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## Articles and Statements

### Lateral flow Immunoassay of Sudan I with Direct Calibration Dependence Based on the use of two Kinds of Conjugated Gold Nanoparticles

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#### Abstract

A novel lateral flow immunoassay based on the use of two kinds of conjugated gold nanoparticles' preparations was developed and applied to the detection of the toxic food colorant Sudan I. The gold nanoparticles were conjugated with monoclonal antibodies specific to Sudan and a Sudan-ovalbumin conjugate. Before the immunochromatographic process, the two conjugates were incubated with a tested sample. The results obtained a direct calibration curve for the detection of low-molecular-weight antigen, in contrast to common reverse concentration dependencies. In the absence of the target compound, minimal coloration of the test line was observed. An increase in the Sudan content led to staining and the appearance of a test line, which could be observed by the naked eye. The total duration of the assay is 15 minutes. The visual limit of Sudan I detection was 0.25 ng/mL, and the instrumental limit of detection (LOD) was 7.6 pg/mL. These values were at least three-four points lower than the established permissible level of the contaminant content in foodstuffs. This reserve in sensitivity provides the possibility of working with highly diluted samples and thus excludes the influence of their matrixes on the assay results. The direct dependence of the coloration on the concentration of Sudan I in the proposed assay means it is easy to identify the presence of the contaminant at extremely low concentrations. The common nature of the proposed approach allows its use in lateral flow immunoassays of different compounds.

**Keywords:** immunoassay, lateral flow tests, gold nanoparticles, immunochromatography, on-site tests, Sudan I.

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## 1. Introduction

Sudan I and related compounds (Sudans II-IV) are illegal food additives. Their intense red hue improves the coloration of food products and, correspondingly, their market potential. They are classified as class 3 carcinogens according to the International Agency for Research on Cancer. Thus, most countries have banned their use in food production (European Food Safety Authority). The EC maximum allowable level for Sudans is 0.5–1 mg/kg (European Food Safety Authority). However, they are often detected in chilli-, curry-, curcuma-, and palm oil-containing foodstuffs (Calbiani et al., 2004; Calbiani et al., 2004a; Oplatowska-Stachowiak, Elliott, 2015), indicating that unscrupulous manufacturers continue to use Sudans.

Many methods have been proposed for the determination of Sudan dyes in foodstuffs as recently reviewed (Oplatowska-Stachowiak, Elliott, 2015; Rebane et al., 2010): HPLC (Liu et al., 2007; Ertaş et al., 2007; Yu et al., 2015; Khalikova et al., 2015), electrochemical sensors (Li et al., 2015; Li et al., 2015a; Wang et al., 2015), and capillary electrophoresis (Mejia et al., 2007). The main disadvantages of the approaches listed above are the high cost of the equipment, the significant duration of one analysis, and difficulties in screening a large number of samples.

Immunoassay techniques, due to their simplicity and productivity, are often used in food control laboratories. Among these, lateral flow immunoassays (LFIAs) focus on screening out-of-laboratory purposes because of they are faster and less labor-intensive compared with alternate immunotechniques (Wong, Tse, 2009; Quesada-González, Merkoçi, 2015). Despite the availability of ELISA for Sudan dyes (Chang et al., 2011; Liu et al., 2012; Shan et al., 2012; Anfossi et al., 2009; Qi et al., 2015), only two LFIAs for Sudan I have been developed, namely a semi-quantitative assay in the work of Wang et al. (Wang et al., 2013), and a quantitative photometric assay in the work of Berlina et al. (Berlina et al., 2016).

Both assays rely on a traditional competitive scheme when hapten conjugated with protein is immobilized on the membrane and an anti-hapten antibody is conjugated with gold nanoparticles. This format is typical for the LFIA of low-molecular-weight compounds. In this case, the brightest test line occurs in the absence of analyte and thus the limit of detection is determined when the color intensity in the test area decreases. However, this method of data processing is suitable when there is a detector. For out-of-laboratory conditions, the best choice is a cut-off test, i.e. control of the coloration disappearance, but such assay protocols have significantly worse sensitivity.

To resolve this problem, we propose an assay involving the application of two kinds of conjugated gold nanoparticles. It allows receipt of direct calibration dependence when an absence of the competitor leads to an absence of coloration.

## 2. Materials and methods

### 2.1. Chemicals and reactants

Mouse monoclonal antibodies of Sudan I and Sudan–ovalbumin conjugate (synthesized using succinimide/carbodiimide technique) were obtained as described previously (Berlina et al., 2016). Goat polyclonal antibodies against mouse immunoglobulins G were from IMTEK (Moscow, Russia). Gold (III) chloride hydrate was purchased from Fluka (St. Louis, MO, USA). Methanol was purchased from Fluka (St. Gallen, Switzerland). Sodium citrate dihydrate, potassium carbonate, Tris, and Tween-20 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from MP Biomedicals (Santa Ana, CA, USA). All other reagents were from Chimmed (Moscow, Russia). Deionized water, with a resistance of 18.2MΩ·cm at 22°C (Simplicity Water Purification system, Millipore, Bedford, MA, USA) was used to prepare all the aqueous solutions for the syntheses and assays. The Amicon Ultra (30 kDa) ultracentrifugation tubes were from Millipore (Bedford, MA, USA).

CNPH-backed nitrocellulose membranes (CNPH90) were purchased from Advanced Microdevices (MDI, Ambala Cantt, India). The following membrane compounds for manufacturing test strips were purchased from Millipore (Bedford, MA, USA): CFSP223000 adsorption pads and fiberglass macroporous CFCP203000 conjugate pads.

### 2.2. Synthesis of gold nanoparticles (Ji et al., 2007)

0.2 mL of 5% H<sub>2</sub>AuCl<sub>4</sub> was added to 97.5 mL of water (filtered through a 0.22- $\mu$ m filter). The solution was heated to its boiling point, and then 1.5 mL of 1 % sodium citrate was added. The resulting mixture was boiled for 20 minutes and then cooled.

### 2.3. Conjugation of antibodies or Sudan-ovalbumin with gold nanoparticles

In this step, 3  $\mu$ L of anti-Sudan antibody with a concentration of 3.8 mg/mL, or 0.3 mL of Sudan-ovalbumin with a concentration 3.5 mg/mL were dialyzed against a 50 mM carbonate buffer, pH 9.0 using Amicon Ultra (30 kDa) ultracentrifugation tubes. The volume upper the separation membrane was added to 1 mL of freshly synthesized gold nanoparticle solution and this mixture was left for 45 minutes to incubate at room temperature with stirring. Then, 15 $\mu$ L of a 10 % BSA aqueous solution was added to the mixture, followed by incubation for 15 minutes under the same conditions. The conjugate was separated by centrifugation at 10,000 g at +4° C for 15 minutes. For the stabilization and long-term storage 50 mL of 50 mM potassium phosphate buffer, pH 7.4, with 0.1 M NaCl, 0.25% BSA, 0.25% Tween-20, 1% saccharose, and 0.05% NaN<sub>3</sub> (TTBSA) were added to the resulting product.

### 2.4. Manufacturing of test strips

Goat anti-mouse IgG polyclonal antibody (0.5 mg/mL in Milli-Q water) was immobilized onto the nitrocellulose CNPH 90membrane as the test line, as described previously (Berlina et al., 2013). The volume of the reagent loaded zone was 0.1  $\mu$ L per 1 mm. The membranes were dried at room temperature overnight. The multi-membrane composite was then assembled and cut into individual 3-mm wide strips. The strips were packed and stored at +4 °C. The antibody and Sudan-ovalbumin conjugate with colloidal gold with OD<sub>520nm</sub> = 10 were mixed in 10% BSA in a 1:10 v/v ratio and used when blending with samples with and without Sudan I.

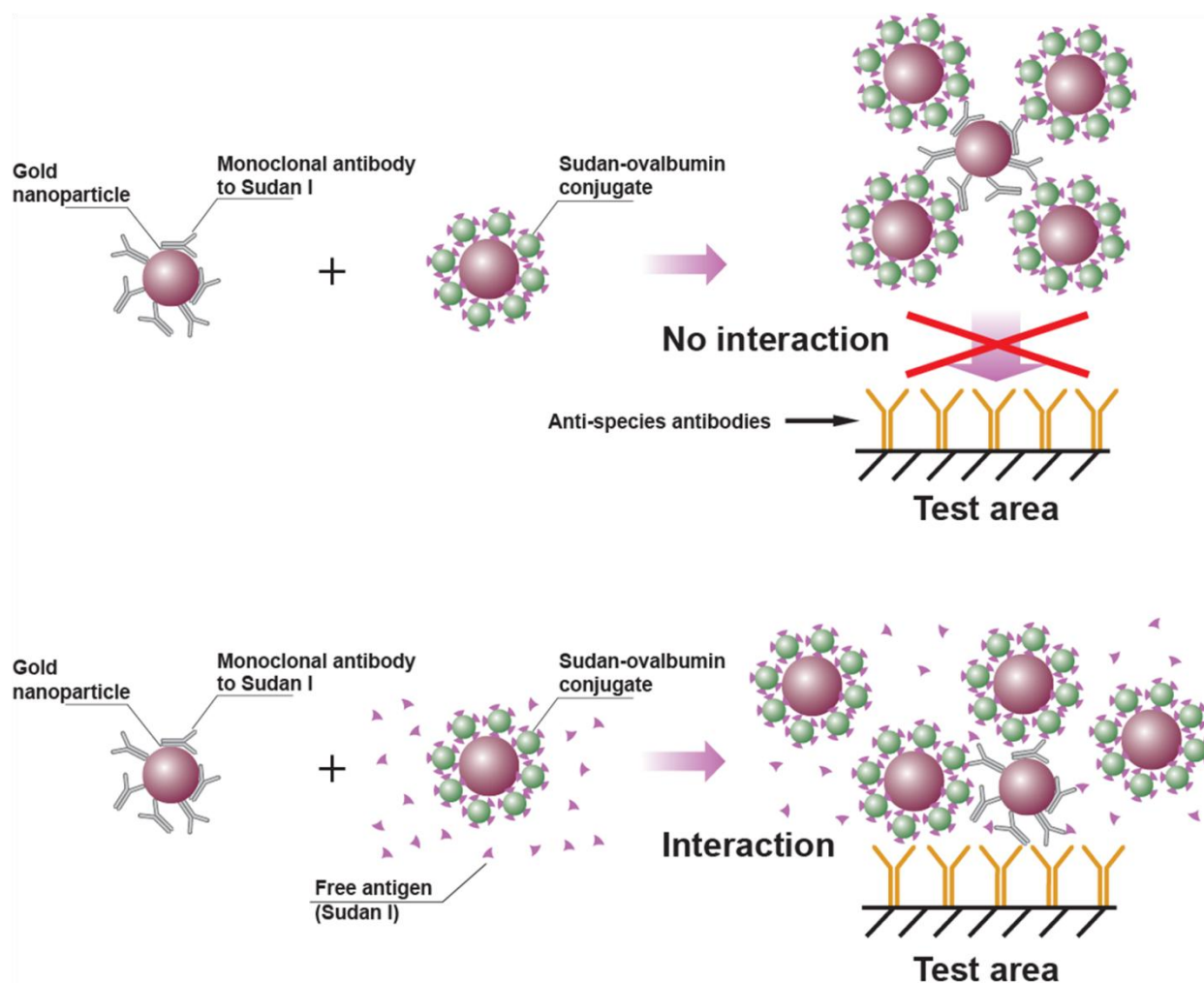
### 2.5. Lateral flow immunoassay

The assay was carried out at room temperature. A sample was added to an Eppendorf tube containing the mixture of conjugates and left for 5 minutes. The test strip was then submerged in the tested sample, incubated for 5 minutes, removed, and placed horizontally for 5-minutes to dry. After the removal of the test strip, 50  $\mu$ L of 50 mM PBS with 0.05% Triton X-100 were added dropwise to the lower part of the test strip (sample pad) to remove the conjugates from the pad.

The resulting appearance of the test line was determined by the naked eye, and the lowest concentration leading to coloration was taken as the visual limit of detection (LOD<sub>v</sub>). To plot a calibration curve, the color intensity was determined by processing the scanned digital images of the strips using TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK) under a 1D regime. The instrumental limit of detection (LOD<sub>i</sub>) was calculated as the concentration that resulted in a reliable difference in color from the background value, i.e., the color intensity in the absence of the analyte was three times higher compared with the standard deviation of the background color.

## 3. Results and Discussion

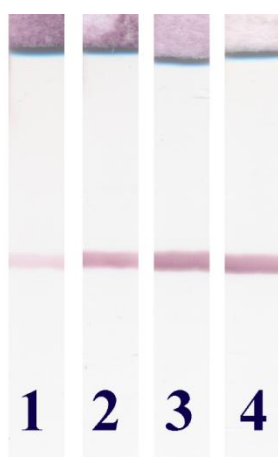
### 3.1. Lateral flow immunoassay design



**Fig. 1.** Scheme of the proposed immunochromatographic assay for Sudan I with the use of two kinds of conjugates.

The mixed conjugates reacted due to an interaction between the Fab fragment of the immobilized antibody on the surface of one gold nanoparticle and a Sudan I derivative conjugated with ovalbumin (Sudan–ovalbumin) on another gold nanoparticle. Thus, the conjugated Sudan–ovalbumin covered the antibodies on the surface of the gold nanoparticle on all sides and prevented the Fc fragment interacting with the anti-mouse IgG immobilized in the test area. This resulted in the absence of visible coloration and is considered as a negative result of the testing. The added Sudan I started to compete with the Sudan–ovalbumin conjugated with gold nanoparticles for the binding sites of antibodies. Thus, Sudan I displaced the Sudan–ovalbumin conjugated to the gold nanoparticles and blocked the Fab-fragments. As a result of this reaction, Fc-binding antibody fragments that were previously blocked by such a big Sudan–ovalbumin conjugate with gold nanoparticles became available for an interaction with an anti-mouse antibody. The result is the binding of mouse anti-Sudan antibodies to goat anti-mouse antibodies immobilized in the test area and the appearance of a colored test line (Fig. 1). The latter signified a positive result of the testing.

For this assay two conjugates of gold nanoparticles were synthesized: the first with an antibody to Sudan I, and the second with a Sudan–ovalbumin conjugate. Both conjugates were mixed in a ratio with 10-fold excess (Sudan–ovalbumin towards antibody) to protect the maximal amount of antibody on the gold nanoparticles. The antibody conjugated to the gold nanoparticles interacted with the target hapten in the conjugate. In the absence of Sudan I in the solution interactions occurred only between the conjugates, and no bright test line was observed (Fig. 2, test strip 1). With an increase in the Sudan I content, the color started to appear due to the opportunity of Fc-fragments antibodies interacting with the anti-species antibody in the membrane's test zone (Fig. 2, test strips 2–4).



**Fig. 2.** Test strips images for different Sudan I concentrations in the samples. Strips 1-4 correspond to Sudan I concentrations of 0 pg/mL, 250 pg/mL, 250 ng/mL, and 1  $\mu$ g/mL, respectively.

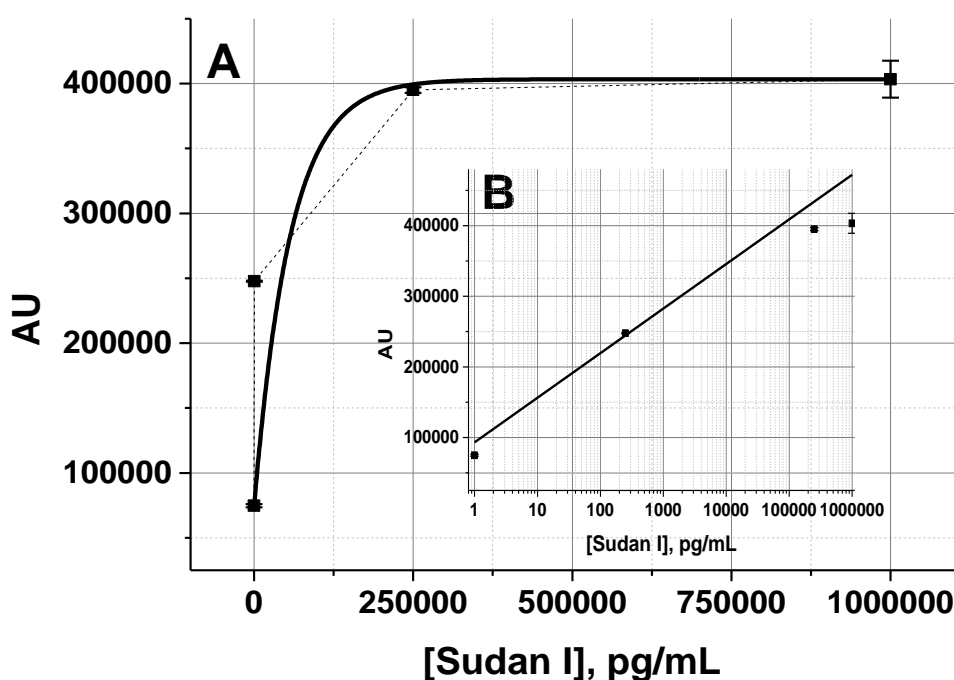
Such a novel and untraditional approach is the way to develop LFIA with a direct calibration for better perception of the obtained data by the naked eye. The effect of steric factors, such as the presence of major competitors (in this case Sudan-ovalbumin conjugated to colloidal gold) and the gradual replacement by the molecules of a low molecular competitor applied in the proposed approach, allow for the adjustment of the interaction with the required parameters. Unlike a traditional competitive scheme, the main difference is the inherent movement of the assay sensitivity to low concentrations of analyte. Visually, the appearance of staining in the case of a low positive result is much better perceived in contrast to the slight decrease of staining intensity in the test zone using a traditional format (Berlina et al., 2016). Moreover, no equipment is needed and this can be used as a cut-off test for the detection of Sudan I by ordinary consumers that are not experts.

### 3.2. Determination of analytical parameters for the proposed LFIA

Various Sudan I solutions were prepared to produce a competitive interaction. The intensity of the color depended directly on the Sudan I concentration (Fig. 3). The  $LOD_v$  (when the test line appeared) was 0.25 ng/mL (which corresponds to 1  $\mu$ g/kg of food taking into account a sample preparation including dilution used in our previous investigation (Berlina et al., 2016)) despite residual staining in the absence of the competitor (Fig. 3A). Analytical characteristics were calculated in accordance with the obtained linear equation (Fig. 3B) and the  $LOD_i$  was considered as 7.6 pg/mL (which corresponds to 0.03  $\mu$ g/kg of food with the same sample pretreatment,  $n=3$ ). The average deviation of detection varied from 0.2 to 3.5 %. The total duration of the assay was 15 minutes.

This study describes the highest sensitivity in lateral flow assay (and immunoassays at all) for Sudan I detection. Compared to the permissible level established by EC 0.5–1 mg/kg of food (Chailapakul et al., 2008) the developed test system is highly sensitive (more than 1,000 times for visual and 33,000 for instrumental detection). Therefore, the developed assay is suitable for screening samples with low levels of contamination or diluted samples containing Sudan I. The application of two conjugates improved the sensitivity of the assay, and showed new prospects in the development of lateral flow assays.





**Fig. 3.** Concentration dependence of the test line coloration in the LFIA for Sudan I: A – exponential calibration curve, B – its linear region.

#### 4. Conclusion

A novel lateral flow immunoassay for Sudan I was developed using two gold nanoparticles conjugates. Interaction of both conjugates at the first stage of assay with followed competition under the influence of steric factors and ultimately binding with anti-species antibody immobilized in the test area are the basis for the new proposed LFIA format with direct calibration of Sudan I. Unlike traditional competitive schemes, the main difference is the inherent movement of the assay sensitivity to low concentrations of analyte. Visually, the difference between negative and positive results is clearer and based on the corresponding absence or presence of the staining in the test zone of the strip. The developed LFIA allows the detection of Sudan I with LOD<sub>v</sub> (when the test line appeared) at 0.25 ng/mL of final solution that corresponds to 1 µg/kg of food before the sample pretreatment. An instrumental limit of detection is 7.6 pg/mL. This level corresponds to 0.03 µg of Sudan I per kg of food.

The application of two conjugates allowed the sensitivity to increase and showed new prospects in the development of immunoassays, especially highly sensitive lateral flow assays. The developed lateral flow assay is suitable for a rapid, non-expensive tool for Sudan I detection even in out-of-laboratory conditions. The proposed approach with the application of two conjugates can be used for further LFIA developments of other food contaminants, including organic hydrophobic compound by the analogy with Sudan I.

#### 5. Acknowledgements

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