JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Low-Level Detections of Sudan I, II, III and IV in Spices and Chili-Containing Foodstuffs Using UPLC-ESI-MS/MS

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ABSTRACT: Sudan dyes are red, synthetic azo dyes that are not allowed in foodstuffs in the European Union (Council Directive 94/36/EC). However, subppm levels of Sudan dye in spices are regularly reported, and it is assumed that these appearances are due to cross-contamination. In this paper, we present a newly developed fast and sensitive method for the quantification of Sudan I, II, III, and IV, using liquid−liquid extraction and UPLC-MS/MS analysis, and giving quantification limits ranging from 2.5 to 200 μg/kg. The method was applied to 21 samples, and 17 of them contained Sudan dye at low concentrations (3.3−8 709 μg/kg). Interestingly, it was observed that the distribution of Sudan dye in the sample is not homogeneous, which may lead to false negatives or to overestimations of the concentration, and that the pretreatment (blending or not) of the sample seriously influences the final result of the analysis.

KEYWORDS: *Sudan dyes, spices, LC-MS/MS, fast-peaks, nonhomogeneous distribution*

INTRODUCTION

Sudan dyes are synthetic fat-soluble azo-compounds, characterized by chromophoric azo groups (—N═N—).[1] The chemical structures of Sudan I−IV are given in Figure 1.

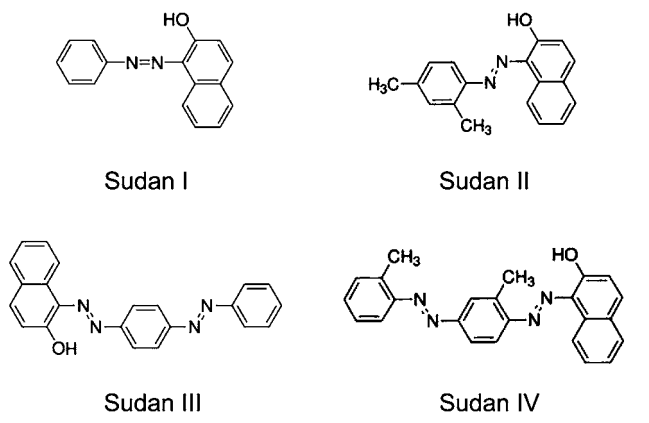

Figure 1. Chemical structures of Sudan I−IV.

They are of intensive red color and are abundantly used by industry for coloring waxes, oils, petrol, solvents, and polishes.[2,3] Because of this intensive red tint, Sudan dyes have also been used fraudulently for enhancing the color of various spices and foodstuffs, like chili powder, curry, or chili sauces.[4,5] However, the breakage of diazo bonds leads to the formation of active aromatic amines that can form DNA adducts entailing mutations.[6] Sudan I for instance has been found to produce tumors in the liver of mice, Sudan II increases the incidence of bladder carcinomas, and Sudan IV enhances the risk of formation of local sarcomas.[7] Additionally, the European Food Safety Authority (EFSA) considers Sudan dyes as suspected to be genotoxic and carcinogenic, and they have been declared suspected carcinogens and classified group 3 compounds by the International Agency for Research on Cancer.[7] Therefore, Sudan dyes are not allowed as food additives in the European Union by the Council Directive 94/36/EC. Despite of this prohibition the RASFF (Rapid Alert System for Food and Feed) portal[8] showed, for the year 2011, 18 entries with detections of Sudan dye (mainly Sudan I and IV) in different foodstuffs. Of those detections, only five measured between 27.3 and 631 mg/kg, the other samples showing only low-level ranges of 0.1 to 3.8 mg/kg. Interestingly, levels of several 100−1000 mg/kg of Sudan I are needed to enhance the color of foodstuffs.[9] For this reason, it seems likely that Sudan dyes detected result from cross-contaminations during the extraction of the spices from the plant or during the transport and the storage of the spice,[9] rather than from intentional adulterations. In order to differentiate between adulterations and cross-contaminations, the European Union fixed an action limit of 0.5 mg/kg for Sudan dyes in foodstuff (Commission Directive 2006/33/EC). Nevertheless, Sudan dyes remain suspected carcinogens, and detection of sub-ppm Sudan concentrations is necessary in order to take measures allowing the industry to further reduce the levels of Sudan dyes in foodstuffs.

Several methods for the analysis of Sudan I−IV in various foodstuffs have been published in the last years, mainly based on LC-DAD (diode array detection) or LC-MS/MS[3]. Recently, in order to further decrease the limits of detection (LDs) and increase the specificity of the analyses, more innovative methods were published including partial filling micellar electrokinetic chromatography (MEKC),[10] solid-phase extraction (SPE) using single-hole hollow molecularly imprinted polymers,[11] SPE using ionic liquid modified polymeric microspheres,[12] and enzyme linked immunosorbent assay (ELISA) with newly developed polyclonal antibodies.[13] These newly published methods are very promising and give excellent

Received: January 10, 2013
Revised: February 8, 2013
Accepted: February 8, 2013
Published: February 8, 2013

detection limits, except for the method using MEKC where the LDs of Sudan I–IV were quite high with values ranging from 1.14 to 1.42 mg/kg, though they use in-house synthesized polymers or antibodies that are not yet commercialized and thus the methods are not ready for being used in routine conditions. Nonetheless, the achievements of the recent studies allowed reducing the detection limits to some ppbs,[1,2] but that gain in sensitivity raised new concerns, like the phenomenon of the "fast-peaks" first mentioned in 2007 by Mölder et al.[14] In fact, due to photochromic E–Z isomerism, Sudan III and Sudan IV can produce two peaks on the chromatograms with different retention times, which may lead to underestimations of the concentration in case the "fast-peak" is missed or it can lead to false positive detection of Sudan I or II when analyzing with LC-DAD as the "fast-peaks" of Sudan III and IV have retention times similar to Sudan I and II.[3] This phenomenon can also be identified in chromatograms published in other studies[15−18] though it is expressly mentioned in only one of them where the "fast-peak" is characterized as a minor metabolite of Sudan.[16]

The aim of the present study was to develop a sensitive, robust, and fast method for the analysis of low-level concentrations of Sudan I–IV that will be employable for routine applications where high sample throughput is required without affecting the accurateness and the sensitivity of the method. The analyses were performed with ultraperformance liquid chromatography (UPLC), coupled to tandem mass spectrometry operated in positive electrospray mode (ESI-MS/MS), as this technique gives the best detection limits of papers published to date[1,3] and allows Sudan to be detected at low ppb levels. This detection mode also allowed sample preparation to be reduced to the strict minimum what resulted in a significant gain of time and a significantly increased sample throughput. In samples with large amounts of natural pigments and lipids like spices or chili sauces, a significant matrix effect will occur through ion suppression or ion enhancement. In previous studies, the matrix effect was bypassed with matrix-matched calibrations[19] or by spiking the samples with stable isotope labeled internal standards.[20] The two approaches have been evaluated in the present study in order to adapt the method to various matrix conditions and to be able to analyze many different matrixes in one single run. Furthermore, the risk of underestimation of the concentration through "fast-peaks" has been assessed by investigating how much it influences the accuracy of the results, and how this phenomenon could be avoided in routine conditions. The developed method was fully validated and applied to 21 spices and chili foodstuff samples.

EXPERIMENTAL SECTION

Chemicals. Sudan I (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol) with a purity of 97% was purchased from Sigma-Aldrich (Bornem, Belgium), Sudan II (1-(phenylazo)-2-naphthol) with a purity of >99% was purchased from Acros Organics (Geel, Belgium), Sudan III (1-(4-phenylazophenylazo)-2-naphthol) with a purity of >99% was purchased from Merck (Overijse, Belgium), and Sudan IV (*o*-tolyazo-*o*-tolylazo-beta-naphthol) with a purity of >99% was purchased from Sigma-Aldrich (Bornem, Belgium). Sudan I-d_4 and Sudan IV-d_6, used as internal standards (istd), were at purity of 99.5% and purchased from Fluka (Bornem, Belgium). Acetonitrile of HPLC-grade, used for the extraction of the dyes from the spices and foodstuffs, and acetonitrile and water of ULC-grade, used for the preparation of the mobile phases in LC-MS/MS analyses, were purchased from Biosolve (Paris, France). Chloroform of analytical grade, used for the preparation of stock solutions of Sudan III and IV, was purchased from Merck (Overijse, Belgium). Formic acid of HPLC-grade, used for peak sharpening, was purchased from Merck (Overijse, Belgium).

Stock solutions of Sudan I, Sudan II, and Sudan I-d_4 at 500 mg/L were prepared in acetonitrile, and stock solutions of Sudan III, Sudan IV, and Sudan IV-d_6 at 500 mg/L were prepared in chloroform:acetonitrile 10:90. Working solutions containing Sudan I–IV at 50 mg/L and 5 mg/L were prepared weekly in acetonitrile and stored at 4 °C in the dark. A working solution containing Sudan I-d_4 and Sudan IV-d_6 at 10 mg/L was prepared monthly.

N_2 (desolvation gas) was produced in-house by a nitrogen generator (NitroFlow Lab, Parker, Richland, MI, USA) at a purity of 97%, and argon (cone and collision gas) at a purity of 99.9999% was purchased from Air Liquide (Luxembourg).

Samples. The samples analyzed in this study were spices and foodstuffs known for their risk of containing Sudan dyes,[3] e.g., curry (3 samples), curry paste (2 samples), turmeric powder (2 samples), paprika powder (4 samples), chili powder (3 samples), chili flakes (2 samples), and chili sauce (4 samples). All samples were purchased on the local market.

Sample Preparation. A 1 g (chili powder and chili flakes), 5 g (curry, turmeric powder, and paprika), or 20 g (curry paste and chili sauce) aliquot of the sample was weighed into a 100 mL volumetric flask, and the flask filled with acetonitrile. Samples were stirred with a stir bar for 1 h. Then, the mixtures were filtered on cellulose filter papers (Whatman #597 1/2, Dassel, Germany) and on 0.45 μm PTFE-syringe filters (VWR, Leuven, Belgium). A total of 1 mL of the filtrate was introduced into an injection vial, and 10 μL of the mix of the internal standards [Sudan I-d_4 and Sudan IV-d_6] at 10 mg/L were added. Then, 10 μL of this solution were injected into LC-MS/MS for analysis. All samples were analyzed in duplicate (two aliquots of the same sample individually extracted and injected).

LC-MS/MS Analysis. All analyses were performed on an Acquity TQD UPLC-MS/MS system (Waters, Milford, MA, USA). The chromatographic separation of the four Sudan dyes was done on an Acquity UPLC BEH C18 column (particle size: 1.7 μm; column size: 2.1 × 100 mm; Waters, Dublin, Ireland), using acetonitrile with 0.1% formic acid (phase A) and water with 0.1% formic acid (phase B) as mobile phases. The flow rate was set at 0.4 mL/min and the gradient was as follows: Linear gradient from 60% A to 100% A in 5 min, isocratic elution at 100% A for 2 min, followed by a return to the initial conditions in 1 min. Total runtime was 8 min. The column temperature was set at 30 °C and the sample temperature at 10 °C.

For MS/MS detection, the source temperature was set at 100 °C and the desolvation temperature at 450 °C. The desolvation gas (N_2) temperature was set at a flow rate of 300 L/h and the cone gas (Ar) at a flow rate of 100 L/h. The electrospray voltage was set at 3 kV. MRM (multiple reaction monitoring) transitions, cone voltages and collision energies are given in Table 1. The dwell time was set at 0.05 s for all compounds.

Validation. The validation process carried out in this study is based on the guidelines of the International Union of Pure and Applied Chemistry.[21]

Linearity, Specificity, and Matrix-Effect. Blank samples (samples with amounts of Sudan below LD) of chili powder, curry, and chili sauce were spiked with different amounts of Sudan dye (0, 2, 5, 20, 50, 200, and 500 μg/L in the final extract) and analyzed as described above. Quantification of the peaks was done by integrating the area of the peaks and dividing it by the area of the peak of Sudan I-d_4 (for Sudan I and II) or the peak of Sudan IV-d_6 (for Sudan III and IV). In order to assess the specificity of the method, the absence of parasite peaks on the chromatograms was verified (in quintuple: five aliquots of the same sample individually extracted and injected). Linearity was checked by calculating the correlation coefficient (r^2), and the matrix-effect was investigated by comparing the slopes of the matrix-matched calibration curves with the slope of a nonmatrix-matched calibration curve.

RSD, Accuracy, and Recovery. Blank samples of chili powder, curry, and chili sauce were spiked with 200 μg/kg of each Sudan dye, extracted, and analyzed as described above. The quantification of the

Table 1. MRM Parameters of the Analysis and Whether the Transition Was Used for Quantification (Q) or Qualification (q)

compound	parent ion (m/z)	product ion (m/z)	cone (V)	collision energy (V)	
Sudan I	249.0	93.0	25	22	Q
	249.0	156.0	25	16	q
Sudan II	277.0	121.0	25	11	Q
	277.0	156.0	25	15	q
Sudan III	353.0	120.0	35	23	Q
	353.0	156.0	35	21	q
Sudan IV	381.5	91.0	30	30	Q
	381.5	156.0	30	20	q
Sudan I-d_4	254.0	237.0	20	25	Q
	253.0	160.0	25	16	q
Sudan I-d_6	387.5	224.0	20	37	Q
	387.5	162.0	30	20	q

compounds was done on the basis of peak areas normalized with the areas of the respective internal standard, and comparison with a calibration curve. Tests were carried out in quintuple (five aliquots of the same sample individually extracted and injected). RSD was calculated by dividing the standard deviation of the five measures by the arithmetic mean of the values. Accuracy was determined by measuring the degree of closeness of the measured concentration to the spiked concentration, and recovery was calculated by dividing the measured amount of Sudan dye by the amount of spiking.

LD and LQ. The limit of detection was defined as being the lowest concentration where both the quantifying and the qualifying transition presented a signal-to-noise ratio of 3. The limit of quantification was defined as being the lowest concentration where both the quantifying and the qualifying transition presented a signal-to-noise ratio of 10, and where the RSD was below 25%. The LD and LQ were defined by spiking blank samples of Chili powder, Curry and Chili sauce with 1, 0.5, 0.1, 0.05, and 0.01 μg/L in the final extract. Tests were carried out in quintuple (five aliquots of the same sample individually extracted and injected).

RESULTS AND DISCUSSION

Validation. All validation data are given in Table 2.

Linearity, Specificity, and Matrix Effect. Calibration was done from 2 to 500 μg/L in the final extract. Correlation coefficients (r^2) are higher than 0.99 for all dyes and for all matrixes, showing the linearity of the method over the entire calibration range. The chromatograms of the blank samples showed a tiny peak for Sudan III in the chili and curry matrix, probably resulting from a slight carry-over from highly concentrated samples injected before the blank samples, but those peaks were more than ten times below the LQs of Sudan III in the considered matrixes and could not be quantified. This shows that the method is specific and should not cause false positive results (when the LQ is considered as cutoff concentration).

The slopes of the calibration curves without istd-correction show a slight matrix effect for Sudan I and II and a serious matrix effect for Sudan III and IV (the shallowest slopes of the curry matrix-calibration being 2.5 and 4.3 times smaller than the steepest slopes of the calibration without matrix). The use of the two internal standards Sudan I-d_5 (for normalizing the areas of Sudan I and II) and Sudan IV-d_6 (for normalizing the areas of Sudan III and IV) allows reducing the difference between these slopes (factors 1.3, 1.4, 1.4, and 1.2 between the shallowest and the steepest slopes for the four compounds). This shows that the matrix effect can be suppressed with the use of two internal standards, and that the calibration curves can be realized by using spiked acetonitrile instead of the more time-consuming matrix calibrations (one calibration for each matrix) or standard additions. Thus, the developed method allows analyzing very different matrixes (e.g., chili flakes, curry, and turmeric powder) in one single run with one single solvent calibration.

Table 2. Validation Data

		Sudan I	Sudan II	Sudan III	Sudan IV
linearity (r^2)	no matrix	0.9934	0.9952	0.9974	0.9933
	chili powder	0.9977	0.9995	0.9978	0.9995
	curry	0.9983	0.9999	0.9962	0.9981
	chili sauce	0.9993	0.9964	0.9960	0.9994
matrix effect (slope without istd-correction)	no matrix	5.66	4.15	4.34	0.91
	chili powder	5.28	4.13	3.60	0.76
	curry	6.56	4.93	1.75	0.21
	chili sauce	4.79	3.71	2.70	0.42
matrix effect (slope with istd-correction)	no matrix	0.027	0.022	0.046	0.016
	chili powder	0.032	0.025	0.052	0.019
	curry	0.025	0.018	0.067	0.019
	chili sauce	0.028	0.022	0.063	0.018
RSD	chili powder	4%	3%	7%	7%
	curry	9%	8%	17%	6%
	chili sauce	4%	4%	19%	9%
accuracy	chili powder	97%	88%	93%	106%
	curry	94%	93%	104%	87%
	chili sauce	99%	98%	94%	100%
recovery	chili powder	99%	90%	105%	101%
	curry	97%	95%	89%	81%
	chili sauce	96%	90%	86%	98%
LQ (μg/kg)	chili powder	50	200	50	200
	curry	10	40	10	40
	chili sauce	2.5	10	2.5	10
LD (μg/kg)	chili powder	10	100	10	100
	curry	2	20	2	20
	chili sauce	0.5	5	0.5	5

RSD, Accuracy, and Recovery. The RSDs were all below 20% and thus confirm the repeatability of the developed method. The accuracy of the method is quite good with values ranging from 87% to 106%.

One aim of this study was to develop a fast and robust method that can be used in routine conditions. Therefore, the purification of the sample extract was limited to a minimum, and the RSD and accuracy values presented here show that the method is very accurate, sensitive, and repeatable. However, a current problem of such "quick and dirty" methods is that the column of the LC-part and the ion source of the MS/MS-part get dirty and need a lot of maintenance to ensure a constant sensitivity and accuracy of the instrument. In this study we repeated the RSD and accuracy tests after 250 injections of matrix-loaded samples, and the values were still satisfying: the RSD values were all below 20% and the accuracy values ranged from 91 to 103% (details not shown).

The recovery ranges from 81% to 105% indicate that no noteworthy losses occur during the extraction procedure.

LD and LQ. The detection limits ranged from 0.5 to 10 μg/kg for Sudan I and III and from 5 to 100 μg/kg for Sudan II and IV. These limits are within the limits described by other research groups that worked on the same matrixes, using LC-MS/MS[3] or HPLC-UV.[12] Better limits were only achieved by Stuart and Walker (2006), who used dichloromethane for the

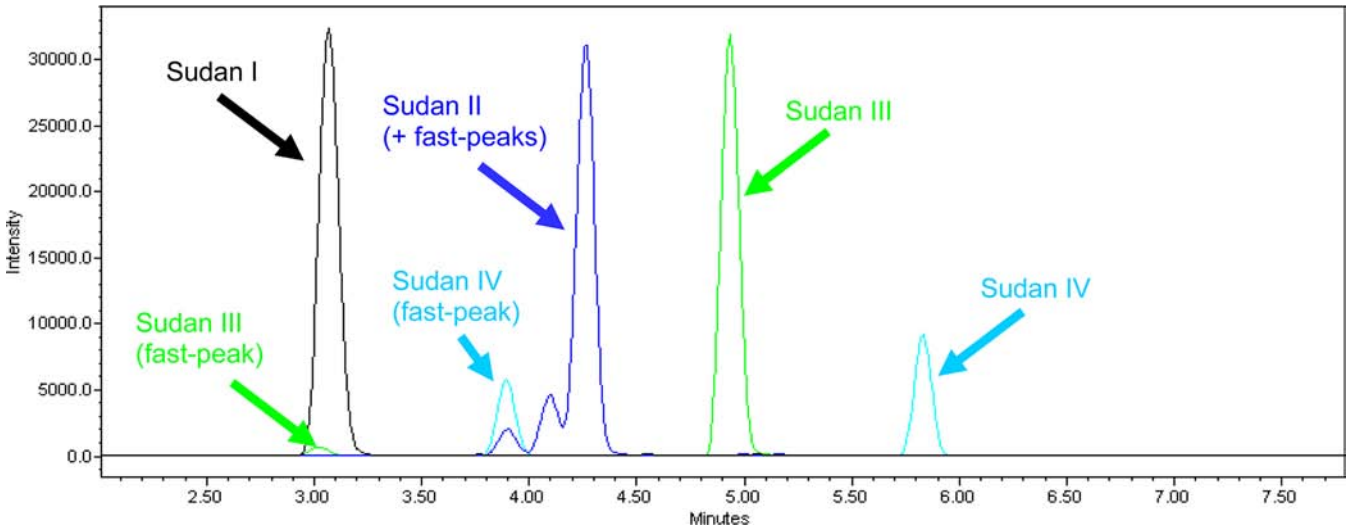

Figure 2. Chromatogram of Sudan I–IV at 50 μg/L, with "fast-peaks".

extraction but who did not give any details about the purification procedure (LDs ranging from 0.35 to 0.6 μg/kg), Liu et al. (LD of 0.003 μg/kg), who worked with flow injection and chemiluminescence determination,[22] Pardo et al. and Sun et al. (LDs ranging from 0.002 to 0.01 and from 0.5 to 1.8 μg/kg), who used gel permeation chromatography cleanup prior to LC-MS/MS analysis,[1,23] and Zhang et al. (LDs ranging from 0.01 to 5.0 μg/kg), who used SPE cleanup with in-house synthesized single-hole hollow molecularly imprinted polymers.[11] This indicates that a previous cleanup step as described in the cited studies might help to further decrease the LDs obtained in the present study, though the sample preparation would become much longer and the method would not be adapted to routine conditions any more, because high sample throughput is required in routine. Better LDs were also achieved by Qi et al., who used ELISA[13] (LDs ranging from 0.1 to 0.8 μg/kg). This method should allow high sample throughput, though it has two drawbacks, first the in-house synthesized polyclonal antibodies used for ELISA that are not yet commercialized, and second a low binding ability of the newly designed antibodies to Sudan II and IV.

The limits of quantification range from 2.5 to 50 μg/kg for Sudan I and III and from 10 to 200 μg/kg for Sudan II and IV. These values are largely sufficient to quantify the Sudan concentrations above the action limit of 500 μg/kg set in the Commission Directive 2006/33/EC and are adequate, too, to quantify the low concentrations of Sudan resulting from cross contaminations.

"Fast-Peaks". Mölder et al. described the appearance of an additional peak for Sudan III and Sudan IV on the chromatograms.[14] As these peaks eluted a few minutes before the main peak of the compounds, they were called "fast-peaks". This observation was confirmed in the present study, as Sudan III produced two peaks, a first peak at R_t = 3.02 min with weak intensity and a second peak at R_t = 4.93 min with strong intensity (average ratio of 0.03 between both peaks) (figure 2). Sudan IV also produced two peaks, a first peak at R_t = 3.84 min and a second peak at R_t = 5.78 min, though both peaks had similar intensities this time (average ratio of 0.7 between both peaks). These so-called "fast-peaks" of Sudan III and IV have only been investigated by Mölder et al.,[14] though they can also be found on chromatograms published by other authors.[15−18] This study confirms the "fast-peaks" of Sudan III and IV, though, for the first time, also showed the existence of "fast-peaks" for Sudan II that displayed two "fast-peaks" at R_t = 3.89 min and 4.09 min, with low intensities compared to the main peak at R_t = 4.26 min. (average ratios of 0.09 and 0.17). Mölder et al. observed that the "fast-peaks" could be prevented when the samples were wrapped in aluminum foil and stored in the dark for 3 to 4.5 h.[14] Therefore, the vials were wrapped in aluminum foil too, and stored in the dark even for 12 h, but the "fast-peaks" were still present, though with a small decrease of the ratio between the two peaks of Sudan IV (decrease to 0.54). Mölder et al. further proposed to perform the entire analytical part of the analysis in darkness, but this is impossible in routine conditions.

The nature of the "fast-peaks" of Sudan was thoroughly discussed in the paper of Mölder et al.,[14] and it will therefore not be repeated here. The point is that, as shown in the present study, the fast-peaks cannot be avoided in routine conditions, and this can affect the accurateness of the results in case the "fast-peaks" are missed or when the sensitivity of the method is too low to allow a reliable integration of the "fast-peaks". This would lead to underestimations of the concentrations. Moreover, because the intensities of both peaks vary with light irradiations and, as the results of this study suggest, with the matrix, the error would not be repeatable. Mölder et al. estimated the error to 10%, though for Sudan IV the error would be much higher, up to 35%, as the intensity of the "fast-peak" is much higher compared to the main peak. However, the results of the present study (see validation part) show that the "fast-peaks" do not affect the accurateness of the analysis when all the peaks of one compound are integrated together as the linearity was confirmed, and the RSD and accuracy are satisfying.

Sample Analysis. A total of 21 samples of various spices and foodstuffs were analyzed in duplicate and in 17 samples, at least 1 Sudan dye was detected (Table 3). Sudan III was by far the most abundant dye as it was detected in 16 samples, though at very low concentrations, except for sample 3A. Sudan I was detected only in turmeric powder, curry, and once in chili sauce (at a concentration < LQ). The highest concentration was measured in turmeric powder. Considering that curry is a mix of spices and turmeric powder is a constituent of its mix, it may

Table 3. Concentrations Measured in the 17 Samples Containing Sudan Dye (in μg/kg)[a]

	sample	Sudan I	Sudan II	Sudan III	Sudan IV
chili powder	1-A	<LD	<LD	67.3	<LD
	1-B	<LD	<LD	<LD	<LD
	2-A	<LD	<LD	52.4	<LD
	2-B	<LD	<LD	<LQ	<LD
	3-A	<LD	<LD	8709.3	<LD
	3-B	<LD	<LD	<LD	1785.6
paprika powder	4-A	<LD	<LD	17.2	<LD
	4-B	<LD	<LD	11.1	<LD
	5-A	<LD	<LD	42.0	<LD
	5-B	<LD	<LD	26.2	<LD
	6-A	<LD	<LD	13.2	<LD
	6-B	<LD	<LD	<LQ	<LD
	7-A	<LD	<LD	17.6	<LD
	7-B	<LD	<LD	22.1	<LD
turmeric powder	8-A	162.0	<LD	71.4	<LD
	8-B	15.1	<LD	17.0	<LD
curry powder	9-A	42.0	<LD	72.9	<LD
	9-B	<LD	<LD	63.6	<LD
	10-A	68.4	<LD	157.8	<LD
	10-B	<LQ	<LD	18.2	<LD
	11-A	47.7	<LD	55.0	<LD
	11-B	<LD	<LD	<LD	<LD
	12-A	<LD	<LD	<LD	110.5
	12-B	<LD	<LD	<LD	<LD
curry paste	13-A	18.0	<LD	38.6	<LD
	13-B	<LD	<LD	3.3	<LD
chili sauce	14-A	<LD	<LD	265.8	<LD
	14-B	<LQ	<LD	<LD	872.9
	15-A	<LD	<LD	11.2	<LD
	15-B	<LD	<LD	3.7	<LD
	16-A	<LD	<LD	21.0	<LD
	16-B	<LD	<LD	7.6	<LD
	17-A	<LD	<LD	<LD	<LD
	17-B	<LD	<LD	5.5	<LD

[a]LDs and LQs of the different compounds are given in Table 2.

be possible that Sudan I is bound more particularly to turmeric powder. Sudan II was never detected and Sudan IV only in three samples, at concentrations above 100 μg/kg. The detection limits for these two dyes are higher than for the two other compounds, which might be a reason for their low detection frequencies.

Two samples contained Sudan dye above the recommended action limit of 500 μg/kg: sample 3 (chili powder: 8709.3 μg/kg Sudan III and 1785.6 μg/kg Sudan IV) and sample 14 (chili sauce: 872.9 μg/kg Sudan IV). These values are far below the concentrations reported for spices where Sudan has been added fraudulently (340−630 mg/kg, see RASFF portal entries of 2011[8]), but the samples should not be sold on the European market anyway.

The RASFF portal lists only detections of Sudan I and IV for 2011. This is in line with the nondetection of Sudan II in the present study, though it is in contradiction with the fact that most detections in the present study were made for Sudan III. Such observations often suggest that contamination of the samples may have occurred in the laboratory. However, this is unlikely as only 16 out of 21 samples contained Sudan III whereas all samples should have been concerned by contamination as they were analyzed all together. Also, blanks were analyzed and no Sudan III was detected in any of them. In fact, Sudan III is the strongest coloring agent of all Sudan dyes and is used for tainting myelin (in the meat industry) and paraffin oils that serve as lubricants.[24] Therefore, it is completely possible that Sudan III is used in lubricants in the extraction plants and that its presence in the tested samples arises from cross-contaminations in the factories.

Another interesting observation is that duplicates of the samples sometimes show serious variations between the A and B samples (e.g., Sudan III sample 3-A: 8 709.3 μg/kg, 3-B: not detected; sample 10-A: 157.8 μg/kg, 10-B: 18.2 μg/kg). As samples A and B were always prepared simultaneously, it is very unlikely that a contamination of the samples may have occurred. Carry-over during the LC-MS/MS analysis is unlikely too, as, in this case, only samples A would have been contaminated (standards were always injected after a B-sample). This is not always true, e.g., for samples 3-B and 14-B that showed high concentrations of Sudan IV while the samples 3A and 14-A did not contain Sudan IV. Furthermore, the sample injected prior to 3-A (8709.3 μg/kg Sudan III) contained Sudan III below LQ and the sample following 3-A did not contain Sudan III. Thus, no carry-over occurred for the sample with the highest concentration, which makes the hypothesis of carry-over quite improbable.

Differences between sample A and B were also observed for Sudan I and IV, e.g., sample 8 (15.1 μg/kg Sudan I in sample A, 162.0 μg/kg Sudan I in sample B), sample 3 (1 785.6 μg/kg Sudan IV in sample B, no Sudan IV in sample A), and sample 14 (872.9 μg/kg Sudan IV in sample B, no Sudan IV in sample A). A possible explanation to these observations might be an inhomogeneous distribution of Sudan dyes in the samples. In fact, the samples were not homogenized prior to extraction because it was assumed that they were in fact homogeneous under both powder and paste form. As described by Hoenicke[9] and suggested above, cross-contaminations of spices with Sudan dye can occur from red fibers of the transportation bags on the fields or in the factory, inks on the transportation bags or red colored lubricants of the devices used during the plant extraction process, and these contaminations could be restricted to local hotspots in the packed, commercialized spice. In order to investigate this hypothesis, samples 3, 8, and 14 were blended and reanalyzed, and after this treatment, no Sudan dye was detected any more. Probably the local hotspots were diluted in the entire sample through the blending operation and concentrations fell below the limit of detection. This suggests that Sudan dye is inhomogeneously distributed in spices and foodstuffs, and therefore, in order to obtain reproducible results, samples must be thoroughly homogenized prior to extraction.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the "Sécurité Alimentaire" of Luxembourg for providing the samples.

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