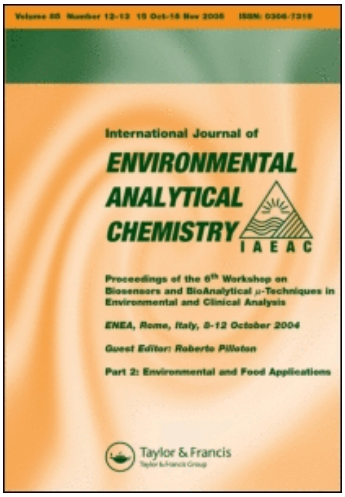

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Analysis of illegal dyes in food by LC/TOF-MS

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A sensitive and accurate methodology was developed for the analysis of seven illegal dyes (Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Orange G, Sudan R and Para Red,) used as additives in food products, such as chilli powder and steak sauces. The analytical methodology consisted of solvent extraction with acetonitrile followed by liquid chromatography time-of-flight mass spectrometry detection. Accurate mass measurements were crucial in order to achieve a high degree of specificity for the target analytes in such complex samples. The dyes were effectively extracted from spice and sauce matrices achieving recoveries higher than 75%. Because of the excellent mass accuracy obtained for the target analytes (better than 2 ppm), no cleanup of the samples was required using this methodology, thus leading to a better precision and reproducibility of the results from the quantitative point of view. Calibration curves were linear and covered two orders of magnitude (from 0.01 to 1 mg L^{-1}) for all the compounds studied with the exception of Para Red. A detailed study of matrix effects is also included in this work, showing a clear improvement when dilution of the extracts was carried out. Method detection limits were in the low mg kg^{-1} range, and the precision, calculated as the relative standard deviation, ranged from 5 to 15%. The methodology was successfully applied to market samples in a survey performed as part of a regional research programme organized by the Andalusian Health Service in Spain, and a positive confirmation for Sudan I was obtained in a chilli powder sample.

Keywords: Sudan dyes; Azo dyes; LC/TOF-MS; Food analysis

1. Introduction

Sudan dyes belong to the azo dye chemical class and are non-ionic fat-soluble dyes used in gasoline, diesel, oils, waxes, shoe and floor polishes, lubricating greases, and polymer-dye production. They have been extensively used in some countries as additives in food (i.e. chilli powder, paprika, steak sauces, etc.) as well. In the summer of 2003, Sudan I was first identified in France in a chilli product from India; later, the colouring was also discovered in other foodstuffs containing chilli. Similarly, 2 years ago, a food alarm was raised in the UK caused by the presence of Sudan dyes and Para Red in food. Soups, sauces, and processed foods that use contaminated chilli powder were found to be the main sources for Sudan dyes.

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Sudan I, Sudan II, Sudan III, and Sudan IV have been classified by the International Agency for Research on Cancer as Group 3 (because of their carcinogenicity to humans) [1]. Para Red is a chemical dye used in printing; it is not a permitted colour under the Colors in Food Regulations 1995, and its use in food is illegal. Sudan dyes are currently illegal and prohibited for use as food additives by the European Commission. In 2003, the decision 2003/460/EC was taken to control the unlawful use of the colouring substance Sudan I in chilli and chilli products. A second decision extending the scope of the measures to cover Sudan II, III, and IV was adopted on 21 January 2004 (December 2004/92/EC) and is still in force. There is now an emergency measure dictating that chilli and chilli products, including curry powder, can only enter an EU country provided there is proof that these illegal chemical dyes are not present. In addition, to date, no maximum residue levels (MRLs) have been established for these compounds in food. For all these reasons, accurate and reliable analytical methods for the determination of synthetic dyes in foodstuff are required for the assurance of consumer health.

Several analytical methods have been developed for the analysis of Sudan dyes. One of the most popular methods is based on liquid chromatography followed by spectrophotometric detection (LC-UV) because of the absorbance properties of azo dyes in the visible region of the spectrum [2–6]. In recent years, several liquid-chromatographic methods coupled with mass-spectrometric techniques have been developed as well for the analysis of Sudan dyes in spices and sauces. In this sense, Sudan I is the most studied dye in the literature, and several modern methods using mass-spectrometric techniques have been proposed for its analysis. For example, a method based on liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry has been reported in the literature for the successful determination of Sudan I in hot chilli, spices, and oven-baked products [7]. Another work using the same analytical technique was developed for the analysis of the same compound, in this case using isotope dilution [8]. However, there are only a few studies reporting the analysis of all four Sudan dyes by LC-MS techniques. One example is the work developed by Calbiani *et al.* [9], where triple quadrupole tandem mass spectrometry was used for the simultaneous unequivocal detection of Sudan I–IV in hot chilli products. The same group developed another method based on accurate mass measurements by LC-Q-TOF for the analysis of the same compounds [10], reporting low detection limits of $mg\,kg^{-1}$. More recently, an LC-MS method combined with UV detection has been applied to the determination of Sudan dyes and other colouring substances in several foodstuff such as soft drinks, ginger, and chilli products [11]. Other recent works have used MS-MS techniques such as ion-trap and desorption electrospray ionization methodologies (DESI-MS-MS) [12, 13].

One of the main drawbacks on the analysis of these compounds in food samples is the high complexity of spice and sauce matrices, where present. In this sense, difficulties for the identification of isobaric ions may occur when using low-resolution LC-MS techniques. This could be solved using tandem mass spectrometry techniques [9] with at least two transitions or using high-resolution techniques [10]. For this reason, we propose in this work the use of a high-resolution MS technique, such as time of flight, which helps to distinguish among interferences in the samples and increase confidence in the identification of the analytes under study by providing elemental compositions [14–19]. Accurate mass measurements greatly increase the confidence of identification because it inherently limits the possible number of candidate compounds and confirms

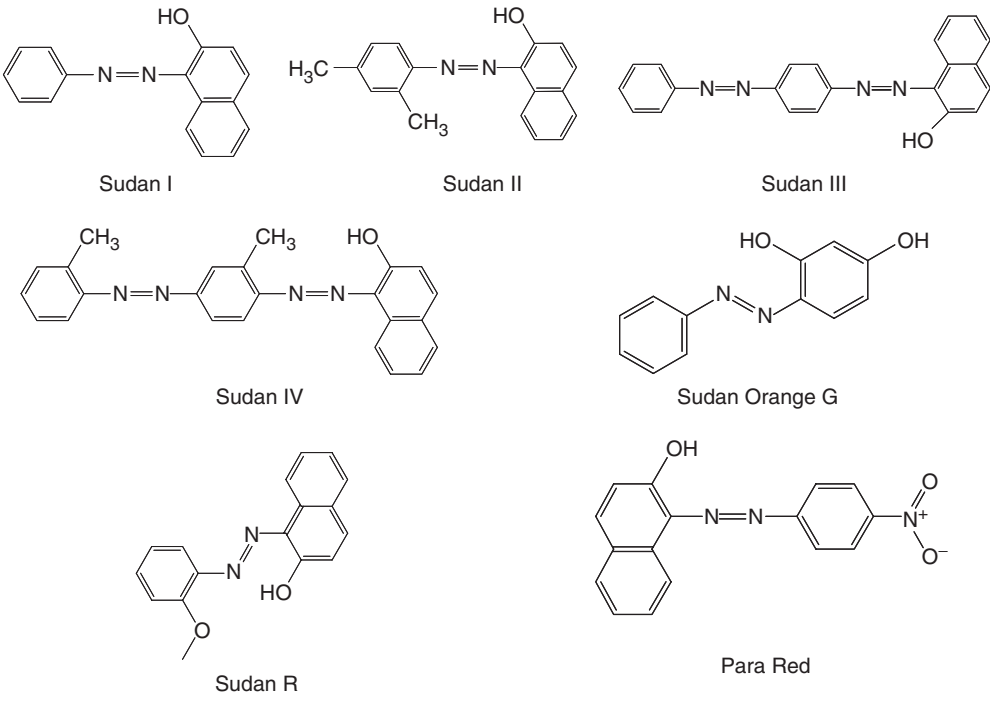

Figure 1. Chemical structures of the selected dyes.

the exact formula for target compounds [20]. This work provides an example of the power of LC/TOF-MS for screening, confirmation, and quantitation analysis of illegal Sudan dyes in food samples.

2. Experimental

2.1 *Chemicals and reagents*

Sudan I (1-(phenylazo)-2-naphthalenol), Sudan II (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol), Sudan IV (1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol), Sudan Orange G (4-phenylazoresorcinol), Sudan R (1-[(2-methoxyphenyl)azo]-2-naphthalenol), and Para Red (1-(4-nitrophenylazo)-2-naphthol) analytical standards were purchased from Institute of Dyes & Organic Products (Zgierz, Poland). Sudan III (1-[4-(phenylazo)phenylazo]-2-naphthol) was obtained from Dr. Ehrenstorfer (Ausburg, Germany). Chemical structures for the dyes studied in this work are shown in figure 1. Individual dye stock solutions (150–300 $\mu g\,mL^{-1}$) were prepared in acetonitrile or methanol and stored at $-18^{\circ}C$ in the dark. HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA) was used to obtain the HPLC-grade water used as mobile phase during the analysis.

2.2 *Sample treatment*

Chilli powder (several brands) and steak sauce (Worcestershire Sauce) commercial samples were purchased from a local supermarket. All of these were stored at ambient temperature before spiking and sample extraction. A previous blank analysis for each product sample was performed in order to ensure that they did not contain any Sudan dyes, and these samples were selected as blanks for spiking, calibration curves, and recovery purposes.

2.3 *Spiking procedure*

2.3.1 Solid samples. One gram of chilli powder was weighed in a watch glass and spread in such a way that it covered the maximum surface of the glass. Then, 1 mL of the standard dye mixture in acetonitrile of the desired concentration was carefully added, and the sample was homogenized. The mixture was left at room temperature until all the solvent evaporated and the sample was dry. The dry powder was then transferred to a 15 mL disposable screw-capped polypropylene tube for subsequent extraction with the organic solvent. The final spiking concentration levels ranged from 0.1 to 0.5 mg kg^{-1} in the chilli powder samples.

2.3.2 Liquid samples. Several millilitres of steak sauce were introduced into a 25 mL volumetric flask. A standard solution of a mixture of the dyes in acetonitrile of the desired concentration was added, and the flask was brought to the mark with the remaining of the steak sauce. The solution was sonicated in an ultrasonic bath to achieve sample homogeneity. Then, it was allowed to stand at room temperature for 1 h, before it was kept at $-18^{\circ}C$, until extraction. The spiking levels for the sauces ranged from 0.1 to 0.5 mg kg^{-1}.

2.4 *Extraction procedure for recovery studies*

In order to achieve satisfactory results, different extraction conditions were tested (type of extraction solvent, sample amount, solvent volume, and extraction times). The following conditions were found to be optimal for each of the matrices tested.

2.4.1 Solid samples. The extraction method for dyes in chilli powder samples was carried out as follows. A representative 1 g portion of solid sample previously homogenized was weighed in a 15 mL disposable screw-capped polypropylene tube, and 10 mL of acetonitrile added. Then, the soution was mixed in the vortex for 30 s and subsequently sonicated in a water bath for 20 min. Afterwards, it was centrifuged at 3700 rpm for 4 min to sediment the solids, and 1 mL of the supernatant extract was filtered with a 0.45-μm PTFE filter (Millex FG, Millipore, Milford, MA) before injection on the LC/TOF-MS. In order to inject the sample in the same organic solvent percentage as the mobile phase, 300 μL of the filtered extract was mixed with 700 μL of water. Thus, a dilution of 3 : 7 took place for these types of samples. However, for recovery calculations, no dilution of the extract with water was carried out; the extract

in acetonitrile was directly injected to achieve sufficient sensitivity for the lowest spiking level of 0.1 mg kg^{-1}.

2.4.2 Liquid samples. The extraction method for the steak sauces was performed as follows: 2 mL of sample previously homogenized was mixed with 2 mL of acetonitrile in a 15 mL disposable screw-capped polypropylene tube for 30 s in the vortex. Afterwards, the mixture of sauce and acetonitrile was sonicated in a water bath for 15 min. The aqueous phase containing the sauce and the acetonitrile phase were not miscible, so the analytes were extracted into the acetonitrile and remained in this top layer. Finally, 1 mL of the supernatant extract was filtered with a 0.45-μm PTFE filter prior to analysis. Similar to the analysis of chilli powder samples, the filtered extract was diluted with water before injection into the LC/TOF-MS system.

2.5 *Calibration curves*

For the calibration curves, direct spiking of blank matrices, extracted as mentioned before, was performed at concentration levels from 0.01 to 1 mg L^{-1}: acetonitrile extracts were evaporated to almost dryness and reconstituted with the mixture of the dyes to the desired concentration up to 1 mL. In this way, all the calibration levels are accounted from matrix enhancement/suppression and can be used for quantitation purposes to calculate recovery values.

2.6 *LC/TOF-MS analyses*

Chromatographic separation of the dyes was achieved using an HPLC System (consisting of vacuum degasser, autosampler and a binary pump) (Agilent Series 1100, Agilent Technologies, Santa Clara, CA) equipped with a reverse-phase C_8 analytical column of 150 mm × 4.6 mm and 5-μm particle size (Zorbax Eclipse XDB-C8). Column temperature was maintained at 25°C. The injected sample volume was 20 μL. Mobile phases A and B were acetonitrile and water with 0.1% formic acid, respectively. The optimized chromatographic method started with the initial mobile phase composition of 30% A and 70% B, increasing in a linear gradient to 100% A in 20 min, and then held at 100% A constant in an isocratic mode for 5 min. The flow rate used was 0.6 mL min^{-1}. This HPLC system was connected to a time-of-flight mass spectrometer Agilent MSD TOF (Agilent Technologies, Santa Clara, CA), equipped with an electrospray interface operating in positive ion mode, using the following operation parameters: capillary voltage: 4000 V; nebulizer pressure: 40 psig; drying gas: 9 L min^{-1}; gas temperature: 300°C; fragmentor voltage: 170 V; skimmer voltage: 60 V; octopole DC 1: 37.5 V; octopole RF: 250 V. Accurate mass spectra were recorded across the range 50–1000 m/z. The data recorded were processed with Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software. The instrument performed the accurate mass internal mass calibration automatically using a dual-nebulizer ion source combined with an automated calibrant delivery system, which introduced the internal reference masses (121.0509 and 922.0098) at a very low flow rate.

Table 1. Main ions and fragments obtained for the selected dyes by LC/TOF-MS (effect of fragmentor voltage is also shown).

		Relative abundance (%)		
	m/z	170 V	200 V	230 V
Sudan I	249[a]	100	100	37
	156 ($C_{10}H_6NO$)	7	12	100
	128 (C_9H_6N)	<5	<5	78
Sudan II	277[a]	100	100	20
	156 ($C_{10}H_6NO$)	6	49	99
	128 (C_9H_6N)	<5	14	56
	121	7	42	100
Sudan III	353[a]	100	100	100
Sudan IV	381[a]	100	100	100
Sudan Orange G	215[a]	100	100	97
	122 ($C_6H_4NO_2$)	<5	15	100
Sudan R	279[a]	100	100	41
	156 ($C_{10}H_6NO$)	5	37	100
	128 (C_9H_6N)	<5	10	65
Para Red	294[a]	100	100	52
	156 ($C_{10}H_6NO$)	6	22	100
	128 (C_9H_6N)	<5	10	54

[a]Protonated molecule.

3. Results and discussion

3.1 *LC/TOF-MS detection of Sudan dyes*

The fragmentor role in LC-MS is critical to obtain an efficient structural information of the target analytes, as well as the best balance between sensitivity and fragmentation, since the production of alternative confirmatory ions occurs at the expense of the protonated molecule ion intensity. For this reason, LC/TOF-MS parameters were optimized in terms of fragmentation in order to obtain additional information from characteristic fragments of the compounds. The accurate mass of these characteristic fragment ions can be used along with that of the protonated molecule for confirmation purposes in case of a positive sample. In this work, three different fragmentation voltages were studied: 170, 200, and 230 V. All the typical fragment ions obtained and their relative abundances are shown in table 1 for all the analytes studied. For Sudan III and Sudan IV, there is no difference in fragmentation between the three fragmentor values, as no fragmentation occurs, even at the highest fragmentation voltage, and only the protonated molecule can be observed. For Sudan Orange G, a small increase in fragmentation can be noted at 200 V, and this enhancement becomes more evident at 230 V. In the case of Sudan I and Sudan II, when the fragmentation voltage increases from 170 to 200 V, the relative abundance of the main fragment ions is higher, which could lead to an important loss of sensitivity; when the voltage is at 230 V, the fragmentation is even higher. Finally, Sudan R and Para Red gave abundant fragmentation information at fragmentor voltages of 200 and 230 V. In spite of these results, and in order to achieve a good sensitivity for quantitation, the optimal fragmentor voltage was set at a low voltage of 170 V. Nevertheless, when a positive confirmation was needed, the fragmentor voltage was set at 230 V to obtain confirmation on at least one fragment ion.

Table 2. LC/TOF-MS accurate mass measurements for the protonated molecules of the dyes studied in chilli-powder matrix-matched standards (fragmentor voltage: 170 V; spiking level: 0.1 mg kg^{-1}).

Compound	Formula	Selected ion	m/z experimental	m/z calculated	Error	
					mDa	ppm
Sudan I	$C_{16}H_{12}N_2O$	$[M+H]^+$	249.1021	249.1022	−0.1	−0.5
Sudan II	$C_{18}H_{16}N_2O$	$[M+H]^+$	277.1332	277.1335	−0.3	−1.2
Sudan III	$C_{22}H_{16}N_4O$	$[M+H]^+$	353.1392	353.1396	−0.5	−1.3
Sudan IV	$C_{24}H_{20}N_4O$	$[M+H]^+$	381.1705	381.1709	−0.5	−1.2
Sudan Orange G	$C_{12}H_{10}N_2O_2$	$[M+H]^+$	215.0816	215.0815	0.1	0.4
Sudan R	$C_{17}H_{14}N_2O_2$	$[M+H]^+$	279.1127	279.1128	−0.1	−0.3
Para Red	$C_{16}H_{11}N_3O_3$	$[M+H]^+$	294.0874	294.0873	0.1	0.2

The accurate mass measurements were carried out using the following procedure: the m/z for every analyte (using a mass window of 0.2 Da) was extracted from the total ion chromatogram (TIC) in order to obtain an extracted ion chromatogram (called XIC). The background of this chromatogram was subtracted, and the accurate mass spectrum was obtained for every compound. The accurate mass of the protonated molecule was used for quantitation purposes in all cases, and the accurate mass of a fragment ion was used for further confirmation. These accurate masses were then processed through the software, which provided a list of possible elemental formula. Once all the possible elements and a minimum and maximum number of each of those were set along with a threshold value for errors (i.e. 5 ppm), a list of empirical formulae ordered by error (ppm) was automatically provided together with the double bond and ring equivalent number (DBE). The mass accuracy values obtained for the protonated molecules of the studied dyes on matrix-matched standards (using a chilli-powder extract fortified at 0.1 mg kg^{-1}) are shown in table 2. The errors obtained were less than 2 ppm in all cases. The widely accepted accuracy threshold for confirmation of elemental compositions is routinely established as 5 ppm, so the mass measurement, along with the characteristic retention time, provides in this case a highly reliable identification of the target analytes. Figure 2 shows an extracted ion chromatogram of the dyes in a chilli-powder extract spiked at 0.1 mg kg^{-1} level. Good separation is achieved for all the compounds studied. Although no analytes elute until 13 min in the chromatographic run, the selected gradient enables separation of other matrix interferences usually present in the samples.

3.2 *Recoveries of extraction*

To evaluate the effectiveness of the extraction method, different recovery studies were carried out by spiking raw blank samples (chilli powder and steak sauces) at two different concentration levels (0.1 and 0.5 mg kg^{-1}) with the target analytes. Samples were then extracted according to the procedures described in section 2 and analysed by LC/TOF-MS. The chromatographic areas of the analytes obtained after the extraction of spiked raw samples were compared with the areas for neat standards spiked directly in the same type of matrix, corresponding to the same amount of analyte extracted. Recoveries were then calculated, dividing both areas and multiplying by a factor of 100. In table 3, recovery results obtained for all the analytes are summarized. As can be observed, the recoveries obtained were higher than 75% for all the dyes analysed in both chilli powder and steak sauce. Para Red was not studied at a concentration level of

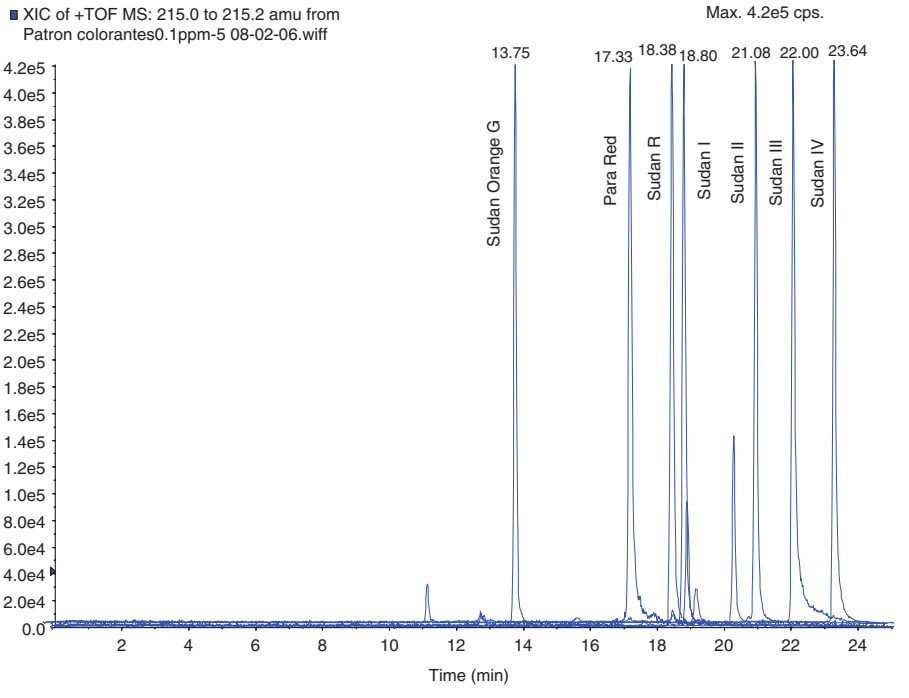

Figure 2. Extracted ion chromatogram corresponding to the analysis of a chilli-powder extract spiked at 0.1 mg kg^{-1} with the selected dyes.

Table 3. Recovery values and relative standard deviations (RSDs) for the target dyes extracted from chilli-powder and steak-sauce samples (peak areas compared with matrix-matched standards; analytes spiked directly in blank matrix extracts; see section 2).

	Amount added (mg kg^{-1})							
	Chilli powder				Steak sauce			
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Sudan I	84	4.7	105	8.6	93	4.4	114	5.8
Sudan II	83	5.8	108	7.9	116	15.3	113	11.1
Sudan III	105	14.6	88	5.4	97	15.2	116	12.8
Sudan IV	77	15.4	108	13.4	89	10.6	110	15.3
Sudan Orange G	76	11.5	75	4.6	100	4.1	118	5.1
Sudan R	79	5.3	106	6.5	90	1.6	116	5.2
Para Red	ns[a]	ns	76	6.7	81	9.1	93	10.3

[a]ns: not studied.

0.1 mg kg^{-1} in chilli powder because of the low sensitivity that this compound presents in LC-MS. Relative standard deviation (RSD) average values were approximately 10% ($n = 5$) for both levels of concentration. Moreover, no differences were found between solid and liquid matrices in terms of recovery values. These results evidence the feasibility of the developed extraction method for both types of matrices.

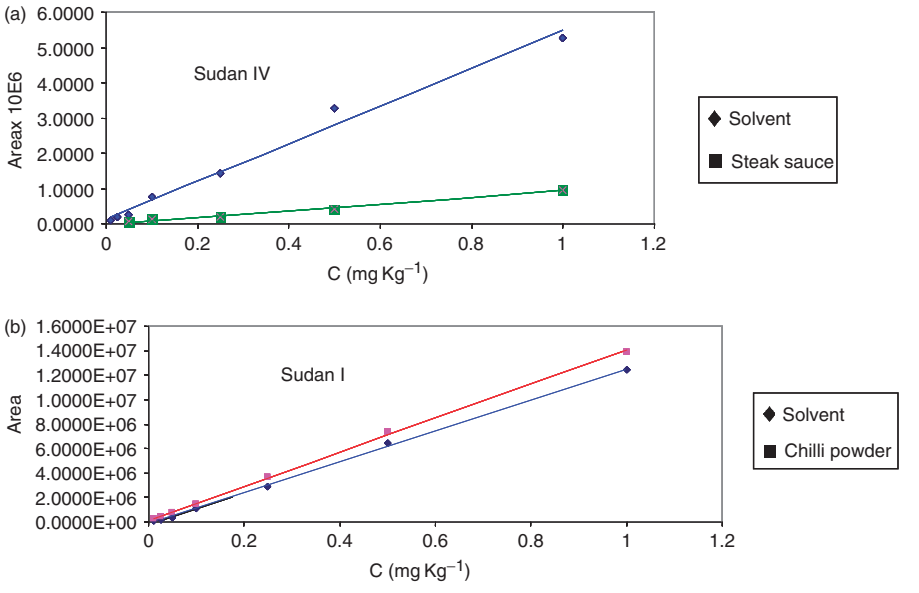

Figure 3. (a) Calibration plot obtained by LC/TOF-MS for a spiked steak sauce (matrix-matched standards) versus standards prepared in pure solvent for Sudan IV. (b) Calibration plot obtained from a spiked chilli-powder sample (matrix-matched standards) vs. standards prepared in pure solvent for Sudan I.

3.3 *Matrix effects*

The occurrence of matrix effects in LC-MS is well known, especially when electrospray ionization (ESI) is used. The phenomenon of signal suppression is related to the ionization system, and therefore not to the analyser used [19]. Matrix effects can both enhance and reduce the response when compared with standards prepared in solvent. A preliminary study to evaluate the signal suppression was performed by comparison of calibration curve slopes obtained with solvent-based standards to those with matrix-matched standards (figure 3). In steak sauce, for example, the matrix effect was more acute, and the signal suppression oscillated between 30% for Sudan Orange G to 81% for Sudan IV. Figure 3a shows an example of the slope obtained for Sudan IV in steak sauce compared with the slope for the same standard in pure solvent. As can be observed, steak sauce showed considerable matrix induced suppression, with Sudan IV being the worst case (for the other analytes studied, this effect was lower but still present; results not shown here). On the other hand, for chilli-powder samples, the matrix effect observed was not so intense, and sometimes it was even nonexistent, as is the case of Sudan I (figure 3b). Similar to steak sauce matrixes, Sudan IV was the dye that presented more signal suppression in chilli powder samples (60% of suppression), but this value is still lower than the 81% observed in the steak sauce. This could be because of the dilution of chilli powder, as the extraction process for solid samples implies an initial 1 : 10 dilution (compared with the 1 : 1 dilution for liquid samples), which helps to minimize the matrix effects.

A more detailed experiment was carried out to study in more detail the signal suppression in this type of matrixes. In this case, a series of standard solutions were prepared using different matrix percentages (1 : 2, 1 : 3, 1 : 5, and 1 : 10), for both

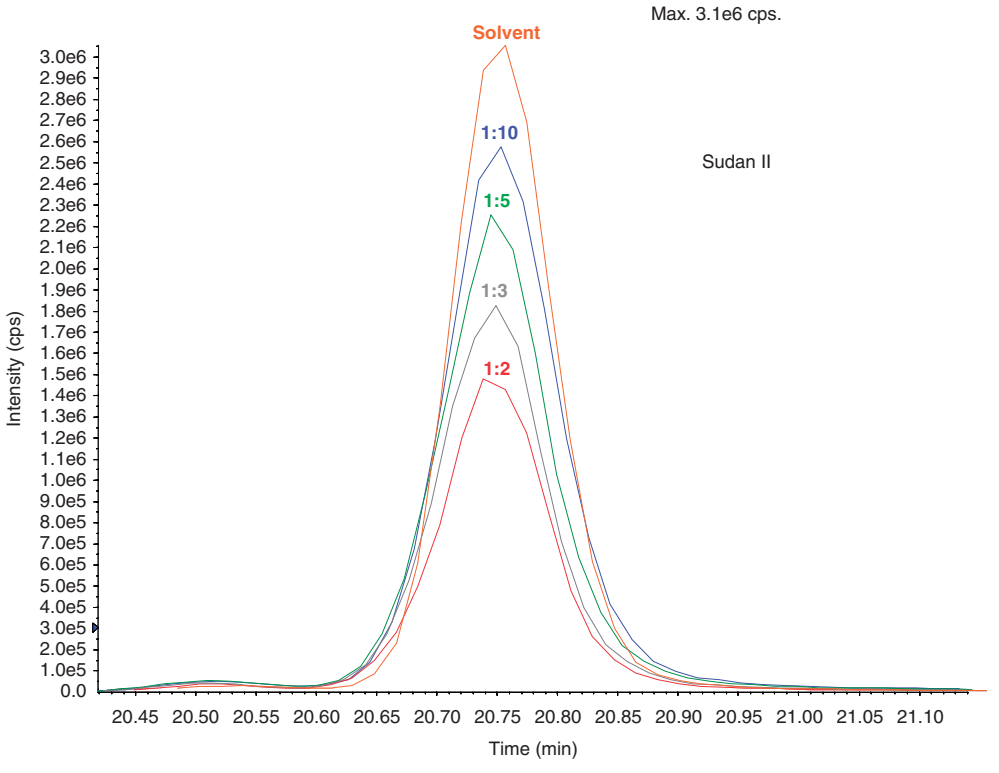

Figure 4. Effect of the dilution of the matrix on the signal suppression of Sudan II in steak sauce.

matrices, at a unique concentration of $1\,mg\,kg^{-1}$ for all the target dyes. The chromatographic response for every one of these solutions was evaluated and compared with a $1\,mg\,kg^{-1}$ standard in pure solvent. An example of this can be observed in figure 4, where the effect of signal suppression for Sudan II in steak sauce at different percentages was plotted. As matrix dilution increases (1 : 10), the signal height increases, and even at this level of dilution, there still exists a slight signal suppression, as compared with a neat standard in pure solvent. This behaviour occurred for all compounds studied. This fact reveals the importance of matrix effects in LC-MS, and the need to use matrix-matched calibration for quantitation purposes. Moreover, it shows that a simple dilution of the extracted sample can minimize such matrix suppression.

3.4 *Analytical performance*

To evaluate the usefulness of LC/TOF-MS for quantitative analyses in this type of matrices, the analytical performance of the proposed method was studied in terms of linearity and limits of detection. Quantitation of the sample extracts was accomplished using a calibration curve based on matrix-matched standards: blank sample extracts (from chilli powder and steak sauces) were evaporated until near dryness under a nitrogen flow and then reconstituted with standard solutions of a mixture of the dyes at different concentrations ranging from 0.01 to $1\,mg\,L^{-1}$ in order to obtain a wide

Table 4. Calibration data for the target dyes in chilli-powder and steak-sauce samples spiked from 0.01 to 1 mg L^{-1} (five calibration data points were used).

	Matrix	Calibration equation	R^2
Sudan I	Chilli powder	$y = 1 \times 10^7 x + 128\ 301$	0.9993
	Steak sauce	$y = 1 \times 10^7 x - 162\ 321$	0.9964
Sudan II	Chilli powder	$y = 8 \times 10^6 x - 120\ 896$	0.9932
	Steak sauce	$y = 7 \times 10^6 x - 7655$	0.9989
Sudan III	Chilli powder	$y = 2 \times 10^6 x + 32\ 477$	0.9906
	Steak sauce	$y = 3 \times 10^6 x + 14\ 980$	0.9994
Sudan IV	Chilli powder	$y = 2 \times 10^6 x - 32\ 443$	0.9942
	Steak sauce	$y = 945\ 131x - 6985.2$	0.9878
Sudan Orange G	Chilli powder	$y = 2 \times 10^7 x - 289\ 597$	0.9974
	Steak sauce	$y = 2 \times 10^7 x - 505\ 106$	0.9967
Sudan R	Chilli powder	$y = 3 \times 10^7 x + 143\ 949$	0.9963
	Steak sauce	$y = 1 \times 10^7 x + 65\ 027$	0.9998
Para Red[a]	Chilli powder	$y = 799\ 494x + 14\ 905$	0.9909
	Steak sauce	$y = 1 \times 10^6 x + 5325$	0.9989

[a]Para Red was studied from 0.1 to 10 mg L^{-1}.

Table 5. Instrumental and matrix limits of detection of the dyes in chilli-powder and steak-sauce samples.

	Instrumental LODs (μg L^{-1})		Matrix LODs (μg kg^{-1})	
Analyte	Chilli powder	Steak sauce	Chilli powder	Steak sauce
Sudan I	3	1	100	6.6
Sudan II	2	1	70	6.6
Sudan III	10	3	333	20
Sudan IV	20	10	660	66
Sudan Orange G	2	1	66	6.6
Sudan R	3	1	100	6.6
Para Red	50	10	1700	66.7

range of concentrations. The exception was for Para Red, which was analysed from 0.1 to 10 mg L^{-1} because of its lower sensitivity.

Linearity was studied in both solvent and matrix-matched standard solutions at five different concentration levels. Quantitation was carried out using the peak area from the extracted ion chromatograms (XIC) of the protonated molecule using a mass window of 0.2 Da. Table 4 shows the calibration equations obtained by LC/TOF-MS for the dyes in chilli powder and steak sauce, and their correlation coefficients. As can be observed, the linearity of the analytical response within the studied range of two orders of magnitude is good, with correlation coefficients higher than 0.99 in all cases except for Sudan IV in steak sauce, which was 0.98.

The limits of detection (LOD) were estimated from the injection of matrix-matched standard solutions with concentration levels giving a signal-to-noise ratio of 3. The results are summarized in table 5. Both instrumental and matrix limits of detection are presented. Instrumental detection limits refer to the concentration in spiked extracts, whereas matrix limits of detection take into account all the dilutions performed during the extraction procedure of the raw sample. Sudan I, II, Orange G, and R were the most sensitive compounds with the lowest LODs in both matrices. In general, the best LODs were observed in the sauce matrix studied, which

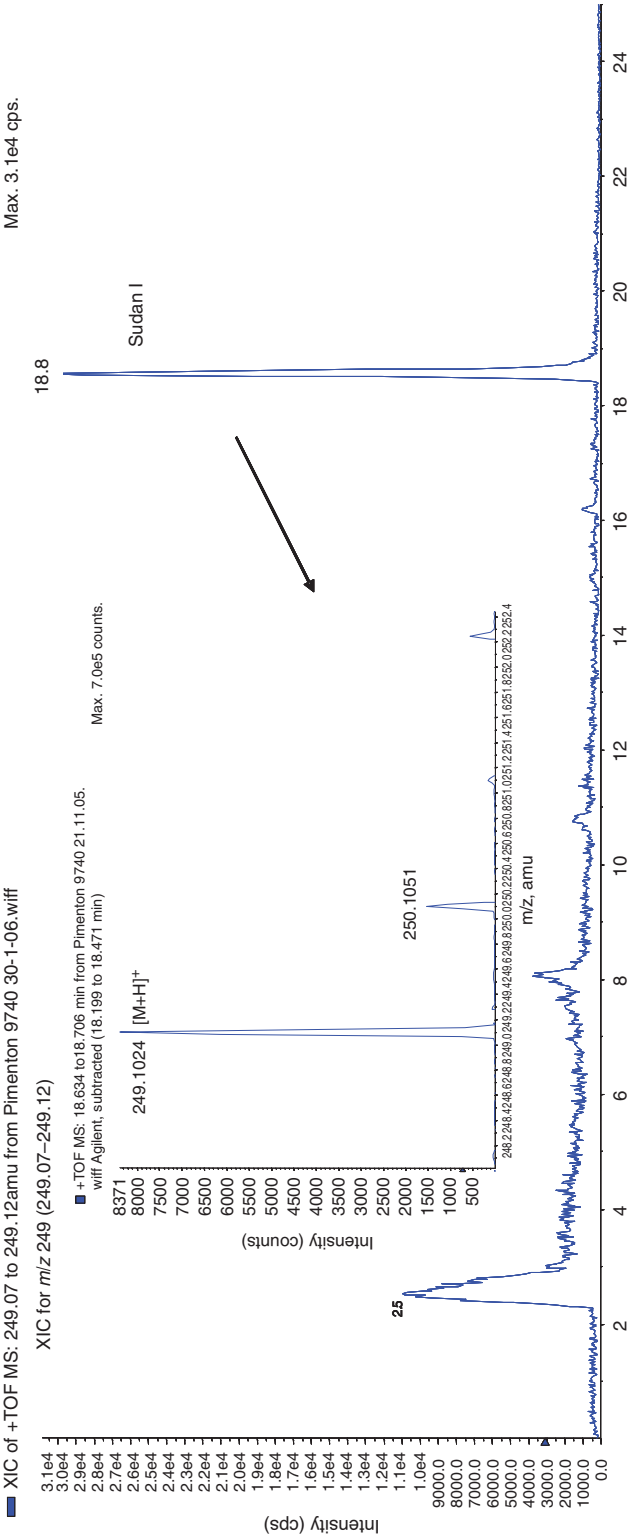

Figure 5. Extracted ion chromatogram of Sudan I in a market chilli-powder sample and its corresponding mass spectra (extracted mass window of 0.05 Da).

could be because of the lower dilution factor of the extraction method compared with chilli-powder samples. The limits of detection for Para Red are appreciably higher because of its low ionization in the electrospray source in positive mode, as compared with the other dyes analysed. The presence of the electron-withdrawing nitro group in its molecule makes nitrogen protonation unfavourable. The limits of detection reported in this study are comparable with those reported in previous studies [10] and suggest that this is a good approach for the identification and quantitation of banned dyes in food samples.

3.5 *Analysis of market samples*

To evaluate the usefulness of the proposed methodology, it was applied to the analysis of real samples in a survey performed as part of a regional research programme organized by the Andalusian Health Service (SAS) in Spain. The samples analysed were chilli powder and different types of sauces. All samples were extracted and analysed as described in section 2. From a total of 70 samples, only one positive finding was detected: the case of a chilli powder sample (similar to paprika) containing $0.128\,mg\,kg^{-1}$ of Sudan I. The correct retention time and the exact mass were used as identification criteria. Figure 5 shows this positive finding where the accurate mass for the protonated molecule of Sudan I was extracted with a narrow mass window of 0.05 Da. A peak at a retention time of 18.8 min was observed, which matched the exact retention time of Sudan I. The experimental exact mass obtained was 249.1024, which, compared with the theoretical exact mass for Sudan I (249.1022), resulted in an error of 0.6 ppm, well below the threshold acceptable value for errors, set at 5 ppm. For an additional confirmation, the analysis was repeated at a higher fragmentor voltage of 230 V. Under these conditions, the characteristic 156 and 128 fragments shown in table 1 were also found, thus making the presence of Sudan I evident and unequivocal in this sample. A high selectivity was obtained in this case by using extracted ion chromatograms with very narrow accurate mass windows. Thus, most of the interferents present in the matrices did not show up in the chromatograms, since they were excluded in the extracted ion mass window.

In conclusion, a novel method based on solvent extraction and analysis by LC/TOF-MS has been developed for the determination of Sudan dyes and Para Red in chilli powder and steak-sauce samples. The method allows for the simultaneous and highly sensitive detection of these illegal additives in food spices. It also offers a high degree of confidence for positive results using the accurate mass measurements of the protonated molecules, as well as a high degree of selectivity for the target analytes in such complex matrices. Moreover, low detection limits were achieved, thus making this methodology a highly reliable and fast tool for the identification of banned dyes in food samples.

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