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Hapten Synthesis, Antibody Development, and a Highly Sensitive Indirect Competitive Chemiluminescent Enzyme Immunoassay for Detection of Dicamba

Jingqian Huo,[†,‡,⊥] Bogdan Barnych,[‡,⊥] Zhenfeng Li,[‡] Debin Wan,[‡] Dongyang Li,[‡] Natalia Vasylieva,[‡] Stevan Z. Knezevic,[§] O. Adewale Osipitan,[§] Jon E. Scott,[§] Jinlin Zhang,[*,†] and Bruce D. Hammock[*,‡]

[†]College of Plant Protection, Agricultural University of Hebei, Baoding 071001, PR China

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

[§]Haskell Agricultural Laboratory, University of Nebraska—Lincoln, Concord, Nebraska 68728, United States

Supporting Information

ABSTRACT: Although dicamba has long been one of the most widely used selective herbicides, some U.S. states have banned the sale and use of dicamba because of farmers complaints of drift and damage to nonresistant crops. To prevent illegal use of dicamba and allow monitoring of nonresistant crops, a rapid and sensitive method for detection of dicamba is critical. In this paper, three novel dicamba haptens with an aldehyde group were synthesized, conjugated to the carrier protein via a reductive-amination procedure and an indirect competitive chemiluminescent enzyme immunoassay (CLEIA) for dicamba was developed. The assay showed an IC_{50} of 0.874 ng/mL which was over 15 times lower than that of the conventional enzyme immunoassay. The immunoassay was used to quantify dicamba concentrations in field samples of soil and soybean obtained from fields sprayed with dicamba. The developed CLEIA showed an excellent correlation with LC-MS analysis in spike-and-recovery studies, as well as in real samples. The recovery of dicamba ranged from 86 to 108% in plant samples and from 105 to 107% in soil samples. Thus, this assay is a rapid and simple analytical tool for detecting and quantifying dicamba levels in environmental samples and potentially a great tool for on-site crop and field monitoring.

KEYWORDS: *dicamba, hapten synthesis, polyclonal antibody, chemiluminescent enzyme immunoassay*

INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid), a widely used broad-spectrum herbicide first registered in 1967, is mainly applied on corn and Triticeae crops for controlling annual, perennial, and biennial weeds.[1] Dicamba is also used for the control of weeds in pastures; range land; and noncrop areas such as fence rows and roadways, where it often is used in combination with a phenoxy herbicide or with other herbicides.[2] Dicamba has found widespread use because of its high efficiency and low toxicity. The release of dicamba-resistant genetically modified plants (soybean and cotton) by Monsanto is another important factor that promoted an increase the use of dicamba worldwide.[3−5] However, dicamba from the old formulations was shown to drift after application. It was reported to vaporize from the treated fields and spread to neighboring nonresistant crops.[6−8] Because of the crop damage and farmers' complaints, Arkansas and Missouri banned the sale and use of dicamba in 2017,[9] and in 2018, the U.S. Environmental Protection Agency (EPA) implemented additional restrictions on the sale and use of dicamba in the United States (https://www.epa.gov/ingredients-used-pesticide-products/registration-dicamba-use-dicamba-tolerant-crops). A lower-volatility formulation of dicamba offered by Monsanto was approved by the U.S. EPA, but the properties of this formulation have not been evaluated by experts outside of Monsanto. In addition, there are reports of suspected illegal use of dicamba. Therefore, it is important to develop an efficient and sensitive analytical method for environmental monitoring that can aid in proper use and monitoring of this herbicide.

At present, the detection and analysis of dicamba (Table 1) are mainly done by chromatographic methods, which include gas[10] and liquid chromatography[11,12] and capillary electrophoresis coupled with ultraviolet (UV)-spectroscopy or tandem-mass-spectrometry (MS)[13−15] detection (UPLC-MS/MS). These methods are not field portable, often require tedious sample preparation, and require expensive equipment. Over the years, enzyme-linked immunosorbent assays (ELISAs) have gained popularity and stand out from the various analytical methods for detection of pesticides and other small molecules.[16−18] The reasons for that are the high throughput capacity of ELISAs in generating quantitative analytical data. Clegg et al.[19] developed the first ELISA for dicamba, based on a polyclonal antibody. It was validated in spiked water samples and its performance was compared with that of GC-MS. However, the immunoassay developed by Clegg et al. had relatively low sensitivity (IC_{50} of 195 ng/mL), which was

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Table 1. Some Reported Assays for the Determination of Dicamba

number	detection methods	IC_{50}	LOD or LOQ	samples	ref
1	LC-MS/MS	—	0.126 ng/g	soil	Xiong et al.[11]
2	positive-ESI LC-MS/MS	—	1.0−3.0 mg/kg	raw agricultural commodities	Guo et al.[12]
3	HPLC-UV	—	0.2 μg/g	soil	Voos et al.[13]
4	CE-UV	—	3.0 ng/mL	water	Hadi et al.[14]
5	HPLC-UVD	—	6.0 μg/kg	food crops	Shin et al.[15]
6	immunoassays, polyclonal antibodies	195 μg/L	2.3 μg/L	water	Clegg et al.[19]

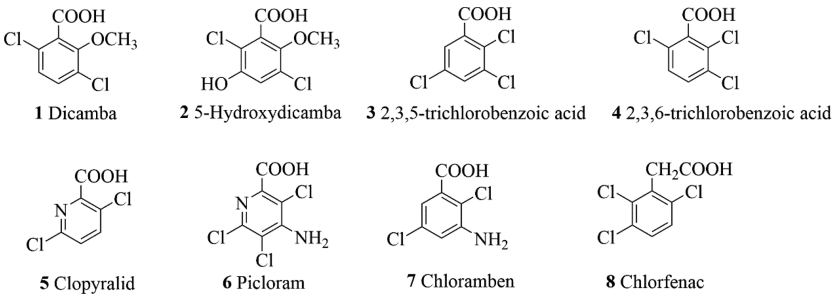
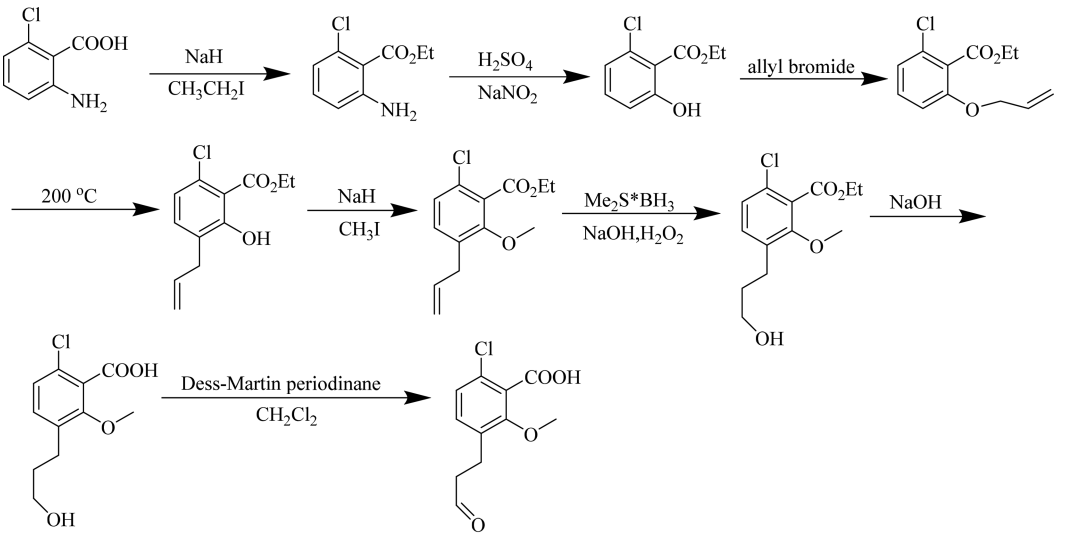
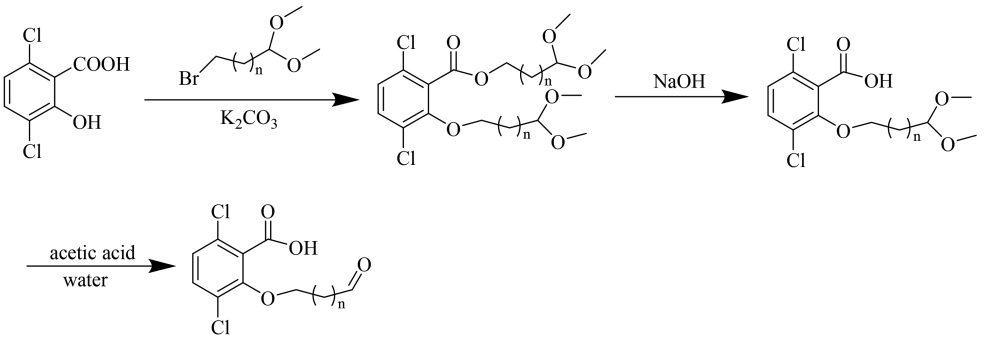


Figure 1. Structures of dicamba, **1**, and structurally related compounds **2**−**8**, which were tested for cross-reactivity.

Scheme 1. Synthetic Route for Dicamba Haptens



Hapten JQ-00-21



n=1 Hapten JQ-00-25
n=3 Hapten JQ-00-24

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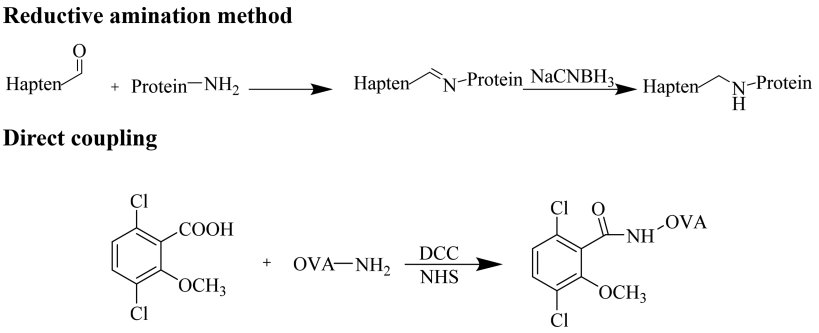
Scheme 2. Preparation of the Hapten−Protein Conjugates[a]



[a]Protein is BSA, OVA, or Thy.

probably because dicamba was coupled to the carrier protein via carboxylic acid, an important structural feature for the recognition of dicamba and because of the homologous nature of their immunoassay. Generally, the position and nature of the spacer arm are two important factors that influence immunoassay performance,[20−22] and they are given particular consideration in the current study. In addition, applying a highly sensitive substrate, such as a chemiluminescent substrate, is another common method for improving the sensitivity of the immunoassay. Chemiluminescent enzyme immunoassays (CLEIAs) are often more sensitive compared with conventional ELISAs with colorimetric readout and have been widely used in analytical fields.[23−26]

An immunoassay with better sensitivity capable of detecting dicamba in environmental samples following its application is still needed in order to assess the efficiency of application and for evaluation of whether all of a field was successfully treated. In addition, an assay with high sensitivity for the detection of dicamba in the areas where dicamba drift may occur is also of great interest. A previous study on dicamba drift showed that an average of 0.56 g of acid-equivalent dicamba per hectare (0.1% of the applied rate) was found 21 m away from a treated plot.[27] The same study showed that as low as 0.01% of the dicamba standard application rate noticeably affects the development of nonresistant plants. This taken together with the potential need to dilute samples to reduce matrix effects, an immunoassay with high sensitivity is needed to address the problem of dicamba drift. In this study, we report the design and synthesis of three novel dicamba haptens with the aim of improving the sensitivity of the immunoassay. Two excellent polyclonal antibodies (#1000 and #998) were produced, and a quantitative indirect competitive chemiluminescent enzyme immunoassay (CLEIA) selective to dicamba was developed on the basis of these antibodies. The performance of the CLEIA for dicamba was evaluated on spiked and real soil and soybean-plant samples and validated by LC-MS. The CLEIA developed here provides a sensitive and convenient method for detecting dicamba in environmental samples.

MATERIALS AND METHODS

Chemicals and Reagents. The chemicals and reagents used for the synthesis of haptens were of analytical grade and were purchased from Sigma or Thermo Fisher Scientific. Bovine-serum albumin (BSA), ovalbumin (OVA), thyroglobulin (Thy), 3,3′,5,5′-tetramethylbenzidine (TMB), luminol, and 4-iodophenol (PIP) were purchased from Sigma. Goat anti-rabbit-IgG−horseradish peroxidase was supplied by Abcam. Standards for dicamba, **1**, and its analogues, 5-hydroxydicamba, **2**; 2,3,5-trichlorobenzoic acid, **3**; 2,3,6-trichlorobenzoic acid, **4**; clopyralid, **5**; picloram, **6**; chloramben, **7**; and chlorfenac, **8** (Figure 1) were purchased from Sigma, Thermo Fisher Scientific, or Chem Service, Inc. OriginPro 8.1 (OriginLab) was used for processing of the analytical data.

Synthesis of Haptens. All reactions were carried out under an atmosphere of dry nitrogen. All chemicals purchased from commercial sources were used as received without further purification. Analytical thin-layer chromatography (TLC) was performed on Merck TLC silica-gel 60 F254 plates. Flash chromatography was performed on silica gel (230−400 mesh) from Macherey Nagel. NMR spectra were recorded on a Varian VNMRS 600 or Inova 400 instrument. Multiplicity is described with the abbreviations b, broad; s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; and m, multiplet. Chemical shifts are given in parts per million (ppm). ^{1}H NMR spectra are referenced to the residual solvent peak at δ = 7.26 ($CDCl_3$). ^{13}C NMR spectra are referenced to the solvent peak at δ = 77.16 ($CDCl_3$). HRMS spectra were recorded on a Thermo Electron LTQ-Orbitrap XL Hybrid MS in ESI. The synthetic route for the haptens is shown in Scheme 1, and synthesis details are listed in the Supporting Information.

Conjugation of Hapten to the Protein. Haptens JQ-00-21, JQ-00-24, and JQ-00-25 were coupled to carrier proteins (BSA, OVA, or Thy) using Schiff-base formation, as previously described.[28] Briefly, carrier protein (BSA, OVA, or Thy; 50 mg) was dissolved in 10 mL of carbonate buffer (pH 9). Then, a solution of the appropriate hapten (JQ-00-21, JQ-00-24, or JQ-00-25; 0.05 mmol) in DMSO was added with gentle stirring. The mixture was stirred for 1 h at room temperature (RT); this was followed by addition of 100 μL of 5 M cyanoborohydride in 1 N NaOH. The reaction mixture was allowed to react for 3 h at RT (Scheme 2). The resulting conjugates were dialyzed in PBS over 72 h at 4 °C and stored at −20 °C for further use. The hapten−Thy conjugate was used for immunization, and the hapten−BSA and hapten−OVA conjugates were used as coating antigens.

3,6-Dichloro-2-methoxybenzoic acid (dicamba, JQ-00-26) was coupled to carrier protein (OVA) using a diimine-carbonization method.[19] Briefly, a mixture of 3,6-dichloro-2-methoxybenzoic acid (22.1 mg), DMF (500 μL), *N*-hydroxysuccinimide (NHS, 11.5 mg), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (20.6 mg) was stirred overnight at RT. The supernatant was collected by centrifugation at 13 800*g* for 5 min and then added dropwise to the 10 mL solution of OVA (112.5 mg) in phosphate buffer (pH 8.0). The reaction was continued at RT for 4 h, and the resulting conjugates were dialyzed in PBS over 72 h at 4 °C and stored at −20 °C for further use.

Production of the Antibody against Dicamba. The immunogens JQ-00-21−Thy, JQ-00-24−Thy, and JQ-00-25−Thy were used to produce polyclonal antibodies #997, #998, #999, #1000, #1001, and #1002. The services of Antagene Inc. were used for the rabbit immunizations according to their protocol. Briefly, two New Zealand white rabbits were immunized with each of the immunogens emulsified with complete Freund's adjuvant. The animals were boosted with an additional 100 μg of immunogen, which was emulsified with Freund's incomplete adjuvant. Booster injections were

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Table 2. Antiserum Titer Responses against Various Coating Antigens[a]

	JQ-00-21−Thy				JQ-00-24−Thy				JQ-00-25−Thy			
	#997		#998		#999		#1000		#1001		#1002	
	dilution fold		dilution fold		dilution fold		dilution fold		dilution fold		dilution fold	
coating antigen (dilution 1000-fold)	1000	10 000	1000	10 000	1000	10 000	1000	10 000	1000	10 000	1000	10 000
JQ-00-21−BSA	+++	+++	+++	+++	+++	+	+++	+	—	—	—	—
JQ-00-21−OVA	+++	+++	+++	+++	+++	+	+++	+	—	—	—	—
JQ-00-24−BSA	+++	++	+++	+++	+++	+++	+++	+++	—	—	—	—
JQ-00-24−OVA	+++	+++	+++	+++	+++	+++	+++	+++	—	—	—	—
JQ-00-25−BSA	—	—	—	—	++	—	++	—	+++	+++	+++	+++
JQ-00-25−OVA	—	—	—	—	++	—	++	—	+++	+++	+++	+++
JQ-00-26−OVA	—	—	—	—	—	—	—	—	—	—	—	—

[a]—, absorbance <0.3; +, absorbance 0.3−0.6; ++, absorbance 0.6−0.9; +++, absorbance >0.9.

given at 20 day intervals. The rabbits were bled 10 days after the fourth immunization, and the serum was collected.

Indirect Competitive-Inhibition ELISA and CLEIA. A checkerboard procedure was first used to determine the optimal dilution of coating antigen and antibody.

ELISA. A microtiter plate was coated with 100 μL/well coating antigen in carbonate buffer (pH 9.6) overnight at 4 °C and then was blocked with 3% skim milk in PBS (10 mM, pH 7.4). The plate was washed three times with PBST (PBS containing 0.05% Tween 20), and then 50 μL of dicamba standard (or sample) and an equal volume of the antibody solution, all dissolved in PBS, were added to the wells and incubated for 1 h at RT. The plate was washed five times with PBST, and 100 μL of goat anti-rabbit-IgG−horseradish peroxidase was added per well at a 10 000-fold dilution before incubation for 1 h at RT. After the plates were washed five times with PBST, 100 μL of TMB-substrate solution (12.5 mL of 100 mM, pH 5.5, citrate−acetate buffer containing 200 μL of 0.6% TMB dissolved in DMSO and 50 μL of 1.0% H_2O_2) was added per well, and the plate was incubated for 15 min at RT. Finally, the reaction was stopped by adding 50 μL/well 2 M H_2SO_4, and the absorbance was read at 450 nm on an Infinite M1000 PRO.

CLEIA. The procedure of the CLEIA was similar to that of the ELISA. The microtiter plate used in CLEIA was a 96-well white microplate, and the blocking agent was 2% BSA. After the competitive reaction and five washes with PBST, 100 μL of the luminol-substrate system (1 mL of 125 mmol/L luminol in DMSO, 1 mL of 125 mmol/L PIP in DMSO, and 10 μL of 30% H_2O_2 added to 8 mL of 0.05 mmol/L Tris-HCl buffer, pH 8.5) was added per well, and the chemiluminescence intensity (relative light units, RLU) was determined using an Infinite M1000 PRO.

Cross-Reactivity (CR). The selectivities of antibodies #1000 and #998 were evaluated by testing their cross-reactivities (CRs) with a set of dicamba structural analogues. Relative CR was calculated by the following formula:

$$CR\ (\%) = [IC_{50}(\text{dicamba})/IC_{50}(\text{tested compound})] \times 100$$

Analysis of Spiked Samples. The spike-and-recovery study was performed using soybean plants and soil. These blank samples were not sprayed with dicamba and were proved to be free of dicamba by LC-MS.

Soybean leaves were frozen in liquid nitrogen, ground, and fortified with dicamba (1 mg/mL in methanol) to final concentrations of 20, 50, and 150 ng/g. Soil samples were fortified with dicamba (1 mg/mL in methanol) to final concentrations of 5, 15, and 45 ng/g and mixed well. These fortified samples (1.0 g) were extracted using 2 mL of 20 mM PBS containing 50% methanol. After vortexing, the mixture was centrifuged at 1500*g* for 15 min, and the supernatants were collected and diluted with 20 mM PBS. All the spiked samples were passed through a 0.22 μm filter and then subjected to CLEIA and LC-MS.

For the LC-MS procedure, samples were injected to an Agilent SL liquid-chromatography system, and the separation was carried out on a Kinetex C18 column (30 × 4.6 mm, 2.6 μm). The column temperature was set up at 50 °C. Water (solution A) and acetonitrile containing 0.1% (v/v) acetic acid (solution B) were used as the mobile phase with a flow rate of 0.6 mL/min. The volume of sample injection was 5 μL, and the run time was 3 min. The gradient is given in Table S1.

The LC system was connected to a 4000 Qtrap mass spectrometer. The instrument was operated in negative-ESI mode and multiple-reaction-monitoring mode. The optimized ion-source parameters and MRM method are shown in Tables S2 and S3, respectively. 12-(3-Cyclohexyl-ureido)-dodecanoic acid with a final concentration of 200 nmol/L was mixed with the analytes and an internal standard to account for ionization suppression.

Analysis and Validation of Real Samples Based on CLEIA and LC-MS. The dicamba-resistant soybean plants were sprayed with 56, 5.6, and 0.56 g/ha dicamba, and the soil samples were collected from the same dicamba-treated soybean field. The amounts of dicamba in the soybean and soil samples were analyzed by CLEIA and LC-MS at the same time. The extraction and analysis followed the same procedures as those used with the spiked samples.

RESULTS AND DISCUSSION

Design and Synthesis of Haptens. Dicamba is a small molecule; therefore, it must be conjugated to a large carrier protein in order to elicit an immune response. Generally, it is important to preserve the key functional groups of the target compound for generating a specific antibody, and therefore it is prudent to attach the handle as distal as possible from the key functional groups.[29] Usually, the length of the linker between the hapten and carrier protein is three to five carbon atoms. The dicamba molecule contains a carboxylic group, which can be directly conjugated to the carrier protein to produce antibodies. Although this method is simple and requires no synthetic chemistry, such a strategy may result in antibodies with low sensitivity because of the significant structural differences between free and conjugated dicamba. Coupling the dicamba via the carboxylic group may also lead to antibodies that mainly recognize the chlorobenzene part of the antigen, with the carboxylic acid functionality being poorly recognized by the antibody. A polyclonal antibody developed using the immunogen generated by the above-mentioned conjugation method was previously developed, and its IC_{50} for dicamba was about 200 ng/mL.[19] We hypothesized that exposing dicamba's carboxylic group in the antigens may result in antibodies with better characteristics, allowing for the development of a more sensitive immunoassay for this pesticide. In addition, retaining a polar carboxylic acid reduces the chance that the hapten will fold back into the hydrophobic protein core.

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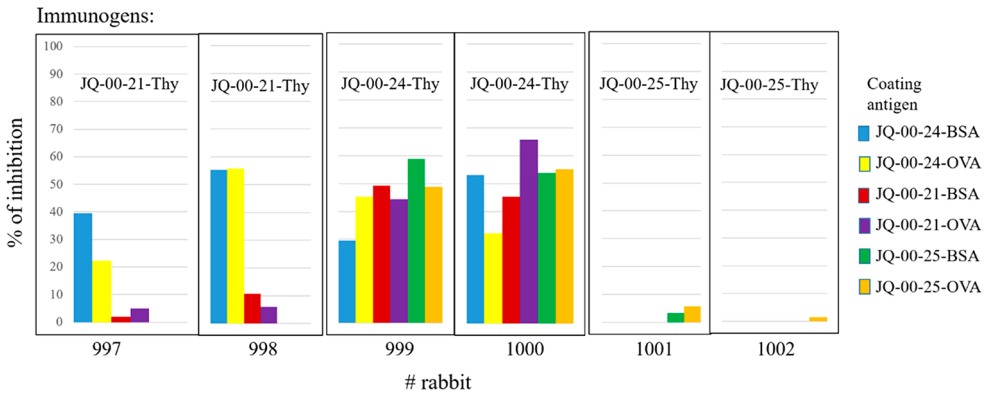

Figure 2. Screening for successful pairs of coating antigen/serum. Criterion of success is ≥50% inhibition at 50 ng/mL dicamba. Absence of the bar indicates that the selected serum did not recognize the corresponding coating antigen.

Table 3. Eight-Point Competitive ELISA Results for the Best Serum/Coating Antigen Pairs

			dilution		curve parameters		
rabbit	immunogen	coating antigen	coating antigen (μg/mL)	antiserum	maximum absorbance	minimum absorbance	IC_{50} (ng/mL)
#998	JQ-00-21−Thy	24−BSA	0.5	1/8000	0.99	0.15	23.0
#998	JQ-00-21−Thy	24−OVA	0.5	1/8000	1.13	0.29	18.4
#999	JQ-00-24−Thy	25−BSA	0.5	1/8000	0.96	0.09	19.6
#1000	JQ-00-24−Thy	24−BSA	0.5	1/80 000	1.87	0.85	220.8
#1000	JQ-00-24−Thy	21−OVA	0.5	1/8000	0.85	0.10	12.3
#1000	JQ-00-24−Thy	25−BSA	5.0	1/4000	1.15	0.24	23.1
#1000	JQ-00-24−Thy	25−OVA	5.0	1/4000	1.26	0.21	40.5

Reductive amination, a common method for the conjugation of haptens to carrier proteins, can be easily performed by reacting the aldehyde group of the hapten with the amino group of the protein, followed by reduction of the labile Schiff-base intermediate into a stable secondary amine in the presence of sodium cyanoborohydride.

In this research, three novel haptens of dicamba were designed and synthesized. Each hapten had an aldehyde group that was used for the conjugation to the carrier protein by the above-mentioned method. The conjugation of the three haptens with BSA (Figure S1) showed that about five to six haptens were conjugated per molecule of protein. As a result of this conjugation, the carboxylic group, a key group in the structure of dicamba, was exposed following conjugation to the surface of the protein. We also synthesized the previously described coating antigen via direct conjugation of dicamba to OVA.

Screening of the Sera and Coating-Antigen Combinations. The titers of the six antisera against the seven coating antigens were measured. As shown in Table 2, all antisera had low titers to coating antigen JQ-00-26−OVA, which indicated that the carboxylic group in the coating antigen was important for the recognition of the antiserum. Antisera #1001 and #1002, generated from immunogen JQ-00-25−Thy, had no or low titers to all the heterologous coating antigens, but antisera #999 and #1000, generated from immunogen JQ-00-24−Thy, had high to moderate titers to the heterologous coating antigens. The difference between JQ-00-25−Thy and JQ-00-24−Thy is in the length of the linker, pointing out the effect of this factor on antibody specificity. Antisera #997 and #998, generated from JQ-00-21−Thy, also had high to moderate titers to the homologous and heterologous coating antigens, even when the antisera were diluted 10 000-fold.

The combinations of antisera and coating antigens that had good recognition with each other were screened in a three-point competitive format (0, 50, and 500 ng/mL dicamba). The results showed (Figure 2) that some combinations had good inhibition with dicamba, and the pairs showing inhibition of ≥50% with 50 ng/mL of dicamba were then tested in an eight-point competitive format (Table 3). From the resulting data, we could see that the IC_{50} value for a homologous pair was generally higher than that for the heterologous assays. For example, in the homologous competitive assay of serum #1000, the IC_{50} value was 220.8 ng/mL (JQ-00-24−BSA), whereas the IC_{50} was 12.3 ng/mL in the heterologous assay (JQ-00-21−OVA). The combinations #1000/JQ-00-21−OVA and #998/JQ-00-24−OVA were chosen for the following studies because they showed the highest sensitivity.

After optimization of antiserum and coating-antigen concentrations, the following IC_{50} values were obtained for the above two combinations: 26.9 ng/mL (#998/JQ-00-24−OVA, Figure 3), with a linear range of 3.85−188.17 ng/mL, and 14.7 ng/mL (#1000/JQ-00-21−OVA, Figure 4), with the linear range of 3.44−62.9 ng/mL.

Indirect Competitive Chemiluminescent Enzyme Immunoassay for Dicamba. Many reports have shown that the sensitivity of immunoassays could be significantly improved using the chemiluminescent readout. Therefore, in order to improve the sensitivity of our assay, a competitive chemiluminescent enzyme immunoassay (CLEIA) was developed on the basis of the combination of antiserum #1000 and coating antigen JQ-00-21−OVA, which had the highest sensitivity according to the results of the ELISA. It is known that the assay parameters, such as pH, ionic strength, organic solvent, and others, influence immunoreactions. Therefore, these parameters were optimized with the goal of decreasing the IC_{50} and increasing the ratio of the maximum relative

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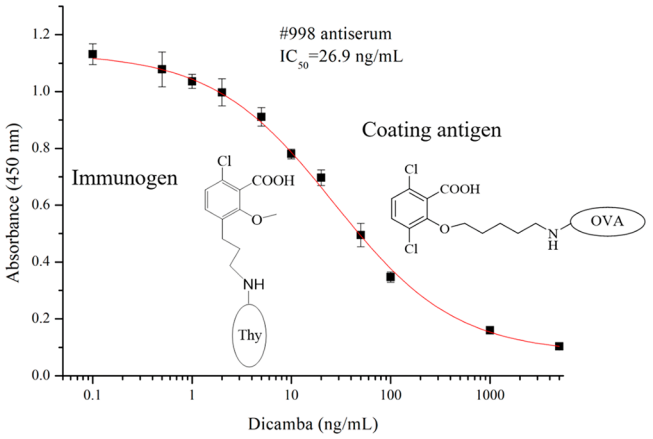

Figure 3. Inhibition curve for dicamba using antiserum #998. Coating antigen JQ-00-24−OVA, 0.35 μg/mL; antiserum, 1:4000; goat anti-rabbit-IgG−horseradish peroxidase, 1:10 000. Each point was tested in triplicate.

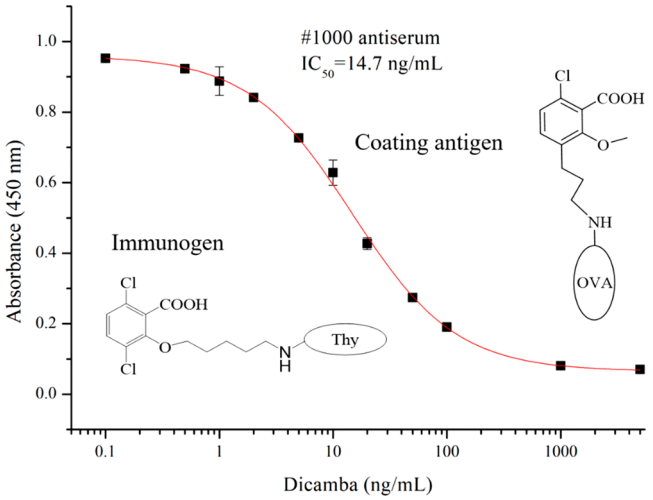

Figure 4. Inhibition curve for dicamba using antiserum #1000. Coating antigen JQ-00-21−OVA, 0.5 μg/mL; antiserum, 1:2000; goat anti-rabbit-IgG−horseradish peroxidase, 1:10 000. Each point was tested in triplicate.

chemiluminescence (RLU_{max}) to IC_{50} (RLU_{max}/IC_{50}). The best combination of fold antiserum concentration (diluted 6000-fold) and coating antigen (diluted 20 000-fold, 175 ng/mL) was determined first using a checkerboard titration. The CLEIA for dicamba was carried out with four different concentrations of PBS, and the results (Figure S2) showed that the chemiluminescence intensity and the sensitivity of the assay were influenced by ionic strength, and the lowest IC_{50} and highest RLU_{max}/IC_{50} were obtained at 20 mM PBS. Next, the effect of pH on the performance of CLEIA was determined (Figure S3), and higher IC_{50} values were observed at pH 5 and 6 (IC_{50} = 3.91 and 3.95 ng/mL, respectively). Overall the assay showed the best performance at pH 7.4. Because of the relatively weak effect on the immunoreaction, methanol is often used in ELISA to improve the solubility of the analyte. In order to evaluate the effect of methanol on the performance of CLEIA, four different PBS solutions containing methanol were studied. As shown in Figure S4, negligible effects on CLEIA were observed at a methanol concentration of 10%. To summarize, the optimal parameters for CLEIA performance were 10% methanol, pH 7.4, and 20 mM PBS.

A standard curve was established using the optimal conditions obtained from the above study for CLEIA (Figure 5). The standard curve had a good correlation coefficient of

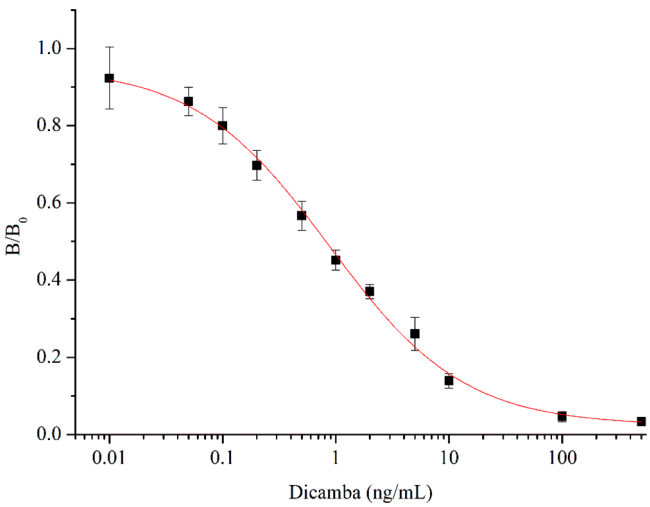

Figure 5. Standard competitive-binding curve of antiserum-#1000-based CLEIA for dicamba under optimized parameters. Coating antigen JQ-00-21−OVA, 175 ng/mL; antiserum #1000, 1:6000.

0.997 and a limit of detection of 0.126 ng/mL. This assay had an IC_{50} of 0.874 ng/mL, with a linear range of 0.131−5.818 ng/mL. The IC_{50} of CLEIA was over 15 times lower than that of the ELISA (IC_{50} = 14.7 ng/mL).

Cross-Reactivity (CR). Although antibodies #1000 and #998 were obtained using different immunogens, they had similar CR. As shown in Table 4, the antibodies were rather

Table 4. Cross-Reactivity of the Antisera #998 and #1000 against Dicamba Structural Analogues

	cross-reactivity (%)	
compound	#998	#1000
dicamba	100	100
5-hydroxydicamba	<0.1	<0.1
2,3,5-trichlorobenzoic acid	<0.1	<0.1
2,3,6-trichlorobenzoic acid	55	33
clopyralid	<0.1	<0.1
picloram	<0.1	<0.1
chloramben	<0.1	<0.1
chlorfenac	<0.1	<0.1

specific toward dicamba, because negligible CR was observed with all compounds except for the structurally close 2,3,6-trichlorobenzoic acid (52% for antibody #1000 and 33% for antibody #998). In the previous[19] reported study, the antibody cross-reacted with 5-hydroxydicamba, 2,3,5-trichlorobenzoic acid, and 2,3,6-trichlorobenzoic acid by about 9.3, 8.4, and 12.8%, respectively. Although, compound 2,3,6-trichlorobenzoic acid had a high CR, currently it is not widely used for weed control and is not likely to be found in environmental samples.

Matrix Effect. Matrix effects are important factors to consider in an immunoassay. The matrix often has a significant effect on the performance of the immunoassay, which then

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alters the quality of the results.[30] Dilution of the sample with assay buffer is the most common method to reduce or eliminate the matrix effects on an immunoassay. Here, dicamba-resistant soybean and soil were selected for matrix-effect evaluation. The blank samples were confirmed to be free of dicamba by LC-MS analysis (LOQ = 0.1 ng/mL, LOD = 0.03 ng/mL). Soybean leaves and soil samples were diluted 10-, 20-, 40-, 80-, and 160-fold in the assay buffer, respectively. Serial dilutions of dicamba standards were prepared in the above-mentioned diluted samples. The results (Figure 6A)

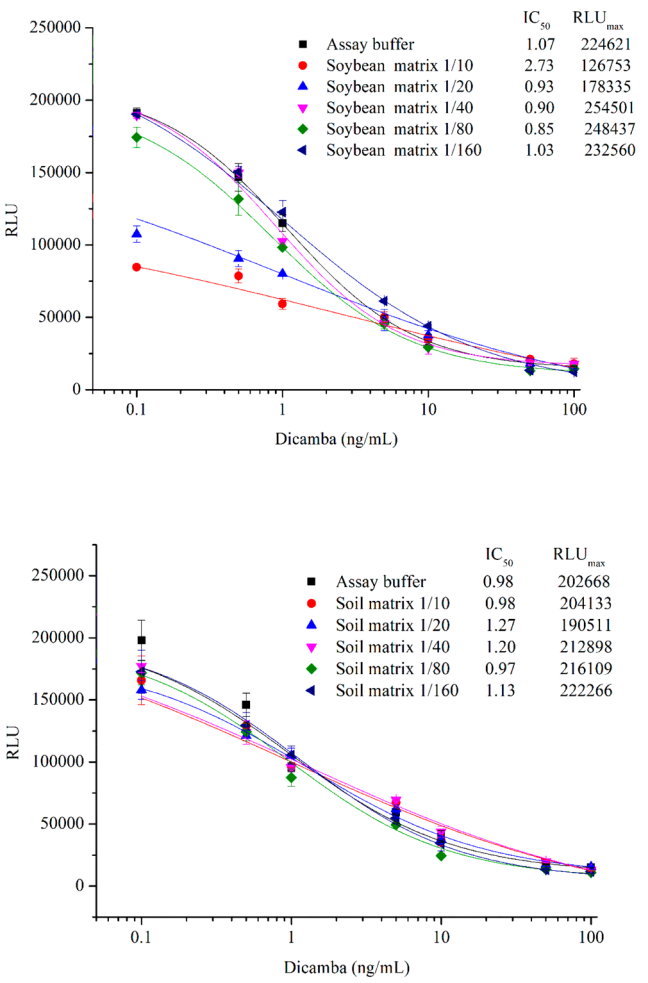

Figure 6. Effect of soybean and soil matrix on the performance of antiserum-#1000-based CLEIA.

showed that the RLU_{max} values of 10- and 20-fold diluted soybean samples were lower than those of other dilutions. Additionally, the 10-fold diluted soybean sample had a higher IC_{50}, which indicated that a higher concentration of the soybean matrix affected antibody binding. The maximum chemiluminescence-intensity and IC_{50} values showed no significant differences among the 40-, 80-, and 160-fold diluted matrix samples and assay buffer. Thus, a 40-fold dilution factor was chosen for the developed assay. The soil-matrix results (Figure 6B) showed that neither the maximum chemiluminescence intensity nor the IC_{50} were significantly affected, indicating that the assay method developed in this study was resistant to soil-matrix effects.

Validation Study. The accuracy and reliability of the developed CLEIA for detecting dicamba were evaluated by applying this method to the quantification of dicamba in spiked soybean-plant and soil samples that were confirmed to be free of dicamba by LC-MS. The soybean and soil samples were spiked with three different concentrations of dicamba and analyzed by both CLEIA and LC-MS. As shown in Table 5, the average recovery rate from the soybean plant measured using CLEIA and LC-MS ranged from 86 to 108% and from 76 to 117%, respectively. For the soil, the average recovery rate ranged from 105 to 107% (CLEIA) and 107 to 116% (LC-MS). It is worth noting that developed CLEIA method provides quantitative data on the total amount of dicamba present in the soil, which may differ significantly from the amount that is bioavailable.

In addition, eight soybean-plant samples and six soil samples collected from a field sprayed with dicamba were analyzed with CLEIA and LC-MS. Soybean samples 1−3 were sprayed with 1/10, 1/100, and 1/1000 of the dicamba standard rate (560 g/ha) and were collected 1 day after treatment. Soybean samples 4−8 were sprayed with 1/10 of the dicamba standard rate and were collected 7, 14, 21, 39, and 67 days after treatment, respectively. Soil samples 1−3 were sprayed with 1/10, 1/100, and 1/1000 of the dicamba standard rate and were collected 1 day after treatment. Soil samples 4−6 were sprayed with 1/10 of the dicamba standard rate and were collected 7, 14, and 21 days after treatment, respectively. As shown in Table 6, correlation was observed between these two methods. These data show that the concentration of dicamba decreases over time after application, consistent with previously reported half-life time of 1 to 4 weeks.[31] Alternatively, the observed time-dependent decrease of dicamba concentration might be at least partially due to its drift, but this factor was not evaluated in the current study. Most importantly, the developed CLEIA method was able to detect dicamba on soybean treated at 1/1000 the dicamba standard application rate (560 g/ha), which corresponds to average drift concentrations found 21 m away from a treated field and which can cause slight abnormalities in nonresistant plants.[27]

A good correlation between the CLEIA and LC-MS results was observed for both the spike-and-recovery studies and the

Table 5. Spike-Recovery Results for Soybean-Plant and Soil Samples Determined by CLEIA and LC-MS[a]

sample	spiked concentration (ng/mL)	CLEIA (ng/mL)	average recovery (%)	CV (%)	LC-MS (ng/mL)	average recovery (%)	CV (%)
soybean plant	20	21.69 ± 2.40	108	11.07	23.37 ± 2.42	117	10.36
	50	49.07 ± 5.03	98	10.26	49.57 ± 2.48	99	5.00
	150	128.80 ± 10.58	86	8.22	113.7 ± 7.22	76	6.35
soil	5	5.34 ± 0.094	107	8.78	5.35 ± 0.34	107	6.27
	15	15.7 ± 0.09	105	8.62	17.4 ± 0.51	116	2.95
	45	47.64 ± 0.086	106	8.17	49.19 ± 2.93	109	5.96

[a]Antibody #1000 was used to analyze the spiked samples.

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Table 6. Quantification of Dicamba in the Real Samples of Soybean Plant and Soil Determined by CLEIA and LC-MS[a]

sample	number	treatment dose (g/ha)	collection time (days after treatment)	CLEIA (ng/g)	CV (%)	LC-MS (ng/g)	CV (%)
soybean plant	1	56	1	1043.95 ± 64.13	6.14	997.92 ± 84.86	8.50
	2	5.6	1	27.45 ± 1.91	6.97	28.67 ± 0.56	1.95
	3	0.56	1	4.76 ± 0.17	3.66	4.66 ± 0.26	5.64
	4	56	7	56.95 ± 7.42	13.02	64.17 ± 3.55	5.54
	5	56	14	177.81 ± 8.54	4.81	179.79 ± 6.74	3.75
	6	56	21	109.22 ± 7.35	6.73	109.79 ± 10.72	9.77
	7	56	39	ND	ND	2.35 ± 0.19	7.97
	8	56	67	ND	ND	2.63 ± 0.26	9.93
soil	1	56	1	165.68 ± 2.79	1.68	168.25 ± 12.8	7.61
	2	5.6	1	6.67 ± 0.46	6.96	6.87 ± 0.37	5.40
	3	0.56	1	ND	ND	0.80 ± 0.17	21.48
	4	56	7	86.63 ± 13.64	15.74	84.25 ± 1.89	2.24
	5	56	14	19.8 ± 1.42	7.19	17.87 ± 0.77	4.32
	6	56	21	2.58 ± 0.50	19.29	3.07 ± 0.11	3.68

[a]Antibody #1000 was used to analyze these samples.

real samples. Thus, the developed CLEIA method showed good accuracy and reliability for the detection and quantification of dicamba in environmental samples. This method will be instrumental in evaluating the drift propensity of new dicamba formulations as well as for rapid analysis of a large number of environmental samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b07134.

Details of hapten synthesis; MALDI-TOF spectra for BSA, hapten JQ-00-21−BSA, hapten JQ-00-24−BSA, and hapten JQ-00-25−BSA; effects of ionic strength, pH, and methanol on the performance of CLEIA for dicamba; LC gradient; mass-spectrometric source parameters; and MRM method (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +86-0312-7528575. Fax: +86-0312-7528575. E-mail: zhangjinlin@hebau.edu.cn (J.Z.).

*Tel.: 5307520492. Fax: 5307521537. E-mail: bdhammock@ucdavis.edu (B.D.H.).

ORCID

Dongyang Li: 0000-0002-5603-3608

Jinlin Zhang: 0000-0001-7768-9406

Bruce D. Hammock: 0000-0003-1408-8317

Author Contributions

⊥J.H. and B.B. contributed equally to this work.

Notes

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