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Development of a Highly Sensitive and Specific Enzyme-Linked Immunosorbent Assay for Detection of Sudan I in Food Samples

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A highly selective and sensitive indirect competitive enzyme-linked immunosorbent assay (ELISA) for Sudan I was developed. Two hapten derivatives with different lengths of carboxylic spacer at the azo-bound para-position were synthesized and coupled to carrier proteins. The hapten−bovine serum albumin (BSA) conjugates were used as immunogens, while the hapten−ovalbumin (OA) conjugates were applied as coating antigens. The antisera which were obtained from four immunized rabbits were characterized in terms of sensitivity and specificity. At optimal experimental conditions it was found that IC_{50} and LOD values of seven pairs based on four antisera and two coating antigens were in the range of 0.3−2 ng/mL and 0.02−0.1 ng/mL, respectively. The most sensitive ELISA could be established with Sudan I-propionic acid−OA coating antigen and the antiserum which was obtained with the corresponding immunogen. The cross-reactivity values of the four antisera with Sudan II, III, and IV was estimated with 0.1−14.3%. No cross-reactivity was found with six edible colorants Sunset yellow, Amarant, Kermes, Indigotin, Bright blue and Lemon yellow, indicating high specificity for Sudan I. Six food samples were fortified with Sudan I and extracted by simple sample preparation. The methanolic extracts after dilution with methanol:water (5:95, v/v) were analyzed by the developed ELISA. Assay precision and accuracy was estimated by determination of three replicates. Acceptable recovery rates of 92.5−114% and intra-assay coefficients of variation of 5.9−24.8% were obtained. The data were validated by conventional HPLC method. As revealed, both methods were highly correlated ($r = 0.9851$, $n = 7$), demonstrating the applicability of the developed ELISA for Sudan I analysis in food samples.

KEYWORDS: Sudan I; food samples; analysis; polyclonal antisera; ELISA; HPLC

INTRODUCTION

With the social development and the rising of living standard, people pay more attention to food quality, especially to possible adverse health effects of additives in food products. In Europe, the Regulation (EC) No. 178/2002 of the European Parliament and of the Council laid down the general principles and requirements of food law (*1*). In May 2003, France gave a warning against chilli powders from India that was found to be contaminated with Sudan I (*2*). In February 2005, the British Food Standard Agency (FSA) declared the withdrawal of foods polluted by Sudan dyes (*3*). In China, from February until May 2005, Sudan I was also found in batches of roasted chicken wings and chicken burgers on sale and in some commercial products such as piccalilli and chilli sauce which led to a great panic (*4*).

Sudan I [1-(phenylazo)-2-naphthol] belongs to Sudan dyes which include Sudan I, II, III, and IV (molecular structures are shown in **Figure 1**). These chemicals are inexpensive fat-soluble azo-compounds which are mainly used as coloring additives in manufacturing of some products, such as oils, wax products, and ball point inks since their bright and vivid colors can improve the luster of commercial products (*5*). Sudan I is classified as a carcinogen by the International Agency for Research on Cancer (IARC) (*6*). There is evidence that Sudan I is potentially carcinogenic in rodents and causes damage to genetic material since it can react with a given sequence of DNA in vitro (*7*). Therefore, Sudan I and other Sudan dyes are not permitted as additives in food production, though they can be used fraudulently to intensify the colors of bell pepper and chilli powders. However, they are still illegally utilized. Therefore,

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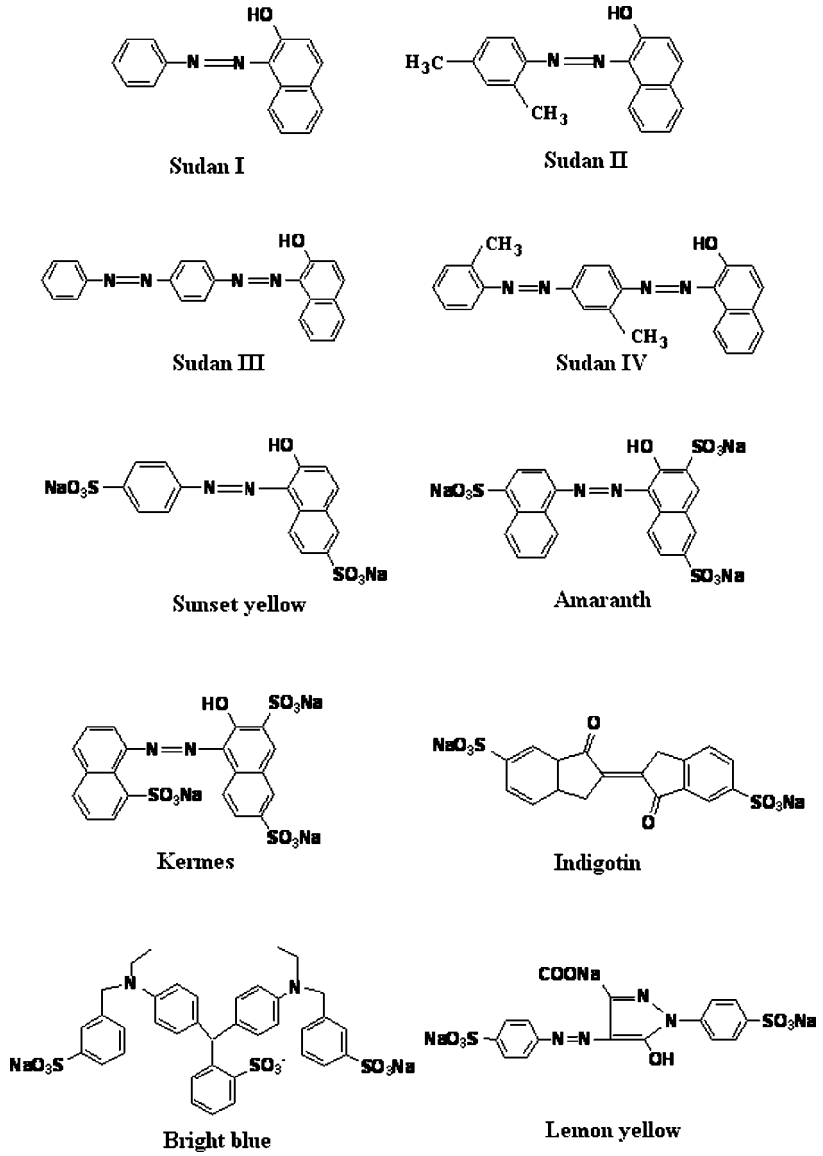

Figure 1. Molecular structures of analytes which were used for cross-reactivity testing.

control of Sudan dyes in food products is very crucial and the development of a simple, economic, and rapid detection method is urgently required.

The standard analysis method is based on liquid chromatography approved by the European Commission (*8*). Other liquid chromatographic methods associated with different detectors such as UV (*9*), APCI-MS (*10*, *11*), ESI-MS (*12*), DAD (*13*), DAD-ESI-MS (*14*), ESI-MS/MS (*15*), capillary LC/Q-TOF-MS (*16*), CL (*17*), etc., for the analysis of Sudan dyes in different food samples have been reported. Generally, chromatographic methods are expensive and time-consuming, mainly because of extensive sample preparation. Therefore, other techniques for detecting Sudan dyes were developed. One approach to reduce the costs for sample preparation is molecular imprinting. Using methacrylic acid and 4-vinylpyridine as functional monomers and ethylene glycol dimethacrylate as cross-linker, an imprinted polymer was prepared for Sudan I. It could successfully be used for the extraction of the target analyte followed by HPLC detection (*18*). The method showed good selectivity; however, the course of washing steps for complex samples was time-consuming. Sudan I, II, III, and IV were also separated by capillary electrophoresis combined with UV-detection, with an LOD of 96 ng/mL (*19*). Recently, Huang et al. found that Sudan dyes react with silver nitrate to produce silver nanoparticles, resulting in obvious plasmon resonance scattering signals at 452 nm, which was used to determine 0.2−2.4 μM Sudan I; however, sensitivity was low and selectivity rather poor (*4*).

Immunoassays are analytical methods which are based on the specific interaction between an antibody and corresponding antigen. The most significant advantages of ELISAs over the traditional instrumental methods are their high sensitivity and specificity, simple sample preparation, high throughput, and therefore low cost per sample. Immunoassays, especially enzyme-linked immunosorbent assays (ELISAs) have been widely used for the determination of both large and small analytes such as proteins, microorganisms (*20*, *21*), antibiotics (*22*), hormones (*23*), pesticides (*24*), halogenated and aromatic pollutants (*25*, *26*), and even heavy metals (*27*) in the biological, medical, agricultural, and environmental area.

To our knowledge, there is no immunoassay available for the detection of Sudan I, a notorious illegal additive in food products. In this study, Sudan I derivatives with different lengths of linking spacer bearing a carboxylic group at the end were synthesized and then covalently coupled to different proteins to prepare both immunogens and coating antigens. The polyclonal antisera (pAb) against Sudan I raised from immunized rabbits were characterized and used to develop a competitive ELISA. The proposed ELISA was used for the analysis of

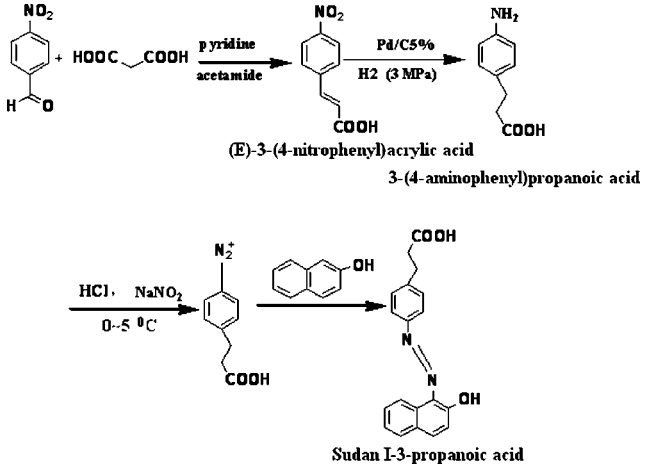

Figure 2. Synthesis of Sudan I-3-propionic acid.

fortified food samples and obtained data validated by a conventional HPLC method.

MATERIALS AND METHODS

Chemicals. 4-(4-Aminophenyl)butyric acid, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 3,3′,5,5′-tetramethylbenzidine (TMB), Tween-20, Sudan I, II, III, IV (HPLC grade), methanol (MeOH) (HPLC grade), casein, bovine serum albumin (BSA), ovalbumin (OA), goat anti-rabbit IgG-horseradish peroxidase conjugate (GaRIgG-POD), and complete and incomplete Freund's adjuvants were purchased from Sigma (St. Louis, MO). 4-Nitrobenzaldehyde, malonic acid, pyridine, sulfuric acid, acetic acid, 30% hydrogen peroxide, palladium-C catalyst (5%), silica gel G and F, physiological saline, dichloromethane (DCM), 2-naphthol, and hydrochloric acid were purchased from Chang Zhen Chemical Company (Chengdu, China). Sunset yellow, Amarant, Kermes, Indigotin, Bright blue, and Lemon yellow used for testing cross-reactivity were generous gifts from the Center of Disease Control of Sichuan Province, China.

Apparatus. ELISA reader Sunrise Remote/Touch Screen, Columbus plus (Tecan, Grödig, Austria), microtiter plate washer M12/2R, Columbus plus, (Tecan), spectrophotometer UV-2300 (Techcomp, Shanghai, China), microtiter plate shaker KJ-201C (Oscillator, Jiangsu Kangjian Medical Apparatus, Co., Ltd., China), pH meter PS-10 (Sartorius, Göttingen, Germany), electronic balance BS 124S (Sartorius). Microtiter plates were from Jiangsu (Haimen, China). Deionized-RO water supply system DZG-303A was purchased from AK Company (Chengdu, Joint Company between Chengdu and Taiwan).

Buffers and Solutions. (1) Coating buffer: 0.05 M carbonate buffer, pH 9.8; (2) coating antigen stock solution: 1 mg/mL of coating antigen prepared with coating buffer; (3) assay buffer: 0.01 M phosphate-buffered saline (PBS) pH 7.4, containing 145 mM NaCl; (4) washing buffer (PBST): assay buffer with 0.1% (v/v) of Tween-20; (5) blocking solution: 1% of casein in assay buffer; (6) acetate buffer: 100 mM sodium acetate acid buffer, pH 5.7; (7) substrate solution (TMB + H_2O_2): 200 μL of 10 mg/mL TMB dissolved in DMF, 20 μL of 5% H_2O_2, and 1 mL of acetate buffer were added to 20 mL of pure water; (8) stop solution: sulfuric acid (5%).

Sudan I stock solution (1 mg/mL) was made by dissolving Sudan I red powder in DMF. Sudan I standard solutions at the concentrations of 0.1, 0.3, 1.0, 3.0, 10, 30, and 100 ng/mL were prepared by diluting the stock solution with MeOH:water (5:95, v/v).

Synthesis of Sudan I Derivatives. Sudan I-3-propanoic acid (Sudan I-C3) was synthesized in three steps as shown in **Figure 2**. Briefly, 3-(4-nitrophenyl)acrylic acid was first synthesized by reacting malonic acid (5.72 g) and 4-nitrobenzaldehyde (7.50 g) in freshly distilled pyridine with acetamide as a catalyst. Then the synthesized 3-(4-nitrophenyl)acrylic acid (0.25 g) was reduced by hydrogen with palladium-C catalyst (*28*), producing 3-(4-aminophenyl)propanoic (0.15 g). 3-(4-Aminophenyl)propanoic acid (0.10 g) was reacted with 2-naphthol (0.09 g) to produce Sudan I-3-propanoic acid. The total synthesized red powder of Sudan I-3-propanoic acid was 0.18 g.

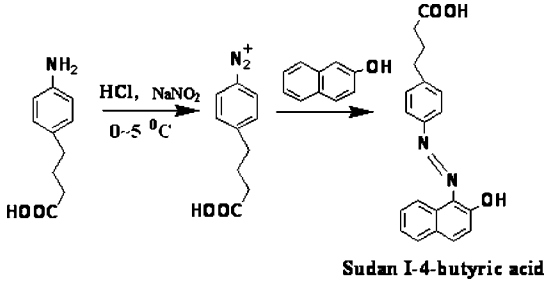

Figure 3. Synthesis of Sudan I-4-butyric acid.

Sudan I-4-butyric acid (Sudan I-C4) was synthesized using 4-(4-aminophenyl)butyric acid as starting compound (**Figure 3**), e.g., 4-(4-aminophenyl)butyric acid (0.10 g) was reacted with 2-naphthol (0.09 g) to produce Sudan I-4-butyric acid. The total synthesized red powder of Sudan I-4-butyric acid was 0.17 g.

Preparation of Immunogens and Coating Antigens. The carboxylic acid derivatives Sudan I-C3 and Sudan I-C4, respectively, were conjugated to BSA and OA by the DCC/NHS ester method as described in the literature (*29*). Briefly, equimolar amounts (0.15 mmol) of Sudan I-derivative, NHS, and DCC were dissolved in 300 μL of DMF and the mixture was incubated overnight at 25 °C. The solution was centrifuged at 12 000 rpm (e.g., 13 400*g*) for 10 min and the supernatant added slowly to 100 mg of protein dissolved in 5 mL of 0.13 M $NaHCO_3$ under stirring. After incubation for 4 h at 25 °C, the solution was centrifuged and the supernatant intensively dialyzed in 0.01 M $(NH_4)_2CO_3$ for 4 days with several changes of the dialyzing buffer solution. Finally, the Sudan I−protein conjugates were lyophilized and stored in the refrigerator until use. While the OA conjugates served as coating antigens the BSA conjugates were used as immunogens for antibody preparation.

Production of Polyclonal Antisera. Two adult New Zealand rabbits were immunized with either Sudan I-C3−BSA or Sudan I-C4−BSA, respectively. Each animal received a dose of 1 mg of immunogen which was dissolved in physiological saline and emulsified with the same volume of Freund's complete adjuvant. Injections were made intradermally at 10 sites on the back of the animal. For booster immunizations, the same volume of Freund's incomplete adjuvant was used. Four subsequent injections were given at 4-week intervals. Bleeding of animals was performed 10 days after the last immunization and the collected antisera, named C3-I, C3-II, C4-I, and C4-II were stored at −60 °C until use. The antisera at appropriate dilutions were directly used for the development of the ELISA.

Indirect Competitive ELISA. An indirect competitive ELISA format was adopted for analyzing Sudan I. The ELISA procedure was as follows. Coating antigen stock solution (1 mg/mL) was diluted with carbonate buffer, pH 9.8, and 200 μL/well added to a 96-well microtiter plate. The plate was incubated overnight at 4 °C and then washed with PBST using an automated plate washer. Some binding sites not occupied by the coating antigen were then blocked by the blocking buffer (280 μL/well) for 1 h at room temperature. After the plate was washed as before, standard solutions or samples (100 μL/well) and diluted antiserum (100 μL/well) were added and incubated for 1 h at room temperature. After washing, GaRIgG-POD was added (200 μL/well) and the plate incubated for 1 h at room temperature. Then, the plate was washed and the substrate solution (200 μL/well) added. After incubation with shaking for about 20 min, sulfuric acid (5%, 80 μL/well) was added and the absorbance measured at 450 nm using a microplate reader. Calibration curves were constructed in the form of $(B/B_0) \times 100\%$ vs log C, where B and B_0 was the absorbance of the analyte at the standard point and at zero concentration of the analyte, respectively.

Cross-Reactivity. The specificity of the generated antisera was investigated by cross-reactivity (CR) experiments. Nine compounds were selected (**Figure 1**) for testing CR. Sudan II, Sudan III, and Sudan IV are fat-soluble, and their use for food production is prohibited. The remaining six chemicals are water-soluble and as edible additives widely

Table 1. Optimized ELISA Parameters Including Coating Antigen, Antibody, and Peroxidase-Labeled Second Antibody (GaRIgG-HRP) and the Corresponding IC_{50} Value

coating antigen			antibody		GaRIgG-HRP	IC_{50}[c] (ng/mL)
name	dilution	ng/well	name	dilution	dilution	
C3–OA[a]	1:10000	20	C3-I	1:100000	1:15000	0.45–1.7
	1:30000	6.7	C3-II	1:150000	1:20000	0.3–1.5
	1:50000	4	C4-I	1:1000000	1:30000	>100
	1:30000	6.7	C4-II	1:70000	1:20000	0.7–2.0
C4–OA[b]	1:5000	40	C3-I	1:25000	1:20000	0.7–2.0
	1:10000	20	C3-II	1:50000	1:20000	1.0–1.5
	1:10000	20	C4-I	1:100000	1:15000	0.7–1.5
	1:30000	6.7	C4-II	1:50000	1:20000	0.7–1.5

[a] C3–OA: Sudan I-C3–ovalbumin conjugate. [b] C4–OA: Sudan I-C4–ovalbumin conjugate. [c] Values were calculated from standard curves ($n = 6$) which were run on 6 consecutive days.

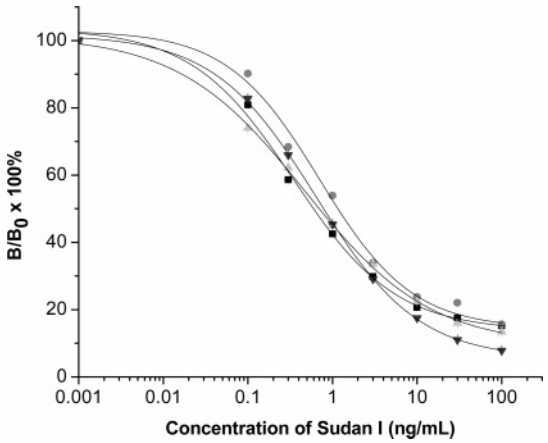

Figure 4. ELISA standard curves for Sudan I with homogeneous combinations of coating antigen and antiserum. ▼ coating antigen C3–OA, 1:30 000 (e.g. 6.7 ng/well); antibody C3-I, 1:150 000; GaRIgG-HRP, 1:20 000; IC_{50} 0.64 ng/mL. ■ coating antigen C3–OA, 1:30 000 (e.g. 6.7 ng/well); antibody C3-II, 1:150 000; GaRIgG-HRP 1:20 000; IC_{50} 0.35 ng/mL; ● coating antigen C4–OA, 1:10 000 (e.g. 20 ng/well); antibody C4-I; 1:100 000; GARIgG-HRP, 1:20 000; IC_{50} 0.70 ng/mL; ▲ coating antigen C4–OA 1:30 000 (e.g. 6.7 ng/well); antibody C4-II, 1:150 000; GARIgG-HRP 1:20 000; IC_{50} 0.76 ng/mL. Each point represents the mean of ± SD of three replicates.

used in food manufacturing. Standard solutions were prepared in the concentration range of 0.001−1000 ng/mL with MeOH:water (5:95, v/v) and applied to the ELISA. CR was expressed as percent IC_{50} values based on 100% response of Sudan I. The IC_{50} can be considered a measure (inverse) of the affinity of an antibody for a given analyte.

Food Samples. Six different food samples, chilli sauce A, chilli sauce B, chilli powder, tomato sauce, preserved tomato juice, and fresh tomatoes were collected from local supermarket in Chengdu (China). While fresh tomatoes had to be homogenized before spiking with Sudan I, the other five samples were directly used.

Details of the fortification experiment were listed in **Table 3**. Briefly, 1−5 g of sample was weighed into a glass tube with a glass stopper. Appropriate amount of Sudan I dissolved in MeOH was added into the tube to prepare a final concentration of 1−10 μg Sudan I/g of sample. The fortified samples were vigorously shaken for 10 min and kept at 4 °C overnight. Then, 5−50 mL of MeOH was added into the tube. The extraction was performed by sonification for 20 min, followed by centrifugation at 12 000 rpm (e.g., 13 400 g) for 5 min. The supernatant was collected and measured by ELISA and HPLC. For ELISA analysis, based on individual sample type, the extracts were diluted at 1:100 to 1:1000 with MeOH:water (5:95, v/v) and directly applied to ELISA using the combination C3−OA coating antigen and C3-II antiserum (see **Table 1**). For HPLC detection, the extracts were filtered by a 0.45 μm filter cellulose acetate membrane filter (Alltech, Unterhaching, Germany) before injection. For each sample, three separate extractions were performed and each sample was determined in triplicate. Unspiked samples were extracted in the same way and used as blanks.

HPLC-UV Analysis. Analysis was performed according to the procedure of Calbiani et al. (*15*). Sudan I standard solutions or sample extracts were passed through a 0.45 μm filter prior to HPLC detection. A HPLC system (Alltech) with a C18 column (250 mm × 4.6 mm, 5.0 μm particle size, Alltech) was equilibrated with mobile phase consisting of 0.1% formic acid in MeOH and 0.1% aqueous formic acid (85:15, v/v) at a flow rate of 1 mL/min. The volume of standard or extract in each analysis was 20 μL. Sudan I was monitored at 480 nm by UV detector. The HPLC work station software (Alltech) was used for the instrument control and data analysis. Peak areas were used for quantification. The calibration curve for Sudan I was constructed with standards of 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μg/mL.

RESULTS AND DISCUSSION

Characterization of Sudan I Derivatives and Protein Conjugates. For the production of high quality antibodies and the development of highly sensitive and specific immunoassays, it is inevitably necessary to modify the molecular structure of the target analyte properly, e.g., to synthesize a hapten derivative which bears a spacer to be coupled covalently to the carrier protein. Both the position at the hapten molecule where the spacer is attached and the length of the spacer may play an important role for a successful antibody generation. Generally, the molecular structure of the hapten should be left unchanged as far as possible, and further a spacer length of 3−5 C-atoms is considered most suitable (*30*, *31*). Concluding, in this study the benzene ring of Sudan I was modified at the para position of the azo bond, i.e., the spacer is attached as far as possible from the naphthol moiety. Two Sudan I-derivatives with different spacer length were synthesized (**Figures 2** and **3**). The structures of Sudan I-C3 and Sudan I-C4 were confirmed by the NMR method. The data from the NMR method are as follows: Sudan I-C3 ^{1}H NMR (200 MHz, $CDCl_3$): δ 14.30 (b, 1 H), 8.55 (d, $J = 4.2$ Hz, 1 H), 7.73−7.50 (m, 4 H), 7.41−7.14 (m, 4 H), 6.89 (d, $J = 3.96$, 1 H), 3.45 (b, 1 H), 3.05 (t, $J = 7.50$ Hz, 2 H), 2.76 (t, $J = 7.46$ Hz, 2 H); Sudan I-C4 ^{1}H NMR (200 MHz, $CDCl_3$): δ 14.32 (b, 1 H), 8.58 (d, $J = 8.57$ Hz, 1 H), 7.75−7.51 (m, 4 H), 7.41−7.09 (m, 4 H), 6.90 (d, $J = 6.89$, 1 H), 3.51 (b, 1 H), 2.76 (t, $J = 7.46$ Hz, 2 H), 2.44 (t, $J = 6.86$ Hz, 2 H), 2.07 (m, 2 H).

All conjugates (Sudan I-C3−BSA, Sudan I-C4−BSA, Sudan I-C3−OA, and Sudan I-C4−OA) were characterized by UV-spectroscopy. The estimated molar ratio of Sudan I-C3−BSA and Sudan I-C4−BSA was 37 and 17, respectively. A ratio of 20 was observed for both OA conjugates.

Optimization of ELISA Conditions. To improve the sensitivity, the assay conditions including type and concentration

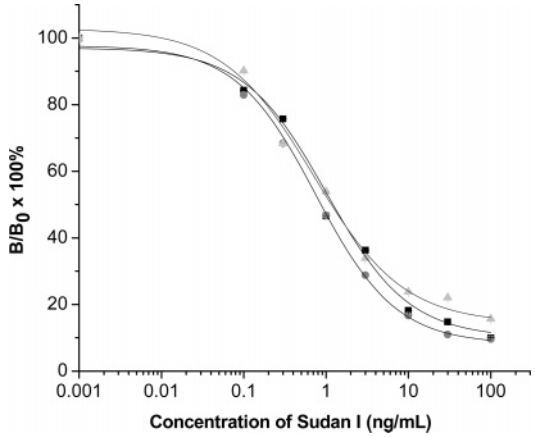

Figure 5. ELISA standard curves for Sudan I with heterogeneous combinations of coating antigen and antiserum. ▲ coating antigen C3–OA, 1:30000 (e.g. 6.7 ng/well); antibody C4-I, 1:70 000; GaRIgG-HRP, 1:20 000; IC_{50} 0.70 ng/mL ● coating antigen C4–OA, 1:10 000 (e.g. 20 ng/well); antibody C3-I, 1:50 000; GaRIgG-HRP 1:20 000; IC_{50} 0.75 ng/mL; ■ coating antigen C4–OA, 1:10 000 (e.g. 20 ng/well); antibody C3-II, 1:50 000; GaRIgG-HRP 1:20 000; IC_{50} 1.0 ng/mL. Each point represents the mean of ± SD of three replicates.

Table 2. Cross-reactivity of the Polyclonal Antisera C3-I, C3-II, C4-I, and C4II with Sudan I–IV and Other Six Edible Colorants[a]

	CR (%)			
compound	C3-I	C3-II	C4-I	C4-II
Sudan I	100	100	100	100
Sudan II	5.3	0.5	1.4	3.4
Sudan III	5.8	14.3	5.0	9.5
Sudan IV	2.4	0.5	0.1	1.9
Sunset Yellow	<0.01	<0.01	<0.01	<0.01
Amaranth	<0.01	<0.01	<0.01	<0.01
Kermes	<0.01	<0.01	<0.01	<0.01
Indigotin	<0.01	<0.01	<0.01	<0.01
Bright Blue	<0.01	<0.01	<0.01	<0.01
Lemon Yellow	<0.01	<0.01	<0.01	<0.01

[a] Note: C3-I and C3-II are antisera obtained from two rabbits immunized with immunogen of Sudan I-C3–BSA; while C4-I and C4II are antisera obtained from the rabbits immunized with Sudan I-C4–BSA.

of the coating antigen, dilution of the antiserum and secondary labeled antibody, etc. should be carefully optimized. In the present investigation, it was performed according to two criteria, i.e (1). to get an IC_{50} value as low as possible and (2) an absorbance in the range of 0.8−1.5 absorption units for the zero standard concentration (blank).

In this study, based on two different coating antigens (Sudan I-C3−OA and Sudan I-C4−OA) and four antisera (C3-I, C3-II, C4-I, and C4-II), eight possible combinations were tested. The experimental conditions including coating antigen concentration and dilution of antiserum and secondary antibody were optimized for each combination (**Table 1)**. With exception of the pair C3−OA coating antigen and C4-I antiserum which could be used at very high dilutions, with all combinations quite comparable optimal conditions were observed. In detail, coating antigen concentration and antiserum and secondary antibody dilutions varied in the range of 6.7−40 ng/mL, 1:25 000−1:150 000 and 1:15 000−1:20 000, respectively. The calculated IC_{50} values were lesser than 2 ng/mL. With an IC_{50} value of 0.3−1.5 ng/mL, the combination of C3−OA conjugate and C3-II antiserum was superior. The aberrant data obtained with pair C3−OA/C4-I are difficult to interpret. Obviously, there is a great amount of high affinity C3−OA-recognizing antibodies present in the generated antiserum which leads to significantly lower affinity for Sudan I compared to other tested combinations. In addition, no significant differences in assay sensitivity were observed between homogeneous and heterogeneous combinations of coating antigens and antisera. This can easily be followed from **Figures 4** and **5**, in which typical standard curves for Sudan I were constructed in the concentration range of 0.1−100 ng/mL and the values of LOD at a signal-to-noise ratio of 3 (S/N = 3) was within 0.02−0.1 ng/mL.

Specificity of the Antisera. Specificity was evaluated by CR testing. Obtained CR values were summarized in **Table 2**. It became obvious that the CR pattern was very similar for all antisera. Accordingly, no recognition of the tested edible colorants was observed, i.e., the CR was below 0.01%. Even the chemicals Sunset Yellow and Amaranth which share both the naphthol ring and attached azo group with Sudan I were not recognized by the antibodies because of the large sulfonic acid group(s), obviously. Further, Sudan I showed by far the highest binding followed by Sudan III with 5.0−14.3%. This is not surprising because these two compounds exhibit highest structural homology among all Sudan dyes. With a CR of only 0.1−5.3% for Sudan II and Sudan IV these compounds were less recognized. This should be caused by the methyl group(s) attached in ortho and para positions to the linking azo group. To summarize, high specific antisera were obtained against Sudan I after immunization with Sudan I derivatives which contained either a C3- or C4-spacer arm attached to the benzene ring.

Fortification Experiment. In this study, six food samples were fortified with Sudan I at a concentration of 1−10 μg/g of sample and extracted with pure MeOH. Unspiked samples served as blanks. For the determination with ELISA, the extracts were 100−1000-fold diluted with MeOH:water (5:95, v/v) depending on the individual sample (**Table 3**). Generally, a simple dilution step is a good means to eliminate or reduce the

Table 3. Concentration of Sudan I in Spiked Food Samples as Determined by ELISA (n = 3)

sample type	weight of sample (g)	Sudan I added (μg)	spiked concn (μg/g)	volume of MeOH (mL)	dilution of extract	concn measured ± SD (μg/g)	intra-assay RSD (%)	recovery (%)
fresh tomato juice	1	1	1	5	100	1.14 ± 0.09	7.9	114.0
preserved tomato juice	1	2	2	5	200	2.07 ± 0.22	10.6	103.5
tomato sauce	1	2	2	5	100	2.06 ± 0.51	24.8	103.0
chilli sauce A	5	10	2	50	100	2.08 ± 0.39)	18.8	104.0
chilli sauce B	5	10	2	50	400	3.27 ± 0.76	23.2	103.0[a]
chilli powder	1	10	10	10	1000	9.25 ± 0.55	5.9	92.5

[a] The concentration of Sudan I in unspiked chilli sauce B as measured by ELISA was 1.21 μg/g of sample. Thus the recovery rate was calculated as: (3.27 − 1.21)/2 = 103.0%. No detectable Sudan I was found in the other blank samples.

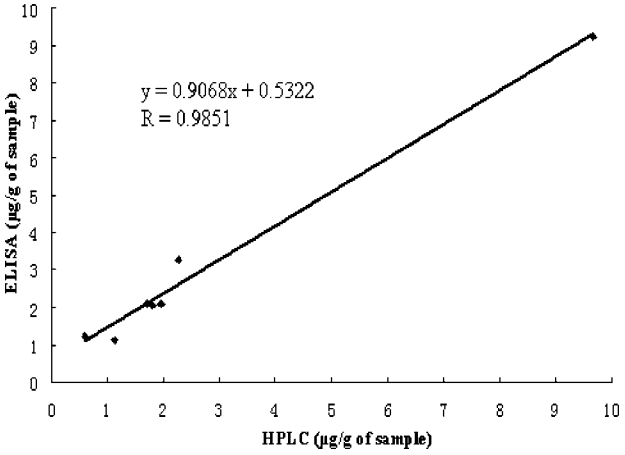

Figure 6. Correlation of ELISA and HPLC analysis of fortified food samples.

matrix effect on the assay. The found matrix effects were different among the collected samples. Correspondingly, fresh tomato juice, preserved tomato juice, and chilli sauce A could be analyzed after only 100-fold dilution while tomato sauce, chilli sauce B, and chilli powder had to be diluted at 1:200, 1:400, and 1:1000, respectively. Regarding blanks only in chilli sauce B Sudan I was found at a concentration of 1.21 μg/g of sample. Other samples were not contaminated. Assay precision and accuracy was estimated by measuring three replicates. Acceptable recovery rates of 92.5−114% and intra-assay coefficients of variation of 5.9−24.8% were obtained. On the other hand, for six fortified samples, the inter-assay coefficients of variation were less than 30%.

Comparison of ELISA and HPLC Determination. The HPLC calibration curve for Sudan I was constructed in the range of 0.2−10 μg/mL with an LOD of 0.5 ng/mL ($n = 3$). The linear equation was $y = 59875x + 3348.5$. To validate the data obtained by ELISA all fortified food samples including the unspiked chilli sauce B were also measured by HPLC. With the selected parameters (see Materials and Methods) the retention time of Sudan I was 24 min. As revealed, both methods were highly correlated ($r = 0.9851$, $n = 7$) (**Figure 6**).

Conclusion. In this work, for the first time, antibodies against Sudan I were prepared in rabbits and used to establish an indirect competitive ELISA. The highly sensitive assay proved useful for the analysis of Sudan I in different food extracts. As the main advantage, the high selectivity of the ELISA became evident. Using the optimized assay, other Sudan dyes such as Sudan II, III, and IV exhibited a cross-reactivity below 15%, while six tested edible colorants of similar molecular structure did not show any binding to the raised antibodies. In addition, interference caused by the sample matrix could be easily overcome by a simple dilution step before immunochemical analysis.

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