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Production of the Polyclonal Antibody against Sudan 3 and Immunoassay of Sudan Dyes in Food Samples

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Supporting Information

ABSTRACT: In this study, 4-aminophenylacetic acid was covalently coupled to aniline to synthesize an intermediate hapten and the intermediate hapten was coupled to β-naphthol to synthesize a tentative hapten of Sudan 3. The hapten was coupled to bovine serum albumin as the immunogen to produce the polyclonal antibody. The obtained antibody was highly specific to Sudan 3, Sudan 1, and Para red, but showed relative low binding ability to Sudan 2, Sudan 4, and Sudan red G. After evaluation of different coating antigens, a heterologous indirect competitive immunoassay was developed to multidetermine the six red dyes in food samples. The cross reactivities to the six analytes were in a range of 21−105%, and the limits of detection were in a range of 0.1−0.8 ng/mL depending on the compound. Intra- and interassay recoveries from the standard fortified blank samples were in a range of 74.5−96.3% with coefficients of variation lower than 15.1%.

KEYWORDS: *Sudan dyes, Sudan 3, polyclonal antibody, immunoassay, food*

INTRODUCTION

Sudan dyes (Sudan 1, 2, 3, 4, Para red, and Sudan red G) are a class of synthetic azo dyes that are usually used in industry or printing. Their chemical structures are shown in Figure 1. Among these red dyes, Sudan 1 (**1**) has been shown to cause cancer in animals and human beings,[1−3] so it is classified a category 3 carcinogen by International Agency for Research on Cancer.[4] Para red (**5**) is chemically similar to **1**, so the independent scientific experts of UK Food Standards Agency advised that it would be prudent to assume that **5** could also be a genotoxic carcinogen.[5] In two recent publications,[6,7] it was shown that **1**, Sudan 2 (**2**), Sudan 3 (**3**), Sudan 4 (**4**), and **5** can be reduced to the carcinogenic aromatic amines by some prevalent species of human intestinal bacteria. This means the residues of these red dyes in foods are not safe for human beings, so many countries have forbidden the use of Sudan dyes as food colorants.[8] However, due to their intensive red-orange color they were still illegally used as food additives to enhance and/or maintain the appearance of chili-, curry-, curcuma-, and palm oil containing foods.[9]

Therefore, it is very important to monitor the presence of these red dyes in foodstuffs, and the development of specific and sensitive analytical methods to determine their residues is the aim of many researchers. By now, many instrumental methods, such as HPLC,[10−14] GC/MS,[14] and LC−MS/MS,[15−17] have been reported to determine these red dyes in various foods. Though these methods can qualitatively and quantitatively determine these dyes, they are time-consuming, and sophisticated extraction procedures and expensive instruments are required. Compared with those methods, enzyme linked immunosorbent assay (ELISA) is a low cost, rapid, and sensitive method capable of screening a large amount of samples in a single test.

By now, there have been several publications involving the ELISA method for the detection of Sudan dyes.[9,18−23] In these reports, **1** was usually used as template to synthesize different haptens and the produced antibodies could recognize one,[19] two,[18,20] three,[9,22] or at most four analytes.[21] Recently, we generated the polyclonal antibody against **5**, and the obtained antibody simultaneously recognized the six red dyes.[23] All the reported antibodies showed high cross reactivity to **1**, **3** and **5**, but showed low or negligible cross reactivity to **2**, **4**, and Sudan red G (**6**). Except **1** and **5**, other Sudan dyes (such as **3**) have not been used to produce antibody for immunoassay of these red dyes so far.

For development of a multianalyte ELISA, the best way is to obtain an antibody showing broad specificity to all the target analytes. For production of a broad specific antibody toward the six red dyes, the key step is to design and synthesize a generic hapten. As shown in Figure 1, the core structure of these red dyes is 1-phenylazo-2-naphthalenol. The specific antibody toward the core structure should recognize the six analytes simultaneously. However, the previous reports have shown the antibodies of Sudan 1 and Para red whose molecules only contain the core structure achieved the unsatisfactory results.[9,18−23] As shown in Figure 1, the molecule of Sudan 3 also contains the core structure and its molecule is bulkier and more complex than that of Sudan 1 and Para red. Moreover, the molecules of Sudan 1 and Para red could all be regarded as a part of Sudan 3. Therefore, Sudan 3 was used as the template to produce polyclonal antibody in this study with the aim of multi-immunoassay of these red dyes in foods.

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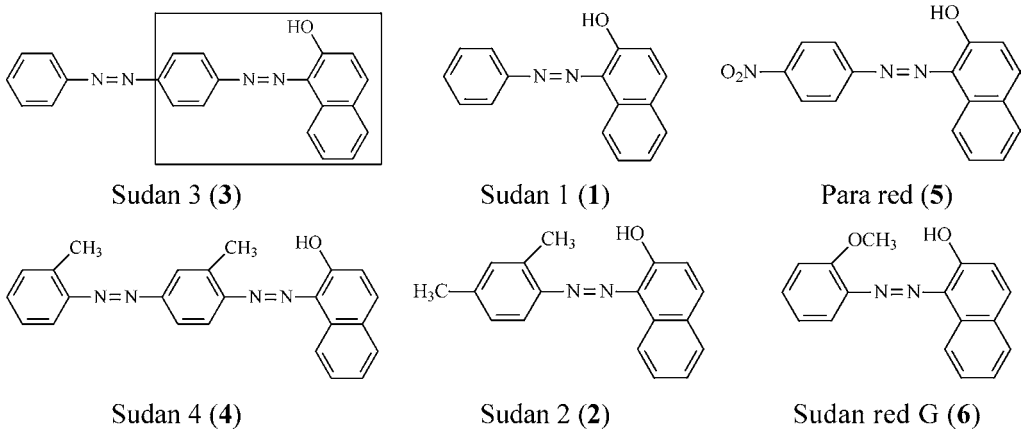

Figure 1. Chemical structures and analytes symbols of the six common Sudan dyes.

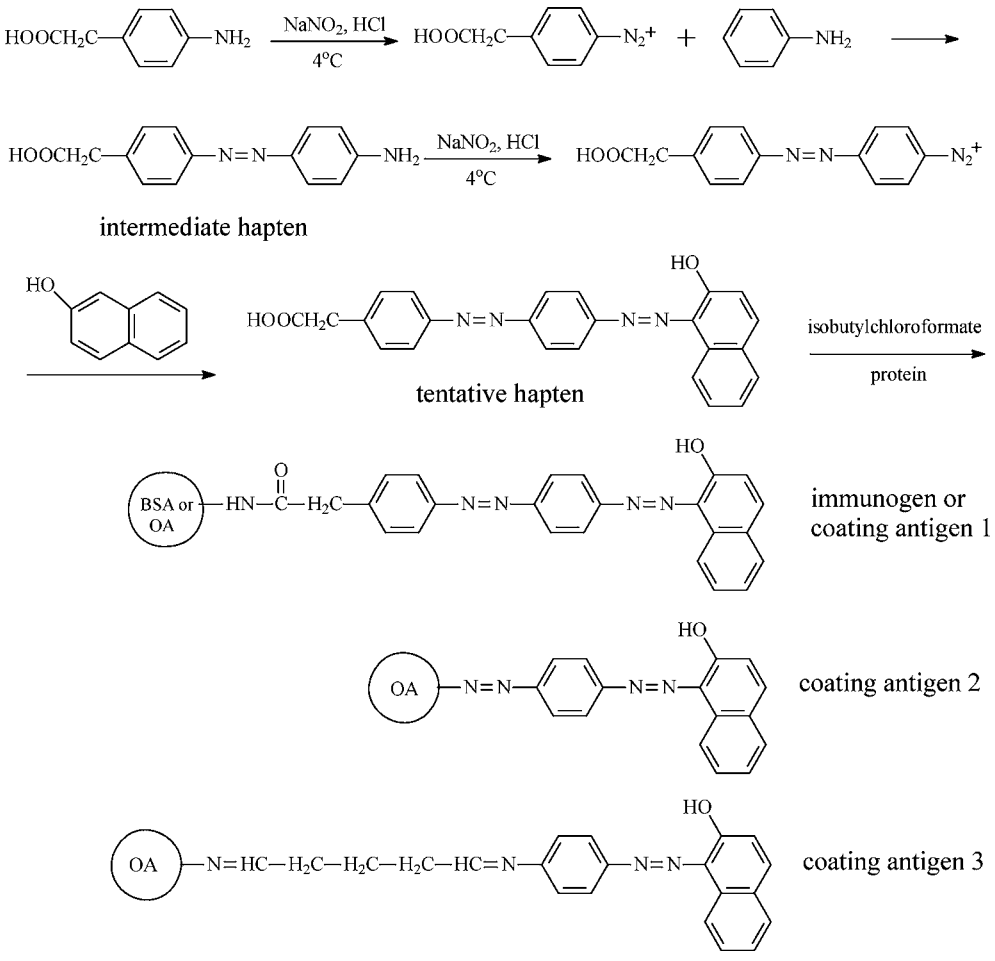

Figure 2. Synthetic route for the hapten and the conjugates of Sudan 3.

MATERIALS AND METHODS

Reagents and Materials. Sudan 1, Sudan 2, Sudan 3, Sudan 4, Para red, bovine serum albumin (BSA), ovalbumin (OA), and Freund's adjuvants were purchased from Sigma-Aldrich (Shanghai, China). Sudan red G was obtained from Shanghai JingChun reagent company (Shanghai, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). 4-Aminophenylacetic acid, aniline, β-naphthol, and other chemical reagents were all analytical grade or better from Beijing chemical company (Beijing, China).

Stock solutions of each compound (100 μg/mL) were prepared in acetonitrile with ultrasonication and stored at −20 °C in the dark to be stable for 3 months. Working solutions of these red dyes with series concentrations (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 ng/mL) were diluted from stock solutions with PBS. All the working solutions were stored at 4 °C to be stable for at least 4 weeks. PBS (pH 7.2) was prepared by dissolving 0.2 g of KH_2PO_4, 0.2 g of KCl, 1.15 g of Na_2HPO4, and 8.0 g of NaCl in 1000 mL of demineralized water. Washing buffer (PBST) was PBS buffer containing 0.05% Tween. Coating buffer was carbonate buffer (0.1 M, pH 9.6). Substrate buffer was 0.1 M citrate (pH 5.5). The substrate system was prepared by

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adding 200 μL of TMB in DMSO (1%, w/v) and 64 μL of 0.75% (w/v) H_2O_2 into 20 mL of substrate buffer.

Synthesis of the Hapten. The synthesis of the hapten (derivative of **3**, **3**D) was divided into two steps. The synthetic route is shown in Figure 2. The first step was to couple 4-aminophenylacetic acid to aniline by use of the diazotization method. About 150 mg (1 mmol) of 4-aminophenylacetic acid was dissolved in 5 mL of 2 M hydrochloric acid with stirring, and the solution was cooled down to 4 °C. Then a solution of 0.1 M $NaNO_2$ was continuously added dropwise into the above solution. During this process, the reaction procedure was monitored by use of potassium iodide−starch test paper until the test paper turned blue (solution 1). About 94 mg (1 mmol) of aniline was dissolved in 2 mL of sodium bicarbonate solution, and the solution was cooled down to 4 °C (solution 2). Solution 2 was continuously added dropwise into solution 1 with gentle stirring. The mixture was stirred for 30 min at 4 °C to obtain the red sediment. Then the pH of the solution was adjusted to 3.0 with concentrated hydrochloric acid prior to another 30 min stirring. Finally, the mixture was filtered under vacuum, and the obtained red deposit was washed with 50 mL of water and subsequently dried to yield the intermediate hapten.

The second step was to couple the intermediate hapten to β-naphthol by use of the diazotization method (Figure 2). The reaction procedure was carried out as described above just replacing 4-aminophenylacetic acid with the intermediate hapten (120 mg, 0.5 mmol) and replacing aniline with β-naphthol (70 mg, 0.5 mmol). Finally, the tentative hapten **3**D was obtained (IR (KBr) ν_{max} 3550, 3200−2500, 3031, 1618, 1558, 1506, 1448, 1225, 1209, 1143, 983, 835, 687, 752 cm^{-1}).

Preparation of 3D Conjugates. The hapten was coupled to carrier protein by use of the mixed anhydride method. The preparation route is shown in Figure 2. About 3 mL of *N*,*N*-dimethylformamide dissolving 21 mg of hapten and 30 μL of triethylamine were added into a glass jar. Then 30 μL of isobutyl chloroformate was added and the mixture was stirred for 60 min at 4 °C. The obtained solution was added dropwise into 3 mL of PBS containing 74 mg of BSA or 40 mg of OA, and the mixture was stirred for 12 h at 4 °C. The obtained immunogen (**3**D-BSA) and coating antigen (**3**D-OA) were dialyzed against three changes of PBS for 3 days and stored at −20 °C until used. The conjugates, carrier protein, **3** and **3**D were scanned respectively on a UV/vis spectrophotometer to identify the conjugation and the hapten/protein coupling ratios were determined according to a previous 2,4,6-trinitrobenzene sulfonic acid method.[24]

Production of the Polyclonal Antibody. Six New Zealand white rabbits were kept at the Animal Experiment Center of College of Animal Science and Technology, Agricultural University of Hebei. The animal experiments were performed according to the Regulation Guideline for Experimental Animals issued by the Ministry of Science and Technology of China. The immunogen **3**D-BSA was used to produce the polyclonal antibody. The rabbits were immunized with an emulsion of **3**D-BSA (0.5 mg of protein per animal) in Freund's complete adjuvant on the dorsal region subcutaneously the first time and then were boosted with an emulsion of **3**D-BSA in Freund's incomplete adjuvant at 3 week intervals. Through seven boosters, the serum from each rabbit was collected and the antibody specificity was monitored by use of indirect competitive ELISA procedure described below. Finally, the rabbits were exsanguinated and the sera were collected, and the IgG fraction was isolated according to a previous saturated ammonium sulfate precipitation method[25] to develop the indirect competitive ELISA.

Development of the ELISA Method. In the present study, coating antigen **3**D-OA, two coating antigens of **5** (Figure 2) previously prepared[23] in our lab, and the obtained antibody were incorporated into three combinations to choose the best reagent combination. The optimal dilutions of each coating antigen and antibody were determined by use of a checkerboard procedure, in which the wells with an absorbance of 1.0 were defined as the optimum coating antigen concentrations and antibody dilutions. Then, the indirect competitive ELISA was developed. Briefly, each well of a microtiter plate was coated with 100 μL of coating antigen and incubated overnight at 4 °C, then blocked with 1% fetal calf serum. The plate was washed three times with PBST. Then 50 μL of optimal antibody dilution and 50 μL of **3** solution with series concentrations were added into the wells (in triplicate) for incubation for 1 h at 37 °C. The plate was washed as above. Then, 100 μL of horseradish peroxidase labeled goat anti-rabbit IgG was added for incubation for 30 min at 37 °C. After washes, 100 μL of TMB substrate system was added for incubation for 15 min at 37 °C. Finally, the reaction was stopped by addition of 50 μL of 2 M H_2SO_4 to each well, and the plate was read on an ELISA plate reader at 450 nm to obtain the OD values.

The other five Sudan dyes and four other competitors (2-naphthol, sunset yellow, fancy red, butter yellow) were also determined as described above. The half inhibition concentration (IC_{50}) and the limit of detection (LOD) for these competitors were determined as the concentrations showing 50% and 10% of inhibition respectively. The cross reactivity (CR) was calculated as follows: CR (%) = $IC_{50(Sudan3)}/IC_{50(competitor)} \times 100\%$. The competitive inhibitory curves were developed by plotting the concentrations (Log C) verse the B/B_0 values (mean OD values of each standard at various concentrations divided by that of the zero standards).

Sample Preparation. *Chili Oil Sample.* The extraction method was modified from a previous report.[13] Briefly, about 2.0 g of homogenized chili oil was added into a 50 mL polypropylene centrifuge tube. Then 10 mL of acetonitrile was added, and the mixture was stirred on a high-speed blender for 5 min. After centrifugation for 5 min at 3000 rpm, the acetonitrile phase was transferred into a clean tube. The residue sample was extracted again. Then the acetonitrile phase was combined and evaporated to dryness on a rotary evaporator at 50 °C. The dry residue was reconstituted in 2 mL of methanol/PBS (20:80, v/v) and filtered through a 0.22 μm Millipore membrane filter prior to ELISA analysis.

Egg Sample. The extraction of Sudan dyes from egg sample was according to our previous report.[23] Briefly, 2.5 g of homogenized egg yolk and 6 g of anhydrous sodium sulfate were added into a 50 mL polypropylene centrifuge tube. Then 20 mL of acetonitrile was added, and the mixture was stirred on a high-speed blender for 5 min. After centrifugation for 5 min at 3000 rpm, the supernatant acetonitrile phase was collected and evaporated to dryness on a rotary evaporator at 50 °C. The dry residue was reconstituted in 2.5 mL of methanol/PBS (20:80, v/v) and filtered through a 0.22 μm Millipore membrane filter prior to ELISA analysis.

Sample Analysis. Twenty-eight unknown chili oil samples and twenty unknown eggs purchased from different markets of China were analyzed by the developed ELISA method. Furthermore, four egg samples which were determined to be **4** positive[12,17,23] were also analyzed by the ELISA. The extracts of these samples were confirmed with our previously reported HPLC method.[23]

RESULTS AND DISCUSSION

Hapten 3D and Immunogen. As shown in Figure 1, the six Sudan dyes contain the same central group, 1-phenylazo-2-naphthalenol. The molecule of **1** is just the central group, and the molecule of **5** is almost same as the central group. The antibody against this structure should recognize all these red dyes. However, the reported antibodies of **1** and **5** showed high CRs to **1**, **3**, and **5**, but showed low or negligible CRs to **2** and **4**.[9,18−23] **3** also contains the central group, and its molecular structure is bulkier and more complex than that of **1** and **5**. In order to verify if the antibody of **3** is better than the antibodies of **1** and **5**, this study was conducted.

There is no chemical group available in the molecule of **3** to be used to couple with carrier protein directly. Therefore, a novel protocol for synthesis of the hapten of **3** was designed (Figure 2). The first step was to couple the diazotized 4-aminophenylacetic acid to aniline at the para position to synthesize an intermediate hapten. The second step was to couple the diazotized intermediate hapten to β-naphthol at the

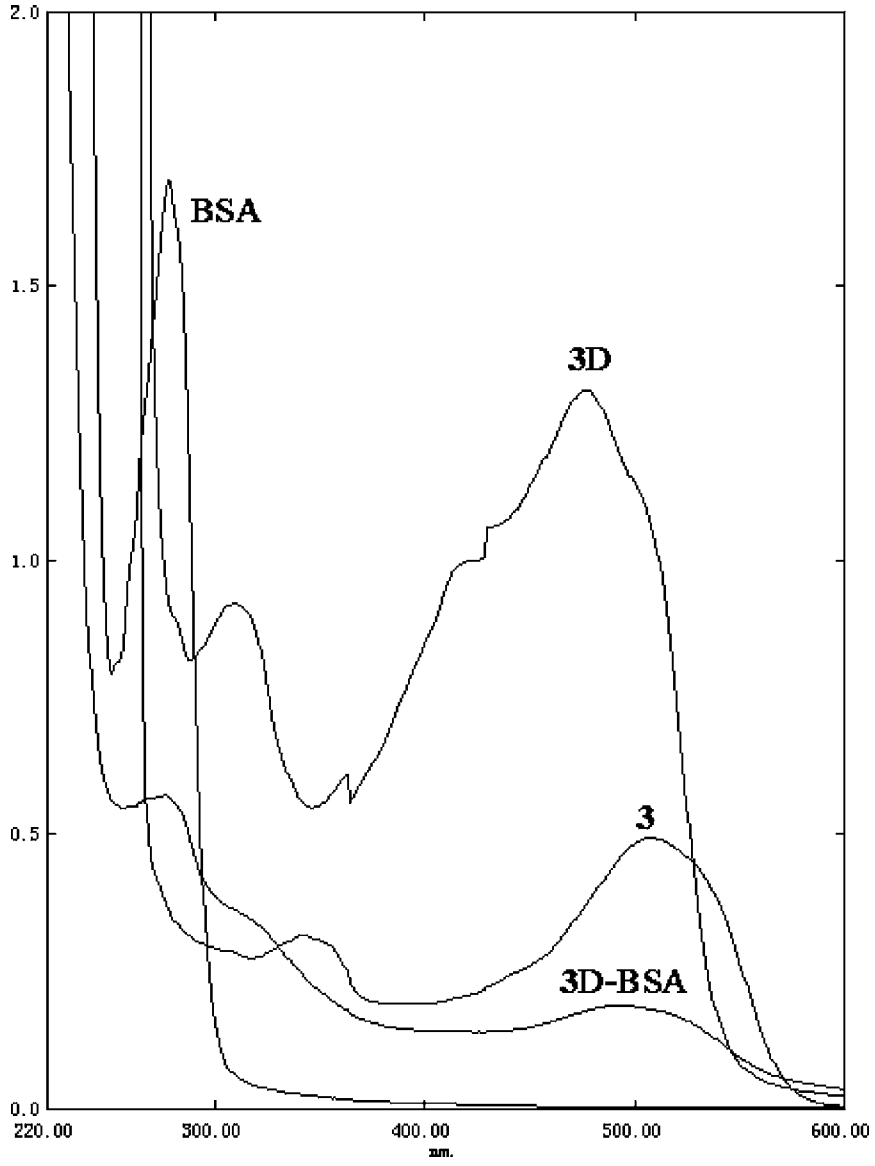

Figure 3. UV spectra of **3** (Sudan 3), **3**D (the hapten), BSA, and **3**D-BSA.

α-position to obtain the tentative hapten **3**D (Figure 2). In the previous reports,[9,18−22] the haptens of **1** were all synthesized by coupling different aromatic diazonium salts to β-naphthol. The successful synthesis of the hapten **3**D was proven by the following data. First, the intermediate hapten and the tentative hapten were all red compounds, indicating that an azo bond was present. Second, the melting point of the hapten (145−150 °C) was different from the melting points of 4-aminophenylacetic acid (196−201 °C), aniline (−6 °C), and β-naphthol (123−124 °C), indicating that it was a new compound. Third, its UV spectrum contained the characteristic peak of **3** (Figure 3), indicating that the general structure of **3** was present. Fourth, the IR data showed that the compound contains a carboxyl group and a methylene group besides the original chemical groups of **3** (hydroxyl group, benzene ring, and naphthalene ring). This hapten synthesis procedure was equivalent to introducing a free carboxyl group at the molecular end of **3**, with which to link with carrier protein.

Then **3**D was coupled to carrier protein by use of the mixed anhydride method. During the conjugation, the red color was always present throughout the process of coupling, purification, and dialysis. Furthermore, the UV spectrum of **3**D-BSA contained the characteristic peaks of **3** and BSA (Figure 3). These findings suggested the successful conjugation. The coupling ratio in immunogen was 20, and that in coating antigen was 13. In this immunogen, the central group of the six red dyes presented to the immune system was far from the carrier, so the produced antibody should recognize the six analytes simultaneously.

Antibody Performances. This is the first report of the production of the antibody against Sudan 3. Among the sera from the six rabbits, the serum showing the highest titer was used for the subsequent experiments. After the polyclonal antibody was obtained, its specificity for these red dyes was determined by the indirect competitive ELISA method. The antibody showed negligible CR to sunset yellow, fancy red, and butter yellow, but showed a CR of 6% to 2-naphthol.

The antibody simultaneously recognized the six red dyes with IC_{50} values in the range of 2.14−26.8 ng/mL and CRs in the range of 8−100% (see Supporting Information). In **3**D-BSA, there was a benzene ring between the central group and the carrier, and the complex benzene ring could also stimulate

lymphocyte to generate the specific antibody. This part of the antibody perhaps influences the discrimination ability of the antibody for structural changes, thus showing binding ability to the chemically similar competitors.

However, the antibody was highly specific to **1**, **3**, and **5** with CRs higher than 91%, but showed low CRs to **2**, **4**, and **6** (8−15%). This phenomenon was similar to the previous reports.[9,18−23] This is because of the different chemical structures of the six analytes. As shown in Figure 1, the molecules of **1**, **3**, and **5** can all be regarded as a part of the hapten **3**D and the central group in their molecules can be clearly recognized by the antibody, thus generating the high CRs. For **2**, **4**, and **6**, there is a methyl or a methoxy substitute on the central structure in their molecules, and these substitutes influenced the antibody's recognition for them, thus generating the low CRs.

Heterologous ELISA Method. Among different ELISA formats, heterology in the coating antigen has been commonly used to improve the method performance.[23,26−30] The ELISA performance can be improved in heterologous format only when a combination of antibody/coating antigen is appropriate. In other words, decreasing the recognition of the antibody for the coating hapten is important. Recently, Xu et al. prepared an antibody to *O*,*O*-diethyl organophosphorus pesticides based on hapten 1, and they also synthesized other two haptens to develop the heterologous immunoassay.[30] Results showed that using the coating antigen containing a hapten 1-different hapten could better improve the assay performances than using the coating antigen containing a hapten 1-similar hapten. Therefore, they suggested that using only a partial structure of the target molecule (or immunizing hapten) as the coating hapten may be a good strategy.

In the present study, **3**D-OA (coating antigen 1) and other two coating antigens based on **5** (coating antigens 2 and 3) were incorporated into three combinations with the antibody to optimize the optimal reagent combination. Results showed that the assay sensitivity and specificity were significantly improved in heterologous formats with CRs in the range of 15−105% and IC_{50} in the range of 1.2−11.3 ng/mL (see Supporting Information). This is the advantage of heterologous ELISA. Among the three coating antigens, the hapten structure in coating antigens 2 and 3 is a part of **3**D, and this result was consistent with the previous findings.[30]

In addition, the performances when using coating antigen 2 (IC_{50} of 1.2−5.7 ng/mL and CRs of 21−105%) were better than that when using coating antigen 3 (IC_{50} of 1.7−11.3 ng/mL and CRs of 15−100%). The hapten in coating antigens 2 and 3 is the same, but there is a long spacer arm in coating antigen 3, and the long spacer arm perhaps makes coating antigen 3 show more competitive ability than coating antigen 2, thus decreasing the antibody recognition to the competitors. In consideration of sensitivity and specificity, the antibody incorporating coating antigen 2 was used for the subsequent experiments. The competitive inhibitory curves for the six analytes are shown in Figure 4A (**3**, **5**, **2**, **4**, **6**) and Figure 4B (**1**) with analyte concentrations in the range of 0.05−50 ng/mL.

Sample Preparation and ELISA Determination. A very important step in the evaluation of an analytical procedure is to assess the matrix effect, which should be minimized by the sample preparation. During the experiments, the stock solution of **1** was diluted with the extracts of blank chili oil and blank egg to prepare the matrix matched **1**. As shown in Figure 4**B**,

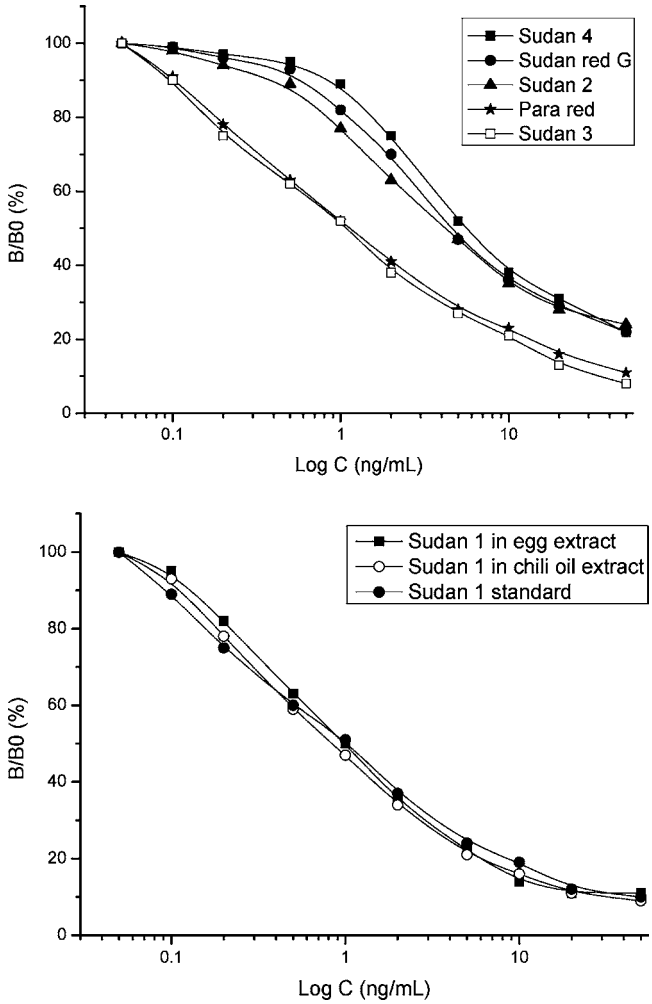

Figure 4. Standard competitive inhibitory curves of Sudan 3, Para red, Sudan 2, Sudan 4, and Sudan red G (A), and the standard and matrix matched competitive inhibitory curves of Sudan 1 (B).

the matrix-matched inhibitory curves of **1** were similar to that of **1** standard, revealing that the extraction methods were satisfactory. Therefore, the LODs for these analytes in chili oil and egg were calculated as the LODs for their standards, i.e. in a range of 0.1−0.8 ng/mL (see Supporting Information). The LODs for **1**, **3**, and **5** were similar to the previous reports,[9,18−22] and those for **2**, **4**, and **6** were similar to our previous report.[23] Then, blank chili oil and egg samples fortified with the six analytes at two levels were extracted and determined by the ELISA. The intra-assay recoveries, interassay recoveries, and coefficients of variation (CV) are shown in Table 1.

In order to assess the detection capability of the ELISA, the 48 unknown samples (28 chili oil samples and 20 eggs) and the four **4**-positive eggs[12,17,23] were analyzed by the ELISA. No unknown sample was determined as positive, and the results were confirmed with a HPLC method[23] to be negative samples. The four **4**-positive eggs were determined as positive samples, and the results calculated as **3** were 6.2, 27, 48, and 5.5 ng/g. The residue levels of **4** in the four eggs in the previous papers were 35.4 and 155 ng/g,[12] 300 ng/g,[17] and 35.6 ng/g,[23] respectively. Taking into account the CR and recovery of the ELISA to **4**, the ELISA results were comparable to those of the instrumental methods. This means if one sample contains any of these red dyes, the ELISA method can only detect the

Table 1. Recoveries of the Six Sudan Dyes from Standard Fortified Samples

		chili oil				egg			
		interassay		intra-assay		interassay		intra-assay	
analyte	added (ng/g)	recovery (%)	CV (%)	recovery (%)	CV (%)	recovery (%)	CV (%)	recovery (%)	CV (%)
Sudan 3	2	86.3	8.5	84.2	7.6	81.3	6.4	79.6	5.4
	20	91.5	10.6	87.0	5.3	84.6	7.5	80.5	6.5
Sudan 1	2	76.4	13.9	81.3	13.4	92.0	9.3	96.1	6.3
	20	83.9	6.8	76.5	8.5	96.3	5.7	90.8	6.7
Para red	2	79.0	15.1	79.8	9.2	76.1	6.3	89.2	8.2
	20	83.2	10.9	82.1	9.0	82.3	5.1	81.4	9.0
Sudan 2	2	85.5	8.7	74.5	7.2	92.5	8.7	83.1	11.3
	20	81.6	7.4	78.9	10.6	76.4	9.1	84.6	6.4
Sudan 4	2	79.8	9.6	82.6	6.9	81.0	10.4	79.5	11.2
	20	76.1	13.2	81.5	9.2	86.3	7.6	72.6	8.5
Sudan red G	2	82.8	11.0	81.0	7.3	77.2	8.3	86.2	9.4
	20	81.3	14.4	82.7	9.8	78.6	4.8	79.1	9.2

analyte amount as **3** equivalent and cannot verify the specific analyte. Therefore, the ELISA positive results needed to be further confirmed by some other instrumental method, such as HPLC.

In previous reports on immunoassay of Sudan dyes, Sudan 1 and Para red were usually used as haptens to produce the antibodies. This study first synthesized a novel hapten of Sudan 3 to produce the polyclonal antibody, and a heterologous indirect competitive ELISA was developed to multidetermine the six red dyes in chili oil and egg. From the analysis of blank fortified samples, unknown samples, and real positive samples, this developed ELISA method could be used as a practical screening tool to inspect the residues of these red dyes in foodstuffs.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of data on antibody performances when using different coating antigens. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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