



APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

Authors:

Brian R. Holder

Wilhad M. Reuter

PerkinElmer, Inc.
Shelton, CT

Analysis of Illegal Dyes in Chili Spices via UHPLC-MS/MS

Introduction

Presently, synthetic azo (Para Red, Dimethyl Yellow, Sudan I, II, III and IV)

and non-azo (rhodamine B) dyes are primarily used in industry for the coloring of solvents, oils and waxes. Though once used as natural food colorings, food safety regulators have banned the use of these synthetic dyes, as metabolic studies have suggested that the degradation products of these dyes could potentially be genotoxic and/or carcinogenic.¹

With strict regulations imposed in Europe and the United States, it is illegal to use any amount of these dyes in food; however, there remain instances of these dyes being used in other countries, particularly in those in which the spices originate. It is believed that the presence of these illegal dyes in spices are often due, in part, to cross contamination or the inadvertent blending of adulterated materials.²

In response to illegal dyes having been detected in various foods, regulators are now conducting more surveillance programs and introducing rapid alerts to prevent foods tainted with illegal dyes from being introduced into the market. Although globally there are currently no published legal limits for illegal food dyes, in virtually all countries, any detectable amount is deemed unacceptable. Therefore, there exists a need to develop highly sensitive methods for the reliable detection of any amount of illegal food dyes, particularly in spices.

Conventional LC-MS/MS assays have been able to detect 10-100 ppb of illegal dyes in food spices.² Herein, we present a reliable UHPLC-MS/MS method for the analysis of seven common, illegal dyes in chili spices, with analyte LOQs ranging from 0.012 to 0.469 ppb. This method includes a simple dye extraction via a strong organic solvent, with the filtrate being directly injected onto a reverse phase C8 column. This method enables the rapid analysis of the seven analyzed dyes within four minutes, with excellent chromatographic separation and good recoveries.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer QSight™ LX50 UHPLC System was used with a PerkinElmer QSight 210 MS/MS detector. All instrument control, analysis and data processing was performed using the Simplicity 3Q™ software platform.

Method Parameters

The LC and MS/MS method/source parameters are shown in Tables 1, 2, and 3, respectively.

Table 2. MS/MS Method Parameters.

Experiment Group	Illegal Dye	Precursor Ion (m/z)	Product Ion (m/z)	CE (V)	CCL2 (V)	Dwell Time (ms)
1.20 – 2.10 min	Rhodamine B	443.7	399.2	-50	-120	50
	Rhodamine B*	443.7	355.0	-76	-122	50
2.30 – 3.19 min	Para Red	294.5	128.1	-40	-40	40
	Para Red*	294.5	156.1	-26	-44	40
	Dimethyl Yellow	226.3	77.1	-20	-54	40
	Dimethyl Yellow*	226.3	105.1	-41	-54	40
	Sudan I	249.4	93.1	-58	-76	40
	Sudan I*	249.4	128.0	-37	-50	40
2.90 – 3.99 min	Sudan II	277.4	121.1	-49	-118	40
	Sudan II*	277.4	128.1	-44	-52	40
	Sudan III	353.5	120.1	-31	-54	40
	Sudan III*	353.5	197.1	-27	-84	40
	Sudan IV	381.5	91.1	-44	-96	40
	Sudan IV*	381.5	224.4	-27	-86	40

*= Qualifier ion; **CE (V)** = Collision Energy; **CCL2 (V)** = Collision Cell Lens 2

Table 3. MS/MS Source Parameters.

Parameter	Setting
Ionization Mode	ESI, Positive
Drying Gas	120
HSID Temperature (°C)	320
Nebulizer Gas	275
Electrospray Voltage (V)	4500
Source Temperature (°C)	420

Solvents and Standards

All solvents, reagents, and diluents used were HPLC-grade or better. For all dilutions, HPLC-grade water, acetonitrile and/or methylene chloride were used. The illegal dye standards,

Table 1. LC Method Parameters.

Column	PerkinElmer Brownlee 3.0 x 100-mm C8 SPP, 2.7 µm (Part# N9308427)				
Mobile Phase	Solvent A: 5 mM ammonium formate in water with 0.1% formic acid Solvent B: Acetonitrile with 0.1% formic acid				
	Step	Time (min)	Flow rate (mL/min)	%A	%B
	1	Initial	0.8	75	25
	2	3.00	0.8	0	100
	3	4.00	0.8	0	100
	4	4.50	0.8	75	25
	5	8.00	0.8	75	25
Analysis Time	4 min; re-equilibration time: 4 min				
Pressure	5000 psi/344.7 bar				
Oven Temp.	35 °C				
Injection Volume	3 µL				

including rhodamine B, Para Red, Dimethyl Yellow, Sudan I, Sudan II, Sudan III and Sudan IV, were purchased from Sigma-Aldrich® Inc., Milwaukee, WI. All 50-ppm stock dye standards were prepared using methylene chloride:acetonitrile (4:6 v/v) and refrigerated prior to use.

Standard and Sample Preparation

Equal volumes of the 50-ppm stock dye standards were combined in a 1.5 mL microcentrifuge tube for a final volume of 750 µL, affording a 12.5-ppm working standard. A 100-fold dilution of the working standard was performed in acetonitrile:water (1:1 v/v), yielding a 1 mL, 125-ppb stock calibration standard, which was serially diluted in two-fold steps, yielding seven freshly-prepared calibration standards, ranging from 0.12-62.5 ppb.

Five commercially available chili spices (labeled ChiliSpice 1-5) were purchased from local stores. 1 g of each sample was weighed into a 50 mL centrifuge tube with 10 mL of acetonitrile added. The suspension was shaken vigorously on a shaker for 10 minutes, followed by the addition of 10 mL of HPLC-grade water. The suspension was shaken for an additional 10 minutes and subsequently filtered into a clean 50-mL centrifuge tube, using a 0.22 μ m syringe filter. The filtrate was centrifuged for 10 minutes at 3,000 rpm and the supernatant extract was collected. A 500- μ L aliquot of each extract was then transferred into a 2-mL glass vial and analyzed using the LC-MS/MS system. The extraction was repeated for the five chili spices and then analyzed for illegal dyes.

For a stock matrix-matched standard, an aliquot of the ChiliSpice 5 extract was spiked to 1250-ppb with the working standard. ChiliSpice 5 was selected for the matrix blank upon an initial screening, which showed no quantifiable amounts of the analyzed dyes (Rhodamine B, Para Red, Dimethyl Yellow, Sudan I, Sudan II and Sudan III). The stock matrix-matched standard was then used for the preparation of a 125-ppb matrix-matched working

calibration standard. From this, 0.12-62.5 ppb calibration standards were freshly prepared via serial dilution.

All standards and samples were submitted for LC-MS/MS analysis and run in triplicate.

For matrix effect assessment, a 2,500-ppb standard was prepared by a five-fold dilution of the 12.5-ppm working standard, using acetonitrile:water (1:1 v/v). 1 mL of the 2,500-ppb standard was then further diluted to prepare 1,250 and 500 ppb standard. A 5 μ L aliquot of the 500, 1,250 and 2,500 ppb was used to separately spike 500 μ L of acetonitrile:water (1:1 v/v) diluent for final dye concentrations of 5, 12.5 and 25 ppb, respectively. This step was then repeated using Chili 5 matrix extract. The spiked modes were compared for any possible matrix effects.

Results and Discussion

As shown in Figure 1 A/B, good chromatographic separation of the seven illegal dyes was achieved in under four minutes. The relative MS response of rhodamine B was clearly the strongest.

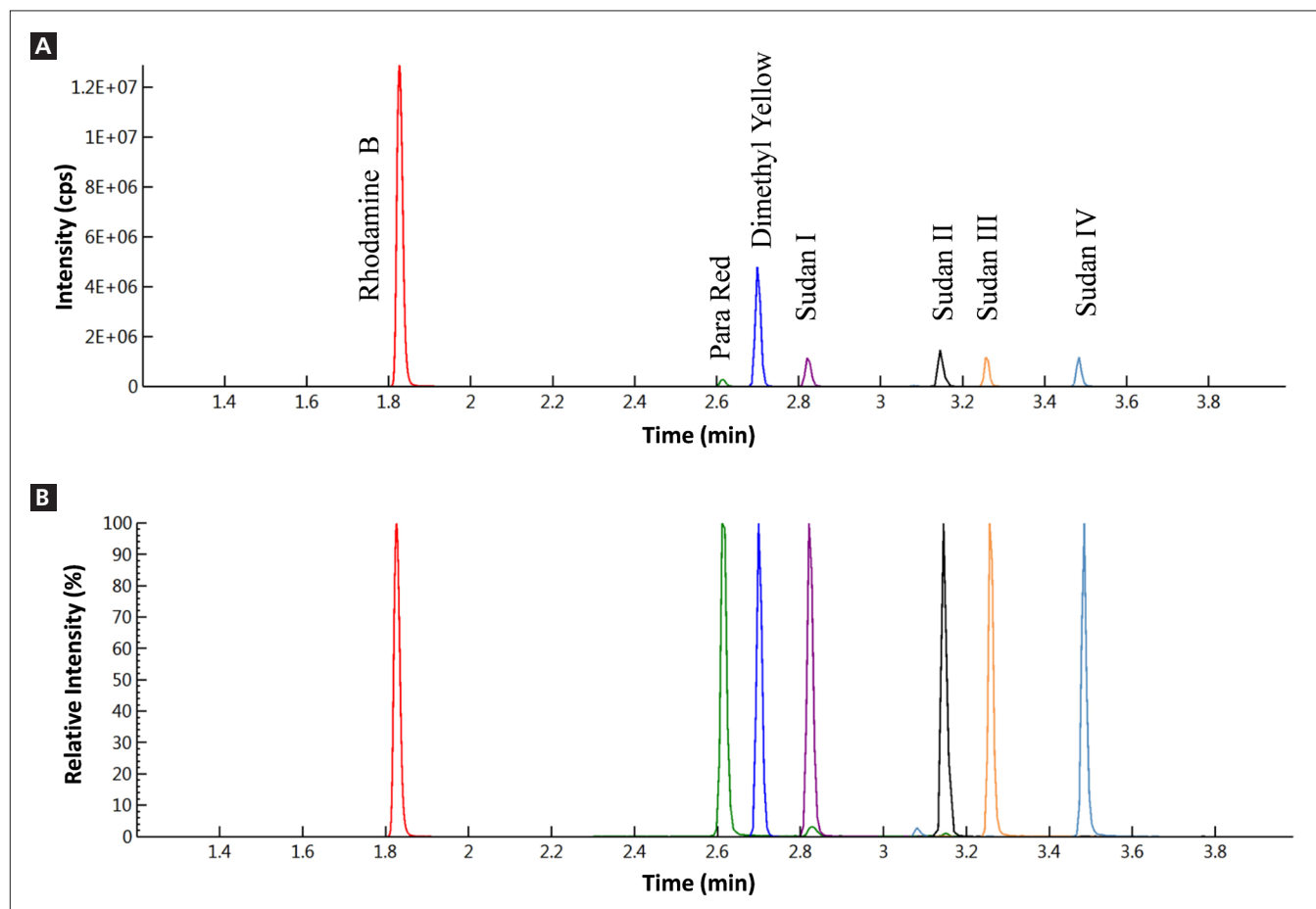


Figure 1. Overlaid MRM chromatograms of the seven illegal dyes in the 62.5-ppb standard. Panel A: The absolute intensity of quantifiers for the individual dye components. Panel B: Normalized view, highlighting the effective chromatographic separation of these dyes.

The chromatographic overlays of six replicate injections of an illegal dye standard demonstrated excellent reproducibility (Figure 2).

Of the five evaluated chili spices, most contained some detectable level of illegal dye. Both rhodamine B and Sudan II were not detected in any of the chili spices (chromatograms not shown). With the exception of the solvent blank and ChiliSpice 5, the chromatograms of the remaining chili spices having no detectable level of dye are not presented. Per Figure 3, Para red was detected in three of the five chili spices (ChiliSpice 1, 2 and 4). No Para red was detected in either ChiliSpice 3 or 5. Both Dimethyl Yellow and Sudan I were detected in ChiliSpice 1 and 2. No detectable levels of Dimethyl Yellow or Sudan I was observed in ChiliSpice 3, 4 and 5. In

ChiliSpice 2, the level of Sudan I was comparable to the 0.243 ppb standard. Sudan III was detected in three of the chili spices, namely ChiliSpice 1, 2 and 4. In ChiliSpice 2, the level of Sudan III was comparable to the 0.243 ppb standard. Sudan III was not detected in ChiliSpice 3 and 5. Sudan IV was detected in all chili spices, though only one (ChiliSpice 3) was at a quantifiable level.

Thereupon, ChiliSpice 5 was chosen for the matrix-matching of calibration standards, serving as the matrix blank, as it contained only a small detectable level of Sudan IV and the MS background was otherwise comparable to that of an acetonitrile:water (1:1 v/v) blank.

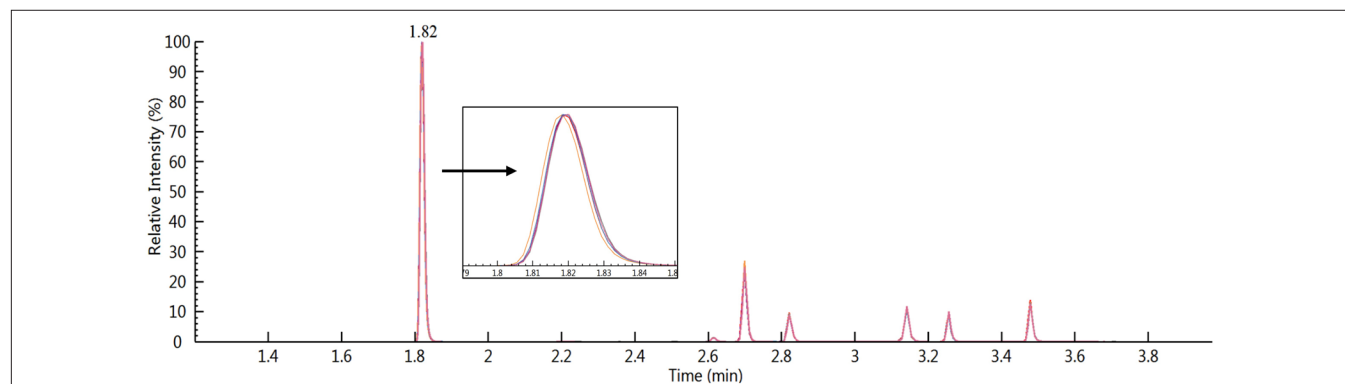


Figure 2. Overlay of six replicate injections of the 62.5-ppb illegal dye standard. Inset: Magnified view for rhodamine B.

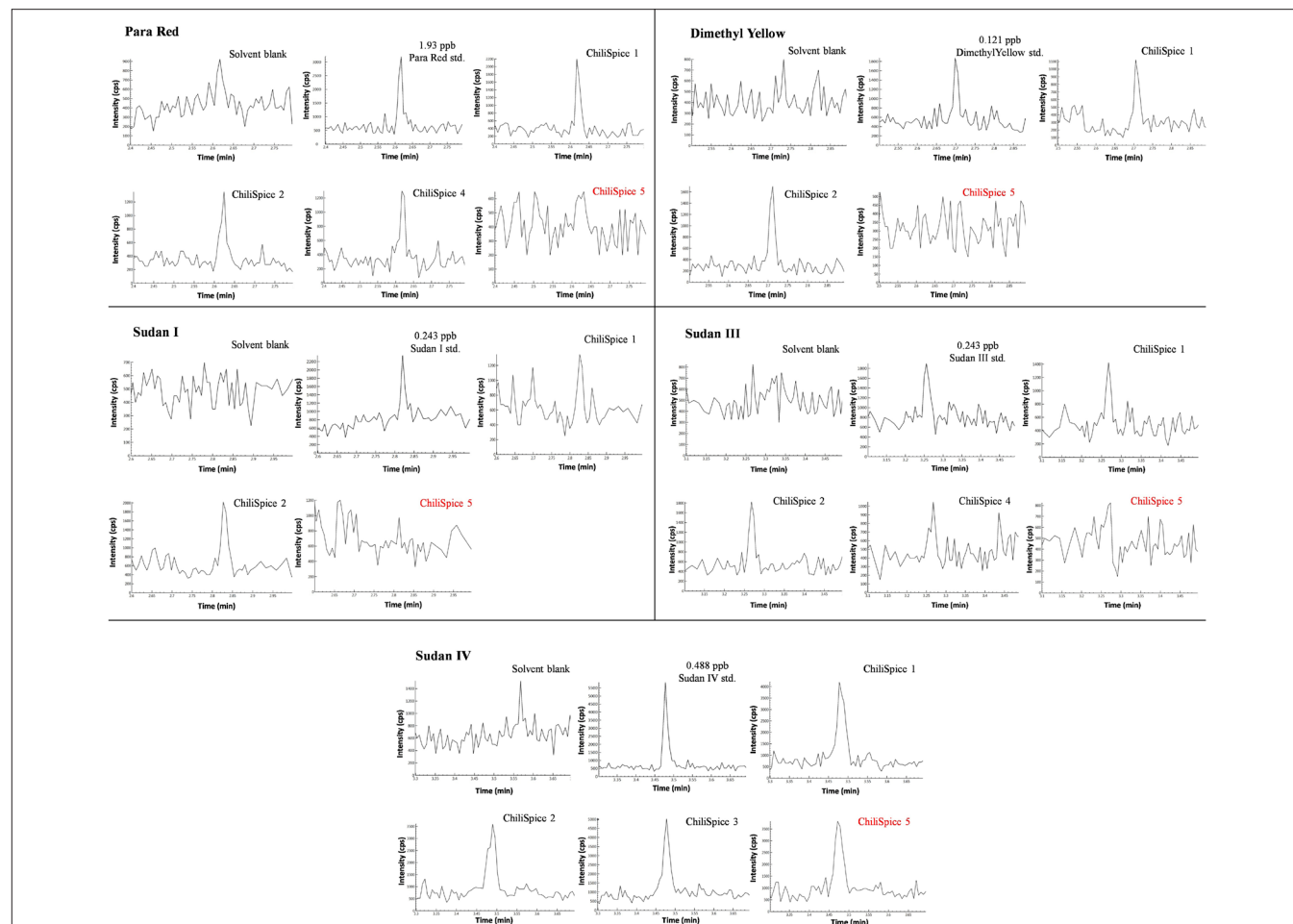


Figure 3. Individual MRM chromatograms of Para Red, Dimethyl Yellow, Sudan I, III and IV in solvent blank, dye standard and the five analyzed chili spices. With the exception of solvent blank and ChiliSpice 5, MRMs without any detectable dye peaks are not shown. Also, chromatograms for Rhodamine B and Sudan II are not shown as these dyes were not detected in any of the chili spices.

The comparative linear-fit results are shown in Figure 4, comparing with and without matrix-match standards, for Para Red, Dimethyl Yellow and Sudan I. The R^2 values for all dyes were in the range of 0.992-0.999, demonstrating very good linearity. Comparing the linear-fits with and without matrix-matched standards, both in slope and overall response, it was evident that there was only a marginal matrix effect ChiliSpice 5 as the matrix-matched standard. The recoveries for Para Red,

Dimethyl Yellow and Sudan I at the highest matrix-matched standard level were 113.4, 92.2 and 95.1%, respectively.

Table 4 presents the detected amounts of the seven illegal dyes in each of the analyzed chili spices. While there were detectable amounts of some of the illegal dyes in all five chili spices, only ChiliSpice 2 and ChiliSpice 3 showed quantifiable amounts of dyes, all being Sudan dyes. Again, as ChiliSpice 5 showed the least amount of detectable dye (only a small amount of Sudan IV), it was chosen for matrix-matching.

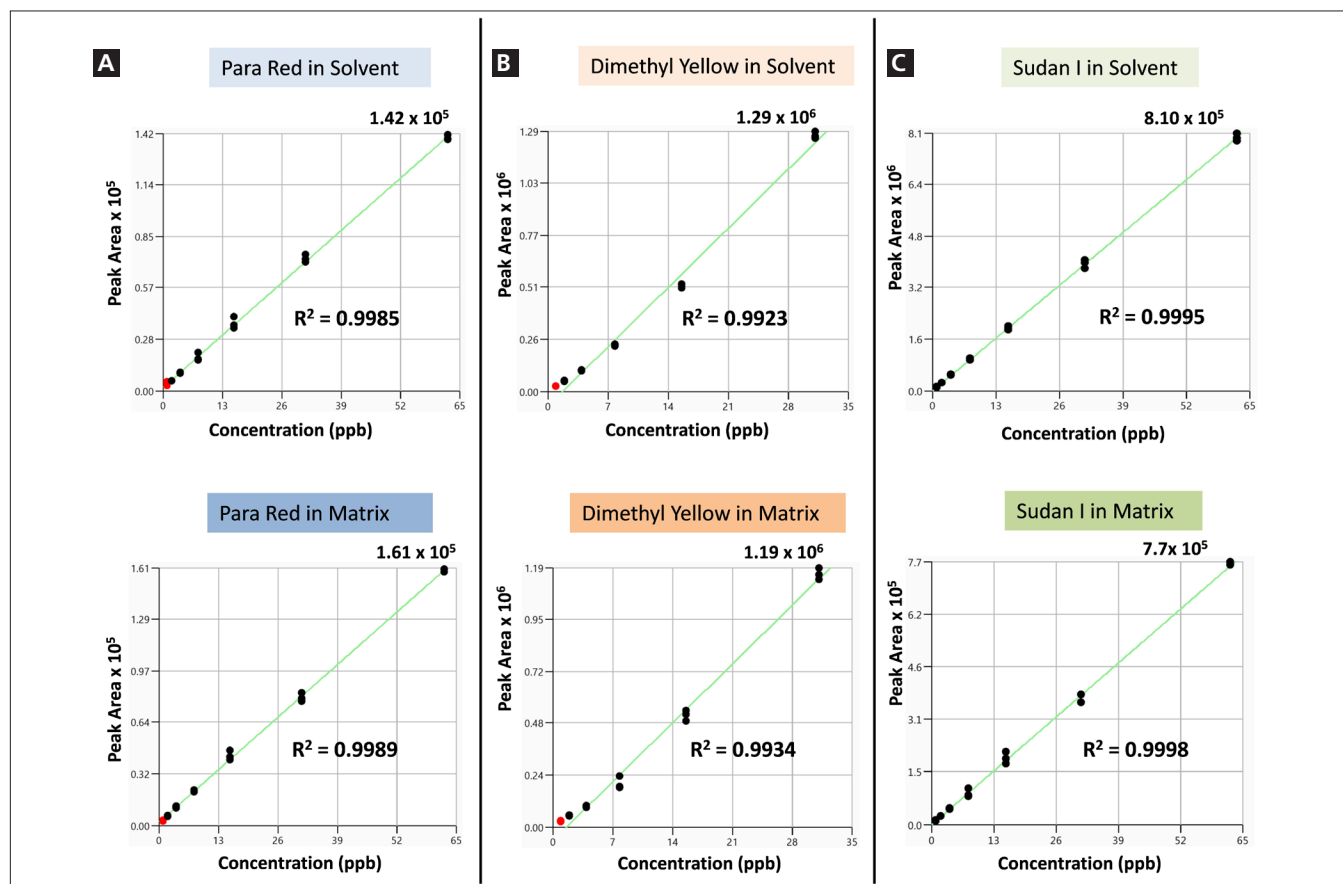


Figure 4. Linear-fit calibration curves for 1.93-62.5 ppb Para Red (A), 1.93-31.3 ppb Dimethyl Yellow (B) and 0.975-62.5 ppb Sudan I (C); Above: in acetonitrile:water (1:1); Below: in ChiliSpice 5 matrix extract.

Table 4. Detected amounts of illegal dyes present in the five chili spices.

	Concentration (ppb)						
	Rhodamine B	Para Red	Dimethyl Yellow	Sudan I	Sudan II	Sudan III	Sudan IV
ChiliSpice 1	X	D	D	D	X	D	D
ChiliSpice 2	X	D	D	0.243	X	0.243	D
ChiliSpice 3	X	X	X	X	X	X	0.488
ChiliSpice 4	X	D	X	X	X	D	D
ChiliSpice 5	X	X	X	X	X	X	D

X = not detected; D = detected

Matrix Effect on Precision and Accuracy

To further assess the impact of possible matrix effects on dye recoveries and to further validate the LC-MS/MS method, quality control (QC) dye standards at 5, 12.5 and 25 ppb, with three replicates each, were prepared with and without ChiliSpice 5 matrix blank (Table 5). The earlier standard curves were used to determine the experimental concentration of the QC standards.

Comparing the recoveries with and without matrix, the experimental QC concentrations were all found to be close to the theoretical values, with accuracies of 92.2-113.6% and CVs of 1.14-15.2%. These results further demonstrate that there is very little matrix effect, particularly ion suppression, for these illegal dyes in chili spice matrix.

Table 5. Percentage recoveries of Para Red, Dimethyl Yellow and Sudan I in QC Standards with and without matrix.

Para Red without Matrix				Para Red in ChiliSpice 5 Matrix		
Spiking	Mean Spiking	Inter-assay	Accuracy	Mean Spiking	Inter-assay	Accuracy
Theoretical (ppb)	Experimental (ppb)	CV (%)	(%)	Experimental (ppb)	CV (%)	(%)
5.0	5.04	10.43	99.20	4.57	4.89	91.41
12.5	12.05	2.19	103.58	12.41	15.20	99.28
25.0	25.60	2.44	97.56	25.31	7.69	101.24
Dimethyl Yellow without Matrix				Dimethyl Yellow in ChiliSpice 5 Matrix		
Spiking	Mean Spiking	Inter-assay	Accuracy	Mean Spiking	Inter-assay	Accuracy
Theoretical (ppb)	Experimental (ppb)	CV (%)	(%)	Experimental (ppb)	CV (%)	(%)
5.0	5.39	2.58	92.28	5.77	1.97	115.34
12.5	10.96	1.84	112.29	13.91	12.35	111.28
25.0	22.51	3.46	96.54	27.57	5.97	110.29
Sudan I without Matrix				Sudan I in ChiliSpice 5 Matrix		
Spiking	Mean Spiking	Inter-assay	Accuracy	Mean Spiking	Inter-assay	Accuracy
Theoretical (ppb)	Experimental (ppb)	CV (%)	(%)	Experimental (ppb)	CV (%)	(%)
5.0	5.10	4.63	97.94	5.45	2.39	108.97
12.5	10.80	2.21	113.64	13.97	10.54	111.74
25.0	21.81	1.14	112.77	26.57	9.36	106.27

Illegal Dye LOQ Determination

As shown in Table 6, the calculated LOQs ($S/N = 10$) for the seven dyes in the ChiliSpice 5 matrix extract ranged from 0.012-0.469 ppb. These were based on a low-level calibration standard.

As a small, but detectable, amount of the Sudan IV dye was present in the ChiliSpice 5 matrix, the validity of the LOQ for Sudan IV may be drawn into question. Thereupon, its actual LOQ is possibly a bit higher than indicated. For further investigations, finding a chili spice containing no detectable illegal dye would be preferred for matrix-matching.

For analyte ID confirmation, the qualifier/quantifier ion ratios for the lowest, detectable calibration standard in ChiliSpice 5 matrix are shown in Table 7. All ion ratios were within a 20% tolerance limit (green), verifying the identification of all seven illegal dyes. For Sudan III, the >1 result suggests that the quantifier and qualifier can be switched for subsequent investigations.

Table 6. Calculated LOQs for the seven illegal dyes in matrix (based on a low level calibration standard).

Component	LOQ (ppb)
Rhodamine B	0.012
Para Red	0.972
Dimethyl Yellow	0.092
Sudan I	0.451
Sudan II	0.469
Sudan III	0.309
Sudan IV	0.388

Table 7. Matrix-derived ion ratios for the illegal dyes.

Analyte Component	Mass Transition Q1/Q2	Ion Ratio Area
Rhodamine B	443.7/399.2	
Rhodamine B*	443.7/355.0	0.44
Para Red	294.5/128.1	
Para Red*	294.5/156.1	0.86
Dimethyl Yellow	226.3/77.1	
Dimethyl Yellow*	226.3/105.1	0.99
Sudan I	249.4/93.1	
Sudan I*	249.4/128.0	0.78
Sudan II	277.4/121.1	
Sudan II*	277.4/128.1	0.78
Sudan III	353.5/120.1	
Sudan III*	353.5/197.1	1.33
Sudan IV	381.5/91.1	
Sudan IV*	381.5/224.4	0.56

Conclusions

- A reverse phase LC-MS/MS method has been developed and demonstrated to be effective for the analysis of seven illegal dyes in chili spices, using a PerkinElmer QSight LX50 UHPLC/QSight 220 MS/MS system.
- The described method, along with the selection of a C8 SPP column, provided exceptional chromatographic repeatability and peak resolution in under four minutes.
- Very good linear calibration fits, in both solvent and matrix, were demonstrated, as well as good recoveries with little matrix effect.

References

1. Opinion of the Scientific Panel on Food Additives, Flavorings, Processing Aids and Materials in Contact with Food on a request from the Commission to Review the toxicology of a number of dyes illegally present in food in the EU Question number EFSA-2005-082. EFSA Journal (2005) 263. [online] <www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/263.pdf>. (accessed September 20, 2017)
2. The world spice Congress 2012: Sustainability and Food Safety-Global Initiatives: Illegal Dyes. [online] www.worldspicecongress.com/uploads/files/28/sess02-c.pdf (accessed September 20, 2017).