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Multi-determination of Para red and Sudan dyes in egg by a broad specific antibody based enzyme linked immunosorbent assay

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ABSTRACT

Para red and Sudan dyes (Sudan 1, 2, 3, 4, and Sudan G) have been proven to show the potential carcinogenic effect to human. This study first reported an indirect competitive immunoassay (ELISA) for simultaneous detection of the six red dyes in egg. Two immunogens of Para red were prepared by coupling the deoxidized Para red to carrier protein by using of diazotization method and glutaraldehyde method, respectively. The obtained polyclonal antibodies showed broad specificity toward Para red and the five Sudan dyes with crossreactivity in a range of 26%–108%. After evaluation of different coating antigen/antibody combinations, a heterologous ELISA was developed to simultaneously determine these red dyes in egg. The limits of detection for these analytes were in a range of 0.2–0.6 ng/g depending on the compound. Intra- and inter-assay recoveries from the standards fortified blank samples were in the range of 77.2%–96.0% with coefficients of variation lower than 10.3%.

1. Introduction

Sudan dyes are a class of synthetic azo dyes that are usually used in industry or printing. However, Sudan 1 (S1) has been proven to cause cancer in animals and human beings (Pinheiro, Touraud, & Thomas, 2004; Stiborova, Martinek, Rydlova, Hodek, & Frei, 2002). Therefore, it was classified as a category 3 carcinogen by International Agency for Research on Cancer (IAGC, 1975). Para red (PR) is also an azo dye used in printing that is chemically similar to S1. The independent scientific experts of UK Food Standards Agency have advised that it would be prudent to assume that PR could be a genotoxic carcinogen (UK Food Standards Agency, 2008). Recently, some researchers have reported that S1, Sudan 2 (S2), Sudan 3 (S3), Sudan 4 (S4) and PR can be reduced to potential carcinogenic aromatic amines by some prevalent species of human intestinal bacteria (Xu, Heinze, Chen, Cerniglia, & Chen, 2007; Xu, Heinze, Paine, Cerniglia, & Chen, 2009). Therefore, these red dyes at any level are not safe for human being and many countries have forbidden the use of them as food colorants (Commission decision of EC, 2003). However, these red dyes have been used as additives in food products by some factories for many years because of their intensive red-orange color, which is a risk to human health.

Therefore, the development of specific and sensitive analytical methods to inspect these red dyes in food is the aim of many researchers. By now, there have been many reports involving HPLC (Ertas, Ozer, & Alasalyar, 2007; Li, Yang, Zhang, & Wu, 2009; Wu, Yang, Zhao, HuangFu, & Shen, 2009), GC/MS (He et al., 2007), or LC-MS/MS (Hou, Li, Cao, Zhang, & Wu, 2010; Mazzotti et al., 2008) method for the determination of these red dyes. Though the methods can qualitatively and quantitatively determine these dyes, they are time-consuming, and the sophisticated extraction procedures and the expensive instruments are required. Compared with those instrumental methods, ELISA is a low cost and sensitive method capable of screening large amount of samples in a single test.

The most common way of development of a multi-analyte ELISA is to produce an antibody showing broad specificity to all target analytes. This conception has been used to determine nitrofurans (Li, Liu, Zhang, Li, & Wang, 2010), fluoroquinolones (Wang et al., 2007) and sulfonamides (Franek, Diblikova, Cernoch, Vass, & Hruska, 2006). There have been several papers reporting ELISA method for the detection of these red dyes, but the antibodies were produced against the derivatives of S1 and the obtained antibodies could only recognize one (Han et al., 2007), two (Xu, Wei et al., 2010; Xu, Zhang et al. 2010), three (Ju, Tang, Fan, & Chen, 2008;

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Wang et al., 2009) or four of the six analytes (Anfossi, Baggiani, Giovannoli, & Giraudi, 2009). Furthermore, the crossreactivity of those antibodies for Sudan red G (SG) was not determined. Therefore, there has been no paper involving ELISA method for the simultaneous determination of PR and the five Sudan dyes. The chemical structures of six common red dyes are shown in Fig. 1.

Then, the development of an ELISA for multi-determination of the six red dyes is highly desirable. From observation of their chemical structures (Fig. 1), it was found that PR is a crude hapten and the antibody against PR maybe recognizes all the compounds. Therefore, PR was selected to prepare the generic hapten for production of the class generic antibody and an indirect competitive ELISA method was then developed to simultaneously determine the six red dyes in egg yolk. Furthermore, the ELISA results were confirmed with an HPLC method.

2. Experimental

2.1. Materials

Para red (PR), Sudan 1 (S1), Sudan 2 (S2), Sudan 3 (S3), Sudan 4 (S4), bovine serum albumin (BSA), Ovalbumin (OA) and Freund's adjuvants were purchased from Sigma–Aldrich (Shanghai, China). Sudan red G (SG) was from Shanghai JingChun reagent company (Shanghai, China). The substrate 3, 3′, 5, 5′- tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). Other chemical reagents were all analytical grade or better from Beijing chemical company (Beijing, China). Microtiter plates were supplied by Greiner Bio-one (Germany).

Stock solution of each compound and the mixed stock solution (1 mg/mL) were prepared in acetonitrile under ultrasonic assistant and stored at −20 °C in the dark to be stable for 3 months. Working solutions with series concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/mL) were diluted from stock solutions with PBS. All the working solutions were stored at 4 °C to be stable for at least 4 weeks. PBS (0.01 mol/L, pH 7.2) was prepared by dissolving 0.2 g KH_2PO_4, 0.2 g KCl, 1.15 g Na_2HPO4, and 8.0 g NaCl in 1000 mL demineralized water. Washing buffer (PBST) was PBS buffer containing 0.05% Tween. Coating buffer was carbonate buffer (0.1M, pH 9.6). Substrate buffer was 0.1 M citrate (pH 5.5). The substrate system was prepared by adding 200 μL TMB in DMSO (1%, w/v) and 64 μL 0.75% (w/v) H_2O_2 into 20 mL substrate buffer.

2.2. Synthesis of PR conjugates

The nitryl group in the molecule of PR was first deoxidized to amidogen as an active chemical group, and then the hapten was coupled to carrier protein by using of diazotization method (Method 1) and glutaraldehyde method (Method 2), respectively. The synthesis process is shown in Fig. 2. Eighty milligrams of zinc powder and 10 mL of 1 M HCl were added into an acetonitrile/methanol solution containing 60 mg of PR. Then the mixture was stirred at 80 °C until the solution turned wine red from bright red. The solution (Solution A) was cooled down to 4 °C for preparation of PR conjugates.

Method 1:1 mL of 0.5 M $NaNO_2$ was added into solution A dropwise, then the pH value of the mixture was adjusted to 1.0 with 1 M HCl, and the mixture was stirred for 2 h at 4 °C. Then, the excessive $NaNO_2$ was removed using ammonium sulfamate and the pH value of the mixture was readjusted to 7.5 with 2 M NaOH. Finally, the mixture was added into 10 mL of PBS solution containing BSA (160 mg) or OA (80 mg) dropwise, and the mixture was allowed to react for 12 h at 4 °C to prepare the immunogen (PR-N-BSA) or coating antigen (PR-N-OA). The obtained conjugates were further purified by passing through a Sephadex G25 cartridge and the eluate with wine red color was collected to be dialyzed against PBS for 3 days at 4 °C.

Method 2: Solution A was dropped slowly into 10 mL of PBS solution containing BSA (160 mg) or OA (80 mg), then 100 μL of 25% glutaraldehyde was added. The solution was mixed round for 4 h at room temperature to prepare the immunogen (PR-C-BSA) or coating antigen (PR-C-OA). The purification and dialysis for the two conjugates were same as described in Method 1.

PR, the two carriers, and the conjugates were scanned respectively on a UV spectrophotometer to identify the conjugation, and the coupling ratios of hapten to protein (hapten density) were determined by using of a previous method (Sashidhar, Capoor, & Ramana, 1994).

2.3. Production of the antibody

Six New Zealand white rabbits were feed at Animal Experiment Center of College of Animal Science and Technology, Agricultural University of Hebei. The animal experiments were performed according to the Regulation Guideline for Experimental Animals issued by the Ministry of Science and Technology of China. The two immunogens were all used to produce the polyclonal antibodies. The six rabbits numbered R1 to R6 were divided into two groups randomly. The rabbits of group 1 (R1 to R3) were immunized with PR-N-BSA and that of group 2 (R4 to R6) were immunized with PR-C-BSA. The rabbits were immunized with the emulsion of PR-BSA (0.5 mg protein per animal) in Freund's complete adjuvant on the dorsal region subcutaneously at first time, then were boosted with PR-BSA in Freund's incomplete adjuvant at a 3 week-interval. After 6 boosters, the rabbits were exsanguinated and the serum was collected. Finally, the IgG was isolated using the saturated ammonium sulfate precipitation method for development of the indirect competitive ELISA.

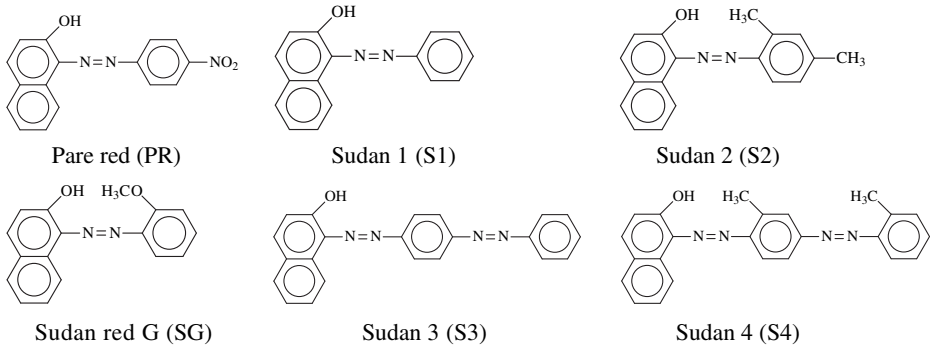

Fig. 1. Chemical structures of Para red and Sudan dyes.