Illegal Dyes in Food and Spices – A 2006 LGC LC-UV/Visible Method Reviewed and Updated for 19 Dyes

KM Gray[a,b,c], MJ Walker[a,c], MJS Burn[a], M Mazur[a], K Niedzwiedzka[a], K Liszka[a] and D Thorburn Burns[c,d]

a Government Chemist Programme, LGC, Queen's Road, Teddington, Middlesex, TW11 0LY, UK
b Corresponding Author – kirstin.gray@lgcgroup.com
c These authors contributed equally to the publication of this work
d Institute of Global Food Security, The Queen's University of Belfast, BT9 5HN, UK

Summary

Intermittent findings of illegal dyes in foods continue to be a feature of international trade. Herein the validation of a 2006 LC-UV/Visible method for a limited number of such dyes in chilli powder has been reviewed and a new multi-dye general screening method is proposed. Both methods apply 90:10 acetonitrile:acetone extraction at 40℃ and reverse phase gradient elution liquid chromatography (LC) with UV/Visible detection. No clean-up, other than filtration, and no concentration stage is required. Of the 23 dyes investigated for the new screening method in chilli powder, canned chicken in a curry sauce, fennel, palm oil, paprika and turmeric, 19 are adequately dealt with by the new method. Recovery, linearity and within-day precision data are reported and although limits of detection (LOD) were not extensively investigated they appear to be consistent with previous LOD data.

The dyes covered by the proposed general screening method are Orange II, Sudan I–IV, Sudan Black B, Sudan Red 7B, Sudan Red G, Methanil yellow, Dimethyl yellow, Auramine O, Bixin, Fast Garnett GBC, Rhodamine B, Oil Orange SS, Orange G, Sudan Orange G, Naphthol Yellow, Acid Red 73, Toluidine Red, Sudan Red B, and Para Red.

Poor sensitivity was exhibited for Orange G, Naphthol Yellow, Congo Red and Acid Red 73. Turmeric proved to be a very challenging matrix for which the proposed general screening method in its present format is not applicable. While the remaining dyes are adequately resolved by the general screening method some display close retention times. For these, the small number of problematic dyes and when confirmation is required, mass spectrometric detection is recommended.

Introduction

Synthetic dyes have been widely used as colouring agents to colour various materials such as waxes, shoe/floor polishes, plastics, oils, textiles as well as food and drinks. The synthetic dyes, compared to most natural dyes, have higher stability and lower production costs. Due to

their chemical structures some synthetic dyes can have adverse effects on health including allergenic and asthmatic reactions and DNA damage. Some are suspected carcinogens and mutagens[1]. For these reasons the use of most synthetic dyes in food products is now forbidden in Europe and by many national and international organisations. Annex II to Regulation (EC) No 1333/2008 lays down a European Union list of food additives approved for use in foods and their conditions of use and includes a positive list of permitted colours in which the dyes under consideration here do not feature[2]. Therefore, any amount of non-permitted dyes in the food chain is undesirable and potentially in breach of Regulation (EC) 178/2002 (General Food Law)[3] which makes it an offence to sell food that is injurious to health, unfit for human consumption or non-compliant with legislation governing food safety. The Standing Committee on the Food Chain and Animal Health, SCoFCAH, at a meeting held in Brussels on 23 June 2006 decided that in order to adopt a consistent approach an action limit of 500ppb (parts per billion, μgkg^{-1}) should be applied to illegal dyes in food ingredients such as spices and palm oil. The Standing Committee added that such an approach should not be seen as Member States accepting adulteration and therefore the food industry should continue to investigate sources of contamination when they are found below $500\mu gkg^{-1}$ and take measures to reduce levels where possible[4]. Recent correspondence (August 2015) with the Food Standards Agency confirmed that no further update of the SCoFCAH position had occurred.

The illegal use of banned dyes in foodstuffs, especially in spices, persists[5], as reported via the Rapid Alert System for Food and Feed[6] in the EU, are detailed in Table 1 (RASFF Search criteria – Subject: Unauthorised Colour, Notified from 01/04/2014 to 31/03/2015).

Table 1 – Unauthorised Colours RASFF Recalls 01/04/2014-31/03/2015

Classification	Date of Case	Country	Product Category	Subject
Alert	09/03/15	Germany	Fats and Oils	Unauthorised colour Sudan IV (1.9 $mgkg^{-1}$) in red palm oil from unknown origin, via the Netherlands
Information for Attention	27/02/15	United Kingdom	Herbs and Spices	Unauthorised colour Rhodamine B (7 $mgkg^{-1}$) in fennel seeds from Pakistan
Alert	27/01/15	Netherlands	Soups, Broths, Sauces and Condiments	Unauthorised colour methyl yellow in bean curd products from Taiwan

Classification	Date of Case	Country	Product Category	Subject
Information for Follow-up	28/11/14	Luxembourg	Herbs and Spices	Unauthorised colour Sudan IV (44 μgkg^{-1}) in yellow curry from Germany
Information for Follow-up	28/11/14	Luxembourg	Soups, Broths, Sauces and Condiments	Unauthorised colour Rhodamine B (17 μgkg^{-1}) in madras curry from Belgium
Alert	20/11/14	Netherlands	Fats and Oils	Unauthorised colour Sudan IV (6400 μgkg^{-1}) in palm oil from Ghana
Alert	15/04/14	Belgium	Fats and Oils	Unauthorised colour Sudan IV (0.76 $mgkg^{-1}$) in red palm oil from Guinea

To protect consumers and legitimate businesses food products must be monitored and tested regularly for assurance that they are free of illegal contaminants. Reliable methods are required for the detection of low levels of these colorants. The Government Chemist and academic colleagues have a long standing interest in such analyses[7-10]. On the emergence of Sudan I as a contaminant of chilli powder in 2003 and a similar major incident involving many food products withdrawn in 2005[11] the determination of Sudan dyes in foods received much attention[12]. In 2006 the Government Chemist validated and released a LC-UV/Visible method for the quantitative determination of Sudans I-IV, Para Red and Rhodamine B dyes in chilli powder, the "2006 Method"[13]. In 2012, prompted by problems with dimethyl yellow in an oleoresin/surfactant matrix[14] the Government Chemist took a renewed interest in illegal dyes. A review of the literature for the most commonly used dyes and matrices of interest showed that dye and/or matrix specific methods are available for many dyes in spices and spice mixtures[15-26]. Most commonly examined were the Sudan dyes. Recoveries for these and the other dyes examined were in the range 60-119%, the most common matrix studied being chilli. However, no widely applicable general simultaneous screening method was available for the most-used illegal dyes.

Herein are described some key aspects of the "2006 Method" and based on this a more general screening method is now proposed. The proposed new screening method was investigated for the detection of 23 of the dyes most often implicated in food recalls, see Table 2.

The matrices investigated, namely chilli powder, canned chicken in a curry sauce, fennel, palm oil, paprika and turmeric were chosen on the basis that they were the most-likely products to be adulterated with illegal dyes.

Table 2 – Dyes that have been Included in a Cross-Section of UK Laboratory Analytical Suites

To 2009	2010 and later
Sudan I – IV	**Acid Red 73**
Sudan Orange G	Azorubin (E122, carmoisine)
Sudan Red VII	Chrysodine#
Sudan Red 7B	**Congo Red**
Sudan Red G	**Naphthol Yellow**
Sudan Red B	Malachite Green#
Sudan Black B	Leucomalachite Green#
Auramine O#	**Oil Orange SS**
Bixin# (E160b)	Ponceau MX#
Butter (dimethyl) Yellow	Ponceau 3R#
Fast Garnet GBC	Red 2G#
Metanil Yellow#	
Nitroanaline	
Orange II#	
Orange G	
Para Red	
Rhodamine B#	
Toluidine Red	

Notes:

a not all laboratories analyse for all of these dyes

b **Bold** entries were studied for this paper

c Bixin (E160b) and Azorubine (E122, carmoisine) are authorised colours but are not permitted in spices

d # denotes water-soluble dyes, the remainder are oil-soluble

How Does the Proposed Method Compare with the "2006 Method"?

The "2006 Method" for the determination of 6 illegal dyes in chilli powder used a 90:10 acetonitrile:acetone extraction solvent at 40°C with no clean-up other than filtration. No concentration stage was required and separation was by reverse-phase gradient elution LC with UV/Visible detection. The method exhibited typical limits of detection (LOD) in the range 0.1 to 0.8mgkg^{-1}. For the proposed general screening method a similar approach was adopted although LOD was not extensively investigated. However, with sample dilution of 5g to 50mL the lowest standard of 0.1µgmL^{-1} is equivalent to 1.0mgkg^{-1} which is consistent with previous LOD data.

In 2006 a fixed detection wavelength of 510nm was chosen as a compromise value however it was known that better sensitivity was possible using the maximum absorbance wavelengths for each dye and in the general screening method several wavelengths are now preferred. The additional dyes required an optimised gradient to achieve improved resolution as described below.

Of the 23 dyes investigated for the multi-dye screening method Orange G, Naphthol Yellow, Congo Red and Acid Red 73 gave very poor recoveries due to low sensitivity, however they are of less practical concern as during the last year there were no RASFF notifications involving these dyes.

It did not prove possible to develop a screening method for turmeric as serious matrix interference effects were found using all the chromatographic conditions investigated.

The method described has been optimised to screen for 19 dyes in chilli powder, canned chicken curry in a curry sauce, fennel, palm oil, and paprika. Some of these dyes, while baseline resolved, still exhibit close retention times, R_t (minutes). Sudan Orange G (R_t=21.09) elutes close to Para Red (R_t=21.12) and Toluidine Red (R_t=22.62) elutes close to Sudan Red G (R_t=22.65).

For dyes/matrices not covered by the screening method, or where confirmation is required different chromatographic conditions or, preferably, mass spectrometry detection should be applied.

Materials and methods followed by the screening method are detailed in Appendix 1.

Review of the "2006 Method" – Validation in a Chilli Matrix

The "2006 Method" validation studies were carried out on Sudan I-IV, Para Red, Rhodamine B and Orange II using the international Harmonised Guidelines for Single-Laboratory Validation of Methods of Analysis[27]. The "2006 Method" was tested for linearity of response, limit of detection, same day and daily precision, bias, recovery, and the effects of different brands of chilli powder. Because of the lack of reference materials the validations were based on blank chilli powder samples spiked with standard solutions. It was originally found that a standard UV/Visible detector operated at a specific wavelength had superior signal to noise properties compared to a diode array detector (DAD) and 510nm was used as the best compromise. A guard column/cartridge were also found to be useful; an ODS Waters Novapak column with guard cartridge were used (4µm particle size, 150mm long, 3.9mm diameter).

The original chromatographic elution conditions are given in Table 3 below.

The linearity, in 2006, of calibration for the dyes studied in the concentration ranges 1-20 mg L^{-1} and 0.1-2 mg L^{-1} was excellent, correlation coefficients being greater than 0.9995 for all except Orange II. As this dye eluted close to the solvent peak it could not be determined at the low concentrations employed in the validation studies.

Table 3 – Elution Gradient ("2006 Method")

Time (min)	0	7	24	27
A: 10mM ammonium acetate, pH 3.6 with glacial acetic acid	60 %	40 %	2 %	80 %
B: Acetonitrile	40 %	60 %	98 %	20 %

In the original work (2006) limits of detection (LOD, 3*sd*) were determined using two low concentration standards and spiked extracts. The standard deviations were calculated from data from six replicate injections. The four sets of data are in Table 4.

Table 4 – Dye Detection Limits (2006 data)

Test Solution	**Rhodamine B**	**Para Red**	**Sudan I**	**Sudan II**	**Sudan III**	**Sudan IV**
	mgkg^{-1} Chilli Powder					
0.2 mg/L	0.6	0.1	0.3	0.5	0.2	0.2
0.4 mg/L	0.4	0.3	0.2	0.4	0.3	0.2
Spiked extract 1[a]	0.4	0.2	0.3	0.4	0.5	0.4
Spiked extract 2[b]	0.4	0.4	0.4	0.8	0.6	0.4

a Final dye concentration equivalent to 0.2 mgmL^{-1}
b Final dye concentration equivalent to 0.4 mgmL^{-1}

LOD values cited in Table 4 were largely independent of whether spiked extracts or standard solutions were used. The results for Sudan II-IV may be influenced by the chilli matrix components which elute late in the chromatograms and cause background interference.

The 2006 precision was estimated from triplicate recoveries at three different spike concentrations (4, 20 and 100 mgkg^{-1}). The recoveries were repeated on two following days at the 4 and 100 mgkg^{-1} levels in order to assess the day-to-day precision. The combined sets of results are shown in Tables 5 and 6.

To examine whether the type or batch of chilli powder had any effect on recovery experiments were carried out on three commercial samples and a laboratory blend of two chilli powders. The results in Table 7 show that the recoveries at both levels were comparable and largely independent of the variety of chilli powder.

Table 5 – Precision at 4 mgkg^{-1} (2006 data)

Dye	Mean (mgkg^{-1})	Variance Components (as SD)			Precision (mgkg^{-1})		Precision (% RSD)*	
		Between-day	Between-extract	Residual	s_r §	s_I	RSDr	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

§ s_r is a combination of the residual and between-extract terms

Table 6 – Precision at 100 mgkg^{-1} (2006 data)

Dye	Mean (mg kg^{-1})	Variance components (SD)			Precision (mgkg^{-1})		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%

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

§ s_r is a combination of the residual and between-extract terms

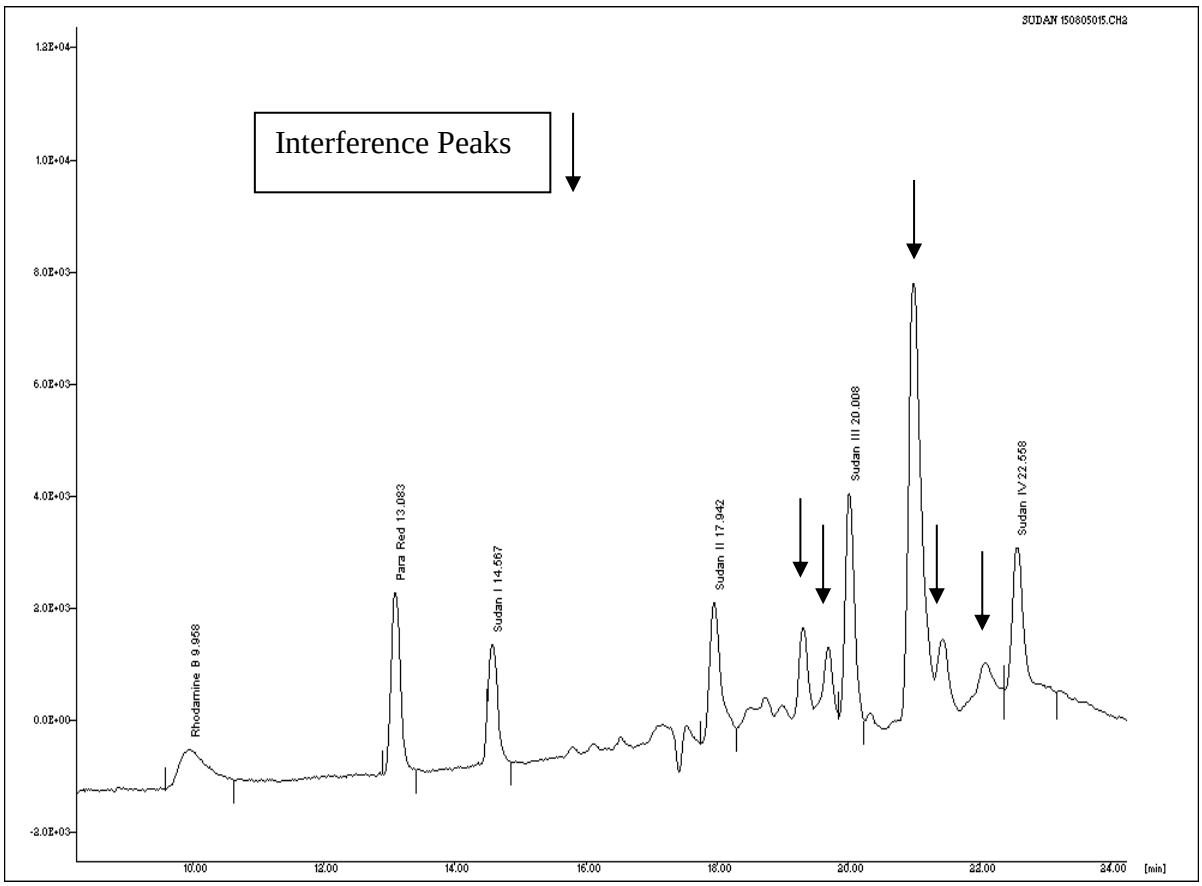
Table 7 – Recovery of Dyes from Chilli Powder at Two Concentrations (2006 data)

Product and Dye Concentration		Rhodamine B	Para Red	Sudan I	Sudan II	Sudan III	Sudan IV
	$mgkg^{-1}$	**Average % Recovery**					
NATCO Chilli	20	84	95	93	104	90	82
	100	90	96	89	88	85	88
NATCO Extra Hot Chilli	20	86	96	93	104	86	82
	100	91	96	91	90	87	86
TRS Chilli	20	89	95	92	103	89	90
	100	89	95	89	82	87	90
Blended Chilli*	20	88	95	93	95	83	81
	100	92	97	93	91	87	89

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

Although most extracts are strongly coloured there is little indication of matrix interference in the extract chromatograms as can be seen in the chromatogram for a sample of spiked extract of chilli powder, see Figure 1. The main coloured compounds in chilli appear to be 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 Sudan 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.

Figure 1 – Chromatogram Showing the Main Peaks from the Chilli Matrix (2006 data)



Proposed Multi-Dye Screening Method

Preliminary Studies

The solubility of the newly-studied dyes (Table 2 bold entries) 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 chlorinated solvents chloroform and dichloromethane. Dichloromethane was chosen for the oil soluble dyes and ethanol/water (80:20) for the water soluble dyes for the preparation of stock solutions (1mgmL^{-1}).

Acetone or methanol was found to extract the dyes from chilli powder and give a lower amount of co-extracted compounds than did dichloromethane. Soxhlet extraction was compared to shaking and sonification of the chilli/solvent mixture. The recovery of dyes was similar by both techniques; shaking and sonification was selected as it was less time-consuming. The best recoveries were found using a solvent mixture of 90:10 acetonitrile:acetone at 40^{o}C.

Further Matrix Studies

With the exception of Congo Red, which had proved very challenging for this method, 22 dyes (3 to 6 per matrix) were spiked into five matrices (chilli powder, canned chicken in a curry sauce, fennel, palm oil and paprika) in triplicate at concentrations of 5, 10 and 20 mgkg^{-1} except for Acid Red 73, Naphthol Yellow and Orange G which, due to their low sensitivity, were spiked at 50, 100, 200, mgkg^{-1}. The extracts were examined by LC/DAD (380, 430, 470, 510, and 550 nm).

Calibration linearity was satisfactory for all the dyes successfully chromatographed with correlation coefficients, (R^2), between 0.9925 and 0.9997 for 0.1 to 2.0µgml^{-1}. Within day precision data for 22 dyes in 5 matrices, expressed as standard deviations, are given in Table 8 and recovery data in Table 9. Precision is acceptable for all but Orange G. At the most sensitive wavelengths the recoveries were not found to be significantly concentration-dependant; most of the recoveries were mainly within the range 60-110%. Acid Red 73 showed poor recovery throughout and Congo Red was not included in these experiments.

Table 8 – Proposed Screening Method – Precision Data

Matrix	λ nm	Dye	Spiking Concentration (mgkg^{-1})	Standard Deviation per Spiking Concentration
Chilli Powder	470	Orange II	5	20.2
			10	3.2
			20	1.0
	510	Sudan I	5	8.0

Matrix	λ nm	Dye	Spiking Concentration ($mgkg^{-1}$)	Standard Deviation per Spiking Concentration
			10	2.9
			20	3.6
		Sudan II	5	6.4
			10	5.9
			20	6.9
		Sudan III	5	12.5
			10	21.4
			20	1.3
		Sudan IV	5	3.8
			10	19.0
			20	4.5
Canned Chicken in a Curry Sauce	550	Sudan Black B	5	7.9
			10	5.8
			20	0.4
		Sudan Red 7B	5	8.8
			10	3.6
			20	4.2
		Sudan Red G	5	10.6
			10	3.1
			20	2.1
Fennel	430	Methanil yellow	5	0.7
			10	6.5
			20	3.9
		Dimethyl yellow	5	3.8
			10	5.1
			20	1.5
		Auramine O	5	4.1
			10	11.4
			20	1.9
	470	Bixin	5	14.1
			10	12.2
			20	3.1
	380	Fast Garnett GBC	5	6.5
			10	3.3
			20	1.7
	550	Rhodamine B	5	10.9
			10	1.3
			20	1.7
Palm Oil	470	Oil Orange SS	5	6.8
			10	2.5

Matrix	λ nm	Dye	Spiking Concentration ($mgkg^{-1}$)	Standard Deviation per Spiking Concentration
			20	1.1
		Orange G	50	74.1
			100	9.6
			200	77.5
	430	Sudan Orange G	5	5.7
			10	5.9
			20	1.7
Paprika	430	Napthol yellow	50	1.9
			100	1.6
			200	1.2
	510	Acid Red 73	50	0
			100	0
			200	19.3
		Toluidene Red	5	11.9
			10	7.0
			20	8.7
		Sudan Red B	5	13.5
			10	10.5
			20	3.5
	470	Para Red	5	3.3
			10	1.8
			20	1.5

n = 3 replicates at each concentration

Table 9 – Proposed Screening Method – Recoveries of Dyes from Selected Matrices

Matrix	Dyes	Concentrations ($mgkg^{-1}$)	% Recovery	
			Mean	Range
Chilli Powder	Orange II	5, 10, 20	121.1	91.2-129.1
	Sudan 1		92.4	80.7-107
	Sudan II		92.8	80.3-106.5
	Sudan III		104.9	80.3-131.0
	Sudan IV		95.7	71.7-121.3
Canned chicken in a curry sauce	Sudan Black B	5, 10, 20	81.6	72.5-86.7
	Sudan Red 7B		88.5	82.4-93.3
	Sudan Red G		90.2	83.9-105.7
Fennel	Methanil yellow	5, 10, 20	76.4	60.5-85.1
	Dimethyl yellow		80.7	71.8-89.1

Matrix	Dyes	Concentrations ($mgkg^{-1}$)	% Recovery	
			Mean	**Range**
	Auramine O		82.1	73.1-103.3
	Bixin		94.4	80.0-112.5
	Fast Garnett GBC		81.9	73.1-85.5
	Rhodamine B		78.1	54.9-90.0
Palm Oil	Oil Orange SS	5, 10, 20	76.7	71.1-89.0
	Orange G	50, 100, 200	152.2	31.9-332.0
	Sudan Orange G	5, 10, 20	87.1	79.6-97.4
Paprika	Naphthol Yellow	50, 100, 200	48.1	29.7-72.4
	Acid Red 73	50, 100, 200	3.70	0.0-33.1
	Toluidine Red	5, 10, 20	97.4	76.1-114.2
	Sudan Red B	5, 10, 20	94.6	82.0-112.3
	Para Red	5, 10,20	90.4;	85.6-97.0

Problems were encountered with the analysis of turmeric due to a large interfering matrix peak at a similar retention time to several dyes of interest. Various clean-up procedures were tried and the chromatographic conditions altered but acceptable results could not be obtained for this matrix. Interestingly, although the canned chicken in curry sauce contained turmeric its concentration was evidently not high enough to cause problems.

Conclusions

Intermittent findings of illegal dyes in food continue to be a feature of international trade. A simple method developed and validated by the Government Chemist in 2006 for 7 illegal dyes in one matrix, chilli powder, has been extended into a general screening method for 19 illegal dyes in 5 matrices, chilli powder, canned chicken in a curry sauce, fennel, palm oil and paprika. The limits of detection, recovery and precision are consistent with the previous method and are considered adequate for a screening method. The following dyes are adequately resolved: Orange II, Sudan I-IV, Sudan Black B, Sudan Red 7B, Sudan Red G, Methanil yellow, Dimethyl yellow, Auramine O, Bixin, Fast Garnett GBC, Rhodamine B, Oil Orange SS, Orange G, Sudan Orange G, Naphthol Yellow, Acid Red 73, Toluidine Red, Sudan Red B, and Para Red. Two pairs exhibit close retention times, Sudan Orange G/Para Red and Toluidine Red/Sudan Red G and for these care should be taken to report the correct compound.

Some dyes, Orange G, Naphthol Yellow, Congo Red and Acid Red 73 did not prove amenable to the general screening method in these matrices and turmeric proved to be a very challenging matrix for which the general screening method in its present format is not applicable. For these, the small number of problematic dyes/matrices and when confirmation is required, mass spectrometric detection is recommended.

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Appendix 1
Proposed 19-Dye Screening Method

Materials

Reagents (ammonium acetate, glacial acetic acid) were analytical reagent grade, solvents (acetone, acetonitrile, dichloromethane and ethanol) were of HPLC grade and deionised water was 18 MΩcm^{-1} (Elga Purelab Ultra™).

The dyes investigated along with CAS numbers, suppliers and grades are described in Table A1.

Table A1 – Dyes Investigated for the Proposed Screening Method

Dye	CAS Number	Supplier	Product Code	Grade or Claimed Purity
Sudan I	842-07-9	Sigma Aldrich	103624-25G	≥95 %
Sudan II	3118-97-6	Sigma Aldrich	199656-25G	90 %
Sudan III	85-86-9	Sigma Aldrich	S4131-25G	≥ 85 %
Sudan IV	85-83-6	Sigma Aldrich	198102-25G	≥ 80 %
Sudan Orange G	2051-85-6	Acros Organics	190170250	"pure"
Sudan Red 7B	6368-72-5	Sigma Aldrich	201618-10G	95 %
Solvent Red I / Sudan Red G	1229-55-6	Sigma Aldrich	17373-25G	reagent for Ph. Eur.
Sudan Red B	3176-79-2	Fluka	86010	Microscopy grade
Sudan Black B	4197-25-5	Fisher Scientific	S/8630/46	"pure"
Auramine O	2465-27-2	Sigma Aldrich	861030-25G	85 %
Butter (dimethyl) Yellow	60-11-7	Acros Organics	151400250	indicator grade, IR authentic, ≤ 3 % Loss on Drying
Fast Garnet GBC	97-56-3	Acros Organics	153260250	≥96 %
Metanil Yellow	587-98-4	Acros Organics	41372	≥88 %
Orange II	633-96-5	Acros Organics	416560250	≥85 %
Orange G	1936-15-8	Acros Organics	416550100	≥80 %
Para Red	6410-10-2	Sigma Aldrich	100994-25G	95 %
Rhodamine B	81-88-9	Sigma Aldrich	R6626-25G	≥95%
Toluidine Red	2425-85-6	Sigma Aldrich	19 975-3	70 %
Acid Red 73	5413-75-2	Fluka	49823	≥97 %
Congo Red	573-58-0	Fisher Scientific	C/7020/46	"pure"
Naphthol Yellow	483-84-1	Fluka	49547-25MG	≥99 %
Oil Orange SS	2646-17-5	Fluka	79285-25MG	≥98 %
Bixin (Annatto)	1393-63-1	Food grade annatto was characterised for its bixin content against a previous reagent grade Bixin		

Stock Standard Dye Solutions

1 Individual Stock Standard Solutions – 1mgml^{-1}

Accurately weigh 100mg of each dye into a separate 100ml volumetric flask and dilute to volume with dichloromethane or ethanol/water (80:20) depending on whether the dye is oil- or water-soluble (see Table 2 for guidance)

2 Intermediate Mixed Standard Solutions – 20 µgml^{-1}

Two separate intermediate standard solutions were prepared on the basis of the retention times of the dyes on the column chosen so that dyes with similar retention times were in different intermediate calibration solutions, see Table A2. Four dyes, Acid Red 73, Congo red, Naphthol yellow and Orange G were found, in preliminary studies (see above) to have poor sensitivity in this method and are not included in Table A2.

Table A2 – Intermediate Calibration Solutions, 20 µgml^{-1}

Intermediate Calibration Solution 1	Intermediate Calibration Solution 2
Sudan I	Sudan IV
Sudan II	Sudan Red 7B
Sudan III	Solvent Red I / Sudan Red G
Sudan Orange G	Sudan Black B
Sudan Red B	Butter (dimethyl) Yellow
Auramine O	Fast Garnet GBC
Orange II	Metanil Yellow
Rhodamine B	Para Red
Toluidine Red	Oil Orange SS
	Bixin (Annatto)

Pipette 1.00ml of each of the stock solutions into the appropriate 50ml volumetric flask and dilute to volume with acetonitrile:acetone, 90:10v/v.

3 Calibration Standard Solutions

Prepare sets of mixed calibration standards (0.1, 0.2, 0.5, 1.0 and 2.0 µgml^{-1}) by pipetting appropriate volumes of the mixed intermediate calibration solution into an appropriate volumetric flask and making up to volume with acetonitrile:acetone, 90:10v/v extraction solvent.

Proposed Screening Method – Outline Standard Operating Procedure (SOP)

As a result of these studies an outline of the proposed screening method is suggested below.

Organisations making use of this proposed method should ensure that validation takes place for the method and a full SOP is drafted including a section dealing with health and safety issues.

1 Sample Preparation Prior to HPLC

1.1 Weigh 3-5g of sample into a 125ml plastic bottle and add sufficient acetonitrile:acetone 90:10v/v extraction solvent for a tenfold dilution of the sample. For example for a 3 g sample 30 ml of solvent should be added.

1.2 Shake the mixture to ensure all the sample is well mixed with solvent then place the bottle in an ultrasonic bath for 30 seconds.

1.3 Place the bottle in a shaking water bath, set at 40±0.5°C and shake for 30 minutes. If a shaking water bath is not available, place the bottle on a shaker and return to a water bath for 5 minutes between shaking periods of 5 minute to maintain the temperature.

1.4 Immediately after extraction filter the contents of the bottle through a GF/A filter.

1.5 Centrifuge the solution (at a suggested 4000rpm) for 10 minutes.

1.6 Transfer an aliquot of the filtrate into a HPLC auto-sampler vial ready for analysis which it is recommended should be carried out without delay.

2 HPLC Conditions

2.1 HPLC system: Gradient pump, Jasco 1580; auto-sampler, Jasco AS 1559; Column oven, Jasco CO965; UV/Visible detector to measure at 380, 430, 470, 510, and 550nm, Jasco 1575 (or equivalents).

2.2 Column: Waters symmetry C18 5µm 250x4.6mm.

2.3 Injection volume: 20µl.

2.4 Column temperature: 25°C.

2.5 Flow rate: $1 ml min^{-1}$

2.6 Mobile phase A: 10 mM ammonium acetate in de-ionised water, pH adjusted to 3.0 with glacial acetic acid.

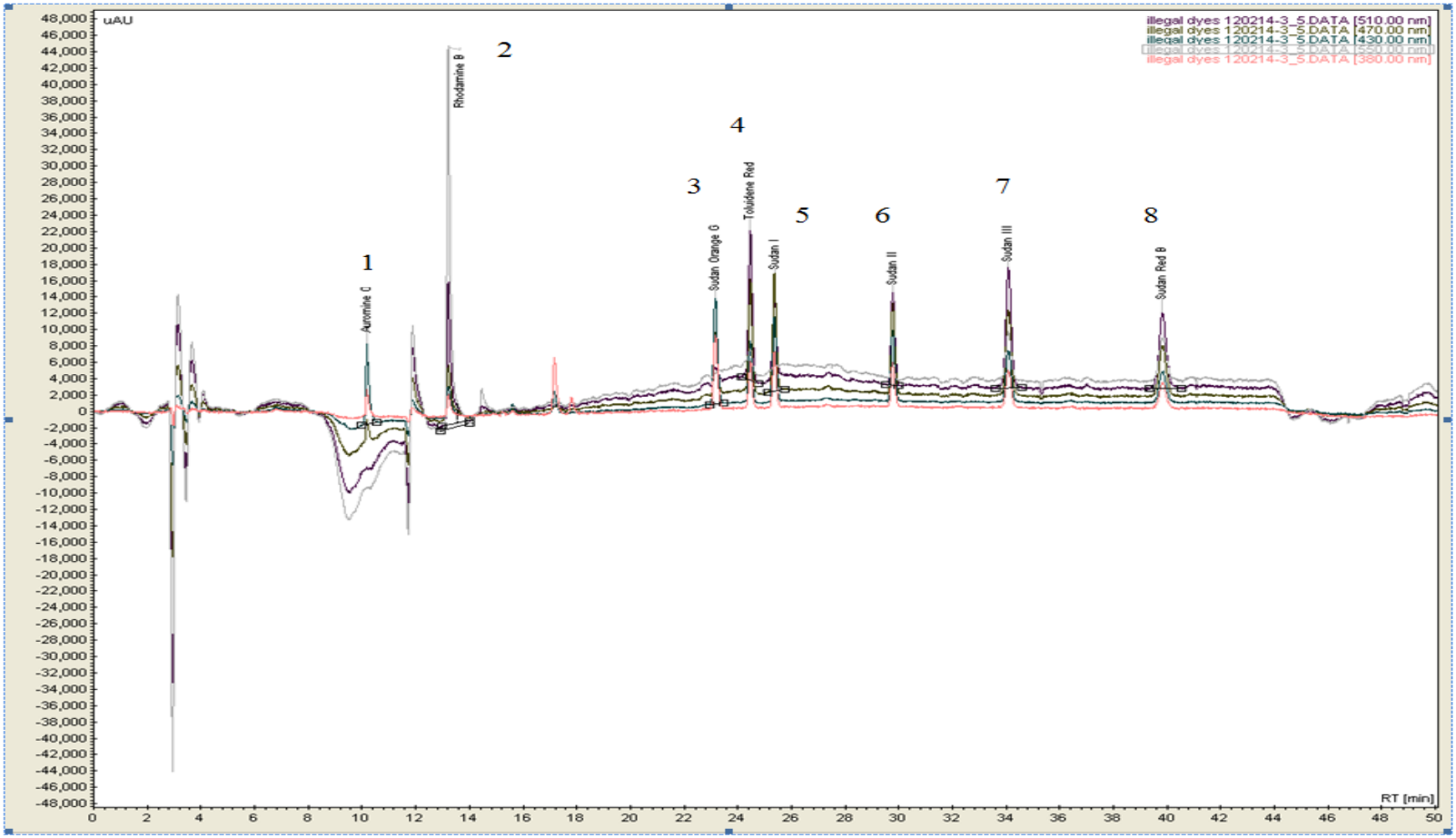
Mobile phase B: acetonitrile adjusted to pH 3.0 with glacial acetic acid.

2.7 Gradient:

Time (min)	**% A**	**% B**
0	80	20
4	80	20
10	40	60
24	2	98
40	2	98
50	80	20

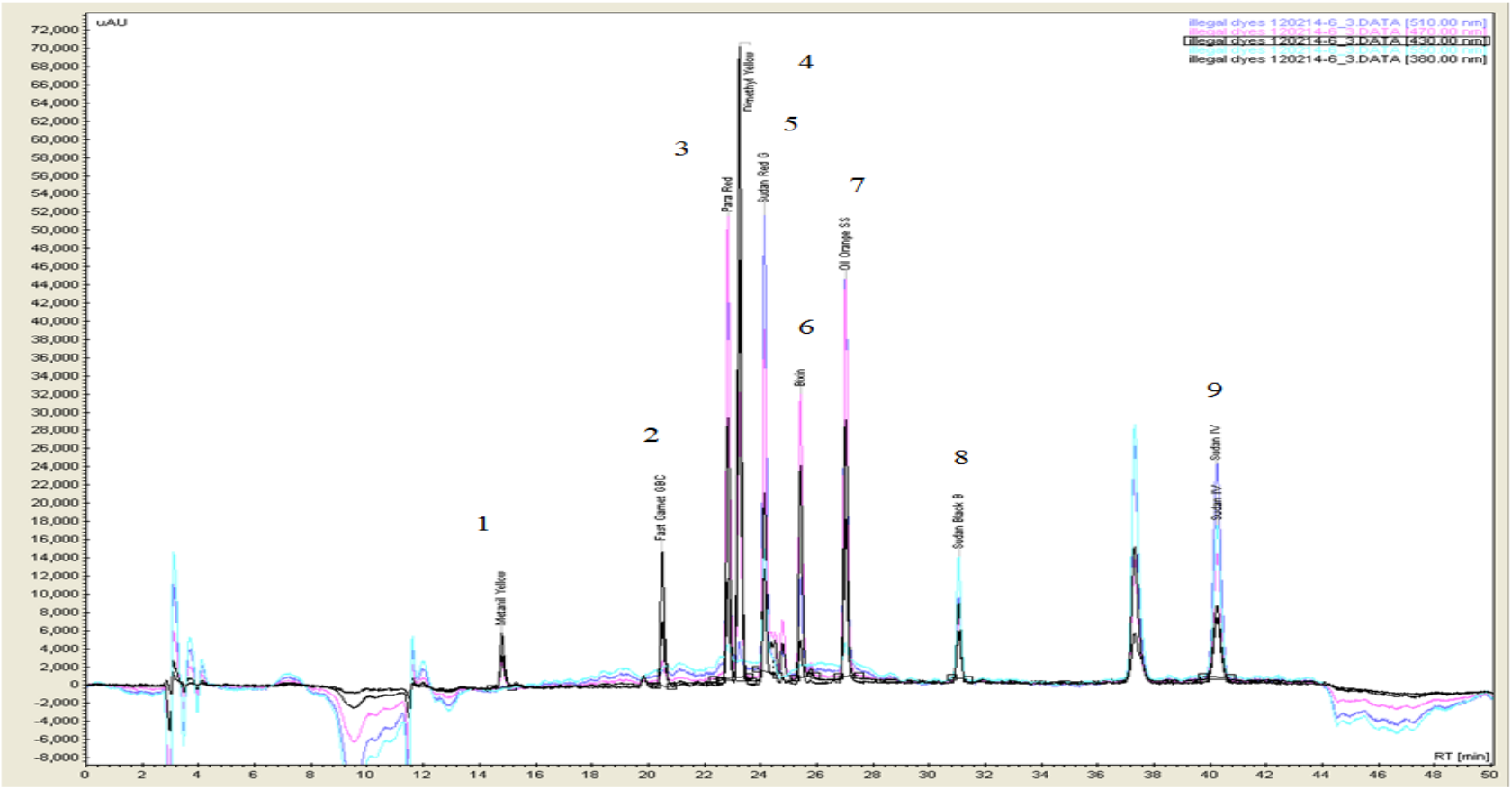
Example chromatograms are shown in figures A1 and A2 below.

Figure A1 – Solvent Standard Chromatogram – Detection at 380, 430, 470, 510 and 550 nm



1 Auramine O, 2 Rhodamine B, 3 Sudan Orange G, 4 Toluidene Red, 5 Sudan I, 6 Sudan II, 7 Sudan III, 8 Sudan Red B

Figure A2 – Solvent Standard Chromatogram – Detection at 380, 430, 470, 510 and 550 nm



1 Metanil Yellow, 2 Fast Garnet GBC, 3 Para Red, 4 Dimethyl Yellow, 5 Sudan Red G, 6 Bixin, 7 Oil Orange SS, 8 Sudan Black B, 9 Sudan IV