A Rapid and Sensitive Analysis Method for Sudan Reds in Curry and Chili Powder Using LC/MS/MS

Application

Food Safety

Authors

Yanyan Fang
Agilent Technologies, Inc.
412 Ying Lun Road
Pu Dong, Shanghai 200131
China

Jeffry Keever
Agilent Technologies, Inc.
200 Regency Forest Drive, Suite 330
Cary, NC 27518-8695
USA

Dustin Yaworsky and Jerry Zweigenbaum
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

A method was developed using the Agilent G6410 Triple Quadrupole LC/MS for Sudan Reds in different matrices, including curry powder, red pepper powder, and chili sources. The analytical performance of the method was evaluated for four different matrices and the results show little or no matrix effects. Linearity of response over three orders of magnitude was demonstrated (r > 0.99). In addition, good reproducibility of the two required product ion ratios was obtained to meet the EU identification points needed for confirmation.

Introduction

The group of color additives known as Sudan dyes consists of a number of red colorants, for example, Sudan I through IV. This group, together with other dyes, such as Para Red, are synthetically produced azo dyes. Because their degradation products are considered to be carcinogens and teratogens, the EU and the U.S. do not permit the use of these colorants as food additives. However, in some countries, these dyes are still occasionally used to intensify the color of bell pepper and chili powders.

The red dyes Sudan I, II, III, and IV are oil-soluble azo dyes used legally in the leather and fabric industries. The International Agency for Research on Cancer (IARC), a part of the World Health Organization, has assessed the Sudan dyes as Group 3 genotoxic carcinogens. [1] The industrial dye Para Red is chemically similar to Sudan I and is also a dye not permitted for use in food.

The EU issued Decision 2003/460/EC requiring as a condition of import that all hot chili and hot chili products be tested for Sudan I. [2] The Decision was amended in January of 2004 (2004/92/EC) to include Sudan II, III, and IV. [3] This requirement remains in effect. The highly selective and sensitive triple quadrupole (QQQ) is used to meet the testing requirement.

Experimental

Reagents and Materials

Ethyl acetate from Burdick and Jackson (Morristown, NJ)

Methanol HPLC-grade from Burdick and Jackson

Water at 18 MΩ from a Milli-Q Synthesis System by Millipore (Billerica, MA)

Syringe filter (0.2 µm, PTFE) from Agilent, p/n 5185-5843

Samples

1. Curry powder (House of Spices [India] Inc.)
2. Red pepper powder (Korean Farm, Inc.)
3. Red pepper powder (Oriental Mascot, Summit Import Corp.)
4. Worcestershire sauce (Lea & Perrins)

Overview of Method

Standard Preparation

1. Standard solution preparation: 10 mg Para Red, Sudan Red I, Sudan Red II, Sudan Red III, and Sudan Red IV were dissolved into 100.0 mL acetontrile/water (90:10) to a final concentration of 100 ppm.
2. Diluents solution preparation: using acetontrile to obtain concentrations at 1 pmm, 0.1 ppm, 10 ppb, 5 ppb, 2 ppb, and 100 ppt.

Sample Preparation

1. ***Curry powder*** was spiked with 10 µL of **standard solution** into 1 g of powder, diluted with 5 mL ethyl acetate, and allowed to ultrasonicate for 10 min. The solution was filtered with a 0.22-µm syringe filter and evaporated to dryness under a nitrogen stream at 40 °C (used below). The residue was reconstituted in 1 mL acetonitrile. This was filtered again before injection. No additional clean up of the sample solution was performed. This gave a spiked sample at a concentration at of 1 ppm. This spiking procedure was repeated to obtain spiked samples at levels of 100, 10, 1, and 0.1 ppb.
2. ***The two red pepper chili powders:*** The procedure used to prepare the curry powder was also used to prepare the spiked samples.
3. ***Worcestershire sauce*** was spiked with 10 µL of standard into 1 mL of sauce. The sauce, at about pH 3, was diluted with 5 mL ethyl acetate and allowed to ultrasonicate for 10 min. The solution was centrifuged for 2 min (8,000 rpm) and the top solvent layer (ethyl acetate) was transferred to a clean tube and evaporated under a nitrogen stream at 40 °C. The residue was redissolved in 1 mL acetonitrile and put in an ultrasonic bath for 1 min. This solution was filtered before injection.

LC/MS/MS Conditions

All analyses were performed with the Agilent 6410 Triple Quadrupole LC/MS equipped with an electrospray ionization source operated in positive mode. The HPLC was the Agilent 1200 LC system equipped with binary pump and well plate autosampler. See Table 1 for the conditions.

Table 1. LC/MS Conditions

HPLC	
Column	ZORBAX XDB C18 2.1 mm × 100 mm, 1.8 µm Agilent p/n: 928700-906
Flow rate	0.4 mL/min
Mobile phases	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Gradient	0.1–4 min, 70~98% B 4–5 min 98% B 5–7 min 70%
Total run time	10 min including re-equilibration
Temperature	40 °C
Injection	5 µL
MS Source Settings	
Source	ESI
Ion polarity	Positive
Drying gas temp.	350 °C
Drying gas flow rate	10 L/min
Nebulizer	45 psi
V_{cap}	3500 V
MRM parameters	As shown in Table 2

The MS analysis was divided into five segments each containing one of the analytes. The appropriate fragmentor and collision energy for the analyte eluting in that segment was contained therein. Segment 1 started at 0 min, segment 2 at 2.0 min, segment 3 at 2.8 min, segment 4 at 3.6 min, and segment 5 at 4.2 min.

Results and Discussion

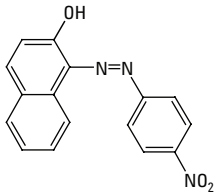
Each of the dyes was analyzed by LC/MS/MS in product ion scan and examined for spectral quality. Appropriate transition ions for quantitative analysis and confirmation were selected. The structure of each of the compounds is shown below. The azo bond cleaves under the collision-induced dissociation conditions and produces unique fragment ions.

Optimization of MS Condition

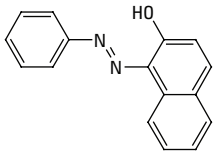
Optimization usually consists of simply finding the fragmentor voltage that maximizes the abundance of the precursor ion and the collision energy that maximizes the abundance of the transition ion. Optimization of the fragmentor was done by stepping through the voltages and recording intensity as shown for Sudan Red I in Figure 1. The other compounds were optimized by the same process. Figure 2 shows the product ion MS/MS spectra for Sudan Red I at two collision energies (CE). It can be seen from the spectra that the transition *m/z* 156 gives a maximum intensity at 15 V and *m/z* 93 at 25 V. Typically the most abundant ion is used for quantitation to maximize precision and accuracy at the lowest levels and the less abundant ion is used as the qualifier for confirmation. However, for the highest selectivity, unique transition ions are sought. In the example of Sudan Red I the transition *m/z* 93 is the aniline radical cation produced by cleavage of the azo bond and hydrogen transposition. [4] This also produces the transition *m/z* 156, both unique. Measuring CE vs intensity for the two most abundant ions of all the compounds resulted in the voltages shown in Table 2 along with the other MS settings.

Dye Structures

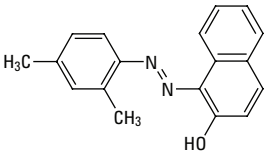
Para red



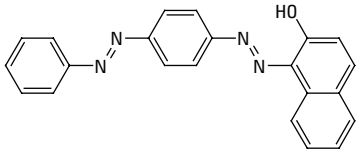
Sudan Red I



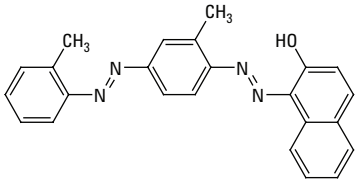
Sudan Red II



Sudan Red III



Sudan Red IV



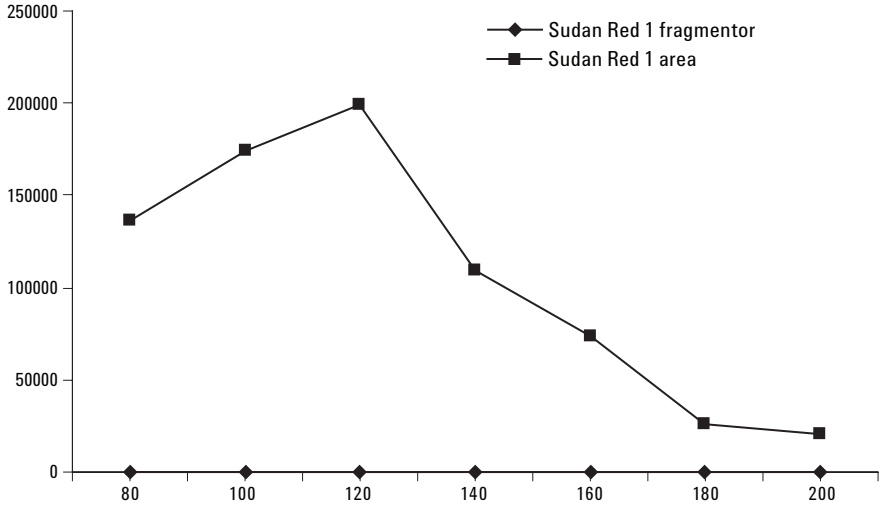


Figure 1. Optimization of fragmentor voltage for Sudan Red I.

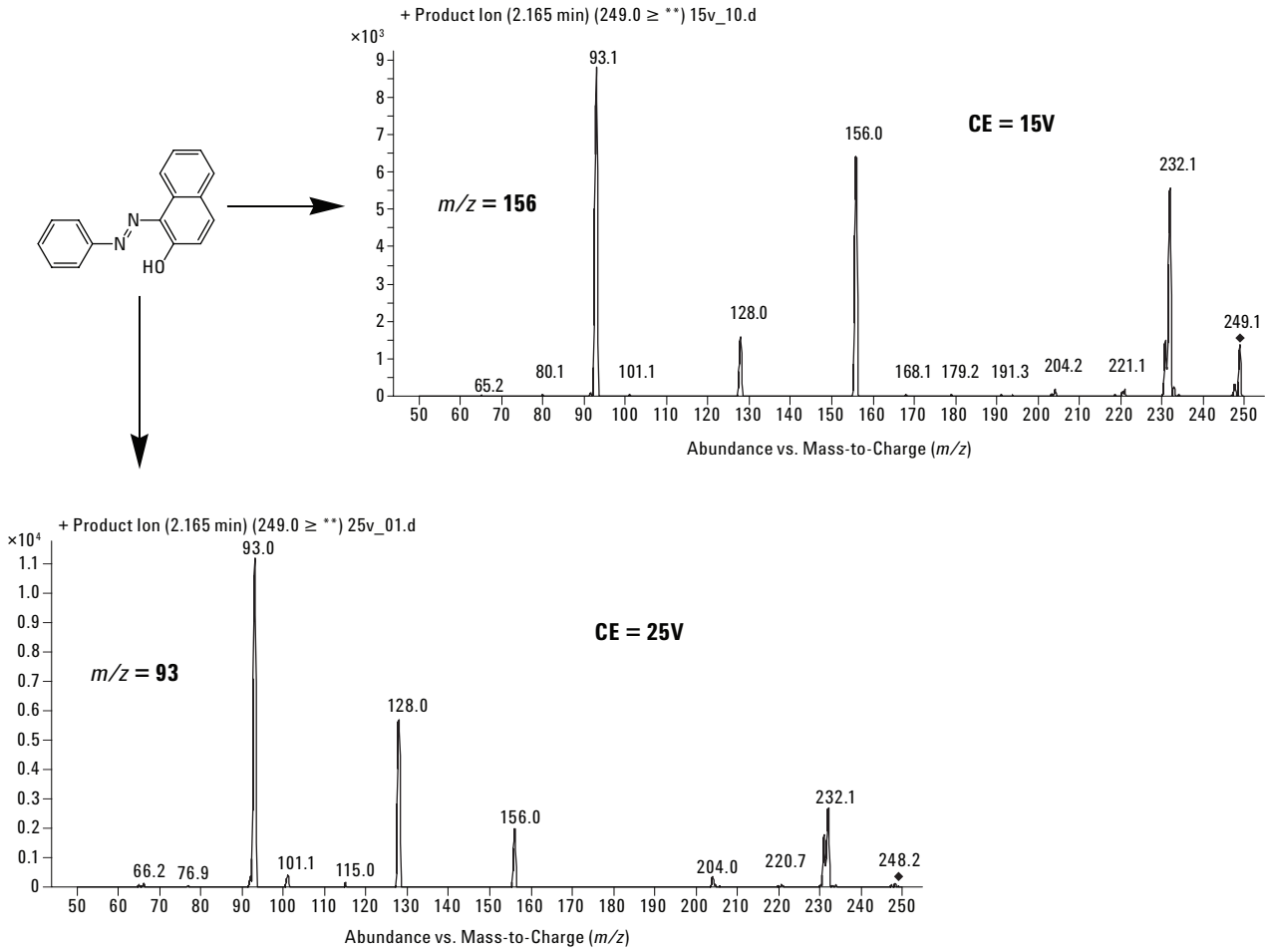


Figure 2. MS/MS spectra of Sudan Red I at collision energies (CE) 15 and 25 V.

Table 2. MRM MS/MS Parameters

Analyte	Transition	Dwell (msec)	Fragmentor voltage	Collision energy	MS2 Res.	Gain
Para Red	294→156	200	120 V	15 V	Unit	1
	294→128	200	120 V	30 V	Unit	1
Sudan Red I	249→156	200	120 V	15 V	Unit	1
	249→93	200	120 V	25 V	Unit	1
Sudan Red II	277→156	200	100 V	10 V	Unit	1
	277→121	200	100 V	20 V	Unit	1
Sudan Red III	353→77	200	120 V	25 V	Unit	1
	353→197	200	120 V	20 V	Unit	1
Sudan Red IV	381→91	200	120 V	30 V	Unit	1
	381→225	200	120 V	15 V	Unit	1

Chromatography and Sensitivity

Figure 3 shows the chromatographic separation achieved for the five analytes allowing the segmentation of the MS/MS conditions. The sensitivity of the methodology is shown with the spike of the chili powder at 1 ppb. This is shown with a blank chili powder (Figure 4) and spike where each transition of the quantifier and qualifier ion for all five analytes is displayed (Figure 5).

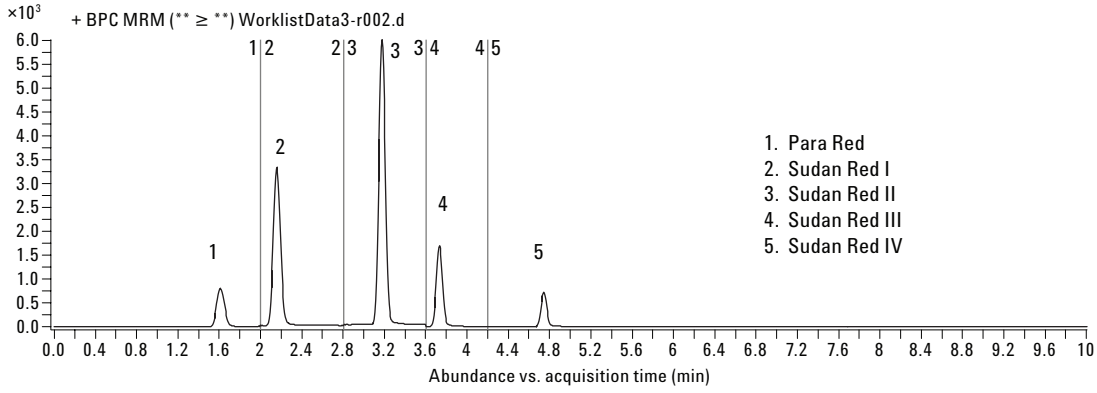


Figure 3. MRM chromatogram of Sudan Reds and Para Red.

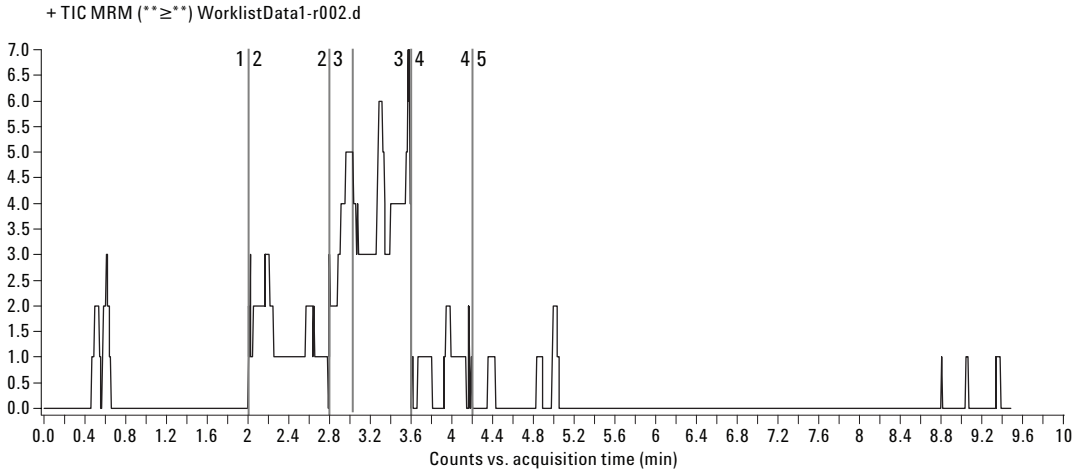


Figure 4. Blank chili pepper extract showing no response for any analyte.

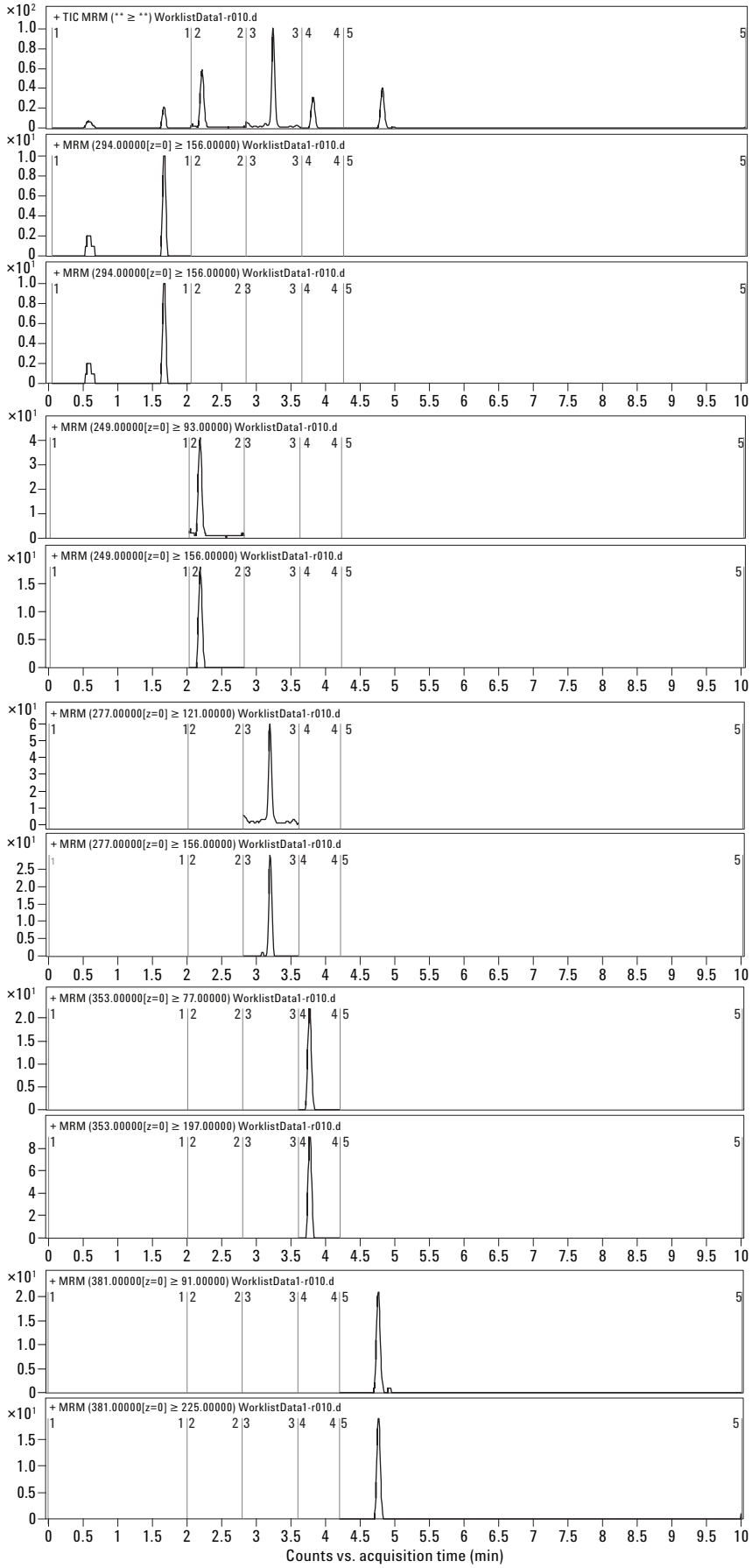


Figure 5. Chili pepper spiked at 1 ppb. Total MRM chromatogram on top and then each transition, quantitation and qualifier of the respective five analytes.

Confirmation by Ion Ratios

The EU Decision 2002/657/EC set confirmation criteria to include two MS/MS transition ions and their relative ratios (to the most abundant of the two). [5] The criteria for the tolerances of those ratios were given by the relative intensity. This is shown in Table 3 and demonstrates that the higher the relative ratio, the tighter the tolerances for acceptance.

We have measured these ratios for each of the analytes in both solvent and matrix and found the matrix has little effect on the measured ratios. Using Sudan Red I as the example, the ratios of the two ions are given in Table 4. In addition, the repeatability of the method was tested using a 1 ppb spike in curry powder. The results of 20 repeats shown in Table 5 demonstrate that the method can perform well within the accepted tolerance of 25%. All of the samples used for the validation study met the relevant identification criteria.

Table 3. Maximum Permitted Tolerances for Relative Ion Intensities Using LC-MS^n

Relative intensity between two transition ions (% of most intensive ion)	Tolerance for LC-MS^n (%)
> 50	± 20
> 20–50	± 25
> 10–20	± 30
≤ 10	± 50

Table 4. Ratio of Quantitation vs Qualifier Transition Ion in Tested Matrices at 100 ppb Spike of Sudan Red I

Matrix	Ratio
Solvent	46.8
Curry powder (Madras)	48.5
Red pepper powder (Korean Farm)	46.5
Red pepper powder (Oriental Mascot)	46.6
Worcestershire sauce (Lea & Perrins)	47.7

Table 5. Repeatability of Sudan Red I Ion Ratios at 1 ppb in Curry Powder

	Quantitation ion (249-93)	Qualifier ion (249-156)	Ratio
1	130	58	45.0
2	141	65	46.0
3	145	71	48.9
4	152	62	41.0
5	135	66	49.1
6	146	66	45.3
7	134	64	47.7
8	139	69	49.5
9	137	71	51.8
10	147	64	43.9
11	143	61	42.4
12	156	65	41.7
13	147	64	43.5
14	144	64	44.7
15	148	65	44.1
16	152	63	41.2
17	143	68	47.7
18	140	66	46.7
19	141	62	44.2
20	142	62	43.8
Average	143.1	64.8	45.4
STD (%)	4.49	4.87	6.55

Linearity, Sensitivity, and Recovery

Pearson's Correlation Coeffcient (R^2) was used as a measure of the standard curve linearity where an R^2 value of at least 0.99 was deemed acceptable. The linearity of the dyes in each of the food matrices was at least 0.99 except where the 1 ppm standard showed saturation, probably of the electrospray current. When that concentration is omitted, all calibrations in all matrices have an $R^2 > 0.99$. An example of this is shown in Figure 6, where red chili pepper is spiked with Sudan Red I.

The detection limit of each of the dyes was estimated by analyzing them in a standard at 0.2 ppb. The total MRM chromatogram is shown in Figure 7. Because each of the signals has little noise, it is difficult to determine with accuracy an s/n of 3:1.

Increasing the gain on the electron multiplier would have given both greater signal and noise and made this determination more accurate. It is reasonable to estimate that the limit of detection (LOD) is close to this concentration.

Finally, recovery of the sample preparation procedure was examined for the Worcestershire sauce since this was a liquid-liquid extraction. This was done with a replicate of three for each level of 100 and 1 ppb. These results are given in Table 6 and show reasonable recoveries.

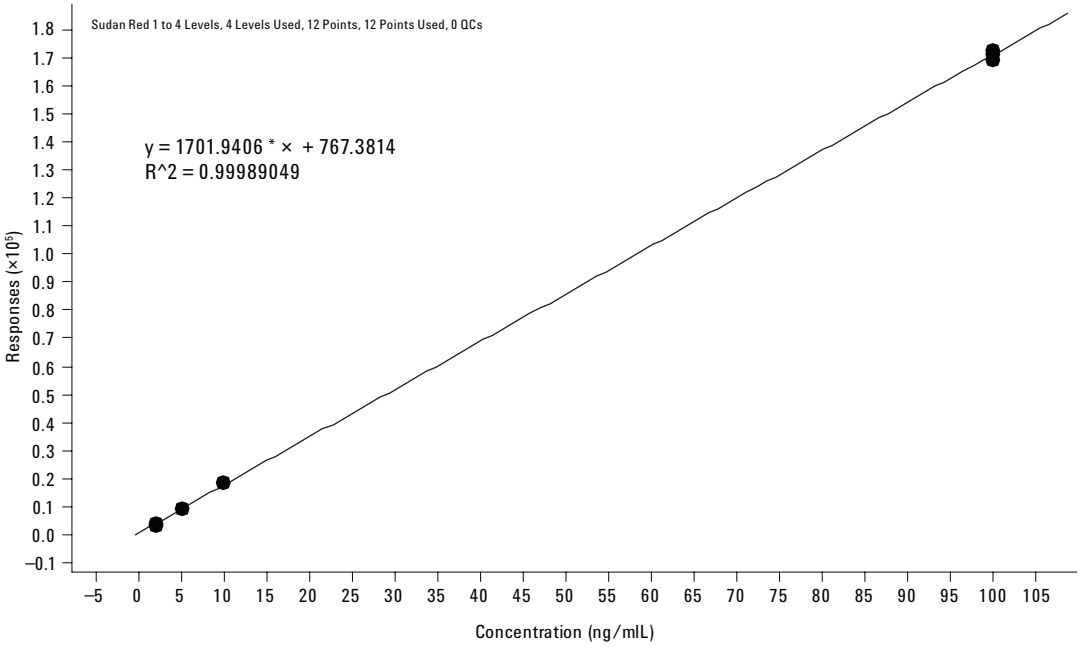


Figure 6. Standard curve of 1 ppb – 100 ppb Sudan Red I in chili powder.

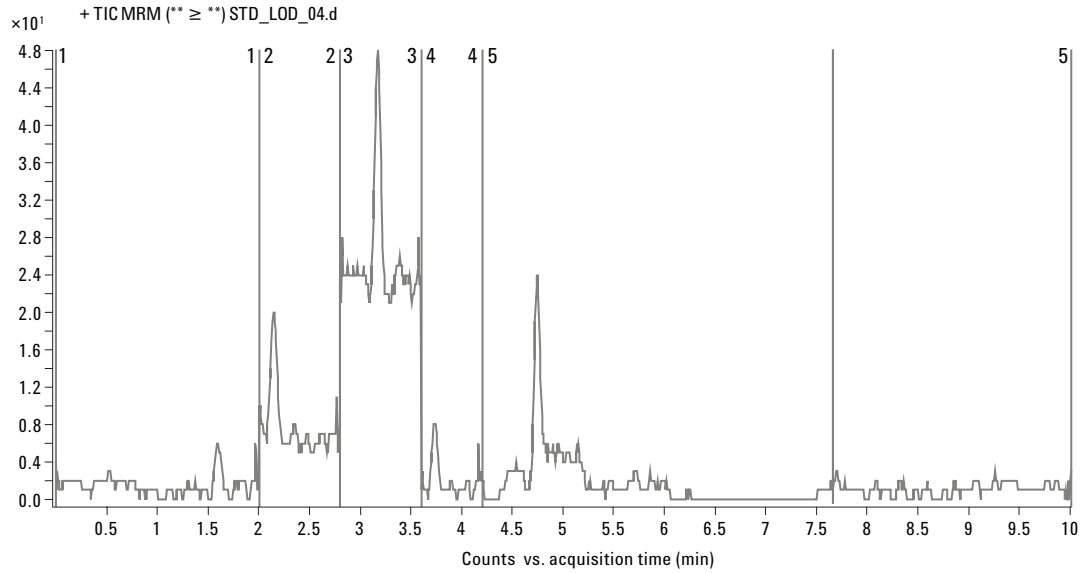


Figure 7. MRM chromatogram of each of the dyes at 0.2 ppb in solvent, representing a response close to the detection limit.

Table 6. Recovery of the Five Dyes in the Worcestershire Sauce

	Para Red (%)	Sudan Red I (%)	Sudan Red II (%)	Sudan Red III (%)	Sudan Red IV (%)
100 ppb recovery (n = 3)	57.8	71.0	63.4	62.1	70.2
1ppb recovery (n = 3)	54.5	66.2	50.1	54.1	51.2

Conclusions

The method described herein for the analysis of four Sudan Red compounds and Para Red in four different matrices has been shown to be highly effective in meeting the criteria for quantitation and confirmation. Optimization of the method was simple, because few parameters in the mass spectrometer need adjustment. In addition, basic requirements for a validated method have been shown. These include sensitivity, repeatability, linearity, and recovery. This shows the Agilent 6410 Triple Quadrupole LC/MS system to be a highly effective instrument for the analysis of Sudan Reds and other azo dyes in food spices.

References

1. IARC *Summaries and Evaluations;* Sudan I; International Agency for Research on Cancer: 1975.
2. 2003/460/EC: Commission Decision of 20 June 2003 on emergency measures regarding hot chili and hot chili products (notified under document number C[2003] 1970) OJ L154/114 (21.6.2003).
3. 2004/92/EC: Commission Decision of 21 January 2004 on emergency measures regarding chili and chili products (notified under document number C[2004] 68) OJ L27/52 (30.1.2004).
4. F. Calbiani, M. Careri, L. Elviri, A. Mangia, I. Zagnoni, "Accurate Mass Measurements for the Confirmation of Sudan Azo Dyes in Hot Chili Products by Capillary Liquid Chromatography-Electrospray Tandem Quadrupole Orthogonal-Acceleration Time of Flight Mass Spectrometry." *Journal of Chromatography A* **2004**, 1058, (1–2), 127–135.
5. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C[2002] 3044), OJ L221/8 (17.8.2002) In 2002; Vol. L221, p 8.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
February 19, 2008
5989-7308EN

Agilent Technologies