Promulgated, Sep 30, 2017
Amended, Dec 8, 2017
Amended, May 22, 2019
TFDAA0062.02

Method of Test for Sudan Dyes in Foods

1. Scope

This method is applicable to the determination of 4 Sudan dyes (Sudan I, Sudan II, Sudan III and Sudan IV) in muscle, eggs and salted yolks of poultry products.

2. Method

After extraction and purification, analytes are determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph-tandem mass spectrometer.

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: CORTEC C18, 1.6 μm, 2.1 mm × 15 cm, or an equivalent product.

2.1.2. Centrifuge: centrifugal force > 5000 ×g.

2.1.3. Shaker.

2.1.4. High speed dispersing device: SPEX SamplePrep 2010 Geno/Grinder®, > 1000 rpm, or an equivalent product.

2.1.5. Vortex mixer.

2.1.6. Nitrogen evaporator.

2.2. Chemicals

Acetonitrile, HPLC grade;
Acetone, HPLC grade;
Formic acid, reagent grade;
EMR-Lipid extraction powder, AR grade;
Sodium citrate, AR grade;
Sodium hydrogen citrate, AR grade;
Magnesium sulfate anhydrous, AR grade;
Sodium chloride, AR grade;
Deionized water, resistivity ≥ 18 MΩ·cm (at 25°C);
Sudan I, Sudan II, Sudan III and Sudan IV, reference standards.

2.3. Apparatus

2.3.1. Volumetric flask: 100 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Ceramic homogenizer (note 1): Bond Elut QuEChERS P/N 5982-9312,

or an equivalent product.

2.3.4. Extraction powder (note 2): containing 1 g of sodium citrate, 0.5 g of sodium hydrogen citrate, 4 g of magnesium sulfate anhydrous and 1 g of sodium chloride)

2.3.5. Clean-up centrifuge tube I (note 2): containing 1 g of EMR-Lipid extraction powder, or an equivalent product.

2.3.6. Clean-up centrifuge tube II (note 2): containing 1.6 g of magnesium sulfate anhydrous and 0.4 g of sodium chloride.

2.3.7. Membrane filter: 0.22 μm, PVDF.

Note 1: Ceramic homogenizer can be used depending on the viscosity of the sample.

Note 2: Commercial extraction/clean-up kit can be used as needed.

2.4. Mobile phase

2.4.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.4.2. Solvent B

Dilute 1 mL of formic acid with acetonitrile to 1000 mL, and filter with a membrane filter.

2.5. Standard solution preparation

Transfer about 100 mg of Sudan dye reference standards accurately weighted to each 100-mL volumetric flask, dissolve and dilute to volume with acetone as the standard stock solutions. Store at 4°C.

2.5.1. Mix appropriate volume of the standard stock solutions, and dilute with acetonitrile as the standard solutions(note) for section 2.7 to establish calibration curves.

Note: The concentrations of Sudan I, Sudan II and Sudan III are 0.16~2 μg/mL, and those of Sudan IV are 0.4~5 μg/mL for the muscle and egg matrices. Concentrations of Sudan I, Sudan II and Sudan III are 0.04~1 μg/mL, and those of Sudan IV are 0.1~2.5 μg/mL for the salted yolk matrix.

2.5.2. Mix appropriate volume of the standard stock solutions, and dilute with acetonitrile as the standard solutions(note) for section 2.8.2 to perform the standard addition method.

Note: The concentrations of Sudan I, Sudan II and Sudan III are 2~8 μg/mL, and those of Sudan IV are 5~20 μg/mL for the muscle and egg matrices. The concentrations of Sudan I, Sudan II and Sudan III are 0.5~2 μg/mL, and those of Sudan IV are 1.25~5 μg/mL for the salted yolk matrix.

2.6. Sample solution preparation

2.6.1. Muscle

Transfer about 2 g of the homogenized sample accurately weighed into a centrifuge tube. Add 8 mL of deionized water, 10 mL of acetonitrile, extraction powder and a ceramic homogenizer, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hand for 1 min, and centrifuge at 5000 ×g for 5 min. Take 0.5 mL of the supernatant, add 0.5 mL of acetonitrile, mix well, and filter with a membrane filter. Take the filtrate as the sample solution.

2.6.2. Eggs

After removing eggshells, transfer about 2 g of the mixed egg white and yolk sample accurately weighed into a centrifuge tube. Add 8 mL of deionized water, 10 mL of acetonitrile, extraction powder and a ceramic homogenizer, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or by hand for 1 min, and centrifuge at 5000 ×g for 5 min. Transfer 5 mL of the supernatant to a clean-up centrifuge tube I pretreated with 2 mL of deionized water, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 1 min, and centrifuge at 5000 ×g for 5 min. Transfer the supernatant to a clean-up centrifuge tube II, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 1 min, and centrifuge at 5000 ×g for 5 min. Take 0.5 mL of the supernatant, add 0.5 mL of acetonitrile, mix well, and filter with a

membrane filter. Take the filtrate as the sample solution.

2.6.3. Salted yolks

Transfer about 0.5 g of the homogenized sample accurately weighed into a centrifuge tube. Add 5 mL of acetone and a ceramic homogenizer, shake for 10 min, and centrifuge at 5000 ×g for 5 min. Transfer the supernatant to a clean-up centrifuge tube I pretreated with 2 mL of deionized water, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 1 min, and centrifuge at 5000 ×g for 5 min. Transfer the supernatant to a clean-up centrifuge tube II, cap the centrifuge tube, and shake vigorously several times by hands to prevent coagulation of salt. Then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 1 min, and centrifuge at 5000 ×g for 5 min. Take 1 mL of the supernatant, and evaporate to dryness by gently flushing with a stream of nitrogen. Dissolve the residue with 1 mL of acetonitrile, mix well, and filter with a membrane filter. Take the filtrate as the sample solution.

2.7. Calibration standard curve

Accurately add 50 μL of the standard solutions from section 2.5.1 to blank samples, and follow the procedure described in section 2.6 to obtain the calibration standard solutions. Operate LC-MS/MS according to the following conditions. Establish the calibration standard curve of each Sudan dye by peak areas of each Sudan dye vs. the added amounts(note).

Note: The amounts of the calibration standard curve for Sudan I, Sudan II and Sudan III are 8~100 ng, and those for Sudan IV are 20~250 ng in the muscle and egg matrices. The amounts of the calibration standard curve for Sudan I, Sudan II and Sudan III are 2~50 ng, and those for Sudan IV are 5~125 ng in the salted yolk matrix.

LC-MS/MS operating conditions(note)

Column: CORTECS C18, 1.6 μm, 2.1 mm × 15 cm.

Mobile phase: a gradient program of solvent A and solvent B is as follows:

Time (min)	A (%)	B (%)

0 → 1.0	60 → 60	40 → 40
1.0 → 5.0	60 → 15	40 → 85
5.0 → 10.0	15 → 15	85 → 85
10.0 → 11.0	15 → 0	85 → 100
11.0 → 15.0	0 → 0	100 → 100
15.0 → 15.1	0 → 60	100 → 10
15.1 → 20.0	60 → 60	10 → 10

Flow rate: 0.25 mL/min.

Injection volume: 5 μL.

Ion spray voltage: 2.2 kV.

Ionization mode: ESI^+.

Ion source temperature：120°C.

Desolvation temperature: 400°C.

Cone gas flow rate: 50 L/hr.

Desolvation flow rate: 850 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are as follows:

Analyte	Ion pair Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Sudan I	249 > 156*	30	28
	249 > 128	30	14
Sudan II	277 > 121*	30	24
	277 > 156	30	20
Sudan III	353 > 197*	50	25
	353 > 156	50	19
Sudan IV	381 > 91*	50	24
	381 > 244	50	22

*The quantitative ion.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.8. Identification and quantification

2.8.1. External calibration method

Accurately inject 5 μL of the sample solution and the standard solutions

into LC-MS/MS separately, and operate according to the conditions in section 2.7. Identify each Sudan dye based on the retention time and the relative ion intensities(note). Calculate the amount of each Sudan dye in the sample by the following formula:

$$\text{The amount of each Sudan dye in the sample (ppb)} = \frac{C}{M}$$

Where,

C: the amount of each Sudan dye in the sample solution calculated by the calibration standard curve (ng)

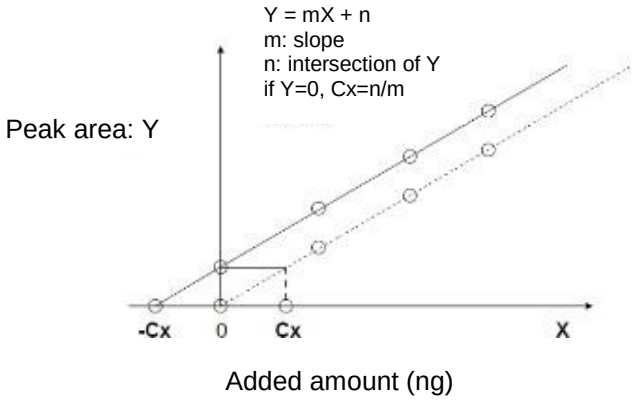
M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≦ 10	± 50

2.8.2. Standard addition method

Accurately add 100 µL of the standard solutions from section 2.5.2 to the sample(note) separately, and follow the same procedure described in section 2.6 to obtain the sample solutions. Take 100 µL of the sample solutions, separately dilute to 1 mL with acetonitrile, and operate according to the conditions in section 2.7. Establish a linear regression curve (y = mx + n) of each Sudan dye by the peak areas of each Sudan dye vs. the added concentrations. Calculate the amount of each Sudan dye in the sample by the following formula:



The amount of each Sudan dye in the sample (ppb) $= \frac{C}{M}$

Where,

C: the amount of each Sudan dye in the sample solution calculated from n/m (ng)

M: the weight of the sample (g)

Note: The added amounts of Sudan I, Sudan II and Sudan III in the muscle and egg matrices are 0~800 ng, and those for Sudan IV are 0~2000 ng. The added amounts of Sudan I, Sudan II and Sudan III in the salted yolk matrix are 0~200 ng, and those for Sudan IV are 0~500 ng.

Remark

1. Limits of quantification (LOQs) are 4 ppb for Sudan I, Sudan II and Sudan III, and 10 ppb for Sudan IV.
2. Further validation should be performed when interference compounds appear in samples.

Reference

Piątkowska, M., Jedziniak, P. and Żmudzki, J. 2014. Determination of illegal dyes in eggs by liquid chromatography-tandem mass spectrometry. Bull. Vet. Inst. Pulawy 58: 247-253.

Reference chromatogram

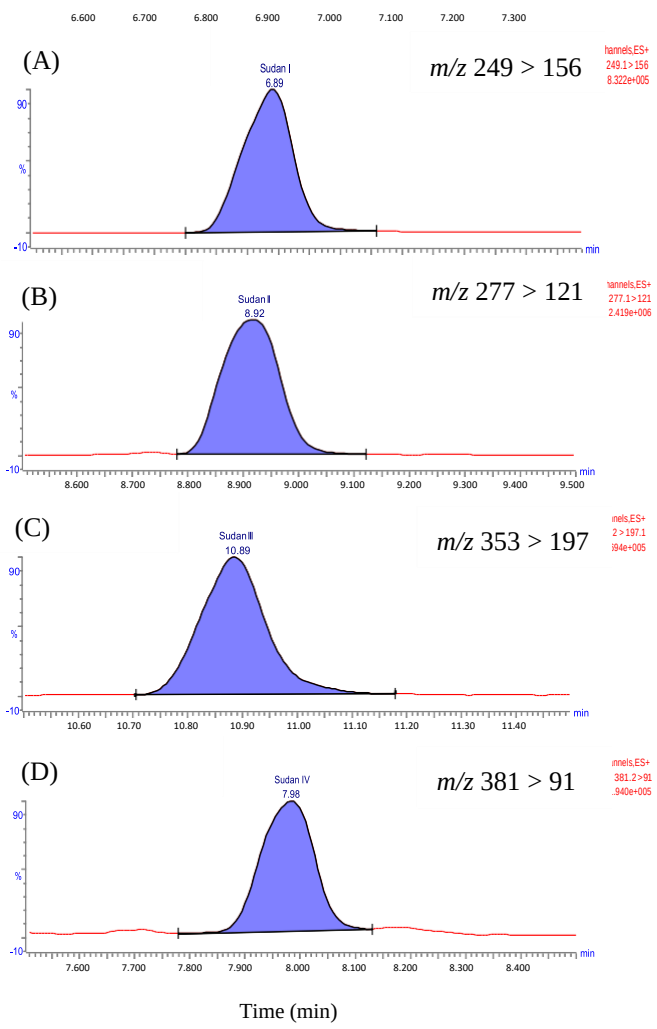


Figure. MRM chromatograms of Sudan I (A), Sudan II (B), Sudan III (C) and Sudan IV (D) analyzed by LC-MS/MS.