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Assay of the Set of All Sudan Azodye (I, II, III, IV, and Para-Red) Contaminating Agents by Liquid Chromatography−Tandem Mass Spectrometry and Isotope Dilution Methodology

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A high-throughput mass spectrometric method is presented for the simultaneous detection of Sudan I, II, III, IV, and Para-Red azodyes in foodstuff. The method is based on the use of deuterium-labeled internal standards and atmospheric pressure chemical ionization (APCI) in a triple-quadrupole instrument. The gas-phase breakdown pattern of each labeled and unlabeled analogue displays the naphthoic moiety as a common fragment. The search for the parents of this common species (parent ion scans) allows, by flow injection and in a single run, the evaluation of the presence of each polluting species spiked in typical foodstuffs. A detailed assay of each azodye was performed by LC-APCI and isotope dilution method, through the multiple reaction monitoring approach, using deuterium-labeled internal standards. Sudan dyes can be quantified above the threshold of 10 ppb except for Sudan Para-Red, for which the limit of quantification was 20 ppb, likely due to the different ionization efficiency.

KEYWORDS: Mass spectrometry; multiple reaction monitoring (MRM); isotope dilution; Sudan azodyes

INTRODUCTION

The development of protocols for the identification of the set of Sudan azodyes illicitly added to foodstuffs has a twofold goal: the fast identification of the analytes to perform high-throughput safety controls and the setup of sensitive and reliable approaches for their assay, at very low level, in various edible matrixes.

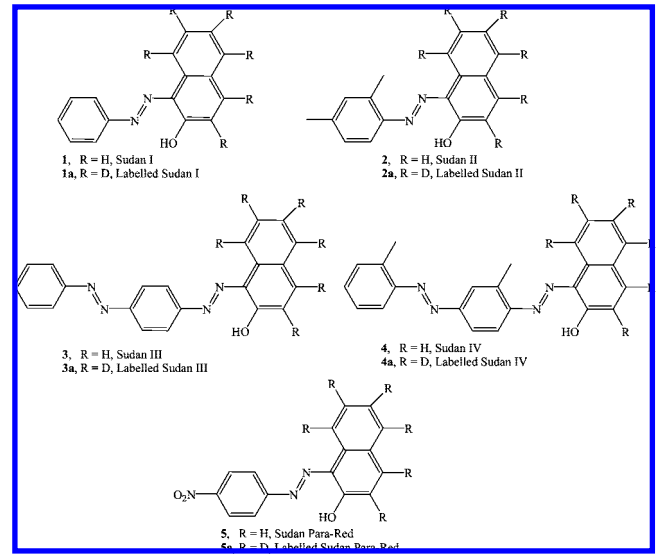
Accordingly, we propose a direct MS/MS parent ion scan approach on flow injected samples, for a preliminary check of the presence of the polluting species, and an isotope dilution method for the simultaneous quantification of Sudan I, II, III, IV, and Para-Red by multiple reaction monitoring (MRM), in the MS/MS mode, based on the use of suitable deuterium-labeled internal standards. The latter has already been applied with success to the assay of Sudan I (*1*); meanwhile, other classical analytical methods, mostly based on MS analysis, have been proposed (*2*–*4*).

The risks of human exposure to azodyes are widely documented; they include, among others, the development of liver carcinoma (*5*) as a consequence of the splitting of the azo function into dangerous aromatic amines (*6*). It has been, quite recently, reported that a similar action can be performed by skin bacteria enzymes (*7*); hence, the risk is extended to people undergoing tattooing (*8*). The widespread use of azodyes **1**−**5** in foods has prompted the European Union to issue a number of directives forbidding their use (*9*).

None of the directives issue any safety threshold; therefore, the use of labeled internal standards coupled with an MS/MS parent ion scan on a common ionic species from the fragmentation of each individual analyte could be enough to assess the safety of the analyzed item at the sensitivity of the method.

On the other hand, an accurate evaluation of the absolute amount of each analyte, easily achievable by the isotope dilution

Chart 1



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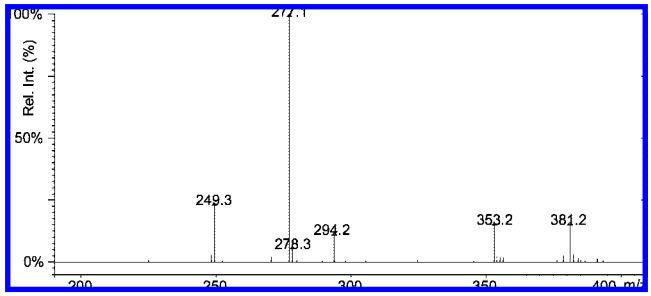

Figure 1. Parent ion scan MS/MS spectrum of the fragment *m*/*z* 156 present in the APCI spectra of **1**−**5** azodyes extracted from baked products and sampled by flow injection.

Table 1. MRM Experiments: Transitions Monitored[a]

	analyte	monitored transition
1	Sudan I (**1**)	***m/z* 249 → *m/z* 93** *m/z* 249 → *m/z* 156
2	d_6-Sudan I (**1a**)	***m/z* 255 → *m/z* 93** *m/z* 255 → *m/z* 162
3	Sudan II (**2**)	***m/z* 277 → *m/z* 156** *m/z* 277 → *m/z* 121
4	d_6-Sudan II (**2a**)	***m/z* 283 → *m/z* 162** *m/z* 283 → *m/z* 121
5	Sudan III (**3**)	***m/z* 353 → *m/z* 77** *m/z* 353 → *m/z* 120
6	d_6-Sudan III (**3a**)	***m/z* 359 → *m/z* 77** *m/z* 359 → *m/z* 120
7	Sudan IV (**4**)	***m/z* 381 → *m/z* 91** *m/z* 381 → *m/z* 156
8	d_6-Sudan IV (**4a**)	***m/z* 387 → *m/z* 91** *m/z* 387 → *m/z* 162
9	Sudan Para-Red (**5**)	***m/z* 294 → *m/z*156** *m/z* 294 → *m/z* 277
10	d_6-Sudan Para-Red (**5a**)	***m/z* 300 → *m/z* 162** *m/z* 300 →*m/z* 283

[a] Analyte identification is provided by the two transitions listed in the third column obtained from the product ion scan spectra. Quantitative assay is based on the relative intensity of the transitions indicated in bold. In all cases, except for Sudan II (row 3), the selected process corresponds to the formation of the most abundant daughter ion.

method, provides clues to assess the risks of human exposure to contaminated foods.

EXPERIMENTAL PROCEDURES

Chemicals. Sudan I, II, III, IV, and Para-Red (**1**−**5**, **Chart 1**) and solvents were commercially obtained (Sigma-Aldrich, St. Louis, MO). The deuterated Sudan (**1a**−**5a**, Chart 1) molecules were obtained by modification of an existing literature method (*10*).

Sample Preparation. To 1 g of milled food (powdered chili pepper, tomato sauce, sausage, baked products) were added selected amounts of **1**−**5** and/or 50 ng of **1a**−**5a**, and the mixtures were homogenized. Twenty milliliters of acetone was added and mixed for 5 min by vortex apparatus. The solution was filtered and evaporated to dryness under reduced pressure; the residue was dissolved in 5 mL of *n*-hexane/ethyl acetate, 95:5 v/v, loaded into a silica Sep-Pak cartridge (Waters, Milford, MA) (*11*), and eluted with 10 mL of the same *n*-hexane/ethyl acetate solvent mixture; the eluate, evaporated to dryness, afforded a residue, which was dissolved in 1 mL of acetonitrile/acetone, 50:50 v/v, and analyzed by LC-MS.

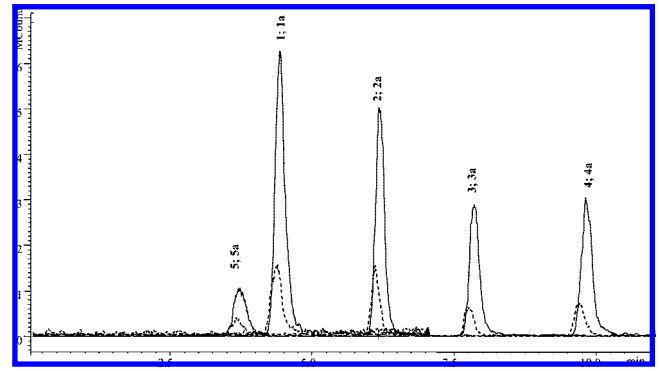

Figure 2. MRM chromatogram of standard solution at 200 ppb of **1**−**5** and 50 ppb **1a**−**5a** (dotted line).

Table 2. Calibration Curve Parameters of the Standard Solutions of **1**−**5**

analyte	equation	correlation factor, R^2
Sudan I (**1**)	$y = 0.0188x + 0.0805$	0.9958
Sudan II (**2**)	$y = 0.0178x + 0.1172$	0.9983
Sudan III (**3**)	$y = 0.0209x + 0.1164$	0.9941
Sudan IV (**4**)	$y = 0.0211x + 0.1399$	0.9966
Sudan Para-Red (**5**)	$y = 0.0255x + 0.0820$	0.9969

Mass Spectrometry. The LC-MS analysis was carried out with a triple-quadrupole mass spectrometer LC 1200 (Varian), equipped with an APCI source interfaced with an HPLC Prostar 210 (Varian Inc., Palo Alto, CA). The chromatographic analysis was performed with a C_{18} column, 5.0 cm × 2.0 mm (Pursuit, Varian Inc.). The flow rate was fixed at 0.25 mL min^{-1} using the following eluents and linear gradient: solvent A (H_2O, 0.1% formic acid), solvent B (CH_3CN); from 60% B to 98% B in 10 min; 2 min at 98% B isocratic; from 98% B to 40% A in 3 min. The corona needle current was fixed at 7.5 μA, the drying gas (N_2) at 12 psi and 200 °C, the nebulizing gas (N_2) at 60 psi and 450 °C, the auxiliary gas (N_2) at 17 psi, the housing temperature at 50 °C, and the electron multiplier at 1350 V. The scan time was 0.25 s/scan, and the resolution was set using a mass peak width of 0.9. The collision gas pressure (Ar) was fixed at 2 mTorr, and the collision energy was −30 V for **1**, **1a**, **2**, **2a**, **5**, and **5a** and −15 V for **3**, **3a**, **4**, and **4a**. The overall MS experiment was composed of two segments: the first from 0 to 7.5 min for the analysis of **1**–**1a**, **2**–**2a**, and **5**–**5a** and the second from 7.5 to 12.0 min for the analysis of **3**–**3a** and **4**–**4a**.

High-resolution electrospray ionization (ESI) experiments were carried out in a hybrid Q-Star Pulsar-i (MSD Sciex Applied Biosystem, Toronto, Canada) mass spectrometer equipped with an ion spray ionization source. Samples were introduced by direct infusion (3 μL/min) of the sample containing the analyte (5 ppm), dissolved in a solution of 0.1% acetic acid, acetonitrile/water 50:50 at the optimum ion spray (IS) voltage of 4800 V. The source nitrogen (GS1) and the curtain gas (CUR) flows were set at pressures of 20 and 25 psi, respectively, whereas the first declustering potential (DP1), the focusing potential (FP), and the second declustering potential (DP2) were kept at 50, 220, and 10 V relative to ground, respectively.

Analytical Parameters. The limit of detection (LOD) and the limit of quantitation (LOQ) for each foodstuff were calculated by applying the equations (eqs 1 and 2), following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry.

$$S_{LOD} = S_{RB} + 3\sigma_{RB} \quad (1)$$

$$S_{LOQ} = S_{RB} + 10\sigma_{RB} \quad (2)$$

S_{LOD} is the signal at the limit of detection, S_{LOQ} is the signal at the limit of quantitation, S_{RB} is the signal of the uncontaminated food matrixes, and σ_{RB} is the standard deviation for uncontaminated matrixes. The concentrations were calculated by the standard curve.

The recovery for each foodstuff was calculated from the area of the signal obtained by analyzing an uncontaminated product added to a

Table 3. Analytical Parameters for Assessing the Accuracy of the Method

Sudan I							
ppb	calcd concn	RSD % (av)	accuracy % (av)	ppb	calcd concn	RSD % (av)	accuracy % (av)
	Powdered Chili Pepper					Sausage	
40	42.9 ± 1.4	3.3	107.2	40	41.4 ± 1.6	4.1	103.5
300	308.8 ± 9.7	3.1	102.9	300	306.2 ± 7.4	2.4	102.1
	Tomato Sauce					Baked Products	
40	42.7 ± 3.5	8.1	107.0	40	39.1 ± 3.0	7.6	97.7
300	310.8 ± 17.3	5.5	103.6	300	297.9 ± 12.7	4.3	99.3

Sudan II							
ppb	calcd concn	RSD % (av)	accuracy % (av)	ppb	calcd concn	RSD % (av)	accuracy % (av)
	Powdered Chili Pepper					Sausage	
40	37.8 ± 1.7	4.5	94.7	40	41.4 ± 2.7	6.5	103.5
300	288.8 ± 13.0	4.5	96.2	300	303.7 ± 13.9	4.5	101.2
	Tomato Sauce					Baked Products	
40	42.5 ± 2.9	6.8	106.2	40	40.2 ± 3.8	9.6	100.5
300	318.0 ± 13.1	4.1	106.0	300	311.0 ± 13.5	4.3	103.6

Sudan III							
ppb	calcd concn	RSD % (av)	accuracy % (av)	ppb	calcd concn	RSD % (av)	accuracy % (av)
	Powdered Chili Pepper					Sausage	
40	39.60 ± 1.10	2.5	99.1	40	44.6 ± 2.5	5.8	111.6
300	285.8 ± 6.4	2.2	95.2	300	316.9 ± 26.3	8.3	105.6
	Tomato Sauce					Baked Products	
40	43.8 ± 3.3	7.5	109.6	40	40.2 ± 3.8	9.6	100.5
300	293.5 ± 6.4	2.1	97.8	300	311.0 ± 13.5	4.3	103.6

Sudan IV							
ppb	calcd concn	RSD % (av)	accuracy % (av)	ppb	calcd concn	RSD % (av)	accuracy % (av)
	Powdered Chili Pepper					Sausage	
40	40.0 ± 1.6	4.0	100.0	40	41.6 ± 4.3	10.3	104.0
300	307.2 ± 4.2	1.3	102.4	300	305.2 ± 14.4	4.7	101.7
	Tomato Sauce					Baked Products	
40	39.9 ± 2.6	6.6	99.9	40	44.1 ± 0.8	2.0	110.3
300	302.8 ± 6.0	1.9	100.9	300	297.9 ± 12.3	4.1	99.3

Sudan Para-Red							
ppb	calcd concn	RSD % (av)	accuracy % (av)	ppb	calcd concn	RSD % (av)	accuracy % (av)
	Powdered Chili Pepper					Sausage	
40	43.69 ± 1.90	4.3	109.2	40	46.40 ± 6.21	13.5	116.1
300	304.4 ± 20.5	6.7	101.4	300	349.50 ± 13.58	3.9	116.5
	Tomato Sauce					Baked Products	
40	46.7 ± 2.1	4.6	116.9	40	45.7 ± 2.9	6.3	114.2
300	303.6 ± 17.5	5.7	101.2	300	309.1 ± 14.5	4.6	103.0

known amount of **1**−**5**. The concentration was estimated by using an external calibration curve built from six standard solutions at 12.5, 25, 50, 100, 200, and 400 ppm.

RESULTS AND DISCUSSION

The use of stable isotopes, as internal standards, in MS assays of analytes in complex natural and biological matrices represents an extremely accurate method of quantitative chemical analysis. The technique of isotope dilution is being used to improve precision and accuracy by reducing the problems arising from calibration procedure, sample preparation, and matrix effects. A known exact quantity of labeled internal standard is added to the sample of unknown concentration. When mass spectrometry is used, the choice of the labeled reference compounds should take into account the possibility of gas-phase isotope isomerization, that is, the possibility that heavy and light atoms might randomize within the framework of the molecule, either before or concomitantly with the breakdown of a specific bond.

The **1a**−**5a** species, used as reference compounds in the present application, afford by high-resolution ESI-MS $[M + H]^+$ clusters containing 92−93% of d_6-labeled isomers. Their gas-phase chemistry allows a reliable use of the selected isotope dilution method.

The MS/MS spectra of reference compounds (**1a**−**5a**) are characterized by the formation of the aromatic amines and β-naphthol fragments, by analogy with the behavior of d_6-Sudan I (*1*). The origin of the other ionic species can be easily rationalized (*12, 13*).

The so-called precursor ion scan method was exploited to fulfill one of the goals of the present work, that is, the fast preliminary check for the presence of Sudan dyes in foodstuffs.

Table 4. Reproducibility[a] (RSD %) and Analytical Parameters of the Proposed Method

analyte	food matrix	LOD	LOQ	recovery	reproducibility (RSD %) 300 ppb	reproducibility (RSD %) 40 ppb
Sudan I	powdered chili pepper	2.8	4.5	93.4	1.3	6.6
	sausage	3.1	7.3	85.7	2.8	4.1
	baked product	3.4	7.8	87.7	1.9	6.6
	tomato sauce	3.8	9.6	50.4	6.0	4.7
Sudan II	powdered chili pepper	3.0	4.1	98.9	4.3	5.8
	sausage	4.1	6.3	79.9	1.4	4.4
	baked product	3.9	5.2	88.2	14.8	3.2
	tomato sauce	4.5	8.6	50.2	4.0	5.3
Sudan III	powdered chili pepper	3.2	6.2	76.6	3.1	6.3
	sausage	4.1	7.2	73.9	2.2	8.1
	baked product	4.2	6.1	74.0	2.1	4.9
	tomato sauce	5.8	9.5	49.2	1.9	7.6
Sudan IV	powdered chili pepper	3.2	5.4	69.7	0.6	3.5
	sausage	4.3	6.3	68.0	1.9	8.1
	baked product	4.0	5.6	77.0	17.8	7.1
	tomato sauce	6.3	8.2	48.5	1.4	6.6
Sudan Para-Red	powdered chili pepper	10.5	16.3	60.6	2.7	5.5
	sausage	14.2	18.0	65.2	4.4	5.8
	baked product	12.2	15.6	69.9	4.9	6.8
	tomato sauce	15.2	19.3	52.0	2.9	5.7

[a] The reproducibility of the measurements was obtained by extracting each sample three times over a period of 1 week.

This scanning mode, available in modern tandem mass spectrometers, was initially developed as a tool for identifying metastable transitions (*14*) and is now currently used to screen multiple analytes in complex mixtures (*15*). A typical experiment was represented by the identification of Sudan azodyes (**1**−**5**, **Chart 1**) spiked, at 1 ppm level, into commercially available baked products, such as cracker biscuits, by direct flow injection in the APCI-MS/MS mode. Accordingly, the common ionic species at *m*/*z* 156, corresponding to the β-naphthol fragment present in the tandem mass spectra of all the **1**−**5** analyzed dyes, was selected to perform the precursor ion scan measurement. All of the spiked analytes were identified as shown in the spectrum reported in **Figure 1**. It should be noted that no relationship exists between the relative intensity of the ionic species displayed by the spectra and the actual concentration of each analyte.

The detailed assay of the polluting species is better carried out by the MRM method, coupled with an online LC separation of the analytes. The reliability of the selected approach was proved by the specificity, good sensitivity, and good-to-excellent LOD and LOQ parameters experimentally determined.

The specificity of analyte identification was guaranteed by the concomitant determination of two transitions originating from the $[M + H]^+$ protonated molecular ion byproduct ion scan mode (**Table 1**). The selection of the species to be used in the quantitative determinations was based both on the relative intensity of the daughter ions and on the specificity of the examined transition. In all cases but Sudan II (**3**, **Table 1**), the most abundant transitions was selected, whereas in the case of compound **3** the choice was guided by the need to avoid possible interferences; hence, the *m*/*z* 277 → *m*/*z* 156 transition was considered.

In the presence of traces of contaminants, namely, in the parts per million to parts per billion range, a crucial step is represented by the cleanup of the analytes after the extraction from the matrixes, because the crude extracts of some foodstuffs show several interferences due probably to the presence of fats and other intrinsic components. The procedure can be conveniently carried out by means of commercial SPE silica gel cartridges, using *n*-hexane/ethyl acetate, 95:5 v/v, as eluant. In these conditions very short-time, that is, 12 min, chromatographic runs can be adopted, thus allowing fast investigations of large numbers of samples (**Figure 2**).

The calibration curve, built by sampling, three times, each of the six solutions at different concentrations, showed a good linearity (**Table 2**). The concentrations of the analytes in the standard solutions ranged from 12.5 to 400 ppb (12.5, 25, 50, 100, 200, 400 ppb), whereas those of the internal standards were fixed at 50 ppb, with the exception of Sudan Para-Red, for which the calibration curve was built using five solutions (25, 50, 100, 200, 400 ppb) because of its poor ionization efficiency with respect to the other Sudan analytes. In any case, the calibration curve gave excellent correlation (R^2) coefficients (**Table 2**). It should be noted that the Sudan Para-Red substrate is characterized by the presence of a powerful electron-withdrawing group placed in the para position of the benzene ring linked to the azo double bond, which is suggested as the protonation site of the analytes, to account for the observed gas-phase chemistry (*13*).

The developed methodology has been applied to different homemade food matrixes free of any possible contaminant. Sausages, powdered chili pepper, baked products, and tomato sauces were spiked with azodyes at two different concentrations: 40 and 300 ppb. These values well represent the matrix contaminations in the lower part and in the higher part of the calibration curve. It should be noted that all of the spiked matrixes have been processed in the same way, thus unifying the methodology when foods of different origin are submitted to a laboratory screening.

The precision of the applied methodology is evident from the data reported in **Table 3**. The RSD % values are in all cases under 14%. **Table 3** illustrates also the real amount of dyes found per each spiked matrix: when the latter results were compared with the amount spiked in the food, the values of accuracy for the two concentrations were in all cases between 94 and 117%. The above-found percentages for accuracy and

precision underscore the strength of the isotope dilution procedure, despite the fact that the amounts of contaminants are in the parts per billion range.

The good results obtained are confirmed by the low levels of LOQ; indeed, all Sudan dyes may be quantified above the threshold of 10 ppb except for Sudan Para-Red, for which the LOQ is 20 ppb, probably due to the scarce ionization efficiency above-mentioned (**Table 4**).

The recovery of the analytes is >68% for Sudan I−IV in all matrixes except for the tomato sauce, for which the recovery is around 50%. Sudan Para-Red shows recoveries near 60% for all matrixes (**Table 4**); this difference could be due to the strong interactions that this molecule could give with the silica gel of the cartridge. The sample preparation conditions have been chosen to provide the best instrumental response. **Table 4** shows in addition the values of the analytical parameters LOD and reproducibility, the latter calculated by extracting three times each foodstuff over a period of 1 week.

The RSD values are in almost all situations under 10%, confirming the goodness of the method; furthermore, the values of LOQ and LOD allow a determination of **1**−**5** in the lower parts per billion range.

The present method can confidently replace all other existing approaches for a simultaneous determination of the set of Sudan azodyes encountered as polluting species in foodstuffs and banned by the international board.

The results presented above show that the determination and quantification of the set of all Sudan azodyes, likely contaminating foodstuffs containing hot chili pepper derivatives and banned by the European Union, can be carried out by means of the classic tools of tandem mass spectrometry, that is, parent ion scan and multiple reaction monitoring with the isotope dilution method. The protocols devised for food safety control can be easily extended to other applications when the quantification of this type of analyte is required.

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