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Development and in-house validation of a liquid chromatography–electrospray–tandem mass spectrometry method for the simultaneous determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli products

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Abstract

An accurate method based on the use of reversed-phase (RP) liquid chromatography–tandem mass spectrometry interfaced with electrospray (LC–ESI–MS/MS) was devised for the determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli food samples. A simple sample treatment procedure entailing the use of an extraction step with acetone without clean-up was developed. A C18 column with an aqueous formic acid/methanol mixture as the mobile phase was used under isocratic conditions. Mass spectral acquisition was done in positive ion mode by applying selected reaction monitoring of three fragmentation transitions per compound to provide a high degree of selectivity. The method was in-house validated in terms of detection limits (LOD), quantitation limits (LOQ), linearity, sensitivity, accuracy, recovery, and selectivity on two kinds of hot chilli sauces. Good results in the low ng/g level were obtained for LOD and LOQ of all analytes in matrices. Under both intra-day repeatability (R.S.D. between 1 and 13%) and intermediate precision (about 5–15% R.S.D. for both chilli sauce matrices) conditions, precision proved to be typical of determinations based on electrospray LC–MS and acceptable for routine monitoring purposes. Extraction recoveries for all four azo-dyes in chilli tomato sauce ranged from 92 to 103% at a spiking level of 5 μg/kg, whereas values between 72 and 97% were calculated in chilli tomato and cheese sauce at the same concentration level. The applicability of the method to the determination of Sudan azo-dyes in hot chilli products was demonstrated.

Keywords: Food analysis; Sudan azo-dyes; Azo-dyes

1. Introduction

Azocompounds are by far the most widely used synthetic organic colorants [1] and more than 2000 of such substances are listed in the color index (CI) [2]. The genetic toxicity of some azo-dyes has been confirmed [3] and structure-activity relationships have been assessed [4,5]. Among organic colorants most of the azo-dyes are recognized to be carcinogens [6].

The Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laid down the general principles and requirements of food law [7], establishing to suspend the placing on the market or use of a food or feed that is likely to constitute a serious risk to human health. In particular, discovery of the azo dye Sudan I in hot chilli and hot chilli products originating from India and marketed in the EU does not comply with the EU food safety requirements. As a consequence of this discovery, in June 2003, the EC Commission has adopted a decision on emergency measures concerning hot chilli and hot chilli products intended for human consumption [8]. The Food Standards Agency also alerts for the contamination with Sudan I dye of various meat preparations on the market in UK and issues warnings about frozen meat products, spice mix and chips containing contaminated chilli powder [9].

On January 2004, the EU Food Regulations 2003 amended the conditions for the import of chilli and chilli products and the EC decided to extend the Commision Decision

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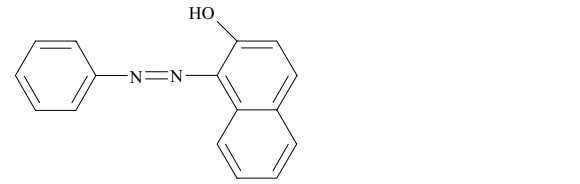
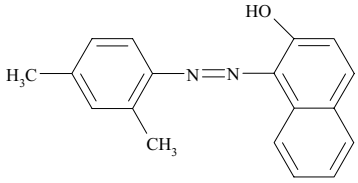
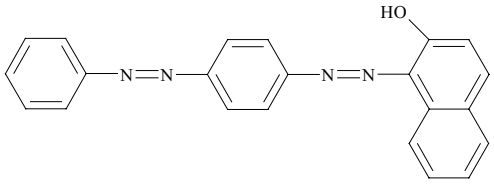


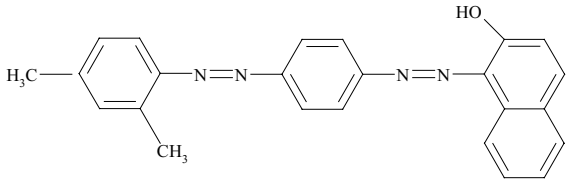
Sudan I (M.W. 248.28)



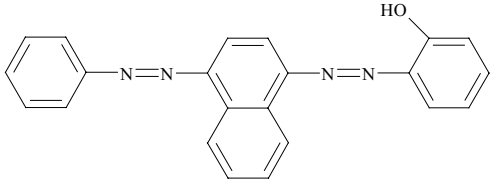
Sudan II (M.W. 276.34)



Sudan III (M.W. 352.40)



Sudan IV (M.W. 380.45)



Disperse Orange 13 (M.W. 352.40)

Fig. 1. Chemical structures of the azo-dyes investigated and of the internal standard Disperse Orange 13.

2003/460/EC requirement to cover Sudan II, Sudan III and Scarlet Red or Sudan IV [10].

Belonging to the azo-dye class, Sudan dyes (Fig. 1) are non ionic fat-soluble dyes used in the gasoline, diesel, lubricating grease and polymer dye production, and as dye for food (chilli) and tattoos.

Sudan I is not a permitted colour under the Colours in Food Regulations 1995 [11]. It is considered to be a genotoxic carcinogen [3,12] and its presence is not permitted in foodstuffs for any purpose at any level. Sudan II is the dimethyl derivative of Sudan I and it has been tested in mice by bladder implantation, resulting in a high incidence of bladder carcinomas [13]. Sudan III and Sudan IV are fat-soluble dye predominantly used for demonstrating presence of triglycerides in frozen sections. In addition, Sudan III and Sudan IV are commonly used for coloring waxes, oils and spirit varnishes. As regards to Sudan III and the dimethyl derivative Sudan IV, no LD_{50}/LC_{50} information relating to normal routes of occupational exposure is available. Sudan I, Sudan II, Sudan III and Sudan IV have been classified by IARC in the Group 3, i.e. not classifiable as to their carcinogenicity to humans.

In this context, the rationale for this paper is on the basis that these colourants could be found in food products exported in European countries. For this reason, sensitive, selective and accurate analytical methods should be developed in order to identify and quantify such substances in complex matrices as processed foodstuffs. As reported in the literature, dealing with the analytical chemistry of Sudan dyes, the usually proposed methods for the determination of these compounds are based on liquid chromatography (LC) with spectrophotometric UV [14] or fluorimetric [13] detection. In the past, commercial Sudan azo-dyes have also been studied by thermospray, particle beam and electrospray (ESI) LC–mass spectrometry (MS) techniques [15].

In this work we developed and validated a LC–ESI–tandem mass spectrometry (MS/MS) method for the simultaneous identification and determination of Sudan I, Sudan II, Sudan III and Sudan IV in chilli tomato sauce and chilli tomato and cheese sauce. Single-laboratory validation procedure was followed to demonstrate data reliability and thus, that the analytical method proposed fits for its intended purpose. In particular, validation protocol included evaluation on detection limits, quantitation limits, linearity, accuracy (precision and trueness), recovery and selectivity. The method proposed requires minimal sample preparation, and provides a well-resolved analyte peak without any interferences.

2. Experimental

2.1. Chemicals

Sudan I (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol; CAS Registry Number (Nr): 842-07-9; CI 12055); Sudan II (1-(phenylazo)-2-naphthol; CAS Nr 3118-97-6; CI 12140); Sudan III (1-(4-phenylazophenylazo)-2-naphthol; CAS Nr 85-86-9; CI 26100); Sudan IV (*o*-tolyazo-*o*-tolylazo-beta-naphthol; CAS Nr 85-83-6; CI 26105) and Disperse Orange 13 (4-[4-(phenylazo)-1-naphthylazo]phenol, CAS Nr 6253-10-7; CI 26080) were obtained from Sigma-Aldrich (Milan, Italy). Stock solutions (1 mg/mL) of Sudan I and Sudan II were prepared in methanol, whereas stock solutions (1 mg/mL) of Sudan III, Sudan IV and Disperse Orange

13 were prepared in ethyl acetate and stored at 4 °C in the dark.

Water was purified with a Milli-Q Element A-10 water purification system (Millipore, Bedford, MA, USA). Methanol, ethyl acetate and acetone (HPLC-grade purity) were purchased from Carlo Erba (Milan, Italy). Analytical reagent grade formic acid was from Carlo Erba.

2.2. Samples

The following commercial samples were purchased in big trades: chilli tomato sauce (three samples deriving from different batches), chilli tomato and cheese sauce (three samples deriving from different batches). For each kind of sauce, one sample in which Sudan dyes were demonstrated to be absent was considered as the blank and used for validation purposes.

2.3. Liquid chromatography–tandem mass spectrometry

HPLC elution was performed on a Luna C18(2) narrow-bore column (250 mm × 2.1 mm, 5 μm) (Phenomenex, Torrance, CA, USA) held at a temperature of 35 °C and using an isocratic solvent system (0.1% (v/v) formic acid in methanol/aqueous formic acid 0.1% (v/v) (97/3, v/v)). The flow-rate was 200 μL/min. The mobile phase was delivered by a Waters 2690 series Alliance quaternary pump (Waters, Milford, MA, USA) equipped with a 120-vial capacity sample management system. The injection volume was 20 μL.

A Quattro LC triple quadrupole instrument (Micromass, Manchester, UK) equipped with an electrospray interface and a Masslynx v. 3.4 software (Micromass) was used for data acquisition and processing.

The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at a flow-rate of 50 and 555 L/h, respectively. Optimal operating parameters of the ESI interface were optimized by infusing standard solutions of Sudan I, Sudan II, Sudan III and Sudan IV in the mobile phase (0.1 μg/mL) at a flow-rate of 5 μL/min using a Harvard syringe pump (Harvard Apparatus Inc., Holliston, MA, USA). LC–MS/MS determinations were performed by operating the mass spectrometer in positive ion (PI) mode. The optimum conditions of the interface were as follows: electrospray voltage 3.0 kV, cone voltage 25 V, rf lens 0.2 V, source temperature 110 °C, desolvation temperature 280 °C. Mass spectra were acquired over the scan range *m/z* 200–500 using a step size of 0.1 Da and a rate of 0.2 scans/s. The quadrupoles were tuned to unit mass resolution and mass drift was periodically checked.

Operating in MS/MS mode, product-ion scan mass spectra of protonated molecules of Sudan dyes were acquired in the 30–450 Da mass range. Qualitative analysis was performed in SRM (selected reaction monitoring) mode by monitoring three transitions for each compound. SRM transitions, cone voltages and collision energies are reported for each analyte and the Internal Standard (IS) in Table 1. The dwell time and the interchannel delay were set at 0.1 and 0.01 s, respectively. For validation and quantitative purposes, one transition for each contaminant was monitored, as indicated in Table 1.

Table 1
Compound-specific LC–ESI–MS/MS parameters for the four azo-dyes and the internal standard Disperse Orange 13

Analyte	SRM trace (*m/z*)	Cone voltage (V)	Collision energy (eV)
Sudan I	249 → 93[a]	25	24
	249 → 128		25
	249 → 156		15
Sudan II	277 → 106	28	40
	277 → 121[a]		18
	277 → 156		15
Sudan III	353 → 77	45	28
	353 → 120[a]		23
	353 → 196		23
Sudan IV	381 → 91	45	26
	381 → 106		40
	381 → 224[a]		20
Disperse Orange 13	353 → 93	47	20

[a] Fragmentation transition monitored for quantitative purposes.

2.4. LC–MS/MS method validation

Validation process of the LC–MS/MS method was carried out following the EURACHEM guidelines [16].

Matrix-matched detection limits (y_D) and quantitation limits (y_Q) were evaluated for the analytes as signals based on the mean value ($\bar{y}_b$) and the standard deviation (s_b) of the blank signal. For $\bar{y}_b$ and s_b determination, 10 independent sauce sample blanks were measured for each kind of matrix. The concentration values of detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the corresponding signals y_D and y_Q through a calibration plot $y = f(x)$ onto the concentration axis.

Linearity of the method was evaluated starting from a concentration value upper to LOQ for all the dyes in matrix-matched solutions. Linearity studies were accomplished by verifying homoscedasticity by means of the Bartlett test and by calculating the goodness of fit of the calibration curve applying the lack-of-fit and Mandel's fitting test [17]. A *t*-test was carried out to verify the significance of the intercept (confidence level 95%).

Method accuracy was evaluated both in terms of precision and trueness. Precision was calculated in terms of intra-day repeatability as R.S.D.% at two concentration levels for the analysis of spiked sample extracts. The confidence interval (CI) referred to concentration of analytes in samples (μg/kg) was also calculated.

Between-day precision was calculated in terms of R.S.D.% ($n = 15$) on three different days. For this purpose, homoscedasticity at 95% confidence level was verified.

Trueness was evaluated by calculating the recovery function, which allows to evidence both constant and propor-

tional systematic errors [17]. To perform this investigation, we used two kinds of sauces (chilli tomato sauce and chilli tomato and cheese sauce) which were shown not to contain detectable residues of the azo-dyes of interest as analytical blanks. First the calibration function of the fundamental analytical procedure was determined:

$$y = a_c + b_c x_c$$

The analytical calibration procedure was then performed on the blank sauce samples spiked at six equispaced concentration levels. The analytical results x_f were then calculated using the measured signal values y_f and the analysis function, i.e. the calibration function solved for x:

$$x_f = \frac{y_f - a_c}{b_c}$$

By plotting the "found concentrations" (x_f) versus the original calibration concentrations (x_c), the recovery curve, which is mathematically described by the recovery function (linear regression line), was calculated:

$$x_f = a_f + b_f x_c$$

The intercept and the slope of the recovery function were then compared with 0 and 1, respectively.

Method selectivity was evaluated by measuring the analytes of interest in test portions to which specific interferences among those likely to be present in samples were deliberately introduced. The effect of interferences was then evaluated verifying if their presence enhance or inhibit detection or quantification of the analytes. Interferents investigated were: fatty acids (oleic acid, palmitic acid, linoleic acid, stearic acid, linolenic acid), stigmasterol, sitosterol, α-tocopherol.

Extraction recoveries were calculated by spiking both blank sauce matrices with the four dyes at the final concentration of 1 and 5 μg/kg. Extraction procedure was repeated three times.

Disperse orange 13 was used as internal standard, both for validation and for quantitative purposes.

All statistical analyses and tests were carried out by using the statistical package SPSS 10.0 for Windows (SPSS, Bologna, Italy).

2.5. Sample preparation and LC–MS/MS analysis

Sample treatment was performed as follows. Before extraction, samples were homogenized for 1 min with a T25 basic homogenizer (Ika Labotechnik, Staufen, Germany). One gram sample was weighed and 50 μL of 2 μg/mL internal standard solution followed by 10 mL acetone were added. After mixing for 2 min on a vortex the extract was centrifuged at 4500 rpm for 10 min. A 3-mL portion of clarified solution was removed and added to 1 mL of deionized water. Extract was filtered through a 0.45-μm nylon syringe filter (Supelco, Bellefonte, PA, USA) before injection onto the LC–MS system (injection volume 20 μL).

For quantitative analysis of Sudan I and Sudan II in sauce samples, calibration graphs were constructed over the range 25–175 μg/L, respectively.

3. Results and discussion

3.1. ESI–MS, ESI–MS/MS and LC–ESI–MS/MS analysis

In this work, ESI–MS and ESI–MS/MS behaviour of Sudan dyes was investigated in PI mode. Operating at a cone voltage of 25 V, the full-scan ESI(+)–MS mass spectra of the analytes showed only the protonated molecule $[M + H]^+$, allowing confirmation of the molecular mass.

Under ESI(+)–MS/MS conditions, the product-ion mass spectra of Sudan dyes showed a characteristic fragmentation pattern for all the analytes (Fig. 2). In the case of Sudan I and Sudan II, a fragment corresponding to the loss of an $OH^\bullet$ was observed at m/z 232 and m/z 260, respectively with a relative intensity greater than 30%, whereas in the case of Sudan III and Sudan IV the relative intensity of such fragments was lower than 10%. The fragment ion at m/z 156 common to all dyes seems to originate from the cleavage of the C–N bond on the opposite side of the naphtalene group with hydrogen transfer to form a radical cation of the type $[C_{10}H_8N_2]^{\bullet+}$.

As for chromatographic separation, data available in literature are very scant. Nagase et al. reported the separation of Sudan I and Sudan II in water by a reversed-phase (RP) LC method with spectrophotometric detection involving the use of C18 (250 mm × 4.0 mm) column in less than 15 min [14]. The chromatographic method developed in this work allowed us to achieve both a good retention of Sudan dyes in a reasonable time and a separation of these substances from matrix constituents ensuring at the same time mass spectrometer compatibility. Preliminary trials were performed by analyzing both standard solutions and sample extracts in RP

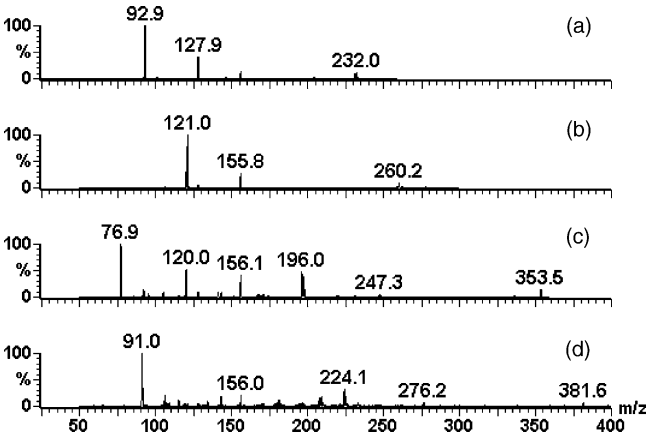


Fig. 2. ESI(+)–MS/MS product-ion mass spectra of: (a) Sudan I; (b) Sudan II; (c) Sudan III; (d) Sudan IV. Collision energy 25 eV, argon pressure 1.57×10^{-3} mbar.

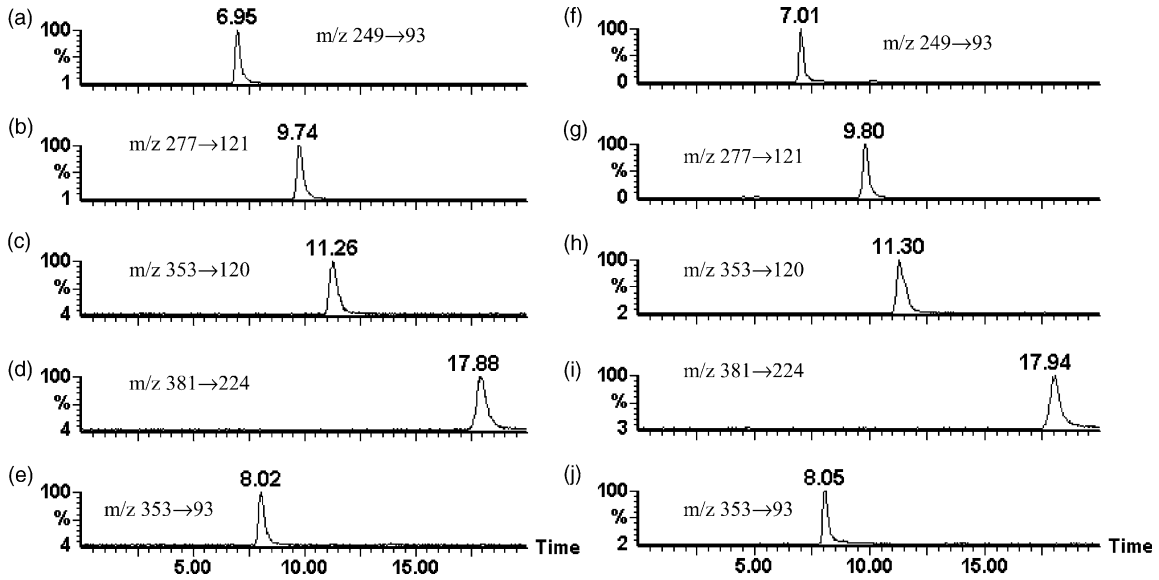


Fig. 3. LC–ESI–MS/MS SRM traces obtained from 125 μg/L standard solution of: (a) Sudan I; (b) Sudan II; (c) Sudan III; (d) Sudan IV; (e) Disperse Orange 13 internal standard (100 μg/L; left column) and from a blank chilli tomato and cheese sauce sample spiked with 125 μg/L each (1685 μg/kg sample) of (f) Sudan I; (g) Sudan II; (h) Sudan III; (i) Sudan IV; (j) Disperse Orange 13 internal standard (100 μg/L; right column; injection volume 20 μL).

partitioning mode using a C18 narrow-bore column and an eluent made up of 0.1% (v/v) formic acid aqueous solution and 0.1% (v/v) formic acid in methanol with a percentage of aqueous phase varying between 1 and 3%. Optimal conditions were obtained in correspondence to the use of an organic/aqueous solvent ratio of 97/3 (v/v) in the mobile phase. Under optimized LC conditions good separation was achieved within 17 min, the four contaminants eluting with high repeatability (retention time of Sudan I 6.97 ± 0.10 min, Sudan II 9.71 ± 0.19 min, Sudan III 11.16 ± 0.29 min, Sudan IV 17.72 ± 0.62 min, $n = 10$). Good performance in terms of efficiency ($n = 20{,}700$) and of asimmetry factor ($T = 1.06$) was exhibited by the column, these values being referred to the peak of Sudan IV.

Fig. 3 depicts the LC–ESI(+)–MS/MS chromatographic separations of the four azo-dyes on standard solution at 125 μg/L and on the chilli tomato and sauce matrix spiked at the same concentration level (1685 μg/kg sample).

3.2. LC–ESI–MS/MS method performance

To support regulatory action a method has to be demonstrated to be accurate, sensitive and able to identify contaminants with high selectivity. For this purpose, we paid attention to the validation of the analytical method according to European guidelines evaluating method accuracy in terms of precision and trueness, recovery, selectivity, detection limits, quantitation limits, linearity and sensitivity.

Among the established requirements, detection limits and quantitation limits of the compounds investigated were thus calculated upon statistic criteria. Detection and quantitation limits of approximately 3–10 μg/kg and 5–17 μg/kg were determined in chilli tomato sauce (Table 2). The method developed showed also good detectability in the case of chilli tomato and cheese sauce, LOD and LOQ values being lower than 25 and 50 μg/kg, respectively.

As for linearity, using least-square regression the calibration functions were calculated for both matrices (Table 3). Homogeneity of variance of replicates at different concentration levels was proved at 95% confidence level ($P > 0.05$). Further, since intercept was demonstrated to be not significant, the best fit was obtained using a linear regression model $y = b_1 x$. From the results of the Mandel test performed on these data, significance values greater than 0.05 indicated a good linear fitting. Slope values obtained for Sudan I and Sudan II attest better method sensitivity for these contaminants with respect to Sudan III and Sudan IV.

Method accuracy of the LC–ESI–MS/MS method was then tested both in terms of precision and trueness. Under both intra-day repeatability (R.S.D. between 1 and 13%) and intermediate precision (about 5–15% R.S.D. for both chilli sauce matrices) conditions, precision proved to be typ-

Table 2
Limits of detection and limits of quantitation of the LC–MS/MS method for hot chilli samples

Analyte	Matrix: chilli tomato sauce		Matrix: chilli tomato and cheese sauce	
	LOD[a] (μg/kg)	LOQ[b] (μg/kg)	LOD[a] (μg/kg)	LOQ[b] (μg/kg)
Sudan I	4	7	11	15
Sudan II	3	5	3	7
Sudan III	5	11	9	19
Sudan IV	11	17	24	48

[a] Calculated as concentration corresponding to signal: $y_D = \bar{y}_b + 2ts_b$.
[b] Calculated as concentration corresponding to signal: $y_Q = \bar{y}_b + 10s_b$.

Table 3
Matrix-matched calibration curves established in (A) chilli tomato sauce and (B) chilli tomato and cheese sauce extracts using LC–ESI–MS/MS[a]

Analyte	Concentration range (μg/kg)	Matrix	Homoscedasticity	Mandel test	$b_1 \pm s_{b1}$	r^2 ($n = 15$)
			P[b]	P[c]		
Sudan I	7–2100	A	0.072	0.125	11.2 ± 0.2	0.998
		B	0.054	0.09	9.6 ± 0.1	0.991
Sudan II	5–1700	A	0.056	0.06	25.9 ± 0.3	0.999
		B	0.057	0.06	27.0 ± 0.5	0.986
Sudan III	10–3400	A	0.057	0.148	4.76 ± 0.09	0.997
		B	0.077	0.148	1.94 ± 0.03	0.986
Sudan IV	17–5500	A	0.064	0.08	1.8 ± 0.2	0.995
		B	0.187	0.08	0.95 ± 0.02	0.981

[a] Calibration function: $y = b_1x$.
[b] 95% Confidence level.
[c] 99% Confidence level.

ical of determinations based on electrospray LC–MS and acceptable for routine monitoring purposes. Before calculating RSD under intermediate precision, homoscedasticity of the data was attested by *P*-values greater than 0.05. Figures of merit referred to intra-day repeatability are reported in Table 4 for both matrices.

Trueness was assessed by investigating matrix effect. For this purpose, recovery functions were calculated in order to ascertain the influence of the matrix for the determination of all four Sudan dyes in hot chilli samples. In fact, matrix effects can be responsible of constant or proportional systematic deviations of the analytical result from the "true" value. In all cases the *t*-test performed on the intercept provided a *P*-value at the 95% confidence level higher than 0.05 demonstrating that constant systematic errors did not occur. In the case of the slope, since the *t*-calculated resulted to be higher than the *t*-tabulated at the 95% confidence level (2.11; d.f. = 17) (data not shown), it can be inferred that the matrix-matched calibration curves are significantly different from that obtained using standard solutions. This effect could be attributed to ion suppression, which can occur when a coeluted compound suppresses the ionization of the analyte in the MS source resulting in a systematic proportional error. Nevertheless, accurate sample quantitation was feasible using matrix-matched calibration curves, which allowed us to overcome matrix effect and avoid time-consuming sample purification treatments.

As for selectivity, the presence of interferents considered was not found to enhance or inhibit detection or determination of the analytes, proving reliability of the LC–MS/MS method both for confirmation and for quantitation purposes.

The mean recoveries of all four azo-compounds ranged from 85 to 101% and from 92 to 103% in chilli tomato sauce at a spiking level of 1 and 5 μg/kg, respectively. Values in the 62–106% and 72–97% range were calculated in chilli tomato and cheese sauce at the fortification level of 1 and 5 μg/kg, respectively.

3.3. Sample analysis

The LC–ESI–MS/MS method developed and validated was then applied to the identification and determination of Sudan dyes in four hot chilli products for control purposes.

With the aim to adding confidence to the identity of the analyte, three fragmentation transitions per compound were monitored, thus complying with MS confirmation criteria

Table 4
Precision: intra-day repeatability of the LC–ESI–MS/MS method

Analyte	Concentration (μg/L)	Matrix: chilli tomato sauce		Matrix: chilli tomato and cheese sauce	
		$X_m \pm$ CI[a]	R.S.D. (%)	$X_m \pm$ CI[a]	R.S.D. (%)
Sudan I	40	39.6 ± 1.1	2	40.1 ± 1.2	8
	160	164 ± 5	3	149 ± 12	9
Sudan II	30	29.1 ± 0.7	2	31.9 ± 1.5	7
	130	126 ± 5	4	131 ± 3	11
Sudan III	60	58 ± 2	3	61 ± 2	5
	250	242 ± 10	1	254 ± 8	9
Sudan IV	100	107 ± 11	1	106 ± 7	9
	400	414 ± 20	10	405 ± 11	13

[a] Average peak area value ± confidence interval (95%).

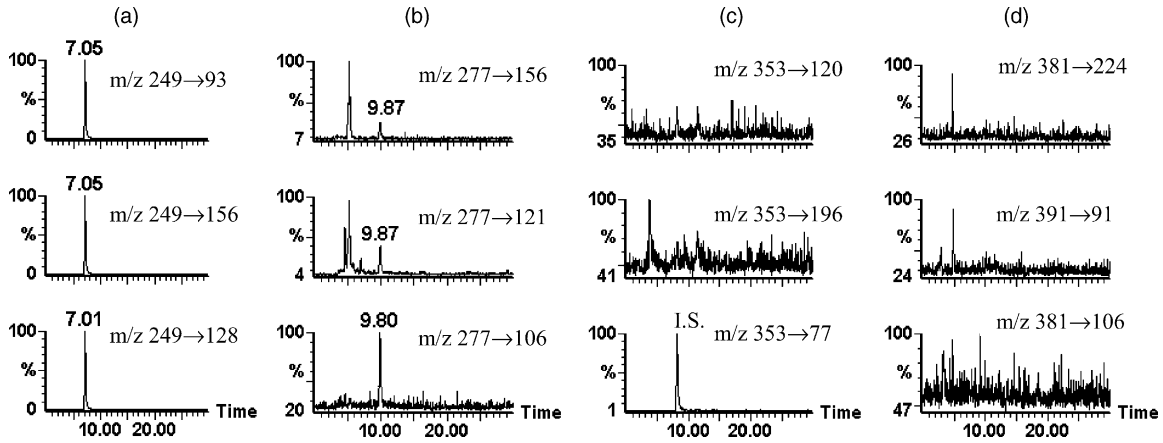


Fig. 4. LC–ESI–MS/MS SRM analysis of a positive chilli tomato and cheese sauce sample. Analyte identified: (a) Sudan I; (b) Sudan II. Sudan I (a) was determined at 1.41 ± 0.06 mg/kg, Sudan II (b) at concentration lower than LOQ. (c) and (d) traces are referred to Sudan III and Sudan IV.

Table 5
LC–ESI–MS/MS quantitative analysis of Sudan dyes in positive hot chilli sauce samples ($n = 3$)

Sample	Analyte			
	Sudan I (mg/kg)	Sudan II (mg/kg)	Sudan III (mg/kg)	Sudan IV (mg/kg)
Chilli tomato sauce	1.24 ± 0.05	n.d.[a]	n.d.[a]	n.d.[a]
Chilli tomato and cheese sauce	1.41 ± 0.06	n.q.[b]	n.d.[a]	n.d.[a]

[a] Not detected.
[b] Detected but not quantified (concentration lower than LOQ equal to 6.6 μg/kg).

as established by 1999/333/EG [18]. Further, to compliance criteria for unambiguous identification of Sudan dyes in real positive samples, product-ion mass spectra were run to confirm the presence of these substances. In particular, relative ion intensities corresponding to samples did not differ more than 20% from those referred to standard solutions as required for LC–MS^n methods, allowing us reliable confirmation of the identity [19].

After confirmation, the quantitative assay of azo-dyes identified in the samples was performed using suitable calibration curves (Table 5). Among the samples investigated, Sudan I was detected in two out of four samples considered at concentrations above 1.24 mg/kg, whereas Sudan II was detected only in one chilli tomato and cheese sauce sample. Fig. 4 illustrates the use of ESI–MS/MS for confirmatory studies in the detection of the dyes investigated in one chilli tomato and cheese sauce sample.

4. Conclusions

Using RPLC under isocratic conditions and ESI(+) detection mode, a reliable LC–MS/MS method for qualitative and quantitative analysis of Sudan dyes in hot chilli food products was devised. Selectivity of the MS/MS technique coupled with chromatographic separation was proved to provide unambiguous identification and accurate determination of such compounds in complex matrices such as hot chilli products without need of laborious clean-up procedures. Further, validation data demonstrate that this method is also convenient for analysis of Sudan dye residues in food products at trace levels, since detection limits and quantitation limits in the low μg/kg were calculated in the test materials investigated and good repeatability was proved.

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