Rapid and Sensitive Immunochromatographic Method-Based Monoclonal Antibody for the Quantitative Detection of Metalaxyl in Tobacco

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ABSTRACT: In this study, we prepared a monoclonal antibody (mAb) against metalaxyl (Met) with a half-maximum inhibitory concentration (IC50) of 0.54 ng/mL based on a new hapten, and a gold nanoparticle-based immunochromatographic assay (GICA) was developed for the rapid detection of Met residues in tobacco. Under optimal conditions, even with the naked eye, one can see the semiquantitative analysis results. The naked eye detection limit of Met in tobacco is 25 μg/kg, and the detection threshold is 100 μg/kg. In addition, the cross-reactivity test shows that the mAb has good specificity for Met, and the GICA results have a good correlation with the indirect competitive enzyme-linked immunosorbent assay and liquid chromatography with tandem mass spectrometry test results, which show that the method is feasible and reliable and are more convenient and quicker than the methods using instrumentation for detection. Therefore, GICA may provide a useful tool for the rapid screening and detection of Met residues in tobacco.

1. INTRODUCTION

In order to control or prevent diseases and to maintain the quantity and quality of agricultural products, a large number of pesticides have been introduced. Currently, among pesticide types, fungicides represent the largest proportion. The commonly used fungicides include triazoles and amides, both of which are chiral pesticides. Amide fungicides are a large class of newly developed fungicides used in agriculture and to treat fruits and vegetables worldwide. They have been widely used for decades in the preservation of foods and in food processing. In terms of chemical structure, they can be roughly divided into carboxylic acid amides, such as mandelic acid and phenylamides; more than 30 varieties have been used in practice and new varieties are constantly being added to this list. Among them, metalaxyl (Met) is a widely used phenylamide fungicide with high efficiency, low toxicity, and low residual effects. Met is an important chiral acetalanilide fungicide; its activity is derived almost entirely from the R-enantiomer. Its mechanism of action is mainly by inhibiting ribosomal RNA synthesis. Therefore, Met is often used around the world to inhibit plant diseases caused by pathogens and acts on a variety of crops, including seeds, vegetables, and even ornamental plants. However, it is mainly used to control downy mildew found in fruits and vegetables and late blight in potatoes. Tobacco is a momentous cash crop and has a huge consumer market worldwide. However, many diseases, such as powdery mildew and brown spot, can threaten the growth of tobacco. In consequence, fungicides are also used to safeguard tobacco from these diseases. However, in recent years, because of the unreasonable use of fungicides, the residues of fungicides in tobacco products exceed the standard, which affects not only the life safety of consumers but also the tobacco trade. Therefore, it is urgent and necessary to establish a method for the rapid detection of fungicide residues in tobacco.

As the use of fungicides in agriculture has gradually increased, the problems they cause have also attracted worldwide attention. Because of the high toxicity and long-term persistence of fungicides, they can often pollute the environment through a variety of ways, including spraying, soil seepage, and storage and discharge of wastewater, which may cause pollution of the ground and surface water and cause public health problems. In addition, studies have reported

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that Met may have an effect on mammalian metabolic mechanisms,\textsuperscript{12} therefore, the abuse of fungicides also poses a potential risk to wildlife and human health.\textsuperscript{13} At present, many countries have strict restrictions on the levels of Met residues in food. The maximum Met residue limit set by the Chinese Ministry of Agriculture is no more than 2 mg/kg in vegetables and no more than 5 mg/kg, but not more than 10 mg/kg in beverages. Therefore, the qualitative and quantitative determination of fungicide residues in tobacco, because of its importance globally, is very important.

At present, the detection of phenylamide fungicides mainly relies on instruments for analysis, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC). Because of the high accuracy and sensitivity of the instrumental analysis methods, they are widely used to detect Met in food materials such as potatoes,\textsuperscript{14} cucumbers,\textsuperscript{15} tomatoes,\textsuperscript{16} other crops,\textsuperscript{17} and seasonal crops.\textsuperscript{14} Compared with HPLC and GC, which are expensive and time-consuming and require tedious sample preparation, immunosassay methods such as gold nanoparticle-based immunochromatographic assay (GICA) method are time-saving, highly specific, and sensitive. The sample preparation for this method is simple, and it provides a visual readout very quickly. We compared the GICA method with several other instrumental analysis methods. From Table 1, it can be seen that the detection results of GICA and traditional methods are almost the same. Therefore, it can be said that it is expected to replace the traditional instrumental analysis method and provides an easy, rapid, and accurate method for detecting fungicide residues.\textsuperscript{18}

Regardless of the method used, currently there are no reports on the detection of fungicide residues in tobacco.

In this research, we prepared a monoclonal antibody (mAb) for the qualitative and quantitative detection of Met residues in tobacco. Tobacco samples have components other than analytes that can significantly interfere with the analyte analysis process and affect the accuracy of the analysis results. These effects and interferences are called matrix effects.\textsuperscript{19} Therefore, the matrix effect of using colloidal gold test paper to detect Met in tobacco will be a main obstacle in this study.

2. RESULTS AND DISCUSSION

2.1. Hapten Design and Characterization. The relative molecular mass of Met is 279.33 Da, which is a small molecule and is incapable of eliciting an immune response alone. Furthermore, there are no active groups such as carboxyl groups or amino groups in the structure of Met, implying that it cannot be directly coupled to a carrier protein. Therefore, in this research, a COOH active group was introduced through derivation without any change in the structure of Met itself. The hapten was constructed as described above, and the structure of the product was confirmed by liquid chromatography–mass spectrometry (LC–MS) (Figure 3). The retention time of Met-hapten was 8.40 min and the m/z ratio was 264.117, confirming the structure of Met-hapten as C\textsubscript{14}H\textsubscript{19}N\textsubscript{1}O\textsubscript{4} (M\textsubscript{w} = 265.117 Da).

2.2. Characterization of the Antigen. As mentioned earlier, the resulting hapten is not immunogenic and needs to bind to carrier proteins [keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and ovalbumin (OVA)]. In this
EDC-KLH. Similarly, this phenomenon also exists in the Met-EDC-BSA UV diagram in Figure 4b, indicating the successful synthesis of Met-EDC-BSA.

2.3. Characterization of the mAb. In this study, a total of five cell lines were obtained, including 1C12, 2B5, 2E1, 5H1, and 5H4, among which 2E1 was the best. Therefore, the mAb 2E1 with a higher titer was chosen for the subsequent optimization study. The optimal concentrations of the coated antigen and the antibody were 0.3 and 0.1 μg/mL, respectively. The buffer pH, ionic concentration, and methanol content all play a vital role in enzyme-linked immunosorbent assay (ELISA) analysis. This is mainly because the concentration of sodium chloride (NaCl) can affect the sensitivity of the antibody for the antigen. Methanol can increase the solubility of the antibody, but a too high concentration will cause the antibody to denature. The pH can change the conformation of the antibody and the antigen and affect the combination of the two.20 In addition, the pH, organic solvent content, and ionic strength have different effects on the dissolution conditions of different analytes except for proteins. Therefore, optimizing the buffer plays a crucial role in improving the sensitivity of the mAb in immunoassays. In this study, the NaCl content of 0.4–6.4% was determined to evaluate the effect of ionic strength. It can be seen from Figure 5a that as the NaCl concentration increases, the absorbance value decreases significantly. When the NaCl content is ≥3.2%, the maximum absorbance value (A_{max}) > 1.0. When the NaCl content is 0.8%, the absorbance at 450 nm/half-maximum inhibitory concentration (A_{450nm}/IC_{50}) value is the highest. As shown in Figure 5b, when methanol is absent, the A_{450nm}/IC_{50} value is the highest. As shown in Figure 5c, when the pH value of the buffer is 7.4, the A_{450nm} value is >1.0 and the A_{450nm}/IC_{50} value is the highest. Therefore, a standard curve was established using 0.4% NaCl concentration, 10% methanol content, and an assay buffer at pH 7.4. Under these optimal conditions, the established Met standard curve shows IC_{50} = 0.54 μg/kg (Figure 5d).

2.4. Antibody Specificity. Cross-reactivity (CR) is significant for the analysis of actual samples because they may induce false positive results due to the fact that the actual samples may contain unknown components with similar structures to the analyzed substances. Table 2 lists the IC_{50} and CR values of Met structural analogues used in this study. It can be seen from Table 2 that this mAb does not cross-react with the other three fungicides with similar structures to Met, indicating that the mAb obtained in this study has good specificity for Met and can be used in the production of test samples.

2.5. GICA. Tobacco samples were analyzed using a gold standard test strip. As mentioned earlier, the gold nanoparticle (GNP) solution with a diameter of 20 nm produces a uniform and stable burgundy red color. Therefore, for testing, 150 μL of a sample solution was mixed with 50 μL of GNP-labeled mAb in the wells of the microtiter plate. The mixed solution was placed at room temperature for 3–5 min, and then dropped into the test strip detection hole to allow the solution to flow from the sample pad to the absorption pad through capillary action. After waiting for 5–10 min, due to the visibility of the GNPs, the test strip results could be observed directly with the naked eye.21 We usually use the following two indicators for qualitative analysis: the critical value is the sample concentration when the T line is colorless and the P_{LOD} value is the visual limit value when the T line is a light color and the lowest detection concentration.22 For quantitative
analysis, the results were measured by a hand-held bar scanner. The strip scanner can directly give a $T/T_0$ value within 30 s (where the $T$ value represents the color intensity of a given sample and $T_0$ represents the color of the blank sample). In this study, we established a standard curve with a series of Met concentrations and their corresponding $T/T_0$ values.

Prior to testing the samples, in order to ensure the best test results, we optimized the sample resuspension solution. In this experiment, we selected three different reagents [polyethylene pyrrolidone (PVP), BSA, and mannitol].21 The results show that the suspension containing the PVP reagent has the best color development effect and the most stable (Figure 6). Therefore, a suspension buffer containing PVP was selected as the tobacco sample resuspension buffer and used in subsequent experiments.

Under the optimal conditions described above, we tested a range of samples (0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100 μg/kg) to establish a GICA method for detecting Met residues in tobacco. It can be seen from Figure 7a that in tobacco samples, the critical value of the test paper is 100 μg/kg, and the $V_{LOD}$ value is 25 μg/kg. These results can be used for the semiquantitative analysis of unknown samples. If the result is positive, the Met content of the samples is greater than or equal to 100 μg/kg; if the result is weakly positive, the Met content is between 25 and 100 μg/kg; and if the result is negative, the Met content is less than 25 μg/kg.

Based on the standard curve established by the tobacco matrix, the calculated detection limit ($C_{LOD}$) is 2.47 μg/kg. Therefore, a combination of GICA and a hand-held strip scanner can be used to detect Met residues in tobacco.

2.6. Indirect Competitive-ELISA, Liquid Chromatography with Tandem Mass Spectrometry, and GICA Determination for Spiked Samples. In order to verify the GICA test results and demonstrate the feasibility of our test method, we performed addition and recovery tests by indirect competitive-ELISA (ic-ELISA), liquid chromatography with tandem mass spectrometry (LC−MS/MS), and test strip determination and performed three repeated tests on each sample with a single batch of test strips and test strip reader measurements. As shown in Table 3, the average recovery range of the GICA test results was 91.6 ± 2.2 to 99.7 ± 2.8; the CV value range was 5.7−7.8%; the average recovery range using the ic-ELISA and LC−MS/MS were 90.42 ± 6.5 to 112.2 ± 8.2 and 92.32 ± 5.5 to 109.45 ± 8.1, respectively; and the CV value range was 7.2−7.7 and 6.0−7.9%, respectively. The highest standard relative deviation of the three was 7.8, 7.7, and 7.9% (Table 3), which shows that our test strip detection method had a good correlation with the result of ic-ELISA and LC−MS/MS and is feasible and reliable. Therefore, the GICA proposed in this study can be used to test Met residues in tobacco samples.

### Table 2. CR of mAb

| amide fungicides | IC$_{50}$ (μg/kg) | CR (%) |
|------------------|-------------------|--------|
| Met              | 0.54              | 100    |
| oxadixyl         | >1000             | <0.02  |
| benalaxyl        | >1000             | <0.02  |
| furalaxyl        | >1000             | <0.02  |

### 3. CONCLUSIONS

To sum up, in this study, we successfully developed a new Met hapten and prepared a specific mAb with high sensitivity to Met by immunizing mice and cell fusion. The antibody has an IC$_{50}$ of only 0.54 μg/kg. Subsequently, we also developed a GICA method for the rapid detection of Met residues in tobacco, and we also performed experiments to optimize the...
reaction conditions for strip analysis, and under the optimal conditions, we determined that the detection limit and the lower detection limit of Met in tobacco were 100 and 25 μg/kg, respectively. In addition, we determined that the recovery of the spiked tobacco samples by the strip method was 91.6−99.7%, which had a good correlation with the result of ic-ELISA and consistent with the LC−MS/MS test results. Therefore, it can be seen that GICA can be used as a method for the rapid screening and detection of Met residues in tobacco, which can be used for the large-scale screening of Met residues in tobacco samples.

4. MATERIALS AND METHODS

4.1. Materials and Apparatus. Met was purchased from J&K Scientific Ltd. (Beijing, China). Carrier proteins, including BSA, OVA, KLH, peroxidase-labeled goat antimouse immunoglobulin (IgG-HRP), Freund’s complete adjuvant (FCA), and Freund’s incomplete adjuvant (FIA), were obtained from Jackson ImmunoResearch Laboratories, Inc. (Bar Harbor, Maine, USA). All other reagents and solvents were purchased from Aladdin (Shanghai, China) and were of analytical grade and above.

The main apparatuses used in this work are as follows: a UV−vis scanner was purchased from Bokin Instruments (Tsushima, Japan) and a Multiskan MKS microplate reader was purchased from Thermo Labsystems Company, Ltd. (Beijing, China). Other instruments included a vortex machine bought from Shanghai Huix Analytical Instrument Factory (Shanghai, China), a membrane distributor obtained from New Genesis Gene Technology (Beijing, China), and a water bath acquired from Shanghai Instrument Group (Shanghai, China).

4.2. Antibody Preparation and Methods. 4.2.1. Hapten Synthesis. Substances with a molecular weight of less than 10 kDa are generally referred to as small molecules, which do not directly induce an immune response in mice and, therefore, need to be bound to a carrier protein (KLH, BSA, or OVA) to induce the desired effect and produce the relevant antibodies.

Table 3. Recovery of Met in Tobacco by GICA, ic-ELISA, and LC−MS/MS (n = 3)

| samples    | spiked level of Met (μg/kg) | GICA recovery (%) ± SD | CV (%) | ic-ELISA recovery (%) ± SD | CV (%) | LC−MS/MS recovery (%) ± SD | CV (%) |
|------------|-----------------------------|------------------------|--------|---------------------------|--------|---------------------------|--------|
| tobacco    | 20                          | 91.6 ± 5.2             | 5.7    | 90.42 ± 6.5               | 7.2    | 92.32 ± 5.5               | 6.0    |
|            | 40                          | 95.3 ± 6.5             | 6.8    | 94.3 ± 7.3                | 7.7    | 98.53 ± 7.8               | 7.9    |
|            | 80                          | 99.7 ± 7.8             | 7.8    | 112.2 ± 8.2               | 7.3    | 109.45 ± 8.1              | 7.4    |
ies. However, small-molecule chemical compounds cannot be directly coupled to carrier proteins but need to be coupled to the carrier protein through active groups such as OH, NH₂, or COOH.²⁰ In this study, we designed a synthesis method for the Met-haptenas follows: hapten are synthesized from Met (Figure 1). Met (2 g) was added to an aqueous solution (200 mL) containing 600 mg of KOH, and the mixture was stirred at 100 °C overnight. Water was added, and the aqueous layers were extracted with EA. The pH of the solution was adjusted to neutral with 6 N HCl aqueous solution and then concentrated to dryness. The obtained crude product was purified by chromatography on a silica cartridge to obtain the hapten as a white solid, and the structure of the product was confirmed by LC–MS/MS.

4.2.2. Antigen Synthesis. The coating antigen Met-EDC-BSA and the immunogen Met-EDC-KLH were prepared using the active ester method, with some modifications made on the basis of the former.²⁴ The synthesis method is as follows: 5.4, 3.6, and 5.1 mg, respectively, of Met-hapten, N-hydroxysuccinimide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were weighed and dissolved in dimethylformamide, with continuous stirring at room temperature for 4–6 h. Then, the solution was added dropwise to 1 mL of KLH solution (10 mg/mL) and continued stirring for 6–8 h. Finally, the mixed solution was put into a dialysis bag and dialyzed against 0.01 M saline (PBS) (pH 7.4) for 72 h, and the final product was stored at −20 °C. The synthesis method of the Met-EDC-BSA conjugate is similar to the above method. The final product was characterized by UV–vis spectrophotometry.

4.3. Immunization and Cell Fusion. 5–6 week old BALB/c female mice were selected to be immunized with Met-EDC-KLH. The immunization program was similar to our earlier report.²⁵ During the first immunization, 100 μg of Met-EDC-KLH was immunized subcutaneously per mouse with an equal amount of FCA. Half-dose boosters were repeated every 3 weeks in FIA. One week after the end of the third immunization, mouse serum was screened by ic-ELISA. In the fifth immunization, without the use of immune adjuvants, mice with the highest inhibitory effect on Met were selected for intraperitoneal injection at a dose of 25 μg of Met-EDC-KLH. After 3 days, the mice were sacrificed and used in cell fusion experiments.²⁶

Mouse spleen cells were fused with SP 2/0 mouse myeloma cells, and the process is as previously described above.²⁷ The fused cells were screened by three rounds of subcloning and ic-ELISA to obtain a purified cell line which could produce antibody stably, and the optimal cell lines were amplified in culture and cryopreserved in liquid nitrogen.

4.4. Antibody Preparation and Characterization. The selected cell lines were cultured on a large scale and collected by centrifugation after 7–10 days. These cells were injected into the abdominal cavity of parafilm-embedded BALB/c mice. One week later, the collected ascites were purified by the precipitation of caprylic acid-ammonium sulfate and then dialyzed with PBS at 4 °C for 3 days. Finally, the resulting pure mAb was stored at −20 °C for future use.²⁷

The sensitivity and specificity of mAb were evaluated using ic-ELISA. To improve the stability and sensitivity, we evaluated different variables such as concentration of sodium chloride (0.4, 0.8, 1.6, 3.2, and 6.4%), methanol concentration (0, 10, 20, 30, and 40%; calculated by volume fraction), and different pH values (pH 4.7, 6.0, 7.4, 8.8, and 9.6) of the standard dilution buffer solution (PBS). Under the best conditions, the absorbance values for Met at 450 nm at different concentrations (0, 0.2, 0.5, 1, 2, 5, and 10 μg/kg) were measured to construct a seven-point standard curve and obtain IC₅₀ from the standard curve to determine the antibody sensitivity.²⁸

4.5. Cross-Reactivity. The specificity of an antibody can be determined by evaluating the cross-reaction between the antibody and a group of structurally similar chemicals with respect to their affinity for Met. Because of the highest similarity between Met, oxadixyl, benalaxyl, and furaxyl, a cross-reaction between Met and the other three substances was also performed. The CR calculation formula is as follows

$$CR\% = \frac{(IC_{50}\text{ of Met})}{(IC_{50}\text{ of structural analogue})}$$

Therefore, IC₅₀ values for the Met structural analogues, including oxadixyl, benalaxyl, and furaxyl, were also measured in this study.

4.6. Preparation of Colloidal Gold-Labeled mAb. 4.6.1. Synthesis of GNPs. GNPs with a diameter of 20–25 nm were prepared by citrate reduction, and the steps are as follows: first, 100 mL of 0.01% chloroauric acid solution was boiled and then 5 mL of 1% trisodium citrate was quickly added during continuous stirring. After waiting for 1 min, the solution turned dark red. It was then allowed to boil for a further 15 min, and then the volume was adjusted to 100 mL with ultrapure water. Finally, the solution was cooled to room temperature and stored at 4 °C. The solution could be characterized by UV–vis spectrophotometry and transmission electron microscopy, and the wavelength of the UV absorption peak was 520 nm (Figure 2).

4.6.2. Preparation of GNP-Labeled mAbs. The antibodies used in this study were labeled using the GNP method as follows: first, before addition of the mAb, the pH of 10 mL of GNP solution was adjusted to 8.0 with 0.1 M potassium carbonate solution. While stirring, an appropriate amount of anti-Met mAb was added dropwise to the colloidal gold solution, and the resulting solution was stirred at room temperature for 2 h. Then, 1 mL of 10% BSA was added to mask the unbound sites of gold to avoid nonspecific adsorption of GNPs and ensure the stability of gold. After the solution was incubated under continuous agitation for 2 h, the solution was centrifuged three times (4000 rcf) to remove the excess mAb and BSA, and the supernatant was discarded to obtain the GNP-labeled mAb precipitate. In the end, the precipitate was resuspended in 1 mL of 0.02 M PBS (including 0.1% Tween-20) and kept at 4 °C until use.

4.6.3. Assembly of the Immunochromatographic Strip. The immunochromatographic strip consists of four parts: conjugated pad, nitrocellulose membrane (NC), sample pad, and absorption pad. Before assembly of the test strip, GNPs labeled with Met antibodies were added to the binding pad, (7–10 μL per pad), and dried in an oven at 37 °C for 2 h. The goat antimouse IgG (10 μg/mL) antibodies and coated antigen (1 mg/mL) were sprayed onto the control line (C line) and the detection line (T line) of the NC membrane with a spray film tester (BioDot Inc., Irvine, CA, USA) and dried at 37 °C for 120 min for later use. Then, in sequence, the sample pad, the processed GNP-antibody binding pad, the NC film, the adhesive plate, and the absorbent pad were assembled and dried at 37 °C for 4 h. Last, the entire assembly was cut into a strip of 3 mm width with a CM4000Gu guillotine cutting pad, the processed GNP-antibody binding pad, the NC membrane with the sample control and detection lines (Figure 3).
module (BioDot Inc., Irvine, CA, USA) and then stored in a self-sealing plastic bag until use.29

4.6.4. Sample Pretreatment. The tobacco matrix contains a variety of pesticide residues, and most of the current tobacco pretreatment methods are based on the QuEChERS method, which involves impregnating the pulverized tobacco sample with water, extracted with acetonitrile, then salted out and centrifuged to separate the layers, and finally purified with adsorbents such as propylethylenediamine. The specific steps are as follows:

The chopped tobacco sample (2 g) was taken in a 50 mL centrifuge tube with a stopper, and 10 mL of water was added to soak the sample and then allowed to stand for 10 min. Then, 10 mL of acetonitrile was added to the centrifuge tube along with 100 µL of Met standard solution (20.0 mg/L). It was then shaken on a vortex mixer at a speed of 2000 rpm for 1 min and placed at -20 °C for 10 min. Then, 4 g of anhydrous magnesium sulfate (MgSO4), 1 g of sodium chloride (NaCl), 1 g of sodium citrate (Na3C6H5O7), and 0.5 g of disodium hydrogen citrate (Na2C6H8O7) were added to the centrifuge tube, which was immediately placed on the vortex mixer and shaken at 2000 rpm for 2 min. After centrifugation at 4000 rcf for 3 min, one milliliter of the supernatant was removed into a tube, which was immediately placed on the vortex mixer and shaken at 2000 rpm for 2 min. After centrifugation at 4000 rcf for 3 min, one milliliter of the supernatant was removed into a tube, which was immediately placed on the vortex mixer and shaken at 2000 rpm for 2 min. Then, 4 g of anhydrous sodium citrate (Na 3C6H5O7), and 0.5 g of disodium hydrogen citrate (Na2C6H8O7) were added to the centrifuge tube, which was immediately placed on the vortex mixer and shaken at 2000 rpm for 2 min and then centrifuged at 4000 rcf for 3 min. One milliliter of the supernatant was removed into a 15 mL centrifuge tube, containing 5 mL of an aqueous solution of sodium chloride (20:100, w/w) and 1 mL of n-hexane on a vortex mixer and shaken at 2000 rpm for 2 min. After centrifugation at 2000 rcf for 3 min, n-hexane was removed, and the remaining solution was tested.

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Notes

The authors declare no competing financial interest.

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