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Development of Competitive Direct ELISA for Gossypol Analysis

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Anti-gossypol monoclonal antibody was purified from cell culturing supernatant by ammonium sulfate precipitation and Protein A AffinityPak. The antigen (i.e., gossypol) was labeled with horseradish peroxidase through Schiff-base formation. Both the purified antibody and the enzyme-labeled gossypol were employed to develop a competitive direct enzyme-linked immunosorbent assay (cdELISA) for gossypol analysis. I_{50} value, the concentration of gossypol causing 50% inhibition of the maximum ELISA signal in the competitive standard curve, was 0.067 μg/mL, whereas the detection limit for gossypol was 0.005 μg/mL. We also observed a good correlation ($R^2 = 0.96$, $P < 0.05$) between the cdELISA method and the AOCS official method for "free" gossypol (extractable gossypol and gossypol derivatives by 70% acetone) analysis of cottonseed meals. This indicates that the newly developed cdELISA could be a valuable and feasible alternative for determination of "free" gossypol, especially when the available sample is limited with relatively low gossypol concentration.

KEYWORDS: Gossypol; monoclonal antibody; purification; immunoassay; ELISA

INTRODUCTION

Cotton has long been known as nature's unique food and fiber plant. It is produced worldwide in tropical and subtropical regions. Both the vegetation and the reproduction parts of the cotton plants contain gossypol, a polyphenolic aldehyde compound, which was known to have a wide spectrum of biological activities, such as antioxidant, anticancer, anti-HIV, antiviral, and antimicrobial activities (*1*−*4*). However, consuming high doses of gossypol can cause problems such as cardiac arrhythmia, pulmonary edema, and malnutrition, leading to the death of laboratory animals (*5*, *6*). Currently, conventional used methods for gossypol analysis include spectrophotometric means as defined by the American Oil Chemists' Society (AOCS) official method (*7*, *8*) and the use of high performance liquid chromatography (HPLC) (*9*−*12*). While both methods demonstrated good reproducibility, they share some common limitations: (a) sample size is required more than 0.1 g; and (b) detection limits are 5 ppm for the AOCS method and 0.5 ppm for the HPLC method. In addition, lack of the detective specificity of the AOCS method tends to result in a higher value of gossypol content than that obtained from the HPLC method. In contrast, enzyme-linked immunosorbent assay (ELISA) possesses advantages over the detection limit, specificity, sample preparation, and efficiency associated with both the AOCS and the HPLC methods.

In our previous studies, the monoclonal antibody against gossypol was obtained by culturing the anti-gossypol monoclonal antibody cell line, which resulted in a novel method using a monoclonal antibody-based competitive indirect ELISA for gossypol analysis (*13*). As compared to the AOCS and HPLC methods, this indirect ELISA has already demonstrated to be an efficient method with a detection limit of 20 ppb gossypol and less than 100 mg sample required. However, it still needed a labeled secondary antibody to quantify the gossypol. To make the immunoassay method simpler and faster, a competitive direct ELISA (cdELISA) for gossypol determination was developed by directly labeling gossypol with horseradish peroxidase. This direct ELISA was further compared to the AOCS official method and the indirect ELISA method for the analysis of "free" gossypol in cottonseed meals.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), Tween 20, NaCl, KCl, KH_2PO_4, Na_2HPO_4, ammonium sulfate, goat anti-mouse peroxidase

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conjugated IgG+IgM (H+L), Rosewell Park Memorial Institute 1640 (RPMI 1640), fetal bovine serum (FBS), and Coomassie brilliant blue R-250 were purchased from Sigma Chemical Co. (St. Louis, MO). One step ABTS (2,2′-azinobis [3-ethylbenzthiazoline 6-sulfonic acid]-diammonium salt) peroxidase substrate, ImmunoPure (Protein A) IgG Purification Kit, BCA Protein Assay Kit, and ImmunoPure Horseradish Peroxidase (HRP) were bought from Pierce (Rockford, IL). Microtiter plates (Maxisorp) used for direct ELISA, sodium cyanoborohydride ($NaBH_3CN$), and dialysis tubing (Nominal MWCO 6000−8000) were bought from Fisher Scientific (Pittsburgh, PA). Ready Gels (10% Tris-HCl, 50 μL, 10 wells) and Prestained Protein Standards (MW from 10 to 250 kDa) were purchased from Bio-Rad Laboratories (Hercules, CA). Gossypol, a variety of cottonseed meals, and their corresponding data of "free" gossypol content analyzed by the AOCS method were generously provided by the laboratories of the Agricultural Research Service, USDA-SRRC, New Orleans, Louisiana. Microtiter plates used for indirect enzyme-linked immunosorbent assay (ELISA) were Immulon 2 HB (Dynex Technologies, Inc., Chantilly, VA).

Purification of Antibody. *Preparation of Crude Antibody.* Antibodies were produced by culturing the anti-gossypol monoclonal antibody cell line at 10^5-10^6 cells/mL in RPMI with addition of 10% FBS, splitting the culture every 2−3 days and collecting the supernatant by centrifugation to remove the cells (*13*).

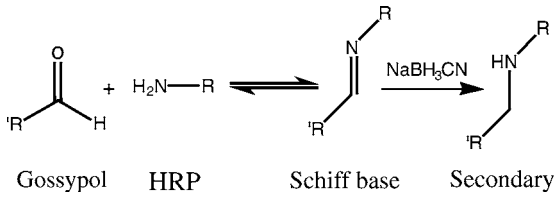
Ammonium Sulfate Precipitation. The supernatant from above was centrifuged at 4400 rpm for 30 min at 4 °C to remove the debris, and precipitated with 50% ammonium sulfate overnight at 4 °C, then centrifuged again at 4400 rpm for 30 min at 4 °C. The precipitate was dissolved in phosphate-buffered saline (PBS, containing 140 mM NaCl, 1.8 mM KH_2PO_4, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.3) and dialyzed (MWCO 6000−8000) against PBS for 24 h. The obtained crude antibody was then further purified.

Antibody Purification by Affinity Column Chromatography. ImmunoPure (Protein A) IgG Purification Kit was used for crude antibody purification according to the procedures provided by the manufacturer. The crude antibody was applied to the Protein A AffinityPak Column equilibrated in advance with binding buffer. First, the Protein A column was washed with binding buffer to remove the unbound impurities, then eluted with elution buffer for the desorption of the bound IgG. Second, the fractions showing the highest absorbance values at 280 nm were collected. After those fractions were desalted by the D-Salt Excellulose plastic desalting columns (included in the ImmunoPure (Protein A) IgG Purification Kit), they were pooled together and concentrated by 30% polyvinylpyrrolidinone K-30 at 4 °C.

Determination of Purity of Antibody. Purity of the antibody was determined via SDS-PAGE (10% polyacrylamide) with prestained protein standards (10−250 kDa) as markers (*14*). Electrophoresis was carried out at 150 V for 2 h. The gel was stained with 0.25% Coomassie Brilliant Blue R-250, and destained by washing overnight with a mixture of methanol−acetic acid−water (5:1:4, v/v).

Assay of Protein Concentration. The change of protein concentration during the purification was determined using the BCA Protein Assay Kit following the procedures described by the manufacturer.

Assay of Antigen-Binding Activity. The antigen-binding activity of the purified antibody was analyzed by indirect ELISA checkerboard (*13*). Briefly, 100 μL/well of gossypol-BSA conjugate (100, 10, 1, 0 μg/mL diluted in PBS) was added to an Immulon 2 HB microtiter plate and incubated overnight at 4 °C. The solution was then removed, and blocking solution (200 μL/well of 1% BSA in PBS) was added and incubated for 30 min at 37 °C. After removing the solution and washing, a solution of 10% methanol in PBS (50 μL/well) together with 50 μL/well of serially diluted antibody (1/10, 1/100, 1/1000 diluted in PBS) was added to each well, and incubated for 45 min at 37 °C. Following the incubation, the solution was removed and wells were washed with 3 × 200 μL of PBST. Next, 100 μL/well of 1/10 000 diluted goat anti-mouse peroxidase conjugated IgG+IgM in PBS was added and incubated for 45 min at 37 °C. After the unbound chemicals were washed away, ABTS substrate (100 μL/well) was added, and the absorbance at 405 nm was measured after 30 min in the dark at room temperature using a SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).



Gossypol HRP Schiff base Secondary amine

Figure 1. Gossypol–protein conjugate formation via a Schiff base intermediate.

Label of Gossypol with Horseradish Peroxidase. Gossypol−horseradish peroxidase conjugates were produced via a Schiff base intermediate as shown in **Figure 1** (*15*, *16*). Gossypol (5 mg) was dissolved in 2 mL of methanol and mixed with 15 mL of horseradish peroxidase (HRP) solution (50 mg of HRP in PBS, pH 7.3) with the addition of 60 mg of sodium cyanoborohydride ($NaBH_3CN$), and the reaction mixture was stirred for 12 h at room temperature in the dark under a stream of nitrogen. After the reaction, the mixture was then dialyzed (MWCO 6000−8000) against PBS for 24 h at 4 °C to remove free gossypol.

Competitive Direct ELISA for Gossypol Determination. *Checkerboard Titration.* Two-dimensional titrations, in which various dilutions of the purified antibody were titrated against various amounts of gossypol−HRP conjugates, were used to have an estimate of their appropriate concentrations for competitive assays. A microtiter plate (Maxisorp) was coated with 100 μL/well of the purified antibody serially diluted (1/5, 1/10, 1/100, 1/1000) with 50 mmol/L of Na_2CO_3−$NaHCO_3$ solution (pH 9.6) and incubated overnight at 4 °C. After the coating solution was removed, blocking solution (200 μL/well of 1% BSA in PBS, containing 0.1% NaN_3) was added, and the wells were washed with PBST after 2 h incubation at 37 °C. The plate was then filled with 50 μL/well of solution (10% methanol in PBS), along with 50 μL/well of serially diluted gossypol−HRP conjugates (1/10, 1/100, 1/1000, 1/10 000 diluted in PBST), and incubated for 1 h at 37 °C. The wells were then washed using 3 × 200 μL PBST, and 100 μL per well of ABTS substrate solution was added and incubated for 30 min in the dark at room temperature. The absorbance was measured at 405 nm.

Assay Protocol. Microtiter plates (Maxisorp) were coated with 100 μL/well of the purified antibody diluted (1/100) with 50 mM of Na_2CO_3−$NaHCO_3$ solution (pH9.6) and incubated overnight at 4 °C. After the coating solution was removed, the wells were washed with 3 × 200 μL PBST and blocked with 200 μL BSA (1% in PBS, containing 0.1% NaN_3) for 2 h at 37 °C. After the blocking solution was removed, the wells were washed with 3 × 200 μL PBST. Next, 50 μL/well of the serially diluted standards (100, 10, 1, 0.1, 0.01, 0.001, and 0 μg/mL of gossypol in 10% methanol) or samples, together with 50 μL/well of diluted gossypol−HRP conjugates (1/100 diluted in PBST), were added to the wells, and the plates were incubated for 1 h at 37 °C. The wells were then washed using 3 × 200 μL PBST, and 100 μL per well of ABTS substrate solution was added. After 30 min incubation at room temperature, the absorbance at 405 nm was measured.

Gossypol Analysis of Cottonseed Meals. "Free" gossypol content from seven cottonseed meal samples was analyzed using this direct ELISA. The "free" gossypol of these cottonseed meals was extracted by 70% acetone (*7*). Briefly, the ground cottonseed meals (Wiley mill; 20 mesh) were accurately weighted and mixed with 50 mL of 70% aqueous acetone in a 250 mL Erlenmeyer flask. The flask was stoppered with a polyethylene stopper, and shaken vigorously on a mechanical shaker for 1 h at room temperature. The slurry was then centrifuged at 1000 rpm for 5 min, and the supernatant was diluted into appropriate concentrations with 10% methanol in PBS, and applied into the direct ELISA for gossypol analysis.

Data Analysis. Standard and samples were run in triplicate. The gossypol standard curve was obtained by plotting absorbance against the logarithm of standard gossypol concentrations. The software package Softmax (Molecular Devices Corp., Sunnyvale, CA) was used to calculate the four parameters for fitting the sigmoidal curve equation:

$$Y = (A - D)/[1 + (X/C)^B] + D$$

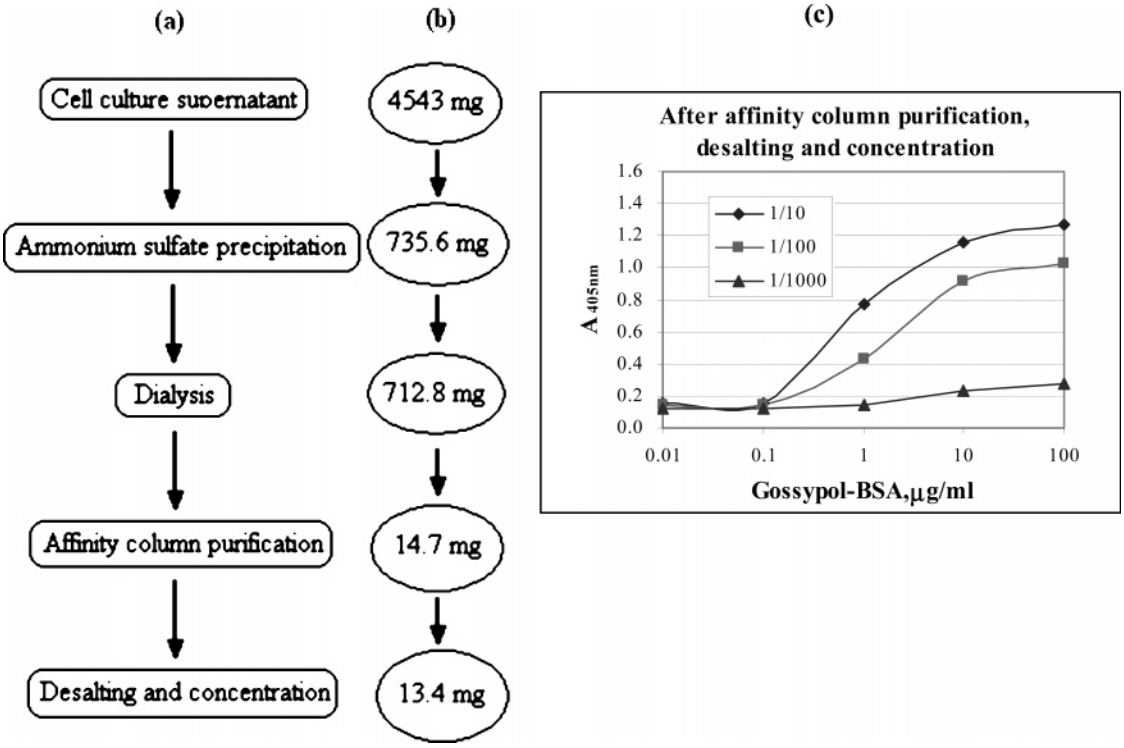


Figure 2. (**a**) Stepwise purification of antibody, (**b**) protein quantification by BCA assay, and (**c**) checkerboard ELISA of antibody obtained after purification.

where A is the asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), B is the curve slope at the inflextion point, C is the gossypol concentration giving 50% inhibition of A_{max}, called I_{50} value, D is the asymptotic minimum, X is gossypol concentration, and Y is the corresponding absorbance at 405 nm. The samples were determined by interpolating their mean absorbance values in the standard curve run on the same plate.

The correlation among the results from the direct ELISA, the indirect ELISA, and the AOCS official method was evaluated by means of one-way analysis of variance (ANOVA) for repeated measure design.

RESULTS AND DISCUSSION

Purification of Antibody. The anti-gossypol antibody was purified from cell culturing supernatant of anti-gossypol monoclonal hybridomas via 50% saturated ammonium sulfate precipitation (**Figure 2**). After dialysis to remove small molecules, the antibody was purified by the Protein A AffinityPak Column, which could efficiently purify IgG antibody (our anti-gossypol monoclonal antibody was identified as IgG) (*13*). Protein A is bacterial cell wall proteins that bind to a domain in the Fc region of antibodies (*17*). This interaction is quite strong, but the affinity is sensitive to changes of pH. At neutral or slightly basic pH values, antibodies bind tightly to protein A; however, the interaction between the antibody and protein A can be dramatically weakened by lowering the pH. The purification of antibodies on protein A affinity column is based on these pH-sensitive changes in affinity. The IgG antibody was initially absorbed onto the Protein A affinity column when washing with IgG binding buffer (pH 8.0), then desorbed when eluting with IgG elution buffer (pH 2.8, amine-containing buffer). Fractions showing high absorbance at 280 nm were collected, desalted, and concentrated.

The content of protein determined by BCA assay gradually decreased along with the subsequent purification steps from 4543 to 13.4 mg as shown in **Figure 2a**,**b**. The antigen-binding capacity of the antibody was estimated by the indirect ELISA checkerboard after purification (**Figure 2c**), in which 1/100 dilution of anti-gossypol antibody could give absorbance around 1.0 at 10 μg/mL of gossypol-BSA coating concentration.

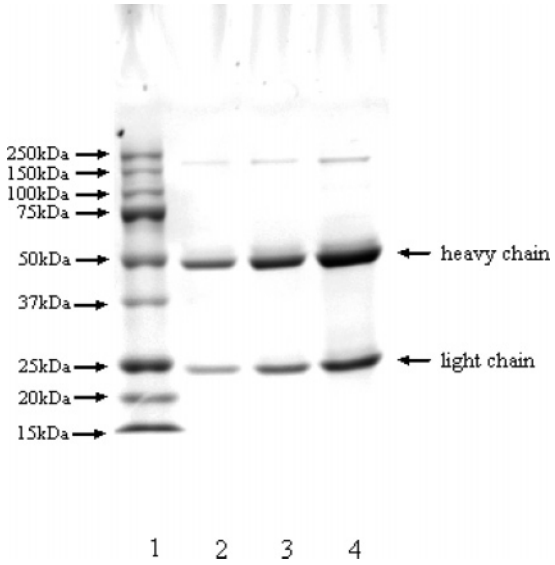


Figure 3. SDS-PAGE of the purified antibody. Line 1, protein standard markers; line 2, the purified antibody of 10 μL; line 3, 20 μL; line 4, 30 μL.

The purity of the final preparation of antibody was estimated by SDS-PAGE (**Figure 3**). The SDS-PAGE showed the homogeneity of the purified antibody. Observation of two bands equivalent to 50 and 25 kDa, respectively, on SDS-PAGE under reducing conditions suggested that one was the heavy chain (MW about 50 kDa) and another was the light chain (MW approximately 25 kDa).

Synthesis of Gossypol−Horseradish Peroxidase Conjugates. Prerequisites for an efficient cdELISA not only require an antibody with a high affinity toward the antigen, but also demand an enzyme that is linked with the antigen remaining a high activity.

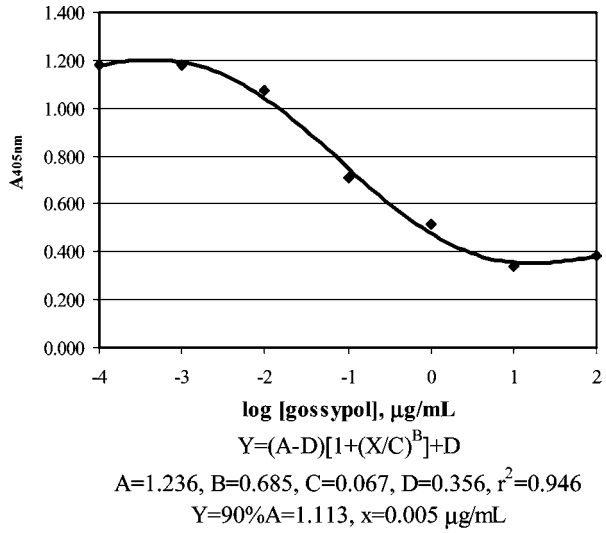


Figure 4. Competitive direct ELISA standard curve.

The most widely used enzymes for labeling are horseradish peroxidase (HRP), alkaline phosphatase (AP), and β-galactosidase. In this study, horseradish peroxidase was selected to label gossypol because HRP had been proved with high turnover number and stability (*18*). Because gossypol (MW 518) is a reactive terpenoid aldehyde, its carbonyl groups can react readily and reversibly with ϵ-amino groups of protein such as HRP to form Schiff bases. In the reaction, pH is the most important factor and the greatest reaction efficiency can be obtained at pH 9−10 (*15*, *19*). However, gossypol can be rapidly oxidized under alkaline conditions by atmospheric oxygen (*20*). The pH of the reaction was therefore selected around pH 7.3. The products formed were stabilized with the reducing agent $NaBH_3$-CN, which could convert the Schiff base to a more stable secondary amine (*19*), and conduced to the formation of more stable gossypol−HRP with more gossypol groups per horseradish peroxidase in the conjugates (*21*).

Development of the Competitive Direct ELISA. Direct ELISA can be regarded as the simplest form of ELISA, of which there are two formats. One is antibody-coated ELISA, and the other is antigen-coated ELISA. In this study, the first format was used for the development of a direct ELISA. The purified antibody was diluted in a coating buffer, carbonate/bicarbonate buffer (pH 9.6). After coating, any excess free antibody was removed by a washing step with a neutral buffered solution (PBST). The blocking solution (1% BSA in PBS, containing 0.1% sodium azide) was then added. After another washing step, a known amount of HRP labeled gossypol with gossypol standards or samples was added. After washing away the unbound materials, the ABTS substrate was added and the color developed was reversely proportional to the amount of antigen from standards or samples.

Figure 4 shows the representative gossypol standard curve prepared in 10% methanol for the cdELISA, which corroborated this cdELISA for gossypol analysis. The midpoint value (I_{50}), concentration causing 50% inhibition of the color, is 0.067 μg/mL, and the detection limit, defined as 10% inhibition of the color, is 0.005 μg/mL. The detection limit from the direct ELISA is much lower than that obtained by the indirect ELISA, in which the detection limit was 0.024 μg/mL under the test conditions (*22*).

Gossypol Analysis of Cottonseed Meals. This cdELISA was further used for "free" gossypol analysis of cottonseed meals, and the results were compared to those determined by the indirect ELISA and the AOCS official methods. One-way ANOVA showed the significant differences among the three methods for gossypol analysis ($P < 0.05$). Gossypol content determined by the AOCS method was significantly higher than that by the direct ELISA method ($P < 0.05$), and that by the indirect ELISA method ($P < 0.05$). However, no significant difference was observed between the direct ELISA method and the indirect ELISA method, which demonstrated the consistency of the two ELISA methods based on the same principle (antigen−antibody interactions). **Figure 5** shows the correlation of "free" gossypol content determined by three methods. The linear correlations ($R^2 = 0.96$, $P < 0.05$ for AOCS vs direct ELISA method; $R^2 = 0.94$, $P < 0.05$ for AOCS vs indirect ELISA method) proved the reliability of the ELISA method being an alternative means of the AOCS method for gossypol analysis.

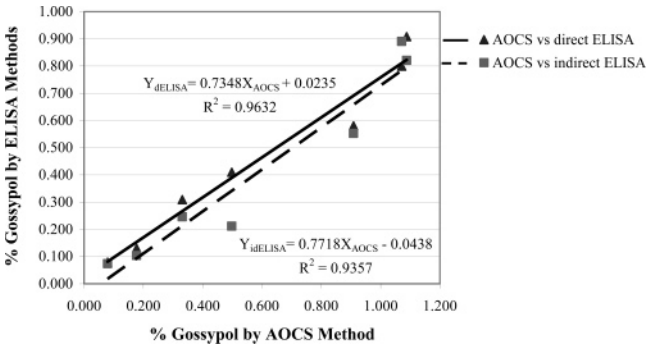


Figure 5. Correlation between the AOCS (free gossypol, Ba 7-58) method, the direct and the indirect ELISA for the analysis of gossypol.

As for the lower values from the ELISA method as compared to the AOCS method, it may be attributed to the following reasons. The ELISA method determines more specifically the gossypol and gossypol derivatives, which are recognized by the anti-gossypol monoclonal antibody, whereas the AOCS official method determines "free" gossypol and all other possibly extracted nongossypol aldehydic compounds by a colorimetric reaction. The latter thus may produce a false positive signal resulting in an overestimation of gossypol content (*23*, *24*). In addition, our previous study (*13*) showed that the monoclonal antibody used in ELISA had higher affinity to gossypol than did the gossypol−amine complex. The presence of gossypol−amine complexes in the extracts (formed with amino acids, small peptides, and proteins, etc.) would be expected to result in a lower response, so this may be another reason for the underestimation of the gossypol concentration.

With the need for a rapid method for an "in field" determination of gossypol, this microtiter plate direct ELISA method can be easily converted to a dipstick ELISA, a fast, easy to perform, and inexpensive method which allows qualitative on-site determination of analytes (*25*−*27*). Dipstick immunoassays usually follow the direct ELISA procedural schemes but use a membrane as antibody-coating support and rely on color development as seen with the naked eye, which will be useful for cotton breeders, cotton product processors, and entomologists conducting cotton research. This work is also under further investigation in our lab.

In conclusion, as compared to the AOCS official method and the HPLC method, the current cdELISA method has significantly improved the detection limit of gossypol and analytical efficiency. The previously published competitive indirect ELISA employs a labeled secondary antibody to quantify the analyte (*13*), while the direct ELISA avoids the addition of enzyme labeled second antibody, which makes the direct ELISA simpler

and more practical. Moreover, this direct ELISA gives a lower detection limit of 0.005 μg/mL versus 0.024 μg/mL in indirect ELISA (*22*), which makes the rapid assay of gossypol possible with higher sensitivity, especially feasible for samples with low gossypol content. Therefore, the direct ELISA method based on HRP labeled gossypol can be used as a convenient and sensitive approach for rapid "free" gossypol assay.

ABBREVIATIONS USED

cdELISA, competitive direct enzyme-linked immunosorbent assay; ABTS, (2,2′-azinobis [3-ethylbenzthiazoline 6-sulfonic acid]-diammonium salt); FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffer saline; PBST, phosphate buffered saline with Tween 20; SDS, sodium dodecyl sulfonate; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase.

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