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Simultaneous determination of fifteen illegal dyes in animal feeds and poultry products by ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

With the increasing presence of illegal dyes, such as sudan reds and malachite green, in animal feeds and food products during the last few years, there is an urgent need of accurate quantitative determination methods for these illicit compounds. Here we established an accurate method for the simultaneous determination of 15 illegal dyes in animal feeds, meat, eggs and other food products using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS). The samples were extracted with a simple procedure using acetonitrile and solid phase extraction cleaning up. The application of C_{18} rapid column can achieve satisfactory separation of the 15 dyes within 16 min; and multiple reaction monitoring of positive ions ensure confirmative detection of these illegal dyes. With the developed method, a sample can be analyzed in less than 2 h. Dyes spiked in feeds, poultry meat and eggs in the range of 0.1–5.0 mg kg^{-1} were tested in terms of linearity, sensitivity, repeatability and recovery. Recoveries for the compounds ranged from 60 to 140%. Intra- and inter-day precisions (RSDs) were less than 15%. Limit of quantification ranged from 0.01 to 5.61 μg kg^{-1} for different dyes. The developed UHPLC–MS/MS method could be used as a qualitative and quantitative technique for the simultaneous determination of illegal dyes in animal feeds and poultry products.

1. Introduction

Food quality is often closely associated with its color. The use of food colorants is an age-old practice to enhance aesthetical appeal of food products [1]. The use of synthetic organic dyes has been recognized as the most reliable and economical method of restoring or providing color to processed food products. Azo compounds are by far the most widely used synthetic colorants [2]. They are synthetic fat-soluble organic colorants with characteristic chromophoric azo (N=N) groups [3]. Currently, there are over 3000 azo dyes in use and they account for 65% of the commercial dye market [4]. Examples of azo dyes include sudan I–IV, sudan red B, sudan red G, sudan red 7B, sudan orange G, para red and toluidine red. Some azo dyes, especially the sudan group dyes and their degradation products, have been recognized as carcinogens [5], thus the application of such dyes has been prohibited in many countries. Malachite green (MG), another harmful dye, is a triphenylmethane compound, which is used as fungicide and antiseptic in aquaculture and fisheries. When absorbed by fish, it is metabolically reduced to the lipophilic leucomalachite green (LMG), which is known to have a long residual time in edible fish tissues and exists as a potential health and environmental hazard [6]. Nowadays MG is not allowed for veterinary application in most countries due to its carcinogenic and mutagenic properties. Other dyes such as rhodamine B, and rhodamine 6G are also considered to be potentially carcinogenic [7]. As a result, these dyes are classified into category 3 by the International Agency for Research on Cancer [8] and are banned as food additives in the European Union [9,10]. Any level of these dyes is considered to be unsafe for human consumption. However, there were many examples of unlawful uses of these dyes in food products in the past years, for example, sudan I was identified in a chili product from India in France in 2003; later, in 2005, a food alarm was raised in UK caused by the presence of sudan dyes and para red in food; in 2006, sudan dyes were detected in eggs and MG and LMG contaminated fishes were found in China. Thus, it is necessary to develop accurate and reliable analytical methods for the confirmative determination of illegal dyes in foodstuff to ensure consumer health.

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Various analytical methods have been reported for the determination of dyes in edible animal tissues and spices, including liquid chromatography [11–14], flow injection chemiluminescence [15–17], enzyme-linked immunosorbent assays [18,19], capillary electrophoresis [20], and elctrochemical methods [21–24]. The most popular method involves liquid chromatography separation followed by spectrophotometric detection. For example, Francisco et al. reported a rapid method for the determination of sudan dyes in chili using liquid chromatography with photodiode array detector [25]. There were also reports on the detection of malachite green and its metabolite leucomalachite green by spectrophotometric methods [26,27]. The major drawback of these spectroscopic methods is low specificity, multiple dyes in complex sample matrix may not be completely detected.

With increasing demand for the confirmation of dyes present in food, liquid chromatography tandem mass spectrometry (LC–MS/MS) based methods are more and more widely used. Because of their high sensitivity and selectivity, LC–MS based methods provide the capability of multi-component validation, quantification and microanalysis [28]. Liquid chromatography with triple quadrupole (LC–QQQ-MS) or ion trap (LC–IT-MS) were used for the simultaneous determination of azo-dyes in chili products, spices [7,29,30], malachite green, leucomalachite green and rhodamine 6G in fish [31–34]. LC–TOF-MS based methods have also been reported because of the high specificity provided by TOF mass analyzer [35,36].

Most researchers pay more attentions to dye residues in foodstuffs because of their direct relationship with human health. However, as we know, many of harmful additives were added into feeds, and their metabolites and other harmful residues stay in animal body and eventually turn into edible food products. So it is very important to detect these harmful additives in feeds to prevent them from entering the food chain. However, to the best of our knowledge, only relatively few methods have been developed to analyze sudan dyes and MG added in animal feeds. There were no reports on simultaneous determination of azo and non-azo dyes such as malachite green in animal feeds and products. Animal feeds have more complex matrices than food products since feed samples typically contain significant amounts of inorganic salts, metal ions, carbohydrate, proteins, and other additives, these matrices can interfere with the detection of dyes. In addition, the concentrations of dyes in animal feeds are generally higher than those in food products. Thus, we have to develop methods, especially sample extraction and cleanup procedure that are suitable for feed samples. In this study we developed a qualitative and quantitative method for the simultaneous detection of fifteen different azo and non-azo dyes in animal feeds, eggs and poultry meat by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS). The proposed method can realize fast separation of the dyes in a 16-min gradient elution. Sub ppb level sensitivity was achieved for most of the dyes tested. The method is validated by evaluating recovery, selectivity, linearity, accuracy and repeatability.

2. Experimental

2.1. Reagents and chemicals

Sudan I (96.1%), sudan II (96.1%), sudan III (91.2%), sudan IV (82.5%), sudan B (90.5%), sudan G (93.5%), sudan 7B (89.0%), sudan orange G (98.8%), para red (95.5%), toluidine red (68%), rhodamine B (92.0%), rhodamine 6G (99.0%), malachite green (98.0%), leucomalachite green (94.0%) and canthaxanthin (96.1%) were purchased from the Dr. Ehrenstorfer GmbH Company (Augsburg, Germany) and were used without further purification (Table 1). Standard stock solutions of each dye (0.5 mg mL^{-1}) were prepared by dissolving the dyes in acetonitrile with the exception of para red and toluidine red which were dissolved in acetone. The standard solutions were stored in darkness at −20 °C. Mixed standard stock solution was prepared by diluting the standard stock solution of each dye with acetonitrile to a final concentration of 20 μg mL^{-1}. Working standard solutions were prepared daily by diluting the mixed standard stock solution with acetonitrile in appropriate proportions.

HPLC grade methanol and acetonitrile were obtained from Fisher Scientific International (Hampton, NH). HPLC grade formic acid and ammonium acetate were purchased from Dima Technology (Richmond Hill, Canada). Guarantee grade acetic acid was obtained from the Sinopharm Chemical Reagent Company (Shanghai, China). All other chemicals were analytical grade. Water used was ultrapure water (Milli-Q, Millipore Corporation, Billerica, MA).

2.2. Apparatus and procedures

Ultra-high performance liquid chromatography tandem mass spectrometry was performed on an Agilent 1200 UHPLC system coupled with a 6460 Mass Selective Detector (Agilent Technologies, Fermont, CA). Neutral Alumina Cartridges (500 mg, 6 mL) for sample purification were also purchased from Agilent. A Desktop Constant Temperature Oscillator (Jing Hong, Shanghai, China) was used to promote sample extraction.

2.3. Preparation of feeds and poultry products

Three types of commercially prepared feed samples (premix, concentrate and complete feeds) were made at the pilot mill of the Ministry of Agricultural Feed Industry Center (Beijing, China). Poultry eggs and meat were purchased from local supermarkets. Before extraction, eggs and chicken meat were homogenized in a homogenizer for 5 min (Da Kang, Tianjin, China).

2.4. Sample extraction and purification

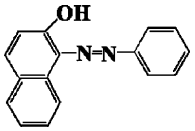
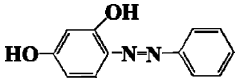
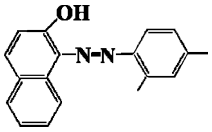
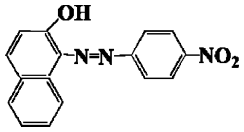
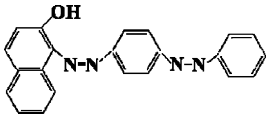
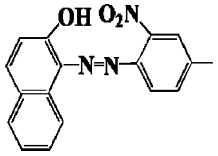
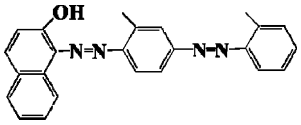
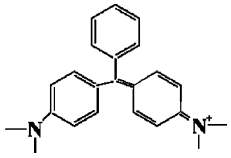
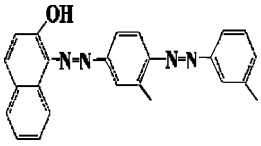
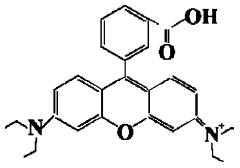
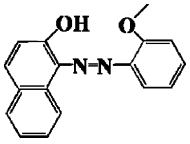
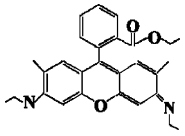
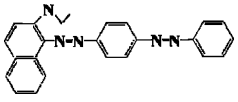
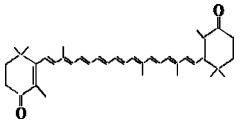
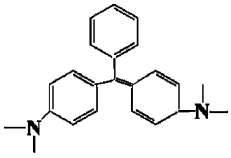
Dye-spiked samples were prepared in a 150 mL conical flask by mixing a fixed amount of matrix (2 g for complete feed, 1 g for concentrate and premix or 2 g for eggs and meat) with a series of the 15-dye mixed standard solutions at various concentrations. Sample extraction was achieved by adding 20 mL acetonitrile into the dye-spiked sample and shaking for 20 min followed by refrigerated centrifuging at 7012 × *g* for 10 min at 4 °C. The supernatant was then subjected to solid phase extraction for further purification.

The neutral alumina column was preconditioned with 3 mL acetonitrile. Then, 1 mL of the extracted supernatant was slowly passed through the column. The analytes were eluted with 3 mL methanol–acetic acid eluent (95:5, v/v). The solution was evaporated to dryness in 50 °C water bath under nitrogen. Finally, the residue was reconstituted with 1 mL acetonitrile and filtered via 0.1 μm microporous film, then 5–20 μL of the solution was injected into the UHPLC system.

2.5. UHPLC–MS/MS conditions

Chromatographic separation of the dye mixture was achieved on an Agilent Eclipse XDB-C_{18} column (2.1 mm × 50 mm, 1.8 μm). The column temperature was 35 °C. The mobile phase consisted of solutions A (0.1% aqueous formic acid solution with 20 mM ammonium acetate) and B (acetonitrile). A gradient program was used for elution: 30% solution B (initial), with 30–50% solution B (from 0 to 2 min), 50–60% solution B (from 2 to 6 min), 60–90% solution

Table 1
Chemical structure of the fifteen dyes tested.

Compound	Structure	Compound	Structure
Sudan I		Sudan orange G	
Sudan II		Para red	
Sudan III		Toluidine red	
Sudan IV		Malachite green	
Sudan B		Rhodamine B	
Sudan G		Rhodamine 6G	
Sudan 7B		Canthaxanthin	
Leucomalachite green			

Source: The structure of dyes was obtained from the manufacturers.

B (from 6 to 10 min), and 90% solution B (from 10 to 16 min). After 16 min, the ratio was reduced to 30% solution B. A 9-min equilibration was necessary before the next injection, so the total run time is 25 min. The mobile phase was delivered at a flow rate of 0.4 mL min^{-1}. The amounts of dyes in the spiked samples were quantified using a calibration curve developed with the mixed dye solution.

The optimized electron spray ionization condition was: gas temperature 350 °C, gas flow 5 L min^{-1}, sheath gas temperature 400 °C, sheath gas flow 12 L min^{-1} and capillary voltage 3500 V. High-purity nitrogen was used as the nebulizing gas. Positive ions were monitored. Multiple reaction monitor mode was applied for quantitative and qualitative analysis. The values of delta electron megavolt (EMV) were set at 400 V (0–5 min), 400 V (5–10 min) and 500 V (10–25 min), respectively.

3. Results and discussion

3.1. Optimization of UHPLC–MS/MS conditions

To achieve the highest selectivity and sensitivity, mass spectrometry parameters including ionization mode, capillary voltage, source temperature, sheath gas flow, nebulizer pressure, fragmentor voltage and collision energy were optimized by direct injection of solutions of individual dyes. In most cases, the most abundant (precursor) ions of the dyes were their molecular ions $[M+H]^+$. The characteristic ion and optimal MS/MS parameters including fragmentor voltage and collision energy for each dye were listed in Table 2.

Chromatographic parameters such as choice of column, mobile phase composition, flow rate, and column temperature were also

Table 2
Tandem mass spectrometry parameters of the fifteen dyes tested.

	Retention time (min)	Molecular weight ($g\,mol^{-1}$)	Quantitative transition (m/z)	Qualitative transition (m/z)	Fragmentor voltage (V)	Collision energy (V)
Sudan I	7.6	248	249/93	249/93	120	25
				249/156	120	15
Sudan II	9.8	276	277/121	277/121	100	20
				277/156	100	15
Sudan III	10.9	352	353/77	353/77	140	25
				353/156	140	20
Sudan IV	12.5	380	381/91	381/91	100	30
				381/224	100	20
Sudan B	12.5	380	381/156	381/156	100	30
				381/224	100	30
Sudan G	7.3	278	279/123	279/123	110	25
				279/108	110	15
Sudan 7B	12.4	379	380/183	380/183	110	10
				380/169	110	20
Sudan orange G	3.4	214	215/93	215/93	140	20
				215/198	140	15
Malachite green	2.9	365	329/208	329/208	100	45
				329/313	100	40
Leucomalachite green	9.2	330	331/239	331/239	100	25
				331/315	100	30
Rhodamine B	3.2	479	443/399	443/399	140	40
				443/355	140	50
Rhodamine 6G	3.8	479	443/415	443/415	130	30
				443/415	130	40
Para red	6.6	293	294/156	294/156	120	15
				294/128	120	25
Toluidine red	7.7	307	308/156	308/156	120	10
				308/128	120	25
Canthaxanthin	14.9	564	565/203	565/203	140	25
				565/133	140	15

tested to obtain the best separation of the 15 dyes. Agilent Eclipse XDB column C_{18} (2.1 mm × 50 mm, 1.8 μm) was compared with Agilent Eclipse Plus C_{18} column (2.1 mm × 100 mm, 1.8 μm). The Eclipse XDB column C_{18} showed better performance in terms of peak shape, resolution and retention time. Our studies indicate that 0.4 mL min^{-1} and 35 °C were the optimal flow rate and column temperature regarding resolution and total run time. Under optimal conditions, total ion chromatogram for the 15-dye mixture was shown in Fig. 1(A), typical quantitative daughter ion chromatograms of the 15 dyes were shown in Fig. 2.

3.2. Optimization of sample extraction and purification

The development of an efficient extraction procedure is the most important step for accurate determination of dyes in animal feeds, eggs and meat. A viable extraction protocol can recover the maximum amounts of dyes from the matrix with minimum interference. Three extract solvents including acetonitrile, ethyl acetate–acetonitrile (50:50, v/v) and methylene chloride were compared. Acetonitrile yielded the most reproducible results and highest recovery for the 15 dyes. Moreover, most of the interference proteins present in feed samples were precipitated in the presence of acetonitrile, and can be efficiently removed by the subsequent refrigerated centrifugation procedure. Thus, acetonitrile was chosen as the extraction solvent.

Most other interfering compounds can be removed in the subsequent clean-up procedure using solid phase extraction. Previous reports on the detection of azo dyes [37] and malachite green [38] used a clean-up procedure with neutral alumina column. The neutral alumina column was preconditioned with acetonitrile, and the analytes were eluted with acetonitrile. However, in our study, the major drawback of using acetonitrile as eluent was that some compounds had low recoveries, especially rhodamine B, sudan orange and sudan 7B. To solve this issue, we tested several different eluents including acetonitrile, methanol–water (95:5, v/v), acetonitrile–formic acid (95:5, v/v), methanol–acetic acid (95:5,

Table 3
Linear relationships and sensitivity for detection of 15 dyes from complete feed sample using tandem mass spectrometry.

	Linear range ($ng\,mL^{-1}$)	Linear equation	R^2	Limit of quantification ($\mu g\,kg^{-1}$)
Sudan I	0.5–200	$y = 186.6x - 26$	0.999	5.24
Sudan II	1–200	$y = 794.0x - 273$	0.999	0.37
Sudan III	0.5–200	$y = 392.1x - 373$	0.999	2.08
Sudan IV	0.5–200	$y = 1617.4x - 590$	0.999	2.12
Sudan B	0.5–200	$y = 298.0x - 63$	0.999	3.52
Sudan G	1–200	$y = 1629.2x - 1578$	0.999	0.80
Sudan 7B	1–200	$y = 1780x + 2953$	0.996	0.16
Sudan orange G	0.5–200	$y = 163.3x + 218$	0.989	2.90
Malachite green	0.1–20	$y = 220.0x - 75$	0.998	0.20
Leucomalachite green	0.05–20	$y = 1382.2x + 1393$	0.988	0.01
Rhodamine 6G	0.1–20	$y = 463.4x + 244$	0.994	0.06
Rhodamine B	0.1–20	$y = 502.39x + 87$	0.988	0.04
Para red	5–2000	$y = 711.2x - 3246$	0.987	5.61
Toluidine red	1–200	$y = 379.5x - 926.$	0.997	0.50
Canthaxanthin	0.5–200	$y = 342.0x + 377$	0.998	0.16

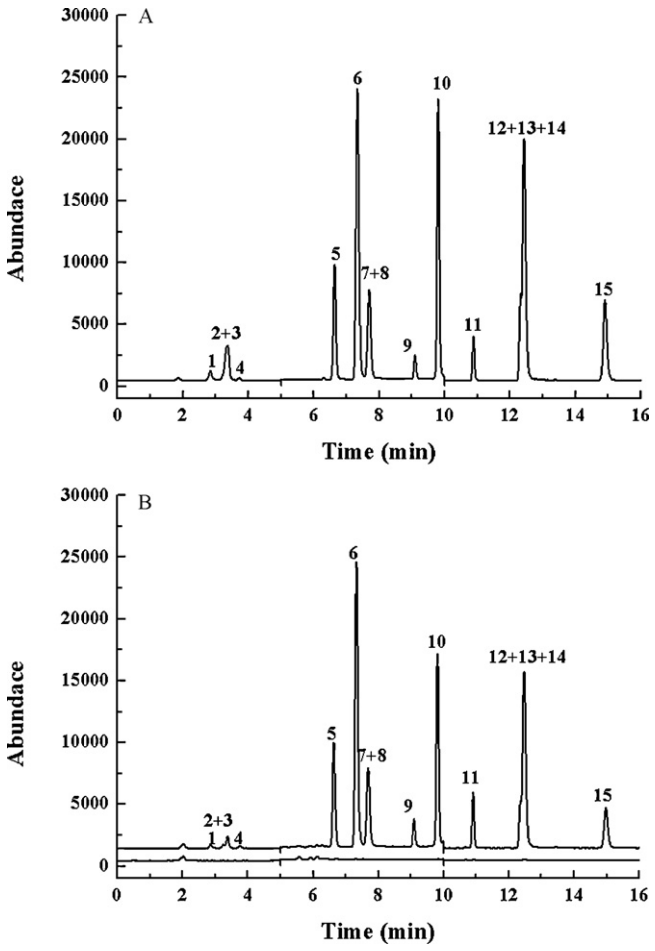


Fig. 1. Total ion chromatogram (TIC) for (A) the 15-dye mixed standard; (B) complete feed sample spiked with 15-dye mixed standard and blank feed sample. (1) Malachite green; (2) rhodamine B; (3) sudan orange G; (4) rhodamine 6G; (5) para red; (6) sudan G; (7) sudan I; (8) toluidine red; (9) leucomalachite green; (10) sudan II; (11) sudan III; (12) sudan 7B; (13) sudan IV; (14) sudan B; (15) canthaxanthin.

v/v) and methanol–acetic acid (90:10, v/v). The results indicate that 5% acetic acid–methanol solution has the highest and most stable dye recoveries (Fig. 3). Thus, for the final clean-up procedure, the neutral alumina column was preconditioned with 3 mL of acetonitrile; and the analytes were eluted with 3 mL methanol–acetic acid (95:5, v/v).

3.3. Method validation

The linearity, sensitivity, as well as precision and accuracy of the method were validated by a series of experiments described below. Linearity was studied by analyzing mixed standard solution of the dyes at several concentrations ranging from 0.05 to 2000 ng mL^{-1}. Most of the dyes showed satisfactory linearity within the concentration range of 1.0–20 ng mL^{-1} except para red, which had a significantly higher concentration range (Table 3). Linear regression analysis showed that the correlation coefficients of all the standards were better than 0.987. Limit of quantitation (LOQ), which was defined as the concentration at 10 times the signal intensity of noise, was determined by examining UHPLC–MS/MS spectra of dye-spiked complete feed. The LOQ values were in the range of 0.04–5.61 μg kg^{-1} (Table 3). To evaluate the reproducibility of the method, inter- and intra-assay reproducibilities were tested. For example, the recoveries of dyes spiked in complete feed were determined by six repeats at intermediate concentration (0.1 mg kg^{-1}). The results indicated that the recoveries of the dyes ranged from

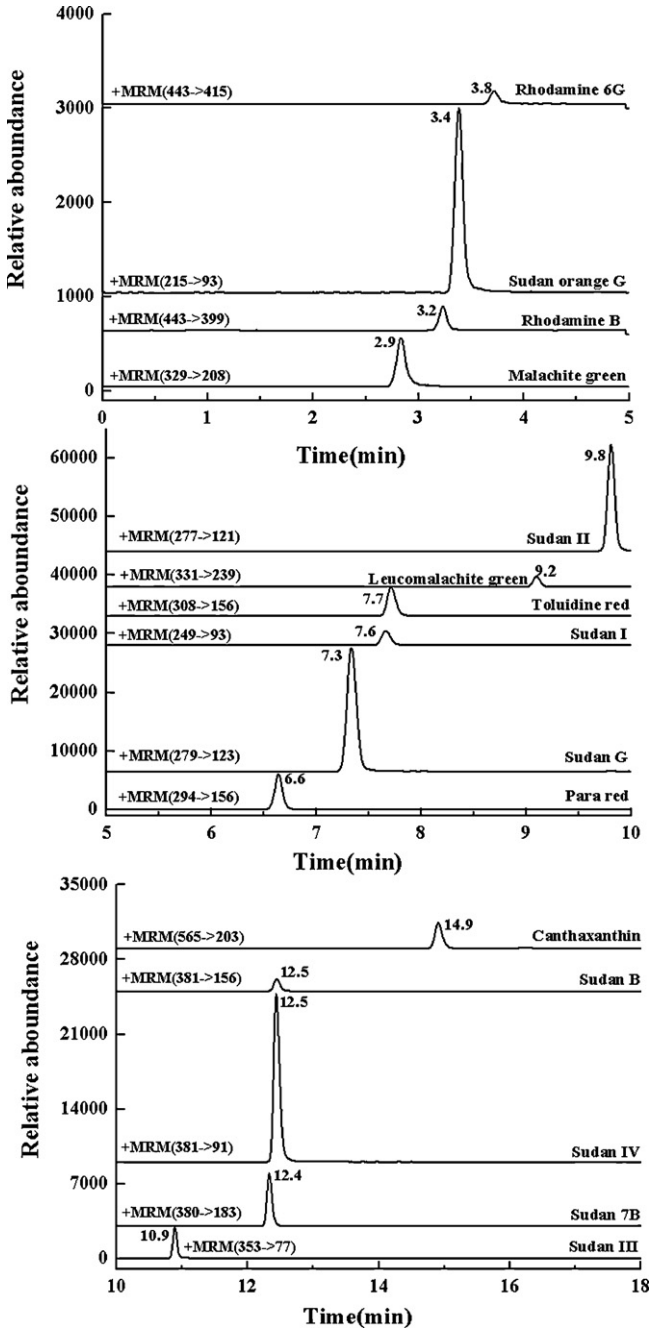


Fig. 2. Quantitative daughter ion chromatograms of the 15-dye standards.

70% to 120% and coefficients of variation were less than 15% for both intra-assay (within a day) and inter-assay (over a period of five consecutive days) measurements. For the meat and egg samples, the methods also showed satisfactory reproducibility.

3.4. Analysis of spiked feeds and poultry products samples

The validated method was applied to the recovery analysis of the 15 dyes using blank feed samples obtained from the pilot plant to validate the capability of the method for routine monitoring of animal feeds. Dyes were spiked in complete feed at 1.0 and 0.1 mg kg^{-1}, premix feed at 5.0 mg kg^{-1}, and concentrate feed at 2.0 mg kg^{-1}. Six replicates were tested for each concentration. Total ion chromatogram for the complete feed sample spiked with the 15-dye mixture and a blank feed sample were shown in Fig. 1(B). The pres-

Table 4
Percentage recoveries and relative standard deviations of the dyes after clean-up in different matrices ($n = 6$).

	Meat		Eggs		Complete feed		Concentrate	Premix
	100 μg kg^{-1}	200 μg kg^{-1}	100 μg kg^{-1}	200 μg kg^{-1}	100 μg kg^{-1}	1 mg kg^{-1}	2 mg kg^{-1}	5 mg kg^{-1}
Sudan I	88.0 (11.5)	73.4 (8.2)	88.8 (5.3)	75.5 (5.6)	84.1 (3.6)	89.1 (6.0)	81.7 (7.6)	98.9 (10.6)
Sudan II	67.1 (7.1)	82.1 (11.6)	89.8 (2.4)	87.8 (9.6)	109.8 (9.0)	96.0 (8.8)	73.6 (8.2)	107.2 (14.6)
Sudan III	93.6 (10.2)	90.0 (11.8)	123.6 (4.7)	96.7 (14.2)	108.0 (11.0)	137.2 (5.4)	129.2 (2.7)	81.8 (11.9)
Sudan IV	119.9 (5.1)	106.0 (10.0)	10.5 (8.9)	93.1 (18.9)	98.3 (5.2)	100.4 (12.7)	71.4 (6.7)	92.2 (9.6)
Sudan B	97.7 (10.8)	98.4 (8.0)	12.7 (9.5)	76.8 (11.4)	89.3 (5.7)	99.1 (8.4)	69.3 (8.0)	87.3 (8.0)
Sudan G	81.0 (15.7)	82.3 (6.6)	112.6 (5.3)	65.1 (4.1)	114.0 (5.2)	93.7 (6.5)	84.3 (9.2)	105.6 (3.7)
Sudan 7B	65.3 (5.8)	103.0 (5.4)	129.4 (2.8)	99.0 (5.1)	95.7 (7.6)	118.7 (13.2)	68.9 (6.9)	128.9 (11.9)
Sudan orange G	72.4 (8.6)	71.5 (10.4)	68.0 (4.6)	62.5 (1.4)	61.5 (5.6)	60.0 (6.6)	69.5 (11.3)	66.2 (6.9)
Malachite green	65.3 (11.0)	64.5 (8.2)	N.A.	N.A.	99.2 (6.1)	86.5 (10.8)	77.2 (12.6)	99.0 (11.3)
Leucomalachite green	72.8 (3.3)	116.9 (8.2)	76.6 (8.6)	106.6 (3.9)	70.9 (3.8)	100.1 (4.1)	106.8 (10.0)	83.5 (8.0)
Rhodamine B	120.6 (13.2)	139.0 (12.5)	103.4 (12.8)	93.9 (13.6)	138.7 (13.8)	102.8 (9.2)	79.0 (14.7)	110.6 (9.3)
Rhodamine 6G	89.8 (10.8)	82.1 (3.7)	101.6 (4.8)	106.1 (4.8)	105.6 (5.0)	93.6 (13.0)	99.0 (12.6)	125.1 (4.5)
Para red	113.5 (10.4)	81.6 (9.1)	125.7 (3.0)	83.7 (8.0)	86.7 (4.8)	72.5 (7.7)	81.8 (8.6)	118.8 (10.9)
Toluidine red	136.0 (5.9)	79.3 (7.5)	109.5 (3.0)	72.3 (4.6)	109.7 (8.0)	87.2 (11.1)	85.0 (10.9)	131.8 (11.8)
Canthaxanthin	65.2 (9.4)	114.1 (6.1)	79.3 (10.5)	94.1 (9.8)	79.7 (5.0)	95.4 (1.2)	60.5 (6.8)	110.4 (11.0)

N.A.: NO Analysis

ence of the dyes was confirmed by comparing the peak intensity ratio of two product ions of each dye with the ratio obtained from standard samples. The most abundant product ion in the mass spectra was used for quantification and determination of the recoveries and coefficients of variation for each dye. The dyes in spiked samples were quantified by external standards using calibration curves. Spike level, spike recovery and correlation coefficient were evaluated. The actual spiked quantity and the measured concentration in the three feed matrices showed good consistency (Table 4). The recoveries ranged from 60 to 140% and coefficients of variation were less than 15%.

The UHPLC–MS/MS methods established above were also applied to the detection of dyes-spiked eggs and poultry meat. Dyes were spiked in chicken meat and eggs at 100 and 200 μg kg^{-1}. Six replicates were tested for each concentration. Spike levels, spike recoveries and correlation coefficients were also evaluated. The results in Table 4 show that most of the samples analyzed yielded reliable results. The recoveries for the most of dyes ranged from 60 to 140% and coefficients of variation were less than 15%. However, the recoveries of sudan IV and sudan B from eggs spiked at 100 μg kg^{-1} were about 10%. The reason may be that acetonitrile used for extraction caused agglomerating and precipitation of the sample, and thus resulted in incomplete extraction of certain dyes

Table 5
Methods for the determination of dyes in edible animal products.

Sample	Analyte	Sample treatment	Determination technique	Recovery	Limit of quantification	Reference
Feedstuffs, eggs and chicken	Sudan I–IV, sudan orange G, sudan B, sudan 7B, sudan G, para red, toluidine red, rhodamine B rhodamine 6G, malachite green, leucomalachite green, canthaxanthin	A simple procedure using acetonitrile and SPE clean-up	UHPLC–ESI-MS/MS	60–140%	0.01–5.61 μg kg^{-1}	This work
Spices	Sudan I–IV, sudan orange G, sudan 7B, sudan G, para red, rhodamine B	An extraction step with acetonitrile without further clean-up	LC–ESI–MS/MS	60–140%	0.2–10 μg kg^{-1}	[7]
Hot chili products	Sudan I–IV, sudan orange G, sudan 7B, sudan G, sudan B, para red,	A clean-up procedure by gel permeation chromatography	LC–ESI-MS/MS	81.7–92.9%	0.4–5.0 μg kg^{-1}	[30]
Chili and hot chili food	Sudan I–IV, sudan orange G, sudan 7B, para red	Pressurized liquid extraction with acetone followed by gel permeation chromatography clean-up	LC–ESI-MS/MS	94–105%	0.006–0.036 μg kg^{-1}	[29]
Fish tissues	Rhodamine 6G, malachite green	A buffer-acetonitrile extraction followed by liquid–liquid extraction	LC–ESI-MS/MS	No relative information	2 μg kg^{-1}	[32]
Chili foods	Sudan I–IV	A buffer-acetonitrile extraction or n-hexane followed by SPE clean-up	HPLC	93.2–103%	13.2–19.1 μg kg^{-1}	[37]
Eggs	Sudan I–IV	Extraction with n-hexane/acetone (95:5, v/v) and SPE clean-up	HPLC	79.8–95.7%	12.3–13.8 μg kg^{-1}	[14]
Catfish or trout tissue	Malachite green, leucomalachite green	A buffer-acetonitrile extraction followed by SPE clean-up	HPLC	Catfish: 75.4% and 61.3%; trout tissues: 82.6% and 48.6%	0.5 μg kg^{-1}	[38]

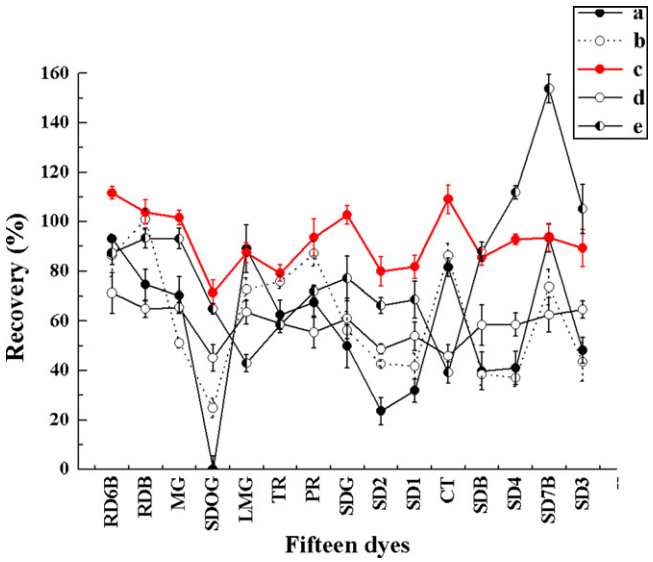


Fig. 3. Recovery of individual dyes from complete feed using five different eluents: (a) acetonitrile; (b) methanol–water (95:5, v/v); (c) methanol–acetic acid (95:5, v/v); (d) methanol–acetic acid (90:10, v/v); (e) acetonitrile–formic acid (95:5, v/v). MG, malachite green; RDB, rhodamine B; SDOG, sudan orange G; RD6G, rhodamine 6G; PR, para red; SDG, sudan G; SD1, sudan I; TR, toluidine red; LMG, leucomalachite green; SD2, sudan II; SD3, sudan III; SD7B, sudan 7B; SD4, sudan IV; SDB, sudan B; CT, canthaxanthin.

from the sample. Further experiments were needed to improve extraction of dyes from egg samples. In addition, as the unused dye in poultry feeds, malachite green was not spiked in eggs.

Table 5 was listed to compare the proposed method with previous reported methods such as the sample treatment, determination technique, LOD/LOQ, and efficiency, et al. From this table, we can see the following advantages of the proposed method: (1) more illegal dyes can been analyzed simultaneously with the approach, not only red dyes such as a series of sudan dyes can be detected, green dyes such as malachite green also can be detected; (2) more matrices including feeds, egg, and meat can been analyzed with the same approach and obtain equally good LOD and recovery; (3) the LOD/LOQ of most of the dyes are equal or lower than those from the previous reports, especially we can analyzed 15 dyes simultaneously in animal feeds, eggs and meats, while most previous reports only detected limited types of dyes in a single test; and (4) the pretreatment procedure is very simple, a single organic SPE process can realize efficient extraction of the dyes, while previous reports used complicated sample extraction procedure, like liquid to liquid extraction.

3.5. Analysis of real samples

Fifteen (feed, meat and egg) samples collected from different markets of local city were applied for validating the proposed method. No peaks of target analytes were detected in any matrices. The results indicate the safety of the feeds since China completely banned the use of these dyes as feed additives. In addition, 10 kg of commercially prepared poultry feeds made at the pilot plant of the Ministry of Agricultural Feed Industry Center (Beijing, China) were added 200 mg sudan I standard and mixed completely. The positive sample was observed trace amount of sudan I at level of 17.6 mg kg^{-1}. The recovery for sudan I was 88%, which indicated that the method was reliable and could be used for the determination of trace dyes in real samples.

4. Conclusion

Using UHPLC with ESI(+) MS/MS detection, a reliable UHPLC–MS/MS method for qualitative and quantitative analysis of 15 illegal dyes in animal feeds and poultry products was developed. Selectivity MS/MS technique coupled with chromatographic separation achieved unambiguous identification and accrate determination of the illicit compounds in complex matrixes such as feeds, chicken, and eggs. Furthermore, the developed method has been validated for routine analysis using real market samples. Based on this work, it is possible to widen the scope of the developed method to other animal products and other matrixes.

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