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Establishment of an immunoaffinity chromatography for simultaneously selective extraction of Sudan I, II, III and IV from food samples

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ABSTRACT

The establishment of an immunoaffinity chromatography (IAC) for simultaneously selective extraction of four illegal colorants Sudan dyes (Sudan I, II, II and IV) from food samples was described. The IAC column was constructed by covalently coupling monoclonal antibody (mAb) against Sudan I to CNBr-activated Sepharose 4B and packed into a common solid phase extraction (SPE) cartridge. It was observed that IAC column was able to separately capture Sudan I, II, III and IV with maximum capacity of 295, 156, 184 and 173 ng, respectively. The extraction conditions including loading, washing and eluting solutions were carefully optimized. Under optimal conditions, the extraction recoveries of the IAC column for Sudan I–IV at two different spiked concentrations were within 95.3–106.9%. After 50 times repeated usage, 64% of the maximum capacity was still remained. Six food samples randomly collected from local supermarket without spiking Sudan dyes were extracted with IAC column and detected by high performance liquid chromatography (HPLC). It was found that there was no detectable Sudan II, III and IV in all six food samples, but Sudan I with the content of 2.7–134.5 ng g^{-1} was detected in three food samples. To further verify the extraction efficiency, other three negative samples were spiked with Sudan I–IV at the concentrations of 20 ng g^{-1} and 50 ng g^{-1}, which were then extracted with IAC column. The extraction recoveries and relative standard deviation (RSD) were 68.6–96.0% and 4.8–15.2%, respectively, demonstrating the feasibility of the prepared IAC column for Sudan dyes extraction.

1. Introduction

Sudan dyes including Sudan I, II, III and IV (Fig. 1) belong to the family of industrial azo dyes that are traditionally used in waxes, inks, plastics, oils and polishes [1]. Recently, these dyes have been found in food products imported by European and several other countries [2]. Sudan dyes are added to food products such as chilli powder and sauce because the red hue mimics the color of the natural products. Sudan dyes are recognized as potential carcinogens [1]. Consequently, adulteration of any food product by Sudan dyes constitutes a risk to public health. The Food and Drug Administration (FDA) and European Union (EU) classify Sudan dyes as illegal food-additives because of the associated health risks [3]. International Agency for Research on Cancer (IARC, 1975) has classified Sudan dyes as category 3 carcinogens to humans and due to this fact, any national and international food regulation act does not permit the use of these colorants as food-additives.

The standard analysis method for the determination of Sudan dyes is based on liquid chromatography approved by the European Union [4]. Other liquid chromatographic methods associated with different detectors such as ultra-violet (UV) [2], atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) [5,6], electrospray ionization-mass spectrometry (ESI-MS) [7], diode array detector (DAD) [8], diode array detector-electrospray ionization-mass spectrometry (DAD-ESI-MS) [9], electrospray ionization-tandem mass spectrometry (ESI-MS/MS) [10], capillary liquid chromatography (LC)/quadrupole time-of-flight mass spectrometry (Q-TOF-MS) [11], chemiluminescence (CL) [12], electrochemical detection [13], etc. for the analysis of Sudan dyes in different food samples have been reported. Generally, chromatographic methods are reliable and sensitive, but they are high cost and time-consuming, mainly because of complicated, expensive instrument and extensive sample preparation.

Considering the complexity of matrix and the very low concentration of Sudan dyes in real samples analysis, a sample preparation step is required for extraction or enrichment of the analytes.

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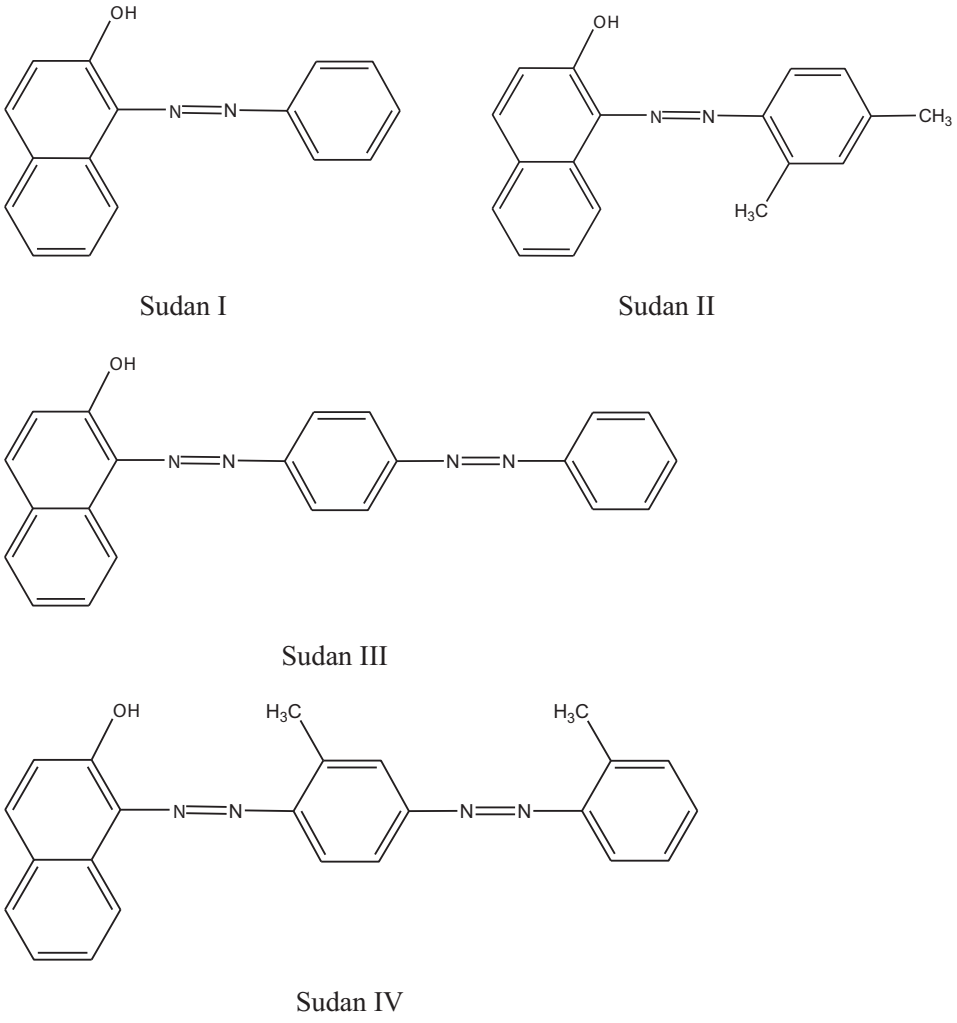

Fig. 1. Molecular structure of Sudan I, II, III and IV.

Conventional sample preparation method for Sudan dyes analysis is solid–liquid extraction [14]. This method requires large amounts of organic solvent, it is often time-consuming and potentially toxic. Other sample preparation techniques, such as ultrasonic-assisted extraction [9,10] and centrifugal sedimentation [11,15] were also reported for Sudan dyes analysis. In recent years, solid phase extraction (SPE) packed with Alumina N was used for clean-up of the food samples prior to the analysis [16,17]. Although this procedure is easy to operate and inexpensive, it requires long time of extraction and large amounts of organic solvent for elution. In addition, activation of Alumina N has great effect on the recoveries of the target analytes. Therefore, the development of simple, rapid and high efficient sample preparation techniques is significant for the analysis of Sudan dyes in foodstuff.

Immunoaffinity chromatography (IAC) is a separation method that takes advantage of the specific and reversible interaction between antibody and antigen [18,19]. Antibodies produced against a target analyte (antigen) are immobilized on a solid support. The immobilized antibodies will specifically retain the antigen from a solution passed through the support. Bound antigen can then be eluted and the support regenerated for reuse. IAC offers unique and powerful techniques, which enables selective extraction and enrichment of individual compounds or classes of compounds in one step. Most types of IAC were proposed to specifically capture only one kind of analyte. Recently, the development of multiple-anlytes IAC for the extraction of several compounds from different matrix has been getting great attention. The multiple-anlytes IAC was usually prepared by immobilizing two to three narrow-specificity antibodies or one kind of broad-specificity antibody on the solid support [20–24].

In our previous study, we have successfully produced monoclonal antibody (mAb) against Sudan I and developed a corresponding enzyme-linked immunosorbent assay (ELISA) for the detection of Sudan I [25]. Taking advantage of the plenty of mAb that we prepared which displayed some extent cross-reactivity with Sudan II, III and IV, the aim of this work is to establish an IAC column for simultaneous extraction and enrichment of Sudan I–IV from foodstuff prior to the analysis by HPLC. The purified mAb was covalently coupled to CNBr-activated Sepharose 4B and packed into a common SPE column. The extraction conditions of the IAC column for Sudan I–IV were optimized and the IAC column was characterized in terms of extraction recovery and stability. Then IAC column was applied for the extraction and enrichment of Sudan dyes in real food samples. To our knowledge, it is the first report for the simultaneous extraction of Sudan dyes with IAC column.

2. Experimental

2.1. Reagents, solutions and apparatus

Sudan I, II, III, IV and dimethylformamide (DMF) were obtained from Sigma (St. Louis, MO, USA). CNBr-activated Sepharose 4B was

purchased from Pharmacia Biotech (Uppsala, Sweden). Methanol and acetonitrile (HPLC grade) were obtained from Dima (Buchs, Switzerland). The other reagents are all analytical grade.

Solutions: (1) 0.01 mol L^{-1} phosphate-buffered saline (PBS), pH 7.4; (2) 0.1 mol L^{-1} Tris–HCl, pH 8.0; (3) 0.1 mol L^{-1} $NaHCO_3$, pH 8.3, containing 0.5 mol L^{-1} NaCl; (4) 0.1 mol L^{-1} acetate buffer, pH 4.0, containing 0.5 mol L^{-1} NaCl; (5) 0.1 mol L^{-1} Tris–HCl, pH 8.0, containing 0.5 mol L^{-1} NaCl; (6) Individual Sudan dye stock solution at concentration of 1 mg mL^{-1} was prepared by weighing 1 mg of one kind of Sudan dye into a small glass bottle and dissolving with 1 mL of DMF; (7) Sudan I–IV mixed stock solution was prepared by weighing 1 mg of Sudan I, together with 1 mg of Sudan II, 1 mg of Sudan III and 1 mg of Sudan IV, into a small glass tube with stopper and dissolving with 1 mL of DMF, so that the concentration of Sudan I–IV was individually at 1 mg mL^{-1}.

Apparatus: Ultraviolet visible spectrophotometer (UV-2300) was purchased from Techcom Com (Shanghai, China). Deionized-RO water machine (DZG-303A) was purchased from AK Company (a conjunctive company between Chengdu and Taiwan, Chengdu, China). The C18 cartridges (GracePureTM SPE C18-Low, 200 mg/3 mL) were from Grace Davison Discovery SciencesTM (Shanghai, China).

2.2. Purification of monoclonal antibody

The mAb against Sudan I was produced according to the procedures described in our previous study [25]. The obtained antiserum was purified using a saturated ammonium sulfate method. To 2 mL of antiserum diluted with the same volume of saline, 4 mL of saturated $(NH_4)_2SO_4$ solution (pH adjusted to 7.4) was added drop wise under gently magnetic stirring at 4 °C. The obtained turbid solution was transferred to a centrifuge tube and allowed to stand for more than 1 h or overnight. Then the solution was centrifuged at 13,000 rpm (e.g. 14,400 × *g*) for 10 min at 4 °C. The supernatant was discarded and the precipitates were dissolved in 2 mL of saline. To this solution, 1 mL of saturated $(NH_4)_2SO_4$ solution was added under magnetic stirring and allowed to stand for 1 h. Then the solution was centrifuged at 13,000 rpm for 10 min at 4 °C and the supernatant was discarded. The precipitates were dissolved in 1 mL of saline and dialyzed in 0.01 mol L^{-1} PBS. The immunoglobulin (IgG) concentration in the obtained solution was measured with a 1 cm cell using a UV-2300 spectrophotometer and calculated based on the UV absorption difference between 280 and 260 nm. The formula used for the calculation was: $C_{protein}$ (mg mL^{-1}) = $1.45A_{280nm} - 0.74A_{260nm}$ [26].

2.3. Preparation of IAC column

The IAC column was prepared by covalently coupling purified mAb against Sudan I to CNBr-activated Sepharose 4B and packed into a common SPE cartridge (GracePureTM SPE C18-Low, 200 mg/3 mL).

The covalent immobilization of mAb on solid support was performed according to the manufacturer's instructions and related literature [26,27]. Briefly, 0.2 g freeze–dried powder of CNB-activated Sepharose 4B was weighed into a clean beaker with 20 mL of 1 mmol L^{-1} HCl to remove the protecting suspended groups. The suspension was transferred to a sintered glass filter and the gel was washed with 200 mL of 1 mmol L^{-1} HCl in three to four fractions. The outlet of the filter was sealed, then 1.5 mL coupling buffer (0.1 mol L^{-1} $NaHCO_3$ containing 0.5 mol L^{-1} NaCl, pH 8.3) was added to the gel. The obtained gel solution was pipetted into a conical flask containing 1.0 mL (~9.2 mg) purified antibody and the reaction proceeded in the incubator shaker (KJ-201c oscillator, Kangjian Medical Apparatus, Jiangsu, China) at room temperature for 3 h. The resultant immuno-sorbent was transferred to a common SPE cartridge with frits placed on both top and bottom. The effluent was collected and measured with UV spectrophotometer. The column was washed with the coupling buffer until the UV absorbance of the effluent at 280 nm was zero. The excess active groups on the immuno-sorbent were capped with the blocking buffer (0.1 mol L^{-1} Tris–HCl buffer, pH 8.0). To ensure complete blocking, the outlet of the column was sealed and the gel was immersed in two column volumes of the blocking buffer and allowed to stand for 2 h. Then the column was washed extensively with of 0.1 mol L^{-1} acetate buffer containing 0.5 mol L^{-1} NaCl (pH 4.0), and 0.1 mol L^{-1} Tris–HCl buffer containing 0.5 mol L^{-1} NaCl (pH 8.0). Finally, the column was washed with 0.01 mol L^{-1} PBS (pH 7.4) and stored in 0.01 mol L^{-1} PBS containing 0.02% NaN_3 at 4 °C until use. For comparison, a blank column without antibody immobilization was prepared as the same way described above.

2.4. Detection of Sudan I–IV by HPLC–UV

As the aim of this study is to prepare an IAC column for simultaneous extraction of Sudan dyes from food samples, to evaluate the prepared IAC column, the analytical method for the simultaneous detection of Sudan dyes should be established. The detection of Sudan I–IV was performed by HPLC–UV and the protocol of HPLC–UV was as follows. Sudan I–IV mixed standard solutions or sample extracts were passed through a 0.45 μm cellulose acetate membrane filter (Alltech, Unterhaching, Germany) prior to HPLC detection. A HPLC system (Alltech, Deerfield, IL, USA) with a C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size, Alltech, Deerfield, IL, USA) was equilibrated with mobile phase consisting of methanol:acetonitrile (20:80, v/v) at a flow rate of 1 mL min^{-1}. Twenty microliters sample loop was used and the detection of Sudan dyes was realized with a UV–vis 201 detector integrated into the HPLC at the wave length of 505 nm. The HPLC work station software (Alltech, Deerfield, IL, USA) was used for the instrument control and data analysis. Peak areas were used for quantification. The standard curves for Sudan dyes were constructed with Sudan I–IV mixed standard solutions in which the individual compound was at the concentrations of 0.02, 0.05, 0.1, 0.2, and 0.5 mg L^{-1} prepared by diluting the Sudan I–IV mixed stock solution with mobile phase. The intra- and inter-day precision was estimated by the relative standard deviation (RSD) of peak area through injection of standard solution replicate during one day and two weeks, respectively.

2.5. Maximum capacity of IAC column for individual Sudan dye

In IAC column, as only one kind of antibody (e.g. mAb against Sudan I) was coupled to CNBr-activated Sepharose 4B, for simultaneous extraction of Sudan I–IV, the first thing needed to do is to investigate whether the column can capture Sudan I–IV and how much of the maximum capacity of IAC column for individual Sudan dye.

The maximum capacity of IAC column for Sudan I (e.g. the maximum amount of Sudan I to be retained by immobilized antibodies in IAC column) was tested at first, which was determined by continuously loading Sudan I standard solution onto the IAC column so that excess amount of Sudan I would saturate all accessible sites of the IAC. 1 mL of Sudan I standard solution at concentration of 500 ng mL^{-1} (total Sudan I: 500 ng), which was prepared with 10% methanol from Sudan I stock solution, was loaded on the column. Then the column was washed with 3 mL of 10% methanol and eluted with 2 mL of pure methanol. All fractions in the loading, washing and eluting steps were collected and evaporated to dryness by low-pressured rotation evaporator (RE-52CS, Ya-Rong Equipmental Com., Shanghai, China) at 45 °C. The residue was reconstituted

in 200 μL of HPLC mobile phase and 20 μL of solution was taken for HPLC analysis.

The procedure of testing the maximum capacity of IAC column for Sudan II, Sudan III and Sudan IV was the same as that described above.

2.6. Optimization of extraction conditions for IAC column

In the case that IAC column can be used for simultaneous extraction Sudan I–IV, to obtain high extraction efficiency, the extraction conditions such as loading, washing and eluting solutions should be carefully optimized. In this study, to find the optimal extraction conditions, six loading solutions (10%, 20%, 30% of methanol; 10%, 20%, 30% of DMF), two washing solutions (10% of methanol and 10% of DMF with the volume of 3.0 mL and 5.0 mL) and six eluting solutions (50%, 60%, 70%, 80%, 90% and 100% of methanol with the volume of 1.0 mL and 2.0 mL) were examined. 1 mL of Sudan I–IV mixed standard solutions in which the individual compound was at the concentration of 50 ng mL^{-1} (e.g. individual Sudan dye: 50 ng; total Sudan dyes: 200 ng) was loaded onto the IAC column. Then 3 mL (or 5 mL) of washing solution was applied to the column to remove the unspecific binding. Finally, 1 mL (or 2 mL) of eluting solution was loaded onto the column to elute the target analytes bound on the column. All eluates in the eluting step were collected and evaporated to dryness by low-pressured rotation evaporator at 45 °C. Then the residues were dissolved in 200 μL mobile phase and analyzed by HPLC.

2.7. Extraction recovery of IAC column

Under optimal extraction conditions, the recovery of the IAC column was measured by loading two different concentrations of Sudan I–IV mixed standard solution: 2.0 and 5.0 ng mL^{-1}. Each concentration was loaded onto the column with the volume of 10 mL (individual Sudan dye: 20 ng and 50 ng, respectively). The eluting solution in the eluting step was collected and evaporated to dryness by low-pressured rotation evaporator at 45 °C. The residues were dissolved in 0.2 mL mobile phase and analyzed by HPLC. The same recovery testing was performed three times.

2.8. Regeneration of IAC column and testing of stability

After eluting the analyte bound on the column, the column was regenerated by alternatively washing with no less than three column volumes of 0.1 mol L^{-1} acetate buffer (containing 0.5 mol L^{-1} NaCl), pH 4.5, and 0.1 mol L^{-1} Tris–HCl buffer, pH 8.5 (containing 0.5 mol L^{-1} NaCl) for three cycles. Finally, the column was washed with 0.01 mol L^{-1} PBS (pH 7.4) and to be ready for next extraction. The stability of IAC column was tested by running a newly prepared IAC column for continuously measuring the maximum capacity for Sudan dyes within one month. The maximum capacity of the IAC column for Sudan dyes was tested two times per week.

2.9. Extraction of Sudan dyes from real food samples by IAC column

Six food samples (e.g. tomato sauce, chilli powder, chilli sauce 1, chilli sauce 2, chilli sauce 3 and chilli sauce 4) were randomly collected from local supermarket. All six food samples without spiking Sudan dyes were firstly extracted with acetonitrile. The extract was evaporated to dryness and re-dissolved with 10% of methanol and then loaded on IAC. Details of the IAC extraction were as follows. 1.0 g of sample was weighed into a glass tube with a glass stopper. Then, 10 mL of acetonitrile was added into the tube. The extraction was performed by sonication for 30 min, followed by centrifugation at 12,000 rpm (e.g. 13,400 × g) for 5 min. The solvent was evaporated to dryness by nitrogen stream and the residue was re-dissolved with 1 mL of 10% of methanol, which was then loaded onto the IAC column for cleanup and enrichment. After washing, the IAC column was eluted. The eluate was evaporated to dryness and reconstructed with 0.2 mL of mobile phase. 20 μl of extract was injected into HPLC column for quantitative detection. For each sample, three separate extractions were performed and each sample was determined in triplicate.

It was found that there were no detectable Sudan dyes in three food samples (e.g. tomato sauce, chilli sauce 2 and chilli sauce 4), which can be used as blank samples for spiking experiment. To further verify the IAC extraction for real food samples, three blank samples were respectively spiked with Sudan I–IV mixed solution and extracted by IAC column. Briefly, 1.0 g of blank sample was weighed into a glass tube with a glass stopper, then 1.0 mL of mixed Sudan dyes solution in which the individual compound was at the concentration of 20 ng mL^{-1} (or 50 ng mL^{-1}) was added. The concentration in final spiking sample was 20 ng (or 50 ng) of individual Sudan dye/g of sample. The spiked samples were vigorously shaken for 10 min and kept at 4 °C overnight. Then, 10 mL of acetonitrile was added into the tube. The other extraction processes were the same as those described for unspiked samples.

2.10. Comparison of the extraction of IAC with SPE

To compare the extraction efficiency of IAC with SPE, the process of SPE cartridge extraction for Sudan dyes was also performed. The SPE C18 cartridge was consecutively conditioned with methanol and pure water, then dried for 30 min under vacuum. The Sudan dyes standard solution (or sample extract in 10% of methanol) was loaded onto C18 cartridge. Washing the column with 3 mL of 10% methanol and eluting the column with 2 mL of pure methanol, the eluate was evaporated to dryness and reconstructed with 0.2 mL of mobile phase. Twenty microliter of extract was injected into HPLC column for quantitative detection. For each sample, three separate extractions were performed and each sample was determined in triplicate.

3. Results and discussion

3.1. Purification of antibody

There are many methods for antibody purification. In this study, the saturated $(NH_4)_2SO_4$ precipitate method was employed for antibody purification due to its simplicity and avoiding loss of antibody activity. By measuring absorbance at 280 and 260 nm, the concentration of purified antibody was calculated to be 9.2 mg mL^{-1}.

3.2. Preparation of IAC column

The solid support is a critical parameter for the preparation of IAC column. Generally, an immunoaffinity support should be mechanically and chemically stable, porous, inert, easily activated to allow antibody attachment, and possess good flow properties. Commonly used support materials include silica, agarose, cellulose, and synthetic polymers. In this study, CNBr-activated Sepharose 4B was used as a solid support because the activated functional ligands on the surface make the particles to be easy for antibody immobilization. In addition, Sepharose 4B with the properties of good chemical stability, low back pressure resistance and poor non-specific interactions is also considered to be ideal particles used in off-line applications. The IAC column was constructed by packing the antibody immobilized Sepharose 4B into a common SPE cartridge. The effluent was collected and measured with UV spectrophotometer. The antibody bound on the column was calculated

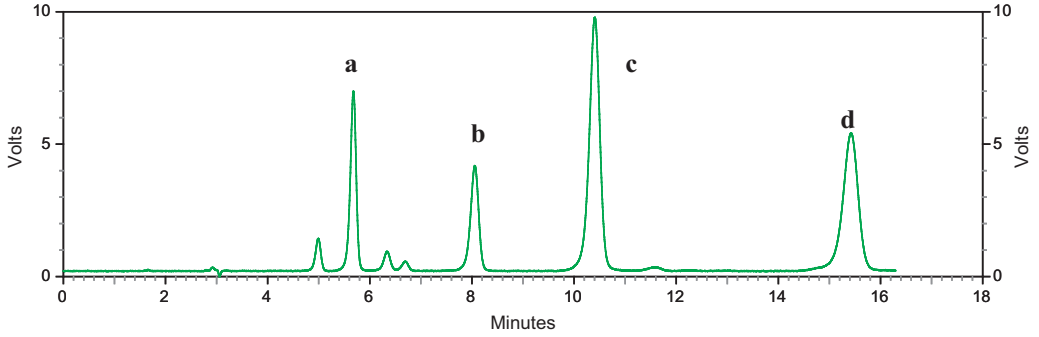

Fig. 2. HPLC chromatogram of (a) Sudan I, (b) Sudan II, (c) Sudan III, and (d) Sudan IV. The concentration of the mixed standard was 0.5 mg L^{-1}.

Table 1
Regression equation, correlation coefficient, linear range, LOD, LOQ, intra-day and inter-day precision of HPLC for the detection of Sudan I-IV.

Analyte	Regression equation	Correlation coefficient	Linear range (μg mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Intra-day precision[a] (%)	Inter-day precision[a] (%)
Sudan I	$y = 41571x + 184$	0.9999	0.01–0.5	5.7	18.8	1.92	3.75
Sudan II	$y = 42920x + 349$	0.9998	0.01–0.5	4.4	14.5	0.87	4.32
Sudan III	$y = 85452x + 439$	0.9999	0.005–0.5	2.7	8.9	1.55	3.76
Sudan IV	$y = 79880x + 22$	0.9998	0.01–0.5	6.0	19.8	0.98	4.62

[a] For the precision study, 20 μl of the mixed standard solution at concentration of 0.1 μg mL^{-1} was employed.

by subtracting the antibody in the effluent from initial antibody. The coupling efficiency of Sudan I antibody on solid support was estimated according to the ratio of Ab coupled to solid support/initial Ab. In this study, three IAC columns were prepared and the coupling efficiencies were found to be 92.5%, 91.8% and to 93.2%, e.g. about 8.5 mg of mAb was covalently coupled to 0.2 g Sepharose 4B gel packed into one IAC column.

3.3. Detection of Sudan I–IV by HPLC–UV

To establish the HPLC method for simultaneous detection of Sudan I–IV, the wave length must be firstly confirmed. To find out appropriate wave length for Sudan dyes, the UV screening spectra of Sudan I–IV at concentration of 1 mg L^{-1} were made and it was found that that Sudan I–IV has a strong absorbance at 505 nm. So λ = 505 nm was selected as detection wave length for HPLC analysis. Other appropriate conditions for HPLC analysis were mentioned in Section 2.5. Under optimal analytical condition, Sudan I–IV can be completely separated. As shown on chromatography (Fig. 2), which was obtained from the mixed standard solution at concentration of 0.5 mg L^{-1}, the retention time (t_R) for Sudan I, II, III and IV was 5.6 min, 8.0 min, 10.4 min and 15.4 min, respectively. It was also observed that there were some small peaks appeared at retention time of 5 min, 6.3 min 6.7 min, which maybe result from the compounds in solvent. The standard curves for Sudan dyes were constructed with Sudan I–IV mixed standard solutions in which the individual compound was at the concentrations of 0.02, 0.05, 0.1, 0.2, and 0.5 mg L^{-1}. The regression equation, correlation coefficient, linear range, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day precisions of HPLC for Sudan I–IV were summarized in Table 1. It was seen that the values of LOQ of HPLC for Sudan I, II, III and IV were 18.8, 14.5, 8.9 and 19.8 ng mL^{-1}, respectively.

3.4. Maximum capacity of IAC column for individual Sudan dye

Usually, the multiple-analytes IAC was prepared by immobilizing two to three narrow-specificity antibodies or one kind of broad-specificity antibody onto the solid support. In this study, the mAb against Sudan I was employed for the preparation of IAC column. In our previous study, the cross-reactivity (CR) values of the mAb with Sudan I, II, III and IV were 100%, 9.5%, 33.9% and 0.95%, respectively. It seems that there was no big specific recognition of the antibody for Sudan II and IV. However, whether the column can capture Sudan I–IV and how much of the maximum capacity of IAC column for individual Sudan dye was mainly dependent on the experimental data.

The maximum capacity of the IAC column for individual Sudan I, II, III and IV was determined by continuously loading Sudan dye standard solution onto the IAC column as described in Section 2.6 and shown in Table 2. It can be seen that values of the maximum capacity of the IAC column for Sudan I, II, III and IV were 295, 156, 184 and 173 ng, respectively, which clearly indicated that the prepared IAC column can specifically capture Sudan I, II, III and IV. In another word, the IAC column can be used for simultaneous extraction of Sudan dyes from food samples. The difference of the specific recognition of the mAb for Sudan dyes observed in IAC and ELISA might be mainly due to different immuno-format. In indirect ELISA, the antibody was in the solution, which was limited amount competed by antigen in aqueous and antigen coated on solid support; while in IAC column, the antibody were abundant amount and covalently linked to the solid support, which can capture all the structurally relative compounds utmost.

3.5. Optimization of extraction conditions for IAC

The extraction conditions should be optimized so that in the loading step, all target analyte can be specifically bound on the column, while in the washing step, the possibly non-specific adsorption should be removed completely, and moreover, in the eluting step, all the bound analyte should be eluted and the Ab activity should be remained as much as possible. In this study, when 1 mL of Sudan I–IV mixed standard solutions in which the individual

Table 2
Maximum capacity of the IAC column for Sudan I, II, III and IV.

	Maximum capacity (ng)	RSD (%) ($n = 3$)
Sudan I	295	2.1
Sudan II	156	7.4
Sudan III	184	10.4
Sudan IV	173	3.5

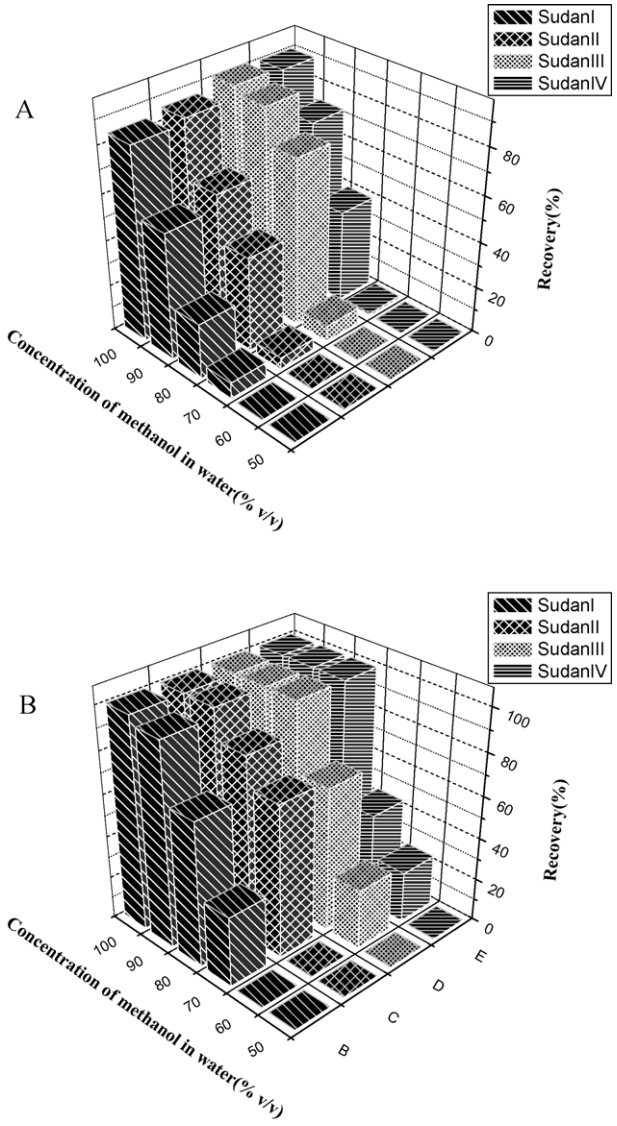

Fig. 3. The effect of the percentage of methanol in eluting solution on the recovery of Sudan I, II, III and IV by IAC extraction. (A) 1 mL of eluting solution; (B) 2 mL of eluting solution.

compound was at the concentration of 50 ng mL^{-1} (e.g. individual Sudan dye: 50 ng; total Sudan dyes: 200 ng) was loaded onto the IAC column, six loading solutions (10%, 20%, 30% of methanol; 10%, 20%, 30% of DMF), two washing solutions (10% of methanol and 10% of DMF with the volume of 3.0 mL and 5.0 mL) and six eluting solutions (50%, 60%, 70%, 80%, 90% and 100% of methanol with the volume of 1.0 mL and 2.0 mL) were examined. It was found that, in the loading step, for all six loading solution, 85–96% of Sudan dyes was bound on the column. As lower organic solvent used and high extraction efficiency (96%), 10% of methanol was selected as loading solution. In washing step, it was observed that either with 3 mL 10% methanol or 3 mL 10% DMF to wash the column, there was no Sudan dyes in the effluent fractions, e.g. no Sudan dyes was removed from the column. As 10% methanol had the higher capability to remove non-specific binding, 10% methanol with the volume of 3 mL was chosen as washing condition. In most off-line IAC procedures, to elute the high hydrophobic analyte from the column, a high percentage of an organic solvent was required [28]. Because the Sudan dyes are the compounds with very high hydrophobic property, high concentration of organic solvent is necessary. As shown in Fig. 3, with the increase of methanol concentration from 50% to 100%, the values of Sudan dyes extraction recovery from the IAC were increased from 2% to 100%. At volume of 2 mL of 90% of methanol, the extraction efficiency of the IAC column for Sudan dyes was within 93–100%. Therefore, 2 mL of 90% of methanol was used as eluting condition. In conclusion, the optimal extraction conditions for IAC column were: 10% methanol as loading solution, 3 mL of 10% methanol as washing condition and 2 mL of 90% methanol as eluting condition.

Table 3
The extraction recovery of the IAC column for Sudan I, II, III and IV.

	Spiked (ng)	Found (ng)	RSD (%) (n = 3)	Recovery (%)
Sudan I	20	18.9	8.2	94.5
	50	53.5	7.8	107.0
Sudan II	20	23.7	9.1	118.8
	50	43.6	7.5	87.2
Sudan III	20	16.2	12.8	81.0
	50	42.3	5.2	84.6
Sudan IV	20	17.9	13.5	89.5
	50	37.5	8.1	75.0

3.6. Extraction recovery of IAC column

Ten milliliters of Sudan I–IV mixed standard solutions at concentrations of 2 ng mL^{-1} and 5 ng mL^{-1} (e.g. individual Sudan dye: 20 ng and 50 ng) were respectively loaded on IAC column. The Sudan dyes content in the fraction of eluting step was detected and compared with that of loading amount. As shown in Table 3, the Sudan dyes recoveries from the IAC column were found to be 75.5–118.8% with the relative standard deviation of 5.2–13.5%, respectively, indicating high extraction efficiency of the column.

3.7. Regeneration of IAC column and testing of stability

The regeneration and storage of the IAC column should be addressed. After each extraction, the IAC column should be washed alternatively with no less than three column volumes of 0.1 mol L^{-1} pH 4.5 acetate buffer containing 0.5 mol L^{-1} NaCl and 0.1 mol L^{-1} Tris–HCl buffer, pH 8.5, containing 0.5 mol L^{-1} NaCl for three cycles. Finally, the column was washed with 0.01 mol L^{-1} PBS. For short term (2–3 days) storage, the IAC column was simply kept in 0.01 mol L^{-1} PBS at 4 °C; while for relatively long-term storage (over a week), it was kept in 0.01 mol L^{-1} PBS containing 0.02% NaN_3 at 4 °C.

The antibodies against small molecular compounds are normally difficult to obtain and are very expensive. Therefore, the prepared IAC columns are expected to be used for many times. Actually most antibodies are tolerant to some extent of organic solvent without significant loss of activity. The IAC column with the maximum capacity of 295, 156, 184 and 173 ng for Sudan I, II, III and IV was tested for its stability. It was observed that within one month for 50 cycles repeated usages, about 64% of the maximum capacity was still remained, indicating high stability of the IAC column.

3.8. Extraction of Sudan dyes from food samples by IAC column

Six food samples including one sample of tomato sauce, one sample of chilli powder and four samples of chilli sauce without spiking Sudan dyes were extracted with acetonitrile. The extract was evaporated to dryness and re-dissolved with 10% of methanol. Loading the methanolic solution to the IAC column and washing the column with 10% methanol, the IAC was eluted with 90% methanol. The eluate was evaporated to dryness and reconstructed with 0.2 mL of mobile phase for HPLC analysis.

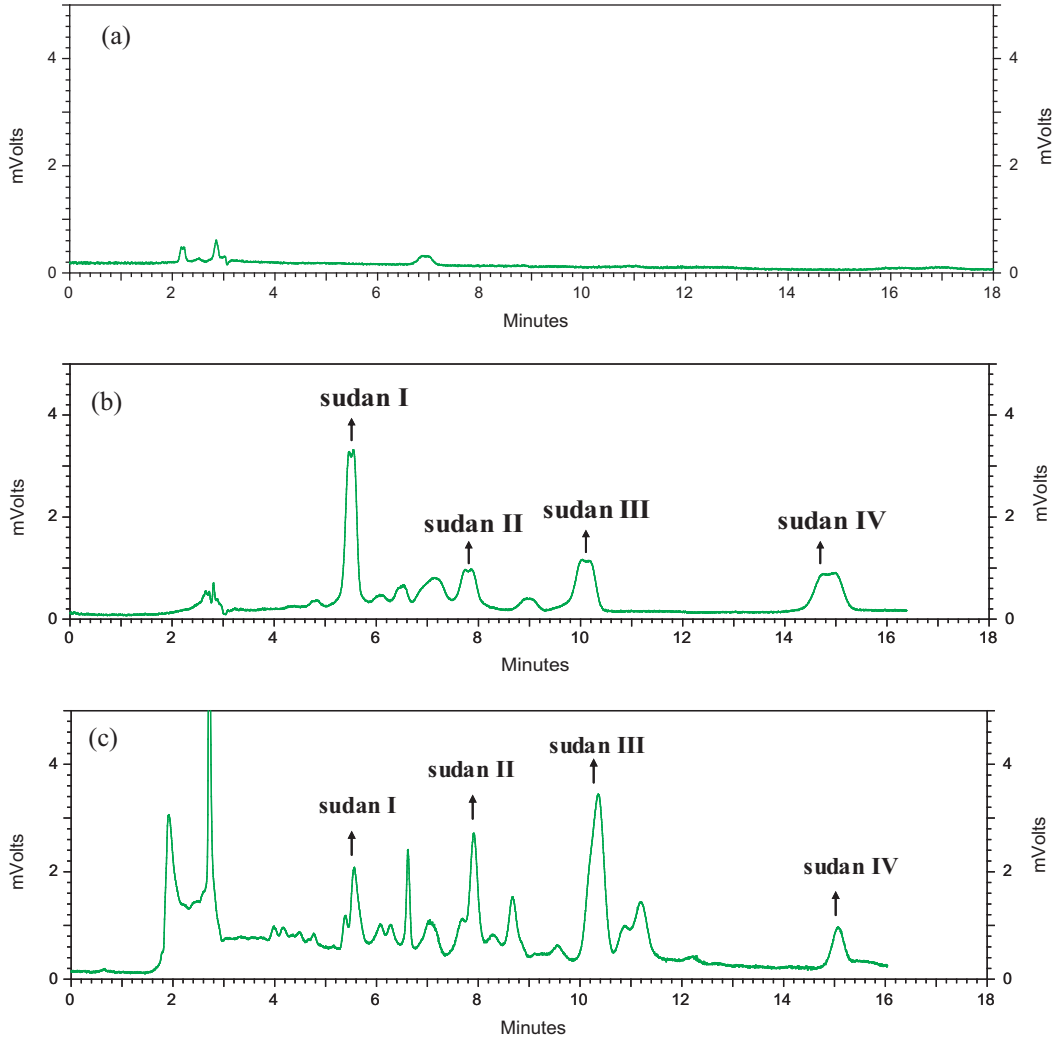

Fig. 4. HPLC chromatograms of chilli sauce sample 2: (a) blank sample after IAC extraction; (b) spiked at 50 ng g^{-1} Sudan I–IV after IAC extraction; (c) spiked at 50 ng g^{-1} Sudan I–IV after SPE extraction.

It was found that there was no detectable Sudan II, III and IV in all six food samples, but in chilli powder, chilli sauce 1 and chilli sauce 3, Sudan I was detected, in which the Sudan I concentrations were found to be 2.7 ng g^{-1}, 14.3 ng g^{-1} and 134.5 ng g^{-1} with the relative standard deviation (RSD) of 5.2%, 14.3% and 13.7% (n = 3), respectively. However, no detectable Sudan I was observed in other three samples, e.g. tomato sauce, chilli sauce 2 and chilli sauce 4.

To further verify the IAC extraction for real food samples, three negative samples (tomato sauce, chilli sauce 2 and chilli sauce 4) were used as blanks for spiking experiment. Each blank was spiked with 1 mL of Sudan I–IV mixed solution in which the individual Sudan dye was at the concentration of 20 ng mL^{-1} and 50 ng mL^{-1} and extracted by IAC column. The HPLC chromatogram of blank chill sauce sample 2 was shown in Fig. 4(a). It was seen that only three very small peaks were appeared at retention time of 2.2 min, 2.8 min and 6.9 min, which obviously indicated that matrix effect could be almost eliminated. The HPLC chromatogram of chill sauce sample 2 spiked at 50 ng g^{-1} Sudan I–IV after IAC extraction was illustrated in Fig. 4(b), in which the peaks Sudan I–IV were clearly appeared. However, in Fig. 4(b), there were some small peaks appeared at retention time of 2.7 min, 4.9 min, 6.2 min, 6.6 min, 7.1 min and 9.0 min. It might be that the peaks at 2.7 min and 7.1 min resulted from the matrix compounds, while the peaks at 4.9 min, 6.2 min and 6.6 came from the compounds in the solvent for preparing mixed standard solution. It seemed hard to explain the peak at retention of 9.0 min. Anyhow, it was apparent that after IAC extraction, the target analytes were specifically retained on IAC column and the matrix effect was almost removed. It should be mentioned that the Fig. 4(b) was the HPLC chromatogram obtained from the eluate of the IAC column after 20 times repeatedly used, that is the part reason why the peaks of the Sudan dyes in Fig. 4(b) were a little bit broader than those in Fig. 2. The main reason of peak broadening in Fig. 4(b) may be due to a little bit sticky of the eluate after high folds of extraction and enrichment by IAC cartridge comparing to that in standard solution used in Fig. 2. The spiking results were shown in Table 4. It can be seen from Table 4 that the recoveries of Sudan I, II, III and IV spiked at the concentration of 20 ng g^{-1} and 50 ng g^{-1} in tomato sauce, chilli sauce 2 and chilli sauce 4 were in the range of 68.6–96.0% with the RSD in 4.8–15.2%, demonstrating the feasibility of the prepared IAC column for Sudan dyes extraction.

3.9. Comparison of the extraction of IAC with SPE

Whether the SPE cartridge can capture Sudan I–IV and how much the maximum capacity of the SPE column for individual Sudan dye were tested as the procedures described in Section of 2.5. At this case, the SPE column instead of IAC column was uti-

Table 4
The recoveries of Sudan I, II, III and IV spiked in three negative food samples at concentrations of 20 ng g^{-1} and 50 ng g^{-1} after IAC extraction.

		Spiked conc. (20 ng g^{-1})			Spiked conc. (50 ng g^{-1})		
		Tomato sauce	Chilli sauce 2	Chilli sauce 4	Tomato sauce	Chilli sauce 2	Chilli sauce 4
Sudan I	Found (ng g^{-1})	14.8	17.2	17.6	40.2	37.3	40.5
	RSD (%) (n = 3)	7.4	8.7	9.6	11.5	8.0	5.4
	Recovery (%)	74.0	86.0	88.0	80.4	74.6	81.0
Sudan II	Found (ng g^{-1})	15.4	19.2	17.4	42.2	39.3	41.5
	RSD (%)	12.5	8.7	5.9	8.4	5.1	4.8
	Recovery (%)	77.0	96.0	87.	84.4	78.6	83.0
Sudan III	Found (ng g^{-1})	16.4	17.6	14.3	38.5	36.5	34.3
	RSD (%)	9.6	14.1	7.8	5.5	10.1	12.7
	Recovery (%)	82.0	88.0	72.0	77.0	73.0	68.6
Sudan IV	Found (ng g^{-1})	15.6	18.3	17.7	36.4	38.5	39.5
	RSD (%)	11.2	8.7	15.2	9.3	12.6	13.1
	Recovery (%)	78.0	92.0	89.0	72.8	77.0	79.0

lized. It was observed that the SPE column can also capture Sudan I, II, III and IV at the same time with the maximum capacities of 238, 170, 219 and 147 ng, respectively. The reason that Sudan I–V could be adsorbed on C18 cartridge column might be mainly due to the high hydrophobicity of the Sudan dyes. The C18 cartridge was also applied for the extraction of Sudan I–IV from three spiked food samples (e.g. tomato sauce, chilli sauce 2 and chilli sauce 4 were spiked with Sudan I–IV mixed solution so that the final concentration of individual Sudan dye was 50 ng mL^{-1}, see Section 2.9), and the typical HPLC chromatogram of the spiked chill sauce sample 2 after SPE extraction was shown in Fig. 4(c). It was found that the recoveries of the SPE extraction for Sudan I–IV from three spiked food samples were in the range of 65.7–86.3% with the RSD in 6.7–14.3%. It seemed that the extraction efficiency of the SPE column for Sudan I–IV was similar to that of IAC column, however, from Fig. 4(c) and comparing it with Fig. 4(b). It was observed that in the Fig. 4(c), the baseline was higher than that in Fig. 4(b), and besides the peaks of Sudan dyes, there were many strong peaks appeared, such as at 2.0 min, 2.8 min, 5.4 min, 6.2 min, 6.4 min, 6.6 min, 7.0 min, 8.8 min, 11.2 min. Apparently, the clean-up effect of the IAC extraction was better than that of the SPE extraction, which might be mainly due to the specific recognition of the antibody bound on the support materials for the target analytes and the sufficient removal of the matrix effect.

4. Conclusions

By covalently coupling mAb against Sudan I on CNBr-activated Sepharose 4B and packing the immunosorbents into a SPE cartridge, the IAC column was prepared. It was confirmed that the prepared IAC column can specifically capture not only Sudan I, but also Sudan II, III and IV as well, so it can be used for simultaneously selective extraction and enrichment of Sudan I–IV prior to the analysis by HPLC. The extraction conditions in the processes of loading, washing and eluting were optimized and the IAC column was characterized in terms of extraction recovery and reusability. The recoveries values of Sudan dyes at two spiked concentrations were within 95.3–106.9%. After 50 times repeated usage, 64% of the maximum capacity was still remained. The prepared IAC was applied for the extraction of Sudan dyes from real food samples and the satisfied results were obtained. The proposal IAC column provides an alternative fast and effective tool for simultaneous extraction Sudan dyes from food samples.

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