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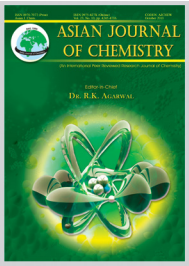

Molecularly Imprinted-Solid Phase Extraction for Multi-Residues Analysis of Sudan Dyes in Sausage

FENGXIA QIAO* and PING CHEN

Department of Biochemistry, Baoding University, Baoding 071000, P.R. China

*Corresponding author: Tel: +86 312 597-2186; E-mail: qiaofengxia@126.com

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A Sudan II molecularly imprinted polymer (MIP) was synthesized and used as the selective sorbent for solid phase extraction (SPE) of Sudan dye (I, II, III and IV) residues in sausage samples. It showed higher affinity to Sudan dyes and could selectively enrich them from sausage matrix. Under the optimized solid phase extraction condition, the extracts were sufficiently clean for further chromatographic analysis with no interferences from template leakage and sausage matrix. Good linearity were obtained from 0.03-10 μg/g with the correlation coefficients $r^2 \geq 0.9994$. The recoveries of four Sudans in sausages at three spiked levels were in the range of 96.8-103 %.

Key Words: Solid-phase extraction, Molecularly imprinted polymer, Sudan dyes, Sausage sample.

INTRODUCTION

Sudan dyes (I, II, III and IV) are a family of lipophilic azo dyes and generally used for solvent, oil, car wax and other synthetic materials. Sudan dyes are fraudulently used to enhance the colour of the food. Sudan dyes have a potential risk of genotoxicity and they are classified in group 3 of IARC (International Agency for Research on Cancer) as to their carcinogenic risk to humans[1]. During the recent years, due to the continuing illicit use of Sudan dyes as food colourants, their determination in different food matrices has received increasing attention all over the world[2].

At present, several methods had been proposed for the determination of Sudan dyes in food products including high-performance liquid chromatography[3], liquid chromatography-mass spectrometry[4], gas chromatography-mass spectrometry[5] *etc*. To isolate and concentrate the compounds of interest from the complex sample matrix, solid-phase extraction (SPE)[6] and solvent extraction[1,7] was commonly used as the sample pretreatment procedure. Though ionic liquid multi-walled carbon nanotube composite film was introduced into Sudan analysis to improve the sensitivity and selectivity[8], liquid-solid extraction was still the dominating sample preparation procedure. Until now, various types of solid phase extraction had been used for sample cleanup and the large majority of sorbent was C_{18}[2], which was lack of special selectivity. Molecularly imprinted solid phase extraction (MISPE) had been proposed to improve the selectivity of the isolation of Sudan dyes[6,9,10], but the most of these reports were mainly focused on mono-content of Sudan dye analysis. Higher selective pretreatment method for multi-residues analysis of Sudan dyes was still desired greatly.

The aim of this work is to develop a high selective molecularly improved solid phase extraction method for multi-residues analysis of four Sudan dyes in Sausage. The results showed that Sudan II-imprinted MIP show high affinity to the four Sudan dyes and the eluents of molecularly improved solid phase extraction were clean enough for further chromatographic analysis with no interferences from template leakage and sausage matrix. The proposed method could be potentially applied for multi-analysis of Sudan dyes residues in other biological samples.

EXPERIMENTAL

Sudan (I, II, III and IV) were obtained from Chemical Reagent Factory of Tianjin Fu Chen (Tianjin, China). Ethylene glycol dimethacrylate (EDMA) was obtained from Shanghai Trading Co. Ltd. (Shanghai, China). α, α'-Azobis(isobutyronitrile) (AIBN) was purchased from Beijing Chemical Reagent Co. (Beijing, China). Methacryclic acid (MAA) from Tianjin No. 1 Chemical Reagent Factory (Tianjin, China) was purified by distillation to remove inhibitor. All the other reagents used in the experiment were of the highest grade commercially available.

The chromatographic analysis was carried out on a Shimadzu HPLC system equipped with two LC-20AT solvent delivery units, a SUS20A gradient controller and a SPD-20A UV-VIS Detector (Shimadzu, Kyoto, Japan). An N-2000 chromatography data workstation (Zheda Zhineng Co. Ltd.,

Hangzhou, China) was used as a data acquisition system. The analytical column was packed with C_{18} stationary phase (150 mm × 4.6 mm I.D., 5 μm, RStech, Korea) and the mobile phase was methanol-formic acid (99.7:0.3, v/v) with a flow rate of 1.0 mL/min. The detection wavelength of the detector was set at 475 nm.

Preparation of molecularly imprinted polymers: 2 mmol Sundan II, 8.0 mmol MAA, 50 mmol EDMA and 0.2 g α,α′-azobis(isobutyronitrile), were dissolved in 25.0 mL chloroform. Polymerization was performed by thermal-initiated polymerization at 60 ºC for 24 h. The obtained polymers were grinded and sieved through a 32 μm sieve and then suspended in acetone until the upper solution became clear. After removed template by methanol-acetic acid (9:1, v/v), the polymer was washed with methanol for 12 h to wash away the residue of acetic acid. Non-imprinted polymer (NIP, in the absence of a template) was prepared and treated in an identical manner.

Solid phase extraction breakthrough volume and molecularly imprinted polymer imprinted factor determination: For breakthrough volume experiment, different volumes of Sudan dyes solution (2.0 μg/mL) were loaded on the SPE columns (300 mg of MIP) and the sample effluents were determined by HPLC. For imprinted factor, MIP and NIP were packed into a 15 cm of HPLC column respectively and methanol was applied as the mobile phase for HPLC analysis. The imprinted factor was defined as $I = \frac{k_{MIP}}{k_{NIP}}$ where $k = \frac{(t_s - t_0)}{t_0}$, t_s was the retention time of Sudan II and t_0 was the retention time of acetone.

Sample pretreatment: Sausage samples were pestled and 0.5 g of it was accurately weighed and transferred into a centrifuge tube and then added into 3 mL hexane for vortex 2 min. After ultrasonic-assisted extraction for 1 min and centrifuge for 5 min. the supernatant was filtered by 0.45 μm membrane. Repeated the extraction processes for three times and the filtrates collected together and evaporated at 30 ºC to dryness under vacuum and then the residues were reconstituted into 1 mL methanol for further MISPE procedure.

Procedure of molecularly imprinted solid phase extraction: 300 mg of molecularly imprinted polymer were packed into empty SPE columns (6 mL) and then preconditioned with 3 mL chloroform and 5.0 mL methanol. After loaded 1.0 mL of sample solution, the cartridges were washed with 1.5 mL methanol-water (1:4, v/v) solution and eluted with 3 mL dichloromethane-acetic acid (95:5, v/v). The eluents were evaporated at 30 ºC to dryness under vacuum and then reconstituted into 0.3 mL mobile phase for further HPLC analysis.

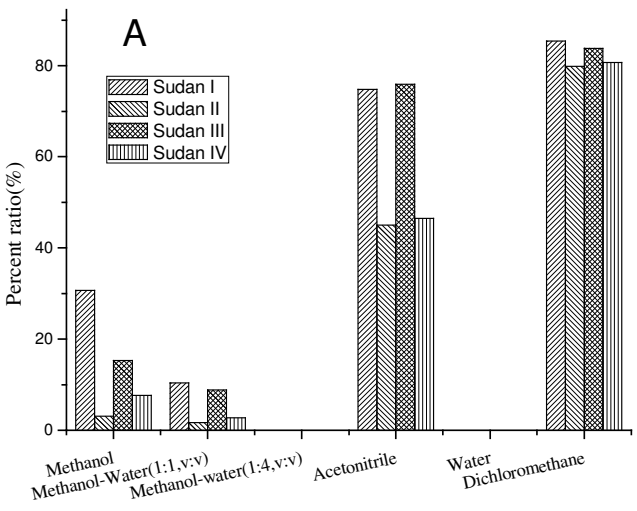
RESULTS AND DISCUSSION

Preparation of imprinted polymers: In order to obtain excellent Sudan II-imprinted polymers with specific molecular recognition to Sudan I, II, III and IV, different monomers such as methacrylic acid, 2-hydroxyethyl methacrylate and acrylamide were investigated. The result revealed that the MIP prepared with methacrylic acid as a functional monomer showed better molecular recognition ability for Sudan dyes. Though using trifunctional crosslinker (TRIM) as crossing reagent could obtained higher rigidity polymer than EDMA, its stretches in solvent was too easily to be employed as SPE sorbent. After optimization, a mol ratio of 1:4:25 (Sudan II: MAA: EDMA) was used to prepare the Sudan II-MIP.

Evaluation of selectivity: In order to investigate the binding capacity and selectivity of the imprinted polymers, breakthrough experiment and imprinted factor were investigated. The results of breakthrough experiment showed that the breakthrough volume of 2.0 μg/mL Sudan solution was 10.5 mL on the MIP column while only 1.5 mL on NIP column, which indicated that the MIP had higher binding capacity for the target analytes. The imprinted factor was 1.59 (the k of Sudan II was 2.77 on the MIP column while 1.74 on the NIP column), which indicated that the MIP had higher selectivity than NIP.

Optimization of HPLC condition: The type of mobile phase was the main key factor for complete separation of four Sudan dyes. The mobile phase consisted of methanol and aqueous formic acid could provide a better separation condition for the four Sudan dyes separation[10]. Therefore, methanol combined different content formic acid solutions were investigated in this work. The baseline separation of four Sudan dyes was obtained when the formic acid-methanol mobile phase was of 0.3:99.7 (v/v). The temperature had no obvious effect on the four Sudan dyes separation result.

Optimization of molecularly imprinted solid phase extraction: In order to eliminate the matrix interferences and not losing the analytes, methanol, acetonitrile, methanol-water (1:1,v/v), methanol-water (1:4, v/v), dichloromethane and water as washing solution were investigated by applying 1 mL of each solution on MIP cartridges (0.5 mL of 2.0 μg/mL Sudan dyes solution were preloaded). The result in Fig. 1(A) showed that methanol-water (1:4, v/v) and water had the lowest elution ability and no Sudan dyes were washed out. Considered the purification efficiency and recoveries of target analytes, methanol-water (1:4, v/v) was selected as the washing solution. When the volume of methanol-water (1:4, v:v) was increased to 2 mL, 2.2 % Sudan I would be washed out while others Sudan dyes still remained on cartridge. Therefore, 1.5 mL of methanol-water (1:4, v/v) is used as washing solution for further work.



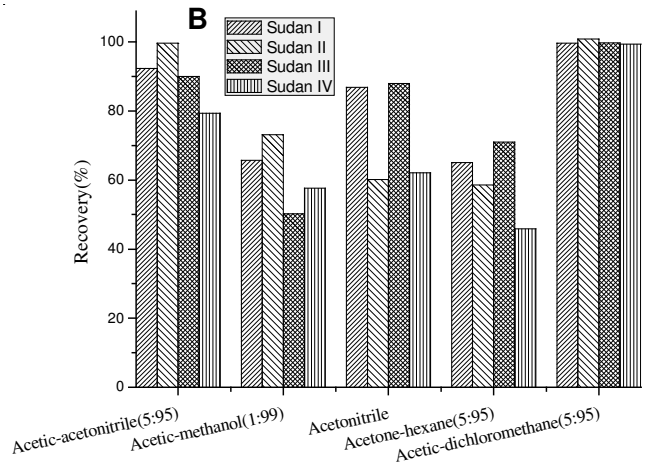


Fig. 1. Washing (A) and elution solvents (B) on extraction efficiency of molecularly imprinted solid phase extraction

Different elution solution including acetic-dichloromethane (5:95, v/v), acetonitrile, acetone-hexane (5:95, v/v), acetic acid-acetonitrile (5:95, v/v) and acetic acid-methanol (1:99, v/v) were investigated. The results in Fig. 1(B) showed that acetic acid-dichloromethane (5:95, v/v) was the best elution solution which allowed the adsorbed analytes to be fully desorbed. To obtain the optimum eluent volume, varying volumes (0.5-6.0 mL) of dichloromethane-acetic acid (95:5, v/v) were evaluated and its elution ability was rapidly increased with the increasing volume before 2 mL and then only slightly increased even further increasing the volume to 6 mL. Combination of analysis time and extraction efficiency, 3 mL of dichloromethane -acetic acid (95:5, v/v) as elution solvent was used for further work.

Analytical performance: Calibration curves of the four Sudans were constructed using the areas of the chromatographic peaks measured at nine increasing concentrations, in a range of 0.03-10 μg/g. Each analyte exhibited good linearity with $r^2 \geq 0.9994$ in the studied range and the LODs based on S/N = 3 were ranged from 0.006-0.011 μg/g (Table-1). The intra-day precision and accuracy of the method evaluated as RSD were ranged from 2.7-3.1 % and the inter-day reproducibility was below 4.9 % in all cases.

Analysis of real samples: To testify the practicability of the proposed molecularly imprinted solid phase extraction method, C_{18} sorbent was also employed in this work for the four Sudan dyes analysis in sausage samples. The result showed that the molecularly imprinted solid phase extraction could obtain higher recovery (96.8-103 %) than that on C_{18}-SPE (48.9-78.0 %) and the chromatogram of molecularly imprinted solid phase extraction was more clear (Fig. 2). Six kinds of sausage products collected from the local markets were evaluated and no residuals of Sudan dyes were detected, which revealed that the abuse of Sudans in sausages at local area is not extensive. The recovery at three spiked level (Table-2) and clean chromatogram (Fig. 2) validated the high selectivity and usability of the proposed molecularly imprinted solid phase extraction method.

TABLE-1
LINEARITY AND LOD OF SUDAN DYES

Analytes	Linearity	r^2	LOD (μg/g)
Sudan I	$y = 4.41 \times 10^4 x - 1.74 \times 10^3$	0.9999	0.007
Sudan II	$y = 6.32 \times 10^4 x - 2.23 \times 10^3$	0.9994	0.006
Sudan III	$y = 7.33 \times 10^4 x - 2.53 \times 10^3$	0.9997	0.009
Sudan IV	$y = 5.41 \times 10^4 x - 2.66 \times 10^3$	1.0000	0.011

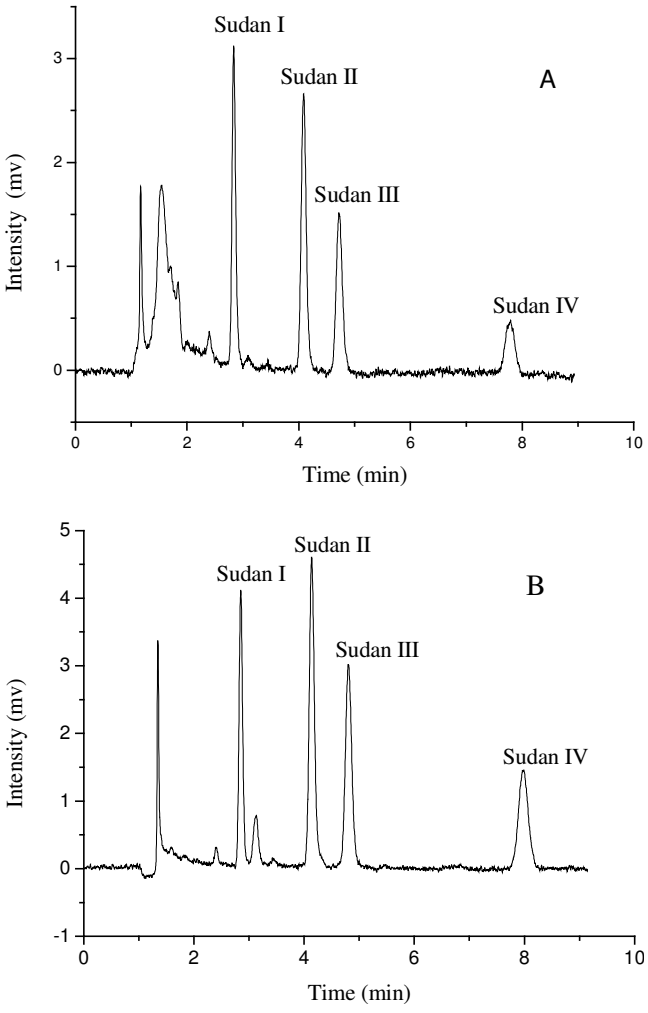


Fig. 2. Chromatograms of the spiked sausage samples. (A: C_{18}-SPE, B: molecularly imprinted solid phase extraction; spiking level: 0.5 μg/g)

TABLE-2
RECOVERIES OF SUDAN DYES IN SAUSAGE SAMPLES (n = 5)

Analytes	0.063 (μg/g)		0.50 (μg/g)		2.50 (μg/g)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Sudan I	103	3.1	100	3.7	98.0	3.9
Sudan II	98.4	4.9	99.9	4.1	102	4.0
Sudan III	103	3.6	101	2.1	96.8	2.0
Sudan IV	98.4	4.6	100	4.7	99.6	4.6

Conclusion

A simple and selective MISPE-HPLC method was developed for the multi-residues analysis of Sudan dyes in sausage samples. Under the optimized condition, the eluents were clean enough for further chromatographic analysis with no interferences from template leakage and sausage matrix. Good linearity were obtained from 0.03-10 μg/g and the recoveries of four Sudans at three spiked levels were in the range of 96.8-103 %. The proposed method was suitable for Sudan dyes analysis in complicated samples.

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