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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 speed of analysis. A competitive indirect ELISA was developed, which showed an IC_{50} of $3.8 \mu\text{g L}^{-1}$. Two competitive direct ELISAs, were also developed, which differed in that the antibody is added before or simultaneously with the other reagents; the first showed an IC_{50} of $8.3 \mu\text{g L}^{-1}$ and the second one showed an IC_{50} of $4.9 \mu\text{g L}^{-1}$. Nevertheless, considering the dilution of extracts which is needed to offset matrix interference, the limits of detection of the three formats were substantially the same ($10 \mu\text{g kg}^{-1}$). The antibodies in all three test formats were able to recognize Sudan I and partially Sudan II, III, and IV and no cross-reactivity was observed with five approved food colours. Twenty food samples, including chilli powder, paprika, ketchup, and egg, were extracted by simple sample preparation and very limited dilution. Extracts were analyzed using 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, III and IV (Figure 1) are non-ionic fat-soluble dyes used as additives in gasoline, grease, oils, plastics, printing inks, and floor polishes (Mejia et al., 2007) These dyes are classified by the International Agency for Research on Cancer as category 3 carcinogens to humans (IARC, 1975), and universally the use of Sudan I in foodstuffs is not permitted by food regulations (EC Regulation No 178/2002, Di Donna et al., 2004). Unfortunately, these dyes are still illegally used to intensify and maintain the colour of food products such as chilli, curry, and palm oil-containing foodstuffs. From May 2003, warnings about chilli powder from Asia and alerts concerning a variety of foodstuffs contaminated with Sudan dyes were issued throughout Europe (Han et al., 2007) and, more recently, Sudan I was found in China in various commercial foodstuffs, including chilli sauce, meat and eggs (He 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. The need for a method is 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 were published in 2007. The vast majority of analytical methods which have been described are based on HPLC with optical or mass spectrometric detection (Di Donna et al, 2004; Ma et al, 2006; Cornet et al., 2006; Mazzetti et al, 2004), however, some gas chromatographic and capillary electrophoresis methods have also been reported (Mejia et al., 2007; He et al., 2007). Generally, chromatographic methods are expensive and time-consuming, therefore other techniques for detecting Sudan dyes have been developed. Among these, Han et al., (2007) described the first enzyme immunoassay 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,

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3 mainly because of the need for diluting the sample extracts from 1:100 to 1:1000 before analysis, in
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5 order to offset the matrix effect. Very recently, the preparation of a monoclonal antibody towards
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7 Sudan I, III and Para red and its use in the development of a sensitive competitive indirect ELISA
8
9 has been described (Ju et al, 2008). The determination of Sudan I in eggs by an immunoassay
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11 exploiting the resonance scattering of gold nanoparticles has also been reported (Jiang et al, 2008).
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13 Nevertheless, the extraction of the dye from eggs requires a two-day protocol.
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20 In this study, a Sudan I hapten derivative was synthesized, with the objective of firstly raising a
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22 polyclonal antibody characterized by low selectivity towards the other three Sudan dyes (II, III, and
23
24 IV) and secondly to develop a competitive immunoassay able to detect the entire group of banned
25
26 Sudan dyes. Different assay formats were compared: a direct and an indirect competitive ELISA
27
28 were developed, and the simultaneous or subsequent addition of reagents was evaluated. Moreover,
29
30 the extraction step and the solvent used to dissolve Sudan dyes were optimized with the aim of
31
32 minimizing matrix interference without the contemporary need for an excessive dilution of extracts.
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34 The assay which was developed was validated through comparison with an HPLC-UV method and
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36 proved to allow the determination of Sudan dyes in very different foodstuffs (chilli powder, ketchup
37
38 and egg yolk) .
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45 **MATERIALS AND METHODS**

46 **Materials**

47
48 All Sudan dyes, 4-aminobenzoic acid, 2-naphthol, bovine serum albumin (BSA), goat anti-rabbit
49
50 immunoglobulin HRP-labelled antibody, horse-radish peroxidase (HRP) N,N'-
51
52 dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and 3,3',5,5'-
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54 tetramethylbenzidine liquid substrate (TMB) were purchased from Sigma- Aldrich (St. Louis, MO,
55
56 USA). Sephadex G-25 resin and low chromatography apparatus were from GE Healthcare (Milan,
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3 Italy). Dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (HPLC grade) and all
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5 other chemicals and microtiter plates were obtained from VWR International (Milan, Italy).
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8 Polyclonal antibodies (ammonium sulphate precipitated IgG fraction) were obtained from Davids
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10 Biotechnologie (Germany). Antibodies were prepared using the standard immunization protocol
11
12 (Davids Biotechnologie, Germany) and sera were collected after 70 days from the first injection.
13
14

15 Sudan dye stock solutions were prepared by dissolving the powders in chloroform and immediately
16
17 diluting them in acetonitrile to the final concentration of 20 mg L⁻¹. Standard solutions were
18
19 prepared by daily diluting the stock solutions with DMSO:water (10:90, v/v).
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22 **Synthesis of hapten and hapten-protein conjugates**

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24 The hapten used for immunization, antigen coating and enzyme labelling is depicted in Figure 1.
25
26 Carboxy-Sudan I (CSudan I) was synthesized as previously reported, (Ju et al, 2008). CSudan I was
27
28 then conjugated with BSA and HRP by the DCC/NHS ester method, as reported in the literature
29
30 (Hosoda et al., 1979). Briefly, equimolar amounts of CSudan I, DCC and NHS were dissolved in
31
32 anhydrous DMF and the mixture was incubated at room temperature for 1 hour. Proper amounts of
33
34 the mixture were then added to protein solutions prepared in 0.13 M NaHCO₃, to obtain a final
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36 molar ratio of 50:1; 100:1; 200:1 (CSudan I:BSA), 20:1; and 10:1 (CSudan I:HRP). BSA
37
38 conjugates were incubated overnight at room temperature, while HRP conjugates were reacted for 1
39
40 hour at room temperature. Separation of conjugates from by-products and the excess of reagents
41
42 was carried out by gel filtration low pressure chromatography on Sephadex G-25 resin (mobile
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44 phase: phosphate buffer). Finally, sodium azide was added to BSA conjugates, which were stored
45
46 at -18°C. The 200:1 conjugate was used as the immunogen, while conjugates with lower
47
48 substitution degree were used as coating antigens. HRP conjugates were stored at 4°C, with 33%
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50 (v/v) of glycerol added.
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Competitive indirect ELISA

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3 Wells were coated with 200 μl of 0.2 mg L^{-1} CSudan I-BSA (50:1) in a carbonate-bicarbonate
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5 buffer (50 mM pH 9.6). The plates were incubated overnight at 4°C. After the plates were washed
6
7 three times with washing solution (0.05% Tween 20), 300 μl of blocking solution (phosphate
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9 buffer, containing 0.1% BSA, 5% sucrose, and 4% polyvinylpyrrolidone) was added to each well
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11 and the plates were further incubated for 1 hour at room temperature. The plates were washed and
12
13 they were either used immediately or stored at 4°C until use. Antiserum was diluted 1:30000 (v/v)
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15 in 20 mM phosphate buffer at pH 7.4, containing 1% (p/v) of BSA (PBSA). A volume of 100 μl of
16
17 diluted antiserum was added to 100 μl of Sudan I at concentrations ranging from 0 to 100 ng L^{-1} and
18
19 incubated in coated wells for 15 min. After 3 well washes with washing solution 200 μl of a diluted
20
21 goat-antirabbit immunoglobulin conjugated to horseradish peroxidase was used to detect the
22
23 immobilized rabbit antibody. The incubation time was 30 min, followed by three well washes.
24
25 Colour development was obtained by a 30 min incubation with TMB (200 μl per well). One
26
27 hundred microlitres of sulphuric acid (1M) was used as a stop solution and absorbance was
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29 recorded at 450 nm. All standards and samples were measured in duplicate.
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39 **Competitive direct ELISA (simultaneous addition of reagents)**

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41 The immunoreactive solid phase was obtained by coating wells with 300 μl of a goat-antirabbit IgG
42
43 solution (10 mg L^{-1}) (Giraudi et al., 2000). Antiserum and CSudan I-HRP were diluted in PBSA.
44
45 A volume of 100 μl of diluted antiserum (1:15000, v/v) was added to 50 μl of 0.75 mg L^{-1} of
46
47 CSudan I-HRP (20:1) and to 50 μl of Sudan I at concentrations ranging from 0 to 100 $\mu\text{g L}^{-1}$. The
48
49 mixture was incubated 15 min in immunoreactive wells. Then the wells were washed with washing
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51 solution. Colour development and absorbance readings were obtained as described above.
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58 **Competitive direct ELISA (pre-immobilization of the antibody)**

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60 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

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3 incubated overnight at 4°C. After the plates were washed three times with washing solution, they
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5 were used immediately. A volume of 100 µl of CSudan I-HRP (20:1) at 0.2 mg L⁻¹ was added to
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7 100 µl of Sudan I at concentrations ranging from 0 to 100 µg L⁻¹. The mixture was incubated for 15
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9 min in wells coated with the anti-Sudan I antibody, then wells were washed with washing solution.
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11
12 Colour development and absorbance readings were obtained as described above.
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17 **Calculations**

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19 Sudan I concentrations were determined by interpolation on the calibration curve, where the signal
20
21 was plotted against the log of analyte concentration. For each experiment, a calibration curve was
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23 determined by a nonlinear regression analysis of the data of the standards using the four-parameter
24
25 logistic equation (Findlay et al., 2000).
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29 Relative cross-reactivity was calculated as follows:
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$$31 \text{CR\%} = (\text{IC}_{50} \text{ Sudan I} / \text{IC}_{50} \text{ Sudan dye}) * 100$$

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33 where IC₅₀ is the Sudan dye concentration which cause 50% inhibition of the maximum observed
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35 signal.
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38 **Samples and sample preparation**

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40 The following commercial samples: 5 kinds of ketchup, 5 kinds of eggs, 10 kinds of chilli powder
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42 and paprika were purchased in large stores. One sample for each category that did not show any
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44 detectable residues of target dyes when analyzed by HPLC was taken as the blank for matrix
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46 interference experiment and fortification experiments. Fortified samples were prepared by adding
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48 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
49
50 before performing the extraction.
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54 Sudan dye extraction was carried out as reported in the literature (Ma et al., 2006) with slight
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56 modifications. Egg yolk was manually separated and gently mixed before extractions. Briefly, 20 g
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3 of homogenized sample were weighed and sonicated for 15 min with 50 ml of DMSO. Egg yolk
4 and ketchup samples were previously mixed with 20 g of sodium sulphate to eliminate water. After
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6 being centrifuged for 15 min at 3000 rpm, extracts were diluted at 1:10 with PBSA and filtered
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8 through a 0.45 µm nylon membrane filter. Based on our experience, the use of a nylon membrane is
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10 mandatory to eliminate matrix interference from chilli powder and paprika samples.
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16 17 **HPLC analysis**

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19 To evaluate the accuracy and to validate the method, a comparative study using both the developed
20
21 ELISA method and a HPLC reference procedure (Thompson et al. 2002) was performed. Samples
22
23 were extracted as described above, except that DMSO extracts were diluted 1:5 (v:v) with the same
24
25 solvent and filtered through a 0.45 µm nylon membrane filter, according to the literature (Ma et al.,
26
27 2006). Each extract was injected in triplicate. An HPLC system (LaChrom Elite, VWR-Hitachi,
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29 Darmstadt, Germany) equipped with a C-18 Chromolith Performance 100-4.6 mm column (VWR,
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31 Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile and water at a volume
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33 ratio of 80:20, delivered to the column at a rate of 1 ml min⁻¹. Detection was made by a UV detector
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35 (472 nm). Sudan I concentrations were calculated by interpolation on an external calibration curve.
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43 **RESULTS AND DISCUSSION**

44 **Production of polyclonal antibodies**

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46 Two rabbit antisera were raised against CSudan I-BSA (200:1). These were tested by a non-
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48 competitive indirect ELISA, following the protocol described in the experimental section, with the
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50 difference that no Sudan I was added. Both antisera show very similar performance (Figure 1) and
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52 high titres on the coating antigen (IC₅₀ at 1:80 000 and 1:100 000, v/v, antibody dilution for rabbit 1
53
54 and 2 respectively). The non-specific binding of antisera towards the carrier protein was also
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56 evaluated, but resulted negligible in comparison with the binding towards the hapten (Figure 1).
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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 was selected as the most suitable on the basis of the IC₅₀ value that was the lowest. Since Sudan dyes are very hydrophobic compounds, they should be dissolved and kept in 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 et al, 2007; Ma et al., 2006; Cornet et al., 2006; Mazzetti et al., 2004). The application as the analyte solvent in immunochemical methods of analysis narrowed the field, but acetonitrile and dimethylsulfoxide (DMSA) 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. The 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 (IC₅₀ 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 (IC₅₀ below 10 µg L⁻¹). The increase of the organic solvent amount from 5 to 10% produced a parallel doubling of the IC₅₀ value, whereas when the solvent percentage was increased up to 20%, the IC₅₀ 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 min was selected as a satisfying compromise between the lowest IC₅₀ and a blank signal around 1-1.5 UA. Figure 3 shows a typical

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3 inhibition curve obtained under optimized conditions. The IC_{50} value of the assay was $4 \mu\text{g L}^{-1}$ with
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5 a detection limit of $0.2 \mu\text{g L}^{-1}$ and a dynamic range of $0.4\text{-}100 \mu\text{g L}^{-1}$.
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9

10 **Competitive direct ELISA**

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12 Competition experiments were carried out under various combinations of antibody and enzyme
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14 tracer concentrations. Two formats were optimized: the first involving the simultaneous reaction
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16 between antibody, tracer and analyte (dissolved in 10% DMSO) and a second one, which was based
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18 on a long pre-immobilization reaction of the antibody, followed by a short incubation of the mixture
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20 of the analyte and the tracer into functionalized wells. Figure 3 shows typical inhibition curves
21
22 obtained under optimized conditions. Antibody dilutions, enzyme tracer concentrations and
23
24 incubation times are summarized in Table 1, together with the analytical performance of the
25
26 optimized assay in both formats. As expected, the pre-immobilization of the antibody allowed the
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28 use of lower amounts of antibody and competitor (the enzyme tracer) and consequently the
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30 improvement of assay sensitivity (lower IC_{50} value). Nevertheless, the preparation of functionalized
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32 wells by the pre-immobilization of the antibody involves overnight incubation before carrying out
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34 the assay or the stabilization of functionalized wells.
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43 **Selectivity of the assays**

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45 Since, from a legal point of view, the occurrence of all four Sudan dyes (I-IV) should be assessed in
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47 food, the assay would be virtually able to cross-react with the four target analytes. To assess the
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49 influence of the assay format on the selectivity, cross-reactivity towards Sudan II has been
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51 measured by the three developed assay formats. The manipulation of the format of the
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53 immunoassay slightly influences the selectivity: the competitive direct ELISA with the pre-
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55 immobilized antibody is the most selective assay ($CR\%=26\%$), whereas the other two formats show
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57 about the same selectivity ($CR\%=34\%$ for the direct assay and 35% for the indirect ELISA).
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3 Since the format finally chosen to validate the method was the direct ELISA with the simultaneous
4 addition of reagents, a more extensive study of selectivity was made using this assay format.
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6 Results reported in Table 2 show that Sudan II is well recognized. Sudan III and IV and other Sudan
7
8 dyes demonstrate a slight recognition, which means that the assay would be able to measure these
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10 compounds only at levels ten-times higher than Sudan I. This finding is not very far from the
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12 performance obtained by some instrumental methods of analysis based on chromatographic or
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14 electrophoretic determination of Sudan dyes (Mejia et al., 2007; Ma et al., 2006).
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22 Several dyes, whose use is permitted in food (Department of Food Biosciences, University of
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24 Reading, UK), were also tested for cross-reactivity. The interference with the assay was found to be
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26 negligible for all tested compounds.
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32 **Better choice of format and its validation**

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34 To evaluate matrix interference on the three developed assays, blank samples of chilli powder,
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36 ketchup and egg yolk were analyzed in six replicates. Negligible interference was observed when
37
38 extracts were analyzed by means of the direct ELISA format with the simultaneous addition of
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40 reagents. The other two formats needed a simple 1/2 (v/v) dilution of samples with the PBSA
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42 containing 10% of DMSO before the analysis to offset the matrix effect.
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48 The LOD was calculated from interpolation of the Sudan I standard curve as the analyte
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50 concentration corresponding to the mean signal of the zero standard (obtained by averaging the
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52 signal of eight replicate sets) minus three times its standard deviation (according to IUPAC, ISO,
53
54 and AOAC harmonized guidelines) (Thompson et al, 2002). The LOD was found to be $0.2 \mu\text{g L}^{-1}$
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56 for both the indirect and the direct ELISA with the pre-immobilization of the antibody. The direct
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58 ELISA with the simultaneous addition of reagents had lower sensitivity (calculated LOD $0.4 \mu\text{g L}^{-1}$
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60¹). This result can be simply explained by remembering that in the direct ELISA (with the

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3 simultaneous addition of reagents) standards and samples are diluted 1/4 (v/v) in wells, while in the
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5 other two formats they are diluted 1/2. For the same reason, no further dilution was required to
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7 offset matrix interference in the direct ELISA with the simultaneous addition of reagents. On the
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9 contrary, the other two formats required an additional 1/2 (v/v) dilution, before analysis.
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11 Consequently, in the end, the limits of detection of the three developed formats, compared to the
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13 solid sample, were substantially the same ($10 \mu\text{g kg}^{-1}$).
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19 Therefore, the direct competitive ELISA with the simultaneous addition of reagents, being rapid,
20
21 sensitive and the easiest to carry out was chosen as the format to be fully validated. The LOD, re-
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23 calculated for each different matrix by measuring and averaging data from the five blank samples,
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25 confirmed the previous estimation ($10 \mu\text{g kg}^{-1}$) as regards ketchup and chilli powder.
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31 The precision of the method was determined by extracting and analyzing replicates of Sudan I
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33 artificially contaminated samples (ketchup, chilli powder,), which were fortified with a Sudan I
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35 concentration of 25 (low level), 125 (medium level), and 625 (high level) $\mu\text{g kg}^{-1}$.
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41 The assay was carried out in six replicates on the day for the evaluation of within-assay precision
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43 and on six different days for the evaluation of the between-assay precision. The values of RSD%
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45 were calculated at each nominal concentration level and ranged from 2 to 16%, which fulfilled FDA
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47 requirements for the validation of bioanalytical methods according to FDA guidance (Viswanathan
48
49 et al., 2007).
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54
55 To evaluate the accuracy of the method, chilli powder and ketchup samples fortified with Sudan I
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57 (concentrations of 25, 125, and $625 \mu\text{g kg}^{-1}$), Sudan II, Sudan III, and Sudan IV (concentrations of
58
59 125 and $625 \mu\text{g kg}^{-1}$) were extracted and analyzed. Results are summarized in Table 3. Recovery
60
values ranged from 70 to 103% for Sudan I, thus indicating a good accuracy of the assay when

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3 applied to real samples of very different compositions. Recovery values for Sudan II, III, and IV
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5 reflect their cross-reactivity and confirm that these dyes could be only detected in highly
6
7 contaminated samples.
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12 Fortification experiments were also conducted on egg yolk samples. This matrix has not been
13
14 included in the work of Ma et al. (2006), thus no information regarding the possibility of extracting
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16 Sudan dyes from this kind of sample by means of the same protocol applied to other matrices was
17
18 available. To our knowledge, this is also the first time that Sudan I has been measured via an
19
20 immunoenzymatic method in egg yolk. Therefore, a blank sample of egg yolk was fortified with 25,
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22 250 and 2500 $\mu\text{g kg}^{-1}$ of Sudan I and extracted in triplicate according to the procedure described in
23
24 the experimental section. Samples were analyzed both by the developed direct ELISA method and
25
26 by HPLC and the obtained recovery values for both methods are reported in Table 4. The fortified
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28 sample at the lowest concentration could only be measured by means of the ELISA. The medium
29
30 and high level fortified samples gave satisfactory recoveries and good agreement between the
31
32 results obtained via the two methods. Generally speaking, the developed ELISA showed an
33
34 increased imprecision of data, in comparison with fortification experiments made on samples
35
36 belonging to different classes (chilli powder, paprika and ketchup), even if the relative standard
37
38 deviation remained in the range considered acceptable for screening methods (Viswanathan et al.,
39
40 2007). The LOD was, in this case, higher than for other considered matrices ($15 \mu\text{g kg}^{-1}$), due to the
41
42 greater data imprecision.
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51 52 53 **Comparison of ELISA and HPLC determination**

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

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6 Comparable results were obtained by the two methods: the linear regression analysis (Figure 4)
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8 yielded a good correlation between the methods ($y=1.19x-10.0$, $r^2 = 0.991$, $n=20$). A limited under-
9
10 estimation of the Sudan I concentration was observed at high contamination levels (above $500 \mu\text{g}$
11
12 kg^{-1}), probably due to the low solubility of the analyte when diluted 1/10 in the PBSA for the
13
14 analysis. In fact, if the two highest fortified samples are not included in the comparison between
15
16 ELISA and HPLC, the agreement between methods further improves ($y=1.09x-1.24$, $r^2 = 0.995$,
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18 $n=18$). Thus, very highly contaminated samples, which can occur in the case of the illegal use of
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20 Sudan dyes (Mazzetti et al, 2004), should be diluted in DMSO before diluting them in PBSA, in
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22 order to prevent Sudan I precipitation and low recoveries.
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29 These results proved that the ELISA can be applied as screening methods for the detection of Sudan
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31 I in foodstuffs of very different kinds, with good accuracy and precision. The assay has good
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33 sensitive, sample extracts need a very limited dilution before being analyzed, and therefore the LOD
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35 in samples remains in the $10 \mu\text{g kg}^{-1}$ range. In addition, the method is simple, rapid (the extraction
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37 requires 30 min and the assay is completed in 45 min), and applicable to very different matrices
38
39 with very slight modifications, thus proving itself to be suitable as a first level screening method or
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41 for routine quality controls of various foodstuffs.
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FIGURE CAPTIONS

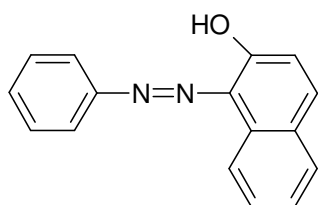
Figure 1: Structure of the analytes; the hapten derivative used for immunization and preparation of the coating antigen and the enzymatic tracer; and analytes used in a cross-reactivity study.

Figure 2: Non-competitive binding of antisera raised against CSudan towards the coating antigen, CSudan-BSA (Δ rabbit 1, \circ rabbit 2) and towards the carrier protein, BSA (\blacktriangle rabbit 1, \bullet rabbit 2)

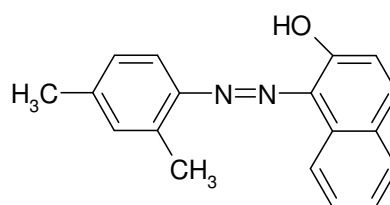
Figure 3: Typical inhibition curves, obtained under optimized conditions for the three ELISA formats developed (\square Competitive Direct ELISA, simultaneous addition of reagents; \circ Competitive Direct ELISA, pre-incubation of antibodies; Δ Competitive Indirect ELISA).

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 ($y=1.19x-10.0$, $r^2 = 0.991$)

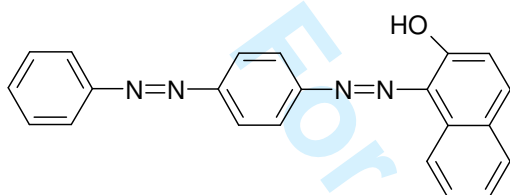
Figure 1



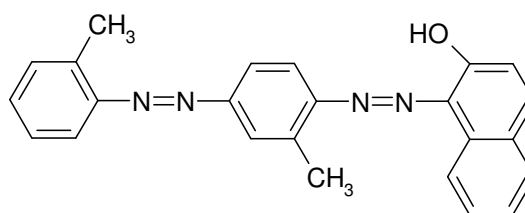
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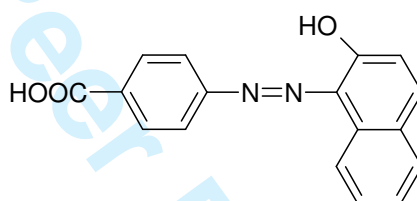
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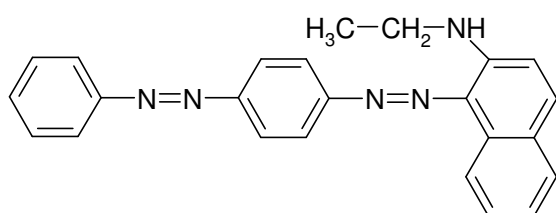
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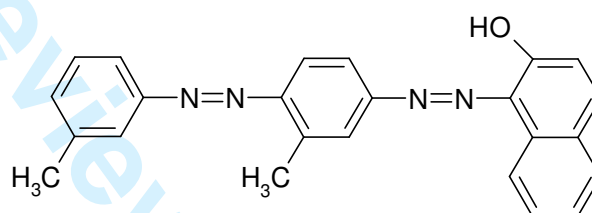
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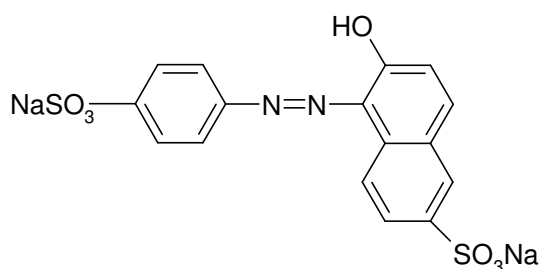
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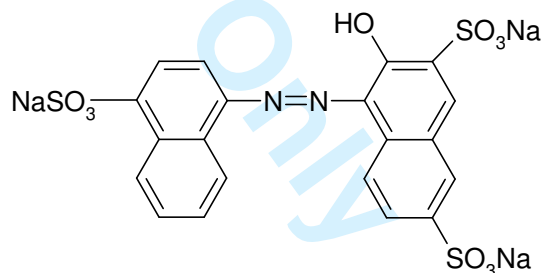
Sudan Red B



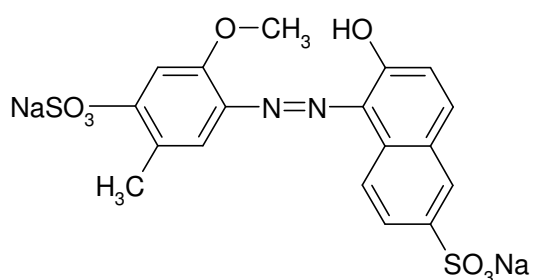
Sudan Red 7B



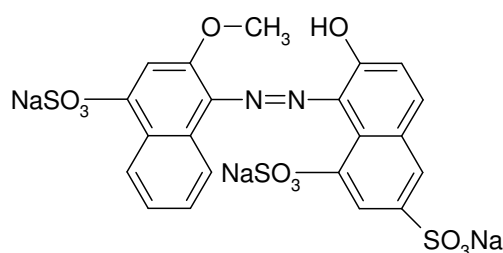
Sunset yellow



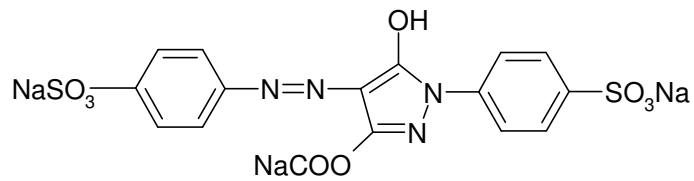
Amaranth



Tartrazine



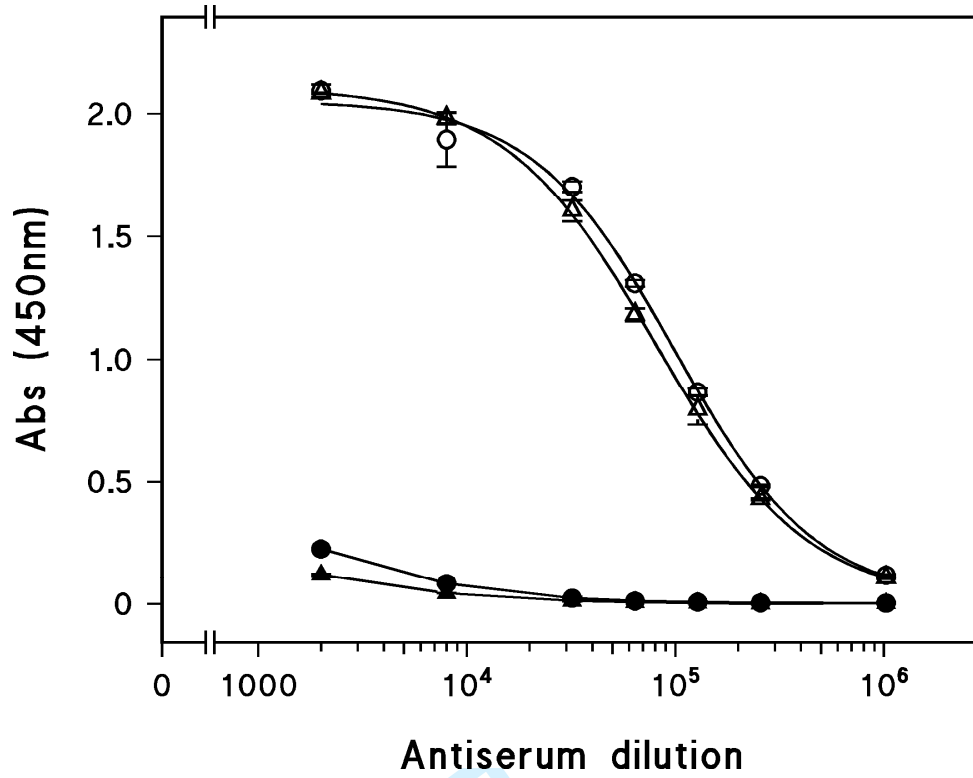
Allura Red



New Coccine

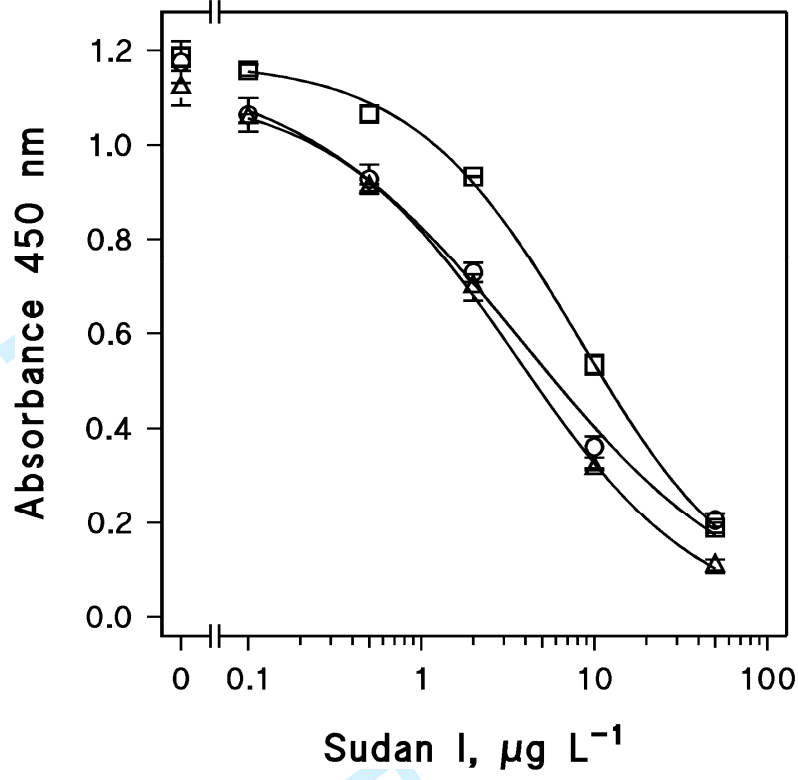
For Peer Review Only

Figure 2



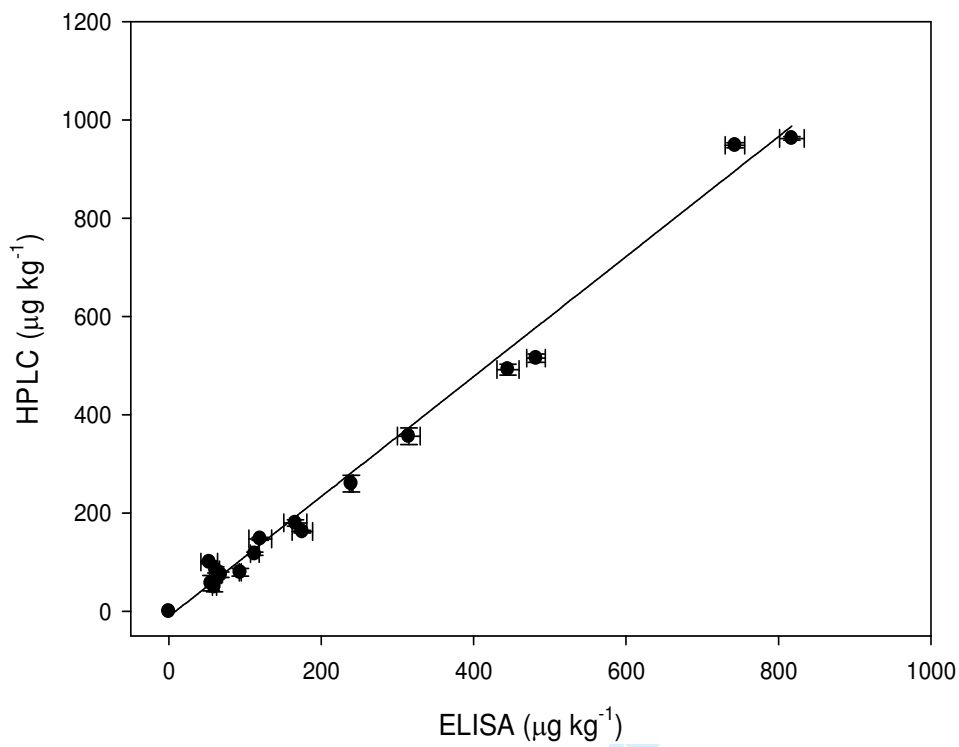
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Figure 3



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Figure 4



TABLES

Table 1 Assay characteristics of the three competitive ELISA formats

	antibody dilution in wells	competitor concentration (ng well)	IC₅₀ (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 cocchine	< 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

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^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 ($\mu\text{g}/\text{kg}$)	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