Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1058 (2004) 127–135
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Accurate mass measurements for the confirmation of Sudan azo-dyes in hot chilli products by capillary liquid chromatography–electrospray tandem quadrupole orthogonal-acceleration time of flight mass spectrometry

F. Calbiani, M. Careri, L. Elviri*, A. Mangia, I. Zagnoni

Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università degli Studi di Parma, Parco Area delle Scienze 17/A, 43100 Parma, Italy

Abstract

The potential of capillary liquid chromatography (microLC)–quadrupole/time-of-flight mass spectrometry (Q-TOF MS) for the confirmation of Sudan I, II, III and IV azo-dyes as contaminants in hot-chilli food products was demonstrated. Using the microLC–electrospray ionization (ESI)–Q-TOF MS technique, accurate mass measurements of Sudan dyes were performed both on standard solutions and on matrices. Precision of exact mass measurements was calculated taking into account the ion statistics according to the number of ion sampled in the measurement. Accurate mass measurements by MS/MS experiments were performed to elucidate azo-dye fragmentation patterns. Selectivity of the microLC–Q-TOF MS method was assessed by evaluating matrix suppression effects by pre-column injection of blank hot chilli tomato sauce matrices. The results were compared with those obtained on a LC–triple quadrupole–MS system. Confirmation of Sudan I present in hot chilli tomato sauce samples was obtained by accurate mass measurements. In real samples trueness of exact mass measurements was estimated to be 1.6 and 4.4 ppm when calculated for hot chilli tomato sauce and hot chilli tomato with cheese sauce samples, respectively; precision was calculated around 9.5 ppm.

Keywords: Sudan azo-dyes; Food analysis; Capillary liquid chromatography; Quadrupole/time-of-flight mass spectrometry

1. Introduction

The recent alarm on the contamination of hot chilli and hot chilli products originating from India and marketed in European Countries contaminated by the azo-dye Sudan I demanded for the development of reliable and accurate analytical methods for the identification and quantification of such compound in foodstuffs.

As a consequence of the non-compliance of the presence of this contaminant in hot chilli and hot chilli products with the EU food safety requirements, in June 2003 the European Commission has adopted a decision on emergency measures concerning hot chilli and hot chilli products intended for human consumption [1] and on January 2004 the EU Food Regulations 2003 decided to extend the Commission Decision 2003/460/EC requirement to cover Sudan II, Sudan III and Scarlet Red or Sudan IV [2].

As reported in the literature dealing with the analytical chemistry of Sudan dyes, recently a method based on liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry has been devised for the analysis of Sudan I in foodstuffs [3]. To the best of our knowledge, there has been no report of the analysis of all four Sudan dyes in foodstuffs apart from the very recent paper published by our researcher group [4]. In that work the development of a liquid chromatography–electrospray–ionization–triple quadrupole tandem mass spectrometry (LC–ESI–QqQ–MS/MS) method for the simultaneous iden-

* Corresponding author.
E-mail address: lisa.elviri@unipr.it (L. Elviri).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.159

tification and determination of Sudan I, II, III and IV in chilli tomato sauce and chilli tomato and cheese sauce has been reported [4]. Single-laboratory validation procedure carried out demonstrated that the analytical method proposed fits for the intended purpose. In particular, detection limits and quantification limits in the low μg/kg were obtained, making the method suitable for the analysis of Sudan dye residues in food products at trace levels [4].

Even if low-resolution triple quadrupole MS is a powerful detection system for both qualitative and quantitative analysis, difficulties may occur in the unequivocally identification of isobaric ions in complex matrices or in the interpretation of collision induced dissociation (CID) mass spectra. The use of high-resolution MS techniques such as the quadrupole orthogonal-acceleration time of flight (Q-TOF) analyzer allows to distinguish among isobaric ions and increase confidence in the identification of the analytes of interest by providing the elemental composition [5–7]. In addition, accurate mass measurements of product ions in MS/MS mode help to elucidate both unknown and structural rearrangements of fragment ions.

The results reported in this paper refer to the development of a method based on the use of capillary liquid chromatography (microLC) coupled with tandem quadrupole orthogonal-acceleration time of flight (Q-TOF) mass spectrometry for the analysis of Sudan I, II, III and IV (Fig. 1). Capillary LC was used since it benefits from improved sensitivity with respect to packed LC when MS measurements are performed on the TOF analyzer. Matrix suppression effects were evaluated and trueness and precision of exact mass measurements were calculated. Good selectivity was demonstrated the main feature of microLC–ESI–Q-TOF–MS coupling for trace analysis of contaminants.

2. Experimental

2.1. Chemicals

Sudan I (1-(phenylazo)-2-naphthol; CAS Registry Number (Nr): 842-07-9; CI 12055); Sudan II (1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 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) were obtained from Sigma-Aldrich (Milan, Italy). Stock solutions (1 mg/ml) of Sudan I and II were prepared in methanol, whereas stock solutions (1 mg/ml) of Sudan III and IV were prepared in ethyl acetate and stored at 4 °C in the dark.

Reserpine (Sigma-Aldrich) was prepared in acetonitrile at 100 μg/ml and diluted to 1 μg/ml when used as lock mass solution.

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

2.2. Liquid chromatography–tandem mass spectrometry

LC elution was performed on a Symmetry C18 capillary column (0.32 mm × 150 mm, 5 μm) (Waters, Milford, MA, USA) using a gradient solvent system [(A) 0.1%, v/v, formic acid in water/acetonitrile 85/15, v/v; (B) 0.1%, v/v, formic acid in acetonitrile/acetone 80/20, v/v]. The flow-rate was 5 μL/min. Gradient elution was as follows: solvent (B) was delivered by a linear gradient from 35 to 80% for 5 min, followed by an isocratic elution of this solvent mixture for

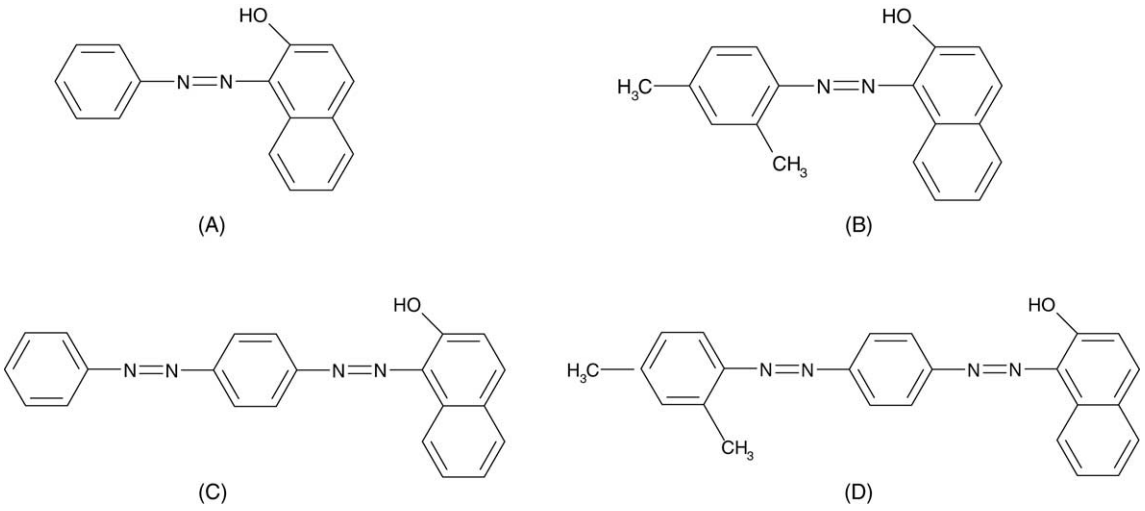

Fig. 1. Chemical structure of investigated dyes: (A) Sudan I (MW 248.0949), (B) Sudan II (MW 276.1261), (C) Sudan III (MW 352.1323), Sudan IV (MW 380.1636).

10 min, and a linear gradient to 90% in 2 min. Solvent (B) was thus maintained at 90% for 13 min before column reequilibration (15 min). The mobile phase was delivered by a CapLC pump (Waters) equipped with a 48-vial capacity sample management system. Ten microliters of standard or sample solution were loaded on a C18 Symmetry 300^{TM} (Waters) precolumn (0.32 mm × 10 mm, 5 μm) and eluted with the mobile phase on the chromatographic column to increase on-column analyte amount and avoid column contamination.

The LC system was coupled to the Micromass® Q-tof micro^{TM} mass spectrometer (Waters) equipped with a Z-spray electrospray interface. A Masslynx v. 3.5 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 10 and 600 l/h, respectively. Q-TOF external calibration was performed using a 0.1% (v/v) aqueous phosphoric acid solution and fifth-order non-linear calibration curve was usually adopted. Optimal operating parameters of the ESI interface were optimized by infusing standard solutions of Sudan I, II, III and 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). The optimum determined conditions by operating the mass spectrometer in positive ion (ESI+) mode of the interface were: electrospray voltage 3.0 kV, cone voltage 27 V for Sudan I and II and 45 V for Sudan III and IV, rf lens 0.5 V, source temperature 50 °C (range tested 50–100 °C), desolvation temperature 100 °C.

To perform the lock mass correction in the single MS mode acquisition, lock mass solution (1 μg/ml reserpine in acetonitrile) was infused using a syringe pump (Harvard Apparatus) at a flow-rate of 5 μl/min. The acquired data were converted to centroid (80% of the top peak area) to generate accurate mass spectra.

Continuum mode TOF mass spectra were acquired over the *m*/*z* 200–630 range using an acquisition time of 1 s and an interscan delay of 0.1 s. Operating in MS/MS mode, product–ion mass spectra of protonated molecules of Sudan dyes were acquired in the TOF analyzer from *m*/*z* 30 to 450. Qualitative analysis was performed by applying collision cell energy (CE) between 14 and 22 eV depending on the compound and using argon as collision gas at a pressure of 11 psi. Product ion mass spectra were lock mass corrected using the exact theoretical mass value of the precursor $[M+H]^+$ ions to ensure correct mass measurement of fragment ions.

2.3. 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.

2.4. Validation procedure

A validation procedure aimed at analyte confirmation was carried out by evaluating detection limits, accuracy of mass spectral data and selectivity.

Detection limit (LOD) was evaluated as the minimum concentration of analyte that provides a spectrum in which an exact mass measurement is feasible with a trueness value of 5 ppm independently from the sample (standard solution or real samples).

Selectivity was assessed by evaluating matrix effects both in terms of signal intensity and of exact mass measurements. For assessment of ion suppression/enhancement effects, a mixture standard solution (0.1 μg/ml) was infused in the ESI source in continuum using a tee system. At the same time, injection of matrices by pre-column concentration was carried out. Selectivity related to accuracy in the exact mass measurements for confirmation purposes was investigated by spiking blank matrix extracts (i.e. hot chilli tomato and hot chilli tomato with cheese sauces) with a standard solution of dyes (0.1 μg/ml).

Accuracy was evaluated in terms of trueness and precision according to ISO 5725 [8].

Precision of exact mass measurements was calculated upon ion statistics following the relationship [9,10]:

$$\lambda_{ppm} = \frac{10^6}{CRS^{1/2}} \quad (1)$$

where λ_{ppm} is a suitable expression of statistical error (e.g. the 95% confidence limit in ppm), C is an instrumental constant, R is the resolution of the mass analyzer and S is the number of ions sampled in the measurements. Experiments were carried out following the parameters of acquisition time and number of spectra combined reported on Table 1. In particular, the signal rates for the analytes and the lock mass were kept less than 200 counts/s and approximately equal.

Trueness was evaluated as the mean of the deviation in the measured mass of a theoretical exact mass from the actual mass. For this purpose, standard solutions of dyes were infused at different acquisition times and 12 determinations for each analysis were performed (Table 1).

2.5. Sample preparation and LC–MS analysis

Sample treatment was performed as reported in a previous paper [4]. Briefly, 1 g sample was weighed and mixed on a vortex for 2 min with 10 ml acetone. The extract was centrifuged at 4500 rpm for 10 min. A 1-ml portion of clarified solution was removed and added to 1 ml of HPLC-grade water. The extract was filtered through a 0.45 μm nylon syringe filter (Supelco, Bellefonte, PA, USA) before pre-column concentration (10 μl) and injection onto the LC–MS system.

Three replicated extractions for each kind of sample and five replicated analyses for each extract were performed using a lock mass solution of reserpine continuously post-column infused at 2 μl/min to produce a signal rate of ∼200 counts/s.

Table 1
Mean mass measurement errors and 95% confidence limits for Sudan compounds obtained by standard infusion

Anal. no.	Scan time (s)	Estimate signal rate (counts/s)[a]	Number of spectra combined	S (number of ions sampled)[b]	$1/S^{1/2}$	Mean error in measured mass (ppm)[c]	λ_{ppm} (ppm)[d]
Sudan I							
1	0.24	124.6	5	149.5	0.08178	−0.05	7.31
2	0.48	124.4	5	298.6	0.05787	0.83	5.65
3	0.96	128.4	5	616.2	0.04028	0.59	4.28
4	1.92	115.6	5	1110.3	0.03001	−0.02	2.42
5	0.24	124.6	20	598.0	0.04089	−0.06	2.60
6	0.48	124.4	20	1194.2	0.02893	−1.06	2.46
Sudan II							
1	0.24	135.9	5	163.1	0.07831	−0.52	6.21
2	0.48	144.3	5	346.4	0.05373	0.02	7.93
3	0.96	148.7	5	713.7	0.03743	−1.02	3.42
4	1.92	129.7	5	1245.6	0.02833	−0.93	1.90
5	0.24	135.9	20	652.2	0.03915	−0.85	2.91
6	0.48	144.3	20	1385.4	0.02686	−0.78	3.27
Sudan III							
1	0.24	116.1	5	139.3	0.08472	3.08	6.16
2	0.48	131.1	5	314.7	0.05637	0.67	3.98
3	0.96	131.9	5	632.9	0.03974	1.58	2.37
4	1.92	115.7	5	1110.3	0.03001	0.58	2.37
5	0.24	116.1	20	557.2	0.04236	2.33	2.95
6	0.48	131.1	20	1258.8	0.02818	1.36	2.27
Sudan IV							
1	0.24	151.9	5	182.3	0.07406	0.29	5.79
2	0.48	153.8	5	369.2	0.05204	−0.04	4.84
3	0.96	158.8	5	762.5	0.03621	−0.40	4.32
4	1.92	149.1	5	1431.6	0.02642	0.31	2.99
5	0.24	151.9	20	729.2	0.03703	−0.08	3.88
6	0.48	153.8	20	1477	0.02802	−0.06	2.61

[a] Combined signal for 100 spectra/(100 × acquisition time).
[b] Signal rate × acquisition time × number of spectra combined.
[c] Statistical mean of differences between actual and observed mass, 12 determinations.
[d] $t_{0.05,\,12}$ × standard deviation; ($t_{0.05,\,12}$ = 1.78).

Ion suppression induced by the lock mass solution was evaluated by injecting a standard mixture of Sudan dyes at a concentration of 0.1 μg/ml with and without infusion of 1 μg/ml reserpine solution.

Real samples were analyzed both in MS full scan and MS/MS product–ion scan mode under the experimental conditions reported in Section 2.2.

Precision and trueness were assessed on real samples. Precision was calculated by using Eq. (1), whereas trueness was evaluated as difference between the detected mean exact mass value of three replicate analyses and the theoretical exact mass.

3. Results and discussion

3.1. Chromatographic separation

Under RP partitioning mode using a C18 capillary column with gradient elution of 0.1% (v/v) formic acid aqueous/acetonitrile solution and 0.1% (v/v) formic acid in acetonitrile/acetone solution was successfully used to separate Sudan azo-dyes. Pre-column concentration of 10 μl of standard or sample solutions allowed us to improve detectability by increasing on-column analyte amount. Under optimized LC conditions good separation was achieved within 27 min, the four contaminants eluting with high repeatability (retention time of Sudan I 11.75 ± 0.20 min, Sudan II 16.60 ± 0.14 min, Sudan III 21.18 ± 0.24 min, Sudan IV 26.82 ± 0.52 min, n = 10). Fig. 2 depicts the LC–ESI(+)–Q-TOF MS extracted ion chromatograms of blank hot chilli tomato sauce sample spiked with Sudan dyes (0.1 μg/ml) in MS acquisition mode.

3.2. ESI–Q-TOF MS and ESI–Q-TOF MS/MS

The effect of different interface and mass analyzer parameters, such as source temperature, cone voltage, electrospray voltage and instrumental set mass, was investigated by infusion of standard solution (0.1 μg/ml) of Sudan dyes. The ESI voltage in the 1.8–3.0 kV range was found to greatly affect response of the four Sudan dyes with a maximum response at 3.0 kV. By increasing this parameter up to 4.0 kV, a stable state was observed, thus indicating Sudan dyes were almost completely ionized at 3.0 kV. Cone voltage was varied from 10 to 80 V and the optimum value was chosen in such a

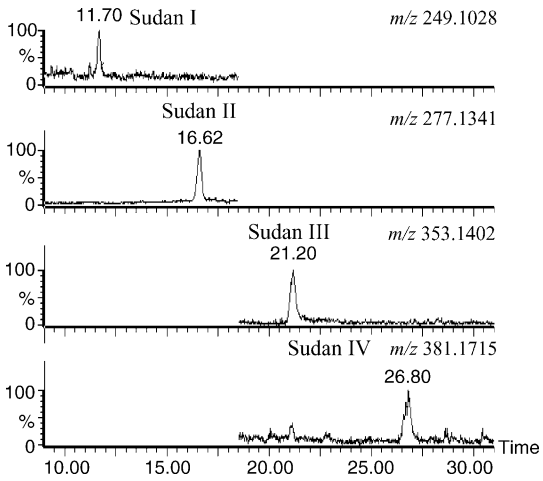

Fig. 2. LC–ESI(+)–Q-TOF MS extracted ion chromatograms of blank hot chilli tomato sauce sample spiked with Sudan dyes (0.1 μg/ml) in MS acquisition mode. For operative conditions see Section 2.

way to avoid in-source fragmentation and obtain the highest protonated molecule signal for each analyte. In particular, the optimum values were found to be 27 V for Sudan I and II and 45 V for Sudan III and IV, respectively. By increasing cone voltage to 45 V, Sudan I and II started to undergo in-source fragmentation, whereas in the case of the more stable protonated molecules of Sudan III and IV a cone voltage of 60 V should be applied to fragment these ions. As for the source temperature, the results obtained showed that this parameter had large influence on the detector responses of the analytes, which were found to be five-fold improved varying from 50 to 100 °C. However, even though for all the analytes maximum response intensity was observed at 100 °C, the use of high temperature values was not feasible because of systematic precipitation of the most hydrophobic compounds Sudan III and IV in the ESI capillary. This behavior could be explained by invoking both a significant evaporation of acetone in the eluent at temperatures greater than 50 °C and the use of an ESI capillary with reduced i.d. (50 μm). This phenomenon was not observed when using a more conventional ESI capillary (i.d. 100 μm) [4].

For this reason, a compromise between this drawback and sensitivity was adopted setting a source temperature of 50 °C. Instrumental set mass was optimized in the 240–400 Da in order to increase ion transmission and thus sensitivity. Since Sudan I is the most frequently azo-dye found in foodstuffs, the operating value was chosen as that optimal for this contaminant, i.e. 250 Da.

CID of Sudan dyes had been previously carried out using a triple quadrupole mass spectrometer instrument [4]. Compared to the triple quadrupole instruments, Q-TOF MS/MS has the unique capability of determining accurate mass on the fragment ions generated in the collision cell. This ability is particularly important in the structural elucidation of unknowns. In this work, using Q-TOF analyzer, MS/MS fragmentation was carried out both in the collision cell and performing in-source CID experiments. Preliminarily, MS/MS mass spectra of Sudan dyes were recorded by fragmenting in the collision cell the $[M+H]^+$ ions of analytes under accurate mass measurement conditions. Fig. 3 shows the MS/MS product–ion mass spectra of all Sudan dyes and the corresponding fragmentation pathways. Sudan I (collision energy 18 eV) and Sudan II (collision energy 14 eV) showed a stable fragment ion at *m/z* 232.1959 (3.8 ppm) and 260.3264 (4.6 ppm), respectively attributed to the loss of a radical hydroxyl group. No further fragmentation of these ions was observed under the experimental conditions chosen. In-source fragmentation confirmed these findings. In fact, by performing in source-CID of the protonated molecule and selecting the ions at *m/z* 232 and 260, respectively, fragmentation was obtained operating at collision energy higher than 45 eV.

The MS/MS product–ion mass spectra of all analytes showed a signal at *m/z* 156.0451 that was attributed to the $[C_{10}H_6NO]^{\bullet+}$ ion with a trueness in the −1.3 to 5.7 ppm range. In this case, the use of the high-resolution Q-TOF mass analyzer allowed us to identify the fragment ion at *m/z* 156 with more increased confidence with respect to the QqQ system [4]. This characteristic signal, which predominates in the spectrum of Sudan I, resulted from cleavage of the N=N double bond on the side of the naphtalen-ol group. For all azo dyes the same cleavage gave rise to the fragments at *m/z* 93.0566, 120.0803, 196.0873 and 224.1191 for Sudan I, II, III and IV, respectively (Fig. 3). In the case of Sudan I and II, one hydrogen transposition is supposed to be involved in the breakdown of the N=N double bond. In the case of Sudan III and IV, two mechanisms involving one and two hydrogen transfer, respectively, seem to occur. As for Sudan III, a further fragmentation of the peak at *m/z* 197 deriving from direct cleavage of the C–N bond lead to the formation of the fragment ions at *m/z* 77.0370 and 120.0555, respectively. By fragmentation of the same C–N bond, the protonated molecular ion of Sudan III give rise to the 248.0934 *m/z* ion. Analogously, considering the protonated molecule of Sudan IV at *m/z* 381, cleavage of the N–C bond on one side of the azo linkage provided two signals at *m/z* 91.0536 and 276.1144, respectively. In particular, the ion at *m/z* 91.0536 (−5.2 ppm) resulted from the release of a methyl group with transfer of one hydrogen.

The in-source CID of Sudan I followed by MS/MS fragmentation of the *m/z* 156 ion showed two fragment ions at *m/z* 128.0504 and at *m/z* 101.0353 attributed to the loss of CO (−3.1 ppm) and of HCN (−4.9 ppm), respectively (Fig. 4A). In addition, cleavage of C–N and N–C bonds on either side of the azo-linkage was observed. In particular, the ion at *m/z* 232 of Sudan I gave rise to the ions at *m/z* 77.0388 (−3.5 ppm) and at *m/z* 127.0550 (−1.6 ppm), respectively (Fig. 4B). Sudan II showed a spectral behavior analogous to that of Sudan I (data not shown). The MS/MS fragmentation of Sudan III performed on the ion at *m/z* 196 generated in the ESI source showed different product ions at *m/z* 167.0775, 141.0721, 120.0571, 92.0504 and 77.0392 (Fig. 4C). The fragment ions at *m/z* 120 and 77 were previously identified. The fragment

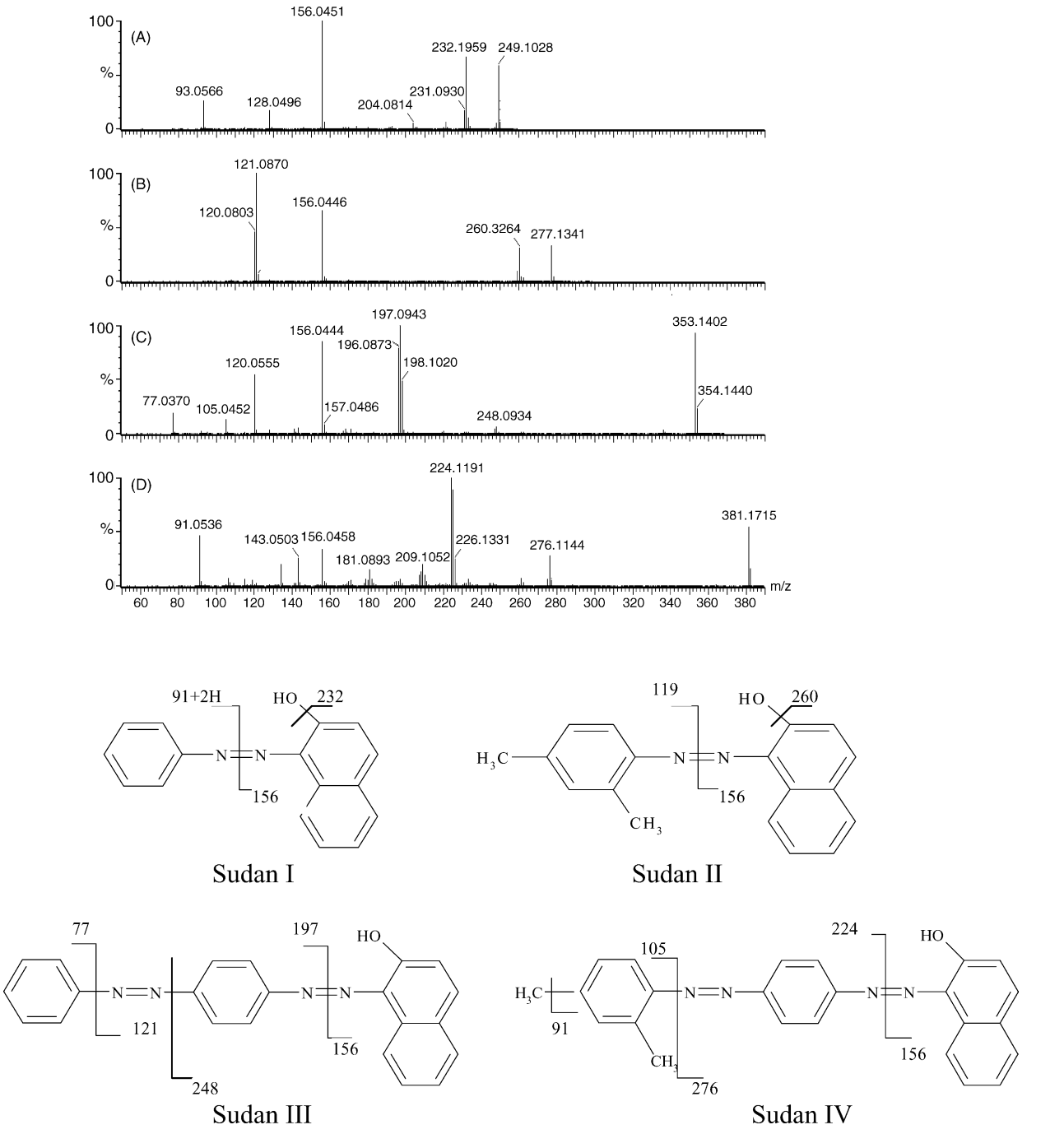

Fig. 3. Q-TOF MS–MS product–ion spectra of standard solution (0.1 μg/ml) mixture of (A) Sudan I, (B) Sudan II, (C) Sudan III and (D) Sudan IV and fragmentation pathways of the four azo-dyes.

ion at *m/z* 92.0504 raised from the cleavage of the N=N double bond (4.3 ppm). The MS/MS fragmentation of Sudan IV performed on the ion at *m/z* 276 generated in the ESI source showed two ions at *m/z* 156 and 128, the identification of which was previously discussed. The fragmentation of ion at *m/z* 224 (Fig. 4D) resulted in the formation of ions at *m/z* 180, 106 and 91. The signal at *m/z* 106.0652 is attributable to the N=N azo-bond cleavage with a loss of methyl group and one hydrogen transfer (4.7 ppm). The $[C_{12}N_2H_8]^+$ ion accounts for the fragment at *m/z* 180.0685 (−1.1 ppm), whereas the transfer of one hydrogen lead to the formation of the ion at *m/z* 181.0768 (−1.1 ppm).

3.3. LC–ESI–Q-TOF MS method performance

Method performance parameters evaluated for qualitative purposes were accuracy of MS data, detection limits and selectivity.

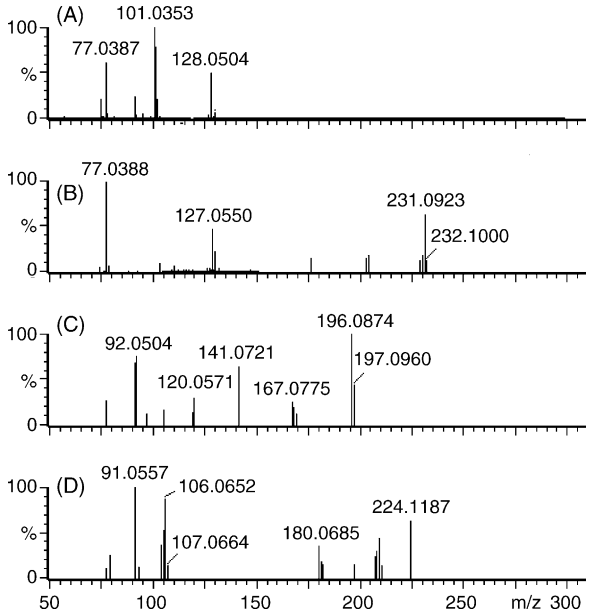

Fig. 4. In source CID Q-TOF MS–MS spectra of (A–B) *m/z* 156 and *m/z* 232 fragment ions of Sudan I, (C) *m/z* 196 fragment ion of Sudan III, (D) *m/z* 224 fragment ion of Sudan IV.

Accuracy of MS data is critical for Q-TOF MS measurements. By performing replicated exact mass measurements, we evaluated accuracy on the measured mass in terms of both random error and systematic error. Accuracy was thus evaluated considering the two components, i.e. precision and trueness [8].

By using the approach reported in the literature [9,10], good results in terms of mean error in the measured mass (trueness) and λ_{ppm} (precision) were obtained (Table 1). In particular, the mean errors in mass measurements were approximately 0.04 ppm for Sudan I, −0.7 ppm for Sudan II, 1.6 ppm for Sudan III and 0.003 ppm for Sudan IV (Table 1).

A non-weighted least-square linear regression was used to fit the precision (λ_{ppm}) versus $1/S^{1/2}$ and a linear calibration well approximated the data (Table 2), the slopes of the calibration curves representing the $10^6/CR$ term in Eq. (1). Comparable slope values were obtained for all the analytes investigated suggesting that the 95% confidence limits for a mass measurement are very similar for all the dyes depending only on the number of ion sampled for each analyte.

Detection limits (LOD) were calculated on both standard solutions and matrices (Table 3). Instrumental detection limits were in the 0.02–0.05 μg/ml range, whereas LODs ranged from 0.4 to 1.1 μg/g when calculated on matrix. No difference in terms of detectability was observed for all the analytes when considering hot chilli tomato sauce and hot chilli tomato and cheese samples. By comparing these results to those obtained using LC–QqQ–MS/MS method (LODs calculated on matrices 4–24 μg/kg) [4], it can be inferred that the method previously developed is demonstrated to be more powerful to detect such contaminants in foodstuffs at trace level.

Table 2
Non-weighted least-square linear regression of precision (λ_{ppm}) vs. $1/S^{1/2}$

Analyte	b_1 ($\pm s_{b_1}$)	r^2 ($n=6$)
Sudan I	89 ± 5	0.908
Sudan II	96 ± 12	0.756
Sudan III	71 ± 2	0.969
Sudan IV	92 ± 7	0.787

Calibration fitting: $Y = b_1 X$.

Table 3
Limits of detection calculated for Sudan dyes using LC–Q-TOF MS

Analyte	Instrumental (μg/ml)	Matrix (μg/g)[a] (hot chilli tomato sauce)
Sudan I	0.05	0.9
Sudan II	0.02	0.4
Sudan III	0.03	0.9
Sudan IV	0.05	1.1

[a] Referred to 1 g sample extracted with 10 ml solvent (see Section 2).

Selectivity was assessed by studying matrix effect, since interferences deriving from matrix components can adversely affect confirmation (i.e. accuracy of exact mass measurements) and detection (signal intensity) of analytes. This effect arising from co-eluting species or co-elution of salts was evaluated for each analyte by using both matrices under investigation. By monitoring MS response for each *m/z* channel referred to protonated molecules, no drops or enhancements of ESI(+) response was observed for all the analytes. These findings suggested the absence of matrix effects in terms of signal intensity in the experimental conditions chosen. However, when analyzing Sudan I and II at higher source temperature (i.e. 100 °C), variations of the analyte signals were observed in agreement with the results obtained on the LC–QqQ–MS/MS system [4]. In particular, the response of these azo-dyes resulted to be enhanced by both matrices. The different behavior observed for these dyes at 50 and 100 °C could be explained on the basis of a different matrix ionization efficiency with respect to that regarding analytes. As for Sudan III and IV, since as above discussed it was not possible to operate at source temperatures higher than 50 °C, matrix effect was not investigated.

As for the evaluation of ion suppression caused by the lock mass solution, no decrease was observed on the peak area of analytes to demonstrate the absence of ion suppression phenomena.

Concerning selectivity of the method in the azo-dye confirmation, the experiments performed did not evidence matrix interferents affecting trueness and precision of the exact mass measurements.

3.4. Application

The microLC–ESI(+)–Q-TOF MS method developed was applied to the confirmation of Sudan azo-dyes in the samples under investigation.

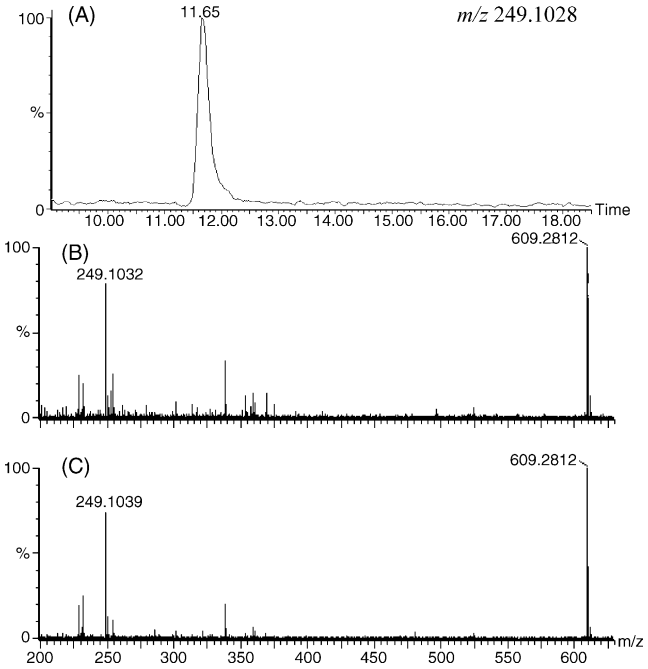

Fig. 5. LC–ESI(+)–Q-TOF MS (A) extract ion chromatogram of Sudan I (theoretical exact mass 249.1028) in a positive hot chilli tomato sauce and spectra in a positive (B) hot chilli tomato (calculated exact mass 249.1032, trueness 1.6 ppm) and (C) hot chilli tomato with cheese sauce (calculated exact mass 249.1039, trueness 4.4 ppm) samples. For operative conditions see Section 2.

The TOF analyzer provides full-scan mass spectrum to enable unequivocal confirmation of the presence of the azo-dyes in two out of six sauce samples analyzed. In particular, Sudan I was identified in one batch of hot chilli tomato sauce and in one batch of hot chilli tomato with cheese sauce samples (Fig. 5). Further, by performing microLC–MS/MS product–ion experiments we exploited the knowledge gained from the precursor azo-dye fragmentation pathway to increase confidence in analyte identity in the real samples investigated. The product–ion mass spectra of Sudan I obtained both for hot chilli tomato sauce and hot chilli tomato sauce with cheese (Fig. 6) showed a fragmentation pattern matching that of the analyte in a standard solution (Fig. 3) in terms of the product ions and relative intensities (±15%). Among criteria used for confirmation, a retention time criterion of within 2% was also satisfied.

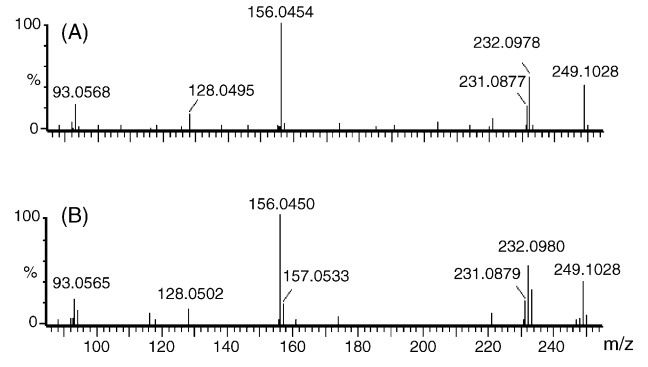

Fig. 6. LC–ESI(+)–Q-TOF MS/MS product–ion spectra of Sudan I in (A) a positive hot chilli tomato sauce and in (B) a positive hot chilli tomato with cheese sauce samples. For operative conditions see Section 2.

Trueness of the exact mass measurements in the samples was estimated to be 1.6 and 4.4 ppm when calculated for hot chilli tomato sauce and hot chilli tomato with cheese sauce samples respectively. In the case of Sudan I, estimate of precision by means of the calibration curves reported in Table 2 lead to adequate λ_{ppm} values, i.e. of 9.5 and 9.6 referred to the hot chilli tomato and hot chilli tomato with cheese sauce samples respectively. According to Eq. (1), these values are related to the number of ions sampled for Sudan I in the real samples (i.e. S = 104 in the case of hot chilli tomato sauce and S = 106 in the case of hot chilli tomato with cheese sauce) and independent of matrix components.

4. Conclusions

The microLC–ESI–Q-TOF MS method developed was demonstrated to be a powerful confirmation method for contaminants in complex foodstuff matrices. Complementary information to that obtained by LC–ESI–QqQ–MS technique was obtained. The high resolution of the Q-TOF MS mass analyzer allowed us to obtain unambiguous confirmation of Sudan I in real samples by means of the accurate mass by MS and MS/MS experiments. Useful information for elucidating spectral fragmentation was obtained by performing both in-source CID and fragmentation in the collision cell of the Q-TOF MS system.

Acknowledgements

This work was financially supported by MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Italy), COFIN 2002 Project "Food safety. New analytical methods for the control of natural trace contaminants".

References

[1] Commission Decision of 20 June 2003 on emergency measures regarding hot chilli and hot chilli products, notified under document number C(2003) 1970, (2003/460/EC), OJ L. 154/114, 21.6.2003.
[2] Implementation of Commission Decision 2003/460/EC of the 21st January 2004.
[3] F. Tateo, M. Bonomi, J. Agric. Food Chem. 52 (2004) 655.
[4] F. Calbiani, M. Careri, L. Elviri, A. Mangia, L. Pistarà, I. Zagnoni, J. Chromatogr. A 1042 (2004) 123.
[5] M.L. Gross, J. Am. Soc. Mass Spectrom. 5 (1994) 57.

[6] H.R. Morris, T. Paxton, A. Dell, J. Langhorne, M. Berg, R.S. Bordoli, J. Hoyes, R. Bareman, Rapid Commun. Mass Spectrom. 10 (1996) 889.
[7] A. Shevchenko, I. Chernushevich, W. Ens, K.G. Standing, B. Thompson, M. Wilm, M. Mann, Rapid Commun. Mass Spectrom. 11 (1997) 1015.
[8] ISO 5725-1: 1994, Accuracy (trueness and precision) of measurement methods and results, General principles and definitions.
[9] A. Tyler, E. Clayton, B. Green, Anal. Chem. 68 (1996) 3561.
[10] K.F. Blom, Anal. Chem. 73 (2001) 715.