Determination of Banned Dyes in Spices by Liquid Chromatography–Mass Spectrometry

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Abstract

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A simple and rapid multiresidue method for the determination of nine banned synthetic dyes in various spices has been developed. Reversed phase HPLC coupled with mass spectrometry (tandem in time – ion trap mass analyser) was employed for the examination of crude acetonitrile extract acidified with acetic acid. The detection limits of Para Red, Sudan Orange G, Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Red 7B and Rhodamine B were in the range of 0.02–0.1 mg/kg, the recoveries ranged from 75.7 to 92.3% with repeatability of 0.9–11.3%. Rather worse performance characteristics were obtained with Tropaeolin 000, obviously due to its more polar nature as compared to other dyes involved in this study. In spite of that, the developed method can be used for a reliable control of a wide range of dyes used for illegal colouring of various spices.

Keywords: Sudan dyes; Para Red; Rhodamine B; chilli; curry; liquid chromatography – mass spectrometry

A wide range of lipophilic and moderately polar synthetic dyes is used in industry for colouring various materials such as mineral oils, waxes, solvents, textile and leather garments, etc. The use of these dyes in foods is not allowed because of the health concerns related to their intake. In spite of this fact, in May of 2003 France provided the information through the Rapid Alert System for Food and Feed (RASFF) on the discovery of the dye Sudan I in hot chilli products originated from India, its levels being as high as 4000 mg/kg (ASTA 2005). By the end of this year, 119 notifications had been provided to the RASFF system (RASFF 2003). With regard to the growing problem, the European Commission issued a decision on emergency measures (2003/460/EC) whereby the Member States prohibit the import of hot chilli and hot chilli products unless the analytical report accompanying the consignment demonstrates that the products do not contain any Sudan I. In a short time, this Commission decision was amended by Decision 2004/92/EC for Sudans II–IV. In the following period, Sudan I and IV were observed in some commodities examined, such as chilli and chilli products, curcuma, curry, sumac and palm oil (RASFF 2004).

In addition to this types of Sudan, several other illegal dyes have also been discovered in imported spices and spice products (RASFF 2005). Under scrutiny are Para Red, Sudan Orange G, Sudan

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Red 7B and Rhodamine B. They are not approved in foods, according to the European Parliament and Council Directive 94/36/EC on colours for use in foodstuffs (EFSA 2005). The overview of the structures of the compounds under concern in this study is shown in Figure 1.

It should be noted that in the opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food there is insufficient data on any of the illegal dyes, Sudan I–IV, Para Red, Rhodamine B, and Orange II, found so far in foods in the EU to perform a full risk assessment. However, there is experimental evidence that Sudan I is both genotoxic and carcinogenic; a similar toxic potential was recognised in Rhodamine B. For the other dyes mentioned above, conclusive evidence on their toxicity for humans is also lacking, nevertheless, because of the structural similarities to Sudan I, it would be prudent to assume that they are potentially genotoxic and possibly carcinogenic as well (EFSA 2005).

Various methods have been employed for the detection (screening) and accurate determination of synthetic dyes in foodstuffs. As individual chemicals are to be controlled in a particular food commodity, chromatographic and/or electrophoretic separation steps have to be involved in the respective procedure.

As shown in the overview presented in Table 1, the existing methods largely differ in their scope as well as in the performance characteristics achievable.

The aim of our study was to develop and validate a simple and rapid procedure enabling an accurate measurement of even low levels of all dyes banned under the current concern in EU.

MATERIALS AND METHODS

Samples. Dried chilli and curry (either ground or powdered) free of synthetic dyes were used for the method development. All samples were purchased from Czech retail markets and/or obtained from Czech importers.

Standards. The overview of the target dyes is shown in Table 2. The concentrations of the stock solutions prepared from the solid standards were in the range of 0.1–1 mg/ml. With regard to the relatively different polarities of the analytes, various solvent/solvent mixtures had to be used for their preparation. Calibration mixtures containing the

Table 1. Chromatographic methods used for identification/quantification of synthetic dyes in spices and other foodstuffs

Analytes (dyes)	Commodity	Analytical method				References
		extraction solvent	pre-concentration	identification/quantification	LOD (mg/kg)	
Sudan I–IV	hot chilli products	acetone	none	HPLC-(ESI+)-MS/MS	0.003–0.024	Calbiani *et al.* (2004a)
Sudan I	foodstuff	acetonitrile	none	FIA – (APCI+)-MS/MS	0.007–0.009	Di Donna *et al.* (2004)
Sudan I–IV	sauce, spices	acetonitrile	none	HPLC-PDA	0.2–2.0	Cornet *et al.* (2006)
Sudan I	hot chilli, spices, oven-baked products	96% ethanol	none	HPLC-(APCI+)-MS	0.06–3	Tateo &Bononi (2004)
Sudan I	chilli powder	chloroform	MISPE	HPLC-UV	not provided	Puoci *et al.* (2005)
Sudan I–IV, Tartrazine, Amaranth, Ponceau 4R, Sunset yellow FCF	ginger, chilli powder	dimethyl-sulphoxide	none	HPLC-PDA-(ESI+)MS	0.001–0.5	Ma *et al.* (2006)
Sudan I–IV	hot chilli products	acetone	none	μLC-(ESI+)-Q-TOF MS	0.0004–0.0011	Calbiani *et al.* (2004b)
Sudan I–IV	red pepper or tomato products	methanol:acetone: dichloroethane	none	HPLC-PDA	0.15–0.25	Daood & Biacs (2005)

MISPE – Molecularly Imprinted Solid Phase Extraction; FIA – Flow Injection Analysis

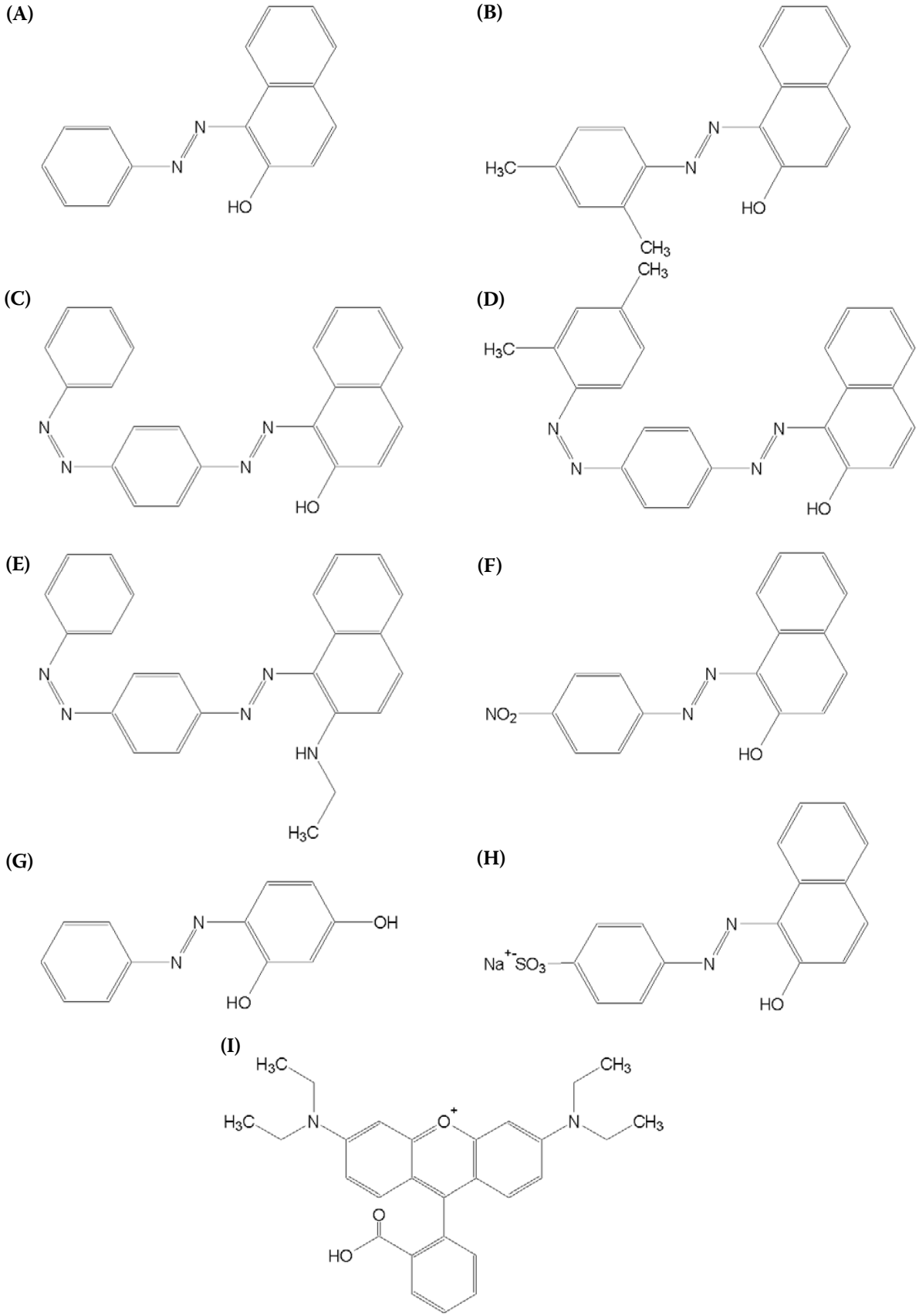

Figure 1. Structures of banned synthetic dyes most reported as used for adulteration: (A) Sudan I, (B) Sudan II, (C) Sudan III, (D) Sudan IV, (E) Sudan Red 7B, (F) Para Red, (G) Sudan Orange G, (H) Tropaeolin 000, (I) Rhodamine B

Table 2. Standards of target analytes

Synthetic dye	CAS number	Supplier	Purity	Stock solutions solvents
Tropaeolin 000	633-96-5	Fluka	n.i.	25% methanol
Rhodamine B	81-88-9	Fluka	n.i.	methanol
Para Red	6410-10-2	Sigma-Aldrich	95%	1% acetic acid: acetonitrile (5:95)
Sudan Orange G	2051-85-6	Sigma-Aldrich	85%	1% acetic acid: acetonitrile (5:95)
Sudan I	842-07-9	Sigma-Aldrich	97%	1% acetic acid: acetonitrile (5:95)
Sudan II	3118-97-6	Aldrich	90%	1% acetic acid: acetonitrile (5:95)
Sudan III	85-86-9	Sigma	≥ 80%	1% acetic acid: acetonitrile: 2-propanole (5:90:5)
Sudan IV	85-83-6	Aldrich	≥ 80%	1% acetic acid: acetonitrile: 2-propanole (5:80:15)
Sudan Red 7B	6368-72-5	Aldrich	95%	1% acetic acid: acetonitrile: 2-propanole (5:80:15)

n.i. – not indicated

analytes in the range of 0.005 and 10 µg/ml (each substance) were prepared in HPLC mobile phase (5% acetic acid:acetonitrile (2:98, v/v)). Prior to use, the solutions were stored in a refrigerator at 4°C.

Chemicals. The solvents for chromatography were HPLC gradient grade acetonitrile (Sigma-Aldrich, Germany), deionised water was produced by Milli-Q apparatus (Millipore, United States), and acetic acid, glacial 99.99+%, was obtained from Aldrich (United States). HPLC gradient grade 2-propanole was purchased from Merck (Germany).

Instruments. HPLC-MS system consisted of Agilent 1100 series (Agilent, USA) coupled with Finnigan LCQ Deca mass spectrometric detector (Thermo Finnigan, USA) equipped with APCI (Atmospheric Pressure Chemical Ionization) and/or ESI (ElectroSpray Ionization) ionisation source. The sheath gas (nitrogen) and the auxiliary gas (nitrogen) were delivered by laboratory nitrogen generator (Peak Scientific, USA). The chromatographic data were collected and processed using the software Xcalibur 1.1 version.

Method. Sample preparation: crude samples were finely ground by the electric blender Waring Commercial 38BL40 (Dynamic Corporation of America, USA) and 5 g of the representative sample were shaken for 30 min with 40 ml 1% acetic acid: acetonitrile mixture (5:95, v/v). The suspension was then filtered and made up to 50 ml. Prior to injection, filtration was carried out by passing the solution through PTFE syringe filter (0.45 µm, Teknokroma, Spain).

HPLC conditions – eight separation columns were tested in the optimalisation process:

(*i*) non-endcapped – Lichrospher® 100 RP-18; Lichrospher® 100 RP-8; SUPELCOSIL™ LC-18-DB;

(*ii*) endcapped – Lichrospher® 100 RP-18e; HyPURITY AQUASTAR; Purospher Star RP-18e;

(*iii*) embedded – Synergi 4u Fusion – RP 80; SUPELCOSIL™ ABZ+Plus.

Since the best separation was obtained on the Purospher Star RP-18 endcapped column (125 × 3 mm; 5 µm; Merck, Germany), this column was used in all follow-up experiments.

The mobile phase consisted of (A) acetonitrile and (B) 5% acetic acid in water. Gradient elution was realised as follows: solvent (A) was maintained at 43% for 2.5 min, followed by the linear gradient to 90% A in 1.5 min and linear gradient to 98% A in 2 min. These conditions were held for 8 min. To recondition the column, 5 min post-run with the initial mobile phase composition was performed. The mobile phase flow rate was 0.5 ml/min; 20 µl of extract was injected. The column temperature was 40°C.

MS conditions: mass spectrometric detector equipped with ion trap mass analyser (tandem in time) was coupled to the HPLC system. Ionisation of the effluent components was carried out in the negative mode for Tropaeolin 000 and Para Red, for the other analytes positive ionisation mode was used. The APCI probe was heated to 220°C (350°C for Tropaeolin 000). The temperature on the heated capillary was 165°C, sheath gas flow and aux gas flow were 1.2 and 3.0 l/min, respectively. Selected reaction monitoring (SRM – MS/MS) and

consecutive reaction monitoring (CRM – MS/MS/MS) were used for the quantitation and confirmation of the target analytes. MS detector setting details are shown in Table 3.

Validation procedure. The validation procedure aimed at the determination of performance characteristics was carried out by evaluating the detection limits (LODs), selectivity, accuracy, and repeatability of measurements.

LODs were evaluated as the minimum concentration of the analyte that provides a signal to noise ratio equal to 3.

Selectivity was assessed by evaluating the matrix effects in terms of both the signal intensity and the exact mass measurements. For the assessment of ion supression/enhancement effects, responses of the mixtures of standard mixture solutions in the concentration range of 5–10 000 ng/ml were compared with responses of the matrix matched standard solutions at the same levels. The matrix matched standard solutions were prepared by evaporating blank chilli extract and subsequently dissolving the residue in the standards mixture (10 000 ng/ml). All lower concentration levels were obtained by the dilution this matrix matched standards solution with blank chilli extract.

The accuracy of results was tested by repeated measurements of a spiked blank sample (2 mg/kg). The recovery of the method was obtained by comparing the analytes response in the spiked sample with the matrix matched standard solution at the same concentration level. The repeatability of the method for the individual analytes was obtained by calculating relative standard deviations from recoveries.

RESULTS AND DISCUSSION

Although most of the food products notified through the RASFF for the occurrence of unauthorised colouring substances contained Sudan I, Sudan IV, and/or Para Red, the availability of analytical procedures enabling also the determination of other banned synthetic dyes that issue

Table 3. MS detector setting

Analyte	Parent mass (MS^1)	Daughter mass (MS^2)	Confirmatory mass (MS^3)	Discharge current (μA)	Isolation width (m/z)	Activation amplitude	Activation	Activation time (ms)
		(m/z)						
Tropaeolin 000	327.2	171.2		0	2	36	0.34	40
	327.2	171.2	107.2	0	2	33	0.33	35
Rhodamine B	443.5	399.4		3	2	55	0.5	30
	443.5	399.4	355.4	3	2	50	0.5	30
Sudan Orange G	215.2	198.6		3	2	30	0.3	30
	215.2	198.6	169.4	3	2	46	0.4	40
Para Red	292.2	264.3		2	2	33	0.28	30
	292.2	264.3	233.5	2	2	39	0.35	40
Sudan I	249.2	232.6		8	2	35	0.3	40
	249.2	232.6	204.4	8	2	70	0.6	40
Sudan II	277.1	260.5		5	2	35	0.3	30
	277.1	260.5	244.5	5	2	56	0.5	40
Sudan III	353.2	336.3		5	2	32	0.25	25
	353.2	336.3	231.4	5	2	50	0.4	30
Sudan Red 7B	380.1	183.4		4	2	25	0.25	30
	380.1	183.4	142.1	4	2	38	0.35	35
Sudan IV	381.2	225.2		5	2	35	0.3	30
	381.2	225.2	209.3	5	2	40	0.35	35

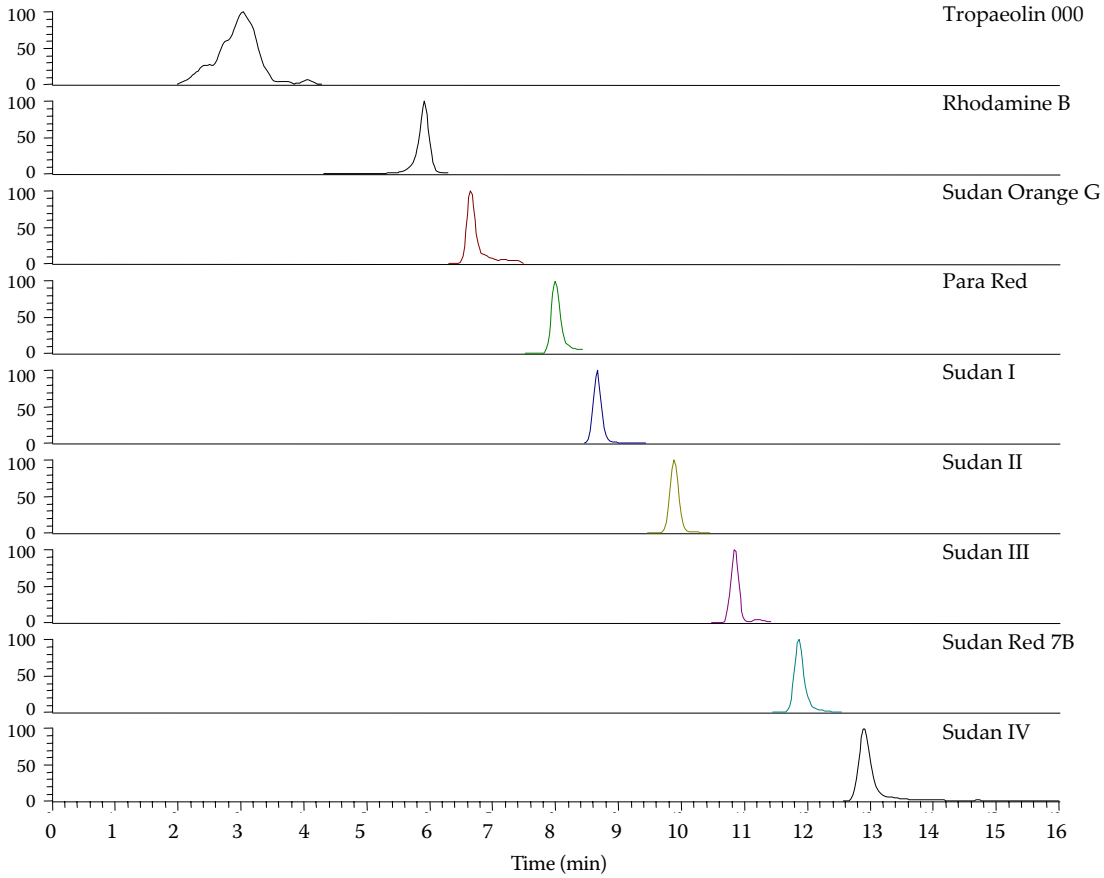

Figure 2. HPLC-MS/MS chromatogram of matrix matched standard, analytes at level 100 ng/ml (corresponds to contamination 1 mg/kg)

were occasionally shown to be present in spices, seasonings, and/or tomato-based products was obviously urgent.

Considering low volatility of the target analytes (Figure 1), HPLC separation is obviously the method of choice for this particular purpose. Although widely available UV/PDA detectors can be employed under certain conditions, problems due to their low selectivity when analysing products with high contents of natural pigments (carotenoids) can be encountered due to similarity of their spectral characteristics with the target analytes. To obtain better selectivity and lower detection limits of examined colouring substance, a mass spectrometric detector was employed in our study. In the first phase of our experiments, the separation step was optimised. While good and rapid separation of Sudan and Para Red dyes was easily achieved on all tested reversed phase columns (see HPLC conditions), irreversible sorption of Rhodamine B occurred on some of them. This problem was eliminated when Purospher Star RP-18e (LichroCart 125 × 3 mm; 5 µm) endcapped column was used (Figure 2). Good peak shapes and baseline resolution were obtained for most dyes when 5% acetic acid: acetonitrile mobile phase was used (gradient elution). The only compound with which no significant improvement of the peak shape was obtained was Tropaeolin 000. Lower pH value attainable e.g. by the addition of formic acid would suppress ionisation of sulphonic group contained in this compound, however, this solution was not feasible in view of the overall performance (the reduction of the analyte signal, hence an increase of LODs, occurred when formic acid was added).

Identification/quantification of target dyes was carried out by mass spectrometric detector employing tandem in time ion trap mass analyser (ITD). Both electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces were tested.

Table 4. Optimised method performance characteristics of optimised method (n = 6)

Analyte	Recovery (%)	Repeatability (%)	LOD (mg/kg)	Linearity – R^2 correlation coefficient within calibration range (0.05–100 mg/kg)
Tropaeolin 000	40.4	25.6	0.50	0.9707
Rhodamine B	89.5	5.5	0.05	0.9915
Sudan Orange G	91.4	0.9	0.10	0.9992
Para Red	92.3	3.5	0.02	0.9983
Sudan I	89.9	6.7	0.02	0.9991
Sudan II	87. 7	8.6	0.05	0.9991
Sudan III	76.8	9.8	0.05	0.9959
Sudan Red 7B	75.7	9.2	0.05	0.9962
Sudan IV	76.6	11.3	0.05	0.9992

Better, i.e. lower detection limits, could be attained for all analytes when using the latter ionisation set-up.

The only exception was Tropaeolin 000, this sulphonated dye provided a distinctly better response in ESI mode.

In Table 4, method performance characteristics are summarised as obtained in the validation process.

Matrix effects (i.e. changes in signal intensity caused by coeluting co-extracts) were found far two of tested dyes, Rhodamine B and for Tropaeolin 000. While in the case of Rhodamine B ion enhancement occurred, a significant ion suppression could be observed for Tropaeolin 000 at concentrations below 5 mg/kg.

The confirmation of the analyte identity was made in the same run as the quantitative determination. Because in ion trap mass analyser only one abundant ion is yielded by fragmentation in MS^2 (quantitation mass), to get a sufficient sensitivity for confirmation MS^3 mode was chosen.

Using the method mentioned above, over 20 samples (sweet chilli, curry, and hot chilli) were analysed. In three of them (sweet chilli), Para Red dye was determined (35–40 mg/kg).

CONCLUSIONS

A simple and rapid HPLC-MS/MS method was developed for the analysis of nine synthetic dyes in various spices. Not only detection limits fairly lower as compared to the procedures employing UV detectors can be achieved by this method but also the confirmation (two MS transitions) of target analytes is possible within a single run. The new method is suitable for the control of requirements established in EU legislation on the measures related to the potential occurrence of the banned dyes in food supply.

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