

**Analysis of Illegal
Dyes in Chilli Powder
by LC-UV**

**Statutory Analysis
Government Chemist
Programme
Ad Hoc Project 1**

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1. Abstract

Sudan I – IV are suspected genotoxic carcinogens. To protect consumers it is illegal to add them to foods within the European Community and elsewhere, so reliable methods are required for detection of low levels of these colours. A method is described for the quantitative determination of Sudans I-IV, Para Red and Rhodamine B dyes in chilli powder. The dyes are extracted with a mixed solvent of 90:10 v/v acetonitrile and acetone at 40 °C. Sample extracts are filtered and analysed by reverse phase HPLC using a gradient elution system of acetonitrile and ammonium acetate buffer, pH 3.6, with a UV/Visible detector at a fixed wavelength of 510 nm.

The method has been validated for the dyes at concentrations from 0.1 to 100 mg/kg of chilli powder. Limits of detection were typically in the range 0.1-0.8 mg/kg. Recoveries were estimated to be in the range 82 to 111 %, with an average precision measured as relative standard deviation of 3-4 %.

2. Introduction

2.1 Background

Chilli powder is an important spice ingredient used by the food manufacturing industry in the formulation of a wide range of products, particularly for oriental dishes, curries, sauces and pickles. The quality of individual batches of chilli powder, which in many cases dictates their economic value, is often arbitrarily judged by the brightness, purity and intensity of the spice's colour.

This has led certain irresponsible traders, to add synthetic colourants to their spices to improve the appearance, and the apparent quality and freshness of their products. Several incidents have been reported within Europe, notably in early summer 2003 and 2005 (UK Foods Standards Agency information), where imported chilli powders and derivative foods were found to be adulterated with the Sudan dyes or related colourants.

Many of the azo dyes are suspected to be genotoxic and, or carcinogenic, according to the European Food Safety Authority, and are illegal additives in foods within the European Community. The European Commission made clear in 2005 that the presence of any dyes not permitted for use in food would be unacceptable at any level. The limit of detection for common HPLC methods at that time was between 0.5 and 1mg/kg. Where an illegal dye was found above this level, using this technique, either in the spice itself or a food in which it had been used, the product would need to be withdrawn from the market. The Commission did not set a limit of detection for testing with LC-MS but had advised that if testing was carried out using this method and an illegal dye was found, companies had to take action to withdraw the food concerned^{1,2}

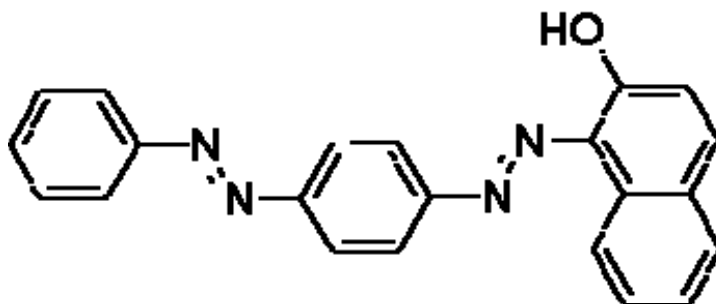
Reliable, accurate and sensitive analytical methods are necessary to help police this area of food manufacture and to ensure that all products are free of these illegal and potentially dangerous colourants.

2.1.1 Illegal Dyes Properties

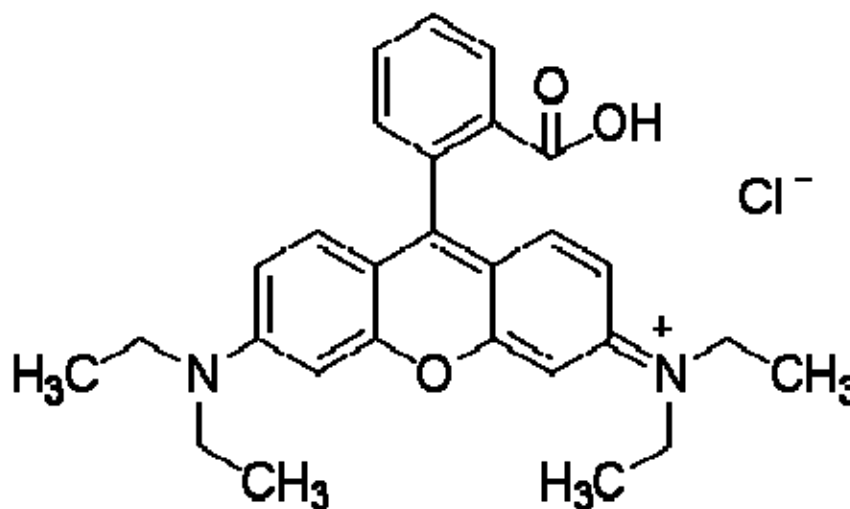
The Sudan dyes I-IV, Orange II and Para Red have molecular structures that are characterised by the nitrogen:nitrogen azo bridge between aromatic groups as illustrated in the structure for Sudan III in Figure I. Their relatively high molecular weights and low polarity provides these dyes with oil

soluble properties. In the dye Orange II the phenyl ring is sulfonated which renders this compound water soluble. The structure of Rhodamine B which is not a Sudan dye is also shown in Figure 1. The basic amine groups and partial ionisation of the molecular affords the Rhodamine B compound solubility in both oil and water.

Figure I – Molecular Structure of Sudan III and Rhodamine B



Sudan III



Rhodamine B

3. Method Development

3.1 Solvent for the Preparation of Standards

The solubility of the dyes in a range of common organic solvents was investigated. The dyes were found to be sparingly soluble in acetonitrile and methanol, more soluble in toluene and very soluble in the chlorinated solvents chloroform and dichloromethane. The solvent dichloromethane was chosen for preparation of stock solutions (1 milligram/millilitre) of Sudans I-IV, Para Red and Rhodamine B. For the dye Orange II ethanol was used to prepare the stock solution.

3.2 Extraction Conditions

Acetonitrile or methanol were found to extract the dyes efficiently from chilli powder samples. These were preferred to dichloromethane because the amount of co-extracted compounds from the chilli matrix was reduced. Soxhlet extraction was compared to shaking and sonication of the chilli/solvent mixture. The recovery of the dyes was similar by the two techniques, shaking and sonication was selected because it was less time-consuming. Best recoveries were found using the shaking method with a solvent mixture of 90:10 acetonitrile/acetone at a temperature of 40 °C.

3.3 Choice of Detector

A PDA detector allows the 7 dyes to be monitored at their individual maximum absorbance wavelengths. However the PDA used had an inferior signal to noise ratio compared to the standard UV/Visible HPLC detector. To achieve the best detection limits for the dyes a UV/Visible was selected and operated at wavelength of 510 nm.

3.4 Choice of HPLC Column

The 15 cm Octadecylsilica (ODS) column was found to give the best separation of the 7 dyes. The only disadvantage of this column is that the Rhodamine B peak is broad and tailing. A short phenylhexyl column can be used to improve Rhodamine B peak shape but with a slight loss in peak resolution. For confirmation of the dyes in a sample extract it is recommended that samples are analysed on both columns.

4. Method Validation

Validation was conducted in accordance with the International Harmonised Protocol for the Validation of Analytical Methods³. The procedure was tested for linearity of response, limit of detection, same day and daily precision, bias, recovery and for the effect of different brands of chilli powder. Because of the lack of reference materials the validation procedures were solely based on blank chilli powder samples that had been spiked with standard solutions.

4.1 Linearity of Calibration

A range of multi-standard solutions from 0.2 to 50 mg/L were prepared and these were run on the HPLC under the conditions detailed in Section 5.2.1. With the exception of Rhodamine B, the 50 mg/L solutions of the dyes were found to be too strong, producing signals that saturated the detector amplifier. Consequently the linearity of each calibration was determined over the

range from 0.2 to 20 mg/L. The linearity for the dyes as shown in Table 1 is excellent for this concentration range.

Table 1: Calibration equations for the Dyes

	Equation of Calibration 0.2 to 20mg/L*	Correlation coefficient R ²	Equation of Calibration 0.2 to 2 mg/L	Correlation coefficient R ²
Sudan I	y=57570x+513	1.0000	y=59592x-916	0.9997
Sudan II	y=46829x-445	0.9999	y=47526x-884	0.9989
Sudan III	y=84937x-5027	0.9999	y=82523x-990	0.9995
Sudan IV	y=71087x-3442	0.9999	y=70061x-1173	0.9992
Para red	y=72511x-1520	0.9999	y=73063x-515	0.9999
Rhodamine B	y=60916x-18098	0.9997	y=52835x-1416	0.9996
Orange II	y=42474x-24974	0.9942		

* for Rhodamine the calibration extends to 50 mg/L

Where y : detector response

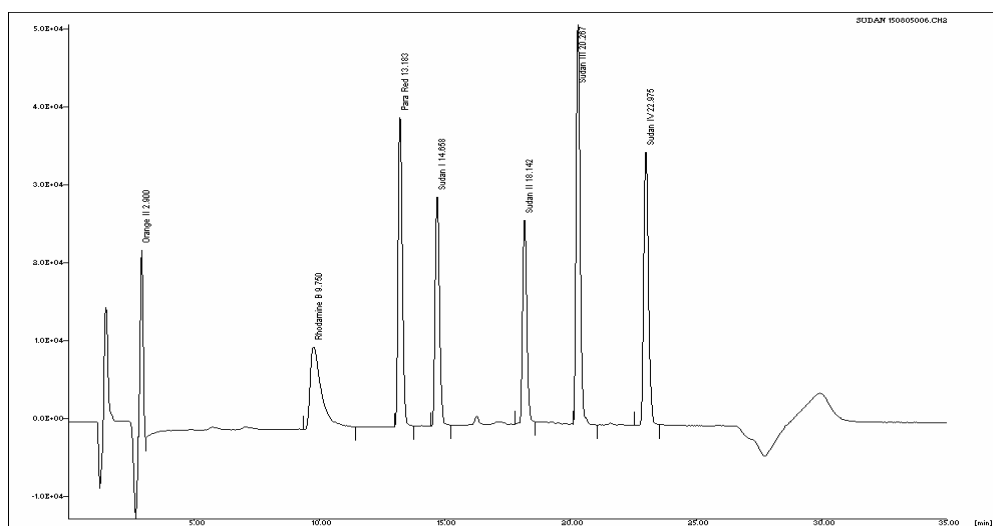
x : concentration of dye in solution (mg/L)

Calibration equations are also given for the range 0.2 to 2 mg/L. This narrower range of calibration was selected in order to determine more accurately the amount of each dye in the LOD experiments where the solutions analysed were at the low end of the concentration range.

The dye Orange II was included in all standard solutions, calibrations, recovery and precision experiments.

However, under the HPLC conditions used in this method Orange II is not retained on the column and it elutes early, close to a solvent peak in all chromatograms, as can be seen in Figure 2

Figure 2: Chromatogram of a Mixed Dye Standard.



Because of this interference it was not possible to determine the dye at the low concentrations employed in the validation experiments. As a result there are no performance data for this analyte.

4.2 Detection Limit

Limit of detection (LOD) was determined using both low concentration standards and spiked extracts. The standard deviation of six replicate injections (stdev.) was determined in terms of sample concentration and the LOD was taken to be equal to three times the standard deviation. The corresponding LODs for each test solution are reported in Table 2.

Table 2: Detection limits for the Dyes

Test Solution	Rhodamine B	Para red	Sudan I	Sudan II	Sudan III	Sudan IV
	mg/kg Chilli Powder					
0.2 mg/L standard solution	0.6	0.1	0.3	0.5	0.2	0.2
0.4 mg/L standard solution	0.4	0.3	0.2	0.4	0.3	0.2
Spiked extract. Final dye concentration equivalent to 0.2 mg/mL	0.4	0.2	0.3	0.4	0.5	0.4
Spiked extract. Final dye concentration equivalent to 0.4 mg/mL	0.4	0.4	0.4	0.8	0.6	0.4

The four sets of LOD values for Rhodamine B, Para Red and Sudan I in the table are very similar and appear to be largely independent of whether standard solutions or spiked extracts were used in the measurement. In contrast, results for Sudans II – IV show higher LODs for the spiked extracts. This is explained because the chilli matrix components tend to elute late in each chromatogram and can therefore cause background interference for these analytes.

4.3 Precision

Recoveries at three different spike concentrations (4, 20 and 100 mg/kg) were carried out within the same day. Each recovery was performed in triplicate enabling precision to be estimated. The recoveries at the 4 and 100 mg/kg level were repeated in duplicate on two following days in order to assess the day to day precision of the method. The combined statistical results are shown in Tables 3 and 4. The method precision s_r and intermediate precision s_I are calculated from the following equations:-

$$s_r^2 = s_E^2 + s_e^2 \quad (1)$$

$$s_I^2 = s_d^2 + s_E^2 + s_e^2 \quad (2)$$

s_r^2 - method repeatability variance

s_I^2 - intermediate (between-day, within-laboratory) variance

s_E^2 - variance due to extraction

s_e^2 – residual variance or between injection variance

s_d^2 – day to day variance

The variance components were determined by both ANOVA (Analysis of Variance), according to ISO 5725:1994 Part 3, and REML (Restricted Maximum Likelihood Estimation) software packages.

As is to be expected the precision values for the 100 mg/kg concentration are in general far better than those for the low level 4 mg/kg spike. This is in part a reflection of the size of the analyte signal being measured but also the ability of the operator or quantitative software to determine the start, end and base of each dye peak. The latter is more difficult with Rhodamine B because the peak is broad and unsymmetrical. Poorer precision is also found with Sudans II and IV because of background interferences from co-eluting chilli components.

Table 3: Precision Data at the 4 mg/kg.

Dye	Mean (mg/kg)	Variance components (as SD)			Precision (mg/kg)		Precision (% RSD)*	
		Between- day	Between- extract	Residual	s_r	s_I	RSD _r	RSD _I
Para Red	3.99	0.08	0.11	0.12	0.17	0.19	4.2%	4.7%
Rhodamine B	3.88	0.12	0.18	0.28	0.33	0.35	8.5%	9.1%
Sudan I	3.89	0.12	0.00	0.15	0.15	0.19	3.8%	4.9%
Sudan II	4.76	0.42	0.00	0.18	0.18	0.46	3.8%	9.6%
Sudan III	3.76	0.06	0.06	0.06	0.09	0.11	2.3%	2.8%
Sudan IV	3.99	0.36	0.01	0.45	0.45	0.57	11.3%	14.4%

*All values are derived from duplicate analysis of three extracts on each of three days. % RSD_x is calculated as s_x/mean .

Table 4: Precision Data at 100 mg/kg.

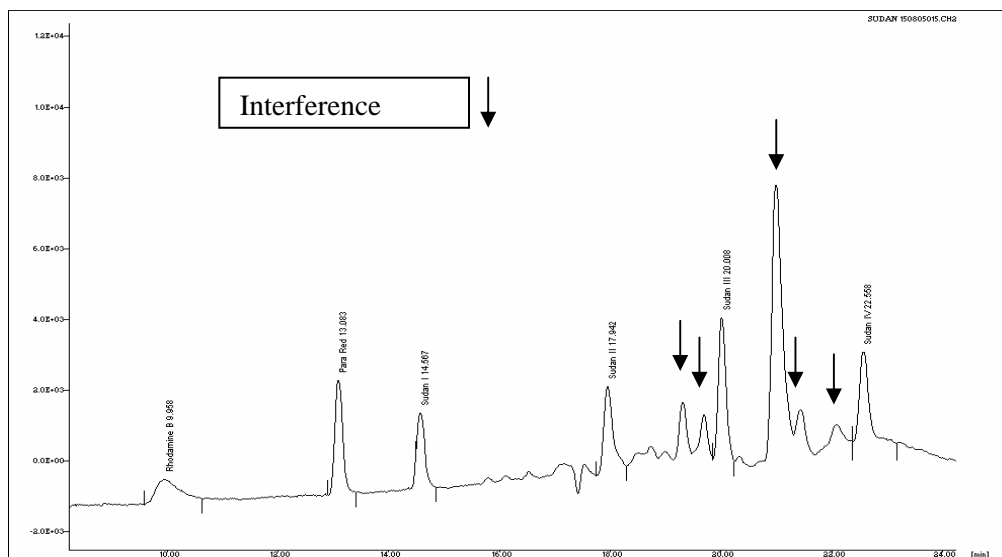
Dye	Mean (mg/kg)	Variance components (SD)			Precision (mg/kg)		Precision (% RSD)*	
		Between- day	Between- extract	Residual	s_r	s_I	RSD _r	RSD _I
Para Red	98.48	3.05	0.04	1.37	1.37	3.34	1.4%	3.4%
Rhodamine B	99.19	6.78	0.05	1.96	1.96	7.06	2.0%	7.1%
Sudan I	96.20	2.76	0.04	1.33	1.33	3.06	1.4%	3.2%
Sudan II	95.24	2.78	0.43	1.43	1.50	3.16	1.6%	3.3%
Sudan III	92.60	1.85	0.05	1.26	1.26	2.24	1.4%	2.4%
Sudan IV	91.64	4.23	0.06	4.34	4.34	6.06	4.7%	6.6%

The day to day variation in precision is only evident for the high concentration recoveries. Percentage recovery is progressively lower for the dyes with longer retention times. This is a trend that has been observed in earlier work and probably arises because the more non-polar dyes are extracted less efficiently by the acetonitrile/acetone solvent.

4.4 The Effect of Chilli Powder Varieties

To establish whether the type or batch of chilli powder has any effect, recovery experiments were performed on four additional samples, three commercial varieties and a laboratory blend of two chilli powders.

Figure 3: Main Interfering Peaks in Chilli Powder



The results in Table 5 show that recoveries at both levels are comparable and are independent of the variety of chilli powder being analysed. Characteristically, most extracts are strongly red coloured, yet there is little indication of matrix interferences in the extract chromatograms. The limited number of interference peaks are shown in Figure 3. Literature information and practical experience has shown that the main coloured compounds in chilli are the carotene – fatty acid, mono- and di-esters. These components appear to be removed by the HPLC guard column. The matrix components that do chromatograph tend to be observed in the longer retention time region of the chromatogram. The principal interfering peak located between Sudans III and IV (for an ODS column) has been assigned to unesterified capsanthin. The concentration of free capsanthin varies between chilli powder types. However, because this component is always well separated from the dye analytes, there appears to be no direct effect on the method performance or recovery data.

Table 5: Recovery of Dyes from Different Varieties of Chilli Powder

% Recoveries at two levels- four chilli powders

Level		Rhodamine	Para Red	Sudan I	Sudan	Sudan	Sudan
		B			II	III	IV
		Average % Recovery					
NATCO Chilli	20 mg/kg	84	95	93	104	90	82
	100 mg/kg	90	96	89	88	85	88
NATCO extra Hot chilli	20 mg/kg	86	96	93	104	86	82
	100 mg/kg	91	96	91	90	87	86
TRS Chilli	20 mg/kg	89	95	92	103	89	90
	100 mg/kg	89	95	89	82	87	90
Blended Chilli*	20 mg/kg	88	95	93	95	83	81
	100 mg/kg	92	97	93	91	87	89

* The blank chilli powder varieties TRS and Rajah were blended at LGC.

5. Analytical Procedure

5.1 Experimental

Dye standards

Sudan I ~97% pure, Sigma-Aldrich.

Sudan II ~90% pure, Sigma-Aldrich.

Sudan III ~90% pure, Sigma.

Sudan IV ~80% pure, Aldrich.

Rhodamine B ~90% pure, Sigma.

Para Red unknown purity, Acros.

Orange II >85% purity, Acros.

Equipment & Chemicals

125 mL plastic bottles, Bibby Sterilin Ltd.

Ultra-sonication bath, Kerry Ltd.

Orbital shaker, Luckham Rotatest.

Water bath, Techne TE 8J.

Plastic filter funnels, 65 mm diameter.

110 mm GF/A filter papers, Whatman.

Acetone, Analytical grade, Fisher.

Dichloromethane, HPLC Grade, Fisher.

Acetonitrile, HPLC Grade, Fisher.

Solvent extractant: mix 90 volumes of acetonitrile with 10 volumes of acetone.

Glacial acetic acid, Fisher.

Ammonium acetate, ANALAR grade, BDH.

Deionised water, Elga.

Blank Samples

Small sachets (100 – 400 g) of Chilli powders were bought at local retailers. These included NATCO Chilli powder, NATCO extra hot Chilli powder, TRS Chilli powder and Rajah brands

Heath & Safety

Organisations making use of this draft method should ensure that a section dealing with health and safety issues is included in their protocol.

5.2 Method

Weigh accurately 3, 4 or 5 g (w) of chilli powder sample into a 150 mL plastic bottle. Add sufficient mixed solvent for a ten fold dilution of the sample ($10 \times w$ in mL) to the bottle and sonicate the mixture for 30 seconds. Place the bottle in a thermostatted shaker at 40 ± 0.5 °C. Shake and extract for a total period of 30 minutes. If the shaker and waterbath are separate units, return the bottle to the waterbath for 5 minutes between shaking periods to maintain the extraction temperature. Immediately after extraction pass the contents of the bottle through a GF/A filter and collect the filtered extract. Place 2 mL of extract in a HPLC vial for analysis. Because the lipid may separate from the extract solution on cold storage it is recommended that HPLC analyses are carried out immediately.

Analyse the samples under the HPLC conditions described in section 5.1.1

5.2.1 HPLC Conditions

Equipment : Jasco 1580 pump, Jasco DG1580-54 degasser, Jasco LG-2080-C4 quaternary gradient unit, AS1559 autosampler, CO965 column oven and Jasco 1575 UV/Visible detector

Column : 4 μ 150 mm 3.9 mm ODS Waters Novapack column with guard cartridge.

Injection volume: 20 μ L

Column temperature : ambient

Flow rate : 1 mL/min

Mobile phase A : 10 mM ammonium acetate in de-ionised water, pH adjusted to 3.6 with glacial acetic acid.

Mobile phase B: acetonitrile

Gradient analysis: This is given in Table 6.

Table 6-HPLC Gradient Conditions

Time (min)	Mobile Phase Composition	
	% A	% B
0	60	40
7	40	60
24	2	98
27	80	20

6. Conclusions

The method as presented does not incorporate an extraction cleanup or concentration stage. Even so the detectability is excellent and is similar for all the dye analytes studied. Typically, limits of detection (LOD) in the range 0.1 to 0.8 mg/kg were found for the dyes, the values for the later eluting Sudans being higher because of the interference from the chilli components. The fixed wavelength of 510 nm is a compromise value which gives generally acceptable LOD values for all the dye analytes studied. However, better sensitivity is possible using the maximum absorbance wavelengths for each dye, 480 nm, 500 nm, 550 nm for Sudan I, Sudan II and Rhodamine B respectively. Within-a-day and daily precision is good and recoveries are high, indicating no bias for the analytical method. The method has been validated for chilli powder containing 0.1 to 100 mg/kg. A higher upper limit to the concentration range is possible if the procedure is modified to include larger extract volumes or dilutions.

The method can be used to determine or confirm the presence of high concentrations of Orange II dye in chilli powder. However if the concentration is less than 100 mg/kg then quantitation of this analyte becomes unreliable because of interference by the solvents.

7. References

1. Food Standards Agency – information
www.food.gov.uk/safereating/chemsafe/sudani/sudanitimeline
and FSA Board Meeting Minutes 12 May 2005
2. Opinion of the European food Safety Authority:
www.efsa.eu.int/science/afc/afc_opinions/1127_en.html
3. Harmonised Guidelines for Single-Laboratory Validation of Methods of Analysis. Thompson. M, Ellison SLR and Wood R. Pure Appl. Chem., Vol. 74, No5, pp. 835-855,2002.
4. ISO 5725:1994 Part 3: Intermediate measures of precision of a standard measurement method. ISO, Geneva (1994)