Analysis of Illegal Dyes in Paprika Powder by LC-MS/MS

Statutory Analysis Government Chemist Programme Ad Hoc Project 1

October 2006

LGC/GC/2007/006

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Date:

10 October 2006

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1. Abstract

Sudan dyes are synthetic industrial colours that are suspected genotoxic carcinogens. Their use as food colour additives, at any concentration, is forbidden by the European Community and many of the equivalent national and international organisations. To protect the consumer, food products need to be monitored and tested regularly for assurance that they are free of these illegal contaminants. This requires the use of reliable, specific and sensitive methods of analysis.

This report explores the feasibility of using a generic LC-MSMS method based on standard addition for the quantitative determination of Sudans I-IV, Sudan Orange G, Sudan Red G, Sudan Red B, Sudan red 7B, Para Red, Toluidine Red, Dimethyl Yellow, Orange II and Rhodamine B dyes in paprika powder. The dyes are extracted with a solvent of 100 % acetonitrile at ambient temperature. Sample extracts are filtered, cleaned on C18 SPE cartridge and analysed by reverse phase HPLC using an isocratic elution system using buffered water/acetonitrile and detection by mass spectrometry.

The method has been validated for the dyes at concentrations from 100 to 1000 ug/kg of paprika powder. Limits of detection were typically around 10 ug/kg for nine of the dyes, while recoveries were estimated to be in the range 80 to 110 %. Precision is more variable and highly dependent on the nature of the dye and the level of spiking. Most of these nine have precision measured as a relative standard deviation between 3-13%, whereas for Sudan III, Sudan Red 7B and Toluidine Red the equivalent precision was in the range 3-40 %.

The dyes Rhodamine B, Orange II, Para Red and Sudan Orange G have poor performance data and cannot be analysed in paprika spice by this method.

2. Introduction

2.1 Background

Paprika is an important spice ingredient used by the food manufacturing industry in the formulation of a wide range of processed foods, particularly for continental dishes, curries, sauces and pickles. The colour and appearance of the bulk spice often dictates its economic price being interpreted as an indication of its freshness and quality of the product.

This has led certain irresponsible traders to supplement the colour of spices with synthetic dyes in order 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 paprika 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 or carcinogenic, according to the European Food Safety Authority[1-2], and are illegal additives in foods within the European Community. Current agreements between the member states of the European Community have stressed that food products containing greater than 0.5 –1.0 mg/kg of any illegal dye are not fit for sale and should be withdrawn from the market.

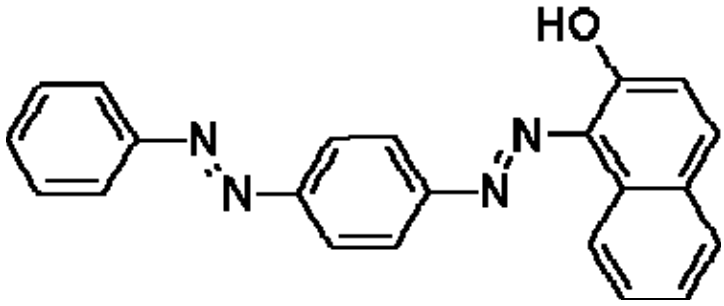
Methods based on LC-MSMS are particularly useful here because they can provide far lower detection limits compared to the equivalent LC-UV/visible procedures and because by monitoring several characteristic ions simultaneously there is extra confirmation that a particular Sudan dye has been detected. Reliable, accurate and sensitive analytical methods are necessary to help police this

area of food manufacture and to ensure that all products are totally free of these illegal and potentially dangerous colourants.

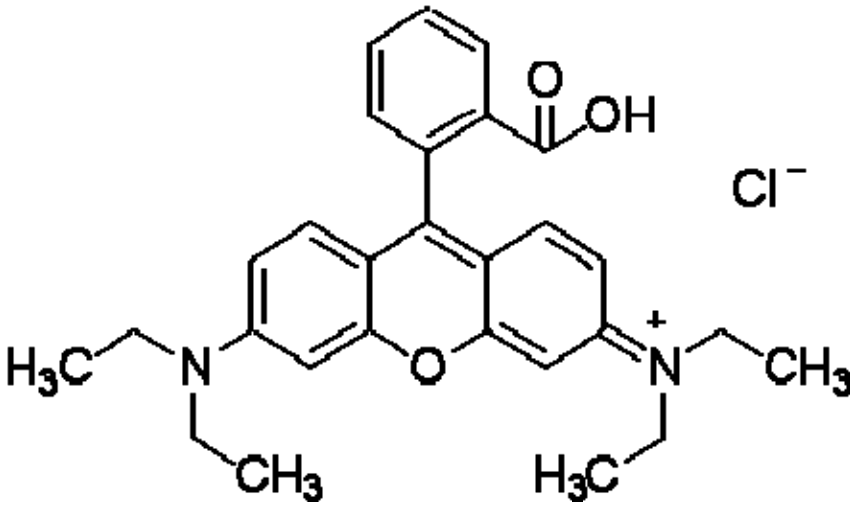
2.2 Illegal Dyes Properties

The Sudan dyes I-IV, Sudan Red B, Sudan Red 7B, Sudan Orange G, Sudan Red G, Dimethyl Yellow, Orange II, Para Red and Toluidine 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 Figure1. The basic amine groups and partial ionisation of the molecular affords the 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, Sudan Red B, Sudan Red 7B, Sudan Red G, Sudan orange G, Dimethyl Yellow, Para Red, Toluidine Red and Rhodamine B. For the dye Orange II ethanol was used to prepare the stock solution.

3.2 Extraction Conditions

In order to reduce the amount of co-extracted material, particularly oil and carotenes, the 90:10 acetonitrile /acetone solvent used in the LC-UV/Visible method for the dyes in chilli powder was changed to 100 % acetonitrile. Also, for similar reasons, the extractions were performed at ambient temperatures rather than at the elevated temperature of 40 ºC as described in the UV/Visible method.

3.3 Cleanup by SPE Cartridge

Despite the use of milder extraction conditions and a more selective extractant solvent it was still found necessary to clean the final spice extract by solid phase extraction before LC-MS/MS analysis was conducted. A C18-silica cartridge was found to remove most of the natural oils and coloured materials from the paprika extract that were potential interferences in the chromatographic analysis. One important disadvantage of the recommended SPE cleanup of the dyes was that two of the analytes, Rhodamine B and Orange II, were removed by this process. In order to analyse these dyes a sample of neat extract (untreated by SPE) was also analysed alongside the cleaned extracts by LC-MS/MS.

3.4 Internal Standards

Internal standards are particularly important in LC-MSMS analysis because they can be used to compensate for both matrix effects and recovery efficiency. Ideally a deuterated analogue for each analyte in the analysis should be added to extracts. However, only Sudan I D5 and Sudan IV D6 materials were found to be commercially available These were added at the 25 ug/L (100uL of 7-8 ug/mL standard solution for 3 gram sample size) level prior to the final analysis for all sample extracts and standards.

3.5 Final Extract Solvent

Chromatograms for standard solutions of the dyes Sudan III, Sudan IV, Sudan Red B and Sudan Red 7B were found to contain two resolved peaks, a main peak and a smaller additional peak at shorter retention time. Because it was not possible to discriminate between the two peaks by altering any of the mass spectrometer parameters it was concluded that the interfering compound must be a structural isomer of each parent dye. A literature search indicated that this was a pH dependent effect and that the addition of ammonia to the sample extract might resolve the issue. This proved to be the case and consequently all standard and sample extracts were prepared in 4% v/v ammonia/methanol prior to the end analysis.

Figure 2: Sudan 3 standard in acetonitrile

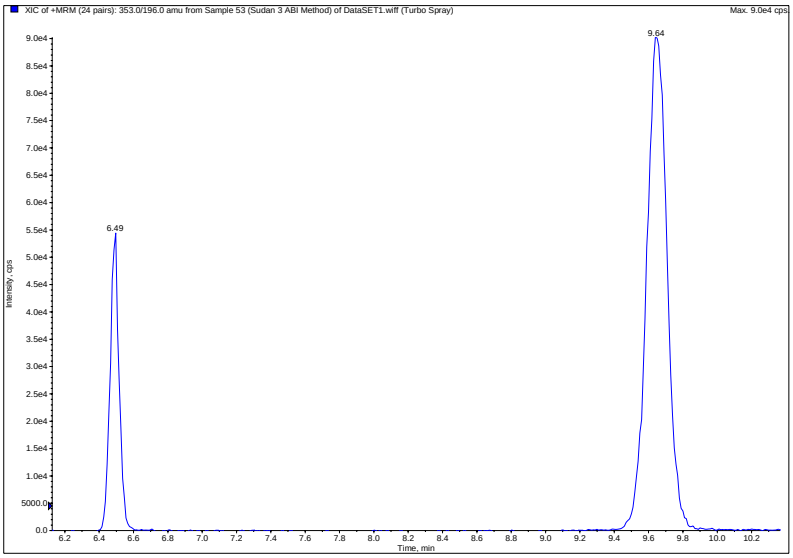
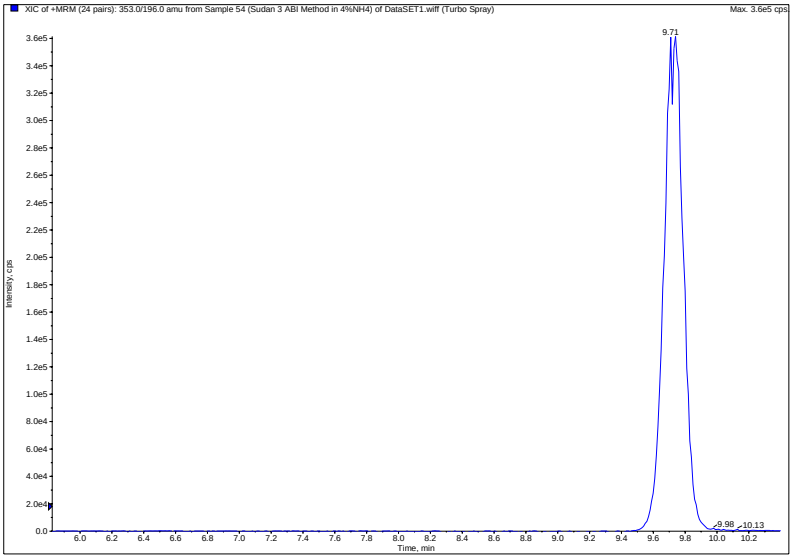


Figure 3: Sudan 3 after reconstitution in methanol with 4% ammonia v/v



The effect of this change of solvent is illustrated by the chromatograms in Figures 2 & 3.

3.6 Choice of HPLC Column

Preliminary work showed that good separation of the dyes was possible on the following HPLC method:-

Column: Phenomenex Synergi Max RP 150 x 2mm, 4µm

Flow rate: 0.3 ml/min

Mobile Phase: 30% Ammonium Acetate (10mM pH 3.8), 70% Acetonitrile

Mode: Isocratic

Injection Volume: 5 µl

With this arrangement the large tailing peak found for the readily ionisable analyte, Rhodamine B caused interferences for the later closely eluting dyes. Also it was found that the column was unable to offer any separation of the isomeric dyes Sudan IV and Sudan Red B. Chromatographic separation of these two dyes is particularly important because they share the same precursor and daughter ions, which means they cannot be resolved by the mass spectrometer alone. The use of a polar phenylhexyl column eliminated these problems. Although with this column, under isocratic conditions, there was generally less separation of closely eluting dyes compared to the above reverse phase column, it was found that the combined resolution of the column and mass spectrometer was sufficient to limit any significant analyte-analyte interferences.

3.7 Choice of Mass Spectrometer Conditions

The optimum mass spectrometer parameters for each dye were found by experiment. A standard containing all the dyes was continuously infused into the mass spectrometer source at a controlled rate. The mass spectrometer excitation parameters were then altered sequentially until the best response for the precursor and daughter ions was achieved for each dye. It was intended that, during the analysis, these conditions would be changed at specific intervals to suit the dye which was eluting from the column at any given time. However, this instrument facility proved inoperable so a set of standardised conditions (for Sudan I) were employed for the whole of the analysis.

A modification of this experiment was also used to provide information on the interference effects caused by co-eluting compounds from the matrix of the sample. A blank matrix sample was injected onto the column in the normal way, at the same time as the standards were being infused at a constant rate into the detector. Any rises or dips in the dye signal response (normally a flat response) was taken as an indication of the effect of co-eluting compounds from the sample. This highlighted not only the retention times where the main co-extractives of the sample were eluting but also pinpointed the dyes that were most likely to suffer matrix interferences.

4. Method Validation

Validation was conducted in accordance with the International Harmonised Protocol for the Validation of Analytical Methods[3]. The procedure was tested for linearity of response, limit of detection**,** same day and daily precision, bias and recovery. Because of the lack of reference materials the validation procedures were solely based on blank paprika powder samples that had been spiked with standard solutions.

4.1 Linearity of Calibration

A range of multi-standard matrix solutions from 1 to 1000 ug/L were prepared by spiking blank paprika extracts. These matrix standard solutions were also spiked with two internal standard compounds Sudan I D5 and Sudan IV D6 at the 25 ug/L level. These were run on the HPLC under the conditions detailed in 5.1.1. All dye calibrations were linear and were found to have excellent correlation up to a concentration of 200 ug/L. Beyond this point the sensitivity tailed off for most analytes with the exception of Orange II and Sudan Orange G. An explanation for this effect is that with strong solutions the higher concentrations of the analytes are competing for the same charge in the excitation chamber of the ion trap mass spectrometer. If it were possible to calibrate the instrument using single dye standards this effect may have been eliminated.

Table 1: Calibration equations for the Dyes

Dye	MRM (t1) Calibration Equation	Correlation Coefficient	MRM (t2) Calibration Equation	Correlation Coefficient
Sudan I	y=0.055 x - 0.0545	0.9997	y=0.0563x + 0.6662	0.9875
Sudan II	y=0.0726x + 0.0453	0.9993	y=0.2223x – 0.0208	0.9996
Sudan III	y=0.0546x – 0.191	0.9913	y=0.1794x – 0.636	0.9958
Sudan IV	y=0.0402x – 0.0328	0.9957	y=0.064x – 0.0584	0.9987
Sudan Red B	y=0.0572x – 0.17	0.997	y=0.0835x – 0.1909	0.9988
Sudan Red 7B	y=0.9353x - 2.43	0.9981	y=0.8012x – 0.219	0.9984
Sudan Red G	y=0.828x + 0.336	0.9987	y=0.2399x – 0.0438	0.9998
Sudan Orange G	y=0.0011x + 0.0261	0.9922	y=0.001x + 0.0075	0.9929
Dimethyl Yellow	y=0.0179x – 0.0078	0.9998	y=0.456x – 0.0169	0.9999
Para Red	y=0.0048x – 0.0172	0.995	y=0.0015x + 0.1072	0.47
Toluidine Red	y=0.4258x + 0.803	0.9901	y=0.294x + 0.0439	0.9997
Rhodamine B	y=0.4676x + 0.657	0.9979	y=0.5156x + 0.1512	1.00
Orange II	y=0.0005x + 0.001	0.996	y=0.0037x – 0.0087	0.9953

y= Ratio of the analyte peak/internal standard peak

x = analyte concentration in ug/L

In the calibrations for dyes Sudan III, Sudan IV, Sudan Red B and Sudan Red 7B the peak area of the dye is ratioed with the peak area of the internal standard Sudan IV D6. All other dye peaks are ratioed to the internal standard peak for Sudan I D5. Calibration equations and correlation coefficients are also given for the second daughter peak studied for each dye referred to in the table as MRM (t2).

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4.2 Detection Limit

For simplicity the detection limit was taken as corresponding to the lowest matrix calibration standard for which a valid peak area could be accurately measured. For the dyes Sudans I-IV, Sudan

Red B, Sudan Red 7B, Sudan Red G, Dimethyl Yellow, Toluidine Red and Rhodamine B the lowest matrix solution that satisfied this criterion was the 1 ug/L standard.

Taking into account the ten fold dilution of extracts used in the procedure this corresponds to a detection limit for the original paprika sample of 10 ug/kg. Table 1 shows that the sensitivity (slope of the calibration) of Orange II, Sudan Orange G and Para red is poorer than the other ten dyes and the corresponding detection limit limits in paprika are estimated to be in the range 20-150 ug/kg.

It should be remembered that the mass spectrometer instrument was optimised only for Sudan I. The facility to switch mass spectral conditions during the elution in order to optimise the detection of each dye was not available here. If it were possible to use this procedure improved detection limits would follow.

4.3 Precision

Recoveries at five different spike concentrations (100, 200, 500, 1000 and 5000 ug/kg) were carried out within the same day. Each recovery was performed five times enabling precision to be estimated. The extractions and analyses were repeated again on a further two days. Unfortunately due to the length of the procedure and time required for analysis the days were not concurrent. The data set includes replicate results for single extractions on each of three days. The appropriate statistical model is

$$y_{ij}=\mu + D_i + e_{ij} \quad (1)$$

where μ is the population mean, D_i is the day effect for day i and e_{ij} a residual error (actually corresponding to the between-injection variation). D and e are assumed normally distributed with mean zero and variances σ_d^2, σ_e^2 respectively. (Experimental estimates of these components of variance will be denoted s_d^2, s_e^2). None of these variances corresponds exactly to the required estimates of method repeatability variance s_r^2 and intermediate (between-day, within-laboratory) variance s_I^2; these are estimated from the experimental data using

$$s_r^2 = s_e^2 \quad (2)$$

$$s_I^2 = s_d^2 + s_e^2 \quad (3)$$

The estimates in eq. 2 and 3 are valid whether the particular terms are statistically significant or not, and in view of the marginal significance of some between-extraction effects, estimation used the same model for all dyes and spiking levels. The variance components s_d^2, s_e^2 were obtained using maximum likelihood estimation (strictly, Restricted Maximum Likelihood estimation, REML) to estimate the variance components. REML does not generate negative variance estimates, and automatically leads to small contributions from insignificant factors. The REML results for combined variance estimates are expected to be nearly unbiased, providing a consistent approach with no negative estimates and little bias. Here, the REML approach agreed very well with the unbiased estimates from the classical ANOVA method (ISO 5725 part 3[3]). The variance estimates and precision data tabulated below (Tables 1 to 8) are accordingly those from the REML method.

The precision data were calculated separately for each dye. Repeatability and intermediate precision figures provide, respectively, the precision estimates for independent replicate injections analysed once each in the same run, and for independent injections analysed once each on different days.

4.4 Table 2. Precision data at 1000µg/kg* for partially cleaned extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	913.53	85.35	26.90	26.90	89.49	2.94	9.80
Sudan II	788.45	94.38	37.24	37.24	101.46	4.72	12.87
Sudan III	799.59	201.83	275.86	275.86	341.81	34.50	42.75
Sudan IV	913.67	100.44	96.82	96.82	139.51	10.60	15.27
Sudan Red 7B	1290.41	418.05	36.77	36.77	419.67	2.85	32.52
Sudan Red G	925.71	128.81	68.56	68.56	145.92	7.41	15.76
Toluidine Red	963.64	153.31	102.97	102.97	184.68	10.69	19.17
Dimethyl Yellow	947.82	119.76	80.51	80.51	144.31	8.49	15.23
Sudan Red B	992.56	142.87	131.24	131.24	194.00	13.22	19.55

* 5 replicates at each spiking level on three separate days

4.5 Table 3. Precision data for 1000µg/kg* for neat extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	888.43	0.01	114.46	114.46	114.46	12.88	12.88
Sudan II	459.04	92.60	100.61	100.61	136.74	21.92	29.79
Sudan III	1023.63	360.35	190.28	190.28	407.50	18.59	39.81
Sudan IV	784.09	178.65	116.35	116.35	213.20	14.84	27.19
Para Red	577.09	412.68	80.18	80.18	420.40	13.89	72.85
Rhodamine B	1784.54	977.97	202.86	202.86	998.78	11.37	55.97
Sudan Red 7B	1254.92	131.41	185.76	185.76	227.54	14.80	18.13
Sudan Red G	1104.94	109.69	147.90	147.90	184.14	13.39	16.67
Toluidine Red	1234.69	393.36	198.83	198.83	440.75	16.10	35.70
Dimethyl Yellow	787.22	279.28	160.49	160.49	322.11	20.39	40.92
Sudan Red B	1182.16	153.83	86.44	86.44	176.45	7.31	14.93

* 5 replicates at each spiking level on three separate days

The complete statistical report is given in the Annex.

The data in the selected Tables 2 and 3 show that accuracy (closeness of the mean to the 1000 ug/kg spiking level) and precision (relative standard deviations) of the analysis, for the majority of dyes, is better for partially cleaned extracts compared to the neat extracts. This is to be expected because the

calibration standard solutions were prepared exclusively by spiking the partially cleaned blank extracts.

The data is better for the instances when the dye analyte is the same as or it is closely related to one of the internal standard compounds employed, in terms of molecular structure, chemical properties and retention time. This is the situation found for the dyes Sudan I, Sudan IV and Sudan Red B. Where the match between analyte and internal standard is not so good, but the dye has sensitive daughter ions, the performance data is satisfactory. However, for the dyes Para Red, Sudan Orange G and Orange II where the sensitivity is relatively poor it was found that the inadequate or incomplete recovery results prevented the measurement of accuracy and precision.

The dyes Rhodamine B and Orange II are removed by the cleanup methods used for the other dyes so recovery measurements could only be performed via the analysis of neat extracts. Despite the high sensitivity offered by Rhodamine B its performance data was poor. The likely reasons for this are that the analyte is not a Sudan dye and therefore it does not match well to the internal standards and the matrices for samples and standards are different and not comparable. Work needs to be conducted to find cleanup procedures for the water soluble dyes Rhodamine B and Orange II.

The data in the tables also highlight considerable day to day variations in accuracy and precision. This may be because of contamination introduced onto the HPLC column and into the mass spectrometer from multiple injections of fairly crude extracts during each analytical sequence. Development work has shown that some matrix components are retained on the guard and main HPLC columns leading to problems of contamination in subsequent analyses.

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.

Sudan Red 7B, 95% purity, Sigma Aldrich

Sudan Red B Fluka.

Sudan Red G Fluka.

Dimethyl Yellow, 85% purity, Acros.

Toluidine Red 90% , Sigma Aldrich.

Sudan Orange G, 98% purity, Acros.

Sudan 1 D5, 99.5% pure, Dr. Ehrenstorfer GmbH.

Sudan IV D6, 95% pure, Dr. Ehrenstorfer GmbH.

Equipment & Chemicals

125 mL plastic bottles, Bibby Sterilin Ltd.

Automatic pipette, Gilson M100.

Ultra-sonication bath, Kerry Ltd.

Orbital shaker, Luckham Rotatest.

Plastic filter funnels, 65 mm diameter.

110 mm GF/A filter papers, Whatman.

Ammonia, Analytical grade, Fisher.

Methanol, HPLC grade, Fisher.

Acetonitrile, HPLC grade, Fisher.

Deionised water, Elga.

Blank Samples

Small bottles (50 g) of paprika powders were bought at local retailers.

Health and Safety

In putting this method into routine operation a COSHH risk assessment should be carried out and suitable precautions inserted into a Health and Safety section of the written procedure.

Method

Weigh accurately 3, 4 or 5 g (w) of chilli powder sample into a 150 mL plastic bottle. Add sufficient acetonitrile for a ten fold dilution of the sample (10*w in mL) to the bottle and sonicate the mixture for 30 seconds. Place the bottle on a shaker and shake for 30 minutes. Add volumes of the two internal standard solutions using an automatic pipette appropriate for a final extract containing 25 ug/L of the deuterated compounds. 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 and label these as neat extracts for each sample. Take 10 mL of neat extract and place it on a 5gram C18 SPE. Allow the sample to drain into a 100 mL rotary evaporator flask. Elute the dyes with a further 30 mL of mixed solvent. Remove the solvent on a rotary evaporator at 40°C. Redissolve the residue in 10 mL of mixed solvent. Transfer 2 mL of extract to an HPLC vial. Label

the vial as a cleaned extract of each sample. Analyse the samples immediately. If this is not possible vials can be stored in refrigerator overnight.

Analyse the samples under the HPLC conditions described in the following section.

5.1.1 HPLC Conditions

Agilent 1100 Autosampler Properties

Autosampler Model: Agilent 1100 Wellplate Autosampler

Syringe Size (µl): 100

Injection Volume (µl): 5.00

Draw Speed (µl/min): 200.0

Eject Speed (µl/min): 200.0

Needle Level (mm): 0.00

Equilibration Time (sec): 30

Contents of Custom Injector Program

1: WASH NEEDLE in flush port for 15 sec.

2: DRAW def. amount from sample def. speed def. offset

3: WASH NEEDLE in flush port for 15 sec.

4: INJECT

5: WAIT EQUIL. default time

6: VALVE bypass

7: WAIT 0.20 min.

8: VALVE mainpass

9: VALVE bypass

10: VALVE mainpass

Agilent 1100 LC Pump Method Properties

Pump Model: Agilent 1100 LC Quaternary Pump

Step Table:

Step	Total Time(min)	Flow Rate(µl/min)	A (%)	B (%)	C (%)	D (%)
0	0.10	500	65.0	0.0	15.0	20.0
1	1.00	500	65.0	0.0	15.0	20.0
2	14.00	500	65.0	0.0	15.0	20.0

A = Methanol (Primar Grade)

C = Water (Elga)

D = Acetonitrile (Primar Grade)

Column: Phenomenex SYNERGI 4u Polar-RP 80A 150mm X 2mm

5.2 MS Conditions

ABI QTrap 4000 MS/MS Method Properties
Period 1 Experiment 1:

Scan Type: MRM (MRM) - multi reaction monitoring
Polarity: Positive
Ion Source: Turbo Spray
Resolution Q1: Unit
Resolution Q3: Unit
Settling Time: 0.0000 msec
MR Pause: 5.0070 msec

Analyte	Q1 Mass (m/z)	Q3 Mass (m/z)	Dwell(msec)	Collision Energy
Sudan 1 T1	249	232	20	25
Sudan 1 T2	249	156	20	25
Sudan 2 T1	277	156	20	25
Sudan 2 T2	277	121	20	25
Sudan 3 T1	353	196	20	37
Sudan 3 T2	353	156	20	37
Sudan 4 /Sudan Red B T1	381	224	20	38
Sudan 4 /Sudan Red B T2	381	156	20	38
Orange 2 T1	329	312	20	30
Orange 2 T2	329	156	20	30
Para Red T1	294	277	20	25
Para Red T2	294	156	20	25
Rhodamine B T1	443	399	20	70
Rhodamine B T2	443	355	20	70
Sudan Orange G T1	215	198	20	28
Sudan Orange G T2	215	122	20	28
Sudan Red 7B T1	380	183	20	25
Sudan Red 7B T2	380	169	20	25
Sudan Red G T1	279	156	20	18
Sudan Red G T2	279	123	20	18
Toluidine Red T1	308	156	20	25
Toluidine Red T2	308	152	20	25
Dimethyl Yellow T1	226	133	20	36
Dimethyl Yellow T2	226	120	20	36
Sudan 1 d5 T1	254	237	20	25
Sudan 1 d5 T2	254	156	20	25
Sudan 4 d6 T1	387	224	20	37
Sudan 4 d6 T2	387	282	20	37

6. Conclusions

The demand for low parts per billion detection limits, wide linearity, good precision and accuracy has been achieved here for at least nine of the dyes on the partially cleaned extracts analysed in this study. It is the general case that the LC-MSMS results for neat extracts of the spice are inferior to equivalent data from cleaned extracts, especially in terms of accuracy and precision. The differences are less marked for the cases where the analyte is the same as, or is closely matched to the internal standard used in relation to retention time, chemical and mass spectral properties. Further for analytes having high sensitivity daughter ions e.g. Sudan Red G, Toluidine Red, the performance data is substantially better than that exhibited by the relatively insensitive analytes Para Red and Sudan Orange G.

The importance of extract cleanup is readily demonstrated from the fact that raw signals for standard solutions of Para Red are about 20 times higher than from the same concentration of the dye in neat extract matrices. This illustrates the potential gain in detectability that should accrue if it were possible to exclude most of the food matrix compounds from the final extracts. It is also true for the other dyes that the more the sample is cleaned and purified the better will be the results of the quantitative analyses. Improved cleanup procedures have now been developed within LGC for the matrices chilli, paprika, curry powder, turmeric and so improvements over the prescribed LC-MSMS method should be now possible.

The dyes Rhodamine B and Orange II have different chemical properties to the normal Sudan dyes and are not retained in the extracts following the normal cleanup procedures employed. Consequently, analytical work could only be performed through the analysis of neat extracts. Here it needs to be emphasised that for these two dyes the matrix of the neat extracts and that used for matrix standard calibrants (partially cleaned) are different and therefore high and consistent recovery values are less likely to be encountered. As work is needed to develop specific cleanup procedures for Rhodamine B and Orange II, it is predicted that several analytical methods will be required if all thirteen analytes need to be analysed in the same spice sample.

In the design of the validation procedures, the standard addition approach i.e. spiking actual samples rather the matrix blanks with known amounts of each dye, was not tackled directly. However from the general quality of the recovery results on spiked blank matrices found in this study, the suggestion is that this technique should be highly successful for most dyes. The recovery experiments conducted at the highest spike level were investigated specifically to underpin the analysis of samples containing up to 1ug/g of the analytes. This follows the recommendation of Thomson et al[5] who proposed that for accurate analyses, standard addition spikes should be at least 5 times the incurred concentration of the dye in the sample. Unfortunately, as mentioned earlier, at this spiking concentration the diluted sample extracts were outside the calibration range of the LC-MS/MS. Hence the 5 ug/g recovery data were discarded in this report.

However, this finding does not necessarily preclude the use of standard addition techniques over the desired concentration range. The reason is that it is anticipated that typical samples, having either a routine or referee status, will be analysed in two stages. Firstly samples will be screened to reveal which dyes are present and their approximate level of contamination This will then allow customised analytical methods to be used for quantitation purposes. The ability to tailor methods, have fewer determinants and calibrants, control the scale of the cleanup, vary the concentration or dilution factors used should allow any problems arising from detectability, linearity and matrix interferences to be eliminated in advance .

In summary this work indicates that there is unlikely to be a single generic procedure that is suitable for both screening and quantification of all the dyes in any one matrix or that covers the wide range of concentration required, from the detection limit up to a concentration of 5ug/g. What is more feasible is that, once information on the contaminants in the sample are known, a selected method

can be chosen from several available options for the purposes of quantitative analysis. The likelihood is that, for trace level applications in uncommon matrices, the method chosen will include both the standard addition technique and LC-MSMS end analysis. Further method development work needs to be conducted to perfect the cleanup of paprika samples and also to apply the analytical procedure detailed here to other sample types. A choice of sample preparative and cleanup procedures should solve the specific analytical problems encountered with the dyes Rhodamine B, Orange II, Para Red, and Sudan Orange G.

7. References

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8. Annex

8.1 Sudan Red and other colourants in spices: Assessment of precision data

8.1.1 Data

Data were provided in Excel spreadsheet The sheet included raw data and estimated recovery data for neat and partially cleaned extracts. Data on spiked samples consisted of single injections. The analysis had been conducted over three days.

The data thereby allowed an estimate of the between and within-day variability for the method as a whole over the concentration range studies (100, 200, 500 and 1000 µg/kg).

8.1.2 Experimental

Statistical calculations were carried out in R version 2.1.01. REML variance estimates used the lme function in package nlme, as described by Pinheiro and Bates[2.] Repeatability and intermediate precision estimates were calculated from the REML values using Excel 2003 (Microsoft, Redmond, CA)

8.1.3 Data pre-treatment

Missing observation were coded NA.

8.1.4 Preliminary Inspection

Data (grouped by concentration, dye and finally by day) were inspected visually via boxplots and values deleted where necessary (rejection was at the 99% level of confidence).

8.1.5 Precision estimates

The data set includes replicate results for single extractions on each of three days. The appropriate statistical model is

$$y_{ij}=\mu + D_i + e_{ij} \quad (1)$$

where μ is the population mean, D_i is the day effect for day i and e_{ij} a residual error (actually corresponding to the between-injection variation). D and e are assumed normally distributed with mean zero and variances σ_d^2, σ_e^2 respectively. (Experimental estimates of these components of variance will be denoted s_d^2, s_e^2). None of these variances corresponds exactly to the required estimates of method repeatability variance s_r^2 and intermediate (between-day, within-laboratory) variance s_I^2; these are estimated from the experimental data using

$$s_r^2 = s_e^2 \quad (2)$$

$$s_I^2 = s_d^2 + s_e^2 \quad (3)$$

The estimates in eq. 2 and 3 are valid whether the particular terms are statistically significant or not, and in view of the marginal significance of some between-extraction effects, estimation used the same model for all dyes and spiking levels. The variance components s_d^2, s_e^2 were obtained using maximum likelihood estimation (strictly, Restricted Maximum Likelihood estimation, REML) to estimate the variance components. REML does not generate negative variance estimates, and automatically leads to small contributions from insignificant factors. The REML results for combined variance estimates are expected to be nearly unbiased, providing a consistent approach with no negative estimates and little bias. Here, the REML approach agreed very well with the unbiased estimates from the classical ANOVA method (ISO 5725 part 3). The variance estimates and precision data tabulated below (Tables 1 to 8) are accordingly those from the REML method.

The precision data were calculated separately for each dye. Repeatability and intermediate precision figures provide, respectively, the precision estimates for independent replicate injections analysed once each in the same run, and for independent injections analysed once each on different days.

Table 1. Precision data at 100µg/kg* for partially cleaned extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	94.83	6.60	5.95	5.95	8.89	6.27	9.37
Sudan II	89.02	8.18	7.07	7.07	10.81	7.94	12.15
Sudan III	103.18	0.00	40.13	40.13	40.13	38.90	38.90
Sudan IV	110.57	18.90	11.98	11.98	22.38	10.84	20.24
Sudan Red 7B	134.02	26.27	35.79	35.79	44.39	26.71	33.12
Sudan Red G	96.90	3.24	7.99	7.99	8.63	8.25	8.90
Toluidine Red	106.87	0.00	19.78	19.78	19.78	18.50	18.50
Dimethyl Yellow	90.61	20.38	18.34	18.34	27.41	20.24	30.26
Sudan Red B	102.25	11.23	18.55	18.55	21.69	18.14	21.21

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

Table 2. Precision data at 200µg/kg* for partially cleaned extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	180.98	11.50	6.99	6.99	13.46	3.86	7.44
Sudan II	165.70	16.67	12.84	12.84	21.04	7.75	12.70
Sudan III	182.37	27.33	71.04	71.04	76.11	38.95	41.74
Sudan IV	194.89	10.97	20.23	20.23	23.01	10.38	11.81
Sudan Red 7B	267.46	58.09	43.81	43.81	72.76	16.38	27.20
Sudan Red G	185.11	10.15	12.20	12.20	15.87	6.59	8.57
Toluidine Red	190.71	0.00	24.05	24.05	24.05	12.61	12.61
Dimethyl Yellow	178.80	44.23	39.44	39.44	59.26	22.06	33.14
Sudan Red B	211.06	18.66	23.54	23.54	30.04	11.16	14.23

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

Table 3. Precision data at 500µg/kg* for partially cleaned extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	473.62	23.92	19.81	19.81	31.06	4.18	6.56
Sudan II	409.33	61.36	32.68	32.68	69.52	7.98	16.98
Sudan III	472.94	191.08	175.70	175.70	259.58	37.15	54.89
Sudan IV	483.94	32.93	25.76	25.76	41.81	5.32	8.64
Sudan Red 7B	655.66	193.65	107.73	107.73	221.60	16.43	33.80
Sudan Red G	467.23	52.81	34.56	34.56	63.11	7.40	13.51
Toluidine Red	490.68	58.27	62.04	62.04	85.11	12.64	17.35
Dimethyl Yellow	454.06	69.96	33.36	33.36	77.51	7.35	17.07
Sudan Red B	538.40	95.17	58.31	58.31	111.61	10.83	20.73

*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 1000µg/kg* for partially cleaned extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	913.53	85.35	26.90	26.90	89.49	2.94	9.80
Sudan II	788.45	94.38	37.24	37.24	101.46	4.72	12.87
Sudan III	799.59	201.83	275.86	275.86	341.81	34.50	42.75
Sudan IV	913.67	100.44	96.82	96.82	139.51	10.60	15.27
Sudan Red 7B	1290.41	418.05	36.77	36.77	419.67	2.85	32.52
Sudan Red G	925.71	128.81	68.56	68.56	145.92	7.41	15.76
Toluidine Red	963.64	153.31	102.97	102.97	184.68	10.69	19.17
Dimethyl Yellow	947.82	119.76	80.51	80.51	144.31	8.49	15.23
Sudan Red B	992.56	142.87	131.24	131.24	194.00	13.22	19.55

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

Table 5. Precision data at 100µg/kg* for neat extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	98.63	6.46	7.06	7.06	9.57	7.16	9.71
Sudan II	34.22	15.70	1.59	1.59	15.78	4.65	46.11
Sudan III	96.33	0.00	30.35	30.35	30.35	31.51	31.51
Sudan IV	95.15	29.60	23.35	23.35	37.70	24.54	39.62
Para Red	104.80	74.56	12.56	12.56	75.61	11.99	72.14
Rhodamine B	86.92	19.86	28.95	28.95	35.11	33.31	40.39
Sudan Red 7B	143.68	16.13	34.91	34.91	38.46	24.30	26.77
Sudan Red G	97.34	9.43	7.80	7.80	12.24	8.01	12.57
Toluidine Red	93.80	17.56	16.92	16.92	24.38	18.04	25.99
Dimethyl Yellow	87.38	30.74	24.35	24.35	39.21	27.87	44.88
Sudan Red B	127.28	0.00	40.26	40.26	40.26	31.64	31.64

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

Table 6. Precision data at 200µg/kg* for neat extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	192.01	1.67	16.02	16.02	16.11	8.35	8.39
Sudan II	68.83	15.53	8.53	8.53	17.72	12.39	25.74
Sudan III	221.53	64.26	22.89	22.89	68.21	10.33	30.79
Sudan IV	201.10	27.41	40.63	40.63	49.01	20.20	24.37
Para Red	192.59	70.27	41.74	41.74	81.73	21.67	42.44
Rhodamine B	277.52	130.45	93.99	93.99	160.79	33.87	57.94
Sudan Red 7B	263.22	19.50	34.16	34.16	39.34	12.98	14.94
Sudan Red G	189.11	21.38	24.94	24.94	32.85	13.19	17.37
Toluidine Red	203.96	0.00	44.97	44.97	44.97	22.05	22.05
Dimethyl Yellow	186.31	72.65	48.64	48.64	87.43	26.11	46.93
Sudan Red B	228.31	32.25	33.74	33.74	46.67	14.78	20.44

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

Table 7. Precision data for 500µg/kg* for neat extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	455.80	34.49	24.04	24.04	42.04	5.27	9.22
Sudan II	203.16	0.00	37.61	37.61	37.61	18.51	18.51
Sudan III	536.71	192.80	158.00	158.00	249.27	29.44	46.44
Sudan IV	473.69	52.98	103.55	103.55	116.32	21.86	24.56
Para Red	335.46	204.59	51.38	51.38	210.95	15.32	62.88
Rhodamine B	891.03	442.69	145.53	145.53	465.99	16.33	52.30
Sudan Red 7B	672.73	0.06	155.40	155.40	155.40	23.10	23.10
Sudan Red G	553.17	27.21	67.05	67.05	72.36	12.12	13.08
Toluidine Red	3986.24	5595.39	1103.51	1103.51	5703.17	27.68	143.07
Dimethyl Yellow	402.99	140.60	22.66	22.66	142.42	5.62	35.34
Sudan Red B	614.79	78.18	99.06	99.06	126.20	16.11	20.53

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

Table 8. Precision data for 1000µg/kg* for neat extracts

Dye	Mean	Variance components (as SD)		Precision (µg/kg)		Precision (%RSD)	
	(µg/kg)	Between-Day	Residual	s_r	s_I	RSD_r	RSD_I
Sudan I	888.43	0.01	114.46	114.46	114.46	12.88	12.88
Sudan II	459.04	92.60	100.61	100.61	136.74	21.92	29.79
Sudan III	1023.63	360.35	190.28	190.28	407.50	18.59	39.81
Sudan IV	784.09	178.65	116.35	116.35	213.20	14.84	27.19
Para Red	577.09	412.68	80.18	80.18	420.40	13.89	72.85
Rhodamine B	1784.54	977.97	202.86	202.86	998.78	11.37	55.97
Sudan Red 7B	1254.92	131.41	185.76	185.76	227.54	14.80	18.13
Sudan Red G	1104.94	109.69	147.90	147.90	184.14	13.39	16.67
Toluidine Red	1234.69	393.36	198.83	198.83	440.75	16.10	35.70
Dimethyl Yellow	787.22	279.28	160.49	160.49	322.11	20.39	40.92
Sudan Red B	1182.16	153.83	86.44	86.44	176.45	7.31	14.93

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

Inspection of the precision data (partially cleaned extraction *vs*. neat extraction) showed no clear trends. Of note is the exceptionally high RSD_I value of 143.07% for Toluidine Red (500µg/kg neat extracts). Subsequent investigation revealed the mean concentration for days 1,2, and 3 to be 10240, 509, 514µg/kg respectively. The 'Day '1 replicate concentration values (8512, 12822, 8844, 9610 and 11413µg/kg) indicate a possible dilution error, or other such inaccuracy. Excluding the 'Day 1' data, the RSD_r and RSD_I values revert to 9.57%.

8.2 References

1 R Development Core Team (2005). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

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