenty blank chicken breast samples were tested, and the corresponding

### Table 2. Cross-Activity of mAb

| Chemicals | Structure | IC₅₀ (μg/mL) | CR (%) |
|-----------|-----------|-------------|--------|
| CBX       | ![CBX Structure](image) | 1.84         | 100    |
| CYA       | ![CYA Structure](image) | 1.85         | 99.5   |
| QCA       | ![QCA Structure](image) | 25.5         | 7.30   |
| OLA       | ![OLA Structure](image) | >200         | <1.0   |
| MEQ       | ![MEQ Structure](image) | >200         | <1.0   |
| QCT       | ![QCT Structure](image) | >200         | <1.0   |
| MQCA      | ![MQCA Structure](image) | >200         | <1.0   |

![Figure 5. Schematic diagram of strip for GICA. (a) Planar view of the strip; (b) Schematic illustration in negative, weakly positive, and positive sample.](image)

![Figure 6. Optimization for GICA. (a) Coating antigen on the T line; (b) mAb amount for GNP labeling; (c) sample suspension solution.](image)
CBX and CYA concentrations were calculated using the matrix standard curve. The average plus three times the standard deviation was defined as the calculated LOD (cLOD). According to this calculation, the cLOD was 2.92 μg/kg for CBX and 2.68 μg/kg for CYA. Therefore, the proposed GICA and hand-held strip scanner could be applied to screening of CBX or CYA residues in chicken samples on site.

Validation of GICA with LC–MS/MS. To evaluate the accuracy, recovery tests were performed. Samples were spiked with CBX (or CYA) at concentrations of 4, 8, or 12 μg/kg. From Table 3, we can see that the recovery rate ranged from 98.8% to 107.8% for CBX and 98.9% to 109.6% for CYA. Therefore, the proposed GICA method could be applied for rapid detection of CBX and CYA residues in chicken breast samples in the field.

Table 3. Analysis of CBX and CYA in Spiked Chicken Breast by GICA and LC–MS/MS (n = 3)

| analytes | spiked level (μg/mL) | GICA recovery rate (%) ± SD | CV (%) | LC–MS/MS recovery rate (%) ± SD | CV (%) |
|----------|---------------------|----------------------------|--------|-------------------------------|--------|
| CBX      | 4                   | 89.5 ± 8.9                 | 9.9    | 93.9 ± 8.9                    | 9.4    |
|          | 8                   | 98.8 ± 12.1                | 12.2   | 96.3 ± 8.0                    | 8.3    |
|          | 12                  | 107.8 ± 7.5                | 9.0    | 99.6 ± 7.3                    | 7.4    |
| CYA      | 4                   | 98.9 ± 14.4                | 14.6   | 106.6 ± 10.4                  | 9.9    |
|          | 8                   | 109.6 ± 10.3               | 9.4    | 114.4 ± 8.5                   | 7.6    |
|          | 12                  | 108.1 ± 9.7                | 9.0    | 106.3 ± 7.3                   | 6.8    |

89.5 ± 8.9 to 109.6 ± 10.3% for CBX and CYA analysis, with coefficients of variation (CVs) ranging from 9.0 to 14.6% in chicken breast. The LC–MS/MS results further confirmed the proposed GICA, with recovery rates ranging from 93.9 ± 8.9 to 114.4 ± 8.5%, and CVs ranging from 6.8 to 9.9%. These results further confirmed the feasibility of the proposed GICA method.

CONCLUSIONS

In this work, we designed a new hapten for production of a mAb against CBX and CYA. Based on this antibody, a GICA was established for semiquantitative and quantitative screening of CBX and CYA residues in chicken breast samples. The detection process could be completed within 15 min. Recovery tests validated the accuracy, and the LC–MS/MS method further validated the results. In conclusion, the developed GICA method could be applied for rapid detection of CBX and CYA residues in chicken breast samples in the field.

EXPERIMENTAL SECTION

Materials and Reagents. Standards, including CBX, CYA, OLA, QCT, and MEQ, were purchased from J&K Scientific Ltd. (Beijing, China). The carrier proteins KLH and OVA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Coupling agent (25% glutaraldehyde solution, m/v), immunologic adjuvants, including Freund’s complete adjuvant (FCA) and Freund’s incomplete adjuvant (FICA) were also purchased from Sigma-Aldrich. The horseradish peroxidase (HRP)-labeled goat antimouse immunoglobulin was obtained from Kangcheng Bioengineering Co. (Shanghai, China). The reagents used for cell fusion, including polyethylene glycol (PEG, MW 1450), RPMI-1640 medium, fetal bovine serum, and hypoxanthine–aminopterin–thyminde (HAT) and hypoxanthine–thyminde supplements were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). The materials used for assembling the strip sensor, including absorption pad (SAP-Z80), sample pad (CB-SB08), NC membrane, and polyvinyl chloride (PVC) sheet (DB-6), were all obtained from JieYi Biotech. Co. Ltd. (Shanghai, China). The handheld GICA analyzer was purchased from Wuxi Determine Biotech. Co. Ltd. (Wuxi, China). Chicken breast samples were bought from Auchan (Wuxi, China).

Hapten Design. The scheme of CBX—hapten synthesis is shown in Figure 1b, and comprised three steps, as follows.

Synthesis of 2-Methylquinoxaline 1,4-Dioxide. The compounds 2-methylquinoxaline (5.00 g, 34.7 mmol) and 3-chlorobenzoperoxoic acid (29.9 g, 174 mmol) were dissolved in dichloromethane (50.0 mL). After stirring overnight at room temperature, this reaction mixture was poured into ice/water. The aqueous layer was extracted with dichloromethane. The combined organic layers were washed with brine, dried over Na2SO4, and concentrated. The residue was purified on a silica column to give 2-methylquinoxaline 1,4-dioxide (4.10 g) as a yellow solid with a yield of 67.2%.

Synthesis of 2-Formylquinoxaline 1,4-Dioxide. A solution of 2-formylquinoxaline 1,4-dioxide (1.00 g, 5.68 mmol) in 1,4-dioxane (10.0 mL) was added to selenium dioxide (1.26 g, 11.4 mmol) and stirred overnight at 100 °C. The reaction mixture was then poured into ice/water, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na2SO4, and concentrated. The residue was purified on a silica column to give 2-formylquinoxaline 1,4-dioxide (800 mg) as a yellow solid with a yield of 72.7%.

Synthesis of 2-(Hydrazonomethyl)quinoxaline 1,4-Dioxide (Hapten). A solution of 2-formylquinoxaline 1,4-dioxide (500 mg, 2.63 mmol) in dichloromethane (5 mL) was added dropwise to hydrazine hydrate (410 mg, 7.88 mmol) and stirred overnight at room temperature. The reaction mixture was poured into ice/water; then, the aqueous layer was
extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated. The residue was purified on a silica column to give the hapten (300 mg) as a yellow solid with a yield of 55.9%.

The structure of the hapten was characterized by 1H NMR spectroscopy and LC–MS/MS analysis.

**Antigen Preparation**. The antigen was synthesized using the glutaraldehyde method. In detail, 20 mg hapten was dissolved in 2 mL dimethylformamide and then 600 μL of glutaraldehyde, diluted ten times using PBS (0.1 M, pH 7.4), was added dropwise. Thirty minutes later, the activated solution was divided into three equal volumes and each was added drop by drop into a carrier protein solution (KLH, BSA or OVA, 5 mg/mL in 0.1 M carbonate buffer pH 9.6). After 2 h of stirring the reaction at room temperature, the conjugates were dialyzed six times against 0.01 M PBS (pH 7.4). Then, hapten–KLH, hapten–BSA, and hapten–OVA were obtained and characterized using an UV–visible spectrophotometer (Agilent, Santa Clara, CA, USA). The hapten–KLH was used as the immunogen, with hapten–BSA and hapten–OVA used as the coating antigens.

**Immunization Schedule**. Female BALB/c mice (6–8 weeks old) were immunized to produce antibodies against CBX and CYA. Before immunization, the immunogen was emulsified with isomeric FCA or FICA. Each mouse was then injected subcutaneously with 100 μg hapten–KLH emulsified with FCA for the first immunization. Every twenty one days, the mouse was given a booster injection of 50 μg hapten–KLH emulsified with FICA. After four immunizations, the serum of each mouse was assessed using ic-ELISA. The detailed ic-ELISA procedure has been described in our previous publication. The mouse with the highest titer and the highest inhibition with CBX and CYA was sacrificed for cell fusion. Three days before cell fusion, this mouse was given a booster injection of 50 μg hapten–KLH without any immunologic adjuvant.

**Cell Fusion and Hybridoma Screening**. As described in our previous reports, spleen cells and sp 2/0 cells were fused at a ratio of 1:5 using the PEG method. Before being inoculated intraperitoneally with 25 μg hapten–KLH without any immunologic adjuvant.

**Gold Immunochromatographic Assay. Preparation of GNPs**. GNPs with a diameter of 25 nm were synthesized using the citrate reduction method. Briefly, 100 mL of chloroauric acid solution (HAuCl₄·3H₂O, 0.01%, w/v) was boiled under continuous stirring; then, 2.0 mL fresh trisodium citrate solution (1%, w/v) was added quickly into the boiled solution. Five minutes after the color changed to wine red, the solution was centrifuged at 8000 rpm, 4 °C for 45 min. The supernatant was then removed, and the sediment was resuspended in 1 mL 0.01 M PBS solution with 0.02% sucrose.

**Strip Fabrication**. The strip for the GICA was made up of four parts, comprising the sample pad, NC membrane, control line, and test line. The strips were stored in a dry environment for further use.

**Preparation of GNP-Labeled mAb**. First, the mAb was diluted to 0.2 mg/mL using 0.2 M borate buffer solution (pH 8.8), then, 40 μL of 0.1 M potassium carbonate solution was added into 10 mL of the GNP solution. Next, 400 μL mAb was added to the GNP solution, and the mixture was allowed to stand for 45 min at room temperature. Finally, 500 μL BSA solution (10%, w/v) was added to the mixture and incubated for 2 h at room temperature. Then, the solution was centrifuged at 8000 rpm, 4 °C for 45 min. The supernatant was then removed, and the sediment was resuspended in 1 mL 0.01 M PBS solution with 0.02% sucrose.

**Antibody Subtype**. The antibody subtype was determined using a mAb isotyping ELISA kit. The different HRP-labeled secondary antibodies included IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM.

**Affinity**. As described in our previous publications, the antibody was diluted from the 1 μg/mL stock to form a gradient of 8-fold serial dilutions. The coating antigen (hapten–OVA) concentrations were 1, 0.3, and 0.1 μg/mL. The mAb affinity was tested by ic-ELISA. By fitting the antibody concentration to the absorption values, the antibody concentration when the absorbance is half of the initial value ([Ab]₀) can be calculated. The affinity constant (Kₘ) was calculated using the following equation, where n is the ratio of two corresponding antigen concentrations.

\[
K_m = \frac{-n - 1}{2(n[Ab]_0 - [Ab]_1)}
\]

**Cross-Reactivity**. Other quinoxalines, including CYA, OLA, MEQ, QCT, MQCA, and QCA, were used to evaluate the cross-reactivity of the mAb. Similarly, the IC₅₀ values of each quinoxaline were determined. The CR % could be obtained from the following equation, as described in previous reports.

\[
CR\% = \frac{(IC_{50}\ value\ of\ CBX)}{(IC_{50}\ value\ of\ each\ structural\ analogue)}
\]
GICA Procedure. To perform the test, 150 μL of standard (CBX or CYA) or sample solution was mixed with 50 μL GNP-labeled mAb in a microplate well. The mixture was incubated for 3 min at room temperature. The strip was then dipped into this well, so that the solution flowed from the sample pad to the absorption pad due to capillary action. After 5 min, the results could be visualized by the naked eye. For quantitative analysis, the strip was tested using a hand-held strip scanner, which could give the T/C value (the ratio of color intensity between the T line and the C line) directly.

Analysis of Spiked Chicken Breast Using the Strip Test. Chicken breast samples were purchased from a local supermarket and were proven to be CBX and CYA-free by CBX or CYA chicken breast standards. Each sample was pretreated as follows: 5.0 g of minced chicken breast was weighted into a 50 mL centrifuge tube; then, 20 mL of acetonitrile was added, and the tube was vibrated for 3 min. Then, the mixture was centrifuged at 5000 rpm for 5 min, after which 4 mL of the supernatant was removed and dried using pressured blowing concentrators at 45 °C. The residue was dissolved in 4.0 mL PBS (0.01 M, pH 7.4) containing 3% acetonitrile was added, and the tube was vibrated for 3 min. Each sample was pretreated as follows: 5.0 g of minced chicken breast was weighted into a 50 mL centrifuge tube; then, 20 mL of acetonitrile was added, and the tube was vibrated for 3 min. Then, the mixture was centrifuged at 5000 rpm for 5 min, after which 4 mL of the supernatant was removed and dried using pressured blowing concentrators at 45 °C. The residue was dissolved in 4.0 mL PBS (0.01 M, pH 7.4) containing 3% acetonitrile was added, and the tube was vibrated for 3 min. Then, the mixture was centrifuged at 5000 rpm for 5 min, after which 4 mL of the supernatant was removed and dried using pressured blowing concentrators at 45 °C. The residue was dissolved in 4.0 mL PBS (0.01 M, pH 7.4) containing 3% acetonitrile was added, and the tube was vibrated for 3 min. Then, the mixture was centrifuged at 5000 rpm for 5 min, after which 4 mL of the supernatant was removed and dried using pressured blowing concentrators at 45 °C. The residue was dissolved in 4.0 mL PBS (0.01 M, pH 7.4) containing 3% acetonitrile was added, and the tube was vibrated for 3 min. Then, the mixture was centrifuged at 5000 rpm for 5 min, after which 4 mL of the supernatant was removed and dried using pressured blowing concentrators at 45 °C. The residue was dissolved in 4.0 mL PBS (0.01 M, pH 7.4) containing 3%



Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is financially supported by National Key R&D Program (2018YFC1602901), the National Natural Science Foundation of China (21977038, 51902136, 21874058, 21771090, and 21673104), and grants from Natural Science Foundation of China (21977038, 51902136, 21874058, and 21673104). This work is also financially supported by the Innovative Project of Postgraduate Education in Jiangsu Province (SJLX15_0542).

REFERENCES

(1) Wang, X.; Martínez, M.-A.; Cheng, G.; Liu, Z.; Huang, L.; Dai, M.; Chen, D.; Martínez-Larañaga, M.-R.; Anadón, A.; Yuan, Z. The critical role of oxidative stress in the toxicity and metabolism of quinoxaline 1,4-di-N-oxides in vivo. Drug Metab. Rev. 2016, 48, 159–182.
(2) Cheng, G.; Sa, W.; Cao, C.; Guo, L.; Hao, H.; Liu, Z.; Wang, X.; Yuan, Z. Quinoxaline 1,4-di-N-Oxides: Biological Activities and Mechanisms of Actions. Front. Pharmacol. 2016, 7, 64.
(3) Huang, Q.; Isan, A.; Guo, P.; Luo, X.; Cheng, G.; Hao, H.; Chen, D.; Jamil, F.; Tao, Y.; Wang, X. Evaluation of the safety of primary metabolites of cyadox: Acute and sub-chronic toxicology studies and genotoxicity assessment. Regul. Toxicol. Pharmacol. 2016, 74, 123–136.
(4) Duan, Z.; Yi, J.; Fang, G.; Fan, L.; Wang, S. A sensitive and selective imprinted solid phase extraction coupled to HPLC for simultaneous detection of trace quinoxaline-2-carboxylic acid and methyl-3-quinoxaline-2-carboxylic acid in animal muscles. Food Chem. 2013, 139, 274–280.
(5) Zhao, Y.; Yue, T.; Tao, T.; Wang, X.; Huang, L.; Xie, S.; Pan, Y.; Peng, D.; Chen, D.; Yuan, Z. Simultaneous Determination of Quinoxalines in Animal Feeds by a Modified QuEChERS Method with MWCNTs as the Sorbent Followed by High-Performance Liquid Chromatography. Food Anal. Methods 2017, 10, 2085–2091.
(6) Yan, D.; He, L.; Zhang, G.; Fang, B.; Yong, Y.; Li, Y. Simultaneous Determination of Cyadox and Its Metabolites in Chicken Tissues by LC-MS/MS. Food Anal. Methods 2012, 5, 1497–1505.
(7) Merou, A.; Kaklamanos, G.; Theodoridis, G. Determination of Carbadox and metabolites of Carbadox and Olaquindox in muscle tissue using high performance liquid chromatography—tandem mass spectrometry. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 2012, 881–882, 90–95.
(8) Xie, J.; Zeng, W.; Gong, X.; Zhai, R.; Huang, Z.; Liu, M.; Shi, G.; Jiang, Y.; Dai, X.; Fang, X. A "Two-in-One" Tandem Immunoaffinity Column for the Sensitive and Selective Purification and Determination of Trace/Ultra-Trace Olaquindox and Its Major Metabolite in Fish Tissues by LC-MS/MS. Food Anal. Methods 2019, 1–10.
(9) Zeng, L.; Song, S.; Zheng, Q.; Luo, P.; Wu, X.; Kuang, H. Development of a sandwich ELISA and immunochromatographic strip for the detection of shrimp tropomyosin. Food Agric. Immunol. 2019, 30, 606–619.
(10) Lin, L.; Jiang, W.; Xu, L.; Liu, L.; Song, S.; Kuang, H. Development of IC-ELISA and immunochromatographic strip assay for the detection of flunixin meglumine in milk. Food Agric. Immunol. 2018, 29, 193–203.
(11) Chen, Y.; Liu, L.; Xu, L.; Song, S.; Kuang, H.; Cui, G.; Xu, C. Gold immunochromatographic sensor for the rapid detection of twenty-six sulfonamides in foods. Nano Res. 2017, 10, 2833–2844.
(12) Chen, Y.; Wang, Y.; Liu, L.; Wu, X.; Xu, L.; Kuang, H.; Li, A.; Xu, C. A gold immunochromatographic assay for the rapid and simultaneous detection of fifteen β-lactams. Nanoscale 2015, 7, 16381–16388.
(13) Chen, Y.; Guo, L.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Ultrasensitive Immunochromatographic Strip for Fast Screening of 27 Sulfonamides in Honey and Pork Liver Samples Based on a Monoclonal Antibody. J. Agric. Food Chem. 2017, 65, 8248–8255.
(14) Guo, L.; Wu, X.; Liu, L.; Kuang, H.; Xu, C. Gold Nanoparticle-Based Paper Sensor for Simultaneous Detection of 11 Benzimidazoles by One Monoclonal Antibody. Small 2018, 14, 1701782.
(15) Peng, J.; Liu, L.; Xu, L.; Song, S.; Kuang, H.; Cui, G.; Xu, C. Gold nanoparticle-based paper sensor for ultrasensitive and multiple detection of 32 (fluoro)quinolones by one monoclonal antibody. Nano Res. 2017, 10, 108–120.
(16) Kong, D.; Liu, L.; Song, S.; Suryoprahowo, S.; Li, A.; Kuang, H.; Wang, L.; Xu, C. A gold nanoparticle-based semi-quantitative and quantitative ultrasensitive paper sensor for the detection of twenty mycotoxins. Nanoscale 2016, 8, 5245–5253.
(17) Wang, Z.; Sun, L.; Liu, L.; Kuang, H.; Xu, L.; Xu, C. Ultrasensitive detection of seventeen chemicals simultaneously using paper-based sensors. Mater. Chem. Front. 2018, 2, 1900–1910.
(18) Wang, Z.; Wu, X.; Liu, L.; Xu, L.; Kuang, H.; Xu, C. An immunochromatographic strip sensor for sulindafm and its analogues. J. Mater. Chem. B 2019, DOI: 10.1039/c8mb00280d.
(19) Fu, X.; Chen, L.; Choo, J. Optical Nanoprobes for Ultrasensitive Immunoassay. Anal. Methods 2017, 9, 124–137.
(20) Huang, X.; Zhan, S.; Xu, H.; Meng, X.; Xiong, Y.; Chen, X. Ultrasensitive fluorescence immunoassay for detection of ochratoxin
A using catalase-mediated fluorescence quenching of CdTe QDs. *Nanoscale* 2016, 8, 9390–9397.

(21) Li, C.; Wen, K.; Mi, T.; Zhang, X.; Zhang, H.; Zhang, S.; Shen, J.; Wang, Z. A universal multi-wavelength fluorescence polarization immunoassay for multiplexed detection of mycotoxins in maize. *Biosens. Bioelectron.* 2016, 79, 258–265.

(22) Le, T.; Xu, J.; Jia, Y.-T.; He, H.-Q.; Niu, X.-D.; Chen, Y. Development and validation of an immunochromatographic assay for the rapid detection of quinoxaline-2-carboxylic acid, the major metabolite of carbadox in the edible tissues of pigs. *Food Addit. Contam.* 2012, 29, 925–934.

(23) Jiang, W.; Beier, R. C.; Wang, Z.; Wu, Y.; Shen, J. Simultaneous Screening Analysis of 3-Methyl-quinoxaline-2-carboxylic Acid and Quinoline-2-carboxylic Acid Residues in Edible Animal Tissues by a Competitive Indirect Immunoassay. *J. Agric. Food Chem.* 2013, 61, 10018–10025.

(24) Cheng, L.; Jianzhong, S.; Zhanhui, W.; Wenxiao, J.; Suxia, Z. A sensitive and specific ELISA for determining a residue marker of three quinoxaline antibiotics in swine liver. *Anal. Bioanal. Chem.* 2013, 405, 2653–2659.

(25) Peng, J.; Kong, D.; Liu, L.; Song, S.; Kuang, H.; Xu, C. Determination of quinoxaline antibiotics in fish feed by enzyme-linked immunosorbent assay using a monoclonal antibody. *Anal. Methods* 2015, 7, 5204–5209.

(26) Le, T.; Yu, H.; Niu, X. Detecting quinoxaline-2-carboxylic acid in animal tissues by using sensitive rapid enzyme-linked immunosorbent assay and time-resolved fluoroomunoassay. *Food Chem.* 2015, 175, 85–91.

(27) Peng, T.; Wang, J.; Xie, S.; Yao, K.; Zheng, P.; Ke, Y.; Jiang, H. Label-free gold nanoclusters as quenchable fluorescent probes for sensing olaquindox assisted by glucose oxidase-triggered Fenton reaction. *Food Addit. Contam., Part A* 2019, 36, 752–761.

(28) Liu, L.; Peng, J.; Xie, Z.; Song, S.; Kuang, H.; Xu, C. Development of an icELISA and Immunochromatographic Assay for Methyl-3-Quinoxaline-2-Carboxylic Acid Residues in Fish. *Food Anal. Methods* 2017, 10, 3128–3136.

(29) Cheng, L.; Shen, J.; Wang, Z.; Jiang, W.; Zhang, S. A sensitive and specific ELISA for determining a residue marker of three quinoxaline antibiotics in swine liver. *Anal. Bioanal. Chem.* 2013, 405, 2653–2659.

(30) Le, T.; Zhu, L.; Shu, L.; Zhang, L. Simultaneous determination of five quinoxaline-1,4-dioxides in animal feeds using immunochromatographic strip. *Food Addit. Contam.* 2015, 33, 244–251.

(31) Nguyen, T.-H.; Greinacher, A. Effect of pH and ionic strength on the binding strength of anti-PF4/polyanion antibodies. *Eur. Biophys. J. Biophys. Lett.* 2017, 46, 795–801.

(32) Kuang, H.; Liu, L.; Xu, L.; Ma, W.; Guo, L.; Wang, L.; Xu, C. Development of an enzyme-linked immunosorbent assay for dibutyl phthalate in liquor. *Sensors* 2013, 13, 8331–8339.

(33) Bergmann-Leitner, E. S.; Mease, R. M.; Duncan, E. H.; Khan, F.; Waitumbai, J.; Angov, E. Evaluation of immunoglobulin purification methods and their impact on quality and yield of antigen-specific antibodies. *Malar. J.* 2008, 7, 129.

(34) Harlow, E.; Lane, D. Antibody purification on protein a or protein g columns. *Cold Spring Harbor Protoc.* 2006, 2006, 82–84.

(35) Yao, K.; Wen, K.; Shan, W.; Xie, S.; Peng, T.; Wang, J.; Jiang, H.; Shao, B. Development of an immunoaffinity column for the highly sensitive analysis of bisphenol A in 14 kinds of foodstuffs using ultra-high-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2018, 1080, 50–58.

(36) Zhang, X.; Wang, G.; Yang, L.; Zhang, W.; Lin, J.; Li, C. Determination of eight quinolones in milk using immunofluorometric microextraction in a packed syringe and liquid chromatography with fluorescence detection. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2017, 1064, 68–74.

(37) Guo, L.; Xu, L.; Song, S.; Liu, L.; Kuang, H. Development of an immunochromatographic strip for the rapid detection of maduramicin in chicken and egg samples. *Food Agric. Immunol.* 2018, 29, 458–469.