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Enhanced Sensitive Immunoassay: Noncompetitive Phage Anti-Immune Complex Assay for the Determination of Malachite Green and Leucomalachite Green

Jie-Xian Dong,[†,‡] Chao Xu,[†] Hong Wang,*[,†] Zhi-Li Xiao,[†] Shirley J. Gee,[‡] Zhen-Feng Li,[†] Feng Wang,[†] Wei-Jian Wu,[†] Yu-Dong Shen,[†] Jin-Yi Yang,[†] Yuan-Ming Sun,*[,†] and Bruce D. Hammock[‡]

[†]Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China

[‡]Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California, Davis, California 95616, United States

Supporting Information

ABSTRACT: To develop a more sensitive immunoassay for malachite green (MG) and leucomalachite green (LMG), we identified the immunocomplex binding phage-borne peptides for use in the noncompetitive phage anti-immunocomplex assay (PHAIA). An anti-LMG monoclonal antibody (mAb) was used to select immunocomplex binding peptides from a circular random eight-amino-acid phage-displayed library. After three rounds of panning-elution, five peptides that bound the LMG−mAb immunocomplex were obtained. One of the phage-borne peptide clones that resulted in an assay with the highest sensitivity was chosen for further research. The concentration of LMG producing 50% of the saturated signal and the limit of detection of the assay were 7.02 and 0.55 ng/mL, respectively, with a linear range of 1.35 to 21.56 ng/mL. The PHAIA based on the same antibody was 16 times more sensitive compared to the competitive immunoassay. PHAIA was used to analyze LMG, MG, and two mixtures of spiked fish samples, with validation by high-performance liquid chromatography (HPLC) with fluorescence detector. Results showed a good correlation (R^2_{LMG} = 0.9841; R^2_{MG} = 0.993; $R^2_{Mixture}$ = 0.9903) between the data of PHAIA and HPLC, thus the assay was an efficient method for monitoring food safety.

KEYWORDS: *malachite green, leucomalachite Green, phage anti-immunocomplex assay*

INTRODUCTION

The antimicrobial and antiparasitic triphenylmethane dye malachite green (MG) is widely used in aquaculture because it is active against skin flukes and gill flukes as well as protozoas and fungi.[1,2] MG is readily absorbed by fish, then metabolized to the colorless, lipophilic leucomalachite green (LMG).[3] Because both MG and LMG are suspected carcinogens, mutagens, and teratogens in humans, residues are not permitted in food of animal from many countries, including the United States, China, Canada, and the European Union (minimum required performance limit of 2 μg/kg).[4−6] Nevertheless, it is still used worldwide because it is cheap and effective. Therefore, a sensitive analytical method is needed to monitor its use in aquatic production.

Immunoassays can have a low limit of detection (LOD), which permits quantification at trace levels. It is a simple tool for detection and quantification of analytes, allowing relatively fast, high-throughput analysis.[7] According to the format, immunoassays fall into two main categories, competitive and noncompetitive assays. In theory, noncompetitive immunoassays are potentially superior to their competitive counterparts in term of precision, working range, sensitivity, and kinetics.[8] However, the noncompetitive assay are fundamental limited by the fact that the target antigen must have at least two epitopes, a drawback that essentially eliminates measurement of low molecular weight compounds. To overcome this limitation, several novel noncompetitive immunoassay approaches for small molecules have been proposed in the past few years. These were based on anti-idiotype antibody,[9,10] split recombinant variable region fragments of antibody,[11−15] and anti-metatype antibody.[16−18] It is quite difficult and time-consuming to prepare these antibodies requiring immunization with antibody or analyte−antibody complexes, making these methods rarely successful except in special cases. However, to circumvent the problems, the phage anti-immune complex assay (PHAIA) technology was developed for noncompetitive assays of small analytes. In this technique, selection of a phage-borne peptide from the phage-displayed library utilizes the analyte−antibody immune complex for selection. The phage clones, which recognize the immune complex instead of the free antibody, are the second binding reagents using an antiphage antibody coupled to horseradish peroxidase (HRP) to generate the signal. The noncompetitive PHAIA technology has been used in the detection of clomazone,[19] molinate and atrazine,[20] phenoxybenzoic acid,[21] and brominated diphenyl ether 47.[22] Significantly enhanced sensitivity was obtained compared to the chemically synthesized competing hapten-based enzyme-linked immunosorbent assays (ELISA).

So far, all of the immunoassays reported previously for MG and LMG are based on conventional competitive immunoassays.[23−27] To expand the sensitive immunoassay for MG and

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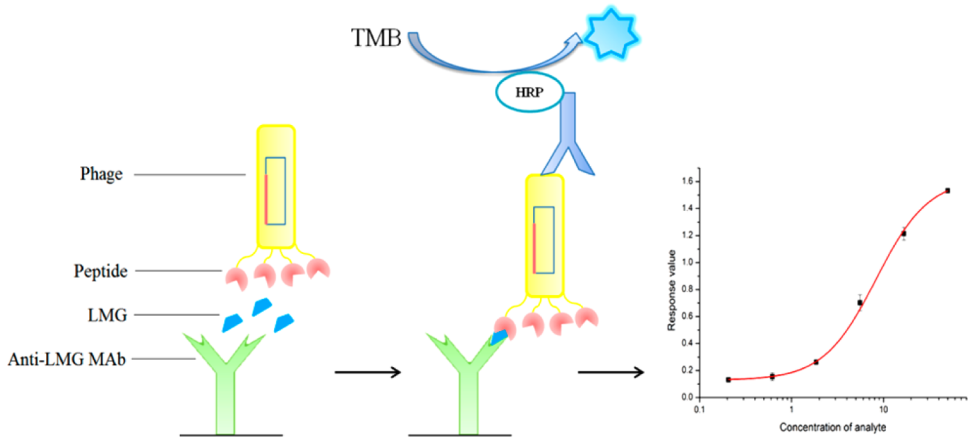

Figure 1. Schematic model of phage anti-immune complex assay (PHAIA). The peptide displayed on phage clones recognizes the immune complex to form the sandwich-model immunoassay. The detectable signal is amplified with antiphage antibody conjugated horseradish peroxidase(HRP), which catalyzes 3,3′,5,5′-tetramethylbenzidine(TMB) to produce a blue colored solution.

LMG, we developed the noncompetitive PHAIA with the anti-LMG monoclonal antibody (mAb), as shown in Figure 1. The phage-displayed peptides that were selective for the LMG-immunocomplex were chosen from the circular random eight-amino-acid library. One of the phage clones was used to establish noncompetitive immunoassays for quantitative estimation of MG and LMG in fish samples.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless otherwise specified. MG, LMG, crystal violet (CV), leucocrystal violet (LCV), brilliant green (BG), parafuchsin (PA), methylene blue (MB), bovine serum albumin (BSA), ovalbumin (OVA), 3,3′,5,5′-tetramethylbenzidine (TMB), and protein A-based affinity columns to purify IgG were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse anti-M13 mAb-HRP was obtained from GE Healthcare (Piscataway, NJ, U.S.A.). Polyethylene glycol 8000 (PEG 8000), Tween-20, tryptone, yeast extract, acetonitrile (ACN, HPLC grade), and dichloromethane (DCM) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Bond Elut PRS columns were obtained from Agilent Technologies (Palo Alto, CA, U.S.A.). *Escherichia coli* (*E. coli*) ER2738 was obtained from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.).

Phage Displayed Peptide Library. A random phage-displayed disulfide-constrained peptide library with an estimated diversity of 3.4×10^{13} independent clones was constructed on the M13KE vector that expressed random peptide of eight amino acids.[28] These peptides are flanked by two cysteine residues and linked to the N terminus of the major pIII phage coat protein.

Synthesis of Hapten and Production of Anti-LMG mAb. 2-(3-(bis(4-(Dimethylamino)phenyl)methyl)phenoxy)-acetic acid (LMGH8) and LMGH8-BSA/OVA were synthesized and conjugated as described by Shen et al.[27] with 2-(3-formyl-phenoxy)acetic acid instead of 2-(4-formylphenoxy)acetic acid. The anti-LMG mAb was identified by competitive ELISA following immunization with LMGH8-BSA, cell fusion, hybridoma selection, ascites growth, and mAb purification processes, which were carried out as reported by Campbell.[29]

Biopanning Procedure. Two wells of a microtiter plate (Nunc-Immuno 96-MicroWell Plate) were coated with purified anti-LMG mAb (10 μg/mL) in 100 μL of 0.1 mol/L $NaHCO_3$ (pH 8.6) at 4 °C overnight. Two additional microtiter plates coated with 100 μL of 3% BSA and mAb, respectively, in 0.1 mol/L $NaHCO_3$ were used for preabsorption eliminating binding to BSA or mAb. Nonspecific binding was blocked by incubation with 300 μL of 0.1 mol/L $NaHCO_3$ containing 5 mg/mL BSA for 1 h at room temperature. Panning-elution was conducted by diluting the phage library (1×10^{10} pfu/mL) with phosphate-buffered saline (PBS). The diluted phage was then added to the BSA preabsorption plate. Following room temperature incubation for 1 h, the solution was transferred to a preabsorption plate containing mAb and incubated at room temperature for 1 h. Then, the unbound phage peptides were transferred into the other wells of the mAb coated plate that had been preincubated with 300 ng/mL LMG and washed seven times with PBS containing 0.1% (v/v) Tween-20 (PBST), followed by incubation at room temperature for 1 h. The wells were washed 10 times with PBST. The bound phage was eluted with 100 μL of 0.1 mol/L glycine-HCl (pH 2.2) per well and neutralized with 4.5 μL of 1 mol/L Tris (pH 9.0). After 10 min incubation, the elution solution was collected, and *E. coli* ER2738 was infected by adding the elution solution for amplification and titration. The amplified phage was used for a subsequent round of panning. The concentration of coating antibody was reduced to 5 and 1 μg/mL in the second and third rounds of panning. The ability to bind to the LMG−mAb immune complex was tested after three rounds of panning-elution selection. Individual plaques were picked from LB/IPTG/X-gal plates.

Phage ELISA Screening. A noncompetitive phage ELISA was set up to screen phage capable of binding the LMG immune complex. The microtiter plates were coated with the anti-LMG mAb or BSA and blocked as described before. Equal volumes of LMG solution (50 ng/mL) diluted with PBST were mixed with the culture of individual amplified phage clones. The mixture was then added to wells (100 μL/well) and incubated at room temperature for 1 h. After seven washes with PBST, 100 μL of a 1/5000 dilution of anti-M13 monoclonal labeled HRP was dispensed into each well. One hour later, the plates were washed another seven times, and 100 μL of peroxidase substrate, which contains 12.5 mL of 0.1 mol/L citrate acetate buffer with pH 5.5, 0.2 mL of TMB (6 mg/mL in dimethyl sulfoxide solution), and 0.1 mL of 1% H_2O_2, was added into each well. The enzymatic reaction was stopped with

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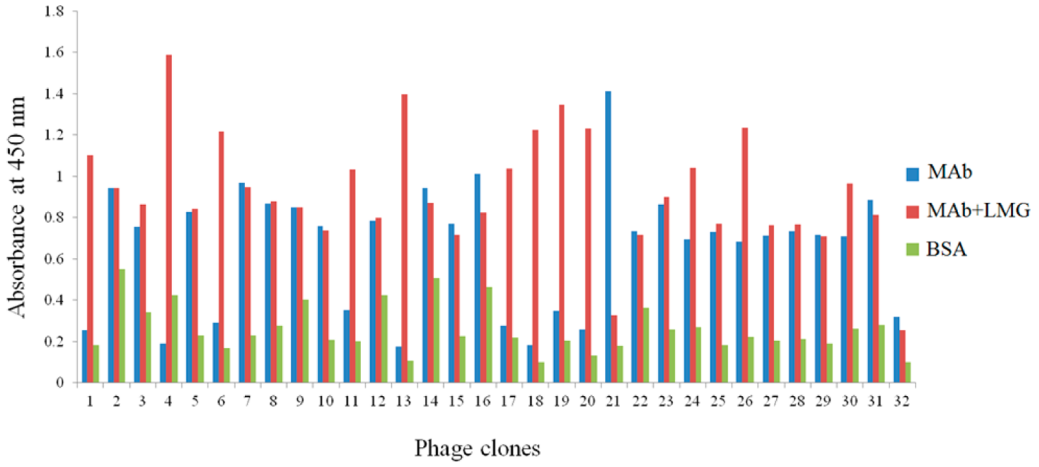

Figure 2. Screening of positive clones by noncompetitive phage ELISA. Clones 1, 4, 6, 11, 13, 17, 18, 19, 20 reacting specifically with the leucomalachite green-monoclonal antibody (LMG-mAb) immunocomplex showed little or negligible signal with the bovine serum albumin (BSA) or uncombined mAb.

50 μL of H_2SO_4 (4 mol/L) after 10 min, and the absorbance at 450 nm was recorded in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). The positive clones demonstrating high absorbance in wells coated with the immune complex and low absorbance in antibody or BSA-coated wells were selected and used for further analysis.

DNA Sequencing and Analysis for the Positive Clones. The positive clones as described above were further amplified and used for single-stranded DNA isolation as introduced in the Ph.D. peptide library kit instruction manual (New England Biolabs, Berverly, MA, U.S.A.). The product of phage DNA was submitted for DNA sequencing using the primer 96gIII (CCCTCATAGTTAGCGTAACG) (Division of Biological Sciences, Automated DNA Sequencing Facility, University of California, Davis, CA, U.S.A.). The program of DNAMAN 4.0 (Lynnon Biosoft, Quebec, Canada) was used to translate and align the amino acid residue sequences of the phage-display peptide from the inserted DNA sequences.

Preparation of Purified Phage Suspensions. Phage clones showing different amino acid residue sequences were selected and individually amplified as described above. After two steps of precipitation with PEG 8000-NaCl (20% (w/v) PEG-8000/2.5 mol/L NaCl), the phage particles were suspended with 0.5 mL of Tris-buffered saline (TBS, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) and stored at 4 °C.

Noncompetitive Phage ELISA Protocol. For checkerboard titration, 100 μL of various concentrations of the purified anti-LMG mAb (1 μg/mL, 0.5 μg/mL, 0.25 μg/mL, and 0.125 μg/mL) was used for coating as described before. The plates were blocked with 3% skim milk in PBS for 1 h at room temperature. After the plates were washed three times by PBST, the dilutions of purified phage suspensions were added to the mAb-coated wells in the presence or absence of LMG (50 ng/mL). Following the procedure of incubation, addition of anti-M13-HRP conjugate and color development as described in phage ELISA screening section, the concentration of coating antibody and phage particle combinations that resulted in an absorbance at about 1 were selected for the further assay.

After the confirmation of the coating antibody and phage particle dilutions, different concentrations of LMG (0−50 ng/mL in PBST solution) were mixed with equal volumes of phage to establish the noncompetitive standard curve for each clone.

Cross-Reactivity Assay. The specificity of the noncompetitive assay was evaluated by using some triphenylmethane analogues. We determined the average compound concentration corresponding to the midpoint of the curve (which corresponds to the concentration of analyte producing 50% saturation of the signal [SC_{50}]) and compared the values to the value from a standard curve for LMG run on the same plate. Cross-reactivity was calculated as follows: 100 × SC_{50} (LMG)/SC_{50} (cross-reactive compound).

Matrix Effect and Assay Precision. The PHAIA was used to detect MG and LMG in tilapia samples obtained from a local California market. Fish samples were minced and then homogenized. The homogenates (2.5 g) were spiked with MG, LMG, or the two-mixture standard solution (5, 10, or 20 ng/g). These samples stood for 5 min or more at room temperature prior to extraction. ACN (10 mL) was added and the sample was homogenized for 30 s at room temperature. Alumina (2.5 g) was added, and the samples were shaken for an additional 2 min. The samples were then centrifuged at 4000 rpm for 5 min and the supernatant was transferred to a clean tube. ACN (10 mL), 0.75 mL of hydroxylamine hydrochloride (20% mass ratio in deionized water), 1.25 mL of *p*-toluenesulfonic acid (0.05 mol/L), and 2.5 mL of ammonium acetate buffer (0.125 mol/L, pH 4.5) were used to re-extract the pellet. After shaking for 2 min, the samples were centrifuged again. Then, the supernatant was combined with the previous supernatant fraction. The extraction was repeated once more. Potassium borohydride (0.2 mol/L; 2.25 mL) was added to the supernatant fraction. After a 10 min incubation, DCM (5 mL) was added. These combined fractions were shaken vigorously for 15 s and centrifuged at 4000 rpm for 5 min. The lower organic layer was then transferred into a clean borosilicate glass tube and dried on a vacuum rotary evaporator at 45 °C. The dry residue was redissolved in 2.5 mL of ACN for solid-phase extraction.

The PRS solid phase extraction columns (Agilent Technologies, Palo Alto, CA, U.S.A.) were preconditioned with 5 mL of ACN. The sample extract was loaded onto the column followed by a 2 mL ACN wash. Two milliliters of the sample eluent solution (ACN/ammonium hydroxide, 90:10, v/v) was added to the column and the eluents were collected into a borosilicate glass tube. The extract was evaporated to dryness under a stream of nitrogen. Finally, the dry residue was

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reconstituted and sonicated for 10 min in 2 mL of PBST for the noncompetitive PHAIA and HPLC analysis. The matrix effects determination was conducted according to the procedure described above using a pure extract to dilute the LMG.

HPLC Analysis. HPLC was carried out on an Agilent 1290 series HPLC system, equipped with an online-degasser, a binary pump, and an autosampler. A fluorescence detector (model G1321B, Agilent, U.S.A.) was adjusted at wavelengths of 265 and 360 nm for excitation and emission. The analysis procedure was run with an isocratic mobile phase consisting of ACN and 0.125 mol/L ammonium acetate (4:1, v/v) at a flow rate of 1.3 mL/min. A Phenomenex Luna 5 μm C18 column (250 mm × 4.6 mm; Torrance, CA, U.S.A.) was used. The injection volume was 20 μL.

RESULTS AND DISCUSSION

Purified Anti-LMG mAb Production and Identification. A hybridoma 6D1 that stably secretes mAbs against LMG was obtained from ascites and purified by a protein A affinity column. The half inhibitory concentration (IC_{50}) of the purified mAb was determined to be 113.12 ng/mL by the indirect competitive ELISA.

Screening of the Peptides by Phage ELISA. In this study, we used the circular random eight-amino-acid library which was displayed on the pIII coat protein of filamentous phage. Compared with the other major coat protein, pVIII, pIII can tolerate the insertion of longer peptides because pIII is the longest coat protein and only 3−5 peptides copies are displayed.[30] We believed that the space between each peptide makes the immunocomplex more accessible for binding. The principle of noncompetitive immunoassay requires the phage-displayed peptides to recognize the antibody−analyte complex rather than any other part of antibody. After three rounds of panning, 32 clones were picked randomly and tested for binding to the anti-LMG mAb-coated wells in the presence or absence of LMG. Nine of 32 clones showed negligible binding to the uncombined antibody, although bounding specifically to the immunocomplex in the presence of 50 ng/mL LMG (Figure 2).

Analysis of Positive Peptides Amino Acid Sequences. After the isolation and sequencing of single stranded DNA from the positive phage clones, five different sequences were obtained (NCB1, NCB4, NCB6, NCB11, NCB13). The deduction of the amino acid sequences are shown in Table 1. The clones NCB 4, NCB 6, and NCB 13 shared the consensus motif of LPXHL, while HEXHXH was common to NCB1 and NCB11, where X represents any amino acid. The results indicated that the two motifs of LPXHL and HEXHXH contributed significantly to the LMG immune complex recognition.

Table 1. Peptide Sequences Isolated with LMG Immunocomplex

phage clones	amino acid sequence
NCB-1	C LNHEFHLHC (2)
NCB-4	CT Y RLPLHLC (1)
NCB-6	CTFTLPRHLC (1)
NCB-11	CVPHEHHTHC (4)
NCB-13	CLFTLPRHLC (1)

PHAIA for LMG. The PHAIA sensitivity is associated with the concentration of coating mAb and the number of phage particles. To determine the optimal concentration of mAb and phage particles, a checkerboard procedure was performed for each clone. Serially diluted phage particles were added to the plates coated with purified antibody (1, 0.5, 0.25, and 0.125 μg/mL) in the absence or presence of LMG (50 ng/mL). The best results were obtained with antibody concentration of 0.5 μg/well and phage concentrations of 2.40×10^{12} pfu/mL for clone NCB1, 1.20×10^{12} pfu/mL for clone NCB4, and 1.27×10^{12} pfu/mL for NCB13, whereas antibody concentrations of 0.25 μg/well and phage concentrations of 1.12×10^{12} pfu/mL for clone NCB6, and 3.00×10^{12} pfu/mL for clone NCB11, were identified (Figure S1−S5).

The assay with clone NCB1 demonstrated the highest sensitivity (SC_{50} = 7.02 ng/mL), followed by clones NCB4, NCB13, NCB6, and NCB11 (SC_{50} values of 8.39, 10.58, 13.00, and 16.15 ng/mL, respectively). For each of the five clones, standard curves were estimated using the optimized conditions. The noncompetitive standard curves are presented in Figure 3.

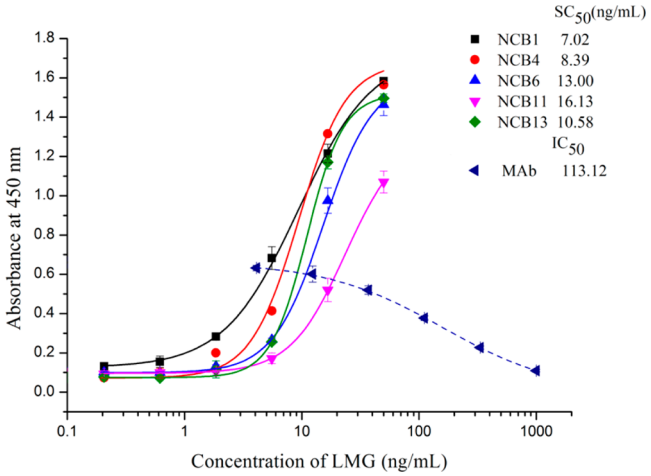

Figure 3. Noncompetitive ELISA for LMG performed with different phage-displayed peptide and competitive ELISA based on mAb for LMG. Results are the average of three replicates. The full lines are the standard curves of phage anti-immune complex assay. The dotted line is the inhibitory curve determined by competitive model. The 50% saturation of the signal (SC_{50}) and the half inhibitory concentration (IC_{50}) was calculated using a logistic plot equation with OriginPro 8.5 (OriginLab, Northampton, MA).

The linear range of NCB1 was 1.35 to 21.56 ng/mL. By altering the traditional competitive assay to the noncompetitive model, we improved the sensitivity of the assay by 16-fold using the same mAb. The sensitivity improvement is consistent with previously reported results.[19]

Cross-Reactivity. The cross reactivity of other structurally related compounds was determined to identify the specificity of the PHAIA, and the results are listed in Table 2. LCV and MG (6.89% and 2.93%, respectively) showed low cross-reactivity. The cross-reactivity was less than 0.01% for other compounds, similar to the competitive assay.

Matrix Effect. Due to the presence of the extraction agent, the sample component, and other ions, false positive or negative results from the matrix could be observed in the immunoassay. For the extraction of lipophilic LMG, it is necessary to minimize the matrix effect by using solid-phase extraction (SPE).[23] In our study, the maximum absorption with

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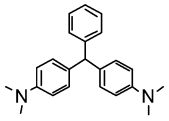
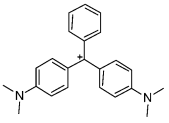
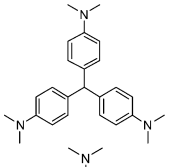
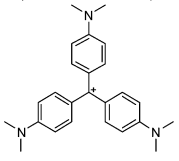
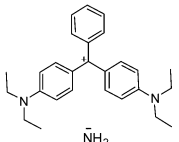
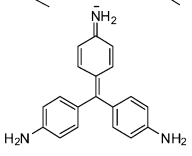
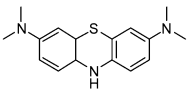
Table 2. Cross-Reactivity of LMG-PHAIA[a]

Analogues	Structure	Cross-reactivity(%)
LMG		100
MG		2.93
LCV		6.89
CV		<0.01
BG		<0.01
PA		<0.01
MB		<0.01

[a]The specificity was presented by cross-reactivity using the malachite green (MG), leucocrystal violet (LCV), crystal violet (CV), brilliant green (BG), parafuchsin (PA), methylene blue (MB).

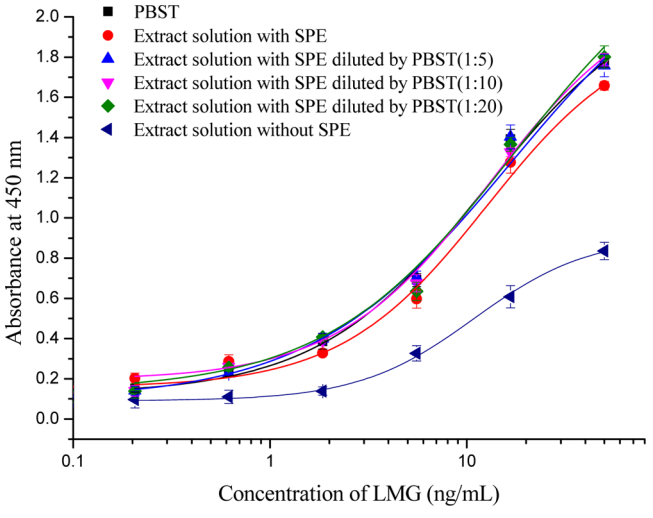


Figure 4. Standard curves of LMG in PBST and in sample extracts of different dilution with or without solid-phase extraction (SPE) (n = 3). The matrix effect was studied by comparison of standard curves obtained in PBST, in sample extracts cleaned up with SPE of 1:1, 1:5, 1:10, 1:20 dilution, and in sample extracts without SPE.

Table 3. Recovery of Spiked Fish Samples Determined by LMG-PHAIA (n = 3)

analyte	spike level (ng/g)	mean ± SD (ng/g)	average recovery (%)	CV (%)
LMG	0	ND		
	5	3.77 ± 0.58	75.33	15.33
	10	8.20 ± 0.76	82.03	9.24
	20	17.60 ± 1.52	88.00	8.64
MG	0	ND		
	5	4.14 ± 0.32	82.80	7.67
	10	8.07 ± 0.65	80.67	8.09
	20	16.89 ± 0.69	84.45	4.11
LMG + MG(1:1)	0	ND		
	5	3.78 ± 0.56	75.60	14.82
	10	7.92 ± 0.74	79.20	9.29
	20	16.79 ± 1.01	83.93	5.99

ND means not detectable.

50 ng/mL LMG in 1:1, 1:5, 1:10, and 1:20 extract dilutions was 1.66 ± 0.02, 1.76 ± 0.06, 1.79 ± 0.03, and 1.80 ± 0.05 (n = 3 per dilution), respectively, compared to 1.77 ± 0.02 in PBST (Figure 4). The SC_{50} values were 8.09, 7.44, 7.64, and

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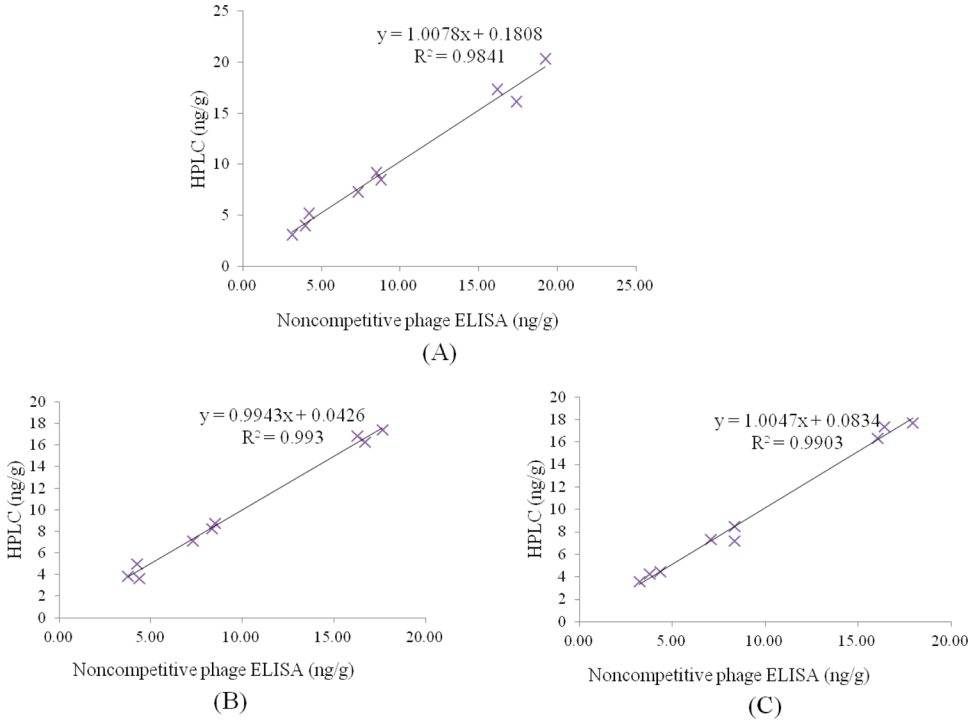

Figure 5. Correlation analysis between the PHAIA and HPLC (n = 3). (A) Samples spiked with LMG. (B) Samples spiked with MG. (C) Sample spiked with a mixture LMG and MG (1:1). All the sample extracted by the same procedure were divided into two equal parts and tested by PHAIA and HPLC.

7.66 ng/mL compared with 7.37 ng/mL for PBST, indicating that the matrix effects of the three fish samples were completely eliminated after pretreatment by PRS solid phase extraction. Although the 1:10 dilution showed less matrix effect, the higher limit of quantitation makes it unattractive. Thus, LMG was extracted at a proportion of 1:5, and the extracts were diluted 5-fold with PBST to minimize matrix effects.

Analysis of Spiked Samples by PHAIA and HPLC. The accuracy and precision of the PHAIA format were evaluated by determination of MG and LMG in spiked fish samples as well as by spiking with the mixtures of the two analytes. The MG was reduced to LMG by potassium borohydride so that it could be detected by the specific anti-LMG mAb. As shown in Table 3, the mean recovery rate measured using the LMG-PHAIA standard curves were 75.33−88.00% for LMG, 80.67−84.45% for MG, and 75.60−83.93% for the LMG and MG mixture. The comparison of results with HPLC shown in Figure 5 indicated that good correlations were obtained at the 5, 10, and 20 ng/mL spiked levels.

The current work described a sensitive detection of MG and LMG using a noncompetitive PHAIA assay. The peptide-based sandwich can work as a simple method to convert competitive assays to more versatile noncompetitive formats for small molecules which will provide significantly improved sensitivity (~16-fold in this work) and more adaptable to other immunoassay detection systems. However, the biological *E. coli* infectivity of phage might be a concern in some laboratories and industry. Further research will focus on the novel phage-free peptide ELISA by using the chemical synthetic peptide or genetic fusion peptide.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Checkerboard assays of NCB1, NCB4, NCB6, NCB11, and NCB13 phage clones. Plates were coated with affinity-purified antibody at 1 μg/mL (a), 0.5 μg/mL (b), 0.25 μg/mL (c), and 0.125 μg/mL (d). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: gzwhongd@163.com (H.W.). Tel.: (+86)-020-8528-3448.

*E-mail: ymsun@scau.edu.cn (Y.-M.S.).

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Notes

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■ ABBREVIATIONS USED

ACN, acetonitrile; BG, brilliant green; BSA, bovine serum albumin; CV, crystal violet; DCM, dichloromethane; ELISA, enzyme-linked immunosorbent assays; HRP, horseradish peroxidase; IC_{50}, half inhibitory concentration; LCV, leucocrystal violet; LMG, leucomalachite green; LMGH8, 2-(3-(bis(4-(dimethylamino)phenyl)methyl)phenoxy)acetic acid; LOD, low limit of detection; mAb, monoclonal antibody; MB, methylene blue; MG, malachite green; OVA, ovalbumin; PA,

parafuchsin; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% (v/v) Tween-20; PEG 8000, polyethylene glycol 8000; PHAIA, phage anti-immune complex assay; SC_{50}, concentration of analyte producing 50% saturation of the signal; TBS, Tris-buffered saline; TMB, 3,30,5,50-tetramethylbenzidine

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