Development of enzyme-linked immunosorbent assays for Sudan dyes in chilli powder, ketchup and egg yolk

UNIVERSITÀ DEGLI STUDI DI TORINO

DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR SUDAN DYES IN CHILLI POWDER, KETCHUP AND EGG YOLK.

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ABSTRACT

This study aimed at developing sensitive competitive enzyme-linked immunosorbent assays (ELISAs) for the banned Sudan dyes, using polyclonal antibodies. Three different formats were developed and characterized in terms of sensitivity, selectivity and rapidity. A competitive indirect ELISA was developed, which showed an IC_{50} of 3.8 µg L^{-1}. Two competitive direct ELISAs, were also developed, which differ in the fact that the antibody is added before or simultaneously with the other reagents; the first showed an IC_{50} of 8.3 µg L^{-1} and the second one showed an IC_{50} of 4.9 µg L^{-1}. Nevertheless, considering dilution of extracts which is needed to offset matrix interference, the limits of detection of the three formats were substantially the same (10 µg kg^{-1}). The antibodies in all three test formats were able to recognize Sudan I and partially Sudan II, III, and IV; no cross-reactivity was observed with five edible dyes. Twenty food samples, including chilli powder, paprika, ketchup, and egg, were extracted by simple sample preparation and very limited dilution. Extracts were analyzed by the developed competitive direct ELISA with the simultaneous addition

of reagents. A good correlation was observed (y=1.19x-10.0, r^2 = 0.991, n=20) when the data was compared with that obtained through conventional HPLC method.

KEYWORDS: Sudan dyes, ELISA, chilli powder, egg yolk

INTRODUCTION

Sudan I, II, II and IV (Figure 1) are non-ionic fat-soluble dyes used as additives in gasoline, grease, oils, plastics, printing inks, and floor polishes (Mejia E. et al., 2007) These dyes are classified by the International Agency for research on Cancer as category 3 carcinogens to humans (IARC, 1975), and the use of Sudan I in foodstuffs is forbidden in global food regulation acts (EC Regulation No 178/2002, Di Donna L. et al., 2004). Unfortunately, they are still illegally utilized to intensify and maintain the colour of food products such as chilli-, curry-, and palm oil-containing foodstuffs. From May 2003, warnings against chilli powder from Asia and a variety of foodstuffs polluted by Sudan dyes were issued throughout Europe (Han D. et al., 2007) and, more recently, Sudan I was found in China in various commercial foodstuffs, including chilli sauce, meat and eggs (He L. et al., 2007). This illegal use of Sudan dyes is a severe danger to public health, therefore simple, rapid and reliable analytical methods for the determination of these banned dyes in foodstuffs are required, as also testified by the large number of papers concerning the development of extraction and detection methods for Sudan dyes in food (particularly, in chilli powder) which was published in 2007. The vast majority of described analytical methods are based on high performance liquid chromatography, with optical or mass spectrometric detection (Di Donna L. et al, 2004; Ma M et al, 2006; Cornet V. et al., 2006; Mazzetti M. et al., 2004), however, some gas chromatographic and capillary electrophoresis methods have also been reported (Mejia E. et al., 2007; He L. et al., 2007). Generally, chromatographic methods are expensive and time-consuming, therefore other techniques for detecting Sudan dyes have been developed. Among these, Deng and co-authors. described the first enzyme immunoassay (Han D. et al., 2007) and applied it to the detection of Sudan I in some food samples (tomato sauce and juice, chilli sauce and powder). They prepared two hapten derivatives with different lengths of carboxylic spacers and used them to synthesize the immunogen and the coating antigen. The optimized indirect competitive ELISA showed itself to be sensitive and specific for Sudan I. However, when applied to samples, limits of detection were in the mg/kg range, which is dramatically higher than the detection capability of the

chromatographic methods, mainly because of the need for diluting the sample extracts from 1:100 to 1:1000 before analysis, in order to offset the matrix effect. Very recently, the preparation of a monoclonal antibody towards Sudan I, III and Para red and its use in the development of a sensitive competitive indirect ELISA has been described (Ju C. et al, 2008). The determination of Sudan I in eggs by an immunoassay exploiting the resonance scattering of gold nanoparticles has also been reported (Jiang Z. et al, 2008). Nevertheless, the extraction of the dye from eggs requires a two-day protocol.

In this study, a Sudan I hapten derivative was synthesized, with the objective of raising a polyclonal antibody characterized by low selectivity towards other three Sudan dyes (II, III, and IV) to develop a competitive immunoassay able to detect the entire group of banned Sudan dyes. Different assay formats were compared: a direct and an indirect competitive ELISA were developed, and the simultaneous or subsequent addition of reagents was evaluated. Moreover, the extraction step and the solvent used to dissolve Sudan dyes were optimized with the aim of minimizing matrix interference without the contemporary need for an excessive dilution of extracts.

The developed assay was validated through comparison with an HPLC-UV method and proven to allow the determination of Sudan dyes in very different foodstuffs (chilli powder, ketchup and egg yolk).

MATERIALS AND METHODS

Materials

All Sudan dyes, 4-aminobenzoic acid, 2-naphthol, bovine serum albumin (BSA), goat anti-rabbit immunoglobulin HRP-labelled antibody, horse-radish peroxidase (HRP) N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and 3,3'5,5'-tetramethylbenzidine liquid substrate (TMB) were purchased from Sigma- Aldrich (St. Louis, MO, USA).

Sephadex G-25 resin and low chromatography apparatus were from GE Healthcare (Milan, Italy)

Dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (HPLC grade) and all other chemicals and microtiter plates were obtained from VWR International (Milan, Italy).

Polyclonal antibodies (ammonium sulphate precipitated IgG fraction) were obtained from Davids Biotechnologie (Germany). Antibodies were prepared using the standard immunization protocol (Davids Biotechnologie, Germany) and sera were collected after 70 days from the first injection.

Sudan dye stock solutions were prepared by dissolving the powders in chloroform and immediately diluting them in acetonitrile to the final concentration of 20 mg L^{-1}. Standard solutions were prepared by daily diluting the stock solutions with DMSO:water (10:90, v/v).

Synthesis of hapten and hapten-protein conjugates

The hapten used for immunization, antigen coating and enzyme labelling is depicted in Figure 1. Carboxy-Sudan I (CSudan I) was synthesized as previously reported (Ju C. et al, 2008).

CSudan I was then conjugated with BSA and HRP by the DCC/NHS ester method, as reported in the literature (Hosoda H. et al., 1979). Briefly, equimolar amounts of CSudan I, DCC and NHS were dissolved in anhydrous DMF and the mixture was incubated at room temperature for 1 hour. Proper amounts of the mixture were then added to protein solutions prepared in 0.13 M $NaHCO_3$, to obtain a final molar ratio of 50:1; 100:1; 200:1 (CSudan I:BSA), 20:1; and 10:1 (CSudan I:HRP). BSA conjugates were incubated overnight at room temperature, while HRP conjugates were reacted for 1 hour at room temperature. Separation of conjugates from by-products and the excess of reagents was carried out by gel filtration low pressure chromatography on Sephadex G-25 resin (mobile phase: phosphate buffer).

Finally, sodium azide was added to BSA conjugates, which were stored at -18°C. The 200:1 conjugate was used as the immunogen, while conjugates with lower substitution degree were used as coating antigens. HRP conjugates were stored at 4°C, with 33% (v/v) of glycerol added.

Competitive indirect ELISA

Wells were coated with 200 µl of 0.2 mg L^{-1} CSudan I-BSA (50:1) in a carbonate-bicarbonate buffer (50 mM pH 9.6). The plates were incubated overnight at 4°C. After the plates were washed three times with washing solution (0.05% Tween 20), 300 µl of blocking solution (phosphate buffer, containing 0.1% BSA, 5% sucrose, and 4% polyvinylpyrrolidone) was added to each well and the plates were further incubated for 1 hour at room temperature. The plates were washed and they were either used immediately or stored at 4°C until use.

Antiserum was diluted 1:30000 (v/v) in 20 mM phosphate buffer at pH 7.4, containing 1% (p/v) of BSA (PBSA). A volume of 100 µl of diluted antiserum was added to 100 µl of Sudan I at concentrations ranging from 0 to 100 ng L^{-1} and incubated in coated wells for 15 minutes. After 3 well washes with washing solution 200 µl of a diluted goat-antirabbit immunoglobulin conjugated to horseradish peroxidase was used to detect the immobilized rabbit antibody. The incubation time was 30 minutes, followed by three well washes. Colour development was obtained by a 30 min incubation with TMB (200 µl per well). One hundred microlitres of sulphuric acid (1M) was used as a stop solution and absorbance was recorded at 450 nm. All standards and samples were measured in duplicate.

Competitive direct ELISA (simultaneous addition of reagents)

The immunoreactive solid phase was obtained by coating wells with 300 µl of a goat-antirabbit IgG solution (10 mg L^{-1}) (Giraudi G. et al., 2000).

Antiserum and CSudan I –HRP were diluted in PBSA. A volume of 100 µl of diluted antiserum (1:15000, v/v) was added to 50 µl of 0.75 mg L^{-1} of CSudan I-HRP (20:1) and to 50 µl of Sudan I at concentrations ranging from 0 to 100 µg L^{-1}. The mixture was incubated 15 minute in immunoreactive wells. Then the wells were washed with washing solution. Colour development and absorbance readings were obtained as described above.

Competitive direct ELISA (pre-immobilization of the antibody)

The same immunoreactive solid phase used for the format with the simultaneous addition of reagents was used in this case. In addition, 200 µl of diluted antiserum (1:50000, v/v) in PBSA was incubated overnight at 4°C. After the plates were washed three times with washing solution, they were used immediately.

A volume of 100 µl of CSudan I-HRP (20:1) at 0.2 mg L^{-1} was added to 100 µl of Sudan I at concentrations ranging from 0 to 100 µg L^{-1}. The mixture was incubated for 15 minutes in wells coated with the anti-Sudan I antibody, then wells were washed with washing solution. Colour development and absorbance readings were obtained as described above.

Calculations

Sudan I concentrations were determined by interpolation on the calibration curve, where the signal was plotted against the log of analyte concentration. For each experiment, a calibration curve was determined by a nonlinear regression analysis of the data of the standards using the four-parameter logistic equation (Findlay J et al., 2000).

Relative cross-reactivity was calculated as follows:

$CR\% = (IC_{50}$ Sudan I / IC_{50} Sudan dye)*100

where IC50 is the Sudan dye concentration which cause 50% inhibition of the maximum observed signal.

Samples and sample preparation

The following commercial samples: 5 kinds of ketchup, 5 kinds of eggs, 10 kinds of chilli powder and paprika were purchased in large stores. One sample for each category that did not show any detectable residues of target dyes when analyzed by HPLC was taken as the blank for matrix interference experiment and fortification experiments. Fortified samples were prepared by adding 25, 125, and 625 µg kg^{-1} of Sudan I and 125, 625 µg kg^{-1} of Sudan II, III, and IV to a blank sample before performing the extraction.

Sudan dye extraction was carried out as reported in the literature (Ma M. et al., 2006) with slight modifications. Egg yolk was manually separated and gently mixed before extractions. Briefly, 20 g of homogenized sample were weighed and sonicated for 15 min with 50 ml of DMSO. Egg yolk and ketchup samples were previously mixed with 20 g of sodium sulphate to eliminate water. After being centrifuged for 15 min at 3000 rpm, extracts were diluted at 1:10 with PBSA and filtered through a 0.45 µm nylon membrane filter. Based on our experience, the use of a nylon membrane is mandatory to eliminate matrix interference from chilli powder and paprika samples.

HPLC analysis

To evaluate the accuracy and to validate the method, a comparative study using both the developed ELISA method and a HPLC reference procedure (Thompson M. et al. 2002) was performed. Samples were extracted as described above, except for the fact that DMSO extracts were diluted 1:5 (v:v) with the same solvent and filtered through a 0.45 µm nylon membrane filter, according to the literature (Ma M. et al., 2006).

Each extract was injected in triplicate. A liquid chromatographic system (LaChrom Elite, VWR-Hitachi, Darmstadt, Germany) equipped with a C-18 Chromolith Performance 100-4.6 mm column (VWR, Darmstadt,Germany) was used. The mobile phase consisted of acetonitrile and water at a volume ratio of 80:20, delivered to the column at a rate of 1 ml min^{-1}. Detection was made by a UV detector (472 nm). Sudan I concentrations were calculated by interpolation on an external calibration curve.

RESULTS AND DISCUSSION

Production of polyclonal antibodies

Two rabbit antisera were raised against CSudan I-BSA (200:1). These were tested by a non-competitive indirect ELISA, following the protocol described in the experimental section, with the difference that no Sudan I was added. Both antisera show very similar performance (Figure 1) and

high titres on the coating antigen (IC_{50} at 1:80 000 and 1:100 000, v/v, antibody dilution for rabbit 1 and 2 respectively). The non-specific binding of antisera towards the carrier protein was also evaluated, but resulted negligible in comparison with the binding towards the hapten (Figure 1). Arbitrarily, rabbit 1 antiserum was used for this study.

Competitive indirect ELISA

Checkerboard assays, in which antiserum was titrated against varying amounts of two coating antigens (CSudan I-BSA 100:1 and CSudan I-BSA 50:1), were used to select appropriate antigen coating and antibody dilutions for the competitive indirect assay. An antibody diluted at 1:30000 (v/v) and the coating antigen CSudan-BSA 50:1 at 0.2 mg L^{-1} were selected as the most suitable on the basis of the IC_{50} value that was the lowest.

Since Sudan dyes are very hydrophobic compounds, they should be dissolved and kept in some organic solvent. A rapid literature review permitted us to identify a wide variety of solvents used to dissolve and extract Sudan dyes from food samples for determination by chromatographic techniques (Mejia E. et al, 2007; Ma M. et al., 2006; Cornet V. et al., 2006; Mazzetti M. et al., 2004). The application as the analyte solvent in immunochemical methods of analysis narrowed the field, but acetonitrile and dimethylsulfoxide, remained as possible candidates. Even if DMSO is rarely associated with ELISA measurement, both organic solvents were considered as the Sudan diluent to set up the standard curve. Solvent effect on the developed assay performance was studied by diluting the Sudan I standard solution in PBSA/DMSO and PBSA/acetonitrile (5, 10 and 20%, v/v) and carrying out the standard curve in the optimized condition. Low amounts of acetonitrile (below 10%) allowed an increase in the absolute signal to be observed, but was associated with limited detectability (IC_{50} above 20 µg L^{-1}). On the contrary, the presence of DMSO (below 10%) lowered the signal of the blank, but allowed us to reach decidedly higher sensitivity (IC_{50} below 10 µg L^{-1}). The increase of the organic solvent amount from 5 to 10% produced a parallel doubling of the IC_{50} value, whereas when the solvent percentage was increased up to 20%, the IC_{50} increased

more dramatically. Finally, 10% (v/v) of DMSO in PBSA was chosen as the solvent to prepare the dilute Sudan I standards. The time of incubation for the competitive reaction was then optimized and 15 minutes was selected as a satisfying compromise between the lowest IC_{50} and a blank signal around 1-1.5 UA. Figure 3 shows a typical inhibition curve obtained under optimized conditions. The IC_{50} value of the assay was 4 µg L^{-1} with a detection limit of 0.2 µg L^{-1} and a dynamic range of 0.4-100 µg L^{-1}.

Competitive direct ELISA

Competition experiments were carried out under various combinations of antibody and enzyme tracer concentrations. Two formats were optimized: the first involving the simultaneous reaction between antibody, tracer and analyte (dissolved in 10% DMSO) and a second one, which was based on a long pre-immobilization reaction of the antibody, followed by a short incubation of the mixture of the analyte and the tracer into functionalized wells. Figure 3 shows typical inhibition curves obtained under optimized conditions.

Antibody dilutions, enzyme tracer concentrations and incubation times are summarized in Table 1, together with the analytical performance of the optimized assay in both formats. As expected, the pre-immobilization of the antibody allowed the use of lower amounts of antibody and competitor (the enzyme tracer) and consequently the improvement of assay sensitivity (lower IC_{50} value). Nevertheless, the preparation of functionalized wells by the pre-immobilization of the antibody involves overnight incubation before carrying out the assay or the stabilization of functionalized wells.

Selectivity of the assays

Since, from a legal point of view, the occurrence of all four Sudan dyes (I-IV) should be assessed in food, the assay would be virtually able to cross-react with the four target analytes. To assess the influence of the assay format on the selectivity, cross-reactivity towards Sudan II has been

measured by the three developed assay formats. The manipulation of the format of the immunoassay slightly influences the selectivity: the competitive direct ELISA with the pre-immobilized antibody is the most selective assay (CR%=26%), whereas the other two formats show about the same selectivity (CR%=34% for the direct assay and 35% for the indirect ELISA).

Since the format finally chosen to validate the method was the direct ELISA with the simultaneous addition of reagents, a more extensive study of selectivity was made using this assay format. Results reported in Table 2 show that Sudan II is well recognized. Sudan III and IV and other Sudan dyes demonstrate a slight recognition, which means that the assay would be able to measure these compounds only at levels ten-times higher than Sudan I. This finding is not very far from the performance obtained by some instrumental methods of analysis based on chromatographic or electrophoretic determination of Sudan dyes (Mejia E. et al., 2007; Ma M. et al., 2006).

Several dyes, whose use is permitted in food (Department of Food Biosciences, University of Reading, UK), were also tested for cross-reactivity. The interference with the assay was negligible for all tested compounds.

Better choice of format and its validation

To evaluate matrix interference on the three developed assays, blank samples of chilli powder, ketchup and egg yolk were analyzed in six replicates. Negligible interference was observed when extracts were analyzed by means of the direct ELISA format with the simultaneous addition of reagents. The other two formats needed a simple 1/2 (v/v) dilution of samples with the PBSA containing 10% of DMSO before the analysis to offset the matrix effect.

The LOD was calculated by interpolation on the Sudan I standard curve as the analyte concentration corresponding to the mean signal of the zero standard (obtained by averaging the signal of eight replicate sets) minus three times its standard deviation (according to IUPAC, ISO, and AOAC harmonized guidelines) (Thompson M. et al, 2002) and it was 0.2 μg L^{-1} for both the indirect and the direct ELISA with the pre-immobilization of the antibody. The direct ELISA with the

simultaneous addition of reagents had lower sensitivity (calculated LOD 0.4 μg L^{-1}). This result can be simply explained by remembering that in the direct ELISA (with the simultaneous addition of reagents) standards and samples are diluted 1/4 (v/v) in wells, while in the other two formats they are diluted 1/2. For the same reason, no further dilution was required to offset matrix interference in the direct ELISA with the simultaneous addition of reagents. On the contrary, the other two formats required an additional 1/2 (v/v) dilution, before analysis. Consequently, in the end, the limits of detection of the three developed formats, compared to the solid sample, were substantially the same (10 μg kg^{-1}).

Therefore, the direct competitive ELISA with the simultaneous addition of reagents, being rapid, sensitive and the easiest to carry out was chosen as the format to be fully validated.

The LOD, re-calculated for each different matrix by measuring and averaging data from the five blank samples, confirmed the previous estimation (10 μg kg^{-1}) as regards ketchup and chilli powder.

The precision of the method was determined by extracting and analyzing replicates of Sudan I artificially contaminated samples (ketchup, chilli powder), which were fortified with a Sudan I concentration of 25 (low level), 125 (medium level), and 625 (high level) μg kg^{-1}.

The assay was carried out in six replicates on the day for the evaluation of within-assay precision and on six different days for the evaluation of the between-assay precision. The values of RSD% were calculated at each nominal concentration level and ranged from 2 to 16%, which fulfilled FDA requirements for the validation of bioanalytical methods according to FDA guidance (Viswanathan C. et al., 2007).

To evaluate the accuracy of the method, chilli powder and ketchup samples fortified with Sudan I (concentrations of 25, 125, and 625 μg kg^{-1}), Sudan II, Sudan III, and Sudan IV (concentrations of 125 and 625 μg kg^{-1}) were extracted and analyzed. Results are summarized in Table 3. Recovery values ranged from 70 to 103% for Sudan I, thus indicating a good accuracy of the assay when applied to real samples of very different compositions. Recovery values for Sudan II, III, and IV

reflect their cross-reactivity and confirm that these dyes could be only detected in highly contaminated samples.

Fortification experiments were also conducted on egg yolk samples. This matrix has not been included in the work of Ma M. et al. (2006), thus no information regarding the possibility of extracting Sudan dyes from this kind of sample by means of the same protocol applied to other matrices was available. To our knowledge, this is also the first time that Sudan I has been measured via an immunoenzymatic method in egg yolk. Therefore, a blank sample of egg yolk was fortified with 25, 250 and 2500 µg kg^{-1} of Sudan I and extracted in triplicate according to the procedure described in the experimental section. Samples were analyzed both by the developed direct ELISA method and by HPLC and the obtained recovery values for both methods are reported in Table 4. The fortified sample at the lowest concentration could only be measured by means of the ELISA. The medium and high level fortified samples gave satisfactory recoveries and good agreement between the results obtained via the two methods. Generally speaking, the developed ELISA showed an increased imprecision of data, in comparison with fortification experiments made on samples belonging to different classes (chilli powder, paprika and ketchup), even if the relative standard deviation remained in the range considered acceptable for screening methods (Viswanathan C. et al., 2007). The LOD was, in this case, higher than for other considered matrices (15 µg kg^{-1}), due to the greater data imprecision.

Comparison of ELISA and HPLC determination

A total of 20 samples (representative for all categories previously indicated) were analyzed with the developed direct competitive ELISA method. Since no positive samples were found in the market, they were randomly fortified with Sudan I at different levels of contamination (from 50 to 2500 µg kg^{-1}) and blindly analyzed by the two methods.

Agreeing results were obtained via the two methods: the linear regression analysis (Figure 4) yielded a good correlation between the methods (y=1.19x-10.0, r^2 = 0.991, n=20). A limited under-

estimation of the Sudan I concentration was observed at high contamination levels (above 500 µg kg^{-1}), probably due to the low solubility of the analyte when diluted 1/10 in the PBSA for the analysis. In fact, if the two highest fortified samples are not included in the comparison between ELISA and HPLC, the agreement between methods further improves (y=1.09x-1.24, r^2 = 0.995, n=18). Thus, very highly contaminated samples, which can occur in the case of the illegal use of Sudan dyes (Mazzetti M. et al, 2004), should be diluted in DMSO before diluting them in PBSA, in order to prevent Sudan I precipitation and low recoveries.

These results proved that the developed ELISA can be applied as screening methods for the detection of Sudan I in foodstuffs of very different kinds, with good accuracy and precision. The assay is really sensitive, because sample extracts need a very limited dilution before being analyzed; therefore LOD in samples remains in the ten µg kg^{-1} range. In addition, the method is simple, rapid (the extraction requires 30 minutes, and the assay is completed in 45 minutes), and applicable to very different matrices with very slight modifications, thus proving itself to be suitable as a first level screening method or for routine quality controls of various foodstuffs.

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FIGURE CAPTIONS

Figure 1: Structure of the analytes; the hapten derivative used for immunization and preparation of the coating antigen and the enzymatic tracer; and analytes used in a cross-reactivity study.

Figure 2: Non-competitive binding of antisera raised against CSudan towards the coating antigen, CSudan-BSA (△ rabbit 1, ○ rabbit 2) and towards the carrier protein, BSA (▲ rabbit 1, ● rabbit 2)

Figure 3: Typical inhibition curves, obtained under optimized conditions for the three ELISA formats developed (□ Competitive Direct ELISA, simultaneous addition of reagents; ○ Competitive Direct ELISA, pre-incubation of antibodies; △ Competitive Indirect ELISA).

Figure 4: Correlation of results obtained by both ELISA and reference HPLC method for the Sudan I detection on ketchup, egg yolk, and chilli and paprika powders. The linear regression analysis yielded a good correlation between methods (y=1.19x-10.0, r^2 = 0.991)

TABLES

Table 1 Assay characteristics of the three competitive ELISA formats

	antibody dilution in wells	**competitor concentration (ng well)**	**IC_{50} (µg L^{-1})**	**Time requested to carry out the assay (min)**
Competitive Indirect ELISA	1/60000	40	3.8	75
Competitive Direct ELISA (simultaneous addition of reagents)	1/30000	37.5	8.3	45
Competitive Direct ELISA (pre-immobilization of the antibody)	1/50000	20	4.9	45

Table 2 Cross-reactivity of target Sudan dyes, compounds structurally related to Sudan I, and dyes legally used in food determined by competitive direct ELISA

Compound	**Cross-reactivity (%)**
Sudan I	100
Sudan II	34
Sudan III	7
Sudan IV	9
Sudan red B	6
Sudan red 7B	3
Tartrazine	< 0.1
Amaranth	< 0.1
Allura red	0.1
New coccine	< 0.1
Sunset yellow	< 0.1

Table 3 Recovery of Sudan dyes from artificially contaminated samples as determined by ELISA detection

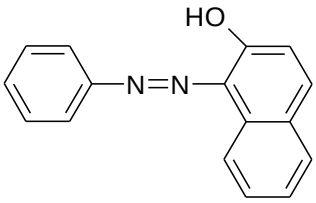
Sample	Fortified concentration (ng/kg)	Sudan I		Sudan II		Sudan III		Sudan IV	
		Recovery (%)	RSD%	Recovery (%)	RSD%	Recovery (%)	RSD%	Recovery (%)	RSD%
Ketchup	25	70	16.2	- [a]		- [a]			
	125	82	4.4	35	2.8	nd		nd	
	625	89	3.3	25	2.4	5	18.4	6	21.7
Chilli powder	25	90	11.8	- [a]		- [a]			
	125	88	3.4	38	7.9	nd		nd	
	625	103	2.3	20	4.0	5	20.8	6	23.9

[a] not measured

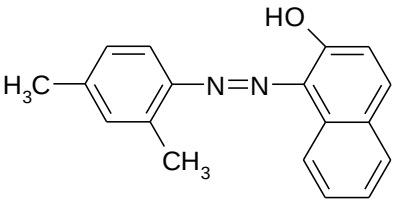
Table 4 Recovery of Sudan I from artificially contaminated egg yolk samples as determined by the reference HPLC method and by ELISA detection

Fortified concentration (µg/kg)	**HPLC**		**ELISA**	
	Recovery (%)	**RSD%**	**Recovery (%)**	**RSD%**
0	nd		nd	
25	nd		97	22.7
250	74	7.7	78	13.7
2500	96	0.6	82	19.9

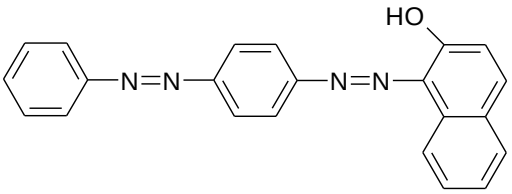
Figure 1



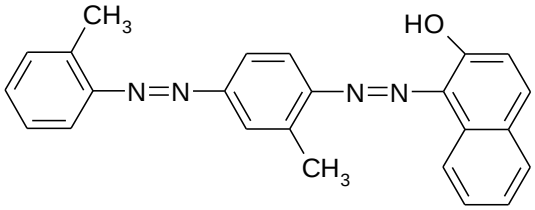
Sudan I



Sudan II



Sudan III



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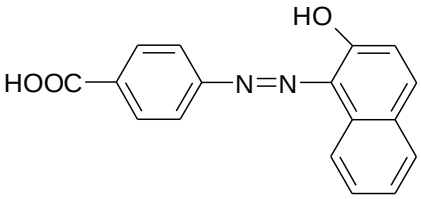
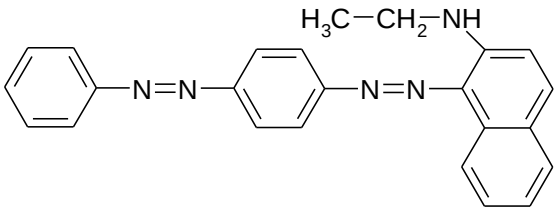
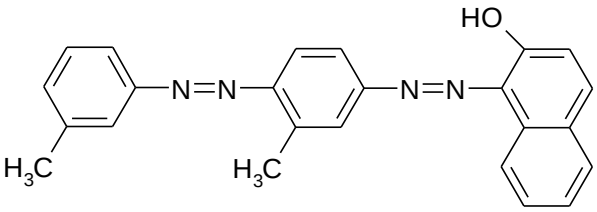


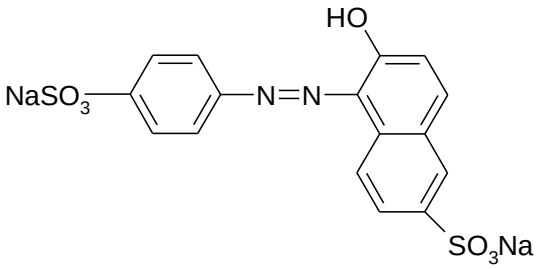
Hapten



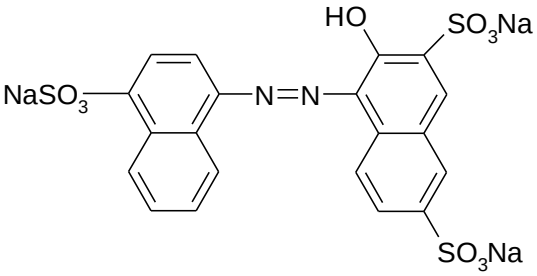
Sudan Red B



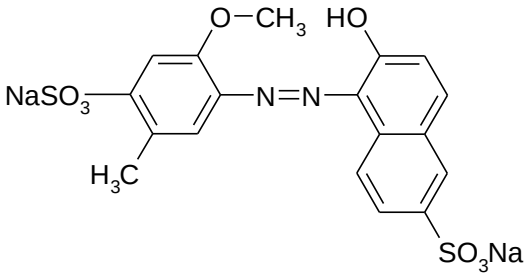
Sudan Red 7B



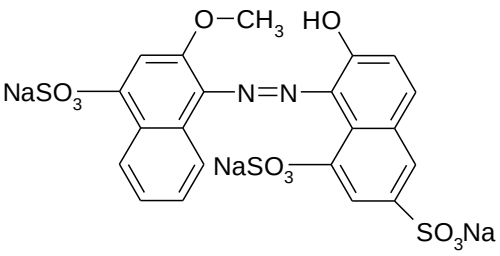
Sunset yellow



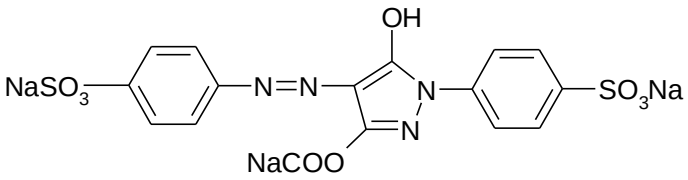
Amaranth



Tartrazine



Allura Red



New Coccine

Figure 2

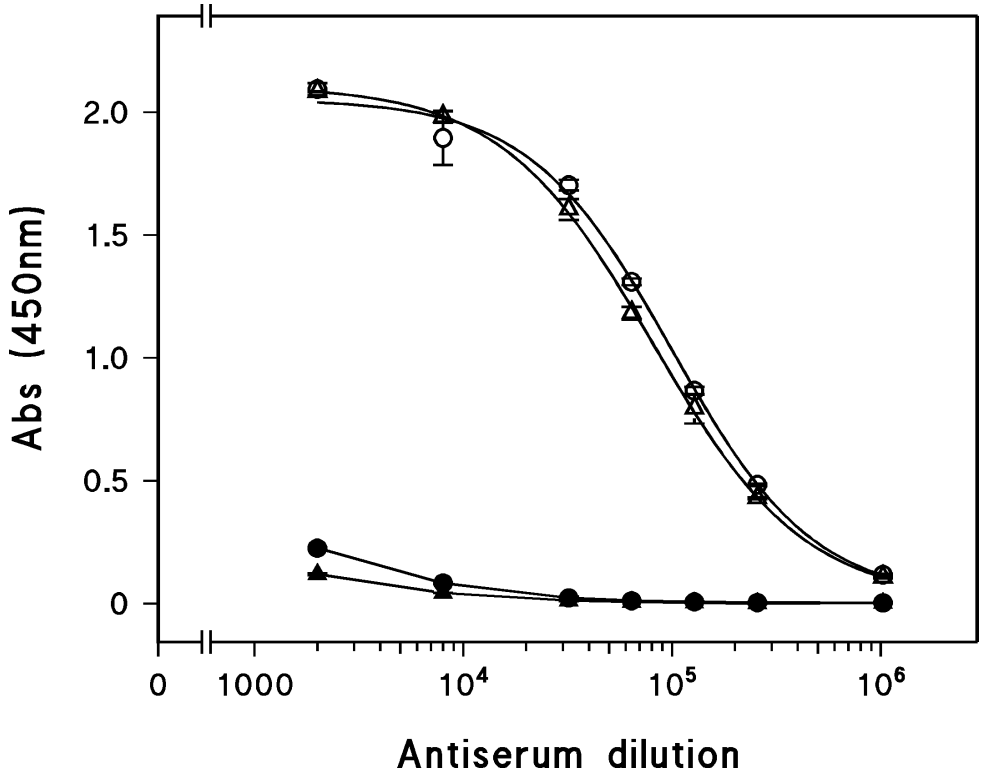


Figure 3

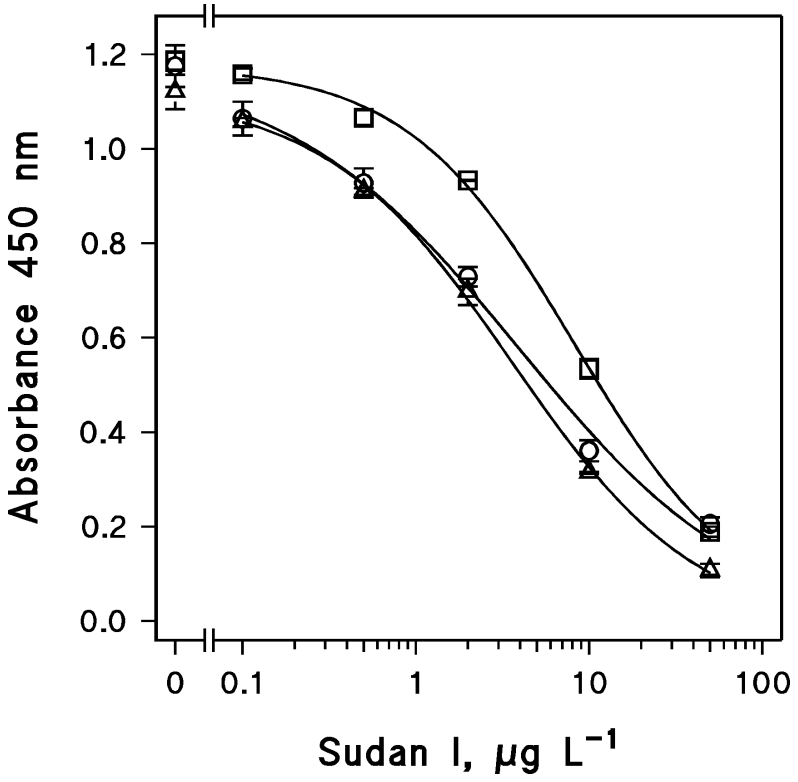
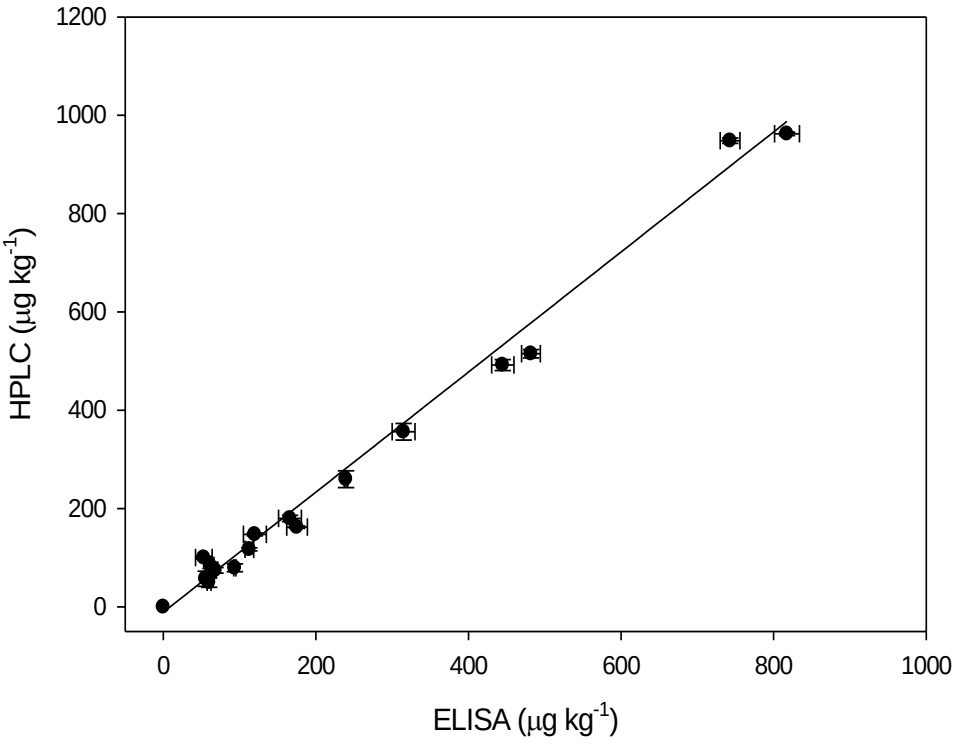


Figure 4


1200
1000
800
600
400
200
0
HPLC (µg kg-1)
0
200
400
600
800
1000
ELISA (µg kg-1)