Development and Validation of a Confirmatory HPLC Method for Simultaneous Determination of Sudan Dyes in Animal Tissues and Eggs

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) analytical method for the simultaneous determination of six Sudan dyes (Sudan Red G, Sudan I, Sudan II, Sudan III, Sudan Red 7B, Sudan IV) in animal tissues and eggs was developed. Samples were extracted with acetonitrile followed by a cleanup using a C_{18} solid-phase extraction column. Chromatographic separation was achieved on a Zorbax SB-C_{18} column under gradient conditions. The analytes were detected at 510 nm by HPLC with diode array detection. Specificity, decision limit (CCα), detection capacity (CCβ), accuracy, and precision were determined during validation process. Recoveries for six Sudan dyes from three animal tissues and eggs were 77.2–98.0% with excellent relative standard deviations. CCα and CCβ were in the range of 7.7–9.0 µg/kg and 9.1–10.3 µg/kg, respectively. The limits of quantitations were between 12.8 µg/kg and 15.0 µg/kg.

Introduction

It is well-known that Sudan dyes have been classified as Category 3 carcinogens by the International Agency for Research on Cancer (IARC) (1,2), and the use of Sudan I in foodstuff is prohibited in Global Food Regulation Act (3).

Several methods have been widely used for the determination of Sudan dyes in water and food by reversed-phase high performance liquid chromatography (HPLC) with UV or diode array detection (DAD) (4–13), mass spectrometry (MS) (1,9,14), tandem mass spectrometry (MS–MS) detection (2–3,15–17) and chemiluminescence (CL) (18). Other analytical procedures, including molecularly imprinted solid-phase extraction (SPE) (19), solid-phase spectrophotometry (20), and CL method (21), also have been reported. However, many HPLC methods with low sensitivity (4–7) are not suitable to quickly detect trace levels of Sudan dyes in animal tissues and eggs. In order to improve the sensitivity of HPLC for Sudan dyes in food, SPE procedure using alumina-*N* (8–10) and humic acid-bonded silica (12) had been adopted. The methods with alumina-*N* SPE procedure can be better in removing impurities, but the sample pre-treatment is very complex. The method using humic acid-bonded silica SPE is simple and sensitive, but humic acid-bonded silica need about 48 h to prepare. An HPLC method had been developed for determination of Sudan dyes in eggs with high sensitivity (13). However, the method is only a kind of screening method, and the suspected sample must be confirmed by gas chromatography with mass spectrometry (GC–MS). Liquid chromatography combined with MS or MS–MS has also been successfully applied to determine Sudan dyes in food with high sensitivity but requires much more demanding and cost-intensive analytical equipment.

In this paper, we described a confirmatory HPLC method for simultaneous determination of six Sudan dyes (Sudan Red G, Sudan I, Sudan II, Sudan III, Sudan Red 7B, Sudan IV) in animal products. A C_{18} SPE was developed in purification procedure for the first time. It is proven that the C_{18} SPE procedure has a good matrix clean-up and assures an adequate selectivity without the use of MS detection through validation of the method. Moreover, a new HPLC operating conditions that can simultaneously separate of six Sudan dyes was developed in this paper. Validation parameters tested included decision limit (CCα), detection capacity (CCβ), specificity, linearity, accuracy, and precision. Finally, the method was applied to the analysis of Sudan dyes residues in duck eggs from local markets.

Experimental

Reagents and materials

Acetonitrile and methanol were HPLC-grade. Hexane, formic acid, and anhydrous sodium sulfate were analytical-grade. All

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standards were obtained from Sigma (Deisenhofen, Germany). The purity of Sudan Red G, Sudan I, Sudan II, Sudan III, Sudan Red 7B, and Sudan IV were 95%, 97%, 90%, 96%, 95%, and 97%, respectively. Water was purified with a Milli-Q reverse osmosis system (Millipore, Milford, MA). The Bond Elut C_{18} cartridges (500 mg, 3 mL) were purchased from Varian (Palo Alto, CA).

Standard solutions

Individual stock standard solutions (40 mg/L) of Sudan I–IV, Sudan Red G, and Sudan Red 7B were prepared by dissolving the pure reference standards (taking account of the stated purity of the product) in acetonitrile. Solutions for sample spiking (500, 750, 1000 μg/L of the six Sudan dyes) were prepared by diluting the stock solution with methanol. Working standards for calibration curves were prepared weekly. These solutions were stored at 4°C.

Food samples

Chicken muscle, duck muscle, and duck egg were obtained from local markets and pre-examined for the absence of Sudan dyes (Sudan Red G, Sudan I, Sudan II, Sudan III, Sudan Red 7B, Sudan IV) by the following method. All samples were mixed homogeneously and stored in –20°C refrigerator.

Instrumentation and separation conditions

A Waters 2690 instrument with 996-photodiode array detector (Milford, MA) was used for the experiments. Separation was carried out on a Zorbax SB-C_{18} (150 mm × 4.6 mm, 5 μm) column maintained at 30°C. The LC mobile phase consisted of water (solvent A) and 0.1% (v/v) formic acid in methanol (solvent B). The linear gradient program was: 75% B at beginning linear ascend to 100% within 15 min, then keep 100% for 5 min, and back to 75% within 1 min, equilibration for 4 min. The DAD detector was set at 510 nm, which is an average maximum absorption wavelength for all of these Sudan dyes.

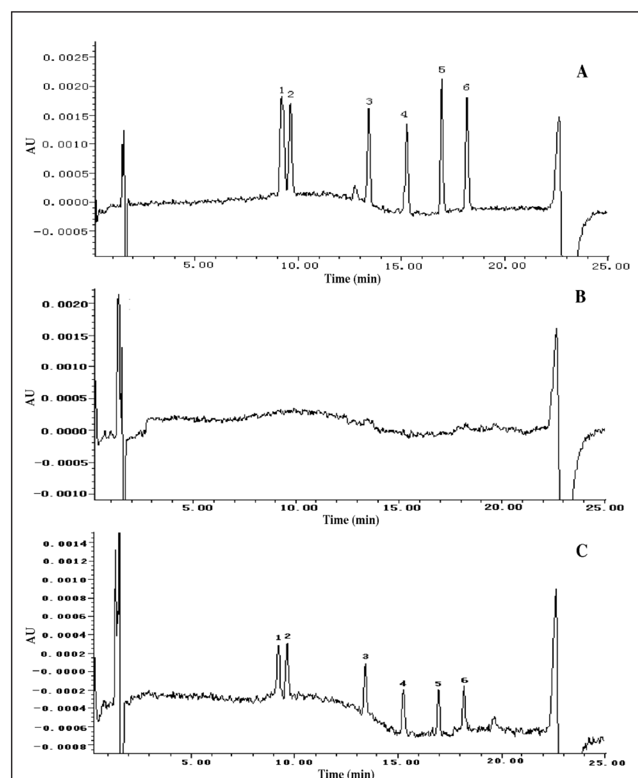

Figure 1. (A) The chromatogram of six Sudan dyes standard at 100 μg/L. (B) The chromatogram of duck egg blank sample. (C) The chromatogram of duck egg sample spiked with Sudan dyes at 20 μg/kg. Peaks 1 = Sudan Red G, 2 = Sudan I, 3 = Sudan II, 4 = Sudan III, 5 = Sudan Red 7B, 6 = Sudan IV.

Sample preparation

Five grams of sample and 10 g of anhydrous sodium sulphate were weighed into a 50-mL polypropylene centrifuge tube, homogenized with 15 mL of acetonitrile for 1 min using a high-speed Ultra-Turrax T25 blender (IKA, Staufen, Germany). After centrifugation for 2 min at 3000 rpm, the supernatant (organic phase) was transferred into a clean centrifuge tube, and the remainder of the sample was extracted once more with 15 mL of acetonitrile. Then, 10 mL of the extracts were mixed with 10 mL of water and applied to a Bond Elut C_{18} cartridge pre-washed with 5 mL of methanol and 5 mL of water. The cartridge was air-dried by aspiration for 2 min. Sudan dyes were eluted from the cartridge with 12 mL hexane. The eluate was accurately evaporated to dryness in a rotary evaporator at 40°C, and the residue was dissolved in 1 mL methanol. The resulting solution was filtered through 0.45-μm nylon membrane filter, and 50 μL of the filtrate were injected into the HPLC for the determination of Sudan dyes.

Validation study

The evaluation of the suitability of the whole procedure for the determination of six Sudan dyes in animal products was carried out according to the European Commission Decision 2002/657/EC (22). To verify the specificity, 20 blank samples from each tissue were analyzed.

Repeatability (precision) and recovery (accuracy) of Sudan dyes were measured in blank samples that were fortified at 10 μg/kg, 15 μg/kg, and 20 μg/kg.

$CC\alpha$ was calculated as three times the signa-to-noise ratio at the time window in which the analyte is expected in the chromatograms of 20 blank samples. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the $CC\beta$.

The stability of Sudan dyes was determined in three different ways: (a) in solvent (stock solutions), (b) in matrix (fortified three samples at 20 μg/kg), and (c) in sample final extracts prior to the HPLC–DAD analysis.

Results and Discussion

Optimization of the method

HPLC operating conditions

After optimization of the gradient parameters and concentration of formic acid in mobile phase, we utilized water and 0.1% formic acid in methanol as mobile phase to separate Sudan dyes on Zorbax SB-C_{18} column under gradient

conditions. The flow rate was 1.0 mL/min, and the monitoring wavelength was 510 nm, which is an average maximum absorption wavelength for all of these Sudan dyes. The chromatogram of six Sudan dyes (100 µg/L) separated with this condition is shown in Figure 1A.

Extraction method development

The extraction efficiencies were studied to adjust the following parameters: the amount of anhydrous sodium sulphate and the volumes of acetonitrile for extracting Sudan dyes from the matrix. Fortified samples were mixed with different amount of anhydrous sodium sulphate (0, 5, 10, 15, and 20 g) and extracted three times each with 15 mL of acetonitrile. The recoveries increased with amount of anhydrous sodium sulphate from 0 to 10 g and remained constant when the amount of anhydrous sodium sulphate was more than 10 g. Therefore, 10 g of anhydrous sodium sulphate was added to the sample during the extraction procedure.

It was found that 91–93% of Sudan Red G, Sudan I, Sudan II, Sudan III and 82–84% of Sudan Red 7B, Sudan IV were recovered in the first extract; 6–8% of Sudan Red G, Sudan I, Sudan II, Sudan III and 14–15% of Sudan Red 7B, Sudan IV in the second and 1–3% in the third. As satisfactory recoveries were given by the first and second extractions, Sudan dyes were extracted twice using 15 mL of acetonitrile.

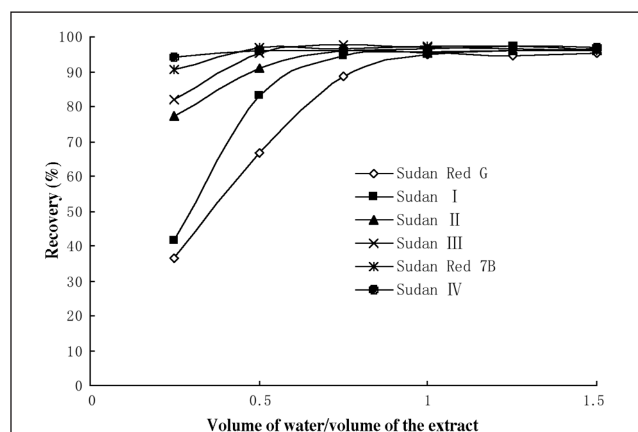

Figure 2. Recoveries of Sudan dyes from different ratio of the extract and water.

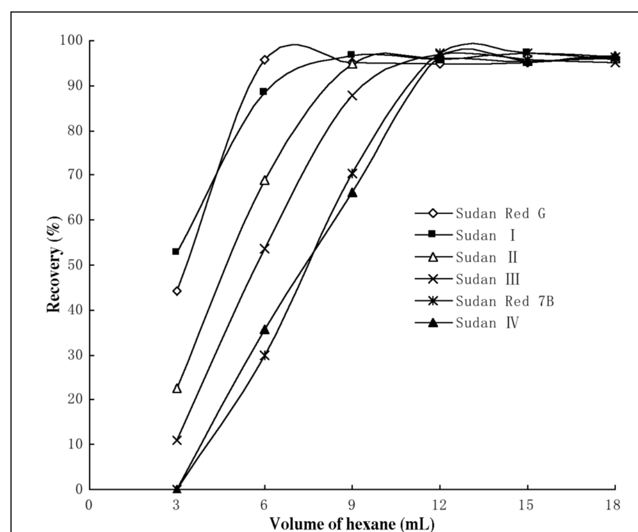

Figure 3. Recoveries of Sudan dyes from different volume of hexane as eluent.

SPE procedure

The extract from the blank sample without a cleanup step had been directly injected into the HPLC. Interfering peaks were found around the retention time of Sudan Red 7B and Sudan IV. Therefore, we used a C_{18} cartridge, and several conditions of SPE were optimized to eliminate interferences.

The influence of the ratio of the extract and water was investigated. Sudan dyes (0.4 µg each) were added to 10 mL extract from blank sample, then mixed with 2.5, 5, 7.5, 10, 12.5, and 15 mL of water, respectively, and treated with the cartridge as described in the sample preparation. As shown in Figure 2, high recovery of the six Sudan dyes was obtained from C_{18} cartridges when 10, 12.5, or 15 mL of water were used. We therefore added 10 mL of water to the extracts prior to sample loading.

During the method development, we wished to elute the Sudan dyes with acetonitrile, methanol or the mobile phase, so that the eluents can be directly injected into HPLC system without additional treatment. However, the presence of chemical interference from co-extracts was observed affecting the chromatographic determination. After many trials, it was found that hexane used as eluent solvent produced the cleanest chromatograms. Moreover, the volume of hexane was investigated. As shown in Figure 3, the recoveries increased with volume of hexane from 0 to 12 mL and kept constant when the volume of hexane was more than 12 mL. So, 12 mL of hexane was chosen to elute the Sudan dyes from the SPE column.

Validation of the method

The results of the linearity at seven levels (15, 50, 100,200, 500, 1000, and 2000 µg/L) are reported in Table I. The linear correlation coefficients (r^2) of the calibration curves were more than 0.9999. The chromatogram of a standard mixture is shown in Figure 1A.

The specificity was evaluated by the analysis of 20 blank samples. No interferences were observed in the region of interest where the analytes were eluted. The chromatogram of blank duck egg is shown in Figure 1B.

Table I. Retention Time (t_R), Linearity*, and Linear Correlation Coefficients of Six Sudan Dyes

Analyte	t_R(min)	b†	a‡	r^2
Sudan Red G	9.107	200.16 ± 0.42	–902 ± 86	1.0000
Sudan I	9.527	173.49 ± 0.30	–876 ± 185	1.0000
Sudan II	13.342	151.59 ± 0.40	–535 ± 75	0.9999
Sudan III	15.301	148.18 ± 0.37	–1786 ± 274	0.9999
Sudan Red 7B	16.991	193.91 ± 0.24	–1040 ± 166	0.9999
Sudan IV	18.223	203.15 ± 0.15	–1455 ± 244	0.9999

* Linear range investigated: 15, 50, 100, 200, 500, 1000, and 2000 µg/L.
† b = slope (± SD of slope).
‡ a = intercept (± SD of intercept).

For Sudan dyes for which no permitted limit has been established, repeatability and recovery of the method should be measured by the analysis of six fortified samples at 1, 1.5, and 2 times the minimum required performance limit (MRPL) based on European Commission Decision 2002/657/EC. However, these MRPLs of Sudan dyes have not yet been established; an auxiliary parameter had to be used to carry out this validation study (23). The concentrations of the analytes in the three repeatability series studied were then chosen in such a way that the lowest concentration was within the range of the assumed minimal concentration that could still be confirmed, and the next levels were set at 1.5 and 2 times of that concentration (23). The results of repeatability and recovery are shown in Table II. The chromatogram of fortified sample is shown in Figure 1C. The mean recoveries (n = 18) ranged from 77.2 to 98.0% for Sudan dyes with relative standard deviation between 2.3 and 14.9%.

The values of the CCα and CCβ are presented in Table III. Additionally, limits of quantitations (LOQs), traditional analytical parameter, were between 12.8 μg/kg and 15.0 μg/kg, which is better than most of HPLC methods (4–9,11).

The stock standard solutions, prepared in acetonitrile, were stable for at least three months stored at 4°C. The stock solutions were analyzed every month, and the instrumental responses were compared with the peak areas obtained at the moment of solution preparation (t = 0). The acceptance criterion was a response comprised between 95 and 105% of the initial one.

Table II. Repeatability and Recovery for the Determination of Six Sudan Dyes in Animal Tissues and Eggs (n = 18)

		Chicken muscle		Duck muscle		Duck eggs	
Fortified level	Analyte	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
10 μg/kg	Sudan Red G	91.8	12.5	92.4	10.2	96.0	12.2
	Sudan I	93.6	10.6	89.6	5.7	89.0	8.8
	Sudan II	86.7	6.6	87.3	5.6	98.0	11.2
	Sudan III	90.5	7.6	92.9	12.6	91.0	11.1
	Sudan Red 7B	77.2	12.7	80.6	10.5	83.0	14.8
	Sudan IV	83.1	11.5	80.0	10.2	86.0	14.9
15 μg/kg	Sudan Red G	88.6	7.5	97.2	6.2	93.3	7.9
	Sudan I	91.1	6.0	87.5	5.1	90.7	5.6
	Sudan II	91.9	7.8	88.0	6.0	94.0	6.0
	Sudan III	88.7	6.5	91.2	5.0	96.7	6.8
	Sudan Red 7B	85.6	8.7	83.7	5.7	84.0	10.8
	Sudan IV	84.3	8.5	89.1	11.6	84.7	9.5
20 μg/kg	Sudan Red G	89.2	6.3	89.0	6.8	90.0	7.5
	Sudan	92.0	5.8	90.1	5.0	93.0	5.5
	Sudan II	88.2	2.3	85.6	4.8	92.0	7.7
	Sudan III	92.0	6.6	84.0	4.6	94.0	5.3
	Sudan Red 7B	82.1	7.8	78.8	8.0	79.5	10.8
	Sudan IV	83.0	12.9	82.2	9.1	78.5	13.6

Fortified samples at 20 μg/kg stored at −20°C were analyzed over a three-week period. For the period of study, we did not observe obvious degradation of Sudan dyes. Finally, to check the stability of purified extracts of fortified samples at 20 μg/kg stored at 4ºC, they were reanalyzed by HPLC–DAD after 24 h and 48 h, and similar results were observed.

Application of the method

The method was applied to determination of 80 duck egg samples from local markets. There were two duck egg samples containing Sudan IV with the concentration of 37.9 μg/kg and 164.2 μg/kg. The chromatograph of positive sample (37.9 μg/kg) is shown in Figure 4.

Conclusions

In this paper, an analytical method to determine Sudan dyes in animal products was developed using a combination of clean up on a C_{18} cartridge and HPLC–DAD. The newly developed sample pre-treatment procedure effectively removed the potential matrix interferences from endogenous substances of animal products. The present method is simple, rapid, and reliable but also permits the simultaneous determination of six different kinds of Sudan dyes with good recoveries (77.2–98.0%), relative standard deviation (2.3–14.9%), CCα (7.7–9.0 μg/kg), and CCβ (9.1–10.3 μg/kg).

Table III. CCα and CCβ* (μg/kg) Obtained for 6 Sudan Dyes in Animal Products†

	Chicken muscle		Duck muscle		Duck egg	
Analyte	CCα	CCβ	CCα	CCβ	CCα	CCβ
Sudan Red G	8.4	10.3	8.3	9.9	8.0	10.0
Sudan I	7.8	9.4	8.2	9.3	8.1	9.4
Sudan II	9.0	9.9	8.8	9.6	8.1	9.9
Sudan III	8.5	9.8	8.3	10.2	8.4	10.1
Sudan Red 7B	8.3	9.9	7.7	9.1	7.7	9.8
Sudan IV	7.8	9.5	7.9	9.3	7.8	10.0

* CCβ calculated as CCα + 1.64.
† Intra-laboratory reproducibility (SD) of the lowest concentration level.

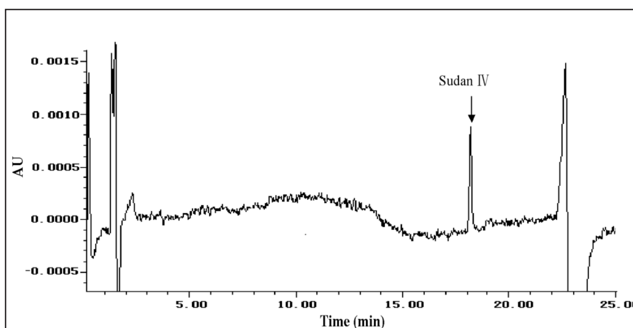

Figure 4. The chromatogram of positive duck egg sample.

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