Application Note 1023

Determination of Sudan Dyes I–IV in Curry Paste

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Key Words
Acclaim Phenyl-1 Column, Acclaim PA2 Column, 2D-LC, Food

Introduction

Sudan dyes are a class of synthetic dyes that are mainly used for industrial applications such as the coloring of plastic. These dyes are banned as a food-coloring agent because they are classified as carcinogens. For economical reasons, however, Sudan dyes are sometimes illegally used to color food to improve its appearance. Therefore, methods are needed to determine if food products have been adulterated with these dyes.

Typically, Sudan dyes are determined by reversed-phase chromatography with UV or mass spectrometry (MS) detection, but complex food samples usually require extensive off-line sample preparation, such as solvent extraction, solid-phase extraction (SPE), sample evaporation, and/or sample reconstitution.

Previously, Dionex™ (now part of Thermo Scientific™) Application Note (AN) 287 demonstrated that two-dimensional (2D) high-performance liquid chromatography (HPLC) combined with on-line SPE can successfully determine Sudan dyes I, II, III, and IV in chili oil.[1] The sample was first extracted off line with methylene chloride and acetonitrile. The extracted sample was then partially separated on the first dimension column, and the dyes were subsequently trapped using an on-line SPE cartridge between the two dimensions. The trapping required a third pump to dilute the mobile phase from the first dimension to ensure that the dyes were completely trapped on the SPE cartridge. The dyes were then sent to the second dimension for separation prior to MS detection.

The method shown here is a different 2D-HPLC approach for determining Sudan dyes I, II, III, and IV in curry paste that requires significantly less time, and thereby reduces the cost per analysis. After sample extraction using acetonitrile and subsequent filtration, the sample is injected into the first dimension for partial separation. By ensuring that the solvent strength of the sample entering the first dimension is low, sufficient sample can be injected to enable a sensitive method. Similarly, by ensuring that the mobile phase strengths of the fractions from the first dimension entering the second dimension are low enough, the Sudan dyes can be simply trapped on the second dimension prior to separation and UV detection.

This method requires neither an SPE column between the two dimensions nor the third pump to dilute the mobile phase from the first dimension. The same UV detector is used for both dimensions. To determine Sudan dyes in curry paste, only sample extraction and filtration are performed off line. The remaining steps are automated using a Thermo Scientific Dionex UltiMate™ 3000 x2 Dual Rapid Separation LC (RSLC) system controlled by Chromeleon™ Chromatography Data System (CDS) software.

Goal

To determine Sudan dyes I, II, III, and IV in curry paste by 2D-HPLC without off-line SPE sample pretreatment

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Equipment

- UltiMate 3000 x2 Dual RSLC system, including:
  - SRD-3600 Integrated Solvent and Degasser Rack
  - DGP-3600RS Dual-Gradient Rapid Separation Pump
  - WPS-3000RS Rapid Separation Wellplate Sampler
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment
  - DAD-3000RS Rapid Separation Diode Array Detector
  - 750 µL Static Mixer (P/N 6040.5750)
  - 150 µL Static Mixer (P/N 6040.5110)
  - Thermo Scientific Dionex Viper™ UHPLC Finger-tight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 65 mm, SST (P/N 6040.2357)
  - Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 250 mm, SST (P/N 6040.2385)
  - Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 350 mm, SST (P/N 6040.2375)
  - Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 450 mm, SST (P/N 6040.2365)
  - Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 550 mm, SST (P/N 6040.2355)
  - Viper UHPLC Fingertight Fitting incl. Capillary for 10-32 Fitting, i.d. 0.18 mm/0.007", Length 650 mm, SST (P/N 6040.2395)
  - Sample Loop, 500 µL
  - Buffer Tubing 250 µL Assay (P/N 6820.2421)
  - Syringe, 500 µL (P/N 6822.0004)
  - Analytical Flow Cell, SST, 13 µL (P/N 6082.0100)
  - Pod for 2-Position 6-Port HT Valve, <1034 bar, 15,000 psi (P/N 6730.0006)
  - Valve Actuator Kit HT for Right Side, <1034 bar, 15,000 psi (P/N 6730.0001)
  - Valve Actuator Kit HT Left Side, <1034 bar, 15,000 psi (P/N 6730.0002)
- Chromeleon CDS software version 6.80, SR9 or higher

Reagents and Standards

- Water, HPLC grade (Fisher Scientific)
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific)
- Methanol (CH_3OH), HPLC grade (Fisher Scientific)
- 2-Propanol (C_3H_7OH), HPLC grade (Fisher Scientific)

Preparation of Solutions and Reagents

Mixed Stock Standard Solution (5 mg/L Sudan I, II, and 10 mg/L Sudan III, IV)

Sudan I, II, III and IV standard solutions (50 mg/L each) were provided by a customer and used for preparation of a mixed stock standard solution. Add 1 mL each of 50 mg/L Sudan I and II solutions into a 10 mL volumetric flask. Add 2 mL each of 50 mg/L Sudan III and IV solutions to the same volumetric flask. Bring to volume with acetonitrile.

Working Standards Solutions

Add the appropriate volume of the mixed stock standard solution per Table 1 into separate 10 mL volumetric flasks and bring to volume with acetonitrile.

Sample Preparation

Weigh 10 g of a curry paste sample into a 50 mL glass bottle, add 20 mL of acetonitrile, and shake. Put the bottle in an ultrasonic bath for 10 min, then filter the sample using a 0.2 µm syringe filter before sample injection.

Prepare the spiked sample in the same manner. Add the mixed stock standard solution to the bottle containing the curry paste before adding acetonitrile.

Chromatographic Conditions

First Dimension	
Column:	Thermo Scientific Acclaim™ PolarAdvantage II (PA2), 3 µm Analytical, 4.6 × 150 mm (P/N 063191)
Mobile Phase:	A: Water B: Acetonitrile C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Table 1. Working standard preparation.

Dye	Working Standard Concentration (µg/L)				Volume of Mixed Stock Standard Solution for a 10 mL Preparation (mL)			
	Level 1	Level 2	Level 3	Level 4	Level 1	Level 2	Level 3	Level 4
Sudan I	20	40	60	80	0.04	0.08	0.12	0.16
Sudan II	20	40	60	80				
Sudan III	40	40	120	160				
Sudan IV	40	40	120	160				

Table 2. Gradient program and valve switching.

First Dimension (Left Pump)					Valve Switching			Second Dimension (Right Pump)				
Time (min)	Flow Rate (mL/min)	% A (Water)	% B (CH_3CN)	% C (C_3H_7OH)	Time (min)	Right Valve Position	Left Valve Position	Time (min)	Flow Rate (mL/min)	% A (Water)	% B (CH_3CN)	% C (CH_3OH)
0.00	1.0	80	20	0	0.00	1-2	1-2	0.00	1.0	85	0	15
2.00	1.0	5	95	0	6.773	6-1	1-2	13.0	1.0	85	0	15
13.5	1.0	5	95	0	7.042	1-2	1-2	14.0	1.0	0	0	100
14.00	0.7	0	5	95	7.947	6-1	1-2	15.0	1.0	0	0	100
19.00	0.7	0	5	95	8.164	1-2	1-2	16.0	1.0	0	45	55
20.0	0.7	5	95	0	9.583	6-1	1-2	25.0	1.0	0	45	55
22.0	0.7	5	95	0	9.811	1-2	1-2	—	—	—	—	—
22.5	1.0	80	20	0	11.990	6-1	1-2	—	—	—	—	—
25.0	1.0	80	20	0	12.290	1-2	1-2	—	—	—	—	—
—	—	—	—	—	13.500	6-1	6-1	—	—	—	—	—

Second Dimension	
Column:	Acclaim Phenyl-1, 3 µm, Analytical, 4.6 × 150 mm (P/N 071969)
Mobile Phase:	A: Water B: Acetonitrile C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm

Viper Fingertight Fitting System Connections

The tubing used in this application is precut and has Viper fingertight fittings. This flexible stainless steel capillary tubing has zero dead-volume connections. The list of the Viper fingertight fitting system tubing and part numbers needed for making the connections is provided in the Equipment Section. Figure 1 shows how the tubing connections were made for a successful installation of this application, which employs 2D chromatography. The left pump is used for the first dimension and therefore is connected to the autosampler. The column oven and the detector are shared by both dimensions.

Method Description

This method uses 2D chromatography to determine Sudan dyes in curry paste without off-line sample preparation. Inject the sample into the first dimension and partially separate using an Acclaim PA2 column. Configure the UV detector in line with the first dimension (left valve, 1-2 position) to monitor where the Sudan dyes are eluting; configure the 750 µL static mixer off line (right valve, 1-2 position). To collect a peak from the first dimension, switch the right valve to the 1-6 position to put the 750 µL static mixer in line. To send a peak to the second dimension and trap it on the head of the Acclaim Phenyl-1 column, switch the valve back to the 1-2 position. Perform this valve switching for each Sudan dye peak.

Once all Sudan dye peaks are cut from the first dimension and trapped on the second dimension, switch the left valve to the 1-6 position to put the second dimension in line with the UV detector, then start the separation on the second dimension. During the separation on the second dimension, wash the first dimension with 2-propanol.

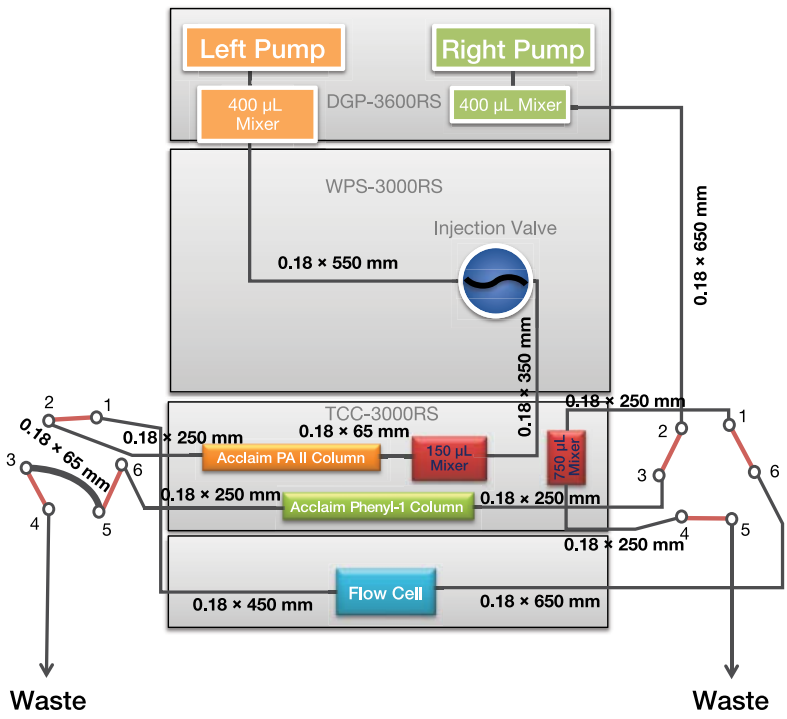

Figure 1. System configuration.

Acetonitrile, the strongest elution solvent in this application, is used for standard and sample preparation. This limits the injection volume because acetonitrile can overload the column; but if the volume of acetonitrile is kept low, method sensitivity is limited. To improve method sensitivity, increase injection volume without overloading the column by placing a 150 µL static mixer between the sample injection and the separation column, and use a low concentration of acetonitrile in the starting mobile phase. Figure 1 shows the location of the mixer. The sample or standard that is injected will be diluted in the mixer with the starting mobile phase before arriving at the head of the column. Using this configuration, the maximum injection for this application is 300 µL.

Figure 2 shows the chromatography of the first dimension using a 300 µL injection volume with and without a 150 µL static mixer installed. Note the peak doubling, a sign of column overload, that occurs without the 150 µL static mixer installed.

Separation and Peak Cutting

Optimize separation of the first dimension on the Acclaim PA2 column by injecting the sample and spiked sample. After Sudan dyes are eluted, wash the column with 2-propanol to remove the strongly retained compounds. Reduce the flow rate during the wash step to reduce column backpressure due to the high viscosity of 2-propanol.

Determine the start and end times for collecting each Sudan dye from the first dimension by using the peak width at baseline and peak retention time of the standard injection. Once Sudan dye peaks are detected by the UV detector, switch the right valve to place the 750 µL static mixer in line with the first dimension to collect the Sudan dye peak before it is sent to the second dimension column. While the peak collections are performed, run the second dimension at a low concentration of methanol to dilute the acetonitrile from the first dimension mobile phase. This enables the second dimension column to trap the Sudan dye peaks.

Some compounds other than Sudan dyes come from the first dimension, so they must be resolved from the Sudan dyes in the second dimension. Four reversed-phase columns were evaluated for the second dimension: the Acclaim 120 C18, Acclaim PA, Acclaim PA2, and Acclaim Phenyl-1 columns. The Acclaim Phenyl-1 column's selectivity differed enough from that of the Acclaim PA2 column in the first dimension to yield the best results in the second dimension.

First Dimension

Column:	Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water B: Acetonitrile C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Second Dimension

Column:	Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water B: Acetonitrile C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm

Samples: 1. Sudan dyes standard mixture with 150 µL mixer
2. Sudan dyes standard mixture without 150 µL mixer

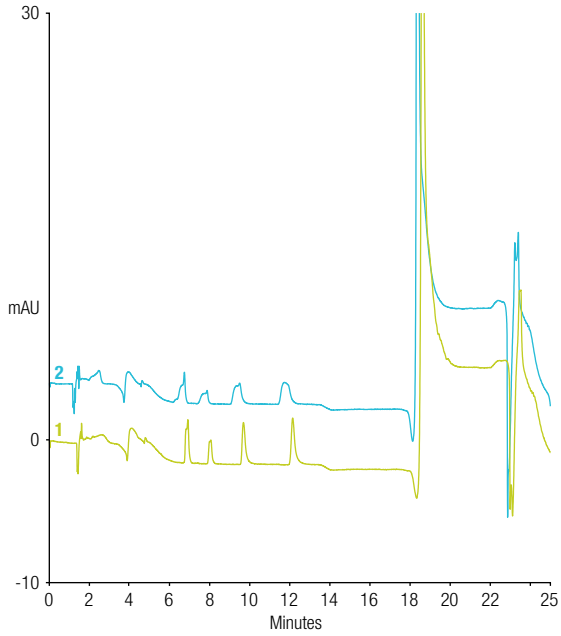

Figure 2. Overlay of chromatograms of 300 µL injections of a mixture of Sudan dyes on the first dimension with and without a 150 µL mixer in the system.

Table 3. Working standard concentrations and calibration results.

Analyte	Concentration (µg/L)				Calibration Result			
	Level 1	Level 2	Level 3	Level 4	Points	r^2	Offset	Slope
Sudan I	20	40	60	80	12	0.99979	-0.0032	23.5069
Sudan II	20	40	60	80	12	0.99877	0.0077	12.8136
Sudan III	40	80	120	160	12	0.99970	-0.0954	10.7585
Sudan IV	40	80	120	160	12	0.99960	-0.1003	9.8593

Results and Discussion

Method Calibration

The method was calibrated before the sample analysis. Four concentration levels of working standard were prepared and triplicate injections were made for each concentration level. The method showed linear peak area response versus concentration. Working standard concentrations and calibration results are shown in Table 3. Figure 3 shows an overlay of chromatograms of each concentration of working standard.

First Dimension

Column:	Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Second Dimension

Column:	Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm
Samples:	Sudan I, II, III, and IV working standards

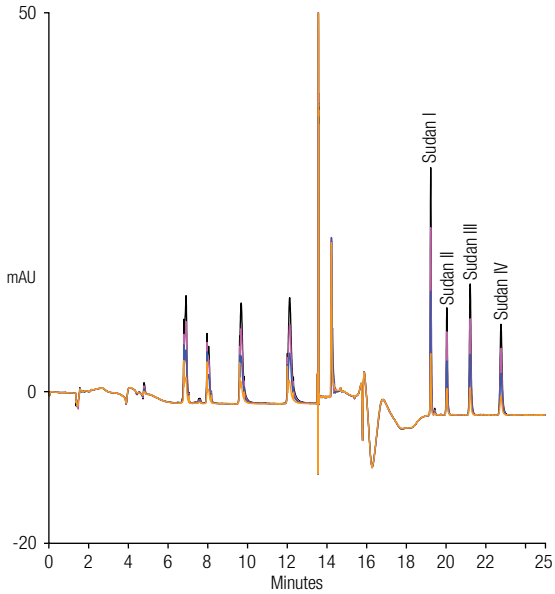

Figure 3. Overlay of chromatograms of working standards.

Method Detection Limit (MDL)

The MDL was estimated from the signal-to-noise (S/N) ratio of spiked samples. Three types of curry paste were purchased from a local supermarket for sample analysis. The samples were spiked with a mixture of Sudan dyes to yield the same concentration as Working Standard Level 1. Table 4 shows the calculated Sudan dye concentrations at a S/N ratio of 3.

Table 4. Estimated MDLs.

Analyte	Estimated MDL, S/N = 3 (µg/L)		
	Fresh Curry Paste	Panang Curry Paste	Red Curry Paste
Sudan I	0.13	0.14	0.12
Sudan II	0.31	0.33	0.31
Sudan III	0.61	0.65	0.61
Sudan IV	0.92	0.98	0.93

Sample Analysis

Each of the three types of curry paste was extracted using acetonitrile in a 1:2 ratio of sample to acetonitrile. After sample extraction and filtration, the sample was injected without further sample pretreatment. Each sample was analyzed five times. No Sudan dyes were found in the samples (Figures 4 through 6).

First Dimension

Column: Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase: A: Water
B: Acetonitrile
C: 2-Propanol
Gradient: See Table 2
Flow Rate: See Table 2
Inj. Volume: 300 µL
Temperature: 30 °C
Detection: UV, 478 nm

Second Dimension

Column: Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase: A: Water
B: Acetonitrile
C: Methanol
Gradient: See Table 2
Flow Rate: See Table 2
Temperature: 30 °C
Detection: UV, 478 nm

Samples: 1. Fresh curry paste
2. Spiked fresh curry paste

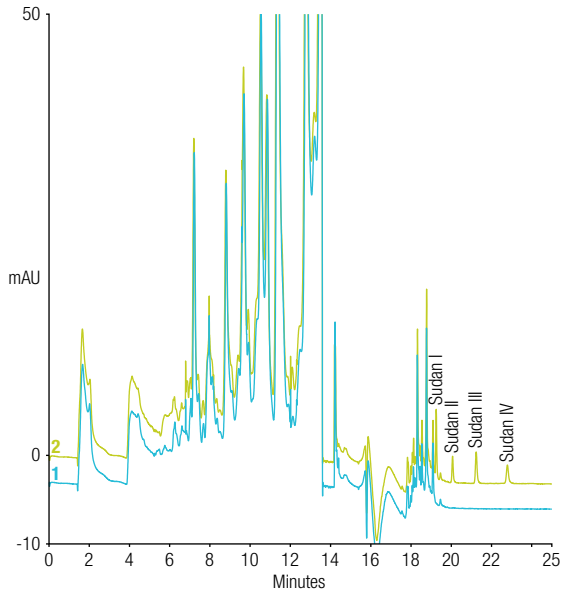

Figure 4. Overlay chromatograms of fresh curry paste and spiked fresh curry paste samples.

First Dimension

Column:	Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Second Dimension

Column:	Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm

Samples: 1. Panang curry paste
2. Spiked Panang curry paste

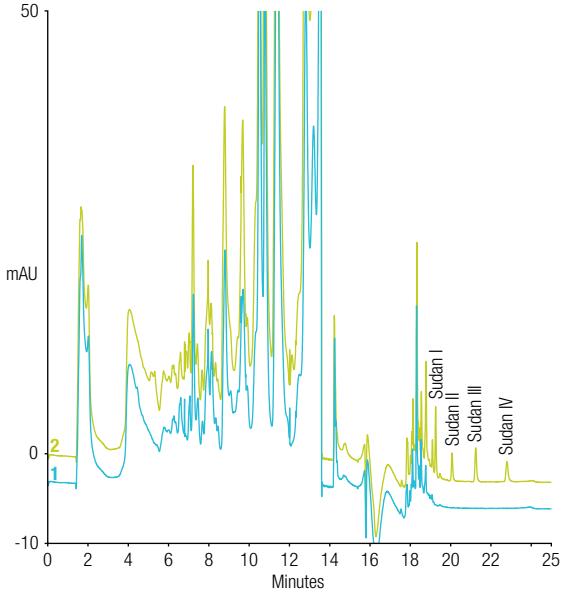

Figure 5. Overlay chromatograms of Panang curry paste and spiked Panang curry paste samples.

First Dimension

Column:	Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Second Dimension

Column:	Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water
	B: Acetonitrile
	C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm

Samples: 1. Red curry paste
2. Spiked red curry paste

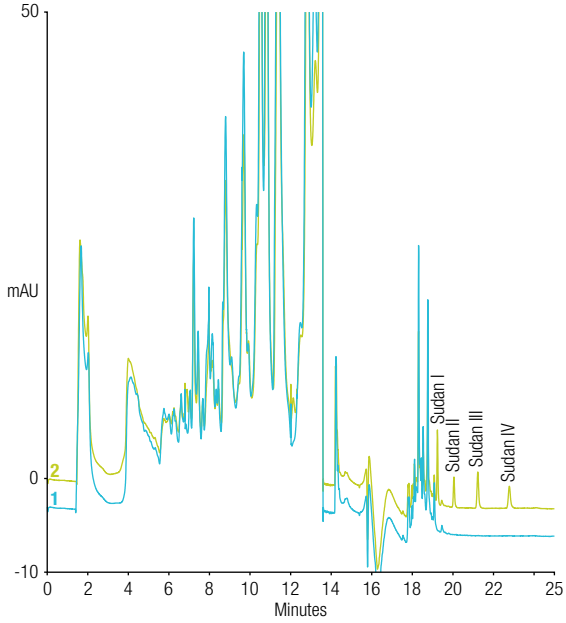

Figure 6. Overlay chromatograms of red curry paste and spiked red curry paste samples.

Method accuracy was determined by spiking the Sudan dyes standard mixture into each of the curry paste samples to yield the same concentration as Working Standard Level 1 after sample preparation. Each spiked sample was injected five times to evaluate reproducibility and recovery. The Sudan dyes' peak area RSDs ranged from 0.57% to 1.79% and the recoveries ranged from 90.5% to 110%. These results are shown in Tables 5, 6, and 7.

A UV spectral library of Sudan dyes was created by injecting a standard mixture of Sudan dyes. The wavelength scanning was performed from 380 to 800 nm. UV spectra of the spiked samples were recorded and compared to the library by matching to evaluate the peak purity. The purity of each Sudan dye peak was also evaluated using the peak purity index and the match value of spectra taken across the peak. Table 8 shows all the peak purity results.

Table 5. Fresh curry paste and spiked fresh curry paste results.

Injection No.	Sample (µg/L)				Spiked Sample (µg/L)			
	Sudan I	Sudan II	Sudan III	Sudan IV	Sudan I	Sudan II	Sudan III	Sudan IV
1	ND	ND	ND	ND	19.8	19.1	43.6	39.9
2	ND	ND	ND	ND	19.6	19.3	43.6	39.3
3	ND	ND	ND	ND	19.7	19.2	43.4	40.3
4	ND	ND	ND	ND	19.5	19.1	43.1	39.8
5	ND	ND	ND	ND	19.6	19.0	43.1	39.8
Average	—	—	—	—	19.6	19.2	43.4	39.8
RSD	—	—	—	—	0.60	0.62	0.61	0.90
Recovery (%)	—	—	—	—	98.0	96.0	109	99.5

Table 6. Panang curry paste and spiked Panang curry paste results.

Injection No.	Sample (µg/L)				Spiked Sample (µg/L)			
	Sudan I	Sudan II	Sudan III	Sudan IV	Sudan I	Sudan II	Sudan III	Sudan IV
1	ND	ND	ND	ND	19.8	18.9	43.7	41.5
2	ND	ND	ND	ND	19.7	19.2	44.4	41.4
3	ND	ND	ND	ND	19.8	19.2	43.9	41.4
4	ND	ND	ND	ND	19.7	19.4	44.0	40.4
5	ND	ND	ND	ND	19.5	19.0	44.2	40.9
Average	—	—	—	—	19.7	19.1	44.0	41.1
RSD	—	—	—	—	0.57	0.98	0.64	1.08
Recovery (%)	—	—	—	—	98.5	95.5	110	103

Table 7. Red curry paste and spiked red curry paste results.

Injection No.	Sample (µg/L)				Spiked Sample (µg/L)			
	Sudan I	Sudan II	Sudan III	Sudan IV	Sudan I	Sudan II	Sudan III	Sudan IV
1	ND	ND	ND	ND	18.2	18.4	43.8	40.0
2	ND	ND	ND	ND	18.0	19.0	44.2	39.7
3	ND	ND	ND	ND	18.1	18.6	43.4	40.2
4	ND	ND	ND	ND	18.2	18.6	44.3	40.9
5	ND	ND	ND	ND	17.8	18.1	42.9	39.0
Average	—	—	—	—	18.1	18.5	43.7	40.0
RSD	—	—	—	—	0.94	1.68	1.30	1.79
Recovery (%)	—	—	—	—	90.5	92.5	109	100

Table 8. Peak purity results and matches with the library (scanned range from 380 to 800 nm) for spiked samples.

Sample	Analyte	Match	RSD Match	PPI	RSD PPI	Match with the Library
Spiked Fresh Curry Paste	Sudan I	1000	0.06	457.6	0.11	999.97
	Sudan II	1000	0.10	472.8	0.18	999.99
	Sudan III	1000	0.01	491.0	0.01	1000.00
	Sudan IV	1000	0.05	502.2	0.10	999.99
Spiked Panang Curry Paste	Sudan I	998	0.61	459.8	0.39	999.94
	Sudan II	1000	0.10	472.9	0.18	999.99
	Sudan III	1000	0.02	491.2	0.04	999.99
	Sudan IV	990	2.54	505.2	0.37	999.31
Spiked Red Curry Paste	Sudan I	999	0.13	457.2	0.17	999.91
	Sudan II	999	0.12	473.1	0.22	999.98
	Sudan III	1000	0.01	491.0	0.02	999.99
	Sudan IV	990	2.37	505.5	0.38	999.05

First Dimension

Column:	Acclaim PA2 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water B: Acetonitrile C: 2-Propanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Inj. Volume:	300 µL
Temperature:	30 °C
Detection:	UV, 478 nm

Second Dimension

Column:	Acclaim Phenyl-1 (3 µm, 4.6 × 150 mm)
Mobile Phase:	A: Water B: Acetonitrile C: Methanol
Gradient:	See Table 2
Flow Rate:	See Table 2
Temperature:	30 °C
Detection:	UV, 478 nm

Samples: 1. Acetonitrile blank before spiked sample injection
2. Acetonitrile blank after 90 spiked sample injections

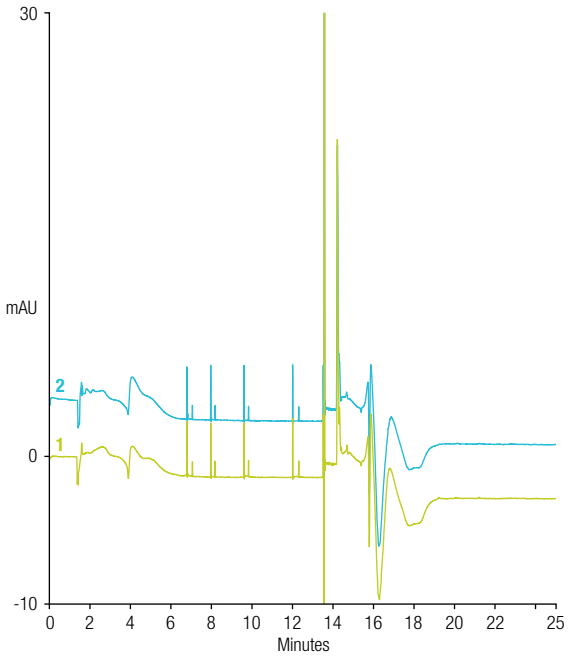

Figure 7. Overlay of chromatograms of an acetonitrile blank before and after 90 spiked sample injections.

The carryover after sample injection was also evaluated by injecting the acetonitrile blank before sample injection and after 90 spiked sample injections. There were no Sudan dyes or other peaks found in the acetonitrile blank after the spiked sample injections. Figure 7 shows the overlay of chromatograms of an acetonitrile blank before and after 90 spiked sample injections.

Conclusion

This study demonstrates a 2D-LC method for determination of Sudan dyes I, II, III, and IV in three different curry pastes using an UltiMate 3000 x2 Dual HPLC system. The sample is partially separated in the first dimension, then the portions of the chromatogram containing peaks of interest are sent to the second dimension where they are further resolved. The second dimension uses a column with selectivity different from that used in the first dimension. The total runtime is 25 min. The off-line sample preparation step is only a simple sample extraction using acetonitrile followed by filtration. The two-dimensional method precludes the need for more rigorous off-line SPE sample preparation. This results in significant time and labor savings, and thereby reduces the cost per analysis.

Reference

1. Dionex (now part of Thermo Scientific) Application Note 287: Two-Dimensional HPLC Combined with On-Line SPE for Determination of Sudan Dyes I–IV in Chili Oil. Sunnyvale, CA, 2011. [Online] www.dionex.com/en-us/webdocs/111142-AN287-HPLC-Sudan-Dyes-Chili-Oil-21Sept2011-LPN2919.pdf (accessed Sept. 17, 2012).

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