Talanta 99 (2012) 125–131

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for detection of Sunset Yellow FCF in food samples

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ARTICLE INFO

Article history:
Received 28 December 2011
Received in revised form
11 May 2012
Accepted 16 May 2012
Available online 26 May 2012

Keywords:
Sunset Yellow FCF
Azo dye
Food additives
Antibody
ELISA

ABSTRACT

Sunset Yellow FCF is widely used as food additives to make foods more attractive. Due to its abuse and potential risk to human health, Sunset Yellow FCF is precisely limited to use in food. To monitor the illegal use of Sunset Yellow FCF, a polyclonal antibody-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) with satisfactory sensitivity and specificity was developed. A carboxyl group was introduced to Sunset Yellow FCF, then the modified hapten was coupled with carrier proteins to synthesize the immunogen and coating antigen. The IC_{50} value of 0.52 ng mL^{-1} and detection limit of 25 pg mL^{-1} (in buffer) were achieved by this method. The cross-reactivity values of the antibodies with six structurally related colorants were less than 1.5%, indicating the high selectivity. Three kinds of food samples (beverage, dried beancurd, braised pork) and serum were chosen to evaluate the application of the immunoassay in real systems. The limits of detection (LOD) in the above three food samples were 0.12, 0.04 and 1.11, respectively (mean+3SD). The recovery (94%–106%), intra-assay ($<5\%$) and inter-assay ($<12\%$) coefficients of variation in foods and serum samples were also acceptable. The results suggest that this ELISA method is a specific, sensitive and simple method for the determination of Sunset Yellow FCF additives.

1. Introduction

Color additives are used in a wide variety of foods such as dairy products, beverages, cereals, snack foods and ice creams. Their bright and vivid colors make food more attractive and appetitive. Colorants include natural and synthetic color additives. Synthetic color additives are organic pigments using artificial synthesis methods and mainly made by coal tar from aniline dyes as raw material. Due to their low cost, gorgeous color and strong tinting ability, synthetic pigments are widely used in the food processing industry.

Azo dyes are the largest group of synthetic colorants (60–70%) and can be used to color a large number of different substrates, such as synthetic and natural textile fibers, plastics, leather, paper, mineral oils, waxes, and even foodstuffs and cosmetics (with selected types) [1]. Sunset Yellow FCF ($C_{16}H_{10}N_2Na_2O_7S_2$, Mw=452.38, Fig. 1), one of the azo dyes, is also called evening yellow and edible yellow No. 3. It is one of the synthetic pigments that could be added in foods (GB2760-2011), which is also widely used in pharmaceuticals and cosmetics [2]. However, Sunset Yellow FCF is reported to be attributable to hepatocellular damage and renal failure [3]. It also causes reproductive toxicity in mice [4] and has potential immunotoxicity [5]. For these reasons, in China and most countries, Sunset Yellow FCF is strictly used as additives in certain food products. However, it is still illegally utilized in foods by some merchants, particularly in cooked meat products and broad bean paste. Therefore, for the assurance of consumer health, it is crucial to control Sunset Yellow FCF dyes in food products and necessary to develop a simple, economic and rapid analytical method.

Presently, the most common analytical techniques that frequently used for the determination of Sunset Yellow FCF include high performance liquid chromatography (HPLC) [6–8], thin-layer chromatography (TLC) [9], capillary electrophoresis [10], nAu–CPE (gold nanoparticles carbon paste electrode) [11], microchip electrophoresis [12], photoacoustic spectroscopy [13], microemulsion electrokinetic chromatography [14], double-beam photometer [15], spectrophotometric method [16], diffuse reflectance spectroscopy [17] and ratio derivative voltammetry [18]. Most of these methods are highly cost and time-consuming because of their complicated, expensive instruments and extensive sample preparation. So there is an urgent need for a rapid, high capacity and sensitive screening method for additives.

Enzyme-linked immunosorbent assay (ELISA) methods have been used for the detection of various additives in real systems because of their rapidity, mobility, high sensitivity and low detection limit [19–23]. In this study, we developed an indirect

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http://dx.doi.org/10.1016/j.talanta.2012.05.029

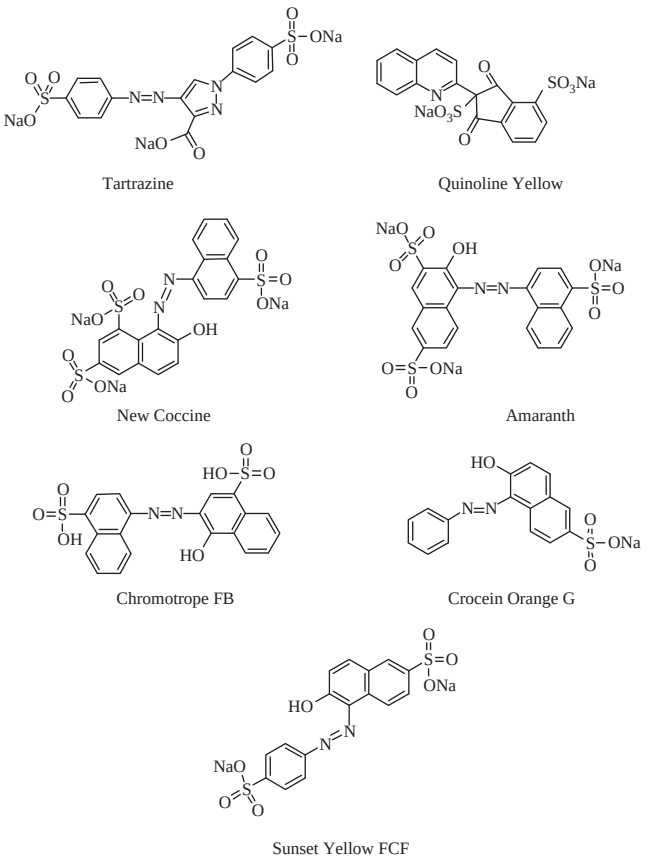

Fig. 1. Sunset Yellow FCF and the related azo dyes evaluated in this research.

competitive ELISA to detect the residue of Sunset Yellow FCF based on polyclonal antibodies preparation. To our knowledge, there is no research about the production of antibodies against Sunset Yellow FCF or development of ELISA for its determination in real samples (beverage, dried beancurd, braised pork and serum). The antibodies obtained were characterized by the ELISA method and showed excellent specificity. The sensitivity with the 50% inhibition concentration value (IC_{50}) is 0.52 ng mL^{-1} in buffer. The results suggest that this ELISA is a specific, accurate and sensitive method, which is suitable to detect residues of Sunset Yellow FCF.

2. Materials and methods

2.1. Chemicals, apparatus, buffers and solutions

2.1.1. Chemicals and apparatus

Sunset Yellow FCF, Tartrazine, Quinoline Yellow, New Coccine, Amaranth, Chromotrope FB, bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (cFA) and Freund's incomplete adjuvant (iFA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) and N-hydroxysuccinimide (NHS) were from Academy of Military Medical Sciences (Military Medical Institute, Beijing, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Shanghai Sangon Biological Engineering Technology (Sangon Biotech Co., Ltd., Shanghai, China). Dimethylformamide (DMF) and tributylamine of chemical grade were supplied by Guangfu Fine Chemical Research Institute (Guangfu Chemical Co., Tianjin, China). Isobutyl chloroformate was from J&K Chemical (J&K Scientific Ltd., Guangdong, China). EDC (1-Ethyl-3-[3–dimethylaminopropyl]-carbodiimide hydrochloride), Crocein Orange G and 5-sulfoanthranilic acid, sodium 2-naphthol-6-sulfonate hydrate were from TCI (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan).

ELISA was measured by enzyme immunoassay microplate reader Model 680 from Bio-Rad Laboratories Headquarters (Bio-Rad, Hercules, USA). Polystyrene microtiter plates (96-well) were from Jet Bio-filtration Products, Co., Ltd. (Jet Biofil, Beijing, China). UV–vis spectra of conjugates were obtained by SHIMADZU UV-1800 (SHIMADZU, Kyoto, Japan). ^{1}H (400 MHz) and ^{13}C (400 MHz) NMR spectra were recorded on a Bruker AVANCE-400 NMR spectrometer (Bruker BioSpin, Zurich, Switzerland) in DMSO.

2.1.2. Buffers and solutions

(1) Phosphate-buffered saline (PBS, pH 7.4) was composed of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4 and 2.7 mM KCl; (2) washing buffer (PBST): a PBS solution containing 0.05% (v/v) of Tween 20; (3) coating buffer: 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6); (4) blocking buffer: PBS mixed with 1% of OVA and 0.05% (v/v) Tween 20; (5) substrate buffer (TMB+H_2O_2): 400 mL of 0.6% TMB-DMSO mixed with 100 mL of 1% H_2O_2 in citrate-acetate buffer (pH 5.5); (6) enzymatic stopping solution: 2.0 M hydrochloric acid.

2.2. Synthesis of Sunset Yellow FCF hapten

In order to synthesize the immunogen and coating antigen, the Sunset Yellow FCF was modified and served as the hapten (Fig. 2). A mixture of 5-sulfoanthranilic acid (0.001 mol) and $NaNO_2$ (0.0011 mol) were added to 3.3 mL of 12.0 M HCl. The reaction was kept at 0–5 °C for 1 h. The product obtained was then mixed with 0.001 mol sodium 2-naphthol-6-sulfonate hydrate in 200 μL of DMF. The pH of the mixture was adjusted to 10 with 10 M NaOH solution. The final reaction was kept at 4 °C for 5 h. The

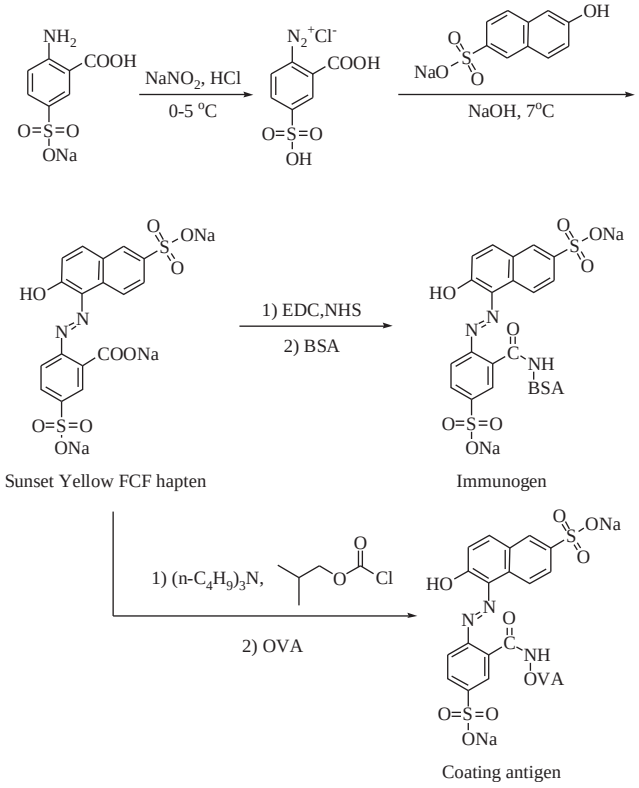

Fig. 2. Synthesis of Sunset Yellow FCF hapten, immunogen and coating antigen.

product was filtered to collect precipitate, which was then used as the Sunset Yellow FCF hapten.

2.3. Preparation of immunogen and coating antigen

As shown in Fig. 2, the hapten was attached with BSA by EDC method and used as the immunogen. Four milliliter of PBS containing 0.06 mol hapten was mixed with 3 mL of PBS containing 0.6 mmol EDC and 0.3 mmol NHS, and reacted for 2 h at room temperature. Then the mixture was added dropwise to 6 mL of PBS containing 0.0017 mmol BSA. The conjugation mixture was then stirred at 4 °C overnight.

The coating antigen was synthesized by coupling the hapten with OVA (Fig. 2). Firstly, the hapten (0.05 mmol) was dissolved in 3 mL of DMF and mixed with 1 mL of PBS solution. Then tributylamine (13 μL) was added to the above solution and stirred at 4 °C. After 0.5 h, isobutyl chloroformate (9.1 μL) was also added to the solution and stirred at 4 °C for 1 h. The blended solution was added dropwise to 12 mL of PBS buffer containing 0.0014 mmol OVA. The conjugation mixture was then stirred at 4 °C overnight. Finally, the solution containing immunogen or coating antigen were lyophilized and stored in the refrigerator (−20 °C) until further use.

2.4. Production of polyclonal antibodies

Polyclonal antibodies were obtained from two male New Zealand white rabbits weighing 2–2.5 kg. Multiple-site injection method was used for preparing polyclonal antibodies against Sunset Yellow FCF. Before the first immunization, each rabbit was exsanguinated from ear to take 1 mL of blood as the negative group. For the initial immunization, each rabbit was subcutaneously injected with the mixture of immunogen (0.5 mg), normal saline (0.5 mL) and FA (0.5 mL) at back. Subsequently, booster injections were performed every two weeks to inject the emulsion made of 0.5 mL of normal saline and 0.5 mL iFA which contained 0.25 mg immunogen. Seven days after the last injection, all rabbits were exsanguinated by heart puncture under general anesthetic and euthanized by lethal injection before recovery. The blood was stored at room temperature for 2 h and then overnight at 4 °C. The next day, the antiserum was separated from blood cells and particulate material by centrifuging at 13,000 rpm for 15 min. The supernatant in the centrifuge tube that served as antiserum was collected and stored in the refrigerator (−20 °C) until use.

2.5. The optimization of the immunoassay

The checkerboard procedure was used to optimize the concentrations of coating antigen and the antiserum. Each polystyrene microtiter well was coated with 100 μL of coating antigen in different concentrations ($5-0.001\ \mu g\ mL^{-1}$) and incubated at 36 °C for 2 h, then the excess coating antigen was removed. The plates were washed three times with PBST, blocked with 250 μL/well of blocking buffer and incubated at 4 °C overnight. Plates were washed three times again, then the antisera at different dilutions were added (dilution ratio of 1:2500-1:32,0000, 100 μL/well) and incubated at 36 °C for 0.5 h. After three washing, goat anti-rabbit IgG-HRP (1:2000, 100 μL) was added into each well and incubated for 0.5 h at 36 °C. Finally, three washing was followed and substrate solution was added (100 μL/well). After incubation for 10 min at 36 °C, 2 M HCl (100 μL/well) was used to stop the enzymatic reaction, and the absorbance at 450 nm was recorded. Concentration of antibody and coating antigen with the absorbance value correspondent with their dilution ratio was selected as the optimal condition for the further experiments.

2.6. Indirect competitive ELISA procedure

An indirect competitive ELISA format was adopted for analyzing Sunset Yellow FCF. The concentrations of coating antigen and antibody were selected by the experiments above. The ELISA procedures were also basically same except that after removing blocking buffer, 50 μL of competitors in diluted antiserum or buffer were added to each well successively. The competitors were Sunset Yellow FCF reference substances, and their concentration was 0.0001 to 10,000 $\mu g\ mL^{-1}$. The absorbance at 450 nm was measured using an automatic microplate reader. The inhibition ratio was calculated as follows:

$$\%\text{inhibition} = \%B/B_0$$

where B is the absorbance of the well containing competitor and B_0 is the absorbance of the well without competitor. The sigmoid curve which was regarded as inhibition curve was fitted to a logistic equation. The IC_{50} values which represented the concentration of competitor that leads to a 50% decrease of the maximum signal were determined.

2.7. Cross-reactivity study

The specificity of the polyclonal antibody was investigated by testing cross-reactivity (CR) with six functionally and structurally similar analogs (Tartrazine, Quinoline Yellow, New Coccine, Amaranth, Chromotrope B, Crocein Orange G, Fig. 1). The IC_{50} values of these compounds was compared to that of Sunset Yellow FCF to calculate the cross-reactivity (CR%) values according to the equation:

$$CR\% = IC_{50}\text{ value of Sunset Yellow FCF}/IC_{50}\text{ values of other pigments} \times 100\%.$$

2.8. Accuracy and precision evaluation

The accuracy and precision of the immunoassay developed were also evaluated. According to the LOD value of the standard calibration curve, 0.01, 0.5 and 10 $ng\ mL^{-1}$ Sunset Yellow FCF solutions were chosen to test the accuracy and precision. The experiments performed for analysis were repeated six times a day and continued for three days.

2.9. Detection of Sunset Yellow FCF in real samples

Beverage, dried beancurd and braised pork without contamination of Sunset Yellow FCF collected from local supermarkets and blank serum were used to evaluate the application of the immunoassay. For the preparation of beverage sample for analysis, beverage was boiled to remove the CO_2 and then diluted. For dried beancurd and braised pork, 5 g samples were triturated and dissolved in 20 mL of 70% ethanol. The mixture was shaken for 10 min and then placed in boiling water bath for 3 h to remove ethanol. After cooling to room temperature, the mixture was centrifuged and the supernatant was diluted appropriately. As to the serum samples, proper dilution was performed and the gotten serum sample was stored together with the other prepared samples at 4 °C before use.

3. Results and discussion

3.1. Sunset Yellow FCF hapten synthesis

The synthesis of haptens was the key step in the whole procedure of immunoassay. Sunset Yellow FCF itself cannot

trigger immune response in animals because of its low molecular mass, and it must be connected with appropriate carrier protein to be immunogenic. The necessary condition in the connection of haptens with carrier protein is the presence of special functional groups like hydroxyl, carboxyl and amino group. As shown in Fig. 1, most of the studied colorants share the naphthalenesulfonate and hydroxyl group in similar site, so these two functional groups are important for hapten synthesis. A diazotization reaction (Fig. 2) was designed to synthesize the Sunset Yellow FCF hapten. The whole characteristic group was retained, and a carboxyl group was introduced which was used to couple with the carrier protein. The structure of hapten was testified by NMR spectra. ^{1}H NMR (400 MHz, DMSO): δ=8.374 (s, 1H, aromatic), 8.35 (s, 1H, aromatic), 8.135 (d, J=8 Hz, 1H, aromatic), 7.769–7.930 (m, 4H, aromatic), 6.638 (d, J=9.2 Hz, 1H, aromatic), 2.508 (s, 1H, hydroxyl). The NMR results (Fig. 3) demonstrated that the hapten was synthesized successfully.

3.2. Synthesis of immunogen and coating antigen

The Sunset Yellow FCF hapten bearing a carboxylic group was covalently coupled with a carrier protein (BSA or OVA). The conjugates of Sunset Yellow FCF–BSA and Sunset Yellow FCF–OVA were used as immunogen and coating antigen, respectively. The identification of conjugates was carried out by UV spectrophotometer (SHIMADZU UV-1800) and the UV spectra were shown in Fig. 4. It was observed that the characteristic peak of BSA located at 280 nm, and that of the hapten was at 310 and 500 nm. After removing the extra free Sunset Yellow FCF hapten, the UV absorbance spectrum of Sunset Yellow FCF hapten–BSA still showed the overlap of BSA and the hapten at 280, 310 and 490 nm, which demonstrated the successful coupling the hapten to BSA. The UV spectra of coating antigen were similar to those in Fig. 4 and therefore not showed herein.

3.3. Characterization of polyclonal antibodies

In order to characterize the antibody produced in this research, the sensitivity, cross-reactivity and stability were determined according to the indirect competitive ELISA described above. Before that, the optimum conditions such as the concentration of coating antigen and dilution of antiserum should be selected to improve the sensitivity of the method. Therefore, checkerboard titration method was used to optimize the above assay conditions. The optimal concentration of coating antigen was found to be 1 μg mL^{-1} and the best dilution of antiserum was 1:40,000.

The sensitivity of the immunoassay was detected under optimal assay conditions and expressed by IC_{50} value. The representative inhibition curve for Sunset Yellow FCF was described in Fig. 5, which showed that the IC_{50} value was 0.52 ng mL^{-1} and the limit of detection (LOD, IC_{20} value) was 25 pg mL^{-1}, indicating excellent sensitivity. The standard curve was showed in Fig. 6, which exhibited good linearity (R^2=0.9997, n=4) with Sunset Yellow FCF concentration from 0.01–10 ng mL^{-1}.

Specificity of antibody was defined as the selectivity targeted antibody with other analytes. The assay specificity was evaluated

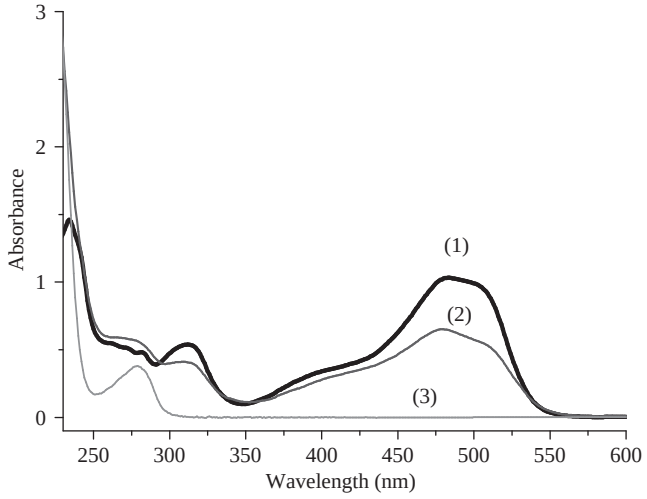

Fig. 4. UV spectrum of: (1) Sunset Yellow FCF hapten, (2) immunogen (Sunset Yellow FCF–BSA), (3) BSA.

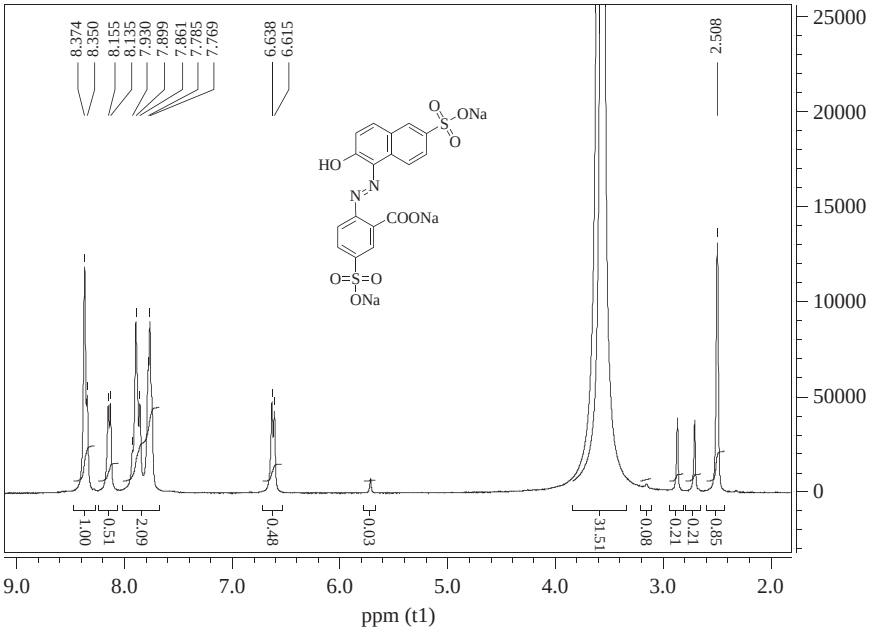

Fig. 3. ^{1}H NMR [400 MHz, DMSO] spectrogram of Sunset Yellow FCF hapten.

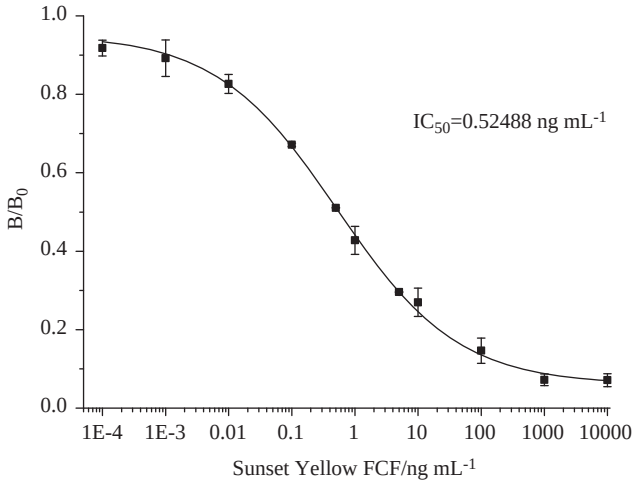

Fig. 5. Inhibition curve of Sunset Yellow FCF. Data are expressed as the mean ± SD (n=4).

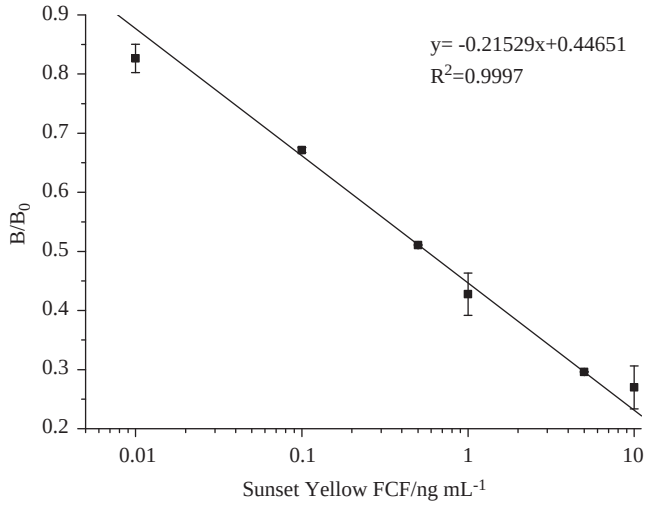

Fig. 6. Linear standard curve of indirect competitive ELISA. Each data point represents the mean ± SD of 4 determinations.

Table 1
Cross-reactivity evaluation of related pigments.

Competitor	IC_{50} (ng mL^{-1})	Cross-reactivity (%)
Sunset Yellow FCF	0.52	100.0
Tartrazine	878.22	0.06
Quinoline Yellow	896.81	0.06
New Coccine	35.63	1.47
Amaranth	83.88	0.62
Chromotrope B	250.63	0.21
Crocein Orange G	944.05	0.05

by cross-reactivity (CR) of the antibody with six structurally related compounds of Sunset Yellow FCF, including Tartrazine, Quinoline Yellow, New Coccine, Amaranth, Chromotrope B and Crocein Orange G. As shown in Table 1, all of the colorants displayed low cross-reactivity (< 1.5%), indicating a high selectivity of the obtained antibody. Besides, the high specificity also demonstrated that the difference in naphthalene rings and substituent groups was an important structure factor to influence the antibody specificity.

Series of Sunset Yellow FCF standard solutions (0.01, 0.5 and 10 ng mL^{-1}) were measured to determine the accuracy and precision of the immunoassay. Intra-assay variation was obtained based on the six times' measurements within one day and inter-assay variation was calculated by the results of three days. As shown in Table 2, intra-assay variation was 9.7%–15.6%, and inter-assay variation was less than 14%. These results certified that the developed ELISA was stable and credible for determining the concentration of Sunset Yellow FCF.

Table 2
Accuracy and precision evaluation of Sunset Yellow FCF detection in buffer system.

Sunset Yellow FCF solution (ng mL^{-1})	Measured level (ng mL^{-1})	Intra-assay variation (%)	Inter-assay variation (%)
0.01	0.011	13.2	13.6
	0.012	12.1	
	0.009	15.6	
0.5	0.497	9.7	13.4
	0.468	10.7	
	0.522	10.7	
10.0	9.845	13.7	9.8
	9.845	11.9	
	10.918	11.4	

Table 3
Dilution factors of the food samples tested in this study.

Matrix	Dilution factors[a]	IC_{50} (ng mL^{-1})
Buffer	–	0.52
Beverage	1:50	0.90
	1:100	**0.19**
	1:200	4.22
Beancurd	**1:50**	**0.28**
Braised pork	1:100	1.70
	1:200	1.46
	1:100	2.59
	1:250	**1.99**
	1:500	2.05

[a] The matrices were further diluted after pretreatment to obtain the most similar IC_{50} values to that of buffer system. The dilution factors were the volume ratio of the matrix and buffer.

3.4. Performance of ELISA in real samples

Three types of matrix systems, including beverage, dried beancurd and braised pork were estimated in this research to investigate the matrix effect on Sunset Yellow FCF analysis. The matrix interference may cause false positives, which is considered as one of the common challenges of immunoassay. However, these matrix interferences could be reduced in many ways, such as dilution of sample extract and sample cleanup procedures like solid-phase extraction or matrix-matched standards. Among the above methods, dilution was a commonly used way to reduce the interferences because of its simplicity and convenience [24].

In order to obtain the most similar IC_{50} values to that of buffer system, the matrices were further diluted after preliminary treatment as indicated above. The dilution factors were showed in Table 3, beverage (1:100), dried beancurd (1:50) and braised pork (1:250) were selected for the detection of sensitivity and recovery.

Based on the determination of 20 different blank samples, the LODs in beverage, dried beancurd and braised pork were 0.12, 0.04 and 1.11 ng mL^{-1}, respectively. Compared to the LOD results in buffer (0.025 ng mL^{-1}), it was obvious that the food samples showed different matrix effect on the detection of Sunset Yellow FCF and among them, braised pork exhibited the greatest effect.

Table 4
Comparison of various methods for Sunset Yellow FCF determination.

Methods	Instruments	Sensitivity	Food samples	Reference
Proposed method	ELISA microplate reader	LOD = 0.025 ppb	Beverage dried beancurd, braised pork	Proposed method
HPLC	Varian ProStar Chromatograph	LOD = 143 ppb	–	[6]
HPLC	Shimadzu LC-10 system, Diode array detector	LOD = 27 ppb	Soft drinks, candies	[7]
HPLC	Agilent 1100 HPLC system	LOD = 50 ppb LOQ = 150 ppb	Solid juice/jelly powder soft drinks	[8]
HP-TLC	Flatbed HP ScanJet 3970, Macherey-Nagel TLC Scanner software	LOD = 5.21 ng/spot LOQ = 10.21 ng/spot	Dried concentrated juices	[9]
Capillary zone electrophoresis	CE fused-silica capillary column, Beckman capillary electrophoresis system	LOD = 1.7 ppm LOQ = 5.5 ppm	Melon beverage orange/ pineapple jelly	[10]
nAu–CPE	Model PGSTAT35 Autolab Potentiostat/Galvanostat, Three electrode system	LOD = 3×10^{-8} mol L^{-1}	Soft drinks	[11]
Microchip electrophoresis	CE microchip EC detector	LOD = 3.6 μM	Soft drink, spiked colored film-coated candy	[12]
Photoacoustic spectroscopy	Monochromator, chopper, photoacoustic cell	LOD = 28 ppb	Gelatin/juice powder	[13]
Microemulsion electrokinetic chromatography	MDQ capillary Electrophoresis system	LOD = 0.78 ppm	Soft drink, popsicle	[14]
Spectrophotometric method	Double beam spectrophotometer	Linear range: 2–40 ppm	Chocolates, wool yarn, soft drink	[15]
Spectrophotometric method	UV–vis spectrophotometer ion selective electrode	–	Beverages	[16]
Diffuse reflectance spectroscopy	Double beam spectrophotometer, spectrofluorimeter	Linear range: 5–40 ppm	Chocolates, wool yarn, soft drink	[17]
Ratio derivative voltammetry	Electroanalyser three-electrode cell	Determination range: 30–160 ppm	Orange juice, fruit juice, merida orangeade	[18]

Table 5
Results of recovery and coefficient of variation for Sunset Yellow FCF determination from real samples by ELISA.

Samples	Inter-assay						Intra-assay				
	Level ($ng\ mL^{-1}$)	n	Negative ($pg\ mL^{-1}$)	Measured ($ng\ mL^{-1}$)	Recovery (%)	CV (%)	n	Negative ($pg\ mL^{-1}$)	Measured ($ng\ mL^{-1}$)	Recovery (%)	CV (%)
Beverage	1	5	3.49 ± 0.40	1.02 ± 0.05	102	4.90	15	3.13 ± 0.55	1.06 ± 0.05	106	4.51
	5	5	3.31 ± 0.23	5.05 ± 0.02	101	0.40	15	2.67 ± 0.58	5.02 ± 0.03	100	0.60
Beancurd	0.05	5	24.97 ± 3.84	0.07 ± 0.001	100	2.00	15	21.41 ± 5.36	0.07 ± 0.002	103	3.79
	0.5	5	27.51 ± 3.99	0.51 ± 0.02	96	4.17	15	20.16 ± 6.62	0.51 ± 0.03	98	5.49
Braised pork	2	5	666.72 ± 102.32	2.63 ± 0.01	98	0.51	15	626.61 ± 117.22	2.67 ± 0.07	102	3.54
	5	5	399.33 ± 37.68	5.20 ± 0.10	96	2.08	15	551.46 ± 108.87	5.61 ± 0.23	103	4.49
Serum	0.05	5	3.28 ± 0.57	0.05 ± 0.002	100	4.98	15	3.11 ± 0.78	0.05 ± 0.006	100	11.90
	0.5	5	3.32 ± 0.60	0.47 ± 0.01	94	1.91	15	3.05 ± 0.67	0.48 ± 0.04	96	8.58

However, in China, the acceptable maximal addition of Sunset Yellow FCF in various foods was 0.025–0.6 g kg^{-1} (GB2760-2011) and the LOD value was 0.28 mg kg^{-1} by the standard HPLC analysis method (GB/T 5009.35-2003). Apparently, the gotten results of this study were all well acceptable according to the standard level whether it was applied in buffer or food samples. The comparison of the proposed method with other methods which were already published for the determination of Sunset Yellow FCF was listed in Table 4. It showed that the proposed method exhibited better LODs in food samples. Additionally, the ELISA method needed lower cost and simpler operation, which displayed better performance than other methods.

To study the application of the developed ELISA method in real systems, the above three food samples and serum were fortified at different levels of Sunset Yellow FCF for the recovery, intra-assay and inter-assay variation determination. As shown in Table 5, the recoveries of Sunset Yellow FCF ranged from 94%–106%, the intra-assay variation ($<5\%$) and inter-assay variation ($<12\%$) were also acceptable. It was indicated that the developed ELISA method was especially suitable for the analysis of Sunset Yellow FCF in food samples and even in the biological samples with minute amount of it.

4. Conclusions

In this study, we successfully synthesized the Sunset Yellow FCF hapten and its immunogen and coating antigen. The antibody against Sunset Yellow FCF was prepared and the developed indirect ELISA for the detection of Sunset Yellow FCF showed a highly sensitive and specific with IC_{50} value of 0.52 ng mL^{-1} and low levels of cross-reactivity ($<1.5\%$). The matrix effect of beverage, dried beancurd and braised pork on the Sunset Yellow FCF immunoassay was also studied and the LOD values in above three food samples were 0.12, 0.04 and 1.11 ng mL^{-1}, respectively. Finally, the performance of the developed ELISA method in foods and serum was studied, the recovery rate of real tissues was 94% to 106%. In conclusion, a new feasible ELISA method with high sensitivity, simplicity and rapidity for the detection of Sunset Yellow FCF was established.

Acknowledgment

We would like to thank the financial support of National Natural Science Foundation (Nos. 81173017, 31101277), the

National High-Tech Research and Development Program of China (863 Program, Nos. 2010AA10Z402, 2007AA06A407), Tianjin Science and Technology Program (No. 09ZCKFSH07500), the Scientists-Company Cooperation Project of the Ministry of Science and Technology of China (SQ2009GJA0002591) and the Fundamental Research Funds for the Central Universities (Nos. 65011751, 65011121).

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