Scientific Research

Determination of Eight Sudan Dyes in Chili Powder by UPLC-MS/MS

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ABSTRACT

Sudan dyes are synthetic azo dyes which are widely used in industry. Although they are not allowed in foodstuffs, they have been found contaminating in different food products and their presence is regularly reported. It is assumed that these appearances are due to cross-contamination or adulteration. In this paper, we present a newly developed fast and sensitive method for the quantification of eight Sudan dyes, using liquid-liquid extraction and UPLC-MS/MS analysis. The calibration curves were linear over the range of 0.1 - 25 mg/kg. Mean recovery for the eight Sudan dyes ranged from 80.7% to 104.4%, and with the inter-day and intra-day precisions ranged from 2.24% to 12.2%. The method was successfully applied in the determination of Sudan dyes in chili powder of 10 samples.

1. Introduction

Sudan dyes are dynthetic fat-soluble azo-compounds, characterized by chromophoric azo groups (-N=N-) [1]. The chemical structures of Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Red G, Sudan Red 7B, Sudan Black B and Sudan Yellow are given in **Figure 1**.

These dyes are extensively used as colorants in food, cosmetics, waxed, solvents, textiles, and so on. Lots of them are found in various foodstuffs, like chili powder, curry, or chili sauces fraudulently [2]. These azo dyes are metabolized to possible carcinogenic colorless amines that can form DNA adducts entailing mutations [3]. Sudan I has been found to be carcinogenic in the rat and to produce tumors in the liver of mice. Sudan II increases the incidence of bladder carcinomas, and Sudan IV increases the risk of formation of local carcomas [4,5]. Besides, Sudan III is included in different laboratory techniques for tissue ceroid and lipofucsin analysis [6]. Also, Sudan Black B, which is considered slightly hazardous in the case of skin contact and toxic to mucus membranes, is extensively used in diagnostic methods [7]. Sudan I, II, and III as well as Sudan Red 7B, and Sudan Black B reaction products have been declared suspected carcinogens and classified group 3 compounds by the International Agency for Research on Cancer [8, 9]. Therefore, Sudan dyes are not safe for humans, and a sensitive and convenient method for the determination of Sudan dyes is required.

Several methods based on liquid chromatography with UV or MS detection [10-12] or on gas chromatography mass spectrometry have been developed for analysis of Sudan I-IV. Although these methods have been successfully applied to analysis of the dyes at trace levels in food products, they often require complicated pretreatment, for example, more than one solvent extraction, solid-phase extraction, rotary evaporation, etc. The extraction and concentration procedures are time-consuming and the results might be interfered in matrix effect. Until now, the methods for the analysis of Sudan Red G, Sudan Red 7B, and Sudan Black B are very few [13], and no report on the analysis of Sudan Yellow. To our knowledge, this is the first research on the determination of eight Sudan dyes.

In this study, a sensitive, robust, and fast method based on UPLC-ESI-MS/MS was developed to determine eight Sudan dyes in chili powder. The technique of extract ion current of mass spectrometry was to eliminate the interference from the co-eluted substances in the complex sample. The matrix effect was discussed, and the determination was accurate. The validated method was successfully applied in the quantification of eight Sudan dyes in 10 samples.

2. Experimental

2.1. Reagents

Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Red G, Sudan Red 7B, Sudan Black B and Sudan Yellow were
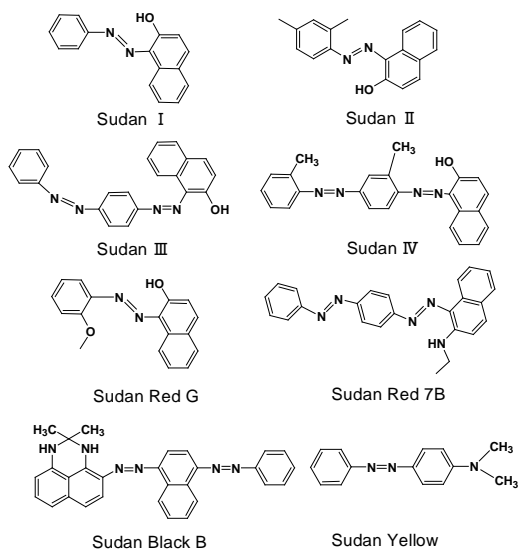
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Figure 1. Chemical structures of Sudan dyes.

purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile of HPLC-grade, used for the preparation of the mobile phases in LC-MS/MS analyses and extraction of the dyes from the chili powder, was purchased from Dikma (Fair Lawn, NJ, USA). Ultra pure water from a Millipor Milli-Q system (Milford, MA, USA) was used for the preparation of mobile phases. Formic acid of HPLC-grade was purchased from Beijing Chemical Reagents Company (Beijing, China).

Stock solutions of Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Red G, Sudan Red 7B, Sudan Black B and Sudan Yellow at 100 μg/mL were prepared in acetonitrile, and stored at 4˚C in the dark.

2.2. Sample Preparation

A 0.5 g aliquot of the chili powder was weighed into a 25 mL volumetric flask, and the flask filled with acetonitrile. Samples were stirred with a stir bar for 1 h. Then, the mixtures were centrifuged at 10,000 g for 10 min, and filtered on 0.45 μm filters. Then, 2 μL of the substance was injected into LC-MS/MS for analysis. All samples were analyzed in duplicate (two aliquots of the same sample individually extracted and injected).

2.3. LC-MS/MS Analysis

Liquid chromatography was performed with an Acquity UPLC system (Waters, Milford, MA, USA). Chromatographic separation of eight Sudan dyes was done on a Phenomenex XB-C18 column (2.1 mm × 100 mm, 2.6 μm particles), using acetonitrile with 0.1% formic acid (phase A) and water with 0.1% formic acid (phase B) as mobile phases. The flow rate was set at 0.3 mL/min and the gradient was as follows: Isocratic elution at 70% A for 5 min, linear gradient from 70% A to 85% A in 10

min, followed by a return to the initial condition in 1 min. Total runtime was 20 min. The column temperature was set at 30˚C and the sample temperature at 10˚C.

LCQ Advantage mass spectrometry (Thermo, USA) was performed with electrospray ionization in positive-ion mode (ESI⁺). Capillary column temperature was set at 300˚C. The source voltage was set at 3500 V, and the sampling S-lens RF amplitude and collision energy were optimized for each molecule. Other conditions were as listed in **Table 1**.

2.4. Method Validation

Blank samples (samples with amounts of Sudan below LD) of chili powder were spiked with different amounts of Sudan dye (0.01, 0.025, 0.05, 0.25, 0.5, 1.0, and 2.5 μg/mL in the final extract) and analyzed as described above. Linearity was checked by calculating the correlation coefficient, and the matrix effect was investigated by comparing the standard solution at a concentration of

Table 1. MRM parameters of the analysis.

Dye	parent ion (m/z)	product ion (m/z)	S-lens RF amplitude (V)	collision energy (eV)
Sudan I	249.1	93.1 (a)	74	31
		232.1 (b)		13
		128.1 (b)		27
Sudan II	277.1	121.1 (a)	74	19
		106.1 (b)		39
		120.1 (b)		40
Sudan III	353.1	77.1 (a)	102	33
		92.1 (b)		32
		197.1 (b)		18
Sudan IV	381.2	91.1 (a)	110	32
		224.1 (b)		21
		106.1 (b)		35
Sudan Red G	279.1	108.1 (a)	74	33
		123.1 (b)		19
		80.1 (b)		50
Sudan Red 7B	380.2	115.0 (a)	90	50
		183.1 (b)		15
		169.1 (b)		32
Sudan Black B	457.2	194.1 (a)	152	33
		193.1 (b)		48
		246.1 (b)		26
Sudan Yellow	226.1	77.1 (a)	90	21
		120.1 (b)		32
		51.1 (b)		51

(a) Ion for quantification; (b) Ion for qualification.

0.05 μg/mL with the standard solution in chili powder.

Blank samples of chili powder were spiked with 0.25, 0.5, and 1.0 mg/kg of each Sudan dye, extracted, and analyzed as described above. Precision was calculated by dividing the standard deviation of the five determination by the arithmetic mean of the values on three different validation days. Recovery was determined by dividing the measured amount of Sudan dye by the amount of spiking.

3. Results and Discussion

3.1. Optimization of Instrumental Conditions

UPLC-ESI-MS/MS was used for qualitative and quantitative analysis of the eight dyes in chili powder. Each standard solution of 10 μg/mL for parameter tuning was introduced directly into the electrospray ionization (ESI) source in combination with mobile phase at a flow rate of 5 mL/min in both positive and negative ionization modes. Positive mode resulted in better sensitivity. The quantification was accomplished by calculating the detector's responses of daughter ion in multiple reaction monitoring (MRM) mode. The parameters of eight Sudan dyes were showed in **Table 1**.

3.2. Method Validation

The possibility of matrix effects was investigated by comparing responses obtained from the standard solution with or without matrix. The results were shown in **Figure 2**. Significant matrix effects were observed, including ion suppression and ion promotion. Because of the matrix effect, calibration plots were prepared using a series of calibration solutions prepared in blank extract instead of standard solutions in the analysis.

A typical chromatogram of the analyses was shown in **Figure 3**. The extracted ions current technique in the mass spectrometry can significantly enhance the selectivity and sensitivity. The linearity of the calibration curves was obtained between the concentrations and the area of each Sudan dye. The correlation coefficients for the calibration plots were better than 0.99 for all eight Sudan
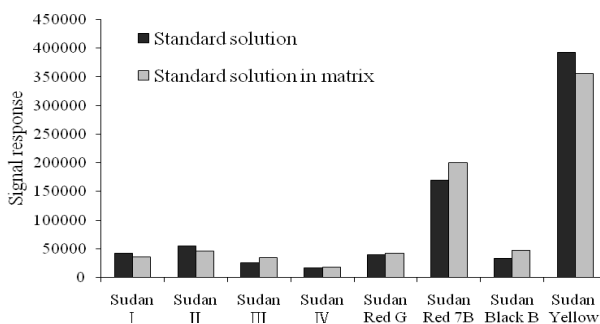

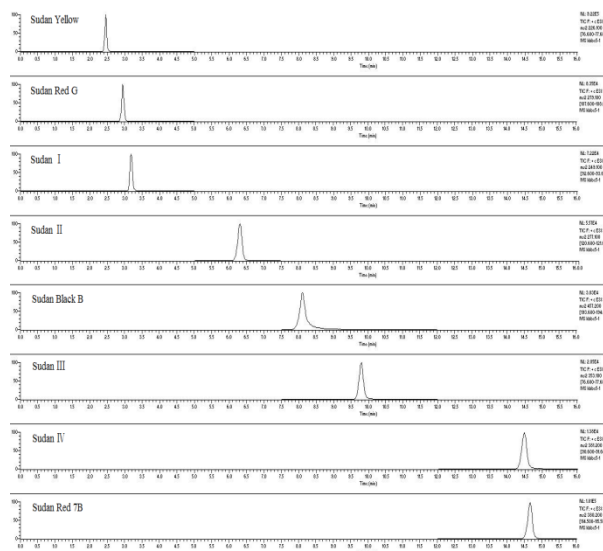**Figure 2. Matrix effects on LC-MS/MS analysis.**

Figure 3. Extracted ion chromatogram of eight Sudan dyes.

Table 2. LOD and LOQ of LC- MS/MS analysis.

Dye	LOD	LOQ
	(mg/kg)	(mg/kg)
Sudan I	0.03	0.10
Sudan II	0.01	0.05
Sudan III	0.003	0.01
Sudan IV	0.01	0.05
Sudan Red G	0.01	0.05
Sudan Red 7B	0.003	0.01
Sudan Black B	0.002	0.005
Sudan Yellow	0.001	0.002

dyes, showing the linearity of the method over the entire calibration range. The LODs (S/N = 3) and LOQs (S/N = 10) of the method for eight Sudan dyes in chili powder were in the ranges of 0.001 - 0.03 and 0.002 - 0.1 mg/kg, respectively (**Table 2**). This showed that the method was specific and should not cause false positive results.

As shown in **Table 3**, the intra-day precision ranged from 2.49% to 10.16% and inter-day precision ranged from 2.24% to 12.20% for each amount spiked at 0.25, 0.50, and 1.00 mg/kg. Mean recovery was 80.7% - 103.1% with intra-day and 85.6% - 104.4% with inter-day. These results demonstrate that the method was repeatable and no noteworthy losses occur during the extraction procedure.

3.3. Analysis of Real Samples

The method was successfully applied in the determination of eight Sudan dyes in chili powder. A total of 10 samples of chili powder were analyzed in duplicate. No Sudan dyes were detected in these samples.

Table 3. Precision and recovery of LC- MS/MS analysis (n = 5).

Dye	amount spiked	Intra-day		Inter-day	
	(mg/kg)	Recovery	Precision	Recovery	Precision
Sudan I	0.25	87.7%	4.75%	90.7%	5.27%
	0.50	90.0%	3.97%	93.2%	3.16%
	1.00	94.0%	7.05%	90.4%	5.80%
Sudan II	0.25	86.1%	4.59%	91.9%	3.43%
	0.50	88.5%	2.49%	91.7%	2.24%
	1.00	91.1%	5.27%	89.7%	4.64%
Sudan III	0.25	80.7%	7.60%	86.3%	5.50%
	0.50	82.5%	2.91%	89.3%	3.12%
	1.00	89.2%	5.80%	88.9%	5.06%
Sudan IV	0.25	81.1%	6.27%	85.6%	7.58%
	0.50	101.6%	3.97%	104.4%	4.76%
	1.00	103.1%	4.49%	100.1%	12.20%
Sudan Red G	0.25	90.8%	7.31%	93.4%	5.06%
	0.50	90.5%	3.83%	93.5%	2.88%
	1.00	96.2%	5.42%	93.3%	4.14%
Sudan Red 7B	0.25	92.4%	10.16%	99.7%	6.24%
	0.50	101.8%	3.21%	100.5%	3.15%
	1.00	91.7%	5.97%	95.9%	7.68%
Sudan Black B	0.25	88.3%	4.10%	89.2%	4.40%
	0.50	89.6%	4.23%	89.1%	3.25%
	1.00	93.7%	7.20%	91.4%	4.07%
Sudan Yellow	0.25	98.8%	5.77%	94.2%	4.34%
	0.50	98.7%	3.37%	94.8%	2.42%
	1.00	100.7%	5.88%	93.0%	3.60%

4. Conclusion

A sensitive UPLC-ESI-MS/MS assay was developed to determine eight Sudan dyes in chili powder. The method had simple pretreatment, and offered high selectivity. Sensitivity, precision and recovery of variation were acceptable. The method could be used for routine analysis.

5. Acknowledgements

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