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Development of an enzyme-linked immunosorbent assay specific to Sudan red I

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ABSTRACT

To obtain antibodies to develop an enzyme-linked immunosorbent assay (ELISA) for the analysis of Sudan red I, haptens were designed and synthesized via four different strategies: (i) attachment of a spacer at the *para* position of the benzene ring, (ii) attachment of a spacer at the naphthol part, (iii) attachment of a spacer at the hydroxyl group of the Sudan red I molecule, and (iv) use of a fragment of the target molecule. A total of 10 haptens were used to generate immunogens, coating antigens, and polyclonal antibodies. One of the heterologous ELISAs developed exhibited an IC_{50} of 1.6 ng/ml, a limit of detection (LOD) of 0.03 ng/ml, and a dynamic range between 0.1 and 14 ng/ml. The assay had 13% cross-reactivity with Para red and negligible cross-reactivity with other structure-related compounds. This ELISA was much more specific than those published previously. This assay was used to determine Sudan red I residues in tomato sauce and chili powder samples after simple pretreatment. The results were validated by comparison with high-performance liquid chromatography (HPLC). The average recoveries of Sudan red I by ELISA and HPLC were in ranges of 70–97% and 82–114%, respectively, indicating suitability of the developed ELISA for screening of Sudan red I in foods.

Synthetic Sudan red dyes I, II, III, and IV (Fig. 1) are widely used as coloring agents in chemical industries such as oils, fats, plastics, waxes, petrol, shoes, printing inks, shoe and floor polishing, and spirit varnishing [1,2]. Besides the chemical industry, they have been used without authorization and illegally in the food industry to enhance and maintain the appearance of food products such as in chili-, curry-, curcuma-, and palm oil-containing foodstuffs [3–5]. Sudan red I (1-phenylazo-2-naphthol), one of the most frequently used Sudan dyes, is considered to be a genotoxic carcinogen [6] and is classified as a category 3 carcinogen by the International Agency for Research on Cancer [7]. Its presence is prohibited in foodstuffs for any purpose at any level worldwide.

Unfortunately, a variety of foodstuffs contaminated with Sudan dyes (particularly Sudan red I) have been detected recently throughout Europe and Asia [8–11]. Because the illegal use of the dyes has major economic consequences for worldwide food industries as well as an adverse impact on public health, a number of articles concerning the development of extraction and detection methods for Sudan dyes in foodstuffs have been published. Most of the analytical methods applied for Sudan red dye determination in foodstuff samples are based on high-performance liquid chromatography (HPLC)[1] coupled with different detection methods, including mass spectrometry [12–14], ultraviolet–visible (UV–VIS) detection [15], and diode array detection (DAD) [16]. There have been only a few reports on the gas chromatography–mass spectrometry (GC–MS) analysis of these compounds, including a recent report [11] on the determination of Sudan dyes in eggs by silylation prior to GC–MS analysis. Several molecularly imprinting techniques have also been proposed and proven to be promising in the trace analysis of Sudan red dyes [17–19].

Although liquid chromatography–mass spectrometry (LC–MS) is able to separate and quantify four Sudan dyes simultaneously with high sensitivity and good recovery [20,21], complex extraction and cleanup steps need to be taken, especially in the analysis of high-fat samples. Moreover, the scope of the instrumental methods is limited to the quantitative detection in the laboratory

[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; UV–VIS, ultraviolet–visible; DAD, diode array detection; GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; LOD, limit of detection; ELISA, enzyme-linked immunosorbent assay; DCC, *N,N′*-dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horseradish peroxidase; BSA, bovine serum albumin; OVA, ovalbumin; IgG, immunoglobulin G; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PBST, PBS plus 0.05% Tween 20; IC_{50}, half-maximum inhibition concentration; AU, arbitrary units; CV, coefficient of variation.

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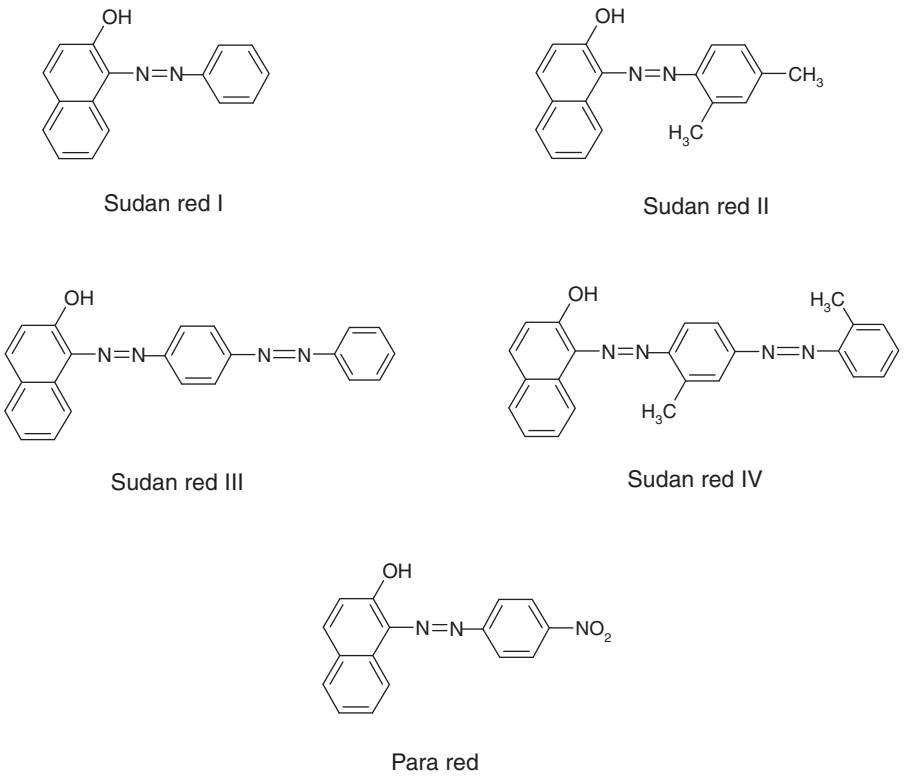

Fig. 1. Chemical structures of Sudan red dyes (I, II, II, and IV) and Para red.

because of the expensive instruments. On the other hand, simple, sensitive, and low-cost immunoassay techniques for Sudan red dyes have been developed recently for screening purpose [22–26]. Because the used haptens were designed following the same strategy to bind different lengths of hydrocarbon linkers to the benzene ring of Sudan red I, all of the developed immunoassays showed high sensitivity to Sudan red I (limit of detection [LOD] of 0.01–0.2 ng/ml) but showed different specificity to the other Sudan red dyes. One of the described enzyme-linked immunosorbent assays (ELISAs) presented very high cross-reactivities with Sudan red III and Para red (Fig. 1) [24].

This study aimed to develop a more specific ELISA for Sudan red I. Thus, a number of new haptens were designed and synthesized following strategies different from those published previously [22–26], including attachment of a spacer at the *para* position of the benzene ring, attachment of a spacer at the naphthol part, attachment of a spacer at the hydroxyl group of the Sudan red I molecule, and use of a fragment of the target molecule. In addition, the influence of different hapten structures on immunoassay sensitivity and specificity is studied. Finally, the determination of Sudan red I in tomato sauce and chili powder samples is presented to discuss the developed ELISA validation protocol, including the sensitivity, recovery, and reproducibility in comparison with HPLC.

Materials and methods

Chemicals and instruments

Sudan red I, *N,N′*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 3,3′,5,5′-tetramethylbenzidine (TMB), horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin (OVA), goat anti-rabbit immunoglobulin G (IgG)–HRP, ethyl bromoacetate, 5-bromovaleric acid ethyl ester, and complete and incomplete Freund's adjuvants were purchased from Sigma–Aldrich (St. Louis, MO, USA). 6-Hydroxy-2-naphthoic acid was obtained from Energy Chemical (Shanghai, China). 4′-Amino-biphenyl-4-carboxylic acid was obtained from Avra Synthesis (India). Tween 20, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 4-aminobenzoic acid, 4-aminophenylacetic acid, 3-(4-aminophenyl)propionic acid, 4-(4-aminophenyl)butyric acid, aniline, 1-amino-2-naphthol, and 2-naphthol were purchased from Beijing Chemical (Beijing, China). Sudan red (II, III, and IV), Sudan red G, Para red, and Sunset yellow used for testing cross-reactivity were generous gifts from Beijing Entry–Exit Inspection and Quarantine Bureau (China).

ELISA was carried out on 96-well polystyrene microplates (Xia Men, China). The result was read spectrophotometrically at a wavelength of 450 nm with a Labsystems Dragon Wellscan MK3 microplate reader (Helsinki, Finland).

Hapten synthesis

Four types of haptens used for immunization and coating antigen and the synthetic routes for haptens **S1**–**S8** are presented in Fig. 2. Haptens **S1**–**S6** were synthesized by the same procedure as that reported by Ju and coworkers [24] using corresponding commercial start materials. Haptens **S7** and **S8** were synthesized following the Williamson ether synthesis procedure [27]. The following subsections describe the synthesis procedure and the identification of haptens.

*4-[(2-Hydroxy naphthalen-1-yl) diazenyl] benzoic acid (hapten **S1**)*

To a stirring solution of 1.37 g of 4-aminobenzoic acid in water (50 ml) cooled in an icewater bath was added dropwise a solution of 12 ml of concentrated hydrochloric acid, followed by adding 0.7 g of sodium nitrite dissolved in 5 ml of water. The mixture was stirred for 10 min, and then 2-naphthol (1.44 g) dissolved in 30 ml of sodium hydroxide solution (10%, w/v) cooled with