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Analytical Methods

Simultaneous quantitative determination of Sudan dyes using liquid chromatography–atmospheric pressure photoionization–tandem mass spectrometry

M.R.V.S. Murty, N. Sridhara Chary, S. Prabhakar, N. Prasada Raju, M. Vairamani *

National Centre for Mass Spectrometry, Indian Institute of Chemical Technology, Hyderabad 500 007, India

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ABSTRACT

Atmospheric pressure photoionization–tandem mass spectrometry (APPI–MS/MS) method has been developed for quantitative determination of Sudan I to IV dyes. This study demonstrates the applicability of a simple isocratic normal phase HPLC method using isopropanol (0.3%) in *n*-hexane as the mobile phase for the separation of these dyes. A simple extraction procedure using *n*-hexane has been applied for the extraction of these dyes from spiked samples of chilli powder and tomato sauce. The quantitative determination of Sudan I to IV is obtained from the spiked tomato sauce and chilli powder samples by external standard method under single reaction monitoring (SRM) mode. The study includes a detailed investigation on LOD, LOQ, linearity and recovery of Sudan I to IV dyes. The LOD ranged from 5–18 μg/l and LOQ ranged from 10–24 μg/l. The present method can be a powerful analytical tool for the simultaneous quantitative determination of Sudan dyes present in food products.

1. Introduction

Sudan dyes (Sudan I, II, III and IV) belong to the family of industrial azo dyes that are traditionally used in waxes, inks, plastics, oils and polishes (Nagase, Osaki, & Matsueda, 1989; Stiborova, Martínek, Rýdlová, Hodek, & Frei, 2002). Recently, these dyes have been found in food products imported by European and several other countries (Commission Decision, 2003; Mazzetti et al., 2004). Sudan dyes are added to food products such as chilli powder and sauce because the red hue mimics the colour of the natural products. Sudan dyes are recognised as potential carcinogens (Puoci et al., 2005; Stiborova et al., 2002). Consequently, adulteration of any food product by Sudan dyes constitutes a risk to public health. The Food and Drug Administration (FDA) and European Union (EU) classify Sudan dyes as illegal food-additives because of the associated health risks (Commission Decision, 2003). International Agency for Research on Cancer (IARC, 1975) has classified Sudan dyes as category 3 carcinogens to humans and due to this fact, any national and international food regulation act does not permit the use of these colourants as food-additives.

Sudan I is known to cause tumours in the liver or urinary bladder of mice and rabbits and considered to be a possible carcinogen and mutagen for humans (Moller & Wallin, 2000; Stiborova et al., 2002). Sudan I to IV dyes are not permitted colourants and considered to be genotoxic carcinogens and their presence is not permitted in foodstuff for any purpose at any concentration level. Hence, the consignments of chilli and food products imported should be accompanied by an analytical report showing the absence of such Sudan compounds by a suitable technique.

The electron ionisation (EI) mass spectra of Sudan I, II, III, and IV are available in the commercial mass spectral libraries; however, there have been only a few reports on the GC–MS analysis of these compounds, which includes a recent report (He et al., 2007) on the determination of Sudan dyes in eggs by silylation prior to GC–MS analysis. Thermospray, particle beam and electrospray ionisation techniques with liquid chromatography–mass spectrometry were reported for the analysis of azo dyes (Straub, Voyksner, & Keever, 1992). The particle beam EI mass spectrometric analysis resulted in molecular ion information and various fragment ions to characterise a few commercial azo dyes. Several methods were developed to detect the presence of these compounds using high performance liquid chromatography (HPLC) (Chen, Mou, Hou, Riviello, & Ni, 1998; Cornet, Govaert, Moens, Loco, & Degroodt, 2006; Mazzetti et al., 2004; Pielesz, Baranowska, Rybak, & Wlochowicz, 2002; Zhang, Zhang, Gong, Gopalan, & Lee, 2005; Zhang, Zhang, & Sun, 2006) with various detectors and capillary electrophoresis (Mejia, Ding, Mora, & Garcia, 2007). Sudan dyes were also studied by HPLC–ESI–MS (Ma, Luo, Chen, Su, & Yao, 2006) and HPLC–APCI–MS (Tateo & Bononi, 2004).

* Corresponding author. Present address: Institute of Pesticide Formulation Technology, Sector-20, Udyog Vihar, Gurgaon 122 016, India. Tel.: +91 124 2347788; fax: +91 124 2348489.
E-mail address: mvairamani@hotmail.com (M. Vairamani).

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The assay of Sudan I in foodstuffs at a concentration of 8–10 μg/kg was determined by APCI–MS/MS method (Donna, Maiuolo, Mazzotti, Luca, & Sindona, 2004). HPLC–ESI–MS/MS method under selected reaction monitoring (SRM) mode was reported for the simultaneous identification and quantification of Sudan I to IV present in the concentration range of 3–48 μg/kg in various food products (Calbiani et al., 2004a; Mazzetti et al., 2004). A method using HPLC–ESI–QTOF–MS instrument (Calbiani, Careri, Elviri, Mangia, & Zagnoni, 2004b) was also demonstrated to be a powerful confirmation method for the unambiguous identification of these dyes in complex food matrices, but the HPLC–ESI–MS/MS method reported by the same group was mentioned to be more sensitive to detect these analytes.

ESI and APCI techniques have become the standard ionisation interfaces for HPLC–MS/MS systems used for qualitative or quantitative analysis of small molecules. ESI usually produces little fragmentation whilst forming both protonated and deprotonated ions of most polar compounds in the positive and negative ionisation modes, respectively. In contrast to ESI, APCI generates ions from less polar compounds in combination with a corona discharge assisted by a heated nebulizer and can be operated at higher LC flow rates. APCI is generally less susceptible than ESI to matrix effects. However, APCI may produce in-source fragmentation of thermally unstable compounds. Another technique, atmospheric pressure photoionization (APPI), introduced by Bruins and co-workers (Robb, Covey, & Bruins, 2000), is a relatively new and novel ionisation interface for the HPLC–MS/MS system. It offers an alternative method of producing ions and sending them into the mass spectrometers. In APPI, the ionisation is initiated by 10 eV photons emitted by a krypton discharge lamp. The photoionization lamp replaces the discharge needle used in APCI; otherwise the body of the APPI ion source is very similar to the heated nebulizer body in APCI. A substance of favourable ionisation energy (IE) called dopant is introduced into the source (Kauppila et al., 2002). The initial reaction in the APPI is the formation of a radical cation of the dopant by 10 eV photons. For this reaction to occur, the IE of the dopant has to be lower than the energy of the photons. The dopant radical cations formed may then ionise the analyte through charge exchange. Alternatively, the dopant radical cation can ionise solvent molecules by proton transfer; the protonated solvent molecules can then protonate the analyte molecules. Eventually, either a radical cation or a protonated molecule of the analyte is detected (Kauppila et al., 2002).

In this work, we have investigated the potential for using APPI as an alternative to ESI and APCI, and demonstrated its applicability to real samples for the determination of Sudan dyes present in food products. The study is focussed on the investigation of dopant-assisted ionisation on the formation of radical cations using normal phase HPLC–APPI–MS/MS method. The instrumental limits of detection (LOD) and limits of quantification (LOQ), linearity and recovery of Sudan dyes from chilli powder and tomato sauce have also been obtained.

2. Experimental

2.1. Materials

Sudan I (1-(phenylazo)-2-naphthalenol), Sudan II (1-(2, 4-dimethylphenyl) azo-2-naphthalenol), Sudan III (1-(4-phenylazophenylazo)-2-naphthol and Sudan IV (*o*-tolylazo-*o*-tolylazo-2-naphthol) were obtained from Sigma–Aldrich (Steinheim, Germany). HPLC grade solvents were obtained from E-Merck (Mumbai, India). Tomato sauce and chilli powder were purchased from local market.

2.2. Preparation of standard solutions

Stock solutions (0.1 mg/ml in *n*-hexane) were separately prepared for all the analytes and diluted further with *n*-hexane to obtain the required concentration. To study the linearity, solutions having the concentration range 25–1000 μg/l were prepared. Solutions having the concentrations of 500 and 1000 μg/l containing each of Sudan I to IV were also prepared for spiking in to the tomato sauce and chilli powder. One millilitre of the solution was added to each matrix (1 g), homogenised for 2 min. and kept at 70 °C for 1 h before extracting with solvent.

2.3. Extraction procedure

The spiked matrix (1 g) was extracted with *n*-hexane by the addition of 2 ml *n*-hexane, followed by 2 min. vortex. The extraction was repeated with 2 ml and 1 ml of *n*-hexane. The supernatant solutions were pooled, dried over Na_2SO_4 and centrifuged at 5000 rpm for 10 min. The volume of the total solution, if necessary, was made up to 5 ml with *n*-hexane to achieve constant final volumes for correct estimation of concentration of the analytes in the solution. The solutions were directly subjected to LC–MS analysis.

2.4. Chromatographic conditions

Analyses were performed on a Waters 2690 series Alliance HPLC (Waters, Milford, MA) with a quaternary pump equipped with a 120-vial capacity sample management system. A Nova-Pak Silica LC column (150 × 3.9 mm, 4 μm, Waters, Ireland) with a solvent flow rate of 150 μl/min was used. The sample injection volume was set at 20 μl and injected by autosampler. Isopropanol (0.3%) in *n*-hexane was used as the mobile phase under isocratic mode. A Valco-T union was incorporated after column to introduce dopant in to the source during dopant-assisted ionisation. The dopant flow was optimised and admitted in to the source at a flow rate of 40 μl/min using an integrated infusion pump.

2.5. Mass spectrometry conditions

A bench top triple quadrupole mass spectrometer, Quattro micro (Waters, Manchester, UK) equipped with an APPI interface, and a Masslynx v.4.1 software was used for data acquisition and processing. The APPI system is composed of a heated nebulizer to vapourise the sample prior to inducing ionisation, and a power supply for krypton lamp to continuously generate 10 eV photons for photoionization.

The nebulizing gas (nitrogen) and desolvation gas (nitrogen) were delivered at a flow rate of 50 and 400 l/h, respectively. Argon was used as the collision gas and the collision cell pressure was 2×10^{-3} mbar. Operating parameters of the positive ion APPI interface were optimised by infusing 1 μg/ml standard solutions of each Sudan I to IV separately at a flow rate of 10 μl/min using an integrated syringe pump. The optimum APPI interface conditions were: repeller voltage 0.92 V, cone voltage 25 V, extractor 3 V, rf lens 0.2 V, source temperature 100 °C, APPI probe temperature 300 °C. The full scan mass spectra were acquired under continuum mode over the scan range *m/z* 200–500 using a step size of 0.1 Da and at a rate of 0.2 scans/s.

The product ion mass spectra were acquired in the mass range of 30–500 Da. Parameters for the multiple reaction monitoring (MRM) method were optimised using product ion mass spectra. For quantitative analysis only one transition was used, however, three transitions for each analyte can be used for qualitative analysis. MRM transitions at different collision energies are reported for each analyte in Table 1. The dwell time and the inter-channel delay were set at 0.1 and 0.01 s, respectively. During LC–MS/MS

Table 1
MRM transitions at different collision energies for each analyte.

Analyte	MRM transitions (*m/z*)	Collision energy (eV)
Sudan I	248→115	35
	248→143[a]	25
	248→143 / 171	15
Sudan II	276→115	35
	276→105[a]/143	25
	276→247	15
Sudan II	352→143	35
	352→247/143	25
	352→247[a]	15
Sudan IV	380→143/91	35
	380→91	25
	380→261[a]	15

[a] Transition monitored for quantitative analysis.

analyses four different time windows (0–14.5, 0–14, 13–16 and 14–18 min) were used to determine all the analytes.

2.6. Quantification and method evaluation

The sensitivity of the method was evaluated by estimating the LOD and the LOQ at a signal to noise ratio (S/N) of 3 and 10, respectively, for the standard solutions. Quantification was based on external standard calibration method. Six concentrations of each analyte in the range of 25–250 μg/l were used to construct the calibration curves. For extraction recovery calculations, matrices were spiked with known amounts of each analyte at two final concentration levels (100 and 200 μg/kg) and subjected to extraction as described above. The linearity of the method was investigated by calculation of the regression line by the method of least squares and expressed by the squared correlation coefficient (R^2). The relative standard deviation (RSD) at two concentration levels were determined ($n = 3$).

3. Results and discussion

3.1. APPI–MS and APPI–MS/MS

APPI–MS and APPI–MS/MS spectra of Sudan dyes were recorded in the positive ion mode. The full scan APPI–MS spectra of the Sudan dyes show abundant $[M+H]^+$ ion which confirms the molecular mass and no radical cation was observed even in the absence of protic solvent. This indicates that the main ionisation process is a proton transfer reaction between either by the protic solvent impurities or the moisture and the analyte molecules. The formation of $[M+H]^+$ ion in APPI indicates the greater proton affinity of these analytes. In the case of Sudan I and II, a less intense fragment corresponding to the loss of an ·OH was also observed at *m/z* 232 and *m/z* 260, respectively. This shows that less stable molecular ions are also formed. The abundance of the $[M+H]^+$ ion was found to be more with methanol compared to *n*-hexane as solvent.

The product ion mass spectra of $[M+H]^+$ ion (Fig. 1) under APPI–MS/MS conditions showed a characteristic fragmentation pattern for all the analytes. The product ion at *m/z* 156 is found in all the cases which is expected from the cleavage of C–N bond on the opposite side of the naphthalene group with hydrogen transfer to form $[C_{10}H_8N_2]^+$ as reported earlier (Calbiani et al., 2004a). But the recent report (Donna et al., 2007) on the Sudan azo dye fragmentation by deuterium labelling experiments under ESI–MS/MS revealed that the ion at *m/z* 156 is an even-electron nitrene ion obtained by the naphthol moiety. The scission of the aza bond was expected to be assisted by proton migration from the naphthol to the aza moiety through the formation of a reactive intermediate. The loss of a hydroxyl radical and a water molecule is the common fragmentation pattern observed in all these compounds. The fragmentation pathways in APPI–MS/MS are similar to that reported earlier under ESI–MS/MS (Calbiani et al., 2004b; Donna et al., 2007).

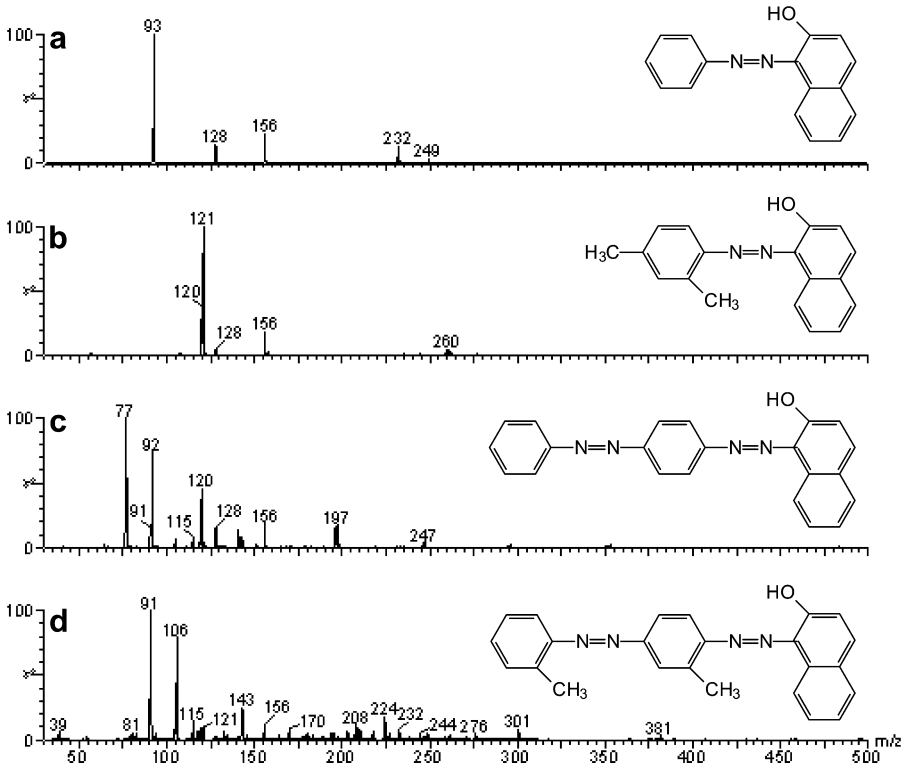

Fig. 1. APPI–MS/MS product ion mass spectra of: (a) Sudan I; (b) Sudan II; (c) Sudan III and (d) Sudan IV.

3.2. Effect of dopant

The increase in the ionisation efficiency was expected with use of dopant and was first introduced in connection with photoionization–ion mobility spectroscopy (PI–IMS) in which acetone, benzene, toluene and xylene were successfully applied as dopants (Kauppila, Kostiainen, & Bruins, 2004). The use of benzene was not suggested in view of its high toxicity. In most of the literature reports on APPI, acetone, toluene and anisole have been used as dopants (Kauppila et al., 2004). Acetone is soluble in water and gives protonated acetone ions during APPI and hence, works well for the analytes with high proton affinity (PA). Toluene has been successfully used for the ionisation of low PA analytes, because it allows charge exchange in solvents with low PA. In solvents having high PA such as acetonitrile and methanol, the toluene radical cation readily gives up its proton to the solvent, which promotes proton transfer instead of charge exchange. Samples that have low IEs and low PAs can be ionised efficiently by means of charge exchange between the anisole radical cation and the analyte in APPI.

To study the effect of dopant on the ionisation mechanism, experiments were conducted initially without the dopant and then acetone, toluene and anisole were used as dopants. Sudan I (1 mg/l) was used as the model compound for this study. Ten microlitres each of acetone, toluene and anisole were separately added to Sudan I solution in *n*-hexane and these solutions were directly infused in to APPI source at the rate of 10 μl/min. The *n*-hexane and acetone containing solutions resulted in similar ionisation pattern and produced only the protonated ion i.e., $[M+H]^+$ for Sudan I at *m/z* 249. However, ions due to loss of water and hydroxyl radical were not observed when acetone was used as dopant. In the case of both toluene and anisole as dopants $[M+H]^+$ ion was observed with the formation of an ion at *m/z* 248 representing $M^{+\cdot}$ for Sudan I. But toluene was found to give better sensitivity towards the $M^{+\cdot}$ ion. Hence the experiments were further continued with toluene as dopant. The concentration of toluene in the solutions was linearly increased. This resulted in significant increase in the formation of radical cations ($M^{+\cdot}$) with decrease in $[M+H]^+$ ion for Sudan I (Fig. 2). The abundance of $M^{+\cdot}$ increased to almost 95% with about 7% of toluene in the solution. But the $[M+H]^+$ ion was gradually decreased with increase in toluene concentration in the sample solution. This showed that the concentration of the dopant plays significant role in the ionisation mechanism and optimum concentration should be used. The study was extended to other Sudan dyes with a view to study the formation of radical cations and their fragmentation mechanism using toluene as dopant.

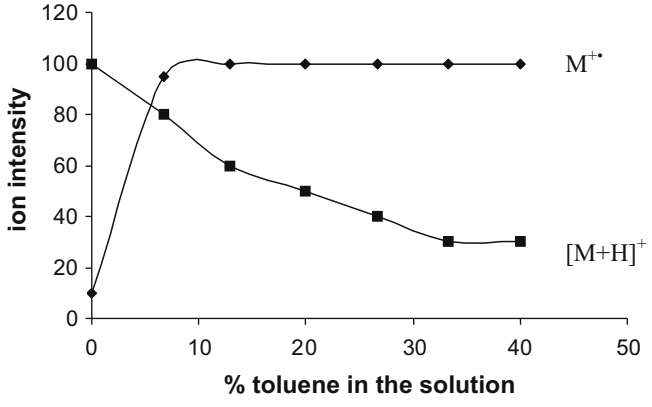

Fig. 2. Effect of toluene as dopant on the formation of $M^{+\cdot}$ and $[M+H]^+$.

3.3. Dopant-assisted APPI–MS and APPI–MS/MS

The dopant-assisted APPI–MS behaviour of Sudan I to IV was investigated in the positive ion mode. At a cone voltage of 25 V, the full scan APPI–MS spectra showed abundant radical cation ($M^{+\cdot}$) for all the four compounds, which allowed the confirmation of their molecular mass. The product ion spectra for the $M^{+\cdot}$ of the compounds were acquired at various collision energies in the range of 5–35 eV. Under APPI–MS/MS conditions the product ion mass spectra (Fig. 3) of $M^{+\cdot}$ ion of Sudan dyes showed a characteristic fragmentation pattern. The product ions of the radical cations are similar to that of the major ions in the EI spectra of these compounds. This shows that the present dopant-assisted APPI of Sudan I to IV is comparable to low energy EI of the same compounds. In the case of Sudan I and II the $[M-H]^+$ ion is clearly seen with a relative intensity greater than 10% whereas in the case of Sudan III and IV the intensity of $[M-H]^+$ ion is not significant. The loss of ·OH from the molecular ion was found for both Sudan I and II whereas it was not observed for Sudan III and IV. The loss of H_2O was not observed as obtained in ESI–MS/MS for any of these compounds. The fragment ion $[C_{10}H_6OH]^+$ at *m/z* 143 obtained by the C–N cleavage is the most abundant common fragment ion for all the analytes. The characteristic ion at *m/z* 115 is obtained by the loss of CO from the ion at *m/z* 143. The ions characteristic of a hydroxyl group on an aromatic ring i.e., $[M-CO]^{+\cdot}$ and $[M-{\cdot}CHO]^+$ are also found in all these compounds, though these ion intensities are less in the case of Sudan III. The C–N cleavage product ions $[HOC_{10}H_6N_2]^+$ and $[C_6H_5N_2]^+$ at *m/z* 171 and *m/z* 105, respectively have also been found in all the compounds.

3.4. Normal phase HPLC method

Several HPLC methods are available in the literature for the separation of Sudan dyes and the methods mostly adopted reversed phase chromatography and the reported HPLC methods can be used for the study using APPI–MS. In this study a simple normal phase HPLC method was developed for the first time for the successful separation of Sudan I to IV dyes using 0.3% isopropanol in *n*-hexane as the mobile phase. The dopant was allowed to mix with column eluent before entering the APPI probe. It was observed that the flow rate of the mobile phase and the dopant significantly affect the ionisation of the analytes as the concentration of dopant is critical. Conditions were optimised to use the method with and without dopant. In the case of dopant-assisted ionisation there is no effect on the separation of the analytes as the dopant is added after the separation. But the absence of dopant mostly results in the formation of $[M+H]^+$ ion during APPI of Sudan compounds. During our initial experiments without the dopant we achieved excellent quantitative results, which are comparable with earlier reported results using HPLC–ESI–MS/MS method (Tateo and Bononi, 2004). Hence, this study was focussed on the quantitative determination of Sudan dyes using normal phase HPLC–APPI–MS/MS with toluene as dopant. Under optimised conditions (see Section 2) good separation of the analytes was achieved within 18 min. Sudan I, II, III and IV eluted at retention times 13.73, 13.16, 15.47 and 16.92 min, respectively.

Quantitative analysis was performed in Single Reaction Monitoring (SRM) mode by monitoring one transition for each compound as shown in Table 1, whereas qualitative analysis was performed in Multiple Reaction Monitoring (MRM) mode by monitoring three transitions for each analyte. The transitions were selected according to highest sensitivity for the analyte of interest in order to provide a simultaneous screening procedure for other Sudan dyes that may be present. Different time windows, where possible, for the analytes were selected to improve the sensitivity in the SRM acquisition mode.

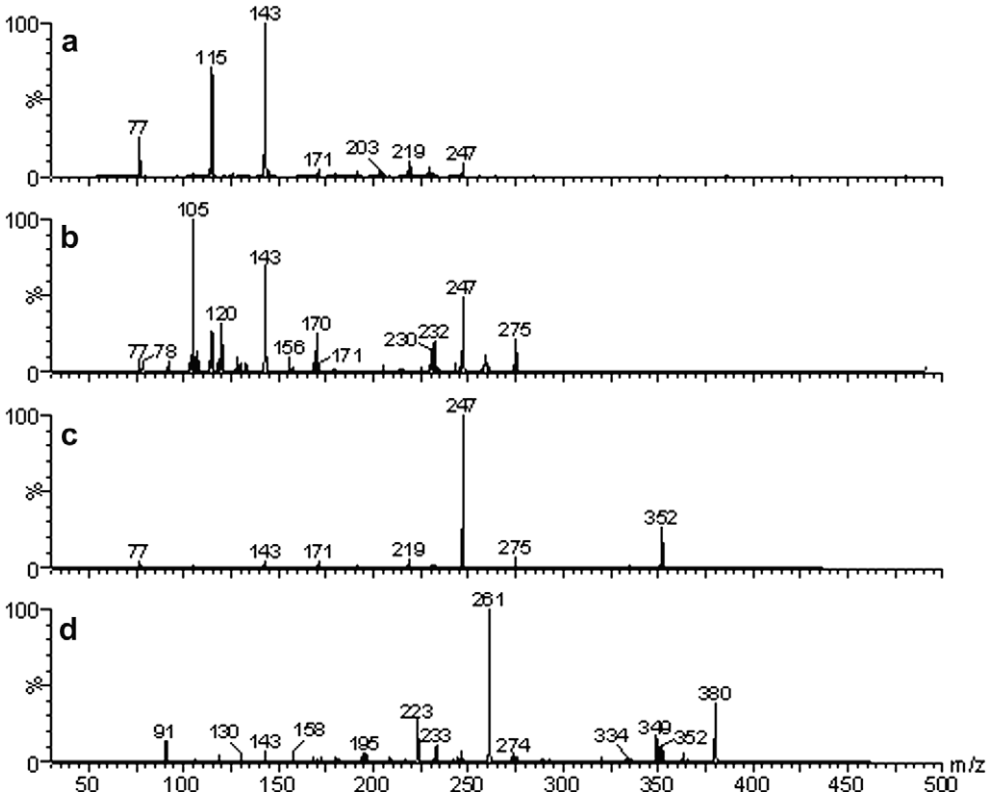

Fig. 3. Dopant (toluene) assisted APPI–MS/MS product ion mass spectra of: (a) Sudan I; (b) Sudan II; (c) Sudan III and (d) Sudan IV.

The instrumental LOD and LOQ values were calculated based on the peak-to-peak S/N ratio using the baseline near the analyte peak obtained by the analyses of standard solutions. In the present method, the LOD values were determined for each analyte separately under SRM mode at a S/N ratio of 3. The LOD values were found to be in the concentration range of 5–18 μg/l. Similarly, the LOQ values obtained at a S/N ratio of 10 are in the concentration range of 10–24 g/l (Table 2).

The linearity of the method for the estimation of Sudan dyes was also evaluated by analysing standard samples at six different concentration levels (in the range of 25–1000 μg/l) in triplicate. The average peak areas were plotted against the concentration of the analyte, resulting in a linear plot. The regression line was obtained by the least squares method, and expressed by the squared correlation coefficient (R^2) shown in Table 2. With these encouraging results, we proceeded to analyse Sudan I to IV simultaneously in commonly used foodstuff matrices such as chilli and sauce.

Several procedures cited in this article include solvent extraction, solid-phase extraction and gel-permeation chromatography for the extraction of Sudan and other azo dyes from chilli and food products. We have selected chilli powder and tomato sauce as matrices, spiked with Sudan I to IV compounds and solvent extraction method was chosen. When the commercial samples of chilli powder and tomato sauce were subjected to solvent extraction, we could not detect the presence of these compounds; hence these matrices were identified as suitable control samples as well as for spiking experiments to measure the recovery of analytes of interest by solvent extraction. These matrices were subjected to solvent extraction by using *n*-hexane as solvent after spiking with the desired concentration of the analytes. The recovery of the spiked analytes was monitored by the present method. To evaluate the percent of recovery of the analytes of interest spiked in the matrix during extraction with *n*-hexane, analyses were carried out at two concentration levels of 100 and 200 μg/kg each of Sudan I to IV in the selected sample matrices. The recoveries were calculated by external standard method from the calibration curves. For this purpose, the calibration curves were constructed using the data obtained by analysing the standard solutions in the concentration range of 25–250 μg/l. The recoveries at different concentration levels were calculated from the calibration curves and are shown in Table 2. The recoveries of Sudan I to IV are found to be in the range of 62–89% with relative standard deviation (RSD) <8% ($n = 3$), and the recoveries increased with the increase in concentration of analyte present in the sample matrix. The recovery was observed to be higher from tomato sauce compared to chilli powder samples. The repeatability of this method was also evaluated and the RSD is <5% ($n = 3$).

Table 2
The instrumental LOD, LOQ, correlation coefficients and % recovery of the analytes.

Analyte	LOD (μg/l)	LOQ (μg/l)	Correlation coefficient R^2	Chilli powder matrix % recovery		Tomato sauce matrix % recovery	
				100 μg/kg	200 μg/kg	100 μg/kg	200 μg/kg
Sudan I	5	10	0.9970	71	74	82	89
Sudan II	8	14	0.9989	76	82	78	87
Sudan III	15	20	0.9926	67	78	75	84
Sudan IV	18	24	0.9992	62	70	73	79

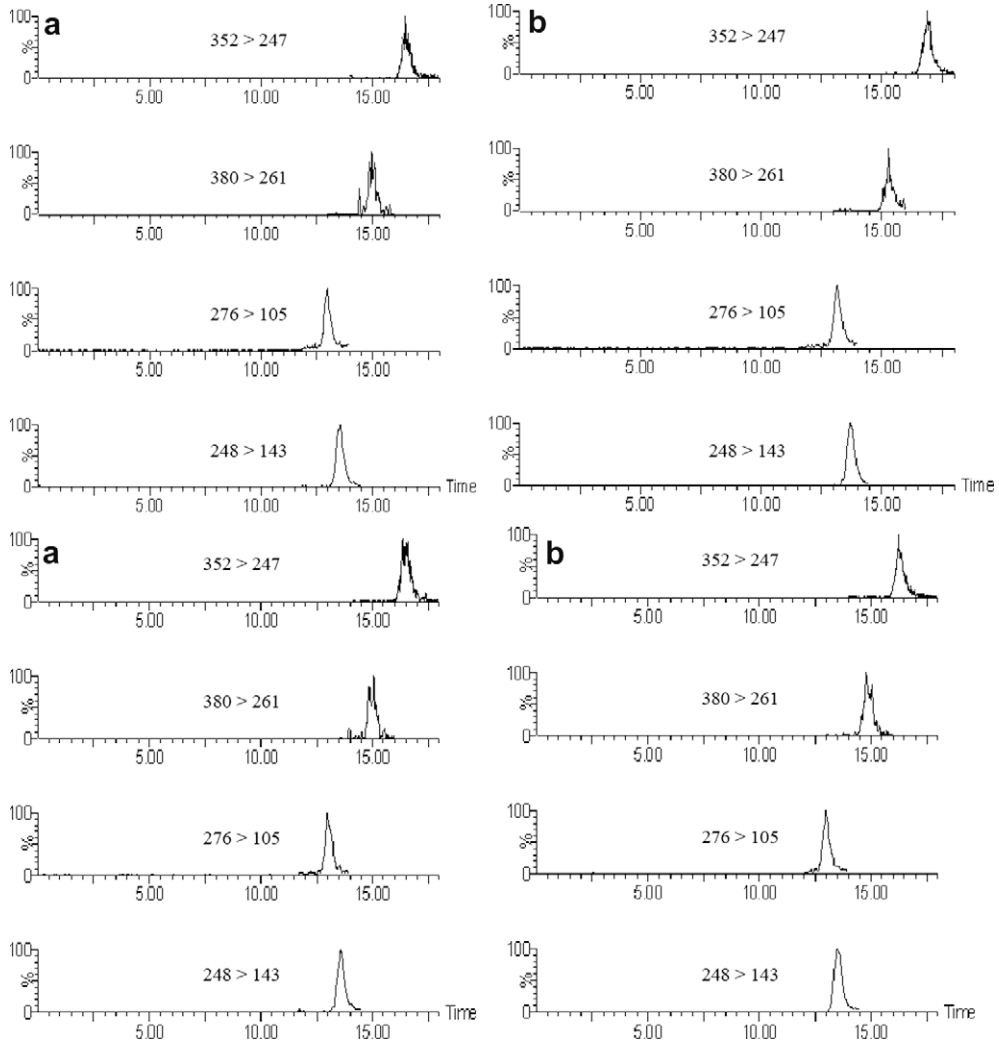

Fig. 4. The SRM chromatograms obtained at a concentration level of: (a) 100 μg/kg and (b) 200 μg/kg with each Sudan I to IV from chilli powder (top) and tomato sauce (bottom) samples, respectively.

Fig. 4 depicts the SRM chromatograms obtained from chilli powder samples spiked at a concentration level of: (A) 100 μg/kg and (B) 200 μg/kg with each Sudan I to IV. It can be observed that the separation of the analytes was not affected by the matrix and also there was no interference from the matrix. Similarly, Fig. 4 depicts the SRM chromatograms obtained from tomato sauce samples spiked at a concentration level of: (A) 100 μg/kg and (B) 200 μg/kg with each Sudan I to IV. In this case also there was no interference or matrix effect on the separation of the analytes irrespective of the concentration level of the analytes.

4. Conclusions

In this study a simple normal phase HPLC–APPI–MS/MS method was developed for the quantitative determination of Sudan I to IV under SRM mode with good separation of all the analytes. The quantitative procedure thus functions simultaneously as a screening procedure for all the Sudan I to IV dyes whilst avoiding interference from the other coeluents from the matrix and offers improved detection limits through the monitoring of the more sensitive transitions. The LOD, LOQ and % recoveries of the analytes were comparable with the best mass spectrometric methods reported earlier. This novel APPI method has been successfully applied to analyse samples spiked with Sudan I to IV dyes and can be used for the determination of these compounds in real samples.

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