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PHD THESIS

DEVELOPMENT AND PRELIMINARY APPLICATION OF IMMUNOCHROMATOGRAPHY TEST STRIPS FOR THE DETECTION OF DOUBLE RESIDUES OF AFLATOXIN B1 AND ZEARALENONE

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The dissertation contains the results of own research. The use of ideas, results and texts of other authors have references to the relevant source WANG YANAN Yanan Wang

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ANNOTATION

Yanan Wang «Development and Preliminary Application of Immunochromatography Test Strips for the Detection of Double Residues of Aflatoxin B1 and Zearalenone» − Qualifying Educational and Scientific Work on the Rights of the Manuscript. Dissertation for the degree of the Doctor of Philosophy in the specialty 211 « Veterinary medicine» − Sumy National Agrarian University, Sumy, 2022

Based on scientific demonstration and experimental research, this thesis is devoted to studying Development and Preliminary Application of Immunochromatography Test Strips for the Detection of Double Residues of Aflatoxin B1 and Zearalenone, to the establishment of colloidal gold immunochromatographic test strip for the determination of aflatoxin B1 (AFB1) and zearalenone (ZEN) dual residue, so as to provide technical support for the rapid detection of AFB1 and ZEN dual residue in cereal food and feed and ensure the safety of cereal food and feed.

Mycotoxins widely exist in the natural environment, mainly pollute all kinds of grain food and feed, seriously threaten the safety of world food and feed, and do harm to human health and animal husbandry development, which has aroused great concern all over the world. Among the more than 400 mycotoxins reported so far, AFB1 and ZEN are the two most important mycotoxins with wide pollution and great harm. Aflatoxins (AFs) are a toxic secondary metabolite produced by *Aspergillus*, and produced under natural conditions mainly include AFB1, AFB2, AFG1 and AFG2.

Nowadays, the established immunoassay methods include gold immunochromatographic assay (GICA), enzyme-linked immunosorbent assay (ELISA), fluorescence immunoassay (FIA) and immunosensor (IS), etc. In contrast, GICA technology has the advantages of fast, simple, on-site operation, multiple detection and large amount of screening samples. It has become an important research topic of AFB1 and ZEN dual residue detection, and shows a broad

development prospect, which is also the purpose of this study.

The preparation of monoclonal antibodies (mAbs) against AFB1 and ZEN with high sensitivity and high specificity is the key to solve the problem of poor quality and unstable source of antibodies. Because the molecular structures of AFB1 and its homologues AFB2, AFG1 and AFG2 all contain dihydrofuran and oxanaphthone, there are only slight differences in the structures of hexacyclic C7-position lactone and pentacyclic C2-position lactone, and the other structures are exactly the same, it is difficult to prepare a high specific antibody for single recognition of AFB1. Similarly, since ZEN and its homologues have slight differences in molecular structure only on the carbonyl or hydroxyl group at C6'-position and the single bond or double bond at C1'-C2'-position of macrolide ring, it is also very difficult to prepare a single highly specific antibody that recognizes ZEN. The synthesis and screening of artificial immunogens are the basis for the preparation of highly specific antibodies, but the specificity of antibodies not only depends on the physical and chemical properties of the immunogen, but is also closely related to immunization methods and antibody screening methods. This study tries to use small dose (30 μg/mL), long immunization interval (4 weeks), multiple sites on the back (4 to 6 points), and multiple frequency (5 times) immunization methods. At the same time, try to use heterologous indirect non-competitive ELISA (inELISA) and heterologous indirect competition ELISA (icELISA) screen mAbs, and prepare the desired AFB1 mAbs and ZEN mAbs by the above methods.

The establishment of colloidal gold immunochromatographic test strip detection method is the path to solve the rapidity, simplicity, on-site detection and multivariate detection of detection technology. AFB1 mAbs and ZEN mAbs with high-sensitivity and high-specificity are used as the labeling target, colloidal gold is used as the labeling material, two mAbs are labeled with nano-gold and two detection lines are set to realize the dual residue detection of AFB1 and ZEN. By comparing and analyzing the sensitivity of gold labeled antigen competition mode and gold labeled antibody competition mode, the gold labeled antibody

competition mode is selected in this paper. In this study, colloidal gold is prepared by trisodium citrate reduction method, gold labeled antibodies are prepared by mey's series stabilization method, and the detection conditions of test strip are optimized, including nitrocellulose membrane selection, gold labeled pad selection, sample pad selection, optimal concentration combination of gold labeled antibody and coated antigen, determination of methanol concentration in sample treatment solution, etc. Finally, the AFB1 and ZEN dual residue colloidal gold immunochromatographic test strip detection method was established, its performance is determined and preliminarily applied, and confirmed by HPLC-MS/MS, so as to provide technical support for the detection of AFB1 and ZEN dual residue in food and feed.

Research has established the method for the preparation and identification of artificial immunogens and coating antigens to obtain high-specific antibodies against AFB1 and ZEN. According to the molecular structure characteristics of AFB1, the carbonyl at C1, the active hydrogen at C2, the hydroxyl at C3, and the C3-C4 bifuran ring are selected as the active sites or active groups, and 6 kinds of artificial immunogen AFB1-BSA and coating antigen AFB1-OVA synthesis methods, including oxime active ester method (OAE), methylation of ammonia (MOA), mixed anhydride (MA), semi acetal (SA), epoxide (EP) and enol ether derivation (EED), have been established. According to the molecular structure characteristics of ZEN, the carbonyl at C6', the active hydrogen at C7', the hydroxyl at C2, and the C5 at the benzene ring are selected as the active sites or active groups, and 5 kinds of artificial immunogens ZEN1-BSA and coating antigen ZEN-OVA synthesis methods, including oxime active ester method (OAE), condensation mixed anhydride method (CMA), formaldehyde method (FA), 1,4-butanediol diglycidyl ether method (BDE) and amino glutaraldehyde method (AGA), have been established. The artificial immunogens are identified by UV, SDS-PAGE and animal immune effect. OAE method is the best method to prepare AFB1 high specific antibody, and AGA method is the best method to prepare ZEN high specific antibody.

Research has established animal immunization method and screening method for positive hybridoma cell lines. The factors affecting animal immune effect include dosage, injection route, time interval, adjuvant use, immunization times and individual differences, among which, immunization dose and time interval are the key factors. After continuous attempts, we have reached the same conclusion as some scholars that low-dose immunogen can induce the production of specific antibodies, and a longer time interval can improve the affinity of antibodies. Therefore, this study established a low-dose (30 μg/mL), long-term interval (4 weeks), multiple points on the back (4-6 points), and multiple frequency (5 times) animal immunization methods. This study successfully established the screening method for positive hybridoma cells, including 4 steps. The first is to use homologous inELISA to determine the antibody titer to correctly evaluate the immunogenic immunoreactivity; the second is to use heterologous inELISA to determine the antibody titer to evaluate the recognition of antibodies; the third is to use a heterologous icELISA to determine the IC50 value of the antibody to AFB1 to correctly evaluate the sensitivity of the antibody; the fourth is to use a cross-reactivity test to determine the cross-reactivity between the antibody and hapten analogs to correctly evaluate the antibody specificity. Using the above method, 3 positive hybridoma strains of AFB1 mAb 2A11, 2F6 and 3G2, and 2 positive hybridoma strains of ZEN mAb 2B6 and 4D9 are screened. The mAbs are prepared by inducing ascites in vivo, and the immunological characteristics of each mAb are identified, including karyotype identification by colchicine blocking method, the class and subclass determination by mouse mAb homotype kit, the stability determination by hybridoma cell passage culture method, the affinity constant (Ka) determination by batty saturation method, the sensitivity determination by icELISA, and the specificity determination by cross-reaction test.

Research has established AFB1 single residue (ZEN single-residue) and AFB1 and ZEN dual residue colloidal gold immunochromatographic test strip detection methods and performance identification methods. The AFB1 single residue colloidal gold immunochromatographic test strip is constructed with nano-

gold labeled AFB1 mAb, and the test strip parameters are optimized. Similarly, ZEN single residue colloidal gold immunochromatographic test strip is assembled. AFB1 mAb and ZEN mAb are labeled with nano-gold, and a certain proportion of mixed gold labeled mAbs are configured according to the detection sensitivity of single residual colloidal gold immunochromatographic test strip. The corresponding immunogens AFB1-BSA (OAE) and ZEN-BSA (AGA) are used to set up two detection lines on the nitrocellulose (NC) membrane, and the dual residue colloidal gold immunochromatographic test strip of AFB1 and ZEN is assembled and established. The performances of the prepared colloidal gold immunochromatographic test strip are determined, including the visual detection limit by visual method, the machine-readable detection limit and IC50 value by the test strip reader scanning method (BioDot-TSR3000 strip reader), the stability determination by intra batch and inter batch tests, and the validity period determination according to the test results.

The 6 immunogens of AFB1 were identified by UV and SDS-PAGE, and the results showed that the 6 immunogens were synthesized successfully, and the molecular binding ratios of AFB1 to BSA were 8.64∶1 (OAE), 6.88∶1 (MOA), 10.78∶1 (MA), 4.46∶1 (SA), 6.38∶1 (EP) and 2.31∶1 (EED), respectively. Balb/c mice were immunized with 6 immunogens of AFB1. The results showed that AFB1-BSA (OAE) had the best effect among the 6 immunogens. The titer of AFB1 pAb reached 1:(1.6×10^3), and the IC50 value was 10.14 μg/kg. AFB1 pAb had the strongest specificity, could recognize AFB1 100%, and its CR values with AFB2, AFG1 and AFG2 were 6.32%, 3.76% and less than 1.0%, respectively. Similarly, the 5 immunogens of ZEN were identified by UV and SDS-PAGE, and the results showed that the 5 immunogens were synthesized successfully, and the molecular binding ratios of ZEN and BSA were 17.2:1 (OAE), 14.6:1 (CMA), 9.7:1 (FA), 8.3:1 (BDE) and 11.6:1 (AGA), respectively. Among the 5 immunogens of ZEN, ZEN-BSA (AGA) had the best immune effect. The titers of ZEN pAb reached 1:(1.6×10^3), and the IC50 value was 18.77 μg/kg. It had the strongest specificity and could recognize ZEN 100%, and its CR values with α-

ZAL, α-ZAL, α-ZOL, β-ZOL, ZON were 1.48%, 1.36%, 3.57%, 1.65% and 4.86%, respectively. Therefore, this study screened out that the OAE method was the best method for preparing AFB1 highly specific antibodies, and the AGA method was the best method for preparing ZEN highly specific antibodies.

The immunological characteristics of the selected AFB1 mAb 2A11, 2F6 and 3G2 hybridoma cell lines were identified. The results showed that the best was 2A11, which belonged to the IgG1 subtype with kappa light chain, and could stably secrete antibodies after 5 passages. The titers of AFB1 mAb in cell culture supernatant was 1: (6.4×10^2) and in ascites was 1: (5.12×10^5) by inELISA. Its Ka was 1.05×10^9 L/moL, and its IC50 value for AFB1 was 6.28 μg/kg. It could recognize AFB1 100%, and its CR values with AFB2, AFG1 and AFG2 were 4.35%, 2.30% and less than 1.0%, respectively. Similarly, two positive hybridoma cell lines of ZEN mAb 2B6 and 4D9 were identified, and the results showed that the best of them was 2B6, which belonged to the IgG1 subtype with kappa light chain, and could stably secrete antibodies after 5 passages. The titers of ZEN mAb in cell culture supernatant was 1: (5.12×10^2) and in ascites was 1: (5.12×10^5) by inELISA. Its Ka was 7.69×10^9 L/moL, and its IC50 value for ZEN was 10.38 μg/kg. It could recognize ZEN 100%, and its CR values with α-ZAL, α-ZAL, α-ZOL, β-ZOL, and ZON were 1.52%, 1.28%, 2.64%, 1.83%, and 4.27%, respectively. Therefore, this study produced high-titer, high-sensitivity and high-specificity AFB1 mAb and ZEN mAb, which could be used for immunoassays for the detection of AFB1 and ZEN residues.

The performances of AFB1 (or ZEN) single residue test strip and AFB1 and ZEN dual residue test strip were measured. The results showed that the visual LOD of AFB1 single residue test strip was 1.0 μg/L (5.0 μg/L for ZEN), the machine-readable LOD was 0.24 μg/L (1.51 μg/L for ZEN), and the machine-readable IC50 value was 1.11 μg/L (4.97 μg/L for ZEN). There was no CR with other compounds. The results of 6 batches of AFB1(or ZEN) single residue strip were measured and showed good stability. The validity period was 6 months at 4 ℃ in refrigerators and 25 ℃ room temperature. The visual LODs of AFB1 and ZEN dual residue test

strip were 1.0 ng/mL and 5.0 ng/mL for AFB1 and 5.0 ng/mL respectively, and the machine-readable LODs for AFB1 and ZEN were 0.23 μg/L and 1.53 μg/L respectively, and the machine-readable IC50 value for AFB1 and ZEN were 1.15 μg/L and 4.91 μg/L respectively. The test results of 6 batches of AFB1 and ZEN dual residue test strip of different batches were completely consistent and showed good stability. The validity period was the same as the single residue test strip. The AFB1 and ZEN dual residue test strip were used for preliminary application and verified by HPLC-MS/MS. The 20 known positive samples including 12 AFB1 positive samples and 8 ZEN positive samples were tested. The results showed that the positive coincidence rate was 100%. A total of 60 natural samples of corn, rice, flour, and feed were tested. 39 positive samples were detected, including 22 positive samples for AFB1 and 17 positive samples for ZEN, which were consistent with the HPLC-MS/MS detection results, with a coincidence rate of 100 %.

Thus, the results of this study focused on the synthesis of ideal immunogens, which solved the problem of poor immunogenicity of AFB1 and ZEN; preparation high-titer, high-sensitivity and high-specificity AFB1 mAbs and ZEN mAbs, which solved problems of the unstable source of antibodies and unstable quality of antibodies; the establishment of AFB1 (or ZEN) single residue and AFB1 and ZEN dual residue detection test strip, which solved the problems of rapidity, simplicity, multiple detection and on-site detection; preliminary application and verification by HPLC-MS/MS of AFB1 (or ZEN) single residue and AFB1 and ZEN dual residue detection test strip, which solved the problems of practicability and reliability. Finally, technical support was provided to realize the rapid detection of AFB1 and ZEN dual residue and ensure the safety of food and feed.

We recommend using the materials of the dissertation work when studying the courses " Veterinary microbiology", Veterinary sanitary examination" for masters of the Faculty of Veterinary Medicine of Sumy NAU. And for the courses "Veterinary microbiology “for masters of the Henan Institute of Science and Technology (HIST).

***Key words*:** Aflatoxin B1, zearalenone, monoclonal antibody with high specificity, immunochromatographic test strip, dual residue rapid detection, micromicets, microflora, physical chemical properties, organic synthesis, food poisoning, resistance, mycotoxins, bacteriological research, allergic studies, biochemical indicators, dystrophy of hepatocytes, necrosis

АНОТАЦІЯ

Янань Ван «Розробка та впровадження імунохроматографічних тест-смужок для виявлення подвійних залишків афлатоксину В1 та зеараленону» − Кваліфікаційна наукова праця на правах рукопису. Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 211 «Ветеринарна медицина» − Сумський національний аграрний університет, м. Суми, 2022.

Мікотоксини широко поширені в природному середовищі, головним чином контамінують усі види зернових продуктів харчування та кормів, серйозно загрожують безпеці харчових продуктів і кормів у світі та завдають шкоди здоров'ю людини та розвитку тваринництва, що викликало велику стурбованість у всьому світі. Забезпечення тварин якісними кормами – головна умова підтримання здоров'я поголів'я та отримання максимального рівня їхньої продуктивності. Але із підвищенням температури у весняний період якість кормів може погіршуватися. І в першу чергу це пов'язано із активним ростом мікроскопічних грибів на поживних субстратах (силосі, сіні, фуражному зерні та інших кормах). Серед мікотоксинів, які детально вивчені на сьогоднішній день, є два найбільш значимі: AFB1 (афлатоксин B1) і ZEN (зераленон). Останні забруднюють навколишнє середовище та завдають великої шкоди. Оцінивши недостатню вивченість зазначених питань враховуючи актуальність питання метою дисертаційної роботи було створення та впровадження імунохроматографічного діагностикуму з використанням моноклональних антитіл мічених колоїдним золотом (GICA; Colloidal Gold Immunochromatograohic Assay) у вигляді тест-смужок для

одночасного виявлення залишків мікотоксинів AFB1 та ZEN, щоб забезпечити технічну підтримку їх швидкого виявлення й гарантування безпечного споживання зернових харчових продуктів і кормів.

Більшість країн світу ввели чіткі положення щодо максимально допустимих залишків речовин (MRL) AFB1 в продуктах харчування.

Створення імунохроматографічного методу виявлення з застосуванням тест-смужок колоїдного золота є шляхом до вирішення питань швидкості і гарантії безпечної зернової продукції. Наукова новизна результатів дослідження полягає в тому що вперше порівняно та проаналізовано імунореактивність гаптенних білків AFB1 та ZEN, синтезування з них антигенів із застосуванням сучасних методів і хімічних сполук, відібрано найкращі антигени (6 видів штучного антигену AFB1 та 5 видів штучних антигенів ZEN) для отримання високоспецифічних антитіл до AFB1 та ZEN. Підібрано найбільш ефективний метод імунізації тварин-донорів для отримання високоспецифічних антитіл і методи скринінгу отриманих із застосуванням відповідних технологій гібридомних клітинних ліній, а також виготовлено високочутливі та високоспецифічні моноклональні антитіла до AFB1 та ZEN.

Результати досліджень впроваджуваються в практику провідних держав світу, а технологію отримання діагностичних смужок рекомендовано до уваги комерційним фірмам Китаю й інших країн для розробки відповідних тест-систем.

В експериментальних дослідженнях використана незначна доза для імунізації (30 мкг/см3), тривалий інтервал між введеннями антигену (4 тижні), тваринам-донорам в ділянці спини (4–6 місць) а також багаторазова імунізація (5 разів). Для контроля активності отриманих сироваток було використано непрямий конкурентний імуноферментний аналіз (inELISA) і непрямий конкурентний імуноферментний аналіз (icELISA) з моноклональними антитілами до AFB1 і ZEN.

В експерименті було досліджено 60 натуральних зразків кукурудзи, рису, борошна, кормів. Як результат, було виявлено 39 позитивних зразків, у тому числі 22 позитивних зразки з AFB1 та 17 позитивних зразків з ZEN. Крім того, ефективність розробленого методу одночасного виявлення токсинів AFB1 та ZEN із застосуванням імунохроматографічної тест-смужки з моноклональними антитілами міченими колоїдним золотом, була підтверджена традиційним методом рідинної хроматографії з тандемною мас-спектрометрією (100% збіг результатів) під час виявлення цих токсинів в продуктах харчування й кормах.

Запропонувано методи синтезування ідеальних антигенів, що вирішило проблему гаптенних характеристик AFB1 і ZEN; приготування високочутливих і високоспецифічних моноклональних антитіл до AFB1 і ZEN, які вирішли проблеми нестабільного джерела антитіл і нестабільної їх якості; створила тест-смужки для виявлення одного з залишків AFB1 (або окремо ZEN) і тест-смужки для виявлення обох залишків AFB1 і ZEN одночасно, останнє вирішило проблеми швидкості, простоти, багаторазового виявлення та виявлення на місці без використання дорогого обладнання. Контрольні дослідження проб зернових продуктів на наявність AFB1 або ZEN у варіанті тест-смужок з одним із компонентів і з двома одночасно показали повний збіг з результатами рідинної хроматографії з тандемною мас-спектрометрією, показали свою практичність і надійність. Впровадження методу одночасного виявлення залишків AFB1 і ZEN тест-смужками дає підставу для розробки діагностикумів з метою виявлення подібних продуктів, крім того, застосування цього методу гарантує безпеку зернових харчових припасів і кормів під час споживання кінцевого продукту людьми або тваринами.

AFB1 mAbs і ZEN mAbs з високою чутливістю та високою специфічністю використовували як мішень для маркування, колоїдне золото як матеріал для мічення, дві mAb були мічені нанозолотом, було встановлено

дві лінії виявлення для реалізації подвійного визначення залишків AFB1 і ZEN.

Шляхом порівняння та аналізу чутливості режиму конкуренції антигену, міченого золотом, і режиму конкуренції антитіл, мічених золотом було вибрано режим конкуренції антитіл, мічених золотом.

Колоїдне золото готували методом відновлення тринатрійцитратом, мічені золотом антитіла готували методом стабілізації серії Мея. Умови виявлення тест-смужки було оптимізовано, включаючи вибір нітроцелюлозної мембрани, вибір міченої золотом пластини, вибір пластини для зразка, вибір оптимальної комбінації концентрації міченого золотом антитіла та антигену з покриттям, визначення концентрації метанолу в розчині для обробки зразка тощо. Було встановлено імунохроматографічний метод виявлення з використанням тест-смужок AFB1 та ZEN із подвійним залишком колоїдного золота. Була визнана та попередньо доведена його ефективність, а також підтверджена HPLC-MS/MS, щоб забезпечити технічну підтримку для виявлення подвійних залишків AFB1 та ZEN в продуктах харчування та кормах.

Дослідження обгрунтували метод приготування та ідентифікації штучних імуногенів і антигенів покриття для отримання високоспецифічних антитіл проти AFB1 і ZEN. Відповідно до характеристик молекулярної структури AFB1, карбоніл у C1, активний водень у C2, гідроксил у C3 та біфуранове кільце C3-C4 вибиралися як активні центри або активні групи, крім того відбирали 6 видів штучного імуногену AFB1-.

В результаті експериментальних досліджень були встановлені методи синтезу BSA та антиген покриття AFB1-OVA, включаючи метод активного ефіру оксиму (OAE), метилювання аміаку (MOA), змішаний ангідрид (MA), напівацеталь (SA), епоксид (EP) та похідне енольного ефіру (EED).

Відповідно до характеристик молекулярної структури ZEN, карбоніл у C6', активний водень у C7', гідроксил у C2 та C5 у бензольному кільці були видібрани як активні центри або активні групи, а також 5 видів штучних

імуногенів. Дослідженнями було встановлені методи синтезу ZEN1-BSA та антигену покриття ZEN-OVA, включаючи метод активного ефіру оксиму (OAE), метод конденсації змішаного ангідриду (CMA), метод формальдегіду (FA), метод 1,4-бутандіол дигліцидилового ефіру (BDE) та метод аміноглутаральдегіду (AGA). Штучні імуногени ідентифікували за УФ, SDS-PAGE та імунним ефектом тварин. Було визначено, що метод OAE є найкращим методом для отримання високоспецифічних антитіл AFB1, а метод AGA є найкращим методом для отримання ZEN високоспецифічних антитіл.

Дослідження встановили ефективність методу імунізації тварин і методу скринінгу позитивних клітинних ліній гібридоми. Фактори, що впливають на імунний ефект тварин, включають дозу, шлях ін'єкції, інтервал часу, використання ад'ювантів, час імунізації та індивідуальні відмінності, серед яких доза імунізації та інтервал часу є ключовими факторами. Після проведення порівняльних дослідів доведено, що низькі дози імуногену можуть індукувати вироблення специфічних антитіл, а довший проміжок часу може покращити афінність антитіл. Дослідженнями встановлено низьку дозу (30 мкг/мл), тривалий інтервал (4 тижні), багатоточкову імунізацію в ділянку спини (4-6 точок) і багаторазову (5 разів) імунізацію тварин.

Це дослідження успішно запровадило метод скринінгу позитивних гібридомних клітин, що включає 4 етапи.

Перший етап полягає у використанні гомологічного inELISA для визначення титру антитіл для правильної оцінки імуногенної імунореактивності; другий полягає у використанні гетерологічного inELISA для визначення титру антитіл з метою оцінки розпізнавання антитіл; третій — використання гетерологічного icELISA для визначення значення IC50 антитіла до AFB1, щоб вірно оцінити чутливість антитіла; четвертий — використання тесту на перехресну реактивність для визначення перехресної реактивності між антитілом і аналогами гаптену, щоб вірно оцінити специфічність антитіла.

Використовуючи вищевказаний метод, скринінгували 3 позитивні гібридомні штами AFB1 mAb 2A11, 2F6 і 3G2 і 2 позитивні гібридомні штами ZEN mAb 2B6 і 4D9. mAb готували шляхом індукування асциту in vivo, також ідентифікували імунологічні характеристики кожного mAb, включаючи ідентифікацію каріотипу за допомогою методу блокування колхіцину, визначення класу та підкласу за допомогою набору гомотипу мишачого mAb, визначення стабільності за допомогою методу пасажу гібридомних клітин, визначення константи афінності (Ka) за допомогою методу насичення, визначення чутливості за допомогою icELISA та визначення специфічності за допомогою тесту перехресної реакції.

Дослідження довели ефективність методу визначення одного залишку AFB1 (однозалишковий ZEN) і AFB1 і подвійного залишку колоїдного золота за допомогою імунохроматографічних тест-смужок і методу ідентифікації ефективності. Імунохроматографічний тестовий папір з одним залишком колоїдного золота AFB1 складається з mAb AFB1, поміченого нанозолотом та оптимізованих нами параметрів тест-смужків. Подібним чином збирається імунохроматографічний тестовий папір ZEN з одним залишком колоїдного золота. AFB1 mAb і ZEN mAb мічені нанозолотом, а певна частка змішаних mAb, мічених золотом, конфігурується відповідно до чутливості виявлення однієї імунохроматографічної тест-смужки із залишковим колоїдним золотом. Відповідні імуногени AFB1-BSA (OAE) і ZEN-BSA (AGA) були використані для створення двох ліній виявлення на нітроцелюлозній (NC) мембрані, а також було зібрано та встановлено імунохроматографічну тест-смужку з подвійним залишком колоїдного золота AFB1 і ZEN.

Було проведено визначення характеристики підготовленої імунохроматографічної тест-смужки з колоїдним золотом, включаючи межу візуального виявлення візуальним методом, машинозчитувану межу виявлення та значення IC50 за допомогою скануючого пристрою для зчитування тест-смужок (стріп-рідер BioDot-TSR3000), визначення

стабільності за допомогою внутрішньосерійні та міжсерійні випробування та визначення терміну придатності за результатами випробувань.

Нами було ідентифіковано 6 імуногенів AFB1 за допомогою УФ та SDS-PAGE, і результати показали, що 6 імуногенів були успішно синтезовані, а коефіцієнти молекулярного зв'язування AFB1 з BSA становили 8,64∶1 (OAE), 6,88∶1 (MOA), 10,78∶1 (MA), 4,46∶1 (SA), 6,38∶1 (EP) і 2,31∶1 (EED) відповідно.

Мишей Balb/c імунізували 6 імуногенами AFB1. Результати показали, що серед 6 імуногенів найкращий ефект мав AFB1-BSA (OAE). Титр AFB1 pAb досягав 1:(1,6×103), а значення IC50 становило 10,14 мкг/кг. AFB1 pAb мав найсильнішу специфічність, міг розпізнавати AFB1 на 100%, а його значення CR для AFB2, AFG1 і AFG2 становили 6,32%, 3,76% і менше 1,0% відповідно. Подібним чином, 5 імуногенів ZEN були ідентифіковані за допомогою УФ та SDS-PAGE. Результати досліджень показали, що 5 імуногенів були успішно синтезовані, а молекулярні співвідношення зв'язування ZEN і BSA становили 17,2:1 (OAE), 14,6:1 (CMA).), 9,7:1 (FA), 8,3:1 (BDE) і 11,6:1 (AGA) відповідно. Серед 5 імуногенів ZEN найкращий імунний ефект мав ZEN-BSA (AGA). Титри ZEN pAb досягали 1:(1,6×103), а значення IC50 становило 18,77 мкг/кг. Він мав найсильнішу специфічність і міг розпізнавати ZEN на 100%, а його значення CR для α-ZAL, α-ZAL, α-ZOL, β-ZOL, ZON становили 1,48%, 1,36%, 3,57%, 1,65% і 4,86%, відповідно.

Таким чином, це дослідження показало, що метод OAE був ефективним методом для отримання високоспецифічних антитіл AFB1, а метод AGA був ефективним методом для отримання високоспецифічних антитіл ZEN.

Були ідентифіковані імунологічні характеристики відібраних ліній гібридомних клітин AFB1 mAb 2A11, 2F6 і 3G2. Результати показали, що найкращим був 2A11, який належав до підтипу IgG1 з легким каппа-ланцюгом і міг стабільно секретувати антитіла після 5 пасажів. Титри AFB1 mAb у супернатанті клітинної культури становили 1: (6,4 × 102), а в асциті – 1: (5,12 × 105) за допомогою ELISA. Його Ka становив 1,05×109 л/моль, а

значення IC50 для AFB1 становило 6,28 мкг/кг. Метод розпізнає AFB1 на 100%, а його значення CR для AFB2, AFG1 і AFG2 становили 4,35%, 2,30% і менше 1,0% відповідно. Аналогічно було ідентифіковано дві позитивні гібридомні клітинні лінії ZEN mAb 2B6 і 4D9. Результати досліджень показали, що найкращою з них була 2B6, яка належала до підтипу IgG1 з легким каппа-ланцюгом і могла стабільно секретувати антитіла після 5 пасажів. Титри ZEN mAb у супернатанті клітинної культури становили 1: (5,12 × 102), а в асциті – 1: (5,12 × 105) за методом ELISA. Його Ka становив 7,69×109 л/моль, а значення IC50 для ZEN становило 10,38 мкг/кг. Метод розпізнає ZEN на 100%, а його значення CR для α-ZAL, α-ZAL, α-ZOL, β-ZOL і ZON становили 1,52%, 1,28%, 2,64%, 1,83% і 4,27% відповідно. Таким чином, у цьому дослідженні були отримані mAb AFB1 і ZEN mAb з високим титром, високою чутливістю та високою специфічністю, які можна було використовувати для імунологічних аналізів для виявлення залишків AFB1 і ZEN.

Визначали ефективність тест-смужки AFB1 (або ZEN) з одним залишком і тест-смужки AFB1 і ZEN з подвійним залишком. Результати показали, що візуальний LOD тест-смужки з одним залишком AFB1 становив 1,0 мкг/л (5,0 мкг/л для ZEN), читаний з приладів LOD становив 0,24 мкг/л (1,51 мкг/л для ZEN), а зчитуване значення IC50 становило 1,11 мкг/л (4,97 мкг/л для ZEN). Не реєстрували CR з іншими сполуками. Було визначено результати 6 партій AFB1 (або ZEN) однієї залишкової смужки, яка показала високу стабільність. Термін придатності склав 6 місяців при 4^0 C в холодильниках та кімнатній температурі до 25^0 C. Візуальні LOD тест-смужки з подвійним залишком AFB1 і ZEN становили 1,0 нг/мл і 5,0 нг/мл для AFB1 і 5,0 нг/мл відповідно, а зчитані з приладів LOD для AFB1 і ZEN становили 0,23 мкг/л і 1,53 мкг/л. відповідно, а зчитані з приладів значення IC50 для AFB1 і ZEN становили 1,15 мкг/л і 4,91 мкг/л відповідно.

Результати випробувань 6 партій тест-смужок із подвійним залишком AFB1 і ZEN різних партій були повністю відповідними та показали високу

стабільність. Термін придатності був таким же, як і для тест-смужки з одним залишком. Тест-смужки AFB1 і ZEN з подвійним залишком використовували для попереднього нанесення та перевіряли за допомогою ВЕРХ-МС/МС. Було протестовано 20 відомих позитивних зразків, у тому числі 12 позитивних зразків AFB1 та 8 позитивних зразків ZEN. Результати показали, що позитивний збіг склав 100%. Всього було досліджено 60 натуральних зразків кукурудзи, рису, борошна, кормів. Було виявлено 39 позитивних зразків, у тому числі 22 позитивних зразки для AFB1 та 17 позитивних зразків для ZEN, які узгоджувалися з результатами виявлення ВЕРХ-МС/МС із рівнем збігу 100 %.

Таким чином, результати нашої роботи були зосереджені на синтезі ідеальних імуногенів, що вирішило проблему низької імуногенності AFB1 і ZEN; приготування високотитрових, високочутливих і високоспецифічних mAb AFB1 і ZEN mAb, які попередили виникнення нестабільного джерела антитіл і нестабільної якості антитіл. Створення тест-смужки для виявлення одного залишку AFB1 (або ZEN) і тест-смужки для виявлення подвійних залишків AFB1 і ZEN дало змогу швидко, просто, багаторазово виявити залишки мікотоксині. Попереднє застосування та перевірка за допомогою ВЕРХ-МС/МС тест-смужки для виявлення одного залишку AFB1 (або ZEN) і тест-смужки для виявлення подвійного залишку AFB1 і ZEN довело практичність та надійність цього методу. Таким чином, отримана ефективна технічна підтримка для реалізації швидкого виявлення подвійних залишків AFB1 і ZEN і забезпечення безпеки харчових продуктів і кормів. Матеріали дисертаційної роботи використовуються при викладанні курсів «Ветеринарна мікробіологія», «Ветеринарно-санітарна експертиза» для магістрів факультету ветеринарної медицини Сумського НАУ та курсу «Ветеринарна мікробіологія» для магістрів Інституту науки Хенань і технології (HIST).

Ключові слова: афлатоксин В1, зеараленон, моноклональне антитіло з високою специфічністю, імунохроматографічна тест-смужка, подвійний залишковий швидкий виявлення, мікроміцети, мікрофлора, фізико-хімічні властивості, органічний синтез, харчові отруєння, резистентність, мікотоксини, бактеріологічні дослідження, алергологічні дослідження, біохімічні показники, дистрофія гепатоцитів, некроз.

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Methodological recommendations:

15. **Wang, Y.,** Fotina, H. Methodical guidelines “Implementation of modern methods of Aflatoxin B1 and Zearalenone detection”, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology"and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 53pp. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021). (*The applicant analyzed the research results, prepared and issued materials for methodical recommendations).*

CONTENT

p.

LIST OF SYMBOLS

- AFB1 aflatoxin B1
- AFB2 aflatoxin B2
- AFG1 Aflatoxin B1
- AFG2 Aflatoxin B2
- AFM1 Aflatoxin M1
- AFs aflatoxins
- AGA amino glutaraldehyde
- AuNCs gold nanoclusters
- AuNFs gold nanoflowers
- AuNRs gold nanorods
- BDE 1,4-butanediol diglycidyl ether
- BS biosensor analysis
- CA chromatographic analysis
- CMA condensation mixed anhydride
- CTN citrinin
- DON deoxynivalenol
- EED enol ether derivative
- ELISA enzyme-linked immunosorbent assay
- EP epoxide
- FA formaldehyde
- FB fumonisin
- FIA fluorescence immunoassay
- GICA Colloidal Gold Immunochromatograohic Assay
- HPLC high performance liquid chromatography
- HPLC-MS/MS high performance liquid chromatography tandem mass spectrometry
- IA immunoassay

- IS immunosensor
- LC/MS liquid-mass spectrometer
- MA mixed anhydride
- MOA methylation of ammonia
- OAE oxime active ester
- OTA ochratoxin A
- PAT patulin
- PCA physicochemical analysis
- SA semi aceta
- TAFs total aflatoxins
- TLC thin layer chromatography
- ZEN zearalenone
- ZON zearalanone
- α-ZAL α-Zearalenol
- α-ZOL α-Zearalenol
- β- ZOL β-Zearalenol,
- β-ZAL β -Zearalenol

INTRODUCTION

Actuality of theme. Mycotoxins are a class toxic secondary metabolites produced by fungi such as *Aspergillus* and *Fusarium*, and mainly pollute plant-derived agricultural products, food and feed, including corn, wheat, barley, rice, and oats, etc., and the contamination can occur in all aspects of crop production, harvesting, storage, processing, among others, which seriously threatens to human health and the development of animal husbandry. Therefore, mycotoxins have attracted widespread attention worldwide [1, 2]. Aflatoxin B1 (AFB1) and zearalenone (ZEN) are the two most common mycotoxins. On account of their acute poisoning, chronic poisoning, carcinogenicity, mutagenicity, teratogenicity, neurotoxicity, immunotoxicity, reproductive toxicity and other toxic effects on the body's health, so far, most countries and regions in the world now have strict limits on AFB1 and ZEN maximum residue limits (MRLs) in food and feed. However, with the growing concern of deepening and research on food safety issues, people found that AFB1 and ZEN in contaminated food and feed often coexist and have synergistic and additive effects, single detection of AFB1 or ZEN cannot meet the needs of the food and feed industry. Thus, the establishment of AFB1 and ZEN dual residue detection has become a development trend [3, 4]. Currently, the established AFB1 and ZEN dual residue detection methods mainly include physico-chemical analysis methods and immunoassays. Owing to the defects of physico-chemical analysis mtheods, such as labor-consuming and time-consuming, lengthy, requires complicated sample preparation procedures, expensive instruments, and skilled technicians, they are not suitable for rapid and on-site operation. Conversely, immunoassays based on antigen-antibody specificity and sensitivity reaction have been rapidly developed and widely used[5, 6]. Among them, colloidal gold immunochromatography assay (GICA), as one of the immunoassay methods, compared with other immunoassays, has become a hot topic in AFB1 and ZEN dual residue detection research due to its strong selectivity,

high sensitivity, speedability and simplicity, large sample screening, on-site operation [7, 8], which is also the purpose of this thesis.

Connection of work with scientific programs, plans, topics. The materials of the dissertation work are part of comprehensive scientific research of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Livestock Products of the Sumy National Agrarian University according to the following thematic plans of research works: "System of monitoring methods of control and veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration No. 0114U005551, 2014-2019); "Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023).

And this dissertation is part of the "12th Five-Year" National Science and Technology Support Program "Research and Demonstration of Rapid Detection Technology for Livestock and Poultry Products" (No.2014BAD13B05), the “13th Five-Year” National Key Research and Development Plan Program “Food Safety Technology Research and Development” (No.2019YFC1605705) and the Program for Innovative Research Team (in Science and Technology) at the University of Henan Province (20IRTSTHN025).

The aim and objectives of the study. The aim of this study is to establish a colloidal gold immunochromatographic test strip detection method for AFB1 and ZEN dual residue, so as to provide rapid detection technical support for ensuring the safety of cereal food and feed.

For the purpose were assigned the following tasks:

1. According to the characteristics of the molecular structure of AFB1 and ZEN, develop different methods of immunogen synthesis. Through the identification of UV, SDS-PAGE and immune effect of animals, choose the best methods of immunogen synthesis to obtain AFB1 and ZEN specific antibodies,

respectively, to solve the problems of low immunogenicity and low antibody specificity of AFB1 and ZEN low molecular weight compounds.

2. Through the selection of animal immunization methods, cell fusion and positive hybridoma cell line screening technologies, to prepare mAb AFB1 and mAb ZEN with high sensitivity and high specificity, respectively, and identify their immunological characteristics to solve the problems of poor quality and unstable antibody source.

3. Through the preparation and identification of colloidal gold, prepare and identify gold-labeled monoclonal antibodies, optimize the detection conditions of the test strips, compose and determine the efficiency of the test strips, develop the detection methods of the double-residue AFB1 and ZEN test strips to solve the problems of low speed, poor simplicity, inability to detect in situ, and inability to multiplex detection in current detection technology.

4. To evaluate through preliminary practical application and HPLC-MS/MS verification, the practicability and reliability of the AFB1 and ZEN dual residue test strip detection methods.

Object of study. Development and preliminary application of dual residue immunochromatographic test strip for AFB1 and ZEN in food and feed.

Subject of study. Synthesis and identification of artificial immunogens and coating antigens of AFB1 and ZEN, screening of immunogen synthesis methods for the preparation of high specific antibodies against AFB1 and ZEN; Selection of immunization methods for Balb/c mice, screening of positive hybridoma cell lines and identification of immunological characteristics; Preparation and identification of colloidal gold, preparation and identification of gold labeled-AFB1 mAbs and gold labeled-ZEN mAbs, optimization of test strip detection conditions, establishment of detection method and performance determination of AFB1 and ZEN dual residue test strip; Preliminary practical application and HPLC-MS/MS verification of AFB1 and ZEN dual residue test strip.

Research methods. Mycological (AFB1 and ZEN detection), clinical (history taking, clinical examination), microbiological (microscopic, biological),

cytobiological (cells detection), toxicological (degree of toxicity and harmlessness), immunological (changes in gene and protein levels of inflammatory factors) and statistical (processing of research results).

Scientific novelty of the obtained results. For the first time, the immunoreactivity and antibody characteristics of immunogens synthesized by different methods of AFB1 and ZEN are compared and analyzed, and the best immunogens for preparing high specific antibodies and class broad-spectrum specific antibodies of AFB1 and ZEN are selected; The animal immunization method for the preparation of highly specific antibodies and the screening method of positive hybridoma cell lines are established, and the highly sensitive and highly specific mAbs against AFB1 and ZEN are prepared; The detection method of AFB1 and ZEN dual residue test strip was established, and its practicability and compliance were verified.

The practical significance of the obtained results. The preparation methods of high-specific antibodies and broad-spectrum specific antibodies to AFB1 and ZEN provide a reference for the preparation of high-quality antibodies to other small molecule hapten compounds; The establishment of AFB1 and ZEN dual residue test strip detection method not only provides ideas for the research and development of similar detection products, but also provides technical support for ensuring the safety of cereal food and feed.

The main provisions of the dissertation were included in the Methodological Recommendations of Implementation of modern methods of Aflatoxin B1 and Zearalenone detection, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology"and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

Research results are introduced to the "Veterinary microbiology" and Veterinary sanitary examination" sections when creating educational and

methodological complexes and are used in distance learning of students based on the "Moodle" platform.

Personal contribution of the applicant.

Under the guidance and help of the supervisors, PhD student had consulted a large amount of literature on the subject of the dissertation, summarized and analyzed the latest progress of the subject research, the main problems solved by the research, and the expected research goals. PhD student had systematically designed research plans and technical routes, selected appropriate technical methods, and carried out relevant experimental research, and performed statistics and analysis on the test data, discussed and summarized the test results, and drew the correct research results and conclusions of the subject.

Approbation of dissertation results.

The main provisions and results of the research were reported and received general scientific approval at the annual scientific reports and conferences of faculty and graduate students of Sumy National Agrarian University, Faculty of Veterinary Medicine (2018-2021); Fifth Annual Regional Scientific Symposium One Health Concept, Kyiv (2019). Food Quality and Safety, Health and Nutrition Congress. Ohrid, Macedonia (2019). journal of Sumy National Agrarian University (2018, 2019).

Publications. For the materials of the dissertation, 15 scientific works were published, including: in scientific and professional publications of Ukraine - 3, Scopus publication 5, publications in Chines journal – 2, in conference materials– 4, and 1 methodical recommendation.

The structure and scope of the dissertation. The dissertation is presented on 218 pages of computer text, illustrated with 33 tables and 60 figures and consists of annotation, introduction, review of literature, materials and methods, results of own research, generalization, analysis and discussion of research results, conclusions, proposals, list used sources, applications. The list of used sources of literature includes 295 names.

CHAPTER 1.

LITERATURE REVIEW ON THE TOPIC AND CHOICE OF RESEARCH DIRECTIONS

1.1 Definition of mycotoxins

Mycotoxins, a term derived from Greek "mykes" and Latin "toxicum", are a class of small molecular weight secondary metabolites produced by some fungi during the growth and process, which can lead to toxic effects on vertebrates at low concentrations. Molds are a part of fungi, which are mainly related to the pollution of plant-derived food and feed. Thus, in the fields of food hygiene and feed hygiene, mycotoxins are usually known as mold toxins [9, 10].

1.2 Kinds and sources

Mycotoxins widely exist in the natural environment and mainly pollute various plant-derived foods and feeds. So far, about 400 kinds of mycotoxins have been reported, the more common and harmful mycotoxins are aflatoxins (AFs), ZEN, ochratoxin A (OTA), deoxynivalenol, (DON), T-2 toxin, Fumonisin (FB), citrinin (CTN), patulin (PAT), etc. [11, 12]. Different toxins derived from different kinds of fungi, AFs and OTA are produced by *Aspergillus*, mainly including *A.flavus* and *A.parasiticus,* etc.[13, 14]. ZEN, DON, FB, T-2 toxin are produced by *Fusarium*, mainly including *F.poae*, *F.culmorum*, *F.porotrichioides*, *F.culmorum*, *F.equiseti*, *F.graminearum*, *F.moniliforme*, *F.proliferatum* and *F.verticillioides*, etc.[15, 16]. CTN and PAT are produced by *Penicillium*, mainly including *P.griseofulvum* and *P. expansum,* etc. [17, 18].

1.3 Pollution status

With the rapid development of global agriculture and animal husbandry, food and feed are seriously contaminated by mycotoxins, and reports of mycotoxins contamination are increasing all over the world. Contamination of mycotoxins can occur in all aspects of crop production, harvest, storage and processing. The degree of damage is extremely huge and the coverage area is wide, which critically threatens the safety of food and feed in the world. The pollution caused by mycotoxins is not only related to the storage temperature and humidity of food and agricultural products, but also related to the weather conditions during the cultivation of agricultural products. Therefore, it is very difficult to prevent and control the pollution caused by fungi, which is a worldwide problem [19]. On the basis of the United Nations Food and Agriculture Organization (FAO), about 25% of crops are contaminated by fungi and their toxins every year, and about 2% of crops lose their nutritional and economic value due to serious pollution, resulting in the direct and indirect losses of tens of billions of dollars. Meanwhile, the contamination of food, feed, dairy products, fruits, nuts and medicinal plants by mycotoxins can bring about mildew, resulting in a mass of agricultural products being reduced or discarded, give rise to huge economic losses [20, 21]. As the largest grain production and consumption country, grain mycotoxin pollution is serious and widespread in China. Cheng et al. investigated the contamination of 5 *fusarium* toxins and 4 AFs in threshed wheat in 4 provinces of China in 2018. The results showed that the threshed wheat in the 4 provinces was contaminated by 5 kinds of *Fusarium* toxins more seriously and had a significant correlation [22]. Additionally, Li et al. studied the statistics of mycotoxin contamination in China's grains from 2009 to 2019. The results showed that rice affected by mycotoxin contamination lighter; Wheat was mainly contaminated by *fusarium* toxins, especially DON and ZEN; Maize was susceptible to various mycotoxins, such as AFs, ZEN, DON and so on. Barley, oats, millet and sorghum were also polluted to varying degrees [23].

1.4 Toxic effects

Mycotoxins are extremely harmful to humans and animals, result in great concern in many countries around the world, mainly include AFs, ZEN, DON, OTA, T-2 toxin, FB, PAT, etc. [24]. Mycotoxins can be transmitted into the human food chain through contaminated food, animals ingested infested feed, etc., causing serious harm to humans and animals. The main manifestations are liver toxicity, renal toxicity, hematopoietic toxicity, immune toxicity, and reproductive toxicity. etc. [25, 26]. The most harmful is that mycotoxins are carcinogenic and teratogenic toxicity. On account of the degree of risk, the International Agency for Research on Cancer (IARC) classifies mycotoxins into class I carcinogens (AF), class II carcinogens (OTA, FB1, FB2), and class III carcinogens (PAT), etc. [27, 28]. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have carried out risk analysis on AFs, OTA, FB and other mycotoxins. Furthermore, the weekly maximum tolerated intake and the daily maximum tolerated intake have been tentatively stipulate, and most countries around the world have also formulated limit standards for mycotoxins.

1.5 Research Progress on pollution status, toxic effect, residue limit and detection technology of AFB1

1.5.1 Pollution status. Discovery of AFB1

As early as the 1960s, about 100,000 turkeys died of poisoning in the South and East of England. The wide coverage, the high mortality rate, and the large economic loss had become a sensational event, which had attracted widespread attention from the British government and society. Originally, because the cause of the disease could not be found, it was called Turkey-X disease. After careful investigation by the researchers, it was confirmed that the death of the turkey was related to the peanut meal that was fed, which was imported from Brazil. Researchers took samples of feed from sick-dead turkey farms and imported

peanut meal raw materials for analysis and testing, and found a type of fluorescent substance, and then confirmed that this type of substance lead to the poisoning and death of turkeys. Thereafter, the researchers conducted in-depth and meticulous research work for more than 2 years. Through cultivation, screening, extraction, concentration, and identification, they found that this type of substance was AFB1 produced by *A.flavus* in peanut meal [29, 30].

1.5.2 Source of AFB1

AFs are a class toxic secondary metabolites produced by the genus *Aspergillus*, which mainly include *A.flavus* and *A.parasiticus*. *A.flavus* mainly produces B-group AFs, including AFB1 and AFB2, and AFB2a is a metabolite of AFB1; *A.parasiticus* mainly produces B-group and G-group AFs, including AFB1, AFB2, AFG1, AFG2, etc., and AFG2a is a metabolite of AFG1 [31]; The metabolites of animals after ingesting AFB1 are aflatoxin M1 (AFM1) and M2 (AFM2), which can be present in animal milk and dairy products [32]. Others, *A.fumigatus* can also produce a small amount of AFs, mainly AFB1 [33]. Up to now, 20 kinds of AFs have been found, and the chemical molecular structures of 18 kinds among them have been identified. In addition to 4 kinds AFs produced under natural conditions, which mainly include AFB1, AFB2, AFG1, and AFG2, AFs are oxidized in the body by enzymes such as CYP450 in the cell to form toxic metabolites, including M1, M2, P1, and Q1, H1, GM, B2a, G2a and R0, etc. [34]. According to the different fluorescent colors of ultraviolet radiation, AFs are divided into two categories: B-group (AFB) and G-group G (AFG), in which AFB exhibits blue fluorescence and AFG exhibits green fluorescence [35].

1.5.3 Physico-chemical property of AFs

AFs mainly include AFB1, AFB2, AFG1, AFG2. The molecular structures of the four major AFs all contain dihydrofuran and oxadione, of which dihydrofuran is the basic toxin structure, and oxadione is the main structure that give rise to cancer and is related to toxicity [36]. AFs are all crystalline substances, colorless,

odorless, and tasteless, hardly soluble in water, and easily soluble in moderately polar solvents such as chloroform, methanol and dimethyl sulfoxide. The physicochemical parameters of 4 common AFs, AFB1, AFB2, AFG1, and AFG2, are shown in Table 1.1 AFB1 is slightly soluble in water with a saturation concentration of 10 to 20 mg/L. It is easily soluble in polar organic solvents such as methanol and dimethylformamide, but hardly soluble in non-polar organic solvents such as hexane and ether. AFB1 can emit blue fluorescence under ultraviolet light, and it has three ultraviolet absorption peaks, 223 nm, 265 nm and 362 nm, respectively [37, 38].

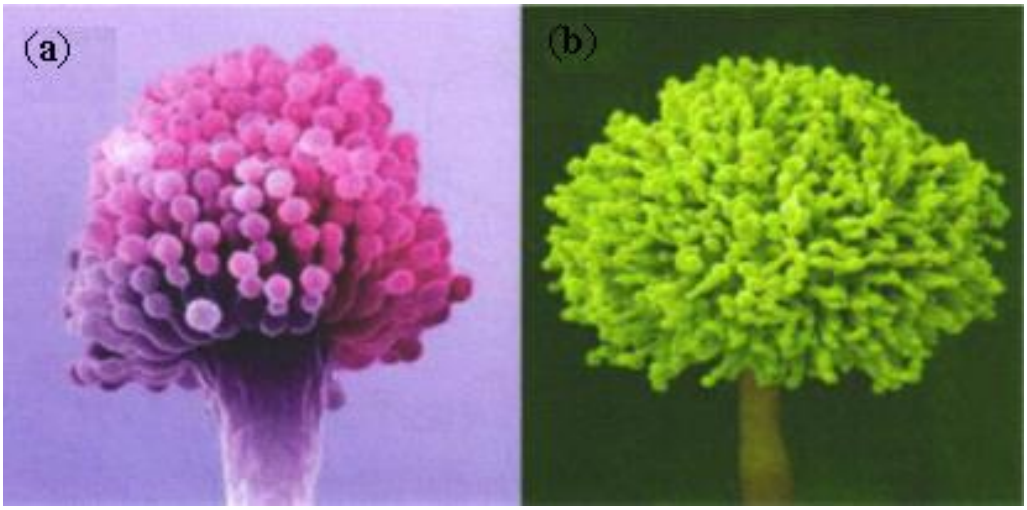


Figure 1.1 Morphological characteristics of *Aspergillus* under electron microscope.(a) *A.parasiticus.* (b) *A.flavus.*

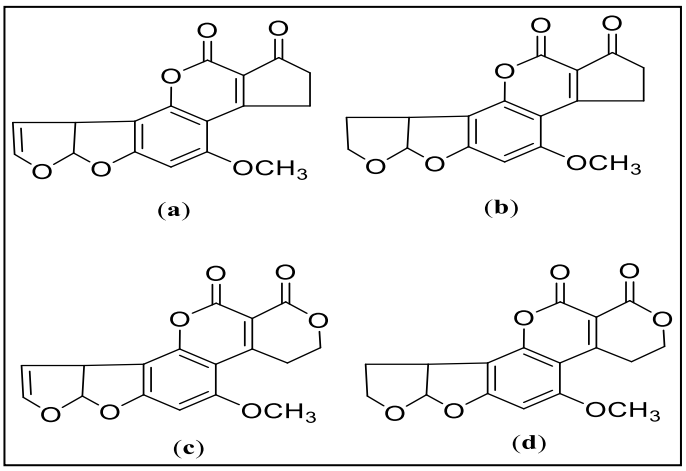
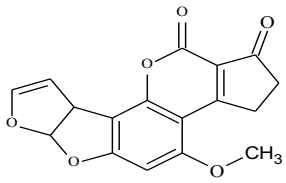
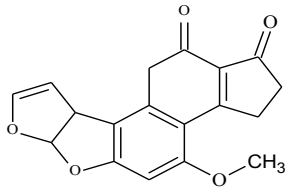
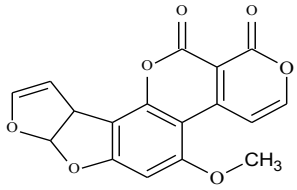
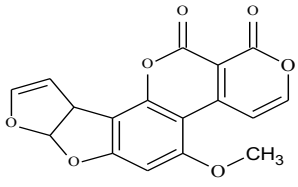


Figure 1.2 The structure of several major aflatoxins. (a) AFB1. (b) AFB2. (c) AFG1. (d) AFG2.

Table 1.1

The physicochemical characteristics of AFB1, AFB2, AFG1, and AFG2

toxin type	structural formula	Molecular formula	molecular weight	melting point	solubility	UV peak （nm）	IR peak （cm^{-1}）
AFB_1		$C_{17}H_{12}O_6$	312	268-269	Soluble in organic solvents, slightly soluble in water	223 265 362	1760,1684, 1632,1598, 1562
AFB_2		$C_{17}H_{14}O_6$	314	286-289	Soluble in organic solvents, slightly soluble in water	222 265 362	1760,1685, 1625,1600
AFG_1		$C_{17}H_{12}O_7$	328	244-246	Soluble in organic solvents, slightly soluble in water	243 257 362	1760,1695, 1630,1595
AFG_2		$C_{17}H_{14}O_7$	330	237-240	Soluble in organic solvents, slightly soluble in water	245 265 365	1760,1694, 1627,1597

1.5.4 Pollution status of AFB1

Globally, 3/4 of the population is directly or indirectly related to AFs pollution, especially in tropical and subtropical regions, due to the high temperature and high humidity environment, it is conducive to the growth of

A.flavus and the contamination is more serious [39, 40]. In case of China, the pollution and overall distribution of AFs are serious in the south, followed by the central region, lighter in the north, and less pollution in the cold regions of the northeast and northwest [41]. *A.flavus* grows rapidly under suitable temperature and humidity conditions, is vigorous and easy to survive, thus the pollution is also prone to occur. It mainly pollutes crops such as wheat, corn, rice, grains, etc., and oil crops such as peanuts and soybeans, etc. Among them, the most serious contamination is peanuts and corn, which bring great harm to agriculture, animal husbandry, and edible oil processing industry.

Foods contaminated by AFs are often diverse, and the detection found that several toxins can exist simultaneously. On the basis of the survey conducted by FAO, the average occurrence ratio of AFB1 and AFB2 is about 4:1, and the average occurrence ratio of the total amount of AFs and AFB1is about 1:0.8 on average [42]. The survey results by Gao et al. [43] in 2011 showed that the AFs content in 197 peanut samples collected from 6 provinces including Jilin were determined by high performance liquid chromatography (HPLC), 115 were positive, and the average concentration was 91.74 μg/kg. Among the 4 AFs, AFB1 had the highest positive rate and the highest average content, 58.38% and 77.77 ug/kg, respectively. The positive rates and average content of the remaining three toxins AFB2, AFG1, and AFG2 decreased sequentially. It is concluded that AFs contamination of peanuts in China is relatively common, 4 kinds of AFs exist and AFB1 contamination is the main one. Cheng et al. [44] investigated and reported the distribution of AFs in feed ingredients in China in 2013. A total of 2423 samples were tested, and the detection was carried out by two methods: the preliminary screening by enzyme-linked immunosorbent assay (ELISA) kit and the verification of the liquid-mass spectrometer (LC/MS). It was found that AFs pollution mainly existed in cottonseed meal, peanut meal, corn and corn alcohol grains. From a regional perspective, AFs pollution was the most serious in South China. The 4 AFs, AFB1, AFB2, AFG1 and AFG2, were present in varying degrees, and AFB1 had the highest positive rate and the highest content. Gao et al.

[45] further studied the relationship between the 4 AFs, and the consequence showed that AFB1 and AFB2 pollution were closely related, and AFB2 was accompanied by AFB1, and the pollution rate and concentration of the two are positively changed. When the concentrations of AFB1 was high, it inhibited AFG1 and AFG2. In accordance with the total aflatoxins (TAFs) was twice that of AFB1, when the TAFs limit in maize was above 4 μg/kg, the simultaneous formulation of the AFB1 and TAFs limits is of great significance compared with only the AFB1 limits.

1.6 Toxic effects of AFB1

1.6.1 Toxicity of AFB1

AFB1 is a highly toxic substance and its toxicity is 10 times that of potassium cyanide, 68 times that of arsenic, and 416 times that of melamine. AFB1 has liver toxicity, reproductive toxicity, neurotoxicity, etc. [34, 46, 47]. Maximum toxicity of AFB1 is carcinogenicity, which can give rise to primary liver cancer in the body. Liver is the main target organ. Furthermore, AFB1 can also result in gastric cancer, bowel cancer, kidney cancer, breast cancer, ovarian cancer, etc. [48, 49].

1.6.2 Organ toxicity of AFB1

Humans and animals infected with AFB1 mainly injure the organs, and the main target organ after being ingested is the liver, which initially causes hepatitis and hepatic necrosis and other diseases, and eventually leads to liver cancer [50]. Common clinical symptoms are hepatalgia, nausea and vomiting, etc. In severe cases, hyposarca, coma, convulsions, and even death from acute poisoning may occur; Other organs such as lungs, stomach, kidneys, spleen, rectum, etc. can also have degenerative diseases [51].

Carcinogenic toxicity of AFB1 AFB1 is also the strongest carcinogen found so far. IARC (International Agency for Research on Cancer, IARC) listed

AFB1 as a possible human carcinogen in 1987, and reclassified AFB1 as a class I carcinogen in 1993, and was identified as the strongest carcinogenic toxins by far [52]. Regarding the carcinogenic toxicity mechanism of AFB1, some scholars believe that AFB1 can interfere with the synthesis of DNA by preventing the synthesis of some enzymes, such as superoxide dismutase, catalase, and glutathione sulfur transferase. Eventually, cells cannot synthesize proteins, leading to disorder in the life cycle of organisms [53]. Some scholars have also pointed out that AFB1 not only affects the synthesis of DNA, but also inhibits the synthesis of RNA in organisms. The most important one is the synthesis of 45SRNA. Furthermore, AFB1 can combine with tRNA to form a complex AFB1-tRNA, which can inhibit the activity of tRNA and some amino acids, such as leucine, arginine, etc., and block protein synthesis [54].

Immunotoxicity of AFB1 AFB1 can be combined with DNA or RNA to restrain the synthesis of proteins, enzymes, etc. in the body, leading to dysfunction and decline of the body's organs, thereby result in immunosuppression and affecting the body's immune function. As AFB1 infects the body, it will interfere with the synthesis of DNA and RNA, and then inhibit the absorption and utilization of some essential amino acids, which not only cause immunosuppression, but also emaciate the body and malnutrition. Additionally, on account of the liver damage caused by AFB1 poisoning, it will also inhibit the production of complement, thus affect the body's immune function [55].

On the basis of the toxicology study of AFs, the toxicity of AFB1, AFB2, AFG1 and AFG2 4AFs is in the order of AFB1 > AFG1 > AFB2 >AFG2. In terms of animal sensitivity, the toxic effects of different species, ages, and sexes are different, and the order is chicken> duckling> rabbit> cat> sheep> cattle> pig [56, 57].

1.7 AFB1 residue limit standard

Due to the high toxicity and harmful effects of AFB1, most countries all over the world have stipulated strict maximum residue limit (MRL) standards. China

strictly restricts the MRL of AFB1, but does not impose restrictions on TAFs (AFB1+AFB2+AFG1+AFG2). Since AFB1 is closely related to other AFs contamination, AFB2 pollution is accompanied by AFB1, and high concentrations of AFB1 have an inhibitory effect on AFG1 and AFG2. Therefore, there are two methods for the immune detection and evaluation of food AFs contamination. One method is to adopt the AFB1 limit standard in some countries including China. However, there are some deficiencies in solving the simultaneous existence of multiple toxins and having toxic additive effects and detection standard. Another method is to employ the TAFs total limit standard in some countries. The national limit standard of AFB1 in Chinese food is shown in Table 1.2

Table 1.2

Maximum levels of AFB_1 in foodstuffs in China

Food category	Food name	Limited standard （μg/kg）
Cereals and their products	Corn, corn flour (dregs, flakes) and corn	20
	Rice, brown rice, rice	10
	Wheat, barley, other grains	5.0
	Wheat flour, oatmeal, other hulled cereals	5.0
Beans and their products	Fermented soy products	5.0
Nuts and seeds	Peanut and its products	2.0
	Other cooked nuts and seeds	5.0
Grease and its products	Vegetable oils (except peanut oil and corn oil)	10
	Peanut oil, corn oil	20
condiments	Soy sauce, vinegar, brewed sauce	5.0
Special dietary food	Infant formula (based on powdered products)	0.5
	Older infants and young children formula	0.5
	Infant formulas for special medical purposes	0.5
	Cereal supplements for infants and young children	0.5

1.8 The maximum residue limits of AFB1 and TAFs in some foods of various countries

Among various AFs, AFB1 is the most toxic and widely polluted (accounting for more than 50%), so most countries in the world have made clear provisions on the maximum residue limits (MRL) of AFB1 in food *[58]*. However, with people's increasing attention to food safety issues, the deepening of toxicity research on TAFs and the continuous progress of detection technology, in order to solve the simultaneous existence of multiple toxin pollution with toxic superposition effect and the corresponding lack of detection standards, it has become a development trend to formulate TAFs (AFB1 + AFB2 + AFG1 + AFG2) limit standards and corresponding detection methods *[59]*. In response to this new limit requirement and its role in international food trade, many countries in the world have launched study on TAFs standards and testing methods. By 2013, 91 countries have adopted the TAFs limit standard [60]. Table 1.3 shows the comparative analysis of AFs residue limits in some foods of different countries.

Table 1.3

Comparative analysis of AFs maximum residue limits in some foods of different countries

varieties of food	MRLs of AFs (μg/kg)					
	China[a]	America[b]	Japan[c]		EU(European Union)[d]	
	AFB1	TAFs	TAFs	AFB1	AFB1	TAFs
Cereals and Products	20	20	10	ND [e]	2	4
Peanuts and Products	20	20	10	ND	2	4
milk and dairy products	0.5 (M1)	0.5 (M1)	10 (M1)	ND	ND	4
Nuts and Products	20	20	10	ND	2	4

Note: [a].GB 2761-2005. [b]. FDA compliance policy guidelines. [c]. Positive List System. [d]. (Eu)No 165/2010. [e]. Not Detected.

1.9 Research progress of AFB1 residue detection technology

The detection methods of AFB1 in food and feed mainly include physicochemical analysis (PCA) and immunoassay (IA). PCA has the advantages of sensitivity, specificity, accuracy, etc., but it has disadvantages such as expensive equipment, high technical requirements, high cost, long cycle, and inability to operate on-site. Therefore, it is difficult to promote and apply [61]. While IA has become the development trend of AFB1 detection technology in the future because of its advantages such as specificity, sensitivity, rapidity, simplicity, large sample screening and on-site operation [62], It plays an important role in the rapid detection of AFB1.

1.9.1 Physicochemical analysis

Currently, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) and other PCA methods are mainly used in various countries.

TLC The basic principle of TLC is that after extraction, concentration and thin-layer separation, the sample is excited to develop color at 365 nm, in which AFB1 produces blue-violet fluorescence, so as to determine the content of AFB1, which is a semi-quantitative analysis method [63]. This method was established by Broadbent et al. [64] in 1963. After continuous development, it is divided into one-way expansion method and two-way expansion method according to its expansion method, and the latter has better sensitivity. In 1990, this method was listed as the standard method for the detection of AFB1 by the Association of official analytical chemists (AOAC). The Chinese national testing standards stipulate that this method should be used to detect AFB1, such as the determination of AFB1 in food (GB/T 5009.22-2003) and in feed (GB/T8381.2008/ISO 6651-2001) [65, 66]. TCL has the advantages of simple instruments and equipment, simple operation, and

easy popularization, and is a classic method for AFB1 detection. Its disadvantages are complicated sample preparation, cumbersome operation, time-consuming and labor-intensive, many interference factors, and poor accuracy of determination results [67]. Robb et al. [68] used this method to detect the content of AFB1 in food samples, and the LOD for AFB1 reached 0.5 ug/kg. Otta et al. [69] reported in 2000 that over pressured-layer Chromatography (OPLC) was established, and its LOD for AFB1 reached 1.0 ug/kg, which met the EU MRL standard of AFB1 in food.

HPLC The principle of HPLC is that after the sample is purified, AFB1 is separated by reversed-phase C_{18} chromatographic column under suitable mobile phase, and the qualitative and quantitative detection of AFB1 is realized under the action of fluorescence detector according to the fluorescence characteristics of AFB1. The method was established by Rao et al. [70] in 1973 and can also be used to detect AFB2, AFG1 and AFG2 with a LOD of 1.0 μg/kg. Subsequently, with the continuous development of HPLC, normal phase HPLC (NP-HPLC) and reversed phase HPLC (RP-HPLC) were derived. Among them, RP-HPLC has better sensitivity and stability, and RP-HPLC is more favored by users, but RP-HPLC has selectivity for TAFs molecules, i.e., it has high sensitivity for AFB2 andAFG2, the fluorescence intensity of AFB1 and AFG1 is easy to quench in aqueous solvent, resulting in low sensitivity or even undetectable. Pre-column or post column derivatization is needed to enhance their fluorescence [71, 72]. Generally, the derivatization methods are divided into pre-column derivatization and post-column derivatization. Trifluoroacetic acid is usually used for pre-column derivatization, which can enhance the fluorescence intensity of AFB1, thereby improving the detection sensitivity of AFB1 [73, 74]. Whereas, post-column derivatization mainly includes electrochemical derivatization and photochemical derivatization to increase the fluorescence intensity of AFB1, thereby solving the fluorescence quenching phenomenon of AFB1 in aqueous solution and improving the detection sensitivity [75].

HPLC has the advantages of high resolution, reliable results, high sensitivity,

automatic operation, suitable for qualitative, quantitative and multivariate analysis of large quantities of samples. It is regarded as the most authoritative and easily accepted method in the current quantitative detection of AFB1 at home and abroad. Its disadvantage is that the instruments and equipment are expensive, the technical level is high, and the samples need to be pretreated, which is not suitable for rapid and on-site detection [76]. Asghar et al. [77] reported that HPLC method was established by post-column derivation to detect AFB1, AFB2, AFG1 and AFG2, and the results displayed that the limit of quantification (LOQ) of AFB1, AFB2, AFG1 and AFG2 were 0.080, 0.073, 0.062 and 0.066 ng/g, respectively. Because HPLC has accurate quantification and high sensitivity, can analyze a variety of substances at the same time, and is not limited by the boiling point, thermal stability and molecular weight of the sample. Therefore, HPLC has been widely used all over the world and has been recognized as an official detection method by Association of Official Analytical Chemists (AOAC).

HPLC-MS/MS The basic principle of HPLC-MS/MS is to use appropriate interface technology to connect HPLC with tandem mass spectrometry. The sample is separated in a liquid chromatograph. After ionization, the ion fragments are separated by mass by a mass spectrometer and obtained by the detector. Mass spectrogram analysis, it combines the two advantages of HPLC's high-efficiency separation ability and MS's high sensitivity to realize the qualitative and quantitative analysis of a variety of compounds. The advantages of LC-MS/MS are fast, efficient, high resolution, micro-injection and automation. Compared with HPLC, there is no need for pre-column or post-column derivatization. The operation is relatively simple and convenient, and it has great advantages, and is more and more widely used. Its disadvantages are that the instruments and equipment are expensive and the level of professional technicians is high [78, 79]. Since LC-MS/MS has the advantages of wide detection range, reliable qualitative analysis results, multiple detection, fast analysis time, high detection sensitivity, and high degree of automation, it has become the most reliable detection method and is mainly used for laboratory confirmation analysis [80, 81].

Mccullum et al. [82] used HPLC-MS/MS to detect food AFs, and the results showed that the detection range was 0.006 μg/kg to3.0 μg/kg, and the detection limits for AFB1, B2, G1, and G2 were 0.0012 μg/kg, 0.0012 μg/kg, 0.0012 μg/kg and 0.0031 μg/kg, the spiked recovery rate was 97.0% to108.0%. Deng et al [83] used HPLC-MS/MS to detect AFB1, T-2 toxin, OTA, and DON in grains, and the results showed that the limit of detection (LOD) and limit of quantification (LOQ) were 0.1 to 2.0 μg/kg and 0.3 to 5.0 μg/kg, respectively. The method showed that the recovery rate, coefficient of variation (CV) and relative standard deviation (RSDs) were between 72.2% to 98.4%, 2.8% to 10.6%, and 5.5% to 15.4%, respectively, through the recovery test of standard addition. This method was used to analyze 40 kinds of food samples, and the results showed that DON was not detected, and the proportions of AFB1, T-2 and OTA were 30.8%, 17.5% and 33.3%, and the content concentrations were 0.58 to 0.89 μg/kg, 0.55 to 1.34 μg/kg, and 0.36 to 1.51 μg/kg, respectively.

1.9.2 Immunoassay analysis

Currently, the established AFB1 immunoassay methods include enzyme linked immunosorbent assay (ELISA), Colloidal Gold Immunochromatograohic Assay (GICA), fluorescence immunoassay (FIA) and immunosensor (IS), etc.

ELISA The basic principle of ELISA to detect small molecule haptens is to coat the antigen (or antibody) on the microplate, and add the antibody and the test hapten (or enzyme-labeled hapten and the test hapten), the antigen and the test hapten (or the enzyme-labeled hapten and the hapten to be tested) jointly compete for the antigen binding site of the antibody. After washing the plate, only the antigen-antibody complex of the antigen and antibody (or the enzyme-labeled hapten and antibody) is left on the reaction and bound. The amount of the complex is negatively correlated with the amount of the hapten to be tested. The color is developed by the enzyme substrate, and the hapten to be tested is qualitatively and quantitatively detected according to the shade of the color [84, 85]. ELISA analysis methods include indirect competitive ELISA (icELISA) and direct competitive

ELISA (dcELISA) two technical modes, which have the advantages of fast and convenient, simple operation, low cost, and large amount of screening samples. There are three main disadvantages of ELISA. First, antibodies and enzymes are both biologically active substances that need to be stored at low temperatures, which are easily affected by the environment and reaction conditions, and have poor stability; Second, there are non-specific reactions, and false positives are prone to detection results; Third, the complicated sample pretreatment operations require degreasing, desalination, pH adjustment, etc. [86, 87]. Li et al. [88] established icELISA for detecting AFB1 with mAb secreted by the screened 10 C9 cell line, and the detection range was 2.1 to 3.2 μg/kg, the recovery was 87.5% to 102.0%. Kim et al [89] established an icELISA method for detecting AFB1 and the total amount of and TAFs with the mAb secreted by the screened 8H10 cell line, with the detection range of 0.2 to 25 μg/kg, the recovery was 79.18% to 91.27%. Kolosova et al. [90] established a dcELISA for detection of AFB1, and the detection range was 0.1 to 10.0 μg/kg, 50% inhibition concentration (IC50) was 0.62 μg/kg, the recovery was 94% to 113%, respectively.

At present, many domestic and foreign companies have developed commercialized and standardized AFB1 detection ELISA kits, which have good performance in sensitivity, specificity, accuracy, stability, applicability, etc., and have become an important method for AFB1 detection. Zheng et al [91] used Singapore Biomin's AFB1 ELISA kit to detect samples such as corn, sorghum, wheat, rice, soybean, peanut and cottonseed. The LOD was 4.0 μg/kg, the detection range was 4.0 to 40.0 μg/kg, the ELISA kit was valid for one year, and its sensitivity was comparable to that of HPLC. Iqbal et al. [92] employed the AFB1 ELISA kit of Romer Labs Technology Company to detect 120 brown rice samples and verified them with TLC, HPLC and LC/MS-MS physical and chemical detection methods. The results exhibited that the LOD of the ELISA kit was 1.0 μg/kg, the detection range was 1.0 to 40.0 μg/kg, the spiked recovery rate was 83.2% to 90.4%, 88 positives were actually detected, and the AFB1 value of the sample was 1.24 to11.68 μg/kg. The detection results are completely consistent

with those of TLC, HPLC and LC/MS-MS. The detection sensitivity of ELISA kit is better than TLC, but slightly lower than that of HPLC and LC/MS-MS.

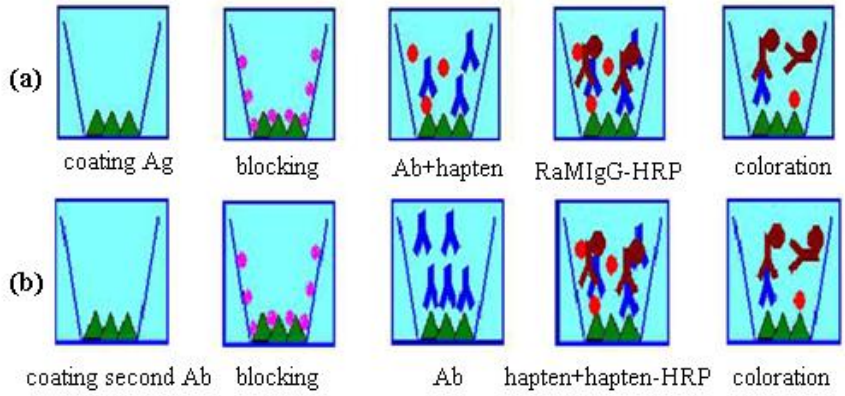


Figure 1.3 The operation procedure of icELISA and dcELISA. (a) The operation procedure of icELISA. (b) The operation procedure of dcELISA.

GICA The basic principle of GICA to detect small molecule hapten is that when the sample contains no small molecule hapten, the free gold-labeled antibody binds to the hapten immobilized on the membrane to form a red band, and the test result is negative; when the sa gold immunochromatographic assay mple contains small molecule hapten, it binds to the free gold-labeled antibody, inhibiting the binding of gold-labeled antibody to the hapten immobilized on the membrane, and not to form a red band, and the test result is positive. The content of small molecule hapten in the sample determines the depth or presence of the red band on the membrane. The advantages of GICA are rapid, simple, strong specificity, good stability, on-site detection, and large sample for screening. Its disadvantages are that it can only be detected qualitatively or semi-quantitatively, and cannot be accurately quantified; the detection sensitivity is not as good as ELISA, HPLC and LC/MS-MS [93, 94]. Sojinrin et al. [95] developed AFB1 detection test strip, the LOD was 0.3 μg/kg, and the detection result was in full agreement with HPLC. Ji et al [96] developed AFB1 immunochromatographic detection test strip. The LOD was 0.5 pg/mL, the detection range for AFB1 was 0.5 to 25 pg/mL, and the IC50 was 4.17 pg/mL.

GICA effectively combines the excellent separation ability of chromatographic analysis with the high specificity of immunoassay, and its portability. It provides an ideal platform for on-site detection that needs to achieve specificity, sensitivity and rapidity, and has realized commercialization and large-scale application. However, the detection limit, sensitivity, quantitative determination and high-throughput detection still need to be improved. With the research and application of new antibody preparation technologies such as single chain antibody and single domain antibody, as well as new nano labeling materials such as carbon nanoparticles, selenium nanoparticles, silicon nanoparticles, magnetic nanoparticles and luminescent quantum dot particles, GICA technology will continue to be developed and improved, and will be more widely used in clinical diagnosis, food safety and environmental monitoring[97, 98].

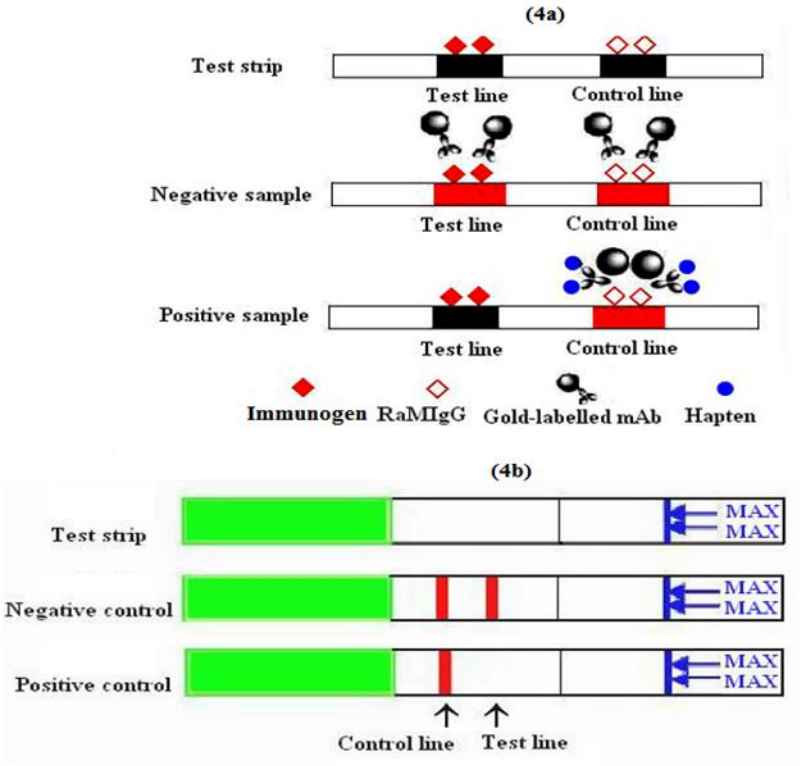


Figure 1.4 Schematic diagram of GICA principle and test results. (a) GICA principle. (b) Test results.

FIA Nowadays, established FIA for AFB1 detection mainly includes Fluorescence polarization immunoassay (FPIA) and time-resolved fluorescence immunoassay (TRFIA).

(1) Fluorescence polarization immunoassay

The basic principle of FPIA to detect small molecule hapten is to label antigen or antibody with fluorescent material. After antigen-antibody specific binding reaction, according to the difference of fluorescence polarization degree of antigen-antibody conjugate, the content of small molecule compound in test sample is measured by competitive method. The FPIA includes three technical modes: single-reagent FPIA, stopped-flow FPIA, and organic medium FPIA. The advantages of FPIA are high throughput, fast speed, easy operation, low detection cost, quantitative detection and rapid screening of samples in large quantities, which play an important role in mycotoxin detection. Its shortcomings include three points. One is that there are false positives due to the matrix effect, the other is the high cost of the instrument due to the need for special fluorescence polarization equipment, and the third is that the use of cup-type detection devices leads to large reagents and low detection efficiency [99, 100]. Sheng et al [101] used a broad-spectrum AFB1 mAb to establish FPLA, and its IC50 value for AFB1 was 23.33 μg/kg, LOD was 13.12 μg/kg, and CR for AFBl, AFB2, AFGl and AFG2 were 100%, 65.7%, 143% and 23.5%, respectively, which can be used for the quantitative detection of AFB1. Beloglazova et al. [102] established FPIA to detect AFB1 residues in beer samples and the LOD of AFB1 was 1.0 μg/kg, and the recovery rate was 89% to 114%.

(2) Time-resolved fluorescence immunoassay

The basic principle of TRFIA to detect small molecule haptens is to use lanthanide elements (Eu, Tb, Sm, Dy, etc.) long-lasting fluorescent markers, and then measure the fluorescence intensity after turning off the excitation light. The advantages of TRFIA are simple operation, high sensitivity, wide detection linear range, good repeatability, long reagent storage time, no pollution hazards, etc., which is currently recognized as the most sensitive immunological analysis method. Its shortcomings include three points. One is that the purity of the reagent is relatively high; the other is that it is difficult to synthesize lanthanide ion chelates; the third is that it is difficult to label multiple targets and the multiplex detection is

limited [103, 104]. Hu et al. [105] reported that anti-AFB1 monoclonal antibody 9B11-D7 was prepared by immunization and cell fusion, and Eu^{3+} was adopted to label goat anti-mouse immunoglobulin G (GaMIgG) to establish a TRFIA detection method for AFB1. The IC50 and LOD were 94.73 pg/mL and 3.55 pg/mL, respectively, the detection range was 3.55 to 1110 pg/mL, and the cross-reaction rates with AFM1, AFB2, AFG1, and AFG2 were 31.26%, 37.6%, 127.46%, and 35.74%, respectively, and had no CR with other analogs. In addition, the results of the standard addition test showed that TRFIA had high accuracy and precision, with an average sample recovery rate of 93.71% to 97.80%, and a coefficient of variation of 1.25% to 3.73%. Wang et al. [106] used Eu^{3+} nanospheres to label a broad-spectrum AFB1 monoclonal antibody, and established TRFIA for the detection of AFB1 and TAFs. The method detects AFBl, AFB2, AFGl and AFG2, the LOD was 0.16 μg/kg, and the detection range was 0.16 to 30.0 μg/kg, the recovery of standard addition was 83.9% to 113.9%, and the coefficient of variation (CV) was 3.5% to 8.8%. Compared with HPLC, ELISA, and GICA, this method has the advantages of sensitivity, accuracy, and speed, and can better meet the needs of detection of AFB1 and TAFs contamination residues in food.TRFIA technology has demonstrated outstanding advantages and huge application prospects in the field of international ultra-micro analysis technology. However, due to the late start of China's research in this field, it still faces two technical problems that need to be solved urgently. One is most of the detection reagents. For example, chelating agents and enhancing liquids need to be imported, and they rely too much on foreign products; second, domestic analytical instruments still have the disadvantages of cumbersome software operation and complex interfaces, which need to be overcome [107].

The basic principle of IS to detect small molecule hapten is that IS is composed of antigen or antibody and transducer. The small molecule hapten in the sample reacts with the specific antibody immobilized on the surface of the sensor to form an antigen-antibody conjugate. The amount of conjugate determines the charge signal of IS, the transducer achieves the purpose of detection according to

the change in the strength of the charge signal. According to the principle of sensing technology, IS can be divided into four categories: optical immunosensor, electrochemical immunosensor, thermal immunosensor and mass immunosensor [108, 109]. At present, optical immunosensor [110] and electrochemical immunosensor [97] for AFB1 detection have been established.

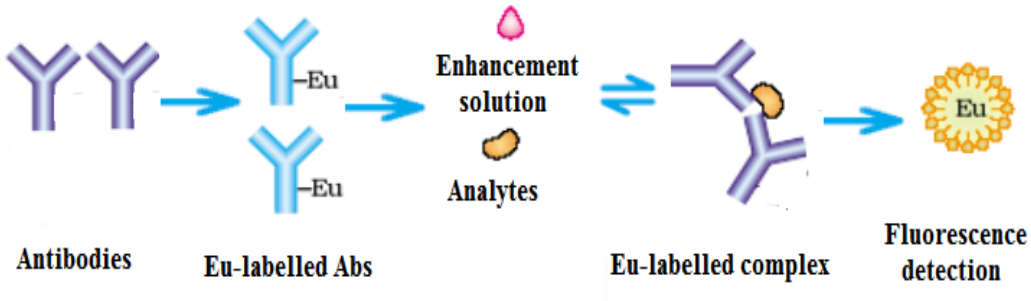


Figure 1.5 Schematic diagram of TRFIA working principle.

The advantages of IS are easy to carry, simple to operate, low cost, and automatic detection. Its disadvantages are that the antigen-antibody immobilization technology is not mature enough, the sensitivity and precision need to be further improved, it is difficult to achieve multiple detection and mass production [111]. Azri et al. [112] established AFB1 electrochemical immunosensor, the LOD was 0.3 pg/mL, linear range was 0.0001 to 10 ng/mL, and the spiked recovery rate of peanut samples was 80% to 127%. Kong et al. [113] developed semi-quantitative and quantitative IS with nano-gold, and realized the multiple detection of mycotoxins. Which can detect 20 kinds of mycotoxins at the same time. Among them, the sensitive and specific AFB1 mAb can be selected to detect AFBl, AFB2, AFG1, and AFG2, the LOD was 0.25 μg/kg, and the detection range was 0.25 to 4.0 μg/kg.

1.10 Research Progress on pollution status, toxic effects, residue limit and detection technology of ZEN

Discovery of ZEN ZEN, also known as the F-2 toxin, and it is chemically named 6-(10-hydroxy-6-oxycarbenyl)-β-ryanoic acid-μ-lactone, which is a non-steroidal estrogenic compound with a toxic estrogen effect. In 1962, Stob et al.

isolated and purified ZEN from the culture broth of F.graminearum for the first time, and initially verified its estrogen-like toxicity through animal experiments [114]. ZEN is similar to estrogen in chemical structure, and similar in biological activity to estrogen. It can bind to estrogen receptors and activate estrogen response elements, resulting in a series of estrogen-like effects [115].

Source of ZEN ZEN is a toxic substance produced by members of the genus Fusarium contaminated by wheat, barley, rice, maize, sorghum and other cereal crops, agricultural products and their by-products. ZEN is mainly produced by F. graminearum. In addition, F. equiseli，F. channellatus，F. trifolium, F. roseum, F. avenaceum, F. culmarum, F.graminis, F. remitecim can also produce ZEN [116, 117]. Owing to the strong viability of Fusarium, it is both parasitic and saprophytic. From the beginning of the field crops to mature, and then to the harvest, drying and storage of food, there is Fusarium infection, which is also accompanied by the pollution of ZEN. Therefore, ZEN pollution distribution is very extensive [118].

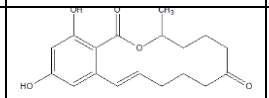
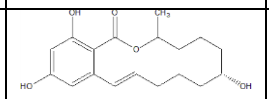
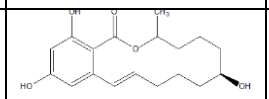
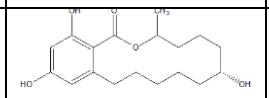
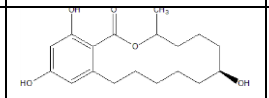
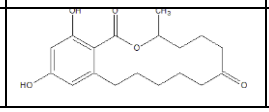
Physico-chemical property of ZEN In 1966, Urry et al. used nuclear magnetic resonance and mass spectrometry techniques to clarify the physico-chemical properties of ZEN for the first time. ZEN is a phenolic dihydroxybenzoic acid lactone structure, a white crystal, molecular formula is C18H22O5, melting point is 161 to 163 ℃, relative molecular mass is 318.36. ZEN dissolved in alkaline solution. Under alkaline environmental conditions, the ester bond opens by itself. When the concentration of the alkali decreases, the ester bond can be restored. ZEN is soluble in solvents such as alcohols, ether, benzene, chloroform, dichloromethane, ethyl acetate and acids. ZEN has maximum ultraviolet wavelength absorption at 236 nm, 274 nm and 316 nm, and the maximum absorption wavelength of infrared spectrum is 970 cm-1 [119, 120].

At present, a total of 15 derivatives of ZEN have been found, and the common ones are α-Zearalenol (α-ZOL), β-Zearalenol (β-ZOL), α-Zearalenol (α-ZAL), β-Zearalenol (β-ZAL) and zearalanone (ZON). In plants, ZEN is mainly metabolized into α-ZOL, while in animals, ZEN can be metabolized into α-ZOL and β-ZOL, and α-ZOL and β-ZOL are further metabolized into α-ZAL, β-ZAL

and ZON [121, 122]. The Physico-chemical properties of ZEN and its derivatives are shown in Table1.4.

Table 1.4

Physical and chemical properties of ZEN and its main derivatives

Compounds	Structural formula	Molecular formula	Molecular weight	Melting Point （℃）	UV peak (nm)
ZEN		$C_{18}H_{22}O_5$	318.4	161-163℃	236,274,316
α-ZOL		$C_{18}H_{24}O_5$	320.4	158-161℃	236
β-ZOL		$C_{18}H_{24}O_5$	320.4	137-139℃	
α-ZAL		$C_{18}H_{26}O_5$	322.4	182-184℃	254,302,357
β-ZAL		$C_{18}H_{26}O_5$	322.4	174-176℃	254,302,357
ZON		$C_{18}H_{24}O_5$	320.4	184-186℃	216,264,302

ZEN's pollution status ZEN is the most extensively polluted area in the world and the toxin that results in the greatest economic loss of global grains. In Europe, South America, North America, Asia, Africa and Oceania and many other countries or regions, cereals and agricultural by-products are polluted by ZEN to varying degrees [123]. Placinta et al. [124] summarized and analyzed the reports of *fusarium* toxin contamination worldwide, and found that ZEN was detected in grains and feeds in many countries.

The contamination involves wheat, barley, corn, feed, grains, oats, oatmeal and rye, among which, the highest content of oats can reach 15mg/kg. The results of a sample survey of ZEN contamination in feed in various Asian regions indicate that the average amount of ZEN contamination in East Asia is 396 to 969 μg/kg, Southeast Asia is 199 to 219 μg/kg, and South Asia is 76 to 1182 μg/kg [125, 126]. Most areas of China have mild climate and heavy rainfall, so that maize and other grains and their products provide a suitable environment for mold growth during the production, harvest, processing, transportation, and storage. Thereby, ZEN

pollution is also more common and serious. Li et al. [127] used a magnetic bead immunoassay-coupled biotin-streptavidin system (BAS-MBI) to conduct a ZEN contamination investigation on 405 maize, maize products and pig feed samples in China from 2016 to 2018. In 2016, 54 of 133 samples were positive, with a positive rate of 40.6%; ZEN levels ranged from 1.8 to 1100.0 ng / g, with an average of 217.9 ng/g. In 2017, 35 of 143 samples were positive, with a positive rate of 24.5%; ZEN levels ranged from 1.1 to 722.6 ng/g, with an average of 166.7 ng/g. In 2018, 41 of 129 samples were positive, with a positive rate of 31.8%. ZEN levels ranged from 1.3 to 947.8 ng/g, with an average of 157.0 ng/g. Statistics indicate that about 20% of ZEN positive samples exceed the MRL of ZEN.

1.11 Toxic effects of ZEN

The toxic effects of ZEN include reproductive toxicity, immunotoxicity, genotoxicity, cytotoxicity and potentially carcinogenic, which mainly give rise to the body's own obstacles and lead to the body organs to occur lesions [128], especially its estrogen effect is the most harmful to the body's reproductive system [129].

Reproductive toxicity The reproductive toxicity of ZEN is mainly manifested as estrogen syndrome, which affects the reproductive function of animals and causes reproductive dysfunction of female animals [130]. For female animals, ZEN results in poisoning in female animals since ZEN has a similar molecular structure to endogenous estrogen, which can bind to estrogen receptors, activate estrogen response elements, and form receptor dimerization reactions, and then a series of estrogen-like effects have appeared, which disrupts the reproductive hormones in the body and destroys the reproductive system [131]. For male animals, a small amount of estrogen can maintain the balance of the endocrine system in the body, but excessive estrogen will have a greater impact on

the fertility of male animals [132]. Among various animals, pigs are most sensitive to the reproductive toxicity of ZEN [133, 134].

Immunotoxicity Any dose of ZEN can affect the body's immune system, directly stimulate the body's immune response or cause toxic effects on the body. After the animal's body is invaded by low concentrations of ZEN, it will promote the gene expression of cytokines, thereby inducing inflammation; High concentrations of ZEN can affect the function of the thymus, inhibit the growth of thymic epithelium, injure the immune organs and immune system, and give rise to disorders of the immune function of the animal body [135]. Furthermore, ZEN also participates in the body's immune regulation, reducing immunity and causing immune toxicity [128]. Hueza et al. [129] administered ZEN to ovariectomized rats by gavage 3.0 mg/kg daily for 28 days to study the toxic effect of ZEN on the immune function of mice. The results displayed that ZEN can cause thymus atrophy, histology and thymocyte phenotype changes, the percentage of B cells in the spleen decreased, and the antibody production and hydrogen peroxide release of macrophages were impaired. Therefore, it is concluded that ZEN is an immunotoxic compound similar to estrogen and some endocrine disruptors.

Genotoxicity ZEN inhibits DNA and protein synthesis, interferes with cell division, affects the expression of genetic material, and results in genotoxicity. After ZEN and its derivatives invade the animal body, the genetic material produced will affect the synthesis of DNA, break the DNA chain of the cell, and affect the DNA replication function of the cell to a certain extent [136]. Gao et al. [137] reported the genotoxic effects of ZEN on Sprague Dawley (SD) rats and F1 female offspring. SD rats and F1 female offspring were exposed to feed containing ZEN 20 mg/kg for 21 days. The results showed that the feed intake and weight of pregnant rats and female offspring were significantly reduced. The birth weight and survival rate of F1 neonatal rats were significantly reduced. F1 female adult rats found obvious follicular atresia and thinning of the uterine layer. The conclusion of the study is that prenatal ZEN exposure in rats affects maternal and fetal development, and may lead to reproductive disorders in F1 adult female rats.

Cytotoxicity ZEN can reduce cell viability and oxidative damage by inhibiting DNA and protein synthesis in the cell. Studies have found that ZEN has a significant inhibitory effect on porcine ovarian granulosa cells. The color of the nucleus of the apoptotic cells is brown, and the gap between the cells is significantly enlarged. The dose of ZEN is directly proportional to the number of apoptotic cells and cytotoxicity, and high doses of ZEN have a greater toxic effect on cells [138, 139].

Carcinogenic toxicity ZEN has similar effects to human endogenous estrogen. It can stimulate the growth of tumor cells in estrogen-dependent tissues and organs, and has potential carcinogenicity to humans and animals. Zeng et al. [140] studied the effects of ZEN and estradiol on the proliferation and apoptosis of human breast tumor cells MCF-7. The results showed that ZEN can quickly restore the proliferation activity of MCF-7 cells, promote mitotic index, and inhibit MCF-7 cell apoptosis caused by estrogen depletion. Tomaszewski et al. [141] reported that ZEN has a tumor-promoting effect on estrogen-dependent tissues similar to estrogen. It detected the endometrium of patients with endometrial hyperplasia and endometrial cancer and the endometrium of normal people. The former detected the presence of ZEN, while the latter did not detect the presence of ZEN, thus supporting the research conclusion that ZEN is potentially carcinogenic.

1.12 The MRL of ZEN

With the continuous deepening of research on ZEN residual toxicity and harmful effects, most countries have formulated ZEN MRL, which effectively prevent food safety problems caused by the pollution of crops. On the basis of different pollution situations, the ZEN MRL formulated by various countries are different. The ZEN MRL in the main agricultural products of different countries are shown in Table 1.5.

Table1.5

Maximum residue limits of zearalenone in main agricultural products of different countries

Country	Food name	MRLs （μg/kg）
China	Grain, grain products	60
EU	Raw corn	200
	Unprocessed grains other than corn	100
	Edible grain food, grain flour	75
	Processed grain baby food	20
Russia	Wheat, wheat flour, wheat germ	1000
Australia	Grain, grain products	50
France	Grain, vegetable oil	200
Italy	Grain, grain products	100
Brazil	Corn	200

1.13 Research Progress of ZEN Detection Technology

At present, the detection methods of ZEN in food and feed mainly include chromatographic analysis (CA), immunoassay (IA), and biosensor analysis (BS).

1.13.1 Chromatographic analysis

CA is a method of separating and purifying the target by using the principle of detecting the difference in the partition coefficient or solubility of the target between the stationary phase and the mobile phase, and finally detecting the content of the target. Chromatography is the most commonly used detection method for the detection of small molecules, including thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography

(HPLC), high performance liquid chromatography-mass spectrometry (LC-MS) and so on.

TCL is an early method used in ZEN detection, but due to its complicated operation process, poor specificity, low sensitivity, poor reproducibility, experimenters need to be exposed to a large number of toxic and harmful reagents, etc., this method has been gradually phased out.

GC is used for ZEN detection and has the advantages of high sensitivity, high peak resolution and well reproducibility. However, its shortcomings are that due to the unique structure of ZEN, it must be derivatized before analysis, and the derivatization reagents are sensitive to humidity, which limits the practical application of this type of method.

HPLC is currently the most authoritative and commonly used method for detecting ZEN both domestic and international. Its advantage is that it has strong accuracy and sensitivity, but its disadvantage is that the equipment is expensive, the operation is cumbersome, and it cannot be used for on-site detection.

LC-MS or LC-MS/MS is currently the most sensitive and reliable method for mycotoxin detection. It has the advantages of low detection limit, high resolution, and multiple detection. Its disadvantage is that the equipment is expensive and cannot be detected on site. Nevertheless, it is still an important direction for the development of the detection field in recent years. Examples of chromatographic analysis method to detect zearalenone are shown in Table1. 6.

1.13.2.Immunoassay

Currently, the ZEN immunoassay methods used mainly include ELISA and GICA. ZEN's ELISA detection method was established earlier and is also a relatively mature method. According to different enzyme-labeled objects, it can be divided into two modes: icELISA and dcELISA.

Table 1.6

Examples of determination of zearalenone by chromatography analysis method

Method type	Pretreatment	Method names	LOD (μg/kg)	Linear range (μg/kg)	Recovery Rate (%)	Literature
TCL	Grinding filter	UV detection	200	500-20000	74-100	[142]
HPLC	Immunoaffinity column purification	Fluorescence detection	10	15-65	95	[143]
	Acetonitrile extraction , Oasis PRiME HLBSolid phase extraction column purification	Fluorescence detection	3.7	10-2000	83.0-101.3	[144]
GC-MS/MS	Ethyl acetate extraction, trimethylsilane derivatization	Mass spectrum	1	2-40	82-86	[145]
	Trimethylsilane derivatization	Mass spectrum	0.19	0.27-6.4	29-67	[146]
LC-MS/MS	Solid phase extraction	Mass spectrum	0.1	0.2-50	82-103	[147]
	Solid phase extraction	Mass spectrum	0.015	0.01-0.1	87-109	[148]

The ELISA method has high sensitivity, good specificity, simple extraction method, does not need large-scale equipment, and has low requirements for operators. It is very suitable for screening large quantities of samples and has been widely used in ZEN rapid detection. China National Standard GB / T 19540-2004 "Determination of Zearalenone in Feed" stipulates that ELISA is a standard method for detecting ZEN residues in compound feed and grain raw materials for

feed. In order to improve the detection performance of ELISA for ZEN, Huang et al. [149] established a biotin-avidin amplified ELISA (BA-ELISA) with a LOD of 0.35 μg/kg and a linear range of 0.54 to 7.99 μg/kg. Compared with traditional ELISA, the sensitivity of BA-ELISA is increased by 6 times.

GICA is a new type of immunological rapid detection technology that integrates monoclonal antibody technology, immune technology, chromatography technology, material labeling technology and other technologies, which is an immunological detection technique that was established and rapidly developed in the 1970s and is widely used. It is a technique that can detect ZEN semi-quantitatively. On account of this technology has the advantages of specificity, sensitivity, simplicity, speed, low cost, no need for equipment, and the naked eye can determine the results, it has played an important role in the detection of residues such as ZEN. Since the traditional preparation of colloidal gold is synthesized by chemical reduction, the purity of gold nanoparticles and the rate of antibody labeling are low, in order to further improve the detection performance of GICA, Urusov et al. [150] used laser ablation technology to prepare colloidal gold and established a new GICA method. Compared with traditional GICA, its sensitivity was increased by 2.5 times. Examples of immunoassay detection of zearalenone are shown in Table1.7.

Biosensors Biosensor is a type of chemical substance detection device that uses biologically active materials such as enzymes, antibodies, antigens, microorganisms, tissues, nucleic acids, organelles, and whole cells as identification elements to convert interactions into measurable signals. In the light of the types of sensors, biosensors are mainly divided into four types: electrochemical biosensors, optical biosensors, thermometric biosensors and piezoelectric biosensors [156, 157]. Biosensors have the advantages of good selectivity, high sensitivity, fast analysis speed, low cost, and can be applied to field detection. Nonetheless, owing to the late start of biosensor research, its application in food safety detection is still very limited.

Table 1.7

Examples of determination of zearalenone by immunoassays

Method type	Pretreatment	Method name	LOD (μg/kg)	Linear range (μg/kg)	Recovery rate (%)	Literature
ELISA	Extraction, centrifugation	inELISA	1.0	1.0-200.0	96.5	[151]
	Extraction, centrifugation	dcELISA	0.2	0.2-100.0	87-112	[152]
	Extraction, centrifugation	BA-ELISA	0.35	0.54-7.99	86.6-93.7	[149]
GICA	Extraction, centrifugation	GICA	20	-	91.30-97.07	[153]
	Extraction, centrifugation	GICA	5	-	-	[154]
	Extraction, centrifugation	GICA	15	-	-	[155]

With the progress and development of sensing technology, the development of biosensors is very rapid.

It can detect targets in complex samples without complicated pretreatment, and has broad development prospects in food safety control [158]. Examples of ZEN detection by biosensors are shown in Table 1.8.

Table 1.8

Examples of determination of zearalenone by biosensors

Method name	Signal output	LOD (μg/kg)	Linear range (μg/kg)	Literature
Colorimetric aptamer sensor	Colorimetric/Visual	10	10.0-250.0	[159]
Colorimetric aptamer sensor	Colorimetric/Visual	10	20-8000	[160]
Fluorescence quenching sensor	Fluorescence intensity	0.32	1.3-100.0	[161]
Fluorescence quenching sensor	Fluorescence intensity	0.5	0.5-64.0	[162]

1.14 Co-contamination and synergistic toxicity of AFB1 and ZEN

1.14.1 Co-contamination of AFB1 and ZEN

Molds and mycotoxins contamination of grain, grain food, feed raw materials and compound feed products are common all over the world. In the process of growth and harvest, especially under environmental stress such as flood and insect pest, maize, wheat, rice, peanut and barley are very likely to suffer from airborne or insect-borne toxic fungi. These fungi produce a large number of mycotoxins in the process of metabolism and pollute crops. At the same time, the temperature and humidity of mould-contaminated grain and feed products in the process of processing and storage are often conducive to the propagation of moulds, thus aggravating mycotoxins contamination [163]. There are three main reasons. One is that a mold may produce a variety of toxins. such as, *Aspergillus flavus* will produce aflatoxin, ochratoxin and citrinin, and *Fusarium graminearum* will produce zearalenone and vomiting toxin. Second, a crop will be polluted by two or more molds at the same time, producing a variety of toxins. Third, the types and severity of mycotoxin pollution are different in different regions, different environments and different feeds. When using different feed ingredients from different regions to prepare livestock and poultry diets, it causes simultaneous contamination of multiple mycotoxins [164].

According to the investigation report on mycotoxin pollution in feed and raw materials in China from 2016 to 2018, the contents of AFB1, ZEN and DON in feed and feed raw materials such as corn, corn by-products, wheat, bran, meal and full price feed were determined by immunoaffinity column HPLC. The contents of AFB1, ZEN and DON in feed and feed raw materials such as corn, corn by-products, wheat, bran, meal and full price feed were determined by immunoaffinity column HPLC. In 2016, among 1304 samples, the positive rate was 97.55% (1272/1304), the positive rate of 1 type of mycotoxin was 15.26% (199/1304), and

the positive rate of 2 types of mycotoxins was 12.88% (168/1304), the positive rate of 3 types of mycotoxins detected was 69.41% (905/1304) [165]. In 2017, in 1304 samples, the positive rate was 98.45% (1284/1304), the positive rate of 1 type of mycotoxin was 13.15% (136/1304), and the positive rate of 2 types of mycotoxins was 27.18% (281/1304), the positive rate of 3 types of mycotoxins was 58.12% (601/1304) [166]. In 2018, among 986 samples, the positive rate was 99.49% (5/986), the positive rate of 1 type of mycotoxins was 12.07% (120/986), and the positive rate of 2 types of mycotoxins was 22.51% (222/986), the positive rate of 3 types of mycotoxins detected was 64.91% (640/986) [167]. The 2016-2018 survey report on mycotoxin contamination in feed and raw materials in China is shown in Figure 14. As can be seen from the above results, from 2016 to 2018, mycotoxin pollution in feed materials and compound feeds such as corn, corn by-products, wheat and bran, meal and full-price feeds, etc. was widespread, and the pollution of two or more mycotoxins was more serious. Therefore, a single mycotoxin detection and prevention and control can no longer meet the needs of the feed industry.

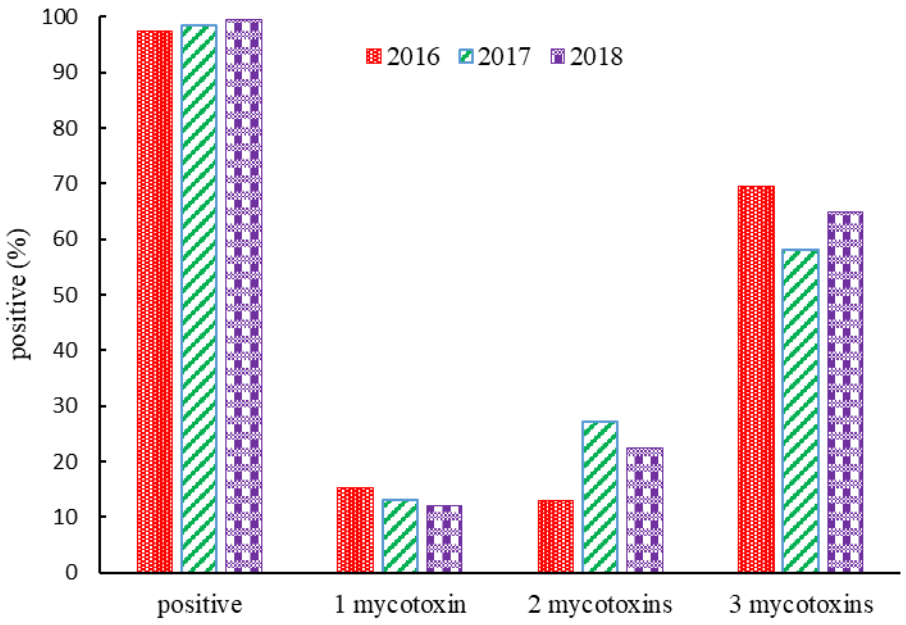


Figure 1.6. Mycotoxin contamination composition of feed and feed ingredients in China from 2016 to 2018.

Zhu et al. used up-conversion luminescence competitive inhibition immunochromatography to determine the contents of AFB1, ZEN and DON in 2237 samples of feed ingredients and compound feed from Shandong Province in China in 2019. The results indicated that the mycotoxin contamination rate of feed ingredients was 75.64%, the AFB1 contamination rate was 39.20%, and the over-standard rate was 7.18%; the ZEN contamination rate was 43.23%, and the over-standard rate was 12.82%; the DON contamination rate was 62.58%, and the over-standard rate was 1.79%. Cross-contamination of the two toxins is more common. The cross-contamination rate of DON and ZEN was 37.19% (832/2237), the cross-contamination rate of ZEN and AFB1 was 24.77% (554/2 237), and the cross-contamination rate of DON and AFB1 was 28.83% (645/2 237) (Figure 1.7).

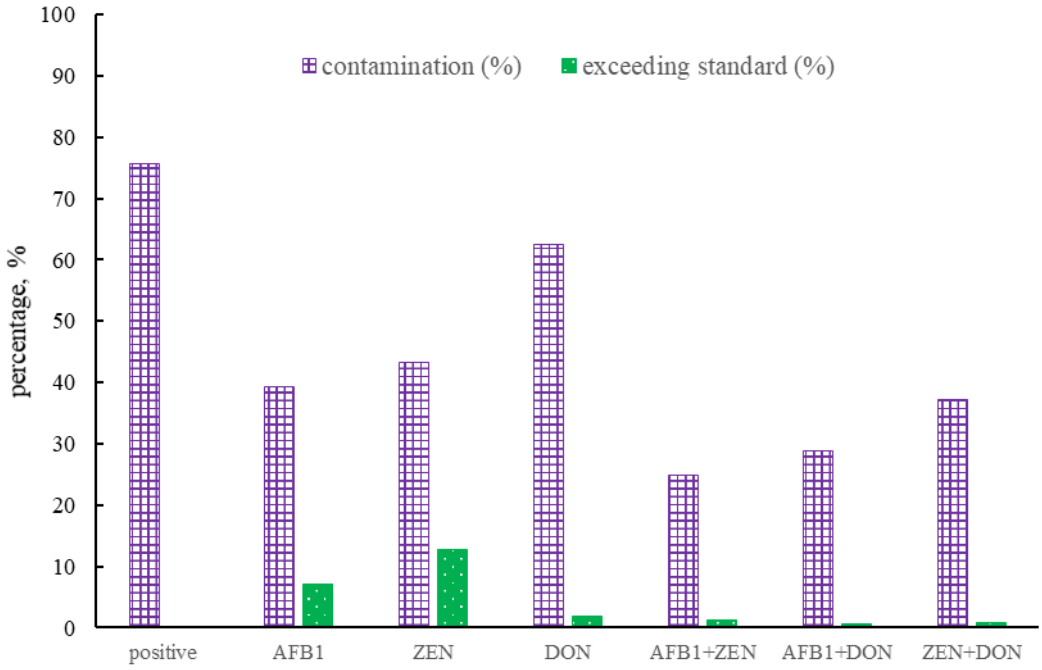


Figure 1.7. Mycotoxin contamination of feed materials and compound feeds in Shandong Province in 2019.

1.14.2 Synergistic toxicity of AFB1 and ZEN

According to the previous relevant reports, the synergistic or cumulative effects of multiple mycotoxins are more harmful to animal health and production performance than a single mycotoxin. Huang et al [168] reported the effects of aflatoxin B1 (AFB1) and zearalenone (ZEN) cross-contamination on the metabolism, immune function and antioxidant status of dairy goats. The results showed that compared with the control group, adding AFB1 and ZEN to the diet significantly reduced the feed intake and milk production of dairy goats; significant decrease in red blood cell count, hematocrit, average red blood cell volume, average red blood cell hemoglobin, and average platelet volume; serum alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities, total bilirubin (TBIL), interleukin-6 and malondialdehyde (MDA) activities were significantly increased; serum superoxide dismutase (SOD), glutathione peroxide (GSH-Px) activity and total antioxidant capacity (T-AOC) were significantly reduced.

Cross-contamination of AFB1 and ZEA can reduce the performance, immunity and antioxidant capacity of dairy goats. Sun et al. [169] reported the toxic effect of AFB1 and ZEN cross-contamination on BRL 3A hepatocytes. The results indicate that AFB1+ZEN has a synergistic toxic effect on BRL 3A cells. These toxins reduce cell viability by inducing the production of intracellular reactive oxygen species (ROS) and promoting the apoptosis of Buffalo Rat Liver (BRL) 3A cells, which are normal rat hepatocytes. The coexistence of AFB and ZEN in agricultural products is more hepatotoxic than alone.

1.15 Research Progress of GICA

1.15.1 Overview of GICA

GICA is a novel rapid immunoassay technology, which combines monoclonal antibody technology, immunoassay technology, chromatography

technology, material labeling technology, etc. It is a widely used immunological detection technology that established and rapidly developed in the 1980s. The technology can be semi-quantitative or use a scanner to achieve quantitative detection of a variety of biological macromolecules or small molecular compounds, such as antigens, antibodies, small molecular hapten and so on. The technology has the advantages of specificity, sensitivity, simplicity, rapidity, low cost, no equipment required, and naked eye can determine the results. It has been widely used in disease diagnosis, antibody evaluation, Physico-chemical analysis, microbial detection, food safety residue detection and other fields. The main components of GICA include the preparation of colloidal gold, the preparation of gold-labeled antibodies, the assembly of test strip, the optimization of technical parameters, and the determination of detection performance, etc. [170]/

1.15.2 Technical principle of GICA for detection of small molecule compounds

According to the size and binding method of the molecule to be tested, GICA can be divided into two technical modes: immunofiltration (sandwich method) and immunochromatography (competitive method). The sandwich method means that different epitopes of the antigen to be tested are combined with two antibodies to form an antibody 1-antigen-antibody 2 sandwich structure on the test strip, which is suitable for macromolecular analytes [171]. The competition method is generally used for small molecule detection, since small molecule compounds commonly have only one antigenic site, they cannot be combined by two different monoclonal antibodies simultaneously, and the sandwich method detection mode cannot be adopted. Therefore, the competition method is selected for detection. Immunochromatographic test strip for detecting small molecule hapten, usually the small molecule monoclonal antibodies labeled with gold particles are fixed on the binding pad. The detection line (T line) is for the immobilization of macromolecular protein antigens, and the quality control line (C

line) is fixed Anti-gold-labeled monoclonal antibody secondary antibody. When the test strip detects the sample to be tested, if the sample to be tested does not contain the hapten to be tested, the gold-labeled antibody flows to the T line and reacts with the immobilized artificial antigen, the gold particles aggregate and develop color, and the flowing gold-labeled antibody reacts with the secondary antibody responds to the C line and color development. If the sample contains the hapten to be tested, the hapten will react with the gold-labeled antibody to form an antigen-gold-labeled antibody complex, as the fluid flows to the T line, the antibody site is occupied by the hapten to be detected, and the gold-labeled antibody cannot react with the artificial antigen at the T line, the gold-labeled antibody flows through reacts with the fixed second antibody at the C line and develops color. The final result is determined that the positive sample has only one C line, while the negative sample has two red lines (T line and C line) [172, 173]. Figure 1.8 shows the schematic of GICA competition method for detection of small molecule compounds.

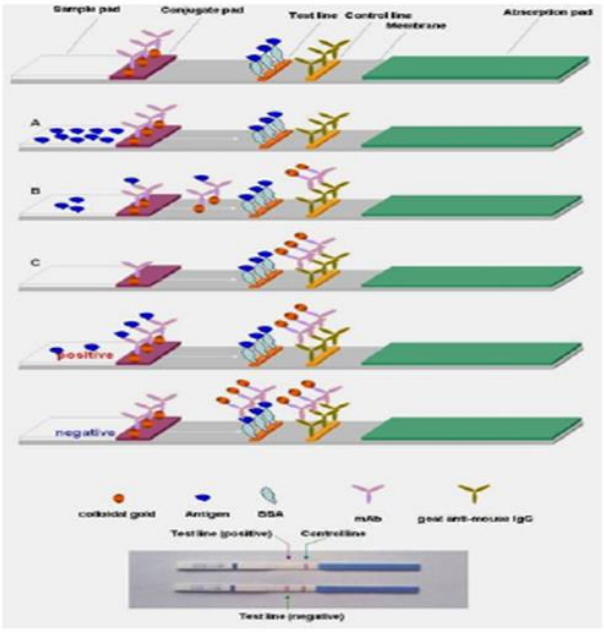


Figure 1.8. The schematic for GICA for the competitive immunoassay.

1.15.3 Basic structure of GICA test strip

GICA test strip is composed of a sample pad, a binding pad, a chromatographic film and a water absorption pad stacked and pasted on the support base plate in turn. The material of sample pad and bonding pad is fiber wool or glass wool, which are treated with different buffers respectively. The sample pad is used to quickly absorb the sample solution to be tested and provide a buffer system for antigen antibody reaction. The adsorption of the binding pad consists of labeled biomaterials, such as gold nanoparticles (AuNPs) labeled antibodies, which bind to the detection target in the sample solution to form immune complexes. The chromatographic membrane is usually nitrocellulose membrane (NC membrane), and fixed with two or more different biological materials (such as antigens or antibodies), forming the detection line (T line) and quality control line (C line), is applied to intercept labeled immune complexes, display the detection results. The absorbent pad is an absorbent cardboard, which provides power for the reaction through the siphon effect, and can also absorb the sample solution flowing through the chromatographic membrane. In addition to the basic structure of the immunoassay strip, some auxiliary materials, such as outer plastic film or plastic shell, need to be added to assemble a complete immunoassay strip, such as strip or card, as shown in Figure 1.9.

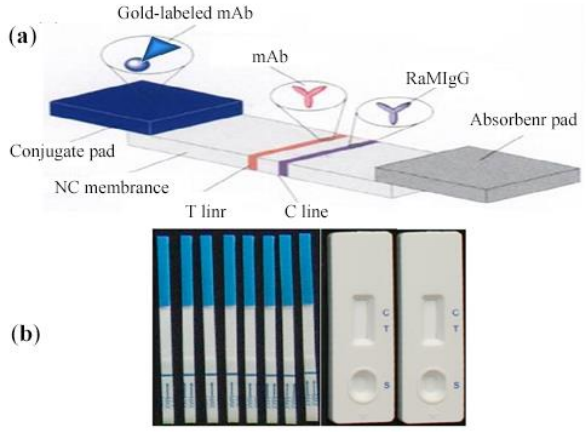


Figure 1.9 The schematic diagram and products of GICA. (a) The schematic diagram of GICA. (b) The products of GICA.

1.15.4 GICA research progress

Research progress of colloidal gold preparation technology. Colloidal gold is a stable colloidal solution formed by the reduction of chlorauric acid solution. It is used to label specific antibodies of small molecule haptens. Through the antigen-antibody reaction, colloidal gold aggregates in the T line of NC membrane, showing a reddish brown color. Finally, the detection result is determined on the basis of the presence and intensity of the color. The particle size of AuNPs is 20-30 nm. Owing to the small particle size, low molar extinction coefficient and low brightness, it is difficult to achieve the ideal detection sensitivity. Therefore, the selection of appropriate particle size is the key to the preparation of AuNPs. Li et al.[174] synthesized AuNPs with 4 particle sizes of 20, 60, 100 and 180 nm, and analyzed the effects of 4 AuNPs probes with different particle sizes on ochratoxin A and bovine serum albumin using biofilm layer interference technology. The affinity of the conjugate was further expressed by immunokinetic analysis. The results indicate that the 100 nm AuNPs probe has high molar extinction coefficient and strong affinity, which makes GICA exhibit superior sensitivity. However, the size of the gold particles at 180 nm AuNPs is too large, which has obvious steric hindrance, which is not conducive to the immunorecognition reaction between the labeled probe and the antigen. On the basis of the traditional colloidal gold method, by improving the particle size and shape of AuNPs, thereby improving the detection sensitivity, the current research progress mainly included gold nanoflowers, AuNFs, gold nanoclusters, AuNCs and gold nanorods, AuNRs. The immune layer is shown in Figure 1.10.

AuNFs is a modified colloidal gold nanoparticle with a diameter of about 100 nm. The surface of the nanoparticle has a radial or flowerlike structure. AuNFs have star, polybranched and sea urchin shapes. This multi-branched structure provides a large specific surface area, which improves optical brightness and binding affinity, thus improving the detection sensitivity of GICA. Huang et al. [175] established AuNFs-GICA, which was used to rapidly and simultaneously

detect fumonisinB1 and DON in traditional Chinese herb. The visual detection limit of this method was 5 ng/mL, and the detection results were compared with HPLC and ELISA have good correlation. Peng et al. [176] compared three gold nanomaterials with the same particle size, namely spherical AuNPs, short-tip AuNFs (tip length 7-8 nm) and long-tip AuNFs (tip length 13-15 nm). The results indicated that the long-tip AuNFs showed the highest signal intensity in GICA, less antibody consumption, the strongest light absorption capacity and the highest affinity (Figure10 a). Serebrennikova et al. [177] improved on the traditional colloidal gold method, prepared gold nanoflowers with a diameter of 100 nm and used them in GICA, the sensitivity of the method was increased by 5 times.

AuNCs is a kind of precious metal nanoclusters, which has become a promising fluorescent labeling material due to its optical stability, superior biocompatibility, large stoke-shift and strong photoluminescence. Peng et al [178] developed a gold nanocluster strip based on high fluorescence green for simultaneous quantitative determination of clenbuterol hydrochloride and ractopamine in pig urine with LOD of 0.003 and 0.023 μg/L, respectively, overcoming the limitation of low sensitivity of traditional ICA (Figure10 b).

AuNRs is one of the precious metal nanomaterials that have been studied more in recent years. Its variable aspect ratio enables AuNRs to exhibit large specific surface area, different absorption spectra or different colors, which is very suitable for rapid visualization screening. Pan et al. [179] applied AuNRs as a signal probe in ICA rapid detection for the first time. The detection limit of zearalenone in grain samples was 40 g/kg, which verified the feasibility and superiority of AuNRs as a visual detection signal probe. It provides an application reference for rapid research in the field of food safety (Figure 10 c). Additionally, in order to further improve the detection performance of GICA, more and more labeling materials have been applied, including quantum dots, time-resolved fluorescent microspheres, upconversion luminescent particles, latex microspheres, carbon nanoparticles, colloidal selenium nanoparticles, magnetic nanoparticles.

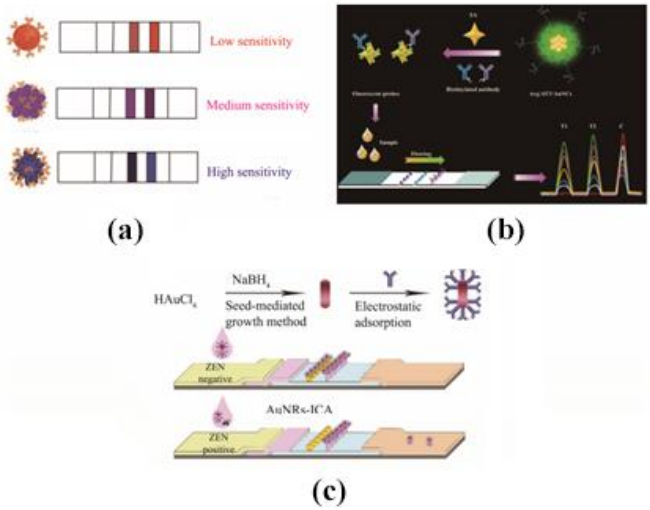


Figure 1.10 Chart of immunochromatography assay for AuNFs, AuNCs and AuNRs [180]. (a) AuNFs. (b) AuNCs. (c) AuNRs.

Particles, liposome nanoparticles, silica nanoparticles, etc. However, the immunochromatography technology based on gold nanomaterials is relatively mature and has the characteristics of simple preparation, easy labeling, high stability, excellent photoelectric performance, green environmental protection and low cost. Therefore, it is favored by the majority of manufacturers, and more and more researchers have carried out in-depth discussion on gold nanomaterials. It further promoted the development and application of GICA technology [181].

Research progress of high-throughput detection technology During the production, processing, packaging, transportation, and storage of agricultural products, food, and feed, they are often contaminated by a variety of harmful compounds, especially mycotoxins, pesticides, veterinary drugs, and special additives. Thus, the detection method of a single target is not only easy to cause missed detection, but also has low detection efficiency. The development of multi-residue immunochromatographic analysis methods and the realization of multiple detection are the main directions of GICA's future research.

Multiple detection with multiple labeled antibodies and several test lines Use AuNPs to label a variety of diverse antibodies to prepare different gold-labeled antibodies, and then mix them in a certain appropriate ratio to form a mixed gold-labeled antibody. A test line is set for a test target to form a test paper

with multiple test lines and a quality control line. Wang et al [13] successfully established a multiple immunochromatographic test strip method by spraying three detection lines and one quality control line on the nitrocellulose membrane, which realized the simultaneous detection of three types of 17 hormone drugs. The sensitivity can reach 0.005ng/mL.

Kong et al [14] established a multiple detection test strip containing five detection lines and one quality control line for the detection of five biotoxins, and prepared five mycotoxin gold-labeled antibodies, respectively. Five mycotoxin antigens are coated on the chromatographic membrane of the test strip to form 5 detection lines, which compete with the 5 mycotoxins in the sample to be tested and bind to the 5 gold-labeled antibodies, thereby realizing the multivariate analysis of mycotoxins in grains, As shown in Figure1.11.

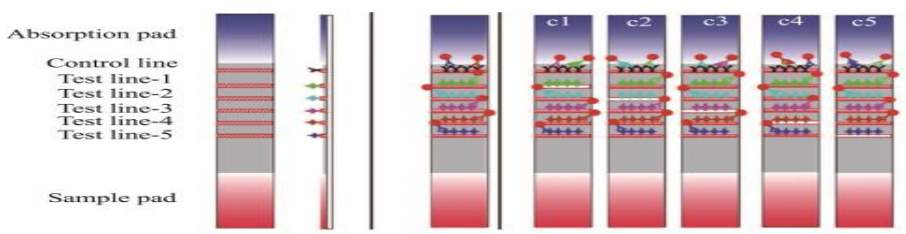


Figure 1.11. Schematic diagram of multiple detection with multiple detection limits for labeling multiple antibodies [113].

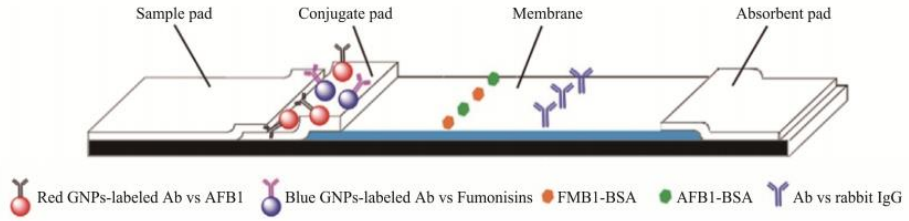
1.16 Multiple detection with labeled broad-specific antibody and one test line

Since multiple detection lines are set on one NC membrance, there are often mutual interferences between them, which affects the detection results. Therefore, it is very limited to expand the detection capability by adding more detection lines on a single NC. It is an ideal method to realize multiple detection by labeling a broad-spectrum specific antibody against a type of residue by AuNPs. In this study, a broad-spectrum specific ZEN mAb for ZEN has been prepared before, and this

method can realize the multiple detection of ZEN and its 5 derivatives. [182]. Chen et al. [183] designed a hapten containing a sulfa core structure by analyzing the structure of sulfa antibiotics, and obtained mAbs that can simultaneously

identify 27 sulfa antibiotic drugs, and the corresponding test strip can perform multiple detection of 27 sulfa antibiotic drugs. However, this type of multi-residue detection method with a single antibody has limitations, that is, it cannot distinguish between different analytes and specific concentrations. In response to this problem, Di et al. [184] established color-coded immunochromatography using multicolor AuNPs. This method couples red and blue AuNPs with two mycotoxin antibodies, respectively, and the detection line is composed of a mixture of two antigens. After the end of the immune response, since the red and blue labeled probes form different combinations on the detection line, so as to display different colors, as shown in (Figure 1.12).

Figure 1.12. Colour-encoded immunochromatography assay[184].



Wu et al. [185] synthesized gold nanomaterials with four colors (Figure 1.13), namely gold nanospheres (AuNSs), gold nanocacti (AuNCs), gold nanoflowers (AuNFs) and hyperbranched Au plasmonic blackbodies (AuPBs). The four gold nanomaterials were used to simultaneously detect FB1, zearalenone (ZEN), OTA, and aflatoxin B1 (AFB1), and the detection limits were 3.27, 0.70, 0.10, and 0.06 ng/mL, respectively.

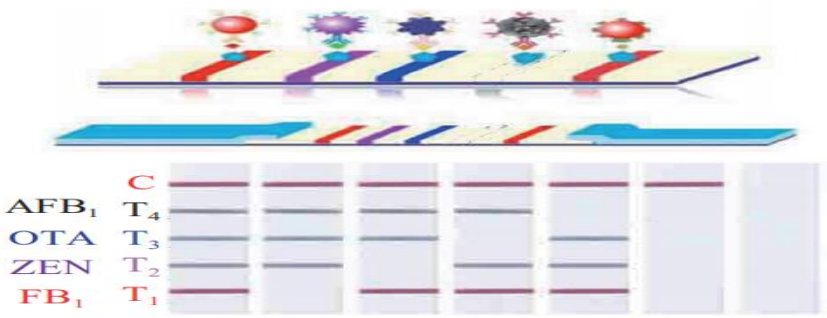


Figure1.13. Schematic illustration of multicolor AuNP-based multiplex ICTS nanosensor [185]

1.17 Multiple detection with multiple labeled antibodies and multiple channels

Some researchers and manufacturers use multiple test cards or multiple channels to share a single sample well to achieve multiple detection. By assembling multiple single test strip into one card, a multi-link card is prepared to realize the simultaneous detection of multiple drugs. For example, the triple test card for chlorpyrifos, diazinon and malathion is of this type, which can realize the simultaneous detection of three pesticides [186] (Figure 1.14).

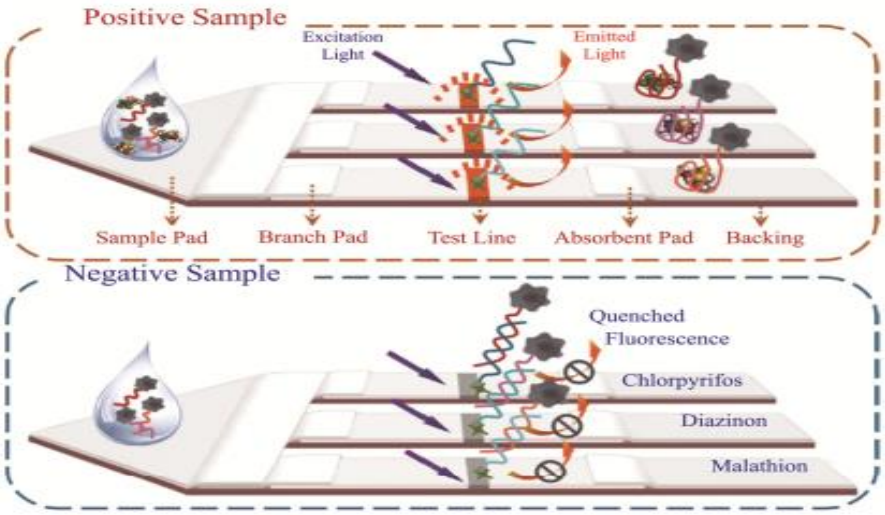


Figure1.14 Multiple detection chart of triple card[188]

Zhao et al. [187] established a ten-channel up-conversion luminescence GICA for rapid and simultaneous detection of 10 prevalent food-borne pathogens in food and water. The sample loading window in the middle of the test box is connected to the 10 test strip channels, and the sample solution can be dripped into the window to chromatographically flow to the 10 test strip at the same time, realizing fast and high-throughput monitoring of food-borne pathogens in food, as shown in Figure 1.15.

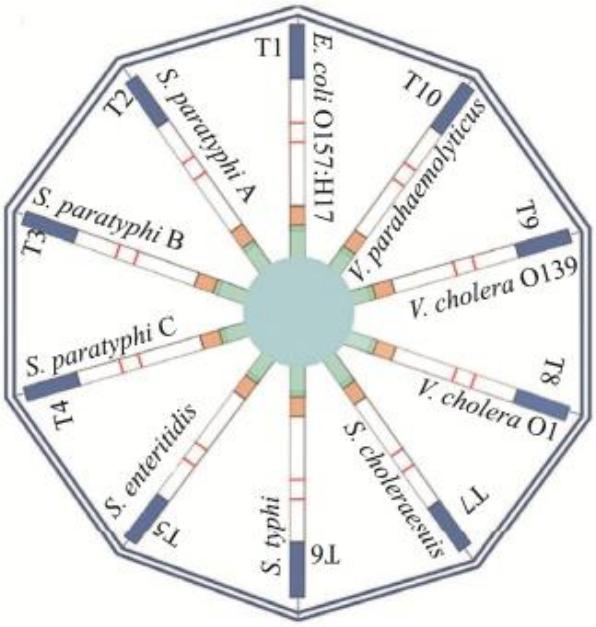


Figure1.15 Multichannel multivariate detection chart [189].

1.18 Conclusions from literature review

AFB1 and ZEN are the two most common toxins in mycotoxins, which mainly contaminate plant-derived agricultural products, food and feed such as corn, rice, wheat, barley, oats, sorghum, and so on. AFB1 and ZEN pollution can occur in all aspects of crop growth, harvesting, storage, processing, etc., and pollution area is extensive, the residue exceeds the standard seriously. AFB1 and ZEN pollution have acute poisoning, chronic poisoning, carcinogenicity, mutagenicity, teratogenicity, neurotoxicity, immunotoxicity, reproductive toxicity and many other toxic effects on humam and animal health, espeially, AFB1 and ZEN pollution residues often coexist and have synergistic and additive effects, it seriously threatens human health and the development of animal husbandry. Therefore, the establishment of AFB1 and ZEN dual residue detection and the strengthening of AFB1 and ZEN contamination monitoring have become important means to ensure food and feed safety.

Because traditional physico-chemical analysis methods are expensive and length, require complicated sample preparation procedures, expensive instruments, and skilled technicians, their application has been greatly restricted. Instead, immunoassay methods based on specific antigen-antibody reactions have been widely used in mycotoxin detection due to their high selectivity, strong sensitivity, rapid and simple sample screening, and portable operation. Among them, colloidal gold immunochromatographic assay (GICA) is one of the immunoassay methods. Compared with other immunoassays, it has strong selectivity, high sensitivity, rapidity, simplicity, large sample screening, and on-site operation. It has have become a hot topic in the research of AFB1 and ZEN double residue detection, which is also the purpose of this thesis.

In this study, through the design and modification of AB1 and ZEN hapten molecules, antigen synthesis and identification, an ideal immunogen is prepared to solve the problem of poor immunogenicity; through animal immunization and hybridoma technology, screening and preparation of strong immunoreactivity, high sensitivity and highly specific mAbs evaluate their immunological properties to solve the problem of unstable antibody sources and unstable quality; through the preparation of colloidal gold, the preparation of gold-labeled antibodies, the assembly of test strip, and the optimization of technical parameters, AFB1 and ZEN double-residue test strip are prepared, and their performances are measured to solve the problem of rapidity, simplicity, on-site operability; through the preliminary practical application of the developed AFB1 and ZEN double-residue detection test strip and the performance verification with ELISA and HPLC-MS/MS to solve the problems of practicability and reliability. In the end, it will provide test basis and technical support for realizing the rapid detection of AFB1 and ZEN dual residue and ensuring the safety of food and feed.

CHAPTER 2
OBJECTS AND METHODS

2.1 Research materials

The dissertation work is carried out in accordance with the programs of research work of Sumy National Agrarian University: "System of monitoring methods of control and veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration No. 0114U005551, 2014-2019); "Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023).

And dissertation work is carried out in accordance with the programs of research work of Henan Institute of Science and Technology part of the "12th Five-Year" National Science and Technology Support Program "Research and Demonstration of Rapid Detection Technology for Livestock and Poultry Products" (No.2014BAD13B05), The work was carried out for the “13th Five-Year” National Key Research and Development Plan Program “Food Safety Technology Research and Development” (No.2019YFC1605705) and the Program for Innovative Research Team (in Science and Technology) at the University of Henan Province (20IRTSTHN025).

So, the work was carried out for the period from 2018 to 2022 at the departments of veterinary examination, microbiology, zooghygiene and safety and quality of animals products, the department of epizootology and parazitology and the department of virology, pathanatomy and bird diseases of Sumy National Agrarian University. In addition, veterinary studies were conducted at Henan, China.

Object of study. Development and preliminary application of dual residue immunochromatographic test strip for AFB1 and ZEN in food and feed.

Subject of study. Synthesis and identification of artificial immunogens and coating antigens of AFB1 and ZEN, screening of immunogen synthesis methods for the preparation of high specific antibodies against AFB1 and ZEN; Selection of immunization methods for Balb/c mice, screening of positive hybridoma cell lines and identification of immunological characteristics; Preparation and identification of colloidal gold, preparation and identification of gold labeled-AFB1 mAbs and gold labeled-ZEN mAbs, optimization of test strip detection conditions, establishment of detection method and performance determination of AFB1 and ZEN dual residue test strip; Preliminary practical application and HPLC-MS/MS verification of AFB1 and ZEN dual residue test strip.

Research methods. Mycological (AFB1 and ZEN detection), clinical (history taking, clinical examination), microbiological (microscopic, biological), cytobiological (cells detection), toxicological (degree of toxicity and harmlessness), immunological (changes in gene and protein levels of inflammatory factors) and statistical (processing of research results).

Consumables. Gloves, masks; test tubes, petri dishes, syringes, measuring cups, measuring cylinders, beakers, Erlenmeyer flasks, volumetric flasks, pipettes, funnels, glass slides, coverslips; microtiter plates, cell culture plates, cell culture Bottle; centrifuge tube, pipette tip; filter paper, pH test paper, lens cleaning paper, kraft paper, gauze, absorbent cotton; medicine spoon, test tube holder, scissors, etc.

Chemicals and reagents AFB1，AFB，AFG1, AFG2 standard (solvent-free), ZEN standard (solvent-free), α-ZA), β-ZAL, α-ZO), β-ZOL, and ZON standard solutions in methanol, DON and OTA standard (Sigma-Aldrich, St. Louis, MO, USA). Carboxymethoxylamine hemihydrochloride (CMO), N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide (EDC), N-Hydroxysuccinimide (NHS), Hydroxylamine hydrochloride, succinic anhydride, 1,4-butanediol diglycidyl ether (BDE), phenacetin, 3,3,5,5-tetra-methylbenzidine (TMB), urea peroxide, and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), culture media RPMI-1640

with L-glutamine, goat anti-mouse IgG conjugated with horseradish peroxidase (GaMIgG-HRP), and a mouse mAb isotyping kit (Rockford, IL, USA). Dioxane, isobutyl chloroformate (IBCF), dimethylformamide (DMF), formaldehyde (FA), glutaraldehyde (GA), ethylenediamine (EDA), tri-n-butylamine (TNBA) and dimethyl sulphoxide (DMSO) (J&K Chemicals. Ltd., Shanghai, China). Fetal bovine serum (FBS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd, Hangzhou, China). Methanol, acetonitrile, petroleum ether, ethyl acetate, trifluoroacetic acid, chloroform, and Polyvinylpyrrolidone K30 (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China). Chloroauric acid, trisodium citrate, sodium azide, polyethylene glycol 20000, boric acid (China National Pharmaceutical Group Corporation, Bijjing, China). All other chemicals and reagents are standard commercial products of analytical grade or better.

Solutions (1) Phosphate buffer salution (0.01 M PBS, pH 7.4): NaCl (137 mmol), $Na_2HPO_4 \cdot 12H_2O$ (10 mmol), KCl (2.68 mmol), and KH_2PO_4 (1.47 mmol). (2) Carbonate buffer Solution (0.05 M CBS, pH 9.6): Na_2CO_3 (15 mmol) and $NaHCO_3$ (35 mmol). (3) Washing buffer: PBS containing 0.05% Tween-20 (PBST). (4) Blocking buffer: Swine serum (5%, *v/v*) in PBST. (5) The substrate buffer: A mixture of part A (500 mL) and part B (500 mL) solutions. Part A contained (per 1 L of water) 3.15 g citric acid, 6.966 g anhydrous sodium acetate, 0.08 g phenacetin, and 0.05 g urea peroxide adjusted to a pH of 5.0 using HCl, and Part B had 1.27 g of TMB dissolved in 500 mL of methanol and 500 mL of glycerol. (6) Stopping solution: 2 mol/L H_2SO_4. (7) Basal medium: 10.4 g RPMI-1640, 2.0 g $NaHCO_3$ dissolved in 1000 mL ddw. (8) Complete medium RPMI-1640/10: 78 mL RPMI-1640, 20 mL FBS, 1 mL antibiotics, and 1 mL HEPES. (9) HAT medium: complete medium, 1% 100×HAT solution. (10) HT medium: complete medium, 1% 100×HT solution. (11) Cell freezing solution: dimethyl sulphoxide (DMSO, 10%, *v/v*) in complete medium. (12) 1% chloroauric acid solution: 1.0 g chloroauric acid is dilute to 100 mL ddw, filtered with 0.2 μm filter membrane, and stored at 4 ℃. (13) 1% trisodium citrate solution: 0.5 g trisodium citrate dilute in 50 mL ddw, filtered with 0.2 μm membrane filter, ready to use. (14)

10% NaCl solution: 10.0 g NaCl dilute in 50 mL ddw and store at 4 ℃. (15) 0.2 mol/L K_2CO_3 solution: 2.764 g K_2CO_3 dilute to 50 mL ddw, filter with 0.2 μm filter membrane, and store at 4 ℃. (16) 10% BSA solution: 10.0 g BSA, 0.03 g NaN_3, dilute to 100 mL ddw, filter with 0.2 μm membrane, and store at 4 ℃. (17) Gold resuspension: 0.7627 g $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g BSA, 3.0 g sucrose, 0.03g NaN_3, constant volume to 100 mL ddw, 0.2 μm filter membrane filtration, 4 ℃ storage. (18) Sample pad treatment solution: 1.0 g BSA, 0.3 mL Tween-20, 5.0 g sucrose, 0.03 g NaN3, constant volume to 100 mL ddw, filtered with 0.2 μm membrane, and stored at 4 ℃. (19) Gold standard pad treatment solution: 0.7627 g $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g BSA, 0.3 mL Tween-20, 0.03 g NaN_3, dilute in 100 mL ddw, store at 4 ℃.

Equipments MULTISKAN MK3 microtiter reader (Thermo Co., Shanghai, China). DU-800 UV–visible spectrophotometer (Beckman-Coulter, Fullerton, CA, USA). Hybrid quadrupole–time of flight mass spectrometer (Q/TOF, HRMS; SYNAPT HDMS, Waters, UK). 700 MHz Avance III spectrometer (1H-NMR; Bruker, Billerica, MA, USA). 303A-1 electric heating constant temperature incubator (Beijing Zhongxing Weiye Instrument Co., Ltd., Beijing, China). Exceed DZG-303A ultrapure water polishing system (Chengdu Kangning Special Experiment Pure Water Equipment Factory, Chengdu, China). JY300C electrophoresis apparatus and gel imaging system (Beijing Junyi Dongfang electrophoresis equipment Co., Ltd., Beijing, China). LDZX-30KB vertical pressure steam sterilizer (Shanghai Shenan Medical Instrument Factory, Shanghai, China). SW-CJ-2 FD superclean bench (Suzhou Purification Equipment Co., Ltd. Suzhou, China). Galaxy S-type CO_2 incubator (RS-Biotech, Ayrshire, UK). TS100-F inverted microscope (Nikon Company, Tokyo, Japan). ME204E electronic balance, FE20-Five Easy Plus pH Meter (Mettler Toledo International Trading Co., Ltd., Shanghai, China). XMTD-8222 Electric thermostatic water sink (Shanghai Jinghong Experimental Equipment Co., Ltd., Shanghai, China). ZNCL-BS19 Heating Magnetic Stirrer (Shanghai Lingke Industrial Development Co., Ltd., Shanghai, China). WH-866 Vortex Mixer (Shanghai Kanghua Biochemical

Instrument Manufactory, Shanghai, China). 3K-30 Multifunctional Refrigerated Centrifuge (Sigma, USA). H-7650 Transmission electron microscope (Hitachi Limited, Tokyo, Japan). XYZ-3210 3D spray point platform (Biodot, USA).

Animals and cells Balb/c mice (License number: SCXK (YU) 2015-0004) (Henan Experimental Animal Center, Zhengzhou, China). NS0 myeloma cells (The Key Laboratory of Animal Immunology of the Ministry of Agriculture, Zhengzhou, China).

2.2 Synthesis of immunogen and coating antigen of AFB1 and ZEN

Synthesis of immunogen and coating antigen of AFB1 According to the active sites on the molecular structure of AFB1, oxime active ester (OAE) [190, 191], methylation of ammonia (MOA) [192], mixed anhydride (MA) [193, 194], semi acetal (SA) [195], epoxide (EP) [196] and enol ether derivative (EED) [197] were proposed to be used to prepare immunogens and coating antigens.

(1) OAE method

Briefly, 5 mg of AFB1 and 10 mg of CMO were dissolved in 500 μL of pyridine, shake for 24 h at 25 ℃ and avoid light, and freeze-dry the reaction product for 24 h to obtain a white powder called AFB1 activator AFB1O. 1 mg AFB1O, 1.073 mg DCC and 0.598 mg NHS were dissolved in anhydrous tetrahydrofuran, shake at 30 ℃ for 24 h, then centrifuge at 4000r/min for 15 min, and wash the precipitate with anhydrous tetrahydrofuran for 2 to 3 times, and then supernatant were combined, and after the tetrahydrofuran in the supernatant was completely evaporated, the residue was dissolved in 0.2 mL of DMF. The solution was slowly added dropwise to BSA solution, and reacted overnight at room temperature. The reaction product was dialyzed with PBS under stirring at 4°C for 3 days, and AFB1-BSA was obtained and stored at -20 ℃ for later use (as shown in Figure 2.1).

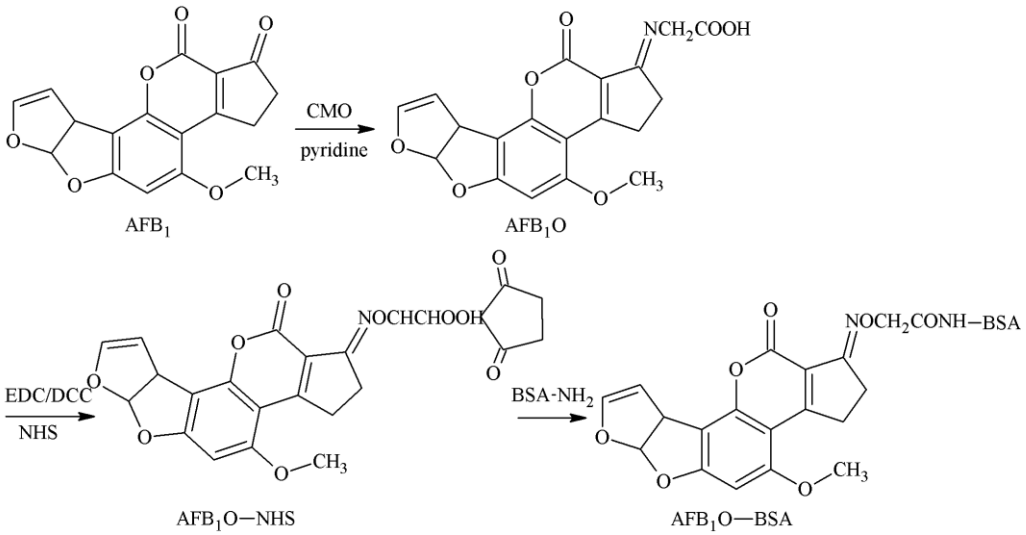
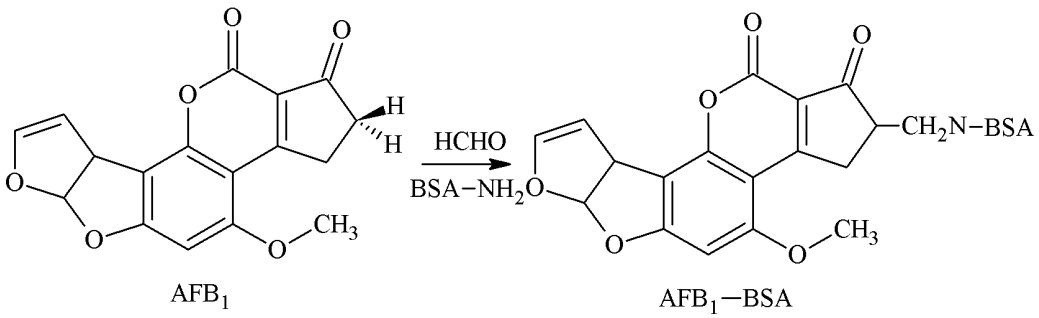


Figure 2.1 Synthesis scheme of AFB1-BSA artificial antigen via OAE method.

(2) MOA method

Briefly, first, dissolve ethylenediamine (EDA) in 0.1 mol/L pH4.8 2-morpholineethanesulfonic acid (MES) buffer and react in an ice bath. Then add the above solution to the mixed solution of BSA and EDC (MES buffer), magnetically stir the reaction for 2 h at room temperature, and finally add 2 mol/L acetic acid to terminate the reaction. The solution was dialyzed to obtain cProtein. Second, dissolve cProtein and AFB1 in 0.1 mol/L MES buffer (containing dimethylformamide), stir slowly, add formaldehyde, and react at 37 ℃ for 24 h.



(see Figure 2.2)

Figure 2.2 Synthesis scheme of AFB_1-BSA artificial antigen via MOA method.

(3) MA method

Briefly, 5mg AFB1 was dissolved in 1 mL methanol, 20 mL 0.1 mol/L citric acid was added, and the reaction was stirred at 30 ℃ for 24 h to obtain the product AFB2a. Then added 15 μL of tri-n-butylamine and 45 μL of isobutyl chloroformate to this solution, and the reaction was stirred for 0.5 h at 0 °C in an ice bath. To this solution, 1 mL BSA activation solution was added at a concentration of 20 mg/mL dropwise. The reaction solution was stirred at 4 °C for 4 h, then dialyzed with PBS at 4 °C for three days. The conjugates AFB2a-BSA was obtained and stored at 4 ℃ for use. (Figure 2.3)

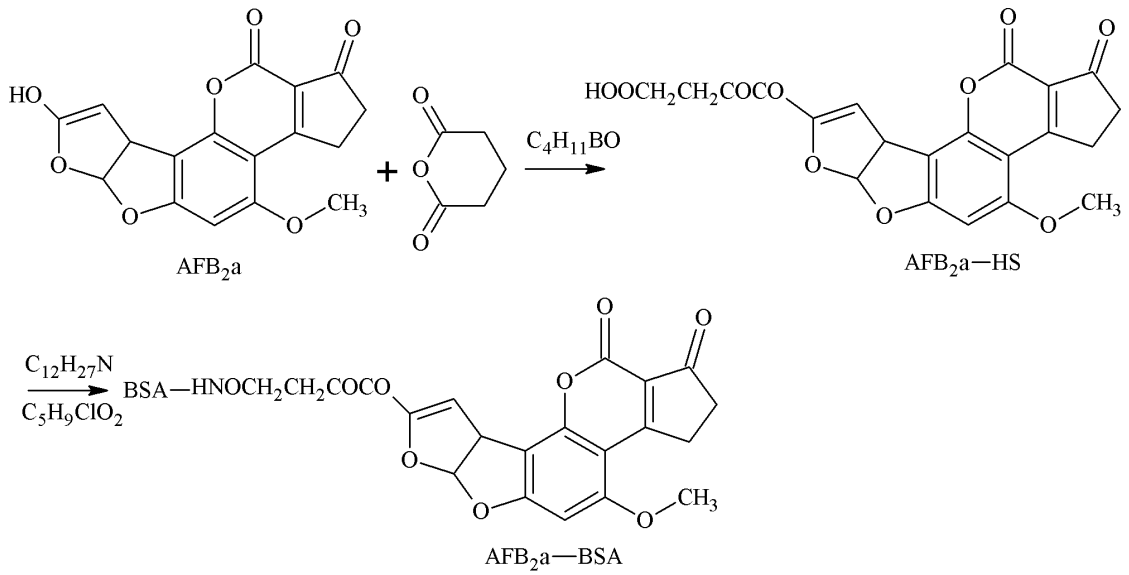


Figure 2.3 Synthesis scheme of AFB2a-BSA artificial antigen by mixed anhydride method.

(4) SA method

Briefly, 4 mg AFB1 was dissolved in 2 mL acetone, 40 μL 10% H_2SO_4 was added, and the mixture was stirred at 56 °C for 4 h. After the product was evaporated to dryness, 5 mL H_2O was added, extracted twice with 25 mL

chloroform, and then the organic layer was washed with 20 mL H_2O, to keep the organic layer, and evaporate the organic solvent to obtain a yellow solid product. 1.0 mg of product, 2 mL of 0.5% BSA solution was added to it, and reacted at 37°C for 30 min. After that, 100 μL of 6.5 mmol $NaHB_4$ was added and reacted at 4°C for 30 min. At 4°C, PBS solution was dialyzed for 3 days and the conjugates were obtained and stored at 4°C for use.(Figure 2.4). The coating antigen AFB1-OVA (SA) was prepared via the same method.

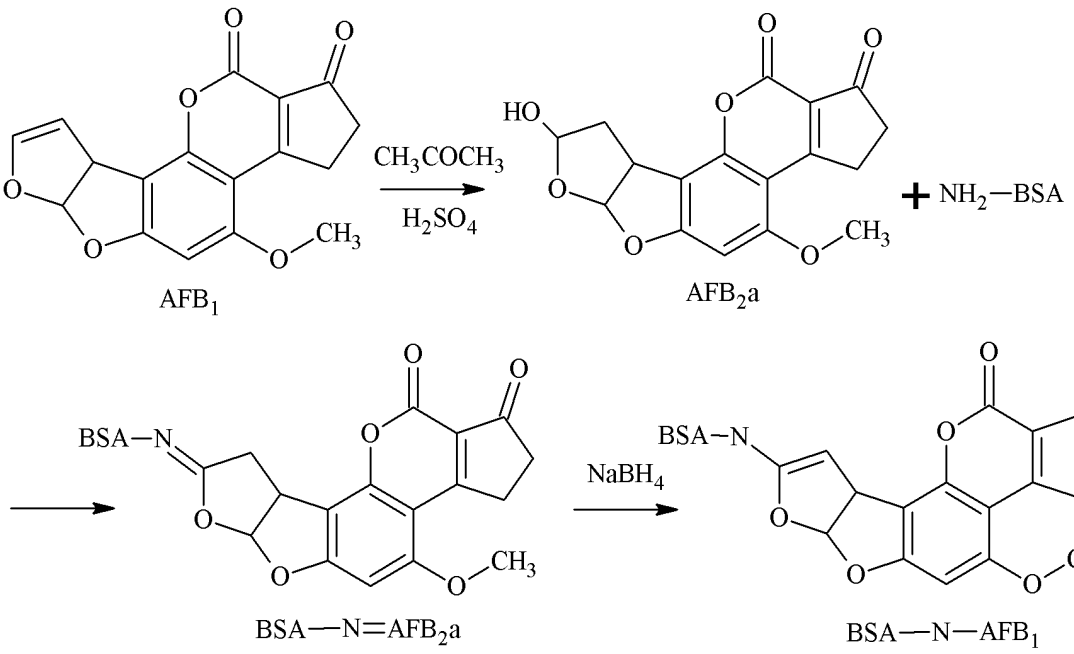


Figure 2.4 Synthesis scheme of AFB1-BSA artificial antigen by SA method.

(5) EP method

Briefly, 1 mg AFBI was dissolved in 2 mL dichloromethane, and an excess of m-chloroperoxybenzoic acid was added. 20 mg BSA was added to the reaction mixture, and the reaction was stirred overnight at room temperature. After that, the reaction solution was centrifuged at 5000 rpm for 20 min, and the supernatant was retained. The supernatant was dialyzed with PBS for 3 days and the conjugates were obtained and stored at 4 °C. The synthetic route of AFB1-BSA (EP) is shown

in Figure 2.5. Notably, the coating antigen ZEN-OVA (EP) was prepared via the same method.

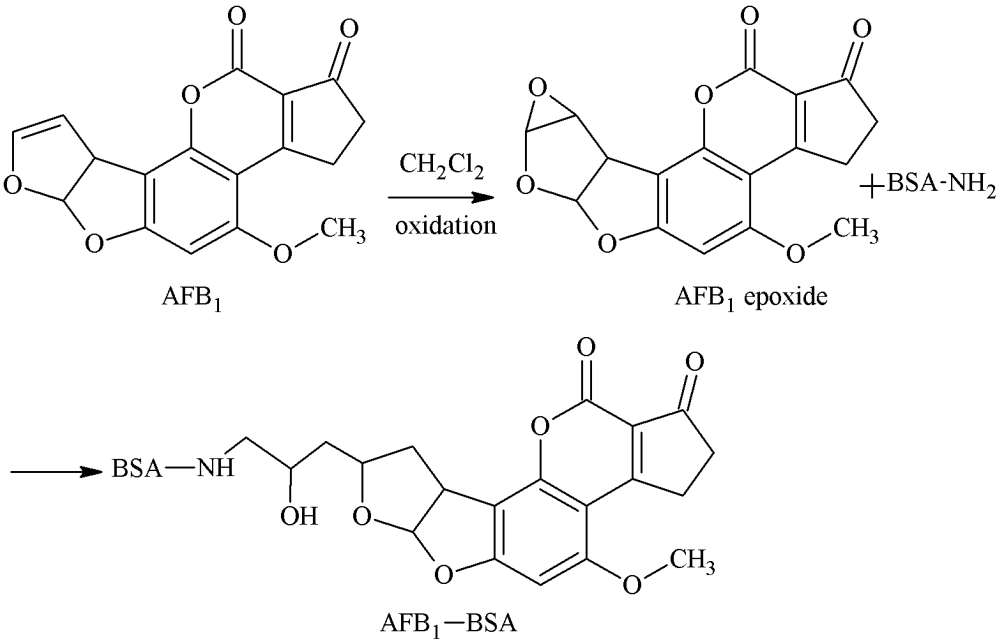


Figure 2.5 Synthesis scheme of AFB1-BSA artificial antigen by EP method.

(5) EED method

Briefly, 10 mg of AFB1 was dissolved in 4 mL of acetonitrile, 2 mL of 10% glycolic acid was added, and the reaction was stirred at room temperature for 20 min, and then purified on a C18 column to obtain AFB1-Glycolic Acid (AFB1-GA). AFB1-GA was dissolved in 0.5 mL of dioxane solution, and then 1.073 mg of DCC and 0.598 mg of NHS were added and stirred for 24 h under ice bath conditions. The reaction solution was added dropwise to 20 mg BSA solution in ice bath slowly, and stirred under ice bath conditions overnight. The mixture was dialyzed using PBS at 4 ℃ for 3 days, and the conjugates AFB1-BSA were obtained and stored in -20 ℃ for later use. The synthetic route of AFB1-BSA (EED) is shown in Figure 2.6. Similarly, the coating antigen AFB1-OVA (EED) was synthesized via the same way.

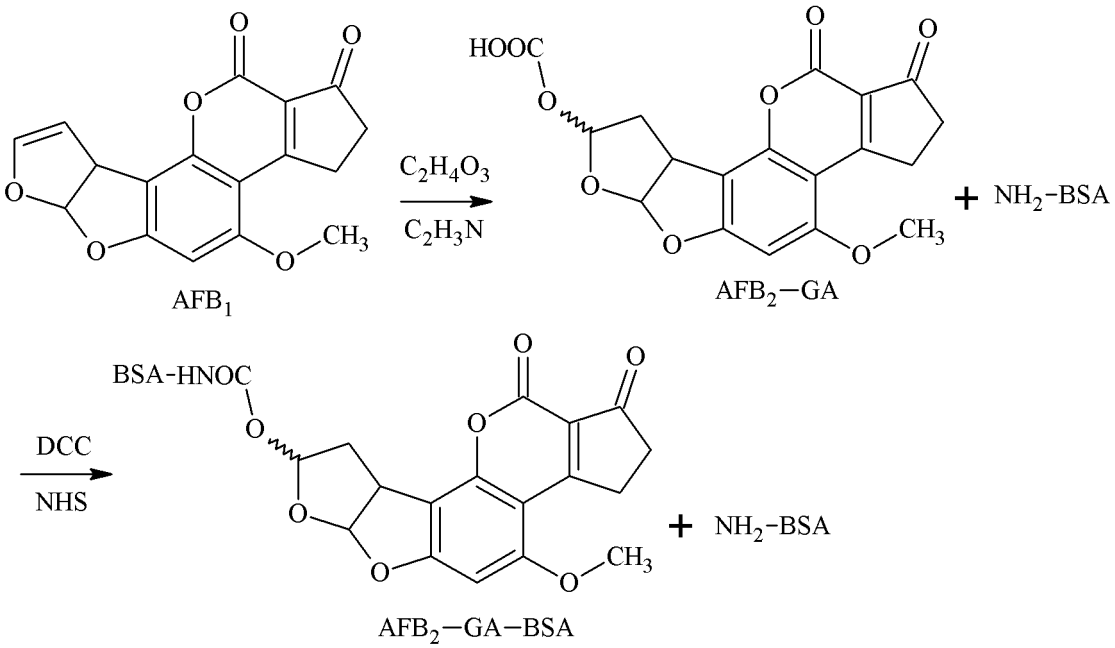


Figure 2.6 Synthesis scheme of AFB1-BSA artificial antigen by enol ether derivative method

2.3. Synthesis of immunogen and coating antigen of ZEN

According to the molecular structure and active site of ZEN, the immunogen and coating antigen were synthesized via oxime active ester (OAE) [198], condensation mixed anhydride (CMA) [199], formaldehyde (FA) [200], 1,4-butanediol diglycidyl ether (BDE) techniques [201], and the amino glutaraldehyde method (AGA) [202].

(1) OAE method

Briefly, according to the initial molar ratio of ZEN and BSA (50:1), 5 mg (0.0157 mmol) ZEN was dissolved in 2 mL pyridine, followed by the addition of 10 mg (0.09117 mmol) CMO. After sealing, the reaction proceeded while stirring using a magnetic bar at room temperature for 24 h to obtain the yellow solution product. The product was dried using nitrogen, and then we added 3.0 mL deionized water, adjusted pH to 8.0 using 0.1 mol/L NaOH, and extracted with an equal volume of ethyl acetate thrice. The aqueous phase was discarded. The organic phase was collected and dried using nitrogen to obtain hapten ZENO,

which was light yellow oil. ZENO was dissolved in 2 mL dioxane, after which 2.5 mg NHS and 5 mg EDC were added, stirred at 4 °C for 4 h to prepare the hapten activation solution. To the hapten activation solution, 1 mL cBSA activation solution was added at a concentration of 20 mg/mL dropwise. The reaction solution was stirred at 4 °C for 4 h, then dialyzed with PBS at 4 °C for three days. The dialysate was changed once a day and stored at 4 °C for subsequent applications. The synthetic route of ZEN-BSA (OAE) is shown in Figure 2.7 The coating antigen ZEN-OVA (OAE) was prepared via the same method.

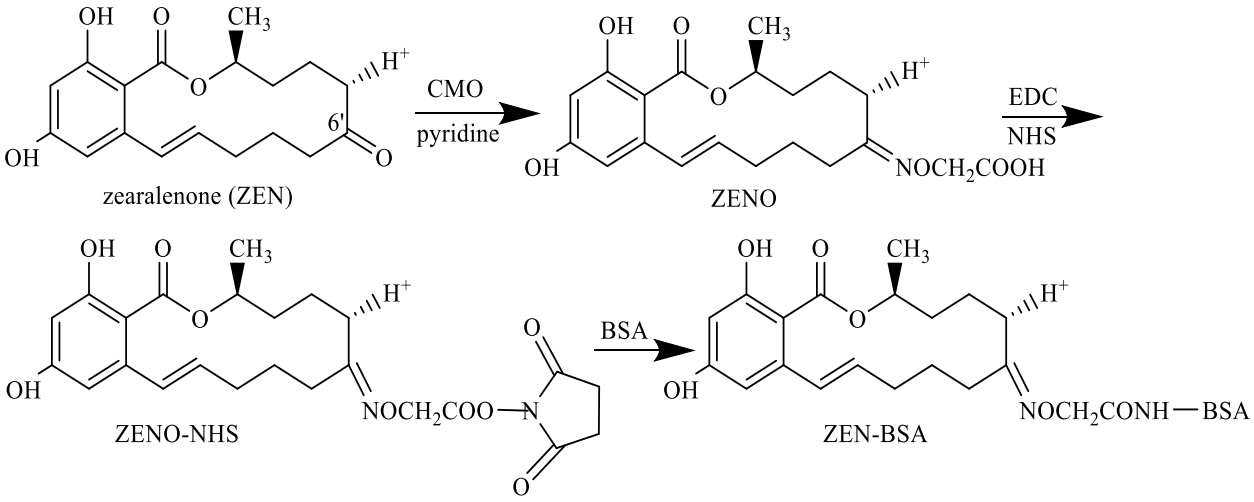


Figure 2.7 Synthesis route of the ZEN-BSA by the OAE method.

(2) CMA method

Briefly, according to the initial molar ratio of ZEN and BSA (50:1), 5 mg (0.0157 mmol) ZEN was dissolved in 2 mL pyridine, and 5.5 mg (0.0785 mmol) of hydroxylamine hydrochloride was added. The reaction was stirred at room temperature for 24 h, after which pyridine and unreacted hydroxylamine hydrochloride were removed via rotary evaporation to obtain the primary product. The initial product was dissolved in 0.5 mL pyridine, 7.8 mg (0.785 mmol) succinic anhydride was added, followed by 1 mL tetrahydrofuran. The reaction was stirred at room temperature for 24 h. The solvent was removed via rotary

evaporation to obtain the hapten product, which was dissolved in 2 mL of dioxane, then added 15 μL of tri-n-butylamine and 45 μL of isobutyl chloroformate. The reaction was stirred for 0.5 h at 0 °C in an ice bath. Eventually, the hapten activation solution was formed, which we added dropwise to 1 mL of cBSA activation solution at a concentration of 20 mg/mL. The reaction was stirred at 4 °C for 4 h. We dialyzed the reaction with PBS at 4 °C for three days. The dialysate was changed once a day. The resulting ZEN-BSA conjugate was stored at 4 °C for subsequent use. The synthetic route of ZEN-BSA (CMA) is shown in Figure 2.8 Notably, the coating antigen ZEN-OVA (CMA) was prepared via the same method.

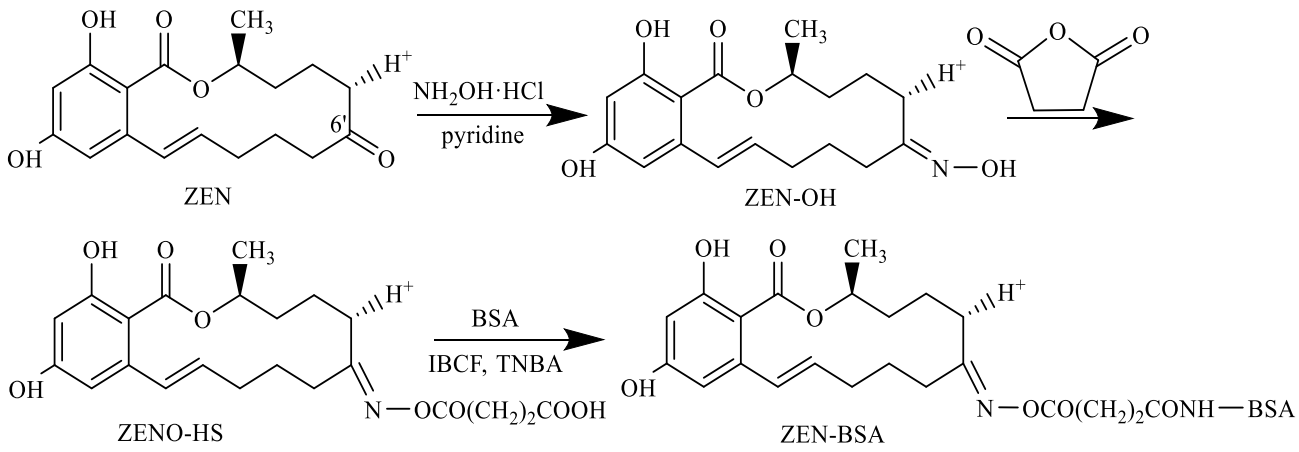


Figure 2.8. Synthesis route of the ZEN-BSA by the CMA method.

(3) FA method

Briefly, the experiment was designed according to an initial molar ratio of 50:1 (ZEN to BSA). Exactly 5 mg (0.0157 mmol) ZEN was dissolved in 1 mL of dimethylformamide (DMF), then added dropwise to 1 mL at a concentration of 20 mg/mL cBSA activation solution, 60 μL 37% formaldehyde aqueous solution (containing formaldehyde 0.8 mmol) was added, and the reaction was magnetically stirred at room temperature for 24 h. The reaction solution was dialyzed with PBS at 4 °C for three days, and the PBS was changed once a day. The dialyzate was collected and stored at 4 °C for subsequent use. The synthetic route of ZEN-BSA

(FA) is shown in Figure 2.9. Similarly, the coating antigen ZEN-OVA (FA) was prepared via the same method.

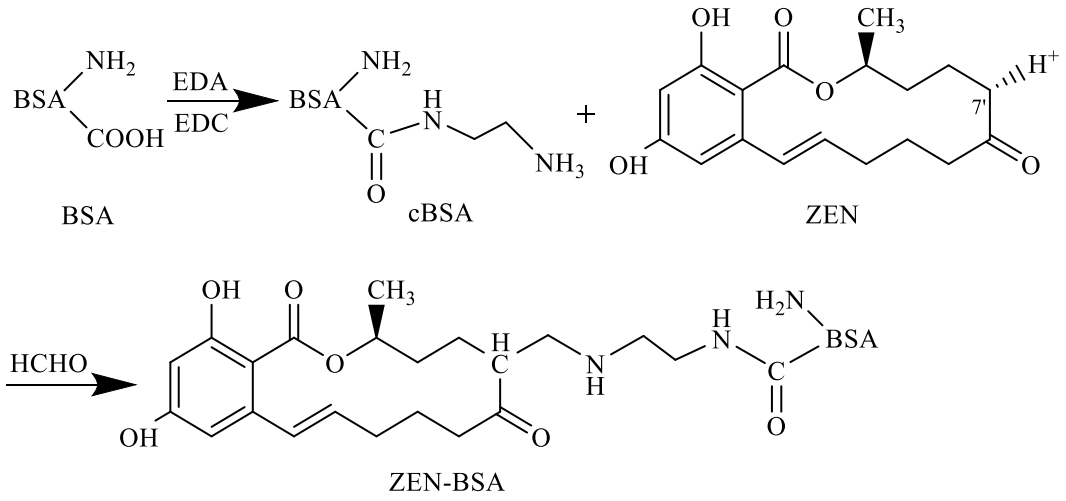


Figure 2.9. Synthesis route of the ZEN-BSA by the FA method.

(4) BDE method

Briefly, the experiment was designed according to the initial molar ratio of 50:1 (ZEN to BSA), 5 mg (0.0157 mmol) of ZEN was dissolved in 0.5 mL of dimethylformamide (DMF), 31 μL (0.157 mmol) 1,4-butanediol diglycidyl ether (BDE) was dissolved in 0.5 mL of double-distilled water (ddw) and added dropwise to the above solution. Then, pH value was adjusted to 10.8 with 1 mol/L NaOH and magnetically stirred at room temperature for 4 h; this formed the hapten activation solution. The hapten activation solution was added dropwise to 1 mL of cBSA activation solution with a 20 mg/mL concentration, the pH value was adjusted to 10.8 with 1 mol/L NaOH, and the reaction was stirred at 4 °C for 4 h. The reaction solution was dialyzed with PBS at 4 °C for three days. The dialysate was changed once a day. After collection, the dialysate was stored at −20 °C for subsequent use. The synthetic route is shown in Figure 2.10. The coating antigen ZEN-OVA (BDE) was prepared via the same method.

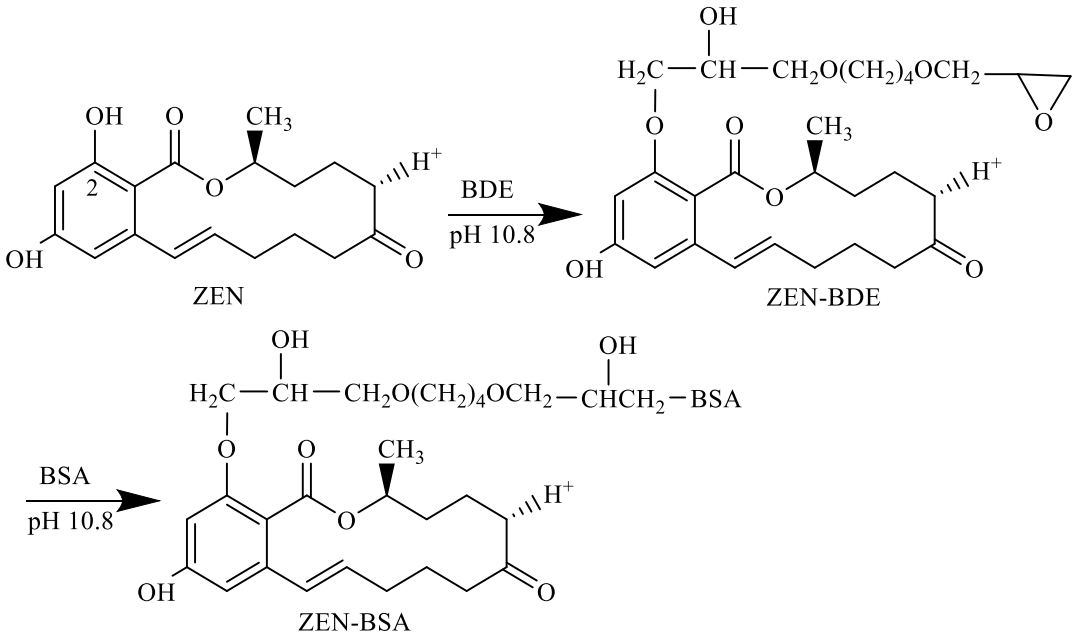


Figure 2.10 Synthesis route of the ZEN-BSA by BDE method.

(5) AGA method

Briefly, in the first step, 0.1 mmol ZEN, 0.1 mmol ZrO $(NO_3)_2$, and 5 mL acetonitrile were added into a reaction tube under nitrogen atmosphere and then the mixture was stirred under refluxed condition for 16 h, and obtained 5-NO_2-ZEN. In the second step, 0.3 mmol 5-NO_2-ZEN was dissolved in 5 mL hydrochloric acid, and the iron powder were added into the solution. Subsequently, the reaction was conducted at room temperature for 2 h. After being filtered and concentrated, the 5-NH_2-ZEN was obtained. 20 mg (0.00015 mmol) of BSA was dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.8). 5 mg (0.015 mM) of 5-NH2-ZEN was dissolved in 500 μL of methanol, 60 μL of 2% GA solution was then added and stirred for 4 h at room temperature. After the reaction, the reaction solution was added dropwise to the BSA solution, and stirred for 12 h at room temperature. The reaction mixture was dialysed against PBS for three days, and the resulting ZEN-BSA conjugate was stored at 4 °C for subsequent use. The coating antigen ZEN-OVA (AGA) was prepared via the same method.

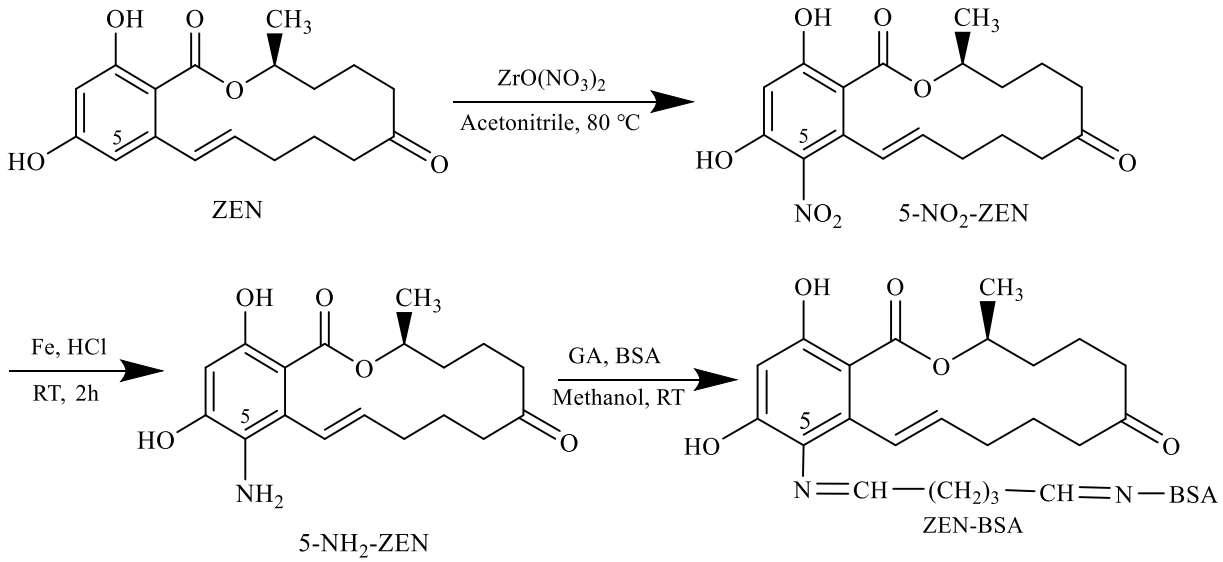


Figure 2.11 Synthesis route of the ZEN-BSA by AGA method.

2.4 Identification of AFB1 and ZEN antigens

UV identification PBS was used as blank control to calibrate the baseline. We prepared a 1mg/mL BSA solution and diluted immunogen to a 1 mg/mL protein concentration. The concentration of the AFB1 or ZEN standard was made to 20 μg/mL. We did a scan within the wavelength range of 200 to 450 nm, analyzed the scanning spectrum, and calculated the molecular binding ratio of conjugates based on Lambert–Beer law, A = εCL (where A is the absorbance value, ε is the molar extinction coefficient, (constant value), C is the solute concentration, and L is the optical path) [203].

SDS-PAGE identification Here, 5% concentration gel and 12% separation gel were selected for electrophoresis analysis. The test voltage for the concentrated gel was 100 V, while that for the separation gel was 60 V. Also, 10 μL/hole sample volume and 10 μg/hole protein content were used. After the Coomassie brilliant blue staining, the molecular binding ratio of conjugates was calculated by ultraviolet gel imaging system analysis software [204].

Preparation of AFB1 pAb and ZEN pAb Balb/c mice were immunized with 11 kinds of immunogens including 6 kinds of AFB1-BSA and 5 kinds of ZEN-BSA, divided into 11 groups, 5 mice in each group. The immunization

method was subcutaneously injected into the neck at multiple sites. The immunization dose was 50 μg/head, calculated according to the amount of BSA in each immunogen. Mice were immunized once every four weeks, five times. In the first immunization, immunogen was dissolved in sterilized PBS and emulsified with the same amount of FCA. In the enhanced follow-up immunization, immunogen was dissolved in sterilized PBS and fully emulsified with an equal amount of FIA and this was administered four weeks after the first immunization. Later, 21 days after the last immunization, the tail was cut off for blood collection. The serum was separated to obtain pAb.

2.5 Identification of pAb

Titers measurement The pAb titers for AFB1 or ZEN were tested through indirect non-competitive ELISA (inELISA) using the procedure described by Han et al. [205]. The coating antigen AFB1-OVA or ZEN-OVA was diluted in CBS at 2 μg/mL, and 100 mL/well added to the 96-well microplate, incubated at 37.8 °C for 2 h. After three times wash with PBST, unbound active sites were blocked with 250 μL/well of blocking buffer at 37.8 °C for 1 h or 4 °C overnight. After subjecting the microplate to another wash, 50 mL/well of pAb with appropriate dilution was added and incubated for 15 min at 37.8 °C.

After another washing procedure, GaMIgG-HRP (50 mL/well) was added, followed by incubation for 30 min at 37.8 °C. After six times wash, we added onto the microplate (60 μL/well) freshly prepared TMB solution, followed by incubation at room temperature for 10 min.

The reaction was stopped by adding 2 mol/L H_2SO_4 (100 mL/well), then measurements of absorbance were taken at 450 nm. Pre-immunization serum and PBST was used as a negative control and blank control, respectively, which we included in all assays. The pAb titers were defined as the reciprocal of the dilution that resulted in an absorbance value, twice the blank value. Each pAb sample and

negative sample were repeated for six times, and the average value was taken. Among the all groups of mice immunized with corresponding immunogen, the mice with the highest titers in each group were selected for evaluating the immune effects of the immunogen.

Sensitivity identification We determined the sensitivity at 50% inhibition concentration (IC50) of AFB1 or ZEN, whereas the IC50 value was determined via indirect competitive ELISA (icELISA) using the procedure described by Imtiaz et al. [206]. The icELISA method was the same as the inELISA except that after blocking, a competition step was introduced by adding 50 mL/well of the analyte, followed by 50 mL/well of appropriate antibodies.

The inhibition rate B/B0% of different concentrations of AFB1 to AFB1 pAb (or ZEN to ZEN pAb) was assessed by icELISA, where B denoted the absorbance value of different AFB1 (or ZEN) concentrations, and B0 represented the absorbance value of AFB1 (or ZEN) 0 concentration. The inhibition curve was drawn using B/B0% as the ordinate and the logarithm of different AFB1 (or ZEN) concentrations as the abscissa. We then deduced the regression equation and calculated the IC50 for AFB1 (or ZEN). Each pAb sample was repeated three times and the average value was taken.

Among the four groups of mice immunized with four immunogens, the mice with the lowest IC50 value for AFB1 (or ZEN) in each group were selected to evaluate the sensitivity of each pAb.

Specificity assessment The cross-reactions test (CR) as described by Ertekin et al. [207] was employed to assess the specificity of each pAb. According to the CR, AFB1 and its analogs including AFB2, AFG1, AFG2, AFM1, AFM2, and ZEN and its structural analogs including α-ZAL, β-ZAL, α-ZOL, β-ZOL, ZON were selected as inhibitors, respectively. The IC50 values for AFB1 (or ZEN) and other inhibitors were determined by icELISA. The percentage of IC50 value for AFB1 (or ZEN) to IC50 value for each inhibitor was considered the cross-reaction percentage (CR%). The CR% was calculated using the formula: CR (%) = [IC50 (AFB1 or ZEN)/IC50 (AFB1 analogs or ZEN analogs)] ×100%. One sample of

each pAb with the lowest IC50 value for AFB1 or ZEN was selected from the mice immunized with each immunogen for specificity determination. Each sample was repeated for three times and the average value was taken.

2.6 Preparation of AFB1 mAb and ZEN mAb

Four days before cell fusion, the mouse with the highest titer, the lowest IC50 value, and the minimum CR value in each group was administered an intraperitoneal booster injection of 200 μg of corresponding immunogen without any adjuvant. Then, the mouse was sacrificed, and the spleen was harvested to generate hybridomas.

Cell fusion and screening of positive hybridoma cell lines were performed using common operation methods [208, 209] with some modifications. Briefly, the splenocytes were isolated and fused with NS0 myeloma cells at a 10:1 ratio using PEG 1500 as a fusing agent.

The fused cells were put into 96-well culture plates where mouse peritoneal macrophages were prepared as feeder cells from young Balb/c mice the day before and grown with a selective HAT medium. After 10 to 14 days, an inELISA and an icELISA were used to screen the positive hybridoma colonies obtained from supernatants.

The positive clones were then transferred to 24-well plates to culture. After seven days, positive hybridomas were subcloned thrice using the limiting dilution method. The colonies of interest were then frozen in a culture medium containing 10% DMSO, cryopreserved in liquid nitrogen, then defrosted thrice to screen and identify the stable hybridoma cell lines.

The in vivo-induced ascites method was used to produce several AFB1 or ZEN ZEN mAbs [206]. Briefly, a mature female Balb/c mouse was intraperitoneally injected with 0.8 mL of paraffin ten days before receiving an intraperitoneal injection of the positive hybridoma cells (1 to 5×10^6 cells). Ascites

fluid was collected two weeks later. The saturated ammonium sulfate precipitation method was used to purify the AFB1 or ZEN mAb and then stored at −20 ℃ for subsequent analysis.

2.7 Assessment of AFB1 mAb and ZEN mAb

Karyotypes of mAbs. The colchicine blocking method was used to identify Karyotype [210].

Isotypes of mAbs. A commercially available mouse mAb isotype kit was used to determine the class and subclass of each mAb isotype.

Stability of mAbs The cryopreserved hybridoma cells were resuscitated and passaged once every ten days, five times, and an inELISA and an icELISA were used to detect the antibody titers and IC50 values of AFB1 or ZEN in the supernatants at different passages to determine the stability of the hybridoma cells secreting an antibody [211].

Affinity of mAbs. The Batty saturation method was used to determine the affinity, as follows:$Ka = (n-1)/[2(n[Ab']t-[Ab]t)]$, where $n = [Ag]/[Ag']$, [Ag]t and [Ag']t indicate the different concentrations of a coating antigen, and [Ab]t and [Ab']t represent the corresponding 50% Amax value of the AFB1 or ZEN mAb concentration when the coating antigen is of different concentrations [212].

Sensitivity of mAbs. An icELISA was used to determine the IC50 value of the AFB1 or ZEN mAb, and the IC50 value represents the sensitivity of mAbs against analytes [213].

Specificity of mAbs. The method for assessment of the specificity of mAbs was the same as that of pAbs, as described above for assessment of the specificity of AFB1 (or ZEN) pAbs.

2.8 Optimization of ELISA conditions for mAbs evaluation

To promote the performance of the icELISA, a checkerboard titration procedure was employed to determine the optimal dilutions of all corresponding

coating antigens, AFB1 mAbs (or ZEN mAbs) and GaMIgG-HRP, in which the well with an absorbance value of 1.0 at 450 nm was defined as theirs optimal working concentrations for icELISA. The ratio of Amax /IC50 (Amax represents maximal absorbance, and IC50 was calculated via regression equation) was used as the evaluation criteria, and some physicochemical parameters, such as the methanol concentration (5, 10, 20, 30, 40 and 50%, v/v), ionic strength (5, 10, 20, 30, 40 and 50 mM PBS/NaCl), and pH value (5.0, 6.0, 7.0, 7.4, 8.0 and 9.0) of the assay buffer were tested and optimized [214, 215]. The AFB1 (or ZEN) standard stock solution was diluted in 30% methanol-PBS (30:70, v/v) at various concentrations (0.33, 1.0, 3.0, 9.0, 27.0, 81.0, and 243.0.0 μg/L), and a seven-point standard curve of icELISA under the above optimized conditions was achieved by plotting the gradient concentrations (Log C) versus the B/B0% values (proportion of mean absorbance value of the standards (B) divided by that of the zero standards (B0) in triplicate experiments), and then a four-parameter logistic regression equation was deduced and the IC50 was calculated [216, 217].

Preparation and identification of colloidal gold Colloidal gold was prepared by sodium citrate reduction method [31,32], the method was as follows: 198 mL of ultrapure water and 2 mL of 1% chloroauric acid aqueous solution were added to a 250 mL erlenmeyer flask to configure a total volume of 200 mL and a final concentration of 0.01 % chloroauric acid aqueous solution. Then put the chloroauric acid aqueous solution on a heated magnetic stirrer and heated it until the liquid boils. After boiled for 5 minutes, added 1% $Na_3C_6H_5O_7 \cdot 2H_2O$ solution dropwise. The color of the solution changed from light yellow to purple red from light to dark. When it was a transparent wine color, boiled for another 10 min and let it cooled down at room temperature. After the solution was cooled to room temperature, made up to the original volume with ultrapure water, filtered with a 0.22 μm microporous membrane, added sodium azide (NaN_3) with a mass concentration of 0.05%, and stored at 4 ℃ for later use. Colloidal gold was identified by Visual observation, UV scanning and projection electron microscopy scanning [218].

Visual observation method: The color of colloidal gold with different particle sizes was different. As the particle size increased, the color of colloidal gold become darker, ranging from wine red to dark red.

The corresponding color changes of colloidal gold with various particle sizes are shown in Table 2.1.

Table.2.1

The corresponding color changes of colloidal gold with different particle sizes

Colloidal gold particle size (nm)	2-5	5-20	20-40	＞40
Color	orange-yellow	Red wine color	Dark red	Blue

Placed the colloidal gold in a clean glass instrument and observed the color of the colloidal gold with the naked eye. If the color was translucent wine red, the color quality of the colloidal gold was good.

UV scanning method: The different colloidal gold particle size resulted in different UV absorption peak positions, different colloidal gold particle uniformity had different absorption peak width, colloidal gold particle uniformity was good, the absorption peak width was small, and the uniformity was poor, the absorption peak width was large. To Take a certain amount of colloidal gold solution and perform ultraviolet scanning in the wavelength range of 400-600 nm to identify the quality of colloidal gold. The UV absorption of colloidal gold with different particle sizes is shown in Table 2.2.

Table.2.2

Uv absorption wavelength of colloidal gold with different particle sizes

Colloidal gold particle size (nm)	2-5	5-20	20-40	60
maximum absorption wavelength (nm)	516	520	530	600

Transmission electron microscope scanning method: To use H-7650 transmission electron microscope to observe the particle size and shape of colloidal gold. Take 1 drop of colloidal gold and drop it on a nickel mesh pre-covered with formvar and Poly-L-lysine, incubated for 10 min, dried at room temperature, and then observed the particle size, shape, uniformity, etc. of the colloidal gold under a transmission electron microscope. If the size of the colloidal gold was uniform and the shape was regular and spherical, the quality of the colloidal gold was better.

2.9 Preparation of colloidal gold-labeled mAb

Screening of the optimal pH value for labeling The pH value of colloidal gold had a greater impact on the labeled antibody. When the pH value of colloidal gold was equal to the isoelectric point of the antibody, the antibody was in an electrically neutral state, and the electrostatic interaction with colloidal gold was small, and its surface tension was the largest and in the hydrated state, it was easily adsorbed by colloidal gold. A protein layer was formed on the surface of colloidal gold to prevent the contact between colloidal gold and keep the colloidal gold in a stable state. If the pH value of colloidal gold was lower than the isoelectric point of the protein, coagulation will occur. Conversely, when the pH was higher than the isoelectric point of the protein, the protein will not bind to the colloidal gold. In addition, the factors that affect the stability of the gold-labeled antibody were the amount of antibody used [219]. Too little antibody was not enough to keep the colloid stable, and too much antibody will cause unnecessary waste.

500 μL colloidal gold was added to 10 centrifuge tubes (1.5 mL) respectively, then 0.2 mol/L K_2CO_3 solution was added 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 μL to each tube, and 20 μL of AFB1 mAb (or ZEM mAb) with a concentration of 1 mg/mL was added to each tube, and the reaction was allowed to stand for 10 min. Then 30 μL of 10% NaCl was added to each tube and let it stand for 2 h to observe the color change of each tube. Compared with the blank colloidal gold, the lowest pH value corresponding to the color of the solution did not change was the optimal pH value of the colloidal gold. A more accurate

measurement method was the UV scanning method, and the method was as follows. After the antibody was added, the mixed solution with no change in color and no coagulation was centrifuged at 5000 r/min for 10 min, and the supernatant was collected. The inELISA was used to determine the supernatant, and the addition of K_2CO_3 as the abscissa, and the absorption value as the ordinate to draw a graph, the pH value corresponding to the addition of K_2CO_3 when the minimum absorption value appears was the optimum pH value.

Determination of the optimal labeling concentration of colloidal gold and mAb The gold-labeled antibody was prepared by the Mey's series stabilization method [220], which was as follows. Briefly, 50 μL/well of ddw was added to 9 wells of the microplate, started from the first well, 50 μL of AFB1 mAb was added and double diluted to the 8th well in turn, the 9th well was the blank control (BC). Then 50 μL of colloidal gold solution with appropriate pH was added to each well, let stand for 10 min at 37 ℃. Later, 100 μL of 0.1% NaCl solution was added and let stand for 10 min at 37 ℃. After reaction, the color development of each well was observed. Due to the binding reaction between AFB1 mAb and gold particles, when the amount of AFB1 mAb was small and the gold particles were abundant, the color of the solution would change from red to blue, and precipitation would appear. Choose the color of the solution to be red without precipitation, and the actual amount of colloidal gold required for AFB1 mAb was an increase of 20% on this basis. The same method was used to determine the actual amount of colloidal gold required for ZEN mAb.

Preparation and purification of gold-labeled mAb According to the above results, the actual amount of colloidal gold required for AFB1 mAb (or ZEN mAb), mixed colloidal gold and AFB1 mAb (or ZEN mAb), let stand for 0.5 h at 37℃, then 10% BSA (w/v) was added. After following incubation for another 30 min, the mixture was centrifuged at 12,000 r/min for 30 min at 4 ℃, discard the supernatant, and resuspend the precipitate with colloidal gold resuspension, repeated centrifugation once, filtered with 0.45 μm membrane, and stored at 4 ℃ for future use [221].

Selection of resuspension for gold-labeled mAb The gold-labeled antibodies were resuspended with 0.01 mol/L PBS buffer (pH 7.4), 0.01 mol/L Tris-HCl buffer (pH 7.4) and 2 mmol/L $Na_2B_4O_7$ buffer (pH 7.4), and performed high-speed low-temperature centrifugation, to observe the state of the purified gold-labeled antibodies and whether there was precipitation [222].

Selection of preservation solution for gold-labeled mAb To prepare resuspension solution containing 1% BSA and 0.05% NaN_3, resuspension solution containing 10% sucrose and 0.05% NaN_3, and resuspension solution containing 1% BSA, 10% sucrose, and 0.05% NaN_3, respectively, then used them as a gold-labeled antibody storage solution, stored the gold-labeled antibody at 4°C, recorded the storage time and the parameter changed of the prepared test strip, selected the group with a longer storage time and less changes in the parameters of the test strip as gold Label antibodies preservation solution [222].

2.10 Optimization and assembly of test strip

Selection of nitrocellulose membrane Since different nitrocellulose (NC) membrane had different pore diameters, flow rates and other properties, it was necessary to test NC membranes of different manufacturers and specifications on the current market in order to screen the most suitable NC membrane for this experiment. Millipore 135, Millipore 180, Prima 40, Prima 85, AE 99, CN 140, HF 135, HF 180, Immunopore FP, and PALL 170 were 10 kinds of commonly used NC membranes were tested. by comparing the migration rate, whether there were flow obstacles, whether there was a detection line for dispersion, color depth of detection line, etc., select the best NC film for this experiment [223, 224].

Selection of the gold-labeled pad The quality of the gold-labeled pad had a vital impact on the entire colloidal gold detection system. 4 kinds different gold-labeled pads, including SB06, SB08, 8964, and 6613, were selected for testing. According to the release of gold-labeled antibodies of different gold-labeled pads,

whether in case of precipitation, selected the best gold-labeled pad for this experiment.

Selection of the sample pad SB06 and SB08 glass fiber were chosen as the candidate sample pads, and extracted the diluent by dripping the negative sample extraction diluent and the positive spiked sample to extract the diluent, and selected the optimal sample pad according to the extraction effect.

Treatment of sample pad and gold-labeled pad To cut the glass fiber cotton into two specifications of 300×7.5mm and 300×15mm respectively, soak the glass fiber cotton of 300×7.5 mm in the gold-labeled pad treatment solution, and dried at 42 ℃ for 1 h, and the pad was gold-labeled pad for use. 300×15mm glass fiber cotton was soaked in the sample pad treatment solution and dried at 45 ℃ for 4 h, and the pad was the sample pad for use.

Selection of optimal concentration combination of gold-abeled antibody, coated antigen and secondary antibody First, to determine the working concentration of the mouse-derived secondary antibody for the NC membrane C line, selected goat (or rabbit) anti-mouse IgG (GaMIgG or RaMIgG) as the secondary antibody, configured the working concentration with PBS to 1.0 mg/mL, and coated it on the C line of the NC membrane. Then configured the T line with PBS to detect the antigen AFB1-BSA concentration in order of 2.0, 1.0, 0.5, 0.25, 0.125, 0.625 mg/mL, coated on the T line of the NC membrane. While configured the gold-labeled AFB1 mAb with the gold-labeled antibody preservation solution, and the dilution ratio of AFB1 mAb was 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32, sprayed on the gold label pad. The square matrix method was used to select the best concentration combination, and AFB1 standard was use as a positive control, and the concentration that can obtain the best sensitivity of the test strip was selected as the best working concentration of AFB1-BSA and gold-labeled AFB1 mAb. Similarly, the best working concentration of ZEN-BSA and gold-labeled ZEN mAb was selected.

Assembly of test strip The test strip was generally composed of a PVC plastic bottom plate, a NC membrane, a colloidal gold pad, a sample absorption

pad, and an absorbent pad. The PVC bottom plate was used to provide an assembly platform, and the NC membrane had a migration rate effect. C line coated with secondary antibody, T1 line (AFB1) and T2 line (ZEN) coated with two antigens AFB1-BSA and ZEN-BSA, respectively. The colloidal gold pad was coated with gold-labeled AFB1 mAb and gold-labeled ZEN mAb (The ratio of gold-labeled ZEN mAb and gold-labeled ZEN mAb was 1:1 mixing), and the sample pad provided the place where the sample to be tested is added. When assembling, the joints of each part should overlap by 2-3mm. The assembly diagram was shown in

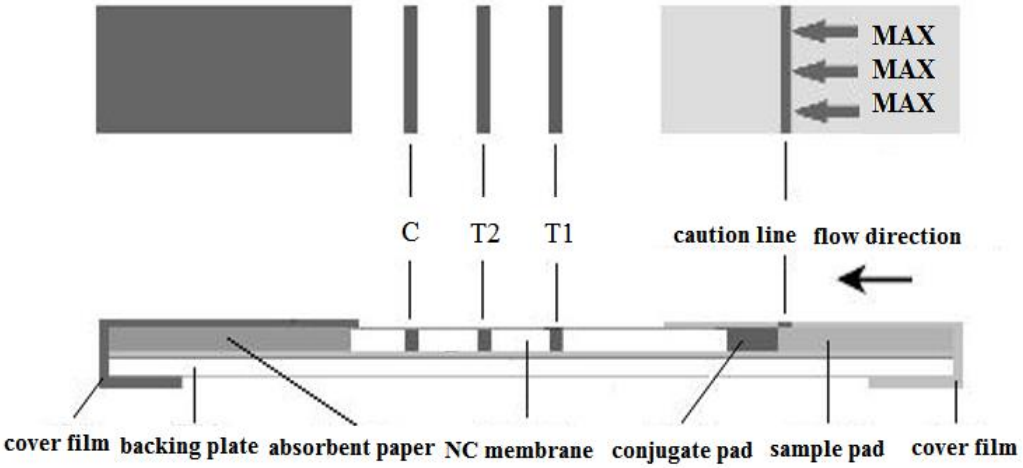


Figure 2.12.

Figure 2.12 The plane schematic diagram of dual test strip.

2.11 Optimization of sample processing methods

Selection of extraction solvent. To compare the extraction effect of ethyl acetate, acetonitrile and methanol. 1.0 g of corn flour sample was added into a centrifuge tube (10 mL), 5 mL of extractant was added, vortexed for 5 min, centrifuged at 4000 r/mim for 2 min. Then 100 μL of the supernatant was added into a centrifuge tube (1.5 mL), 400 μL of 0.2 mol/L PBS (pH 7.4) was added and mixed well for later use.

Selection of extraction time 1.0 g of corn flour sample was added into a centrifuge tube (10 mL), 5 mL of extractant was added to the centrifuge tube, and

vortexed for 3 min, 5 min and 7 min, respectively, and centrifuged at 4000 r/min for 2 min. Then 100 μL of the supernatant was added into a centrifuge tube (1.5 mL), then 400 μL of 0.2 mol/mL PBS (pH 7.4) was added and mixed well for later use.

Judgment of test strip test results The test strip test results had different judgment methods and different judgment results. Two methods were commonly used. One was visual inspection without any instrument, which could realize semi-quantitative detection, and the other was scanning with Bio Dot-TSR3000 strip reader. The color gradation of the T-line of the test paper could be quantitatively detected [225, 226].

(1) Visual inspection The test strip was used to detect the sample, the red band appeared on the detection line T1 and T2 at the same time, and the sample was negative, i.e., the content of AFB1 and ZEA in the sample did not exceed the detection limit. If T1 developed color but T2 did not developed color, the sample was single positive, i.e., the ZEA content in the sample exceeded the detection limit. If T1 did not develop color and T2 developed color, the sample was single positive, i.e., the AFB1 content in the sample exceeded the detection limit. If T1 and T2 were not colored, the sample was double positive, i.e., the sample was both AFB1 and ZEA content exceeded the detection limit. As shown in Figure 2.13.

(2) Strip reader scanning method To scan the T line of test strip with the Bio Dot-TSR3000 strip reader to obtain the relative optical density (ROD) curve of the T1 line and the T2 line and the peak area G/D×A of the optical density curve. The test paper detected the standard samples with different AFB1 (or ZEN) concentrations set to obtain the corresponding G/D×A value. To be taking the log value of the AFB1 (or ZEN) standard concentration as the abscissa, and the G/D×A value (B) measured under different AFB1 (or ZEN) standard concentration and G without AFB1 (or ZEN) standard The ratio of /D×A value (B0) was the ordinate (B/B0), drew the standard curve of the test strip, and fitted the regression equation, i.e., the test strip could quantitatively detect the concentration of AFB1 (or ZEN) in the sample.

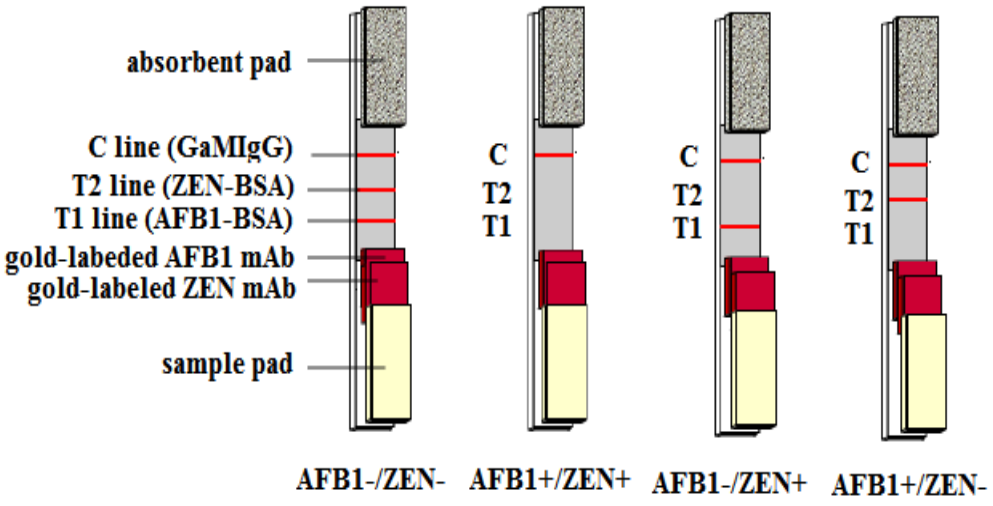


Figure 2.13 Schematic diagram of AFB1 and ZEN by dual test strip.

2.12 Measurement of test strip performance

The LOD and detection range of dual test strips [227] (1) The LOD and detection range of the visual method To prepare AFB1/ZEN mixed standard solutions with sample extracts, the final concentrations were at 0/0, 0.3125/3.125, 0.625/6.25, 1.25/12.5.0, 2.5/25.0, 5.0/50.0, 10.0/100.0, 20.0/200.0 ng/m L. 200μL/well was added into the microplate, and the dual test strips were used to detect the two toxins at the same time. Repeated 6 times for each concentration to determine the LOD and detection range of the test strips. (2) The LOD and detection range of the scanning method of the strip reader The Bio Dot-TSR3000 strip reader was enployed to scan the T1 line and T2 line of the test strips, and used the peak area G/D×A data of the optical density curve to draw a standard curve. When G/D×A was inhibited by 10%, the corresponding target concentration was determined as the LOD of the test strip, i.e., the LOD of the test strip. The detection range was calculated according to the deduction formula.

The specificity of dual test strip AFB1 and its analogs including AFB2, AFG1, AFG2, AFM1, AFM2 (or ZEN and its analogs including α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZON) and other mycotoxins including DON, OTA, T-2 toxin were selected as competitors, and the standard solutions with concentrations of 1.0, 2.0,

4.0, 8.0, 16.0, 32.0, and 64.0 ng/mL with the sample extract were prepared. 200 μL/well of each standard solution was added into the microplate, and used the dual test strip to simultaneously detect the above-mentioned competitors. Repeated 6 times for each concentration to test the specificity of the dual test strip [228].

Reproducibility of dual test strip 6 batches of dual test strip were from different batches, and were used to test the concentrations of the AFB1/ZEN mixed standard solution at 0/0, 0.3125/3.125, 0.625/6.25, 1.25/12.5.0, 2.5/25.0, 5.0/50.0, 10.0/100.0, 20.0/200.0 ng/mL, respectively, and each concentration was repeated 6 times. The test results were used to determine the reproducibility of the dual test strip.

Determination of accuracy and precision of dual test strip The additive recovery test was usually used to evaluate the accuracy of the test strip. The AFB1 and ZEN standard solution were added to the crushed corn samples to make the final concentrations of 2.0, 4.0, 8.0 ng/g and 5.0, 10.0, 20.0 ng/g respectively, stirred well, and then processed the samples according to the sample treatment method and tested with dual test strip. Each concentration was repeated 10 times, the recovery rate of dual test strip was measured to evaluate its accuracy, and the coefficient of variation (CV) was calculated to evaluate the precision of the dual test strip [229].

Determination of the validity period of dual test strip Due to the good stability of the dual test strip, the validity period was 12 months when stored in an ordinary refrigerator (2 to 8 ℃), and the validity period was 6 months when stored in a dry condition at room temperature (25 ℃). In order to shorten the test strip storage validity period, this test used accelerated stability test to evaluate the stability of the dual test strip.

The dual test strips were stored at 45 ℃. The test was based on the Arrhenius formula to calculate different temperatures and accelerated test days. The relationship between storage at 45 ℃ for 40 d was equivalent to storage at room temperature 25 ℃ for 1 year. Therefore, in this experiment, the same batch of dual test strips were stored in the dark at 45 ℃, and dual test strips were tested

on the 0th, 10th, 20th, 30th, 40th, and 50th d, and the dual test results of different storage time were observed to determine the quality [230].

False positive rate and false negative rate of dual test strip The dual test strip was used to detect 20 positive samples (AFB1≥5 ng/mL, ZEN≥50 ng/mL) and 20 negative samples (AFB1≤5 ng/mL, ZEN≤50 ng/mL), calculated the false positive rate and false negative rate of the test strip, the calculation formula is as follows, false positive rate (%) = number of detected positives/20×100. False positive rate (%) = number of detected negatives/20×100.

2.13 Comparative analysis of the results of dual test strip and HPLC-MS/MS

30 samples were obtained from the Agricultural Quality Standards and Testing Technology Research Center of Henan Academy of Agricultural Sciences, including 10 corn samples, 10 peanut samples and 10 feed samples. The detection targets were AFB1 and ZEN, and HPLC-MS/MS was attached. The test results were tested with the developed AFB1 and ZEN dual test strip, and each sample was tested repeatedly for 3 times to obtain the average value. The concentrations detected by HPLC-MS/MS in each sample were used as the abscissa, and drew a scatter plot with the concentration detected by dual test strip as the ordinate to generate a regression curve, and the R^2 of the regression curve was used to evaluate the two detection methods. The relevance of the test results was used to evaluate the reliability of the dual test strip [231, 232]. Meanwhile, the author collected 20 corn samples, 20 peanut samples and 20 feed samples from the market, and tested them with dual test strip and HPLC-MS/MS, and determined the coincidence rate according to the test results.

Data statistics and image processing Origin Pro 2018 (OriginLab corparation, Northampton, USA) and Excel software (Microsoft Corparation, Redmond, WA, USA) were used for plotting the standard curves and the data analysis. ChemSketch 12.0 (Advanced Chemical Development Co., Ltd, Victoria, Canada) was used to sketch the chemical formulas.

CHAPTER 3
RESULTS OF OWN RESEARCH

3.1 Identification of AFB1 and ZEN immunogens

3.1.1 Identification of AFB1 immunogens

3.1.1.1 UV identification

The results were shown in Figure.3.1. In the range of 200 to 500 nm, the characteristic peak of BSA was at 278 nm and the characteristic peak of AFB1 was at 363 nm. The immunogens AFB1-BSA synthesized by OAE, MOA, MA, SA, EP and EED methods all contained the characteristic peaks of BSA and AFB1, indicating that the immunogens AFB1-BSA could be synthesized by the above six methods. The calculation results of molecular binding ratio of BSA and AFB1 were shown in Figure Table.3.1.

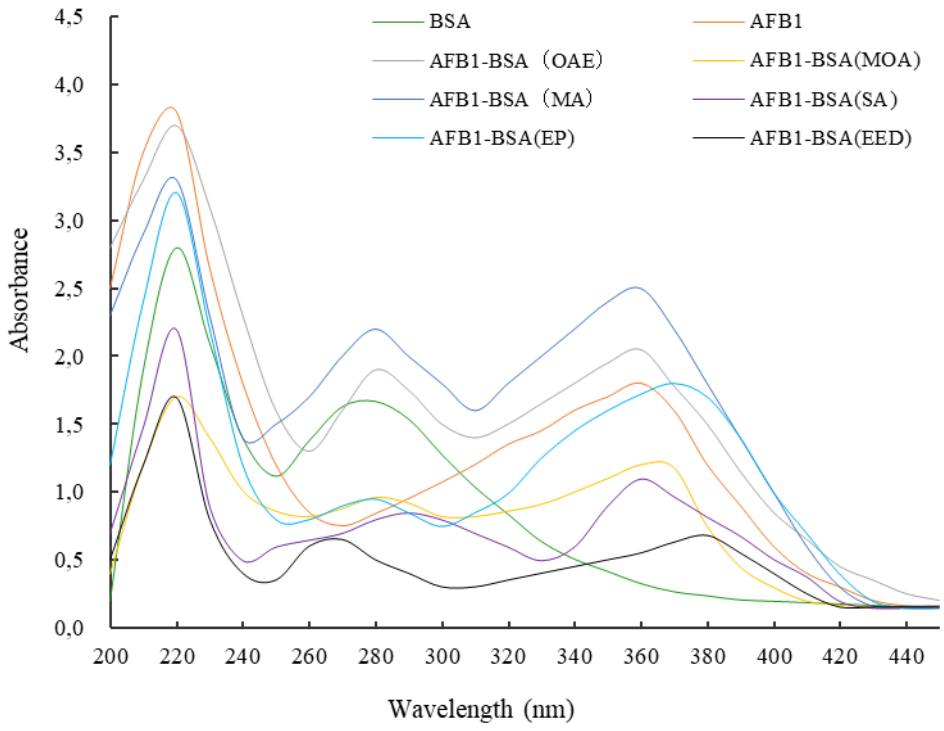


Figure.3.1 UV spectra of BSA, AFB1 and AFB1-BSA synthesized by different coupling methods.

Table.3.1

Molecular binding ratio of AFB1-BSA prepared by six methods

Synthesis methods	Initial molar ratio of AFB_1 to BSA	Molecular binding ratio of AFB_1-BSA	Usage ratio of AFB_1 (%) [a]
OAE	50:1	8.64:1	17.28
MOA	50:1	6.88:1	13.76
MA	50:1	10.78:1	21.56
SA	50:1	4.46:1	8.92
EP	50:1	6.38:1	12.76
EED	50:1	2.31:1	4.62

Note: [a] Compared to the molecular weight of BSA and AFB_1, BSA is 66.446, AFB1 is 312, BSA is much larger than AFB_1, so the utilization rate of BSA is 100% when the utilization ratio is calculated.

3.1.1.2 SDS-PAGE identification

The results were shown in Figure.3.2. The bands of six immunogens AFB1-BSA lagged behind those of BSA, indicating that the molecular weight of AFB1-BSA was greater than that of BSA, so it could be judged that the synthesis of AFB1-BSA was successful.

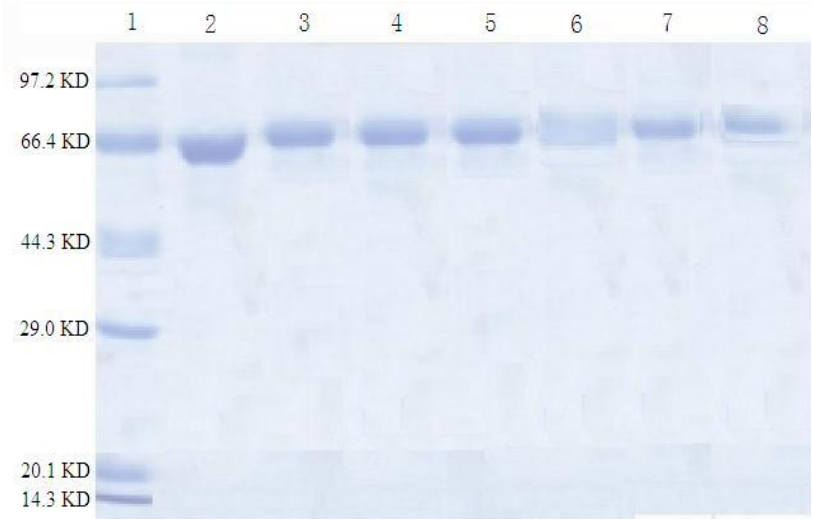


Figure.3.2 The SDS-PAGE photo of AFB1-BSA immunogens. 1.Maker; 2.BSA; 3. AFB1-BSA(OAE); 4. AFB1-BSA(MOA); 5. AFB1-BSA(MA); 6.AFB1-BSA(SA); 7. AFB1-BSA(EP); 8. AFB1-BSA(EED).

3.1.1.3 Analysis of immunological characteristics of AFB1 pAbs

(1)Titers measurement. The results of titers measurement were shown in Figure.3.3. After 5 rounds immunization, one mouse with the highest titer was selected from each group of 5 mice, and a total of 6 mice were selected. The titers were measured and compared by indirect noncompetitive ELISA (inELISA), and the titers reached 1:(1.6×10^3), which showed that the immunogen AFB1-BSA synthesized by the six methods all had good immunogenicity. The evaluation the immunoreactivity of the 6 groups of immunogens according to their titers. According to the strength of the immune response, the order was OAE, MA, MOA, SA, EP and EED. The OAE group and MA group had the best immune response effect, and the titers reached 1: (6.4×10^3), respectively.

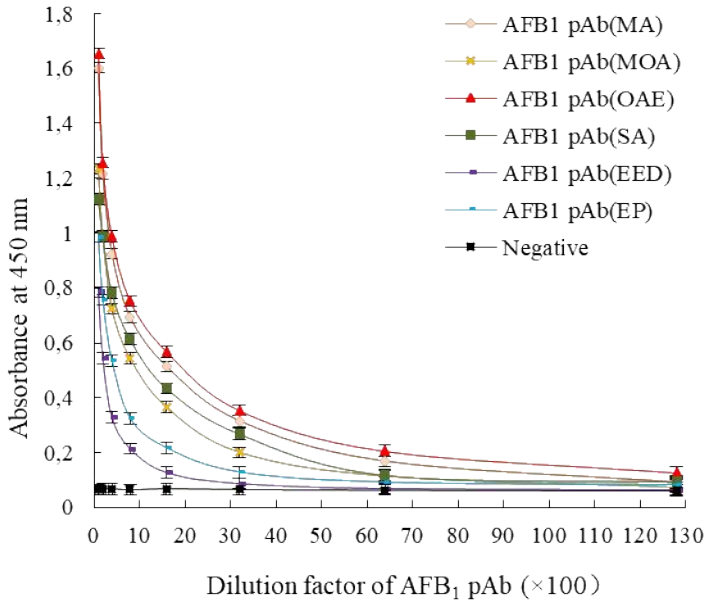


Figure.3.3 The titers of AFB1 pAbs were measured by inELISA. Each point represents the mean of three replicates (n = 3).

(2) Sensitivity measurement

The results were shown in Figure.3.4 The indirect competitive ELISA (icELISA) inhibition curve of 6 immunized mice had a good linear relationship. The OAE group had the best sensitivity, with an IC50 of 10.14 μg/L. The

sensitivities of the other groups were inferior to that of the OAE group. The regression equation, R^2 value and IC50 value of AFB1 pAb to AFB1 icELISA determination curve were shown in Table.3.2.

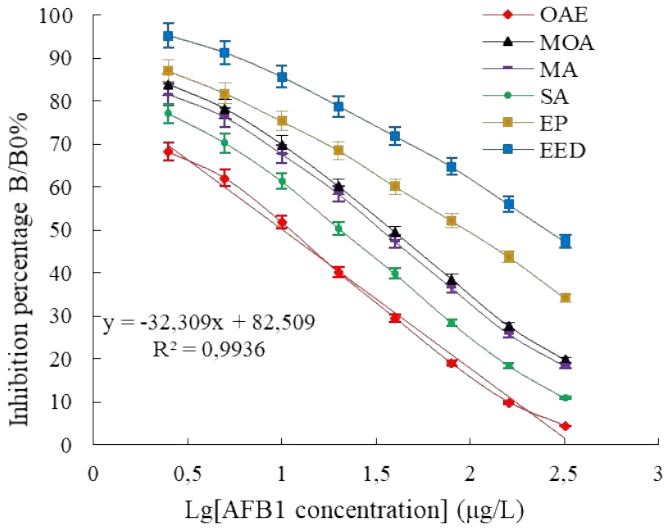


Figure.3.4 The sensitivity measurement of AFB1 pAb to AFB1 by icELISA. The values indicate the mean of three independent assays (n = 3).

Table.3.2

The regression equation, R^2 and IC_{50} of AFB1 pAb to AFB1 by icELISA

Group	Regression equation	R^2 value	IC50 (μg/kg)
OAE	y=－32.309x＋82.509	0.9936	10.14
MOA	y=－31.822x＋99.59	0.9943	36.18
MA	y=－31.546x＋97.263	0.9938	31.49
SA	y=－32.875x＋92.292	0.9966	19.36
EP	y=－25.245x＋99.481	0.9932	91.21
EED	y=－22.979x＋107.8	0.9894	307.81

(3) Specificity measurement

The results were shown in Table.3.3. The antibodies prepared by the six methods could recognize AFB1 100%, and the OAE method had better specificity

and sensitivity with an IC50 of 10.14 μg/kg and a CR of 86.46% with AFB2, and the CR of AFG1 and AFG2 were 44.13% and 14.72%, respectively. The antibodies prepared by other methods had good specificity and could recognize AFB1 100%, but their sensitivity is not as good as the antibodies prepared by the OAE method. The results showed that the best antigen synthesis method for preparing antibodies against AFB1 with high sensitivity and strong specificity was the OAE method.

Table.3.3

The percent cross-reactivity of AFB1 pAb with AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2

AFs	AFB_1 pAb(OAE)		AFB_1 pAb(MOA)		AFB_1 pAb(MA)		AFB_1 pAb(SA)		AFB_1 pAb(EP)		AFB_1 pAb(EED)	
	IC_{50} (μg/kg)	CR (%)	IC_{50} (μg/kg)	CR (%)	IC_{50} (μg/kg)	CR (%)	IC_{50} (μg/kg)	CR (%)	IC_{50} (μg/kg)	CR (%)	IC_{50} (μg/kg)	CR (%)
AFB1	10.14	100	36.18	100	31.49	100	19.36	100	91.21	100	307.81	100
AFB2	16.56	61.23	46.03	78.61	46.85	67.22	23.83	81.26	144.14	63.28	590.13	52.16
AFG1	23.39	43.35	＞1000	＜0.5	＞1000	＜0.5	35.67	54.27	＞1000	＜0.5	＞1000	＜0.5
AFG2	70.11	14.72	＞1000	＜0.5	＞1000	＜0.5	79.41	24.38	＞1000	＜0.5	＞1000	＜0.5
AFM1	63.08	16.08	741.39	4.88	615.04	5.12	541.74	3.68	＞1000	＜0.5	＞1000	＜0.5
AFM2	716.67	1.42	＞1000	＜0.5	＞1000	＜0.5	＞1000	＜0.5	＞1000	＜0.5	＞1000	＜0.5

3.1.2 Identification of ZEN immunogens

3.1.2.1 UV identification

In the range of UV 220–450 nm, BSA had characteristic absorption peaks for UV at 278 nm, and ZEN had characteristic absorption peaks for UV at 236 nm, 274 nm, and 316 nm, respectively (Figure.3.5). The immunogens ZEN-BSA synthesized via the five methods (OAE, CMA, FA, BDE, and AGA) all showed the haracteristic absorption peaks of BSA and ZEN, or the characteristic absorption peaks were shifted. Thus, the above four methods could synthesize the artificial immunogen ZEN-BSA. According to the Lambert-Beer law, the formula A=εCL (where: A denotes the absorbance value read by the instrument; ε denotes the molar extinction coefficient, which is a constant value; C denotes the solute concentration in the solution; L represent the optical path, as determined by the

instrument), was applied to calculate the molecular binding ratio of ZEN and BSA (see results in Table.3.4).

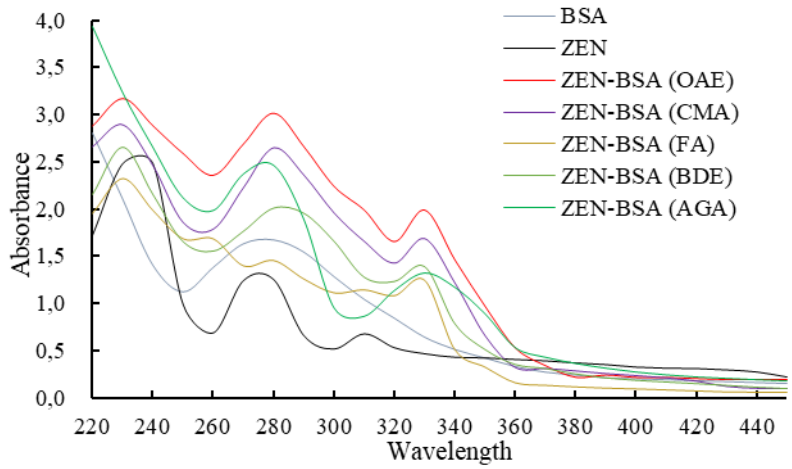


Figure.3.5 UV spectra of ZEN-BSA synthesized via the five methods.

Table.3.4

Molecular binding ratio of ZEN-BSA prepared via the five methods

Synthesis methods	Initial molar ratio of ZEN to BSA	Molecular binding ratio of ZEN-BSA	Usage percentage of ZEN* (%)
OAE	50:1	17.2:1	34.4
CMA	50:1	14.6:1	29.2
FA	50:1	9.7:1	19.4
BDE	50:1	8.3:1	16.6
AGA	50:1	11.6:1	23.2

* Since the molecular weight of BSA (66446) was much larger than that of ZEN (318.36), the utilization rate of BSA was set to 100% when calculating the utilization rate of BSA and ZEN.

3.1.2.2 SDS-PAGE identification

All the electrophoretic bands of the artificial immunogen ZEN-BSA synthesized via the four methods were lagging behind the BSA bands, indicating that the molecular weight of ZEN-BSA was greater than BSA. This proved the successful synthesis of ZEN-BSA (Figure.3.6). Detected by the gel imaging system, the molecular weight of BSA was 66446 Da, whereas the molecular weight of ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) was 71803 Da, 70994 Da, 69482 Da, and 699023 Da, respectively. The molecular binding ratios of ZEN and BSA in ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE) were 17.17:1, 14.57:1, 9.73:1, and 8.26:1, respectively, which concurred with the UV identification results.

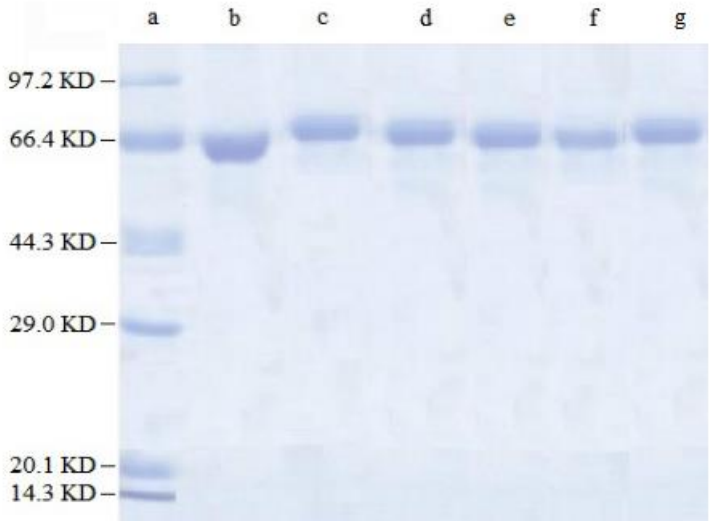


Figure.3.6 SDS-PAGE image of ZEN-BSA synthesized via the four methods. a: Maker; b: BSA; c: ZEN-BSA(OAE); d: ZEN-BSA(CMA); e: ZEN-BSA(FA); f: ZEN-BSA(BDE); g: ZEN-BSA (AGA).

3.1.2.3 Analysis of immunological characteristics of ZEN pAbs

(1) Titers measurement The results of titers measurement were shown in Figure.3.7. After five round immunization, one immunized mouse with the highest inELISA titers from each group was selected for comparative analysis. The inELISA titers of immunized mice with ZEN-BSA (OAE), ZEN-BSA (CMA),

ZEN-BSA (FA), ZEN-BSA (BDE), and ZEN-BSA (AGA) were 1:(6.4×10^3), 1:(6.4×10^3), 1:(1.6×10^3), 1:(1.6×10^3), and1:(6.4×10^3), respectively, all of which attained beyond 1:(1×10^3) (Figure 9). Following the above results, the five immunogens synthesized showed satisfactory immunogenicity. According to the inELISA titers, the immune effects of the four immunogens were evaluated in the order of ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (AGA), ZEN-BSA (BDE), and ZEN-BSA (FA).

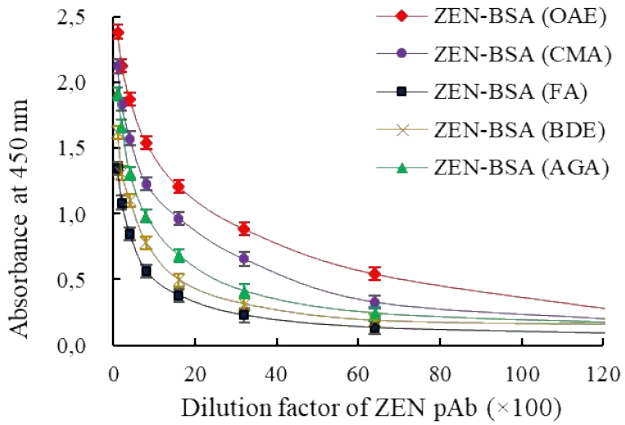


Figure.3.7. The titers of ZEN pAbs were measured by inELISA. Each point represents the mean of three replicates (n = 3).

(3) Sensitivity measurement The icELISA curves of the five immunized mice with the highest inELISA titers selected by each group showed a good linear relationship (Figure.3.8).

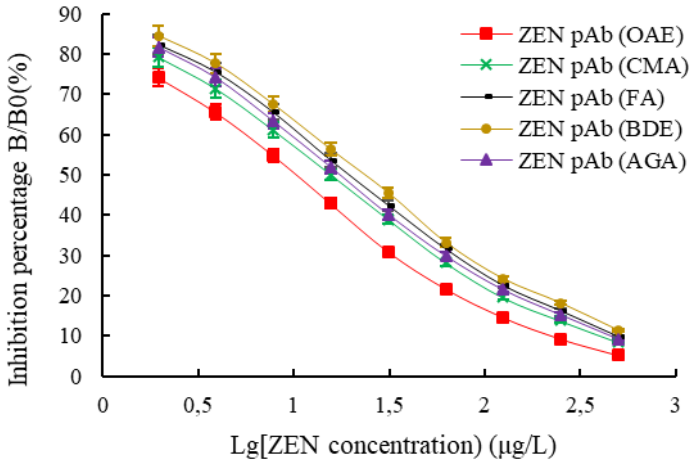


Figure.3.8. Sensitivity measurement of ZEN pAb to ZEN via icELISA. The values indicate the mean of three independent assays (n = 3).

The regression equation, R^2 value, and IC50 value of the inhibition curve are shown in Table.3.5. The IC50 value for ZEN by icELISA of each immunogen ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), ZEN-BSA (BDE), and ZEN-BSA (AGA) were 10.34 μg/L, 16.29 μg/L, 20.92 μg/L, 24.36 μg/L, and 12.77 μg/L, respectively. Using IC50 value for ZEN, in evaluating the immune effect of the four artificial immunogens, the order was reported as follows: ZEN-BSA (OAE), ZEN-BSA (AGA), ZEN-BSA (CMA), ZEN-BSA (FA), and ZEN-BSA (BDE).

(4)

Table.3.5

The regression equation, R^2 and IC50 value for ZEN via icELISA

Group	Regression equation	R^2	IC50 (μg/L)
ZEN-BSA (OAE)	y=−30.294x+80.733	0.9792	10.34
ZEN-BSA (CMA)	y=−31.071x+87.658	0.9895	16.29
ZEN-BSA (FA)	y=−31.894x+92.116	0.9918	20.92
ZEN-BSA (BDE)	y=−32.173x+94.599	0.9922	24.36
ZEN-BSA (AGA)	y=−31.678x+90.341	0.9899	18.77

(2) Specificity measurement ZEN pAb produced by immunizing mice with ZEN-BSA (OAE), ZEN-BSA (CMA), ZEN-BSA (FA), ZEN-BSA (BDE), and ZEN-BSA (AGA) could all recognize ZEN 100% (Table.3-6). The ZEN pAbs from the mice immunized with ZEN-BSA (OAE) and ZEN-BSA (CMA) had similar characteristics, i.e., they had class broad-specificity for ZEN and its analogs.

The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (OAE) was 10.34 μg/L, and the CR values of α-ZAL, β-ZAL, α-ZOL, β-ZOL, and ZON were 36.53%, 16.98%, 64.33%, 20.16%, and 10.66%, respectively. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (CMA) was 16.29 μg/L, and the CR values of α-ZAL, β-ZAL, α-ZOL, β-ZOL, and ZON were 23.55%, 12.18%, 51.86%, 18.62%, and 9.64%, respectively. In terms of class broad-specificity, the ZEN pAb from mice immunized with ZEN-BSA (OAE) was more prominent.

Whereas, the ZEN pAbs from the mice immunized with ZEN-BSA (FA) and ZEN-BSA (BDE) exhibited similar characteristics, i.e., they had better specificity for ZEN but with poor class broad-specificity for ZEN analogs. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (FA) was 20.92 μg/L, and the CR values of α-ZAL, β-ZAL, α-ZOL, β-ZOL, and ZON were all less than 1%. The IC50 value for ZEN by icELISA of the ZEN pAb from the mice immunized with ZEN-BSA (BDE) was 24.36 μg/L, except for the higher CR (36.57%) with ZON, and the CR values of α-ZAL, β-ZAL, α-ZOL, and β-ZOL were both less than 3%. In terms of specificity, the ZEN pAb from mice immunized with ZEN-BSA (FA) was more prominent. Surprisingly, the IC50 value for ZEN by icELISA of the ZEN pAb derived from the mice immunized with ZEN-BSA (AGA) was 18.77 μg/L, and the CR values of α-ZAL, β-ZAL, α-ZOL, β-ZOL, and ZON were all less than 5%. The results demonstrated that the preparation of the class broad-specificity antibodies for ZEN and its analogs can be achieved by immunizing animals with the immunogen ZEN-BSA prepared by OAE method, while the preparation of highly specific antibodies for ZEN could be achieved by immunizing animals with the immunogen ZEN-BSA prepared by the AGA method.

Table 3.6

The cross-reaction of ZEN pAb with ZEN and its analogs

Compound	ZEN pAb (OAE)		ZEN pAb (CMA)		ZEN pAb (FA)		ZEN pAb (BDE)		ZEN pAb (AGA)	
	IC50 (μg/L)[a,b]	CR (%)[a]	IC50 (μg/L)[a,b]	CR (%)[a]	IC50 (μg/L)[a,b]	CR (%)[a]	IC50 (μg/L)[a,b]	CR (%)[a]	IC50 (μg/L)[a,b]	CR (%)[a]
ZEN	10.34	100	16.29	100	20.92	100	24.36	100	18.77	100
α-ZAL	31.95	32.36	69.17	23.55	2682.05	0.78	1441.42	1.69	1268.24	1.48
β-ZAL	68.73	15.04	133.74	12.18	2582.72	0.81	2511.34	0.97	1380.15	1.36
α-ZOL	18.14	57.0	31.41	51.86	3670.18	0.57	990.24	2.46	525.77	3.57
β-ZOL	57.89	17.86	87.49	18.62	4358.33	0.48	1331.15	1.83	1137.58	1.65
ZON	109.48	9.13	168.98	9.64	2273.91	0.92	66.61	36.57	386.21	4.86

Note. [a] All of the data were calculated from triplicate assays. [b] The compound standard solution was prepared in 70% methanol-PBS (7:3, *v/v*).

3.2 Preparation and assessment of AFB1 mAbs and ZEN mAbs

3.2.1 Preparation and assessment of AFB1 mAbs

3.2.1.1 Screening of immunogens and immunization method

Based on the purpose of this study and the results of immunogen identification and immunological characteristics analysis of AFB1pAb, the OAE method was selected to prepare immunogen AFB1-BSA, and the Balb/c mice were inoculated with method of small dose (30 μg/head), long interval (4 weeks) and two routes (subcutaneous injection on the back and intraperitoneal injection), and generated high sensitivity and high specificity AFB1 mAbs desired.

3.2.1.2 Selection for potential cell fusion in immunized mice

An inELISA was carried out to measure the titers of AFB1 pAbs from immunized five mice (as shown in Figure.3.9), all of them had a better positive immune response with high titers (>103), whereas the No.1 mouse (M1) had the most efficient immune response with titers of 1:(1.28 × 104).

An icELISA was adopted to measure the IC50 values (Figure.3.10) and CRs of AFB1 pAbs to AFB1 and its analogs including AFB2, AFG1, AFG2, AFM1, and AFM2 (Table.3.7).

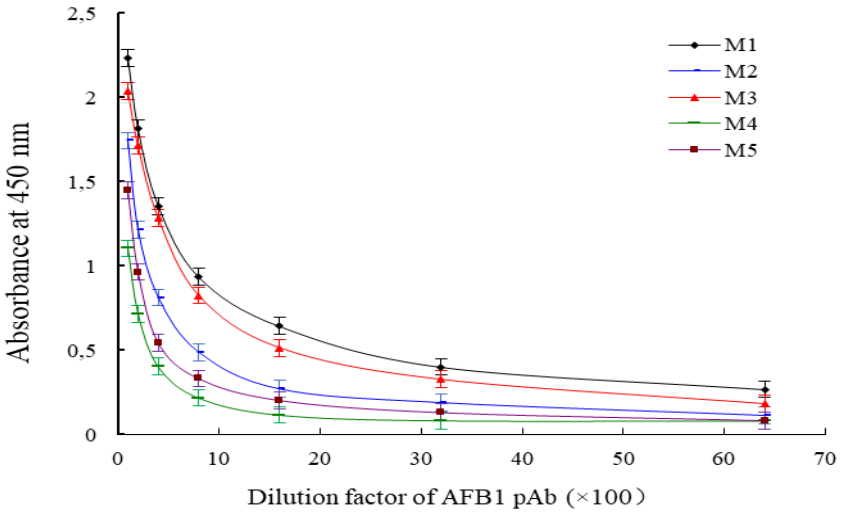


Figure.3.9. The titer curves of AFB1 pAb determined by an inELISA.

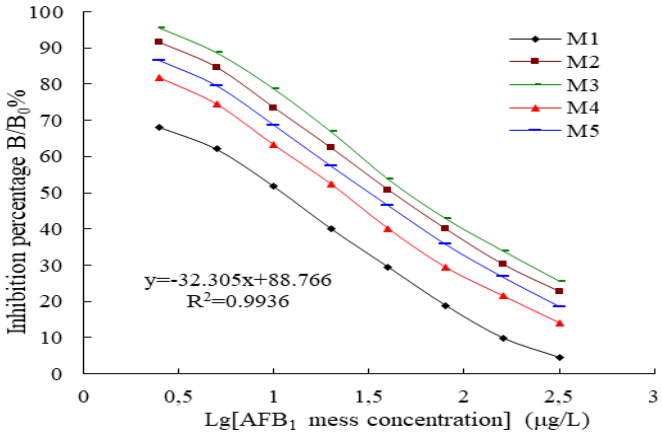


Figure.3.10. The inhibitory curves of AFB1 pAb against AFB1 dermined by an icELISA.

The results showed that ZEN pAbs from the mouse M1 had the lowest IC50 value (10.14 μg/L) and the smallest CRs with AFB1 analogs. Due to M1 mice performed the best and had the strongest immune response, the lowest IC 50 values

and the smallest CRs values, so that the M1 mouse was chosen for the cell fusion experiment.

Table.3.7

The cross-reactivity of AFB1 pAbs to AFB1 and its analogs

Compound	M1		M2		M3		M4		M5	
	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]
AFB1	10.14	100	45.02	100	57.23	100	23.30	100	33.62	100
AFB2	160.44	6.32	605.11	7.44	837.92	6.83	323.16	7.21	544.01	6.18
AFG1	269.68	3.76	1092.72	4.12	2127.51	2.69	626.34	3.72	836.31	4.02
AFG2	＞1000	＜1.0	＞1000	＜1.0	＞1000	＜1.0	＞1000	＜1.0	＞1000	＜1.0
AFM1	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1
AFM2	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1	＞5000	＜0.1

Note. a All of the data were calculated from triplicate assays. b The compound standard solution was prepared in 70% methanol-PBS (7:3, v/v).

3.2.1.3 Screening of positive clones and establishment of hybridoma cell lines

On the 5th day after cell fusion, the fusion effect was observed. Among the 384 wells of the four microplates, 322 clones were observed, and the fusion rate was 83.9%. The titers of AFB1 antibodies in culture supernatants were measured by an inELISA, 51 positive wells were detected, and the positive rate was 15.86%. Of 51 positive wells, 10 strong positive and obvious inhibitive wells were screened by an icELISA, and calculated the strong positive rate was 19.6%. After subcloning three times and expanded cultivation, three hybridoma cells were screened out, named 2A11, 2F6 and 3G2 respectively, and subsequently identified. The results of cell fusion and positive hybridoma screening were shown in Figure.3.11.

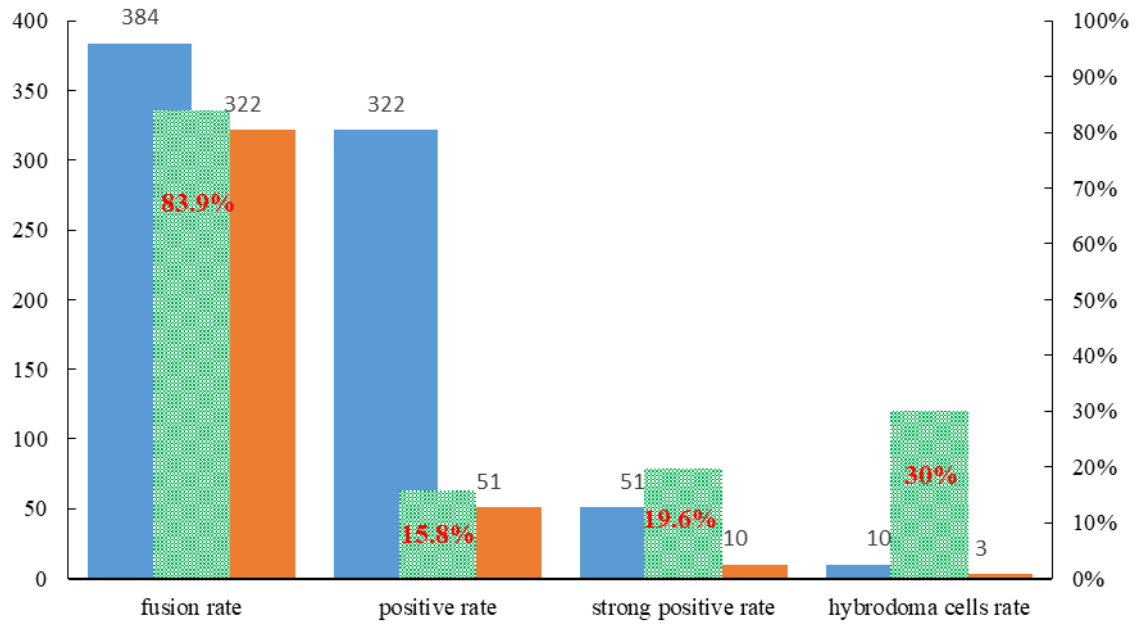


Figure.3.11. The results of cell fusion and positive hybridoma screening.

3.2.1.4 Assessment of immunological characteristics of AFB1 mAbs

(1) Karyotype The chromosomes of myeloma NS0 cells were between 62 and 68, and those of mouse spleen cells were 40. However, hybridoma cell chromosomes were between 96 and 102 (an average of 98.4), indicating that the cell lines fused by the two parents were the hybridoma cells since their chromosomes were more than those of the individual parents (Figure.3.12).

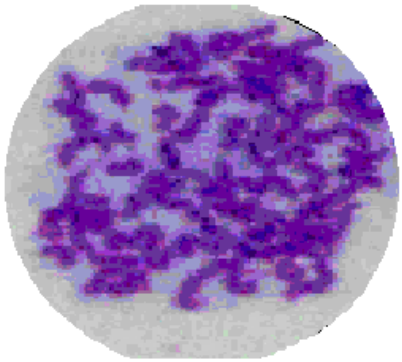


Figure.3.12. The chromosome of hybirdoma cell.

(2) Isotype Using a mouse mAb isotyping kit, 2A11 and 2F6 mAbs were determined to be of the IgG1 isotype possessing a kappa light chain, and 3G2 mAb was to be of the IgG2a isotype possessing a lambda light chain (Figure.3.13).

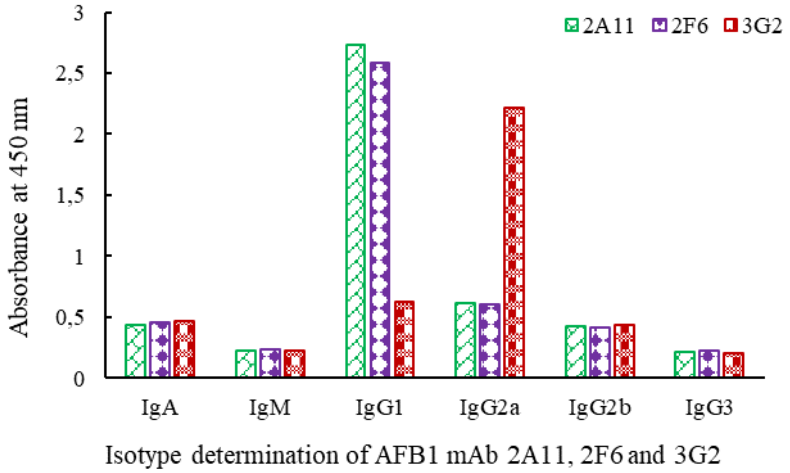


Figure.3.13 Isotype determination of AFB1 mAb 2A11, 2F6 and 3G2.

(3) Affinity The antibody affinity reflects its binding strength with an antigen and the affinity constant (Ka) which represents the antibody affinity. In this study, the Batty saturation method, a simple, rapid, and reliable method, was used. The Ka of 2A11, 2F6 and 3G2 were 1.05×10^{9}, 9.64×10^{8}, and 7.71×10^{8} L/moL, respectively, which were high affinity antibodies, of which 2A11 had the highest affinity (Figure.3.14).

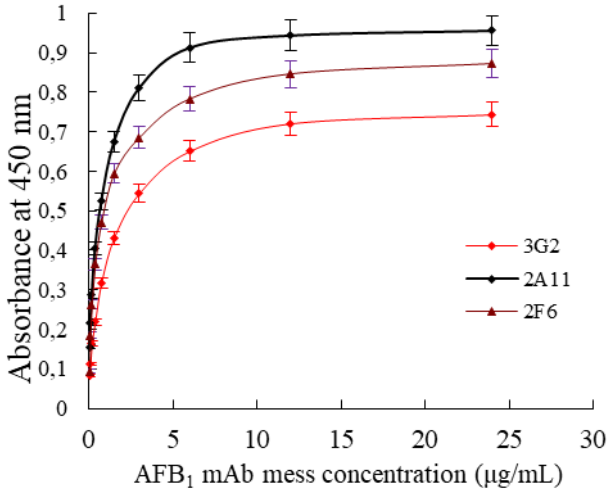


Figure.3.14 The Ka determination curves of AFB1 mAb 2A11, 2F6 and 3G2.

(4) Stability Stability verification showed that the three hybridoma cell lines secreted mAbs. The titer and IC50 values of AFB1 mAbs against AFB1 in the supernatants of each generation were determined after five repeated freeze-thaw cultures, but there was no significant difference. Therefore, the mAb-secreting hybridoma cells were genetically stable.

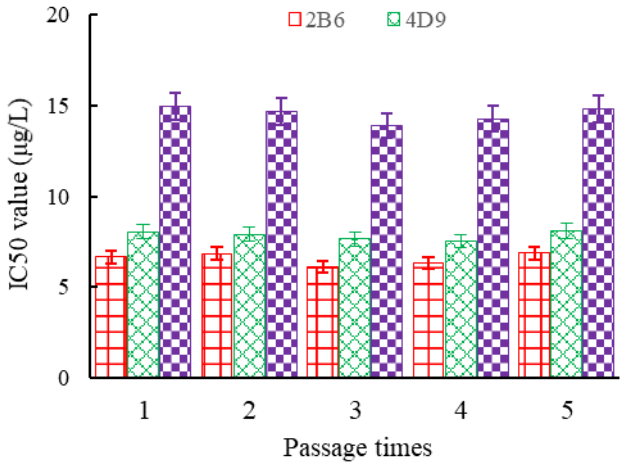


Figure.3.15 The titers of the AFB1 mAbs secreted by three hybridomas after five freeze/thaw cycles.

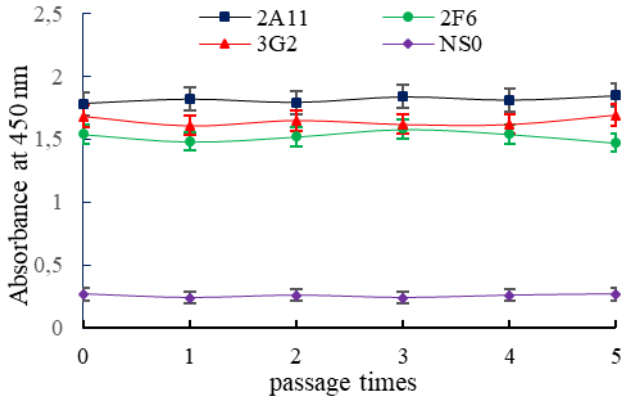


Figure.3.16 IC50 values of the AFB1 mAbs secreted by two hybridomas after five freeze/thaw cycles.

(5) Titers measurement An inELISA was employed to measure the titers of AFB1 mAb 2A11, 2F6 and 3G2, the results were exhibited in Table.3.8.

Table.3.8

The titers of AFB1 mAbs produced by three hybridomas

AFB1 mAbs	Titers in cell culture supernatants [a]	Titers in ascites [a]
2A11	1:（6.4×10^2）	1:（5.12×10^5）
2F6	1:（1.28×10^3）	1：（1.28×10^5）
3G2	1:（3.2×10^2）	1:（2.56×10^5）

Note. a All of the data were calculated from triplicate assays.

（6）Sensitivity and specificity Sensitivity and specificity were key in assessing the quality of antibodies since they determine immunoassay performance. Sensitivity was one of the important indicators for evaluating the antigen–antibody reaction mode, i.e., the immunoassay method, represented by an IC50 value. In this study, icELISA conditions, such as the best working concentration of coating antigen and antibody, concentration of organic solvent, ionic strength, and pH value, were optimized (detailed results are provided in the section titled “Optimization of the icELISA”). Therefore, the standard curve of an icELISA was established, and the IC50 and CRs values of the mAbs were determined. (as shown in Table.3.9). The IC50 values of AFB1 mAb 2A11, 2F6, and 3G2 were 6.22, 7.85, and 14.36 μg/L, respectively. The AFB1 mAb 2A11 had the lowest IC50 value against AFB1 analogs (AFB2, AFG1, AFG2, AFM1, and AFM2). Thus, AFB1 mAb 2A11 was selected for further testing to establish an immunochromatographic test strip method for detecting AFB1 residues in food and feed.

Table.3.9

The IC50 values and CRs of three AFB1 mAbs against AFB1 analogs

Compound	2A11		2F6		3G2	
	IC50 (μg/L) [a,b]	CR (%) [c]	IC50 (μg/L) [a,b]	CR (%) [c]	IC50 (μg/L) [a,b]	CR (%) [c]
AFB1	6.22	100	7.85	100	14.36	100
AFB2	144.32	4.31	168.82	4.65	263.97	5.44
AFG1	272.81	2.28	310.28	2.53	469.28	3.06
AFG2	>1000	<1.0	>1000	<1.0	>1000	<1.0
AFM1	>10000	<0.1	>10000	<0.1	>10000	<0.1
AFM2	>10000	<0.1	>10000	<0.1	>10000	<0.1
Zearalenone	>10000	<0.1	>10000	<0.1	>10000	<0.1
Deoxynivalenol	>10000	<0.1	>10000	<0.1	>10000	<0.1
T-2 toxin	>10000	<0.1	>10000	<0.1	>10000	<0.1
Ochratoxin A	>10000	<0.1	>10000	<0.1	>10000	<0.1

Note. [a] All of the data were calculated from triplicate assays, and the average coefficient of variation (CV) was below 10%. [b] The compound standard solution was prepared in 70% methanol-PBS (7:3, v/v). [c] All of the data were calculated using the CR of AFB1 mAbs against AFB1 as 100%.

3.2.1.5 Analysis of affecting factors on the performances of AFB1 mAb 2A11 in icELISA mode

(1) Effects of homologous and heterologous coating antigens on sensitivity of AFB1 mAb 2A11 in icELISA mode The IC50 value of AFB1 mAb 2A11 was determined by the homologous icELISA mode and the five heterologous icELISA modes, and the combination with the lowest IC50 value was selected to establish the icELISA method. The results showed that, among the five heterologous

icELISA combinations, the two combinations of AFB1 mAb 2A11 (AFB1-BSA by OAE) and coating antigen (ZEN-OVA by SA), the AFB1 mAb 2A11 (AFB1-BSA by OAE) and coating antigen (ZEN-OVA by MA) had the better effect than that of the homologous icELISA, and the combinations of AFB1 mAb 2A11 (AFB1-BSA by OAE) and coating antigen (ZEN-OVA by SA) was obviously better than that of AFB1 mAb 2A11 (AFB1-BSA by OAE) and coating antigen (ZEN-OVA by MA). Whereas the other three heterologous icELISA combinations were not as effective as the homologous icELISA combination. (Figure.3.17).

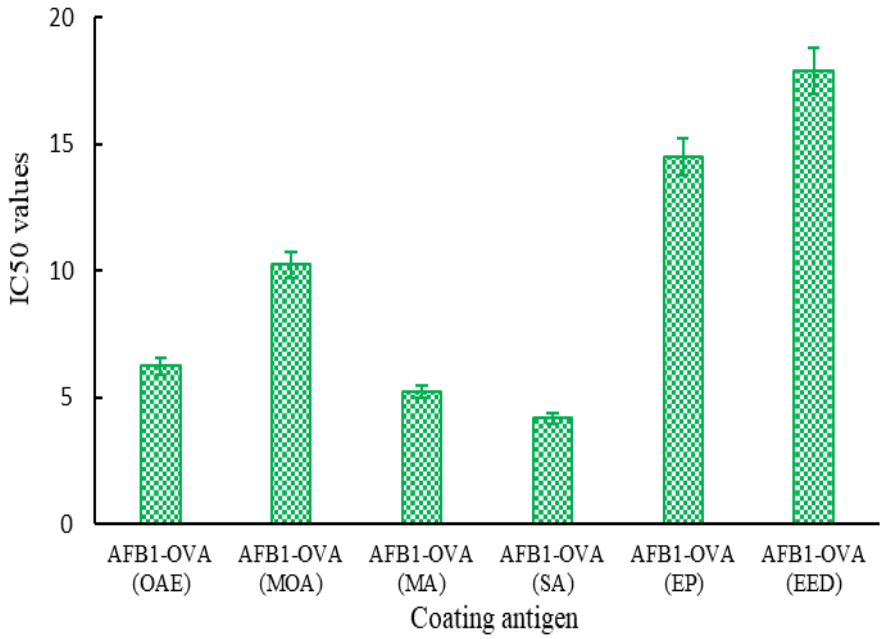


Figure.3.17 Effects of homologous and heterologous coating antigens on sensitivity of ZEN mAb 2B6 in icELISA mode.

(2) Effects of organic solvent type and concentration on sensitivity of AFB1 mAb 2A11 in icELISA mode Methanol, Tween-20, acetonitrile, and acetone, which may interfere with antigen-antibody binding and increase the solubility of analytes, were tested for their effects on the AFB1 mAb 2A11 in icELISA mode (Figure.3-18).

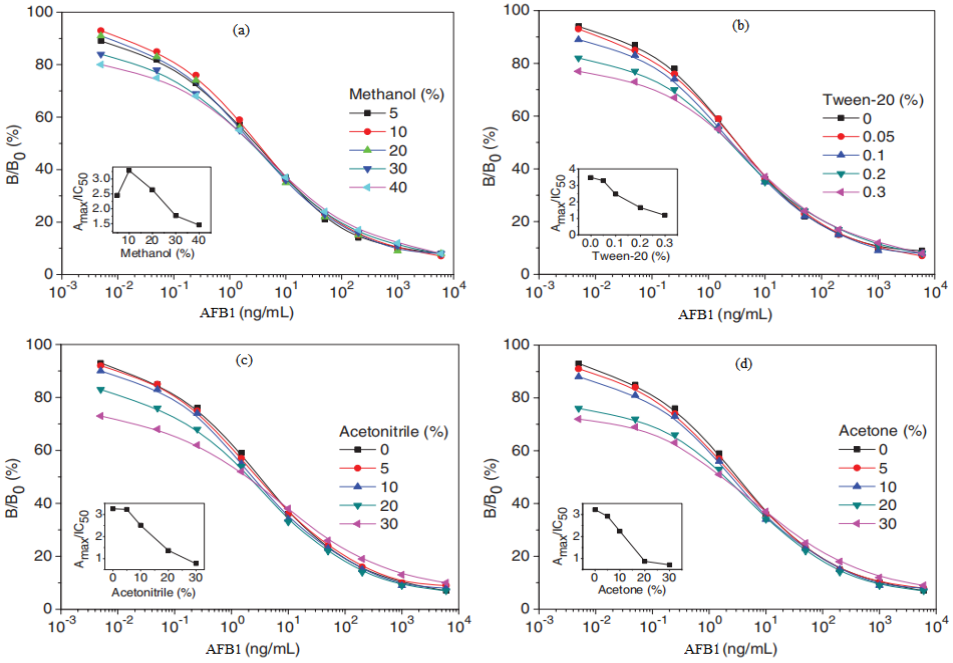


Figure.3.18 Effects of organic solvent type and concentration on sensitivity of AFB1 mAb.

Figure.3-18 (a)shows the normalized dose-response curves at various solvent concentrations. First, the influence of methanol from 5% to 40% (v/v) on the immunosorbent assay was studied. The results showed that the IC50 value was increased at methanol concentrations greater than 20%, whereas it was slightly affected by methanol ranging from 5% to 20%. If higher solvent concentrations were necessary in some assays, up to 20% of methanol could be used because the sensitivity decrease was acceptable at this concentration. Tween-20, acetonitrile, and acetone were shown in Figure.3.18 (b), (c) and (d). The results showed that the IC50 and Amax values of the immunoassay were varying notably when increasing the amounts of organic solvents. These results indicated that the solvents not only inhibited the interaction between the mAb and analyte, but also weakened the enzyme activity. It indicated that a reproducible inhibition curve could not be observed, but only when the concentration of the solventwas not higher than 10%. Therefore, Methanol was a suitable organic solvent, and its concentration was less than 30%.

3.2.2 Preparation and assessment of ZEN mAbs

3.2.2.1 Screening of immunogens and immunization method

According to the molecular structure and active sites of ZEN, five artificial immunogen synthesis methods were designed, and they were identified by UV, SDS-PAGE and comparative analysis of the characteristics of ZEN pAb produced by immunized animals. The results showed that the OAE method was the best method for preparing broad-specificity antibody for ZEN and its analogs, and the AGA method was the best method for preparing ZEN high-specificity antibody. Since the purpose of this study was to prepare highly specific ZEN antibodies and established an immunoassay method, the immunogen prepared by the AGA method was selected for subsequent experiments. Additionally, it should be noted that in order to prepare ZEN mAbs with high specificity and high sensitivity, Balb/c mice were immunized with a small dose (30 μg/head), long interval (4 weeks) and two routes (subcutaneous injection on the back and intraperitoneal injection).

3.2.2.2 Selection for potential cell fusion in immunized mice

An inELISA and an icELISA were used to measure the titers, the IC50 values, CR of ZEN pAbs from the five immunized mice after the five inoculations. Although the five immunized mice had a better positive immune response with high titers (>10^3) (Figure.3.19), the fifth (No. 5) mouse had the most efficient immune response, with titers of 1:(6.4×10^3).

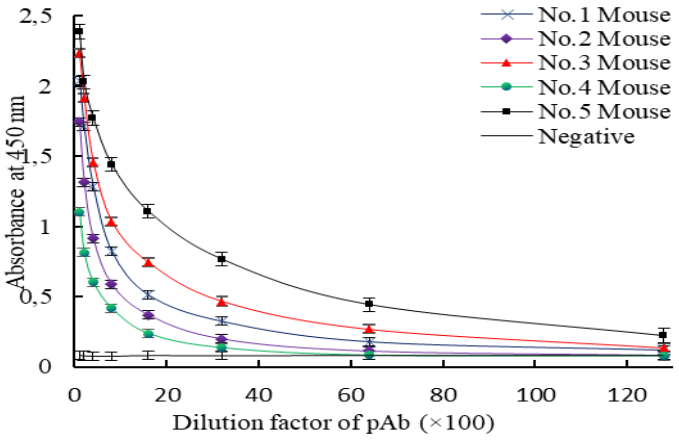


Figure.3.19 The inELISA titers measurement for the ZEN mAb.

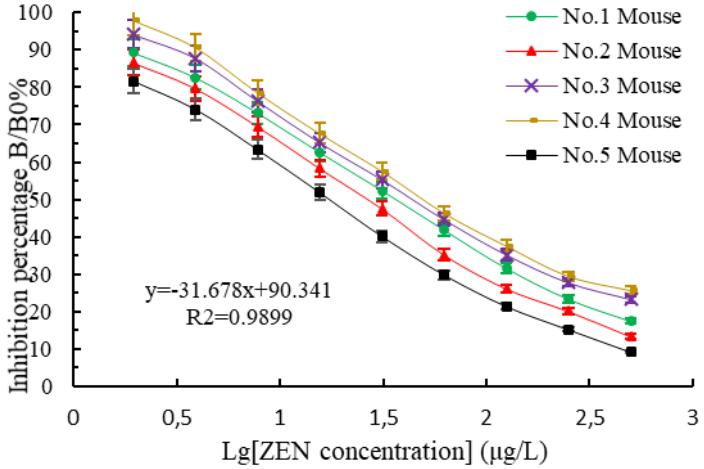


Figure.3.20 Sensitivity measurement of ZEN mAb to ZEN using an icELISA.

The inhibition curves (Figure.3.20) showed that the fifth mouse had the lowest IC50 value (18.77 μg/L). The five mice showed similar specificity towards the ZENs, with the fifth mouse having the smallest CRs (＜10%) (see Table.3.10). Thus, the fifth mouse was chosen for the cell fusion experiment.

Table.3.10

The cross-reactivity of ZEN pAbs with ZEN and its homologues

Compound	ZEN pAb (No.1 Mice)		ZEN pAb (No.2 Mice)		ZEN pAb (No.3 Mice)		ZEN pAb (No.4 Mice)		ZEN pAb (No.5 Mice)	
	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]	IC50 (μg/L) [a,b]	CR (%) [a]
ZEN	38.14	100	28.08	100	50.87	100	60.04	100	18.77	100
α-ZAL	1199.37	3.18	1210.35	2.32	1579.81	3.22	1631.52	3.68	1268.24	1.48
β-ZAL	1433.84	2.66	1270.59	2.21	1772.47	2.87	2257.14	2.66	1380.15	1.36
α-ZOL	654.20	5.83	615.79	4.56	954.41	5.33	1083.76	5.54	525.77	3.57
β-ZOL	1297.28	2.94	1210.35	2.32	1919.62	2.65	2077.51	2.89	1137.58	1.65
ZON	413.67	9.22	348.82	8.05	532.11	9.56	613.91	9.78	386.21	4.86

Note. [a] All of the data were calculated from triplicate assays. [b] The compound standard solution was prepared in 70% methanol-PBS (7:3, v/v).

3.2.2.3 Screening of positive clones and establishment of hybridoma cell lines

The growing hybridoma cell clones were observed 12 days after the cell fusion. A total of 321 of the 384 wells of four 96-well cell culture plates had hybridoma cells, at a fusion rate of about 83.6%. An inELISA and an icELISA were used to screen the culture supernatants of all of the wells simultaneously. A total of 51 wells were positive at a rate of about 15.9%. After subcloning thrice using a limiting dilution, the two stable hybridoma cells 2B6 and 4D9 that had a high-titer and a lower IC50 value in relation to ZEN were used to produce mAbs. The titers and the IC50 values of the mAbs from culture supernatants and ascites were shown in Table.3.11.

Table.3.11

The titers and IC50 values of the ZEN mAbs produced by two hybridomas

ZEN mAbs	Titers of Supernatants [a]	Titers of Ascites [a]	IC50 Values of Supernatants (μg/mL) [a,b]	IC50 Values of Ascites (μg/mL) [a,b]
2B6	5.12×10^2	5.12×10^5	6.72	6.54
4D9	2.56×10^2	1.28×10^5	10.47	9.75

Note. [a] All of the data were calculated from triplicate assays. [b] The ZEN standard solution was prepared in 70% methanol–PBS (7:3, *v/v*).

3.2.2.4 Assessment of immunological characteristics of ZEN mAbs (1) Karyotype

The chromosomes of myeloma NS0 cells were between 62 and 68, and those of mouse spleen cells were 40. However, hybridoma cell chromosomes were

between 96 and 102 (mean, 98.4), indicating that the cell lines fused by the two parents were the hybridoma cells since their chromosomes were more than those of the individual parents.

(2) Isotype Using a mouse mAb isotyping kit, both mAbs were determined to be of the IgG1 isotype possessing a kappa light chain (Figure.3.21).

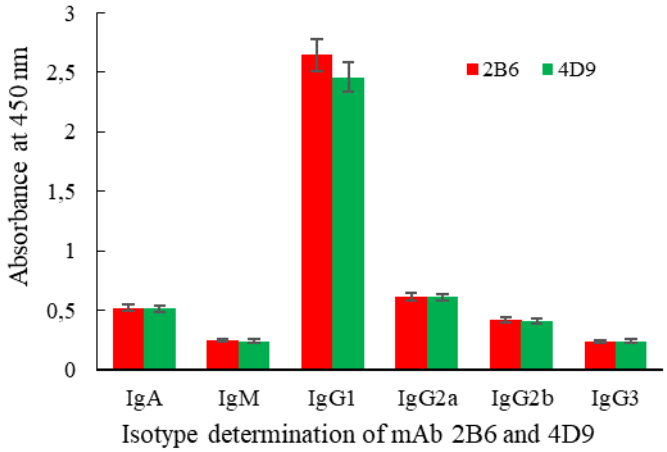


Figure.3.21 Isotype determination.

(3) Affinity The antibody affinity reflects its binding strength with an antigen and the affinity constant (Ka) represents the antibody affinity. In this study, the Batty saturation method was used. The Ka of 2B6 and 4D9 were 7.69×10^9, and 4.95×10^9 L/mol, respectively, which were high affinity mAbs, of which 2B6 had the highest affinity (Figure.3.22).

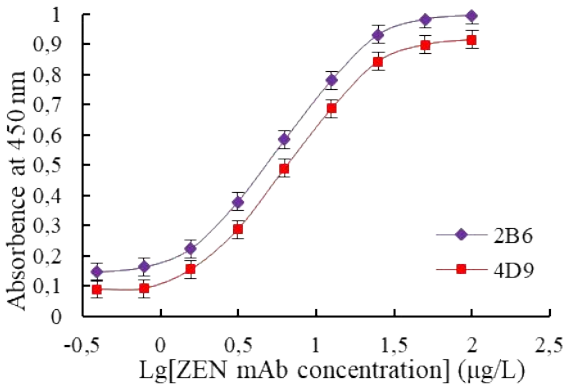


Figure.3.22 The Ka determination curves.

(4) Stability Stability verification showed that the three hybridoma cell lines secreted mAbs. The titer and IC50 values of ZEN mAbs against ZEN in the supernatants of each generation were determined after five repeated freeze-thaw cultures, but there was no significant difference. Besides, the mAb-secreting hybridoma cells were genetically stable (Figure.3.23 and Figure.3.24).

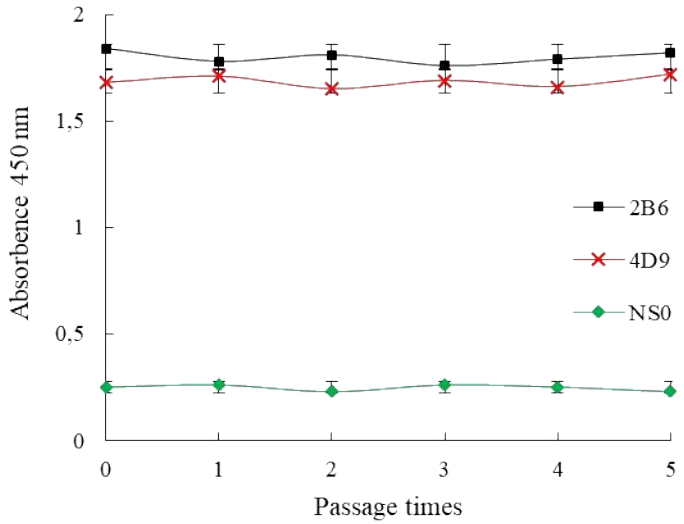


Figure.3.23 The titers of the ZEN mAbs secreted by three hybridomas after five freeze/thaw cycles.

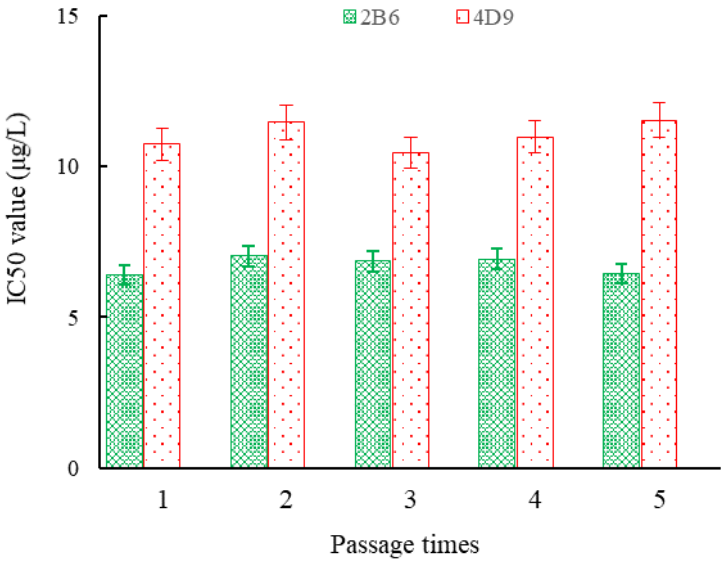


Figure.3.24 IC50 values of the ZEN mAbs secreted by two hybridomas after five freeze/thaw cycles. Each point represents the average of three separate assays in triplicate.

(5) Sensitivity and specificity Sensitivity and specificity were key in assessing the quality of antibodies since they determine immunoassay performance. Sensitivity was one of the important indicators for evaluating the antigen–antibody reaction mode, that is, the immunoassay method, represented by an IC50 value. In this study, icELISA conditions, such as the best working concentration of coating antigen and antibody, concentration of organic solvent, ionic strength, and pH value, were optimized (detailed results are provided in the section titled "Optimisation of the icELISA"). Therefore, the standard curve of an icELISA was established, and the IC50 and CRs values of the antibodies were determined (Table.3.12). The IC50 values of mAb 2B6 and mAb 4D9 were 6.54 μg/L and 9.75 μg/L, respectively. The mAb 2B6 had the highest sensitivity to six ZENs (ZEN, α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON). Therefore, mAb 2B6 was selected for further testing to establish an immunochromatographic test strip method for detecting ZEN residues in food and feed.

Table.3.12

The sensitivity (IC50) and specificity (CR) of two mAbs against ZENs

Compound	2B6		4D9	
	IC50 (μg/L) [a,b]	CR (%) [c]	IC50 (μg/L) [a,b]	CR (%) [c]
ZEN	6.54	100	9.75	100
α-ZAL	430.26	1.52	598.16	1.63
β-ZAL	510.94	1.28	722.22	1.35
α-ZOL	247.73	2.64	340.91	2.86
β-ZOL	357.38	1.83	485.08	2.01
ZON	153.16	4.27	204.92	4.88
Aflatoxin B1	>10000	<1	>10000	<1
Deoxynivalenol	>10000	<1	>10000	<1
T-2 toxin	>10000	<1	>10000	<1
Ochratoxin A	>10000	<1	>10000	<1

Note. a All of the data were calculated from triplicate assays, and the average coefficient of variation (CV) was below 10%. b The compound standard solution was prepared in 70% methanol-PBS (7:3, v/v). c All of the data were calculated using the CR of ZEN mAbs against ZEN as 100%.

3.2.2.5 Condition optimization of the icELISA

Some challenges, such as the type of coated antigen, optimal working concentration of the antigen and the antibody, the concentration of the organic solvent, ionic strength, and pH value, were often encountered during the establishment of the icELISA method.

(1) Effects of homologous and heterologous coating antigens on sensitivity of ZEN mAb 2B6 in icELISA mode

The IC50 value of ZEN mAb 2B6 was determined by the homologous icELISA mode and the four heterologous icELISA modes, and the combination with the lowest IC50 value was selected to establish the icELISA method. The results showed that, among the four heterologous icELISA combinations, the combination of mAb 2B6 (ZEN-BSA by AGA) and coating antigen (ZEN-OVA by FA) had the best effect, and mAb 2B6 had the lowest IC50 value for ZEN. Obviously better than the homologous IcELISA, but the other three heterologous icELISA combinations are not as effective as the homologous icELISA combination. (Figure.3.25).

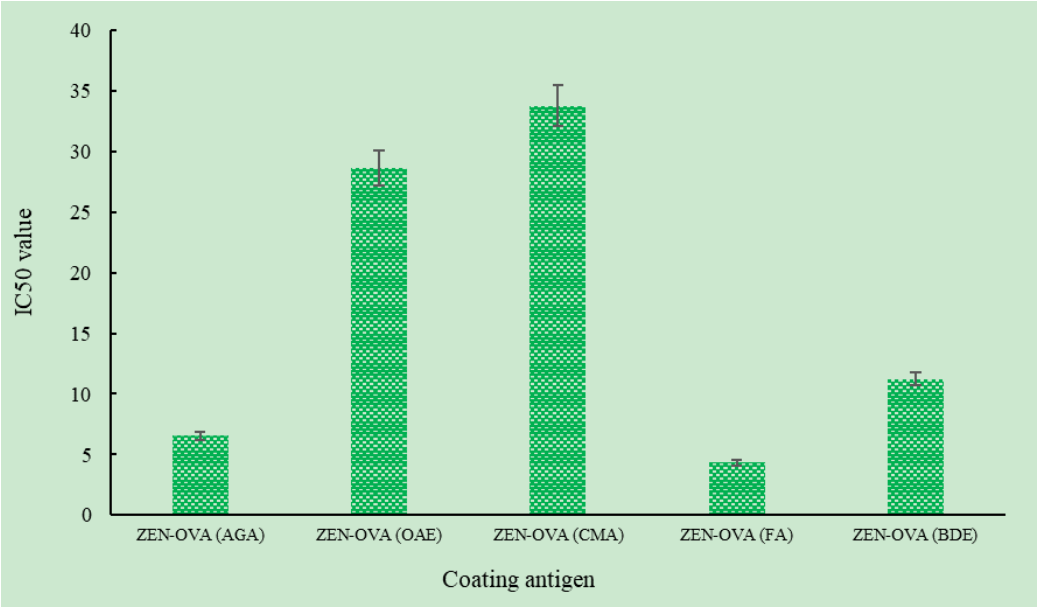


Figure.3.25 Effects of homologous and heterologous coating antigens on sensitivity of ZEN mAb 2B6 in icELISA mode.

(2) The effects of methanol concentration on sensitivity of ZEN mAb 2B6 in icELISA mode

Methanol was widely used in an icELISA as an organic cosolvent to extract the mycotoxins from the food and feed matrix. An appropriate methanol level not only affects ELISA sensitivity but also helps to dissolve analytes. The Amax and the Amax/IC50 value decreased when the methanol concentration exceeded 30%, but there was no significant effect when the methanol concentration was below 30% (Figure.3.26).

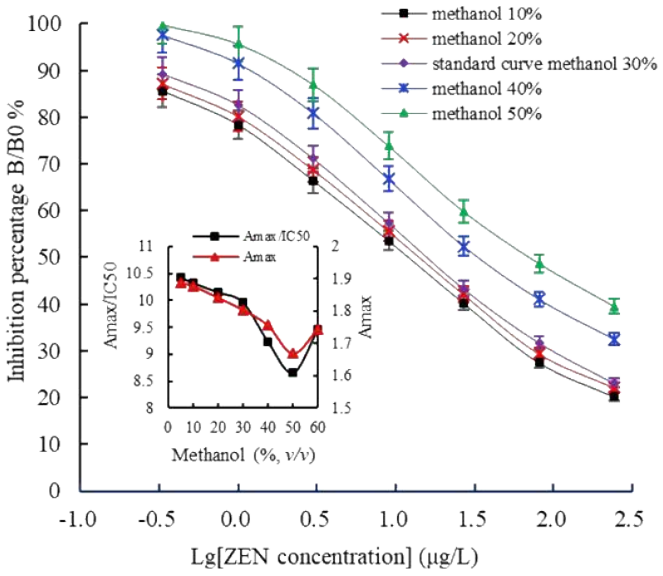


Figure.3.26 The effects of methanol concentration on on sensitivity of ZEN mAb 2B6 in icELISA mode. Each value represents the mean of three replicates.

(3) The effects of ionic strength on sensitivity of ZEN mAb 2B6 in icELISA mode

The effects of phosphate ions were estimated by comparing inhibition curves obtained under various conditions with that of a control. Sensitivity was obviously altered with the increase of phosphate ion concentration (Figure.3.27). Sensitivity was obviously altered with the increase of phosphate ion concentration (Figure 3

B). When phosphate ions moved up from 1 to 10 mM, the IC50 value improved from 0.31 to 0.28 ng/mL. This sensitivity improvement may be due to the dispersion and weakening of the nonspecific binding derived from mAbs. Concentrations of phosphate ion higher than 20 mM resulted in lower absorbance and sensitivity drop as the higher ionic strength may weaken the antibody–hapten interaction. Therefore, 10 mM PBS was the preferred assay buffer, and was employed for the remainder of the study. In practice, the ionic strength of matrix samples can be adjusted by simple dilution with distilled water or concentrated buffer.

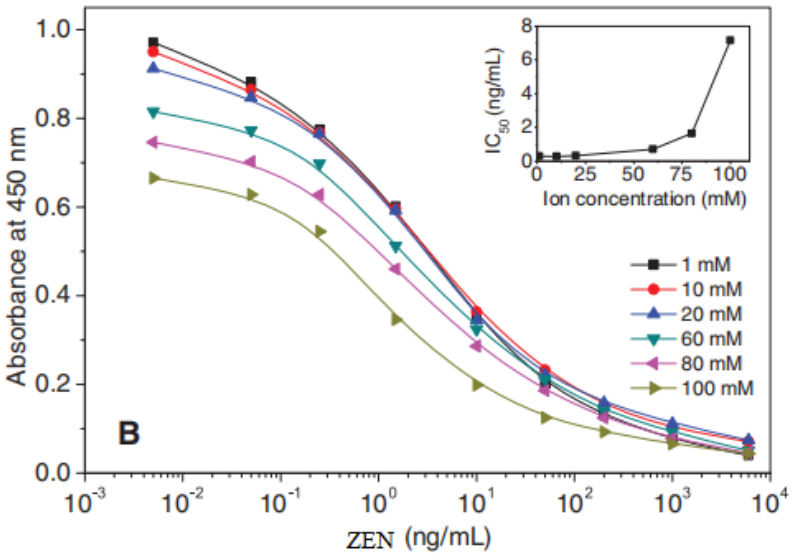


Figure.3.27. The effects of ionic strength on sensitivity of ZEN mAb 2B6 in icELISA mode.

(4) The effects of pH values on sensitivity of ZEN mAb 2B6 in icELISA mode The pH between 5.0 and 9.0 had no significant effect on the Amax/IC50 and Amax. However, the Amax/IC50 and Amax were highest at a pH of 7.4, indicating full binding between antibodies and the antigen. Therefore, choosing a PBS with a pH value between7.2 and 7.4 was t he most suitable in an icELISA system (Figure.3.28) 。

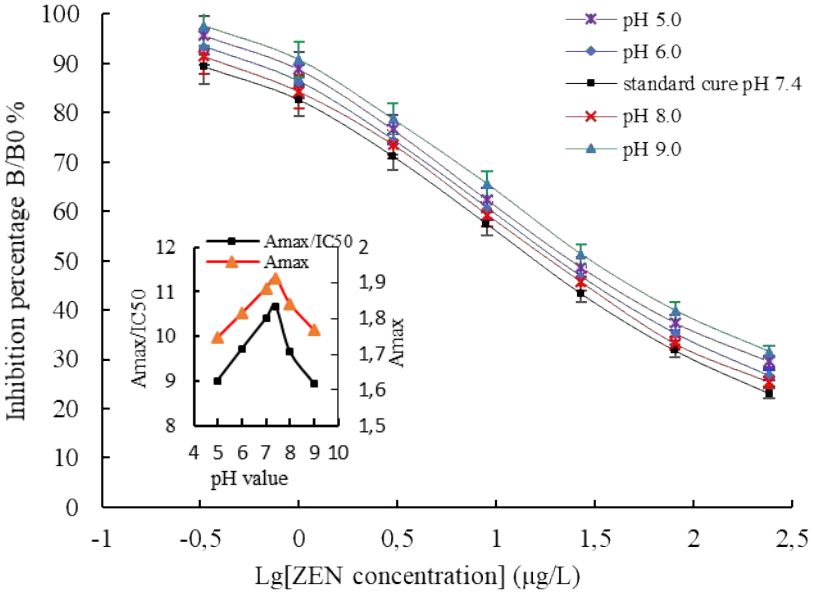


Figure.3.28 The effects of pH value on an icELISA. Insets indicate the fluctuations of Amax/IC50 (Y-principal axis) and Amax (Y-secondary axis) as a function of pH value.

3.3 Establishment of dual test strip detection method

3.3.1 Identification of colloidal gold

(1) Visual observation Observed by naked eyes, the colloidal gold solution was translucent wine red, there was no floating matter in the upper layer, and no sediment in the lower layer. The solution was not turbid under strong light, had good light transmittance, and no astigmatism occurs (Figure 3.29).

Figure.3.29 Identification of colloidal gold by visual observation.

(2) UV scanning The maximum absorption peak (λmax) of the colloidal gold solution was at 523.8 nm, and the maximum absorption value (Amax) was 0.941. There was only one absorption peak and the peak width was small. According to the research results of Cvak et al. [3], the size and distribution of colloidal gold particles were uniform, with a particle size of 25 nm (Figure 3.30).

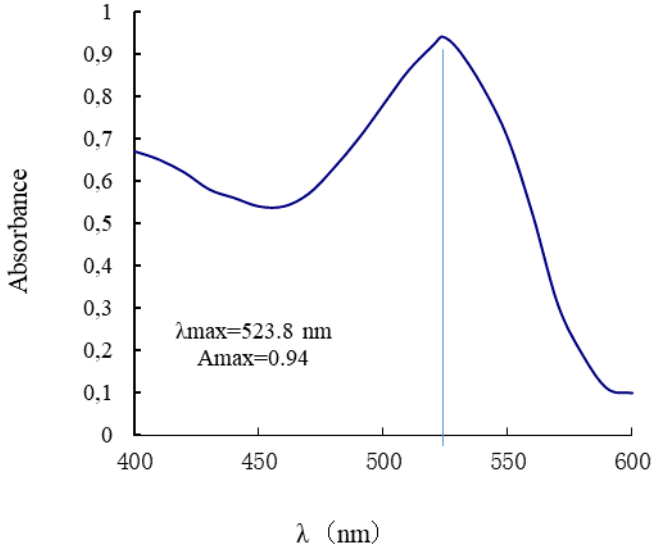


Figure.3.30 Identification of colloidal gold by UV scanning.

(3) Electron microscopy scanning.

The prepared colloidal gold was uniformly distributed under the transmission electron microscope and had a regular shape. 100 particles were randomly measured and their particle size were 25±1.0nm, which were basically consistent with the UV scanning result (Figure 3.31). The results showed that 1L of 0.01% chlorauric acid solution required 17 mL of 1% trisodium citrate solution.

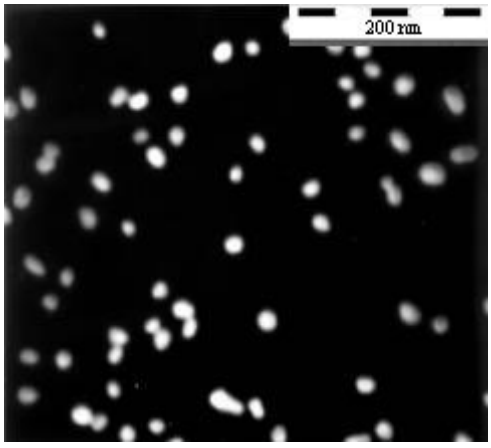


Figure.3.31 Identification of colloidal gold by electron microscope.

3.3.2 Development of AFB1 (and ZEN) test strip

(1) Determination of the optimal pH of colloidal gold The pH of colloidal gold was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 with 0.2 mol/L K_2CO_3. After adding AFB1 mAb and NaCl, the gold-labeled mAb solutions in each tube were scanned at 523.8 nm by UV. The absorbance value was as the ordinate, and the pH was as the abscissa, and absorbance curve was drew. The results were shown in Figure 3.32.

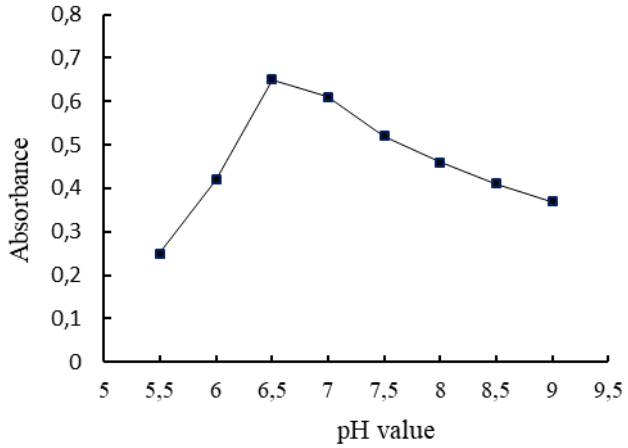


Figure.3.32 Determination of optimal pH for colloidal gold labeling AFB1 mAbs.

When the pH of the colloidal gold solution was 6.5, the curve reached the highest point, and the combination was close to saturation. Thus, the optimal binding pH of AFB1 mAbs and colloidal gold was determined at 6.5. Similarly, the optimal pH of ZEN mAbs and colloidal gold was 7.0 (Figure 3.33)

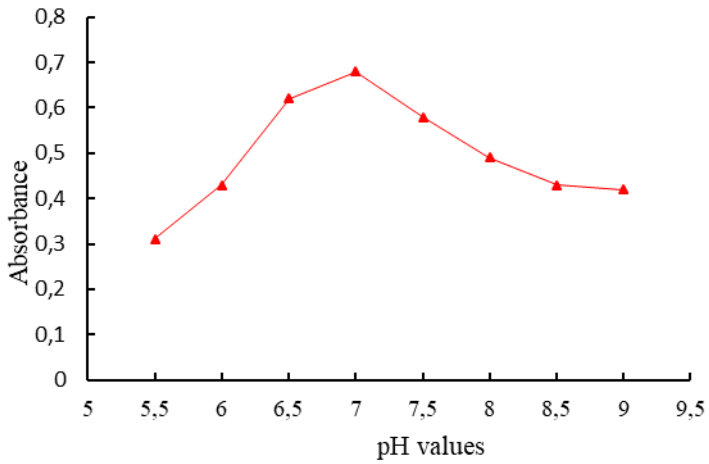


Figure.3.33 Determination of optimal pH value for colloidal gold labeling ZEN mAbs.

(2) Determination of the best concentration of colloidal gold labeling AFB1 mAbs and ZEN mAb

Mey's series stabilization method. The results were shown in Table 3.13.

Table.3.13

Selection of optimal labeling concentration of AFB1 mAb and colloidal gold

Serial number	1	2	3	4	5	6	7	8	BC
AFB1 mAb (μg/mL）	50	25	12.5	6.25	3.125	1.5625	0.7825	0.391	0
0.01% colloidal gold (μL)	25	25	25	25	25	25	25	25	25
10% NaCl （μL）	100	100	100	100	100	100	100	100	100
Coloration	Wine	Wine	Dark red	Light red	Light blue	Dark blue	Dark blue	Dark blue	Dark blue

The lowest concentration of AFB1 mAb labeled with colloidal gold was 6.25 μg/mL, which was an increase of 10%, and the optimal labeling concentration of AFB1 mAb was about 7.0 μg/mL. The optimal labeling concentration of ZEN mAb was about 3.5 μg/mL, which was selected by the same way.

UV scanning method. The UV scan results were shown in Figure 3.34.

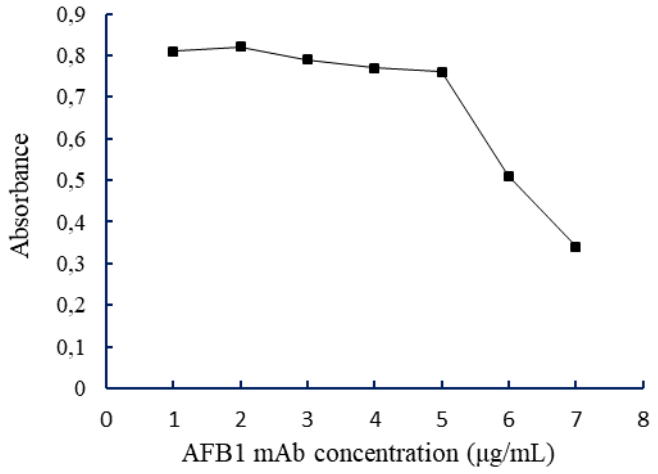


Figure.3.34 Determination of optimal labeling concentration of AFB1 mAbs. 1.100 μg/mL. 2. 50 μg/mL. 3.25 μg/mL. 4. 12.5 μg/mL. 5. 6.25 μg/mL. 6. 3.125 μg/mL. 7. 1.5625 μg/mL. 8. 0.391 μg/mL.

The results showed that the absorbance value reached a higher position at the AFB1 mAb concentration of 6.25 μg/mL. Because excessive antibody will cause the sensitivity to decrease, 7.0 μg/mL (6.25×110%≒7.0) was determined to be the optimal labeling amount for AFB1 mAbs. Figure 3.35 showed that the optimal labeling concentration of ZEN mAb was 3.5 μg/mL.

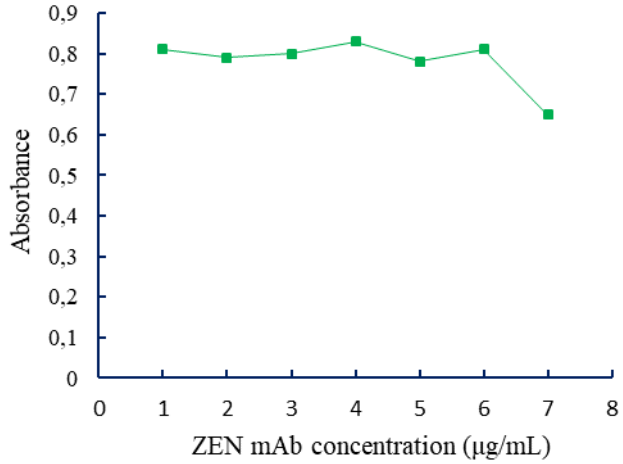


Figure.3.35 Determination of optimal labeling concentration of ZEN mAbs. 1.100 μg/mL. 2. 50 μg/mL. 3.25 μg/mL. 4. 12.5 μg/mL. 5. 6.25 μg/mL. 6. 3.125 μg/mL. 7. 1.5625 μg/mL. 8. 0.391 μg/mL.

(3) Selection of resuspending solution and preservation solution for gold-labeled mAb

Selection of resuspending solution for gold-labeled mAb. The selection results were as follows. After the gold-labeled antibody solution was reconstituted in Tris-HCl buffer, centrifuged at 5000 r/min for 10 min, black flakes appeared at the bottom of the centrifuge tube, indicating that the gold-labeled antibody was not stable enough in the Tris-HCl buffer and precipitated phenomenon. After the gold-labeled antibody solution was reconstituted in PBS buffer, it was centrifuged at 5000 r/min for 10 min, and it was found that there were red colloidal gold residues on the wall of the centrifuge tube, indicating that the PBS buffer had insufficient fluidity and wall hanging. After re-dissolved the gold-labeled antibody solution in the borate buffer, centrifuge at 5000 r/min for 10 min, and there was no abnormal phenomenon, indicating that the gold-labeled antibody had good activity.

Therefore, 2 mmol/L borate buffer (pH7.4) was selected as a colloidal gold resuspension solution.

Selection of preservation solution for gold-labeled mAb. The results were shown in Table 3.14.

Table.3.14

Selection of preservation solution for gold-labeled mAb

Preservation time（month）	1	2	3	4	5	6
Borate buffer (containing 1%BSA and 0.05%NaN_3)	√[a]	√	√	×[b]	×	×
Borate buffer (containing 10% sucrose and 0.05%NaN_3)	√	√	×	×	×	×
Borate buffer (containing 1%BSA,10% sucrose, and 0.05%NaN_3)	√	√	√	√	√	√

Note: [a] means normal. [b] Indicates that there is coprecipitation.

The gold-labeled antibodies stored in 2 mmol/L borate buffer containing 1% BSA and 0.05% NaN_3, and 2 mmol/L borate buffer containing 10% sucrose and 0.05% NaN_3 as the preservation solution showed precipitation at 4 months and 2 months, respectively. Whereas, the gold-labeled antibody stored in mmol/L borate buffer (containing 1% BSA, 10% sucrose, and 0.05% NaN3) was the preservation solution, and the stability of the gold-labeled antibody was very good, and there was no precipitation in 6 months. Therefore, 2 mmol/L borate Buffer (containing 1% BSA, 10% sucrose, 0.05% NaN3) was chosen to be used as a gold-labeled antibody storage solution.

(4) Optimization and assembly of test strip

Selection of nitrocellulose membrane. AFB1-BSA and ZEN-BSA were coating on different types of NC membranes respectively, and were dripped the corresponding gold-labeled antibody on the surface, the different types of NC

membranes all showed clear red bands, and there was no obvious difference. In comparison, the color development results of 5 min and 10 min showed that the color of Millipore 135 membrane was more clear. Therefore, Millipore 135 membrane was selected as the NC membrane of the test strip detection area.

Selection of the gold-labeled pad. After the gold-labeled antibody was added to the gold-labeled pad and dried, the degree of release of the gold-labeled antibody was observed for 10 min. The results showed that the SB06, SB08 and 6613 gold-labeled pads could not completely release the gold-labeled antibody within 10 minutes, and coagulation occurred. On the contrary, the 8964 gold-labeled pad could completely release the gold-labeled antibody within 10 minutes without coagulation. Therefore, glass fiber 8964 was selected as the gold standard pad for this experiment.

Selection of the sample pad. Comparing SB08 and SB06 sample pads' absorption and carrying capacity for sample treatment solution, the results showed that SB08 sample pad has greater absorption and carrying capacity for sample treatment solution, i.e., more sample treatment solution can be added to make competitive reaction to be more complete. Therefore, SB08 was selected as the sample pad in this study.

Determination of optimal concentration combination of gold-labeled antibody and coating antigen. The optimal working concentration combination of gold-labeled AFB1 mAb and coating antigen AFB1-BSA was determined by the chessboard method. The results were displayed in Table 3.15, which indicated that the optimal working concentrations of gold-labeled AFB1 mAb and AFB1-BSA were 1:4 and 1.0 mg/mL, respectively. Similarly, the best working concentration of gold-labeled ZEN mAb and ZEN-BSA were 1:4 and 2.0 mg/mL, respectively (Table 3.16).

Table.3.15

Determination of optimal combination of gold-labeled mAb and AFB1-BSA

gold-labeled AFB1 mAb	AFB1-BSA (mg/mL)						
	4.0	2.0	1.0	0.5	0.25	0.125	0.0625
1∶1	++	++	++	+	-	-	-
1∶2	++	++	++	+	-	-	-
1∶4	++	++	++	+	-	-	-
1∶8	++	++	+	-	-	-	-
1∶16	++	+	-	-	-	-	-
1∶32	+	-	-	-	-	-	-

Note: “++” means clear red color of test line. “+” means lighter red color of test line. “-” means no red color of test line.

Table.3.16

Determination of optimal concentration combination of gold-labeled ZEN mAb and ZEN-BSA

gold-labeled ZEN mAb	ZEN-BSA (mg/mL)						
	4.0	2.0	1.0	0.5	0.25	0.125	0.0625
1∶1	++	++	+	-	-	-	-
1∶2	++	++	+	-	-	-	-
1∶4	++	++	+	-	-	-	-
1∶8	++	+	+	-	-	-	-
1∶16	++	+	-	-	-	-	-
1∶32	+	-	-	-	-	-	-

Note: “++” means clear red color of test line. “+” means lighter red color of test line. “-” means no red color of test line.

The AFB1-BSA(OAE) and AFB1 optimal working concentrations were used to further investigate the use effects as coating antigen of other immunogens including AFB1-BSA(MOA), AFB1-BSA(MA), AFB1-BSA(SA), AFB1-BSA (EP), and AFB1-BSA (EED) (Table 3.17).

Table.3.17

Determination of optimal coating antigen for AFB1 test strip

gold-labeled AFB1 mAb	AFB1-BSA (1.0 mg/mL)					
	AFB1-BSA (OAE)	AFB1-BSA (MOA)	AFB1-BSA (MA)	AFB1-BSA (SA)	AFB1-BSA (EP)	AFB1-BSA (EED)
1:4	++	+	+	+	-	-

Note: "++" means clear red color of test line. "+" means lighter red color of test line. "-" means no red color of test line.

The results showed that the best combination for AFB1 test strip was AFB1 mAb and AFB1-BSA (OAE). The best combination for ZEN test strip was ZEN mAb and ZEN-BSA (AGA or FA) (Table 3.18).

Table.3.18

Determination of optimal coating antigen for ZEN test strip

gold-labeled AFB1 mAb	AFB1-BSA (2.0 mg/mL)				
	ZEN-BSA (AGA)	ZEN-BSA (OAE)	ZEN-BSA (CMA)	ZEN-BSA (FA)	ZEN-BSA (BDE)
1:4	++	+	+	++	+

Note: "++" means clear red color of test line. "+" means lighter red color of test line. "-" means no red color of test line.

(5) Determination of optimum methanol content in sample treatment solution

When the methanol content in the sample treatment solution is less than 20%, the T line of the AFB1 test strip and the ZEN test strip show clear color; when the methanol content is 30%, the T line of the AFB1 test strip shows clear color, but

the T line of the ZEN test strip T The color of the line is lighter; when the methanol concentration is 40%, the T line of the AFB1 test strip is lighter, and the T line of the ZEN test strip is not colored; when the methanol concentration is greater than 50%, the T lines of the two test strips are both No color development. Therefore, the final methanol content in the sample treatment solution should not exceed 20%.

3.3.3 Assembly of single and dual test strip

(1) Assembly of single (AFB1 or ZEN) test strip The plane schematic diagrams of single (AFB1 or ZEN) test strip and dual (AFB1 and ZEN) were shown in Figure 3.36 and Figure 3.37.

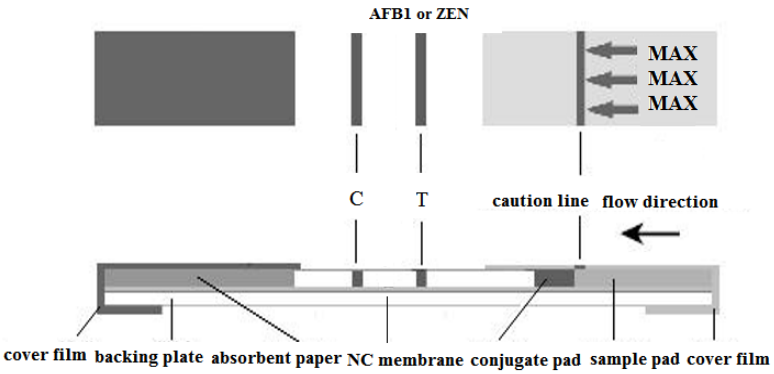


Figure.3.36 The plane schematic diagram of single (AFB1 or ZEN) test strip.

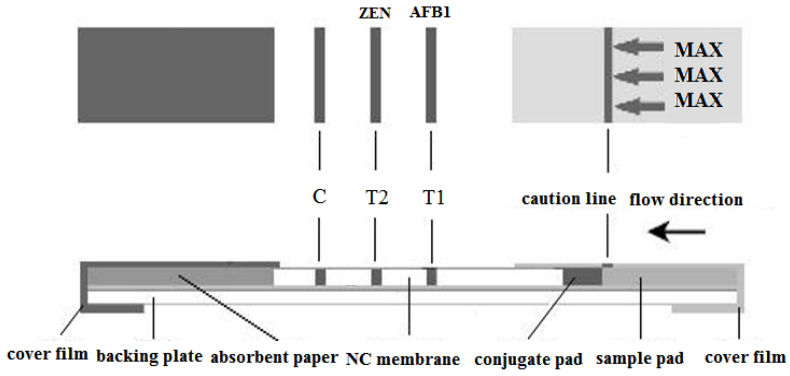


Figure.3.37 The plane schematic diagram of dual AFB1 (or ZEN) test strip.

3.4 Performance measurement of single (AFB1 or ZEN) and dual (AFB1 and ZEN) test strip

3.4.1 Performance measurement of single (AFB1 or ZEN) test strip

(1) The sensitivity measurement of single (AFB1or ZEN) test strip Visual method. The results were shown in Figure 3.38.

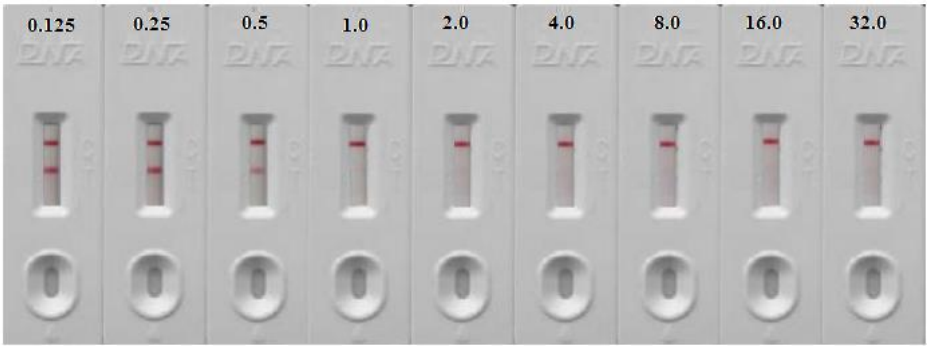


Figure.3.38 Sensitivity measurement of AFB1 test strip.

When the AFB1 concentration in the sample treatment solution reached 0.5 μg/L, the color of the T line became significantly lighter than the negative control. When the AFB1 concentration in the sample treatment solution reached 1.0 μg/L, the T line color basically disappeared. Therefore, the LOD of AFB1 test strip for AFB1 was 1.0 μg/L. Similarly, the LOD of ZEN test strip for ZEN was 5.0 μg/L (Figure 3.39).

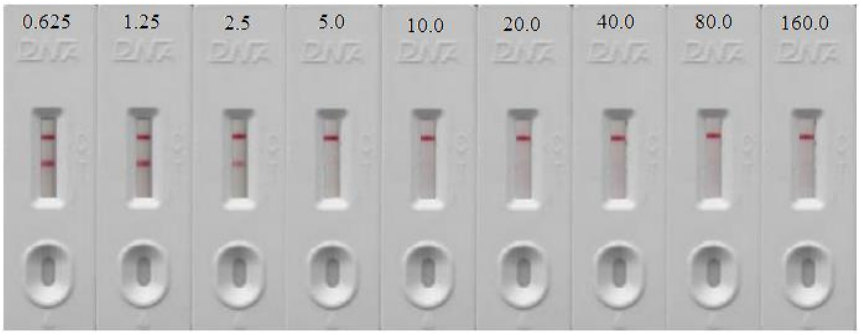


Figure.3.39 Sensitivity measurement of ZEN test strip.

Test strip reader scanning method. The BioDot-TSR3000 strip reader was carried out to scan the T line of the AFB1 test strip, and the scanning graph was shown in Figure 3.40, the scanning data was shown in Table 3.19, and the drawn standard curve was shown in Figure 3.41. The linear regression equation of AFB1 was y=−52.495x+47.525, the IC50 was 1.11 ng/mL, the LOD was 0.24 ng/mL. For the ZEN test strip, the scanning graph was shown in Figure 3.42, the scanning data was shown in Table 3.20, and the drawn standard curve was shown in Figure 3.43. The linear regression equation of ZEN was y=−34.237x+73.854, the IC50 was 4.97 ng/mL, and the LOD was 1.51 ng/mL.

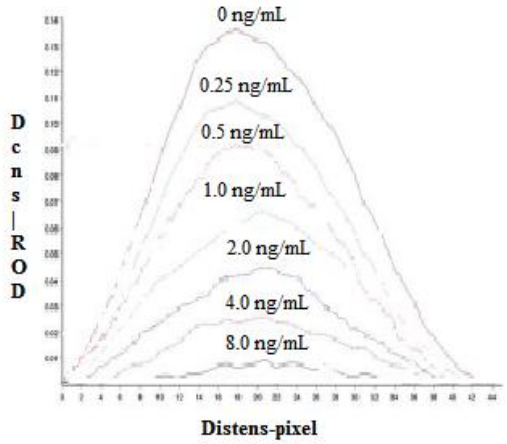


Figure.3.40 Relative optical density (ROD) curves of AFB1 standard by BioDot-TSR 3000.

Table.3.19

The scanning data of T line of AFB1 test strip by Bio Dot-TSR 3000

AFB1 concentration (ng/mL)	Lg[AFB1 concentration]	G/D×A－ ROD(pixel)	G/D×A－ ROD(pixel)%
0	-	16.65	100
0.25	-0.602	13.24	82.60
0.5	-0.301	10.12	65.78
1.0	0	7.25	43.54
2.0	0.301	4.31	25.89
4.0	0.602	2.06	12.37
8.0	0.903	1.26	7.57

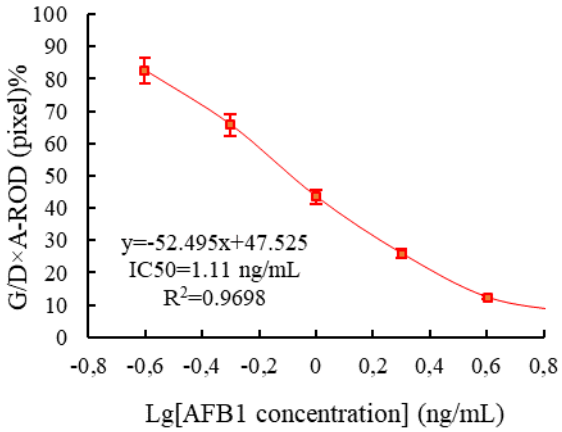


Figure.3.41 The standard curve of AFB1 test strip detected by BioDot-TSR3000.

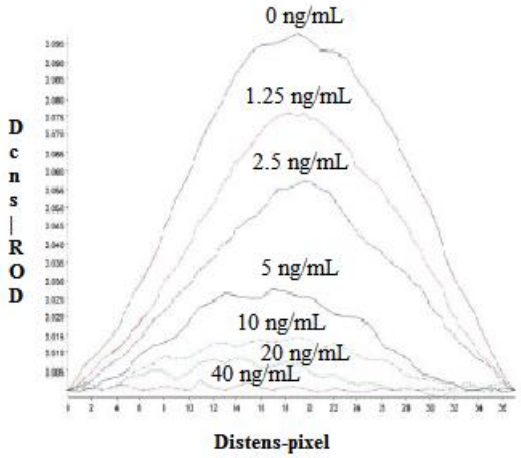


Figure.3.42 Relative optical density (ROD) curves of ZEN standard by BioDot-TSR3000.

Table.3.20

The scanning data of T line of AFB1 test strip by Bio Dot-TSR3000

AFB1 concentration (ng/mL)	Lg[AFB1 concentration]	G/D×A－ROD(pixel)	G/D×A－ROD(pixel)%
0	-	15.52	100
1.25	0.099	14.22	91.61
2.5	0.398	13.25	85.36
5.0	0.699	11.85	76.33
10.0	1	10.14	65.36
20.0	1.301	8.02	51.67
40.0	1.602	6.50	41.88

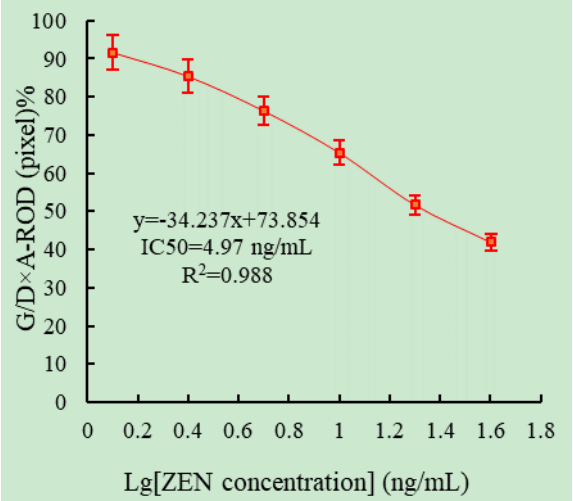


Figure.3.43. The standard curve of ZEN test strip detected by BioDot-TS R3000.

(2) The specificity measurement of AFB1 test strip and ZEN test strip

The determination results of AFB1 test strip were shown in Table 3.21 and ZEN test strip were shown in Table 3.22 The results showed that AFB1 test strip for AFB1 and ZEN test strip for ZEN had high specificity respectively, and had no cross reaction with their analogs and other mycotoxins.

Table.3.21

Specificity measurement of AFB1 test strip

Toxins concentration (ng/mL)	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0
AFB1	-	-	-	+	+	+	+	+	+
AFB2	-	-	-	-	-	-	-	-	-
AFG1	-	-	-	-	-	-	-	-	-
AFG2	-	-	-	-	-	-	-	-	-
AFM1	-	-	-	-	-	-	-	-	-
AFM2	-	-	-	-	-	-	-	-	-
ZEN	-	-	-	-	-	-	-	-	-
DON	-	-	-	-	-	-	-	-	-
OTA	-	-	-	-	-	-	-	-	-
T-2	-	-	-	-	-	-	-	-	-

Note: “-” means negative. “+” means positive.

Table.3.22

Specificity measurement of ZEN test strip

Toxins concentration (ng/mL)	1.25	2.5	5.0	10.0	20.0	40.0	80.0	160.0	320.0
ZEN	-	-	+	+	+	+	+	+	+
α-ZAL	-	-	-	-	-	-	-	-	-
β-ZAL	-	-	-	-	-	-	-	-	-
α-ZOL	-	-	-	-	-	-	-	-	-
β-ZOL	-	-	-	-	-	-	-	-	-
ZON	-	-	-	-	-	-	-	-	-
AFB1	-	-	-	-	-	-	-	-	-
DON	-	-	-	-	-	-	-	-	-
OTA	-	-	-	-	-	-	-	-	-
T-2	-	-	-	-	-	-	-	-	-

Note: “-” means negative. “+” means positive.

(3) Stability measurement of AFB1 test strip and ZEN test strip

Six batches of AFB1 test strip and ZEN test strips of different batches were reproducible for corn samples, rice samples and compound feed samples. The test results were completely consistent, indicating that AFB1 test strip and ZEN test strip had good stability (Table 3.23 and Table 3.24).

Table.3.23

The stability measurement of AFB1 test strip

Batches	AFB1 concentration (μg/L)											
	Corn sample				Rice sample				Compound feed sample			
	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
1	-	+	+	+	-	+	+	+	-	+	+	+
2	-	+	+	+	-	+	+	+	-	+	+	+
3	-	+	+	+	-	+	+	+	-	+	+	+
4	-	+	+	+	-	+	+	+	-	+	+	+
5	-	+	+	+	-	+	+	+	-	+	+	+
6	-	+	+	+	-	+	+	+	-	+	+	+

Table.3.24

The stability measurement of ZEN test strip

Batches	ZEN concentration (μg/L)											
	Corn sample				Rice sample				Compound feed sample			
	2.5	5.0	10.0	20.0	2.5	5.0	10.0	20.0	2.5	5.0	10.0	20.0
1	-	+	+	+	-	+	+	+	-	+	+	+
2	-	+	+	+	-	+	+	+	-	+	+	+
3	-	+	+	+	-	+	+	+	-	+	+	+
4	-	+	+	+	-	+	+	+	-	+	+	+
5	-	+	+	+	-	+	+	+	-	+	+	+
6	-	+	+	+	-	+	+	+	-	+	+	+

(4) Validity period measurement of AFB1 test strip and ZEN test strip

The results of the validity period of the test strip were shown in Table 3.25.

Table.3.25

The validity period measurement of the test strip

Time （d）	25 ℃			4 ℃			37 ℃		
	false negative (%)	false positive (%)	T line color	false negative (%)	false positive (%)	T line color	false negative (%)	false positive (%)	T line color
30	0	0	****	0	0	****	0	0	****
60	0	0	****	0	0	****	0	0	****
90	0	0	****	0	0	****	0	0	****
120	0	0	****	0	0	****	0	0	****
150	0	0	****	0	0	****	2	0	***
180	0	0	****	0	0	****	4	0	**

Note: “*” means the intensity of color.

The results showed that the appearance and accuracy of the test strip had not changed within 6 months under the conditions of 4℃ refrigerator and 25℃ room temperature, and there were no false positives and false negatives. The color of the test strip became lighter after being stored at 37 ℃ for the 150th day, with a false negative rate of 2%. The color of the test strip was lighter on the 180th day, with a

false negative rate of 4%, indicating that the stability of the test strip becomes worse.

3.4.2 Performance measurement of dual (AFB1 and ZEN) test strip

(1) Sensitivity measurement of dual test strip

Visual method. The results were shown in Figure 3.44. The results showed that when the T1 line was at AFB1 ≥ 1.0 ng/mL and the T2 line was at ZEN ≥ 5.0 ng/m, the T1 and T2 lines were clearly colored, and the sample showed a positive result. Therefore, the visual sensitivity of AFB1 and ZEN of the dual test strip were 1.0 ng/mL and 5.0 ng/mL, respectively.

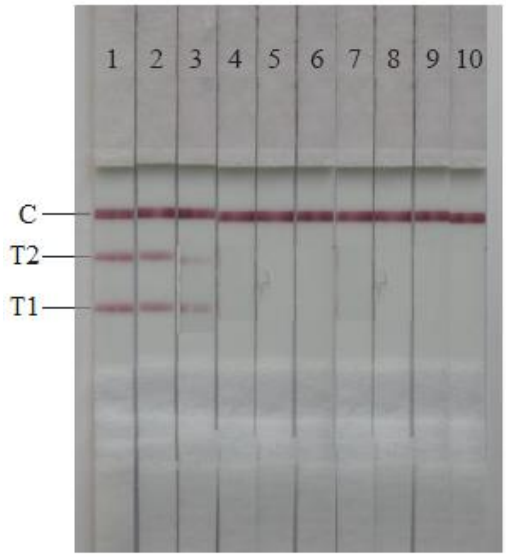


Figure.3.44 Sensitivity measurement of dual test strip. 1.AFB1/ZEN, 0/0. 2.AFB1/ZEN, 0.25/1.25. 3.AFB1/ZEN, 0.5/2.5. 4.AFB1/ZEN, 1.0/5.0. 5.AFB1/ZEN, 2.0/10.0. 6.AFB1/ZEN, 4.0/20.0. 7.AFB1/ZEN, 8.0/40.0. 8.AFB1/ZEN, 16.0/80.0. 9.AFB1/ZEN, 32.0/160.0. 10.AFB1/ZEN, 64.0/320.0. (ng/m L).

Test strip reader scanning method. The scanning results of T1 line and T2 line were shown in Figure 3.45.

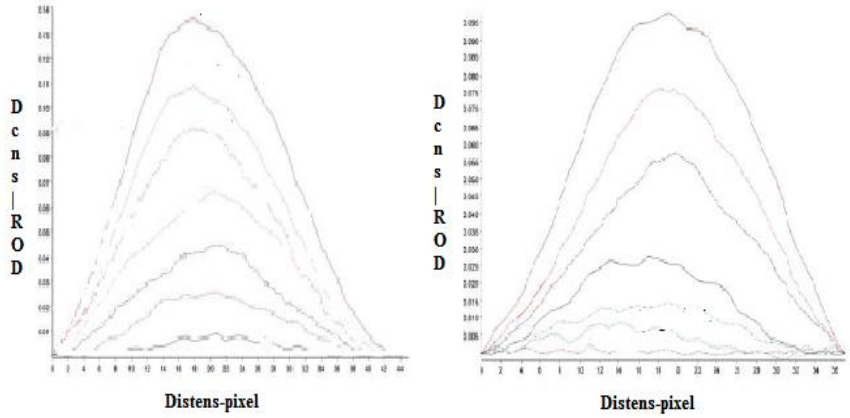


Figure 3.45 T1 and T2 scanning results of dual test strip by Bio Dot-TSR 3000.

The drawn standard curve was shown in Figure 3.46. The linear regression equation of AFB1 was y=−52.526x+46.762, the IC50 was 1.15 ng/mL, the LOD was 0.23 ng/mL. Similarly, for the ZEN test strip, the linear regression equation of ZEN was y=−34.215x+73.644, the IC50 was 4.91 ng/mL, and the LOD was 1.53 ng/mL. The above scanning results and calculated results of the deduced formula were basically consistent with that of AFB1 test strip and ZEN test strip resipectively.

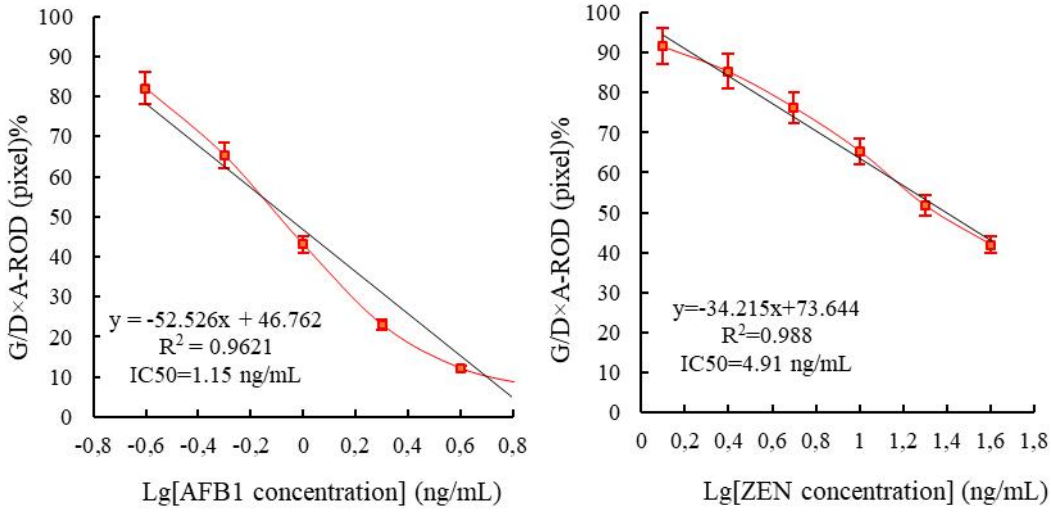


Figure.3.46 The standard curves of dual test strip detected by BioDot-TSR 3000.

(2) Stability measurement of dual test strip

Six batches of dual test strip of different batches were reproducible for corn samples, rice samples and compound feed samples. The test results were completely consistent (Table 3.26), indicating that the AFB1/ZEN dual test strip had good stability.

Table.3.26

The stability measurement of dual test strip

Batches	Concentration (AFB1/ZEN, μg/L)											
	Corn sample				Rice sample				Compound feed sample			
	0.5/2.5	1.0/5.0	2.0/10.0	4.0/20.0	0.5/2.5	1.0/5.0	2.0/10.0	4.0/20.0	0.5/2.5	1.0/5.0	2.0/10.0	4.0/20.0
1	-	+	+	+	-	+	+	+	-	+	+	+
2	-	+	+	+	-	+	+	+	-	+	+	+
3	-	+	+	+	-	+	+	+	-	+	+	+
4	-	+	+	+	-	+	+	+	-	+	+	+
5	-	+	+	+	-	+	+	+	-	+	+	+
6	-	+	+	+	-	+	+	+	-	+	+	+

(3) Determination of the validity period of dual test strip The results of the validity period of dual test strip were shown in Table 3.27. The results showed that the appearance and accuracy of the test strip had not changed within 6 months at 4℃ and 25℃, T1 line and T2 line colored clearly and there were no false positives and false negatives. The measurement results of storage at 37 ℃ for 4 months were the same as storage at 4 ℃ and 25 ℃ for six months, but the measurement results over 4 months (150 days) found that the color of T1 line and T2 line became lighter, and there were two false negative samples. The results on the 180th day showed that the color of T1 line and T2 line was lighter, and there were five false negative samples. Therefore, the dual test strip can be stored in the refrigerator (4 ℃), room temperature (25 ℃) for 6 months, and high temperature (37 ℃) for 4 months.

Table.3.27

The validity period measurement of dual test strip

Time（d）	25 ℃				4 ℃				37 ℃			
	false negative (%)	false positive (%)	T1 color	T2 color	false negative (%)	false positive (%)	T1 color	T2 color	false negative (%)	false positive (%)	T1 color	T2 color
30	0	0	****	****	0	0	****	****	0	0	****	****
60	0	0	****	****	0	0	****	****	0	0	****	****
90	0	0	****	****	0	0	****	****	0	0	****	****
120	0	0	****	****	0	0	****	****	0	0	****	****
150	0	0	****	****	0	0	****	****	2	0	***	***
180	0	0	****	****	0	0	****	****	5	0	**	**

Note: “*” means the intensity of color.

3.5 Comparative analysis of the results of dual test strip and HPLC-MS/MS

The results of AFB1 and ZEN dual test strip detecting corn positive samples were shown in Table 3.28. Among the 20 positive sample, 12 samples were positive for AFB1, with a positive value of ≥1.0 ng/mL, HPLC-MS/MS measurement results were 1.0-11.9 ng/mL, and the positive coincidence rate was 100%. 8 samples were positive for ZEN, the positive value was ≥5.0 ng/mL, the determination result of HPLC-MS/MS was 5.0-367.6 ng/mL, and the positive coincidence rate was 100%.

The test results of actual samples were shown in Table 3.29. A total of 39 positive samples were detected out of 60 samples, the positive rate was 65%, which was consistent with the detection results of HPLC-MS/MS, and the coincidence rate was 100%. Of the 39 positive samples, 22 samples were AFB1 positive samples, and the positive value of dual test strip was ≥1.0 ng/mL, the positive value of HPLC-MS/MS was 1.0-14.3 ng/mL. There were 17 samples ZEN positive samples, the positive value of dual test strip was ≥5.0 ng/mL, the positive

value of HPLC-MS/MS was 5.0 to 510.6 ng/mL, and the positive coincidence rate was 100%.

Table.3.28

Results comparison of 20 corn positive samples detected by dual test strip and HPLC-MS/MS

Sample number	dual test strip						HPLC-MS/MS					
	AFB1			ZEN			AFB1			ZEN		
	Result	PN	PV (ng/mL)	Result	PN	PV (ng/mL)	Result	PN	PV (ng/mL)	Result	PN	PV (ng/mL)
20	+	12	⩾1.0	+	8	⩾5.0	+	12	1.0-11.9	+	8	5.0-367.6

Note: “PN” means positive number. “PV” means positive value.

Table.3.29

Comparison of detection results of different positive samples by dual test strip and HPLC-MS/MS

Sample type	Sample number	dual test strip						HPLC-MS/MS					
		AFB1			ZEN			AFB1			ZEN		
		Results	PN	PV (ng/mL)	Results	PN	PV (ng/mL)	Results	PN	PV (ng/mL)	Results	PN	PV (ng/mL)
Corn	15	+	9	⩾1.0	+	7	⩾5.0	+	9	1.0-12.5	+	7	5.0-410.7
Rice	15	+	3	⩾1.0	+	2	⩾5.0	+	3	1.0-9.4	+	2	5.0-235.4
Flour	15	+	2	⩾1.0	+	2	⩾5.0	+	2	1.0-6.6	+	2	5.0-176.3
Feed	15	+	8	⩾1.0	+	6	⩾5.0	+	8	1.0-14.3	+	6	5.0-510.6
Total	60		22			17			22			17	

Note: “PN” means positive number. “PV” means positive value.

CHAPTER 4

SUMMARY AND ANALYSIS OF RESULTS

4.1 Synthesis and Identification of AFB1 and ZEN Immunogens

Synthesis and Identification of AFB1 Immunogen

(1) Synthesis method of AFB1 immunogen. The molecular weight of AFB1 is 312.27, which belongs to the small molecule hapten and has no immunogenicity. According to the hapten-carrier effect theory, the specific antibody against the hapten can only be obtained by combining with the macromolecular protein carrier to form an artificial antigen. Therefore, the design of antigen synthesis method is crucial [233]. According to the hapten-carrier effect theory, in the immunogen synthesis method, the main factors that affect the characteristics of the antibodies produced by the immunogen include: The complexity of the hapten molecular structure, the selection of the position of the active site, the length and molecular space structure of the spacer arm, the molecular binding ratio of the hapten to the carrier protein, etc. [234]. The complexity of hapten molecular structure refers to whether the hapten molecular structure contains benzene ring and number, heterocycle and number, branch structure and number. Generally, haptens with complex structures are easy to induce the body to produce antibodies, while haptens with simple structures are more difficult to induce the body to produce antibodies [235]. Szurdoki et al. [236] reported that the success rate of antibody preparation was 33.3% with a benzene ring in the hapten molecular structure, while the success rate without a benzene ring was only 9.1%. Regarding the selection of active sites, on the one hand, different active sites will lead to changes in the distribution of electrons in the hapten molecule, thereby changing its electrochemical properties, thereby changing its immune activity, and affecting the affinity of producing antibodies; on the other hand, because different active sites

lead to different spatial structures of molecules, the exposed antigenic determinants are also different, which in turn affects the specificity of antibodies [237]. Therefore, try to use different sites to synthesize immunogens separately, and then screen out the best immunogens for target antibody preparation through comparative analysis. The choice of the spacer arm includes the length and structure of the spacer arm. Regarding the determination of the length of the spacer arm, Tuomola et al. [238] and Jung et al.[239] proposed that the optimal length of the spacer arm is 3 to 6 carbon atoms. The short spacer arm is not conducive to the full exposure of the hapten, and the long spacer arm will cause the folding of the alkyl chain due to hydrophobic interaction, which will cause the hapten molecule to be covered by the carrier protein, which is not conducive to the recognition of antigen-presenting cells; for the selection of spacer arm structure, Bruun et al. [240] and kolosova et al. [241] believe that the spacer arm should not contain structures with high immune activity, such as aromatic ring, conjugated double bond or heterocycle, so as to reduce the over recognition of the generated antibody to the spacer arm and reduce the recognition ability of the target molecule. As for the determination of molecular binding ratio, the research results of Vikas et al. [242] and Peterson et al. [243] show that when the molecular binding ratio is large, the carrier effect is weakened because too much hapten is covered on the carrier protein, which is not conducive to the binding between the carrier protein and lymphocytes; When the molecular binding ratio is too small, the immunogen is not enough to cause the body to produce immune response; The best molecular binding ratio of hapten to carrier protein should be 5 to 20 : 1.

In this study, according to the molecular structure characteristics of AFB1, C1 carbonyl, C2 active hydrogen, C3 hydroxyl and C3-C4 bifuran ring were selected as active groups. Through different chemical reaction methods, the available carboxyl, hydroxyl, aminomethyl and other active groups were introduced to realize the coupling with carrier protein to synthesize immunogens. On the basis of referring to a large number of literatures, this study used six methods to synthesize the immunogen AFB1-BSA of AFB1: oxime active ester

(OAE), methylation of ammonia (MOA), mixed anhydride（MA）, hemiacetal method (semi acetal, SA), epoxide method (epoxide, EP) and enol ether derivative method (enol ether derivative, EED). OAE method is to synthesize afb1-bsa (OAE) in the form of monoamide bond between afb1o and BSA under the action of coupling agent Dicyclohexylcarbodiimide (DCC) by carbonyloxime at C1 position of AFB1 active site to afb1o and introducing carboxyl active group [190, 191]. The principle of MOA method is to use the α-active hydrogen at the C2 position of AFB1, through the Mannich reaction, make the α-active hydrogen and the amino group of BSA undergo aminomethylation reaction, and couple in the form of Mannich base to synthesize AFB1-BSA (MOA) [192]. The principle of MA method is that AFB1 is converted into AFB2a under the action of H2SO4. Using the active site C3 hydroxyl group to react with acid anhydride, the product is the half-ester compound AFB2a-HS. After that, carboxyl reactive groups were introduced, and under the action of the coupling agent isobutyl chloroformate (IBC), AFB1O and BSA formed a single amide bond to synthesize AFB1-BSA (MA) [193, 194]. The principle of SA method is using the 3-position aldehyde group of AFB2a was selected as the active site, and the aldehyde group formed an unstable Schiff-base with the amino group of the carrier protein. Under the action of NaBH4, the AFB1-BSA antigen was synthesized via a monoamide bond as the spacer arm [195]. The principle of EP method is to take dichloromethane as solvent, oxidize C3-C4 double furan ring double bond to form AFB1 epoxide, react with the primary amine of BSA to form secondary amine, introduce a hydroxyl on the epoxide and couple with BSA in the form of monoamide to synthesize afb1-bsa (EP) [196]. The principle of EED method is that AFB1 molecular structure contains active site C3-C4 bifuran ring, which can react with glycolic acid to produce AFB1 enol ether derivative (afb1-ga) with active group carboxyl group, and afb1-bsa (EED) is synthesized by coupling its carboxyl group with BSA [197].

(2) Animal immunization method. The specificity of antibodies not only depends on the physicochemical properties of the immunogen, but also is closely

related to the immunization method [244]. Due to the complex immunochemistry, antibodies produced by animals immunized with the same immunogen have markedly different immunological properties. Influencing factors for immunization methods include injection route, dose, interval, adjuvant use, frequency, and individual differences in animals, of which dose and interval are key factors. Some scholars have pointed out that low-dose immunogen induces a narrower antibody spectrum specificity, while high-dose immunogen induces a wider antibody spectrum specificity [245]. Immunogens cause the production of memory B cells, which require a certain immune interval to clone and proliferate to produce several specific antibodies. Moreover, a certain immunization interval can improve the affinity of the antibody. In general, the interval between immunizations of animals is not less than two weeks [246]. In this study, a small dose (30 μg/mL), a longer immunization interval (4 weeks), multiple sites (4-6 points) on the back, and multiple frequency (5 times) immunization methods were used.

(3) Analysis of the immune effect of AFB1 immunogen. The purpose of this study was to screen out high-quality antibodies with high sensitivity and specificity to AFB1 to meet the detection technical requirements under the limited standard of AFB1, and the preparation of immunogens is the premise of obtaining high-quality antibodies. At present, the research on the synthesis method of AFB1 immunogen is still at the empirical level, and the trial and error method is mostly used. Although a variety of immunogen identification methods have been established, the immunogenicity of the prepared immunogen is ultimately confirmed by the effect of animal immunization [247]. In this paper, on the basis of referring to a large number of relevant research literatures, AFB1 was used as the starting material for the reaction, and six methods including OAE method, MOA method, MA method, SA method, EP method and EED method were used to synthesize immunogen. Immunogen identification by UV, SDS-PAGE and antibody characterization by animal immunization. In this study, the molecular binding ratios of AFB1 and BSA in the immunogens prepared by different synthetic methods were 8.64:1 (OAE), 6.88:1 (MOA), 10.78:1 (MA), 4.463:1 (SA), 6.38 :1

(EP) and 2.31:1 (EED). The results of immunizing animals with 6 different immunogens and analyzing the characteristics of the antibodies produced showed that the OAE method was the best. The AFB1 pAb antibody produced has a high titer, with an indirect ELISA titer of 1: (1.6×103), good sensitivity to AFB1, IC50 of 10.14 μg/L, strong specificity, it can recognize AFB1 100%, and has certain CR with afb2, AFG1, afg2, amf1 and afm2, which are 6.32%, 3.76%, less than 1.0%, less than 0.1% and less than 0.1% respectively. Li et al [245] reported that high specificity AFB1 mAbs 1B5 and 2F12 were also prepared by OAE method, and their IC50 for AFB1 were 0.012 μg/L and 0.01 μg/L, respectively. The CR of AFB1 mAb 1B5 with AFB2, AFG1, AFG2, and AMF1 was 4.0%, 3.0%, <0.1%, and <0.1%, respectively, but no CR with AFM2 was reported. The CR of AFB1 mAb 2F12 with AFB2, AFG1, AFG2, and AMF1 was 5.0%, 2.0%, 0.2%, and <0.1%, respectively, and CR with AFM2 was also not reported. Simultaneously, the study found that the OAE method has significant advantages, such as easy construction of the reaction system, mild reaction conditions, simple operation steps, and high product yield. However, in terms of the advanced nature of the technical route adopted in this study, the research and application of molecular simulation technology and computer-aided technology need to be improved [248, 249].

In summary, according to the molecular structure of AFB1, this study designed six immunogen synthesis methods, identified and characterized the AFB1 pAb produced by immunizing animals by UV and SDS-PAGE. The results show that OAE method is an effective way to realize the preparation of AFB1 antibody with high immunoreactivity, high sensitivity and high specificity among the six established immunogen synthesis methods, which laid a material and technical foundation for the establishment of AFB1 specific immune detection method.

Synthesis and identification of ZEN immunogen

(1) ZEN immunogen synthesis method. ZEN is a metacyclic acid lactone compound containing the main characteristic structures of 2,4-dihydroxybenzene ring and macrolide ring. ZEN and its five analogs are structurally identical except

for slight differences in the carbonyl or hydroxyl group at the C6' position of the macrolide ring and the single or double bond at the C1'-C2' position. Therefore, the special chemical properties of ZEN have laid a good foundation for the preparation of five types of specific antibodies to ZEN and its analogs, but also brought difficulties to the preparation of highly specific antibodies to ZEN [153]. The specificity of antibodies mainly depends on the recognition of immunogenic determinants by immune cells. The spatial conformation and characteristic structure exposure of haptens on carrier proteins are closely related to the immune recognition of immune cells. Thus, in hapten design, its main characteristic structure must be exposed to the maximum extent as part of the immunogen to obtain the desired target antibody [250, 251]. As a consequence, ZEN immunogen synthesis is the key to the preparation of ZEN highly specific antibodies.

In this study, on the basis of the molecular structure of ZEN, oxime active ester (OAE), condensation mixed anhydride (CMA), formaldehyde (FA), 1,4-butanediol diglycidyl ether (BDE) and amino glutaraldehyde (AGA) were used to synthesize immunogens and detect antigens. OAE method is to select the C6 'carbonyl group on Zen molecule as the active site, synthesize Zen oxime (Zeno) through oximation reaction with CMO, introduce the carboxyl group of active group, and combine the carboxyl group of hapten Zeno with the amino group of BSA through monoamide bond couple under the action of EDC [252]. CMA method is to select the C6 'carbonyl group on ZEN molecule as the active site, introduce hydroxyl group through condensation reaction with hydroxylamine hydrochloride, and the hydroxyl group reacts with succinic anhydride to introduce the active group carboxyl group. The introduced carboxyl group can react with isobutyl chloroformate in the presence of tri-n-butylamine to produce an active intermediate mixed anhydride, and then react with the primary amino group on the protein carrier to synthesize the immunogen ZEN-BSA (CMA) through single amide bond coupling [253]. FA method takes the C7 'active hydrogen on Zen molecule as the active site, the hapten containing active hydrogen and the amino group of protein undergo Mannich condensation reaction under the action of

formaldehyde, and the artificial antigen ZEN-BSA (FA) is synthesized by double ammonia bond coupling [250]. BDE method uses the C2 hydroxyl active site on Zen molecule. Both ends of 1,4-butanediol diglycidyl ether contain an ethylene oxide group respectively. One end of ethylene oxide group can react with the hydroxyl of hapten to form ether bond, and the other end of ethylene oxide group can react with the amino group of carrier protein to form secondary amine, which is combined into artificial antigen ZEN-BSA (BDE) through long-chain couple [153]. AGA method is to select the C5 position of benzene ring on Zen molecule as the active site, introduce nitro through nitration reaction, and after that introduce amino active group through reduction reaction. The introduced amino active group is coupled with the amino group of BSA through Mannich reaction in the form of mannich base to synthesize afb1-bsa (AGA) [202].

(2) Animal immunization method. The same as AFB1 immunogen, its purpose is to prepare high specific antibody. Therefore, this study also adopts the immune method of low dose (30 μg/mL), long immune interval (4 weeks), multiple points on the back (4-6 points) and multiple frequencies (5 times).

(3) Analysis of the immune effect of ZEN immunogen. As far as the current research progress is concerned, to realize the single immune detection of ZEN residues, it is necessary to improve the specificity of ZEN antibodies. The preparation of ZEN highly specific antibodies is mainly completed by immunizing animals with synthetic immunogens by FA method and AGA method. Burkin et al. [254] reported that the immunogen ZEN-BSA was synthesized by FA method, and ZEN pAb was prepared by immunizing rabbits, which could specifically recognize ZEN. The IC50 for ZEN was 2.0 μg/L, and the CR with α-ZAL, β-ZAL, α-ZOL and ZON were 0.15%, 0.02%, 0.12% and 31.7%, respectively. Gao et al [250] reported that the immunogen ZEN-BSA was synthesized by FA method, and the molecular binding ratio was 12:1. ZEN pAb and ZEN mAb were prepared by immunizing Balb/c mice, and ZEN pAb could specifically recognize ZEN.

The IC50 for ZEN was 233.35 μg/L, and the CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were 2.25%, 5.65%, 3.14%, 1.96% and 6.79%, respectively;

ZEN mAbs specifically recognize ZEN. The IC50 for ZEN was 55.72 μg/L, and the CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were 0.63%, 0.92%, 0.65%, 0.94% and 1.48%, respectively.

Teshima et al. [202] reported that the immunogen ZEN-BSA was synthesized by AGA method, and ZEN mAb was prepared by immunizing Balb/c mice. ZEN mAb could specifically recognize ZEN, and its IC50 for ZEN was 11.2 μg/L. CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON was less than 0.1%, 0.1%, 0.9%, 0.1% and 4.0%, respectively. In this study, AGA method was used to prepare ZEN highly specific antibody, and its IC50 for ZEN was 18.77 μg/L. The CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON was 1.48%, 1.36%, 3.57%, 1.65% and 4.86%, respectively [211].

To sum up, in this study, ZEN was used as the reaction starting material, and the immunogen was synthesized by OAE method, CMA method, FA method, BDE method and AGA method.

Immunogen identification by UV and SDS-PAGE and comparative analysis of antibody characteristics by animal immunization showed that: OAE method is the best method to prepare broad-spectrum specific antibodies to ZEN and its homologues, and its IC50 for ZEN is 11.67 μg/L. The CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON was 36.53%, 16.98%, 64.33%, 20.16% and 10.66%, respectively. AGA method is the best method to prepare ZEN highly specific antibody, and its IC50 for ZEN is 18.77 μg/L. The CR with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON was 1.48%, 1.36%, 3.57%, 1.65% and 4.86%, respectively.

4.2 Preparation and identification of AFB1 mAb and ZEN mAb

Preparation and identification of AFB1 mAb

(1) Screening method for AFB1 mAb-positive hybridoma cell lines. The screening of hybridoma cells is a key step in the preparation of high-quality monoclonal antibodies. As a typical response to artificial hapten carrier conjugates, B lymphocytes produce different antibody molecules that recognize different parts

of the conjugate. In addition, each B lymphocyte produces a specific type of antibody molecule [255]. Therefore, it is particularly important to establish a reliable screening system for positive hybridoma cells.

Multiple hybridoma cell lines can be obtained by one cell fusion. Although the antibodies secreted by them can react with the same immunogen, if the immunogen has more than one antigenic determinant, the antibodies secreted by these hybridoma cell lines may be different. Even if the generated antibodies are directed against the same epitope, different types of antibodies will still show different affinity for the same epitope due to the differences in types and subclasses of immunoglobulins [256]. Accordingly, in the screening of positive hybridoma, there is more than one hybridoma cell in most holes at the first detection, that is, several cells may exist at the same time, and their binding strength to hapten may be different. It is also unknown whether the number of cells that can secrete high affinity antibody is dominant in several cells. Moreover, cells secreting high affinity antibodies tend to grow slowly. Thus, in order to avoid the omission of positive hybridoma cell lines secreting high affinity antibodies, a gradient two-step screening method was used in this study. High concentration AFB1 was used in the initial screening, which can avoid the omission of cells secreting high affinity antibodies and not dominant in the pores. After three subclones, most of the wells are single cells, and the binding strength to AFB1 can be clearly seen through the competitive reaction. Thereby, low concentration AFB1 is used in the second screening. At this time, if low concentration AFB1 can still significantly inhibit the color development of a well supernatant, it indicates that the antibody secreted by the well cells has a high binding strength to AFB1. The screening results showed that the positive hybridoma gradient two-step screening method could screen more cell lines with higher affinity. To this end, the present study employed a heterologous inELISA against the immunogen AFB1-BSA (OAE) using AFB1-OVA (SA) as the coating antigen to measure cell culture supernatant antibody titers. Heterologous icELISA was used to determine the ability of positive hybridomas to recognize AFB1 and cross-react to AFB1 analogs. The gradient

two-step screening method was used to screen out three positive hybridomas with strong immunoreactivity, high sensitivity and high specificity of AFB1 mAbs 2A11, 2F6 and 3G2. Among them, the best-performing AFB1 mAb 2A11, the titers of cell culture supernatant and ascites fluid were 1∶(6.4×102) and 1∶(5.12×105), respectively, and the IC50 for AFB1 was 6.28 μg/L. The CR with AFB2, AFG1, AFG2, AFM1 and AFM2 was 4.35%, 2.30%, less than 1.0%, less than 0.1% and 0.1%, respectively.

In this study, a hybridoma cell screening system was successfully established. The author believes that the system has four characteristics. First, it can accurately measure the antibody titer of the cell culture supernatant after cell fusion and correctly evaluate the immunoreactivity; Second, it is able to fully recognize AFB1, that is, to exclude the interference of bridge antibodies, and to correctly evaluate the recognition of antibodies; Third, it can accurately determine the ability of the antibody to recognize AFB1, determine the IC50 value of the antibody to AFB1, and correctly evaluate the sensitivity of the antibody; Fourth, it is able to accurately measure the cross-reactivity of the antibody with AFB1 analogs, and correctly evaluate the specificity of the antibody to AFB1.

(2) Production method of AFB1 mAb. In this study, AFB1 mAb was prepared by in vivo ascites-induced method [257]. Freund's incomplete adjuvant and liquid paraffin were selected as pretreatment reagents for ascites preparation. The experimental results showed that in the production of ascites, the mice treated with incomplete Freund's adjuvant produced a total of about 3 mL/mouse, while the liquid paraffin-treated mice produced a total of about 1.5 mL/mouse. There are two reasons for this result. First, the volume of ascites extracted in each time, the mice treated with incomplete Freund's adjuvant were more than the mice treated with liquid paraffin. Secondly, the mice treated with incomplete Freund's adjuvant can extract ascites 3-4 times, while those treated with liquid paraffin are mostly 1-3 times. However, the cost of Freund's incomplete adjuvant is much higher than that of liquid paraffin, and different laboratories can choose to use it according to their own needs. In addition, it is worth noting that the existing monoclonal antibody

preparation methods include in vivo induction of ascites, in vitro culture in cell culture medium, in vitro production in bioreactors, single cell technology, and phage display technology [258].

Due to the advantages of simple operation, low cost, short cycle, high antibody concentration and high yield, the in vivo induced ascites method is still widely used in most laboratories [259]. Nevertheless, this method is still acceptable. For example, the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) clearly states that, "under any circumstances, the use of ascites for antibody production should no longer be accepted" (Barroso et al., EURL ECVAM recommendations for antibodies of non-animal origin). Although the in vivo ascites induction method was still used in this study, the authors suggest that, for animal protection and animal ethics considerations, the in vitro culture method should be advocated instead of the in vivo ascites induction method for the production mAbs in future studies.

(3) Purification of AFB1 mAb. In addition to specific antibodies, there are many non-antibody proteins such as albumin, globulin, and lipids among the monoclonal antibodies prepared by the in vivo ascites-induced method. Hence antibodies need to be separated and purified to remove impurities and other components before use. Currently, commonly used antibody purification methods include ammonium sulfate precipitation, absolute ethanol precipitation, octanoic acid-ammonium sulfate precipitation, affinity chromatography and euglobulin precipitation, etc. The corresponding methods are selected according to different needs. When the requirements for protein purity are not high, the saturated ammonium sulfate method can be selected. This method can remove part of the impurity protein, but the purity is not high, and can also be used as the pretreatment of other purification methods. Ammonium octanoate sulfate method has better effect on the extraction of IgG1 and IgG2a, while the effect on IgG3 and IgA is relatively weak. The purity of IgG extracted by affinity chromatography is relatively high, but this method is easier to form affinity-coupled ligands and needs to be eluted with eluent during the purification process, which may damage the

activity of antibody proteins, and the cost of affinity column is high. The euglobulin precipitation method is more suitable for the purification of IgG3 and IgM, especially the extraction rate of IgG3 can reach more than 90% [260, 261]. The subtypes of AFB1 mAbs 2A11, 2F6, and 3G2 obtained in this study were IgG1, IgG1, and IgG2a, respectively. Therefore, the ammonium caprylate sulfate method, which has a better effect and lower cost in purifying IgG1 and IgG2a, was selected. The principle of octanoic acid sulfuric acid purification is to use octanoic acid to combine with other proteins except IgG in ascites under acidic conditions and form a precipitate. The supernatant is mainly IgG, and then the supernatant is precipitated with ammonium sulfate, IgG with higher purity can be obtained [262].

(4) Genetic stability evaluation of AFB1 mAb-positive hybridoma cell lines. Hybridoma cells are fusion cells of spleen cells and tumor cells, and their ability to secrete antibodies is easily changed by external factors. Therefore, long-term evaluation of the genetic stability of the selected hybridoma cell lines is required. In this study, the hybridoma cell lines were continuously subcultured for 5 generations, and the culture supernatant of hybridoma cells was collected every 10 days, and the stability was determined by competition method. The results showed that the cell line had better activity and stability, and could secrete antibodies continuously and stably. Additionally, in order to prepare AFB1 mAb in large quantities, the cryopreserved hybridoma cell lines were recovered several times in this study, and were cultured for a long time to collect cells and injected into mice to prepare ascites in large quantities, and then determine the changes in the IC50 value of AFB1 mAb in ascites. The results showed that the hybridoma cell line could stably secrete AFB1 mAb.

(5) Affinity evaluation of AFB1 mAb. The mAb affinity refers to the binding strength between a single determinant on the surface of an antigen and a single binding site of the corresponding antibody. It is the most essential description of the interaction between an antigen and an antibody, and is often expressed by an affinity constant (Ka). There are various methods to determine Ka, including equilibrium dialysis, fluorescence quenching, non-competitive ELISA

saturation method, icELISA method, thiocyanate elution (TCE), etc. [263, 264]. In recent years, with the development of science and technology and the improvement of experimental conditions, some new measurement methods such as reflectometric interference spectroscopy (RIS), electrophoretic band migration method, surface plasmon resonance (SPR), quartz crystal microbalance (QCM) have appeared one after another[265, 266]. Among the above methods, the Batty saturation method is often used to measure the Ka of specific antibodies produced by small molecule haptens, but this method has been questioned by quite a few scholars for a period of time after it was proposed. The main problem is that most scholars believe that the process of antigen immobilization and coating makes the antigen-antibody complex reaction transferred from the homogeneous pure liquid phase system to the solid-liquid interface. This makes it possible to alter the kinetics of the reaction and limit the application of the law of mass action [267]. However, some scholars have proved theoretically and experimentally that the law of mass action is still applicable when the experimental conditions of the ELISA reaction are strictly controlled. Moreover, the Ka value of anti-CEA IgG measured by this method is completely consistent with the Ka value measured by equilibrium dialysis method. So far, Batty saturation method has been recognized by the world and is currently the most common method for determination of mAb affinity [268, 269]. In this study, the Ka of AFB1 mAb 2A11, 2F6 and 3G2 determined by Batty saturation method were 1.05×10^{9}, 9.64×10^{8} and 7.71×10^{8} L/moL, respectively. On the basis of the evaluation method of mAb affinity[270], high-affinity mAbs with a Ka of 10^{7} to 10^{12} L/moL. The three AFB1 mAbs prepared in this study are all high-affinity mAb.

(6) Establishment and optimization of icELISA method. There are two main ELISA modes for the analysis of small molecule compounds, one is indirect competitive ELISA (icELISA). The principle of icELISA is that the antigen is coated on a solid phase carrier, and the antigen to be tested is mixed and incubated with a known amount of enzyme-labeled antibody at the same time. Since the solid-phase antigen and the antigen to be tested have the same epitope, they

compete with each other indirectly to form an immune complex with enzyme labeled antibody, and then add enzyme reaction substrate to determine the content of small molecule hapten to be tested according to the color development degree. The icELISA mode can be expressed as: Ag+Ag*+Ab=Ag Ab+Ag*Ab+Ag+Ag*+Ab, where Ag is the free antigen to be tested, Ag* is the coated antigen, and Ab is the specific antibody, Ag Ab is a complex of free antigen and antibody, and Ag*Ab is a complex of coated antigen and antibody. Ag* is coated on the solid phase carrier, Ag* and Ag compete for the limited sites on the Ab, and the excess Ag and Ag* are eluted after the reaction is equilibrated. The binding of Ag Ab and Ag*Ab was displayed by the enzyme system, and then the content of Ag in the sample was estimated [271, 272]. The second is direct competitive ELISA (dcELISA), which is based on the principle that the specific small molecule compound antibody is coated on the solid phase carrier, and the drug antigen to be tested is mixed with the known amount of enzyme labeled antigen for incubation. Among them, the drug to be tested and the enzyme labeled drug with the same antigenic determinant are competitively combined with the solid-phase antibody to separate the free and bound enzyme labeled antigen antibody complex. Add enzyme reaction substrate and determine the content of small molecular compound to be tested according to the color development degree. The dcELISA mode is expressed as: Ag+Ag*+Ab=Ag Ab+Ag*Ab+Ag+Ag*+Ab, where Ag is the free antigen to be tested, Ag* is the enzyme-labeled antigen, Ab is the specific antibody, AgAb is the free antigen and specific antibody complex, Ag *Ab is the complex of enzyme-labeled antigen and specific antibody. The Ab was coated on the solid-phase carrier, Ag and Ag * competed for the limited sites on ab. after the reaction reached equilibrium, the excess Ag and Ag * were eluted. The binding of Ag AB and Ag * AB was displayed by horseradish peroxidase (HRP) and its substrate color development system, and then the content of Ag in the sample was calculated quantitatively [273, 274]. Since the enzyme-labeled antibodies in the icELISA mode are standardized and commercialized reagents, the establishment of the icELISA method is relatively simple and stable. However, the

enzyme-labeled hapten in the dcELISA mode is a self-made reagent. The cross-linking method of the hapten and HRP is different, and the activity and stability of the enzyme are also different. Thus, the establishment of dcELISA method is difficult and lacks stability. Therefore, this study adopts the established icELISA method to determine the sensitivity and specificity of the antibody.

The reagents required by the icELISA system used for AFB1 determination in this study mainly include coated antigen, blocking solution, AFB1 mAb, enzyme-labeled secondary antibody, enzyme reaction substrate and stop solution. Afb1-ova was selected as the coating antigen, PBS solution containing 5% porcine serum as the blocking solution, AFB1 mAb 2A11 specific antibody, GaMIgG-HRP as the enzyme labeled secondary antibody, TMB as the enzyme reaction substrate and 2 mol/L H_2SO_4 as the termination solution to establish the detection method of AFB1 icELISA. Since both AFB1 mAb and GaMIgG-HRP in icELISA are biologically active substances, therefore, the determination results of icELISA are easily affected by factors such as the type and concentration of coating antigen, antibody concentration, enzyme-labeled secondary antibody concentration, incubation temperature, incubation time, buffer pH, and buffer organic solvent content [275]. In this study, the parameters of icELISA were optimized, mainly including four aspects: first, the types of coating antigens were screened by combination method. AFB1 mAb 2A11 was used as antibody, AFB1-OVA (OAE), AFB1-OVA (MA), AFB1-OVA (SA), AFB1-OVA (MOA), AFB1-OVA (EP) and AFB1-OVA (EED) were used as coating antigens, and the IC50 values of AFB1 mAb 2A11 were measured respectively, the combination with the lowest IC50 value was selected to establish icELISA. The results showed that among the six coating antigen and antibody combinations, the IC50 of AFB1 mAb 2A11 and AFB1-OVA (SA) was the lowest. Therefore, AFGB1-OVA (SA) was selected as the coating antigen. Second, the checkerboard method was used to determine the optimal working concentrations of the coating antigen, antibody and enzyme-labeled secondary antibody, and AFB1-OVA (SA) was selected as the coating antigen. When the working concentration of AFB1-OVA (SA) was 2.0 μg/mL, the

working concentration of AFB1 mAb 2A11 was 1:3200 and that of GaMIgG-HRP was 1:1000. The third is to determine the appropriate concentration of methanol, the organic solvent of the buffer. The appropriate methanol concentration not only does not affect the sensitivity of icELISA, but also helps to dissolve AFB1. When the methanol concentration exceeds 30%, the Amax and Amax/IC50 values decrease, but when the methanol concentration is lower than 30%, the effect is not significant. The fourth is to determine the appropriate pH value of the buffer. When the pH of the buffer was between 5.0 and 9.0, there was no significant effect on Amax/IC50 and Amax, while at pH 7.4, Amax/IC50 and Amax were the highest, indicating that the antibody was fully bound to the antigen. Thus, it is appropriate to select PBS with pH value of 7.2 to 7.4 as the buffer in this study.

(7) Sensitivity and specificity evaluation of AFB1 mAb. Sensitivity is one of the important indicators to evaluate the antigen-antibody reaction pattern, expressed by IC50 value, the lower the value of IC50, the higher the sensitivity [276]. In this study, under the optimal assay conditions, the icELISA standard curve of AFB1 was established, and the IC50 values of AFB1 mAbs 2A11, 2F6 and 3G2 were determined. The results are shown in Table 3.9. The IC50 values of AFB1 mAbs 2A11, 2F6 and 3G2 were 6.28, 7.85 and 14.36 μg/L, respectively, of which mAb 2A11 was the most sensitive to AFB1. Specificity represents the ability of an antibody to recognize the corresponding antigen or hapten, which is determined by the complementarity between the spatial structure of the hypervariable region of the antibody molecule and the antigenic determinant. It is expressed by the CR value，the lower the value of CR, the stronger the specificity [277]. Table 3.9 shows the CR values of three AFB1 mAbs, 2A11, 2F6 and 3G2, the results show that the CR values of mAbs 2A11, 2F6 and 3G2 are 0.1%~4.35%, 0.1%~4.65% and 0.1%~5.44%, respectively. Among them, mAb 2A11 had the lowest CR value. At the same time, the IC50 value of the three mAbs for ZEN, DON, T-2 toxin and OTA were all greater than 10000 μg/L, and the CR values were all less than 1%, indicating that the prepared AFB1 mAbs are CR-free with

other mycotoxins. Since mAb 2A11 has the lowest IC50 value and lowest CR values, it was selected for the follow-up study.

So far, there have been several reports using immunogens developed by OAE and SA methods to obtain AFB1 mAbs with high sensitivity and specificity for AFB1. A comparison between mAb 2A11 in this study and other AFB1 mAbs reported in recent literature is shown in Table 4.1. mAb 2A11 has high affinity, IC50 value of 6.28 μg/L, high specificity, and CR values ranging from 0.1% to 4.35%, as expected. Therefore, mAb 2A11 can be used to establish an immunoassay for the analysis of AFB1 in food and feed. However, as reported by Li et al.[245], mAb 2A11 has higher IC50 values (0.012 μg/L) than mAb 2F12, but lower CR values (0.1% to 5.0) than 2F12 [278], mAb 2F12 was derived from the OAE method. This result suggests that antigen-antibody recognition is based on interactions generated by steric criteria and molecular electronic properties, so the positional space or number of epitopes may play an important role in antigen-antibody interactions [278]. As the dose of the immunogen was increased, the epitopes became more diverse. Thus, the opportunity to select a variety of monoclonal antibodies has arisen, which means that low doses of immunogens help to obtain narrow-spectrum antibody specificities, while high doses of immunogens help to develop broad-spectrum antibody specificities. Zhang et al. [279] used the SA method to prepare the immunogen AFB1-BSA (SA) to obtain AFB1 mAb 3A12 with an IC50 value of 6.1 μg/mL for AFB1. The CR values of AFB2, AFG1, AFG2, and AFM1 were 7.8%, 20.2%, 0.6%, and less than 0.1%, respectively, and a highly specific mAb for AFB1 was prepared. It is believed that AFB1 has two characteristic structural regions which are different from AFB2, AFG1, AFG2 and AFM1, namely the difuran ring and the cyclopentenone ring. When AFB1-BSA (OAE) was used as the immunogen, due to the shielding effect of the carrier protein or the linking arm and the great similarity in the structure of AFs, the obtained antibody had a high CR value with AFG1 and AFG2. AFB2a is the hemiacetal form of AFB1, which can exist in the form of dialdehyde phenate in aqueous solution, and its aldehyde group and the amino group of BSA can generate

unstable Schiff base. Under the action of reducing agent sodium borohydride (NaBH), the C=N double bond was reduced to form stable AFB2a-BSA. Because AFB2a greatly retains the difference structure between B-group cyclopentenone and G-group lactone, it can effectively induce the body to produce highly specific AFB1 antibody and reduce the CR with AFG1, AFG2, AFM1, and AFM2. However, compared with AFB1 mAb 2A11 and 3A12, the CR value of AFB1 mAb 2A11 and AFB1 analogs was significantly lower, that is, the specificity of AFB1 mAb 2A11 was significantly better than that of 3A12. Therefore, the OAE method was still used in this study to prepare AFB1 highly specific mAb.

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Preparation and identification of ZEN mAb

(1) Preparation of ZEN mAb. In this study, a heterologous inELISA against the immunogen ZEN-BSA (AGA) and AFB1-OVA (FA) as the coating antigen was used to measure the antibody titer of the cell culture supernatant. Heterologous icELISA was used to determine the ability of positive hybridomas to recognize ZEN and cross-react to ZEN analogs. The gradient two-step screening method was used to screen out two positive hybridomas with strong immunoreactivity, high sensitivity and high specificity, 2B6 and 4D9. Among them, ZEN mAb 2B6 had the best performance, and the titers of cell culture supernatant and ascites fluid were 1:(5.12×10^2) and 1:(5.12×10^5), respectively. The IC50 for ZEN was 10.38 μg/L, and the CRs for α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were 1.52%, 1.28%, 2.64%, 1.83% and 4.27%, respectively. Therefore, ZEN mAbs with high immunoreactivity, high sensitivity and high specificity were obtained in this study.

Table 4.1

Comparison of IC50 and CR of AFB1 and its analogs by OAE and SA method in this study and other previously reported AFB1 pAbs and mAbs

References	AFB1 antibody	Coupling method	Immunoassay format	IC50 of AFB1 (μg/L)	CR (%) [a]				
					AFB2	AFG1	AFG2	AFM1	AFM2
This study (2021)	AFB1 pAb	OAE	icELISA	10.14	6.32	3.76	＜1.0	＜0.1	＜0.1
	AFB1 mAb 2A11	OAE	icELISA	6.28	4.35	2.30	＜1.0	＜0.1	＜0.1
	AFB1 pAb	SA	icELISA	19.36	8.14	18.44	＜1.0	＜0.1	＜0.1
Li et al. (2017) [21]	AFB1 mAb 2F12	OAE	icELISA	0.01	5.0	2.0	0.2	＜0.1	＜0.1
	AFB1 mAb 1B5	OAE	icELISA	0.012	4.0	3.0	＜0.1	＜0.1	＜0.1
Zhang et al. (2011) [60]	AFB1 mAb 3A12	SA	icELISA	6.1	7.8	22.2	0.6	＜0.1	＜0.1

Note. [a] All of the data were calculated using the CR of AFB1 as 100%.

(2) Identification of ZEN mAbs Genetic stability evaluation. After 5 subcultures, the titer and IC50 value of ZEN monoclonal antibody in cell culture supernatant and ascites fluid did not change significantly. The results showed that hybridoma cells had genetic stability. Affinity evaluation. In this study, the Batty

saturation method was used to determine the Ka of ZEN mAbs 2B6 and 4D9, which were 7.69×10^9, and 4.95×10^9 L/mol, respectively, both of which were high-affinity antibodies. Establishment and optimization of icELISA method. In this study, the parameters of icELISA were optimized, mainly including four aspects. First, the types of coated antigens were screened by combination method. Using Zen mAb 2B2 as antibody and zen-ova (AGA), zen-ova (OAE), zen-ova (CMA), zen-ova (FA) and zen-ova (BDE) as coating antigen, the IC50 value of Zen mAb 2b6 was determined, and the combination with the lowest IC50 value was selected to establish iceelisa. Second, the optimal working concentration of the coated antigen, antibody and enzyme-labeled secondary antibody was determined by the checkerboard method. ZEN-OVA (FA) was selected as the coating antigen. When the working concentration of ZEN-OVA (FA) was 2.0 μg/mL, the working concentration of ZEN mAb 2B6 was 1:1600, and the working concentration of GaMIg G-HRP was 1: 1000. The third is to determine the appropriate concentration of methanol in the buffer, the methanol concentration should be less than 30%. The fourth is to determine the appropriate pH value of the buffer solution, when the appropriate pH value of the buffer solution is 7.4. Sensitivity and specificity evaluation. In this study, the icELISA standard curve of ZEN was established under the optimal assay conditions, and the IC50 values of ZEN mAb 2B6 and 4D9 were determined, which were 10.38 μg/L and 17.23, respectively. Obviously, the sensitivity of ZEN mAb 2B6 was significantly better than that of 4D9. The CR assay results showed that the CR of ZEN mAb 2B6 with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were 1.52%, 1.28%, 2.64%, 1.83% and 4.27%, respectively. The CR of 4D9 with α-ZAL, β-ZAL, α-ZOL, β-ZOL and ZON were 1.63%, 1.35%, 2.86%, 2.01% and 4.88%, respectively. Apparently, the sensitivity of ZEN mAb 2B6 is significantly better than that of 4D9. On account of ZEN mAb 2B6 has the lowest IC50 value and lowest CR value, mAb 2B6 was selected for follow-up studies. A comparison between the monoclonal antibody 2B6 in this study and other ZEN monoclonal antibodies reported in recent literature is shown in Table 4.2. The IC50 value of monoclonal antibody 2B6 is 10.38μg/L, which is

highly sensitive to ZEN. At the same time, the CR value of monoclonal antibody 2B6 was between 1.35-4.88 μg/L, which was highly specific to ZEN and achieved the expected purpose of the study. Monoclonal antibody 2B6 can be used to establish immunoassays for ZEN analysis in grain, feed and other samples [211]. In terms of sensitivity, ZEN mAb 2B6 is by far the best compared to antibodies prepared by similar methods. However, in terms of specificity, ZEN mAb 2B6 has higher CR values than ZEN pAb reported by Burkin et al. [254], ZEN mAb reported by Gao et al. [250] and ZEN mAb7-1-144 reported by Teshima et al.[202]. Therefore, the specificity of ZEN mAb 2B6 needs to be further improved. In future experiments, it is necessary to comprehensively consider the physical and chemical properties of hapten, immunogen synthesis method, coupling rate, immunization procedure, antibody screening method and other factors to prepare ZEN mAb with better sensitivity and specificity.

Table.4.2

Comparison of IC50 values and CR values of ZEN pAbs by the FA and the AGA method in this study and other previously reported ZEN pAbs and ZEN mAbs

References	ZEN antibody	Coupling method	Immunoassay format	IC50 of ZEN (μg/L)	CR (%) [a]				
					α-ZAL	β-ZAL	α-ZOL	β-ZOL	ZON
This study (2021) [211]	ZEN pAb	FA	icELISA	20.92	0.78	0.81	0.57	0.48	0.92
Burkin et al.(2002) [254]	ZEN pAb	FA	icELISA	31.7	0.12	-	0.15	0.02	-
Gao et al. (2012) [250]	ZEN pAb	FA	icELISA	233.35	2.25	5.65	3.14	1.96	6.79
	ZEN mAb [#]	FA	icELISA	55.72	0.63	0.92	0.65	0.94	1.48
Teshima et al. (1990) [202]	7-1-144 mAb	AGA	icELISA	11.2	＜0.1	＜0.1	0.9	＜0.1	4.0

Note. [a] All of the data were calculated using the CR of ZEN as 100%. - No data. [#] No name.

4.3 Preparation and performance measurement of AFB1 and ZEN dual test strip

Preparation of AFB1 and ZEN dual test strip (1) Technical advantages and limitations of GICA. Faulk et al. [280] founded colloidal gold immunochromatography assay (GICA) in 1971. Beggs et al. [281] first applied GICA technology to detect the small molecule drug human chorionic gonadotropin (HCG) in 1990 and achieved success. Compared with other detection techniques, although GICA is a semi-quantitative detection method, it has three major advantages that other techniques do not have. First, it is fast and simple, and the test results can be obtained within 10 minutes, without any additional equipment and reagents; second, it can be operated on-site, and can be detected anytime, anywhere, and is not limited by site, environmental conditions, etc.; third, the amount of screening samples Large, multiple detection can be achieved. At present, GICA technology has developed into an important method for the detection of environmental pollutants such as heavy metal ions, biological toxins, pesticides and veterinary drug residues in food, and major pathogens such as viruses and bacteria, and has shown broad application prospects [282]. Nonetheless, in the realization of multiplex detection, GICA technology still faces three technical difficulties. First, the specificity of antibodies is poor, and there are different degrees of cross-reactivity between antibodies, and the test strip cannot effectively distinguish their respective targets. The second is the poor compatibility of the test strip buffer system. Different antibodies have different properties and require different buffer systems. Multiplex test strip require a unified buffer system for different antibodies, which undoubtedly increases the difficulty of preparing multiplex test strip. Third, it is difficult to choose the sample processing method. Different targets have different solubility and different sample extraction methods, which is also an important factor restricting the development of multiplex detection test strip [283].

In recent years, the use of GICA technology to achieve multivariate detection has achieved remarkable results. In terms of realizing multiplex detection

using gold-labeled single mAb and single detection line mode, Zeng et al. [284] reported that a broad-spectrum and specific mAb of sulfonamides was prepared, and a colloidal gold immunochromatography method was established. It can detect 13 sulfonamides simultaneously with detection limits of 0.42 to 8.64 μg/L. Similarly, Wang et al. [285] reported that a broad-spectrum specific monoclonal antibody for nitrofuran drugs was prepared, and a colloidal gold immunochromatography method was established to simultaneously detect 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), and 1-aminohydantoin (AHD), the detection limit of AOZ is 0.5 μg/L, and the detection limit of SEM, AMOZ and AHD is 0.75 μg/L. In terms of using gold-labeled multiple mAbs and multiple detection lines to achieve multiplex detection, Zhang et al. [286] reported that highly specific OTA mAb and ZEN mAb were prepared, respectively, and a dual-detection colloidal gold immune layer was established. The detection limit of OTA was 0.32 ng/ml and the detection range was 0.53-12.16 ng/ml, and the detection limit of ZEN was 0.58 ng/ml and the detection range was 1.06-39.72 ng/ml. Similarly, Li et al. [287] reported that highly specific AFB1 mAb, OTA mAb and ZEN mAb were prepared, respectively, and a multi-detection colloidal gold immunochromatographic method was established. The detection limits for AFB1, OTA and ZEA were 0.25 ng/mL, 0.5 ng/mL and 1.0 ng/mL, respectively. In this study, based on the continuous progress and maturity of GICA technology, on the basis of preparing high-sensitivity and high-specificity monoclonal antibodies against AFB1 and ZEN, GICA technology was used to establish a double detection technology method for AFB1 and ZEN, which was used for the detection of AFB1 and ZEN in food and feed. Provide technical support for simultaneous detection of dual residue.

(2) Selection of GICA technology mode. According to the principle of antigen-antibody reaction, GICA technology can be divided into two technical modes: gold-labeled antigen and gold-labeled antibody. The technical principle of gold-labeled antigen mode is: when there is no drug hapten in the sample, free

gold-labeled antigen and fixed The monoclonal antibody on the membrane binds to form a red band; when there is a drug hapten in the sample, it competes with the free gold-labeled antigen for binding to the monoclonal antibody immobilized on the membrane. The content of the drug hapten in the sample determines the depth or presence of the red band on the detection line on the membrane [288]. The technical principle of the gold-labeled antibody mode is: when there is no drug hapten in the sample, the free gold-labeled antibody binds to the hapten immobilized on the membrane to form a red band; when there is a drug hapten in the sample, it binds to the free The binding of the gold-labeled antibody inhibits the binding of the gold-labeled antibody to the hapten immobilized on the membrane. The content of the drug hapten in the sample determines the depth or presence of the red band on the detection line on the membrane [289]. Since the sensitivity of the gold-labeled antigen mode is not as sensitive as that of the gold-labeled antibody mode, the gold-labeled antibody mode is mostly used in immunoassays. Similarly, this study also used the gold-labeled antibody model to establish an immunoassay detection method.

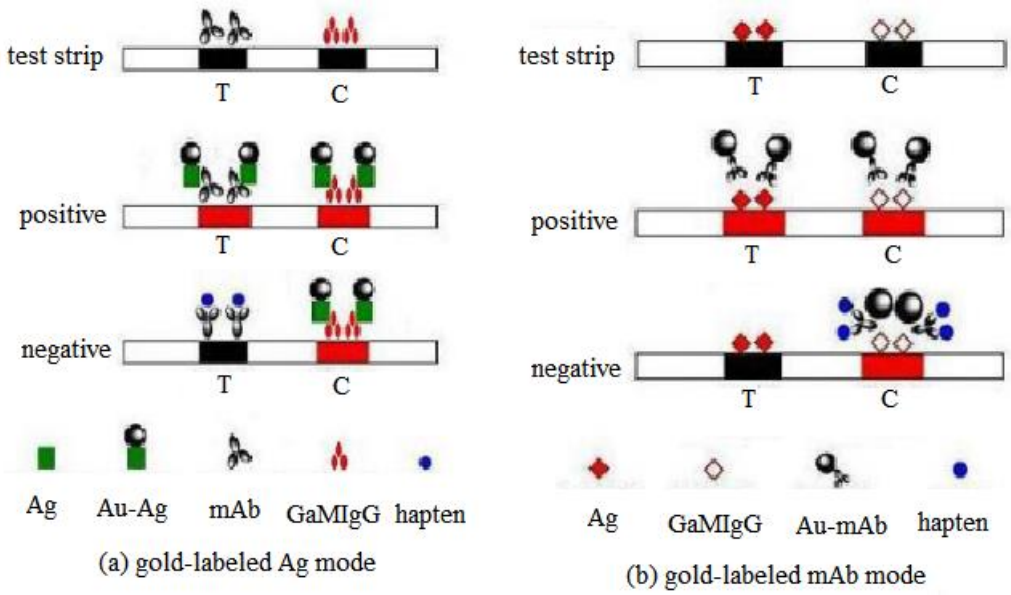

Figure.4.1 Technology model of GICA.

(3) Selection of colloidal gold preparation method. Colloidal gold is a suspension of many individual gold particles reduced by gold ions. The gold particle is an icosahedron containing 11 gold atoms composed of a basic crystal

nucleus and a surrounding double ion layer. The surface of the crystal nucleus is an inner negative ion, and the outer ion layer is dispersed among the colloids. The negatively charged layer surrounded by the surface of the gold particles is called the zeta potential, which can make the colloidal gold particles repel each other and keep the suspension stable [290]. In immunoassay detection, the color development is due to the aggregation of colloidal gold on the T-line or C-line. A particle size that is too small will not produce sufficient color signal, and a particle size that is too large will create steric hindrance that prevents IgG from approaching the zeta potential of the gold particles. The gold-labeled complex has poor stability. The MW of IgG is 16 kDa, the diameter is about 8 nm, and about 4 nm can bind to the surface of gold particles. Molinelli et al. [291] and Shim et al.[223] reported that selecting gold particles with a particle size of 40 nm can obtain better labeling effect of antibodies. However, Li et al. [292] and Sun et al. [293] reported that the effect of selecting gold particles with a particle size of 25 nm to label the antibody was better. In this study, colloidal gold of 21 nm, 25 nm and 42 nm was prepared and labeled with AFB1 mAb and ZEN mAb, respectively, and the sensitivity and stability were compared. The results showed that the gold particles with a particle size of 25 nm had the best effect. Therefore, the particle size of the gold particles selected in this study was 25 nm.

The particle size of colloidal gold is mostly 1-100 nm, and different reducing agents and their addition amount are the main factors affecting the diameter of gold particles. Commonly used reducing agents are white phosphorus, trisodium citrate, tannic acid, ascorbic acid, sodium borohydride, etc. Among them, trisodium citrate is the most commonly used reducing agent at present, due to its low price, stable properties and good reduction effect [294]. In this study, the trisodium citrate reduction method was also selected, and the prepared colloidal gold was identified by observation method, ultraviolet scanning method and electron microscope scanning method. The results showed that 1L of 0.01% chloroauric acid solution required 17 mL of 1% trisodium citrate solution to determine the optimum dosage.

(4) Selection of gold-labeled antibody preparation method. There are two methods to determine the optimal concentration of colloidal gold and IgG. One is Mey's series stabilization method, that is, adding 0.1 mL of antibodies of different concentrations to 1 mL of colloidal gold, adding 0.1 mL of 10% NaCl solution after 5 minutes at room temperature, and observing the color change after 5 minutes. The antibody concentration when the color of the mixture just changes from red to blue is the optimal concentration. The second is the ultraviolet scanning method, that is, the labeled complex is scanned in the range of 400-600 nm to obtain the maximum absorption wavelength, and the absorbance of the complex formed by different antibody concentrations is measured at this wavelength. The absorbance value was plotted against the antibody concentration, and the antibody concentration corresponding to the point where the antibody concentration increased and the absorbance value hardly changed was the optimal antibody concentration. Although the Mey method has the defect of poor accuracy, the method is simple and clear, the results are intuitive, and it is easy to operate, so it is still a good method. In this study, the combination of Mey's series stabilization method and UV scanning method was used to determine the optimal labeling concentration of colloidal gold and IgG. The optimal labeling concentration of AFB1 mAb was 7.0 g/mL, and the optimal labeling concentration of ZEN mAb was 3.5 g/mL.

(5) Optimization of detection conditions for immunoassays. The performance of immunoassays mainly depends on the quality and configuration of all reagents and materials. In order to improve the detection performance of the immunoassay, this study optimized the detection conditions in five aspects. One is the choice of nitrocellulose membrane. 10 kinds of NC membranes were tested, and Millipore 135 membrane was finally selected as the test strip detection area. The second is the choice of gold-label pads. Four kinds of gold-label pads were tested, and 8964 glass fiber was finally selected as the gold-label pad. The third is the selection of sample pads. Two sample pads, SB08 and SB06, were tested, and SB08 was finally selected as the sample pad for this test. The fourth is the selection

of the optimal concentration combination of gold-labeled antibody and coating antigen. AFB1 mAb and corresponding 6 kinds of immunogens and ZEN mAb and corresponding 5 kinds of immunogens are tested respectively, and the optimal dilution of gold-labeled AFB1 mAb is finally determined. The ratio is 1:4, the working concentration of the immunogen AFB1-BSA (OAE) is 1.0 mg/mL, the optimal dilution ratio of the gold-labeled ZEN mAb is 1:4, and the working concentration of the immunogen ZEN-BSA (AGA) is 2.0 mg/mL. The fifth is the determination of methanol concentration in the sample treatment solution. Through the determination of 10%, 20%, 30%, 40%, 50%, 60%, 70% series of methanol concentrations, it is finally determined that the methanol concentration in the sample treatment solution should not exceed 20%. %.

Performance measurement of AFB1 and ZEN dual test strip

(1) Performance of a single test strip. In this study, we first established a single immunoassay test method for AFB1 or ZEN. Its sensitivity is determined by the affinity of the antibody and its corresponding antigen. The mAb used in this study was developed by ourselves and is a high-affinity mAb. Therefore, a single immunoassay paper has better sensitivity, the visual detection limit of AFB1 single test paper is 1.0 μg/L, and the visual detection limit of ZEN single test paper is 5.0 μg/L. Meanwhile, in this study, the sensitivity was determined by Bio Dot-TSR3000 Test strip reader, the IC50 value of AFB1 was 1.11 μg/L, and the detection limit was 0.24 μg/L. ZEN has an IC50 value of 4.97 μg/L and a detection limit of 1.51 μg/L. The specificity is determined by the specificity of the antibody. In this study, the cross-reactivity test was used to identify the specificity of the test strip, and the CR values were determined with AFB1 mAb and AFB1 and its homologues, ZEN mAb and ZEN and its homologues, respectively. The results show that the test strip has high specificity and can be used for the residual detection of AFB1 and ZEN, respectively.

(2) Performance of double test strip. The ultimate goal of this study is to establish a dual test strip for simultaneous detection of AFB1 and ZEN. It was determined that the results could be read within 10 min by the double-coupled test

paper, and the visual detection limit of the double-coupled test paper for AFB1 was 1.0 μg/L, and the visual detection limit for ZEN was 5.0 μg/L. The visual sensitivity of OTA and ZEN in grains was 6 ng/mL and 20 ng/mL, respectively; At the same time, the sensitivity was determined by Bio Dot-TSR3000 Test strip reader in this study. The IC50 value of AFB1 was 1.15 μg/L, and the detection limit was 0.23 μg/L. The IC50 value of ZEN was 4.91 μg/L, and the detection limit was 1.53 μg. /L. By confirming with HPLC-MS/MS, the double test paper prepared in this study for the simultaneous detection of AFB1 and ZEN can be used for the detection of AFB1 and ZEN contamination in natural samples, providing technical support for the detection of AFB1 and ZEN contamination in food and feed. At present, there have been many reports on the GICA method for single residue detection of AFB1 or ZEN. Hu et al. [295] reported that a single residue GICA detection method for AFB1 was established with a detection limit of 0.1 μg/L. Ji et al [155] reported that a single-residue GICA detection method for ZEN was established with a detection limit of 15 μg/L. There are also some reports on the GICA method for the double-residue detection of mycotoxins. Sun et al. [293] reported that a double-residue GICA detection method for OTA and ZEN was established. The detection limit for OTA was 6.0 μg/L, and the detection limit for ZEN was 20.0 μg/L. Zhang et al. [286] reported that a dual-residue GICA detection method for OTA and ZEN was established, with a detection limit of 0.32 μg/L for OTA and a detection limit of 0.58 μg/L for ZEN. However, so far, to our knowledge, there is no report on the establishment of a dual-residue GICA assay for AFB1 and ZEN. Nevertheless, we should further improve the quality of antibodies, immunogens, colloidal gold, gold labeled antibodies and other reagents in subsequent tests, and further optimize the detection conditions such as NC membrane, gold labeled pad, sample pad, buffer and sample treatment solution, so as to improve the performance of AFB1 and ZEN dual detection test paper.

CONCLUSIONS

In the dissertation work, based on research expounded the toxicity and harmful effects of AFB1 and ZEN on human health, compared and analyzed in detail the detection methods, advantages and disadvantages of AFB1 and ZEN double residues in food and feed, and put forward the great significance of establishing the detection method of AFB1 and ZEN residue colloidal gold immunochromatographic dual test strip.

1. Six kinds of AFB1 and five kinds of ZEN artificial immunogens were successfully synthesized. Through UV, SDS-PAGE identification and animal immunization effect analysis, OAE method and AGA method were selected as the best methods to prepare high specific antibodies to AFB1 and ZEN, respectively.

2. Balb/c mice were immunized with AFB1-BSA (OAE) and ZEN-BSA (AGA) respectively, and an animal immune method for preparing highly specific antibodies was established. Three AFB1 mAb and two ZEN mAb cell lines were successfully obtained by cell fusion and positive hybridoma screening techniques. The immunological characteristics showed that AFB1 mAbs and ZEN mAbs had high titer, high sensitivity and high specificity, which could be used for the establishment of AFB1 and ZEN immunoassay methods.

3. The detection methods of AFB1 (or ZEN) single residue and AFB1 and ZEN residue colloidal gold immunochromatographic dual test strip were successfully established. After performance determination, preliminary application and HPLC-MS/MS verification, the method could be used for the simultaneous detection of AFB1 and ZEN residues in food and feed.

4. The research results of this thesis not only realized the rapid detection of double residues of AFB1 and ZEN, but also provided a reference for the multiple detection of other small molecular compounds such as mycotoxins.

PRODUCTION PROPOSALS

1. On the basis of our recearsh Methodological Recommendations “Implementation of modern methods of Aflatoxin B1 and Zearalenone detection”, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology"and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 53pp. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

2. We recommend using the materials of the dissertation work when studying the courses "Veterinary microbiology", "and Veterinary sanitary examination" for masters of the Faculty of Veterinary Medicine of Sumy NAU. And for the courses "Veterinary internal medicine" for masters of the Henan Institute of Science and Technology (HIST).

3. We can recommend, we can recommend that the AFB1 and ZEN dual test strips were suitable for rapid and simultaneous detection of AFB1 and ZEN contamination in food and feed samples.

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APPLICATIONS

Appendix A

List of works published on the topic of the dissertation

LIST OF WORKS PUBLISHED ON THE THEME OF THE DISSERTATION

Scientific works in which the main scientific results of the dissertation are published:

Articles in scientific professional publications of Ukraine:

1. **Wang, Y.** (2021). Design of Hapten Synthesis and Antibody Characterization of G-group Aflatoxins. Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies. Series: Veterinary sciences, 23(102), 130–135. doi: 10.32718/nvlvet10220 https://nvlvet.com.ua/index.php/journal/article/view/4190

2. **Wang, Y.,** Fotina, H., & Fotin, A. (2021). Design of antigen synthesis and identification of its artificial antigen for zearalenone. Ukrainian Journal of Veterinary and Agricultural Sciences, 4(2), 7–12. **DOI:** https://doi.org/10.32718/ujvas4-2.02*(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

3. **Wang, Y**., & Fotina, H. (2020). Design of antigen synthesis and preparation and characterization of specific and eurytopic antibodies against B-group aflatoxins. Bulletin of Sumy National Agrarian University. The Series: Veterinary Medicine, (4 (51), 52-60.DOI: https://doi.org/10.32845/bsnau.vet.2020.4.8

Scopus publication:

4. Wang, Y., Jiang, J., Fotina, H., Zhang, H., & Chen, J. (2020). Advances in Antibody Preparation Techniques for Immunoassays of Total Aflatoxin in Food. Molecules (Basel, Switzerland), 25(18), 4113. https://doi.org/10.3390/molecules25184113 *https://www.mdpi.com/1420-*

3049/25/18/4113 (PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).

5. Wang, Y., Wang, X., Zhang, H., Fotina, H., & Jiang, J. (2021). Preparation and Characterization of Monoclonal Antibodies with High Affinity and Broad Class Specificity against Zearalenone and Its Major Metabolites. Toxins, 13(6), 383. https://doi.org/10.3390/toxins13060383 *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

6. Wang, Y., Wang, X., Zhang, H., Jiang, J., & Fotina, H. (2021). Synthesis of Zearalenone Immunogen and Comparative Analysis of Antibody Characteristics. *International journal of analytical chemistry*, *2021*, 7109383. https://doi.org/10.1155/2021/7109383 *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

7. Wang, Y.; Wang, X.; Wang, S.; Fotina, H.; Wang, Z. (2022). Development of a Highly Sensitive and Specific Monoclonal Antibody Based on Indirect Competitive Enzyme-Linked Immunosorbent Assay for the Determination of Zearalenone in Food and Feed Samples. Toxins, 14, 220. https://doi.org/10.3390/toxins14030220 https://www.mdpi.com/2072-6651/14/3/220 *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

8. Wang, Y., Wang, X., Wang, S., Fotina, H., & Wang, Z. (2022). A Novel Lateral Flow Immunochromatographic Assay for Rapid and Simultaneous Detection of Aflatoxin B1 and Zearalenone in Food and Feed Samples Based on Highly Sensitive and Specific Monoclonal Antibodies. *Toxins*, *14*(9), 615. https://doi.org/10.3390/toxins14090615 https://www.mdpi.com/2072-6651/14/9/615*(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

Chinas publication:

9. Zhang Haitang, Wang Xiaofei, Zhi Aimin, **Yanan Wang** Wang Ziliang (2019). Design of Antigen Synthesis and Preparation and Characterization of Specific and Eurytopic Antibodies Against B-group Aflatoxins. Journal of Nuclear Agricultural Sciences. 33, (12), 2414-2420. DOI: 10.11869/j.issn.100-8551.2019.12.2414 *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

10. HAN Xiao, **WANG Yanan**, JIANG Jinqing, FAN Guoying, LI Renfeng, WANG Ziliang (2021). Research progress on detection technology of swine intestinal coronavirus. MODERN ANIMAL HUSBANDRY 5, (3), 6. http://j.issn.1008-3111.2021.03.006/ *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

Conference papers:

11. **Yanan Wang**. (2018) The development and preliminary application of double residue detection paper cards for aflatoxin b1 and Zearalenone. Sumy national agrarian university. Materials of the All-Ukrainian Student Scientific Conference (November 12-16, 2018).

12. **Yanan Wang**. (2019) Physicochemical properties and toxicological effects of Zearalenone. Sumy national agrarian university. Materials of the All-Ukrainian Student Scientific Conference (November 11-15, 2019).

13. **Yanan Wang** Fotina, H and Jiang J. (2019). Screening of synthetic methods of artificial antigen and analysis of antibodies characteristics of g-group Aflatoxins. 12-14 June, 2019 – Ohrid, Macedonia. *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

14. **Yanan Wang** Fotina, H. (2019). Design of Antigen Synthesis and Preparation and Characterization of Specific and Eurytopic Antibodies against B-

group Aflatoxins. Fourth Annual BTRP Ukraine Regional One Health Research Symposium . 20-24 May 2019. *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

Methodological recommendations:

15. Methodical guidelines “Implementation of modern methods of Aflatoxin B1 and Zearalenone detection”, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology"and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 53pp. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021). (*The applicant analyzed the research results, prepared and issued materials for methodical recommendations).*

Appendix B

Methodical Guidelines

Methodical guidelines “Implementation of modern methods of Aflatoxin B1 and Zearalenone detection”, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology" and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 53pp. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
SUMY NATIONAL AGRARIAN UNIVERSITY

METHODICAL GUIDELINES

"Implementation of modern methods of Aflatoxin B1 and Zearalenone detection",
for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology" and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise".

Sumy- 2021

Compilers:
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Fotina H.A., doctor of veterinary science, professor, professor of Department of Veterinary Examination, Microbiology, Zoohygiene and Safety and Quality of Livestock Products

"Implementation of modern methods of Aflatoxin B1 and Zearalenone detection", for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology" and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise".

Reviewers:
R.V. Petrov, Doctor of Veterinary Medicine, Professor, Head of the Department of Department Of Virusology, Patanatomy And Bird Diseases Named After Professor I.I.Panicar of Sumy NAU;
O.I. Shkromada, Doctor of Veterinary Medicine, Professor, Head of the Department of Obstetrics and Surgery of the Sumy National University of Science.

Recommended for publication by the educational and methodical council of the Faculty of Veterinary Medicine of SNAU, protocol No.5 dated "29" 12. 2021.

Appendix C

Conclusion of the commission on bioethics in English

Laboratory Animal Welfare and Ethical review of Henan Institute of Science and Technology

Application Number: 202004-021 Application Date: Apr 4, 2020 Issue No. 2020HIST021

<table>
<tr><td>Program and No.:</td><td colspan="2">Research and development of key food safety technologies "Application Demonstration of Quality and Safety Assurance Technology for Beef and Mutton Products" (2019YFC1605705)</td></tr>
<tr><td rowspan="2">Applicant</td><td>Name: Yanan Wang</td><td>Organization: College of animal science and veterinary medicine</td></tr>
<tr><td>E-mail: wyn564@126.com</td><td>Telephone Number: 15617198503</td></tr>
<tr><td rowspan="2">Animals</td><td colspan="2">Animal Source: Henan Animal Experiment Center</td></tr>
<tr><td colspan="2">Animal Grade (Normal、SPF or Others) : SPF</td></tr>
<tr><td colspan="3">Experimental objective, necessity and significance and how the program has been designed to achieve the objectives of the research:
Objective: To confirm whether the specific antibody against immunogen is produced after immunizing animals with immunogen, so as to lay the foundation for promoting the project.
Significance: Whether synthetic immunogen has immunogenicity should be verified by animal experiments.
Steps: 1. To synthesize artificial immunogen and to immunize Balb/c mice animal model, to collect blood from the tail to prepare polyclonal antibodies, and evaluated the immune effect;
2. To use hybridoma technology to prepare monoclonal antibodies, and use monoclonal antibodies to establish immunological analysis methods.</td></tr>
<tr><td>Animal Care</td><td colspan="2">SPF conditions, Normal</td></tr>
<tr><td rowspan="2">Animal Disposition</td><td colspan="2">Death conduct:
□ CO_2 suffocated □ Exsanguinations with anesthesia
☑ Cervical dislocation □ Anesthesia overdose
□ Others, detailed description.</td></tr>
<tr><td colspan="2">Not for the death of the animal disposition:
□ Continue to use □ Save in the agency
□ Release to the wild
□ Others, detailed description.</td></tr>
<tr><td colspan="3">Poisonous (harmful) material (infection, radiate, chemical poison and other) being used
□ Yes ☑ No
Declare: No.</td></tr>
</table>

Applicant's Declaration:

1. I will abide by the law and regulation stipulation, and accept the supervision and inspection by the committee and laboratory animal department.
2. The information I have given is accurate, detailed and comprehensive.

Applicant Signature (Seal): Jianhe Liu

September 04, 2019

Approval opinions:

■ Approval □ Not approve

Authorized Personnel Signature (Stamp):

September 10, 2019

Appendix E

Laboratory Biosafety Certificate

河南省教育厅

教科外〔2009〕80号

河南省教育厅
关于公布2008年度河南省高校工程技术研究中心建设项目的通知

各高等学校：

根据《河南省教育厅关于开展2008年度河南省高校工程技术研究中心立项建设的通知》（教科外〔2008〕737号）精神，经学校申报、中心负责人答辩、专家评审、现场考察，教育厅审核并公示，决定依托河南科技大学等学校建设12个河南省高校工程技术研究中心（以下简称工程中心）。现将工程中心及依托单位名单予以公布（附件1），有关事宜通知如下：

附件1

2008年度河南省高校工程技术研究中心建设项目名单

序号	中心名称	依托学校	技术领域	类别
1	轴承工程技术	河南科技大学	制造业	认定
2	动物疫病和残留物防控	河南科技学院	农业与生物	认定
3	生态化工	郑州大学	资源与环境	立项
4	作物抗逆改良	河南大学	农业与生物	立项
5	农业资源与环境	河南农业大学	资源与环境	立项
6	道地中药材保育及利用	河南师范大学	农业与生物	立项
7	矿物加工与矿用材料	河南理工大学	新材料	立项
8	粮食信息与检测技术	河南工业大学	现代服务业	立项
9	高性能土木工程材料与环境	郑州航空工业管理学院	新材料	立项
10	生物质降解与气化	商丘师范学院	资源与环境	立项
11	信阳毛尖茶产业	信阳农业高专	农业与生物	立项
12	怀药提取分离	焦作大学	化学化工	立项