Development of a Fast Analytical Method for the Determination of Sudan Dyes in Chili- and Curry-Containing Foodstuffs by High-Performance Liquid Chromatography–Photodiode Array Detection

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A simple and fast analytical method for the determination of sudans I, II, III, and IV in chili- and curry-containing foodstuffs is described. These dyes are extracted from the samples with acetonitrile and analyzed by high-performance liquid chromatography coupled to a photodiode array detector. The chromatographic separation is carried out on a reverse phase C_{18} column with an isocratic mode using a mixture of acetonitrile and water. An "in-house" validation was achieved in chili- and curry-based sauces and powdered spices. Depending on the dye, limits of detection range from 0.2 to 0.5 mg/kg in sauces and from 1.5 to 2 mg/kg in spices. Limits of quantification are between 0.4 and 1 mg/kg in sauces and between 3 and 4 mg/kg in spices. Validation data show a good repeatability and within-lab reproducibility with relative standard deviations < 15%. The overall recoveries are in the range of 51–86% in sauces and in the range of 89–100% in powdered spices depending on the dye involved. Calibration curves are linear in the 0–5 mg/kg range for sauces and in the 0–20 mg/kg range for spices. The proposed method is specific and selective, allowing the analysis of over 20 samples per working day.

KEYWORDS: Sudan dyes; chili- and curry-containing foodstuffs; HPLC-PDA; food safety

INTRODUCTION

A lot of dyes are used as food additives and include both natural and synthetic substances, covering a wide range of chemical entities. The most common artificial food colors are azo dyes. The list of authorized food colors and the maximum permitted levels in foodstuffs are laid down in the annexes of Council Directive 94/36/EC (*1*). Other azo dyes such as the sudan dyes are nonauthorized and are illegally used in the food industry to enhance and maintain the appearance of food products.

Sudans I, II, III, and IV are phenyl-azoic derivatives widely used in chemical industries for coloring materials such as hydrocarbon solvents, oils, fats, plastics, printing inks, and shoe and floor polishes. Their chemical structures are illustrated in **Figure 1**. Repeated notifications about the detection of the nonauthorized sudan dyes in foods have been disseminated by the European Union rapid alert system since 2003. On the 9th of May 2003, French sanitary authorities detected sudan I in hot chili products imported from India (*2*). As sudan I is classified as a category 3 carcinogen by the International Agency for Research on Cancer (IARC) and as a category 3 mutagen

in Annex I of the Council Directive 67/548/EEC (*3–6*), this synthetic dye constitutes a potential risk for public health if it enters the food chain. Except in some African or Asian countries, their use as additives, at any level, in food products destined for human consumption is prohibited worldwide. Consequently, the Commission Decision 2005/402/EC (*7*) requires that all chili-, curry-, and curcuma-containing food products and palm oil coming into any European Union member state are certified to be free of sudan dyes. Since July 2003, more than 160 products have been recalled for destruction in the United Kingdom because of the detection of sudan dyes. As the illegal use of dyes has major economic consequences for most European Union food industries as well as an impact on public health, suitable analytical screening and confirmatory methods are required for compliance verification of these foodstuffs. At this moment, very few analytical methods have been described in the literature. A method including a Soxtec extraction and a gel permeation chromatography cleanup followed by high-performance liquid chromatography (HPLC) with ultraviolet/visible detection has been recently proposed by Mazzetti et al. for the detection of sudan I in chili powder and chili-containing food products (*8*). A second method using thin-layer chromatography has been developed for the qualitative analysis of sudans I, II, III, and IV in palm oil (*9*). The scope of these two

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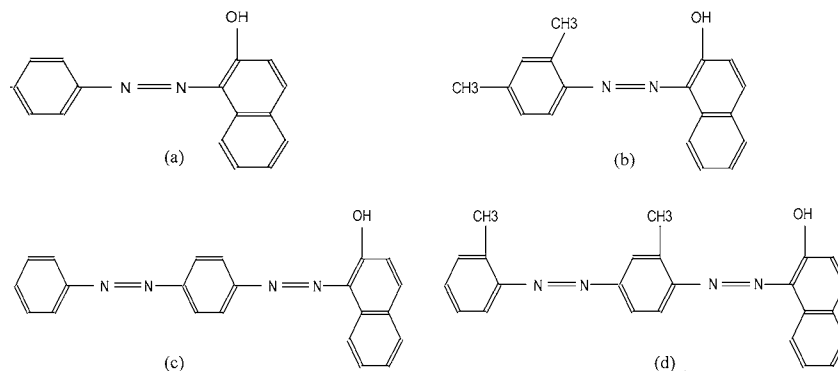
Figure 1. Chemical structures of sudan dyes: (**a**) sudan I, (**b**) sudan II, (**c**) sudan III, and (**d**) sudan IV.

methods is limited either to the detection of one of the four sudan dyes or to the qualitative determination of the dyes. This paper presents a simple analytical method to identify and quantify simultaneously sudans I, II, III, and IV in chili- and curry-based sauces and spices by HPLC coupled to a photodiode array detector (HPLC-PDA).

MATERIALS AND METHODS

Reagents and Materials. Sudan I (>99%), sudan II (>99%), and sudan IV (80%) were purchased from Acros Organics (Geel, Belgium). Sudan III (95%) was supplied by Sigma-Aldrich (Bornem, Belgium). Lichrosolv acetonitrile was of HPLC grade from Merck (Darmstadt, Germany). HPLC grade water was freshly produced by an internal Millipore purification system (Bedford, MA) and filtered with Millipore filters (0.45 μm). Syringe filters (nylon, 0.45 μm, 25 mm) used to filter final extracts were obtained from Euroscientific (Lint, Belgium).

Standard Solutions. Stock solutions of 500 (sudans I and II) and 100 μg/mL (sudans III and IV) were individually prepared in acetonitrile and were stored at 4 °C for 6 months. An intermediate standard solution of 50 μg/mL containing sudans I, II, and III was prepared in acetonitrile. Working standard solutions of 25, 10, 5, and 2.5 μg/mL (sudans I, II, and III) were prepared in volumetric flasks by dilution of the 50 μg/mL solution in acetonitrile. An intermediate standard solution (50 μg/mL) and working standard solutions (25, 10, 5, and 2.5 μg/mL) of sudan IV were prepared following the same procedure. The intermediate standard solutions were kept at 4 °C for 1 month while the working standard solutions were renewed daily.

Food Samples. Chili- and curry-containing sauces and spices were obtained from local supermarkets and preexamined for the absence of sudans I—IV by the method described below. Samples were mixed homogeneously and stored in 50 mL Falcon tubes (VWR, Leuven, Belgium) at −20 °C for sauces or at room temperature for spices.

Extraction Procedure. For sauces, 5 g of sample was weighed in a 50 mL Falcon tube and homogenized with 25 mL of acetonitrile. The extract was vigorously vortex mixed and shaken in a second step for 1 h on a rotative stirrer. After centrifugation at 4000 rpm for 10 min at 4 °C (Eppendorf Centrifuge 5810R, VWR), one part of the extract (±1 mL) was filtered in a vial for HPLC use and 100 μL was injected into the HPLC system. Blank samples were analyzed in the same way. For fortified samples, spiking levels were 0.5, 1, and 2.5 mg/kg: 500 μL of the sudans I, II, and III working standard solutions (5, 10, or 25 μg/mL) and 500 μL of the sudan IV working standard solution (5, 10, or 25 μg/mL) were added to 5 g of blank material, which was then homogenized with 24 mL of acetonitrile.

The spice samples were prepared as follows: A 0.5 g amount of matrix was weighed and homogenized with 10 mL of solvent. The remaining procedure was identical to the sauce samples. The levels of the fortified powdered spices were 2.5, 5, and 10 mg/kg. They were prepared by adding 500 μL of the sudans I, II, and III working standard solutions (2.5, 5, or 10 μg/mL) and 500 μL of the sudan IV working standard solution (2.5, 5, or 10 μg/mL) to 0.5 g of blank material. The fortified samples were homogenized with 9 mL of acetonitrile.

Instrumental Conditions. The HPLC system consisted of a Waters 717 plus autosampler (Waters, Milford, MA) and a Varian 9010 HPLC

pump (Varian, Sint-Katelijne Waver, Belgium) coupled with a Waters 996 PDA detector. The chromatographic separation was performed on a Varian Microsorb-MV reverse phase column (150 mm × 4.6 mm 100-5 C18) protected by a guard column RP SS 10 mm × 2 mm (Varian). The mobile phase was composed by acetonitrile and water (80/20, v/v) at a flow rate of 1 mL/min in an isocratic mode. Sudan dyes I—IV were detected at 478, 496, 510, and 520 nm, respectively. Because of an interference at the retention time of sudan I in curry powders at 478 nm, the dye was detected at 496 nm for this matrix. Chromatograms were recorded and processed by the Millenium[32] software (Waters).

Matrix-Matched Calibration Standards. Because of a matrix effect, matrix-matched calibration standard curves were used for the quantification of sudan dyes in samples (peak height vs standard concentration) and were prepared as follows: 100 μL of the sudans I, II, and III standard solutions (2.5, 5, 10, 25, or 50 μg/mL) and 100 μL of the sudan IV standard solution (2.5, 5, 10, 25, or 50 μg/mL) were added to a 5 mL volumetric flask containing blank extract in order to provide calibration standards of 0.25, 0.5, 1, 2.5, and 5 mg/kg in sauces or 1, 2, 4, 10, and 20 mg/kg in spices.

Validation Scheme. Validation of analytical methods was a prerogative for every ISO/IEC 17025 (*10*) accredited laboratory. An "in-house" validation protocol was applied in accordance with an internal procedure as well as the Commission Decision 2002/657/EC (*11*, *12*), which implements the Council Directive 96/23/EC (*13*) concerning the performance of analytical methods and the interpretation of results in the framework of veterinary drug residues in animal products.

Chili- and Curry-Based Sauces. An in-house total validation was performed in sauces containing chili. The matrix effect was first studied by comparing a calibration curve based on solution standards with a calibration curve based on matrix-matched standards in the concentration range of 0—5 mg /kg ($n = 3$ replicates per concentration level). Correctness of the calibration model was verified in the 0—5 mg/kg range by the Mandel's fitting test (*14*).

Two approaches were considered to determine the limit of detection (LOD) and the limit of quantification (LOQ): measurements on 20 blanks based on 3:1 and 6:1 signal-to-noise ratios and measurements on blank samples fortified at decreased levels from 0.1 to 1 mg/kg (n = 3 replicates per concentration level).

As no reference material was available for the four sudan dyes, recovery of the method was determined on nine blank samples fortified at three different levels of concentration: 0.5, 1, and 2.5 mg/kg (n = 3 replicates per concentration level for three different days). Repeatability and within-lab reproducibility were determined according to the ISO5725-2 (*15*) guidelines and expressed by coefficients of variation (CV) measured on the same nine fortified blank samples (n = 3 replicates per concentration level and analyzed in three independent analytical runs).

Selectivity and specificity were checked by fortifying a blank chili sauce at 5 mg/kg with veterinary drugs, which absorb in the UV band like sulfonamides, malachite green, oxfendazole sulfone, and two other red dyes authorized in the food industry (azorubine E 122 and erythrosine E 127).

For curry-based sauces, a secondary validation was performed in the framework of the Belgian flexible scope (*16*). In this case, the

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Table 1. Identification Parameters of Sudan Dyes in Foodstuffs by HPLC-PDA: Retention Time (t_R) and Maximum Absorption Wavelength [λ_{max}(acn)]

dye	t_R (min)	λ_{max} (acn) (nm)
sudan I	4.576	475–481
sudan II	7.866	493–499
sudan III	11.988	503–509
sudan IV	22.341	517–523

flexible scope allowed the laboratory to extend faster its scope of accreditation for the analytical method with a new matrix. In addition, guidelines for the validation of the analytical methods were proposed by the accreditation organization. Under certain circumstances, a laboratory was allowed to follow a reduced validation protocol. This reduced validation was also called a secondary validation. The recovery, repeatability, and within-lab reproducibility of the method for the curry samples were determined by measurements on 10 blank samples fortified at two levels of concentration: 0.5 and 2.5 mg/kg for sudans I−III and 1 and 2.5 mg/kg for sudan IV ($n = 2$ replicates per concentration level for day 1 and 3; $n = 6$ replicates for day 2).

Chili- and Curry-Based Spices. The same validation protocol was applied for chili- and curry-containing powdered spices. Matrix effect and correctness of the calibration model were checked in the concentra-

tion range of 0−20 mg/kg. LODs and LOQs were determined by comparing measurements on 20 blank samples based on 3:1 or 6:1 signal-to-noise ratios with measurements on blank samples fortified at decreased levels (from 0.8 to 5 mg/kg). The recovery, repeatability, and within-lab reproducibility were measured on nine blank samples spiked at three different levels of concentration: 2.5, 5, and 10 mg/kg ($n = 3$ replicates per concentration level and analyzed in three independent analytical runs). In contrast of curry-based sauces, a total validation was also performed for curry-containing spices. In curry blank spices, an interference with sudan I was observed (**Figure 4**). Because of the large tailing of this interference at 478 nm, the good integration of sudan I was disturbed. The interference can be eliminated by detecting sudan I at 496 nm. At this wavelength, the interference absorbed less. It was proven during method validation that sudan I was correctly detected and integrated at 496 nm. Validation data show a good repeatability and reproducibility of sudan I in curry spices.

Statistics and Interpretation of Results. All validation data were statistically treated with the Microsoft Office Excel Solver software (Frontline System Inc., NV). The validation results were evaluated according to the criteria defined in the Commission Decision 2002/657/EC.

RESULTS AND DISCUSSION

Identification Criteria. To identify sudan dyes by HPLC-PDA, three parameters calculated by the Millenium[32] software
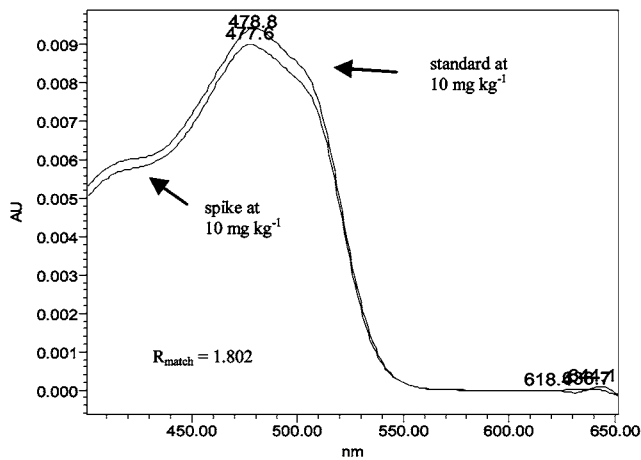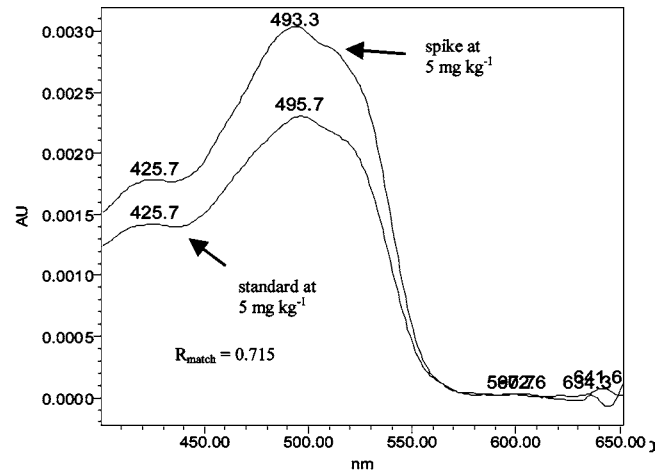
Figure 2. Comparison of UV spectra between a spiked blank sample and a matrix-matched standard. (**a**) Sudan I in chili-based spice, (**b**) sudan II in curry-based spice, (**c**) sudan III in curry-based sauce, and (**d**) sudan IV in chili-based sauce.

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Table 2. Matrix Effect and Linearity Study
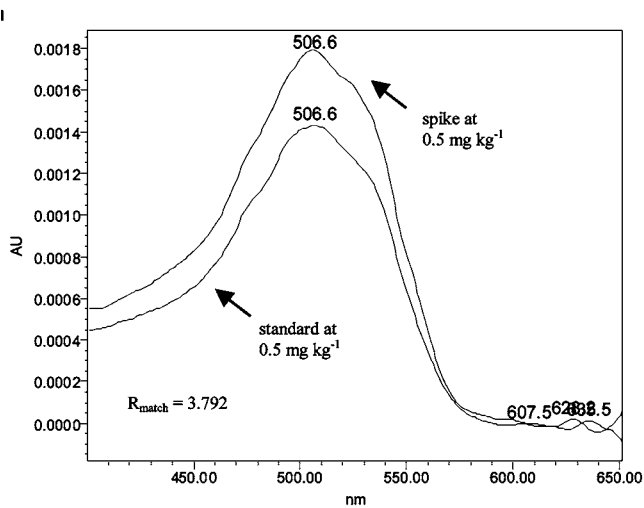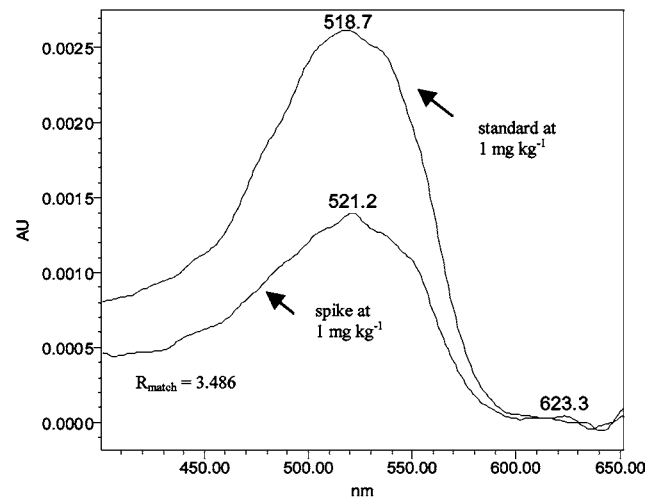
matrix	dye	matrix effect t-Student's test			linearity Mandel's fitting test			
		$t_{observed}$	$t_{0.95(theoretical)}$	k (dL)	$F_{observed}$	$F_{0.99(critical)}$	k_1 (dL)	k_2 (dL)
sauces	sudan I	10.109	2.015	22	0.005	8.285	1	18
	sudan II	15.081			2.929			
	Sudan III	13.251			0.153			
	sudan IV	5.753			0.098			
spices	sudan I	3.402	2.024	19	0.140	8.683	1	15
	sudan II	1.379			0.631			
	sudan III	0.045			0.020			
	sudan IV	1.112			0.002			

Table 3. Limits of Detection (LOD) and Quantification (LOQ) of Sudan Dyes in Sauces and Powdered Spices Determined by Measurements on Blank Samples Spiked at Decreased Levels

	mg/kg			
	sauces		spices	
dye	LOD	LOQ	LOD	LOQ
sudan I	0.20	0.40	1.5	3
sudan II	0.20	0.40	1.5	3
sudan III	0.25	0.45	2.0	4
sudan IV	0.50	1	2.0	4

are considered as follows: the retention time (t_R), the maximum absorption wavelength [λ_{max} (acn)], and the matching coefficient (R). In samples, the presence of sudan dyes is confirmed if the criteria are satisfied as follows: the retention time and the maximum absorption wavelength of the unknown peak observed in the chromatogram have to be equal to those of the corresponding standard with a tolerance of ±5% (t_R) and ±3 nm [λ_{max} (acn)]. The matching parameter that compares the UV spectra of the sample and the standard has to be inferior to 10. If one of these three criteria is not satisfied for a suspected routine sample, it will be reanalyzed and a standard addition
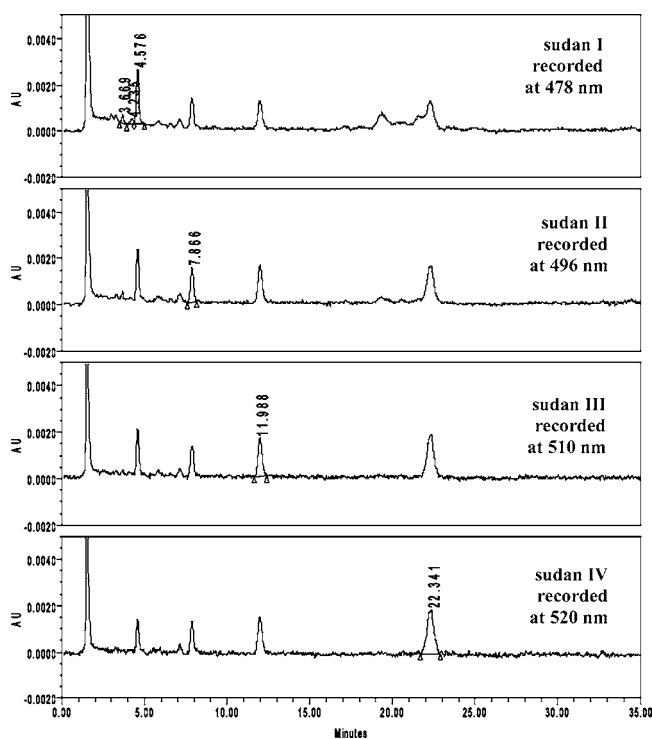
Figure 3. Chromatogram of a blank sauce sample spiked at 0.5 mg/kg for sudan I, sudan II, and sudan III and at 1 mg/kg for sudan IV.
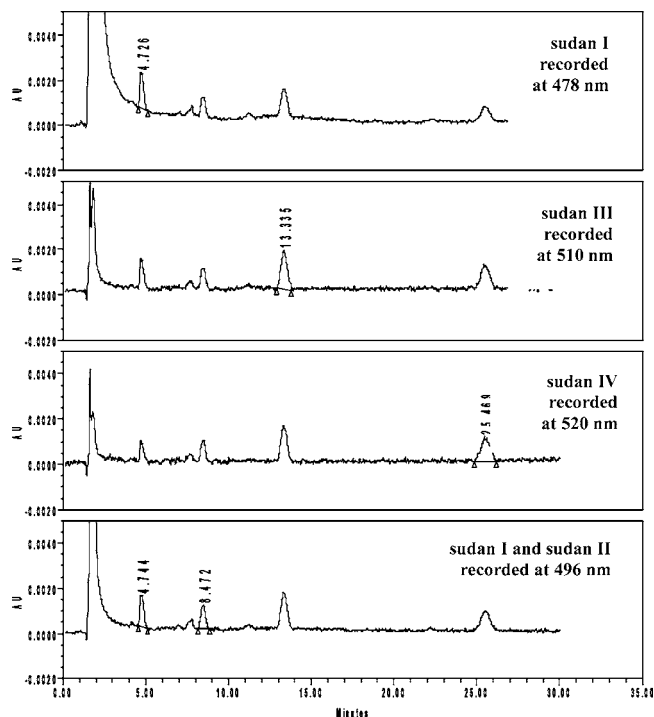
Figure 4. Chromatogram of a 2 mg/kg matrix-matched standard of sudan I, sudan II, sudan III, and sudan IV in curry powdered spice.

will be systematically performed. Retention time and maximum absorption wavelengths of sudans I−IV are presented in **Table 1**. The comparison between the UV spectra of fortified blank samples and the equivalent matrix-matched standards is illustrated in **Figure 2** for each dye.

Selectivity and Specificity. The HPLC-PDA method for the determination of sudans I−IV in foodstuffs is selective and specific. No interference appears at the retention time of each dye when analyzing samples containing other molecules such as veterinary drug residues (malachite green, sulfonamides, or oxfendazole sulfone) or colors used in agrofood industries (azorubine E 122 and erythrosine E 127).

Matrix Effect and Linearity. In sauces, a matrix effect is observed for the four sudan dyes: a t-Student's test ($p = 0.95$, $n_1 = 24$, $n_2 = 24$, $t_{observed} > t_{theoretical}$) shows a significant difference between the slopes of the calibration curve based on solution standards and the calibration curve based on spiked blank samples. In spices, this matrix effect is only noticed for sudan I (t-Student's test, $p = 0.95$, $n_1 = 21$, $n_2 = 21$, $t_{observed} > t_{theoretical}$). The $t_{observed}$ and $t_{theoretical}$ values are presented in **Table 2**. Consequently, the level of sudan dyes in samples will be calculated by interpolation of a matrix-matched standard calibration curve.

Calibration graphs are linear in the concentration range of 0−5 mg/kg in sauces and in the 0−20 mg/kg range in spices, with correlation coefficients >0.995 for each dye. The correctness of the calibration model is also checked by the Mandel's fitting test as the $F_{observed}$ values presented in **Table 2** are inferior to the $F_{critical}$ value ($p = 0.99$, $n = 21$ for sauces, $n = 18$ for spices).

LODs and LOQs. LODs and LOQs in sauces and powdered spices are mentioned in **Table 3**. LODs and LOQs calculated on 20 blank samples are equivalent or inferior to those calculated on spiked samples at decreased levels depending on the matrix (sauces or spices) and on the dye (sudans I, II, III, or IV). To take into account the matrix effect and to certify statistically

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Table 4. Results for Recovery, Intraassay Repeatability (CVr), and within-Lab Reproducibility (CV_Rw) Obtained on 9 or 10 Replicates (*n*) Per Concentration Level Analyzed in Three Independent Analytical Runs According to the ISO 5725-2 Guidelines

	spike level (mg/kg)															
		sauce								powder						
		chili				curry				chili				curry		
parameter	n	0.5	1	2.5	n	0.5	1	2.5	n	2.5	5	10	n	2.5	5	10
						dye: sudan I (478 nm)										
day 1	3	0.44	0.84	2.13	2	0.39		1.99	3	2.35	4.80	9.82	3	2.5	4.84	10.06
day 2	3	0.42	0.86	2.15	6	0.43		2.17	3	2.24	4.75	9.81	3	2.43	4.95	9.68
day 3	3	0.43	0.89	2.20	2	0.40		2.18	3	2.17	4.74	9.63	3	2.42	4.89	9.96
mean		0.43	0.86	2.16		0.42		2.13		2.25	4.76	9.75		2.45	4.89	9.90
SD		0.03	0.03	0.05		0.02		0.08		0.10	0.09	0.18		0.06	0.10	0.24
CVr (%)		**6.44**	**1.99**	**1.79**		**1.94**		**0.92**		**2.68**	**2.15**	**1.86**		**2.45**	**2.15**	**1.93**
CV_Rw (%)		**6.44**	**3.36**	**2.23**		**4.74**		**4.63**		**4.69**	**2.15**	**1.86**		**2.69**	**2.15**	**2.54**
recovery (%)		86	86	86		84		85		90	95	98		98	98	99
						dye: sudan I (496 nm)										
day 1													3	2.55	4.89	10.15
day 2													3	2.48	4.98	9.69
day 3													3	2.44	4.93	9.95
mean														2.49	4.93	9.93
SD														0.06	0.11	0.24
CVr (%)														**1.65**	**2.35**	**1.61**
CV_Rw (%)														**2.68**	**2.35**	**2.67**
recovery (%)														100	99	99
						dye: sudan II (496 nm)										
day 1	3	0.37	0.73	1.86	2	0.31		1.76	3	2.30	4.55	9.69	3	2.48	4.83	9.92
day 2	3	0.36	0.76	1.97	6	0.40		2.01	3	2.81	5.09	10.07	3	2.49	5.00	9.70
day 3	3	0.41	0.80	2.00	2	0.36		2.02	3	2.36	4.95	9.87	3	2.28	5.04	9.93
mean		0.38	0.77	1.94		0.37		1.96		2.49	4.86	9.88		2.41	4.96	9.85
SD		0.03	0.03	0.07		0.04		0.11		0.26	0.27	0.21		0.12	0.14	0.19
CVr (%)		**6.38**	**2.50**	**2.43**		**1.27**		**1.58**		**3.73**	**2.76**	**1.52**		**3.34**	**2.25**	**1.74**
CV_Rw (%)		**8.28**	**4.90**	**4.16**		**12.28**		**7.01**		**11.73**	**6.16**	**2.26**		**5.60**	**2.88**	**1.93**
recovery (%)		76	77	78		74		78		100	97	99		96	99	98
						dye: sudan III (510 nm)										
day 1	3	0.33	0.62	1.55	2	0.30		1.52	3	2.15	4.59	9.66	3	2.52	4.83	10.25
day 2	3	0.31	0.66	1.71	6	0.36		1.78	3	2.60	4.85	9.59	3	2.49	4.77	9.50
day 3	3	0.33	0.68	1.67	2	0.31		1.76	3	2.20	4.56	9.08	3	2.38	4.82	9.69
mean		0.32	0.65	1.65		0.34		1.73		2.32	4.67	9.44		2.47	4.81	9.81
SD		0.03	0.04	0.09		0.03		0.11		0.24	0.18	0.31		0.11	0.11	0.37
CVr (%)		**11.01**	**4.48**	**3.20**		**5.89**		**1.30**		**5.57**	**3.03**	**1.72**		**4.09**	**2.54**	**1.73**
CV_Rw (%)		**11.01**	**5.73**	**5.67**		**11.39**		**8.18**		**11.47**	**4.16**	**3.65**		**4.41**	**2.54**	**4.19**
recovery (%)		64	65	66		68		69		93	93	94		99	96	98
						dye: sudan IV (520 nm)										
day 1	3		0.51	1.31	2		0.44	1.10	3	2.33	4.71	10.02	3	2.51	5.03	10.72
day 2	3		0.47	1.44	6		0.57	1.40	3	2.33	4.45	9.11	3	2.23	4.55	9.03
day 3	3		0.54	1.37	2		0.60	1.52	3	2.08	4.23	8.80	3	2.31	4.72	9.42
mean			0.51	1.37			0.55	1.36		2.25	4.46	9.31		2.35	4.77	9.73
SD			0.04	0.08			0.06	0.15		0.17	0.22	0.59		0.16	0.24	0.80
CVr (%)			5.31	3.75			3.23	2.97		6.02	1.88	2.62		4.48	2.91	2.83
CV_Rw (%)			8.39	5.93			14.36	14.03		8.11	5.64	7.11		7.16	5.65	9.35
recovery (%)			51	55			55	55		90	89	93		94	95	97
CV_{1/2Horwitz} (%)		**8.88**	**8.00**	**6.97**		**8.88**	**8.00**	**6.97**		**6.97**	**6.28**	**5.66**		**6.97**	**6.28**	**5.66**
CV_{2/3Horwitz} (%)		**11.72**	**10.56**	**9.20**		**11.72**	**10.56**	**9.20**		**9.20**	**8.29**	**7.47**		**9.20**	**8.29**	**7.47**
CV_Horwitz (%)		**17.76**	**16.00**	**13.94**		**17.76**	**16.00**	**13.94**		**13.94**	**12.56**	**11.31**		**13.94**	**12.56**	**11.31**

the 3:1 or 6:1 signal-to-noise ratio, LODs and LOQs are determined based on values established with fortified samples. Identification criteria are satisfied for each sudan dye at the LOD value. Chromatograms of a chili sauce sample spiked at a level near the LOQ (0.5 mg/kg for sudans I−III; 1 mg/kg for sudan IV) and of a curry powdered spice matrix-matched standard at a level near the LOD (2 mg/kg for the four dyes) are, respectively, presented in **Figures 3** and **4**.

Recovery and Precision. Validation data about the recovery and the precision of the method are presented in **Table 4**. The overall recovery of spiked blank samples is between 51 and 86% in chili sauces, 55 and 85% in curry sauces, 89 and 100% in chili spices, and 95 and 100% in curry spices depending on the dye (sudans I, II, III, or IV). Recovery correction will be systematically applied. When considering each matrix indepen-

dently (sauces or spices), no significant difference is observed between recoveries measured in chili- and curry-containing foodstuffs.

The method has a good precision since the CVs are within the tolerances set by the Horwitz equation (*17*) for the two matrices: the repeatability CVs (CV_r) are mostly below 1/2 CV_Horwitz while most of the within-lab reproducibility CVs (CV_Rw) are below 2/3 CV_Horwitz.

According to the validation data, no significant difference is shown between the mean recovery, CV_r, and CV_Rw calculated at 478 and 496 nm for sudan I in curry-based spices. In conclusion, the absorption wavelength of 496 nm is chosen for the detection and quantification of sudan I in this matrix.

It can be concluded that the presented method allows an easy and fast determination of four illegal dyes in chili and curry

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foodstuffs. The validation data demonstrate that the method has a good overall recovery, an excellent precision, and low LODs and LOQs.

In the framework of the Belgian monitoring program, this method was successfully tested on over 150 chili and curry products and allowed to highlight the noncompliant samples. In chili-containing sauces and spices, sudan I was the main dye detected. Sudan IV was only found in palm oil coming from Africa while sudan II and sudan III have not yet been detected. None of the four dyes were found in curry-based food products. As the use of nonpermitted dyes in ready-to-eat food is more and more stated, the next purpose of the study will be to extend this method to other illegal dyes of interest like para-red, butter yellow, or rhodamine B.

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