Determination of banned dyes in red spices by ultra-high-performance liquid chromatography-atmospheric pressure ionization-tandem mass spectrometry

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HIGHLIGHTS

- Atmospheric pressure ionization behaviour of Sudan and Rhodamine B dyes.
- MS^n and high-resolution mass spectrometry studies to characterize product ions.
- Matrix effect studies showed better performance in APCI and APPI in front of ESI.
- New UHPLC–APCI–MS/MS method for the determination of eight banned dyes in spices.

GRAPHICAL ABSTRACT
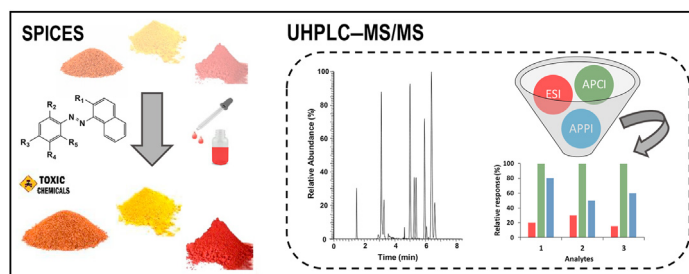
ABSTRACT

This paper reports the development of an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) method to determine eight banned dyes (Sudan I-IV, Sudan Orange, Sudan Red 7B, Para Red, Rhodamine B) in turmeric, curry, and chili products. For this purpose, the feasibility of electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) for the ionization of these compounds was evaluated. The tandem mass spectrometry (MS/MS) fragmentation of all targeted compounds was studied and both multistage mass spectrometry and high-resolution mass spectrometry were used to establish the fragmentation pathways and identify common fragmentation behaviors. Among the most significant ions, the most characteristic and abundant product ions observed on the triple quadrupole were selected to propose a selective and sensitive UHPLC–MS/MS method (multiple reaction monitoring mode, MRM) of these target compounds in spices samples after a quick and easy extraction with acetonitrile. Matrix effect (ME) studies carried out in the three atmospheric pressure ionization sources have demonstrated that APCI showed the best performance with ME values ranging from 2 to 25%. Furthermore, the estimated quality parameters indicated the good performance of the proposed method, providing low method limits of detection (MLODs) (1–48 µg kg^{-1}), good intra-day precision (RSD % < 15%), and accurate quantitation (relative error % < 15%). Finally, the applicability of the developed method was demonstrated by the analysis of turmeric, curry, and chili products. In total, 36 diverse samples coming from different countries were analyzed and although none of these compounds were detected above the MLODs, the analysis of spiked samples showed that the method was able to detect this family of compounds at low µg kg^{-1}.

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1. Introduction

Color is one of the most critical food attributes, being considered one of the parameters that determine the acceptance of products towards the consumer. However, the natural color in many food products may be lost during their manufacturing, and thus, some colored substances are frequently used to re-establish and keep their initial appearance. Natural dyes have been used for centuries to color food, although nowadays, the food industry often would rather artificial/synthetic dyes to natural ones. Although we can find a wide hue of colors in nature, those suitable as food dye are limited. Besides, artificial dyes can be mass-produced at a lower cost in a wide variety of colors, and they might be longer-lasting. However, the use of these synthetic substances to color food is regulated to guarantee food safety [1] since some of them can be harmful to human health. For instance, dyes such as Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Orange G, Sudan Red 7B, Rhodamine B, and Para Red, among others, are banned in food products for human consumption [2] owing to their teratogenicity, genotoxicity [3] and carcinogenic effects.

Para Red and all Sudan dyes are fat-soluble synthetic dyes with similar chemical structure (Fig. 1) that contains aromatic rings and azo groups (−N═N−). Toxicity studies about Sudan I-IV [4,5] have demonstrated that these compounds can induce some forms of liver and bladder cancer in animals. Therefore, they have been classified as Class-III carcinogens by the International Agency for Research on Cancer (IARC) [6]. Furthermore, experts note that it would be wise to assume that Rhodamine B [7], as well as Para Red, Sudan Red 7B, and Sudan Orange, should also be considered as potential genotoxic and carcinogenic compounds [8]. This is because Rhodamine B has shown potential toxicity similar to that of Sudan I-IV, and there is a lack of information on the toxicity of Para Red, Sudan Red 7B, and Sudan Orange. Although the use of this family of compounds as food colorants has been banned [2,9], they are still being used illegally to improve the quality perception of food products. For instance, powdered species have been occasionally adulterated with azo dyes to intensify these products of red color [10], becoming a risk to public health. Nevertheless, Sudan I, III, IV, Rhodamine B, and Para Red have been detected in chili, curry,

and turmeric products at concentration levels ranging from 1 to 8700 μg kg⁻¹ [11−13].

These banned dyes have been determined by liquid chromatography (LC) using reversed-phase chromatography and spectrophotometric detection (LC/UV−Vis) [14,15]. However, the most frequently used technique for their determination is liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) since tandem mass spectrometry allows to overcome limitations observed on other techniques providing high sensitivity, selectivity, and structural information for the identification, confirmation, and characterization of analytes in complex matrices at low concentration levels [16]. Most of these LC−MS/MS methods used electrospray (ESI) as ionization technique [17−19]. However, only a few works have explored the ionization of azo dyes by atmospheric pressure chemical ionization (APCI) [20,21] and atmospheric pressure photoionization (APPI) [22]. Although these studies highlight the advantages of mass spectrometry comparing to the spectrophotometric detection systems (UV/PDA), they do not delve into the atmospheric pressure ionization mechanism of these compounds, and generally, most of the selected ions for the LC−MS/MS method are not fully assigned. Besides, some of these studies only focus on the determination of a few of these dyes in some spice samples.

The present work aimed to study the ionization performance of selected eight azo/non-azo banned dyes in three atmospheric pressure ionization (API) sources (ESI, APCI, and APPI) to identify which one provides the best performance for the selective and sensitive simultaneous determination of these compounds in several spices samples by LC−MS/MS using a triple quadrupole (QqQ) mass analyzer. The compatibility of a simple and fast sample extraction with the use of these API sources was also explored by studying the matrix effect of the co-extractants from spice samples (turmeric, curry, and chile) on the ionization of these target compounds. The feasibility and applicability of the proposed ultra-high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (UHPLC−APCI−MS/MS) method was evaluated by the analysis of 36 spices samples.
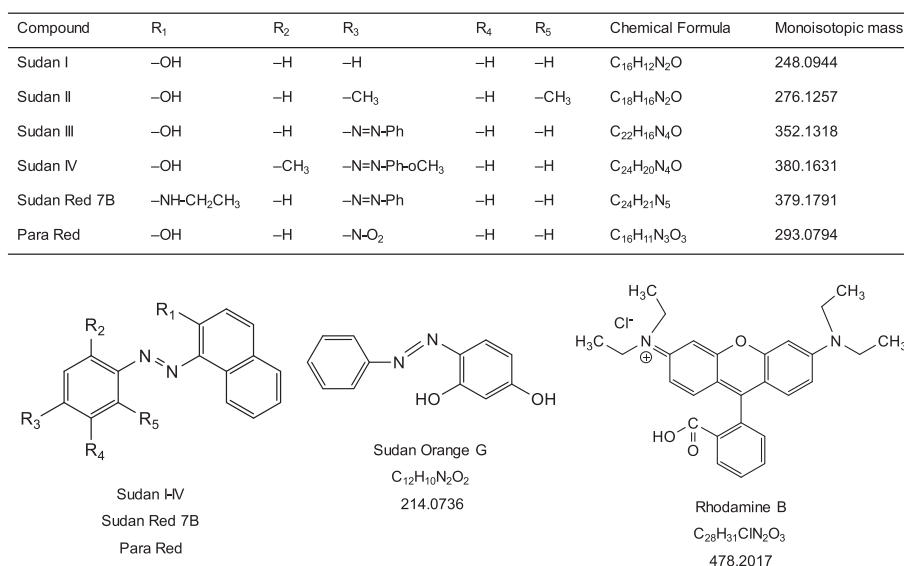
Compound	R₁	R₂	R₃	R₄	R₅	Chemical Formula	Monoisotopic mass
Sudan I	−OH	−H	−H	−H	−H	$C_{16}H_{12}N_2O$	248.0944
Sudan II	−OH	−H	−CH₃	−H	−CH₃	$C_{18}H_{16}N_2O$	276.1257
Sudan III	−OH	−H	−N═N-Ph	−H	−H	$C_{22}H_{16}N_4O$	352.1318
Sudan IV	−OH	−CH₃	−N═N-Ph-oCH₃	−H	−H	$C_{24}H_{20}N_4O$	380.1631
Sudan Red 7B	−NH-CH₂CH₃	−H	−N═N-Ph	−H	−H	$C_{24}H_{21}N_5$	379.1791
Para Red	−OH	−H	−N-O₂	−H	−H	$C_{16}H_{11}N_3O_3$	293.0794

Fig. 1. Chemical structures, and chemical formula of the studied banned dyes.

2. Materials and methods

2.1. Reagents and standards

All chemicals were analytical reagent-grade. Sudan I ($\geq$96%), Sudan II ($\geq$96%), Sudan III ($\geq$96%), Sudan IV ($\geq$96%), Sudan Red 7B ($\geq$96%), Sudan Orange G ($\geq$96%), Para Red ($\geq$97%) and Rhodamine B ($\geq$97%) were purchased from Sigma-Aldrich (Steinheim, Germany). Chemical structures of the studied compounds are shown in Fig. 1.

Individual stock solutions (1000 mg L^{-1}) of Sudan I, Sudan II, Sudan Orange G, Sudan Red 7B, and Rhodamine B were prepared in acetonitrile. In contrast, dimethyl sulfoxide (DMSO) was the solvent used for the stock solutions of Sudan IV and Para Red in and acetone was employed to prepare the Sudan III one. Intermediate individual solutions (300 mg L^{-1}) and a standard mixture solution (50 mg L^{-1}) containing all target compounds were prepared monthly from stock standard solution by appropriate dilution in acetonitrile. All these standard solutions were stored at 4 $^\circ$C until their use. Matrix-matched calibration was used to correct matrix effects over the concentration range (15–10,000 μg kg^{-1} for turmeric and curry, and 50 to 10,000 μg kg^{-1} for chili). Extracts were filtered through 0.22 μm Nylon membrane filters (Whatman, Clifton, NJ, USA) before their injection into the UHPLC−MS/MS system.

Dimethyl sulfoxide EMSURE® ACS ($\geq$99.0%), formic acid ($\geq$98%), ammonium formate ($\geq$98%), ammonium acetate ($\geq$98%), acetic acid ($\geq$99.7%), chlorobenzene and toluene for HPLC ($\geq$99%), anisole anhydrous ($\geq$99.7%), tetrahydrofuran ($\geq$99.7%), and acetonitrile and water of LC−MS grade, were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone (LiChrosolv®, purity $\geq$ 99.8%) was obtained from Honeywell (Charlotte, North Caroline, USA), and sodium chloride ($\geq$99%) from Panreac Química S.A. (Barcelona, Spain). Solvents used as components of the mobile phase were filtered through Nylon membrane filters of 0.22-μm pore size (Whatman, Clifton, NJ, USA) before their use.

Nitrogen (99.95%) used for heated-electrospray ionization (H−ESI), APCI, and APPI was purchased from Linde (Barcelona, Spain), and the high-purity argon (<99.999%) used as a collision-induced dissociation gas (CID gas) in the triple quadrupole instrument was purchased from Air Liquide (Madrid, Spain).

2.2. LC−MS instrumentation and working conditions

For the chromatographic method, a UHPLC system equipped with an Accela 1250 quaternary pump, an Accela autosampler and, a column oven (Thermo Fisher Scientific, San Jose, CA, USA) was used. The reversed phase separation was carried out in an Accucore C_{18} analytical column (100 mm × 2.1 mm id.) packed with superficially porous particles (2.6 μm particle size) purchased from Thermo Fisher Scientific. The chromatographic separation was performed using gradient elution mode with a mobile phase composed of (solvent A) formic acid/ammonium formate aqueous buffer (pH 3.75, 20 mM) and (solvent B) acetonitrile. The gradient elution program started with 60% solvent A (40% solvent B) for 2.5 min, followed by a linear gradient that raised solvent B to 90% (10% solvent A) in 1.5 min. Finally, solvent B was raised to 100% for 2 min, and these conditions were maintained in an isocratic step for 2 min more before returning to initial conditions. The injection volume was 10 μL, the mobile phase flow rate was 600 μL min^{-1}, and the column oven temperature was held at 25 $^\circ$C during the chromatographic run.

The UHPLC system was coupled to a TSQ Quantum Ultra AM (Thermo Fisher Scientific) mass spectrometer equipped with a triple quadrupole mass analyzer. Three API sources (H-ESI, APCI, and APPI) could be swappable in this TSQ mass spectrometer. Working parameters for H-ESI, APCI, and APPI were as follows: vaporizer and

capillary temperatures were 350 $^\circ$C and 250 $^\circ$C, respectively; nebulizer gas and auxiliary gas pressures were 50 and 25 arbitrary units (a.u.), respectively; the electrospray needle voltage was held at ± 3 kV in H-ESI, whereas the corona discharge current was set at +6/-6 μA in APCI, and a krypton lamp emitting photons of 10.6 eV was used in APPI. Finally, the tube lens offset voltage was optimized for each compound, and values ranged from 15 to 50 V. Regarding APPI source, direct photoionization, and dopant-assisted ionization were compared, and several dopants (acetone, toluene, tetrahydrofuran, anisole, and chlorobenzene) were post-column added into the mobile phase using a zero-dead volume T-piece. Chlorobenzene was selected as the most appropriate dopant, and the best response was obtained when chlorobenzene at a flow rate of 5% of the mobile phase flow rate was post-column added.

The mass spectral data were acquired in full scan and product ion scan modes for ionization and fragmentation studies, while multiple reaction monitoring (MRM) mode was used for quantification purposes, operating both quadrupoles (Q1 and Q3) at a resolution of 0.7 m/z full width half maximum (FWHM). In MRM, two transitions were monitored for each compound using 50 ms of dwell time, which was enough to acquire at least 23 data points across each chromatographic peak. The pressure of the collision gas (argon) was set at 1.5 mTorr. The Xcalibur $v2.1$ software (Thermo Fisher Scientific) was used to control the instrument setup as well as to acquire and process the MS data.

The multi-stage fragmentation studies of targeted compounds were carried out in a hybrid mass spectrometer (linear ion trap-Orbitrap), LTQ-Orbitrap Velos (Thermo Fisher Scientific) using the APCI source in both positive and negative ion mode. The LTQ-Orbitrap was also coupled to an Accela UHPLC pump system (Thermo Fisher Scientific). Individual standard solutions, prepared in an adequate solvent mixture, were infused at 10 μL min^{-1} using a syringe pump and introduced in the stream of the mobile phase (formic acid/ammonium formate (pH 3.75, 20 mM):acetonitrile, (1:1, v/v) 300 μL min^{-1} through a Valco zero-dead-volume T-piece to deliver a final concentration of 10 mg L^{-1} into the ionization source. The APCI optimal working conditions were as follows: discharge current, 5 μA; sheath gas pressure, 40 a.u.; auxiliary gas pressure, 10 a.u.; and vaporizer and capillary temperatures 400 and 350 $^\circ$C, respectively. Regarding the linear ion trap parameters, the isolation width was 1.0 m/z, while the activation time and activation Q were fixed at 30 ms and 0.25, respectively. MS^n data were acquired in the Orbitrap in full scan positive and negative ion modes (100−500 m/z) at a resolution of 30,000 FWHM (at m/z 200). The S-lens RF was fixed at 60%, while the maximum injection time and the AGC target were adjusted at 200 ms and 10^6, respectively. The Xcalibur $v2.1$ software (Thermo Fisher Scientific) was used to control the instrument setup and acquire and process the MS and MS^n data.

2.3. Samples and sample treatment

A total of 36 diverse samples (turmeric, curry, and chili powders) were analyzed in this study. Samples coming from different countries (India, Spain, France, Germany, Mexico, and Chile) were purchased at Spanish markets (Table S1).

Sample treatment was carried out as follows: 5 g of sample was weighed in a falcon tube, and 5 mL of acetonitrile were added. After shaking with a vortex for 0.5 min, the sample solution was sonicated for 15 min and centrifugated for 15 min at 4800 rpm. The supernatant extract was filtered through a 0.22 μm Nylon filter and stored at +4 $^\circ$C until its analysis. Finally, 10 μL of the final extract was injected into the UHPLC–MS/MS system.

2.4. Quality control and method validation

Quality control standard solutions and procedural blanks were introduced among the analysis of standards and samples to ensure the quality of the results and control the LC separation and the sensitivity of the LC–APCI–MS/MS system.

To estimate the extraction efficiency (EE, %), both a blank spice powder sample (turmeric, curry, and chile) and the corresponding spiked one were submitted to the same extraction procedure. The quantitative results obtained after their UHPLC–API–MS/MS determination using standards prepared in the mobile phase were compared. Additionally, the matrix effect (ME, %) in the ionization process was estimated for each compound from the relative difference between the peak area observed in the analysis of the spiked blank extract and that obtained from standard mixtures prepared in the mobile phase at the same concentration level.

Instrumental and method limits of detection (ILODs and MLODs) and limits of quantification (ILOQs and MLOQs) were estimated as S/N of 3 and 10, respectively. Moreover, linearity within the working concentrations range (15−10,000 $\mu g\ L^{-1}$ for standard solutions and 15−10,000 $\mu g\ kg^{-1}$ for matrix-matched calibration solutions) was studied. Both intra-day and inter-day precision and the trueness of the method were estimated using spiked blank turmeric and curry samples at medium (150 $\mu g\ kg^{-1}$) and low (100 $\mu g\ kg^{-1}$) concentration levels. Nevertheless, these parameters were evaluated for Sudan II and Sudan III in spiked blank turmeric and Sudan I in curry at concentration levels of 250 $\mu g\ kg^{-1}$ and 200 $\mu g\ kg^{-1}$, respectively. In the case of chili, the low concentration level corresponded to 30 $\mu g\ kg^{-1}$ and the medium concentration one to 100 $\mu g\ kg^{-1}$ for all the compounds except for Sudan I, for which the concentration level used were 100 $\mu g\ kg^{-1}$ (low) and 200 $\mu g\ kg^{-1}$ (medium).

3. Results and discussion

3.1. Liquid chromatography

In this study, the chromatographic separation of target compounds was carried out in a reversed-phase UHPLC (Accucore C_{18}) column, packed with superficially porous particles to take advantage of the ultra-high-performance that this technology offers, which should provide a highly efficient chromatographic separation in a short analysis time.

Several mobile phases and gradient elution programs were tested to optimize the chromatographic separation of Sudan, and Rhodamine B banned dyes. Methanol and acetonitrile were evaluated as organic modifiers showing acetonitrile the best results in terms of chromatographic resolution and analysis time. Therefore, further studies were carried out with acetonitrile-based mobile phases. As aqueous solvent we explored the used of (i) 0.1% acetic acid, (ii) 10 mM ammonium acetate:acetic acid (pH: 4.0), (iii) 0.1% formic acid, (iv) 20 mM ammonium formate:formic acid (pH: 3.75). Results showed that when acetic acid was used, the first eluting compound (Rhodamine B) showed tailing peak and less retention. Nevertheless, formic acid/ammonium formate buffers favored the retention and produced much more symmetric peaks. This fact may be related to the possibility of formate to form an ion-pair specie with the quaternary ammonium of Rhodamine B, which would increase the hydrophobicity of this compound and favor its retention in the C_{18} stationary phase. Additionally, the neutralization of the positive charges (quaternary ammonium and tertiary amine) in this molecule might avoid secondary interactions such as the hydrogen bond with the silanol groups on the surface of the silica particles, thus providing a more symmetric peak.

Thereby, (A) formic acid/ammonium formate aqueous buffer

(pH 3.75, 20 mM), and (B) acetonitrile were selected as mobile phase components in gradient elution mode as described in Section 2.2. In this way, the eight banned dyes were separated in less than 8 min, obtaining good peak shapes and baseline resolution except for Rhodamine B and Sudan Orange G, which partially co-eluted (Fig. 2). Although these compounds can be separated by mass spectrometry based on the differences of their *m/z* values, the signal suppression or enhancement due to the co-elution of these compounds was also studied by injecting individual solutions (100 $\mu g\ L^{-1}$) and a mixture of both compounds (100 $\mu g\ L^{-1}$). The results demonstrated that peak areas showed differences lower than 10%, which were similar to the intra-day precision RSD% indicating that the co-elution of Rhodamine B and Sudan Orange G did not influence their responses.

3.2. Atmospheric pressure ionization behavior studies

Electrospray in positive ion mode has been the ionization technique traditionally chosen for the LC–MS determination of Sudan dyes [11,23] despite the significant matrix effect that this ionization technique usually shows. However, APCI and APPI are generally less affected by the matrix effect, and they might promote efficient ionization of analytes via gas-phase ion-molecule reactions. For these reasons, the ionization behavior of these targeted compounds through the three API sources (ESI, APCI, APPI) has been evaluated in both positive and negative ion modes. In this way, each individual standard solution of these target compounds was injected in the UHPLC–API–MS system using the optimal
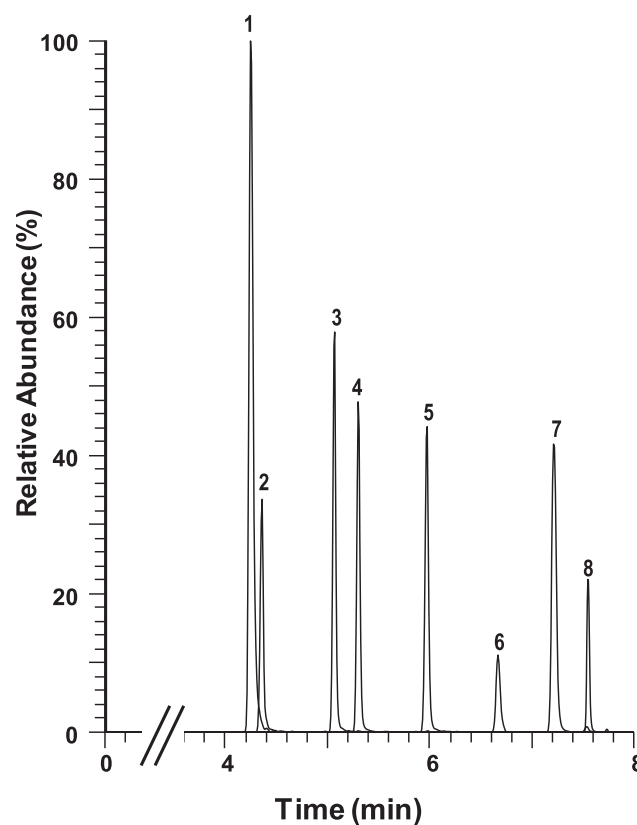

Fig. 2. UHPLC–APCI–MS/MS chromatogram corresponding to a standard mixture solution (5 mg L^{-1}) of targeted compounds obtained under optimal gradient elution conditions. (1) Rhodamine B; (2) Sudan Orange G; (3) Para Red; (4) Sudan I; (5) Sudan II; (6) Sudan III; (7) Sudan Red 7B; (8) Sudan IV.

working conditions for each ionization source (see section 2.2). Table 1 shows the ion assignment and the relative abundance of ions observed in the three API sources (H—ESI, APCI, and chlorobenzene-assisted APPI). All Sudan dyes mainly showed the protonated molecule $[M+H]^+$ in positive ion mode, while Rhodamine B yielded the molecular ion $[M]^+$ as it is a quaternary ammonium salt. However, Para Red showed a better ion signal-to-noise ratio (S/N) in negative-ion mode since the conjugation of para-nitro, and 2-naphthalol groups through the azo functionalization favored the deprotonation of the hydroxyl. Thereby, to monitor the whole set of compounds in a single run with satisfactory sensitivity, a polarity switch before and after the elution area of Para Red was required. It must be pointed out that the chromatographic windows between Sudan Orange and Para Red (t_{window}: 0.65 min) and between Para Red and Sudan I (t_{window}: 0.18 min) were high enough to prevent peak cutting during the polarity switching. Under H—ESI, target compounds did not form any adduct ions neither in-source CID fragment ions (Fig. S1A$_1$: Sudan II and Fig. S1B$_1$: Sudan Red 7B). However, when ionizing Sudan II and Sudan Red 7B under APCI conditions, it was always observed some in-source CID fragment ions (Rel. Ab. < 30%) (Fig. S1A$_2$: Sudan II and Fig. S1B$_2$: Sudan Red 7B). Regarding APPI, it was possible to ionize all target compounds in positive ion-mode without the use of a dopant. The high number of double bonds and electron-donor groups present in the chemical structure of these compounds might lower their ionization potential facilitating the direct photoionization. However, as it is well known, in negative ion-mode, the photoionization mechanism always occurs via dopant-assisted [24]. For this reason, several dopants (acetone, toluene, chlorobenzene, anisole, and tetrahydrofuran) were tested in order to study their effect on the ionization of these analytes. These studies were carried out using the chromatographic conditions previously optimized for the separation of these compounds. All target compounds generated similar type of ions in APPI (Fig. S2). The base peak in the full scan mass spectrum of Sudan dyes was always the ion $[M+H]^+$, and no radical cation via charge exchange was observed in any case, even in the absence of protic dopants. Rhodamine B always produced the $[M]^+$ as a base peak in APPI even when adding dopants with high proton affinities such as acetone and tetrahydrofuran. On the contrary, in negative ion-mode, Para Red yielded the $[M - H]^-$ without the presence of adduct ions or in-source CID fragment ions. Thereby, to compare the ionization efficiency of both direct and dopant-assisted APPI modes (using different dopants), the signal of the base peak of each compound in the respective case was normalized to the most intense signal observed for each compound (Fig. S3). Results showed that dopant-assisted APPI using chlorobenzene provided the highest peak area for most analytes, although the used of toluene as dopant also provided satisfactory responses for Sudan Orange and Sudan I. Hence, as a compromise, further APPI experiments were performed with chlorobenzene as dopant (Fig. S1A$_3$: Sudan II and Fig. S1B$_3$: Sudan Red 7B). The chlorobenzene percentage added into the total mobile phase flow rate was also optimized within the range of 1—10% (*v/v*). The highest responses were obtained at 5% (*v/v*), with no significant improvement at higher chlorobenzene percentages.

3.3. Liquid chromatography-tandem mass spectrometry

The use of tandem mass spectrometry was evaluated to improve the detectability and sensitivity of the UHPLC—MS method and to ensure the accurate identification and quantitative determination of target compounds. For each banned dye, the product ion scan of the ions generated in the API sources was studied using different collision energies. The most abundant and characteristic product ions were selected from these results to develop a selective and sensitive UHPLC–MS/MS method using MRM mode (Table 2). The CID fragmentation in a triple quadrupole could hinder the unequivocal identification of product ions due to multiple collision fragmentation, even more, when there are nitrogen atoms in the chemical structure. For this reason, a LIT–Orbitrap was used to determine the genealogical order of product ions and the corresponding accurate mass, which allowed the determination of the elemental composition and the fragmentation pathway, especially for those product ions that were selected for quantitative and confirmatory purposes. Table S2 and Fig. S4 summarize the product ions observed in the LIT-Orbitrap up to MS^3 using APCI in positive-ion mode for the dyes studied, except for Para Red, which was acquired in negative-ion mode. Although the MS/MS fragmentation of Sudan I-IV, Para Red, and Rhodamine B have previously been studied in low- and high-resolution mass spectrometry instruments and their fragment ions were almost identified [25,26], their fragmentation pathway has not been reported so far. In addition, there is less reported information about the CID fragmentation and the fragmentation pathway of Sudan Red 7B and Sudan Orange G. For this reason, the present work shows a summary of Sudan I-IV results and provides additional information about fragmentation pathways that complement what was already published. At the same time, a more detailed fragmentation study is also included for the other compounds.

Table 1

Assignment of ions generated in H-ESI, APCI and APPI (dopant:chlorobenzene) under optimal conditions.

Compound	ESI		APCI		APPI	
	m/z (Rel. Ab. %)	Ion Assignment	*m/z* (Rel. Ab. %)	Ion Assignment	*m/z* (Rel. Ab. %)	Ion Assignment
Sudan I	249.1 (100)	$[M+H]^+$	249.1 (100)	$[M+H]^+$	249.1 (100)	$[M+H]^+$
					156.1 (16)	$[M+H-C_6H_5O]^+$
Sudan II	277.1 (100)	$[M+H]^+$	277.1 (100)	$[M+H]^+$	277.1 (100)	$[M+H]^+$
			156.1 (10)	$[M+H-C_8H_{11}N]^+$	156.1 (21)	$[M+H-C_8H_{11}N]^+$
			120.1 (12)	$[M+H-C_{10}H_7NO]^+$	120.1 (25)	$[M+H-C_{10}H_7NO]^+$
Sudan III	353.2 (100)	$[M+H]^+$	353.2 (100)	$[M+H]^+$	353.2 (100)	$[M+H]^+$
Sudan IV	381.2 (100)	$[M+H]^+$	381.2 (100)	$[M+H]^+$	381.2 (100)	$[M+H]^+$
					224.2 (14)	$[M+H-C_{10}H_7NO]^+$
Sudan Orange G	215.1 (100)	$[M+H]^+$	215.1 (100)	$[M+H]^+$	215.1 (100)	$[M+H]^+$
Sudan Red 7B	380.2 (100)	$[M+H]^+$	380.2 (100)	$[M+H]^+$	380.2 (100)	$[M+H]^+$
			183.1 (36)	$[M+H-C_{12}H_{11}N_3]^+$	183.1 (65)	$[M+H-C_{12}H_{11}N_3]^+$
Rhodamine B	443.2 (100)	$[M]^+$	443.2 (100)	$[M]^+$	443.2 (100)	$[M]^+$
Para Red	292.1 (100)	$[M-H]^-$	292.1 (100)	$[M-H]^-$	292.1 (100)	$[M-H]^-$

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Table 2
MRM transitions used in UHPLC−APCI−MS/MS.

Compound	Precursor ion (m/z)	Triple quadrupole (QqQ)				LIT-Orbitrap m/z (ppm)	Ion Assignment
		Transition	Product ion (m/z)	NCE (V)	Ion Ratio ± SD[a]		
Sudan I	249.1	Q	93.1	30	1.62 ± 0.04	93.0570 (−3.2)	$[M+H-C_{10}H_5NO]^+$
		q	232.1	15		232.0984 (−4.7)	$[M+H-OH]^{+\bullet}$
Sudan II	277.1	Q	120.1	20	2.49 ± 0.01	120.0811 (3.0)	$[M+H-C_{10}H_7NO]^+$
		q	106.1	45		106.0649 (−1.6)	$[M+H-C_{11}H_9NO]^+$
Sudan III	353.2	Q	92.1	38	1.71 ± 0.04	−	$[M+H-C_{16}H_{11}N_3O]^+$
		q	197.1	16		197.0943 (−2.3)	$[M+H-C_{10}H_6NO]^{+\bullet}$
Sudan IV	381.2	Q	225.1	15	2.40 ± 0.01	225.1256 (−2.1)	$[M+H-C_{10}H_6NO]^+$
		q	106.1	35		106.0650 (−1.1)	$[M+H-C_{17}H_{13}N_3O]^+$
Sudan Orange G	215.1	Q	93.1	25	1.14 ± 0.03	93.0570 (3.0)	$[M+H-C_6H_4NO_2]^+$
		q	198.1	15		198.0782 (−2.9)	$[M+H-OH]^{+\bullet}$
Sudan Red 7B	380.2	Q	115.1	40	3.66 ± 0.05	115.0541 (−1.0)	$[M+H-C_{15}H_{15}N_5]^+$
		q	183.1	15		183.0909 (−4.2)	$[M+H-C_{12}H_{11}N_3]^+$
Rhodamine B	443.2	Q	399.2	50	2.95 ± 0.06	399.1715 (3.1)	$[M-C_3H_8]^+$
		q	355.1	50		355.1077 (−0.1)	$[M-C_3H_8-C_3H_8]^+$
Para Red	292.1	Q	264.1	15	2.01 ± 0.02	264.0662 (−1.6)	$[M-H-N_2]^-$
		q	138.0	25		138.0193 (−2.7)	$[M-H-C_{10}H_6N_2]^-$

[a] SD, standard deviation (n:5), Q: quantitation ion, q: confirmation ion.

Sudan I-IV, Para Red, and Sudan Red 7B share a similar chemical structure based on aromatic rings (phenyl and naphthyl), containing one (Sudan I, Sudan II, and Para Red) or two (Sudan III, Sudan IV, Sudan Red 7B) azo groups. They also have a hydroxyl in R_1, except Sudan Red 7B that shows an aminoethyl group in this last position (Fig. 1). Regarding CID fragmentation in positive ion mode, Sudan dyes containing a β-naphthol group showed the cleavage of the azo double bond as a common fragmentation pathway, which led to two major product ions: the nitrene ion derived from the naphthol moiety and the amine ions derived from the phenyl moiety. These Sudan dyes showed a common product ion at m/z 156.0444 (first-generation ion) corresponding to the nitrene ion when the substituent R_1 was a hydroxyl group. This fact indicates that the hydrogen of the hydroxyl might participate in the fragmentation mechanism favoring the positive charge to remain in the naphthyl moiety after the cleavage of the azo bond. Besides, in the MS^3 mass spectra of m/z 156.0444, a product ion at m/z 174.0550 was observed. This product ion can be due to the formation of an adduct ion with water, which can be explained because the presence of trace amounts of water in the nitrogen used as collision gas and that remaining inside the mass analyzer, as well as due to the longer residence time of ions inside the ion trap [27]. Moreover, the MS^3 mass spectra also showed a product ion at m/z 128.0495, which can be assigned to the loss of a CO unit. This product ion further formed an adduct ion with water yielding the ion at m/z 146.0600. Furthermore, and as discussed before, cleavage of the azo group generally led to both the odd and even amine ions. For instance, Sudan III and Sudan Red 7B have an azo phenyl group as R_3 substituent, and therefore their product ion scan showed the common product ions at m/z 198.1026 $[C_{12}H_{12}N_3]^+$ and m/z 197.0943 $[C_{12}H_{11}N_3]^+$. These ions come from the cleavage of the second azo group to yield the amine ions, $[M+H-C_{10}H_5NO]^+$ (m/z 198.1018) and $[M+H-C_{10}H_6NO]^{+\bullet}$ (m/z 197.0943) for Sudan III and $[M+H-C_{12}H_{10}N_2]^+$ (m/z 198.1021) for Sudan Red 7B (Table S2, Fig. S4). Furthermore, Sudan I and Sudan Orange (m/z 93.0570), as well as Sudan II (m/z 120.0811) and Sudan IV (m/z 225.1256), also showed these losses in the formation of the first-generation product ions, which were selected for quantitation purposes. On the other hand, the cleavage of the two azo groups of Sudan III led to two abundant and characteristic product ions with charge remaining in the phenyl moiety that were selected for quantitation (m/z 92.1) and confirmation (m/z 197.1). However, the product ion at m/z 92.1 was not possible to be confirmed by LIT-Orbitrap experiments because the cut-off phenomena that occur in the ion trap

usually fail to trap ions at the lower end of the m/z range (below ~1/3 the m/z value of the precursor ion) during resonance excitation CID tandem MS. Moreover, the product ion coming from the direct loss of a hydroxy radical was selected as confirmatory ion for Sudan Orange (m/z 198.0788) and Sudan I (m/z 232.0984), whereas for Sudan II and Sudan IV the ion selected for confirmatory purposes was the common first-generation product ion at m/z 106.0649 that corresponded to a dimethyl benzyl radical ion originated by the α-cleavage of the azo group.

Regarding the MS/MS spectrum of Sudan Red 7B in the triple quadrupole, there were observed intense product ions in the region below m/z 170 (Fig. 3A). The shift observed in the maximum of collision energy curves (Fig. 3B) for these low product ions might suggest that they were product ions of higher generation, which might be produced by multiple collisions in the collision cell. This fact could be confirmed in the $APCI-MS^n$ spectra of Sudan Red 7B in positive-ion mode using the LIT-Orbitrap (Fig. 4). For instance, although the product ion selected for quantitation (m/z 115.0541) was present at low abundance on the MS^2 spectra, it could also be observed on the MS^3 spectra of m/z 183.0913 as a second-generation product ion. These product ion (m/z 183.0913) could be assigned to the cleavage of the azo double bond leading to the nitrene ion derived from the naphthol moiety, whereas the m/z 115.0541 could be attributed to the consecutive loss of $C_3H_4N_2$ (68.0369 Da). On the other hand, the Rhodamine B cation, which was also observed in positive APCI ion mode, showed a low CID fragmentation in both triple quadrupole and ion trap. Thus, the main product ion observed at m/z 399.1703 was assigned to a first-generation ion formed by the loss of a propane unit (44.0620 Da) from the molecular ion $[M]^+$. Besides, the ion selected for confirmation purposes (m/z 355.1077) was observed on the MS^3 spectrum (ion trap) of m/z 399.1703 indicating that it was a second-generation product ion coming from the consecutive loss of another propane unit. Regarding Para Red, which showed better sensitivity in negative ion-mode, the MS^2 spectrum (ion trap) of its deprotonated molecule only showed two product ions at m/z 264.0662 and m/z 138.0193 which were selected for quantitation and confirmation purposed, respectively. These first-generation product ions were the result of the cleavage of the azo bond to lose both the naphthyl moiety and N_2, in this last case through a rearrangement [28].

From these fragmentation studies, the ions for quantitation and confirmation purposes were selected and the instrumental limits of detection (ILODs) were estimated for the three UHPLC−API−MS/
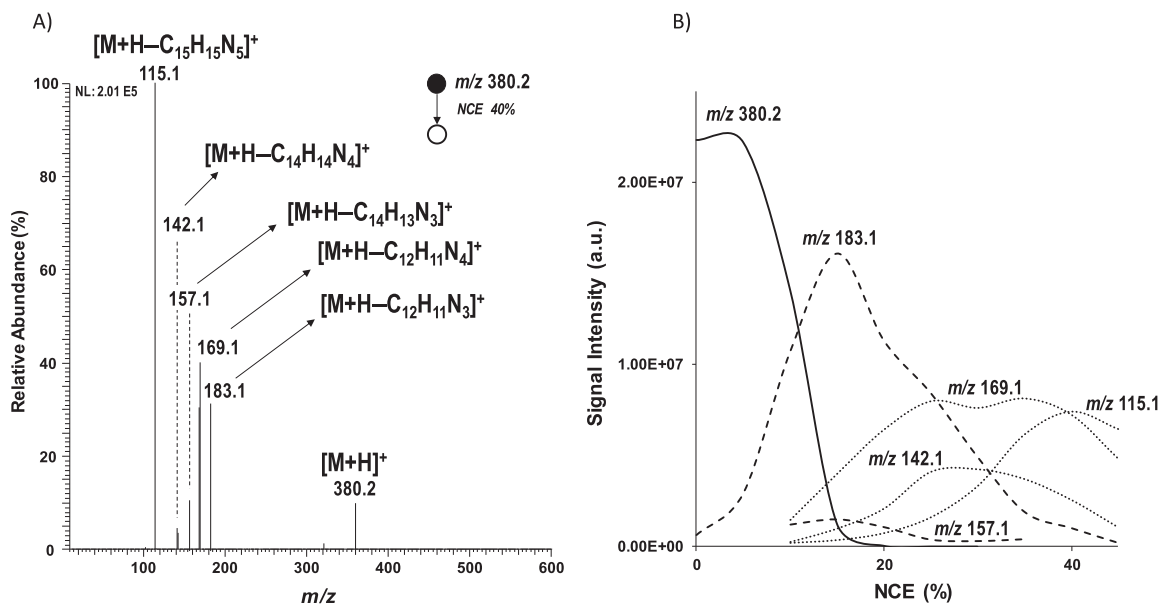
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Fig. 3. Product ion mass spectra and collision energy curves for Sudan Red 7B under positive ion APCI conditions.

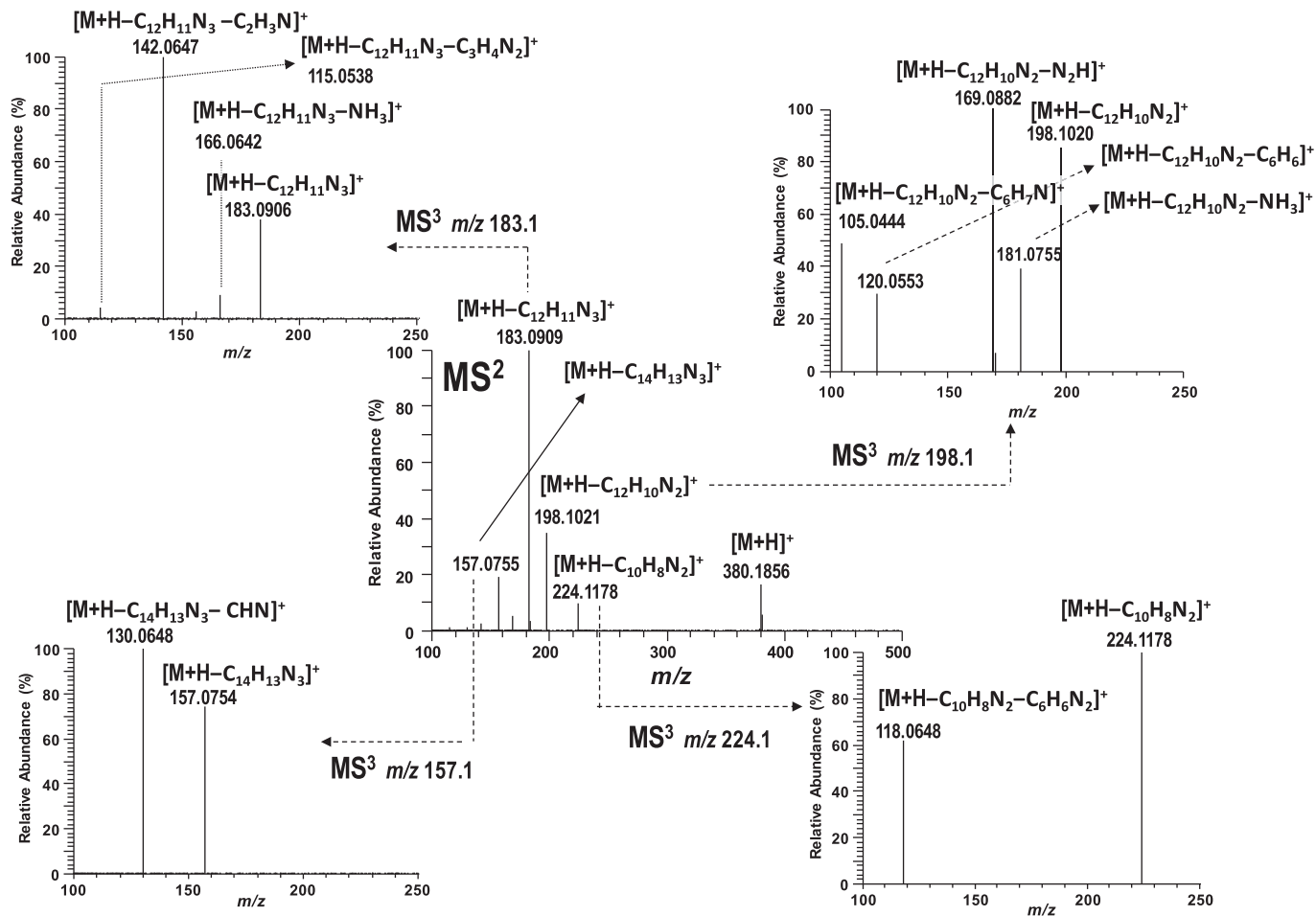**Fig. 4.** MS^n mass spectra of the most important product ions for Sudan Red 7B under positive ion APCI conditions.
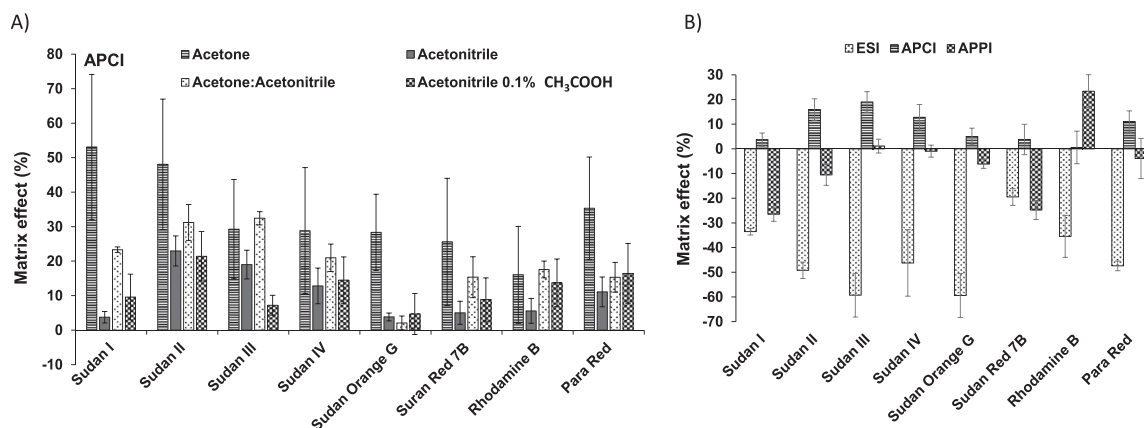
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Fig. 5. Matrix effect (%) using different A) extractant solvent (source: APCI) and B) atmospheric pressure ionization source (extractant solvent: acetonitrile).

MS systems working in MRM mode (Table S3) by injecting standard solutions at low concentration levels. Thereby, in most of the cases the ILODs (based on an S/N of 3) using H−ESI (0.9−30 μg L^{-1}) were only two times higher than those obtained by APCI (0.1−6 μg L^{-1}) and APPI (0.6−15 μg L^{-1}). Besides, the instrumental intra-day precisions estimated at low (15 μg L^{-1}) and medium (50 μg L^{-1}) concentration provided RSD% values lower than 10% in all cases (n = 3).

3.4. Method performance and sample analysis

A quick and cost-effective extraction method was applied in this study to extract targeted dyes from spice samples such as turmeric, curry, and chili. Blank spice powder samples (5 g) were spiked with 20 mg of target compounds and shaken for 12 h to obtain a homogeneous spiked sample. Afterward, banned dyes were extracted using 5 mL of the extractant solvent. For this purpose, acetonitrile and acetone, acetonitrile:acetone (1:1, v/v) and acetonitrile with 0.1% acetic acid were evaluated. The extracts (spiked and blank samples), after centrifugation and filtration, were injected (10 μL) in the UHPLC–API–MS/MS system using the three API sources. The external standard calibration method was used to evaluate the extraction efficiency (EE, %) of the tested solvents and the matrix effect (ME, %) produced in each API source by the corresponding extracts. Results showed that in all cases, the EE% was higher than 95% for all the compounds indicating that all extracting solvents were effective enough to extract these targeted compounds from spice samples. Regarding ME%, blank extracts were spiked (100 μg L^{-1}) with the banned dyes and the quantified concentrations using the three API sources were compared with that

obtained from the analysis of a standard mixture solution prepared at the same concentration level in acetonitrile. As an example, Fig. 5A shows the UHPLC−APCI−MS/MS results obtained when extracting the banned dyes from a spiked turmeric sample using the tested solvents. As can be observed, acetone, and its mixtures with other solvents showed the highest matrix effects for all the compounds, probably owing to the possible coextraction of more hydrophobic compounds by these solvents. Moreover, the addition of 0.1% acetic acid to the acetonitrile coextracted more interfering substances than the pure acetonitrile itself. Therefore, 100% acetonitrile was selected as the most efficient solvent for the simultaneous extraction of the eight studied dyes. Besides, Fig. 5B shows the comparison of the ME% observed using pure acetonitrile as an extracting solvent in H−ESI, APCI, and chlorobenzene-assisted APPI. As can be observed, H−ESI showed the highest ME% values producing an ion suppression of 60%. In contrast, chlorobenzene-assisted APPI showed ion suppression or ion enhancement depending on the target compound with values below 28%. Additionally, these studies were also carried out with curry and chili samples, and generally lower matrix effect (<25%) was observed when using APCI (Table S3). Thus, pure acetonitrile as extracting solvent and APCI as ionization source (easier to operate than APPI) is proposed for the rutine determination of these banned dyes at low concentration levels in spices samples.

Besides, since matrix effect can significantly be affected by the analyte concentration in the sample, which also could affect the method reproducibility and accuracy, the matrix effect was also evaluated throughout the whole working concentration range. Matrix effects were evaluated using both calibration curves obtained from the analysis of standards prepared in the mobile phase

Table 3
Quality parameters of the UHPLC–APCI–MS/MS method estimated in chili.

Compound	mLOD (μg kg^{-1})	mLOQ (μg kg^{-1})	Intra-day precision (RSD, %)		Inter-day precision (RSD, %)		Trueness (RE, %)	
			Low level[a]	Medium level[b]	Low level[a]	Medium level[b]	Low level[a]	Medium level[b]
Sudan I	24	80	2	1	15	3	7	2
Sudan II	6	20	7	6	14	5	12	7
Sudan III	1	2	3	2	9	5	4	2
Sudan IV	3	10	11	6	14	8	9	8
Sudan Orange G	7	22	2	2	5	5	8	5
Sudan Red 7B	2	5	6	2	6	4	6	5
Rhodamine B	1	3	3	1	8	6	12	7
Para Red	1	4	3	2	7	6	5	1

[a] Low conc. level (30 μg kg^{-1} except Sudan I; 100 μg kg^{-1}).
[b] Medium conc. level (100 μg kg^{-1} except Sudan I; 200 μg kg^{-1}).

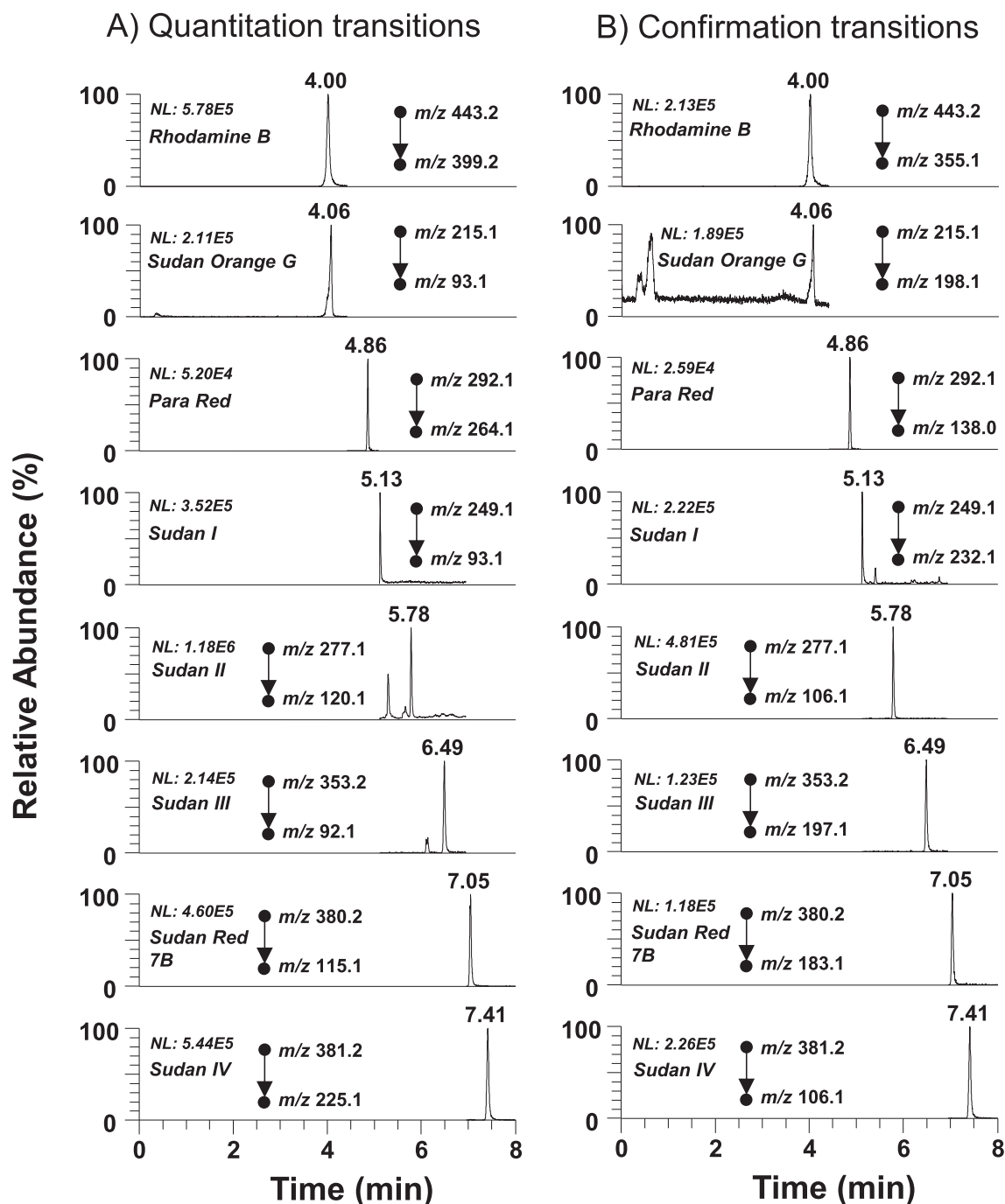## A) Quantitation transitions

B) Confirmation transitions

Fig. 6. UHPLC–APCI–MS/MS extracted ion chromatograms of a blank chili sample spiked at 100 μg L^{-1} for all the compounds.

and matrix-matched method based on spiked blank samples (turmeric, curry, and chili powders) at different concentration levels. Thereby, the differences (in percentage) between the slopes of matrix-matched calibration curves and solvent-based calibration curves were compared. H−ESI studies presented a significant ion suppression showing values from −13% to −83%. Moreover, for the same compounds the ME% value had a significant variation in the different spice sample. For instance, Sudan I showed an average ME % value of 61% (average among the different matrices) with a RSD% of 40%. In contrast, APCI showed the least differences between the slopes being the chili samples the ones that showed the least

matrix enhancement (15−30%), whereas turmeric and curry samples resulted in up to 55% (Fig. S5). However, the average ME% values were more similar than the H-ESI ones for the same compound being for Sudan I the average ME% value 37% with a RSD% of 18%. Although APCI was less affected by matrix effect, matrix-matched calibration was selected for the accurate quantitation of these banned dyes in the desired products.

The applicability of the proposed UHPLC–APCI–MS/MS method was evaluated by determining the eight banned dyes in spice samples in a total of 36 samples (turmeric, curry, and chili products) that were analyzed in triplicate. None of the analyzed samples

showed detectable concentrations of the targeted compounds, so quality parameters of the developed UHPLC–APCI–MS/MS method were calculated from spiked samples of chili (Table 3), turmeric (Table S4), and curry (Table S5) to demonstrate the feasibility of the method on real samples. Thereby, the linearity of the analytical response of target compounds within a working range of two orders of magnitude was satisfactory obtaining correlation coefficients (r) higher than 0.998 for all the compounds. Besides, method limits of detection (MLODs) were estimated under the three API sources. Regarding H−ESI, these values ranged from 4 to 300 μg kg^{-1} due to the high ion suppression observed, whereas APPI resulted in MLOD values from 6 to 50 μg kg^{-1}. Nevertheless, the MLOD values estimated for APCI ranged from 1 to 20 μg kg^{-1} for most of the analytes in chili and curry samples except for Sudan I, which were 24 and 30 μg kg^{-1}, respectively. However, the MLODs were higher in turmeric samples in all cases (up to 48 μg kg^{-1}). These results confirm the good detectability of the developed UHPLC–APCI–MS/MS method. Besides, both intra-day and inter-day precision were determined using the corresponding blank sample spiked at two concentration levels (described in section 2.4). In this way, good intra-day precision was achieved with relative standard deviation (RSD, %) values ranging from 1% to 12% (n = 3), while the inter-day precision values were up to 15%. Furthermore, trueness, expressed as the relative error (RE, %), was also studied at the two same concentration levels. The results were always lower than 15% and 12% (n = 3) in low and medium concentration levels, respectively, in all cases. Fig. 6 shows as an example the UHPLC–APCI–MS/MS extracted chromatogram obtained by spiking a blank chili sample at 100 μg kg^{-1} and as can be seen, the target compounds can be easily detected at this concentration level On the base of these findings, the feasibility of the UHPLC–APCI–MS/MS method has been demonstrated, and it can be proposed for the reliable determination of the eight azo/non-azo banned dyes in chili, turmeric, and curry samples for the control of requirements established in EU legislation.

4. Conclusions

The UHPLC−MS/MS method developed using APCI source has proved to be a reliable and accurate method for the simultaneous determination of seven Sudan dyes and Rhodamine B in spices samples. The use of a UHPLC reversed-phase column and a mobile phase composed of (A) formic acid/ammonium formate aqueous buffer (pH 3.75, 20 mM), and (B) acetonitrile and operating in gradient elution mode provided efficient chromatographic separation and resolution of all target compounds in a short analysis time (<8 min). Furthermore, the observed results on the ionization behavior showed that these compounds generate the same ions in the three API sources. In general, most compounds showed the protonated molecule [M+H]$^+$ as the base peak in positive ion-mode except for the cationic Rhodamine B where the [M]$^+$ predominated the mass spectrum and Para Red that always showed the best ionization efficiency when working in negative ion-mode yielding the [M−H]$^-$ as base peak. Moreover, the multiple-stage MS study in the ion-trap mass analyzer allowed the establishment of the genealogical relationship of product ions and the identification of the most relevant product ions for quantification and confirmation purposes in the determination of banned dyes by UHPLC−MS/MS (MRM mode). Moreover, the identified common product ions as well as those from common losses could be used as diagnostic ions for a fast screening of both target and unknown related compounds in complex food samples. A quick and cost-effective extraction method based on a simple extraction with acetonitrile is proposed to extract simultaneously the targeted dyes from spices samples such as turmeric, curry, and chili since the highest extraction

efficiencies were obtained in addition to the lowest matrix effect values. UHPLC−MS/MS, APCI provided the best sensitivity and the lowest matrix effects for all targeted compounds in the selected samples. The developed UHPLC−APCI−MS/MS method provided low detection limits (1−48 μg kg^{-1}), good repeatability (RSD % < 15), and high accuracy (RE% < 15). The good performance of the developed UHPLC−APCI−MS/MS method and the relevant results obtained in the analysis of chili, turmeric, and curry samples have demonstrated their applicability. It can be proposed for the determination of the targeted eight banned dyes in spices.

CRediT authorship contribution statement

A. Arrizabalaga-Larrañaga: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing − original draft. **S. Epigmenio-Chamú:** Investigation, Validation, Formal analysis. **F.J. Santos:** Methodology, Supervision, Writing − review & editing. **E. Moyano:** Funding acquisition, Project administration, Methodology, Supervision, Writing − review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338519.

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