Food Chemistry 239 (2018) 598–602

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Absence of evidence or evidence of absence? A transfer and depletion study of Sudan I in eggs

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ARTICLE INFO

*Article history:*
Received 4 November 2016
Received in revised form 1 June 2017
Accepted 29 June 2017
Available online 30 June 2017

*Keywords:*
Sudan I
Illegal dyes
Eggs
Residues
Transfer
Depletion study

ABSTRACT

Sudan I is a carcinogenic industrial azo-dye, forbidden for use in food. However, it has been detected in food on several occasions, such as in paprika, used in animal husbandry to enhance egg yolk colour. Therefore, an animal experiment was designed to simulate the transfer of Sudan I to eggs after its unintentional administration to laying hens. A group of laying hens (n = 18) received feed contaminated with Sudan I at the raising concentrations: 0.45 mg/kg, 4.97 mg/kg and 42.1 mg/kg. Residues of Sudan I were detected in egg yolks (0.29 ± 0.03 μg/kg, mean ± SD) only after the administration of the feed contaminated with the dye at the highest concentration. The determined concentrations were much lower than expected based on the compound's lipophilicity. In conclusion, the transfer of Sudan I to eggs was limited and strongly dependent on its concentration in feed.



## 1. Introduction

Consumers' preferences for egg yolk colour vary among populations but colour is still one of the most important criteria when choosing the product (Dvořák, Suchý, Straková, & Kopřiva, 2012). It is so important for consumers that food producers dye egg yolks, using colouring agents in the form of feed additives. In husbandry there are eight dyes approved for poultry: capsanthin (E 160c), beta-apo-8′-carotene (E 160e), ethyl ester of beta-apo-8′-carotene acid (E 160f), lutein (E 161b), cryptoxanthin (E 161c), canthaxanthin (E 161g), zeaxanthin (E 161h) and citranaxanthin (E 161i) (Annon, 2007: List of the authorised additives in feedingstuffs, valid in the European Union, 2004).

Because not all of the dyes are available in synthetic forms, they may be extracted from plants containing them, such as *Calendula, Capsicum annuum* or *Tagetes* petals to produce oleoresins, which are used as feed additives. The direct addition of marigold flowers, peppers, alfalfa, carrots, tomato peel, corn meal, dried alfalfa and grasses, or crab shells enhances the egg yolk colour (Piatkowska, Jedziniak, & Żmudzki, 2014a).

As consumers' interest in organic food grows, enhancing the egg yolk colour with natural products becomes increasingly popular. On the other hand, products, such as paprika pepper, may be adulterated with illegal dyes. These dyes belong to the azo-dye group, used for colouring plastics and other synthetic materials in the industry. The practice shows that, as well as authorised dyes, food producers might use banned, often harmful, substances. They are banned as food additives but are sometimes detected in products such as chilli powder and chilli products, curry, curcuma, red pepper and virgin palm oil (Rapid Alert System for Food and Feed, RASFF ec.europa.eu/food/food/rapidalert/index_en.htm). This is because the spices, during storage, lose their colour and the addition of a stable yellow-orange or red azo-dye gives more intensive colours to the spices and suggests a better quality and freshness of the product. In the period of only five years (2005–2010), there were 516 notifications about the presence of illegal dyes in food containing spices (Kozłowska, Jeruszka-Bielak, Piwowarczyk, & Brzozowska, 2012). Sudan I (1-phenylazonaphth-2-ol) is an azo-dye and is the most frequently used to adulterate peppers, quite often with a smaller quantity addition of Sudan IV (Mishra et al., 2007, RASFF). Adulterated plants may be further unintentionally used in animal husbandry. In the past there were incidents of detection of Sudan dyes in food of animal origin, e.g. in eggs, swine muscle and liver (Chen et al., 2013, Xinyi et al., 2010, china.org.cn). Additionally, recent research on natural contamination has demonstrated that the combination of Sudan I-contaminated soils and application of Sudan I-containing agronomic materials constitutes a major source of Sudan I in fruits during the growth period (at levels of 0.18–2.52 μg/kg, Lian et al., 2014).

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http://dx.doi.org/10.1016/j.foodchem.2017.06.156
0308-8146/

In view of the above, the aim of this study was to evaluate the transfer and depletion of selected Sudan azo-dye in eggs after administration of feed containing contaminated paprika pepper.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Acetonitrile (ACN), hexane and formic acid (99.5%) (HPLC grade) were provided by J.T. Baker (USA). Anhydrous sodium sulphate and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (Germany). Water was purified through a Mili-Q plus system from Millipore (USA). The SPE cartridges Hybrid-SPE™ (30 mg/1 ml) and PTFE syringe filters (0.22 μm) were received from Restek (USA). Analytical standards of Sudan I (≥95%) and Sudan I-$d_5$ were purchased from Sigma-Aldrich (Germany).

### 2.2. Preparation of standard solutions

The stock standard solutions (1000 μg/ml) of Sudan I and Sudan I-$d_5$, were prepared by weighing appropriate amounts of substances and dissolving them in acetonitrile. The solutions were stable in the dark at a temperature below −18 °C for 6 months. Working standard solutions of Sudan I were prepared by dilution of an appropriate amount of stock standard solution with acetonitrile. A solution of internal standard (IS) was prepared separately. Working standard solutions (100, 10, 1, 0.1 and 0.01 μg/kg) were stable in the dark at 2–8 °C for three months.

### 2.3. Sample preparation equipment

A Multiquick 7 K 3000 blender (Braun GmbH, Germany), M20 universal mill (IKA®-Werke GmbH & Co. KG, Germany) and a cement mixer MK-165/130 L/900 W/230 V Profi were used to prepare the experimental feed. Analytical PR/SR precision balances (Mettler Toledo International Inc., Switzerland) were used to weigh the samples. A homogeniser Polytron PT-3100 (Kinematica, Switzerland) operating at 7000 rpm was used to homogenize the egg yolk. Samples were weighed in 50 ml Nunc™ conical sterile polypropylene centrifuge tubes (Thermo Fisher Scientific Inc., USA) and a Waters (Milford, USA) SPE chamber was used for sample clean-up. An ultrasound Sonorex (Bandelin Electronic, Germany) operated at 30 °C and rotator Stuart STR 4 (Bibby Scientific Limited, UK) were used to improve the extraction. A centrifuge set at 4500 rpm, 4 °C MPW-6K15 (MPW Med. Instruments, Poland) was used to remove the precipitated proteins. A VLM Eva EC1/EC2L (VLM, Germany) nitrogen evaporator set at 45 °C was used for sample evaporation.

### 2.4. Determination of Sudan I in feed

The method for the determination of Sudan I in feed is already published elsewhere (Piatkowska et al., 2017). Briefly, the feed sample (3 g ± 0.01) was weighed into a centrifuge tube and working standard solution was added. A further 30 ml quantity of hexane was added and samples were vortex-mixed. Samples were extracted, using a shaker mixer (200 rpm, 30 min), and then placed in an ultrasonic bath (15 min, 30 °C). After the centrifugation (10 min, 4500 rpm, 4 °C), 500 μl of the extract were taken to a glass tube for evaporation. The dry residues were reconstituted with 500 μl of acetonitrile saturated with hexane: DMSO (80:20), vortex-mixed, and filtered through 0.2 μm PTFE syringe filters. Afterwards, 10 μl of the extract were injected into a UPLC-MS/MS system for analysis.

The analytical method was previously in-house validated. The repeatability was in the range of 2.9–9.5%, while the recoveries ranged from 88 to 106%.

### 2.5. Preparation and statistical analysis of experimental feed

The experimental feed was in-house prepared at three concentrations of Sudan I (0.5, 5 and 50 mg/kg), using the commercial complete feed for laying hens ("DJ-Nioska") obtained from Agropol s.j. feed mill (Motycz, Poland). Both red pepper (purchased in a local supermarket) and animal feed were examined for the presence of illegal dyes before they were used in the experiment. Three premixes, each of 2 kg feed containing 400 g of red pepper, were prepared to be mixed with 38 kg of feed. The content of paprika in the feed was calculated to be at the level of one percent (w/w).

To perform the homogeneity study (Thompson, Ellison, & Wood, 2006), ten randomly taken samples of each concentration were taken and analysed in duplicate, using UPLC–MS/MS.

To detect analytical outliers the Cochran test, with 95% confidence, was used. Results were verified by the test for sufficient homogeneity. The material is sufficiently homogeneous if:

$$S_{sam}^2 \leqslant F_1 \sigma_{all}^2 + F_2 s_{an}^2$$

where $s_{sam}^2$ is the sampling variance, $s_{an}^2$ is the analytical variance, $\sigma_{all}^2$ all is the allowable variance (30% of target standard deviation) and $F_1$ and $F_2$ are statistical constants.

### 2.6. Animal study design and sample collection

The animal experiment was carried out under the Decision No 79/2012 of the Local Animal Experimentation Ethics Committee in Lublin (Poland). A flock of 18 laying hens was housed in a deep litter system. The animals were kept under typical conditions of ventilation, temperature and lighting. For the period of acclimatization (eight days), the hens were given water and commercial feed free from Sudan I *ad libitum*. Subsequently, an experimental feed contaminated with Sudan I at three concentrations was given to the laying hens:

- 0.5 mg Sudan I per kg feed 9th–24th day of the experiment,
- 5 mg Sudan I per kg feed 26th–39th day of the experiment,
- 50 mg Sudan I per kg feed 42nd–55th day of the experiment.

Then, for the next 13 days, a withdrawal period (with feed free from Sudan I) was applied. Eggs were collected during the experimental period twice a day. Each day, six randomly chosen eggs were broken and yolk and white were homogenised separately. Samples were kept below −18 °C for no longer than one week and used for the determination of Sudan I in egg yolk and white.

### 2.7. Determination of Sudan I in egg yolks and whites

The egg yolk and white samples were prepared, using a method published elsewhere (Piatkowska, Jedziniak, & Żmudzki, 2014b). Briefly, 2 g (±0.01) of homogenised egg yolk or white was weighed into a 50 ml centrifuge tube and the IS was added. Further quantities (8 ml of acetonitrile and 5 g of anhydrous $Na_2SO_4$) of salt and solvent were added. Samples were vortex-mixed, rotary shaken for 30 min and ultrasonicated for 15 min at 30 °C. After the centrifugation (10 min, 4500 rpm, 4 °C), the samples were passed through Hybrid SPE; an additional 1 ml of 0.1% formic acid in acetonitrile was passed through the cartridges and samples were collected for evaporation ($N_2$, 45 °C). Afterwards, the samples were reconstituted in acetonitrile saturated with hexane: DMSO (80:20), vortex-mixed, and filtered through 0.22 μm PTFE syringe filters. Finally, 10 μl of the extract were injected into the LC-MS/