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A rapid HPLC method for determination of Sudan dyes and Para Red in red chilli pepper

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Abstract

A rapid high-performance liquid chromatography (HPLC) system consisting of an ultraviolet-visible (UV–VIS) detector was developed for the separation and determination of Sudan dyes (I, II, III, and IV) and Para Red in red chilli peppers. The chromatographic separation was achieved on a reverse phase C_{18} column with isocratic elution, using a mobile phase of acetonitrile/methanol (80:20, v/v); detector was set at 506 nm. All four Sudan dyes and Para Red were separated in less than 9 min. Among 80 red chilli peppers screened, only one of them contained 0.10, 0.04, and 0.05 mg/kg Sudans I, III, and IV, respectively. No Sudan II and Para Red were detected in any of the red chilli peppers analysed. The method was 'in-house' validated using red chilli peppers based on following criteria: limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, reproducibility, and linearity in red chilli peppers. Depending on the dye involved, LOD and LOQ were in the range of 1.2–5.4 and 4–18 μg/kg in red chilli, respectively. The recovery, repeatability (expressed as coefficient of variation, CV_r), and reproducibility (CV_R) varied from 89 to 98%, from 0.82 to 4.09%, and from 1.33 to 4.65%, respectively. Linearity obtained for all dyes and Para Red were all $r^2 > 0.9999$ (in the range of 0.01–5 mg/l). The applicability of the method to the determination of Sudan dyes and Para Red in red chilli peppers was demonstrated. This method has potential to be used for illegal Sudan dyes and Para Red in red chilli peppers and some foodstuffs due to its simple, reliable, rapid, and excellent precision.

Keywords: Sudan dyes; Para Red; Red chilli peppers; HPLC-UV-VIS

1. Introduction

Synthetic Sudan dyes (I, II, III, and IV) are non-authorized and illegally used in the food industry to enhance and maintain the appearance of food products such as in chilli-, curry-, curcuma-, and palm oil-containing foodstuffs (Calbiani et al., 2004; Commission Decision, 2005; Cornet, Govaert, Moens, Loco, & Degroodt, 2006). The U.K. Food Standards Agency (FSA) alerts for the contamination with Sudan I dye of various meat preparations on the market in the U.K. and issues warnings about frozen meat products, spice mix, and chips containing contaminated chilli powder (FSA, 2006). Except in some Africans or Asians countries, their use as additives, at any level, in food products destined for human consumption is prohibited worldwide. Recently, Commission Decision (2005) requires that all chilli-, curry-, curcuma-containing food products and palm oil coming into any EU state are certified to be free of Sudan dyes.

Besides foodstuffs, Sudan dyes are widely used as colouring agents in chemical industries such as oils, fats, plastics, waxes, petrol, shoes, printing inks, shoe and floor polishing, and spirit varnishing, among others (Dillion, Combes, & Zeiger, 1994; Rafii, Hall, & Cerniglia, 1997; Society of Dyers & Colourists, 1971).

Sudan I is considered to be a genotoxic carcinogen (Stiborová, Martínek, Rýdlová, Hodek, & Frei, 2002) and classified as a category 3 carcinogen by the International

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Agency for Research on Cancer (IARC, 1975). Its presence is not permitted in foodstuffs for any purpose at any level. Sudan II is the dimethyl derivative of Sudan I and it has been tested in mice by bladder implication, resulting in a high incidence of bladder carcinomas (Pielesz, Baranowska, Rybak, & Włochowicz, 2002). Sudans III and IV are fat-soluble dye predominantly used for demonstrating presence of triacyglycerols in frozen sections. Para Red is chemically very similar to Sudan I. Although there is very limited data available, the U.K. FSA independent scientific experts have advised that, Para Red could be a genotoxic carcinogen (FSA, 2006).

As the illegal use of dyes has major economic consequences for world-wide food industries as well as an impact on public health, simple, reliable, rapid, and cheap-to-run analytical method is required. Although several techniques and instruments have been used by various researchers (Calbiani et al., 2004; Cornet et al., 2006; Nagase, Osaki, & Matsueda, 1989; Mazzetti et al., 2004; Pielesz et al., 2002), the scope of these methods are limited either to the detection of one of the four Sudan dyes or the quantitative determination of the dyes. In addition, it has not been mentioned in any of those reported techniques for simultaneous separation of all four Sudan dyes (I, II, III, and IV) together with Para Red using one absorbance using the same wavelength for detection in less than ten minutes. Therefore, a rapid, reliable, and cheap analytical technique is required for screening Sudan dyes and Para Red in foodstuffs. In our Food Institute, we reported a validated and improved rapid method for separating all four Sudan dyes and Para Red (Fig. 1) in red chilli peppers. Single-laboratory validation procedure was followed to demonstrate data reliability and thus, that the analytical methods proposed fits for its intended purpose. Therefore, the objective of this study is to present a rapid analytical method to separate and quantify simultaneously Sudan dyes (I, II, III, and IV) and Para Red in red chilli peppers by HPLC-UV-VIS method and to discuss its validation protocol including limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, reproducibility, and linearity.

2. Materials and methods

2.1. Samples

Ground eighty red chilli peppers were procured from different regions of Turkish local markets and bazaars. Samples were selected randomly without considering any of their histories.

2.2. Chemicals

Sudan I: 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol; (*catalogue no: 842-07-9*), **Sudan II:** 1-(phenylazo)-2-naphthol; (*catalaogue no: 3118-97-6*), and **Para Red:** 1-*p*-nitrobenzeneazo-2-naphthol; (*catalogue no: 6410-10-2*) were obtained from Sigma–Aldrich Co. Ltd. (Dorset, U.K.). **Sudan III:** 1-(4phenylazophenylazo)-2-naphthol; (*catalogue no: 201-638-6*) and **Sudan IV:** *o*-tolyazo-*o*-tolylazo-betanaphthol; (*catalogue no: 201-635-6*) were obtained from Alfa Aesar (Karlsruhe, Germany). All solvents were purchased from Merck (Darmstadt, Germany), unless otherwise specified.

2.3. Instrument

The high-performance liquid chromatography (HPLC) system consisted of an ultraviolet-visible (UV–VIS) detector (model SPD10AVVP), pump (model LC10ATVP), low pressure gradient unit (model FCV-10ALVP), degasser line (model DGU14A), autosampler (model

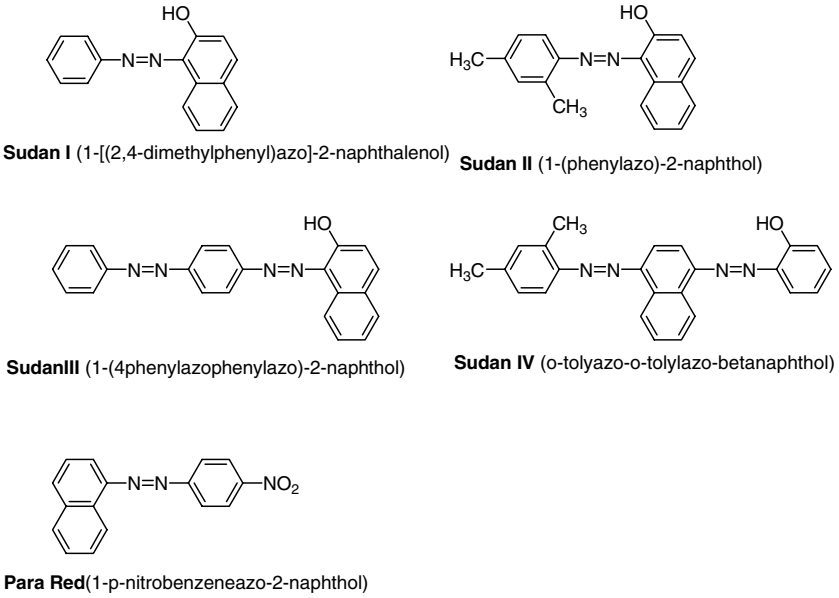

Fig. 1. Chemical structures and common name of Sudan dyes and Para Red.

SIL-10ADVP), column oven (model CTO-10AVP), and system controller (model SCL-10AVP) (Shimadzu, Tokyo, Japan). The chromatographic separation of Sudan dyes and Para Red were performed at 40 °C on a column of ACE C18, 250 mm × 4.6 mm i.d., 5 μm particles (Advanced Chromatography Technologies, Aberdeen, Scotland). The mobile phase of acetonitrile/methanol (80:20, v/v) was delivered at flow rate of 1 ml/min. The detection was monitored at 506 nm for Sudan dyes (I, II, II, and IV) and Para Red. Injection volume was set to 25 μl.

2.4. Stock solution

Stock solution of 100 mg/l Sudan dyes and Para Red were individually prepared in a solvent mixture of acetone/dichloromethane/methanol (3:2:1, v/v/v) and were stored at 4 °C until used. From stock solution, standard solutions in the range of 0.01–5.0 mg/l were prepared using the same solvent used.

2.5. Sample preparation

Acetone, dichloromethane, and methanol (3:2:1, v/v/v) mixture was used for extraction of Sudan dyes and Para Red from red chilli peppers. A 2.5 g sample was weighed into a 50 ml sample tube and diluted with 30 ml of above solvent mixture. The tube was then heated at 40 °C for 30 min, by vortexing for 1 min in every 5 min-intervals. After that, the extract was filtered through a Whatman no. 598 filter paper. The residue was washed several times with the extraction solvent till no colour was left. Finally, all combined supernatants were evaporated to dryness at 40 °C under vacuum. The residue was made up to a final volume of 10 ml with HPLC mobile phase. The sample extract was finally filtered through a GELMAN Acrodisc LC13 PVDV 0.45 μm pore size syringe filter (PALL Life Sciences, Ann Ambor, MI) for the HPLC analysis. As Sudan dyes and Para Red are heat and light sensitive, they should be kept in the dark at low temperature (<40 °C).

2.6. LOD and LOQ

Ten blank chilli peppers spiked at very low level were used for measurement of LOD and LOQ which were determined as follows:

$$\text{LOD} = \text{mean value} + 3 \times \text{standard deviation (SD)}$$
$$\text{LOQ} = \text{mean value} + 10 \times \text{SD}$$

where, mean value is zero.

2.7. Recovery, repeatability, and reproducibility

Ten blank chilli peppers spiked at the levels of 2.5, 5, and 50 mg/kg were used for measurement of recovery in three different concentrations. 250 μl solution from three different levels of concentration (25, 50, and 500 mg/l) were separately added to 2.5 g blank chilli pepper in order to obtain the concentrations of 2.5, 5, and 50 mg/kg, respectively. Spiked samples were incubated at 35 °C for 1 h in the dark in order to remove the remaining solvent before extraction. The repeatability and within-laboratory reproducibility were determined according to the ISO (1994) guidelines and expressed by coefficient of variation (CV_r and CV_R, respectively) measured on the same 10 fortified blank chilli peppers ($n = 3$-4 replicates per concentration level and analysed in three independent analytical runs).

2.8. Linearity and calibration standards

The linearity of the method was calculated using various concentrations of Sudan dyes and Para Red (0.01, 0.05, 0.5, 1.0, 2.5, and 5 mg/l) in triplicate. Quantification of Sudan dyes and Para Red in samples was carried out on the basis of peak areas and comparison with a calibration curve obtained with the corresponding standards.

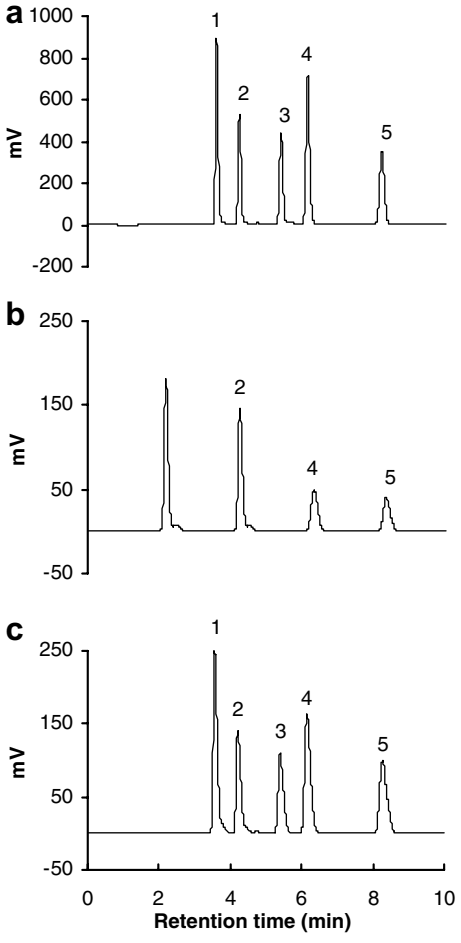

Fig. 2. Typical HPLC-UV chromatograms: (a) blank red chilli pepper spiked with Sudan dyes and Para Red (0.125 mg/kg), (b) red chilli pepper, and (c) standards. Peak no: (1) Para Red, (2) Sudan I, (3) Sudan II, (4) Sudan III, and (5) Sudan IV.

2.9. Validation scheme

Validation of the analytical method was a prerogative for every ISO/IEC 17025 (ISO, 2005) accredited laboratory. An 'in house' validation protocol was applied in accordance with an internal procedure as well as the Commission Decision 2002/657/EC (Commission Decision, 2002).

2.10. Statistical analysis

Results were expressed as mean ± SD ($n = 3$). The SD and coefficient of determinations (r^2) were determined using Microsoft Excel statistical software (Microsoft Corporation, Microsoft Office Excel 2003, Redmond, WA).

3. Results and discussion

3.1. Sudan dyes and Para Red in red chilli pepper

Typical HPLC-UV chromatograms of Sudan dyes and Para Red are presented in Fig. 2. Four Sudan dyes (I–IV) and Para Red were separated for the first time using one wavelength (506 nm) under 9 min. Among 80 red chilli peppers screened in our laboratory, only one of them contained 0.10, 0.04, and 0.05 mg/kg Sudans I, III, and IV, respectively (Table 1). No Sudan II and Para Red were detected in any of the red chilli peppers analysed. Although the use of Sudan dyes and Para Red as additives, at any level, in food products intended for human consumption is prohibited in Turkey and elsewhere due to their carcinogenicity (Commission Decision, 2005), red chilli pepper containing Sudan dyes, even if imported, should not have been placed in the market. For this reason, it is very important to develop a rapid, reliable, sensitive, and cheap method to analyse these synthetic Sudan dyes and Para Red even at trace level in foodstuffs.

3.2. LOD and LOQ

LODs and LOQs of the method for Sudan dyes (I–IV) and Para Red are presented in Table 2. Depending on the dye involved, LODs and LOQs were in the range of 1.2–5.4 and 4–18 μg/kg in red chilli, respectively. Even at very low concentration, Sudan dyes and Para Red gave excellent absorbance at 506 nm (Fig. 2). These values are better than those of Cornet et al. (2006) who found that LODs and LOQs were between 1.5 and 2 and between 3 and 4 mg/kg in spices, respectively.

3.3. Recovery, repeatability, and reproducibility

Sudan dyes and Para Red were spiked into blank red chilli peppers at three different concentrations (2.5, 5, and 50 mg/kg). The recovery, repeatability, and reproducibility varied from 89 to 98%, from 0.82 to 4.09% (CV_r), and from 1.33 to 4.65% (CV_R), respectively (Table 3). These results show that the method has a good precision due to its high recovery and low CV_r and CV_R values. Though Cornet et al. (2006) found the similar overall recoveries

Table 1
Content of Sudan dyes and Para Red in positive sample of red chilli pepper[a]

Sudan dyes and Para Red	(mg/kg)
Sudan I	0.10 ± 0.01
Sudan II	nd[b]
Sudan III	0.04 ± 0.01
Sudan IV	0.05 ± 0.02
Para Red	nd

[a] Data are expressed as mean ± SD ($n = 3$) on a fresh weight basis.
[b] nd, not detected.

Table 2
LOD and LOQ of the method for Sudan dyes and Para Red

	LOD (μg/kg)	LOQ (μg/kg)
Sudan I	1.2	4.0
Sudan II	3.9	13.0
Sudan III	1.2	4.0
Sudan IV	5.4	18.0
Para Red	3.6	12.0

Table 3
Results for recovery, repeatability (CV_r), and within-lab reproducibility (CV_R) in Sudan dyes and Para Red[a]

	Spike level of Sudan dyes and Para Red (mg/kg)															
	Sudan I				Sudan II			Sudan III			Sudan IV			Para Red		
	n	2.5	5	50	2.5	5	50	2.5	5	50	2.5	5	50	2.5	5	50
Day 1	3	2.44	4.77	49.71	2.45	4.79	48.97	2.42	4.85	48.97	2.35	4.57	45.38	2.39	4.63	47.33
Day 2	3	2.46	4.72	48.52	2.45	4.74	48.32	2.41	4.91	48.98	2.40	4.43	45.52	2.41	4.69	46.55
Day 3	4	2.42	4.87	47.23	2.46	4.79	47.15	2.47	4.91	48.90	2.38	4.34	44.30	2.37	4.70	46.71
Mean		2.44	4.79	48.49	2.45	4.77	48.15	2.43	4.89	48.95	2.38	4.45	45.07	2.39	4.67	46.86
SD		0.03	0.10	1.34	0.02	0.07	1.18	0.04	0.20	0.53	0.02	0.12	0.88	0.03	0.08	0.74
CV_r (%)		1.23	2.09	2.76	0.82	1.47	2.45	1.65	4.09	1.08	0.84	2.70	1.95	1.26	1.71	1.58
CV_R (%)		2.06	2.96	3.20	1.90	2.34	3.10	1.66	4.46	1.33	1.90	3.53	2.54	4.35	4.65	4.10
Recovery (%)		98	96	97	98	95	96	97	98	98	95	89	90	96	93	94

[a] Data are expressed on 10 replicates per concentration level analysed in three independent analytical runs.

Table 4
Linearity of Sudan dyes and Para Red

	Regression equation	r^2
Sudan I	$Y = 1.22 \times 10^4 X$	0.999998
Sudan II	$Y = 1.07 \times 10^4 X$	0.999998
Sudan III	$Y = 2.03 \times 10^4 X$	0.999997
Sudan IV	$Y = 1.18 \times 10^4 X$	0.999998
Para Red	$Y = 9.28 \times 10^4 X$	0.999957

Where, X is the concentration and Y is the peak area.

(89–100%) in powdered spices depending on the dye involved, their CV_r and CV_R values were much higher (<11.73%) than those of ours (<4.65%). Therefore, the method used in this study is superior compared to that of Cornet et al. (2006).

3.4. Linearity

The linearity of the method was assessed using various concentrations of Sudan dyes and Para Red (0.01 to 5 mg/kg). The coefficients of determinations (r^2) obtained for Sudan dyes (I–IV) and Para Red were all 0.9999 (Table 4).

4. Conclusions

It can be concluded that the presented method (HPLC-UV) has potential to be used for Sudan dyes (I–IV) and Para Red in red chilli peppers due to its rapidness, simplicity, reliability, and sensitivity. The validated method has a good overall recovery, repeatability, and reproducibility as well as low LOD and LOQ. It can separate all Sudan dyes and Para Red at one wavelength in less than 9 min and also involves minimal sample preparation. The average analysis time (sample preparation, extraction, separation, and quantification) takes approximately 1 h.

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References

Calbiani, F., Careri, M., Elviri, L., Mangia, A., Pistarà, L., & Zagnoni, I. (2004). Development and in-house validation of a liquid chromatography-electrospray-tandem mass spectrometry method for the simultaneous determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli products. *Journal of Chromatography A, 1042*, 123–130.

Commission Decision (2002). Commission decision 2002/657/EC of 12 August 2002 implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Communities, L221*, 8–36.

Commission Decision (2005). Commission decision 2005/402/EC of 23 May 2005 on emergency measures regarding chilli, chilli products, curcuma and palm oil. *Official Journal of the European Communities, L135*, 34–36.

Cornet, V., Govaert, Y., Moens, G., Loco, J. V., & Degroodt, J.-M. (2006). Development of a fast analytical method for the determination of Sudan dyes in chili- and curry-containing food stuffs by high-performance liquid chromatography-photodiode array detection. *Journal of Agricultural and Food Chemistry, 54*, 639–644.

Dillion, D., Combes, R., & Zeiger, E. (1994). Activation by caecal reduction of the azo dye D & C Red No. 9 to a bacterial mutagen. *Mutagenesis, 9*, 295–299.

FSA (2006). <http://www.food.gov.uk>, accessed October 15, 2006.

IARC (1975). *Monographs on the evaluation of the carcinogenic risk of chemicals to man: some aromatic azo compounds* (Vol. 8). Lyon, France: International Agency for Research on Cancer, pp. 224–231.

ISO (1994). *Accuracy (trueness and precision) of measurement methods and results-part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method (ISO 5725-2)*. Geneva, Switzerland: International Standards Organization.

ISO (2005). *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025)*. Geneva, Switzerland: International Standards Organization.

Mazzetti, M., Fascioli, R., Mazzoncini, I., Spinelli, G., Morelli, I., & Bertoli, A. (2004). Determination of 1-phenylazo-2-naphthol (Sudan I) in chilli powder and in chilli-containing food products by GPC cleanup and HPLC with LC/MS confirmation. *Food Additives and Contaminants, 21*, 935–941.

Nagase, M., Osaki, Y., & Matsueda, T. (1989). Determination of methyl yellow, Sudan I and Sudan II in water by high-performance liquid chromatography. *Journal of Chromatography A, 465*, 434–437.

Pielesz, A., Baranowska, I., Rybak, A., & Włochowicz, A. (2002). Detection and determination of aromatic amines as products of reductive splitting from selected azo dyes. *Ecotoxicology and Environment Safety, 53*, 42–47.

Rafii, F., Hall, J. D., & Cerniglia, C. E. (1997). Mutagenicity of azo dyes used in foods, drugs and cosmetics before and after reduction by *Clostridium* species from the human intestinal tract. *Food and Chemical Toxicology, 35*, 897–901.

Stiborová, M., Martínek, V., Rýdlová, H., Hodek, P., & Frei, E. (2002). Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. *Cancer Research, 62*, 5678–5684.

Society of Dyers and Colourists (1971) (3rd ed.). *Colour index* (Vol. 3). Badford, U.K.: Society of Dyers and Colourists.