Determination of Para Red, Sudan Dyes, Canthaxanthin, and Astaxanthin in Animal Feeds Using UPLC

Xiaolin Hou[1], Yonggang Li[2], Guojuan Wu[1], Lei Wang[3], Miao Hong[4], and **Yongnin Wu[4,*]**

[1]Department of Animal Science, Beijing University of Agriculture, Beijing, P.R. China, 102206; [2]Circulation Industry Promotion Center of Ministry of Commerce, Beijing, 100070; [3]Department of Preventative Medicine, College of Veterinary Medicine, China Agricultural University, 100094; [4]Institute of Nutrition and Food Hygiene, Chinese Center for Disease Control and Prevention, 100050

Abstract

A simple high-performance liquid chromatography method was developed for quantitative determination of para red, Sudan I, Sudan II, Sudan III, Sudan IV, canthaxanthin, and astaxanthin in feedstuff. The sample was extracted using acetontrile and cleaned up on a C_{18} SPE column. The residues were analyzed using ultra-performance liquid chromatography coupled to a diode array detector at 500 nm. The mobile phase was acetonitrile–formic acid–water with a gradient elution condition. The external standard curves were calibrated. The mean recoveries of the seven colorants were 62.7–91.0% with relative standard deviation 2.6–10.4% (intra-day) and 4.0–13.2% (inter-day). The detection limits were in the range of 0.006–0.02 mg/kg.

Introduction

Sudan azo-dyes are synthetic colorants widely used in chemical industries. The colorants, known as Sudan I–IV, have been proved through laboratory experiments and assessed as category 3 carcinogens to animals and human beings by the International Agency for Research on Cancer (IARC). Thus, Sudan dyes at any level are harmful to the human health. Para red, which is chemically similar to Sudan I, is another potential carcinogen. Due to the facts, almost all nations have forbidden the use of these colorings as food additives and feed additives (1,2).

Canthaxanthin and astaxanthin are primarily used as a flesh colorant and added to feeds for fish and poultry and may be beneficial to consumer health within limits. Canthaxanthin uptake can cause a deposition in retina, which causes visual problems. A directive has been adopted with a view to reducing concentrations of canthaxanthin from 80 mg/kg to 25 mg/kg in animal feed for salmon and broiler hens and to 8 mg/kg for laying hens (3). Astaxanthin was permitted to be used for salmon and trout for pigmentation. In China, canthaxanthin and astaxanthin were permitted only to be used in poultry and fish feedstuff, respectively (4).

*Author to whom correspondence should be addressed: email E-mail: wuyn@public.bta.net.cn

Several analytical methods have been developed for determination of para red and/or Sudan dyes in foods based on liquid chromatography with UV–vis (DAD) or high-performance liquid chromatography with mass spectrometry (HPLC–MS) (5–22), gas chromatography (5), and thin layer chromatography (TLC) (23). For astaxanthin and canthaxanthin, HPLC (24,25), HPLC–MS (25,26), and TLC (27) methods were also developed.

Feedstuff safety lies in the first element for animal food safety. Recently, colorant additive has been used in laying hens to produce red yolk egg. To safeguard the safety for food, it is necessary to distinguish the colorant additive in feeds. But few papers were published for determination of these colorants in feedstuff. In this paper, a simple ultra-performance liquid chromatography (UPLC) method was introduced for detection of these colorants in feedstuff.

Experimental

Reagents and materials

Para red (95.5%), Sudan I (97.5%), Sudan II (83.3%), Sudan III (91.3%), Sudan IV (91.5%), canthaxanthin (95.2%), and astaxanthin (94.1%) were purchased from Sigma (St. Louis, MO). Stock solutions (1 mg/mL) of Sudan I and Sudan II were prepared in acetonitrile whereas stock solutions (1 mg/mL) of canthaxanthin, astaxanthin, para red, sudan III, and sudan IV were prepared in chloroform, respectively. And the stock solutions were stored at –18ºC in the dark. Standard mix work solutions in a range of 0.1–1.00 µg/mL were diluted in acetonitrile. Acetontrile and formic acid were of HPLC-grade (Dikma, Beijing, China). Ultra-pure water was obtained using a Milli-Q Plus water purification system (Millipore, Bedford, MA). Other reagents were all analytical-grade. The compounded feedstuff was for laying hens.

Sample extraction and clean-up

The compounded feedstuff was homogenized and ground to particles of 0.5 mm or less. A portion (5.00 g) was accurately weighed into a 50-mL centrifuge tube. For recovery studies, an

appropriate amount of mixed working standard solution was added and vortexed for 10 s, and the sample was allowed to stand for 15 min. The fortified sample was extracted with 50 mL of acetonitrile, stirred in an ultrasonic bath for 10 min at room temperature, and then centrifuged at 4000 rpm for 5 min. After centrifugation, 10 mL of supernatant was transferred into another tube and mixed with 10 mL of water.

Table I. Standard Curves and R^2 for the Seven Compounds

Compounds	Standard curves	R^2*
Para red	$y = 49547x + 716.39$	0.9962
Sudan I	$y = 38178x + 2922.6$	0.9963
Sudan II	$y = 26272x + 614.63$	0.9985
Sudan III	$y = 68339x + 1115.70$	0.9971
Sudan IV	$y = 42795x + 1304.84$	0.9993
Astaxanthin	$y = 72036x + 1059.72$	0.9994
Canaxanthin	$y = 68956x + 986.67$	0.9993

* R^2 = Correlation coefficients.

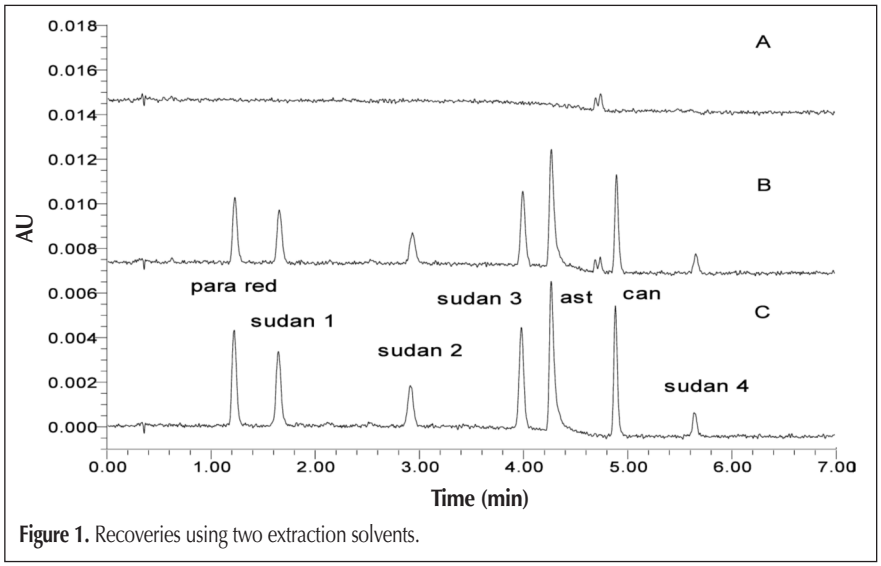

Figure 1. Recoveries using two extraction solvents.

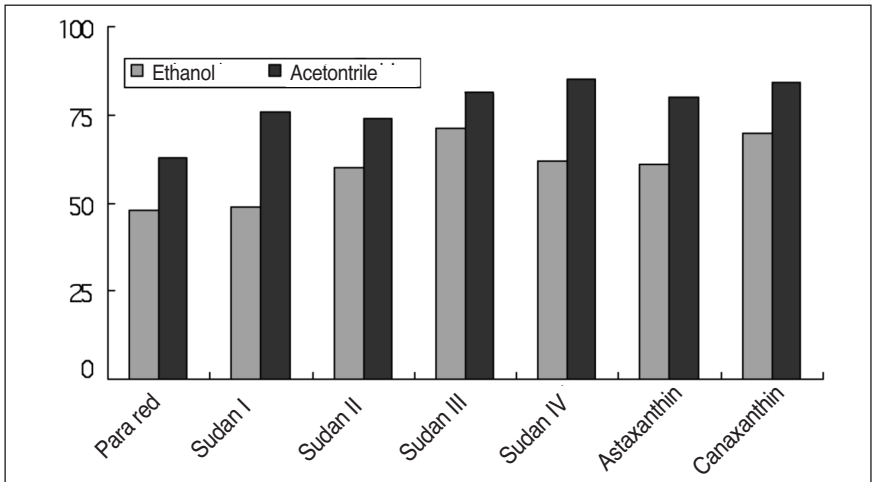

Figure 2. Chromatogram of colorants: (A) blank feedstuff; (B) fortified at 0.2 mg/kg for each colorant, and (C) standard solution of 0.2 mg/L for each colorant.

The mixture (20 mL) was loaded onto an ENVI18 SPE column (500 mg, 3 mL; Supelco, Bellefonte, CA). The column was previously conditioned with 5 mL of acetontrile and 5 mL of acetontrile–water (1:1, v/v). The analyte-containing column was washed with 5 mL of acetonitrile–water (1:1, v/v) and then dried under vacuum for 2 min. The residue was eluted with 10 mL of acetontrile–formic acid (99:1, v/v). The flow rate of load, wash, and elution was all controlled at 20–25 drop per min. The collected elution was dried under a stream of nitrogen gas at ambient temperature. Then the residue was re-dissolved in 1 mL of acetonitrile and filtered through 0.25-µm pore size filter (Jinsheng, Tianjing, China) for LC analysis.

UPLC separation and detection

The LC analysis was performed on a Waters Acquity UPLC system including Acquity UPLC binary pump, sample manager, and photodiode array detector (PDA). The separation was carried on an Acquity UPLC BEH C_{18} (1.7 µm, 2.1 × 50 mm). The mobile phase consisted of (A) acetonitrile–formic acid (100:0.1, v/v) and (B) water. Initial condition was 60% A and 40% B followed by linear gradient to 70% for A in 3 min and linear gradient to 90% for A in 1 min. The condition was held for 3 min. To re-condition the chromatography column, one min post-run with the initial mobile phase composition was performed. The mobile phase flow rate was 0.4 mL/min. The column temperature was set at 35°C. The quantificational wavelength was 500 nm. And the spectrum from 240 to 500 nm for each peak was collected and compared with the standard for its presence. The injection volume was 5 µL.

Standard curves

A set of five standard solutions at the following concentrations was prepared in a range of 0.1, 0.2, 0.5, 0.8, and 1.0 µg/mL. Each of them was analyzed in duplicate. Calibration curves were constructed by plotting peak area as a function of analyte concentration. The sample concentration was measured by comparing the peak area for fortified sample extracts and then calculated from the standard curves.

Method validation

Feedstuff fortified with 0.8, 0.4, 0.2, and 0 mg/kg for the seven colorants were analyzed for accuracy and precision. The precision is expressed as relative standard deviation (RSD) within-laboratory, and the accuracy is expressed as mean recovery, respectively.

Limit of detection (LOD) and limit of quantification (LOQ) were estimated by analyzing 10 blank samples. The LOD and LOQ were calculated as three and 10 times noise from the blank matrix, respectively.

Results and Discussion

Pretreatment

The extraction solvents for these materials were all organic solvents. In the study, several organic solvents, such as acetone, ethanol, acetonitrile, hexane, dichloromethane, or their mixture, were tested. The extraction of hexane, acetone, and dichloramethane was too dirty for further clean up. The extraction of acetonitrile had the least endogenous matrix.

Previously, silica (27) or *N*-alumina (5) solid-phase extraction (SPE) was usually used for clean-up; in this study, the reverse-phase of C_{18} was used. The seven colorants all possess fat-soluble and hydrophobic character, so their retention on a reversed-phase chromatography column is specific. Moreover, canaxanthin and astaxanthin are prone to thermal decomposition; rotary evaporation should be avoided as much as possible. Solvent that is soluble in water was first selected. Acetonitrile possesses a better extraction performance than ethanol for its higher recoveries (Figure 1). Therefore, acetonitrile was finally selected as the extraction solvent. The extraction was directly loaded on the C_{18} SPE column without evaporation to dryness. And the elution solvent was dried under a stream of nitrogen gas at ambient temperature.

UPLC separation and detection

Firstly, constant elution condition was tested. The seven dyes could be baseline separated, but the response of sudan I was too weak. So the gradient program was designed. Under the UPLC condition, a separation of the dyes could be completed in 7 min (Figure 2). Previously, 10–15 min was needed for a separation using HPLC (5–7,12,15,18,24). Since then, the analysis speed was doubled. The maximum absorbance wavelength for para red, sudan I, sudan II, sudan III, sudan IV, astaxanthin, and canaxanthin, respectively, were 450, 476, 493, 512, 520, 475, and 480 nm. In this case, considering the lowest response of sudan IV, wavelength of 500 nm was selected for the quantification of all compounds. To initially validate their presence, the spectrum from 240 to 500 nm for each peak was compared with the standard.

As shown in Table I, under the concentrations of 0.1, 0.2, 0.5, 0.8, and 1.0 µg/mL for each compound, the obtained standard curves were linear with correlation coefficients (R^2) > 0.996 (Table I). For real feedstuff, the samples could be further diluted to be within the range of the standard curves.

Table II. Mean Recoveries and RSDs of the Seven Colorants

Compound	Added level (mg/kg)	Mean Recovery* (%)	Intra-day RSD† (%)	Inter-day RSD‡ (%)
Para red	0.2	64.8	8.7	12.2
	0.4	62.7	6.0	8.7
	0.8	66.4	4.8	4.3
Sudan I	0.2	76.1	6.3	8.1
	0.4	76.8	5.9	6.6
	0.8	74.4	6.0	5.9
Sudan II	0.2	72.2	8.1	10.2
	0.4	70.4	8.6	10.5
	0.8	76.8	4.2	5.0
Sudan III	0.2	80.4	4.5	4.3
	0.4	85.5	3.9	5.3
	0.8	91.0	4.2	6.0
Sudan IV	0.2	90.7	10.4	13.2
	0.4	82.5	6.6	5.1
	0.8	85.9	4.8	4.0
Astaxanthin	0.2	80.2	4.6	5.8
	0.4	85.5	2.6	4.4
	0.8	74.9	3.2	4.8
Canaxanthin	0.2	88.4	6.4	7.2
	0.4	88.6	5.6	6.5
	0.8	85.7	5.5	6.4

* Mean Recoveries (%) (n = 15)
† Intra-day RSD (n = 5). ‡ Inter-day RSD (n = 3).

Accuracy and precision

The accuracy of the method was determined using feedstuff samples fortified at 0.2, 0.4, and 0.8 mg/kg for each colorant (Table II). The mean recoveries of the seven colorants were 62.7–91.0% with relative standard deviation 2.6–10.4% (intra-day) and 4.0–13.2% (inter-day). The typical chromatogram is shown in Figure 2.

LOD and LOQ

The LOD, based on three times the baseline noise from blank tissues, were 0.01, 0.015, 0.02, 0.008, 0.006, 0.006, and 0.06 mg/kg for para red, sudan I, sudan II, sudan III, astaxanthin, canaxanthin, and sudan IV, respectively. The LOQ, based on 10 times the baseline noise from blank tissues, were 0.03, 0.05, 0.06, 0.025, 0.02, 0.02, and 0.2 mg/kg for para red, sudan I, sudan II, sudan III, astaxanthin, canaxanthin, and sudan IV, respectively.

Conclusion

A novel method for determination of para red, sudan I, sudan II, sudan III, sudan IV, astaxanthin, and canaxanthin in feedstuff was developed. The whole procedure was simple, convenient, reproducible, and time-saving. To prevent illegal colorants in animal original food, we strongly recommend the method be used as routine analysis for feedstuff.

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